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# Expression of the Heat Shock Protein Genes in Response to Thermal Stress in the Silkworm *Bombyx mori*

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**The expression of heat shock protein genes (Hsp 70, Hsp 40, Hsp 20.8 and Hsp 20.4) against thermal stress in silkworm *Bombyx mori* was performed through semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR). Upon exposure of silkworm to two temperature regimes (38°C and 42°C), significant change in the expression of Hsp gene was observed as compared to the control. Hsp 70 and Hsp 40 showed increased expression than the small heat shock protein genes Hsp 20.8 and Hsp 20.4. The Hsp 70 showed increased expression during the recovery period as compared to 1 hr thermal treatments (38°C/1 hr and 42°C/1 hr). Whereas, Hsp 40, Hsp 20.8 and Hsp 20.4 genes showed higher expression level at initial stages that later gradually decrease during recovery period. Tissue specific expression of Hsp 70 showed variation in the level of expression amongst the tissues. The mid gut and fat body tissues showed higher expression than the cuticle and silk gland tissue. The Hsp 70, Hsp 40 gene expression was analyzed in thermotolerant (Nistari) and thermo susceptible silk worm strain (NB4D2) and results showed significant variation in their expression level. The Nistari showed higher expression of Hsp 70 and Hsp 40 genes than the NB4D2. These findings provide a better understanding of cellular protection mechanisms against environmental stress such as heat shock, as these Hsps are involved in an organism thermotolerance.**

**Key words:** *Bombyx mori*, Heat shock protein, Thermotolerance, Gene expression

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## Introduction

The impact of abiotic factors are of vital importance to sericulture industry, because they affect the growth and yield of silkworm. Among the abiotic factors temperature plays a major role on growth and productivity of silkworm, as the silkworm is poikilothermic insect (Benchamin and Jolly 1986). In India, the tropical climate prevails in most of the sericulture belt, where temperature goes beyond the ambient during summer, adversely affecting the silkworm rearing. Although multivoltine silkworms are poor in silk content, they are mostly tolerant to high temperature and diseases, whereas bivoltine are of temperate origin, yield good quality silk and prone to various diseases and high temperature.

The heat shock response was discovered in the *Drosophila* as a model system for elucidation of mechanism of thermotolerance, which eventually led to discovery of heat shock proteins (Hsp), their encoding genes and molecular chaperone (Rittosa 1962; Rittossa 1996). Heat shock proteins were expressed nearly by all organisms in response to heat and other stresses, which promote stress tolerance by functioning as molecular chaperones (Lindquist 1993). Enormous studies witnessed both at the biochemical level and in the demonstration of the heat shock protein are responsible for a large component of organism thermotolerance (Morimoto *et al.*, 1994).

The 70 kDa Hsp family (Hsp 70) is categorized into constitutive and inducible forms which contribute to stress tolerance by increasing the chaperone activity in the cytoplasm (Nollen *et al.*, 1999; Dahlgaard *et al.*, 1998). The inducible form of Hsp 70 (Hsp 70i) has been proposed as a predictor or indicator for thermotolerance at either the cell or animal level (Li and Mak, 1989; Flanagan *et al.*, 1995). A strong correlation has been found between Hsp 70 expression and thermotolerance in diverse species (Feder and Hofmann, 1999). For instance, Hsp 70

**Table 1.** Gene sequence, expected product size, primer sequence, annealing temperature of primers used for semi quantitative RT-PCR analysis of the genes in the thermal treated silkworm *Bombyx mori.*

| Genes | cDNA accession number | 5' → 3' primer sequence | Primer annealing temperature | Product size |
|---|---|---|---|---|
| Hsp 70 | DQ311189.1 | FP-GAACACACTCGCTGCACATC | 58°C | 400 bp |
| | | RP-GAGGAGTGCCCAAGATCGAC | | |
| Hsp 40 | AB206400 | FP-TCGGACGATGACATCAAGAA | 54°C | 520 bp |
| | | RP-CCCGGGCGATATCTTCTAAT | | |
| Hsp 20.4 | AF315318 | FP-TTTTGGCCTTGCCTTAACAC | 57°C | 453 bp |
| | | RP-TTCGCTCTGGTCCTTGATCT | | |
| Hsp 20.8 | AF315317 | FP-CTAACCCCGAACGACATGCT | 54°C | 216 bp |
| | | RP-GATGTACCCATCGGCAGTCT | | |

improves thermotolerance in both embryos (Welte *et al.*, 1993) and larvae (Feder, 1996) of *Drosophila melanogaster*, and natural variation of Hsp70 expression in larvae correlates with thermotolerance (Feder and Krebs, 1997). Hsp 40 work as co chaperone for Hsp 70 and approximately 70-amino-acid residue-long J domain is the defining signature sequence of the Hsp 40 family of molecular chaperones that function via protein-protein interaction to coordinate ATP hydrolysis and substrate selection of the Hsp 70 chaperone machine (Kelley, 1998; Mayer *et al.*, 2001). Small heat shock proteins (sHsps) were associated with nuclei, cytoskeleton and membranes and as molecular chaperones. They bind partially denatured proteins, there by preventing irreversible protein aggregation during stress (Sun and Mac Rae 2005).

The effects of temperature on survival and on physiological changes have been studied with silkworm (He and Oshiki 1984; Lohmann and Riddiford 1992; Omana and Gopinathan 1995). They express heat shock proteins in response to heat shock and produce three group proteins similar to that of other insect (Lohmann and Riddiford 1992; Omana and Gopinathan 1995) and Vasudha *et al.*, 2006 studied the differential display of heat shock protein and impact of heat shock on commercial traits of silkworm *B. mori*. The present study was formulated with the objective to study the tissue specific expression of heat shock protein genes in thermotolerant and thermo susceptible silkworm strains.

## Materials and Methods

### Insects

Silkworm strains showing difference in the traits and thermotolerance status were maintained at CSGRC Hosur, Tamil Nadu India. The *B. mori* multivoltine silkworm strains Pure Mysore & Nistari and bivoltine strain NB4D2 were selected for this study. They were reared and maintained in a standard condition of 25±2°C temperature and 75±3% relative humidity (Krishnaswamy 1978).

### Identification of heat shock protein genes and primer synthesis

The heat shock protein genes cDNA sequences were retrived from NCBI nucleotide database (http://www.ncbi.nlm.nih.gov). The cDNA of Hsp genes viz., Hsp 70, Hsp 40, Hsp 20.8 and Hsp 20.4 were have been already cloned and their sequences were deposited in the database. The up and down gene specific primers were designed for cDNA of Hsp genes in the *B.mori* using the software programme of primer3 (http:// frodo.wi.mit.edu/cgibin/primer3). Based on the software programme, the primer binding site and the PCR product size were determined (Table 1)

### Heat shock and RNA isolation

The larvae of fifth instar 3rd day were exposed to 38±1°C for 1 hrs and 42±1°C 1 hr and larvae were recovered 2 hrs at room temperature and simultaneously, control larvae were kept at room temperature. The treated larvae and control larvae were dissected separately and the silk gland, cuticle, fat body and mid gut tissues were frozen in the liquid nitrogen and stored at −80°C until use. Total RNA of tissues was prepared by TRIzol extraction (Invitrogen). Tissues were homogenized in 1 ml TRIzol reagent and centrifuged (12000Xg for 15 min at 4°C). The upper layer was transferred to fresh microfuge tube and 0·2 ml chloroform was added and samples were incubated for 5 min at room temperature. After centrifugation (12 000Xg for 15 min at 4°C), total RNAs were precipitated from the aqueous upper phase using 500 µl isopropanol and then resuspended in 50 µl RNase free water and stored at −80°C. Extracted amounts of RNA were determined by measuring absorbance at 260 nm.

**cDNA preparation**

The first strand cDNA was synthesized by the using 2 μg of total RNA. Then the RNA was treated with 0.5 μl of DNase (Sigma) and 0.5 μl of DNase buffer for 15 minutes at room temperature and the reaction was terminated by heating at 75°C for 10 minutes then added 1 μl 10 mM dNTP, oligo $(dT)_{18}$ (Bangalore Genei) and incubated at 65°C for 5 min. Finally 1X reverse transcriptase buffer (4 μl), 5 mM DTT and M-MLV Superscript III reverse transcriptase (Invitrogen) 1 μl added and final volume to 20 μl. The reaction was initially incubated at 50°C for 1 hr and then terminated by heating at 75°C for 10 min according to the manufacture instruction manual.

**RT-PCR of different tissues and Genome PCR**

Reverse transcriptase PCR (RT-PCR) was done in a 25 μl reaction mixture containing 1X PCR buffer, 1.5 mM $MgCl_2$, 200 mM dNTP mix, 0.5 μM primer. Taq polymerase (0.25 units) and 1 μl of cDNA as a template. The template was prepared by the first strand cDNA synthesis method using total RNA (2 μg) from different tissues. The resulting cDNA was amplified PCR using the Hsp 70 (forward primer 5'-gaacacactcgctgcacatc -3' and reverse primers 5'-gaggagtgcccaagatcgac-3'). The reaction was done at 94°C for 2 min, 32 cycles of 94°C for 30s, 58°C for 30s and 72°C for 1 min. PCR products were electrophoresed on an 1.5% agarose gel containing 0.5 μg per milliter of ethidium bromide and image anlysed with a densito quant image analyzer (Biovis, India).

Genomic DNA was isolated and purified from silk moth using modified Phenol: Chloroform: Isoamyl alcohol (Nagaraju and Nagaraja 1995). The quantity of the DNA was analysed through spectrophotometrically. Genome PCR was done by the same method of RT- PCR except that genomic DNA 20 ng was used as template instead of cDNA.

**Semi Quantitative RT PCR analysis of heat shock protein genes**

The heat shock protein genes transcripts were analyzed for control and temperature treated (38°C / 1 hr, 42°C / 1 hr and 2 hrs recovered) tissue samples of silkworm by semi-quantitative RT-PCR. The nucleotide sequence of the forward and reverse primers for Hsp 70 gene were (5'-gaacacactcgctgcacatc-3' and 5'-gaggagtgcccaagatcgac-3'), Hsp 40 gene (5'-tcggacgatgacatcaagaa-3' and 5'-cccgggcgatatcttctaat-3'), Hsp 20.4 gene (5'-ttttggccttgccttaacac-3' and 5'-ttcgctctggtccttgatct-3') and Hsp 20.8 gene (5'-ctaaccccgaacgacatgct-3' and 5'-gatgtacccatcggcagtct-3'). The βactin gene forward and reverse primers (5'-cactgaggctcccctgaac-3' and 5'-ggagtgcgtaccctcgtag-3') (Bangalore Genei) were used as an internal standard. PCR condition consisted 94°C for 3 min followed by a certain number of cycles of 94°C for 30 s, 54-58°C for 30 s (Table 1), 72°C for 2 min and a final extension of 7 min at 72°C. Similarly this method is applied to analyze the expression of Hsp 70 and Hsp 40 genes in thermotolerant and thermo susceptible strains. After PCR, equal volumes of products were loaded and run on a 1.5% agarose gel containing 0.5 μg per milliliter of ethidium bromide. Images of the gel were obtained under ultraviolet exposure, and the intensity of staining was quantified by Biovis Image analysis software. In this experiment, PCR reactions with 28, 32, 36 and 40 cycles were performed, and it was confirmed that less than 32 cycles for Hsp genes and less than 28 cycles for β-actin could avoid the plateau effects. These experiments were carried out in triplicate. Student's t test was performed for statistical analysis.

## Result

**Genomic and RT PCR**

The analysis of Heat shock protein genes of both genomic DNA and RT-PCR showed no difference in their mobilities (Fig. 1). The bands were excised from the gel and purified through gel purifying column (Bangalore Genie). The purified products were sequenced. The nucleotide sequence of genomic PCR and RT-PCR were compared

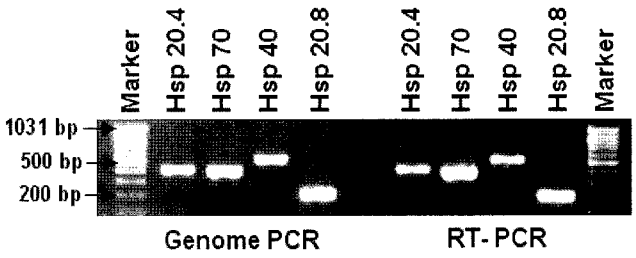


**Fig. 1.** Genomic and RT-PCR of Hsp 20.4, Hsp 70, Hsp 40 and Hsp 20.8 gene of silkworm *Bombyx mori*. The products were resolved in 1.5% agarose gel with standard markers.

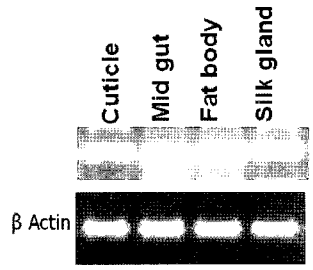


**Fig. 2.** Tissue specific expression of Hsp 70 genes upon heat shock 42°C for 1 hr treated larva of silkworm *Bombyx mori* and β-Actin primer expression as a control. The products were resolved in 1.5% agarose gels.

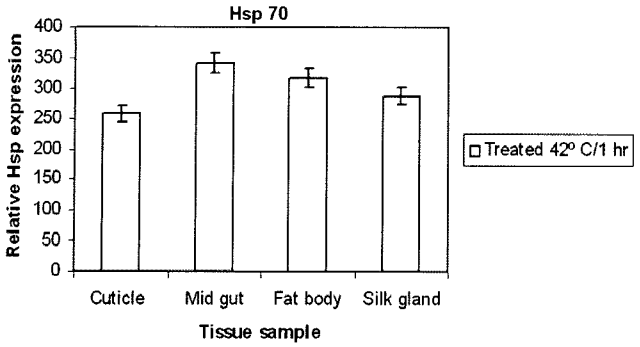


**Fig. 3.** Gene expression of Hsp 70 gene after exposure (42°C for 1 hr) of tissue samples of silkworm *Bombyx mori* L. These experiments were performed in triplicate (mean ± SD), and the bar representing the mean ± SD of the triplicate experiments results.

and there was no sequence variation between them (Data not shown).

The RNAs extracted from treated tissues namely cuticle, silk gland, fat body and mid gut was subjected to RT-PCR to analyze tissue specific variation of Hsp 70 expression of 42°C treated larvae. The tissue specific 42°C for 1 hr showed the apparent increase in the Hsp 70 expression in all tissues. The mid gut and fat body tissue showed increased relative expression than the silk gland and cuticle tissue (Fig. 2 and 3).

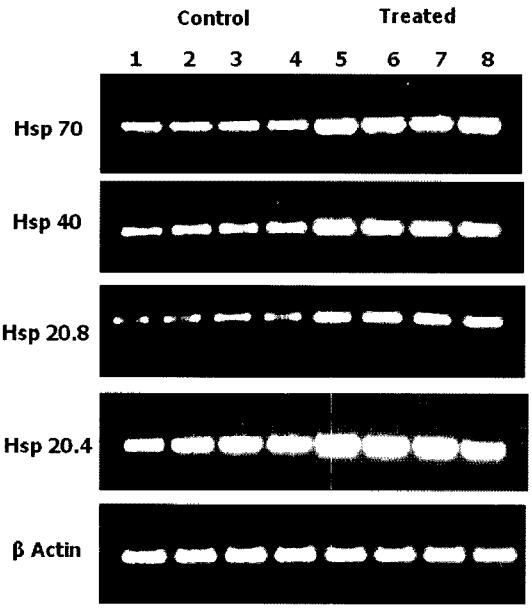


**Fig. 4.** Semi-Quantitative RT-PCR of Heat shock protein genes in silkworm *Bombyx mori* upon heat shock. 1 to 4 were control sample for 5, 6, 7 and 8 sample respectively; 5 was 38°C for 1 hr, 6th was 2 hrs recovery of 38°C / 1 hr treated larvae; 7th was 42°C for 1 hr and 8th was 2 hrs recovery of 42°C / 1 hr treated larvae.

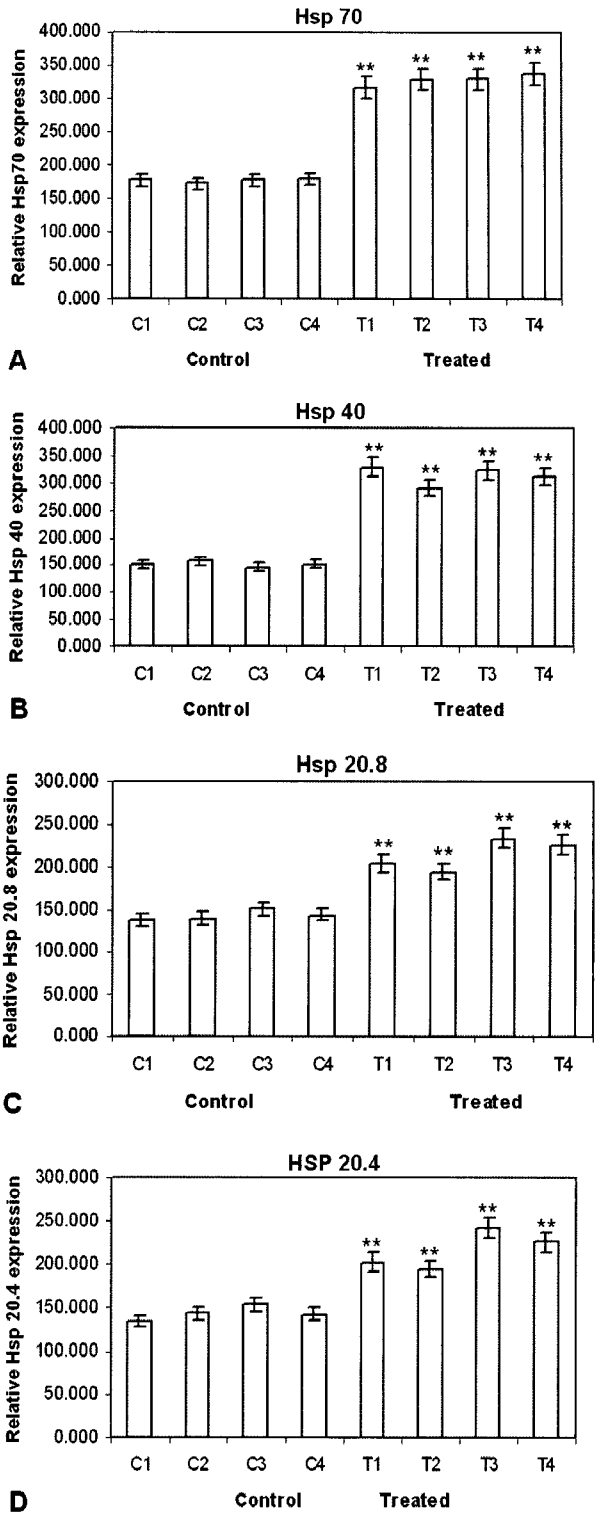


**Fig. 5.** Gene expression of Hsp genes (Hsp 70, Hsp 40, Hsp 20.4 and Hsp 20.8) after exposure to heat shock. 1 to 4 were control sample for 5, 6, 7 and 8th samples respectively; 5 was 38°C for 1 hr, 6th was 2 hrs recovery of 38°C / 1 hr treated larvae; 7th was 42°C for 1 hr and 8th was 2 hrs recovery of 42°C / 1 hr treated larvae. These experiments were performed in triplicate (mean ± SD), and the bar representing the mean ± SD of the triplicate experiments results. The treated were **significantly different from control ($P < 0.01$).

**Expression of heat shock protein genes of different temperature regime**

The effects of different temperature on larvae were examined using treated larvae fat body tissue. Semi-quantitative RT-PCR was performed on selected genes viz., Hsp 70, Hsp 40, Hsp 20.8 and Hsp 20.4, as a control, β actin gene was used (Fig. 4). Changes in expression levels were observed for four genes and showed significant differences in expression level after heat shock. The heat shock protein genes transcripts apparently increased compared to control after heat shock (38°C/1 hr, 38°C/2 hr recoveries, 42°C/1 hr and 42°C/2 hr recoveries). The Hsp 70 and Hsp 40 showed more expression than the Hsp 20.8 and Hsp 20.4 (Fig. 5 A, B, C and D). The increased Hsp 70 gene expression was observed in the treated 38°C/2 hr and 42°C/2 hr recovery sample than the 38°C/1 hr and 42°C/1 hr treatments. In Hsp 40 gene expression increased initially during heat shock at 38°C/1 hr and 42°C/1 hr and decreased in the 38°C/2 hr and 42°C/2 hr recoveries. The similar observation was also noticed in Hsp 20.8 and Hsp 20.4 genes.

**Differential expression of Hsp 70 Hsp 40 in thermotolerant and thermo susceptible race of silkworm**

Semi quantitative RT-PCR analysis of thermotolerant race (Nistari) and thermosusceptible race (NB4D2) showed a significant increase in the mRNA transcript level of Hsp 40 and Hsp 70 in 42°C/1 hr and 42°C/2 hr recovered sample compared to the control (Fig. 6). The increase in expression level of Hsp 70 and Hsp 40 genes was observed in the thermotolerant strain than the thermosusceptible strain. The Hsp 70 expression was increased in the recovery period than the initial temperature treatment of 42°C for 1 hr (Fig. 7A). The Hsp 40 gene expression was initially increased and later decreased during the period of recovery (Fig. 7B).

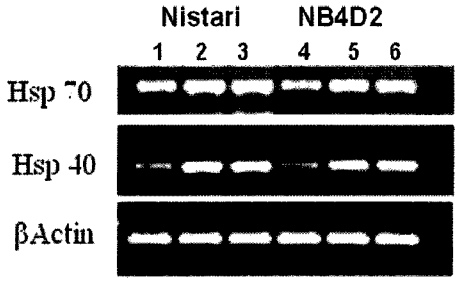


**Fig. 6.** Semi-Quantitative RT-PCR of Hsp 70, Hsp 40 and β actin genes of thermotolerant (Nistari) and thermosusceptible strain (NB4D2) in silkworm *Bombyx mori* upon heat shock. 1&4 were Control; 2&5 were 42°C for 1 hr treated; 3&6 were 2 hrs recoveries of 42°C/1 hr treated larvae of tolerant (Nistari) and Susceptible (NB4D2) races respectively.

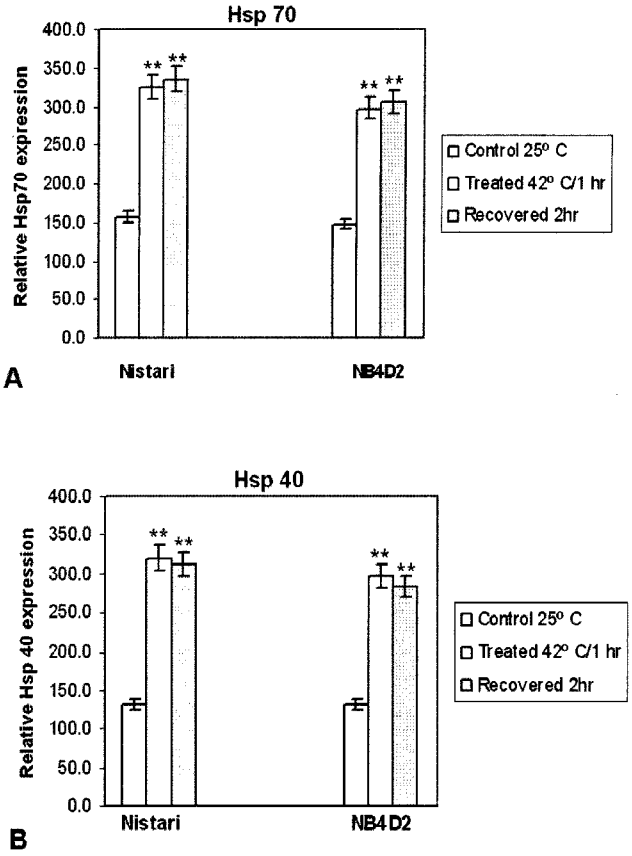


**Fig. 7.** Gene expression of Hsp 70 and Hsp 40 genes after exposure (42°C for 1 hr and 42°C/1 hr treated samples were recovered at 2 hrs) of tissue samples of thermo tolerant and susceptible strains in silkworm *Bombyx mori*. These experiments were performed in triplicate (mean±SD), and the bar representing the mean±SD of the triplicate experiments results. The treated were **significantly different from control ($P<0.01$).

## Discussion

The Genome PCR and RT-PCR result infer us that there was no intronic region in the amplified area. Naturally, some of heat shock protein genes were lacking the intron region. The silkworm *B. mori* Hsp 70, Hsp 20.8 and Hsp 20.4 genes were not possessed any intron. Where as Hsp 40 gene possess two exons and one intervening intronic sequence, the primers were designed for first exonic region. The most obvious characteristic of Hsp gene locus is the presence of long exon without any intron. The thermal stress related protein usually lacks splicing mechanism as splicing process is inhibited by the heat shock (Yost and Lindquist 1986).

Tissue specific heat shock protein gene expression was noticed in silkworm strains. The Hsp 70 expression was observed in all treated tissues viz., cuticle, silk gland, fat body and mid gut. The expression of Hsp 70 is significantly higher in the mid gut and fat body, whereas, cuticle

and silk gland showed less expression. Similar observation in variation of Hsp expression in tissues was observed in other insects like *Heliothis armigera* (Singh and Lakhotia 2000).

Analysis of expression pattern of heat shock protein genes in heat shocked, recovered was compared to control larva. The results exhibited more active induction of Hsp 70, Hsp 40 genes as well as small molecular weight Hsp genes viz., Hsp 20.8 and Hsp 20.4. The results infer that all these Hsp genes were involved in the thermal stress tolerance and work as molecular chaperons during the heat shock. These Hsps were protecting the protein during heat denaturation and other stresses. Small Hsps forms oligomeric complex during heat shock and disaggregation of protein complex is prerequisite for efficient chaperone function (Haslbeck *et al.,* 1999).

The comparison of thermotolerant and thermosusceptible strains showed difference in Hsp expression during and after heat shock. The Hsp 70 and Hsp 40 expression were significantly higher in the thermotolerant strain Nistari compared to the thermosusceptible strain NB4D2. The Hsp 70 is an important molecular chaperones and Hsp 40 is a co-chaperone falling in a DNAJ-like protein, which is an essential cofactor of Hsp 70 and in complex with it, is involved in the restoring of protein confirmation after heat shock (Welch, 1990; Morimoto et al 1990). From the result obtained it could be inferred that the up regulation of hsp gene expression has great impact on an organism's thermotolerance. Similar results were observed indicating that the increase in Hsp 70 gene expression is positively correlated with thermotolerance, which has already been demonstrated in lizards and other organisms (Gehring *et al.,* 1995; Ul'masov *et al.,* 1992; Ul'masov *et al.,* 1997). Additionally, the Hsp expressions studies support the fact that the Nistari is a thermotolerant multivoltine and NB4D2 is a thermosusceptible bivoltine silkworm strain. The multivoltine strains are of tropical origin and they are tolerant to high temperature, whereas bivoltine are of temperate origin and vulnerable to high temperature. The understanding of molecular mechanism of adaptability to temperature and stress is useful for sustenance of the sericulture industry in developing silkworm breeds possessing thermotolerance through conventional or molecular breeding technique.

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