

# The sugar beet gene encoding the sodium/proton exchanger 1 (BvNHX1) is regulated by a MYB transcription factor

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**Abstract** Sodium/proton exchangers (NHX) are key players in the plant response to salinity and have a central role in establishing ion homeostasis. NHXs can be localized in the tonoplast or plasma membranes, where they exchange sodium ions for protons, resulting in sodium ions being removed from the cytosol into the vacuole or extracellular space. The expression of most plant *NHX* genes is modulated by exposure of the organisms to salt stress or water stress. We explored the regulation of the vacuolar *NHX1* gene from the salt-tolerant sugar beet plant (*BvNHX1*) using *Arabidopsis* plants transformed with an array of constructs of *BvNHX1::GUS*, and the expression patterns were characterized using histological and quantitative assays. The 5' UTR of *BvNHX1*, including its intron, does not modulate the activity of the promoter. Serial deletions show that a 337 bp promoter fragment sufficed for driving activity that indistinguishable from that of the full-length (2,464 bp) promoter. Mutating four putative *cis*-acting elements within the 337 bp promoter fragment revealed that MYB transcription factor(s) are involved in the activation of the expression of *BvNHX1* upon exposure to salt and water stresses. Gel mobility shift assay confirmed that the WT but not the mutated MYB binding site is bound by nuclear protein extracted from salt-stressed *Beta vulgaris* leaves.

**Keywords** Abiotic stress · *Arabidopsis* · *Beta vulgaris* · MYB · NHX · Promoter activity · Sodium/proton exchanger · Sugar beet

## Abbreviations

AtNHX1	<i>Arabidopsis</i> NHX1
BvNHX1	Sugar beet NHX1
EMSA	Electrophoretic mobility shift assay
GUS	$\beta$ -Glucuronidase
MS	Murashige and Skoog basal plant medium
MYB	Myeloblastosis viral oncogene homolog
MYC	Myelocytomatosis viral oncogene homolog
NHX1	Sodium/proton exchanger 1

## Introduction

Salinity and drought are the major abiotic stresses limiting the production of crop plants worldwide (Zhu 2001; Munns 2002). Plants exposed to these stress conditions respond in biochemical, physiological and molecular levels to establish a new homeostasis that would enable them to survive the stress condition (Hasegawa et al. 2000; Serrano and Rodriguez-Navarro 2001; Zhu 2001, 2003; Bohnert et al. 2006; Pardo et al. 2006; Sahi et al. 2006). Sodium/proton exchangers (NHX) are major players in maintaining ion homeostasis (Blumwald 2000; Serrano and Rodriguez-Navarro 2001; Pardo et al. 2006; Sahi et al. 2006). These membrane-bound antiporters utilize electrochemical gradient of protons across the tonoplast or plasma membrane to transport sodium or potassium ions into the vacuole or sodium ions outside the cell, respectively (Blumwald 2000; Serrano and Rodriguez-Navarro 2001; Zhu 2003). The overexpression of tonoplast NHXs was shown to improve plant salt tolerance (Yamaguchi and Blumwald 2005; Apse

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and Blumwald 2007). In agreement with this finding, decreased activity of tonoplast NHX resulted in increased salt sensitivity (Apse et al. 2003; Sottosanto et al. 2007).

Vacuolar NHXs were cloned from a number of glyco-philic and halophilic plant species (Blumwald and Poole 1985; Barkla et al. 1995; Fukuda et al. 1998; Hamada et al. 2001; Yamaguchi et al. 2001; Xia et al. 2002; Aharon et al. 2003; Wu et al. 2004; Kagami and Suzuki 2005; Vasekina et al. 2005; Yang et al. 2005; Jiu et al. 2007; Zhang et al. 2008). It was suggested that the activity of vacuolar  $\text{Na}^+/\text{H}^+$  antiporters differ in salt-tolerant and salt-sensitive plants. In agreement with this notion, following salt stress the induction of NHX genes is higher in salt-tolerant plants than in salt-sensitive plants (Chauhan et al. 2000; Hamada et al. 2001; Shi and Zhu 2002; Xia et al. 2002; Yokoi et al. 2002; Zhang et al. 2008). In addition, comparison of the transcriptomes of salt-sensitive *Arabidopsis thaliana* with that of the related tolerant species *Thellungiella halophila* (*sal-suginea*) (Taji et al. 2004; Gong et al. 2005; Wong et al. 2006) suggested that these plants mainly differ in the expression levels and expression patterns of stress-related genes and ion transporters.

A large number of regulatory genes are involved in signaling pathways that are responsive to abiotic stresses (Chen and Zhu 2004). Salt and water stresses involve a number of signaling pathways, some of which are ABA dependent (Hasegawa et al. 2000; Zhu 2002; Chen and Zhu 2004; Yamaguchi-Shinozaki and Shinozaki 2006). A large number of transcription factors have been shown to be associated in the regulation of expression of target stress-inducible genes. Osmotic stress signaling pathway involves the AREB/ABF and DREB2 transcription factors, which bind the ABRE/CE and DRE/CRT *cis*-acting elements, respectively (reviewed by Zhu 2002; Yamaguchi-Shinozaki and Shinozaki 2006). Other transcription factors such as MYB, MYC, NAC and AP2/ERF were also shown to be involved in the regulation of genes activity by these abiotic stresses (Zhu 2002; Yamaguchi-Shinozaki and Shinozaki 2006).

Tonoplasts of the salt-tolerant sugar beet (*Beta vulgaris*) plant were shown to possess high  $\text{Na}^+/\text{H}^+$ -exchange activity (Blumwald et al. 1987; Blumwald and Poole 1987; Pantoja et al. 1990). NHX activity was correlated with the salinity tolerance of *B. vulgaris* cell cultures (Blumwald and Poole 1987). A cDNA encoding BvNHX1 was isolated (Xia et al. 2002). The BvNHX1 transcript levels in both the suspension cell culture and whole plants increased following salt treatment and this increase was concomitant with elevated BvNHX1 protein and vacuolar  $\text{Na}^+/\text{H}^+$  antiporter activity.

Here, the promoter of *BvNHX1* was cloned and its activity was studied in transgenic *Arabidopsis* expressing the *BvNHX1::GUS* construct. Interestingly, the promoter of *BvNHX1* does not contain ABRE or DRE *cis*-acting ele-

ments, common in promoters of osmotic stress regulated genes. The minimal 337 bp promoter sequence that was up-regulated by salt treatment and osmotic stress was identified by constructs containing serial deletions of the promoter. A number of potential *cis*-acting element sequences were mutated to evaluate their possible role in stress-modulated expression. Of these mutations only that in the binding site for the MYB transcription factor abolished the salt stress and water stress response.

## Materials and methods

### Cloning of the *BvNHX1* promoter

Sugar beet genomic DNA was digested with *Dra*I, *Eco*RV, *Pvu*II, or *Stu*I and ligated to GenomeWalker™ adapter using the Clontech GenomeWalker™ Universal Kit (Clontech, Mountain View, CA, USA). Screening for *BvNHX1* upstream sequences was performed using general primer and gene-specific primers according to the protocol in the instructions manual. Two steps of DNA walking were performed. For the first step, the isolation of genomic DNA fragment containing partial promoter sequence and 5' UTR, the primers used were: ptH, GCGAAGCTTCGACGGCCC GGGCTG; ptN AACTGCTCCACCATGGCTTCACAT AC. The primers used for the second step (cloning of distal promoter) were: GSP6-5, CCCAGAAACCCAAGTTACA GAAAAG; BvP1, CAAGGCCCAATGCAAGTGACAAA TG; BvP2, GTGTGGAGGAGAGAGATCGTGTTG.

### Construction of promoter serial deletions

Deletions were prepared by PCR using a combination of either Bv-Short*Eco*RI and each of the five primers designated Bv+NNN*Bam*HI (where NNN stands for the primer position), or Bv-Long*Eco*RI and each of the five Bv+NNN-*Bam*HI primers, or the primer Bv+UTR*Bam*HI. PCR was done using the Takara EX-*Taq*. Products were isolated from gels, digested with *Eco*RI and *Bam*HI, and subcloned into the respective sites of the pCAMBIA 1391Z vector. The serial deletions forward primers were: Bv+1*Bam*HI, ATTGGATCCTCATGTGCGAAGAGGACAAT; Bv+271 *Bam*HI, ATTGGATCCTCTATGGTGCCTGTATG; Bv+535*Bam*HI, ATTGGATCCGTGCATATTTATGATGAGA TAG; Bv+803*Bam*HI, ATTGGATCCACAGACATACAT GCATCTG; Bv+1042*Bam*HI, ATTGGATCCGAGACC GTCTAATTTTAGTAC; Bv+UTR*Bam*HI, ATTGGATC CACGTCTGCCATAATTTTG. The serial deletions reverse primers were: Bv-Short*Eco*RI, CCAGAATTCAGT AGAAAATCAGGAAATAG; Bv-Long*Eco*RI, CCAGA ATTCACACCAACAACCACAAT for the cloning promoter sequences without or with 5' UTR, respectively.

### Site-directed mutagenesis

Site-directed mutagenesis of promoter sequences was performed using the QuikChange II<sup>®</sup> Site-Directed Mutagenesis Kit protocol (Stratagene, La Jolla, CA, USA) employing the following mutation-containing primer pairs (mutation in bold): MYB 1AT, CTTAAAAATTATTGGATTAATTCTCTTCTTACTCGAGAATTGCATTAACCTAATCAC and GTGATTAGTTTAATGCAATTCTCGAGTAAGAAGAGAATTAATCCAATAATTTTTAAG; MYB ST1, CCAAAAAAAAAAAAAATCACTAAAAAATGGATCCATTCATTATTCGATTAATTCTCTTTC and GAAAGA GAATTAATCGAATAATGAATGGATCCATTTTTTA GTGATTTTTTTTTTTTTTTGG; MYC, GGATTAATTCTCTTCTTAATAACTCTAGACATTAACCTAATCACTA AAAAATTTTTG and CAAAAATTTTTTAGTGATTA GTTTAATGTCTAGAGTTATTAAGAAGAGAATTAA TCC; napA, TAAAAATAAACGATTTTTTATAAGACCTCGAGTTTCTTTTATATTCCTTCTGC and GCAG AAGGAATATAAAAGGAACTCGAGGTCTTATAA AAAATCGTTTATTTTTTA. The mutated sequences were designed to contain a unique restriction site to assist in the selection of plasmids containing mutated sequences. First screening was performed by PCR amplification of the promoter sequence using Bv-ShortEcoRI and Bv+271BamHI primers (see above), followed by digestion with the respective restriction enzyme. Mutations were further confirmed by DNA sequencing.

### Plant material and transformation

*Arabidopsis thaliana* (Columbia; Arabidopsis Biological Resource Center, Columbus, OH, USA) were grown at 25°C and 50% humidity under a 12 h light/12 h dark regime. The plants were grown either in pots or in Petri dishes containing 0.5× MS medium solidified by 0.7% agar. Transformation vectors containing the respective constructs were introduced into *Agrobacterium* GV3101 cells, and used for genetic transformation of *Arabidopsis* (Clough and Bent 1998). Homozygous T2 and T3 generation transformed plants with a single transgene insert each were selected on hygromycin and used in this study. *Beta vulgaris* seeds (Genesis seeds Ltd., Rehovot, Israel) were sown in potting mix and were grown at 28°C and 50% humidity under a 16 h light/8 h dark regime.

### GUS activity

Histochemical staining of GUS was performed essentially as described (Jefferson 1987). Quantitative GUS activity was assayed spectrophotometrically using *p*-nitrophenyl- $\beta$ -D-glucuronide (Duchefa Biochemie, Haarlem, The Netherlands) (Jefferson 1987).

### Electrophoretic mobility shift assay (EMSA)

Four-week-old seedlings were treated with 0.2 M NaCl for 24 h. Leaves were harvested, nuclei and nuclear proteins were prepared as previously described (Giuliano et al. 1988). EMSA was performed as described (Shkolnik and Bar-Zvi 2008). Mixtures containing 2.4  $\mu$ g of each forward and reverse oligonucleotides containing WT (ggTAACCA tataTAACCAaaTAACCAatttaaTAACCA, ggTGGTTAttaaatTGGTTAttTGGTTAtataTGGTTA) or mutated (ggCTC GAGtataCTCGAGaaCTCGAGatttaaCTCGAG, ggCTCG AGttaaatCTCGAGttCTCGAGtataCTCGAG) MYB binding sites were annealed by heating for 100°C and slowly cooling to RT. WT and mutated MYB binding sites are shown in CAPS, and linkers in small letters. Annealed oligonucleotides were end-labeled using Klenow fragment of DNA polymerase I and [ $\alpha$ -<sup>32</sup>P]dCTP. Free nucleotides were removed by gel filtration, and the resulting labeled dsDNAs were used as probes for EMSA. Nuclear proteins (1.2  $\mu$ g) were preincubated at RT for 10 min in a reaction mix contained in a final volume of 20  $\mu$ l: 20 mM Hepes KOH pH 8, 50 mM KCl, 0.5 mM EDTA, 5% glycerol, 12.5 ng poly(dI-dC)poly(dI-dC) (GE Healthcare, Piscataway, NJ, USA). End-labeled DNA probe (1  $\mu$ l, 3,000 cpm) was added, and mix was incubated for an additional 20 min. Reaction mixes were loaded onto 5% polyacrylamide gels containing 0.25× TBE buffer (22.25 mM Tris-borate, 0.5 mM EDTA). Samples were electrophoresed at 30 V, gels were dried and exposed to X-ray film.

## Results

### Cloning of the *BvNHX1* promoter

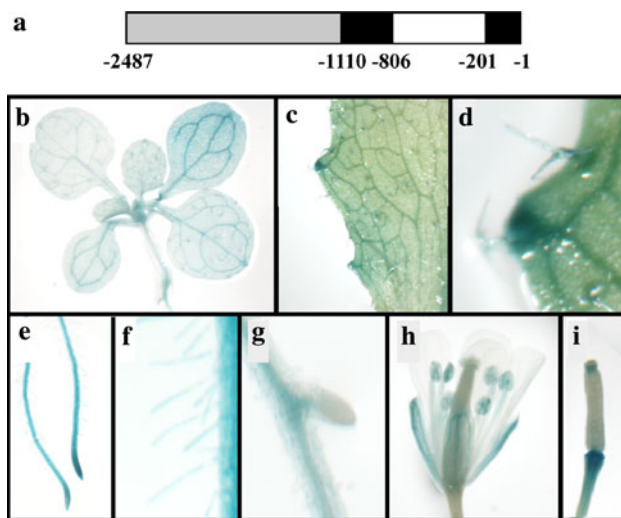
GenomeWalker *B. vulgaris* libraries were screened for upstream sequences using a gene-specific primer, designed from the 5' UTR sequences of *BvNHX1* cDNA (Xia et al. 2002) and a 370 bp upstream of the translation start ATG start codon sequence was localized. The first amplification round yielded a 704 bp fragment. To clone the DNA sequence comprised 704 bp promoter DNA fragment and the 5' UTR, genomic DNA was used as a template for the amplification using ptH and ptN primers, corresponding to the upstream and downstream sequences of this DNA fragment, respectively. PCR reactions using genomic DNA template and ptH and ptN primers to amplify the DNA sequence of the 704 bp promoter fragment cloned in the first round of gene walking and 5' UTR GeneWalking using ptN and GSP6-5 resulted in a DNA fragment corresponding to sequences 1 and upstream of the translation start codon, respectively. The expected size of the PCR product was 1,073 bp. The amplified sequence was

1,678 bp long and sequencing analysis revealed that the DNA encoding the 5' UTR of the *BvNHX1* gene is interrupted by a 605-bp long intron.

A second round of walking was performed using the gene-specific primers BvP1 and BvP2 which were designed from sequences in the 704 bp fragment and yielded a 911 bp DNA fragment. The final combined promoter sequence comprised 2,464 bp upstream the translation start codon, composed of 1,378 bp promoter, 304 bp 5' UTR exon I, 605 bp intron and 200 bp 5' UTR exon II (Fig. 1a).

#### Expression pattern of the *BvNHX1* gene

To study the expression pattern of *BvNHX1*, we produced transgenic *Arabidopsis* expressing the GUS reporter gene driven by the 2,464 bp *BvNHX1* sequence starting from 23 bp upstream of the translation start codon (construct *LI::GUS*). Histochemical GUS staining showed that the promoter was active in all vegetative tissues (Fig. 1b–g). In addition, high expression levels were detected in hydathodes and trichomes (Fig. 1c, d). The promoter was not highly active in guard cells and the roots showed a higher staining intensity at the tips and elongation zones (Fig. 1e). The *BvNHX1* promoter was expressed in root hairs (Fig. 1f) but was not active in emerging lateral roots (Fig. 1g). Sepals and anthers were stained, whereas petals and carpels were not (Fig. 1h). Expression in pedicels was detected at the fruit stage but not at the flower stage (Fig. 1h, i).



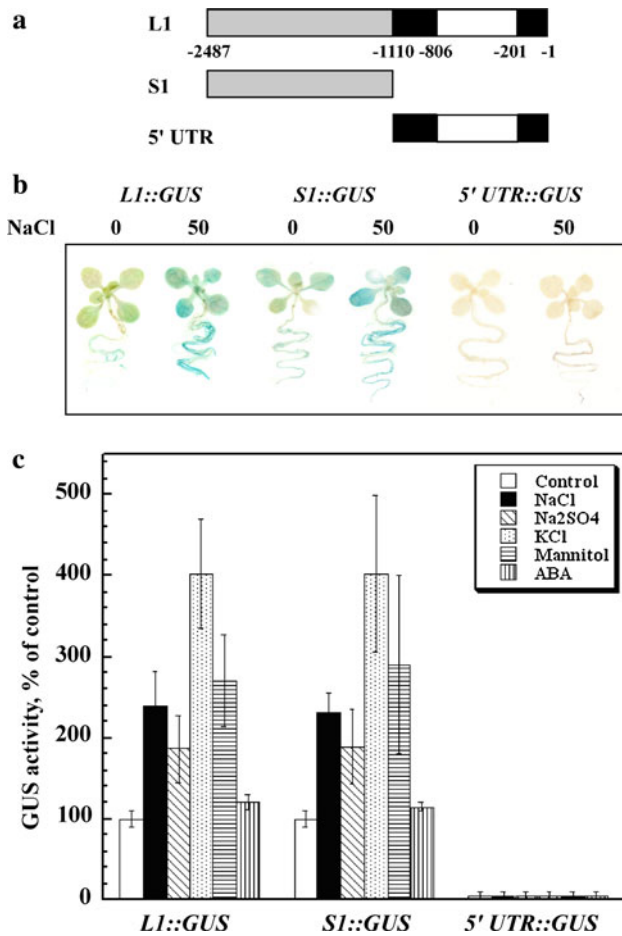
**Fig. 1** Expression pattern of the full-size *BvNHX1* promoter. **a** Scheme of full-size *BvNHX1* promoter structure. Upstream promoter sequence, 5' UTR, and intron are marked in gray, black and white, respectively. Nucleotides are numbered in according to the translation start ATG start sequence. **b–i** *Arabidopsis* plants expressing the 2,487 bp *BvNHX1::GUS* constructs were grown under non-stressed conditions. Histochemical staining of GUS was performed as described in “Materials and methods”. **b** Shoot of soil grown plant. **c** Section of a mature leaf. **d** Close-up of the edge of the leaf shown in (c). **e** Primary roots. **f** Root hairs. **g** Emerging lateral root. **h** Flower. **i** Silique

To study the salt-induced expression of the *BvNHX1* promoter, seeds expressing the *LI::GUS* construct (Fig. 2a) were germinated in the presence of 50 mM NaCl. Seedlings were harvested 2 weeks later, and GUS activity was determined (Fig. 2b, c). The exposure of the seedlings to 50 mM NaCl did not affect seedling growth, but resulted in a marked increase in promoter activity. The expression levels observed with this physiologically relevant long-term mild stress were much higher than those obtained with a brief exposure of non-stressed seedlings to high-salt (>0.25 M) concentrations (not shown) according to the stress application protocol used by others (e.g., Shi and Zhu 2002).  $\text{Na}_2\text{SO}_4$  and mannitol resulted in similar levels of GUS activation to that obtained using NaCl (Fig. 2c), suggesting that the activity can also be induced by osmotic stress. Interestingly, a similar concentration of KCl resulted in higher expression rates. ABA, on the other hand, had a slight, statistically non-significant increase in GUS activity. The various mild stress treatments resulted in less than 30% differences in the size of the seedlings.

Interestingly, similar to DNA sequence encoding the 5' UTR of the *Arabidopsis AtNHX1*, the 5' UTR of *BvNHX1* also contains an intron. To explore whether the 5' UTR and intron play a role in the transcription regulation of the *BvNHX1* gene, we transformed *Arabidopsis* plants with constructs in which the promoter sequence upstream of the transcription start (*SI::GUS*) or containing the 5' UTR + intron (*5' UTR::GUS*) were fused with GUS (Fig. 2a), and the plants were tested for GUS activity. GUS activities assayed in plants transformed with *LI::GUS* and *SI::GUS* were similar and differences were not statistically significant (not shown). The expression pattern of the *SI::GUS* lines, containing a 1,378 bp promoter sequence upstream of the transcription start, was indistinguishable to that of plants transformed with the *LI::GUS* construct both qualitatively (Fig. 2b) and quantitatively (Fig. 2c). On the other hand, the *5' UTR::GUS* construct had GUS activity similar to that of non-transformed control plants (Fig. 2b, c), suggesting that this sequence is not likely to affect the transcription of this gene.

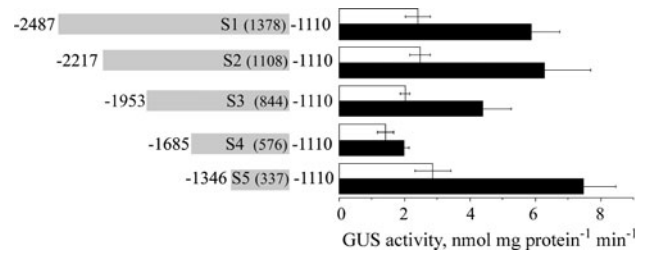
#### Construction of serial deletions

Five constructs comprising upstream sequences fused to reporter gene GUS were made. These constructs, designated *S1::GUS* to *S5::GUS*, contained 1,378, 1,108, 844, 576, and 337 bp promoter sequences upstream of the 5' UTR. T2 and T3 generation plants from homozygous lines containing a single insertion were selected and at least five independent lines of each construct were tested. GUS activity in homogenates prepared from non-stressed and salt-treated seedlings was determined (Fig. 3). Deletions of 270 and 534 bp from the 5' of the promoter sequence (*S2* and



**Fig. 2** Expression of constructs containing *BvNHX1* full-size promoter, promoter sequence upstream of the 5' UTR, and 5' UTR including intron. **a** Constructs used. Upstream promoter sequence, 5' UTR, and intron are marked in gray, black and white, respectively. Nucleotides are numbered in according to the translation start ATG start sequence. **b** Histochemical staining of seedlings germinated and grown on solid medium without or with salt. Homozygous seeds of *Arabidopsis* transformed with the indicated constructs were sown on plates containing 0.5× MS solid medium (0) or the same medium supplemented with 50 mM NaCl (50). Two-week-old seedlings were stained for GUS. **c** Quantitative GUS expression in non-stressed and stressed *Arabidopsis* seedlings. GUS activity was assayed in homogenates of homozygous seedlings described in (b). Data shown represent average activity determined in homogenates prepared from 50 seedlings each of five independent lines of each constructs germinated and grown for 2 weeks on plates containing solid 0.5× MS medium without (white bars) or containing in addition 50 mM NaCl (black bars), 25 mM Na<sub>2</sub>SO<sub>4</sub> (diagonally dashed), 50 mM KCl (dotted), 100 mM mannitol (horizontally dashed), or 20 μM ABA (vertically dashed). Activity is normalized to allow averaging between different transgenic lines and different experiments. GUS activities of *LI::GUS* and *SI::GUS* constructs under control conditions were similar, and were not statistically different

S3, respectively) did not affect the promoter activity. Deletion of an additional 268 bp fragment inhibited the activation capacity of the resulting 567 bp promoter fragment (construct S4) both in the absence and presence of salt. On the other hand, the *S5::GUS* construct containing the short-

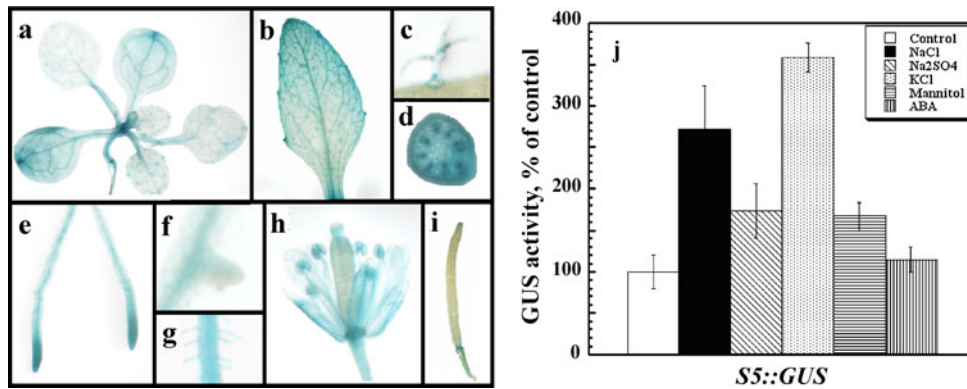


**Fig. 3** Serial deletion analysis of the *BvNHX1* promoter activity. The 5' serial deletions of the upstream promoter sequence (corresponding to the S1 sequence in Fig. 2) were fused to GUS and used to transform *Arabidopsis*. Nucleotides are numbered as in Fig. 2. GUS was assayed in homogenates prepared from 50 seedlings of each of five independent lines of each constructs, germinated and grown for 2 weeks on plates containing solid 0.5× MS medium without (white bars) or containing in addition 50 mM NaCl (black bars)

est, 337 bp long, promoter fragment was sufficient to drive GUS expression to similar levels to that obtained by the *SI::GUS* construct both in the presence and absence of salt (Fig. 4). These results suggested that the transactivation capacity of the S5 promoter fragment was essentially identical to that of the full-length S1.

#### Analysis of potential cis-acting elements in the *BvNHX1* minimal promoter region

The DNA sequence of the S5 construct was used to search for potential binding sites of known *trans*-acting factors through the Plant *cis*-acting Regulatory DNA Elements (PLACE) database (Higo et al. 1999) <http://www.dna.affrc.go.jp/PLACE/> (Fig. 5a). Four of these potential sites were further studied using site-directed mutagenesis (Table 1). The sequences are putative binding sites for MYB 1AT, MYC, MYB ST1, and napA transcription factors. Transcription factors from the MYC and MYB families are known to be also involved in the modulation of salt stress and water stress regulated genes (Zhu 2002; Shinozaki et al. 2003), whereas napA was shown to function in seeds (Stalberg et al. 1996). Site-directed mutagenesis was performed using Stratagene QuikChange II<sup>®</sup>, where the putative *cis*-acting sequence was changed into a restriction enzyme recognition sequence (Table 1). Mutated sequences were confirmed by restriction digests and DNA sequence analyses, and were used for the genetic transformation of *Arabidopsis* plants. Homozygous transgenic plants were selected and assayed for GUS activity. The basal expression levels in non-stressed plants were not affected by the mutation of any of the potential *cis*-acting elements (Fig. 5b). Mutation in the MYB 1AT box (position –1362) almost abolished the induction of promoter activity by NaCl and mannitol, suggesting that the *BvNHX1* promoter is regulated mainly by a MYB 1AT transcription factor. Other mutations did not affect the expression of the GUS



**Fig. 4** Activity of the 337 bp upstream promoter fragment. Homozygous plants expressing the S5 construct (Fig. 3, 337 bp promoter fragment fused to GUS) were assayed for the activity of the reporter gene. **a–i** Histochemical staining of non-stressed plants. **a** Shoot of soil grown plant. **b** Mature leaf of soil grown plant. **c** Trichome. **d** Cross-section of inflorescence stem. **e** Roots. **f** Emerging lateral root. **g** Root

hairs. **h** Flower. **i** Silique. **j** Quantitative analysis using homogenates (five independent lines, 50 seedling each) from seedlings grown for 2 weeks in the 0.5× MS solid medium without (*white bar*) or containing in addition 50 mM NaCl (*black bar*), 25 mM Na<sub>2</sub>SO<sub>4</sub> (*diagonally dashed*), 50 mM KCl (*dotted*), 100 mM mannitol (*horizontally dashed*), or 20 μM ABA (*vertically dashed*)

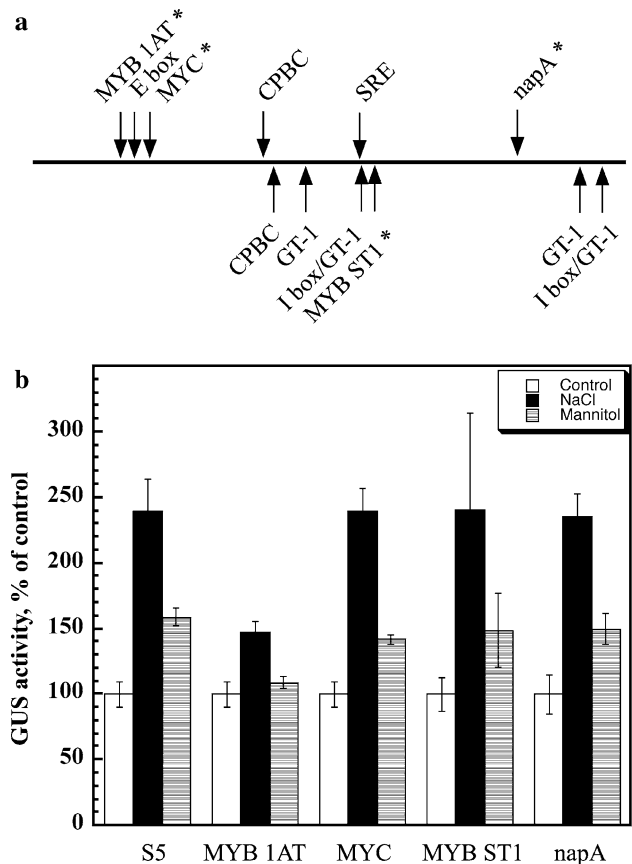
reporter gene compared to the non-mutated S5 promoter fragment.

To determine if the MYB 1AT box found in the heterologous *A. thaliana* species is also recognized in *B. vulgaris*, we challenged nuclear protein preparation extracted from salt-treated *B. vulgaris* seedlings for binding probes containing the WT or mutated MYB 1AT box. Figure 6 shows that *B. vulgaris* nuclear proteins bind WT MYB 1AT DNA sequence, but are incapable of binding the mutated sequence. Moreover, nuclear extract prepared from irrigated leaves did not show DNA binding activity under the conditions tested (Fig. 6) in agreement with Fig. 5.

## Discussion

NHX-like sodium/proton exchangers play important roles in establishing pH and ion homeostasis during salt stress (Pardo et al. 2006; Apse and Blumwald 2007). It has been suggested that an important difference between NHX genes from salt-sensitive and salt-tolerant plants is not in possible differences in the gene sequences but it resides in their expression patterns and the regulation of their expression. Similar to the *Arabidopsis thaliana* *AtNHX1*, *BvNHX1* is expressed in leaves and roots and also in stamen, petal fruit bases and fruit tips (Shi and Zhu 2002). On the other hand, unlike *AtNHX1*, *BvNHX1* is highly expressed in root tips and trichomes, but not in guard cells, at least in *Arabidopsis* (Fig. 1).

The 5' UTR of *BvNHX1* contains a 605 bp intron (Fig. 1a). Introns have been shown in some cases to increase transcriptional activity in plants (Rose 2004) in a process known as intron-dependent enhancement of transcription (IME) (Rose 2008). A recent bioinformatics

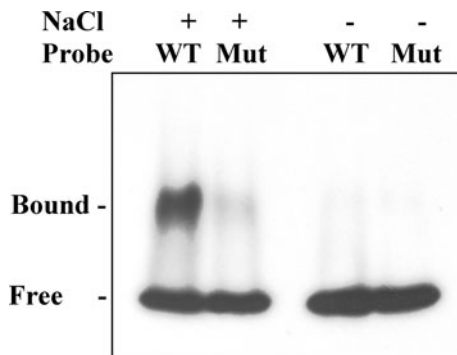


**Fig. 5** Activities of mutated 337 bp *BvNHX1* promoter fragments. **a** Putative *cis*-acting elements in the S5 promoter fragment. Sites in forward or reverse modes are marked *above* and *below* the line, respectively. Mutated sequences are marked by asterisks. **b** GUS activity was assayed in homogenates made from five homozygous lines of *Arabidopsis* containing S5::GUS constructs with the indicated mutations (see also Table 1). Seeds were sowed on 0.5× MS (*white bars*) supplemented with 50 mM NaCl (*black bars*) or 100 mM mannitol (*dashed bars*), and seedlings harvested and analyzed 2 weeks later

**Table 1** Mutations of putative DNA binding sites of transcription factors

Recognition	Location	Orientation	DNA sequence		Restriction site
			WT	Mutated	
MYB 1AT	−1362	+	<b>ATAACCA</b>	<u>CTCGAGA</u>	<i>XhoI</i>
MYC	−1358	±	<b>CAATTG</b>	<u>TCTAGA</u>	<i>XbaI</i>
MYB ST1	−1228	−	<b>TATCCT</b>	<u>GGATCC</u>	<i>BamHI</i>
napA	−1136	+	<b>CAAACAC</b>	<u>CCTCGAG</u>	<i>XhoI</i>

DNA sequences of putative DNA binding motifs (in bold) were mutagenized into a sequence containing restriction site (underlined)



**Fig. 6** *B. vulgaris* nuclear proteins bind WT but not mutated MYB 1AT DNA sequence. End-labeled dsDNA probes containing WT and mutated sequence were incubated with protein nuclear extract prepared from control or salt-treated *B. vulgaris* leaves. Mixes were resolved by native electrophoresis on 5% polyacrylamide, the gel was dried and exposed to X-ray film. Free and Bound, migration of the free and protein bound dsDNA probe, respectively

study suggested that almost 20% of genomic sequences in *Arabidopsis* contain 5' UTR introns (Chung et al. 2006). The *BvNHX1* 5' UTR and its intron do not seem to affect the extent of gene expression, since their removal did not have any effect on the expression driven by 5' upstream sequences in both the absence or presence of salt- and osmotic-stress (Fig. 2). Moreover, the DNA encoding the 5' UTR and intron was not sufficient for driving expression (Fig. 2). Thus, the role of the *BvNHX1* 5' UTR intron remains to be determined.

Activity assays of the 5' deletion series of the *BvNHX1* promoter suggested that nucleotides at positions −2487 to −1954 do not contain major *cis*-acting elements affecting gene expression (Fig. 3). On the other hand, the positions −1953 to −1686 and −1685 to −1347 might contain positive and negative elements, respectively (Fig. 3). Interestingly, a 347 bp promoter fragment (−1346 to −1110) could drive the expression of the GUS reporter gene (Figs. 3, 4) to similar levels to that of the full-length promoter sequence (Fig. 1, 2).

Bioinformatics analysis suggested a number of potential *cis*-acting elements occurring within this 347 bp promoter sequence (Fig. 5). Some of the proposed transcription factors are known to be regulated by salinity and drought.

There are two binding sites for MYB transcription factors: the MYB 1AT binding site [consensus sequence (A/T)AACCA, TAACC in *BvNHX1*] in the promoters of dehydration-responsive gene *rd22* and many other genes (Abe et al. 2003), and the MYB ST1 (Baranowskij et al. 1994), a MYB homolog containing only one repeat that can function as a transcriptional activator. The Myb motif of the MybSt1 protein (GGATA) is distinct from the plant Myb DNA binding domain described so far. MYC (consensus sequence CANNTG, CAATTG in *BvNHX1*) was shown to act in promoters of genes regulated by drought and cold stress (Abe et al. 2003; Chinnusamy et al. 2004; Yamaguchi-Shinozaki and Shinozaki 2006). The *napA* element conserved in many seed-expressed genes (Stalberg et al. 1996). Interestingly the *BvNHX1* promoter lacks a binding site for ABRE, a major transcription factor regulating genes modulated by salt and water stresses (Yamaguchi-Shinozaki and Shinozaki 2005). Mutating the MYB 1AT-binding site eliminated the salt and osmotic activation of the transcription activity of *BvNHX1*, whereas the other mutations did not affect its expression. EMSA assay using nuclear extract prepared from *B. vulgaris* leaves (Fig. 6) confirmed the results obtained with the heterologous *A. thaliana* plants (Fig. 5), e.g. DNA binding activity of the MYB 1AT sequence was induced by salt treatment, and was abolished when the DNA binding site was mutated. These results suggest that the mechanism is conserved between some sensitive and tolerant species. In vitro binding of the tobacco NtMYBJS1 to the AACC core motif was reduced by mutation of this sequence (Galis et al. 2006). The MYB family is the largest transcription factor family in plants with family size of up to 258 members, depending on the prediction algorithm used (Qu and Zhu 2006). The nature of the MYB protein(s) that modulate the activity of this gene is yet to be discovered. Nevertheless, this protein activity is not dependent on ABA. Expression analysis of 163 genes of the *Arabidopsis* MYB transcription factor family revealed that about 25% of them are affected by ABA (Yanhui et al. 2006).

The short promoter sequence identified here could be used as a water deficit-regulated promoter for driving gene expression in transgenic plants.

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