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Research paper

Nucleopolyhedroviruses (NPV) induce the expression of small heat shock protein 25.4 in *Antheraea pernyi*

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ABSTRACT

Nucleopolyhedroviruses (NPVs) is one group of Baculoviruses. The infection of NPV in silkworm is often lethal. To investigate the effective measures to stop the infection of NPV, we cloned cDNA encoding small heat shock protein 25.4 in *Antheraea pernyi* (Ap-HSP25.4). The translated amino acid sequence consisted of 223 residues with a calculated molecular mass of 25.4 kDa and an isoelectronic point (pI) of 4.93. Quantitative real-time PCR was used to investigate the expression patterns and distribution profiles of *Ap-sHSP25.4* before and after challenged with NPV. We found that the inhibitors of eicosanoid synthesis could suppress the transcription of *Ap-sHSP25.4* in the fat body in a dose dependent manner. And arachidonic acid induced the expression of *Ap-sHSP25.4*. Thus, we concluded that sHSPs may be promising candidates to boost insect immunity in practice.

1. Introduction

Baculoviruses are a class of large, double-stranded DNA viruses that infect invertebrate hosts, particularly the larvae of silkworms and other moths (*Lepidoptera*) (Zhang et al., 2015a,b,c). *Baculoviruses* are phylogenetically divided into four genera, i.e., *Alphabaculovirus*, *Betabaculovirus*, *Gammabaculovirus*, and *Deltabaculovirus* (Jehle et al., 2013). *Alphabaculoviruses* are further subdivided into nucleopolyhedroviruses (NPVs) groups I and II based on phylogenetic studies (Jehle et al., 2006). In a typical NPV life cycle, two virion phenotypes are produced. One phenotype is the budded virions (BVs), which play a role in disseminating the infection to all tissues of the host infected with BmNPV. The other phenotype is the occlusion–derived virions (ODVs), which are required for initiating host transmission (Lekha et al., 2015). Infection of silkworms by NPV is often lethal; however, effective measures to stop NPV infection are currently not available (Jiang et al., 2012).

Because *Baculovirus* infection is recognized as a stress factor in host cells, various cellular pathways are activated. The activation of heat shock proteins (HSPs) is a hallmark, universal cellular defensive reaction to various forms of environmental and pharmacological stress, which is characterized by a dramatic up-regulation of members of the ubiquitous chaperone family of HSPs with various molecular masses (Liang et al., 2007; Basha et al., 2012). HSPs have a central role in protein homeostasis and protection against proteotoxic stresses by preventing the misfolding and aggregation of proteins (Parsell et al., 1994) or by directing damaged proteins to the ubiquitin proteasome system for degradation (Nakamoto and Vigh, 2007; Baird et al., 2014).

NPV infection in insects activates the expression of certain genes and the synthesis of some proteins (Lekha et al., 2015; Nobiron et al., 2003). In particular, immune response genes, including arylphorin, cathepsin B, gloverin, lebocin, serpin, HSP 19.9, HSP 20.1, HSP 20.4, HSP 20.8, HSP 21.4, HSP 23.7, HSP 40, HSP 70, and HSP 90, have different levels of expression following NPV infection of two silkworm races, Sarupat and CSR-2 (Ponnuvel, 2014). When *Bombyx mori* (*B. mori*) challenged with NPV, HSP70 and HSP 70 protein cognate gene expression is up-regulated significantly (Hu et al., 2015). Iwanaga et al. (2014) found that BmHSC70-4 accumulates in the nucleus of NPV-infected BmN cells in a very late phase of infection. Additionally, western blot experiments show that BmHSC70-4 is a novel component protein of budded virus (BV) and occlusion-derived virus (ODV), whereas KNK437, an inhibitor of inducible HSPs, reduces BV production and delays viral DNA replication. In NIAS-Bmoyanagi2 cells, expression of the silkworm *B. mori* hsc70 ortholog increased by 1.6-fold 24 h post-infection (h p.i.) with no further increase at 12 h p.i., and the transcripts of HSP 20.1 decreased to approximately 50% at 2 h p.i. with NPV (Sagisaka et al., 2010). Collectively, these data suggest that HSPs likely have important roles in the propagation of NPV.

From work on the wax moth *Galleria mellonella*, Stanley-Samuelson et al (Buyukguzel et al., 2007) found that the nodulation and phenoloxidase activity were likely mediated by eicosanoids.

Abbreviations: HSP, heat shock protein; sHSPs, small heat shock proteins; *A. pernyi*, *Antheraea pernyi*; *AP-sHSP25.4*, small heat shock protein 25.4 in *Antheraea pernyi*; PCR, Polymerase Chain Reaction; RT-PCR, real-time PCR; PVDF, polyvinylidene difluoride; DEX, dexamethasone; NDGA, nordihydroguaiaretic acid; Indo, indomethacin; AA, arachidonic acid.

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Eicosanoids are oxygenated metabolites of polyunsaturated fatty acids, particularly arachidonic acid. Additionally, Yajima et al reported a functional coupling between an immune deficiency pathway and eicosanoid biosynthesis in *Drosophila* (Yajima et al., 2003). Thus, eicosanoids are likely essential elements of immunity in insects.

The Chinese oak silk moth, *Antheraea pernyi* (*Lepidoptera*: *Saturniidae*), is an economically valuable, silk-producing moth that is primarily commercially cultivated in China, India and Korea (Liu et al., 2010). We cloned the small heat shock protein 21 from *A. pernyi* (*Ap-sHSP21*) and found that it was significantly up-regulated after heat shock (Liu et al., 2013a,b). By contrast, in our previous work (Zhang et al., 2015a,b,c), NPV infection led to the up-regulation of *A. pernyi* small heat shock proteins, sHSP21.4 and sHSP20.8, in the midgut, hemocytes and fat body of infected larvae. Injected siRNA constructs silenced *Ap-sHSP21.4* and some immune related genes, including *defensin*, *Toll1* and *lysozyme* after NPV challenge. The influence of NPV infection on the expression of *Ap-sHSP21.4* and *Ap-sHSP20.8* was strongly inhibited by treating experimental larvae with inhibitors of eicosanoid biosynthesis; without inhibitors, treating larvae with AA (the eicosanoid biosynthesis precursor) induced expression of Ap-sHSP21.4 and Ap-sHSP20.8. Despite these studies, understanding of the role of sHSPs as an antiviral mechanism in silkworms remains limited. Therefore, further study is required to determine the antiviral effects of specific molecular mechanisms; hence, we investigated the patterns of expression and distribution profiles of small heat shock protein 25.4 in *A. pernyi* (*Ap-sHSP25.4*; GenBank: KM881572) in response to NPV infection. Additionally, we examined whether the expression of *Ap-sHSP25.4* upon NPV infection was mediated by the biosynthesis of arachidonic acid.

2. Materials and methods

2.1. Experimental insects

The experimental insects, *A. pernyi*, were provided by the Sericultural Research Institute of Henan. Larvae were reared on fresh oak leaves at 25 ± 1 °C with 14 h light:10 h dark (a long day length). Fat bodies were dissected in 0.75% NaCl from third-day fifth instar larvae and stored at −80 °C until used (Wei et al., 2011).

2.2. RNA extraction, cDNA synthesis, PCR primers and conditions

Total RNA was isolated from fat bodies with TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and the first-strand cDNA was obtained with TransScript Synthesis SuperMix (TransGen, Beijing, China). The open reading frame (ORF) sequences of *AP-sHSP25.4* from various insects were aligned in Clustal W (http://www.ebi.ac.uk/Tools/ClustalW). The degenerate oligonucleotide primers F2 and R2 (Table 1) were designed with Primer premier 5.0. PCR was performed using the following amplification program: 5 min at 94 °C, followed by 35 cycles at 94 °C for 30 s, 57 °C for 35 s, 72 °C for 30 s and a final elongation step at 72 °C for 8 min. PCR products were analyzed using 1% agarose gel electrophoresis and were sequenced at Invitrogen, Shanghai.

Table 1
The primers used in this study.

Primer	Sequences (5′–3′)	Application
F1	CGTGGAAGGCGACAAGTATCAG	qPCR
R1	TCTTGCTATCCTCTGGCTGCTT	qPCR
F18S	CGATCCGCCGACGTTACTACA	qPCR
R18S	GTCCGGGCCTGGTGAGATTT	qPCR
F2	ATGATCGCCTTAGTGTTGT	amplification
R2	TTAATATGGAATCGGCAAG	amplification
F3	GGGGGATCCATGATCGCCTTAGTGTTGT	expression
R3	GGGCTCGAGTTAATATGGAATCGGCAAG	expression

Note: Restriction sites are underlined.

2.3. Sequence analysis

Nucleotide sequences were assembled and ORFs were identified with the DNAStar software package (Version 5.02). Signal peptides were evaluated with SignalP 4.0 software (http://www.cbs.dtu.dk/services/SignalP/). sHSP-related protein sequences were retrieved from the Genbank database for multiple sequence alignment, and phylogenetic analysis was performed using ClustalX2 (http://www.ebi.ac.uk/tools/clustalw2/index.html). Phylogenetic and molecular evolutionary analyses were performed using the neighbor-joining method with Molecular Evolutionary Genetics Analysis (MEGA version 6.0) software (Tamura et al., 2013).

2.4. Real-time quantitative PCR

Total RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Then, RNA samples were treated with RQI RNase-free DNase (Takara) to remove any contaminating DNA, following the manufacturer's instructions. One microgram of total RNA was reverse-transcribed in a 20 μl reaction mixture with random hexamer primers using a first-strand cDNA Synthesis Kit (Toyobo) according to the manufacturer's instructions. Real-time PCR was performed in a StepOne Plus Real-Time PCR System using a SYBR® Premix Ex TaqTM kit (TaKaRa). Reaction mixtures (20 μl) contained 10 μl of 2× SYBR® Premix Ex TaqTM buffer, 1 μl of forward and reverse primers, 1 μl of cDNA, and 7 μl of Rnase-free H2O. The PCR procedure was as follows: 95 °C for 10 s, followed by 40 cycles each at 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 with continuous monitoring of fluorescence as the sample was slowly heated from 60 °C to 95 °C. Primers for the *Ap-sHSP25.4* gene and the 18S gene were designed using the online Primer3 internet-based interface (http://frodo.wi.mit.edu/) (Table 1). The dissociation curve analysis was performed for both primer pairs, and all experimental samples had a single, sharp peak at the melting temperature of amplification. The standard curves for *Ap-sHSP25.4* and 18S rRNA of *A. pernyi* were generated with a series of 10-fold dilutions, and the efficiencies of the RT-qPCR primers for the *Ap-sHSP25.4* and 18S rRNA genes were 1.93 and 1.95, respectively. The relative expression level of *Ap-sHSP25.4* gene was calculated according to the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001), and the 18S rRNA gene of *A. pernyi* (GenBank DQ347469) was used as the housekeeping gene. All RT-qPCR experiments were repeated three times. Each independent experiment was conducted in triplicate, and the data were analyzed by Student's *t*-tests. Differences were statistically significant when $P < 0.05$, with those differences indicated by asterisks.

2.5. Expression and purification of recombinant AP-sHSP25.4

In the construction of pET-28a (+)-*Ap-sHSP25.4* expression vectors, with primers F3 and *R*3, PCR was used to amplify *Ap-sHSP25.4* cDNA that encoded the entire ORF (Table 1). The PCR products were digested by *Bam* HI and *Xho* I and then ligated to *Bam* HI/*Xho* I digested pET28a vector to transform competent *E. coli* BL21 (DE3) cells. The cloned PCR products were sequenced to confirm the identity of the cloned gene. The positive clones containing the inserts were incubated at 37 °C in LB medium containing kanamycin (50 μg/ml) to an A600 of 0.6. Recombinant protein expression was induced by the addition of IPTG to a final concentration of 0.4 mM. After incubation for 5 h at 25 °C, bacteria were harvested by centrifugation at 10,000*g* for 10 min at 4 °C, with sonication. Recombinant proteins in the supernatants were purified by affinity chromatography using a 6XHis-Tagged Protein Purification Kit (CW Biotechnology, Beijing, China) according to the manufacturer's instructions.