Construction of hybrid *Autographa californica* nuclear polyhedrosis bacmid by modification of *p143* helicase

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Abstract

We developed a new hybrid nuclear polyhedrosis virus (NPV) bacmid capable of infecting *Spodoptera frugiperda*, *Tricoplusia ni*, and *Bombyx mori*, and *B. mori* cell lines for producing hybrid recombinant baculovirus that can carry a gene of interest and express it in a broad range of hosts. A GFP_{uv}-β1,3-*N*-acetylglucosaminyltransferase 2 fusion gene was expressed successfully in silkworm larvae using this hybrid bacmid. The hybrid NPV bacmid provides an altogether simple and realistically feasible method for large-scale applications using silkworm larvae. It can be easily managed in *E. coli*, which has no biohazard safety concerns, in addition to the baculovirus-based expression system.

Keywords: Hybrid bacmid; insect cell; *Bombyx mori*; silkworm; β1,3-*N*-acetylglucosaminyltransferase

1. Introduction

Baculovirus expression systems (BESs) are widely used to express heterologous genes in cultured insect cells and insect larvae hosts; gene expression is driven by the strong polyhedrin promoter. These systems also have great advantages for the expression of eukaryotic proteins because insect cells can add post-translational modifications to proteins so that they are similar to their native forms and can be handled easily (Croizier et al., 1994; Luckow et al, 1991 and 1993; O'Relly), which is not the case for bacterial expression systems.

The expression of foreign genes using conventional preparations of recombinant baculoviruses requires the cotransfection of plasmid transfer vectors and circular wild-type genomic viral DNA into insect cells. This approach needs at least 40 days, as it requires multiple plaque assays, purification from nonrecombinant parental virus and amplification of the recombinant baculovirus. A more rapid and efficient method to generate recombinant baculoviruses was developed using the site-specific transposition of an expression cassette into a baculovirus shuttle vector (AcNPV bacmid) that can be propagated in *E. coli* (Luckow et al, 1993). This bacmid contains a mini-F replicon, a kanamycin resistance marker and a segment of DNA carrying the *lacZ*α gene with an attachment site for the bacterial transposon Tn7 at its N terminus. Recombinant bacmid DNA can be rapidly isolated from small-scale cultures and then used to transfect insect cells. Viral stocks harvested from the transfected cells can be used to infect insect cells for subsequent protein expression. The time required for the introduction of a foreign gene and isolation of the recombinant baculovirus was reduced significantly from 40

days to 7-10 days by the use of this bacmid. However, this AcNPV bacmid can only be used in cell lines from *Spodoptera frugiperda* and *Tricoplusia ni*, but neither in silkworms nor their cell lines. Recently, Motohashi et al. (2005) have developed the BmNPV bacmid system, which can directly infect silkworms and *B. mori* cell lines. This makes the large-scale production of eukaryotic proteins in silkworms possible; the protein expression level using silkworms is 10- to 100-fold higher than that using insect cells.

The AcNPV bacmid does not replicate in *B. mori* cell lines and the BmNPV bacmid does not replicate in *S. frugiperda* or *T. ni* cell lines. Although AcNPV and BmNPV are closely related to each other, having collinear genome organizations, their host ranges are different. Much research (Arguad et al., 1998; Kamita and Maeda, 1997; Kondo and Maeda, 1991; Maeda et al., 1993; Mori et al., 1992; Muneta et al., 2003) has expanded the host range of AcNPV vectors by producing hybrid-AcNPV (Hy-AcNPV). The *p143* gene is the host-range genetic determinant for AcNPV infectivity in *B. mori* cell lines (Croizier et al., 1994; Maeda et al., 1993). This gene is essential to AcNPV DNA replication and has been suggested to encode a helicase (Lu and Carstens, 1991). Maeda et al. (1993) isolated a hybrid baculovirus of BmNPV and AcNPV that is capable of replicating in both *S. frugiperda* and *T. ni* cell lines. Their nucleotide sequencing analyses revealed that a 572-bp *Sac* I-*Hin*d III fragment, BmScH, is responsible for the expanded host range of AcNPV and that it is localized within BmNPV *dnahel* (Kamita and Maeda, 1997). Two adjacent nucleotides (A and T) appeared to be the minimal essential sequence necessary to expand the host range of AcNPV, encoded by a single

amino acid difference between BmNPV (Asp) and AcNPV (Ser). Muneta et al. (2003) applied this hybrid AcNPV to the production of interleukin-18 in silkworms and obtained 20-fold more interleukin-18 than when using Sf 9 cells. However, although this hybrid baculovirus is able to expand the host range, the method still involved the isolation, purification, and amplification of the recombinant virus, which is tedious and time consuming. This inability of the recombinant virus to infect different host cells requires the same tedious preparations, which causes inexplicable loss for respective hosts each time. Therefore, a bacmid with an expanded host range is desirable for the improvement of qualitative and quantitative efficiency of protein expression.

In this paper, we report a new hybrid nuclear polyhedrosis virus (hybrid NPV) bacmid that is infectious for *S. frugiperda* and *T. ni* cell lines as well as silkworms. As this method utilizes the advantages of a bacmid it can be easily prepared and screened in *E. coli* to produce sufficient DNA for recombinant virus preparation. This method allows recombinant virus production in *S. frugiperda* and *T. ni* cell lines, and large-scale protein expression in silkworms.

2. Materials and Methods

2.1. Cell lines, bacterial cells, plasmids, bacmid and media

The Sf 9 cell line and AcNPV bacmid were purchased from Invitrogen (Carlsbad, CA, USA). The Bm5 cell line was kindly provided by Professor K. S. Boo of the Insect Pathology Laboratory of Seoul National University. *E. coli* DH5α, *E. coli* TOP10, *E.*

coli DH10Bac and Electro Max DH10B were obtained from Invitrogen. Wild-type viral BmNPV DNA was obtained from Funakoshi Co., (Tokyo, Japan), and the transfer vector for the AcNPV bacmid, pUC18, was kindly provided by National Institute of Genetics (Mishima, Japan). Sf 900 II serum-free medium (Invitrogen) supplemented with 1% antibiotic-antimycotic (Invitrogen) and 1% FBS, and TNM-FH medium (Invitrogen) containing 10% FBS were used for Sf 9 and Bm5 cell cultures. Insect cells were grown in 25 cm^2 tissue culture flasks (Falcon). A plasmid vector harboring a GFP_{uv}-β1,3-*N*-acetylglucosaminyltransferase 2 fusion gene fused with the melittin signal sequence (me-β-3GnT2) (Kato et al., 2003 and 2004) was used for evaluation of hybrid bacmid in silkworm larvae. Supplements, if necessary, were added into medium at the following concentrations: ampicillin, 100 μg/ml; kanamycin, 50 μg/ml; 5-bromo-4-chloro-3-indolyl-β-galactopyranoside (X-Gal), 100 μg/ml; gentamycin, 7 μg/ml; tetracycline, 10 μg/ml and isopropyl-β-D-thiogalactopyranoside (IPTG), 40 μg/ml.

2.2. Construction of plasmid carrying DNA helicase to be used in homologous recombination

Helicase cDNA was extracted from wild-type BmNPV DNA by PCR using the oligonucleotide primers BmDhel Sac I-f and BmDhel Hind III-r (Table 1). A 572-bp long helicase fragment was isolated from a 1.5% agarose gel and purified using GFX PCR DNA and gel band Purification kit (Amersham Biosci. Corp., Piscataway, NJ,

USA). The purified 572-bp band was digested by *Sac* I/*Hin*d III restriction enzymes and the resulting fragment, BmScH, was purified again. The purified insert was ligated into pUC18 at the *Sac* I/*Hin*d III sites and transformed into DH5α competent cells (Takara Bio, Otsu, Japan). The pUC18/BmScH transformants were screened on LB plates containing ampicillin, IPTG, and X-Gal in a 37^{o}C incubator. The sequences of all the PCR fragments inserted into pUC18 were confirmed by dideoxynucleotide chain terminating sequence (Sanger et al., 1977) using Thermo Sequenase Cycle Sequencing kit (USB Co., Cleveland, Ohio, USA).

2.3. Construction of hybrid NPV bacmid and confirmation of bacmid DNA

Two hundred nanograms of AcNPV bacmid and 3 μg of pUC18/BmScH in 100 μl of Sf 900 II medium without antibiotics were mixed with 6 μl of Cellfectin in 100 μl of Sf 900 II medium without antibiotics. This mixture was incubated at room temperature for 45 min under sterile conditions. The mixed solution was poured onto a monolayer of Sf 9 cells (1x10^{6} cells/ml) in 6 well plates with a 9.6 cm^{2} surface area (Falcon, NJ, USA) and was incubated for 5 hr at 27^{o}C for transfection. After transfection, the mixture solution was removed and 2.5 ml of Sf 900 II medium with 1% antimycotic antibiotic was added. After 3 days incubation at 27^{o}C, the supernatant containing hybrid NPV was removed and used for further infection. The infection with the hybrid NPV was repeated three times as mentioned above. To investigate the infectivity of hybrid NPV for Bm5 cells, 500 μl of the virus solution was poured onto the monolayer of

$1x10^6$ cells/ml of Bm5 cells in Sf 900 II medium. After a 2-hr incubation at 27°C, the medium was replaced with fresh TNM-FH medium containing 10% FBS, and the cells were incubated for 4 days. The virus was amplified three times as mentioned above.

The above-described virus samples were centrifuged at 20,630xg for 1 hr at 4°C to precipitate virus particles. The supernatant was removed and the pellet was dissolved in 50 μl of TE buffer containing 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA. Using proteinase K treatment at 60°C for 15 min, the viral envelope was removed. One hundred microliters of TE buffer was added and viral DNA was extracted by phenol: chloroform purification. The DNA was precipitated using ethanol and purified by centrifugation. The dried DNA was dissolved in 50 μl of TE buffer. The isolation of hybrid NPV bacmid DNA was confirmed by PCR to check the 572-bp BmScH fragment of the *p143* gene of the AcNPV genome using primers (Table 1). The PCR products were further confirmed by *Nsp*V restriction analysis.

2.4. Construction of hybrid NPV bacmid expressing GFP_{uv}-β3GnT2 fusion protein

Hybrid NPV bacmid DNA was transformed into DH10Bac competent cells containing pHelper, which encodes a transposase and confers resistance to tetracycline. The new DH10Bac/hybrid NPV bacmid/pHelper cells were screened on LB agar plates containing kanamycin and tetracycline. The selected colonies were cultured to prepare fresh competent cells for Bac-to-Bac system.

Fifty micrograms of pDEST8-me-GFP_{uv}-β3GnT2 DNA was transformed into 100

μl of DH10Bac/hybrid NPV bacmid/pHelper competent cells. The transformed cells were added to 500 μl of SOC and incubated at 37°C for 1 hr, and then were cultured on LB agar plates containing gentamycin, kanamycin, tetracycline, IPTG, and X-Gal at 37°C overnight. White colonies were inoculated into LB medium containing gentamycin, kanamycin, and tetracycline and cultured at 37°C and agitated at 150 rpm overnight. Hybrid NPV bacmid/me-GFP_{uv}-β3GnT2 was isolated and confirmed by PCR using me-β3GnT2-Forward and M13-Reverse primers (Table 1).

2.5. Expression of GFP_{uv}-β3GnT2 fusion protein in Sf 9, Bm5, and silkworm larvae

Approximately 200 ng of hybrid NPV bacmid/me-GFP_{uv}-β3GnT2 DNA was transfected into Sf9 and Bm5 cells. At 3-4 days postinfection, the culture was harvested and centrifuged at 2000 x g for 5 min. Precipitated cells were diluted with fresh medium and were observed using a fluorescence microscope to confirm the expression of the fusion protein.

Fifth instars larvae (Ehime Sansyu Co. Ltd., Ehime, Japan) were used for the expression of fusion protein. Twenty microliters of hybrid NPV/me-GFP_{uv}-β3GnT2 ($1x10^7$ pfu/ml) was injected into the dorsal region of the larvae using a syringe with 26-gauge beveled needle. The expression of the fusion protein in silkworms was monitored by examining GFP_{uv} fluorescence using a fluorescent microscope or a UV transilluminator table.

The fusion protein contained in hemolymph was subjected to SDS-PAGE on a

12 % polyacrylamide gel using the Mini-protean II system (Bio-Rad, Hercules, CA). The respective bands were detected using a Molecular-FX multi-imager (Bio-Rad).

3. Results

3.1. Construction of hybrid NPV bacmid

First, we prepared a transfer vector necessary for the construction of the hybrid NPV bacmid. Using PCR primers, 572-bp region from the BmNPV DNA helicase gene was amplified and inserted into pUC18 at the *Sac* I/*Hin*d III restriction enzyme sites to give the plasmid pUC18/BmScH encoding an ampicillin resistance marker and the *lacZ*α peptide (Fig. 1). White colonies containing pUC18/BmScH were picked up and inoculated into 3 ml of LB medium. Plasmid DNA was isolated and confirmed by *Sac* I/*Hin*d III digestion on agarose (data not shown). The BmScH cDNA (Fig. 2) was transferred from pUC18/BmScH into the *p143* cDNA of the AcNPV bacmid by homologous recombination (as described in the Materials and Methods section). BmScH isolated from the hybrid NPV bacmid was confirmed by PCR using primers (Table 1). The hybrid NPV bacmid PCR product was further analyzed by the restriction enzyme *Nsp* V that digests at TT/CGAA nucleotide sequences (Fig. 2), giving two bands of 500 bp and 100 bp. PCR products of hybrid NPV and BmNPV showed the two expected bands of 500 bp and 100 bp, but that of AcNPV was not digested by the *Nsp V* restriction enzyme, giving one band (Fig. 3).

3.2. Application of hybrid NPV bacmid in Bac-to-Bac system

The prepared hybrid NPV bacmid DNA was successfully transformed into DH10Bac competent cells containing pHelper, encoding for transposase, to create DH10B/hybrid NPV bacmid/pHelper transformant cells. pDEST-8/GFP_{uv}-β3GnT2, as a transfer vector, was transformed into DH10B/hybrid NPV bacmid/pHelper competent cells to create a recombinant hybrid NPV-me-GFP_{uv}-β3GnT2 bacmid. A successful transfer of cDNA from the destination vector to the hybrid NPV bacmid took place, which proves that this system works successfully. Using the hybrid NPV-me-GFP_{uv}-β3GnT2 bacmid, cotransfection was performed and recombinant virus was prepared, which can infect both *B. mori* Bm5 cells as well as Sf 9 cells efficiently. The infected Bm5 and Sf 9 cells were bigger in size compared with mock-transfected cells owing to the occluded virions that are embedded within polyhedral inclusion bodies (data not shown). This indicates that the hybrid NPV can be infectious to Sf 9 and Bm5 cells.

To validate the application of hybrid NPV in the Bac-to Bac system, Bm5 cells infected with the hybrid NPV-me-GFP_{uv}-β3GnT2 bacmid virus were observed under a fluorescence microscope and they showed a distinct GFP_{uv} fluorescence (Fig. 4B). The same cells were also viewed under a normal light microscope and showed the expected infected cell structure because of the occluded virions (Fig. 4A). This result indicates that this hybrid bacmid can be applied to the Bac-to-Bac system.

3.3. Expression of fusion protein in silkworm larvae using hybrid bacmid

The efficiency of the hybrid bacmid was further verified by infecting silkworm larvae with the hybrid NPV-me-GFP_{uv}-β3GnT2 bacmid virus. The hybrid NPV-me-GFP_{uv}-β3GnT2 bacmid virus was injected into silkworms using a syringe. The larvae appeared greenish at 48 hr postinfection and the fluorescence intensity further increased with the optimum result at 96 hr postinfection (II in Fig. 5A). At 96 hr postinfection, the larvae were dissected, and hemolymph fluid was collected to check the expression of the fusion protein by SDS-PAGE (12%) (lane 1 in Fig. 5B). A fluorescent band of approximately 75 kDa corresponding to the expected size of the GFP_{uv} fusion protein was observed for GFP_{uv}-β3GnT2.

4. Discussion

We have developed a new hybrid bacmid capable of infecting a broad range of host insect cells. A highly homologous region (BmScH) of 572-bp exists within both the helicase gene of BmNPV and p143 of AcNPV. BmScH was inserted into the AcNPV bacmid using a pUC18-based transfer vector. A unique *Nsp* V restriction enzyme site exists in the 572-bp region of the BmScH fragment of bacmid-BmNPV; thus, homologous recombination between pUC18/BmScH and AcNPV bacmid was easily selected and confirmed (Fig. 2). The hybrid bacmid was infectious to both *S. frugiperda* and *T. ni* cell lines. GFP_{uv} fusion protein was expressed successfully in silkworm larvae using this hybrid bacmid system. The use of the GFP_{uv} fusion protein assisted us to

easily monitor the progress of infection.

We could easily propagate the hybrid bacmid in *E. coli* like any other plasmid and easily screen for the bacmid carrying inserts with a very high efficiency and precision. The new hybrid bacmid was utilized in the Bac-to-Bac expression system. Using the Gateway system, transfer vectors can be prepared with ease and the foreign gene of interest can be transferred to the hybrid bacmid by site-specific homologous recombination.

The hybrid NPV bacmid is altogether simple and realistically feasible for use in large-scale applications. It can be easily managed in *E. coli*, which has no biohazard safety concerns, in addition to the baculovirus-based expression system. The biotechnology industry requires the use of expensive human proteins of medicinal value and also those for other research studies. In this study, we concur with other leading research groups around the world, which are focused on meeting the growing demand in the biotechnology field on this idea.

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Legends for Figures

Fig. 1. BmNPV genome map with restriction sites showing DNA helicase gene ORF78. A 572-bp fragment (BmScH) was isolated from BmNPV genome using PCR with primers flanked by *Sac* I/*Hind* III restriction sites and ligated into pUC18 at the *Sac* I/*Hind* III restriction sites.

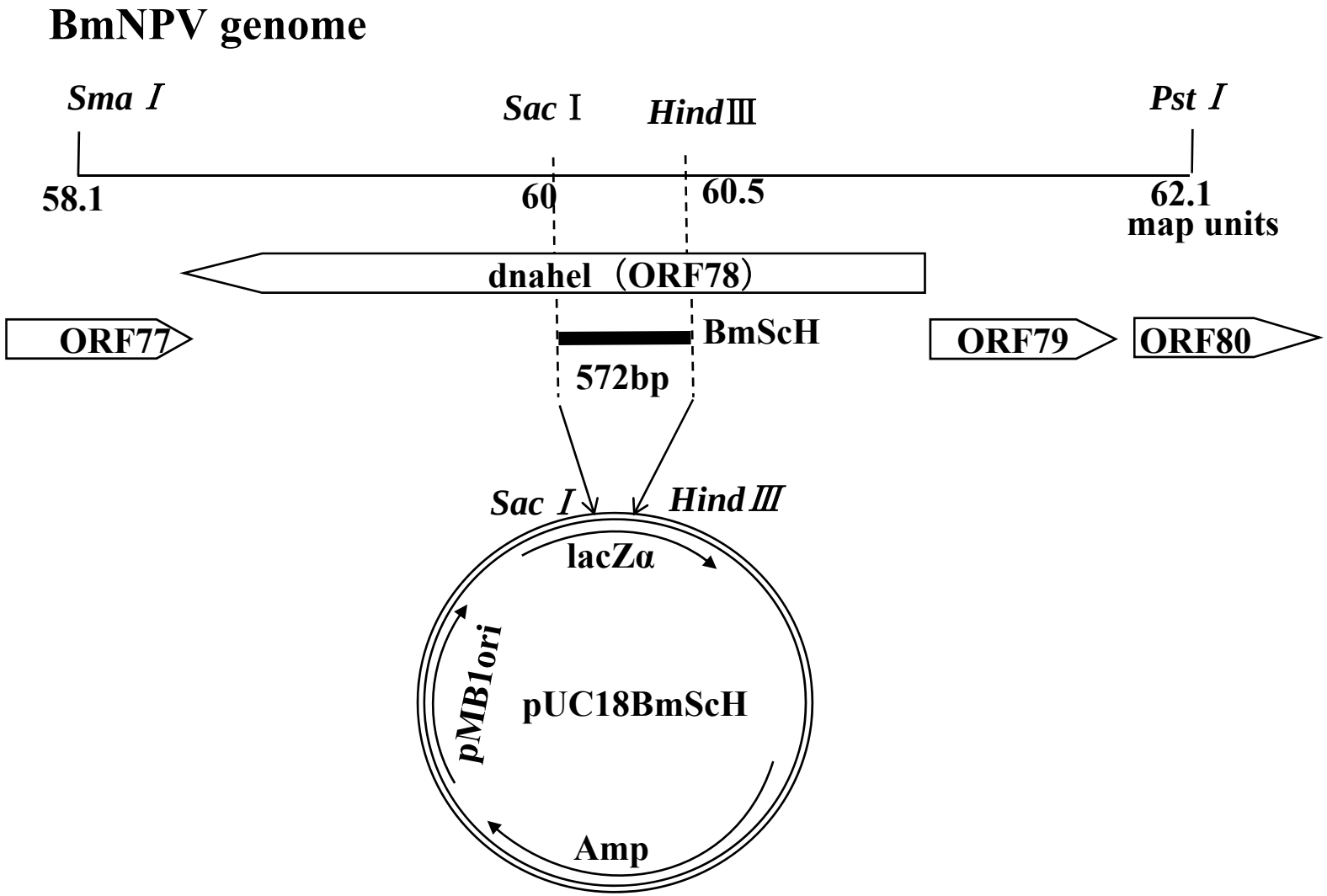
Fig. 2. Highly homologous area in 572-bp BmScH fragment of DNA helicase gene of BmNPV and p143 gene of AcNPV. Asn and Ser denote asparagine and serine, respectively. The *Nsp* V restriction enzyme site is shown in the BmScH fragment.

Fig. 3. 1.5% agarose gel showing PCR products. Lane M denotes λ-Hind III marker. Lanes 1, 3, and 5 denote PCR products from wild-type BmNPV, wild-type hybrid NPV, and wild-type AcNPV, respectively. Lanes 2, 4, and 6 denote *Nsp* V digested PCR products from wild-type BmNPV, wild-type hybrid NPV, and wild-type AcNPV, respectively.

Fig. 4. Bm5 cells at 3 days postinfection with hybrid NPV/me-GFP_{uv}-β3GnT2. A and B were obtained using a light microscope and a fluorescence microscope, respectively.

Fig. 5. Silkworm larvae infected with hybrid NPV/me-GFP_{uv}-β3GnT2 at 3 days postinfection. A. Mock-infected silkworm larvae (I) and hybrid

NPV/me-GFP_{uv}-β3GnT2 infected silkworm (II). B. Fluorescent image of SDS-PAGE (12%) of fluorescent GFP_{uv} fusion protein. M denotes GFP fluorescent marker. Lane 1 denotes GFP_{uv} fusion protein band of 75 kDa from silkworm hemolymph.


BmNPV genome
Sma I
Sac I
HindIII
Pst I
58.1
60
60.5
62.1
map units
dnahel（ORF78）
ORF77
BmScH
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ORF79
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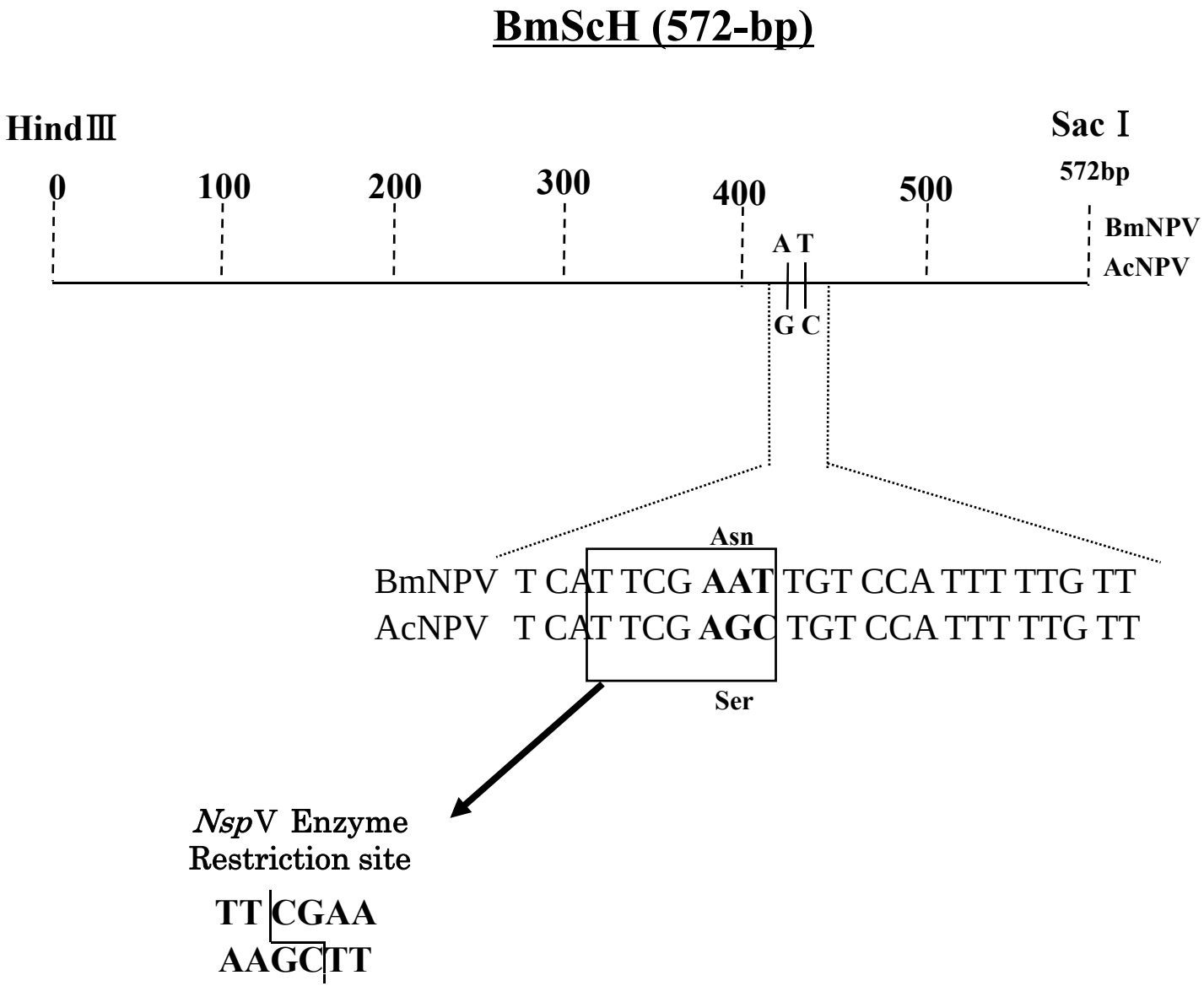
BmScH (572-bp)
HindⅢ
Sac Ⅰ
572bp
0
100
200
300
400
500
BmNPV
AcNPV
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G C
Asn
BmNPV T CAT TCG AAT TGT CCA TTT TTG TT
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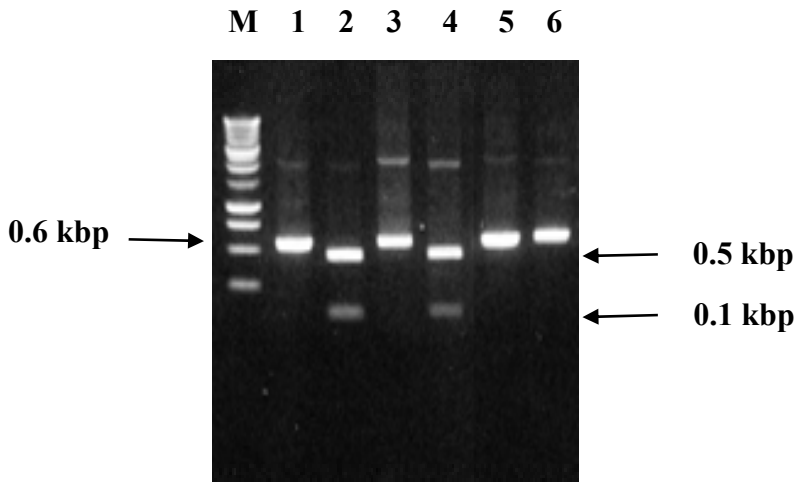
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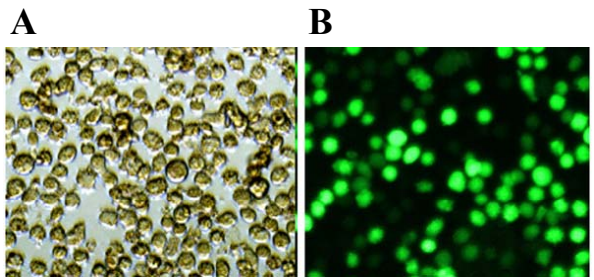
A
B

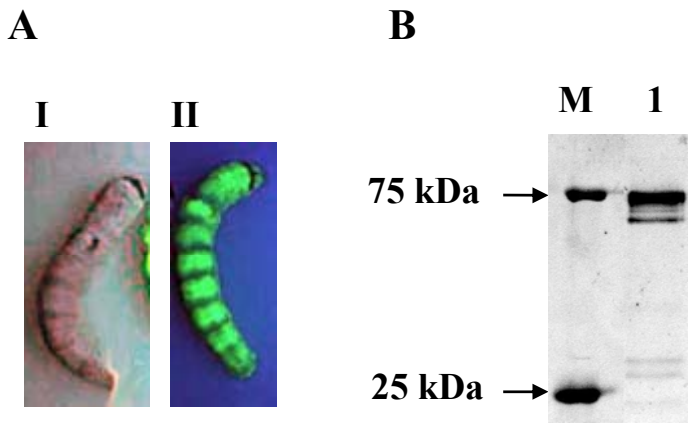
A
I
II
B
M
1
75 kDa
25 kDa

Table 1

Primers used to prepare BmScH fragment for hybrid NPV bacmid and to identify the GFP_{uv}-β3GnT2 cDNA using PCR.

Primer-name	Sequence (5'→3')
BmDhel *Sac* 1-f	ATGCTGAGCTCGTCGGTGCTAGTAGATA
BmDhel *Hin*d III-r	GAATGAAGCTTTGAAACGATGTGACCT
me-β3GnT2-Forward	GGGGTACCATGAAATTCTTAGTCAACGTTGCCCTTGTTTTTATGGTCGTATACATTTCTTACATCTATGCCGGACGCGGGGTTC TCATCATCA C
M13-Reverse	CAGGAAACAGCTATGACC