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ORIGINAL PAPER

Molecular characterization and expression patterns of heat shock proteins in *Spodoptera littoralis*, heat shock or immune response?

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Abstract

The Egyptian cotton leaf worm, *Spodoptera littoralis* (Boisd.), is a major agricultural lepidopterous pest causing extensive damage in a variety of crops including vegetable, cotton, fodder, and fiber crops. Heat shock protein (HSP) family members play important roles in protecting insects against environmental stressors. In this study, we characterized three putative heat shock proteins (SpliHsp70, SpliHsp90, and SpliHSF) from *S. littoralis* and analyzed their expression levels in response to heat, cold, ultraviolet irradiation, *Bacillus thuringiensis*, and *Spodoptera littoralis* nucleopolyhedrovirus treatments. Significant upregulation of *SpliHsp70* was observed in female pupae, while the highest expression levels of *SpliHsp90* and *SpliHSF* were found in female adults. Heat shock triggered increases in *SpliHsp* levels compared to cold treatment. *SpliHsp90* exhibited the highest expression levels during the first 30 min of UV treatment. Both bacterial and viral pathogenic agents effected the regulation of Hsps in *S. littoralis*. These findings suggest that *SpliHsp* genes might play significant roles in the response to biotic and abiotic stress, as well as in the regulation of developmental stages.

Keywords Heat shock protein · *Spodoptera littoralis* · Stress tolerance · Immune response

Introduction

Insects use a variety of tactics against stress. Regulation of protein expression is one of the defense mechanisms of insects against different stress signals. Heat shock proteins (HSPs) are chaperone proteins present in the cells of all organisms, which assist in the folding of nascent polypeptides. The formation of denatured polypeptides and proteins as a result of cellular stress leads to the upregulation of *Hsp* gene expression (Richards et al. 2017). Transcription of HSPs is regulated by the heat shock transcription factor (*HSF*) (Morimoto 1998).

The induction of HSP synthesis is a universal response upon exposure to various environmental conditions, including abiotic stresses such as heat (Joplin and Denlinger 1990; Mahroof et al. 2005; Zhang and Denlinger 2010; Tungjitwitayakul et al. 2015), cold (Rinehart et al. 2007; Tungjitwitayakul et al. 2015), radiation (Nguyen et al. 2009; Sang et al. 2012), insecticides (Li et al. 2017), oxidative stress (Lopez-Martinez et al. 2008), heavy metal (Braeckman et al. 1999; Sonoda et al. 2007; Shu et al. 2011), nutrient deficiencies (Benoit et al. 2011), dehydration (Benoit et al. 2010), and biotic stresses including virus (Lyupina et al. 2010), bacteria (Iryani et al. 2017), and fungus (Richards et al. 2017).

Insect HSPs are known to be responsive to elevated temperatures (Ritossa 1996), due to their increasing gene expression levels after exposure to heat (Zhao and Jones 2012). They are highly conserved proteins that also contribute to cold tolerance in insects (Rinehart et al. 2007). Most insects have evolved impressive strategies to enable their survival at subzero body temperatures (Renault et al. 2002; Lee and Denlinger 1991). In addition to temperature stress, insects fight with intrusive microorganisms by using their innate immune system, which comprises cellular and humoral

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responses (Wu and Yunhong 2018). Previous studies reported stimulation of immune responses by HSPs in insects against pathogens (Wojda and Jakubowicz 2007; Wojda et al. 2009; Dubovskiy et al. 2013; Richards et al. 2017). Ultraviolet (UV) irradiation is an additional abiotic stress, regularly experienced by insects, which can induce oxidative stress (Adams and Shick 2001) and potentially cause death by damaging nucleic acids (DNA and RNA) (Lah et al. 2012). Several studies have indicated that following UV exposure, expression of insect HSPs is strongly induced to help repair the DNA damage within the cell (Wang et al. 2014; Pan et al. 2018).

The Egyptian cotton leafworm, *Spodoptera littoralis* (Boisd.), (Lepidoptera: Noctuidae) is one of the most devastating phytophagous lepidopteran pests, which gives rise to extensive damage in many vegetable, fiber crops, and fodder, in countries in Southeast Asia and around the Mediterranean Basin (Balachowsky 1972; Sneh et al. 1981). Chemical-based control methods are still used as a management strategy for this polyphagous pest, though resistance has been developed to miscellaneous insecticides (Issa et al. 1984a, 1984b; El-Guindy et al. 1989; Rashwan et al. 1992). Therefore, it is crucial to understand *Spodoptera littoralis*' physiology in order to develop alternative pest control strategies, which target the behavior, and physiology of the pest.

In this study, we characterized full-length cDNAs of the *Heat shock protein 70* (*SpliHsp70*) and *Heat shock protein 90* (*SpliHsp90*), and a partial cDNA of *Heat shock transcription factor* (*SpliHSF*) in *S. littoralis*. We construct phylogenetic trees based on amino acid sequences of SpliHsp70 and SpliHsp90 from various species, and examined expression profiles of *SpliHsp*s in different tissues and developmental stages. We also analyzed the regulation of *SpliHsp* expression in response to thermal stress, radiation, and following infection with a gram-positive bacterium (*Bacillus thuringiensis*) and baculovirus (SpliNPV) over time.

Materials and methods

Biological material

S. littoralis laboratory cultures and dissected tissues were used from our previous study (Guz et al. 2013).

RACE PCR and sequencing analysis

Isolation of total RNA was carried out from sixth instar larvae using TRIzol Reagent (Thermo Fisher Scientific, Waltham, MA, USA) according to manufacturer's protocol. To prevent DNAse contamination, DNase digestion was applied to RNA using RNase-Free DNase (Ambion). Complementary DNAs (cDNAs) were synthesized using the Transcriptor First Strand cDNA Synthesis Kit (Roche) according to the manufacturer's recommended protocol.

Contigs encoding *SpliHSP*s and *SpliHSF* were identified from transcriptome analysis of *S. littoralis* (Unpublished data). Gene-specific primers for RACE-PCR were designed using BLAST at the NCBI website to obtain full-length cDNA sequences of *SpliHsp70* and *SpliHsp90* (Table 1). Rapid amplification of cDNA ends (RACE) PCR technique was performed to amplify 5′ and 3′ ends of the *SpliHsp70* and *SpliHsp90* cDNAs using the 5′/3′ RACE Kit, 2nd Generation (Roche), according to the manufacturer's recommended protocol. Sequencing reactions of SpliHSP70 and SpliHSP90 were carried out using DTCS Quick Start Kit (Beckman

Table 1 Primers used in this study

Gene	Primer Sequence (5′-3′)	Application
SpliHsp70	F: CTTGTGAAAGGGCGAAGAGA	RT-qPCR
	R: GCTCTGGTGATGGATGTGTAG	
SpliHsp90	F: CGAGCCCATTGATGAGTATGT	RT-qPCR
	R: GTCTTCCTCACGCTTCTTCTT	
SpliHSF	F: TCAGTGCTATGAAGCAGGAAA	RT-qPCR
	R: CAAGTGACATCAGGAATTGGATAA	
β-Actin	F: ATC ATG TTC GAG ACC TTC AAC	RT-qPCR
	R: GCA CGA TTT CTC TCT CGG	
SpliHsp70	SP1: GCCACCTGGTTCTTGGCGGCA	RACE-PCR
	SP2: TGCCACCATCACTGACAACCTCGA	
	SP3: GAGTCGTTGAAGTACGCTGGAACCG	
	SP5: CTCCAACCAGCTGGCTGACAAGGA	
SpliHsp90	SP1: CTGTCCAGCTTTGATGGGTCGG	RACE-PCR
	SP2: GACGAGGTCAGCCTTGGTCATACC	
	SP3: CTCCCACATGTACTGCTCGTCGTC	
	SP5: CTGCCCTGCTGTCGTCTGGATTCA	

Coulter) and analyzed with CEQ 8800 Genetic Analysis System (Beckman Coulter, Fullerton, CA). Expasy MW/pI tool was utilized to calculate putative molecular weights and isoelectric points of proteins.

Comparison of the deduced amino acid sequences of SpliHsp70, SpliHsp90, and SpliHSF were carried out using BLAST at the NCBI website and EXPASY. CLUSTAL OMEGA was used to align putative amino acid sequences. BOXSHADE was used to identify conserved regions.

To construct phylogenetic trees, amino acid sequences of SpliHsp70 and SpliHsp90 from various species were aligned with MEGA6 (Tamura et al. 2013) suit using ClustalW (Higgins and Sharp 1988).

Expression analyses

RT-qPCR was used to investigate expression profiles of *SpliHsp* and *SpliHSF* genes, in different tissues and developmental stages of *S. littoralis*. Tissue-specific expression was carried out using isolated RNA from ovaries, fat body, Malpighian tubules, hemolymph, midgut, and nervous system. All the tissues from sixth instar larvae were dissected, whereas only the reproductive tract was dissected from adults. To analyze developmental stage-specific expression, total RNA was extracted from egg, neonate, second instar larvae, third instar larvae, fourth instar larvae, fifth instar larvae, sixth instar larvae, pupae, and 5-day-old female and male adults.

To examine gene expression patterns at different temperatures, third instar *Spodoptera* larvae were exposed to 25 °C (control), 0 °C and 42 °C for 1 h. In order to test the effects of UV irradiation on the expression of *SpliHsp*s and *SpliHSF*, a UV light (X-15 N, Spectronics, USA) emitting UV in the 254 nm wavelength (UV-C) was used to irradiate third instar larvae at approximately 300 μW/cm^2 and different time points (0.5, 1, 1.5, 2, and 3 h). Controls were subjected to the same handling procedures without UV exposure. Larvae were immediately frozen in liquid nitrogen after heat and irradiation treatments and stored at – 80 °C.

In order to check immune responsive gene expression profiles of *SpliHsp*s and *SpliHSF*, recently emerged third instar larvae were challenged by 10^6 *B. thuringiensis* and SpliNPV cells, as previously described (Guz et al. 2013). Infected larvae were collected at 12, 24, 48, and 72 h post feeding (hpf). Three biological replicates were collected from the experimental and control groups in each time point, and held at – 80 °C. Total RNA was isolated from larvae using TRIzol Reagent (Thermo Fisher Scientific, Waltham, MA, USA).

RT-qPCR analysis of SpliHsps and SpliHSF expressions

Six infected and age-matched control larvae were tested at the four time points post challenge (12, 24, 48, and 72 h) using RT-qPCR. Primer sequences are given in Table 1. At each time point, three biological replicates were collected from the experimental and control groups, and stored at – 80 °C (Guz et al. 2013). Three technical replicates were included for each biological replicate. cDNA was synthesized from total RNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche) according to the manufacturer's recommended protocol. RT-qPCR reactions were performed using LightCycler 480 SYBR Green I Master kit (Roche). Amplification conditions were as follows: an initial preincubation at 95 °C for 10 min, followed by amplification for 35 cycles at 95 °C for 10 s, 53 °C for 30 s, 72 °C for 1 s, and 1 cycle of cooling at 40 °C for 10 s with the LightCycler 480 (Roche). A standard curve was determined for each set of primers in order to measure the efficiency of each reaction. The SpliHSPs mRNA levels were normalized to β-actin levels (Guz et al. 2013). Developmental, tissue, and sex-specific expression were calculated using ΔCT method, which refers the difference in threshold cycle between SpliHSPs and β-actin genes. Relative expression levels of the SpliHSPs during the heat-cold shocks, UV-C radiation, *B. thuringiensis*, and *SpliNPV* infection were calculated using the relative quantitative method ($2^{-\Delta\Delta Ct}$) (Livak and Schmittgen 2001). SPSS 16 software was used to perform statistical analyses and statistical significance was established using One-way ANOVA and Duncan's multiple range tests. *P* value less than 0.05 was accepted statistically significant.

Results

Characterization of SpliHsp70, SpliHsp90, and SpliHSF cDNAs

The full-length *SpliHsp70* cDNA (GenBank accession nr. KC787696.1) contains an ORF of 1965 bp encoding 654 amino acids with a molecular weight of 71.5 kDa and theoretical pI of 5.32. BLAST analysis revealed that the sequence of SpliHSP70 displayed 100% identity to HSP70 from *Spodoptera litura* and *Helicoverpa armigera*, 99.84% identity to HSP70 from *Sesamia inferens*, *Mythimna separata*, *Agrotis ipsilon*, and *Xestia c-nigrum*. The putative SpliHsp70 contains three Hsp70 family signatures, including IDLGTTYS (aa 10-17), IFDLGGGTFDVSIL (aa 198-211), and IVLVGGSTRIPKVQK (aa 335-349). Furthermore, two characteristic motifs of Hsp70 were found; the deduced ATP/GTP binding site motif AEAYLGKT (aa 132-139) (Saraste et al. 1990), and conserved EEVD motif at the end of *SpliHsp70* (aa 650-653) C-terminus (Fig. 1).

The phylogenetic tree constructed based on the alignment of the deduced amino acid sequences shows that insect Hsp70s are grouped in two main branches. One main branch includes Lepidoptera, Diptera, Coleoptera, and Hemiptera, and the other includes parasitic Hymenopteran Hsps. SpliHsp70 is clustered in Lepidopteran clade and

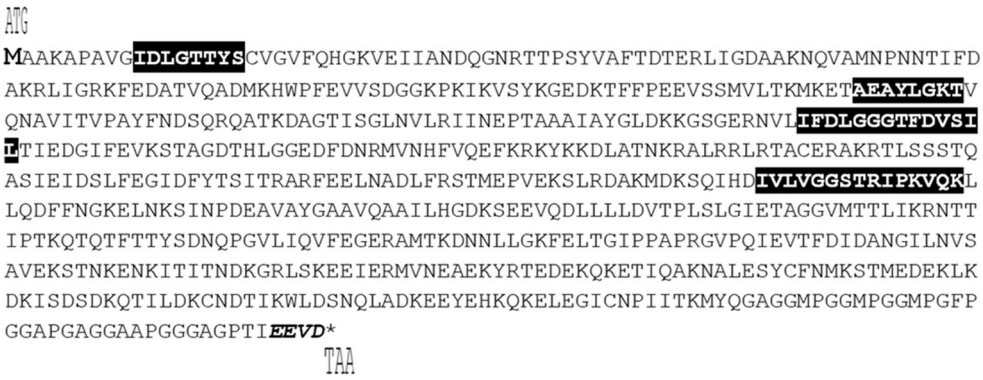

Fig. 1 Deduced amino acid sequence of Hsp70 in *S. littoralis* is shown with numbering beginning at the initiation methionine. Characteristic signature of amino acid sequence of Hsp70 are labeled

phylogenetically close to Hsp70 from *S. litura*, which is also consistent with BLAST analysis. Hsps are clustered into their order clades (Fig. 4a).

The full length of *SpliHsp90* cDNA (GenBank accession nr. KC787695.1) contains an ORF of 2154 bp encoding a 717 amino acid with a molecular weight of approximately 83 kDa and theoretical pI of 5.00. BLAST analysis revealed that the deduced sequence of SpliHSP90 displayed 99.86% identity to HSP70 from *S. litura* and *Spodoptera frugiperda*, 99.58% identity to HSP90 from *H. armigera*, 99.44% identity to HSP90 from *S. exigua* and *Helicoverpa zea*, and 99.30% identity to HSP90 from *Helicoverpa assulta.* Five Hsp90 protein family signatures (NKEIFLRELISN(S/A)SDALDKIR (aa 48-68), LGTIA(K/R)SGT (aa 115-123), IGQFGVGFYS (A/C)(Y/F)LVA(E/D) (aa 139-154), IKLYVRRVFI (aa 360-369), and GVVDS(E/D)DLPLN(I/V)SRE (aa 386-400)) were found in SpliHsp90 (Gupta 1995). Additionally, MEEVD conserved pentapeptide was detected at the C-terminal region (Fig. 2).

Based on the constructed phylogenetic tree, the deduced amino acid sequence of SpliHsp90 is phylogenetically close to Hsp90 from *S. litura* in the first main branch, which is consistent with BLAST analysis. The first main branch consists of Lepidopteran, Coleopteran, and Hemipteran Hsps; the second main branch includes parasitic Hymenoptera; and the third branch includes Dipteran Hsps (Fig. 4b).

The length of partial *SpliHSF* was 497 bp (GenBank accession nr. KC787697.1). A heat shock transcription factor conserved domain family signature was found in SpliHSF using CD-search tool (Marchler-Bauer et al. 2017). BLAST analysis revealed that the partial cDNA sequence of SpliHSF displayed 100% identity to HSF from *S. litura* and *S. frugiperda*, 92.12% identity to HSF from *H. armigera*, and 91.52% identity to HSF from *Trichoplusia ni*. One DNA binding domain (DBD), two hydrophobic heptad repeat domains (HR-A/B and HR-C), and one C-terminal trans-activation domain (CTAD) were found in the SpliHSF sequence (Fig. 3).

Developmental, tissue, and sex-specific expression of SpliHsps

RT-qPCR analysis showed that *SpliHsp70*, *SpliHsp90*, and *SpliHSF* were expressed throughout all the stages of leaf worm's life cycle (Fig. 5a). Significant upregulation of *SpliHsp70* was observed in female pupae, with high expression levels also observed in male pupae and female adults. The highest expression levels for *SpliHsp90* and *SpliHSF* were found in female adults. The expression level of *SpliHsp90* increased progressively throughout development

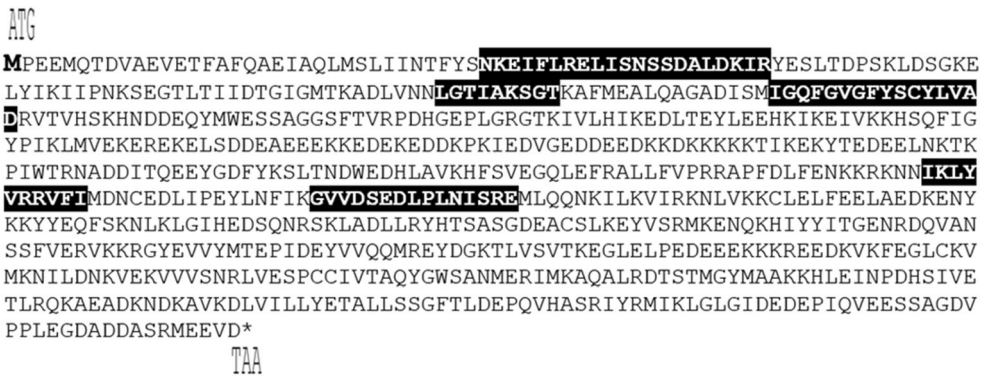

Fig. 2 Deduced amino acid sequence of HSP90 in *S. littoralis* is shown with numbering beginning at the initiation methionine. Five characteristic signatures of amino acid sequence of HSP90 are labeled

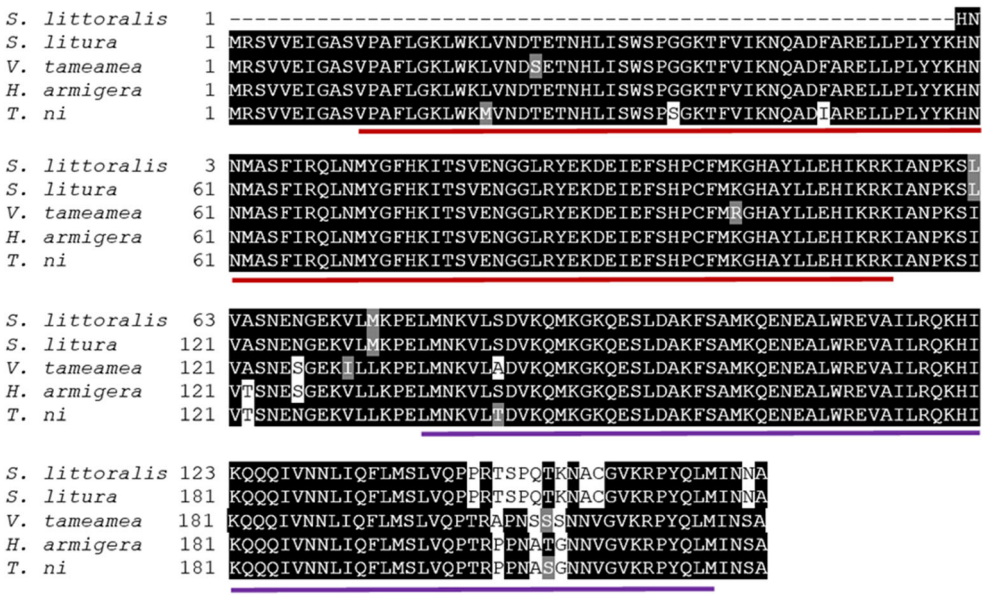

Fig. 3 Multiple alignment of the *S. littoralis* HSF partial amino acid sequence with other insect species. Conserved amino acids are shown in black. The GenBank accession numbers of the HSF sequences are as follows: *S. litura* XP_022822206, *H. armigera* XP_021188844, *Vanessa tameamea* XP_026493788, and *T. ni* XP_026732073. DBD and HR-A/B domains are underlined with red and purple, respectively

and showed a significantly higher level of expression in females than in males at the adult stage. *SpliHSF* transcript levels were statistically significant higher in pupal and adult stages of both males and females compared to the other developmental stages (Fig. 5).

Tissue-specific expression is shown in Fig. 5b. *SpliHsp70* was highly expressed in the midgut and reproductive tract. Interestingly, *SpliHsp90* had higher expression levels in the reproductive tract than *SpliHsp70*. *SpliHSF* was predominantly expressed in nervous system of *S. littoralis*.

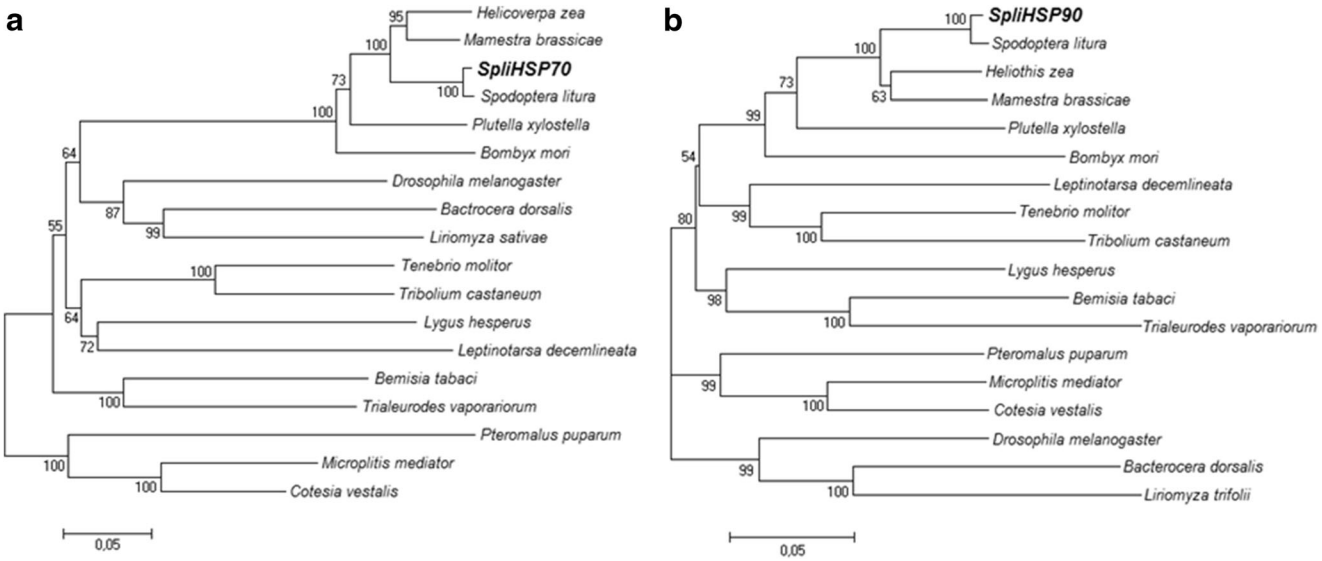

Fig. 4 Phylogenetic analysis of heat shock proteins (Hsps) from various insects. **a** The species and GenBank accession numbers of the Hsp70 sequences used to construct the phylogenetic tree are as follows: *H. zea* ACV32640.1; *M. brassicae* BAF03555.1; *S. litura*, ADV03160.1; *P. xylostella* ADK39311.1; *B. mori* BAF69068.1; *D. melanogaster* NP_731651.1; *B. dorsalis* XP_011208284.1; *L. sativae* AAW32099.2; *T. molitor* AFE88580.1; *T. castaneum* XP_015834944.1; *L. hesperus* JAQ04155.1; *L. decemlineata* XP_023021706.1; *B. tabaci* ADO14473.1; *T. vaporariorum* ACH85201.1; *P. puparum* ACO57618.1; *M. mediator* ABV55505.1; *C. vestalis* AGF34718.1. **b** The species and GenBank accession numbers of the Hsp90 sequences used to construct the phylogenetic tree are as follows: *S. litura*, ADM26738.1; *H. zea* ACV32639.1; *M. brassicae* BAF03554.1; *P. xylostella* AHA36864.1; *B. mori* BAB41209.1; *L. decemlineata* AHB18587.1; *T. molitor* AFN02498.1; *T. molitor* AHF20221.1; *T. castaneum* AHF20221.1; *L. hesperus* AFX84559.1; *B. tabaci* ADO14474.1; *T. vaporariorum* ACH85202.1; *P. puparum* ACO57617.1; *M. mediator* ABV55506.1; *C. vestalis* AGF34719.1; *D. melanogaster* NP_001261362.1; *B. dorsalis* XP_011212529.1; *L. trifolii* ARQ84029.1

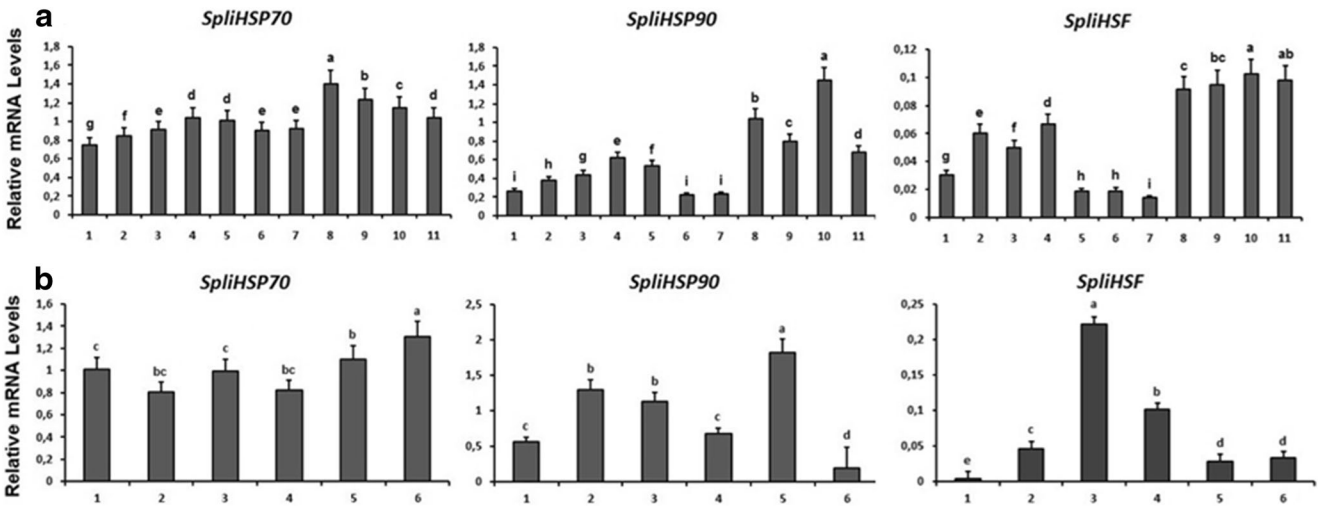

Fig. 5 RT-qPCR analysis of developmental (**a**) and tissue specific (**b**) expression profiles of *SpliHsp*s and *SpliHSF*. **a** 1. Egg, 2. Neonate, 3. 2nd instar larvae, 4. 3rd instar larvae, 5. 4th instar larvae, 6. 5th instar larvae, 7. 6th instar larvae, 8. female pupae, 9. male pupae, 10. female adult, 11. male adult. **b** 1. Fat body, 2. hemolymph, 3. nervous system, 4. Malpighian tubules, 5. reproductive tract, 6. midgut. Developmental, tissue and sex specific expression levels were normalized to β-actin levels using ΔCT method

Expression profiles of SpliHsps during the heat and cold shocks

Expression levels of *SpliHsp*s and *SpliHSF* genes under temperature stress are shown in Fig. 6. Under high-temperature stress (42 °C), all genes were significantly upregulated and the highest expression was observed in *SpliHSF* when compared to control (25 °C). No significant difference has been observed in the genes we analyzed during cold shock. Under cold stress (0 °C) expression levels were downregulated when compared to the heat shock treatment.

Expression profiles of SpliHsps in response to UV-C radiation

SpliHsp gene expression profiles were evaluated in third instar larvae exposed to UV-C radiation at different time points (0.5, 1, 1.5, 2, and 3 h). *SpliHsp90* exhibited the highest expression at first 30 min of exposure, after which expression of this gene showed tendency to decrease over time (Fig. 7). *SpliHsp70* and *SpliHSF* were expressed at all time points, with the highest levels occurring after 60 min of exposure for *SpliHsp70*, and 30–60 min of exposure for *SpliHSF*.

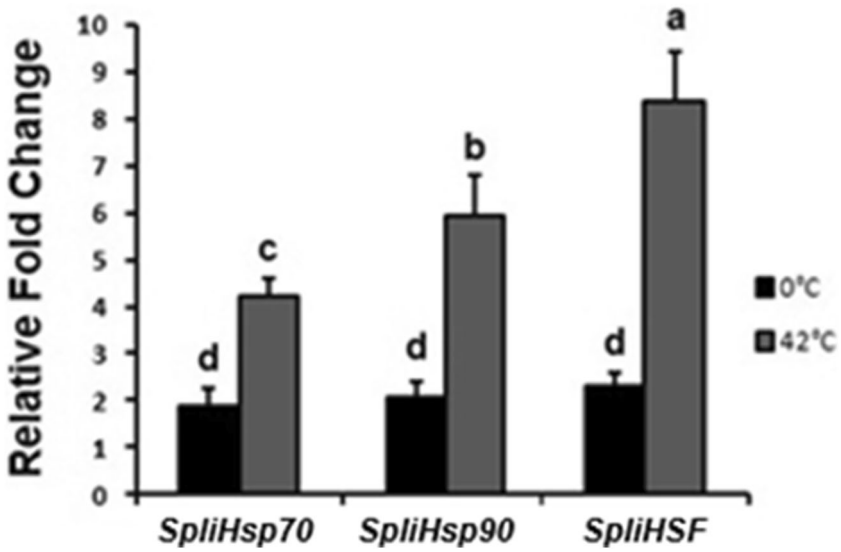

Fig. 6 *SpliHsp* and *SpliHSF* mRNA expression after 1 h heat (42 °C) and cold (0 °C) shock treatments. The bars represent the 2−ΔΔCt method normalized to the β-actin gene expression in three heat/cold treated versus non-treated ones (25 °C)

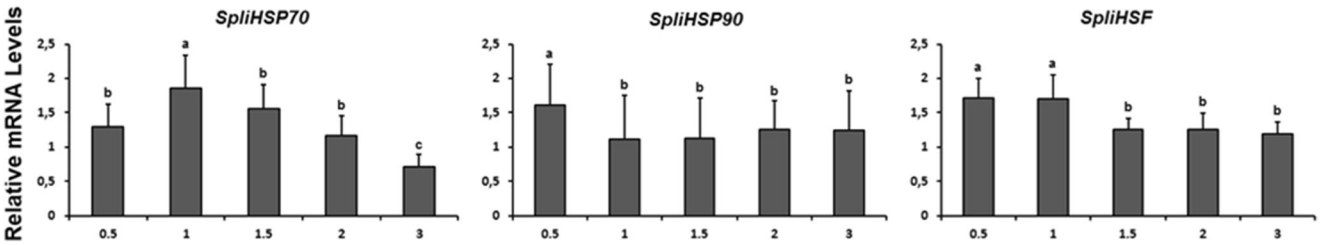

Fig. 7 Effects of UV-C irradiation on mRNA levels of *Hsp*s and *HSF* of *S. littoralis* larvae following different exposure durations. Relative expression of SpliHSPs was analyzed using β-actin reference gene for qPCR normalization in three irradiated larvae versus non-treated ones

SpliHsp expressions in response to *B. thuringiensis* and *SpliNPV* infection

The expression of *SpliHsp* in response to *B. thuringiensis* and SpliNPV infection was analyzed using RT-qPCR in infected third instar larvae (Fig. 8). Both bacterial and viral exposure affected the regulation of *Hsp*s in *S. littoralis*. The results indicated that all transcripts were upregulated following bacterial infection, with the highest expression levels for all transcripts observed at 72 h post infection. In response to SpliNPV infection, *SpliHsp*s were also upregulated and expression values presented similar profiles in the three transcripts. *SpliHsp90* had the most significant expression levels upon viral infection, with the highest expression level found at 12 h post infection. *SpliHSF* showed higher expression levels at 12 h post infection; however, there was no statistically significant difference detected among the 12, 24, and 48 h groups compared to untreated samples. Over the 72-h exposure period, HSP and HSF expression levels were higher upon exposure to bacterial infection, compared to viral infection. In addition, expression of all transcripts was upregulated faster following exposure to *B. thuringiensis* compared to SpliNPV, potentially an earlier immune response to bacterial infection.

Discussion

Insects use their complex defense system to adapt to environmental stress and induce their stress proteins as a universal response against several factors, such as insecticides, microbial agents, extreme temperatures, etc. Expression profiles of the heat-shock proteins in *Spodoptera* species in response to UV, heat shock, cold shock, heavy metals, and virus and bacterial infection have been previously reported for *S. frugiperda* (Landais et al. 2001; Lyupina et al. 2010), *Spodoptera exigua* (Xu et al. 2011; Jiang et al. 2012), and *S. litura* (Shen et al. 2011; Shu et al. 2011). To our knowledge, there is no information about transcriptional regulation of heat shock proteins in *S. littoralis*.

In the present study, we identified two full-length cDNAs encoding *SpliHsp70* and *SpliHsp90*, and a partial cDNA sequence of *SpliHSF* from *S. littoralis*. *SpliHsp70*, *SpliHsp90*, and *SpliHSF* showed high similarity to other known insect *Hsp*s (100% identity) indicating that these large *Hsp*s are highly conserved when compared with small *Hsp* gene families (Denlinger et al. 2001). The main signatures of SpliHsp70, SpliHsp90, and SpliHSF were predicted in the deduced amino acid sequences of Hsp70, Hsp90, and HSF proteins. IDLGTTYS, IFDLGGGTFDVSLLT, and VVLVGGSTRTRKVQS are the typical signature sequences of SpliHsp70, and AEAYLGKT is the putative ATP/GTP binding site motif (Gupta and Singh 1994; Zhang and Denlinger 2010; Zhang et al. 2015), of the deduced amino acid sequence. The conserved EVVD motif found in SpliHsp70 helps to recognize the tetratricopeptide (TPR) domain of the Hsp90/70 organizing protein (HOP) and suggests that SpliHsp70 is a cytosolic homolog (Chen et al. 2015). The EEVD motif is considered to mediate interactions with

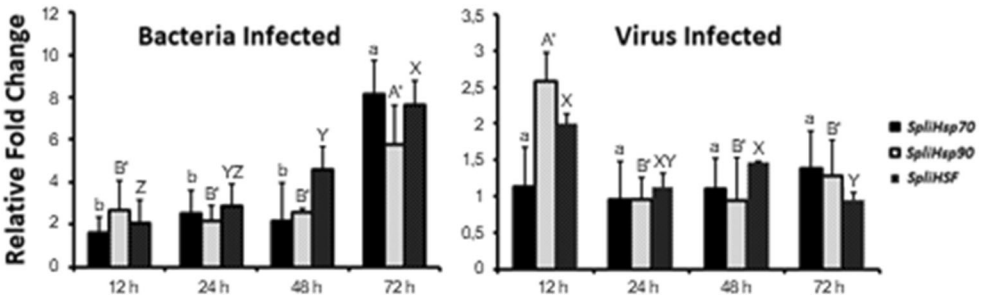

Fig. 8 Expression profiles of *SpliHsp* and *SpliHSF* genes of bacteria (*B. thuringiensis*) and baculovirus (SpliNPV) infected larvae. Relative expression of SpliHSPs was analyzed using β-actin reference gene for qPCR normalization in three infected larvae with *B. thuringiensis* and *SpliNPV* versus non-infected ones

chaperone cofactors and is included in binding with other co-chaperones (Boorstein et al. 1994; Freeman et al. 1995; Johnson et al. 1998). Early studies demonstrated that deletion of the EEVD motif affected ATPase activity (Junprung et al. 2019). The SpliHsp90 protein has five conserved signature sequences including NKEIFLRELISNSSDALDKIR, LGTIAKSGT, IGQFGVGFYSCYLVAD, IKLYVRRVFI, and GVVDSEDLPLNISRE (Gupta 1995). The conserved "MEEVD" pentapeptide located at the C-terminus of SpliHsp90 demonstrates that SpliHsp90 is a cytosolic homolog, similar to SpliHsp70 (Chen et al. 2015; Gupta 1995). The characteristic domains and conserved motifs of SpliHsp90 emphasize that SpliHsp90 is functional and belongs to the Hsp90 family. Evolutionary conserved domains of insect HSFs, as described by Chen et al. (2018), one DNA binding domain (DBD), two hydrophobic heptad repeat domain (HR-A/B and HR-C), and one C-terminal trans-activation domain (CTAD), were found in the SpliHSF sequence. It is considered that the amino-terminal helix-turn-helix DBD is the most conserved functional domain of HSFs throughout evolution (Pirkkala et al. 2001; Chen et al. 2018). While active HSF trimerization formation is facilitated by HR-A/B, inhibition of the formation of trimerization is carried out by HR-C. The last domain, CTAD, includes hydrophobic and acidic residues (Chen et al. 2018). In the present study, the partial amino acid sequence of SpliHSF contains DBD and HR-A/B domains, suggesting that SpliHSF is functional, evolutionarily conserved, and part of the HSF family.

The expression of insect *Hsp*s varies among different developmental stages, depending on species (Xu et al. 2011; Jiang et al. 2012; Sonoda et al. 2006). Shu et al. (2011) analyzed expression levels of *Hsp70* (*Slhsp70*) and *Hsp90* (*Slhsp90*) in *S. litura* from four different life stages including fifth instar larvae, sixth instar larvae, pupae, and adult. The highest levels of *Slhsp70*/*90* expression were detected in adults, *Hsp70* expression in pupae was remarkably lower than in adults, and the lowest expression levels were found in fifth instar larvae. Moreover, the highest level of *Slhsp90* expression was also detected in adults, which was remarkably higher than sixth instar larvae, pupae and the fifth instar larvae. It was also shown that *S. litura Hsp70* was involved in developmental processes, being upregulated around pupation, suggesting that *Hsp70* and *sHsp*s might be the main players in insect midgut metamorphosis (Gu et al. 2012). In our study, the highest expression levels of *SpliHsp70* and *SpliHsp90* were observed in female pupae and female adults respectively. Expression levels of *SpliHsp70* in male pupae and female adults were also high. Among developmental stages, the lowest expression levels of *SpliHsp70*/*90* were detected in egg and in fifth/sixth instar larvae. *SpliHsp70*, *SpliHsp90*, and *SpliHSF* were expressed throughout *S. littoralis* development, suggesting that these genes might be expressed to deal with environmental stress and pupal adult transition (Feder and Hofmann 1999). Inhibition of *HSF* function gives rise to rapid aging and short life span, overexpression of it leads to extended life span in *Caenorhabditis elegans* (Garigan et al. 2002; Hsu et al. 2003; Morley and Morimoto 2004). The higher *SpliHsp* expression levels observed in this study at the beginning of the larval and throughout the pupal stages could imply that these genes are crucial in terms of survival against environmental conditions and regulation of growth and aging in the *Spodoptera* genus. In addition, the highest expression levels of *SpliHsp90* and *SpliHSF* in female adults could indicate important roles in female reproduction, since temperature is one of the main factors that determine the various life activities including development and reproduction.

Although *Hsp* genes are expressed in all insect tissues, they have different expression levels, which are specific to the type of tissue and species. The expression of *SpliHsp70* was detected in all tissues including fat body, hemolymph, nervous system, Malpighian tubules, reproductive tract, and midgut. Similarly, *Hsp70* was specifically expressed in fat body, midgut, epidermis, spermary, and trachea in *S. exigua* (Xu et al. 2011). Although some *Hsp*s are considered as tissue-specific genes (Sharma et al. 2006), *SpliHsp70* is not regulated in a tissue-specific manner for *S. littoralis* and *S. exigua*. It has been stated that *Hsp70* is involved in insect midgut metamorphosis in *S. litura* (Gu et al. 2012). In this study, the highest *SpliHsp70* expression levels were found in midgut, which should be further analyzed using midgut tissues from different developmental time points. On the other hand, the highest *SpliHsp90* expression levels were found in the reproductive tract of *S. littoralis*, a pattern which is consistent with that described in *Bombyx mori* (Saravanakumar et al. 2008). *SpliHsp90* might protect the reproductive tract cells from environmental stress and sustain their regeneration, and it might be involved in reproduction, fecundity, and survival of *S. littoralis*. Protection of the insect immune system against foreign invaders is sustained by humoral and cellular responses and hemocytes, which phagocytize microorganisms (Williams 2007). HSP levels are known to be elevated when non-native proteins exist in the cells (Krebs and Feder 1997). After immune challenge, HSP70 and HSP82 were upregulated in the hemolymph of *D. melanogaster* and *B. mori* (de Morais et al. 2005; Song et al. 2006). In our study, mRNA transcripts were detected in the hemolymph of *S. littoralis*, which might be involved in an immune defence mechanism. In various insects, heat shock proteins have been detected in Malpighian tubules (Shen et al. 2011). Furthermore, after heat shock treatment, expression of different HSPs, including small HSPs, was observed in Malpighian tubules of *D. melanogaster* and *Lucilia cuprina* (Lakhotia and Singh 1989; Tiwari et al. 1995; Lakhotia and Singh 1996; Xu et al. 2011). Malpighian tubules are excretory organs and

have a role in the re-absorption of water or catabolism of the toxic substances, thus the prevalence of SpliHSPs in Malpighian tubules suggests a potential role in heat tolerance and water loss in insects. Heat shock transcription factors play vital roles in the regulation of tolerance to various temperatures. Besides their role in the regulation of longevity in various tissues of *C. elegans* (Morley and Morimoto 2004), it has been reported that *HSF*s have major roles in brain development and regulation of neuronal functions in mammals (Gomez-Pastor et al. 2018). The high expression levels observed in the nervous system suggest that *SpliHSF* might have different roles in *S. littoralis* physiology, although analysis of stage-specific expression in this tissue is required before further conclusions can be drawn.

The *Spodoptera* genus is considered freeze-susceptible, but different species within the genus may present differences in cold tolerance mechanisms (Kim and Kim 1997). Using RNA interference, *Hsp70* was shown to be the predominant contributor to thermotolerance in *S. exigua* (Choi et al. 2014). In fact, a thousand-fold *Hsp70* upregulation has been detected in different insects in response to heat or cold shock (Velazquez et al. 1983; Huang and Kang 2007). In this study, *SpliHsp70*, *SpliHsp90*, and *SpliHSF* demonstrated similar expression profiles against cold shock and heat stress. The results suggest that *S. littoralis* is more tolerant to heat than cold stress and that upregulation of *SpliHsp*s may play a crucial role in thermotolerance of *S. littoralis*.

Ultraviolet C (UV-C) radiation is the most active and shortest wavelength of UV (100–280 nm), which has great impact on organisms and can cause cellular damage (Pattison and Davies 2006). Recent studies implied that *Hsp*s could be prompted by exposure to radiation including UV light (Sang et al. 2012), which might be a significant abiotic factor for insects feeding on plants (Zhao and Jones 2012). In this study, UV-C exposure had no influence on the survival of *S. littoralis*, whereas expression levels of *SpliHsp70*, *SpliHsp90*, and *SpliHSF* were induced by UV-C radiation. *SpliHsp70* displayed the highest increase followed by *SpliHSF*, which is consistent with the reports that HSP70's transcriptional and translational levels were immediately elevated after exposure to UV (Datkhile et al. 2011; Sang et al. 2012; Wang et al. 2014; Pan et al. 2018). However, in *Chouioia cuneae*, the response of *Hsp90* to UV irradiation represented the highest expression level rather than *Hsp70* (Pan et al. 2018). The significant expression levels of *SpliHsp90* and *SpliHSF* occurred after 0.5 h of exposure appear to play a role in protecting cells from damage generated by UV damage. After 0.5 h of UV exposure, expression of *SpliHsp90* decreased significantly, whereas it remained stable for *SpliHSF*. On the other hand, maximum expression of *SpliHsp70* peaked after 1 h, before decreased gradually up to 3 h, a pattern that might be related to the negative feedback on the regulation mechanism of Hsp expression, which helps avoid the accumulation of hazardous substances such as chemicals including heavy metals, ethanol, and other contaminants (Sang et al. 2012). The results implied that these genes could take a part in defense mechanism in *S. littoralis*, which provides cell protection against UV-C light (Simon et al. 1995).

The humoral immune responses of insects generally involve the release of AMPs through three main pathways (Tsakas and Marmaras 2010); the Toll, the Imd, and the Jak-Stat pathways (Rosales 2017). In *Drosophila*, Gram-positive bacteria and fungi mainly induce the Toll signaling pathway, while Gram-negative bacteria activate the Imd pathway and antiviral immunity is mediated by the Jak-Stat pathway (Michel et al. 2001; Dostert et al. 2005; Kemp et al. 2013; Rosales 2017). Insect heat shock proteins greatly influence the functioning of the immune system (Wrońska and Boguś 2020). In this study, we showed that *SpliHsp*s were upregulated in response to challenge with *B. thuringiensis* and SpliNPV, with 10^6 *B. thuringiensis* and 3000 OB of SpliNPV inoculation being enough to induce *SpliHsp*s expression. Furthermore, expression of the different *SpliHsp* genes upon exposure to these microbial pathogens was dependent on time and infection-type. Major protection by three *SpliHsp* genes against *B. thuringiensis* and SpliNPV occurred 72 h and 12 h after treatment, respectively. *SpliHsp* genes could therefore act as an immune response mediator in *S. littoralis*.

In conclusion, we characterized *Hsp* and *HSF* genes from an important lepidopteran pest, *S. littoralis*. The genes analyzed in this study showed different expression profiles in response to elevated temperatures, UV light, and pathogen stress, as well as during different developmental stages and in different tissues. Further work should investigate the effect of the daily and seasonal temperature changes on *SpliHsp*s and *SpliHSF* expression, and the relationship between gene expression and thermal tolerance in wild populations of *S. littoralis*. Understanding these processes might be useful to estimate the distribution and outbreaks of the Egyptian cotton leaf worm. Furthermore, the present results could offer opportunities to analyze detailed function of *Hsp* genes via RNA interference.

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