Characterization of recombinant expression of Bombyx mori bidensovirus ns1 using a modified vector

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INTRODUCTION

A great demand in the post-genome era is to disclose the functions and three-dimensional structures of proteins, which will help us to understand life mystery and provide useful information for further elucidating the details of specific disorders. The three-dimensional structure and function of protein are largely determined by their exact spatial conformation and post-translational modifications. So, an efficient expression system remains an essential requirement for expressing a great amount of proteins with proper folding. To achieve this goal, baculovirus expression vector system (BEVS) is considered as one of the best eukaryotic expression systems for expressing heterologous proteins with specific soluble form and exact function, especially those proteins with post-translational modifications. To date, thousands of soluble recombinant proteins with proper folding and posttranslational modifications, such as IFN-β, αFGF, bFGF, IL-2 and human protein phosphatase 2A catalytic subunit α etc., have been successfully expressed by the BEVS (Smith et al., 1983; Wu et al., 2001; Gujar & Michalak, 2006; Kost et al., 2005; Hitchman et al., 2009; Rubiolo et al., 2012). These recombinant proteins are often expressed at high levels in cultured insect cells or infected larvae and functionally similar to their authentic counterparts.

As known, the genomes of Autographa californica nucleopolyhedrovirus (AcNPV) and Bombyx mori nucleopolyhedrovirus (BmNPV) have been genetically modified to be eukaryotic expression vectors of Ac-bacmid and Bm-bacmid, which are powerful recombinant protein production systems and most popular with scientific researchers. In the BEVS, it is a crucial step to prepare recombinant virus harboring the foreign gene of interest under the control of the strong, late viral polyhedrin promoter for expression of heterologous genes. Traditionally, recombinant baculoviruses are generated between target DNA and wild-type viral genomic DNA in insect cells through homologous recombination. However, only 0.1 to 1% of the resulting progeny are recombinant viruses, and the standard transfection and plaque assay methods take us 4 to 6 weeks to separate the recombinant virus (Fraser, 1989; Miller et al., 1986). Since Bac-to-Bac® expression system was developed, recombinant virus isolation and quantification methods for eliminating some tedious procedure have been simplified (Luckow et al., 1993). Briefly, the Bac-to-Bac® expression system involves site-specific transposition of target gene from a donor plasmid to a genetically modified baculoviral DNA, or ‘bacmid’ in such a way that the target gene is controlled by the polyhedrin promoter. All these genetic manipulations can be easily performed in E. coli which provides a rapid and highly efficient method for the generation of recombinant bacmid.

The recombinant bacmid must still be transfected into cultured insect cells for production of the recombinant virus. However, there is no effective visible marker in BEVS to confirm the successful production of recombinant virus except for the cell morphological change. Multiple rounds of virus titration could be used for the detection of infectious virions in the cultured supernatant, but it is a tedious and time consuming process. To easily identify whether infectious virions are generated in the Sf9 cells transfected with recombinant Ac-bacmid, two tandem cassettes of Cm-PacI-egfp inserted into the ac68 locus of Ac-bacmid were described here and the modified vector was exploited to express BmBDV NS1. BmBDV is a major pathogen causing flacherie disease in silkworms, which results in significant loss in silk industry. To control the spread of viral infection and development of BmBDV genome, it is required to study

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Abbreviations: BmBDV, Bombyx mori bidensovirus; BEVS, baculovirus expression vector system; AcNPV, Autographa californica nucleopolyhedrovirus; BmNPV, Bombyx mori nucleopolyhedrovirus; Sf9, Spodoptera frugiperda 9; egfp, enhanced green fluorescent protein; h.p.t., hour post-transfection; h.p.i., hour post-infection; PBS, phosphate-buffered saline; LC-MS/MS, liquid chromatography-tandem mass spectrometry.
the role of viral genes. *Bm*BDV *ns1* consisted of 951 nucleotides encoding a predicted 316-amino acid protein, which was identified to be a multifunctional protein involved in viral replication (Li et al., 2009). Several phosphorylation sites were predicted in *Bm*BDV NS1 using the NetPhos 2.0 software (http://www.expasy.ch), indicating that *Bm*BDV NS1 is likely to be a phosphoprotein, and the activity of *Bm*BDV NS1 is possibly regulated by its phosphorylation. To disclose further the phosphorylation pattern of *Bm*BDV NS1 and its corresponding role, the overexpression of *ns1* and its series of mutants using the modified BEVS are very helpful to determine these scientific issues.

In this study, *bom*BDV *ns1* was inserted into the genetically modified Aec-Bacmid by site-specific transposition, and successful transfection of Sf9 cells with the resulting recombinant was performed. The resulting recombinant baculovirus was easily monitored by the observation of green fluorescent signals. *Bm*BDV NS1 was expressed in Sf9 cells with the recombinant baculovirus and one phosphorylation site (Thr-184) was identified by LC-MS/MS analysis.

### MATERIALS AND METHODS

**Plasmids, bacmid, virus and cells lines.** Plasmids of pUC18, pFastBacHTB, pMD18T- *egfp* and pMD18T-*ns1* were maintained in our laboratory. *Ae*NPV strain accompanying the Bac-to-Bac® expression system used in this study was purchased from Invitrogen. *Bm*BDV was propagated in the midgut of silkworms. Spodoptera frugiperda 9 (Sf9) cells were grown at 27°C in Grace’s medium supplemented with 10% fetal calf serum, penicillin (50 U/ml) and streptomycin (50 µg/ml). Plasmid pBAGbA and pKOV-Cm were kindly donated by Dr. Kai Yang (State Key Laboratory of Biocontrol, Sun Yat-sen University).

**Construction of recombinant plasmid pUC18-ac68U-Cm-P*<sub>ac68</sub>*-*egfp*-ac68D.** To introduce enhanced green fluorescent protein (*egfp*) into the BEVS, it was required to construct a recombinant plasmid pUC18-ac68U-Cm-*ns1*-egfp-ac68D as shown in the following Fig1 flowchart. In a brief, ac68U indicated the 5’ flanking sequence of Ac-bacmid *egfp* (ac68), which was amplified with *ac68U*-F and *ac68U*-R, and ac68D indicated the 3’ flanking sequence of ac68, which was amplified with *ac68D*-F and *ac68D*-R. Additionally, 1.039-bp Cm resistance gene cassette from pKOV-Cm was amplified with primers Cm-F and Cm-R, and 1299-bp *egfp* cassette under control of ie1 promoter was amplified from pMD18T-*egfp* with P*<sub>ac68</sub>*-F and egfp-R. All primer sets were listed in Table 1. The resulting linear 3349-bp fragment containing Cm gene cassette, *egfp* cassette and *egfp* flanking region was resuspended in distilled water to a final concentration of 200 ng/µl.

**Insertion of two tandem cassettes of Cm-P<sub>ac68</sub>-*egfp* into ac68 locus of Ac-bacmid.** A resistance gene of Cm was used to screen recombinant clones directly from plates, and *egfp* cassette was used to identify effectively proper transfection and infection of Sf9 cells with recombinant baculoviruses. The two tandem cassettes of Cm-P<sub>ac68</sub>-*egfp* replace the ac68 locus of AenPV bacmid by homologous recombination according to the manufacturer’s instructions (Heermann et al., 2008; Li et al., 2008). The homologous recombination between linear DNA fragment and Ac-bacmid in *E. coli* was mediated by λ Red recombinase from plasmid pBAD-gbaA. Several recombinant colonies were grown on the plate containing 20 µg/ml Cm, 100 µg/ml ampicillin, and 50 µg/ml kanamycin, which were selected for further confirmation by PCR. The primer pairs in Fig. 2B show the relative positions in Ac-bacmid, which were used to confirm the correct insertion of two tandem cassettes of Cm-P<sub>ac68</sub>-*egfp* in the ac68 locus.

**Fluorescence microscopy analysis.** To examine whether infectious virions were generated in Sf9 cells transfected with the recombinant Ac-bacmid, green fluorescent signal could be exploited to indicate effectively the generation and the spread of virions among Sf9 cells at different time points. Briefly, the recombinant Ac-bacmid with an insertion of *egfp* cassette in ac68 locus was isolated from the resulting DH10B cells, then 2.0 µg DNA of the recombinant Ac-bacmid was mixed with 6 µl Cellfectin® (Invitrogen) in 200 µl Grace’s medium

### Table 1. Primers used for PCR procedure in this study

<table>
<thead>
<tr>
<th>Primers</th>
<th>Primer sequence (5’-3’)</th>
<th>Enzyme digestion sites</th>
</tr>
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<tbody>
<tr>
<td><em>ac68D</em>-F</td>
<td>GGCGATCATGATTTGAGCAGAAAAATTAAT</td>
<td>Sph I</td>
</tr>
<tr>
<td><em>ac68D</em>-R</td>
<td>TAAGCTTGGCAATAAATTAGTCGGT</td>
<td>Hind III</td>
</tr>
<tr>
<td><em>P</em>&lt;sub&gt;ac68&lt;/sub&gt;-F</td>
<td>AACTGATCTAGTTGATGAAATATGAAAGGA</td>
<td>Pst I</td>
</tr>
<tr>
<td><em>egfp</em>-R</td>
<td>AAGCATCTCTACGTACAGCCTGTCAT</td>
<td>Sph I</td>
</tr>
<tr>
<td>Cm-F</td>
<td>GGATCCCTCGAAATAATACCTTGGAA</td>
<td>BamHI</td>
</tr>
<tr>
<td>Cm-R</td>
<td>CTGCAGAACACCGACATATAGATCCAA</td>
<td>Pst I</td>
</tr>
<tr>
<td><em>ac68U</em>-F</td>
<td>CGGGTACCTCCTTCTGATGCTTTGAAAGATTGC</td>
<td>Kpn I</td>
</tr>
<tr>
<td><em>ac68U</em>-R</td>
<td>GGCGATCTAACTTATAGCAGCGTGGT</td>
<td>BamHI</td>
</tr>
<tr>
<td><em>ac67F</em>-R</td>
<td>TTGGTATGAAAATGTGGCTCATG</td>
<td></td>
</tr>
<tr>
<td><em>ns1</em>-F</td>
<td>ATGGCTAGCTGGAATCGAGTCAA</td>
<td>Nhe I</td>
</tr>
<tr>
<td><em>ns1</em>-R</td>
<td>TACTCGAGCTACATATATTATTTATACG</td>
<td>Xho I</td>
</tr>
</tbody>
</table>

Note: underlined letters indicate restriction enzyme digestion sites.
Recombinant expression of *Bombyx mori* bidensovirus ns1

Unsupplemented at 27°C for 30 min. Subsequently, 800 µl Grace’s medium unsupplemented was added into the mixed DNA-cellfectin solution, which was distributed onto the seeded Sf9 cells by dropping it slowly and evenly. After the mixed DNA-cellfectin solution in each well was mixed gently at 30 min interval and incubated for 6 h, the supernatant was completely removed via aspiration from the wells and the seeded cells were washed twice with non-serum Grace’s medium. Finally, the cells were cultured at 27°C with 2 ml Grace’s medium containing 10% fetal bovine serum. The expression of egfp was observed respectively at 24, 48, 72 and 96 h p.t. (hour post-transfection) through fluorescence microscopy. To determine whether the generated virions were infectious, analysis of viral propagation by supernatant passage in Sf9 cells was performed. In a brief, Sf9 cells were incubated with the harvested supernatant containing BVs for 1 h, then the supernatant was removed and 2 ml fresh Grace’s medium with 10% FBS was added into the Sf9 cells. The expression of egfp was observed respectively at 24, 48, 72 and 96 h p.i. (hour post-infection) through fluorescence microscopy.

**Construction of viral expression vector Ac-bacmid-nsl.** The genetically modified Ac-bacmid vector was used for expression of *Bm* BDV NS1, which was constructed as shown in the following Fig. 4A. Primer pair nsl-F and nsl-R was used to amplify nsl gene from the genome of *Bm* BDV, and the amplified fragment was ligated into pFastBacHTB to generate pFastBacHTB-nsl. The donor plasmid pFastBacHTB-nsl was transformed into DH10B cells harboring the modified Ac-bacmid and helper vector encoding a transposase to generate recombinant Ac-bacmid-nsl by transposition. The transpositions were confirmed by PCR using M13 forward and reverse primers.

The recombinant Ac-bacmid-nsl and the genetically modified Ac-bacmid with no nsl gene were transfected respectively into Sf9 cells with cellfectin Reagent (Invitrogen) according to manufacturer’s instructions. The transfected supernatants were harvested from each well at 96 h p.t. if green fluorescence signal could be observed through fluorescence microscopy. On the contrary, the transfected supernatants were discarded from the wells if no green fluorescent signal was observed.

**Identification of nsl expression mediated by the modified BEVS.** The harvested supernatant was used to infect Sf9 cells for the production of 6×His-NS1 protein. Briefly, 100 µl virus stock was added into the culture medium after a monolayer of cells were cultured in the 75 cm² flask for 24 h. Then, the infected cells pellet was collected at 96 h p.i. and resuspended in phosphate-buffered saline (PBS, pH 7.4), next lysed in SDS-PAGE loading buffer, and finally analyzed by Western blot. Additionally, Nickel-coated beads were used to incubate with cell lysate for enrichment of the target protein, which was also examined by Western blot using anti-6×His tag monoclonal antibodies and anti-NS1 monoclonal antibodies. Anti-NS1 monoclonal antibodies were prepared in Abmart (Shanghai, China). Briefly, Sf9 cells were seeded in one flask with Grace’s medium supplemented with penicillin (50 U/ml) and streptomycin (50 µg/ml), then 100 µl virus stock was added into the flask. After the cells were cultured for 96 h, the cells pellet was collected by centrifugation at 2890 × g for 10 min. The supernatant was removed and the cells pellet was resuspended in 400 µl of lysis buffer (20 mM Tris-Cl pH7.8, 0.5 mM EGTA, 1 mM EDTA, 1 mM MgCl₂, 10% Glycerol, 200 mM NaCl, 0.1% NP40, 1% Triton, 1 mM PMSF). The suspension was sonicated for 15 s in a Kontes sonicator operated at 5 W using a 3-mm tip, and the final suspension should appear milky and homogeneous. The broken cells were incubated on ice for 30 min and pelleted at 11 560 × g for 60 min, and then the supernatant was loaded on a 60 µl Ni-NTA agarose column equilibrated with the lysis buffer, by end-to-end rotation for 1–2 h. Finally, the Ni-NTA agarose was pelleted by centrifugation at 11 560 × g for 1 min and boiled for 15 min for separation of target protein from Ni-NTA agarose. The supernatant sample was subjected

[Diagram: Figure 1. The flowchart showing the construction steps of the pUC18-ac68U-Cm-Pie1-egfp-ac68D.]
to Western blot analysis and the concentration of target protein was assessed by measuring the band density. Against 6×His tag or NS1 monoclonal antibodies were used as primary antibody at a dilution of 1:10000 to incubate with the total protein from the infected SF9 cells. Alkaline Phosphatase horse anti-mouse IgG was used as secondary antibody. The immunoreactive bands were visualized using the BCIP/NBT color reaction.

Identification of phosphorylation sites by LC–MS/MS. Mass spectrum analysis was performed to determine the phosphorylated residues in BmBDV NS1. The total protein of SF9 cells infected with recombinant virus was subjected to 12% SDS-PAGE for the separation of 6×His-NS1, and target protein band was excised from gels according to Western blot result. The strips were placed in a 1.5-ml plastic microcentrifuge tube, washed with MiliiQ water three times, followed by 300 μl of 30% acetonitrile (ACN) several times, and dried in a vacuum centrifuge. The cysteine reduction and alkylation steps consisted of incubation in 100 mM DTT for 30 min at 56°C, 200 mM iodoacetamide (IAA) for 20 min in the dark. The gel pieces were then dried again and rehydrated in a minimal volume of 100 mM NH₄HCO₃ (pH 8.0) for 15 min at room temperature. After they had been washed again with 300 μl of 30% ACN for 5 min, the gel pieces were dried and digested with sequencing grade trypsin (10 ng/μl in 50 mM NH₄HCO₃) for 20 h at 37°C. The peptides were then extracted twice in 100 μl of 60% (v/v) ACN and 0.1% (v/v) Trifluoroacetic acid (TFA), which was subsequently analyzed by liquid chromatography-tandem mass spectrometry (LC–MS/MS) (AB SCIEX). The MS/MS spectra was searched against BmBDV NS1 database using the automatic Mascot algorithm for possible Y, T and S phosphorylation sites.

RESULTS

Construction of the plasmid pUC18-ac68U-Cm-P.ser1-egfp-ac68D

Figure 1 is a flowchart illustrating the construction steps of the recombinant plasmid pUC18-ac68U-Cm-P.ser1-egfp-ac68D, which was confirmed by analysis of PCR and enzyme digestion (data not shown). The results indicated that ac68U, Cm, P.ser1-egfp and ac68D fragments were successfully ligated into pUC18 vector in correct order. Additionally, the sequenced result of the plasmid pUC18-ac68U-Cm-P.ser1-egfp-ac68D was corresponded with the expected sequence.

Replacement of ac68 locus of Ac-bacmid by two Cm-P.ser1-egfp tandem cassettes

Previous studies reported that ac68 was not essential for the propagation of AcNPV (Li et al., 2008; Nie et al., 2012). Therefore, two tandem cassettes of Cm-P.ser1-egfp were used to replace the ac68 locus of Ac-bacmid by homologous recombination in this study, which facilitate the rapid judgement of infectious viruses generated in the SF9 cells transfected with the modified Ac-bacmid or infected with virus supernatant. In ac68 deleted bacmid, a 120-bp fragment inside the ac68 coding region (nt 59, 179–59, 298) was replaced with Cm-P.ser1-egfp by homologous recombination (Fig. 2A). The resulting Ac-bacmid was confirmed by PCR analysis with different primer pairs (Fig. 2B and C). A 1039-bp DNA fragment was amplified from ac68-deleted bacmid, but not from wt bacmid with primers Cm-F/Cm-R. An 1299-bp DNA fragment was amplified from ac68-deleted bacmid, but not from wt bacmid with primers P.ser1-F/egfp-R. A 2338-bp DNA fragment was amplified from ac68-deleted bacmid, but not from wt bacmid with primers Cm-F/egfp-R. An 1707-bp DNA fragment was amplified
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Recombinant expression of *Bombyx mori* bidensovirus ns1 from ac68-deleted bacmid, but not from wt bacmid with primers ac67-F/Cm-R. Primers US-F/DS-R amplified a 3349-bp DNA fragment from ac68-deleted bacmid and an 1201-bp DNA fragment from wt bacmid.

Fluorescence microscopy analysis of the transfected cells

To examine whether the genetically modified Ac-bacmid could offer an easy and rapid convenience for detecting the production of infectious virions, the modified Ac-bacmid containing the egfp cassette inserted in the ac68 locus was transfected into Sf9 cells with Cellfectin®. The signal of green fluorescent protein was visualized in very few cells as early as 24 h p.t., and the number of green fluorescent signal was slightly increased at 48 h p.t. However, the number of green fluorescent signal was insteady and substantially increased in Sf9 cells from 72 h p.t. to 96 h p.t. (Fig. 3A), indicating that virions produced in Sf9 cells were infectious and initiated the next round infection of Sf9 cells. Furthermore, the transfected supernatants were collected and used to infect Sf9 cells to confirm

**Figure 3.** The expression of egfp gene in Sf9 cells.

(A) Fluorescence micrographs of Sf9 cells transfected with the ac68-deleted bacmids. Bacmids used for transfection were indicated on the left and the times after transfection were indicated above. (B) Fluorescence micrographs of Sf9 cells infected with virus supernatant. Viruses used for infection were indicated on the left and the time post infection was indicated above.

**Figure 4.** Strategy for construction of recombinant virus for expression of *Bm*BDV NS1 and identification of recombinant virus through the expression of egfp.

(A) A flow diagram illustrating the steps of producing a recombinant Ac-bacmid. (B) Lane M, DNA marker; Lane 1, PCR product of ns1 amplified from *Bm*BDV genome; Lane 2, pMD18-T-ns1 digested with *Nhe*I and *Xho*I. (C) Lane M, DNA marker; Lane 1, PCR product of ns1; Lane 2, pFastBacHTB-ns1 digested with *Nhe*I and *Xho*I. (D) Lane M, DNA marker; Lane 1, PCR product amplified from wild Ac-bacmid; Lane 2, PCR product amplified from Ac-bacmid-ns1. (E) Fluorescence micrographs of Sf9 cells transfected with recombinant bacmid. Bacmid used for transfection were indicated below and the time after transfection was indicated above.
Identification of BmBDV NS1 expressed in Sf9 cells

The transfected supernatant containing the recombinant virus was harvested, which was used to infect the cultured monolayer Sf9 cells in one 75 cm² flask. Western blot results indicated that only a specific band ~36 kDa was detected in the total protein of Sf9 cells infected with recombinant virus using anti-6×His tag monoclonal antibodies (Fig. 5A lane 2) and anti-NS1 monoclonal antibodies (Fig. 5A lane 3), but not in the protein fraction of Sf9 cells infected with wild virus (Fig. 5A lane 1). Additionally, Ni-NTA agarose was used to enrich the target protein of NS1 from the lysate of Sf9 cells infected with recombinant virus, which was subjected to SDS/PAGE analysis to estimate the abundance of NS1. The result showed that the NS1 protein was detected in the eluted fraction from Ni-NTA agarose pellets, and the abundance of NS1 was about 12.6 µg in 10 µl loading sample (Fig. 5B lane 2). The NS1 band was excised from gel corresponding to Western blot result (Fig. 5 B lane 3) and digested with trypsin and analyzed by LC-MS/MS analysis. The results confirmed that the target protein expressed in Sf9 cells by the genetically modified BEVS was corresponded with BmBDV NS1 protein (Fig. 5C). Furthermore, the MS result indicated that T184 of BmBDV NS1 was a threonine phosphorylation site (Fig. 5D) with an E-value of 0.029 generated by Mascot. E-values <0.05 are considered significant. Besides, our LC-MS/MS analysis also identified that T 181 and T191 of BmBDV NS1 may be the alternative threonine phosphorylations with E-values >0.05.

DISCUSSION

Expression of heterologous genes in insect cells mediated by Baculovirus has become well-established, and BEVS has been widely used to overexpress heterologous genes for its relative ease use and the high chance of obtaining a biologically active protein. However, BEVS is also faced with some challenges such as the expensive production cost, the relative low protein yield and some technological bottlenecks slowing down the overall production processes, especially transfection inefficiency or failure usually leading to the ultimate unsuccessful expression of target genes in insect cells (van, 2011; Hu, 2005). These aspects would take us a lot of time and energy to check each experiment step in turn.
Although some efforts have been made to modify the baculovirus expression vectors by incorporating stronger promoter and integration of enhancer elements and other innovative strategies to improve the efficiency of BEV (Tiwari et al., 2010; Lee et al., 2007), an effective method has not been established for easily identifying successful transfection and infection of S9 cells with recombinant baculoviruses. Based on the above considerations, egfp cassette was introduced in the ac68 locus of Ac-bacmid shuttle vector by homologous recombination in this study. ac68 gene was reported to be an unnecessary gene for the viral propagation (Li et al., 2008; Nie et al., 2012), which was replaced with two designed tandem cassettes, Cm and egfp. Target recombinant colonies were obtained with Cm antibiotic screening and PCR confirmation. The recombinant viros produced in transfected S9 cells were easily and rapidly identified through fluorescence microscopy. Therefore, the modified BEVS was an easy and convenient insect system for heterologous protein expression. BomBDV ns1 was successfully expressed in S9 cell with the modified BEVS, but we are not satisfied with the relatively low yield of BomBDV NS1. Further research was required to improve the expression yield of BomBDV NS1 by seeking the optimal conditions.

NS1 protein was identified to be a multifunctional protein involved in replication of BomBDV (Li et al., 2009). To further elucidate the regulation mechanism of BomBDV NS1, it is a crucial step to determine its posttranslational modifications in viral life cycle of BomBDV. There are evidences that BomBDV NS1 is a phosphoprotein required in a variety of steps during progeny virus production. For example, NS1 of minute virus of mice (MVM), a homolog of the BomBDV NS1, was reported to possess DNA helicase activity, site-specific interaction with target DNA motifs and transcriptional regulation, which were modulated by the phosphorylation state of NS1 (Nüssel et al., 1998, 2003; Dettwiler et al., 1997). It was reported that the NS1 proteins of porcine parvovirus and H-1 parvovirus could be phosphorylated on serine and threonine residues in infected cells (Molitor et al., 1985; Paradiso, 1984). Moreover, as homolog of the BomBDV NS1, Periplaneta fuliginosa densovirus (PFDNV) nonstructural protein NS1 contains an endonuclease activity that is regulated by its phosphorylation. Virology 437: 1–11.


