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A review on heat shock protein gene expressions and its association with Thermo tolerance in the silkworm of *Bombyx mori* (L)

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ABSTRACT

Studies on thermo tolerance in silkworm genetic resources are imperative to know the degree of thermo tolerance of the silkworm breeds enabling them to cope with varied agro climatic conditions in the tropical regions of the country. The inbuilt mechanism of gene expression of heat shock proteins when the breeds are exposed to higher temperature is not understood properly in *Bombyx mori (L)*. Proteins are involved in resisting the higher temperature effect on the cellular physiology and biochemical synthetic process that get abruptly affected during the heat shock period. Their expression profiles in different silkworm breeds when documented through reproducible molecular markers would favor us in updating the theoretical knowledge on the use of these markers for identification of different thermo tolerant varieties and short listing them from the germplasm stocks. The identified markers, can be used by researchers and breeders to be utilized as dependable markers for identification of hardy silkworm breeds /or races from the silkworm genetic resources.

Keywords: Heat shock proteins, *Bombyx mori (L),* Molecular markers, Expression profiles.

1. Introduction

Silkworm *Bombyx mori* belongs to Lepidoptera and is one of the most economically important insects because of the worldwide importance of silk. Sericulture plays a significant role as an agro based cottage industry. It is being practiced traditionally in China, India, Korea, and many other countries in the world.

The domesticated silkworm, *B. mori* has a long history as an organism of economic importance. The archeological and biogenographical evidence show that silkworm rearing was practiced in China about 2500 B.C. The earliest known silk textiles are almost 5000 years old [40] Corroborating well with the origin of domesticated silk moth *B. mori* (*L*) from its wild relative *B. mandarina* about 4600 years ago [25] Sericulture began probably 45 centuries ago in North China along the bank of the Hwang Ho River, in the 12th century B.C. and spread outside via the smuggling of mulberry seeds and silkworm eggs. Thereafter, the secret of silkworm rearing and silk production spread to the neighboring countries and other parts of the world through Silk Road; leading to the establishment of sericulture industry in many countries.

The silkworm is a holometabolous insect. The life cycle of silkworm is about 50 days with four different stages, egg, larva, pupa and adult moth. The larval stage is the only stage at which food is ingested. The range of food selection of this insect is very narrow and limited to only mulberry leaves (Moraceae, genus *Morus*) and hence, the silkworm is classified as a monophagous insect.

Desirable characters of silkworm have been exploited for commercial and research purpose resulting in careful collection, cataloguing and maintenance. At present *B. mori* (*L*) comprises a large number of ecotypes and synthetic inbred lines, which represent high degrees of divergence with respect to geographic origin, morphological, qualitative and quantitative traits of basic biological and economic interest including body size, silk quality, fecundity, pathogen resistance and heat tolerance. It is estimated that around 3000 silkworm genotypes exhibit diverse origin and are maintained as ecotypes and different inbred lines in tropical and temperate countries [61].

The silkworm strains which are incubated in temperate countries like China, Japan and Korea are bivoltine (two generations per year) with diapausing eggs. Whereas the strains available in tropical countries like India are multi or polyvoltine (more than three generations per year) and are non-diapausing. The tropical strains are hardy, can withstand adverse eco-climatic conditions but produce very little quantity of good quality silk.

Silkworm is one of the most sensitive organisms. Intensive and careful domestication over centuries has apparently deprived the insect of opportunities to acquire susceptibility to both biotic and a biotic factors. Biotic factors *viz.* improper nutrition, photoperiod, humidity, temperature and attack of pathogens like bacteria, fungi and virus cause diseases affecting the growth of silkworms and yield of silk produced.

Among the biotic factors, temperature plays a very important role in the silkworm growth and silk yield. The silkworms, being poikilothermic, are more prone to high temperature. The ambient temperature for rearing silkworm is 22 °C to 27 °C whereas in summer the temperature goes beyond the normal rearing temperature leading to loss. The high temperature affects the feeding ability, digestion, absorption leading to the physiological imbalance and poor health of the larvae, reduces survival rate, cocoon quality and fecundity of the breeds. Therefore, as the environment is dynamic, varying conditions bring about profound changes in the physical and biotic factors governing the expression of commercial characters in the silkworm.

2. Temperature and thermo tolerance

All organisms are strongly affected by their surrounding environment, and the environmental factors play an important part in shaping ecology and evolution of biology systems. Environmental stress is especially important at many levels of biological organization [27, 28]. In this context, environmental stress is regarded as an environmental factor causing a change in biological system, which is potentially injurious [35]'.

For ectoderm species, such as insects, temperature has been recognized as a major environmental factor responsible for species abundance and geographic distribution [44]. Thus the capacity to adapt and tolerate extreme temperature is critical for the persistence of populations. When exposed to extreme temperatures, insects may respond in different ways, they could behaviorally avoid extremes by escaping the adverse conditions, or respond through changes in morphology, life history and physiology [28].

Temperature affects nearly all biological processes, including the structure of proteins, biological membranes and rate of biochemical and physiological reactions [22, 42]. At the molecular level small increase in temperature can affect the rates of chemical reactions and disrupt weak macromolecular bonds (hydrogen, Vander Waals, Hydrophobic interactions). Because, these types of weak bonds are pervasive in metabolic structures (protein conformation, nucleic acid structure, liquid viscosity) the regulation of metabolic activity is highly dependent on temperature [26].

Thermo tolerance is the ability of the organisms to withstand temperature extremes which may otherwise prove lethal. The ability of organisms to tolerate and adjust to varying thermal conditions is a key factor affecting both local habitat selection and geographic distribution [83].

2.1 Effect of high temperature on Silkworm

The growth and silk yield of silkworms have been affected by both biotic and a biotic factors. Among the biotic factors, temperature plays a major role in growth and productivity of silkworm, as the silkworm is a poikilothermic insect [4]. The effect of temperature on silkworm and the quantitative characters of silkworm such as cocoon weight, shell weight, pupal weight, silk weight, filament length, and filament thickness and survival rate of larvae in a known environment are of utmost importance in sericulture [91, 92, 93, 94, 37]. Many of these characters are not only controlled by genes but also influenced by environmental factors such as nutrition, incubation, temperature, photoperiod etc. Environmental factor especially temperature and humidity play a very important role in

the life cycle of silkworm in determining the cocoon characters and existence in a particular zone [71]. Silkworm larvae are affected by rearing at temperature higher than 30 °C [86] and more sensitive to temperature during 4th and 5th stages. Studies related to effect of temperature in the 5th instar larvae of silkworm on the nutritional metabolism, dietary efficiency, digestion and utilization of dietary protein indicated that high temperature could decrease the rate of utilization of protein from mulberry leaves [80, 88] and also resulted in low survival rate due to low feeding activity resulting in the physiological imbalance and poor health of larvae. Exposure to high temperature affects the later developmental stages of silkworm considerably reducing the survival rate, cocoon quality and fecundity of the breeds [68, 69]. The effect of temperature on cocoon and post cocoon characters of *B. mori (L)*. Confirmed that rearing temperature largely influenced the silk productivity. The productivity of silkworm in terms of cocoon crop depends on several factors that operate within and outside the body of silkworm. The maintenance of nutritional conditions and ideal rearing temperature becomes crucial for maximization of productivity [81, 84].

3. Role of heat shock protein for thermal stress

'Heat shock protein' first observed as 'chromosomal puffs' in *Drosophila* salivary glands as a response to increase in temperature and exposure to chemicals (DNP, sodium salicylate, sodium azide [72, 73, 74]. The heat shock response was accompanied by the high level of expression of unique set of heat shock proteins. This research led to the discovery of many related proteins in prokaryotic organisms as well as eukaryotic cells, tissues and whole organisms. The scope of this research is enormous, because of the biochemical properties of the proteins (structure, regulation and function), their roles in a variety of diseases, physiology and ecology of organisms, and their role as potential bioindicators of environmental stress [17, 16].

The heat shock proteins can be classified into groups primarily based on size which can vary from 10 -170 kDa. The six major size classes currently recognized are Hsp 100, Hsp 70, Hsp 60, Hsp 40, and the small heat shock proteins which can range from 10-30 kda [22, 57]. This large class of proteins was originally referred to as the 'Heat shock Proteins' and were discovered within the tissues of *Drosophila melanogaster* subjected to a sub lethal heat shock of 5 °C above normal body temperature.

Studies on Heat shock proteins have generated renewed interest because these proteins are not only involved in cellular protection against stress, but also in essential physiological processes in unstressed cells [2]. Heat shock proteins are highly conserved proteins that are found abundant in different cellular compartments including cytosol, mitochondria, nucleus, nuclease, endoplasmic reticulum (ER), liposomes and the plasma membrane. Many Heat shock proteins are always present in the cell while the expressions of Heat shock proteins are increased by stress. This can be separated into three major categories. Including (1) environmental stress such as heat shock , toxic chemicals and heavy metals (ii) non-metals conditions, including the cell cycle, growth factors, serum stimulation, development, differentiation and activation by certain physiological and disease states including oxidative stress, fever, inflammation , infection, myocardial stress and ischemia, neural degenerative diseases and cancer [58] It is now clear that not all Heat shock proteins are stress-inducible and constitutive isoforms, referred to as molecular chaperons, which bind to stabilize protein that is in a non-native conformation (Table 1). In general they are believed to interact with hydrophobic domain of polypeptide unfolded by the stimuli interaction with these unstable protein conformations prevent the formation of large protein

aggregates and facilitate normal protein folding, membrane translocation, and the degradation and removal of damaged proteins [16]. Non-native protein conformations can be present in a cell for several reasons 1) exposure to protein-denaturing stress.2) protein 'immaturity i.e. the protein or protein complex has not yet been fully synthesized 3) Folded or assembled 4) for ease of translocation i.e. it is easier to move an unfolded protein across a membrane than a fully folded one.

Table 1: Major heat shock proteins, cellular localization and their function [60]

Size (kD)	Major Function	Cellular Localization
27-28	*stabilization of microfilaments Cytokine signal* Transduction	*Cytosol and nucleus*
60	protein assembly	Mitochondria
70-73	Protein folding and translocation	Cytosol, nucleus endoplasmic Reticulum, Mitochondria
90	protein translocation Receptor regulation	Cytosol , nucleus endoplasmic Reticulum
100-104	protein folding	Cytosol

As to the role of stress – induced Heat shock proteins more work is needed to understand the exact mechanism by which these proteins provide protection to the cell in particular at the tissue level [23]. Exposure of cells to a mild thermal stress protects them from further exposure to stronger, otherwise lethal, other than heat, these findings have prompted new attempts at manipulation of the Heat shock proteins response in medicine e.g. the development of drugs for the control and prevention of degenerative disease [57]. Heat shock proteins are considered as potential biomarkers of organisms stress and some applications of Heat shock proteins expression for environmental biomonitoring have indeed been reported [38, 39]. Although heat-induced over expression of Heat shock proteins has been demonstrated in almost all species the characterization of proteins under heat shock response in different organisms may be important for the understanding adaptive mechanism to different environment and its role in animal evolution [78, 15, 29]. The expression of the Heat shock proteins genes regulation differs among organisms, depending greatly upon their history and adaptation capacity [24]. Natural variations in the heat-shock response seem to be correlated with distribution along environment gradients of stress [30]. Threshold induction temperature for Heat shock proteins is positively correlated with ambient environmental temperature [14]. Moreover, it may contribute to the understanding of several other physiological and ecological aspects for the use of Heat shock proteins as biomarkers.

3.1 Heat shock protein 90
Members of the Heat shock protein 90 family are highly conserved and these essential proteins are found in all organisms from bacteria to humans. Examples include the cytosolic form in eukaryotes, Heat shock protein 90, the ER form, Grp94, and the *E. coli* homolog HtpG. The Hsp 90 family is a very highly conserved group displaying higher than 50% identity among eukaryotes and ubiquitously expressed in prokaryotypes to eucaryotypes. Heat shock proteins 90 are highly abundant, amounting 1% of soluble proteins in normal physiological conditions [97, 67]. Members of the

Hsp 90 family are present in the cytosol and nucleus of all eukaryotes examined and are also found in the ER (GPR94) of higher eukaryotes, the Hsp 90 proteins are found in complexes with a variety of proteins in the cytoplasm , including progesterone and glucocorticord receptors , tyrosine kinases, and cytoskeletal elements such as actin and tubulin [62].

The presence of Hsp 90, Hsp 70 and other heat shock proteins is important for protecting the functional domains of the denatured protein. The protection provided by the heat shock protein helps in refolding of denatured protein [3]. The Hsp 90 functions in as folding of non native proteins [96] peptide translocation [6] regulation of nuclear receptors and kinases [31, 67] centrosome structure [42] membrane biogenesis [13] and anti apoptic pathway [63] .Furthermore, effects of Hsp 90 on the buffering and release of genetic variation suggests that they may have an impact on evolutionary processes.

In higher eukaryotes and in yeast, the cytosolic Hsp 90 has been known to interact directly with ligand-dependent transcription factors and cell cyclic regulators [76, 79, 9]. Its interaction with regulatory factors is a prerequisite for such factors to interact properly with ligands and target molecules. In addition to the typically dimeric Hsp 90, the functional complexes usually include other heat shock proteins and associated factors [79]. The impact of Hsp 90 on the morphological differentiation of multicellular organisms was demonstrated recently and a role as molecular capacitor of morphological evolution has been proposed [46]. Moreover, Hsp 90 can also play a more general chaperoning role maintaining the structure of heat stable proteins [79].

3.2 Heat shock protein 70
The Heat shock protein 70 is highly conserved among eukaryotes and prokaryotes [34, 51, 78, 1, 18]. This Heat shock protein 70 exhibiting about 50% sequences identity between representatives of such diverse organisms as *E. coli* (DNA) and eukaryotes (Heat shock protein 70s). Members of this group of proteins in several species encoding proteins related to Heat shock protein 70 that contributes the heat shock protein 70 gene family although structurally related, members of this gene family appear to be functionally distinct [64].

Genes encoding HSP 70s traditionally are divided into two groups. Genes in the first group can be induced quickly under stressful conditions or heat inducible (Heat shock protein), but return to a normal expression level under non-stressful conditions, gene in the second group are not stress –inducible [51] and are generally referred to as being constitutively expressed or as heat shock cognates (HSC).

HSP 70 family members mostly composed of 3 domains. The 44 Kda. N-terminal adenosine triphosphatase (ATPase) domain binds and hydrolyses adenosine triphosphate (ATP).The variable 18-Kda peptide –binding domain interacts with unfolded polypeptides and 10 kda , C –terminal domain bears the highly conserved , EEVd terminals sequences present in all eukaryotic Hsp 70 families [35] additional members of the Hsp 70 family include a mitochondrial form ,Hsp 75 and a glucose –regulated protein known as Grp78 or heavy –chain binding protein (Bip) that is located in the endoplasmic reticulum [51, 35]. The genes for these bonofied Hsp 70 proteins lack introns.

Hsp 70 plays an essential role in protein metabolism under stress conditions. Hsp 70 family members also interact with a number of other proteins, promoting specific chapering functions [96] .Some Hsp 70 proteins are weakly expressed at best, under normal conditions but are induced by heat and other stresses allowing cells to cope with acute stressor insults. It has anti-apoptotic activity [95] and is also involved in protein transport [66] and auto regulation of the heat response [57], with respect to the later, Hsp 70 increases the

rate of Hsf1 deactivation during recovery from stress [5] and thus restrains Hsf-1 mediated transcription. Hsp 70 plays a vital role in cell cycle progression and human STRI1 and Hsp 70 have been reported to activate histone transcription during S-phase [104].

Heat shock proteins 70 have been shown to provide essential functions needed for normal growth [15]. They include the folding of newly synthesized polypeptides, membrane translocation, formation and disassembly of protein complexes and degradation of misfolded proteins under adverse environmental conditions [16, 27, 64, 35, 23, 27] HSP 70 can even increase the cell survival by restoring cellular homeostasis [62].

3.3 Heat shock protein 40
Hsp 40 acts as co-chaperons for Hsp 70 proteins in a wide variety of cellular processes in organisms. The domain in Hsp 40 proteins that is responsible for regulation of Hsp 70 ATPase activity is the J-domain, showing its presence in all Hsp 40 family members. The J domain is about 75 amino acids in length and can be found at various locations within Hsp 40 proteins [9]. The J-domain was first identified in *E. coli* DNA J and contains a conserved HPD tripeptide that represents the signature motif of the Hsp 40 protein family [100]. The N-terminal J-domain is separated from the C-terminal part by glycine/phenylalaninine-rich domain (the G/F-rich domain). The N-terminal J-domain that spans over the first 75 N-terminal amino acids is the most conserved between species. Three amino acids, HPD, at positions 32-34 in the J-domain of Hsp, are highly conserved and essential for the interaction between J-domain –containing proteins and Hsp70 [70].The HPD motif plays a critical role in the regulation of Hsp70 function because mutations in it block the ability of Hsp40s to regulate Hsp70 ATPase activity [87]. A truncated protein restricted to the J-domain alone is defective in stimulating the Hsp 70ATPase activity [55, 33]. For efficient stimulation, the adjacent G/F rich domain is required. However, prokaryotic and eukaryotic mutants carrying only these two domains have reduced efficiencies compared with the wild type proteins. This suggests that parts that of the C terminus are also involved in stimulating Hsp 70 ATPase activities.

The major function of Hsp 40 proteins is to regulate adenosine triphosphate ATP dependent polypeptide binding by Hsp 70 [48, 91, 10, 43]. Hsp 40 with HSp 70 involved in many process ,such as preventing protein aggregation , enhancing the folding newly translated protein and translocation of proteins across organelle membranes [12] .

3.4 Small Heat shock proteins
The small HSPs form a structurally divergent protein family with members present in Archaea, Bacteria, and Eukarya [11, 8]. They are 15-30 kDA in size but with ability to oligomerize into particles of varying monomer number [47] Proteins in this group possess a conserved domain of 90-100 amino acid residues, pia –crystalline signature sequence [85]. This domain is preceded by an N- terminal domain, which is highly variable in size and sequence, and is followed by a short, poorly conserved –terminal extension. Some sHSP genes contain an intron which delineates the N-terminal and pia – crystalline domains [36, 75] with a two domain structure [99, 89]. The C-terminal extension of sHSPs appears relatively unstructured [10] and is known to undergo numerous modifications, including truncation (the amino terminal extension modulates oligomerization, subunit dynamics and substrate binding, whereas the flexible carboxyl terminal extension promotes solubility, chaperoning and oligomerization, the latter by inter subunit linkage [85].

The sHsps protect cells during stress by offering resistance to apoptosis, cytosketel modulation, thermo tolerance, protection against oxidative stress, cell growth [41, 65, 49] and regulation of apoptosis [53] .There is ample evidence that some of them not only prevent denaturation of proteins but assist in their renaturation as well [45, 32]. The ATP-independent chaperone like activity of these proteins is directed towards preventing protein aggregation early in denaturation, rather than the active refolding of compromised proteins [90]. Heat stable esterase proteins were identified in hemolymph and mid tissues of different thermo tolerance breeds *viz.* Nistari, Pure Mysore indicating the role of these protein during thermal stress [82].

4. Heat shock protein transcription
The transcription of the major heat shock genes can be increased over 100-fold upon heat shock and other stresses [19, 50, 59] and is mediated by a heat shock transcription factor (Hsf) Heat shock transcription factor recognizes a target sequence localized in the promoter region of heat –inducible genes , which was first described by Pelham and called "Pelham box" or " Heat –shock" element (HSE)" [64] At normal body temperature, heat shock proteins are expressed at low basal level and maintain the heat shock transcriptional factor (Hsf) in a repressed state. Relief of repression occurs via the titration of the Hsps by the stress-introduced unfolding and denaturation of native proteins [51, 105]. This results in the activation of Hsf, which trimerizes and binds to specific regions in the promoter of Hsf genes. The activation of Hsf genes and the accumulation of Hsf contributes in enhancing cell survival against subsequent protein-denaturing heat shock and in turning off Hsf.

Besides transcriptional regulation, regulatory mechanism at level of RNA processing [101] translation and mRNA stabilization also have great effect on heat shock gene expression [103].

Tissue specific variation in heat shock protein gene expression was noticed in silk worm races. The Hsp70 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. The results indicated sensitivity of different tissues and their variation in expression.

The results exhibited more active induction as Hsp 70, Hsp 40 genes as well as small molecular weight Hsp genes viz., Hsp 20.8, Hsp 20.4 and Hsp 90. The results infer that all these Hsp genes were involved in the thermal stress tolerance and work as molecular chaperons during the heat shock .The Hsp were protecting the protein during heat denaturing and other stresses. Small Hsps forms oligomeric complex is prerequisite for efficient chaperone function [21].

5. Conclusion
Different molecular proteins were observed as key proteins involved in defense mechanism against thermal shock to silkworm races, their expression profiles warranted a further study analyzing the sequence pattern of the genes for understanding the role of Heat shock protein (HSP)

6. Acknowledgement

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