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Genome-Wide Identification and Characterization of *Hsf* and *Hsp* Gene Families and Gene Expression Analysis under Heat Stress in Eggplant (*Solanum melongema* L.)

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Abstract: Under high temperature stress, a large number of proteins in plant cells will be denatured and inactivated. Meanwhile Hsfs and Hsps will be quickly induced to remove denatured proteins, so as to avoid programmed cell death, thus enhancing the thermotolerance of plants. Here, a comprehensive identification and analysis of the *Hsf* and *Hsp* gene families in eggplant under heat stress was performed. A total of 24 *Hsf*-like genes and 117 *Hsp*-like genes were identified from the eggplant genome using the interolog from Arabidopsis. The gene structure and motif composition of *Hsf* and *Hsp* genes were relatively conserved in each subfamily in eggplant. RNA-seq data and qRT-PCR analysis showed that the expressions of most eggplant *Hsf* and *Hsp* genes were increased upon exposure to heat stress, especially in thermotolerant line. The comprehensive analysis indicated that different sets of *SmHsps* genes were involved downstream of particular *SmHsfs* genes. These results provided a basis for revealing the roles of *SmHsps* and *SmHsp* for thermotolerance in eggplant, which may potentially be useful for understanding the thermotolerance mechanism involving *SmHsps* and *SmHsp* in eggplant.

Keywords: eggplant; heat shock factor (Hsf); heat shock protein (Hsp); heat stress; thermotolerance

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1. Introduction

Plants live in complex environments where multiple abiotic stresses, such as salt, drought and extreme temperature, may seriously restrict their growth and development [1]. As sessile organisms, plants cannot move to avoid these stresses and, thus, they have developed mechanisms, such as enhanced expression of tolerance-related genes, in response to heat stress [2,3]. To survive and acclimatize under adverse environment conditions, plants have established self-defense mechanisms during the course of long-term evolution. Previous studies have shown that under heat stress (HS), plant cells respond rapidly to high temperatures by inducing the expression of genes encoding heat shock proteins (Hsps), which are involved in preventing heat-related damage and confer plant thermotolerance in strawberry, walnut, barley and grapevines [4–7]. Many Hsps function as molecular chaperones in preventing protein misfolding and aggregation, consequently maintaining protein homeostasis in cells and inducing acquired thermotolerance in plants [8]. The expression of Hsps is controlled and regulated by specific types of transcription factors called heat shock factors (Hsfs), which normally exist as inactive proteins [9].

Currently, many plant *Hsf* and *Hsp* genes from various species have been isolated and comprehensively studied. Based on their approximate molecular weights and sequence homologies, Hsps are classified into five families, namely, the small *Hsp* (*sHsp*), *Hsp60s*,

Hsp70s, *Hsp90s* and *Hsp100s* [10]. The expression of *sHsp* is positively correlated with thermostability [11]. As chaperones, *Hsp60* proteins participate in the folding and aggregation of many proteins transported to organelles, such as chloroplasts and mitochondria [12]. *Hsp70* chaperones, together with their co-chaperones, make up a set of prominent cellular machines that assist with a wide range of protein folding processes in almost all cellular compartments [13]. In Arabidopsis TU8 mutants, the downregulation of *Hsp90* expression leads to mutants that are more sensitive to heat. In *Arabidopsis thaliana* seedlings, fungi producing *Hsp90* inhibitors increase the expression of the *Hsp101* and *Hsp70* genes, resulting in the enhancement of plant heat resistance [14]. Arabidopsis has at least 21 *Hsf*s [15]. *HsfA1a*, *HsfA1b* and *HsfA1d* act as the main positive regulators of the heat shock response [16] and HsfA2 can enhance the thermotolerance of plants [17]. Above all, *Hsfs* and *Hsps* play crucial roles in plant thermotolerance. The *Hsf* and *Hsp* gene families have been extensively studied in the model plant *Arabidopsis thaliana* and in non-model plants, such as rice (*Oryza sativa*) [18], poplar (*Populus trichocarpa*) [19], maize (*Zea mays*) [20] and Chinese cabbage (*Brassica rapa* L. ssp. *pekinensis*) [21].

Eggplant (*Solanum melongema* L.) is an important economic solanaceous crop, ranking third, after potato and tomato. Eggplant is primarily cultivated in East Asia, South Asia, the Middle East and northern Africa. The optimal temperature for eggplant growth and development ranges from 22 °C to 30 °C. With global warming, the temperature in subtropical and tropical regions is often above 35 °C, resulting in serious heat injury in eggplant, including limited plant growth, reduced productivity and damaged quality [2]. Thermotolerance is an important agronomic trait for eggplants, but the molecular mechanisms of heat tolerance remain elusive. Hsfs and Hsps play core roles in the signal transduction pathways involved in plant response to heat stress. Due to the vital regulatory functions of *Hsf* and *Hsp* genes in plant responses to heat stress, *Hsf* and *Hsp* genes in eggplant under heat stress were studied. The eggplant genome was sequenced and assembled [22], enabling the characterization of the eggplant *Hsf* and *Hsp* families and their responses to heat stresses at the molecular level. Therefore, genome-wide identification of *Hsf* and *Hsp* genes in eggplant was conducted to infer their expansion and evolutionary history. RNA-seq data and quantitative real-time RT-PCR analyses were used to explore their expression difference in the thermotolerant line 05-4 and the thermosensitive line 05-1 as elicited by naturally increased temperature. The results provide a relatively complete profile of the *Hsf* and *Hsp* gene families in eggplant and elucidate their relationship with thermotolerance, which provides a foundation for further functional research on these genes in eggplant. Furthermore, these findings could potentially be useful for understanding the mechanism of thermotolerance mediated by Hsfs and Hsps in eggplant.

2. Materials and Methods

2.1. Identification and Classification of Hsf and Hsp Family Members in Eggplant

Published Arabidopsis *Hsf* and *Hsp* sequences [23] were retrieved and used as queries in BLAST searches against the eggplant genome database (http://eggplant.kazusa.or.jp/, accessed on 6 June 2021) to identify potential eggplant *Hsfs* and *Hsps*. All output genes identified according to Arabidopsis *Hsf* and *Hsp* sequences were collected and confirmed using Pfam (http://pfam.xfam.org/search, accessed on 6 June 2021) and SMART (http://smart.embl-heidelberg.de/, accessed on 6 June 2021). The isoelectric points and molecular weights were predicted using the Compute pI/Mw tool from ExPASy (http://web.expasy.org/compute_pi, accessed on 6 June 2021).

2.2. Phylogenetic Analysis

Alignments of the full eggplant Hsf and Hsp proteins were performed using clustal X2.1 [24]. Phylogenetic trees were constructed using the neighbor-joining (NJ) method in MEGA (version 5.0) [25] with bootstrap values from 1000 replicates indicated at each node. To identify signature domains, the Hsf and Hsp protein sequences were compared with Arabidopsis and tomato. *SmHsfs* and *SmHsps* (*sHsp*, *Hsp60s*, *Hsp70s*, *Hsp90s* and *Hsp100s*)

were named based on the subfamily classification and their phylogenetic relationships with the corresponding *AtHsfs* and *AtHsps* and gene names of eggplant *sHsps* were revised according to their molecular weights in the eggplant genome database based on Hirakawa et al. [22].

2.3. Gene Structures, Conserved Motifs and Protein Functional Network Analysis

The exon and intron structures were illustrated using the Gene Structure Display Server (GSDS, http://gsds.cbi.pku.edu.cn accessed on 6 June 2021) [26] by aligning the predicted cDNA sequences with their corresponding genomic DNA sequences. The conserved motifs in the encoded proteins were analyzed using the MEME online program (http://meme.sdsc.edu, v4.9.0, accessed on 6 June 2021) [27]. MEME was run locally with the following parameters: number of repetitions = any, maximum number of motifs = 20 and optimum motif width = 6–100 residues for *Hsf*, *sHsp*, *Hsp60*, *Hsp70* and *Hsp100*. The STRING protein interaction database (http://string-db.org/, accessed on 6 June 2021) was used to analyze the interaction networks of Hsf and Hsp proteins in the highly specific protein and parameter selection model plant species *Arabidopsis thaliana*.

2.4. Plant Materials, Growth Conditions and Stress Treatments

In the present study, two inbred eggplant lines (selected by the Vegetable Research Institute, Guangdong Academy of Agricultural Sciences, Guangzhou, China), the thermotolerant line 05-4 and the thermosensitive line 05-1, were used. Eggplant seedlings were cultivated under 25/20 °C day/night conditions and a 16/8 h day/night photoperiod in a growth chamber until the four true leaves period for treatments. For the HS treatment, the seedlings of 05-1 and 05-4 with four leaves were directly placed in the 42 °C light incubator (RXZ-1000B3, Jiangnan Instrument Factory, Ningbo, China). For the heat treatment used for RNA-seq, the 3rd mature leaves of treated seedlings were collected at 0 and 6 h after HS treatment and 10 plants were used for each treatment. For the heat treatment used for the qRT-PCR, the 3rd mature leaves from two different lines were harvested at 0 and 6 h. The samples were harvested, immediately frozen in liquid nitrogen and stored at −80 °C for RNA extraction. Three biological replicates were performed and each replicate had 10 plants.

2.5. RNA Extraction and Quantitative Real-Time PCR Analysis

Total RNA was extracted using a TransZol Plant kit (TransGen Biotech/TransBionovo, Beijing, China) and the cDNA was synthesized according to the manufacturer's instructions (Takara, Dalian, China). Primers with amplicon lengths of 80–150 bp were designed using Primer5 software. All primer sequences are listed in Table S14. Real-time qRT-PCR was conducted on a Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) using the SYBR Premix Ex Taq kit (TaKaRa, Dalian, China) according to the manufacturer's instructions. The 10 µL reaction system contained 5 µL of SYBR Green Supermix (2×), 4 µL of cDNA template (30 ng/µL) and 0.5 µL of each primer (10 µM). The qRT-PCR reaction was performed using the following parameters: pre-denaturation at 95 °C for 30 s, followed by 39 cycles of denaturation at 95 °C for 5 s, annealing at 60 °C for 15 s and extension at 72 °C for 15 s. The fluorescent signal was measured at the end of each cycle and the melting curve analysis was performed by heating the PCR product from 65 °C to 90 °C to verify the specificity of the primers. Three independent biological replicates were performed and the qPCR of each replicate was performed in triplicate. The relative expression levels of eggplant *Hsf* and *Hsp* genes were calculated using the $2^{-\triangle Ct}$ method [28]. The *SmEF1a* genes were used as internal controls.

3. Results

3.1. Genome-Wide Identification and Analysis of Hsf and Hsp Gene Family Members in Eggplant

To search for *Hsf* and *Hsp* genes in eggplant, we used the conserved Hsf and Hsp domain consensus sequences of several proteins as BLASTP queries against the eggplant

genome database (http://eggplant.kazusa.or.jp/, accessed on 6 June 2021). In addition, homology searches using identified protein sequences of *Arabidopsis thaliana* were performed. After automated database searching and a manual review, 24 and 117 genes were identified as members of the *Hsf* and *Hsp* families in eggplant, respectively, whose classification and naming were based on the rules of the *Hsp* gene families from Arabidopsis and tomato, including *sHsp*, *Hsp60*, *Hsp70*, *Hsp90* and *Hsp100*. The *Hsf* and *Hsp* gene families in eggplant were relatively large compared with those in Arabidopsis and those in tomato and rice, respectively. The numbers of identified genes in the *Hsf*, *sHsp*, *Hsp60*, *Hsp70*, *Hsp90* and *Hsp100* families of eggplant were 24, 39, 21, 30, 17 and 10, respectively (Table 1).

Table 1. Numbers of *Hsf* and *Hsp* genes in Arabidopsis, eggplant, tomato and rice.

Family	Arabidopsis	Eggplant	Tomato	Rice
Hsf	22	24	23	25
Hsp20	27	39	23	39
Hsp60	18	21	16	20
Hsp70	19	30	22	24
Hsp90	7	17	8	9
Hsp100	8	10	13	10

As shown in Supplementary Materials Table S1, the amino acid lengths for Hsfs ranged from 111 (*SmHsfA1c*) to 496 (*SmHsfA1b*), with deduced molecular weights from 12.2 kDa to 54.8 kDa and the predicted isoelectric points of Hsfs were divergent, ranging from 4.60 (*SmHsfA3*) to 9.64 (*SmHsfA1d*). The length of sHsp proteins ranged from 87 (*Sm10.2-sHsp*) to244 amino acids (*Sm27.2-sHsp*) and the predicted molecular weights were between 10.2 kDa (*Sm10.2-sHsp*) and 27.2 kDa (*Sm10.2-sHsp*). In addition, the predicted pI-values of sHsp proteins ranged from 4.56 (*Sm10.2-sHsp*) to 10.49 (*Sm12.7-sHsp*). The amino acids lengths were consistent with the molecular weights of Hsp60s. The amino acid number and molecular weight for Smcpn60-4 was the highest, while that for SmCpn60-7.3 was the lowest and the predicted pI-values ranged from 5.26 (*SmCpn60-a1*) to 10.29 (*SmCpn60-7.3*). The deduced length of the Hsp70 proteins ranged from 85 (*SmmtHsc70-3*) to 914 (*SmHsp70-18*) amino acids and the highest- and lowest-molecular-weight SmHsp70s were *SmHsp70-18* (103.1 kDa) and *SmHsp70-5* (11.5 kDa), respectively, while the pI values ranged from 4.52 (*SmmtHsc70-3*) to 9.35 (*SmHsp70-19*). The length of Hsp90 proteins ranged from 137 (*SmHsp90-4.4*) to 782 (*SmHsp90-6*) amino acids, the predicted molecular weights of Hsp90s were between 16.2 kDa (*SmHsp90-4.4*) and 89.6 kDa (*SmHsp90-7.1*) and the predicted isoelectric points ranged from 4.78 (*SmHsp90-5*) to 9.55 (*SmHsp90-2.1*). The longest amino acids lengths and highest molecular weights in Hsp100s were *SmHsp100-ClpB1*, with 979 amino acids and 110.2 kDa, respectively. In contrast, the smallest was *SmHsp100-ClpC3*; the predicted isoelectric points ranged from 5.38 (*SmHsp100-ClpB3*) to 9.07 (*SmHsp100-ClpC1*) and these proteins were distributed from the alkaline to acidic.

3.2. Phylogenetic and Sequence Structure Analysis of Hsf and Hsp Proteins in Eggplant

To evaluate the evolutionary relationship of the eggplant Hsf and Hsp proteins, a phylogenetic analysis of each family was performed based on the full-length amino acid sequences from Arabidopsis, eggplant and tomato and each family could be classified into different subfamilies. The *SmHsf* family contained three subfamilies: type A (18 genes), type B (5 genes) and type C (1 gene). Based on the phylogenetic tree, class HsfA had the maximum number of subclasses among the three classes and was closer to tomato Hsf proteins, which coincided with the botanical classification (Table S2). A total of 39 *sHsp* genes could be grouped into 12 distinct subfamilies, containing 6 groups of cytosolic *sHsp* genes, C-I, C-II, C-III, C-IV, C-V and C-VI and 2 groups of mitochondrial *sHsp* genes, MT I and MT II. Notably, the C-I *sHsp* group in the eggplant genome was large, containing 24 genes, compared with 6 in Arabidopsis (Table S3). The *Hsp60* family was divided into 4 subfamilies, including cytosol-localized Cpn60 (12 genes), mitochondrion-localized Hsp60

(4 genes) and chloroplast-localized Cpn60-a (2 genes) and Cpn60-b (3 genes) (Table S4). The *Hsp70* family contains genes encoding 19 cytosolic *Hsp70s*, 4 binding proteins (BIPs, Hsp70 homologs in the ER), 3 mitochondrial Hsp70s (mtHsc70s) and 2 chloroplastid Hsp70s (cpHsc70s) (Table S5). Seventeen *Hsp90* family genes could be divided into cytoplasm (Cyt), mitochondrial (MT), endoplasmic reticulum (ER) and chloroplast, containing 8, 3, 2 and 1 proteins, respectively (Table S6). The Hsp100 family can be classified into ClpB, C, D and X classes as follows: 3 ClpB proteins (designated as B1, B2 and B3), 4 ClpC proteins (C1, C2, C3 and C4), 1 ClpD protein (D1) and 2 ClpX proteins (X1 and X2) (Table S7).

3.3. Structure of Hsf and Hsp Genes and Conserved Motifs of Hsf and Hsp Proteins in Eggplant

To obtained further insights into the structural diversity of *Hsf* and *Hsp* genes in eggplant, we used the Multiple Expectation maximization for Motif Elicitation (MEME) [27] to predict the conserved motifs shared among the related proteins within these families. In each family, 20 putative motifs were identified. The details of these motifs are listed in Tables S8–S13. Most of the closely related members in the phylogenetic tree shared common motif compositions.

The exon/intron structures of eggplant *Hsf* and *Hsp* members were analyzed based on their coding sequences and the corresponding genome sequences. The eggplant *Hsfs* shared highly conserved exon/intron structures with 0–3 intron phases (Figure 1A). The intron phases were remarkably well conserved among family members. Most of the eggplant *sHsps* did not contain introns and only a few had 1–3 introns (Figure 1B). Interestingly, in the *Hsp60* family, two members, *SmCpn60-8* and *SmHsp60-3,* had no introns in their coding regions, while the other eggplant Hsp60s contained several introns (1–22) (Figure 1C). In the *Hsp70* family, cytosolic *Hsp70s* had 0–13 introns, ER-localized BIPs had 4–7 introns, mitochondrion-localized *mtHsc70s* had 0–4 introns and chloroplast-localized *cpHsc70s* had 6 introns, while truncated *Hsp70ts* had no introns (Figure 2A). With the exception of *SmHsp90-1.1*, each *Hsp90s* member contained 1–17 introns (Figure 2B). The number of exons and introns of *Hsp100* family members differed greatly. For example, *SmHsp100-ClpX1* contained up to 16 introns, but *SmHsp100-ClpB3* and *SmHsp100-ClpC3* only had 4 introns (Figure 2C).

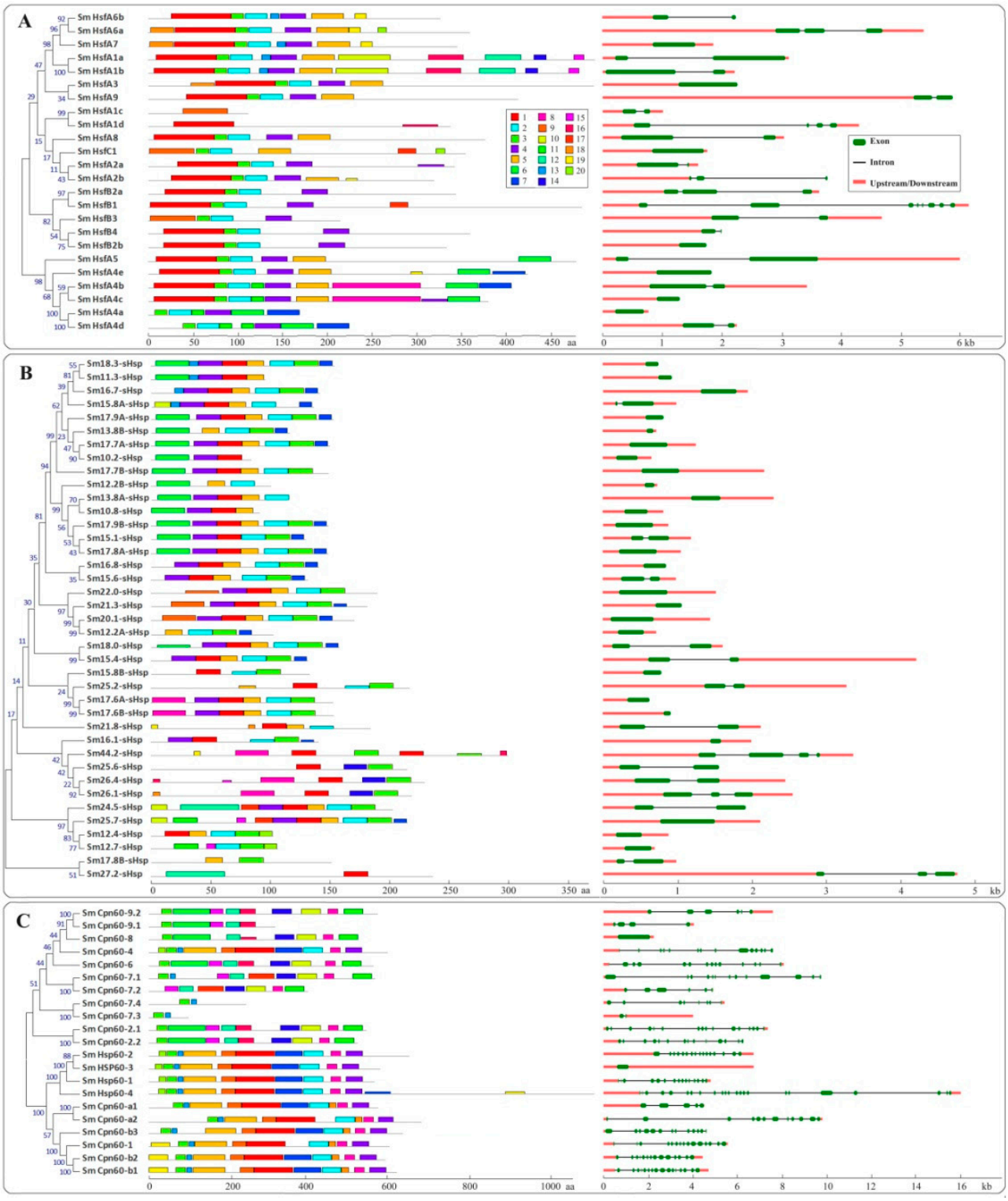

Figure 1. Phylogenetic relationships, gene structures and motif compositions of *Hsf*, *sHsp* and *Hsp60* family members in eggplant. Multiple alignment of the Hsf (**A**), sHsp (**B**) and Hsp60 (**C**) proteins from eggplant (Sm) was performed with MEGA 5.0 using the neighbor-joining (NJ) method with 1000 bootstrap replicates (left panel). A schematic representation of conserved motifs (obtained using MEME) in the Hsf and sHsp proteins is displayed in the middle panel. Different motifs are represented by differently colored boxes. Details of the individual motifs are in Tables S8–S10. Exon/intron structures of the *Hsf* and *sHsp* genes are shown in the right panel. Green boxes represent exons and black lines represent introns.

Figure 2. Phylogenetic relationships, gene structures and motif compositions of the Hsp70, Hsp90 and Hsp100 family members in *S. melongena* (Sm). Multiple alignment of the Hsp70 (**A**), Hsp90 (**B**) and Hsp100 (**C**) proteins from *S. melongena* (Sm) was performed with MEGA 5.0 using the neighbor-joining (NJ) method with 1000 bootstrap replicates (left panel). A schematic representation of conserved motifs (obtained using MEME) in the Hsp70 (**A**), Hsp90 (**B**) and Hsp100 (**C**) proteins is displayed in the right panel. Different motifs are represented by differently colored boxes. Details of the individual motifs are in Tables S11–S13. The exon/intron structures of the Hsp70 (**A**), Hsp90 (**B**) and Hsp100 (**C**) genes are shown in the middle panel. Green boxes represent exons and black lines represent introns.

3.4. Expression Patterns of Eggplant Hsf and Hsp Genes

To examine the heat response for *Hsfs* and *Hsps* in eggplant, an RNA sequencing profile (data not shown) in leaves of thermosensitive line 05-1 and thermotolerant line 05-4, at 0 and 6 h after HS treatment, was used. *Hsf* and *Hsp* genes were selected according to annotations and their expression profiles were analyzed. We analyzed the transcription levels of 18 *Hsf*, 25 *sHsp*, 6 *Hsp60*, 18 *Hsp70*, 11 *Hsp90* and 6 *Hsp100* genes in the leaves. As shown in Figure 3, for the thermosensitive line 05-1, 16 genes (89%) of the *Hsf* family were upregulated and two members, *SmHsfA4e* and *SmHsfB3*, were downregulated under HS conditions, which were more than in the thermotolerant line 05-4, in which 17 *Hsf* genes (94%) were upregulated by HS and only *SmHsfA8* was downregulated. In contrast to line 05-1, *SmHsfA4e* and *SmHsfB3* were strongly induced in treated 05-4 leaves. In the leaves of the thermotolerant line 05-4, among the upregulated members, the expression levels of most A (A1a, A1b, A3, A4a, A4b, A4d, A4e, A5, A6a and A6b), B1 and B2a were higher than those of other members under HS.

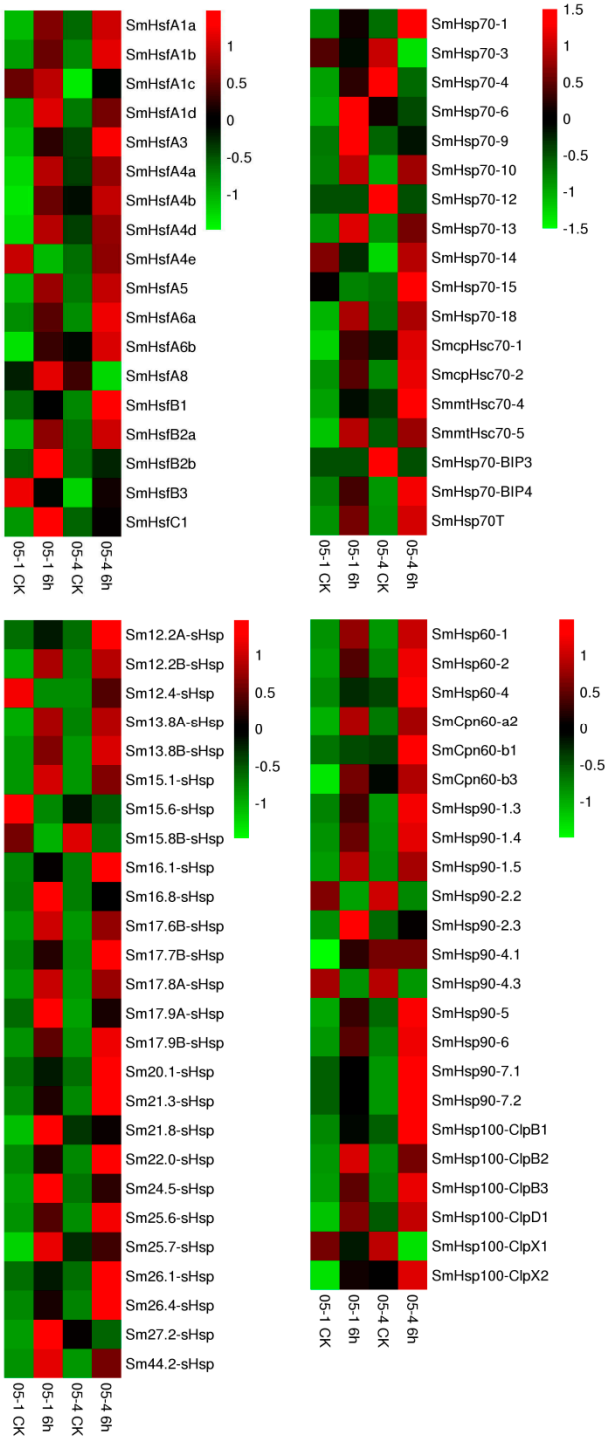

Figure 3. Expression analysis of eggplant *Hsf* and *Hsp* genes. Raw data were from RNA-seq data, in response to HS treatment in 05-1 and 05-4 leaves. HS treatment: 42 °C for 6 h; 05-1: eggplant thermosensitive line; 05-4: eggplant thermotolerant line.

A strong response to HS in all of the 25 *sHsp* genes from both lines (05-1 and 05-4) was observed, in which a majority of these genes were upregulated and only *Sm15.6-sHsp* and *Sm15.8-sHsp* were downregulated. After high-temperature treatment, the expression of *SmHsp60-1*, *SmHsp60-2*, *SmCpn60-a2* and *SmCpn60-b3* was increased in the two inbred lines, while *SmHsp60-4* and *SmCpn60-b3* was increased in 05-4 and no significant difference could be observed in 05-1. Among the 18 *Hsp70* genes, the expression was remarkably changed in response to heat treatment in the thermosensitive 05-1 and thermotolerant

05-4 leaves and these genes were upregulated in both plants. Among these upregulated genes, the expression quantity of *SmHsp70-1*, *SmcpHsp70-2* and *SmHsp70-BIP4* was higher in 05-4, compared with 05-1. However, *SmHsp70-14* and *SmHsp70-15* were increased in the thermotolerant line, but decreased in the thermosensitive line. Considering the Hsp90 genes, the expression levels of most genes (*SmHsp90-1.3*, *SmHsp90-1.4*, *SmHsp90-1.5*, *SmHsp90-2.3*, *SmHsp90-5*, *SmHsp90-6*, *SmHsp9-7.1* and *SmHsp90-7.2*) were increased and only *SmHsp90-2.2* and *SmHsp90-4.3* were downregulated in the two lines. Among the upregulated *Hsp90* genes, gene expression levels of six genes in 05-4 were obviously higher than in 05-1. After heat treatment, *SmHsp100-ClpB1*, *SmHsp100-ClpB2*, *SmHsp100-ClpB3*, *SmHsp100-ClpD1* and *SmHsp100-ClpX2* expressions in the two lines were significantly increased. Among these genes, *SmHsp100-ClpB1*, *SmHsp100-ClpB3* and *SmHsp100-ClpX2* showed higher expression in 05-4 than in 05-1, but *SmHsp100-ClpB2* was more abundant in the thermosensitive line.

3.5. Validation of Hsf and Hsp Gene Expression Levels by qRT-PCR

To verify the accuracy of the transcriptome sequencing, the expressions of 12 randomly selected genes were validated using quantitative real-time RT-PCR (qRT-PCR). The results showed that the expression pattern of each tested gene was similar to that of the transcriptome sequencing and the increase rate of all these *Hsf* and *Hsp* genes in the thermotolerant line 05-4 were significantly higher than those in thermosensitive line 05-1 (Figure 4).

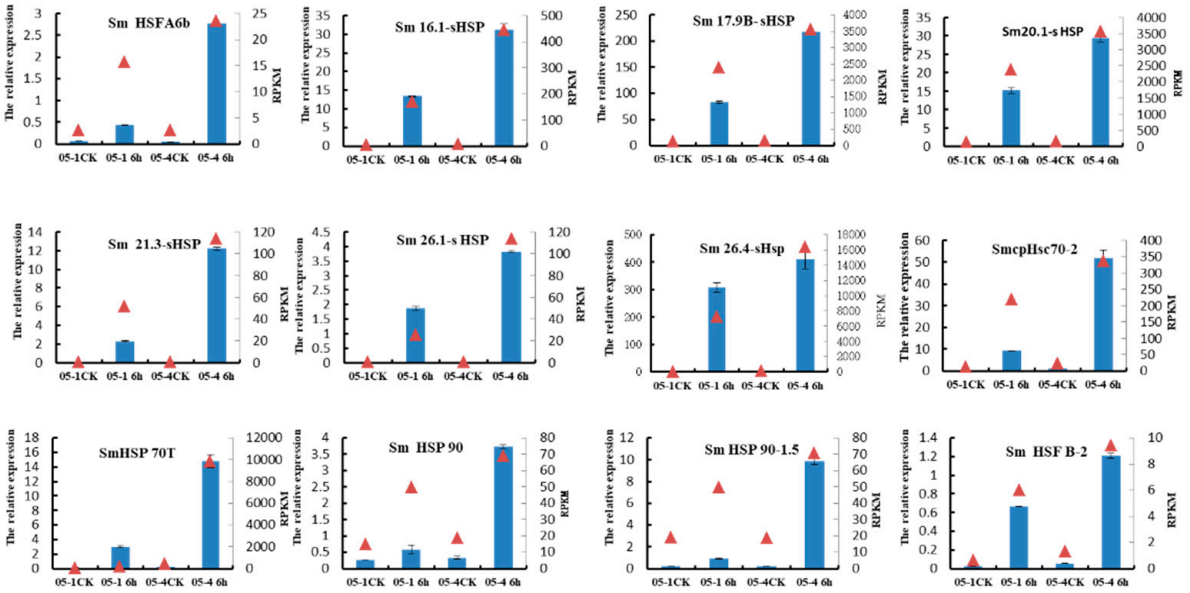

Figure 4. Comparison of transcripts expression results from RNA-seq and qRT-PCR analysis. Abscissa: Sample number; the ordinate (left): the relative expression of gene validated using qRT-PCR, represented by bar chart; coordinates (right): RPKM value obtained from the transcriptome sequencing, represented by triangle scatter diagram.

4. Discussion

Many studies have suggested that *Hsfs* and *Hsps* play central roles in plant developmental and defense processes [29,30]. Benefiting from genome availability, the functions of the *Hsf* and *Hsp* family genes have been characterized in many plants. Although *Hsfs* and *Hsps* exist in all living organisms, their numbers vary in different plants. There are 22 *Hsfs* in Arabidopsis, 25 *Hsfs* in rice [18], 30 *Hsfs* in maize [20], 25 *Hsfs* in pepper [31] and 52 *Hsfs* in soybean [32]. Compared to the 27 *sHsp* genes in Arabidopsis [33], there are 35, 51 and 27 *sHsp* genes in pepper [31], soybean [34] and Chinese cabbage [35], respectively. Previous studies have identified 18 *Hsp70* genes in Arabidopsis and 32 genes in rice [36]. The grapevine genome contains at least seven genes encoding members of the

Hsp90 super family [37]. Zhang et al. (2015) reported 28 *Hsf*, 37 *sHsp*, 28 *Hsp60*, 20 *Hsp70* and 5 *Hsp100* genes in the poplar genome [19]. However, with the limited investigations into the molecular mechanism of heat tolerance, little is known about the *Hsf* family in eggplant.

In the present study, we identified 24 *Hsf* genes, 39 *sHsp* genes, 21 *Hsp60* genes, 30 *Hsp70* genes, 17 *Hsp90* genes and 10 *Hsp100* genes based on the eggplant genome (Table 1). Although the total number of *Hsf* and *Hsp* genes was similar to that of Arabidopsis [18,38–40], rice [18,41] and tomato [42], the members of some specific *Hsf* and *Hsp* subclasses in eggplant were different from the other three species. Two members were identified that belonged to subclass HsfC2 in rice, while no eggplant *Hsf* members were classified into subclass HsfC2 and the same events were also observed in *Arabidopsis thaliana* [18] and pepper [31]. Rice is the model plant use for the monocot lineage and we inferred that the gene duplications led to the unique HsfC2 subclass in monocot species [17,42], which was the most marked difference between monocots and eudicots. In contrast, similar to tomato and *Arabidopsis thaliana* [18,43], eggplant also has members that were partitioned into the HsfA6 subclass, but no rice *Hsf* members were classified into subclass HsfA6 [44]. This finding suggested that *Hsf* genes were doubled and gained new functions during the evolution of the eggplant genome. Another interesting observation was that the subclass *HsfA9* had 1 member in eggplant, compared with 4 members in pepper [31] and *Eucalyptos grandis* (Myrtaceae) contained at least 17 closely related *HsfA9*-encoding genes [17], suggesting a gene loss event during the evolutionary process of eggplant. However, there were two *HsfA4* subclass genes in eggplant, more than in pepper *CaHsfA4*, which showed that some *Hsfs* might have the similar functions, as in maize [20]. The reasons for the increase in the *HsfA9* genes need further investigation.

The phylogenetic analysis revealed that eggplant *Hsf* and *Hsp* members were more closely related to those from tomato than to those from Arabidopsis, which was consistent with the fact that both eggplant and tomato are members of the Solanaceae family [45]. Based on the previous analysis of the evolution of *Hsfs* and *Hsps* in Chinese cabbage [21,35], rice [46] and soybean [47], *Hsf* and *Hsp* genes essentially cover all the subfamilies and are relatively stable and conserved in the evolutionary process of eggplant and most of the *Hsf* and *Hsp* gene families were closely related to the evolutionary species.

Divergences in coding regions, particularly those that change the function of the gene, reflect amino acid altering substitutions and/or alterations in exon–intron structure [19]. The differences in intron and exon structure play important roles in the evolution of family genes. Structural analyses showed that the eggplant *Hsf* genes contained 0–7 introns and there were significant differences in the intron length; similar results were also obtained in cucumber [48], rice [49] and chickpea [50], but this result was different from that of pepper [31], for which all members have one intron. The number of introns of the *Hsp* gene family members in eggplant also showed differences, similar to the results of previous studies on poplar *sHsp*, *Hsp60*, *Hsp70* and *Hsp100* [19]. Qiao (2015), researching the pear *Hsf* and Guo (2015), researching the pepper *Hsp20*, showed a lack of conserved motifs among all the family genes and none of these genes contained the whole sequence, consistent with the eggplant *Hsfs* and *Hsps* in the present study [31,51]. We speculated that the deletion of introns and domains leads to structural changes during evolution, leading to functional diversity in *Hsf* and *Hsp* genes in eggplant; however, this theory needs experimental confirmation.

Hsfs, as transcriptional activators of *Hsps*, cooperate with *Hsps* to form a network responding to various stresses. These factors play a broad role in the tolerance to multiple environmental stress treatments apart from heat stress [52,53]. The comprehensive analysis of the expression for individual *Hsf* and *Hsp* members under HS was necessary for further functional analyses in plant thermotolerance [23,54]. The present study showed that most members of the eggplant *sHsp*, *Hsp60*, *Hsp70*, *Hsp90* and *Hsp100* families were induced by HS treatment in lines 05-1 and 05-4 and only a few members were significantly downregulated. Several studies have indicated that the expression and accumulation of

heat shock proteins and heat shock transcription factors can enhance the thermostability of tomato [55], wheat [56] and rice [57]. *Hsfs* are activated under HS conditions and subsequently bind the HSE elements of the promoters of the *Hsp* genes to regulate the expression of downstream *Hsp* genes [17]. The accumulation of the *Hsps* effectively reduces the damage from HS and enhances thermotolerance by binding denaturing proteins and preventing them from irreversible aggregation [58]. Thus far, only *sHsp* has been shown to play a major role in improving plant thermotolerance in the form of molecular chaperones and cell membrane stabilizing factors [59]. However, the specific mechanisms of other *Hsp* genes are less well established. Previous studies have shown that the response of plants to high temperature was a quantitative trait controlled by multiple genes; some normal genes were closed and some stress tolerance-related genes were induced under high-temperature stress, thus altering plant morphogenesis, physiological functions and biochemical and molecular structures, which in turn influenced the growth of plants [60]. In addition, heat shock proteins are different from other stress proteins and have their own unique characteristics. In the present study, *Hsps* (*sHsp*, *Hsp60*, *Hsp70*, *Hsp90* and *Hsp100*) showed species diversity, universal distribution and instantaneous response and structural conservation. For example, the synthesis of heat shock protein was fast, beginning between the first few minutes and tens of minutes and the expression lasted for up to several hours, occasionally continuing for 12 or more hours (Figure 4). Similar results were also observed in poplar [19] and grape [61].

In Arabidopsis, there are four members of the HsfA1 family, A1a, A1b, A1c and A1d [62]. Studies have shown that HsfA1a can directly sense heat stress and become activated and the same treatments also induced the binding to *Hsp18.2* and *Hsp70* promoters, as examined by chromatin immunoprecipitation [63]. Overexpressing *HsfA1a* enhances diverse stress tolerance by promoting stress-induced *Hsp18.2* and *Hsp70* gene expression [64]. In addition, *AtHsfA1* was also related to drought stress [65] and programmed cell death [66]. Thus, in eggplant, *HsfA1* may also play a similar function to *AtHsfA1* and simultaneously communicate with *Hsps*. Increasing evidence suggests that *Hsp* is one of the most important heat stress proteins regulated by *Hsf* and is the material basis of the response of plant cells to high temperature damage [67–69]. Once exposed to high temperature, most of *Hsf* and *Hsp* genes in eggplant were induced to express rapidly and the expression level of these genes in the thermotolerant line was much higher than that in the thermosensitive line. Therefore, the Hsf–Hsp involved protein degradation pathway is also the main pathway of eggplant response to high temperature stress and may play an important role in the production of heat-tolerance in eggplant. The results provide a foundation for further functional research of these genes in eggplant, which could potentially be useful for elucidating the mechanism of thermotolerance in eggplant, even in other solanaceous plants.

5. Conclusions

In the present study, 24 *Hsf* genes and 117 *Hsp* genes (including *sHsp*, *Hsp60*, *Hsp70* and *Hsp100*) were identified from the eggplant genome. The phylogeny, gene structure, expression profiles and heat stress responses of these genes were analyzed. The total number of *Hsf* and *Hsp* genes of eggplant was similar to that of Arabidopsis, tomato and rice, covering all the subfamilies, and the gene structure and motif composition were relatively stable and conserved in the evolutionary process. *SmHsf* genes, as key transcriptional activators of *Hsp* genes, regulated different subfamilies of *Hsps* in eggplant. Most of *Hsf* and *Hsp* genes are highly induced by HS in eggplant leaves, which indicated these genes participate in the response to heat stress. The expression levels of these genes in the thermotolerant line were enhanced significantly higher than those of in the thermosensitive line under HS in eggplant, which may be the main reason for strong thermotolerance in thermotolerant eggplant. According to the above results, it is expected to evaluate the thermotolerance of different eggplant resources by analyzing the expression change of specific *Hsf* and *Hsp* genes under HS and even the thermotolerance of other solanaceae species resources. The present study was undertaken to establish a solid

foundation for functional research on the eggplant *Hsf* and *Hsp* gene families and broaden our understanding of the mechanism of thermotolerance mediated by *Hsf* and *Hsp* genes in solanaceous plants.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/horticulturae7060149/s1, Table S1: *Hsf* and *Hsp* gene families in eggplant. Table S2: Phylogenetic analysis of Hsf proteins in eggplant, Arabidopsis, and tomato. Table S3: Phylogenetic analysis of sHsp proteins in eggplant, Arabidopsis, and tomato. Table S4: Phylogenetic analysis of Hsp60 proteins in eggplant, Arabidopsis, and tomato. Table S5: Phylogenetic analysis of Hsp70 proteins in eggplant, Arabidopsis, and tomato. Table S6: Phylogenetic analysis of Hsp90 proteins in eggplant, Arabidopsis, and tomato. Table S7: Phylogenetic analysis of Hsp100 proteins in eggplant, Arabidopsis, and tomato. Table S8: Sequence logos for the conserved motifs of Hsf proteins in Arabidopsis and eggplant. Table S9: Sequence logos for the conserved motifs of sHsp proteins in Arabidopsis and eggplant. Table S10: Sequence logos for the conserved motifs of Hsp60 proteins in Arabidopsis and eggplant. Table S11: Sequence logos for the conserved motifs of Hsp70 proteins in Arabidopsis and eggplant. Table S12: Sequence logos for the conserved motifs of Hsp90 proteins in Arabidopsis and eggplant. Table S13: Sequence logos for the conserved motifs of Hsp100 proteins in Arabidopsis and eggplant. Table S14: Primers used in qRT-PCR analysis.

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