A THESIS

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Construction and Characterization of Novel Baculovirus Expression Vector System Using *Bombyx mori* Nucleopolyhedrovirus

누에나방(*Bombyx mori*) 핵다각체병바이러스를 이용한 새로운 베큘로바이러스 발현시스템 구축과 특성 구명

By

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Major in Entomology

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ABSTRACT

A novel recombinant bacmid, bEasyBm, that enables the easy and fast generation of pure recombinant baculovirus without any purification step was constructed. In the bEasyBm, attR recombination sites were introduced to facilitate the generation of a recombinant viral genome by *in vitro* transposition. Moreover, an extracellular RNase gene from *Bacillus amyloliquefaciens*, *barnase*, was expressed under the control of the *Cotesia plutellae* bracovirus early promoter to negatively select against the non-recombinant background. The bEasyBm bacmid could be replicated only in host insect cells when the barnase gene was replaced with the gene of interest by *in vitro*

transposition. When bEasyBm was transposed with pDualBac-EGFP, the resulting recombinant virus, EasyBm-EGFP, showed high levels of EGFP expression efficiency compared to that of non-purified recombinant virus BmGOZA-EGFP, which was constructed using the bBmGOZA system. In addition, no non-recombinant backgrounds were detected in unpurified EasyBm-EGFP stocks. Based on these results, a high-throughput system for the generation of multiple recombinant viruses at a time was established.

Also, an easy, fast and mass purification system was developed using baculovirus expression vector system (BEVS) in which Expression by translational fusion of the polyhedrin-enhanced green fluorescence protein (EGFP) led to the formation of granular structures and these fluorescent granules were easily precipitated by high-speed centrifugation. Cry1Ac foreign protein fused with the partial polyhedrin and EGFP gene at the C-terminus, including an enterokinase (EK) site between EGFP and Cry1Ac protein, was expressed in insect cells. Cells infected by BmPolh19EG-1Ac or BmPolh32EG-1Ac produced fluorescent granules. The Cry1Ac fusion protein was purified from granule-containing cells in three steps: cell harvest, sonication, and EK digestion. Through final enterokinase digestion, Cry1Ac presented mainly in the supernatant, and this supernatant fraction also showed a pure Cry1Ac band. These results suggest that the combined procedure of polyhedrin fusion expression and enterokinase digestion can be used for rapid and easy purification of other proteins.

Key words: baculovirus expression vector system, EasyBm, *in vitro* transposition, barnase, protein purification, polyhedrin-EGFP fusion protein, enterokinase

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INTRODUCTION

Baculovirus expression vectors have been extensively used for the expression of foreign genes because they enable high-level expression and support most eukaryotic post-translational modifications (O'Reilly, 1997). A number of methods for the convenient and rapid generation of recombinant baculoviruses expressing a gene of interest have been reported (Airenne et al., 2003; Possee et al., 2008; Schlaeppi et al., 2006; Yao et al., 2007). A major drawback of these methods is the tedious efforts required to purify recombinant viruses from non-recombinant backgrounds.

The recently constructed novel recombinant bacmid, bEasyBac, enables the rapid generation of recombinant virus without any processes needed to purify target recombinant viruses from a non-recombinant background (Choi et al., 2012b). In this recombinant bacmid, the barnase gene from *Bacillus amyloquefaciens*, which is lethal to insect cells, was introduced into the *Autographa californica* nucleopolyhedrovirus (AcMNPV) genome under the control of early promoters from *Cotesia plutellae* bracovirus (CpBV). In addition, bacteriophage lambda site-specific attachment (*att*) sites were introduced into the genome for the fast generation of a recombinant viral genome by *in vitro* transposition. The bEasyBac bacmid can only replicate in transfected insect cells when the CpBV promoter-barnase cassette is replaced with the gene of interest by *in vitro* transposition. This bacmid is suitable for the high-throughput expression of heterologous

genes because the replication of the non-recombinant bacmids in insect cells is blocked by host cell death at the early stage of viral replication. Therefore, time-consuming selection steps are not required.

Most baculovirus expression vectors have been based on AcMNPV or *Bombyx mori* nucleopolyhedrovirus (BmNPV). For the large-scale production of foreign proteins, BmNPV-based vectors have a unique advantage of having an advanced system, the silkworm *B. mori*, which can be used for *in vivo* expression (Kawakami et al., 2008). The silkworm can be easily mass-cultured at low cost by an automatic feeding machine and use of advanced artificial diets (Maeda, 1989).

Although methods for generation of recombinant BmNPV directly in silkworm using BmNPV bacmid have been recently reported (Lee et al., 2007; Motohashi et al., 2005), they are not suitable for the high-throughput expression of heterologous genes because these systems require time-consuming steps for purification and amplification of recombinant viruses. In this study, *att* sites and barnase gene were introduced under the control of CpBV early promoter into the BmNPV genome for the fast generation of a recombinant viral genome by *in vitro* transposition, and the effectiveness of this system for the high-throughput expression of foreign genes was investigated.

Baculovirus expression vector systems (BEVSs) typically produce soluble proteins that accumulate within the infected cell or are secreted into the growth medium (O'Reilly et al., 1992). The most common BEVS purification method is to use an affinity tag, such

as a histidinetag (His6), calmodulin-binding peptide (CBP), FLAG epitope (FLAG), streptactin-binding peptide(Strep), or hemagglutinin (HA) conjugated to a highly soluble protein like glutathione S-transferase (GST), maltose-binding peptide (MBP), thioredoxin (TRX), or the IgG-binding domain of protein A (ProA) (Abdulrahman et al., 2009; Hunt, 2005).

As the other method of purification, fusion expression of polyhedrin and a foreign protein forming the occlusion body has several advantages, including ease of isolation of the recombinant virus and purification of recombinant occlusion bodies (OBs). Furthermore, several protease cleavage recognition sites that include an enterokinase, factor Xa protease, thrombin, TEV protease, or PreScission protease site are usually inserted between the fusion and target proteins to facilitate purification of large fusion proteins (Hunt, 2005).

Recently, an improved purification system using polyhedrin fusion protein that incorporates a foreign protein within the polyhedrin matrix and forms a granular structure by self-assembly among polyhedrin has been studied (Roh et al., 2010). It was expected that introducing enhanced green fluorescence protein (EGFP) as a visible marker in fusion protein could enhance the efficiency and convenience of purification by observing the occlusion bodies fluorescing under ultraviolet (UV) light.

The polyhedrin amino acid sequence contains the KRKK sequence at positions 32-35 and functions as a minimal nuclear localization signal (NLS) (Jarvis et al., 1991).

Fusion expressions with polyhedrin amino acids 19 to 110 and 32 to 110 lead to localization of recombinant protein into the nucleus and mediate its assembly (Bae et al., 2013).

In this study, partial-length polyhedrin amino acids 19 to 110 and amino acids 32 to 110 were used for translation fusion protein which consisted of partial-length polyhedrin, EGFP, and a target protein at the N-terminus where enterokinase site was inserted between EGFP and the target protein. For the characterization, *cry* gene of *Bacillus thuringiensis* was expressed and purified by using developed BEVS and purification systems.

LITERATURE REVIEW

1. Biology of baculovirus

Baculoviriade is a large family of insect pathogenic viruses that were initially used as biopesticides to control insect pests (Contreras-Gomez et al., 2014). Baculoviruses are a large and diverse group of rod-shaped, enveloped, double-stranded DNA viruses that replicate in the nucleus of their host cells (Au et al., 2013). The viral particles range from 30 to 60 nm in diameter and from 250 to 300 nm in length. Baculovirus genome consists of a double-stranded covalently closed circular DNA that is 80 to 180 kbp in size, depending on the species (Miele et al., 2011).

Most baculoviruses with possible exceptions have two virion phenotypes, which are the occlusion-derived virions (ODVs) and the budded virions (BVs) (Jehle et al., 2006). The BVs serve to spread the virus within the host whereas ODVs ensure the survival of the virus in the external environment and contribute to its spread from insect to insect (Hunter-Fujita FR et al., 1998). ODVs are occluded in a crystalline protein matrix, the occlusion body, and set off infection in the midgut epithelium of the ingesting insect (Jehle et al., 2006). The BVs are formed after the initial infection via budding through the plasma membrane of the infected cell. They typically consist of a single nucleocapsid enveloped within a structure that is derived from the plasma membrane of the host modified by viral proteins (Jehle et al., 2006). In contrast, ODVs consist of a

single or multiple nucleocapsids contained in an envelope that differs in origin and composition relative to the envelope of the BVs (Jehle et al., 2006).

Based on the different morphologies of the occluded virion particles, baculoviruses have been divided into nucleopolyhedrovirus (NPV) and granuloviruses (GV) (Guarino, 2011; Jehle et al., 2006). Both these are specific to the larval stage of their insect hosts. The NPV occlusion body (OB) contains multiple virus particles. Some of the NPVs have a single nucleocapsid within each virus particle (SNPV), while others have multiple nucleocapsids (MNPV), depending on the virus species (van Oers, 2011). The occlusion body diameter of NPVs ranges between 0.15 and 15 μm (Hunter-Fujita FR et al., 1998). The NPVs are subdivided into type I and type II (Herniou and Jehle, 2007). The type I NPVs contain the GP64 fusion protein that is required for the virus to gain entry into the host cells and for cell-to-cell transmission (Monsma et al., 1996). Type II NPVs lack the GP64 and contain instead a generic fusion protein, the F protein, that has the same role as the GP64. The F protein is found also in betabaculoviruses, deltabaculoviruses and some vertebrate viruses (Pearson et al., 2000).

The occlusion bodies (OBs) of granuloviruses (GVs) range in length from 0.15 to about 0.3 μm and usually contain a single enveloped nucleocapsid (Hunter-Fujita FR et al., 1998). In granuloviruses, only one singly enveloped virion is occluded per inclusion body (i.e. the “capsule”) of an oval shape. The major matrix protein of GVs is granulin, a polypeptide of 25 to 30 kDa that is similar to polyhedrin. The basic structure and

composition of the virions of the GVs is very similar to that of the NPVs. The double-stranded, circular DNA genome of the GVs is similar in size (50–100 Da), density, and other hydrodynamic characteristics to the genomes of the NPVs (Miele et al., 2011).

Based on phylogenetic evidence and other characteristics, a new classification system for baculoviruses recognizes four genera (Fig. 1). These are Alphabaculovirus, Betabaculovirus, Gammabaculovirus, and Deltabaculovirus. The genus Alphabaculovirus includes all lepidopteran-specific nucleopolyhedroviruses, both the single nucleocapsid (SNPV) type and the multiple nucleocapsid (MNPV) type (Jehle et al., 2006). The genus Betabaculovirus comprises the members of the existing lepidopteran-specific genus Granulovirus. The genus Gammabaculovirus comprises the Hymenopteran-specific NPVs and the genus Deltabaculovirus is reserved for the Diptera-specific baculoviruses (Jehle et al., 2006).

Unlike in most other DNA viruses, baculovirus gene expression takes place in four phases (Jarvis, 2009; van Oers, 2011). These are the immediate-early phase, the delayed-early phase, the late phase and the very late phase. The immediate-early phase is associated with the expression of viral transregulators and genes that do not need transregulators for effective transcription. The genes expressed are mostly implicated in establishing the infection. The delayed-early phase is associated with the expression of genes implicated in the replication of the virus and manipulation of the host. The genes expressed in the delayed-early phase commonly require the presence of viral

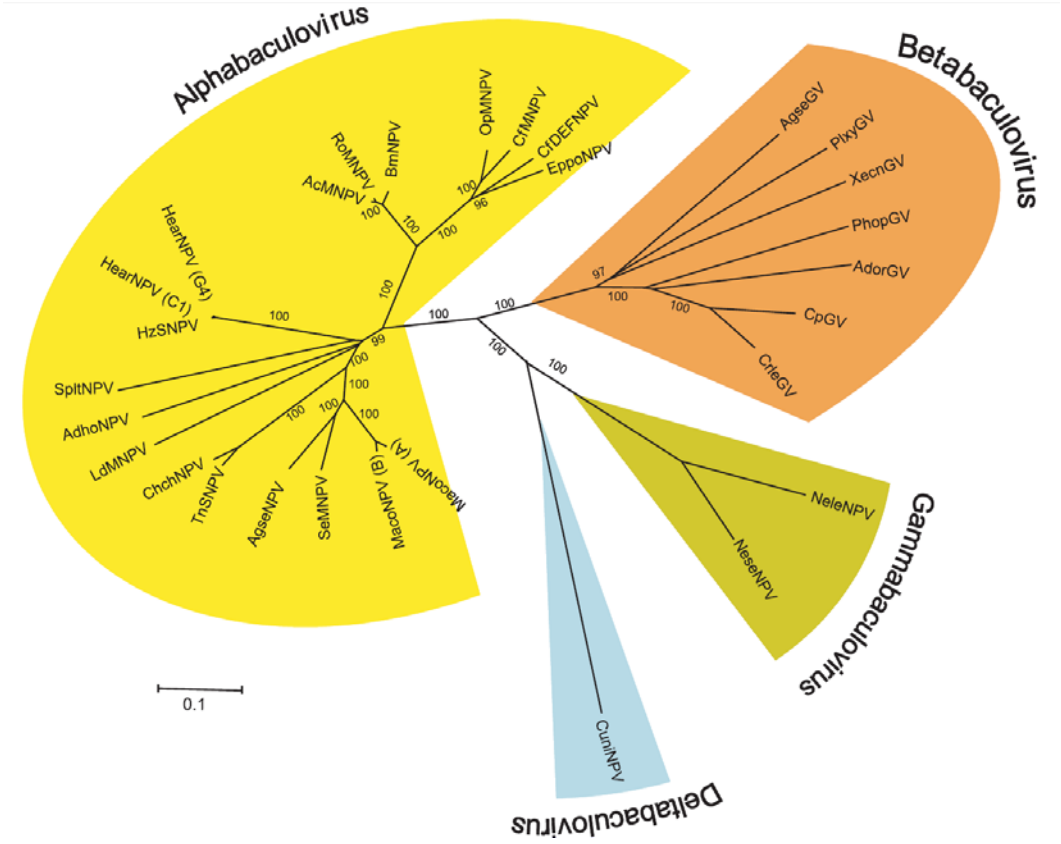


Fig. 1. Neighbour-joining tree of the amino acid alignment of 29 baculovirus core genes.

(Jehle et al., 2006)

transregulators for efficient transcription. The late phase is associated with the production of nucleocapsids. The transition from the early to the late phase is characterized by a termination of DNA replication and protein synthesis in the host cell. Budded viruses are produced and disseminate the infection throughout the host.

In the very late phase of gene expression, the virions become occluded and the proteins polyhedrin and P10 are produced in large amounts (van Oers, 2011). Polyhedrin forms the matrix of the OBs. The role of P10 is unclear, but seems to be related to the release of the OBs from the nucleus (Carpentier DCJ and LA, 2009; van Oers, 2011). Viral proteases lyse the host cell and degrade the chitinous exoskeleton of the insect so that the occluded progeny virus is dispersed into the surroundings for horizontal transmission (Contreras-Gomez et al., 2014).

2. Baculovirus expression vector system (BEVS)

BEVS have been used extensively for the production of heterologous proteins (Cruz PE et al., 1998; Kost et al., 2005; Metz and Pijlman, 2011; Morais et al., 2001; Wu et al., 1994). Many insect cell lines are highly susceptible to infection by *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) and therefore many expression vectors based on this baculovirus have been developed (Luckow et al., 1993). In fact, AcMNPV is the most widely used baculovirus vector for producing recombinant proteins in insect cells. Other baculoviruses such as *Bombyx mori* nucleopolyhedrovirus (BmNPV)

(van Oers, 2011) and *Trichoplusia ni* single nucleopolyhedrovirus (TnSNPV) (Joshi et al., 2001) are used to a lesser extent than AcMNPV.

A key characteristic of these baculoviruses is their ability to produce occlusion bodies (OBs) made of polyhedrin. Polyhedrin and also P10 are the proteins involved in the horizontal transmission of infection in a larval population, but are not needed to produce budded virions (BVs), the virus form associated with a systemic infection of the host and the insect cells in culture (van Oers, 2011). Therefore, the polyhedrin and P10 genes can be replaced by a foreign gene under the control of the very late polh and p10 promoters to drive the expression of foreign proteins in insect cells in culture (Merrington et al., 1997; Smith et al., 1983).

Heterologous genes driven by AcNPV late and very late promoters are typically abundantly expressed (Roy et al., 1997). This circumstance was originally exploited for producing the first recombinant baculoviruses by standard homologous recombination procedures using transfer plasmids carrying the foreign genes. These baculoviruses were designed to express chimeric genes consisting of the polyhedrin promoter and the foreign coding sequence. Expression cassettes comprising the gene of choice flanked by baculoviral sequences of the polyhedrin region were provided on the transfer plasmids and integrated into the circular baculovirus genome by homologous recombination in *Spodoptera frugiperda* insect cells (usually Sf9 or Sf21 cell lines). Integration occurred into the polyhedrin locus, thereby eliminating the native polyhedrin gene, and thus giving

rise to occlusion-incompetent recombinants. A recombination frequency of ~0.1% and a tedious isolation procedure of recombinant clones by their distinctive occlusion-negative plaque phenotype (visualized in plaque assay), however, made the integration process of foreign genes laborious and difficult (Trowitzsch et al., 2010).

Integration of DNA fragments into the baculoviral genome was significantly improved by using linearized rather than circular baculoviral DNA in the co-transfection experiment with the transfer plasmid harboring the gene(s) of choice (Kitts et al., 1990). Homology regions present on the baculoviral DNA and the transfer plasmid allowed integration of the expression cassettes via recombination within the insect cell. Heterologous gene products were only produced from re-circularized, replication competent viral DNA. This strategy increased the efficiency of recombinant baculovirus production from ~0.1% to ~20%. Later, this approach was further improved by using not only one but several restriction sites for linearization, thereby reducing background. One restriction site was placed within an essential viral gene, which was thus truncated. The missing piece (i.e. a complete gene) was then replenished from the transfer plasmid upon productive homologous recombination. Multiple-site linearization of parental virus DNA and concomitant functional inactivation of this essential viral gene lead to an increase in efficiency of recombinant virus production to over 90% (Kitts and Possee, 1993). A number of companies undertook to commercialize linearized baculoviruses and the corresponding transfer plasmids (Pharmingen Baculogold, Novagen BacVector series,

OET FlashBac systems and others). Still, the baculovirus plaque assay to identify positive recombinants remained an essential part of the method, somewhat complicating its handling (Trowitzsch et al., 2010).

Two further approaches to generate recombinant baculoviruses by transposition were described. In an *in vitro* transposition system (BaculoDirect), a gene of choice is transferred from a plasmid into viral DNA utilizing purified transposase. Upon transposition, a negative selection marker gene is eliminated from the parental viral DNA, thus allowing only insect cells transfected with recombined viral DNA to survive. In an alternative approach, viral DNA carrying a lethal mutation in a gene product (ORF1629) essential for virus replication is propagated in *E. coli* as a bacterial artificial chromosome (BAC) and purified. A recombination event in insect cells co-transfected with the mutated baculovirus genome and a transfer plasmid carrying the gene of interest and the wild-type viral ORF, reconstitutes the essential gene activity upon integration into the viral DNA (Zhao et al., 2003). In both cases tedious plaque assays are in theory no longer necessary. Apart from purifying clonal viral populations, the plaque assay is also commonly used to determine viral titers, i.e. the number of infectious viral particles (plaque forming units, pfu) present in a defined volume of viral supernatant. Also for this purpose, useful alternatives to the time intensive (5–7 days) plaque assay were developed based on an immunological assay or a PCR reaction, which can also be used on automated platforms (Bahia et al., 2005; Chambers et al., 2004; Kitts and Green, 1999;

Kwon et al., 2002; Lo and Chao, 2004; Shen et al., 2002). Examples of BEVS commercially available are as follows.

1) Bac-to-Bac

The Bac-to-Bac system is based upon site-specific transposition of the foreign gene to be expressed from a plasmid vector into a baculovirus genome, maintained as a bacmid in *E. coli* cells. The donor plasmids (pFastBAC series) contain the foreign gene to be expressed which is inserted under the control of a baculovirus gene promoter, usually derived from the polyhedrin gene. *E. coli* cells (DH10Bac™, Invitrogen) that contain the baculovirus genome as a bacmid are then transformed with the donor plasmid and site-specific transposition generates the recombinant bacmid DNA. Transposition occurs between the mini-attTn7 target site in the bacmid DNA and a mini-Tn7 element in the donor plasmid. Recombinant bacmid containing colonies are obtained by plating onto selective media, before amplifying a stock of *E. coli* cells. The recombinant bacmid DNA is then isolated from the *E. coli* cells using a simple alkaline lysis procedure and the resulting DNA is used to transfect insect cells. The culture medium harvested from the transfected cells contains recombinant virus only and requires no further selection prior to virus amplification (King et al., 2007).

2) BaculoDirect

BaculoDirect system(Invitrogen) is that the foreign gene is inserted directly from a Gateway entry clone into a linearized baculovirus genome that has been modified to contain attR sites. The gene of interest is simply cloned into a suitable Gateway entry vector and is then mixed with the BaculoDirect linear DNA and Gateway LR Clonase™. The resulting recircularized baculovirus DNA containing the foreign gene is then transfected into insect cells to generate recombinant virus (Fig. 2A) (King et al., 2007).

3) flashBAC

The flashBAC(Oxford Expression Technologies) system utilizes a baculovirus (AcMNPV) genome that lacks part of an essential gene (ORF 1629) and contains a bacterial artificial chromosome (BAC) at the polyhedrin gene locus, replacing the polyhedrin coding region. The essential gene deletion prevents virus replication within insect cells but the BAC allows the viral DNA to be maintained and propagated, as a circular genome, within bacterial cells (Fig. 2B) (King et al., 2007; King and Possee, 1991).

The BEVS has seen a new development in recent years with the MultiBac system, which allows the synthesis of multisubunit protein complexes using a single baculovirus vector (Berger et al., 2013). MultiBac technology can be combined with the OmniBac transfer plasmid that can be used universally to generate recombinant baculoviruses,

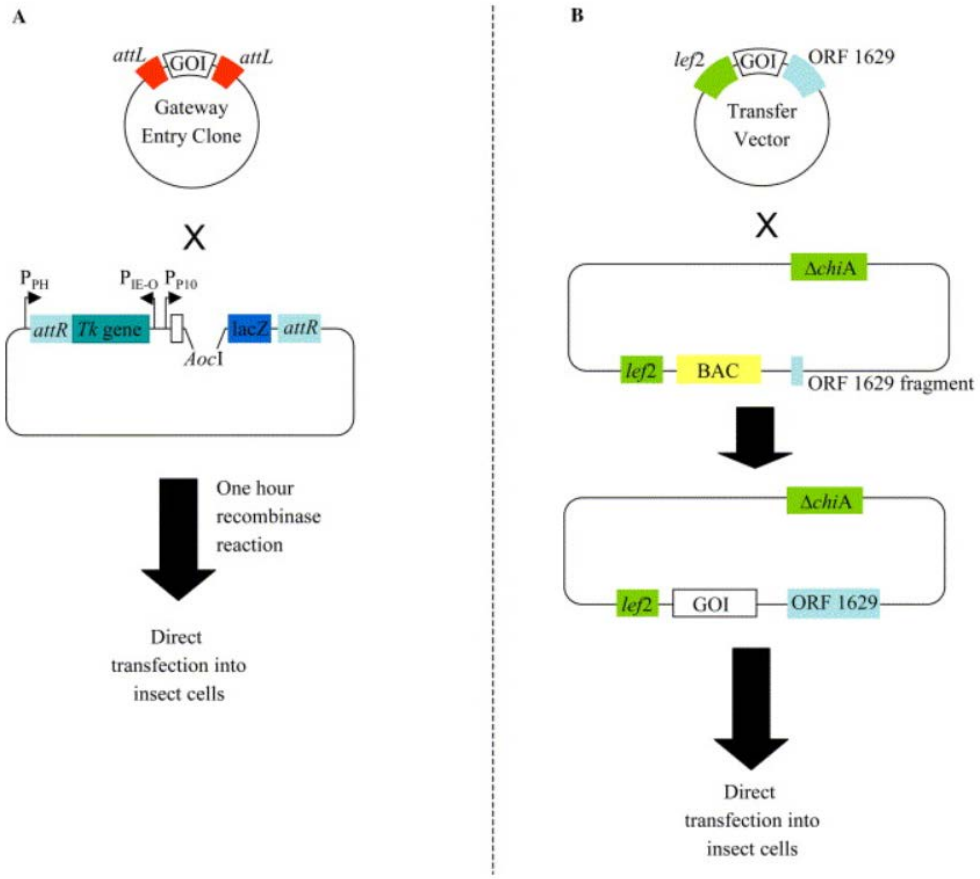


Fig. 2. Schematic representation of (A) Baculodirect and (B) flashBac recombinant baculovirus generation strategies (Hunt, 2005).

either by homologous recombination using linearized genomes or by applying bacmid technology (Raj et al., 2014). Alternatives are multilocus baculovirus vectors, in which large foreign genes can be inserted at several loci within the baculovirus genome (Galibert and Merten, 2011; Kanai et al., 2013).

3. Protein purification methods

Recombinant expressed and purified proteins are a prerequisite for a wide range of downstream applications such as activity assays, interaction analysis, and crystallization approaches (Maertens et al., 2015). Tagging protein purification is the most widely used method of all protein purification methods. Several different tags have therefore been developed to facilitate rapid single-step purification of proteins that are commercially available (Stevens, 2000; Terpe, 2003).

Tags used to improve the production of recombinant proteins can be roughly divided into purification and solubility tags. The former are used along with affinity binding to allow rapid and efficient purification of proteins, while the latter refer to tags that enhance the proper folding and solubility of a protein. Table 1 and table 2 list some of the common purification tags that are used for protein expression (Malhotra, 2009).

Tags can be placed at either the N- or C-terminus of a target protein. One advantage of placing a tag on the N-terminal end is that the construct can take advantage of efficient translation initiation sites on the tag. Solubility tags based on highly expressing proteins

Table 1. Common affinity tags (Malhotra, 2009)

Tag	Size	Affinity matrix
His-tag	6–10 His	Immobilized metal ions - Ni, Co, Cu, Zn
GST (glutathione-S-transferase)	211 aa	Glutathione resin
FLAG-tag	8 aa (DYKDDDDK) (22 aa for 3xFLAG)	Anti-FLAG mAB
Strep-II-tag	8 aa (WSHPQFEK)	Strep-Tactin (modified streptavidin)
Protein A (staphylococcal Protein A)	280 aa	Immobilized IgG
MBP (maltosebinding protein)	396 aa	Cross-linked amylose
CBP (calmodulinbinding protein)	26 aa	Immobilized calmodulin
CBD (chitin-binding domain)	51 aa	Chitin
HaloTag	~300 aa	Chloroalkane

Table 2. Common solubility tags (Malhotra, 2009)

Tag	Size	Affinity matrix
MBP	396 aa	Maltose-binding protein
NusA	495 aa	N-utilization substance
Trx	109 aa	Thioredoxin
SUMO	~100 aa	Small ubiquitin modifier
GB1	56 aa	IgG domain B1 of streptococcus Protein G
SET/SEP	<20 aa	Hydrophilic solubility enhancing peptide sequences
HaloTag	~300 aa	Mutated dehalogenase

such as MBP, Trx, and NusA are also more efficient at solubilizing target proteins when positioned at the N-terminal end (Sachdev and Chirgwin, 1998), though recent high-throughput studies have shown than the MBP tag is still quite effective when positioned at the C-terminal end (Dyson et al., 2004). Another advantage of placing a tag on the N-terminal site is that the tag can be removed more cleanly, since most endoproteases cut at or near the C-terminus of their recognition sites.

While placing a tag, care should be taken to preserve the positioning of any signal sequences or modification sites. Sequences at termini of the fusion protein should be examined for effects on the stability of the final construct, especially at the N-terminal end, which should be inspected for the host cell's N-end rule degradation signals (Bachmair et al., 1986; Wang et al., 2008). It is also useful to examine the sequence of the tagged protein for any inadvertently created interaction or cleavage sites using motif databases such as PROSITE (Malhotra, 2009).

Tags can interfere with the structure and function of the target protein, and provision must be made to remove tags after the expression and purifications steps. Multiple cleavage sites can be engineered into the expression construct to remove individual tags at different stages of purification. Table 3 lists some of the endoproteases used for tag removal (Malhotra, 2009).

By far, the most popular are glutathione S-transferase (GST) and polyhistidine (His6) tags.

Table 3. Cleavage sites used for the removal of fusion partners (Hunt, 2005; Malhotra, 2009)

Excision site	**Cleavage enzyme**
Asp Asp Asp Asp Lys↓	Enterokinase
Ile Glu/Asp Gly Arg↓	Factor Xa protease
Leu Val Pro Arg↓Gly Ser	Thrombin
Glu Asn Leu Tyr Phe Gln↓Gly	TEV protease
Leu Glu Val Leu Phe Gln↓Gly Pro	PreScission protease
Recognizes SUMO tertiary structure and cleaves at the C-terminal end of the conserved Gly–Gly sequence in SUMO	SUMO protease (catalytic core of Ulp1)

1) GST tag

GST is an abundantly expressed 26 kDa eukaryotic protein, and GST cloned from *Schistosoma japonicum* was shown to promote solubility and expression as an N-terminal fusion (Smith and Johnson, 1988). When positioned at the C-terminal end, GST is less efficient at improving protein solubility but still functions well as an affinity tag. GST binds to resin immobilized glutathione, and this property is used for affinity purification of GST tagged proteins. After the fusion protein is bound to the resin, it can be eluted under rather mild conditions using free reduced glutathione (10–40 m*M*) at neutral pH. Resins used for GST fusion purification, such as Glutathione-Sepharose beads, are relatively cheap, have high binding capacity (5–10 mg of GST/ml of resin), and can be regenerated and reused multiple times (Malhotra, 2009).

2) His-tag

The His-tag (also called 6xHis-tag) is one of the simplest and most widely used purification tags, with six or more consecutive histidine residues. These residues readily coordinate with transition metal ions such as Ni^{2+} or Co^{2+} immobilized on beads or a resin for purification. IMAC is the preferred choice as a first step during the purification of His-tagged proteins, though small batch reactions or spin columns with IMAC beads can be used for expression tests or small-scale preparations. Metal ions are immobilized using

linkages such as Ni(II)-nitrilotriacetic acid (Ni-NTA) or Co^{2+}-carboxymethyl-aspartate on resins and beads available from many commercial sources. IMAC media typically has high binding capacities (5–40 mg of His-tagged protein/ml of media), is relatively low cost, and can easily be sanitized. For nickel binding media, the metal ion can often be stripped (using buffers with EDTA) and recharged for multiple use cycles. Some cobalt based resins (such as Talon, Clontech Inc.) use proprietary linkages that are more durable and cannot be recharged; such resins can be reused three and four times, but offer the advantage of being more specific for polyhistidine tags and almost no metal leakage during protein elution. IMAC can also be used under denaturing conditions, since the His-tag does not need a specific protein conformation for metal binding; indeed, binding to IMAC resins is stronger under denaturing conditions as the His-tag becomes more exposed (Malhotra, 2009).

MATERIALS AND METHODS

1. Insects, insect cells and baculoviruses

Bombyx mori larvae were provided from National Academy of Agricultural Science in Korea and were reared on artificial diets under 25°C, 65±5% relative humidity and 16 h : 8 h light dark cycles. *B. mori* cell line Bm5 was maintained in TC-100 medium (JBI, Korea) supplemented with 10% heat-inactivated (56°C, 30 min) fetal bovine serum (FBS) (JBI, Korea), incubated at 27°C and sub-cultured every 4-5 days. Wild-type BmNPV and all of the recombinant BmNPVs used in this study were propagated in Bm5 cells maintained in TC-100 medium.

2. Plasmid, bacmid, *Escherichia coli* and cloning

Bacmid DNA and plasmid DNA were prepared by HiSpeed Plasmid Midi Kit (QIAGEN, Germany) and QIAprep Spin Miniprep Kit (QIAGEN, Germany), respectively. *E. coli* strain JM109 (Takara, Japan) was used for the transformation of high-copy plasmids. Transformation of high-copy plasmids was carried out by a heat-shock protocol using chemically treated competent cells according to the manufacturer's instruction. For transformation of low-copy bacmids, *E. coli* strain DH10b (Invitrogen, USA) was used. Low-copy bacmids were transformed into electro-competent cells by electroporation as follows. Competent cells (200 μl) were mixed with 1 μg bacmid DNA and single shocked

in a 0.1 cm electroporation cuvette (Bio-Rad, USA) using a Bio-Rad Gene Pulser under the following conditions: volts, 1.8 kV; resistance, 200 Ω; and capacitance, 25 μF. All of the restriction endonucleases and modifying enzymes were from Roche Applied Science (Germany).

3. PCR and primers

For the amplification of the barnase-barstar cassette, oligonucleotides BaBa-XhoF (5'-CCCGCTCGAGATGGCACAGGTTATCAACACG-3') and BaBa-EcoRR (5'-CCCGGAATTCTTAAGAAAGTATGATGGTGA-3') were used in the PCR. Additionally, the kanamycin resistance gene-MiniF replicon cassette was amplified using oligonucleotides Ka-F (5'-ATTGTCGCACCTGATTGCC-3') and Mini-R (5'-GTCATCTGCATCAAGAACTAG-3'). For PCR, the recombinant bacmid DNAs were subjected to 33 PCR cycles (1 min at 94°C, 30 sec at 55°C and 1 min at 72°C) followed by a 7 min final extension at 72°C using *AccuPower*® PCR Premix (Bioneer, Korea) and C1000™ Thermal Cycler (Bio-Rad, USA).

4. Construction of plasmid vectors

To construct pBmKSK3-attBLacZ, 467 bp of the *lacZ* gene fragment was amplified from pGEM-5zf(-) (Stratagene, USA) using oligonucleotides attB1LacZ-EcoRF (5'-GGCGAATTCACAAGTTTGTACAAAAAAGCAGGCTTTCCATTCGCCATTCAGG

C-3') and attB2LacZ-KpnR (5'-GGCGGTACCACCACTTTGTACAAGAAAGCTGGGTAGCG CAACGCAATTAATGTG-3') and digested with *Eco*RI. This PCR-amplified and *Eco*RI-digested attB1-*lacZ*-attB2 cassette was introduced into the *Eco*RI and *Sma*I sites of pBmKSK3 (Choi et al., 2000). The pBm101-MF plasmid was constructed by inserting the *Sac*I-digested pMiniF-Kan (Je et al., 2001) into the *Sac*I site of pBm101 (Hong et al., 2001).

5. *In vitro* transposition

The transposition reaction was carried out using Gateway® LR Clonase™ II or BP Clonase™ II Enzyme Mix (Invitrogen, USA). In the transposition reaction, 150 ng of donor vector was mixed with 150 ng of bEasyBm bacmid DNA. In total, 2 μl of LR Clonase™ II or BP Clonase™ II Enzyme Mix was added to the transposition reaction, and the mixture was mixed well by vortexing briefly twice. The reaction was centrifuged briefly and incubated at 25°C overnight (approximately 12-16 h). A total of 1 μl of proteinase K solution was added to the reaction to terminate transposition and incubated at 37°C for 10 min.

For the verification of generation efficiency of recombinant virus, bEasyBm was carried out *in vitro* transposition with pDualBac-EGFP or pDualBac-Luc as a donor vector, expressing EGFP or luciferase, respectively. The reactive solution of transposition was transfected into Bm5 cells. Homologous recombination was carried out between

bEasyBm DNA (0.25~1 ng) and pBmKSK3-EGFP transfer vector (2~4 μl) (Table 4). Reaction solution was injected into 5th instar larvae of *B. mori* to verify the generation of recombinant virus.

6. Transfection

Approximately 5×10^4 Bm5 cells per well were seeded in a 24-well tissue culture plate and incubated at 27°C for 30 min to allow the cells to attach. In total, 11 μl of LR recombination reaction was added to 100 μl of incomplete TC-100 medium in a polystyrene tube. In another polystyrene tube, 10 μl of Cellfectin^{TM} (Invitrogen, USA) was mixed with 100 μl of incomplete TC-100 medium. The two solutions were gently mixed, and the matrix was incubated at room temperature for 45 min. The attached cells were washed once with 1 ml of incomplete TC-100 medium and refreshed with 0.5 ml of the same medium. The 1/5 or 1/10 fractions of Cellfectin-DNA complexes were added drop-wise per well to the medium covering the cells while the plate was gently swirled. After incubation at 27°C for 5 h, each well was refreshed with 2 ml of TC-100 medium supplemented with 10% FBS, and the transfected cells were incubated at 27°C. At 5 days post-transfection (p.t.), the transfection supernatant was harvested by centrifugation at 500×g for 5 min and stored at 4°C.

7. RNA and RT-PCR

Table 4. Quantities of bEasyBm DNA and transfer vector used for the generation of recombinant virus in larvae of *Bombyx mori*

bEasyBm (μg)	pBmKSK3-EGFP (μg)	Injection volume (μl)
1	4	200
0.5	4	200
0.5	2	200
0.25	4	200
0.25	2	200

Total RNA was isolated from Bm5 cells transfected with recombinant bacmids at 4 days p.t. using TRIZOL Reagent (Invitrogen, USA) according to the manufacturer's instructions. RT-PCR was carried out using *AccuPower*® RT/PCR Premix (Bioneer, Korea) in a 20 μl volume according to the manufacturer's instructions. To amplify the *gp64* gene, the oligonucleotides gp64-F (5'-CCAAACATGAACGAAGTC-3') and gp64-R (5'-GACACTGTGCTTCATCG-3') were used. The *vp39* gene was amplified using oligonucleotides Bm-vp39-F (5'- AGGCGGCTACACCTCCA-3') and Bm-vp39-R (5'-GTATGATGCAAGCCGAA-3').

8. Infection of cells with baculoviruses

Insect cells were seeded in a 6-well plate at a density of 5×10^5 cells/well and incubated at 27°C for 30 min to allow cells to attach. Attached cells were washed twice with 3 ml of incomplete TC-100 medium and inoculated with each recombinant virus at a multiplicity of infection (MOI) of 10 plaque-forming units (PFU)/cell. After incubation at 27°C for an hour with gentle rocking, the medium was replaced with 3 ml of fresh medium and incubated at 27°C. At 3 days post-infection (p.i.), infected cells were harvested by centrifugation at 500×g for 5 min, washed twice with phosphate buffered saline (PBS) and stored at 4°C.

9. Quantification of EGFP

Insect cells infected with recombinant viruses were harvested and washed, as mentioned above. Cells were sonicated in PBS containing 1/100 volume of protease inhibitor cocktail (Sigma, USA) and centrifuged at 22,000×g for 15 min. The fluorescence of the resulting supernatant was measured using a SPECTRAmax GEMINI XS Microplate Spectrofluorometer (Molecular Device Inc., USA) with an excitation filter of 450 nm and an emission filter of 510 nm. EGFP levels were calculated from measured relative fluorescence units (RFU) and compared with a standard curve ($r^2 = 0.999$), which was plotted as a log function of 2-fold serial dilutions of EGFP (BD Biosciences, USA). SigmaStat Ver. 2.0 software (SPSS Inc., USA) was used for detecting significant differences by Duncan's multiple range test.

10. Luciferase activity assay

Insect cells infected with recombinant virus were harvested and washed, as mentioned above. The intracellular luciferase assay was performed using a Luciferase Assay System (Promega, USA) according to the manufacturer's protocols.

11. Expression and purification of Polyhedrin-EGFP fusion protein

Bm5 cells (5×10^6) were infected with recombinant virus in 100-mm diameter dish at 10 MOI (multiplicity of infection). Infected cells at 3 days p.i. were harvested by centrifugation at 1,000×g for 10 min, and suspended with 5 ml of PBS. The suspension

was sonicated at 30 sec (output level 3, duty cycle 30%) using Sonifer 450 (Branson, USA), pellet was harvested by centrifugation at 25,000×g for 10 min. The pellet was washed two times with 0.01% of Tween20, and suspended with 1 ml of PBS. EK Max^{TM} enterokinase (Invitrogen, USA) was treated on the suspended pellet at the rate of 1 unit per 30μg (the amount of total protein in the PBS resuspension), and incubated at 37° for 16 hr. Supernatant was harvested by centrifugation at 25,000×g for 10 min.

12. SDS-PAGE and measurement of EGFP

The cellular lysates at 3 days p.i. and the samples collected at the purification steps were analyzed on 12% SDS-PAGE gels and stained with Coomassie Brilliant Blue. The fluorescence of the collected samples was measured using a SPECTRAmax GEMINI XS Microplate Spectrofluorometer as mentioned above.

13. Bioassay

For the evaluation of insecticidal activity of *Bacillus thuringiensis* Cry1Ac purified from granule of polyhedrin-EGFP-Cry1Ac fusion protein, bioassay was carried out against diamondback moth (*Plutella xylostella*). Purified Cry1Ac endotoxin protein was diluted with 1 M of NaCl and 0.001% Triton X-100. The endotoxin protein was divided in five different concentration of 5 ng/cm^2 to 70 ng/cm^2. 100 μl of each dilution solution was treated on a disc of chinese cabbage leaf (1×1 cm^2), in which thirty 3rd–instar larvae

were introduced for 24 hours. After then, the larvae were laid on artificial diet without any treatment. The mortality for *P. xylostella* was calculated by counting the dead larvae at 24 h intervals for 3 days.

RESULTS

1. Construction of EasyBm system for high-throughput recombinant virus generation

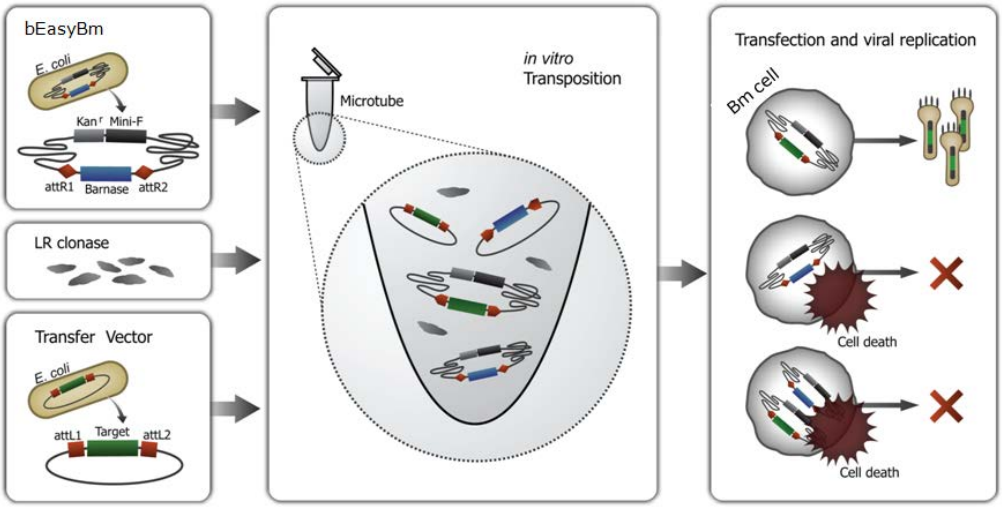
1) Construction of recombinant virus, vBpLacZ

To express a target gene utilizing high-throughput recombination, the EasyBm system was developed by *in vitro* transposition using a transposable element (att). The flow chart of the EasyBm system is presented in Fig. 3. For this purpose, pBmKSK3-attBLacZ was co-transfected with bBpGOZA DNA, and recombinant virus vBpLacZ was purified from infected Bm5 cells by plaque purification (Fig. 4).

vBpLacZ has *lacZ* gene in place of the *polyhedrin* gene between the att sites on BmNPV genome for *in vitro* transposition, which can be expressed in *E. coli*. In addition, the other *polyhedrin* gene was introduced at the *p10* gene site, allowing expression of Polyhedrin under the control of the p10 promoter. The internal structure of vBpLacZ genome was confirmed by PCR using specific primers (Fig. 5), restriction endonuclease digestion pattern (Fig. 6), and sequence analysis.

Transfection of genomic vBpLacZ DNA into Bm5 cells resulted in generation of inclusion body with the same structure as wild-type BmNPV, supporting the idea that virus could replicate normally in the host cells (Fig. 7).

2) Construction of transfer vector, pBm101-MF



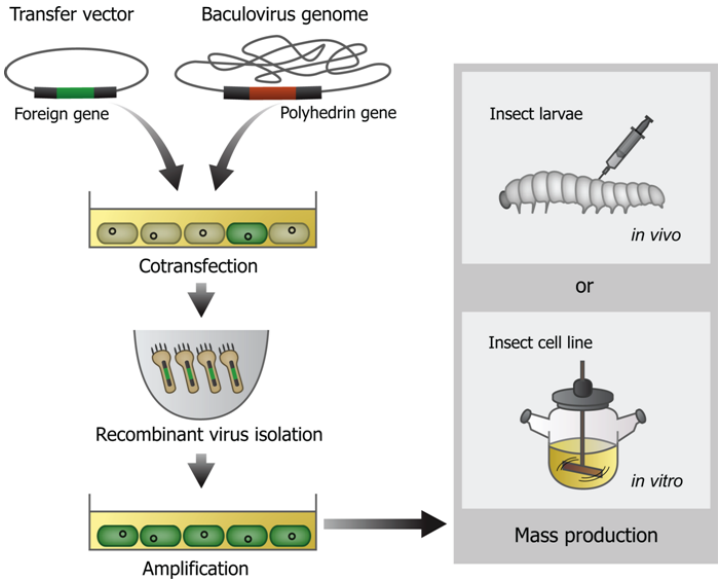


Fig. 3. Flow chart of high-throughput recombinant virus generation using EasyBm system.

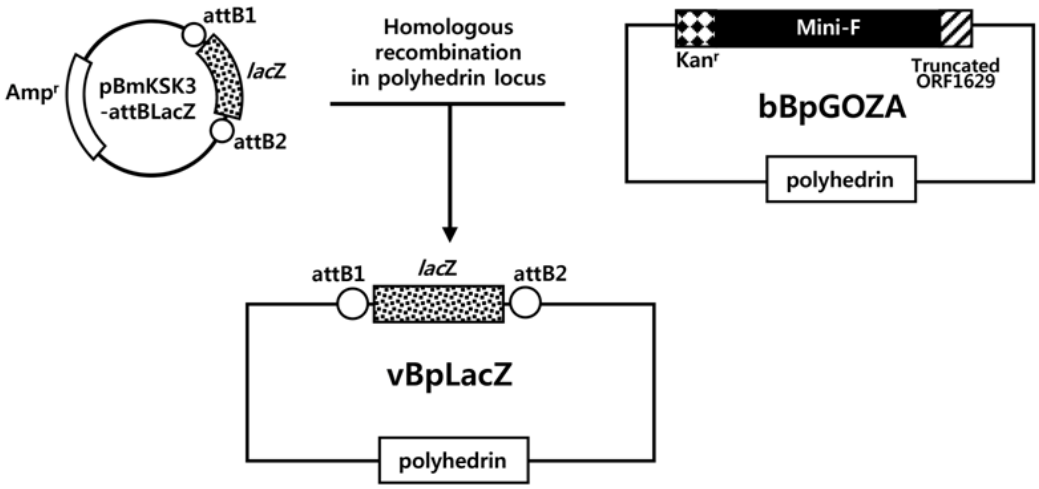


Fig. 4. Construction map of the recombinant virus, vBpLacZ.

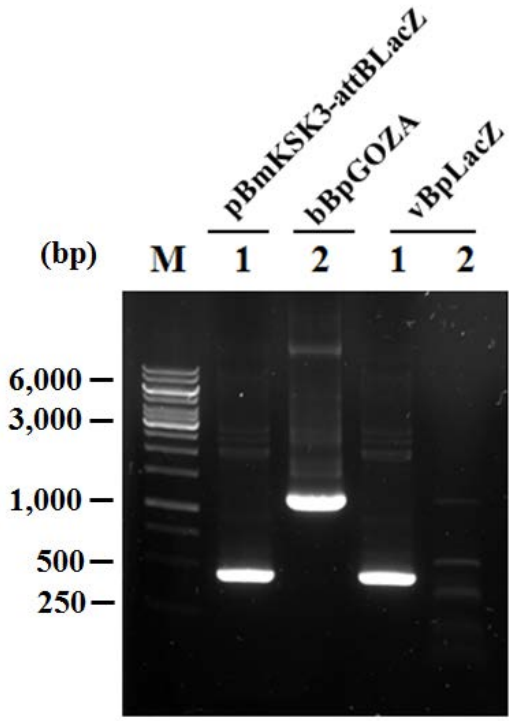


Fig. 5. Verification of internal structure of the recombinant virus, vBpLacZ, by PCR using specific primer sets. Lane: M, 1kp ladder; 1, primers LacZF and LacZR 2, primers MiniFKan-Fw and MiniFKan-Re.

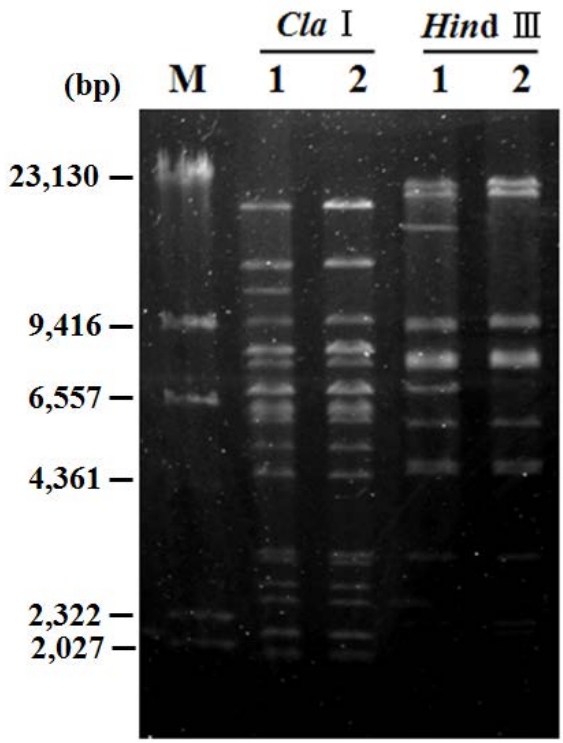


Fig. 6. Confirmation of genome structure of the recombinant virus, vBpLacZ, by restriction endonuclease digestion pattern. Lane: M, λ DNA digested with *Hind*III; 1, bBpGOZA; 2, vBpLacZ.

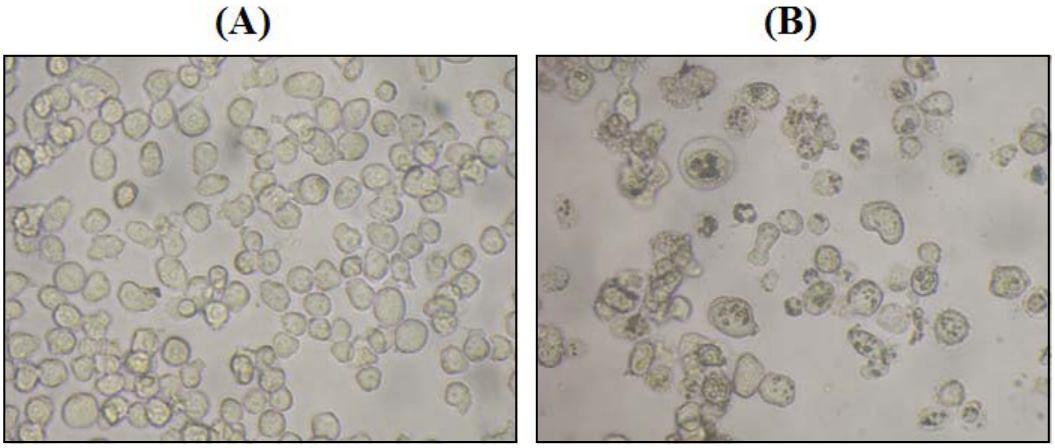


Fig. 7. Phase-contrast microscopy of mock-infected Bm5 cells (A) and Bm5 cells infected with vBpLacZ (B) (Magnification: × 600).

A transfer vector, pBm101-MF, was constructed by insertion the cassette of MiniF-replicon and kanamycin resistance gene in pMini-Kan into the pBm101 transfer vector, which was based on p10 promoter (Fig. 8). pBm101-MF can be replicated and selected in *E. coli* on account of MiniF-replicon and kanamycin resistance gene in the p10 region. The internal structure of pBm101-MF was confirmed by restriction endonuclease digestion pattern (Fig. 9) and sequence analysis.

3) Construction of recombinant bacmid, bBmTenLacZ

Recombinant virus vBpLacZ DNA was co-transfected with pBm101-MF into Bm5 cells. After 5 days, viral DNA was extracted from the transfected cells and used to transform *E. coli* strain DH10b cells. The recombinant bacmid, bBmTenLacZ, was purified by the selection of blue colonies in blue/white screening of the transformants (Fig. 10).

bBmTenLacZ can selectively replicate in *E. coli* because of the presence of the replication origin of *E. coil* (MiniF-replicon) and kanamycin gene in the p10 region of the BmNPV genome. Furthermore, bBmTenLacZ has *lacZ* gene expressed in *E. coli* between *in vitro* transposition sites (attB) in place of *polyhedrin* gene, allowing a target bacmid to be selected in blue/white screening. However, Polyhedrin is not generated in Bm5 cells transfected with bBmTenLacZ due to the absence of *polyhedrin* gene on the bacmid, although virus can normally replicate (Fig. 11).

The internal structure of bBmTenLacZ genome was confirmed by PCR using specific primers (Fig. 12), restriction endonuclease digestion pattern (Fig. 13), and sequence analysis.

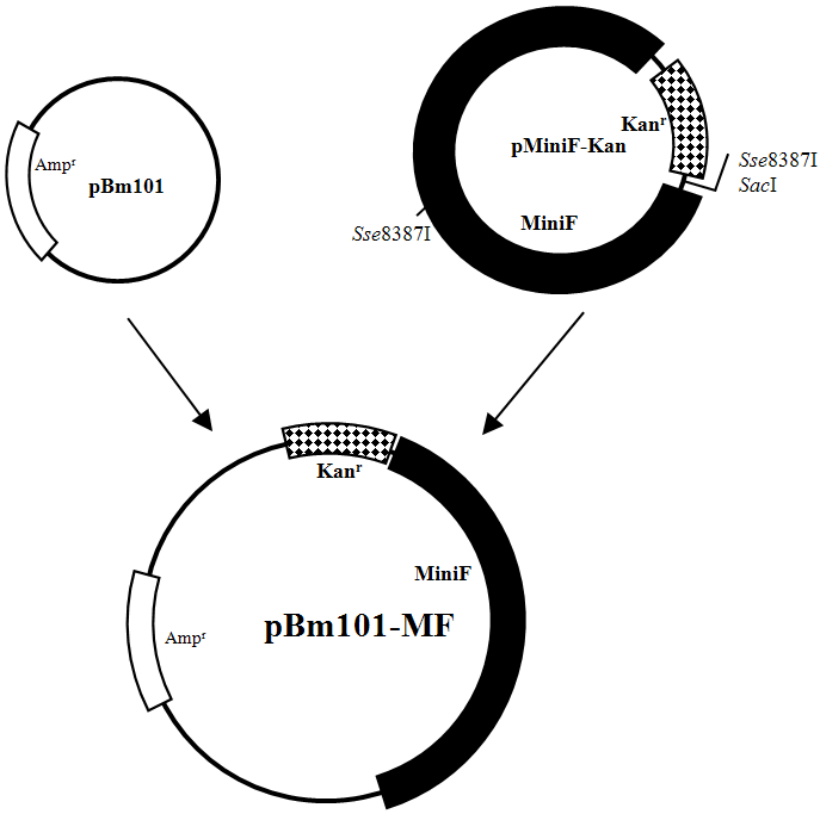


Fig. 8. Construction map of the transfer vector, pBm101-MF.

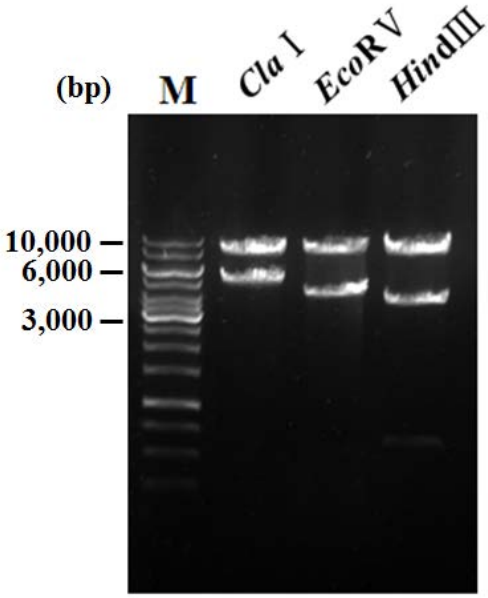


Fig. 9. Confirmation of internal structure of the transfer vector, pBm101-MF, by restriction endonuclease digestion pattern. Lane: M, 1 Kb DNA Ladder.

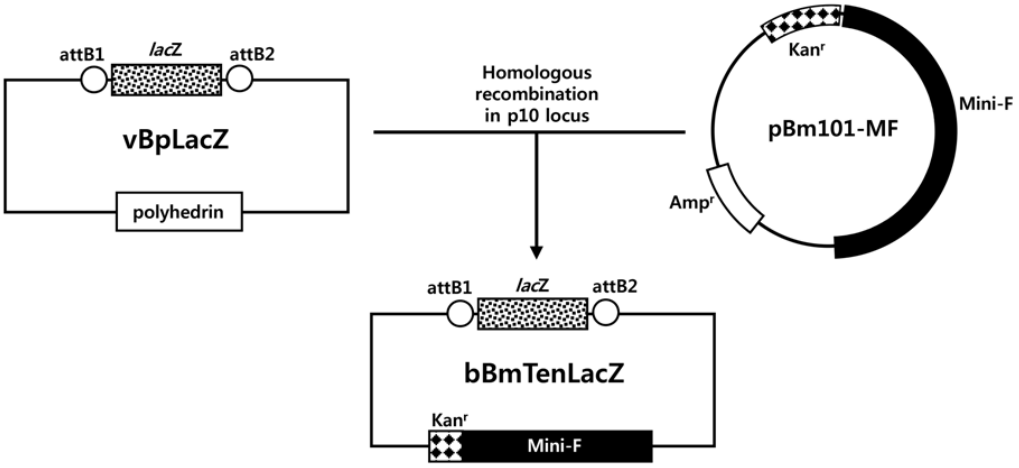


Fig. 10. Construction map of the recombinant bacmid, bBmTenLacZ.

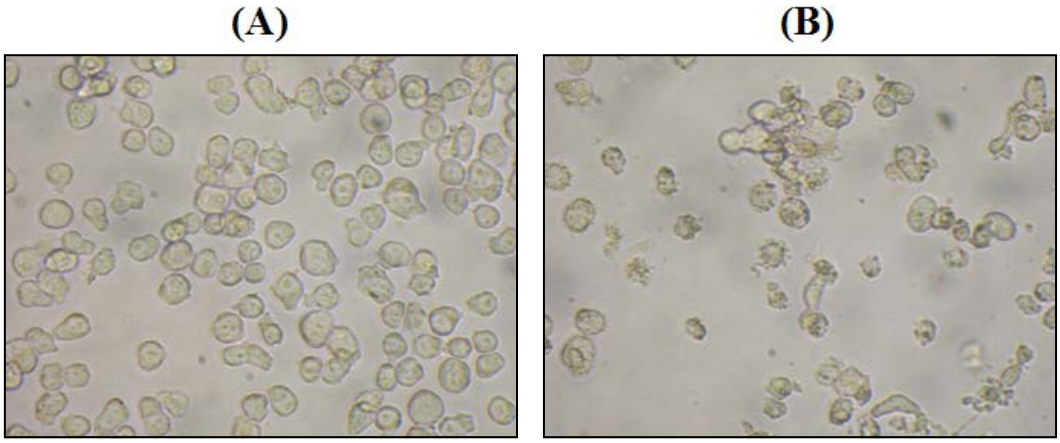


Fig. 11. Phase-contrast microscopy of mock-infected Bm5 cells (A) and Bm5 cells infected with bBmTenLacZ (B) (Magnification: × 600).

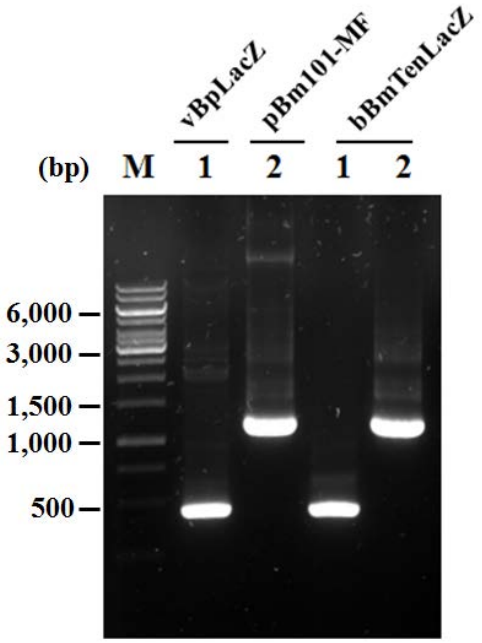


Fig. 12. Verification of internal structure of the recombinant bacmid, bBmTenLacZ, by PCR using specific primer sets. Lane: M, 1kp ladder; 1, primers LacZF and LacZR; 2, primers MiniFKan-Fw and MiniFKan-Re.

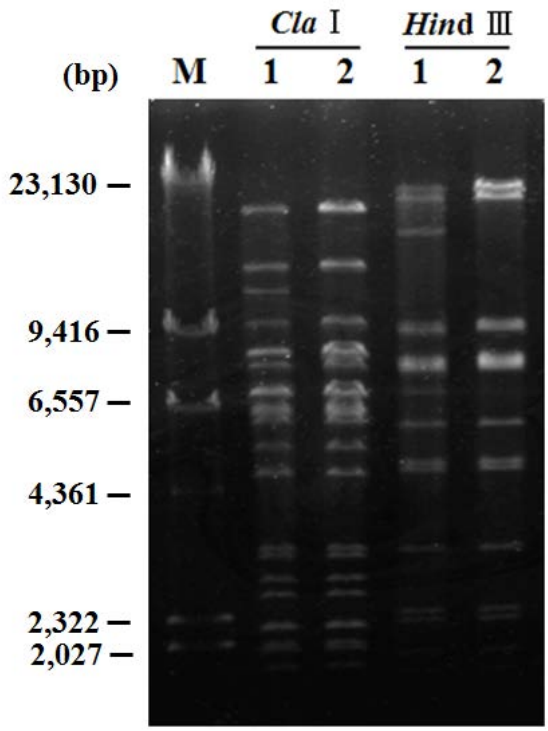


Fig. 13. Confirmation of genome structure of the recombinant bacmid, bBmTenLacZ, by restriction endonuclease digestion pattern. Lane: M, λ DNA digested with *Hind*III; 1, bBpGOZA; 2, bBmTenLacZ.

4) Construction of recombinant bacmid, bEasyBm

bBmTenLacZ including *lacZ* gene between *in vitro* transposition sites (attB) was transposed using BP Clonase with a donor vector, pDONR-3005ProBarnaseII-Amp, harboring the *Cotesia plutellae* bracovirus (CpBV) ORF3005 promoter-Barnase cassette between *in vitro* transposition sites (attP). The transposed DNAs were transformed into *E. coli* DH10b, and the recombinant bacmid, bEasyBm, was constructed by the selection of white colonies in blue/white screening of the transformants (Fig. 14).

bEasyBm can replicate selectively in *E. coli* because the p10 region of BmNPV genome harbors the replication origin of *E. coli* (MiniF-replicon) and *kanamycin* gene. In addition, the *polyhedin* gene has been replaced with ORF3005 promoter-Barnase cassette between *in vitro* transposition sites (attR). Hence, the replication of bEasyBm in insect cells is blocked by host cell death at the early stage of viral replication.

The internal structure of the bEasyBm genome was confirmed by PCR using specific primers (Fig. 15), restriction endonuclease digestion pattern (Fig. 16), and sequence analysis.

2. Activities and efficiency of bEasyBm system

1) Cytopathic effect of barnase gene

To examine the cytopathic effect of barnase expressed by bEasyBm under the control of the CpBV ORF3005 promoter, bEasyBm DNA was tranfected into Bm5 cells, and the growth of transfected cells was investigated (Fig. 17). While a reduced cell growth rate was observed at 1-2 days post-transfection (p.t.), no obvious difference on cell growth

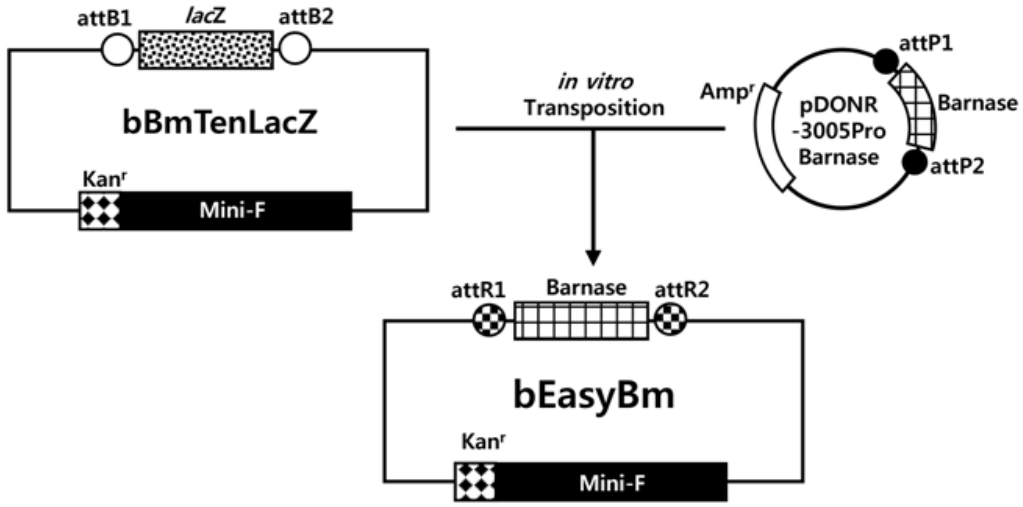


Fig. 14. Construction map of the recombinant bacmid, bEasyBm.

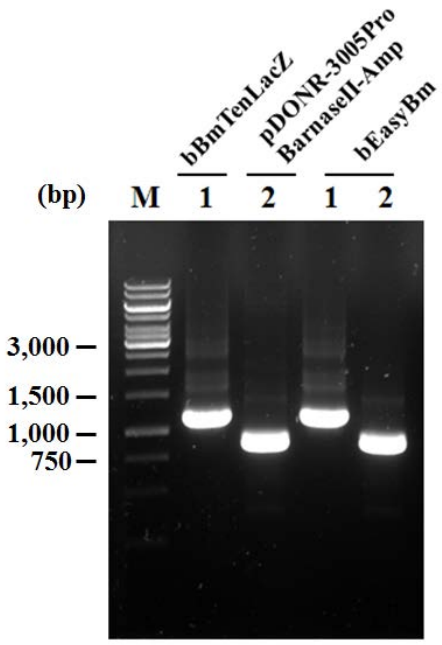


Fig. 15. Verification of internal structure of the recombinant bacmid, bEasyBm, by PCR using specific primer sets. Lane: M, 1kp ladder; 1, primers MiniFKan-Fw and MiniFKan-Re; 2, primers BaBa-XhoF and BaBa-EcoRR.

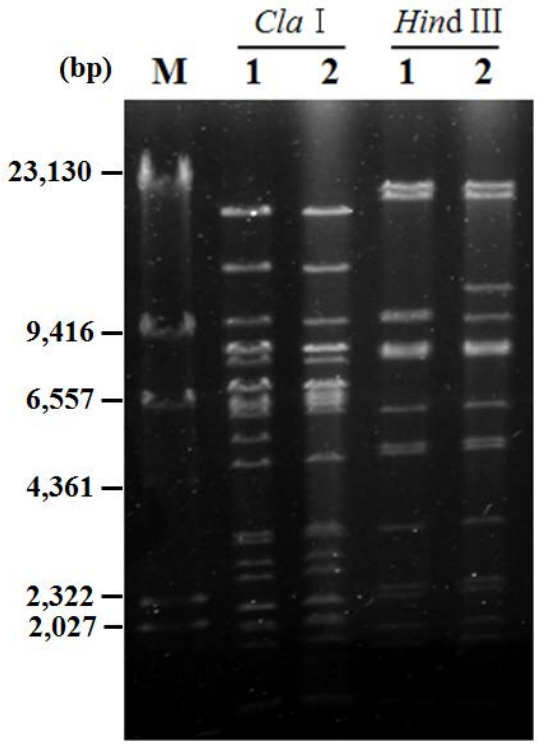


Fig. 16. Confirmation of genome structure of the recombinant bacmid, bEasyBm, by restriction endonuclease digestion pattern. Lane: M, λ DNA digested with *Hind*III; 1, bBmTenLacZ; 2, bEasyBm.

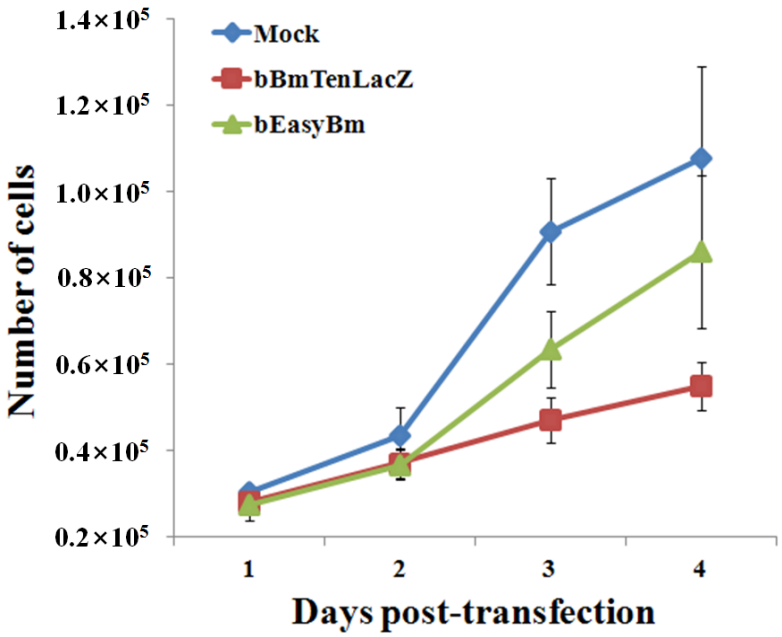


Fig. 17. Growth of Bm5 cells transfected with the recombinant BmNPV genome, bEasyBm, expressing Barnase under the control of CpBV ORF3005 promoter.

rate was observed after 3 days p.t. in Bm5 cells transfected with bEasyBm compared with mock-transfected Bm5 cells. In contrast, Bm5 cells transfected with bBmTenLacZ showed dramatically reduced growth rate compared with mock-transfected cells at all of the time points investigated.

2) Cytotoxic effect of barnase on baculovirus genome

To investigate the replication of bEasyBm in insect cells, bEasyBm or bBmTenLacZ DNA was transfected into Bm5 cells to produce recombinant virus particles, and the resulting transfection medium was passaged four times in Bm5 cells. At each passage, the total RNA was extracted from infected Bm5 cells, and RT-PCR analysis using primer sets specific for the *gp64* and *vp39* gene, which are essential for BmNPV replication, was performed. Although both of the genes were amplified from the total RNAs extracted from Bm5 cells infected with bBmTenLacZ, neither of the *gp64* or *vp39* gene-specific primer sets amplified and specific fragments from the total RNA extracted from Bm5 cells infected with bEasyBm (Fig. 18).

3) Recombination efficiency of recombinant bacmid, bEasyBm, through *in vitro* transposition

To assess the generation efficiency of recombinant virus using bEasyBm as a parental viral genome by *in vitro* transposition between attR sites contained in bEasyBm

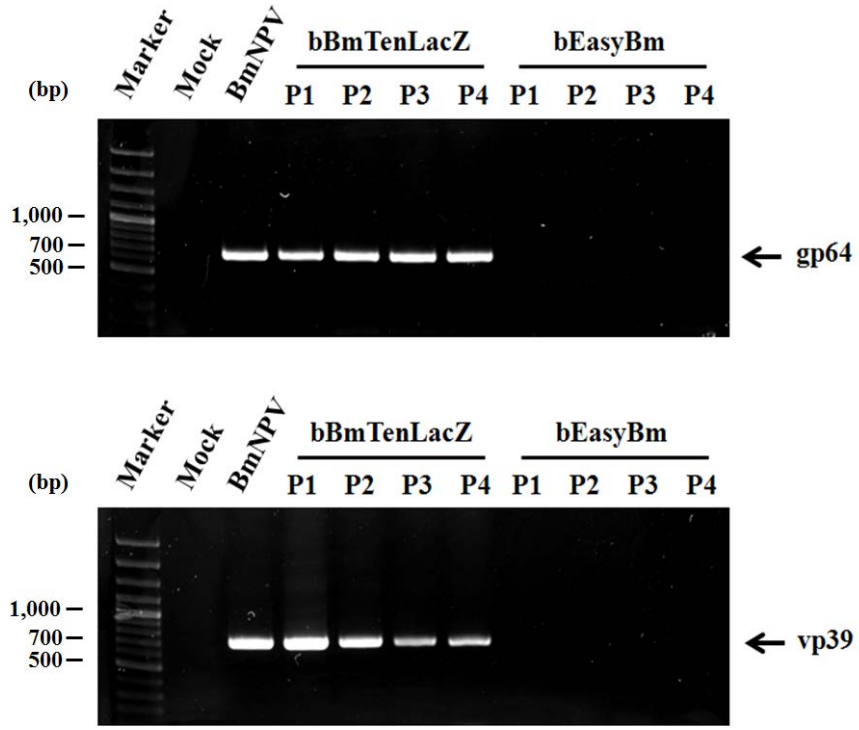


Fig. 18. Detection of viral mRNAs from Bm5 cells transfected with the recombinant BmNPV genome, bEasyBm, along serial passage. Passage numbers are indicated as 'Pn', 'P' for passage and 'n' for number.

and attL sites contained in the donor vector pDualBac, an LR recombination reaction was performed with 150 ng of bEasyBm DNA purified from *E. coli* and 150 ng of donor vector, pDualBac-EGFP or pDualBac-Luc, in which the *egfp* or *luciferase* reporter gene, respectively, was expressed under the control of the *polyhedrin* promoter. When the 1/5 fractions (equivalent to 30 ng of bEasyBm and donor vector) were transfected into Bm5 cells, recombinant viruses EasyBm-EGFP and EasyBm-Luc expressing EGFP and luciferase, respectively, were generated with an efficiency of approximately 83.3-91.7% (Fig. 19).

To verify the absence of non-recombinant backgrounds in non-purified viral stocks, the transfection media of the recombinant viruses EasyBm-EGFP and BmGOZA-EGFP, which were generated by co-transfection of bBmGOZA and pDualBac-EGFP into Bm5 cells, were passaged five times in Bm5 cells. After the fifth passage, the viral DNAs of EasyBm-EGFP and BmGOZA-EGFP were extracted from infected Bm5 cells, and PCR analysis was carried out for the barnase gene (EasyBm-EGFP) or the kanamycin resistance gene-Mini-F replicon (Mini-F) cassette (BmGOZA-EGFP), which had been replaced by the *egfp* gene through *in vitro* transposition or homologous recombination. Although the kanamycin resistance gene-Mini-F replicon cassette was amplified from viral DNA of BmGOZA-EGFP, barnase gene-specific primers did not amplify any specific fragments from the viral DNA of EasyBm-EGFP after the first passage (Fig. 20).

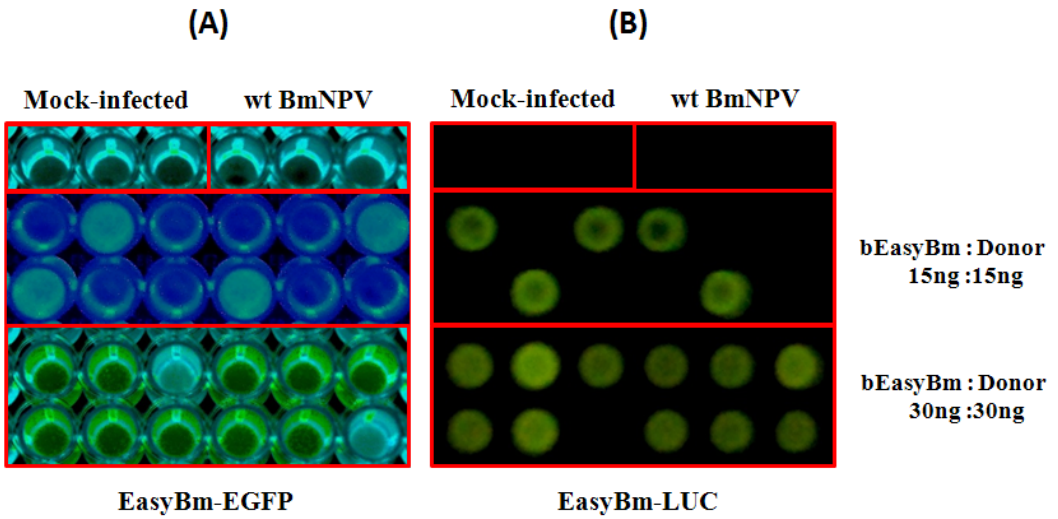
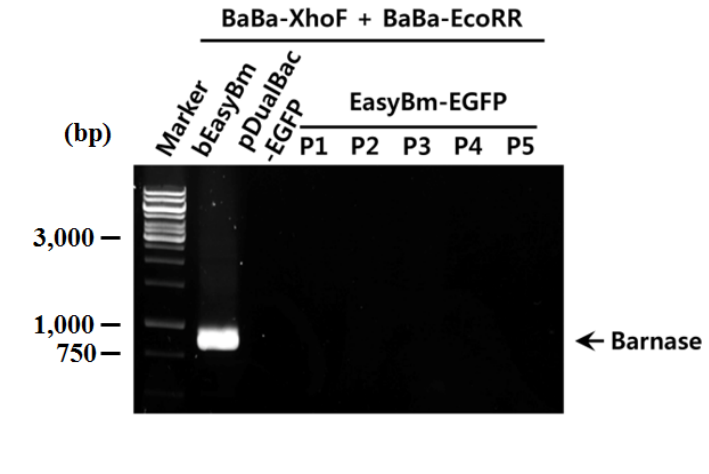
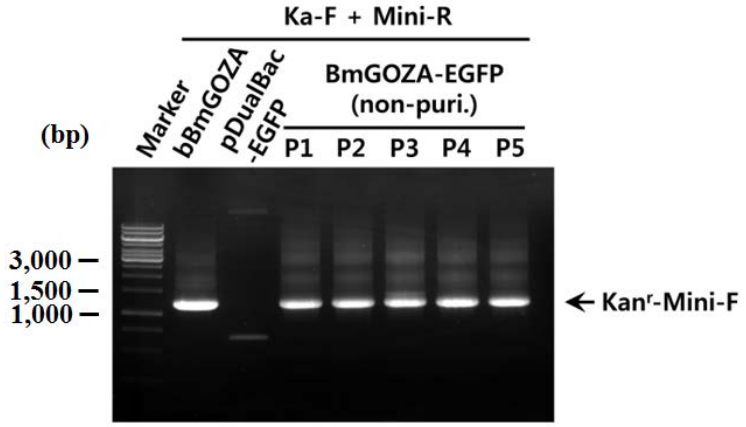


Fig. 19. *in situ* Assay of EGFP (A) and luciferase (B) in Bm5 cells infected with EasyBm-EGFP and EasyBm-Luc, respectively. Quantities of bEasyBm genome and donor vector used for transposition were indicated at right of each pannel.



(A)



(B)

Fig. 20. Verification of the genomic structure of the recombinant viruses EasyBm-EGFP (A) and BmGOZA-EGFP (B) throughout serial passages. Passage numbers are indicated as 'Pn', 'P' for passage and 'n' for number.

4) Expression efficiency of foreign genes using bEasyBm through *in vitro* transposition

To investigate the expression efficiency of foreign genes using bEasyBm, Bm5 cells were infected with recombinant viruses (EasyBm-EGFP and non-purified BmGOZA-EGFP), and the expression of EGFP in infected Bm5 cells during serial passages was compared (Fig. 21). While Bm5 cells infected with non-purified BmGOZA-EGFP showed dramatically reduced levels of EGFP after the 3rd passage, Bm5 cells infected with EasyBm-EGFP showed high levels of EGFP from 1st passage.

5) Generation efficiency of foreign genes using *in vivo* system, *B. mori*

For the investigation of the generation efficiency of recombinant virus *in vivo* system, homologous recombination was carried out between transfer vector pBmKSK3-EGFP and bEasyBm DNA. The transfection reaction mixture was injected into 5^{th}-instar *B. mori* larvae in various quantities. Haemolymp of the larvae was harvested to verify the generation of recombinant virus at 7 days after their injection. RT-RCR revealed recombinant virus was observed as range of 16.7~100% (Fig. 22). Especially, recombinant viruses were generated 100% in efficiency when 1 μg of bEasyBm DNA and 4 μg of transfer vector were co-transfected for homologous recombination.

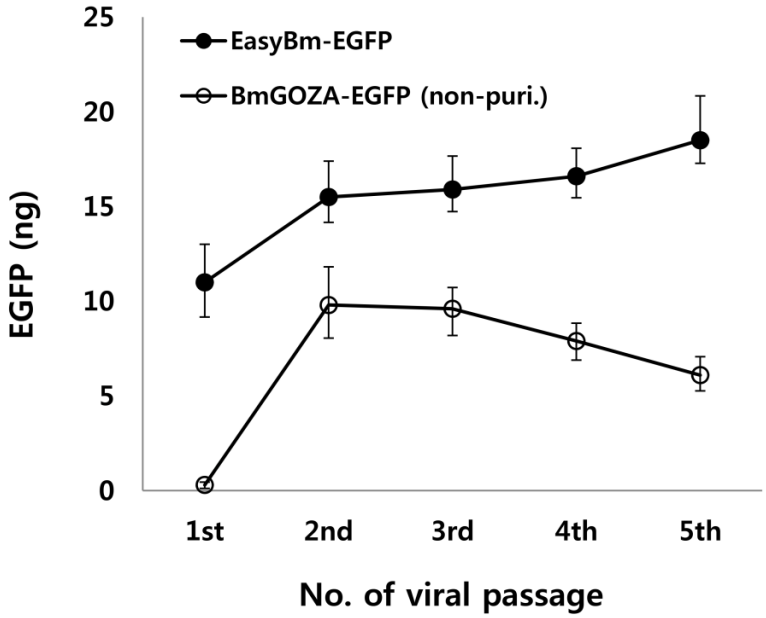
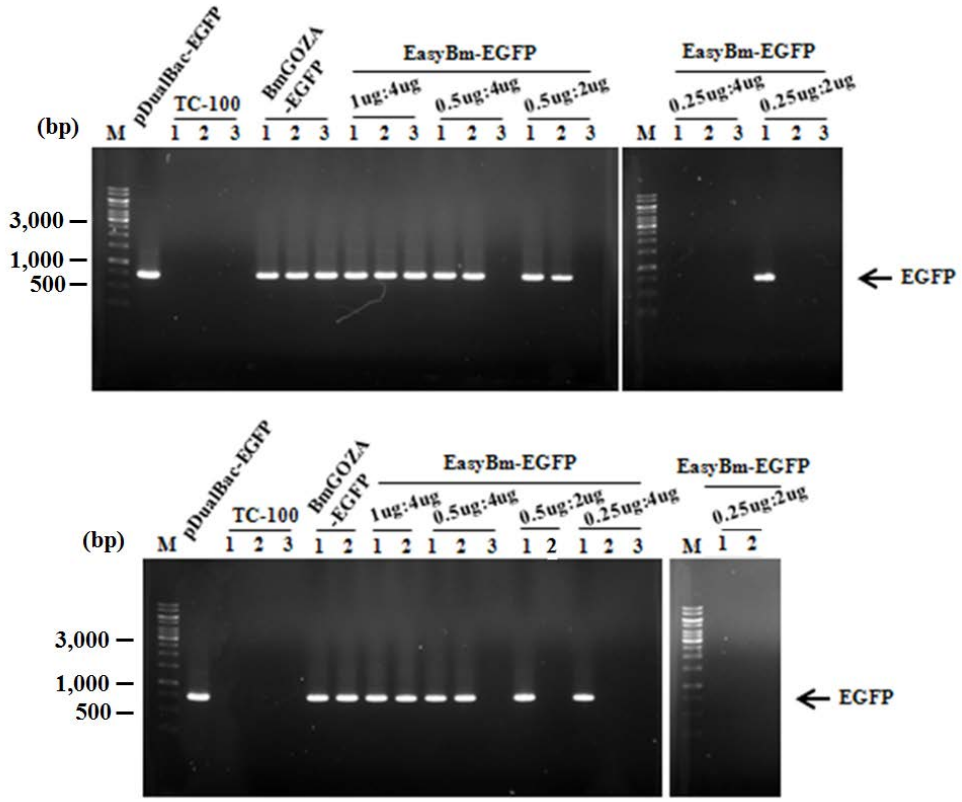


Fig. 21. Expression of EGFP in Bm5 cells infected with the recombinant BmNPVs along serial passage. EasyBm-EGFP and BmGOZA-EGFP were constructed using bEasyBm and bBmGOZA as parent viral genomes, respectively.



(A)

bEasyBm (μg)	pBmKSK3-EGFP (μg)	Generation efficiency (%)
1	4	**100**
0.5	4	66.7
0.5	2	60.0
0.25	4	**16.7**
0.25	2	20.0

(B)

Fig. 22. Detection (A) and generation efficiency (B) of the recombinant virus EasyBm-EGFP in 5th instar larvae of *B. mori* injected with transfection reaction mixture of bEasyBm DNA and pBmKSK3-EGFP with various quantities.

3. Construction of easy purification system using polyhedrin-EGFP fusion protein

1) Construction of transfer vector pPolh19-110-EGEK

For the easy purification system that recombinant protein can be purified abundantly and rapidly by simple methods, such as sonication and centrifugation, through the monitoring of the granules of polyhedrin-EGFP fusion protein, a transfer vector for the expression of fusion protein between polyhedrin-EGFP and target protein was constructed (Fig. 23). In this study, two kind of transfer vector was constructed to minimize the size of fusion protein (Fig. 24)

1 kb of AcMNPV polhyhedrin amino acid 19-110+EGFP+EK site cassette (Polh19-110-EGEK) was amplified in three steps using PCRs with specific primers (Fig. 25). For the PCR, pB9-Ac19-110-eGFP kindly provided from Prof. Soo-Dong Woo (Chungbuk National University) was used as a template, and PCR was performed with primers as follows: Ac19-110-BglF (5'-CCGAGAT CTATGAAGTACTACAAAAATTT AGGTGC-3') for forward primer, EGFP-EK-R1 (5'-TCATCGTCATCCTTGTACAGC TCGTCCATGCCG-3'), EGFP-EK-R2 (5'-CCCTTGTCATCGTCATCGTCATCCTTGT ACAGCTCG-3') and EGFP-EK-PstR (5'-CCGCTGCAGG ACCCTTGTCATCGTCATC GTCATC-3') for reverse primers, respectively. The PCR amplified Polh19-110-EGFP was cloned into pGEM-T Easy vector to construct pGT-Polh19-110-EGEK(+) (Fig. 26).

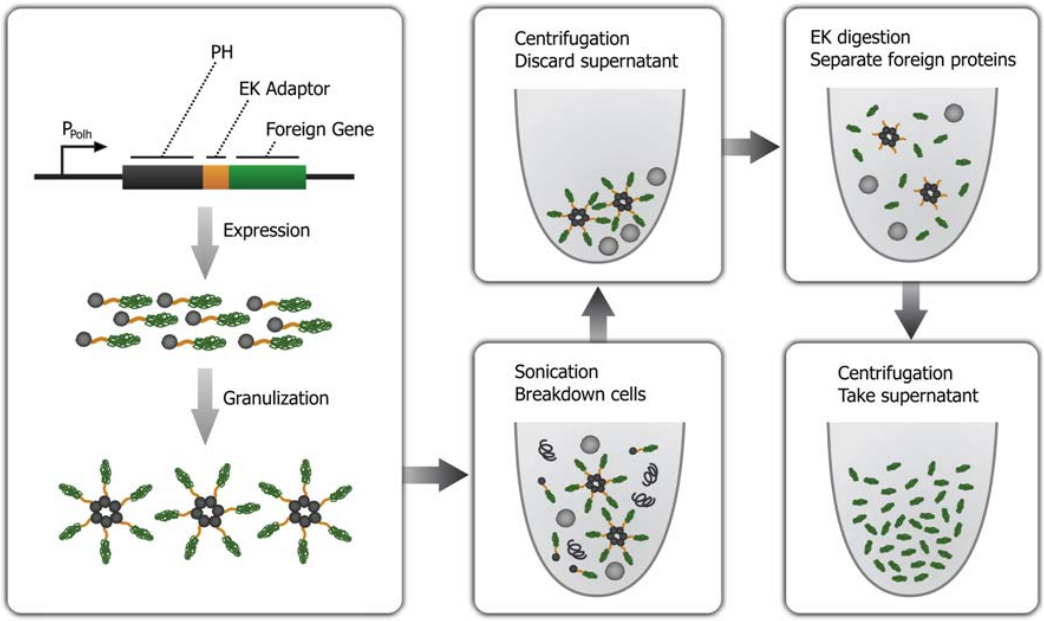


Fig. 23. Flow chart of easy purification system using polyhedrin fusion protein.

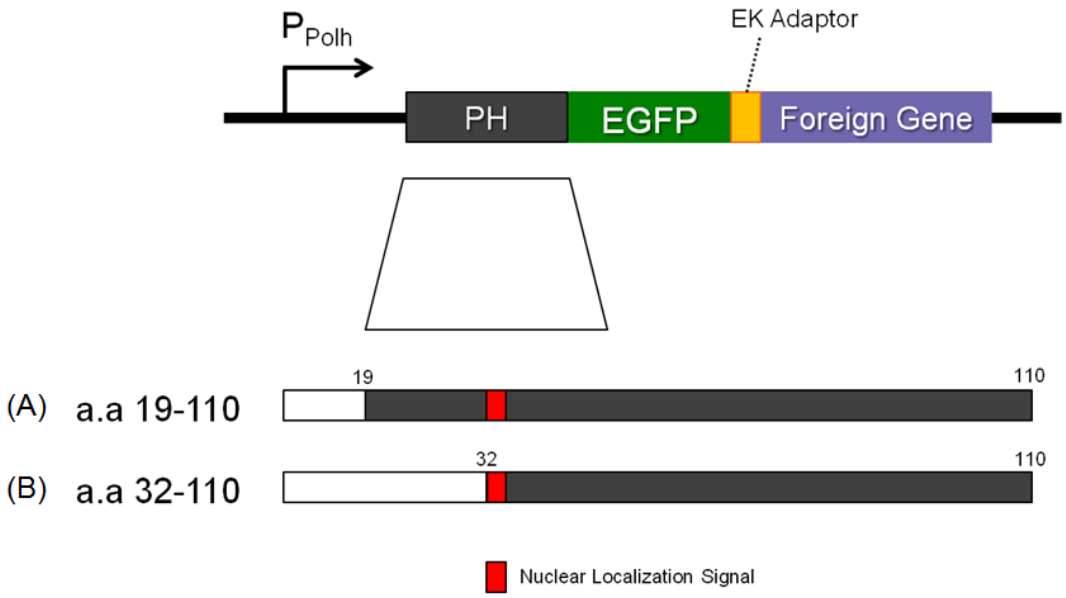


Fig. 24. Structure of transfer vectror expressing partial polyhedrin protein using amino acid 19 to 110 (A) and amino acid 32 to 110 (B).

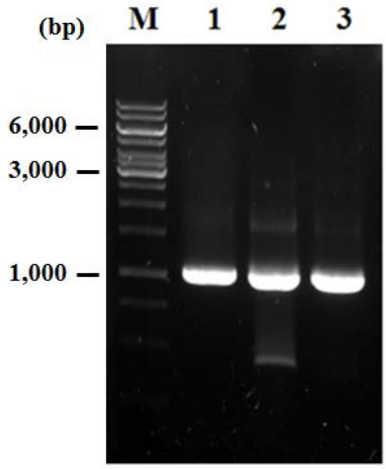


Fig. 25. PCR amplification of AcMNPV Polh. 19-110 + EGFP + EK site cassette using specific primer sets. Lane: M, 1kp ladder; 1, primers Ac19-110-BglF and EGFP-EK-R1; 2, primers Ac19-110-BglF and EGFP-EK-R2; 3, primers Ac19-110-BglF and EGFP-EK-PstR.

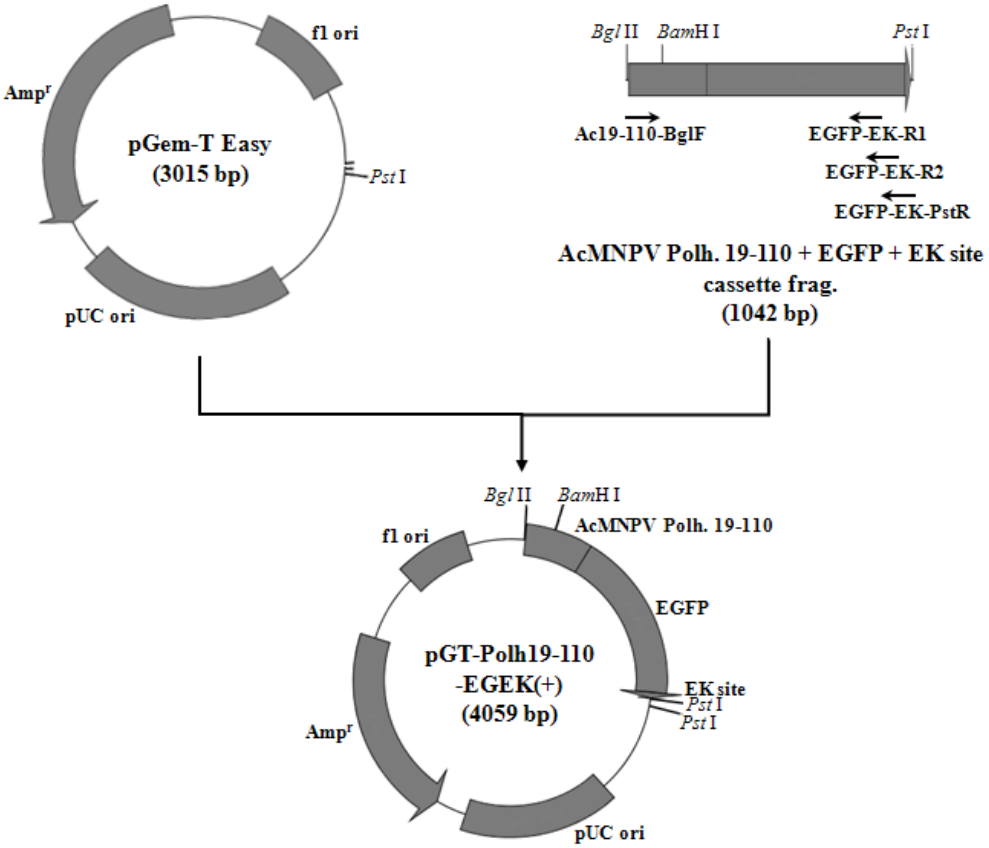


Fig. 26. Construction map of the transfer vector, pGT-Polh19-110-EGEK(+). The 1.04 kb of PCR amplified AcMNPV Polh. 19-110+EGFP+EK site cassette fragment was inserted into pGem-T Easy to obtain pGT-Polh19-110-EGEK(+). Solid arrows indicate primer positions used in PCR amplification.

pGT-Polh19-110-EGEK(+) has a fusion structure between 19 to 110 amino acid of AcMNPV polyhedrin acid in N-terminus of full-length EGFP and enterokinase site for simple separation of target protein in C-terminus of that. The internal structure was confirmed by restriction endonuclease digestion pattern and sequence analysis.

About 1 kb of Polh19-110-EGEK cassette eluted from pGT-Polh19-110-EGEK(+) by restriction enzymes (*Bgl*II and *Pst*I) was inserted into pDualBac digested by *Bam*HI and *Pst*I, resulting in a transfer vector for pPolh19-110-EGEK (Fig. 27)

2) Construction of transfer vector pGT-Polh32-110-EGEK(+)

To minimize the size of fusion protein, other transfer vector was constructed using amino acids 32 to 110 of AcMNPV polyhedrin as a fusion partner. 1 kb of AcMNPV polhyhedrin amino acid 32-110+EGFP+EK site cassette (Polh32-110-EGEK) was amplified in three steps by using PCR with specific primers (Fig. 28). For PCR, pB9-Ac32-110-eGFP kindly provided from Prof. Soo-Dong Woo (Chungbuk National University) was used as a template, and PCR was performed with the following primers: Ac32-110-BglF (5'-CCGAGATCTATGAAGCGCAAGAAGCACTT CG-3') for forward primer, EGFP-EK-R1, EGFP-EK-R2 and EGFP-EK-PstR for reverse primers, respectively. The PCR amplified Polh32-110-EGFP was cloned into pGEM-T Easy vector to construct pGT-Polh32-110-EGEK(+) (Fig. 29).

About 1 kb of Polh32-110-EGEK cassette eluted from pGT-Polh32-110-EGEK(+) by

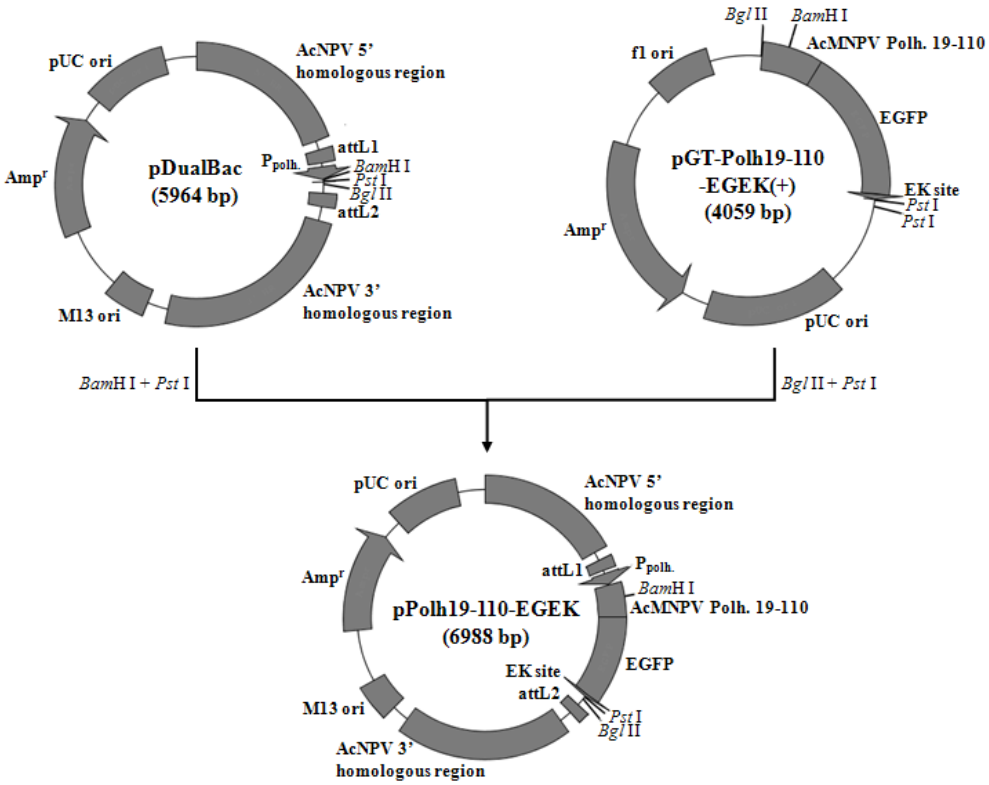


Fig. 27. Construction map of the transfer vector, pPolh19-110-EGEK. The 1.04 kb of AcMNPV Polh. 19-110+EGFP+EK site cassette fragment was inserted into pDualBac to obtain pPolh19-110-EGEK.

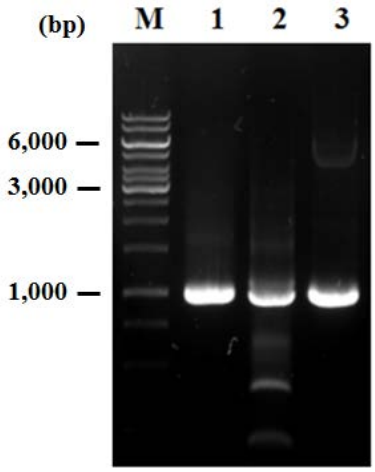


Fig. 28. PCR amplification of AcMNPV Polh. 32-110+EGFP+EK site cassette using specific primer sets. Lane: M, 1kp ladder; 1, primers Ac32-110-BglF and EGFP-EK-R1; 2, primers Ac32-110-BglF and EGFP-EK-R2; 3, primers Ac32-110-BglF and EGFP-EK-PstR.

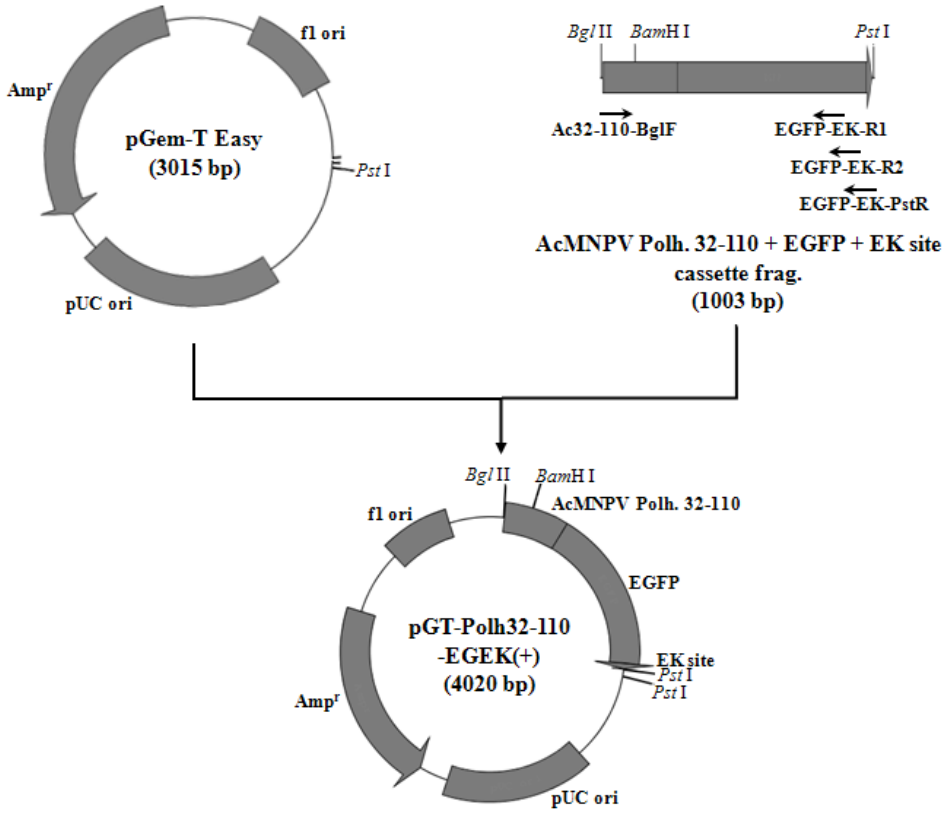


Fig. 29. Construction map of the transfer vector, pGT-Polh32-110-EGEK(+). The 1.0 kb of PCR amplified AcMNPV Polh. 32-110+EGFP+EK site cassette fragment was inserted into pGem-T Easy to obtain pGT-Polh32-110-EGEK(+). Solid arrows indicate primer positions used in PCR amplification.

restriction enzymes *Bgl*II and *Pst*I was inserted into pDualBac and digested using *Bam*HI and *Pst*I, resulting in a transfer vector for pPolh32-110-EGEK (Fig. 30). pPolh32-110-EGEK has a fusion structure between amino acids 32 to 110 AcMNPV polyhedrin in the N-terminus of full-length EGFP and enterokinase site for pure separation of target protein in the C-terminus. The internal structure was confirmed by restriction endonuclease digestion pattern and sequence analysis (Fig. 31).

3) Purification efficiency of foreign protein in *in vitro* system using insect cells

For the investigation of purification efficiency of foreign protein using granule of fusion protein between polyhedrin-EGFP and target foreign protein, *B. thuringiensis* (Bt) endotoxin Cry1Ac as a foreign protein was cloned into transfer vectors pPolh19-110-EKEG or pPolh32-110-EKEG. To construct a transfer vector expressing polyhedrin-EGFP-Cry1Ac fusion protein, *cry1Ac* gene was amplified from pBt91 vector (kindly provided from Dr. Beom Seok Park, RDA) by PCR using primers, Fw-ModCry1Ac (5'-GATGGACAACAACCCAAAC-3') and Re-ModCry1Ac (5'-GGCGAATTCTTAAAGA TTGTACTCAGCCTC-3'). The amplified *cry1Ac* gene was digested by *EcoR*I, and inserted into the *Box*I and *EcoR*I site of pPolh19-110-EGEK or pPolh19-110-EGEK to obtain pPolh19-110-EGEK-ModCry1Ac or pPolh32-110-EGEK-ModCry1Ac. The internal structure was confirmed by restriction endonuclease digestion pattern and sequence analysis (Figs. 32 and 33).

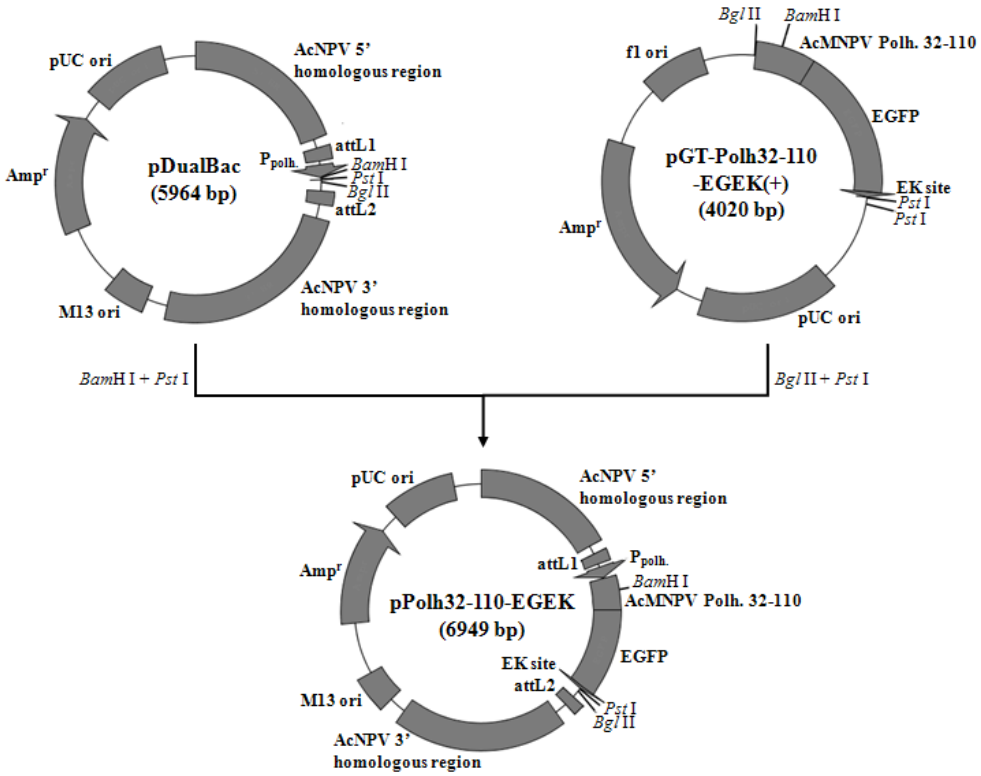


Fig. 30. Construction map of the transfer vector, pPolh32-110-EGEK. The 1.0 kb of AcMNPV Polh. 32-110+EGFP+EK site cassette fragment was inserted into pDualBac to obtain pPolh32-110-EGEK.

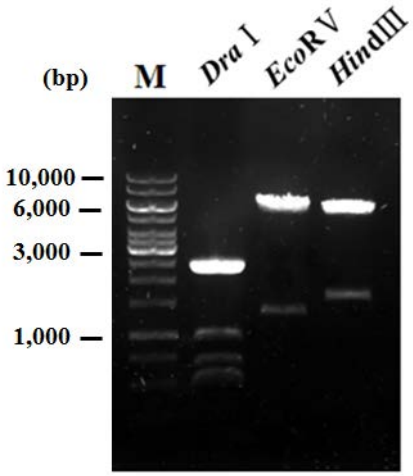
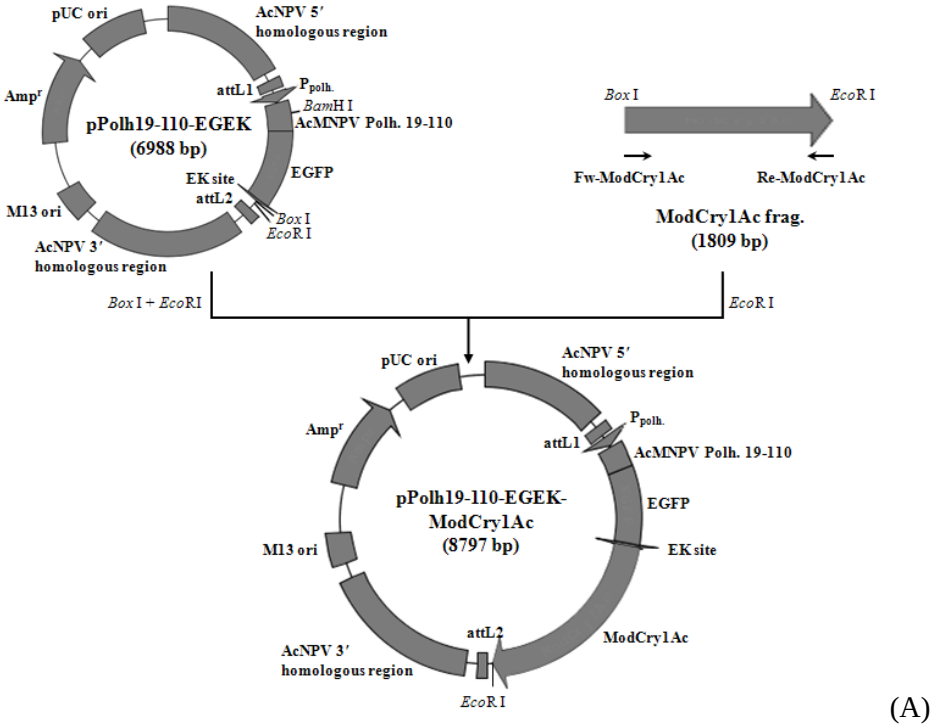
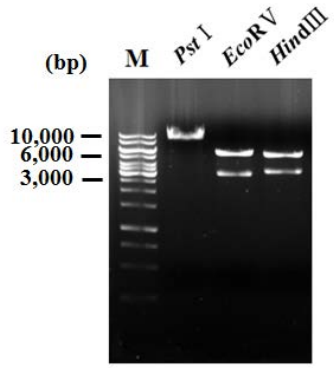


Fig. 31. Confirmation of internal structure of the transfer vector, pPolh32-110-EGEK, by restriction endonuclease digestion pattern. Lane: M, 1 Kb DNA Ladder.

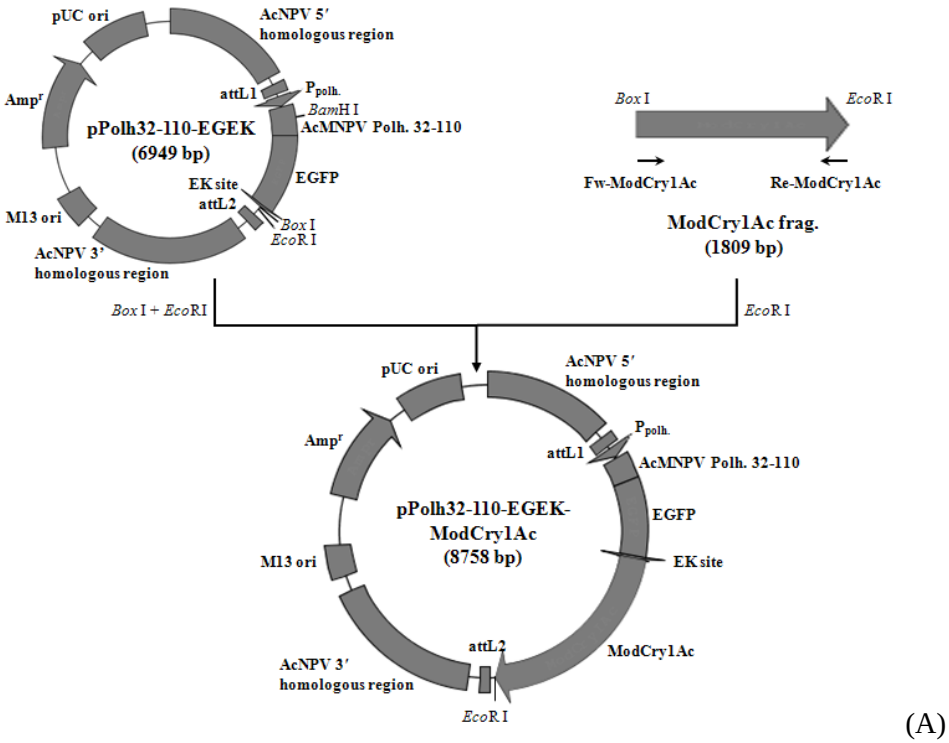


(A)

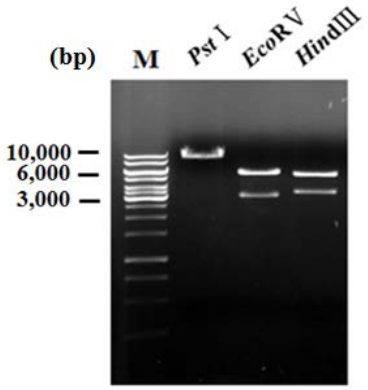


(B)

Fig. 32. Construction map (A) and restriction endonuclease digestion pattern (B) of the pPolh19-110-EGEK-ModCry1Ac. The 1.81 kb of PCR amplified ModCry1Ac gene fragment was inserted into pPolh19-110-EGEK to obtain pPolh19-110-EGEK-ModCry1Ac. Solid arrows indicate primer positions used in PCR amplification.



(A)



(B)

Fig. 33. Construction map (A) and restriction endonuclease digestion pattern (B) of the pPolh32-110-EGEK-ModCry1Ac. The 1.81 kb of PCR amplified ModCry1Ac gene fragment was inserted into pPolh32-110-EGEK to obtain pPolh32-110-EGEK-ModCry1Ac. Solid arrows indicate primer positions used in PCR amplification.

For the construction of BmPolh19EG-1Ac or BmPolh32EG-1Ac expressing polyhedrin-EGFP-Cry1Ac fusion protein, *in vitro* transposition and transfection with pPolh19-110-EGEK-ModCry1Ac or pPolh32-110-EGEK-ModCry1Ac was performed using the EasyBm system. The internal structure was confirmed by restriction endonuclease digestion pattern and PCR analysis (Figs. 34 and 35).

As a result of infecting recombinant virus BmPolh19EG-1Ac or BmPolh32EG-1Ac into Bm5 cells, strong fluorescence was observed in the infected cells with BmPolh19EG-1Ac or BmPolh32EG-1Ac after 3 days of infection (Fig. 36). In the cells infected with BmEGFP that did not express Polyhedrin but formed soluble EGFP under the control of the polyhedrin promoter, EGFP was evenly dispersed on their nucleous and cytoplasm. In cells by infected BmPolh19EG-1Ac or BmPolh32EG-1Ac, polyhedrin-EGFP-Cry1Ac fusion protein was observed as granular forms in infected cells.

SDS-PAGE of infected Bm5 cells revealed 29 kDa of Polyhedrin and 27 kDa of EGFP in wild-type BmMNPV and BmEGFP, respectively, whereas approximately 100 kDa of Polyhedrin-EGFP-Cry1Ac fusion protein was expressed in infected cells with either BmPolh19EG-1Ac or BmPolh32EG-1Ac (Fig. 37).

For the localization of EGFP fluorescence, all EGFP photographs were taken in each purification step on a UV -transilluminator in a dark room (Fig. 38). Strong fluorescence was observed in the precipitated pellet by centrifugation of cells infected with BmEGFP,

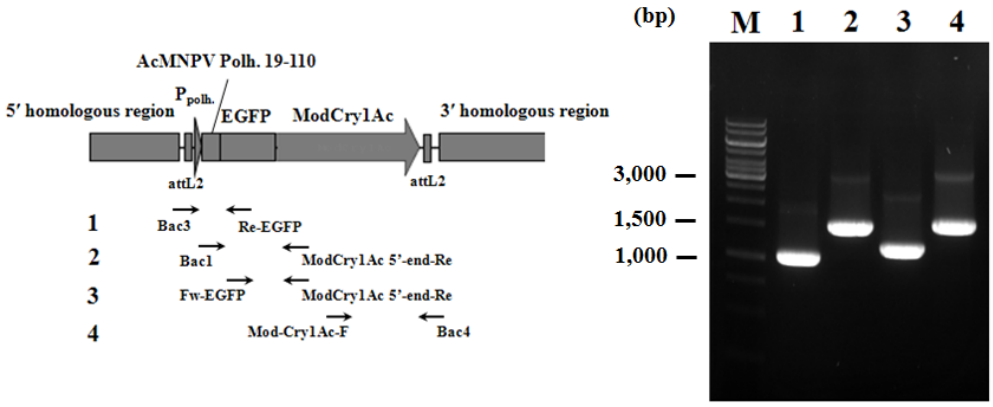


Fig. 34. Verification of the genome structure of recombinant virus, BmPolh19EG-1Ac expressing polyhedrin-EGFP-Cry1Ac fusion protein under the control of polyhedrin promoter by PCR using specific primers. Lane: M, 1kp ladder; 1, primers Bac3 and Re-EGFP; 2, primers Bac1 and ModCry1Ac 5'-end-Re; 3, primers Fw-EGFP and ModCry1Ac 5'-end-Re; and 4, primers Mod-Cry1Ac-F and Bac4.

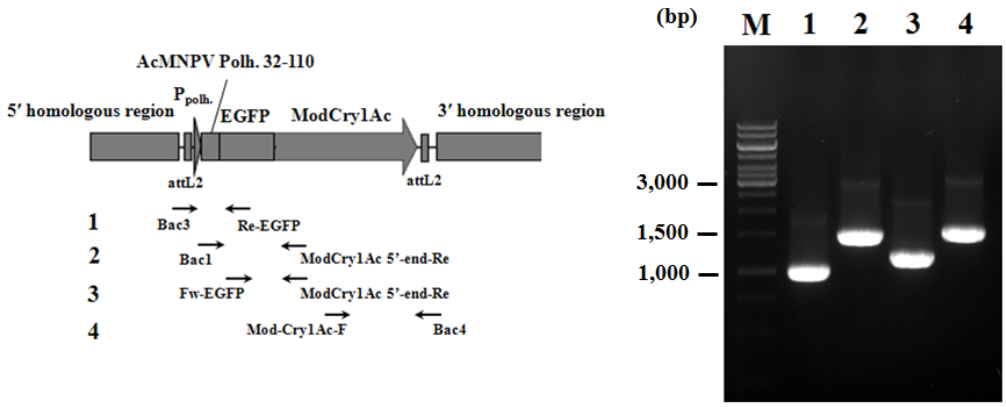


Fig. 35. Verification of the genome structure of recombinant virus, BmPolh32EG-1Ac expressing polyhedrin-EGFP-Cry1Ac fusion protein under the control of polyhedrin promoter by PCR using specific primers. Lane: M, 1kp ladder; 1, primers Bac3 and Re-EGFP; 2, primers Bac1 and ModCry1Ac 5'-end-Re; 3, primers Fw-EGFP and ModCry1Ac 5'-end-Re; and 4, primers Mod-Cry1Ac-F and Bac4.

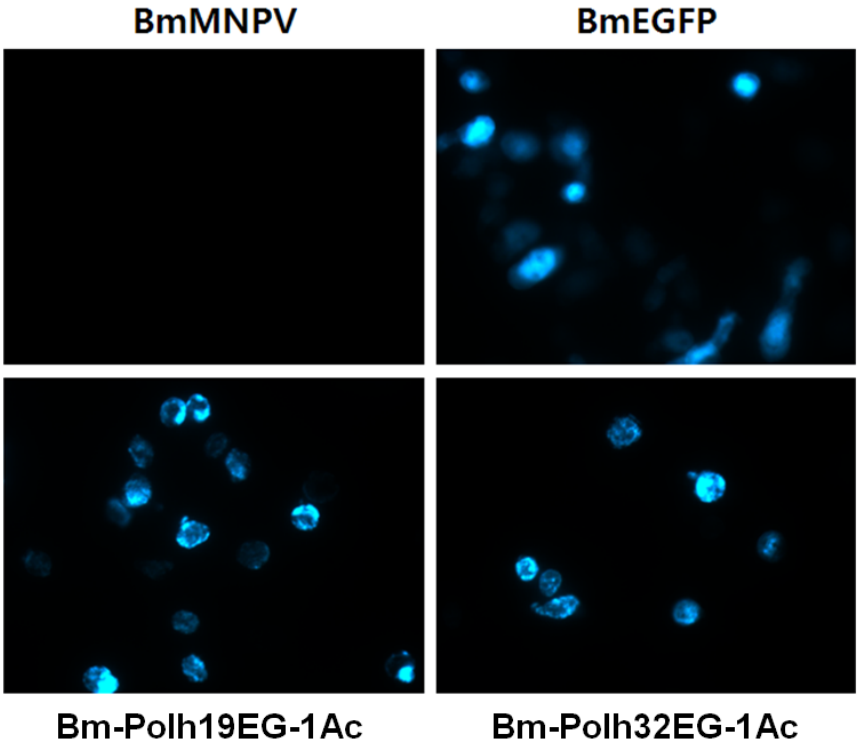


Fig. 36. Fluorescence microscopy of Bm5 cells infected with the recombinant viruses, BmPolh19EG-1Ac or BmPolh32EG-1Ac expressing polyhedrin-EGFP-Cry1Ac fusion protein under the control of polyhedrin promoter (Magnification: ×400).

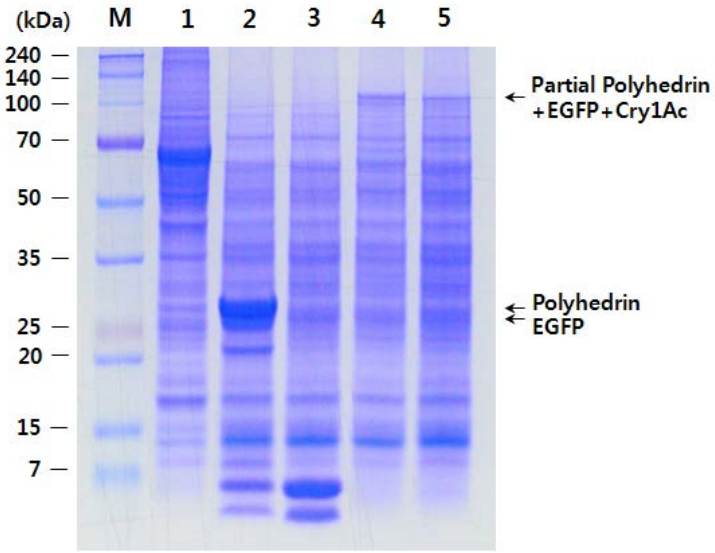


Fig. 37. SDS-PAGE analysis of Bm5 cells infected with the recombinant viruses, BmPolh19EG-1Ac or BmPolh32EG-1Ac expressing polyhedrin-EGFP-Cry1Ac fusion protein under the control of polyhedrin promoter. At 3 days p.i., total cellular lysates were separated by 12% SDS-PAGE, and the gels were then stained with Coomassie Brilliant Blue. Lane: M, protein molecular weight marker; 1, mock-infected Bm5 cells; 2, wild-type BmNPV-infected Bm5 cells; 3, BmEGFP-infected Bm5 cells; 4, BmPolh19EG-1Ac-infected Bm5 cells; 5, BmPolh32EG-1Ac-infected Bm5 cells.

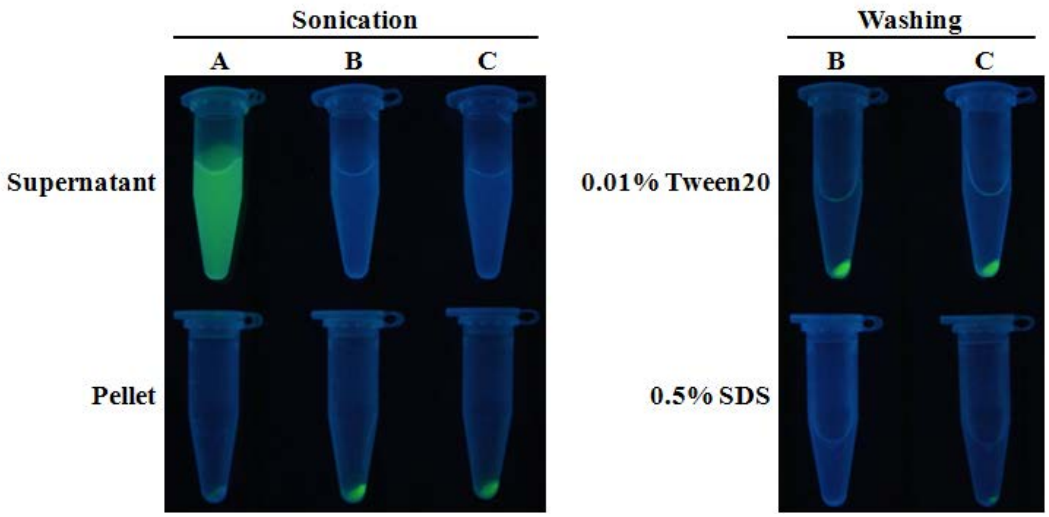


Fig. 38. Localization of EGFP fluorescence during purification of the partial polyhedrin-EGFP-Cry1Ac fusion protein. A, BmEGFP; B, BmPolh19EG-1Ac; C, BmPolh32EG-1Ac. All EGFP photographs were taken on a UV-transilluminator in a dark room.

whereas fluorescence was distributed evenly throughout the supernatant after sonication of the pellet. However, in the cells infected with BmPolh19EG-1Ac or BmPolh32EG-1Ac fluorescence was still measured even in precipitated pellet subsequently to the sonication, and strong fluorescence was observed in the pellet even after washing in 0.01% Tween-20 .

To examine the expression efficiency of foreign protein using granules formed by fusion protein between polyherin-EGFP and target protein, recombinant viruses BmPolh19EG-1Ac or BmPolh32EG-1Ac were infected into Bm5 cells, and fluorescence on infected cells was detected at 4 days p.i.. Fusion of amino acids 19 to 110 was about 41.5% more efficient than that of amino acids 32 to 110 (Fig. 39).

To investigate the purification efficiency of foreign protein using granules formed by fusion protein between polyhedrin-EGFP and target foreign protein, quantification of the purified Cry1Ac protein from the granules of polyhedrin-EGFP-Cry1Ac fusion protein was carried out using Bradford method (Figs. 40 and 41). As a result, the fusion of amino acids 19 to 110 was about 39.2% more efficient than that of amino acid 32 to 110 (Fig. 42)

4. Production of insecticidal protein and its insecticidal activity

For the production and characterization of insecticidal protein, granules of

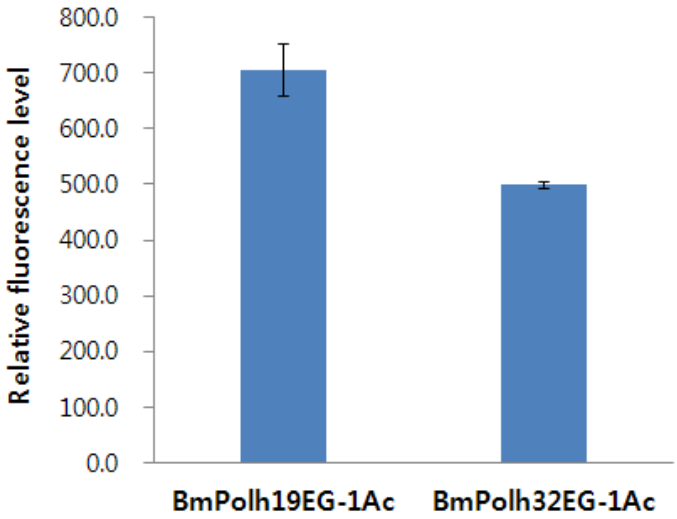


Fig. 39. Expression of partial polh.-EGFP-Cry1Ac fusion protein in Bm5 cells infected with recombinant viruses, BmPolh19EG-1Ac and BmPolh32EG-1Ac.

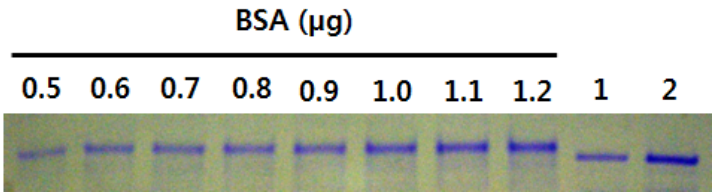


Fig. 40. Quantification of the purified partial Polh.(a.a. 19-110)-EGFP-Cry1Ac fusion protein. Lanes: 1, 5 μl of the purified fusion protein 2, 10 μl of the purified fusion protein.

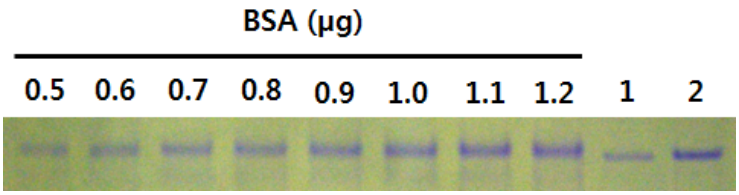


Fig. 41. Quantification of the purified partial Polh.(a.a. 32-110)-EGFP-Cry1Ac fusion protein. Lanes: 1, 5 μl of the purified fusion protein 2, 10 μl of the purified fusion protein.

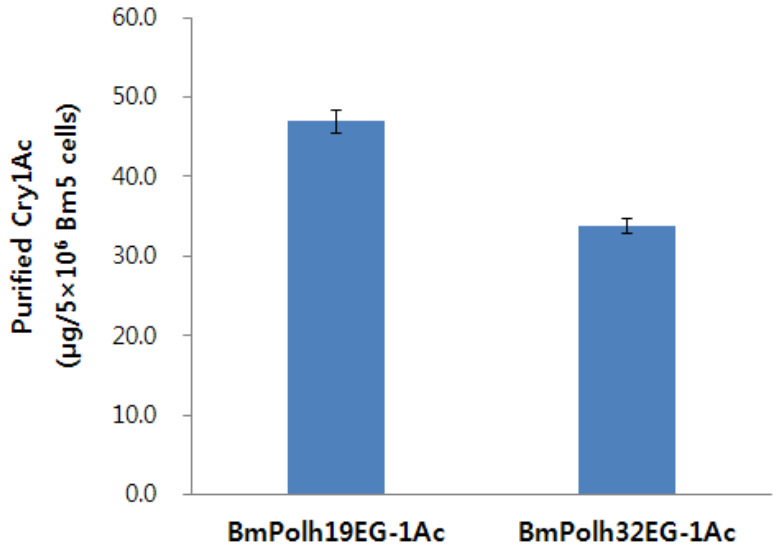


Fig. 42. Cry1Ac protein purified from granules of partial polh.-EGFP-Cry1Ac fusion protein expressed in Bm5 cells infected with recombinant viruses, BmPolh19EG-1Ac and BmPolh32EG-1Ac.

polyhedrin-EGFP-Cry1Ac fusion protein expressed from infected cells with recombinant virus BmPolh19EG-1Ac were sonicated and washed three times with 0.01% Tween 20.

After their centrifugation, polyhedrin-EGFP-Cry1Ac fusion protein was harvested from the pellet and enterokinase was used to treat granules of polyhedrin-EGFP-Cry1Ac fusion protein. The resulting was purification of Cry1Ac as a target protein in the pellet obtained after centrifugation (Figs. 43 and 44).

To evaluate the insecticidal activity of the expressed and purified Cry1Ac protein, bioassay was performed using *Plutella xylostella* larvae, comparing with Cry1Ac endotoxin protein isolated from Bt *kurstaki* HD-73 strain as a control group. Cry1Ac endotoxin protein purified from granules of polyhedrin-EGFP-Cry1Ac fusion protein had almost similar level of insecticidal activity to that of the control group (Figs. 45 and 46).

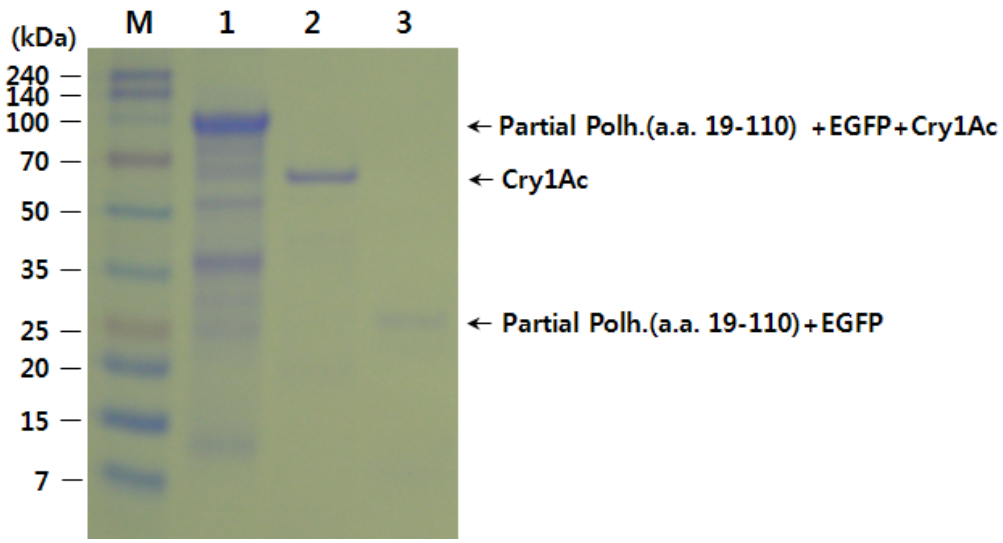


Fig. 43. Protein profiles during purification of the partial Polh.(a.a. 19-110)-EGFP-Cry1Ac fusion protein at each step. Lanes: M, Protein molecular weight marker; 1, after sonication and washing step; 2, supernatant fraction after enterokinase digestion step; and 3, pellet fraction after enterokinase digestion step.

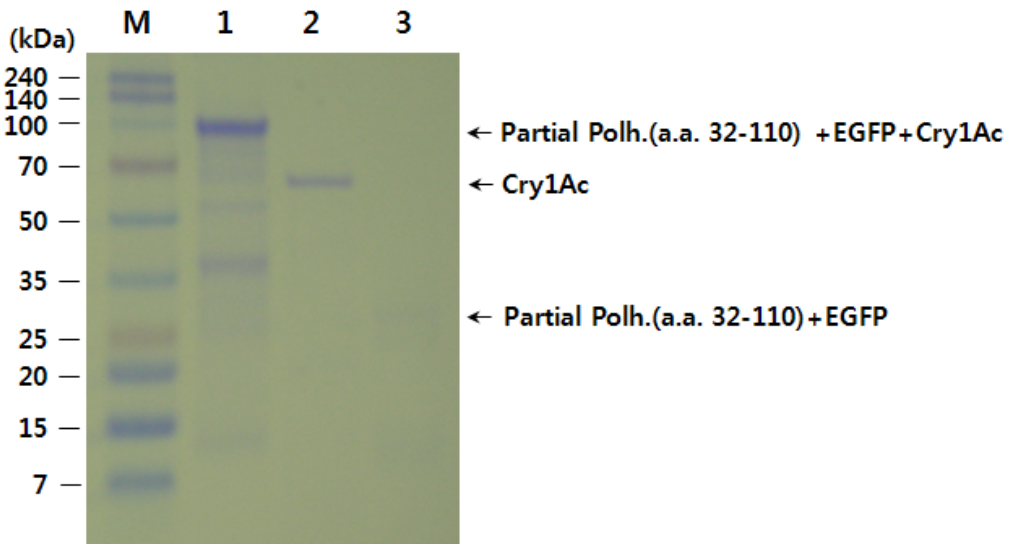


Fig. 44. Protein profiles during purification of the partial Polh.(a.a. 32-110)-EGFP-Cry1Ac fusion protein at each step. Lanes: M, Protein molecular weight marker; 1, after sonication and washing step; 2, supernatant fraction after enterokinase digestion step; and 3, pellet fraction after enterokinase digestion step.

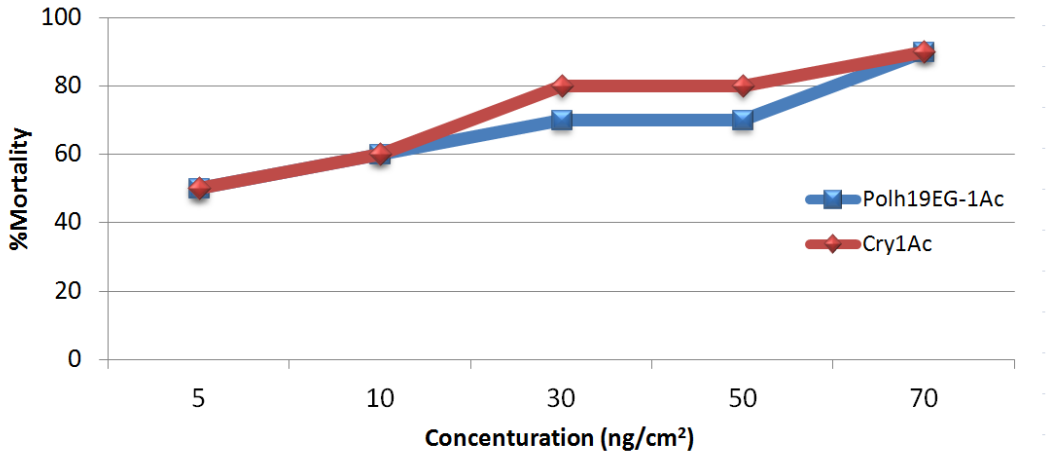


Fig. 45. Dose-response of 3rd instar larvae of *Plutella xylostealla* against Cry1Ac protein purified from granule of polyhedrin-EGFP-Cry1Ac fusion protein.

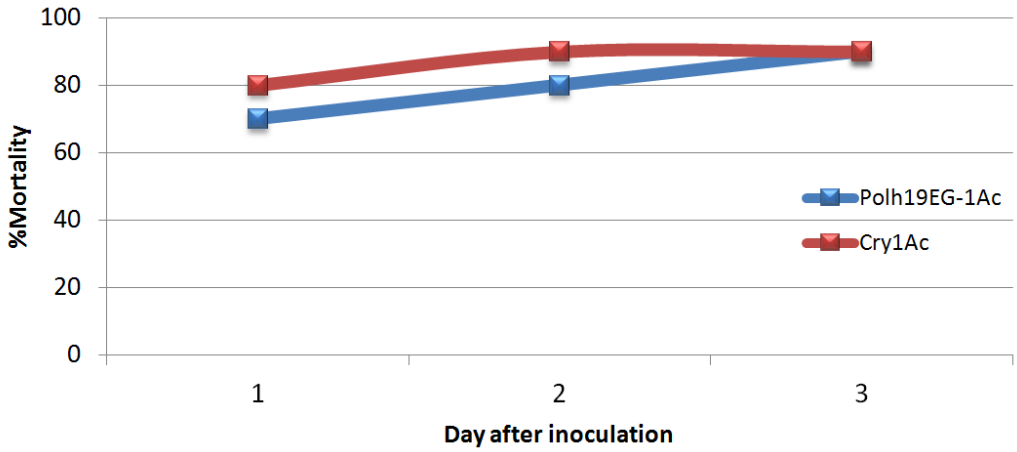


Fig. 46. Time-response of 3rd instar larvae of *Plutella xylostealla* against Cry1Ac protein purified from granule of polyhedrin-EGFP-Cry1Ac fusion protein at concentration of 70 ng/cm^2.

DISCUSSION

A major bottleneck in expressing foreign genes using baculovirus expression vector systems is that these systems require somewhat complex and laborious processes for purification of target recombinant viruses from mixed populations produced by either transposition in *E. coli* or homologous recombination in insect cells. Previously, to achieve the fast and easy generation of recombinant baculovirus by removing extra steps for purification, a recombinant bacmid, bEasyBac, was constructed which is the recombinant AcMNPV genome that replicates in *E. coli*, but not in insect cells, and which contains bacteriophage lambda site-specific attachment (att) sites for *in vitro* transposition (Choi et al., 2012a).

Although cultured insect cells have been used most frequently as the host for production of foreign proteins in baculovirus expression vector system, infected insect larvae can also be used. In this regard, one of the significant advantages of the BmNPV based expression system is that its host, *B. mori*, has been characterized extremely well and methods for mass rearing of *B. mori* larvae are also well established. In addition, there are a number of possible advantages to produce foreign proteins in infected insect larvae. First, proteins expressed in insect larvae could be subject to a more diverse range of post-translational modifications than those expressed in cultured cells, because there are a variety of different tissues having typically different capacities for post-translational

modifications in insect larvae. This could have benefits for the production of functional or authentic proteins (Je et al., 2001). Second, the yields obtained in insect larvae could be up to 500-fold higher than those obtained in cultured cells (Choi et al., 2000; Maeda, 1989; Reis et al., 1992). Finally, expression using insect larvae represents a relatively low cost alternative to the production of a foreign protein using cultured cells (Choi et al., 2000; Je et al., 2001).

In the bEasyBm constructed in this study, the barnase gene was introduced into the BmNPV genome under the control of CpBV early promoter. Barnase, a major extra-cellular ribonuclease isolated from *B. amyloquefaciens*, is lethal to cells in which it is expressed (Hartley, 1989; Qin et al., 2005). CpBV promoter used for the expression of barnase showed activity earlier than the polyhedrin promoter, and the activity of some of these promoters was superior to the AcMNPV ie-1 promoter (Choi et al., 2009). The bEasyBm can only replicate in transfected insect cells when the CpBV promoter-barnase cassette is replaced with the gene of interest by *in vitro* transposition. Therefore, no plaque purification or drug selection steps are required, and only recombinant virus is obtained after transfection of the *in vitro* recombination reaction between bEasyBm DNA and donor vector harboring the gene of interest because the replication of non-recombinant bEasyBm in insect cells is blocked by host cell death at an early stage of viral replication.

Insect cells showed cytopathic effects with reduced cell growth at 1-2 days p.t. when

transfected with bEasyBm. However, thereafter, the cells recovered growth rates comparable to mock-transfected cells because of absence of viral replication and transmission through the death of insect cells in which barnase was expressed by bEasyBm. The replication of bEasyBm in transfected cells was not observed RT-PCR for baculovirus genes essential for viral replication was done using the total RNA extracted from transfected cells. This lack of replication was further proven by the observation that the non-recombinant bEasyBm background was not detected in non-purified viral stocks of EasyBm-EGFP. Additionally, while the expression efficiency of non-purified stocks of BmGOZA-EGFP was reduced, the expression efficiency of non-purified stocks of EasyBm-EGFP was consistently maintained at a high level in serial passages. Futhermore, target recombinant viruses were generated with approximately 83.3-91.7% efficiency when the insect cells in a 12-well plate format were concomitantly transfected with 30 ng of the recombination reaction.

Also, recombinant virus using homologous recombination in *in vivo* larvae of *B. mori* was generated according to increasing quantity of bEasyBm DNA and transfer vector DNA. Especially, an efficiency of 100% was evident when 1 μg of bEasyBm DNA and 4 μg of transfer vector were co-transfected.

Purification of foreign protein in granules of polyhedrin fusion protein with EGFP as a visible marker may be more easy to do and efficient. Nevertheless, the final fusion protein exceeded 55 kDa because polyhedrin and EGFP consisting of the fusion protein,

except for a target protein, were 28 kDa and 27 kDa, respectively. Increasing molecular weight makes expression efficiency lower, which means that to stably produce a target protein in large-scale, the size of fusion protein needs to be minimized. Therefore, amino acids 19 to 110 and amino acids 32 to 110 that lead to localization of recombinant protein into the nucleus and mediate its assembly were used in this study, and improved transfer vectors, pPolh19-110-EGFP and pPolh32-110-EGEK, were constructed.

Purification efficiency in *in vitro* system was detected using granules of fusion protein between polyherin-EGFP and target protein. Localization of EGFP fluorescence in the infected cells with BmEGFP suggested that EGFP was only soluble, and not precipitated after cell lysis. On the other hand, in the infected cells with BmPolh19EG-1Ac or BmPolh32EG-1Ac, strong fluorescence was observed in the pellet, similar to BmEGFP, and the pellet still fluoresced after sonication, which was different from infected cells with BmEGFP. Thus, Polyhedrin-EGFP-Cry1Ac fusion protein was existed in granule form after sonication and washing in 0.01% Tween-20. Moreover, the approximately 39.2% purification efficiency of Polyhedrin amino acids 19 to 110 as fusion partner was higher than that of amino acids 32 to 110; it was assumed that the fusion of amino acids 19 to 110 could be more efficient, considering that the purification efficiency of target protein was affected by fusion protein expressed from infected cells,

Concerning the expression efficiency of foreign protein in *in vivo* system, the 3th instar of *B. mori*, most EGFP protein was localized on fat body than hemolymph, which

indicated that the EGFP protein was not a secretory protein with signal peptide. Even though the efficiency of EGFP expression at 4 days p.i. was little higher than that of 3 days p.i., the mortality of larvae (0%) at 3 days p.i. was lower than that at 4 days p.i.(88.2%). Thus, it may be efficient to harvest foreign protein at 3 day p.i. because a protein in dead larvae was affected so that it could not be harvested.

In the bioassay against *Plutella xylostella* larvae, Cry1Ac endotoxin purified from granule of polydedrin-EGFP showed almost same insecticidal activity to that of Bt *kurstaki* HD-73 strain as a control group. This suggested that the target protein expressed in the fusion protein with polyhedrin-EGFP maintained its characteristics.

In conclusion, bEasyBm has an effective benefit, allowing for a high-throughput baculovirus expression vector without purifying recombinant viruses. Also, a target protein with an innate characteristic can be produced efficiently through the purification of granules of fusion protein between polyhedrin-EGFP and foreign protein.

For the further study, it is suggested that the bEasyBm and the purification method using granules of fusion protein should be applied to large-scale expression and purification of various functional proteins for their characterization.

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누에나방(*Bombyx mori*) 핵다각체병바이러스를 이용한 새로운 베큘로바이러스 발현시스템 구축과 특성 구명

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김 양 수

초 록

어떠한 별도 분리과정 없이 순수한 재조합 베큘로바이러스만을 쉽고 빠르게 생성할 수 있는 새로운 재조합 박미드인 bEasyBm을 제작하였다. bEasyBm은 *in vitro* transposition에 의해 재조합 바이러스 게놈 생성이 가능하도록 attR recombination sites를 도입하였다. 또한 *Bacillus amyloliquefaciens*의 extracellular RNase 유전자인 *barnase*를 재조합되지 않은 바이러스 백그라운드를 제거하기 위해서 프루텔고치벌 Bracovirus (*Cotesia plutellae* bracovirus; CpBV)의 조기발현 프로모터의 조절 하에 발현되도록 하였다. bEasyBm은 *barnase* 유전자가 *in vitro* transposition에 의해 목적 유전자로 치환될 때에만 숙주인 곤충 세포에서 복제될 수 있었다. bEasyBm과 pDualBac-EGFP가 *in vitro* transpostion을 통해 만들어진 재조합 바이러스 EasyBm-EGFP은 bBmGOZA 시스템을 이용하여 분리과정 없이 만들어진 재조합 바이러스 BmGOZA-EGFP에 비해 높은 EGFP

발현율을 보였다. 또한 별도의 순수 분리과정을 거치지 않은 EasyBm stocks에서는 재조합되지 않은 백그라운드가 검출되지 않았다. 이러한 결과를 바탕으로 다수의 바이러스를 동시에 생성할 수 있는 고효율(high-throughput) 시스템을 구축하였다.

한편 베큘로바이러스 발현 시스템을 통해 발현된 폴리헤드린-EGFP 융합단백질은 과립구조를 형성하고 고속의 원심분리를 통해 쉽게 침전될 수 있어 이를 이용한 쉽고 빠른 대량의 단백질 정제 시스템을 개발하였다. 이번 연구에서는 C-말단에 폴리헤드린 일부와 EGFP, 그리고 EGFP와 엔테로카이네이즈 절단 위치로 연결된 Cry1Ac 단백질을 곤충세포에서 발현시켰다. 그 결과 재조합 바이러스인 BmPolh19EG-1Ac와 BmPolh32EG-1Ac에 감염된 세포들은 형광을 띠는 과립체를 생성하였다. Cry1Ac 단백질은 세포 수거, 초음파 분쇄, 엔테로카이네이즈 처리 등 3단계 과정을 거쳐 과립체를 포함한 세포로부터 정제될 수 있었다. 엔테로카이네이즈 처리로 원심분리 상층에는 Cry1Ac을 확인하였고, 순수한 Cry1Ac만을 분리할 수 있었다. 이상의 결과들을 통해 폴리헤드린과 융합 발현 및 엔테로카이네이즈 처리를 결합한 방법을 이용하여 외래단백질을 쉽고 빠르게 정제할 수 있음을 확인하였다.

주요어: baculovirus expression system, EasyBm, *in vitro* transposition, barnase, protein purification, polyhedrin fusion expression, enterokinase

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