Insect science

Insect Science (2019) 26, 44–57, DOI 10.1111/1744-7917.12505

ORIGINAL ARTICLE

Genome-wide identification and characterization of *HSP* gene superfamily in whitefly (*Bemisia tabaci*) and expression profiling analysis under temperature stress

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Abstract Heat shock proteins (HSP) are essential molecular chaperones that play important roles in the stress stimulation of insects. *Bemisia tabaci*, a phloem feeder and invasive species, can cause extensive crop damage through direct feeding and transmission of plant viruses. Here we employed comprehensive genomics approaches to identity HSP superfamily members in the Middle East Asia Minor 1 whitefly genome. In total, we identified 26 *Hsp* genes, including three *Hsp90*, 17 *Hsp70*, one *Hsp60* and five *sHSP* (small heat shock protein) genes. The *HSP* gene superfamily of whitefly is expanded compared with the other five insects surveyed here. The gene structures among the same families are relatively conserved. Meanwhile, the motif compositions and secondary structures of BtHsp proteins were predicted. In addition, quantitative polymerase chain reaction analysis showed that the expression patterns of *BtHsp* gene superfamily were diverse across different tissues of whiteflies. Most *Hsp* genes were induced or repressed by thermal stress (40°C) and cold treatment (4°C) in whitefly. Silencing the expression of *BtHsp70-6* significantly decreased the survival rate of whitefly under 45°C. All the results showed the *Hsp*s conferred thermo-tolerance or cold-tolerance to whiteflies that protect them from being affected by detrimental temperature conditions. Our observations highlighted the molecular evolutionary properties and the response mechanism to temperature assaults of *Hsp* genes in whitefly.

Key words cold stress; comparative genomics; HSP; phylogenetic; thermal stress; whitefly

Introduction

When organisms, from archaebacteria to eubacteria, from plants to animals, are exposed to cold, heat or some other environment stresses, they can synthesize a group of proteins called heat shock proteins (Hsps) (Arya *et al.*, 2007), which are considered to play important roles in thermal adaptation and in other proteotoxic stress tolerances (Bedulina *et al.*, 2013; Colinent *et al.*, 2013). The Hsps are a set of remarkably well characterized proteins which function as molecular chaperones in protecting cellular proteins during the process of protein biosynthesis and refolding (King & MacRae, 2015), and originally discovered from *Drosophila* as a response to high temperature (Ashburner & Bonner, 1980). Generally, the Hsps are divided into five different families based on their molecular weight and sequence homology, including HSP100, HSP90, HSP70, HSP60 and small HSPs (sHSPs) (Lindquist & Craig, 1988; Feder & Hofmann, 1999). Among them, Hsp70 is one of the most highly conserved protein families, which respond to a range of adverse conditions and also interact with a series of other proteins in producing phenotypic effects under stress (Bettencourt *et al.*, 2008). Usually, the HSP70s are divided into two groups based on expression profiles (Kiang &

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Tsokos, 1998), including heat shock cognate 70 (HSC70) which is constitutively expressed in normal conditions and heat shock 70 (HSP70) which is inducible in stressed conditions. These proteins are found in multiple subcellular compartments, playing critical roles in the mitochondria, endoplasmic reticulum (ER), cytosol, lysosomes and extracellular compartments (Stetler *et al.*, 2010). The first member of the HSP90 gene family has been identified in *Drosophila*, and there appears to be only one gene in this family, *Hsp82* (Blackman & Meselson, 1986). Generally, HSP90 exists in the cytoplasm of diverse kinds of cells under normal and stress conditions. As chaperone proteins, they play important roles in the maintenance of folding state by conjuncting with denatured proteins (Lindquist & Craig, 1988). Although most *Hsp60*s are involved in mitochondria that function as housekeeping genes, subcellular stress can also induce the expression in rats (Truettner *et al.*, 2009). The sHsps are those HSPs between 17 to 30 kDa, usually bellow 30 kDa (deJong *et al.*, 1993). Unlike other HSP families, the sHsps are ubiquitous, diverse molecular chaperones that bind to the proteins and prevent them from aggregation during thermal stress (Bruey *et al.*, 2000). HSPs serve as molecular chaperones during periods of stress by binding to other proteins. Meanwhile, the genes encoding those proteins are able to accumulate abundant transcripts immediately upon different kinds of stresses. When a more suitable environment is available, the *Hsp* genes are again repressed.

As ectotherm animals, insects are susceptible to impact by environment stresses, such as heat or cold stress, and pathogen infection. Insect *Hsp* genes encode molecular chaperones that help repair stress injuries via transportation and degradation of aggregated proteins in the organism (Rinehart *et al.*, 2007; Shu *et al.*, 2011; Kim *et al.*, 2014). Although HSP expression patterns have been identified in *Drosophila* species and some model insects (Feder *et al.*, 1992; Kregel, 2002; Rinehart *et al.*, 2007; Tower, 2011), there is a lack of information on a group of important pests regarding Hsp expression patterns. The whitefly, *Bemisia tabaci*, is a species complex with more than 35 cryptic species (Li *et al.*, 2010; De Barro *et al.*, 2011; Alemandri *et al.*, 2012) that causes severe economic losses to agricultural production by direct feeding, excreting honeydew and transmitting plant viruses. Within the species complex, the Middle East Asia Minor 1 (hereafter MEAM1) and Mediterranean (hereafter MED) species are highly invasive and cause considerable economic damages to many important crops worldwide (Oliveira *et al.*, 2001; Liu *et al.*, 2007; McKenzie *et al.*, 2009). The invasive species is widely distributed in tropical and subtropical zones. It has been reported that temperature tolerance might be an important factor in this colonizing success (Díaz *et al.*, 2015). A growing literature has investigated the effect of temperature on the performance of whitefly. Xiao *et al.* (2016) found that the high temperature tolerance of the two invasive whiteflies was related to *Hsp90* and *Hsp70* genes. Lü and Wan (2011) demonstrated that the messenger RNA (mRNA) expression of *Hsp23* and *Hsp70* play a key role for heat tolerance in females and promotes a higher survival rate under heat shock conditions. Despite increasing demonstration of the role of HSPs in response to temperature stress in whitefly, some issues remain unclear, for example, the evolutionary feature of the Hsp superfamily, the association between gene structure and function, and the role of HSPs for cold tolerance in whitefly.

In present work, we investigated *Hsp* gene expression in whitefly under extreme temperature conditions. Twenty-six *Hsp* genes, including three *Hsp90*, 17 *Hsp70*, one *Hsp60* and five *sHSP* genes were identified in the *B. tabaci* genome, and the expression patterns in response to 4°C (cold), 40°C (heat) were examined. In addition, silencing the expression of *BtHsp70-6* significantly decreased the survival rate of whitefly under 45°C, while the percentage of survival of whitefly subjected to –4°C did not significantly change. Our observations highlighted the molecular evolutionary properties and the response mechanism to temperature assaults of *Hsp* genes in whitefly.

Materials and methods

Dataset resources and identification of Hsp genes

The sequencing of cryptic species MEAM1 (mtCOI GenBank accession no. GQ332577) of the whitefly *B. tabaci* complex genome was completed recently (Chen *et al.*, 2016), and the genome information can be accessed from (http://www.whiteflygenomics.org/cgi-bin/bta/index.cgi). To identify *B. tabaci Hsp* gene superfamily members, the published HSP proteins of *Drosophila ananassae*, *Nilaparvata lugens* and *Athalia rosae*, were downloaded from InsectBase (http://www.insect-genome.com/) (Yin *et al.*, 2016). Afterward, these published HSP proteins were used as queries to search against the genome of *B. tabaci* with BlastP and tBlastN programs with a stringent *E* value cut-off ($\leq e^{-20}$). All significant hits were subjected to Pfam (http://pfam.sanger.ac.uk/) (Finn *et al.*, 2014) and National Center for Biotechnology Information (NCBI) Conserved Domain Database (http://www.ncbi.nlm.nih.gov/cdd) (Marchler-Bauer *et al.*, 2015) to confirm the presence of the conserved domain. Subsequently, the non-redundant candidates

of HSP protein superfamily in *B. tabaci* were determined by Interproscan (Quevillon *et al.*, 2005). The same approaches were performed in the genome files of *Plutella xylostella* and *Tribolium castaneum* downloaded from the InsectBase (Yin *et al.*, 2016) to obtain the putative HSP protein superfamily members. Finally, the molecular weight and isoelectric point of *B. tabaci* full-length Hsp protein were calculated by Compute pI/Mw tool from ExPASy (http://web.expasy.org/cgi-bin/compute_pi/pi_tool) (Bjellqvist *et al.*, 1994). Subcelluar localization was predicted by CELLO v2.5 (http://cello.life.nctu.edu.tw/) (Yu *et al.*, 2004). The nomenclature and description of Hsps were referred to Chen *et al.* (2006).

Phylogenetic analysis and classification of Hsp genes

For phylogenetic analysis, all putative HSP proteins were aligned by Muscle 3.52 (Edgar, 2004) with default option and then manually refined by BioEdit v7.2.5 (Hall, 2015). Subsequently, the unrooted phylogenetic trees of all Hsp proteins were generated by MEGA 5.2 (Tamura *et al.*, 2011) using a neighbor joining (NJ) method with the following parameters: poisson correction model, pairwise deletion, and bootstraps test with 1000 replications (random seed). To verify the reliability of the NJ method, the phylogenetic trees were reconstructed by maximum likelihood (ML) methods using PhyML software (Guindon & Gascuel, 2003).

Structural features analysis of BtHsps

To analyze the genomic organizations of *B. tabaci Hsp* (*BtHsp*) genes, the information of the gene structures were extracted from the gff file of *B. tabaci* genome. Gene Structure Display Server (http://gsds.cbi.pku.edu.cn/) (Hu *et al.*, 2015) was then applied to graphically portray the numbers and positions of coding sequence (cds)/intron. To further evaluate the structural diversity of BtHSP proteins, the conserved motifs were detected by online program Multiple Expectation Maximization for Motif Elicitation (Bailey *et al.*, 2009). The parameters were set as follows: distribution of motifs, zero or one per sequence; maximum number of motifs, 20; number of repetitions, any; and optimum motif width from 30 to 70 residues for Hsp90, Hsp70 and Hsp60 family members. Due to the short protein sequences of the sHsp family, the optimum motif width was set between 10 and 40. In addition, the secondary structures α-helix and β-sheet of BtHSP proteins were predicted by Jpred4 (http://www.compbio.dundee.ac.uk/jpred/) (Drozdetskiy *et al.*, 2015).

Whitefly sampling

The cryptic species MEAM1 (mtCOI GenBank accession no. GQ332577) of the whitefly *B. tabaci* complex was reared on cotton plants (*Gossypium hirsutum* L. cv. Zhemian 1793) in insect-proof cages at 26 $\pm$ 1°C, 16 : 8 h L : D in a temperature-controlled room.

Temperature treatments

To characterize the expression of *Hsp*s in whiteflies under temperature stress, the whiteflies were kept at 4°C (cold) or 40°C (hot) for 1 h and 4 h as temperature treatment groups. All cold or hot temperature treatments were applied on *B. tabaci* adults. The mixed-sex adult whiteflies (newly emerged 5–7 days) were placed in test tubes (25 mm $\times$ 5 mm diameter) covered with gauze. Temperature treatments were implemented in climatic chambers (Sanyo, MLR-350H, Sanyo Electric Co., Ltd., Osaka, Japan) which offered precise control of temperatures within $\pm$ 0.5°C of the set value. Controls were treated identically without being stressed. Then, the total RNA of 200 mixed-sex adult whiteflies was extracted for each treatment group.

RNA interference and survival rate analyses

Double-stranded RNA (dsRNA) was synthesized using AmpliScribeTM T7-Flash Transcription kit (Epicentre, ASF3507) with specific primers. For RNA interference, adult whiteflies were fed with 15% sucrose with 200 ng/μL *dsHsp70-6*. The sucrose diet containing 200 ng/μL *dsgfp* was used as a negative control. Then whiteflies were first fed on a 15% sucrose diet with dsRNA for 2 days and then subjected to temperature stress. Each group of 20 adults were used for survival estimates. The number of adults was counted after recovery at 25°C for 3 h to exclude those individuals that were in suspended animation. Each treatment was assayed in 20 replicates.

To investigate the effect of *Hsp* genes on survival rates of whiteflies, the treatments were divided into two different groups: (P+CS) the pre-treatment at low temperature (4°C) for 1 h and recovery at 25°C for 1 h followed by cold (–4°C) shock for 1 h; (P+HS) the pre-treatment at high temperature (40°C) for 1 h and recovery at 25°C for 1 h followed by heat (45°C) shock for 1 h.

RNA isolation and quantitative real-time PCR (qPCR)

Total RNAs of head and body were extracted from both stress-treated and control samples using SV Total

RNA isolation system (Promega, Z3100). The quantity and quality of RNAs were evaluated by a NanoDrop2000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Approximately 1 μg RNA was reverse-transcribed using the SYBR PrimeScript reverse transcription-PCR (RT-PCR) kit II (Takara, RR037A). qRT-PCRs were conducted on the CFX96^{TM} Real-Time system with SYBR green detection. The gene-specific primers are list in Table S1. In each qRT-PCR experiment, each gene was run in triplicate from three biological replicates. Whitefly *β-actin* (forward primer 5′-3′: TCTTCCAGCCATCCTTCTTG; reverse primer 5′-3′: CGGTGATTTCCTTCTGCATT) was used as endogenous control gene to normalize all data.

Statistical analyses

For qPCR results, the comparative cycle threshold (Ct) method ($2^{-\Delta\Delta Ct}$) was carried out to calculate the relative expression levels (Livak & Schmittgen, 2001). Statistical analysis was carried out using the data obtained from three separate complementary DNA (cDNA) sets of three independent biological samples. The heatmap for expression profile was generated with Mev 4.0 software (Saeed *et al.*, 2003). Student's two-tailed *t*-test was used to compare the survival rate between different treatments. The differences between treatments were considered significant when $P < 0.05$. All statistical analyses were conducted using SPSS 20.0 (SPSS Inc., Chicago, IL, USA)

Results

Genome-wide identification of the Hsp gene superfamily in Bemisia tabaci

After BlastP and tBlastN searches, 26 non-redundant genes were identified as *B. tabaci* Hsp proteins in whitefly genome. In parallel, 23 and 20 HSP proteins were also identified in the genomes of *Plutella xylostella* and *Tribolium castaneum*, respectively. The numbers in the genomes of *B. tabaci* and the other five surveyed insect species are listed in Table 1. The predicted proteins encoded by 26 *BtHsp* genes were initially classified based on the NCBI Conserved Domain Database (NCBI CDD) analysis, which divided them into four families, including *Hsp90*, *Hsp70*, *Hsp60* and *sHsp*. The Hsp genes from *B. tabaci* were abbreviated as *BtHsp*, followed by the family designation number (90 and 70), and subsequently by an individual gene number. *Hsp90A* and *Hsp90B* represent the HSP proteins located in cytosolic and ER, respectively. HSC70 represented the constitutive 70 kDa Hsps, whereas HSP70 indicated the inducible ones. In addition, the small HSP proteins were named as sHsp followed by their molecular weight (Li *et al.*, 2009).

As illustrated in Table 1, Hsp70 family was the largest one followed by HSP90 family in *B. tabaci*. Compared with the other five well characterized insect species, *Bemisia tabaci Hsp* gene superfamily was the most expanded one with 26 members. The detailed information on BtHSP superfamily is listed in Table 2. The amino acid length of BtHSPs ranged from 171 (BtHSP19.4) to 811 (BtHSP70-1), and the molecular weight correlated well with the family they belonged to. Most Hsp proteins were predicted to locate in cytoplasm; however, a few members were also positioned in different organelles such as ER, mitochondria and nucleus.

Phylogenetic relationship analysis of all Hsps

To assess the phylogenetic relevance between *B. tabaci* HSP proteins and other HSPs, all Hsp proteins were aligned to generate unrooted trees. As shown in Figure 1A, BtHSP proteins can be divided into four families including HSP90, HSP70, HSP60 and HSP10. All the proteins from the same family of different insect species clustered

Table 1 *Hsp* genes identified in six sequenced insect genomes.

Order	Organism	Genome size (Mb)[†]	Family						Total
			HSP100	HSP90	HSP70	HSP60	sHSP	HSP10	
Homoptera	*Bemisia tabaci*	615.02	–	3	17	1	5	–	26
Diptera	*Drosophila ananassae*	230.99	–	3	1	4	9	2	19
Hymenoptera	*Athalia rosae*	163.83	–	3	4	2	2	1	12
Lepidoptera	*Plutella xylostella*	390.10	–	1	6	–	16	–	23
Hemiptera	*Nilaparvata lugens*	1140.79	–	1	9	4	2	2	18
Coleoptera	*Tribolium castaneum*	165.94	–	3	12	–	5	–	20

[†]The information comes from http://www.ncbi.nlm.nih.gov/genome/.

Table 2 Information on *Hsp* gene superfamily in *Bamisia tabaci*.

Family	Gene identifier	Gene name	CDS	Mw (kDa)	pI	Subcelluar location	Strand
Hsp 90	XM_019042324.1	*BtHsp90A1*	2151	82.3	4.98	Cytoplasmic	minus
	XM_019042743.1	*BtHsp90A2*	2163	83	4.99	Cytoplasmic	plus
	XM_019043097.1	*BtHsp90B1*	2379	90.5	4.95	Endoplasmic reticulum	minus
Hsp70	XM_019043796.1	*BtHsp70-1*	2436	90.7	5.39	Cytoplasmic	minus
	XM_019045108.1	*BtHsc70-1*	1971	72.7	5.16	Endoplasmic reticulum	plus
	XM_019048607.1	*BtHsp70-2*	2106	76.3	5.75	Cytoplasmic	minus
	XM_019051375.1	*BtHsp70-3*	1536	56	5.22	Cytoplasmic/Golgi	plus
	XM_019053413.1	*BtHsc70-2*	1956	71.5	5.32	Cytoplasmic	plus
	XM_019053433.1	*BtHsp70-4*	1959	70.9	5.53	Cytoplasmic	plus
	XM_019054622.1	*BtHsp70-5*	2052	74.4	5.61	Cytoplasmic	plus
	XM_019054623.1	*BtHsp70-6*	2019	73.4	5.61	Cytoplasmic	plus
	XM_019055446.1	*BtHsp70-7*	1845	67.8	5.3	Cytoplasmic	minus
	XM_019055501.1	*BtHsp70-8*	1848	68.2	5.81	Cytoplasmic	plus
	XM_019055502.1	*BtHsp70-9*	1848	68.6	6.16	Cytoplasmic	plus
	XM_019055503.1	*BtHsp70-10*	1848	68.7	5.61	Cytoplasmic	plus
	XM_019056010.1	*BtHsp70-11*	1941	70.9	5.59	Cytoplasmic	minus
	XM_019056134.1	*BtHsp70-12*	1962	71	5.41	Cytoplasmic	minus
	XM_019058712.1	*BtHsp70-13*	1890	68.8	5.63	Cytoplasmic	minus
	XM_019060092.1	*BtHsc70-3*	2079	75.8	5.71	Mitochondrial	plus
	XM_019061270.1	*BtHsp70-14*	1875	68.7	5.68	Cytoplasmic	plus
Hsp60	XM_019053486.1	*BtHsp60*	1731	61.1	5.39	Mitochondrial	plus
sHsp	XM_019051537.1	*BtsHsp19.5*	522	19.5	7.89	Extracellular/Nuclear	minus
	XM_019059161.1	*BtsHsp21.5*	588	21.7	6.52	Nuclear	minus
	XM_019059246.1	*BtsHsp22.6*	597	22.6	5.68	Nuclear	minus
	XM_019060691.1	*BtsHsp21.6*	591	21.6	6.52	Cytoplasmic/Nuclear	plus
	XM_019062080.1	*BtsHsp19.4*	516	19.4	6.09	Extracellular/Nuclear	minus

together. This corresponds well with the initial classification of BtHsps based on NCBI CDD results. Because of the short sequences of sHsp family members, a separate phylogenetic tree was constructed (Fig. 1B). Meanwhile, the topologies of phylogenetic trees reconstructed by ML method with PhyML (Supplementary Figs. S1, S2 and S3) approximately coincided with those of NJ method, which proved the reliability of our results.

Phylogenetic and gene organizations analysis of BtHsps gene superfamily

To obtain further insight into the evolutionary relationships among BtHsp superfamily members, their gene structures were predicted and compared based on the phylogenetic analysis. Figure 2A displayed that BtHsps can be classified into four groups with high bootstraps values. As illustrated in Figure 2, most *Hsp70* genes had no intron except *BtHsp70-3*, *BtHsp70-13* and *BtHsp70-14* which separately possessed seven, six and three introns. *Hsc70* genes contained 11 (*BtHsc70-3*) to 12 (*BtHsc70-1*) introns. Furthermore, there was considerable difference between the gene structures of BtHsp90A subgroup and BtHsp90B subgroup. In contrast, the gene structures of the BtsHsp family were highly conserved.

Phylogenetic and protein structures analysis of Hsps in B. tabaci

To explore the structural diversity of BtHsp superfamily, conserved motifs analysis was performed. We searched 20 putative motifs in each family as shown in Figure 3 and the details of these motifs are listed in Supplementary Data Sheet 1. In general, the BtHsp proteins from the same family shared similar motifs. The motifs were highly conserved within closely related BtHSP members. Moreover, some HSP proteins from sister branches even had common motif compositions. Such phenomena were correlated with the gene structures and phylogenetic relationships.

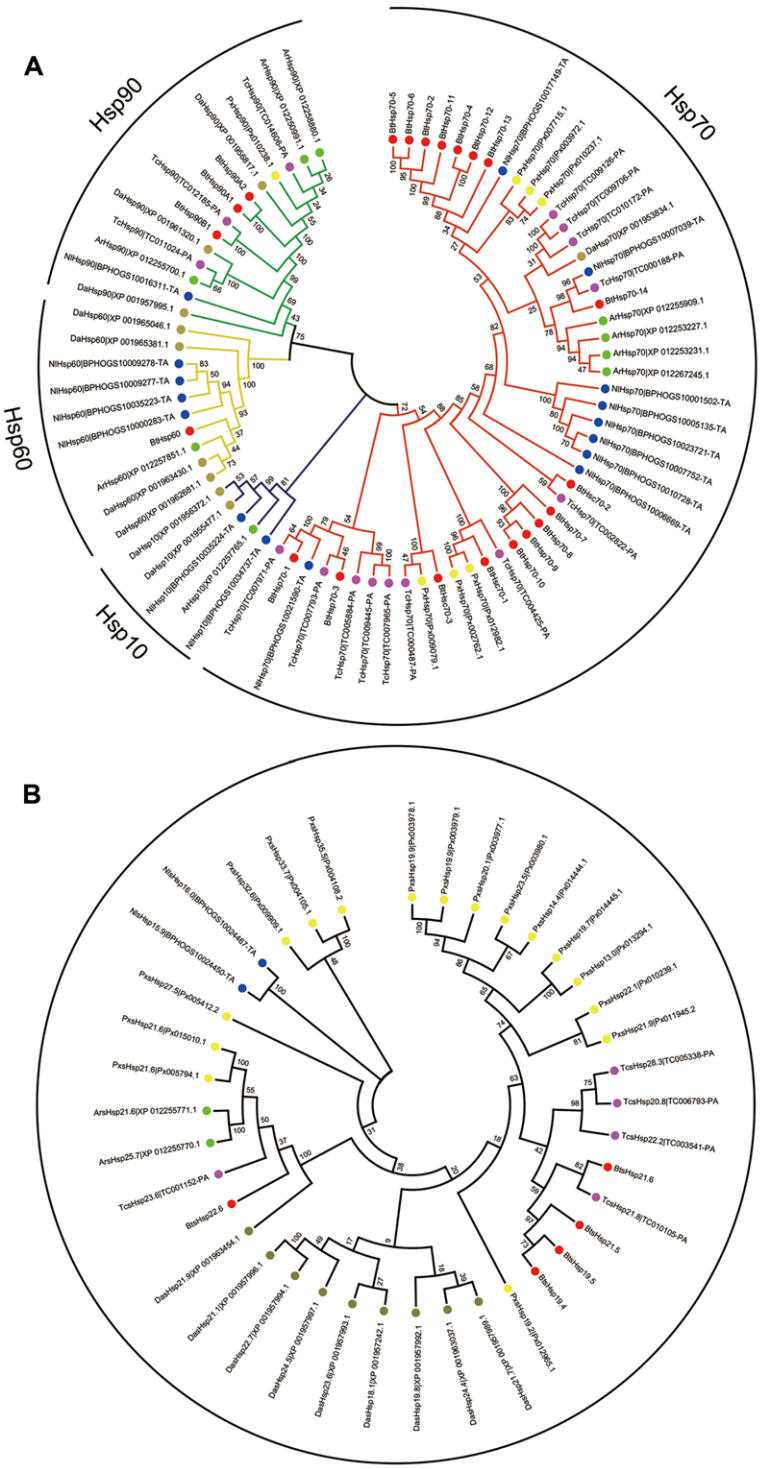

Fig. 1 Phylogenetic analysis of all heat shock proteins (Hsps) from *Bemisia tabaci*, *Plutella xylostella*, *Tribolium castaneum*, *Drosophila ananassae*, *Athalia rosae* and *Nilaparvata lugens*. The unrooted phylogenetic trees of Hsp proteins (A) and small Hsps (sHsps) (B) from the six insects surveyed here were constructed using MEGA 5.2 by Neighbor-Joining method. Numbers on branches are bootstrap portions from 1000 replicates. The specific colors indicate different families.

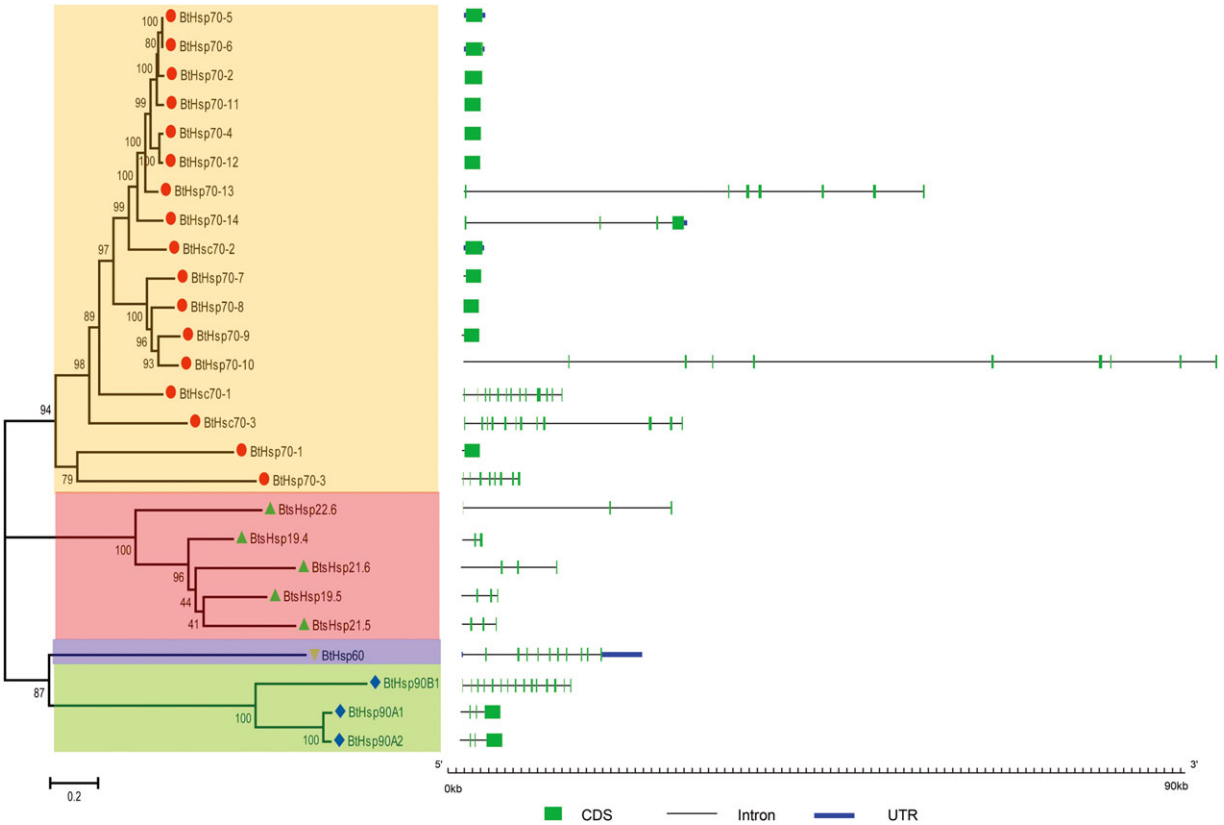

Fig. 2 Phylogenetic relationships and gene structures analysis of the *Bemisia tabaci Hsp* (*BtHsp*) gene superfamily. The unrooted phylogenetic tree was constructed using MEGA 5.2 by Neighbor-Joining method and the bootstrap test was performed with 1000 replicates. The colored shadow marks the different BtHsp families. CDS/Intron structures of *BtHsp* genes. The green boxes, gray lines and blue box, respectively, represent the cds, intron, and untranslated regions.

Subsequently, the deduced amino acid sequences from the full-length BtHSP sequences were aligned in each family. Taking the BtsHSP family as an example, Figure 4 suggests that all BtsHsp proteins included the conserved α-crystalline domain with chaperone function located in the C-terminal region. There were nine β-sheet sandwich structures in the region. In Figures S4, S5 and S6, the sequence alignments of HSP70, HSP90 and HSP60 families were displayed, respectively. For HSP70 proteins, the peptide-binding domain and the conserved C-terminal motifs were highlighted. Similarly, there existed histidine kinase-like adenosine triphosphatase (ATPase) domain in the N-terminal domain and chaperone motifs in the C-terminal domain of HSP90 proteins. Finally, the BtHSP60 protein contained two GroEL-like equatorial domains and one GroEL-like apical domain.

Tissue-specific expression profiles of BtHsps in whitefly

To investigate the function of Hsp proteins in whitefly, qPCR was performed to examine the expression patterns in different tissues of whitefly (Fig. 5). Overall, a majority of the 26 *BtHsp* genes can be detected in whitefly. However, the expression levels of *BtHsp70-2*, *BtHsp70-7*, *BtHsp70-11*, *BtHsp70-12* and *BtHsp19.4* were quite low in all tissues of whitefly. The *Hsp90* gene family members had similar expression patterns, with abundant transcripts in the body. *BtHsp60*, *BtHsp19.5*, *BtHsp21.5* and *BtHsp21.6* were strongly expressed in the body but not in the head. Surprisingly, belonging to the sHSP family, *BtHsp22.6* was strongly expressed in both tissues. In addition, the three constitutively expressed *Hsc70* genes exhibited high-level accumulation in all the tissues of whiteflies surveyed.

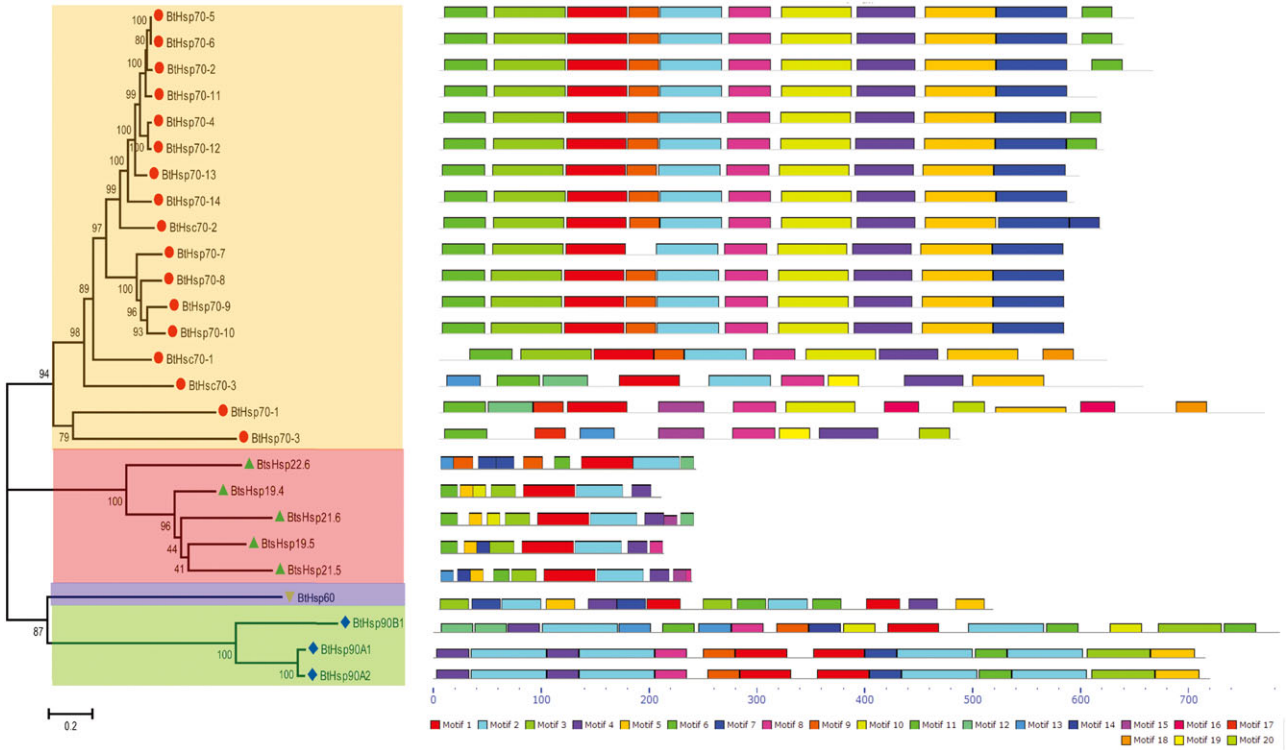

Fig. 3 Phylogenetic relationships and protein motif analysis of *Bemisia tabaci* heat shock proteins (BtHsps). The unrooted phylogenetic tree was constructed using MEGA 5.2 by Neighbor-Joining method and the bootstrap test was performed with 1000 replicates. The colored shadow marks the different BtHsp families. All motifs were identified by MEME database with the complete amino acid sequences of BtHsps. Lengths of motifs for each BtHsp protein are exhibited proportionally.

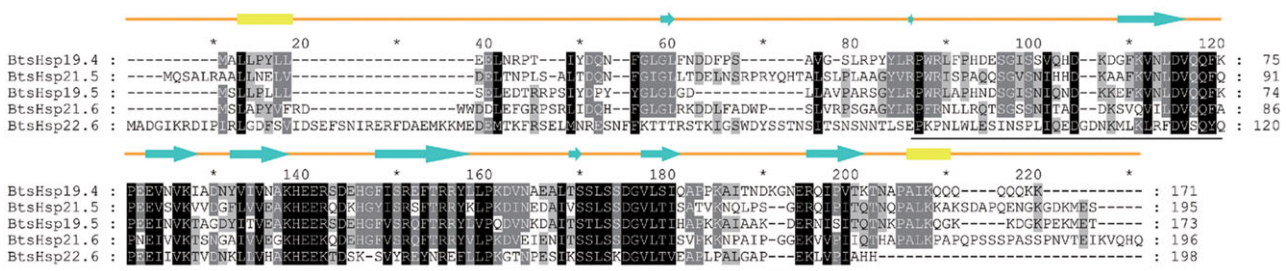

Fig. 4 Multiple sequence alignment of the *Bemisia tabaci* short heat shock protein (BtsHsp) family. The secondary structures of BtsHsp proteins are shown above the alignment. α-helices and β-sheets are represented by yellow boxes and blue arrows, respectively. The conserved domain of sHsp proteins is marked with a line under the sequences.

Differential expression of BtHsp genes under stress treatments

A large body of literature has indicated *Hsp* genes are involved in response to diverse environment stresses. Therefore the stress responses of whitefly *Hsp* gene superfamily under different treatments were investigated using qPCR. As shown in Figure 6A, only a few *Hsp* genes were up-regulated after heat treatment for 1 h and 4 h, including *BtHsp70-4*, *BtHsp70-5*, *BtHsp70-6*, *BtHsp70-11*, *BtHsp70-12*, *BtHsp70-13*, *BtHsp19.4* and *BtHsp19.5*. In contrast, all the members of *BtHsp90* and *BtHsp60* gene families were down-regulated at any time point under heat stress. Interestingly, nearly all *Hsp* genes were strongly induced when whiteflies were subjected to low temperature treatment at 4°C except

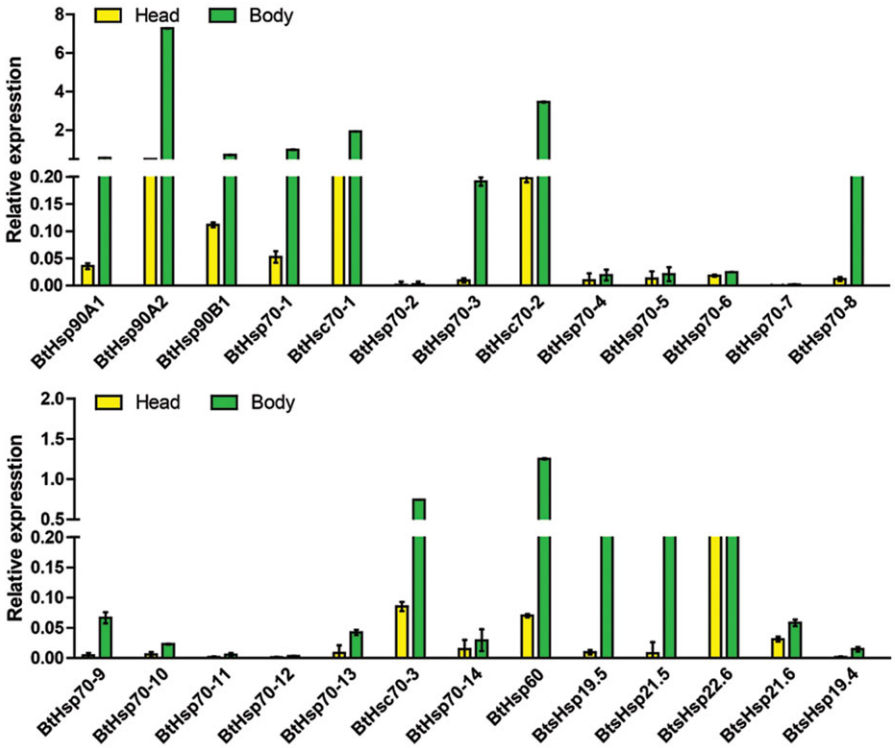

Fig. 5 Quantitative real-time polymerase chain reaction (qPCR) analysis of expression profiles of *BtHsp* gene superfamily in different parts of *Bemisia tabaci*. Gene names are shown on the *x*-axis and the expression levels on the *y*-axis. Different parts of whitefly insect are shown in different colors.

BtHsp70-10 which was down-regulated at every time point (Fig. 6B).

Effect of Hsp gene superfamily on the survival rate of whitefly

To further examine *BtHsp* involvement in whitefly temperature stress response, *BtHsp70-6* was selected as a candidate for RNA interference. Expression pattern of BtHsp genes indicated that *BtHsp70-6* was the only gene among the HSP70 protein family dramatically up-regulated in both heat and cold treatments, which suggests a major function. Figure 7A shows the expression of *hsp70-6* was significantly suppressed (nearly 50%) in whiteflies after feeding dsRNA for 2 days, as revealed by qPCR. Figure 7B displays that the silencing of *BtHsp70-6* did not affect the tolerance to lower lethal temperature of whitefly, since the survival rates of the *dsgfp* group and *dsHsp70-6* group were 54% and 45.75%, respectively. Nevertheless, the survival rate of *dsHsp70-6* group under a higher lethal temperature at 45°C sharply decreased to 29% compared with that of 40% in the *dsgfp* group.

Discussion

Expanded Hsp gene superfamily in whitefly

The release of the recently completely sequenced genome of *B. tabaci* MEAM1 makes it possible to identify stress-responsive related gene families through comparative genomics approaches (Chen *et al.*, 2016). In this study, we found 26 *Hsp* genes encoding four types of insect *Hsp*s in the *B. tabaci* genome by comprehensive bioinformatic methods. These findings indicated that *Hsp* gene superfamily in *B. tabaci* had expanded compared to those in *Drosophila ananassae*, *Athalia rosae*, *Nilaparvata lugens* (Yin *et al.*, 2016), *Plutella xylostella* (You *et al.*, 2013) and *Tribolium castaneum* (Consortium *et al.*, 2008). The scale of the *Hsp* gene superfamily is species-specific, not proportional to the sizes of genomes. For instance, *N. lugens* genome only contained 18 *Hsps* albeit its genome size is largest in the six insects surveyed here. This could be explained by gene loss in *Athalia rosae* and *N. lugens* while there is gene duplication in *B. tabaci*. Our results show that HSP70 was the largest clade among the four types of insect Hsps, which was consistent with

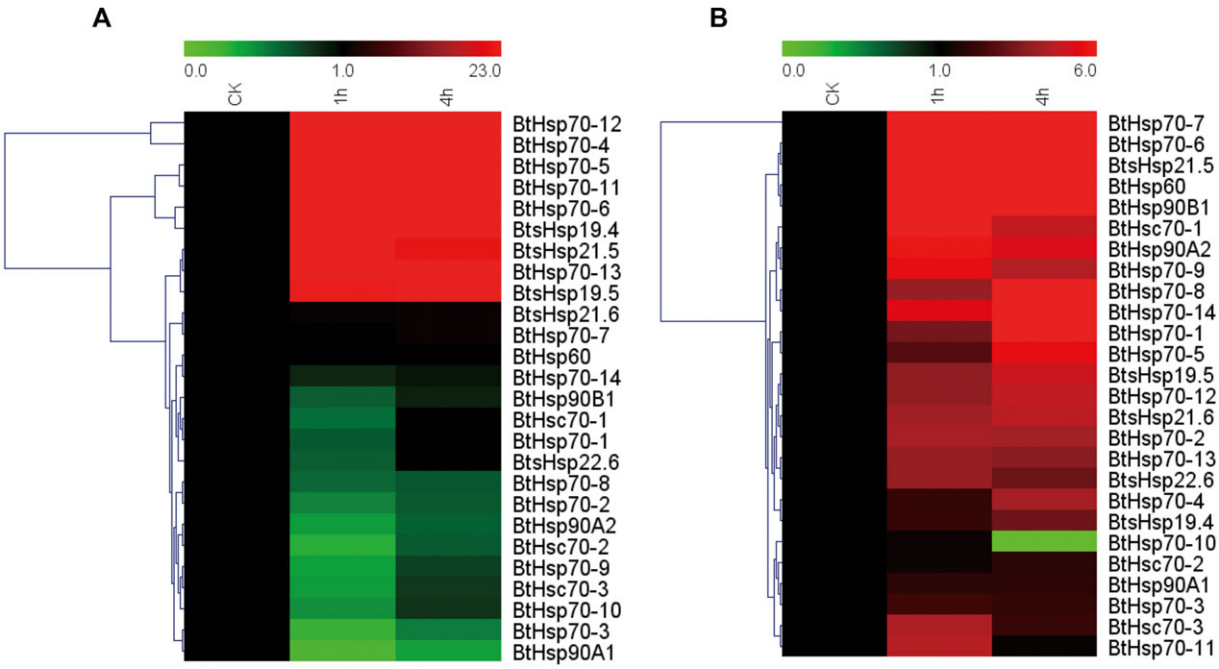

Fig. 6 Differential gene expression under diverse stresses in *Bemisia tabaci*. The heat map shows the real-time quantitative reverse transcription polymerase chain reaction (q-RT-PCR) analysis results of *BtHsp* genes in whitefly subjected to heat stress (A) and cold treatment (B). The colors of the bar vary from green to red representing the scale of relative expression levels. Each column represents a sampling time point or different part of the whitefly insect, and each row represents a *BtHsp* gene member. The clustering results of gene expression patterns are shown on the left.

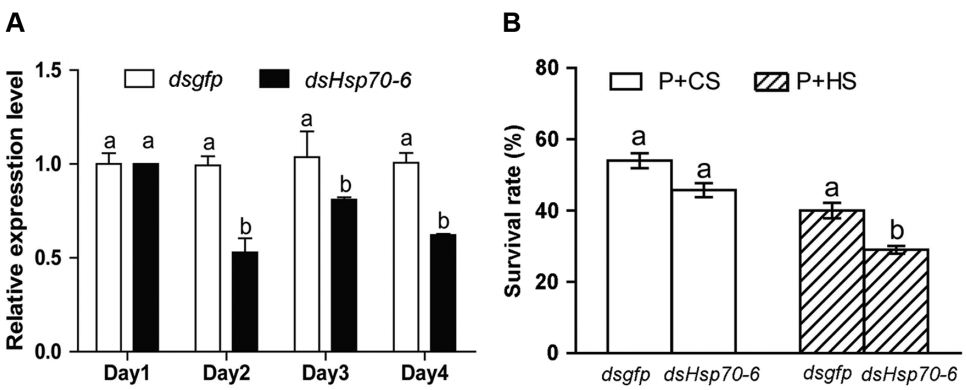

Fig. 7 Survival rate of *Bemisia tabaci* under thermal and cold stress after RNA interference (RNAi) treatments. (A) RNAi efficiency of *hsp70-6* after feeding double-stranded RNA (dsRNA) for different time intervals. (B) Survival rates: percentage of surviving adults after thermal and cold stress. The survival rate of whitefly treated with *dsgfp* or *dsHsp70-6* in different treatment groups. When significant differences were evident from the Student's two-tailed *t*-test, different letters are used to show significant differences among treatments. Each column shows means ± standard errors.

the finding that HSP70 family is one of the major and abundant HSP families (Csermely *et al.*, 1998). Furthermore, HSP10 family was absent in genomes of *B. tabaci*, *Plutella xylostella* and *Tribolium castaneum*. This may be caused by gene loss events during the course of evolution across different species. It has been reported that HSP10 proteins serve as the co-factor of HSP60 to assist in the folding of newly synthesized proteins imported into mitochondria (Frydman, 2001; Hartl & Hayer-Hartl, 2009; Hartl *et al.*, 2011). Thus, HSP10 is important for the

regulation of mitochondrial function and structure (Gupta, 1995; Lau *et al.*, 1997). Combining with the fact that only one Hsp60 existed in the whitefly genome, we hypothesized the maintenance of mitochondria structure and function relied on other kinds of chaperone proteins in *B. tabaci*. What's more, analysis of the phylogenetic tree revealed that Hsp homologs among different species clustered separately, which meant the duplication events in Hsp superfamily occurred after the radiation of insects.

Conserved sequence features of Hsp superfamily members in B. tabaci

To point out the details of *Hsp* gene superfamily expansion and divergence in *B. tabaci* genome, the gene structures and motif compositions were compared among BtHsp superfamily members. Overall, a high level of structural identity was observed among the Hsps from the same family. In the case of cds/intron structural characteristics, 11 out of 17 *Hsp70* genes were intronless while others were with variable numbers of introns. The phylogenetic tree of BtHsps superfamily indicated BtHSP70 family was the earliest one that expanded from the evolutionary branch. As a result, the structure may be more complex due to the longest evolutionary history. Generally, the intronless genes are archetypical in the prokaryotic genomes (Huang *et al.*, 2015). There are three pivotal mechanisms explaining the formation of intronless genes in the eukaryotic genomes. First, the gene transferred horizontally from ancient prokaryotes to eukaryotes. Then, duplication events arose in existing intronless genes, and the last one is in retroposition of intron-containing genes (Zou *et al.*, 2011). Research has shown that the similarities among HSP70 family members are greater from different organisms than that from the same species in some cases (Lindquist & Craig, 1988). Such phenomena indicates early gene duplication events and maintenance of this multigene family have occurred over evolutionary history.

The secondary structure prediction results show that Hsp proteins from the same family shared similar motif compositions. Different types of Hsp proteins had specific domains which were important in their function. HSP70 proteins contained a highly conserved 44 kDa ATPase domain and a 25 kDa domain in the N and C terminal, respectively (Bukau & Horwich, 1998). The C-terminal motif was different with diverse subcellular localizations (Boorstein *et al.*, 1994). V/IEEVD motif is featured in cytoplasmic Hsp proteins. Hsp proteins in ER are ended with KE/DEL. But BtHSP70-1 and BtHSP70-3 does not have any known signature motifs in the C-terminal end. Therefore, these two HSP70 proteins are in the phylogenetic clades far away from other BtHSP70s. Taken together, the structural divergence and conservation were closely involved with the evolutionary relationship and expansion of whitefly HSP superfamily.

Specific BtHsp genes are important in response to temperature stress

It has been reported that HSP superfamily members were differently expressed in diverse tissues of insects. This may be correlated with the facts that *Hsp* genes were significant in the development and response to stresses of insects. In the present study, we checked the expression profiles of *Hsp* genes in different tissues of whiteflies. To date, many reports have shown that Hsp proteins are related to heat and cold stress (Waters *et al.*, 2008). Therefore, we verified the expression patterns of *Hsp* gene superfamily under different temperature stresses in whiteflies via qPCR. HSP70 is a powerful indicator of a heat shock response, and it is a highly conserved protein that acts as a molecular chaperone. Our results show that most *Hsp70* genes were induced by thermal and cold stress. The Hsc70 family members did not respond to any temperature stress, which is consistent with earlier work (Luo *et al.*, 2015). Meanwhile, the *BtHsp90* family members show similar expression patterns under all treatments. Compared with heat and cold treatment of whiteflies, we can find two conclusions: the numbers of *Hsp* genes that are involved in response to cold temperature treatment are much more than that in high temperature treatment; the expression level of *hsp* genes caused by thermal stress were significantly higher than low temperature treatment. These results confirmed that the induction of *Hsp* genes are related to widely recognized temperature stress responses.

To further clarify that Hsps contributed to temperature stress, we blocked the expression of *BtHsp70-6* which was greatly induced both under cold and heat stresses through RNA interference. Surprisingly, the silencing of *BtHsp70-6* significantly decreased the survival rate of whiteflies subjected to the high lethal temperature of 45°C. However, this phenomenon was not observed in whiteflies treated with a low lethal temperature of –4°C. Therefore, we hypothesized that *BtHsp70-6* may be a major gene involved with thermotolerance rather than cold tolerance, since under different temperature stresses, the expression level of *BtHsp70-6* was significantly higher in heat stress treatment than in cold stress treatment.

To sum up, the large *BtHsp* gene superfamily possessed distinctive expression changes under various kinds of stimuli. Perhaps it is the conservation and diversity of structure that lead to gene family expansion and

functional constraint or differentiation. The *Hsps* conferred thermotolerance or cold-tolerance to whiteflies that protect them from being affected by detrimental temperature conditions.

Acknowledgments

Financial support for this study was provided by the National Natural Science Foundation of China (31390421) and the National Basic Research Program of China (2014CB138404).

Disclosure

The authors declare that there is no conflict of interest.

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Manuscript received February 12, 2017
Final version received May 22, 2017
Accepted June 1, 2017

Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Phylogenetic relationships of heat shock proteins (Hsps) from *Bemisia tabaci*, *Plutella xylostella*, *Tribolium castaneum*, *Drosophila ananassae*, *Athalia rosae* and *Nilaparvata lugens*. The unrooted phylogentic tree was constructed using PhyML software by Maximum Likelihood method with the LG model. The bootstrap test was performed with 1000 replicates.

Fig. S2. Phylogenetic relationships of small heat shock proteins (sHsps) from *Bemisia tabaci*, *Plutella xylostella*, *Tribolium castaneum*, *Drosophila ananassae*, *Athalia rosae* and *Nilaparvata lugens*. The unrooted phylogentic tree was constructed using PhyML software by Maximum Likelihood method with the LG model. The bootstrap test was performed with 1000 replicates.

Fig. S3. Phylogenetic relationships of all heat shock proteins (Hsps) from *Bemisia tabaci*. The unrooted phylogentic tree was constructed using PhyML software by Maximum Likelihood method with the LG model. The bootstrap test was performed with 1000 replicates.

Fig. S4. Multiple sequence alignments of the *Bemisia tabaci* heat shock protein 70 (BtHSP70) family. The secondary structures of BtHSP70 proteins are shown above the alignment. α-helices and β-sheets are represented by yellow boxes and blue arrows, respectively. The conserved domain of HSP70 proteins is marked with a line under the sequences.

Fig. S5. Multiple sequence alignments of the *Bemisia tabaci* heat shock protein 90 (BtHSP90) family. The secondary structures of BtHSP90 proteins are shown above the alignment. α-helices and β-sheets are represented by yellow boxes and blue arrows, respectively. The conserved domain of HSP90 proteins is marked with a line under the sequences.

Fig. S6. The secondary structures of *Bemisia tabaci* heat shock protein 60 (BtHSP60) protein. α-helices and β-sheets are represented by yellow boxes and blue arrows shown above the sequence, respectively. The conserved domain of HSP60 proteins is marked with a line under the sequences.

Supplementary data sheet 1

1. Sequence logos for the conserved motifs of HSP90 proteins in *Bemisia tabaci*.
2. Sequence logos for the conserved motifs of HSP70 proteins in *Bemisia tabaci*.
3. Sequence logos for the conserved motifs of HSP60 proteins in *Bemisia tabaci*.
3. Sequence logos for the conserved motifs of HSP60 proteins in *Bemisia tabaci*

Supplementary data sheet 2

The information of Hsp sequences in the six surveyed species

Table S1. Gene-specific primers for q-RT-PCR used in this study.