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Expression Patterns of Calreticulin from *Bombyx mori* After Immune Challenge

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Abstract.- Calreticulin is a 46-kDa chaperone protein with multifunction, mainly involved in directing proper conformation of proteins, controlling calcium level, and participating in immune responses. In previous study, calreticulin from *Bombyx mori* (BmCRT) was identified, and BmCRT was significantly induced by some stimuli of intracellular calcium disturbance. In this study, we report that BmCRT mRNA and protein were detected in all organs of 5th larvae stage. *BmCRT* mRNA was specially abundant expressed in testis, ovary and hemocytes, but BmCRT protein was higher in testis and fat body than other tissues. To study the involvement of BmCRT in insect immune response, *B. mori* larvae were challenged by *Escherichia coli*, *Micrococcus lysodeikticus*, *Beauveri bassiana* or nucleopolyhedrovirus, respectively. After 1, 4 and 12 h microorganism injection, BmCRT mRNA and protein expression level in fat body were detected by qPCR and western blot methods. BmCRT expression in fat body can be induced by microorganisms, but with different expression patterns by different microorganisms. And BmCRT protein was induced later than the mRNA expression. Our results indicated BmCRT in fat body may be involved in humoral immune response against pathogenic microorganisms, and helpful to understand the comprehensive function of insect calreticulin.

Key words: Calreticulin, expression profile, *Bombyx mori*, humoral immunity.

INTRODUCTION

Calreticulin (CRT) is a multifunctional protein, first isolated from endoplasmic reticulum (ER) of rabbit skeletal muscle in 1974 (Michalak *et al*., 1999). Besides localize in the ER, calreticulin is also found to be localized to the outer cell surface of a variety of cell types, in the cytosol, and in the extracellular matrix (ECM). When localized to ER, calreticulin has functions in proper folding of proteins and glycoproteins and regulation of calcium metabolism (Somogyi *et al*., 2003). The non-ER calreticulin (localized outside the ER) has been reported participating in wound healing, immune response, fibrosis and other physiological processes (Gold *et al*., 2010). Calreticulin has been reported in mammals (Michalak *et al*., 2009; Wang *et al*., 2012b), invertebrates (Choi *et al*., 2002; Gao *et al*., 2008) and plants (Jia *et al*., 2009; Vitale, 2009), but not found in yeast or prokaryotes based on their genome database.

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In insects, calreticulin from *Drosophila melanogaster* (DmCRT) was first reported, later it was confirmed participating in the olfactory system, anesthetic sensitivity and phagocytosis of apoptotic cell in *D. melanogaster* (Gamo *et al*., 2003; Kuraishi *et al*., 2007; Stoltzfus *et al*., 2003). A 47 kD protein from *Galleria mellonella* was identified as calreticulin (GmCRT), which was involved in non-self recognition in cellular defense reactions (Choi *et al*., 2002). Calreticulin from *Pieris rapae* (PrCRT) hemocytes was involved in immune-related phagocytosis of yeast cells and cellular encapsulation (Asgari and Schmidt, 2003; Wang *et al*., 2012a). In parasitoid, calreticulin was also identified from the expression products of polydnavirus and venom proteins (Asgari *et al*., 2003; Crawford *et al*., 2008; Zhu *et al*., 2010). Venom calreticulin from *Costesia rubecula* or *Pteromalus puparum* could inhibit host hemocyte spreading and cellular encapsulation in vitro (Wang *et al*., 2013; Zhang *et al*., 2006). These results indicated that calreticulin mainly involved in the cellular response of insect immunity, little is known about calreticulin whether involved in humoral response in insects.

Calreticulin from the silkworm, *Bombyx mori*, was isolated from fat body using two-dimensional gel electrophoresis and mass spectrometry methods

(Dong *et al*., 2008). Calreticulin from *Bombyx mori* (BmCRT) had an endoplasmic reticulum retentional HDEL motif at its C-terminus and a predicted molecular mass of 45,801 Da (Goo *et al*., 2005). When treated with ER stress-inducing drugs, the expression level of BmCRT was not significantly induced. However, BmCRT in *B. mori* culture cell line BM5 was significantly induced by some stimuli of intracellular calcium disturbance (Goo *et al*., 2005). In this study, we tested the tissue expression level of BmCRT in mRNA and protein level. And we analyzed BmCRT mRNA and protein expression level in fat body of silkworm larvae at different times after microbial-challenged. Our results indicated BmCRT in fat body could be induced in response to different microorganisms. These results can provide us new insights into that calreticulin maybe also activate of the humoral immune system in fat body.

MATERIALS AND METHODS

Experimental insects

Silkworm (*Bombyx mori*, Dazao) larvae were reared with fresh mulberry leaves at 25±1°C with a photoperiod of 12:12 h (light : darkness). Dazao larvae were breeding to the third day of the fifth instar for tissue dissection or microbes immune-challenged.

Tissue dissection

Dazao larvae from third day of the fifth instar were paralyzed on ice for 30 min, and then feet were cut off for collecting hemolymph. The hemolymph was centrifuged at 800 g for 10 min at 4°C; the pellet was collected for hemocytes sample. Fat body, epidermis, midgut, silk gland, ovary, testis and Malpighian tubule were dissected or collected, respectively. Each tissue sample had two copies, one for RNA extraction and another one for protein extraction. Each biological treatment was repeated 3 times.

Immune-challenged by four microorganisms

The larvaes on third day at fifth instar were used for microorganism injection. Larvae were injected with PBS (as control), heat-killed *Escherichia coli* (DH5α, Gram-negative bacterium, 10^4 cells/larvae), *Micrococcus lysodeikticus* (Gram-positive bacterium, 1 μg/larvae), *Beauveri bassiana* (Fungi, 10^4 cells/larval) or nucleopolyhedrovirus (NPV, 10^4 virions/larval) at a total 5 μl volume (Liu *et al*., 2009). Fat body from each treatment silkworm group (at least three larvae) was collected at 1, 4 and 12 h after injection. Each treatment was repeated five times.

Total RNA extraction and quantitative real-time PCR (qPCR)

Total RNA was isolated from fat body or other tissues with TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions. DNase treatment was performed to eliminate genomic DNA contamination in RNA. cDNAs were synthesized from mRNAs with random hexamers using M-MLV reverse transcriptase (Takara, Japan). Primers for BmCRT sequence (GenBank accession number: AY297158), as well as for the endogenous reference gene (cytoplasmic actin A3, GenBank accession number: U49854) (Zhao *et al*., 2012), were designed by the online Primer 3 internet based interface (http://frodo.wi.mit.edu) (Table I).

Table I.- Primers used for real-time PCR in this study.

Primer name	Primer sequence (5'---3')
BmCRT-SP	AAGTTCTTCAGCGACCCAGA
BmCRT-AP	GTCCTTCTGCTCCAGTTTGC
BmActin3-SP	ATCACCATCGGAAACGAAAG
BmActin3-AP	GGTGTTGGCGTACAAGTCCT

To detect *BmCRT* mRNA expression level in different tissues of silkworm larvae, semi quantitative RT-PCR was performed. One microliter of transcribed cDNA was used as a template for PCR reactions: 94°C for 3 min, 28 cycles of 94°C for 30 s, 55°C for 35 s, 72°C for 30 s, followed by 72°C for 5 min. The PCR products were separated on 1% agarose gel and photographed with the gel imaging analysis system.

Expression of *BmCRT* mRNA level in fat body after microbial challenge was performed by real-time PCR. Real-time PCR was performed using the Power 2× SYBR Real-time PCR Premixture (25 μl) (Takara) contained 12.5 μl 2× SYBR Premix Ex TaqII (Tli RNase Plus), 1 μl forward and reverse

primers, 2 µl cDNA, and 8.5 µl RNase-free H_2O. The amplification program procedure was: 95°C for 30 s, 40 cycles of 95°C for 5 s, 60°C for 30 s, 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 65 to 95°C. The relative expression level of *BmCRT* gene was calculated according to the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). All the real-time qPCR experiments were repeated five times. Relative fold expressions for *BmCRT* gene were set to 1 for the control treatment (calibrator). Normally distributed data were analyzed using one-way ANOVA analysis by DPS software (version 9.50) (Tang and Zhang, 2013). All the data were presented as relative mRNA expression (means of measurements ± standard error). Differences were considered significant when *P* value was<0.05.

Western blot

The silkworm organs were homogenized in phosphate buffered saline (PBS), and then centrifuged at 12,000x g for 10 min at 4°C. The pellet was removed, and the supernatant was collected for protein analysis. The protein concentrations were measured using the modified Bradford method using bovine serum albumin (BSA) as a standard. Total protein samples (10 µg each) from various tissues or treated fat body of *B. mori* were subjected to SDS-PAGE with 4 % stacking gel and 10 % separating gel, and proteins were then transferred to a polyvinylidene difluoride (PVDF) membrane by Mini-Trans-Blot electrophoretic transfer system (Bio-Rad, Hercules, CA). Membranes were blocked with 5% non-fat milk (diluted with PBS containing 0.1% Tween-20) (PBST) overnight at 4 °C. Then membranes were washed with PBST three times and subsequently incubated with PrCRT polyclonal antibody (Wang *et al*., 2012a) (diluted 1:1000) for 2 h at room temperature. After washing with PBST, membranes were incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (diluted 1:5000) for 1 h at room temperature. The immunoblot signal was detected by TMB Stabilized Substrate for HRP (Promega). Membranes were scanned with a Bio-Rad GS-800 imager.

RESULTS AND DISCUSSION

Tissue distribution of BmCRT

Semiquantitative RT-PCR results indicated *BmCRT* mRNA was highest expressed in testis, also high expressed in ovary, hemocytes, silk gland and fat body, but with a low level in Malpighian tubule, epidermis and midgut (Fig. 1A). While Goo *et al*. (2005) reported *BmCRT* gene was the dominant expression in fat body by Northern blot method. Maybe we detected the silkworm at different larvae stage. *PrCRT* mRNA was expressed in all organs tested, with significantly higher levels in hemocytes and Malpighian tubule (Wang *et al*., 2012a). Calreticulin amino acid sequence exhibited high homology (87.4 % identity), so we chose polyclonal antibody against PrCRT as primary antibody to detect BmCRT protein. Western blot results showed BmCRT protein levels were slightly higher in testis and fat body than other tissues (Fig. 1B). PrCRT protein was constitutively expressed in tissues (Wang *et al*., 2012a). GmCRT protein was detected highly in hemocytes but not in plasma and fat body (Choi *et al*., 2002). It is interesting that calreticulin in different insects have a different expression pattern, suggesting that BmCRT may be involved in many different processes in different tissues. The high level of BmCRT in fat body (one of the main tissue/organs in insect metabolism and innate immunity) suggesting that BmCRT may be involved in silkworm metabolism, immune response, and so on. The mRNA and protein level of BmCRT in testis is consistency, but in fat body is not consistency. Maybe BmCRT is transported from other tissues, and accumulated in fat body.

Expression pattern of BmCRT in fat body at 1 h after immune-challenged

Calreticulin has been reported that it could be induced by microorganisms (Wang *et al*., 2012a). To determine the expression of BmCRT in *B. mori* fat body after immune challenges, real-time PCR and western blot were performed. At 1 h after injection, *BmCRT* mRNA levels in fat body were significantly up-regulated when larvae were immune-challenged by *B. bassiana, M. lysodeikticus* or NPV, with the highest expression after *B. bassiana* injection (Fig. 2A). However, *BmCRT*

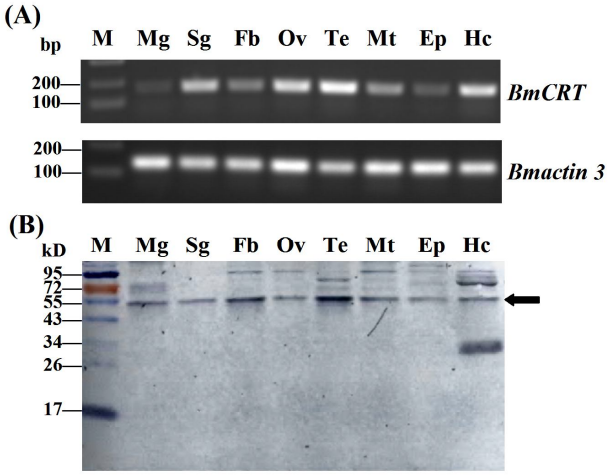

Fig. 1. Tissue distribution of BmCRT. (A) Analysis of *BmCRT* mRNA expression in *B. mori* larvae by semiquantitative real-time PCR. The silkworm cytoplasmic actin A3 gene (Bmactin3, GenBank accession No. U49854) was used as the endogenous reference gene. (B) Analysis of BmCRT protein in *B. mori* larvae by Western blot. Total proteins (10 μg) of each tissue were analyzed on 10 % SDS-PAGE, and BmCRT protein was detected by immunoblotting using anti-PrCRT rabbit polyclonal antibody. The arrow indicates the specific band of BmCRT. M: protein marker; Mg: Midgut; Sg: silk gland; Fb: fat body; Ov: ovary; Te: testis; Mt: Malpighian tubule; Ep: epidermis; Hc: hemocytes.

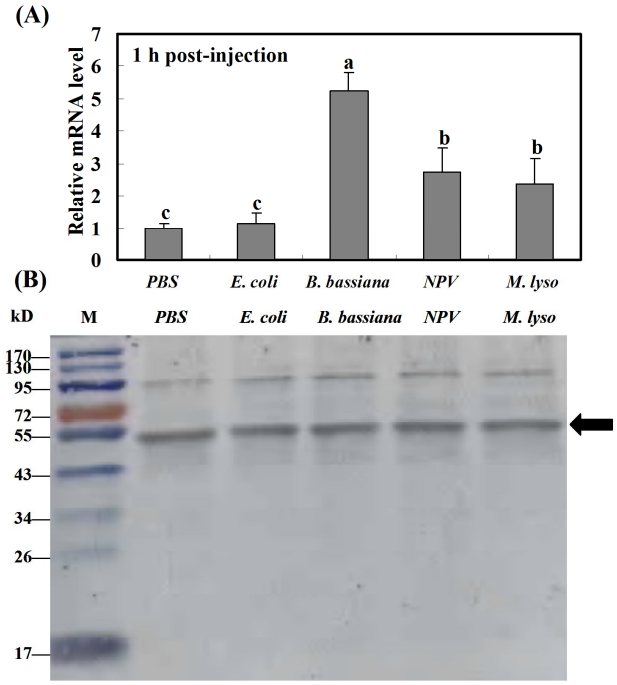

Fig. 2. Induced expression of BmCRT in *B. mori* larvae fat body after 1 h post-injection by microorganisms. Three-day fifth-instar *B. mori* larvae were injected with PBS (as a control), heat-killed *E. coli* (10^4 cells/larvae), *B. bassiana* (10^4 cells/larvae), NPV (10^4 virions/larval) or *M. lysodeikticus* (1 μg/larvae) at a total 5 μl volume. Fat body was collected at 1 h post-injection. (A) Expression of *BmCRT* mRNA in fat body was determined by real-time PCR. The bar represents the mean of three individual measurements ± SE. *BmCRT* mRNA in the PBS-injected fat body was designated as the calibrator. (B) BmCRT proteins in each treated fat body were analyzed by immunoblotting using anti-PrCRT antibody as the primary antibody.

mRNA in fat body did not change significantly after challenge by *E. coli* (Fig. 2A). The phenomenon also occurred in PrCRT when challenged by *E. coli* (Wang *et al.*, 2012a). BmCRT protein in fat body after 1 h immune-challenged had no significantly different change compared with control (Fig. 2B). The expression change of BmCRT protein was later than BmCRT transcript expression. These results suggest that BmCRT can be induced in early response to different microorganisms.

Expression pattern of BmCRT in fat body at 4 h after immune-challenged

qPCR results showed *BmCRT* mRNA in fat body were significantly up-regulated at 4 h after injection by *B. bassiana,* or NPV (Fig. 3A). *BmCRT* mRNA did not change significantly after injection by *E. coli* or *M. lysodeikticus* compared with control (Fig. 3A). Western blot results indicated BmCRT protein level in fat body at 4 h after injection by *E. coli*, *B. bassiana* or NPV were significantly increased than control or injected by *M. lysodeikticus* (Fig. 3B). The calreticulin genes (calreticulin, calreticulin like and calreticulin like 2) from channel catfish, *Ictalurus punctatus*, were induced high level expression at 4 h after infection by *Edwardsiella ictaluri*. Expression of calreticulin from *Fenneropenaeus chinensis* (FcCRT) was induced significantly after 3 h of heat shock treatment, reached the maximum at 4 h and dropped after that time (Luana *et al.*, 2007).

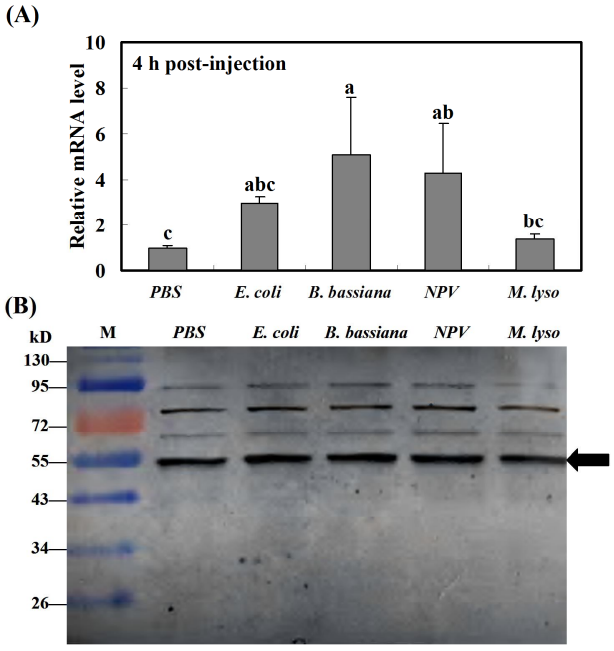

Fig. 3. Induced expression of BmCRT in *B. mori* larvae fat body after 4 h post-injection by microorganisms. Three-day fifth-instar *B. mori* larvae were injected with PBS (as a control), heat-killed *E. coli* (10^4 cells/larvae), *B. bassiana* (10^4 cells/larvae), NPV (10^4 virions/larval) or *M. lysodeikticus* (1 μg/larvae) at a total 5 μl volume. Fat body was collected at 4 h post-injection. (A) Expression of *BmCRT* mRNA in fat body was determined by real-time PCR. The bar represents the mean of three individual measurements ± SE. *BmCRT* mRNA in the PBS-injected fat body was designated as the calibrator. (B) BmCRT proteins in each treated fat body were analyzed by immunoblotting using anti-PrCRT antibody as the primary antibody.

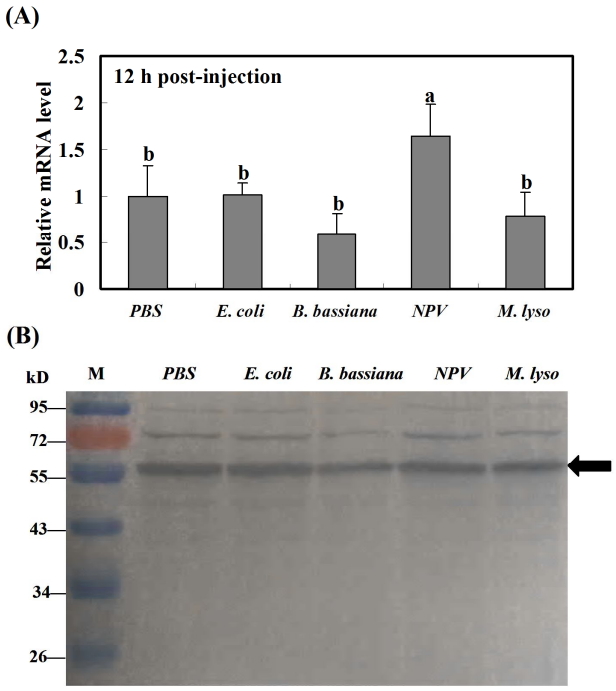

Fig. 4 Induced expression of BmCRT in *B. mori* larvae fat body after 12 h post-injection by microorganisms. Three-day fifth-instar *B. mori* larvae were injected with PBS (as a control), heat-killed *E. coli* (10^4 cells/larvae), *B. bassiana* (10^4 cells/larvae), NPV (10^4 virions/larval) or *M. lysodeikticus* (1 μg/larvae) at a total 5 μl volume. Fat body was collected at 12 h post-injection. (A) Expression of *BmCRT* mRNA in fat body was determined by real-time PCR. The bar represents the mean of three individual measurements ± SE. BmCRT mRNA in the PBS-injected fat body was designated as the calibrator. (B) BmCRT proteins in each treated fat body were analyzed by immunoblotting using anti-PrCRT antibody as the primary antibody

In hepatopancreas, the expression of FcCRT in white spot syndrome virus (WSSV) challenged group was up-regulated significantly at 5 h post-challenge and then its expression dropped (Luana *et al*., 2007).

Expression pattern of BmCRT in fat body at 12 h after immune-challenged

At 12 h after injection, *BmCRT* mRNA expression challenged by NPV was maintained a high level than control (Fig. 4A); *BmCRT* mRNA after challenged by *E. coli*, *B. bassiana* or *M. lysodeikticus* was with the same expression level with the control (Fig. 4A). However, BmCRT protein levels at 12 h after challenged by *B. bassiana* was decreased significantly compared with control (Fig. 4B). When after 12 h challenged by *E. coli*, NPV or *M. lysodeikticus*, BmCRT protein was maintained the same level of control (Fig. 4B). The expression of FcCRT at 14 h to 23 h after WSSV challenged was significantly lower than that in control shrimp (Luana *et al*., 2007). After 24 h polydnavirus HdIV injection, cDNA microarray results indicated transcript level was decreased in the hemocytes (Barat-Houari *et al*., 2006). The

transcript level of *PrCRT* in hemocytes was significantly decreased from 1 h up to 48 h post-parasitization by parasitoid wasp *P. puparum* compared to the non-parasitized control (Wang *et al*., 2012a). Venom protein PpCRT significantly inhibited PrCRT expression from 4 to 12 h post-injection (Wang *et al*., 2013). These results indicated calreticulin was involved in innate immune response and as a target protein for microorganism's invader.

In our study, BmCRT expression in fat body can be induced in response to different microorganisms, but with different expression patterns. This may be related to the surface features of different invaders. The specific surface components of microorganism can stimulate the specific innate immunity pathway to exert relevant immune response. In the present study, BmCRT was induced by the activation of the humoral immune response in silkworm fat body, since the fat body is capable of a humoral response in insect immunity. Previous studies showed that calreticulins in hemocytes played important roles in cellular immunity responses (encapsulation, phagocytosis) (Asgari and Schmidt, 2003; Wang *et al*., 2012a; Zhang *et al*., 2006). Calreticulin in insect humoral immune response was rarely reported. How calreticulin enhances insect innate (cellular and humoral) immune response was unclear. Just in mammalian species, calreticulin was reported as a receptor for C1q, mannose-binding lectins and ficolins, which connect calreticulin to innate immune processes (Naresha *et al*., 2009; Paidassi *et al*., 2011). Future work is to study how BmCRT or other insect calreticulin participates in immune response against pathogenic microorganisms.

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