Cloning and Characterization of the HSP70 Gene, and Its Expression in Response to Diapausas and Thermal Stress in the Onion Maggot, Delia antiqua

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The cytosolic members of the HSP70 family of proteins play key roles in the molecular chaperone machinery of the cell. In the study we cloned and sequenced the full-length cDNA of Delia antiqua HSP70 gene, which is 2461 bp long and encodes 643 aa, with a calculated molecular mass of 70.787 Da. We investigated gene copies of cytosolic HSP70 members of 4 insect species with complete genome available, and found that they are quite variable with species. In order to characterize this protein we carried out an alignment and a phylogenetic analysis with 41 complete protein sequences from insects. The analysis divided the cytosolic members of the family into two classes, HSP70 and HSC70, distinguishable on the basis of 15 residues. HSP70 class members were slightly shorter in length and smaller in molecular mass relative to the HSC70 class members, and the conservative and functional regions in these sequences were documented. Mainly, we investigated the expression of Delia antiqua HSP70 gene, in response to diapausas and thermal stress. Both summer and winter diapausas elevated HSP70 transcript levels. Cold-stress led to increased HSP70 expression levels in summer and winter-diapausing pupae, but heat-stress elevated the levels only in the winter-diapausing pupae. In all cases, the expression levels, after being elevated, gradually decreased with time. HSP70 expression was low in non-diapausing pupae but was up-regulated following cold- and heat-stresses. Heat-stress gradually increased the mRNA level with time whereas cold-stress gradually decreased levels after an initial increase.

Keywords: Cloning, Delia antiqua, Diapause, Expression, Genomic, HSP70, HSC70, Insect, Phylogeny, Thermal stress

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Introduction

Heat shock genes are a subset of a larger group of genes coding for molecular chaperones (Sorensen et al., 2003). In insects there are four heat-shock gene families: the small HSP (sHSP) family with molecular masses ranging from 15-30 kDa, the HSP60 family with molecular masses of approximately 60 kDa, the HSP70 family with molecular masses of approximately 70 kDa, and the HSP90 family with higher masses (Dentinger et al., 2002). The HSP70 family is one of the most highly conserved gene families and its proteins are the most widely studied stress proteins (Gupta and Golding, 1993; Boorstein et al., 1994). The members of the HSP70 family are nuclear genes, and their proteins have been localized to various cellular compartments, including the cytosol, mitochondria, chloroplast, and endoplasmic reticulum. Cytosolic members include the heat-inducible HSP70 and the constitutively expressed heat shock cognate 70 (HSC70), both of which play key roles in the molecular chaperone machinery (Caplan, 2003; Sorensen et al., 2003). Although the expression patterns of these two genes are quite different, their proteins share common structural features. These proteins consist of two domains: a 44-kDa N-terminal ATP-binding domain, and a 30-kDa C-terminal substrate-binding domain that bears the highly conserved HEVD terminal sequence, unique to the cytosolic forms of HSP70 family members (Dang and Lee, 1989; Kang and Tsokos, 1998). Little is known about the evolutionary relatedness and characteristic difference between HSP70 and HSC70 proteins in insects; as earlier phylogenetic studies only involved one insect species, Drosophila melanogaster (Boorstein et al., 1994; Gupta and Singh, 1994; Gupta et al., 1994).

To survive seasonally recurring environmental stresses, such as cold, heat or dry seasons, most insects enter diapause (Rinhardt et al., 2000). Initiated by environmental cues, diapause is characterized by developmental arrest, decreased
metabolism (Tauber et al. 1986) and an increase in resistance to stresses (Adedeji and Dentlinger, 1984). Diapausa
ing individuals demonstrate striking differences in gene expression when compared with non-diapausing ones; i.e., most genes are silenced but a few are highly upregulated during diapausa (Dentlinger, 2002). Whereas some studies have found that HSP70 is upregulated during insect diapausa (Rinchart et al. 2000; Yocum, 2001), others have not found the same pattern (Goto et al. 1998; Tachibara et al. 2005). This inconsistency needs to be elucidated with additional species. The onion maggot Delia antiqua can be easily induced in laboratory into both summer diapausa (SD) and winter diapausa (WD) occurring at a comparable developmental stage, the pupal stage (Ishikawa et al. 2000; Chen et al. 2005a, 2005b), thus is an ideal model species for diapausa research.

In the present study, we clone the HSP70 cDNA of the onion maggot Delia antiqua, investigate 4 insect species of genomes that have been completely sequenced, characterize HSP70 and HSC70 protein sequences of insects by bioinformatics analyses, and examine HSP70 expression in response to both summer and winter diapausa as well as to thermal stress through diapausa and in non-diapausing pupae.

Materials and Methods

Experimental insects and treatment. The non-diapausing (ND) colony of D. antiqua was reared on an artificial diet at 20°C with a 16L:8D photoperiod and 50-70% relative humidity, as described by Ishikawa et al. (1983). At the fourth day after pupariation (D4) pupae were subject to cold stress at -10°C with 0L:24D, or heat stress at 35°C with 16L:8D, both for 2, 4 or 6 days. To induce SD, larvae were maintained at 25°C with 16L:8D. Newly formed puparia were kept under the same conditions as the larvae until day 15 after pupariation (D15) and then transferred to 16°C and 16L:8D to trigger diapausa termination (Ishikawa et al. 2000). A few frequently occurring non-diapausing pupae, which could be discriminated by direct observation through the semi-transparent pupal case (Nomura and Ishikawa, 2000), were eliminated at D6. Diapausa pupae were subject to cold stress at -15°C with 0L:24D, or heat stress at 35°C with 16L:8D, both for 2, 4 or 6 days. To induce WD, larvae were reared at 15°C with 12L:12D (Nomura and Ishikawa, 2000), and pupae were kept under this condition throughout. The pupae at D40 were treated for 3, 10 or 15 days either at -20°C with 0L:24D or 35°C with 16L:8D. Pupae collected at various stages were snap frozen in liquid nitrogen and stored at -80°C prior to RNA extraction.

cDNA synthesis, cloning and sequencing. mRNA was isolated from the pupae using a MicroPoly(A)Pure™ kit (Ambion). The first-strand cDNA was synthesized from the mRNA using the oligo(dT)16 primer from a 5′/3′ RACE Kit (Roche Applied Science). After purification with a SUPREC™2 kit (TaKaRa), a 185 bp of HSP70 cDNA fragment was amplified by PCR from cDNA using degenerate primers (“5’-GYBMTGACHAARGYR CAA-3’ for forward; 5’-CKWCCYTTRCCTTYTDTAT-3’ for reverse”), designed using consensus mRNA sequences of HSP70 genes (in sensu stricto) from 17 insect species showed in Fig. 2. The 185 bp of fragment ranges bp 1518-1702 in the final HSP70 cDNA sequence in the present study (GenBank accession number DQ014057). The fragment was then purified with a PCR Purification Kit (Qiagen) and cloned into a pGEM-T vector (Promega). The HSP70 fragment was amplified from positive clones by PCR with vector-specific primers and sequenced using BigDye Terminator v.1.1 Cycle Sequencing Kit (Applied Biosystems) on an ABI PRISM 377 DNA Sequencer (Perkin-Elmer).

For 3′ RACE (rapid amplification of cDNA ends), the cDNA was amplified with the oligo(dT)-anchor primer and a HSP70-specific forward primer (5′-ATCTATGGCTTTCTCTAAAP-3′, designed based on the cDNA fragment obtained earlier). The PCR product was reamplified using the anchor primer mentioned above and another gene-specific forward primer (5′-AGATGAGACCGGCGTAC-3′). For 5′ RACE, template cDNA was synthesized with a HSP70-specific reverse primer (5′-AACGTGGCAAGCTCTCAG-3′). After purification, the 5′ end of the cDNA was attached with a poly(A)-tail using dATP and terminal deoxynucleotidyl transferase. Then, the cDNA was amplified by PCR using the anchor primer and a gene-specific primer (5′-TTGCAATCAGATGTCAT-3′). The PCR product was amplified once more with the anchor primer and another gene-specific primer (5′-TAGGTGGTCCTTTCGCA-3′). The second-round PCR products for both 3′ and 5′ RACE were cloned and sequenced as described above. The initial HSP70 cDNA fragment and cDNA ends derived from the 3′ and 5′ RACE were edited and assembled with BioEdit (Hall 1999).

Quantitative real-time PCR (Q-RT-PCR). Total RNA was isolated from the pupae using a Nuclease-MiniMax kit (Qiagen) and treated with RNase-Free DNase I (Qiagen). cDNA was reverse-transcribed from total RNA using random primers with a RNA PCR kit (TaKaRa) and purified with a SUPREC™2 kit (TaKaRa). The reverse transcribed cDNA samples were used for real-time PCR, which was performed on an ABI PRISM 7700 thermal cycler (PE Biosystems). A HSP70 cDNA fragment (189 bp) was amplified using gene-specific primers 5′-TGACAAAAAGCAACAACCTC-3′ and 5′-GGCGAATCGAATGTCG-3′. The 18S rRNA gene was chosen as a reference for normalizing the HSP70 mRNA levels. Primers for 18S rRNA were the same as those used by Chen et al. (2005a), which amplified a 333 bp fragment. One cDNA sample diluted to 1 × 10^3, 1 × 10^4, 1 × 10^5, and 1 × 10^7, was employed as an internal standard. Q-RT-PCR was performed in 20 μl reactions containing 1 μl of template cDNA or the standard, 1× QuantiTect SYBR Green PCR premix (Qiagen), and 0.3 μM of each primer. Thermal cycling conditions were 95°C for 15 min, 45 cycles of 95°C for 15 s, 58°C for 30 s and 72°C for 30 s, then 40°C for 30 s and 95°C for 1 min with ramp time of 15 s. After Q-RT-PCR, the absence of unwanted by-products was confirmed by automated melting curve analysis and agarose gel electrophoresis of the PCR product. The relative molar amounts of HSP70 and 18S rRNA transcripts were calculated based on crossing point analysis, using standard curves generated from the cDNA standards. HSP70 mRNA levels were normalized with those of 18S rRNA in the same samples quantified in the same manner, and the final relative mRNA levels of HSP70 were averages of five replicates. Regression analysis was carried out with the software Statistica (release 4.5, StatSoft, Inc.)
Bioinformatics analyses. The HSP70 family of protein sequences was retrieved by PSI-BLAST (Altschul et al., 1997) searches against the Arthropoda protein database at NCBI (https://www.ncbi.nlm.nih.gov) using the HSP70Ba and HSC70-4 protein sequences of D. melanogaster (accession numbers NP_753176.1 and NP_524356.1, respectively) as queries. The search resulted in a list of similar sequences, which were added by the next round of PSI-BLAST iteration searches. The search continued until no new sequence with an alignment score above the default threshold was retrieved. The cytosolic HSP70 and HSC70 protein sequences were subsequently recognized from the retrieved collection through their characteristic EEVD C-terminus residues (Kiang and Tsokos, 1998; Gupta et al., 1994) or via their high similarity with EEVD-ended sequences for incomplete sequences. The sequences were aligned via Clustal X alignment (Thompson et al., 1997).

Four insect species of nuclear genomes that have been completely sequenced were investigated (Table 1). To localize the members of the HSP70 gene family within each genome, we used MapViewer at NCBLA TBLASTN search against each genome assembly was applied using D. melanogaster HSP70Ba and HSC70-4 protein sequences mentioned above as queries, whereas a BLASTN search was performed with the nucleotide sequences of these two genes as queries. Subsequently, a 200-kb genome sequence flanking each hit, or close hits, was downloaded from the corresponding strand of the chromosome and analyzed as Chen et al. (2005c; 2006).

In order to identify biologically significant motifs and domains for each divergent protein sequence we used the program InterProScan (Zdobnov et al., 2001) to search against InterPro, a database of protein domains and functional sites (http://www.ebi.ac.uk/interpro). The signal peptide was predicted using both neural network (NN) and hidden markov model (HMM) methods with the program SignalP v3.0 (Bendtzen et al., 2004; Nielsen et al., 1997). The protein alignment was used to explore HSP70- and HSC70-specific residues and in subsequent phylogenetic analysis. Sequence identity and molecular mass were calculated using BioEdit.

In order to understand the evolutionary relationships of cytosolic HSP70 copies in insects we performed a phylogenetic analysis using protein sequences with PAUP* v4.0b10 (Swofford, 2001). Maximum parsimony was used for phylogenetic inference. We performed an heuristic search employing step-wise addition with 200 random taxon addition sequence replicates and 10 best trees held at each step. All characters were given equal weight and gaps were treated as “missing”. As endoplasmic reticulum (ER) HSP70 homologue is the sister of cytosolic HSP70 genes (Boorstein et al., 1994), we used the ER HSP70 sequences of Drosophila melanogaster (NP_727564.1) and Apis mellifera (XP_593906.2) as a combined outgroup. The node support was assessed using 1000 bootstrap pseudo-replicates with full-heuristic algorithm.

Results

HSP70 cDNA in Delia antiqua. Cloning and sequencing of the cDNA fragment amplified by the degenerate primers yielded a 285 bp expressed sequence tag. Based on the tag sequence, we designed 5 gene-specific primers and amplified both the 5' and 3' ends of its cDNA. The full-length HSP70 cDNA of Delia antiqua, assembled from the tag sequence and both 5' and 3' ends, is 2461 bp long and contains a unique open reading frame (ORF) spanning nucleotides 183-2114. It has a polyA tail at its 3' end, and a polyadenylation signal (AAATAA) at nucleotides 2424-2729. The polypeptide deduced from the ORF comprises 643 aa, with a calculated molecular mass of 70,787 Da and an estimated isoelectric point of 5.65. The deduced sequence was confirmed to belong cytosolic HSP70 gene via alignment with sequences of other insects, and is 85.0% identical to D. melanogaster cytosolic HSP70Ba (NP_731761.1). The cDNA and its deduced protein sequence were deposited in GenBank with accession numbers DQ017057 and AAY28732, respectively.

Protein sequence properties and phylogenetic relationships. We obtained 144 cytosolic protein sequences from insects (61 for D. melanogaster alone) from databases searches. From these, 85 belong to HSP70 and 59 belong to HSC70 protein classes. After removing redundant or unconfirmed, or very similar sequences in each species, 24 HSP70 (including Delia antiqua HSP70) and 17 HSC70 complete protein sequences were subject to subsequent protein sequence characterization and phylogenetic analysis.

Using InterPro searches and primary literature we identified 3 signature motifs, 2 nuclear localization signals, an ATP-binding domain, a substrate binding domain and α- and β-domain in the substrate binding domain of cytosolic HSP70 proteins from insects (Fig. 1). We recognized 15 aa, residues that distinguish HSP70 from HSC70 proteins in our alignment. All 41 cytosolic HSP70 sequences terminate with the motif EEVD. No signal peptide, characteristic of a secreted protein, unlike that found for mitochondrial and ER localized proteins, was detected for these protein sequences. The length of HSP70 protein sequences range from 633 aa. (Manduca sexta) to 645 aa. (Chironomus yoshimatsui), making them slightly shorter than those of HSC70 that range from 649 aa. (Bombyx mori) to 659 aa. (Catera subtruculata). The molecular masses of HSP70 proteins range from 69.5 kDa (Mand. sexta) to 71.2 kDa (Chi. yoshimatsui), slightly smaller than those of HSC70 that ranges from 71.1 kDa (Apis mellifera) to 71.9 kDa (Trichopoda ni). The length and mass differences are mainly due to two non-conservative regions recognized in the present study (a and b, Fig. 1), which are slightly shorter in HSP70 proteins.

Genomic analysis revealed that D. melanogaster has 8 HSP70 and 2 HSC70 class of genes (Table 1). Six out of 8 HSP70 genes (Aa=Ah, Bb=Bc, Ba and Bb) are actually very similar with only 0-2 aa, difference. The HSC70-2 and HSC70-3, which were treated as HSC70 class of genes, are decided to be a gene of HSP70 class, and a gene functioning in endoplasmic reticulum (with termination sequence KDEL) via sequence and phylogenetic analysis in the present study, respectively. Relatively less, Anopheles gambii has only 1 HSP70 and 2 HSC70 class of genes, and Tribolium castaneum has 3 HSP70 and 1 HSC70 class of genes. Surprisingly, we did not find any HSP70 class of gene in Apis mellifera but 2 HSC70 genes,
Table 1. Cytosolic HSP70 genes present on the complete genomes of 4 insect species, with *Delia antiqua* HSP70 gene

<table>
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<tr>
<th>Gene8</th>
<th>Accession number mRNA</th>
<th>Accession number a.a.</th>
<th>Chromosomal location 8</th>
<th>Exon no 8</th>
<th>aa. length</th>
<th>Mass (kDa)</th>
<th>Genome size (bp)</th>
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8 The gene names are given temporarily in the study with old name in parentheses.

9 Sequence with complete coding region (CDs) only, sequence with incomplete CDs only, and all others are full-length of cDNA.

+1, forward strand, preceded by chromosomal name; −, reverse strand, followed by location in a given chromosome. N/A indicates unavailability of the chromosomal information.

9 Total number of exons in CDs. The numbers in parentheses include exons in untranslated regions.

and the HSP70 gene earlier named for the species is decided to be a HSC70 gene in the present study. There are 1-3 exons in coding region with a genome size smaller than 10 kb throughout all these cytosolic HSP70 genes.

Maximum parsimony analysis of the sequence data resulted in the most parsimonious trees shown in Fig. 2 with tree length = 2049, consistency index = 0.605, homoplasy index = 0.395, retention index = 0.765 and rescaled consistency index = 0.463. Among the 729 a.a. characters used, 251 characters were constant, 121 were parsimony-uninformative and 357 were parsimony-informative. The complete ingroup, HSP70 and HSC70 are separately monophyletic with 100%, 99% and 95% bootstrap supports, respectively (Fig. 2). However, in both HSP70 and HSC70 clades, there is no >50% bootstrap value to support the relationships among different orders of insects. This is mainly due to high conservation of HSP70 sequences and different number of copies in different species.

Expression of HSP70 through diapause. When onion maggot larvae were reared at 25°C and 16L:8D, 98% of the pupae enter summer diapause (SD) at 2.2 days after pupariation (Ishikawa et al., 2000). Although the SD period is quite variable at 25°C and 16L:8D, lasting between 15-45 days, pupae complete SD and start postdiapause development soon after the temperature decreases to 16°C (Ishikawa et al., 2000). The relative amounts of HSP70 transcripts, normalized to 18S rRNA levels, were very low in both pre-diapause (0.03-0.21 for D0.5, D1 and D2) and postdiapause pupae (0.55-0.12 for D18, D21 and D24) (Fig. 3). At the onset of SD (D3) the amount increased to 0.86; interestingly, three days after, the amount steeply rose to 4.53 (D6), 5.27 times higher than that for D3. Subsequently the amounts gradually decreased from 3.23 (D9), 2.41 (D12) to 2.04 (D15) with a slope rate of 0.276/ day.

Almost all the pupae enter winter diapause (WD) when...
Fig. 1. The representative of the global alignment of cytosolic HSP70 (with P as suffix) and HSC70 (with C as suffix) amino acid sequences of insects, showing their difference, domains and functional regions. For comparison, completely conserved amino acid residues are marked below the alignment with "**". A "-" corresponds to a gap and the last residue in each line is assigned a number. ▼ stands for distinguishable residues between HSP70 and HSC70. The conservative regions are indicated by: "I, II, III" for three HSP70 protein family signatures (document # P500297 and P500299 of database Prolific, and Liu et al. 2004); α, β for two non-conservative regions recognized in the present study; "++" for ATP/ATP binding site (Liu et al. 2004); "+++" for nuclear localization signal (Liu et al. 2004), respectively. Backward "-" and forward "++" arrows separately indicate ATP-Binding and substrate binding domains (Liu et al. 2004). The substrate binding domain is divided into codomain and β-domain (Choi and Li, 1998), separated by "=". The abbreviations of the sequence names: HSP70, DmDrosophila melanogaster (NP_751161.1), CcCChironomus tentans (ANJ18137.1), CaC-Caenorhabditis elegans (NP_523456.1), CaC-Chilobasis (AANI14255.1), CaC-Canarian (AAC23392.1), and MosC - Mus. ocellata (Q9QUD7).
Fig. 2. The most parsimonious tree (2049 steps, see Results for parameter values) inferred from cytosolic HSP70 protein sequences and rooted with endoplasmic reticulum HSP70 homologues. Bootstrap percentages of 1000 replicates are shown above the branches where they exceed 50%. The abbreviations of genera: *Ano*: *Anopheles*, *Ant*: *Antennaria*, *Cer*: *Ceratitis*, *Chi*: *Chironomus*, *Co*: *Cotesia*, *Dro*: *Drosophila*, *Loc*: *Locusta*, *Lom*: *Lonomia*, *Man*: *Manduca*, *Tr*: *Trichoplusia* and *Tri*: *Trichoplusia*.

larvae are maintained at 15°C and 12L:12D (Nomura and Ishikawa, 2000). WD starts on day 4.1 after puparium and lasts for about 99 days with a post-diapause period of 23.5 days (Nomura, 2001). In a similar pattern to that observed under SD, the relative mRNA levels in the post-diapause stages (D2, D3 and D4) were relatively low (0.15, 0.31 and 0.68), respectively, but increased to 2.52 in the early WD stage (D29; Fig. 3). Then the expression level gradually decreased from 1.97 (D54), to 1.29 (D79), to 0.88 (D105), with a slope rate of −0.022/day (Fig. 3). The transcript amounts in three post-diapause stages, 0.49 (D112), 0.3 (D119) and 0.10 (D126) are comparable to the levels found in pre-diapause stages. The transcript amounts in pre-diapause and post-diapause pupae in SD and WD are similarly low compared to those in ND (between 0.14 at D2 and 0.45 at D6, Fig. 3).

**Thermal stress response.** Cold-stressing (−15°C in complete darkness) the SD pupae led to increased HSP70 expression levels from 3.30 (D5) to 4.06 (D7); however, heat-stressing (35°C and 16L:8D) the SD pupae from D5 did not significantly influence the expression level in the first two days (Fig. 3). After D7, the mRNA levels in cold- and heat-stressed pupae
gradually decreased with treatment duration, consistent with the pattern of unstressed SD pupae. The slope rates of HSP70 decrease for cold-stressed (−0.384/day) and heat-stressed pupae (−0.106/day) were 1.4 times faster and 0.6 times slower than that for unstressed pupae (−0.276/day), respectively (Fig. 3).

Cold-stressing (−20°C in complete darkness) and heat-stressing (35°C and 16L:8D) the WD pupae increased Cold-stressing (−10°C and 0L:24D) and heat-stressing (35°C and 16L:8D) ND pupae led to 3 and 2 fold increases in HSP70 expression levels, respectively, from D4 to D6. After the D6 stage, mRNA levels in cold-stressed pupae gradually decreased with treatment duration with a slope rate of −0.286/day, whereas those in heat-stressed pupae gradually increased with a slope rate of 0.300/day.
Discussion

In the present study, we cloned the cDNA of *Delia antiqua* cytosolic HSP70 gene, which is 2461 bp long and encodes a peptide of 643 aa, with molecular mass of 70,787 Da. The protein sequence deduced from the cDNA is most similar to *D. melanogaster* HSP68 gene with 85.2% identity. We investigated complete nuclear genomes of 4 insect species, and found the cytosolic HSP70 gene copy number changes with different species. All 41 complete HSP70 sequences investigated terminate with EEVD, which supports the claim by Kiang and Tsokos (1998) and Gupta et al. (1994) that considered this motif the signature feature of cytosolic HSP70 proteins across all organisms. These sequences are consistently 633-656 aa, long with molecular masses ranging from 69.5-71.9 kDa, and carrying no signal peptide. Three additional HSP70 family signatures, as well as an ATP/GTP binding site, 2 nuclear localization signals, an ATP-binding domain, and a substrate binding domain (identified from InterProScan searches; Guy and Li, 1998; Liu et al., 2004) are all conserved in insect cytosolic members of HSP70 family proteins. In this study we additionally recognized two highly-variable regions located at the beginning and at the end of all cytosolic HSP70 protein sequences.

This is the first comparative study of insect HSP70 and HSC70 proteins. In previous studies (Lo et al., 2004; Boorestein et al., 1994; Gupta et al., 1994; and Gupta and Singh, 1994), only one insect species, *D. melanogaster* was involved. Our phylogenetic analysis clearly differentiates cytosolic HSP70 proteins into two classes, HSP70 and HSC70. Fifteen characteristic residues can reliably distinguish the two classes of protein sequences in insects. In addition, the length of HSP70 sequences (633-645 aa) is shorter than that of HSC70 (649-656 aa), and the molecular masses of HSP70 (69.5-71.2 kDa) is smaller than that of HSC70 (71.1-71.9 kDa). The differences are mainly due to two non-conservative regions recognized in the present study (a and b, Fig. 1). We also found that the cytosolic HSP70 sequences are very conserved with 74.6-98.7% sequence identity in HSP70 class and 83.4-97.8% identity in HSC70 class. They do not seem good marker genes for insect phylogeny analysis due to their high conservation and variable gene copies.

We designed the real-time PCR primers from the *Delia antiqua* HSP70 sequence in its gene specific region determined by *D. melanogaster* HSP68 and its most homologous copies. Therefore, the expression really come from the *Delia antiqua* HSP70 gene cloned if there is more than 1 copy of cytosolic HSP70 class of gene in the species. In *Delia antiqua* we found that HSP70 is upregulated during WD, and that the transcript level declines to pre-diapause levels when diapause is terminated. These results are comparable to those found for HSP70 in *Sarcophaga bullata* WD (Rinehart et al., 2000). The expression pattern of HSP70 in *Delia antiqua* is comparable to that of some other genes we investigated in *Delia antiqua*, *D. hydei*, TCP-1, and HSP90, which are all upregulated in response to WD (Chen et al., 2005a; Kayukawa et al., 2005; Chen et al., 2005b). In contrast, HSP70 is not upregulated as a function of diapause in *Drosophila virgina* and *Lucilia sericata* (Goto et al., 1998; Tachibana et al., 2005). HSP70 is slightly upregulated in the diapausing adults of Colorado potato beetle *Leptinotarsa decemlineata* (Yocem, 2001), and is only expressed in the diapausing prerad larval instar larva of the gypsy moth *Lymantria dispar* after exposure to low temperature (Yocem et al., 1991). Variation among species is also found in the expression of other heat stress genes, HSP23 and HSP90. These genes are not regulated in *Drosophila virgina* and *L. sericata* during WD (Goto and Kimura, 2004; Tachibana et al., 2005), whereas HSP23 is highly upregulated and HSP90 is down-regulated in *Sarcophaga bullata* (Yocem et al., 1998; Rinehart and Denlinger, 2000). These results indicate that gene expression in response to WD diapause is species-specific.

This is the first study on HSP70 expression in response to SD, and, thus, generalizations relative to this gene's pattern of expression will only be possible once SD is further investigated in other species. In *Delia antiqua* under SD, HSP70 transcript levels rise in the first three days and then gradually decrease with time. This pattern is not mimicked by *HSP90* transcripts where levels increase sharply in the first few days and then gradually increase with time (Chen et al., 2005b), or by *D. hydei* transcript levels that rise in the first few days and then remain at similar levels until SD termination (Chen et al., 2005a).

HSP70 transcripts are usually undetectable or constitutively expressed at low levels under normal (non-diapausing) conditions, but transcription is induced by heat and cold stress in a variety of insect species (Goto et al., 1998; Rinehart et al., 2000; Tachibana et al., 2005). Our results are consistent with these earlier reports. In contrast to the results obtained for other species (Rinehart et al., 2000; Yocem et al., 1991; Denlinger et al., 1992), we showed that during diapause, heat stress significantly raised HSP70 expression in *Delia antiqua*. However, in some of the previous studies (Yocem et al., 1991; Denlinger et al., 1992), cold stress did not obviously affect HSP70 expression within a short period after the treatment (2 days for SD, and 5 days for WD). After this period, and despite the continuation of the temperature shocks, the expression levels gradually decreased in both cold and heat stress treatments for both SD and WD.

To date, HSP70 is the better-studied HSP gene regarding its response to various stresses (Sorensen et al., 2003). HSP70 has been found to be up-regulated in response to high larval rearing density (Sorensen and Loeschke, 2001), high inbreeding coefficient (Kristensen et al., 2002), desiccation (Hayward et al., 2004), and nutrient deprivation (Salvucci et al., 2000). In addition, increased levels of HSP70 following heat-stress were found to correlate with increased longevity in transgenic *Drosophila melanogaster* lines carrying extra copies of the gene (Tatar et al., 1997). A single study looked at the expression of both HSP70 and HSC70 genes following...
temperature stresses. While in non-diapausing pupae of the flesh fly, Sarcophaga crosyntaphus, heat-stresses only elevated the levels of the inducible HSP70, cold stresses up-regulated both the inducible and the constitutive HSC70 (Rinehart et al., 2000). It appears that our current knowledge of the function of these proteins in insects is still quite incomplete. Future comparative and functional studies should attempt to target both HSP70 and HSC70 genes. Like the earlier studies mentioned above, the expression experiments in the present study also only targeted the inducible HSP70 gene.

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References


