Proteomic Analysis of Differentially Expressed Proteins Involved in BmNPV Resistance in the Fat Body of Silkworm, *Bombyx mori*

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To investigate the mechanism of nucleopolyhedrovirus resistance of silkworm, we bred a near-isogenic silkworm line, designated BC_9, from the parental resistant strain NB and the susceptible strain 306, that is resistant to infection by nucleopolyhedrovirus. Proteomic techniques were employed to search for candidate genes playing a role in the antivirus response, based on differential protein expression profiles in the fat bodies of these strains. Four proteins were identified, two of which are possibly related to energy metabolism, the third one may have a function similar to integrase, and the fourth one is completely novel. Thus, our strategy of the combined use of near-isogenic silkworm line and proteomic techniques is effective for discovering new genes in the antivirus response of insects.

Key words: Bombyx mori, Nucleopolyhedrovirus, Fat Body

Introduction

Sericulture-related processing and manufacturing has thousands of years of history, and today remains a vital industry for many developing countries, including China, India, Thailand, Vietnam, Cambodia.

Sericulture industry is negatively affected by many factors, such as bad weather, pests, poisoning, and diseases. Domestic silkworm, *Bombyx mori* L. (Lepidoptera: Bombycidae), is the primary factory to generate raw silk, and it can be infected by a number of pathogens. BmNPV (*Bombyx mori* L. nucleopolyhedrovirus) is one of the key pathogens that can cause severe diseases of viral infection. For many decades, numerous attempts have been made to prevent and control viral diseases, but not very successfully, mainly due to the lack of an understanding of the mechanism of virus invasion and host-antivirus response (Popham *et al.*, 2004).

Recently, remarkable achievements have been accomplished in the characterization of BmNPV (Jehle *et al.*, 2006; Rahman and Gopinathan, 2004; Blissard, 1996; Vialard *et al.*, 1995). A rough picture of the infection cycle of BmNPV can be drawn like this: the enveloped viruses, occluded in a proteinaceous polyhedrin crystal, can be eaten by silkworm as food contaminates. The polyhedrin crystal is dissolved in the midgut, leading to the release of viruses and the infection of midgut cells. Enveloped virions without the polyhedrin crystal coat will be replicated, assembled, and released in large quantities during the early period of infection, and they can easily invade other types of cells, causing systematic and fatal infection. Later on the polyhedrin crystal will be formed, and the enveloped viruses will be coated and released once the silkworm has decomposed (Washburn *et al.*, 2001; Engelhard and Volkman, 1995; Kirkpatrick *et al.*, 1998). Despite of this rapid progress, the mechanism of entry, intracellular trafficking, replication, assembly, and release of BmNPV still remain elusive, as well as its interaction with the host cell.

Because viruses like BmNPV can cause a severe threat to the life of the silkworm, it would be reasonable to expect that the silkworm has some kind of antivirus mechanism allowing survival. It may lack adaptive immune responses widely utilized by vertebrates (Hoffmann, 2003), yet its strategy of defense is still comparably complex and effective, such as physical blockades (cuticle, peritrophic matrix, epithelial barriers), protease cascades leading to coagulation and melanization, phagocytosis and encapsulation (Lavine and Strand, 2002; Ligoxygakis *et al.*, 2002; Lehane, 1997; Yamakawa and Tanaka, 1999), cell-mediated antiviral immune response (Washburn *et*

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al., 2000), apoptosis or programmed cell death (Narayan, 2004), hemolin (Terenius, 2008), antimicrobial peptides like alloferons (Chernysh *et al.*, 2002; Lavine and Strand, 2002; Hoffmann, 1995), lipase and serine proteases (Ponnuvel *et al.*, 2003; Nakazawa *et al.*, 2004), NADPH oxidoreductase (Selot *et al.*, 2007), phenol oxidase (Popham *et al.*, 2004). Recently we have also discovered some genes that are related to BmNPV resistance in silkworm (Xu *et al.*, 2005; Yao *et al.*, 2003).

We have already discovered a silkworm strain, named NB, which is highly resistant to BmNPV (Chen *et al.*, 2003). To facilitate identification of the genes and factors in NB that are involved in this prominent antivirus response, we constructed a near-isogenic silkworm line (NIL) by introgression, which is resistant to BmNPV but is otherwise almost genetically identical to the parental strain 306 that is BmNPV-susceptible. On the other hand, fat body represents a major site for virus replication, and plays a key role in triggering the insect immune response against pathogens (Hultmark, 1993). In the present study we used proteomic tools to investigate the different protein expression profiles in fat body of NB, 306, and their NIL BC_9. Some proteins have been identified that were only expressed in BC_9 and NB but not in 306, indicating their possible roles in the resistance response to BmNPV.

Material and Methods

Silkworm strains

Highly BmNPV-resistant silkworm strain NB ($LD_{50} = 2.5 \cdot 10^8$ viruses/larva) and highly BmNPV-susceptible silkworm strain 306 ($LD_{50} = 3.4 \cdot 10^5$ viruses/larva) were used in this study. These strains were preserved and denominated by our laboratory.

Construction of near-isogenic line

The near-isogenic line was constructed according to Chen *et al.* (2003). The line BC_9 ($LD_{50} = 2 \cdot 10^8$ viruses/larva) was obtained by crossing progeny of strain 306 and NB back to 306. The backcrosses were conducted for nine generations followed by two generations of self-crossing. BmNPV was administered via mouth ($5 \cdot 10^6$/larva) to larvae to select the individuals that showed the BmNPV-resistant phenotype.

Collection of the fat body sample

All larvae from NB, 306, and BC_9 were fed on mulberry leaves, raised to the fifth instar at the same time, and kept in a photoperiod of 12 h light/12 h dark. The fat bodies of ten silkworms of each line were collected, respectively.

Protein sample preparation

500 mg fat body were frozen in liquid nitrogen and ground. The proteins were precipitated in cold acetone containing 10% TCA (trichloroacetic acid) and 5 mM DTT (dithiothreitol). After incubation at −20 °C for 2 h, the mixture was centrifuged at 4,100 × *g* for 30 min at 4 °C. The resultant pellet was washed twice with acetone containing 5 mM DTT, 1 mM PMSF (phenylmethanesulfonyl fluoride), and 2 mM EDTA (ethylenediaminetetraacetic acid), and lyophilized in a SpeedVac instrument. The resultant powder was suspended in 0.5 mL lysis buffer containing 7 M urea, 2 M thiourea, 4% CHAPS, 0.2% Bio-Lyte, pH 3–10 (Bio-Rad, Hercules, CA, USA), 1 mM PMSF, 2 mM EDTA, and 65 mM DTT, pH 8.5. The solution was sonicated for 3 min followed by centrifugation at 35,000 × *g* for 30 min. Protein concentration was measured with the RC DC Protein Assay kit (Bio-Rad).

2-D Gel electrophoresis (2-DE)

Isoelectric focusing electrophoresis was carried out with 17-cm (pH 3–10) IPG strips, at 20 °C, according to the manufacturer's instructions (Bio-Rad). Approx. 0.8 mg protein was loaded onto each gel, and six repeat experiments were done. Briefly, the strips were rehydrated at 50 V for 13 h. The isoelectric focusing was performed for 1 h at different voltages, *i.e.* 250, 500, 1,000, and 10,000 V, respectively, then at 10,000 V until it reached a total of 60 kVh. The focused strips were equilibrated for 15 min in the solution containing 6 M urea, 20% (v/v) glycerol, 2% (w/v) SDS (sodium dodecyl sulfate), 0.05 M Tris/HCl, pH 8.8, and 2% (w/v) DTT with traces of bromphenol blue. Then free thiol groups were alkylated for 15 min in the same solution containing 2.5% (w/v) iodoacetamide instead of DTT. The treated strips were placed on the immediate top of the 12% uniform SDS-polyacrylamide gels, and PAGE was performed at 2.5 W/gel for 30 min and 15 W/gel until bromophenol blue reached the bottom. The gels were visualized by Coomassie blue staining.

Image analysis

Gels were scanned by a Typhoon 8600 scanner (GE Healthcare, Milpitas, CA, USA) with a resolution of 600 dpi. The images were further processed by PDQuest 7.2 2-D gel analysis software (Bio-Rad). The images were cropped to frame the same clusters of spots. Six representative gels per silkworm strain were used to create a match-set. Spots were detected and matched automatically to a master gel selected by the software, then were edited manually. The spot boundary tool was applied to detect large spots. The patterns in sections of the gels in appropriate magnification were checked, and spots were added manually to the master gel to allow matching unique spots present in the individual gels. The means of the logarithmic ratios method was used for normalization. For gel comparison, a statistical approach was applied when determining differentially expressed proteins using the PDQuest software. Student's t-test was performed with 95% significance level to determine which proteins were differentially expressed.

In-gel digestion

Gel pieces indicated in Fig. 1 were extracted manually using a pipette with a trimmed polypropylene tip. Destaining and reduction/alkylation/digestion of the extracted proteins were done according to adapted procedures (Mirza *et al.*, 2000). Destaining and clean up were done by subsequently incubating the gels in water, 50 mM ammonium bicarbonate, 50% acetonitrile, and 100% acetonitrile, After reduction with DTT and derivatization with iodoacetamide, the gel pieces were treated with freshly prepared trypsin (Promega, Madison, WI, USA) solution (20 μg/mL in 40 mM ammonium bicarbonate/10% acetonitrile) and incubated at 37 °C overnight. The gels were extracted with 5% formic acid in 1:1 (v/v) water/acetonitrile. All solvents and reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA).

MALDI-TOF-MS identification

One μL of peptide extract was mixed with 1 μL of 10 mg/mL α-cyano-4-hydroxycinnamic acid (Sigma-Aldrich) saturated with 50% acetonitrile in 0.1% trifluoroacetic acid, and subjected to matrix-assisted laser desorption/ionization-time-of-flight (MALDI-TOF) mass spectrometry (MS). Standard peptide and trypsin were used for calibration according to the manufacturer's instruction (Ultraflex Tof/Tof; Bruker Daltonics, Billerica, MA, USA).

Protein identification

Mascot software (Matrix Science Ltd, London, UK) was used to search the NCBI and a silkworm EST database. The mass tolerance was set as 0.3 Da, and peptide modification was limited to alkylation of cysteine and oxidation of methionine. Peptide was identified with a Mowse score >79 ($P < 0.05$).

Results

Differential protein analysis

Fig. 1 shows that at least 480 protein spots could be easily identified in the 2-D gels. The genes responsible for BmNPV resistance should only exist or be expressed, respectively, in the BC_9 and NB strains, not in the 306 strain. Therefore, only those genes showing a 10-fold difference in their corresponding protein levels (intensity in gel) were considered to be involved in the anti-BmNPV function of silkworm, and were selected for further analysis.

As indicated in Fig. 1, four protein candidates were identified that showed marked expression in strains NB and BC_9, but were below the detectable level in 306. Fig. 2 shows a 3-D view, zoomed in to the corresponding gel region, to unambiguously pinpoint the candidates.

Mass spectrometry analysis

Protein spots 1–4 in Fig. 1 were extracted from the 2-D gels and analysed by MS. The MS data are shown in Table I.

Protein 1 was identified in a silkworm EST database which we constructed. One translated EST has a very high score with the MS data of protein 1, score = 123 and $P < 0.01$. With this EST, we searched to a *Bombyx mori* unigene through the NCBI database, the accession number of the unigene is Bmo.2272. Its function is not known but may be similar to a putative phosphoglycerate kinase from *Tribolium castaneum*.

Protein 2 was identified as a silkworm arginine kinase (AK) with a high score of 97. Protein 3 was unidentified. Protein 4 was identified with

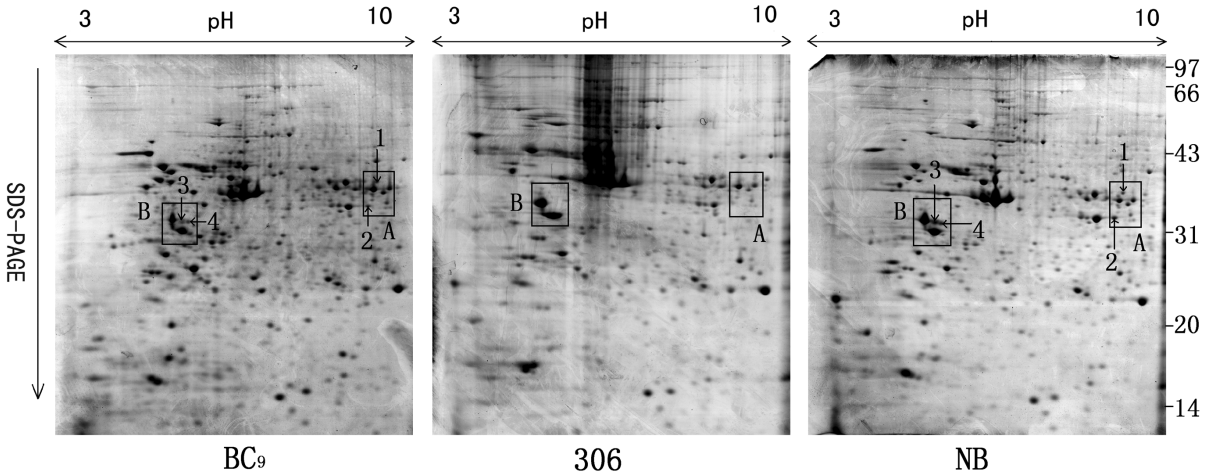

Fig. 1. 2-D Gel analysis of protein expression. NB, highly BmNPV-resistant silkworm strain; 306, highly BmNPV-susceptible silkworm strain; BC_9, near-isogenic line. Proteins (1 and 2 in A, 3 and 4 in B) were identified and are indicated in open rectangles that show marked difference in expression profile. The gels were visualized by Coomassie blue staining.

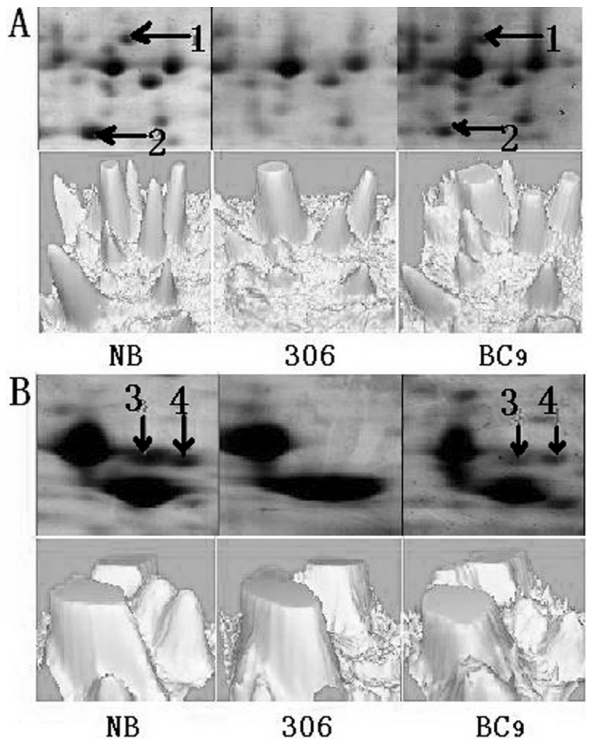

Fig. 2. Zoomed view of proteins with different expression profiles. NB, highly BmNPV-resistant silkworm strain; 306, highly BmNPV-susceptible silkworm strain; BC_9, near-isogenic line. The corresponding sections of A and B in Fig. 1 were zoomed in to project the four candidates. (A) Zoomed view; (B) 3-D view. The arrowheads indicate the four proteins.

a silkworm complete cDNA database which we collected from GenBank and BGI-Silkworm Genome Database (http://silkworm.genomics.org.cn/), and it is a kind of Pol polyprotein, the score is 78.

Discussion

To achieve stable and sustained growth in sericulture industry, it is necessary to take full advantage of knowledge about the intimate interaction between pathogens and domesticated silkworm *Bombyx mori*. Recently, the amazing advancement in biotechnology, like widely applied proteomic techniques, has begun to enable us to systematically explore the numerous factors and genes utilized by silkworm to fight against a variety of pathogens. For example, 23 of 34 antibacterial genes have been identified (Xia *et al.*, 2004), that are mainly expressed in fat bodies and hemocytes. In this study, we also chose the silkworm fat body to investigate the mechanism of resistance to BmNPV of silkworm.

To facilitate the identification of anti-BmNPV-related genes, we have successfully bred a NIL of *Bombyx mori*, by 9 times of backcrossing the BmNPV-resistant progeny to its parental strain 306. This NIL is almost genetically identical to strain 306, but retains the anti-BmNPV phenotype.

Table I. Summary of MS analysis of four candidate proteins.

Spot no.	Protein name	NCBI accession	Monoisotopic masses (matched)
1	None	AU002884	1300.76, 1943.04, 1966.04, 1418.82, 1673.81, 1776.90, 1648.81, 1434.64, 1450.64, 1819.02
2	Arginine kinase (AK)	NP 001037402.1	1578.87, 837.45, 2033.00, 1217.61, 1761.93, 2219.16, 1507.75, 1050.54, 1718.85, 974.49, 1899.00, 1136.62, 1008.52, 1797.00, 815.47, 2020.01
3	None	None	909.25, 913.84, 945.85, 1189.97, 1199.04, 1211.94, 1318.08, 1548.14, 1561.22, 1884.42, 1994.48, 2035.56, 2051.55, 2067.56, 2186.67, 2245.78, 2292.77, 2308.82, 2324.79, 2907.40, 3177.30, 3193.35, 3199.36, 3209.34, 3225.38, 3729.07
4	Pol polyprotein	BAA92690.1	2035.03, 3728.88, 2246.15, 1470.76, 1882.91, 2309.18

As a first step to systematically characterize strain NB, we employed 2D-PAGE and MALDI-TOF-MS to screen for differentially expressed genes. In this study, four proteins were preliminarily identified that displayed prominent differences in their levels in these strains. Although, the true nature of these candidate genes needs to be further characterized, some clues about their function can be deduced by bioinformatics. Protein 1 has high sequence similarity to phosphoglycerate kinase (PGK), and protein 2 to arginine kinase (AK). These kinases are mainly involved in energy metabolism (Krishnan *et al.,* 2003), for example, PGK transfers the high-energy phosphate group of 1,3-bisphosphoglycerate to ADP to generate ATP. This may suggest that regulation of the energy metabolism may be required for resisting infection of BmNPV, which is further supported by other reports that AK in silkworm is up-regulated when infected by viruses (Bing *et al.*, 2006; Astrofsky *et al.*, 2002). Therefore, it will be very interesting to further explore the structure and function of these two proteins, especially their possible roles in energy metabolism, which may lead to new discoveries to better control virus infection. Protein 4 shows similarity to a Pol polyprotein, which is the core domain of integrase, but its function in virus infection is unknown. Protein 3 is completely new because it has no corresponding candidate in public databases. Experiments are underway in our laboratory to characterize these exciting genes.

In conclusion, we successfully identified four candidate genes that may be involved in the anti-BmNPV function of *Bombyx mori*. In our study, combined usage of NIL and proteomic tools has proved to be an effective way to search for new proteins involved in host-antivirus response, and may be applied in other research.

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Astrofsky K. M., Roux M. M., Klimpel K. R., Fox J. G., and Dhar A. K. (2002), Isolation of differentially expressed genes from white spot virus (WSV) infected Pacific blue shrimp (*Penaeus stylirostris*). Arch. Virol. **147**, 1799–1812.

Bing W., Li F. H., Dong B., Zhang X. J., and Zhang C. S. (2006), Discovery of the genes in response to white spot syndrome virus (WSSV) infection in *Fenneropenaeus chinensis* through cDNA microarray. Mar. Biotechnol. (NY) **8**, 491–500.

Blissard G. W. (1996), Baculovirus-insect cell interactions. Cytotechnology **20**, 73–93.

Chen K. P., Yao Q., Wang Y., and Cheng J. L. (2003), Genetic basis of screening of molecular markers for nuclear polyhedrosis virus resistance in *Bombyx mori* L. Int. J. Indust. Entomol. **7**, 5–10.

Chernysh S., Kim S. I., Bekker G., Pleskach V. A., Filatova N. A., Anikin V. B., Platonov V. G., and Bulet P. (2002), Antiviral and antitumor peptides from insects. Proc. Natl. Acad. Sci. USA **99**, 12628–12632.

Engelhard E. K. and Volkman L. E. (1995), Developmental resistance in fourth instar *Trichoplusia ni* orally inoculated with *Autographa californica* M nuclear polyhedrosis virus. Virology **209**, 384–389.

Hoffmann J. A. (1995), Innate immunity of insects. Curr. Opin. Immunol. **7**, 4–10.

Hoffmann J. A. (2003), The immune response of *Drosophila*. Nature **426**, 33–38.

Hultmark D. (1993), Immune reactions in *Drosophila* and other insects: a model for innate immunity. Trends Genet. **9**, 178–183.

Jehle J. A., Blissard G. W., Bonning B. C., Cory J. S., Herniou E. A., Rohrmann G. F., Theilmann D. A., Thiem S. M., and Vlak J. M. (2006), On the classification and nomenclature of baculoviruses: a proposal for revision. Arch. Virol. **151**, 1257–1266.

Kirkpatrick B. A., Washburn J. O., and Volkman L. E. (1998), AcMNPV pathogenesis and developmental resistance in fifth instar *Heliothis virescens*. J. Invertebr. Pathol. **72**, 63–72.

Krishnan P., Gullen E. A., Lam W., Dutschman G. E., Grill S. P., and Cheng Y. C. (2003), Novel role of 3-phosphoglycerate kinase, a glycolytic enzyme, in the activation of L-nucleoside analogs, a new class of anticancer and antiviral agents. J. Biol. Chem. **278**, 36726–36732.

Lavine M. D. and Strand M. R. (2002), Insect hemocytes and their role in immunity. Insect Biochem. Mol. Biol. **32**, 1295–1309.

Lehane M. J. (1997), Peritrophic matrix structure and function. Annu. Rev. Entomol. **42**, 525–550.

Ligoxygakis P., Pelte N., Ji C., Leclerc V., Duvic B., Belvin M., Jiang H., Hoffmann J. A., and Reichhart J. M. (2002), A serpin mutant links toll activation to melanization in the host defence of *Drosophila*. EMBO J. **21**, 6330–6337.

Mirza U. A., Liu Y. H., Tang J. T., Porter F., Bondoc L., Chen G., Pramanik B. N., and Nagabhushan T. L. (2000), Extraction and characterization of adenovirus proteins from sodium dodecylsulfate polyacrylamide gel electrophoresis by matrix-assisted laser desorption/ionization mass spectrometry. J. Am. Soc. Mass Spectrom. **11**, 356–361.

Nakazawa H., Tsuneishi E., Ponnuvel K. M., Furukawa S., Asaoka A., Tanaka H., Ishibashi J., and Yamakawa M. (2004), Antiviral activity of a serine protease from the digestive juice of *Bombyx mori* larvae against nucleopolyhedrovirus. Virology **321**, 154–162.

Narayan K. (2004), Insect resistance: its impact on microbial control of insect pests. Curr. Sci. **86**, 800–814.

Ponnuvel K. M., Nakazawa H., Furukawa S., Asaoka A., Ishibashi J., Tanaka H., and Yamakawa M. (2003), A lipase isolated from the silkworm *Bombyx mori* shows antiviral activity against nulceopolyhedrosisvirus. J. Virol. **77**, 10725–10729.

Popham H. J. R., Shelby K. S., Brandt S. L., and Coudron T. A. (2004), Potent virucidal activity in larval *Heliothis virescens* plasma against *Helicoverpa zea* single capsid nucleopolyhedrovirus. J. Gen. Virol. **85**, 2255–2261.

Rahman M. M. and Gopinathan K. P. (2004), Systemic and *in vitro* infection process of *Bombyx mori* nucleopolyhedrovirus. Virus Res. **101**, 109–118.

Selot R., Kumar V., Shukla S., Chandrakuntal K., Brahmaraju M., Dandin S. B., Laloraya M., and Kumar P. G. (2007), Identification of a soluble NADPH oxidoreductase (BmNOX) with antiviral activities in the gut juice of *Bombyx mori*. Biosci. Biotechnol. Biochem. **71**, 195–200.

Terenius O. (2008), Hemolin – A lepidopteran anti-viral defense factor? Dev. Comp. Immunol. **32**, 311–316.

Vialard J. E., Arif B. M., and Richardson C. D. (1995), Introduction to the molecular biology of baculoviruses. Methods Mol. Biol. **39**, 1–24.

Washburn J. O., Haas-Stapleton E. J., Tan F. F., Beckage N. E., and Volkman L. E. (2000), Co-infection of *Manduca sexta* larvae with polydnavirus from *Cotesia congregata* increases susceptibility to fatal infection by *Autographa californica* M nucleopolyhedrovirus. J. Insect Physiol. **46**, 179–190.

Washburn J. O., Wong J. F., and Volkman L. E. (2001), Comparative pathogenesis of *Helicoverpa zea* S nucleopolyhedrovirus in noctuid larvae. J. Gen. Virol. **82**, 1777–1784.

Xia Q., Zhou Z., Lu C., Cheng D., Dai F., Li B., Zhao P., Zha X., Cheng T., Chai C., Pan G., Xu J., Liu C., Lin Y., Qian J., Hou Y., Wu Z., Li G., Pan M., Li C., Shen Y., Lan X., Yuan L., Li T., Xu H., Yang G., Wan Y., Zhu Y., Yu M., Shen W., Wu D., Xiang Z., Yu J., Wang J., Li R., Shi J., Li H., Li G., Su J., Wang X., Li G., Zhang Z., Wu Q., Li J., Zhang Q., Wei N., Xu J., Sun H., Dong L., Liu D., Zhao S., Zhao X., Meng Q., Lan F., Huang X., Li Y., Fang L., Li C., Li D., Sun Y., Zhang Z., Yang Z., Huang Y., Xi Y., Qi Q., He D., Huang H., Zhang X., Wang Z., Li W., Cao Y., Yu Y., Yu H., Li J., Ye J., Chen H., Zhou Y., Liu B., Wang J., Ye J., Ji H., Li S., Ni P., Zhang J., Zhang Y., Zheng H., Mao B., Wang W., Ye C., Li S., Wang J., Wong G. K., and Yang H. (Biology Analysis Group) (2004), A draft sequence for the genome of the domesticated silkworm (*Bombyx mori*). Science **306**, 1937–1940.

Xu J. P., Chen K. P., Yao Q., and Liu X. Y. (2005), Fluorescent differential display analysis of gene expression for NPV resistance in *Bombyx mori* L. J. Appl. Entomol. **129**, 27–31.

Yamakawa M. and Tanaka H. (1999), Immune proteins and their gene expression in the silkworm, *Bombyx mori*. Dev. Comp. Immunol. **23**, 281–279.

Yao Q., Li M. W., Wang Y., Wang W. B., Lu J., Dong Y., and Chen K. P. (2003), Screening of molecular markers for NPV resistance in *Bombyx mori* L. J. Appl. Entomol. **127**, 134–136.