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Cloning and characterization of vacuolar Na^+/H^+ antiporter gene promoter from wheat (*Triticum aestivum*) cv. Kharchia Local

ETIKA GOYAL[1,2], AMIT KUMAR SINGH[1], MAHESH M MAHAJAN[1,3], ARUN KUMAR[4] and KANIKA KUMAR[1]*

[1]*ICAR-National Institute for Plant Biotechnology, New Delhi 110 012, India*
[2]*Department of Chemical Engineering, Texas A&M University, Texas, USA*

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ABSTRACT

Soil salinity is one of the significant abiotic stresses in plants. Few efficient stress-inducible promoters from plants, especially from monocotyledonous crops like wheat (*Triticum aestivum* L.) are available to drive stress-inducible expression of transgene. Therefore, an experiment was conducted at ICAR-National Institute for Plant Biotechnology, New Delhi, during 2017–18 to clone and characterize stress-inducible promoter from wheat. Results found a 418 bp long *NHX1* promoter (*TaUNHX*) from a bread wheat landrace, Kharchia Local. *In silico* analysis of *TaUNHX* predicted several *cis*-acting regulatory elements, including CAAT, DOF, GATA motifs and some essential stress-responsive elements. To analyze the activity of *TaUNHX*, Agrobacterium-mediated transient GUS assay in tobacco (*Nicotiana tabacum* L.) leaves and stems was studied under various abiotic stresses like salt (300 mM NaCl), drought (20% PEG) and ABA (100 μM). Stress-responsive nature of this promoter can be used to drive the expression of transgene following exposure to various stresses. The study's finding has significant implications as the characterized promoter can be used to develop transgenics where the transgene expression can be induced following exposure to stress.

Keywords: Abscisic acid, *Agrobacterium tumefaciens*, Drought stress, Salt stress, Transient GUS assay

Abiotic stresses cause severe loss of productivity in cereals. To improve stress tolerance in crop plants, various transgenics have been developed. *In vivo* characterization of plant promoters has been carried out in transgenic plants (Zhu *et al.* 1996). This method of characterization is slow and labor-intensive. To overcome these limitations, transient expression analysis has been widely used and is a convenient alternative for stable transformation (Yang *et al.* 2000, Guan *et al.* 2022). The number of efficient promoters has been evaluated for their ability to drive the expression of transgenes (Park *et al.* 2010). Few of these promoters have been successfully used to develop stress-tolerant transgenics. Apart from constitutive promoters, stress-inducible promoters have also been cloned and characterized for their efficiency in driving transcript expression (Goyal *et al.* 2013, Tiwari *et al.* 2014). The use of these promoters to drive the expression of the transgene is known to reduce the risk of phenotypic abnormalities and yield penalty.

Several stress-responsible promoters like *SbGSTU* from *Salicornia brachiata*; *Em* from wheat (*Triticum aestivum* L.), *Osem*, *Rab16A,* 4XABRE and 2XABRC from rice (*Oryza sativa* L.), *Rab17* and *ZmGAPP* from maize (*Zea mays* L.), *HVA1* and *HVA22* from barley (*Hordeum vulgare* L.), Pr_{AlSAP} from *Aeluropus littoralis* and *SlPEAMT* from *Suaeda liaotungensis* have been cloned (Hattori *et al.* 1995, Shen *et al.* 1995, 1996, Ganguly *et al.* 2011, Song *et al.* 2012, Saad *et al.* 2015, Hou *et al.* 2016, Li *et al.* 2016, Tiwari *et al.* 2016).

Among the stress responsive gene vacuolar Na^+/H^+ antiporter gene is well known. It is involved in the compartmentalization of Na^+ into the vacuole to maintain a low content of cytosolic Na^+ under salt stress (Wang *et al.* 2016). Even though *NHX1* is a vital gene, there is a dearth of information about its promoter, especially from monocot crops. Therefore, the present study aimed to clone and characterize the promoter of the vacuolar *NHX1* gene from the salt-tolerant bread wheat genotype. To the best of our knowledge, it is the first report of cloning and characterization of the promoter of *NHX1* from the ancient wheat landrace Kharchia Local.

MATERIALS AND METHODS

Plant growth material and germination: Seeds of wheat cv. Kharchia Local were germinated and seedlings were raised in hydroponics using Hoagland solution with 16/8 h light/dark at 24/18°C, 150 mW/mm^2 of irradiance, and

[1]ICAR-National Institute for Plant Biotechnology, New Delhi; [2]Department of Chemical Engineering, Texas A&M University, Texas, USA; [3]Bio Genome, India; [4]ICAR-Indian Agricultural Research Institute, New Delhi. *Corresponding author email: kumarkanika@rediffmail.com

Table 1 Sequences of the primers used in the present study

Name	Primer sequences (F, Forward primer; R, Reverse primer)
NHXG-WRP1	5′ CAGGACAAGCGCGGCGGTGGTGGACTC 3′
NHXG-WRP2	5′ CCCGGTGTACTTGAGAGCGAGGGC 3′
NHXPF/R	F: 5′ ATCCGCGGCTGAATCTGTTGTCCGGT 3′ R: 5′ TAGAGCTCGCCGGTCGCCACCTCGGC 3′

(Restriction sites are underlined)

60% air humidity. The plants were grown at the National Phytotron Facility, ICAR-Indian Agricultural Research Institute, New Delhi during 2017–18. After 10 days of germination, leaf samples were collected and stored at -80°C until further use. Seeds of tobacco were germinated and raised in plastic pots containing soil rite mix at National Phytotron Facility, ICAR-IARI, New Delhi.

Construction of genome walking libraries: Genomic DNA was extracted using the DNeasy® Plant Extraction Kit (Qiagen, Hilden, Germany) from 100 mg leaves samples. The upstream region of the *NHX1* gene was amplified using Genome Walker™ Universal Kit (Clontech, Palo Alto, CA, USA) following the manufacturer's protocol. *NHX1* promoter-specific primers (NHXGW1 and NHXGW2) were designed from nucleotide sequences available in the NCBI GenBank database with Accession No. KT273926 (Goyal *et al.* 2017). Advantage 2 polymerase mix (Clontech, USA) was used to perform primary and secondary PCR using the primers sets AP1 and NHXGW1, and AP2 and NHXGW2, respectively (Table 1).

Isolation, cloning and analysis of the upstream region of the NHX1 gene: PCR amplified, gel eluted amplicon was ligated to pGEM®-T Easy vector (Promega, Madison, USA) and transformed into *E. coli* DH5α cells using a standard protocol. The transformants were identified by blue-white screening and confirmed by colony PCR. To confirm the amplification of specific fragments, sequencing was performed. Online databases *PLACE/Signal Scan* (*http://www.dna.affrc.go.jp/)* and PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) were used to identify the *cis*-acting regulatory elements present in the obtained nucleotide sequence. The predicted promoter was named *TaUNHX*.

Construction of GUS construct in the binary vector: *TaUNHX* was amplified by PCR using primer pair (NHXPF/R) containing *Sac*II and *Sac*I restriction sites in forward and reverse primer for sub-cloning into the pORE R2 promoter less binary vector (Table 1, Fig 1). The amplified PCR product and pORE R2 vector was digested with restriction enzymes *Sac*I and *Sac*II simultaneously. The digested products were ligated using T_4 DNA ligase following purification (Fermentas, Hanover, USA).

TaUNHX::GUS: E. coli DH5α cells were transformed

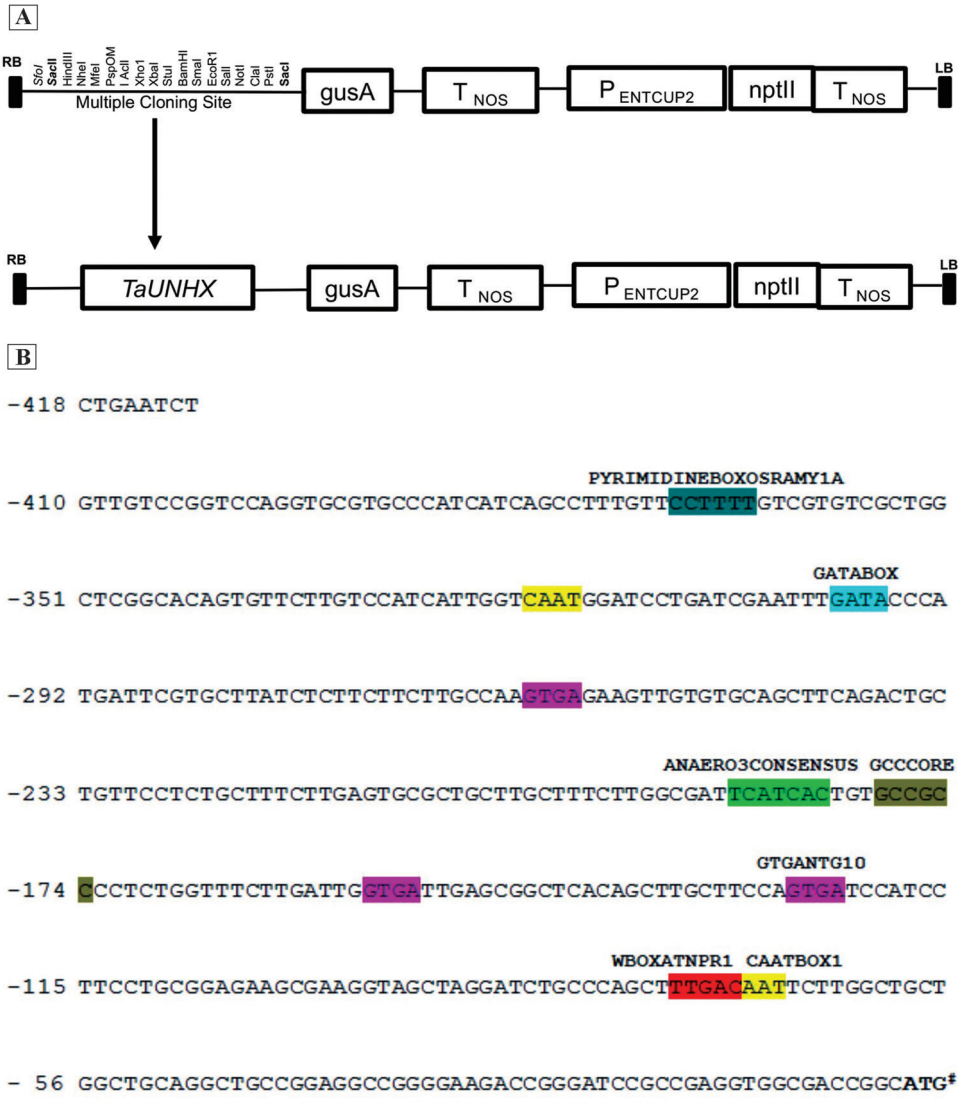

Fig 1 (A) Linear map of T-DNA of pORE R2 promoterless binary vector. (B) *T. aestivum NHX1* promoter (*TaUNHX*) sequence (acc. No. KY002680) showing various *cis*- acting elements. Highlighted boxes represent different *cis*-acting regulatory elements. A of ATG (translational start site) was used as +1 for counting the number of nucleotides in *TaUNHX* sequence.

with *TaUNHX::GUS* using the standard protocol (Goyal *et al.* 2013). The plasmid construct (*TaUNHX::GUS*) isolated from *E. coli* DH5α cells was further mobilized into *Agrobacterium tumefaciens* strain GV3101 by the freeze-thaw method. Transformants were selected on kanamycin (50 µg/ml) and rifampicin (25 µg/ml) supplemented YEM agar. Positive clones were identified using colony PCR.

Agro-infiltration and stress treatment: A. tumefaciens strain GV3101 containing the construct (*TaUNHX::GUS*) was used for agro-infiltration as described by Goyal *et al.* (2013). The agro-infiltrated plants were given different treatments: control 1 (without stress); control 2 (negative control, i.e. plant infiltrated only with infiltration buffer); salt stress (300 mM NaCl); drought stress (20% w/v PEG) and ABA stress (100 µM ABA). These were then incubated in a growth chamber for 24 h. The experiment was conducted using 3 biological replicates and repeated twice independently.

Histochemical GUS staining: The histochemical GUS activity assay was performed 72 h after agro-infiltration, as reported by Goyal *et al.* (2013). The plant tissues were observed and photographed using a microscope (Wild M8, Heerbrugg, Switzerland).

RESULTS AND DISCUSSION

Plants' response to various physiological and environmental stresses involves spatial or temporal expression of a specific gene(s), which is controlled through numerous mechanisms. Controlled expression of these genes is significant for any organism to survive and flourish. This is achieved through a highly precise and fine-tuned gene regulatory network. Activating these regulatory network pathways can enable plants to respond to environmental stimuli and tolerate various stresses. The role of vacuolar *NHX1* under salt stress is well known, and its *in-silico* analysis has also been carried out. In the present study,

Table 2 Location of motifs with respect to the translational start site (TSS) detected in upstream region of *TaUNHX* using *PLACE/ Signal Scan*

Motif		Role	Sequence*	Location**
ANAERO2CONSENSUS	S000478	FP	AGCAGC	-209
ANAERO3CONSENSUS	S000479	FP	TCATCAC	-230
ARR1AT	S000454	TA	NGATT	-4, -127, -226, -258, -265
CAATBOX1	S000028	C	CAAT	-92, -260, -267
CACTFTPPCA1	S000449	C	YACT	-76, -99, -156, -204, -234, -292
CCAATBOX1	S000030	C	CCAAT	-92, -260
DOFCOREZM	S000265	OP	AAAG	-42, -50, -196, -217, -342
EBOXBNNAPA	S000144	L	CANNTG	-20, -154
GATABOX	S000039	C	GATA	-119, -138
GCCCORE	S000430	P	GCCGCC	-240
GTGANTG10	S000378	C	GTGA	-157, -264, -277, -293, -233
IBOX	S000124	L	GATAAG	-136
IBOXCORE	S000199	L	GATAA	-137
MYB1AT	S000408	D	WAACCA	-250
MYBCORE	S000176	OP	CNGTTR	-7
MYCCONSENSUSAT	S000407	T	CANNTG	-20, -154
NODCON2GM	S000462	NS	CTCTT	-141
OSE2ROOTNODULE	S000468	NS	CTCTT	-141
POLLEN1LELAT52	S000245	OP	AGAAA	-218, -253
PYRIMIDINEBOXOSRAMY1A	S000259	H	CCTTTT	-49
RAV1AAT	S000314	OP	CAACA	-8, -59
RAV1BAT	S000315	OP	CACCTG	-20
RHERPATEXPA7	S000512	OP	KCACGW	-131
WBOXNTCHN48	S000508	BF	CTGACY	-95
WBOXNTERF3	S000457	BF	TGACY	-95
WRKY71OS	S000447	GB	TGAC	-96

FP, fermentative pathway; TA, transcriptional activator; C-*cis,* regulatory element; OP, other processes; L, light; P, photosynthesis; D, drought/dehydration; T, temperature; NS, nodule specificity; H, hormone responsive; BF, biotic factor; GB, gibberellin.

*N=A/T/G/C; W=A/T; Y= C/T; K=G/T; ** -location with respect to TSS.

its promoter was cloned from wheat cv. Kharchia Local and functionally validated in tobacco (Goyal *et al.* 2017). Kharchia Local is a landrace known for its tolerance to salt stress. The sequence of upstream region of the *NHX1* gene exhibited homology with the 5′end sequence of the *NHX1* gene. The sequence analysis confirmed that cloned sequence was the upstream region of the *NHX1*. This sequence was named *TaUNHX*.

Characterization of cis-regulatory elements in the TaUNHX promoter region: In-silico analysis is the most common method to identify putative regulatory elements in plant promoters. *TaUNHX* was analyzed to identify different *cis*-acting regulatory elements using PLACE and PlantCARE databases. The *cis*-acting regulatory elements are DNA sequences where transcription factors bind, thereby regulating gene expression under different conditions. They control many essential biological processes, including development, response to hormones, response to abiotic and biotic stresses, etc. In plants, several cis-regulatory elements are essential for expressing stress-responsive genes (Goyal *et al.* 2013). The *TaUNHX* sequence was submitted to GenBank under accession no. KY002680. The complete sequence of *TaUNHX,* along with other *cis*-acting regulatory elements is shown in Table 2.

TaUNHX promoter sequence contained multiple stress-responsive *cis*-acting regulatory elements, including TATA and CAAT box, which are essential for the initiation of transcription and enhanced transcription level. CAAT box is commonly found in the promoter and enhancer region of the promoter (Park *et al.* 2010, Srivastava *et al.* 2014). The presence of this motif, in the present case, suggests that the amplified region is a promoter. Other motifs, including light and hormone-responsive, transcriptional activator and sugar signaling, etc. were also found in *TaUNHX* (Table 2). GATA motif known to confer tissue-specific expression was also predicted in this promoter (Srivastava *et al.* 2014). The mutation in the GATA box significantly reduces the efficiency of the promoter, suggesting that it is essential for its activity. GATA family of transcriptional factors (GATA) regulates tissue-specific gene expression and recognizes the GATA consensus sequence in mammalian systems. The role of GATA motifs in the light-regulated promoters has also been reported earlier (Hatton *et al.* 1995).

Many other essential potential *cis*-acting elements identified in the present study were ARR1AT element (element involved in cytokinin responsiveness), MYBCORE element (activator of a reporter gene), PYRIMIDINEBOXHVEPB1 element (element involved in abscisic acid responsiveness), POLLEN1LELAT52 element (pollen-specific activator), NODCON2GM and OSE2ROOTNODULE element (elements responsible for nodule specificity), WBOXNTERF3 element (wound responsive), GARE and P-Box motif (gibberellin responsive element) and MYB1AT element (element involved in MYB recognition, responsive to abscisic acid signaling during dehydration stress). Dehydration, responsive element MYCCONSENSUSAT, found in this promoter, is a binding site of ICE1 (inducer of CBF expression) that regulates the transcription of CBF/DREB1 genes under cold stress in *Arabidopsis.* Another class of *cis*-acting regulatory element, i.e. CAAT box identified in the *TaUNHX* promoter, is known to increase promoter activity under heat shock (Bienz *et al.* 1986, Srivastava *et al.* 2014). DOFCOREZM, a transcription factor, was also predicted in the cloned promoter, which is a core site for binding Dof proteins with one zinc finger and unique to plants (Fig 1).

Drought and salt stress lead to an increase in ABA accumulation, which may trigger adaptive responses in plants (Bienz *et al.* 1986). The presence of either single or multiple ABREs *cis*-acting regulatory elements is sufficient to confer ABA-mediated osmotic stress tolerance (Kim *et al.* 2011, Banu *et al.* 2014). The *cis*-acting regulatory part like, G-box and ABREs elements regulate many stress-responsive genes (Liu *et al.* 2014, Wang *et al.* 2014). A G-box element, which has a significant role in plant development, response to hormone and fungal infection and light, and an active E-box element which is critical for the activity of promoters was also identified in the present study (Banu *et al.* 2014, Srivastava *et al.* 2014). Therefore, it will be advantageous to analyze the *cis*-acting elements to improve our understanding of the regulatory gene networks of the promoter in stress-responsive cascades. Previously, functional *NHX1* promoter has been cloned from *Arabidopsis*, *Nitraria sibirica* and rice (Shi and Zhu 2002, Fukuda *et al.* 2011, Wang *et al.* 2016). The upstream region of *TaUNHX* was compared with these promoter sequences using PlantCare. It was observed that few *cis*-acting regulatory elements were common among them. These were related to hormone, light, wound, defense, and stress responsiveness.

Validation of the activity of TaUNHX promoter: Agrobacterium-mediated transient expression assay has been used extensively for functional validation of plant promoters. Nearly 4-week-old tobacco leaves were used for agro-infiltration and analyzing promoter activity under various stress conditions. TaUNHX-driven *GUS* gene activity was analyzed using histochemical assay under control and different stress conditions. Blue spots because of active GUS were observed in control 1 (without stress, Fig 2), whereas no *GUS* expression was observed in the negative control (control 2, Fig 2). This suggested that the *TaUNHX* sequence drove the expression of GUS and was, therefore, functional in tobacco leaves and stems. To further analyze the activity of this promoter under various stresses, *GUS* expression was performed under salt (300 mM NaCl), ABA (100 µM ABA) and drought (20% PEG) stresses. Blue spots were visible in leaves and stem under all three stresses (Fig 2c-g). This indicated that the *TaUNHX* promoter was also active under various stresses. Previously, Shi and Zhu (2002) reported that *AtNHX1* promoter activity is enhanced by NaCl, KCl and ABA treatments, as observed in the present study. The similar pattern of expression was observed in a halophyte *Nitraria sibirica* (Wang *et al.* 2016). A similar pattern was observed with *Zmap* promoter following exposure to drought stress (Jin *et al.* 2019). Our

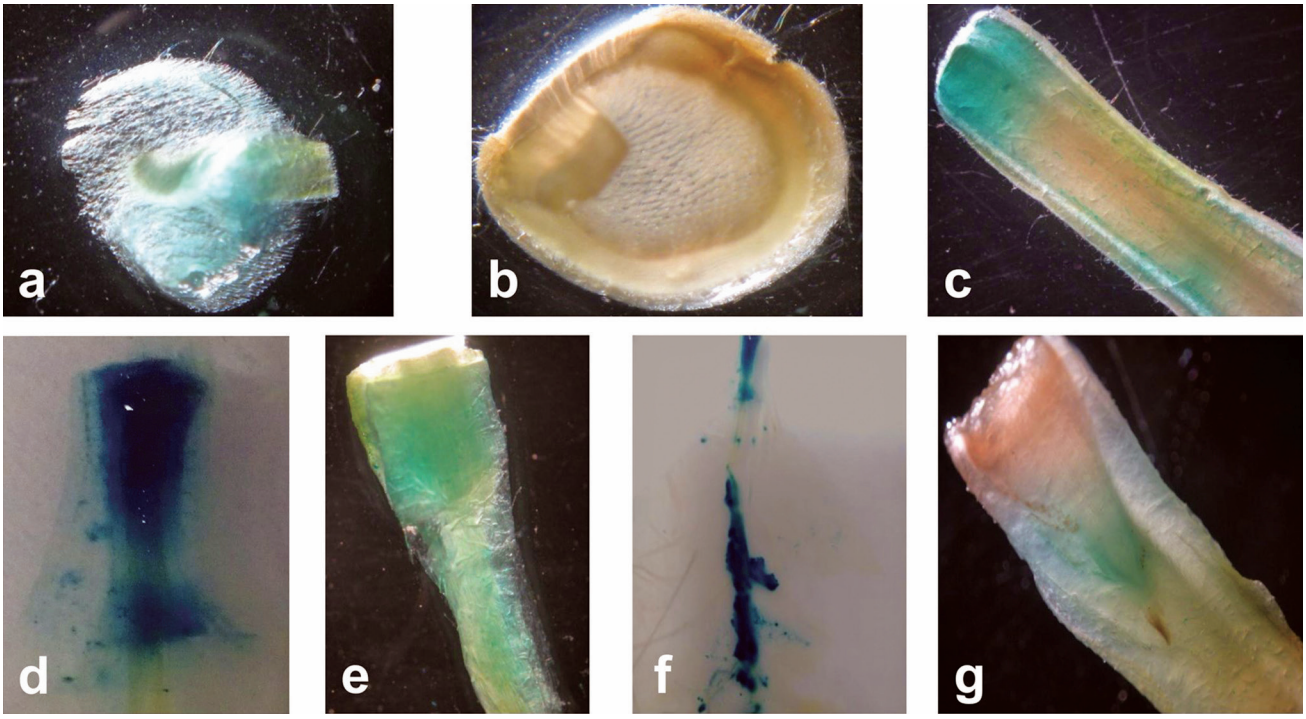

Fig 2 Microscopic view of the GUS staining of the agro-infiltrated tobacco stems and leaf base. (a), Control 1; (b) Control 2; (c-d) Transient GUS expression under salt stress; (e-f) GUS staining under drought stress; (g) *TaUNHX: GUS* GUS expression under ABA stress.

results suggest that the *cis*-acting elements identified in the promoter sequence are responsible for its activity under salt, ABA and drought stress inferring that *TaUNHX* is a stress-responsive promoter.

In conclusion, functional validation of *TaUNHX* revealed that this promoter contains many universal *cis*-acting regulatory elements, which are essential for its activity. This promoter is active under various abiotic stresses like salt, drought and ABA. Therefore, *TaUNHX* can produce stress-responsive transgene expression in crop plants.

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