ORIGINAL ARTICLE

Molecular characterization of *Brassica napus* stress related transcription factors, *BnMYB44* and *BnVIP1*, selected based on comparative analysis of *Arabidopsis thaliana* and *Eutrema salsugineum* transcriptomes

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Abstract
Many studies have been performed to identify regulatory circuit underlying plant stress tolerance. However, the reliability of some findings has been criticized because of exclusive use of stress sensitive plant species such as *Arabidopsis thaliana*. Sensitive plant species often harbor narrow defensive mechanisms and have relatively low capacity for adaptive responses. Therefore, it is useful to employ tolerant model plants, such as *Eutrema salsugineum*, to provide comprehensive insights into various mechanisms involved in response to abiotic stresses. In this study, comparative transcriptome and regulatory network analysis of stress-sensitive (*A. thaliana*) and -tolerant (*E. salsugineum*) model plants uncovered regulatory hierarchies underlying response to abiotic stresses and suggested the transcription factor genes, *MYB44* and *VIP1* as the candidate hub genes to perform molecular analyses on their *Brassica napus* homologs, *BnMYB44* and *BnVIP1*. The full-length coding sequence of *BnMYB44* and *BnVIP1* with 891 and 969 bp long were cloned and sequenced. They shared high similarity with their counterparts in other plants at nucleotide and amino acid levels. The expression patterns of *BnMYB44* and *BnVIP1* genes of the two *B. napus* cultivars under drought and salt stress conditions coupled with the data obtained from the physiological measurements as well as analysis of the *BnMYB44* and *BnVIP1* promoters suggested that *BnMYB44* and *BnVIP1* genes may contribute to responses to drought and salt stresses in *B. napus*.

Introduction

Crop plants frequently encounter adverse environmental factors that limit their growth and productivity. Drought and salinity stresses are known as the most crucial environmental constraints for agricultural production worldwide [1, 2]. To cope with such stresses, plants reprogram their genome activities which essentially cause changes in expression and

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function of genes [3–5]. Protein families of transcription factors (TFs), protein kinases (PKs) and protein phosphatases (PPs) are the core regulatory components underlying downstream functions involved in plant stress responses [5–8]. For example, expression change of only one regulatory gene, namely histidine kinase 1 (*ATHK1*) may lead to alter activity of more than six-hundred downstream genes in *A. thaliana* [9]. Thus, understanding the roles of regulatory components will be of great benefit to improve crop stress tolerance.

Several studies have been performed to identify important regulatory genes involved in plant stress tolerance. However, the reliability of some findings has been criticized because of exclusive use of stress sensitive plant species such as *A. thaliana* [10]. In contrast to stress tolerant plant species, sensitive plant species often harbor narrow defensive mechanisms and have relatively low capacity for adaptive responses [10, 2]. Therefore, it is useful to employ model plants that can provide comprehensive insights into various mechanisms conferring tolerance to abiotic stresses.

Eutrema salsugineum (also known as *Thellungiella halophila*) is widely used as a model plant to study abiotic stress responses and tolerance [11–15]. This stress tolerant species is a close relative of Arabidopsis, and its genes show high homology to those of Arabidopsis at the coding sequence level [16, 17]. Interestingly, *E. salsugineum* does not recruit complex adaptation mechanisms such as dramatic morphological changes, and its stress tolerance is simply derived from enhancement of mechanisms involved in controlling water status, metabolic rates, ion homeostasis and so on, which are also present in stress sensitive species [11, 16, 18]. A large number of *E. salsugineum* homologs of Arabidopsis stress-inducible genes are highly expressed under normal growth conditions [19, 20]. The availability of whole-genome sequence and transcriptome data of *E. salsugineum* [21] along with various Arabidopsis genomic resources has opened a new vista for a better understanding of genome function under abiotic stress conditions in *Brassicaceae* family, including the agronomically important *Brassica* genus.

Rapeseed (*B. napus* L.) is a globally important oilseed crop. Rapeseed production is often limited by abiotic stresses in arid and semi-arid regions. Identification of regulatory genes involved in response mechanisms to abiotic stresses will facilitate development of rapeseed cultivars with improved stress tolerance [22–24]. *B. napus* is an obvious target crop to exploit comparative genomics data between Arabidopsis and *E. salsugineum*. Several abiotic stress responsive genes have been identified in *B. napus* based on homology with Arabidopsis [25–29]; however, their significance in relation to other co-expressed genes in the context of a network system have remained unclear. Comparative transcriptome analysis of Arabidopsis and *E. salsugineum* has the potential to uncover regulatory hierarchies underlying response and tolerance to abiotic stresses

and suggests candidate hub genes to be characterized in *B. napus*.

This study aimed to use comparative transcriptomics and experimental approaches to identify and characterize key regulatory genes that respond to drought and salt tolerance in *B. napus*. A gene regulatory network was constructed based on comparative analysis of the microarray data from *Arabidopsis thaliana* and *E. salsugineum*. The transcription factor genes namely *MYB44* and *VIP1* were selected from the regulatory network to perform molecular analyses on their *B. napus* homologs, *BnMYB44* and *BnVIP1*. The coding sequence of *BnMYB44* and *BnVIP1* were isolated, cloned and sequenced. Afterwards, the expressions of *BnMYB44* and *BnVIP1* genes in response to drought and salt stresses were analyzed in two *B. napus* cultivars with different levels of tolerance to drought and salt. Finally, the expression data coupled with the measurements of physiological characteristics as well as the results of promoter analysis of the genes of interest provided an insight into the role and significance of *BnMYB44* and *BnVIP1* in response to drought and salt stresses in *B. napus*.

Materials and methods

Comparative analysis of microarray data from *Arabidopsis thaliana* and *Eutrema salsugineum*

The microarray raw data from *A. thaliana* and *E. salsugineum* were retrieved from European Bioinformatics Institute (EBI) array expressed sub-division (http://www.ebi.ac.uk/arrayexpress/experiments/E-GEOD-19740/). Two independent leaf samples per species collected at the growth stage 6.10 (i.e. about 35.9 days after sowing in which, 10% flowers to be produced are open) according to Boyes et al. [30] were used to perform two color microarray assay.

The microarray expression data were normalized by Robust Multi-array Averaging (RMA) algorithm [31] using FlexArray software 1.6.1 [32]. In order to identify Differentially Expressed Genes (DEGs) between leaves of *A. thaliana* and *E. salsugineum*, a Bayesian *t* test was applied followed by the false discovery rate (FDR, q-value) method [33] to correct p-values for multiple testing. All the DEGs at q-value ≤ 0.05 were subjected to further analysis to specify those genes encoding regulatory proteins. To do so, first, a local database was made comprising Arabidopsis transcription factors collected from PlantTFact (http://plantgrn.noble.org/PlantTFcat/) [34] as well as a complete set of Arabidopsis protein kinases and protein phosphatases obtained from Plant Protein Phosphorylation Database (P3DB) (http://www.p3db.org/) [35]. The DEGs were then searched against the local database to specify those genes encoding TFs, PKs and PPs.

The regulatory genes were subjected to Gene Set Enrichment (GSE) analysis to extract significant sub-networks based on Fisher exact test (p-values ≤ 0.05), using Pathway Studio webtool [36, 37], as previously described [38–40]. After that, all the significant sub-networks were merged by "union selected pathways" of Pathway studio software 7 to construct the hierarchical regulatory network. In order to delineate significance of the DEGs in the regulatory network, topological features including betweenness and closeness centrality as well as edge betweenness centrality [41], were determined for the network using Cytoscape software 3.1.1 [42, 43].

Cloning and sequencing of *B. napus MYB44* and *VIP1* genes

Total RNA was extracted from leaf samples of *B. napus* cv. SLM046 using column RNA isolation kit (DENA Zist Asia, Iran) according to the manufacturer's protocol. RNA quantification and integrity were double-checked by agarose gel electrophoresis and spectrophotometry. DNA contamination was removed from RNA using Fermantas DNaseI treatment (Thermo scientific, USA). First strand cDNA was synthesized from total RNA by Viva 2-steps RT-PCR kit (Vivantis, Malaysia). Since the genome sequence of *Brassica napus* was not publicly available at the time of primer design, the primers were designed based on *Brassica rapa* orthologs of *MYB44* and *VIP1* identified by searching *B. rapa* genome sequence (http://www.phytozome.net) against Arabidopsis *MYB44* and *VIP1* (Table 1). The two pairs of primers were used to clone two fragments with overlapping region to cover up the full-length coding sequence of *BnVIP1*.

PCR reaction was carried out as follows: initial denaturation at 94 °C for 5 min followed by 35 cycles of 1 min at 94 °C, 1 min at 56.5 °C and 1 min at 72 °C, and a final extension at 72 °C for 10 min. The PCR products were analyzed by agarose gel electrophoresis (1% w/v). Subsequently, the PCR products were purified and cloned into pTZ57R/T vector (InsTAclone PCR Cloning kit, Fermentas, USA). Transformation of competent cells of *E. coli* strain DH5α was performed by electroporation. The cloned amplicons were finally sequenced.

The coding sequences were subjected to similarity searches against non-redundant nucleotide and protein database using BLASTN and BLASTX (E-value $\leq 10^{-5}$), respectively, using CLC Genomics workbench 3.6 software. Also, the structure and synteny of *BnMYB44* and *BnVIP1* were assigned using Blat tool of GenoScope (http://www.genoscope.cns.fr/spip/), the *B. napus* genome sequence database. Moreover, protein features of the deduced amino acid sequences of *BnMYB44* and *BnVIP1* genes were specified using CLC Genomics Workbench software 3.6.

Plant materials, growth conditions and stress treatments

The *B. napus* cultivars, SLM046 and Zarfam, with different responses to abiotic stresses [25, 44–46], were used to perform drought and salt stress experiments. The drought experiment was conducted in factorial completely randomized design with three replicates. Plants were grown in plastic pots filled with sterilized sandy-clay soil under growth room conditions (16 h light/8 h dark; 24 °C day/8 °C night). Plants with five true leaves were subjected to drought stress by withholding water to reach soil moisture to 40% of field capacity (FC). Sampling was carried out under non-stress along with three drought stress treatments including when soil moisture reached to 40% of FC as well as maintaining that for 6 and 24 h. The non-stress condition was performed

Table 1 The list of primers used for isolation and expression analysis of *BnMYB44* and *BnVIP1* genes

Gene	Primer	Amplicon length on cDNA (bp)
BnMYB44	BMF1 5′-CATCTCTCTCACAACAAAACAG-3′	943
	BMR1 5′-CTACTCGATTCTTCCAACTCC-3′	
	MqF 5′-CGTGCTGGTGGTGTTATGTTG-3′	116
	MqR 5′-CGGTTTGACTCCTCGCTGAC-3′	
BnVIP1	BVF1 5′-GGGTAGGTATAATAAACTACTG-3′	713
	BVR1 5′-GTATACTGGTGAGCTGGAGAG-3′	
	BVF2 5′-ATCACCGGAGCAATTCTATGG-3′	622
	BVR2 5′-CATCCAATATTGCAGCCCC-3′	
	VqF 5′-GCTTCTCAGTTGATTCCGATTC-3′	113
	VqR 5′-GTGATGATGATGATGACCTTTCC-3′	
Bnelf1α	elf-F 5′-AGCCGCAAGTCCTCCTCTCAG-3′	186
	elf-R 5′-TTCATCTCAGCAGCCTCCTTCTCG-3′	

by regularly irrigating plants to keep soil moisture around FC during the experiment.

The salt stress experiment was also carried out in factorial completely randomized design with three replicates. The surface-sterilized seeds of the *B. napus* cultivars, SLM046 and Zarfam, were sown in sandy soil under a long-day photoperiod (16 h light/8 h dark) with 24 and 8 °C day and night temperature, respectively. 10-day-old seedlings were then transferred to a hydroponic culture system supplemented with half-strength Hoagland solution (pH ≈ 6.0) under growth room conditions. Plants with five true leaves were exposed to salt stress by adding 150 mM NaCl solution to the medium. Sampling was carried out under non-stress as well as three salt stress treatments including 3 h, 24 h and 7 days after adding salt solution.

Physiological measurements

In order to evaluate drought tolerance of the *B. napus* cultivars, a number of physiological characteristics were measured under all the drought stress conditions. Relative Water Content (RWC) was determined according to the method of Slatyer and Shmueli [47]. Proline content was measured using the protocol described by Bates et al. [48]. Cell membrane damage caused by drought stress was estimated by measuring Electrolyte leakage (EL) following the method described by Sairam and Srivastava [49]. In addition, the concentration of Na^+ and K^+ ions in leaf and root tissues of the *B. napus* cultivars were determined under all the salt stress conditions according to Munns et al. [50] protocol in order to obtain K^+/Na^+ ratio as an indicator for salt tolerance. The data were subjected to analysis of variance followed by comparison of means using Least Significant Difference (LSD) test at p-value ≤ 0.05.

Expression profiling of *BnMYB44* and *BnVIP1* under stress conditions

Real time quantitative RT-PCR (qRT-PCR) was used to detect expression levels of *BnMYB44* and *BnVIP1* in leaf and root tissues of the cultivars during drought and salt stresses. Total RNA extraction and cDNA synthesis were done as described earlier for isolating the genes. The specific primers, listed in Table 1, were designed for each gene. qRT-PCR was performed on a Bioer thermal cycler (Bioer Technology Co., Ltd., China) by using RealQ PCR Master Mix kit (Ampliqon, Denmark) according to the manufacturer's instructions. qRT-PCR reactions were also performed for the reference gene, *B. napus Elongation Factor 1α* (*Bnelf1α*; GenBank Accession number: FJ529181) for normalization of expression data. Three independent biological samples were used to do qRT-PCR. Each reaction was also repeated two times as technical replicates. The relative expression

levels of the target genes were calculated using the $2^{-\Delta\Delta CT}$ method [51].

Analysis of *BnMYB44* and *BnVIP1* promoters

1500 bp upstream of *BnMYB44* and *BnVIP1* genes (including 5′UTR), as putative promoter regions were obtained from GenoScope (http://www.genoscope.cns.fr/spip/). The promoter sequences were searched for cis regulatory elements (CREs) using PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/; [52]). Then, a statistical test for pairwise comparison of the promoters of *BnMYB44* and *BnVIP1* genes was performed according to the method of Shamloo-Dashtpagerdi et al. [53] to identify CREs with significantly different number of occurrences between the promoters, which may contribute to different expression patterns of the genes.

Results

Comparative analysis of microarray data

The microarray data obtained from leaf samples of Arabidopsis and *E. salsugineum* were analyzed to identify differentially expressed regulatory genes. Considering q-value ≤ 0.05, we found 3706 genes that their expressions significantly differed between leaves of Arabidopsis and *E. salsugineum*. Of those, 564 genes fell into the three major groups of regulatory genes encoding transcription factors, protein kinases and protein phosphatases (Supplementary 1). 307 transcription factors belonging to 50 different families were found with different magnitudes and directions of expression changes indicating that there is a great difference in the regulatory functions of Arabidopsis and *E. salsugineum* transcriptomes. bZip family with 31 members was the largest group of transcription factors (Supplementary 1). In addition, 226 protein kinase and 31 protein phosphatase genes belonging to 52 and 22 families, respectively, showed significantly different expressions between Arabidopsis and *E. salsugineum*. The receptor like cytoplasmic kinase VII family with 18 members was the largest protein kinase family. Among protein phosphatase families, the protein phosphatase 2C family was the largest group containing eight members (Supplementary 1).

Regulatory network analysis

The hierarchal gene regulatory network (Fig. 1a) was predicted using the differentially expressed regulatory genes in order to identify major stress-responsive regulatory genes. The network clearly identified the regulatory genes involved in the drought and osmotic stress pathways. The network
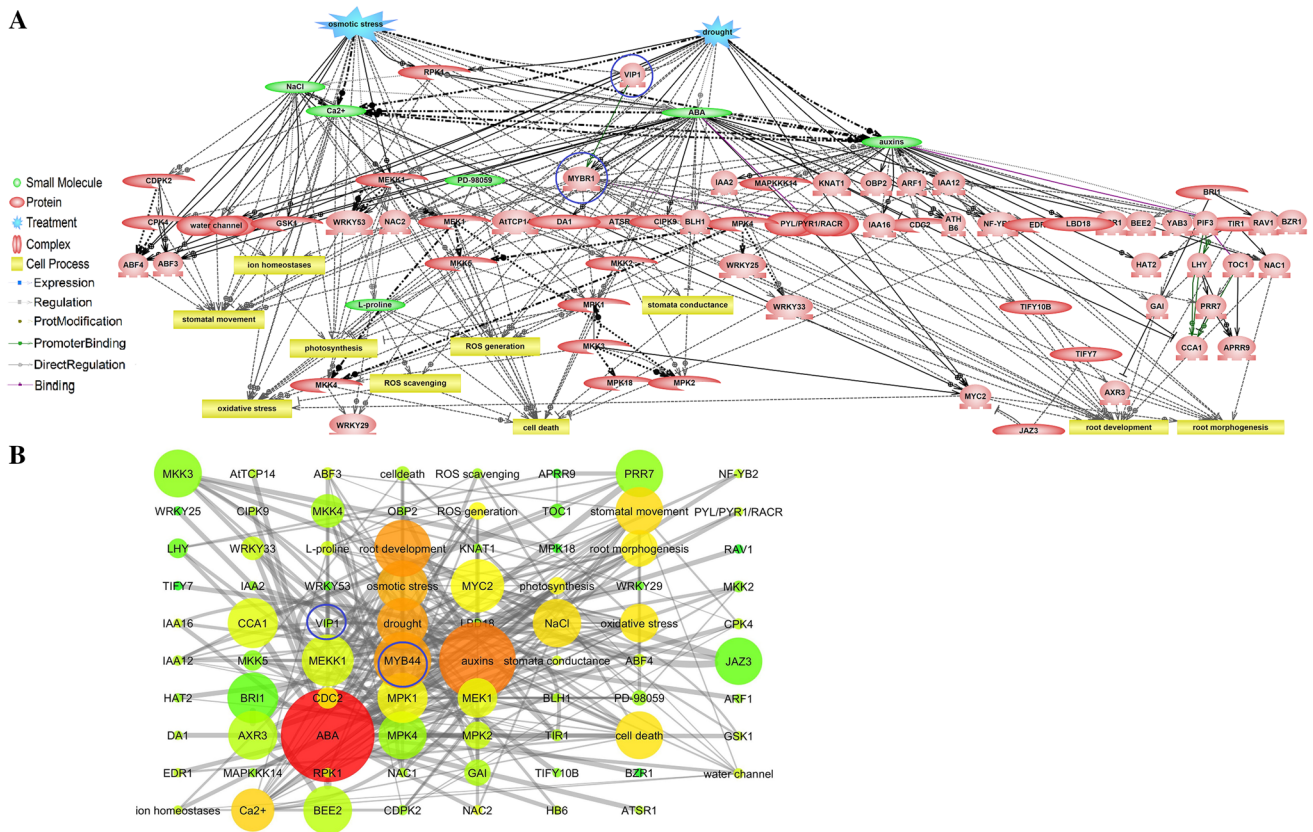
Fig. 1 Regulatory network analysis. **a** The hierarchical regulatory network based on the differentially expressed regulatory genes between *Arabidopsis thaliana* and *Eutrema salsugineum*. **b** The centrality analysis of the gene network: the nodes with bigger size indicate higher betweenness centrality. Red color represents the highest closeness centrality, while green color indicates the lowest value of closeness centrality. The edges with more thickness represent high values of edge betweenness centrality. The positions of *MYB44* and *VIP1* have also been shown. (Color figure online)

represented that signaling pathways of ABA and auxin hormones as well as Ca^{2+} ion govern most of the abiotic stress responses. The protein kinase gene, *RPK1*, and the transcription factor gene, *VIP1* were positioned at the forefront of the regulatory genes.

Topology analysis revealed that the network contained a total number of 81 nodes and 261 edges (supplementary 2). Among different components of the network, ABA, auxins, osmotic stress and drought showed the largest number of connectivity (more than 20 connections). Also, transcription factor *MYB44* had the largest number of connectivity (19 connections) among the genes. A summary of topology analysis of the regulatory network was illustrated in Fig. 2b. The nodes with bigger size indicated higher betweenness centrality and had greater influence. Betweenness is a measure of the relevance of a given node obtained by counting the number of shortest paths going through it to quantify the importance of a node in a network [41, 42]. In addition, the closeness centrality is scaled using gradient color-coding system in which, red color represents the highest closeness centrality, while green color indicates the lowest value of closeness

closeness centrality. The closeness of a node is defined as the inverse of the average distance from all other nodes [41, 54]. The edges with more thickness represent high values of edge betweenness centrality. Edge betweenness is defined as the number of shortest paths between pairs of nodes that run through that edge and is known as a standard measure of the impact of a node or a linkage in a network [41, 54]. Accordingly, topology analysis uncovered that ABA, auxin and NaCl nodes obtained high values for the centrality indices. Of those, ABA with the highest values was presumed as the main node in the network (Fig. 1b). Moreover, among the functional classes within the network; root development, stomatal movement, cell death and root morphogenesis had the maximum values for the centrality indices, respectively, illustrating the main strategies to cope with abiotic stresses based on the network's output. The results also revealed that the transcription factor *MYB44* achieved the highest centrality values implying its pivotal role in the regulatory network. As a result, *MYB44* and its direct regulator, the transcription factor gene *VIP1*, were selected to perform molecular cloning and characterization of their *B. napus* homologs.
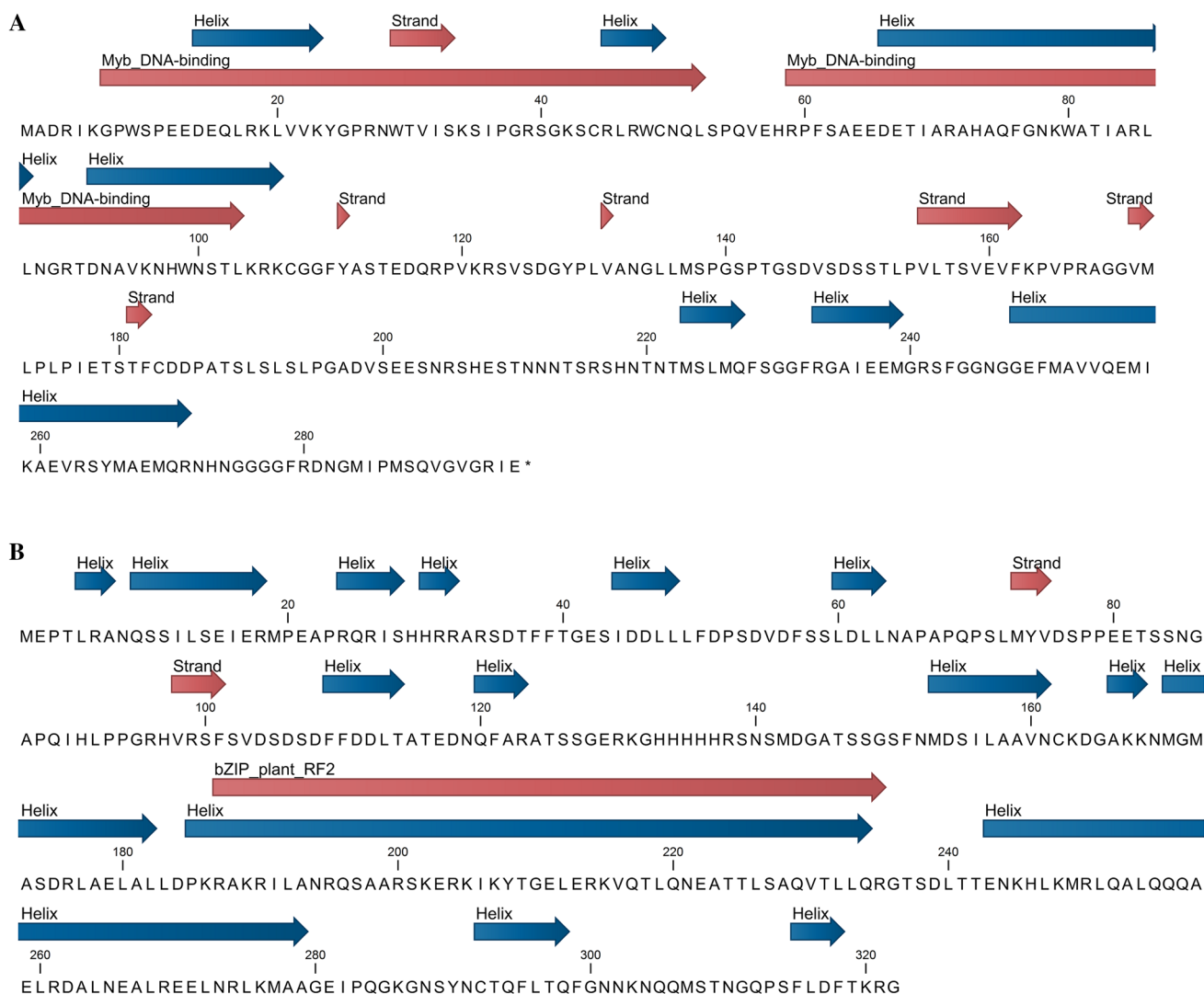
A

Helix — Strand — Helix — Helix
Myb_DNA-binding
20   40   60   80
MADR I KGPWS PEEDEQLRKL VVKYGPRNWTV I SKS I PGRSGKSCRL RWCNQL SPQVEHRPF SAEEDET I ARAHAQFGNKWAT I ARL

Helix Helix
Myb_DNA-binding   Strand   Strand   Strand   Strand
100   120   140   160
L NGRTDNAVKNHWNST LKRKCGGF YASTEDQRPVKRSVSDGYP L VANGL LMSPGSPTGSDVSDSST LPVL TSVEVFKPVPRAGGVM

Strand   Helix   Helix   Helix
180   200   220   240
LPLP I ETSTFCDDPATSLSLSLPGADVSEESNRSHESTNNNTSRSHNTNTMSLMQFSGGFRGA I EEMGRSFGGNGGEFMAVVQEM I

Helix
260   280
KAEVRSYMAEMQRNHNGGGGFRDNGM I PMSQVGVGR I E *

B

Helix Helix   Helix Helix   Helix   Helix   Strand
20   40   60   80
MEPTLRANQSS I LSE I ERMPEAPRQR I SHHRRARSDTFFTGES I DDLLLFDPSDVDFSSLDLLNAPAPQPSLMYVDSPPEETSSNG

Strand   Helix   Helix   Helix   Helix Helix
100   120   140   160
APQ I HLPPGRHVRSF SVDSDSDFFDDLTATEDNQFARATSSGERKGHHHHHRSNSMDGATSSGSFNMDS I LAAVNCKDGAKKNMGM

bZIP_plant_RF2
Helix   Helix   Helix
180   200   220   240
ASDRLAEL ALLDPKRAKR I LANRQSAARSKERK I KYTGELERKVQTLQNEATTLSAQVTLLQRGTSDLTTENKHLKMRLQALQQQA

Helix   Helix   Helix
260   280   300   320
ELRDALNEALREELNRLKMAAGE I PQGKGNSYNCTQFLTQFGNNKNQQMSTNGQPSFLDFTKRG

Fig. 2 The structural features of the deduced amino acid sequences of *BnMYB44* and *BnVIP1*. **a** BnMYB44 protein. The MYB-like DNA binding domains at positions 6–52 and 58–103 residues have been illustrated. **b** BnVIP1 protein. The b-ZIP domain was found at 205–297 amino acid residues

Cloning and sequence analysis of *BnMYB44* and *BnVIP1* genes

BnMYB44 (GenBank Accession Number: KT071672) was 891 bp in length and encoded a deduced protein of 296 amino acid residues with a predicted molecular weight of 32.457 kDa and isoelectric point of 9.02. BnMYB44 protein contained two conserved MYB-like DNA binding domains, identified at positions 6th -52nd and 58th -103rd amino acid residues, indicating that BnMYB44 protein is a member of MYB R2R3 subfamily whose members have two conserved DNA-binding domains [55]. The amino acid sequence and some structural features of BnMYB44 protein were shown in Fig. 2a.

Blast search showed that the *BnMYB44* gene had high homology (more than 80%) with other plant *MYB44* genes at nucleotide and amino acid levels. The structural analysis revealed that the *BnMYB44* gene was located on chromosome 7 of *B. napus* A genome and consisted of one exon, which resembled the genomic structure of *AtMYB44* gene [56]. Synteny analysis showed that orthologs of the *BnMYB44* in *B. napus* progenitor species, *B. rapa* and *B. oleracea*, were located on the chromosome 7 of their corresponding genomes.

The two isolated fragments of *BnVIP1* gene were assembled resulting in an open reading frame of 969 bp (GenBank Accession Number: KR537434). The deduced protein consisted of 322 amino acids with molecular weight of

35.988 kDa and isoelectric point of 7.89. One bZIP domain was detected within the protein of BnVIP1 (from 187th to 235th amino acid residue) implying that BnVIP1 is a member of the bZIP transcription factor family (Fig. 2b). *BnVIP1* gene and its homologs from other members of Brassicaseae family including *E. salsugineum, A. lyrata, Camelina sativa* and *(A) thaliana* shared more than 80% nucleotide sequence homology in the coding regions. At the amino acid level, BnVIP1 protein showed 88, 83, 75 and 73% identity with its homologs in *(B) rapa, E. salsugineum, A. lyrata* and *(A) thaliana*, respectively. The results also revealed that *BnVIP1* gene was located on the chromosome 8 of *(B) napus* C genome, originated from its progenitor, *B. oleracea*. The genomic structure of *BnVIP1*, similar to its Arabidopsis counterpart [57], contained four exons with different lengths. Moreover, orthologs of the *BnVIP1* in *B. rapa* and *B. oleracea* were located on the chromosome 8 of their corresponding genomes.

Physiological responses of *B. napus* to drought stress

A number of physiological characteristics were measured in the *B. napus* cultivars, SLM046 and Zarfam, under non-stress and drought stress conditions. The results showed that drought stress significantly affected all the physiological characteristics (Table 2). While no variation was observed between the cultivars for the physiological characteristics under non-stress conditions, the cultivars showed significantly different physiological responses to drought stress treatments (Table 2). RWC significantly decreased during drought stress conditions, however the cultivars exhibited different rates of reduction of RWC under more severe drought stress treatments (D2 and D3). SLM046 (stress-tolerant cultivar) had significantly higher amount of RWC than Zarfam, under such conditions. High accumulation of Proline content occurred during drought stress conditions, although there were different levels of proline accumulation between the cultivars. SLM046 showed significantly higher proline contents compared to Zarfam under more severe drought stress treatments. Cell membrane damage, as measured by EL, significantly increased under drought stress conditions. Zarfam showed much higher amount of EL than SLM046 indicating higher degree of cell membrane injury during drought stress (Table 2). Altogether, evaluation of the physiological characteristics revealed distinct responses of the *B. napus* cultivars to drought stress, as SLM046 was more efficiently capable of coping with drought stress effects and consequently showed more tolerance to drought compared to Zarfam. This finding provided physiological evidence to explain possible variation between the cultivars in the expression patterns of *BnMYB44* and *BnVIP1* genes in response to drought stress.

Expression patterns of *BnMYB44* and *BnVIP1* under drought stress

The expression patterns of *BnMYB44* and *BnVIP1* in response to drought stress were analyzed by qRT-PCR in the time course studies. Different expression profiles were observed for the genes not only between the cultivars but also between leaf and root tissues during drought stress period. In leaves of SLM046, *BnMYB44* expression was more quickly induced in response to drought stress and also showed significantly higher level of increase compared to that of the other *B. napus* cultivar (Fig. 3a). In roots of SLM046, *BnMYB44* expression was significantly upregulated 6 h after maintaining soil moisture at 40% of field capacity (D2) and then declined to the level with no significant difference with its transcript level under non-stress conditions (Fig. 3b). On the other hand, drought stress caused a significant downregulation in the expression of *BnMYB44* in root tissue of Zarfam.

The expression of *BnVIP1* gene in leaves of SLM046, rapidly and significantly responded to drought stress and showed the maximum level of transcript accumulation at the

Table 2 Physiological responses of *B. napus* to drought stress

	RWC (%)		Proline (µM/gFW)		EL (%)	
	SLM046	Zarfam	SLM046	Zarfam	SLM046	Zarfam
NS	90.52	93	0.58	0.63	14.3	14.32
D1	84.07	81.47	4.32	3.68	59.71	59.7
D2	80.2	71.01	5.71	4.29	60.77	70.86
D3	65.72	60.67	24	18.36	69.52	86.11
CV (%)	3.08		9.8		3.46	
LSD (5%)	4.18		1.31		3.46	

Mean comparison of relative water content (RWC), proline and electrolyte leakage (EL) measured in the *B. napus* cultivars, SLM046 and Zarfam under non-stress (NS) and three drought stress treatments including the time that soil moisture reached to 40% of field capacity (D1) as well as maintaining that for 6 h (D2) and 24 h (D3). For each trait, LSD value at $\alpha = 0.05$ has been calculated. Means with a difference greater than the given LSD value are significantly different at $\alpha = 0.05$
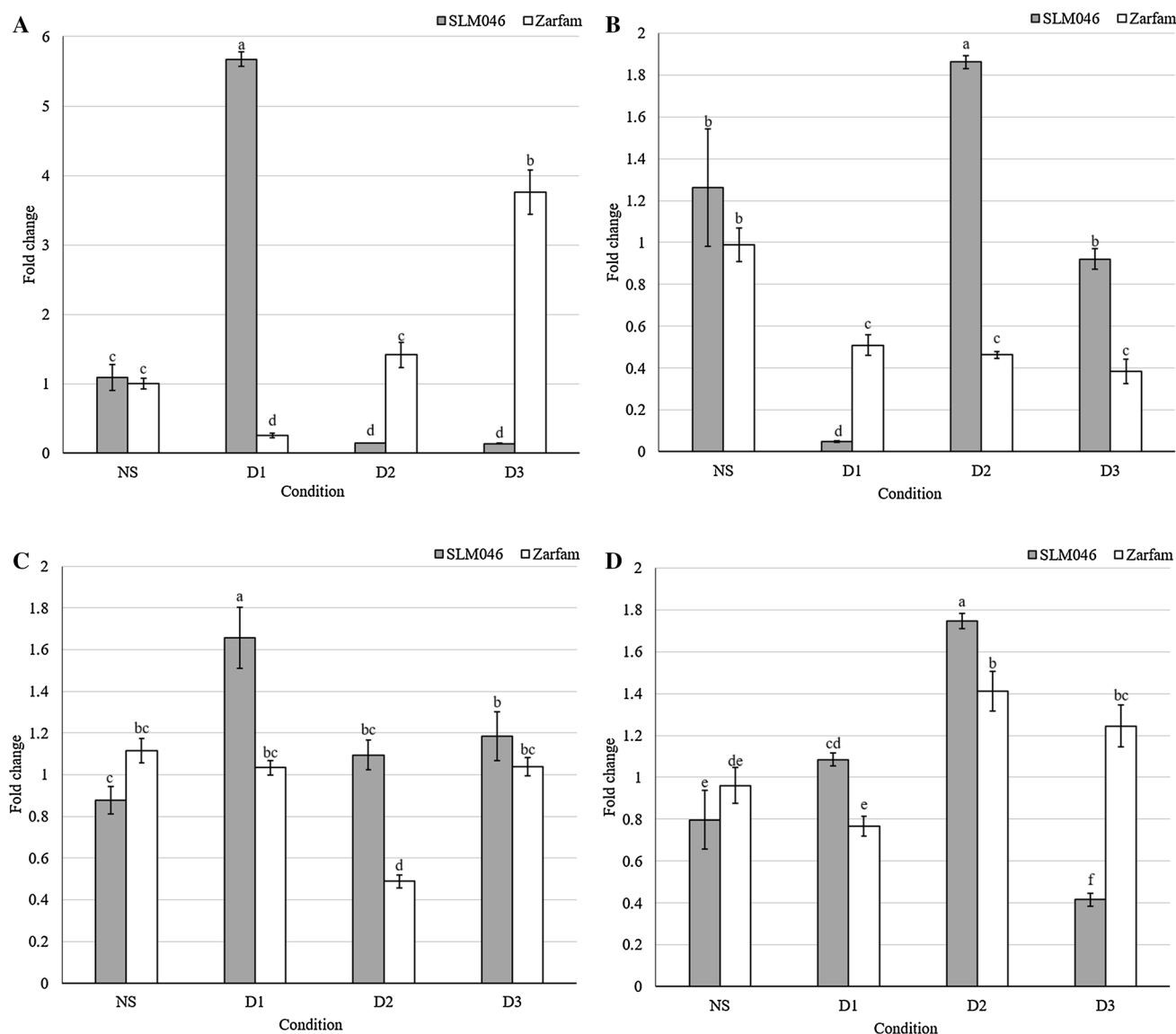
Fig. 3 The expression of *BnMYB44* and *BnVIP1* in response to drought stresses. **a** *BnMYB44* in leaves. **b** *BnMYB44* in roots. **c** *BnVIP1* in leaves. **d** *BnVIP1* in roots. The expression was determined by Real time quantitative RT-PCR. Three drought stress treatments include the time that soil moisture reached to 40% of field capacity (D1) as well as maintaining that for 6 h (D2) and 24 h (D3). Different letters indicate significant differences according to Duncan's test ($P < 0.01$)

first drought stress treatment (D1) (Fig. 3c). On the contrary, no induction was detected for the expression of *BnVIP1* during the drought stress period in leaves of Zarfam. The highest level of *BnVIP1* expression in roots of both cultivars was observed 6 h after maintaining soil moisture at 40% of field capacity (D2), however, Zarfam had a lower level of transcript accumulation (Fig. 3d).

Ion homeostasis status under salt stress

Salt stress had a significant effect on sodium and potassium contents of leaves and roots of the *B. napus* cultivars,

SLM046 and Zarfam. Consequently, K^+/Na^+ ratio, as a reliable indicator for salt tolerance in Brassica species [58], significantly decreased due to salt stress (Table 3). Significant variation was also observed between the cultivars for K^+/Na^+ ratio indicating their different levels of salt tolerance (Table 3). SLM046 showed higher leaf K^+/Na^+ ratios than those of Zarfam under salt stress treatments. While Zarfam showed a higher level of root K^+/Na^+ ratio than that of SLM046 under non-stress conditions, salt stress caused a dramatic fall in root K^+/Na^+ ratios of Zarfam which eventually resulted in slightly higher root K^+/Na^+ ratios for SLM046 under 3 and 6 h after salt stress induction. Overall,

Table 3 Ion homeostasis of *B. napus* under salt stress

	Leaf		Root	
	SLM046	Zarfam	SLM046	Zarfam
NS	21.7	12.91	11.28	23.32
S1	12.48	9.28	2.46	2.37
S2	3.02	2.95	1.47	1.04
S3	1.1	0.64	0.62	1.14
CV (%)	17.44		22.8	
LSD (5%)	2.44		1.1	

Mean comparison of K^+/Na^+ ratio measured in the *B. napus* cultivars, SLM046 and Zarfam under non-stress (NS) as well as three salt stress treatments including 3 h (S1), 24 h (S2) and 7 days (S3) after adding salt solution. For each trait, LSD value at $\alpha = 0.05$ has been calculated. Means with a difference greater than the given LSD value are significantly different at $\alpha = 0.05$

SLM046 exhibited more effective ion homeostasis, possibly associated with more tolerance to salt stress compared to Zarfam. This result helped to find out whether the expression patterns of *BnMYB44* and *BnVIP1* genes under salt stress may be associated with salt tolerance of the cultivars.

Expression patterns of *BnMYB44* and *BnVIP1* under salt stress

The expression patterns of *BnMYB44* and *BnVIP1* in response to salt stress were examined by qRT-PCR in leaves and roots of the *B. napus* cultivars. The results revealed significant differences for the expression trends of the genes between the cultivars and also between leaf and root tissues (Fig. 4). In leaves of SLM046 (the salt tolerant cultivar), *BnMYB44* expression rapidly and sharply increased and reached its maximum level at 3 h following salt stress (S1) in which the transcript accumulation of *BnMYB44* was almost seven times higher than that of non-stress conditions (Fig. 4a). *BnMYB44* expression was then significantly downregulated and showed the minimum transcript level at 24 h (S2) and 7 days (S3) after salt stress application. Similar trend was detected for *BnMYB44* expression in roots of SLM046, as *BnMYB44* expression exhibited a tenfold increase at S1 compared to non-stress conditions, and then significantly reduced during S2 and S3 treatments (Fig. 4b). On the other hand, *BnMYB44* expression in leaves of Zarfam showed no induction during salt stress period. Moreover, salt stress caused a significant reduction in *BnMYB44* expression in roots of Zarfam, although no significant variation was observed for its expression across the salt stress treatments (Fig. 4b).

BnVIP1 expression was significantly upregulated in leaves of SLM046 at 3 h post salt stress and then declined to finally reach the level with no significant difference of that under non-stress conditions at 7 days post salt stress

(Fig. 4c). On the contrary, *BnVIP1* expression was not induced in leaves of Zarfam by salt stress and exhibited no significant changes during salt stress (Fig. 4c). *BnVIP1* expression generally showed a declining trend in roots of both cultivars during salt stress period (Fig. 4d). No significant difference was detected for *BnVIP1* expressions between roots of the cultivars in each of the salt stress treatments (Fig. 4d).

Analysis of *BnMYB44* and *BnVIP1* promoters

The promoter regions of *BnMYB44* and *BnVIP1* genes were analyzed to eventually identify CREs relevant to stress responsiveness. The total number of 136 and 94 CREs from 37 to 26 different types were identified within *BnMYB44* and *BnVIP1* promoter sequences, respectively (Supplementary 3). The results showed that BnMYB44 promoter contained several hormone-responsive CREs associated with activity and regulation of ABA, gibberellin, salicylic acid and auxin suggesting that *BnMYB44* may be involved in hormone crosstalk. In addition, several biotic and abiotic stress responsive CREs involved in anaerobic, fungal, heat, low temperature and wounding stresses were found in BnMYB44 promoter indicating its possible contribution in response to environmental changes (Supplementary 3). Interestingly, one VIP1 response element (VRE) motif (TTGACC) was also detected within the *BnMYB44* promoter.

In contrast to *BnMYB44, BnVIP1* promoter contained few hormone related CREs (Supplementary 3). Also, there were a number of stress responsive CREs involved in fungal and abiotic stresses within *BnVIP1* promoter, however they were fewer than the ones within *BnMYB44* promoter (Supplementary 3). This is in accordance with the level of responsiveness of *BnMYB44* and *BnVIP1* to drought and salt stresses. The transcript accumulation of *BnMYB44* and *BnVIP1* genes under drought and salt stress conditions revealed that *BnMYB44* gene was significantly more responsive to these stresses than *BnVIP1* gene.

According to the statistical pairwise test [53], eleven CREs with significantly different numbers between the two promoters of *BnMYB44* and *BnVIP1* genes were identified (Table 4). The six significant CREs, involved in various biological processes, namely BOXII, G-BOX, AS-2-BOX, E-box, GT1-MOTIF and ABRE were more abundant in the promoter of *BnMYB44*. On the other hand, the CREs including CAAT-BOX, HSE, GAG-MOTIF, CGTCA-MOTIF and TGACG-MOTIF had higher number of occurrences in the *BnVIP1* promoter. The result of the pairwise comparative promoter analysis coupled with the expression data of *BnMYB44* and *BnVIP1* genes will be further discussed in order to explain biological significance of some of the differentially significant CREs.
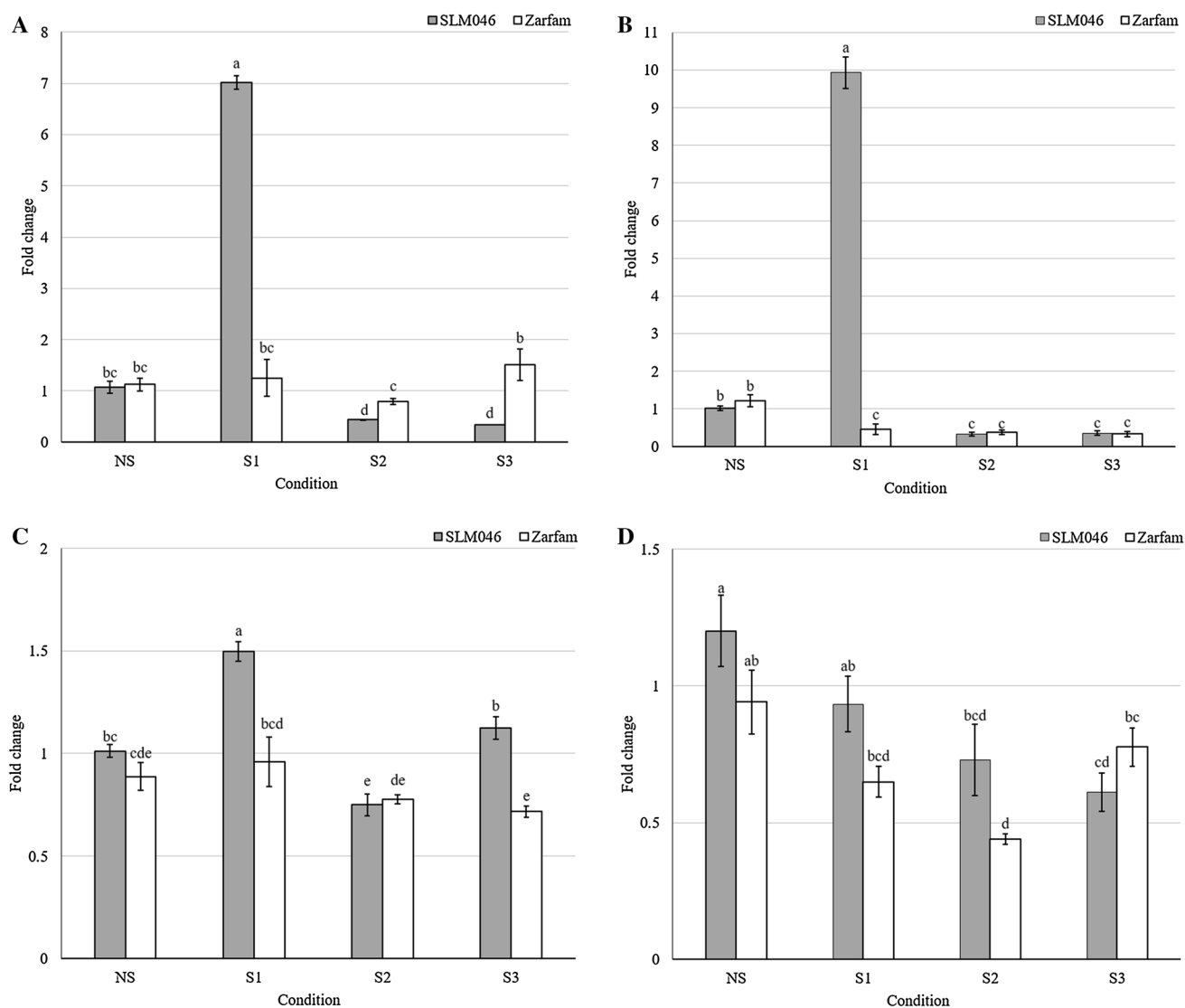
Fig. 4 The expression of *BnMYB44* and *BnVIP1* in response to salt stresses. **a** *BnMYB44* in leaves. **b** *BnMYB44* in roots. **c** *BnVIP1* in leaves. **d** *BnVIP1* in roots. The expression was determined by Real time quantitative RT-PCR. Three salt stress treatments include 3 h (S1), 24 h (S2) and 7 days (S3) after adding salt solution. Different letters indicate significant differences according to Duncan's test (P < 0.01)

Table 4 The list of the CREs with significantly different numbers between the promoter sequences of *BnMYB44* and *BnVIP1*

CRE	Function	The number of the CRE		q-Value
		BnMYB44	*BnVIP1*	
BOXII	Light responsive element	10	0	0/002
G-BOX	Light responsive element	10	2	0/004
AS-2-BOX	Involved in shoot-specific expression	3	0	0/01
CAAT-BOX	Cis-acting element/enhancer	16	23	0/001
E-box	Role in circadian clock	6	2	0/01
GT1-MOTIF	Light responsive element	4	0	0/006
HSE	Heat stress responsiveness	1	2	0/01
ABRE	Abscisic acid responsiveness	3	2	0/01
GAG-MOTIF	Light responsive element	1	3	0/007
CGTCA-MOTIF	MeJA-responsiveness	0	2	0/008
TGACG-MOTIF	MeJA-responsiveness	0	2	0/009

Discussion

Transcriptomics based networks have been widely used to elucidate plant responses to abiotic stresses [8, 59–62]. In this study, the regulatory network derived from the microarray data from Arabidopsis and *E. salsugineum*, shed light on the significance of some regulatory components involved in metabolisms and signaling of ABA and auxin hormones, root development and morphogenesis, stomatal movements, oxidative stress, cell death and Ca^{2+} signaling. Based on the topological features of the network, MYB44 transcription factor was known as the network bottleneck and the network central protein. *MYB44* and its upstream regulator gene, *VIP1* had significantly higher levels of expressions in *E. salsugineum* compared to Arabidopsis. Previous findings have indicated that *E. salsugineum* homologs of Arabidopsis stress-responsive genes are highly expressed under normal growth conditions [19, 20]. Arabidopsis MYB44 is a R2R3-MYB transcription factor gene expressed in different plant tissues and after various treatments [63]. Arabidopsis plants overexpressing *MYB44* gene showed tolerance to abiotic stresses [64]. Moreover, transgenic rice plants harboring *AtMYB44* gene exhibited significantly enhanced tolerance to drought stress [65]. As shown in the regulatory network, VIP1 transcription factor was located upstream of MYB44 (Fig. 1a). Arabidopsis VIP1 is a bZIP protein known for its role in nuclear import of T-DNA during Agrobacterium-mediated plant transformation [57]. Tsugama et al. [66] suggested that VIP1 functions as a regulator of osmo-sensory signaling in Arabidopsis through up-regulating of osmotic stress responsive genes. The phosphorylated form of VIP1 translocates from cytoplasm to nucleus in a stress dependent manner and regulates target genes such as *MYB44* [67, 68].

This study has provided evidence indicating that *BnMYB44* and *BnVIP1* genes may be involved in adaptive responses to abiotic stresses in *B. napus*, however it is essential to perform overexpression and knockout experiments to resolve the direct involvement of these genes in tolerance mechanisms to abiotic stresses. The expression analysis showed that *BnVIP1* was quickly induced under drought stress in the drought tolerant *B. napus* cultivar (SLM046). Accordingly, a strong and quick upregulation of *BnMYB44* expression was observed in SLM046 compared to the drought sensitive *B. napus* cultivar (Zarfam). Arabidopsis Plants overexpressing *VIP1* also accumulate high levels of *MYB44* transcripts [68]. VRE is known as the specific binding site of Arabidopsis VIP1 protein and exists within the promoters of VIP1 target genes such as *Trxh8* and *MYB44* [68]. The existence of one VRE motif

within the *BnMYB44* promoter suggested that *BnMYB44* transcription may be influenced by *BnVIP1*.

The different expression patterns of *BnVIP1* and *BnMYB44* genes between the cultivars during drought stress were somewhat associated with the physiological responses of the cultivars. The results indicated that SLM046 exhibited higher cell membrane stability and leaf relative water content under drought stress conditions in comparison with Zarfam. High relative water content and proline over-accumulation have been considered as the beneficial drought and salinity tolerance indicators in *B. napus* [69, 70]. This was evidenced by higher level of proline concentration in leaves of the drought tolerant cultivar during drought stress compared to the drought sensitive cultivar. Osmotic stress-induced proline accumulation is partially modulated by an ABA signaling pathway [71]. Furthermore, a meaningful relationship has been found between leaf relative water content and stomatal closure in various plant species [72]. stomatal closure in response to abiotic stress conditions is a powerful strategy to reduce water loss and to adjust water status in plants [73]. Stomatal closure is mainly under control of an ABA-positive signaling gene, *SnRK2* (SNF1-related protein kinase 2) which is kept inactive by the type 2C protein phosphatase genes (PP2Cs) known as negative regulators of ABA signaling [74, 75]. It has been previously reported that MYB44 transcription factor plays a role in the ABA-mediated signaling pathway in Arabidopsis via reducing the expression of PP2Cs genes [64]. Arabidopsis plants overexpressing *MYB44* exhibited enhanced stomatal closure [63] and reduced level of superoxide [76].

BnMYB44 and *BnVIP1* genes showed significantly different expression profiles between roots of the drought tolerant and sensitive *B. napus* cultivars. Interestingly, it has already been unveiled that an ABA receptor, PYL8, recruits MYB44, MYB77 and MYB73 transcription factors to reinforce auxin signaling and thereby promoting lateral root growth under water limited conditions in Arabidopsis [77]. In addition, Arabidopsis MYB44 is involved in regulation of root architecture by modulating antagonistic interaction between salicylic acid and jasmonic acid signaling [78]. It is required to measure root morphological characteristics of the cultivars as well as performing in-depth analyses about possible functions of *BnVIP1* and *BnMYB44* in relation to root architecture of *B. napus*.

Homeostasis of sodium and potassium ions is of particular importance for plant salt tolerance [79]. The results demonstrated that the *B. napus* cultivars had different levels of salt tolerance evidenced by K/Na ratio. The significant differences observed in the salt-induced expression levels of *BnMYB44* and *BnVIP1* between the *B. napus* cultivars may be related to salt stress response of the cultivars. The cultivar with higher salt tolerance (SLM046) generally showed higher levels of *BnMYB44* and *BnVIP1* transcripts

during salt stress period. Further investigations are required to determine the regulatory roles of *BnVIP1 and BnMYB44* under salt stress conditions. The previous reports discovered that *AtMYB44* gene was activated under salinity [63] and Arabidopsis plants overexpressing *AtMYB44* showed enhanced salt tolerance [76]. It has recently been reported that salt stress induces the eviction of H2A.Z containing nucleosomes from the promoter region of *AtMYB44* leading to transcript accumulation of this gene [80].

Computational-based promoter analysis can provide valuable knowledge for understanding and prediction of gene expression pattern [81–83]. The expression profiles of *BnVIP1* and *BnMYB44* under drought and salt stress conditions revealed that *BnMYB44* was more responsive to such stresses. In accordance with this finding, the stress related CREs were generally more over-represented within the *BnMYB44* promoter. In particular, some of the CREs with significantly different number of occurrences between the *BnVIP1* and *BnMYB44* promoters may contribute to the level of responsiveness of these genes. The number of occurrences of b-ZIP transcription factor binding sites including G-BOX, BOXII and ABRE within the *BnMYB44* promoter was significantly greater than those within the promoter of *BnVIP1*. G-BOX and ABRE are the ABA responsive CREs that have been found in promoter regions of several ABA signaling genes such as *MYB2* and *MYC2* [84, 85]. These CREs are involved in early response to abiotic stresses to activate a broad range of adaptation mechanisms [86]. The greater number of occurrences of these CREs within the *BnMYB44* promoter, compared to *BnVIP1* promoter, may be connected with its faster induction under stress conditions. The greater numbers of CAAT-Box element, which is a common enhancer [87], within the *BnVIP1* promoter may likely be involved in transcribing *BnVIP1* at sufficient level to provide cytoplasmic concentration of VIP1 as a protein at the forefront of the signaling pathways.

Conclusion

In conclusion, the comparative transcriptome analysis of stress-sensitive (Arabidopsis) and -tolerant (*E. salsugineum*) model plants resulted in the regulatory network as a reliable source of the regulatory genes for further investigations in the agronomically important species such as *B. napus*. This study is the first report on cloning and expression analysis of *B. napus* transcription factor genes, *BnMYB44* and *BnVIP1*, aimed at elucidating their functions in response to drought and salt stresses. The expression patterns of *BnMYB44* and *BnVIP1* genes of the two *B. napus* cultivars under drought and salt stress conditions along with the data obtained from the measurements of the stress-related physiological characteristics as well as analysis of the *BnMYB44* and *BnVIP1*

promoters suggested that *BnMYB44* and *BnVIP1* genes may contribute to responses to drought and salt stresses in *B. napus*. Further detailed analyses are required to fully understand the roles and importance of *BnMYB44* and *BnVIP1* genes in stress regulatory pathways.

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