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Identification of immune response-related genes in the Chinese oak silkworm, *Antheraea pernyi* by suppression subtractive hybridization

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ABSTRACT

Insects possess an innate immune system that responds to invading microorganisms. In this study, a subtractive cDNA library was constructed to screen for immune response-related genes in the fat bodies of *Antheraea pernyi* (Lepidoptera: Saturniidae) pupa challenged with *Escherichia coli*. Four hundred putative EST clones were identified by suppression subtractive hybridization (SSH), including 50 immune response-related genes, three cytoskeleton genes, eight cell cycle and apoptosis genes, five respiration and energy metabolism genes, five transport genes, 40 metabolism genes, ten stress response genes, four transcription and translation regulation genes and 77 unknown genes. To verify the reliability of the SSH data, the transcription of a set of randomly selected immune response-related genes were confirmed by semi-quantitative reverse transcription-PCR (RT-PCR) and real-time quantitative reverse transcription-PCR (qRT-PCR). These identified immune response-related genes provide insight into understanding the innate immunity in *A. pernyi*.

1. Introduction

Innate immunity is a system for insects to defend against invading microbes and consists of cellular and humoral immunity (Elrod-Erickson et al., 2000; Iwanaga and Lee, 2005; Royet et al., 2005; Siva-Jothy et al., 2005). Humoral immune responses include antimicrobial peptides, enzymic cascades and the production of reactive oxygen species and nitrogen species (Marmaras and Lampropoulou, 2009; Tsakas and Marmaras, 2010). The cellular immunity refers to the phagocytosis, cytotoxicity, nodulation, melanization and encapsulation of foreign invaders conducted by hemocytes (Lavine and Strand, 2002). Both humoral immunity and cellular immunity could activate prophenoloxidase (pro-PO) cascade. The zymogen Pro-PO was triggered to be converted into its active form phenoloxidase (PO) quickly by pattern recognition proteins such as PGRP, βGRP and C-type lectins. However, this process was negatively regulated by serine protease inhibitors (serpins) (Cerenius and Soderhall, 2004; Ling and Yu, 2005; Liu et al., 2012).

Over the past years, insect innate immunity had been widely studied and genome-wide identification of immune response-related genes in insects had been described (Irving et al., 2001; Sackton et al., 2007), such as in *A. gambiae* (Christophides et al., 2002), *Aedes aegypti* (Waterhouse et al., 2007), *Apis mellifera* (Evans et al., 2006), *Tribolium castaneum* (Zou et al., 2007) and *B. mori* (Tanaka et al., 2008).

A. pernyi is one of the most well-known species among wild silkworms and commercially cultivated for silk production in China, India, and Korea (Liu et al., 2010). The pupae of *A. pernyi* are considered to be a source of high-quality protein foods containing all the essential amino acids (Zhou and Han, 2006). Moreover, *A. pernyi* has become an excellent natural bioreactor for the production of recombinant proteins (Huang et al., 2002). To investigate the mechanisms *A. pernyi* uses in response to pathogen infection, a subtractive cDNA library was constructed to screen for immune response-related genes in the fat bodies of pupae challenged with *E. coli*.

2. Materials and methods

2.1. Insects

A. pernyi was originally from the Sericultural Research Institute of Henan and reared on the leaves of oak until the pupation. The pupae were collected and kept at room temperature.

2.2. E. coli challenge

One million *E. coli* cells in 10 μl sterile saline (0.85% NaCl) were inactivated at 85 °C for 1 h and then injected into 5-d old *A. pernyi*

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pupae. The control pupae were injected injected with 10 μl sterilized saline. Fat bodies were collected at 0.5, 3, 6, 12, 24, 48 and 72 h post-infection and frozen immediately in liquid nitrogen and stored at −80 °C.

2.3. RNA extraction and cDNA synthesis

Total RNA was extracted with Trizol reagent (Invitrogen, USA) according to the manufacturer's instructions. The RNA integrity was evaluated by gel electrophoresis. The mRNA was purified using Oligotex™-dT30 (Super) mRNA Purification Kit (TaKaRa, China). The Super SMART PCR cDNA Synthesis Kit (Clontech, USA) was used for double-stranded cDNA synthesis according to the manufacturer's instructions.

2.4. Construction of subtractive cDNA library

PCR-Select cDNA Subtraction Kit (Clontech, USA) was used to generate a cDNA library of differently expressed genes in pupae upon bacterial infection. In brief, the infected sample was defined as "tester" and the control sample as "driver". Tester and driver cDNAs were digested with *Rsa* I endonuclease to yield blunt ends appropriate for ligation. The digested tester sample was subdivided into two portions, and each was ligated with a different adaptor (Adaptor 1 or 2R). Then two separate tester samples were added to an excess of driver sample, and hybridized again by adding a fresh volume of driver. As cDNAs present in both the tester and driver populations reannealed to form double-stranded cDNAs, any remaining single stranded molecules should represent uniquely expressed genes. These single stranded tester cDNAs were then targeted for PCR enrichment via adaptor-mediated priming. The amplified DNA fragments were ligated into a pMD-19 T vector (TaKaRa, China) for cloning.

2.5. Expressed sequence tags sequencing and annotation

Four hundred positive clones were randomly selected from the cDNA library and sequenced using the M13 forward primer by an Applied Biosystems 3730 analyzer (Sunbiotech, China). Raw sequences were first trimmed to remove vector sequences and low-quality sequences using the program "Crossmatch". ESTs with length less than 60 bp were also discarded. The high-quality sequences were assembled and clustered using the program CAP3 with the default options (http://pbil.univ-lyon1.fr/cap3.php). EST sequence annotation was performed using the blast tools in NCBI web site (http://blast.ncbi.nlm.nih.gov/Blast) and DNAStar package (DNAStar, USA).

2.6. Sequence alignment and phylogenetic analysis

The deduced amino acid sequences of cecropin and lysozyme were translated according to the corresponding cDNA sequences, and the amino acid sequences of the cecropin or lysozyme from different organisms used for phylogenetic analysis were downloaded from GenBank database. Multiple sequence alignments were carried out using Clustal X software (Thompson et al., 1997). A Neighbor-joining (NJ) tree was constructed by MEGA version 5 with bootstrap test of 1000 replicates (Tamura et al., 2011).

2.7. Detection of the transcription profile for candidate genes by semi-quantitative RT-PCR

Total RNA from the fat bodies of pupae was extracted with Trizol reagent (Invitrogen, USA). The RNA sample was treated with RNase-Free DNase (Promega, USA) to remove genomic DNA contaminants and was then used to synthesize the first strand cDNA by TUREscript cDNA Synthesize Kit (Aidlab, Beijing) following the manufacturer's instructions. The synthesized cDNA was used as

Table 1
A list of all PCR primers used in this study.

Primer name	Forward primer (5–3′)	Reverse primer (5-3′)	Purpose
Beta-1,3-glucan recognition protein	AAGCGGGTCATTGGTCTC	GTTGAAGGTTTCGGTGGG	RT-PCR, qRT-PCR
Peptidoglycan recognition protein A	TATTGGGATATTGGTTCAG	CATTACTCACTCCGCATC	RT-PCR, qRT-PCR
Peptidoglycan recognition protein	AATGAATTTCGTCGTGGGC	CGGTGGCGAGGACTTGTT	RT-PCR
Hemolin like protein	CAGGCGTCAGATGAAGGT	GCAGCGATCAAGTAAGTCC	RT-PCR, qRT-PCR
Hemolin	GGAAAGCGAGTCGGTGAG	CATAGAGTGCGTCTGTGCC	RT-PCR, qRT-PCR
Immunlectin A	GCACAGAACGCTCATCTACT	TCTCGTGCTATTTCCAACA	RT-PCR, qRT-PCR
Attacin-1	GGGTGGGAAACTGAAT	CCAAGAGGTCCTAAAGTG	RT-PCR
Basic attacin-1	GGAAGAAGTTCGATACGC	ACGGCAATTATGGTTTAT	RT-PCR
Cecropin B	TGCCTTCGTCAACAGT	GCTTTGGCTTCTCCTA	RT-PCR
Defense protein 1	CGACGACTCATTTCAT	GGTACGGAGTGTTTCTA	RT-PCR
Gallerimycin	GGGGAAGAAGAAATAGGT	AATTTAGGCATTGGCATC	RT-PCR
Lebocin like protein 2	GTGTTCACCCGGAGCATC	AGTTATAGGCGTAAATTGGA	RT-PCR, qRT-PCR
Lebocin-like protein 3	CAGTAAGCATCGTGGTGG	AATTACGGCGATTGTAGC	RT-PCR
Gloverin	GACGGGTTATTCGGTAAA	GAGATCAAGAGCGGCATT	RT-PCR
Protease inhibitor protein 2	AGGCAATGAGTCACTTTCG	TGCAGTCTTCAGGCAAAA	RT-PCR
Protease inhibitor protein 4	ATTATCGCTTGACGAGTGC	TGTGGTGGTTGGTGGG	RT-PCR
Protease inhibitor protein 7	GAAACACGAAAACTGGG	GAATATGCCGAAATACTTAA	RT-PCR
Serpin like protein 2	GTCCACCACCGGATTTCC	ATCCGTCTGGAGGGTTAC	RT-PCR
Serpin-5B like protein	TCATGTTTACATTCCCTATCAC	TCCTCTGGGCGTCACCTT	RT-PCR
Microvitellogenin	CCTATCTAAAATCTTCAT	CGTTTGTTTTGTATAATC	RT-PCR
Lectin5	ACCCAAATCAGAGGAAGA	AGCCCACCTGTGATACCC	qRT-PCR
Cecropin-like protein 4	TCAGGCTACAGCATTGGC	CGGTTGATGAAGGGTTATTT	qRT-PCR
Lysozyme	AGCAAACTAAGTAAATCTC	CACAATGTAAACACGAAA	qRT-PCR
Protease inhibitor protein 3	TGAGGTGTACTTATGGAATGCT	CTCCACTAAGGCCACGCT	qRT-PCR
Protease inhibitor protein 5	ACCTGTCCCGTAACTCC	AACTTGTCACTGGTGGG	qRT-PCR
Protease inhibitor protein 6	TTGCACCAACATTAAATTCCAG	CACTTGCCGTCGCTGATT	qRT-PCR
Protease inhibitor protein 8	GCCGAGTAACATTGGATA	CAGTTGTTGAAGGCAGAT	qRT-PCR
Serine protease like protein 1	CGATATGTTTACCGAAGCC	TTGGTCTAAGATGTTGTGCC	qRT-PCR
Serpin like protein 4	GATGGACCTGGCTCTGTT	CGTCGATACTGTCCGATGA	qRT-PCR
Dopa decarboxylase	ATCTGGCTGCATGTGGAT	ACCCTGTTGGTCGTGTTT	qRT-PCR
Glutathione S-transferase theta	AGCCGTCCTCCTCACA	CAGGTTTCAAGTGCTCCC	qRT-PCR
18S rRNA	CGATCCGCCGACGTTACTAC	GTCCGGGCCTGGTGAGATT	RT-PCR, qRT-PCR