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Research Article
In silico Study of Promoter Regions in Vacuole-type Sodium/ Hydrogen Exchanger Genes from Canola (*Brassica napus* L.)

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Abstract
Background and Objective: Vacuole-type NHX proteins are important for plants' ability to tolerate abiotic stress, especially salt stress. **Materials and Methods:** To gain information about the mechanisms of molecular regulation of *NHX* genes under abiotic stresses in canola (*Brassica napus* L.), *in silico* method was used to identify cis-acting regulatory elements present in 2.5 kbp upstream regions of identified vacuole-type *NHX* genes from the canola genome (*BnNHXs*). **Results:** A total of 51 cis-acting regulatory elements were identified that showed remarkable differences in frequency and site-specific distribution and they fell into five groups: Light-responsive elements, stress response, hormonal regulation, cellular development and elements with unknown function. The site-specific distribution of stress response and hormonal regulation elements indicated that they were most dense at -1600 to -1800 bp and at -800 bp, that is, far from the transcription start site. The most common motifs were the G-Box and Box 4 cis-elements, followed by the MBS, HSE and ARE motifs from the stress response group and the GARE-motif and ABRE from the hormonal regulation group. The results indicated that regulation of expression of *BnNHXs* under abiotic stresses involves TC-rich repeats, heat shock elements (HSE), LTR, anaerobic responsive element (ARE), Box-W1, MBS, CCAAT-box, ABA-responsive elements (ABRE), CGTCA-motif, TGACG-motif and ERE. **Conclusion:** This study provided information on the mechanisms by which *BnNHX* genes are regulated under abiotic stresses in canola.

Key words: Gene expression, gene regulation, phylogeny, promoter, signaling, transcription factor

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

It is estimated that there is need to increase food production[1] by 58% by 2050. Crops-especially wheat, rice, canola and maize-are crucial in food production for a billion people around the world, but a variety of biotic and abiotic stresses threaten the safety of food production[2]. Abiotic stresses inhibit crop growth mainly by creating an osmotic effect and leading to the accumulation of toxic ions in the plant cell[3]. For many years, scientists have been seeking to improve crop tolerance under, but lack of understanding of the complex molecular basis of plant responses stresses has limited progress in this field.

Various studies have demonstrated many cis-acting elements, for example, ABRE[4], CE1[5], CE3[6], MYBR[7], MYCR[8], DRE[9], CTR[10], LTRE[11], NACR[12], ZFHDR[12], ICEr1[13] and ICEr2[13]. The ABRE and DRE/CRT are two major elements found at the promoter regions of stress-induced genes, being involved in ABA-dependent and ABA-independent gene expression pathways[14]. In recent decades, advances in DNA sequencing technology have created various genome-scale data sets that provide an opportunity for analysis[15]. Several computer programs have been developed to analyze cis-acting elements in plants; for example PLACE[16], PlantCARE[17], AGRIS[18], TRANSFAC[19] and Plant PAN[20]. Using these platforms, several studies have successfully been conducted to analyze the promoter regions and their regulatory elements of genes responsive to various stimuli. Huang and Wu[21] used computational approaches based on ABRE to identify ABA-regulated genes throughout the genome, they identified 137 ABA-regulated candidate genes and confirmed their results by reverse transcription polymerase chain reaction (RT-PCR). Gomez-Porras *et al.*[22] performed analyzed two ABA-responsive cis-elements in *Arabidopsis* and rice using an *in silico* method and found that two elements, ABRE and CE3, show distinctive patterns in these plants. Li *et al.*[23] presented a computational method using cis-regulatory motifs to identify osmotic stress-responsive genes in *Arabidopsis*. They used known cis-regulatory elements to train an artificial neural network (ANN) algorithm and confirmed the efficiency of this method using RT-PCR. *In silico* methods have been used to demonstrate that the TFs DREB1/CBF, prominent in responding to cold stress, use combinations of cis-regulatory motifs for governing sets of cold-stress responsive genes[24]. In *Arabidopsis* and rice, computational based analysis in sucrose transporter gene families has revealed that the cis-regulatory elements associated with plant development, plant hormonal regulation and stress response are involved in regulating these gene families[25]. In another study, an *in silico* study of cis-acting elements to analyze abiotic-stress responsive genes in the chloroplast genome revealed important cis-element involved in responding to stresses[26]. Kaur *et al.*[27] investigated the promoter regions of pathogenesis-related genes using computational approaches. They showed that CpG islands are more numerous in monocots and found a high frequency of cis-elements involved in the response to stress and hormonal regulation. In the pea, an *in silico* analysis of DNA helicase 45-a high-salinity responsive gene-showed that the cis-regulatory elements ABRE, MBS, G-box, GARE-motif and TGA-element are present at the 5'-UTR sequence of the gene. These studies show that *in silico* tools can be effective for the analysis and characterization of cis-acting regulatory elements under the influence of specific conditions.

Among the main plant transporter proteins responsible for resistance to biotic and abiotic stresses are NHX-type Na^+/H^+ exchanger proteins, also known as sodium/hydrogen (Na^+/H^+) antiporter proteins. The NHXs are important in detoxification of the cell from excessive Na^+ via sequestration of sodium within the vacuole and export of sodium from the cell[28]. They are grouped into three categories based on subcellular localization: Plasma membrane class, endosomal class and vacuole class[28]. It has been demonstrated that NHXs are involved in salt stress response[29,30], ion and pH hemostasis[31], potassium hemostasis[32] and cellular vesicle trafficking[32]. Several recent studies have reported that cloning one of these genes confers more tolerance to salt stress, for example in wheat[33], tobacco[34], tomato[35], poplar[36] and cotton[37].

Canola, *B. napus* is well-known for its vegetable oil and is widely cultivated worldwide[38]. It is of economic importance and can be used for biodiesel production[39]. Abiotic stresses restrict canola cultivation and lower its growth and performance[40-43]. Therefore, engineering canola to be more stress tolerance is essential to enable this plant to produce a high yield to meet growing demands. However, engineering plants requires comprehensive information about the molecular mechanisms of gene regulation in response to environmental stresses. Given the importance of canola, the key role of *NHX* genes in tolerating stresses and advances in genome sequencing of *B. napus*, computational based methods were used to systematically study the gene structure, regulatory regions, protein motifs and phylogenetic relationship of *NHX* genes in *B. napus*, with a focus on identifying cis-acting regulatory elements involved in responding to a biotic stresses. There has been little or no analysis of cis-elements of vacuole-type *NHX* genes incanola. It is expected that our findings will shed light on the regulatory mechanisms at transcription level by which the *NHX* genes from canola are expressed under abiotic stresses.

MATERIALS AND METHODS

Genome-wide identification of vacuole-type *NHX* genes from canola (*Brassica napus* L.) genome and structure analysis: To extract the *NHX* gene sequences, *AtNHX1* to *AtNHX4* gene sequences of *Arabidopsis thaliana* encoding vacuole-type Na^+/H^+ exchanger proteins were retrieved from The Arabidopsis Information Resource (https://www.arabidopsis.org/) and blasted against the *B. napus* genome (https://www.ncbi.nlm.nih.gov/genome/?term=brassica%20napus). The chromosomal regions in the *B. napus* genome operating as a Na^+/H^+ exchanger were extracted and saved in the FASTA format. The Nucleotide Database (https://www.ncbi.nlm.nih.gov/nucleotide?cmd=search) was searched to locate the locus of the genome, chromosome and positions on the chromosomes for the identified *NHX* genes. The genomic sequences identified as putative vacuole-type *NHX* genes were structurally analyzed using GENESCAN (http://genes.mit.edu/GENSCAN.html) for the coding sequence (CDS), exon and intron arrangement. The IBS v.1.0 was used to illustrate sequences, domains and other structures (http://ibs.biocuckoo.org/).

Motif characterization and phylogenetic analysis of identified vacuole-type NHX proteins: Genomic sequences identified from the *B. napus* genome were blasted (BLASTx) against non-redundant protein sequences (nr) to find protein sequences predicted for the identified *B. napus NHX* genes. The candidate NHX sequences were confirmed by a cut off more than 10^{40} for E-value and scanning for the presence of trans membrane helices (TMHs) using TMHHM v.2.0 (http://www.cbs.dtu.dk/services/TMHMM/).

To confirm vacuole-type protein sequences, the amino acid sequences of NHX-type Na^+/H^+ exchanger proteins from 11 species[44] were retrieved and used to construct a phylogenetic tree. The NHX protein sequences of *B. napus* and other species were aligned using Clustal X2.1[45]. The PhyML v.3.0[46] was used to construct a phylogenetic tree with 1000 bootstrap replicates and the maximum likelihood method. Species used to construct phylogenetic tree were *A. thaliana*, *Eucalyptus grandis*, *Medicagotruncatula*, *Vitisvinifera*, *Glycine max*, *monocotyledonous*, *Oryza sativa*, *Sorghum bicolor*, *Brachypodiumdistachyon*, *Zea mays*, *Physcomitrella patens* and *Populustrichocarpa*.

Prediction of promoter regions and analysis of cis-acting regulatory elements: Using genomic sequences of *NHX* genes found in the *B. napus* genome, a 2.5 kbp upstream region relative to the translation start site (ATG) was extracted for each identified gene. The translation start site (ATG) was specified in the *B. napus* genomic sequence for annotated Na^+/H^+ exchanger genes at https://www.ncbi.nlm.nih.gov/genome/?term=brassica%20napus. The TSS Plant[47] was used to predict core and proximal promoter regions in a 2.5 kbp upstream region of the identified *NHX* genes and PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) was used to find putative cis-acting regulatory elements. Because of the importance of accuracy in determining core promoter regions, DAMBE was used to calculate GC skew for 2.5 kbp upstream regions[48], to further confirm the TSS Plant results. Window size and step size were set to 100 and 1 bp, respectively.

RESULTS

Genome-wide identification of vacuole-type *NHX* genes from *Brassica napus* genome, chromosomal distribution and gene structure analysis: To identify the vacuole-type *B. napus NHX* genes, vacuole-type *AtNHX* gene sequences were used from *A. thaliana* to perform BLASTn against the canola genome. The four *AtNHX* genes from *Arabidopsis* (*AtNHX8*, *AtNHX2*, *AtNHX1* and *AtNHX3*) hit homology (i.e., showed >70% homology) with five loci from the canola genome. These loci were named *BnNHX1* to *BnMHX5* for the low side NC_027758.2, NC_027761.2, NC_027767.2, NC_027771.2 and NC_027773.2, respectively. Genomic length of identified putative *NHX* genes (*BnNHXs*) varied from 4076 bp in *BaNHX1* to 4460 bp in *BaNHX5* (Table 1).

The *BnNHXs* distributed among five chromosomes-including A2, A5, C1, C5 and C7. The *BnNHX1, 2, 4* and *5*-were near the end of the chromosome, while *BnNHX3* was near the middle regions of the chromosomes (Fig. 1a).

Gene structure analysis showed that *BnNHX1*, 2, 3, 4 and 5 had 12, 11, 11, 6 and 9 introns, respectively. Intron phases were similar in all identified genes. Also, the exon length showed similar variation among the genes, varying from 46-329 bp in each gene (Fig. 1b).

Phylogenetic and motif analysis of the BnNHX proteins: To demonstrate function and the evolutionarily relationship of *BnNHXs* identified in the canola genome, first the presence of TMH in BnNHX proteins was analyzed. The TMH was 10 in BnNHX3 and 12 in the remaining BnNHX proteins. All BnNHX proteins possessed NhaP-type Na^{+}/H^+ and K^{+}/H^+ antiporters, with C-terminal TrkAC and CorC domains. Phylogenetic analysis of BnNHX proteins with NHX protein families from 11 species (92 sequences) indicated that the BnNHX proteins were grouped in vacuole-type Na^+/H^+ exchanger proteins. The

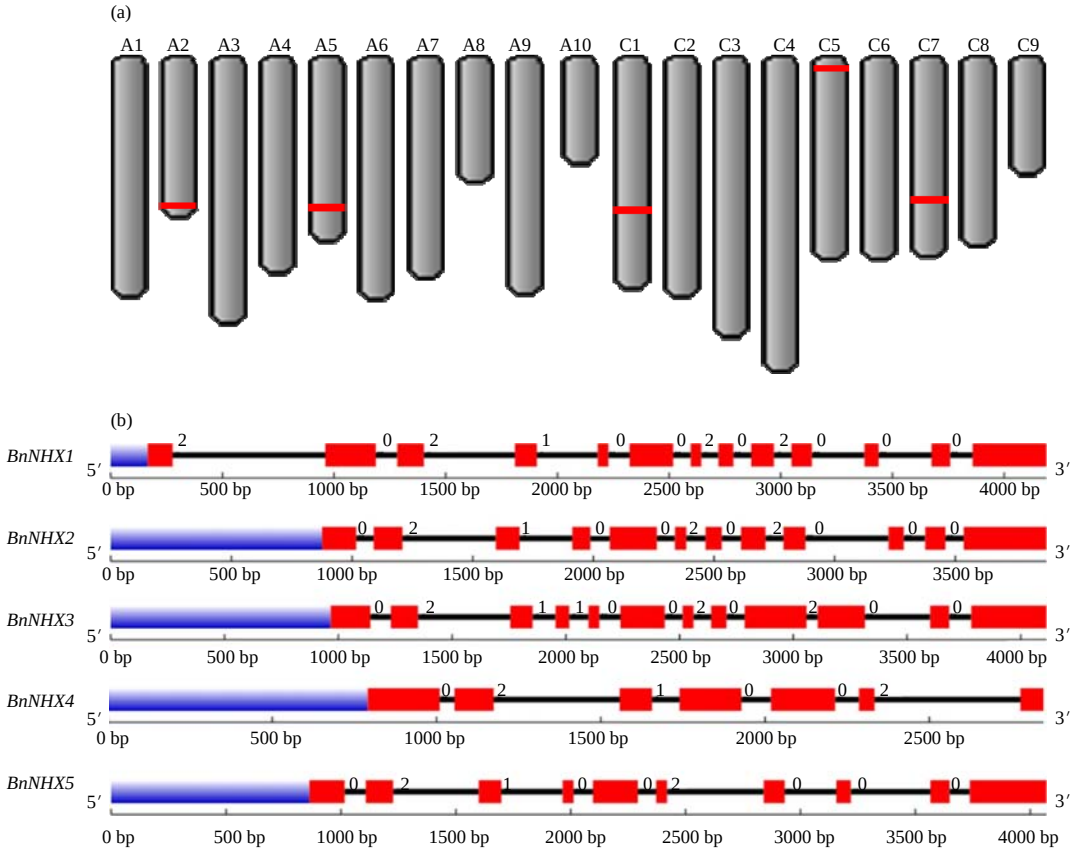

Fig. 1(a-b): Map and structure of *NHX* genes found in *Brassica napus* genome (*BnNHX1* to *BnNHX5*). (a) Location of sodium/hydrogen exchanger genes on *Brassica napus* chromosomes and (b) Exon-intron arrangement of sodium/hydrogen exchanger genes identified in *Brassica napus* genome. Blue and red rectangles depict the up and downstream region and exon, respectively. Lines between exons indicate introns and numbers (0, 1 and 2) indicate the intron phase

Table 1: Blast results for sodium/hydrogen exchanger genes identified in the *Brassica napus* genome

Locus on the genome	Location on chromosome	Position on chromosome	Assigned name	Number of base pairs (genomic sequence)	AtNHX8 (AT1G14660.1) homology to the chromosome region	AtNHX2 (AT3G05030.1) homology to the chromosome region (%)	AtNHX1 (AT5G27150.1) homology to the chromosome region (%)	AtNHX3 (AT5G55470.1) homology to the chromosome region
NC_027758.2	A2	31454014-31457198	*BaNHX1*	4460	Not found	75	81	79%
NC_027761.2	A5	30810495-30813537	*BaNHX2*	4087	Not found	80	75	Not found
NC_027767.2	C1	29429401-29432554	*BaNHX3*	4397	Not found	74	81	Not found
NC_027771.2	C5	43507604-43510623	*BaNHX4*	4076	84%	79	75	Not found
NC_027773.2	C7	26173784-26176990	*BaNHX5*	4345	Not found	75	78	79%

results of phylogenetic analysis confirmed that putative *NHX* genes identified in canola (*BnNHXs*) are for vacuole-type Na^+/H^+ exchangers. The NHX-type Na^+/H^+ exchanger proteins could be divided into three groups, based on their location: vacuole-type Na^+/H^+ exchanger, endosomal-type type Na^+/H^+ exchanger and plasma membrane-type Na^+/H^+ exchanger. Most of the NHX proteins were located in vacuole-type Na^+/H^+ exchanger proteins (Fig. 2, 3).

Prediction of the promoter region and analysis of cis-acting regulatory elements: Upstream regions up to 2.5 kbp from the translation start site, marked as ATG, in each BnNHX were analyzed to determine the core promoter (-60 to +40 bp in relation to the transcription start site) and proximal promoter regions (-300 bp in relation to the transcription start site). These promoter regions spanned -466 to -806, -237 to -577, -483 to -823, -1234 to -1574 and -752 to -1092 for *BnNHX1*-5,

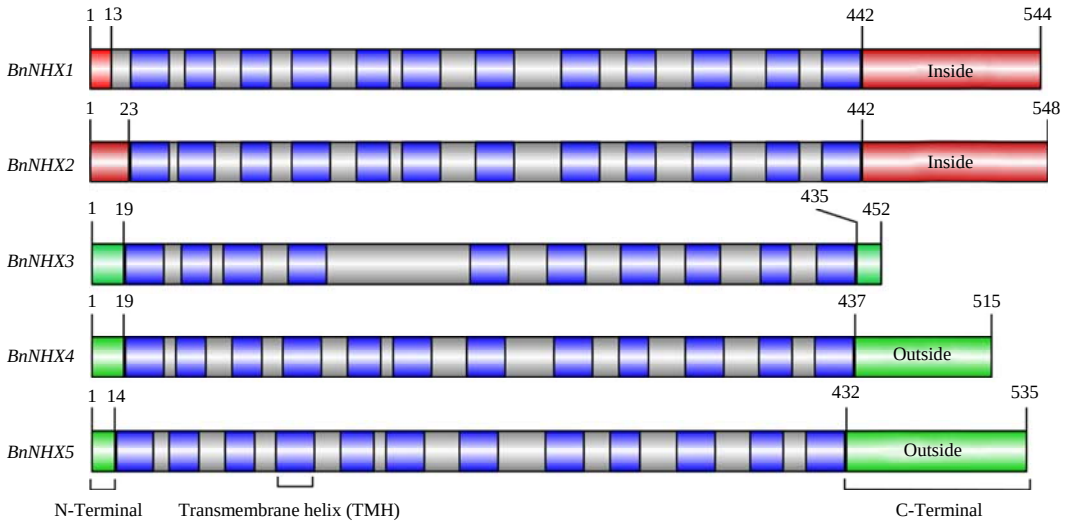

Fig. 2: Locations and number of transmembrane helices in *BnNHX*-vacuole-type proteins

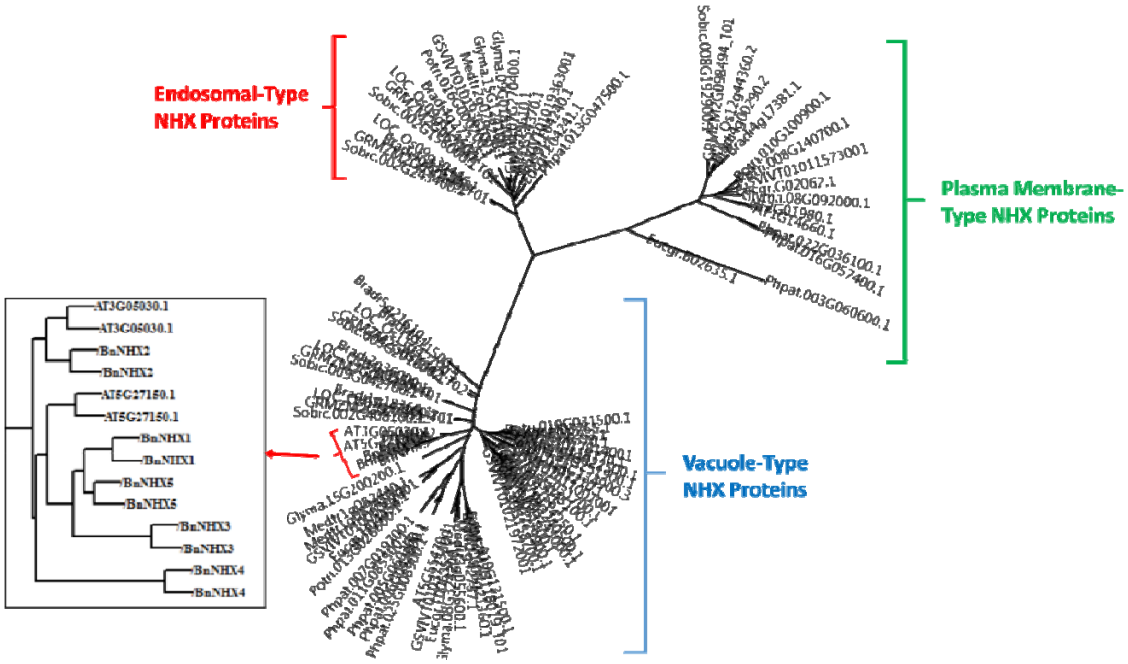

Fig. 3: Phylogenetic analysis of *BnNHX* genes identified in canola (*Brassica napus*). The NHX members from 11 species were *Arabidopsis thaliana*, *Eucalyptus grandis*, *Medicago truncatula*, *Vitis vinifera*, *Glycine max*, *Monocotyledonous*, *Oryza sativa*, *Sorghum bicolor*, *Brachypodium distachyon*, *Zea mays*, *Physcomitrella patens* and *Populus trichocarpa*. The red arrow indicates the position of *BnNHX* on phylogenetic tree

respectively. The *BnNHXs* showed a TATA-box, core promoter element at -473, -256, -509, -1240 and -820 and a CAAT-box common element in core promoter region at -454, -351, -648, -1408 and -975 (Fig. 4). GC-skew was calculated for further confirmation of the identified promoter regions. The GC skew was significant around the transcription start site and declined immediately after that site.

After determining the promoter region at 2.5 kbp upstream of the genes, those sequences were scanned to identify regulatory motifs. No CpG island was detected in those regions, meaning there is a low probability of epigenetic effects in the regulation of these genes. However, many cis-acting regulatory elements were detected in the 2.5 kbp upstream regions in *BnNHXs*. The position-specific distribution

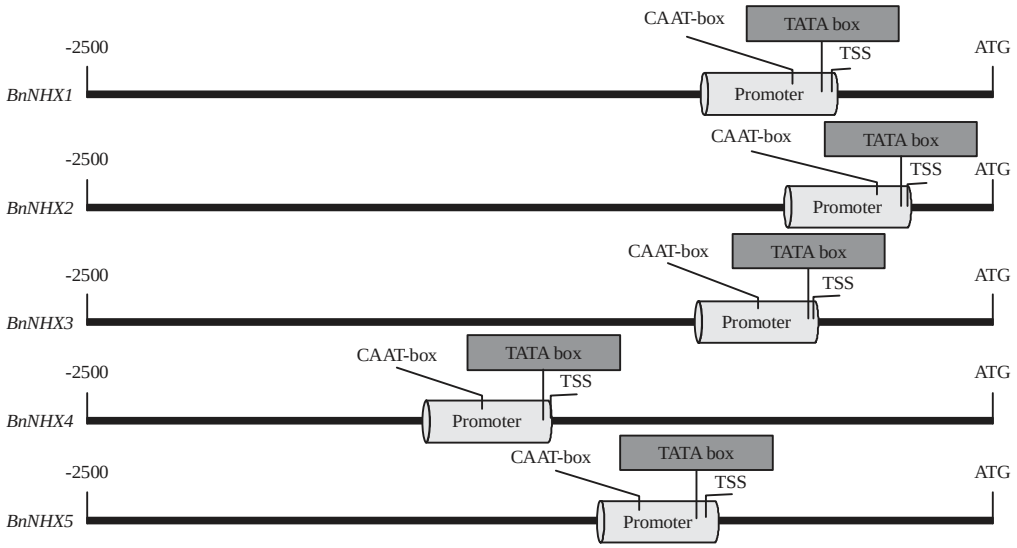

Fig. 4: Promoter regions (core and proximal regions) found at 2.5 kbp upstream regions of *BnNHX* genes

of cis-acting regulatory elements indicated that the frequency and density of the motifs varied among *BnNHX* genes. In the *BnNHX1* and *BnNHX2* genes, there were high-density regions around -100, -300, -1300 and -1700 relative to the transcription start site. In the *BnNHX3* genes there was a higher frequency of cis-acting regulatory elements around the -200, -1200 and -1600 bp upstream regions relative to the transcription start site. The upstream region of the *BnNHX4* gene showed a high frequency compared to the *BnNHX1-3* genes, meanwhile, all motifs were had a similar frequency of distribution between ATG to -2500 bp upstream. In the *BnNHX5* gene, the regions around +700, +500, -600 and -1300 indicated a high number of cis-acting regulatory elements (Fig. 5).

A total of 51 cis-acting regulatory elements were identified in *BnNHX* genes (Table 2). These elements were functionally categorized into five groups: Light-responsive elements, stress response, cellular development, hormonal regulation and elements with unknown function. The light-responsiveness group included cis-acting regulatory elements involved in the regulation of light responsiveness, through elements and modules (or parts of elements and modules). The stress response group included cis-acting elements involved in defense and stress responsiveness, heat-stress responsiveness, low-temperature responsiveness and anaerobic induction; it also involved a fungal elicitor responsive element, an MYB binding site involved in drought inducibility and aMYBHv1 binding site. The hormonal regulation group included cis-acting elements involved in responsiveness to auxins, salicylic acid, ethylene and gibberellin. The cellular development group includes motifs involved in the binding site of the AT-rich DNA binding protein (ATBP-1), cis-acting elements conferring high transcription levels and involved in cell cycle regulation, cis-acting regulatory elements involved in circadian control, zein metabolism and meristem-specific activation and an elicitor-responsive element, enhancer and cis-acting regulatory element required for endosperm expression (Fig. 6a). In total, motifs with a relatively high frequency were G-Box and Box 4 with 31 and 21 frequencies from the light-responsive group, HSE and MBS with 16 frequencies and ARE with 14 frequencies from the stress responsive group, GARE-motif with 12 frequencies and ABRE with 11 frequencies from the hormonal regulation group and Skn-1-motif and 5UTR Py-rich stretch with 11 frequencies from the cellular development group (Fig. 6b-e).

After the light-responsive group, the stress-responsive group, at 21%, constituted the major part of all identified cis-acting regulatory elements and the hormonal-regulation group, at 15% was the next highest. Stress response motifs included TC-rich repeats, HSE, low temperature response (LTR), ARE, Box-W1, MBS and CCAAT-box (Fig. 6b). Hormonal regulation motifs included TGA-element, TCA-element, ABRE, CGTCA-motif, CGTCA-motif, TGACG-motif, ethylene responsive element (ERE), P-box and GARE-motif (Fig. 6d). Site-specific distribution of stress-responsive motifs indicated that these motifs were more frequent at -1600 to -1800 bp from the transcription start site. Hormonal-regulated motifs were more frequent at -800 and -1600 to -1700 bp from the transcription start site (Fig. 7).

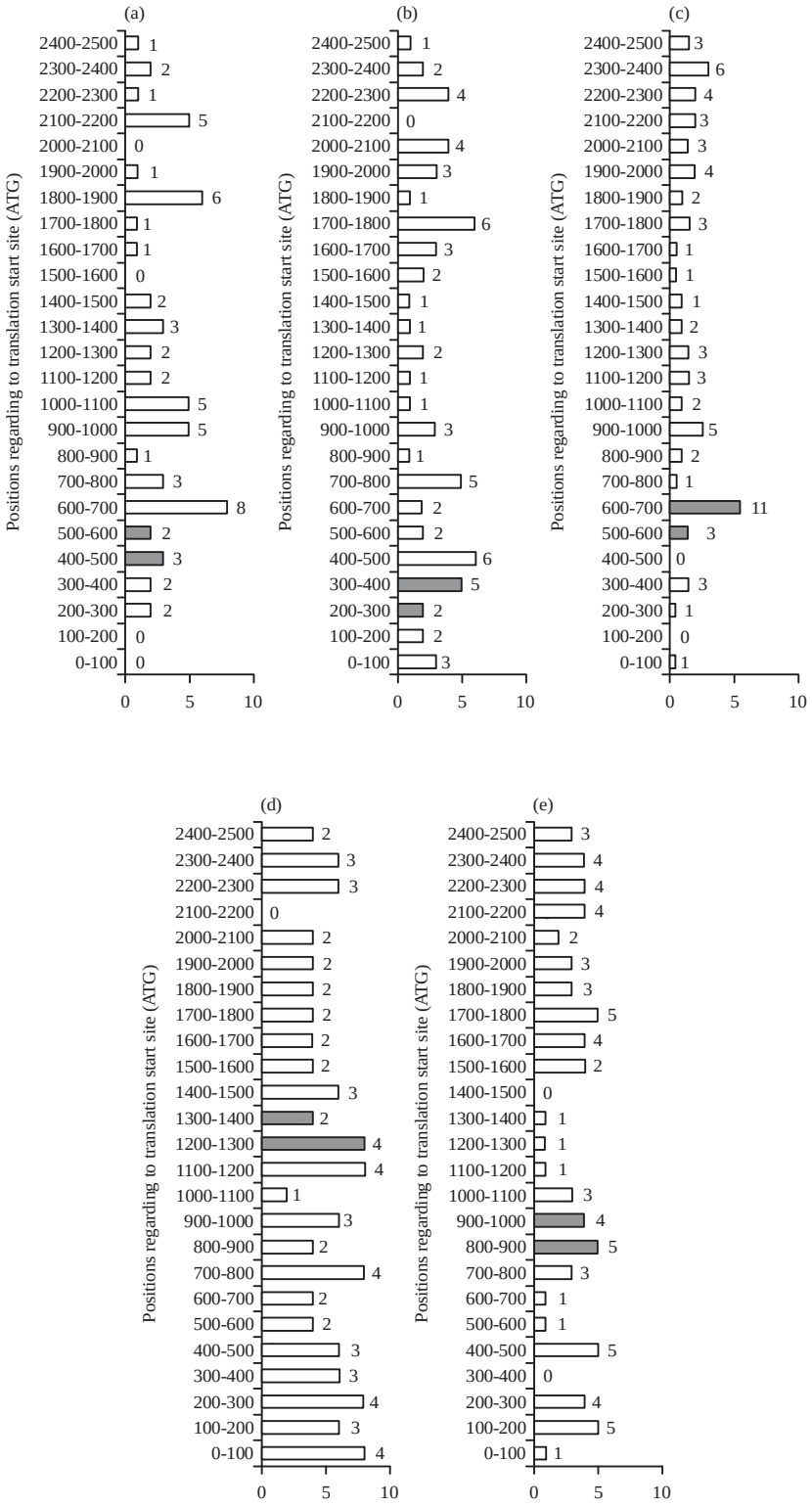

Fig. 5(a-e): Position-specific distribution of cis-acting regulatory elements found at 2.5 kbp upstream regions of *BnNHX* genes. (a) *BnNHX1*, (b) *BnNHX2*, (c) *BnNHX3*, (d) *BnNHX4* and (e) *BnNHX5*

Yellow bars indicate proximal and core promoter region

Table 2: Collection of cis-acting regulatory elements found at 2.5 kbp upstream of *BnNHX* genes in canola (*Brassica napus*)

Cis-acting regulatory element	Sequences	Functions	Position regarding to ATG
AT-rich element	ATAGAAATCAA	Binding site of AT-rich DNA binding protein (ATBP-1)	-960
5UTR Py-rich stretch	TTTCTTCTCT	Cis-acting element conferring high transcription levels	-600 to -1500
MSA-like	(T/C)C(T/C)AACGG(T/C)(T/C)A	Cis-acting element involved in cell cycle regulation	-2332
Circadian	CAAATTAATC	Cis-acting regulatory element involved in circadian control	-1261 to -2190
O2-site	GGTAGAGTAG	Cis-acting regulatory element involved in zein metabolism regulation	-820 to -1780
CCGTCC-box	CCGTCC,CCGTCC	Cis-acting regulatory element related to meristem specific activation	-920
EIRE	TTCGACC	Elicitor-responsive element	-1063
TA-rich region	TATATATATATATATATATATA	Enhancer	-1222
Skn-1-motif	GTCAT	Cis-acting regulatory element required for endosperm expression	-1222
TGA-element	AACGAC	Auxin-responsive element	-830 to -1992
TCA-element	GAGAAGAATA	Cis-acting element involved in salicylic acid responsiveness	-600 to -1000
ABRE	CACGTG	Cis-acting element involved in the abscisic acid responsiveness	-700 to -2000
CGTCA-motif	CGTCA	Cis-acting regulatory element involved in the MeJA-responsiveness	-1700 to -2400
TGACG-motif	GCAGT	Cis-acting regulatory element involved in the MeJA-responsiveness	-1700 to -2200
ERE	ATTTCAAA	Ethylene-responsive element	-270 to -700 and -1800 to -2200
P-box	GCCTTTTGAGT	Gibberellin-responsive element	-801 to -1937
GARE-motif	AGACAAA	Gibberellin-responsive element	-200 to -800 and -1800 to -2100
ACE	ACGTGGA	Cis-acting element involved in light responsiveness	-32 to -900 and -2000 to -2200
G-Box	CACGTC	Cis-acting regulatory element involved in light responsiveness	-900 to -2300
4cl-CMA2b	TCTCACCAACC	Light responsive element	-620
3-AF1 binding site	AAGAGATATTT	Light responsive element	-40 to -100 and -1200
GT1-motif	GGTTAA	Light responsive element	-80 to -600 and -1700 to 2500
Box I	AAA CTTT	Light responsive element	-250 to -700 and -1800 to -2200
Sp1	CCTCCCTCT	Light responsive element	-600 to -1200
As-2-Box	GATAatGATG	Light responsiveness	309-2073
ATCC-motif	CAATCCTC	Part of a conserved DNA module involved in light responsiveness	1806-
ATC-motif	AGTAATCT	Part of a conserved DNA module involved in light responsiveness	971-50
ATCT-motif	AATCTAATCT	Part of a conserved DNA module involved in light responsiveness	-500 to -2500
Box 4	ATTAAT	Part of a conserved DNA module involved in light responsiveness	-400 to -2000
TCCC-motif	TCTCCCT	Part of a light responsive element	-771
L-Box	AACCAACC ACTCT	Part of a light responsive element	-631
CATT-motif	GCATTC,GCATTC	Part of a light responsive element	-432 to -1955
GA-motif	ATAGATAA	Part of a light responsive element	-200 to -2000
I-box	aAGATAAGA	Part of a light responsive element	-178 to -328
GAG-motif	GGAGATG	Part of a light responsive element	-300 to -2400
TCT-motif	TCTTAC	Part of a light responsive element	-500 to -2000
AT1-motif	ATTAATTTTACA	Part of a light responsive module	-1000 to -1300
AE-box	AGAAACAT	Part of a module for light response	-200 to -2500
TC-rich repeats	AATT CTTTTG	Cis-acting element involved in defense and stress responsiveness	-380, -823 and -1600 to -2300
HSE	AAAAAATTTC	Cis-acting element involved in heat stress responsiveness	-200 to 800 and -1500 to -2300
LTR	CCGAAA	Cis-acting element involved in low-temperature responsiveness	-674 and -1500 to -2500
ARE	TGGTTT	Cis-acting regulatory element essential for the anaerobic induction	-300 to -500 and -1100 to -2300
Box-W1	TTGACC	Fungal elicitor responsive element	-447 and -960
MBS	GTCAAT	MYB binding site involved in drought-inducibility	-960 and -1600 to -2000
CCAAT-box	CAACGG	MYBHv1 binding site	-306, -1003, -1823, -1734 and -2335
TATCCAT/C-motif	TATCCAT	Unknown	-2257
AC-I	TCTCACCAACC	Unknown	-633
Box E	ACCCATCAAG	Unknown	-660
CTAG-motif	ACTAGCAGAA	Unknown	-107, -977 and -1165
W box	TTGACC	Unknown	-450 to -950
AAGAA-motif	gGTAAGAA	Unknown	-90 to 700 and -1300 and -1900

DISCUSSION

In this study of canola, a total of five vacuole-type *BnNHX* genes were found and characterized. The gene length was about 4kbp; the CDS length showed a similar pattern in all *BnNHX* genes but the CDS number differed among the genes (Fig. 1b). These genes encode BnNHX proteins varying from 452 amino acids (aa) to 548 aa in length and containing 10 TMHs in BnNHX3 and 12 TMHs in the remaining BnNHX proteins (Fig. 2). These proteins were phylogenetically clustered in the same cluster of vacuole-type Na^+/H^+ transporter proteins, along with five *NHX* genes from *Arabidopsis*, poplar, common grape wine, stiff brome and *Physcomitrella*, whereas, other species showed four, six and eight *NHX* genes in vacuole-type clusters (Fig. 3).

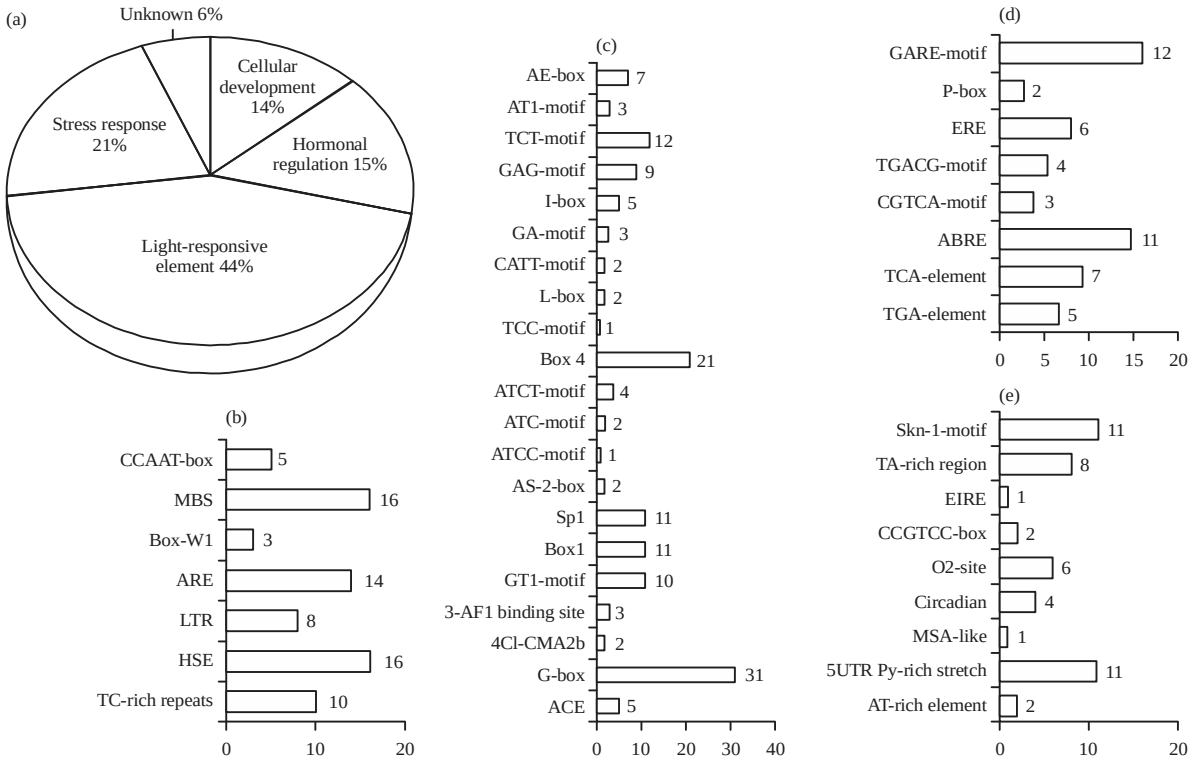

Fig. 6(a-e): Pie distribution and frequency of identified motifs in 2.5 kbp upstream region of *BnNHXs* in canola (*Brassica napus*), (a) Functional categorization, (b) Frequency of stress-responsive elements, (c) Frequency of light-responsive elements, (d) Frequency of motifs involved in hormonal-regulation elements and (e) Frequency of motifs involved in cellular development

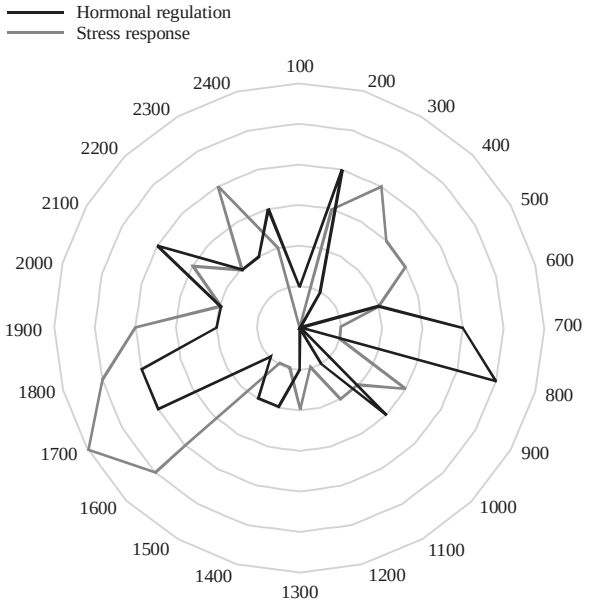

Fig. 7: Site-specific distribution of cis-acting regulatory elements grouped in the stress response and hormonal regulation group across the 2.5 kbp upstream regions of *BnNHX* genes

Under abiotic stress, vacuole-type Na^+/H^+ exchangers are mostly induced, particularly in root cells where they function to sequester excessive sodium into vacuole. The NHX-type Na^+/H^+ exchangers, especially vacuole-type ones, have been shown to be one of the most important responsive proteins to abiotic stresses, especially salt stress[49-52]. In Arabidopsis, the *AtNHX1* gene (AT5G27150.1) is located on chromosome five and has a length of 4259 bp; it encodes a vacuolar Na^+/H^+ anti-porter with 12 TMHs that mediates transport of sodium and potassium into the vacuole. Plants transformed with the *AtNHX1* gene have shown significant tolerance[50-53]. Additionally, various plant species have shown over sensitivity and remarkable growth reduction when their *AtNHX1* gene have been knocked out[54,55]. The *AtNHX2* (AT3G05030) is located on chromosome three with length of 4486 bp encodes vacuole-type proteins with 12 TMHs. This transporter is essential to uptake potassium into vacuole. The *AtNHX3* (AT5G55470) has a length of 346 bp and is located on chromosome five and *AtNHX4* has a length of 4128 bp and is located on chromosome three[56,57]. In poplar, the *PtNHX1-5* genes have a gene length of 4000-5000 bp and encode PtNHX proteins with 10 TMHs[44]. In *Physcomitrella*, the PpNHX1-5

genes, in *Oryza sativa* the *OsNHX1-4* genes and in *Zea mays* the *ZmNHX1*-4 genes all have a different genomic length and encode vacuole-type Na^+/H^+ exchanger proteins with lengths of 450-750 aa[49]. In spite of some differences in the length of genes (<500 bp) and the Na^+/H^+ exchanger proteins, the NhaP-type Na^+/H^+ and K^+/H^+ antiporter domain was common among vacuole-type Na^+/H^+ exchanger proteins. Furthermore, the number of TMHs was the same in canola and the studied plants. The results implied that vacuole-type Na^+/H^+ exchanger proteins have been conserved during evolution.

All *BnNHXs* were found to have the TATA-promoter located at a different distance from the translation start site (ATG) in each gene. Moreover, all *BnNHX* genes showed CAAT-box in the promoter region (Fig. 4). The CAAT-box acts as a binding site for the RNA transcription factor NF-Y and is an essential element for initiating transcription in the genes harboring this element. It seems that this element is present in the regulatory promoter[58]. Promoter regions identified further were confirmed by GC-compositional strand bias (GC-skew=(C-G)/(C+G)). It has been demonstrated that GC-skew around the transcription start site shows the specific model. In *Arabidopsis* and rice, GC-skew was significant in the transcription start site of genes and declined after that site[59]. In this study, the significant GC-skew around the transcription start site for the *BnNHX* genes identified in the canola genome.

To explore the molecular mechanisms behind vacuole-type *BnNHX* genes in responding to abiotic stresses, a pattern recognition program was used to identify cis-acting regulatory elements at the 2.5 kbp upstream region relative to the translation start site. Eukaryotic gene expression is governed mainly by TFs binding to specific conserved patterns on the regulatory regions of the gene. High-throughput experimental works and bioinformatic tools now provide a rapid and reliable way to study regulatory mechanisms. In our study, the 2.5 kbp upstream of vacuole-type *BnNHX* genes analyzed by scanning those regions for the presence of cis-acting regulatory elements and discussed those elements in the light of other studies. Because our focus was on *BnNHXs* in responding to abiotic stress, we looked at stress-responsive elements as well as hormone-responsive elements involved in responding to the stresses. These results could not show which mediating molecules perform the sensing and cascade events that lead to activation or suppression of the TFs that bind to the regulatory regions of *BnNHX* genes. However, the identified *BnNHX* genes appeared to contain various cis-acting regulatory elements, suggesting that the *BnNHX* genes are regulated by various signaling pathways. Identification of cis-acting elements is an important step toward understanding the regulatory mechanisms of gene expression. The cis-acting regulatory elements as being responsible for regulation of vacuole-type *BnNHX* genes were identified under abiotic stresses.

The stress-responsive group constituted the second highest frequency of elements, mainly distributed at -1600 to -1800 bp, far from the transcription start site (Fig. 6, 7). In this group, HSE, MBS and ARE motifs showed high frequency. Heat shock elements (HSEs) containing a AGAAnnTTCT sequence lie on the regulatory regions of myriad genes that are responsive to temperature stress. Heat stress TFs (HsFs) are TFs that bind to HSE, mediating the response to heat stress. The HsFs are also induced under salinity and drought stress[60]. Over expression of HsFs has been shown to confer more tolerance to salt and drought stress[61,62]. The MBS is a drought-inducible cis-acting element that acts as a binding site for MYB transcription factor, which in turn have shown expression changes under salinity in different plants, suggesting their responsiveness to salt stress[63,64]. The ARE is responsible for gene expression under anaerobic conditions. It has suggested that, in the absence of hypoxia conditions, this motif is required in responding to cold and dehydration[65]. However, we found no reports of the presence of this motif in promoter regions of salt-responsive genes. A TC-rich repeat is involved in defense and stress responsiveness; it has been found at the promoter of *osa-MIR396c* salt responsive transcript[66], the transcription factor gene *TaMYB33* responsive to salt, drought and abscisic acid[67] and copper-containing amine oxidase genes[68]. The LTR motif is specifically involved in responding to low temperature, but has also been found at promoter regions of salt-responsive genes[69]. The CCAAT-box provides a binding site for MYBHv1. This cis-element has been found at the promoter regions of DoGMP1 from *Dendrobium officinale*. The DoGMP1 contributes to the response to salt stress[70]. The fungal elicitor responsive element, Box-W1, was found at a low frequency at the promoter region and Manimaran *et al.*[71] reported that this cis-acting element is present in the 1.5 kb promoter region of *OsNF-YC13* that is involved in responding to salt stress. These findings indicate that a few of the cis-elements kind provides the responsiveness of the *BnNHXs* to various abiotic stresses, including heat, low temperature, salt, dehydration and anaerobic conditions.

Motifs involved in hormonal regulation constituted the third most frequent group of cis-acting elements (Fig. 6). In this group, the TGA-element was responsive to auxins, the TCA-element was responsive to salicylic acid, the ABRE was responsive to abscisic acid (ABA), the CGTCA-motif and TGACG-motif were responsive to methyl jasmonate, the ERE

was responsive to ethylene and P-box along with the GARE-motif were responsive to gibberellin. In this group, the GARE-motif and ABRE showed a high frequency in the promoter regions. Plant hormones, along with other signaling pathways such as phospholipids and calcium ions are vital in appropriate and integrative response to stresses[72]. The ABA, methyl jasmonate and ethylene have been proposed as factors regulating the adaptive responses to abiotic stresses, while auxin, salicylic acid and gibberellin are thought to be involved in growth and development[72]. The ABRE was found at a high frequency after the GARE-motif (Fig. 6). This motif has been shown to be involved in the ABA-dependent expression of genes under stressful conditions[73,74]. The genes harboring ERE are regulated in the presence of ethylene. It has been suggested that, under salt stress, ethylene modulates salt-responsive gene expression (e.g., *AtERF4*, *Cor6.6*, *rd17*, *RD21A* and *VSP2*, which are up-regulated and *BBC1*, *Lea* and *AtNAC2*, which are down-regulated)[75-77]. The presence of CGTCA- and TGACG-motif implied that methyl jasmonate may have a role in regulating the *BnNHX* genes. A high level of methyl jasmonate was reported in salt-tolerant rice[78]. Some reports indicated crosstalk between ABA and methyl jasmonate at MYC2 transcription factor[79,80], a factor that is involved in regulation of gene expression under salt stress[81]. Thus, the presence of cis-elements relating to ABA, ethylene and methyl jasmonate imply these hormone have roles in vacuole-type *BnNHXs* in response to abiotic stresses.

CONCLUSION

Vacuole-type Na^+/H^+ exchanger proteins are important in plants' tolerance of salt stress. In this study, five *BnNHX* genes from canola (*B. napus*) were identified and analyzed for chromosomal distribution, gene structure, motifs and phylogenetic relationship. Identification of regulatory cis-acting elements is an essential step to elucidate the gene regulatory mechanisms. The main propose of the study was to identify cis-acting regulatory elements at 2.5 kbp upstream regions of the identified *BnNHX* genes. The motifs involved in responding to abiotic stress were concentrated mostly at --800 and -1600 to -1700 bp relative to the transcription start site. It seems that, under various stresses, the expression of *BnNHX* genesis regulated by ABA-independent, ABA-dependent, ethylene and methyl jasmonate regulatory elements. These results contribute information about the regulatory mechanism of vacuole-type *NHX* genes in response to abiotic stresses by *in silico* study of cis-acting regulatory elements at the promoter region. This information could be used for further study of regulatory mechanisms and genetic engineering of canola to abiotic stresses.

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