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ORIGINAL PAPER

Investigation of the expansin gene family in sugar beet (*Beta vulgaris*) by the genome-wide level and their expression responses under abiotic stresses

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Abstract
Sugar beet (*Beta vulgaris* ssp. *vulgaris*) is primarily used in sugar production worldwide. Expansins are a gene family of cell wall proteins effective in regulating cell wall structure. They also participate in developmental stages, including cell and leaf growth, root development, and fruit ripening. This study comprehensively characterizes the expansin gene family members found in the sugar beet genome. In addition, in silico expression analysis of sugar beet expansin genes under variable abiotic stress conditions and expression profiles of expansin genes under combined drought and heat stresses by the qRT-PCR method were evaluated in the study. A total of 31 sugar beet expansin genes were identified. *BvuEXLA-02* and *BvuEXLB-02* genes can have abiotic stress tolerance roles besides their roles in normal development. Determining the properties of sugar beet expansin, family members can help enable the cellulose hydrolysis mechanism and raise plant biomass. Elucidating expression profiles of the sugar beet expansin genes under variable stress conditions can support improving plant productivity. The results of the current study may also contribute to the deep understanding of sugar beet expansin genes in the future.

Keywords Sugar beet · Expansin · Genome-wide characterization · Gene expression

Introduction

Sugar beet (*Beta vulgaris* ssp. *vulgaris*), a biennial plant, is regarded in the family of Chenopodiaceae. It is the most widely used plant in the sugar industry worldwide and is one of the two primary sources of sucrose (sugar) in human consumption along with sugar cane (Erdal et al. 2007; Holtgräwe et al. 2014). The sugar ingredient of sugar beet is much higher than sugar cane and is especially used in human food, animal feed, and the animal feed industry (Erdal et al. 2007). Besides, sugar production from sugar beet creates waste products. Ethanol production, a renewable energy source from sugar beet waste, further increases the interest in sugar beet production and makes the production important.

Expansin family members are a cell wall protein first determined in cucumber hypocotyls (Sampedro et al. 2006; Santiago et al. 2018). It is a superfamily of proteins mainly found in plants, bacteria, fungi, and amoeba (Armijos-Jaramillo et al. 2018). They play a role in loosening, remodeling with in vivo cell expansion, and joining by means of disrupting the non-covalent hydrogen bonds between cellulose and hemicellulose polymers in the plant cell wall in a pH-dependent manner. In addition, expansins show activity under plant biotic and abiotic stress factors including drought, salt, and pathogenicity. They are commonly assigned in the root development, control of plant cell growth, leaf growth, cell decomposition, cell wall thawing, fruit maturing, seed germination, and other developmental processes (Sampedro et al. 2006; Liu et al. 2016; Santiago et al. 2018). The proteins have two domains in front of the signal peptide: Domain I and Domain II. Domain I is the N-terminal domain (D1, ~ 15 kDa) and has a six-helix double psi beta-barrel structure (DPBB-double psi beta-barrel). Domain I involves the His-Phe-Asp (HFD, histidine-phenylalanine-aspartate) motif and a highly protected, cysteine-rich region that helps to stabilize the binding domain by constituting disulfide bonds. Domain II is the C-terminal domain (D2, ~ 10 kDa) and has four tryptophan residues at the C-terminal, and is also widely associated

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with the family of pollen allergens (Cosgrove 2000, 2005; Krishnamurthy et al. 2015; Santiago et al. 2018). Expansin proteins are divided into four families according to the nomenclature based on their phylogenetic relationship: alpha expansin (α-expansin, EXPA), beta expansin (β-expansin, EXPB), expansin-like A (EXLA), and expansin-like B (EXLB) (Kende et al. 2004). EXPA and EXPB constitute two large families of plant expansin genes and they have roles in cell expansion and other developmental processes that do not require enzymatic activity. EXPAs diverge from other expansins by having a large α-insertion in domain I and deletion in its surroundings. In addition, the preserved HFD motif is found in EXPA and EXPB, but not in EXLA and EXLB (Krishnamurthy et al. 2015; Ding et al. 2016).

Plant growth, product yield, membrane integrity, and photosynthesis are influenced by drought which is a severe abiotic stress factor (Praba et al. 2009). Severe water deficiency suppresses cell proliferation by inhibiting water flow to elongating cells. High temperature is another important abiotic stress factor and has mainly negative effects on plant growth, yield, quality of products, and seed germination (Hasanuzzaman et al. 2013). In fact, plants are often exposed to a combination of natural abiotic stress factors. Combined heat and drought stresses have more severe effects on plant growth stages and productivity than the effects of these stresses alone (Mittler 2006).

The genome size of the sugar beet is 758 Mbp and its whole genome sequence was published in 2014 (Dohm et al. 2014). Expansin gene family members were identified in sugar beet and their expression levels were studied in normal tissues and rhizomania of sugar beet, caused by Beet necrotic yellow vein virus (BNYVV) in a previous study (Fernando et al. 2018). As far as we know, expansin gene family members have not yet been comprehensively elucidated regarding their properties and abiotic stress responses in sugar beet. This study aims to characterize the expansin gene family members in sugar beet plants using genomic data. Besides, expression profiles of expansin genes under variable abiotic stress conditions were determined in the current study by in silico method. Moreover, real-time PCR method (qRT-PCR) was applied to evaluate the roles of expansin genes under combined drought and heat stress conditions in drought-sensitive and drought-tolerant sugar beet cultivars. This study may offer insight into future studies to increase sugar beet biomass.

Material and methods

Identification, characterization, and chromosomal position of expansin genes in sugar beet

Peptide sequences of the sugar beet were obtained from the sugar beet genome database (https://bvseq.boku.ac.at/) (Dohm et al. 2014). Expansin peptide sequences were determined by Pfam Domain Search analysis in QIAGEN CLC Genomics Workbench 11.0.1 (QIAGEN, Aarhus, Denmark) (https://digitalinsights.qiagen.com/). Sequences that have both domains of DPBB-1 and Pollen_allerg_1 (PF03330 and PF01357) were determined as expansin proteins (Lv et al. 2020). Determined peptide sequences were classified into four expansin subgroups (alpha expansin, beta expansin, expansin-like alpha, and expansin-like beta) using BLASTp. Expasy Prot Param tool server was used to specify gene characteristics such as amino acid length and estimated isoelectric point of the expansin sequences (Gasteiger et al. 2005). BLASTp search was performed using the sugar beet genome database to determine the chromosomal distributions of the expansin genes determined in the sugar beet. Afterward, the localization of these genes on chromosomes was displayed by using Tbtools (v 1.09876) Program (Chen et al. 2020).

Determination of gene structure, preserved motifs, and homology modeling

The exon and intron organization of the expansin gene family members were identified by Gene-Structure Display Server (http://gsds.gao-lab.org/) which was performed by comparing coding (CDS) and genomic sequences (Hu et al. 2015). Multiple Em for Motif Elicitation (MEME) database (https://meme-suite.org/meme/tools/meme) was used as a motif scanning tool to define the preserved motifs (Bailey and Elkan 1994). The maximum motif number was determined as 20, and the width of motifs was selected as between ≥ 2 and ≤ 300. The estimated three-dimensional structure of the expansin proteins was predicted by the Phyre2 database (ProteinHomology/AnalogY Recognition Engine; http://www.sbg.bio.ic.ac.uk/phyre2) using the intensive mode (Kelley et al. 2015).

Sequence alignment and phylogenetic tree analysis

Sugar beet expansin proteins were aligned on the Mega7 program by the ClustalW method (Kumar et al. 2016). The maximum likelihood method was utilized to create a phylogenetic tree after alignment. In this method, 1000 bootstraps (which is the boot tab of 1000 repetitions) and

the Jones-Taylor-Thornton (JTT) substitution model were selected. Subsequently, the phylogenetic tree was transferred to the Interactive Tree of Life (iTOL; https://itol.embl.de/) and visualized with the same web-based tool (Letunic and Bork 2019).

Gene ontology (GO) analysis

Functional analyses of expansin proteins in sugar beet were performed by the Blast2GO program (Conesa and Götz 2008). Firstly, matches with the existing arrays in the program (BLASTp) were identified. Afterward, maps were generated by BLAST results (MAPPING). Finally, an information file comprising these arrays was created (ANNOTATION). GO classification categorized expansin proteins in sugar beet into biological function, cellular component, and molecular function.

Identification of miRNAs targeting expansin genes in sugar beet

Plant miRNA precursors were determined using miRBase v22.1 (http://www.mirbase.org/) to find miRNAs targeting sugar beet expansin genes (Kozamara et al. 2019). Besides, all plant miRNAs and expansin gene transcripts were aligned by using the psRNATarget (Plant Small RNA Target Analysis Server) (https://www.zhaolab.org/psRNATarget/) database and all plant and sugar beet miRNAs were identified (Dai et al. 2018). Then Cytoscape software was used for a schematic representation of the miRNAs targeting expansin genes in sugar beet (Shannon et al. 2003).

Calculation of homologous and non-homologous substitution rates

To calculate homologous (Ks) and non-homologous (Ka) substitution rates, firstly, the ClustalW-based multiple sequence alignment program was applied to align orthologous pairs among expansin genes in sugar beet. Then, the amino acid sequences of orthologous pairs were aligned between sugar beet expansins and expansin proteins from *Arabidopsis* (*Arabidopsis thaliana)*, poplar (*Populus trichocarpa)*, rice (*Oryza sativa)* and maize (*Zea mays)*. Afterward, PAL2NAL (http://www.bork.embl.de/pal2nal) tool was utilized to predict (Ks) and (Ka) substitution rates by aligning amino acid sequences and original complementary DNA (cDNA) sequences (Suyama et al. 2006). The time of divergence and duplication of each expansin gene (T, million years ago, Mya) was calculated by the formula of $T = Ks/2\lambda$ ($\lambda = 6.5 \times 10^{-9}$) (Lynch et al. 2000). Tandem and segmental duplications were visualized using TBtools (v 1.09876) (Chen et al. 2020).

Expression profiling of sugar beet expansin genes using transcriptome data

Transcriptome data analysis was achieved to determine the expression profiles of sugar beet expansin genes. Essential data for RNA-Seq analysis was acquired from SRA (Sequence Read Archive) open resources. The accession numbers of the Illumina HiSeq and Roche454 readings are as follows: SRP044105 (Minoche et al. 2015), SRP115884 (Li et al. 2019), SRP235645 (Xing et al. 2020). All readings were in ".sra" format and the raw sequence data were downloaded. Afterward, these data were converted to "fastq" format. Insufficient quality readings (Phred quality (Q) score < 20) were eliminated. Reading quality was checked by performing FastQC analysis on all remaining clean reads. CLC Genomic Workbench v11 program was used for normalizing and transforming all readings, and then, by converting RPKM values to log2 with a hierarchical clustering map (heatmap), gene expression measurements were made with the Permut Matrix program. Expansin genes whose expression are frequently increased under different stress conditions were selected according to transcriptome data, and gene expression profiles under combined drought and heat stress conditions were examined by qRT-PCR analysis.

Growth conditions of sugar beet and stress treatments

Drought-sensitive and drought-tolerant sugar beet cultivars were kindly supplied from Sugar Institute (Türkiye Şeker Fabrikaları A. Ş., Ankara). The seeds of the cultivars were cleaned and waited in distilled water for 2 h. The seeds were transferred to the plastic pods, including vermiculite. Then, pods were kept in climate cabinet conditions (24 ± 2 °C with a 16 h light and 8 h dark photoperiod, 400 µmol m^{-2} s^{-1} light intensity and 55%–80% relative humidity) and flooded by Hoagland solution (Hoagland et al. 1950) every day for 4 weeks. For combined drought and heat stresses, a Hoagland solution containing 20% polyethylene glycol 6000 (PEG-6000) was applied to the plants, then plants were kept at 50 °C in a climate cabinet. Plant leaf samples were collected at 0, 1 h, and 2 h after the stress treatment (Altunoğlu et al. 2019). 0 hour samples were used as control samples in the experiments. Liquid nitrogen was utilized to freeze samples immediately, and then samples were kept at − 80 °C for further analysis.

Determination of expression patterns of expansin genes under combined drought and heat stresses by qRT-PCR

Total RNA was obtained from leaf samples by Trizol reagent (ABP Biosciences, USA) following the manufacturers'

instructions and experience in previous studies in our laboratory (Baloğlu et al. 2015; Altunoglu et al. 2018). The purity and concentration of the isolated samples were measured by a spectrophotometer (MultiScanGo, Thermo Fisher Scientific, USA). *DNase I* enzyme (RNase-free, Thermo Fisher Scientific, USA) was used to digest DNA from the RNA samples. cDNA production from RNA samples was achieved by RevertAid First Strand cDNA Synthesis Kit (ThermoFisher Scientific, USA). Primers for expansin genes were designed by the NCBI Primer Blast tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). Primer sequences of reference genes (*GAPDH* and *Actin1*) (Ghaemi et al. 2020; Li et al. 2021) and sugar beet expansin-specific genes were presented in Supplementary Material 1. After cDNA production, the qRT-PCR reaction was carried out by using SYBR Green master mix (Roche Applied Science, Germany) in the Qiagen Rotor-Gene 6000 Real-Time PCR device with the following temperature cycles: 5 min at 95 °C for the first denaturation followed by 10 s at 95 °C for denaturation, 20 s at 54 °C for annealing and 10 s at 72 °C for elongation, which was repeated 40 cycles. Also, melting curve analyses were added for each PCR to control non-specific amplifications. Ct (cycle threshold) values were normalized by the control status and reference gene. ΔCT and ΔΔCT [$\Delta CT = CT_{sample} - CT_{reference}$ and $\Delta\Delta CT = \Delta CT_{stressed\ sample} - \Delta CT_{control}$ (0 h)] were estimated and differences between relative expression profiles were stated as $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen 2001). Three biological and three technical replicates were used for every sample in the experiments.

Statistical analysis was conducted using the One-way Anova method in the Minitab 18 package program. If the p-value was < 0.05, the expression difference between control and stressed samples was interpreted as meaningful.

Results and discussion

Gene and protein properties of the expansins

Based on comprehensive searches, 31 peptide sequences were determined as sugar beet expansin genes. It was determined that 19 sequences belong to EXPA, 2 to EXPB, 7 to EXLA, and 3 to EXLB subgroup. The properties (localization on chromosomes, protein lengths, estimated isoelectric points, and stability index) of the sugar beet expansin gene family members were shown in Supplementary Material 2. According to the results, their amino acid lengths were between 179 and 272 aa. Isoelectric points (*pI*) of expansin proteins changed between 4.5 and 10.39. While *pI* values were higher than 7 in most EXPA subgroup members (15 proteins in 19), *pI* values were variable in EXPB subgroup members. All EXLA subgroup members were in basic character (> 7), whereas all EXLB subgroup members were in acidic character (< 7). Molecular weights of the proteins showed variation between 19.92 and 29.33 kDa. Except for BvuEXPA-04 and BvuEXPA-05, all proteins exhibited a stable state. It has been observed that 29 genes exist on chromosomes. However, *BvuEXPA-18* and *19* genes were at the scaffold level. The highest gene density was found on chromosome 9 with 9 genes. It was followed by the fifth and second chromosomes containing 7 and 4 genes, respectively. The first and eighth chromosomes containing only one gene, were also identified as chromosomes with the lowest number of genes (Fig. 1a).

When considering the previous studies, there were 92 expansin genes belonging to the sugarcane plant and 51 were in the EXPA subgroup (Santiago et al. 2018). The total amount of expansin proteins found in apple fruit was 41, 34 of which belong to EXPA subgroup (Zhang et al. 2014a, b). In addition, it has been reported that 26 out of 36 expansins in *Arabidopsis* belong to EXPA (Li et al. 2002), 27 out of 36 expansins in poplar (Sampedro et al. 2006), 20 out of 29 expansins belong to EXPA in grapevine (Dal Santo et al. 2013). In the current study, the number of expansin family members was compatible with the expansin gene family numbers in the studied plants. 19 EXPA subgroup members were determined in the sugar beet genome, consistent with previous studies. Based on this fact, it can be said that the EXPA subgroup of proteins primarily represents plant expansin proteins. *pI* values of expansin members in common wheat (*Triticum aestivum* L.) were between 4.64 and 9.78 and displayed a weakly alkaline character (Han et al. 2019). According to the study about expansin proteins in jujube (*Ziziphus jujuba* Mill.), it was observed that all members of jujube EXPA, EXPB, and EXLA subgroup members had *pI* values above 7.0, whereas the *pI* values of the most members of the EXLB subgroup were below 7.0 (Hou et al. 2019). These findings are pertinent to those obtained from expansins in sugar beet, and it can be said that members of different subgroups have different physiological characteristics for activity.

Gene structure, preserved motifs, and homology modeling

The gene structure of sugar beet expansins was shown in Fig. 2. According to the analysis, all sugar beet expansin genes contain introns. It was observed that *BvuEXPA*s generally have 2 introns. *BvuEXLA*s generally had 4 introns, whereas all the *BvuEXPB* genes and most of *BvuEXLB*s had 3 introns. Based on exon structure, most *BvuEXPA*s had 3 exons, while all *BvuEXPB*s had 4. *BvuEXLA*s and *BvuEXLB*s usually possessed 5 and 4 exons, respectively. The study conducted on wheat (*Triticum aestivum* L.) determined that the members of the same subgroup had very similar patterns

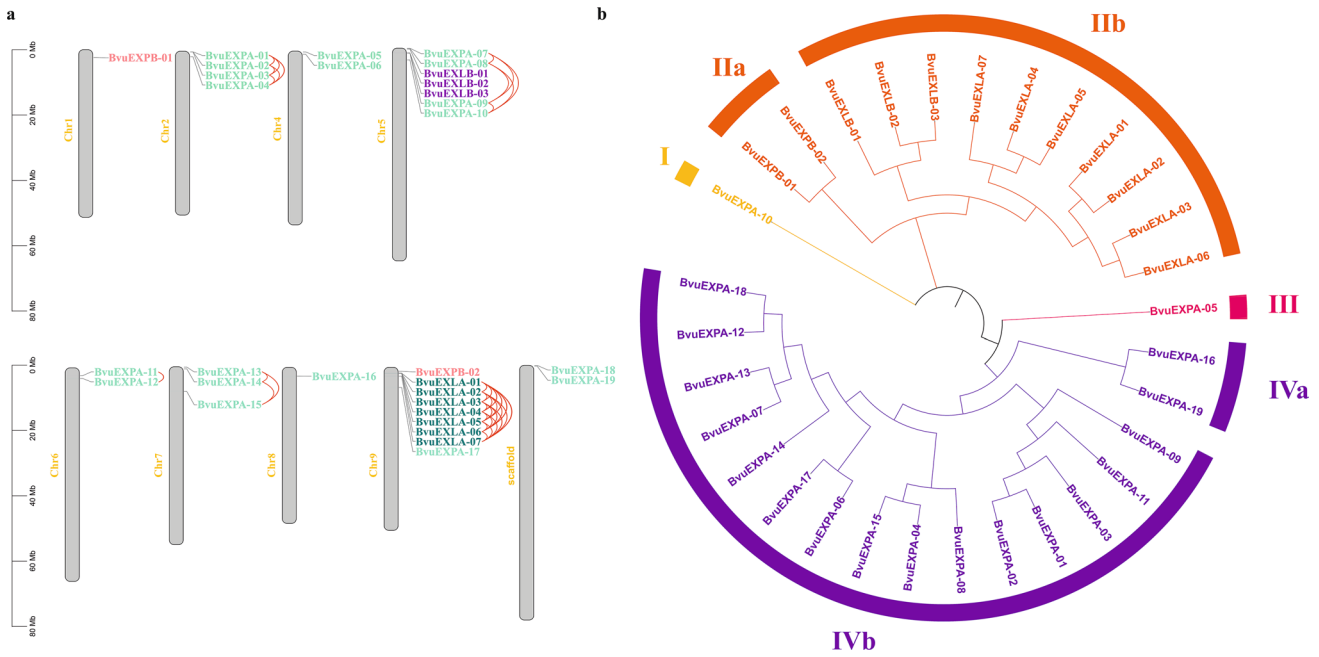

Fig. 1 **a** Visualization of chromosomal position for expansins of sugar beet and tandem duplications **b** Phylogenetic tree visualization of sugar beet expansin family

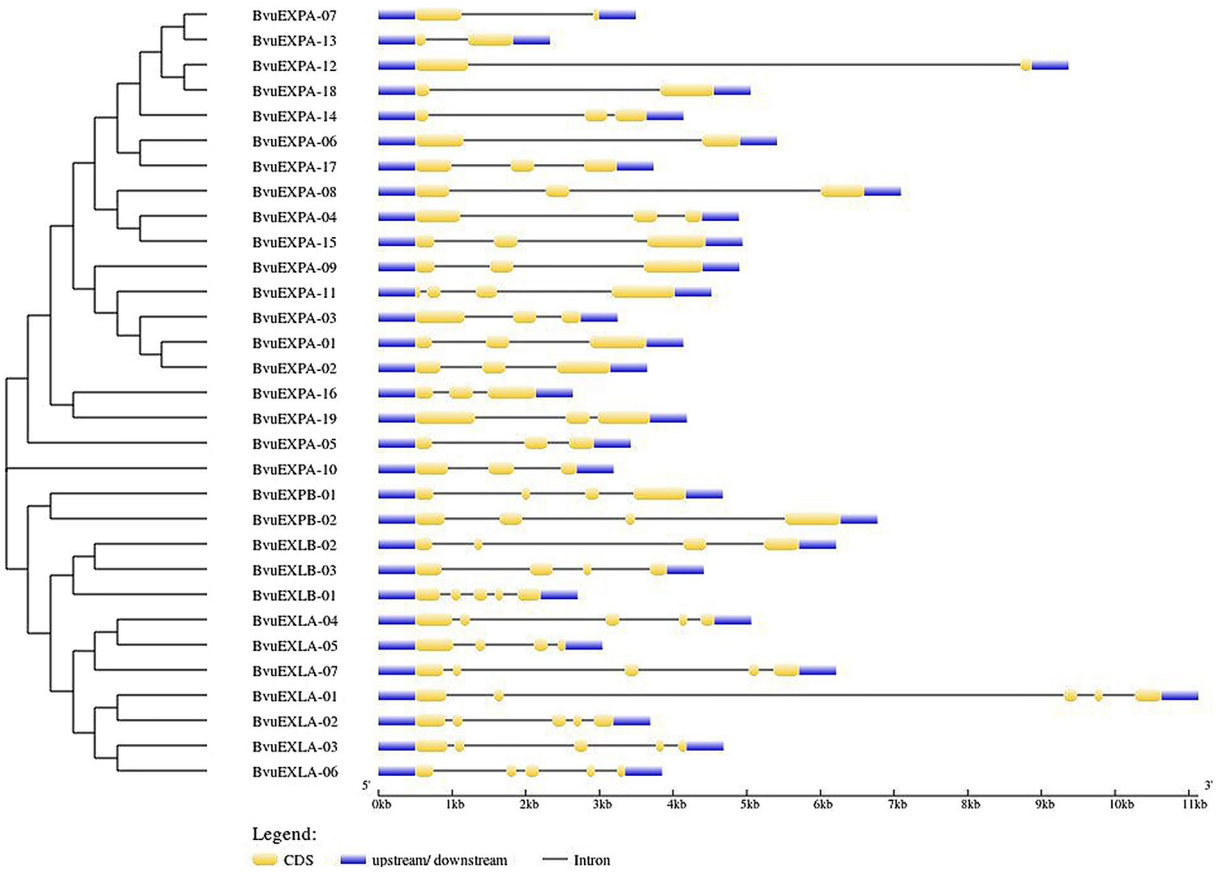

Fig. 2 Gene structure displaying of *BvuEXP* genes

and many of them contained the same number of exons. In this plant, most of the *EXPB* subgroup members have four exons, while many *EXPA* subgroup members have three exons, and other subgroups (*EXLA* and *EXLB*) generally have two or four exons (Han et al. 2019). Among tomato expansin genes, 15 of the genes belonging to 25 *EXPA* subgroups had a 3-exon/2-intron structure, and 9 of them had a 2-exon/1-intron structure. In addition, all *EXPB* subgroup members consist of a 4-exon/3-intron structure, while *EXLB* subgroup members generally consist of a 4-exon/3-intron structure (Lu et al. 2016). Also, it has been shown that *EXPA* subgroup members in the sugarcane genome contained 1 or 2 introns; the *EXPB* subgroup members had 3 introns, but *EXLA*s contained 3 introns (Santiago et al. 2018). Generally, our results are convenient with the results about the expansin gene structure in other plants. In conclusion, this may show that expansin genes share a conserved structural architecture through evolution.

The conserved motifs of sugar beet expansin proteins were shown in Supplementary Material 3. Twenty different motifs were screened for the expansin genes. Expansin subgroups displayed conserved and similar motif content. Motifs 1 and 4 were found to be typical for all subgroups of expansin proteins. Motif 3 was common in all expansin proteins except *Bvu*EXLA-05. All *Bvu*EXPA and *Bvu*EXPB proteins possessed a second motif containing the His-Phe-Asp (HFD) motif unique to *Bvu*EXPA and *Bvu*EXPB. *Bvu*EXLA and *Bvu*EXLB have not contained the HFD motif and these structural differences were convenient with the literature (Krishnamurthy et al. 2015; Ding et al. 2016; Lv et al. 2020). Except for *Bvu*EXPA-10, all *Bvu*EXPA's contained the 8th motif. Motif 20 was observed only in *Bvu*EXPA-16 and 19. The 16th motif that contains the conserved CDRC motif was found only in the *Bvu*EXLA subgroup except for *Bvu*EXLA-05. Hence, sugar beet expansin-like alpha proteins were supported to belong to the EXLA subgroup of the expansin family (Sampedro and Cosgrove 2005). Just *Bvu*EXLAs contained Motif 15. Merely *Bvu*EXLA and *Bvu*EXLB proteins were observed to include motifs 10 and 11 (excluding *Bvu*EXLB-01) and 12 (excluding *Bvu*EXLA-05).

Homology modeling of the sugar beet expansin proteins was performed in the Phyre2 program. A total of 18 *Bvu*EXP proteins (*Bvu*EXPA-01-03-04-05-07-08-09-10-12-13-14-16-18-19, *Bvu*EXLA-04, *Bvu*EXLB-01-02-03) showed high homology and 85–97% of residues were modeled at 90% confidence interval under intensive mode. The β-sheet structure was predominant in all modeled expansin proteins and especially two α-helices were conspicuous (Supplementary Material 4). According to the three-dimensional model of VpEXPA2 protein from papaya fruit, it was determined that the catalytic domain included six β sheets connected by small loops and two α-helices structures. The cellulose-binding domain (CBD) contained eight β-strands assembled in two β-sheets, constructing a β-sandwich pattern. This domain mainly included aromatic (Phe, Trp, and Tyr) residues (Gaete-Eastman et al. 2015). Moreover, based on the structural analysis of three alpha-expansin proteins from beach strawberry (*Fragaria chiloensis*), it was revealed that all three proteins include two domains: The 'catalytic' domain in all analyzed structures displayed highly conserved ß-barrel folds and a CBD in all three structures had an antiparallel β-sandwich pattern. These two domains were in contact through a short loop nearly 10–12 Å in length (Valenzuela-Riffo et al. 2019). When we compare the three-dimensional (3D) structures of sugar beet expansins and modeled proteins in those previous studies, the 3D structures were very similar. These studies can explain the current study's findings about the dominance of β-sheets and one or two α-helices in the predicted 3D structures of the sugar beet expansin proteins. The 3D similarity among the expansin proteins from different plants can provide evidence for the conserved structure of expansin proteins for function.

Sequence alignment and phylogenetic tree analysis

According to Fig. 1b, the evolutionary relationships among the sugar beet expansin family members have been analyzed using the maximum likelihood method in the Mega7 program at 1000 bootstrap values. The sugar beet expansin proteins were classified into 4 main clusters. While the first cluster contained only *Bvu*EXPA-10, the second cluster was divided into two subclasses. IIa included all the *Bvu*EXPB subgroup and IIb included the *Bvu*EXLA and *Bvu*EXLB subgroup members in distinct branches. The third cluster contained only BvuEXPA-05 and the fourth cluster was divided into two sub-classes. IVa class included the *Bvu*EXPA-16 and *Bvu*EXPA-19 and IVb class contained the remaining *Bvu*EXPA subgroup members. It is possible to deduce from the conserved motif content of those proteins that proteins with similar motifs were clustered and grouped into the same branch. This situation was supported by *Bvu*EXPA-16 and *Bvu*EXPA-19 proteins, which were the proteins that had motif 20, in IVa class. *Bvu*EXPA-10 protein in the first cluster differed from other *Bvu*EXPAs phylogenetically because it did not contain the eighth motif. In addition, EXPA, EXPB, EXLA, and EXLB protein subgroup members were divided into the same classes or branches of the cluster in the phylogenetic tree. Therefore, the results of the phylogenetic tree and the preserved motif analyses are compatible.

Gene ontology (GO) analysis

Based on gene ontology annotation analysis, all sugar beet expansin proteins were found to have binding activity as a molecular function. In the biological processes section, expansins were seen to play a major role in cellular processes. It has also been shown to be functional in reproduction, reproductive, multiple organism, and developmental processes. In addition, detailed analysis displayed that this biological process was usually plant-type cell wall organization for many expansin proteins. The cellular component of sugar beet expansin proteins was determined as a cellular anatomical entity from the program. When considering the detailed analysis for cellular components, *Bvu*EXPA proteins were usually located in the extracellular region, cell wall, membrane, and integral component of the membrane of plant cells. As for the *Bvu*EXPLA subgroup of proteins, this was an extracellular region, plant-type cell walls, and plasmodesma. All *Bvu*-EXPLB proteins were in the extracellular region (Supplementary Material 5a and 5b). Based on GO analysis of maize expansin genes, it was observed that 86% of maize expansin genes were categorized in the cell envelope functional category, and approximately 43% of expansins were classified in the stress response events (Zhang et al. 2014a, b). According to the GO analysis on tobacco and *Arabidopsis* expansin proteins, all expansin proteins were found in the extracellular region corresponding to the plant cell wall. The biological process of the EXPA subgroup of proteins in *Arabidopsis* and tobacco was determined as plant-type cell wall organization; in addition, sexual reproduction was defined as the biological process of expansin proteins (Ding et al. 2016). Wheat (*Triticum aestivum*) expansin proteins were categorized in the cell envelope functional category and GO categories of stress and immune responses (Li et al. 2016). These findings in different plants about biological roles and localization of expansin proteins are relevant to findings in sugar beet expansins and those results supported their roles in cell wall modification by the expansion of the wall (Cosgrove 2000). Besides, it was determined that EXPB subgroup members support pollen tube invasion of the stigma by solving the middle lamella (Sampedro et al. 2015) which can be attributed to their defined roles in sexual reproduction or reproductive processes in GO categories. In addition, in accordance with the binding activity of sugar beet expansins as molecular function, it has been reported that expansin proteins target hydrogen bonds that bind cellulose and hemicellulose, mainly xyloglucan, causing the cell wall polymers to shift and thereby relax the cell wall (Dal Santo et al. 2013; Bashline et al. 2014; Fukuda 2014).

Identification of targeting miRNAs

MicroRNAs (miRNA) are non-coding RNA molecules with an approximate length of 21–24 bases that significantly regulate gene expression in organisms (Eldem et al. 2013). miRNAs are involved in an interaction with mRNA that causes translation breakdown and repression (Yang et al. 2007). For this reason, they play a significant role in gene regulation under stress states through damaging gene transcripts in plants. The most targeted sugar beet expansin transcripts were *BvuEXPB-02* (37 miRNAs), *BvuEXLB-02* (23 miRNAs) and *BvuEXPA-15* (22 miRNAs). athmiR-156 h was shared by *BvuEXPA-15* and *BvuEXPB-02* transcripts while zma-miR2275a-5p was shared by *BvuEXPA-15* and *BvuEXLB-02* transcripts. The *BvuEXPB-02* transcript was mostly targeted by miR478, miR395, miR169, and miR529. miR390, miR156, and miR167 targeted the *BvuEXLB-02* transcript, while the *BvuEXPA-15* transcript was mostly targeted by miR478, miR447, and miR2275. miR478 was the most abundant miRNA (Supplementary Material 6a-6b). In *Arabidopsis*, miR390 contributes to the formation of aerial plant organs such as leaves and flowers by controlling gene regulation of auxin (Yoon et al. 2010). In rice, miR390 stimulates the growth of lateral roots by induced auxin triggers (Lu et al. 2018). It was suggested that miR395 in wild bananas (*Musa itinerans*) might play a significant role in tolerance to cold stress (Liu et al. 2018). The expression of the sulfate transporters in *Arabidopsis* was mainly regulated by miR395 based on sulfur starvation or abundance (Kawashima et al. 2011). Based on a genome-wide analysis of drought-related miRNAs in maize lines, it was observed that miR156-*SPL* (Squamosa Promoter-Binding Protein-Like) which is a miRNA-mRNA module, displayed a negative regulatory relationship and participated in the inhibition of metabolism in drought-exposed leaves in the sensitive maize line (Liu et al. 2019). In another study, miR156 controls stem thickness, shoot branching, trichome density, flowering period, and forage yield in alfalfa (Aung et al. 2015a, b). Besides, the alfalfa plant with SPL13 RNAi knockdown and miR156 overexpression displayed upregulated response to heat stress (40 °C) (Matthews et al. 2019). Another study resulted that the miR529c–*SPL* module has a role in bryophyte growth in an identical pathway to the miR156-*SPL* module in seed plants. They found that inhibition of *SPL2* transcription factor expression by miR529c prevented the reproductive transition in liverwort (*Marchantia polymorpha*) (Tsuzuki et al. 2019). A previous study found that miR395 and miR169 were downregulated under salinity stress in the leaf tissues of salt-water cordgrass (*Spartina alterniflora*) (Qin et al. 2015). It can be said that abundant miRNAs targeting sugar beet expansin genes have significant roles in normal development and stress tolerance in plants. miRNAs targeting expansin gene transcripts in the sugar

beet genome were first identified in the current study. It is expected that studying these types of miRNAs in various other plants may contribute to enhancing the understanding of the roles of these molecules in different tissues of the plants.

Calculation of homologous and non-homologous substitution rates

The rate of change (Ka/Ks) of non-homologous (Ka) to homologous (Ks) was determined to understand the evolutionary processes in the *BvuEXP* gene family (Supplementary Material 7a). While *BvuEXP* genes showed 72 pairs of tandem duplications (Fig. 1a), 268 pairs of *BvuEXP* genes displayed segmental duplications (Fig. 3). The rate of change (Ka/Ks) was an average Ka/Ks value of 0.15 for *BvuEXP*s showing tandem duplication while the average Ka/Ks value of 0.03 for *BvuEXP*s showing segmental duplications. According to this, the tandem duplication and segmental duplication events could have occurred approximately an average of 61 million years ago (Mya) and 212 Mya, respectively (Supplementary Material 7b). Besides, orthologous expansin genes were revealed between sugar beet and model organisms, including *Arabidopsis* (620 pairs), poplar (456 pairs), rice (600 pairs), and maize (577 pairs). Nonetheless, the average ratio of Ka/Ks was determined as 0.02 for *Arabidopsis*, 0.05 for poplar, 0.01 for rice, and 0.01 for maize from the genomes with orthologous *BvuEXP* genes in the sugar beet genome. When considering the average divergence times of orthologous expansin genes between sugar beet and the model organisms, the nearest divergence was revealed with poplar expansin genes (178 million years ago, Mya). *BvuEXPs* have diverged before an average of 211 Mya with *Arabidopsis,* 338 Mya with rice, and 350 Mya with

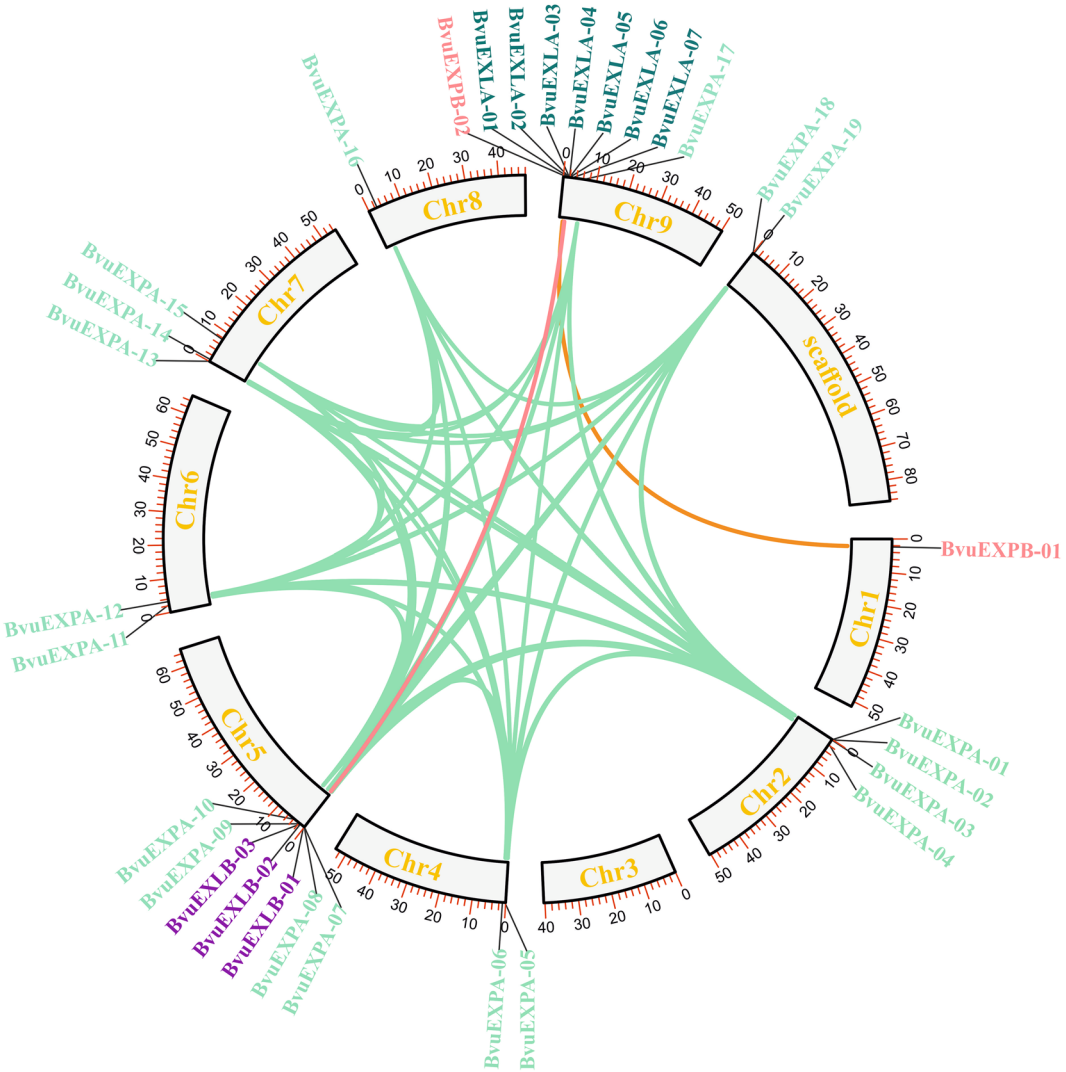

Fig. 3 Visualization of segmental duplications of expansin genes

maize expansin genes, respectively (Supplementary Material 7c). Gene duplication has a role in genome evolution and enables the generation of new genes and functions (Kong et al. 2007). It can be hypothesized that expansin genes in the sugar beet genome expand mainly by segmental duplication than tandem duplication events. In addition, all the Ka/Ks ratios of expansin genes were lower than 1, which may be the reason for the strong selection pressure for sugar beet expansins. In the soybean genome, it was determined that 14.7% of the expansin genes displayed tandem duplication, while 68% of the genes participated in segmental duplication events. In the same study, tandem and segmental duplication events occurred in 27.8% and 50% percentages of the *Arabidopsis* expansin genes. 55.2% of the rice expansin genes showed tandem duplication while 27.6% displayed segmental duplication (Zhu et al. 2014). The study on the moso bamboo plant revealed 14 pairs of tandem duplication and 31 pairs of segmental duplication (Jin et al. 2020). As dicotyledonous plants, sugar beet, soybean, and *Arabidopsis* exhibited segmental duplication dramatically during their expansion. Besides, the most orthologous genes were determined between sugar beet and *Arabidopsis* expansin genes. Therefore, among the expansin genes in the sugar beet genome and *Arabidopsis* genome, it can be said that more genes originated from a common ancestor and evolved from there. However, there are no comprehensive studies in the literature to determine the gene orthologous of the expansin genes with other plants, 20 homologous genes were determined between *Arabidopsis* and jujube (*Ziziphus jujuba* Mill.) (Hou et al. 2019).

Expression profiles of sugar beet expansin genes under abiotic stresses using transcriptome data and qRT-PCR

In silico expression profiles of *BvuEXP,* genes were evaluated using available RNA-seq data. This data included leaf samples of sugar beet under heat, salt, and light stresses (SRP044105) (Minoche et al. 2015), sugar beet leaf and root samples of 12th, 24th., 48th, 72nd hours of salt stress application (SRP115884) (Li et al. 2019), and root samples of sugar beet after 1st, 6th, 12th, and 24th hour of abscisic acid (ABA) application and controls (SRP235645) (Xing et al. 2020). The heat maps of the *BvuEXP* genes were created according to data coming from these SRA readings (Fig. 4). When looking at the leaf samples under heat stress, *BvuEXPA-05-10-16-17-19, BvuEXLA-01-02-03-05*, and *BvuEXLB-03* genes had expression in both controls and stressed samples. While expression of *BvuEXPA-01-03-04-11-15,* and *BvuEXLB-02-03*

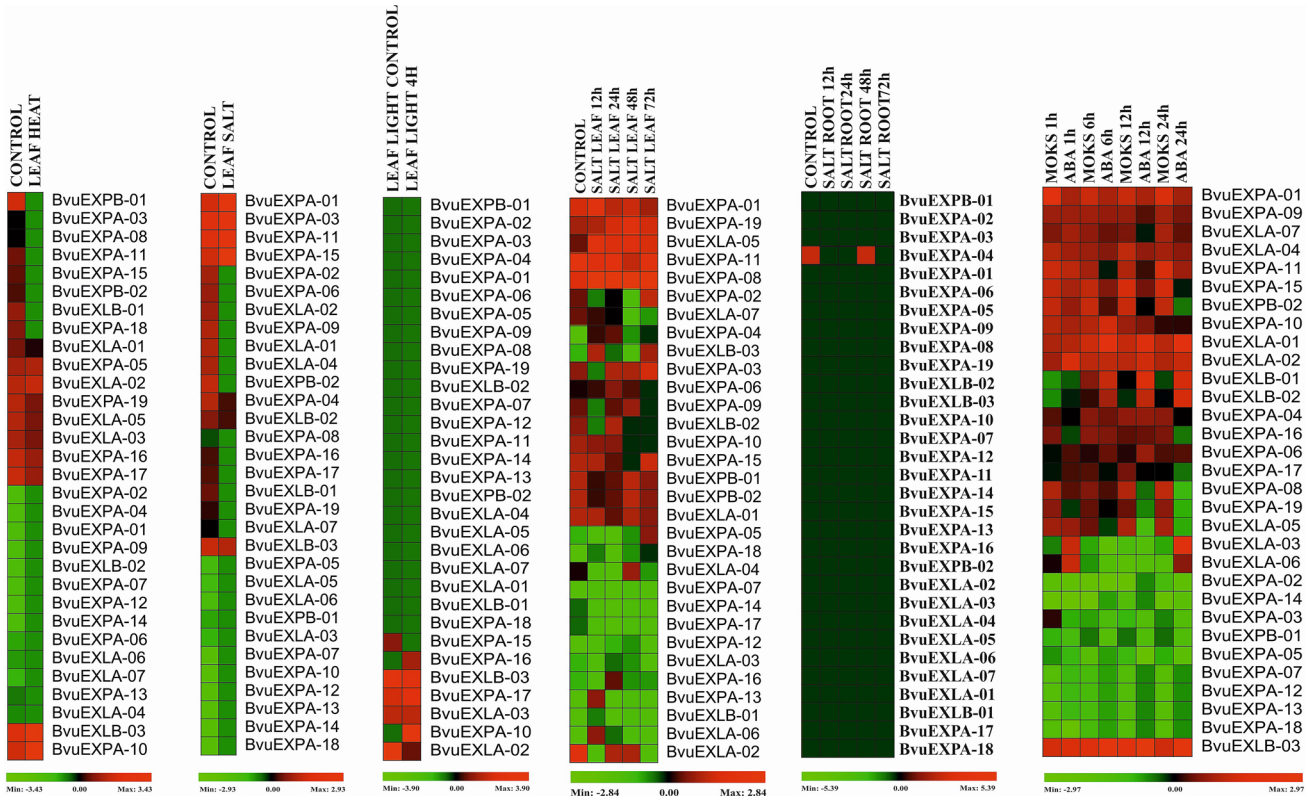

Fig. 4 In silico heat maps of *BvuEXP* genes in variable tissues and conditions

genes were determined in both control and leaf tissues under salt stress, expression of *BvuEXPA-10–16* genes was observed in only leaf tissues under light stress. When considering the other data, including different hours of salt stress application, it was observed that the expression of *BvuEXPA-04-05-06-13-16, BvuEXLA-03-04-06, BvuEXLB-01-03* genes was determined in different hours of the stress treatment in leaf tissues. Only *BvuEXPA-04* gene expression was determined in root in control and 48th hour of salt-stressed samples. According to the ABA application data, *BvuEXLA-03-06* and *BvuEXLB-02* genes had expression during different hours of ABA treatment.

According to the heat map analyses obtained from the RNA-seq data, some expansin genes whose expression generally increases under various abiotic stress conditions were selected and gene expression levels in drought-sensitive and drought-tolerant sugar beet cultivars (with combined drought and heat stresses applied) studied with qRT-PCR (Fig. 5) (Supplementary Material 8). The genes for these analyzes were chosen with care to represent each subfamily of the expansin genes. According to the gene expression results, *BvuEXLB-02* gene expression was enhanced significantly in the second hour of combined drought and heat stress treatment in drought-tolerant cultivars. Moreover, *BvuEXLA-02* gene expression was upregulated significantly after the first hour of stress in drought-tolerant and sensitive cultivars. From this view, *BvuEXLA-02* and *BvuEXLB-02* genes may have roles in the combined drought and heat tolerance of the plant. Considering the RNA-seq data results, *BvuEXLA-02* gene expression was determined in both control and heat-stressed samples. Besides, according to the RNA-seq data, *BvuEXLB-02* gene expression was observed

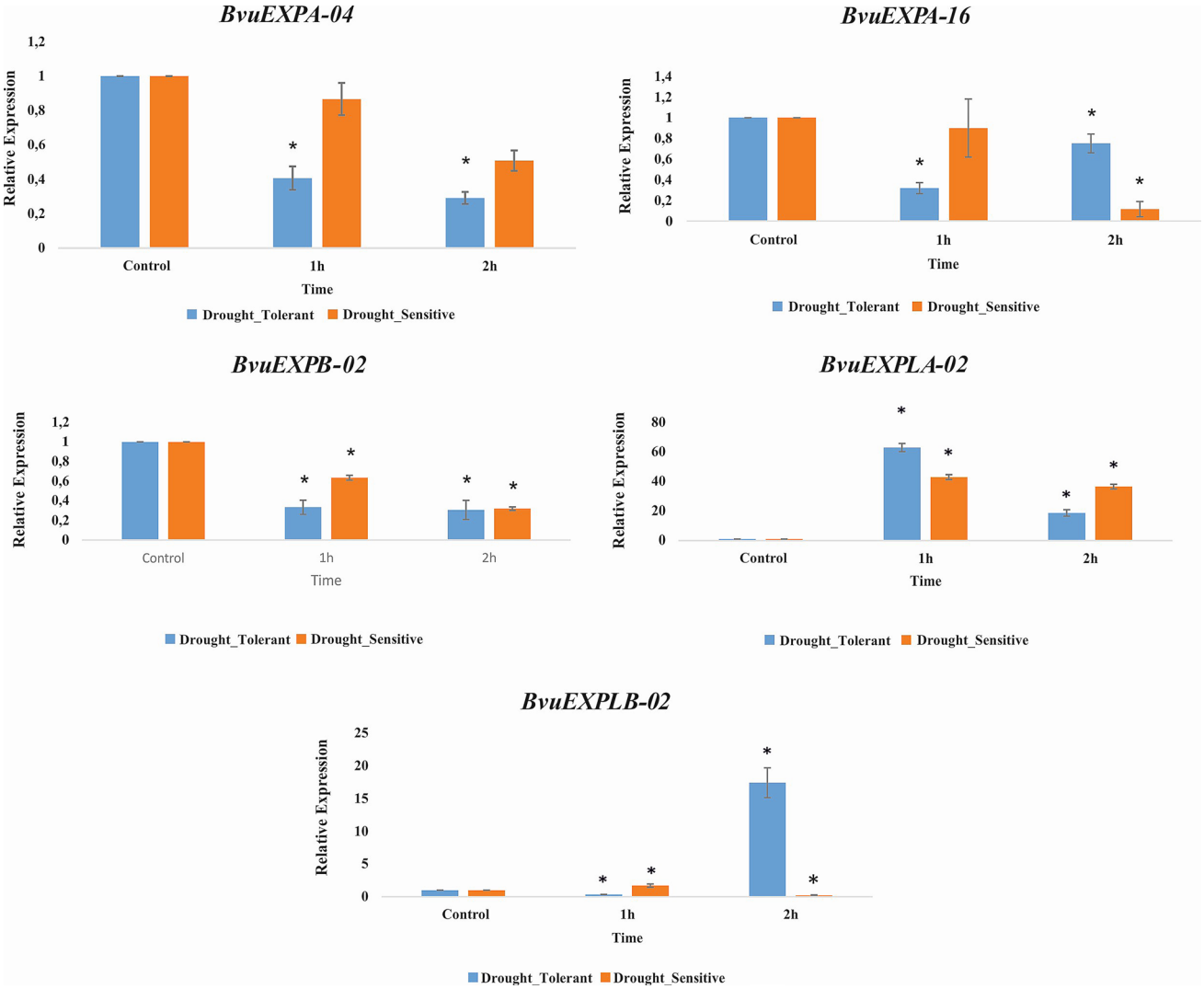

Fig. 5 Expression profiles of *BvuEXP* genes under combined drought and heat stresses (*$p < 0.05$, *GAPDH* and *Actin1* genes were used as reference genes)

in both control and salt-stressed leaf tissues and in different ABA-treated root tissues. Moreover, it was determined that the gene orthologous to the *BvuEXLA-02* gene in *Arabidopsis* was the *EXLA2* gene (AT4G38400-NP_195553.1) and it was induced significantly by salinity and cold stresses and by the exogenous treatment of ABA (Abuqamar et al. 2013). However, expressions of *BvuEXPA-04* and *BvuEXPB-02* genes were downregulated according to qRT-PCR analysis in all studied stress hours in drought-tolerant and drought-sensitive cultivars. Considering the RNA-seq data, the expression of the *BvuEXPA-04* gene in leaf samples increased at different times of salt stress compared to the control, while the expression of only this gene was found in the root sample under the same stress. On the other hand, it was observed that the expression of the *BvuEXPB-02* gene was high in leaf samples under both control and salt stress in the same data. Moreover, *BvuEXPA-16* was especially down-regulated in both drought-sensitive and drought-tolerant cultivars compared to the controls in the second hour of the combined drought and heat stress treatment according to the qRT-PCR analysis. However, in RNA-seq data analysis, it was observed that the expression of the *BvuEXPA-16* gene increased in sugar beet leaf tissue samples under light and salt stresses compared to the control. According to these results, it can be said that *BvuEXPA-04, BvuEXPA-16,* and *BvuEXPB-02* genes respond differently to various stress conditions. In addition, it can be concluded that these genes are especially involved in the response to salt stress. The fact that these genes are members of different expansin subfamilies and therefore have different biochemical properties may be related to their different responses to different stress conditions.

Considering the RNA-seq data, orthologous gene analysis, and qRT-PCR experiments suggest that among the studied genes, *BvuEXLA-02* and *BvuEXLB-02* genes can play a role in the plant's response to different abiotic stress conditions. Moreover, the increased expression of those genes under combined drought and heat stress in drought-resistant sugar beet cultivars also suggests that they may play a role in the plant's stress tolerance mechanism.

Conclusions for future biology

Sugar beet is one of the most widely used plants worldwide in the sugar production industry. This study includes determining and characterizing the expansin genes found in the sugar beet genome. In addition, in silico expression analysis of sugar beet expansin genes under variable abiotic stress conditions and expression profiles of expansin genes under combined drought and heat stresses in cultivars by the qRT-PCR method were evaluated in the study.

The current study provides crucial insights into the expansin gene family within the sugar beet genome, drawing on a comprehensive analysis of their characterization and potential roles under varying abiotic stress conditions. In a world that is becoming increasingly warm, the role of abiotic stress tolerance is becoming increasingly important. We noted that specific expansin genes, such as *BvuEXLA-02* and *BvuEXLB-02*, may play a role in abiotic stress tolerance, suggesting a possible pathway for enhancing the resilience of this crop in challenging environmental conditions. The defined speisific expansin genes can be specifically edited using genetic editing techniques such as CRISPR-Cas9 to enhance drought and heat stress resistance. Moreover, a deeper understanding of these expansin genes may further our grasp of cellulose hydrolysis mechanisms, which can stimulate an increase in plant biomass providing opportunities to enhance biofuel production from sugar beet pulp. Additionally, our findings encourage a broader study of expansin gene families in industrial crops. Ultimately, this study extends our understanding of sugar beet genetics and stress physiology as well as paving the way for future innovative research. In the future, expansin genes able to play a significant role in agriculture, biofuel production, and sustainable development in general.

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Declarations

Conflict of interest The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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