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Differential Expression of Heat Shock Proteins during Heat Stress in Tropical Tasar Silkworm, *Antheraea mylitta*

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A B S T R A C T

Heat shock proteins (HSPs) usually act as molecular chaperones to prevent proteins from being denatured in response to high temperature and other extreme conditions. These are highly conserved from prokaryotes to eukaryotes. In this study, we analysed the differential expression pattern of *hsp21* and *hsp70* transcript during thermal stress in tropical tasar silkworm. We found expression of *hsp21* and *hsp70* were upregulated ~2.5 and 4 folds respectively in the temperature treated larvae in comparison to the control larvae. This result strongly suggests that the expression of HSP21 and HSP70 proteins are imperative for the tolerance towards the thermal stress.

Introduction

Heat shock proteins (HSPs) are a class of highly conserved proteins that are ubiquitously found in all types of organisms, from prokaryotes to eukaryotes. They are called as HSPs because of their increased expression during exposure to increased temperatures and other stresses. They are molecular chaperones, involved in many cellular functions such as protein folding, transport, maturation and degradation. Under physiological conditions, HSP are expressed in a constitutive manner, and exert fundamental house-keeping and homeostatic functions (Mallouk *et al.*, 1999). Besides these functions HSPs also mediates the adaptive and cytoprotective responses of cells. HSPs can be divided into five families, including HSP100, HSP90, HSP70, HSP60, and sHSP (Bar-Lavan *et al.*, 2016). This classification is mainly based on the molecular weight as well as the homologous relationship of HSPs. All of these families are conserved, except for sHSP family that is more diverse than other four (Li *et al.*, 2009). sHSPs have been widely investigated in various insect species including *Bombyx mori* but till now no work has been done in *Antheraea mylitta*. Investigating the production of heat shock

proteins by tasar silkworms in response to a heat shock might provide an insight into the cytoprotective aspect of their tolerance of thermal stress. The information obtained is also likely to provide a clue to the performance of a selection line in the field and assist in the selection of more thermo-tolerant lines.

A. mylitta, a Lepidopteran insect of the Saturniidae family produces tasar silk of commercial importance. It feeds mainly on the tender leaves of *Terminalia arjuna*, *T. tomentosa*, and *Shorea robusta* (Jolly *et al.,* 1974; Peigler, 1993, 1994). The tasar cocoons are also reported to be the largest among all the silk-producing insects in the world (Akai, 2000). In this study, for the first time we are reporting differential expression of HSPs during heat stress in Tropical Tasar Silkworm, *A. mylitta*.

Materials and Methods

Collection of tissues

Fifth instar larvae of temperature treated and control group of tropical tasar silkworm were collected from rearing field of Central Tasar Research and Training Institute, Ranchi. Ten temperature treated larvae were gradually exposed to high temperature, 45^{o}C/4 h for three days and ten larvae of control group were kept at room temperature. After the third day of exposure, two individuals were collected randomly. Instantly, collected tissues were processed for RNA extraction.

Extraction of RNA from silkworm tissue

Tissue samples of 100 mg were homogenized using liquid nitrogen.1ml of TRIzol™ Reagent per 100 mg of tissue was added and further homogenized using mortar and pestle. After proper homogenization, lysate was centrifuged at 12,000 × g at 4°C for five minutes. Clear supernatant was transferred to a new tube and incubated for 5 min to permit complete dissociation of the nucleoprotein complex. 0.2 ml of Chloroform was added and incubated for 5 min. Sample was centrifuged for 15 min at 12,000 rpm/4^{o}C. Aqueous phase containing the RNA was transferred to a new tube without disturbing the interphase or organic layer. 500µl of Isopropanol was added to the aqueous phase and incubated for 10 min. After incubation, sample was centrifuged at 12,000 rpm for 10 min at 4^{o}C. Total RNA was precipitated which forms a white gel like pellet at the bottom of the tube. Supernatant was discarded and pellet was resuspended in 75% ethanol. Sample was vortexed briefly and centrifuged at 7500 x g for 5 min at 4^{o}C. Supernatant was discarded and RNA pellet was air dried for 5 – 10 min (Chomczynski, 1993). Final pellet was resuspended in 20-50 µl of 0.1 mM EDTA solution and stored in -80^{o}C for further reaction.

Purification of extracted RNA

Sample volume was made upto 500 µl and it was transferred to a Spin Cartridge with a Collection Tube. Cartridge with sample was centrifuged at 12,000 rpm for 1 min at room temperature. The flow-through was discarded and reinserted the Spin Cartridge into the same Collection Tube. Wash Buffer I of 700 µL was added to the Spin Cartridge. Centrifuge was performed at 12,000 rpm for 1 min at room temperature. Discard the flow-through and the Collection Tube. Insert the Spin Cartridge into a new Collection Tube. Added 500 µL of Wash Buffer II with ethanol to the spin cartridge and centrifuged at 12,000 rpm for 1 min at room temperature. Flow-through was discarded and spin cartridge was inserted into the same collection tube. To dry the membrane, cartridge was centrifuged 12,000 rpm for 1 minute at room temperature. Collection Tube was discarded and spin cartridge was inserted into a recovery tube.

Added 30–100 µL of RNase–Free water to the center of the spin cartridge and incubated at room temperature for 1 minute. Spin Cartridge was centrifuged with the recovery tube for 2 minutes at 12,000 rpm at room temperature (Chomczynski and Sacchi, 1987; Boom *et al.,* 1990). Purified RNA was stored in -80^{o}C for further process.

First strand cDNA synthesis

RNA sample of 1 µg (1 µl), 2 µl of d(T)$_{23}$, 1 µl of 10mM dNTP and 6 µl of nuclease free water were mixed together for the preparation reaction mixture. RNA/primer was denatured for 5 minutes at 65°C and cooled down to ice cold condition. 10X AMV buffer of 2 µl, 1 µl of AMV RT, 0.2 µl RNase inhibitor and 16.8 µl of nuclease free water were mixed together in the same vial and incubated at 42^{o}C for one hour. After cDNA synthesis, enzyme was inactivated at 80^{o}C for 5 min. After reverse transcription, cDNA was stored at -20^{o}C.

Expression analysis of hsp21 and hsp70 genes

Totally two pairs of HSP21 primers (Hsp21A-F1, Hsp21A-R1, Hsp21A-F2 and Hsp21A-R2) and two pairs of HSP70 primers (Hsp70A-F1, Hsp70A-R1, Hsp70A-F2 and Hsp70A-R2) were designed based on the conserved regions of *hsp21*and *hsp70* gene specific transcript sequences in NCBI. The cDNA synthesized using the 1µg of total RNA from temperature treated and control group of *A. mylitta* were used as a PCR template. The PCR program included an initial denaturation at 94 °C for 2 min followed by 30 cycles of denaturation at 94°C for 30s, annealing at 58°C for 45s, and elongation at 72°C for 90s, followed by extension at 72 °C for 10 min. The amplified products were electrophoresed in agarose gel. Differential expression of *hsp21* and *hsp70*transcripts between two groups were analysed based on the intensity of bands observed in agarose gel over UV transilluminator using imageJ.

Results and Discussion

Tissue collection from silkworm

The physiological response of fifth instar larvae of *A. mylitta* have been studied in response to thermal stress. The differential expression of transcript coding for HSP21 and HSP70 in the tissues reflects tissue specific changes in different developmental stages and in response to the imposed thermal stress. The insects living under different environmental conditions in which the intensity of dominant factors and their combination vary to different degrees causing reversible or irreversible changes in the metabolism of insects. These changes cause survival or death of an organism. In this study, fifth instar larvae were gradually exposed to 45^{o}C/4 h for three days. During the exposure, mortality and dehydration stress were not observed in the silkworm. After the third day of exposure, tissue samples were collected immediately after temperature stress and processed instantly for RNA extraction.

Expression of hsp21 and hsp70 transcript after heat shock

Total RNA was extracted using Trizol from the collective tissues after thermal stress given to fifth instar larvae and it was purified through affinity coloumn. Extracted RNA was confirmed by formaldehyde agarose gel electrophoresis through the presence of 18s and 28s ribosomal RNA (Fig. 1).

The mRNA sequences of *hsp21* and *hsp70* genes were retrieved from NCBI GenBank JQ708200 and GU945198 respectively. The primers were designed for the specific quantification of *hsp21*and *hsp70* expression during heat stress (Table 1).

Figure.1 Agarose gel electrophoresis of RNA extracted from fifth instar of *A. mylitta* larvae. M, marker; C, control; TT, Temperature Treated

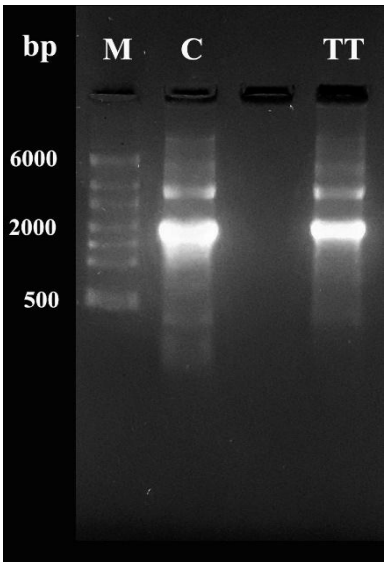

Figure.2 Agarose gel electrophoresis based quantification of heat shock protein transcript expression in temperature treated fifth instar *A. mylitta* larvae. (A) Expression analysis of *hsp21* transcript with temperature treated (TT) and control (C) cDNA templates. (B) Expression analysis of *hsp70* transcript. M is a 1kb DNA in (A) and 300bp DNA ladder in (B) used as a molecular weight standard. Graphs of relative band intensity of the amplified transcripts for each gels

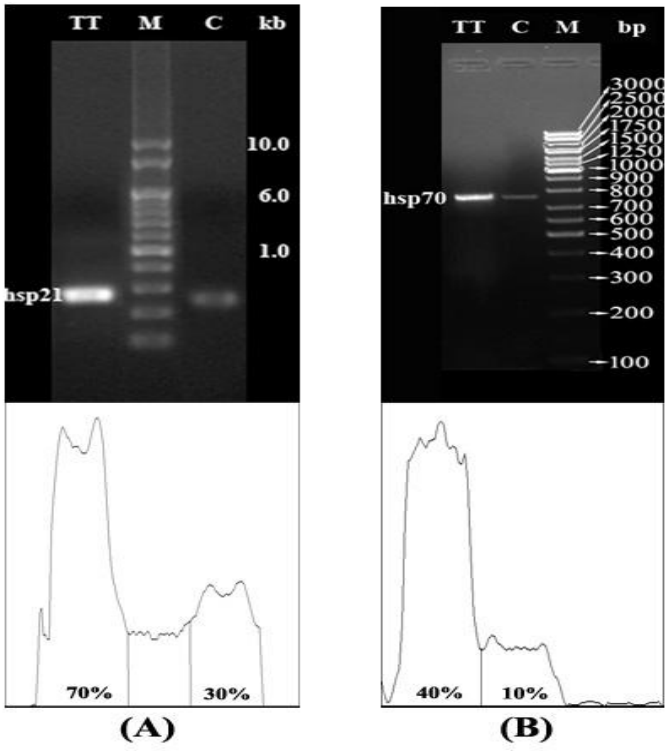

Table.1 Description of *hsp21* and *hsp70* gene and its specific primers

Gene	Description	Gene Bank Accession No.	Sequence (5′-3′)	Location	RT-PCR product size (bp)
hsp21	Heat shock protein 21kDa	JQ708200	A-F1: CGAGGAGAAGAAAGATGAAC A-R1: CTTCTCAGTTTCTTCGGTTT	402 – 421 636 – 617	234
			B-F2: CGTTGACACCTGAAGATTTA B-R2:TTAATGCTTGATCCGACATC	179 – 198 296 - 277	117
Hsp70	Heat shock protein 70kDa	GU945198	A-F1: CAGCTATTGGAATTGATCTCGGT A-R1: GGATTCATTCGCAAGTCCTTCTT	005 – 027 764 – 742	759
			B-F2: GGGACAGATTCACGACGTTG B-R2: TCATCACTCCACCAGCTGTT	978 – 997 1225 - 1206	247

Prior to performing expression analysis of *hsp21* and *hsp70*, we optimized the primers annealing temperature. Among four pairs of primers, Hsp21A-F1 and Hsp21A-R1 showed amplification for *hsp21* and *hsp70* transcript was amplified with Hsp70A-F1 and Hsp70A-R1. The designed primers amplified the partial *hsp21* and *hsp70* transcript of length 230 and 760bp. The *hsp21* and *hsp70* mRNA expression was observed in both high temperature treated and control larvae but the expression of *hsp2*1 and *hsp70* were found to be upregulated ~2.5 and 4 folds respectively in the temperature treated larvae (Fig. 2). This strongly suggests that the expression of HSP21 and HSP70 proteins are imperative for the tolerance towards the thermal stress.

Tolerance of *B. mori* of high temperatures is influenced by both environmental and genetic factors (Kumar *et al.,* 2011). The better survival of a silkworm strain in the field is governed by molecular mechanisms in their cells. Therefore, the expression of *hsp21* gene in the tissue was up-regulated after heat shock, which indicates their products are possibly used to protect silkworms against heat shock.

In order to study the correlation between transcriptional and translational products of *Hsps* in *B. mori*, Li *et al.,* (2012) exposed 4 day old 5th instar larvae of thermotolerant (Nistari) and thermo sensitive (Jingsong) to a heat shock of 45°C for 35 min and 41°C for 60 min and after recovering for 2 and 4 h the level of expression of *Hsps* were measured. This revealed a higher expression of *Hsp19.9*, *Hsp20.4* and *Hsp21* in the thermo sensitive breed than in the tolerant breed. As in *Locusta migratoria* (Wang and Kang, 2005), the phenotypic variation in the thermotolerance of silkworm is also heritable (Kato *et al.,* 1989) and hence controlled by genetic factors. Further, Li *et al.,* (2012) show that thermotolerance varies with breed, sex, treatment and recovery period in silkworm. Similarly, Heredia-Middleton *et al.,* (2008) report different thermal profiles in the expression of *Hsp70* in three different clonal lines of rainbow trout.

The expression of *hsp21* and *hsp70* were 2.5 and 4.0 folds higher in the temperature treated line than the control line. Previous reports indicate that the increase in mRNA level of *Hsp70* after heat shock of 1.5 to 4-fold is significant (Snutch *et al.,* 1988; Requena *et al.,* 1992; Qin *et al.,* 2003), even though it can vary from 1- to 1000-fold (Lindquist, 1986). Hence, *Hsp70* has a prominent role in heat tolerance; it is a major molecular chaperon involved in protecting organisms from extreme temperatures, by chaperoning unfolded proteins (Parsell and Lindquist, 1993). Besides its protein protecting role under stress, high levels of *Hsp70* are known to protect intact larvae from thermal inactivation by alcohol dehydrogenase and thermal inhibition of feeding (Feder and Krebs, 1998).

Since, breeding continuously aims to produce new breeds with the desired traits, silkworm breeders' aiming to produce a new breed/selection line tolerant of high temperatures can use *hsp21* and *hsp70* gene expression as a marker for the identification of thermo-tolerant lines.

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