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Expression analysis of silkworm genes related to juvenile hormone during the BmCPV infection

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ABSTRACT: *Bombyx mori* cytoplasmic polyhedrosis virus (BmCPV) is one of the most important pathogens of silkworm diseases, which infects the midgut epithelial cells of silkworm larvae. Previous results on isobaric tags for relative and absolute quantitation and digital gene expression showed that certain genes and proteins related to the synthesis, metabolism, and signaling pathway of juvenile hormone (JH) were expressed differentially during BmCPV infection. To investigate the relationship between BmCPV infection and JH regulatory mechanisms, quantitative real-time PCR was used to further detect the relative expression levels of some genes. Results showed that the genes involved in JH synthesis and signaling pathway were up-regulated in the BmCPV-infected midgut of silkworm, whereas those involved in JH metabolism were mainly down-regulated in the midgut after BmCPV infection. JH binding protein (JHBP) is the receptor protein that binds to JH, and its up-regulation of 40.7 times in BmCPV-infected silkworm at 120 hpi was closely related to the high concentration of JH. Thus, BmCPV infection could increase JH concentration by inducing synthesis or reducing the degradation of JH. High concentration of JH in the late stage of BmCPV-infected silkworm prolonged the larval stage of silkworm by participating in signal transduction pathway, providing a condition conducive to virus replication. The results would provide not only insights into the response mechanism of hormone regulation in silkworm during virus infection but also new ideas regarding the biological activities of candidate genes and defense mechanism in silkworm against BmCPV infection.

KEYWORDS: *Bombyx mori*, cytoplasmic polyhedrosis virus, quantitative real-time PCR, juvenile hormone

INTRODUCTION

Silkworm (*Bombyx mori*) is an important economic insect and has been used as a new laboratory animal model of human diseases such as phenylketonuria, Parkinson's disease, diabetes mellitus, and drug screening. However, virus infection is a major cause of silkworm death, resulting in enormous damage to the sericultural industry [1]. The midgut of silkworm is the first barrier against foreign substances such as pathogens and pesticides [2]. BmCPV specifically invaded midgut columnar cells and could form polyhedrons and cause white wrinkles in the posterior midgut as a chronic disease [3]. No specific therapeutic agent for BmCPV infection exists to date.

To explore the defense mechanism of silkworm against BmCPV infection at the molecular level, we analyzed the differential expression of proteomes and transcriptomes of silkworm midgut during BmCPV infection by using isobaric tags for relative and absolute quantitation and digital gene expression profiling. The results showed that the expressions of specific genes and proteins involved in the synthesis and metabolism of juvenile hormone (JH) significantly differed after the silkworm infection by BmCPV [4–6].

JH is a sesquiterpenoid that is synthesized and released by corpora allata of insects. JH regulates various development [7] revealed by transcriptome sequencing, reproduction [8], and physiological phenomena [9] in insects in cooperation with the steroid 20-hydroxyecdysone (20E). JH activity is elevated early in each larval instar to maintain larval shape and characteristics, whereas the titer of 20E consistently increases at the end of each larval instar to trigger the transition instar from larva to larva. In the final larval instar, the JH titer decreases significantly, whereas the 20E titer increases to a high level sufficient to initiate metamorphic transition from larva to pupa [10, 11]. During this period, cell apoptosis and cell proliferation occurs in the insect. Apoptosis plays an important role in insect metamorphosis and development and is a form of insect antiviral immune response [12].

In this study, to explore the relationship between BmCPV infection and JH regulatory mechanisms in *Bombyx mori*, we extracted the total RNA in the silkworm midgut after BmCPV infection. Several vital genes involved in JH biosynthesis, metabolism, and signaling pathway were selected, and their relative expression levels were detected by using Quantitative real-time PCR (qRT-PCR). The results provide not only insights into the relationship between BmCPV infection and JH regulation mechanism but also new ideas for

studying host resistance to BmCPV infection.

MATERIALS AND METHODS

Silkworm rearing

Domestic silkworm strain DaZao (p50) used in this study were provided by the National Center for Silkworm Genetic Resources Preservation of the Chinese Academy of Agricultural Sciences. The larvae were reared at standard temperature, suitable humidity and under a photoperiod of 12 h of light and 12 h of dark up to fourth molting.

Virus inoculation

BmCPV viral stock was suspended in distilled water at a concentration of 10^8 polyhedra per ml. One hundred microliters of viral suspension were spread evenly on mulberry leaves of approximately 4 cm^2 each in size. After slightly dried, these leaves containing virus were fed to 60 5th-instar larvae of newly molted silkworm. The infection dose was calculated to be 1×10^5 polyhedra per larva. The control larvae were fed with the same amount of mulberry leaves with sterilized water spread on them. Then, all larvae were fed with fresh mulberry leaves after 6 h.

Midgut collection

Midguts of both the BmCPV-infected and control larvae were collected at different post-inoculation times (pi) (12, 24, 48, 72, 96, and 120 hpi) by dissecting the larva on ice. The midguts were quickly washed in diethylpyrocarbonate (DEPC)-treated 0.9% physiologic saline solution to remove the attached leave pieces and then immediately frozen in liquid nitrogen. The midguts of 5 larvae were pooled as a sample, then stored at −80 °C for following experiments. All the experiments were carried out in 3 biological repetitions under the same condition.

RNA extraction

Total RNA from the midgut of the BmCPV-infected larvae and the control larvae were extracted by using RNA extraction Kit (Purchased from BLKW Biotechnology Co, Ltd, Beijing, China) according to the manufacturer's protocol and then diluted with RNA-free water. Total RNA concentration was determined using a Biophotometer (Eppendorf, Hamburg, Germany) by measuring absorbance at 260 nm and 280 nm (A260:A280). The integrality of total RNA was determined through 2% agarose gel electrophoresis. Total RNA was diluted to 1 g/l for subsequent reverse transcription and then stored at −80 °C.

qRT-PCR analysis

One microgram of total RNA of BmCPV-infected and control was used to synthesize the first strand cDNA using the PrimeScript Reverse Transcriptase kit (TaKaRa, Dalian, China) for qRT-PCR. These genes' primers used in this study were in Table S1, and β-actin A3 was used as a reference gene. The primers of reference gene were ActinF (5′- CCGTATGCGAAAGGAAATCA-3′) and ActinR (5′-TTGGAAGGTAGAGAGGGAGG-3′). qRT-PCR was carried out in an ABI PRISM 7300 Sequence Detection System (Applied Biosystems, Foster, USA) using SYBR Green Supermix (TaKaRa, Dalian, China) according to the instructions of the manufacturer. The thermal cycle conditions were 95 °C for 2 min for denaturation, followed by 40 cycles of 95 °C for 15 s, and 60 °C for 31 s for annealing, and then an extension. $2^{-\Delta\Delta Ct}$ method was used to calculate the differential expression of the target genes, and all data were given in terms of relative mRNA expression as mean ± SE.

RESULTS AND DISCUSSION

In the final larval instar of silkworm (p50), the JH titer decreased significantly, whereas the 20E titer increased to a high level sufficient to initiate metamorphic transition from larva to pupa. The high-performance liquid phase-ultraviolet (HPLC-UV) method was used to detect the titers of JH in the experimental group after the silkworm was infected with BmCPV and the control group. But it could not be detected due to its low concentration in the haemolymph of 5th-instar silkworm. Quantitative real-time PCR was used in this study to detect the relative expression of some genes (JHAMT and FAMet) that participated in JH synthesis, genes (JHE, JHEH, and JHDH) that participated in JH metabolism, and genes (JHBP and Kr-h1) that participated in JH signaling pathway (Table S1). The results showed that the genes involved in JH synthesis and signaling pathway were mainly up-regulated in the BmCPV-infected midgut of silkworm. At the same time, the genes involved in JH metabolism were mainly down-regulated in the midgut after BmCPV infection except at 120 hpi.

JH biosynthesis includes the mevalonate (MVA) pathway and an isoprenoid branch in *Bombyx mori*. The MVA pathway is called the upstream pathway of JH biosynthesis, the synthesis process utilizing acetyl-coA to generate farnesyl diphosphate (FPP) as the JH precursor. The synthesis process from FPP to JH is the downstream step of JH biosynthesis, also known as the isoprenoid branch [13]. Two essential enzymes are involved in this process: farnesoic acid (FA) O-methyltransferase (FAMeT) [14] and JH acid methyltransferase (JHAMT) [15, 16]. FA is epoxidated to form JH acid, which is methylated by JHAMT to synthesize JH, by Cyp15C1. FA is methylated by FAMeT to form methyl farnesoate, which is oxidized by Cyp15A1 to produce JH [17]. JHAMT (BGIBMGA010391) presented a notably low expression in the midgut of 5th-instar larvae after BmCPV infection and control larvae. The enzyme was hardly detected by the qRT-PCR system. At the transcription level,

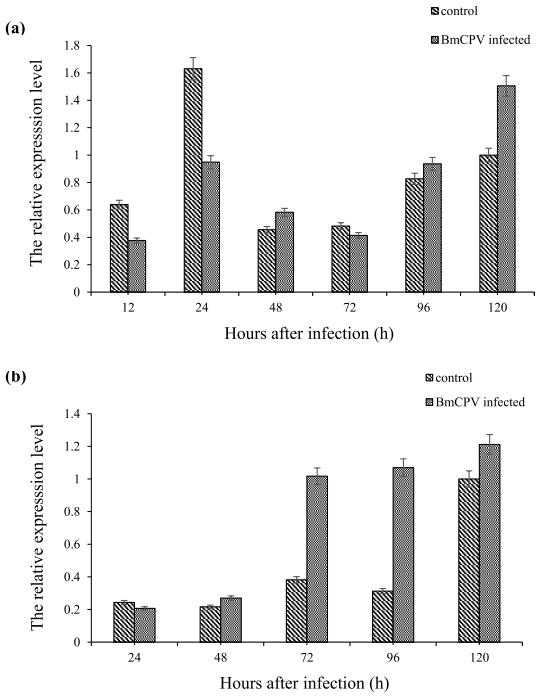

Fig. 1 The relative expression level of (a) *BmFAMeT* (BGIBMGA002604) and (b) FAMeT (BGIBMGA002314) in the midgut from BmCPV-infected and control silkworm larvae.

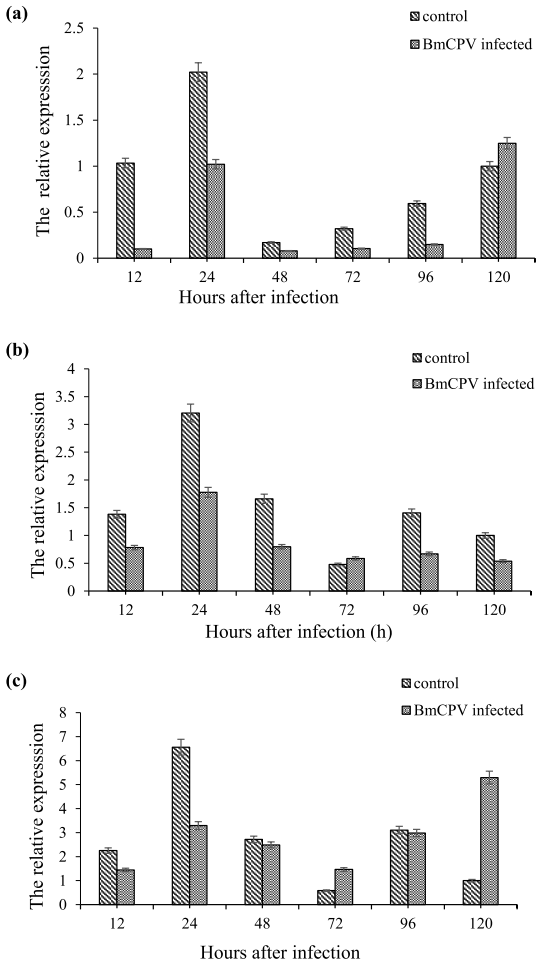

Fig. 2 The relative expression level of (a) *BmJHE*, (b) *BmJHEH*, and (c) *BmJHDK* in the midgut from BmCPV-infected and control silkworm larvae.

FAMeT (BGIBMGA002604) was down-regulated after BmCPV infection at 12 and 24 hpi and was slightly up-regulated after BmCPV infection at 72 and 96 hpi (Fig. 1a). There might be some relationship between BGIBMGA002604 and BmCPV infection. Another FAMeT (BGIBMGA002314) was a novel protein found by iTRAQ analysis after BmCPV infection and had a higher proteomic level at a later stage after BmCPV infection [6]. What is more, it was significantly up-regulated at the mRNA levels (Fig. 1b) in the silkworm midgut. So, BGIBMGA002314 may play an important role in the process of BmCPV infection by a bypass pathway of juvenile hormone synthesis.

The expressions of genes related to the JH metabolism pathway also changed at the transcription level due to BmCPV infection in *Bombyx mori* midgut. JH metabolism was catalyzed by JH esterase (JHE), JH epoxide hydrolase (JHEH), and JH diol kinase (JHDK) in hemolymph and cytoplasm [7, 18]. JHE (BGIBMGA000772), which converts JH to JH acid, showed down-regulation with 0.1, 0.5, 0.5, 0.3, and 0.25-fold change in qRT-PCR anlysis at 12, 24, 48, 72, and 96 hpi, respectively (Fig. 2a). JHEH could hydrolyze JH to JHd and JH acid to JH acid diol in the cytoplasm. BGIBMGA013930, as a JHEH, presented down-regulated expression in the midgut of silkworm during BmCPV infection (Fig. 2b). JHDK catalyzes the conversion of JH diol to JH diol phosphate, which is involved in the catabolism and inactivation of lipid-soluble hormones. BGIBMGA008814 and BGIBMGA008815 are genes coding for JHDK, whose mRNA expression levels were down-regulated in the BmCPV-infected midgut at 12, 24, 48, 72, and 96 hpi (Fig. 2c). BGIBMGA008815-PA exhibited down-regulated expression at the mRNA and proteomic levels in the silkworm midgut after BmCPV infection [6].

On the contrary, it showed that JHE and JHDK were up-regulated at 120 hpi in Fig. 2a and Fig. 2c. The upward tendency may be due to the higher concentration of JH in the BmCPV infected midgut than the controls which may feedback modulate its own degrading enzyme. By increasing the mRNA of JHE and JHDK at 120 hpi, more JHE and JHDK could be made to keep JH level from rising too high. Moreover, JH binding protein (JHBP) is the receptor protein that binds to JH and brings it into the target cells to prevent non-specific absorption. So, JHBP could sequester

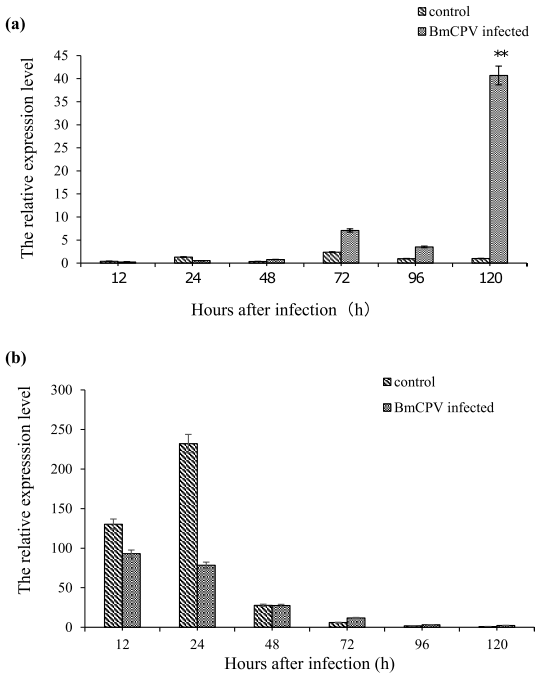

Fig. 3 The relative expression level of (a) *BmJHBP* and (b) *BmKr-h1* in the midgut from BmCPV-infected and control silkworm larvae.

away the excess JH. The higher concentration of JH, the more JHBP is needed. Therefore, the JHBP up-regulation of 40.7 times in BmCPV-infected silkworm at 120 hpi (Fig. 3a) was closely related to the high concentration of JH. These results showed that the concentration of JH was high in the midgut of BmCPV-infected silkworm at 120 hpi.

JH signal transduction pathway is involved in the mechanism of JH in insect molting, metamorphosis, and growth [19] with the identification and analysis of genes related to this pathway [20–22], and it is becoming increasingly important research hotspots. Certain molecules such as JHBP and Krüppel homolog 1 (Kr-h1) were involved in JH signaling pathway. BGIBMGA011553 coding for JHBP was first down-regulated at 12 and 24 hpi and exhibited significant up-regulation after BmCPV infection at the transcription level. The results showed that JHBP expression was up-regulated up to 6.8, 3.5, and 40.7 times in *Bombyx mori* infected with BmCPV compared with the control at 72, 96, and 120 hpi (Fig. 3a). Kr-hl encodes a transcription factor that contains C_2H_2 zinc finger structures and was first identified in *Drosophila melanogaster*; it is also a vital response gene mediating signal transmission downstream of JH signaling [23]. The expression level of Kr-h1 was 2.0, 1.8, and 2.3-fold change at 72, 96, and 120 hpi in BmCPV-infected midgut, respectively (Fig. 3b). The zinc finger transcription factor, Kr-h1, was identified as the anti-metamorphic factor which directly mediates transcriptional suppression via binding to the Kr-h1 binding site (KBS) on the promoters of several 20E primary-response genes. Meanwhile, JH biosynthesis was also prevented by 20E [24]. So, JH and 20E have antagonistic actions and the crosstalk between them through Kr-h1 to prevent 20E-induced metamorphosis.

The titer of 20E was detected by HPLC-UV method, and the results showed that the average concentration of 20E in the BmCPV-infected silkworm was higher than that in the control in the early stage of infection. The increase of 20E concentration could reduce the concentration of JH. So, the slightly down-regulation of Kr-h1 and JHBP might be due to the increase of 20E concentration at 12 and 24 hpi, but it was 0.3926 μg/ml in the hemolymph of the silkworm infected with BmCPV, which was about 1/3 of that in the normal silkworm at 72 hpi. As a result, the expression of Kr-h1 was significantly up-regulated at 72, 96, and 120 hpi in BmCPV-infected silkworm which was similar to that of JHBP after BmCPV infection. The high concentration of JH in the late stage of BmCPV-infected silkworm prolonged the larval stage of silkworm by participating in signal transduction pathway, providing a condition conducive to virus replication.

Several studies reported that JH biosynthesis-related genes were up-regulated, and JH metabolism-related genes were down-regulated after microsporidia infection. Ma et al [25] observed that the expression of JH biosynthesis-related genes of FAMeT and JHAMT were up-regulated, and JH metabolism-related genes were down-regulated after Nosema bombycis infection. Chen [26] revealed that JHBP0303, which is a member of the JHBP family, exhibited a constantly high expression in silkworm fat body after infection with the gram-positive bacterium *Staphylococcus aureus*, gram-negative bacterium *Serratia marcescens*, fungus *Beauveria bassiana*, or phosphate buffer solution injection. Other related studies showed that the JHBP gene was also up-regulated after *Bombyx mori* nuclear polyhedrosis virus (NPV) infection. Additional JH acid could stimulate the transcriptional activity of BmNPV ie-1 promoter in insect cells [27].

In this study, we observed that the related genes involved in JH synthesis and signaling pathway were mainly up-regulated, whereas those involved in JH metabolism were down-regulated in the midgut after BmCPV infection. Virions replicated largely in the midgut after the silkworm was infected by BmCPV, and this condition might induce silkworm to synthesize more JH and reduce the enzyme degradation of JH to increase its concentration. High concentration of JH prolonged the larval stage of silkworm by participating in signal transduction pathway, and this condition was conducive to virus replication. The infection of BmCPV altered the expressions of genes involved in JH biosynthesis, metabolism, and signaling pathway, and such

results could affect the physiological development, ecdysis, and metamorphosis of infected silkworm larvae. Therefore, BmCPV infection generally causes hypogenesis and emaciation, prolongs pupation, and reduces the body size of infected larvae.

Appendix A. Supplementary data

Supplementary data associated with this article can be found at http://dx.doi.org/10.2306/scienceasia1513-1874.2022.xxx.

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Appendix A. Supplementary data

Table S1 Sequences of primers for qRT-PCR.

Pathway	Gene ID	Gene	Primer
JH synthesis	BGIBMGA002604	Farnesoic acid O-methyltransferas (FAMeT)	5′ CGGTGGAGAAACTGAAGATGG 3′ 5′ TGTAGCAGACGCCGTGAGA 3′
	BGIBMGA002314	Farnesoic acid O-methyltransferas (FAMeT)	5′ CGGTGGGTGTCAGCGTCT 3′ 5′ GCTCTGCCAACATACAAGGTCTC 3′
	BGIBMGA010391	Juvenile hormone acid methyltransferase (JHAMT)	5′ ACCACGGCTTCGGTAGGAC 3′ 5′ GTGCGGTAAACATCAAAGATAGGA 3′
JH metabiolism	BGIBMGA013930	Juvenile hormone epoxide hydrolase (JHEH)	5′ CCAGCGGCGACATTCCTTGAG 3′ 5′ TCAGGCTTCGTGGCTTGAATATGC 3′
	BGIBMGA000772	Juvenile hormone esterase 1 (JHE)	5′ CTGCTGCTCACCTTCTCACCTTATC 3′ 5′ ATTAGTGGAGTTGACGCCAAGGATG 3′
	BGIBMGA008814	Juvenile hormone diol kinase (JHDK)	5′ TCCGATAAGAGTGGAGTCGTAGA 3′ 5′ GCCATTCGTCCTGAGTAACCT 3′
JH signaling	BGIBMGA011553	Juvenile hormone binding protein e96h-0303	5′ GTCCAGGCTGGCTTGCACATG 3′ 5′ ATTCACCAACCAGTGTCACAGAGC 3′
	BGIBMGA003160	Kruppel homolog 1 (Kr-h1)	5′ ACGAGGAGCGAGTTCATCAGT 3′ 5′ CGCCAGTATGGGTTCGGTAG 3′