ACTA ABP BIOCHIMICA POLONICA

Vol. 61, No 4/2014
787–794
on-line at: www.actabp.pl

Regular paper

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

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***ns1* gene of *Bombyx mori* bidensovirus (*Bm*BDV) consisted of 951 nucleotides encoding a deduced 316-amino aicd protein. In this study, the gene was cloned and fused in frame with a N-terminal 6×His tag under control of the polyhedrin promoter, which was transposed into the mini-attTn7 locus of a modified baculovirus vector. Transfection of Sf-9 cells with the resulting recombinant DNA was performed to prepare recombinant virus and the resultant supernatant of transfection with fluorescent signal was harvested. Western blot analysis revealed that NS1 protein was successfully expressed in Sf9 cells infected with the recombinant virus and was confirmed by LC-MS/MS analysis. Moreover, the expressed NS1 is a phosphorylated protein and the phosphorylation site is Thr-184. These results showed that the activity of *Bm*BDV NS1 may be regulated by phosphorylation.**

Key words: *Bm*BDV, NS1, baculovirus expression vector, Sf9, *egfp*

Received: 19 May, 2014; revised: 07 October, 2014; accepted: 11 October, 2014; available on-line: 06 November, 2014

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 protein with a 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, aFGF, 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 (*Ac*NPV) and *Bombyx mori* nucleopolyhedrovirus (*Bm*NPV) 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 genomic viral 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-P_{ie1}-*egfp* inserted into the *ac68* locus of Ac-bacmid were described here and the modified vector was exploited to express *Bm*BDV NS1.

*Bm*BDV 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 *Bm*BDV genome, it is required to study

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Abbreviations: *Bm*BDV, *Bombyx mori* bidensovirus; BEVS, baculovirus expression vector system; *Ac*NPV, *Autographa californica* nucleopolyhedrovirus; *Bm*NPV, *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, *Bm*BDV *ns1* was inserted into the genetically modified Ac-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-P_{ie1}-*egfp* and pMD18T-*ns1* were maintained in our laboratory. *Ac*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 pBAD-gbaA 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_{ie1}-egfp-ac68D. To introduce enhanced green fluorescent protein (*egfp*) into the BEVS, it was required to construct a recombinant plasmid pUC18-ac68U-Cm-P_{ie1}-egfp-ac68D as shown in the following Fig.1 flowchart. In a brief, ac68U indicated the 5' flanking sequence of Ac-bacmid *orf68* (*ac68*), which was amplified with *ac68*U-F and *ac68*U-R, and ac68D indicated the 3' flanking sequence of *ac68*, which was amplified with *ac68*D-F and *ac68*D-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-P_{ie1}-*egfp* with P_{ie1}-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 *ac68* flanking region was resuspended in distilled water to a final concentration of 200 ng/μl.

Insertion of two tandem cassettes of Cm-P_{ie1}-*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_{ie1}-*egfp* replace the *ac68* locus of *Ac*NPV 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_{ie1}-*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

Primers	Primer sequence (5'–3')	Enzyme digestion sites
*ac68*D-F	GGGCATGCATGTTGCAGCAAAAATTAAAT	*Sph* I
*ac68*D-R	TAAAGCTTGGCAAATTAAAATTAGCTGCGT	*Hin*d III
P_{ie1}-F	AACTGCAGTAGGTTATTGATAAAATGAACGGA	*Pst* I
egfp-R	AAGCATGCTTACTTGTACAGCTCGTCCAT	*Sph* I
Cm-F	GGATCCCTTCGAATAAATACCTGTGA	*Bam*H I
Cm-R	CTGCAGAACCAGCAATAGACATAAGC	*Pst* I
*ac68*U-F	CGGGTACCTTCTTCCATGTCTTTGAAAGATTGC	*Kpn* I
*ac68*U-R	CGGGATCCATTAACATTGACCGTTTGATCGT	*Bam*H I
ac67-F	TTGTTAGTCAAATAGTCGCTCATGG	
ns1-F	ATGCTAGCATGGAATCGAAGTCAAATTT	*Nhe* I
ns1-R	TACTCGAGCTACCCATAATATTTATTATATACG	*Xho* I

Note: underlined letters indicate restriction enzyme digestion sites.

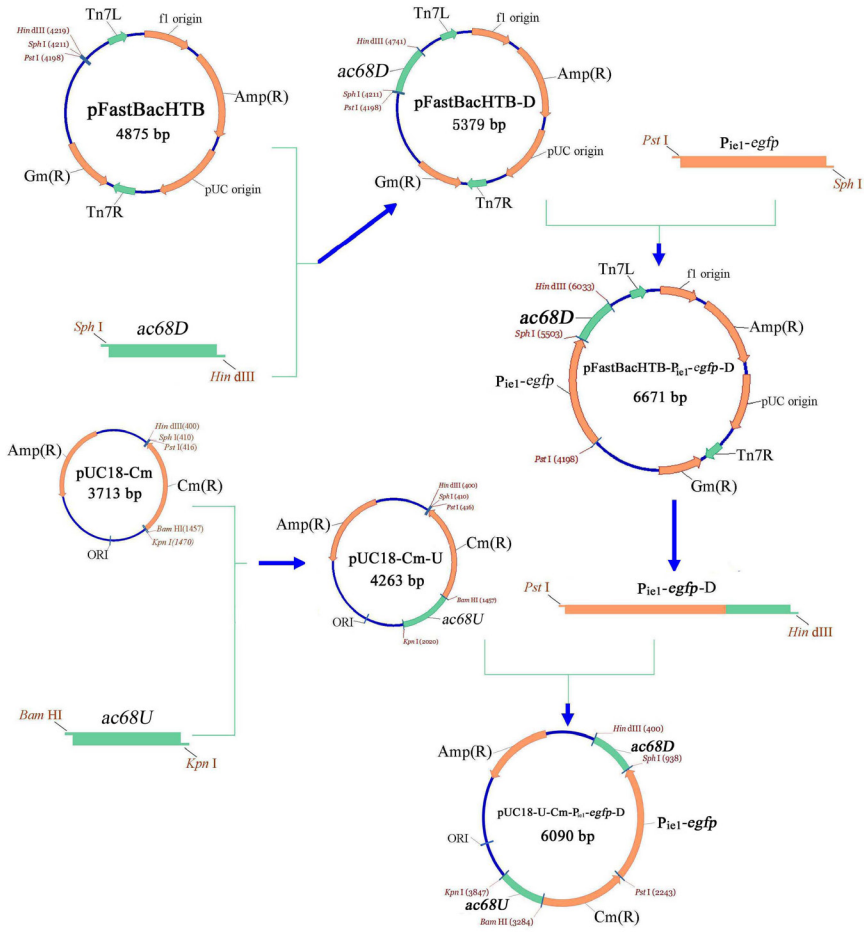

Figure 1. The flowchart showing the construction steps of the pUC18-*ac68*U-Cm-P_{ie1}-*egfp*-*ac68*D.

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-*ns1*. 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 *ns1*-F and *ns1*-R was used to amplify *ns1* gene from the genome of *Bm*BDV, and the amplifed fragment was ligated into pFastBacHTB to generate pFastBacHTB-*ns1*. The donor plasmid pFastBacHTB-*ns1* was transformed into DH10B cells harboring the modified Ac-bacmid and helper vector encoding a transposase to generate recombinant Ac-bacmid-*ns1* by transposition. The transpositions were confirmed by PCR using M13 forward and reverse primers.

The recombinant Ac-bacmid-*ns1* and the genetically modified Ac-bacmid with no *ns1* 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 ns1 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 cm^2 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 $MgCl_2$, 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 bufer, 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

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 *Bm*BDV 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 MilliQ 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 NH_4HCO_3 (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 NH_4HCO_3) 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 *Bm*BDV NS1 database using the automated Mascotalgorithm for possibleY, T and S phosphorylation sites.

RESULTS

Construction of the plasmid pUC18-*ac68*U-*Cm*-P_{ie1}-*egfp*-*ac68*D

Figure 1 is a flowchart illustrating the construction steps of the recombinant plasmid pUC18-*ac68*U-*Cm*- P_{ie1}-*egfp*-*ac68*D, which was confirmed by analysis of PCR and enzyme digestion (data not shown). The results indicated that *ac68*U, Cm, P_{ie1}-*egfp* and *ac68*D fragments were successfully ligated into pUC18 vector in correct order. Additionally, the sequenced result of the plasmid pUC18-*ac68*U-Cm-P_{ie1}-*egfp*-*ac68*D was corresponded with the expected sequence.

Replacement of *ac68* locus of Ac-bacmid by two *Cm*-P_{ie1}-*egfp* tandem cassettes

Previous studies reported that *ac68* was not essential for the propagation of *Ac*NPV (Li *et al.*, 2008; Nie *et al.*, 2012). Therefore, two tandem cassettes of Cm-P_{ie1}-*egfp* were used to replace the *ac68* locus of Ac-bacmid by homologous recombination in this study, which facilitate the rapid judgement of infectious virions 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_{ie1}-*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_{ie1}-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

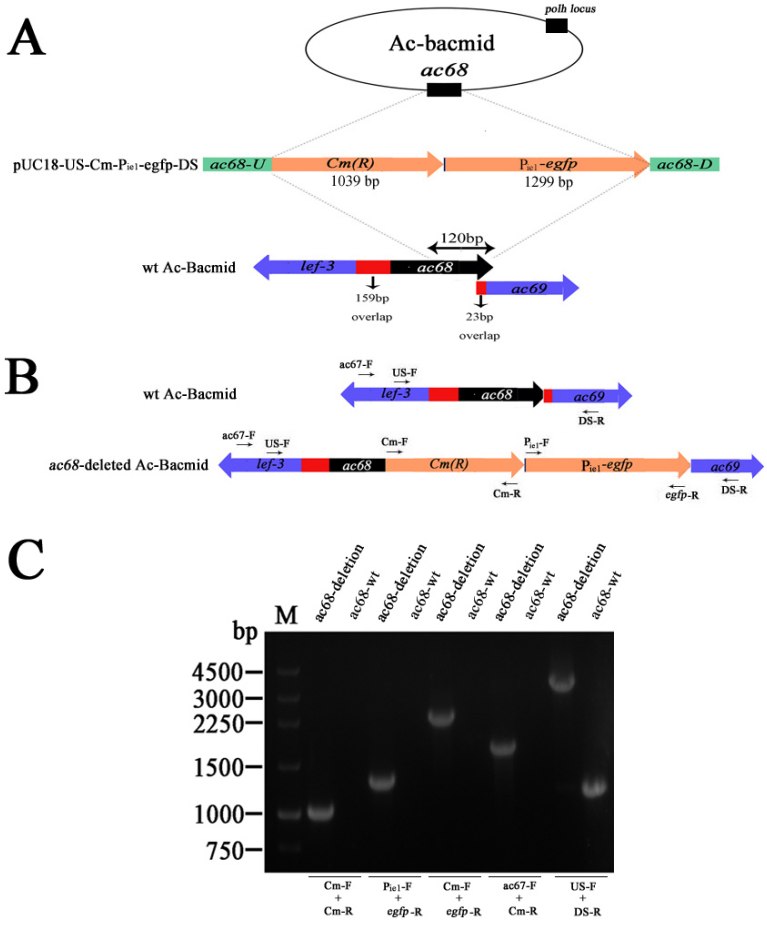

Figure 2. Strategy for construction of *ac68*-deleted Ac-bacmid and confirmation by PCR analysis.
(**A**) Schematic diagram showing the structure of *ac68* locus in wild-type and *ac68*-deleted bacmid and the replacement of a 120 bp fragment with two tandem cassettes of Cm-P_{ie1}-*egfp*. (**B**) Positions of primer pairs used in the confirmation of the *ac68* gene deletion and correct insertion of Cm-P_{ie1}-*egfp*. (**C**) PCR analysis with different primer pairs. Marker: molecular weight marker with sizes indicated. The bacmid templates are shown above each lane and the primer pairs used are shown below.

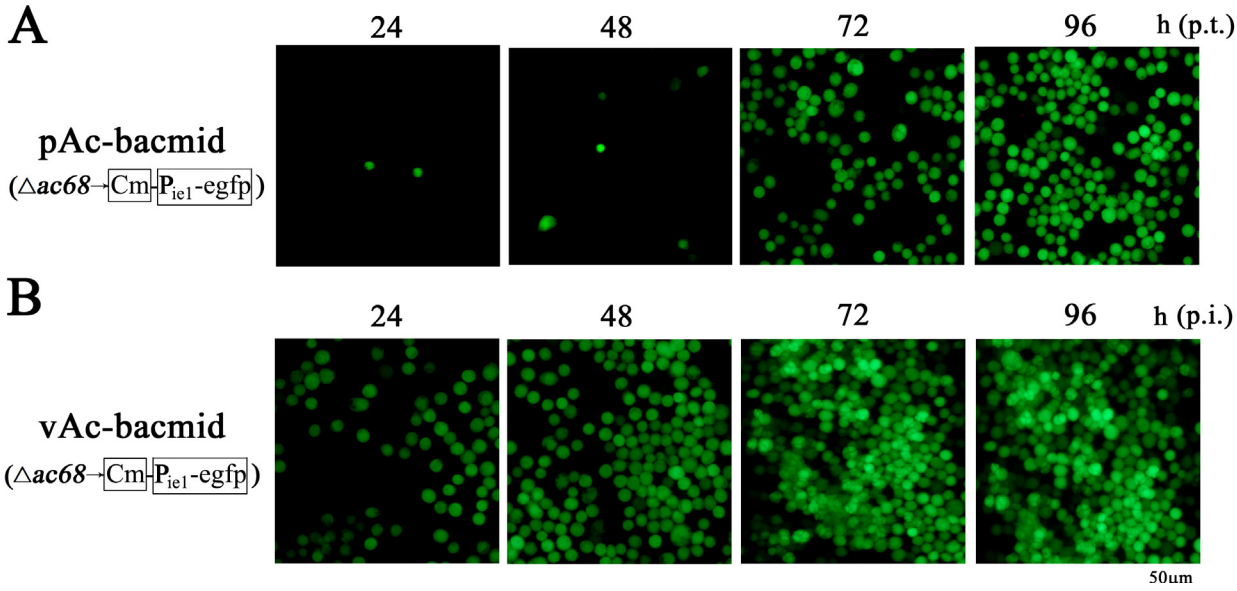

Figure 3. The expression of *egfp* gene in Sf9 cells.
(**A**) Fluorescence micrographs of Sf9 cells transfected with the *ac68*-deleted bacmids. Bacmids used for transfection were indicated on the left and the times after transfection were indicated above. (**B**) Fluorescence micrographs of Sf9 cells infected with virus supernatant. Viruses used for infection were indicated on the left and the time post infection was indicated above.

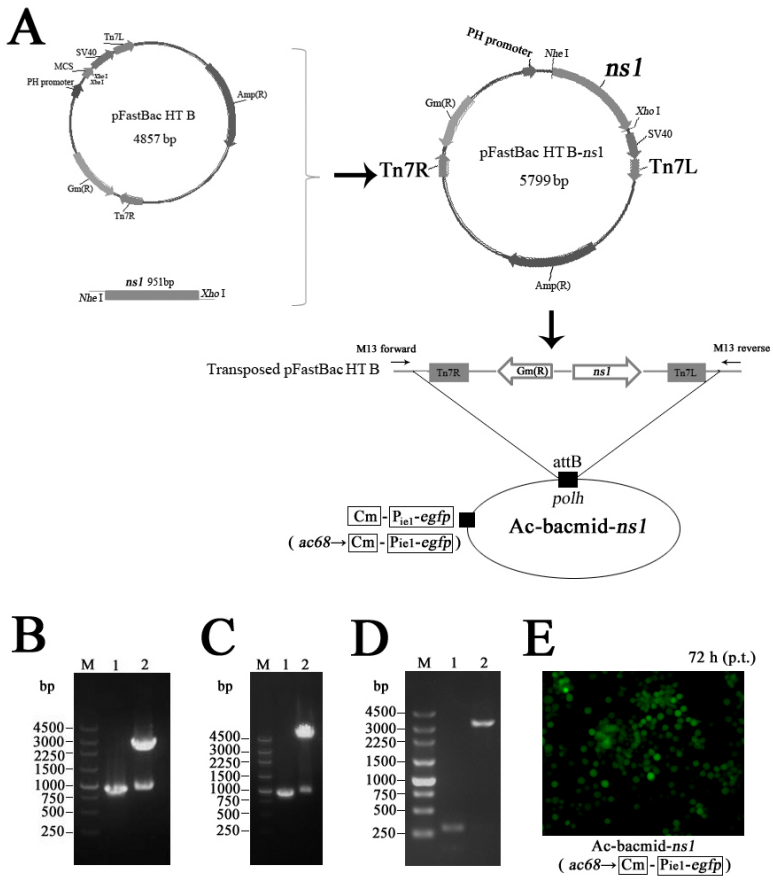

Figure 4. Stategy for construction of recombinant virus for expression of *Bm*BDV NS1 and identification of recombinant virus through the expression of *egfp*.
(**A**) A flow diagram illustrating the steps of producing a recombinant Ac-bacmid. (**B**) Lane M, DNA marker; Lane 1, PCR product of *ns1* 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.

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 vey 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

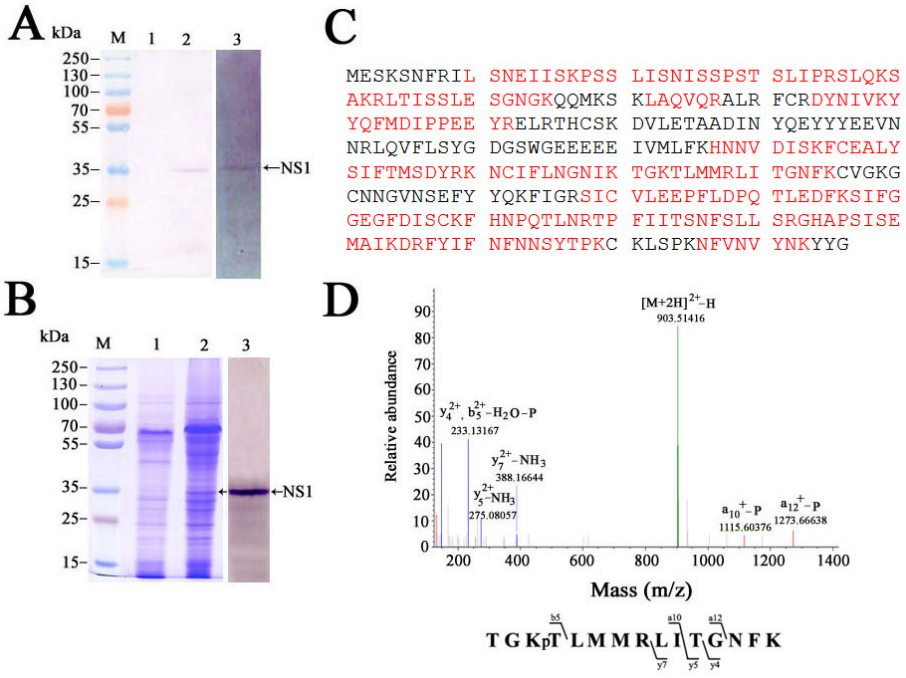

Figure 5. Identification of NS1 expressed in Sf9 cells by Western blot and Mass Spectrometry
(**A**) Western blot analysis of NS1 expressed in Sf9 cells infected with recombinant virus; Lane 1: blank control; Lane 2: NS1 was examined using anti-6×His tag monoclonal antibodies; Lane 3: NS1 was examined using anti-NS1 monoclonal antibodies. (**B**) The enriched NS1 was subjected to SDS/PAGE anslysis; Lane M: pre-staining protein marker; Lane 1: blank control; Lane 2: the enriched NS1 indicated with arrow; Lane 3: The enriched NS1 was examined using anti-NS1 monoclonal antibodies. (**C**) Peptide sequences identified by mass spectrometry. The *Bm*BDV NS1 deduced amino acid sequence is shown, and the matched peptide sequences are indicated with red letters. (**D**) MS results showing T184 phosphorylation. The graph represents the LC–MS/MS production spectrum of the parent ion of phosphorylated peptide 181–195 from trypsin digested *Bm*BDV NS1. The ions are labeled in the graph. The x- and y-axis show mass-to-charger at ration (*m/z*) and relative abundance (%) of the ions, respectively. The bottom panel shows the digested peptide, which contains phosphorylated threonine at residue 184. The a-, b- and y- type fragment ions observed are indicated with the peptide sequence.

the infectivity of these virions. The results demonstrated that green fluorescent signal appeared in some Sf9 cells infected with the infectious supernatants at 24 h p.i., and the green fluorescence signal was sharply intensified from 48 h p.i. to 96 h p.i. (Fig. 3B), indicating that the cultured Sf9 cells were subjected to primary infection and the next round infection.

Preparation of recombinant virions used for expression of *Bm*BDV *ns1*

A schematic outline for producing recombinant virus in Sf9 cells is shown in Fig. 4A. Briefly, the plasmid pMD18-T-*ns1* was digested with *Nhe*I and *Xho*I (Fig. 4B), which generated a 951-bp DNA fragment. Then, it was ligated into pFastBacHTB vector to generate the transfer vector pFastBacHTB-*ns1* (Fig. 4C). The transposition events can be achieved between the pFastBacHTB-*ns1* and the genetically modified Ac-bacmid (Δ*ac68*→Cm-P_{ie1}-*egfp*), which were confirmed by PCR with M13 forward and reverse primers (Fig. 4D). A specific band about 3.2 kb was amplified from the recombinant Ac-bacmid-*ns1* (Fig. 4D lane 2). Meanwhile, 300-bp DNA fragment was amplified from wild-type Ac-bacmid (Fig. 4D lane 1). The recombinant virions were produced in the supernatant of cultured Sf9 cells, which was confirmed through fluorescence microscopy (Fig. 4E).

Identification of *Bm*BDV NS1 expressed in Sf9 cells

The transfected supernatant containing the recombinant virions was harvested, which was used to infect the cultured monolayer Sf9 cells in one 75 cm^2 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 *Bm*BDV NS1 protein (Fig. 5C). Furthermore, the MS result indicated that T184 of *Bm*DNV 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 *Bm*DNV NS1 may be the alterative 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 Sf9 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 virions produced in transfected Sf9 cells were easily and rapidly identified through fluorescence microscopy. Therefore, the modified BEVS was an easy and convenient insect system for heterologous protein expression. *Bm*BDV *ns1* was sucessfully expressed in Sf9 cell with the modified BEVS, but we are not satisfied with the relatively low yield of *Bm*BDV NS1. Further research was required to improve the expression yield of *Bm*BDV NS1 by seeking the optimal conditions.

NS1 protein was identified to be a mulfunctional protein involved in replication of *Bm*BDV (Li *et al*., 2009). To further elucidate the regulation mechanism of *Bm*BDV NS1, it is a crucial step to determine its posttranslational modifications in viral life cycle of *Bm*BDV. There are evidences that *Bm*BDV NS1 is a phosphoprotein required in a variety of steps during progeny virus production. For example, NS1 of minue virus of mice (MVM), a homolog of the *Bm*BDV 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 (Nüesch *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 *Bm*BDV NS1, *Periplaneta fuliginosa* densovirus (*Pf*DNV) nonstructural protein NS1 contains an endonuclease activity that is also regulated by its phosphorylation (Han *et al*., 2013). Additionally, there are many predicted phosphorylation sites in the amino acid sequence of the *Bm*BDV NS1 using the Netphos 2.0 software (http://www.expasy.ch). Therefore, the *Bm*BDV NS1 is very likely to be a phosphoprotein in viral life cylce. *Bm*BDV *ns1* was successfully expressed in Sf9 cells using the modified baculovirus expression vector, and the NS1 band was excised from the gels for LC-MS/MS analysis corresponding with Western blot result. The genetically modified Ac-bacmid was verified to be a feasible and convenient eukaryotic expression vector for expressing heterologous genes, and T184 of *Bm*BDV NS1 was found to be phosphorylated by LC-MS/MS analysis. Although the phosphorylation of *Bm*BDV NS1 expressed in Sf9 cells did not represent true states of *Bm*BDV NS1 during a wide range of stress responses, it provides important clues for studying its role mechanism in viral life cycles.

In this study, an effective method for accurately identifying recombinant baculovirus produced in cultured insect cells was established to facilitate the BEVS for production of desired proteins. Using this system, *Bm*BDV *ns1* with translational modification was successfully expressed and our results indicated that its activity may be regulated by phosphorylation. Further research is required to explore the phosphorylation pattern of *Bm*BDV NS1 and their roles in viral life cycles.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (31270192, 31000080, 31272507 and 31402016), the National basic Research Program of China under Grant No. 2012CB114604, and the Startup Scientific Research Fund from Jiangsu University for Senior Professionals (No. 09JDG057).

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