

# The Homingbac baculovirus cloning system: An alternative way to introduce foreign DNA into baculovirus genomes

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Received 17 July 2006; received in revised form 24 October 2006; accepted 30 October 2006

Available online 4 December 2006

## Abstract

An *in vitro* baculovirus cloning system has been developed for direct cloning of foreign DNA into baculovirus genomes. This system is called the “Homingbac system” because it uses homing endonucleases. The Homingbac system was engineered into the baculoviruses *AcMNPV*, *BmNPV*, *PxMNPV*, *RoMNPV*, *HaSNPV* and *H<sub>2</sub>SNPV*. All Homingbac viruses were designed to retain the polyhedra phenotype so that they could be inoculated *per os* to insects. This is the first time a common *in vitro* baculovirus cloning system has been made for multiple baculovirus species that include both groups I and II nucleopolyhedroviruses (NPVs). In this study, the Homingbac system was demonstrated by directly cloning a PCR-amplified  $\beta$ -glucuronidase gene cassette into a parent Homingbac virus. This new collection of groups I and II NPV Homingbac viruses are a significant expansion of *in vitro* cloning technology and are new tools for making recombinant baculoviruses.

Published by Elsevier B.V.

**Keywords:** Baculovirus; Expression vector system; Homing endonuclease; Cloning system

## 1. Introduction

Baculoviruses are insect-specific viruses that are widely known to the scientific community in the form of commercial baculovirus express vectors (BEVs). Baculoviruses also have an established application as insecticides against agricultural and forestry pests (Cook et al., 2003; Moscardi, 1999; Zhang, 1994). In their wild type form, baculoviruses produce two virus phenotypes; the budded virus (BV) and the occlusion-derived virus (ODV). BVs are produced first in the infection cycle and transmit virus infection within the insect host tissues or in insect cell cultures. ODVs are produced later in infection and are occluded in polyhedral or granular occlusion bodies (OBs). OBs are critical for baculovirus insecticides because they transmit virus between insect hosts by the *per os* route.

The performance of baculovirus insecticides has been improved by genetic modification and the addition of foreign genes such as insect-specific neurotoxin genes (American-Cyanamid-Company, 1994; Hughes et al., 1997; Prikhod'ko et al., 1996; Tomalski and Miller, 1991). There is the need to evaluate a number of foreign genes in recombinant baculovirus insecticides. Commercial BEVs lack the *polyhedrin* gene and do not produce OBs. Thus, they are not suitable for *per os* infection or the generation of recombinant baculovirus insecticides. For the current study, there was the desire to develop a new, orally infectious baculovirus cloning system. This cloning system also had to be applicable in several baculovirus species that infect agriculturally important insect pests. An additional objective was to make this cloning system for both groups I and II NPVs (Herniou et al., 2001).

Most baculovirus cloning systems are based on either homologous recombination (Maeda et al., 1985; Pennock et al., 1984; Smith et al., 1983) or bacmid transposition (Luckow et al., 1993). Homologous recombination is inefficient and the number recombinants that can be made are limited. Bacmid-based cloning is very efficient but did not meet the present requirements for several reasons. Bacmid baculoviruses include bacteriophage DNA

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and marker genes along with the introduced foreign gene. These extra genes were undesirable for recombinant viruses that were to be evaluated as insecticides. In addition, there was the need to engineer a baculovirus cloning system into several baculovirus species and in a location outside of the *polyhedrin* gene locus. Bacmids have been observed to be genetically unstable when engineered outside of the *polyhedrin* locus of some baculovirus species (Pijlman et al., 2003).

The specialized needs of this study led to the design of an enzyme-based, *in vitro* baculovirus cloning system. Baculoviruses have circular dsDNA genomes ranging in size from 90 to 180 kbp in size depending on species. Baculovirus genomes can be cut using molecular biology techniques and foreign DNA inserts ligated in. This *in vitro* approach to baculovirus cloning was developed in the mid-1990s using unique restriction endonuclease (REN) sites that naturally occur in the *AcMNPV* baculovirus genome (Lu and Miller, 1996). Subsequently, enzymes called Homing endonucleases (HENs) offered new potential for *in vitro* baculovirus cloning systems. HENs are DNA cutting enzymes that were first isolated from *Chlamydomonas* green alga species (Marshall and Lemieux, 1992). HEN binding sites are 15–37 bp in length (Lambowitz and Belfort, 1993; Turmel et al., 1997) and are thus unlikely to occur in baculovirus genomes. HENs also bind to non-palindromic DNA sequences and when they cleave DNA, they produce asymmetric cohesive ends. This permits directional cloning of DNA fragments.

The first HEN-based *in vitro* baculovirus cloning system was called *Ac-Omega* (Ernst et al., 1994) and was based on the HEN, I-SceI. The 18 bp, I-SceI recognition site was engineered into the genome of *AcMNPV*. I-SceI sites were then engineered onto the ends of DNA inserts that were to be cloned into *Ac-Omega* baculoviruses. A later improvement of this strategy took advantage of the BstX I REN (Ernst et al., 1998). BstX I cleavage sites (Fig. 1b) could be designed with cohesive ends that were compatible with I-SceI cleavage sites. The smaller 12 bp size of BstX I recognition sites made them easier to engineer onto the ends of DNA inserts destined to be cloned into *Ac-Omega* viruses.

In the present study, an *in vitro* HEN-dependant baculovirus cloning system was developed and it was called the Homingbac system. This Homingbac system incorporates the features of the *Ac-Omega* cloning system but uses the HEN, I-CeuI, which has a 26 bp recognition site (Fig. 1a). As with the *Ac-Omega* virus system, the BstX I REN was used to clone DNA inserts. The Homingbac cloning system has the distinction of being engineered into more than one baculovirus species including the group I NPVs, *AcMNPV*, *BmNPV*, *PxMNPV*, and *RoMNPV* and the group II NPVs *HaSNPV* and *HzSNPV*. All these viruses were made into parent Homingbac viruses by replacing their *wt UDP-ecdyglycosyltransferase (egt)* genes (O'Reilly and Miller, 1989) with a I-CeuI flanked *green fluorescent protein (GFP)* gene cassettes. The parent Homingbac-GFP viruses all have a green fluorescent marker phenotype and produce orally infectious polyhedra. The Homingbac cloning strategy (Fig. 1d) involves cutting parent Homingbac-GFP viruses with I-CeuI to liberate the GFP cassette and ligating in a foreign gene with

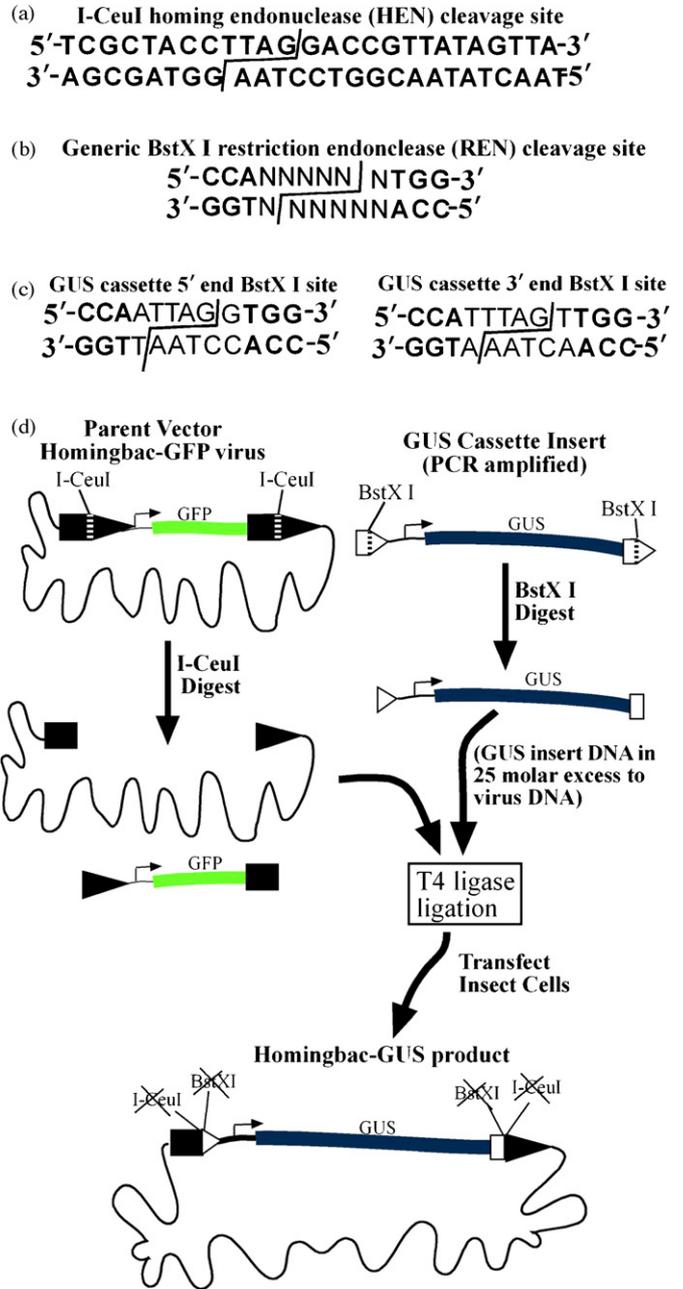


Fig. 1. Elements of the Homingbac cloning system. The Homingbac cloning system uses a parent Homingbac-GFP baculovirus with I-CeuI HEN sites. Foreign genes are cloned into the I-CeuI sites with the BstX I REN. (a) The DNA binding site of I-CeuI is a non-palindromic 26 bp region. I-CeuI cleavage near the center of this site produces a 3' overhang cohesive end that is non-palindromic. This permits directional cloning of DNA inserts. (b) The DNA binding site of BstX I is a 12 bp region with the center 6 bp being non-specific. BstX I cleaves DNA at the non-specific region and produces 3' overhang cohesive ends. This feature allows one to engineer BstX I cleavage sites to produce cohesive ends that are compatible with I-CeuI cohesive ends. (c) In demonstration of the Homingbac system, PCR primers were used to engineer BstX I sites onto the 3' and 5' ends of a GUS cassette. (d) In this demonstration, a parent Homingbac-GFP virus is cut with I-CeuI to liberate the GFP marker gene cassette. A PCR-generated GUS cassette is cut with BstX I and combined at great molar excess in a ligation with the I-CeuI-cut parent virus. After ligation with T4 ligase, the resulting Homingbac-GUS product lacks both the GFP marker gene and I-CeuI sites. The non-palindromic nature of the I-CeuI and BstX I cleavage sites ensures that the GUS cassette goes in a single orientation.

compatible BstX I sites (Fig. 1c). In this paper, the Homingbac cloning system was demonstrated by cloning a PCR-amplified  $\beta$ -glucuronidase (GUS) reporter gene cassette into a parent Homingbac-GFP virus.

## 2. Materials and methods

### 2.1. Viruses and cell lines

MNPV refers to multiple capsid nucleopolyhedrovirus and SNPV refers to single capsid nucleopolyhedroviruses. The baculoviruses *Autographa californica* MNPV (*AcMNPV*) isolate HR3 (Brown et al., 1979), *Plutella xylostella* MNPV (*PxMNPV*) isolate ep/h10 (Lynn and Ferkovich, 2004), *Rachoplysia ou* MNPV (*RoMNPV*) isolate tnr (Lynn and Ferkovich, 2004) and their recombinants were grown in *Spodoptera frugiperda* cell lines *Sf-9* (Summers and Smith, 1987). *BmNPV* isolate Thailand strain 3 (T3) (Maeda and Majima, 1990) virus and recombinants were grown in *BmN* cells (Kamita et al., 2003). These cell lines were cultured in TNM-FH medium (Hink, 1970) containing 10% v/v fetal bovine serum (FBS). The *HaSNPV* China isolate (See Acknowledgments) and *Helicoverpa zea* SNPV (*HzSNPV*) isolate hv1 (Lynn and Ferkovich, 2004) viruses and their recombinants were propagated in the *Helicoverpa zea* cell line *H2AM1* (McIntosh and Ignoffo, 1983). This cell line was propagated in ExCell400 media supplemented with 5%, v/v fetal bovine serum. All cell lines were grown at 27 °C.

### 2.2. DNA constructs used to make Homingbac baculoviruses

I-CeuI HEN linkers were made by annealing the DNA oligomers; I-CeuI-upper (5'-TCGATAACTATAACGGTCCTAAGGTAGCGA-3') and I-CeuI-lower (5'-TCGATCGCTACCTTAGGACCGTTATAGTTA-3'). The annealed oligomers contained the 26 nt I-CeuI recognition sequence (underlined) and produced 5' overhangs that allowed ligation to *Sal*I or *Xho*I REN cut DNA. The plasmid pBS-I-CeuI was made by inserting an I-CeuI linker into the *Sal*I REN site of the blue script (pBS) plasmid (Promega Corp., Madison WI, USA).

The plasmid pBS-I-Ceu-GFP contains a *GFP* reporter gene flanked by I-CeuI HEN sites. The *GFP* reporter gene (*GFPmut1*) (Cormack et al., 1996) had been previously cloned downstream of a 166 bp *AcMNPV gp64* early/late baculovirus promoter in the plasmid pBac-5-EGFP (Slack et al., 2001). The *GFP* open reading frame (ORF) and its baculovirus promoter were cut from pBac-5-EGFP by REN digestion with *Xho*I and *Sal*I. This 982 bp fragment was ligated with I-CeuI linkers and cloned into the I-CeuI site of plasmid pBS-I-CeuI. The resulting pBS-I-Ceu-GFP plasmid was further modified into plasmid baculovirus shuttle vectors.

Shuttle vectors were made by cloning viral DNA regions on either side of the GFP cassette in pI-CeuI-GFP. PCR and the Deep Vent<sup>TM</sup> polymerase (New England Biolabs (NEB) Inc., Ipswich MA, USA) were used to amplify viral DNA regions.

The *AcMNPV*, *BmNPV*, *PxMNPV*, and *RoMNPV*-specific shuttle vector pAc<sup>egt</sup>USDS-GFP-I-CeuI was made from

PCR amplified upstream and downstream regions of the *AcMNPV* UDP-ecdyglycosyltransferase gene (*egt*) (O'Reilly and Miller, 1989). The *egt* gene upstream region PCR product was made using primers; Ac-egt-US-LP-*Hind*III (5'-AATAAAGCTTATACATGTTTTATTTTACAATAC-3') and Ac-egt-US-RP-*Hind*III (5'-TGCTAAGCTTCGAATAACTGTACTGGTAATTTG-3'). These primers introduced *Hind*III sites (underlined) that were then used to clone the 1798 bp *egt* upstream region into the *Hind*III site of pI-CeuI-GFP upstream of the GFP cassette. The *egt* gene downstream region PCR product was made using primers; Ac-egt-DS-LP-*Xba*I (5'-ACACTCTAGATTACGTCAATAAATGTTATTAC-3') and Ac-egt-DS-RP-*Kpn*I (5'-AAATGGTACCAATAGTTAATAGCTGTCTACCCG-3'). These primers introduced *Xba*I and *Kpn*I sites (underlined) that permitted cloning of a 1408 bp *egt* downstream region into downstream of the GFP cassette in pI-CeuI-GFP.

The *HaSNPV* and *HzSNPV*-specific shuttle vector pHa<sup>egt</sup>USDS-GFP-I-CeuI, was made by PCR amplifying the upstream and downstream regions of the *HaSNPV* *egt* gene (Chen et al., 2001). The *egt* gene upstream region PCR product was made using primers; Ha-egt-US-LP-*Hind*III (5'-TAAACAAGCTTGAACAATACACATATGTT-3') and Ha-egt-US-RP-*Hind*III (5'-AAAAATAAGCTTACGTCCGTTATGGGTCAGAG-3'). These primers introduced *Hind*III sites (underlined) that were then used to clone a 2460 bp *egt* upstream region into the *Hind*III site of pI-CeuI-GFP. The *egt* gene downstream region PCR product was made using primers; Ha-egt-DS-LP-*Xba*I (5'-TAGATTTCTAGAAATTATTTACGCATGACTACC-3') and Ha-egt-DS-RP-*Kpn*I (5'-CACGACGGTACCCAACCTAAGTACTACAAAATA-3'). These primers introduced *Xba*I and *Kpn*I sites (underlined) allowing a 1521 bp *egt* gene downstream region to be cloned into pI-CeuI-GFP.

Plasmid shuttle vectors were co-transfected with *wt* viral DNA onto insect cells to produce parent Homingbac-GFP viruses through homologous recombination. Homingbac-GFP viruses were isolated by plaque assay (Hink and Vail, 1973) and were screened for green GFP fluorescent plaques as previously described (Slack et al., 2001). Homingbac-GFP viruses were also confirmed by PCR using the *egt* loci-specific primers. For *AcMNPV* and related viruses the primers Ac-egt-LP5 (5'-TTACATAATCAAATTACCAGTACAG-3') and 169-Ac-egt RP5 (5'-GGTTGGTGAGCGTTTATAAAGGCTG-3') were used. For *HaSNPV* and related viruses the primers Ha-egt-LP2 (5'-TATAAGATGCAGCATCCCGTCCGTTG-3') and Ha-egt-RP (5'-CGTAGCTGCGCAATAGGAGACGGGTA-3') were used.

### 2.3. The GUS cassette and PCR amplification with BstX I primers

PCR primers were designed to amplify a GUS reporter gene cassette containing a late viral promoter from the *AcMNPV p6.9* gene (Wilson et al., 1987), a GUS gene ORF and a 3' untranslated region (UTR) from the *AcMNPV ie-1* gene (Guarino and Summers, 1987). These elements were previously cloned

together into a pBS plasmid vector called pBS-p6.9GUS. This plasmid was used as PCR template for the GUS cassette. Specifically, the plasmid contained 332 bp of the *AcMNPV* *p6.9* gene promoter (−1 to −332 relative to the start of the *p6.9* ORF), the 1812 bp GUS gene ORF, and 379 bp of the *AcMNPV* *ie-1* gene 3' UTR (+19 to +398 relative to the end of the *ie-1* gene ORF).

The PCR primers were designed to incorporate *Bst*X I sites onto the ends of the GUS cassette. The primers were *Bst*X I-GUS-LP2 (5'-GTTTTCCCAACCAATTAGGTGGTAAAACGACGGCCAGT-3') and *Bst*X I-GUS-RP1 (5'-TCAATACGCCAACTAAATGGATCTTCTGCGGGCCA-3'). The *Bst*X I sites are underlined. The GUS cassette was amplified as a 2676 bp PCR product. Primer *Bst*X I-GUS-LP annealed to the vector and included 97 bp of vector sequence on the 5' end of the cassette. There was also an additional, 52 bp of vector sequence downstream of the GUS ORF between the *ie-1* 3' UTR. Primer *Bst*X I-GUS-LP annealed to the *ie-1* 3' UTR.

The GUS cassette was amplified by PCR using Deep Vent polymerase in and the following reaction mix; 1x Thermpol™ buffer (NEB), 2 mM MgSO<sub>4</sub>, 320 μM dNTP mix, 1 μM of each primer, and 50 ng of DNA template. PCR was performed in a Perkin-Elmer Cetus DNA thermocycler 480 for 35 cycles of 95 °C 1:00 min, 62 °C 1:30 min, 70 °C 3:00 min.

### 3. Results

To begin this project, parent Homingbac-GFP viruses were produced. Plasmid shuttle vectors were designed to recombine with the *egt* loci and to replace the *egt* ORF with an I-CeuI flanked GFP cassette (See Section 2). Plasmid shuttle vector, pAc<sup>egt</sup>USDS-GFP-I-CeuI was made for *AcMNPV* and the related viruses, *BmNPV* (Gomi et al., 1999), *PxMNPV* (Kariuki et al., 2000), *RoMNPV* (Harrison and Bonning, 2003). Plasmid shuttle vector, pHa<sup>egt</sup>USDS-GFP-I-CeuI, was made for *HaSNPV* and the related virus *HzSNPV* (Chen et al., 2002).

Parent Homingbac-GFP viruses were screened by plaque assay and selected for GFP fluorescent viruses (Fig. 2a). The *AcMNPV* *gp64* promoter driving expression of GFP cassette is group I NPV-specific (Blissard and Rohrmann, 1989). However, it is notable that this promoter also is functional in the group II NPVs *HaSNPV* and *HzSNPV*. PCR was used to confirm the replacement of the *egt* ORF with the GFP cassette (Fig. 2b). Primers specific to the upstream and downstream regions of the *egt* ORF were used. For *AcMNPV*-related viruses, primers Ac-*egt*-RP-5 and Ac-*egt*-LP-5 produced a 1663 bp *wt* product and a 1202 bp Homingbac-GFP product. Likewise, for *HaSNPV*-related viruses, primers Ha-*egt*-LP2 and Ha-*egt*-RP produced a 1750 bp *wt* product and a 1180 bp Homingbac-GFP product. From these data, it was confirmed that six parent Homingbac-GFP viruses had been made. These viruses included *AcHomingbac-GFP*, *BmHomingbac-GFP*, *PxHomingbac-GFP*, *RoHomingbac-GFP*, *HaHomingbac-GFP* and *HzHomingbac-GFP* (Fig. 2).

After creating parent Homingbac-GFP viruses, an experiment was designed to demonstrate the Homingbac system could be used for *in vitro* cloning. The goal was to clone a PCR product directly into a parent Homingbac-GFP virus (Fig. 1d).

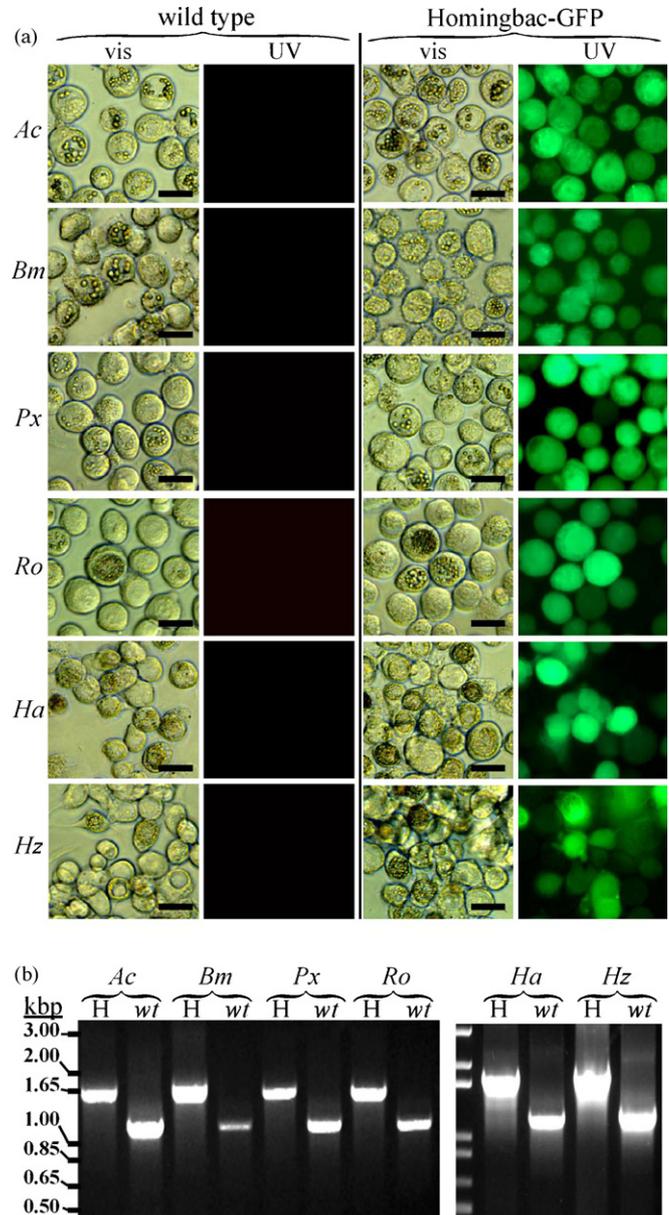


Fig. 2. Parent Homingbac-GFP baculoviruses. The baculoviruses *AcMNPV* (*Ac*), *BmNPV* (*Bm*), *PxMNPV* (*Px*), *RoMNPV* (*Ro*), *HaSNPV* (*Ha*) and *HzSNPV* (*Hz*) were made into Homingbac-GFP viruses. (a) These viruses were confirmed by GFP-specific fluorescence. Virus-infected cells were visualized by inverted microscope at 36 h post infection under visible light (vis) and under 470 nm excitation and 495 nm emission (UV). The scale bars in the visible images are 10 μm and baculovirus species are indicated on the left side. Recombinant viruses retain the polyhedra phenotype due to recombination in the *egt* locus. (b) PCR primers specific to the *egt* loci were used to distinguish recombinants. Primers either amplified the *egt* gene from *wt* viruses (*wt*) or amplified the GFP cassette from Homingbac-GFP viruses (H). The PCR products were fractionated on 1 X TAE, 0.8%, w/v agarose gels along side a size standard and stained with ethidium bromide. PCR primers are described in the Section 2.

A large stock of *AcHomingbac-GFP* virus DNA was made for this experiment. Twenty T-flasks (150 cm<sup>2</sup> area) of *Sf-9* cells (4 × 10<sup>8</sup> total cells) were infected with the *AcHomingbac-GFP* virus at 0.1 plaque forming units/cell. The BV-containing media (20 ml/flask) was collected at 5 days post infection and separated from cells by centrifugation for 5 min, 1000 × *g*. Virus

DNA purification was based on established methods (O'Reilly et al., 1992) and was as follows. BVs were pelleted from media by ultracentrifugation at  $110,000 \times g$  for 1.5 h at  $8^\circ\text{C}$  in a Beckman SW-28 swinging bucket rotor. BV pellets were suspended over night at  $37^\circ\text{C}$  in  $300\ \mu\text{l}$  of TE buffer (10 mM Tris-OH, 1 mM EDTA, pH 8.0) supplemented with 0.25% w/v SDS and 0.5 mg/ml proteinase K. Liberated viral DNA was fractionated by CsCl density gradient ultracentrifugation using described methods (Slack and Lawrence, 2002). Except for that the DNA was not ethanol precipitated and the CsCl was removed by dialysis in 15 K molecular weight cut off dialysis tubing. A 1 ml volume of DNA solution was dialyzed at  $4^\circ\text{C}$  for 2 h against 1 l of TE followed by overnight against 4 l of TE. The viral DNA yield of  $320\ \mu\text{g}$  was diluted in TE ( $300\ \text{ng}/\mu\text{l}$ ) and stored at  $4^\circ\text{C}$ .

Using the purified viral DNA, the Homingbac *in vitro* cloning method was tested (Fig. 3). A GUS cassette DNA fragment was amplified by PCR for ligation into I-CeuI-cut parent Homingbac-GFP virus DNA. Primers were designed to incorporate unique BstX I REN sites onto the ends of the GUS cassette (See Section 2 and Fig. 1c). For this experiment, the GUS cassette PCR product was fractionated by agarose gel electrophoresis before and after BstX I digestion (Figs. 3b and 4b).

The parent *AcHomingbac*-GFP virus DNA was cut with I-CeuI HEN at  $37^\circ\text{C}$  followed by heat inactivation at  $65^\circ\text{C}$  (Fig. 3a). It was confirmed by agarose gel electrophoresis that the GFP cassette had been excised by I-CeuI (Fig. 4a). I-CeuI-cut virus DNA and BstX I-cut insert DNA were then ligated together (Fig. 3c).

The ligation was then transfected onto insect *Sf-9* cells (Fig. 3d). During the development of this method, it was discovered that the polyethylenimine-based DNA transfection reagent; Exgen500<sup>TM</sup> from Fermentas<sup>1</sup> was excellent for the transfection of insect cells (Ogay et al., 2006). Exgen500<sup>TM</sup> was specifically chosen for this method because unlike other transfection reagents, it tolerates the presence of protein components from the I-CeuI digestion and ligation. Baculovirus DNA is fragile and additional DNA purification steps would have reduced virus DNA viability. Exgen500<sup>TM</sup> also tolerates antibiotics in the cell culture media. This allowed most of the cloning procedures to be done on the bench prior to transfection onto insect cells. The successful cloning of the GUS cassette and the creation of *AcHomingbac*-GUS viruses was confirmed by observing GUS positive virus plaques (Fig. 4d). In addition, PCR was used to screen for *AcHomingbac*-GUS viruses (Fig. 4c).

The time for digestion, ligation and transfection was 2 days and recombinant *AcHomingbac*-GUS virus was detectable after 3 days based on blue color in the cell culture media.

#### 4. Discussion

In this study, the Homingbac system was successfully engineered into six baculovirus species. The Homingbac cloning

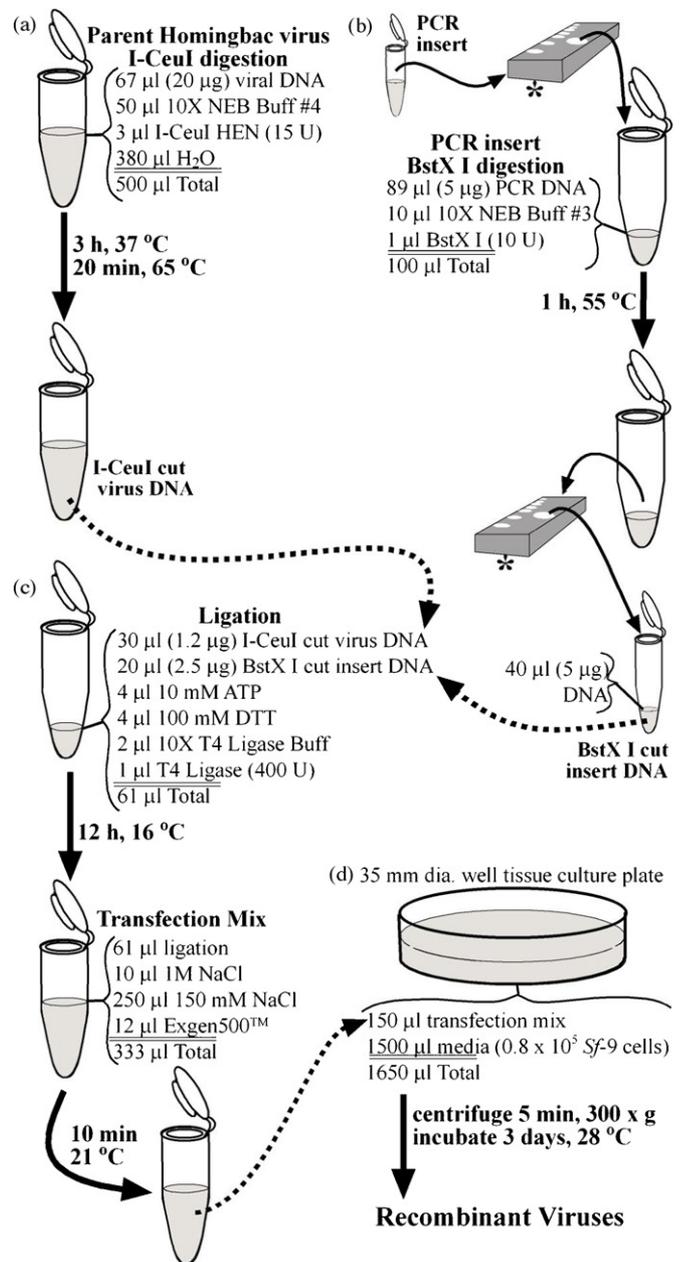


Fig. 3. The detailed Homingbac cloning protocol. The figure shows a detailed protocol for the Homingbac cloning system. All buffers and enzymes were from New England Biolabs Inc. The three major steps of Homingbac cloning protocol were (a) cutting the parent Homingbac baculovirus DNA with I-CeuI, (b) cutting the PCR-amplified DNA insert with BstX I, (c) ligating the insert and virus DNA with T4 ligase and (d) transfecting the ligation onto insect cells. The I-CeuI-cut viral DNA was not purified and was used directly in the ligation. (\*) The GUS cassette insert DNA was fractionated by agarose gel electrophoresis before and after BstX I digestion. The 2.67 kbp sized GUS cassette DNA fragment was cut from agarose gels and purified using Qiaex<sup>TM</sup> II "glass milk" from Qiagen Inc. The cell culture media was TNM-FH supplemented with 10% FBS, 100 U penicillin and 0.25  $\mu\text{g}$  amphotericin B.

system has been proven to work by cloning a GUS cassette into a parent *AcHomingbac*-GFP virus. The GUS cassette was chosen as a test insert for the cloning system because recombinants are easily detected. In ligations, the molar ratio of PCR insert DNA to virus vector DNA was 25–1. This was appar-

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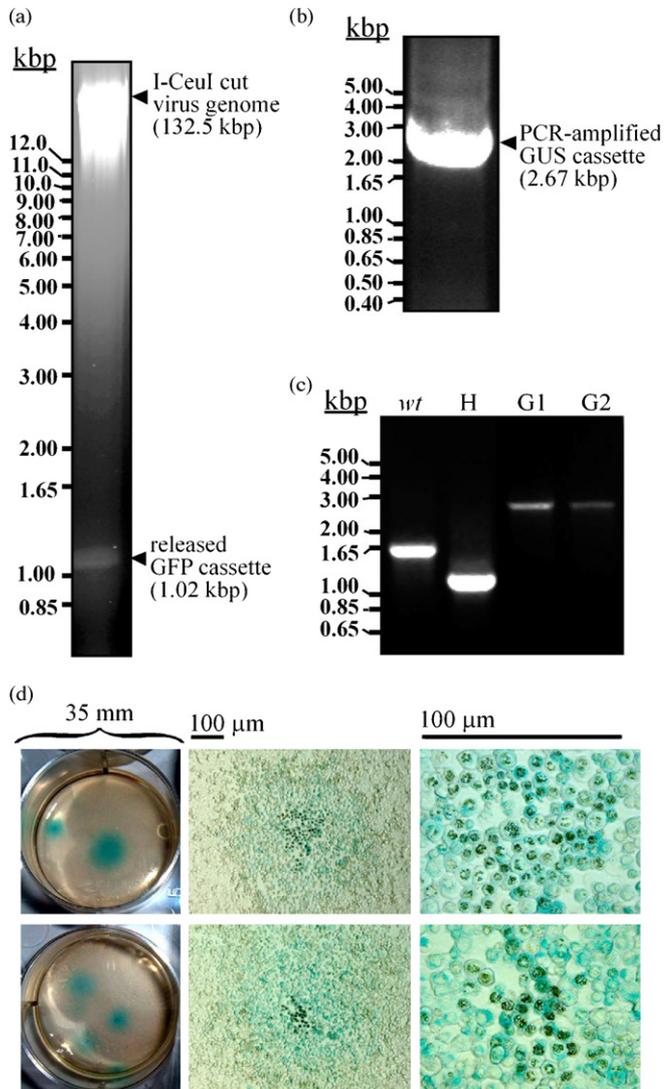


Fig. 4. Evidence of successful Homingbac cloning. (a) I-CeuI-cut *AcHomingbac-GFP* virus DNA was fractionated in 0.5% 1X TAE agarose gel, stained with ethidium bromide and visualized using a Typhoon 9400™ fluorescent scanner (GE Healthcare). The excised GFP cassette and the remaining viral genome were visualized. (b) The GUS gene, BstX I flanked PCR product was fractionated in and purified from a 0.8% 1X TAE agarose gel. (c) After I-CeuI-cut virus DNA was ligated with BstX I-cut insert DNA, the ligation was transfected onto insect cells. Plaque isolates were obtained and screened by PCR using the same primers as in Fig. 2. An agarose gel is shown of PCR results from *wt* *AcMNPV* (*wt*), *AcHomingbac-GFP* (H) and two *AcHomingbac-GUS* recombinants (G1 and G2). (d) These new recombinant viruses were produced GUS positive blue plaques (left panels) and also had occlusion bodies (right panels). Blue plaques were visualized by adding the GUS-specific color indicator substrate, 5-bromo-4-chloro-3-indoyl β-D-glucopyranoside (X-Glc) at 150 μg/ml concentration.

ently sufficient to drive the ligation to overwhelmingly favor inserting the GUS cassette instead of the GFP cassette that had been liberated from the parent Homingbac-GFP virus after I-CeuI digestion. The percentage of GUS positive virus clones could not be determined because background parent Homingbac viruses were below the limit of detection in the present experiments. In spite of these results, this cloning system is not likely to be 100% efficient and the isolation of single virus clones

by end point dilution is strongly recommended. The polyhedra OB phenotype of these Homingbac viruses makes it easy to see infected cells under the microscope. Polyhedral OBs can also be easily purified (O'Reilly et al., 1992) and counted under the microscope. One can then dilute OBs and use insects to amplify OB-derived viral clones. It is suggested that one add the heat denaturable shrimp alkaline phosphatase to the I-CeuI digested viral DNA and that one add more I-CeuI after ligations such to eliminate any parent Homingbac viruses. The quality of ligations can also be monitored by PCR and in addition, one may include control ligations without insert.

Some may not wish to purify parent Homingbac virus DNA by CsCl density gradient. Success has been had using virus DNA that had been purified with cell culture DNA midi kits from Qiagen Inc. In addition, it has been found that DNA from polyhedral OBs is higher in quality (See O'Reilly et al., 1992 for purification protocol) than BV-derived DNA.

Although this system has been developed for making recombinant baculovirus insecticides, the polyhedra-positive Homingbac viruses also have potential for use in BEV systems. Whole insects can be excellent protein production "factories" and *per os* inoculation can be done under non-sterile conditions. Polyhedra are extremely stable and can preserve the integrity of virus clones much better than BV media stocks. Accumulating defective interfering viruses also do not occur when viruses are passed through insects by the *per os* route.

The parent Homingbac viruses described in this paper, require that foreign genes be cloned as cassettes that include baculovirus promoters. This is ideal for PCR sub-cloning of foreign genes from recombinant baculovirus constructs that have been made in commercial BEVs. Thus, one can use the Homingbac system to quickly convert to orally infectious recombinant baculoviruses when needed.

Preliminary data has recently shown that I-CeuI sites do not interfere with baculovirus transcription or translation when located between baculovirus promoters and gene ORFs. In further development of the Homingbac system, the orientation of I-CeuI sites is being re-engineered such that the baculovirus promoter region is upstream of the I-CeuI cloning site. These constructs will permit the cloning of PCR-amplified gene ORFs into baculovirus genomes downstream of baculovirus promoters. The next generation of this Homingbac cloning system will enable baculoviruses to be used for *in vitro* cloning projects such as cDNA expression libraries.

## Acknowledgements

The authors wish to thank Dr. Arthur McIntosh for providing a *Helicoverpa armigera* cell line, Dr. Yan Dong Hui of the Chinese Academy of Forestry, Beijing for providing *HaSNPV* virus, Dr. George Katima of the University of California at Davis for providing a *Bombyx mori* cell line and *BmNPV* virus. We also thank Dr. Guido Caputo of the Great Lakes Forestry Centre for providing *Spodoptera frugiperda* cell lines. We thank Dr. Min-ju Chang who provided helpful suggestions for revisions in the manuscript. Tim Ladd is also acknowledged for testing the Homingbac system and providing additional insights.

This project was supported by a Science & Technology Centre of Ukraine grant (STCU project 119) and was part of collaboration between the Uzbekistan Academy of Sciences of Uzbekistan, UC Davis and the USDA.

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