Cloning and expression of five heat shock protein genes in relation to cold hardening and development in the leafminer, *Liriomyza sativae*

Li-Hua Huang, Chen-Zhu Wang, Le Kang*

State Key Laboratory of Integrated Management of Pest Insects and Rodents, Institute of Zoology, Chinese Academy of Sciences, B5 Datun Road, ChaoYang District, Beijing 100101, China

**A B S T R A C T**

The vegetable leafminer, *Liriomyza sativae* has spread worldwide, causing serious loss of agricultural productivity. Heat shock proteins (HSPs) play important roles in the environmental adaptation of various organisms, and to explore the functions of HSPs in relation to cold tolerance and development in *L. sativae*, three full-length cDNAs of small heat shock protein genes (ls-hsp19.5, ls-hsp20.8 and ls-hsp21.7) and two partial cDNAs of tcp1 (the hsps60 homolog, ls-tcp1x and ls-tcp1y) were cloned, and their transcriptional expression during cold hardening and development was examined by real time quantitative PCR. The open reading frames (ORFs) of ls-hsp19.5, ls-hsp20.8 and ls-hsp21.7 are 516, 543 and 573 bp in length, encoding proteins with molecular weights (M.W.) of 19.5, 20.8 and 21.7 kDa, respectively. The 956 and 323 bp partial cDNAs were respectively sequenced from ls-tcp1x and ls-tcp1y. The expression profiles during cold hardening revealed that ls-tcp1s did not respond to cold stress. However, the three small hspS were significantly induced by cold, and ls-hsp20.8 was more cold-sensitive than the others. These results suggest that different shp members may be responsible for cold stresses of different intensity. The expression of hspS during developmental processes revealed that the mRNA levels of small hspS reached a peak in the pupal stage, whereas the levels of large hspS, including two ls-tcp1s, hsps60 and hsps90 increased gradually with the developmental process. These results suggest that in addition to a heat shock response, these HSPs may be involved in the development of *L. sativae*.

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1. Introduction

The vegetable leafminer *Liriomyza sativae* (Blanchard), a worldwide insect pest, causes serious loss of agricultural productivity (Parrella, 1987). It was originally found in South America but has spread widely from polar to tropical areas (Spencer, 1973; Reitz and Trumble, 2002). Temperature, particularly winter low temperature, is one of the key variables that limit the distribution and spread of *Liriomyza* species in temperate regions (Chen and Kang, 2004, 2005).

In the past decades, a number of studies have been carried out to explore the adaptive mechanism of *L. sativae* to temperature changes. It was found that the leafminer has a supercooling point (crystallization temperature of cells) of about −11 °C (Zhao and Kang, 2000), which allows it to survive at the −2 °C isotherm of the minimum mean temperature of January in North China (Chen and Kang, 2005). Both laboratory and natural cold acclimation increased its cold tolerance (Chen and Kang, 2005). This was suggested to relate to the expression of heat shock protein genes (Huang and Kang, 2007; Huang et al., 2007; Kang et al., 2009).

The heat shock proteins (HSPs) represent a super gene family. On the basis of molecular weight (M.W.) and homology, heat shock proteins are divided into several families, including HSP100, HSP90, HSP70, HSP60, HSP40 and small HSPs (sHSPs, the molecular weights ranging from 12 to 43 kDa) (Kim et al., 1998; Feder and Hofmann, 1999; Sørensen et al., 2003). In response to a variety of stress factors, such as extreme temperatures, organisms commonly synthesize heat shock proteins, which usually act as molecular chaperones, promoting correct refolding and preventing aggregation of denatured proteins (Johnston et al., 1998; Feder and Hofmann, 1999).

Heat shock proteins are suggested to participate in diverse physiological processes. One of the best known functions is to increase heat tolerance and protect organisms from thermal injury and killing (Gehring and Wehner, 1995). Heat shock proteins are also suggested to be involved in the developmental processes of some insects. In *Drosophila melanogaster*, expression of small heat shock proteins was observed during both male gametogenesis (Joannis et al., 1998) and embryonic development (Haass et al., 1990). In addition, heat shock proteins may play roles in insect
diapause, a physiological stage of developmental arrest (Flannagan et al., 1998; Yocum et al., 1998; Rinehart et al., 2000, 2007).

The early study on Liriomyza insects has shown that hsp90, hsp70, hsp40 and some shsps could be induced by cold shock, however, hsp60 had only a slight induction upon heat shock, and did not respond to cold (Huang and Kang, 2007). Moreover, the expression of HSP genes may bring some negative effects on feeding and fecundity in Liriomyza, which indicated that the expression of hsps may relate to physiological processes in Liriomyza (Huang et al., 2007). To further explore the functions of hsps in relation to cold tolerance and development in Liriomyza, we cloned five hsp homologs and analyzed their transcriptional expression during cold hardening and development. The following two questions are addressed in this study. (1) How do different hsps respond to cold hardening? (2) Are they involved in the development of L. sativae?

2. Materials and methods

2.1. Insects

The leafminers were originally collected on celery in Beijing in 1997 and reared in the laboratory at 25 °C with a 12 h light–12 h dark cycle as described by Chen and Kang (2005).

2.2. Cold hardening

Thirty 2-day-old pupae were placed in a 5 ml cryogenic tube, shocked at a target temperature, including 0, −5, −10 and −15 °C, respectively for 1 h. Temperature treatments were achieved by the programmable temperature controller (Polyscience, IL, USA). The pupae were then allowed to recover at 25 °C with a 12 h light–12 h dark cycle as described by Chen and Kang (2005).

2.3. Sampling at different developmental stages

Thirty 3-instar larvae, 2-day-old pupae, and 1-day-old adults were respectively sampled in this experiment.

2.4. Reverse transcription PCR and RACE

Total RNAs were isolated using the RNeasy® Mini Kit (Qiagen, CA, USA), and 2 μg RNAs were used to generate the cDNAs using the oligo(dT)15 primer according to the instruction of Reverse Transcription System (Promega, WI, USA). Degenerate primers (Table 1) were used to amplify the partial segments of HSP genes, the 5′ and 3′ regions of the corresponding cDNAs were obtained by 5′ and 3′ RACE (Rapid Amplification of cDNA Ends) using the SMART RACE cDNA Amplification Kit (Clontech, CA, USA) with gene specific primers GSP1 and GSP2 (Table 1). To ensure that the 5′ and 3′ fragments were from the same gene, specific primer sets flanking the open reading frames (ORFs) were designed, then used to PCR amplify the entire ORF sequences.

2.5. Sequence alignment and identity analysis

The obtained cDNA sequences of HSPs were used to search for analogs in GenBank by BLAST software available at the NCBI website (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). The sequence alignment and identity analysis were carried out using the DNAMAN software package (Lynnon Corporation, Canada), and ORFs were identified with the aid of the ORF Finder software (http://www.ncbi.nlm.nih.gov/orf/orf.html). The molecular weights of the predicted proteins were calculated by the SWISS-PROT (ExPASy server) program “Compute pi/Mw” (http://au.expasy.org/tools/pi_tool.html).

2.6. Real time quantitative PCR

Real time PCR reactions were performed in a 20 μL total reaction volume including 10 μL of 2× SYBR® Premix EX Taq™ (TaKaRa) master mix, 5 μM each of gene specific primers (Table 1), and the cDNA templates. The reactions were carried out on the Mx 3000P detection system (Stratagene, La Jolla, CA) according to the method of Huang et al. (2007). The quantity of each hsp mRNA was normalized to the abundance of β-actin. Subsequently, the normalized value of each hsp was divided by a special control (the amount of hsp70 at 25 °C), and the fold was then used as the relative mRNA levels of each hsp. To correct for plate variation, the hsp70 level at 25 °C was quantified in each plate.

2.7. Statistical analysis

The statistical significance of difference between treatments was analyzed either by t-test (for comparison of two means) or by one-way analysis of variance (ANOVA; Systat Inc.) followed by a Tukey’s test for multiple comparisons. For the ANOVA, hsp expression levels were log-transformed by the method of Tomanek and Somero (1999) to assure homogeneity of variances among different groups. The data were denoted as means ± SE and analyzed using SPSS 11.0 software.

Table 1

<table>
<thead>
<tr>
<th>Genes</th>
<th>Primer sequence (5′→3′)</th>
<th>Fragment length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>shp</td>
<td>GTDCARGGSGAAYCAYAGG</td>
<td>134</td>
</tr>
<tr>
<td>tcp1α</td>
<td>GTBGNMYSACACAAATG</td>
<td>956</td>
</tr>
<tr>
<td>tcp1ζ</td>
<td>GHTYTTYTYTACAAWRNCNG</td>
<td>323</td>
</tr>
</tbody>
</table>

3. Results

3.1. Cloning and characterization of ls-hsps

Three heat shock genes, namely ls-hsp19.5, ls-hsp20.8 and ls-hsp21.7 (GenBank accession nos. DQ452373, DQ452374, DQ452372), were cloned from L. sativae. The ORFs are 516, 543 and 573 bp, encoding 171, 180 and 190 amino acids with molecular weights of 19,468, 20,848 and 21,721 Da, respectively. Their deduced amino acid sequences contain the typical α-crystallin domain (Fig. 1), which...
suggests that they are small heat shock protein genes. The non-
translated regions of these hsp genes contain several typical motifs, such as the polyadenylation signal (AATAAA or ATTAAA, Tabaska and Zhang, 1999) and the AT-rich element (ATTTA) (Fig. 1), which have been shown to afford greater mRNA stability at normal temperatures (Colgan and Manley, 1997; Rubenstein and Lyons, 2001) and to contribute to the maintenance and re-establishment of basal levels of gene expression (Lindquist and Petersen, 1990). A search of the

Fig. 1. Nucleotide sequences for L. sativae small heat shock protein genes and their predicted amino acid sequences. Nucleotide numbering starts with the adenine in the first methionine codon of the putative open reading frame. The highly conserved region, α-crystallin domain, is underlined. The asterisk indicates the translational termination codon. The putative polyadenylation signal is double-underlined, and the AT-rich element is boxed.

`ls-hsp19.5`

suggests that they are small heat shock protein genes. The 3' non-
translated regions of these hsp genes contain several typical motifs, such as the polyadenylation signal (AATAAA or ATTAAA, Tabaska and Zhang, 1999) and the AT-rich element (ATTTA) (Fig. 1), which have been shown to afford greater mRNA stability at normal temperatures (Colgan and Manley, 1997; Rubenstein and Lyons, 2001) and to contribute to the maintenance and re-establishment of basal levels of gene expression (Lindquist and Petersen, 1990). A search of the

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`ls-hsp20.8`

suggests that they are small heat shock protein genes. The 3' non-
translated regions of these hsp genes contain several typical motifs, such as the polyadenylation signal (AATAAA or ATTAAA, Tabaska and Zhang, 1999) and the AT-rich element (ATTTA) (Fig. 1), which have been shown to afford greater mRNA stability at normal temperatures (Colgan and Manley, 1997; Rubenstein and Lyons, 2001) and to contribute to the maintenance and re-establishment of basal levels of gene expression (Lindquist and Petersen, 1990). A search of the

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`ls-hsp21.7`
GenBank sequence repository revealed that the amino acid identities among insect sHSPs varied from 27 to 63%, with the highest similarity between ls-hsp21.7 and D. melanogaster l(2)efl (GenBank accession no. NP_523827). However, all three ls-hsps show very low amino acid identity (about 30%) with the best known shsps (hsp22, hsp23, hsp26 and hsp27) from D. melanogaster.

Partial sequences of ls-tcp1α and ls-tcp1ζ (GenBank accession nos. EU098136, EU098137) were also obtained from L. sativae. They are 956 and 323 bp in length, encoding 318 and 107 amino acids, respectively (Fig. 2). A search of GenBank by Blast X revealed that they have the highest sequence identity to the D. melanogaster TCP1α (NP_524450) and TCP1ζ (NP_573066), respectively. It was suggested that they are TCP1s, distant homologues of HSP60 (Gupta, 1995; Brocchieri and Karlin, 2000).

3.2. Expression of the ls-hsps in cold hardening

The ls-hsps revealed different expression patterns in response to cold hardening. The three small hsps were significantly induced by cold (Fig. 3A) but not the two tcp1s (Fig. 3B). The relative expression levels of three small ls-hsps dramatically increased in cold hardening. For example, as compared with the amount of expression at 25 °C, the expression levels of three ls-hsps (ls-hsp19.5, ls-hsp20.8, and ls-hsp21.7) at -10 °C increased by 3.9, 24.7 and 2.1 fold, respectively (Fig. 3A). Their expression patterns in response to cold differed from each other. Expression of ls-hsp20.8 began to be induced at -5 °C \( (t_{25-5} = 10.5, P < 0.001) \) and reached its peak (82.9 fold) at -10 °C. However, the transcriptional expression of both ls-hsp19.5 and ls-hsp21.7 was not induced until the

![Fig. 2. Partial nucleotide sequences for tcp1α and tcp1ζ of L. sativae and their deduced amino acid sequences.](image_url)
temperature decreased to $-10^\circ C$ ($ls$-hsp19.5, $t_{(25^\circ C - -10^\circ C)} = 3.73$, $P = 0.02$; $ls$-hsp21.7, $t_{(25^\circ C - -10^\circ C)} = 3.79$, $P = 0.019$), and the highest levels were no more than 35 fold. This suggests that $ls$-hsp20.8 is more sensitive to cold than the others. The expression profiles of $ls$-tcp1s revealed that the expression levels of these two genes did not change upon cold exposure (1 h at 0, $-5$, $-10$ and $-15^\circ C$, respectively; $ls$-tcp1$\alpha$, $F = 2.35$, $P = 0.13$; $ls$-tcp1$\zeta$, $F = 0.66$, $P = 0.63$) (Fig. 3B).

3.3. Expression profile of $ls$-hsp at different developmental stages

To profile the expression of $ls$-hsp during the developmental process in $L$. sativae, mRNA levels of the five $ls$-hsp were analyzed at larval, pupal and adult stages, respectively. The relative levels of former identified hsps ($hsp60$ and $hsp90$, Huang and Kang, 2007) were also analyzed. The expression levels of $\beta$-actin did not change significantly at different developmental stages of $L$. sativae (Fig. 4), which suggests that $\beta$-actin can be used as an internal standard in quantitative PCR. The mRNA levels of the three small $ls$-hsp in larva and adult were low and nearly the same as each other, except for $ls$-hsp21.7, whose level decreased significantly in the adults.
Small HSPs are probably the most diverse in structure and function amongst the various superfamilies of stress proteins (Franck et al., 2004). Different organisms have different numbers of sHSPs, ranging from only one in Saccharomyces cerevisiae (Petko and Linquist, 1986) up to 50 in higher plants (Mansfield and Key, 1995; Leroux and Candido, 1997; Huang et al., 2008). The expression of these two genes indicated that neither sHSPs, ranging from only one in pupa (ls-hsp19.5, t(pupa–pupa) = 5.24, P = 0.006; t(pupa–adult) = 5.89, P = 0.004; ls-hsp20.8, t(pupa–pupa) = 12.67, P < 0.001; t(pupa–adult) = 8.75, P = 0.001; ls-hsp21.7, t(pupa–pupa) = 9.70, P = 0.01; t(pupa–adult) = 12.42, P < 0.001) (Fig. 5A). The expression of tcp1s exhibited different patterns from the small hsp60. Their mRNA levels remarkably increased with the progress of development, except for ls-tcp1Δ, which did not increase beyond the pupal stage (hsp60, t(larva–pupa) = 5.36, P = 0.006; t(pupa–adult) = 3.69, P = 0.02; ls-tcp1α, t(larva–pupa) = 6.63, P = 0.003; t(pupa–adult) = 8.73, P = 0.001; ls-tcp1Δ, t(larva–pupa) = 4.80, P = 0.009; t(pupa–adult) = 2.56, P = 0.063) (Fig. 5B).

The levels of hsp90 increased gradually with development and reached its maximum in the adults (t(larva–pupa) = 4.42, P = 0.012; t(pupa–adult) = 5.37, P = 0.006) (Fig. 5C).

4. Discussion

Cytosolic HSP60 is less understood as compared with the mitochondrial versions. In this study, we cloned two L. sativae tcp1s, which were suggested to be present in the cytosol (Willison and Kubota, 1994) and a member of HSP60 (Burston and Clarke, 1995; Leroux and Candido, 1997; Huang et al., 2008). The expression profiles of these two genes indicated that neither responded to cold. Our previous studies on Liriomyza have shown that the mitochondrial hsp60 is slightly induced by heat, and does not respond to cold (Huang and Kang, 2007). Therefore, it appears that hsp60s are insensitive to temperature hardening. Similar phenomena have also been observed in other insects. For instance, the expression levels of mitochondrial HSP60 are not influenced by heat and cold in Trichinella spiralis (Wong et al., 2004). Furthermore, the expression of HSP60 does not respond to various types of stresses, such as H2O2 (Martinez et al., 2002), acidic and oxidative stress (Wong et al., 2004), and is critical for embryo formation. The physiological function of heat shock proteins during development suggest that Hsp transcription is highly regulated during development. Therefore, in addition to their involvement in the heat shock response, these HSPs may be involved in development. The physiological function of heat shock proteins during development remains the subjects of further investigation.

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References


In conclusion, the heat shock proteins of L. sativae showed different transcriptional expression profiles in response to cold and at different development stages. The regular expression patterns of heat shock protein genes during development suggest that Hsp transcription is highly regulated during development. Therefore, in addition to their involvement in the heat shock response, these HSPs may be involved in development. The physiological function of heat shock proteins during development remains the subjects of further investigation.

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