



Article

Genome-Wide Identification of Heat Shock Transcription Factor Family and Key Members Response Analysis to Heat Stress in Loquat

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Abstract: *Eriobotrya japonica* (loquat) is an evergreen fruit tree of the apple tribe in *Rosaceae* with high edible and medicinal value. The yield and quality of loquat fruits are significantly influenced by environmental stress, particularly heat stress during fruit ripening. In this study, thirty *EjHSFs* were identified in the loquat genome. Twenty-nine *EjHSFs* were unevenly distributed across sixteen chromosomes, except Chr-6. A synteny analysis revealed that twenty-six *EjHSF* genes had undergone duplication events. Twenty-nine *EjHSF* genes were found to be in sync with *HSF* in apples while also diverging with other *Rosaceae* species. A phylogenetic analysis revealed that *EjHSFs* could be divided into three categories, including eighteen *EjHSF-A*, ten *EjHSF-B*, and two *EjHSF-C*. Twenty-nine members of the *EjHSF* family showed high homology to those of *Malus domestica* and *Gillenia trifoliata*. A promoter analysis retrieved thirty-three CAREs that were functionally relevant and connected to the expression of *HSFs*. Among these, the promoters of twenty-three *EjHSF* genes possessed at least one STRE element that could be activated by heat shock. Eleven of these *EjHSFs* were highly expressed in loquat tissues, namely *EjHSF-B4a*, *EjHSF-A4a*, *EjHSF-A4d*, and *EjHSF-C1a* in roots; *EjHSF-B4b* in roots and inflorescence; *EjHSF-C1b* in stems and roots; *EjHSF-A2a* in three tissues; *EjHSF-A8b* in four tissues; and *EjHSF-A4c*, *EjHSF-B1a*, and *EjHSF-B2b* in six tissues. Moreover, fifteen *EjHSFs* were differentially expressed (eleven upregulated and four downregulated) in fruits under heat stress treatment in the color-changing period. Among them, *EjHSF-A2a* and *EjHSF-A2b* upregulated transcriptional abundance over 300 times after heat treatment; *EjHSF-B2b* consistently displayed an increasing trend over time; and *EjHSF-B1a* was significantly downregulated. Hence, these results suggest that *EjHSFs* take part in loquat tissue development and may be involved in the fruit's heat stress response. These findings enhance the understanding of *EjHSFs'* role within loquats and the heat stress response of its fruit and provide target genes for heat stress improvement.

Keywords: loquat; HSF; heat stress; expression profile



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1. Introduction

Loquat (*Eriobotrya japonica*), belonging to the *Rosaceae* family, is widely cultivated in Southern China for its delicious fruit and medicinally valuable leaves [1]. The growth of loquat fruit is sensitive to fluctuations in various abiotic and biological stresses [2]. Previous studies have demonstrated that heat stress during the color-changing period significantly impacts both the yield and quality of loquat fruit. Metabolomic, transcriptomic, and small RNA analyses investigated loquat fruit in response to heat stress revealing the involvement of 36 hormone types that are crucial for fruit heat tolerance [3]. Furthermore, a differential protein analysis revealed 26 proteins associated with the defense response, carbohydrate

and energy metabolism, photosynthesis, and other functional categories. Additionally, five miRNAs exhibited responsiveness to high temperatures in loquat fruits [4].

Heat shock transcription factors (HSFs) are known regulators of HSP expression and confer thermotolerance [5]. These HSFs possess several conserved functional domains, including an N-terminal deoxyribonucleic acid-binding domain (DBD), an oligomerization structural domain (OD), a nuclear localization signaling domain (NLS), a nuclear export signaling domain (NES), and a C-terminal transcriptional activation domain (CAD) [6,7]. The DBD specifically binds to heat stress elements located within the target promoter, and the OD region connects with the DBD through a hydrophobic amino acid sequence known as the HR-A/B region [8]. Furthermore, the NLS and NES play assembling roles in the nuclear import complex involving the target protein and receptor-mediated export complex with NES receptor exportin- α [9]. AHA motifs, located in the CAD of HSF plants, play a crucial role in their activator function and exhibit specificity toward HSFA-specific motifs. HSFs are classified into three families based on the length of the flexible linker region between the DBD and HR-A/B region and the number of amino acid residues: subfamilies A (A1–9), B (B1–4), and C (C1–2) [10].

The comprehensive identification of HSF has been accomplished in various plant species by developing and utilizing genomic data. For instance, fifty-six HSFs have been identified to respond to heat stress and other biotic stresses in wheat (*Triticum aestivum*) [11]. Several HSFs have been identified in various plants since the first HSF was identified in tomatoes and functions associated with heat stress. *TrHSFB2a* of white clover (*Trifolium repens*) negatively regulates drought, heat, and salt stress tolerance in transgenic *Arabidopsis* [12]. Overexpression of *PsnHSF21* confers salt tolerance in *Populus simonii* $\times$ *P. nigra* trees [13]. Moreover, AtHOPs participate in folding and stabilizing HSFA1a alongside being essential for initiating transcriptional responses associated with thermomorphogenesis [14]. Overexpression of apple *MdHSF4*, *MdHSFB2A*, and *MdHSFA1D* imparts heat tolerance and rescues the heat-sensitive phenotype of *MdWRKY75-Ri3* [15]. Grape *VdHSFA2* and *VvHSFA2* are positive regulators in grape thermotolerance; meanwhile, they induce a thermotolerance response by targeting the *MBF1c* promoter [16]. These findings highlight the significant role of HSFs in response to environmental stresses. However, little is known about the presence or potential functions of HSFs within *Eriobotrya japonica*.

In this study, a bioinformatics analysis was conducted to identify and characterize members of the loquat's HSF gene family, discovering 30 genes. Furthermore, an extensive expression analysis of these genes was performed at various growth stages and under heat-stress conditions. The findings present a preliminary analysis of HSFs in loquat and demonstrate their roles in response to high-temperature stress resistance and their potential application in genetic breeding.

2. Materials and Methods

2.1. Identification of HSF Genes in *Eriobotrya japonica*

The genomic data of *Eriobotrya japonica* cv Jiefangzhong (CNP0001531), formerly released by Su et al. (2021) [1], were obtained from the China National GeneBank database (<https://db.cngb.org/cnsa>, accessed on 14 September 2023). HSF protein domain data (Pfam accession number: PF00447.20) were obtained from the protein families database (<http://pfam.xfam.org/>, accessed on 14 September 2023) [17]. The HMM profile was constructed for the known HSF using the “hmmsearch” function in HMMER-3.3.2 software. All identified HSFs with e-values less than or equal to 1×10^{-5} were considered for downstream analysis [18]. ProtParam (<https://web.expasy.org/protparam/>, accessed on 15 September 2023) was used to predict the theoretical molecular weights (MWs) and isoelectric point (pI) values of these proteins [19]. The CELLO2GO program (<http://cello.life.nctu.edu.tw/cello2go/>, accessed on 15 September 2023) was used to predict the subcellular localization of the *EjHSF* genes [20].

2.2. Phylogenetic Tree Construction and Gene Structure Analysis

The HSF protein sequences of *Eriobotrya japonica*, *Arabidopsis thaliana*, *Oryza sativa*, *Malus domestica*, *Prunus persica*, and *Gillenia trifoliata* [18,21–24] were used for multiple sequence alignment using MEGA X software. The phylogenetic tree was constructed with the NJ method (1000-replicates bootstrap). MEME suite (<https://meme-suite.org/meme/>, accessed on 16 September 2023) was used to identify the motifs with e-values of less than 1×10^{-20} and number the 10–50 amino acids according to the corresponding e-values [25]. The gene structure of the *EjHSFs* was analyzed using genome annotation files.

2.3. Cis-Element Analysis

The *EjHSFs* upstream of the 2000 bp sequence was extracted to predict the *cis*-acting elements using PlantCARE database (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>, accessed on 16 September 2023) [26].

2.4. Gene Duplication Events

Circos 0.69-9 software was used to analyze gene duplication events in *EjHSFs* via the MCScanX function [27]. The HSF gene annotation files and whole-genome sequences of *Eriobotrya japonica*, *Arabidopsis thaliana*, *Oryza sativa*, *Malus domestica*, *Prunus persica*, and *Gillenia trifoliata* were downloaded from the Joint Genome Institute database (<https://genome.jgi.doe.gov/portal/>, accessed on 17 September 2023). Synteny analysis of the *EjHSFs* and the other five species of HSF genes was performed using the MCScanX function in Circos 0.69-9 software.

2.5. Expression of *EjHSF* Genes

Trim Galore v0.6.10 software was used to prune the transcriptome reads, and the reads were then used for alignment with the *Eriobotrya japonica* genome using Hisat2 [3]. The count of each gene's reads was calculated using StringTie v2.1.5 software and converted into FPKM to evaluate the gene expression. The FPKM values of the *EjHSF* genes were transformed via log₂, which was visualized by constructing heat maps using TBtools-ll v2.009 software [28]. The tissue-specific expression levels for thirty *EjHSF* genes were referenced from Su et al.'s study (2021) [1]. This included the gene expression in different tissues (root, stem, young leaf, mature leaf, flower, inflorescence, pollen, young fruit, mature fruit, and seed tissues) of cultivated 'Jiefangzhong'. The heat stress expression levels for thirty *EjHSF* genes were referenced from Chen et al.'s study (2022) [3]. Fruits in the color-changing period of 'Wuduiyadanben' from 8-year-old loquat trees planted in the National Germplasm Bank of Loquat (Fuzhou, China) were exposed to 40 °C in temperature-controlling boxes (Fuzhou, China) from 10:30 a.m. to 12:00 a.m. wherein the ambient temperature fluctuated around 28 ± 2 °C, and the illumination intensity was $\geq 1100 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ on 7 April 2016. Fruits with 0, 0.5, 1, and 1.5 h heat treatment were collected for physiological and molecular analyses. Peel tissues were separated immediately after the fruits were collected, frozen in liquid nitrogen, and stored at -80 °C for further analysis [3].

3. Results

3.1. Identification and Chromosomal Distribution of *EjHSFs*

Thirty *EjHSFs* were identified based on the *Eriobotrya japonica* genome (PF00447.20). As indicated in Table 1 and Figure 1, these genes were unevenly distributed across the 16 chromosomes of the reference genome of 'Jiefangzhong' loquat, except for *EjHSF-A4d*, which was not located on any chromosome. Chromosome 14 harbored the highest number of *EjHSF* genes (five), while chromosomes 1, 3, 7, 9, 10, 11, 13, and 17 contained a single copy of an *EjHSF* gene. In addition, three *EjHSFs* were found on both chromosomes 2 and 5, and two genes were identified on each of chromosomes 4, 8, 12, 15, and 16. Sixteen *EjHSFs* were localized on the upper arm of the chromosome, while thirteen members resided on the lower arm.

Table 1. Summary of heat shock transcription factors (EjHSFs) in Eriobotrya japonica.

GeneID	GeneName	Chr	Start	End	pI	Mw/kDa	Protein Length/aa	Subcellular Location
Ej00038938	EjHSF-A1a	chr05	15,417,691	15,423,371	5.53	73,313.98	659	Nuclear
Ej00005590	EjHSF-A1b	chr10	5,669,409	5,673,397	5.12	59,636.99	534	Nuclear
Ej00081356	EjHSF-A2a	chr14	39,162,493	39,164,514	5.36	44,862.03	402	Nuclear
Ej00011860	EjHSF-A2b	chr08	6,128,697	6,130,531	4.85	45,921.50	411	Nuclear
Ej00044128	EjHSF-A3a	chr15	46,335,175	46,337,696	5.05	73,528.70	654	Cytoplasmic
Ej00008404	EjHSF-A3b	chr12	40,198,212	40,200,771	5.01	61,348.73	553	Nuclear
Ej00050337	EjHSF-A4a	chr09	837,765	841,420	5.50	49,328.06	434	Nuclear
Ej00001403	EjHSF-A4b	chr17	9,213,388	9,217,290	5.23	49,924.67	440	Nuclear
Ej00028977	EjHSF-A4c	chr05	7,022,296	7,024,714	5.23	47,357.55	420	Nuclear
Ej00000248	EjHSF-A4d	utg0_pilon	3,628,917	3,630,868	5.86	47,689.09	423	Nuclear
Ej00014341	EjHSF-A5a	chr11	39,174,040	39,177,497	5.29	53,735.29	485	Nuclear
Ej00040219	EjHSF-A6a	chr16	18,736,545	18,738,152	4.85	40,867.55	355	Nuclear
Ej00083177	EjHSF-A7a	chr03	36,894,500	36,896,975	5.70	47,676.50	418	Cytoplasmic
Ej00044555	EjHSF-A7b	chr04	6,817,911	6,820,245	5.94	45,551.86	400	Nuclear
Ej00062710	EjHSF-A8a	chr13	1,248,507	1,251,313	4.90	46,788.41	412	Nuclear
Ej00056238	EjHSF-A8b	chr16	1,718,798	1,721,539	4.76	47,005.87	414	Nuclear
Ej00045930	EjHSF-A9a	chr14	25,583,993	25,586,505	4.87	53,371.73	479	Nuclear
Ej00017485	EjHSF-A9b	chr02	313,403	315,310	5.22	54,382.91	484	Nuclear
Ej00027446	EjHSF-B1a	chr15	30,589,331	30,592,234	6.35	32,374.30	294	Nuclear
Ej00019640	EjHSF-B1b	chr02	15,089,357	15,105,668	5.48	36,813.31	331	Nuclear
Ej00094962	EjHSF-B2a	chr04	29,569,081	29,570,621	4.69	36,024.64	334	Nuclear
Ej00006997	EjHSF-B2b	chr07	36,221,462	36,222,856	6.39	34,474.80	307	Nuclear
Ej00050877	EjHSF-B2c	chr01	34,673,165	34,674,594	8.84	33,814.23	297	Nuclear
Ej00023119	EjHSF-B3a	chr14	7,081,222	7,084,794	7.56	27,874.44	243	Nuclear
Ej00004040	EjHSF-B3b	chr12	30,632,183	30,636,337	8.46	27,793.39	243	Nuclear
Ej00081580	EjHSF-B4a	chr14	37,493,345	37,495,029	7.81	43,447.70	384	Nuclear
Ej00045578	EjHSF-B4b	chr08	9,403,046	9,404,710	7.13	43,143.32	381	Nuclear
Ej00066400	EjHSF-B5a	chr05	5,238,626	5,240,893	8.86	21,733.83	190	Nuclear
Ej00047075	EjHSF-C1a	chr14	27,775,187	27,776,352	5.45	37,333.45	334	Nuclear
Ej00017302	EjHSF-C1b	chr02	2,833,348	2,834,528	6.14	37,977.44	337	Nuclear

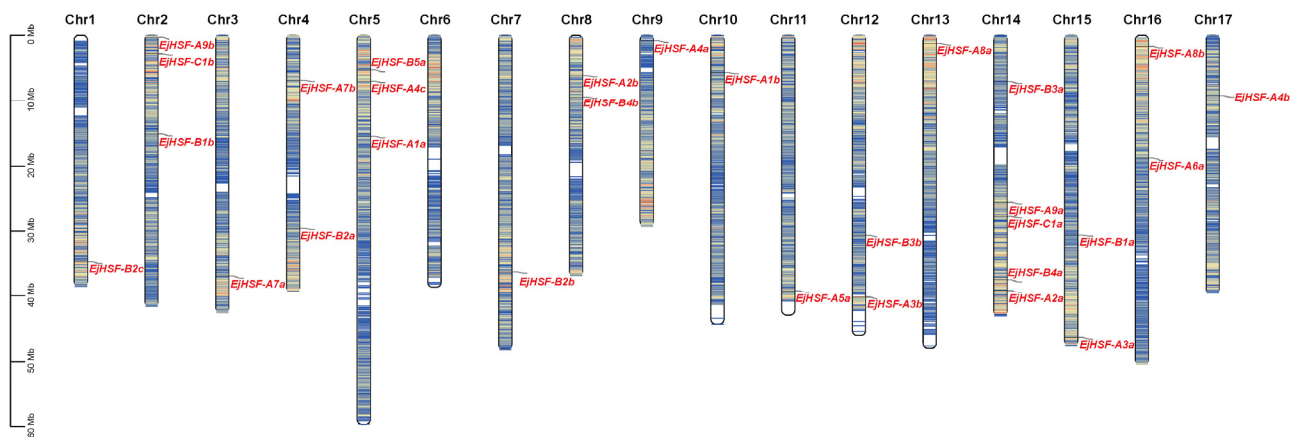


Figure 1. The localization of HSF family genes on loquat chromosomes. The scale represents the megabases (Mbs), the numerical value represents the chromosome number (LG for linkage group), and the chromosomes' differences in color represent the gene density, the color ranges from white to red, the higher the density.

Out of the 30 members analyzed, 18 (60%), 10 (33.3%), and 2 (6.7%) belonged to the HSP-A, HSP-B, and HSP-C families, respectively. EjHSF-A1a exhibited the longest protein length of 659 aa while EjHSF-B5a exhibited the shortest of 190 aa. The MWs of these proteins ranged from 21.7 kDa (EjHSF-B5a) to 73.5 kDa (EjHSF-A3a), and their pI values ranged

from 4.69 (EjHSF-B2a) to 8.86 (EjHSF-B5a). Based on the subcellular location prediction results, twenty-eight EjHSF members were predicted to be localized in the nucleus, and EjHSF-A3a and EjHSF-A7a were predicted to be localized in the cytoplasmic compartment.

3.2. Synteny Analysis

Gene duplication is a natural process that generates new genes with useful functions, promoting species development [18]. The loquat genome's homology blocks are represented by gray lines in Figure 2. The red lines between chromosomes denote that twenty-six EjHSFs had undergone duplication events, while only three genes (*EjHSF-A5a*, *EjHSF-B5a*, and *EjHSF-A4c*) did not experience duplication events. The syntenic genes were located on different chromosomes from their partner, except *EjHSF-A9a* and *EjHSF-A2a*, which were located on the same chromosome (Chr-14).

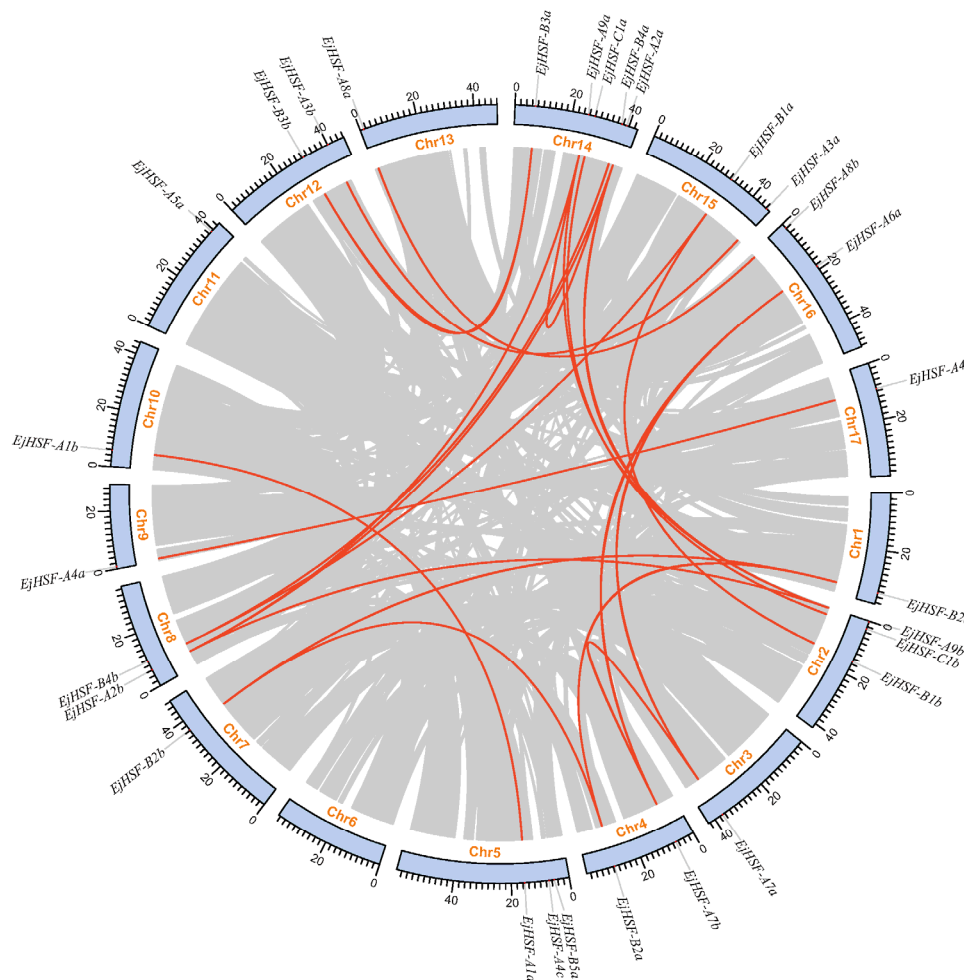


Figure 2. Chromosomal distribution and duplication events. The 29 putative segmental duplicated pairs of the *EjHSF* genes were investigated using MCScanX and linked by the colored lines, respectively. The gray line indicates all the putative segment duplication pairs in the loquat genome while the red line links the *EjHSF* segment duplication pairs.

A synteny analysis was performed to understand the phylogeny of the *EjHSF* gene family, comparing it with *HSF* genes from *Eriobotrya japonica* (30), *Arabidopsis thaliana* (25), *Oryza sativa* (25), *Malus domestica* (47), *Prunus persica* (45), and *Gillenia trifoliata* (34) species (Figure 3). The analysis revealed that twenty-nine *EjHSFs* were in sync with *Malus domestica* (both from the apple tribe of *Rosaceae*) while diverging with *Prunus persica* and *Gillenia trifoliata* (other *Rosaceae* species). In addition, twenty-three *EjHSF* genes were in sync with

Vitis vinifera genes and twenty-one with *Arabidopsis thaliana* genes. Moreover, seventeen and fifteen *EjHSF* genes were in sync with *Ananas comosus* and *Oryza sativa* genes, respectively (Supplementary Table S1). Several *EjHSFs* were associated with multiple *HSF* genes in *Malus domestica*; for example, *EjHSF-A6a* was associated with four *HSFs* in *Malus domestica*.

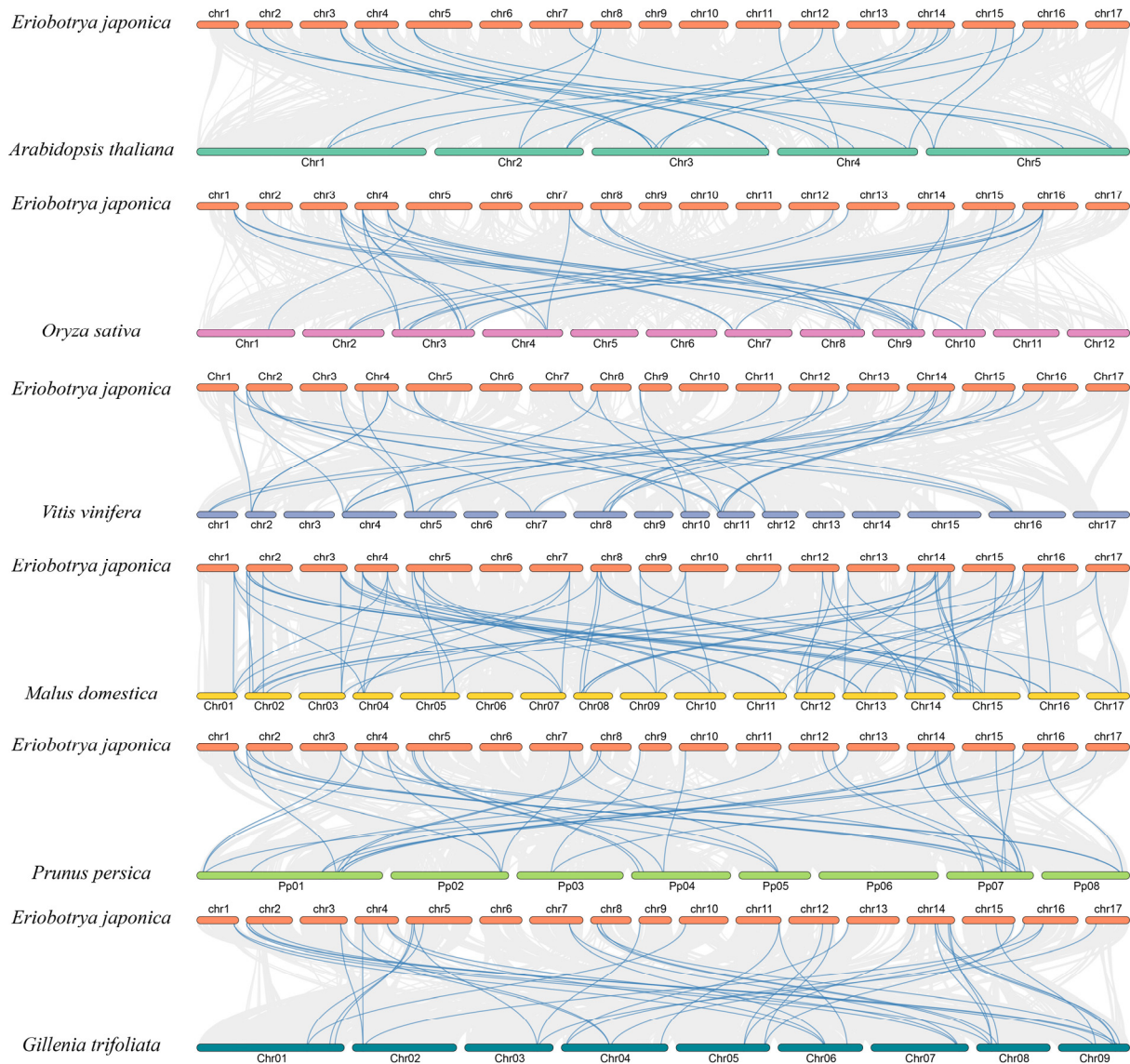


Figure 3. Synteny analysis of *HSF* genes between loquat and five other plant species. The gray line in the background represents the collinear blocks between *Eriobotrya japonica* and six other plant species (*Arabidopsis thaliana*, *Oryza sativa*, *Vitis vinifera*, *Malus domestica*, *Prunus persica*, and *Gillenia trifoliata*) while the blue line exhibits the syntenic *HSF* gene pairs.

3.3. Phylogenetic Classification of *EjHSFs*

The classification and evolutionary relationships of the *EjHSF* gene family in loquat were elucidated. Furthermore, the phylogenetic tree was constructed for the 206 *HSF* protein sequences, including *Eriobotrya japonica* (30), *Arabidopsis thaliana* (25), *Oryza sativa* (25), *Malus domestica* (47), *Prunus persica* (45), and *Gillenia trifoliata* (34) (Figure 4). The thirty members of the *EjHSF* gene family were clustered into three categories based on the clustering bootstrap value: classes A, B, and C. Among these, 18 members belonged to subgroup A, which was further divided into nine subclusters (A1, A2, A3, A4, A5, A6, A7, A8, and A9), and 10 members belonged to subgroup B, which was classified into five subclasses (B1, B2, B3, B4, and B5). Two members (*EjHSF-C1a* and *EjHSF-C1b*) belonged to C1 subclus-

ters. Except for EjHsf-C2 subfamilies, every other EjHsf subfamily contained 1–4 *EjHsf* genes. Twenty-nine members of the *EjHsf* family had high homology with *Malus domestica* and *Gillenia trifoliata*. For example, EjHsf-A2a and EjHsf-A2b had high homology (99%) with MdHsfA2b and MdHsfA2a, respectively; EjHsf-B2c had high homology (93%) with MdHsfB2a; and EjHsf-B1a had high homology (92%) with Gtr09g3859. Proteins within each class typically exhibited similar biological functions, enabling valuable predictions regarding the future biological functions of additional members within the *EjHsf* gene family.

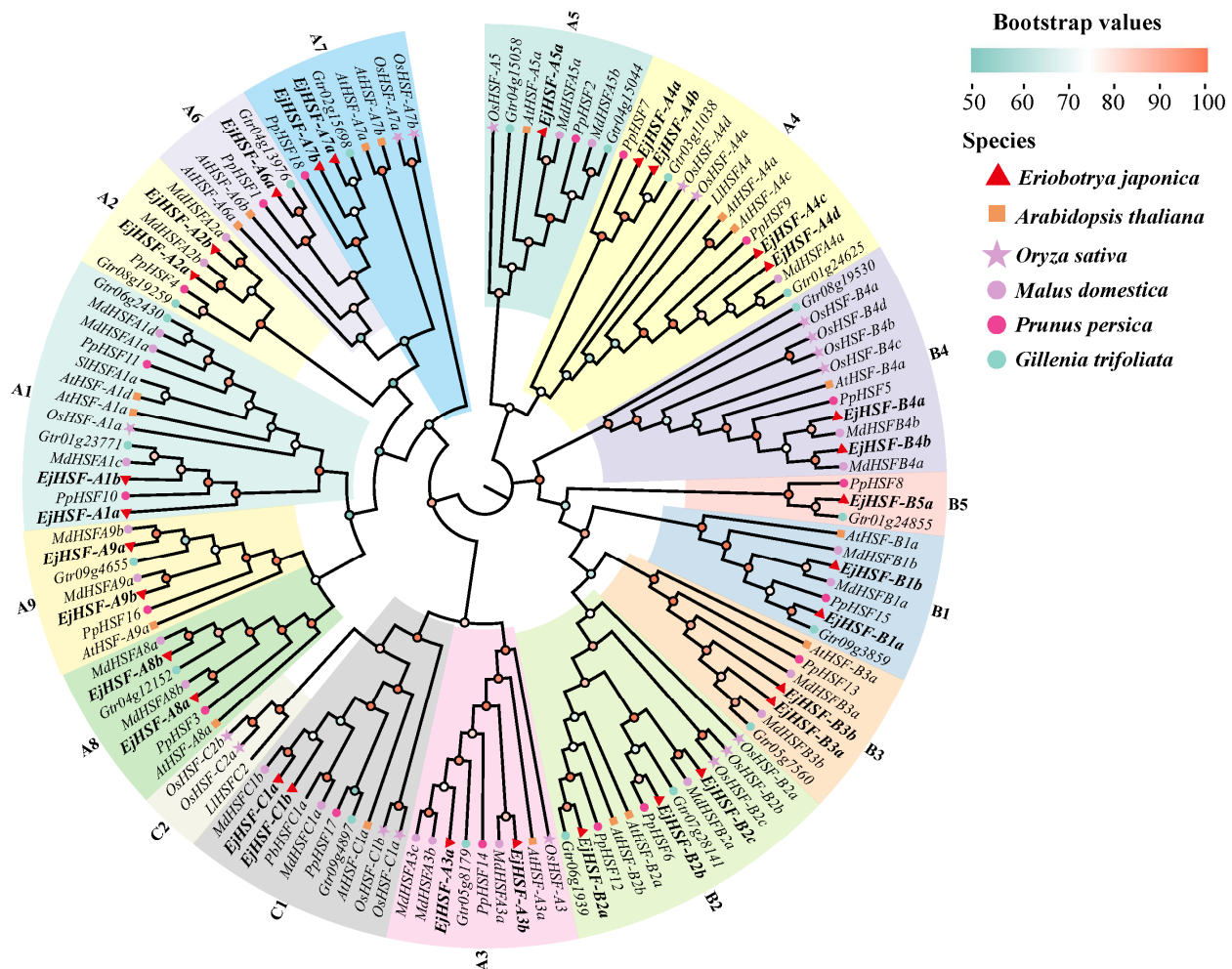


Figure 4. Protein-based phylogenetic tree of HSF gene family. ▲, ■, ☆, ●, ●, and ● represent the phylogenetic analysis of HSFs in *Eriobotrya japonica* (Ej), *Arabidopsis thaliana* (At), *Oryza sativa* (Os), *Malus domestica* (Md), *Prunus persica* (Pp), and *Gillenia trifoliata* (Gt), respectively. The other genes mentioned in the chart are *Lilium longiflorum* (Ll), *Solanum lycopersicum* (Sl), and *Pyrus betulifolia* (Pb).

3.4. Conserved Motifs and Gene Structure Analysis of *EjHsf*s

The structural diversity of the *EjHsf*s was evaluated by analyzing all their motifs using MEME Suite. Ten distinct motifs were identified in the *EjHsf* proteins (motifs 1–10). Motifs 1, 2, 4, and 5 were consistently found together, suggesting a close association with SQUAMOSA promoter binding-like (SPL) proteins. Members of the same subfamily of *EjHsf*s exhibited similar motif compositions. For example, subfamily B contained motifs 1, 2, 4, 5, and 7; subfamily C contained motifs 1, 2, 3, 4, and 5; and subfamily A exhibited nine motifs (motifs 1–6 and 8–10). Additionally, several motifs were located at specific positions. For example, motif 5 was consistently located at the beginning of the motifs, except for EjHsf-A1a and different motifs were positioned at the end. Motif 2

always appeared between motifs 5 and 1 and motif 4 followed after motif 1 (Figure 5A,B; Supplementary Table S2).



Figure 5. The motif and gene structure organizations of *EjHSF* members. (A) The evolutionary tree of loquat HSF family members. (B) Protein motif locations of loquat HSF members. The protein sequences were subjected to Motif Search based on homology. Boxes represent motifs, and different colors represent different motifs. (C) Conserved binding sites of *EjHSF* genes. Different colored boxes represent different types of conserved binding sites. (D) Gene structures of *EjHSF* genes. Intron–exon size distribution of HSF family genes. The nucleotide sequences of these genes were used for analysis. The red box represents exons, and the line represents introns.

All *EjHSFs* contained HSF-DNA-binding sites, and *EjHSF*-A1a, *EjHSF*-A4a, *EjHSF*-A4b, and *EjHSF*-A7a contained methyltransf-12, Tup-N, TolA binding tri, and macoilin, respectively (Figure 5C). The structural and taxonomic diversity of the HSF genes in loquat was explored by comparing the genomic DNA sequences. Differences in the exon–intron structures revealed that thirty *EjHSF* genes had varying numbers of exons, ranging from two to six. Most *EjHSF* genes (22 (73.3%)) contained two exons. *EjHSF*-A3a exhibited the highest number of six exons and five introns. Furthermore, *EjHSF*-A2a and *EjHSF*-A2b were classified into the A2 subfamily with identical exon and intron structures consisting of four exons and three introns (Figure 5D). Generally, *EjHSFs* belonging to the same subfamily presented similar gene structures and potential functional diversification among its members.

3.5. Analysis of cis-Acting Elements in *EjHSF* Promoters

The promoter regions of *EjHSFs* were analyzed to gain insights into their tissue-specific expression patterns and stress responses. This study retrieved 2000 upstream sequences (the upstream regulatory region) for all thirty HSFs from the loquat genome using the gene coordinates. Upon searching against the PlantCARE databases, this study obtained 33 *cis*-acting regulatory elements (CAREs) that were functionally relevant and connected to the expression of HSFs (Figure 6). The promoter CAREs were categorized into three groups: abiotic and biotic stresses, phytohormone responsiveness, and plant growth and development-related elements. Most individual *EjHSF* genes in loquat encompassed

various phytohormone response elements, such as abscisic acid (ABA) response elements (ABRE), SA hormone response elements (TCA-element), and MeJA hormone response elements (CGTCA-motif and TGACG-motif). Additionally, CAREs associated with low temperature (LTR), drought (MYC, MBS, as-1), anaerobic conditions (ARE), and other abiotic and biotic stress-related defenses were found in most of the *EjHSF* genes. The *cis*-element number of the *EjHSF-A8b* promoter reached 78. Among these, the number of light elements (G-box) and ABA response elements (ABRE) were 12 and 10, respectively.

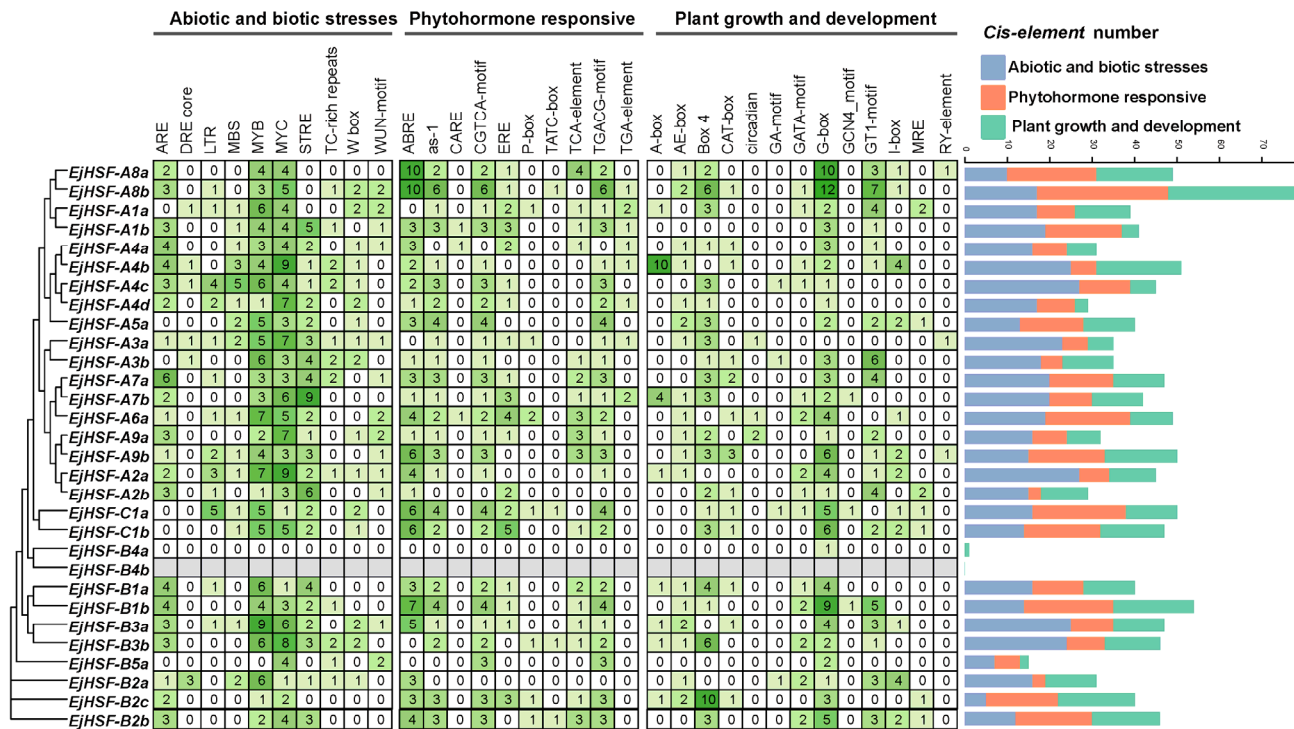


Figure 6. Distribution of cis-acting elements in promoters of *EjHSF* gene family members. The *cis*-element number of each *EjHSF* gene promoter shows in each box with green color, the greener the color, the greater the number of *cis*-element.

The *EjHSF* promoters contained more than one CARE, except for the *EjHSF-B4b* promoter, which did not contain an element. Twenty-eight *EjHSF* promoters contained drought elements (MYC) and light elements (G-box). The *EjHSF-B4a* promoter contained only one G-box element. Twenty-seven *EjHSF* promoters contained drought elements (MYB). STRE elements, activated by heat shock, were found in twenty-three *EjHSF* promoters, indicating that these genes may regulate HSFs. The *cis*-element number of the *EjHSF-A7b* promoter reached nine. Twenty-five *EjHSF* promoters contained MeJA response elements (CGTCA-motif and TGACG-motif), and twenty-four promoters contained ABA response elements (ABRE) and drought elements (as-1). These findings suggest that specific *cis*-acting elements may regulate gene expression in different tissues, such as seeds and meristematic tissues. Furthermore, it is speculated that *EjHSF* genes might participate in plant growth developmental processes and respond to various abiotic and biotic stresses.

3.6. Expression Patterns of *EjHSFs* in Various Tissues

Further analysis was conducted using the available RNA-seq data from ten tissue types of loquat to investigate the specific expression patterns of the thirty *EjHSF* genes (Figure 7; Supplementary Table S3). Among them, eleven *EjHSF* genes were highly expressed in different tissues. For instance, *EjHSF-B1a* revealed high expression levels in stem, root, flower, inflorescence, young fruit, and mature fruit tissues, especially in mature fruit and stem tissues. *EjHSF-A4c* were highly expressed genes in stem, root, young leaf, mature leaf,

young fruit, and mature fruit tissues. *EjHSF-A8b* showed highly expressed genes in stem, root, flower, and young fruit tissues, while *EjHSF-A2a* was highly expressed in seed, flower, and stem tissues. *EjHSF-B2b* exhibited significantly higher expression levels in root, young leaf, flower, pollen, young fruit, and mature fruit tissues, especially young fruit. Meanwhile, *EjHSF-B4b* was highly expressed in root and inflorescence tissues. *EjHSF-C1b* was highly expressed in stem and root tissues. Conversely, *EjHSF-A4a* was the highest expressed in roots. The *EjHSF-A4d*, *EjHSF-B4a*, and *EjHSF-C1a* genes indicated high transcriptional abundance in roots but exhibited low expression levels in other tissues, suggesting potential developmental roles. Meanwhile, five *EjHSFs* (*A7b*, *A3b*, *A2b*, *B5a*, and *A6a*) exhibited low expression levels in all tested tissues. It was suggested that these genes may be highly expressed in other tissues.

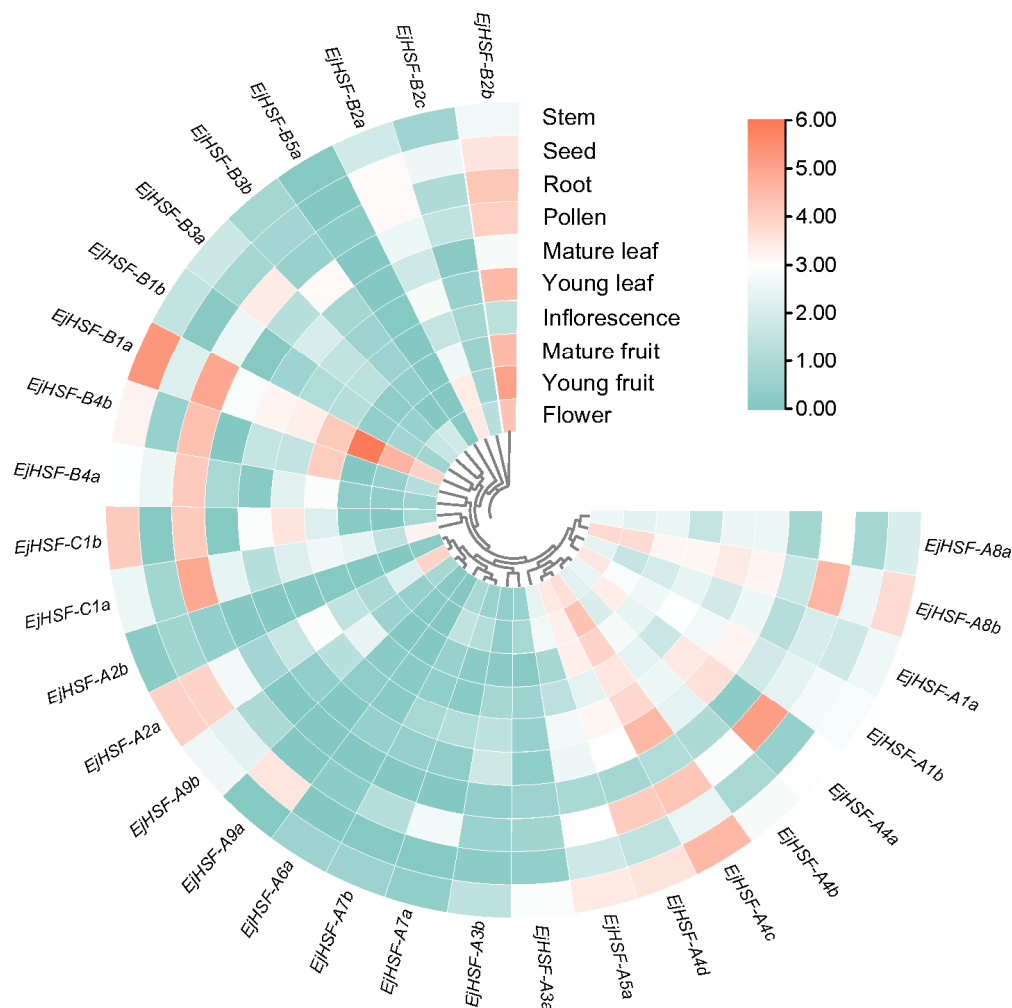


Figure 7. Expression heatmap of *EjHSF* genes in different tissues (root, stem, young leaf, mature leaf, flower, inflorescence, pollen, young fruit, mature fruit, and seed tissues). FPKM values of *EjHSF* genes were transformed via log2.

3.7. Expression Profiles of *EjHSF* Genes Under Heat Stress

This study collected and analyzed the expression data under high-temperature stresses to investigate the expression profiles of *EjHSF* genes under heat-stress conditions. Among the different heat stress durations applied (Figure 8; Supplementary Table S4), eleven *EjHSFs* (*A3a*, *A5a*, *A2a*, *A2b*, *A7a*, *B2c*, *A7b*, *B1b*, *B2a*, *B2b*, and *A8b*) were upregulated to different degrees, while four *EjHSFs* (*B1a*, *A1b*, *A4d*, and *A8a*) were downregulated. Both *EjHSF-A2a* and *EjHSF-A2b* were upregulated by up to 300 times compared with the control. Meanwhile, four *EjHSFs* (*B2c*, *B2b*, *A7a*, and *A7b*) were continuously increased (log2-based

fold change >1) based on the processing time under heat-stress conditions. *EjHSF-A3a* and *EjHSF-A5a* initially exhibited higher expression, followed by decreased expression 1 h after the initiation of treatment. *EjHSF-B1a* was significantly downregulated under heat stress, whereas fourteen *EjHSFs* (*A1a*, *B3a*, *B3b*, *A3b*, *C1a*, *A9a*, *B4a*, *C1b*, *A6a*, *B4b*, *A9b*, *B5a*, *A4a*, and *A4b*) consistently displayed low expression levels regardless of the presence of heat stress. Meanwhile, *EjHSF-A4c* consistently displayed an increasing trend over time regardless of whether heat stress was present.

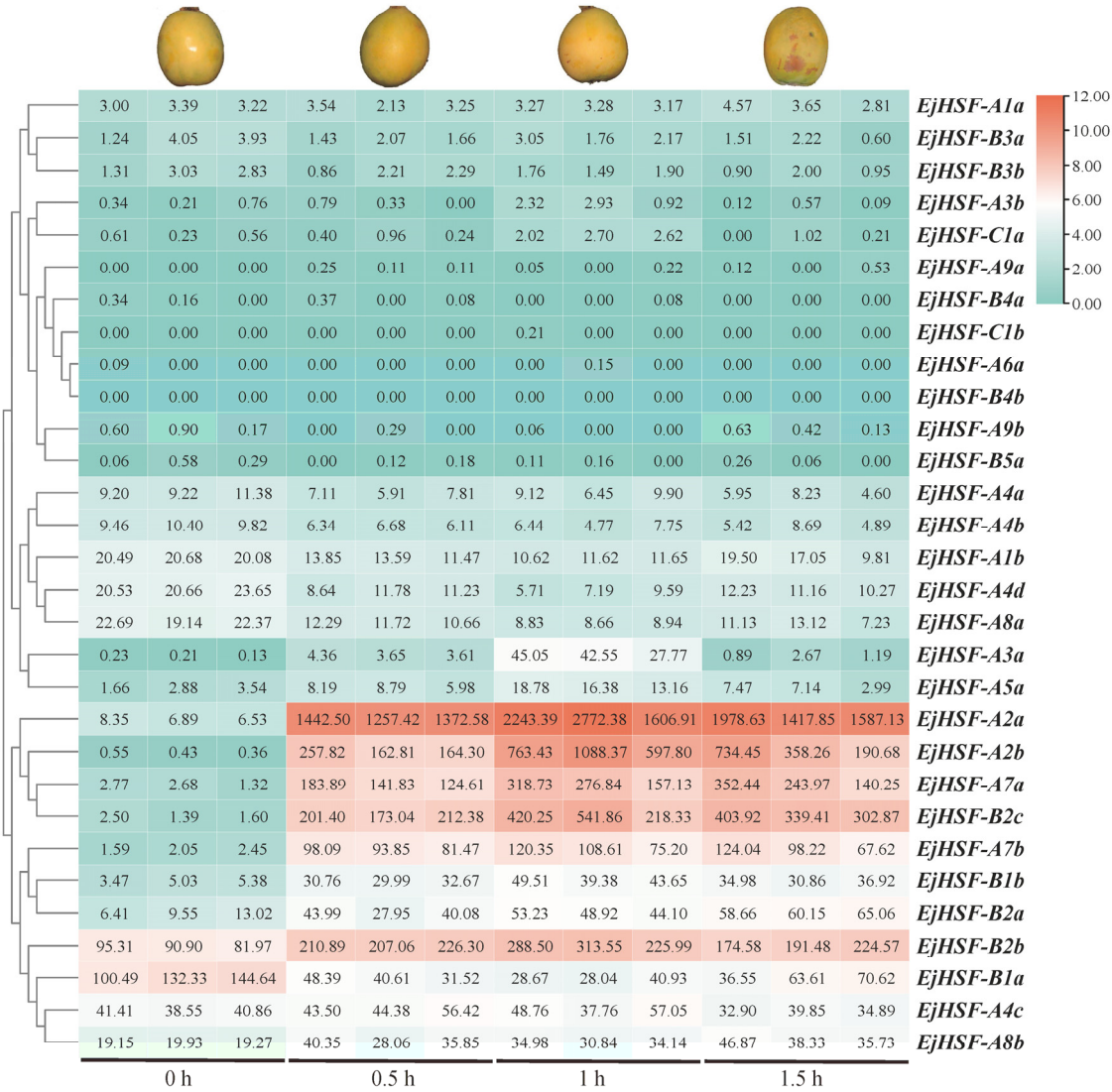


Figure 8. Heatmap revealing differential expression patterns of *EjHSF* genes in the color-changing period of ‘Wuduiyadanben’ loquat fruit under control conditions and 40 °C heat treatment. 0 h: control group, 0.5 h: 0.5 h under heat stress, 1 h: 1 h under heat stress, and 1.5 h: 1.5 h under heat stress. The number in the box represents the raw TPM (transcripts per kilobase of exon model per million mapped reads). The different colors represent FPKM-normalized log2-converted counts, where red represents high expression, and green represents low expression.

4. Discussion

With the publication of the loquat genome sequence, a series of *HSF* families have undergone genome-wide identification in the last years, such as rye (*Secale cereale* L.) [29], peanut (*Arachis hypogaea* L.) [30], peach (*Prunus persica*) [31], and pineapple (*Ananas comosus*) [32]. The loquat is an important economic fruit crop and medicinal plant. However, the yield and quality of loquat fruits are significantly influenced by various environmental

stresses. Thus, characterizing gene families in the loquat genome is crucial for understanding their role in the plant's response to environmental stresses. In this study, thirty *EjHSF* genes were identified in the *Eriobotrya japonica* genomic sequence, including eighteen *EjHSF-A*, ten *EjHSF-B*, and two *EjHSF-C* genes, which reflected a similar distribution proportion to that seen among *HSF* genes found in other higher plants' genomes.

EjHSF genes revealed an uneven distribution among chromosomes, suggesting genetic divergence during evolution. Twenty-nine *EjHSF* genes were distributed across sixteen chromosomes, with most members located on Chr-14, while no *EjHSF* gene was found on Chr-6. A similar phenomenon was observed in peaches, where eighteen *PpHSFs* were distributed among chromosomes 1–8, except Chr-6 [31]. Similarly, pineapples had nineteen *AcHSF* genes mapped onto its eleven chromosomes, while three *AcHSF* genes were located on a scaffold instead of specific chromosomes [32]. Soybeans exhibited a distribution of thirty-eight *HSF* genes across fifteen chromosomes, excluding chromosomes 2, 6, 7, 12, and 18 [33]. This observation reveals that some *Rosaceae* plant genes are not located on Chr-6.

Phylogenetic analysis divided the identified *EjHSF* genes into 15 families comprising 30 members. Gene families are genes from a common ancestor, often retaining similarities [34]. *EjHSF-A4c* and *EjHSF-A4d* demonstrated closer similarity to *LIHSFA4* from *Lilium longiflorum* plant species, which positively regulates the response against *B. cinerea* infection via the *LIWRKY33-LIHSFA4-L1CAT2* regulatory module [35]. Consequently, *EjHSF-A4c* and *EjHSF-A4d* may also participate in the disease response. Similarly, *EjHSF-A1a* and *EjHSF-A1b* belonged to the same subfamily as *Solanum lycopersicum* *SIHSFA1a*. *SIHsfA1a* maintains pollen thermotolerance and cellular homeostasis by enhancing antioxidant capacity and protein repair and degradation, ultimately improving pollen viability and fertility [36]. Hence, further investigation is needed to explore the potential involvement of *EjHSF-A1a* and *EjHSF-A1b* in pollen development. Moreover, *EjHSF-C1a* and *EjHSF-C1b* shared the same cluster as *Pyrus betulifolia*'s *PbHSFC1a*, known for its ability to improve drought tolerance by coordinating ABA biosynthesis and H_2O_2 signaling pathways [15]. This suggests that *EjHSF-C1a* and *EjHSF-C1b* might participate in drought tolerance.

Gene duplication is essential for evolution because it facilitates the production of new genes and gene functions. Twenty-six *EjHSF* genes had undergone duplication events. Similar results were found in chickpeas and peas [18]. Synteny analysis is a method used to compare the arrangement of genes and their relative positions on the chromosomes of different species. Synteny analysis revealed homology between the loquat *HSF* family and other plants belonging to the *Rosaceae* family, such as *Malus domestica*, *Prunus persica*, and *Gillenia trifoliata*. High homology was observed between *EjHSFs* and *Malus domestica* (97%), indicating the conservation of *HSF* gene expression during plant evolution in these two species, and these processes are likely regulated via similar mechanisms in both species [18]. However, significant divergence was noted when loquat *HSF* genes were compared with species such as *Arabidopsis thaliana* (86%), *Ananas comosus* (74%), and *Oryza sativa* (65%). Kanwar et al. [18] found that many pea *PsHSF* genes were conserved between peas and *Arabidopsis*, and eleven *PsHSFs* exhibited synteny with *AtHSFs*, providing evidence that duplication events played a crucial role in genome expansion. This study discovered that twenty-nine *EjHSFs* showed synteny with thirty-one apple *MdHSFs*, suggesting that *EjHSFs* might have similar functions to the apple genes.

Most *EjHSF* genes examined in the gene structure analysis contained two exons and one intron, suggesting their contribution to transcriptional regulation under stress conditions. *EjHSF-A3a* was an exception, possessing six exons and five introns, implying the possible occurrence of gene fragment insertions or deletions during the evolutionary process. Furthermore, *EjHSF* genes within the same subfamily, such as *EjHSF-A2a* and *EjHSF-A2b*, exhibited similar gene structures, supporting their phylogenetic relationships. Subfamily A indicated greater structural differences in the number of introns, which play an important role in gene transcriptional regulation under stress conditions. In loquats, four protein motifs (motifs 1, 2, 4, and 5) were commonly found in the *EjHSF* gene structure,

indicating their close association with SPL proteins. Motif 7 was exclusively involved in class B, while motif 3 was specially contained in classes A and C, but not B. These results suggest that genes from the same subfamily tend to cluster together based on similar genetic compositions and structures to those observed in other plants. In alfalfa, protein motifs 1 and 3 were highly conserved across all *MsHSP* genes, while motifs 7 and 9 were only present in HSF A. Motif 8 was exclusive to HSF class B [37]. Similarly, peaches exhibited motifs 1–3 present in each *PpHSF*, while motif 4 occurred only in classes A and B [31]. Pineapples' classes A and B mostly contained motif 4. In contrast, motif 3 existed solely in class A and C *HSFs*, not class B [32]. Conserved protein motifs constitute the structural basis for the biological function of *HSFs*. The same protein is distributed in different HSF subfamilies among different plants.

In peaches, every promoter contained at least two MYB elements. The next most common elements were MYC, CGTCA- and TGACG-motifs, ARE, ERE, and MBS [31]. The STRE motif was the most common element in petunias, and the other *cis*-acting elements were ARE and Box 4. The *PtHSF01* promoter in kiwifruits contains the highest number of *cis*-acting elements (class A), while the *PtHSF23* (class C) promoter had the fewest *cis*-acting elements [38]. In rye, the *ScHSF* gene covers many phytohormone response elements, including ABR and MeJA hormone response elements (CGTCA-motif and TGACG-motif) [29]. In asparaguses, almost all *AoHSF* members were found to possess both hormone- and stress-related features, with *AoHSF06* identified as containing an RY element related to sprouting [39]. Overall, *HSF* promoters in different plants universally express hormone response- and stress-related elements. The analysis of *cis*-acting elements within the *EjHSF* promoters revealed thirty-three CAREs that were functionally relevant and connected to the expression of *HSFs*. Twenty-eight *EjHSF* promoters contained drought elements (MYC) and light elements (G-box). Twenty-seven *EjHSF* promoters contained drought elements (MYB). Twenty-five *EjHSF* promoters contained MeJA response elements (CGTCA-motif and TGACG-motif), and twenty-four promoters contained ABA response elements (ABRE) and drought elements (as-1). STRE elements, activated by heat shock, were found in twenty-three *EjHSF* promoters, indicating that these genes may regulate *HSFs*. *EjHSF* genes may also be involved in multiple signaling pathways and play a critical role in regulating development, abiotic stress, and hormone synthesis in loquats.

In the tomato, *SlHSEF1a* plays a crucial role in maintaining pollen heat resistance and improving pollen viability and fertility [36]. In the loquat, the expression patterns of *EjHSFs* in different tissues and organs were analyzed, and distinct tissue-specific expression patterns were revealed among different *EjHSFs*. Eleven *EjHSFs* (A2a, A4a, A4c, A4d, A8b, B1a, B2b, B4a, B4b, C1a, and C1b) were highly expressed in different tissues. Ten *EjHSFs* (A4a, A4c, A4d, A8b, B1a, B2b, B4a, B4b, C1a, and C1b) had high expression levels in roots. Among them, *EjHSF-A4a* showed the highest expression. *EjHSF-C* subfamilies exhibited high expression levels in roots but low levels in fruits, indicating their potential role in plant growth regulation. *EjHSF-B1a* exhibited the highest expression levels in mature fruits and stems, while *EjHSF-B2b* showed the highest expression levels in young fruits. Conversely, five *EjHSFs* (A7b, A3b, A2b, B5a, and A6a) revealed low expression across all tissues examined. Further investigation is needed to explore the potential function of *EjHSFs*.

Most plant *HSFs* known to date play crucial regulatory roles in response to environmental stress, particularly heat stress. For example, *CaHSEF2* was activated by *HSEF6a*, which was directly targeted and activated by *CaHDZ15* and further improved the heat resistance of peppers [40]. Grape *VdHSEF2* of the heat-tolerant *Vitis davidii* 'Tangwei' and *VvHSEF2* of the heat-sensitive *Vitis vinifera* 'Jingxiu' are positive regulators in grape thermotolerance, and *VdHSEF2* can confer higher heat resistance than *VvHSEF2* [16]. Overexpression of lily *LHsfA2*, rice *OsHsfA2e*, and tall fescue *FaHsfA2c* enhanced plant heat resistance [41–43]. In the research results, *EjHSF-A2a* and *EjHSF-A2b* belonged to the HSF-A2 subfamily. The phylogenetic analysis showed that *EjHSF-A2a* and *EjHSF-A2b* were highly homologous to *AtHSEF2a*. *EjHSF-A2a* was highly expressed in seed, flower, and stem tissues, while *EjHSF-A2b* exhibited low expression levels in all tested tissues. The

transcriptional abundance of *EjHSF-A2a* and *EjHSF-A2b* changed by over 300 times after 1 h of heat stress treatment, wherein the *EjHSF-A2a* expression level was notably upregulated. Their specific functions have been followed up for further study.

TrHSFB2a of white clover (*Trifolium repens*) was highly homologous to *MtHSFB2b*, *CarHSFB2a*, *AtHSFB2b*, and *AtHSFB2a* and exhibited more strongly induced high expression after heat stress treatment for 3 h and then reduced continuously. Under drought-, salt-, and heat-stress conditions, the overexpression of *TrHSFB2a* in *Arabidopsis* significantly reduced drought, salt, and heat resistance. Meanwhile, silencing *AtHSFB2a* via RNA interference in *Arabidopsis* showed an opposite trend to wild-type *Arabidopsis* plants [12]. These observations were also observed in the transgenic lines developed via the overexpression and RNA interference homolog of *OsHSFB2b* in rice [44] and the overexpression of chickpea *CarHSFB2b* in *Arabidopsis* [45]. Apple plants that overexpressed *MdHSF4*, *MdHSFB2a*, and *MdHSFA1d* exhibited improved heat resistance and rescued the heat-sensitive phenotype of *MdWRKY75-Ri3* [15]. In this study, *EjHSFs-B2* was highly homologous to *MdHSFB2* and *AtHSFB2*. *EjHSF-B2b* exhibited significantly higher expression levels in young fruits and showed high expression in root, young leaf, flower, pollen, and mature fruit tissues. Meanwhile, the expressions of *EjHSF-B2b*, *EjHSF-B2c*, and *EjHSF-B2b* continuously increased with the processing time under heat-stress conditions, suggesting their potentially similar functions.

In the tomato, *SIHSFB1* suppression strongly enhanced the induction of HSP due to the higher activity of other HS-induced HSFs under heat stress (HS), resulting in increased heat resistance compared with the wild type. *SIHSFB1* overexpression stimulates the co-activator function of *SIHSFB1* and, consequently, the accumulation of HS-related proteins under non-stress conditions. Plants with enhanced *SIHSFB1* levels show high heat resistance [46]. Frangkostefanakis et al. [46] indicated that *SIHSFB1* acts as both a co-activator of *HSFA1a* and a transcriptional repressor of other HSFs (*HSFA1b* and *HSFA2*). This dual function explains the activation of chaperones to enhance protection and regulate the balance between growth and stress responses upon deviations from the homeostatic levels of *SIHSFB1* in tomatoes [46]. In this study, *EjHSF-B1a* belonged to the same *EjHSF-B1* subclusters. *EjHSF-B1a* revealed the highest expression levels in mature fruits and stems and high expression levels in other tissues (root, flower, inflorescence, and young fruit tissues). *EjHSF-B1a* was significantly downregulated in loquat fruits under heat stress, while *EjHSF-B1b* expression slightly increased. Whether *EjHSFB1* has the same function as *SIHSFB1* requires further study.

5. Conclusions

In conclusion, this study showed that the diverse expression patterns of *EjHSFs* collectively contribute to the loquat's adaptation to heat-stress conditions, highlighting the important regulatory role of *HSF* genes during *Eriobotrya japonica*'s response to heat stress. This warrants further investigation and attention for genomic improvement and medical applications related to high-temperature resistance.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/horticulturae10111195/s1>, Table S1: Synteny analysis; Table S2: Motif consensus; Table S3: The expression profile of *EjHSFs* in different tissues; Table S4: The expression profile of *EjHSFs* under heat stress [1,3].

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