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# Rapid Expression of *Bm46* in *Bombyx mori* Cell Lines, Larvae and Pupae

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**In this study, ORF 46 of *Bombyx mori* nucleopolyhedrovirus (*Bm46*) fused with *EGFP* was expressed in *Bombyx mori* cell lines, larvae and pupae by BmNPV Bacmid system. *Bm46* and *EGFP* were cloned into donor plasmid pFastBacHTb, which was transformed to competent DH10B cells containing helper and BmNPV bacmid by site-specific transposition. Recombinant bacmid was used to transfected BmN-4 cells to produce the recombinant baculovirus vBm-Bm46-EGFP. Recombination virus was injected into silkworm larvae and pupae. The expression of the fusion protein was monitored by examining green fluorescence using a fluorescent microscope. Intense fluorescence in cells and silkworm was observed at 4 days post-infection, indicating the *Bm46-EGFP* fusion gene was expressed successfully.**

**Key words:** *Bm46,* expression, Baculovirus, BmNPV bacmid, *Bombyx mori* larvae, Pupae.

## Introduction

*Bombyx mori* has long been reared as a beneficial insect in the sericulture industry. A major disease problem next to pebrine in mass rearing of this insect is infection by the baculovirus *B. mori* Nucleopolyhedrovirus (BmNPV) (Khurad *et al.*, 2004). However, baculoviruses have been exploited as efficient vectors for expression of functionally active proteins in insect cells. The insect cell-baculovirus expression (BES) system has become a popular method of recombinant protein production because of its overproduction of functional heterologous proteins, which typically follow a strong polyhedron (*polh*) promoter and are the products of posttranslational modification, a necessity for many eukaryotic proteins (Luckow *et al.*, 1991). Recently, the BmNPV bacmid system have been developed, which can directly infect silkworm *B. mori* cell lines (Motohashi *et al.*, 2005). This makes the large scale production of eukaryotic proteins in silkworms possible.

The levels of protein expression using the silkworm or its pupa is 10-100-fold higher than that using *B. mori* cells, indicating that the silkworm or its pupa is an optimal system for the mass production of recombinant proteins. From this point, BmNPV BES using *B. mori* silkworm larvae or pupae is the most suitable combination for the large-scale production of eukaryotic proteins (Enoch *et al.*, 2007).

BmNPV ORF46 (*Bm46*) is located at 42221-42706 nt in the genome of BmNPV T3 strain (Gomi *et al.*, 1999). It contains 486 nts and is predicted to encode a putative 161 amino acid peptide with a deduced molecular weight of 19.1 kDa. Since function of *Bm46* gene is still unknown, supplies of gene product, proteins, for investigating its function is in great demand. In this paper, a recombinant virus expressing *Bm46* under the control of the polyhedron (*polh*) promoter was constructed. The recombinant virus was injected the silkworm larvae and pupae to produce the fusion protein Bm46-EGFP. The co-expression of EGFP allowed identification of the recombinant baculoviruses in insect cells. Moreover, complicated and time-consuming assays including Western blotting and ELISA can be bypassed.

## Materials and methods

### Cells and virus

DH10Bac containing BmNPV bacmid was kindly provided by Professor Park of Shizuoka University (Japan). Plasmid pEGFP-N1 and TG1 strain were stored in our lab.

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The vector pFasBacHTb, BmN-4 cell were kindly provided by Chuanxi Zhang of Zhejiang University (China). BmNPV (T3 strain) virus whose genome has been entirely sequenced was propagated in BmN (BmN-4) cells, maintained at 27°C in TC-100 insect medium supplemented with 10% (v/v) fetal bovine serum (Gibco-BRL). The titration of virus and other routine manipulations were performed according to the standard protocols (O'Reilly *et al.*, 1992).

### Construction of recombination donor plasmid pFas BacHTb-Bm46-EGFP

The entire *Bm46* was amplified from the BmNPV T3 genome with the primers: Bm46F (*Bam*HI site): 5'-CGGATCCATGAAACTTCCCGTT-3' and Bm46R (*Xho*I site) : CCTCGAGCTTGTACAGTTTTAT-3'. The PCR product was first cloned into pGEM-T-Easy vector (Promega) (pT-Bm46), and then cloned into the vector pFasBacHTb with *Bam*HI and *Xho*I. The *EGFP* coding region was amplified from the pEGFP-N1 plasmid with the primers: up (*Xho*I site): 5'-ACTCGAGATGGTGAGCAAGGGCG-3' and down (*Hin*dIII site): 5'-GAAGCTTTTACTTGTACAGCTCG-3'. To get the fusion protein Bm46-EGFP, *EGFP* gene was cloned into the recombinant vector pFasBacHTb-Bm46 with *Xho*I and *Hin*dIII The resultant plasmid was designated pFasBacHTb-Bm46-EGFP.

### Recombinant virus production in BmN-4 cell

Transposition was carried out by transforming the donor plasmid pFasBacHTb-Bm46-EGFP into *E. coli* BmDH-10Bac cells harboring a transposition helper plasmid. Colonies containing recombinant bacmids were identified by disruption of the lacZα gene. High molecular weight mini-prep DNA was prepared from selected *E.coli* clones containing the recombinant bacmid, and this DNA was then used to transfect insect cells.

The approximately 100 ng of recombinant bacmid was transfected into BmN-4 cells using Cellfectin (Gibco-BRL). The cells were observed using a fluorescence microscope (Leica) to confirm the expression of the fusion protein. After 4 days, the cultured medium was harvested and centrifuged at 2000 × *g* for 5 min, and then the supernatant was collected. The resulting recombinant virus vBm-Bm46-EGFP was stored at 4°C.

### Expression of Bm46-EGFP in *B. mori* silkworm larvae and pupae

Fifth instars larvae and pupae were used for the expression of fusion protein. Bm46-EGFP fusion protein expression in silkworm was carried out using direct injection of recombinant virus into the dorsal of the larvae and pupae with a 10 microlitres syringe. The expression of the fusion protein in silkworms was monitored by examining EGFP fluorescence using a fluorescent microscope (Olympus).

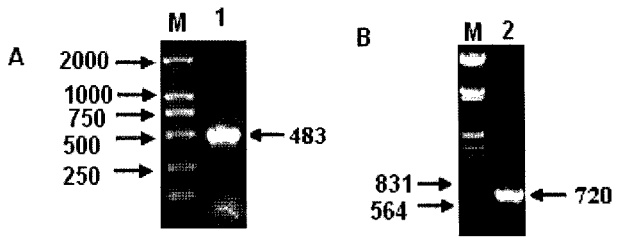


**Fig. 1.** PCR production of *Bm46* and *EGFP* genes. (A) M: DNA marker 1: 483 bp PCR production of *Bm46* gene using primers F and R. (B) M: DNA marker 2: 720 bp PCR production of *EGFP* gene using up and down primers.

## Results

### Generation of recombinant bacmid

Using PCR primers, 483-bp region from the BmNPV genome and 720-bp fragment from pEGFP-N1 plasmid were amplified (Fig. 1) and cloned into donor plasmid pFasBacHTb, which has the mini-Tn7 containing an expression cassette consisting of a Gen gene, a strong polh promoter, a 6×His-tag, a multiple cloning site, and an SV40 poly (A) signal inserted between the left and right arms of Tn7.

Recombinant bacmids are constructed by transposing a mini-Tn7 element from a pFasBacHTb donor plasmid to the mini-attTn7 attachment site on the bacmid when the Tn7 transposition functions are provided by a helper plasmid (Fig. 2). If transposition has occurred, the PCR product produced by these primers was 2,430 bp plus the size of *Bm46-EGFP* gene (Fig. 3).

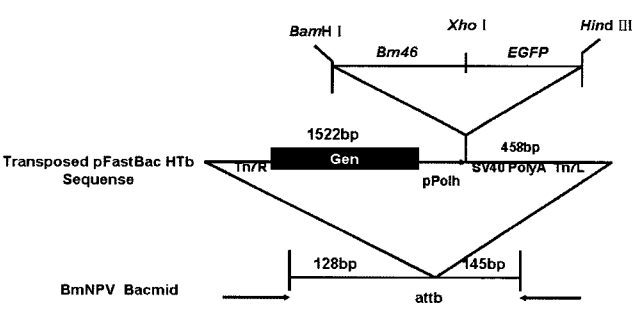


**Fig. 2.** Construction of recombinant baculovirus. The gene of *Bm46-EGFP* was cloned into a pFasBacHTb donor plasmid, and the recombinant plasmid was transformed into DH10Bac competent cells which contain the bacmid with a mini- attTn7 target site and the helper plasmid. The mini-Tn7 element on the pFasBacHTb donor plasmid can transpose to the mini-attTn7 target site on the bacmid in the presence of transposition proteins provided by the helper plasmid.

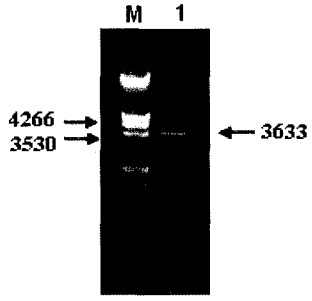


**Fig. 3.** PCR product of recombinant baculovirus. M: DNA maker. 1: PCR product of recombinant bacmid using M13 primers.

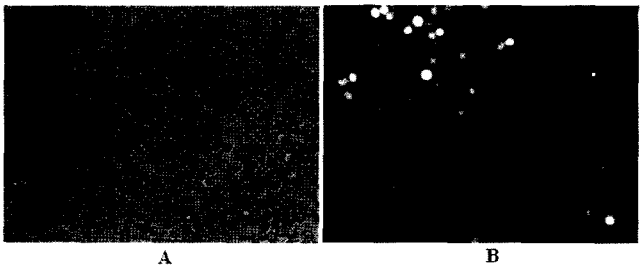


**Fig. 3.** Images of recombinant virus-infected BmN-4 cells at 4 days post-infection. (A) Phase contrast images; (B) fluorescence images. All images were magnified at 200×.

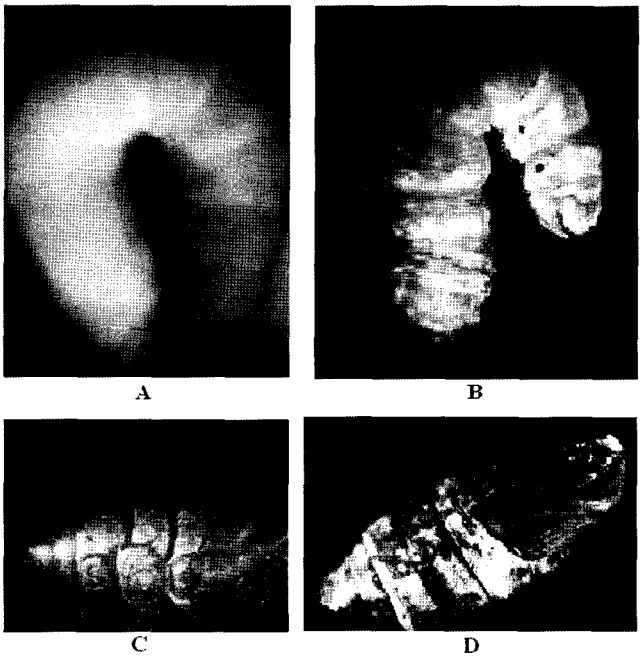


**Fig. 5.** Silkworm larvae and pupae infected with recombination virus at 4 days post-infection. The silkworm larvae and pupae were injected recombinant baculovirus vBm-Bm46-EGFP using syringe. At 4 days post-infection, the infected larvae, pupae (B and D) and mock-infected (A and C) silkworm were viewed under a fluorescence microscope. All images were magnified at 10×.

### Expression of Bm46-EGFP in silkworm larvae and pupae

Recombinant bacmid was transfected into BmN-4 cells to produce the recombinant baculovirus. Infection of *B. mori* derived BmN-4 cells with the recombinant virus resulted in the expression of *Bm46-EGFP* gene from 2 days post infection (d p.i.), with maximal accumulation of the expressed protein at 4 d p.i. (Fig. 4). *B. mori* larvae and pupae that injected recombination virus appeared greenish at 2 days post infection and the fluorescence intensity further increased at 4 d p.i. (Fig. 5).

## Discussion

The silkworm, *Bombyx mori*, is a good experimental insect because it can be easily mass-cultured in a laboratory by using advanced artificial diets. It is now considered to be one of the most thoroughly studied lepidopteran insects in the field of physiology, biochemistry and genetics (Kenichi *et al.*, 2003). The silkworm has also been used to study NPVs because viral infection often causes large losses in silk production. In addition, recent advances in baculovirus will further facilitate the elucidation of molecular mechanisms underlying the infection process of BmNPV.

The conventional preparation of a recombinant baculovirus that express exogenous genes requires a lot of time and effort, because multiple rounds of purification and amplification of viruses are necessary. However, using BmNPV bacmid expression system greatly reduces the time it takes to identify and purify a recombinant virus from 4 to 6 weeks (typical for conventional methods) to within 7 to 10 days (Luckow *et al.*, 1993). In the present study, we reported the production of BmNPV *ORF46* using BmNPV bacmid system in silkworm larvae and pupae for the first time. The expression of Bm46 fused with EGFP was rapidly detected using a fluorescence microscope without employing complicated assay methods (Rucker *et al.*, 2001). Perhaps the greatest advantage of BmNPV bacmid system is that it permits the rapid and simultaneous isolation of multiple recombinant viruses, and is particularly suited for the expression of Bm46-EGFP for structure/function studies.

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