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Gene expression patterns in response to pathogen challenge and interaction with hemolin suggest that the Yippee protein of *Antheraea pernyi* is involved in the innate immune response

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ABSTRACT

Yippee was first identified as a protein that physically interacts with the Hemolin protein of *Hyalophora cecropia*. In this study, we identified a gene with a 366 bp open reading frame (ORF) that encodes a 121 amino acid protein containing a conserved Yippee domain. We named this gene *Ap-Yippee* (*Yippee* gene from *Antheraea pernyi*), and investigated the role of the protein in the host immune response. A recombinant Ap-Yippee protein was expressed in *Escherichia coli* cells, and polyclonal antibodies were produced against the recombinant protein. Real-time PCR and a Western blot analysis revealed that Ap-Yippee is expressed in the hemocytes, Malpighian tubules, midgut, silk gland, epidermis, and fat bodies of *A. pernyi*, with the highest expression level observed in Malpighian tubules. The fifth instar larvae of *A. pernyi* were challenged by injecting them with nucleopolyhedrovirus (AP-NPV), the Gram-negative bacterium *E. coli*, the Gram-positive bacterium *Micrococcus luteus*, or the entomopathogenic fungus, *Beauveria bassiana*. These challenges with diverse pathogens resulted in differential expression patterns of the protein. A knockdown of the *Ap-Yippee* gene by small interfering RNA (siRNA) transfection had a significant influence on the expression of the hemolin in the pupae which was confirmed by qRT-PCR and Western blot. Furthermore, a possible protein-protein interaction between *Ap-Yippee* and Hemolin was explored by Far-Western blotting. Therefore, our data suggest that the Ap-Yippee protein is involved in a pathway that regulates the immune response of insects.

1. Introduction

Insects and other invertebrates have robust innate immune systems, which include both humoral and cellular immunity to defend against invading microbes (Iwanaga and Lee, 2005; Wu et al., 2010). Upon microbial challenge, immune effector genes, such as the immunoglobulin superfamily member, Hemolin, as well as anti-microbial peptides (e.g., lysozymes) are up-regulated primarily within in the fat body and hemocytes (Duvic et al., 2012; J. Li et al., 2012; J.F. Li et al., 2012; Sun et al., 2015).

Hemolin is a bacteria-inducible immunoglobulin-like protein, and has been reported to be involved in a diverse range of cellular interactions (Schmidt et al., 2010), including the regulation of embryonic development (Bettencourt et al., 2002), metamorphosis (Roxstrom-Lindquist et al., 2005; Yu and Kanost, 1999), and various immune responses (Eleftherianos et al., 2007; Li et al., 2005; Yu and Kanost, 2002).

Drosophila Yippee was initially identified as a protein that interacts with the blood protein Hemolin of the moth *Hyalophora cecropia* (Roxstrom-Lindquist and Faye, 2001). Subsequently, Yippee was found to be highly conserved among eukaryotes (Hosono et al., 2004). The YPEL (Yippee-like protein) family of proteins are found in virtually all eukaryotes, including fungi, plants, and animals, and all members exhibit extremely high levels of sequence homology (Hosono et al., 2010). A previous study demonstrated that Yippee is an intracellular protein that contains two cysteine pairs, 52 amino acids apart (Cys-x2-Cys-x52-Cys-x2-Cys) that could be part of metal (zinc) binding pocket (Roxstrom-Lindquist and Faye, 2001). Conservation of the zinc-finger-like metal-binding domains and the subcellular localization suggests an important role for Yippee in the cell cycle (Liang et al., 2010). The Yippee protein is known to be important in eukaryotes, that is involved in cell proliferation and growth and studies describing the various functions of this protein have been reported since 2001

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(Hosono et al., 2010, 2004). However, little is known about the function of Yippee in innate immunity.

Antheraea pernyi (Lepidoptera: Bombycoidea) is one of the most well-known species of wild silkworms that is commercially cultivated for silk production in China, India, and Korea (Liu et al., 2010). Since Yippee has been shown to interact with the bacteria-inducible immunoglobulin-like protein Hemolin, we performed Far-Western blotting between *Ap-Yippee* and Hemolin to determine whether these two proteins directly bind to each other and, if not, whether a third protein mediates the formation of a physical complex between them. Far-Western blotting is widely used to (1) identify the binding partners of a bait protein (Cantor et al., 2001; Gupta et al., 2007); and (2) confirm one-to-one protein–protein interactions identified by high-throughput screening or other methods (Feldman et al., 2005; Wu et al., 2006).

We then investigated the expression patterns and distribution profiles of Yippee in *A. pernyi* (Ap-Yippee) in response to various microorganism challenges in this study. Additionally, we used RNAi to elucidate the relationship between Yippee and Hemolin. The results of our study provide further insight into the role of Yippee in the innate immune response of *A. pernyi*.

2. Materials and methods

2.1. Experimental insects

A. pernyi was provided by the Sericultural Research Institute of Liaoning, China, for all experiments. The *A. pernyi* larvae were fed fresh oak leaves and housed at room temperature with a 10:14-h light: dark photoperiod with 70% relative humidity until they pupated.

2.2. RNA extraction and cloning of the Yippee gene

The total RNA was isolated from the fat bodies using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and the first-strand of the cDNA was synthesized using the TransScript Synthesis SuperMix (TransGen, Beijing, China). Degenerate primers (Oligonucleotide primers) (Table 1) were designed using the Primer Premier 5.0 software package to amplify the cDNA fragment of the *Yippee* gene. PCR was performed using an amplification program as follows: 5 min at 94 °C, followed by 35 cycles at 94 °C for 30 s, one cycle at 55 °C for 40 s, one cycle at 72 °C for 30 s, and a final elongation step at 72 °C for 5 min. We analyzed the PCR products by 1% agarose gel electrophoresis, and they were then sequenced by Invitrogen (Invitrogen, Shanghai, China).

Table 1
Oligonucleotide primers used for PCR in this study.

Primer name	Purpose	Primer sequence (5′–3′)
Yippee F	Oligonucleotide primers	AACTTGCAAAAATGGGAAGAATAT
Yippee R		GACTTAACTCAAAAACAACAGATA
F YP	Protein expression	GGCGGATCCATGGGAAGAATATTT
R YP		CGCCTCGAGGTATACTAGGTTAAC
F He-RT	Real-time PCR	TATGATGGCGAAGGCTGGT
R He-RT		AGGTTCTATTGTGGCGGGTG
F YP-RT		TGGCAGAGCTTCTTGTTCCATAAA
R YP-RT		TAAACCCAGCCGAGCTTAGTACCGC
F18S		CGATCCGCCGACGTTACTACA
R18S		GTCCGGGCCTGGTGAGATTT
F YP-siRNA	RNAi	CCUUACGAAUCGUGCUCAATT
R YP-siRNA		UUGAGCACGAUUCGUAAGGTT
Negative control F		UUCUCCGAACGUGUCACGUTT
Negative control R		ACGUGACACGUUCGGAGAATT

Note: single underline (———) indicates a *BamH*I recognition site. Double underline (═══) indicates an *Xho*I recognition site.

2.3. Nucleotide sequence analysis

NCBI bioinformatics tools (available at http://blast.ncbi.nlm.nih.gov/Blast.cgi) were used to detect the conserved domains of Ap-Yippee. Additionally, the molecular weight of the Ap-Yippee protein was calculated by ExPASy (http://web.expasy.org/compute_pi/). Multiple sequence alignments were performed using the Clustal X program with its default parameters (Livak and Schmittgen, 2001).

2.4. Prokaryotic expression and protein purification

To express the recombinant Ap-Yippee protein in *Escherichia coli*, a pair of specific primers (F YP and R YP) was designed to amplify the 564 bp DNA fragment containing the entire ORF. The ORF sequence was cloned into pMD-19T, digested with restriction enzymes (Bam HI and Xho I), and then ligated into the pET-28a (+) vector (Novagen, USA). The insertion of the recombinant plasmid, pET-28a-*Ap-Yippee*, was confirmed by DNA sequencing, and it was then transformed into *E. coli* BL21 (DE3) (Novagen, USA) for protein expression. After a short-term culture for 4 h and isopropyl-β-D-thiogalactopyranoside (IPTG) induction at a final concentration of 0.8 mM, the cells were harvested by centrifugation at 8000*g* for 5 min. The cell pellets were resuspended in a binding buffer (20 mM Tris–HCl, 500 mM NaCl, 5 mM imidazole, pH 7.9) and disrupted by sonication on ice. After centrifugation at 12,000*g* for 20 min at 4 °C, the recombinant proteins were purified using the express® Ni-NTA Fast Start Kit (Qiagen, Germany) according to the manufacturer's protocol. The recombinant proteins were analyzed by 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting.

2.5. Antibody preparation

The 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-Yippee protein that was homogenized in complete Freund's adjuvant. A booster injection was administered one week later. The rabbit serum was collected seven days after the final immunization, and stored at −80 °C. A monoclonal anti-6-His antibody (Qiagen, Germany) was used to verify protein expression and the molecular weight.

2.6. Expression analysis using quantitative RT-PCR

The total RNA was extracted from hemocytes, fat bodies, midguts, epidermis, silk glands, and Malpighian tubules of three larvae using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. The RNA samples were then reverse transcribed into cDNA. Oligonucleotide primers specific for the *Ap-Yippee* sequence and the endogenous control (18S rRNA, accession number: DQ347469) were designed by the Primer 5.0 software based on known sequences (Table 1). Real-time PCR was performed in 25 μL reactions containing 12.5 μL of 2× SYBR Premix Ex TaqII (Takara), 1 μL each of the forward and reverse primers, 2 μL of the cDNA, and 8.5 μL of RNase-free H_2O. The amplification program was performed as follows: 95 °C for 30 s; 39 cycles at 95 °C for 15 s, 1 cycle at 58 °C for 30 s, and 1 cycle at 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