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ORIGINAL ARTICLE

HSF and *Hsp* Gene Families in sunflower: a comprehensive genome-wide determination survey and expression patterns under abiotic stress conditions

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Abstract

Sunflowers belong to the Asteraceae family, which comprises nutrimental and economic oilseed plants. Heat shock proteins (Hsps) are protein families vital for all organisms' growth and survival. Besides the ordinary conditions, the expression of these proteins ascends during abiotic stress factors such as high temperature, salinity, and drought. Using bioinformatics approaches, the current study identified and analyzed HSF and Hsp gene family members in the sunflower (*Helianthus annuus* L.) plant. HSF, sHsp, Hsp40, Hsp60, Hsp70, Hsp90, and Hsp100 domains were analyzed in the sunflower genome, and 88, 72, 192, 52, 85, 49, and 148 genes were identified, respectively. The motif structures of the proteins in the same phylogenetic tree were similar, and the α-helical form was dominant in all the protein families except for sHsp. The estimated three-dimensional structure of 28 sHsp proteins was determined as β-sheets. Considering protein-protein interactions, the Hsp60-09 protein (38 interactions) was found to be the most interacting protein. The most orthologous gene pairs (58 genes) were identified between *Hsp70* genes and *Arabidopsis* genes. The expression analysis of selected genes was performed under high temperature, drought, and high temperature-drought combined stress conditions in two sunflower cultivars. In stress conditions, gene expressions were upregulated for almost all genes in the first half and first hours at large. The expressions of *HanHSF-45* and *HanHsp70-29* genes were raised in two cultivars under high temperature and high temperature-drought combined stress conditions. This study presents a blueprint for subsequent research and delivers comprehensive knowledge of this vital protein domain.

Keywords *Helianthus annuus* L. · Hsp (Heat shock proteins) · HSF (Heat shock factors) · Genome-wide determination · Bioinformatics analysis · Gene expression

Introduction

Sunflower (*Helianthus annuus* L.) is in the Asteraceae family and is one of the most cultivated oilseed plants in the world after soybean, rapeseed, and peanuts (Hu et al. 2010; Debaeke et al. 2017). The agriculture of sunflowers is carried out in two types: oil and appetizer (Fernández-Martínez et al. 2009; Alberio et al. 2015). Although sunflower production worldwide is comprehensive, Ukraine, Russia, the EU, Argentina, and China are among the countries with the largest share of sunflower agriculture. Turkey follows China in sunflower production (Konyalı 2017).

Heat shock proteins (Hsps) are induced by many stress factors such as high and low temperature, UV light, NaCl, wounding, and drought, ubiquitous in prokaryotic and eukaryotic cells (Boston et al. 1996; Süle et al. 2004). Despite these proteins being frequently located in the cytosol, they are in many cell regions, such as the endoplasmic reticulum (ER), chloroplast, mitochondria, and nucleus. It has been proven that different sites' proteins have distinctive and dynamic roles in cell homeostasis (Wang et al. 2004a; Kotak et al. 2007; Gupta et al. 2010; Fortunato et al. 2023). It has been established that Hsps are blamed for the folding, assembly, transport, and degradation of proteins during

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average cell growth and development stages. They also perform critical chaperone functions, like the three-dimensional folding of newly created proteins and damaged or misfolded proteins under stress conditions (Patil et al., 2022; Wang et al., 2004b).

Heat shock transcription factors (HSFs) reside in the cytoplasm as inactive bodies and regulate the transcription of genes coding Hsps (Hu et al. 2009; Åkerfelt et al. 2010; Guihur et al. 2022). Plant HSF proteins are characterized by short peptide motifs (AHA) whose N- (amino-terminal) DNA binding domain (DBD), C- (carboxyl terminal) is essential for activator role in many occasions (Döring et al. 2000; Scharf et al. 2012; Lü et al. 2022). HSFs regulate gene expression in maintaining cellular homeostasis, controlling the heat stress response, and many more environmental factors. Under stress conditions, they activate transcription and accumulation of Hsps, which serve as chaperones to fold damaged proteins and to save new ones (Kim et al. 2021).

Hsps are grouped based on their molecular weights as sHsp, Hsp40, Hsp60, Hsp70, Hsp90, and Hsp100. Small heat shock proteins (sHsps) are one of the most common subgroups of Hsp, and the plant sHsps are primarily stimulated by heat stress. Hsp40 is referred to as J-proteins based on the existence of the highly protected J domain. Hsp40 proteins perform as molecular co-chaperones, and the J domain induces Hsp70 ATPase activity (Wall et al. 1994). Hsp60 proteins, also called molecular chaperonins, assist in efficiently folding new synthesized and misfolded polypeptides. Hsp60 also binds to the substrates of unfolded proteins, preventing aggregation (Boshoff 2015). Hsp70 regulates stress-sensitive signaling mechanisms, protein disruption, protein folding, and protein replacement at the membranes (Kampinga and Craig 2010; Nillegoda and Bukau 2015). Hsp90 has been detected in plants' cytosol, nucleus, ER, mitochondria, and chloroplasts. Hsp90 expression has increased with high and low temperature, salt, wounding, UV, bacterial or viral infection, and other stress factors. The primary functions of Hsp90 proteins are to maintain cell viability via participating in the construction, stabilization, maturation, and activation of signaling proteins (Conner et al. 1990; Nover and Miernyk 2001). Hsp100/Clp is a (100 kDa) hexameric protein family belonging to the ATPase superfamily with comprehensive functional properties. Besides regular chaperone functions to inhibit protein aggregation and misfolding, the Hsp100/Clp family has an active role in protein degradation (Adam and Clarke 2002; Suzuki et al. 2012; Mittler et al. 2012).

An abiotic stress factor is an environmental variable such as light, high temperatures, droughts, salts, and pH that adversely affects the growth of plants (Altunoğlu et al. 2019). Plants are sessile against stress factors, but they have adapted themselves to most of the unfavorable conditions in evolution and have acquired complex mechanisms (Mittler 2006). Plants grown in the territory are exposed to different abiotic stress conditions. According to The Intergovernmental Panel on Climate Change (IPCC), it has been reported that the frequency and duration of drought will increase in the coming years due to global warming. The 1.1°C rise in global warming gives rise to high evaporation in all water resources, extreme weather events, changes in the climate system, and the disappearance of sea ice (Qwais 2022). Due to high-temperature stress, the degradation of the cell membrane permeability and decrease in ion flow and lipids peroxidation leads to the aggregation of reactive oxygen species (ROS) such as hydrogen peroxide (McClung and Davis 2010; Suzuki et al. 2012). Accumulated hydrogen peroxide induces several genes including *HSF* and *Hsp* genes. However, ascorbic acid, glutathione, and other antioxidant molecules also take part in plant defense against ROS (Larkindale and Knight 2002; Neill 2002; Bose et al. 2014). A plant in drought has to struggle simultaneously with salinity and high-temperature stress. As a result of exposure to high temperature-drought, plants undergo physiological changes such as high respiration and low photosynthesis rates, closed stomata, and elevated leaf temperature, as well as metabolic changes such as starch breakdown and change in proline level (Mittler, 2006). In drought conditions, the expression of stress-related genes is induced, resulting in protein synthesis (Mahalingam 2015). Proteins of tolerance to drought stress are functional proteins that participate in gene expression regulation. Proteins involved in ABA biosynthesis, transcription factors, phosphatases, and protein kinases that are regulatory proteins play a role in regulating gene expression. Late embryogenesis proteins (LEA), chaperones, antifreeze proteins, and mRNA binding proteins are directly involved in stress tolerance (Cechin et al. 2006; Kim et al. 2010; Sarvepalli et al. 2018). It is thought that plants' molecular and metabolic reactions to the combination of high temperature and drought differ from the stress conditions applied separately (Mahalingam 2015; Lawas et al. 2018). In former studies, it has been argued that Hsps give rise to plant resistance to abiotic stress conditions (Zhang et al. 2010; Lawas et al. 2018; Altunoğlu et al. 2019).

Hsps have been defined in many organisms such as poplar (Yer et al. 2018), jujube (Ceylan et al. 2021), orange (Shafqat et al. 2020), potato (Tang et al. 2016), lettuce (Kim et al. 2021), maize (Lin et al. 2011), soybean (Chung et al. 2013), and rice (Hu et al. 2009). In these genome-wide studies, data compatible with our study were presented, such as the organization of genes, protein motifs, phylogenetic analyses, and predicted three-dimensional structures of the proteins. Like the current paper, it was determined that the most crowded Hsp family was the Hsp40 and Hsp100 families. Also, Kim et al. (2021) found that Hsp and Hsf domains with similar motifs and gene organization were located on the same phylogenetic tree (Kim et al. 2021). The sunflower's genome was published in 2017 and updated in 2020 (Badouin et al. 2017). According to our knowledge, identification and defining features of HSF and Hsp family members in sunflowers using whole genome data have not been performed yet. The present study was carried out to prove whether *Hsp* and *HSF* genes have a potential role against abiotic stress conditions including high temperature, drought, and high temperature-drought combined stress in sunflowers. First, *Hsp* and *HSF* genes were systematically identified and characterized by bioinformatics approaches. Then, gene expression profiles of the genes were analyzed in qRT-PCR based on transcriptomic data against abiotic stress conditions in Saray (drought-resistance) and Turay (drought-sensitive) cultivars of sunflower. The study results will supply additional beneficial information for the functional analysis of *Hsp* and *HSF* genes in sunflowers.

Material and methods

Identification of *HSF* and *Hsp* genes in sunflower genome

Extensive research was performed to determine the *Hsp* and *HSF* genes in the sunflower genome based on our group's previous studies with the Hsp family (Yer et al. 2016a, b, 2018; Altunoğlu et al. 2019; Ceylan et al. 2021). Briefly, protein sequences of HSF, sHsp, Hsp40, Hsp60, Hsp70, Hsp90, and Hsp100 from other plants were derived from the heat shock protein database (HSPIR) (http://pdslab.biochem.iisc.ernet.in/hspir/) (Kumar et al. 2012). BLAST P search was performed for these sequences on the NCBI (National Biotechnology Information Center) database, and also, The Hidden Markov Model (HMM) statistical model (Baloglu et al. 2014) was compared with all proteins in the sunflower genome. The statistical matches which met the expectation value of $\geq e^{-50}$ and identity value of $\geq e^{-60}$ were saved as significant. Following that, the analysis of HSF and Hsp protein conserved region was performed on CLC Genomic Workbench v11.0 (Qiagen, Germany), and domain analysis of proteins was carried out using Pfam databases (https://pfam.xfam.org/) (Finn et al. 2016). Finally, the isoelectronic value, molecular weight, amino acid length, and the instability index of each one of the proteins were acquired using the PROTPARAM tool (http://web.expasy.org/protparam) (Gasteiger et al. 2005).

Chromosomal distribution and estimation of the gene structure of *HSF* and *Hsp* genes

The sequences of *HSF* and *Hsp* genes were analyzed in the Genome Data Viewer (https://www.ncbi.nlm.nih.gov/genome/gdv/) to determine the chromosomal position of the genes. Then, the MapChart program (Voorrips 2002) was used to visualize the location of genes on the chromosome. To elucidate the structure of sunflower *HSF* and *Hsp* genes, the exon-intron sites of genes were defined using the Gene Structure Display Server (http://gsds.cbi.pku.edu.cn/) (Hu et al. 2015).

Identification of conserved motifs, sequence alignment, and phylogenetic analysis of HSF and Hsp proteins

To analyze the conserved motif of sunflower HSF and Hsps proteins, the MEME Suit web portal (Bailey et al. 2009), a motif-based search tool, was utilized to identify the protected motifs. Ten motif models were chosen for HSF and Hsp proteins, and the optimum width of the motif was selected between ≥ 2 and ≤ 300.

The sequences of the proteins were uploaded into the MEGAX software (Kumar et al. 2018), and the multiple sequence alignment was achieved using the ClustalW alignment method (Larkin et al. 2007). By using the maximum likelihood method (Milligan 2003) with 1000 iterative bootstrap assays, the phylogenetic trees were constructed, and their visualization was performed using the iTOL (Interactive Tree Of Life) tool (Letunic and Bork 2007).

Prediction and correlation analysis and ontology of HSF and Hsp proteins

The HSF and Hsp protein sequences were aligned to STRING 11.5 v (Mering 2003) online server that predicts protein-protein interaction via the K-mean algorithm. *H. annuus* was preferred as the organism, and the analysis was carried out with medium confidence (0.4).

The functional analysis of *HSF* and *Hsp* genes was performed on the OmicsBOX program (Conesa and Götz 2008). The protein sequences of sunflower HSF and Hsps were utilized in the program, and the functional investigation was achieved in stages like BLAST, InterPro, mapping, annotation, charting, and graphing. After the investigation, molecular function, cellular location, and biological function of HSF and Hsps were stated for each gene.

Computation of tandem-segmental duplications and determination of gene orthologous

While computing tandem and segmental analysis of *HSF* and *Hsp* genes, genes in the same location of the phylogenetic tree are identified as paralog genes. Paralogous genes are segmental if they are on duplicated chromosomes and tandem if they are on the same chromosome (Ünel 2018). The coding sequences of *HSF* and *Hsp* genes were loaded into the database (http://services.cbu.uib.no/tools/kaks), and the results were inspected with phylogenetic trees, and segmental and tandem relationships between the genes were revealed.

Moreover, using protein BLAST in NCBI, the orthologous relations of sunflower *HSF* and *Hsp* genes with *Arabidopsis*, rice, poplar, and maize were performed. Among the results, rates with an expectation value of e=0 and matching the ≥80% identity limitation were approved between the sequences indicating identity.

In addition, multiple amino acid sequence alignment of HSF and Hsps was carried out using the ClustalW method by aligning orthologous gene pairs between the duplicated genes in the sunflower genome, and *Arabidopsis*, rice, poplar, and maize. Afterward, computing of homologous (Ks) and non-homologous (Ka) exchange ratio with coding sequence and amino acid sequence of *HSF* and *Hsp* genes was fulfilled using the PAL2NAL (http://www.bork.embl.de/pal2nal) online tool (Suyama et al. 2006). Therefore, utilizing the formula ($T = \mathrm{Ks}/2\lambda$ ($\lambda = 6.5\times10^{-9}$), the separation times of orthologous genes in the evolution period were calculated as million years ago (mya) (Lynch and Conery 1979; Yang et al. 2008).

Defining of miRNAs targeted to *HSF* and *Hsp* genes in sunflower

To determine miRNAs that target the *HSF* and *Hsp* genes in the sunflower genome, miRBase v22.1 (http://www.mirbase.org/) (Kozomara and Griffiths-Jones 2014) software was performed. Using the psRNA Target Server (https://www.zhaolab.org/psRNATarget/), all putative plants and sunflower miRNAs were determined by aligning all recognized miRNA with *HSF* and *Hsp* gene transcripts in the sunflower (Dai et al. 2018). miRNAs targeting *HSF* and *Hsp* gene transcripts were pictured via Cytoscape software (Shannon et al. 2003).

Estimated three-dimensional (3D) structures of HSF and Hsp proteins

BLAST P search was carried out in the Protein Data Bank (PDB) to identify the estimated three-dimensional structure of the HSF and Hsp proteins. Predicted 3D forms of sunflower HSF and Hsps were performed using the PHYRE2 database (http://www.sbg.bio.ic.ac.uk/phyre2) (Kelley et al. 2015).

Expression analysis of *HSF* and *Hsp* genes using transcriptomic data

To check *HSF* and *Hsp* gene expressions in sunflowers, transcriptome data from the Sequence Read Archive (SRA, https://www.ncbi.nlm.nih.gov/sra) was downloaded from NCBI. The SRA data of PRJNA483306, PRJNA398727, and PRJNA632976 were converted to the "fastq" format. Then, FastQC experiment was performed to check the quality of the readings. Normalization and transformation of all data were studied on the CLC Genomic Workbench v 11.0 platform. Afterward, heat maps were generated using the Permut Matrix program that utilizes gene expression attained by converting RPKM values to log2 (Caraux and Pinloche 2005).

Plant materials and stress treatments

Two different sunflower cultivars, Saray (drought-resistant) from the Directorate of the Trakya Agricultural Research Institute and Turay (drought-sensitive) from the Directorate of the Aegean Agricultural Research Institute, were utilized. The seeds were cleaned and held in distilled water overnight. Then, the seeds were sown in containers that contained vermiculite. They were grown with Hoagland solution at 24 ± 2°C and 16/8 light-dark period, 60% humidity, and 400 μmol $\mathrm{m}^{-2}\mathrm{s}^{-1}$ light intensity for approximately 1 month in the climate cabinet (Supplementary Fig.1). Drought, temperature, and high temperature-drought combined stresses were implemented to the plants. 20% Hoagland solution, including polyethylene glycol 6000 (PEG-6000), was utilized for drought stress. The temperature of the climate cabinet was adjusted at 50°C for high-temperature stress (Baloglu et al. 2014; Altunoğlu et al. 2019). The leaf samples from sunflowers were gathered at 0, 30 min, 1 h, and 2 h of stress treatment as in former papers (Baloglu et al. 2014; Altunoğlu et al. 2019). Samples harvested at zero hours were accepted as control. All collected samples were kept at −80°C until RNA isolation.

RNA isolation and quantitative real-time PCR (qRT-PCR) experiments

Total RNA was isolated using a RiboEx reagent (GeneAll, South Korea) protocol reported in our group's past studies (Baloglu et al. 2014; Altunoğlu et al. 2019). The concentration on integrity was checked with the spectrophotometer (MultiScanGo, Thermo Fisher Scientific, USA) and agarose gel electrophoresis. To purify isolated RNA samples from DNA contamination, DNase (RNase-free, Thermo Fisher Scientific, USA) was performed based on the manufacturer's instructions. Afterward, cDNA synthesis was achieved from total RNA using iScript cDNA Synthesis Kit (Bio-Rad, USA). For the standardization of qRT-PCR analysis, the amount of cDNA was used at the same quantity approximately 50 ng. Primers for *HSF* and *Hsp* genes were designed by the NCBI Primer Blast tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). qRT-PCR was performed using

Qiagen Rotor-Gene 6000 “Real-Time” PCR device. SYBR Green Supermix (Bio-Rad, Hercules, USA) was utilized for the reaction. Three biological and technical repeats were practiced from every sample. The ingredients for qRT-PCR assay were 10 µL in total, including 0.5 µL forward primer, 0.5 µL reverse primer (25 pmol/µL), 2 µL cDNA (50 ng/µL), 5 µL SYBR Green Supermix, and nuclease-free water. qRT-PCR protocols were demonstrated by first heating at 95°C for 5 min, followed by 45 cycles of 95 °C for 10 s, 58°C for 20 s, and 72 °C for 10 s. Every sample’s melting curve was analyzed by holding 97°C for 5 s and 65 °C for 1 min and cooling it to 4°C for 10 s. *18S rRNA* (GenBank ID: X515 42.1) and *GAPDH* were preferred as reference genes (Baloglu et al. 2014; Arslan et al. 2021). Computed ΔCT (ΔCT = CT sample − CT reference) and ΔΔCT (ΔΔCT = ΔCT stressed sample − ΔCT control) values were utilized to ascertain the variation in gene expression ratios, as $2^{-\Delta\Delta CT}$ and graphics of expression of the genes were visualized (Livak and Schmittgen 2001). The Minitab 18 software calculated statistical differences between control and stressed samples. After the univariate-ANOVA test, it was anticipated that the differences in expression of the related genes with a p-value of < 0.05 were statistically prominent.

Results and discussion

The current paper comprises two parts, bioinformatics analyses and gene expression assays. In the initial part, *HSF* and *Hsp* genes in the sunflower genome were identified and characterized including genes distribution on chromosomes, the genes organization, phylogenetic relationships of domains, conserved motif regions, gene ontology analysis, protein-protein interactions, and estimated three-dimensional structures. Besides, genes targeted by miRNAs, tandem and segmental duplications, separation rates, and orthologous relationships with other species were determined.

In the latter part, the heat maps of sunflower *HSF* and *Hsp* genes expression evaluated according to SRA data were detected and potential resistant gene candidates were analyzed at gene expression levels. Sunflower Saray (drought resistant) and Turay (drought sensitive) cultivars were exposed to high-temperature stress, drought stress, and high temperature-drought combined stress. Gene expression profiles of samples at 0 min, 0.5 h, 1 h, and 2 h were formed.

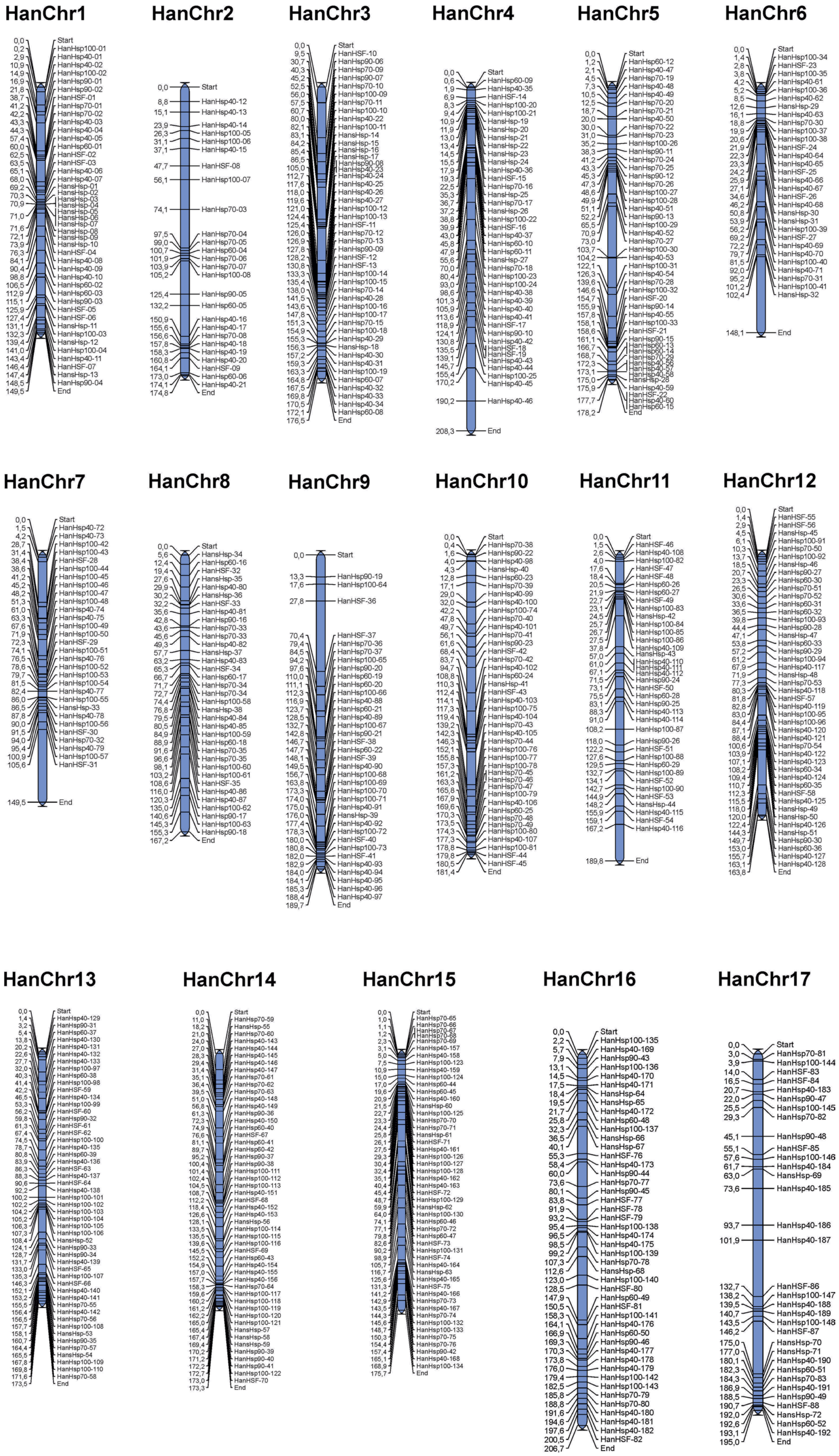
Identification of *HSF* and *Hsp* genes in sunflower genome

As a result of considerable research, 88, 72, 192, 52, 85, 49, and 148 genes consisting of HSF, sHsp, Hsp40, Hsp60, Hsp70, Hsp90, and Hsp100 protein subgroups were identified, respectively. *HSF* and *Hsp*s were shortened as *HanHSF* and *HanHsps*, called from the initial letters of *Helianthus annuus* (Supplementary Table 2.1–2.7). It was specified that the amino acid length of HanHSFs changed between 115 and 965 aa; most of them were acidic. It has been seen that protein lengths of the HansHsp proteins were between 100 and 511 aa; most were basic. The size of HanHsp40 proteins varied between 110 and 2542 aa; 118 of the protein were basic, and 74 were acidic. The amino acid content of HanHsp60 proteins altered from 92 and 1649 aa; 79% of proteins were acidic. It was found that the protein length of the HanHsp70s was 78 to 118 aa long; most proteins were acidic. HanHsp90 proteins were 193 to 1393 aa in length; most proteins were acidic. The amino acid length of HanHsp100 varied between 87 and 1604 aa; most were acidic. According to molecular weights and amino acid lengths, the molecular weights of the sunflower *HSF* and *Hsp* genes were between 9967.55 and 183,600.25 Da. Several previous researches are associated with identifying and characterizing *HSF* and *Hsp* genes in plant genomes. Based on previous papers, 283 *Hsp* genes were identified in watermelon (Altunoğlu et al. 2019), 390 in poplar (Yer et al. 2016b, 2018), 113 in foxtail millet (Singh et al. 2016), 753 in wheat (Kumar et al. 2020), 95 in cucumber (Chen et al. 2021), 165 in lettuce (Kim et al. 2021), 474 in jujube (Ceylan et al. 2021), and 141 in eggplant (Gong et al. 2021). The Hsp40 family has the most proteins among the Hsps. While the *Hsp40* gene was found in 192 genes in the sunflower genome, it was the most crowded Hsp domain with 100 genes in cucumber (Ünel 2018), 273 genes in wheat (Kumar et al. 2020), 174 genes in jujube (Ceylan et al. 2021), 91 genes in maize (Lin et al. 2011), 64 genes in grapes (Ji et al. 2019), 101 genes in watermelon (Altunoğlu et al. 2019) and 111 genes in rice (Guo et al. 2008). Hsp proteins are abundant in many plants due to their role in plant defense. In addition, it is known that Hsp40 proteins are the most crowded protein family, and the J domain of Hsp40 proteins works together by inducing Hsp70 ATPase activity (Wall et al. 1994; Laufen et al. 1999; Kampinga and Craig 2010).

Chromosomal distribution and estimation of the gene structure of *HSF* and *Hsp* genes

The *HanHSF* and *HanHsp* genes were distributed over 17 chromosomes in the sunflower genome, and most of them were localized on chromosomes 13 and 14 (Fig. 1). There is no *HansHsp* gene on the second chromosome, *HanHsp60* and *HanHsp90* genes on chromosomes 6 and 7, and *HanHsp70* on chromosome 11. All genes of *HanHSF*, *HanHsp40*, and *HanHsp100* were distributed over all chromosomes (Supplementary Fig.2.1–2.7).

When the exon-intron organization of *HSF* and *Hsp* genes was analyzed, it was specified that most genes in all domains contained the intron regions. However, it was observed


HanChr1
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◂**Fig. 1** Chromosomal localization of all *HSF* and *Hsp* genes on sunflower chromosome. Chromosomal intervals are determined as Mbp. The highest distribution of genes was on the 13th and 14th chromosomes of sunflower

that 6, 27, 52, 9, 50, 9, and 14 genes in *HSF*, *sHsp*, *Hsp40*, *Hsp60*, *Hsp70*, and *Hsp100*, respectively, do not comprise introns (Supplementary Fig.3.1–3.7). Most *HanHSF* genes have been identified to contain one or two intron regions like previous *HSF* gene exon-intron organization in rubber trees, radishes, physic nuts, common beans, and potatoes (Tang et al. 2016; Liu et al. 2019; Zhang et al. 2022). *HansHsp* genes were detected as an intronless domain with the highest rate in the current study. Under stress conditions (Jo and Choi 2015; Deshmukh et al. 2016), *sHsp* genes are recognized to be quickly and highly induced because most *sHsp* genes are considered intronless (Deshmukh et al. 2016; Chen et al. 2018). Hsp40, Hsp60, Hsp70, Hsp90, and Hsp100 proteins act as chaperones and help to fold many proteins in the cell (Asea et al. 2016). According to exon-intron organizations, it was seen that most of these genes contained multiple intron regions. It can be attributed to the roles of introns, which are vital in the alternative splicing, positive regulation of gene expression, and initiation and termination of transcription (Wu and Lindsay 1996; Fedorova and Fedorov 2003; Rose 2008; Jo and Choi 2015).

Identification of conserved motifs, sequence alignment, and phylogenetic analysis of HSF and Hsp proteins

The phylogenetic trees were constructed, and a conserved motif search was fulfilled to assess the evolutional relations among the HanHSF and HanHsps (Supplementary Fig.4.1–4.7). Like the current study, ten conserved motif scans were analyzed in tea (Chen et al. 2018), tobacco (Song et al. 2019), potato (Tang et al. 2016), rubber tree (Li et al. 2019), bean (Zhang et al. 2022), and jujube (Ceylan et al. 2021). When the conserved motifs of HanHSF and HanHsp proteins were examined in the evolutionary process, it was seen that the dominant motifs were different in all groups. Although they have dissimilar amino acid sequences, motifs 1 and 2 were found in all dominant motifs in all domains.

After investigation of the phylogenetic trees constructed by the maximum likelihood method (Supplementary Fig.5.1–5.5), it was seen that HanHSF (Fig. 2A), HansHsp, HanHsp70, and HanHsp90 proteins (Fig. 2B) were divided into three main clusters. Two main groups were determined for HanHsp60 proteins, and the phylogenetic tree of HanHsp40 and HanHsp100 included four main collections. It has been observed that proteins with similar motifs and exon-intron organization are settled in the same branch of the tree. To give an example from the present study, when the motifs of the HansHsp-29, HansHsp-40, HansHsp-45, and HansHsp-71 proteins were analyzed, four proteins have motif 5-1-7-3-2 organization, and it has been detected that they were located in the IIIa branch of the phylogenetic tree and demonstrated similarity in exon-intron structures.

Prediction and correlation analysis and ontology of *HSF* and *Hsp* genes

Molecular, biological functions and cellular localization of sunflower *HSF* and *Hsp*s were identified via ontology analysis (Supplementary Fig.6.1–6.5). HanHSF family members have several biological functions such as cellular process, metabolic process, regulation of biological processes, biological regulation, and response to stimuli. They are distributed in the cell membrane, organelles, organelle membrane, and endoplasmic reticulum despite these proteins mostly being present in nuclei. Transcription factors (TF) that promote or suppress genes play a major role in the expression of genes (Latchman 1997; Sarvepalli et al. 2018). It was noticed that the molecular function of HanHSF proteins included transcription regulatory activity, binding, and catalytic activity. Considering protein-protein interactions (Supplementary Fig.7.1–7.5), the interaction of HanHSFs with proteins such as alanine-2-oxoglutarate aminotransferase 2 (AOAT2), aspartate aminotransferase (ASP3), and tryptophan synthase (TRPB) gets vital roles in the metabolic process, and these proteins have roles in biological regulation (Yin et al. 2010; Romney et al. 2017). HansHsps have roles in cellular processes, response to stimulus, regulation of biological processes, metabolic process, biological regulation, and positive regulation of biological processes; their molecular function is binding and catalytic activity. They are mainly found in the cytoplasm, nucleus, and membrane. HansHsp proteins are known to have high protein-protein interactions with T-complex proteins and their subunits. These proteins interact with 15% of all cellular proteins and play a role in the folding of actin and tubulin. The HanHsp40 ontology analysis (Fig. 3A) revealed that their biological functions were in cellular process, metabolic process, stimulus-response, biological regulation, and localization; the molecular function was divided into two groups: binding and catalytic activity. Their localization spreads over a wide area such as membrane, cytosol, chloroplast, nucleus, organelle membrane, endoplasmic reticulum, and organelle membrane. The proteins with which HanHsp40 proteins interact highest are CRR23 (inorganic carbon transport protein), A0A251SWU7 (inner membrane translocase subunit), and TIM50 (Haloacid dehalogenase-like hydrolase (HAD) superfamily protein. Based on their interactions, both molecular and biological functions of HanHsp40 were directly proportional.

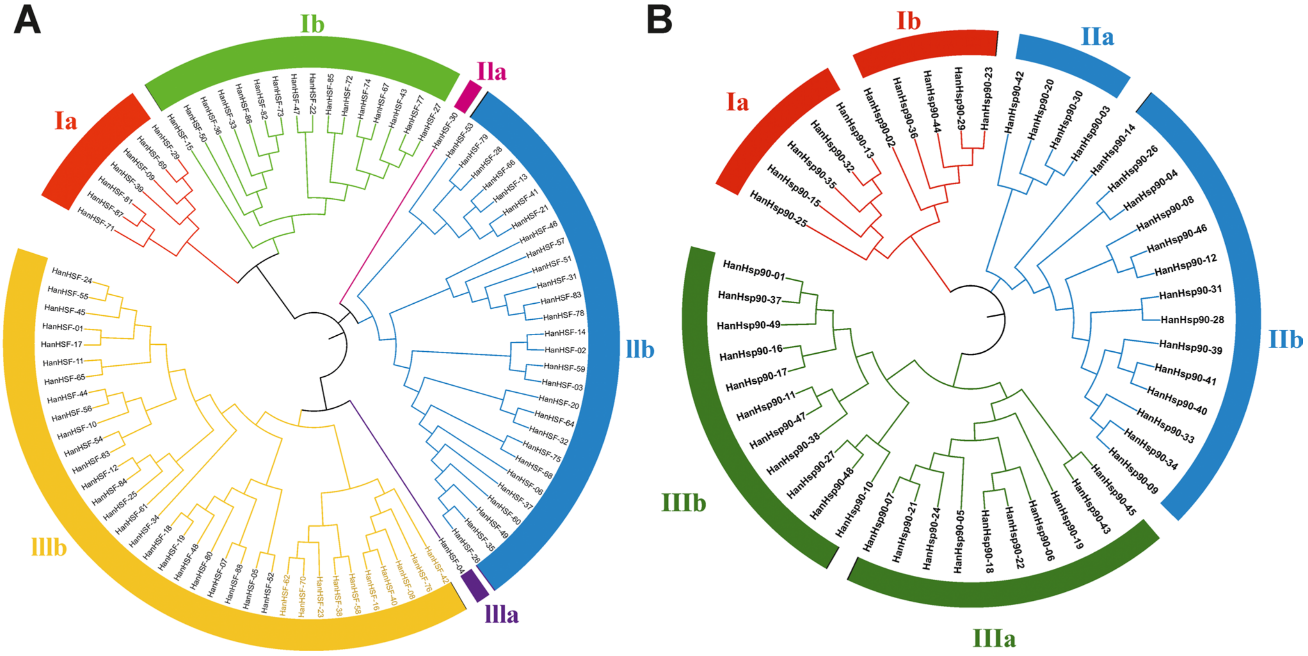

Fig. 2 Phylogenetic comparison of sunflower HanHSF proteins with three clusters (A), sunflower HanHsp90 proteins with three clusters (B). The construction of phylogenetic trees was done using the maximum likelihood method with 1000 repetitive bootstrap assays

Like HanHsp40, the molecular function of HanHsp60 is binding and catalytic activity, and they are frequently located in the chloroplast, membrane, mitochondrial matrix, and endosome membrane. Cellular and metabolic processes were identified for the molecular function of HanHsp60 members. Mostly, HanHsp60 proteins interacted with phosphorylation, phosphatidylinositol, phospholipase, and ATPTEN1 related to the calcium/lipid binding phosphatase gene (Fig. 4) (Hirayama et al. 1995; Sanchez and Chua 2001; Gupta et al. 2002; Kang et al. 2011). It has been determined that the expression of these proteins is induced under stress situations such as dehydration, high salinity, and low temperature (Gupta et al. 2002). The ontology analysis of *HanHsp60* genes is compatible with previous studies, including jujube (Ceylan et al. 2021), watermelon (Altunoğlu et al. 2019), and poplar (Yer et al. 2018). HanHsp70 ontology analysis discovered that their biological functions were in cellular process, response to stimulus, metabolic process, regulation of biological processes, signaling, and biological regulation; their molecular function was divided into three groups: binding, protein folding chaperone, and catalytic activity. They are distributed in the membrane, nucleus, endoplasmic reticulum chaperone complex,

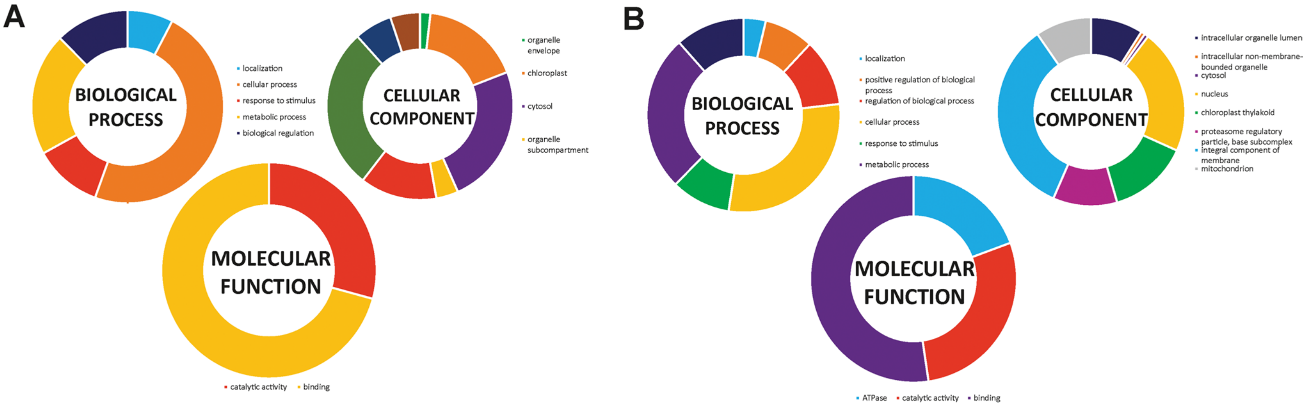

Fig. 3 Gene ontology analysis of (A) *HanHsp40* genes and (B) *HanHsp100* genes by Omicsbox program with three steps (biological process, cellular component, and molecular function)

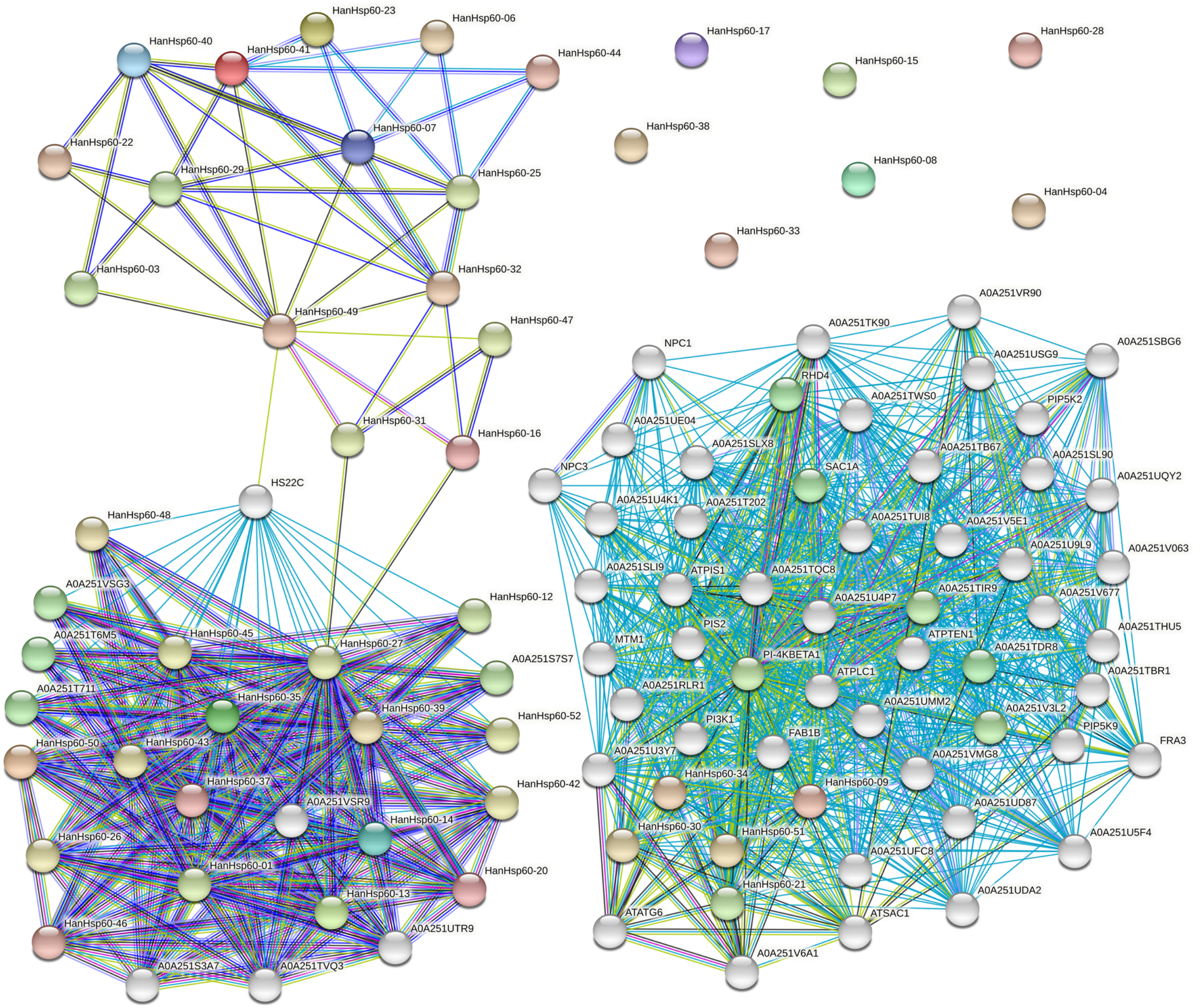

Fig. 4 Protein-protein interactions of HanHsp60 proteins. Medium confidence (0.4) was preferred for analysis. The HanHsp60-09 protein (38 interactions) was found to be the most interacting protein

cytosol, chloroplast, and endoplasmic reticulum lumen. The protein-protein interaction of HanHsp70 was mostly proteins about the cellular process or binding function like cyclin-dependent kinase E1 (CDKE1) and protein kinase family. HanHsp90 proteins are distributed in the perinuclear part of the cytoplasm, cytosol, mitochondrial large ribosomal subunit, chloroplast membrane, plasma membrane, stroma, Golgi membrane, nucleus, vacuole membrane, cell wall, and endoplasmic reticulum. HanHsp90 domain members get many biological functions in cellular and metabolic processes, response to stimulus, biological regulation, localization, multicellular organism process, and developmental process; the molecular function was primarily binding, catalytic activity, and structural molecular activity. HanHsp90 proteins primarily interact with ribosomal proteins and subunits that affect the functions of these proteins. HanHsp100 take part in the regulation of biological processes, localization, regulation and positive regulation of biological processes, response to stimulus, metabolic process, and biological process (Fig. 3B). ATPase, binding, and catalytic activity were determined as their molecular function. They are mainly located in the membrane, nucleus, thylakoid, intracellular organelle, cytosol, mitochondria, proteasome, and organelles. The proteins with which the Hsp100 proteins interact were ankyrin proteins, proteins related to proteasomes and their subunits, and proteins belonging to the ATPase family. These proteins participate in cellular and metabolic processes (Liberek et al. 1991; Becerra et al. 2004).

Computation of tandem-segmental duplications and determination of gene orthologous

Gene duplications among organisms supply informative findings like *HSF* and *Hsps*. Gene duplications can explain the differentiation of gene families at the genome level. The percentage of segmental and tandem duplications and their divergence has been calculated mutually in the study (Supplementary Table 3). The sunflower genome shows 41% (36/88) of segmental duplication and no tandem duplication. For Hsp proteins, it was estimated that 14% (81/598) tandem duplication and 27% (161/598) segmental duplication. As in previous studies, the percentage of distribution of segmental duplication was calculated more than tandem duplications (Tuskan et al. 2006; Zhang et al. 2015; Yer et al. 2018). The results presume a predominantly high correlation in evolution between *HSF* and *Hsp* genes in various organisms. The conserved sequence of the genes in the genome can provide this case.

Sunflower *HSF* and *Hsp* genes were compared with model organisms, including *Arabidopsis thaliana*, *Oryza sativa*, *Populus trichocarpa*, and *Zea mays*, to elucidate the orthologue relationship between sunflower *HSF*, *Hsp* genes, and model organisms (Fig. 5, Supplementary Table 4.1–4.7). Orthologous genes have functionally similar properties in different species. In addition, non-synonymous (Ka) vs. synonymous (Ks) substitution rates (Ka/Ks) were calculated for *HSF* and *Hsp* genes. The Ka/Ks ratios of all sunflower *HSF* and *Hsp* gene family members were calculated below 1 (< 1). The most orthologous gene pairs were identified between *HanHsp70* genes and *Arabidopsis* with 58 gene pairs, and the least orthologous genes were discovered among *HanHSF*-maize and *HansHsp*-rice with 6 genes. It was calculated that the first divergence occurred between *HanHsp90* genes and maize with an average of 229 Mya, and the final separation happened between *HanHsp100* genes and *P. trichocarpa* with an average of 23 Mya. Determination of divergence period distinct organism assume essential roles to deduce the evolutional correlation among studied species. Since *O. sativa* and *Z. mays* plants are monocots, the initial separation in all *Hsps* and *HSF* genes occurred in these plants, while the final separation occurred in *A. thaliana* and *P. trichocarpa* plants, which are dicots such as sunflower. In former studies, consistent with the current study, it was found that the initial and the latest divergence were respectively with monocots and dicots in cucumber and watermelon that are dicotyledons, too (Ünel 2018; Altunoğlu et al. 2019).

Identification of miRNAs targeted to *HSF* and *Hsp* genes in sunflower

miRNAs are involved in gene silencing mechanisms, especially under stress (Yang et al. 2007; Xin et al. 2010; Khraiwesh et al. 2012). HanHSF-88, HansHsp-05, HanHsp40-80, HanHsp60-45, HanHsp70-48, HanHsp90-28, and HanHsp100-122 were the most commonly targeted transcripts among the domains, and it was determined that mir167, miR1858, miR414, miR3437, miR397, miR2630, and miR396 were the miRNAs targeting these transcripts at most, respectively (Fig. 6) (Supplementary Fig. 8.1–8.5).

mir167 is more abundant in dicotyledons than in monocotyledons and participates in regulating plant growth, vegetative and reproductive organ growth, flowering time, and stress

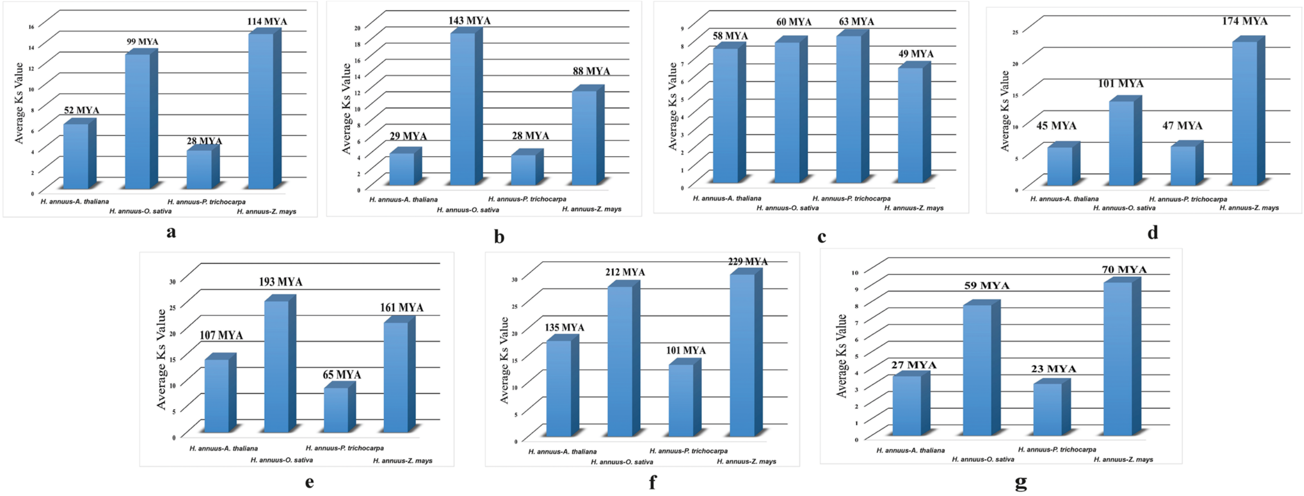

Fig. 5 Gene orthologous of *HanHSF* (a), *HansHsp* (b), *HanHsp40* (c), *HanHsp60* (d), *HanHsp70* (e), *HanHsp90* (f), and *HanHsp100* (g) genes in other plants and presumed divergence times. Among the results of sunflower and others, expectation value and matching respectively were approved e=0 and ≥ 80% identity limitation. The initial separation was detected between *HanHsp90* genes and *Z. mays* genes (229 Mya) and the last of divergence was appeared between *HanHsp100* genes and *P. trichocarpa* genes (23 Mya)

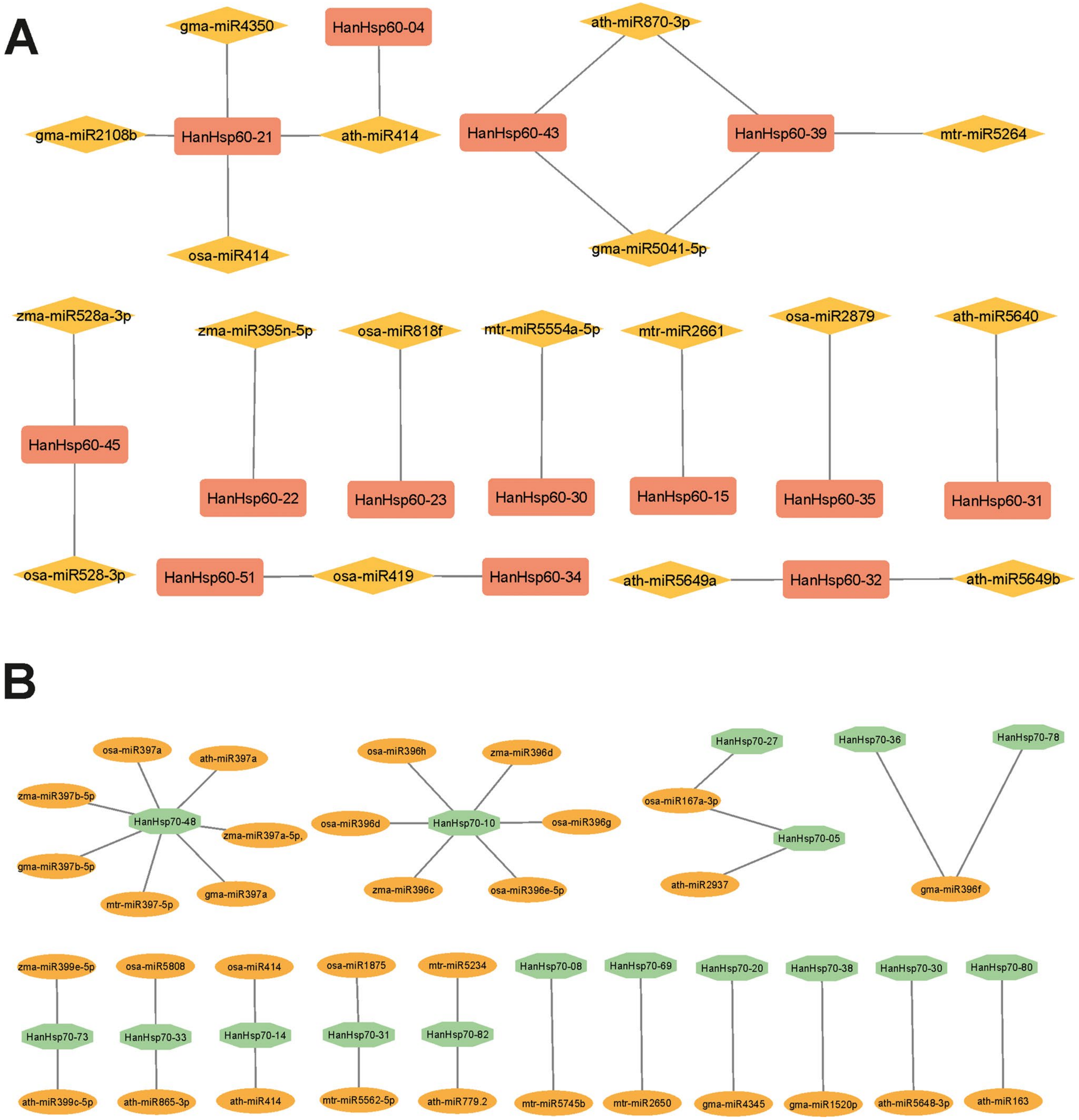

Fig. 6 miRNAs targeting *HanHsp60* genes (A), miRNAs targeting *HanHsp70* genes (B). It was determined that the most targeted genes by miRNAs were the transcripts of *HanHsp60-45* and *HanHsp70-48* genes

response (Zheng et al. 2019; Liu et al. 2021). It was observed that mir167 regulates gene expression under high salinity, drought (Khraiwesh et al. 2012; Phookaew et al. 2014), and high-temperature stress (Sailaja et al. 2014). Previous studies showed that miR1858 responds to environmental stresses such as heavy metal, high salinity, pathogen infection, endogenous oxidation stress, apoptosis (Jia et al. 2009), and cellular processes such as fruit ripening and lateral root growth (Xu et al. 2014). miR414 was recognized transcriptional regulators like WRKY, MYB, B3, and TCP and contributed to plant growth, development, physiological and morphological changes, metabolic processes, and defense mechanism (Wittung-Stafshede et al. 2003; Guleria and Yadav 2011). In a study about *Arabidopsis*, it was noticed that miR397 has abiotic stress-regulating and post-embryogenic roles (Dong and Pei 2014; Liu et al. 2021) and was concerned with flowering (Feng et al. 2020), flower, seed, and fruit development (Burklew et al. 2014). Research about radish (*Raphanus sativus* L.) stated that miR2630 attended cellular and developmental processes such as root development and fruit ripening and response mechanism to biotic and abiotic stress conditions (Xu et al. 2013). It was involved in cellular metabolic processes in cowpea (*Vigna unguiculata* L.) (Gul et al. 2017). In addition, miR2630 was related to the protein kinase metabolism of maize (Zhao et al. 2013; Yousuf et al. 2021). It was determined that miR396 positively affects plant root development and growth factors in *Medicago truncatula* (Bazin et al. 2013). In other studies about *Arabidopsis*, miR396 regulates growth factors (Mecchia et al. 2013) and interacts between the cell cycle and the developmental processes of cells (Rodriguez et al. 2010). The consequences extracted from former research analyzing the functions of miRNAs that target various *HSF* and *Hsp* genes in the sunflower family imply that HSF and Hsp proteins have an essential function in the stress tolerance of the plant both in normal tissue growth and under stress.

Estimated three-dimensional protein structures of HSF and Hsp proteins

To identify the predicted structure of HSF and Hsp proteins, protein modeling on the Phyre2 database was performed at ≥90 confidence level. Protein homology was found through the normal mode option for 3D modeling. Coverage rates changing from ≥80 were received. Except for HansHsp, it appeared that α-helix structures were dominant in the predicted 3D structure of HSF and Hsps (Supplementary Fig.9.1-9.6). The β sheet structure effectively provides oligomerization and stabilization both by establishing intermolecular hydrogen bonds necessary for chaperone function and by establishing hydrophobic contacts for sHsp proteins (Bondino et al. 2012; Haslbeck and Vierling 2015; Zhou et al. 2019). The N-terminal of HSF (Pirkkala et al. 2001), histidine-proline-aspartic (HPD) motif of Hsp40 (Genevaux et al. 2003; Knox et al. 2011), and M domain of Hsp100 (Asea et al. 2016) (Fig. 7) mainly consist of α-helix structure. A study about chaperonins demonstrated that their α-helical structure facilitates the folding of a protein faster (Motojima 2015). Therefore, the predicted structure of Hsp60, Hsp70, and Hsp90 proteins was identified as α-helix. The results of the current study are compatible with poplar (Yer et al. 2016b, 2018), watermelon (Altunoğlu et al. 2019), and jujube (Ceylan et al. 2021).

Expression analysis of sunflower *HSF* and *Hsp* genes by transcriptomic data and their expression profiles under abiotic stress applications by qRT-PCR

RNA-seq data were derived from the SRA, and the data of three projects (PRJNA483306 (9 SRA), PRJNA398727 (12 SRA), and PRJNA632976 (12 SRA)) were used in the transcriptomic data analyses for sunflower *HSF* and *Hsp* genes (Supplementary Fig. 10.1-10.7). PRJNA483306 showed that sunflowers were exposed to drought and salt stress, and PRJNA398727 included drought stress applications in the sunflower seedlings. PRJNA632976 was related to the sunflower flower RNA-seq data for different periods. Evaluation of gene expression profile is one of the most favored methods to recognize the expression of analyzed genes against stress conditions. Corresponding to RNA-seq data, strongly expressed *HSF* and *Hsp* genes under heat, drought, and high temperature-drought combined stresses were qualified with qPCR. According to transcriptome data of determined genes, the gene expression profile was raised, particularly in the transcriptome data of drought and salt stress transcriptome data.

HanHSF-45, HanHSF-59, HansHsp-18, HansHsp-58, HanHsp40-147, HanHsp40-186, HanHsp60-22, HanHsp70-28, HanHsp90-33, HanHsp100-93, and HanHsp100-130 displayed increased gene expression, and genes were mainly selected from transcriptome data of PRJNA483306, PRJNA398727, and PRJNA632976. Expression of both *HSF* genes demonstrated similar patterns, increasing almost every hour under heat stress and high temperature-drought combined stress conditions in Saray and Turay cultivars (Fig. 8, Supplementary Fig. 11.1-11.7). Especially under the high temperature-drought combined stress conditions, *HanHSF-45* gene expression increased for both cultivars at 0.5 h and 1 h and decreased at 2 h compared to the control. It has been reported in the study with the Arabidopsis AT2G26150, which is the orthologous gene of *HanHSF-45*, that it is upregulated in different studies, such as the current research in leaf tissue samples taken under cold and drought conditions (Vanderauwera et al., 2005) and seedlings under high-temperature stress conditions (Lin et al., 2011). *HansHsp-18* and *HansHsp-58* genes were selected after analyzing the transcriptome data. In the Saray cultivar, the peak increase in gene expression appeared with a 22-fold rising for the *HansHsp-18* gene under heat stress conditions. The expression of the *HansHsp-58* was pointed out to increase in both varieties under heat-stress conditions. According to orthologue genes of the *HansHsp-58*, the gene expression of AT5G37670, AT5G59720, and AT1G52560 in *Arabidopsis* (Ikeda et al., 2011) and the gene expression of Glyma.04G054400 and Glyma.07G200200 in two different varieties of soybean increased against heat stress condition (Song et al., 2019). *HanHsp70-29* gene expression increased in both Saray and Turay varieties under heat and high temperature-drought combined stress. In a study about high light stress, the expression of the At4g24280, an orthologous gene of *HanHsp70-29*, upregulated under stress conditions

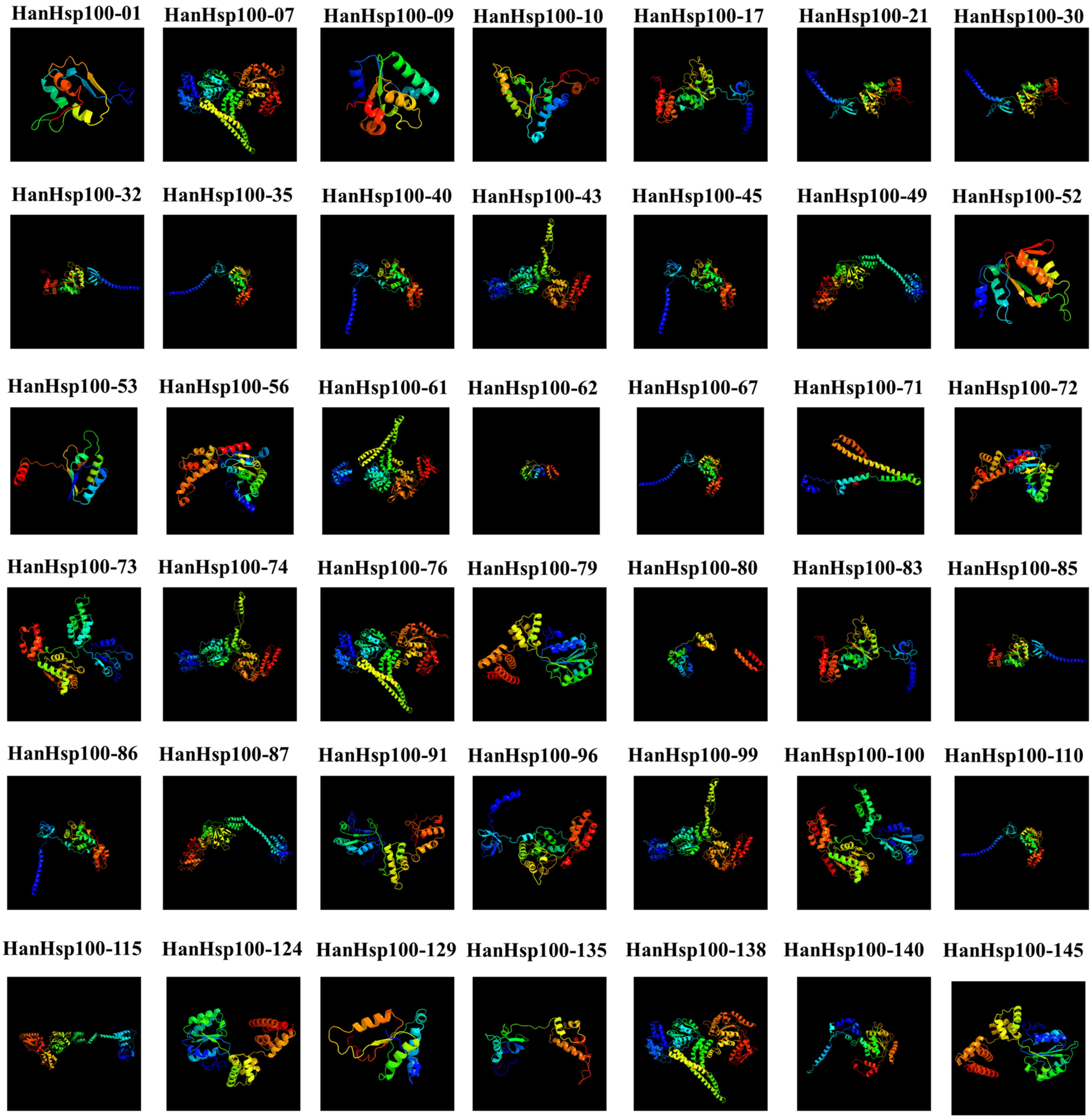

Fig. 7 Estimated three-dimensional structure of sunflower Hsp100 proteins with > 90% confidence level. α-helix structures were dominant in the predicted 3D structure of HSF and most of the Hsps.

(Giacomelli et al., 2006). According to transcriptomic data and sunflower *HSF* and *Hsp* gene expression results, *HanHsp70-29* plays a crucial role under heat and high temperature-drought combined stress conditions. The gene expression results of the *HanHsp70-29* are consistent with the present study of the differences in the expression of the At4g24280, which is orthologous to this gene, under high light stress (Giacomelli et al., 2006). It was concluded that *HanHsp100-93* and *HanHsp100-130* could be potential marker genes by displaying a significant decrease in gene expression at all hours in both cultivars under drought-stress conditions. *HanHsp40-147* and *HanHsp40-186* showed a significant increase in gene expressions in both Saray and Turay cultivars under high temperature and high temperature-drought combined stress conditions. It was determined that the expression of the AT3G13310

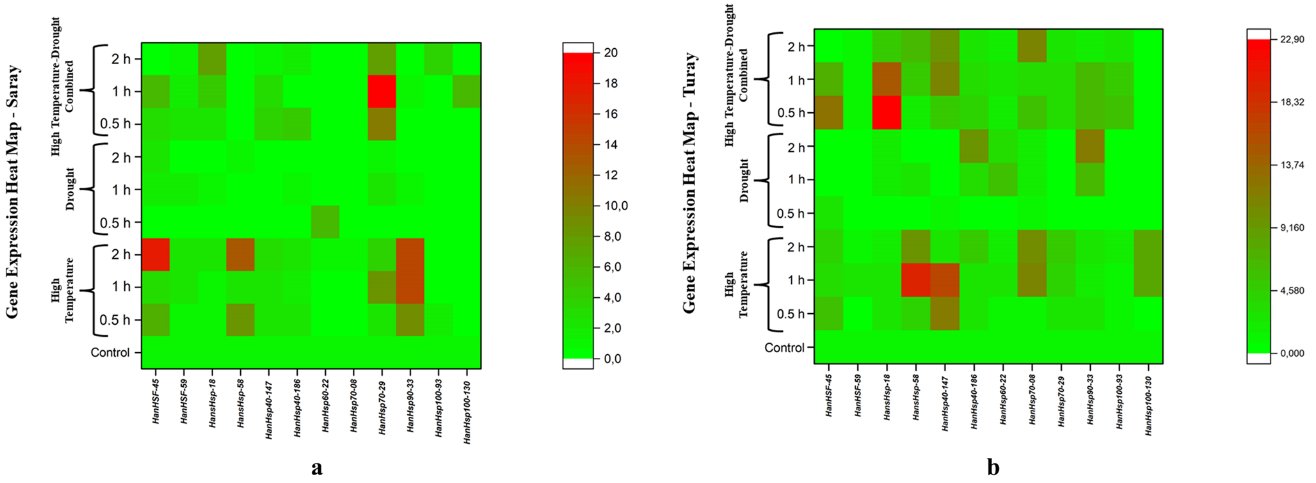

Fig. 8 Heatmaps of *HSF* and *Hsps* genes expression of Saray (**a**) and Turay cultivars (**b**) of sunflower against high-temperature, drought, and high temperature-drought stresses by qRT-PCR ($p < 0.05$ was accepted as statistically prominent)

(Ikeda et al., 2011), which is the orthologous gene of the *HanHsp40-147*, increased under high-temperature stress conditions applied to the plant. The gene expressions of the orthologous AT5G01390.4 and AT2G20560 genes of the *HanHsp40-186* increased under the applied abiotic stress conditions (Ikeda et al., 2011). Especially in the Turay cultivar, *HanHsp60-22* gene expression increased under all stress conditions. At the same time, *HanHsp90-33* gene expression upregulated in both cultivars under heat stress conditions, which increased in the Turay cultivar compared to the Saray cultivar under drought and high temperature-drought combined stress conditions.

By comparing Saray (drought-resistance) and Turay (drought-sensitive) cultivars, it was specified that the expression of Turay cultivar genes upregulated more under high temperature and high temperature-drought combined stress conditions. In drought stress conditions, the gene expression in Turay cultivar increased especially in the 1 h and 2 h (Supplementary Fig. 11.1-11.7). For Saray and Turay cultivars, *HansHsp-58* gene under high-temperature stress conditions, *HanHsp100-93* and *HanHsp100-130* genes under drought stress conditions, and *HanHSF-45* and *HanHsp70-29* genes under both high temperature and high temperature-drought combined stress conditions have similar gene expression profiles which indicate that these genes can be designated as potential marker genes. Consistent with the current study, it has also been determined in the literature that the gene expressions of sensitive cultivars increase under stress conditions. In the research conducted on two sugar beet varieties resistant and sensitive to water stress, it was designated that gene expression upregulated more in the sensitive variety (Rodrigues et al., 2010). More variation in gene expression in sensitive cultivars is thought to be due to insufficient stress avoidance mechanisms or to alleviate the effects of stress in these plants (Muthusamy et al., 2016).

In the current paper, *HanHSF* and *HansHsp* gene expression generally upregulated at 30 min and 1st hour like in previous studies in watermelon (Altunoğlu et al., 2019) and poplar (Yer et al., 2016b, 2018) related to *Hsp* genes. Expressions of *Hsp* genes were revealed to be the precursor gene family to respond to stress against the stress conditions they are exposed to, and herein, it is compatible with our study.

Conclusion

In the study, a total of 598 *HanHsps* and 88 *HanHSF* genes in the *Helianthus annuus* genome were determined. It was observed that the genes demonstrated a high distribution in the 13th and 14th chromosomes. Most of the HSF and Hsps were acidic, with the exception of the HansHsp and HanHsp40 proteins, which were mostly basic. It had been noted that proteins with similar motifs and exon-intron organization are found in the same branch of the phylogenetic tree. Except for HansHsp proteins, the predicted three-dimensional structures of all HanHsps and HSF proteins were determined to be dominated by α-helical structure. While the most orthologous gene pairs were identified between *HanHsp70* genes and *Arabidopsis* genes (58 gene pairs), the last separation occurred between *HanHsp100* genes and *P. trichocarpa* genes (23 Mya). It was identified that gene expression profiles of *HanHSF-45* and *HanHsp70-29* genes increased under high temperature and high temperature-drought combined stress conditions in Saray and Turay sunflower cultivars.

The recent paper presents an elaborative genome-wide detection of HSF and Hsp family in the sunflower genome that can contribute to their function. Given the seriousness of global warming, this article may be an initial stage towards growing resistant sunflower, an essential and high commercial value agricultural oilseed against temperature, drought, and high temperature-drought combined stress factors. In addition, the current study offers new viewpoints on the function of *HSF* and *Hsp* gene superfamilies. They deliver extensive knowledge for upcoming analysis detecting functional principles of HSF and Hsp proteins.

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Author contributions Planned and designed the experiment: YCA, YC, EH; performed the experiments: YC, EH; analyzed the data: YC, YCA, EH; writing and editing: YC, YCA. All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Data availability All data generated or analyzed during this study are included in this published article and its supplementary information files.

Declarations

Ethics approval This article does not contain any studies with human participants or animals performed by any of the authors.

Consent to participate and for publication Not applicable

Conflict of interest The authors declare no competing interests.

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