Cloning and interspecific altered expression of heat shock protein genes in two leafminer species in response to thermal stress

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Abstract

Studies have demonstrated differences in temperature tolerance between two *Liriomyza* species, *L. huidobrensis* and *L. sativae*. To investigate whether the heat shock proteins (Hsps) in the two species have different expression profiles during temperature stress, we cloned *hsp90*, *70*, *60*, *40* and *20*, and analysed their expression profiles across temperature gradients by real-time quantitative PCR and Western blotting. The results revealed that the number of TATA-box-like elements and A/T-rich insertion/deletions within the 5′ UTRs of the *hsps* are different in the two species. The temperatures for onset (T_{on}) or maximal (T_{max}) induction of *hsp* expression in *L. huidobrensis* were generally 2.5–10 °C lower than those in *L. sativae*, and the T_{on} were highly consistent with the temperature limits of the northern boundary of the range of these two leafminer species. These studies confirmed, in terms of gene expression levels, that *L. huidobrensis* is more cold tolerant than *L. sativae*, which is more heat tolerant, and suggest that the T_{on} (or T_{max}) of *hsps* can represent the differences in temperature tolerance of these two leafminer species, and may be used to determine their natural geographical distribution limits.

Keywords: heat shock protein, expression profile, temperature tolerance, leafminer, geographical distribution.

Introduction

Liriomyza huidobrensis and *Liriomyza sativae* (Diptera: Agromyzidae) are important insect pests on vegetables and ornamental plants. They can severely reduce yields, transport viral and fungal diseases (Civelek & Önder, 1999) and even kill plants at high densities (Spencer & Steyskal, 1986). Originating from South America (Spencer, 1973), they have spread to Europe, Africa and Asia. In China, these two leafminer species were first found in Hainan Island in 1993 and Yunnan province in 1994, respectively, and have since spread to more than 15 provinces (Kang, 1996; Chen & Kang, 2002).

Temperature is one of the most important factors that determines the distribution and abundance of species (Worner, 1998; Bale *et al.*, 2002). The *L. huidobrensis* pupa has an average supercooling point of about −21 °C (Chen & Kang, 2002), which is much lower than that of *L. sativae* (−11 °C; Zhao & Kang, 2000). Moreover, the highest survival temperature limit for *L. huidobrensis* is lower than that of *L. sativae* (32 and 37 °C, respectively; Zhou *et al.*, 2001). Overall, *L. huidobrensis* is more cold tolerant but less heat resistant than its congener. In nature, the leafminer populations in cool areas and cool seasons are dominated by *L. huidobrensis*, which has expanded its distribution in northern China more quickly than *L. sativae* in recent years (Chen & Kang, 2002). Indeed, population displacement was also observed between *L. huidobrensis* and *L. trifolii* (MacDonald, 1991). Early on, researchers speculated that this displacement was a result of differential pesticide resistance (Parrella *et al.*, 1984; MacDonald, 1991). However, recent studies indicate that differential temperature tolerance is one of the important reasons for population displacement in these leafminers (Chen & Kang, 2002, 2005a). Because of the use of greenhouses in large-scale agricultural development in China, distributions of *L. huidobrensis* and *L. sativae* have expanded into Northern temperate regions, where their cold tolerance has declined remarkably, but there still remains a differentiation in the levels of cold tolerance between the species (Chen & Kang, 2004, 2005b). To date, the mechanisms behind the

different cold tolerance between the two species have not been examined at molecular level.

Many candidate genes have been identified for thermoresistance. The *Methusalah* (Lin *et al.*, 1998) and *Turandot* (Ekengren & Hultmark, 2001) genes are connected to heat resistance, and *Dca* (Goto, 2000), *frost* (Goto, 2001) and *hsr-omega* (Singh & Lakhotia, 1984) are potential candidate genes related to cold tolerance. Recent studies have suggested that the heat shock proteins (Hsps) may be some of the most important genes involved in temperature responses (Feder & Hofmann, 1999; Hoffmann *et al.*, 2003). Heat shock proteins usually act as molecular chaperones, promoting correct refolding and preventing aggregation of denatured proteins (Johnston *et al.*, 1998; Feder & Hofmann, 1999) in response to a variety of stress factors such as heat, cold, osmotic and oxidative stresses, heavy metal, irradiation, viral infection and high population densities (Lindquist, 1986; Feder & Hofmann, 1999; Sørensen *et al.*, 2003). On the basis of molecular weight and homology of amino acid sequences, Hsps can be divided into several families including Hsp90, 70, 60, 40 and small Hsps (sHsps) (Feder & Hofmann, 1999; Sørensen *et al.*, 2003). Although there is a strong indication that heat shock proteins lead to thermotolerance or thermoprotection (Mitchell *et al.*, 1979), the functional significance of the heat shock response is only partially understood. Among the Hsp families, Hsp90 and Hsp70 are the most abundant in the cell. Hsp90 appears to be involved in the negative regulation of proteins such as steroid receptors, tyrosine kinases, elongation factor eF-2α, protein kinase C, casein kinases, actin and tubulin (Lindquist & Craig, 1988). The Hsp70 proteins have diverse functions in protein folding, translocation across membranes, assembly and metabolism (Pelham, 1986). The function of sHsp has not yet been fully revealed, but it appears to play roles in the organization of cytoskeletons and protection of insects during the diapause (Yocum *et al.*, 1998; Rinehart & Denlinger, 2000).

A few insect models have been used to study the mechanism and evolution of thermotolerance. The two leafminer species studied here originated from South America (Spencer, 1973), have spread worldwide and differ greatly in temperature tolerance (Zhao & Kang, 2000; Chen & Kang, 2002), creating an ideal situation with which to study the mechanism of temperature tolerance. Although Hsps have been proposed to participate in cold and heat shock responses, the mechanisms as to how Hsps respond to temperature stress in insects are not well understood. In the present study, we try to compare differences in Hsp expression profiles between these two leafminer species in an attempt to examine the roles of Hsps in relation to temperature tolerance. We discuss our findings in the context of relationships among the Hsp expression features and natural distribution patterns of these leafminers.

Results

Cloning and characterization of hsp genes

Heat shock proteins are a superfamily including Hsp90, 70, 60, 40 and sHsps. To clone all the five *hsp* genes in the two leafminer species, degenerate primers were designed according to other insect *hsps*, and the full-length cDNAs were obtained by 5′ and 3′ RACE.

The full-length cDNAs of *hsp90* in the two leafminer species were obtained. Both contain a 2154 bp open reading frame (ORF) encoding 714 amino acids (aa). Their molecular weights (MWs) are 81 691 and 81 578 Da and have the GENBANK accession numbers AY851367 and AY851368 for *L. huidobrensis* and *L. sativae*, respectively. The full aa sequences of *Liriomyza hsp90s* were aligned with those of other insects such as *Drosophila melanogaster*, *Bombyx mori*, *Apis mellifera*, *Locusta migratoria* and *Tribolium castaneum* (Supplementary Material Fig. S1). The alignment displayed a high degree of conservation (81–97%).

The ORFs of *Liriomyza hsp70s* comprise 642 and 638 aa with the calculated masses of 70 738 (AY842476) and 70 236 Da (AY842477) in *L. huidobrensis* and *L. sativae*, respectively. The *hsp70* aa sequence of *L. huidobrensis* is highly homologous (97% identity) to that of *L. sativae*, and their high identities with other insects were also observed: 85% to *D. melanogaster*, 81% to *B. mori*, 74% to *T. castaneum*, and 73% to both *A. mellifera* and *L. migratoria*. A conserved 'EEVD' motif was found at the C-terminals of *Liriomyza hsp90* (Fig. 1A) and *hsp70* (Fig. 1B). From the alignment of insect *hsp70s* (Supplementary Material Fig. S2), we found another typical motif (GGMP)$_n$ (Fig. 1B), which is close to the 'EEVD' motif. However, the 'GGMP' repeats are lost in the *hsp70* of *Liriomyza* and *Drosophila*.

A 1716 bp ORF is present in *Liriomyza hsp60s*, and encodes 572 aa with deduced MWs of 60 924 (AY845949) and 61 209 Da (AY851366) in *L. huidobrensis* and *L. sativae*, respectively. The aa identity of *hsp60* is 97% between the two leafminer species and above 73% among all the other insects. The *Liriomyza hsp60s* were characterized by a conserved 'GGM' motif located at the C-terminals (Fig. 1C). Many ATP/Mg^{2+} binding sites were found to spread all over the *hsp60* domain (Supplementary Material Fig. S3).

The *Liriomyza hsp40s* contain 1020 and 1017 bp ORFs, and encode 340 and 339 aa with MWs of 38 163 (DQ452364) and 37 915 Da (DQ452365) in *L. huidobrensis* and *L. sativae*, respectively. The aa identity is very high (90%) between the two leafminer species, and is about 60% compared with those of *D. melanogaster*, *B. mori*, *A. mellifera* and *L. migratoria* (Supplementary Material Fig. S3). The N-terminal 65 aa (position 4–68), which constitute the most conserved region of *hsp40*, are referred to as the DnaJ domain (Fig. 1D).

The sHsps are very diverse in structures, and their MWs range from 12 to 43 KDa (MacRae, 2000; Franck *et al.*,

Figure 1. Salient features of the coding regions of the five heat shock protein (*hsp*) genes. The full amino acid sequences of *hsp90* (A), *70* (B), *60* (C), *40* (D) and *20* (E) genes were aligned and the typical motifs or domains were boxed and indicated on the top. For complete alignments refer to the Supplementary Material. Dashes denote identity to that on the top, and dots indicate alignment. LH90, *Liriomyza huidobrensis hsp90*; LS90, *Liriomyza sativae hsp90*; DM90, *Drosophila melanogaster hsp90* (P02828); TC90, *Tribolium castaneum hsp90* (XP_967904); AM90, *Apis mellifera hsp90* (XP_623939); BM90, *Bombyx mori hsp90* (NP_001036876); LM90, *Locusta migratoria hsp90* (AAS45246); LH70, *L. huidobrensis hsp70*; LS70, *L. sativae hsp70*; DM70, *D. melanogaster hsp70* (P02825); TC70, *T. castaneum hsp70* (XP_966611); AM70, *A. mellifera hsp70* (XP_392933); BM70, *B. mori hsp70* (NP_001037396); LM70, *L. migratoria hsp70* (AAP57537); LH60, *L. huidobrensis hsp60*; LS60, *L. sativae hsp60*; DM60, *D. melanogaster hsp60* (AAQ23524); TC60, *T. castaneum hsp60* (XM_966537); AM60, *A. mellifera hsp60* (XP_392899); MP60, *Myzus persicae hsp60* (CAB58441); LH40, *L. huidobrensis hsp40*; LS40, *L. sativae hsp40*; DM40, *D. melanogaster hsp40* (Q24133); BM40, *B. mori hsp40* (NP_001036990); AM40, *A. mellifera hsp40* (XP_394545); LM40, *L. migratoria hsp40* (DQ355966); LH20, *L. huidobrensis hsp20*; LS20, *L. sativae hsp20*; DM20, *D. melanogaster hsp20* (NP_523827); BM20, *B. mori hsp20* (AAG30945); AM20, *A. mellifera hsp20* (XP_392405); LM20, *L. migratoria hsp20* (ABC84493).
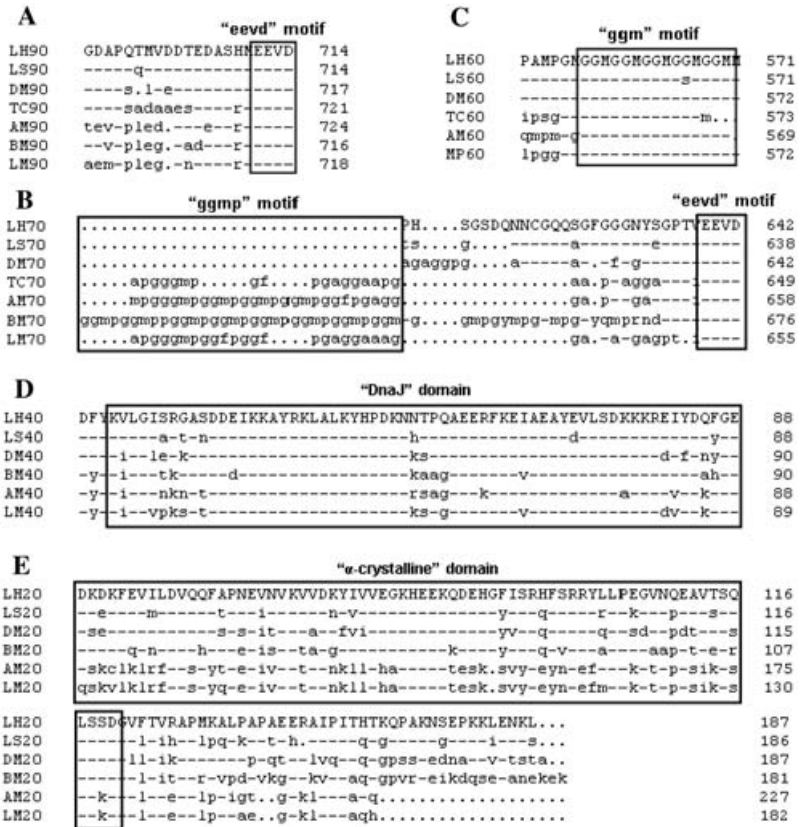

2004; Taylor & Benjamin, 2005). The ORFs are 561 bp long, encoding 187 aa with mws of 21 401 (DQ452370) and 21 251 Da (DQ452371) in *L. huidobrensis* and *L. sativae*, respectively. The alignment of sHsps revealed a conserved region in the middle, which constitutes an α-crystalline domain (Fig. 1E). The N- and C-terminals are both highly variable (Supplementary Material Fig. S5). The aa identity is 73% between the two leafminer species and is higher than those among leafminers and other insects: *D. melanogaster* (60%), *B. mori* (51%), *A. mellifera* (28%) and *L. migratoria* (27%). The *Liriomyza shsps* show the highest homologous (60% identity) to *l(2)efl* of *D. melanogaster*, indicating that they are the homologues of *l(2)efl*.

The coding regions of *Liriomyza hsps* are highly conserved. However, the 5′ UTRs are comparatively variable except for *hsp90*, in which only two nucleic acids have changed between the two species (Fig. 2A). Many TATA-box-like elements were identified within the 5′ UTRs of the five *Liriomyza hsps*, and the amounts varied among the *Liriomyza* species. The TATA-box-like element of *hsp60*, located at position –50 in *L. huidobrensis*, has disappeared in *L. sativea* as a result of two nucleic acid mutations at positions –54 and –56 (Fig. 2C), and similar situations have taken place in both *Liriomyza hsp40* (Fig. 2D) and *hsp20* (Fig. 2E). In general, the TATA-box-like elements are more abundant in *L. huidobrensis* than in *L. sativea* because of

mutations. The insertion/deletions have taken place in the 5′ UTRs of *Liriomyza hsps*. Comparing with the 5′ UTRs of *L. huidobrensis hsp40*, a 78-bp insertion was found to be present at position –44 in *L. sativea* (Fig. 2D). The insertions (> 14 bp) were also found in other *L. sativea hsps* including *hsp70* and *hsp20*. These insertions/deletions are A/T abundant (69.6–84.4%).

Expression profiles of the hsp genes

The relative mRNA levels of five *hsps* were quantified by real-time quantitative PCR at temperature gradients from –20 to 45 °C. The results revealed that the five *hsps* could not be induced by 1 h shock at temperatures ranging from 2.5 to 27.5 °C (Fig. 3). Most *hsps* were significantly up-regulated when temperature stress was enhanced, except that *hsp60* did not respond to the cold. The expression of the five *hsps* was inhibited when temperatures were lower than –17.5 °C or higher than 42.5 °C, both of which exceed the tolerance limits of the two leafminer species. The intensities of temperature response varied greatly among different *hsp* members. For example, the highest increases were 1432- and 254-fold in *hsp70* and *hsp20*, respectively. However, the increase was less than 40-fold in other *hsp* members. *Hsp90*, *hsp70* and *hsp40* showed very similar expression profiles and all three reached the highest amounts at exactly the same heat shock conditions. Two
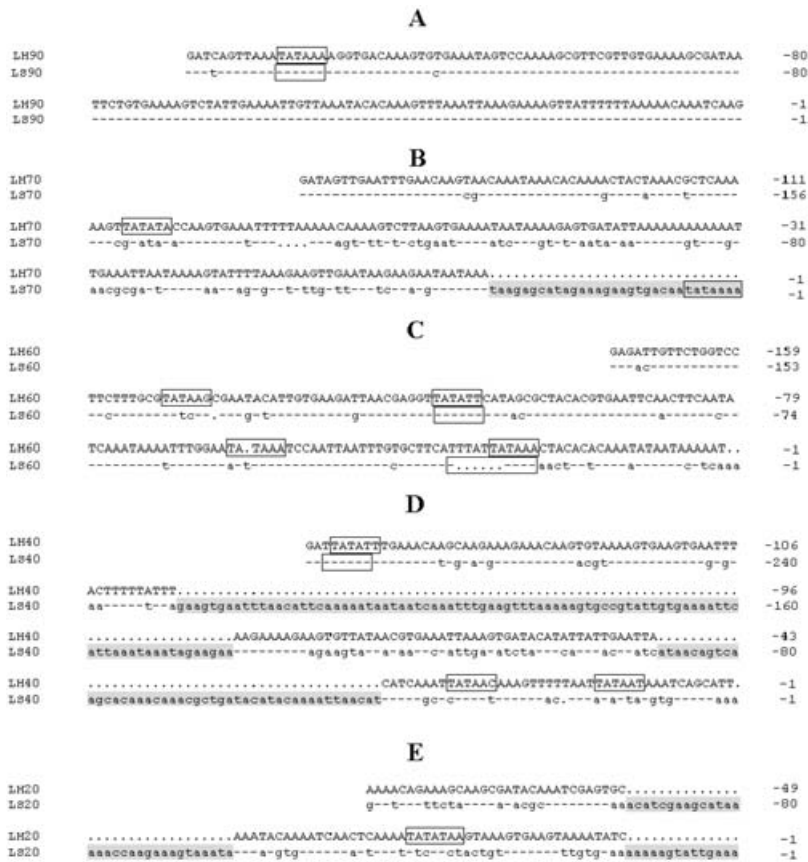
Figure 2. Alignment of the 5′ UTRs of *Liriomyza* heat shock protein (*hsp*) genes. The TATA-box-like elements are indicated in the box, and the insertions are shaded in grey. Dashes denote identity to that on the top, and dots indicate alignment. The proximal nucleic acid to initiative code (ATG) was numbered −1. Abbreviations as in Fig. 1. (A) *hsp90*; (B) *hsp70*; (C) *hsp60*; (D) *hsp40*; (E) *hsp20*.

obvious characteristics were revealed by the *hsp* expression profiles: (1) *hsp60* does not respond to the cold, whereas other *hsps* can be induced by both heat and cold temperature stresses; and (2) the temperature responses of *hsp70* and *hsp20* are more intense than those of the others.

Interspecific differences of hsp gene expression

To find the temperatures for onset (T_{on}) and maximal (T_{max}) induction of *hsp* expression, the relative mRNA levels of *hsps* were compared with each other and an interspecific difference was observed in most comparisons. T_{on} and T_{max} varied positively with the temperature tolerance. *Hsp90* began to be induced at −5 °C in *L. huidobrensis*, and peaked at −15 °C, whereas in *L. sativae*, T_{on} and T_{max} were −2.5 and −12.5 °C, respectively. Both T_{on} and T_{max} shifted by 2.5 °C between the two leafminer species at the cold temperature stress. Under heat stress, T_{on} and T_{max} of *hsp90* in *L. huidobrensis* decreased by 10 and 2.5 °C, respectively, as compared with those in *L. sativae* (Fig. 3). The other *hsps* showed the same trend of temperature shifts between the two leafminer species. The largest temperature shifts by cold stress occurred in *hsp70* and *hsp20*, both which were about 5−7.5 °C, and the most significant shifts (7.5−10 °C) by heat shock took place in *hsp90* and *hsp40*. Generally, the common trend was that T_{on} and T_{max} in

L. huidobrensis were 2.5−10 °C lower than those in *L. sativae* whenever the leafminers were shocked by cold or heat temperature stress.

Verification at protein levels

To test whether the shifts in T_{on} (or T_{max}) took place at the protein level, we examined the synthesis of Hsp proteins by Western blotting. Hsp70 and Hsp60 were chosen as Hsp representatives, as they represent two different patterns at the mRNA level. In *L. huidobrensis*, the protein level of Hsp70 started to increase at −7.5 °C, reached a maximum at −17.5 °C and then declined (Fig. 4). *L. sativae* Hsp70 showed a similar pattern as that in *L. huidobrensis*, except that T_{on} and T_{max} were −2.5 and −10 °C, respectively. T_{on} for Hsp70 protein synthesis under heat stress were 32.5 and 35 °C in *L. huidobrensis* and *L. sativae*, respectively, whereas both T_{max} were 42.5 °C, which was slightly different from the mRNA profiles. The temperature shifts for Hsp60 protein synthesis in the two species revealed the same pattern as those of the mRNA expressions (Fig. 5). The levels of Hsp60 proteins at low temperatures were nearly the same as those of the controls (25 °C), suggesting that the synthesis of Hsp60 proteins was not significantly induced in the cold. These results revealed that the expression profiles of Hsps at the mRNA and protein levels are
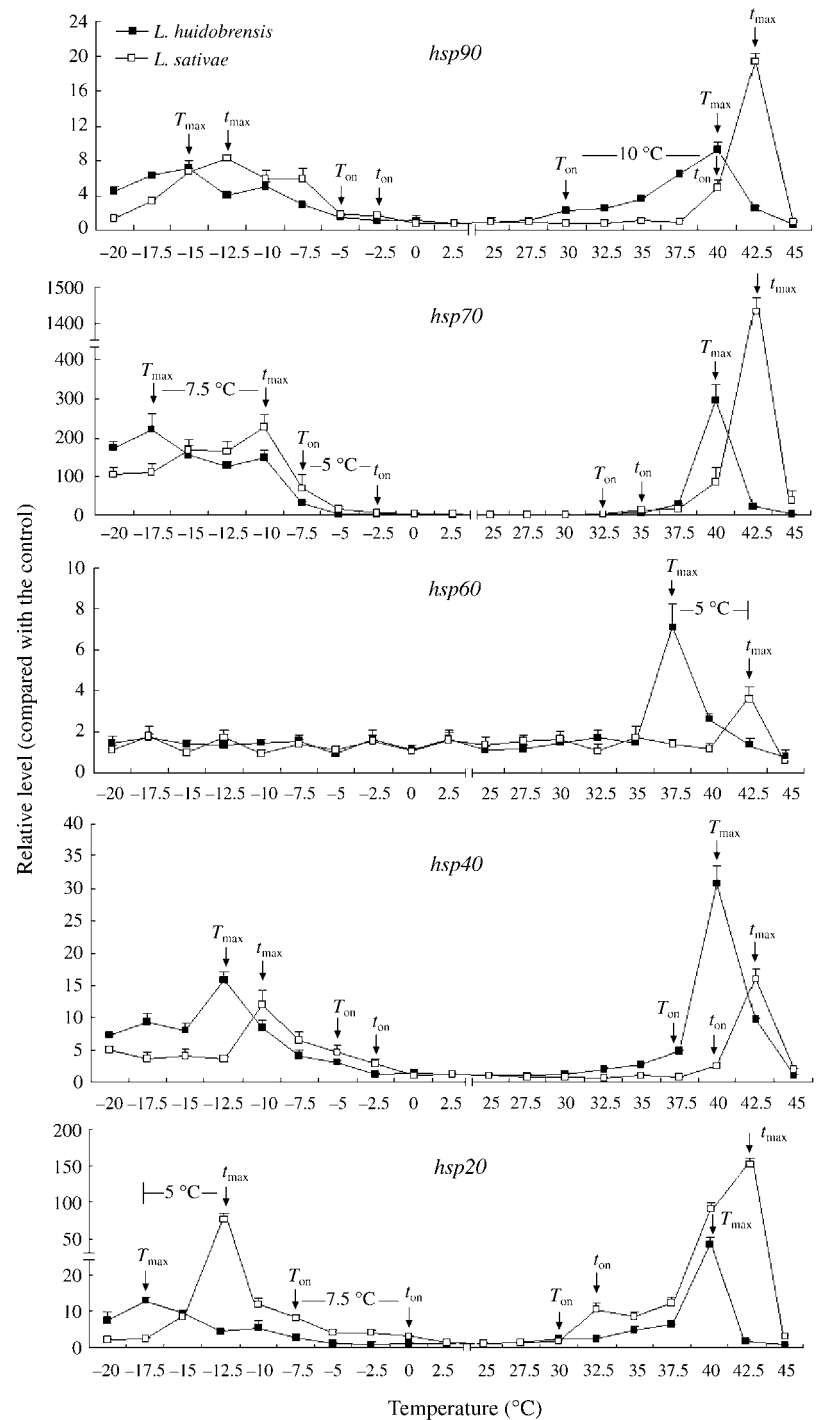
Figure 3. The mRNA expression profiles of five heat shock protein (*hsp*) genes in two leafminer species. The relative levels of *hsp* mRNAs were examined at the temperature gradients (–20–45 °C). The *Liriomyza hsps* were not induced by 1 h shock at temperatures from 2.5 to 27.5 °C, and the data between 2.5 °C and 25 °C are therefore omitted from the figure. The first temperature at which the expression level was significantly higher than that of the 25 °C treated samples was described as the onset temperature (T_{on}) of the synthesis of a particular *hsp*, and the temperature at which the expression level was significantly higher than those of all the others was denoted as T_{max}. T_{on} and T_{max} are marked by the arrow '→', and the remarkable temperature shifts of T_{on} and T_{max} are indicated on the curves. The letters 'T' and 't' represented the temperatures used in *Liriomyza huidobrensis* and *L. sativae*, respectively. The relative level of each *hsp* represented the increased folds compared with the mRNA amount of the untreated control. The data were denoted as mean ± SEM.

highly in agreement with each other, and the shifts of T_{on} (or T_{max}) at the protein level also represented the different temperature tolerances of the two leafminer species.

Discussion

The coding regions of five *hsp* genes are highly conserved in these two leafminer species, and a considerable number of special motifs are found in these regions. The tetrapeptide 'EEVD' and 'DnaJ' domains are, respectively, located at the C-terminal of Hsp70 and N-terminal of Hsp40 in *Liriomyza*. The two characteristic segments were postulated to interact with each other to stimulate the ATPase activity of Hsp70 (Michels *et al.*, 1999). The 'EEVD' motif is also present at the C-terminal of Hsp90, but the detailed function is still unclear. Our results reveal that the expression profiles of
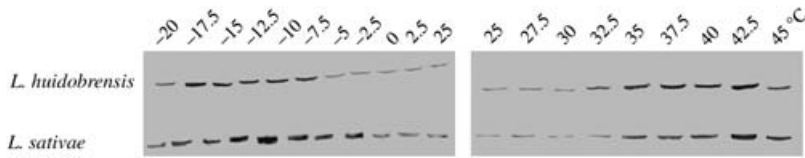
Figure 4. Western blotting of Hsp70 protein in two leafminer species.

Figure 5. Western blotting of Hsp60 protein in two leafminer species.

Hsp90, Hsp70 and Hsp40 are similar to each other. This is in agreement with the earlier finding that these Hsp members compose a chaperone complex (Tomanek & Somero, 2002). The C-terminal repeats $(GGM)_n$, which are characterized by mitochondrial Hsp60 (Tsugekils *et al.*, 1992), are present in *Liriomyza* Hsp60, indicating that *Liriomyza* Hsp60s are versions of mitochondrial Hsps. However, all the other *Liriomyza* Hsp proteins are located in the cytosol according to the amino acid identities. Hsp60 did not respond to the cold, whereas the other Hsp members could be induced by both cold and heat. The distinct difference between Hsp60 and other Hsps may relate to their subcellular locations. Another $(GGM)_n$-like motif (GGMP-repeats) is found to be present in Hsp70 of many insect species, such as *T. castaneum*, *A. mellifera*, *L. migratoria* and *B. mori*, but it seems to be absent in dipteran insects including *Drosophila* and *Liriomyza*. The similar 'GGM' or 'GGMP' repeats, which are located in both Hsp70 and Hsp60, indicate that they may be involved in protein–protein interactions. Indeed, the multiple structural similarities between Hsp70 and other Hsps (Hsp90, Hsp60 and Hsp40) suggest that Hsp70 may play a key role in the Hsp chaperone system.

Many regulatory elements present in the *hsp* promoter region such as HSE, 'TATA-box' and 'CAAT homology' (Hunt & Morimoto, 1985; Wu *et al.*, 1986, 2001; Yamada *et al.*, 2001; Grace *et al.*, 2004) play an important role in the *hsp* expression. Many TATA-box-like elements are found to exist in the 5′ UTRs of *Liriomyza hsps*, and their amount in *L. huidobrensis* was generally more than those in *L. sativae*. We also observed that several A/T-rich deletions (14–78 bp) are present within the 5′ UTRs of *L. huidobrensis hsps* including *hsp70*, *hsp40* and *hsp20*. The stereochemical properties of A/T-rich simple sequences are unusually important. (McClellan *et al.*, 1986), and have been reported to act as upstream enhancer elements *in vivo* to increase mRNA expression (Struhl, 1985). These findings suggest that the variation in the promoter region may be important in establishing specific patterns of *hsp* expression.

The two leafminer species differ in their *hsp* expression profiles, especially in their T_{on} (or T_{max}). Tomanek & Somero

(1999) have suggested that T_{on} (or T_{max}) may be useful indicators to interpret the limits of temperature tolerances. Recent findings support these relationships. The low-latitude species *D. virilis* exceeds the high-latitude species *D. lummei* in the temperature threshold for heat-shock factor activation (Garbuz *et al.*, 2003). Shifts in T_{max} have also been observed in Hsps. *D. melanogaster* and *D. ambigua* differ with respect to their maximal induction of Hsp70 protein synthesis and accumulation by 3−4 °C, and the former is more heat-resistant (Gehring & Wehner, 1995). A common trend was found that T_{on} (or T_{max}) of five *hsps* in *L. huidobrensis* were generally 2.5–10 °C lower than those in *L. sativae* whenever they were shocked by cold or heat. Furthermore, in cold stress, the *L. huidobrensis* hsps have lower T_{on} (or T_{max}), indicating that they can withstand more severe cold to activate the expression of Hsps. However, the situation is reversed at high temperatures: T_{on} (or T_{max}) are lower in *L. huidobrensis* than in *L. sativae*. Therefore, *L. huidobrensis* is more susceptible to heat than *L. sativae*. These observations conform well to previous evidence that *L. huidobrensis* is more cold tolerant while *L. sativae* is more heat resistant (Zhao & Kang, 2000; Chen & Kang, 2002). It seems that the lower the T_{on} (or T_{max}), the more cold tolerant (or less heat resistant) organisms are, and vice versa.

Temperature, particularly low winter temperature, may be a critical factor in determining the geographical range of insects (Worner, 1998; Bale *et al.*, 2002). Chen & Kang (2004, 2005a) investigated the distribution limits of these two leafminer species in China, and found that a −2 °C isotherm in January conforms to the northern boundary of *L. sativae*, and that *L. huidobrensis* has expanded to a belt between the isotherms of −4 to −6 °C in January An interesting phenomenon is that the T_{on} of *hsp90, 70, 40* and *20* are 0 ~ −2.5 °C in *L. sativae* and −5 ~ −7.5 °C in *L. huidobrensis*, and T_{on} in the two leafminer species were coincident with their distribution limits at the northern boundary. A possible explanation for such coherence is that temperatures around T_{on} may result in continual over-expression of *hsps*, which may restrain the development of insect populations. The induction of Hsp may provide

protection against the immediate damage from environmental stress, but this protection may carry a heavy cost measured as harm to the organism. This type of trade-off has been reported in many insect species. For example, the extra-copy larvae of *D. melanogaster* produce more Hsp70 protein in response to high temperature than excision larvae, but the abnormally high concentrations decrease growth, development and survival to adulthood (Krebs & Feder, 1997). High population density also induces the continual over-expression of *hsps* (*hsp90*, *20.7*, *20.6* and *20.5*) in gregarious locusts, resulting in smaller body size and lower fecundity than in solitary locusts (Wang *et al.*, 2007). Generally, the deleterious fitness effects will restrain dispersal and ultimately determine distributions of insects (Jenkins & Hoffmann, 1999). Therefore, T_{on} may be critical in determining the natural geographical distribution limits of insects.

Earlier studies indicated that *hsp* genes are strongly correlated with thermal tolerance (Mitchell *et al.*, 1979; Feder & Hofmann, 1999; Hofmann *et al.*, 2003). However, the direct relationship and relative importance of each *hsp* gene are poorly understood. Variation in the *hsp* promoter and coding regions has been found among geographical populations (Frydenberg *et al.*, 1999, 2003; Zatsepina *et al.*, 2001). This suggests that the *hsp* genes are selected by thermal pressure. Therefore, the DNA sequence variation among natural populations along the latitudinal gradients may give us a clue to understanding the evolution and function of *hsp* genes. Modern molecular genetic techniques have made it possible to directly manipulate the *hsp* genes. One method is to change gene expression. Extra copies of *hsps* can be introduced into the genome to increase the expression levels. Twelve extra copies of *hsp70* have been integrated in the genome of *D. melanogaster*, and this extra-copy strain was found to increase stress tolerance (Feder *et al.*, 1996; Roberts & Feder, 2000; Gong & Golic, 2006). Besides, a mutation in the promoter region also affects gene expression. The mutant strains can be generated by homologous recombination, and the consequences can be observed by phenotypic changes. Another method is to knock out the *hsp* genes. The *hsp70* genes have been successfully knocked out in *Mus musculus* (Hunt *et al.*, 2004), *Caenorhabditis elegans* (Olsen *et al.*, 2006) and *D. melanogaster* (Gong & Golic, 2004). It provides a direct approach to study the role of each *hsp* gene in thermal adaptation.

In conclusion, the expression of *Liriomyza hsps* has revealed two distinct patterns: (1) both cold- and heat-induced *hsps* (including *hsp90*, *70*, *40* and *20*), and (2) one only heat-induced *hsp* (*hsp60*) exist. Among the Hsp superfamily, *hsp70* and *hsp20* are more susceptible to temperature stress than the others. The T_{on} (or T_{max}) represent the temperature tolerance differentiation of the two leafminer species, and are highly in agreement with their distribution limits at the northern boundary, suggesting that T_{on} (or T_{max}) are good temperature indicators and may be used to determine natural geographical distribution limits.

Experimental procedures

Insect samples and temperature treatments

Laboratory-reared populations of *L. huidobrensis* and *L. sativae* were originally collected on celery in Beijing in 2001 and 1997, respectively. The two leafminer species were maintained at 25–26 °C following protocols outlined by Chen & Kang (2002, 2005a). Two-day-old pupae were collected for further treatments. About 30 pupae were placed in a 5 ml cryogenic tube, and pretreated for 1 h at different temperatures from −20 to 45 °C with a gradient of 2.5 °C. After the treatment, the leafminers were allowed to recover at 25 °C for 1 h, and then frozen quickly in liquid nitrogen, and stored at −70 °C. Temperature alteration was achieved by submersing the tubes in a glycol bath (Programmable Temperature Controller, Polyscience®, Niles, IL, USA). Each treatment was repeated four times.

Cloning the full-length cDNA of hsps

Total RNAs were isolated using the RNeasy® Mini Kit (Qiagen, Valencia, CA, USA), and 2 μg RNA was used to generate the cDNAs. Degenerate primers (Table 1) were used to amplify the partial segments of *hsps*, and then 5′ and 3′ RACE were applied to obtain the full cDNA lengths following the manufacturer's instructions (SMART RACE cDNA Amplification Kit, Clontech, Mountain View, CA, USA). To make sure that the 5′ and 3′ fragments were from the same gene, specific primer sets flanking the ORFs were designed, then used to PCR amplify the full-length cDNAs using 5′ RACE ready cDNA templates.

Real-time quantitative PCR

The PCR reactions were performed in a 20 μl total reaction volume including 10 μl of 2 × SYBR® Premix EX Taq™ master mix (TaKaRa, Kyoto, Japan), 5 μM each of gene-specific primers (Table 1) and 1 μl cDNA templates. They were carried out on the Mx 3000P detection system (Stratagene, La Jolla, CA, USA). The thermal cycler parameters were as follow: 10 s at 95 °C, then 40 cycles of 5 s at 95 °C, 20 s at 58 °C and 20 s at 72 °C, then one cycle of 30 s at 95 °C, 30 s at 58 °C and 30 s at 95 °C in order to produce the melting curves, which can be used to judge the specificity of the PCR products. β-actin was cloned in both *L. huidobrensis* (DQ452368) and *L. sativae* (DQ452369) and used as the housekeeping gene. A standard curve was derived from the serial dilutions to quantify the copy numbers of target mRNAs. The amount of each *hsp* mRNA was then normalized to the abundance of β-actin. Subsequently, the normalized values of each *hsp* mRNA in the stressed samples were divided by those in the untreated controls, and the folds were used as the relative levels of each *hsp* mRNA. The *hsp70* level of *L. huidobrensis* shocked at 25 °C was quantified in each plate in order to correct the plate variation.

Protein extraction and Western blotting

The samples were treated as per the fore-mentioned method except that the recovery time was prolonged to 3 h to ensure the synthesis of different Hsp proteins. The sample was homogenized in the extraction buffer containing 120 mM Tris.HCl (pH 6.8), 10% (v/v) glycerol, 3.4% sodium dodecyl sulfate (SDS), 100 mM

Gene	Species	Primer sequence (5′→3′)	Fragment length (bp)
Primers used in the cDNA cloning			
hsp90		TTCATYGGBTAYCCNATYAA	1553
		TTAATCNACYTCYTCCAT	
hsp70		AGATYATYGCCAAYGACCAG	1449
		CGDCCCTTGTCGTTCTTGAT	
hsp60		GCKGGDGAYGGNACNACNWC	595
		TCDCCRAADCCRGGNGCTTTKA	
hsp40		GCNGARGCNTAYGANGTGCT	485
		TTBGTDCCNKCCTTCCAKCC	
hsp20		GTDGARGGSAAVCAYGARGA	134
		GTSARNACVCCRTCDGADGA	
β-actin		CAGWSCAAGMGWGGTATCCT	403
		GGATCTTCATSAGGTAGTCRG	
Primers used in quantitative PCR			
hsp90	*L. huidobrensis*	CATCACAATACGGTTGGTCTGC	92
		CTTGCCACTCATGTAGCCCAT	
	L. sativae	AGCACTGCGTGACTCATCAACC	198
		ACTTGAGGGCTGTCCAATGAGA	
hsp70	*L. huidobrensis*	CTTTGACTTGGGTGGCGGTA	197
		GACGCAAGGCTCTGGGATT	
	L. sativae	GGAACCACATACTCCTGCGT	127
		CATCACCAATCAACCGCTCT	
hsp60	*L. huidobrensis*	ATTCGTCGTGGTGTCATGTTGG	110
		GCTGAGATGGTGGCTACTTGAG	
	L. sativae	GTCAGCAACAGTTTTGGCACG	89
		CACCACGACGAATTTCTACAGG	
hsp40	*L. huidobrensis*	ATTAGGCGGTGGTGCTTTTCG	167
		GAGCCAAGGACATGCGTGAGA	
	L. sativae	CGCAAGCGTCAAAGTCAAGAT	171
		CCATCCGGGCTTCACATTTA	
hsp20	*L. huidobrensis*	AGTAGAGGGGAAGCACGAGGA	154
		CTTCATAGGGGCACGCACA	
	L. sativae	ATGTGGTAGTGGAAGGCAAGC	98
		GTTGACGCCCTTAGGTAGACG	
β-actin	*L. huidobrensis*	TGACTGAAGCCCCATTGAACC	236
		GCGACCAGCCAAGTCCAAAC	
	L. sativae	ACCTTCAACACACCCGCTATG	168
		AGCCAAGTCCAAACGCAAGA	

1,4-dithiothreitol (DTT), and 2% β-mercaptoethanol. The supernatant was removed and stored at −70 °C for later use. The protein concentrations were determined using the Micro-BCA assay (Pierce, Rockford, IL, USA) according to the manufacturer's instructions. 30 μg of the proteins was separated on SDS/10% polyacrylamide gels for 60 min at 80 V. Subsequently, the proteins were transferred on to Millipore nitrocellulose membrane (0.45 μm) at 30 V overnight (Millipore, Billerica, MA, USA). The membrane was blocked in bovine serum albumin (BSA) buffer for 1 h, washed three times in Tris-buffered saline (TBS), and then incubated for 1 h at 37 °C with the monoclonal mouse antibody against Hsp70 (clone BRM-22; Sigma, St Louis, MO, USA; 1 : 5000 dilution) or, in the case of Hsp60, with the rabbit anti-Hsp60 polyclonal antibody (product # SPA-805; Stressgen (San Diego, CA, USA); 1 : 5000 dilution). After washing in TBS buffer, a secondary antibody, peroxidase-conjugated goat anti-mouse or goat anti-rabbit, was added at 1 : 10 000 dilution, and the membrane was incubated for 1 h at 37 °C. For detection, the membrane was washed three times in TBS buffer (10 min each), placed in a Petri dish, and overlaid with a solution of enhanced chemiluminescent reagent (Pierce) according to the manufacturer's instructions. The signal was detected by Bio-Rad's Quantity One® system (Hercules, CA, USA).

Sequence alignment and statistical analysis

The full-length cDNAs of *hsps* in these leafminers were used as quires to search for other insect *hsps* in GENBANK by BLAST software available at the NCBI website (http://www.ncbi.nlm.gov/BLAST/). The sequence alignment and identity analysis were carried out with Cluster X (Thompson *et al.*, 1997). The ORF was identified using ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) and the amino acid molecular weight was calculated by the SWISS-PROT (ExPASy server) program 'Compute pI/Mw' (http://au.expasy.org/). The statistical significance of differences among treatments was analysed by one-way analysis of variance (ANOVA; Systat, Inc., San Jose, CA, USA) followed by a Tukey's test for multiple comparisons. For the ANOVA, data were log-transformed; incubation temperature was used as the independent variable and expression level of *hsps* as the dependent variable. We describe the first temperature at which the expression level was significantly higher than that of 25 °C treated samples as the onset temperature (T_{on}) of the synthesis of a particular Hsp protein. The temperature at which the expression level was significantly higher than those of all the others was denoted as T_{max}. The data were denoted as mean ± SEM, and the treatment differences were considered

significant at $P < 0.05$. We have not indicated significant differences on the figures, but discuss these in the text.

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Supplementary material

The following supplementary material is available for this article online:

Figure S1. Alignment of *Liriomyza hsp90* amino acid sequences with those of other insects.

Figure S2. Alignment of *Liriomyza hsp70* amino acid sequences with those of other insects.

Figure S3. Alignment of *Liriomyza hsp60* amino acid sequences with those of other insects

Figure S4. Alignment of *Liriomyza hsp40* amino acid sequences with those of other insects.

Figure S5. Alignment of *Liriomyza* small *hsp* amino acid sequences with those of other insects.

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