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Analysis of transcripts of heat shock protein genes in silkworm, *Bombyx mori* (Lepidoptera: Bombycidae)

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Abstract. Silkworm is a poikilothermic insect, whose growth and development is significantly affected by high temperatures. The current study aimed to identify bivoltine breeds tolerant of the high temperature conditions that occur in the tropics. Percentage pupation at high temperatures and heat shock responses of silkworms were used as measures of thermotolerance. Thermotolerance of 20 silkworm breeds was assessed by rearing them at 36°C. Based on percentage pupation, three breeds, namely Nistari (multivoltine), SK4C (bivoltine) and CSR2 (bivoltine) were designated tolerant, moderately tolerant and susceptible, respectively. To understand the heat shock responses and the molecular mechanisms underlying thermotolerance, the tissue specific expression profiles of the nine heat shock protein (*Hsp*) genes were determined in the three breeds after a heat shock of 1 h at 36°C and a 2 h recovery period by performing real-time qPCR. The level of expression of *Hsp* genes was significantly increased in heat shocked tissues and gradually decreased during the recovery period. The greatest increase in the expression of *Hsp* genes was recorded in fat body followed by mid gut and silk gland. Of the three breeds, Nistari showed the highest expression of *Hsp* genes and SK4C a moderate expression relative to CSR2. The qPCR results showed that the transcript levels of *sHsp20.4* and *20.1*, and *Hsp70* were increased by 10.3, 9.7 and 2.3 times, respectively, in Nistari compared to CSR2. Similarly the expression of *sHsp20.4* and *20.1*, and *Hsp70* were increased by 3.5, 2.3 and 1.5 times, respectively in SK4C compared to CSR2. The expression levels of *Hsps* during heat shock corresponded to the percentage pupation recorded for the three breeds at a high temperature. It is suggested that the *Hsps* and their levels of expression may play an important role in increasing the survival of silkworm larvae at high temperatures. This study identified SK4C as a bivoltine breed, which is highly tolerant of high temperature measured in terms of percentage pupation (of the bivoltine breeds) and higher levels of expression of *Hsp* genes compared to CSR2. The importance of SK4C as a thermotolerant bivoltine parent for breeding new bivoltine hybrids tolerant of high temperatures is discussed.

INTRODUCTION

Silkworm, *Bombyx mori*, is a domesticated insect recognized for its silk producing ability and as a model system for biological experiments. Continuous domestication has made silkworm sensitive to abiotic and biotic stresses, which can adversely affect the sericultural industry. Among the abiotic factors, temperature is an important determinant of growth and productivity as silkworms are poikilothermic insects (Benchamin & Jolly, 1986). During summer, the temperature in tropical countries like India, where sericulture is important, goes beyond tolerable limits leading to poor growth of silkworm and silk yield. Generally, multivoltine (nondiapausing) silkworms are more tolerant of high temperatures than bivoltine (diapausing) strains, but produce inferior quality silk. On the other hand, bivoltine silkworms produce high quality silk but are less tolerant of high temperatures. Because of the hot climatic conditions prevailing in India, the success and spread of silkworm rearing is dependent on the introduction of F_1 hybrids between native multivoltine strains (high temperature tolerance) as female parents and bivoltine strains (high quality silk) as male parents as they are hardy and can survive and reproduce in fluctuating environmental climatic conditions (Lakshmi et al., 2011). However, cocoon and silk yield, and quality of the silk yarn of such F_1 hybrids is inferior to that of temperate bivoltine silkworm hybrids (Kumar et al., 2001; Singh & Kumar, 2010). Because of these drawbacks, it is important to produce bivoltine breed because of their potential for increasing the production and quality of silk under the tropical conditions prevailing in India. However, attempts to use bivoltine silkworm breeds throughout the sericulture belt of India resulted in serious crop losses, especially in the hot and humid climatic conditions in the tropics (Kumar et al., 2001). Therefore, in India, the challenge is not only to develop stress and disease-resistant breeds but also breeds that produce a large quantity of high quality silk and thrive when the temperatures are high (Chavadi et al., 2006). Based on the survival of silkworm breeds at high temperatures, many Silkworm breeders' selected different bivoltine breeds as parents in order to develop hybrids and double hybrids (Shirota,

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1992; Kumar et al., 2002, 2003, 2004, 2006; Rao et al., 2007; Krishna Rao et al., 2003). These breeds, however, were not widely adopted by the Indian sericulture industry as they did not perform well under field conditions (Singh & Kumar, 2010). For this it is important to develop a bivoltine breed tolerant of the high temperature conditions in the tropics. The performance of any new silkworm strain in the field depends on molecular mechanisms in cells involved in what is referred to as the heat shock response (Howrelia et al., 2011).

The heat shock response is a universal phenomenon in which there is a rapid synthesis of a group of proteins known as heat shock proteins (*Hsps*) in response to thermal stress (Lindquist, 1986). *Hsps* serve as molecular chaperons that assist in the folding and translocation of newly synthesized proteins, as well as in coping with stress-induced denaturation of other proteins (Nover & Scharf, 1997). Minimal temperature for induction of *Hsps*, involvement of different *Hsps* and their relative importance in stress tolerance differs from organism to organism (Parsell & Lindquist, 1993). Cells that are pre-sensitized to sub-lethal temperatures show an increase in tolerance of high temperatures, a phenomena called inducible thermotolerance. There is sufficient evidence that inducible thermotolerance develops due to the accumulation of *Hsps* (Lozovskaya & Evgen'ev, 1984; Lindquist, 1986; Morimoto et al., 1999; Margulis & Guzhova, 2000). Based on their molecular weight, heat shock proteins are categorised as *Hsp100*, *Hsp90*, *Hsp70*, *Hsp60* and low molecular weight proteins (Nover & Scharf, 1997).

Hsp90 is abundant, highly conserved and makes up about 1–2% of the total protein in a normal eukaryotic cell (Lindquist & Craig, 1988). They are constitutively expressed (absence of stress) and able to perform folding of non-native proteins (Welch et al., 1982), peptide translocation (Brugge et al., 1981) and signal transduction (Pandey et al., 2000) with the aid of several different co-chaperones (Pratt & Toft, 2003). The *Hsp90* gene of *B. mori* and *Spodoptera frugiperda* is characterized by a single long exon and is induced at high temperatures in *S. frugiperda* and ubiquitously expressed in several organs or tissues of *B. mori* (Landais et al., 2001).

Hsp70 is characterized by a highly conserved N-terminal ATPase domain functionally coupled to a less conserved C-terminal peptide binding domain (Fink, 1999). There are two categories of *Hsp70*, the constitutive form *heat shock cognate 70* (*Hsc70*), which is expressed under normal physiological conditions and inducible *Hsp70*, which is expressed under various stress conditions. The gene encoding *Hsc70* has an intron, whereas *Hsp70* gene lacks an intron, which is heat inducible (Fink, 1999; Boutet et al., 2003). *Hsc70* assists in folding of newly synthesized polypeptide chains and *Hsp70* assists in the refolding of denatured and misfolded proteins induced by stress (Morimoto, 1998). Members of the *Hsp70* family cooperate with cofactors of the DnaJ/*Hsp40* family in an ATP-dependent manner. In addition to ATP hydrolysis, *Hsp40* co-chaperone is also involved in directing the substrate proteins either to refold, or be degraded to *Hsp70*. Moreover, there is a highly significant correlation between *Hsp70* expression and thermotolerance in *Drosophila melanogaster* (Welte et al., 1993; Feder, 1996), lizards, ants (Ul'masov et al., 1992; Gehring et al., 1995) and many other organisms (Feder & Hofmann, 1999).

Small heat shock proteins (*sHsps*) are 15–30 kDa in weight and occur as a homo-/heteromeric complex made of 2 to 40 subunits (Kappé et al., 2003). They consist of a conserved α-crystallin domain of approximately 90–100 amino acid residues and a conserved β-sheet in their secondary structure. Several subunits of sHSP form a large oligomer with the help of the β-sheet structures, sandwiched in a secondary structure of sHSP (Kim et al., 1998; Montfort et al., 2001). Under thermal stress this stable multimeric structure composed of monomers of sHSPs binds to the proteins and protects them from thermal denaturation through chaperone activity (Lee et al., 1995; Montfort et al., 2001; Nakamoto & Vigh, 2007). Sakano et al. (2006) isolated and characterized six genes encoding *sHsps* in *B. mori*. They also found that all *sHsps* are up regulated after heat shock, except *sHsp21.4*.

One-dimensional gel electrophoresis revealed that in response to heat shock, *B. mori* produces three groups of heat shock proteins, including HSP82, HSP70 and sHSP (Lohmann & Riddiford, 1992). The heat shock responses of the multivoltine silkworm breeds, C.Nichi and Pure Mysore, and the bivoltine breed, NB4D2, as indicated by the heat shock response of different tissues differs in terms of the presence of additional proteins (Joy & Gopinathan, 1995). By exposing larvae to 40°C for 1 h and using O'Farrel's two-dimensional gel electrophoresis, Hsieh et al. (1995) identified 3 heat shock specific protein spots with molecular weights of about 70kD in the thermotolerant silkworm strain Nong (multivoltine), which are absent in the susceptible strain C-54 (bivoltine). Vasudha et al. (2006) report differential expression of heat shock proteins (*Hsps*) in different instars of bivoltine silkworm breeds viz., NB4D2, NP2, KSO1, CSR2 and CSR4, and confirm that the resistance to heat shock increases as larval development proceeds from first instar > second instar > third instar > fourth instar > fifth instar. Moghaddam et al. (2008) studied the proteome of heat tolerant (multivoltine) and heat susceptible (bivoltine) silkworms in response to heat shock using advanced protein analysis techniques, like 2D electrophoresis and mass spectrometry. This study demonstrate that the protein spot intensity of sHSPs is lower in multivoltine than in bivoltine breeds after heat shock treatment at 45°C. Velu et al. (2008) report a higher expression of *Hsp70* and *Hsp40* genes at the tissue level after a heat shock of 1 h at 38°C and 42°C in the thermotolerant breed, Nistari (multivoltine) than the thermo susceptible breed, NB4D2 (bivoltine), using RT-PCR. The expression of *Hsp70* and *Hsp40* genes was highest in the fat body followed by the mid gut and silk gland. Li et al. (2005) analyzed the expression of the small heat shock protein gene *BmHsp* 19.9 in silkworms using RT-PCR and found varying levels of this protein in testis, ovary, silk gland and

pupae. Li et al. (2014) performed digital gene expression analysis on the mid gut of the thermotolerant silkworm variety "932" (bivoltine) and thermosensitive variety "HY" (bivoltine) after exposure to high temperature and suggest that *Hsps* and the levels of their expression may be important in the resistance of silkworms to high temperature stress. Most of the studies on the heat shock response of *B. mori* involve using multivoltine breeds as they are thermotolerant and bivoltine breeds as they are thermo susceptible. Few reports (Vasudha et al., 2006; Li et al., 2014) are available on the heat shock response of bivoltine breeds as thermotolerants.

In the present study, 20 silkworm breeds were reared at a high temperature and based on the percentage that pupated, three breeds viz., Nistari, SK4C and CSR2, were designated tolerant, moderately tolerant and susceptible, respectively. Nistari is multivoltine and of tropical origin, a high percentage of which pupate at high temperatures (Li et al., 2012) and synthesize heat stable esterase (Moorthy et al., 2007a). It is widely reared in North-East India. SK4C is a bivoltine breed, which is widely reared in North-East India. Its female parent is productive bivoltine breed "SK4" and male parent "Cambodge", which is tolerant of both high temperatures and high humidity. Cambodge is multivoltine and originates from Cambodia, has the dominant gene, *Stu* (for sturdiness or robustness), which accounts for its tolerance of high temperatures and humidity (Murakami & Ohtsuki, 1989). SK4C is a near isogenic line of SK4. It was obtained by selecting larvae based on their surviving high temperature (33°C) and humidity (80–90%) and it has an esterase isozyme banding pattern similar to its multivoltine parent (Moorthy et al., 2007b). CSR2 is bivoltine and is widely reared in Southern India. It was bred from Japanese hybrids highly productive in favourable environmental conditions and as a consequence is not tolerant of high temperatures and humidity (Sreekumar et al., 2011). In the present study the tolerance of these three silkworm breeds was measured in terms of percentage pupation and expression of *Hsp* genes at high temperatures. The information generated provides better understanding of the relationship between *Hsps* expression and thermotolerance in the silkworm.

MATERIAL AND METHODS

Rearing at a high temperature

Twenty silkworm breeds, eighteen bivoltine and two multivoltine, were selected based on information on their thermotolerance. Larvae of these breeds were reared from hatching until they reached 2nd day of 5th instar at 25 ± 2°C and 75 ± 3% relative humidity as suggested by Krishnaswami et al. (1978). There were 9 replications of this experiment with 100 larvae per replication. On the 3rd day of 5th instar, the larvae were exposed to 36°C in a SERICATRON (Environment chamber with temperature and humidity control) for 6 h / day until they started to spin a cocoon. The larvae were fed mulberry leaves twice a day and the mature larvae were mounted on plastic mountages for spinning cocoons. Cocoon harvesting was carried out on the 7th day of spinning. Cocoons were deflossed and defective ones were rejected. The number of live and healthy cocoons was used to determine the percentage pupation. Based on this measurement, CSR2, Nistari and SK4C were categorized as susceptible, tolerant and moderately tolerant, respectively (Table 1).

Heat shock and tissue collection

On the 3rd day of 5th instar, CSR2, Nistari and SK4C larvae were separately exposed to 36°C for 1 h and then some of them were allowed to recover for 2 h at 25 ± 2°C. Larvae not subjected to a heat shock were used as controls. The treated (larvae exposed to a heat shock for 1 h at 36°C), recovered (2 h at 25 ± 2°C after heat shock) and control larvae were dissected separately, the silk gland, mid gut and fat body tissues of 5 larvae from each treat-

Table 1. Morphological characters and percentage pupation of twenty silkworm breeds.

Sl. No.	Breed	Larval marking	Cocoon shape	Cocoon colour	Percentage pupation (%)
1	ATR16	Plain	Dumbbell	White	19.22
2	ATR29	Marked	Dumbbell	White	40.44
3	BHR2	Marked	Dumbbell	White	43.89
4	BHR3	Marked	Dumbbell	White	59.67
5	B37	Plain	Oval	White	12.56
6	CSR2	Plain	Oval	White	12.22
7	CSR17	Plain	Oval	White	27.89
8	CSR46	Plain	Oval	White	40.67
9	CSR47	Marked	Dumbbell	White	28.33
10	CSR50	Plain	Oval	White	36.33
11	CSR51	Marked	Dumbbell	White	18.56
12	D6(P)	Marked	Dumbbell	White	28.22
13	D6(P)N	Marked	Dumbbell	White	44.40
14	NN6D	Plain	Oval	White	44.22
15	S-38	Plain	Oval	White	22.67
16	SK3	Plain	Oval	White	32.44
17	SK4	Marked	Dumbbell	White	38.56
18	SK4C	Marked	Dumbbell	White	60.89
19	Nistari*	Marked	Spindle	Yellow	85.11
20	Cambodge*	Plain	Spindle	Yellow	84.00

* multivoltine breeds.

ment were collected and pooled to minimize variation, frozen in liquid nitrogen and stored at −80°C until used.

Total RNA extraction and cDNA preparation

Total RNA was extracted from 100 mg of silk gland, mid gut and fat body using TRIzol reagent (Invitrogen, USA), as per manufacturer's instructions. The frozen tissues of silk gland, mid gut and fat body were separately ground to a fine powder in liquid nitrogen with the immediate addition of TRIzol reagent (1 ml). To assess the quantity and integrity of RNA, the total RNA was denatured in formaldehyde, formamide and electrophoresed on 2.0% agarose gels. The first strand cDNA was synthesized using the DNase treated RNA sample (2 µg), and adding 1 µl oligo $(dT)_{18}$ (0.01 mM) (Eurofin India Pvt Ltd, Bangalore) followed by incubation at 70°C for 3 min. Finally, 1X reverse transcriptase buffer (4 µl), 10 mM dNTP (2 µl), 5mM DTT (2 µl) and M-MLV Superscript III reverse transcriptase (Invitrogen, USA) (0.5 µl) were added to obtain a final volume of 20 µl. The reaction mixture was incubated at 42°C for 60 min and terminated by heating at 75°C for 10 min according to the manufacturer's protocol.

cDNA quantification

The expression of nine *Hsp* genes in different tissue/breed/treated or recovery combinations was analyzed by performing qRT-PCR with reverse transcribed product and gene-specific primers. The total RNA isolated from heat shocked and recovered tissues were calibrated relative to the values recorded for the control larvae. The calibrator was included on each plate. The fluorescent ROX dye (Takara) was used as an internal control of pipetting errors and well-to-well differences. Each sample was tested in triplicate. One µl of first strand cDNA was used in a 20 µl reaction mixture using the specific primers designed for Real time PCR (qPCR). The primer details are listed in Table 2. The reactions were conducted on a STRATAGENE Mx 3005P real time PCR system. The relative expression levels of each gene at different times were normalized using the Ct values obtained for the β-actin amplifications run on the same plate. The mean value ± SD was used for the analysis of relative levels of expression of genes for each tissue/breed/treatment using the ΔΔCt method. A non-template control (NTC) sample was also run to detect any contamination. The two-way analysis of variance (ANOVA) was performed with SPSS software with breeds and tissues as factors for each *Hsp* gene.

Table 2. Primer pairs used for quantitative RT-PCR.

Gene	Accession No.	Primer pairs
Hsp19.9	NM_001043519	TGTACGGCTGAATCTGTGGA
		TTGGATTGGTCCTTCACCTC
Hsp20.1	NM_01043476	GCCAACGATGTCCAGAGATT
		CTGCCTCTCCTCGTGCTTAC
Hsp20.4	AF315318	TTTTGGCCTTGCCTTAACAC
		TTCGCTCTGGTCCTTGATCT
Hsp20.8	AF315317	CTAACCCCGAACGACATGCT
		GATGTACCCATCGGCAGTCT
Hsp21.4	AB 195972	CCGAAATGAGGAAGATGGAA
		GAATGAGCGGCGAGTTTAAG
Hsp23.7	NM_ 01043477	GGACGAGCACGGATACATTT
		CCGGGCCAGTTTTAGTGATA
Hsp40	AB206400	TCGGACGATGACATCAAGAA
		CCCGGGCGATATCTTCTAAT
Hsp70	DQ311189	GAACACACTCGCTGCACATC
		GAGGAGTGCCCAAGATCGAC
Hsp90	AB060275	TTCCCAGTTCATTGGCTACC
		TCTTGCGCTTCTTGTTTTCA

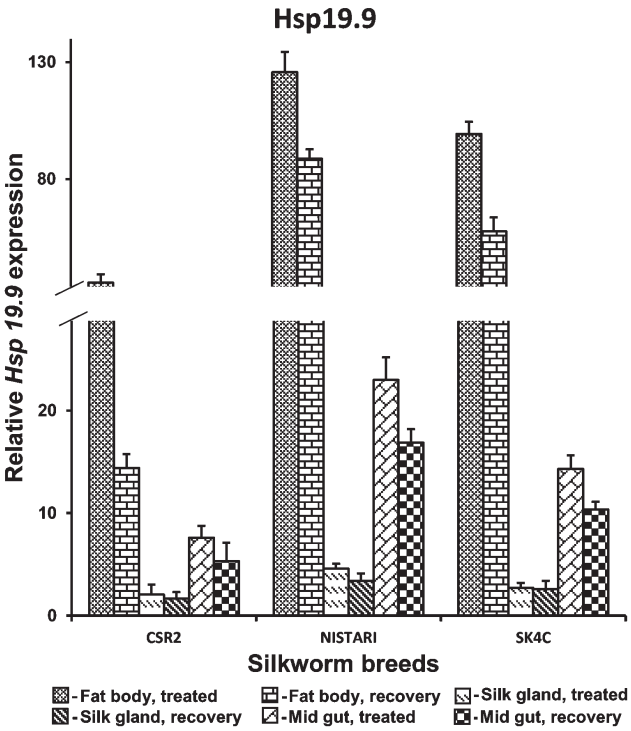

Fig. 1. The relative levels of expression of *Hsp19.9* in different tissue/breed/treated or recovery combinations. This experiment was performed in triplicate and the quantity of *Hsp19.9* mRNA normalized by using the transcript quantity of β-actin in non-heat shocked tissue at 0-hour.

RESULTS

Selection of silkworm breeds

Of the twenty silkworm breeds subjected to a heat shock Nistari was the most tolerant and CSR2 the least tolerant. In addition, among the bivoltine breeds SK4C survived best. The survival of the larvae of Nistari was 1.3 and 6.9 times greater than that of SK4C and CSR2, respectively. Similarly, 4.8 times more larvae of SK4C survived than of CSR2. Thus, CSR2 (12.22%), Nistari (85.11%) and SK4C (60.89%) were categorized as susceptible, tolerant and moderately tolerant of heat shock, respectively. The morphological characters and percentage pupation of these silkworm breeds are presented in Table 1.

Expression of *Hsps* in tissues of the different breeds subjected to a heat shock

The relative mRNA levels of nine *Hsp* genes were quantified using real time qPCR of the fat body, mid gut and silk gland tissues of 3 day old 5th instar larvae of CSR2, Nistari and SK4C breeds after a heat shock of 1 h at 36°C followed by 2 h of recovery at 25 ± 2°C. Non-heat shock samples at 0 h with both genes of interest and β-actin genes were used to calibrate variation in RNA/cDNA extractions. The results indicate that the expression of the *Hsp* gene was not the same in all the tissues. It increased, however, after heat shock and decreased during the recovery period. The high-

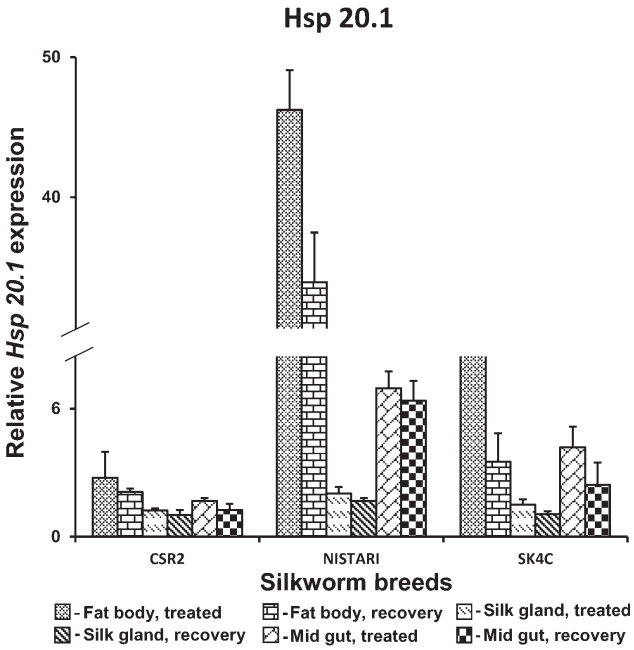

Fig. 2. The relative levels of expression of *Hsp20.1* in different tissue/breed/treated or recovery combinations. This experiment was performed in triplicate and the quantity of *Hsp20.1* mRNA normalized by using the transcript quantity of β-actin in non-heat shocked tissue at 0-hour.

est increase was recorded in the fat body followed by mid gut and silk gland of the three selected silkworm breeds.

The quantity of *Hsp* gene transcript in the different tissues after heat shock was in the order *sHsp20.4* > *Hsp70* > *Hsp40* > *sHsp23.7* > *sHsp20.8* > *sHsp19.9* > *sHsp20.1* > *Hsp90* > *sHsp21.4* (Figs 1–9). Even though the expression of *Hsp* in the different tissues was same, the level of expression differed greatly. For example, the level of expression of *sHsp20.4* and *Hsp70* in the mid gut was 10 times and 4.7 times greater than in the silk gland, and 0.32 and 0.85 times less than in the fat body in Nistari, respectively (Figs 3 and 8). Similar differences were recorded for the fat body, mid gut and silk gland tissues of the selected breeds with respect to each *Hsp* member indicating the expression of *Hsp* genes is tissue specific. Moreover, for the fat body

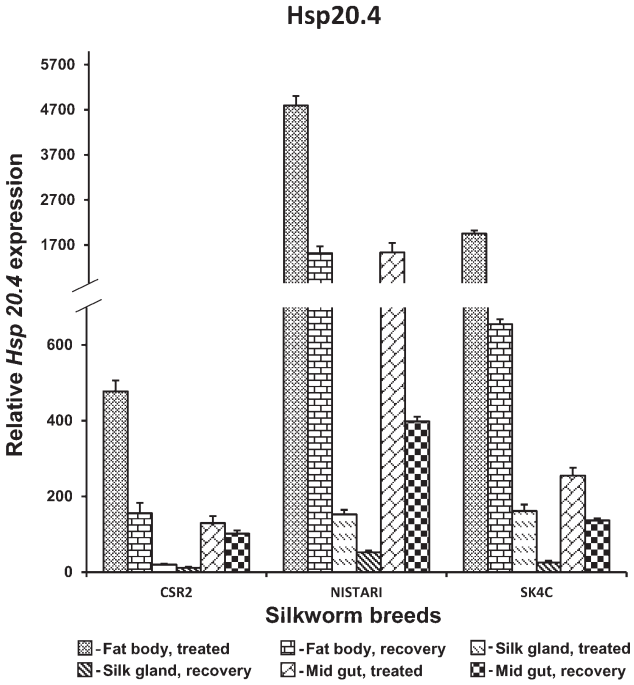

Fig. 3. The relative levels of expression of *Hsp20.4* in different tissue/breed/treated or recovery combinations. This experiment was performed in triplicate and the quantity of *Hsp20.4* mRNA normalized by using the transcript quantity of β-actin in non-heat shocked tissue at 0-hour.

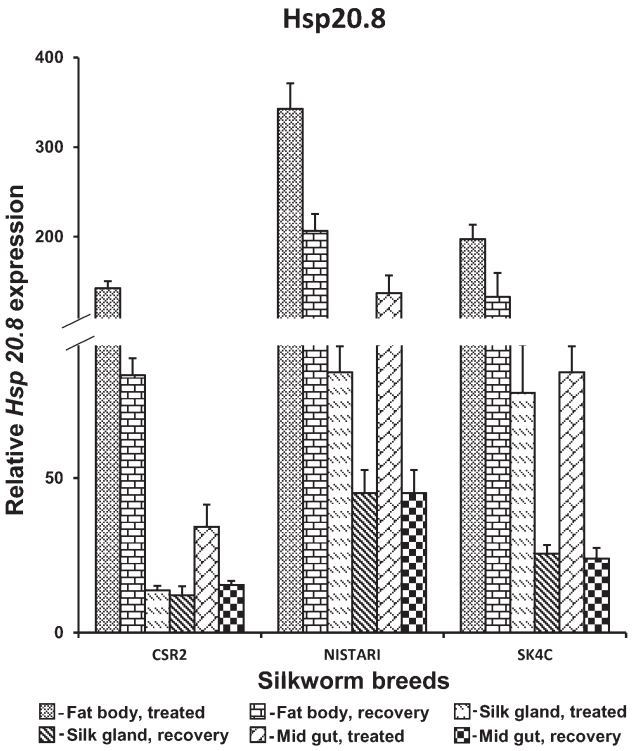

Fig. 4. The relative levels of expression of *Hsp20.8* in different tissue/breed/treated or recovery combinations. This experiment was performed in triplicate and the quantity of *Hsp20.8* mRNA normalized by using the transcript quantity of β-actin in non-heat shocked tissue at 0-hour.

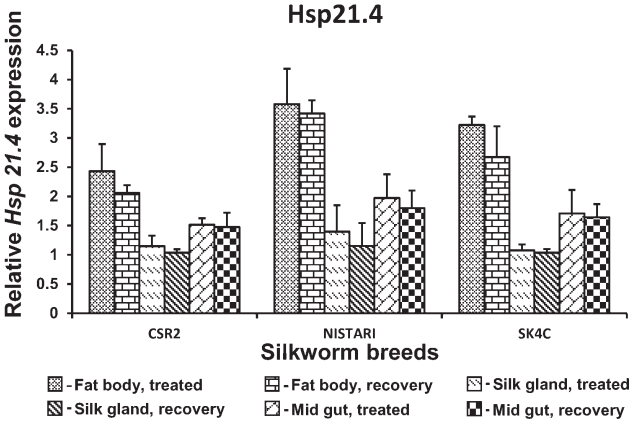

Fig. 5. The relative levels of expression of *Hsp21.4* in different tissue/breed/treated or recovery combinations. This experiment was performed in triplicate and the quantity of *Hsp21.4* mRNA normalized by using the transcript quantity of β-actin in non-heat shocked tissue at 0-hour.

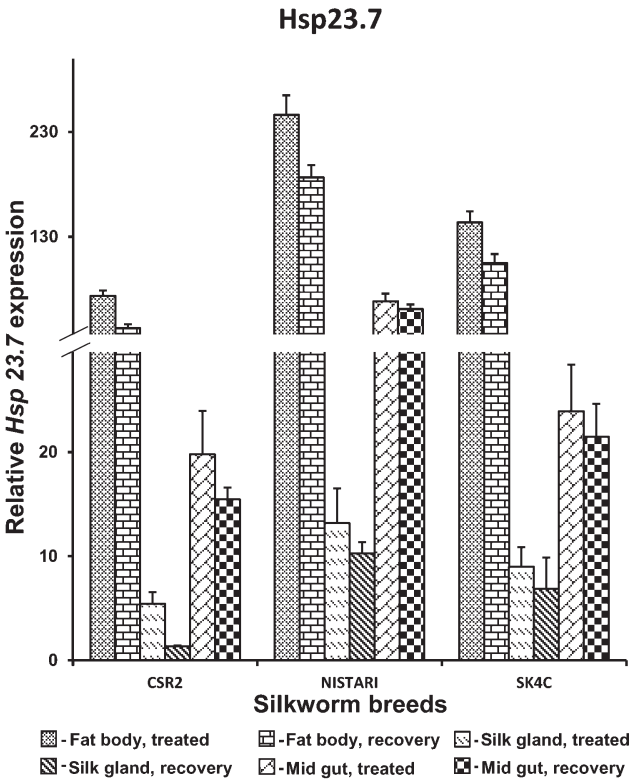

Fig. 6. The relative levels of expression of *Hsp23.7* in different tissue/breed/treated or recovery combinations. This experiment was performed in triplicate and the quantity of *Hsp23.7* mRNA normalized by using the transcript quantity of β-actin in non-heat shocked tissue at 0-hour.

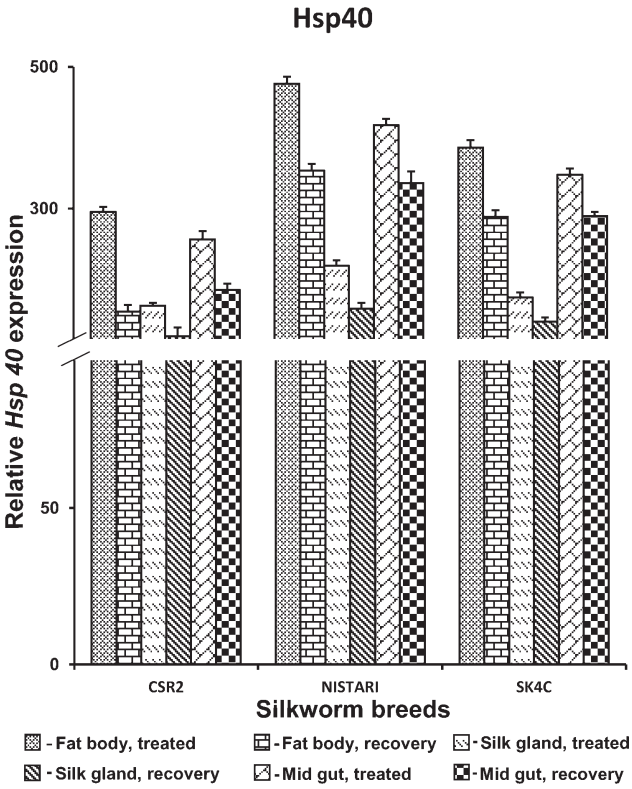

Fig. 7. The relative levels of expression of *Hsp40* in different tissue/breed/treated or recovery combinations. This experiment was performed in triplicate and the quantity of *Hsp40* mRNA normalized by using the transcript quantity of β-actin in non-heat shocked tissue at 0-hour.

in Nistari the highest expressed gene *sHsp20.4* differed from lowest expressed gene *Hsp21.4* by more than 1338 times. Similarly, for the fat body of SK4C *sHsp20.4* was expressed 605 times more than *Hsp21.4* (Fig. 5). These results indicate that the *Hsp* genes are differently expressed at the tissue level.

Expression of *Hsps* in larvae recovering from heat shock

The expression of *Hsp* genes in the tissues of larvae recovering from heat shock was considerably less than in the tissue of larvae that have just experienced a heat shock. The profile of expression of *Hsp* in recovering larvae and those that have just experienced a heat shock was the same but the level of expression differed. The level of expression of *Hsps* in the tissues of recovering larvae was highest in fat body followed by mid gut and silk gland. The genes that were expressed most in the fat body of larvae recovering from heat shock were *sHsp20.4* and *Hsp70*, which were expressed 9.7 and 2.7 times more, respectively, in Nistari than in CSR2. Similarly, *sHsp20.4* and *Hsp70* were 4.2 and 1.4 times more expressed in the fat body of larvae recovering from heat shock of SK4C than CSR2 (Figs 3 and 8).

Expression of *Hsps* in breeds of silkworm that are susceptible, tolerant and moderately tolerant to heat shock

The expression of all nine *Hsps* in CSR2 (susceptible), Nistari (tolerant) and SK4C (moderately tolerant) were significantly up-regulated. Generally, the expression of *Hsp* genes was high in Nistari and low in CSR2, with an intermediate level of expression in SK4C (Figs 1–9). The highly expressed *Hsp* genes viz., *sHsp20.4* and *Hsp70,* were 9.4 and 2.3 times, respectively, more highly expressed in Nistari than CSR2. Similarly, the expression of *sHsp20.4* and *Hsp70* was 3.5 and 1.5 times, respectively, more highly expressed in SK4C than CSR2. However, the increase in the expression of other *Hsp* members was not less than 1.4 and 1.2 times in Nistari and SK4C, respectively, compared to CSR2. Two-way ANOVA with tissues and breeds as factors revealed significant differences ($P < 0.05$) in the expression of all the *Hsp* genes (except *Hsp90*) (Table 3).

DISCUSSION AND CONCLUSIONS

The objective of this study was to identify breeds of bivoltine silkworms that can be reared at the high temperatures prevailing in the tropics. The thermotolerance of different breeds of *B. mori* is measured by recording the percentage pupation of larva reared at a high temperature (Huang et al., 1979; He & Oshiki, 1984). Therefore, 3 day old 5th instar larvae of 20 silkworm breeds were exposed to 36°C for 6 h every day until they started to spin cocoons. Using a 6 h heat shock everyday was thought to best mimic the daily fluctuation in temperature experienced in the tropics. The thermotolerant ability of multi- and bivoltine silkworm breeds measured in terms of percentage pupation differed. The percentage pupation recorded for multivoltine breeds

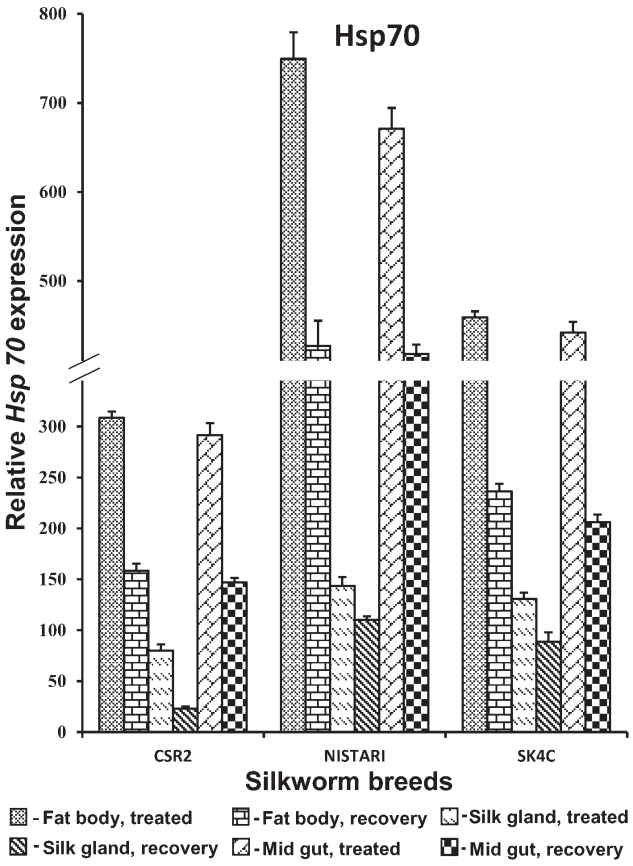

Fig. 8. The relative levels of expression of *Hsp70* in different tissue/breed/treated or recovery combinations. This experiment was performed in triplicate and the quantity of *Hsp70* mRNA normalized by using the transcript quantity of β-actin in non-heat shocked tissue at 0-hour.

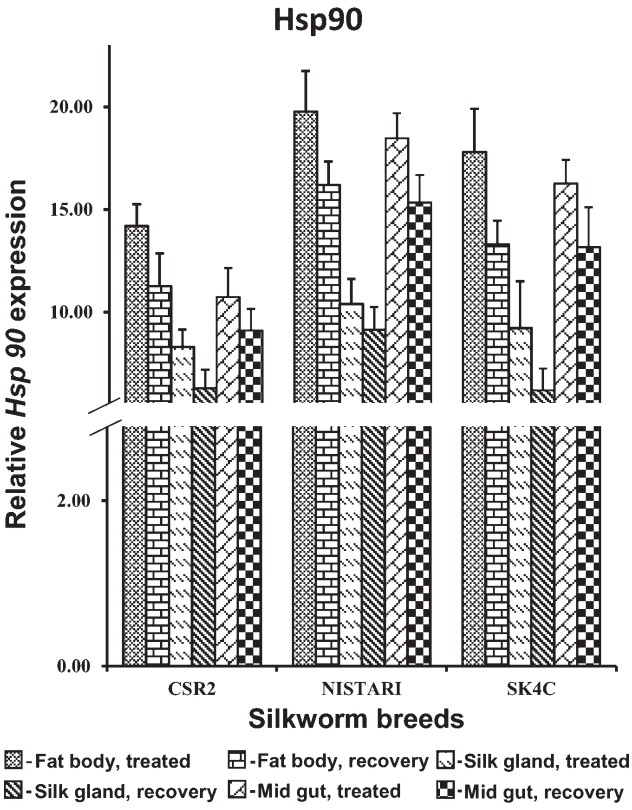

Fig. 9. The relative levels of expression of *Hsp90* in different tissue/breed/treated or recovery combinations. This experiment was performed in triplicate and the quantity of *Hsp90* mRNA normalized by using the transcript quantity of β-actin in non-heat shocked tissue at 0-hour.

was higher than that recorded for bivoltine breeds. The percentage pupation of the bivoltine breeds varied markedly indicating it might be possible to select bivoltine breeds for better thermotolerance. For the 20 silkworm breeds tested, the highest percentage pupation was recorded for Nistari and the lowest for CSR2. These two breeds experience very different natural thermal regimes; Nistari is a multivoltine breed native to tropical areas whereas CSR2 is a bivoltine breed native to temperate areas, which provides a high cocoon yield but is very sensitive to high temperatures. Generally, multivoltine breeds are better able to survive a heat shock than bivoltine breeds (Hsieh et al., 1995; Joy & Gopinathan, 1995). Koundinya et al. (2003) report that Nistari is the most tolerant of high temperatures of the 11 multivoltine breeds they tested. Based on percentage pupation, Srivastava et al. (2007) classified 15 multivoltine breeds as 6 tolerant, 5 moderately tolerant and 4 susceptible. Joy & Gopinathan (1995) report high percentage survival for the multivoltine silkworm breeds, C.Nichi and Pure Mysore, and low percentage survival for the bivoltine breed, NB4D2, after a heat shock of 1 h or 2 h at 39°C or 41°C. Similar results are reported by Hsieh et al. (1995) after a heat shock of 1h at one of several high temperatures.

For the bivoltine breeds the highest percentage pupation of 60.89% was recorded for SK4C, which is 4.8 times greater than that recorded for CSR2. Though, SK4C is not a native of the tropics it is more tolerant of heat stress than other bivoltine breeds and was produced by using "Cambodge" (multivoltine) as one of the parents and screening every generation for individuals that survived better at high temperatures and humidity (Moorthy et al., 2007). Therefore, it is possible that SK4C inherited its tolerance of high temperatures from its multivoltine parent. Earlier studies indicate that bivoltine breeds like KA and their hybrids are to some extent tolerant of 38°C (Pillai & Krishna Swami, 1987). Kumar et al. (2003) studied the effect of high temperature on the quantitative traits of bivoltine breeds and reports 3 line produced oval cocoons and 3 produced dumbbell cocoons that are more tolerant and also studied the tolerance of their hybrids and double hybrids.

Tolerance of *B. mori* of high temperatures is influenced by both environmental and genetic factors (Kumar et al., 2012). The better survival of a silkworm strain in the field is governed by molecular mechanisms in their cells. Investigating the production of heat shock proteins by 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 breed in the field and assist in the breeding of more heat-tolerant breeds. Therefore, the expression of 9 *Hsp* genes in the fat body, mid gut and silk gland tissues of Nistari, CSR2 and SK4C in response

Table 3. Two-way ANOVA on each *Hsp* gene expression in silkworm larvae using breed and tissue as factors.

Source	*DF*	Mean Square	*F*	*P* value
Hsp90				
Breed	2	108.2	25.5	0.0001**
Tissue	2	255	60.1	0.0001**
Breed * Tissue	4	10	2.3	0.067 NS
Error	45	4.2		
Hsp70				
Breed	2	291985.3	26.8	0.0001**
Tissue	2	474519	43.6	0.0001**
Breed * Tissue	4	39708.3	3.64	0.012*
Error	45	10884.6		
Hsp40				
Breed	2	77942.3	34.2	0.0001**
Tissue	2	142355.3	62.5	0.0001**
Breed * Tissue	4	8737.7	3.84	0.009**
Error	45	2277.7		
Hsp23.7				
Breed	2	23522	97.2	0.0001**
Tissue	2	78123.9	322.9	0.0001**
Breed * Tissue	4	9284.1	38.3	0.0001**
Error	45	241.9		
Hsp21.4				
Breed	2	1.679	16.2	0.0001**
Tissue	2	14.55	140.6	0.0001**
Breed * Tissue	4	0.508	4.91	0.002**
Error	45	0.103		
Hsp20.8				
Breed	2	39401.4	25.1	0.0001**
Tissue	2	109159.5	69.5	0.0001**
Breed * Tissue	4	6214.3	3.95	0.008**
Error	45	1571.9		
Hsp20.4				
Breed	2	7483063.5	16.02	0.0001**
Tissue	2	11383226.8	24.4	0.0001**
Breed * Tissue	4	3145581.4	6.73	0.0001**
Error	45	466933.9		
Hsp20.1				
Breed	2	1108.845	136.8	0.0001**
Tissue	2	1175.669	145.1	0.0001**
Breed * Tissue	4	739.429	91.2	0.0001**
Error	45	8.106		
Hsp19.9				
Breed	2	4854.991	37.7	0.0001**
Tissue	2	23796.682	184.6	0.0001**
Breed * Tissue	4	2921.418	22.7	0.0001**
Error	45	128.885		

NS no significant difference; * significant; ** highly significant.

to a heat shock of 1 h at 36°C and after being allowed to recover for 2 h at 25 ± 2°C was determined. The expression of *Hsp* genes was up-regulated in all the tissues of the silkworm breeds tested after heat shock, which indicates their products are possibly used to protect silkworms against heat shock. Two-way ANOVA of the results revealed significant differences ($P < 0.05$) in the levels of expression of most of the *Hsp* genes (except *Hsp90*) at the tissue level and among breeds. Irrespective of the level of thermotolerance of the different breeds, the level of expression of *Hsp* genes was more pronounced in fat body than other tissues. This may be due to the anatomical position of the fat body, which is located beneath the cuticle whereas the mid gut and silk gland are surrounded by fat body. Hence any thermal stress first affects the fat body and then other tissues (Kajiwara et al., 2006). Moreover, the fat body in insects is described as the invertebrate liver, which functions as a storage tissue and site of synthesis of numerous biological substances required for the proper physiological functioning of silkworms. Further, these results indicate the sensitivity of different tissues to heat stress. Similar observations on the variation of *Hsp* expression in tissues are recorded for *B. mori* (Velu et al., 2008) and other insects like *Heliothis armigera* (Singh & Lakhotia, 2000).

In the susceptible (CSR2), tolerant (Nistari) and moderately tolerant (SK4C) the transcription of the *Hsp* gene increased most in Nistari, less so in SK4C and least in CSR2. That is greater levels of *Hsp* synthesis were recorded under heat shock in the tolerant than in the susceptible breed. 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 *Hsp70* in the thermo sensitive breed than in the tolerant breed. As in *Locusta migratoria* (Wang & 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.

Compared to CSR2 the levels of expression of *sHsp* were more pronounced in Nistari with a 10.3 times increase in *sHsp20.4*, 9.7 times increase in *sHsp20.1* and a 1.3 times increase in *sHsp21.4*. Other than *sHsp*, the expression of *Hsp70* was 2.3 times higher in the tolerant than the susceptible breed. 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 & 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 & Krebs, 1998). Small heat shock proteins (s*Hsps*) bind partially denatured proteins, thereby preventing irreversible protein aggregation during stress (Sun & Mac Rae, 2005). Small *Hsps* functions by transferring bound proteins to ATP dependent chaperones, such

as *Hsp70*, and refolding (Richter et al., 2010). Small *Hsps* form oligomeric complexes during heat shock and the disaggregation of protein complexes is a prerequisite for efficient chaperone function (Haslbeck et al., 1999).

Keshan et al. (2014) report that the up-regulation of *Hsp90* gene is associated with the ability of silkworms to survive mild and mild-to-severe heat stress during the pupal stage. In *Drosophila* cells *Hsp70* appears to be the primary protein involved in thermotolerance (Parsell et al., 1993). Carmel et al. (2011) report a high positive correlation between the levels of *Hsp40* expression and induced thermotolerance in fruit flies, which implies a significant contribution of the *Hsp40* gene to thermoadaptation. Coulon-Bublex & Mathelin (1991) report the appearance of a 70kDa stress response protein after heat shock in the diapausing embryos of *B. mori*. Howrelia et al. (2011) suggest that thermotolerance can be achieved by the induction of *Hsp72* in the larval haemolymph by subjecting the *B. mori* cross-breed PM × CSR2 to several heat shock treatments. Sakano et al. (2006) report an increase in the mRNA transcripts levels of *sHsps* (*sHsp19.9*, *sHsp20.1*, *sHsp20.4*, *sHsp20.8* and *sHsp23.7*) in fat body of *B. mori* after heat shocks of different durations. But the amount of *sHsp21.4* mRNA is decreased by increasing in the duration of the heat shock. In our experiment expression of *sHsp21.4* was also low. The splicing process is inhibited by a heat shock and hence for the maximal synthesis during thermal stress, the *Hsp* genes lack introns (Yost & Lindquist, 1986). The *sHsp21.4* gene is speculated to have one intron and hence a low expression. Li et al. (2012) record the up-regulation of *sHsps*, including *Hsp20*.4 and *Hsp20*.8 in the posterior silk gland of silkworms at high temperatures. *BmHsp27.4*, a novel gene in *B. mori*, has an important role in its response to high-temperature stress (Wang et al., 2014). Li et al. (2014) report higher mRNA levels of *Hsp19.9*, *Hsp23.7*, *Hsp40* and *Hsp90* after a 24 h continuous heat shock in a tolerant variety (bivoltine) of silkworm than in a susceptible variety (bivoltine). Further Li et al. (2014) suggest that these *Hsps* and their levels of expression may play important roles in providing resistance to high temperature stress in different varieties of silkworm. The expression of different *Hsps* differs within and between tissues. Therefore, in this study the expression of nine *Hsp* genes was analyzed in three tissues of three breeds that differed in their thermotolerance. The expressions of all nine *Hsp* genes were up-regulated in all the tissues tested. The levels of expression of the nine *Hsp* genes were correlated with the thermotolerance of the silkworm breeds studied. Thus the levels of expression of all the nine *Hsp* genes collectively facilitate the survival of silkworm larvae at high temperatures.

Molecular mechanisms underlying the up-regulation of heat shock proteins are different in Nistari and CSR2 because they have completely different genetic backgrounds and natural thermal regimes. Similarly, Garbuz et al. (2008) show that although a 30-min heat shock treatment increases *Hsp70* synthesis two to three fold in *Stratiomys japonica* (naturally thermo-tolerant species), it resulted in the production of significantly less *Hsp70* in *Oxycera pardalina*, a cold environment species belonging to the same family (Stratiomyidae, Diptera). But in case of SK4C, it was produced by crossing female SK4 with male Cambodge and the F_1 males were then backcrossed with the females of cyclical backcross parent, SK4, to produce BC_1, the males of which were backcrossed with SK4. This backcrossing was repeated for six generations, followed by two generations of self-crossing. Larvae of each generation were screened at 33°C (Moorthy et al., 2007b). The genetic differences between the parents are believed to be responsible for the thermotolerance of SK4C. After a heat shock at 36°C for 1 h the level of expression of *Hsps* in SK4C was lower than in Nistari and higher than in CSR2. Similar results are also reported by Lin et al. (2014) for a near isogenic line. Further, Lin et al. suggest that there are many quantitative trait loci for thermotolerance on several chromosomes of silkworms as in *D. melanogaster* (Morgan & Mackay, 2006). It is likely that SK4C did not inherit all of the quantitative trait loci for thermotolerance of Cambodge. Therefore, the intermediate expression of *Hsps* in SK4C is due to a partial inheritance of the quantitative trait loci for thermotolerance of Cambodge. In order to survive at the high temperatures used for screening SK4C has to use the quantitative trait loci for thermotolerance it inherited from Cambodge. Therefore, of the bivoltine breeds, SK4C had best percentage survival and its level of expression of *Hsps* was almost twice that recorded for CSR2 larvae. In particular, the level of expression of *Hsp70* was 1.5 times higher in SK4C than CSR2 larvae. Of the *sHsp*, the levels of expression of *sHsp20.4* and *sHsp20.1* were 3.5 and 2.3 times higher in SK4C than CSR2. The higher level of expression of *Hsp* enhances the thermotolerance of SK4C by protecting the organism from protein injury due to thermal stress.

As the thermotolerance of the different breeds differ there is scope for breeders' to select for silkworm breeds that are more tolerant of heat stress. Earlier attempts to identify a bivoltine breed with high thermotolerance that could be used as a parent for breeding for high temperature tolerance were based solely on percentage pupation at a high temperature (Kumar et al., 2002, 2003, 2004; Krishna Rao et al., 2003). This study indicates that SK4C was able to survive high temperatures by using the quantitative trait loci for thermotolerance it inherited from its thermotolerant parent. That the percentage survival at a high temperature of SK4C was the highest of the bivoltine breeds and its levels expression of nine *Hsp* genes were higher than that recorded for CSR2 support this. The level of expression of *Hsp* genes in the fat body, mid gut and silk gland after a heat shock is the way silkworms respond to a heat shock and survive the fluctuating environmental conditions it experiences in the field (Chavadi et al., 2006). Therefore, SK4C with it high level of expression of *Hsp* genes (compared to CSR2) is better able to survive high temperature conditions than other bivoltine breeds. In addition, the DNA profile of SK4C, with 5 microsatellite markers associated with thermotolerance, is exactly the same as that of thermotolerant silkworm breeds (Moorthy et al., 2013). Indian sericulture is largely dependent on cross breeds produced

by using multivoltine breeds as the thermotolerant parent, which yield poor quality silk. Therefore, by producing bivoltine × bivoltine hybrids using SK4C as the thermotolerant parent, the hybrid will be bivoltine and will produce high quality silk. Since, breeding continuously aims to produce new breeds with the desired traits, silkworm breeders' aiming to produce a new bivoltine breed tolerant of high temperatures can use SK4C as the thermotolerant bivoltine parent and so amalgamate its thermotolerance with other economically important traits.

In conclusion, the thermotolerance and expression of *Hsp* genes varied in different silkworm breeds. Their thermotolerant ability was associated with the level of expression of *Hsp* genes. These findings provided a better understanding of the relationship between *Hsp* expression and thermotolerance in silkworms. In addition, the results indicate that it is the s*Hsp* (except *sHsp21.4*) and *Hsp70* genes that are mainly involved in the tolerance of thermal stress by working as molecular chaperons to protect silkworms from heat shock. The SK4C breed with the highest percentage pupation at high temperatures (among bivoltines) and a high level of expression of *Hsp* genes after heat shock survives better than other bivoltine breeds at high temperatures. Therefore, it can be utilized as a parent for breeding new bivoltine breeds/hybrids that are tolerant of high temperature conditions.

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