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Characterization of three heat shock protein 70 genes from *Liriomyza trifolii* and expression during thermal stress and insect development

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Abstract

Heat shock proteins (HSPs) participate in diverse physiological processes in insects, and HSP70 is one of the most highly conserved proteins in the HSP family. In this study, full-length cDNAs of three HSP70 genes (*Lthsc70*, *Lthsp701*, and *Lthsp702*) were cloned and characterized from *Liriomyza trifolii*, an important invasive pest of vegetable crops and horticultural crops worldwide. These three HSP70s exhibited signature sequences and motifs that are typical of the HSP70 family. The expression patterns of the three *Lthsp70*s during temperature stress and in different insect development stages were studied by real-time quantitative PCR. *Lthsp701* was strongly induced by high- and low-temperature stress, but *Lthsc70* and *Lthsp702* were not very sensitive to temperature changes. All three *Lthsp70*s were expressed during insect development stages, but the expression patterns were quite different. The expression of *Lthsc70* and *Lthsp702* showed significant differences in expression during leafminer development; *Lthsc70* was most highly expressed in female adults, whereas *Lthsp702* was abundantly expressed in larvae and prepupae. *Lthsp701* expression was not significantly different among leafminer stages. These results suggest that functional differentiation within the *Lt*HSP70 subfamily has occurred in response to thermal stress and insect development.

Keywords: *Liriomyza trifolii*, heat shock protein 70, thermal stress, developmental stage, expression profile

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Introduction

Thermal stress tolerance is essential for the completion of insect life cycles, successful overwintering, and habitat exploration (McDonald *et al.*, 1999; Hoffmann *et al.*, 2003; Chidawanyika & Terblanche, 2011). Insects exposed to thermal stress may exhibit alterations in behavior, morphology, life history, and physiological characteristics (Taylor, 1981; Duman, 2003; Kelly *et al.*, 2012; Lu *et al.*, 2014). Among these strategies, the production of heat shock proteins (HSPs) has

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been widely studied and is one of the best predictors of insect tolerance to temperature stress (Feder & Hofmann, 1999). Furthermore, HSPs participate in diverse physiological processes (Haass *et al*., 1990; Johnston *et al*., 1998; Lu *et al*., 2014; Lu *et al*., 2016) and are presumably involved in the development of some insects (Denlinger, 2002; Rinehart *et al*., 2007; MacRae, 2010; Cheng *et al*., 2016).

Insect forms of HSPs are divided into several families based on molecular weight and homology; these include HSP90, HSP70, HSP60, HSP40, and small heat shock proteins (sHSPs) (Lindquist & Craig, 1988; Moseley, 1997; Feder & Hofmann, 1999; Sørensen *et al*., 2003). HSPs function as molecular chaperones in response to a variety of stress factors, promote proper protein folding, and prevent the aggregation of denatured proteins (Gehring & Wehner, 1995; Johnston *et al*., 1998). Among HSPs, the HSP70 family is highly conserved (Boorstein *et al*., 1994; Mayer & Bukau, 2005) and can be subdivided into two groups (Karlin & Brocchieri, 1998). One group is quickly and abundantly induced in response to various stress conditions and returns to a basal level of expression in the absence of stress. The other HSP70 group is not stress-inducible; it is generally constitutively expressed and is referred to as heat shock cognate protein 70 (HSC70) (Kiang & Tsokos, 1998; Tang *et al*., 2012; Sun *et al*., 2016; Zhang *et al*., 2015; Shen *et al*., 2015).

The genus *Liriomyza* is an important insect pest worldwide (Spencer, 1973), and *L. trifolii* is a highly invasive insect that has caused great losses in agricultural and horticultural crops (Johnson *et al*., 1983; Parrella *et al*., 1985; Reitz *et al*., 1999). *L. trifolii*, *L. sativae*, and *L. huidobrensis* are serious pests of vegetable crops in some regions of China (Kang *et al*., 2009; Xiang *et al*., 2012). *L. sativae* is the dominant leafminer in mainland China, whereas *L. huidobrensis* has occurred primarily in cooler climates since its initial outbreak in the 1990s (Wen *et al*., 1996, 1998). *L. trifolii*, which was initially discovered in Guangzhou, has since been reported in more than ten provinces (Wang *et al*., 2007; Lei *et al*., 2007; Gao *et al*., 2017). Several species displacement events have been reported between these *Liriomyza* spp. (Abe & Kawahara, 2001; Reitz & Trumble, 2002a, b; Abe & Tokumaru, 2008; Gao & Reitz, 2016). Generally, the distribution and abundance of insect species are delineated by their adaptability to climatic stress (Bale, 2002; Bale *et al*., 2002); furthermore, low-temperature tolerance is a critical factor impacting the distribution and spread of *Liriomyza* spp. in temperate regions.

Since the initial discovery of HSPs in *Drosophila melanogaster* (Tissières *et al*., 1974), research on insect forms of HSP70 has primarily focused on the structural characteristics and expression during different experimental conditions (Velazquez & Lindquist, 1984; Flaherty *et al*., 1990; Flaherty *et al*., 1994; Fung *et al*., 1996; Rinehart *et al*., 2000; Shim *et al*., 2006; Sonoda *et al*., 2006; Morano *et al*., 2014; Qiao *et al*., 2015; Wang *et al*., 2014). In *Liriomyza* spp., an earlier study involving *L. sativae* and *L. huidobrensis* demonstrated that *hsp90*, *hsp70*, *hsp40*, and *shsp* could be induced by temperature stress; however, *hsp60* was only slightly induced during heat shock and did not respond to cold stress (Huang & Kang, 2007). Moreover, the expression of HSP genes can result in negative effects on feeding and fecundity in *L. huidobrensis*, which is further evidence that HSPs function in the physiology of *Liriomyza* spp. (Huang *et al*., 2007). In *L. sativae*, the expression of *hsps* during development was investigated; the transcription of small *hsps* peaked during the pupal stage, whereas the expression of *tcp1* (two genes), *hsp60*, and *hsp90* gradually increased during development (Huang *et al*., 2009). Compared with congener species, the study of HSPs in *L. trifolii* has been relatively limited (Zheng *et al*., 2010; Ji *et al*., 2013). Recently, five *Lthsps* were studied and showed expression profiles similar to homologous genes in *L. sativae* and *L. huidobrensis*; although *L. trifolii* occurs primarily in southern China, the results indicated that this pest has the potential to survive in more northern provinces (Chang *et al*., 2017a). In another study, *Lthsp21.7* expression was induced by temperature stress and varied with different insect developmental stages (Chang *et al*., 2017b). In the current study, we identify three genes encoding HSP70s in *L. trifolii* and describe their genomic structure. The expression of these three genes was monitored during thermal stress and during insect development with the aim of understanding molecular mechanisms of environmental tolerance and development in *Liriomyza* spp.

Materials and methods

Insects

L. trifolii were reared in the laboratory at 26 ± 1°C with a 16:8 h (L:D) photoperiod as described previously (Chen & Kang, 2002). Beans (*Vigna unguiculata*) were seeded in plastic pots (12 cm in diameter) and transferred to screened cages (40 × 40 × 65 cm) when 5–6 true leaves were present. Bean leaves exhibiting tunnels caused by larval feeding were collected for pupation; the resulting pupae were transferred into glass tubes for experimental treatments.

Temperature treatments

Two-day-old pupae ($n = 30$) were placed in glass tubes, and exposed to hot (30, 32.5, 35, 37.5, 40, 42.5, 45°C) or cold stress (−5, −7.5, −10, −12.5, −15, −17.5, −20°C) for 1 h in a constant temperature controller (DC-3010, Ningbo, China). A set of pupae maintained at 25°C was regarded as a control group. After temperature stress, pupae were allowed to recover at 25°C for 1 h, frozen in liquid nitrogen, and stored at −70°C. Each treatment was repeated four times.

Developmental stage and sex

Developmental stages included third instar larvae, prepupae, 2-day-old pupae, 10-day-old pupae, male adults, and female adults ($n = 30$). Each experimental treatment was comprised of four independent biological replicates, except for prepupae (three repetitions).

RNA isolation and cloning experiments

Total RNA was extracted from *L. trifolii* using the SV Total RNA isolation system (Promega, Fitchburg, WI, USA). The integrity and purity of RNA was determined by agarose gel electrophoresis and spectrophotometry (Eppendorf Bio Photometer plus, Hamburg, Germany). Three HSP70 genes were selected based on the analysis of our transcriptome data (unpublished). The partial segments of the three HSP70 genes were amplified using the corresponding pair of specific primers (table 1), and 5′- and 3′-RACE were utilized to obtain the full-length cDNAs (SMART RACE cDNA Amplification Kit, Clontech, CA, USA).

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Table 1. Primers used in the cDNA cloning and real-time quantitative PCR.

Gene		Primer sequences(5′→3′)	Fragment length (bp)
Primers for cDNA cloning and genome amplification			
hsc70	F	AACCCCATCGTATGTAGCCTTTA	835
	R	ATCCATAGTGCTGCGGAATAAAT	
	5′	TCACCAGCAGTGGACTTGACCTCG	810
	3′	ATCCCGATGAGGCTGTGGCTTATG	1106
	ORF-F	CTAATACGACTCACTATAGGGCA	2220
	ORF-R	CCTAAACTTAAAACGTAGCATGT	
hsp701	F	CTTCGTTCAAATCCCAGAGCCTT	479
	R	TTAGTCATAACTCCACCAGCCGT	
	5′	CAAGGCTCTGGGATTTGAACGAAG	875
	3′	TGAAACGGCTGGTGGAGTTATGAC	958
	ORF-F	TATCAAAAGTAATAAATAGAAAGAA	2056
	ORF-R	GTAATTTAGGAATGACAGTCTTT	
hsp702	F	TGGTGAGGACTTTGACAACAGAT	745
	R	GGTCACATCTATTTTAGGCACTC	
	5′	GCTACATAACTGGGAGTGGTACGGT	161
	3′	AAAGGTCGTTTGTCACAGGCGGAT	524
	ORF-F	CAAAAAGGAAAATATTTCTACAAT	1990
	ORF-R	GAATAAATAGAAAAGTGTTGTGAA	
Primers for qRT-PCR			
hsc70	F	GTCATTACTGTGCCCGCTTACT	103
	R	GTGGGCTCATTGATAATACGCA	
hsp701	F	CAAATCCCAGAGCCTTGAGAC	173
	R	GCTACGGAATAAGTCAGCACAAA	
hsp702	F	TTTGAGTGGCGACAAGAGTAGTG	256
	R	GAATGCCAGTTAGGTTGAAGGTG	
Actin	F	TTGTATTGGACTCTGGTGACGG	73
	R	GATAGCGTGAGGCAAAGCATAA	

F, forward; R, reverse; 5′, 5′-RACE primer; 3′, 3′-RACE primer; ORF-F, genome amplification forward primer; ORF-R, genome amplification reverse primer.

Characterization of genomic DNA

Genomic DNA of *L. trifolii* was extracted using the AxyPrep™ Multisource Genomic DNA Kit (Axygen, New York, NY, USA). The sequences of the full-length cDNAs were used to design pairs of specific primers (table 1) for amplifying *Lthsp70* genomic fragments. The complete open reading frame (ORF) sequences of three genes were confirmed by 5′-RACE and genomic DNA. Amplified products were purified using a gel extraction kit (Axygen, New York, NY, USA), cloned into PGEM-T Easy vector (Promega, Fitchburg, WI, USA), and transformed into competent *Escherichia coli* DH5α cells. After confirmation by PCR, positive clones containing target genes were isolated and sequenced.

Quantitative real-time reverse transcriptase PCR

Total RNA was extracted as described above and 0.5 µg was reverse-transcribed into cDNA using the Bio-Rad iScript™ cDNA Synthesis Kit (Bio-Rad, CA, USA). Quantitative real-time reverse transcriptase PCR (qRT-PCR) was performed in 20 µl reaction volumes using conditions described previously (Chang *et al.*, 2017a) and the gene-specific primers in table 1. Reactions were carried out using a CFX-96 real-time PCR system (Bio-Rad Laboratories, Berkeley, USA). Treatments included four replicates, and each reaction was performed in triplicate. The quantity of the three *Lthsp70* mRNAs was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001), and *ACTIN* was used as a reference gene (Chang *et al.*, 2017b).

Sequence alignment and data analysis

Full-length cDNAs sequences of the three *Lthsp70* genes were used as queries to search for other insect *hsps* using the BLAST programs available at the NCBI website (http://www.ncbi.nlm.gov/BLAST/). Sequence alignments were performed using Clustal X software (Thompson *et al.*, 1997), and ORFs were identified using ORF Finder (https://www.ncbi.nlm.nih.gov/orffinder/). Sequence analysis tools of the ExPASy Molecular Biology Server (Swiss Institute of Bioinformatics, Switzerland) were used to analyze deduced *Lt*hsp70 sequences. Phylogenetic trees of HSPs were generated with MEGA 6.0 (Tamura *et al.*, 2013) using the neighbor-joining method with the following parameters: Poisson correction model, pairwise deletion, and 1000 bootstrap replicates (random seed).

One-way analysis of variance (ANOVA) was used to detect the significant differences in mRNA levels among treatments, followed by Tukey's multiple comparison ($P < 0.05$) and analysis with SPSS v. 16.0 (SPSS, Chicago, IL, USA). For the ANOVA, data were transformed for homogeneity of variances test.

Results

Cloning and characterization of three hsp70s from L. trifolii

Three heat shock genes were cloned from *L. trifolii* and designated *Lthsc70*, *Lthsp701*, and *Lthsp702* (GenBank accession nos. KY933450, KY933451, and KY933452, respectively). The full-length cDNAs of *Lthsc70*, *Lthsp701*, and *Lthsp702* were 2332, 2261, and 2078 bp and encoded predicted proteins containing 651, 645, and 632 amino acids, respectively. The predicted protein products of *Lthsc70*, *Lthsp701*, and *Lths702* had molecular weights of 71.20, 70.81, and 69.24 kDa and isoelectric points of 5.31, 5.70, and 5.70, respectively.

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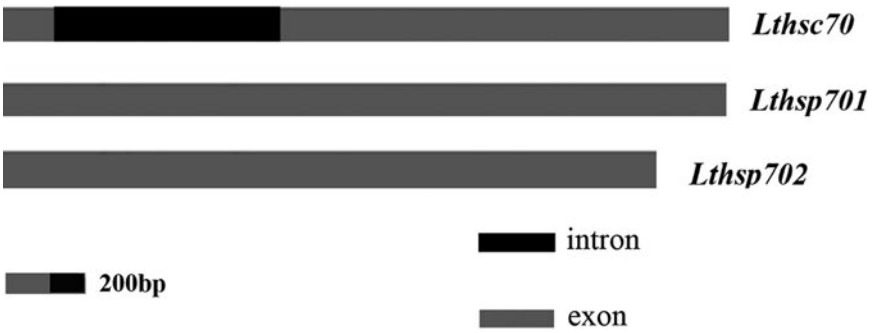

Fig. 1. Schematic representation of three *Lthsp70* genomes. Light gray and black rectangles are used to highlight the exons and introns, respectively.

Three signature sequences of the HSP70 family (Gupta & Singh, 1994) were identified in the deduced amino acid sequences of the three *Lthsc*/*Lthsp70* genes; these were IDLGTTYS, IFDLGGGTFDVSIL, and IVLVGGSTRIPKVQR/N/S (see sequences bound by rectangles, fig. S1). The three *L. trifolii* HSP70 proteins also contained the conserved EEVD motif (Pockley *et al*., 2008) at their *C*-termini (fig. S1). Furthermore, three other motifs typical of HSP70 were identified: an ATP/GTP binding site (AEAY/FLGK/TT) (Saraste *et al*., 1990), two bipartite nuclear localization signals: [(KKDLTTNKRALRR and KRALRRLRTACERAKRT) in *Lt*Hsc70 and (KKDLRSNPRA/SLRR and PRA/SLRRLRTA/EAER/KAKRT) in *Lt*HSP701 and *Lt*HSP702] (Knowlton & Salfity, 1996) and a non-organellar consensus motif RARFEEL (Zhang & Denlinger, 2010) (fig. S1).

The genomic DNA sequence of *Lthsc70* was identified and deposited in GenBank as accession no. KY933449. We found that *Lthsp701* and *Lthsp702* had no introns, whereas *Lthsc70* had a 1273-bp intron, which mapped close to the *N*-terminus (fig. 1). The nucleotide sequences at the splice junctions in *Lthsc70* are consistent with the canonical GT-AG rule (Mount, 1982).

A TATA-box-like regulatory element was identified in the 5′ UTR of *hsp701*, but was absent in the 5′ UTRs of *hsc70* and *hsp702* (fig. 2).

Phylogenetic analysis

The deduced amino acid sequences of the three *Lthsp70s* displayed a high degree of identity with published *Lt*HSP70 (Chang *et al*., 2017a) and orthologs in congener leafminers (fig. S1). To further examine the relationships between insect HSP70s, a phylogenetic tree was generated using the amino acid sequences of 20 HSP70 family members in orders Diptera, Lepidoptera, and Hemiptera (table S1; fig. 3). The resulting phylogenetic tree was divided into two distinct clusters containing HSC70 and HSP70. *Lt*HSP701 and *Lt*HSP702 grouped in the HSP70 cluster along with the previously published *Lt*HSP70, *Lh*HSP70, and *Ls*HSP70 (fig. 3). Interestingly, *Lt*HSP701 and *Lt*HSP702 were located on different phylogenetic branches (fig. 3). *Lt*HSC70 grouped with HSC70s in orders Diptera, Hemiptera, and Lepidoptera.

Expression of the three Lthsp70s in response to temperature

The expression of the three *Lthsp70*s was evaluated in response to temperature stress by qRT-PCR. The expression levels of all three *Lthsp70*s were significantly increased after exposure to cold stress relative to the control group at 25°C (*hsc70*:$F_{7,24} = 7.669$, $P < 0.001$;*hsp701*:$F_{7,24} = 37.055$, $P < 0.001$; *hsp702*:$F_{7,24} = 7.446$, $P < 0.001$). Expression of *Lthsp701* was highest at −15°C (T_{max}), which was 258.84-fold greater than the control (fig. 4b). *Lthsc70* and *Lthsp702* were expressed at much lower levels than *Lthsp701* during cold stress. At T_{max} temperatures of −15 and −17.5°C, *Lthsc70* and *Lthsp702* were expressed at levels 2.29- and 7.66-fold higher than the control group, respectively (fig. 4a, c).

Compared with the control group (25°C), expression of the three *Lthsp70*s was significantly increased after exposure to heat stress (*hsc70*:$F_{7,24} = 3.602$, $P < 0.05$;*hsp701*:$F_{7,24} = 14.348$, $P < 0.001$;*hsp702*:$F_{7,24} = 12.590$, $P < 0.001$). Expression of *LtHsp701* was highest at 42.5°C (T_{max}), which was 497.21-fold greater than the control (fig. 4e). Expression levels of *Lthsc70* and *Lthsp702* were lower than *Lthsp701* in response to heat stress; both genes showed the highest expression levels (T_{max}) at 40°C and were 2.76- and 12.18-fold greater than the control (fig. 4d, f).

Expression of Lthsp70s during L. trifolii *development*

qRT-PCR was also used to monitor the expression of the three *Lthsp70*s in different developmental stages of *L. trifolii*, including third instar larvae, prepupae, 2-day-old pupae, 10-day-old pupae, male adults, and female adults. *Lthsc70*

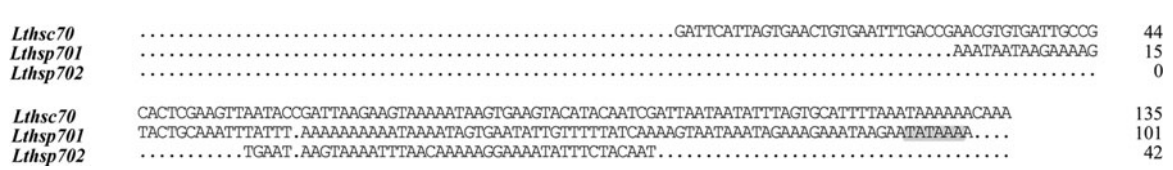

Fig. 2. Alignment of 5′UTRs of three *Lthsp70s*. The TATA-box-like elements are indicated by shading and the dots indicate alignment.

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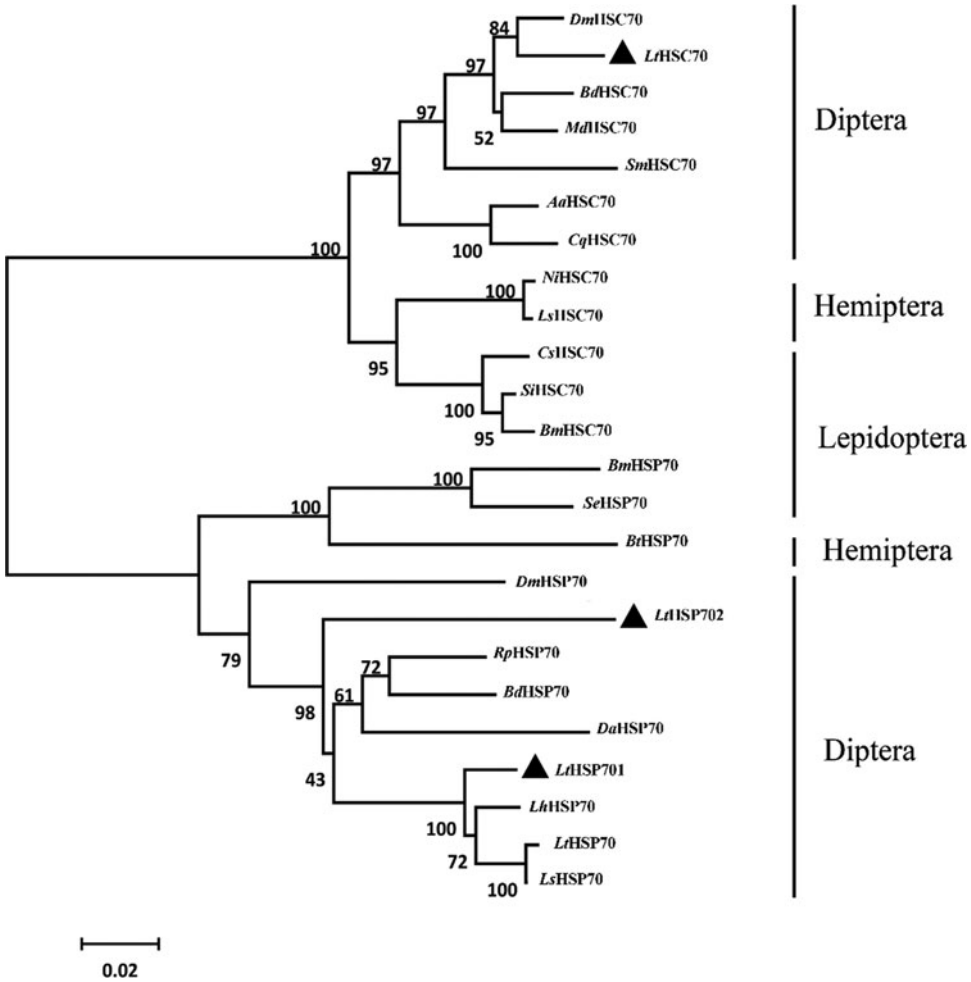

Fig. 3. Neighbor-joining phylogenetic tree of insect HSP70s. The *Liriomyza trifolii* HSP70s are labeled with triangles. Numbers on the branches are the bootstrap values obtained from 1000 replicates (only bootstrap values >50 are shown). The accession numbers and abbreviation for the species names are listed in Table S1.

and *Lthsp702* exhibited significant differences in expression levels during development (*Lthsc70*:$F_{5,17} = 3.345$, $P < 0.05$; *Lthsp702*:$F_{5,17} = 12.151$, $P < 0.001$), whereas no significant differences were observed for *Lthsp701* ($F_{5,17} = 0.504$, $P = 0.769$). Expression of *Lthsc70* was highest in female adults, which was 2.86-fold higher than the control group (male adults). Moreover, the expression of *Lthsc70* has significant difference between female adults and 10-day-old pupae (fig. 5a). The expression level of *Lthsp702* was significantly upregulated in larvae and prepupae, which were 29.54- and 41.59-fold higher than the control group (fig. 5c). Although *Lthsp701* showed a slight increase in expression in pupal stages (fig. 5b), the difference was not significant.

Discussion

HSPs participate in a variety of physiological processes and are known to increase the thermal tolerance of diverse organisms (Burton *et al.*, 1988; Parsell & Lindquist, 1993; Parsell *et al.*, 1993). In insects, HSPs are produced during normal developmental stages and diapause (Joplin & Denlinger, 1990; Li *et al.*, 2007; Xiao *et al.*, 2011). HSP70 is one of the most conserved and intensively studied members of the HSP family (Cui *et al.*, 2010).

In this study, three genes encoding HSP70s were identified in *L. trifolii*, namely, *Lthsc70*, *Lthsp701*, and *Lthsp702*. The deduced protein products of these three genes contained signature sequences and motifs typical of the HSP70 family. The *C*-termini of the three *Lt*HSP70s contain the conserved EEVD motif, which enables HSC70/HSP70 to interact with chaperones in the cytoplasm (Daugaard *et al.*, 2007). The *N*-termini of the *Lt*HSP70s contained a conserved sequence with an ATP–GTP binding site, which is associated with conformational switching and ATPase activity (Siligardi *et al.*, 2005; McLaughlin *et al.*, 2006).

With respect to genomic structure, a 1273-bp intron was discovered in *Lthsc70*; in contrast, *Lthsp701* and *Lthsp702* lacked introns. A negative correlation between intron size and gene expression level was suggested previously, and genes lacking introns or containing smaller introns were more highly expressed than genes with large introns (Castillo-Davis *et al.*, 2002; Comeron, 2004). It also remains

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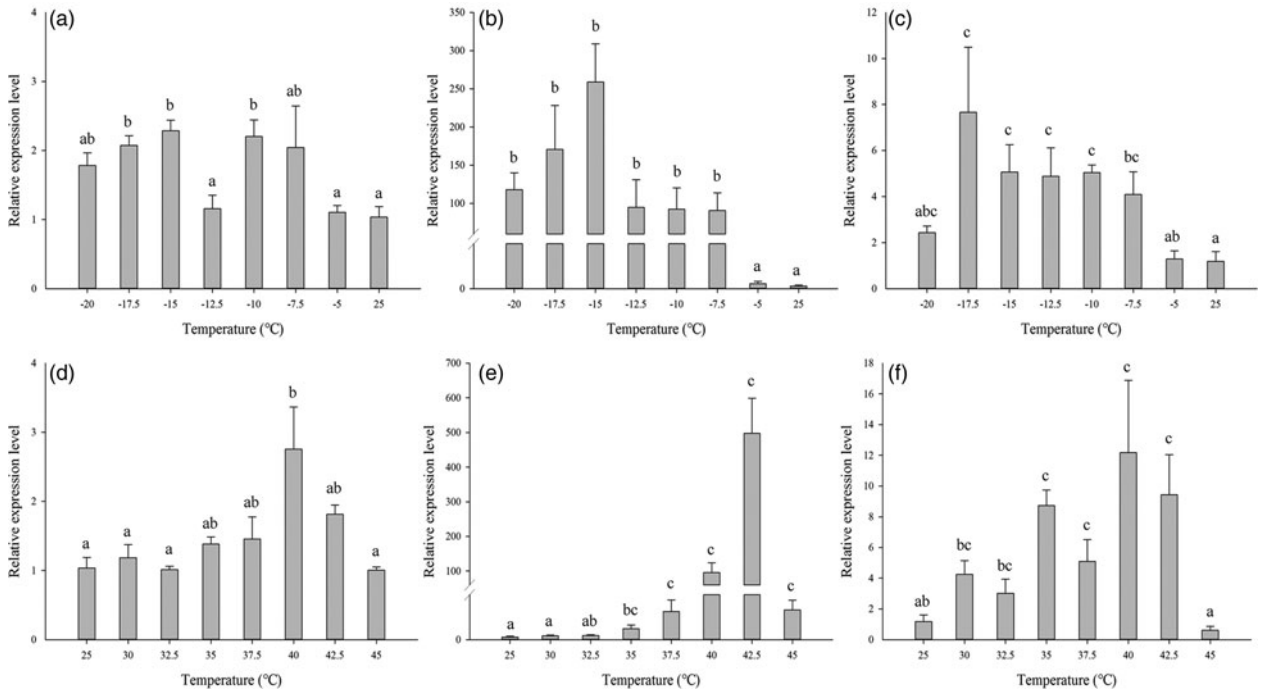

Fig. 4. Relative expression levels of three HSP70 genes in *Liriomyza trifolii* under low- and high-temperature treatments. The relative level of *hsp* expression represented the fold increase as compared with the expression in controls. Relative expression levels of *hsc70* under cold stress (A), *hsp701* under cold stress (B), *hsp702* under cold stress (C), *hsc70* under heat stress (D), *hsp701* under heat stress (E), *hsp702* under heat stress (F). The data were denoted as mean ± SE.

plausible that HSP-encoding genes that lack introns (e.g. *LtHsp701* and *LtHsp702*) might be more sensitive to environmental stress. Another notable difference in genomic structure was the presence of a TATA-box-like motif in the 5′UTR of *Lthsp701*, which was absent in *Lthsc70* and *Lthsp702*. We previously identified five TATA-box-like elements in *Lthsp70*, whereas orthologs in *L. huidobrensis* (*Lhhsp70*) and *L. sativae* (*Lshsp70*), contained one and two TATA-box elements, respectively (Huang & Kang, 2007; Chang *et al*., 2017a). The position, spacing, and number of TATA elements can influence *hsp* expression patterns (Hunt & Morimoto, 1985; Wu *et al*., 2001; Grace *et al*., 2004).

The predicted amino acid sequences of *Lthsc70*, *Lthsp701*, and *Lthsp702* shared considerable sequence similarity to HSP70 in other insect species. Interestingly, the dendrogram generated by the NJ method placed *Lt*HSC70, *Lt*HSP701, and *Lt*HSP702 in different branches. This contrasts with the findings reported for *Macrocentrus cingulum* and *Plutella xylostella* (Xu *et al*., 2010; Zhang *et al*., 2015), where intraspecific HSPs clustered together. Thus, in addition to cellular localization and expression patterns (Mahroof *et al*., 2005; Renner & Waters, 2006; Daugaard *et al*., 2007), there may be additional ways to classify HSP70. It is interesting to speculate that HSP genes in insects have originated from an ancestral gene

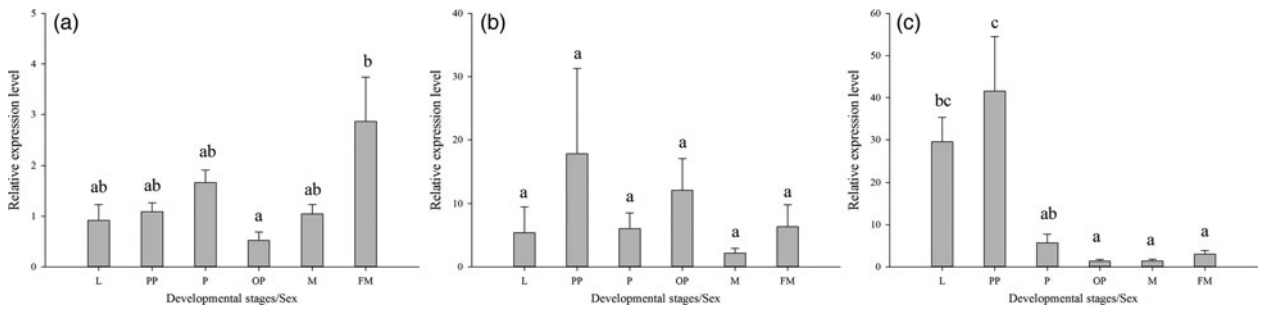

Fig. 5. Relative expression levels of three HSP70 genes in different developmental stages of *Liriomyza trifolii*. The relative level of *hsp* expression represented the fold increase as compared with the expression in controls. Relative expression levels of *hsc70* (A), *hsp701* (B), *hsp702* (C). The data were denoted as mean ± SE. Abbreviations: FM, females adult; M, males adult; L, third instar larvae; PP, prepupae; P, 2-day-old pupae; OP, 10-day-old pupae.

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but have since diverged after a long evolutionary period. In this context, HSPs can be regarded as useful markers for phylogenetic analysis in insects (Lu *et al.*, 2016).

Multiple studies have shown that genes encoding HSPs are induced by temperature stress; furthermore, *hsp70* and *hsp20* are generally more susceptible to thermal stress than other *hsps* (Huang & Kang, 2007). In general, *hsp70* can be induced by high and low temperatures, whereas *hsc70* is insensitive to thermal stress. Interestingly, our results indicate that *Lthsc70* was induced by thermal stress, which is consistent with *hsc70* in *M. cingulum* (Xu *et al.*, 2010), *Pteromalus puparum* (Wang *et al.*, 2008), and *P. xylostella* (Sonoda *et al.*, 2006). However, our results contrasted with other studies (Rinehart *et al.*, 2000; Bettencourt *et al.*, 2008; Zhang & Denlinger, 2010). Therefore, insect forms of *hsc70* display species-specific transcriptional changes in response to heat stress and can be induced or constitutively expressed. The three *Lthsp70*s varied in terms of temperature sensitivity (fig. 4) and suggest a synergistic effect of different HSP70 family members with respect to thermal tolerance.

The role of HSPs in the regulation of insect growth and development has become an important area of inquiry. In the current study, the three *Lthsp70s* showed some level of expression in all developmental stages and in both sexes. The expression of *Lthsp702* was highest in larvae and pre-pupae, whereas expression of *Lthsc70* was highest in female adults. The latter result is similar to the findings reported for *L. sativae* where *Lshsp* expression was highest in adults; however, sexes were not discriminated in the former study (Huang *et al.*, 2009). In this study, female adults had a slightly larger body size vs. male adults, which could possibly explain the results with *Lthsc70* expression. It remains possible that evolution may favor the expression of HSPs in female adults to preserve fecundity and reproduction; however, the tradeoffs between thermal tolerance and fitness costs should not be ignored (Huang *et al.*, 2007; Zhou *et al.*, 2010; Lü *et al.*, 2014). Functional connections between hormones and heat shock regulatory systems have been previously suggested (Lezzi, 1996). The expression of selected HSPs was induced by ecdysone in *Drosophila* and steroid hormones in mammals (Ryan & Hightower, 1998). Therefore, the expression of HSP genes during *L. trifolii* development may influence the secretion of hormones.

The expression of *Lthsp702* in larvae and prepupae was significantly higher than the control group (fig. 5), which consisted of male adults. The larvae of *L. trifolii* cause damage by feeding inside foliar tunnels, which form a microenvironment that provides some protection from temperature fluctuations. In the thermal stress experiments, we removed larvae from leaves, and the removal process may have elevated HSP gene expression. In the case of prepupae, the larvae simply exit the leaves, and the soft puparium may be more sensitive to temperature fluctuations. We observed a no negligible increase in *Lthsp701* and *Lthsp702* expression during the transition from larvae to prepupae, though there were no significant difference between those two developmental stages (fig. 5). Thus, in addition to diapause, metamorphosis may also induce HSP gene expression. Many tissues and organs are degraded and re-constructed during insect metamorphosis. It is quite possible that HSPs, which function as chaperones (Feder & Hofmann, 1999), facilitate the reconstruction of new tissues and organs during the pupal stage. The metamorphosis period is characterized by large physical changes, and the change in protein structure was previously shown to result in high expression levels of *hsp*s (Huang *et al.*, 2009). Interestingly, *Lthsp701* was also expressed at different developmental stages; however, there was no significant difference in *Lthsp701* expression between stages, which indicates that *Lthsp701* may not function in the leafminer growth and development.

In conclusion, the genes encoding *Lt*HSP70 in *L. trifolii* showed different expression profiles in response to temperature stress and during different leafminer development. This study provides new insights into the potential role of Hsp70s in insect behavior and development. Future studies are underway to further investigate the function of HSPs in the physiology of *L. trifolii.*

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Supplementary material

The supplementary material for this article can be found at https://doi.org/10.1017/S0007485318000354.

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