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Research

# A Pattern Recognition Receptor C-type Lectin-S6 (CTL-S6) is Involved in the Immune Response in the Silkworm (Lepidoptera: Bombycidae)

**Dongxu Shen,[1,2,*] Meijin Tong,[1,2,*] Jiyun Guo,[1,2] Xianghan Mei,[1,2] Dingguo Xia,[1,2] Zhiyong Qiu,[1,2] and Qiaoling Zhao[1,2,3]**

[1]Jiangsu Key Laboratory of Sericultural Biology and Biotechnology, School of Biotechnology, Jiangsu University of Science and Technology, Zhenjiang, Jiangsu 212018, China, [2]Key Laboratory of Silkworm and Mulberry Genetic Improvement, Ministry of Agriculture and Rural Affairs, Sericultural Research Institute, Chinese Academy of Agricultural Sciences, Zhenjiang, Jiangsu 212018, China, and [3]Corresponding author, e-mail: qlzhao302@126.com

*These authors have made equal contribution to this study.

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## Abstract

Insect innate immunity is initiated by the special recognition and binding of the foreign pathogens, which is accomplished by the pattern recognition receptors (PRRs). As an important type of PRRs, C-type lectins (CTLs) play various roles in insect innate immunity, including pathogen recognition, stimulation of prophenoloxidase, regulation of cellular immunity and so on. In this study, we have cloned the full-length cDNA of a CTL gene named *CTL-S6* from the silkworm, *Bombyx mori*. The open reading frame (ORF) of *B. mori CTL-S6* encodes 378 amino acids, which contain a secretion signal peptide. The mRNA of *CTL-S6* exhibited the highest transcriptional level in the midgut. Its transcriptional level increased dramatically in fat body and hemocytes upon *Escherichia coli* or *Micrococcus luteus* challenge. Purified recombinant CTL-S6 could bind to bacterial cell wall components, including peptidoglycan (PGN, from *Bacillus subtilis*) and lipopolysaccharide (LPS, from *E. coli* 0111:B4), and recombinant CTL-S6 was involved in the encapsulation and melanization of hemocytes. Furthermore, the addition of recombinant CTL-S6 to the hemolymph of silkworm resulted in a significant increase in phenoloxidase activity. Overall, our results indicated that *B. mori* CTL-S6 may serve as a PRR for the recognition of foreign pathogens, prophenoloxidase pathway stimulation and involvement in the innate immunity.

**Key words:** C-type lectin, *Bombyx mori*, innate immunity, recognition, melanization

Insects lack the unique acquired immune system of vertebrates and rely on their own innate immune system to resist the invasive pathogens (Kingsolver and Hardy 2012, Kanost and Jiang 2015). Insect innate immune system composes of hemolymph-mediated humoral immunity and hemocytes-mediated cellular immunity. Initiation of insect innate immune system relies on the recognition of components called pathogen-associated molecular patterns (PAMPs) on the surface of microbial cell walls, by insect pattern recognition receptors (PRRs) (Takahashi et al. 2015). The recognition of special components on microorganisms surface leads to the stimulation of signaling pathways and then produce high-efficiency effect factors for the clearance and elimination of the invasive pathogens (Basbous et al. 2011).

PRRs, as highly conserved receptor proteins, could recognize a variety of ligands, including polysaccharides, glycoproteins, and glycolipids (Steiner 2004). C-type lectins (CTLs) comprise the large superfamily of glyco-binding proteins in a $Ca^{2+}$-dependent manner, which exist in vertebrates, insects and other invertebrates (Weis et al. 1991). CTLs function in cell adhesion, recognition of pathogen and elimination or initiation of immune responses, and they are defined by at least one C-type lectin-like domain (CTLDs), also known as carbohydrate recognition domain (CRD) (Cambi et al. 2005, Geijtenbeek and Gringhuis 2009). CTLD normally consisted of 110–130 amino acid residues and formed a spherical domain containing two α-helices, five β-sheets, and long loops that maintained stability by disulfide bonds (Weis et al. 1992, Zelensky and Gready 2005).

CTLs are usually linked with the capacity of carbohydrate recognition and binding via characteristic motifs that are located in the CTLD, such as the Glu-Pro-Asn (EPN) motif or Gln-Pro-Asp (QPD) motif (Drickamer 1992). CTLD with EPN motif defined as mannose-type sugar binding, that could specifically bind to mannose; while CTLD with QPD motif defined as galactose-type

sugar binding that specifically binds to galactose (Drickamer 1992). For rat mannose-binding protein A (MBP-A), $Glu^{185}$, and $Asn^{187}$ as the residues contained in the EPN motif, they together with $Glu^{193}$, $Asn^{205}$, and $Asp^{206}$ involved in the formation of $Ca^{2+}$-binding site 2 (Weis et al. 1992). In mammals, mutation of EPN to QPN can change the sugar-binding specificity of CTLs (Kolatkar and Weis 1996). It needs to be noted that the presence or absence of EPN motif or QPD motif does not totally define the sugar ligands binding specificity of CTLs in insects. A large portion of CTLs do not contain complete motifs, but they still could bind to various polysaccharides (Xia et al. 2018).

CTLs play multiple roles in the recognition and binding of sugar ligands, mediating cell adhesion, prophenoloxidase activation, as well as regulation of antimicrobial peptide synthesis. For example, *Manduca sexa* IML-2 as a CTL identified early in lepidopteran insects, was able to bind various polysaccharides including LPS, LTA, mannan, laminarin, Lipid A (Yu and Kanost 2003; Shi and Yu 2012). *Drosophila melanogaster* DL2 and DL3 could accelerate the encapsulation and melanization due to recruit hemocytes and agglutinated *Escherichia coli* (Ao et al. 2007). *Ostrinia furnacalis* IML-10 enhanced cellular aggregation by directly binding to the hemocytes surface and further enhanced cellular encapsulation (Song et al. 2020). *Helicoverpa armigera* CTL-3 cooperated with β-integrin located on the surface of hemocytes to promote the cellular encapsulation on beads (Wang et al. 2017).

The silkworm is another typical lepidopteran model and important economic insect. A total of 23 CTLs genes were identified and characterized in silkworm *Bombyx mori*, and some of them have done relevant functional research (Rao et al. 2015). Among these, *B. mori* IML-3 (LPS-binding protein, LBP) could promote the nodulation and melanization of hemocytes to *E. coli* in blood cavity, and then complete the clearance of bacteria (Koizumi et al. 1999). *Bombyx mori* IML-4 (LEL-2) and IML-5 (LEL-1) could bind to smooth or rough strains of gram-negative bacteria (Takase et al. 2009).

In this study, we have reported a member designed as CTL-S6 from the CTLs family in *B. mori*. *CTL-S6* mRNA was mainly detected in the midgut and the transcription level of *CTL-S6* was increased significantly upon bacterial infection. We produced and purified the recombinant CTL-S6 and investigated its sugars binding specificity and function in the immune response. Our results implied that recombinant CTL-S6 exhibited strong binding affinity to bacterial PGN, but weakly binding to LPS. Furthermore, recombinant CTL-S6 was involved in the stimulation of prophenoloxidase pathway, encapsulation, and melanization of hemocytes.

## Materials and Methods

### Biological Materials

The silkworm strain QiuFeng was provided by the Chinese Academy of Agricultural Sciences (Zhenjiang. China) and fed with fresh mulberry leaves under a photoperiod of 12:12 (L:D) h at 25 ± 1°C and 70–85% relative humidity. For *Escherichia coli* (strain DH5α) culture, a single colony grown in the Luria–Bertani (LB) solid culture plate at 37°C was subcultured into the liquid medium until $OD_{600}$ reaches 0.6~0.8. After centrifugation at 2,500 g and washed with PBS, and the bacterial cells were resuspended by sterile PBS for subsequent use.

### Multiple Sequences Alignment and Phylogenetic Analysis

Prediction of encoded amino acids sequences, molecular weight, and isoelectric point was carried out using the EXPASY (Expert Protein Analysis System) website (http://www.expasy.org). Signal peptides were predicted using Signal P 5.0 server (http://www.cbs.dtu.dk/services/SignalP/). Prediction of conserved domains and transmembrane regions were performed in SMART (http://smart.embl-heidelberg.de/smart/ set_mode.cgi). Multiple sequences alignment of CTL-S6 with other insect CTLs was performed in the Clustal Omega website (https://www.ebi.ac.uk/Tools/msa/clustalo/). And phylogenetic analysis of CTL-S6 with other insect CTLs was carried out using MEGA 6.0 software at the following parameters (Tamura et al. 2013, Shen et al. 2018).

### Expression Profile Analysis of *B. mori* CTL-S6

To investigate the changes of *B. mori CTL-S6* transcript levels in different tissues of silkworm, heads, midguts, Malpighian tubule, fat body, and epidermis from third-instar day 1 larvae were collected by dissection, and hemocytes from hemolymph were collected by centrifugation at 1,000 g for 2 min, then RNA samples from the above tissues were individually extracted by using TRIzol Reagent (Sangon Biotech (Shanghai) Co., Ltd., China). And the first-strand cDNA was synthesized from 1 μg of RNA samples following the manufacturer's instructions for QuantScriptRT Kit (TIANGEN, Biotech (Beijing) Co., Ltd, China). The Quantitative Real-time PCR (qRT-PCR) experiment was performed with NovoStartSYBR qPCR SuperMix (Novoprotein, Nanjing, China) on LightCycler 96 Real-Time PCR Cycler (Roche, Switzerland). The *B. mori GAPDH* gene was used as the housekeeping gene. The qRT-PCR analysis was performed following the conditions: pre-denaturation at 95°C 10 min, followed by 40 cycles of denaturation at 95°C for 10 s, annealing at 56°C for 10 s, extension at 72°C for 10 s. The melt curve was generated from 58°C to 95°C in increment of 0.5°C very 5 s. The relative transcript level of *CTL-S6* was quantified with the $2^{-\Delta\Delta Ct}$ method. Each qRT-PCR analysis was performed with three biological and three technical replicates.

To determine the expression patterns of *B. mori CTL-S6* after foreign pathogen infection, third-instar day 0 larvae from each group were injected individually with 2 μl of sterile phosphate-buffered saline (PBS) containing formaline-killed *E. coli* ($1 \times 10^4$ cells/μl) or *Micrococcus luteus* ($1 \times 10^4$ cells/μl), and sterile PBS was used as control. The larvae were fed with fresh mulberry leaves after injection. Twenty-four hours later, each three larvae from treated and control group were collected, and RNA samples preparation, cDNA synthesis, and qRT-PCR assay were conducted as described above.

### Recombinant CTL-S6 Expression and Purification

The specific primers were designed to clone the sequence encoding the mature CTL-S6 (Supp Table S1 [online only]). The forward primer containing an *Nco* I site and provided the start codon, the reverse primer with a *Xho* I site at the 3′-end of stop codon. The PCR products were ligated into pMD18-T vector for sequencing analysis and then digested and ligated into the same restriction sites of pET28a vector (Novagen). The constructed plasmid was transformed into *E. coli* BL21 (DE3) strain competent cells. For recombinant CTL-S6 expression, a single colony was incubated at 37°C in LB liquid medium containing kanamycin (50 μg/ml) until $OD_{600}$ reached 0.6, then isopropyl β-D-thiogalactoside (IPTG) was added at a final concentration of 0.2 mM, and recombinant protein was

expressed at 37°C for 6 h. Bacterial cells were harvested and resuspended with the lysis buffer (50 mM sodium phosphate, 300 mM NaCl, 10 mM imidazole, pH 8.0). Cells were sonicated and the inclusion bodies were obtained by centrifugation. The recombinant CTL-S6 was purified by two steps of denaturation with buffers (50 mM Tris–HCl, 50 mM NaCl, 1 mM EDTA, 100 mM 2-hydroxy-1-ethanethiol, 7 M Guanidine Hydrochloride, pH=8.0; 50 mM Tris–HCl, 50 mM NaCl, 1 mM EDTA, 10 mM 2-hydroxy-1-ethanethiol, 8 M Urea, pH=8.0).Purified recombinant CTL-S6 was renatured in a dialysis buffer (20 mM Tris–HCl, 150 mM NaCl, pH 8.0). The purified recombinant protein was blotted with immunoblotting using anti-His IgG (Sangon Biotech (Shanghai) Co., Ltd., China) as the primary antibodies.

## Binding of rCTL-S6 to Different Microorganisms Cell Wall Components

Binding of biotinylated microorganisms cell wall components to rCTL-S6 was examined using the Enzyme-Linked Immunosorbent Assays (ELISA). LPS (from *E. coli* 0111:B4) or PGN (from *B. subtilis*) were diluted to 40 μg/ml with sterile water. Flat bottom 96-well assay plates (F605032, Sangon Biotech (Shanghai) Co., Ltd., China) were coated with 2 μg/well of components overnight and uncoated wells are used as controls. After blocking with BSA (100 μl/well, 10 mg/ml) at 37°C for 2 h and rinsed three times with TBS (containing 0.1% Tween-20, 200 μl/well), rCTL-S6 diluted into different concentrations added to each well and then incubated at 37°C for 2 h. Bound recombinant CTL-S6 was detected by using mouse monoclonal anti-polyhistidine antibody (1:2000 in TBS containing 1 mg/ml BSA, 100 μl/well, 37°C for 2 h) as the primary antibody and goat anti-mouse IgG conjugated to HRP (1:5000 in TBS containing 1 mg/ml BSA, 100 μl/well, 37°C for 2 h, Sangon Biotech (Shanghai) Co., Ltd., China) as the secondary antibody. After rinsed with TBS three times again, the EL-TMB Chromogenic Reagent kit (C520026, Sangon Biotech (Shanghai) Co., Ltd., China) using for color measurement. Absorbance at 450 nm of each well was measured by a Microplate Spectrophotometer (Bio Tek, America).

## Effect of rCTL-S6 on Encapsulation and Melanization of Hemocytes

His-tagged recombinant CTL-S6 was used to coat Ni-NTA agarose beads (Qiagen, Valencia, CA). Recombinant CTL-S6 coated agarose beads were rinsed three times with sterile TBS and resuspended in sterile TBS at a concentration of 80~100 beads/μl. Ni-NTA agarose beads without protein coating were used as a control. Encapsulation assays were carried out in a 24-well cell culture plate (Corning, Corning Incorporated, United States) that coated with 1% agarose as described (Yu et al. 2005, Ling and Yu 2006). Ten microliters of fresh hemolymph of the day 1 fifth instar larvae was collected into 450 μl cell culture medium containing 50 μg/ml of tetracycline. Two microliters of rCTL-S6 coated beads or plain nickel agarose beads were added to each well, and the cell culture plate was placed horizontally without shaking at room temperature for incubation. The beads that encapsulated and melanized were observed and counted under microscopy after 2 and 4 h incubation.

## Stimulation of Prophenoloxidase Activation by rCTL-S6 in *B. mori* Larval Hemolymph

Day 1 fifth instar larvae of *B. mori* were disinfected with 75% ethyl alcohol and then placed on ice to anesthetize. Hemolymph were collected from cut abdomen legs and centrifuged at 1,000 g for 1 min to remove hemocytes was examined using Dopamine was used as substrate to examine the phenoloxidase (PO) activity as described previously (Jiang et al. 2003). Aliquots of the plasma (1 μl) were mixed with sterile PBS or purified recombinant CTL-S6 (1 μl, 100 μg/ml), followed by the addition *E. coli* suspension ($1.6 \times 10^5$cells/ml). The overall volume of each mixture was adjusted to 10 μl with sterile PBS. After incubation at room temperature for 10 min, the PO activity was examined on a microplate reader (Bio Tek, America). All


**Fig. 1.** Sequence alignment of *B. mori* CTL-S6 with other insect CTLs. The GenBank accession numbers of the amino acid sequences used are as below: AgCTL1, XP_309985.4; AaCTL1, XP_001653784.1; MsIML-2, XP_030038244.1. The predicted signal peptide was shaded in gray. The C-type lectin-like domain (CTLD) of each protein was shown in light blue font. The conserved cysteine residues in CTLD were marked with red font. The consistent amino acid residues in the sequences were shown on a pink background. The key residues that were critical for the definition of sugar-binding specificity was marked by a yellow background. The disulfide bond formed by cysteine was assigned above sequences from N to C termini of amino acid sequences.

of the treatments were performed in triplicate. One unit of PO activity was defined as the amount of enzyme producing an increase in absorbance ($OD_{490}$) of 0.001 per min.

## Results

### cDNA Cloning and Sequence Analysis of *B. mori* CTL-S6

As a typical CTL protein, the ORF of *B. mori* CTL-S6 encodes 378 amino acids residues containing a CTLD and a predicted secretion signal peptide consisting of 19 residues. The calculated molecular weight and isoelectric point of the mature CTL-S6 are respectively 40.5 kDa and 7.76. The amino acid sequences of CTL-S6 had four conserved Cys residues that define CTLD and containing the NPG motif, which was a non-canonical motif (Fig. 1). To investigate the evolutionary relationships between *B. mori* CTL-S6 and other insects CTLs, we retrieved other insects CTLs amino acids sequences from GenBank and constructed phylogenetic tree by using neighbor-joining method. We found that CTL-S6 formed a cluster with orthologs from the other species in Hymenoptera (*Cerapachys biroi*) and Lepidoptera (*M. sexta*, *Danaus plexippus*, *Papilio xuthus*), and formed 1:1 orthologous group with *M. sexta* CTL-S5 (Fig. 2).

### Expression profiles of *B. mori* CTL-S6

Firstly, we analyzed the mRNA level of *B. mori CTL-S6* in different tissues using qRT-PCR methods. The results of qRT-PCR showed that the *CTL-S6* transcript was at the highest level in the midgut, and then in the head and epidermis (Fig. 3). To check the expression pattern of *B. mori CTL-S6* after infected by foreign pathogens, we analyzed the transcript level of *CTL-S6* after *B. mori* third-instar larvae were infected by *E. coli* or *M. luteus*. As shown in Fig. 3B, the transcript level of *B. mori CTL-S6* increased dramatically in the tissues, including fat body and hemocytes after the larvae immune challenged by bacteria.

### Recombinant Expression and Purification of *B. mori* CTL-S6

For further investigation of the function of *B. mori* CTL-S6, we constructed pET28a-CTL-S6 vector for recombinant expression of CTL-S6 and the recombinant CTL-S6 was purified by Ni-NTA

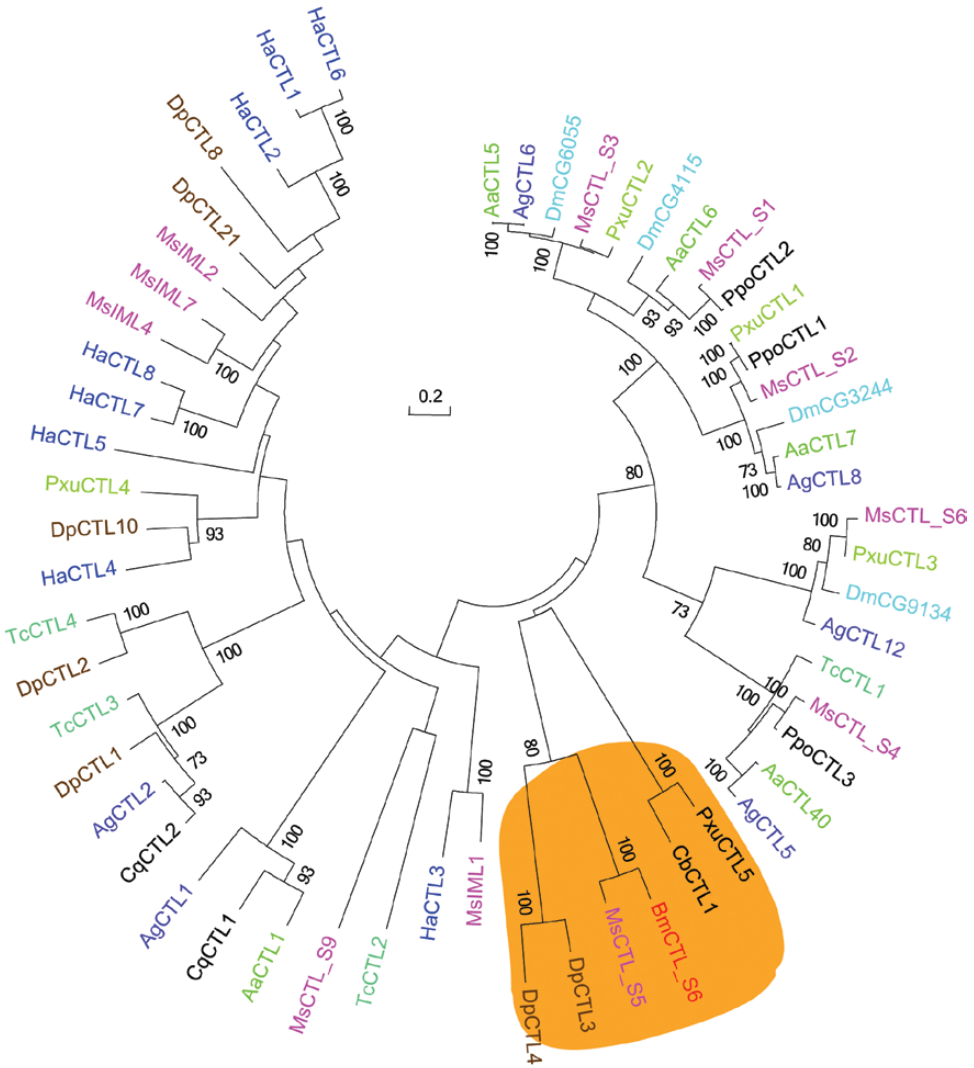


**Fig. 2.** Phylogenetic analysis of *B. mori* CTL-S6 with other insect CTLs. A total of 56 CTLs sequences from other insects were used to construct the phylogenetic tree. The branches specific for *B. mori* CTL-S6 was shaded in orange. Bootstrap values only greater than 70 were shown. The abbreviations are: Aa for *A. aegypti*, Ag for *A. gambiae*, Bm for *B. mori*, Cb for *Cerapachys biroi*, Cq for *Culex quinquefasciat*, Dm for *D. melanogaster*, Dp for *D. plexippus*, Ha for *H. armigera*, Ms for *M. sexta*, Ppo for *Papilio polytes*, Pxu for *Papilio xuthus*, and Tc for *T. castaneum*.

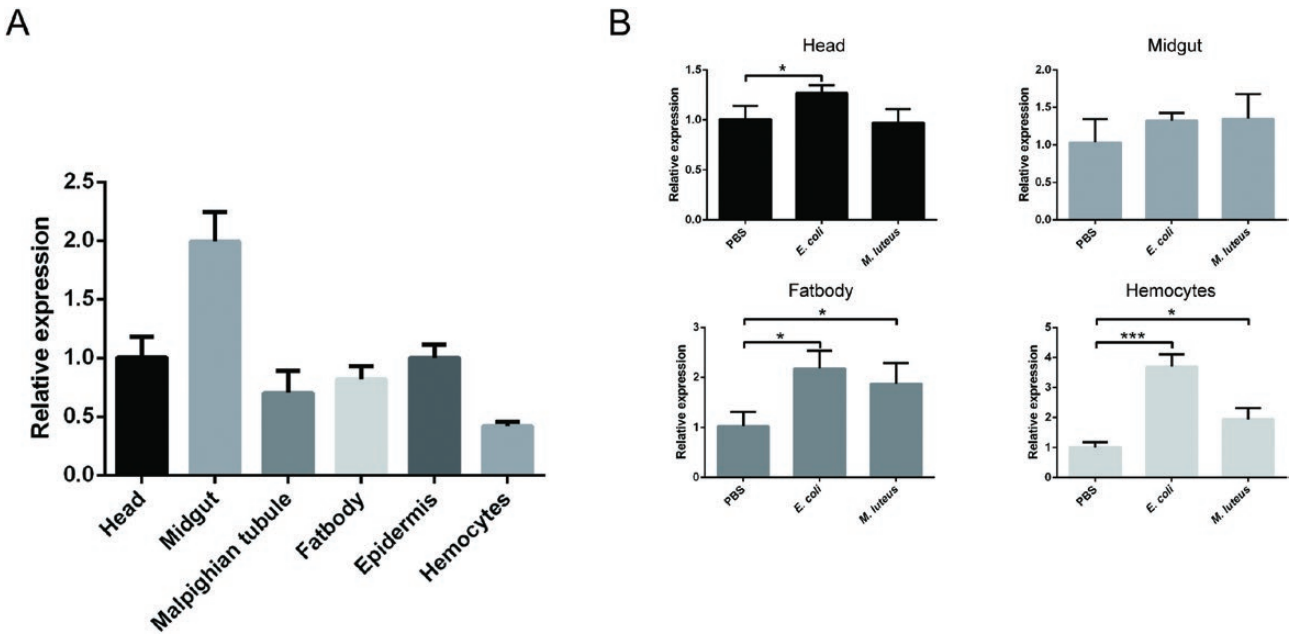


**Fig. 3.** Expression profile analysis of *B. mori CTL-S6*. (A) Expression profiles of *B. mori CTL-S6* in various tissues. RNA was individually extracted from various tissues and was converted into first-strand cDNA used for the template of qRT-PCR assay. The *GAPDH* was used as a reference gene. (B) Expression profiles of *B. mori CTL-S6* upon bacterial challenge. Third-instar day 1 larvae were infected with *E. coli, M. luteus* or sterile PBS (as control). RNA of different tissues was prepared from each group 24 h after infection. The bars represent mean ± SD. ($n = 3$). Asterisk denotes significantly difference (unpaired *t* test; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$).

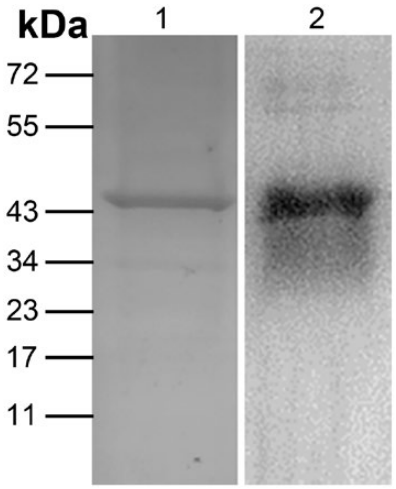


**Fig. 4.** Purification and identification of recombinant mature CTL-S6. Purified recombinant CTL-S6 from *E. coli* was analyzed by SDS–PAGE (1) and Western blotting (2). For immunoblotting, the proteins were blotted onto a PVDF membrane followed by immunoblotting using mouse monoclonal anti-polyhistidine antibody as primary antibodies. Molecular weight (kDa) and the positions of standard protein are marked on the left.

affinity chromatography. The result of SDS–PAGE shown in Fig. 4 indicated that recombinant CTL-S6 was successfully expressed and purified. It has an apparent molecular weight of around 43 kDa, and the purified recombinant CTL-S6 with hexahistidine tags was detected by commercial antibodies (Fig. 4).

## Recombinant CTL-S6 Could Bind to Sugars

We conducted a plate ELISA to examine the binding ability of recombinant CTL-S6 to bacterial LPS or PGN, since glycoconjugates that bearing on the microbial surfaces are the most possible targets recognized and bound by CTLs. Binding of rCTL-S6 to biotinylated LPS or PGN was detected by mouse monoclonal anti-polyhistidine antibody and goat anti-mouse IgG conjugated to HRP. As shown in Fig. 5, the plate coated with bacterial cell wall components indicated that rCTL-S6 could bind to PGN, which exhibiting a dose-dependent pattern, and the absorbance value corresponding to each protein concentration fits into a logarithmic curve. Moreover, the results indicated that rCTL-S6 exhibited stronger binding ability to PGN from *B. subtilis* compared to LPS from *E. coli*.

## Recombinant CTL-S6 Enhanced Melanization of Hemocytes

Hemocytes-mediated encapsulation and melanization were the important immune response that defense against invading parasites in insects. Then we conducted an encapsulation assay in vitro by using Ni-NTA agarose beads coated with rCTL-S6 with hexahistidine tags to test whether rCTL-S6 was involved in encapsulation and melanization. As shown in Fig. 6, agarose beads coated with rCTL-S6 were surrounded by many hemocytes and began to melanized after 2 h incubation. After 4 h incubation, more hemocytes gathered around the agarose beads and more beads were encapsulated and melanized. However, almost no hemocyte was observed around the plain agarose beads, and almost no melanization was observed for the agarose beads without protein coating.

## Recombinant CTL-S6 Increased PO Activity of *B. mori* Hemolymph

To test whether recombinant CTL-S6 participated in the stimulation of prophenoloxidase of *B. mori* hemolymph, renatured rCTL-S6/*E. coli* elicitor, alone or both rCTL-S6 and *E. coli* complex was added to *B. mori* hemolymph. Incubation of *E. coli* elicitor or rCTL-S6 alone with hemolymph resulting in a significant increase

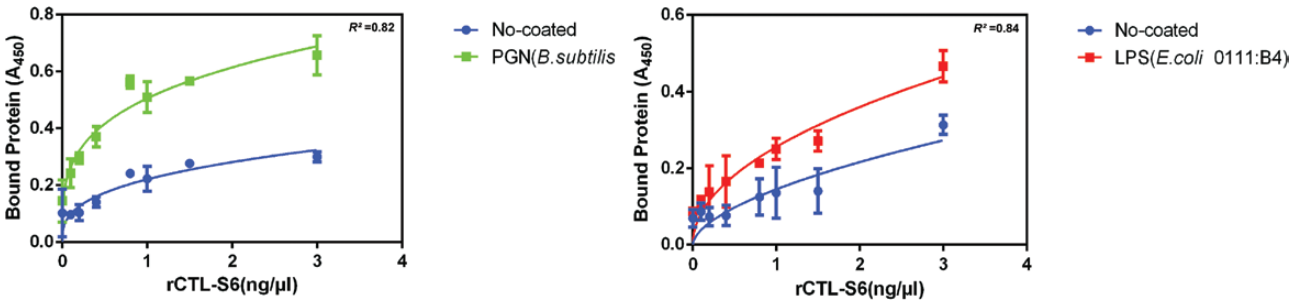


**Fig. 5.** Binding activities of recombinant CTL-S6 to biotinylated PGN and LPS. The plates were coated with PGN from *B. subtilis* or LPS from *E. coli* 0111:B4. No coated wells were used as control. Increasing concentrations of rCTL-S6 added into each well and the binding ability assay was conducted as described in section 2.5. Solid lines represent the fitted curves formed by nonlinear regression method with the one-site binding model. The bars indicate the mean of three individual measurements ± SD.

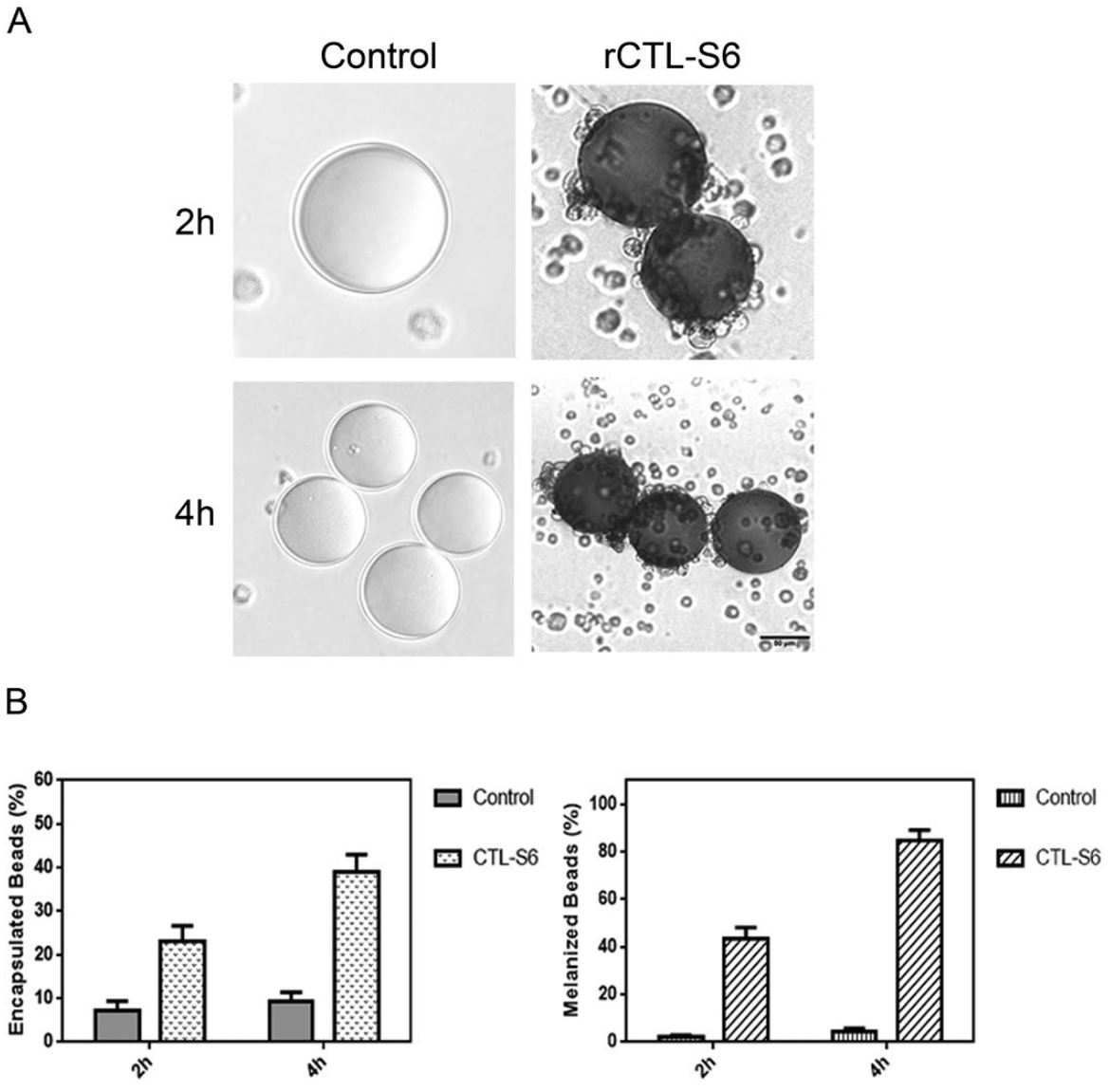


**Fig. 6.** Recombinant CTL-S6 enhanced encapsulation and melanization of hemocytes. (A) Nickel agarose beads coated with recombinant CTL-S6 were incubated with hemocytes from *B. mori* larvae. No coated beads were used as a control. Agarose beads were observed after 2 h or 4 h incubation by inverted microscopy. (B) The percentage of encapsulation or melanized beads, respectively. A total of 100 beads were counted at 2 h and 4 h after incubation, respectively. The occur of encapsulation was defined when the beads were coated with more than ten hemocytes. The columns indicate the mean of three individual counts ± SD.

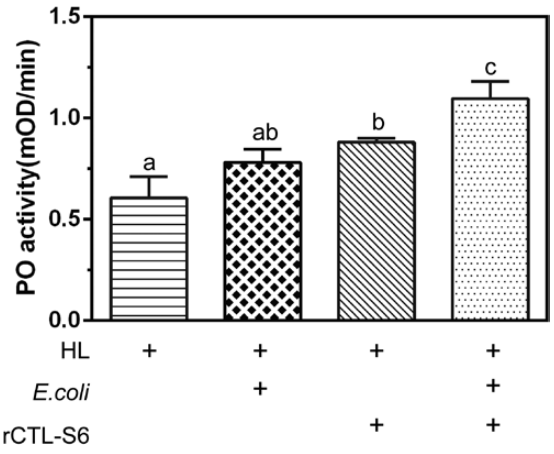


**Fig. 7.** Enhancement of PO activity of *B. mori* hemolymph after the addition of recombinant CTL-S6. As described in section 2.7, hemolymph from fifth instar day 1 larvae were individually incubated with *E. coli* cells ($1.6 \times 10^5$cells/ml) or recombinant CTL-S6 (100 μg/ml) at room temperature for 10 min. The bars represent mean ± SD ($n = 3$). The difference between the samples was analyzed by the ANOVA and Tukey's multiple comparisons test. Bars marked with different letters mean significantly different.

in PO activity. Moreover, both rCTL-S6 and *E. coli* was incubated with hemolymph led to a considerable increase compared to that induced by *E. coli* or rCTL-S6 alone (Fig. 7). These results suggested that rCTL-S6 was involved in prophenoloxidase activation pathway, possibly through the recognition and binding of the invading foreign exogenous pathogen such as *E. coli*.

## Discussion

As a category of important PRRs, CTLs were reported to contribute in the innate immunity of invertebrate, e.g., agglutinating microorganisms, enhancement of encapsulation and nodulation of hemocytes, stimulation of prophenoloxidase, antimicrobial activity, and opsonization (Jiravanichpaisal et al. 2006, Brown et al. 2018, Xia et al. 2018). In the present study, we selected a potential CTL gene named *CTL-S6* from *B. mori* genome database for further investigation. In our work, bioinformatics analysis, tissue-specific distribution, and immune inducibility of *CTL-S6* were performed, and recombinant CTL-S6 protein was expressed and purified by prokaryotic expression system of *E. coli* and affinity chromatography, proposing to verify its key molecular roles during the immune immunity of *B. mori*. Phylogenetic analysis revealed that *B. mori* CTL-S6 was tightly grouped with orthologs from the other species from Lepidoptera and Hymenoptera, and was closest to *M. sexa* CTL-S5. We speculate that CTL-S6 seems to evolve from a common ancestor gene with *M. sexa* CTL-S5. The mature protein of CTL-S6 contained 359 amino acids residues with a CTLD of 130 amino acids. It was unknown that if the other region was important to the structure feature and function of CTL-S6. *CTL-S6* mRNA was detected in multiple tested tissues, especially exhibiting the highest transcriptional level in the midgut, but its transcriptional level in fat body and hemocytes was dramatically induced by bacterial infection. This result implied that CTL-S6 may play key roles in maintaining gut homeostasis and immune response against pathogens. Among the protein superfamily of CTLs, CTL-S was the original type of CTLs that has only a single CTLD and does not contain other types of conserved domains. In our study, CTL-S6 with a canonical CTLD and four conserved Cys residues that critical to CTLD, but not contain an EPN motif or QPD motif instead of NPG motif. CTLD with QPD motif was defined as galactose-type sugar-binding affinity, which specifically bind to galactose (Drickamer 1992), while CTL-S6 with NPG motif was predicted to bind to galactose (Rao et al. 2015).

As an important family of PRRs, CTLs served as receptors to recognize various components on the surface of microbial cell walls, such as LPS, PGN, LTA, mannan, glucan, and so on. For example, IML-4 (dual CTLDs) from *M. sexa* was capable to bind bacterial LPS and LTA (Yu et al. 2006). Ha-lectin containing two tandem CRDs from *H. armigera* could bind Mannose, Galactose, Sucrose, Curdlan, and PGN (Chai et al., 2008). Although CTL-S6 had only a single CTLD with the NPG motif, it seems like that exhibit a broad binding spectrum to various bacterial microorganisms and microbial surface components. Our ELISA results showed that CTL-S6 could bind to PGN (*B. subtilis*) and LPS (*E. coli* 0111:B4), while had a higher affinity for PGN than LPS. *B. mori* CTL-S3 exhibited binding affinity to Laminarin (Laminaria digitate), LPS (*E. coli* 055:B5), LTA (*B. subtilis*), PGN (*E. coli* K12), and PGN (*S. aureus*) (Zhan et al. 2016). Five CTLs all with a single CTLD from *Armigeres subalbatus* exhibited binding affinity to multiple microbial cell wall components, such as PGN, LPS, LTA, lipid A, zymosan, and laminarin (beta-1,3-glucan) (Shi et al. 2014). Further investigation is required for accurate binding ability of CTL-S6 to each kind of cell wall component.

In addition, some CTLs have unique functions, such as regulating cellular immunity (nodulation, encapsulation, phagocytosis) or antimicrobial activity. For example, *O. furnacalis* IML-10 could accelerate aggregation of hemocytes by binding to the surface of them and further improve their encapsulation capacity (Song et al. 2020). Recombinant Ha-lectin protein had no antimicrobial activity, but it could inhibit the growth of *Bacillus thuringiensis* in vivo and may serve as a regulator to promote the phagocytosis of hemocytes (Tian et al. 2009). Although recombinant CTL-S6 was capable of binding to cell wall components (PGN and LPS), it has not exhibit agglutination or antimicrobial activity (data not shown). We speculated that the reason for CTL-S6 does not possess agglutination or antimicrobial activity may be our lower protein concentration. Furthermore, we have shown that recombinant CTL-S6 participated in the encapsulation of hemocytes and prophenoloxidase stimulation. It was likely that CTL-S6 may bind to the surface of hemocytes, and then promote cells aggregation with or without other factors. In the cotton bollworm, HaCTL3 enhanced hemocytic encapsulation and melanization cooperated with Haβ-integrin that located on the surface of hemocytes together involved in the hemocytic encapsulation (Wang et al. 2017).

In summary, we characterized and cloned a CTL gene called *CTL-S6* with a single CTLD from *B. mori* genome, which was detected in multiple tested tissues. It exhibited binding affinity to microbial cell wall components, including PGN or LPS. Moreover, BmCTL-S6 was involved in the encapsulation and melanization of hemocytes and prophenoloxidase stimulation pathway. It may function as a PRR in the recognition and clearance of invading pathogens.

## Supplementary Data

Supplementary data are available at *Journal of Insect Science* online.

Table S1. Primers for plasmid construction and qRT-PCR.

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## Author Contributions

Conceived and designed the experiments: DXS, QLZ. Investigation: DXS, MJT, XHM. Performed the experiments: DXS, MJT, JYG . Analysis the data: DXS, MJT, DGX, ZYQ. Manuscript preparation&editing: DXS, MJT, QLZ.

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