

RESEARCH REPORT

Gene expression of HSP90 and HSP70 in four silkworm hybrids (*Bombyx mori* L.) in response to severe thermal shockSF Mousavi¹, SH Hosseini Moghaddam², N Ghavi Hossein-Zadeh¹, SZ Mirhosseini²¹Department of Animal Science, Faculty of Agricultural Sciences, University of Guilan, Rasht, Iran²Department of Animal Science and Department of Sericulture, Faculty of Agricultural Sciences, University of Guilan, Rasht, Iran

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Abstract

Usually silkworm egg producers provide several silkworm hybrids with different qualities of productivity and viability traits. This study was conducted to compare the expression of HSP70 and HSP90 genes among different silkworm hybrids. In the fourth day of fifth larval instar of two Japanese maternal parents (103×104 and 107×110) and two Chinese maternal parents (110×107 and 104×103), silkworm larval fat body was sampled from heat shock (45 °C for 35 min) and non-heat shock groups. Sampling was done at 0, 2, 4 and 24 h after heat shock. Gene expression of HSP70 and HSP90 (target genes) were measured by qRT-PCR using RPL27a as reference gene. The prolonged heat shock (8 h at 39 °C) was utilized to examine the thermal tolerance of larvae in comparison with control group (24°C). The results showed both HSP70 and HSP90 have been up-regulated in the treated larvae. The hybrids with Japanese female parents (107×110 and 103×104) were more sensitive than two others; moreover in these hybrids, HSP90 and HSP70 were expressed significantly more than others after heat exposure. The maximum expression was occurred in the time zero, then a decreasing trend was observed over time. Comparison of larval mortality among four hybrids revealed that (103×104) and (110×107) hybrids had the highest and the lowest mortality rate.

Key Words: gene expression; heat shock proteins; real-time PCR; silkworm hybrids; thermotolerance**Introduction**

Sericulture has an important role in village economy in countries like Iran where sustainable rural development is very important. High sensitivity of commercial hybrids to high temperature is the main barrier in its expansion in hot and dry climates. Therefore, factors affecting silkworm tolerance should be considered in the silkworm breeding and introduction of new hybrids (Hosseini Moghaddam, 2005). Moreover, substantial studies have been carried out by researcher to evaluate the effect of heat stress on various organisms due to probability of sudden and rapid climate change when the earth is getting warmer.

When insects expose to extreme temperature, they respond to this temperature change by arising heat shock proteins (HSPs) as molecular chaperones to protect of protein folding process. HSPs family is divided according to their molecular

weight, function and sequence homology into several groups including HSP100, HSP90, HSP70, HSP60, HSP40, small HSP (sHSPs) and HSP10 (Zhao and Jones, 2012).

Among these HSPs, high molecular weight HSP70s as a housekeeping molecule has different responsibilities in insects such as developmental processes and fecundity (Jensen *et al.*, 2014), development (Huang *et al.*, 2009), diapause (Rinehart *et al.*, 2007), longevity (Choi *et al.*, 2014), and metamorphosis (Zheng *et al.*, 2010). HSP70s genes were up-regulated in response to cold or heat stress in a variety of insects, including Coleoptera (Mahroof *et al.*, 2005), Diptera (Gray, 2013) and Lepidoptera (Jiang *et al.*, 2012; Choi *et al.*, 2014; Shen *et al.*, 2014). In a study on flash fly (*Sarcophaga crassipalpis*) in response to hypoxia, HSP90 showed little response, however, HSP70 was the most responsive and increased several hundred fold in the cells (Michaud *et al.*, 2011). Results of previous studies on the *Chilo suppressalis*, *Liriomyza trifolii*, and *Pteromalus puparum* showed that HSP70 can be related to thermotolerance and survivability (Cui *et al.*, 2010; Zheng *et al.*, 2010). In *Drosophila melanogaster*,

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family of HSP70's could not make thermotolerance against severe thermal shock (Bettencourt *et al.*, 2008). The well-defined role of HSPs in acquired thermotolerance in the silkworm and other insects is not clearly known yet (Manjunatha *et al.*, 2010).

HSP90 that forms about 1 - 2 % of cellular proteins is one of the most abundant proteins in the living cells. When insects are under normal conditions, the HSP90 expressed at low levels (Wegele *et al.*, 2004). HSP90 acted as a protective against thermal stress in some insects including *Plutella xylostella* and *Liriomyza huidobrensis*, (Sonoda *et al.*, 2006; Huang and Kang, 2007). Zhang *et al.* (2009) reported that among different tissues of silkworm, sometimes the HSP90 gene expression was up-regulated and sometimes was down-regulated.

The commercial silkworms are usually two-way cross hybrids (direct and reciprocal crosses) which are synthesized by two parents including Japanese (e.g. 103, 107) and Chinese (e.g. 104, 110) strains. According to previous report (Hosseini Moghaddam, 2005) the productivity traits (cocoon weight and cocoon shell weight) of Japanese maternal parents (103x104 and 107x110) were higher than Chinese maternal parents (110x107 and 104x103) and conversely for viability traits. The purpose of this research is the comparison of gene expression of two high molecular weight HSPs in four commercial silkworm hybrids (including two maternal Chinese and two maternal Japanese parents), besides the relationship of gene expression with thermal tolerance of silkworm larvae.

Materials and Methods

Silkworm rearing, thermal treatment and sampling

Two Iranian silkworm hybrids: 110x107 and 104x103 (first parent is female and a Chinese cocoon shape strain) and their reciprocal 103x104 and 107x110 (first parent is female and a Japanese cocoon shape strain) were reared in the Iran Silk Research Center. The 103 and 104 silkworm strains are originally from Japan and the strains 107 and 110 were isolated from a Korean hybrid under FAO/UNDP TCP project (1992-1997).

Fifth instar larvae were transferred to the silkworm Laboratory, Faculty of Agricultural Sciences, University of Guilan to continue rearing and heat shock treatments. Sampling was done from fat body. This tissue is under larval skin that can easily affected by heat exposure. Fat body as a site for energy storage tissue in the silkworm, synthesize many biological proteins like heat shock proteins that these substances are expected to be active in the fat body (Kajiwara *et al.*, 2006).

While all process was performed on ice, the fat body free of muscle was collected in the fourth day of fifth larval instar from both heat shock (45 °C for 35 min) and non-heat shock larvae at 0, 2, 4 and 24 h after heat treatment. Before collection of fat body, the surface of dissected larva was washed by ice-cold insect physiological salt solution (0.7 % NaCl) (Chavadi *et al.*, 2006). Three independent fat body samples were mixed together to minimize variation and to get enough amount of samples. They were store immediately at -80 °C for further use.

RNA Extraction, cDNA synthesis and Real time-PCR

RNA extraction procedure was carried out by TRIzol reagent (Invitrogen, USA) based on the supplier's instructions. The quality and quantity of extracted RNA was evaluated using NanoDrop-2000 spectrophotometer (Thermo Scientific) and the quality of agarose gel electrophoresis bands. DNase I treatment (TAKARA, Japan) was applied to avoid possible contamination of genomic DNA. The cDNA of all samples were made using cDNA synthesis kit (Thermo Scientific) based on manufacturer protocol. Specific primers of HSP70 and HSP90 were designed using online software Primer 3 (Table 1). Real time-PCR was carried out using Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific). Data was normalized using Livak (2001) formula ($2^{-\Delta\Delta Ct}$). A factorial experiment (4x4) with completely randomized design was utilized to evaluate the effects of different hybrids (110x107, 104x103, 103x104, 107x110) and the times after heat shock (0, 2, 4, 24 h), as fixed effects, and their interactions on gene expression data. Statistical analysis of data was performed using the GLM procedure of SAS 9.0. Least squares means were used to identify the main effects.

Table 1 Specific primer sequences for Real-time PCR

Gene	NCBI Reference	Primer Sequence	PCR Product length	Annealing temperature
RPL27a	NM_001044057	Right : TGACAGGTTGTTTGGGGAG Left: CAGACGAGGCTGAAGTATGC	144	60
HSP70	NM_001043931.1	Right: GTGCTTCATGTCTGCTGAA Left: TCGCCTTGAACCCTAACAAAC	100	60
HSP90	NM_001043411.1	Right: AGGCCTTCGAACTTACCTT Left: ATGGCAAGACCCTTGTATCG	103	54.7

Table 2 Least-squares means of HSP70 and HSP90 gene expression in the studied silkworm hybrids at four times after thermal exposure

Hybrids	Times	Expression of HSP70	Expression of HSP90
107×110	0	1549.54 ^a	2183.49 ^a
107×110	2	646.83 ^b	595.96 ^b
107×110	4	1.002 ^e	83.54 ^c
107×110	24	0.01 ^e	1.85 ^c
110×107	0	277.47 ^c	32.78 ^c
110×107	2	7.51 ^e	7.26 ^c
110×107	4	0.06 ^e	0.13 ^c
110×107	24	0.007 ^e	0.04 ^c
103×104	0	1598.42 ^a	278.11 ^a
103×104	2	26.07 ^d	49.90 ^c
103×104	4	21.11 ^d	2.15 ^c
103×104	24	0.09 ^e	0.43 ^c
104×103	0	97.57 ^d	26.99 ^c
104×103	2	85.62 ^d	20.87 ^c
104×103	4	2.28 ^e	10.06 ^c
104×103	24	0.07 ^e	0.01 ^c
SE		27.78 [*]	80.86 [*]

^{a-e} Different superscripts within a column indicate significant differences ($p < 0.0001$)

^{*} Standard error for all least squares means of gene expression

Evaluation of silkworm viability and thermal tolerance

In order to measure thermotolerance of different silkworm hybrids, larvae were exposed to 39 °C for 8 h (9 am to 5 pm) from second to seventh day of fifth instar as long-term heat stress; in other word six days from one day after forth molting to one day before larval cocooning. While larval mortality was recorded daily, mortality rate was measured after two days (LO1) and also at the end of heat shock period (LO2). Because, it is expected that sensitive genotypes will respond more quickly to the initial thermal shock and die; therefore, the first time measurement of mortality rate (LO1) was considered as a thermotolerance criterion. In the same time, larval mortality was recorded for non-heat stress groups. LO2 is not a good criterion to rank the silkworm hybrids; since long-term heat exposure may cause to be killed most of larvae in each tray.

Average larval mortality in response to heat shock was reported as log-transformed values to assure normality. A factorial experiment (4×2) with completely randomized design was utilized to evaluate the effects of different silkworm hybrids (110×107, 104×103, 103×104, 107×110) and heat exposure (with or without thermal shock), as fixed effects, and their interactions on larval mortality (LO1 and LO2). Statistical analysis of data was carried out using the GLM procedure of SAS 9.0. Least squares means were used for identifying the main effects.

Results

The results showed that gene expression of HSP90 and HSP70 in hybrids with Japanese

maternal parents) (103×104 and 107×110) were significantly ($p < 0.0001$) higher than hybrids with Chinese maternal parents (104×103 and 110×107). Table 2 shows that the fluctuation of HSP expression for both genes is almost the same. In other word, the expression of HSP90 was in good accordance with HSP70. Least-squares means of HSP70 and HSP90 gene expression (Table 2) indicated that there was a high significant difference ($p < 0.0001$) in the HSP70 and HSP90 expression between two reciprocal hybrids (110×107 vs. 107×110 and 103×104 vs. 104×103) immediately after heat exposure (Fig. 1).

Expression of both genes immediately after heat shock (time zero) was significantly higher than other times ($p < 0.0001$) in all genotypes, afterwards, gradually decreased to the lowest level after 24 h (Table 2). In this time down-regulation was observed in almost all genotypes, in fact, HSP70 and HSP90 expression reduced over time. Four h after thermal exposure, while HSP70 expression in the three hybrids declined but still 103×104 had higher expression. The hybrid 104×103 had significantly lower expression among hybrids ($p < 0.0001$) which proposed that this hybrid need less HSP70 and HSP90 in thermal shock conditions.

In another experiment a prolonged heat shock (8 h at 39 °C for six days) was implemented to examine the thermotolerance of larvae. The mortality rate (in %) was considered as a measure of thermotolerance. Mortality of all treatment groups was higher than control groups. The hybrid 110×107 and 103×104 had the lowest and the highest mortality rates, respectively. In fact, 103×104 as a sensitive hybrid had the highest losses and 110×107 hybrid as a resistant hybrid had the lowest

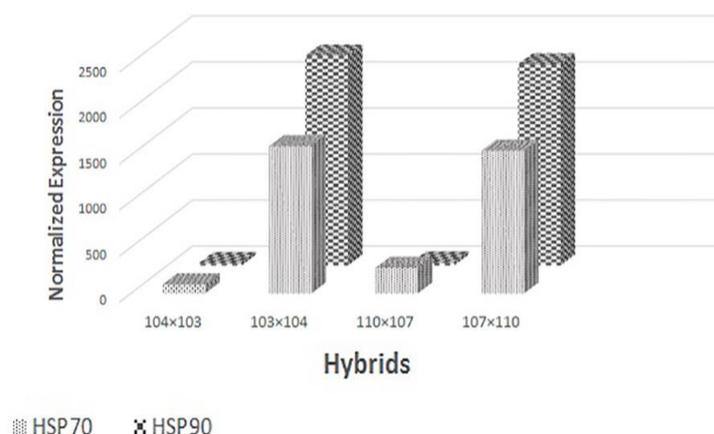


Fig. 1 HSP70 and HSP90 gene expression immediately after heat exposure (time zero) in the studied silkworm hybrids

losses (Fig. 2). The same result was obtained control group; however differences was not significant (Table 3). 110x107 and 107x110, two silkworm hybrids that their parents were originally from Korea, were more resistant than hybrids 103x104 and 104x103 with Japanese parents.

Among four genotypes, the sensitive hybrids (103x104 and 107x110) had the highest gene expression immediately after heat exposure (zero time) implying that these genotypes need more HSP70 and HSP90 under severe thermal conditions. In fact, hybrids with Japanese maternal parents (107x110 and 103x104) were sensitive and hybrids with Chinese maternal parents (110x107 and 104x103) were relatively resistant. Therefore, sensitive hybrids immediately after heat exposure need both HSP70 and HSP90 proteins to reduce thermal effects.

Discussion

A large difference was observed between the genotypes in terms of HSP expression. Generally, the results showed that the hybrids with Japanese maternal parents had more HSPs expression than Chinese maternal parents. There are some reports

on genetic differences of HSP70 or HSP90 among silkworm genotypes such as comparison of Nistari and Jingsong (Hosseini moghaddam *et al.*, 2008), Nistari and NB4D2 (Velu *et al.*, 2008), C. Nichi, Pure Mysore and NB4D2 strains (Joy and Gopinathan, 1995) and Pure Mysore and NB4D2 (Sosalegowda *et al.*, 2010). Velu *et al.* (2008) reported little differences in HSP70 expression between two resistant and sensitive strains on agarose gel (semi-quantitative) after a mild heat shock (41 °C for one hour) but Li *et al.* (2011) using qRT-PCR method showed that expression of HSP70 in Jingsong (a thermosensitive commercial strains) was significantly more than Nistari breed (a thermotolerant multivoltine breed in tropical region). It means that thermo-sensitive breed induced strongly HSP70 mRNA after heat shock treatments. This result was consistent to our study which among four genotypes, the sensitive hybrids (103x104 and 107x110), as Japanese maternal parents, had the highest gene expression. Garbuz *et al.* (2002) reported that among *Drosophila* species and strains originating from different climatic zones, some of species and strains exhibited positive correlation between HSP70 expression and thermotolerance and for others negative correlation.

Table 3 Average larval mortality in the four silkworm hybrids for both thermal treatment and control groups

Hybrids	LO 1*	LO 2
107 x110 (Treatment)	0.70 ± 0.07 ^c	1.84 ± 0.02 ^a
107x110 (Control)	0.13 ± 0.02 ^d	0.93 ± 0.01 ^d
110 x107 (Treatment)	0.50 ± 0.08 ^{cd}	1.66 ± 0.01 ^b
110x107 (Control)	0.09 ^d	0.57 ± 0.005 ^e
103x104 (Treatment)	1.66 ± 0.07 ^a	2.02 ± 0.003 ^a
103x104 (Control)	0.34 ± 0.02 ^d	1.23 ± 0.07 ^c
104 x103 (Treatment)	1.19 ± 0.03 ^b	1.91 ± 0.005 ^a
104 x103 (Control)	0.09 ^d	0.7 ± 0.04 ^d

^{a-e} Different superscripts within a column indicate significant differences ($p < 0.0001$)

* Average larval mortality is log-transformed values

(LO1: First period of measuring mortality; LO2: Second period of measuring mortality)

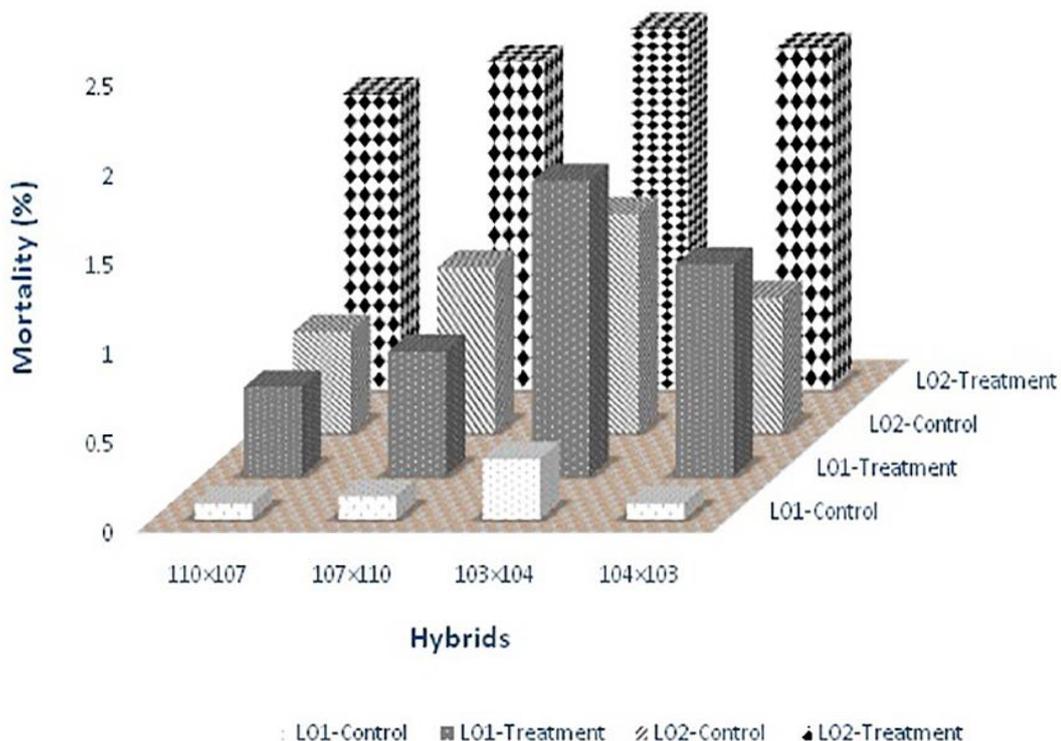


Fig. 2 Larval mortality percentage by treatment and control groups in the studied silkworm hybrids (LO1: First period of measuring mortality; LO2: Second period of measuring mortality; Average larval mortality is log-transformed values)

In the current study HSP70 and HSP90 expression was up-regulated in the fat body of silkworm larvae after severe (45 °C) but non-lethal heat shock. Li *et al.* (2011) observed higher expression of HSP70 in fat body rather than testis and ovary in a thermosensitive strain of silkworm. Velu *et al.* (2008) reported mid gut and fat body tissues had higher HSP70 expression than the cuticle and silk gland tissues. Keshan *et al.* (2014) studied HSP90 expression in various tissues of silkworm and indicated that a higher HSP90 expression in all examined larval tissues after mild (39 °C) and mild to severe (42 °C) heat treatments. The highest expression was observed just immediately after heat exposure (time zero) in all silkworm hybrids. To our knowledge, this is the first study where the differences of HSP gene expression were evaluated for reciprocal crosses in the silkworm.

In the silkworm, high thermotolerance in fifth instar larvae reflects its adaptation to high temperature (Manjunatha *et al.*, 2010). A large difference was also observed between the genotypes in terms of heat tolerance. The hybrids with Japanese maternal parents were more sensitive than the Chinese ones, which was in line with other researches (Vasudha *et al.*, 2006; Firdose and Reddy, 2009). Hsieh *et al.* (1995) by comparing Chinese and Japanese maternal parents demonstrated that Feng, a Chinese strain, was the

most tolerant strain followed by Japanese races, Kuo and J-09, while another Chinese strain, C-54 was most susceptible. In different studies, different genotypes had different reactions to the heat stress. This diversity among silkworm varieties is related to their genetic background and related silkworm breeding plan. Our results showed that this kind of differences could be detected in molecular levels. Further investigations of these differences can help us to understand the mechanisms of protecting cells against environmental stresses and also identify variation of HSPs genes expression among susceptible/ tolerant strains and hybrids of silkworm.

Conclusion

The results of this study showed that both HSP70 and HSP90 have been up-regulated in the treated larvae. The hybrids with Japanese female parents (107x110 and 103x104) were more thermo-sensitive than two others, moreover HSP90 and HSP70 were also significantly expressed in these two hybrids after heat shock. Comparison of larval mortality among four hybrids revealed that the hybrid 103x104 had the highest mortality rate and 110x107 had the lowest mortality rate. High expression of HSPs in thermo-sensitive hybrids showed importance of HSPs in survivability. Generally, it can be concluded that the expression patterns of high molecular weight HSP can

determine the various levels of thermotolerance in the different silkworm genotypes.

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