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Cloning, Expression and Analysis of a Novel Defense Gene from *Antheraea pernyi*

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Abstact.- In this study, we first reported the defense gene (*ApDef*) in lepidoptera insect, the Chinese oak silkworm *Antheraea pernyi* (Lepidoptera: Saturniidae). It consists of 677 bp with a putative open reading frame (ORF) of 492 bp encoding 163 amino acids residues. A signal peptide with 18 amino acids and a typical reeler domain was found in ApDef protein. Phylogenetic analysis indicated that *ApDef* gene had the closest genetic distance with *Antheraea mylitta* Defense. The results of real-time quantitative PCR (qRT-PCR) showed that this gene was highly expressed in fat body, low expressed in hemocytes and midgut, and not expressed in epidermis. After being challenged by three types of pathogens (*Escherichia coli*, *Micrococcus luteus* and *Beauveria bassiana*), the expression levels of *ApDef* were up-regulated significantly compared with the control, and reached the maximum at 12 h post infection. These data indicated that *ApDef* may play an important role in innate immune responses of *A. pernyi*
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Key words: Defense gene, Reeler domain, *Ap Def* Gene, *Antheraea pernyi*, qRT-PCR, Innate immune, Chinese Oak silkworm.

INTRODUCTION

Immune response is usually divided into two branches, innate immunity and acquired immunity. Unlike vertebrates, invertebrates don't have complex and memory property acquired immune system, but only rely on the innate immune system to resist the invasion of pathogens and infection (Kimbrell and Beutler, 2001; Jiravanichpaisal *et al*., 2006). Insects, as the largest groups in invertebrates, living in a complex environment, have the quite perfect innate immune system, which can mediate immune process quickly and efficiently through specific proteins (Gillespie *et al*., 1997; Lavine and Strand, 2002; Cherry and Silverman, 2006). Pathogen-associated molecular patterns, PAMP, were widely existed on the surface of pathogenic microorganisms, such as lipopolysaccharide, LPS (Gram negative bacteria); peptidoglycan, PGN (Gram negative bacteria) andβ-1,3-glucan (Fungi) (Medzhitov and Janeway, 2002). Insects can identify the PAMP effectively through their membrane receptors and conservative or induced specific proteins, then regulate and stimulate the humoral and cellular immune response through activating a series of signal pathways, such as Toll, IMD, JAK-STAT, JNK, Ras/Rho, NO and so on (Williams *et al*., 2005, 2006; Dijkers and O' Farrell, 2007; Lamprou *et al*., 2007; Sackton *et al*., 2007).

A type of immune-related defense gene, with an typical reeler domain, have been identified in lepidopteran insects, such as *Samia ricini*, *Antheraea mylitta*, *Bombyx mori*, *Lonomia oblique*, *Hyphantria cunea* and *Manduca sexta* (Shin *et al*., 1998; Bao *et al*., 2003; Zhu *et al*., 2003; Veiga *et al*., 2005; Gandhe *et al*., 2007; Bao *et al*., 2011). The reeler domain was initially identified from a secreted glycol protein in the mouse (D'Arcangelo *et al*., 1995). In mammals, it plays an important role in the development of the central nervous system (Quattrocchi *et al*., 2002). In insects, it can be induced by pathogenic microorganism challenge and participated in both cellular (nodule formation) and humoral (melanization cascade) immune responses (Bao *et al*., 2011). So we speculated that AsDef protein in insects may be involved in various signaling pathways for different types of microbial infection.

Antheraea pernyi (Lepidoptera: Saturniidae) is an economically valuable silk-producing insect and commercially cultivated mainly in China, India and Korea. It contained a large number of bioactive peptides/proteins, which have an obvious inhibitory effect against bacteria, fungi, virus and cancer cells (Kavanagh and Reeves, 2004; Strand, 2008; Liu, *et*

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al., 2010). Here, we cloned and characterized a defense gene with a typical reeler domain from *A. pernyi*, and described its expression profiles after the different pathogens challenge. This will provide the foundation for further functional research on insects reeler domain Defense proteins.

MATERIALS AND METHODS

Experimental animals

The *A. pernyi* larvae were kindly provided by the Sericultural Research Institute of Henan and reared on the leaves of oak until pupation (Wei *et al*., 2011). The heat-inactived gram-negative bacterium (*Escherichia coli* 5 µL, 10^9 cfu/mL), gram-positive bacterium (*Micrococcus luteus* 5 µL, 0.5 mg/mL) and fungi (*Beauveria bassiana* 5 µL, 10^9 cfu/mL) were used for injecting into the abdomen of *A. pernyi* larvae (Wang *et al*., 2014). All the microorganism pellets were diluted in the sterilized PBS. The larvae injected with sterilized PBS were used as negative control. Six larvae from each group were dissected to collect fat body, midgut, hemocytes, malpighian tubule and epidermis at different time points after injection, respectively. These tissues were frozen immediately in liquid nitrogen and stored at -80°C.

Total RNA extraction and cloning of the ApDef *gene*

Total RNA was extracted with TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions. The purity and quantity of the extracted RNA were quantified by the ratio of OD_{260}/OD_{280} by a NanoDrop 1000 spectrophotometer (NanoDrop Technologies Wilmington, DE). Using 500 ng of total RNA per sample to generate first strand cDNA with PrimeScript™ One Step RT-PCR Kit Ver.2 (Takara, Japan) following the manufacture's instruction. Oligonucleotide primers (Table I) were designed by Primer Premier 5.0 software based on the reported defense sequences from *A. mylitta*. PCR conditions were denaturation at 94°C for 5 min, followed by 30 cycles of 94°C for 30 s, annealing at 55 °C for 40 s and extension at 72 °C for 1 min. 5'- and 3'-RACE were performed according to the manufacture's introduction. The PCR products were separated on 1% agarose gel and purified with Gel Extraction Mini Kit (Aidlab, China). Purified PCR products were then ligated into the pMD19-T simple cloning vector (Takara, Japan) and transformed into competent cells of *E. coli* strain DH5α. Positive clones were sequenced at Invitrogen, Shanghai.

Table I.- Primers used in this study.

Primers number	Primers sequence
DF1	5'-GGCGGATCCATGTTAAAATTGTCATTTG-3'
DR1	5'-CAGAAGCTTTTATTTTACATTGACATTG-3'
DF2	5'-CGGACTTCAGATATCGTG-3'
DR2	5'-GACGGCGTTATTAGGTTC-3'
5' RACE-G1	5'-CGGGAGCAGTCCAGAGGTATGAGA-3'
5' RACE-G2	5'-GACCAGGATGTGGTTGTGGTTCAG-3'
3' RACE	5'-TGAAGTAACCATCAGCGGCAATA-3'

§ "___" present restriction enzyme cutting site: *Bam* HI; "___" present restriction enzyme cutting site: *Hind* III

Sequence analysis

The molecular weight (MW) and the isoelectric point (pI) of the deduced amino acid sequences were calculated by ExPASy (http://web.expasy.org/compute_pi/);hydrophobicity was analyzed by Protscale (http://web.expasy.org/protscale/); transmembrane structure was analyzed online (http://www.ch.embnet.org/software/TMPRED_form.html); signal peptide was predicted by SignalP (http://www.cbs.dtu.dk/services/SignalP-2.0/); other species sequences were downloaded from NCBI public databases (http://www.ncbi.nlm.nih.gov/); phylogenetic tree was constructed by MEGA version 5.0 using the neighbor-joining (NJ) method with bootstrap test of 1000 replications.

Protein expression, purification and antibody preparation

The PCR products were ligated to pET-28 (a+) after digesting with corresponding restriction enzymes. The recombinant plasmids pET-28*a*-defense were identified by sequencing, then transformed into competent *E. coil* BL21 (DE3) cells for protein expression induced by different concentrations of IPTG. The recombinant proteins were analyzed with 12% SDS polyacrylamide gel electrophoresis (SDS-PAGE) and western-blotting. nickel-nitrilotriacetic acid (Ni-NTA) affinity chromatography (Qiagen, Germany) was used to

purify the recombinant proteins according to the instructions. The purified fusion proteins that were homogenized in complete Freund's adjuvant were used to immunize male New Zealand rabbits for three times at two-week intervals. A boost injection in incomplete Freund's adjuvant was given for another week. Rabbit serum was collected seven days after the last immunization. Antiserum was prepared according to the method described (Harlow and Lane, 1999).

Western blotting

Proteins that prepared for SDS-PAGE were subjected to SDS-PAGE and then transferred to a polyvinylidene difluoride (PVDF) membrane by an electrophoretic transfer system (Bio-Rad). Membranes were blocked with 5% non-fat milk in PBST (PBS contained 0.1% Tween-20) for 2 h at room temperature, then washed with PBST five times at 5 min interval and subsequently incubated with primary antibodies for 2 h at room temperature. After washing with PBST, membranes were incubated with horseradish peroxidase (HRP)-conjugated sheep antirabbit IgG antibody (Sigma) for 1 h at room temperature. The final detection was performed with a HRP-DAB Detection Kit (Tiangen, China).

qRT-PCR analysis

Total RNA of each tissue was extracted with TRIzol reagent (Takara, Japan) according to the manufacturer's instructions. Then, RNA samples were treated with RQ1 RNase-free DNase (Promega, USA) to remove genomic DNA contamination. 5μg of total RNA was reverse transcribed into cDNA with M-MLV reverse transcriptase (Invitrogen, USA). The gene-specific primers (Table I) were designed by Primer Premier 5.0 software. qRT-PCR was carried out to measure the expression levels of *ApDef* in various tissues and different treatments. The PCR reaction was performed in a total volume of 25 μL containing 12.5 μL 2 × SYBR Premix Ex TaqII (Tli RNase Plus) (Takara, Japan), 1 μL of each primer, 1.5 μL of 1:8 diluted cDNA templates and 9 μL RNase-free H_2O. QRT-PCR was performed using an CFX96^{TM} real-time detection system (Bio-Rad, USA) using the following procedure: initial denaturation at 95 °C for 30 s, followed by 40 cycles of denaturation at 95 °C for 5 s, annealing at 58 °C for 30 s, and a final extension at 72 °C for 25 s. The relative quantitative method ($2^{-\Delta\Delta CT}$) was used to determine the expression level changes (Livak and Schmittgen, 2008). All data were represented as mean±SD and analyzed by Student's *t*-test, differences were considered statistically significant when *p*-values less than 0.05.

RESULTS

Sequence analysis of ApDef

A cDNA fragment of 677 bp was obtained by RT-PCR and RACE-PCR (Fig. 1A). Nucleotide sequence analysis revealed that the *ApDef* contained a putative ORF of 492 bp encoding 163 amino acids residues. A signal peptide with 18 amino acids and a typical reeler domain was found in ApDef protein. Sequence alignment showed that *ApDef* had 49.7%, 50.9%, 70.2%, 64.6% and 88.2% identity with *M. sexta*, *B. mori*, *S. ricini*, *L. obliqua* and *A.mylitt ApDef*s, respectively (Fig. 1B). Phylogenetic analysis indicated that *ApDef* gene had the closest genetic distance with *A. mylitta* defense (Fig. 1B).

Prokaryotic expression, protein purification and polyclonal antibody preparation

A recombinant protein with a molecular weight of about 18 kDa from *E. coli* BL21 (DE3) was detected by SDS-PAGE, and its expression level was not influenced by different IPTG concentrations (Fig. 2A). Western blotting of recombinant proteins using the anti-His-tag antibody suggested that a consensus protein of 18 kDa was detected (Fig. 2B). All of these indicated that the recombinant protein obtained stable expression in *E. coli* cells. Soluble fusion proteins were successfully purified and detected by SDS-PAGE showing a single band in the gel (Fig. 2C).

Expression of ApDef *in various tissues*

qRT-PCR and immunoblot were carried out to determine the expression levels of *ApDef* in different tissues including the fat body, midgut, hemocytes, malpighian tubule and epidermis of *A. pernyi*. Actin gene was used for normalization. Interestingly, the results for qRT-PCR and western

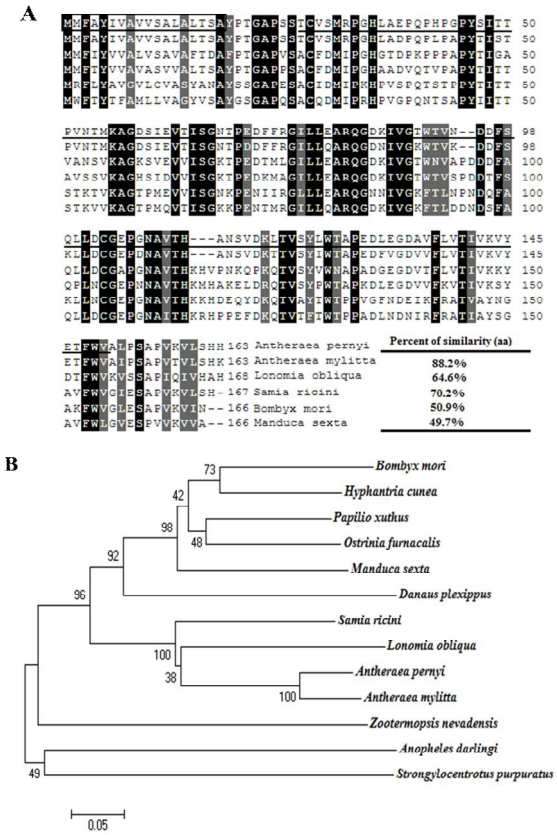

Fig. 1. Sequence and phylogenetic analysis of ApDef protein.
(A) Sequence alignment of Defense proteins from *A. pernyi* and other organisms. Identical and similar amino acids were presented in black and gray, respectively. Comparisons of similarity in full-length amino acid sequence between Defenses proteins were shown in the lower corner. The signal peptide is boxed, Reeler domain is dark underlined. (B) Phylogenetic analysis based on the defense amino acid sequences from various insects. The phylogenetic tree was constructed using the neighbor-joining algorithm method and bootstrap values (1000 repetitions) of the branches were indicated. The origins and accession numbers of the Defenses sequences were: *Antheraea mylitta* (Q0Q028); *Lonomia obliqua* (Q5MGQ0); *Samia ricini* (Q765V4); *Strongylocentrotus purpuratus* (XP_003727312); *Danaus plexippus* (EHJ65746); *Ostrinia furnacalis* (AGV28583); *Manduca sexta* (Q86RS3); *Bombyx mori* (NP_001243939); *Antheraea pernyi* (KJ913669); *Hyphantria cunea* (O96382)、 *Zootermopsis nevadensis* (KDR14834); *Anopheles darling* (ETN58185); *Papilio xuthus* (BAM17850).

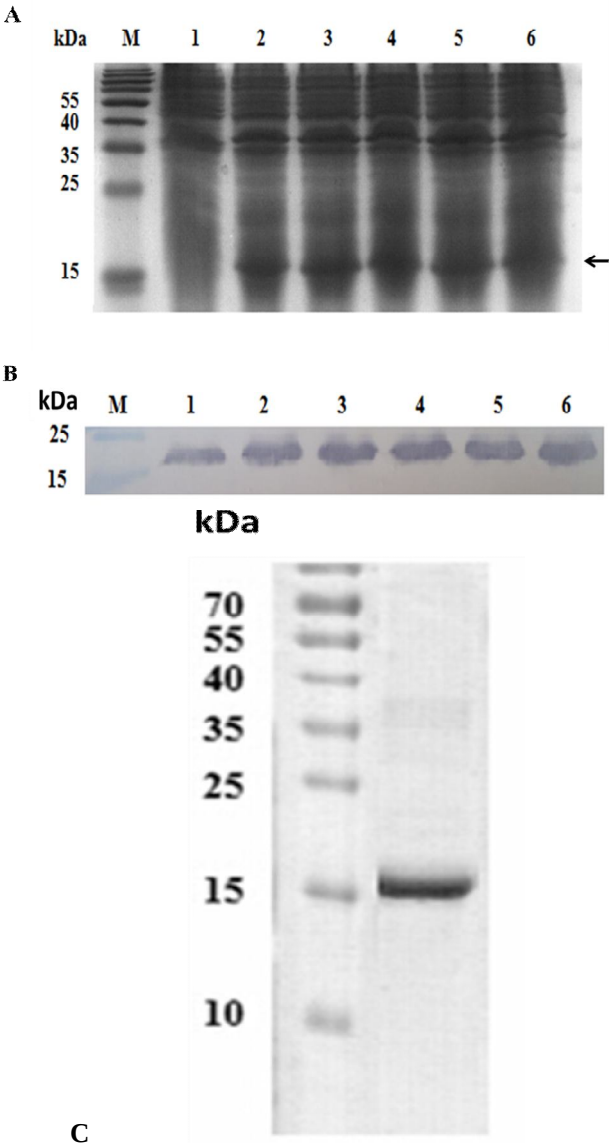

Fig. 2. Analysis of recombinant ApDef protein. (A) The fusion protein detected on 12% SDS-PAGE gels and (B) the corresponding western blot analysis. (C) The purified protein. The gels were revealed by Coomassie blue R-250 staining. Bacterial proteins were collected after 4 h of IPTG induction. M: molecular weight marker; Lane 1: before induction; Lanes 2-6: after induction by 0.3, 0.6, 0.9, 1.2, and 1.5 mM IPTG.

blot analysis were just consistent. They both showed especially high expression levels of *ApDef* in fat body, while low in midgut, hemocytes, malpighian tubule and epidermis tissues (Fig. 3).

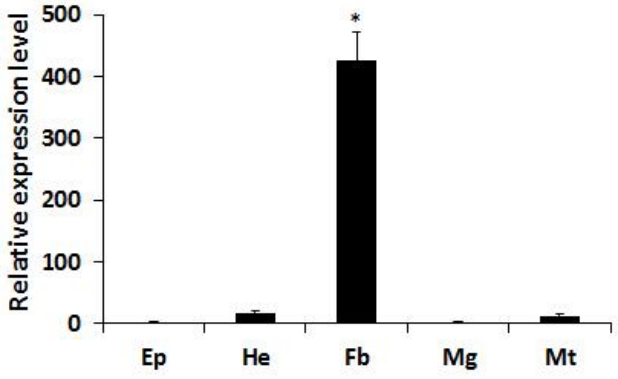

Fig. 3. Expression profiles of *ApDef* mRNA in different tissues. Ep: epidermis; He: hemocyte; Fb: fat body; Mg: midgut; Mt: malpighian tubule. All data were analyzed by one-way analysis of variance. The values were presented as mean±SD of independent experiments done in triplicates and analyzed by Student's *t*-test, **P*<0.05 when compared to control values.

Induced expression profiles of ApDef

To further understand the induced expression profiles of s *ApDef* gene in response to a microbial infection, qRT-PCR was performed by fat body. Fat body was prepared after 0, 1.5, 3, 6, 12, 24 and 48 h challenge by three types of pathogens (*E. coli*, *M. luteus* and *B. bassiana*), respectively. Results revealed that the expression levels of *ApDef* were obviously increased from 1.5 to 48 h after infection and reached the maximum at 12 h (Figs. 4A, 4B and 4C). These results suggested that *ApDef* played an important role in defending against many types of pathogenes infection.

DISCUSSION

In this study, we reported for the first time the cloning, characterization and identification of a defense gene from *A. pernyi*. Based on homologous alignment and phylogenetic analysis, the defense protein from *A. pernyi* was highly homologous to that of *A. mylitta*. ApDef protein has an obvious signal peptide sequence and a reeler domain, which are similar with other insect defense protein. This will provide effective data to analyze the biological functions of *A. pernyi* defense.

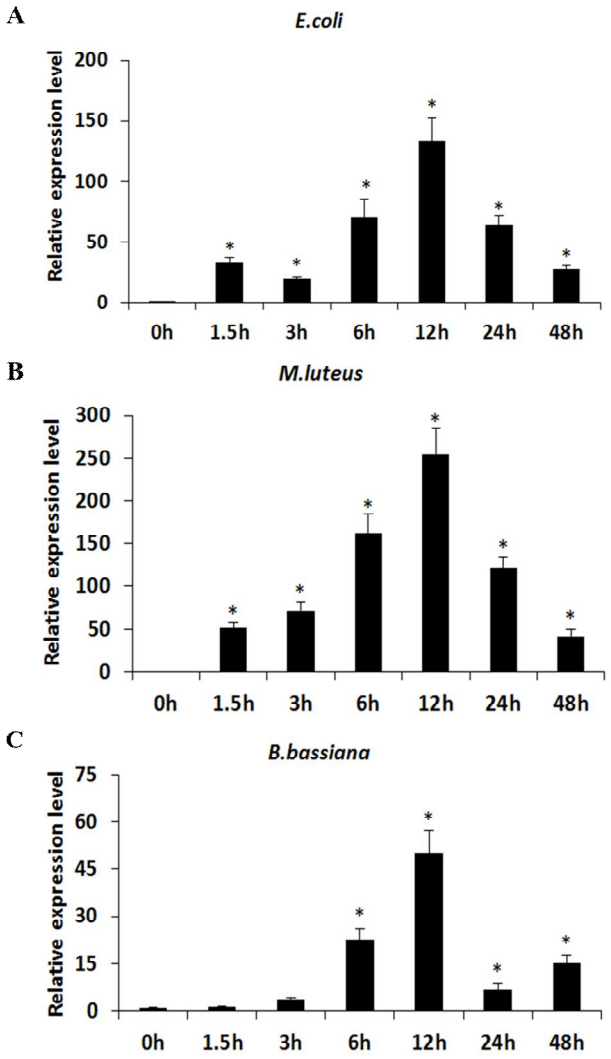

Fig. 4. Induced expression profiles of *ApDef* in fat body at the 5th instar larvae of *A. pernyi*. Fifth-instar larvae (day 3). Fat body was collected at 0, 1.5, 3, 6, 12, 24 and 48 h post treatments, respectively. The group injected with PBS was used as the calibrator. (A) injected with Gram-negative bacterium (*E. coli*); (B) injected with Gram-positive bacterium (*M. luteus*); (C) injected with fungi (*B. bassiana*). All data were analyzed by one-way analysis of variance. The values were presented as mean±SD of independent experiments done in triplicates and analyzed by Student's *t*-test, **P*<0.05 when compared to control values.

Compared with the other examined tissues, the expression levels of *ApDef* were highest in fat body. To investigate the role of *ApDef* in response to

microbial infection, the expression of *ApDef* was determined after being challenged by three different types of pathogens. The transcriptional expression profiles of *ApDef* indicated that its expression level was significantly up-regulated in fat body post bacterial infection. Also, among all types of pathogens used in experimental treatments, we found the transcriptional level of *ApDef* reached the highest post *M. luteus* injection, Therefore, we considered the *ApDef* showed stronger bactericidal activity in response to the Gram-positive bacterium than to the Gram-nagative bacterium and fungi. All the results suggested that *ApDef* played an important role in defending against the pathogens infection. Although the characterization and expression of *ApDef* were investigated, their physiological functions still need further study.

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