

Article

cDNA Cloning and Expression Analysis of Pattern Recognition Proteins from the Chinese Oak Silkworm, *Antheraea pernyi*

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Abstract: Pattern recognition receptors play an important role in insect immune defense. We cloned the β -1,3-glucan recognition protein, lectin-5 and C-type lectin 1 genes of *Antheraea pernyi* and examined the expression profiles of immune-stimulated pupae. After infection with *Bacillus subtilis*, *Escherichia coli*, *Antheraea pernyi* nuclear polyhedrosis virus (ApNPV) and *Saccharomyces cerevisiae*, respectively, the pupae showed different gene expression levels in the different tissues examined (midgut, fatbody, epidermis, testis, and hemocytes). Ap β GRP and Aplectin-5 was induced by all the microorganisms, and mainly in epidermis and hemocytes, but not in testis; Aplectin-5 was also expressed in fatbody. Ap C-type lectin 1 was, on the contrary, highly expressed in testis and also in fatbody, but not in hemocytes. Unlike Ap β GRP and Aplectin-5, Ap C-type lectin 1 was not induced by Gram-positive bacteria. The results suggest that the cloned lectins may have different functions in different tissues of *A. pernyi*.

Keywords: insect immunity; *Antheraea pernyi*; β GRP; lectin; gene expression

1. Introduction

Lepidopteran immune resistance to foreign pathogenic microorganisms relies on the recognition of conserved microbial structures of the microorganisms. In turn, this leads to downstream reactions: cellular responses including phagocytosis and nodule formation, humoral responses such as antimicrobial peptide synthesis, and melanization after activation of the prophenoloxidase system [1,2]. For the insect's humoral immune system, pattern-recognition receptors play an important role. For example, peptidoglycan-recognition proteins can recognize bacterial surfaces and activate relevant immune pathways to resist bacterial invasion. Many other pattern recognition receptors such as hemolin, complement-like proteins and scavenger receptors have also been found in Lepidoptera.

A β -1,3-glucan recognition protein (β GRP) was originally isolated from the silkworm *Bombyx mori* by Ochiai *et al.* [3], and has since then been found in other invertebrates, such as the moths *Manduca sexta* [4] and *Plodia interpunctella* [5], and also in crayfish [6] and earthworms [7]. β -GRPs contain a conserved structural domain with a glucan-binding region and can recognize lipopolysaccharide of Gram-negative bacteria, phosphorus acid (of Gram-positive bacteria) and β -1,3-glucan cell walls of fungi, activating immune pathways, and promoting the activation of phenoloxidase [7] and antimicrobial-peptide synthesis [8].

The C-type lectin superfamily is a major type of pattern recognition receptors, which are calcium-dependent carbohydrate-binding proteins [9,10]. They take part in immune responses such as recognition of bacteria and fungi, activation of prophenoloxidase, and hemocyte nodule formation [11–15]. Insect lectins differ from most animal C-type lectins by having two tandem C-type carbohydrate recognition domains (CRDs) instead of one [14,16]. Different CRDs in the same lectin may have different functions [17].

The Chinese oak silkworm, *Antheraea pernyi*, is an important silk producer, but is commonly struck by infections, which has serious impact on sericulture. A greater understanding of immunity in this species may lead to a selection of more disease-resistant strains. In this paper, the β GRP, lectin-5 and C-type lectin 1 genes of *A. pernyi* were cloned, and their expression profiles following infection with different microorganism were investigated. Also, their *in silico*-deduced secondary structures and microorganism recognition specificities are described.

2. Results and Discussion

2.1. cDNA Cloning of Ap β GRP, Aplectin-5 and ApCTL1

The full length cDNAs of Ap β GRP, Aplectin-5 and Ap C-type lectin 1 (ApCTL1) were obtained by RT-PCR after reverse transcription of total RNA of *A. pernyi* fatbody with conserved primers followed by 5'RLM-RACE and 3'RLM-RACE. Ap β GRP consists of 2245 nucleotides and has an open reading frame containing 1470 nucleotides (positions 34–1503). Sequence analysis of this protein by a Simple Modular Architecture Research Tool (SMART) suggests that it has a signal peptide of 17 amino acids and contains a conservative glycosidic hydrolase domain belonging to the glycoside hydrolase family 16 (Glyco_hydro_16) (Figure 1), which has the ability to bind β -1,3-glucan.

Figure 1. Nucleotide and amino acid sequences of Ap β GRP. *Italic amino acids indicate the signal peptide of Ap β GRP, the boxed amino acids indicate Glyco_hydro_16.*

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1  gtctcgggata tca caa aag cga aggaaggga aat gtt cgg aag tgctat atg ttt gtt ggc gct ggt cgc gtt c
1  M F G S A I C L L A L V A F
76  tgcaatggacagcctccgcaata cac cgt acc acc agc aaa act aga agc cat ata ccctaaaggcttgagggtt
26  C N G Q P P Q Y T V P P A K L E A I Y P K G L R V
151 actgtaccagatgatggtttctctctattcgcctttcaccgggaaact caa cga gga gat gga agg cct aga agc g
51  T V P D D G F S L F A F H G K L N E E M E G L E A
226 ggtcattggctcgggatata cacaaaagcgaagggaaggaaatggat ctt cag aga tagaaa cgc ccagct caag
76  G H W S R D I T K A K E G K W I F R D R N A Q L K
301 at tgg gga caa aat tta ttt ctg gac cta tgt cataaaaaga tgg ttt ggg ata tag gca gga taa tgg aga atgg
101 I G D K I Y F W T Y V I K D G L G Y R Q D N G E W
376 acagt aactgaata cgt gga cga gca ggg aaa tgt ggt gga cgt ggt caa tccccc acc gaa acc tta caact ggt
126 T V T E Y V D E Q G N V V D V V N P P P K P S T G
451 ccagt cca aact cct ccc acc acc tgt ggt cac tta ccc tcc agt tac gaa gcc gcctgt acc cga ttgtc aggt g
151 P V Q T P P P P V V T Y P P V T K P P V P D C Q V
526 tcagcgacagtcgtgcaaggctgtggttaa agt gtg cgc ggg atc gct tgt gtt cag cga aga att tga taag acc
176 S A T V V Q G R G K V C A G S L V F S E E F D K T
601 agcct taa aga cat gcc cga ttg gac agc aga aat taa att ccc tga aga acc ggatta ccc gtt caa ttt ata t
201 S L K D M P D W T A E I K F P E E P D Y P F N L Y
676 actacgaa tgg tactgt cgc atta agt ga cgg gtc gtt ggt cat caa acc tgt act ttt gga atc aag ttt ctac
226 T T N G T V A L S D G S L V I K P V L L E S S F Y
751 gaagg caagat caa tga aga act gga cct gac aaa cca atg tac aggt caa agt ggg aac gac cga gtc caggcgg
251 E G K I N E E L D L T N Q C T G Q V G T T E C R R
826 caagcgtcggg cgt cagat cct gcc gcc ggt gtt atc gcc caa act cacaac gaa gcc gaa aat cgggataaaa
276 Q A S G A Q I L P P V L S A K L T T K R K F G I K
901 tatgg ccg tat cga aat ccg agc cca act acc cgt tgg caa ctggct cgt acctgaaat taa ttt gga acc cct c
301 Y G R I E I R A Q L P V G N W L V P E I N L E P L
976 gaaaa cgt ata tgg gtc ccg tgc gta cga gtc tgg tct cat gag agt ggc ctt tgc gaa aggtaa tgc ggt ctt c
326 E N V Y G S R R Y E S G L M R V A F A K G N A V F
1051 gcgaa gaa att gta tgg tgg ccc tgt gct ttc cga tactgagccatt cagaactat gct gct taa aga aaa gatt t
351 A K K L Y G G P V L S D T E P F R T M L L K E K I
1126 gggat caa caa ctggaa taaggat ttt tcata ctta cac ttt aat ttggaa acccaga tgt gat cga gat gta cgt c
376 G I N N W N K D F H T Y T L I W K P D V I E M Y V
1201 gatgg gga acagta cgg gtc gat tag tcc cgg gga agg att cta cgc gat cgg aag gaa gaa cgc ggt gcc gca c
401 D G E Q Y G S I S P G E G F Y A I G R K N A V P H
1276 gccgcgcagtggtctcccggaactat tctggcgcctctcgatcaaat ttt ctatata tgcgttggggttgccgcgtg
426 A A Q W L S G T I L A P L D Q I F Y I S L G L R V
1351 gg cgg cgt gaa tga ctt cgc gga cgg cacc ga caagccgtggcgc caa caagaa caa caaggc gat gct ttc ctt c
451 G G V N D F A D G T D K P W R N K N N K A M L S F
1426 tggaa tga tcc gga ggc gtt ggt ccc cac ctg gta cga cgc gaa cct caa agtagatta cgt cag agt tta cgc g
476 W N D R E R W L P T W Y D A N L K V D Y V R V Y A
1501 ctttag
501 L *

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The full-length Aplectin-5 cDNA is 589 nucleotides and has an open reading frame (ORF) of 522 bp, which codes for 174 amino acids and with a predicted signal peptide of 29 amino acids (Figure 2).

ApCTL1 has 1035 nucleotides and an open reading frame (ORF) of 924 bp which codes for 308 amino acids with a predicted signal peptide of 24 amino acids (Figure 3). Domain analysis by SMART suggests that these two proteins contain conservative carbohydrate recognition domains (CRDs). However, since ApCTL1 has two CRDs as compared to one in Aplectin-5, we can deduce that Aplectin-5 and ApCTL1 belong to different subfamilies, which also is reflected in Figure 5.

Figure 2. Nucleotide and amino acid sequences of Aplectin-5. Italic amino acids indicate the signal peptide and the boxed amino acids indicate the carbohydrate recognition domain.

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1 atggggctccttcaaaacgaggcgtgaagaagcactttagaatgttcgctatattcatattgctgggtgtoaacgct
1 M G S F K R G V K K H F R M F A I F I L L V S I A
76 aacgttatttgcaaggctcctgatggatgtaatacaacaacgcagtcgggtcacgggttacaaaattgatgtacata
26 N V I C K A P D G Y V I N N A V G H G Y K L M Y I
151 gca caacca tgggacagggcttgtgaaaaatgtcttgaagatggcgccaaattagcagta cccaaatcaaggaa
51 A Q P W D R A C E K C L E D G A K L A V P K S E E
226 gagtttaagtttatacagaaaatcgctcgtggaatgcactaccgctcgattatcgggtccgattacaatctagtg
76 E F K F I Q K I V R G M H Y P S I I G S D Y A N L V
301 gca tggctaggataaataat ttggtaaat tatacaaca tggaggaacgttgatggg gaaacataaacgcacg
101 A W L G I N N L V N Y T T W R N V D G E N I N D T
376 ggg tat cacagg tgggctggacttaacggacaaggctatagtgatgacccggcagagcca cactgctggcatt
126 G Y H R W A G L N G Q G Y S D D P A E P H C A G I
451 gacgggatcaacaatggcctacgtgacttcgggtgtcaaaaacgcctccatacata tgcgagacacaaacataa
151 D G I N N G L R D F W C Q K R L P Y I C E T Q T *

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Figure 3. Nucleotide and amino acid sequences of ApCTL1. Italic amino acids indicate the signal peptide and the two sets of boxed amino acids indicate carbohydrate recognition domains.

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1 atggcagcatacatcggtgtgtttttattatctactgtgtctcttgatcagca ccaagctccggcgagacaacaa
1 M A A Y I V C F L L F T V S L V S A P A P A R T K
76 ggtataccgtagtgattatacgtataataaaaaaacgatgctgcttctacaaattaca tatagaaacaggacgga
26 V Y R S D Y T Y N K K T D A F Y K L H I E T R T E
151 gagaagcgcaactcaggtttgttcgcttgaaggcgcgcagctgatgat tcccttcgcagcgtgata tacaact
51 R S A T Q V C S V E G A Q L M I P S Q R D I I Q L
226 aca cggaaatgtaaaacaattccctgatataagggaattatgtatgggtcgg ttcgacggtaaaagaacatgaatc
76 H G M L K Q F P D I G N Y V W V G S D G K E H E S
301 cgctgaagaacaccaattat tgattatcgccaacaaaacgcagatattcga aactgggtttccgctggtc acgaga
101 A E E T P I I D L S P T N A D I R T G F P W S R E
376 atgtgatgtagtaacgcgccaggcgatgtcgattcgttccc atgctatcg gcaactaccggttatttgcaagtg
126 C D V V T R Q G D V D S F P C Y R Q L P F I C K V
451 ggaggccaaagatgtctctta tga tgaacattgtca cgtttttgggaacaggcta taaatacttcc tagtatgg
151 E A K D A P Y D E A H C H V F G T G Y K Y F P S I G
526 aagttgtataagataccgcgat tgtgtaactcgtggaa cgaagcgtatgc agactg cccagcagacagaa cgctca
176 S C Y K I P R I V Y S W N E A Y A D C R A Q N A H
601 tctactggtcttgaactcagagacagaaatggtagctgt aaaaaatctaac gaa cactgagctcag tgtgacagg
201 L L V L N S E T E M L A V K N L T N T E L S V A G
676 agcaagatttgctggtgtttttctatgctggttttcg agcggataaaacctgt tggaaa tagcagcagagtttttaa
226 A R F A W F F Y A G F R A D K P V G N S T R V F K
751 aacgatattcaatcaaacgttaga ggaagcgtggttacag cgtgtggtcgcc taa cgaaccaaa taa tgcgctgct
251 T I F N Q T L E E A G Y S V W S P N E P N N A L S
826 gaa cgaagactg cggctctgtgtt caagaa cga tggaaa actaaatga tgt tga ctgttcg cagacata cgcttt
276 N E D C G S V F K N D G K L N D V D C S Q T Y A F
901 cgtgtgtgaaaaagagattaa cgtgtgatt
301 V C E K E I N V *

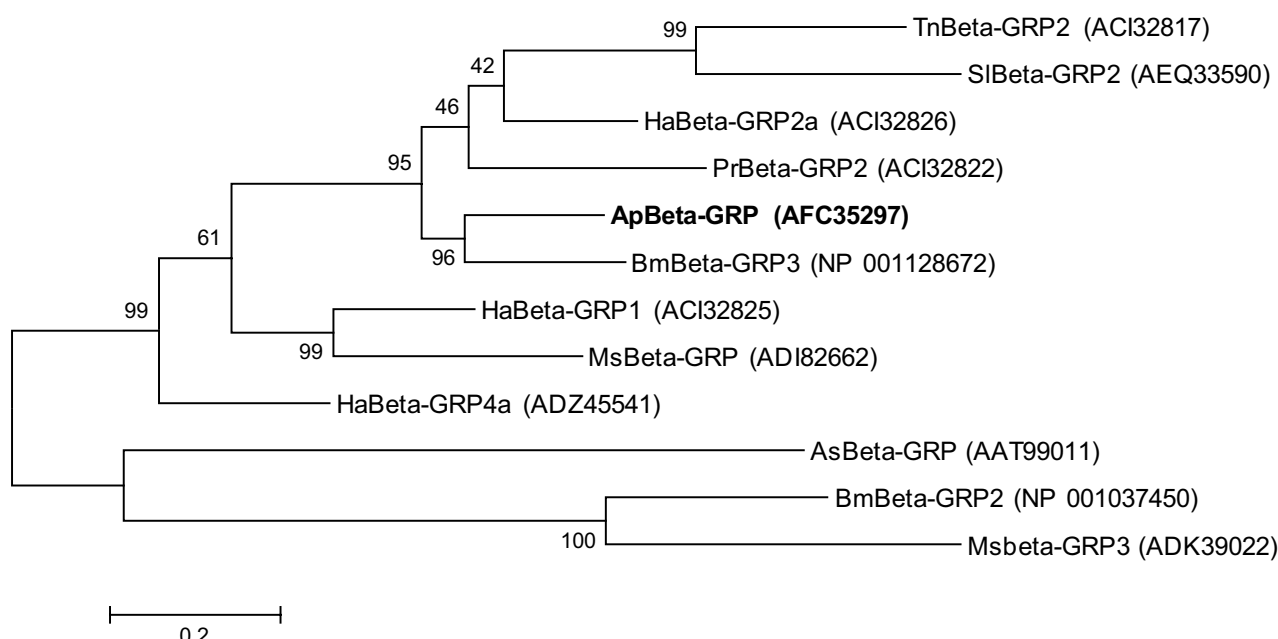
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2.2. Phylogenetic Analysis of ApβGRP, Aplectin-5 and ApCTL1

To examine the relationship of βGRP from *A. pernyi* with other insect βGRPs, 12 amino acid sequences were used for a phylogenetic tree (Figure 4). In this phylogenetic tree, ApβGRP and *Bombyx mori* βGRP-3 belong to the same branch; the similarity of their amino acid sequences is 83%, which would indicate a similar three-dimensional structure [18]. However, sequence analysis indicates that *Bombyx mori* βGRP-3 has no glucan-binding domain, but instead glycosidase activity, which is another way to recognize and bind to β-1,3-glucan [19].

We also constructed a phylogenetic tree composed of Aplectin-5, ApCTL1 and 17 other lectins from different species (Figure 5). In this phylogenetic tree, *A. pernyi* C-type lectin 1 is most similar to *B. mori* C-type lectin-11, and *M. sexta* IML1 and immunolectin-B. *M. sexta* IML1 is known to bind lipopolysaccharides and activate prophenoloxidase [14] and their similarity of about 60% indicates that they may have a similar function.

Figure 4. Molecular phylogenetic analysis of β -1,3-glucan recognition proteins (Beta-GRP) from different species. The evolutionary history was inferred by using the Maximum Likelihood method. The tree with the highest log likelihood (-7627.2903) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Ap, *A. pernyi*; As, *Armigeres subalbatus*; Bm, *B. mori*; Ha, *Helicoverpa armigera*; Hp, *Hepialus pui*; Lo, *Lonomia obliqua*; Ms, *M. sexta*; Pr, *Pieris rapae*; Tn, *Trichoplusia ni*.



2.3. Expression Analysis of Ap β GRP, Aplectin-5 and ApCTL1 in Different Tissues

In order to identify whether the three pattern recognition genes can be induced by different pathogenic microorganisms, *A. pernyi* diapausing pupae were injected with four different microorganisms: Gram-positive bacteria (*Bacillus subtilis*), Gram-negative bacteria (*Escherichia coli*), *A. pernyi* nuclear polyhedrosis virus (ApNPV), and yeast (*Saccharomyces cerevisiae*). Total RNA was extracted from midgut, fatbody, epidermis, testis and hemocytes 24 h after injection, and quantitative RT-PCR was performed. The expression of Ap β GRP was increased by all microorganisms; however, in general, the most significant response was induced by yeast (Figure 6). Also, Ap β GRP was expressed mainly in epidermis and hemocytes.

As shown in Figure 7, Aplectin-5 and ApCTL1 can also be induced by all four kinds of microbes, but their expression profiles differ. Aplectin-5, on the one hand, is most up-regulated in epidermis and

hemocytes, whereas ApCTL1, on the other hand, has high expression in testis. Both genes were up-regulated in fatbody and down-regulated in the midgut.

These results suggest that ApβGRP, Aplectin-5 and ApCTL1 can participate in relevant immune pathways by recognizing surface components of the four different pathogenic microorganisms. Epidermis is the first line defense of insects, and plays a key role in insect immune systems. All three genes are up-regulated in the epidermis by infection of the four microbes in our experiment, and thus are reacting to an early phase of the infection. In addition, ApβGRP and Aplectin-5 are up-regulated in hemocytes, the second line of defense, which protect the insect through phagocytosis, nodule formation and melanization. ApCTL1 is different from the other two pattern-recognition proteins in that it is expressed in testis and therefore may be important for protecting the next generation

Figure 5. Molecular phylogenetic analysis of lectins from different species. The evolutionary history was inferred by using the Maximum Likelihood method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches [20]. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Ap, *A. pernyi*; Bm, *B. mori*; Ha, *Helicoverpa armigera*; Lo, *Lonomia obliqua*; Ms, *M. sexta*.

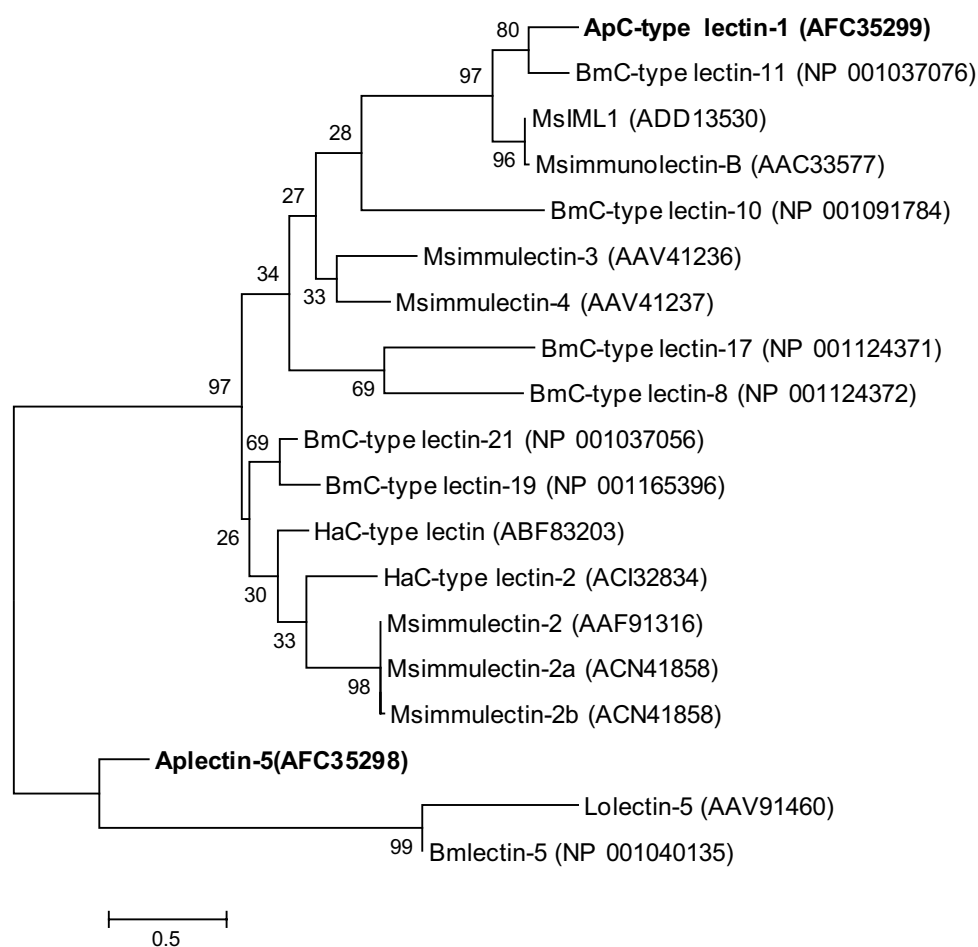


Figure 6. Expression of Ap β GRP at 24 h postinjection in different tissues of *A. pernyi* pupae. Different letters indicate significant differences in expression, $p < 0.05$. Error bars indicate standard deviation of three biological replicates.

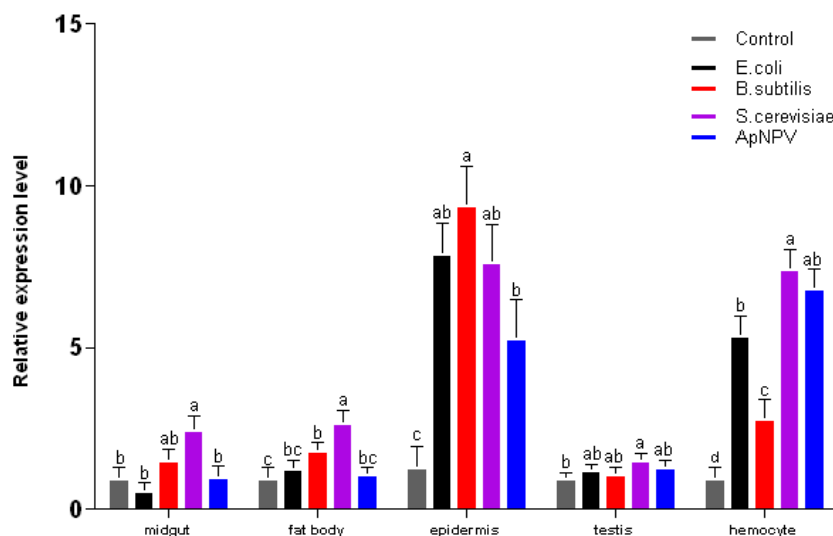
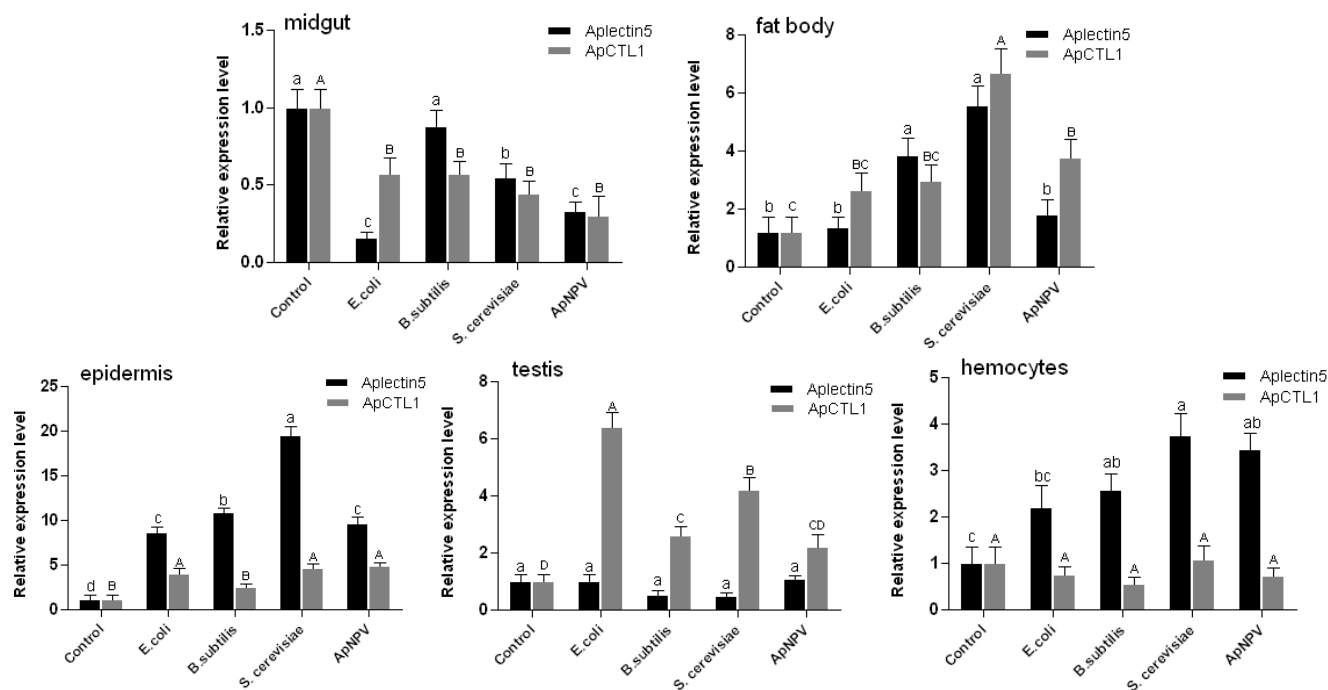


Figure 7. Expression of Aplectin-5 and ApCTL1 at 24 h postinfection in different tissues of *A. pernyi* pupae. Different letters indicate significant differences in expression, $p < 0.05$. Error bars indicate standard deviation of three biological replicates.



3. Experimental Section

3.1. Insects, Injection of Microorganisms and Tissue Sample Collections

Antheraea pernyi pupae were stored at 4 °C and transferred to room temperature 30 min before injection and then kept at room temperature throughout the experiments. The following microorganisms were used: Gram-positive bacteria (*Bacillus subtilis*), Gram-negative bacteria

(*Escherichia coli* Top10), *Antheraea pernyi* nuclear polyhedrosis virus (*ApNPV*) and fungus (the yeast *Saccharomyces cerevisiae*).

The pupae were divided into five groups with six pupae in each (three females and three males), four groups were injected 100 μ L of one of the following microorganisms: *E. coli* Top10, *B. subtilis*, *S. cerevisiae* (all at OD = 0.8, diluted 1:50 in PBS) or 100 μ L *ApNPV* (2.2×10^9 copies). A control group was injected with PBS only. 24 h after infection, the pupae were sacrificed and dissected into different tissues (epidermis, midgut, fatbody, hemocytes and testis). An equal amount of separated tissue from each pool of three males and three females were immersed together in liquid nitrogen and ground using mortar and pestle. Total RNA was isolated from the ground tissue using TrizolA+ Reagent (Tiangen).

3.2. Cloning of β GRP, Aplectin-5 and ApCTL1 Genes

Total RNA was used to clone the fragments of Ap β GRP, Aplectin-5 and ApCTL1 cDNA by RT-PCR (TaKaRa). The primers were designed based on conserved regions of *M. sexta* and *B. mori* as follows:

Ap β GRP -F: 5'-CTCAACGAGGARATGGAAGGC-3',
 Ap β GRP -R: 5'-ACTGTCCAYTCYCCRTTATCCTG-3',
 ApCTL1-F: 5'-CTACCGTTTATTTGCAAAGTGGAGG-3',
 ApCTL1-R: 5'-GTGCTATTTCCAACAGGTTTATCC-3',
 Aplectin-5-F: 5'-TCGGGTCCGATTACAATCTAGTG-3',
 Aplectin-5-R: 5'-GTCATCACTATAGCCTTGTCCGT-3'.

Thermal cycle was 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 58 °C for 30 s and 72 °C for 1 min, and a final extension of 72 °C for 7 min. The PCR products were purified by a PCR purification kit (TaKaRa) and cloned into the pMD-18-T vector (TaKaRa). The positive clones were subjected to sequence analysis.

To obtain the full length cDNA of these three genes, 3'RACE and 5'RACE were employed with the FirstChoice RLM-RACE kit (Ambion). The resulting PCR products were purified by a PCR purification kit (TaKaRa), cloned into the pMD-18-T vector and subjected to sequence analysis. The primers for RACE were as follows:

Ap β GRP, 5'RACE outer primer: 5'-CTCGGGATATCACAAAAGCGAAGGA-3'
 Ap β GRP, 5'RACE inner primer: 5'-GCAATACACCGTACCACCAGCAAAA-3'
 Ap β GRP, 3'RACE outer primer: 5'-GTACCACCAGCAAAACTAGAAGC-3'
 Ap β GRP, 3'RACE inner primer: 5'-GGTCTCGGGATATCACAAAAGC-3'
 Aplectin-5, 5'RACE outer primer: 5'-AACGTTCCCTCCATGTTGTATAATTT-3'
 Aplectin-5, 5'RACE inner primer: 5'-ACCAAATTATTTATACCTAGCCATG-3'
 Aplectin-5, 3'RACE outer primer: 5'-ACAGGTGGGCTGGACTTAACGG-3'
 Aplectin-5, 3'RACE inner primer: 5'-AACATGGAGGAACGTTGATGGTGA-3'
 ApCTL1, 5'RACE outer primer: 5'-GTTTCATCATAAGGAGCATCTTTGGC-3'
 ApCTL1, 5'RACE inner primer: 5'-GCCTGTTCCAAAACGTGACAATGT-3'
 ApCTL1, 3'RACE outer primer: 5'-TTTGCGTGGTTTTTCTATGCTGGTT-3'
 ApCTL1, 3'RACE inner primer: 5'-ACGCTCATCTACTGGTCTTGAAGTC-3'

3.3. Phylogenetic Analysis and Protein Structure Prediction

Evolutionary analyses were conducted in MEGA5 [21]. The evolutionary history was inferred by using the Maximum Likelihood method based on the JTT matrix-based model [22]. Initial tree(s) for the heuristic search were obtained automatically as follows. When the number of common sites was <100 or less than one fourth of the total number of sites, the maximum parsimony method was used; otherwise BIONJ method with MCL distance matrix was used. All positions containing gaps and missing data were eliminated. The total number of positions in the final dataset was 369 for Beta-GRPs and 72 for the lectins. SMART online software, <http://smart.embl-heidelberg.de> [23], was used to predict protein structure.

3.4. Transcript Profiles of Ap β GRP, Aplectin-5 and ApCTL1

Gene expression levels were measured with real-time quantitative PCR and calculated using the $2^{-\Delta\Delta CT}$ method [24] with *A. pernyi* β -actin as internal reference; each type of infection was repeated three times. The primers for transcript profiles were as follows:

Ap β -actin-F: 5'-ACCAACTGGGACGACATGGAGAAA-3'
 Ap β -actin-R: 5'-TCTCTCTGTTGGCCTTTGGGTTGA-3'
 Ap β GRP-F: 5'- ATACGTGGACGAGCAGGGAAATGT-3'
 Ap β GRP-R: 5'-TTTACCACGACCTTGCACGACTGT-3'
 Aplectin-5-F: 5'-CTTCAAACGAGGCGTGAAGAAGCA-3'
 Aplectin-5-R: 5'- AATTTGTAACCGTGACCGACTGCG -3'
 ApCTL1-F: 5'- AGTGGAGGCCAAAGATGCTCCTTA -3'
 ApCTL1-R: 5'- TACGCTTCGTTCCACGAGTACACA -3'

4. Conclusions

Pattern recognition receptors play an important role in the insect immune system, they can interact with pathogen-associated molecular patterns (PAMPs) and initiate reactions in the Toll pathways or imd pathways to produce antimicrobial peptides or protease inhibitors for resistance against pathogenic microorganisms [25,26]. In addition, they also regulate cellular immunity to kill microorganisms through phagocytosis, nodule formation and melanization.

In this study, we cloned the cDNA of Ap β GRP, Aplectin-5 and ApCTL1 from the Chinese oak silkworm, *A. pernyi*. Signal peptide prediction analysis of Ap β GRP suggests that the protein is a secretion protein and phylogenetic analysis of Ap β GRP and β GRPs from other species showed that the β GRP of *A. pernyi* and the β GRP-3 of *B. mori* belong to the same branch. Though their protein domains are different, both of them can be induced by Gram-negative bacteria, Gram-positive bacteria and fungi, respectively [27]. Their expression is up-regulated in midgut, epidermis and fatbody. The functions of the Ap β GRP and the β GRP-3 of *B. mori* may therefore be similar. Protein domain prediction analysis of Aplectin-5 and ApCTL1 showed that they have different structures; Aplectin-5 contains a single CRD while ApCTL1 contains two CRDs, and therefore belong to different subfamilies. Lectins with two or more CRDs can be regarded as an evolutionary innovation by insects,

because they can identify more types of microorganisms, and can agglutinate pathogenic microbes thereby improving the efficiency of phagocytosis and nodule formation [28].

Ap β GRP and Aplectin-5 were induced by all the microorganisms and mainly in epidermis and hemocytes, but not in testis; Aplectin-5 was also expressed in fatbody. ApCTL1 was on the contrary highly expressed in testis and also in fatbody, but not in hemocytes. Unlike Ap β GRP and Aplectin-5 it was not induced by Gram-positive bacteria. The immune system is particularly important in testis because these organs are exposed to the outside environment through the ejaculatory duct and oviduct. In previous investigations, the pattern-recognition protein hemolin was induced in male and female testes of *A. pernyi* pupae after bacterial injection [29] and knock-down of hemolin in *Cecropia* moths led to lethality in next generation embryos, indicating effects on the reproduction [30]. C-type lectins that are expressed in testes in response to Gram-negative bacteria have previously been found in *B. mori* [13]. Also, in fruit flies, the antimicrobial peptide andropin is present in male genitals [31], and some pattern recognition receptors have been found in generative organ of mammals [32].

In conclusion, we have shown that Ap β GRP, Aplectin-5 and ApCTL1 are up-regulated by a variety of pathogens and are differently expressed. The universality emphasizes their important role in the immune system of *A. pernyi*, but their signaling pathways remains to be elucidated.

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