Construction of a cysteine protease deficient *Bombyx mori* multiple nucleopolyhedrovirus bacmid and its application to improve expression of a fusion protein

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Abbreviations: Bacmid, baculovirus shuttle vector; BmMNPV, *Bombyx mori* multiple nucleopolyhedrovirus; bx, signal sequence from *Bombyx mori* bombyxin; CPD-BmMNPV, cysteine protease-deleted BmMNPV; GFP_{uv}, green fluorescent protein when excited with long-wave UV light; GGT2, GFP_{uv}-β3GnT2 fusion protein; PCR, polymerase chain reaction; v-cath, papain-type cysteine protease with cathepsin L-like characteristics; β3GnT2, β1,3-*N*-acetylglucosaminyltransferase 2

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Abstract

The bacmid system of BmMNPV with cysteine protease gene deletion (CPD-BmMNPV bacmid) was constructed using the lambda recombination system. The protease activities of *Bombyx mori* cells and silkworm larvae infected with this CPD-BmMNPV bacmid were reduced by 94% and 85%, respectively. By using this system, a GFP_{uv}-β1,3-*N*-acetylglucosaminyltransferase 2 (GFP_{uv}-β3GnT2) fusion protein was successfully expressed in silkworm larvae with less protein degradation and without larvae liquefaction; β3GnT activity improved 30%. This CPD-BmMNPV bacmid system provides rapid protein production in silkworms and can be used for the production of recombinant eukaryotic proteins without proteolytic degradation.

Keywords: Bacmid; *Bombyx mori* multiple nucleopolyhedrovirus; Cysteine protease; Expression; Silkworm

1. Introduction

The baculovirus expression system is a useful tool for the efficient production of eukaryotic proteins that require correct folding and posttranslational modification such as signal peptide processing (Davis et al., 1992), phosphorylation (Hericourt et al., 2000) and glycosylation (James et al., 1995). However, *Autographa californica* multiple nucleopolyhedrovirus has a papain-type cysteine protease with cathepsin L-like characteristics (v-cath) (Slack et al., 1995), which plays an important role in the degradation of host tissues during virus infection. Slack et al. (1995) reported that v-cath has 35.5 kDa and 32 kDa precursor forms that are processed to a 27.5 kDa mature form.

When insect cells are infected with baculoviruses, v-cath accumulates as a propeptide, and the death of infected cells provokes v-cath activation (Hom et al., 2002). This v-cath protease causes the degradation of expressed recombinant proteins, which hinders their efficient production in baculovirus expression systems (Yamada et al., 1990; Pyle et al., 1995; Pham et al., 1999). Kato et al. (2004) expressed a human glycoprotein, β-1,3-*N*-acetylglucosaminyltransferase-2 (β3GnT2) fused with GFP_{uv} in Sf9 and Tn-5B1-4 cells using a baculovirus expression system, but β3GnT2 activity decreased markedly at 3 days postinjection owing to the presence of v-cath protease. This suggests that the degradation of recombinant proteins due to v-cath protease is a critical drawback in the expression of glycoproteins using a baculovirus expression

system.

To prevent the proteolytic degradation of expressed proteins, Monsma and Scott (1997) generated BacVector-3000, in which the v-cath protease gene was deleted from *Autographa californica* multiple nucleopolyhedrovirus, and reported that the degradation of recombinant proteins was markedly reduced.

Protein expression levels using silkworms or pupae are 10- to 100-fold higher than those using *Bombyx mori* cells or conventional insect cells, indicating that the silkworm or its pupa is one of the most suitable systems for large-scale production of eukaryotic proteins. Recently, Motohashi et al. (2005) have developed the first practical *B. mori* multiple nucleopolyhedrovirus (BmMNPV) bacmid system directly applicable to protein expression in silkworms. This method provides rapid protein production in silkworms within 10 days, is free from biohazards, and thus will be a powerful tool as a future production factory of recombinant eukaryotic proteins because the bacmid does not require any baculovirus amplification step. Using this system, the green fluorescent protein was successfully expressed in silkworm larvae and pupae not only by infection with the recombinant virus but also by direct injection of its bacmid DNA. However, we found that the silkworm larvae were liquefied 5 days postinfection. Ohkawa et al. (1994) reported that BmMNPV also encodes v-cath-like cysteine protease, which has an extremely high homology (96.3%) to *Autographa californica* multiple nucleopolyhedrovirus putative viral cysteine protease (Whitford et al., 1989). Suzuki et al. (1997) constructed BmMNPV virus lacking the cysteine protease gene and produced

firefly luciferase and human growth factor in silkworms very efficiently because of the markedly reduced degradation of these proteins.

In this study, we constructed a cysteine protease-deleted BmMNPV (CPD-BmMNPV) bacmid using the lambda recombination system. The CPD-BmMNPV bacmid provides rapid protein production in silkworms with less degradation of recombinant eukaryotic proteins, showing improved expression of GFP_{uv}-β1, 3-*N*-acetylglucosaminyltransferase 2 (GGT2) fusion protein. These results indicate that the CPD-BmMNPV bacmid is suitable for the future production of recombinant eukaryotic proteins free from biohazards.

2. Materials and Methods

2.1. Bacterial cells, plasmids and media

Escherichia coli DH10Bac was obtained from Invitrogen (Carlsbad, CA, USA). pKD3 and pKD46 were kind gifts from Dr. Mary K. B. Berlyn of Yale University. The vectors for BmMNPV and the wild-type viral BmMNPV DNA were obtained from Funakoshi Co. (Tokyo, Japan), and pENTER/D-TOPO and Gateway pDEST8 were purchased from Invitrogen. The GFP_{uv} gene was obtained by PCR from pBlueBacHis2-GFP_{uv}/β3GnT2 (Kato et al., 2004). The *B. mori* Bm5 cell line was provided by Dr. K. S. Boo from the Insect Pathology Laboratory of Seoul National University. Sf900 II serum-free medium (Invitrogen) supplemented with 0.5%

antibiotic-antimycotic (Invitrogen) and 1% FBS was used for *B. mori* Bm5 cell culture. Supplements, if necessary, were added into the media at the following concentrations: ampicillin, 100 μg/mL; kanamycin, 50 μg/mL; Bluo-gal (Invitrogen), 100 μg/mL; gentamycin, 7 μg/mL; tetracycline, 10 μg/mL and isopropyl-β-D-thiogalactopyranoside, 40 μg/mL. The cysteine protease inhibitor [*trans*-epoxysuccinyl-L-leucylamido-(4-guanidino) butane] was purchased from Wako Pure Chem. Ind. Ltd. (Osaka, Japan).

2.2. Construction of CPD-*BmMNPV bacmid*

A flowchart describing the construction of the CPD-BmMNPV bacmid is shown in Fig. 1. A CPDcat fragment of 1.1 kb was obtained by PCR using the chloramphenicol acetyltransferase gene of pKD3 using Bm98758Fcat and Bm99687Rcat (Table 1) containing 50 bp sequences from start codon and stop codon of v-cath, respectively. The pKD3 template mixed in the PCR product was digested with *Dpn* I restriction enzyme. Red recombinase plasmid pKD46 was transformed to DH10Bac competent cells containing BmMNPV bacmid DNA (Motohashi et al., 2005). The resulting transformed cells were grown on Luria-Bertani (LB) agar plates (Sambrook et al., 1989) containing kanamycin and ampicillin at 30^{o}C overnight. Grown colonies were selected on SOB agar plates containing 0.2% L-arabinose at 30^{o}C overnight. Purified CPDcat fragment (500 ng) was transformed into DH10Bac competent cells containing BmMNPV bacmid

DNA and pKD46 using electroporation. Kanamycin- and chloramphenicol-resistant colonies were selected on LB agar plates at 37°C. After confirming the replacement of the v-cath gene with the cat gene by PCR, the CPD-BmMNPV bacmid containing deleted v-cath gene was isolated. Approximately 100 ng of CPD-BmMNPV bacmid DNA was transfected into Bm5 cells by lipofection. After incubation of the Bm5 cells for 72 h at 27°C, νCPD-BmMNPV was harvested. The harvested recombinant virus was diluted tenfold, and infected into Bm5 cells for amplification. The final amplified recombinant virus was stored for infection into silkworm larvae.

2.3. Infection with recombinant virus

Bm5 cells were infected with 1 x 10^6 pfu/mL νCPD-BmMNPV, and the infected Bm5 cells were incubated for 72 h at 27°C. The cell pellet was separated from 300 μL of culture broth by centrifugation at 2,000 *g* for 15 min. Three hundred microliters of 50 mM Tris-HCl containing 1% Triton X-100 were added to the cell pellet and placed on ice for 15 min. The supernatant was assayed for intracellular protease activity. For silkworm infection, fifth instar larvae (Ehime Sansyu Co. Ltd., Ehime, Japan) were used for the expression of fusion protein. Twenty microliters of 1x10^6 pfu/mL νCPD-BmMNPV suspended in Sf900II serum-free medium were injected into the dorsal region of the larvae using a syringe with 26-gauge beveled needle. Hemolymph (100 μL) was harvested 4 d postinjection and added to 100 μL of 50 mM Tris-HCl

containing 1% Triton X-100 for protease activity assay.

2.4. Construction of vCPD-BmMNPV expressing GGT2 fusion protein

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

To enable the introduction of a signal-peptide-coding region, the GGT2 fusion fragment of pBlueBacHis2-GGT2 (Kato et al., 2003) was amplified using a PCR primer set (Table 1). This PCR product, including the signal sequences, was cloned into the Gateway cloning vector pENTR/D-TOPO (Invitrogen) according to the protocol provided for the TOPO cloning system. The isolated plasmid was designated pENTR/D/bx-GGT2, in which the GGT2 fusion gene fused with the silkworm bombyxin signal sequence (Adachi et al., 1989). Fifty micrograms of pDEST8/bx-GGT2 DNA were transformed into 100 μL of DH10Bac/CPD-BmMNPV bacmid/pHelper competent cells. The transformed cells were added to 500 μL of SOC and incubated at 37°C for 1 h, and then were cultured on LB agar plates containing gentamycin, kanamycin, tetracycline, isopropyl-β-D-thiogalactopyranoside, and 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) at 37°C overnight. White

colonies were inoculated into LB medium containing gentamycin, kanamycin, and tetracycline and cultured at 37°C with agitation at 150 rpm overnight. CPD-BmMNPV bacmid/bx-GGT2 was isolated and confirmed by PCR using the bx-GGT2 and reverse primers (Table 1).

2.5. Expression of GGT2 fusion protein in silkworm larvae

Approximately 200 ng of CPD-BmMNPV bacmid/bx-GGT2 DNA were injected into fifth instar larvae in a similar way as described earlier. The fusion protein contained in the hemolymph was stocked for β3GnT and cysteine protease assays.

2.6. β3GnT and cysteine protease assay

β3GnT activity assay was carried out according to the protocol described by Kato et al. (2004).

Cysteine protease assay at neutral pH was performed as described by Hom et al. (2002). First, the sample (83 μL) was added to 583 μL of 0.2% azocasein in 50 mM sodium phosphate buffer (pH 7.2). The mixture was incubated at 37°C for 4 h, followed by the addition of 27 μL of 100% TCA to stop the reaction. Hemolymph samples were centrifuged at 25,000 *g* for 5 min, and 9 M urea (333 μL) was added to the supernatant before measuring the absorbance at 410 nm. One unit of cysteine protease activity was defined as the amount of enzyme capable of increasing the absorbance by 1 at 410 nm

within 1 h.

2.7. SDS-PAGE

The protein contained in the hemolymph was subjected to SDS-PAGE on a 12 % polyacrylamide gel using the Mini-protean II system (Bio-Rad, Hercules, CA). To detect directly the molecular band of a specific GGT2 protein on SDS-PAGE gel using Molecular Imager FX (Bio-Rad), the samples were only mixed with sample buffer without boiling. In this case, the molecular mass shifted to a slightly lower value compared to boiled samples on SDS-PAGE gel (Aoki et. al., 1996).

3. Results

3.1. Construction of CPD-BmMNPV bacmid

We prepared the CPDcat fragment, which consists of 50 bp fragments of the 5'- (3-52) and 3'- (884-933) regions in v-cath and complete cat gene from pKD3, by PCR with Bm98758Fcat and Bm99687Rcat (Table 1) containing 50 bp sequences from start codon and stop codon of v-cath, respectively. To construct v-cath-deleted bacmid, CPD-BmMNPV bacmid, by homologous recombination with CPDcat fragment, we used the Gam-producing plasmid pKD46 in which the *bet, exo,* and *gam* genes were under the control of an arabinose operon (Datsenko and Wanner, 2000). The Gam

prevents RecBCD-promoted digestion of λ phage DNA, so that Exo and Bet can gain access to DNA ends to promote recombination (Murphy, 1998). This homologous recombination was useful for the construction of the CPD-BmMNPV bacmid.

Deletion of the v-cath gene was confirmed by PCR using the bacmid extracted from kanamycin- and chloramphenicol-resistant colonies as a template. The v-cath gene fragment, including the promoter and signal peptide sequences, was approximately 1.3 kb, but the v-cath deleted fragment containing cat gene was approximately 1.5 kb as was expected (Fig. 2). Elimination of pKD46 occurred because replicon is temperature sensitive and cannot work at 37 ^{o}C.

3.2. Protease activity in Bm5 cells and silkworm larvae using vCPD-BmMNPV bacmid

When νBmMNPV and νCPD-BmMNPV were infected into Bm5 cells, their intracellular protease activities were measured at 72 h postinjection. The protease activities of νBmMNPV- and νCPD-BmMNPV-infected Bm5 cells were 0.34 and 0.02 U/mL, respectively (Fig. 3). The protease activity of νCPD-BmMNPV-infected Bm5 cells was the same level as that of νBmMNPV-infected Bm5 cells with the addition of cysteine protease inhibitor, [*trans*-epoxysuccinyl-L-leucylamido-(4-guanidino) butane, suggesting that the decrease in protease activity of νCPD-BmMNPV-infected Bm5 cells was due to v-cath deletion.

Figure 4A shows a comparison of the liquefaction times of the silkworm larvae.

The larvae infected with νBmMNPV were liquified at 5 d postinjection, whereas the larvae infected with νCPD-BmMNPV almost retained their normal white skin and neither melanized nor melted at 5 d postinjection. For silkworm larvae infected with νCPD-BmMNPV, the protease activity of the hemolymph was 0.32 U/mL (Fig. 4B), which was only 15% in comparison with that of νBmMNPV (2.03 U/mL). For Bm5 cells and silkworm larvae, νCPD-BmMNPV suppressed protease activity by 94% and 85%, respectively, compared with Bm5 cells and silkworm larvae infected with BmMNPV, because of the deletion of cysteine protease in the BmMNPV bacmid.

3.3 Protease activity and β3GnT2 expression in silkworms using CPD-BmMNPV/bx-GGT2 bacmid

Protease activity and GGT2 fusion protein expression in the CPD-BmMNPV/bx-GGT2 bacmid-injected silkworm larvae are shown in Figs. 5 and 6, respectively. When the BmMNPV bacmid was injected, the silkworm larvae melted at 7 d postinjection, but the silkworm larvae injected with CPD-BmMNPV/bx-GGT2 bacmid remained intact and showed no symptoms of liquefaction (Fig. 5A). The protease activities of the silkworm larvae hemolymphs infected with the BmMNPV/bx-GGT2 and CPD-BmMNPV/bx-GGT2 bacmids were 0.74 U/mL and 0.09 U/mL, respectively. Eighty-eight percent of the cysteine protease activity was not

observed in the hemolymph infected with the CPD-BmMNPV/bx-GGT2 bacmid, which is the same result shown in Fig. 3. The protease activity of mock was similar to that of the hemolymph infected with the CPD-BmMNPV/bx-GGT2 bacmid. The difference in the protease activity of the hemolymphs between the νBmMNPV and BmMNPV bacmids may be due to the degree of infectivity.

The maximum β3GnT activity in the hemolymph of the silkworm larvae injected with the CPD-BmMNPV/bx-GGT2 bacmid was 39.3 mU/mL at 4 d postinjection, whereas that in the hemolymph of the silkworm larvae injected with the BmMNPV/bx-GGT2 bacmid was 29.7 mU/mL (Fig. 6A). The low β3GnT activity in the hemolymph injected with the BmMNPV/bx-GGT2 bacmid might be caused by the degradation of β3GnT2 by proteases. The full-length fusion protein disappeared in SDS-PAGE gels at 4 d postinjection due to proteolytic degradation, and low molecular bands appeared (Fig. 6B). However, the fusion protein band in the hemolymph injected with the CPD-BmMNPV/bx-GGT2 bacmid had thickened at 4 d postinjection without degradation.

4. Discussion

Silkworms or pupae are one of the most suitable systems for the efficient production of eukaryotic proteins because of their more extensive expression of functional proteins than insect cells or human-oriented cultured cells. However, until now, silkworms have

been troublesome for protein expression because of the requirement of a long recombination time in baculovirus and because several rounds of virus purification and amplification of viruses in insect cells are required to obtain high titer virus concentrations. The development of the practical BmMNPV bacmid system directly applicable to protein expression in silkworms solved these problems (Motohashi et al., 2005).

Many studies (Kaba et al., 2004; Lee et al., 2006; Wang et al., 2005) have indicated that foreign gene products in silkworm larvae are frequently degraded owing to cysteine proteases originating from the BmMNPV gene. This is a serious problem in terms of the mass production of proteins of interest in silkworm larvae. To overcome this problem, we constructed a practical CPD-BmMNPV bacmid, in which the *v-cath* gene was replaced with the chloramphenicol acetyltransferase gene. We observed that the protease activities of Bm5 cells and silkworm larvae infected with this νCPD-BmMNPV were reduced by 94% and 85%, respectively. The larvae infected with νCPD-BmMNPV maintained their normal white skin without liquefaction even at 5 d postinjection. Compared with the liquefaction induced by virus infection, the liquefaction of the silkworm larvae in this study was delayed by 2 d. The expression level of the fusion protein was also higher than that by injection with the conventional BmMNPV bacmid. This shows that the CPD-BmMNPV bacmid was an efficient tool for expressing eukaryotic genes in the hemolymph of the silkworm larvae.

The use of silkworm larvae as protein factories is very attractive owing to the low

cost of production, as silkworms are easy to treat and have a high safety level in terms of biohazards. We believe that the CPD-BmMNPV bacmid can be utilized as a very effective expression vector for the stable production of useful foreign proteins, particularly proteins susceptible to cysteine protease. Furthermore, for production of secretory proteins in silkworm larvae, it is important to collect hemolymph before the degradation of target-gene products begins. Using this CPD-BmMNPV bacmid as an expression vector, undegraded proteins of interest might be harvested and degradation of proteins of interest during purification from hemolymph might be prevented.

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Legends for figures

Fig. 1. Schematic outline for construction of CPD-BmMNPV bacmid. A CPDcat PCR fragment was obtained from pKD3. DH10Bac cells that were ampicillin- and kanamycin-resistant were selected and then incubated on an LB plate with 0.2% L-arabinose at 30^{o}C to induce Gam, Bet, and Exo from pKD46. The CPDcat PCR fragment was introduced to DH10Bac cells containing BmMNPV DNA and pKD46 by electroporation. The CPDcat PCR fragment and BmMNPV bacmid DNA recombined homologously. Chloramphenicol- and kanamycin-resistant cells were selected under conditions of incubation overnight at 37^{o}C and designated CPD-BmMNPV bacmid.

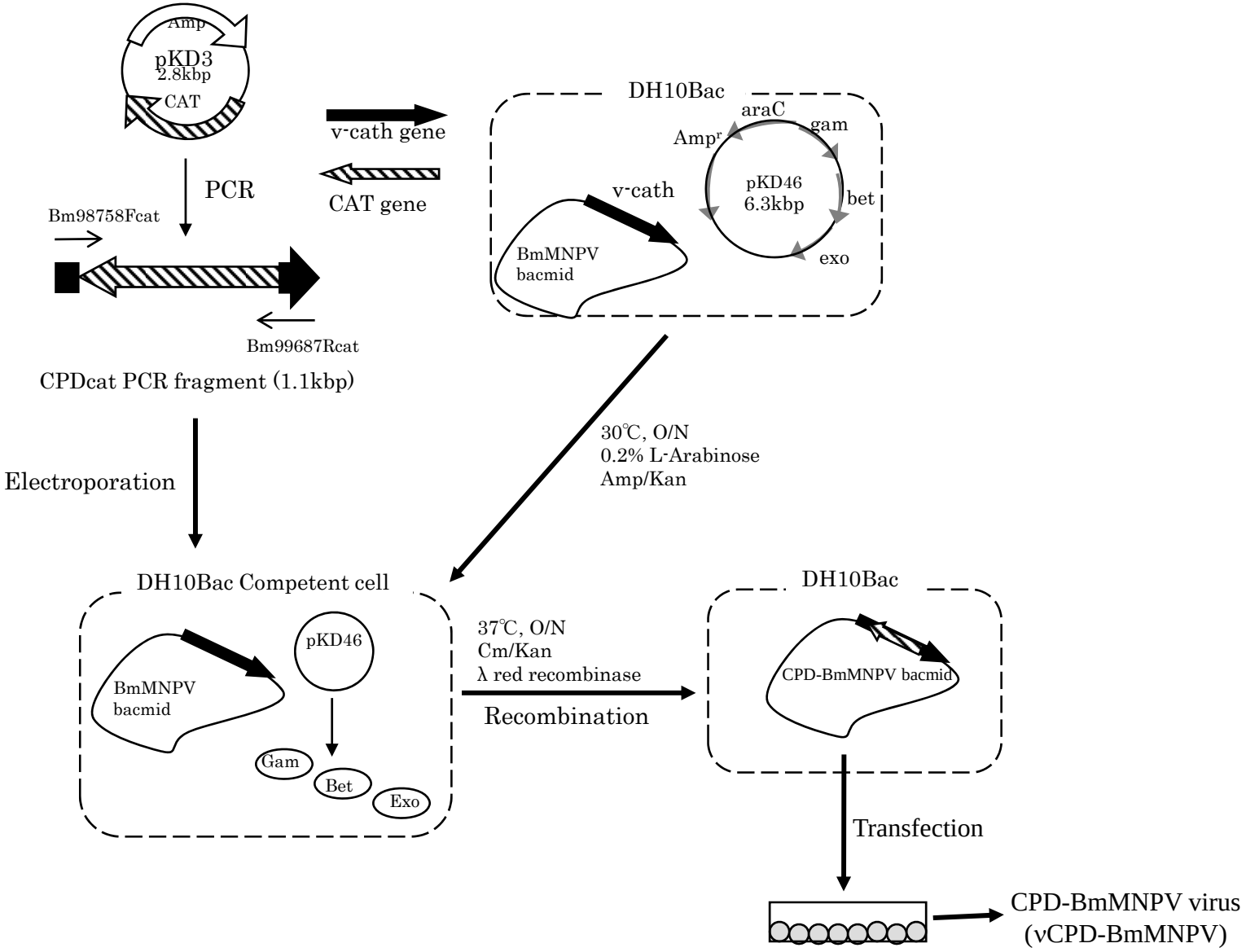
Fig. 2. Confirmation of v-cath gene deletion by PCR. Lane M denotes DNA marker. Lanes 1 and 2 denote PCR products from BmMNPV bacmid and CPD-BmMNPV bacmid, respectively. The v-cath gene fragment was approximately 1.3 kb because of including the promoter and signal peptides, but the v-cath gene fragment containing cat gene was approximately 1.5 kb.

Fig. 3. Protease activity in Bm5 cells infected with BmMNPV and CPD-BmMNPV viruses (A) and hemolymph fluid of silkworm larvae (B), respectively.

Fig. 4. Comparison of larval liquefaction (A) and protease activity (B) infected with BmMNPV and CPD-BmMNPV viruses. The left column in (A) indicates postinjection time (d).

Fig. 5. Comparison of larval liquefaction (A) and protease activity (B) injected with BmMNPV and CPD-BmMNPV bacmids. The left column indicates postinjection time (d). Protease activity in the hemolymph fluid of mock, injected with BmMNPV and CPD-BmMNPV bacmids at 6 d postinjection was measured.

Fig. 6. β3GnT activity (A) and GFP_{uv} fluorescent analysis of GGT2 fusion protein (B) in the hemolymph of silkworms injected with BmMNPV/bx-GGT2 (white bars) and CPD-BmMNPV/bx-GGT2 bacmids (black bars). Protein bands in (B) were detected by GFP_{uv} fluorescence. Bars denote standard deviations (n=3).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37℃, O/N
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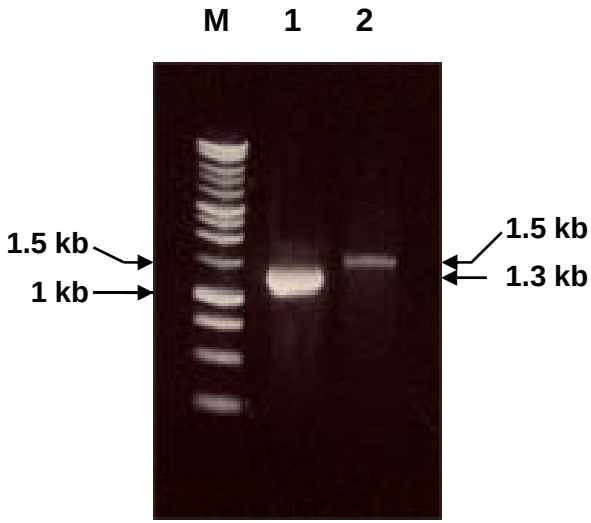
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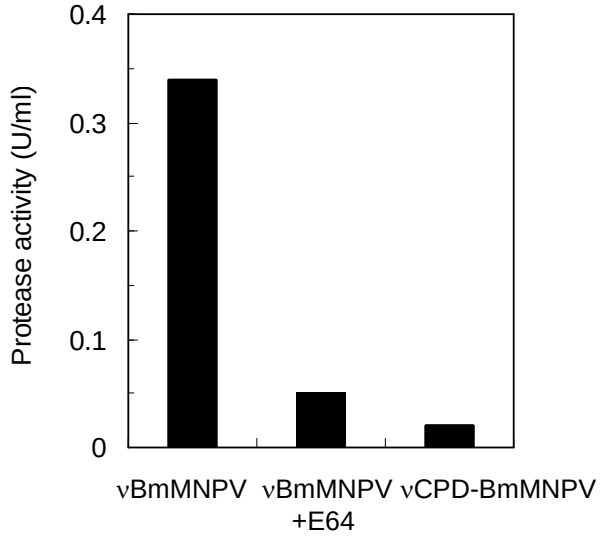
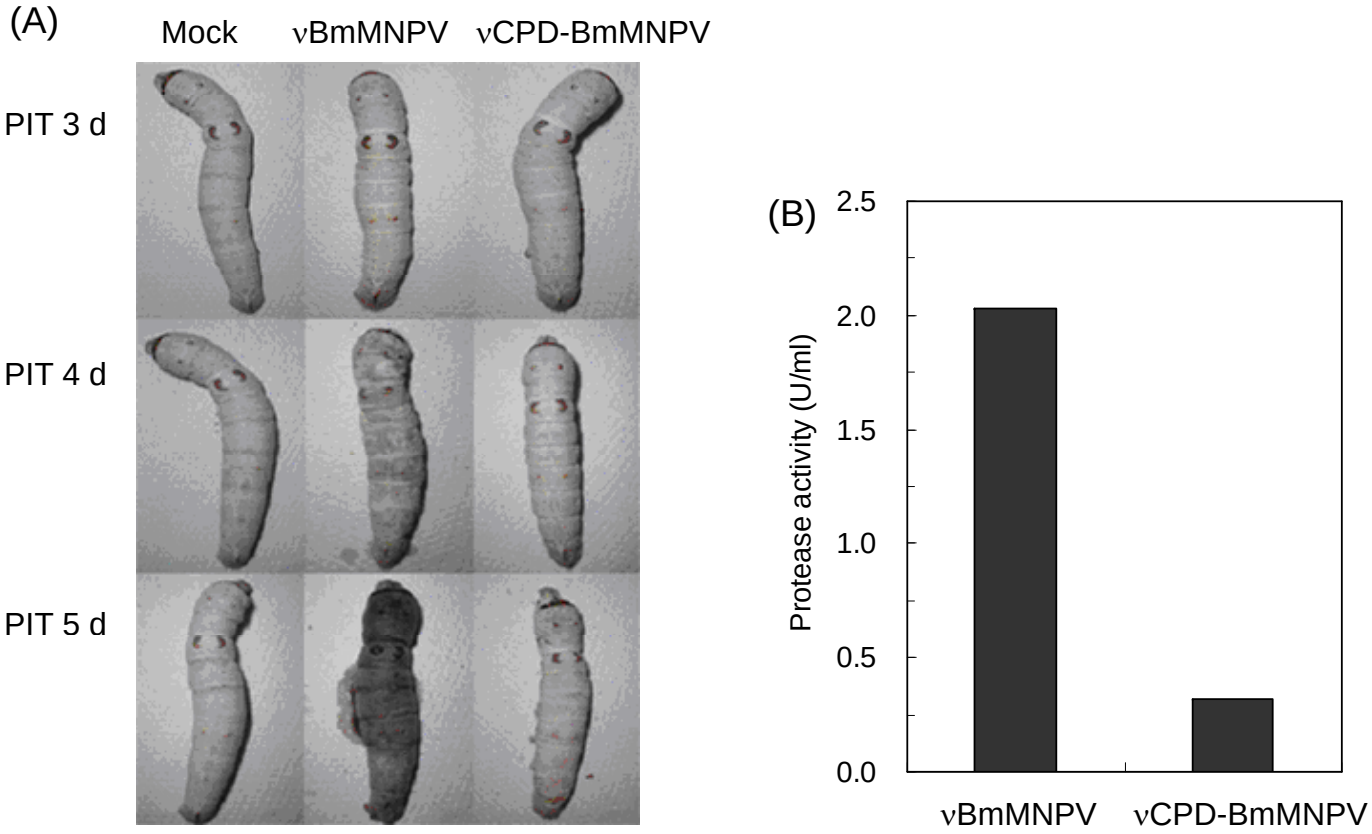
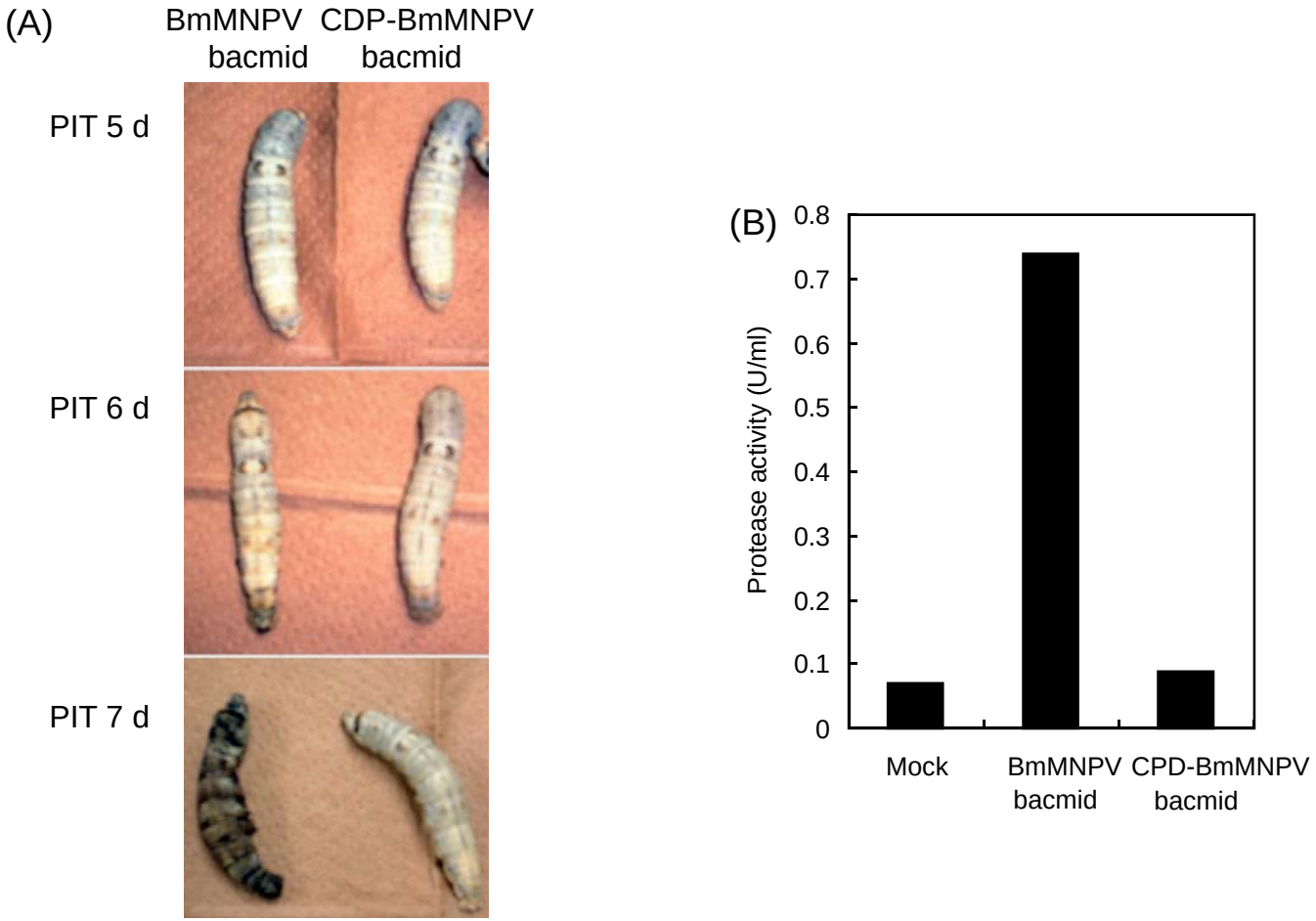
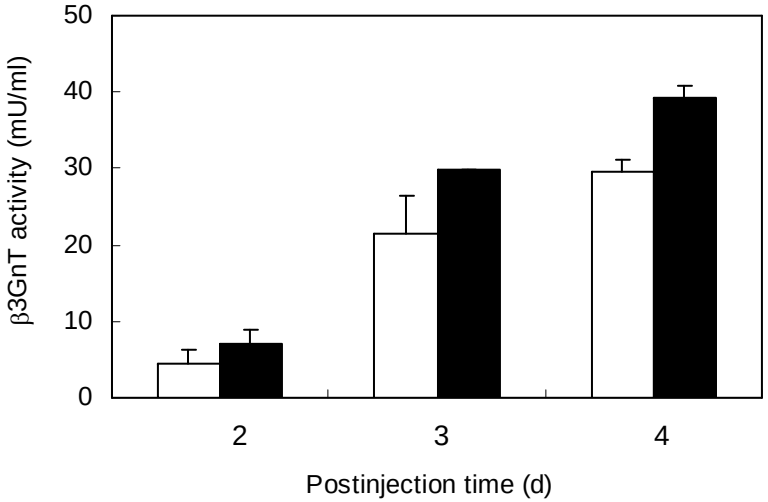
Fig. 3, Hiyoshi et al.




(A)
Mock
νBmMNPV
νCPD-BmMNPV
PIT 3 d
PIT 4 d
PIT 5 d
(B)
Protease activity (U/ml)
2.5
2.0
1.5
1.0
0.5
0.0
νBmMNPV
νCPD-BmMNPV


(A)
BmMNPV bacmid
CDP-BmMNPV bacmid
PIT 5 d
PIT 6 d
PIT 7 d
(B)
Protease activity (U/ml)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Mock
BmMNPV bacmid
CPD-BmMNPV bacmid

(A)


50
40
30
20
10
0
β3GnT activity (mU/ml)
2
3
4
Postinjection time (d)

(B)

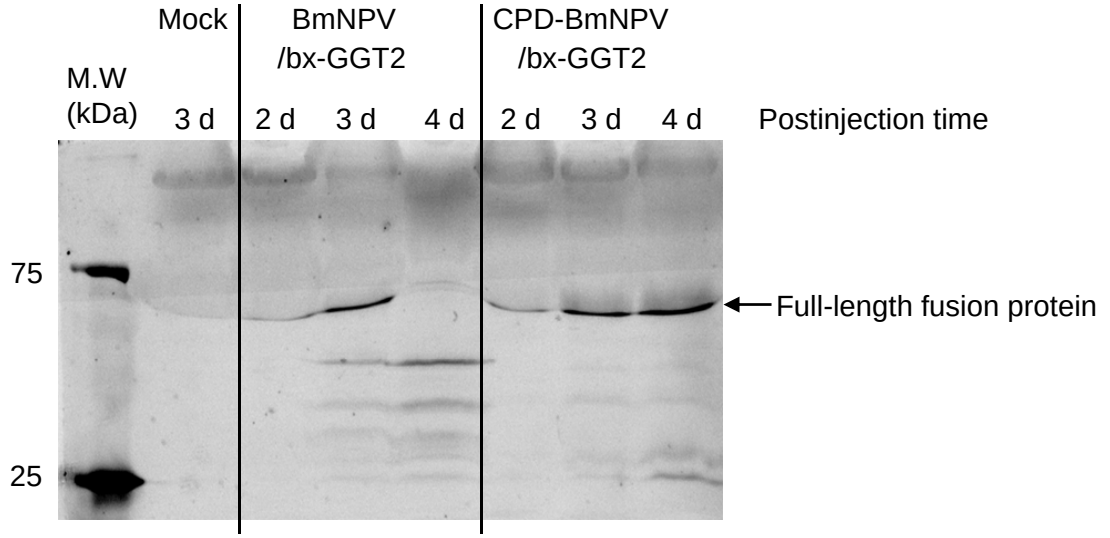

Mock
BmNPV
/bx-GGT2
CPD-BmNPV
/bx-GGT2
M.W
(kDa)
3 d
2 d
3 d
4 d
2 d
3 d
4 d
Postinjection time
75
25
Full-length fusion protein

Table 1

Primers used to prepare CPDcat fragment and signal sequence

Primer-name	Sequence (5'→3')
Bm98758Fcat	GAACAAAATTTTGTTTTATTTGTTTGTGTACGCCGTTGTAAAGAGCGCGG TTGTGTAGGCTGGAGCTGCT v-cath chloramphenicol acetyltransferase
Bm99687Rcat	TAATAAATGACTGCAGTAGACGCAAGTTCGTTTCTCATACCACAAGCGT TCCATATGAATATCCTCCTTA v-cath chloramphenicol acetyltransferase
bx-GFP_{uv}-β3GnT2	CACCATGAAGATACTCCTTGCTATTGCATTAATGTTGTCAACAGTAATGTGGGTGTCAACACAACCGCGG Signal sequence GGTTCTCATCATC GFPuv-β3GnT2
Reverse primer	CGGAATTCTGAAGGGTTTAGAGGCCCTCAAATGGG