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ORIGINAL RESEARCH

Stacking of *Hordeum vulgare* vacuolar sodium/proton antiporter and a *bar* gene in wheat for salt and herbicide tolerance

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Abstract
Abiotic stresses and emerging climate change patterns are forecasted to be the biggest challenge to food security. Salt and drought are the critical abiotic stresses responsible for the wheat yield gap with irrigated and fertile lands. In this study, the *Hordeum vulgare NHX1* gene, which encodes for vacuolar Na+/H+ antiporter, was transformed in two wheat varieties, FSD-2008 and Galaxy. The *HvNHX1* gene expression cassette was developed under a constitutive viral promoter (2X *CaMV35S*). The construct was assembled in pSB219, a monocot transformation vector containing the herbicide tolerance gene (*bar*). The transgenic plants were initially screened by two rounds of *BASTA* selection (2 mg/L and 3 mg/L). PCR later confirmed the putative transgenics. The transformation efficiency was estimated to be 0.4% for Galaxy and 0.2% for FSD-2008, respectively. Expression analysis of the *NHX1* gene in T_2 transgenics and non-transgenic controls through qRT-PCR revealed a 12 fold higher expression of the transgene in Galaxy and onefold higher expression in FSD-2008. Under salt stress, the transgenic lines displayed increased chlorophyll content, reduced electrolyte leakage, and higher relative water content in their leaves than in the control plants. Moreover, under stress conditions (200 mM NaCl), the transgenic lines yielded higher biomass and seed weight than non-transgenic controls. The results demonstrated that the constitutive expression of the *HvNHX1* gene in wheat resulted in better grain yield than parent lines. Additionally, the *bar* gene co-transformed with the *HvNHX1* confers herbicide (BASTA) resistance in salt-tolerant wheat transgenics.

Keywords Salt tolerance · *HvNHX1* · *bar* gene · Vacuolar Na+/H+ antiporter · Wheat · Abiotic stress

Introduction

Cereals (sorghum, barley, corn, rice, and wheat) are imperative staple food crops worldwide. Wheat contributes 35% to staple food crops (IDRC 2010). Europe, North America, and Asia are the biggest producer of wheat. However, Asia and Africa are the key importers too, because the rate of wheat consumption is higher than the production in these regions (OECD/FAO 2019). The United States Department of Agriculture predicted the global wheat production for the year 2020/2021 approximately to be 772.64 million metric tons and an increase in the production rate by 1.14% compared to the previous year (USDA 2021; Grote et al. 2021). Regardless of the world's remarkable and continuously growing agricultural production, a significant part of the world's population still faces food insecurity. The rapidly growing global population rate of 1.05% is an additional threat to food security (United Nations 2021), which is higher than the volatile growth rate of wheat. Referring to a global report by the United Nations, about 746 million people are considered food insecure and malnourished (FAO 2020). The global population growth and wheat production with the increasing consumer demand indicate no balance in the equation.

Wheat yield is further compromised owing to detrimental effects caused by abiotic stresses mainly salinity and drought. Abiotic stresses harm the biomass production and accumulation, grain yield, and survival of crops all across the globe (Singhal et al. 2016). Furthermore, water scarcity is emerging as a new risk to crop productivity globally. Countries with a water supply below 1000 cubic meters per person experience chronic water stress and water scarcity

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below 800 cubic meters (Falkenmark et al. 1989). Insufficient water reservoirs along with poor water management are leading cause of water scarcity all across the globe but primarily in the developing countries (Zhang et al. 2021). Crop production is dependent mainly on both quantity as well as quality of irrigation water besides other factors. At present, agriculture sector primarily of the developing countries is facing irrigation related issues, such as poor water quality, soil salinity, water use efficiency, and low crop yields. Surface water has been predominantly used for irrigation owing to its ideal quality but accumulates tons of salts per hectare in the irrigated land (Qureshi and Perry 2021). Due to surface water scarcity, agricultural requirements are met by exploiting groundwater reservoirs. Therefore, areas with saline groundwater lack excessive pumping and are thus more prone to waterlogging due to the rise in the water table. Contrary to this, in other regions with fresh groundwater, increased pumping through tube wells causes the water table to fall and leads to salt deposition and secondary salinization (Qureshi and Perry 2021; Zhang et al. 2021). At present, nearly greater than 900 million hectares of land is salt-affected globally, from which 20% comprises the irrigated area (Afzal et al. 2020).

Salinity in plants causes membrane disorganization, water stress, ionic imbalance, oxidative stress, decreased cell division and expansion, changes in metabolic processes, gene toxicity and nutritional disorders (Al-shareef and Tester 2019). The mechanism of salt tolerance is complex and multi-genic. It comprises functional proteins, macromolecules, membrane transporters, enzymes involved in the biosynthesis of different osmoregulators, detoxification enzymes (Na+/H+ antiporter gene), transcriptional factors (e.g., *DREB*, *bZIP*, *MYB* and *MYC*), protein kinases, and proteinases (e.g., phosphodiesterase and phospholipase C) which collectively regulate gene expression and signal transduction associated to stress tolerance (Singhal et al. 2016).

Na+ toxicity is the most commonly occurring negative effect of salinity, which results in the accumulation of sodium ions in leaf tissues and eventually leads to necrosis. Consequently, reducing the lifespan of leaves and detrimentally affecting the growth, yield and net productivity of crops (Al-shareef and Tester 2019). Plants naturally deal with Na+ toxicity by employing their Na+ transporters and maintain cellular ionic balance (Uozumi and Schroeder 2010). Compartmentation of sodium ions in plant vacuoles by Na+/H+ antiporter is a vital strategy against salt stress used by plants (Zhang et al. 2015). Therefore, the present study was intended to enhance the natural capability of wheat plants against salt stress by developing transgenic wheat lines constitutively expressing Na+/H+ antiporter from barley.

Plant's intracellular Na+(K+/H+ antiporters (*NHXs*) resides in the vast family of monovalent cation/H+ antiporters and are responsible for maintaining cellular pH and ion-homeostasis (Chanroj et al. 2012). Plants possess two kinds of Na+/H+ antiporters; one is found in the vacuole membrane (*AtNHX1*), whereas the second is located in the plasma membrane (*AtSOS1*). The vacuolar membrane antiporters (*NHX*) are more at the edge, because, in addition to Na+ homeostasis, they also regulate K+ homeostasis (Liu et al. 2010). The first plant vacuolar membrane Na+/H+ antiporter (*AtNHX1*), was isolated from *Arabidopsis* (Gaxiola et al. 1999). Afterward, plants overexpressing *AtNHX1* were engineered, which showed remarkable tolerance to salt stress by accumulating more Na + in their vacuoles (Kumar et al. 2017). Literature has shed light on crucial genes related to *NHX* transporter, which have been effective in enhancing salt tolerance in various species, such as mungbean (Sahoo et al. 2016), cowpea (Mishra et al. 2015), alfalfa (Zhang et al. 2015) and tobacco (Chen et al. 2015).

Barley (*Hordeum vulgare*) is a close relative of wheat but possesses excellent abiotic stress tolerance capabilities. The *HvNHX1* gene has a high degree of homology with *TaNHX1* and can be expressed in wheat. This study developed salt-tolerant wheat plants by overexpressing the barley *HvNHX1* (vacuolar Na+/H+ antiporter) gene. It is the first report on the stacking of *HvNHX1* and *bar* gene in wheat. It is estimated that the transgenic wheat developed through this work can improve the wheat yield up to 10% in salt and drought-affected areas and would provide convenient germplasm for easy removal of weeds from the field using the herbicide BASTA.

Materials and methods

Plant material

Two local hexaploidy wheat varieties (FSD-2008 and Galaxy), cultivated in the Punjab and Khyber Pakhtunkhwa provinces of Pakistan, were selected for transformation with *HvNHX1* and *Bar* genes. The seeds for these varieties were obtained from Wheat Research Institute (WRI), Faisalabad, Pakistan and sown at 10-day intervals in the agricultural fields of Forman Christian College University (FCCU), Lahore, Pakistan to get immature embryos for wheat transformation.

Retrieval of nucleotide sequences and primer designing

The nucleotide sequence of the 1.6 Kb *HvNHX1* gene was retrieved from GenBank (accession no. AB089197). A transient expression vector pGR1 available at the FCCU lab was used to PCR amplify the promoter and terminator. The plasmid (pGR1) is 5.8 kb in size and

Table 1 Sequences of primer pairs used to PCR amplify 2× *CaMV35S* promoter, *HvNHX1* gene and *CaMV* terminator

Primer name	Primer sequence (5′–3′)	Tm
X35SCF	CCGAAGCTTACTCCAAAAATGTCAAAGATACAGTC	61.7
X35SCR	TTACGTCGACGCCCTTACGTCAGTGGAGATGT	61.2
HvNXF1	ACACGTCGACGCCATGGCGTTCGAAGTGGTG	62.5
HvNXR1	GCGATTTAAATTATCATGCCACGATTACGTTTGGATCG	66.6
CMVTRF1	GGCATTTAAATGCTGAAATCACCAGTCTCTCTCTACA	66.2
CMVTRR1	AATGGCGCGCCGCATGCTCCGGTGTGAGGGAACT	68.3

The Tm was calculated without the composite nucleotide sequences (the first three nucleotides and the restriction sites)

Table 2 Sequences of gene junction primer pairs used for the screening of transgenic plants

Primer name	Primer sequence (5′–3′)	Tm
BASTF2	AAGCACGGTCAACTTCCGT	57.5
BASTR2	TCCAGGGACTTCAGCAGGT	59.5
RT2XF1	CATCTCCACTGACGTAAGGGCG	65.8
RTHVR1	CCCGACAGCGCCGAATAATGTG	65.8

Table 3 Sequences of gene-specific and internal control primers used for quantitative RT-PCR

Primer name	Primer sequence (5′–3′)	Tm
18SRTF2	ATGATAACTCGACGGATCGC	58.4
18SRTR1	GACACTAATGCGCCCGGTAT	60.5
HVRTF6	TTCTACTGACCGTGAGGTTGC	60.0
HVRTR6	ATTTGCCAGGGCTGTCACTA	59.3

contains *GUS* with intron under 2× *CaMV35S* promoter followed by *CaMV* terminator. The composite primers containing specific restriction sites were designed manually to amplify the 2×*CaMV35S* promoter, *HvNHX1* gene and *CaMV* terminator from the specific templates (Table 1). The primers for screening the transgenics at Promoter:Gene or Gene:Terminator regions (Table 2) were also designed manually. The "Oligo Calculator" software publicly available at http://biotools.nubic.northwestern.edu/OligoCalc.html was used to determine the melting point (Tm) of primers at salt adjusted option. The melting points were adjusted within a difference of 4 °C. The GC clamp was avoided by limiting a maximum of three G and C at the 3′ end of the primer. The primers used in qRT-PCR study were designed using NCBI primer BLAST. Four to five primer pairs showing the lowest values for self or 3′ self-complementarity were selected. The primer pairs validating the amplification efficiency of > 90% and R^2 value of 0.98 were selected for qRT-PCR analysis (Table 3).

Vector construction and transformation in bacterial strains

The *A. tumefaciens* strain AGL1 and the helper plasmid pAL154 were acquired from Ms. Caroline Sparks, Rothamsted Research, UK. The wheat transformation vector "pSB219", which contained the *bar* gene as a selectable marker, was obtained from the Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), Germany. The vector pSB219 was modified to include 10 rare cutter sites in the multiple cloning region and the *GFP* expression cassette was removed. The modified vector (pSB219M) was maintained in the Top10 strain of *Escherichia coli*. The *HvNHX1* cassette was cloned in the pSB219M vector using *Hind*III and *Sgr*D1 for 2× *35S* promoter, *Sgr*DI and *Swa*1 for *HvNHXI* gene and *Swa*I and *Asc*I for *CaMV* terminator. The pSB219M vector with *HvNHX1* cassette was named pSB219-NHX. The T-DNA region of the vector pSB219-NHX is depicted in Fig. 1. The pSB219-NHX and helper plasmid pAL154 were mobilized into the AGL1 strain of *Agrobacterium tumefaciens* for wheat transformation.

Wheat transformation

The immature seeds of wheat cultivars (FSD-2008 and Galaxy) were collected at 15-day post-anthesis (DPA). The wheat embryonic calli were infected with AGL1 strain of *A. tumefaciens* having pSB219-NHX and pAL154 plasmids. The tissue culture and *Agrobacterium*-mediated transformation protocol were followed as described by Abid et al. (2014) and Hasnain et al. (2020).

Screening of transgenic plants by PCR

Total genomic DNA was isolated by the CTAB method from T_0 plants (Murray and Thompson 1980). The conventional PCR was used to screen the transgenic plants for *HvNHX1* and *bar* gene cassettes using primers listed in Table 2. The PCR profile and composition of PCR reagents is provided in supplementary Tables S1 and S2, respectively. The efficacy of PCR was assessed by positive and negative

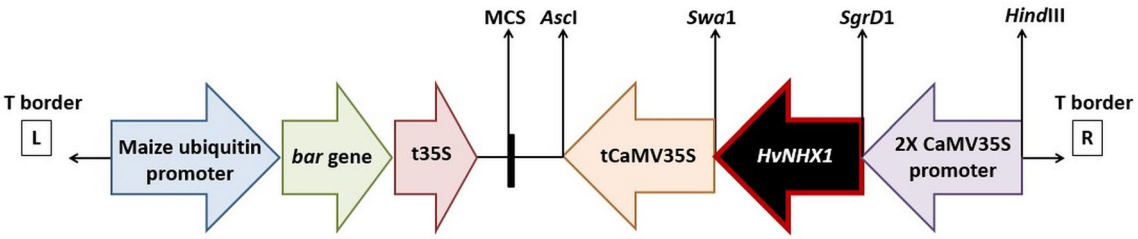


Fig. 1 Schematic representation of T-DNA region of transformation vector pSB219-NHX under the genetic control of 2X *CaMV35S* promoter

controls, which included *HvNHX1* vector construct and genomic DNA from the non-transgenic plant as templates, respectively. The seeds from T_0 plants were collected and sown in the soil to obtain T_1 generation.

BASTA leaf paint assay

Four-week-old T_1 plants were analyzed for BASTA (phosphinothricin) resistance based on *bar* gene expression by painting 0.005% BASTA solution to a specified area on the plant leaf (Akhtar et al. 2020). The dark brown lesions were observed in transgenic and non-transgenic control plants from 7 to 14 days of herbicide leaf paint.

Plant growth conditions and stress treatment

The T_2 *HvNHX1* transgenic lines of FSD-2008 and Galaxy were selected based on BASTA assay, PCR screening and agronomic traits. Four transgenic lines of FSD-2008 (p3-G1, p3-2, p4-G1 and p4-1) and eight for Galaxy (p1-G2, p1-G3, p2-G1 p2-2, p5-3, p5-G1, p6-G3 and p6-6) were selected for expression analysis and stress tolerance assays. Seeds of wild-type and selected transgenic wheat lines were germinated on moist filter paper for 4 days at 28 °C in the dark. Seedlings were then transferred to pots filled with sand. Climate-room conditions were adjusted to the illumination of 1200 mmol photons m-2 s-1 and 12-h/12-h day/night at 18–25 °C (Hasnain et al. 2020).

Two-month-old plants in pots were subjected to a final 200 mM NaCl stress. The NaCl stress was gradually increased by 50 mM every 3rd day till the 200 mM concentration was reached. Finally, plants were exposed to 200 mM NaCl (lethal dose) for 24 h which imposed salt-induced symptoms (Xue et al. 2004; Kumar et al. 2017; Yousefirad et al. 2018).

Expression analysis by real-time PCR

Quantitative real-time PCR (qRT-PCR) assay was performed on T_2 transgenic wheat plants for evaluating the effect of salt stress on the expression of *HvNHX1* gene. After 7 days of stress treatment, leave samples from the 2-month-old plants were collected to extract total RNA. Plant RNA purification reagent (Invitrogen Cat#12322-012) was used for RNA extraction following the manufacturer's instructions. RevertAid H minus first-strand cDNA synthesis kit (Thermo Scientific, USA) was used to reverse transcribe the total RNA using oligo-dT primers according to the manufacturer's protocol. Semi-quantitative RT-PCR was conducted before quantitative RT-PCR to detect *HvNHX1* gene expression in the non-transgenic controls and transgenic plants. The *18S* gene was used as an internal control for template equalization. The primer sequences of *18S* and *HvNHX1* genes used for expression analysis are given in Table 3. Moreover, *bar* gene expression was also detected in the wheat transgenics through semi-quantitative RT-PCR with *bar* gene-specific primers mentioned in Table 2. Supplementary data contains PCR profile (Table S2) and the composition of PCR reagents (Table S4) used for quantitative real-time PCR.

Assays for salt tolerance

The T_2 transgenic lines were selected for assessing tolerance to salinity stress tests. Leave samples from non-transgenic control and T_2 *HvNHX1* transgenic plants were taken before and after 200 mM NaCl stress for determining parameters, such as electrolyte leakage, chlorophyll content, relative water content (RWC) and total protein content.

Total chlorophyll content

Mature leaves from non-transgenic control and T_2 transgenic plants (2 months) were harvested to determine the chlorophyll content. Total chlorophyll was determined by the procedure described by Arnon (1949). Concisely, about 0.2 g of fresh leaf sample was measured and homogenized in 10 ml of 80% acetone using a pestle and mortar. Afterward, the extract was centrifuged at 2500 rpm for 10 min. The supernatant was then transferred to a test tube after raising the volume to 10 ml with 80% acetone. Finally, the absorbance was measured at two wavelengths 645 nm and 663 nm, respectively. Three parallel replicates were conducted for

each sample. Total chlorophyll was measured in mg/g fresh weight with the equation given below:

$$\text{Total chlorophyll} = 20.2(\text{OD } 645) + 8.02(\text{OD } 663) \times W \times V \div 1000.$$

Relative water content (RWC)

RWC was determined according to Lv et al. (2009) using the formula given below. For this purpose, the fresh weight of the leaf segment (FW) was measured, and then the turgid weight (TW) was recorded upon floating the leaf segment on deionized water at 28 °C for 5 h. Finally, the leaf segment was placed in an oven set at 80 °C for 72 h and dry weight (DW) was taken. The experiment was repeated thrice for each sample.

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

Electrolyte leakage (EL)

Electrolyte leakage was estimated thrice for each sample according to the procedure published by Tas and Basar (2009). The leaf segments were dipped in deionized water and electrical conductivity (EC1) was recorded. The electrical conductivity (EC2) was measured again after boiling the samples at 100 °C for 10 min. The electrolytic leakage was determined by placing the EC values in the following formula:

$$\text{Electrolyte leakage } (\%) = (\text{EC1} / \text{EC2}) \times 100.$$

Determination of soluble proteins

Protein concentration was determined according to the Bradford method (Bradford 1976) with slight modifications adopted from Hasnain et al. (2020) and BSA was used as a standard. About 200 mg of fresh leaf sample was briefly measured and homogenized in phosphate buffer solution. The leaf extract was then centrifuged at 5000 rpm for 10 min and the supernatant was transferred to a new eppendorf. The supernatant was then treated with the coomassie blue reagent and incubated at room temperature for 5 min. Finally, the absorbance was recorded at a wavelength of 595 nm. The experiment was repeated three times for each sample.

Morphological characteristics

To determine the transgenic lines growth differences with controls, specific growth parameters like plant height, spike length, number of spikes, fresh and dry weight of plants, and thousand seeds weight were measured under 200 mM salt stress conditions (Hasnain et al. 2020).

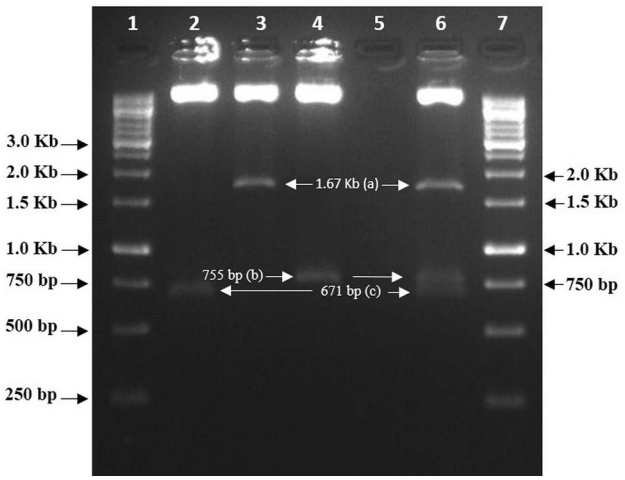
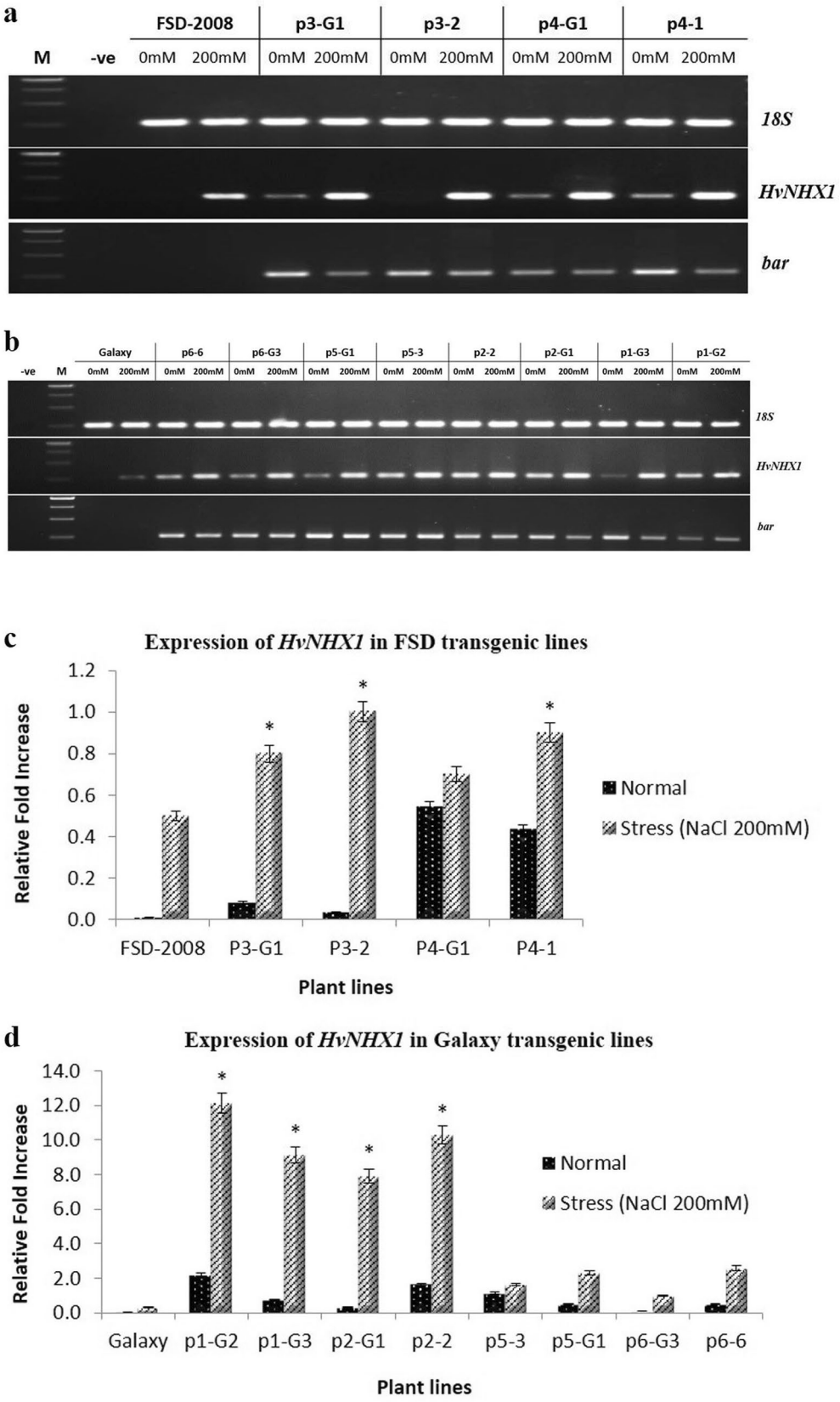


Fig. 2 Restriction analysis of *HvNHX1* gene cassette resolved on 1.5% agarose gel. Lane 1; 1 Kb DNA ladder. Lane 2; *HvNHX1* cassette digested with *Hind*III and *SgrD*1 to excise 671 bp 2× *CaMV35S* promoter. Lane 3; *HvNHX1* cassette digested with *SgrD*1 and *Swa*1 to release 1.67 Kb *HvNHX1* gene. Lane 4; *HvNHX1* cassette digested with *Swa*1 and *Asc*I to observe the release of 755 bp *CaMV* terminator. Lane 5; Blank. Lane 6; *HvNHX1* cassette digested with all the four enzymes (*Hind*III, *SgrD*1, *Swa*1 and *Asc*1) to release the *HvNHX1* gene of 1.67 Kb (fragment **a**), *CaMV* terminator of 755 bp (fragment **b**) and *2X CaMV35S* promoter of 671 bp (fragment **c**). Lane 7; 1 Kb DNA ladder

Statistical analysis

Data analysis was done using ANOVA in SPSS version 17.0, and means were evaluated by Dunnette's test, where p values were significant, i.e., $p < 0.05$.

Fig. 3 BASTA leaf paint assay (Herbicide tolerance) of *HvNHX1-bar* wheat transgenics. Herbicide tolerance analysis of T_1 transgenic and wild type (WT) plants after 14 days of BASTA painting on the surface of plant leaf. Transgenic plants showed BASTA tolerance, while severe necrosis was observed in non-transgenic controls


a
FSD-2008
p3-G1
p3-2
p4-G1
p4-1
M
-ve
0mM 200mM
18S
HvNHX1
bar
b
Galaxy
p6-6
p6-G3
p5-G1
p5-3
p2-2
p2-G1
p1-G3
p1-G2
c
Expression of HvNHX1 in FSD transgenic lines
Relative Fold Increase
Normal
Stress (NaCl 200mM)
FSD-2008
P3-G1
P3-2
P4-G1
P4-1
Plant lines
d
Expression of HvNHX1 in Galaxy transgenic lines
Relative Fold Increase
Normal
Stress (NaCl 200mM)
Galaxy
p1-G2
p1-G3
p2-G1
p2-2
p5-3
p5-G1
p6-G3
p6-6
Plant lines

◂**Fig. 4** Relative expression level of *HvNHX1* in 2-month-old non-transgenic control and T_2 transgenic wheat plants. **a** Semi-quantitative RT-PCR analysis of *18S*, *HvNHX1* and *bar* gene expression in transgenic FSD-2008 wheat lines. **b** Semi-quantitative RT-PCR analysis of *18S*, *HvNHX1* and *bar* gene expression in transgenic Galaxy wheat lines. **c** Real-time qRT-PCR analysis of *HvNHX1* expression in FSD-2008 transgenic lines. **d** Real-time qRT-PCR analysis of *HvNHX1* expression in Galaxy transgenic lines. After equalization with *18S* expression the values are displayed as the means ± SD of three replicates. Asterisk indicates a significant difference from the wild-type control under stress at $p < 0.05$

Results

Confirmation of *HvNHX1* cassette in pSB219

The monocot transformation vector (pSB219) having the *HvNHX1* expression cassette (3 Kb) was named pSB219-NHX. The expression cassette included 2× *CaMV35* promoter, *HvNHX1* gene, and the *CaMV* terminator (Fig. 1). The expression cassette in pSB219-NHX was verified by restriction digestion with *Hind*III, *SgrD*1, *Swa*1 and *Asc*1, respectively. The restriction digestions resulted in the release of three fragments, which included the *HvNHX1* gene (fragment **a**; 1.67 Kb), *CaMV* terminator (fragment **b**; 755 bp) and 2× *CaMV35S* promoter (fragment **c**; 671 bp) as depicted in Fig. 2. The *HvNHX1* cassette in pSB219 was further verified by primer walking utilizing Eurofins, UK's commercial DNA sequencing services.

Wheat transformation and screening of putative transgenics

Approximately two thousand calli of the two wheat verities, FSD-2008 and Galaxy, were inoculated with *A. tumefaciens* transformed with pSB219-NHX. Merely 205 plants were regenerated and reached the BASTA selection stage. Twenty-five putative transgenics survived the BASTA selection, which included 17 plants from Galaxy and eight from FSD-2008. PCR based screening of the 25 putative transgenics using gene junction primers for *HvNHX1* cassette and *bar* gene-specific primers (Table 2) revealed that the entire T-DNA region was integrated into only four Galaxy transgenics and two FSD-2008 transgenics. The transformation efficiency was estimated for the two cultivars and was found to be 0.4% for Galaxy and 0.2% for FSD-2008, respectively.

BASTA leaf paint assay

Herbicide tolerance against BASTA was analyzed in non-transgenic controls and T_1 transgenic plants through leaf paint assay. Two weeks after applying BASTA, leaves of transgenic plants showed tolerance to the herbicide, whereas, dark browning of leaves was observed in non-transgenic plants and is shown in Fig. 3. Transgenic events HVp3 and HVp4 were from the FSD-2008 variety, whereas the remaining four events (HVp1, HVp2, HVp5 and HVp6) belonged to Galaxy.

Expression analysis of *HvNHX1* gene in wheat transgenics

Semi-quantitative RT-PCR of the transgenic lines exhibited that *HvNHX1* and *bar* gene expression was proficiently detected in all the transgenic plants, as shown in Fig. 4a for FSD-2008 and Fig. 4b for Galaxy transgenic lines. The *NHX1* transcripts in salt stressed controls was also detected both in FSD-2008 and Galaxy plants indicating that a homologue of *HvNHX1* also exists in wheat, which is induced under stress conditions. The *HvNHX1* gene transformed in wheat was under the control of 2× *CaMV35S* constitutive promoter and its expression was not supposed to be enhanced by the salt stress conditions. The increase in the *NHX1* transporter transcript level indicated by higher fluorescent intensity of amplification products in the transgenic lines as compared to the controls is most likely due to the additive effect of native and transgenic transporter. Therefore, it may be inferred that the expression of *HvNHX1* at 0 mM salt concentration is actually due to the transgene expression under 2× *CaMV35S* promoter, but the increased expression under high salt is the additive effect of native *NHX* gene which looks like induced under high salt concentration.

The expression analysis of the *HvNHX1* gene in transgenic lines was monitored in 2-month-old T_2 plants both under normal and salt stress conditions (200 mM NaCl) by qRT-PCR. Three out of four transgenic lines of FSD-2008 (p3-G1, p3-2 and p4-1) showed a significant increase in *NHX1* expression under stress, as shown in Fig. 4c. The rise in *NHX1* gene expression under stress spanned from 0.70 to 1.0 fold, with the lowest expression (0.70 fold) detected in transgenic line p4-G1 and highest (1.0 fold) in line p3-2. In the case of transgenic lines of the Galaxy, four lines (p1-G2, p1-G3, p2-G1 and p2-2) exhibited a significant rise in *NHX1* gene expression compared to non-transgenic control both under normal and stress conditions. The gene expression in Galaxy transgenic lines was observed to range between 1.0 and 12.1 folds under high NaCl stress of 200 mM. The highest expression of 12.1 folds was detected in transgenic line p1-G2, whereas the lowest was found in p6-G3, as illustrated in Fig. 4d.

Physiological, biochemical and agronomic trait analysis of *HvNHX1* transgenic wheat lines

The parameters such as electrolyte leakage, total chlorophyll content, relative water content, total soluble

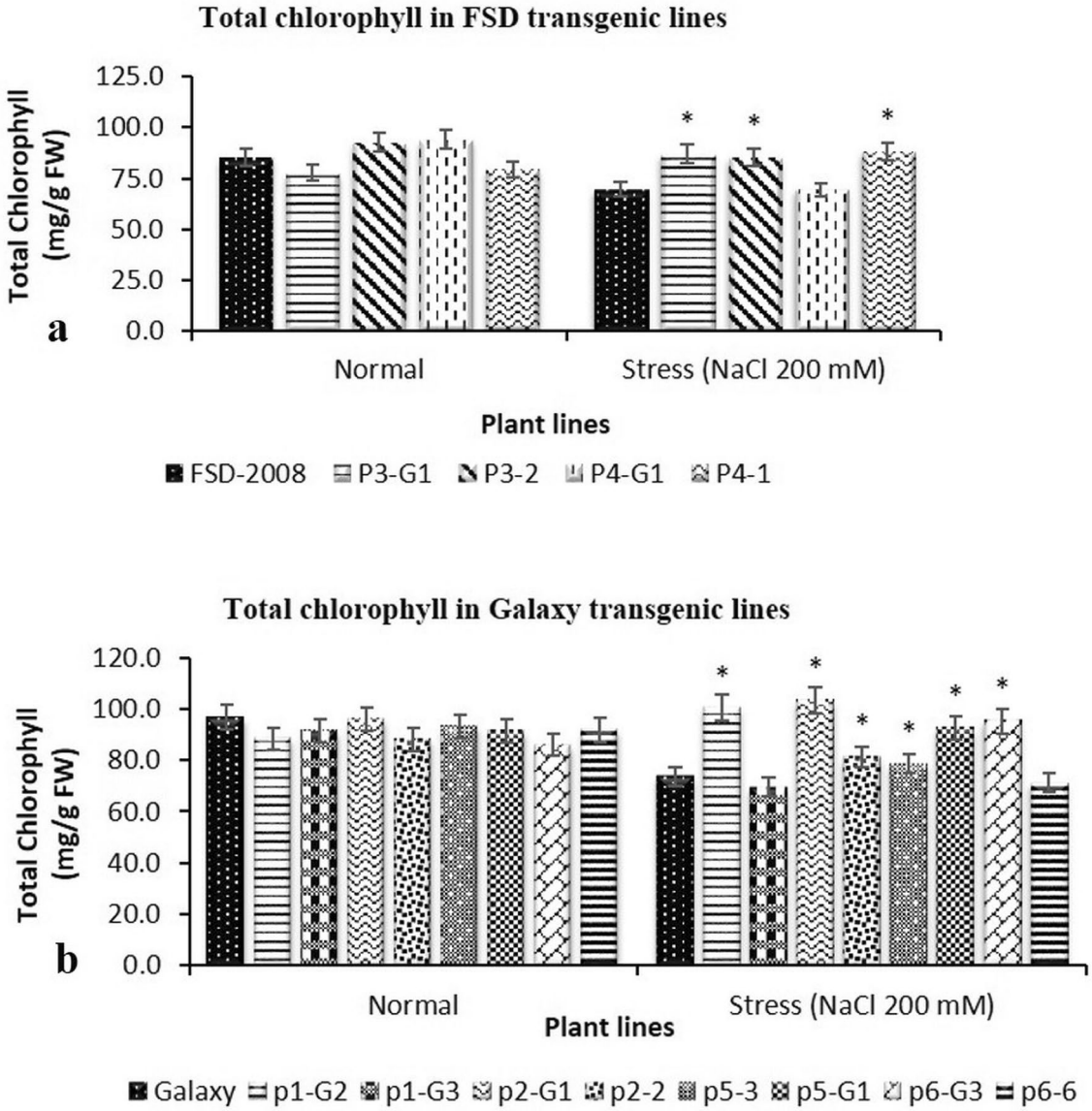


Fig. 5 Total chlorophyll content (mg/g FW) of non-transgenic control and *HvNHX1* T_2 transgenic plants subjected to 200 mM NaCl stress for 7 days. **a** Chlorophyll content of non-transgenic (NT) control and transgenic FSD-2008 lines. **b** Chlorophyll content of NT and transgenic Galaxy lines. Values are represented as the means ± SD of three replicates. Asterisk indicates a significant difference from the non-transgenic control under stress at $p < 0.05$

proteins and morphological characteristics were measured to obtain molecular evidence for salt tolerance of transgenic *HvNHX1* plants.

Total chlorophyll content

Results of total chlorophyll estimation revealed that three transgenic *HvNHX1* lines (p3-G1, p3-2 and p4-1) for FSD-2008 and six lines in the case of the Galaxy (p1-G2, p2-G1, p2-2, p5-3, p5-G1 and p6-G3) showed significantly higher chlorophyll as compared to non-transgenic controls under salt stress condition (Fig. 5a and b). The highest amount of observed chlorophyll was 88 mg/g FW for transgenic line p4-1 in FSD-2008, whereas 103.5 mg/g FW was detected for p2-G1 Galaxy transgenic line. Under stress conditions, the fold increase in total chlorophyll content for all the *HvNHX1* transgenic wheat lines compared to non-transgenic control was recorded to range between 0.9 and 1.4, respectively.

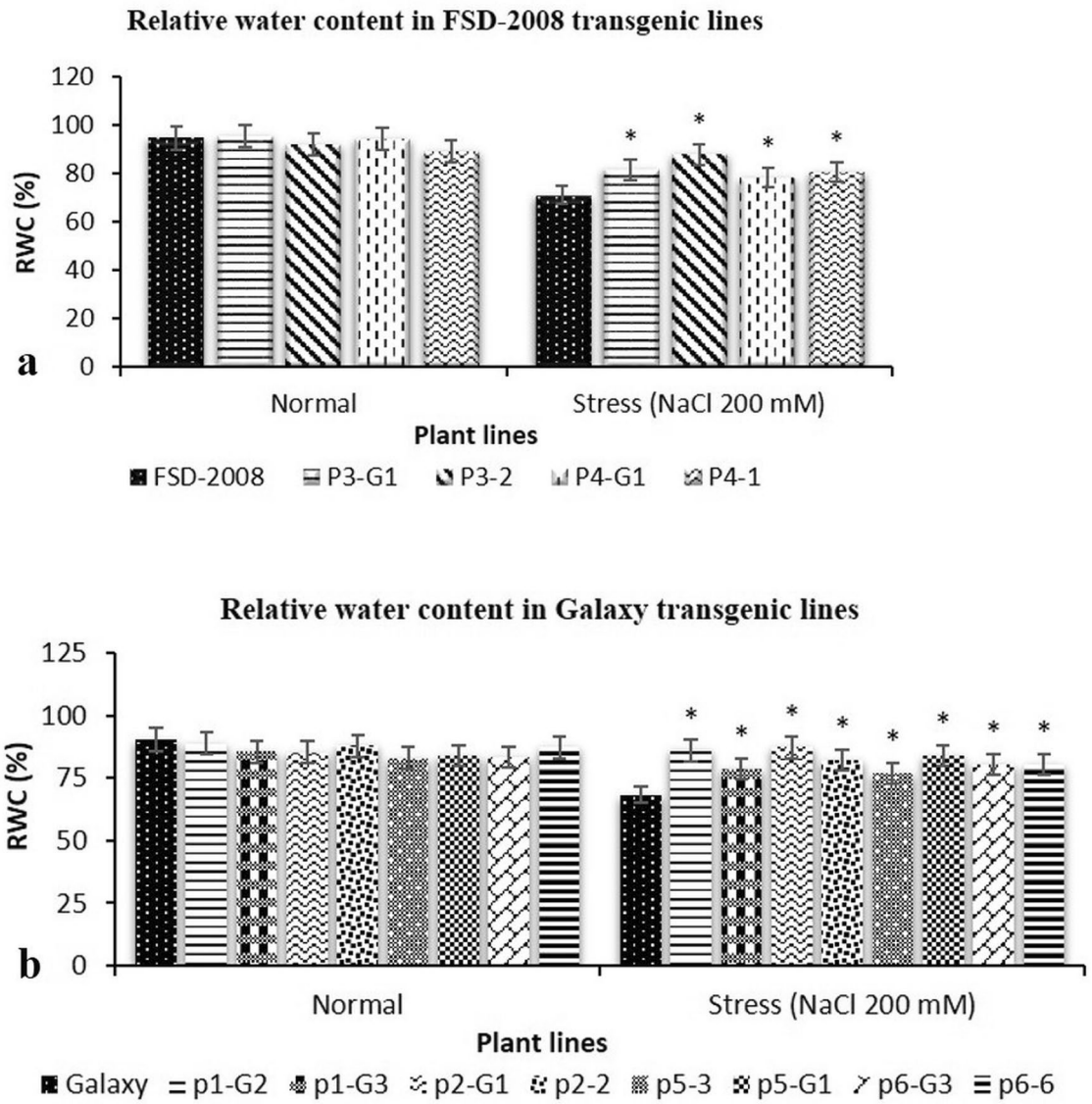


Fig. 6 Relative water content (%) of non-transgenic control and *HvNHX1* T_2 transgenic plants subjected to 200 mM NaCl stress for 7 days. **a** RWC of non-transgenic (NT) and transgenic FSD-2008 lines. **b** RWC of NT and transgenic Galaxy lines. Values are represented as the means ± SD of three replicates. Asterisk indicates a significant difference from the non-transgenic control under stress at $p < 0.05$

Relative water content (RWC)

The maintenance of water balance is vital for plants to survive under stress conditions. Thus, under the 200 mM salt concentration, the relative water content in wildtype (WT) control and transgenic *HvNHX1* plants was measured. The WT control plants showed a decrease of 0.7–0.8 fold in their relative water content (RWC) value, whereas the RWC was not significantly affected (Fig. 6). The transgenic T_2 lines, when compared to WT control under high NaCl stress, displayed a higher potential of 1–1.2 folds to maintain water content in their leaf tissues (Fig. 6a and b). Thus, it has been demonstrated from our results that *HvNHX1* transgenic lines under salt stress retained considerably higher water in their leaves.

Electrolyte leakage

The cell membrane integrity can be inferred by estimating the electrolyte leakage of cytoplasmic solutes from leaf tissues. Under normal conditions, no significant difference was observed in the electrolyte leakage between non-transgenic control and T_2 *HvNHX1* transgenic wheat plants (Fig. 7). Conversely, under high NaCl stress (200 mM) the

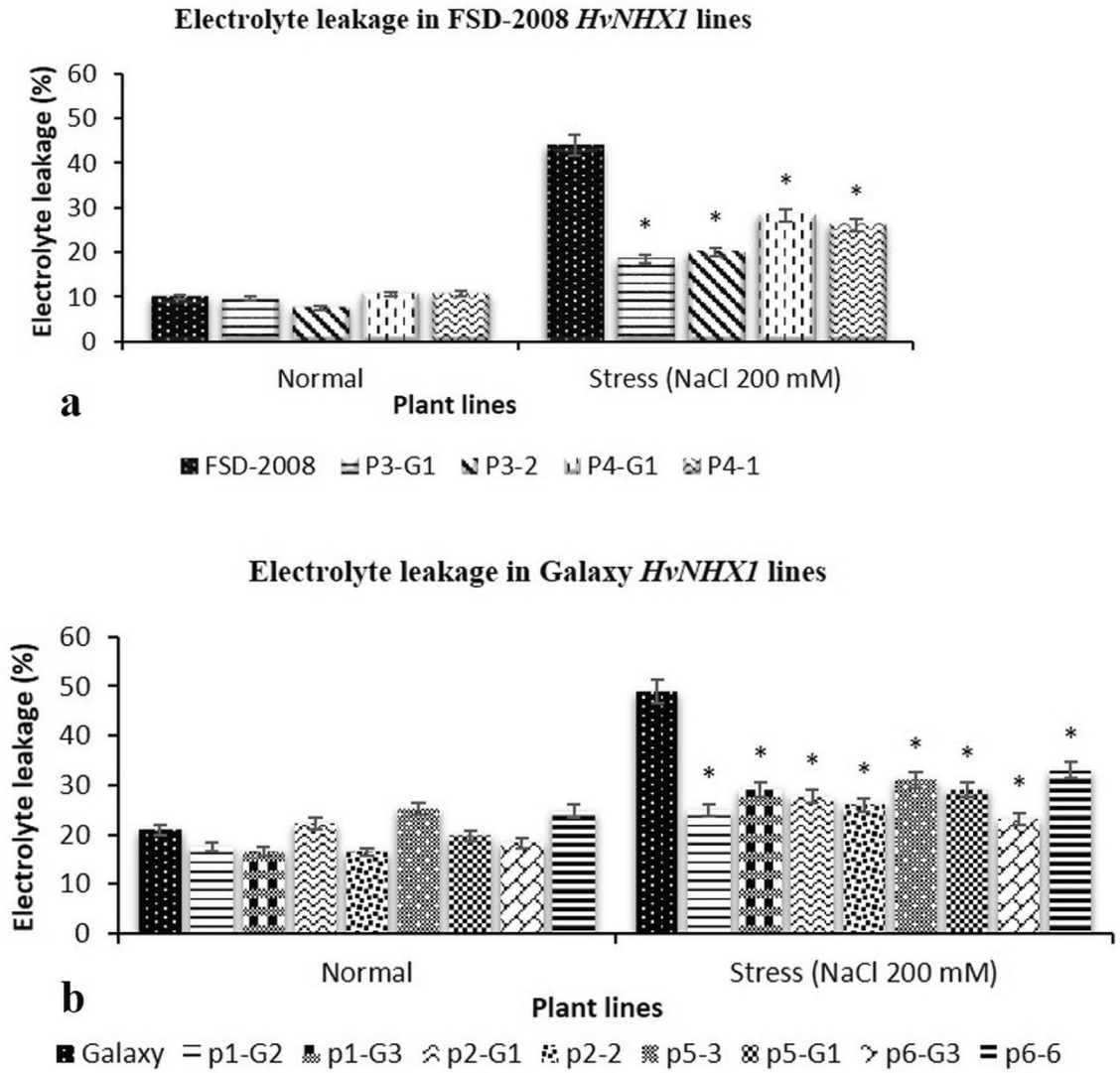


Fig. 7 Electrolytic leakage (%) of non-transgenic control and T_2 *HvNHX1* transgenic plants exposed to 200 mM NaCl stress for 7 days. **a** Electrolyte leakage of NT and transgenic FSD-2008 lines. **b** Electrolyte leakage of NT and transgenic Galaxy lines. Values are represented as the means ± SD of three replicates. Asterisk indicates a significant difference from the non-transgenic control under stress at $p < 0.05$

electrolytic leakage of transgenic plants exhibited a remarkable reduction of 1.6 to 2.4 fold in FSD-2008 (Fig. 7a) and 1.5 to 2 fold in the case of Galaxy (Fig. 7b) when compared to their respective WT controls. Results revealed that under 200 mM salt stress, the T_2 *HvNHX1* transgenic plants retained considerably higher electrolytes and water content in their leaf tissues.

Total soluble proteins

The non-transgenic control and *HvNHX1* transgenic plants were assessed for total soluble protein content under normal and stressed conditions (200 Mm NaCl) to validate the role of *HvNHX1*. No significant difference was detected in the protein concentrations of control and transgenic plants under normal conditions. However, in FSD transgenics, 2 out of 4 lines showed a significant increase in their protein content with a maximum value of 7.4 mg/g FW exhibited by p3-2, as shown in Fig. 8a. Similarly, a significant protein increase was observed in 3 out of 8 Galaxy transgenic lines with the highest value of 9.3 mg/g FW shown by p2-2 (Fig. 8b).

Morphological characteristics

Different agronomical traits were observed in control and transgenic plants after 200 mM NaCl stress (Tables 4, 5). All transgenic lines showed significantly increased plant height when compared to their respective control plants. A

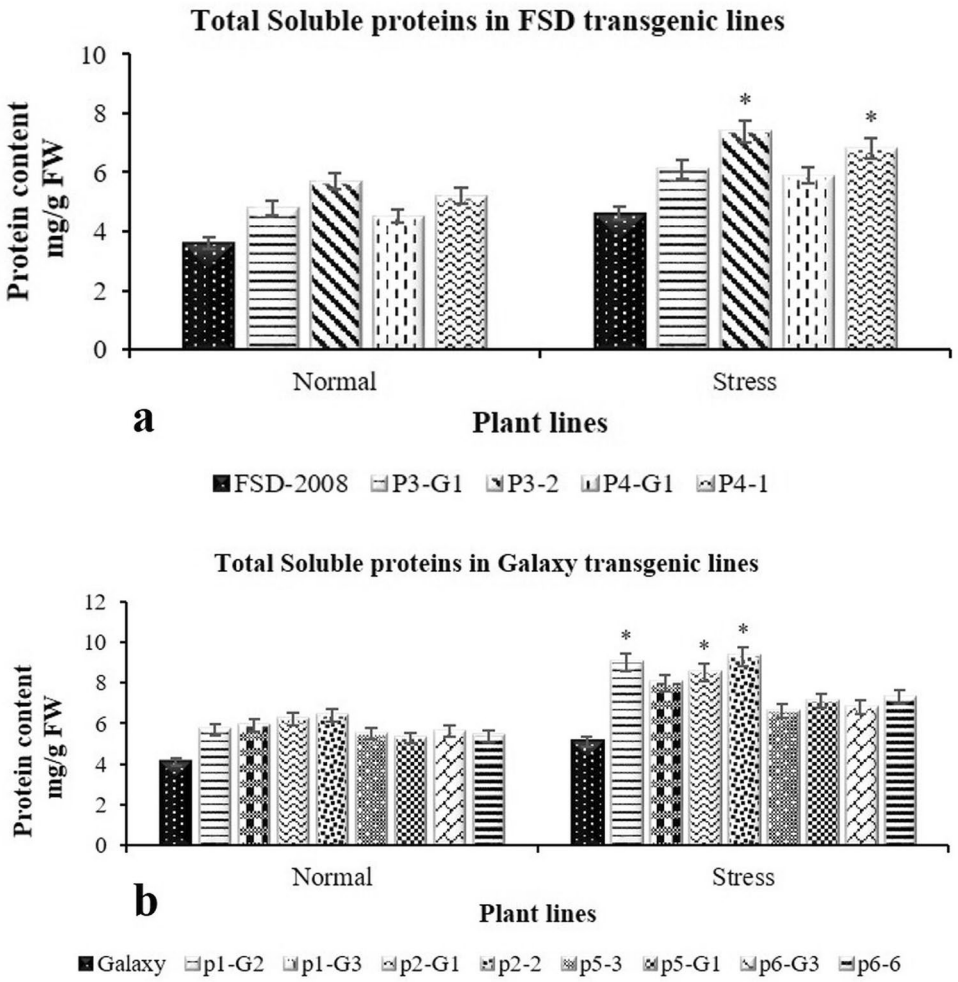


Fig. 8 Total soluble protein content (mg/g FW) of non-transgenic control and transgenic *HvNHX1* T_2 plants subjected to 200 mM NaCl stress for 7 days. **a** Protein content of NT FSD-2008 (control) and transgenic lines. **b** Protein content of NT Galaxy (control) and transgenic lines. Values are represented as the means ± SD of three replicates. Asterisk indicates a significant difference from the non-transgenic control under stress at $p < 0.05$

Table 4 Comparison of agronomic traits of control and transgenic FSD-2008 wheat lines after salt stress of 200 mM

Plant line	Plant height (cm)	No. of spikes	Spike length (cm)	Fresh weight (g)	Dry weight (g)	10 seeds weight (g)
FSD-2008	45 ± 0.6	2 ± 0.6	8.5 ± 0.6	2.1 ± 0.6	0.5 ± 0.6	0.38 ± 0.01
P3-G1	53 ± 0.6*	2 ± 0.6	9 ± 0.6	3.1 ± 0.6	0.9 ± 0.6*	0.49 ± 0.01*
P3-2	56 ± 0.6*	3 ± 0.6	9.5 ± 0.6	4.2 ± 0.6	1.4 ± 0.6*	0.59 ± 0.01*
P4-G1	55 ± 0.6*	2 ± 0.6	8.5 ± 0.6	2.8 ± 0.6	0.7 ± 0.1	0.51 ± 0.01*
P4-1	52 ± 0.6*	2 ± 0.6	9 ± 0.6	2.9 ± 0.6	0.7 ± 0.1	0.55 ± 0.01*

Values are represented as the means ± SD of three replicates. Asterisk indicates a significant difference from the non-transgenic control under stress at $p < 0.05$

maximum height of 56 cm was shown by the p3-2 transgenic line in the case of FSD-2008 (Table 4), whereas among Galaxy transgenic lines, maximum height was exhibited by p1-G2 (63 cm), as depicted in Table 5. Similar findings were observed for seed weight as all transgenic lines displayed increased weight. Maximum weight of 0.59 g was recorded for the FSD-2008 transgenic line (p3-2) and 0.57 g in the case of Galaxy (p1-G2). No significant difference was observed in spike length and spike number for both varieties. However, most of the transgenic lines showed increased spike length in comparison with the control plants. Plant fresh weight was also recorded and out of eight transgenic Galaxy lines, two showed a significant increase in fresh weight, while one line (p5-3) displayed a decrease in weight compared to non-transgenic control. Conversely, no significant change was detected in the case of FSD transgenic lines.

Table 5 Comparison of agronomic traits of control and transgenic Galaxy wheat lines after salt stress of 200 mM

Plant line	Plant height (cm)	No. of spikes	Spike length (cm)	Fresh weight (g)	Dry weight (g)	10 seeds weight (g)
Galaxy	49 ± 0.6	2 ± 0.6	9 ± 0.6	2.3 ± 0.06	0.53 ± 0.01	0.363 ± 0.01
p1-G2	63 ± 0.6*	4 ± 0.6	11.5 ± 0.6	3.2 ± 0.06*	0.66 ± 0.01*	0.57 ± 0.01*
p1-G3	57 ± 0.6*	2 ± 0.6	10 ± 0.6	2.5 ± 0.06	0.57 ± 0.01*	0.51 ± 0.01*
p2-G1	58 ± 0.6*	3 ± 0.6	11 ± 0.6	2.3 ± 0.06	0.59 ± 0.01*	0.49 ± 0.01*
p2-2	60 ± 0.6*	3 ± 0.6	11 ± 0.6	2.7 ± 0.06*	0.61 ± 0.01*	0.53 ± 0.01*
p5-3	56 ± 0.6*	2 ± 0.6	8.5 ± 0.6	2 ± 0.06*	0.55 ± 0.01	0.41 ± 0.01*
p5-G1	59 ± 0.6*	2 ± 0.6	9 ± 0.6	2.4 ± 0.06	0.56 ± 0.01*	0.43 ± 0.01*
p6-G3	54 ± 0.6*	2 ± 0.6	9.5 ± 0.6	2.2 ± 0.06	0.54 ± 0.01	0.39 ± 0.01*
p6-6	57 ± 0.6*	2 ± 0.6	9 ± 0.6	2.5 ± 0.06	0.55 ± 0.01	0.44 ± 0.01*

Values are represented as the means ± SD of three replicates. Asterisk indicates a significant difference from the non-transgenic control under stress at $p < 0.05$

However, two transgenic FSD-2008 lines and five Galaxy transgenic lines showed a significant rise in dry weight compared to non-transgenic control plants (Tables 4, 5).

Discussion

Salt tolerance in plants is a complex multi-genic phenomenon that involves biochemical and physiological responses of numerous stress-associated genes (Kumar et al. 2017). Despite the complexity of the salt tolerance mechanism, utilizing a single stress-responsive gene (*NHX* type Na+/H+ vacuolar antiporter) has been reported to enhance salt tolerance in plants due to its role in ion homeostasis (Zhang et al. 2015; Sahoo et al. 2016). Several transgenic plants overexpressing Na+/H+ vacuolar antiporter have been developed that showed enhanced salt tolerance (Chen et al. 2015; Huang et al. 2017). Another study (Sahoo et al. 2016) has reported enhanced salt tolerance in mungbean due to constitutive expression of the *AtNHX1* gene. Therefore, in the present study, the *NHX1* gene was selected to enhance salt tolerance in wheat and an *HvNHX1* expression cassette was developed in the pSB219 plant transformation vector under the control of 2× *CaMV35S* constitutive promoter (Fig. 1). The constitutive promoter 2X *CaMV35S* has been used in many studies to develop transgenic plants with enhanced tolerance to abiotic stresses (Huma et al. 2015; Ortega-Amaro et al. 2016).

In the present study, two wheat varieties, FSD-2008 and Galaxy, were transformed with *HvNHX1* and *bar* gene by *Agrobacterium*-mediated transformation. A total of six transgenic events were obtained, two from FSD-2008 (P3 and P4) and four from Galaxy (p1, p2, p5 and p6). Two screened plants each from the T_2 lines of the six transgenic events were used for salt stress and herbicide tolerance analysis. The 12 plants from the six transgenic events when subjected to 200 mM salt stress, showed higher *NHX1* expression than non-transgenic controls as depicted in Fig. 4 (Sahoo et al. 2016; Kumar et al. 2017). The *HvNHX1* gene was constitutively expressed in all the wheat transgenics and its expression could be detected in all the transgenic plants tested at 0 mM NaCl concentration. The higher expression of the *NHX* gene in the transgenics subjected to 200 mM NaCl concentration reflected the additive effect of transgenic *HvNHX1* and the wheats native inducible *TaNHX1* gene. The qRT-PCR results indicated that under stress conditions, the cumulative expression of the *HvNHX1* and *TaNHX1* genes observed in transgenic Galaxy lines was between the range of 1–12 folds (Fig. 4d), whereas for FSD-2008, it was 0.5 to 1 fold (Fig. 4c). This variation in expression among different varieties and within transgenic lines of a single variety can be attributed to host response, gene integration event, gene stability and heterologous gene expression in two different wheat lines (Abid et al. 2017). In other words, it can be inferred that expression of the native *TaNHX1* gene in Galaxy upon 200 mM salt stress is several fold higher as compared to FSD-2008 and the transgenic *HvNHX1* is additionally enhancing the expression levels from 0.1 to 2 fold in different transgenic events.

We have assumed from our findings that the transgenic plants overexpressing the *HvNHX1* gene sustained a low cytosolic Na+ concentration to delay stress-induced senescence and protect photosynthesis (Gao et al. 2016). Excessive concentration of sodium ions disrupts ion homeostasis in plant cells and promotes ROS production, which results in the degradation of chlorophyll (Yasar et al. 2006). Thus, salt stress causes detrimental effects on plant growth by affecting photosynthetic activity, transpiration rate and stomatal conductivity. We found that the transgenic *HvNHX1* wheat plants withstand 200 mM salt stress by displaying significantly higher chlorophyll (Fig. 5) and water content (Fig. 6) in their leaves with decreased electrolyte leakage (Fig. 7)

as compared to the non-transgenic controls. Comparable results were demonstrated (Kumar et al. 2017), in which the transgenic mungbean overexpressing *AtNHX1* showed enhanced salt tolerance by maintaining their photosynthesis efficiency and retaining higher water content and electrolytes in their leaves.

Similarly, salt stress triggered a remarkable increase in total soluble proteins in *HvNHX1* transgenic plants (Fig. 8). Plant soluble proteins are vital for cell survival and stabilize membrane under salt stress (Goudarzi and Pakniyat 2009). Galaxy transgenic line p2-2 displayed the highest protein content (Fig. 8b), while the lowest was observed in FSD-2008 p4-G1 (Fig. 8a). It is well understood that plants synthesize new proteins for their survival and average growth under stress conditions. Thus, it can be inferred from the above results and other supporting studies that salt-tolerant transgenic plants exhibit efficient mechanisms of osmotic regulation, which reduces sodium toxicity in the cytosol as compared to non-transgenic control plants (Flowers and Yeo 1995).

Under the above findings, transgenic plants under stressed conditions displayed significantly higher plant height, biomass, and seed weight when compared with non-transgenic controls (Tables 4, 5). Likewise, another study has reported improved biomass and grain yield in transgenic maize plants expressing the *OsNHX1* gene (Chen et al. 2015). In addition to salt tolerance, the T_2 transgenic wheat plants also exhibited herbicide tolerance when 0.005% BASTA (phosphinothricin) was painted on plant leaves, as shown in Fig. 3. Leaves of transgenic plants remained green and unaffected, whereas the non-transgenic controls displayed severe leaf necrosis (Kumar et al. 2017). These interpretations suggested that the T-DNA region containing the *HvNHX1* and *bar* genes transformed in wheat is stable and functional.

Conclusion

In conclusion, the co-expression of *HvNHX1* and *bar* gene enhanced salt and herbicide tolerance in T_2 transgenic wheat plants. Improved salt tolerance can be successfully correlated with increased *NHX1* expression, thus proposing alleviation of sodium toxicity by transporting Na+ ions to plant vacuoles. This transport improved plant growth and grain yield in wheat transgenics under 200 mM salt stress by retaining higher water content and electrolytes in leaves. The restoration of photosynthetic efficiency and membrane stabilization displayed through increased protein production and higher chlorophyll content also adds to the salt tolerance mechanism. The transgenic wheat lines developed in the present study may be helpful to overcome the alarming situation of salinity and drought.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s12892-022-00144-6.

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Author contributions AB and KAM devised and supervised the project. SI and MV performed the experiments. SI, AM and MI contributed to the preparation of all figures and tables. SI wrote the manuscript. SM and AB edited the manuscript. All authors reviewed, revised, and approved the manuscript.

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Availability of data and materials The transparency of data will be maintained. Any information relevant to the research work will be provided when required.

Declarations

Conflict of interest The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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