High-level Expression, Polyclonal Antibody Preparation and Bioinformatics Analysis of Bombyx mori Nucleopolyhedrovirus orf47 Encodes Protein

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***Bombyx mori* nucleopolyhedrovirus (*Bm*NPV) *orf47* gene was characterized for the first time. The coding sequence of *Bm47* was amplified and subcloned into the prokaryotic expression vector pET-30a(+) in order to produce His-tagged fusion protein in the BL21 (DE3) cells. The His-Bm47 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. As the genome of *Bm*NPV is available in GenBank and the EST database of *Bm*NPV is expanding, identification of novel genes of *Bm*NPV was conceivable by data-mining techniques and bioinformatics tools. Structural bioinformatics approach to analyze the properties of *Bm47* encodes protein.**

Key words: *Bm*NPV, *Bm47*, Prokaryotic expression, Fusion protein, Polyclonal antibody, Bioinformatics

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 (Lu et al. 1998). 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 (Baluchamy and Gopinathan 2005). The *Bombyx mori* NPV (*Bm*NPV) 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 (Gomi et al. 1999). Since the sequencing of the genome of the baculovirus *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) (Ayres et al. 1994), 25 other baculovirus genomes have been reported. So far, the functions of several genes in BmNPV have been characterized, such as *orf8* (Imai et al. 2004), *orf60* (Du et al. 2006), *orf42* (Acharya and Gopinathan 2002), *orf79* (Xu et al. 2006), lef7 (Wang et al. 2006), chitinase (Daimon et al. 2007), vfgf (Katsuma et al. 2006), *orf67* (Chen et al. 2007) etc. But the functions of many other genes still remain elusive, including *orf47*.

*Bm*NPV *orf47* (*Bm47*) is located at 42723-43238 nt in the genome of *Bm*NPV T3 strain. It contains 516 nts and is predicted to encode a putative 171 amino acid peptide with a deduced molecular weight of 20.1 kDa. It is transcribed in the opposite orientation to the polyhedrin gene. Though *Bm47* homologues are identified in several lepoidoptera NPVs, it is uncertain whether *Bm47* and its homologues are functional genes.

In this study, we analysed the *Bm47* gene by examining transcription of the gene and expression of its protein product in *Bm*NPV infected *Bm*N-4 cells. Western blot analysis found that about a 15 kDa product of Bm47 was detected in *Bm*NPV infected *Bm*N-4 cells from 12 h p.i..We found that the expression of the fusion protein was incompletely. These results together indicated that *Bm47* was a late gene.

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Materials and Methods

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.

Computer-assisted sequence analysis

The data from all baculovirus genes in this paper were obtained from GenBank. We used the ExPASy Translate tool (http://au. expasy. org/tools/dna. html) to deduce the cDNA's amino acid sequence, and similarity analysis was performed using the BLAST tool in GenBank (blastx) and SIB BLAST Network Service (http://au.expasy.org/tools/BLAST/). Several annotated secondary databases can be viewed as sitting on top of the primary protein sequence sources. We used SMART (http://smart.embl-heidelberg.de) to predict the secondary structure. We used PLOC (http://www.genome.jp/SIT/plocdir/) (Park and Kanehisa 2003) to predict the subcellular location of *Bm*47.

Construction of the prokaryotic expression plasmid pET-30a(+)

The subcloning and transformation steps of *Escherichia coli* were carried out using standard techniques[13]. The open reading frame was amplified from the BmNPV T3 (GenBank accession No. NC 001962) genome template using the polymerase chain reaction (PCR). A sense primer 5-AGGATCCATGTATCAAATTCCCG-3 (containing the *Bam*HI site) and an antisense primer 5-CCTC-GAGTTAATAGTTGTAATAATT-3 (containing the *Xho*I site) were used for the construction of the soluble form of *Bm47* encodes fusion protein with a 6His tag at its N-terminus. The PCR-amplified enzymes(TaKaRa) and subcloned into the pET-30a(+) expression vector (Novagen). Positive clones were first selected by PCR and reconfirmed by restriction digestion. One clone was selected and was used for further study.

Expression and identification of the *Bm47* encodes fusion protein

The recombinant plasmid was transformed into *Escherichia coli* BL21 cells and selected on an LB agar containing 50 μg/ml ampicillin (Sigma). The fusion protein expression was induced by 1.0 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at 28°C when the optical density of the culture at 600 nm reached 0.5.

To identify the His-Bm47 fusion protein,Western blotting was done according to standard protocol (Sambrook et al. 1989). The proteins on the polyacrylamide gel were transferred electrophoretically onto a PVDF membrane using a Trans-blot SD semi-dry electrophoretic transfer

cell (Bio-rad).After overnight blocking in 5% non-fat dry milk,the meebrane was incubated with the anti-6His monoclonal antibody at a dilution of 1 : 500 for 2 h at RT.The immunoreactive protein bands were detected with peroxidase-conjugated goat anti-mouse IgG.

The recombinant protein His-Bm47 was purified and used to immunize New Zealand white rabbits to raise polyclonal antibodies. The immunization procedure was carried out as described (Hu et al. 2002). New Zealand white rabbits received four injections of 200 ìg of purified His-Bm47 fusion protein. Antiserum was collected one week after the last injection. The polyclonal rabbit antibody against His-Bm47 was used for immunoassay.

Transcription analysis of *Bm47*

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, 72 and 96 h p.i. using Trizol (Invitrogen) according to manufacturer's protocol. The RNA was dissolved in 25 μL water and quantified by absorbance measurement at 260 nm. RT-PCR was performed using 1 μg 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 Bm47F and Bm47R. The PCR products were analyzed in 1.0% agarose gel.

Temporal expression of Bm47 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 6, 12, 18, 24, 36, 72, 96 h p.i. and washed three times with PBS. The protein concentrations of the cell extracts were determined by Bradford's method. Protein samples (20 μg) were separated by 12% SDS-PAGE and subsequently subjected to western blot assay.

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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 5% skim milk powder in PBST for 3 h followed by incubation with the anti-Bm47

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.

Results

Sequence analysis of Bm47

The Bm47 ORF contains 516 nts and is predicted to encode a 171-amino acid peptide with a predicted molecular weight of 20.1 kDa. A baculovirus consensus late transcriptional start motif ATAAG was found at 176nt upstream of the start codon ATG (Fig. 1). The canonical
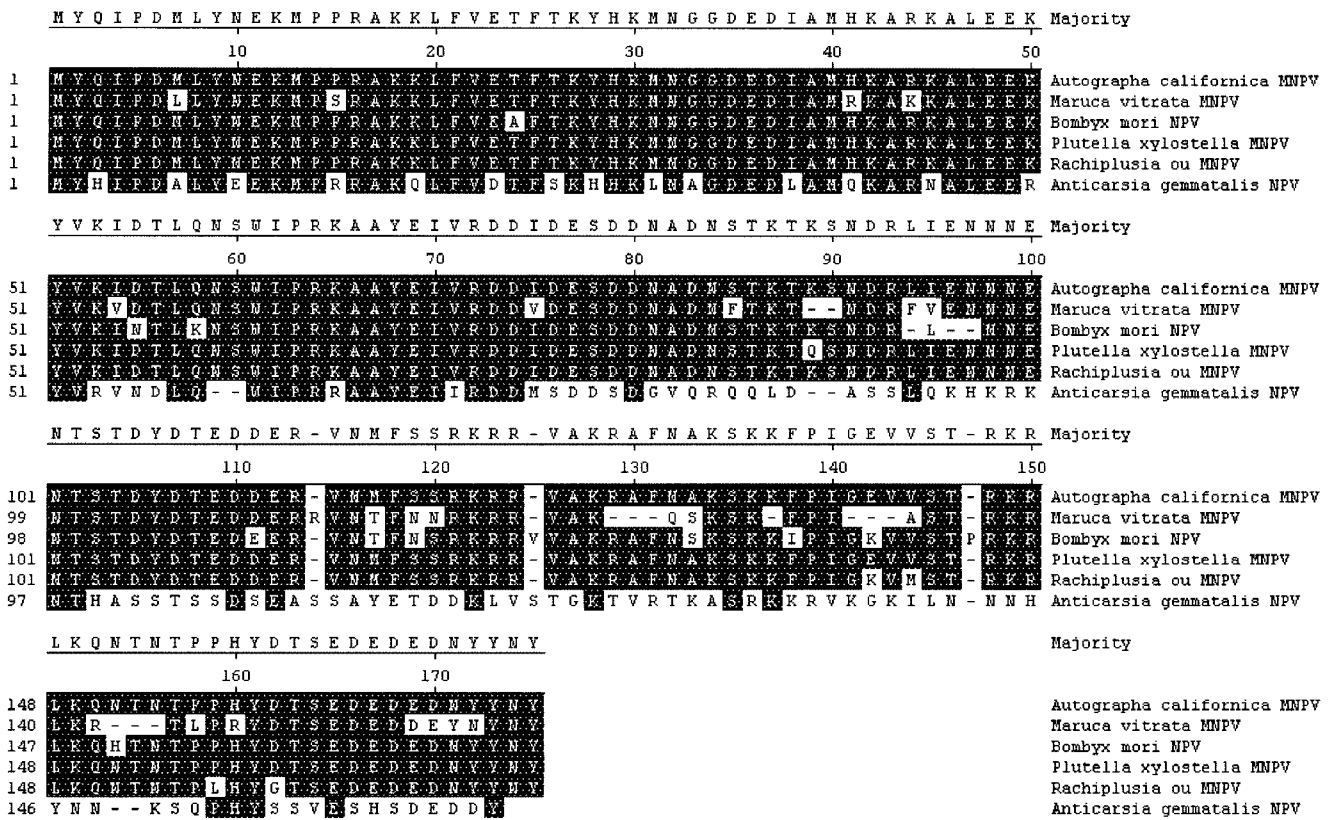
poly (A) signal (AATAAA) was not found at the downstream region of the translational stop codon (TAA). Nine putative phosphorylation sites were found in the putative Bm47 protein including three casein kinase II phosphorylation sites (aa99, aa105 and aa159), five protein kinase C phosphorylation sites (aa105, aa56 and aa85, aa116, aa131, aa142), and a tyrosine kinase phosphorylation site (aa44). We also found two bipartite nuclear localization signals, a N-glycosylation site and ChaB superfamily (aa5-aa65). ChaB family of proteins contain a conserved 60 residue region. This protein is known as ChaB in E. coli and is found next to ChaA which is a cation transporter protein. ChaB may be regulate ChaA function in some way[15].

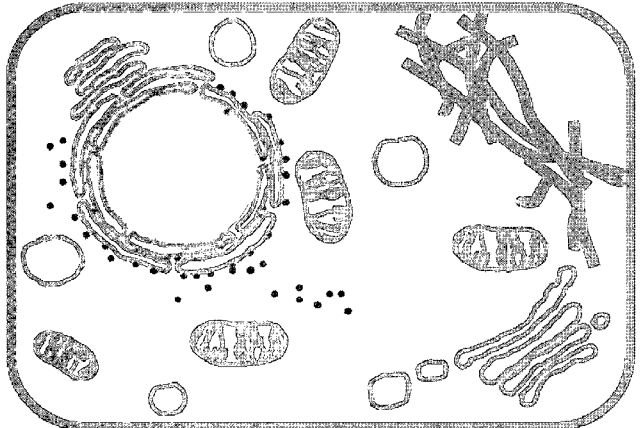Searches of protein databases, GenBank and SWISS-PROT, revealed that the deduced Bm47 protein was

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43441  gaggatatttgaaaaattctacgatcgtagtttacaaaaatataagtcgacacatatagc

43381  taccaagctagcatgttgtgctgttaggaaaaaatacatattgatagactgtaaatggca

43321  gccaaggcctgacgccaacaatagcgacactactactactaatacagaggattccactac

43261  agatacagaaaccgaagacgaaattgcttaattctaattaca atg tatcaaattcccgat
                                                    M   Y   Q   I   P   D

43201  atgttatacaatgaaaaaatgcctcctcgcgccaaaaagttatttgtcgaggcgtttaca
       M   L   Y   N   E   K   M   P   P   R   A   K   K   L   F   V   E   A   F   T

43141  aagtatcataaaatgaacggcggcgacgaagacattgctatgcataaagctagaaaagcg
       K   Y   H   K   M   N   G   G   D   E   D   I   A   M   H   K   A   R   K   A

43081  ctcgaagaaaagtacgttaaaataaatacattgaaaaattcttggattccgcgcaaagcc
       L   E   E   K   Y   V   K   I   N   T   L   K   N   S   W   I   P   R   K   A

43021  gcctacgagatagttagggacgacattgacgaaagcgacgataacgcggacaattctacg
       A   Y   E   I   V   R   D   D   I   D   E   S   D   D   N   A   D   N   S   T

42961  aaaactaaatcaaacgatcgtcttaacaacgaaaacactagcacggattatgacactgaa
       K   T   K   S   N   D   R   L   N   N   E   N   T   S   T   D   Y   D   T   E

42901  gacgaagagcgcgtcaacacgtttaatagtcgtaaacggcgagtcgtcgctaagcgggcg
       D   E   R   V   N   T   F   N   S   R   K   R   R   V   V   A   K   R   A

42841  tttaattcgaaatccaaaaaaattcctatcggcaaagtggtgtcgacgccgcgtaaacga
       F   N   S   K   S   K   K   I   P   I   G   K   V   V   S   T   P   R   K   R

42781  ttaaaacaacacacaaacacaccaccgcattatgacaccagcgaagatgaggacgaagat
       L   K   Q   H   T   N   T   P   P   H   Y   D   T   S   E   D   E   D   E   D

42721  aattattacaactat taa
       N   Y   Y   N   Y   -
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Fig. 1. Nucleotide sequence and deduced amino acid sequence of *Bm47*. The initiate codes and the stop codes are framed in Grey Shading. The predicted motif ChaB is represented in the box.

M Y Q I P D M L Y N E K M P P R A K K L F V E T F T K Y H K M N G G D E D I A M H K A R K A L E E K Majority

        10   20   30   40   50

1   Autographa californica MNPV
1   Maruca vitrata MNPV
1   Bombyx mori NPV
1   Plutella xylostella MNPV
1   Rachiplusia ou MNPV
1   Anticarsia gemmatalis NPV

Y V K I D T L Q N S W I P R K A A Y E I V R D D I D E S D D N A D N S T K T K S N D R L I E N N N E Majority

        60   70   80   90   100

51 Autographa californica MNPV
51 Maruca vitrata MNPV
51 Bombyx mori NPV
51 Plutella xylostella MNPV
51 Rachiplusia ou MNPV
51 Anticarsia gemmatalis NPV

N T S T D Y D T E D D E R - V N M F S S R K R R - V A K R A F N A K S K K F P I G E V V S T - R K R Majority

        110   120   130   140   150

101 Autographa californica MNPV
99 Maruca vitrata MNPV
98 Bombyx mori NPV
101 Plutella xylostella MNPV
101 Rachiplusia ou MNPV
97 Anticarsia gemmatalis NPV

L K Q N T N T P P H Y D T S E D E D E D N Y Y N Y   Majority

        160   170

148 Autographa californica MNPV
140 Maruca vitrata MNPV
147 Bombyx mori NPV
148 Plutella xylostella MNPV
148 Rachiplusia ou MNPV
146 Y N N - - K S Q ... S S V ... S H S D E D D ...   Anticarsia gemmatalis NPV

Fig. 2. Multiple alignments of the amino acid sequences of Bm47 proteins from *Autographa californica* MNPV (ABF30960), *Maruca vitrata* MNPV(YP_950773), *Bombyx mori* NPV(NP_047463), *Plutella xylostella* NPV (YP_758526), *Rachiplusia ou* MNPV (NP_703049), *Anticarsia gemmatalis* NPV (YP_803453). Invariant residues are highlighted in black.

homologous to the putative products from 6 NPV ORFs. The BmNPV *orf47* shared an identity of 100% with AcM-NPV *orf58*. The homologs from the other 5 NPVs shared high identity with Bm47 (Fig. 2). By using PLOC, we found the subcellular location of Bm47 predicted in the nucleus ((Fig. 3). Using SMART secondary structure prediction, we got ChaB family of proteins contain a conserved 60 residue region (Fig. 4). This protein is known as ChaB in E. coli and is found next to ChaA which is a cat-

ion transporter protein. ChaB may be regulate ChaA function in some way (Shijuku et al. 2002).

Expression of the *Bm47* gene in *E.coli*

The open reading frame encoding Bm47 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 pET-30a(+) vector. The 516 bp PCR products were cloned into the pET-30a(+) vector.

The recombinant plasmid pET-Bm47 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-His monoclonal antibody further confirmed that the 15 kDa polypeptide was the His-Bm47 fusion protein (Fig. 5). The fusion protein was purified and used to immunize rabbits for producing the anti-Bm47 antiserum.

Fig. 3. Subcellular location of Bm47 predicted in the nucleus.

Name	Begin	End	E-value
Pfam:ChaB	5	65	1.00e-27
low complexity	73	85	-

Fig. 4. Predicted domains within Bm47 protein.

1   2   3   4   5   M

97.2KD

66.4KD

29.0KD

20.1KD

14.5KD
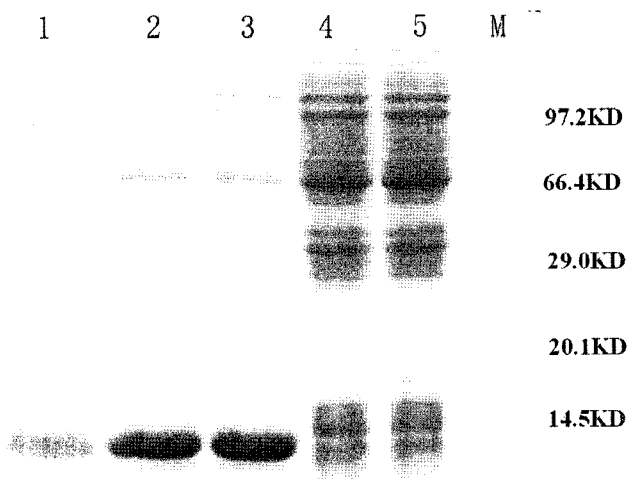
Fig. 5. The expression of pET-Bm47 in *E. coli* cells and Western blotting analysis. M: protein marker.5: BL21 cell extracts; 4: proteins of BL21 transformed with pET-30a(+); 3 and 2: the extracts of BL21 transformed with pET-Bm47; 1: Western blotting analysis using anti-His monoclonal antibody for BL21 transformed with pET-Bm47.

0 3 6 12 18 24 48 72 96

Bm47

action
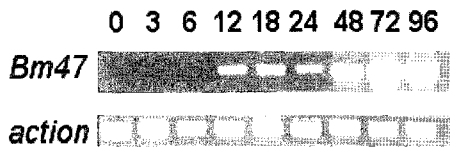
Fig. 6. Transcriptional analysis of *Bm47*. Total RNA was isolated from BmNPV-infected cells at 0, 3, 6, 12, 18, 24, 48, 72 h and 96 h p.i. RT-PCR was used to analyse *Bm47* transcription in each sample. A 516bp band was observed in 12,18, 24, 48, 72 and 96 h p.i. sample respectively.

Transcriptional analysis

Temporal regulation of *Bm47* transcript was examined by RT-PCR analysis using total RNA isolated from BmNPV infected host cells at designated time intervals (0, 3, 6, 12, 18, 24, 48,72 and 96 h p.i.). The result revealed a band with an expected size of 648 bp (Fig. 6). The *Bm47* transcript was detected at 18 h p.i. and remained detectable until 72 h p.i.

Time course of *Bm47* expression in BmNPV-infected cells

A time course of BmNPV infected BmN-4 cells were analyzed by western blot using the polyclonal antibody against Bm47. The result revealed that a specific immunoreactive band with approximately 26 kDa, which first detected at 18 h.p.i. and could be detected until 96 h.p.i. (Fig. 7). The 26 kDa immunoreactive band was not consistent with predicted molecular weight 20.1 kDa, suggesting that the post translational modification of the Bm47 protein occurred.

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 (Jehle et al. 2006). Based on the phylogenetic analysis, 63 genes were conserved among all sequenced lepidopteran NPVs (Herniou et al. 2001). In this study, we described some preliminary characteristic of *Bm47*, a gene that has thus far not been characterized. The search for homologues of *Bm47* revealed that homologues were present in 6 completely sequenced members of lepidopteran NPVs, suggesting *Bm47* and its homologues were specific genes for 7 completely sequenced lepidopteran NPVs and might play an important role in function related to these hosts. In order to study the expression of the Bm47 protein and its possible function, a polyclonal antibody was prepared by immunization of rabbits with a purified His-Bm47 fusion protein. Immunodetection of Bm47 protein in infected cells was performed using the polyclonal antibody against Bm47. A specific immunoreactive band of approximately 26 kDa was first observed at 18 h p.i. and remained detectable up to 96 h p.i. This was consistent with the transcriptional analysis of the *Bm47* 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 *Bm47* might be a late gene and probably used only one late transcription start motif, ATAAG, found 176nt

6   12   18   2-4   36   72   96

Fig. 7. Western blot analysis of *Bm47* gene product in BmN-4 cells. The cells were collected at 6, 12, 18, 24, 36, 72 and 96 h p.i. and 20 μg cell lyses at each interval was subjected to Western blot analysis using anti-His-Bm47 serum. The binding was developed with diaminobenzidine (DAB) as a chromogenic substrate.

upstream of the translation start codon ATG of *Bm47*. We observed that the size of the immuno-reactive protein was larger than the predicted molecular weight of 20.1 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 Bm47 protein. Computer analysis of Bm47 predicted the existence of two bipartite nuclear localization signals. The subcellular location revealed that Bm47 localized in the nucleus. Therefore, Bm47 protein possibly form a protein complex with one of proteins involved into transporting process and was interacted with proteins that were transported to the nucleus.

Although some basic characteristics were described in this paper, much information about this gene keeps unknown. The function of *Bm47* is being further investigated by knockout mutants of *Bm47*.

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