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RESEARCH

Unveiling the Heat Shock Protein Network in Sugar Beet: Comprehensive Genome-Wide Identification, Characterization, and Stress-Induced Expression Patterns

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Abstract
Heat shock proteins (Hsps) are vital for plant responses to abiotic stress, serving as molecular chaperones that maintain protein stability under adverse conditions. This study supplies a comprehensive genome-wide analysis of Hsp family members in the sugar beet (*Beta vulgaris*) genome and Hsp family gene expression patterns in three genotypes: drought-tolerant, drought-sensitive, and wild beet (*Beta maritima*). 334 *Hsp* genes belonging to six major families (sHsp, Hsp40, Hsp60, Hsp70, Hsp90, and Hsp100) were identified. Abundant tandem and segmental duplication events were observed, particularly within the Hsp70 and Hsp100 families, indicating a potential role in stress adaptation. Gene expression analysis under heat, drought, and combined stress conditions revealed significant upregulation of *BvHsp70-22*, *BvHsp90-03*, and *BvHsp60-28* in the drought-tolerant genotype, indicating their critical roles in stress recovery. In contrast, the drought-sensitive genotype displayed upregulation of *BvHsp60-01* and *BvHsp90-03*, exhibiting a potentially less robust stress response. *BvsHsp-34* and *BvsHsp-38* were highly expressed under drought stress in the wild beet, suggesting unique adaptive mechanisms. Synteny and phylogenetic analyses demonstrated conserved genetic linkages between sugar beet *Hsp* genes and orthologs in *Arabidopsis thaliana*, *Oryza sativa*, and *Glycine max*, indicating evolutionary conservation. Gene ontology analysis highlighted their roles in protein folding, binding, and stress response processes. This research sheds light on the molecular mechanisms behind heat shock protein-driven tolerance to abiotic stress in sugar beet, and identifies *BvHsp70-22*, *BvHsp90-03*, and *BvHsp60-28* as promising candidate genes for future breeding strategies aimed at enhancing stress resilience..

Keywords Heat shock protein (Hsp) · Sugar beet · Synteny · Abiotic stress · Gene expression

Introduction

The sea beet (*Beta maritima*), native to the Mediterranean region, is considered the wild ancestor of modern sugar beet (*Beta vulgaris*). Historical records suggest early recognition and usage of beet plants, with references found in eighth-century BCE Mesopotamian writings and later described by classical authors such as Theophrastus and Aristotle. Sugar beet, which spread from the Mediterranean to North Africa and the Mediterranean region of Europe, was initially cultivated for its leaves and later for its roots. In Europe, beets were first used as animal feed and became a popular vegetable for human consumption in the sixteenth century. In the nineteenth century, alchemists and chemists discovered that the beetroot was a source of sugar (Attokaran 2017). Sugar beet, a crop of significant economic importance, is used in various industries such as sugar and alcohol production, animal feed, and fertilizer manufacturing, contributing around 30% to the world's total sugar supply (Dohm et al. 2014; Dutton and Huijbregts 2007). In 2022, 260,998,613 tons of sugar beet were produced worldwide (FAO 2024).

Heat-shock proteins (Hsps) were discovered in *Drosophila melanogaster* cells in 1962 (Ritossa 1962). Besides heat, they are triggered by various other stress factors, including drought, salinity, cold, UV radiation, and oxidative

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stress (Cho and Choi 2009; He et al. 2015; Liu et al. 2010; Rizhsky et al. 2002). Early plant studies, such as those by Lopato and Gleba (1985), demonstrated species-specific expression of Hsps in cell cultures of *Nicotiana* and *Atropa* species (Lopato and Gleba 1985), while Chen and Vierling (1991) identified chloroplast-localized small Hsps in *Petunia hybrida* and *Arabidopsis thaliana* (Chen and Vierling 1991), contributing to the understanding of their functional diversity and organelle specificity. Predicated on their molecular weight and functional roles, Hsps are divided into six categories: small heat shock proteins (sHsps), Hsp40 (J-proteins), Hsp60 (chaperonins), Hsp70, Hsp90, and Hsp100 (Clp proteins) (Kumar et al. 2012). This study used HSPIR (Kumar et al. 2012) for Hsp terminology like used in other plant studies (Kumar et al. 2020b; Wang et al. 2024; Zhao et al. 2023).

Small heat shock proteins (sHsps) are vital throughout plant development, including pollen formation, seed development, and growth from germination to storage organ accumulation (Asea et al. 2016). DnaJ/Hsp40 proteins, which are evolutionarily conserved, are key players in protein translation, folding, unfolding, the translocation process, and destruction by primarily enhancing the ATPase activity of Hsp70s (Qiu et al. 2006). Hsp60 proteins, called chaperonins, facilitate the folding and aggregating proteins destined for organelles, including chloroplasts and mitochondria (Lubben et al. 1989; Suzuki et al. 2009). The Hsp70 family is highly conserved across evolution and is expressed in reply to both biotic and abiotic stresses, performing various cellular functions, including protein biogenesis, stress protection, prevention of protein aggregation, and aiding in protein translocation (Asea et al. 2016; Wang et al. 2014). Hsp90 proteins are essential molecular chaperones involved in folding co-proteins related to signal transduction, including protein kinases and transcription factors (Cowen and Lindquist 2005). Hsp100 proteins, also known as Hsp104/ClpB, have a broad range of functions, including regulating transcription for increased tolerance to high temperatures (Glover and Lindquist 1998).

Plants are continually subjected to various environmental factors, particularly abiotic stresses like drought and heat. When drought and heat stress co-occur, they can trigger stomatal closure, diminish photosynthesis, elevate respiration, and increase leaf temperature. Notably, transcripts that are typically induced during drought—such as those coding dehydrin, catalase, and glycolate oxidase—and those induced during heat stress, like thioredoxin peroxidase and ascorbate peroxidase, tend to be repressed when both stresses are combined. This observation implies that the plant's response to combined drought and heat differs significantly from its reaction to each stress individually (Rizhsky et al. 2002). Heat stress alone has an adverse effect on crop productivity. For instance, in sugar beet, germination rates dropped by up to 65% at 32 °C, and no normal germination occurred at 38 °C, highlighting the critical sensitivity of early developmental stages to elevated temperatures (Malmir et al. 2018).

This underscores the necessity for a deeper understanding of plant responses to heat in order to enhance agricultural yield and secure the food supply (Wang et al. 2021). More thorough research on heat shock proteins (Hsps) is necessary, given the critical role these proteins play in preserving cellular activities under both normal and stressful circumstances (Yer et al. 2018). However, a clear understanding of their genome-wide organization and coordinated function under multiple stress scenarios in sugar beet remains lacking.

To address this knowledge gap, the present study performs a genome-wide identification of six *Hsp* gene families in sugar beet. We analyzed their chromosomal distribution, gene duplication events, and evolutionary relationships. Despite the known importance of heat shock proteins (Hsps) in plant stress tolerance, previous studies in sugar beet have been limited to individual gene families or specific stress conditions. For example, expression of Hsp70 under heat stress has been investigated using RT-PCR (Skaracis et al. 2005), and the role of calcium in Hsp regulation has been explored in suspension cultures (Trofimova et al. 1999). Additionally, Zhang et al. (2024) identified HSF transcription factors involved in salt stress responses (Zhang et al. 2024), but not the *Hsp* genes themselves. To date, there has been no comprehensive genome-wide analysis of all major *Hsp* gene families in sugar beet, nor an integrative evaluation of their expression patterns under multiple abiotic stresses across genotypes with contrasting stress tolerances. Here, we performed a genome-wide identification of *Hsp* genes in sugar beet and analyzed their expression profiles under heat, drought, and combined stress conditions using quantitative real-time PCR. Our study includes wild beet (*Beta maritima*) and drought-sensitive and drought-tolerant sugar beet cultivars. This approach aims to pinpoint candidate *Hsp* genes for use in future breeding programs to enhance climate resilience in sugar crops.

Materials and Methods

Identification of Heat Shock Protein Genes in Sugar Beet Genome

First, the peptide sequences of the members of the sHsp, Hsp40, Hsp60, Hsp70, Hsp90, and Hsp100 families from plants were acquired from the Heat Shock Protein Information Resource (HSPIR, https://pdslab.biochem.iisc.ac.in/hspir/) (Ns et al. 2012). Second, the sugar beet genome and protein sequences were downloaded from the *Beta vulgaris*

Resource Database (https://bvseq.boku.ac.at/) (Dohm et al. 2014). Third, protein sequences homologous to those in sugar beet were identified using BLASTP in CLC Genomics Workbench version 11.0.01 (QIAGEN, Aarhus, Denmark). Finally, the defined sequences were scanned using the Hidden Markov Model (HMM) and selected based on conserved regions, which were verified with the Pfam (https://pfam.xfam.org/) database (Mistry et al. 2021). Hsp proteins were categorized into six major families—sHsp, Hsp40, Hsp60, Hsp70, Hsp90, and Hsp100—based on their conserved domain structures, as defined by Pfam entries specific to each family (Kumar et al. 2020b; Wang et al. 2024; Zhao et al. 2023). Stability, molecular weight, amino acid length, and isoelectric point (pI) values were obtained using the ProtParam tool (https://web.expasy.org/protparam/) (Gasteiger et al. 2005).

Determination of Chromosomal Positions and Gene Structure

Chromosomal positions of the *Hsp* genes in sugar beet were identified using the NCBI Genome Data Viewer (https://www.ncbi.nlm.nih.gov/genome/gdv/) (Rangwala et al. 2021). TBtools (Chen et al. 2020a) was then used to represent various *BvHSP* genes on chromosomes. Intron and exon positions were identified using the Gene Structure Display Server (http://gsds.cbi.pku.edu.cn/) (Hu et al. 2015).

Phylogenetic Evaluation and Determination of Protected Motifs

MEGA 11 (Tamura et al. 2021) was used for Clustal W, and the phylogenetic tree was prepared using the neighbor-joining method with bootstrap analysis for 1000 iterations (Saitou and Nei 1987). Motif discovery of Hsp proteins was conducted utilizing the MEME Suite online tool (https://meme-suite.org/meme/) (Bailey and Elkan 1994).

Computation of Homologous and Non-homologous Change Rates

Orthologous relationships between sugar beet and other species, including *Arabidopsis thaliana*, *Oryza sativa*, *Glycine max*, and *Populus trichocarpa*, were compared using the BLASTP. Sequences with *e*-values ≤ 60 and 65% identity were considered relevant (Baloglu et al. 2014; Unel et al. 2019, 2023). These sequences were aligned using CLUSTALW, and synonymous (Ks) and non-synonymous (Ka) substitutions were calculated using the CODEML (http://www.bork.embl.de/pal2nal/) (Suyama et al. 2006). Duplication and divergence times of each *Hsp* gene were estimated using the formula $T = \mathrm{Ks}/2\lambda$ ($\lambda = 6.5 \times 10^{-9}$) (Lynch and Conery 2000).

Determination of miRNAs for Sugar Beet Hsp Target genes

Sequences of all miRNAs identified in *Arabidopsis thaliana*, *Oryza sativa*, *Glycine max*, and *Populus trichocarpa* were collected from the Plant miRNA Encyclopedia (PmiREN, https://www.pmiren.com/) (Guo et al. 2022b). The psRNA Target Server (https://www.zhaolab.org/psRNATarget/) was used to align these miRNAs with sugar beet *Hsp* gene transcripts to identify miRNA targets (Dai and Zhao 2011).

Homology Modeling

The three-dimensional (3D) structure of *Beta vulgaris* Hsp proteins was predicted using AlphaFold (https://www.alphafold.ebi.ac.uk/) (Jumper et al. 2021). Amino acid sequences of sugar beet Hsp proteins were searched in the Protein Data Bank (PDB) database to identify homologous samples with known 3D structures. Models were visualized using PyMOL (http://pymol.org) (Schrodinger 2015).

Gene Ontology Analyzes

Amino acid sequences of Hsps were loaded into the OmicsBox 2.0 software (https://www.biobam.com/omicsbox/) to determine their biological processes, cellular components, and molecular functions (Götz et al. 2008; Bioinformatics and Valencia 2019).

Expression Patterns of Sugar Beet Hsp Genes Using Transcriptomic Data

For RNA-Seq analyses, data from heat, salt, and light treatments (PRJNA254489), salt leaf and salt root treatments (PRJNA516574), and mock and ABA-treated samples (PRJNA594791) were retrieved from the Sequence Read Archive (SRA). Reads were processed to generate log2 RPKM values using the CLC Genomic Workbench version 11.1, filtered with a combined threshold of $p < 0.05$ and fold change > 2 used to determine statistically and biologically significant changes. The heatmaps were drawn using the PermutMatrix software (Caraux and Pinloche 2005).

Growing of Sugar Beet Plants and Stress Conditions

Seeds of drought-sensitive and drought-tolerant sugar beet cultivars and *Beta maritima* were kindly provided by Sugar Institute (Türkiye Şeker Fabrikaları A. Ş., Ankara). To ensure sterility, the seeds were surface-sterilized by immersion in 75% ethanol (v/v) for 1 min, followed by three washes with distilled water. They were then soaked in distilled water for 24 h. Seedlings were planted in plastic containers (93 mm in diameter, 400 cc volume)

with approximately five seedlings per container, using vermiculite as a growth medium. All seedlings were maintained under uniform conditions: a light intensity of 400 µmol m^{-2} s^{-1}, 24 ± 2 °C temperature, and a 16-h light/8-h dark photoperiod. Irrigation was carried out using ½ Hoagland solution for 30 days (Hoagland and Arnon 1938) (Supplementary Fig. 1).

Thirty-day-old seedlings bearing 6–8 fully expanded true leaves were used to ensure uniform growth and adequate tissue for RNA extraction. For abiotic stress treatments, plants

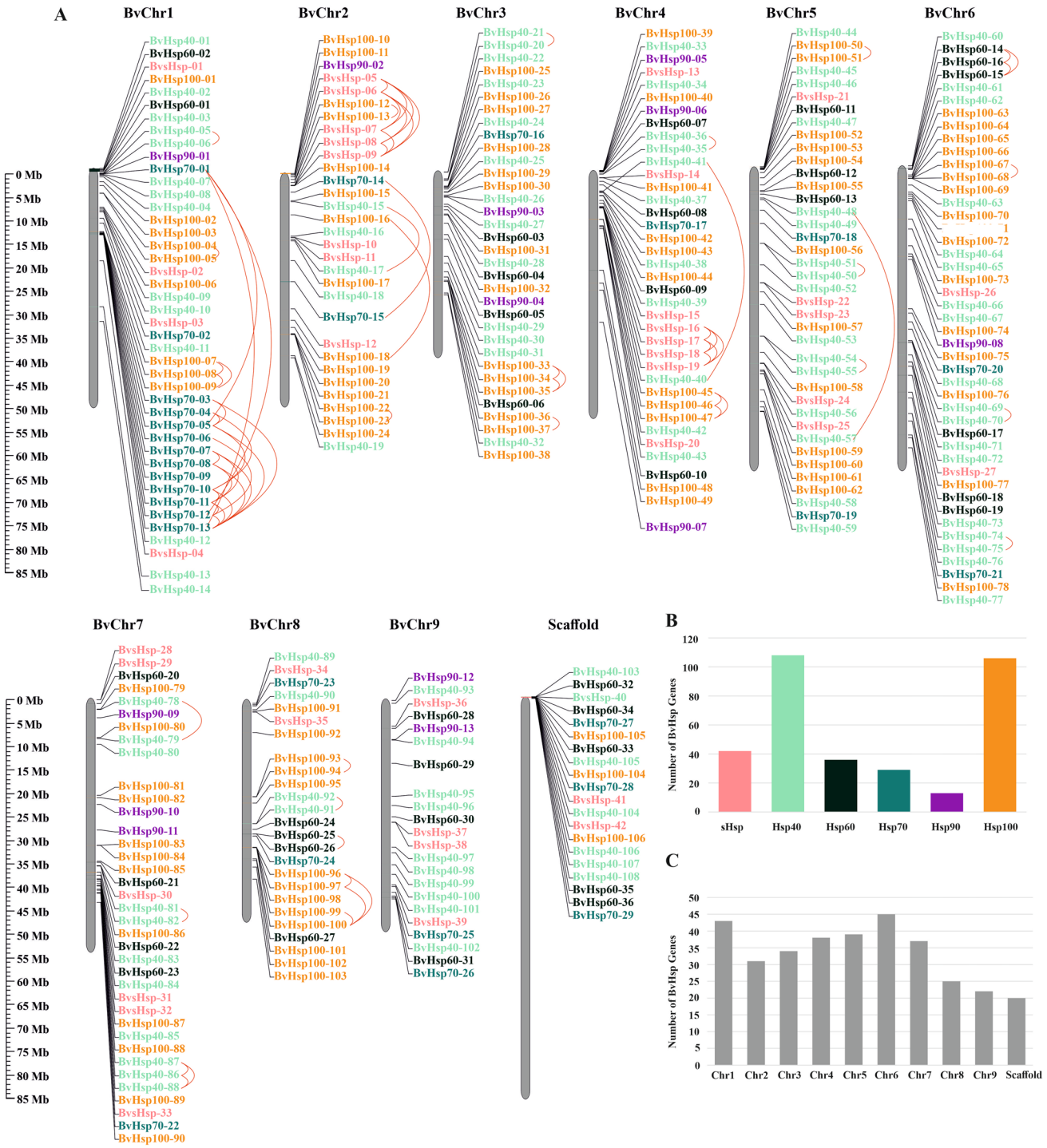

Fig. 1 **A** The localization of *BvHsp* genes on sugar beet chromosomes. **B** Graph shows the number of *BvHsp* genes. **C** Graph shows the localization of *BvHsp* genes on sugar beet chromosomes

were exposed to 20% polyethylene glycol 6000 (drought) (Licaj et al. 2024; Afa et al. 2012; Sushmita and Deeksha 2017; Meher et al. 2018), 50 °C (heat) (Aydogan and Turhan 2022; Khalil et al. 2020), and a combination of drought and heat stresses (50 °C and 20% polyethylene glycol 6000). Leaf tissues (3–4 leaves per plant) were collected at the end of 0, 0.5, 1, and 2 h post-stress treatments, and untreated tissues were used as controls. The experiments were performed in three biological replicates, with all tissues immediately frozen in liquid nitrogen and stored at –80 °C until RNA isolation (Çelik Altunoğlu et al. 2019).

Total RNA Isolation and Quantitative Real-Time PCR (qRT-PCR) Analysis

Total RNA was isolated using Trizol solution (GeneAll, South Korea) following the optimized protocol (Celik Altunoglu et al. 2016). The purified RNA was dissolved in 20 µl of DEPC-treated water, its quality and concentration were assessed via agarose gel electrophoresis and measured at 260/280 nm using the MultiScan Go spectrophotometer (Thermo Fisher Scientific, USA). DNase treatment (RNase-free, Thermo Fisher Scientific, Waltham, MA, USA) was applied to eliminate genomic DNA contamination. Subsequently, the RNA was reverse transcribed into first-strand cDNA using the iScript cDNA Synthesis Kit (BioRad, Hercules, CA, USA), following the manufacturer's protocol.

The NCBI Primer Blast tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) generated primers for *Hsp* genes according to transcriptomic data analysis. Supplementary Table 1 displays the primer sequences for the *Hsp* genes and both reference genes (GAPDH and Actin1) (Ghaemi et al. 2020; Li et al. 2020). The iTaq Universal SYBR Green supermix (BioRad, Hercules, CA, USA) was used in the Biorad CFX96 Touch Real-Time PCR device to perform the qRT-PCR reaction following the cDNA production. The temperature cycles were 30 s at 95 °C for the initial denaturation, followed by 5 s at 95 °C for denaturation, and 30 s at 58 °C for annealing. This procedure was repeated 40 times. After this reaction, the reaction mixture was denatured at 95 °C, and the temperature was lowered to 65 °C. Fluorescence signals were recorded at 530 nm wavelength per second at intervals of 0.5 °C from 65 to 95 °C for the melting curve analysis. For each sample in the trials, three technical and three biological replicates were employed.

The reference gene and control status were used to standardize the Ct (cycle threshold) data. The variations between relative expression patterns were expressed as $2^{-\Delta\Delta Ct}$, and ΔCT and ΔΔCT [ΔCT = CT sample–CT reference and ΔΔCT = ΔCTstressed sample–ΔCTcontrol (0 h)] were computed (Livak and Schmittgen 2001). The Minitab 18 package program's one-way ANOVA approach was used for statistical analysis. If the *p*-value was under 0.05, the expression differences between the stressed and control samples were deemed important. Additionally, only those genes exhibiting a fold change greater than 2 were considered biologically significant.

Results and Discussion

Identification, Characterization, and Gene Duplication of Sugar Beet Hsp Genes

A total of 334 *Hsp* genes were determined in the sugar beet genome, including 42 *BvsHsp*, 108 *BvHsp40*, 36 *BvHsp60*, 29 *BvHsp70*, 13 *BvHsp90*, and 106 *BvHsp100* genes. The expansion of the Hsp40 and Hsp100 gene families in sugar beet, comprising 108 and 106 members, respectively, is consistent with observations in other plant genomes (Unel et al. 2023; Ceylan et al. 2023; Chen et al. 2021b; Baloğlu et al. 2021; Çelik Altunoğlu et al. 2019). This pattern suggests a conserved evolutionary mechanism in plants to enhance proteostasis, particularly under environmental stress. Hsp40 proteins function as essential co-chaperones of Hsp70, facilitating substrate recognition and delivery, thereby increasing the cellular capacity to refold misfolded proteins under heat or drought conditions. Similarly, the Hsp100 family, primarily associated with protein disaggregation and thermotolerance, is crucial for recovering protein function after severe stress (Rosenzweig et al. 2019; Saibil 2013). The expansion of these two gene families likely reflects the evolutionary pressure on plants to maintain protein homeostasis under fluctuating and extreme environments. Hence, their numerical dominance in the sugar beet genome is both functionally and evolutionarily significant. Chromosome 6 has the highest number of *BvHsp* genes of other chromosomes, with 44 genes identified. Additionally, 20 *BvHsp* genes could not be mapped to any chromosome (Fig. 1). This study's findings indicate a significant variance in the number of *Hsp* genes across different species. For example, in *Arabidopsis thaliana*, 19 *sHsp* (Scharf et al. 2001), 89 *Hsp40* (Miernyk 2001), 29 *Hsp60* (Hill and Hemmingsen 2001), 14 *Hsp70* (Lin et al. 2001; Sung et al. 2001), 7 *Hsp90* (Krishna and Gloor 2001), and 8 *Hsp100* (Agarwal et al. 2001) genes have been recorded. In comparison, our research group identified 88 *Hsf* (Heat shock factor), 72 *sHsp*, 192 *Hsp40*, 52 *Hsp60*, 85 *Hsp70*, 49 *Hsp90*, and 148 *Hsp100* genes in sunflower (*Helianthus annuus L.*) (Ceylan et al. 2023). These differences may result from the depth of bioinformatics analysis, the high ploidy level, and the genome size of the plants.

The amino acid lengths, molecular weights, stability, and isoelectric points (pI) of the BvHsp proteins are shown in Supplementary Table 2.1–2.6. The peptide sequence lengths of BvHsp proteins ranged from 90 amino acids (BvHsp60-29, BvHsp60-30, BvHsp60-35) to 5437 amino

acids (BvHsp100-86). Molecular weights varied between 9.7 kDa (BvHsp60-35) and 617.35 kDa (BvHsp100-86). This sequence length and molecular weight diversity may be attributed to the distinct roles of Hsp family members within the organism, reflecting their functional specialization in response to different cellular demands.

Gene duplication can lead to the evolution of new functions, increasing gene diversity. The rates of non-synonymous (Ka) and synonymous (Ks) substitutions, and the ratio of non-synonymous to synonymous substitution rates ratio (Ka/Ks), are commonly used to investigate the selective pressure acting on gene pairs (Li et al. 2009; Lynch and Conery 2000; Long et al. 2003). Tandem (Fig. 1) and segmental (Fig. 2) duplication events in the *BvHsp* gene family were calculated to investigate the evolution situation of *BvHsp* gene families (Supplementary Table 3). Seventy-seven pairs of tandem duplications were identified, with the most occurring on chromosome 1 and *BvHsp70* and *BvHsp100* genes

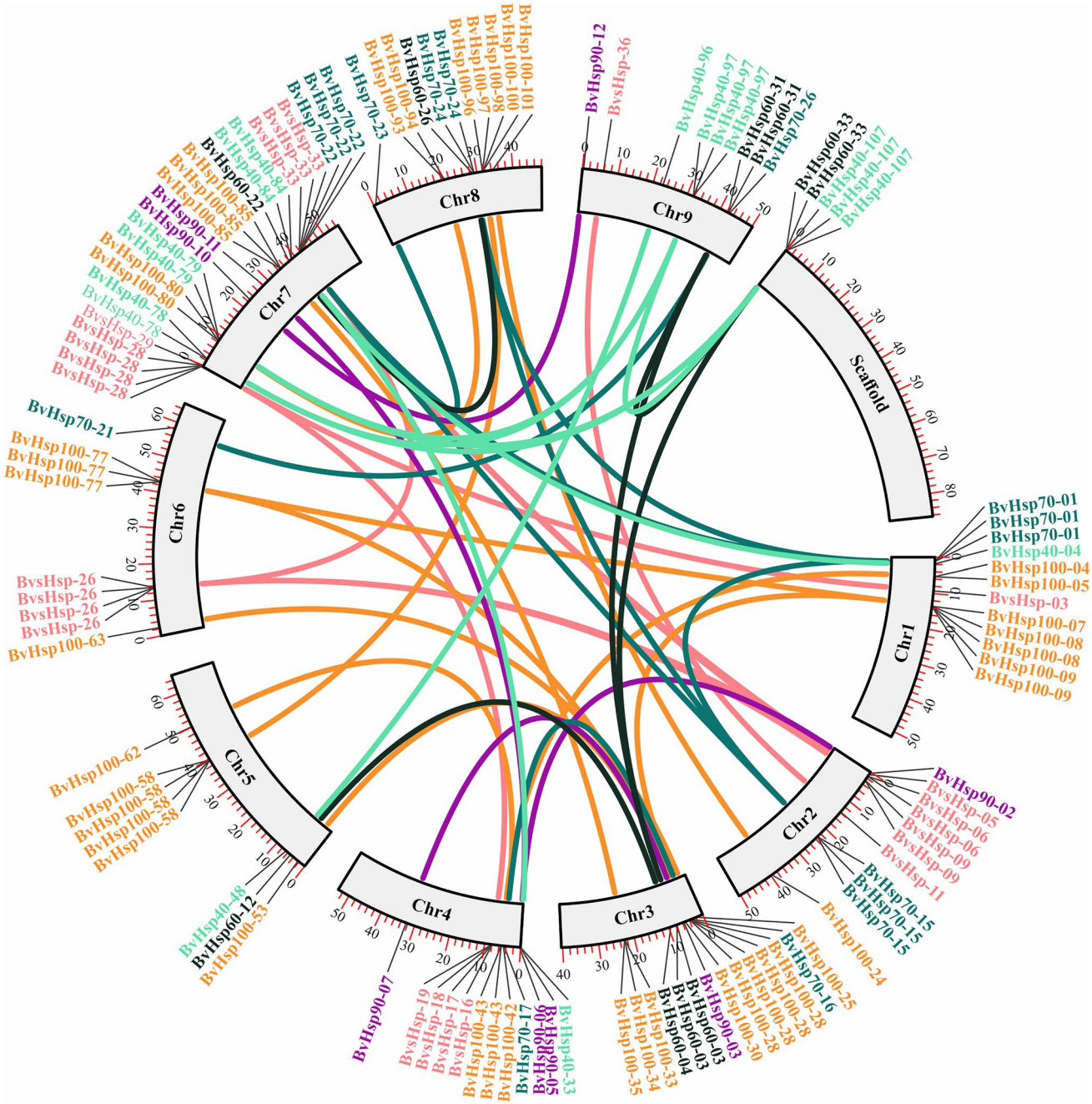

Fig. 2 Segmental duplications of the *BvHsp* genes in the sugar beet genome

(21 pairs each). No tandem duplications were found on chromosome 9 or in *BvHsp90* genes. Fifty-eight pairs were identified for segmental duplications, with the highest number in *BvHsp70* genes (21 pairs). All chromosomes exhibited segmental duplications, with *BvHsp90* genes having the fewest (four pairs) and *Hsp60* genes having five pairs. Similar results have been reported in poplar (Yer et al. 2018) and wheat (Kumar et al. 2020a), where *Hsp90* genes showed limited tandem duplications. The high number of duplications in specific Hsp families might suggest an adaptive advantage in sugar beet, possibly contributing to its stress tolerance. Further functional studies on these duplicated genes could elucidate their roles in stress responses.

Gene Structure Organization

A gene's structure dictates its coding potential, and genes with comparable structures are probably descended from a common ancestor. Introns are critical in regulating gene expression and increasing protein diversity (Greenberg and Soreq 2013; Jo and Choi 2015). It was determined that 14 genes from the sHsp family, 16 genes from the Hsp40 family, 3 genes from the Hsp60 family, 4 genes from the Hsp70 family, 3 genes from the Hsp90 family, and 7 genes from the Hsp100 family do not contain introns (Supplementary Fig. 2.1–2.6). Results are consistent with findings in poplar, where the Hsp40 family also had the highest number of intron-free genes (Yer et al. 2018). This observation aligns with the findings in the sunflower genome, where a high proportion of intronless genes were identified, particularly in the sHsp family (Ceylan et al. 2023). To evaluate whether intronless *Hsp* genes in sugar beet exhibit different evolutionary or functional characteristics, we compared Ka/Ks ratios between intronless and intron-containing gene groups. On average, intronless genes exhibited lower Ka/Ks ratios (mean = 0.36) than intron-containing genes (mean = 0.52), suggesting stronger purifying selection. In both instances, the absence of introns in these genes may facilitate rapid transcriptional responses during stress conditions, as highlighted in previous research (Deshmukh et al. 2016; Chen et al. 2017). In the context of *Hsp* genes in the sunflower genome, 52 genes from the Hsp40 family were reported to lack introns, a much higher number than sugar beet (16). Similarly, in squash (*Cucurbita pepo*), 23 *Hsp40* genes were found to be intronless, further indicating a functional trend across different species where intronless genes may be evolutionarily favored for rapid transcriptional responses under stress (Ceylan 2023). The existence of intronless genes within these families suggests that they may play a pivotal function in facilitating rapid adaptation to abiotic stress, particularly in species such as sunflower and squash that are well adapted to diverse environmental conditions.

The *BvHsp100-86* gene was identified as having the highest number of introns, with 75. The study conducted in poplar showed that *Hsp40* and *Hsp100* genes contained many intron regions (Yer et al. 2018). High intron numbers in *Hsp40* and *Hsp100* genes suggest a potential mechanism for increased gene complexity and regulation (Deshmukh et al. 2016). However, a study on bread wheat reported that the highest number of intronless genes was in the sHsp family, followed by the Hsp40 family (Kumar et al. 2020a). Intronless genes may be genes synthesized from RNA to DNA by the reverse transcriptase enzyme using the retrotransposon mechanism (Wang 2017) or may result from the loss of intron regions by the genomic deletion mechanism (Deshmukh et al. 2016).

Phylogenetic Classification and Protected Motifs of Hsps

Phylogenetic analysis was conducted to understand the evolutionary relationships of BvsHsp, BvHsp40, BvHsp60, BvHsp70, BvHsp90, and BvHsp100 proteins (Supplementary Fig. 3.1–3.6). According to the results, BvsHsp proteins were generally divided into three subgroups in the phylogenetic tree. A comprehensive phylogenetic tree analysis was also constructed for all BvHsp proteins to indicate their relationships (Fig. 3). Most BvHsp proteins clustered within their respective families, except for some BvHsp40 proteins, which grouped with BvHsp100. These results could be attributed to sequence similarities or common intron/exon structures. The absence of introns is a recognized characteristic of *Hsp* genes, and numerous studies have reported intronless genes across various Hsps (Unel et al. 2023; Ceylan et al. 2023; Tao et al. 2015; Wen et al. 2017; Yan et al. 2017; Yu et al. 2016). Intronless Hsp transcripts can move rapidly from the nucleus to the cytoplasm without splicing, which may drive the evolutionary selection of such genes (Huang et al. 2019). This might explain why some *BvHsp40* and *BvHsp100* genes were clustered in the same group despite belonging to different families. In addition, it was determined that the proteins belonging to the genes showing tandem and segmental duplications were in the same group and branch in the phylogenetic tree. This result can provide reliable phylogenetic relationships within a gene family.

Motif analysis of BvHsp proteins was conducted to validate the phylogenetic tree's accuracy (Supplementary Fig. 4.1–4.6). The analysis revealed the presence of Hsp20/alpha-crystallin and ARID domain (ARID/BRIGHT DNA binding domain) in BvsHsp proteins. At the same time, TCP-1/cpn60 chaperonin, PAC3 (proteasome assembly chaperone 3), and RING-type zinc-finger conserved regions were identified in BvHsp60 proteins. Additionally, all BvHsp70 proteins screened for motifs contained the Hsp70 domain, and the Hsp90 domain and the HATPase_c

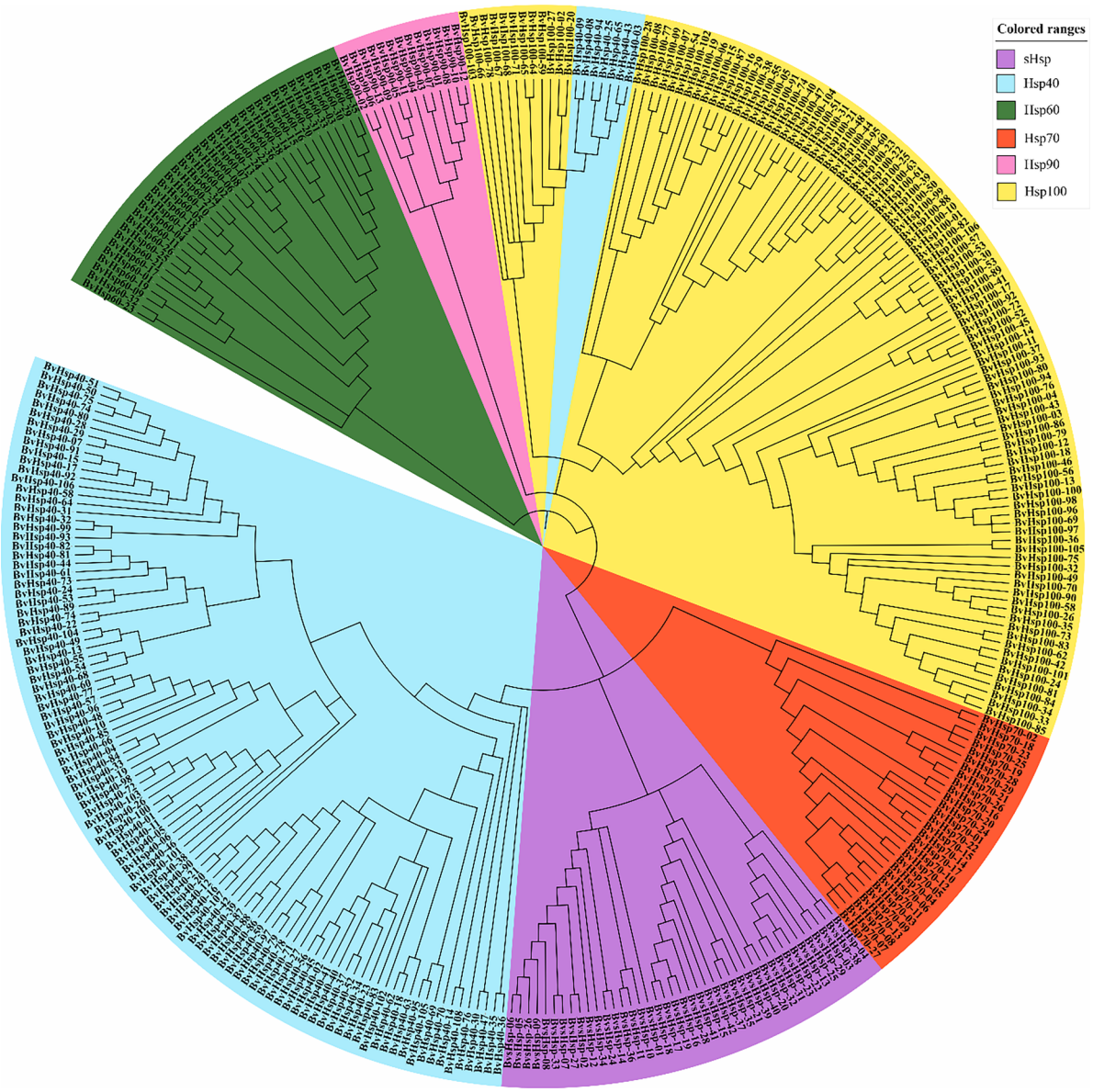

Fig. 3 Phylogenetic comparison within Hsp sub-families in sugar beet, constructed by maximum likelihood method. Different colors represent Hsp sub-families

domain were found in Hsp90 proteins. BvHsp100 proteins exhibited AAA (ATPase family associated with various cellular activities) and AAA_2 domains. Interestingly, non-Hsp domains were also detected in some BvHsp proteins, similar to observations in gin millet (Singh et al. 2016) and bread wheat (Kumar et al. 2020a), which may indicate potential involvement in additional cellular processes beyond classical chaperone activity. However, further functional or expression analyses are needed to clarify the significance of these domain architectures. In general, motifs defining the Hsp group were identified. As a result, the conserved motifs of BvHsp proteins containing the same motif were found structurally similar and determined to be in the same class in the phylogenetic tree.

Synteny Analysis, Determination of Homologous and Non-homologous Exchange and Separation Times

Nadeau and Taylor (1984) first introduced the concept of synteny, describing it as the presence of two or more homologous genes on the same chromosome, where these genes share functional similarities. Over time, the definition of synteny has evolved, now often referring to orthologous

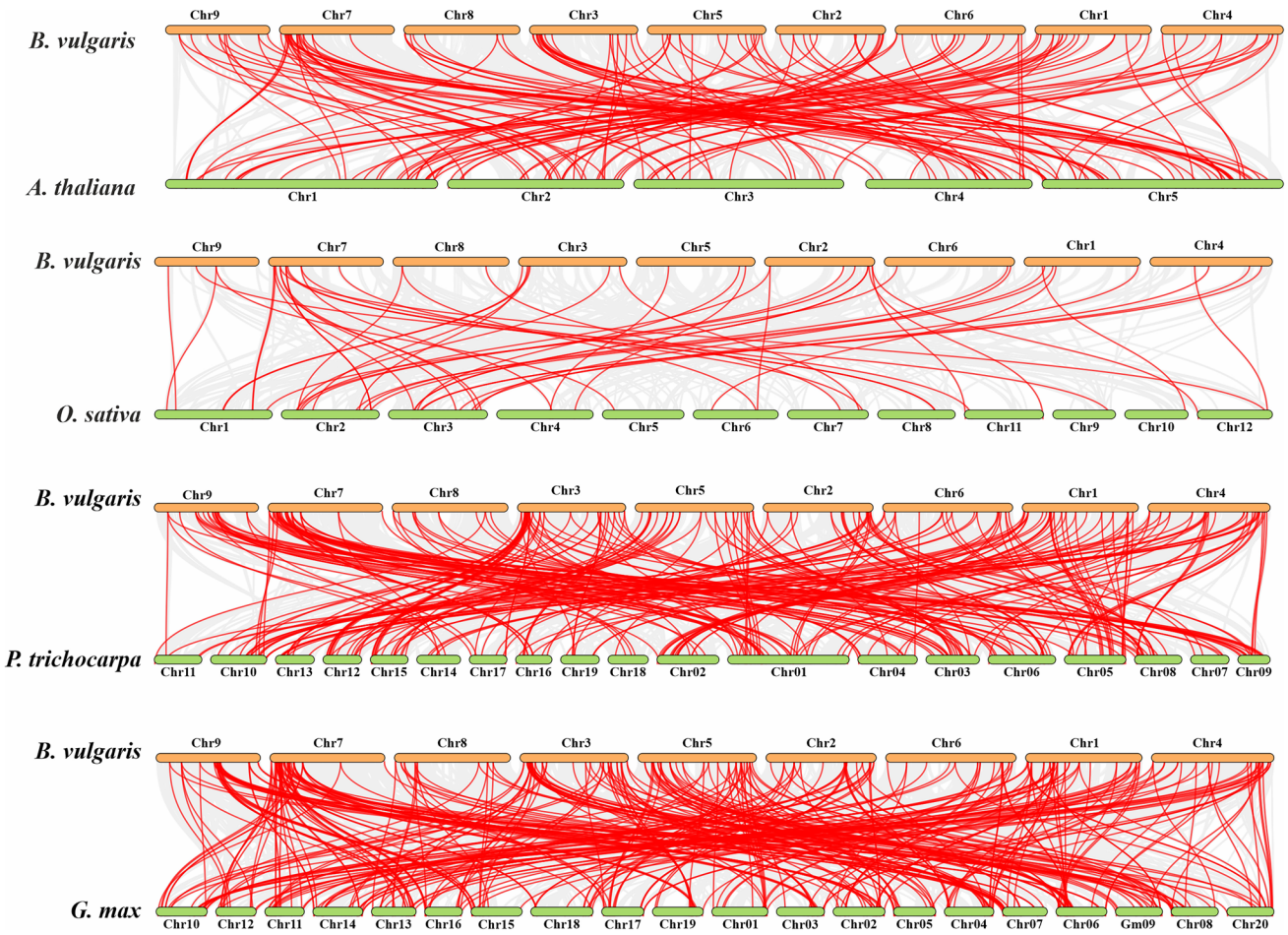

Fig. 4 Synteny analysis of *BvHsp* genes between Arabidopsis, paddy, poplar, and soybean

genes that arise from speciation events, with an emphasis on sequence conservation rather than functional conservation (Nadeau and Taylor 1984; Ghiurcuta and Moret 2014; McCouch 2001; Restrepo-Montoya et al. 2021). Synteny analysis is a powerful method for tracing evolutionary patterns and identifying genes with related functions (Restrepo-Montoya et al. 2021).

In this study, syntenic relationships were mapped between sugar beet (*Beta vulgaris*) and four phylogenetically and functionally diverse plant species: Arabidopsis (*Arabidopsis thaliana*), soybean (*Glycine max*), paddy (*Oryza sativa*), and poplar (*Populus trichocarpa*). These species were selected based on the availability of high-quality, chromosome-level genome assemblies and their established roles as model organisms representing distinct clades and biological traits. *A. thaliana* is a small-genome, fast-growing eudicot model; *O. sativa* represents the monocot lineage and is the most studied cereal crop; *G. max* is a legume with recent polyploidy events and symbiotic nitrogen fixation capabilities; and *P. trichocarpa* serves as a model for woody perennials. This selection ensured reliable orthology inference across both monocot and eudicot lineages and enabled an evolutionarily relevant comparative framework while minimizing the confounding effects of incomplete or low-resolution genomes that are common in algal, moss, or gymnosperm taxa. Thus, the chosen species allowed robust syntenic and phylogenetic analysis while maintaining biological interpretability. The findings are visually represented in Fig. 4, where grey lines denote all orthologous gene connections across chromosomes and red lines specifically highlight the orthologous relationships between *BvsHsp* genes.The most orthologous relationships were found between *BvsHsp* and *P. trichocarpa* (17 pairs), between *BvHsp40* and *P. trichocarpa* (62 pairs), between *BvHsp60* and *G. max* (12 pairs), between *BvHsp70* and *G. max* and *P. trichocarpa* (9 pairs), between *BvHsp90* and *G. max* (5 pairs), and between *BvHsp100* and *G. max* and *P. trichocarpa* (50 pairs). In sugar beet and other species, *BvsHsp-10–30-34*, *BvHsp40-22–35-59–61-81*, *BvHsp60-21*, *BvHsp70-02–22*, *BvHsp90-03–07-08*, and *BvHsp100-11–12-16–54-25–28-29–30-42–58-82–89* genes were identified as common orthologous, and these genes may be highly conserved in the evolutionary process. These

syntenic relationships highlight conserved genetic linkages across different species, which can be crucial for understanding evolutionary processes and gene functions of *Hsp* genes. Considering all *Hsp* genes of sugar beet, the highest number of orthologous was found in soybean with 323 pairs, followed by 269 in poplar, 142 in Arabidopsis, and 49 in paddy. The number of orthologous genes was higher in dicotyledonous sugar beet and dicotyledonous plants (Arabidopsis, poplar, and soybean) than in monocotyledonous plants (paddy). More orthologous genes in dicotyledonous plants than in monocotyledonous paddy suggest evolutionary conservation among dicots. No orthologous gene was identified between sugar beet *Hsp90* genes and the paddy plant. Orthologous genes were identified among other Hsp subgroups, including Arabidopsis, poplar, soybean, and paddy. In a study conducted on monocot bermuda grass (*Cynodon transvaalensis*), also known as dogtooth weed, more orthologous genes were identified in *Hsp20* genes between monocot plants than dicot plants (Cui et al. 2021). In the study conducted on cucumber, more orthologous genes were identified among dicotyledons than monocotyledons in *Hsp* genes (Chen et al. 2021b). In the present study, *BvsHsp-10–30-34* from the sHsp family, *BvHsp40-22–35-59–61-81* from the Hsp40 family, *BvHsp60-21* from the Hsp60 family, *BvHsp70-02* and *BvHsp70-22* from the Hsp70 family, *BvHsp90-03–07-08* from the Hsp90 family, and *BvHsp100-11–12-16–25-28–29-30–42-54–58-82–89* genes from the Hsp100 family were found to be orthologous in monocot and dicot plants. These genes may have arisen before monocotyledons and dicotyledons were differentiated and highly conserved in the evolutionary process. Ka, Ks, and Ka/Ks values were calculated for orthologous of *BvHsp* genes in Arabidopsis, soybean, paddy, and poplar plants. A total of 783 orthologous gene pairs were identified, and further analysis revealed that the Ka/Ks ratio was less than 1 for all pairs (Supplementary Table 4.1–4.6), suggesting that these essential *Hsp* genes in sugar beet are under strong purifying selection.

Identification of miRNAs Targeting Hsp Transcripts

miRNAs play crucial roles in regulating gene expression under stress conditions (Bartel 2004; Bushati and Cohen 2007). In this study, putative miRNAs predicted to target BvsHsp-34, BvHsp-38, BvHsp40-03, BvHsp40-44, BvHsp60-01, BvHsp60-28, BvHsp70-20, BvHsp70-22, BvHsp90-03, BvHsp90-07, BvHsp100-12, and BvHsp100-58 transcripts, whose gene expression levels were examined by qRT-PCR, were determined by in silico predictions (Supplementary Fig. 5.1–5.7, Supplementary Table 5). Due to the current absence of sugar beet (*Beta vulgaris*)–specific miRNAs in the latest release of miRBase, in silico prediction analysis was performed using experimentally validated miRNAs from model plant species (*Arabidopsis*, rice, soybean, and poplar). These model species were selected based on their genomic proximity or relevance to *Beta vulgaris* and because their miRNAs are well annotated and functionally characterized. Although this approach does not ensure species specificity, it provides a comprehensive first-pass identification of conserved miRNA–Hsp regulatory relationships, which can guide future experimental validation. Some predicted miRNA–target relationships mirror stress-responsive patterns reported in other species, supporting their potential relevance. For instance, drought stress in soybeans has been shown to upregulate miR4411 (Mantri et al. 2013) which specifically targets the BvsHsp-34 transcript. Similarly, transgenic potato plants overexpressing miR160a-5p, targeting the BvHsp40-44 mRNA, exhibited enhanced tolerance to abiotic stresses, including drought and salinity (Öztürk Gökçe et al. 2021). In tobacco, miR396a-5p, which targets the BvHsp60-01 transcripts in the current study, promoted resistance to multiple stressors, including salt, cold, and drought (Chen et al. 2015). Additionally, soybeans subjected to drought, salt, and alkali stresses showed increased expression of miR1507a (targeting the BvHsp70-20 transcript) (Li et al. 2011). Under drought stress, miR4358 (targets the BvHsp70-22 transcript) was also upregulated in soybean (Mantri et al. 2013). Furthermore, miR159a, targeting the BvHsp90-03 transcript, has been associated with increased disease resistance in rice plants (Chen et al. 2021a). Under cold stress, miR166a, which targets the BvHsp90-07 transcripts, is expressed more (Zhou et al. 2008). Drought tolerance is conferred by miR827, which targets the BvHsp100-12 transcript (Ferdous et al. 2017). miRNAs that target the *BvHsp* genes are linked explicitly to drought stress. Noncoding small RNAs like miRNAs are pivotal in managing biotic and abiotic stress responses, influencing various cellular and metabolic processes (Chen et al. 2015). These observations emphasize the critical role miRNAs play in sugar beet's stress response mechanisms, particularly in enhancing tolerance to drought, salt, and cold stresses (Chen et al. 2015; Şanlı and Öztürk Gökçe 2021; Li et al. 2011). Identifying the diverse regulatory roles of miRNAs targeting BvHsp transcripts through bioinformatics can provide valuable insights, aiding in developing sugar beet varieties with enhanced stress tolerance and contributing to more sustainable agricultural practices amidst climate change.

Homology Modeling of Hsps

In order to investigate the structural characteristics of crucial Hsps in sugar beet, homology modeling was conducted for proteins encoded by the genes *BvHsp70-20*, *BvHsp70-22, BvHsp90-03*, *BvHsp90-07*, *BvHsp100-12*, *BvHsp100-58*, *BvHsp60-28*, *BvHsp40-03*, *BvHsp40-44*, *BvsHsp-34,* and *BvsHsp-38*, which had their gene expression levels examined via qRT-PCR (Supplementary Fig. 6.1–6.6). These

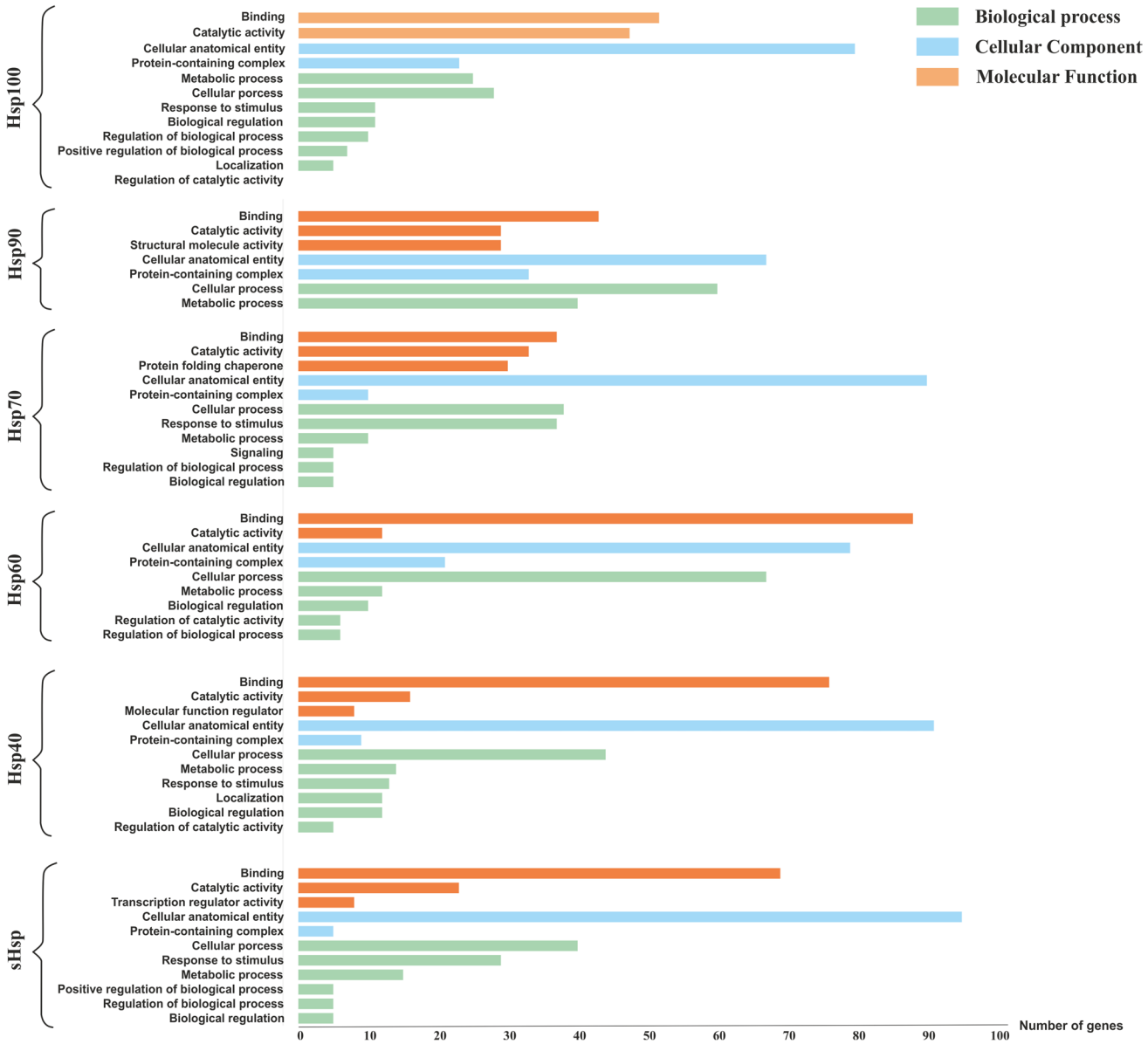

Fig. 5 Functional analyses of *BvHsp* genes

genes were particularly selected due to their high levels of expression according to the transcriptome data analysis. Beginning with the Hsp70 family, these proteins typically consist of a nucleotide-binding domain (NBD), a substrate-binding beta-sheet domain (SBDβ), and an alpha-helical lid domain (SBDα) (Rosenzweig et al. 2019). BvHsp70-20 and BvHsp70-22 displayed significant homology in these domains, highlighting their conserved functional mechanisms essential for ATP-driven protein folding. Next, the Hsp90 family comprises three distinct domains: the amino-terminal domain (NTD), the middle domain (MD), and the carboxyl-terminal domain (CTD) (Harris et al. 2004). BvHsp90-03 and BvHsp90-07 demonstrated structural homology across all three domains, suggesting their critical roles in chaperone activities and regulating signal transduction pathways. Moving to the Hsp100 family, which is divided into two classes: class I includes Hsp104/ClpB proteins that feature an N-terminal domain (N), a middle domain (M), and two nucleotide-binding domains (NBD1 and NBD2) (Braun 2010; Saibil 2013). BvHsp100-12 exhibited homology only in the NBD1 domain, while BvHsp100-58 displayed high homology across the N, NBD1, and NBD2 domains, with some modifications in the middle domain, suggesting potential functional specialization.

In the Hsp60 family, structural and functional studies on *Escherichia coli* have shown that the GroEL protein

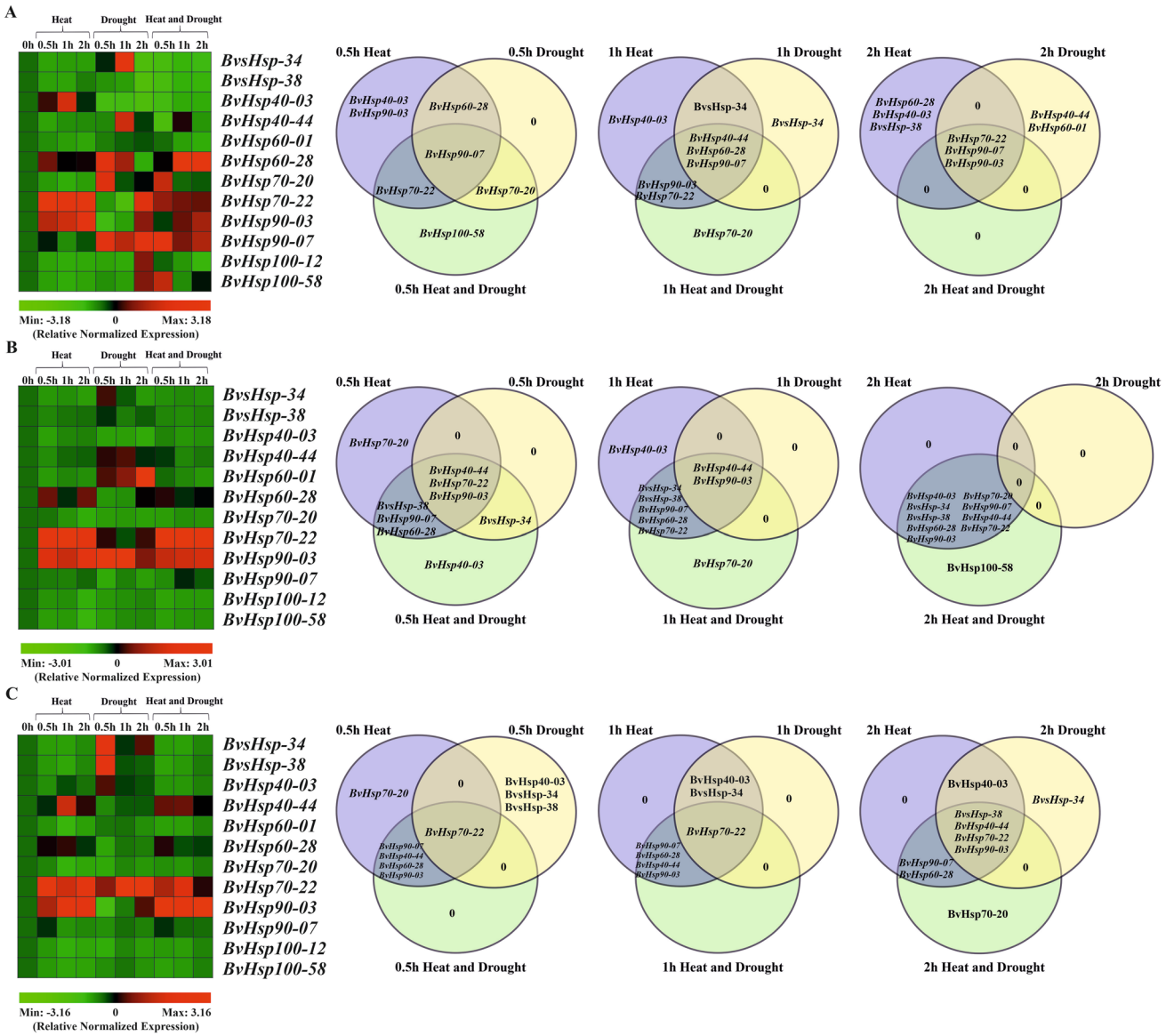

Fig. 6 Heatmap and Venn diagram of the expression of selected *BvHsp* genes. **A** Drought-tolerant genotype. **B** Drought-sensitive genotype. C Wild-type (rate of change > 2 and *p*-value < 0.05 in Venn diagram)

forms a complex tetradecameric structure consisting of two overlapping heptameric rings when bound to GroES. Each GroEL monomer weighs approximately 57 kDa, while GroES monomers weigh around 10 kDa (Gupta et al. 2014). BvHsp60-28 exhibited substantial homology in the apical, intermediate, and equatorial domains of GroEL, indicating a similar structural arrangement essential for its role in protein folding within mitochondria and chloroplasts. The conserved J-domain is a crucial characteristic in the Hsp40 (DNAJ) family, which stimulates ATPase activity when working with Hsp70 proteins (Kampinga and Craig 2010). BvHsp40-03 and BvHsp40-44 were found to possess this J-domain, confirming their role in protein folding and their function as co-chaperones to Hsp70. Focusing on the small heat shock protein (sHsp) family, the defining feature is the alpha-crystallin domain (ACD), composed of approximately 80–100 amino acids, flanked by variable N-terminal and C-terminal regions (Santhanagopalan et al. 2015). BvsHsp-34 and BvHsp-38 contained this conserved ACD, affirming their classification within the sHsp family and their role in preventing protein aggregation during stress.

The three-dimensional structures predicted for the Hsp family members were compared with experimentally determined structures from the Protein Data Bank (PDB), specifically with IDs: 2KHO for Hsp70, 2CG9 for Hsp90, 1QVR for Hsp100, 1AON for Hsp60, 6Z5N for Hsp40, and 1GME for sHsp. This comparison showed a notable homology and similarity between the predicted BvHsp protein structures

and their respective PDB templates, affirming the accuracy of the homology models. These results underscore that the conserved structural domains identified in sugar beet Hsps are likely critical to their roles in stress responses, akin to Hsps in other organisms. By recognizing these structural parallels, their functional mechanisms and potential to enhance plant stress tolerance can be understood more deeply. Effective homology modeling becomes valuable, offering insights into protein interactions and molecular behavior, especially under stressful conditions. This approach could also help predict how these proteins interact with other molecules and proteins, further illuminating stress response pathways in sugar beet (Gupta et al. 2014; Rosenzweig et al. 2019; Saibil 2013).

Gene Ontology

The molecular function, cellular localization, and biological function analysis performed using the OmicsBox program revealed that members of the BvsHsp family are located in intracellular anatomical structures and the cytoplasm, where they play crucial roles such as protein folding and binding (Fig. 5, Supplementary Table 6.1–6.6). Specifically, the Hsp40 family was identified to be primarily localized in the cytoplasm and various intracellular compartments, where it plays a key role in protein folding by binding to unfolded proteins, preventing potential misfolding damage. This function is vital for maintaining cellular homeostasis, especially under stress conditions that increase the risk of protein denaturation. Similarly, the Hsp60 family is heavily involved in protein folding, residing in the cytoplasm and other intracellular compartments, including organelles like mitochondria, ensuring proper protein assembly and refolding. The Hsp70 family not only aids in protein folding but also binds to misfolded proteins, stabilizing them to prevent aggregation, which is crucial during cellular stress. Its presence in the cytoplasm underscores its central role in maintaining protein integrity across various cellular environments. Meanwhile, the Hsp90 family, also predominantly found in the cytoplasm, contributes to cellular metabolism by acting as an organic molecule binder, playing a pivotal role in regulating signal transduction pathways and supporting stress recovery and cell growth. Additionally, the Hsp100 family specializes in organic compound binding and participates in the metabolic processing of organic matter, further emphasizing its involvement in cellular metabolism and stress responses. Together, these Hsp families cover various functions, from facilitating protein folding to supporting essential metabolic processes, highlighting their critical importance in cellular function and adaptation to environmental stresses.

In a similar study on *Cucumis sativus*, Hsp ontology analysis has demonstrated that cucumber Hsp40, Hsp60, Hsp70, Hsp90, and Hsp100 proteins play functional roles comparable to those in sugar beet. For instance, cucumber Hsp40 proteins were involved in cellular processes, response to stimuli, and regulation of cellular components, closely reflecting the roles of BvsHsp40 proteins. Similarly, cucumber Hsp60 proteins, like their sugar beet counterparts, were engaged in cellular and metabolic processes, emphasizing the conserved roles of Hsp proteins across species in stress response mechanisms (Unel et al. 2023). Additionally, Hsp70 proteins in cucumber displayed roles in protein folding and catalytic activity, consistent with the findings for BvsHsp70 proteins in sugar beet, highlighting their involvement in maintaining cellular homeostasis during abiotic stress. In the sunflower genome, both Hsp40 and Hsp70 proteins were identified as essential for protein folding and stress response, with a broader cellular localization that includes the nucleus and chloroplast. Sunflower Hsp90 proteins were more involved in protein synthesis regulation, likely due to their interaction with ribosomal subunits, while sugar beet Hsp90 proteins focused more on metabolic regulation. Sunflower Hsp100 proteins, localized in thylakoids and proteasomes, had broader roles in stress recovery than the primarily metabolic functions observed in sugar beet Hsp100 proteins (Ceylan et al. 2023). Similarly, zucchini Hsp ontology analysis revealed that Hsp40, Hsp60, Hsp70, and Hsp100 proteins showed binding functions alongside contributions to catalytic activity and cellular stress response processes. Zucchini Hsp90 proteins were implicated in metabolic processes and cellular stress mitigation like their sugar beet counterparts (Ceylan 2023). These findings highlight the conserved roles of Hsp proteins across different plant species, underscoring their importance in maintaining cellular stability under environmental stress. In a bread wheat study, Hsps were involved in protein binding, folding, and various cellular processes (Kumar et al. 2020a). Molecular Hsp chaperones were observed in the cytoplasm and organelles like the nucleus, mitochondria, chloroplasts, and endoplasmic reticulum (Wang et al. 2004). The roles identified in this study align with previous findings in other plant species mentioned above, confirming the functional importance of Hsps in stress responses and normal cellular functions.

Gene Expression Analyses of Hsp Genes

Transcriptome data of temperature, salt, light, and ABA hormone treatment were obtained from the NCBI database with accession numbers PRJNA516574 (Li et al. 2019), PRJNA254489 (Minoche et al. 2015) and PRJNA594791 (Xing et al. 2020). *BvsHsp-34*, *BvsHsp-38*, *BvHsp40-03*, *BvHsp40-44*, *BvHsp60-28*, *BvHsp70-20*, *BvHsp70-22*, *BvHsp90-03*, *BvHsp90-07*, *BvHsp100-12*, and *BvHsp100-58* genes were selected from heatmaps (Supplementary Fig. 7.1–7.6) to examine gene expression levels by qRT-PCR

using heat maps. These genes were specifically chosen based on their prominent expression levels ($p < 0.05$ and fold change > 2) observed in transcriptome analysis under various abiotic stress conditions as indicated by RNA-seq. *BvsHsp-34* and *BvsHsp-38* showed high expression, particularly under salt stress, suggesting their significant roles in stress tolerance. Similarly, *BvHsp40-03* and *BvHsp40-44* displayed notable expression under various conditions, reinforcing their involvement in protein folding and stress responses. These results were consistent with those from qRT-PCR analyses. For the Hsp60 family, *BvHsp60-28* showed notable upregulation under temperature and salt stress, confirming its pivotal role in cellular stress adaptation. Similarly, *BvHsp70-20* and *BvHsp70-22*, selected based on their strong expression profiles from transcriptome data, exhibited heightened activity under multiple stress conditions, highlighting their importance in managing abiotic stress. In the Hsp90 and Hsp100 families, *BvHsp90-03*, *BvHsp90-07*, *BvHsp100-12*, and *BvHsp100-58* demonstrated significant upregulation, particularly in response to temperature and salt stresses.

The expression analysis of Hsps in plants under abiotic stress is critical for understanding their role in boosting plant resilience. Hsps (especially sHSPs and Hsp70, Hsp90, and Hsp100) function as molecular chaperones, ensuring proper protein folding and protecting cellular integrity during stress. The differential expression of Hsps in response to stressors like heat, drought, salinity, and oxidative stress is well documented, underscoring their central role in plant stress tolerance mechanisms (Wang et al. 2004). Moreover, studies on tobacco and Arabidopsis revealed that plant responses to the combination of temperature and drought stress differ from those to each stressor applied individually (Rizhsky et al. 2004, 2002). In this study, three beet genotypes (drought-sensitive (DS), drought-resistant (DR) breeding materials, and wild-type *Beta maritima* (WT)) were grown for four weeks. Leaf samples were collected at 0, 0.5, 1, and 2 h from plants exposed to drought, heat, and combined stress. Gene expression levels were analyzed via qRT-PCR, with results summarized in Fig. 6 and detailed in Supplementary Fig. 8.1–8.10, showing significant upregulation of specific *BvHsp* genes under various stress conditions across the genotypes.

Heat stress is a major environmental factor affecting plant growth and productivity. In the drought-tolerant genotype, *BvHsp70-22*, *BvHsp90-03*, *BvHsp40-03*, and *BvHsp60-28* were significantly upregulated under heat stress, suggesting a solid heat stress response. These Hsps likely play crucial roles in protein stabilization and protection against heat-induced damage. Specifically, the upregulation of *BvHsp40-03* in the tolerant genotype may indicate its unique contribution to heat tolerance. These findings align with studies like Zou et al. (2009), where Hsps in rice were differentially expressed under heat stress, highlighting their distinct roles in stress responses (Zou et al. 2009). Similarly, Singh et al. (2016) reported that overexpression of *sHsp* genes in foxtail millet improved heat tolerance, further emphasizing Hsps' potential in crop resilience enhancement (Singh et al. 2016). *BvHsp70-22, BvHsp90-03*, and *BvHsp60-28* were upregulated in the drought-sensitive genotype, signaling an active response to heat stress. However, the absence of *BvHsp40-03*, compared to the tolerant genotype, implies that the sensitive genotype might not respond as effectively to heat stress. This observation suggests that, while the drought-sensitive genotype can initiate a heat stress response, it may lack a full complement of protective mechanisms that would provide greater resilience. Results align with findings from Muthusamy et al. (2017), where specific *Hsp20* genes in wheat were upregulated in response to heat stress, indicating that specific Hsps may determine the extent of stress tolerance (Muthusamy et al. 2017). In contrast, the wild beet exhibited upregulation of *BvHsp70-22*, *BvHsp90-03*, and *BvHsp60-28*, similar to the other genotypes, suggesting a conserved heat stress response mechanism across different beet varieties. However, the absence of *BvHsp40-03* in the wild beet might indicate a different evolutionary strategy or adaptation mechanism, reflecting its response to diverse and fluctuating environmental conditions. Studies have also shown that *BvHsp70-22* and its Arabidopsis orthologue, *AT5G02490.1*, play roles in plant development and abiotic stress responses (Leng et al. 2017). Furthermore, under temperature stress, the expression of *AT5G02490.1* in Arabidopsis increases (Guo et al. 2022a). Similarly, in the soybean, temperature stress enhanced the expression of the *BvHsp90-03* orthologue, *Glyma.09G131500.1* (Huang et al. 2019; Song et al. 2017).

Drought stress triggers complex changes in gene expression, and Hsps are vital players in mitigating the adverse effects of water deficits. *BvHsp90-07* and *BvHsp60-28* were significantly upregulated in the drought-tolerant genotype, with *BvHsp90-07* playing a critical role in drought response by enhancing stress recovery and maintaining cellular homeostasis. Findings are consistent with Kummari et al. (2020), where the promoter region of the pearl millet *Hsp10* gene was activated under drought stress, underscoring the integral role of Hsps in drought tolerance (Kummari et al. 2020). In the drought-sensitive genotype, *BvHsp90-03* and *BvHsp60-01* were upregulated. Interestingly, the upregulation of *BvHsp60-01* rather than *BvHsp60-28* indicates a slight variation in the drought response mechanism, possibly reflecting the genotype's sensitivity to drought. This suggests that *BvHsp60-01* may compensate for the lack of more robust Hsps in the tolerant genotype, though this compensation may not suffice for effective stress management. This result aligns with the findings of Singh et al. (2016), who noted that specific Hsps were upregulated in stress-tolerant

foxtail millet cultivars, indicating that the expression of specific Hsps is closely linked to a plant's ability to withstand drought stress (Singh et al. 2016). In the wild beet (*Beta maritima*), *BvHsp70-22*, *BvsHsp34*, and *BvsHsp38* were upregulated, which may contribute to its resilience in natural environments where drought conditions prevail. The unique upregulation of *BvsHsp34* and *BvsHsp38* in the wild beet, absent in the other genotypes, suggests these Hsps could play a role in specific adaptive responses to fluctuating environmental conditions. These findings align with Zou et al. (2009), where specific Hsps in rice were induced by PEG-induced drought stress, highlighting their vital role in drought tolerance (Zou et al. 2009). Moreover, following a 6-h PEG treatment, the expression of *BvHsp40-03* orthologue, *Potri.005G113100.5*, in poplar was found to increase, indicating its involvement in protein folding, transport, and degradation via Hsp70-binding J domains (Mun et al. 2017). These results, supported by qRT-PCR, affirm that *BvHsp* genes are critical components of sugar beet's drought stress response pathways.

When drought and heat stresses are combined, the physiological response becomes even more complex. In the drought-tolerant genotype, *BvHsp60-28*, *BvHsp70-22*, *BvHsp90-03*, and *BvHsp90-07* were significantly upregulated, indicating a coordinated mechanism to handle the compounded effects of these stresses. These genes likely contribute to enhanced protein stability and recovery from stress, which is critical for sustaining plant vitality under extreme conditions. The tandem involvement of *BvHsp70-22* and *BvHsp90-07* suggests that these proteins may work together to offer comprehensive protection against combined drought and heat stress. This observation is in line with Sewelam et al. (2014), who found that specific Hsps, including sHsps, were strongly upregulated in response to combined stresses, more so than individual stress treatments (Sewelam et al. 2014). This observation is also consistent with the findings of Muthusamy et al. (2017), who reported that specific Hsp20 genes in wheat were upregulated in response to heat and drought stresses separately, highlighting the multifunctional role of Hsps in managing multiple abiotic stresses (Muthusamy et al. 2017). These Hsps are vital in preventing protein aggregation, a crucial function when plants face multiple stressors. *BvHsp70-22*, *BvHsp90-03*, and *BvHsp60-28* were upregulated in the drought-sensitive genotype. However, the absence of *BvHsp90-07* suggests a less robust response to combined stress, which might contribute to the vulnerability of this genotype. The lack of *BvHsp90-07* could be critical, as this protein might be essential for managing the compounded effects of heat and drought. Wild beet (*Beta maritima*) also showed significant upregulation of *BvHsp70-22*, *BvHsp90-03*, and *BvHsp40-44* under combined stress. Interestingly, *BvHsp40-44* was upregulated in wild beet but not in the tolerant and sensitive genotypes, suggesting a unique adaptive mechanism in wild beet that could be tied to its resilience in variable environmental conditions. Sewelam et al. (2014) also observed that certain genes, such as *Hsp17.4* and *Hsp18.2*, were explicitly upregulated in response to combined heat and drought stress, aiding the plant's ability to develop stress memory and recover from repeated stress exposure (Sewelam et al. 2014). This elevated expression in wild beet may enhance its adaptability to environmental challenges. Including *BvHsp40-44* in wild beet's response profile further highlights its potential role in cross-stress tolerance, possibly helping this species thrive in diverse environments. This finding is supported by Reddy et al. (2016), who emphasized the role of Hsps in protein folding and recovery under combined abiotic stresses, which is critical for maintaining cellular function during adverse conditions (Reddy et al. 2016). Additionally, Rizhsky et al. (2002) found that drought and heat stress combined in tobacco led to higher levels of Hsp90, Hsp70, and Hsp100, similar to findings in sugar beet (Rizhsky et al. 2002). The study further noted that combined stress resulted in a more complex induction of reactive oxygen species (ROS)-detoxifying enzymes, indicating that plants may engage different protective mechanisms when facing multiple stresses (Rizhsky et al. 2002). These findings may help explain why *BvHsp* genes in sugar beet play such a vital role, as the simultaneous activation of these proteins likely supports more efficient recovery and protection from cellular damage caused by dual stressors.

All examined genes, except for the *BvHsp100-12* gene, coincided with the transcriptome data when transcriptome analysis and qRT-PCR findings were compared, and their expressions rose noticeably at various stressors and timeframes. Furthermore, among the transcriptome data of temperature, salt, light, ABA, and salt stress application in root tissue, *BvsHsp-29*, *BvHsp40-97*, *BvHsp60-26*, *BvHsp70-15*, *BvHsp90-08*, and *BvHsp100-101* genes were found to be significantly represented. Plant tolerance may depend on these genes.

Functional validation studies provide critical insights into the specific roles of Hsps in stress tolerance. The upregulation of *BvHsp90-07*, *BvHsp70-22*, and *BvHsp90-03* in this study, particularly in the drought-tolerant genotype and wild beet, aligns with functional studies in other species. For example, Mu et al. (2013) demonstrated that the overexpression of the sHsp LimHsp16.45 in Arabidopsis conferred enhanced tolerance to heat and drought by maintaining protein stability and reducing oxidative damage (Mu et al. 2013). Similarly, Song et al. (2014) showed that the overexpression of chrysanthemum Hsp70 improved drought tolerance through increased peroxidase activity and higher proline content (Song et al. 2014). These functional studies suggest that the upregulated *BvHsp* genes in this study likely play similar roles in sugar beet, contributing to the

stabilization of crucial proteins, protection against oxidative stress, and enhancing stress tolerance. The findings also underscore the potential for utilizing these genes in breeding programs to enhance stress resilience in crops, a strategy supported by the broader literature on Hsps.

The observed expression patterns of *BvHsp* genes in this study are consistent with global trends in plant stress biology. Hsp101, for example, is essential for thermotolerance in various crops, including wheat and cowpea (Kumar et al. 2020a; Selinga et al. 2022). The coordinated action of Hsp101 with other sHsps prevents protein aggregation and maintains cellular homeostasis under stress conditions and is well documented. The potential interplay between BvHsp90-03 and BvHsp70-22 with Hsp101-like chaperones could be a vital aspect of the stress response in the sugar beet. This inference is supported by Xu et al. (2013), who demonstrated that overexpression of *GmHsp90* in Arabidopsis enhanced tolerance to various abiotic stresses, indicating the universal importance of Hsps across different plant species (Xu et al. 2013). The conserved nature of these stress responses across species highlights the evolutionary significance of Hsps in plant adaptation.

Comparative analysis of Hsp expression across species reveals that specific Hsps, such as Hsp70 and Hsp90, are universally important in stress responses. The significant upregulation of *BvHsp90-03* and *BvHsp70-22* in sugar beet under stress conditions mirrors findings in other species, indicating a conserved function across different plant species.

The expression of *Hsp* genes is tightly regulated by heat shock transcription factors (Hsfs), which are critical for the rapid induction of Hsps in response to stress. The regulatory framework involving Hsfs is likely responsible for this study's observed upregulation of *BvHsp* genes under stress conditions. Guo et al. (2016) demonstrated that Hsfs are central to conferring thermotolerance through the regulation of Hsps, a mechanism likely conserved in sugar beet (Guo et al. 2016). The interaction between Hsfs and Hsps is crucial for managing reactive oxygen species (ROS) generated during stress. Chen et al. (2020a, b) showed that the Hsf from *Sedum alfredii* enhances cadmium tolerance by regulating ROS scavenger activities alongside Hsp expression, illustrating the multifaceted role of Hsps in stress responses (Chen et al. 2020b). This regulatory interaction is likely a key component in sugar beet's ability to manage heat and drought combined stresses.

Hormonal regulation plays a significant role in modulating the expression of Hsps under stress. For instance, Colombage et al. (2023) found that melatonin treatment upregulated Hsp expression in plants, thereby improving their ability to cope with abiotic stress (Colombage et al. 2023). This suggests that the crosstalk between hormonal signaling pathways and Hsp expression could be an essential aspect of the stress response in sugar beet. The role of abscisic acid (ABA) in regulating *Hsp* expression is also well established, as shown in Zou et al. (2009), where Hsps in paddy were differentially expressed in response to ABA treatment, highlighting the importance of hormonal regulation in stress responses (Zou et al. 2009). This study's detailed expression analysis of *BvHsp* genes under various abiotic stresses highlights their potential roles in stress tolerance mechanisms. The consistent upregulation of specific *Hsp* genes in drought-tolerant and wild beet genotypes underscores the importance of these genes in enhancing plant resilience. While the role of *Hsp* genes in heat and drought stress has been widely explored in model plants, studies focusing on combined stress responses in sugar beet remain limited. However, recent research has begun to shed light on sugar beet's molecular and physiological adaptation mechanisms, primarily under individual stress conditions. For instance, (Putnik-Delić et al. 2017) demonstrated that proline accumulation and transcriptional reprogramming of stress-responsive genes strongly correlate with drought tolerance in sugar beet genotypes (Putnik-Delić et al. 2017). Similarly, Wang et al. (2017) utilized a proteomic approach to show that drought-tolerant sugar beet cultivars uniquely express stress-responsive proteins, such as antioxidant enzymes and metabolic regulators, which are largely absent in drought-sensitive varieties (Wang et al. 2017). Romano et al. (2013) further highlighted the importance of physiological traits like antioxidant capacity and plasma membrane H^+-ATPase activity as indicators of drought resilience, suggesting that both biochemical and biophysical responses contribute to stress adaptation (Romano et al. 2013). In addition to physiological traits, metabolic and regulatory interventions have also been explored. Islam et al. (2022) found that exogenous application of putrescine significantly improved drought tolerance in sugar beet by enhancing the antioxidant defense system and modulating stress-responsive gene expression (Islam et al. 2022). Moreover, Zou et al. (2023) identified 386 drought-responsive long non-coding RNAs (lncRNAs) in sugar beet, providing a rich set of regulatory targets for stress resilience (Zou et al. 2023). However, it is important to note that none of these studies explicitly investigated the role of Hsps under stress conditions.

Thus, our study contributes significantly to this gap by profiling the expression of *Hsp* genes across three genetically distinct sugar beet genotypes under both individual and combined stress conditions. The results not only expand our understanding of the functional roles of specific Hsp families (e.g., BvHsp70-22, BvHsp90-03, BvHsp60-28), but also offer valuable insights into stress-responsive gene regulation with direct implications for sugar beet breeding.

Functional validation studies from other species and the observed expression patterns in sugar beet suggest that these Hsps are likely involved in fundamental protective

mechanisms, such as protein stabilization, ROS scavenging, and the regulation of stress-responsive pathways.

Conclusions

This study provides comprehensive insights into the molecular characterization and expression profiles of *Hsp* genes in sugar beet (*Beta vulgaris*) under abiotic stress conditions. By comparing drought-sensitive and drought-tolerant cultivars with wild beet (*Beta maritima*), the research highlights the differential expression of key *BvHsp* genes, particularly *BvHsp70-22* and *BvHsp90-03*, which were strongly upregulated in tolerant genotypes.

A total of 334 *Hsp* genes were identified, with gene duplication and synteny analyses revealing conserved evolutionary patterns. The expression data, supported by functional evidence from other plant species, underscore the role of *BvHsp* genes in protein stabilization, regulation of stress-responsive pathways, and indirectly ROS scavenging. These findings lay a foundation for breeding and biotechnological approaches aimed at enhancing sugar beet resilience. Future research should prioritize functional validation and explore Hsp regulatory networks to better understand plant stress responses and inform crop improvement under climate stress.

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Declarations

Competing interests The authors declare no competing interests.

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