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A novel immune-related gene HDD1 of silkworm *Bombyx mori* is involved in bacterial response

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ABSTRACT

Insects have evolved an effective immune system to respond to various challenges. In this study, a novel immune-related gene, called *BmHDD1*, was first charactered in silkworm, *Bombyx mori*. BmHDD1 contained an ORF of 837 bp and encoding a deduced protein of 278 amino acids. *BmHDD1* was specifically expressed in hemocytes, and highly expressed at the molting and metamorphosis stages under normal physiological conditions. Our results suggested that BmHDD1 was mainly generated by hemocytes and secreted into hemolymph. Our results also showed that the expression level of *BmHDD1* was significantly increased after 20E injection, which indicated that BmHDD1 might be regulated by ecdysone. More importantly, BmHDD1 was dramatically induced after injected with different types of PAMPs or bacteria, either in hemocytes or fat body. Those results suggested that BmHDD1 plays a role in developing and immunity system in silkworm, *Bombyx mori*.

1. Introduction

Organisms have evolved an effective immune system, which including innate and acquired immunity to respond to various challenges. As the biggest population, insects have developed a special immune system during the long-term evolutionary progress. They have an effective innate immunity to eliminate invading pathogenic microorganisms, but with lacking of adaptive immune system (Lemaitre and Hoffmann, 2007). Innate immunity includes humoral and cellular defenses, and humoral defenses mainly includes soluble effector molecules, such as antimicrobial peptides, polyphenoloxidase, lysozyme, anti-virus factor, lectin, and proteinase inhibitor (Kanost et al., 2004). Cellular immunity includes phagocytosis, encapsulation, and nodule, which are mainly mediated by hemocytes (Oliver et al., 2011; Liu et al., 2013; Satyavathi et al., 2014; Zhang et al., 2014a).

The domesticated silkworm, *Bombyx mori* is an economic insect, can secrete silk to form cocoon. At the same time, it also could be used as a lepidopteron insect model. Although whole-genome sequencing, more than 20,000 genes were identified in silkworm. However, nearly one quarter of them are novel genes (Xia et al., 2004; Xia et al., 2009; Xia et al., 2014).

In this study, a novel gene *BmHDD1* was first cloned and identified in silkworm, *Bombyx mori*. Its temporal and spatial expression profiles were investigated. BmHDD1 recombinant protein was acquired using prokaryotic expression system and purified by Ni-affinity chromatography, and antibody against was generated and verified. The response to the treatment of 20-Hydroxyecdysone (20E) and various bacteria were also surveyed.

2. Materials and methods

2.1. Insect and cell line

The *Bombyx mori* strain Dazao used in this study was from our laboratory and reared with fresh mulberry (Zhang et al., 2015a). Eggs were collected during whole embryo stages. Epidermis, malpighian tubules, midgut, silk gland, head, hemocytes, ovary, testis, and fat body in day 3 of the fifth-instar were dissected or collected. All materials were stored in liquid nitrogen.

The silkworm cell line BmE-SWU3 was generated in our lab (Xu et al., 2015a) and was cultured in Grace medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen).

2.2. RNA isolation and cDNA synthesis

Total RNA was extracted by TRIzol (Takara, Japan) according to the manufacturer's instructions. Subsequently, Total RNAs were treated with DNase I (Takara, Japan) for 30 min at 37 °C to remove the residual

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Table 1
Sequences of the primers were used in this study.

Name	Sequence (5′-3′)	Usage
BmHDD1-F	ATGTACAGACTAGTGTGCTTCCTT	PCR
BmHDD1-R	TTAATGAGACAAGAAGCCATTAC	
GSP1	GCCGTGGTGCGATCAGCAGTAGTG	5′RACE
NGSP1	CCTCAGGTTTCTGGGTCGTAATCA	
GSP2	CGACCCAGAAACCTGAGGAGTTCT	3′RACE
NGSP2	CGACGATTCCACCAGTGGGTTACA	
Bm HDD1-CDS-F	ATGTACAGACTAGTGTGCTTCCTT	Overexpression
Bm HDD1-CDS-R	ATCATTTCGCGCGTACTTAT	
BmHDD1-PE-F	AACGACATTGTTATCGGCC	Prokaryotic expression
BmHDD1-PE-R	ATCATTTCGCGCGTACTTAT	
BmHDD1-qRT-F	ACGTTGAAGTTTATTCTGTGCCC	
BmHDD1-qRT-R	TTCAGTTGTCGCTGTGAAGGTC	
BmGAPDH-qRT-F	CATTCCGCGTCCCTGTTGCTAAT	qRT-PCR
BmGAPDH-qRT-R	GCTGCCTCCTTGACCTTTTGC	

genomic DNA. First-strand cDNA was synthesized with M-MLV reverse transcriptase (Promega, USA) by using 1–5 μg of total RNA in a 20 μL reaction mixture according to the protocol provided by the manufacturer.

2.3. Gene cloning and rapid amplification of cDNA ends (RACE)

The predicated coding-sequence (CDS) and EST sequences were downloaded from SilkDB (http://www.silkdb.org/silkdb/) and KAIKObase (http://sgp.dna.affrc.go.jp/KAIKObase/). Primers were designed and the fragment of the BmHDD1 was acquired by PCR. The PCR condition included 2 min initial denaturation at 94 °C, followed by 25–35 cycles of the following: 30 s denaturation at 94 °C, 30 s annealing at 55 °C, 1 min of extension at 72 °C and a final 10 min extension at 72 °C. Subsequently, 3′ and 5′ RNA ligase-mediated rapid amplification of cDNA ends (RLM-RACE) were performed to obtain its full-length cDNA according to the GeneRacer™ kit (Clontech, USA) manual. All primers used in this study were listed in Table 1. All PCR products were cloned into PMD19-T vector (TaKaRa, Japan) and sequenced at BGI (Beijing, China).

2.4. Bioinformatic and phylogeny analysis

The nucleotide of BmHDD1 was analyzed with the BioEdit program. The open reading frame (ORF) of BmHDD1 was confirmed by the ORF Finder software (http://www.ncbi.nlm.nih.gov/gorf.html). The molecular weight and isoelectric point was calculated using isoelectric point calculator (http://isoelectric.ovh.org/). The signal peptide and transmember domain were predicted by SignalP 4.0 (http://www.cbs.dtu.dk/services/SignalP) and TMpred (http://www.ch.embnet.org/software/TMPRED_form.html) respectively. The SMART (http://smart.embl-heidelberg.de/) was also used to analyze the predict protein.

2.5. Expression and purification of the recombinant protein

The full-length CDS sequence of BmHDD1 without the signal peptide, was cloned to PET22b vector, and transformed into *E. Coli* Rosetta (DE3). 0.1 mM IPTG was used for 24 h at 16 °C to induce the recombinant protein expression. Induced bacteria were centrifuged and washed with PBS, and re-suspended in PB buffer (19 mM NaH_2PO_4, 81 mM Na_2HPO_4, pH 7.4). After homogenized and centrifuged, the supernatant was filtrated and used for further purification. The sample was loading onto Ni-NTA His Bind Resin (Novagen, USA) and washed with imidazole gradient elution buffer. All fractions were collected and subjected to SDS-PAGE.

2.6. Polyclonal antibody preparation

Mice were immunized to prepare the polyclonal antibody against BmHDD1. For the first immunization, 50 μg proteins were used with mixed and homogenized with an equal volume of Freund's complete adjuvant (Sigma). For the following three subsequent immunizations, 75, 100, and 125 μg proteins were used with Freund's incomplete adjuvant respectively. 150 μg proteins were injected directly without any adjuvant in the fifth immunization. Three days after the last immunization, mice were killed and the serum was collected and stored.

2.7. Western blotting assay

Hemocytes and fat body were harvested, and suspended in RIPA Lysis Buffer for protein analysis. Protein concentrations were measured using a BCA protein assay kit (Beyotime Biotech, China). Cell lysate or cell-free hemolymph were subjected to SDS-PAGE and transferred onto PVDF membranes (Millipore, USA). After blocking with 5% BSA, the membranes were incubated gently with primary antibody against BmHDD1 (1:200) or Tubulin (1:1000, Beyotime) at 4 °C overnight. After washed with TBST buffer (20 mM Tris-HCl, 500 mM NaCl, and 0.1% Tween 20), the membranes were incubated with HRP-labeled goat anti-mouse IgG (H + L) (Invitrogen, 1:10000) at room temperature for 2 h. Proteins were visualized by SuperSignal West Femto Maximum Sensitivity Substrate (Thermo SCIENTIFIC) using a western blotting detection instrument (Clinx Science). The western blot bands were quantified using ImageJ software.

2.8. Immunofluorescence assay

Hemocytes were collected in the molting stage of 4th instar larva by cutting a leg and incubated for 15 min at room temperature. After attached, cells were washed and fixed with 4% PFA for 15 min. Samples were permeabilized using 0.5% Triton-X 100 for 15 min. Washed three times, cells were blocked with 10% goat serum for 2 h at 37 °C. Subsequently, the cells were incubated with anti-BmHDD1 serum (1:200) for 1 h at 37 °C. Cells washed three times and incubated with Alexa Fluor®488 goat anti-mouse IgG (H + L) (1:1000, Invitrogen) for 1 h. Hoechst 33342 (1:2000, Invitrogen) was used for nuclear staining. Cells were observed under an inverted fluorescence microscopy (Nikon 80i).

2.9. Bacteria challenge experiment

Two PAMPs (pathogen-associated molecular pattern) molecular, including PGN (Peptidoglycan from *Bacillus subtilis*, SIGMA), and LPS (Lipopolysaccharides from *Escherichia coli* 055:B5, SIGMA), and three types of bacteria, including *S. aureus*, *E. Coli*, and *P. aeruginosa*, were used in this challenging experiment.

0.2 μg PGN, or 1 μg LPS was injected into each larva on the third day of the 5th instar, respectively. PBS buffer was used for control. Hemocytes and fat body were collected at different time points. *S. aureus*, *E. Coli*, and *P. aeruginosa* were stored in our laboratory, and were cultured on Luria-Bertani (LB) broth at 220 rpm at 37 °C on a shaker. The cultured bacteria were inactivated by 0.1% PFA at 37 °C for 1 day. After washed, centrifuged, and re-suspended in the PBS buffer, bacteria were counted by a hemocytometer. On the third day of the 5th instar, larva was injected with either *S. aureus* (10^6 CFU/Larva), *E. Coli* (Two gradients, 10^6, and 10^7 CFU/Larva), or *P. aeruginosa* (10^6 CFU/Larva). The hemocytes, fat body, and cell-free hemolymph were collected at different time points for further analysis.

2.10. Injection of 20E

The second day of the 5th instar larva was chosen for 20E (20-Hydroxyecdysone, Sigma Aldrich, USA) at the dose of 1.5 μg/larva.