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Genome-Wide Identification and Expression Analysis of *HSP70* Gene Family in *Chrysanthemum lavandulifolium* under Heat Stress

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Abstract: As a molecular chaperone, HSP70 is widely involved in complex activities in plants. Under high temperature, drought, high salt, low temperature, heavy metals, and other stresses, HSP70 is rapidly synthesizes, stabilizes protein and biological macromolecular structures, and improves the stress resistance of plants. In this study, 83 *ClHSP70* genes in *Chrysanthemum lavandulifolium* were identified based on the published *Chrysanthemum lavandulifolium* genome database. The genes were divided into six clusters based on a phylogenetic analysis, and the gene structures, conserved motifs, and functional domains were relatively conserved. Only two collinear genes were observed, and they formed a pair of duplicating genes. Multiple abiotic stress and phytohormone response elements were observed on the *ClHSP70* promoter, such as temperature, drought, methyl jasmonate, abscisic acid, and other stress-related elements, and CpG islands were found on some *ClHSP70* promoters, suggesting that they may be related to methylation modifications. Tissue expression analysis showed that the *ClHSP70* genes were highly expressed in the roots. In addition, the gene expression changed significantly after 1 h of heat treatment and plays an important role in plant responses to temperature stress. The results of this study provide insights on the *ClHSP70* gene family members and a theoretical basis for further research on functional analyses of the *ClHSP70* gene family.

Keywords: heat shock protein 70 family; *Chrysanthemum*; genome-wide analysis; heat stress

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1. Introduction

During the process of growth and development, plants are affected and stressed by various environmental factors, such as high (low) temperature, strong (weak) light, drought (flood), high salt, heavy metals, and pathogenic bacterial (or viral) infections. When the influence of these factors exceeds a certain threshold, the growth and development of plants are adversely affected to varying degrees [1]. Heat shock proteins (HSPs) are defense mechanisms induced in plants to adapt to changing environments [2]. Heat shock proteins (HSPs) are a highly conserved protein family that is widely distributed in various organisms, from prokaryotes to eukaryotes [3,4]. HSPs are highly conserved in structure, have molecular chaperone functions, and are involved in biological processes, such as the transport, folding, assembly, renaturation, and degradation of denatured proteins [5,6]. HSPs can be divided into different types according to their sequence similarity and protein molecular weight, such as HSP100, HSP90, HSP70, HSP60, HSP40, and small HSPs (sHSPs)/HSP20s [7].

The *Arabidopsis HSP70* gene family consists of 18 members and is divided into two subfamilies: DNAK and HSP110/SSE [8]. This family mainly includes two functional domains, the nucleotide-binding domain (NBD) and substrate-binding domain (SBD), which are connected by a hinge region. In addition to a signal peptide sequence that guides the localization of HSP70 to different organelles, the NBD also has an active

center that binds and hydrolyzes ATP [9]. The C-terminal polypeptide-binding domain can bind, process, and fold substrate proteins [10]. The molecular chaperone function of HSP70 requires two types of co-chaperones: heat shock protein 40 (HSP40) and nucleoside exchange factor (NEF) [11]. HSP40/DnaJ utilizes its zinc finger or C-terminal domain to interact with abnormally folded substrate proteins, which bind and transmit the substrate protein to HSP70 by interacting with HSP70 [12]. In addition, its J domain also interacts with the HSP70 protein to initiate the ATPase activity of HSP70. This activity induces changes in the conformation of HSP70 that allow HSP70 to bind tightly to the substrate protein and use the energy released by ATP hydrolysis to realize correct folding of the substrate [13]. After this process is complete, NEF binds to the HSP70-ADP-polypeptide complex and decomposes it, the polypeptide substrate is released, and HSP70 changes from the ADP bound state to the ATP-bound state, thus initiating the next cycle [14,15].

HSP70 also plays an important role in plant stress resistance and growth and development regulation [16,17]. In a study of transgenic *Arabidopsis* plants, *Heat Shock Cognate 70-1* (*HSC70-1*) overexpression plants were more tolerant to heat shock of 44 °C for 10 min [18]. Duan et al. showed that TaHSP70 accumulated significantly in wheat seedlings after heat shock [19]. Jung et al. found that among the 32 *HSP70* genes in rice, 24 *HSP70* genes were upregulated at high temperatures, with 14 showing significant increases; moreover, the expression of *OsHSP70* was also significantly changed under salt and drought treatments [20,21]. The cytoplasmic *HSP70* and nuclear *HSP70* genes in *Arabidopsis thaliana* regulate the immune response by interacting with the *SGT1* gene, and deletion mutations lead to the loss of plant defenses against pathogens [22]. Song et al. isolated *HSP70* cDNA from the hexaploid cultivated chrysanthemum 'Zhong Shan Zi Gui' and showed that it has a positive effect on plant stress resistance after heterologous expression in *Arabidopsis* and improves the plant's heat, drought, and salt tolerance [23]. *HSP70-1* affects plant growth and development, immunity, heat shock tolerance, and stomatal closure [18]. AtHSP70-15-deficient *Arabidopsis* plants are characterized by severe growth retardation, impairs stomatal closure, accelerates withering, and enhances tolerance to TuMV [24]. *HSP70-16* affects sepal development, and its loss of function results in abnormal floral organ formation and impaired fertilization and fruiting [25]. Our understanding of the functions of individual *HSP70s* remains limited owing to their high sequence similarity and functional redundancy.

With the development of bioinformatics and the application of sequencing technology, a large amount of genomic and RNA omic data have been collated and released. As a molecular chaperone, HSP70 has gradually become a hotspot, and many species of *HSP70* gene family members have been identified, including 18 gene family members in Arabidopsis [8]: 32 in rice [20]; 61 in soybean [26]; 52 in cabbage [27]; 21 in *Ziziphus jujuba* [28]; and 21 in pepper [29]. Chrysanthemum (*Chrysanthemum morifolium*) is one of the top ten most famous traditional flowers in our country and one of the four major cut flowers in the world. As a perennial plant, chrysanthemum has a high ornamental and economic value. Additionally, it is more vulnerable to heat stress and severely restricted by high temperatures during growth and development [30]. There are few reports about the *HSP70* gene family and its function in the Asteraceae family. Therefore, it is important to identify and analyze the *HSP70* gene family in chrysanthemums. However, the genome of hexaploid chrysanthemum has high heterozygosity and polyploidy and thus is relatively large, and it has not yet been published. The published diploid chrysanthemum genome is often used for research on related gene families. Here, we used the recently published chromosome-level reference genome of *Chrysanthemum lavandulifolium* to identify and analyze the *HSP70* gene family [31].

2. Materials and Methods

2.1. Plant Material and Treatment

C. lavandulifolium was propagated by cuttings and then grown for 5–6 weeks under long-day conditions at 22 °C. Roots, stems, and leaves were collected during this period and used for RNA extraction. After transitioning to short-day conditions for flower trans-

formation, the roots, stems, leaves, buds, and capitulums were collected. Samples from nine tissues were subjected to quantitative real-time polymerase chain reaction (qRT-PCR) assays. In addition, the high temperature treatment was performed using seedlings grown at 22 °C for 5–6 weeks under long-day conditions, planted in nutrient soil and vermicure with 1:3 matrix, transferred the seedlings to 37 °C light incubator. Three *C. lavandulifolium* plants at the same growth level were treated as replicates, and the upper fresh leaves were selected and frozen in liquid nitrogen after treatment for 0, 1, and 6 h, respectively.

2.2. Identification and Sequence Analysis of HSP70 Gene Family in C. lavandulifolium

C. lavandulifolium genome sequences and annotation files were obtained from PRJNA681093, National Center for Biotechnology Information (NCBI), based on the complete *C. lavandulifolium* genome sequence [31]. The Hidden Markov Model was downloaded from the Pfam database (http://pfam.xfam.org/, accessed on 12 July 2022) (Pfam: PF00012). Entire amino acid sequences, CDS sequence assemblies, and corresponding annotations of *Arabidopsis* and rice were downloaded from the EnsemblPlants database (http://plants.ensembl.org/index.html, accessed on 14 July 2022).

The *HSP70* gene family members in *C. lavandulifolium* were identified by performing a BLASTP search against the *C. lavandulifolium* genome database using orthologous amino acid sequences of *A. thaliana* as queries (e-value of 1×10^{-5} as the threshold). The Hidden Markov model (HMM) of the HSP70 protein family was also used to search for candidate amino acid sequences. The genes in these two sets of results represented candidate genes for *ClHSP70*. The final results were analyzed using the NCBI CD-Search program (https://www.ncbi.nlm.nih.gov/Structure/bwrpsb/bwrpsb.cgi, accessed on 4 August 2022), which excludes sequences that do not contain HSP70 conservative domain information. Finally, 83 *HSP70* genes were identified as *ClHSP70* in *C. lavandulifolium*.

The ExPASy database (http://expasy.org/, accessed on 9 September 2022) was used to determine the physical and chemical features of the HSP70s. The predicted subcellular localization of ClHSP70s was analyzed using WoLF PSORT (https://wolfpsort.hgc.jp/, accessed on 9 September 2022).

2.3. Chromosomal Location, Gene Duplication, and Synteny Analysis

The chromosomal locations of *HSP70* family genes were mapped onto the *C. lavandulifolium* linkage map using TBtools [32]. The required files, including the length of all chromosomes and the microsynteny view of gene pairs in *C. lavandulifolium*, were provided using the *C. lavandulifolium* database and TBtools. We then performed a synteny analysis of the *HSP70* family genes in *C. lavandulifolium*. MCscanX in TBtools was used to conduct the dual synteny analysis of *C. lavandulifolium.* A Ka/Ks analysis of the *HSP70* family genes with a collinear relationship was performed using TBtools (Table S2).

2.4. Phylogenetic Analysis

The full amino acid sequences of HSP70 proteins from *A. thaliana*, *O. sativa,* and *C. lavandulifolium* were downloaded from the TAIR (https://www.arabidopsis.org/, accessed on 12 September 2022), RGAP (http://rice.plantbiology.msu.edu/, accessed on 12 September 2022), and NCBI databases. Multiple sequence alignment was performed using MEGA X (https://www.megasoftware.net/, accessed on 13 September 2022) and a phylogenetic tree was constructed using the neighbor-joining (NJ). The bootstrap method with 1000 replicates was selected for the phylogeny test. iTOL (https://itol.embl.de/, accessed on 15 September 2022) was used to construct the phylogenetic tree.

2.5. Analysis of Gene Structures, Motifs, and Conserved Domains

The MEME program (https://meme-suite.org/meme/doc/meme.html, accessed on 20 September 2022) identified conserved motifs of the *ClHSP70* family using the following parameters: any number of repetitions and maximum number of motifs of 10. The NCBI CD-Search program (https://www.ncbi.nlm.nih.gov/Structure/bwrpsb/bwrpsb.cgi, accessed

on 20 September 2022) was used to identify the domain types and positions of all HSP70 protein sequences. The results were then used for domain visualization using TBtools, which was also used to display the *ClHSP70* family gene structures by comparing the coding and genomic sequences.

2.6. Promoter Sequence Analysis of ClHSP70 Family

We downloaded the 2000 bp region upstream of identified *ClHSP70s* from the *C. lavandulifolium* genome. The PlantCARE database (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/, accessed on 23 September 2022) was used to analyze cis-regulatory elements in the *ClHSP70* promoters. The final results were visualized and displayed using TBtools. CpG islands were predicted using Methprimer 2.0 (http://www.urogene.org/methprimer/, accessed on 25 September 2022).

2.7. qRT-PCR

Based on CpG island and cis-element analyses, 15 candidate genes were screened for gene expression patterns. A Quick RNA isolation kit (HUAYUEYANG BIOTECHNOLOGY, Beijing, China) was used to extract total RNA from the different tissues of *C. lavandulifolium*. The extracted RNA was then employed as a template with the Evo M-MLV Reverse Transcription Kit (with gDNA removal reagent for qPCR) (Accurate Biology, Changsha, China) for first-strand cDNA synthesis. Real-time quantitative PCR of *ClHSP70s* was performed on a Light Cycler 96 System (Roche, Basel, Switzerland) using the SYBR Green Premix Pro Taq HS qPCR kit (Accurate Biology, Changsha, China). Primers were designed using Primer Premier 5.0, and primer sequences are presented in Table S3. *Elongation factor 1 alpha* (*EF1α*) was used as an internal reference gene for data normalization. *EF1a* was consistently expressed in cold-stressed potatoes and tomatoes [33,34], cold-treated and heat-treated rice [35], and heat-stressed chrysanthemum [36]. The PCR thermal cycling conditions were as follows: 95 °C for 2 min and 45 cycles of 95 °C for 10 s, 60 °C for 10 s, and 72 °C for 20 s. Three technical replicates and three biological replicates were used for each analysis. Relative expression of *ClHSP70s* was calculated using the $2^{-\Delta\Delta Ct}$ method [37].

3. Results

3.1. Identification of ClHSP70 Genes in C. lavandulifolium

A total of 83 ClHSP70 proteins were identified based on the amino acid sequences of the conserved HSP70 structural domains in *A. thaliana* and *C. lavandulifolium* using BLAST and the Markov hidden model (HMM), and they were named according to their position on the chromosome. The physicochemical properties of the samples were analyzed (Table S1). The coding region length ranged from 353 to 1054 amino acids. Variations in gene molecular weight corresponded to the variation in HSP70 amino acid length, and most HSP70 proteins with theoretical isoelectric point (pl) ranged from 5 to 10. Most HSP70 proteins with an instability index less than 40 were stable proteins, although some proteins with an index higher than 40 were unstable proteins, such as ClHSP70-1 and ClHSP70-2. The grand average hydropathicity of all members was negative, which indicated that they were all hydrophobins, and the values did not vary widely. Subcellular localization predictions revealed the localization of HSP70 in cells. In addition to localization in the cytoplasm, most members may also function in the nucleus, chloroplasts, mitochondria, endoplasmic reticulum, and Golgi apparatus. We speculate that ClHSP70 proteins may function in different compartments and may be involved in a variety of life processes.

3.2. Chromosomal Localization and Collinearity Analysis of ClHSP70 Genes

The distribution of the gene family on the chromosome can be clearly displayed by its location on the chromosome. We determined the chromosomal distribution profile of the *ClHSP70* gene family and mapped it to the genome (Figure 1). The 83 *ClHSP70* genes showed an uneven distribution on nine chromosomes and were arranged in order from top to bottom according to the position of the genes on the chromosome. In addition, the

distribution of *ClHSP70s* had no obvious correlation with the length of the chromosome, among which chromosome 4 had the most at 21 genes, followed by chromosome 6 at 13 *HSP70* genes, with the lowest on chromosome 7 at only two.

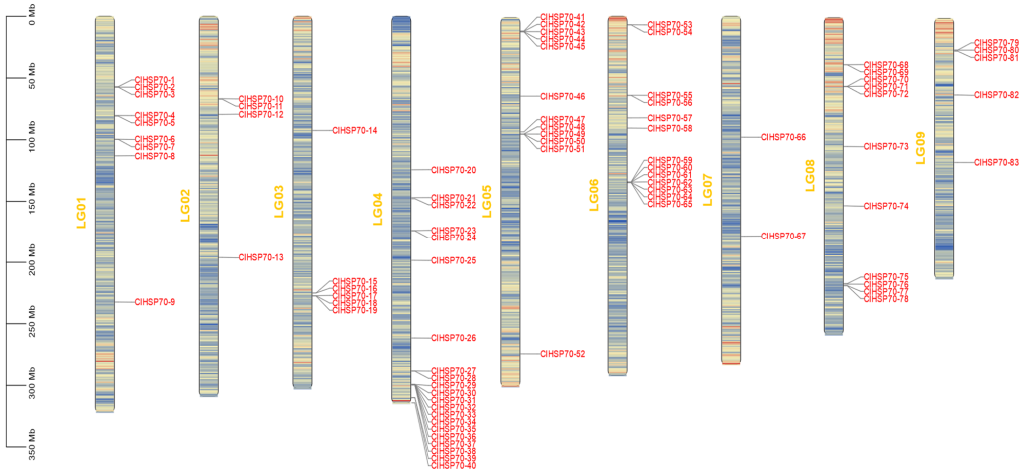

Figure 1. Distribution of the *ClHSP70* gene family members on *C. lavandulifolium* chromosomes. The bars represent the chromosomes. Chromosome numbers are shown on the left. *ClHSP70* genes are marked to the right of the chromosomes. The scale bar on the left indicates the length of the chromosome.

Gene duplication plays an important role in gene family formation and species adaptation and can provide the original genetic material for genes with new functions. We analyzed gene duplication of the *ClHSP70* family in the *C. lavandulifolium* genome, and the results showed that there were only two collinear genes, and they formed a pair of duplicating genes, *ClHSP70-79*/*ClHSP70-15*. Based on this genomic analysis, we found that gene duplication events of *HSP70* were infrequent in *C. lavandulifolium* (Figure 2). The selection pressure analysis of this gene pair during the evolutionary process showed that the Ka/Ks value was less than 1, indicating that purifying selection occurred during the evolution of *ClHSP70* (Table S2).

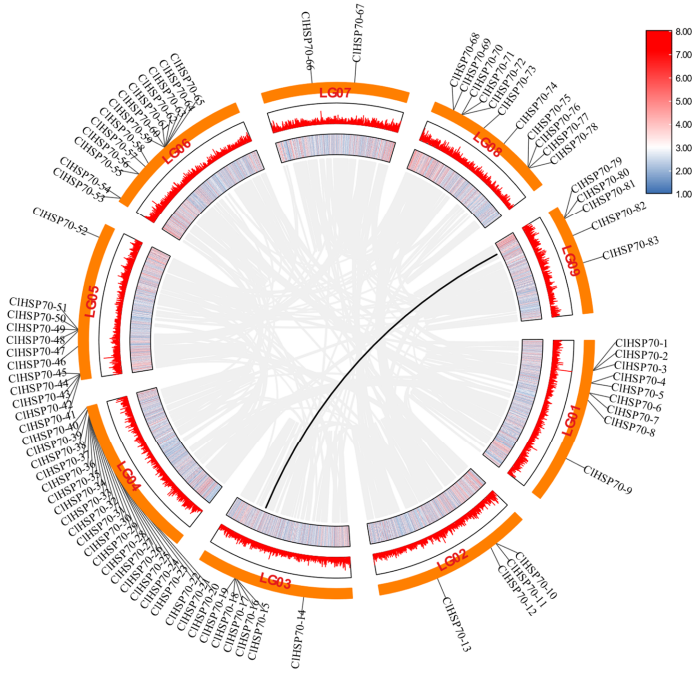

Figure 2. Duplication of the *ClHSP70* gene family members in *C. lavandulifolium*.

3.3. Phylogenetic Relationship ClHSP70 Genes

To study the evolutionary relationship of *ClHSP70s*, we downloaded the amino acid sequences of 18 *Arabidopsis* HSP70 proteins and 32 rice HSP70 proteins from the database. Five subfamilies are included in the *Arabidopsis* AtHSP70 family, and the OsHSP70 family in rice is divided into six subfamilies [20]. Based on the previous classification, we constructed an HSP70 protein phylogenetic tree of multiple species using the HSP70 amino acid sequences of *Arabidopsis*, rice, and *C. lavandulifolium* (Figure 3). We performed a phylogenetic analysis of 83 *C. lavandulifolium*, 32 rice, and 18 *Arabidopsis* HSP70 proteins and grouped them into six clusters. The distribution of ClHSP70 in the evolutionary tree conformed to the grouping of two subclasses of the HSP70 family: DnaK and HSP110/SSE. Except for Clusters V and VI, the other subgroups contained *Arabidopsis*, *C. lavandulifolium*, and rice genes, which reflect the evolutionary conservation of the ClHSP70 family. Cluster V had only two members: *Arabidopsis* HSP70-8 and rice LOC Os03g11910. These members are consistent with the analysis of the rice HSP70 family, indicating that the two are closely related and independent of other branches. Cluster VI consisted of 43 members, all of which were included in the ClHSP70 family. In the process of species evolution, ClHSP70 likely formed a unique branch type of this species and may have unique functional differentiation.

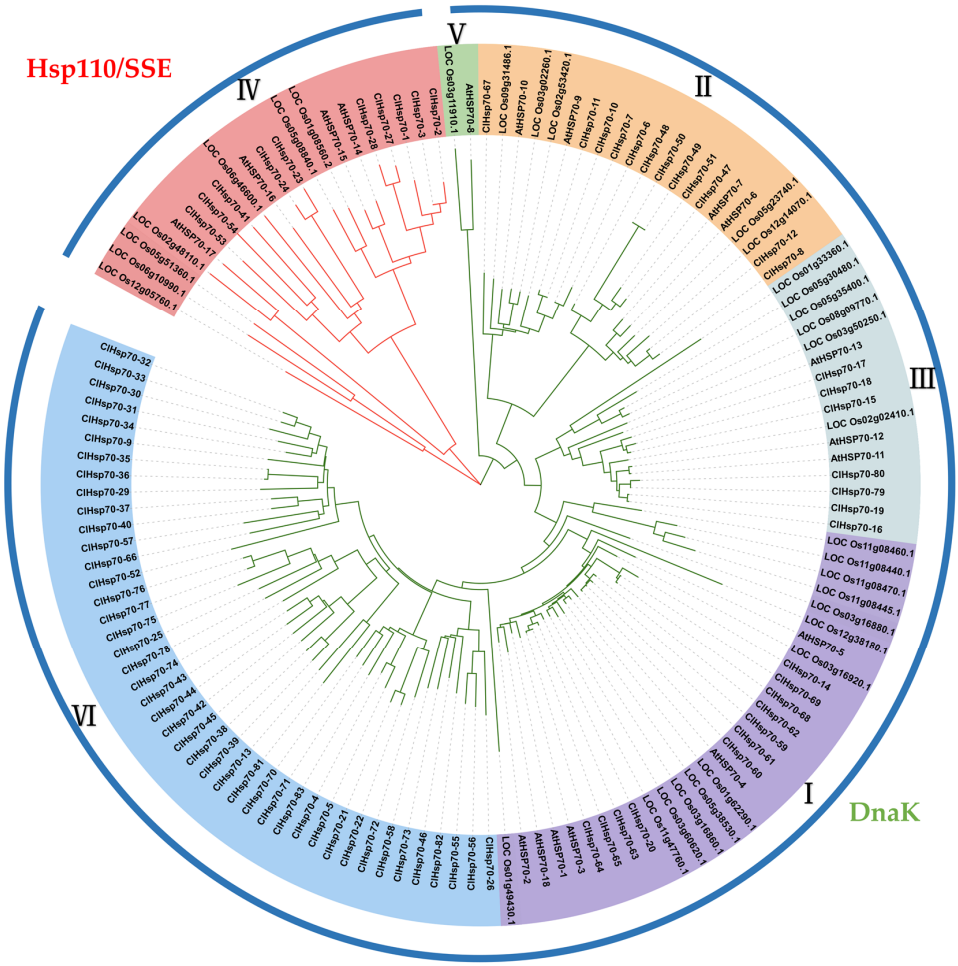

Figure 3. Phylogenetic tree of the HSP70 proteins from *C. lavandulifolium*, *Arabidopsis*, and rice. The red branching line represents subclass Hsp110/SSE, and the green line represents Dnak.

3.4. Conserved Motif, Gene Structure, and Domain Analysis of ClHSP70s

Conserved motif analysis can reveal the diversity of gene family members within a species and differences in their functions. We used the MEME online tool to search for motifs in *ClHSP70s* and drew a visual map of the motifs of *ClHSP70* based on their

phylogenetic relationships. Most closely related members shared similar motif patterns, suggesting possible functional similarities between these *ClHSP70s*, with motif 9 being the most common motif present in all *ClHSP70* members. Except for the duplication and deletion of a few motifs in Clusters I, III, and VI, most members presented motifs 1–10, indicating that this family is highly conserved. *ClHSP70-49* and *ClHSP70-50* had only two motifs, motif9 and motif10, their gene structures have undergone significant changes, and they may have obvious functional differentiation (Figure 4).

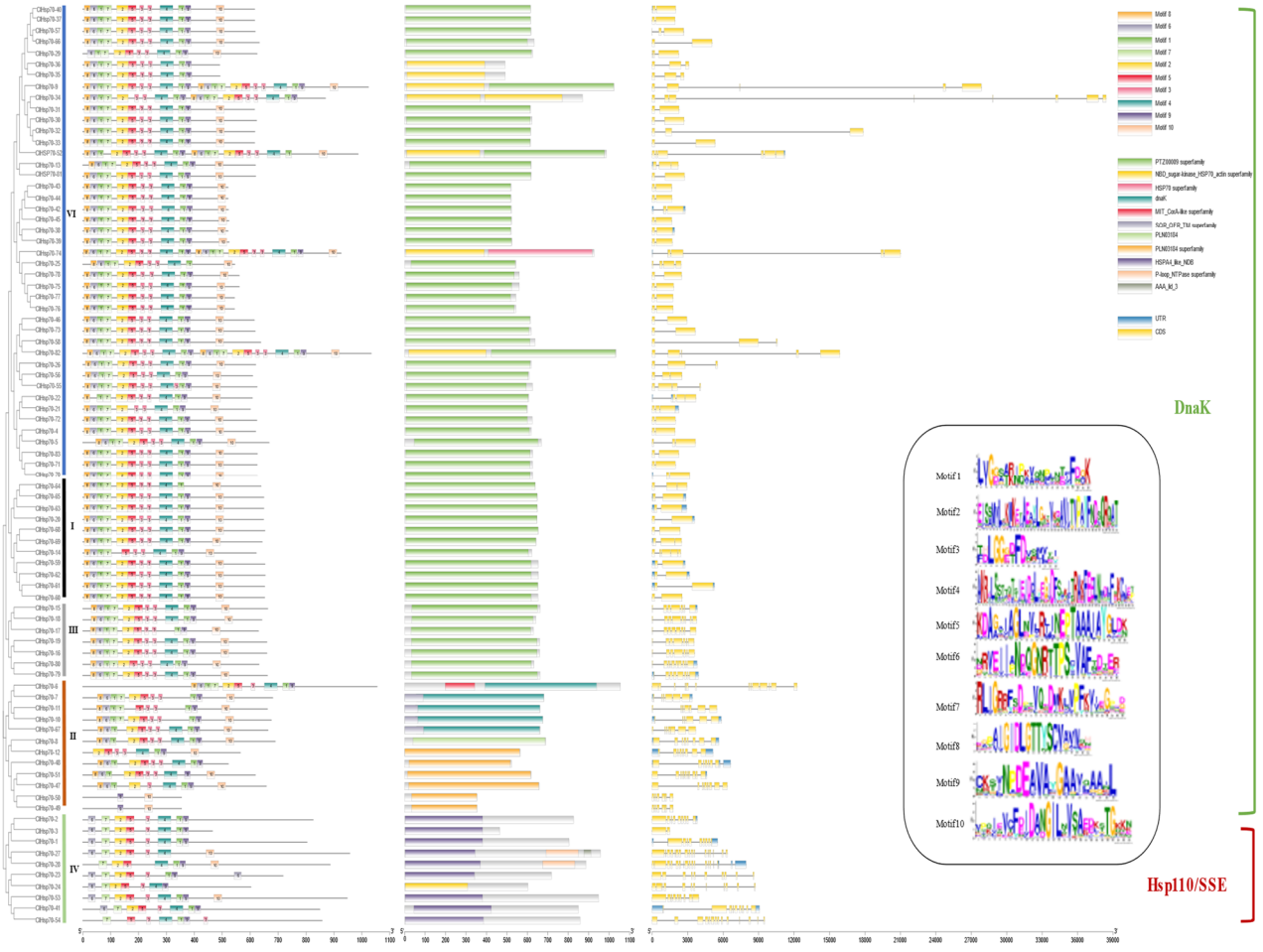

Figure 4. Conserved motif, gene structure, and domain analysis of *ClHSP70s* family genes according to their phylogenetic relationships.

The exon-intron structure of genes reflects the evolution of gene families. We further investigated the gene structure of 83 *ClHSP70* genes. The results showed that the gene structure of the *HSP70* gene family members was diverse among the different genes. The number of introns varied widely and ranged from 0 to 14, and more than half of the *HSP70* genes did not contain a UTR. The gene structures of Clusters I and VI were relatively similar, and the number of introns was between one and three. The gene structure of Cluster III was the most conserved, and the number, relative position, and length of introns among members were the most consistent, indicating that this subfamily may be functionally conserved.

The functional domains of 83 members of the ClHSP70 family were analyzed. PTZ00009 is one of the highly conserved HSP70 superfamily protein domains and represents a typical heat shock protein 70. Most ClHSP70 members have this domain. The Dnak domain is also an important feature for identifying HSP70. The PLN03184 superfamily belongs to the chloroplast HSP70 subfamily, and six ClHSP70 members have this domain. The HSPA4_like_NDB domain is the nucleotide-binding domain of 105/110 kDa heat shock

proteins (including HSPA4 and similar proteins). It belongs to the 105/110 kDa heat shock protein (HSP105/110) subfamily of the HSP70-like family, which includes proteins believed to function generally as co-chaperones of HSP70 chaperones, which act as NEFs to remove ADP from their HSP70 chaperone partners during the ATP hydrolysis cycle. HSP70 proteins in Cluster IV belong to this type. During long-term evolution, along with functional differentiation and diversification, other domains related to the functions of ClHSP70 were formed, such as the SQR_QFR_TM domain related to oxidoreductase and the P-loop_NTPase domain related to ATP hydrolysis.

3.5. cis-Element and CpG Island Analysis of the ClHSP70 Genes in C. lavandulifolium

To further study the role of *ClHSP70* family genes in growth, development, and stress responses, we submitted the sequences 2000 bp upstream of the start codon of *ClHSP70* family genes to the PLANTCARE database for cis-acting element analysis (Figure 5). The results showed that in addition to the most basic TATA-box and CAAT-box elements, the 83 *ClHSP70* gene promoters also contained a large number of other cis-acting elements, including light-responsive cis-acting elements (MRE, GT1-motif, and G-box), development-related cis-acting elements (CN4_motif and CAT-box), stress-related cis-acting elements (LTR, MBS, and TC-rich repeats), and hormone-related cis-acting elements (TGA-element and CGTCA-motif). It is speculated that the *ClHSP70* gene is widely involved in the growth and development of *C. lavandulifolium* and acts on various pathways, such as light response, photosynthesis, seed germination, leaf morphogenesis, and rhizome elongation. In addition, the *ClHSP70* promoter also includes a large number of cis-acting effects related to stress and hormone responses, indicating that the expression of *ClHSP70s* may also be regulated by various environmental factors and endogenous cues.

In addition, we analyzed and predicted the CpG sites of the sequence 2000 bp upstream of the start codon of *ClHSP70s*, which is usually located near the gene promoter and represents a region of DNA. CG-rich genes are involved in DNA methylation and represent an important source of epigenetic improvement. Methylation modification, which does not change the sequence of genes but can change the expression of genes, introduces heritable phenotypic changes and represents an important epigenetic marker. Eighteen *ClHSP70* members were predicted to contain CpG islands (Table 1), suggesting that they may be related to methylation modification of DNA; however, additional treatment of genomic DNA with bisulfite is required to detect the presence of methylation modification by the Methylation-Specific Polymerase Chain Reaction (MSP) method [38].

Table 1. Details of each of the eighteen genes with the CpG island.

NO.	*HSP70* Gene	Island #	Start	End	Length
1	*ClHSP70-11*	1	−51	−302	251bp
2	*ClHSP70-12*	2	−532	−633	102bp
			−1719	−1933	215bp
3	*ClHSP70-15*	1	−230	−364	135bp
4	*ClHSP70-16*	1	−1691	−1861	171bp
5	*ClHSP70-19*	1	−1677	−1841	165bp
6	*ClHSP70-28*	1	−180	−467	288bp
7	*ClHSP70-29*	1	−358	−472	115bp
8	*ClHSP70-40*	1	−1422	−1591	170bp
9	*ClHSP70-46*	2	−625	−791	167bp
			−1496	−1627	132bp
10	*ClHSP70-47*	1	−1407	−1515	109bp
11	*ClHSP70-48*	1	−1391	−1507	117bp
12	*ClHSP70-51*	1	−1391	−1455	121bp
13	*ClHSP70-52*	1	−168	−456	271bp
14	*ClHSP70-53*	1	−332	−434	102bp
15	*ClHSP70-60*	1	−861	−995	135bp
16	*ClHSP70-61*	1	−1582	−1701	120bp
17	*ClHSP70-65*	3	−257	−487	231bp
			−736	−858	123bp
			−1450	−1550	101bp
18	*ClHSP70-72*	1	−692	−833	142bp

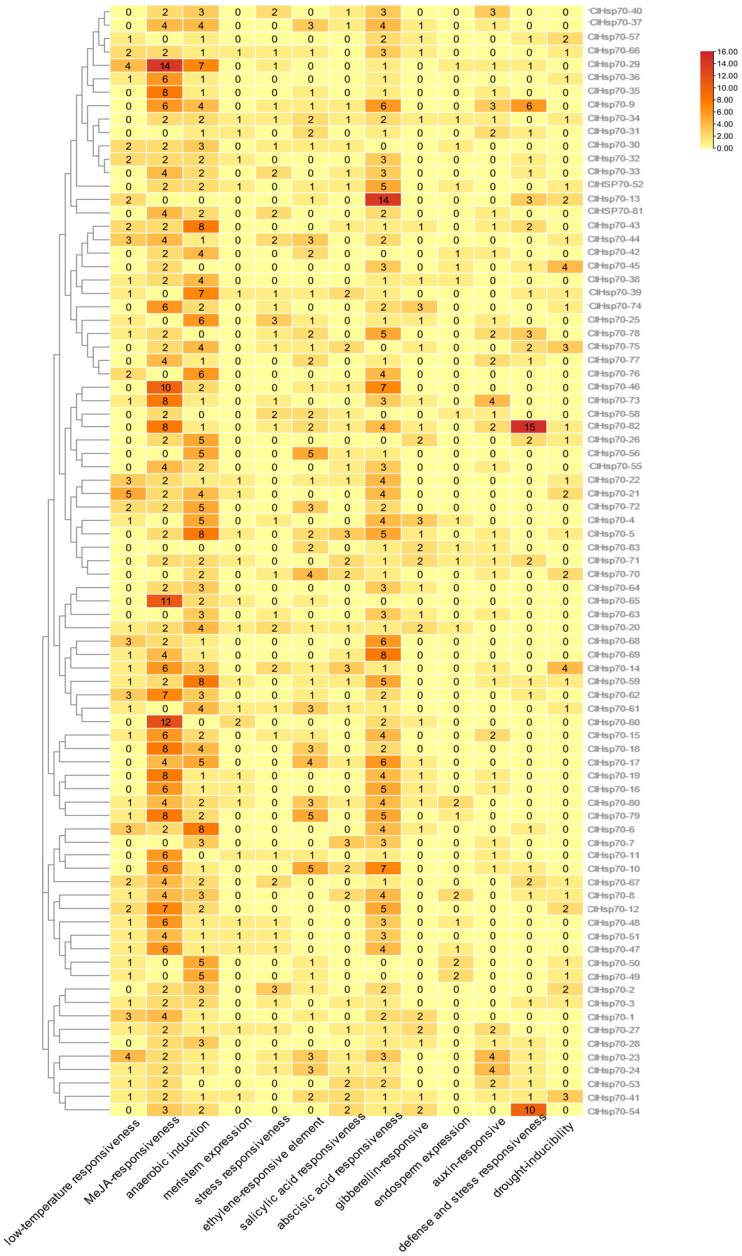

Figure 5. Putative cis-elements analysis of *ClHSP70s*.

3.6. qRT-PCR Analyses

Based on the above analysis, 15 *HSP70* genes were selected for tissue expression-specific analysis to determine their expression patterns in all tissues at different stages of the plant life cycle. Among them, five genes were selected to predict the presence of long CG-rich regions, which have a greater likelihood of methylation. In addition, we focused on stress responses and selected 10 *ClHSP70s* in all clusters with a large number of predictive cis-elements associated with adverse stress responses. We extracted total RNA from nine tissues during the vegetative and reproductive growth periods of *C. lavandulifolium*. The results verified that the expression of *ClHSP70s* in different tissues was significant, among which *ClHSP70-41* and *ClHSP70-52* were the highest in the roots during the vegetative growth period, *ClHSP70-14* and *ClHSP70-59* were the highest in the stem (VSt) during the vegetative growth period, and the gene families were expressed in all tissues. *ClHSP70-6*, *ClHSP70-21*, *ClHSP70-23*, *ClHSP70-28*, *ClHSP70-54*, and *ClHSP70-82*

had the highest expression in roots during reproductive growth, and it was speculated that they may be involved in some functions in roots (Figure 6A).

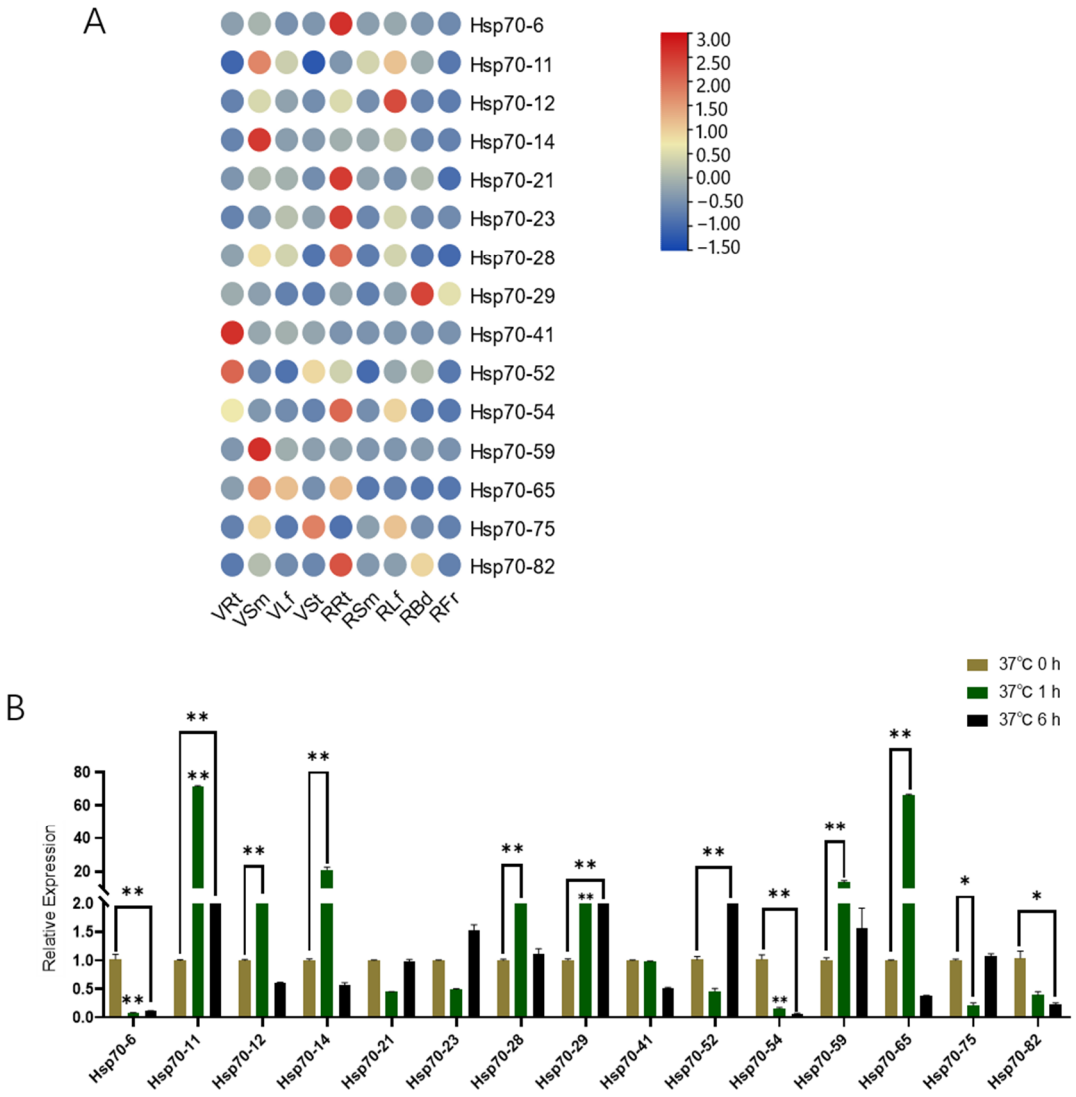

Figure 6. Expression patterns of the *ClHSP70* family. (**A**) Expression analysis of the selected *ClHSP70* genes in different tissues (root, stem, leaf, capitulum) at different stages. Expression of 15 genes: 5 genes were selected due to CpG island predictions, and 10 genes were selected based on their putative cis-elements associated with adverse stress responses. Abbreviations of different growth stages and tissues are as follows: VRt, vegetative growth stage root; VSm, vegetative growth stage stem; VLf, vegetative growth stage leaf; VSt, vegetative growth stage shoot; RRt, reproductive growth stage root; RSm, reproductive growth stage stem; RLf, reproductive growth stage leaf; RBd, reproductive growth stage bud; RFr, reproductive growth stage flower. (**B**) Expression patterns of *ClHSP70* family genes in response to heat stress by 37 °C. Values are the mean ± S.D. * $p \leq 0.05$, ** $p \leq 0.01$, Sidak's test.

As global temperatures increase, high temperatures have become one of the most important issues impacting crop growth. *HSP70* is heat-induced and plays a crucial role in thermal response. After high temperature treatment at 37 °C, *ClHSP70* gene expression in leaves was upregulated compared to that of the control, and most *ClHSP70s* showed a clear response trend after 1 h of heat treatment (Figure 6B). Among them, *ClHSP70-11*, *ClHSP70-65*, *ClHSP70-14*, *ClHSP70-59*, *ClHSP70-12*, *ClHSP70-28*, and *ClHSP70-29* increased rapidly and reached a maximum after 1 h of high temperature stress, indicating that they

play an important role in high-temperature stress. In addition, the expression of some *ClHSP70s* decreased rapidly after 1 h of high temperature stress, and its expression was inhibited by high temperatures. For example, *HSP70-6* and *HSP70-21* correspond to cis-elements containing more low-temperature response-related components, suggesting that their functions may be induced by low temperatures. In conclusion, *ClHSP70* plays an important role under temperature-stress conditions.

4. Discussion

The importance of *HSP70* genes in stress responses has been reported in many plant species. For example, compared with the control group, heat and drought tolerance was significantly improved in *HSP70* transgenic tobacco plants [39], and this result was also verified in *Arabidopsis* [40]. However, chrysanthemums occupy an important position in Chinese flower production. *Chrysanthemum morifolium* is a typical obligate short day (SD) herbaceous perennial species [41], and the realization of annual production is an urgent need of chrysanthemum industry. Furthermore, Chrysanthemum is susceptible to summer heat stress during the annual production process, which greatly affects their production and quality [42]. However, the *HSP70* gene family has not been extensively studied. *C. lavandulifolium* is one of the original species of chrysanthemum exhibiting strong stress resistance, and it is a diploid species in the Asteraceae family [43]. Therefore, understanding the heat tolerance mechanisms of chrysanthemum under heat stress is necessary to develop chrysanthemum varieties suitable for annual production.

Preliminary analysis of the *HSP70* gene family has been performed in the model plants *Arabidopsis* and rice [8,20,21]. In this study, we identified 83 *HSP70* genes in the *C. lavandulifolium*. Because of the size of the genome or evolution diversity, the number of the same gene family varies in different species [26]. In addition, we performed a comprehensive analysis of the *HSP70* family in *C.lavandulifolium* from the aspects of physicochemical properties, chromosomal location, phylogeny, gene structure, conserved motifs and expression profiles. *HSP70* is a multi-genic family. Various members constituting this family were present in different cellular compartments [39,44]. The analysis of subcellular localization of ClHSP70 proteins confirmed it. All of the *ClHSP70* members were divided into two groups, Dnak and Hsp110/SSE, consistent with the model plant *Arabidopsis*. This demonstrated that the *HSP70* gene family has a certain degree of conservation.

In addition, a promoter cis-element analysis was performed, and the cis-elements found in our study were mainly related to hormonal and environmental stresses. Most of these *ClHSP70s* were associated with methyl jasmonate (MeJA) and abscisic acid (ABA) responses, the latter of which is a stress-responsive hormone and a key factor in stress tolerance [45]. Jasmonic acid (JA) and MeJA play important roles in plant stress resistance. Duan et al. (2011) confirmed that *TaHSC70* is expressed 2 h after spraying MeJA [19]. The amount reached a maximum, indicating that TaHSC70 may participate in the basic defense of wheat through the JA signaling pathway. Besides that, there are a large number of cis-elements, such as drought, low temperature, and stress response in the promoter region of *ClHSP70*, which provide more evidence that this gene family plays a key role in environmental stress conditions. Methylation modification of histones is an important epigenetic regulatory method, and it has been observed for an increasing number of non-histone proteins. HSP70 is an evolutionarily conserved chaperone that is known to be modified by phosphorylation, ubiquitination, and glycosylation after translation; moreover, the methylable non-histone modification enzyme METTL21A has been reported to methylate the lysine site of HSP70 protein, thereby affecting protein interactions [46]. In a study on human cancer, it was found that HSP70 methylation is enhanced, whereas methylation is almost undetectable in the corresponding non-tumor tissues, and methylated HSP70 has a growth-promoting effect in cancer cells, demonstrating the key role of HSP70 methylation in human carcinogenesis [47]. In plant studies, the potential function of HSP70 protein methylation modifications is poorly understood, and studying such modifications will provide important insights.

We also used the methylation prediction tool to predict the CpG islands of *ClHSP70* and found that 18 *ClHSP70s* had CpG islands and may have exhibited methylation modification. However, the occurrence of methylation modifications still needs to be verified through relevant experiments. Based on this study, we selected 5 *ClHSP70s* with large CpG island length greater than 200 bp as candidate genes. In addition, we focused on the effects of environmental stress. According to the putative cis-element analysis and related reports on *HSP70s* in *Arabidopsis*, we selected 10 *ClHSP70* as candidate genes, and analyzed the tissue expression of the selected 15 *ClHSP70* candidate genes in *C. lavandulifolium* and found that most of *ClHSP70* was expressed in roots during vegetative growth and reproductive growth. Therefore, we speculated that it may be related to some functions in roots, such as drought and waterlogging stress, which further verified the important role of *ClHSP70* in environmental stress. Chrysanthemum has a certain tolerance to low temperatures but is very sensitive to high temperatures. These plants can grow normally at 18–21 °C, and a high temperature environment above 32 °C will cause slow growth and development, with a long-term high temperature environment causing damage to chrysanthemum [48]. HSP70 protein is synthesized under a variety of environmental stress conditions, such as high temperature, low temperature, hormone, high salt, drought, heavy metal ions, and mechanical damage [49]. Among them, HSP70s play vital roles in response to heat stress [50].

In this study, we explored the heat resistance mechanism of *ClHSP70* and analyzed its expression under high temperature stress. The results showed that most of the *ClHSP70s* had a significant upward regulation trend after 1 h treatment at 37 °C, such as *ClHSP70-11*, *ClHSP70-65*, *ClHSP70-14*, *ClHSP70-59*, and the expression level increased greater than 20-fold after 1 h of heat stress. In addition, all of them are highly expressed in the VSm (vegetative growth stage stem), and indicating that these four genes play an important role under heat stress, they may be used as candidate genes to carry out further research on the mechanism of heat stress in *C. lavandulifolium*. We also found that some *ClHSP70* expression showed a clear downward trend after high-temperature treatment. Interestingly, there were more low-temperature response elements in its promoters, which may be related to the cold tolerance of chrysanthemums. These results were consistent with previous studies showing the pivotal functions of *HSP70s* of other species in response to heat stress. Heat treatment of 37 °C significantly induces a number of *AtHSP70s* in *Arabidopsis* seedlings after 2 h, and the expression of *AtHSP70-4*, *AtHSP70-5*, *AtHSP70-8*, *AtHSP70-7*, *AtHSP70-10*, *AtHSP70-11*, and *AtHSP70-15* was highly induced by heat stress [8]. Ye et al. (2012) analyzed the expression patterns of four *OsHSP70* genes under heat stress, significantly increased expression levels of the four *OsHSP70* genes were detected after 3 h at 42 °C, which indicated these genes are quickly and sharply induced by heat stress [51]. The expression profiles of 61 soybean *HSP70* genes in response to heat (0, 3, 6, 12, 24 h) indicated that 55 of *GmHSP70* genes were upregulated from two-fold to 241-fold after 42 °C treatment. Most members were upregulated after 3 h, and only 6 *GmHSP70* genes were downregulated or nearly unchanged under the heat treatment [26]. After stress treatment at 40 °C, all nine *PsHSP70s* could respond to heat stress, with expression patterns rising first and then falling in Tree peony (*Paeonia suffruticosa*) [52]. Combining the heat map and the expression profiles found that the majority of *ZmHSP70s* was significantly up-regulated in leaves after 1 h of 42 °C heat treatment, indicating that these genes may play an important role in maize resistance to heat stress [53]. These results indicate that there is a functional redundancy and division of labor, in terms of the heat stress regulation of *HSP70* genes. Under normal conditions, the expression level of *HSP70s* in cells is basic and low, while the synthesis rate of *HSP70s* increases significantly, generally reaching the highest level within a short time under high temperature and various stress conditions so as to improve the ability to resist stress of organisms. However, the detailed mechanism requires further investigation.

5. Conclusions

In conclusion, this study aimed to identify the *ClHSP70* gene family and explore the genetic improvement potential of the *HSP70* gene under heat stress conditions. A total of

83 *ClHSP70* genes were analyzed. The cis-elements and CpG island promoter predictions indicated that there are multiple abiotic stress and phytohormone response elements on the *ClHSP70* promoter and methylation modification may have occurred. Tissue expression and heat treatment analyses further verified the important role of *ClHSP70s* in environmental stress. This provides valuable information for further studies on the function of this gene family under heat stress.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/horticulturae9020238/s1, Table S1: Analysis of physical and chemical properties of *ClHSP70s*; Table S2: Analysis of Ka/Ks; Table S3: The primers of qRT-PCR; Table S4: The candidate genes list for expression analysis; Figure S1: The high-quality motifs image in the main text, from motif 1 to motif 10 in order.

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