1 **Specific expression of GFP_{uv}-β1,3-*N*-acetylglucosaminyltransferase 2**
2 **fusion protein in fat body of *Bombyx mori* silkworm larvae using signal**
3 **peptide**

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Abbreviations: AcMNPV, *Autographa californica* multiple nucleopolyhedrovirus; BmMNPV, *Bombyx mori* multiple nucleopolyhedrovirus; BmMNPV-CP⁻, cysteine protease deficient *Bombyx mori* multiple nucleopolyhedrovirus; bx, bombyxin; ER, endoplasmic reticulum; GFP_{uv}, green fluorescent protein when excited with long-wave UV light; β3GnT2, β1,3-*N*-acetylglucosaminyltransferase 2; GGT2, GFP_{uv}-β3GnT2; HMM, hidden Markov model; PCR, polymerase chain reaction; ppae, *Bombyx mori* prophenoloxidase-activating enzyme.

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Abstract

Bombyxin (bx) and prophenoloxidase-activating enzyme (ppae) signal peptides from *Bombyx mori*, their modified signal peptides, and synthetic signal peptides were investigated for the secretion of GFP_{uv}- β 1,3-*N*-acetylglucosaminyltransferase 2 (GGT2) fusion protein in *B. mori* Bm5 cells and silkworm larvae using cysteine protease deficient *B. mori* multiple nucleopolyhedrovirus (BmMNPV-CP) and its bacmid. The secretion efficiencies of all signal peptides were 15-30% in Bm5 cells and 24-30% in silkworm larvae, while that of the +16 signal peptide was 0% in Bm5 cells and 1% in silkworm larvae. The fusion protein that contained the +16 signal peptide was expressed specifically in the endoplasmic reticulum (ER) and in the fractions of cell precipitations. Ninety-four percent of total intracellular β 1,3-*N*-acetylglucosaminyltransferase (β 3GnT) activity was detected in cell precipitations following the 600, 8000, and 114000 x g centrifugations. In the case of the +38 signal peptide, 60% of total intracellular activity was detected in the supernatant from the 114000 x g spin, and only 1% was found in the precipitate. Our results suggest that the +16 signal peptide might be situated in the transmembrane region and not cleaved by signal peptidase in silkworm or *B. mori* cells. Therefore, the fusion protein connected to the +16 signal peptide stayed in the fat body of silkworm larvae with biological function, and was not secreted extracellularly.

Keywords: Bacmid; Baculovirus expression system; BmMNPV; Signal sequence; Silkworm; β 1,3-*N*-acetylglucosaminyltransferase 2

1 **Introduction**

2
3 Recently, it has been shown that insect larvae can be used in the baculovirus
4 expression system instead of insect cell culture for large-scale protein production.
5 Scale-up is performed more easily in insect larvae than in insect cells [1-3]. Moreover,
6 the *B. mori* multiple nucleopolyhedrovirus (BmMNPV) bacmid system was established
7 [4] and time-consuming manipulation including virus amplification and virus titer
8 determination can be omitted by using this bacmid system.

9 To improve the production of proteins in insect cells, various genetic manipulations
10 have been performed; random and site-directed mutagenesis [5], signal peptide and
11 promoter exchange [6], and increase of plasmid copy number [7]. In the case of
12 secreted proteins, processing of signal peptide and glycosylation may be crucial. In
13 both insect cell-based expression systems, enforcement of the secretory pathway in host
14 cells and overexpression of molecular chaperones such as calnexin and calreticulin,
15 enhance the secretion of recombinant proteins [8-12].

16 In this study, using the BmMNPV bacmid, the GFP_{uv}-β1,3-*N*-
17 acetylglucosaminyltransferase 2 (β3GnT2) fusion protein was expressed in *B. mori*
18 Bm5 cells and silkworm larvae. In a previous report, its fusion protein was expressed
19 using the *B. mori* bombyxin (bx) and *B. mori* prophenol oxidase-activating enzyme
20 (ppae) signal peptide from *B. mori*, which was functional in Bm5 cells [13]. In order to
21 investigate secretory efficiency, synthetic signal peptides was predicted by a computer
22 program (SignalP ver.3.0) [14] and its effects on the secretion of the fusion protein were

examined in Bm5 cells and silkworm larvae. Further, characterization of the ability of signal peptides to secretion fusion protein was performed.

Materials and Methods

Signal peptide design. Signal peptides of bx and ppae from *B. mori* were modified with the aid of SignalP 3.0 which is based on artificial neural network and HMM [14]. Signal peptides +38, +34, +28, and +16 were used for reference [15].

Construction of recombinant BmMNPV and BmMNPV bacmids. Fundamentally, construction of recombinant baculoviruses was performed as described in a previous report [13]. Recombinant baculovirus was constructed using the BmMNPV-CP⁻ Bac-To-Bac system [16]. GFP_{uv}-β3GnT2 (GGT2) fusion genes containing different signal sequences were amplified by polymerase chain reaction (PCR) using the primers shown in Table 1. Finally, recombinant BmMNPV-CP⁻/(signal peptide)-GGT2 and its bacmid were constructed. Eight different types of signal peptides were used.

Cultivation of Bm5 cells, bacmid transfection and baculovirus infection. Culture of Bm5 cells was carried out in 25 cm² T-flasks with Sf-900 II Serum Free Medium (SFM) (Invitrogen) supplemented with 1% antibiotic-antimycotic (Invitrogen). Transfection of Bm5 cells with recombinant bacmids was performed according to the procedure described in the Invitrogen manual. Bm5 cells, at a density of approximately 1.0 x 10⁶ cells/ml, were infected with the viral transfection solution. Cell viability and cell number were measured using the 0.4% trypan blue staining method.

Recombinant BmMNPV bacmid injection to silkworm larvae. The *B. mori* fifth-instar larvae (Ehime Sansyu, Ehime, Japan) were reared for 1 day on an artificial feed (Silkmate 2, NOSAN Co., Yokohama, Japan) in a 27°C incubator, and were injected with 50 µl bacmid solution consisting of 8 µg bacmid DNA in 45 µl of SF900 SFM and 5 µl DMRI-C (Invitrogen) per larva. Hemolymph was collected at 1 day after emission of GFP_{uv} fluorescence in silkworm larvae. Five mM phenyl-thiourea was immediately added into collected hemolymph to prevent melanization. Fat body was collected after dissection of silkworm larvae and suspended in 50 mM Tris-HCl (pH 8.0), and then disrupted using a sonicator (VC 130PB, Sonic & Materials, Newtown, CT, USA) for three 30 sec periods with 1 min intervals. Its suspension was used as the fat body extraction.

Confocal laser scanning microscopy analysis. Fat body was collected from silkworm larvae and immediately observed with the confocal laser-scanning microscope (TCS SP2, Leica Microsystems Heidelberg, Germany) equipped with an imaging system.

Cell fractionation. BmMNPV-CP/(signal peptide)-GGT2-infected Bm5 cells and fat body from BmMNPV-CP/(signal peptide)-GGT2 bacmid injected silkworm larvae were collected and suspended in 50 mM Tris-HCL (pH 8.0). The suspension was homogenized and the homogenate was spun at 600 x g for 5 min using a centrifuge (3700, Kubota, Tokyo). The supernatant was then centrifuged at 8000 x g for 5 min. Finally, the supernatant was spun at 114000 x g for 1 h using a centrifuge (himac CS150GXS, Hitachi Koki Co., Ltd., Tokyo). All precipitates were resuspended in 50

1 mM Tris-HCL (pH 8.0). Supernatants and suspension of precipitation were analyzed
2 for GGT2 expression.

3 *SDS-PAGE*. The protein contained in the hemolymph was subjected to SDS-PAGE
4 on a 10% polyacrylamide gel using the Mini-protean II system (Bio-Rad, Hercules, CA).
5 To detect directly the molecular band of a specific GGT2 protein on SDS-PAGE gel
6 using Molecular Imager FX (Bio-Rad), the samples were mixed with sample buffer, but
7 not boiled.

8 *β3GnT assay*. After cultivation, the culture broth was centrifuged at 20000 x g for 5
9 min, and the supernatant was used for the enzyme assay. A lysis buffer containing 50
10 mM Tris-HCl (pH8.0) and 1% Triton X-100 was added to the cell pellet and the mixture
11 was placed on ice for 10 min. This cell lysate was used for the measurement of
12 intracellular enzyme activity. The β3GnT activity assay was carried out according to
13 the protocol described previously [12].

14 15 **Results and Discussion**

16 17 *Mutagenesis of bx and ppae signal peptide*

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19 Signal peptides are indispensable to the secretion of protein into the hemolymph in
20 silkworm larvae. Several studies have reported on the secretion of recombinant protein
21 extracellularly in insect cell culture [17, 18], but there are few reports about the
22 secretion of protein into the hemolymph of silkworm larvae.

1 In this study, mutated bx and ppae signal peptides were designed using SignalP 3.0
2 [14] (Table 2). The bx signal peptide has hydrophilic amino acids ¹²Ser and ¹³Thr in the
3 predicted central hydrophobic core region (h-region). By removing Ser and Thr
4 residues, signal peptide possibility and maximum cleavage site possibility of the bx
5 signal peptide changed to 1.000 and 0.999, from 1.000 and 0.995, respectively.
6 Replacement of the predicted cleavage site from Thr-Gln to Ala-Ala in the bx signal
7 peptide, resulting in the bx-12 signal peptide.

8 Possibilities of signal peptide and maximum cleavage site of ppae signal peptide
9 were 0.962 and 0.446, respectively. The low maximum cleavage site possibility was
10 likely due to an ambiguous cleavage site. Mutation of ¹⁹Val to Ala could lead to a
11 maximum cleavage site possibility of 0.769 from 0.446. This mutated ppae signal
12 peptide was named ppae-1. In addition to this mutation, mutation of ¹⁷Lys to Leu
13 altered the maximum cleavage site possibility from 0.769 to 0.987. This signal peptide
14 was named ppae-12. Its maximum cleavage site possibility increased to 0.996 by
15 deletion of ²⁰Val in the ppae-12 signal peptide. This signal peptide was named ppae-123.

16 Moreover, synthetic signal peptides were also investigated (Table 2). Barash *et al.*
17 previously constructed signal peptides which allow fusion protein to be secreted
18 extracellularly in human embryonic kidney (HEK) 293 cells using HMM [15]. We also
19 investigated the ability of the HMM +38, +34, +28, and +16 signal peptides to secrete
20 the GGT2 fusion protein into the hemolymph of silkworm larvae.

21
22 *Secretion of GGT2 fusion protein in Bm5 cells using synthetic signal peptide*

At first, the capacity of each signal peptide was investigated in Bm5 cells by infection of each BmMNPV-CP/(signal peptide)-GGT2. Bm5 cells were infected with 0.5 ml virus solution and cultivated for 2 - 4 days in a static culture until cell viability reached 50-70%. The secretory efficiency was calculated as the percentage of extracellular activity to the total activity (Table 3). All signal peptides forced Bm5 cells to secrete the fusion protein in a ratio (17-27%) similar to the bx signal peptide which was adopted in a previous report (27%), with the exception of the +16 signal peptide (0%). The fusion protein was expressed intracellularly in each case (data not shown). The secretory efficiency of synthetic signal peptides, +38, +34, +28, and mutated signal peptides ppae-12, ppae-123, and bx-12 were at similar levels to those of bx or ppae (Table 3). Mutated signal peptides based on predictions by the SignalP 3.0 program, ppae-1, -12, -123, and bx-12, did not enhance secretion of the GGT2 fusion protein. However, in the case of the +16 signal peptide, extracellular β 3GnT activity could not be detected (Table 3), suggesting that the fusion protein connected with the +16 signal peptide was hardly secreted in Bm5 cells. Based on the prediction by the SignalP 3.0 program, the +16 signal peptide is not a signal anchor. However, the +16-GGT2 fusion protein remained intracellular and was not secreted into the culture medium. This suggests that the +16 signal peptide might work as a signal anchor in Bm5 cells.

Secretion of the GGT2 fusion protein in silkworm larvae

When the BmMNPV/(bx or ppae)-GGT2 bacmid was injected into silkworm larvae for GGT2 fusion production, more than 90% of total activity was detected in the hemolymph of silkworm larvae [13]. In the present study, using the BmMNPV-CP⁻ bacmid containing the fusion protein connected with the ppae signal peptide, β3GnT activity was measured in both hemolymph and fat body. In the silkworm's organs, fat body showed the most fluorescence intensity; the fusion protein was secreted partly into hemolymph and the rest remained in the fat body of silkworm larvae. Twenty-six to 30% of total expressed fusion protein was secreted into the hemolymph, but the remainder stayed in the fat body (Table 4).

The secretory efficiency of the synthetic signal peptides +38 and +34 were 24% and 30%, respectively (Table 4), which was similar to that of ppae. However, in the case of the +16 signal peptide, the secretory efficiency in silkworm larvae was only 1% (Table 4), suggesting that the fusion protein connected with the +16 signal peptide was hardly secreted in silkworm larvae as well as Bm5 cells.

Expression pattern of the GGT2 fusion protein connected with the +16 signal peptide in Bm5 cells

In both Bm5 cells and silkworm larvae, the GGT2 fusion protein connected with the +16 and +38 signal peptides was observed. BmMNPV-CP⁻/+38-GGT2- or BmMNPV-CP⁻/+16-GGT2-infected cells were cultivated, and both β3GnT activity and expression patterns of the fusion protein were investigated (Fig. 1). Intracellular fusion protein was

observed at 2 - 3 days post-infection in both cases (Fig. 1B), but β 3GnT activity was not detected. The β 3GnT activity can be detected at 4 days post-infection (Fig. 1A). At the same time, upper bands of expressed fusion protein were detected (Fig. 1B). These upper bands correspond to the glycosylated form of the fusion protein. *N*-glycosylation was essential for β 3GnT activity, because treatment of infected cells with tunicamycin abolished β 3GnT activity [19]. In both cases the glycosylation was executed in Bm5 cells, but in BmMNPV-*CP*⁻/+16-GGT2-infected Bm5 cells, the glycosylated active form of the fusion protein remained intracellularly after glycosylation. The lower band might be the non-glycosylated form in BmMNPV-*CP*⁻/+38-GGT2-infected cells [19], but not in BmMNPV-*CP*⁻/+16-GGT2-infected cells, suggesting that the +16 signal peptide might be inclined to make the fusion protein glycosylated compared with the +38 signal peptide.

Localization of the fusion protein in BmMNPV-CP⁻/+16-GGT2-infected Bm5 cells

To investigate why the fusion protein is trapped intracellularly, localization of the expressed fusion protein was confirmed by cell fractionation. Either BmMNPV-*CP*⁻/+38-GGT2- or BmMNPV-*CP*⁻/+16-GGT2-infected cells were cultivated for 5 days and cells were suspended in PBS buffer and subsequently homogenized. The homogenate was centrifuged and fractionized. β 3GnT activity of each fraction is shown in Fig. 2. In BmMNPV-*CP*⁻/+38-GGT2-infected cells, the main activity existed in supernatant from the 114,000 x g spin. However, in BmMNPV-*CP*⁻/+16-GGT2-infected cells, more than

80% of total activity existed in precipitates from the 600 x g and 8000 x g centrifugations. The fractionated fusion protein was analyzed by SDS-PAGE (Fig. 3). The fusion protein in supernatant from the 114000 x g spin of BmMNPV-CP⁺/+38-GGT2-infected cells on an SDS-PAGE gel (Fig. 3, lane 3) was consistent with one in the extracellular fraction (Fig. 3, lane 9), suggesting the fusion protein might exist in the soluble fraction in ER or Golgi apparatus. On the contrary, the little band (Fig. 3, lane 7) from supernatant following the 114000 x g spin of BmMNPV-CP⁺/+16-GGT2-infected cells consistent with glycosylated active form (Fig. 3, lane 9) was detected, while upper glycosylated active forms were precipitated at 8000 x g (Fig. 3, lane 6). β 3GnT activity was only 16% of total intracellular activity in the precipitate from the 114000 x g spin, but β 3GnT activity was 94% of total intracellular activity in precipitates from the 600 x g and 8000 x g spins (Fig. 2). It seemed that the fusion protein connected with the +16 signal peptide might be located in the transmembrane region and associated with either ER or Golgi membrane. In HEK 293 cells, the +16 signal peptide forced cells to secrete approximately 85% alkaline phosphatase activity [16]. The +16 signal peptide was similar to the type II transmembrane peptide judging from the HMM bit score, but in the case of HEK 293 cells Barash *et al.* [15] reported that the recombinant protein continued to be secreted. This suggests that signal peptide is likely to work in a different manner in HEK-293 and Bm5 cells, or substrate recognition of signal peptidase may be different between the two cell lines.

In fat body of both BmMNPV-CP⁺/+38-GGT2 bacmid- and BmMNPV-CP⁺/+16-GGT2 bacmid-injected silkworm larvae, about half of total β 3GnT activity existed in

the precipitated fraction from the 114000 x g spin (Figs. 4A and 4B). In fat body BmMNPV-CP/+38-GGT2 bacmid-injected silkworm larvae, expressed fusion protein with green fluorescence was observed at the border of cells (Fig. 4C). It was supposed that the fat body of silkworm larvae might force secretory proteins to stay in cell membranes or at the cell surface.

Wu *et al.* [20] reported that expression of human acidic fibroblast growth factor or basic fibroblast factor containing the gp67 signal peptide in silkworm larvae was detected in fat body, and these factors were purified from supernatant following the 14000 x g centrifugation of fat body of silkworm homogenate. This suggested that the +16 and gp67 signal peptides forced silkworm larvae to express recombinant protein in fat body. However, in the case of the +16 signal peptide, the mechanism causing the peptide to remain in the fat body might be different. The +16 signal peptide is probably associated with membrane and stopped in secretory pathway. In addition to the +16 and gp67 signal peptides, human granulocyte-macrophage colony-stimulating factor without its signal peptide was *N*-glycosylated in silkworm pupae [21]. Signal peptide is thought to be an interesting thing for protein expression in silkworm, because entrance of expressed protein to the ER is needed for its glycosylation and generally it cannot enter the ER without a signal peptide. Further improvement of signal peptide development will lead to the mass and efficient production of recombinant protein in silkworm.

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Table 1: PCR primers

Name	Sequence (5'-3')
38-F	CACCATGTGGTGGAGGCTTTGGTGGCTTCTTCTTCTTCTTCTTCTTGGCCCATGGTGTGGGCCAAACCGCGGGGTTCATCATC
34-F	CACCATGACGCCACATGGGCTTGGTGGCTTTTCCTTGTGCTTCTTCTTGGCCTTTGGGCTCCCGCTACGGCTAAACCGCGGGGTTCATCATC
28-F	CACCATGATGTGGCTTTGGCTTGTGCTTCTTCTTCTTGCCTTGCCGGCAACGTGCAGGCCAAACCGCGGGGTTCATCATC
16-F	CACCATGAAGCTTCTTCTTATCTTCTTCGTGCTTGTGGTGTGGATGGGCCCCGCCCATCGCAAACCGCGGGGTTCATCATC
ppae-1-F	CACCATGTTTTTAATTTGGACATTCATCGTGGCTGTTCTGGCGATCCAGACCAAAAGTGCTGTTCAACCGCGGGGTTCATCATC
ppae-12-F	CACCATGTTTTTAATTTGGACATTCATCGTGGCTGTTCTGGCGATCCAGACCCTGAGTGCTGTTCAACCGCGGGGTTCATCATC
ppae-123-F	CACCATGTTTTTAATTTGGACATTCATCGTGGCTGTTCTGGCGATCCAGACCCTGAGTGCTCAACCGCGGGGTTCATCATC
bx-12-F	CACCATGAAGATACTCCTTGCTATTGCATTAATGTTGGTAATGTGGGTGTCAGCTGCTCCGCGGGGTTCATCATC
bx-F	CACCATGAAGATACTCCTTGCTATTGCATTAATGTTGTCAACAGTAATGTGGGTGTCAACACAACCGCGGGGTTCATCATC
ppae-F	CACCATGTTTTTAATTTGGACATTCATCGTGGCTGTTCTGGCGATCCAGACCAAAAGTGTTGTTCAACCGCGGGGTTCATCATC
β3GnT2-R	CGGAATTCTGAAGGGTTTAGAGGCCCTCAAATGGG

1 Underlined sequences indicate putative signal peptide coding sequences.

2

1 Table 2: Amino acid sequence of signal peptides

Signal peptide	Amino acid sequence
+38	<u>M W W R L W W L L L L L L L W P M V W A</u> K P R G S
+34	<u>M R P T W A W W L F L V L L L A L W A P A R G</u> K P R G S
+28	<u>M M W L W L V L L L L C L A G N V Q A</u> K P R G S
+16	<u>M K L L L I F F V L V V W M G P A H R K</u> P R G S
bx	<u>M K L I L L A I A L M L S T V M T V S T</u> Q P R G S
bx-12	<u>M K L I L L A I A L M L</u> V M T V S A A P R G S
ppae-1	<u>M F L I W T F I V A V L A I Q T K S A</u> V Q P R G S
ppae-12	<u>M F L I W T F I V A V L A I Q T L S A</u> V Q P R G S
ppae-123	<u>M F L I W T F I V A V L A I Q T L S A</u> Q P R G S
ppae	<u>M F L I W T F I V A V L A I Q T K S</u> V V Q P R G S

2 Underlined sequences indicate putative signal peptide coding regions.

3

4

1 Table 3: Secretory efficiency of each signal peptide in Bm5 cells static culture

Signal peptide	Secretory efficiency (%) [*]
+38	21
+34	24
+28	17
+16	0
ppae-1	27
ppae-12	20
ppae-123	21
bx-12	18
bx	22
ppae	19

2

3 ^{*} Secretory efficiency (%) = $\frac{\text{extracellular activity}}{(\text{extracellular activity} + \text{intracellular activity})} \times 100$

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1 Table 4: Secretory efficiency of each signal peptide in silkworm larvae

Signal peptide	Secretory efficiency (%) [*]
+38	24
+34	30
+16	1
bx	18
ppae	26

2

3 ^{*} Secretory efficiency (%) = $\frac{\text{activity in hemolymph}}{(\text{activity in hemolymph} + \text{activity in fat body})} \times 100$

4

Legends for figures

Fig. 1. GFP_{uv} fluorescent analysis of GGT2 fusion protein in cell lysate from BmMNPV-*CP*⁻/+38-GGT2- or BmMNPV-*CP*⁻/+16-GGT2-infected Bm5 cells on a SDS-PAGE gel (A). Proteins were separated on a 10% SDS-PAGE gel and detected by fluorescent image analyzer. Time course of intracellular β3GnT activity in BmMNPV-*CP*⁻/+38-GGT2- or BmMNPV-*CP*⁻/+16-GGT2-infected cells (B). Aliquots of culture medium were collected every 24 h and culture supernatants were separated from cells. Cell lysates treated with 1% Triton X-100 were analyzed.

Fig. 2. β3GnT activity of each cell fraction in BmMNPV-*CP*⁻/+38-GGT2- (A) and BmMNPV-*CP*⁻/+16-GGT2-infected cells (B). Virus-infected cells were suspended in PBS buffer and homogenized. Cell fractionation was performed according to Materials and Methods.

Fig. 3. GFP_{uv} fluorescent analysis of GGT2 fusion protein in each cell fraction shown in Fig. 2. Lanes 1-4: BmMNPV-*CP*⁻/+38-GGT2-infected cells, lanes 5-8: BmMNPV-*CP*⁻/+16-GGT2-infected cells. Lanes 1, 5: 8000 x g supernatant; lanes 2, 6: 8000 x g precipitation; lanes 3, 7: 114000 x g supernatant; lanes 4, 8: 114000 x g precipitation; lane 9: extracellular fraction of BmMNPV-*CP*⁻/+38-GGT2-infected cells. Arrow indicates an glycosylated active form of the fusion protein in the extracellular fraction.

Fig. 4. β 3GnT activity of each cell fraction in fat body of BmMNPV-*CP*⁻/+38-GGT2 (A) and BmMNPV-*CP*⁻/+16-GGT2 (B) bacmids-injected silkworm larvae. After each bacmid-injected silkworm larvae were reared for 4-6 days, they were dissected and fat body was collected. Fat body from silkworm larvae was suspended in PBS buffer and homogenized. Cell fractionation was performed according to Materials and Methods. (C) Confocal laser-scanning microscopy analysis of fat body of BmMNPV-*CP*⁻/+38-GGT2 bacmid-injected silkworm. Bar denotes 10 μ m.

Supplemented document

In order to examine virus-infected Bm 5 cells and bacmid-injected fat body of silkworm confocal laser scanning microscopy was used. Bm5 cells were infected with recombinant baculovirus followed by 4 days cultivation. Infected cells were collected and washed with phosphate-buffered saline (PBS) containing 1% FBS. Cells were stained with PBS containing 5 mM ER-Tracker Red (Molecular Probes, Eugene, OR, USA) for 30 min at room temperature, respectively. Cells were examined under a confocal laser-scanning microscope (TCS SP2, Leica Microsystems Heidelberg, Germany) equipped with an imaging system.

Most of the green fluorescence overlapped with the ER (SupFig. 1). It seems that most of the fusion protein expressed remained in the ER as the green fluorescence was not detected in the plasma membrane in either case. It was supposed that the insect cells might force secretory proteins to stay in cell membranes or at the cell surface. Sometimes expression of secretory or transmembrane protein was sometimes enhanced in insect cells by co-expression of a chaperone in the ER.

SupFig. 1. Confocal microscopy analysis of BmMNPV-CP/+38-GGT2- or BmMNPV-CP/+16-GGT2-infected cells. Virus-infected cells were cultivated for 5 days, and were stained with ER-Tracker and observed by Confocal laser microscope. Bars denote 10 μ m.

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