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Cloning of heat shock protein genes (*hsp70*, *hsc70* and *hsp90*) and their expression in response to larval diapause and thermal stress in the wheat blossom midge, *Sitodiplosis mosellana*

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ABSTRACT

Sitodiplosis mosellana Géhin, one of the most important pests of wheat, undergoes obligatory diapause as a larva to survive unfavorable temperature extremes during hot summers and cold winters. To explore the potential roles of heat shock proteins (hsp) in this process, we cloned full-length cDNAs of *hsp70*, *hsc70* and *hsp90* from *S. mosellana* larvae, and examined their expression in response to diapause and short-term temperature stresses. Three hsps included all signature sequences of corresponding protein family and EEVD motifs. They showed high homology to their counterparts in other species, and the phylogenetic analysis of hsp90 was consistent with the known classification of insects. Expression of *hsp70* and *hsp90* were highly induced by diapause, particularly pronounced during summer and winter. Interestingly, *hsp70* was more strongly expressed in summer than in winter whereas *hsp90* displayed the opposite pattern. Abundance of *hsc70* mRNA was comparable prior to and during diapauses and was highly up-regulated when insects began to enter the stage of post-diapause quiescence. Heat-stressed over-summering larvae ($\geqslant$30 °C) or cold-stressed over-wintering larvae ($\leqslant$0 °C) could further elevate expression of these three genes, but temperature extremes i.e. as high as 45 °C or as low as −15 °C failed to trigger such expression patterns. Notably, *hsp70* was most sensitive to heat stress and *hsp90* was most sensitive to cold stress. These results suggested that *hsp70* and *hsp90* play key roles in diapause maintenance and thermal stress; the former may be more prominent contributor to heat tolerance and the latter for cold tolerance. In contrast, *hsc70* most likely is involved in developmental transition from diapause to post-diapause quiescence, and thus may serve as a molecular marker to predict diapause termination.

1. Introduction

Diapause is a developmental strategy that allows insects to survive harsh environment conditions and synchronize their growth pace with both abiotic and biotic factors needed for development and reproduction (Chen et al., 2005; Li et al., 2008). The diapause program involves behavioral, morphological and physiological changes that are regulated by a specific set of genes. Because of its central role in insect survival and reproduction, fully understanding the molecular mechanisms underlying diapause is vital for agricultural pest management (Denlinger, 2008).

Heat shock proteins (hsps), a group of highly conserved proteins present in almost all living organisms, function as molecular chaperones by preventing protein aggregation and promoting correct refolding of denatured proteins during environmental stresses such as extreme temperatures, heavy metals, starvation, ultraviolet radiation and anoxia (Cheng et al., 2015; Shu et al., 2011; Sørensen et al., 2003; Tungjitwitayakul et al., 2015). Based on sequence similarity and the molecular size, hsps are divided into four categories: the small hsp family with molecular masses ranging 12–43 kDa, hsp60 family with molecular mass of approximately 60 kDa, hsp70 family with molecular mass of approximately 70 kDa, and hsp90 family with higher molecular masses (Gu et al., 2012; Shen et al., 2015). Hsp70 family contains both stress-inducible and constitutively (hsc70) expressed members that share many common structural features.

In recent years, an increasing number of studies have indicated that *hsp* expression is not only induced by various stresses but also associated with insect diapause (Denlinger, 2002; MacRae, 2010). For example, in flesh fly *Sarcophaga crassipalpis*, transcripts of

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0022-1910/

hsp70 and *hsp23* are highly abundant in diapausing pupae, and declined when diapause is terminated and post-diapause development begins (Hayward et al., 2005; Rinehart et al., 2000). By contrast, expression of *hsp90* in *S. crassipalpis* is down regulated during diapause, but restored once new development is initiated (Rinehart and Denlinger, 2000).

Differential responses of *hsps* to diapause suggest that *hsps* may play different roles in diapausing insects among different species. For instance, *hsp90* is up-regulated during pupal diapause of the onion maggot *Delia antiqua* (Chen et al., 2005) as well as during larval diapause of rice stem borer *Chilo suppressalis* (Sonoda et al., 2006), thus *hsp90* may be related to diapause maintenance in these species. However, it does not respond to prepupal diapause of the solitary bee *Megachile rotundata* (Yocum et al., 2005) nor adult diapause of the fruit fly *Drosophila triauraria* (Goto and Kimura, 2004). In contrast to up-regulation in *S. crassipalpis* and apple maggot *Rhagoletis pomonella* (Lopez-Martinez and Denlinger, 2008), *hsp70* is down-regulated during larval diapause of the moth *Sesamia nonagrioides* (Gkouvitsas et al., 2009a, 2009b) and shows no change when the blow fly *Lucilia sericata* (Tachibana et al., 2005) and bamboo borer *Omphisa fuscidentalis* (Tungjitwitayakul et al., 2008) go through diapause. For most insects, *hsc70* is not affected by diapause (Rinehart et al., 2007; Yocum et al., 2005; Zhang and Denlinger, 2010), but it is induced during deep diapause in *S. nonagrioides* (Gkouvitsas et al., 2009a) and repressed in early diapause in the heads of adult mosquito *Culex pipiens* (Li and Denlinger, 2009).

The orange wheat blossom midge, *Sitodiplosis mosellana* (Géhin) (Diptera: Cecidomyiidae), is one of the most important pests of wheat in the northern hemisphere (Gaafar and Volkmar, 2010; Jacquemin et al., 2014; Miao et al., 2013). Generally, it has one generation per year. In most wheat-growing areas in northern China, adults emerge and lay eggs in late April and early May. The mature larvae drop to the ground from wheat heads during middle to late May, and move into the soil to form round cocoons in which the larvae spend summer, autumn and winter in obligatory diapause. Post-diapause development is initiated in the spring in response to rising soil temperatures, indicated definitively by larvae exiting cocoons. Although several ecological and biochemical aspects of diapause in the wheat blossom midge have been documented (Cheng et al., 2009; Doane and Olfert, 2008; Hinks and Doane, 1988; Wu and Yuan, 2004), little information is available on the underpinning molecular mechanism (Gong et al., 2013).

To unravel the potential contribution of hsps to diapause in *S. mosellana*, in the present study, we cloned three full-length *hsp* genes from larvae, and examined their expression in response to diapause and to thermal stresses during diapause. Our results provide some molecular insight into diapause-related stress tolerance in *S. mosellana*.

2. Materials and methods

2.1. Insect collection

Larvae of *S. mosellana* at different developmental stages, including pre-diapause, diapause, post-diapause quiescent and developing stages, were used in this study. They were maintained at natural ambient conditions in Yangling, Shaanxi Province, China (34°16′N, 108°4′E) and collected according to the method described by Cheng et al. (2009). Specifically, wheat ears containing late instar larvae of *S. mosellana* were harvested and put on soil in a field insectary in late May, 2013. Pre-diapause larvae were simultaneously collected from wheat ears when the wheat was ripe in the experimental field. The soil was watered to maintain moisture for insects entering and breaking diapause. Cocooned larvae were collected monthly, from late June 2013 to late February 2014. We have discovered that almost all cocooned larvae collected in December or later could arouse and begin further development once exposed to 25 °C, indicating that by December they have entered the post-diapause quiescence (data not shown). Post-diapause developing larvae were collected in mid- and late March, when over 80% and 98% of larvae respectively exited cocoons. All larvae collected were frozen immediately in liquid nitrogen and then stored at −80 °C for cloning and real time quantitative PCR.

2.2. Temperature treatments

S. mosellana mainly goes through summer, autumn and winter as cocooned larvae between 3 and 10 cm under the soil surface (Doane and Olfert, 2008). Generally, temperatures in these habitats are lower than 30 °C in hot summer and higher than 0 °C in cold winter in Yangling district of Shaanxi province, China (34°16′N, 108°4′E). Extreme temperatures on the soil surface often exceed 45 °C in summer, and occasionally drop to −15 °C in winter, a temperature still higher than the mean super-cooling point (−23.6 °C) of *S. mosellana* cocoons in this region (Chen et al., 2012). To determine how the *S. mosellana hsp* genes respond to short-term stresses of extreme temperatures, cocoons in August were heat-treated (30–45 °C) and cocoons in December were cold-treated (0 to −15 °C) as described below.

Twenty freshly cocoons collected in late August or late December were put into 1.5 mL cryogenic vials. To generate a heat

Table 1
Primer sequences used in this study.

Purpose	Primer name	Primer sequence (5′–3′)
Conserved cDNA	hsp70 forward	CTTGGGAGGTGAAGACTTTG
	hsp70 reverse	AGAAATCCTTTCGCGTTGT
	hsc70 forward	TCTGGTTTGAATGTGCTCCGTA
	hsc70 reverse	GAGACTTTGGCTTTGATGTT
	hsp90 forward	CTGGTATCGGTATGACTAAAGC
	hsp90 reverse	CGTCGATGCCAAGACCCAA
3′RACE	hsp70 inner	CGGTGGAGTAATGACAAAGCT
	hsp70 outer	CTGGGTAGATTTGAGTTAAGCG
	hsc70 inner	TGAAGAATTGAACGCCGACT
	hsc70 outer	TTGCCTATGGTGCTGCTGT
	hsp90 inner	CATGAAAGCTCAAGCCCTAC
	hsp90 outer	CTCAAGAAACGTGGCTATGAA
5′RACE	hsp70 inner	TGTTCGCTTGGCTCGTT
	hsp70 outer	TCGATACGCCTGGTTGGT
	hsc70 inner	GTTGCTTCAGCGTATGGAGGTGG
	hsc70 outer	CGGAGCACATTCAAACCAGA
	hsp90 inner	CCAAGTAGGCAGAGTAGAAACC
	hsp90 outer	CGTCGCTTACTTCTTGGTCG
Full-length validation	hsp70 sense	GCTGTTGGTATTGATTTGGGTA
	hsp70 antisense	GAGCAAACCTGGCTGACTT
	hsc70 sense	GATTGCGCTATACGTGTTCT
	hsc70 antisense	TAGGCTGTTGTGACAGTGCTT
	hsp90 sense	AACTGAATAAGCAAAGTGAAC
	hsp90 antisense	CGGCGTCATCAACGAGTGG
Real time quantitative PCR	hsp70 forward	ACGCAACGGCTTCAT
	hsp70 reverse	CTAACCGACGCCAAAT
	hsc70 forward	TGAGTTCTTTGCCGTTGA
	hsc70 reverse	CGACTTGTTCCGTGCC
	hsp90 forward	GCGTTACGGGCCAAA
	hsp90 reverse	GAGGGTGAAGAACCAAAGAT
	GAPDH forward	TGCTGACCTGCTGCTGT
	GAPDH reverse	ATGGGTGGGGCTTGTGT

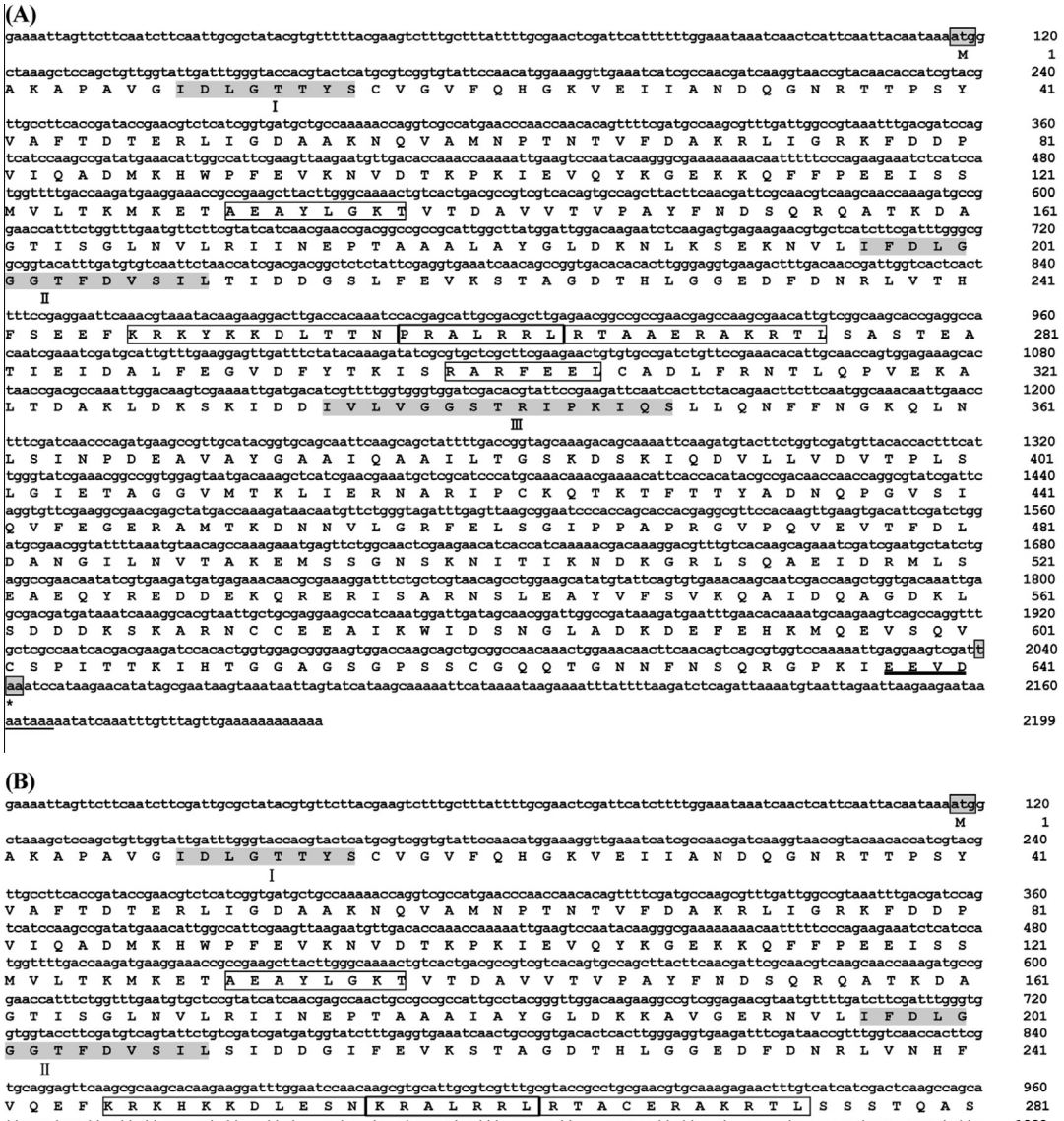

Fig. 1. Nucleotide and deduced amino acid sequences of *hsp70* (A), *hsc70* (B) and *hsp90* (C) in *Sitodiplosis mosellana.* Start codons (atg) and stop codons (taa) were shaded and boxed; the putative polyadenylation signals (aataaa) were underlined. Three signature sequences in hsp70/hsc70 family were shaded and labeled I, II and III; five signature sequences in hsp90 family were shaded and labeled I, II, III, IV and V. The hsp/hsc70 C-termini region (EEVD) and hsp90 C-termini region (MEEVD) were emphasized with bold underlines. Three other typical motifs in hsp70/hsc70 proteins were boxed.

shock, vials containing August cocoons were submerged in water bathes at 30, 35, 40 and 45 °C for 15, 30, 60 and 90 min, respectively. For cold shock, vials containing the December cocoons were placed in incubators with temperature controlled at 0, −5, −10 and −15 °C for 15, 30, 60 and 90 min, respectively. Insects collected prior to temperature treatments served as control. Upon completion of treatments at each time point for every temperature, insects were frozen immediately in liquid nitrogen and then stored at −80 °C for further molecular analysis. All treatments were repeated at least three times.

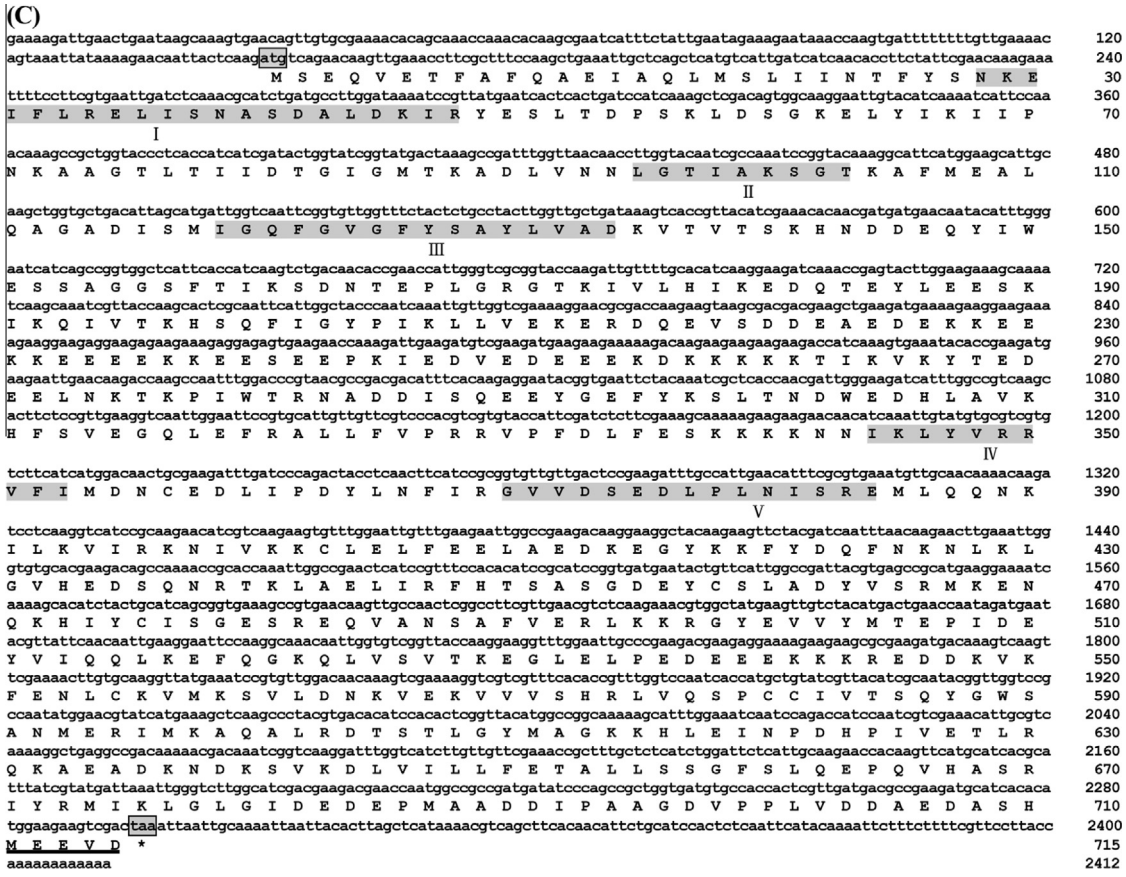

Fig. 1 (*continued*)

2.3. RNA extraction, cDNA synthesis and hsp cloning

Total RNA was isolated from *S. mosellana* larvae using the RNAsimple Total RNA Kit following the manufacturer's instructors (Tiangen, Beijing, China). The quality and quantity of RNA were determined by gel electrophoreses and a spectrophotometer (Nanodrop2000c, Thermo Fisher Scientific, West Palm Beach, FL, USA). Reverse transcriptase reactions were performed with 1.0 μg of total RNA as a template for first-strand cDNA synthesis using PrimeScript™ RT reagent Kit with gDNA Eraser (Perfect Real Time) (TaKaRa, Dalian, China).

To clone cDNAs of *S. mosellana hsp70*/*hsc70*/*hsp90*, primers (Table 1) were designed based upon *hsp70*/*hsc70*/*hsp90* sequences from a transcriptome dataset we obtained earlier from *S. mosellana* larvae. PCR reactions were carried out under the following conditions: 95 °C for 3 min; 30 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min; with a final extension at 72 °C for 10 min. The PCR products obtained were separated on a 1% agarose gel. Bands with expected sizes were excised and purified by a gel extraction kit (CWBIO, Beijing, China), then ligated into a pGEM-T easy vector (Promega, Madison, WI, USA). The constructs were transformed into DH5α competent cells (Transgen Biotech, Beijing, China). Positive transformants were identified by a blue-white screening, then cultured in liquid LB culture medium. Inserts were PCR examined, followed by DNA sequencing analysis (AuGCT DNA-SYN Biotechnology, Beijing, China).

Based on the partial sequence information obtained, *S. mosellana hsp70*/*hsc70*/*hsp90* gene-specific primers for 5′- and 3′-RACE were designed and synthesized (Table 1). The 5′- and 3′-ends of each cDNA were synthesized using 3′-Full RACE Core Set with PrimeScript™ RTase and 5′-Full RACE Kit with TAP (TaKaRa, Dalian, China) according to the manufacturer's instructions. Nested PCR conditions for 3′RACE were as follow: 95 °C for 3 min; 30 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min; with an extra extension at 72 °C for 10 min. For 5′-RACE, the same conditions were applied except that the outer and inner PCR cycle numbers were 20 and 25, respectively. PCR products were separated by electrophoresis, and bands with expected sizes were recovered from the gel, cloned and sequenced. Sequences obtained from RACE-PCR were used to design primers for PCR of the full-length *S. mosellana hsp70*/*hsc70*/*hsp90* cDNAs (Table 1). The amplification conditions were as follows: 94 °C for 7 min, followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 2 min, with a final extension at 72 °C for 15 min. PCR products of the expected sizes were purified, cloned and sequenced as above.

2.4. Sequence analysis

Identities of the cDNA clones were determined by the BLAST search in GenBank (http://www.ncbi.nlm.nih.gov/BLAST). Open reading frames (ORF) were identified by NCBI ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). Molecular weights and theoretical isoelectric points of predicted proteins were calculated by the Compute pI/Mw tool (http://web.expasy.org/protparam/). Multiple sequence alignments of deduced amino acid sequences were carried out using the DNAMAN software package (Lynnon Corporation, Pointe-Claire, Quebec, Canada). The phylogenetic tree was constructed by MEGA 5.0 software using the Neighbor-Joining method (Saitou and Nei, 1987) according to 20 full-length Hsp90 amino-acid sequences from insects.

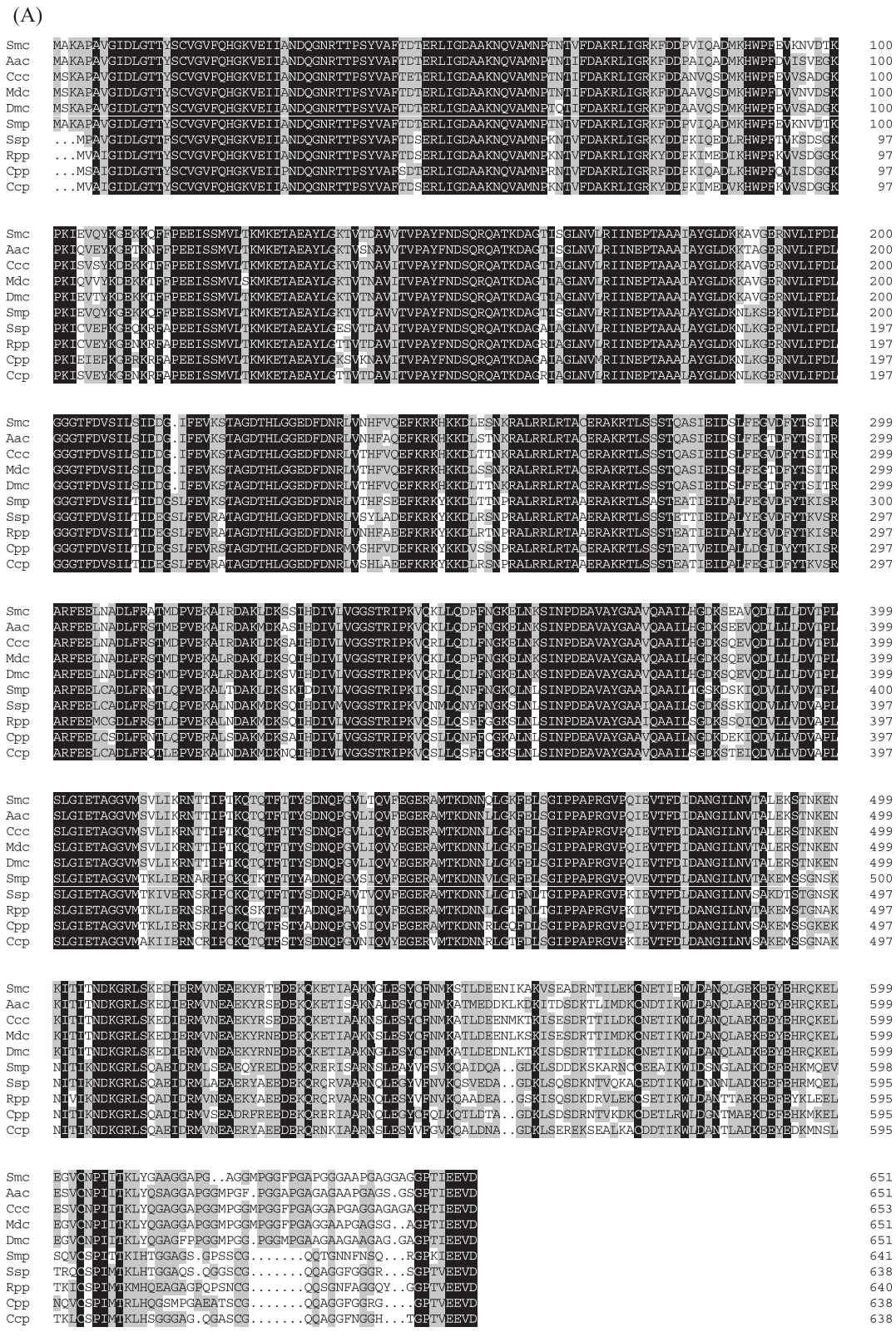

Fig. 2. Multiple sequence alignments of the deduced amino acid sequences of *Sitodiplosis mosellana hsp70* (with p as suffix), *hsc70* (with c as suffix) (A) and *hsp90* (B) with other insect *hsp70*, *hsc70* and *hsp90*. Identical or similar amino acids were shaded black or grey. Abbreviations: Smc = *Sitodiplosis mosellana* hsc70, Aac = *Aedes aegypti* hsc70, Ccc = *Ceratitis capitata* hsc70, Mdc = *Musca domestica* hsc70, Dmc = *Drosophila melanogaster* hsc70, Smp = *Sitodiplosis mosellana* hsp70, Ssp = *Stratiomys singularior* hsp70, Rpp = *Rhagoletis pomonella* hsp70, Cpp = *Culex pipiens* hsp70, Ccp = *Ceratitis capitata* hsp70; Sm = *Sitodiplosis mosellana*, Ag = *Anopheles gambiae*, Cc = *Ceratitis capitata*, Bc = *Bactrocera correcta*, Md = *Musca domestica*.

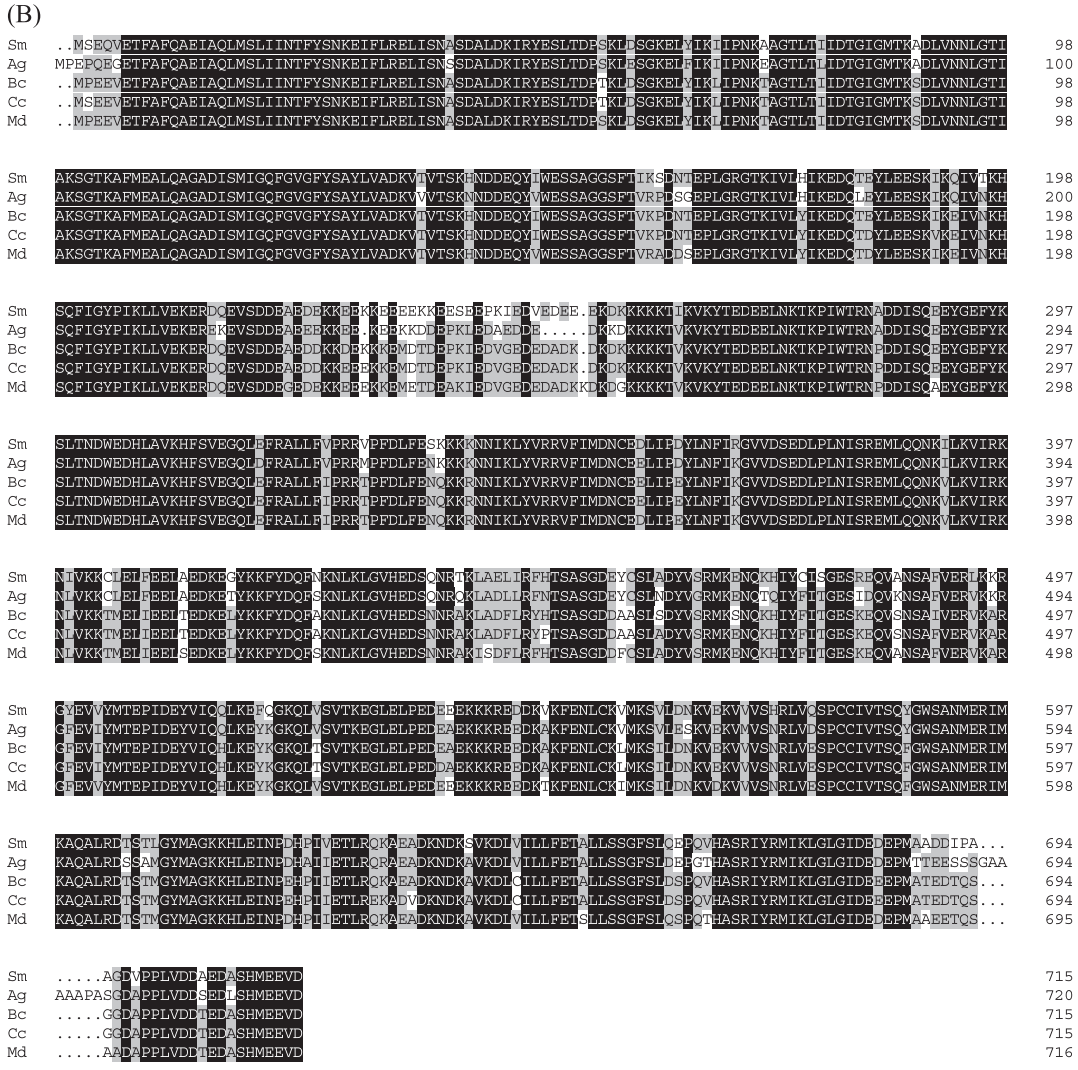

Fig. 2 (*continued*)

2.5. Real time quantitative PCR

Real time quantitative PCR (qPCR) was used to determine expression patterns of three *S. mosellana hsps* in response to diapause and temperature stresses. Total RNA of all samples, including larvae at different stages of diapause and diapausing larvae treated by heat or cold, was extracted and cDNAs were synthesized as described above. The glyceraldehyde-3-phosphate dehydrogenase gene of *S. mosellana* (*gapdh*, GenBank number: KR733066) was chosen as a reference gene, as it displays a stable expression level throughout diapause. The qPCR reactions were performed in 20 μL total reaction volume containing 10 μL of 2× SuperReal PreMix Plus (TIANGEN, China), 0.8 μL each of the gene-specific primers (Table 1), 1 μL of the cDNA template and 7.4 μL of ddH_2O. Reactions were carried out on the iQ5 real-time PCR detection system (Bio-Rad, Hercules, CA) at the following conditions: 95 °C for 15 min followed by 40 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 30 s. The melting curve was used to confirm the specific PCR amplification. Three biological replicates were conducted for each sample.

Transcript levels of each gene were estimated from the Ct (cycle threshold) value (Walker, 2002), and normalized with *gapdh* gene. The relative mRNA levels were calculated using the $2^{-\Delta\Delta CT}$ formula (Livak and Schmittgen, 2001). Data were expressed as means ± SE and analyzed using a one-way ANOVA followed by Tukey's multiple range test for pairwise comparison ($P < 0.05$). All analyses were carried out using SPSS 16.0 software (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Characterization of hsp70/hsc70/hsp90 cDNAs

The full-length cDNA sequence for *hsp70* obtained from *S. mosellana* larvae is 2199 bp long and contains a 1926 bp open reading frame (ORF) starting at nucleotide 117 and ending at nucleotide 2042 (Fig. 1A). The polypeptide deduced from the ORF comprises 641 amino acid residues with a predicted molecular weight of 70.4 kDa and a putative pI of 5.48. It has a putative polyadenylation signal (aataaa) at nucleotides 2161–2166. This *S. mosellana* gene was assigned GenBank accession number KJ813013.

The full-length cDNA sequence of *hsc70* is 2228 bp (Fig. 1B, GenBank accession number KW014659). The 1956 bp ORF starting at nucleotide 117 and stopping at nucleotide 2072 encodes a 651 amino acid protein with a predicted molecular weight of 70.0 kDa and a pI of 5.28. The putative polyadenylation signal is located at nucleotides 2197–2202.

Likewise, the full-length cDNA sequence of *hsp90* is 2412 bp (Fig. 1C, GenBank accession number KJ813012). The 2148 bp ORF starting at nucleotide 150 and terminating at nucleotide 2297 encodes 715 amino acid residues with a calculated molecular weight of 82.0 kDa and a pI of 5.02.

3.2. Characterization of hsp70/hsc70/hsp90 protein sequences

All three signature sequences of the *hsp70* family (Sonoda et al., 2006) were discovered in the deduced amino acid (a.a.) sequences of the cloned *hsp*/*hsc70*: IDLGTTYS (a.a. 9–16), IFDLGGGTFDVSIL (a.a. 197–210), and IVLVGGSTRIPKIQS (a.a. 335–349 in *hsp70*) or IVLVGGSTRIPKVQK (a.a. 334–348 in *hsc70*) (Fig. 1A and B). Also, their C-termini have the conserved EEVD motif (Fig. 1A and B), which enables *hsp*/*hsc70* to interact with other co-chaperones (Daugarrd et al., 2007). In addition, three other typical motifs were identified: an ATP/GTP binding site motif AEAYLGKT (a.a. 131–138) (Saraste et al., 1990), a deduced bipartite nuclear localization signal KRKYKKDLTTNPRALRRL (a.a. 247–264) and PRALRRLRTAAERAKRTL (a.a. 258–275) in *hsp70* or KRKHKKDLESNKRALRRL (a.a. 246–263) and KRALRRLRTACERAKRTL (a.a. 257–274) in *hsc70* (Sonoda et al., 2006), and a non-organellar consensus motif RARFEEL (a.a. 300–306 in *hsp70* and a.a. 299–305 in *hsc70*) (Zhang and Denlinger, 2010).

Similarly, the deduced amino acid sequence of the cloned *hsp90* contains all five *hsp90* family signatures (Gupta, 1995): NKEIFLRELISNASDALDKIR (a.a. 28–48), LGTIAKSGT (a.a. 95–103), IGQFGVGFYSAYLVAD (a.a. 119–134), IKLYVRRVFI (a.a. 344–353) and GVVDSEDLPLNISRE (a.a. 370–384). The pentapeptide MEEVD at the C-terminus (Fig. 1C), the core sequence for binding to the tetratricopeptide repeats (TPRs) domain of *hsp90* co-chaperones (Pearl and Prodromou, 2006), is also well conserved.

3.3. Sequence comparison and phylogenetic analysis

Multiple sequence alignment indicated that the deduced amino acid sequences of *S. mosellana hsp*/*hsc70*/*hsp90* show high homology to their corresponding sequences in other insects (Fig. 2). Specifically, *S. mosellana* hsp70 shares 80% similarity with hsp70s from *Ceratitis capitata* (XP_004536234.1), *Culex pipiens* (AAX84696.1) and *Stratiomys singularior* (ADX42270.1), and 81% similarity with hsp70 from *Rhagoletis pomonella* (ABL06948.1). *S. mosellana* hsc70 displays 91% similarity with hsc70 s of *Aedes aegypti* (ABF18332.1), *Ceratitis capitata* (XP_004518150.1) and *Drosophila melanogaster* (NP_524356.1), and 92% similarity with hsc70 of *Musca domestica* (XP_005183920.1). Additionally, hsp70 and hsc70 from *S. mosellana* were 74% identical, thus similar, yet distinctly different. *S. mosellana* hsp90 is 86% identical with hsp90s from *Anopheles gambiae* (XP_308800.3) and *Ceratitis capitata* (NP_001274755.1), and 87% identical with hsp90s from *Bactrocera correcta* (AGU42458.1) and *Musca domestica* (XP_005176932.1).

Twenty full-length hsp90 amino acid sequences from Diptera, Lepidoptera, Coleoptera and Hemiptera were employed for phylogenetic analysis (Fig. 3). Names and accession numbers of these sequences are given in figure captions. The phylogenetic tree was distinctly divided into two clades. CladeⅠincluded Diptera, which was further divided into Nematocera and Brachycera suborders. CladeⅡ consisted of three branches: Hemiptera, Coleoptera and Lepidoptera. Furthermore, superfamilies Sciaroidea (*S. mosellana*) and Culicoidea were separated within the Nematocera, and infraordo Stratiomyomorpha (*Stratiomys singularior*) and Muscomorpha were separated within the Brachycera. Among Muscomorpha insects, superfamilies Tephritoidea, Muscoidea and Ephydroidea were also well segregated, and Opomyzoidea (*Liriomyza sativae*) were separated from them as another superfamily. Also, superfamilies Noctuoidea and Yponomeutoidea were separated within the Lepidoptera, and superfamilies Tenebrionoidea and Chrysomeloidea were separated within the Coleoptera. These results confirmed that *hsp90* is a good candidate for phylogenetic analysis at these higher taxonomic levels.

3.4. Expression of hsp70/hsc70/hsp90 during diapause

To determine the diapause-associated expression profiles of *S. mosellana hsp70*/*hsc70*/*hsp90*, the transcript levels of each gene were compared in four larval stages relating to diapause: pre-diapause, diapause, post-diapause quiescence and post-diapause development (Fig. 4).

The expression patterns of *hsp70* and *hsp90* were similar in the course of diapause. Transcripts of both genes were dramatically up-regulated upon entry of diapause (June), remained high throughout diapause (June-November) and early-to-mid phase of post-diapause quiescence (December and January), but greatly declined at the late quiescent stage (February), and returned to the pre-diapause level (May of previous year) at the post-diapause developmental stage (March). The highest expression level was observed in July and August (summer) followed by December (winter) for *hsp70*, and vice versa for *hsp90* (Fig. 4A and C).

In contrast, *hsc70* expression did not change after entering diapause. But expression began to increase from November, a transition time from diapause to post-diapause quiescence, with a peak in December and January and thereafter a progressive decrease. Once post-diapause development began, expression returned to the pre-diapause level (Fig. 4B).

3.5. Expression of hsp70/hsc70/hsp90 under high temperature stress during diapause

Three genes showed differential expression patterns when oversummering diapausing larvae were subjected to high temperature treatments. Compared with the control, the expression of *hsp70*

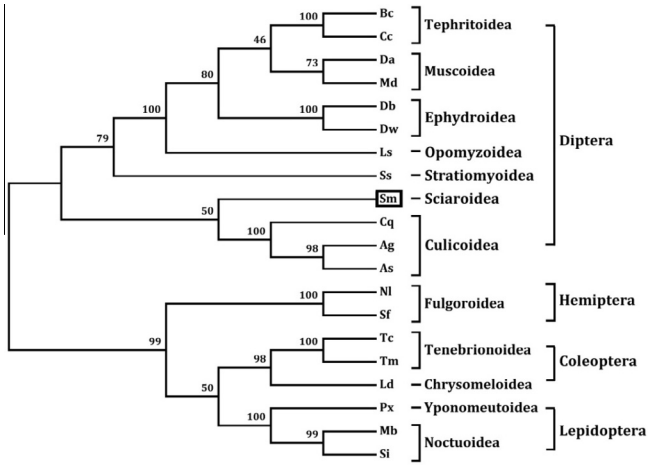

Fig. 3. Phylogenetic neighbor-joining tree built by MEGA 5.0 software for *Sitodiplosis mosellana* hsp90 and hsp90 from other insects. The following abbreviations were used: Bc = *Bactrocera correcta* (AGU42458.1), Cc = *Ceratitis capitata* (NP_001274755.1), Da = *Delia antique* (CAI64494.1), Md = *Musca domestica* (XP_005176932.1), Db = *Drosophila buzzatii* (ABK34943.1), Dw = *Drosophila willistoni* (XP_002062325.1), Ls = *Liriomyza sativae* (AAW49253.2), Ss = *Stratiomys singularior* (AER28025.1), Sm = *Sitodiplosis mosellana* (KJ813012), Cq = *Culex quinquefasciatus* (XP_001865484.1), Ag = *Anopheles gambiae* (XP_308800.3), As = *Anopheles sinensis* (KFB48798.1), Nl = *Nilaparvata lugens* (ADE34169.1), Sf = *Sogatella furcifera* (AFK64820.1), Tc = *Tribolium castaneum* (NP_001094067.1), Tm = *Tenebrio molitor* (AFN02497.1), Ld = *Leptinotarsa decemlineata* (AHB18587.1), Px = *Plutella xylostella* (AHA36864.1), Mb = *Mamestra brassicae* (BAF03554.1), Si = *Sesamia inferens* (AIZ00846.1) .

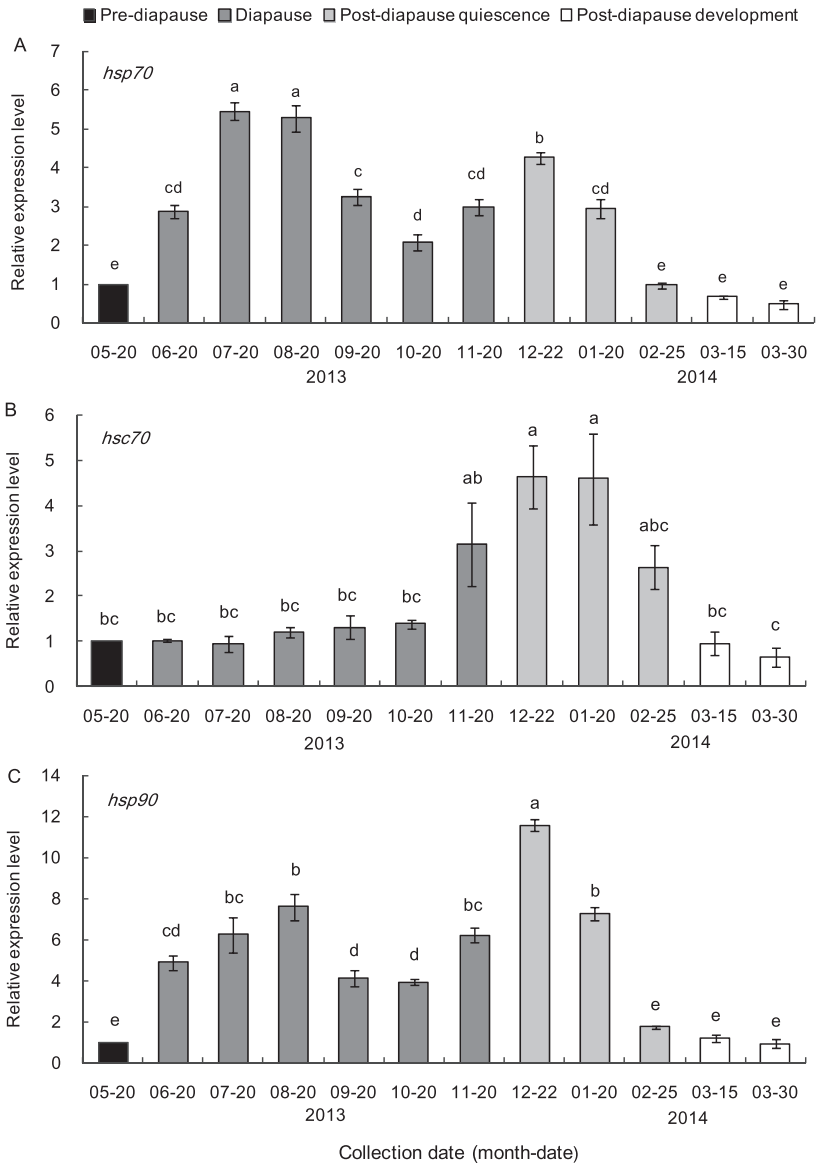

Fig. 4. Expression profiles of *Sitodiplosis mosellana hsp70* (A), *hsc70* (B) and *hsp90* (C) in pre-diapause, diapause and post-diapause larvae. Expression level of each treatment was relative to that of pre-diapause, which was arbitrarily set at 1. Bars represent the average ± SE. One-way ANOVA analysis results for *hsp70*, *hsc70* and *hsp90* were df = 11, $F = 83.68$, $P < 0.001$; df = 11, $F = 8.87$, $P < 0.001$ and df = 11, $F = 70.64$, $P < 0.001$, respectively. Values followed by the different small letters were significantly different by Tukey's multiple range test ($P < 0.05$).

was greatly induced at the temperatures range 30–40 °C, with the highest level at 35 °C (approximately 10.3-fold) (Fig. 5A). Up-regulation of *hsp90*, however, was found at 30–35 °C and the maximum expression value (approximately 4.5-fold) was found at 30 °C (Fig. 5C). For *hsc70*, the expression increased only 2.5-fold at 30 °C (Fig. 5B). Such induction was not observed at 45 °C for any of the *hsps*.

Treatment duration also affected transcript levels of *hsp70*/*90*. At 35 °C, *hsp70*/*90* expression significantly increased at 30 min, and reached a maximum at 60 min (Fig. 6A and C). Expression of *hsc70*, however, did not show a significant change in all treatment times examined (Fig. 6B).

3.6. Expression of hsp70/hsc70/hsp90 under cold stress during diapause

Cold treatment also affected *hsp70*/*90* expression of over-wintering diapausing larvae. Exposure of insects to 0–10°C greatly elevated *hsp70*/*90* expression, with the maximum value at −5 °C with 8.0-fold and 7.7-fold increases, respectively; but −15 °C failed to do so (Fig. 7A and C). Notably, expression level of *hsc70* also increased remarkably at −5 °C (approximately 2.9-fold), but no evident change was observed when treated at 0 °C, −10 °C and −15 °C relative to the control (Fig. 7B).

At −5 °C, significant increases in expression were detected for *hsp70* and *hsc70* at 15 min and 30 min, respectively. They reached a peak at 60 min (Fig. 8 A and B). For *hsp90*, expression level significantly increased as the treatment duration increased (Fig. 8C).

4. Discussion

Hsps play important roles in various stress responses, especially in heat/cold adaptation in insects (Hu et al., 2014; Kang et al., 2009). It has been shown that hsps participate in diapause in a manner that is species-dependent (MacRae, 2010). *S. mosellana* escapes temperature extremes in the summer and in the winter

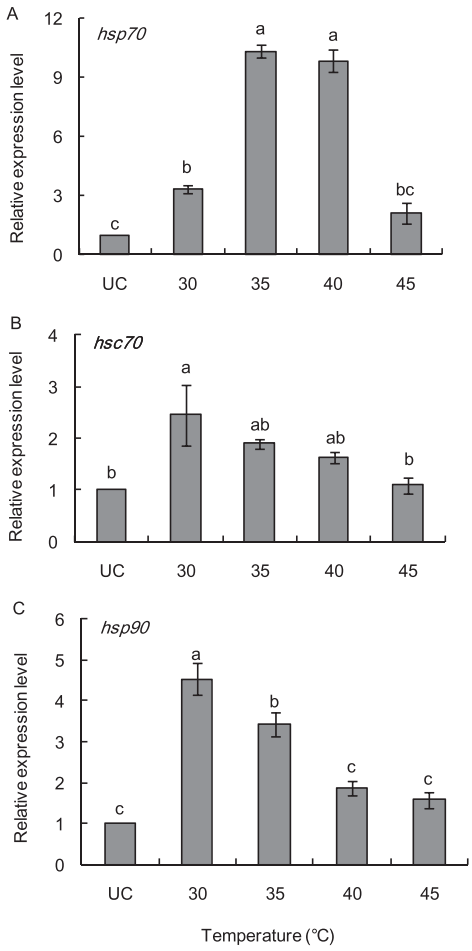

Fig. 5. Expression profiles of *Sitodiplosis mosellana hsp70* (A), *hsc70* (B) and *hsp90* (C) in response to heat shock in diapauing larvae collected in August, exposed to 30–45 °C for 1 h. Expression level of each treatment was relative to that of the untreated control (UC), which was arbitrarily set at 1. Bars represent the average ± SE. One-way ANOVA analysis results for *hsp70*, *hsc70* and *hsp90* were df = 4, $F = 128.33$, $P < 0.001$; df = 4, $F = 4.69$, $P = 0.022$ and df = 4, $F = 35.06$, $P < 0.001$, respectively. Values followed by the different small letters were significantly different by Tukey's multiple range test ($P < 0.05$).

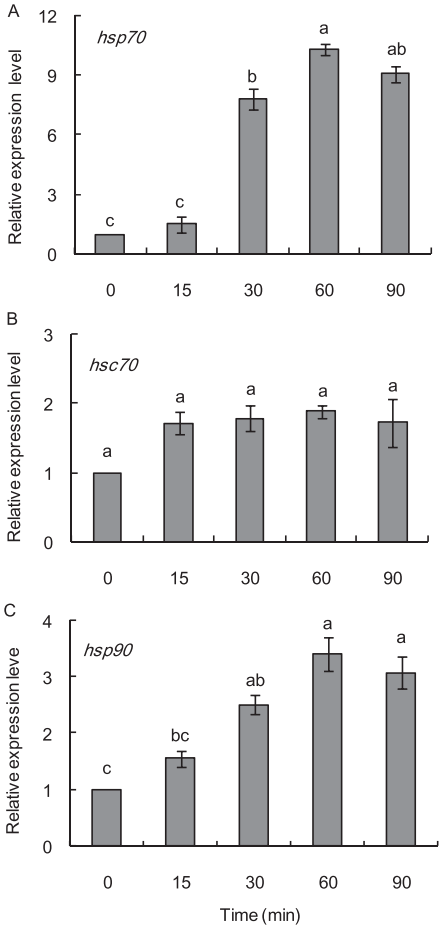

Fig. 6. Expression profiles of *Sitodiplosis mosellana hsp70* (A), *hsc70* (B) and *hsp90* (C) in response to heat shock in diapausing larvae collected in August, exposed to 35 °C for 0–90 min. Expression level of each treatment was relative to that of the untreated control (0 min), which was arbitrarily set at 1. Bars represent the average ± SE. One-way ANOVA analysis results for *hsp70*, *hsc70* and *hsp90* were df = 4, $F = 143.96$, $P < 0.001$; df = 4, $F = 3.33$, $P = 0.056$ and df = 4, $F = 23.01$, $P < 0.001$, respectively. Values followed by the different small letters were significantly different by Tukey's multiple range test ($P < 0.05$).

by undergoing diapause. Thus understanding how *S. mosellana hsp* genes respond to diapause and thermal stresses at the molecular level is of great importance. In the current study, we cloned full-length cDNAs of three hsp genes, *hsp70*, *hsc70* and *hsp90* from *S. mosellana* larvae and determined their expression patterns in response to diapause and further short-term stresses of extreme temperatures. Differing from many molecular studies on insect diapause, insect samples at all developmental stages used here including pre-diapause, diapause, post-diapause quiescence and post-diapause development, were collected from field, presumably more closely reflecting natural situation.

As expected, three *S. mosellana* hsps share high sequence similarities with their counterparts from other insects, and include all conserved signature motifs that facilitate interaction with other proteins and enhance their chaperone function (Figs. 1 and 2) (Daugarrd et al., 2007; Pearl and Prodromou, 2006). The characteristic of highly conserved sequences makes *hsp* genes widely used for evolutionary and phylogenetic analysis. Hsp90 is especially useful in this regard since a single gene copy is present in many insects (Gkouvitsas et al., 2009b; Theodoraki and Mintzas, 2006; Zhang and Denlinger, 2010), thus reducing the complication of dealing with isoforms. Here, the phylogenetic tree was built based on twenty full-length hsp90 amino acid sequences of known insects. Our results showed that Diptera, Lepidoptera, Coleoptera and Hemiptera were well segregated from each other, and even the superfamilies Tephritoidea, Muscoidea, Ephydroidea, Opomyzoidea, Stratiomyoidea, Sciaroidea and Culicoidea were also well separated within the Diptera, further supporting that hsp90 protein allows good phylogenetic analysis at the superfamily level (Zhang and Denlinger, 2010).

Similar with patterns observed in *D. antique* for *hsp70* and *hsp90* (Chen et al., 2005, 2006) and in *S. crassipalpis* for *hsp70* (Hayward et al., 2005), *S. mosellana hsp70* and *hsp90* expression were highly up-regulated from the onset of diapause through diapause and expression decreased after the resumption of overt development (Fig. 4A, C). It has been proposed that diapause up-regulated hsps may be directly involved in cell cycle arrest (Denlinger, 2002). Elevated expression of two small *hsps* and *hsp70* has been directly linked to cell cycle arrest or retardation in *Drosophila melanogaster* (Feder et al., 1992; Krebs and Feder, 1997). Presumably, *S. mosellana* hsp70/hsp90 may also perform that function in regulation of diapause.

S. mosellana hsp70 and *hsp90* are also clearly induced by seasonally high and low temperatures (Fig. 4), suggesting that they may

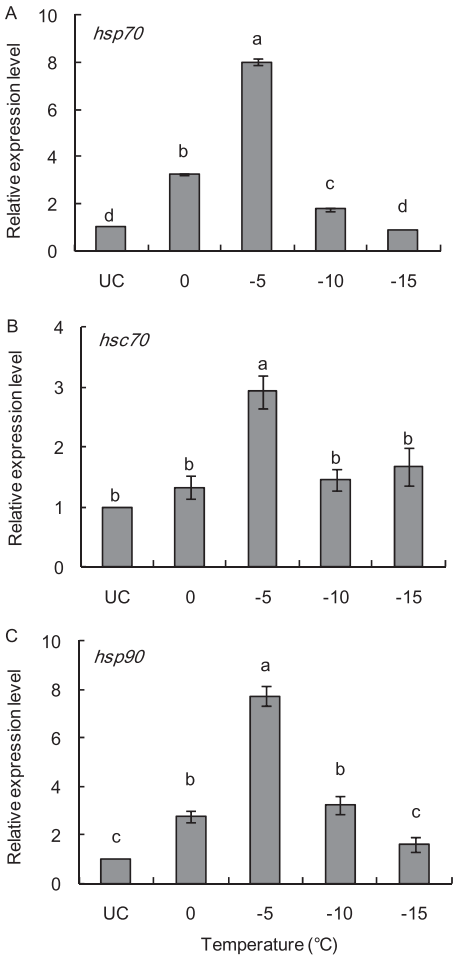

Fig. 7. Expression profiles of *Sitodiplosis mosellana hsp70* (A), *hsc70* (B) and *hsp90* (C) in response to cold shock in cocooned larvae collected in December, exposed to 0–15 °C for 1 h. Expression level of each treatment was relative to that of the untreated control (UC), which was arbitrarily set at 1. Bars represent the average ± SE. Data were analyzed using One-way ANOVA (*hsp70*: df = 4, $F = 2366.62$, $P < 0.001$; *hsc70*: df = 4, $F = 11.18$, $P = 0.001$; *hsp90*: df = 4, $F = 85.46$, $P < 0.001$). Values followed by the different small letters were significantly different by Tukey's multiple range test ($P < 0.05$).

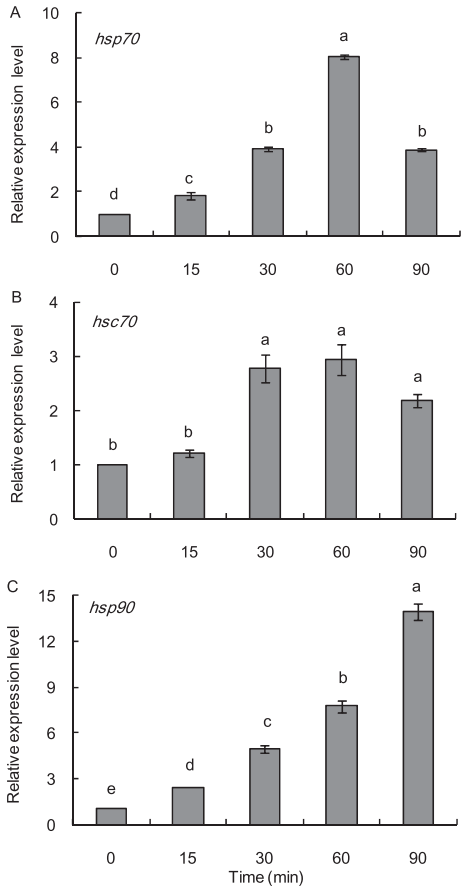

Fig. 8. Expression profiles of *Sitodiplosis mosellana hsp70* (A), *hsc70* (B) and *hsp90* (C) in response to cold shock in cocooned larvae collected in December, exposed to −5 °C for 0–90 min. Expression level of each treatment was relative to that of the untreated control (0 min), which was arbitrarily set at 1. Bars represent the average ± SE. Data were analyzed using One-way ANOVA (*hsp70*: df = 4, $F = 677.25$, $P < 0.001$; *hsc70*: df = 4, $F = 23.94$, $P < 0.001$; *hsp90*: df = 4, $F = 255.67$, $P < 0.001$). Values followed by the different small letters were significantly different by Tukey's multiple range test ($P < 0.05$).

play important roles in survival of over-summering and over-wintering individuals. In *S. crassipalpis*, up-regulation of *hsp70* has been shown to be directly associated with enhanced cold-hardiness of over-wintering individuals (Rinehart et al., 2007). Complementary expression patterns of *hsp70* and *hsp90* in winter and summer (Fig. 4) imply that *hsp70* may be more prominent for heat tolerance and *hsp90* for cold tolerance.

Expression of *S. mosellana hsc70* is not affected by diapause initiation, but winter low temperature, a necessary factor to terminate diapause under natural conditions, apparently induces expression (Fig. 4B), contrasting to other insects including *C. pipiens* (Li and Denlinger, 2009), *S. crassipalpis* (Rinehart et al., 2007), *M. rotundata* (Yocum et al., 2005) and *Helicoverpa zea* (Zhang and Denlinger, 2010). Larval and pupal diapauses are also known to be directly regulated by ecdysteroids, and interestingly in *M. sexta hsc70* expression is regulated by 20-hydroxyecdysone, and a rapid increase of ecdysteroid synthesis in stimulated prothoracic gland is accompanied by increased protein synthesis, including hsc70 (Rybczynski and Gilbert, 1995, 2000). In addition, hsc70 participates in the 20-hydroxyecdysone signal transduction pathway via binding to the ecdysone receptor protein USP, as exemplified by *H. armigera hsc70* (Zheng et al., 2010). Furthermore, ecdysteroid titers in *S. mosellana* diapausing larvae are significantly higher in December and January than other diapause periods (Cheng et al., 2009), which is correlated with the *hsc70* expression pattern we observed here. Presumably, high ecdysteroid titers in *S. mosellana* may be essential for up-regulation of *hsc70*. Because the up-regulation of *S. mosellana hsc70* is initiated at an early stage in the transition from diapause to post-diapause quiescence (Fig. 4B, it thus may serve as a reliable molecular marker denoting this transition.

Heat-stressed over-summering larvae or cold-stressed over-wintering larvae rapidly raised expression of *S. mosellana hsps* within a short period (Figs. 5–8). Similar results were also seen in other insects, such as *D. antiqua* (Chen et al., 2005, 2006) and *Lymantria dispar* (Denlinger et al., 1992; Yocum et al., 1991). Cleary, the maximum level for expression under heat stress is at 35 °C for *hsp70*, and when temperatures reach 45 °C, the expression of all three genes is no longer induced (Fig. 5). One hour exposure to 47.5 °C led to death of *S. mosellana* cocooned larvae (Sokhansanj et al., 1992), indicates that hsp expression cannot prevent death under severe temperature stresses, even for a short period of time. This may explain the mode of action of the cultural control methods that elevate soil temperature; the summer fallow season reduces *S. mosellana* populations by direct sunshine on the soil, while tillage bring the insects to the soil surface, where the temperature in the summer is higher than it is several centimeters below the surface (Barnes, 1941; Yuan, 2004).

In summary, we have demonstrated developmental and environmental regulation of *S. mosellana hsps*. Transcripts of *hsp70*/*90* are diapause up-regulated, while that of *hsc70* is up-regulated during the transition to post-diapause quiescence, a response possibly associated with the high ecdysteroid titers during this period. Expression of *hsp70*/*90* is also highly responsive to heat/cold during diapause, but not to extreme high/low temperature. These results provide some molecular insight into the diapause mechanism and stress tolerance to ecologically relevant environmental temperature in *S. mosellana*.

Acknowledgements

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