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Eicosanoids mediate *sHSP 20.8* gene response to biotic stress in larvae of the Chinese oak silkworm *Antheraea pernyi*

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ABSTRACT

Small heat shock proteins (sHSPs) can regulate protein folding and protect cells from stress. To investigate the role of sHSPs in the silk-producing insect *Antheraea pernyi* (*A. pernyi*; Lepidoptera: *Saturniidae*), cDNA encoding HSP20.8 in *A. pernyi*, termed *Ap-sHSP20.8*, was identified as a 564 bp ORF. The translated amino acid sequence encoded 187 residues with a calculated molecular mass of 20.8 kDa and an isoelectronic point (pI) of 5.98; the sequence showed homology to sHSP chaperone proteins from other insects. *Ap-sHSP20.8* mRNA transcript expression was abundant in the midgut and fat body and found to be both constitutive and inducible by infectious stimuli. Therefore, *Ap-sHSP20.8* may play important roles in *A. pernyi* immune responses under biotic stress. Furthermore, we found that eicosanoids could mediate the induction of *Ap-sHSP20.8* in the fat body and midgut. Our findings show that sHSPs may be promising molecules to target in order to cripple immunity in insect pests.

1. Introduction

Insects and other invertebrates have robust innate immune systems, including humoral and cellular immunity to defend against invading microbes (Stanley et al., 2012; Wu et al., 2010; Iwanaga and Lee, 2005). Upon microbial challenge, immune effecter genes, such as antimicrobial peptides (AMPs) and lysozymes, are mainly up-regulated in the fat body and hemocytes (Duvic et al., 2012; J. Li et al., 2012; J.F. Li et al., 2012; Tanaka et al., 2008; Morishima et al., 1997). A hallmark of universal cellular defense reactions to various environmental and pharmacological stresses is the activation of the heat shock response (HSR). It is characterized by drastic up-regulation of members of the ubiquitous chaperone family of heat shock proteins (HSPs) with various molecular masses which repair denatured proteins and protect organisms from cellular damage (Craig, 1994; Henderson and Henderson, 2009; Ahamed et al., 2010). This cytoprotective action of HSPs during the pathogenesis infection has been suggested to be important. However, little is known about the function of small HSP response to biotic stress. In *Spodoptera frugiperda* Sf9 cells, five HSP70s induced by *Autographa californica multiple nucleopolyhedrovirus* infection and three constitutive cognate HSC70s were found abundant in infected cells (Lyupina et al., 2011). Additionally, envenomation of the ectoparasitoid *Bracon hebetor*, and the expression of sHSP and hsc70 in the lepidopteran host *Plodia interpunctella* were both up-regulated after 4 days by northern blotting (Shim et al., 2008). The red flour beetle, *Tribolium castaneum*, injected with crude lipopolysaccharide (LPS), induced strong expression of HSP (HSP27 and HSP68) mRNA transcripts (Altincicek et al., 2008). All of these data indicate that HSPs play an important role in insect immunity.

In addition to changes in heat shock protein synthesis, infection induces release of arachidonic acid (AA) from plasma membranes and subsequent eicosanoid synthesis, i.e. activation of the AA pathways. The eicosanoid biosynthesis pathways are commonly initiated synthesization of arachidonic acid by phospholipase A2 (PLA2), which can be inhibited by dexamethasone (DEX). The free AA is substrate for COXs and LOXs, and the two enzymes can be inhibited by indomethacin (INDO) and nordihydroguaiaretic acid (NDGA), respectively (Phelps et al., 2003; Stanley, 2011). The structures, biosynthetic pathway and biological significance of eicosanoids in insects have been detailed by Stanley (Stanley, 2011; Stanley et al., 2012; Miller and Stanley, 2004). Eicosanoids mediate various physiological processes of various biological systems, including reproduction, excretion, ion transport, and immune defense of insects (Büyükgüzel, et al., 2010; Miao and Nair, 2003; Park and Kim, 2012a, 2012b; Tunaz et al., 2003; Shrestha and Kim, 2009). For example, in larvae of *Pimpla turionellae* and *Bombyx mori* eicosanoids mediate melanotic nodulation reactions to pathogen

Abbreviations: HSP, heat shock protein; sHSP, small heat shock protein; *A. pernyi*, *Antheraea pernyi*; *AP-sHSP20.8*, heat shock protein 20.8 in *Antheraea pernyi*; ORF, open reading frame; pI, isoelectronic point; LPS, lipopolysaccharide; BmNPV, *Bombyx mori* nuclear polyhedrosis virus; IPTG, isopropyl-β-D-thiogalactopyranoside; PCR, polymerase chain reaction; RT-PCR, real-time PCR; *E. coli*, *Escherichia coli*; PVDF, polyvinylidene difluoride; DEX, dexamethasone; NDGA, nordihydroguaiaretic acid; INDO, indomethacin; AA, arachidonic acid; MDH, malate dehydrogenase; BSA, bovine serum albumin.

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infection (Stanley et al., 1997; Buyukguzel et al., 2007; Durmus et al., 2008). And Bundey et al. (2003) reported that eicosanoids mediate behavioral fever responses to infection in the locust *Schistocerca gregaria*. But, at the molecular level, little is known how eicosanoids regulate the immune response. As in mammals, infection is also associated with local increase in tissue temperature, we propose that eicosanoids are involved in the heat shock protein gene transcription induced by pathogens.

The Chinese oak silk moth, *Antheraea pernyi* (Lepidoptera: Saturniidae) is an economically valuable silk-producing insect that is mainly commercially cultivated in China, India and Korea (Zhou and Han, 2006; Liu et al., 2010). Here, we investigated the expression patterns and distribution profiles of *Ap-sHSP20.8*(GenBank accession number KM881570) in *A. pernyi* challenged by different microorganisms.Additionally, we examined whether arachidonic acid mediates *Ap-sHSP20.8* response to biotic stress, such as that induced by nuclear polyhedrosis virus (NPV).

2. Materials and methods

2.1. Experimental insects

The experimental insects, *A. pernyi*, were provided by the Sericultural Research Institute of Henan. The larvae were reared on fresh oak leaves at 25 ± 1 °C in 14 h light:10 h dark (a long day length) with 70% humidity. The fat bodies and midguts were dissected from 3rd day fifth instar larvae and stored at −80 °C until used (Wei et al., 2011).

2.2. RNA extraction, cDNA cloning and phylogenetic analyses

Total RNA was isolated from fat bodies with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and first-strand cDNA was obtained using TransScript Synthesis SuperMix (TransGen, Beijing, China). Degenerate oligonucleotide primers, *Ap-sHSP20.8* F2 and R2, were designed using the Primer Premier 5.0 software package. PCR was performed using an amplification program as follows: 5 min at 94 °C, followed by 35 cycles of 94 °C for 30 s, 53 °C for 35 s and 72 °C for 30 s, and a final elongation step of 72 °C for 8 min. We analyzed PCR products by 1% agarose gel electrophoresis and then products were sequenced by Invitrogen.

NCBI bioinformatics tools (available at http://blast.ncbi.nlm.nih.gov/Blast.cgi) were used to detect conserved domains in *Ap-sHSP20.8*. Additionally, DNAman software was used to predict secondary structures and search for open reading frames (ORFs). Multiple sequence alignments were performed using the Clustal X program with its default parameters (Livak and Schmittgen, 2001). Phylogenetic and molecular evolutionary analyses were performed using the neighbor-joining method with the Molecular Evolutionary Genetics Analysis (MEGA version 4.1) software package.

2.3. Construction of expression plasmids and recombinant protein expression

To express recombinant Ap-sHSP20.8 protein in *Escherichia coli* BL21 (DE3), a pair of specific primers (*Ap-sHSP20.8* F3 and R3) were designed to amplify the 564 bp fragment encoding the entire ORF. The ORF sequence was cloned into *p*MD-19T and digested with the restriction endonucleases *Bam* HI and *Not* I, and then ligated into the expression vector *p*ET-28a (+) which was digested with the same restriction endonucleases. The expression plasmid *p*ET-28a (+)-*Ap-sHSP20.8* was then transformed into the BL21 (DE3) *E. coli* strain. After short term culture and isopropyl-β-D-thiogalactopyranoside (IPTG) induction at a final concentration of 0.9 mM, bacteria were harvested by centrifugation, resuspended, sonicated and then centrifuged again. The final pellet was resuspended in PBS and subjected to 15% SDS-PAGE. Ap-sHSP20.8 protein in the medium was purified using a 6×-His-Tagged Protein Purification Kit (CW Biotechnology, Beijing, China) according to the manufacturer's instructions, and the purified protein was examined by 15% SDS-PAGE.

2.4. Purification of recombinant Ap-sHSP20.8 protein and antibody preparation

After induction by IPTG at 37 °C for 12 h, *E. coli* BL21 (DE3) cells were collected by centrifugation at 5000×*g* for 10 min. Cell pellets were suspended in lysis buffer (50 mM NaH_2PO4, 300 mM NaCl, pH 8.0, and 1 mg/mL imidazole), stirred on ice for 30 min and then disrupted by sonication. After centrifugation at 10,000×*g* for 30 min at 4 °C, the recombinant proteins were purified by affinity chromatography through Ni-NTA (nickel-nitrilotriacetic acid) agarose resins (Qiagen, Hilden, Germany) following the manufacturer's protocol. The purified recombinant proteins were analyzed by SDS-PAGE and quantified using the BCA protein reagent kit (Aidlab Biotech Co. Ltd., Beijing). Antiserum was prepared according to a previously described method (Harlow and Lane, 1999). New Zealand white rabbits were immunized three times at two-week intervals with 100 μg eluted Ap-sHSP20.8 protein that was homogenized in complete Freund's adjuvant, and a boost injection was given one week later. Rabbit serum was collected seven days after the final immunization and stored at −80 °C.

2.5. Expression analysis using quantitative RT-PCR

Total RNAs from hemocytes, fat bodies, midguts, epidermis, silk glands and malpighian tubules of three larvae were reverse transcribed into cDNAs. Real-time PCR was carried out to measure the expression level of *Ap-sHSP20.8* with specific primers F1 and R1 (Table 1); the housekeeping *18S* rRNA gene (GenBank accession number DQ347469) was used for mRNA expression level normalization. Real-time PCR was performed with a StepOne Plus Real-Time PCR System using the SYBR® Premix Ex Taq™ kit (Takara). Reaction mixtures (20 μL) contained 10 μL 2×SYBR® Premix Ex Taq™ buffer, 1 μL forward and reverse primers, 1 μL cDNA and 7 μL RNAse-free H_2O. The PCR thermocycling protocols were as follows: 95 °C for 10 s, followed by 40 cycles of 95 °C for 15 s, 62 °C for 15 s and 72 °C for 30 s. At the end of the reaction, a melting curve was produced by monitoring the fluorescence continuously while slowly heating the sample from 60 °C to 95 °C. Each independent experiment was conducted in triplicate and the data was analyzed by Student's *t*-test. Differences were considered to be statistically significant when $P < 0.05$ and such differences were indicated by an asterisk. Additionally, total RNA that was extracted from fat bodies and midguts of larvae of the oak silkworm, *A. pernyi*, was treated with *E. coli*, *Beauveria bassiana* and NPV (1.0×10^9 virus particles or 1.0×10^6 bacterial cells or 1.0×10^6 fungal spores were suspended in 10 μL of sterilized 0.85% NaCl, and then were separately injected into each larvae).

Table 1
The primers used in this study.

Primer	Sequences (5′–3′)	Application
Ap-sHSP20.8 F1	GAGAAGAAAGACGAGCACGGGTAT	qPCR
Ap-sHSP20.8 R1	ATGGTGAGAACCCCGTCTGATG	qPCR
18S F	CGATCCGCCGACGTTACTACA	qPCR
18S R	GTCCGGGCCTGGTGAGATTT	qPCR
Ap-sHSP20.8 F2	ATGTCTCTTCTACCATTCGTGTTG	Amplification
Ap-sHSP20.8 R2	CTACTTATTTTCGGCATCCTGGGTT	Amplification
Ap-sHSP20.8 F3	GGGGGATCCATGTCTCTTCTACCATTCGTGTTG	Expression
Ap-sHSP20.8 R3	GGGCTCGAGCTACTTATTTTCGGCATCCTGGGTT	Expression

Note: restriction sites are underlined.