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Bombyx mori nucleopolyhedrovirus *orf25* encodes a 30kDa late protein in the infection cycle

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Abstract: *Bombyx mori* nucleopolyhedrovirus (BmNPV) *orf25* gene was characterized for the first time. The coding sequence of *Bm25* was amplified and subcloned into the prokaryotic expression vector pGEX-4T-2 to produce glutathione S-transferase-tagged fusion protein in the BL21 (DE3) cells. The GST-Bm25 fusion protein was expressed efficiently after induction with IPTG. The purified fusion protein was used to immunize New Zealand white rabbits to prepare polyclonal antibody. Temporal expression analysis revealed a 30-kDa protein, which was detected beginning 24 hours post-infection using a polyclonal antibody against GST-Bm25 fusion protein. The transcript of *Bm25* was detected by RT-PCR at 18-72 h p.i. In conclusion, the available data suggest that *Bm25* encodes a 30kDa protein expressed in the late stage of infection cycle.

Keywords: BmNPV; *Bm25*; Expression temporal; RT-PCR; polyclonal antibody
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1 Introduction

The family Baculoviridae is a large family of viruses that infect invertebrates, particularly insects of the order Lepidoptera. Baculoviruses contain circular double stranded DNA genomes of 80 to 180 kb[1]. They are taxonomically subdivided into two genera, the nucleopolyhedrovirus (NPVs) and the granuloviruses (GVs) according to the morphology of occlusion bodies (OBs). The two viral forms are essential for the natural propagation of the baculoviruses. They have different viral structure compositions despite containing an identical genome[2]. The *Bombyx mori* NPV (BmNPV) is a major pathogen of the mulberry silkworm and cause disastrous effect on output of *Bombyx mori* silk.

The BmNPV genome is 128413bp and potentially encodes 136 genes [3]. So far, the functions of several genes in BmNPV have been characterized, such as *orf8* [4], *orf60* [5], *orf42* [6], *orf79* [7], lef7 [8], chitinase [9], vfgf [10] etc. But the functions of many other genes still remain elusive, including *orf25*.

BmNPV ORF25 (*Bm25*) is located at 24367-25014 nt in the genome of BmNPV T3 strain. It contains 648 nts and is predicted to encode a putative 215 amino acid peptide with a deduced molecular weight of 24.8 kDa. It is transcribed in the opposite orientation to the polyhedrin gene. Though *Bm25* homologues are identified in several lepoidoptera NPVs, it is uncertain whether *Bm25* and its homologues are functional genes.

In this study, we analysed the *Bm25* gene by examining transcription of the gene and expression of its protein product in BmNPV infected BmN-4 cells. RT-PCR analysis indicated that *Bm25* was transcripted at 18h p.i. and western blot analysis found that a 30 kDa product of *Bm25* was detected in BmNPV infected BmN-4 cells from 24 h p.i.. These results together indicated that *Bm25* was a late gene.

2 Materials and Methods

2.1 Cells and viruses

The *Bombyx mori* cell line, BmN-4 originated from ovary, was mantained in TC-100 medium (Gibco, Carlsbad) with 10% fetal bovine serum (Gibco). The BmNPV (Zhenjiang strain) was used for infections as wild type and propagated in BmN cells.

2.2 Computer-assisted sequence analysis

The data from all baculovirus genes in this paper were obtained from GenBank.The protein sequence was analysis using softwares of the ExPASY for the

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prediction of motifs, domains, signal peptide, transmembrane regions.

2.3 Expression of Bm25 and generation of anti-Bm25 antiserum

Two primers, Bm25F (5′-Aggatcca TGACAACGGTTGCTG-3′) with *Bam*HI site and Bm25R (5′-CctcgagCTCAAAGTCCATCAAT-3′) with *Xho*I site (shown in bold) were designed to amplify the *Bm25* gene from the BmNPV T3 (GenBank accession No. NC 001962) genome. The *Bm25* coding region amplified was ligated into pGEM-T easy vector Promega).The insert was retrieved by digestion with *Bam*HI and *Xho*I, and then subcloned into the expression vector pGEX-4T-2 (Amersham Pharmacia). This generated plasmid pGEX-GST-Bm25, in which *Bm25* is in-frame and fused with GST at the N terminus. The recombinant plasmid was transformed into *Escherichia coli* BL21 cells and fusion protein expression was induced by 1 mmol/L IPTG at 27℃ when the optical density of the culture at 600 nm reached 0.5. The recombinant protein GST-Bm25was purified using Glutathione Sepharose column (Amersham Pharmacia) and used to immunize rabbits to raise polyclonal antibodies.

New Zealand white rabbits received four injections of 200 μg of purified GST-Bm25 fusion protein. Antiserum was collected one week after the last injection. The polyclonal rabbit antibody against GST-Bm25 was used for immunoassay.

2.4 Transcription analysis of *Bm25*

BmN-4 cells were infected with BmNPV at a m.o.i of 10. Total RNA was isolated at 0, 3, 6, 12, 18, 24, 48 and 72 h p.i. using Trizol (Invitrogen) according to manufacturer's protocol. The RNA was dissolved in 25μL water and quantified by absorbance measurement at 260 nm.

RT-PCR was performed using 1μg total RNA as template for each time point. The total RNA was first treated with DNase to eliminate any potential genomic DNA contamination. First-strand cDNA was synthesized from total RNA by using 15-nt oligo(dT) primer and AMV reverse transcriptase (Promega) following the manufacturer's protocol. The cDNA mixtures were amplified by PCR using the gene-specific primer Bm25F and Bm25R. The PCR products were analyzed in 1.0% agarose gel.

2.5 Temporal expression of *Bm25* in infected BmN-4 cells

For the time course analysis, BmN-4 cells were infected with BmNPV at m.o.i. of 10. Samples of total cell proteins were harvested from infected cells at 0, 3, 6, 12, 18, 24, 48, 72 h p.i. and washed three times with PBS. The protein concentrations of the cell extracts were determined by Bradford's method. Protein samples (20μg) were separated by 10% SDS-PAGE and subsequently subjected to western blot assay.

Following SDS-PAGE electrophoresis, the protein samples were transferred onto a PVDF membrane by wet electrophoresis transfer with Trans-Blot Cell (Bio-Rad). The membrane was blocked with 4% skim milk powder in PBST for 3 h followed by incubation with the anti-Bm25 polyclonal antiserum diluted 1: 5000 for 2 h at room temperature. After washing, the membrane was incubated with a goat anti-rabbit IgG conjugated to HRP diluted 1: 5000 for 1 h at room temperature. The peroxidase activity was developed with 0.1% H_2O_2 and diaminobenzidine (DAB) as a chromogenic substrate.

3 Results

3.1 Sequence analysis of Bm25

The Bm25 ORF contains 648 nts and is predicted to encode a 215-amino acid peptide with a predicted molecular weight of 24.8kDa. A baculovirus consensus late transcriptional start motif ATAAG was found at 146nt upstream of the start codon ATG (Fig. 1, shown in bold). The canonical poly (A) signal (AATAAA) was not found at the downstream region of the translational stop codon (TAA). Nine putative phosphorylation sites were found in the putative Bm25 protein including three serine phosphorylation sites (aa30, aa98 and aa200), three threonine phosphorylation sites (aa25, aa139 and aa201), and three tyrosine phosphorylation sites (aa104, aa147 and aa209). No signal peptide, transmembrane regions, mitochondrial targeting sequences or membrane retention signals were found by any of the motif search engines employed.

Searches of protein databases, GenBank and SWISS-PROT, revealed that the deduced Bm25 protein was homologous to the putative products from 6 NPV ORFs. The BmNPV *orf25* shared an identity of 100% with AcMNPV *orf34*. The homologs from the other 5 NPVs shared 29%–35% identity with *Bm25* (data not shown).

3.2 Expression of the *Bm25* gene in *E.coli*

The open reading frame encoding Bm25 protein was amplified from BmNPV T3 genomic using PCR. *Bam*HI and *Xho*I sites were designed in the primers to facilitate cloning into the pGEX-4T-2 vector. The 648 bp PCR products were cloned into the pGEX-4T-2 vector (Fig. 2).

The recombinant plasmid pGEX-GST-Bm25 was transformed into *E. coli* BL21 and fusion protein expression was induced by incubation in the presence of 1 mmol/L IPTG. Western blot analysis using the anti-GST monoclonal antibody further confirmed that the 45kDa polypeptide was the GST-Bm25 fusion protein (Fig.3). The fusion protein was purified and used to immunize rabbits for producing the anti-Bm25 antiserum.

3.3 Transcriptional analysis

Temporal regulation of *Bm25* transcript was examined by RT-PCR analysis using total RNA isolated from BmNPVinfected host cells at designated time intervals (0, 3, 6, 12, 18, 24, 48 and 72 h p.i.). The result revealed a band with an expected size of 648 bp (Fig.4). The *Bm25* transcript was detected at 18 h p.i. and remained detectable until 72 h p.i.

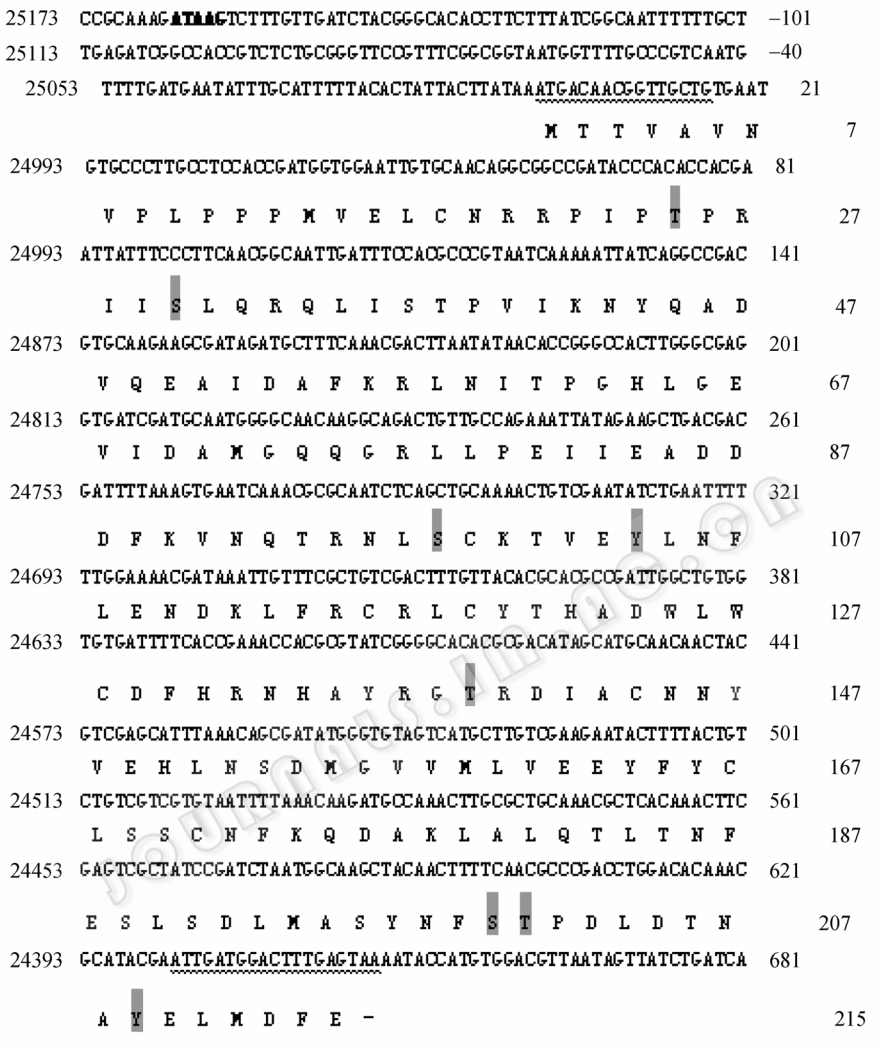

Fig.1 Nucleotide sequence and deduced amino acid sequence of *Bm25*. A baculovirus consensus late transcriptional start motif ATAAG is shown in bold. The primers used in this study are underlined. The putative threonine, tyrosine, and serine phosphorylation sites are shown in Grey Shading.

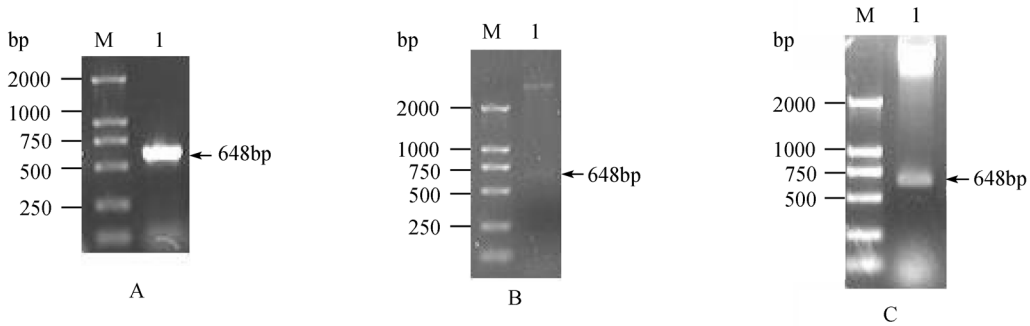

Fig.2 The PCR product of *Bm25* amplification, recombinant pGEM-T easy vector and pGEX-4T-2-Bm25 vector identified by enzyme cleavage. A: the PCR product of *Bm25* amplification; B: recombinant pGEM-T easy vector was identified by *Bam*H I and *Xho* I enzymes; C: pGEX-4T-2-Bm25 was identified by *Bam*H I and *Xho* I enzymes.

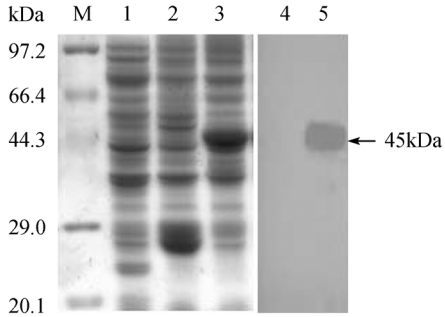

Fig.3 The expression of GST-Bm25 in *E. coli* cells and Western blotting analysis. M: protein marker.1: BL21 cell extracts; 2: proteins of BL21 transformed with pGEX-4T-2; 3: the extracts of BL21 transformed with pGEX-4T-2-Bm25; 4: Western blotting analysis using anti-GST monoclonal antibody for BL21; 5: Western blotting analysis using anti-GST monoclonal antibody for BL21 transformed with pGEX-4T-2-Bm25.

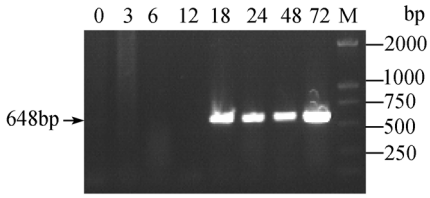

Fig.4 Transcriptional analysis of *Bm25*. Total RNA was isolated from BmNPV-infected cells at 0, 3, 6, 12, 18, 24, 48, 72 h p.i. RT-PCR was used to analyse *Bm25* transcription in each sample. A 648bp band was observed in 18, 24, 48, 72 h p.i. sample respectively.

3.4 Time course of *Bm25* expression in BmNPV-infected cells

A time course of BmNPV infected BmN-4cells were analyzed by western blot using the polyclonal antibody against Bm25. The result revealed that a specific immunoreactive band with approximately 30 kDa, which first detected at 24 h.p.i. and could be detected until 72 h.p.i. (Fig.5). The 30 kDa immunoreactive band was not consistent with predicted molecular weight 24.8 kDa, suggesting that the post translational modification of the Bm25 protein occurred.

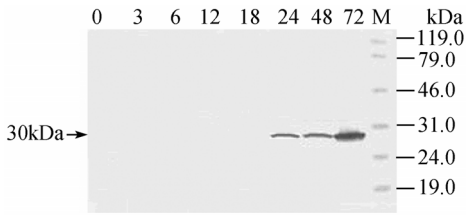

Fig.5 Western blot analysis of *Bm25* gene product in BmN-4 cells. The cells were collected at 0, 3, 6, 12, 18, 24, 48, 72 h p.i. and 20 μg cell lyses at each interval was subjected to Western blot analysis using anti-GST-Bm25 serum. The binding was developed with diaminobenzidine (DAB) as a chromogenic substrate. Protein markers are indicated on the right.

4 Discussion

During the last decade, 29 baculovirus genomes have been sequenced offering a wealth of information on the gene content and phylogeny of the baculovirus genomes [11]. Based on the phylogenetic analysis, 63 genes were conserved among all sequenced lepidopteran NPVs [12]. In this study, we described some preliminary characteristic of *Bm25*, a gene that has thus far not been characterized. The search for homologues of *Bm25* revealed that homologues were present in 6 completely sequenced members of lepidopteran NPVs, suggesting *Bm25* and its homologues were specific genes for 7 completely sequenced lepidopteran NPVs and might play imp ortant role in function related to these hosts.

In order to study the expression of the Bm25 protein and its possible function, a polyclonal antibody was prepared by immunization of rabbits with a purified GST-Bm25 fusion protein. Immunodetection of Bm25 protein in infected cells was performed using the polyclonal antibody against Bm25. A specific immunoreactive band of approximately 30 kDa was first observed at 24 h p.i. and remained detectable up to 72h p.i. This was consistent with the transcriptional analysis of the *Bm25* gene, however, the protein was detectable 6 hours later than the gene transcript , was probably due to the low amount of protein produced shortly after transcription. This result suggested that *Bm25* might be a late gene and probably used only one late transcription start motif, ATAAG, found 146nt upstream of the translation start codon ATG of *Bm25*. We observed that the size of the immuno-reactive protein was larger than the predicted molecular weight of 24.8 kDa, suggesting that the post-translational modification might have occurred. This result was also agreement with the presence of nine potential phosphorylation sites, which were found in the putative Bm25 protein.

Although some basic characteristics were described in this paper, much information about this gene keeps unknown. The function of *Bm25* is being further investigated by knockout mutants of *Bm25*.

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家蚕核型多角体病毒 *orf25* 基因在病毒感染过程中编码一个 30kDa 的晚期蛋白

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摘要: 首次对家蚕核型多角体 *orf25* 基因进行了描述。扩增 Bm25 基因, 亚克隆到原核表达载体 pGEX-4T-2, 在大肠杆菌 BL21 (DE3)中表达含有 GST 标签的融合蛋白。IPTG 诱导后高效表达 GST-Bm25 融合蛋白。纯化的融合蛋白免疫新西兰大白兔制备多克隆抗体。利用制备的抗 GST-Bm25 融合蛋白的多克隆抗体进行表达时相分析显示: 24 h p.i.检测到 30 kDa 的蛋白条带。RT-PCR 方法, 在 18-72 h p.i 检测到 *Bm25* 基因的转录本。结论: 以上数据表明 Bm25 基因编码一晚期表达的 30kDa 蛋白。

关键词: BmNPV; *Bm25*; 表达时相; RT-PCR; 多克隆抗体

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