Genome-Wide Identification and Expression Analysis of HSF Transcription Factors in Alfalfa (Medicago sativa) under Abiotic Stress

Jin Ma 1,2,3,†, Guozhe Zhang 1,2,3,†, Yacheng Ye 1,2,3,†, Linxue Shang 1,2,3, Sidan Hong 1,2,3, Qingqing Ma 1,2,3, Yu Zhao 1,2,3,* and Cuihua Gu 1,2,3,*†

1 College of Landscape and Architecture, Zhejiang Agriculture & Forestry University, Hangzhou 311300, China
2 Zhejiang Provincial Key Laboratory of Germplasm Innovation and Utilization for Garden Plants, Zhejiang Agriculture & Forestry University, Hangzhou 311300, China
3 Key Laboratory of National Forestry and Grassland Administration on Germplasm Innovation and Utilization for Southern Garden Plants, Zhejiang Agriculture & Forestry University, Hangzhou 311300, China

* Correspondence: zhaoyu@stu.zafu.edu.cn (Y.Z.); gucuihua@zafu.edu.cn (C.G.)
† These authors contributed equally to this work.

Abstract: Alfalfa (Medicago sativa) is one of the most important legume forage species in the world. It is often affected by several abiotic stressors that result in reduced yields and poor growth. Therefore, it is crucial to study the resistance of M. sativa to abiotic stresses. Heat shock transcription factors (HSF) are key players in a number of transcriptional regulatory pathways. These pathways play an essential role in controlling how plants react to different abiotic stressors. Studies on the HSF gene family have been reported in many species but have not yet undergone a thorough analysis in M. sativa. Therefore, in order to identify a more comprehensive set of HSF genes, from the genomic data, we identified 16 members of the MsHSF gene, which were unevenly distributed over six chromosomes. We also looked at their gene architectures and protein motifs, and phylogenetic analysis allowed us to divide them into 3 groups with a total of 15 subgroups. Along with these aspects, we then examined the physicochemical properties, subcellular localization, synteny analysis, GO annotation and enrichment, and protein interaction networks of amino acids. Finally, the analysis of 16 MsHSF genes’ expression levels across all tissues and under four abiotic stresses using publicly available RNA-Seq data revealed that these genes had significant tissue-specific expression. Moreover, the expression of most MsHSF genes increased dramatically under abiotic stress, further validating the critical function played by the MsHSF gene family in abiotic stress. These results provided basic information about MsHSF gene family and laid a foundation for further study on the biological role of MsHSF gene in response to stress in M. sativa.

Keywords: Medicago sativa; HSF gene family; expression profile; abiotic stress

1. Introduction

Normal plant growth is often affected by a variety of adverse environmental factors such as drought, high salinity, temperature extremes and other abiotic stresses [1]. Abiotic stress inhibits normal plant growth, development, and function by speeding up chlorophyll degradation, disrupting chloroplast membrane activities, and decreasing photosynthetic efficiency, as shown by a number of studies [2–4]. In response to external stimuli, plants’ bodies produce signals that trigger the phosphorylation of downstream proteins, which in turn trigger a number of transcription factors [5]. Ultimately, plant-associated resistance genes and associated defense systems are induced, thus altering the ability of the plant to adapt to its environment [6–8].

Plants have developed multiple defense mechanisms and strategies to cope with adverse conditions and respond accordingly [9–11]. Under abiotic stress, induction of numerous proteins, including transcription factors (TF), can regulate the expression of specific
functional genes and enhance plant resistance through signal transduction pathways [12]. Reactive oxygen species (ROS) scavenger enzymes and HSP are important functional proteins induced by HS, and their corresponding genes are targets of several HS-responsive TFs [13]. Previous studies have shown that HSFA6b is essential in *Arabidopsis thaliana* as a downstream regulator of the ABA-mediated heat stress response (HSR) [14,15].

HSF is a class of transcription factors that are widely present in eukaryotes [16]. Since the first HSF gene was isolated from *Solanum lycopersicum* in 1990, HSF has been reported in *A. thaliana*, *Oryza sativa*, *Glycine max* and other plants with the continuous improvement of genome sequencing technology [17–21]. The number of HSF gene family members varies widely among plants, with the highest number in *Triticum aestivum* containing 56 [22]. *G. max* [23], *Zea mays* [24], and *A. thaliana* [25] contained 52, 30, and 21, respectively. HSF plays a crucial role in the transmission and receipt of signals, the detection of heat shock components, the regulation of downstream genes, and the induction of plant stress responses [26]. The important role of HSF in plant responses to abiotic stresses can be well established.

Alfalfa (*Medicago sativa*) is one of the most economically valuable crops in the world and the most widely farmed legume fodder grass [27]. However, as it matures and flourishes, *M. sativa* is regularly damaged by a number of abiotic factors, such as salinity, cold and drought, which have a detrimental effect on *M. sativa* quality and output [28–31]. Therefore, it is particularly important to breed *M. sativa* germplasm resources with high resistance to stress. HSF is a class of transcription factors that are widely found in eukaryotes. They are crucial in the transmission and receipt of signals, the identification of heat shock components and the control of downstream genes, and the induction of plant responses to abiotic stresses [26,32–34]. Studies on the HSF gene family have been reported in many species but have not yet undergone a thorough analysis in *M. sativa*. Therefore, in this study, we identified the MsHSF family gene in *M. sativa* by integrating conserved motifs, gene structure, chromosome mapping, promoter cis-elements, and their phylogenetic relationships, and we analyzed the expression of HSF genes in *M. sativa* under four abiotic stresses. These results will deepen our current understanding of the evolutionary relationships and functional differentiation of *M. sativa* HSF genes and provide valuable information for further studies on the role of HSF genes in *M. sativa* for resistance under abiotic stresses.

2. Results

2.1. Identification of HSF Genes in *M. sativa*

In this study, we used the *A. thaliana* HSF protein (AtHSF) as a query to search the *M. sativa* genome database for 25 MsHSF candidate genes. We then used the HMMER3 search to search the *M. sativa* genome database using the HSF-type DBD model to retrieve 22 putative MsHSF candidate genes (PF00447). Finally, we used SMART and NCBI conserved structural domains to delete duplicate genes and proteins without DBD conserved structural domains to obtain 16 MsHSF family members. Based on these genes’ chromosomal positions, they were given the new names *MsHSF01–MsHSF16*.

The predicted physicochemical properties of the amino acid sequences indicated that the 16 HSF genes encode proteins containing 211 (MsHSF06) to 543 (MsHSF09) amino acids with molecular weights (MWs) ranging from 24,541.86 (MsHSF06) to 60,982.41 Da (MsHSF06), with an average molecular weight of 42,922.38 Da. The predicted isoelectric points (pI) ranged from 4.72 (MsHSF05) to 8.16 (MsHSF06), with a mean value of 5.88. Instability index calculations predicted that 14 (87.5%) of the HSF proteins were unstable in vitro, and only MsHSF08 and MsHSF12 predicted proteins with an instability index of less than 40 were classified as stable proteins. In addition, the aliphatic amino acid index (A.I.) ranged from 62.79 (MsHSF08) to 83.61 (MsHSF12), indicating that their thermal stability was less different. The overall mean of the hydrophilic (GRAVY) scores of all HSF proteins was negative, indicating that they are all hydrophilic proteins.
subcellular localization predictions showed that all MsHSF proteins were located in the nucleus (Table 1).

| Protein Name | Gene ID | Chromosome Location | Size (aa) | MW (kDa) | pI | Stability | A.I | GRAVY | Predicted Location |
|--------------|---------|---------------------|-----------|----------|----|-----------|-----|-------|-------------------|
| HSF01        | MsG0180003410.01.T01 | S1:61939996–61942587 | 496       | 55,750.22| 4.91| U         | 69.91| 0.591   | Nuclear           |
| HSF02        | MsG0180003479.01.T01 | S1:92250440–92250760 | 372       | 42,094.06| 8.16| U         | 62.82| 0.715   | Nuclear           |
| HSF03        | MsG0380017281.01.T01 | S3:94430904–94433609 | 381       | 42,838.88| 4.93| U         | 78.5 | 0.547   | Nuclear           |
| HSF04        | MsG0480021749.01.T01 | S4:12500000–1252171 | 480       | 53,424.6 | 4.85| U         | 69.62| 0.55    | Nuclear           |
| HSF05        | MsG0480021648.01.T01 | S4:1504039–72255708  | 671       | 24,541.86| 6.02| U         | 69.72| 0.708   | Nuclear           |
| HSF06        | MsG0480022400.01.T01 | S4:96158543–96121035 | 401       | 45,748.67| 4.99| U         | 80.7 | 0.498   | Nuclear           |
| HSF07        | MsG0480023951.01.T01 | S6:41477418–41479676 | 295       | 33,653.64| 5.66| U         | 68.14| 0.802   | Nuclear           |
| HSF08        | MsG0580024862.01.T01 | S6:94077724–9408033 | 287       | 32,169.91| 6.9 | U         | 62.79| 0.784   | Nuclear           |
| HSF09        | MsG0580025495.01.T01 | S6:19600912–19606188 | 543       | 60,982.41| 5.1 | U         | 71.47| 0.624   | Nuclear           |
| HSF10        | MsG0680032444.01.T01 | S7:41477418–41479676 | 301       | 33,653.64| 5.66| U         | 68.14| 0.802   | Nuclear           |
| HSF11        | MsG0680032449.01.T01 | S7:41477418–41479676 | 319       | 36,439.77| 5.27| U         | 70.34| 0.717   | Nuclear           |
| HSF12        | MsG0680032451.01.T01 | S7:41477418–41479676 | 401       | 45,748.67| 4.99| U         | 80.7 | 0.498   | Nuclear           |
| HSF13        | MsG0680033612.01.T01 | S7:41477418–41479676 | 480       | 53,424.6 | 4.85| U         | 69.62| 0.55    | Nuclear           |
| HSF14        | MsG0680035659.01.T01 | S7:41477418–41479676 | 301       | 33,653.64| 5.66| U         | 68.14| 0.802   | Nuclear           |
| HSF15        | MsG0780040480.01.T01 | S7:41477418–41479676 | 401       | 45,748.67| 4.99| U         | 80.7 | 0.498   | Nuclear           |
| HSF16        | MsG0780040676.01.T01 | S7:41477418–41479676 | 480       | 53,424.6 | 4.85| U         | 69.62| 0.55    | Nuclear           |

2.2. Phylogenetic Analysis of HSF Genes in M. sativa

We created a NJ phylogenetic tree using MEGA6.0 for the amino acid sequences of 16 M. sativa HSF, 21 A. thaliana HSF, and 25 O. sativa HSF in order to study the evolutionary relationships of MsHSF genes (Figure 1). Based on the well-established A. thaliana family classification, HSF are clearly classified into three groups, namely HSF A (green), HSF B (yellow) and HSF C (blue). Members of MsHSFs were identified in all three groups. The largest was MsHSF A, which made up 52.2% of the overall MsHSF, which was broken down into 9 subgroups (A1–A9). HSF A consists of nine proteins, namely MsHSF01, MsHSF04, MsHSF05, MsHSF07, MsHSF09, MsHSF10, MsHSF11, MsHSF12 and MsHSF16. MsHSF proteins were not aggregated into the three subgroups, A4, A7 and A9. MsHSF B was divided into 5 subgroups (B1–B5), accounting for 39.1%, and consisted of 6 members (MsHSF02, MsHSF03, MsHSF06, MsHSF08, MsHSF13, and MsHSF15). MsHSF C was the smallest group. Also, it contained only one MsHSF14. M. sativa HSF proteins clustered more closely with A. thaliana HSF proteins, according to the phylogenetic tree, which is interesting. This finding shows that the HSF proteins of dicotyledons and monocotyledons have distinct evolutionary differences.

2.3. Multiple Sequence Alignment and Protein Modeling Analysis of the HSF Gene in M. sativa

To check for the presence and position of conserved protein structural domains, we used Jalview to perform a multiple sequence alignment on 16 MsHSF proteins. We found that the DBD structural domain, which contains roughly 100 amino acids, is substantially conserved among all members of the MsHSF family (Figure 2). The DBD structural domain has three helix bundles (1–3) and four reverse parallel folds, as predicted from protein secondary structure (1–4). Additionally, we predicted the protein 3D structure of HSF of M. sativa and labeled the starting position of the 1 DBD structural domain in the figure (Figure 3).

2.4. Gene Structure and Conserved Motif Analysis of the HSF Gene in M. sativa

HSF proteins were split into three categories, HSF A, HSF B, and HSF C, based on the phylogenetic analyses mentioned above (Figure 4A). Our analysis of the MsHSF gene structure revealed that HSF genes from the same group typically have a similar number of introns in their structures. There is just one intron and two exons shared by all HSB and HSCF genes in M. sativa (Figure 4B). Except for MsHSF12, which had eight introns, the M. sativa HSF A genes ranged in intron count from one to four. Three genes included two introns; three genes (MsHSF01, MsHSF07) contained four introns; two genes (MsHSF10, MsHSF11) contained one intron. MsHSF09 and MsHSF016 contain four introns.
(MsHSF02, MsHSF03, MsHSF06, MsHSF08, MsHSF13, and MsHSF15). MsHSF C was the smallest group. Also, it contained only one MsHSF14.

M. sativa HSF proteins clustered more closely with A. thaliana HSF proteins, according to the phylogenetic tree, which is interesting. This finding shows that the HSF proteins of dicotyledons and monocotyledons have distinct evolutionary differences.

Figure 1. Model for phylogenetic analysis of HSF in M. sativa. Each subgroup is distinguished by a different color.

2.3. Multiple Sequence Alignment and Protein Modeling Analysis of the HSF Gene in M. sativa

To check for the presence and position of conserved protein structural domains, we used Jalview to perform a multiple sequence alignment on 16 MsHSF proteins. We found that the DBD structural domain, which contains roughly 100 amino acids, is substantially conserved among all members of the MsHSF family (Figure 2). The DBD structural domain has three helix bundles (1–3) and four reverse parallel folds, as predicted from protein secondary structure (1–4). Additionally, we predicted the protein 3D structure of HSF of M. sativa and labeled the starting position of the 1 DBD structural domain in the figure (Figure 3).

Figure 2. Conserved domain alignment of MsHSF members.

We also used MEME to identify up to 10 highly conserved motifs in each HSF protein (Figure 4C). The results show that the relative positions of most of the sequence motifs of the same group are similar. All MsHSF contain motifs 1 and 3, which constitute the most highly conserved part of the DBD, and the absence of motif 2 in some genes indicates that motif 2 is not necessary in the highly conserved part of the DBD. Motifs 7 and 9 were found only in HSF A, and motif 8 was present only in HSF B. These results suggest that the biological functions of the MsHSF proteins that are grouped together may be similar, and that different patterns may be related to different functions in separate subgroups.

Figure 3. Three-dimensional model of the protein of MsHSF family members, with the starting position of the $\alpha 1$ DBD structural domain indicated by 1. A–P in the figure represent the 16 proteins of the M. sativa HSF family, respectively.

2.4. Gene Structure and Conserved Motif Analysis of the HSF Gene in M. sativa

HSF proteins were split into three categories, HSF A, HSF B, and HSF C, based on the phylogenetic analyses mentioned above (Figure 4A). Our analysis of the MsHSF gene structure revealed that HSF genes from the same group typically have a similar number of...
2.4. Gene Structure and Conserved Motif Analysis of the HSF Gene in M. sativa

HSF proteins were split into three categories, HSF A, HSF B, and HSF C, based on the phylogenetic analyses mentioned above (Figure 4A). Our analysis of the MsHSF gene structure revealed that HSF genes from the same group typically have a similar number of

2.5. Chromosome Distribution and Covariance Analysis of HSF Genes in M. sativa

M. sativa HSF genes were unevenly distributed on the six chromosomes (Figure 5). This included chromosomes 1, 3, 4, 5, 6, and 7, with the largest number of genes found on chromosome 6 with 5 genes, followed by 3 genes found on chromosome 4. All the remaining chromosomes contained only 2 MsHSF genes each.
introns in their structures. There is just one intron and two exons shared by all HSFB and HSFC genes in *M. sativa* (Figure 4B). Except for *MsHSF12*, which had eight introns, the *M. sativa* HSF genes ranged in intron count from one to four. Three genes included two introns; three genes (*MsHSF01, MsHSF07*) contained four introns; two genes (*MsHSF10, MsHSF11*) contained one intron. *MsHSF09* and *MsHSF016* contain four introns.

We also used MEME to identify up to 10 highly conserved motifs in each HSF protein (Figure 4C). The results show that the relative positions of most of the sequence motifs of the same group are similar. All *MsHSF* contain motifs 1 and 3, which constitute the most highly conserved part of the DBD, and the absence of motif 2 in some genes indicates that motif 2 is not necessary in the highly conserved part of the DBD. Motifs 7 and 9 were found only in HSF A, and motif 8 was present only in HSF B. These results suggest that the biological functions of the *MsHSF* proteins that are grouped together may be similar, and that different patterns may be related to different functions in separate subgroups.

Figure 4. Phylogenetic relationship tree (A), gene structure (B) and conserved patterns (C) of HSF in *M. sativa*.

Figure 5. Distribution of *MsHSF* genes on the chromosomal scaffolds of *M. sativa*.
Gene duplication events are common in all species; they can generate new functional genes and drive species evolution. Therefore, we used MCScanX genomic homozygosity analysis to explore duplications in the *M. sativa* HSF gene family (Figure 6). Three (*MsHSF10, MsHSF11*, and *MsHSF12*) tandem duplicated genes and two pairs (*MsHSF01, MsHSF02, MsHSF15*, and *MsHSF16*) of codominant genes were detected in the MsHSF family.

Additionally, the proportion of HSF genes shared by *M. sativa* and other species may reflect the evolutionary relationships of the HSF gene family among *M. sativa*, dicotyledons, and monocotyledons. Therefore, in this study, we constructed a comparative homozygous map of three HSF genes from *M. sativa* and three dicots (*A. thaliana, G. max*, and *Medicago truncatula*) and one monocot (*Z. mays*) (Figure 7). A total of 44 direct homologous pairs (including one *M. sativa* gene corresponding to more than one *G. max* gene) were studied between *M. sativa* and *G. max* HSF genes. 20, 11, and 7 pairs of direct homologous genes were presented in *M. truncatula*, *A. thaliana*, and *Z. mays*, respectively. During evolution, most of the HSF genes of *M. sativa* have more than two direct homologs in *A. thaliana*, which further suggests that *M. sativa* has experienced more whole gene duplication events.
between *M. sativa* and *G. max* HSF genes. 20, 11, and 7 pairs of direct homologous genes were presented in *M. truncatula*, *A. thaliana*, and *Z. mays*, respectively. During evolution, most of the HSF genes of *M. sativa* have more than two direct homologs in *A. thaliana*, which further suggests that *M. sativa* has experienced more whole gene duplication events.

![Figure 7. Synthetic analysis of the *M. sativa* genome with the genomes of one monocotyledon and three dicotyledon plants. Gray lines represent alignment blocks between paired genomes, and blue lines indicate synthetic HSF gene pairs.](image)

### 2.6. Analysis of the Promoter Cis-Element of the HSF Gene in *M. sativa*

In order to learn more about how the **MsHSF** gene is regulated, this study examined the cis-acting components of the promoter region. We searched the PlantCARE database for potential cis-acting elements in the 2000 bp upstream sequence of the MsHSF translation start codon (Figure 8). As a result, most are stress response elements, and all promoters contain at least one TATA-box, CAAT-box, and short_function element. The next most common element is the ABRE element (contained in all promoters except MsHSF11), which is associated with the abscisic acid response. Other elements present in MsHSF promoters include the MBS element (involved in drought, high salt, and low temperature responses), TC-rich repeat sequences (involved in defense and stress responses), AE-box, and TGA erythromycin response elements. These findings suggest that MsHSF genes may be involved in multiple transcriptional regulatory mechanisms of plant growth and stress responses.

### 2.7. Expression Profiling of the *M. sativa* HSF Gene in Different Tissues

To determine the expression patterns of individual MsHSF genes in various tissues, a hierarchical clustering heat map was also constructed from public RNA-seq data obtained from NCBI for exploring the transcriptional patterns of MsHSF genes in this study (Figure 9). The blue color in the graph indicates low transcript abundance and the red color indicates high transcript abundance. The analysis showed that there were different HSF genes showing a trend of high expression in six tissues of *M. sativa*. MsHSF06 and MsHSF15 were highly expressed in the roots. The expression of each gene was not significant in the root nodules. In pre-elongating stems, MsHSF05, MsHSF09 and MsHSF14 showed higher expression. However, the gene with higher expression in elongating stems was MsHSF03. In *M. sativa* leaves, MsHSF07, MsHSF08 and MsHSF16 showed higher expression. Interestingly, MsHSF10 was not expressed in any other tissue parts of alfalfa but was expressed in the flowers, a phenomenon that suggests that different MsHSF genes have more obvious specificity in different tissues.
Figure 8. A schematic representation of the cis-acting elements identified in the 2000 bp promoter region upstream of the \( MsHSF \) gene. The different colors represent the number of cis-acting elements contained (A). The number of \( MsHSF \) genes corresponding to the cis-acting element (B).

2.8. Expression Analysis of HSF Genes in \( M. \) sativa in Response to Abiotic Stresses

To investigate the expression levels of MsHSF genes under abiotic stress, we analyzed the expression patterns of MsHSF genes under treatment with cold stress, abscisic acid (ABA), drought, and salt using published transcriptome data (Figure 10). The results of the analysis showed that the genes that functioned in \( M. \) sativa under different stress treatments were slightly different compared to the control. However, the genes that appeared to be the first to function and to be highly expressed in the immediate period of abiotic stress were \( MsHSF09 \) and \( MsHSF15 \), while \( MsHSF07 \) and \( MsHSF08 \) gradually became more highly expressed as the time of stress increased to counteract the external stimuli. By the later stages of stress, it is \( MsHSF04 \), \( MsHSF05 \) and \( MsHSF13 \) that play a role. Interestingly, most of the MsHSF genes were induced to increase in response to abiotic stress, except for \( MsHSF02 \) and \( MsHSF16 \), which were suppressed in response to cold stress, which may be related to cellular trauma in \( M. \) sativa during cold stress.
Figure 9. A heat map representation of MsHSF expression between different tissues. The values in the rectangle represent the magnitude of the gene expression.

2.8. Expression Analysis of HSF Genes in M. sativa in Response to Abiotic Stresses

To investigate the expression levels of MsHSF genes under abiotic stress, we analyzed the expression patterns of MsHSF genes under treatment with cold stress, abscisic acid (ABA), drought, and salt using published transcriptome data (Figure 10). The results of the analysis showed that the genes that functioned in M. sativa under different stress treatments were slightly different compared to the control. However, the genes that appeared to be the first to function and to be highly expressed in the immediate period of abiotic stress were MsHSF09 and MsHSF15, while MsHSF07 and MsHSF08 gradually became more highly expressed as the time of stress increased to counteract the external stimuli. By the later stages of stress, it is MsHSF04, MsHSF05 and MsHSF13 that play a role. Interestingly, most of the MsHSF genes were induced to increase in response to abiotic stress, except for MsHSF02 and MsHSF16, which were suppressed in response to cold stress, which may be related to cellular trauma in M. sativa during cold stress.

2.9. GO Annotation and Enrichment Analysis of M. sativa HSF Protein

Plants have evolved complex mechanisms to sense and respond to biotic and abiotic stresses, and HSF is an important component of these defense systems. We carried out GO annotation and enrichment analysis on 16 MsHSF proteins in order to learn more about the biological functions of this protein (Figure 11). MsHSF was enriched for 55 biological processes, 1 cellular component, and 2 molecular functions in comparison to the entire GO database. According to the GO enrichment data, MsHSF transcription factors are primarily involved in biological processes including responding to abiotic stimuli, responding to temperature stimuli, responding to heat, and responding to xenobiotic stimuli. The findings again suggest that HSF genes play an extremely important role in resisting abiotic stresses.
Plants 2022, 11, x FOR PEER REVIEW 11 of 19

Figure 10. Expression of 16 MsHSF genes in cold (A), drought (B), ABA (C), and salt (D) treatments. The values in the rectangle represent the magnitude of the gene expression.

2.10. Interaction Network Analysis of HSF Proteins in M. sativa

We predicted probable interactions between MsHSF proteins using the STRING database in order to better comprehend MsHSF protein interactions (Figure 12). The findings demonstrate that while certain proteins, like MsHSF10 and MsHSF16, exhibit direct connections, others, including MsHSF10, MsHSF16, and MsHSF08, exhibit more complex multigene interactions. Where it is projected that the major nodes MsHSF01, MsHSF08, and MsHSF13 radiate a significant number of connections to additional nodes.
2.10. Interaction Network Analysis of HSF Proteins in M. sativa

We predicted probable interactions between MsHSF proteins using the STRING database in order to better comprehend MsHSF protein interactions (Figure 12). The findings demonstrate that while certain proteins, like MsHSF10 and MsHSF16, exhibit direct connections, others, including MsHSF10, MsHSF16, and MsHSF08, exhibit more complex multigene interactions. It is predicted that the major nodes MsHSF01, MsHSF08, and MsHSF13 radiate a significant number of connections to additional nodes.

Figure 12. Interaction network of HSF proteins in M. sativa. Nodes represent proteins; central nodes are indicated in blue, and black lines indicate interactions between nodes.

3. Discussion

3.1. The Characteristics of HSF Gene Family in M. sativa

HSF is a particular sort of transcription factor that is crucial for plants’ ability to resist diverse stressors [35]. The highly conserved plant HSF DBD is located at the N-terminal end where it is able to precisely locate and recognize the heat stress element (HSE) in the promoter of the target gene [36–38]. All MsHSF proteins comprised DBD with three helices and four folds, according to multiple sequence alignment and secondary structure prediction (Figure 2). It’s interesting to note that some MsHSF proteins also have additional conserved structural domains; further experimental confirmation is required to determine whether this is a sign of the gene family’s functional diversification. Tertiary structural
analysis showed that the portion of transcription factors interacting with nucleic acids is conserved in the subfamily. HSF B and HSF C members may not have transcriptional activation because they lack AHA motifs, which is consistent with the results of previous studies [39–42].

Similar gene architectures and conserved protein motifs among members of the same phylogenetic group typically indicate a tight phylogenetic relationship [43]. Short sequences involved in significant biological processes are typically referred to as motif [44]. The presence of Motifs 1–2 in every MsHSF raises the possibility that they may have significant biological roles, however this has not yet been established. Because motif 9 is specific to the HSF A subgroup, it is possible that the HSF genes in this subgroup perform a particular role. Additionally, we discovered that the majority of MsHSF share exon-intron architectures and motif distributions within the same evolutionary tree grouping, indicating that genes within a subfamily frequently have comparable biological activities.

Only 16 HSF genes have been found in *M. sativa*, which is less than other plant species and may reflect the lack of expansion of the MsHSF family. MsHSF genes might be further grouped into three categories using homology matching and multispecies matching: A, B, and C. They are clustered in the same way as the members of the *A. thaliana* HSF gene family, with group A having the most genes and group C having the fewest [26]. Three subgroups, A4, A7, and A9, are missing from *M. sativa*, demonstrating that despite HSF family proteins sharing a common ancestor, they have evolved separately in different species. The majority of the alfalfa HSF proteins grouped with the *A. thaliana* HSF proteins but not with the *O. sativa* HSF proteins, suggesting that MsHSF and AtHSF have a tight evolutionary relationship. The HSF proteins of dicotyledons and monocotyledons have evolved in quite different ways.

Gene duplication events have a big impact on how gene families are formed. By supplying the necessary building blocks for the creation of new genes, gene duplication aids in the development of new, functional genes [45]. The majority of gene duplication occurs as tandem and fragmental duplication [46]. The 16 MsHSF genes in the *M. sativa* genome contained four homologous gene pairs, all of which underwent WGD (whole genome duplication) or fragmental duplication events and intense purifying selection pressure. These findings imply that WGD or fragmental replication is essential for MsHSF gene amplification.

Cis-acting elements are nucleotide sequences found upstream or downstream of genes that regulate their transcriptional levels [47]. When plants react to numerous developmental processes and stressors, they work by binding to certain transcription factors [48]. According to studies, cis-acting elements are present in plant-inducible promoters in response to adverse stress. There are numerous hormone-responsive core promoter elements and binding sites spread across the 16 *M. sativa* HSF promoter regions. According to this, MsHSF might be involved in the communication between several hormone signaling pathways. Aside from the heat stress element, the majority of MsHSF also contained the drought response element MBS, the anaerobic induction response element ARE, and the low temperature response element LTR. This shows that this gene family may control the effects of a variety of abiotic stimuli. These findings imply that MsHSF may interact with hormone signaling pathways that control growth and development as well as stress responses in *M. sativa*.

According to GO enrichment analysis, 15 of the 16 MsHSF genes are involved in two biological processes of GO resistance to abiotic stress and synthesis of abiotic stress factors (Figure 11). Several studies have shown that HSF regulates the expression of stress-related proteins, such as heat shock protein (HSP), which plays an important role in the plant stress response, especially heat stress [49–51]. Therefore, we speculate that the MsHSF gene may play a key role in plant resistance to abiotic stresses.
3.2. The Potential Roles of Differentially Expressed MsHSF Genes

In order to protect plants from heat stress, HSPs can raise the denaturation temperature of their proteins. They can also fix damaged proteins, enabling plants to withstand high temperatures [52]. HSFs play a major role in transcriptionally controlling the expression of HSPs. Additionally, the role of the HSFs signaling pathway encompasses many stresses, including cold, osmosis, drought, and salt, in addition to the response to heat stress [53]. It's interesting to note that osmotic pressures such as drought, salinity disruption, and other stresses result in the buildup of ROS, ABA, and H$_2$O$_2$ as well as alterations to cell walls. Ca$^{2+}$ and ROS are the key factors causing abiotic stress response processes. Therefore, we examined the M. sativa transcriptome under ABA, salt, drought, and low temperature stressors. It was discovered that the expression of a considerable number of HSF genes was elevated under these stressful circumstances. This suggests that these HSF genes may be involved in some processes in the response of plants to external stresses. In conclusion, MsHSF genes are an important class of regulatory genes that control the plant’s growth, development, and response to stress.

HSF plays an important role in the plant’s response to abiotic stresses because it can achieve resistance to abiotic stresses by regulating the expression of different genes [54,55]. Among the HSF genes in plants, HSFA is a major transcriptional activator because it is essential to awaken HSR [56–58]. Although there were some subtle differences in the responses of MsHSF genes to different stresses in this study, the first to play a role in resistance was the MsHSF09 gene in HSFA, which reinforces the important role of HSFA in resistance to abiotic stresses. Unlike HSFA, a considerable number of HSFB and HSFC have not been reported as transcriptional activators, but interestingly, in M. sativa, many genes in HSFB, such as MsHSF07, MsHSF08, and MsHSF15, also play important roles in resistance to abiotic stresses, and based on previous studies and analyses, it is clear that genes in HSFB in alfalfa function as transcriptional co-activators of HSFA. Taken together, several MsHSF genes are differentially expressed under abiotic stresses (including heat, salt, or ABA stress), and these results suggest that they may be involved in plant responses to abiotic stresses.

4. Materials and Methods

4.1. Identification and Sequence Analysis of HSF Genes in M. sativa

HSF protein sequences from the A. thaliana TAIR database (https://www.arabidopsis.org/) (accessed on 7 June 2022) were used as a reference sequence, and members of the putative MsHSF gene were sought using M. sativa genomic data from the National Genomics Data Center (https://ngdc.cnbc.ac.cn/) (accessed on 7 June 2022) and a BLASTP search. We then used the native HMMER 3.0 software (Robert, D.F.; Ashburn, VA, USA) [59], using Hidden Markov (HMM) mapping of the HSF protein (PF0047), downloaded from the pfam database (http://pfam.xfam.org) (accessed on 9 July 2022). The potential gene members of M. sativa HSF identified were pooled using these two search techniques. WebCD-search (https://www.ncbi.nlm.nih.gov/cdd) and SMART (http://smart.embl.de/) were used to identify conserved HSF structural domains in all potential MsHSF genes (accessed on 9 July 2022). We finally identified 16 MsHSF genes and renamed them according to their position on the M. sativa chromosome.

We predicted and examined the physicochemical characteristics of all MsHSF potential proteins, including amino acid numbers, molecular weights, and theoretical sites, through the website ExPasy (https://www.expasy.org/) (accessed on 11 August 2022). Cell-PLoc2.0 was used to create subcellular localizations (http://www.csbio.sjtu.edu.cn/bioinf/plant-multi/#) (accessed on 11 August 2022).

4.2. Construction of Phylogenetic Tree and Sequence Comparison

Whole genome information for A.thaliana and O.sativa was downloaded from the NCBI database (https://www.ncbi.nlm.nih.gov/) (accessed 2 August 2022). Using MUSCLE technology, the 16 MsHSF protein sequences, 21 AtHSF proteins, and 25 OsHSF sequences
found were compared to multiple sequences. The comparison parameters were in multiple comparison mode (other parameters were in default mode), and the obtained comparison results were used to construct a neighbor-joining (NJ) phylogenetic tree that we created in MEGA 7.0 using 1000 bootstrap replications, and the phylogenetic tree was created as an illustration using iTOL ([https://itol.embl.de/](https://itol.embl.de/)) (accessed on 2 August 2022).

Intraspecific classification of M. sativa HSF sequences was performed based on interspecific phylogenetic trees, and amino acid sequences of conserved structural domains were compared and modified using Jalview software 2.11.2.4 (Andrew, M.W.; Cambridge, MA, USA) ([http://www.jalview.org/](http://www.jalview.org/)) (accessed on 2 August 2022), and conserved thematic WebLogo were produced.

We also submitted the Jalview output to SOPMA ([https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_sopma.html](https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_sopma.html)) (accessed on 3 August 2022) for protein secondary structure prediction by using default parameters. For tertiary structure prediction, we completed the analysis via the online website SWISS-MODEL ([https://swissmodel.expasy.org/interactive](https://swissmodel.expasy.org/interactive)) (accessed on 3 August 2022).

4.3. Gene Structure and Motif Identification

The conserved amino acid sequences of HSF proteins were analyzed using the online MEME tool ([https://meme-suite.org/meme/](https://meme-suite.org/meme/)) (accessed on 5 August 2022) with the parameters of minimum width of \( \geq \) for 6, maximum width of 50, and number of parentheses of 10; all other parameters were set to default values. Then we obtained the intron-exon distribution of the MsHSF gene from the M. sativa genome using the GFF annotation method, and finally the results were displayed using TBtools software v1.098661 (Chen, C.J.; Guangzhou, China) [60].

4.4. Chromosome Location and Covariance Analysis

Chromosome lengths and gene positions were obtained from M. sativa genome annotation files, and MG2C v.2 ([http://mg2c.iask.in/mg2c_v2.0/](http://mg2c.iask.in/mg2c_v2.0/)) (accessed on 8 August 2022) was used to visualize gene positions on chromosomes. M. sativa protein sequences were aligned to each other or to those of A. thaliana, G. max, Z. mays, or M. truncatula using TBtools software. MCScanX was used along with default parameters to identify homozygous relationships between gene replication events and HSF proteins, and results were visualized using Circos and Dual Synteny Plot in TBtools.

4.5. Identification of Cis-Acting Elements

A 2000 bp promoter region upstream of the MsHSF transcriptional start site was extracted from the M. sativa genome ([http://www.genoscope.cns.fr/brassicanapus/](http://www.genoscope.cns.fr/brassicanapus/)) (accessed on 25 August 2022) and submitted to PlantCARE ([http://bioinformatics.psb.ugent](http://bioinformatics.psb.ugent)) (accessed on 25 August 2022) to identify the three types of regulatory cis-acting elements and finally visualize the results using TBtools.

4.6. Analysis of Tissue-Specific Expression and Abiotic Stress Transcriptome Data

Relevant transcriptome data were downloaded from the public NCBI database to investigate the expression patterns of MsHSF genes in different tissues and under different biotic stresses. M. sativa RNA-Seq data for different tissues (pre-elongated stems, elongated stems, flowers, leaves, roots, and rhizomes) can be downloaded from accession number SRP055547. The NCBI Short Read Long Archive database contains abiotic stress-related transcriptome data (cold, at SRR7091780-SRR7091794; drought, salt, and ABA, at SRR7160313-SRR7160357). The raw data was filtered and the SRA files were converted to FASTQ files by the SRA to Fastq program of TBtools. Gene expression levels were calculated by fragment number per kilobase per million mapped reads (FPKM values). Finally, the Hetmap program of TBtools was imported to generate the associated heat map.
4.7. Protein Interaction Network Prediction and GO Enrichment Analysis

HSF protein sequences were uploaded to the STRING database (https://string-db.org/) (accessed on 19 August 2022) for node comparison, and relationships between important proteins were predicted based on *A. italiana* protein interactions. Finally, Cytoscape (Shannon, P.; California, USA) [61] was used to visualize the generated networks.

The online software EggNOG-Mapper (http://eggnog-mapper.embl.de/) (accessed on 18 August 2022) was used to annotate the GO function of the MsHSF gene. The results were collated using the eggNOG-mapper Helper function of TBtools and the text files used for downstream analysis were exported separately to GO Enrichment for enrichment analysis. Finally, use the online charting tool HIPlot (https://hiplot.com.cn/) (accessed on 19 August 2022) to view and examine the data.

5. Conclusions

From the genomic information of *M. sativa*, we discovered a total of 16 MsHSF genes in this study. We analyzed the physicochemical properties of the 16 MsHSF proteins and found that all the MsHSF genes were localized in the nucleus. Amino acid sequence comparison showed that all MsHSF genes contain conserved DBD structures, after which we classified MsHSF proteins into 2 groups and 15 subgroups based on evolutionary relationships and showed that they are mostly similar within the same group but differ significantly between subgroups. Cis-acting element and GO enrichment analyses suggest that MsHSF genes may be involved in multiple transcriptional regulatory mechanisms for plant growth and stress response. In addition, expression profiling indicated that MsHSF genes could show significant specificity during tissue development and that MsHSF genes play a very important role in response to abiotic stresses. Overall, bioinformatic analysis and expression profiling studies of HSF can help to understand the important role of HSF in abiotic stress responses in alfalfa and provide a basis for exploring ways to understand and regulate these stress responses.

**Author Contributions:** Conceptualization, C.G., J.M., G.Z. and Y.Z.; methodology, J.M.; software, G.Z.; validation, Y.Y. and Y.Z.; investigation, J.M. and Y.Y.; data curation, G.Z. and Q.M.; writing—original draft preparation, J.M. and G.Z.; writing—review and editing, C.G., J.M., G.Z., S.H. and L.S.; funding acquisition, C.G. All authors have read and agreed to the published version of the manuscript.

**Funding:** This work was financially supported by Zhejiang Provincial Natural Science Foundation of China (No. LY21C160001); National Natural Science Foundation of China (31272494); and Zhejiang Provincial Natural Science Foundation of China (No. LY16C170003).

**Institutional Review Board Statement:** Not applicable.

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** All data in this study can be found in the manuscript.

**Conflicts of Interest:** The authors declare no conflict of interest.

**References**

1. Pu, J.; Li, M.; Mao, P.; Zhou, Q.; Liu, W.; Liu, Z. Genome-Wide Identification of the Q-type C2H2 Transcription Factor Family in Alfalfa (*Medicago sativa*) and Expression Analysis under Different Abiotic Stresses. *Genes* 2021, 12, 1906. [CrossRef] [PubMed]
2. Song, Y.; Lv, J.; Ma, Z.; Dong, W. The mechanism of alfalfa (*Medicago sativa* L.) response to abiotic stress. *Plant Growth Regul.* 2019, 89, 239–249. [CrossRef]
3. Mao, P.; Jin, X.; Bao, Q.; Mei, C.; Zhou, Q.; Min, X.; Liu, Z. WRKY Transcription Factors in *Medicago sativa* L.: Genome-Wide Identification and Expression Analysis Under Abiotic Stress. *DNA Cell Biol.* 2020, 39, 2212–2225. [CrossRef] [PubMed]
4. Yuan, Y.; Yu, J.; Kong, L.; Zhang, W.; Hou, X.; Cui, G. Genome-wide investigation of the PLD gene family in alfalfa (*Medicago sativa* L.): Identification, analysis and expression. *BMC Genom.* 2022, 23, 243. [CrossRef] [PubMed]
5. Yang, Y.; Qi, L.; Nian, L.; Zhu, X.; Yi, X.; Jiyu, Z.; Qiu, J. Genome-Wide Identification and Expression Analysis of the SRS Gene Family in *Medicago sativa*. *DNA Cell Biol.* 2021, 40, 1539–1553. [CrossRef]
6. Dong, X.; Deng, H.; Ma, W.; Zhou, Q.; Liu, Z. Genome-wide identification of the MADS-box transcription factor family in autotetraploid cultivated alfalfa and expression analysis under abiotic stress. *BMC Genom.* 2021, 22, 603. [CrossRef]
7. Sheng, S.; Guo, X.; Wu, C.; Xiang, Y.; Duan, S.; Yang, W.; Li, W.; Cao, F.; Liu, L. Genome-wide identification and expression analysis of DREB genes in alfalfa (Medicago sativa) in response to cold stress. *Plant Signal. Behav.* **2022**, *17*, 2081420. [CrossRef]

8. He, F.; Wei, C.; Zhang, Y.; Long, R.; Li, M.; Wang, Z.; Yang, Q.; Kang, J.; Chen, L. Genome-Wide Association Analysis Coupled With Transcriptome Analysis Reveals Candidate Genes Related to Salt Stress in Alfalfa (Medicago sativa L.). *Front. Plant Sci.* **2022**, *12*, 826584. [CrossRef]

9. Westerheide, S.D.; Raynes, R.; Powell, C.; Xue, B.; Uversky, V.N. HSF Transcription Factor Family, Heat Shock Response, and Protein Intrinsic Disorder. *Curr. Protein Pept. Sci.* **2012**, *13*, 86–103. [CrossRef]

10. Nian, L.; Zhang, X.; Yi, X.; Liu, X.; A’in, N.U.; Yang, Y.; Li, X.; Haider, F.U.; Zhu, X. Genome-wide identification of ABA receptor PYL/RCAR gene family and their response to cold stress in *Medicago sativa*. *Physiol. Mol. Biol. Plants* **2021**, *27*, 1979–1995. [CrossRef]

11. Nian, L.; Liu, X.; Yang, Y.; Zhu, X.; Yi, X.; Haider, F.U. Genome-wide identification, phylogenetic, and expression analysis under abiotic stress conditions of LIM gene family in *Medicago sativa*. *PLoS ONE* **2022**, *16*, e0252213. [CrossRef] [PubMed]

12. Panzade, K.P.; Kale, S.S.; Kapale, V.; Chavan, N.R. Genome-Wide Analysis of Heat Shock Transcription Factors in *Ziziphus jujuba* Identifies Potential Candidates for Crop Improvement Under Abiotic Stress. *Appl. Biochem. Biotechnol.* **2021**, *193*, 1023–1041. [CrossRef]

13. Zhang, L.; Chen, W.; Shi, B. Genome-wide analysis and expression profiling of the heat shock transcription factor gene family in Physic Nut (*jatropha curcas* L.). *PeerJ* **2020**, *8*, e9467. [CrossRef] [PubMed]

14. Wang, P.; Song, H.; Li, C.; Li, P.; Li, A.; Guan, H.; Hou, L.; Wang, X. Genome-wide dissection of the heat shock transcription factor family genes in Arabidopsis. *Front. Plant Sci.* **2017**, *8*, 106. [CrossRef]

15. Wan, X.; Yang, J.; Guo, C.; Bao, M.; Zhang, J. Genome-wide identification and classification of the Hsf and sHsp gene families in *Prunus mume*, and transcriptional analysis under heat stress. *PeerJ* **2019**, *7*, e7312. [CrossRef]

16. Li, M.; Xie, F.; Li, Y.; Gong, L.; Luo, Y.; Zhang, Y.; Chen, Q.; Wang, Y.; Lin, Y.; Zhang, Y.; et al. Genome-Wide Analysis of the Heat Shock Transcription Factor Gene Family in *Brassica juncea*: Structure, Evolution, and Expression Profiles. *DNA Cell Biol.* **2020**, *39*, 1990–2004. [CrossRef] [PubMed]

17. 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 melongena* L.). *Horticulture* **2021**, *7*, 149. [CrossRef]

18. Rehman, A.; Atif, R.M.; Azhar, M.T.; Peng, Z.; Li, H.; Qin, G.; Jia, Y.; Pan, Z.; He, S.; Qayyum, A.; et al. Genome wide identification, classification and functional characterization of heat shock transcription factors in cultivated and ancestral cottons (*Gossypium* spp.). *Int. J. Biol. Macromol.* **2021**, *182*, 1507–1527. [CrossRef]

19. Huang, B.; Huang, Z.; Ma, R.; Chen, J.; Zhang, Z.; Yrijála, K. Genome-wide identification and analysis of the heat shock transcription factor family in moso bamboo (*Phyllostachys edulis*). *Sci. Rep.* **2021**, *11*, 16492. [CrossRef]

20. 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]

21. Zhang, J.; Liu, B.; Li, J.; Zhang, L.; Wang, Y.; Zheng, H.; Lu, M.; Chen, J. Hsf and Hsp gene families in Populus: Genome-wide identification, organization and correlated expression during development and in stress responses. *BMC Genom.* **2015**, *16*, 181. [CrossRef] [PubMed]

22. Xue, G.-P.; Sadat, S.; Drentgh, J.; McIntyre, C.L. The heat shock factor family from *Triticum aestivum* in response to heat and other major abiotic stresses and their role in regulation of heat shock protein genes. *J. Exp. Bot.* **2014**, *65*, 539–557. [CrossRef] [PubMed]

23. Lin, Y.; Cheng, Y.; Jin, J.; Jin, X.; Jiang, H.; Yan, H.; Cheng, B. Genome Duplication and Gene Loss Affect the Evolution of Heat Shock Transcription Factor Genes in Legumes. *PLoS ONE* **2014**, *9*, e102825. [CrossRef] [PubMed]

24. Li, H.-C.; Li, G.-L.; Liu, Z.-H.; Zhang, H.-M.; Zhang, Y.-M.; Guo, X.-L. Cloning, Localization and Expression Analysis of ZmHsf-like Gene in Zea mays. *J. Integr. Agric.* **2014**, *13*, 1230–1238. [CrossRef]

25. Zhao, L.; Pan, H.; Sun, M.; Zhang, Q. Molecular cloning of *Arabidopsis thaliana* HSF-A2 gene and agrobacterium-mediated genetic transformation of chrysanthemum morifolium ramat. *Adv. Intl. Soft Comput.* **2010**, *134*, 827–834. [CrossRef]

26. Guo, M.; Liu, J.-H.; Ma, X.; Luo, D.-X.; Gong, Z.-H.; Lu, M.-H. The Plant Heat Stress Transcription Factors (HSFs): Structure, Regulation, and Function in Response to Abiotic Stresses. *Front. Plant Sci.* **2016**, *7*, 114. [CrossRef]

27. Wang, T.; Ren, L.; Li, C.; Zhang, D.; Zhang, X.; Zhou, G.; Gao, D.; Chen, R.; Chen, Y.; Wang, Z.; et al. The genome of a wild Medicago species provides insights into the tolerant mechanisms of legume forage to environmental stress. *BMC Biol.* **2021**, *19*, 96. [CrossRef]

28. Zhang, L.; Jia, X.; Zhao, J.; Hasi, A.; Niu, Y. Molecular characterisation and expression analysis of NAC transcription factor genes in wild Medicago falcata under abiotic stresses. *Funct. Plant Biol.* **2020**, *47*, 327–341. [CrossRef]

29. Xu, C.; He, C.G.; Wang, Y.J.; Bi, Y.F.; Jiang, H. Effect of drought and heat stresses on photosynthesis, pigments, and xanthophyll cycle in alfalfa (*Medicago sativa* L.). *Photosyntethica* **2020**, *58*, 1226–1236. [CrossRef]

30. Guiza, M.; Benabdellahim, M.A.; Brini, F.; Haddad, M.; Saibi, W. Assessment of Alfalfa (*Medicago sativa* L.) Cultivars for Salt Tolerance Based on Yield, Growth, Physiological, and Biochemical Traits. *J. Plant Growth Regul.* **2021**, *10*, 1435-8107. [CrossRef]

31. He, K.; Li, C.; Zhang, Z.; Zhan, L.; Cong, C.; Zhang, D.; Cai, H. Genome-wide investigation of the ZF-HD gene family in two varieties of alfalfa (*Medicago sativa* L.) and its expression pattern under alkaline stress. *BMC Genom.* **2022**, *23*, 150. [CrossRef] [PubMed]
32. Tang, M.; Xu, L.; Wang, Y.; Cheng, W.; Luo, X.; Xie, Y.; Fan, L.; Liu, L. Genome-wide characterization and evolutionary analysis of heat shock transcription factors (HSFs) to reveal their potential role under abiotic stresses in radish (Raphanus sativus L.). *BMC Genom.* 2019, 20, 772. [CrossRef] [PubMed]

33. Guo, M.; Lu, J.-P.; Zhai, Y.-F.; Chai, W.-G.; Gong, Z.-H.; Lu, M.-H. Genome-wide analysis, expression profile of heat shock factor gene family (CaHsfs) and characterisation of CaHsfA2 in pepper (Capsicum annuum L.). *BMC Plant Biol.* 2015, 15, 151. [CrossRef] [PubMed]

34. Ma, C.; 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]

35. 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]

36. Zhang, H.Z.; Yang, J.L.; Chen, Y.L.; Mao, X.L.; Wang, Z.C.; Li, C.H. Identification and expression analysis of the heat shock transcription factor (HSF) gene family in Populus trichocarpa. *PLANT OMICS* 2013, 6, 415–424. [CrossRef]

37. Fan, K.; Mao, Z.; Ye, F.; Pan, X.; Li, Z.; Lin, W.; Zhang, Y.; Huang, J.; Lin, W. Genome-wide identification and molecular evolution analysis of the heat shock transcription factor (HSF) gene family in four diploid and two allopolyploid Gossypium species. *Genomics* 2021, 113, 3112–3127. [CrossRef]

38. 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 common bean (*Phaseolus vulgaris*). *Int. J. Mol. Sci.* 2019, 20, 5233. [CrossRef]

39. Xue, H.; Slavov, D.; Wischmeyer, P. Glutamine activates heat shock transcription factor-1 (HSF1) gene transcription. *FASEB J.* 2012, 26, 6462–67. [CrossRef]

40. Duan, S.; Liu, B.; Zhang, Y.; Li, G.; Guo, X. Genome-wide identification and abiotic stress-responsive pattern of heat shock transcription factor family in *Triticum aestivum* L. *BMC Genom.* 2019, 20, 257. [CrossRef]

41. 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] [PubMed]

42. Zhang, Q.; Geng, J.; Du, Y.; Zhao, Q.; Zhang, W.; Fang, Q.; Yin, Z.; Li, J.; Yuan, X.; Fan, Y.; et al. Heat shock transcription factor (Hsf) gene family in common bean (*Phaseolus vulgaris*) Genome-wide identification, phylogeny, evolutionary expansion and expression analyses at the sprout stage under abiotic stress. *BMC Plant Biol.* 2022, 22, 33. [CrossRef] [PubMed]

43. Yu, X.-Y.; Yao, Y.; Hong, Y.-H.; Hou, P.-Y.; Li, C.-X.; Xia, Z.-Q.; Geng, M.-T.; Chen, Y.-H. Differential expression of the Hsf family in cassava under biotic and abiotic stresses. *Genome* 2019, 62, 563–569. [CrossRef] [PubMed]

44. Shyamli, P.S.; Pradhan, S.; Panda, M.; Parida, A. De novo Whole-Genome Assembly of Moringa oleifera Helps Identify Genes Regulating Drought Stress Tolerance. *Front. Plant Sci.* 2021, 12, 766999. [CrossRef]

45. Shen, C.; Yuan, J. Genome-wide characterization and expression analysis of the heat shock transcription factor family in pumpkin (*Cucurbita moschata*). *BMC Plant Biol.* 2020, 20, 471. [CrossRef] [PubMed]

46. Agarwal, P.; Khurana, P. Functional characterization of HSFs from wheat in response to heat and other abiotic stress conditions. *Funct. Integr. Genom.* 2019, 19, 497–513. [CrossRef]

47. Ducy, P.; Karsenty, G. Two distinct osteoblast-specific cis-acting elements control expression of a mouse osteocalcin gene. *Mol. Cell. Biol.* 1995, 15, 1885–1869. [CrossRef]

48. Wang, R.; Zhong, Y.; Liu, X.; Zhao, C.; Zhao, J.; Li, M.; Hassan, M.U.; Yang, B.; Li, D.; Liu, R.; et al. Cis- regulation of the amino acid transporter genes ZmAAP2 and ZmLHT1 by ZmPHR1 transcription factors in maize ear under phosphate limitation. *J. Exp. Bot.* 2021, 72, 3846–3863. [CrossRef]

49. Binder, R.J.; Blachere, N.E.; Srivastava, P.K. Heat shock protein-chaperoned peptides but not free peptides introduced into the cytosol are presented efficiently by major histocompatibility complex I molecules. *J. Biol. Chem.* 2001, 276, 17163–17171. [CrossRef]

50. Yang, D.; Tu, Y.; Wang, X.; Cao, C.; Hu, Y.; Shao, J.; Weng, L.; Mou, X.; Dong, X. A photo-triggered antifungal nanoplatform with efflux pump and heat shock protein reversal activity for enhanced chemo-photothermal synergistic therapy. *Biomater. Sci.* 2021, 9, 3293–3299. [CrossRef]

51. Altstaff, K.; Radha, V. Influence of heat shock protein (hsp-70) enhancing compound from red alga (Porphyridium purpureum) for augmenting egg production in copepod--A new in silico report. *Mar. Sci. Technol. Bull.* 2021, 10, 186–192. [CrossRef]

52. Simon, S.; Aissat, A.; Degrujillier, F.; Simonneau, B.; Fanen, P.; Arrigo, A.P. Small Hsps as Therapeutic Targets of Cystic Fibrosis Transmembrane Conductance Regulator Protein. *Int. J. Mol. Sci.* 2021, 22, 4252. [CrossRef] [PubMed]

53. Tian, F.; Hu, X.-L.; Yao, T.; Yang, X.; Chen, J.-G.; Lu, M.-Z.; Zhang, J. Recent advances in the roles of HSFs and HSPs in heat stress response in woody plants. *Front. Plant Sci.* 2021, 12, 704905. [CrossRef] [PubMed]

54. Zhou, L.; Yu, X.; Wang, D.; Li, L.; Zhou, W.; Zhang, Q.; Wang, X.; Ye, S.; Wang, Z. Genome-wide identification, classification and expression profile analysis of the HSF gene family in Hypericum perforatum. *PeerJ* 2021, 9, e11345. [CrossRef] [PubMed]

55. Tang, R.; Zhu, W.; Song, X.; Lin, X.; Cai, J.; Wang, M.; Yang, Q. Genome-Wide Identification and Function Analyses of Heat Shock Transcription Factors in *Raphanus sativus*. *Front. Plant Sci.* 2016, 7, 490. [CrossRef]

56. Samtani, H.; Sharma, A.; Khurana, J.P.; Khurana, P. The heat stress transcription factor family in *Aegilops tauschii* Genome-wide identification and expression analysis under various abiotic stresses and light conditions. *Mol. Genet. Genom.* 2022, 2, 11–15. [CrossRef]
57. Saha, D.; Mukherjee, P.; Dutta, S.; Meena, K.; Sarkar, S.K.; Mandal, A.B.; Dasgupta, T.; Mitra, J. Genomic insights into HSFs as candidate genes for high-temperature stress adaptation and gene editing with minimal off-target effects in flax. *Sci. Rep.* 2019, 9, 5581. [CrossRef]

58. Tan, B.; Yan, L.; Li, H.; Lian, X.; Cheng, J.; Wang, W.; Zheng, X.; Wang, X.; Li, J.; Ye, X.; et al. Genome-wide identification of HSF family in peach and functional analysis of PpHSF5 involvement in root and aerial organ development. *PeerJ* 2021, 9, e10961. [CrossRef]

59. Eddy, S.R. What is a hidden Markov model? *Nat. Biotechnol.* 2004, 22, 1315–1316. [CrossRef]

60. Chen, C.; Chen, H.; Zhang, Y.; Thomas, H.R.; Frank, M.H.; He, Y.; Xia, R. TBtools: An Integrative Toolkit Developed for Interactive Analyses of Big Biological Data. *Mol. Plant* 2020, 13, 1194–1202. [CrossRef]

61. Shannon, P.; Markiel, A.; Ozier, O.; Baliga, N.S.; Wang, J.T.; Ramage, D.; Amin, N.; Schwikowski, B.; Ideker, T. Cytoscape: A Software Environment for Integrated Models of Biomolecular Interaction Networks. *Genome Res.* 2003, 13, 2498–2504. [CrossRef] [PubMed]