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Molecular cloning of a phosphotriesterase-related protein gene of silkworm and its expression analysis in the silkworm infected with *Bombyx mori* cytoplasmic polyhedrosis virus

Xiu Wang[1], Kun Gao[2,3], Ping Wu[2,3], Guangxing Qin[2,3], Ting Liu[2,3], Xijie Guo[2,3*]

[1]College of Biotechnology and Chemical Engineering, Jiangsu University of Science and Technology, Zhenjiang, China;
[2]Sericultural Research Institute, Jiangsu University of Science and Technology, Zhenjiang, China;
[3]Sericultural Research Institute, Chinese Academy of Agricultural Sciences, Zhenjiang, China; *Corresponding Author: guoxijie@126.com

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ABSTRACT

***Bombyx mori* cytoplasmic polyhedrosis virus is one of the major viral pathogens for the silkworm. The immune response of silkworm to the virus infection is obscure. A phosphotriesterase-related protein gene of silkworm, *Bombyx mori* (*BmPTERP*) was found in our previous microarry analysis of the midgut infected with the virus. In the present study, we cloned and analyzed the full-length cDNA of *BmPTERP* gene by means of rapid amplification of complementary DNA ends (RACE) and bioinformatic analysis for exploring its functions in interaction between the silkworm and the virus. The nucleotide sequence of the gene is 1349-bp and contains a 131 bp 5'UTR and a 165 bp 3'UTR. The 1053 bp open reading frame encodes a 350 amino acid protein. The deduced protein contains specific hits of phosphotriesterase-related proteins and belongs to the amidohydrolase superfamily. RT-PCR analysis revealed that *BmPTERP* gene was expressed in all the tissues tested, including midgut, hemocyte, gonad, fat body and silk gland. Real-time quantitative polymerase chain reaction analysis indicated that the relative transcript of *BmPTERP* gene in the infected midgut was 19.32 fold lower than that in normal midgut at 72 hours post inoculation.**

Keywords: Silkworm; Cytoplasmic Polyhedrosis Virus; Phosphotriesterase-Related Protein; Gene

1. INTRODUCTION

Phosphotriesterase-related protein (also called phosphotriesterase homology protein, PHP) is a member of amidohydrolase superfamily and exhibits higher sequence identity, and high sequence similarity to phosphotriesterase (PTE) [1]. PTE is a group of bacterial enzyme that catalyzes the hydrolysis of a wide range of organophosphate triesters including organophosphate insecticides and chemical nerve agents [2]. PTE exists as a homodimer with one active site per monomer. The active site is located next to a binuclear metal center, at the C-terminal end of a TIM alpha-beta barrel motif and contains tow zinc ions in native enzyme. However, these ions can be replaced with other metals such as cobalt, cadmium, nickel or manganese and the enzyme still remains active. The hydrolysis reaction of PTE is sufficient to utilize the nucleophilicity of the bridging hydroxide according to theoretical study of the phosphotriesterase reaction mechanism [2].

The PTE has attracted much interest in recent years because of their potential ability in the decontamination of hazardous organophosphate compounds [3]. The most efficient PTEs have been identified from several microbial species, such as *Pseudomonas diminuta*, *Sulfolobus solfataricus* and *Flavobacterium* [4]. The directed evolution research of PTE resulted in improvement in functional expression and enzymatic activity [5,6]. Data collected showed that PTE was evolved from the family of phosphotriesterase-related proteins [7-9]. The research on its structure and expression would elucidate the evolutionary story of PTEs. To date, the phosphotriesterase-related proteins have been isolated from *E. coil* and some other organisms and grouped into a single family [7,9]. Although considerably homologous in sequence and in the structure of the bimetal catalytic site, phosphotriesterase-related proteins differs from PTE and exhibits no phosphotriesterase activity [1,7]. But, subsequent studies reported a weak esterase activity and PTE activity in an *E. coli* PHP mutant [1].

Bombyx mori cytoplasmic polyhedrosis virus (BmCPV), which belongs to the genus *Cypovirus* in the family *Reoviridae*, is a significant virus to the silkworm, often causing severe economic damages to the sericultural industry [10,11]. Unfortunately, to date, the immune mechanism of silkworm to CPV infection remains obscure. In our previous studies, a differentially expressed gene similar to the genes of phosphotriesterase-related proteins was identified in the CPV-infected silkworm by using microarry analysis [12]. In the present study, we first cloned the gene of phosphotriesterase-related protein from silkworm, *Bombyx mori* (*BmPTERP*) by means of RACE method. Furthermore, the expression patterns of *BmPTERP* in midgut and some other tissues after infection with BmCPV were analyzed by quantitative real-time PCR. The results provided useful information for further study of the immune mechanism of silkworm to virus infection.

2. MATERIALS AND METHODS

2.1. Silkworm Strain

The silkworm strain p50 was provided by the National Silkworm Genetic Resource Preservation Center of Chinese Academy of Agricultural Science. The larvae of silkworm were reared with mulberry leaves at standard temperature of 25˚C and under a photoperiod of 12 h light and 12 h dark up to the fourth molting for virus inoculation.

2.2. Virus Inoculation

BmCPV was suspended in disinfected distilled water to a concentration of 10^8 polyhedra per mL. One mL viral suspension was spread totally on 10 pieces of mulberry leaves which were nearly 15 cm^2 each. 25 newly molted fifth instar larvae were fed on it. The control larvae were treated with the same amount of mulberry leaves spread with distilled water. The infection does was calculated as 4×10^5 polyhedra per larva.

2.3. Collection of Midgut and Other Tissues

Midgut of BmCPV-infected and control larvae were collected at 24, 48 and 72 h post-inoculation by dissecting the larvae on ice. The midgut were quickly washed in diethylpyrocarbonate (DEPC)-treated water to remove the attached leaf pieces and then immediately frozen in liquid nitrogen. At the same time, other tissues namely gonad, silk gland, hemocyte and fat body were also collected at 48 h post inoculation and frozen in liquid nitrogen before being stored at –80˚C. The same tissues of five larvae for midgut and of ten larvae for gonad, silk gland, hemocyte and fat body at each time point were mixed for RNA extraction and following experiments.

2.4. Isolation of Total RNA

Total RNA was extracted respectively from midgut, gonad, silk gland, hemocyte and fat body collected at different time point post inoculation by using Trizol reagent (Invitrogen, Carlsbad, CA, US) and subjected to DNase I treatment according to the manufacturer's protocol. The concentration of total RNA was determined by using a Biophotometer (Eppendorf, Hamburg, Germany) to measure the absorbance at 260 nm and 280 nm. RNAs with the A260:A280 value of 1.9 to 2.0 were stored at –80˚C and used for further study.

2.5. Rapid Amplification of cDNA Ends (RACE)

Full-length cDNA of *BmPTERP* gene was synthesized using 2 μg total RNA of midgut as a template with SMART^{TM} RACE cDNA Amplification Kit (Clontech). Specific primers for 5'RACE and 3'RACE were designed based on *BmPTERP* gene cDNA fragment revealed in our previous study. The primer for 5'RACE was 5'-TTACAACTGCCTCCTTCGCGGCCTGA-3' and that for 3'RACE was 5'-CGGCTGCGGTGTCAGTTTCCATCCTC-3'. 5'-RACE was performed in a reaction system of 25 μL containing 17.25 μL PCR-Grade water, 2.5 μL 10 × Advantage 2 PCR buffer, 0.5 μL dNTP Mix (10 μM), 0.5 μL 50 × Advantage 2 Polymerase Mix, 1.25 μL 5'-RACE-Ready cDNA, 2.5 μL UPM (10×), 0.5 μL 5'-specific primer (10 μM) and as the following procedures: 32 cycles of 95˚C for 30 s, 68˚C for 3 min. 3' RACE was performed in a reaction system of 25 μL containing 17.25 μL PCR-Grade water, 2.5 μL 10 × Advantage 2 PCR buffer, 0.5 μL dNTP Mix (10 μM), 0.5 μL 50 × Advantage 2 Polymerase Mix, 1.25 μL 5'-RACE-Ready cDNA, 2.5 μL UPM (10×), 0.5 μL 3'-specific primer (10 μM) with the same procedures. The PCR products were examined by electrophoresis in 1% agarose gel and the fragment sizes were determined relative to marker DNA. The appropriate band was purified, cloned into pGEM-T Easy vector and sequenced by Sangom Biotech Co. Ltd. (Shanghai).

2.6. Sequence Analysis, Multiple Sequence Alignment and Phylogenetic Analysis

The sequences were searched in GenBank with BLASTx for comparative analysis and assembled with the obtained fragments. The sequences were analyzed using the BLAST algorithm at NCBI (http://www.ncbi.nlm.gov/blastn). The gene structure was predicted with Gene Structure Display Server (GSDS, http://gsds.cbi.pku.edu.cn). The deduced amino acid sequence of *Bm-*

PTERP was analyzed with Protparam software (www.expasy.ch/tools/protparam.html) and ProtScale software (http://www.expasy.org/tools/protscale.html). The signal peptide was predicted with the SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP/). Multiple protein sequences were aligned using the MegAlign program by CLUSTAL W method in DNASTAR software package. A phylogenetic tree was constructed by MEGA 4.0 software.

2.7. RT-PCR

Total RNA extracted as described above from different tissues of fifth instar silkworm, including hemocyte, silk gland, fat body, gonad and midgut were used as template to synthesize cDNA using the Prime Script™ RT Reagent Kit (TaKaRa). The PCR reaction was performed as the procedures with denaturation for 2 min at 94˚C, 30 cycles of 30 s at 94˚C, 30 s at 54˚C and 10 min at 72˚C.

2.8. Quantitative Real-Time PCR of *BmPTERP* Gene

Total RNAs extracted respectively from the midgut of BmCPV-infected and control larvae at 24, 48 and 72 h were adjusted with diethylpyrocarbonate (DEPC) H_2O to a concentration of 500 ng/μL. 1000 ng of each of the total RNAs was reverse transcribed in 20 μL of reaction system containing 4 μL 5 × PrimeScript buffer (for Real time), 1 μL PrimerScript RT Enzyme Mix I, 1 μL Oligo dT Primer (50 μM), 1 μL Random 6 mers (100 μM), 2 μL Total RNA, 11 μL RNase Free dH_2O using the Prime Script™ RT Reagent Kit (TaKaRa). Quantitative real-time PCR was performed using 1 μL of diluted first-strand cDNA (1/10) in each 25 μL reaction volume according to the manufacturer's instructions for the SYBR Premix Ex Taq™ (TaKaRa). Specific primers for *BmPTERP* gene and *β*-actin were designed by Primer Premier 5.0 software (Primer Premier, Palo Alto, CA, US). For *BmPTERP* gene, the forward primer was 5'-ATTTAGACCGAACCCTACTTG-3' and the reverse primer was 5'-TCGTGGGACATTAACACTTT-3' and for *β*-actin, they were 5'-AATGGCTCCGGTATGTGC-3' and 5'-TTGCTCTGTGCCTCGTCT-3' respectively. Reactions were run in triplicate for the same pooled samples on an Opticon lightcycler (BioRad, Hercules, CA, US) using the following thermal cycling parameters: 95˚C for 10 s, 40 cycles of 95˚C for 5 s, 60˚C for 20 s, 72˚C for 5 s. Following amplification, melting curves were constructed. Data were analyzed and normalized relative to *β*-actin transcript levels by Opticon Monitor Analysis software (MJ Research, Waltham, MA, US). The relative quantitative method was used to evaluate the differential expression of *BmPTERP* gene [13]. Ct for amplified target product of *BmPTERP* gene and internal control *β*-actin was determined for each sample to normalize the differences in the amount of template and the efficiency of RT-PCR ($\Delta Ct = Ct_{target} - Ct_{\beta\text{-actin}}$). The RNA of the normal silkworm larvae was used as calibrator and the ΔCt for each sample was subtracted from the ΔCt of the calibrator to calculate the difference ΔΔCt. $2^{-\Delta\Delta Ct}$ was used to calculate the relative expression level of *BmPTERP* gene. Relative expression level is expressed as average ± SE.

For other tissues, the quantitative real-time PCR of *BmPTERP* gene were performed with the same procedure described above but using the total RNA from a specific tissue as template.

3. RESULTS

3.1. Occurrence of Infection

Inoculation of BmCPV with the concentration of 4×10^5 polyhedra per larva to the fifth instar silkworm caused infection and disease to all the larvae. It was confirmed by the appearance of white wrinkles on the midgut as typical symptoms at approximately 72 h post-inoculation and the observation of polyhedra under a microscope.

3.2. cDNA Cloning and Sequence Analysis of *BmPTERP* Gene

The full length cDNA of the phosphotriesterase-related protein gene of the silkworm, *Bombyx mori* (*BmPTERP* gene) was cloned by RACE method and then sequenced. The obtained full length cDNA of *BmPTERP* gene is 1349-bp, containing a 131-bp 5'untranslated region (UTR), a 165-bp 3'untranslated region and a 1053-bp open reading frame (ORF). The nucleotide sequence of the cloned gene has been deposited in GenBank with the accession number HQ391899. The ORF which locates in the region from 132-bp to 1184-bp encodes a putative protein of 350 amino acids. A putative polyadenylation signal AATAAA was detected in the 3'UTR 14-bp upstream from the poly(A) tail.

For analysis of the gene structure, the cloned sequence of *BmPTERP* gene was aligned with the genome sequence of *Bombyx mori* by Blastn. It was found that the full cDNA was completely contained in the genome sequence of *Bombyx mori* (GenBank accession number: BABH01012392.1). The result by GSDS (Genes structure display server) showed that the gene sequence of *BmPTERP* gene contains six exons and five introns (**Figure 1**). Each exons-intron boundary comforms to the "GT-AG" rule.

3.3. Characteristic and Phylogenetic Analysis of *BmPTERP*

Analysis by using Protparam software revealed that

the molecular weight of the putative *BmPTERP* was 39.03-KDa and isoelectric point 5.72. Analysis of the amino acid sequence with ProtScale software indicated that *BmPTERP* has stronger hydrophilicity. No typical signal peptide was predicted by SignalP 3.0, which suggested that *BmPTERP* may be a soluble protein. Homology analysis of the protein indicated that *BmPTERP* shares 69% identity to the phosphotriesterase-related protein of *Helicoverpa zea* (ADK73626.1) and 54% identity to the phosphotriesterase-related protein of *Apis mellifera* (XP_395159.2). A phylogenetic tree was constructed (**Figure 2**), showing that *BmPTERP* is clustered well with phosphotriesterase-related protein from *Helicoverpa zea*.

3.4. Expression Analysis of *BmPTERP* Gene in Different Tissues

RT-PCR analysis of the RNA from different tissues of fifth instar larvae of silkworm revealed that the gene of *BmPTERP* was expressed in all the five tested tissues, namely midgut, fat body, silk gland, hemocyte and gonad (**Figure 3**). The highest transcript level was found in fat body and the lowest transcript level in silk gland as compared to other tissues. Furthermore, the gene showed differential expression in the tissues of silkworm infected with BmCPV as compared to the normal ones. At 48 hours post inoculation, significant difference in the gene expression was found in gonads, while almost no changes in the hemocyte (**Figure 4**).

3.5. Expression Analysis of *BmPTERP* Gene in Normal and CPV-Infected Midgut of Silkworm

BmCPV infects the midgut and multiplies mainly in the columnar cells of the midgut of silkworm larvae. Therefore, the differential expression of *BmPTERP* gene in midgut of both CPV-infected and normal silkworm larvae at different time points 24, 48 and 72 h post inoculation was analyzed in details. Representative amplification plots of real-time PCR were used to differentiate transcript level in normal and CPV-infected midget of silkworm. The results showed that the transcript level of *BmPTERP* gene has significant difference between the midgut of CPV-infected and normal larva at 72 h point, while at 24, 48 h, its transcript level has no significant difference. The Ct values and the standard deviations of A (β-action transcript level in BmCPV infected midgets at 72 h point), B (β-action transcript level in normal midgut at 72 h point), C *(PTERPBm* transcript level in normal midgut at 72 h point) and D (*PTERPBm* transcript level in BmCPV infected midgut at 72 h point) were 19.16 ± 0.11, 20.3 ± 0.13, 24.3 ± 0.31, 27.49 ± 0.23, respectively. The qPCR distinguished that the expression of *BmPTERP* gene was obviously down-regulated in the BmCPV-infected silkworm at 72 h post inoculation. Its relative expression in the infected midgut was calculated to be approximately 19.32 fold lower than that in normal midgut at 72 hours post inoculation (**Figure 5**).

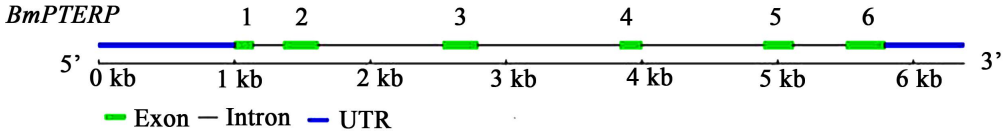

Figure 1. Genomic structure of *BmPTERP* gene. The gene contains six exons and five introns. The sequences of BABH01012392.1 were downloaded according to the result of alignment. On the basis of cDNA sequence of *BmPTERP* gene and downloaded sequence of BABH01012392.1, the gene structure of *BmPTERP* gene was analyzed by gene structure display server, GSDS (http://gsds.cbi.pku.edu.cn/). Exon1, 145bp(4149-4293); exon2, 252bp(4515-4766); exon3, 258bp(5684-5941); exon4, 165bp(6988-7152); exon5, 218bp(8045-8262); exon6, 279bp(8658-8936).

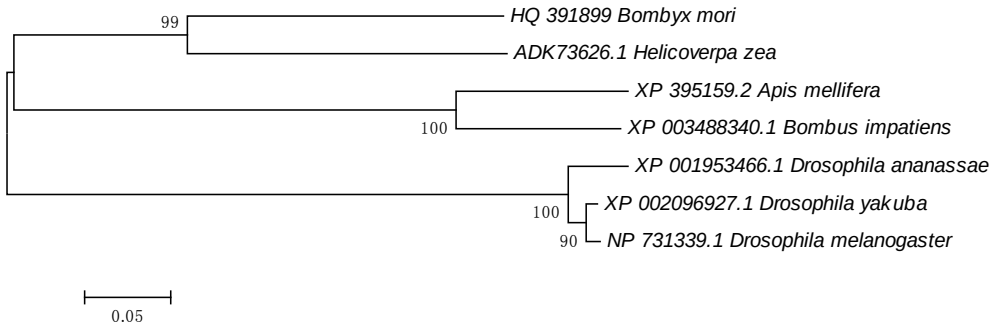

Figure 2. Phylogenetic tree of *BmPTERP* gene of silkworm and homologous sequences from other insects. This was constructed by MEGA 4.0.

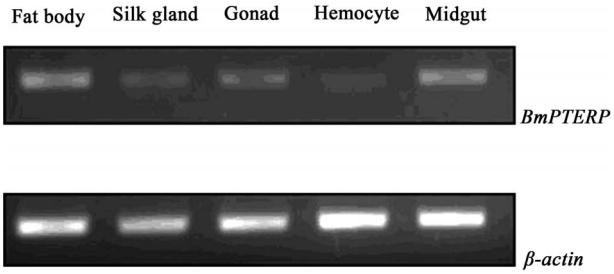

Figure 3. Expression analysis of *BmPTERP* gene in five different tissues. The *BmPTERP* gene was expressed in all the five tested tissues. *β*-actin in corresponding tissue were displayed in the bottom panel as internal control.

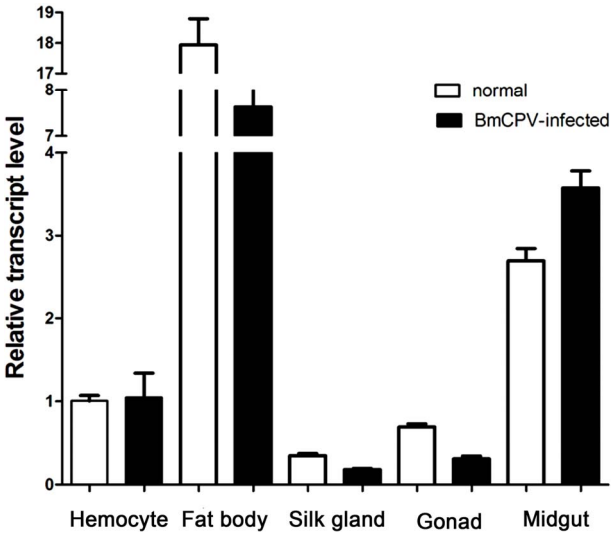

Figure 4. Relative expression analysis of *BmPTERP* gene in different tissues between the BmCPV-infected and normal silkworm at 48 hours post inoculation. The blank and filled columns indicate normal and CPV-infected silkworm respectively. Error bars represent standard deviation of triplicate experiments for the same pooled sample.

4. DISCUSSION

Bombyx mori cytoplasmic polyhedrosis virus (BmCPV) belongs to the genus *Cypovirus* in the family *Reoviridae.* BmCPV infects the midgut epithelium and multiplies in the cytoplasm of columnar cells and forms inclusion bodies which occlude virus particles. The virus contains segmented, double-stranded RNA as the genome [14,15]. However, the molecular mechanism of CPV infection in the silkworm is poorly understood. The purpose of this study is to provide significant information for exploring the molecular mechanism of CPV infection to the silkworm.

In the present study, the gene of a putative phosphotriesterase-related protein was cloned by means of RACE techniques for the first time from the silkworm, *Bombyx mori* and analyzed by bioinformatic method. Sequence analysis convinced that the gene we cloned encodes for a putative protein showing sequence identity with the phosphotriesterase-related protein family and belongs to the amidohydrolase superfamily. We therefore propose that the gene be called *BmPTERP* gene. Homologous analysis showed that the *BmPTERP* from silkworm shared high homologies with other known phosphotriesterase-related proteins, especially the highest with that of *Helicoverpa zea*. Phylogenetic analysis showed that the *BmPTERP* was clustered well with phosphotriesterase-related protein from *Helicoverpa zea*, indicating that these two genes are homologues and might share similar functions. Quantitative real-time RT-PCR showed that the relative transcript level of *BmPTERP* gene in the infected midgut was 19.32 fold lower than that in normal midgut at 72 hr post inoculation. The same tendency was also observed by Wu *et al.* [12] in microarray analy- sis of BmCPV-infected midgut of silkowrm. It was clear that after BmCPV invasion, the expression level of *BmPTERP* gene in midgut changed correspondingly, suggesting that *BmPTERP* gene of silkworm might be correlated to the interaction between the silkworm host and BmCPV infection.

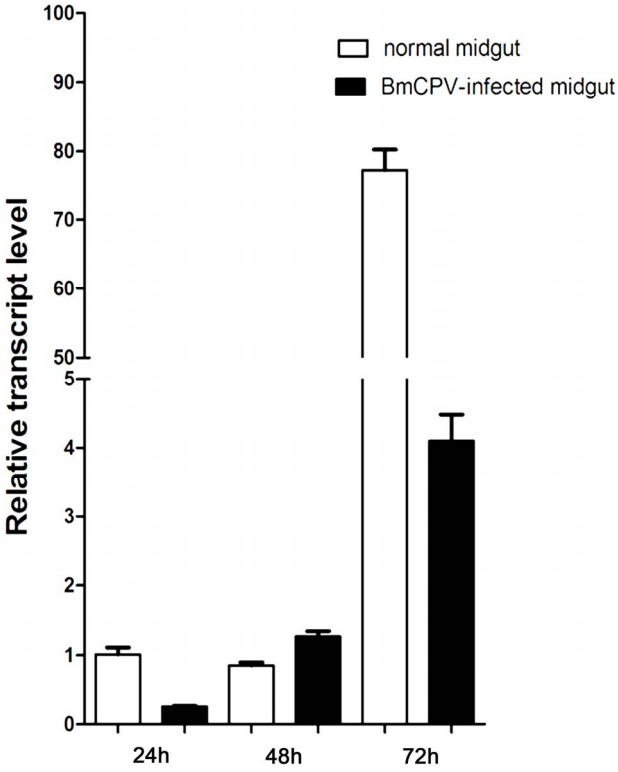

Figure 5. Relative expression of *BmPTERP* gene in BmCPV-infected and normal midgut of silkworm. The relative expression of *BmPTERP* gene in the infected midgut was approximately 19.32 fold lower than that in normal midgut at 72 hours post inoculation. Error bars represent standard deviation of triplicate experiments for the same pooled sample.

Regarding the function of the phosphotriesterase-related protein, only few reports were available at present. In mice, phosphotriesterase has hypothetical function that catalyzes small cytoplasmic molecules which would prove toxic and protects mice against organophosphate neurotoxins [16,17]. Attempts to control agricultural and forest insects and spread of insect-borne diseases, such as malaria, using organophosphate insecticides are being frustrated by the development of resistant strains of insects. Some of these achieve resistance by modification of acetylcholinesterase, others show a phosphotriesterase activity apparently different from that of bacterial phosphotriesterases [18]. However, no activity of the phosphotriesterase-related protein was commonly detected, except for a weak esterase activity and PTE activity in an *E. coli* PHP mutant [1]. Recently, a PHP gene from the thermophilic bacterium *Geobacillus caldoxylosilyticus* TK4 was cloned and overexpressed in *E. coli* [19]. The recombinant protein showed activities with *p*-nitrophenyl acetate and *p*-nitrophenyl butyrate. This is the first reported PHP having an extremely pH- and thermostable esterase activity.

Our result in the present study is the first report that a phosphotriesterase-related protein gene was cloned from the silkworm. While, its *in vivo* function still remains unclear. At present, the only clue comes from its strong homology to PHPs of bacteria and other organisms, but there is not yet any experimental proof that *BmPETRP* is itself a phosphotriesterase-related protein. As the disease of silkworm caused by the infection with BmCPV advanced, the expression of the *BmPETRP* gene was obviously down-regulated. This might be attributed to the facts that a series of physiological and pathological changes takes place due to the infection. Further functional experimental research should be addressed in our future work. If *BmPETRP* does turn out to have the activities of PHP or phosphotriesterase predicted from its homology, it would be very interesting to establish whether insects have homologous genes, whether these genes might confer resistance to organophosphate pesticides and most importantly whether these genes might be involved in the interaction between the silkworm and the BmCPV infecton.

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