Expression Characteristics of *AaHsp90* Gene in *Antheraea assamensis* under Different Temperature and Starvation Stress

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Abstract [**Objectives**] To investigate the effects of different temperature and starvation stress on the expression of *AaHsp90* and reveal the molecular mechanism of adaptation to environment in *Antheraea assama*. [**Methods**] Taking the normal feeding group at 26 ℃ as the control, the expression change of *AaHsp90* was detected by real-time PCR in midgut, fat body and hemlymph after high temperature stress at 38 ℃, low temperature stress at 4 ℃ and starvation stress separately for different time on the third day of the fifth larvae. [**Results**] The expression of *AaHsp90* in midgut, fat body and hemlymph of *Antheraea assama* were increased obviously at first and then decreased sharply with the prolongation of treatment time at 38 ℃. There has a certain inhibitory effect on the expression of *AaHsp90* in midgut, fat body and hemolymph after treatment with 4 ℃ for different time. After treatment with starvation, the *AaHsp90* expression were increased at 12 and 18 h and decreased sharply at 24 h in midgut, fat body and hemolymph of *A. assama*. [**Conclusions**] Comprehensive analysis showed that high temperature and starvation stress can induce the expression of *AaHsp90*, while low temperature stress mainly suppressed its expression. It was suggested that the *AaHsp90* protein may play an important role in the process of adaptation to high temperature and starvation stress in *A. assama*.

Key words Heat shock protein 90, *Antheraea assama*, Temperature stress, Starvation stress, Expressing characteristics

1 Introduction

Heat shock proteins (Hsps) widely exist in plants, animals and microorganisms. It is a specific stress protein produced by organisms under adverse environmental stress, and mainly involved in regulating the correct folding of other proteins in cells and maintaining the physiological activity of mature proteins[1-2]. According to their relative molecular weight, amino acid sequences and biological functions, insect heat shock proteins are mainly divided into four families: small heat shock proteins (sHSPs), Hsp60, Hsp70 and Hsp90[3-4]. Hsp90 is one of the most abundant stress proteins in insects, and it can regulate and maintain the conformation and function of a variety of proteins in cells, so that cells can survive under stress[5-6]. Temperature is a main factor affecting the growth and development of insects. Insects have the ability to survive in abnormal temperature environment, which is the result of stress resistance produced by the body's heat shock response. *Hsp90* gene will change with the change in temperature in insects, and it can enhance the tolerance of the body to cold and heat stress by regulating its expression[7]. Hsp90 protein plays an important role in maintaining the normal physiological metabolism and growth and development of insects. *Drosophila* and *Tribolium castaneum* Hsp83 belong to the Hsp90 protein family, and the *Drosophila Hsp83* gene is required for normal sperm cell formation, survival, growth and development, as well as the normal function of centrosomes[8]. The *T. castaneum Hsp83* gene plays an important protective role in the development of adult ovaries, and inhibition of the expression of the *Hsp83* gene by RNAi technology will lead to the inability of females to produce mature egg cells[9].

Antheraea assamensis is a wild silk insect mainly distributed in northeastern India and the India – Myanmar border[10]. Because the cocoon silk is tough, wear-resistant, natural amber and not easy to fade, it has high development and utilization value. In China, Sericulture and Apiculture Research Institute, Yunnan Academy of Agricultural Sciences took the lead in investigation and collection of wild A. assamensis resources, and research on its biological characteristics[11]. Compared with *Bombyx mori* Linnaeus, *A. assamensis* has the typical characteristics of wild silkworm and can better adapt to the harsh environment in the wild, but its stress resistance mechanism is still unclear. Hsp90, as an important stress-resistant protein in insects, plays an important role in the process of adaptation to various adverse stresses. There are few reports on the physiological role and molecular mechanism of *A. assamensis* larvae in response to temperature and starvation stress. Exploring the changes in heat shock protein *AaHsp90* gene in *A. assamensis* larvae under high temperature, low temperature and starvation stress will not only help to understand the evolutionary mechanism of *A. assamensis* adaptation to harsh environment, but also provide an important theoretical basis for the study of stress resistance and variety breeding of *A. assamensis*.

2 Materials and methods

2.1 Materials *A. assamensis* was obtained from Silkworm Genetic Breeding and Application Innovation Team of Sericulture and

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Apiculture Research Institute, Yunnan Academy of Agricultural Sciences. The larvae were reared with fresh branches and leaves of *Cinnamomum japonicum* until the third day of the fifth instar.

2.2 Instruments and reagents

2.2.1 Main instruments: Constant temperature and humidity incubator, low temperature refrigerator and small high-speed refrigerated centrifuge were purchased from Shanghai Yiheng Scientific Instrument Co., Ltd.; ABI real-time fluorescence quantitative PCR instrument, Bio-rad common PCR amplification instrument and double-beam ultraviolet/visible spectrophotometer were purchased from Guangzhou Shenhua Biotechnology Co., Ltd.; the agarose gel electrophoresis apparatus and gel imaging system were purchased from Kunming BioTek Instrument Co., Ltd.

2.2.2 Main reagents: High-purity total RNA rapid extraction kit (Item No.:RP1202) was purchased from Beijing BioTeke Corporation; PCR amplification Mix (Item No.:TSE101) was purchased from Beijing Tsingke Biotechnology Co., Ltd.; reverse transcription reagent (Item No.:RR047A) and fluorescent quantitative PCR reagent TB Green^{R} Premix Ex Taq^{TM} (Item No.: RR42LR) were purchased from TakaRa.

2.3 Methods

2.3.1 Temperature stress treatment. The third day of the fifth instar *A. assamensis* larvae with similar body size were exposed to low temperature (4 ℃) and high temperature (38 ℃) for 1, 3, 6 and 9 h, respectively. The hemolymph, fat body and midgut of the *A. assamensis* larvae were collected, and the control group was taken at the same time. Each sampling was repeated for 3 times, and 3 silkworms were taken in each repetition. The samples were frozen with liquid nitrogen and stored at −80 ℃ for later use.

2.3.2 Starvation stress treatment. Feeding was stopped from 9 am on the third day of the fifth instar, and the samples were collected after 6, 12, 18 and 24 h of starvation treatment, and the sampling method was the same as that of the temperature stress treatment group.

2.3.3 Extraction of total RNA and preparation of cDNA. Total RNA was extracted from different tissues of AAA according to the operation of high-purity total RNA rapid extraction kit. The concentration of RNA was measured by double-beam UV/Vis spectrophotometer, and cDNA was prepared according to TaKaRa reverse transcription kit for routine PCR amplification and real-time fluorescence quantitative PCR detection.

2.3.4 Design and synthesis of gene primers. The *A. assamensis* β-actin gene reported by Chen Anli *et al.*[12] was used as the reference gene (GenBank accession number: KY676861), and the heat shock protein gene *AaHsp90* was used as the target gene (GenBank accession number: MK 165664.1). A quantitative primer was designed using Primer Premier 5.0, which was synthesized by Sangon Biotech (Shanghai) Co., Ltd. (the sequence is listed in Table 1).

Table 1 Primers used in quantitative real-time PCR

Gene name	Primer sequence
AaHsp90	F: 5'- TCGCGGAGTACATGGAGGAG-3'
	R: 5'-CAGACAGCTCCTTTTCGCGT-3'
β-actin	F: 5'-CAAAACATTCAACACCCCCG-3'
	R: 5'-TGGCGTGAGGCAGTGCGTAA-3'

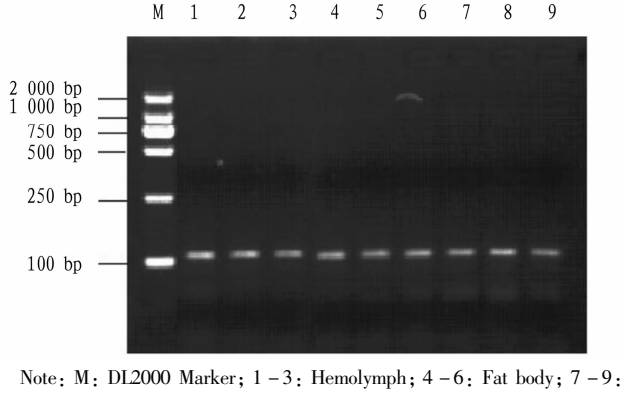
2.3.5 PCR detection of target gene primers. The cDNA obtained by reverse transcription was used as a template for general PCR amplification and quantitative primers for detection of *AaHsp90* gene. The operation was carried out according to that instruction for use of the PCR amplification Mix reagent. Amplification procedure: pre-denaturation at 98 ℃ for 2 min; 30 cycles at 98 ℃ for 10 s, 58 ℃ for 10 s, and 72 ℃ for 15 s; final extension at 72 ℃ for 5 min. PCR products were detected by 2% agarose gel electrophoresis, and then observed and photographed in the gel imaging system.

2.3.6 Real-time fluorescence quantitative PCR detection. The fluorescent quantitative PCR reaction system was prepared according to the instructions of TB Green^{R} Premix Ex Taq^{TM}, and the operation program was set according to the CFX-96 real-time fluorescent quantitative PCR instrument of Bio-Rad Company: pre-denaturation at 95 ℃ for 5 min → (95 ℃, 10 s → 60 ℃, 30 s) for 40 cycles. The relative expression of genes was calculated by $2^{-\triangle\triangle CT}$ method[13]. Excel 2016 and SPSS 21.0 software were used to plot and analyze the data.

3 Results and analysis

3.1 Detection of *AaHsp90* gene in *A. assamensis* by primer

The cDNA obtained by reverse transcription of total RNA from different tissues of *A. assamensis* in the control group was used as template for ordinary PCR amplification and agarose gel detection. The purpose is to determine the specificity of the *AaHsp90* gene quantitative primer and whether there is an obvious primer dimer. Fig. 1 shows that the designed *AaHsp90* gene quantitative primer can amplify the target band from the cDNA template, and there is no obvious primer dimer band, indicating that the designed primer meets the requirements of fluorescent quantitative PCR detection and can be used for subsequent tests.



Note: M: DL2000 Marker; 1−3: Hemolymph; 4−6: Fat body; 7−9: Midgut.

Fig. 1 PCR amplification results of *AaHsp90* gene primer

3.2 Effects of high temperature stress on *AaHsp90* gene expression in *A. assamensis*

The expression of *AaHsp90* gene in different tissues of *A. assamensis* larvae treated with high temperature, low temperature and starvation for different time was quantitatively analyzed by real-time fluorescent quantitative PCR. The relative expression level of *AaHsp90* gene in the control group was set as 1. If the relative expression level of *AaHsp90* gene in different stress treatment groups was significantly greater than 1, it indi-

cated that the gene expression was up-regulated; if the relative expression level was significantly less than 1, it indicated that the gene expression was inhibited.

A. assamensis larvae on the third day of the fifth instar were treated with high temperature of 38 ℃. Fig. 2 shows the relative expression changes in the heat shock protein gene *AaHsp90* in the intestine, fat body and hemolymph. The relative expression of *AaHsp90* gene in the midgut of *A. assamensis* exposed to high temperature for 1 and 3 h was not different from that of the control group. After 6 h of treatment, the expression of *AaHsp90* gene was significantly up-regulated, and its relative expression level was 1.71 times that of the control group. However, the expression of *AaHsp90* gene was inhibited after 9 h of high temperature treatment, and the relative expression decreased to 36% of the control group. The expression of *AaHsp90* gene in fat body was continuously up-regulated after 3 and 6 h of high temperature treatment, and its relative expression level was 2.92 and 3.16 times of the control group. The expression of *AaHsp90* gene in fat body was down-regulated after 9 h of high temperature treatment, and the expression level decreased to 27% of the control group. The expression of *AaHsp90* gene in hemolymph was up-regulated at 1 h of high temperature treatment, and the relative expression level was the highest at 3 h of high temperature treatment, which was 4.75 times that of the control group. After 6 h of treatment, the relative expression of *AaHsp90* gene tended to decrease, but it was still higher than that of the control group, and its relative expression was 1.58 times of that of the control group. After 9 h of treatment, the relative expression of *AaHsp90* gene was the lowest, only 21% of that of the control group.

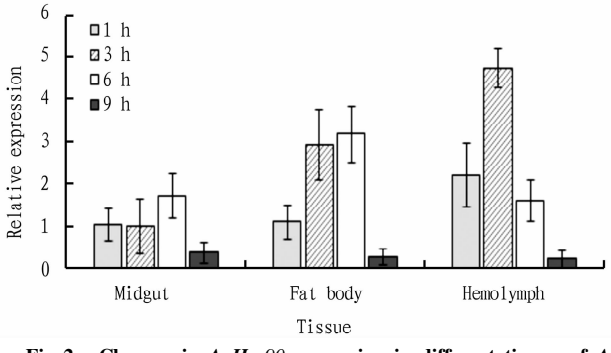


Fig. 2 Changes in *AaHsp90* expression in different tissues of *Antheraea assama* after treatment with 38 ℃ for different time

3.3 Effects of low temperature stress on *AaHsp90* gene expression in *A. assamensis* Fig. 3 shows that the relative expression of *AaHsp90* gene in the midgut of AAA larvae treated at 4 ℃ for 1 h was slightly lower than that of the control group. The expression of *AaHsp90* gene was significantly inhibited after treatment for 3 and 6 h and its relative expression decreased to 38% and 32% of the control group, respectively. After 9 h of treatment, the relative expression of *AaHsp90* gene decreased to the lowest level, which was 12% of the control group. There was no significant difference in the relative expression of *AaHsp90* gene in the fat body between the low temperature treatment group and the control group separately at 1 and 3 h treatment. After 6 and 9 h treatment, the relative expression of *AaHsp90* gene decreased to 21% and 13% of the control group, respectively, and the gene expression was significantly inhibited. The expression of *AaHsp90* gene in hemolymph was down-regulated at 1 h after cold treatment, and the expression of *AaHsp90* gene continued to be down-regulated with the extension of treatment time. At 9 h after cold treatment, the relative expression level of *AaHsp90* gene was extremely low, which was only 9.26% of the control group.

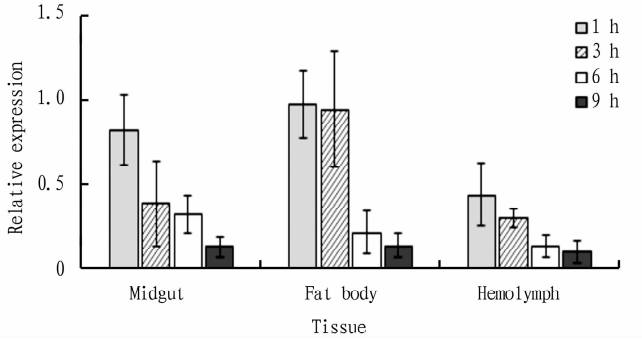


Fig. 3 Changes in *AaHsp90* expression in different tissues of *Antheraea assama* after treatment with 4 ℃ for different time

3.4 Effects of starvation stress on *AaHsp90* gene expression in *A. assamensis* As shown in Fig. 4, the expression of *AaHsp90* gene in the midgut of *A. assamensis* larvae was significantly induced by starvation treatment for 12 and 18 h, and the relative expression of *AaHsp90* gene was the highest at 12 h of starvation, which was 3.43 times that of the control group. However, the expression of *AaHsp90* gene in the midgut was significantly inhibited after 24 h of starvation, and its relative expression decreased to 12% of the control group. The expression of *AaHsp90* gene in fat body and hemolymph was not affected by 6 h starvation. The expression of *AaHsp90* gene in fat body was significantly higher than that of the control at 12 and 18 h of starvation, and the relative expression was 2.91 and 2.27 times of the control. The expression of *AaHsp90* gene in fat body was significantly inhibited by starvation for 24 h, and its relative expression was reduced to 15% of the control group. The expression of *AaHsp90* gene in hemolymph was only slightly up-regulated at 12 and 18 h of starvation, and the relative expression level was 1.46 and 1.37 times of the control group, respectively. The expression of *AaHsp90* gene was severely inhibited at 24 h of starvation.

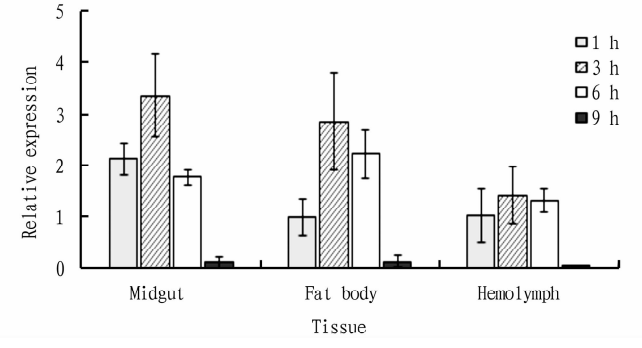


Fig. 4 Changes in *AaHsp90* expression in different tissues of *Antheraea assama* after starvation for different time

4 Discussion and conclusions

Insects live in a changing natural environment and are subject to a variety of external environmental stresses, such as the threat of hunger, sudden changes in temperature and humidity, and so on. It is an important strategy for insects to adapt to the environment by regulating the expression of *Hsp90* gene to cope with adverse stress[2-3]. At present, *Hsp90* genes of many insects have been cloned and identified, and *Hsp90* genes can resist and cope with adverse stresses by enhancing their own expression[14-18]. Under high temperature stress of 36 ℃ and low temperature stress of 4 ℃, the expression of *SfHsp90* gene in female and male adults of *Spodoptera frugiperda* was significantly increased, indicating that *Hsp90* gene played an important role in responding to environmental temperature stress and helping insects adapt to changes in external conditions[14]. The incremental expression of *Hsp90* gene in insects is conducive to their tolerance to temperature. The relative expression of *BcHsp90* gene in adults of *Bactrocera cucurbitae* treated at 38 ℃ and 40 ℃ for 1 and 2 h was significantly higher than that in the control group[15]. The relative expression of *Hsp90* gene of *Grapholitha molesta* increased significantly with the increase of temperature under high temperature stress, and was positively correlated with the degree of temperature stress[16]. The relative expression of *MsHsp90* gene was the highest when *Mythimna separata* was treated at 40 ℃ for 6 h, indicating that *MsHsp90* gene could improve the heat resistance of the body in *M. separata*[17]. After the fifth instar larvae of *Antheraea pernyi* were treated at 42 ℃ for 30 min, the expression of *ApHsp90* gene in hemolymph, midgut and fat body was significantly higher than that of the control group, indicating that *ApHsp90* gene played a protective role in the high temperature resistance of *A. pernyi*[18].

The most suitable temperature for the growth and development of the fifth instar larvae of *A. assamensis* is 26 – 32 ℃. If the temperature is too high or too low, the normal physiological metabolic process will be inhibited, resulting in growth retardation and even death of the larvae[19]. In this study, the expression of *AaHsp90* gene in midgut, fat body and blood lymphocytes of fifth instar *A. assamensis* larvae on the third day increased significantly at first, and then decreased sharply with the extension of treatment time. The expression of *AaHsp90* gene in hemolymph, fat body and midgut of *A. assamensis* larvae was up-regulated at 1, 3 and 6 h of high temperature stimulation, respectively, indicating that the response time of *AaHsp90* gene to high temperature stimulation in different tissues was not consistent. However, the expression of *AaHsp90* gene was significantly inhibited after 9 h of high temperature treatment. These results indicated that short time of high temperature stimulation could effectively enhance the expression of *AaHsp90* gene and improve the ability of *A. assamensis* larvae to resist high temperature. With the increase of high temperature stimulation time, the relative expression of *AsHsp90* gene decreased sharply, and the ability of *A. assamensis* larvae to resist high temperature began to decline. The expression of *AaHsp90* gene was down-regulated in the midgut and hemolymph of *A. assamensis* after 1 h treatment at 4 ℃, and the relative expression of *AaHsp90* gene decreased with the increase of treatment time. The expression of *AaHsp90* gene in the fat body was not significantly different from that in the control group at 1 and 3 h of cold treatment. However, the expression of *AaHsp90* gene in the fat body was significantly inhibited at 6 and 9 h of cold treatment, indicating that the response of *AaHsp90* gene in the fat body to low temperature was later than that in the hemolymph and midgut. The expression of *AaHsp90* gene in different tissues of *A. assamensis* was inhibited by low temperature treatment, which indicated that *A. assamensis* larvae had weak tolerance to low temperature. *A. assamensis* is mainly distributed in tropical or subtropical regions, and its tolerance to high temperature rather than low temperature is the result of its long-term adaptation to the environment.

A. assamensis has not been completely domesticated and has the typical characteristics of wild silkworm, so it will inevitably encounter a certain degree of food shortage in the wild. In order to adapt to the changing environment in the wild, its ability to tolerate starvation stress will also be improved. The results of this study showed that the expression of *AaHsp90* gene in *A. assamensis* midgut, fat body and hemolymph increased first and then decreased sharply after starvation stress. The expression levels of *AaHsp90* gene in midgut, fat body and hemolymph of *A. assamensis* were higher than those of the control group at 12 and 18 h of starvation stress, and the expression levels of *AaHsp90* gene in all tissues were the highest at 12 h of starvation stress, indicating that *AaHsp90* gene played an important role in *A. assamensis* to resist starvation stress. However, the expression of *AaHsp90* gene in all tissues was significantly lower than that in the control group after 24 h of starvation, indicating that the tolerance of *A. assamensis* to starvation was limited. Through analysis, we believed that starvation for 24 h will weaken the nutritional metabolism of *A. assamensis* and inhibit the expression of *AaHsp90* gene to a certain extent.

In summary, the expression of *AaHsp90* gene in the fifth instar larvae of *A. assamensis* increased first and then decreased sharply under the stress of 38 ℃ or starvation, while the expression of *AaHsp90* gene was inhibited under the stress of 4 ℃. The results indicated that AaHsp90 protein played a certain role in the process of *A. assamensis* larvae resisting high temperature and starvation stress.

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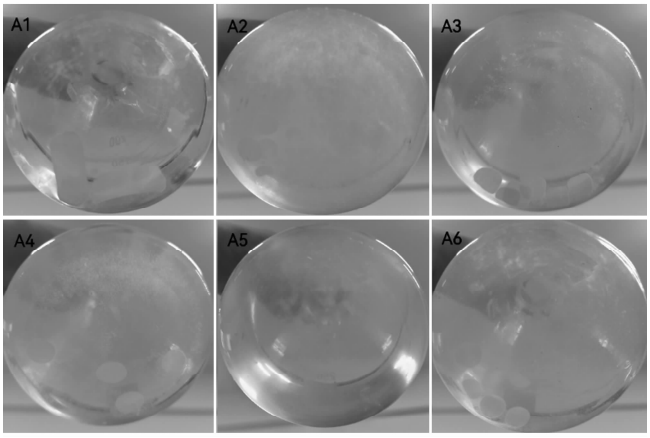

Fig. 2 Degradation effect of strains on filter paper

4 Conclusions

Nine strains capable of degrading cellulose were isolated and purified from the soil piled with rice straw. According to the results of transparent zone, cellulase activity and filter paper disintegration test, it was confirmed that strains A2 and A5 could degrade cellulose very well, and the results of 16s rDNA identification showed that A2 belonged to *Pseudoxanthomonas mexicana* and A5 belonged to *Bacillus cibi*. The results can provide high quality strain resources for the degradation of cellulose in straw, and have important application value for improving the efficiency of straw composting.

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