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Characterization, Evolutionary Analysis, and Expression Pattern Analysis of the Heat Shock Transcription Factors and Drought Stress Response in *Heimia myrtifolia*

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Abstract: Heat shock transcription factors (HSFs) are among the most important regulators of plant responses to abiotic stimuli. They play a key role in numerous transcriptional regulatory processes. However, the specific characteristics of HSF gene family members and their expression patterns in different tissues and under drought stress have not been precisely investigated in *Heimia myrtifolia*. This study analyzed transcriptome data from *H. myrtifolia* and identified 15 members of the HSF family. Using a phylogenetic tree, these members were classified into three major classes and fifteen groups. The amino acid physicochemical properties of these members were also investigated. The results showed that all *HmHSF* genes are located in the nucleus, and multiple sequence alignment analysis revealed that all HmHSF proteins have the most conserved DBD structural domains. Interestingly, a special HmHSF15 protein was found in the three-dimensional structure of the protein, which has a conserved structural domain that performs a function in addition to the unique structural domain of HSF proteins, resulting in a three-dimensional structure for HmHSF15 that is different from other HmHSF proteins. GO enrichment analysis shows that most *HmHSFA*-like genes are part of various biological processes associated with abiotic stresses. Finally, this study analyzed the tissue specificity of *HmHSF* genes in different parts of *H. myrtifolia* by qRT-PCR and found that *HmHSF* genes were more abundantly expressed in roots than in other tissues, and *HmHSF05*, *HmHSF12*, and *HmHSF14* genes were different from other *HSF* genes, which could be further analyzed to verify their functionality. The results provide a basis for analyzing the functions of *HmHSF* genes in *H. myrtifolia* and help to explore the molecular regulatory mechanism of *HmHSF* in response to drought stress.

Keywords: heat shock transcription factors; drought stress; *Heimia myrtifolia*

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1. Introduction

As the global climate gradually deteriorates, localized weather extremes are becoming more frequent, with drought being one of the most obvious climatic features [1]. Extensive research has demonstrated that drought is a significant abiotic stress factor that impacts plant growth and development [2,3]. It frequently causes cell water loss, disconnection of the serum wall and enzyme systems, metabolic imbalances, and ultimately changes the direction of plant growth [4–6]. When plants are subjected to external stress stimuli, they produce a number of signals in the living organism that cause a variety of signaling pathways to be turned on and the activation of subsequent proteins, such as those from HSFs, DREB, and bZIP [7]. The stimulation of plant-specific resistance genes and associated

defense mechanisms by transcription factors alters the ability of plants to adapt to their environment [8].

Transcription factors are a class of proteins with specific structures and regulatory functions that can play a significant role in the growth and behavior of plants in the external environment by activating or repressing gene transcription [9]. The heat shock transcription factor class is an important class of plant stress resistance factors [6,10]. It has a major impact on heat, cold, dehydration, drought, salt, and disease resistance processes in plants, and plays an extremely important role in controlling plant adaptation to stress as well as plant development [11–13]. The highly conserved HSF protein in plants is made up of a structurally and functionally conserved DNA-binding domain at the N-terminus (DBD), an adjacent dichotomous domain for oligomers, a nuclear localization signal, a nuclear export signal, and a deterrent domain [14,15]. The DBD structural domain is the one that has been retained the best in HSF out of all of these conserved structural domains [16]. According to related studies, the HSF family members and their activities were exhaustively examined in *Arabidopsis thaliana* [17], *Ziziphus jujuba* [18], and other model plants [15,19–21]. The findings clearly indicate that HSF plays a remarkably valuable role in the resistance of these plants to abiotic stresses [22].

Heimia myrtifolia loves the sun and has few pests and diseases, making it a rare small shrub that flowers in both summer and autumn [23]. When in bloom, the golden-yellow flowers adorn the branches and add great amenity and accent to the landscape. For this reason, it is widely used in landscaping [24]. However, *H. myrtifolia* has a higher water requirement for its habitat. In the event of a water shortage during its lifetime, the leaves will curl, and, if they are not rehydrated in time, the whole plant will promptly die. Therefore, it is essential to conduct research on drought tolerance in *H. myrtifolia* and to progressively select drought-tolerant *H. myrtifolia* germplasm resources. The group determined the transcriptome of *H. myrtifolia* drought stress in the early stage and carried out a series of analyses. Analytical studies revealed that most *HSF* genes play an important role in the response of *H. myrtifolia* to drought stress. However, the study did not investigate the key *HSF* genes. It is known that *HSF* genes are essential for receiving and transmitting signals, recognizing heat shock components, and controlling downstream genes [25–27]. Therefore, this study first identified family members of the *H. myrtifolia HSF* using a bioinformatic approach based on transcriptome data from *H. myrtifolia*. Subsequently, this study investigated the amino acid physicochemical properties, multiple sequence alignment, GO enrichment, and tissue expression pattern analysis of HSF family members under simulated drought conditions. These findings will help us to better understand the evolutionary links and the functional differentiation of *HmHSF* genes in *H. myrtifolia* and provide corresponding theoretical support for future studies on the molecular biology of *H. myrtifolia HSF* genes.

2. Materials and Methods

2.1. HSF Gene Characterization and Sequencing Analysis in H. myrtifolia

This study retrieved the sequences of the HSF family of *A. thaliana* from the TAIR database (https://www.arabidopsis.org/ (accessed on 3 March 2023)). A BLASTP search was then performed on the transcriptome data of *H. myrtifolia*. In the next step, the built-in HMMER 3.0 program was used to download the HSF family members of *H. myrtifolia* by using the Hidden Markov Model of the HSF protein (PF0047) in combination with the Pafm database (http://pfam.xfam.org/ (accessed on 15 March 2023)). This study summarized the potential genes for *HmHSF* identified by these two search techniques. Finally, the potential HmHSF proteins were validated for HSF-conserved structural domains by Web CD-search and SMART, thus ensuring the accuracy of the screened *HmHSF* members. This study finally identified 15 *HmHSF* genes and renamed them in the order of the original assembly ID of the transcriptome.

2.2. Prediction of the Physicochemical Properties and Protein Structure of HmHSF

This study predicted the subcellular localization of HmHSF proteins using Cell-PLoc 2.0, and, through the ExPasy website, the physicochemical properties of all HmHSF proteins.

The secondary structures of the proteins were analyzed by the online site SOPMA, and then the data were collated using Excel. The prediction of the tertiary structure of proteins was presented by the online tool SWISS-MODEL.

2.3. Phylogenetic Tree Construction and Sequence Comparison

Using the NCBI database (https://ngdc.cncb.ac.cn/ (accessed on 26 March 2023)), this study downloaded all *HSF* genes from *Oryza sativa* and *A. thaliana*. Multiple-sequence protein alignment using the 21 AtHSF proteins, 25 OsHSF sequences, and 15 HmHSF protein sequences was performed using MEGA 6.0 to generate NJ trees for HSF proteins. The phylogenetic tree was post-landscaped and processed using iTOL. This study also used Jalview software to perform a multiple sequence alignment analysis on HSF proteins.

2.4. GO Database Annotation and Enrichment Analysis

This study performed GO functional annotation of the *HmHSF* gene via the online tool eggNOG-mapper. The annotation result files were then imported into the eggNOG-mapper program of TBtools for analysis and aggregation. Finally, this study used the online website HIP-LOT to graph and embellish the data results generated by TBtools.

2.5. Plant Material, Methods, and qRT-PCR Validation

This study grew annual cuttings of *H. myrtifolia* in an artificial climate chamber at Zhejiang Agriculture and Forestry University. The soil moisture content in the cuttings' basins was maintained at 33.5% for one week prior to the drought treatment and replenished each morning at 8 a.m. using a TDR100 portable soil moisture meter. For the drought treatment, one control group (CK) and four treatment groups (T1, T2, T3, and T4) were established, each with three replicates. The relative soil moisture contents were 65–75% (CK), 45–60% (T1), 30–45% (T2), 15–30% (T3), and 5–15% (T4). The field water content was measured each morning at 8 a.m. to maintain it within the control range. After 10 days of drought treatment, roots, stems, leaves, and flowers were collected from the control group and leaves from the different treatment groups. The samples were rapidly frozen in liquid nitrogen and stored at −80 °C.

This study extracted total RNA from the leaves of the control and treatment groups using the FastPure Plant Total RNA Isolation Kit from Vazyme. The first strand cDNA was then reverse-transcribed using HiScriptIII All-in-One RT Perfect Hypermix. Specific primers for 15 pairs of genes were designed using Primer 5 software, with *HmGAPDH* as the internal reference gene (Table S1). qRT-PCR amplification was performed using SYBR Premix Ex TaqTM in 10 µL volumes on an ABI 7300 real-time PCR instrument. Three replicates were performed for each selected gene and the relative expression levels of *HmHSF* genes were analyzed using the $2^{-\Delta\Delta Ct}$ method. The experimental data were analyzed and organized using Excel 2010 and SPSS 20.0.

This study performed K-means clustering analysis of 15 HmHSF genes through the online website HIP-LOT, which was then further embellished using Photoshop 2022.

3. Results

3.1. Identification of HSF Genes in H. myrtifolia

This study searched for 15 *HmHSF* genes in the *H. myrtifolia* transcriptome database (transcriptome data available from NCBI under number PRJNA804698) using BLAST and HMMER search methods. For subsequent analysis, all members were named *HmHSF01-HmHSF15* in the order of their assembly ID (Table 1).

Table 1. Physicochemical property analysis of HmHSF proteins.

Gene Name	Gene ID	Size (aa)	MW (kDa)	pI	Stability	A.I	GRAVY	Predicted Location
HmHSF01	Gene.80304	446	50501.11	5.83	U	65.88	−0.869	Nuclear
HmHSF02	Gene.33060	363	40958.06	4.81	U	81.87	−0.515	Nuclear
HmHSF03	Gene.82041	290	32889.93	6.35	U	59.55	−0.808	Nuclear
HmHSF04	Gene.67227	391	44075.58	5.11	U	61.10	−0.931	Nuclear
HmHSF05	Gene.83400	339	38308.34	8.23	U	70.44	−0.522	Nuclear
HmHSF06	Gene.78293	325	36158.11	4.64	U	65.38	−0.677	Nuclear
HmHSF07	Gene.92484	372	41725.87	9.07	U	71.05	−0.756	Nuclear
HmHSF08	Gene.77897	303	34137.49	6.19	U	68.58	−0.549	Nuclear
HmHSF09	Gene.93333	253	28667.25	9.33	U	61.70	−0.836	Nuclear
HmHSF10	Gene.82043	295	32617.38	8.31	U	66.88	−0.69	Nuclear
HmHSF11	Gene.98726	427	48851.44	5.16	U	69.13	−0.784	Nuclear
HmHSF12	Gene.96475	392	44531.09	6.04	U	77.83	−0.663	Nuclear
HmHSF13	Gene.44218	320	34804.22	5.33	U	72.28	−0.46	Nuclear
HmHSF14	Gene.51133	279	30884.61	7.02	U	62.26	−0.785	Nuclear
HmHSF15	Gene.86874	492	53592.58	4.85	U	61.20	−0.643	Nuclear

The 15 *HmHSF* genes are anticipated to encode proteins with 253 (*HmHSF09*) to 492 (*MsHSF15*) amino acids based on the projected physicochemical features of the amino acid sequences. The molecular weights (MWs), with an average of 39,513.54 Da, varied from 28667.25 (*MsHSF09*) to 53592.58 Da (*MsHSF15*). The anticipated isoelectric point (pI), with a mean value of 6.42, varied from 4.64 (*MsHSF06*) to 9.33 (*MsHSF09*). The stability index indicates that every HSF protein is erratic in vitro. The aliphatic amino acid index (A.I.) also varied between 59.55 (*MsHSF03*) and 81.87 (*MsHSF02*), indicating a minor difference in thermal stability. The hydrophilic (GRAVY) rankings of each HSF protein have a negative overall mean, demonstrating that all of them are hydrophilic proteins. The final discovery was that all HSF proteins were found in the atomic nucleus, as predicted by subcellular localization predictions.

3.2. HmHSF Protein Sequence Analysis

To further investigate the existence and location of the conserved protein structural domain, in this study, we performed a multiple sequence alignment analysis of the 15 HmHSF proteins using Jalview software and found that all 15 HmHSF proteins contain a highly conserved structural domain, the DBD conserved structural domain (Figure 1B), which consists of approximately 100 amino acids (Figure 1D). Subsequently, to further reveal the structural diversity of the HmHSF protein sequences, this study analyzed the 10 most conserved motifs of the HmHSF proteins using MEME software and named them Motif 1–10. The conserved amino acid patterns range in length from 9 to 50. Motif 1 is the longest in this study with 50 conserved amino acids, while motif 9 is the shortest with 9 conserved amino acids. This study visualized the conserved structural domains of HmHSF using TBtools and found that HmHSF proteins in the same broad class have similar conserved motifs. Interestingly, Motif 5 was only found in HSFA-like proteins. These results suggest that specific motifs may be associated with the functions of different subgroups.

3.3. Phylogenetic Analysis of HmHSF Protein

To investigate the evolutionary features among *H. myrtifolia HSF* genes, in this study, we constructed an NJ phylogenetic tree of the amino acid sequences of 15 *H. myrtifolia* HSF proteins, 21 *A. thaliana* HSF proteins, and 25 *O. sativa* HSF proteins using MEGA 6.0 (Figure 2). According to our previous family classification of *A. thaliana* HSF proteins, in this study, we divided the 15 *H. myrtifolia* HSF proteins into three groups, namely HSFA (green), HSFB (yellow), and HSFC (blue). Of these, HSFA was divided into nine subgroups (A1–A9) with seven members, including HmHSF01, HmHSF02, HmHSF04, HmHSF07, HmHSF11, HmHSF12, and HmHSF15, where the *H. myrtifolia* HSF proteins did not aggregate into five subgroups: A3, A6, A7, A8, and A9. HSFB (B1–B4) has four subgroups within

it, consisting of seven members, namely HmHSF03, HmHSF05, HmHSF06, HmHSF09, HmHSF10, HmHSF13, and HmHSF14. *H. myrtifolia* HSFC is the smallest group, containing only one member, HmHSF08, distributed in subgroup C1.

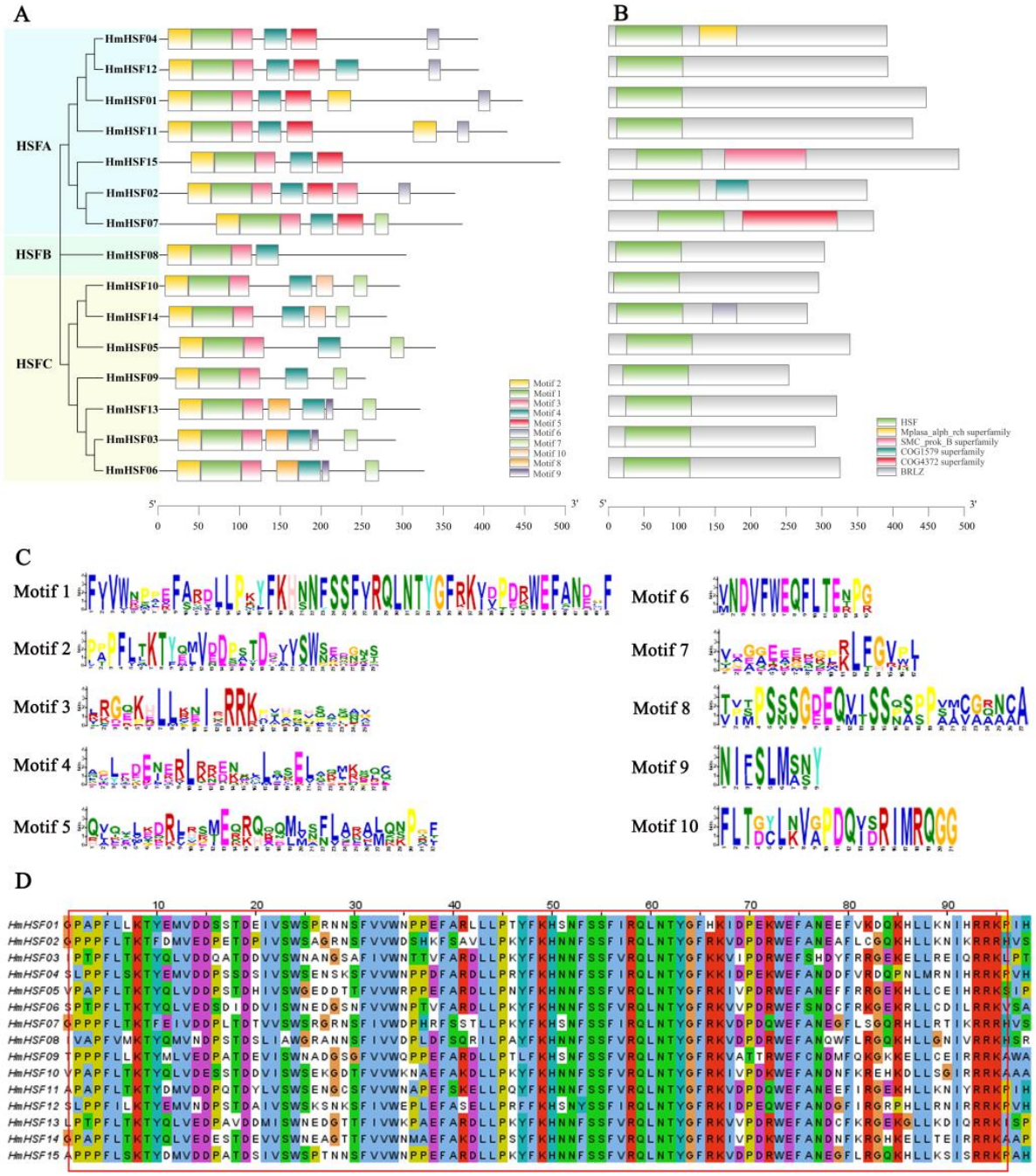


Figure 1. Characterization of *HmHSF* genes. (**A**) Phylogenetic tree of the *HmHSF* gene family. Genes in the *HSFA*, *HSFB*, and *HSFC* groups are shown in blue, green, and yellow, respectively. Different colored boxes represent different conserved motifs. (**B**) Conserved protein structural domains. The green color is the most conserved DBD structural domain of the HSF protein. (**C**) The sequence identity of all identified HmHSF patterns was analyzed. (**D**) DBD structural domain of HmHSF protein multiple sequence alignment. The red boxed part is the most conservative structural domain of HSF, DBD structure.

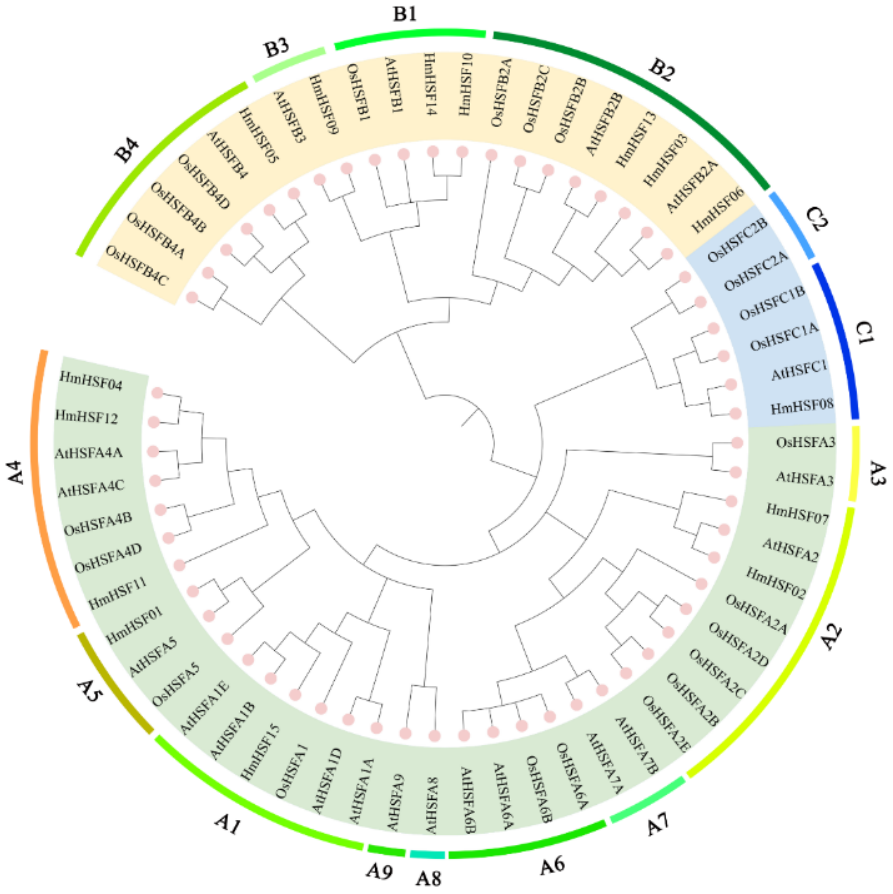


Figure 2. NJ phylogenetic tree of HSF proteins of *H. myrtifolia*, *A. thaliana*, and *O. sativa* constructed using default parameters. The evolutionary tree was divided into three broad categories of different colors: HSFA (green), HSFB (yellow), and HSFC (blue). According to the previous subfamily classification of AtHSF proteins, HSFA contains nine subgroups (A1–A9), HSFB contains four subgroups (B1–B4), and HSFC contains two subgroups (C1–C2).

3.4. Structural and Interaction Network Analysis of the HmHSF Protein

To better understand the interactions between HmHSF proteins, this study used the STRING database to predict their possible interactions (Figure 3). The results showed that the HmHSF15 protein is a central node in the protein interaction network graph and is closely related to other connecting nodes, and, subsequently, this study investigated the HmHSF secondary structure and tertiary structure to further analyze the HmHSF protein (Table S2). The secondary structure analysis showed that the secondary structure of HmHSF02 and HmHSF07 proteins is mainly α-helix, and then randomly becomes a coiled coil; then an extended chain; and lastly, it exhibits β-rotation. The secondary structures of the other 13 HmHSF proteins were mainly random coiling, followed by α-helix, followed by the extension chain, and β-rotation was the least common. Our prediction of protein tertiary structure showed that random coiling and α-helix contribute significantly to the stability of the tertiary structure, while β-rotation plays a crucial role in modification. Interestingly, the tertiary structure models of all HmHSF proteins were highly similar except for HmHSF15 (Figure 4). The difference between the structure of the HmHSF15 protein and the rest of the proteins can be analyzed with the help of the domains in Figure 1B, where it can be clearly seen that HmHSF15 protein has other structural domains besides the conserved structural domain of HSF, which performs other functions and causes changes in the three-dimensional structure of the HmHSF15 protein at the same time.

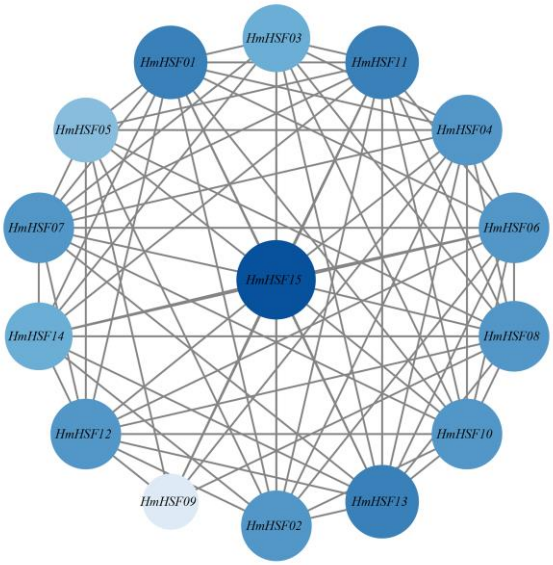


Figure 3. Interaction network diagram of HmHSF proteins. Nodes represent proteins; darker blue indicates stronger connectivity of the protein, and grey lines indicate interactions between nodes.

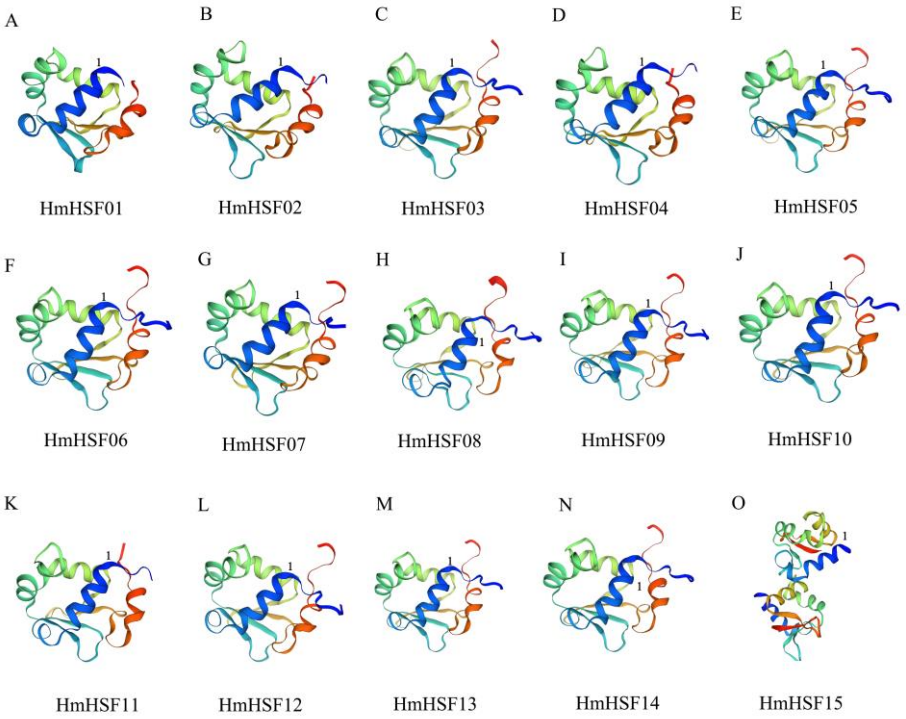


Figure 4. 3D structure model of HmHSF protein. (**A**–**O**) represents HmHSF01-HmHSF15, respectively. Number 1 represents the DBD structure of HmHSF protein.

3.5. Analysis of the GO Annotation and Enrichment

To understand the biological activities carried out by HmHSF proteins, in this study, we performed a GO annotation analysis of 15 *HmHSF* genes, which showed that 13 genes are involved in the biological processes (BP), cellular components (CC), and molecular functions (MF). This study then performed a GO enrichment analysis on 12 *HmHSF* genes (Figure 5), which revealed that *HmHSF* genes are involved in biological functions related to drought stress, such as in the response to abiotic factors, response to temperature stimuli, and response to xenobiotic stimuli. Interestingly, GO enrichment showed that most of the biological processes responding to stress and other related functions were enriched

in *HmHSFA*-like genes; *HmHSFB*-like genes were enriched very rarely, and *HmHSFC*-like genes were not enriched at all. Therefore, *HmHSFA*-like genes play an irreplaceable role in the response to drought stress.

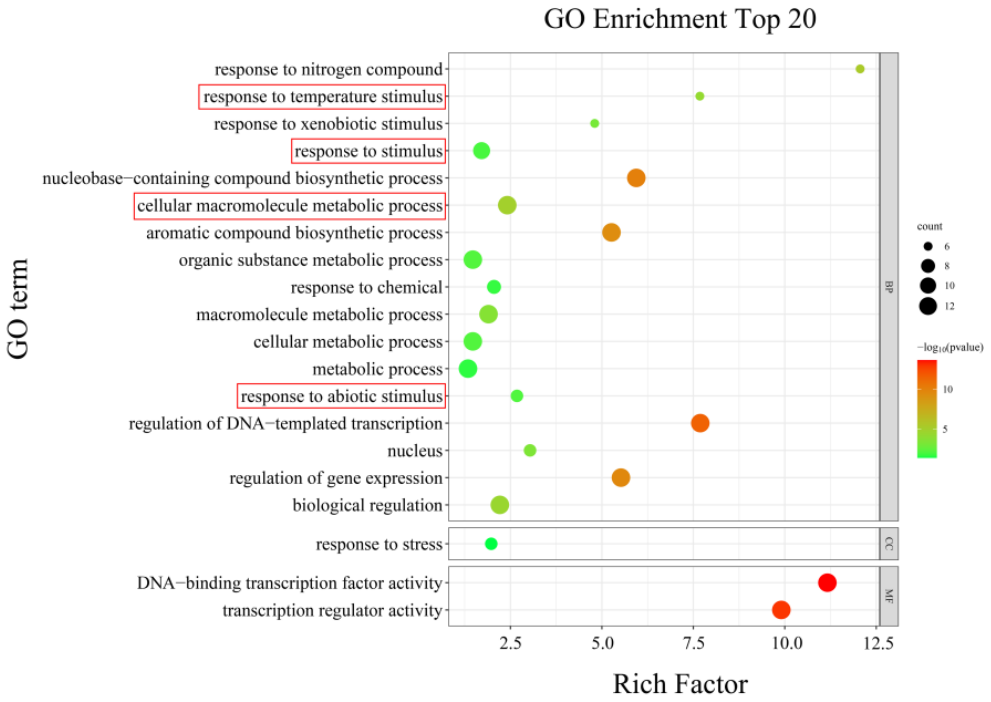


Figure 5. Analysis of the HmHSF proteins' GO enrichment in comparison to the GO database. The red squares represent is the biological processes associated with drought stress.

3.6. Expression and K-Means Clustering Analysis of HmHSF Gene

To investigate the potential biological functions of *HmHSF* genes in the growth and development of *H. myrtifolia*, this study studied the expression of *HmHSF* genes in roots, stems, leaves, and flowers using real-time quantitative PCR to determine the expression patterns of specific *HmHSF* genes in different tissues. The results were also presented via TBtools software in the form of heat maps (Figure 6A). It was found that *HmHSF* genes were significantly expressed in roots except for *HmHSF08* and *HmHSF09*, suggesting that these genes may be required for root tissue development. Notably, all *HmHSF* genes were barely expressed in flowers and stems except for *HmHSF03*, *HmHSF08*, and *HmHSF09*, which were highly expressed in leaves. These findings imply that there is a clear tissue specificity in the expression of *HmHSF* genes in different tissues.

In addition, this study obtained the expression profile of *HmHSF* genes by transcriptome data extraction and screening (Figure 6B), and found that the genes that exerted the earliest response in the initial stage of drought stress were *HmHSF05*, *HmHSF09*, and *HmHSF12*; with the increase in stress duration, *HmHSF02*, *HmHSF06*, *HmHSF07*, and *HmHSF13* were highly expressed, resisting the damage caused by drought stress; finally, the genes that were not expressed in the first two stages during the continuous increase in drought stress also appeared to be highly expressed to resist the stress. In this study, we also performed a K-means clustering analysis of 15 *HmHSF* genes (Figure 6C), and the analysis showed that 15 *HmHSF* genes were clustered into 10 clusters that functioned in the process of drought stress in the form of regulation without passage, respectively. These results suggest that genes of the HmHSF family may play important roles in different stages of drought stress. Notably, *HmHSF05*, *HmHSF12*, and *HmHSF14* played roles in two of the three stages of drought stress, which subsequently warranted further investigation into their functionality in drought stress.

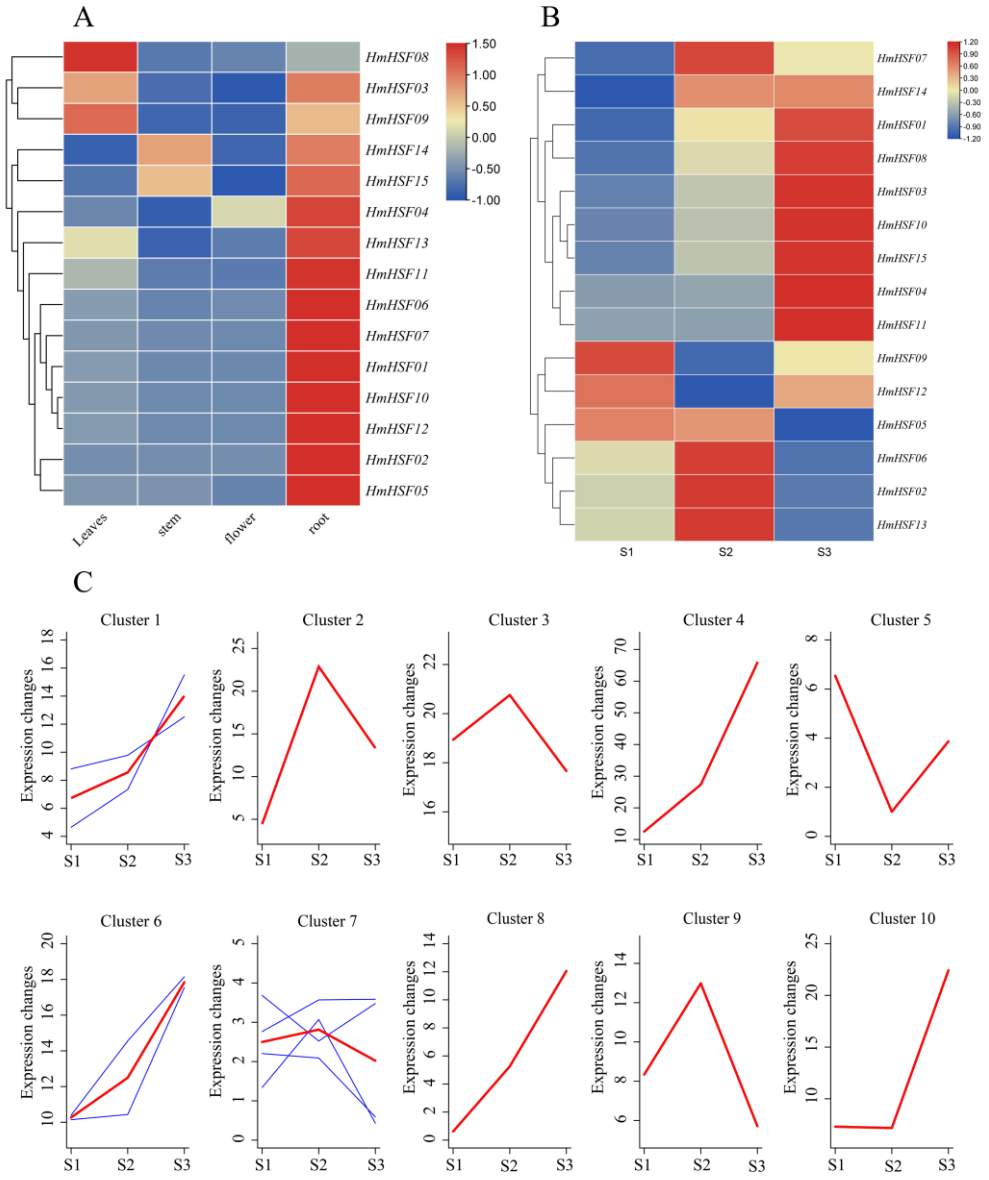


Figure 6. (**A**) The expression of *HmHSF* genes in different tissues using a heatmap, where red represents high expression and blue represents low expression. (**B**) The expression of *HmHSF* genes under drought stress in transcriptome data, with soil water content ranging from 65–75%, 30–45%, and 5–15%. The expression values were visualized in a heatmap, with red representing high expression and blue representing low expression. (**C**) K-means clustering analysis of *HmHSF* gene expression. The red line in the figure indicates the trend of such genes, and the blue line indicates the *HmHSF* genes enriched in this trend.

3.7. Expression of HSF Genes under Drought Stress

The genes *HmHSF05*, *HmHSF12*, and *HmHSF14* were identified by tissue specificity analysis and K-means clustering analysis as being different from other *HmHSF* genes in that they were highly expressed in two tissues (Figure 7). This study further investigated the expression of these genes under drought stress using q-PCR. The analysis showed that the expression trend of the three genes increased and then decreased, with *HmHSF05* and *HmHSF14* being negatively regulated and *HmHSF12* being positively regulated. These results were consistent with the transcriptome data. Therefore, it is hypothesized that *HmHSF* genes may be closely related to the response mechanism of drought stress.

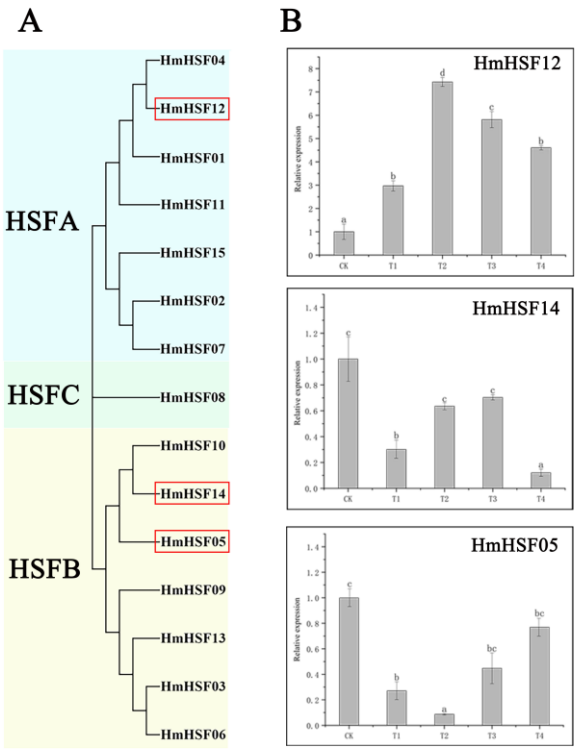


Figure 7. (**A**) The phylogenetic tree of the *HmHSF* gene family is shown. Genes in the *HSFA*, *HSFB*, and *HSFC* groups are shown in blue, green, and yellow, respectively. (**B**) The expression analysis of three *HmHSF* genes under simulated drought stress is presented. Data represent the mean ± SD of three biological replicates. Means indicated by the same letter are not significantly different at $p < 0.05$, as determined by Duncan's multiple range test.

4. Discussion

Knowledge on plant stress under drought stress is important in order to enhance the ornamental value of landscape plants under adversity. HSFs belong to a relatively special category among all transcription factors. Such a class of TFs is essential for plants so that they can withstand diverse biotic and abiotic stresses [28].

Based on the transcriptome data of *H. myrtifolia*, 15 complete HmHSF genes with open reading frames were discovered in this study, and their fundamental characteristics were investigated. *H. myrtifolia* has fewer *HSF* genes than plants such as *A. thaliana* (21), *S. lycopersicum* (25), and *Zea mays* (30); this may be due to the loss of some *HSF* genes during evolution [29–31]. The analysis of the physicochemical properties of *H. myrtifolia* HSF proteins showed that the molecular weight and amino acid content of the proteins did not differ much from each other. Subcellular localization analysis showed that all HSF proteins were localized in the nucleus. This suggests that no differential evolution has occurred during the evolution of HmHSF proteins. Early studies found that the *N*-terminal end of the highly conserved plant HSF DBD is capable of locating and identifying the target gene's heat stress element (HSE) prokinetic region [32,33]. Comparative multiple sequence analysis and protein secondary structure prediction revealed that all HmHSF proteins have DBD structures, which consist of three α-helices and four β-folds. It is noteworthy that HmHSF has other structural domains besides the DBD structural domain; therefore, further experiments are necessary to analyze whether this structural domain affects the function of the HSF gene family. In this study, we also constructed phylogenetic trees for *H. myrtifolia*, *A. thaliana*, and *O. sativa*. The *HmHSF* genes were further grouped into three groups, *HmHSFA*, *HmHSFB*, and *HmHSFC*, using homology matching and multi-species matching. The numbers of genes in the groups *HmHSFA* and *HmHSFB* were the highest, and the numbers of genes in the group *HmHSFC* were the lowest, and this clustering

was the same as that of the *A. thaliana* HSF gene family members [34]. A related study found that the expression of class A *HSF* increased most significantly when *A. thaliana* was subjected to heat stress, and excessive manifestation of the *AtHSFA2* gene significantly increased the basal heat resistance of plants [35]. Although class B *HSF* can also recognize and bind to the thermal initiation element HSE, it is prevented from doing so by the carboxy-terminal characteristic structural domain (-LFGV-), which prevents plants from regulating either their ability to tolerate heat or their ability to initiate normal thermal response gene expression [36,37]. Therefore, whether there are differences in the protein function and mode of action of different groups of *H. myrtifolia HmHSF* needs further study. Notable is the absence of the subgroups A3, A6, A7, A8, and A9 in the *H. myrtifolia* HSF proteins; this finding shows that despite having a common ancestor, HSF family proteins developed independently in various species. Nearly all the *H. myrtifolia* HSF proteins are comparable to those of *A. thaliana* but not *Oryza sativa*, indicating that HmHSF and AtHSF have a closer evolutionary connection [38–40]. In addition, GO annotation and enrichment analysis of these 15 predicted *HmHSF* genes showed that most of them are part of biological processes in response to abiotic stresses, such as the responses to abiotic factors, responses to temperature stimuli, and responses to xenobiotic stimuli.

This study observed that most of these *HSF* genes showed a progressive upward trend in expression with increasing drought levels. About 13% of HSF genes were upregulated and 67% were downregulated, suggesting that they may play a particular role in drought conditions. The majority of the regulated genes belong to the HmHSFA group. This result is interesting because *HSFA* genes often perform irreplaceable roles. These genes include *HSFA1* and *HSFA2*, which have been identified as responsive to drought stimulation in *A. thaliana* and *Solanum lycopersicum* [41]. There are also some related studies showing that some *HSFB*-like genes are implicated in osmotic pressure tolerance in *Arachis hypogaea* [18,42]. These results suggest that *HSF* members have species-specific and stress-specific features when it comes to regulating the roles of genes involved in plant stress responses. In addition, in this study, we found that the expression levels of up-regulated genes reduced rapidly while the expression levels of down-regulated genes increased in the reaction to drought pressure in *H. myrtifolia*. These findings suggest a more efficient function of the down-regulated genes, as they function more effectively in response to drought stress. However, the process by which genes exert their regulatory effects is a complex one, and further research on *HSF* genes is therefore essential. It is noteworthy that HmHSF15 protein has a different three-dimensional structure to other HmHSF proteins. Combined with the analysis of the conserved structural domains, it can be concluded that HmHSF15 protein has other structures besides the specific structural domains of the HSF family, and therefore the HmHSF15 protein has more regulatory functions that deserve attention in the follow-up study. In addition, tissue specificity analysis and K-means clustering analysis showed that the *HmHSF* gene has significant tissue specificity in the growth and development of *H. myrtifolia*. Notably, the *HmHSF05*, *HmHSF12*, and *HmHSF14* genes were highly exo-compressed in two tissues, unlike other *HmHSF* genes, and their specific functions could be demonstrated by subsequent experiments. It can be speculated that the above genes may exhibit enhanced functions in response to stress. In addition, a study on the analysis of promoter cis-acting elements of *A. thaliana HSF* genes revealed that *HSF* genes not only play a role in abiotic stresses but also in biological processes involved in growth and development, biosynthesis, and hormone response.

Therefore, in the subsequent bioinformatics study, the promoter cis-acting elements of the key genes can be continually observed in order to further investigate the response of *HSF* genes in drought and in the growth development of *H. myrtifolia*. Based on bioinformatics, the screening of the key *HSF* genes can be further performed for full-length cloning and genetic transformation, and the response mechanism of the key *HSF* in drought stress can be studied in depth to provide a theoretical basis for the subsequent breeding of a new cold-tolerant *H. myrtifolia* species.

5. Conclusions

This study analyzed and identified 15 *HmHSF* genes. Amino acid sequence alignment revealed that all *HmHSF* genes contain a conserved DBD structure. HmHSF proteins were classified into three groups and fifteen sub-groups based on evolutionary relationships. Proteins within the same group were mostly similar, but differed significantly between subgroups. Additionally, it was found that the HmHSF15 protein has a conserved structural domain in addition to the DBD structure. The specific function of this domain needs to be verified by further experiments. The GO analysis revealed that most *HmHSF* genes are a part of various biological processes associated with abiotic stresses. Finally, it was found that *HmHSF* genes showed very obvious tissue specificity in different tissues of *H. myrtifolia*, especially the three genes *HmHSF05*, *HmHSF12*, and *HmHSF14*, which belonged to the same class of genes in the K-means clustering analysis. Further experiments can be arranged to elucidate the molecular mechanism of the key *HSF* genes against drought stress in *H. myrtifolia*.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/horticulturae9050588/s1. Table S1: qRT-PCR primers for the *HmHSF* genes; Table S2: Secondary structure prediction of HmHSF.

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References

1. Fang, Y.; Xiong, L. General mechanisms of drought response and their application in drought resistance improvement in plants. *Cell Mol. Life Sci.* **2015**, *72*, 673–689. [CrossRef] [PubMed]
2. Joshi, R.; Wani, S.H.; Singh, B.; Bohra, A.; Dar, Z.A.; Lone, A.A.; Pareek, A.; Singla-Pareek, S.L. Transcription Factors and Plants Response to Drought Stress: Current Understanding and Future Directions. *Front. Plant Sci.* **2016**, *7*, 1029. [CrossRef] [PubMed]
3. Ilyas, M.; Nisar, M.; Khan, N.; Hazrat, A.; Khan, A.H.; Hayat, K.; Fahad, S.; Khan, A.; Ullah, A. Drought Tolerance Strategies in Plants: A Mechanistic Approach. *J. Plant Growth Regul.* **2021**, *40*, 926–944. [CrossRef]
4. Batool, M.; El-Badri, A.M.; Hassan, M.U.; Haiyun, Y.; Chunyun, W.; Zhenkun, Y.; Jie, K.; Wang, B.; Zhou, G. Drought Stress in *Brassica napus*: Effects, Tolerance Mechanisms, and Management Strategies. *J. Plant Growth Regul.* **2022**, *42*, 21–45. [CrossRef]
5. Zhang, X.; Lei, L.; Lai, J.; Zhao, H.; Song, W. Effects of drought stress and water recovery on physiological responses and gene expression in maize seedlings. *BMC Plant Biol.* **2018**, *18*, 68. [CrossRef] [PubMed]
6. Nakashima, K.; Yamaguchi-Shinozaki, K.; Shinozaki, K. The transcriptional regulatory network in the drought response and its crosstalk in abiotic stress responses including drought, cold, and heat. *Front. Plant Sci.* **2014**, *5*, 170. [CrossRef]
7. Abdelraheem, A.; Esmaeili, N.; O'Connell, M.; Zhang, J. Progress and perspective on drought and salt stress tolerance in cotton. *Ind. Crop. Prod.* **2019**, *130*, 118–129. [CrossRef]
8. Leng, P.; Zhao, J. Transcription factors as molecular switches to regulate drought adaptation in maize. *Theor. Appl. Genet.* **2020**, *133*, 1455–1465. [CrossRef]
9. Zhang, D.-F.; Zeng, T.-R.; Liu, X.-Y.; Gao, C.-X.; Li, Y.-X.; Li, C.-H.; Song, Y.-C.; Shi, Y.-S.; Wang, T.-Y.; Li, Y. Transcriptomic profiling of sorghum leaves and roots responsive to drought stress at the seedling stage. *J. Integr. Agr.* **2019**, *18*, 1980–1995. [CrossRef]
10. Yao, T.; Zhang, J.; Xie, M.; Yuan, G.; Tschaplinski, T.J.; Muchero, W.; Chen, J.-G. Transcriptional Regulation of Drought Response in Arabidopsis and Woody Plants. *Fron. Plant Sci.* **2021**, *11*, 572137. [CrossRef]

11. Liang, Y.; Wang, J.; Zheng, J.; Gong, Z.; Li, Z.; Ai, X.; Li, X.; Chen, Q. Genome-Wide Comparative Analysis of Heat Shock Transcription Factors Provides Novel Insights for Evolutionary History and Expression Characterization in Cotton Diploid and Tetraploid Genomes. *Front. Genet.* **2021**, *12*, 658847. [CrossRef] [PubMed]
12. Zhou, M.; Zheng, S.; Liu, R.; Lu, J.; Lu, L.; Zhang, C.; Liu, Z.; Luo, C.; Zhang, L.; Yant, L.; et al. Genome-wide identification, phylogenetic and expression analysis of the heat shock transcription factor family in bread wheat (*Triticum aestivum* L.). *BMC Genomics.* **2019**, *20*, 505. [CrossRef] [PubMed]
13. Yu, Y.; Yu, M.; Zhang, S.; Song, T.; Zhang, M.; Zhou, H.; Wang, Y.; Xiang, J.; Zhang, X. Transcriptomic Identification of Wheat AP2/ERF Transcription Factors and Functional Characterization of TaERF-6-3A in Response to Drought and Salinity Stresses. *Int. J. Mol. Sci.* **2022**, *23*, 3272. [CrossRef] [PubMed]
14. Li, W.; Wan, X.-L.; Yu, J.-Y.; Wang, K.-L.; Zhang, J. Genome-Wide Identification, Classification, and Expression Analysis of the Hsf Gene Family in Carnation (*Dianthus caryophyllus*). *Int. J. Mol. Sci.* **2019**, *20*, 5233. [CrossRef]
15. Liu, X.; Meng, P.; Yang, G.; Zhang, M.; Peng, S.; Zhai, M.Z. Genome-wide identification and transcript profiles of walnut heat stress transcription factor involved in abiotic stress. *BMC Genom.* **2020**, *21*, 474. [CrossRef]
16. Chen, X.; Wang, Z.; Tang, R.; Wang, L.; Chen, C.; Ren, Z. Genome-wide identification and expression analysis of Hsf and Hsp gene families in cucumber (*Cucumis sativus* L.). *Plant Growth Regul.* **2021**, *95*, 223–239. [CrossRef]
17. Zhang, Y.; Wang, C.; Wang, C.; Yun, L.; Song, L.; Idrees, M.; Liu, H.; Zhang, Q.; Yang, J.; Zheng, X.; et al. OsHsfB4b Confers Enhanced Drought Tolerance in Transgenic Arabidopsis and Rice. *Int. J. Mol. Sci.* **2022**, *23*, 10830. [CrossRef]
18. Song, X.; Liu, G.; Duan, W.; Liu, T.; Huang, Z.; Ren, J.; Li, Y.; Hou, X. Genome-wide identification, classification and expression analysis of the heat shock transcription factor family in Chinese cabbage. *Mol. Genet. Genom.* **2014**, *289*, 541–551. [CrossRef]
19. Lal, S.V.; Brahma, B.; Gohain, M.; Mohanta, D.; De, B.C.; Chopra, M.; Dass, G.; Vats, A.; Upadhyay, R.C.; Datta, T.K.; et al. Splice variants and seasonal expression of buffalo HSF genes. *Cell Stress Chaperones* **2015**, *20*, 545–554. [CrossRef]
20. Chen, J.; Gao, T.; Wan, S.; Zhang, Y.; Yang, J.; Yu, Y.; Wang, W. Genome-Wide Identification, Classification and Expression Analysis of the HSP Gene Superfamily in Tea Plant (*Camellia sinensis*). *Int. J. Mol. Sci.* **2018**, *19*, 2633. [CrossRef]
21. Jiang, L.; Hu, W.; Qian, Y.; Ren, Q.; Zhang, J. Genome-wide identification, classification and expression analysis of the Hsf and Hsp70 gene families in maize. *Gene* **2021**, *770*, 145348. [CrossRef] [PubMed]
22. Andrási, N.; Pettkó-Szandtner, A.; Szabados, L. Diversity of plant heat shock factors: Regulation, interactions, and functions. *J. Exp. Bot.* **2020**, *72*, 1558–1575. [CrossRef] [PubMed]
23. Jahan, K.; Nie, H.; Yan, X. Revealing the potential regulatory relationship between *HSP70*, *HSP90* and *HSF* genes under temperature stress. *Fish Shellfish Immunol.* **2023**, *134*, 108607. [CrossRef] [PubMed]
24. Lin, L.; Wang, J.; Wang, Q.; Ji, M.; Hong, S.; Shang, L.; Zhang, G.; Zhao, Y.; Ma, Q.; Gu, C. Transcriptome Approach Reveals the Response Mechanism of *Heimia myrtifolia* (Lythraceae, Myrtales) to Drought Stress. *Fron. Plant Sci.* **2022**, *13*, 877913. [CrossRef]
25. Giorno, F.; Guerriero, G.; Baric, S.; Mariani, C. Heat shock transcriptional factors in Malus domestica: Identification, classification and expression analysis. *BMC Genom.* **2012**, *13*, 639. [CrossRef]
26. Huang, B.; Huang, Z.; Ma, R.; Chen, J.; Zhang, Z.; Yrjälä, K. Genome-wide identification and analysis of the heat shock transcription factor family in moso bamboo (*Phyllostachys edulis*). *Sci. Rep.* **2021**, *11*, 16492. [CrossRef]
27. Zhang, H.; Li, G.; Fu, C.; Duan, S.; Hu, D.; Guo, X. Genome-wide identification, transcriptome analysis and alternative splicing events of Hsf family genes in maize. *Sci. Rep.* **2020**, *10*, 8073. [CrossRef]
28. Liu, G.; Chai, F.; Wang, Y.; Jiang, J.; Duan, W.; Wang, Y.; Wang, F.; Li, S.; Wang, L. Genome-wide Identification and Classification of HSF Family in Grape, and Their Transcriptional Analysis under Heat Acclimation and Heat Stress. *Hortic. Plant J.* **2018**, *4*, 133–143. [CrossRef]
29. MMallick, B.; Kumari, M.; Pradhan, S.K.; Acharya, G.C.; Naresh, P.; Das, B.; Shashankar, P. Genome-wide analysis and characterization of heat shock transcription factors (Hsfs) in common bean (*Phaseolus vulgaris* L.). *Funct. Integr. Genom.* **2022**, *22*, 743–756. [CrossRef]
30. Huang, Y.; Li, M.-Y.; Wang, F.; Xu, Z.-S.; Huang, W.; Wang, G.-L.; Ma, J.; Xiong, A.-S. Heat shock factors in carrot: Genome-wide identification, classification, and expression profiles response to abiotic stress. *Mol. Biol. Rep.* **2015**, *42*, 893–905. [CrossRef]
31. Liu, Z.-W.; Wu, Z.-J.; Li, X.-H.; Huang, Y.; Li, H.; Wang, Y.-X.; Zhuang, J. Identification, classification, and expression profiles of heat shock transcription factors in tea plant (*Camellia sinensis*) under temperature stress. *Gene* **2016**, *576*, 52–59. [CrossRef] [PubMed]
32. Chidambaranathan, P.; Jagannadham, P.T.K.; Satheesh, V.; Kohli, D.; Basavarajappa, S.H.; Chellapilla, B.; Kumar, J.; Jain, P.K.; Srinivasan, R. Genome-wide analysis identifies chickpea (*Cicer arietinum*) heat stress transcription factors (Hsfs) responsive to heat stress at the pod development stage. *J. Plant Res.* **2018**, *131*, 525–542. [CrossRef] [PubMed]
33. Kumar, R.R.; Goswami, S.; Singh, K.; Dubey, K.; Rai, G.K.; Singh, B.; Singh, S.; Grover, M.; Mishra, D.; Kumar, S.; et al. Characterization of novel heat-responsive transcription factor (TaHSFA6e) gene involved in regulation of heat shock proteins (HSPs)—A key member of heat stress-tolerance network of wheat. *J. Biotechnol.* **2018**, *279*, 1–12. [CrossRef] [PubMed]
34. Bin, J.; Zhu, M.; Ding, H.; Zai, Z.; Shi, T.; Wang, L.; Yang, X.; Yue, Y. New Insights into the Roles of Osmanthus Fragrans Heat-Shock Transcription Factors in Cold and Other Stress Responses. *Horticulturae* **2022**, *8*, 80. [CrossRef]
35. Xu, P.; Guo, Q.; Pang, X.; Zhang, P.; Kong, D.; Liu, J. New Insights into Evolution of Plant Heat Shock Factors (Hsfs) and Expression Analysis of Tea Genes in Response to Abiotic Stresses. *Plants* **2020**, *9*, 311. [CrossRef]

36. Shyamli, P.S.; Pradhan, S.; Panda, M.; Parida, A. De novo Whole-Genome Assembly of Moringa oleifera Helps Identify Genes Regulating Drought Stress Tolerance. *Fron. Plant Sci.* **2021**, *12*, 766999. [CrossRef]
37. Li, P.-S.; Yu, T.-F.; He, G.-H.; Chen, M.; Zhou, Y.-B.; Chai, S.-C.; Xu, Z.-S.; Ma, Y.-Z. Genome-wide analysis of the Hsf family in soybean and functional identification of GmHsf-34 involvement in drought and heat stresses. *BMC Genom.* **2014**, *15*, 1009. [CrossRef]
38. Ma, J.; Xu, Z.-S.; Wang, F.; Tan, G.-F.; Li, M.-Y.; Xiong, A.-S. Genome-wide analysis of HSF family transcription factors and their responses to abiotic stresses in two Chinese cabbage varieties. *Acta Physiol. Plant.* **2014**, *36*, 513–523. [CrossRef]
39. Ma, G.; Shen, J.; Yu, H.; Huang, X.; Deng, X.; Hu, Z.; Amee, M.; Chen, L.; Cao, L. Genome-wide identification and functional analyses of heat shock transcription factors involved in heat and drought stresses in ryegrass. *Environ. Exp. Bot.* **2022**, *201*, 104968. [CrossRef]
40. Liu, M.; Huang, Q.; Sun, W.; Ma, Z.; Huang, L.; Wu, Q.; Tang, Z.; Bu, T.; Li, C.; Chen, H. Genome-wide investigation of the heat shock transcription factor (Hsf) gene family in Tartary buckwheat (*Fagopyrum tataricum*). *BMC Genom.* **2019**, *20*, 871. [CrossRef]
41. Gong, C.; Pang, Q.; Li, Z.; Li, Z.; Chen, R.; Sun, G.; Sun, B. Genome-Wide Identification and Characterization of Hsf and Hsp Gene Families and Gene Expression Analysis under Heat Stress in Eggplant (*Solanum melongema* L.). *Horticulturae* **2021**, *7*, 149. [CrossRef]
42. Chen, C.; Chen, H.; Zhang, Y.; Thomas, H.R.; Xia, R. TBtools: An Integrative Toolkit Developed for Interactive Analyses of Big Biological Data. *Mol. Plant* **2020**, *13*, 1194–1202. [CrossRef] [PubMed]

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