African Journal of Biotechnology Vol. 8 (16), pp. 3711-3720, 18 August, 2009
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2009 Academic Journals

Full Length Research Paper

Differentially expressed genes in the midgut of Silkworm infected with cytoplasmic polyhedrosis virus

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Accepted 16 June, 2009

Understanding of the responsive and interactive mechanism between the host cells and *Bombyx mori* cytoplasmic polyhedrosis virus (BmCPV) is crucial to the diagnosis of CPV-caused disease and the development of new control measures. In this report, we employed suppression subtractive hybridization to compare differentially expressed genes in the midguts of CPV-infected and normal silkworm larvae. 36 genes and 20 novel ESTs were obtained from 2 reciprocal subtractive libraries. Three up-regulated genes (ferritin, rpL11 and alkaline nuclease) and 3 down-regulated genes (serine protease, trypsin-like protease and inhibitor of apoptosis protein) were identified by quantitative real¬time PCR. The transcript differences of these 6 genes at 6, 12, 24, 48 and 72 h post-inoculation both in CPV-infected and normal midguts were compared. Our results indicated that ferritin and rpL11 were increased during the early stage (6-12h p.i.) of CPV infection, whereas alkaline nuclease was increased during the late stage (24-72h p.i.) of CPV infection. The expression of serine protease and trypsin-like protease is decreased at 24-72 h after CPV infection, while the expression of inhibitor of apoptosis protein is decreased throughout the infective stage. Our results provide new clues for investigating the molecular mechanism of BmCPV infection.

Key words: *Bombyx mori*, cytoplasmic polyhedrosis virus, midgut, suppression subtractive hybridization, quantitative real-time PCR.

INTRODUCTION

Silkworm *Bombyx mori*, a model system for Lepidoptera, has contributed enormously to the study of insect immunology. *B. mori* Cytoplasmic polyhedrosis virus (BmCPV), one of the major viral pathogens for the silkworm, causes enormous damages to the sericultural industry. CPVs are classified as 14 distinct species (electropherotypes) within the genus *Cypovirus*, family Reoviridae (Hill et al., 1999) and the genome consists of 10 discrete double-stranded RNA segments (Rubinstein et al., 1976). The invasive target of BmCPV is the epithelial cells of the midgut. After infection, the debilitating effects of the virus on larval and postlarval stages have been remarkable (Magnoler, 1974). BmCPV is infectious to all larval instars but the younger instar larvae are more susceptible to the infection. The infected silkworms are characterized by hypogenesis, emaciation and sluggishness. As the disease advances, white wrinkles can be observed in the posterior part of the midgut, which is the typical symptom of CPV-caused disease. A series of changes involved in molecular pathology occur to silkworm due to BmCPV infection, such as, proliferation of secondary lysosome, tumefaction in cavity and bursa of endoplasmic reticulum, and increment of ribosome (Zhang et al., 1990). RNA of CPV is synthesized independently of host cell RNA synthesis (Kawase and Kawamori, 1968; Kawase and Furusawa, 1971). The hemagglutinating activity increased significantly in the hemolymph of the 5th instar larvae

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of the silkworm infected with BmCPV (Mori et al., 1989). In resent years, few relative researches have been reported. Therefore, to date, the molecular mechanism of CPV infection is poorly understood.

The purpose of this study is to identify differentially expressed genes between the CPV-infected and non-infected midguts in the silkworm by suppression subtractive hybridization (SSH), a powerful technique for selectively amplifying the differentially expressed cDNA fragments (Diatchenko et al., 1996). We report here our initial results as part of our efforts in exploring the molecular mechanism of BmCPV infection.

MATERIALS AND METHODS

Silkworm strain

The silkworm strain P50, provided by National Center for Silkworm Genetic Resources Preservation of Chinese Academy of Agricultural Sciences was used in this study. The larvae were reared at the standard temperature and under a photoperiod of 12 h of light and 12 h of dark up to fourth molting for virus inoculation.

Virus inoculation

The *B. mori* cytoplasmic polyhedrosis virus (BmCPV) was suspended in distilled water to a concentration of 10^8 polyhedra per ml. 1 ml viral suspension was spread evenly on 10 pieces of mulberry leaves of about 15 cm^2 each, which was fed to 25 newly exuviated fifth instar larvae. Among the 25 larvae, 10 were used for dissecting and collecting midguts and the remaining 15 larvae were reared to ob-serve whether the silkworm is infected or not. The control larvae were treated with the same amount of mulberry leaves spread with distilled water. The infected dose was calculated as 4×10^6 polyhedra per larva.

Midgut collection

Midguts of both the CPV-infected and control larvae were collected at different time points post-inoculation (pi) (6, 12, 24, 48 and 72 h pi) by dissecting the larva on ice. The midgut was quickly washed in diethylpyrocarbonate (DEPC)-treated 0.8% physiologic saline solution to remove the attached leave pieces and then immediately frozen in liquid nitrogen.

Isolation of total RNA and polyA+ RNA

Total RNA from the midguts of the CPV-infected larvae at 6, 12, 24, 48 and 72 h pi as well as from the water-treated larvae were extracted by using Trizol reagent (Invitrogen) and subjected to DNase I treatment according to the manufacturer's protocol. Total RNA concentration was determined using a Biophotometer (Eppendorf) by measuring absorbance at 260 and 280 nm (A260:A280). Equal amount of total RNA from 5 different time point was pooled. PolyA+ RNA was purified from the RNA pool by using an Oligotex mRNA Mid Kit (Qiagen) and 2 µg of poly (A) + RNA was used as the starting material for reverse transcription to construct the subtracted cDNA libraries. RNA samples were stored at –80°C.

Suppression subtractive hybridization (SSH)

SSH was performed using the Clontech PCR-select cDNA subtraction kit (Clontech). Reciprocal forward and reverse subtractions were constructed according to the manufacturer's protocol. The forward subtraction was constructed by using the cDNA of the CPV-infected midgut as the tester and the cDNA of the control as the driver. The reverse subtraction was constructed by using the cDNA of the control as the tester and the cDNA of the CPV-infected midgut as the driver. cDNA was synthesized with 2 µg pooled mRNA and was cut into fragments by Rsa I digestion. cDNA from the testers was then separated into 2 portions and adapters 1 and 2R were added each to one of the 2 parts. Testers were then separately hybridized with excess drivers in a ratio of 1:30 (tester:driver) for the first hybridization and followed by a second hybridization by adding 1 µl driver mixture to the first hybridization solution. Finally, suppression and nested PCR amplification was performed to amplify differentially expressed genes.

Cloning and sequencing analysis of cDNA fragments

PCR products were purified and cloned into the pGEM T-Easy vector (Promega), transformed into JM109 competent cells (TaKa Ra) and screened on Luria broth plates containing ampicillin/X-gal/IPTG. White clones from forward and reverse libraries were randomly chosen and incubated overnight at 37°C for sequence analysis. Sequences similarity (excluding vector sequence) was analyzed by blastx (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Annotation of genes was analyzed by online software AmiGO (http://www. geneontology.org/).

Identification of differentially expressed genes by quantitative real-time PCR

Total RNA from the midgut of CPV-infected and control larvae at 6, 12, 24, 48 and 72 h pi was extracted respectively by Trizol reagent (Invitrogen) and subject to DNase I treatment. The concentration of RNA at each time point was adjusted with DEPC H_2O to 500 ng/µl. A total of 800 ng RNA was reverse transcribed in a 20 µl of reaction sys-tem using the prime script^{tm} 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 manufac-turer's instructions of the SYBR Premix Ex Taq^{TM} (TaKaRa). Specific primers of genes (ferritin, ribosomal protein L11, serine protease, trypsin-like protease, alkaline nuclease and Inhibitor of apoptosis protein) and BmGAPDH, an endogenous control gene were designed by primer premier 5.0 software (Premier). The sequences of the primers were list in Table 1. The final concentration of the primers was 300 nM. Reactions were run in triplicate on an opticon llightcycler (BioRad) using the thermal cycling parameters: 95°C 10 s, 40 cycles of 95°C 5 s and 60°C for 20 s. Following amplification, melting curves were constructed. Data were analyzed and normalized relative to BmGAPDH transcript levels by Opticon monitor analysis software (MJ Research). A relative quantitative method (ΔΔCt) was used to evaluate relative expression difference.

RESULTS

Confirmation of infection

At about 72 h post-inoculation of BmCPV with a dose of 4×10^6 polyhedra per larva, all the inoculated silkworm larvae got infected and diseased with the appearance of white wrinkles on the midgut as typical symptom and further confirmation by observation of polyhedra under a microscope.

Table 1. Sequences of primers used in real time PCR.

Gene	Forward primer	Reverse primer
Serine protease	CAGGTGCTGCGACCGTTAGA	AAACGCCAAGTCAGCCTTCA
Trypsin-like protease	ATCTGACGGCAACCTCCACA	GAAGGCAGGGGCACCCAAGT
Inhibitor of apoptosis protein	TAAGGTAGAAATTATGAGGTGGGT	CGGGCAGGTACAAAGGGACA
Ferritin	CTTTCCCGTTGTTCTCGGTTAT	GCGACCTCCTCCACGATGCT
Ribosomal protein L11	CTTTGCTTTGCTATTAAGTATG	GTAAGACAGGAAAAGTGGGA
Alkaline nuclease	ATCGCTGGATTCGTGGGTAA	TGAAAGGCTGCCATTGAGGA
BmGAPDH	AATGTTGTTGCTGTTCCAGATGTAG	TTCCTCAGCAACCTCCGATG

Differentially expressed genes of two reciprocal SSH libraries

Total 36 genes and 20 novel ESTs were obtained from 148 sequenced cDNA clones of 2 subtraction libraries. Sequences of those ESTs have been submitted to dbEST (database of expressed sequence tags), the GenBank accession is FK939880-FK939934 and GO065780-GO065785.

In the forward subtraction, 20 putative genes and 13 novel ESTs were revealed from 79 sequenced cDNA clones by BLAST algorithm. Very high frequency genes included the ribosome protein genes rpL11 (6) and ferritin (8). Other high frequency genes were alkaline nuclease (4), lipase-1 (3), hypothetical protein 32 (3), lipase-like protein (3) and cytochrome C oxidase subunit I (3). However, most genes appeared at a low frequency of 1 or 2 times (Table 2).

In the reverse subtraction, 16 putative genes and 7 novel ESTs were revealed from 69 sequenced cDNA clones. Very high frequency genes included serine protease (6), trypsin-like protease (7) and alkaliphilic serine protease P-IIc (5). Other high frequency genes were inhibitor of apoptosis protein (4), chymotrypsin-like serine protease (4) and UDP-glucose 4-epimerase (4). Some low frequency genes were specifically detected in the reverse subtraction (Table 3).

The gene ontology (GO) project aims to describe the annotations of gene products of any organism. The 3 separate ontologies of GO are cellular component, biological process and molecular function. According to GO, among 36 genes, 22 genes can be annotated to 5 sub-groups of biological process, described as cellular process (72.73%), biological regulation (13.64%), metabolic process (4.55%), multicellular organismal process (4.55%) and multi-organism process (4.55%) (Figure 1). Similarly, 13 genes can be annotated to 3 sub-groups of molecular function, described as catalytic activity (76.92%), binding (15.38%) and transporter activity (7.69%) (Figure 2).

Confirmation of differentially expressed genes by quantitative real-time PCR

6 genes that showed high frequency in the 2 SSH libraries were selected to be identified by quantitative real-time PCR. We also compared the transcript level of these 6 genes in CPV-infected and normal midgut at 6, 12, 24, 48 and 72 h post-inoculation. 3 up-regulated genes from the forward SSH library were ferritin, rpL11 and alkaline nuclease and other 3 down-regulated genes from the reverse SSH library were serine protease, trypsin-like protease and inhibitor of apoptosis protein (IAP) (Figure 3).

As showed in Figure 3, the transcript level of ferritin in CPV-infected midgut was significantly higher than that in non-infected midgut at all time points. Its expression level exhibited a mountain-like pattern, with the expression peaking at 24 h in CPV-infected midgut of the silkworm (Figure 3a). The expression level of rpL11 in infected midgut was quickly increased at 6 h post-inoculation with a maximal level at 24 h and gradually decreased at 48 and 72 h. The transcript level of rpL11 both in CPV-infected and control midguts had no significant differences at 48 h (Figure 3b). With regard to alkaline nuclease, at 6 and 12 h post-inoculation, its transcript level has no significant difference between CPV-infected and control midguts, while at 24 - 72 h, its transcript level in CPV-infected midgut showed significantly higher than that in control midgut (Figure 3c). As to IAP, its transcript level in CPV-infected midgut was lower than that in non-infected midgut. The transcript level of IAP in CPV-infected midgut at 24 h post-inoculation was increased compared with that at 6 and 12 h, then its expression level was kept at a consistent level without obvious variations, while in normal midgut, its maximal level at 72 h (Figure 3d). The transcript level of serine protease and trypsin-like protease in CPV-infected midgut of silkworm was obviously lower than that in non-infected midgut at 24-72 h post-inoculation, but had no significant differences at 6-12 h (Figure 3e and f).

DISCUSSION

Upon infection with CPV, a serial of furious physiological and pathological changes have taken place in the silkworm. Under these circumstances, expression level of some genes that are responsive to the virus invasion has changed as well. Most genes were functional and played

Table 2. Differentially expressed genes from forward SSH library.

Clone no.	Accession no.	Putative gene products by Blastx or tBlastn	Annotation of genes	% of total sequenced clones
Biological process				
F5	FK939900	ribosomal protein L11 (*B. mori*)	GO:0006412 : translation	7.59
F32	FK939905	ferritin (*B. mori*)	GO:0006880 : intracellular sequestering of iron ion	10.10
F123	FK939929	hypothetical protein 32 (*B. mori*)	GO:0006915 : apoptosis	3.80
F92	FK939924	ribosomal protein L8 (*B. mori*)	GO:0006414 : translational elongation	2.53
F175	FK939934	Bombyx mori ribosomal protein L35A	GO:0006412 : translation	1.27
F75	FK939917	lipase-like protein [*Helicoverpa armigera*]	GO:0006629 : lipid metabolic process	3.80
F44	FK939908	ribosomal protein L24 (*B. mori*)	GO:0044249 : cellular biosynthetic process	5.06
F88	FK939922	ribosomal protein S12 (*B. mori*)	GO:0006412 : translation	2.53
F35	FK939906	RhoGEF domain-containing protein [*Dictyostelium discoideum* AX4]	GO:0009966 : regulation of signal transduction	5.06
F97	FK939925	Ribosomal protein S5 (*B. mori*)	GO:0006412 : translation	2.53
F144	FK939880	elongation factor 1 alpha (*B. mori*)	GO:0006414 : translational elongation	3.80
F109	FK939927	cytochrome C oxidase subunit I (*B. mor)*	GO:0042773 : ATP synthesis coupled electron transport	3.80
F4	FK939899	ribosomal protein L7A (*B. mori*)	GO:0007052 : mitotic spindle organization	2.53
Molecular function				
F52	FK939911	lipase-1 [*Bombyx mori*]	GO:0016298 : lipase activity	3.80
F82	FK939920	putative amino acid transporter (*B. mori*)	GO:0015171 : amino acid transmembrane transporter activity	2.53
F139	FK939931	zgc:158376 (*Danio rerio*)	GO:0005515 : protein binding	1.27
F71	FK939915	similar to KIAA1629 protein [*Pan troglodytes*]	GO:0003700 : transcription factor activity	1.27
F78	FK939918	myosin 1 light chain (*Lonomia oblique*)	GO:0003774 : motor activity	2.53
F104	FK939926	alkaline nuclease [*Bombyx mori*]	GO:0004518 : nuclease activity	5.06
E12	GO065783	juvenile hormone esterase (*B. mori*)	GO:0004453 : juvenile-hormone esterase activity	3.80
Unknown function				
F67	FK939914	GF13620 (*Drosophila ananassae*)	unknown	1.27
F197	FK939897	dispersed gene family protein 1(*Trypanosoma cruzi* strain CL Brener)	unknown	1.27
F84	FK939921	unknown	unknown	2.53
F114	FK939928	unknown	unknown	1.27
F11	FK939903	unknown	unknown	2.53
F80	FK939919	unknown	unknown	1.27
F26	FK939904	unknown	unknown	2.53
F49	FK939909	unknown	unknown	1.27
F51	FK939910	unknown	unknown	2.53

Table 2. contd.

F146	FK939932	unknown	unknown	1.27
F62	FK939913	unknown	unknown	2.53
F59	FK939912	unknown	unknown	1.27
A2	GO065780	unknown	unknown	3.80

Table 3. Differentially expressed genes from reverse SSH library.

Clone no.	Accession no.	Putative gene products by Blastx or tBlastn	Annotation of genes	% of total sequenced clones
Biological process				
R53	FK939892	alkaliphilic serine protease P-IIc=trypsin-like protease (*B.,mori*)	GO:0006508 : proteolysis	7.25
R104	FK939893	trypsin-like protease (*B. mori*]	GO:0032501 : multicellular organismal process	10.14
R50	FK939891	chlorophyllide A binding protein precursor (*B. mori*)	GO:0030682 : evasion or tolerance of host defense response	4.35
R2	FK939882	SLY-1 homologous (*D. melanogaster*)	GO:0006888 : ER to Golgi vesicle-mediated transport	4.35
R199	FK939898	Predicted: similar to 24-dehydrocholesterol reductase (*Tribolium castaneum*)	GO:0006695 : cholesterol biosynthetic process	2.90
R13	FK939888	inhibitor of apoptosis protein (*B. mori*)	GO:0048523 : negative regulation of cellular process	5.79
R7	FK939901	serine protease (*B. mori*)	GO:0006508 : proteolysis	8.70
R72	FK939916	HMG176 [*Helicoverpa armigera*]	GO:0048813 : dendrite morphogenesis	2.90
F89	FK939923	chymotrypsin-like serine protease (*B. mori)*	GO:0006508 : proteolysis	5.79
Molecular function				
R8	FK939884	formiminotransferase cyclodeaminase (FTCD)	GO:0030412 :formimidoyltetrahydrofolate cyclodeaminase activity	2.90
R9	FK939885	similar to ubiquitin-activating enzyme E1-domain containing 1 (*Apis mellifera*)	GO:0008641 : small protein activating enzyme activity	2.90
R16	FK939889	triacylglycerol lipase (*B. mor)*]	GO:0004806 : triacylglycerol lipase activity	5.79
R38	FK939890	lysozyme (*B. mori*)	GO:0003796 : lysozyme activity	4.35
R167	FK939895	UDP-glucose 4-epimerase (*Culex pipiens quinquefasciatus*)	GO:0003978 : UDP-glucose 4-epimerase activity	5.79
A3	GO065785	NADPH oxidoreductase (*B. mori*)	GO:0016651 : oxidoreductase activity, acting on NADH or NADPH	4.35
Unknown function				
R118	FK939894	arylphorin (*B. mori*)	unknown	4.35
R5	FK939883	unknown	unknown	1.45
R10	FK939886	unknown	unknown	1.45
R196	FK939896	unknown	unknown	2.90

Table 3. contd.

R12	FK939887	unknown	unknown	2.90
E8	GO065782	unknown	unknown	4.35
F10	GO065784	unknown	unknown	1.45
A12	GO065781	unknown	unknown	2.90

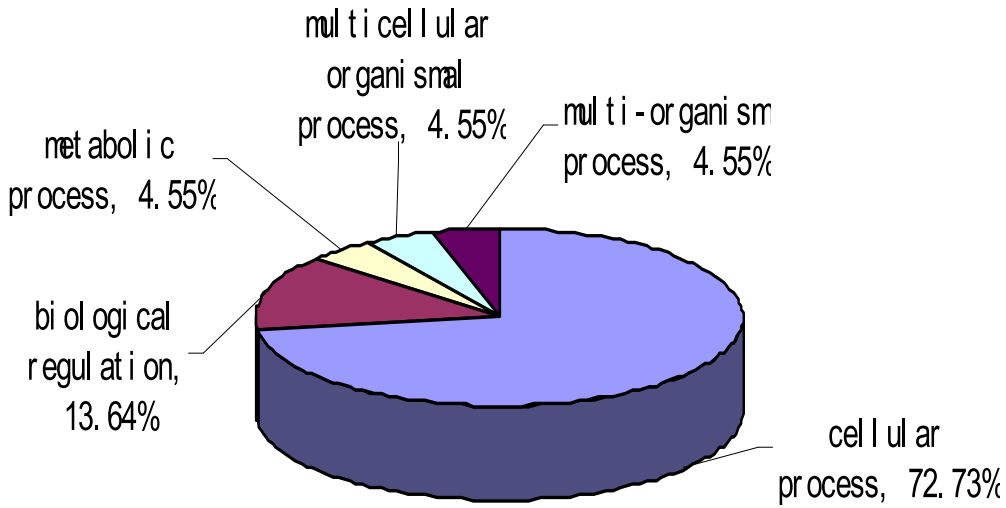


Figure 1. Genes identified to be involved in biological process. According to the Gene Ontology (GO)(http://www.geneontology.org/), a total of 22 genes from the two SSH libraries were involved in biological process, which were further classified into five sub-ontologies: cellular process(72.73%), biological regulation(13.64%), metabolic process (4.55%), multicellular organismal process(4.55%) and multi-organism process(4.55%)

important roles in the regulation of expression and metabolism, cell defense and material transportation. For better understanding of the molecular mechanism of the development of CPV infection, we analyzed the transcript level of six genes at 5 different time courses (6, 12, 24, 48 and 72 h post-inoculation), which could provided a detailed expression differences between CPV-infected and normal mid-guts of the silkworm.

Ribosomal proteins have been reported to participate in various cellular processes besides protein biosynthesis. They do not only act as components of the translation apparatus, but also regulate cell proliferation and apoptosis (Kasai et al., 2003). For example, rpL11 acts as a molecular switch to control L7 binding and plays a pivotal role during protein synthesis (Harms et al., 2008). RpL24 is a key factor for translation re-initiation of downstream ORFs on the polycistronic cauliflower mosaic virus 35S RNA transcription unit (Nishimura et al., 2004). Differential expression of ribosome protein is associated with disease and development (zheng et al., 2006; Vaarala et al., 1998; Campbell and Wilson, 2002; Zhang et al., 2007). RpL11 is differentially expressed during metamorphosis (Dong et al., 2007). Over-expression of rpL11 inhibits c-Myc-induced transcription and cell proliferation (Dai et al., 2007). In our study, rpL11 was up-regulated in CPV-infected midgut especially at 6 - 24 h. Its expression

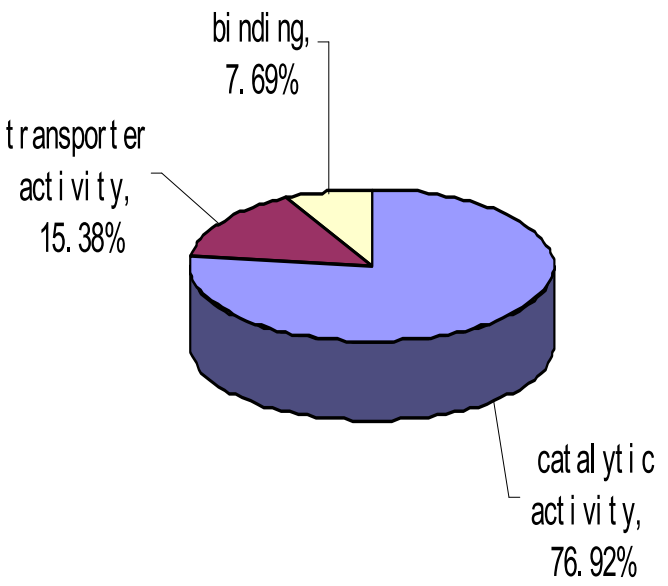


Figure 2. Genes identified to be involved in molecular function. 10 genes from the two SSH libraries were involved in molecular function by the rule of GO, which were further classified into three sub-ontologies: catalytic activity (70%), binding (20%) and transporter activity (10%)

reached the maximum level at 24 h post-inoculation, when progeny cytoplasmic polyhedrosis virus began to assemble (Sun et al., 2006). At this stage, a large number of genes used for translation were expressed to meet the needs of virus replication. Therefore, we conjecture that rpL11 may be one of the response genes to CPV which commandeered for viral replication and morphogenesis at the early stage of CPV invasion.

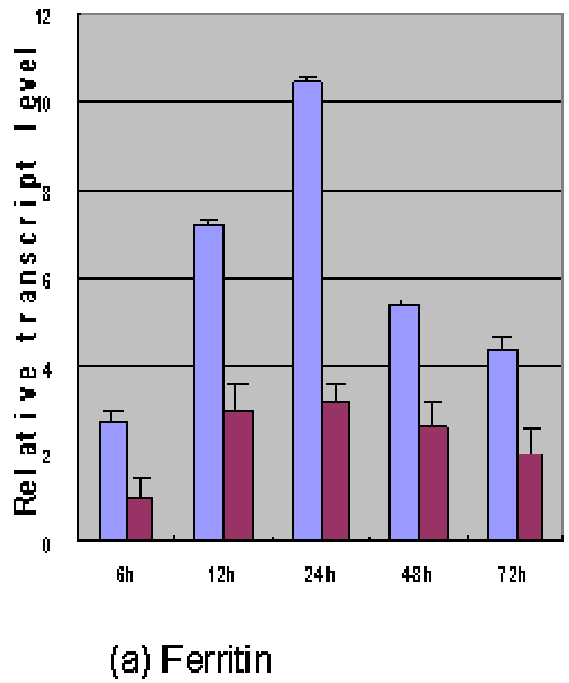
Ferritin is a ubiquitous iron storage protein. Diagnosis of several diseases can be assessed by the increasing level of ferritin (Peatman et al., 2007; Weiss, 2002). A primitive yet effective antimicrobial mechanism is depriving microbial organisms of their nutrients. Such a tactic is commonly referred to as the iron-withholding strategy of innate immunity (Ong et al., 2006). After BmCPV invasion, the expression level of ferritin in CPV-infected midgut quickly increased from 6 h and reached the maximum level at 24 h despite the fact that transcript levels also increased in the normal midgut, but not significantly when compare to the CPV-infected midgut. CPV invasion induced the high transcript level of ferritin at 6-24 h post-inoculation, which made us suppose that ferritin may be other important early response gene to CPV infection as well as rpL11.

We detected one up-regulated gene and 2 down-regulated genes with the function of catalysis in the CPV-infected midgut of silkworm. They encode alkaline nuclease, serine protease and trypsin-like protease, respectively. Molecular character of alkaline nuclease of silkworm revealed that it encoded a 51 kDa precursor protein. Its expression occurred in the middle and posterior midgut tissues and started from day 1 of the 5th instar larvae. The 43 kDa mature protein, post-translated from precursor protein, was produced in this tissue from day 2 (Arimatsu et al., 2007). Alkaline nuclease, which catalyses the hydrolysis of ester linkages within nucleic acids, appears to be involved in viral DNA processing and capsid egress from the nucleus (Shao et al., 1993). In our study, the expression of alkaline nuclease showed consistently higher level from 24-72 h post-infection in the CPV-infected midgut when compared with that in normal midgut. The mechanism of up-regulation of alkaline nuclease at 24-72 h post-infection in the CPV-infected midgut is unclear. We could only speculate that alkaline nuclease may be a late response gene to CPV infection.

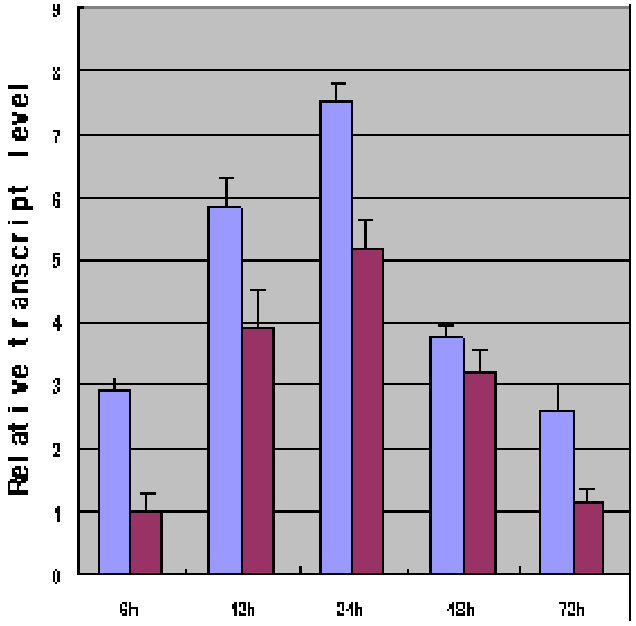
Serine protease and trypsin-like protease are the main digestive proteases in the midgut of silkworm. They were stable to the alkaline environment of the lepidopteran gut (Chougule et al., 2008). The serine proteases are a family of enzymes that cut certain peptide bonds in other proteins. This activity depends on a set of amino acid residues in the active site of the enzyme. In mammals, serine proteases perform many important functions, especially in digestion, blood clotting and the complement system. Recently, it was reported that the serine protease genes were expressed throughout feeding stages and down-regulated in non-feeding stages (Marshall et al., 2008). Trypsin-like protease was related to insecticide resistance in mosquitoes, *Cx. pipiens pallens* (Gong et al., 2005). Down-regulation of trypsin-like protease was also detected in BmDNV-Z (*B. mori* infection with a densonucleosis virus) susceptible strain compared with resistant strain (Bao et al., 2008). In CPV-infected midgut, the expression of these 2 genes was sharply decreased at 24-72 h, while at 6-12 h, these 2 genes have no significant expression differences between CPV-infected and normal midgut. It is suggested that at the late stage of CPV invasion, the serine protease and trypsin-like protease activity was decreased. Invasion of CPV leads to decompensation of protein and amino acid in the silkworm. We supposed that CPV infection destroyed the function of digestion and absorbtion of midgut, which resulted in drastically decrease of proteins in the midgut of silkworm.

IAPs are a family of antiapoptotic proteins and play important roles in both apoptosis and innate immunity (Leu et al., 2008). Growing evidence also indicates that IAPs also modulate cell division, cell cycle progression and signal transduction pathways (Schimmer, 2004). We detected the down-regulation of IAP in CPV-infected midgut. The transcript level of IAP in CPV-infected midgut consistently kept at a relative low level compared with that in normal midgut, which revealed that the IAP may response to CPV infection throughout the infective stage. Continuous multiplication of CPV may damage to the normal signal pathway of the cells. Decrease of IAPs make some given cells enter a cycle of uncontrolled proliferation.

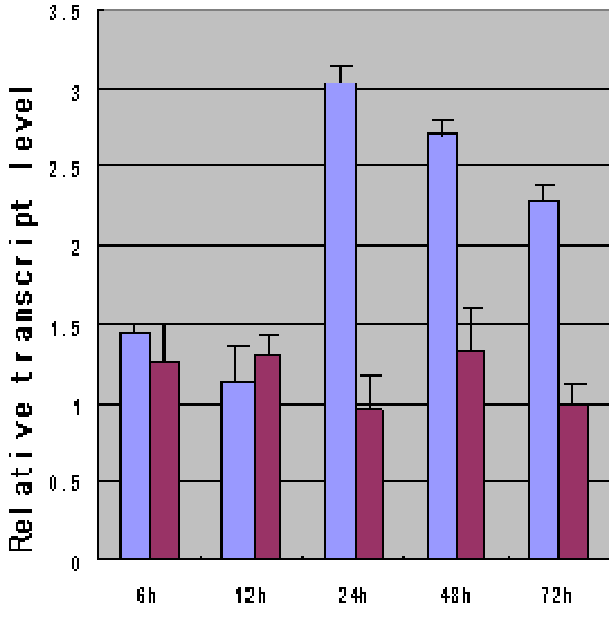
In our study, we also found significant changes of gene expression in control midgut along with the development of the silkworm. The expression of serine protease and



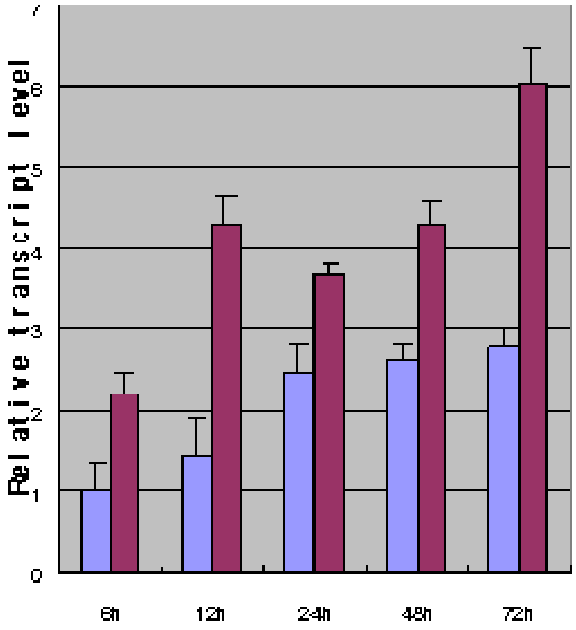
(a) Ferritin



(b) Ribosomal protein L11



(c) Alkaline nuclease



(d) Inhibitor of apoptosis protein

trypsin-like protease showed similarity and exhibited mountain-like pattern in control midgut. The peak transcript level of serine protease was at 24 h and that of trypsin-like protease was at 48 h. The expression of IAP was lowest at 6 h and reached the highest level at 72 h. The 5th instar of silkworm is an active stage, where a series of changes of metabolism and physiology took place. The metamorphism is a complex process and the mechanism of metamorphism has not been clear until now. According to our results, it is speculated that the proteins encoded by those genes are multi-functional and the significant changes in the expression level of those

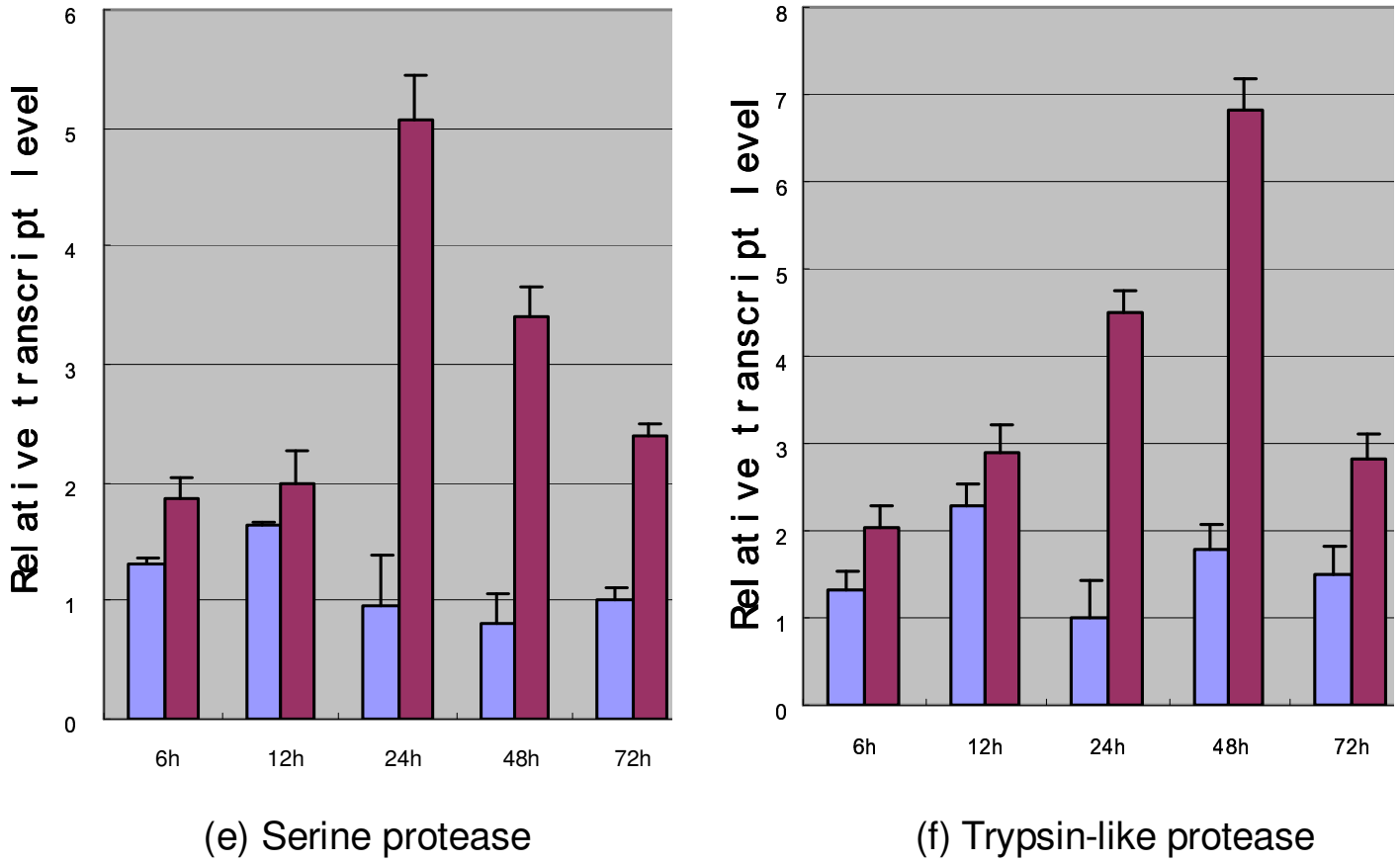


Figure 3. A relative expression analysis of CPV responsive genes in silkworm midguts at five different time points (6, 12, 24, 48 and 72h post-inoculation). Samples were analyzed in triplicate. Ct values of each reaction were normalized to GAPDH, the endogenous control. The mean value ± SD was used for analysis of relative transcript levels for each time point by the ΔΔCt method. In each assay, the expression level is shown relative to the lowest expression level, which is arbitrarily set to one. CPV-infected midgut and normal midgut were shown on the left (blue) and right (purple), respectively. Error bars represent standard deviation.

genes in the control are related to the digestion and absorption of food and the development of the silkworm. While, this paper focuses on the differentially expressed genes between CPV-infected and control midguts at the special time point and significant changes were distinguished.

In summary, our initial data provide some new clues to the molecular events that were induced by the CPV infection of silkworm. Confirmation of other candidate genes and functional analysis of these genes will be required to explore their potential roles in molecular mechanism of the development of CPV infection and innate immunity of insects.

ACKNOWLEDGMENTS

This project was supported by the National Basic Research Program of China (Grant No. 2005CB121004). We thank Drs. Ruiyin Chu and Hong Liu for critical reading and editing of this manuscript.

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