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Full Length Research Paper

Differential expression of early viral gene BmORF51 in *Bombyx mori* nucleopolyhedrovirus infection of resistant and susceptible silkworms

Feng Lin, Qin Yao, Huiqing Chen, Yang Zhou and KePing Chen*

Institute of Life Sciences, Jiangsu University, 301 Xuefu Road, Zhenjiang 212013, P. R. China.

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Open reading frame 51 of *Bombyx mori* nucleopolyhedrovirus (Bm51) is a homologue of autographa californica multiple NPV ORF63. In this study, the expression profiles of Bm51 in the resistant strain NB and the susceptible strain 306 were characterized, and Bm51 gene was amplified from BmNPV genomic DNA by polymerase chain reaction (PCR) and cloned into *Escherichia coli expression* vector pET-30a (+). The recombinant His-tagged Bm51 protein was expressed in *E. coli* BL21 (DE3) and purified by metal chelating affinity chromatography to produce antibodies against Bm51 protein. The amino acid sequence of recombinant protein was confirmed by mass spectroscopic analysis. The transcription and protein product of early viral gene, Bm51, was detected at 6 h post-infection (p.i.) in resistant strain NB by quantitative real-time (qRT)-PCR and western blotting, and the expression of Bm51 in NB reached the maximal level at 36 h p.i. in NB, and then gradually decreased to undetectable level at 72 h p.i. In contrast, the Bm51 protein was undetectable until 12 h p.i. in susceptible strain 306 and the expression of Bm51 progressively increased during the 72 h post-infection.

Key words: *Bombyx mori* nuclear polyhedrosis virus, open reading frame 51 of *Bombyx mori* nucleopolyhedrovirus (Bm51), transcription, protein expression silkworm.

INTRODUCTION

Sericulture has been one of the important economical agriculture activities in Asian countries for hundreds of years. Most serious damages to sericulture can be attributed to virus infection of silkworm that leads to a poor harvest of cocoon. Therefore, it is important for sericulture to prevent virus infection and to breed a silkworm strain with high productivity of cocoon. Four types of virus such as nuclear polyhedrosis, cytoplasmic polyhedrosis, infectious flacherie and densonucleosis are major diseases causing reagents to silkworm (Tanada and Kaya, 1993). The *Bombyx mori* nuclear polyhedrosis virus (BmNPV) infects various tissues, and multiplies in the nucleus forming inclusion bodies as polyhedra, which occludes virus particles. The virus is rod shaped (330 × 45 nm) and contains double-stranded DNA (Watanabe, 2002).

The BmNPV is a major pathogen of the silkworm, and can cause disastrous effect on sericulture (Du et al., 2006). Like autographa californica nucleopolyhedrovirus (AcMNPV), BmNPV is also widely used in baculovirus expression system. The BmNPV (T3 strain) genome has been completely sequenced (Gomi et al., 1999). A number of BmNPV genes have been characterized, such as ie1 (Lu et al., 1996), BRO (Zemskov et al., 2000), Bm8 (Imai et al., 2004), Bm68 (Iwanaga et al., 2002), Bm67 (Chen et al., 2007), Bm122 (Jun-Qing et al., 2009) and Bm79 (Xu et al., 2006). Nevertheless, the functions of many other genes of BmNPV still remain unknown and

*Corresponding author. E-mail: kpchen@ujs.edu.cn. Tel: 86-511-8791923. Fax: 86-511-8791923.

Abbreviations: Bm51, Open reading frame 51 of *Bombyx mori* nucleopolyhedrovirus; **qRT-PCR,** quantitative real-time-polymerase chain reaction; **p.i.,** post-infection; **BmNPV,** *Bombyx mori* nuclear polyhedrosis virus; **AcMNPV,** autographa californica nucleopolyhedrovirus; **AMV,** avian myeloblastosis virus**; IPTG,** isopropyl-β-D-thiogalactopyranoside; **SDS-PAGE,** sodium dodecyl sulfate polyacrylamide gel electrophoresis; **NCBI,** national center for biotechnology information; **MOWSE,** molecular weight search.

the interaction of virus and host determined the outcome of infection. Some *B. mori* genes have been characterized to render BmNPV resistance (Ling et al., 2008; Zhao et al., 2007; Xu et al., 2008), this include Bms3a protein, which have been known to play crucial roles in protein synthesis, and is related to apoptosis (Xu et al., 2008). Previously, we constructed a BmNPV resistant silkworm strain NB with a half lethal dosage 800-fold higher than that of susceptible strain 306 (Jia-ping et al., 2005). In this study, we characterized the transcription and protein product of Bm51 in a highly susceptible silkworm strain 306 and a resistant silkworm strain NB.

MATERIALS AND METHODS

Materials

BmNPV (T3 strain) virus was propagated in BmN cells, which were maintained at 27℃ in TC-100 media supplemented with 10% (v/v) fetal bovine serum (Gibco-BRL, Carlsbad). The titration of virus and other routine manipulations were performed according to standard protocol (O'Reilly et al., 1992). The silkworm *B. mori* was inbred in our laboratory. Highly BmNPV-susceptible silkworm strain 306 and BmNPV-resistant silkworm strain NB were used for this study.

Transcription analysis

The newly metamorphosed 5th instar larvae of 306 and NB were orally fed with 5 µl (TCID50/ml = 109) of BmNPV. Total RNA was extracted from the tissue of midgut at 0, 6, 12, 24, 36, 48 and 72 h post-infection (p.i.) using the Trizol RNA extraction kit (Invitrogen). For cDNA synthesis and polymerase chain reaction (PCR), the total RNA was treated with Dnase I (Takara) to digest genomic DNA contamination. Total RNA (2 µg) from each time point was reverse transcribed by avian myeloblastosis virus (AMV) reverse transcriptase (Takara) with oligo (dT) primers (Takara) to synthesize cDNA, according to the manufacturer's instruction. Reactions without adding AMV reverse transcriptase were used as negative controls. The coding region of the Bm51 gene was amplified by PCR with two primers: Bm51-F, 5'-GCACGGAGAGGCAAAATACT-3'; and Bm51-R, 5'- CACTTCTTTGGCGTAAGGATT-3'. The constitutive gene actinA3 was used as the internal control. Two primers ActinF (reverse: 5'-GGATGTCCACGTCGCACTTCA-3') and ActinR (forward: 5'-GCGCGGCTACTCGTTCACTACC-3') were designed on the basis of the sequence of the *B. mori* actin gene (Accession number: BMU49854). As a control, quantitative real-time (qRT)-PCR was performed on a late gene Bm134 with two primers (Bm134-F, 5'-TCGCATCTCAACACGACTAT-3' and Bm134-R, 5'- TGTAGTCGG CAGTTCTTTTG-3') using the same cDNA.

Quantitative real-time PCR was performed with SYBR Premix ExTaq (Takara) using Mx 3000P (Stratagene, San Diego, CA) for thermal cycling, real-time fluorescence detection and subsequent analysis. The two-step amplification protocol consisted of a 1 min at 94℃ followed by target amplication through 40 cycles at 94℃ for 20 s, 58℃ for 30 s and 72℃ for 15 s. The transcript levels of the target fragment were normalized against actinA3 transcript levels in the same samples.

Prokaryotic expression of Bm51 and preparation of antibody

The Bm51 coding region was amplified from the BmNPV genomic DNA by PCR using an upstream primer (5'-CGGGATCCATG TACAATAAATTTCTGATTTATCTTC-3') with a BamHI site (underlined) and a downstream primer (5'-CCGCTCGAG CATATTATATTTAG CAAGAAGTAATAACA-3') with an XhoI site (underlined). The Bm51 was subcloned into the pET30a (+) expression vector (Novagen, USA) in frame with the N-terminal 6×His tag. The recombinant plasmid, pET-Bm51, was verified by PCR, restriction analysis and DNA sequencing. The recombinant plasmid was transformed into *E. coli* BL21 (DE3) cells for expression induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at 37℃ for 6 h. The 6×His-tagged recombinant Bm51 protein was purified by a Ni2+-NTA column (Novagen) and used to raise polyclonal antibodies in rabbits. The antibody was prepared using standard techniques (Harlow and Lane, 1988), and was purified by protein A affinity column (Millipore).

Mass spectrometry analysis

The Bm51 protein was separated by 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then protein bands were manually excised from gels. Spots from Coomassie gels were washed with 100 ml of 50% acetonitrile/50 mM ammonium hydrocarbonate pH 8.0 (Schevchenko et al., 1996). Gel pieces were then dehydrated with acetonitrile and vacuum dried. After rehydratation in 10 µl of 50 mM ammonium hydrocarbonate pH 8, samples were incubated in the same buffer containing 0.5 µg of porcine trypsin (Promega, France) for overnight (16 – 18 h) at 37℃. Peptide fragments from digested proteins were then crystallized with α-cyano-4-hydroxycinnamic acid as a matrix and subjected to MALDI-TOF MS (Bruker Daltonics, Germany) for peptide mass fingerprinting. The peak lists were the basis for peptide mass fingerprint analyzed by the Mascot software (Matrix Science; http: www. matrixscience.com/search_form_select.html).

Western blot analysis

The 5th instar larvae of 306 and NB were orally fed with 5 µl (TCID50/ml = 109) of BmNPV and the midgut were harvested at 0, 6, 12, 24, 36, 48 and 72 h p.i. Protein samples of the midgut were lysed in SDS-PAGE loading buffer by boiling for 10 min. Then the proteins were separated on 15% SDS-PAGE and subjected to western blot assay. Western blot was performed as described by Towbin et al. (1979).

RESULTS

Transcriptional analysis of Bm51

To determine the expression profile of viral Bm51, qRT-PCR was performed using total RNA isolated from BmNPV-infected larvae of 306 and NB at different times p.i. qRT-PCR analysis showed that the transcripts of Bm51 were detected from 6 to 72 h p.i. and reached the maximal level at 36 h p.i. in BmNPV-infected larvae of NB (Figure 1A). Transcripts of Bm51 were detected at 6 to 72 h p.i. in BmNPV-infected larvae of 306, but the maximal level was at 72 h p.i. (Figure 1A). The results suggest that the expression of Bm51 in NB was higher than that of 306 before 36 h p.i., and then the expression of Bm51 was reduced quickly in NB, but the expression of Bm51 in 306 was continuously increased over 72 h after infection. At 72 h p.i., the expression level of Bm51 in 306 was more than 8-fold higher than that in NB. Meanwhile,

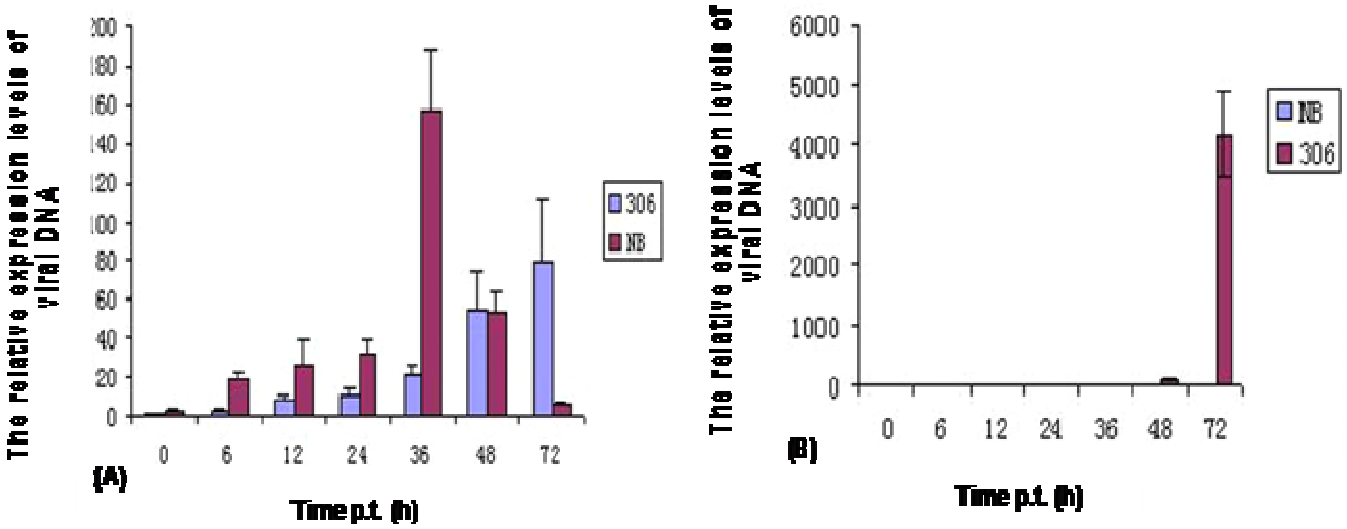

Figure 1. Quantitative real-time PCR analysis of viral DNA. (A) The analysis of the transcription of Bm51 was performed using total RNA extracted from BmNPV-infected larvae of highly susceptible silkworm strain 306 and resistant silkworm strain NB at different time intervals post-infection (p.i.). (B) transcripts of Bm134 were analysed in BmNPV-infected larvae of highly susceptible silkworm strain 306 and resistant silkworm strain NB. Total RNA was isolated from BmNPV-infected midguts at 0, 6, 12, 24, 36, 48 and 72 h p.i. Mean values ± SD of three independent experiments are shown.

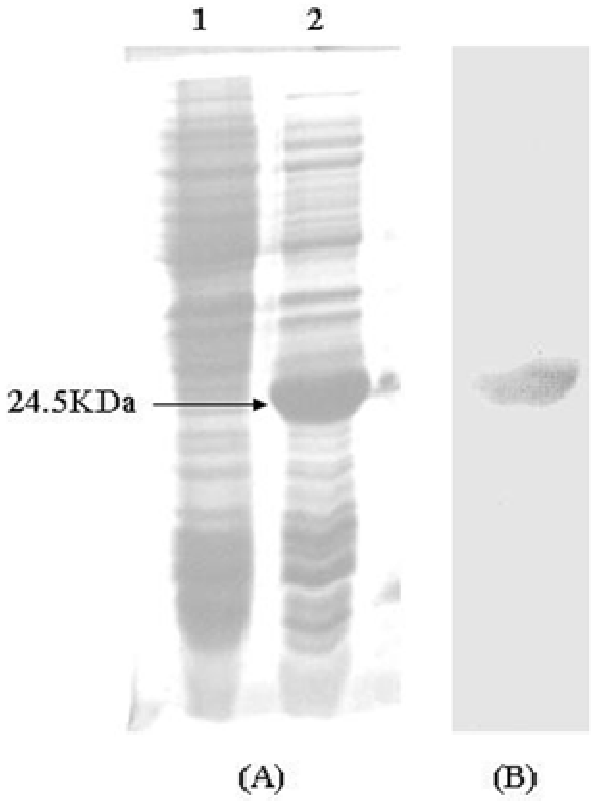

Figure 2. SDS-PAGE and western blot analysis of fusion protein His-Bm51 expressed in *E. coli* BL21 (DE3) cells. (A) SDS-PAGE. Lane1, proteins from BL21 (DE3) cells transformed with pET30a. Lane 2, proteins from BL21 (DE3) cells transformed with pET-BmORF51. (B) Western blot analysis of fusion protein His-Bm51 using His monoclonal antibody.

transcripts of a late gene Bm134 were detected at 48 - 72 h p.i. in BmNPV-infected larvae of 306 but not in NB (Figure 1B).

Production of Bm51 protein and mass spectrometry analysis

For the purpose of detecting Bm51 protein, the 6×His-tagged recombinant Bm51 protein of 24.5kDa was expressed in *E. coli.* Western blot analysis using anti-His antibody confirmed that the 24.5-kDa fusion protein was successfully expressed (Figure 2).

To determine whether the amino acid sequence of recombinant Bm51 matches the predicted one from DNA sequencing results, the MALDI-TOF-TOF mass spectra of tryptic digests of recombinant Bm51 was characterized to identify the recombinant protein. Six peptide fragments were identified in mass spectra (Figure 3). By comparing the masses of identified peptides to the hypothetical tryptic peptides of proteins in non-redundant national center for biotechnology information (NCBI) database using the MASCOT search engine, Bm51 was obviously identified with molecular weight search (MOWSE) score of 98. The identified 6 peptide fragments were matched against the deduced amino acid sequence of Bm51 with 40% sequence coverage.

Western blotting

To study the expression of viral Bm51 in the infected *B. mori*, the tissues of BmNPV-infected 306 and NB were

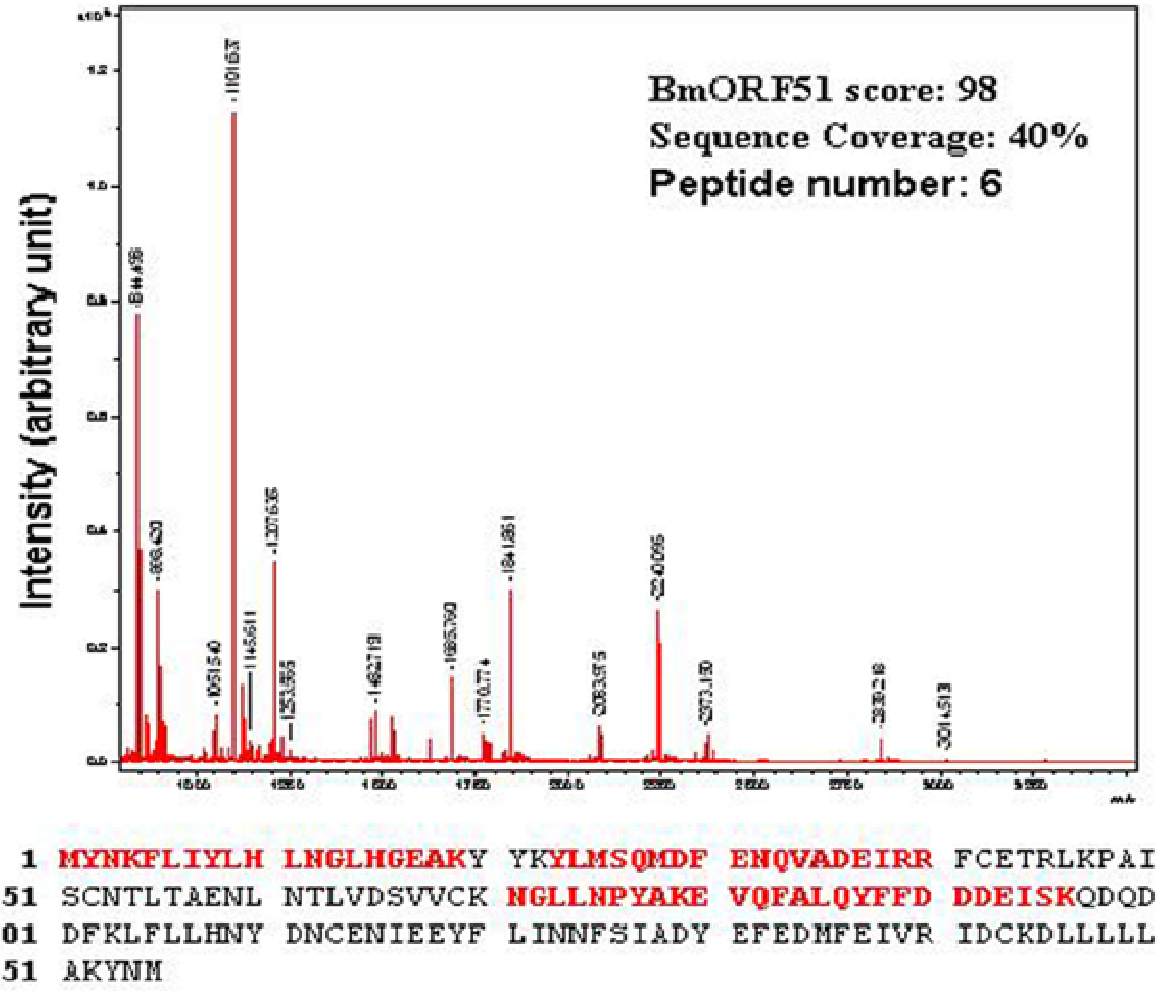

Figure 3. MALDI spectra of tryptic digest of recombinant Bm51. The identified protein, score, amino acid sequence coverage and the number of identified peptides are shown.

collected at various times post-infection and analyzed by western blot using anti-Bm51 antibody. A specific immunoreactive band of approximately 18.5 kDa, which is in agreement with predicted molecular weight of Bm51, was first observed at 6 h p.i. and remained detectable up to 48 h p.i. in BmNPV-infected NB (Figure 4A). But the expression of Bm51 reached maximal levels at 36 h p.i. in NB, and then Bm51 expression level decreased quickly, until undetectable by western blotting at 72 h p.i., which was consistent with the qRT-PCR analysis. In addition, an immunoreactive band in the midguts of BmNPV-infected 306 was first observed at 12 h p.i., and enhanced up to 72 h p.i. (Figure 4B).

DISCUSSION

In this study, we reported the characterization of a BmNPV gene Bm51, which was reported as an early gene (Tian et al., 2009) in the BmNPV resistant silkworm strain NB and the BmNPV susceptible strain 306. To further clarify the function of Bm51, we analyzed the transcription and protein levels of Bm51 in BmNPV-infected NB and 306.

Natural infection of viruses in insects occurs per orally. Steps of the infectious process with viruses include the entering of virions into the gut lumen, the adsorption and fusion of virus particles to the cell plasma membrane of the midgut epithelium, the penetration of virions into the midgut cell where they replicate, and the passing of the virions through the midgut epithelium into the body cavity where they eventually attack the target tissues (Watanabe, 2002). So we extracted the RNA and protein from the midgut of BmNPV-infected NB and 306.

qRT-PCR showed that Bm51 transcription was detected as early as 6 h p.i., and the transcription level gradually decreased to undetectable level after peaking at 36 h p.i. in strain NB. Western blots analysis of total proteins from infected NB indicated that the viral Bm51 protein peaked at 36h p.i and decreased to undetectable level at 72h p.i.,

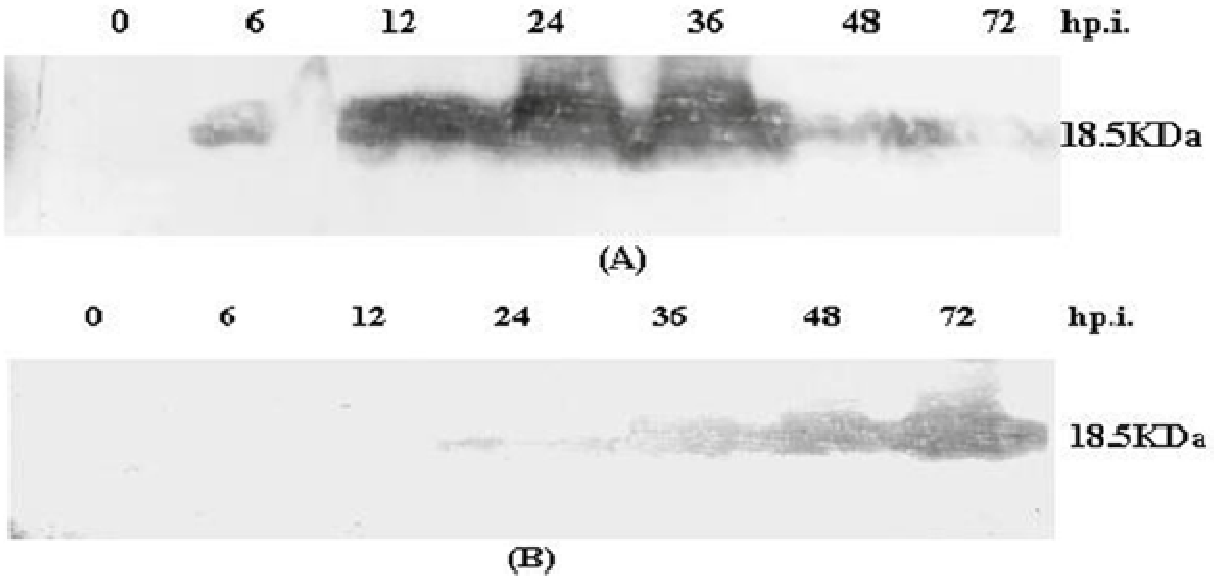

Figure 4. Western blot analysis of the BmORF51 protein in NB (A) and 306 (B). The midguts of BmNPV-infected NB and 306 were collected at 0, 6, 12, 24, 36, 48 and 72 h p.i., and then processed for western blot using anti-Bm51 antibody followed by incubating with a goat antirabbit Immunoglobulin G (IgG) conjugated to horseradish peroxidase (HRP). The signal was detected with 3,3' Diaminobenzidine (DAB) substrate.

which confirmed the qRT-PCR result. Western blots of total protein from infected 306 first detected viral Bm51 protein at 24 h p.i. This could be due to the fact that the western blot was not sensitive enough to detect low levels of protein at early time.

These results not only further confirmed that Bm51 is an early gene, but suggested that the expression of Bm51 in BmNPV-infected NB silkworm may be higher than that of 306 at initial infection stage (36 h p.i.). Due to the interaction of virus and host factors, the expression level of viral Bm51 reversed in 306 and NB strain at 72 h with high viral Bm51 expression in 306 and low Bm51 expression in NB, suggesting that some host factors in BmNPV resistant strain inhibited the expression of viral Bm51.

Baculovirus gene expression can be divided into at least three phases (Okano et al., 2006): early, late and very late. The products of early genes are often involved in DNA replication, late gene expression and host-modification processes (Todd et al., 1996). The low expression level of early viral gene Bm51 at 72 h p.i could lead to low expression of late viral genes, which is consistent with the observation that the expression of viral late gene Bm134 was only detected in strain 306 but not in strain NB.

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