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Research

Construction of the *Antheraea pernyi* (Lepidoptera: Saturniidae) Multicapsid Nucleopolyhedrovirus Bacmid System

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Abstract

In this study, we established the *Antheraea pernyi* multicapsid nucleopolyhedrovirus (AnpeNPV) bacmid system for the construction of a Bac-to-Bac expression system and the generation of virus mutants. The CopyRight pSMART BAC cloning vector harboring the chloramphenicol resistance gene was introduced into the AnpeNPV genome to produce the AnpeNPV bacmid that could be propagated in *Escherichia coli* with stable replication. The enhanced green fluorescent protein (EGFP) was successfully expressed in both Tn-Hi5 cells and *A. pernyi* pupae using the AnpeNPV Bac-to-Bac expression system. To generate the AnpeNPV mutants, we developed the AnpeNPV bacmid/λ Red recombination system that facilitated the deletion of viral genes from the AnpeNPV genome. The genes *cathepsin* and *chitinase* were deleted and a derivative AnpeNPV Bac-to-Bac expression system was constructed. Furthermore, we demonstrated that the novel expression system could be used to express human epidermal growth factor in *A. pernyi* pupae. Taken together, the AnpeNPV bacmid system provides a powerful tool to create the AnpeNPV Bac-to-Bac expression system for protein expression in *A. pernyi* pupae. Further, it helps to knock-out genes from the AnpeNPV genome with λ Red recombination system for identification of the role of viral genes involved in regulating gene expression, DNA replication, virion structure, and infectivity during the AnpeNPV infection process.

Key words: *Antheraea pernyi*, nucleopolyhedrovirus, Bacmid, Bac-to-Bac, λ Red recombination system

Baculoviruses are known to infect invertebrates and exhibit host specificity. Depending on occlusion body morphology, they are divided into nucleopolyhedroviruses (NPVs) and granuloviruses (GVs). More than 600 different types of baculoviruses have been reported from different species of invertebrates belonging to orders Diptera, Hymenoptera, and Lepidoptera. Most of them are NPVs from insects of the order Lepidoptera (Rohrmann 2019). Two types of progeny viruses—occlusion-derived virus (ODV) and budded virus (BV)—are generated during the baculovirus infection process in insects. ODVs cause primary infection and undergo horizontal virus transmission from insect to insect through the oral route. BVs initiate cell-to-cell secondary infection and result in a systemic infection within the insect host (Chambers et al. 2018, Rohrmann 2019). Baculoviruses have double-stranded, circular, and supercoiled DNA genomes with sizes varying from about 80 kb to over 180 kb, possessing between 90 and 180 genes. Depending on the time of gene transcription, the baculovirus genes are divided into early, late, and very late phase. The genes *polyhedrin* and *p10* are very late genes with very strong promoters and are nonessential for BV production in insect cells (Rohrmann 2019). Both genes are used to express foreign genes in baculovirus expression vector systems (BEVSs; Smith et al. 1983, Kitts et al. 1990, Zuidema et al. 1990). Since 1983, BEVSs based on various NPVs have been rapidly developed and universally used to produce heterologous proteins for scientific research or commercial production. They provide a high-level expression, correct conformation, and appropriate posttranslational modification pattern for recombinant protein production (Chambers et al. 2018). Thousands of recombinant proteins have been successfully expressed using BEVSs, and some of these proteins are now commercially available for human and veterinary use (Felberbaum 2015, Yee et al. 2018). *Autographa californica* multiple NPV (AcMNPV) and insect cell lines (Smith et al. 1983, Irons et al. 2018) as well as *Bombyx mori* NPV (BmNPV) and silkworm larvae (Maeda et al. 1985) are some of the widely used BEVSs. Using directly live insect larvae or pupae as 'biofactories' to reduce the high cost of insect cell cultures is a very attractive approach for production of recombinant proteins using BEVSs (Targovnik et al. 2016).

The method of homologous recombination between the viral circular genomic DNA and a transfer vector was originally used in BEVSs to produce recombinant baculoviruses; however, this method had low efficiency and involved time-consuming purification of recombinant baculoviruses from a background of parental viruses.

A major technological improvement of this method happened in 1993 when the bacmid system was developed. In this method, close to 100% recombinant AcMNPVs were generated in *Escherichia coli* and the plaque purification step for obtaining recombinant viruses was eliminated (Luckow et al. 1993). This bacmid is a bacterial artificial chromosome (BAC) vector carrying the entire AcMNPV genome, the kanamycin resistance gene, Tn7 transposition sites (mini-*att*Tn7), and *lacZα* as a reporter gene. When the transfer vector containing the gene of interest flanked by Tn7 transposition elements (mini-Tn7) is transformed into *E. coli* harboring the AcMNPV bacmid, the gene is inserted into the mini-*att*Tn7 cassette of the bacmid by site-specific transposition, and the recombinant AcMNPV bacmid can be identified by white colonies with antibiotic resistance on agar plates (Luckow et al. 1993). Apart from the AcMNPV bacmid, several other bacmids have been constructed from other baculoviruses, including *Spodoptera exigua* multicapsid nucleopolyhedrovirus (SeMNPV; Pijlman et al. 2002), *Helicoverpa armigera* SNPV (HearNPV; Wang et al. 2003), BmNPV (Motohashi et al. 2005), *Cydia pomonella* granulovirus (CpGV; Hilton et al. 2008), and *Agrotis ipsilon* nucleopolyhedrovirus (AgipNPV; Abdallah et al. 2017). The bacmid has now become a powerful tool that is being used not only to construct Bac-to-Bac expression systems but also to generate mutant bacmids with λ Red recombination system for functional studies of baculovirus genes (Ono et al. 2012, Rohrmann 2019).

Antheraea pernyi, also known as Chinese oak silkworm, has been used as a bioreactor for recombinant protein production using a BEVS, which is based on *A. pernyi* multicapsid nucleopolyhedrovirus (AnpeNPV) and silkworm pupae because of the large size of pupae, year-round availability, ease of manipulation, and lack of need for rearing (Fan et al. 2007, Zhao et al. 2019). In this AnpeNPV-based expression system, High Five (BTI-TN-5B1-4, Tn-Hi5) cells, which are nonpermissive cells for AnpeNPV, are used to generate recombinant viruses by homologous recombination between AnpeNPV DNA and a polyhedron-based transfer vector. In this system, low frequency of homologous recombination leads to a small number of recombinant viruses, resulting in time-consuming purification with the probability of losing recombinants. We have recently constructed a linearized derivative of AnpeNPV, which is already being used to significantly improve the efficiency of homologous recombination in AnpeNPV-based BEVS (Zhao et al. 2020). However, the step of purification of recombinant viruses generated using Tn-Hi5 cells is required. Therefore, it is imperative to construct a new bacmid system for not only improving the AnpeNPV-based BEVS but also for performing functional studies of AnpeNPV genes.

In the present study, we constructed a novel AnpeNPV bacmid carrying the bacterial transposon Tn7 target site (mini-*att*Tn7) flanked by *lacZα* using a bacterial BAC vector, pSMART BAC. The newly established Bac-to-Bac expression system is composed of the *E. coli* DH10B carrying the helper plasmid, the AnpeNPV bacmid, and a transfer vector pApBacDual-N1, which harbors two very late promoters (*polyhedrin* and *p10*) from AnpeNPV flanked by the left and right ends of the mini-Tn7 element. The infective recombinant AnpeNPV bacmid was generated by site-specific transposition between the transfer vector and AnpeNPV bacmid in the presence of Tn7-transposase from the helper plasmid (Kitts and Possee 1993). Using the AnpeNPV Bac-to-Bac expression system, we expressed enhanced green fluorescent protein (EGFP) in both Tn-Hi5 cells and *A. pernyi* pupae. Further, a *cathepsin* (*v-cath*) and *chitinase* (*chiA*) gene-defective AnpeNPV bacmid was created using the phage λ Red recombination system and the FLP/FRT (FLP recombinase recognition target) recombinase system (Datsenko and Wanner 2000). The expression of human epidermal growth factor (huEGF) in *A. pernyi* pupae was tested using *v-cath* and *chiA*-defective AnpeNPV bacmid. The AnpeNPV bacmid system is a useful tool for manipulation of the AnpeNPV genomic DNA in *E. coli*, thereby identifying and characterizing genes of AnpeNPV, and obtaining recombinant AnpeNPV in a simpler and faster way.

Materials and Methods

Insects, Viruses, Viral DNA, Cell Lines, and Media

Antheraea pernyi pupae were obtained from the Sericultural Research Institute of Liaoning Province (Fengcheng, China). AnpeNPV L2 (GenBank: EF207986) and a linearized derivative of AnpeNPV (Zhao et al. 2020) were available in our lab and propagated in *A. pernyi* pupae. The AnpeNPV genomic DNA was extracted as per previously described methods (Fan et al. 2007). Tn-Hi5 cells (BTI-TN-5B1-4) were purchased from Thermo Fisher Scientific (Waltham, MA) and cultured in TNM-FH medium (GE Healthcare Life Sciences, Chicago, IL) supplemented with 10% (v/v) fetal bovine serum (Gibco, Billings, MT) containing 0.5% penicillin–streptomycin solution (Gibco) at 27°C. SF-900 II serum-free medium (Cat. no. 10902-088) and Cellfectin II (Cat. no. 10362100) were purchased from Thermo Fisher Scientific.

Bacterial Cells, Plasmids, and Others

The BAC vector pSMART BAC BamHI (42030–1) and *E. coli* BAC-Optimized Replicator v2.0 Electrocompetent Cells (60210–1) were purchased from Lucigen Corporation (Middleton, WI). *Escherichia coli* DH10Bac (10361-012), DH10B (18290-015), and the transfer vector pFastBac Dual (10712-024) were purchased from Thermo Fisher Scientific. The AcNPV bacmid bMON14272 and the helper plasmid with transposase functions pMON7124 were isolated from DH10Bac competent cells. The plasmids pKD46 and pCP20 were purchased from Miaoling Bioscience & Technology Co., Ltd. (Wuhan, China). Plasmid DNA purification kits (PD1311-01) and PCR product purification kits (DC3514) were purchased from BIOMIGA (Hangzhou, China). The antibiotics, antibodies, isopropyl-b-d-thiogalactopyranoside (IPTG), and X-Gal were purchased from BioDee Biotechnology Co., Ltd. (Beijing, China).

Construction of the AnpeNPV Bacmid

A flowchart describing the construction of the AnpeNPV bacmid is shown in Fig. 1. Briefly, pSMART BAC-N1 harboring the chloramphenicol resistance gene (*Cm*) and a multiple cloning site was derived from pSMART BAC BamHI (42030–1, Lucigen) using PCR-driven overlap extension (Heckman and Pease 2007) and self-ligation (Supp Fig. 1 [online only]). A fragment carrying the bacterial transposon Tn7 target site flanked by *lacZα* reporter gene (*lacZα*:mini-*att*Tn7:*lacZα*) was obtained from the AcNPV bacmid bMON14272 (Luckow et al. 1993) by PCR using the primers PlacZ-mini-F/PlacZ-mini-R (Table 1) and cloned into the pMD18-T vector (6011, TaKaRa) for sequencing. It was digested at the restriction sites BglII and AvrII, which were synthetically incorporated into the primers. The digested fragment was then gel-purified and cloned at the BamHI/AvrII restriction sites of pSMART BAC-N1 to generate the plasmid pSMART BAC-N1/*lacZα*:mini-*att*Tn7:*lacZα* (Fig. 1A). Further, two DNA fragments, one containing partial *orf144*, full-length *orf145*, and the polyhedrin promoter from the AnpeNPV genomic DNA (corresponding to 124957–126242 nt) with AvrII and HindIII restriction sites and the other containing partial *polyhedrin* and *orf2* of the AnpeNPV genomic DNA (corresponding to 254–1393 nt) with BamHI and AvrII restriction sites, were amplified by PCR using the primer pairs PAnpeNPV-F1/PAnpeNPV-R1 and PAnpeNPV-F2/PAnpeNPV-R2,

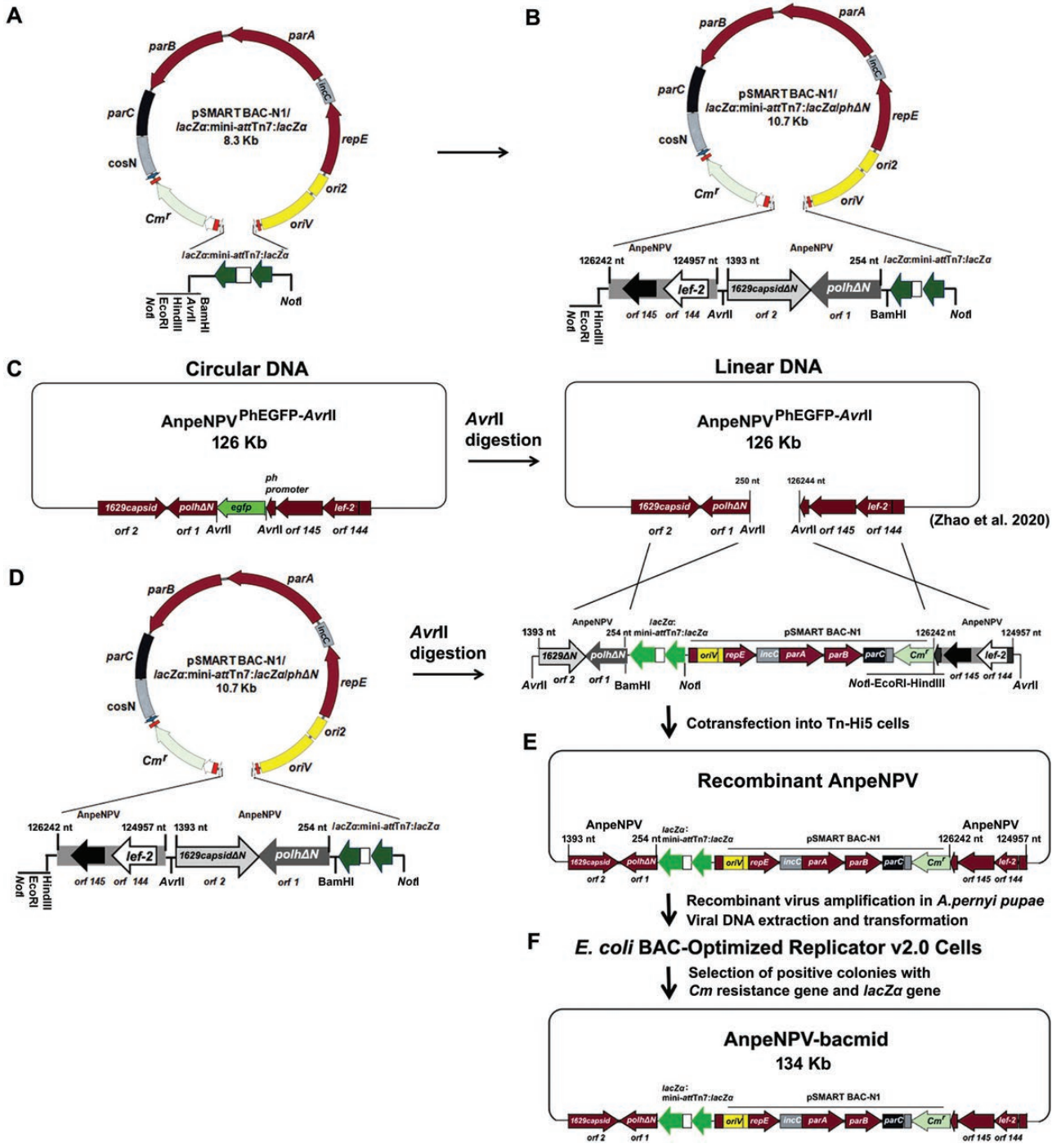

Fig. 1. Schematic representation of the construction strategy of the AnpeNPV bacmid. (A) A fragment carrying the bacterial transposon Tn7 target site flanked by *lacZ*α reporter gene (*lacZ*α:mini-*att*Tn7:*lacZ*α) was inserted into pSMART BAC-N1 to generate the plasmid pSMART BAC-N1/*lacZ*α:mini-*att*Tn7:*lacZ*α. (B) Two DNA fragments, one containing partial *orf144* (*lef-2*), full-length *orf145,* and the polyhedrin promoter of AnpeNPV genomic DNA (correspond to nt 124957–126242*) with AvrII and HindIII restriction sites and the other containing partial *orf1* (*polyhedrin*) and *orf2* (*1629capsid*) (corresponding to nt 254–1393*) with BamHI and AvrII restriction sites were cloned into pSMART BAC-N1/*lacZ*α:mini-*att*Tn7:*lacZ*α to create the transfer vector pSMARTBAC-N1/*lacZ*α:mini-*att*Tn7:*lacZ*α/*phΔN*. *The numbering system begins with the first nucleotide (A) of the initiation codon of *orf1* (*polyhedrin*) based on the sequence of AnpeNPV L2 (GenBank accession number: EF207986). (C) Treatment of the genomic DNA of AnpeNPV$^{PhEGFP\text{-}AvrII}$ with AvrII endonuclease generated the linear AnpeNPV DNA (Zhao et al. 2020). (D) Treatment of pSMARTBAC-N1/*lacZ*α:mini-*att*Tn7:*lacZ*α/*phΔN* with AvrII generated the linearized transfer vector DNA. (E) The recombinant AnpeNPV was obtained by homologous recombination between the linear AnpeNPV DNA (C) and the linearized transfer vector DNA (D) in Tn-Hi5 cells, and the recombinant virus was amplified in *A. pernyi* pupae. (F) The genomic DNA of the recombinant AnpeNPV was extracted from hemolymph of *A. pernyi* pupae and transformed into *E. coli* BAC-Optimized Replicator v2.0 Cells by electroporation. Positive colonies that contained AnpeNPV genomic DNA with chloramphenicol resistance gene and *lacZ*α reporter gene were selected. The bacmid is named AnpeNPV bacmid.

Table 1. Primers used for the construction of vectors in this study

Primer name	Primer Sequences (5′–3′)	Amplicon length (bp)	Restriction site
PlacZ-mini-F	CGAGATCTCCCGGGCTGCAGGAATTCACATAAC	404	BglII
PlacZ-mini-R	ATCCTAGGGATCCGCTAGCGTCTTCGAAGCGCGTAACC		AvrII, BamHI
PAnpeNPV-F1	GACCTAGGGCTGCGACGCGAACTAAATAGC	1286	AvrII
PAnpeNPV-R1	GGAAGCTTATAGGAAATTTTACTACAAAG		HindIII
PAnpeNPV-F2	ACGGATCCTTATTGTCAACTGGAGCGG	1139	BamHI
PAnpeNPV-R2	CCCCTAGGCGGCGACTTGTTAAACCAG		AvrII
PAnpeNPV-F3	GTCTGCAGGAATACACCAAGTTTGGCG	275	PstI
PAnpeNPV-R3	GAGAATTCTGAATTACCATCAAGCGCGG		EcoRI
PAnpeNPV-F4	AAGTCGACGAACTTGCAACCTTAGCAAC	233	SalI
PAnpeNPV-R4	GAGGATCCGCTCAAACGGCAGCATGCTC		BamHI
Pegfp-BamHI	GAGGATCCATGGTGAGCAAGGGCGAGGAGC	720	BamHI
Pegfp-EcoRI	CCGAATTCTTACTTGTACAGCTCGTCCATG		EcoRI

Relevant restriction sites incorporated in the primers are underlined.

respectively (Table 1). The two fragments were then inserted into the plasmid pSMART BAC-N1/*lacZα*:mini-*att*Tn7:*lacZα* multiple cloning site to produce the transfer vector pSMARTBAC-N1/*lacZα*:mini-*att*Tn7:*lacZα*/*phΔN* (Fig. 1B). This transfer vector DNA was linearized with AvrII digestion (Fig. 1D) and used for homologous recombination by co-transfection with the linear AnpeNPV DNA (Zhao et al. 2020; Fig. 1C) into the Tn-Hi5 cells. For co-transfection, 1 μg of the linearized transfer vector DNA and 0.5 μg of the linear AnpeNPV DNA were co-transfected into Tn-Hi5 cells with Cellfectin II according to the manufacturer's instructions. The cell culture supernatant was harvested at 5- to 6-d posttransfection and injected into *A. pernyi* pupae (100 μl for each pupa) to amplify the recombinant AnpeNPV (Zhao et al. 2020; Fig. 1E). After incubation for 10–12 d at 22°C–24°C, hemolymph samples from infected pupae were collected and used to extract viral genomic DNA as previously described (Fan et al. 2007). The viral genomic DNA was detected by PCR using the primer pairs PlacZ-mini-F/PlacZ-mini-R and PAnpeNPV-F1/PAnpeNPV-R1 (Table 1).

Subsequently, 0.2 μg (<2 μl) of the viral genomic DNA was transformed into the BAC-Optimized Replicator v2.0 Electrocompetent Cells (Lucigen, 60210–1) using the electroporator Gene Pulser II (Bio-Rad, Cat 165–2105) with 1-mm cuvettes under the conditions of 1.8 KV, 25 μF, and 200 Ohm (Fig. 1F). Transformed cells were spread on YT plates (8-g Bacto-tryptone, 5-g yeast extract, 5-g NaCl, and 15-g agar/liter) containing chloramphenicol (12.5 μg/ml), X-Gal (100 μg/ml), and IPTG (40 μg/ml), and incubated overnight at 37°C. Colonies appearing blue due to the expression of *lacZα* reporter gene in *E. coli* and representing chloramphenicol-resistant clones were selected and grown in LB containing 12.5 μg/ml chloramphenicol overnight with shaking at 37°C. DNA minipreps were performed using a plasmid DNA purification kit (PD1311-01, BIOMIGA) following standard methods. Bacmid DNA was detected by PCR using the primer pairs PlacZ-mini-F/PlacZ-mini-R, PAnpeNPV-F1/PAnpeNPV-R1, and PAnpeNPV-F2/PAnpeNPV-R2 (Table 1). The bacmid DNA identified through PCR was digested with XhoI restriction enzyme for comparison with the wild-type AnpeNPV DNA to further confirm the presence of the insertion.

Transposition of the AnpeNPV Bacmid

The AnpeNPV bacmid DNA was transformed into DH10B electrocompetent cells harboring the transposition helper plasmid pMON7124 by electroporation as described above. The cells were plated on LB plates containing chloramphenicol (12.5 μg/ml) and tetracycline (10 μg/ml), and the colonies obtained were designated as *E. coli* DH10Bac/AnpeNPV bacmid.

The vector pApBacDual-N1 was derived from pFastBac Dual (Invitrogen, 10712-024). In this vector, the polyhedrin (PH) promoter and p10 promoter from AnpeNPV replaced the PH promoter and p10 promoter of pFastBac Dual. These promoters were used to control the expression of two heterologous genes (Supp Fig. 2 [online only]). The EGFP gene was amplified from the viral DNA of *ApNPV-Δph/egfp*$^{+}$ (Zhao et al. 2019) by PCR using Pegfp-BamHI and Pegfp-EcoRI primers (Table 1) and cloned into the BamHI and EcoRI sites of pApBacDual-N1 to generate the donor plasmid pApBacDual-N1/*phegfp*. In this plasmid, *egfp* was under the control of the polyhedrin promoter.

The site-specific transposition was performed by transforming pApBacDual-N1/*phegfp* DNA into *E. coli* DH10Bac/AnpeNPV bacmid. *Egfp* was inserted into the mini-*att*Tn7 cassette, which resulted in the disruption of the *lacZα* sequence. The transformed cells were plated on LB plates containing chloramphenicol (12.5 μg/ml), gentamicin (7 μg/ml), tetracycline (10 μg/ml), X-Gal (100 μg/ml), and IPTG (40 μg/ml). After incubation at 37°C for 24 h, white colonies were picked and grown in LB medium containing 12.5 μg/ml chloramphenicol and 7 μg/ml gentamicin for extraction of the recombinant bacmid DNA, named as AnpeNPV-bacmid/*phegfp*.

Further, 0.5 μg DNA of AnpeNPV-bacmid/*phegfp* was transfected into Tn-Hi5 cells using Cellfectin II according to the manufacturer's instructions. After 5 d of incubation at 27°C, EGFP-expressing cells were detected under UV light using an inverted microscope (DMI3000B; Leica, Wetzlar, Germany). The culture supernatant was harvested and injected into *A. pernyi* pupae (100 μl for each pupa) to amplify the recombinant virus, which is designated as vAnpeNPV-bacmid/*phegfp*. After 10 d of incubation at 22°C–24°C, hemolymph cells were collected from the pupae and detected by using an inverted microscope.

Deletion of v-cath and chiA Genes in AnpeNPV Bacmid Using λ Red Recombinase

AnpeNPV bacmid DNA was introduced into DH10B electrocompetent cells containing phage λ Red recombinase (pKD46) using electroporation as described above. The transformed cells were plated on LB plates containing chloramphenicol (12.5 μg/ml) and ampicillin (100 μg/ml), and the colonies obtained were designated as *E. coli* DH10B/AnpeNPV bacmid/λ Red recombinase.

To construct the gene-targeting vector for homologous recombination with AnpeNPV, a DNA fragment containing *zeocin* (Zeo) (GenBank: AOS59253.1) flanked by flippase recognition target (FRT) sequences (GenBank: MH976504.1:958–1005) and cloning

sites was synthesized at SBS Genetech Co., Ltd. (Beijing, China), and cloned in the vector pUC57 to generate the plasmid pUC57/*FRT-Zeo-FRT* (Supp Materials and Methods [online only]). To remove the *cathepsin* (*v-cath*, *orf31*) and *chitinase* (*chiA*, *orf32*), two homologous targeting arms corresponding to 28,464–28,738 nt and 31,410–31,642 nt of the AnpeNPV genomic DNA were amplified by PCR using the primer pairs PAnpeNPV-F3/PAnpeNPV-R3 and PAnpeNPV-F4/PAnpeNPV-R4, respectively (Table 1). The resultant fragments were inserted into the vector pUC57/*FRT-Zeo-FRT*, and the gene-targeting vector pUC57/*FRT-Zeo-FRT*/*Δv-cath*/*ΔchiA* was generated.

For recombination, the fragment containing homologous arms with the *FRT-Zeo-FRT* element was amplified from the gene-targeting vector by PCR using PAnpeNPV-F3/PAnpeNPV-R4 primers (Table 1) and purified using a PCR product purification kit (DC3514, BIOMIGA). In total, 0.2 μg of the DNA fragment was transformed into electrocompetent cells of *E. coli* DH10B/AnpeNPV bacmid/λ Red recombinase by electroporation as described above. Positive recombinant colonies were selected after culturing in LB medium containing chloramphenicol (12.5 μg/ml) and zeocin (50 μg/ml). The bacmid DNA of positive colonies was further transformed into electrocompetent cells of *E. coli* DH10B with FLP recombinase (pCP20). The fragment of *zeocin* flanked by FRT was removed from AnpeNPV bacmid with FLP recombinase. The positive colonies were selected on chloramphenicol (12.5 μg/ml) and ampicillin (100 μg/ml) plates after culturing and screened by PCR with the PAnpeNPV-F3/PAnpeNPV-R4 primers (Table 1). The PCR products were further cloned into the pMD18-T vector (6011, TaKaRa) to confirm the sequence of *zeocin*. The resulting bacmid was designated as AnpeNPV bacmid/*Δv-cath:ΔchiA*.

Expression of huEGF in *A. pernyi* Pupae Using AnpeNPV Bacmid/Δv-cath:ΔchiA

The AnpeNPV bacmid/*Δv-cath:ΔchiA* DNA was transformed into DH10B electrocompetent cells with the helper plasmid pMON7124 by electroporation as described above. The resultant cells were designated as *E. coli* DH10Bac/AnpeNPV bacmid/*Δv-cath:ΔchiA*. A gene encoding peptide of huEGF (GenBank: NP 001954, Asn971- Arg1023) fused with 6x His-tag at the C-terminal was synthesized at SBS Genetech Co., Ltd. (Beijing, China) and inserted into pApBacDual-N1 at the BamHI and EcoRI sites to generate the donor plasmid pApBacDual-N1/*phhuegf*. In this plasmid, *huegf* was under the control of the polyhedrin promoter.

The pApBacDual-N1/*phhuegf* DNA was transformed into *E. coli* DH10Bac/AnpeNPV bacmid/*Δv-cath:ΔchiA* cells as per methods described above. The cells were plated on LB plates containing chloramphenicol (12.5 μg/ml), gentamicin (7 μg/ml), tetracycline (10 μg/ml), X-Gal (100 μg/ml), and IPTG (40 μg/ml), and white colonies were selected for culturing for the extraction of the recombinant bacmid DNA, named as AnpeNPV-bacmid/*Δv-cath:ΔchiA/phhuegf*.

Subsequently, 0.5-μg DNA of AnpeNPV-bacmid/*Δv-cath:ΔchiA/phhuegf* was transfected into Tn-Hi5 cells by Cellfectin II as described above. After 5 d of incubation at 27°C, the cell culture supernatant was harvested and injected into *A. pernyi* pupae (100 μl for each pupa) to obtain the recombinant virus, which was designated as vAnpeNPV-bacmid/*Δv-cath:ΔchiA/phhuegf*. After 7–11 d of incubation at 22°C–24°C, hemolymph cells were collected from pupae and detected using western blotting with an anti-His antibody (D291-3, MBL Beijing Biotech Co., Ltd, Beijing, China).

Results

Generation of the AnpeNPV Bacmid

To construct the AnpeNPV bacmid, we firstly engineered the transfer vector pSMARTBAC-N1/*lacZα*:mini-*att*Tn7:*lacZα*/*phΔN*, which contained the unique AvrII restriction site to facilitate the linearization of the plasmid DNA (Fig. 1B). Homologous recombination between the linear AnpeNPV DNA (Zhao et al. 2020; Fig. 1C) and the linearized transfer vector DNA (Fig. 1D) occurred in Tn-Hi5 cells, which generated a recombinant AnpeNPV containing pSMART BAC with transposon Tn7 target site and *lacZα* reporter gene at the polyhedrin locus of AnpeNPV (Fig. 1E). PCR detection of the infected pupae using the primer pairs PlacZ-mini-F/PlacZ-mini-R and PAnpeNPV-F1/PAnpeNPV-R1 exhibited specific bands of 404 and 1,286 bp, respectively (Fig. 2A), confirming that the recombinant AnpeNPV was produced in the infected pupae.

Crude viral genomic DNA extracted from the hemolymph of the infected pupae was transformed into *E. coli* BAC-Optimized Replicator v2.0 Electrocompetent Cells (Fig. 1F). As expected, blue colonies were obtained on YT plates containing chloramphenicol, X-Gal, and IPTG. Bacmids from three colonies were purified and detected using PCR with the primer pairs PlacZ-mini-F/PlacZ-mini-R,

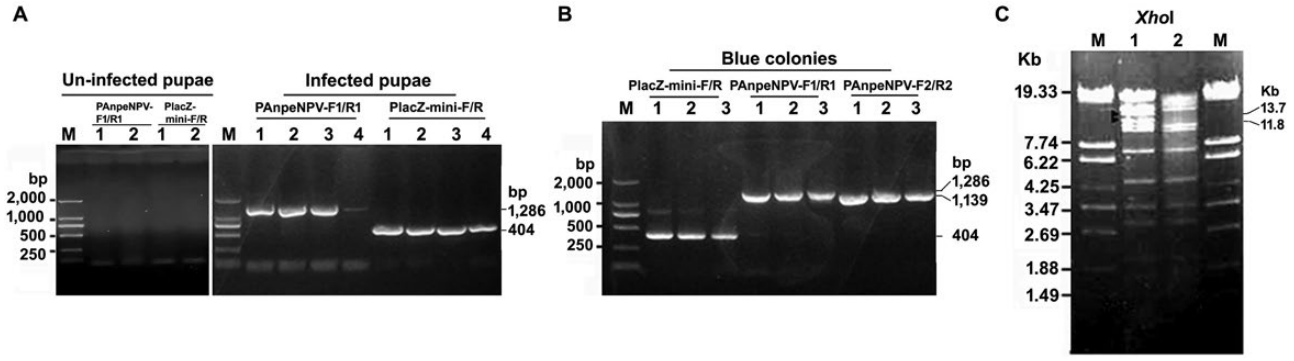

Fig. 2. Identification of the AnpeNPV bacmid. (A) The recombinant AnpeNPV DNA samples isolated from different infected *A. pernyi* pupae (No. 1–4) were subjected to PCR tests using the primer pairs PlacZ-mini-F/PlacZ-mini-R and PAnpeNPV-F1/PAnpeNPV-R1. M-DL 2,000 DNA marker. The genomic DNA from un-infected pupae was used as the negative control. (B) Bacmid DNA from a different blue colony (No. 1–3) was detected by PCR using the primer pairs PlacZ-mini-F/PlacZ-mini-R, PAnpeNPV-F1/PAnpeNPV-R1, and PAnpeNPV-F2/PAnpeNPV-R2. M-DL 2,000 DNA marker. (C) Identification of the AnpeNPV bacmid DNA digested with XhoI endonuclease. M- λ/EcoRT14 I marker. 1-AnpeNPV bacmid DNA digested with XhoI endonuclease. 2-The wild-type AnpeNPV DNA digested with XhoI endonuclease. Black triangle indicates that two specific fragments were observed when the AnpeNPV bacmid DNA was digested with XhoI in comparison with the wild-type AnpeNPV DNA.

PAnpeNPV-F1/PAnpeNPV-R1, and PAnpeNPV-F2/PAnpeNPV-R2. All three samples showed PCR products of the same size, corresponding to 404, 1,286, and 1,139 bp, respectively (Fig. 2B). Restriction analysis with XhoI showed that two specific fragments of 13.7 and 11.8 kb, respectively, were present in the positive bacmid DNA in comparison with the wild-type AnpeNPV DNA (Fig. 2C). It indicated that AnpeNPV genome was successfully integrated into pSMART BAC in the bacmid. The resulting bacmid was named as AnpeNPV bacmid, which contained the AnpeNPV genome without the partial polyhedrin sequence (corresponding to 1–253 nt), the transposon Tn7 target site, *lacZα* reporter gene, and pSMART BAC vector carrying chloramphenicol resistance gene, single-copy replication origin, and inducible medium-copy replication origin (Fig. 1F).

Construction of the AnpeNPV Bac-to-Bac Expression System

Bacmids are widely used to generate expression vectors by introducing the gene of interest into the transposon Tn7 target site in the bacmid. To test if AnpeNPV bacmid could be used to generate the recombinant bacmid by site-specific transposition to infect Tn-Hi5 cells and *A. pernyi* pupae, the specialized donor plasmid pApBacDual-N1/*phegfp* was constructed and transformed into the *E. coli* DH10Bac/AnpeNPV bacmid cells harboring the helper plasmid pMON7124. The recombinant bacmid was obtained and named as AnpeNPV-bacmid/*phegfp*. EGFP was expressed and observed in Tn-Hi5 cells after 120 h of transfection with the AnpeNPV-bacmid/*phegfp* DNA (Fig. 3A). The culture supernatant of transfected cells was further used to infect *A. pernyi* pupae. EGFP expression in the cells from infected pupae was also observed after 6 d of infection (Fig. 3C). These data showed that the AnpeNPV bacmid could be used to produce recombinant viruses by site-specific transposition for protein expression in *A. pernyi* pupae. This indicated that the AnpeNPV Bac-to-Bac expression system, consisting of the transfer vector pApBacDual-N1 and the *E. coli* DH10Bac/AnpeNPV bacmid cells harboring the helper plasmid pMON7124, was successfully constructed.

Construction of the v-cath and chiA-Defective AnpeNPV Bacmid

Using pUC57/*FRT-Zeo-FRT* (Fig. 4A), the gene-targeting vector pUC57/*FRT-Zeo-FRT/Δv-cath/ΔchiA* (Fig. 4B) was constructed for the deletion of *v-cath* and *chiA* genes from the AnpeNPV genome. After transforming the PCR products harboring the FRT-zeocin resistance cassette with AnpeNPV flanking sequences into *E. coli* DH10B/AnpeNPV bacmid/λ Red recombinase cells, zeocin resistance transformants were obtained by replacing *v-cath* and *chiA* genes with the *FRT-Zeo-FRT* element through homologous recombination. The colonies harboring the successfully recombined construct

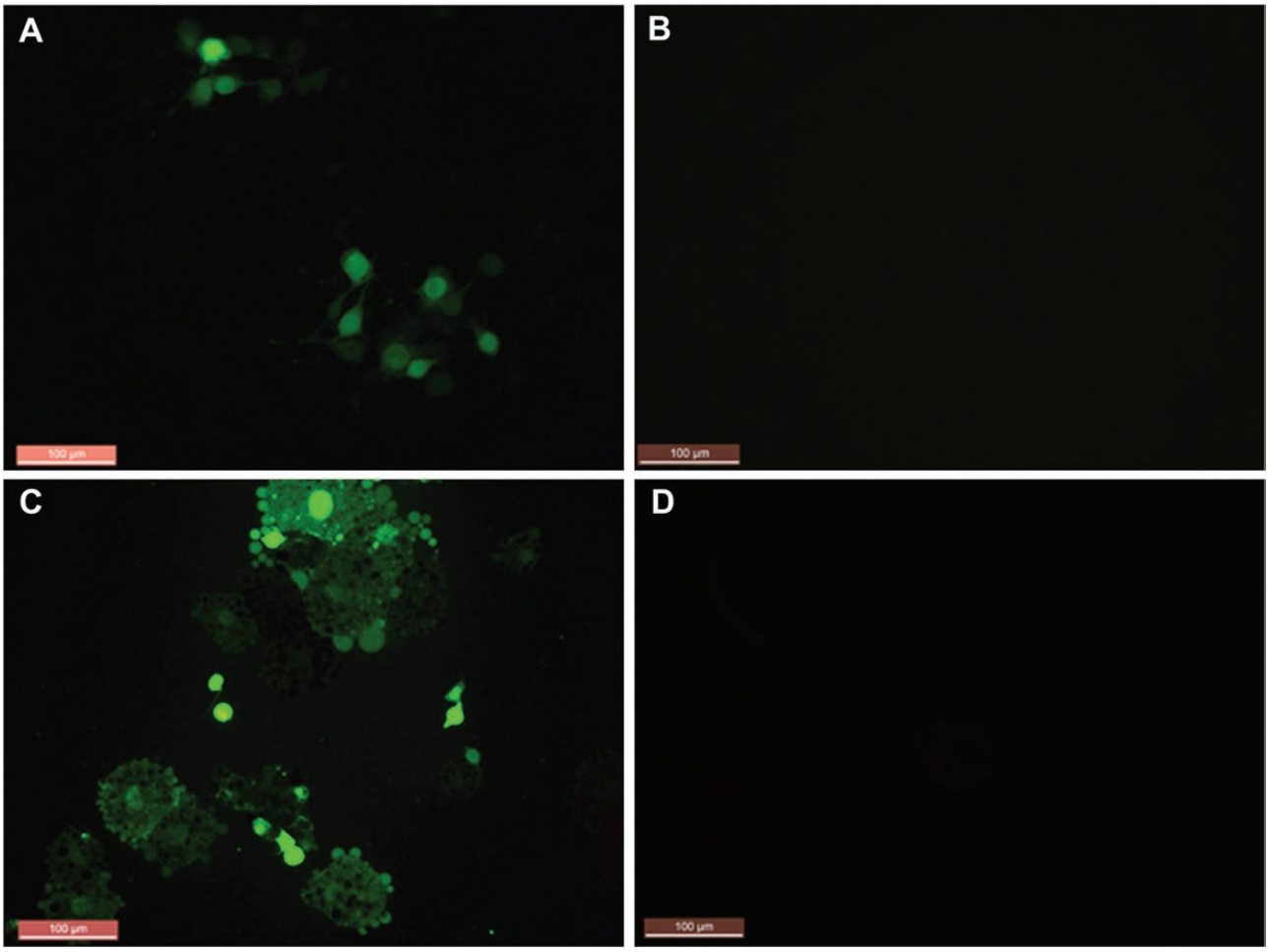

Fig. 3. Expression of EGFP using the AnpeNPV Bac-to-Bac expression system. (A) Tn-Hi5 cells transfected with the AnpeNPV-bacmid/*phegfp* DNA, 120 hpi. (B) Untransfected Tn-Hi5 cells. (C) Hemolymph cells from the vAnpeNPV-bacmid/*phegfp* infected *A. pernyi* pupae, 10 dpi. (D) Hemolymph cells from the wild-type AnpeNPV infected *A. pernyi* pupae. EGFP expression in cells was detected under UV light using an inverted microscope (100 μm).

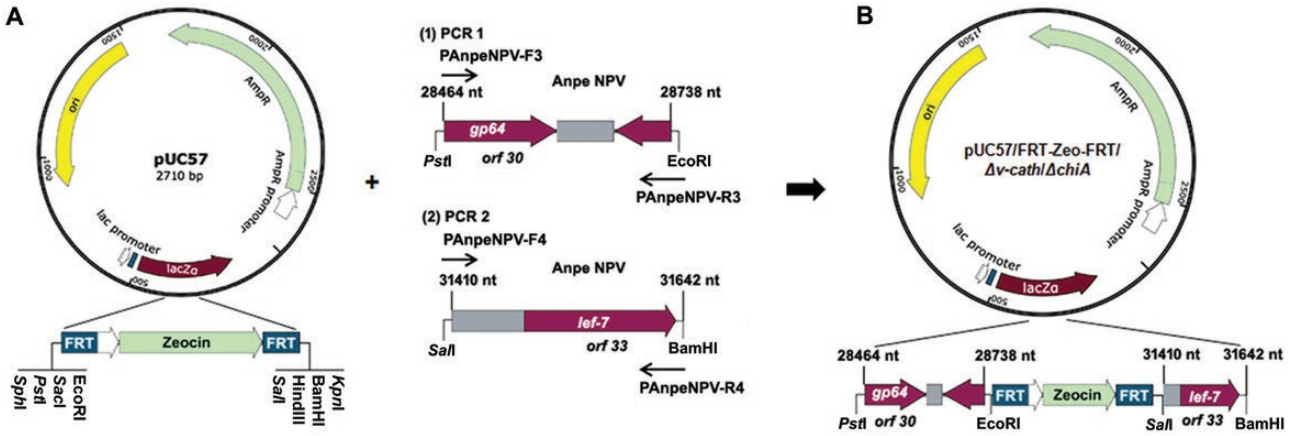

Fig. 4. Construction of the gene-targeting vector for deletion of *cathepsin* and *chitinase* genes from the AnpeNPV genome. (A) Two homologous targeting arms corresponding to nt 28464–28738* and nt 31410–31642* of the AnpeNPV genome were inserted into the vector pUC57/*FRT-Zeo-FRT* to generate the gene-targeting vector pUC57/*FRT-Zeo-FRT*/Δ*v-cath*/Δ*chiA*. *The numbering system begins with the first nucleotide (A) of the initiation codon of *orf1* (*polyhedrin*) based on the sequence of AnpeNPV L2 (GenBank accession number: EF207986). (B) The structure of the gene-targeting vector. The PCR products containing FRT-zeocin resistance cassette flanked by homologous targeting arms were obtained using the primers PAnpeNPV-F3/PAnpeNPV-R4 and used for transformation in *E. coli* DH10B/AnpeNPV bacmid/λ Red recombinase cells.

were selected on LB agar plates containing chloramphenicol and zeocin. PCR analysis with the PAnpeNPV-F3/PAnpeNPV-R4 primers showed DNA fragments of 1,091 bp, which were of the correct size as expected to be obtained from the recombinant bacmid DNA of positive colonies (Fig. 5A). The zeocin resistance cassette was then eliminated by transforming the recombinant bacmid DNA into *E. coli* DH10B with FLP recombinase (pCP20) and counter-selecting the colonies on plates containing both chloramphenicol and ampicillin. The final positive colonies were further detected by PCR analysis (Fig. 5B1) and DNA sequencing (data not shown). As expected, sequencing results indicated that *v-cath* and *chiA* genes were deleted and replaced by an FRT fragment without *zeocin* in the recombinant bacmid, named AnpeNPV bacmid/*Δv-cath:ΔchiA* (Fig. 5B2). The results demonstrated that AnpeNPV bacmid based on pSMART BAC vector could be used to generate gene-defective AnpeNPV bacmid in *E. coli* DH10B using λ Red recombinase and FLP recombinase.

Expression of the Recombinant huEGF

To examine the utility of AnpeNPV bacmid/*Δv-cath:ΔchiA* as an expression vector, *huegf* was used to generate the recombinant virus designated as vAnpeNPV-bacmid/*Δv-cath:ΔchiA/huegf*. The expression of huEGF in *A. pernyi* pupae infected with the recombinant virus was detected by western blotting. The results showed a specific band with a size was higher than the expected size (7.5 kDa) in cell lysates of the infected-*A. pernyi* pupae (Fig. 6). Nothing was detected in the negative control sample from cell lysates of *A. pernyi* pupae infected with the wild-type AnpeNPV. This indicated that AnpeNPV bacmid/*Δv-cath:ΔchiA* could be used for recombinant protein production in *A. pernyi* pupae.

Discussion

In this study, we successfully constructed the AnpeNPV bacmid through homologous recombination between pSMART BAC vector with flanking AnpeNPV homologous arms and the linear AnpeNPV DNA in Tn-Hi5 cells. The AnpeNPV bacmid can be propagated in *E. coli* as a BAC, and it is infectious to both Tn-Hi5 cells and *A. pernyi* pupae as a specific baculovirus. Based on the AnpeNPV bacmid, we developed the AnpeNPV Bac-to-Bac expression system that allowed a rapid generation of recombinant AnpeNPVs. Moreover, we established the AnpeNPV bacmid/λ Red recombination system, which provided an efficient methodology for deletion of genes from the AnpeNPV genome (or inserting genes into AnpeNPV genome) in *E. coli* and for the study of gene function.

Different strategies have been used to construct bacmids from various baculovirus genomes. The AcNPV bacmid was generated by homologous recombination between a transfer vector (pMON14271) containing the mini-F-*lacZα*-mini-attTn7-Kan cassette and the wild-type genomic AcNPV DNA in insect cells (Luckow et al. 1993). The same strategy was used to produce the HearNPV bacmid (Wang et al. 2003) and BmNPV bacmid (Motohashi et al. 2005). For the construction of the CpGV and SeMNPV bacmids, the mini-F-*lacZα*-mini-attTn7-Kan cassette from the AcNPV bacmid was ligated into a unique restriction site within the viral genome and was directly transformed into *E. coli* (Pijlman et al. 2002, Hilton et al. 2008). To generate the AnpeNPV bacmid, a transfer vector carrying the mini-F-*lacZα*-mini-attTn7-Kan cassette from AcNPV bacmid was initially constructed and co-transfected with the wild-type genomic AnpeNPV DNA into Tn-Hi5 cells. However, no correct recombinant AnpeNPV was obtained; this was probably due to low frequency of homologous recombination in nonpermissive Tn-Hi5 cells (data not shown). Previous studies have shown that a relatively low number of complete viral genomes is produced by direct cloning for bacmids (Pijlman et al. 2002, Wang et al. 2003). Linear AnpeNPV DNA has been shown to increase the frequency of homologous recombination with a cognate transfer vector in Tn-Hi5 cells (Zhao et al. 2020). The CopyRight pSMART BAC cloning vector harboring chloramphenicol resistance gene (42030–1, Lucigen) utilizes the single-copy origin of the *E. coli* F plasmid to maintain large inserts of up to 350 kb with the highest stability possible in *E. coli*. In the present study, the pSMART BAC cloning vector was chosen to construct a new transfer vector pSMARTBAC-N1/*lacZα*:mini-*att*Tn7:*lacZα*/*phΔN*. In this vector, AvrII restriction site was introduced between two homologous arms of AnpeNPV, thereby allowing linearization of the transfer vector DNA (Fig. 1B). Homologous recombination between the linear transfer vector DNA and linear AnpeNPV DNA occurred with high efficiency in Tn-Hi5 cells, thereby producing the correct AnpeNPV bacmid that could be

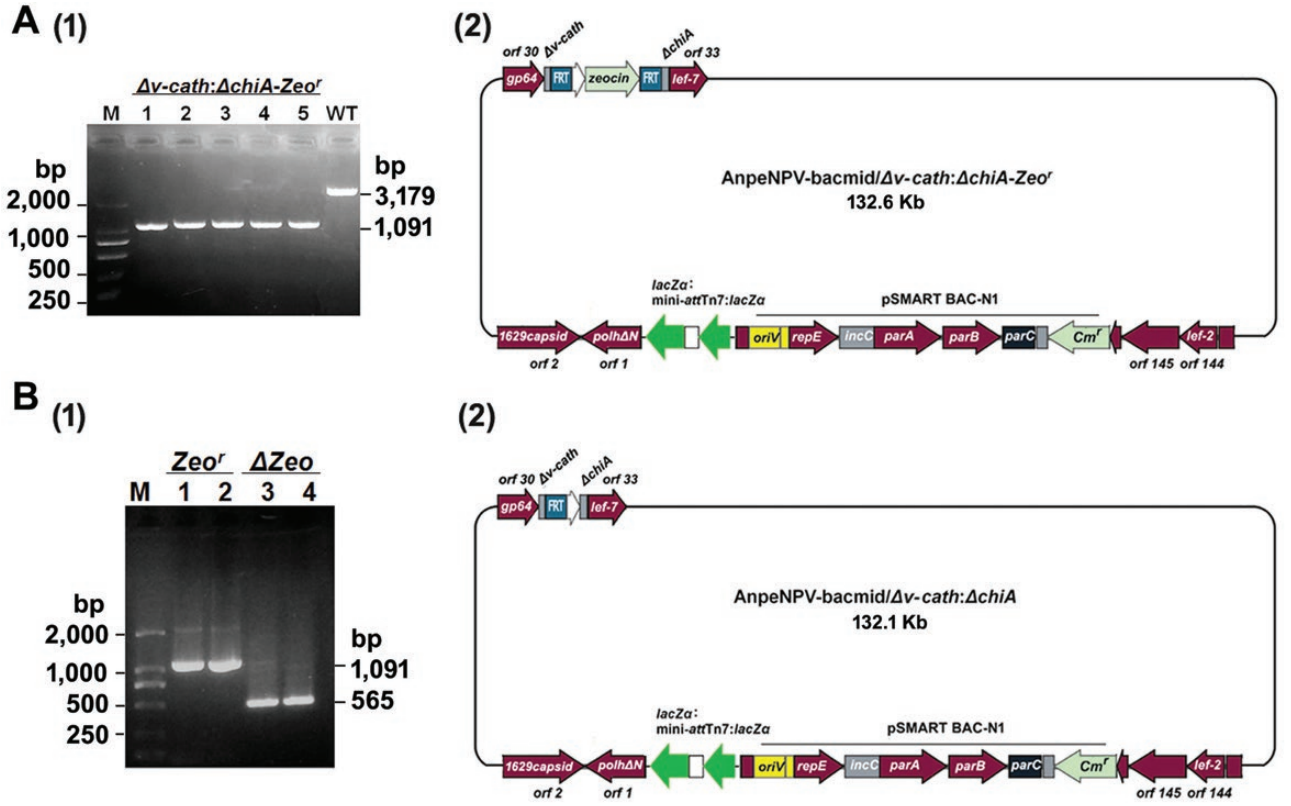

Fig. 5. PCR detection of the *cathepsin* and *chitinase* gene-defective AnpeNPV bacmid. (A1) Bacmid DNA from a different colony with zeocin resistance (No. 1–5) and the AnpeNPV bacmid DNA (WT) were detected by PCR using the primers PAnpeNPV-F3/PAnpeNPV-R4. M-DL 2,000 DNA marker. (A2) The map shows that *cathepsin* and *chitinase* were deleted from the AnpeNPV bacmid, and *zeocin* was inserted between *orf30* and *orf33*. (**B**1) Bacmid DNA from a different colony with zeocin resistance (*Zeo*^{r}, No. 1–2) or without zeocin resistance (Δ*Zeo*, No. 3–4) was detected by PCR using the primers PAnpeNPV-F3/PAnpeNPV-R4. M-DL 2,000 DNA marker. (B2) The map shows that *zeocin* was removed from AnpeNPV bacmid/Δ*v-cath*:Δ*chiA-Zeo*^{r}.

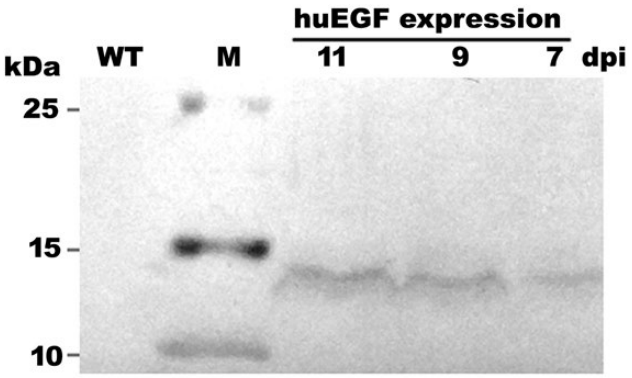

Fig. 6. Western blot analyses of huEGF expression in *A. pernyi* pupae using the AnpeNPV bacmid/Δ*v-cath*:Δ*chiA* as an expression vector. Hemolymph cells from the *A. pernyi* pupae infected with vAnpeNPV-bacmid/Δ*v-cath*:Δ*chiA/huegf* and the wild-type AnpeNPV were detected by western blotting using an anti-His antibody. The 7, 9, 11-dpi of *A. pernyi* pupae infected by vAnpeNPV-bacmid/Δ*v-cath*:Δ*chiA/huegf*. WT-10 dpi of *A. pernyi* pupae infected by the wild-type AnpeNPV.

propagated in *E. coli* BAC-Optimized Replicator v2.0 cells with high yield and quality (Fig. 1F).

A baculovirus bacmid contains the transposon Tn7 target site that allows insertion of the foreign gene of interest by Tn7 transposition using transposase in *E. coli* (Rohrmann 2019). To verify if transposition can also occur properly into the AnpeNPV bacmid, we designed a donor plasmid, pApBacDual-N1/*phegfp*. Transposition events were observed by screening white colonies on agar plates containing X-Gal, IPTG, and three selectable antibiotics after pApBacDual-N1/*phegfp* was transformed into the *E.coli* DH10Bac/AnpeNPV bacmid cells. The production of *lacZ*α was inactivated by the insertion of *egfp* into the mini-attTn7 cassette located on the AnpeNPV bacmid. The recombinant virus generated by the transfection of the recombinant AnpeNPV bacmid DNA into Tn-Hi5 cells could infect both Tn-Hi5 cells and *A. pernyi* pupae. EGFP expression has been detected in Tn-Hi5 cells and tissues and cells of *A. pernyi* pupae (Fig. 3). These data show that the AnpeNPV genomic DNA is stably maintained and that transposition can occur properly into the pSMART BAC-based AnpeNPV bacmid in *E.coli* DH10B.

The bacmid can also be used to delete a target gene from viral genomes with the aid of the λ Red homologous recombination system in *E. coli* for conducting studies on baculovirus gene function. This technique is available for several baculoviruses, including AcMNPV, BmNPV, HearNPV, and CpGV (Wang et al. 2003, Okano et al. 2004, Hilton et al. 2008, Ono et al. 2012). To develop the gene knock-out (KO) technology for AnpeNPV bacmid, we constructed the plasmid pUC57/*FRT-Zeo-FRT* (Fig. 4A) that facilitates the cloning of the homologous arms of any target gene from the AnpeNPV genome. The zeocin resistance gene is used as a marker for selection of positive transformations. FRT sequences are used to eliminate *zeocin* from the integrated site in the recombination bacmid with FLP recombinase. Two nonessential genes, *v-cath* and *chiA*, were successfully deleted from the AnpeNPV bacmid in *E. coli* DH10B cells harboring the plasmid pKD46 with the phage

λ Red recombination system. The zeocin resistance gene could be efficiently removed from the recombinant bacmid DNA in *E. coli* DH10B cells harboring the plasmid pCP20 with FLP recombinase. The gene KO technology is now available for the AnpeNPV bacmid that will allow future studies on AnpeNPV gene function. The expression tests of huEGF showed that the recombinant viruses derived from AnpeNPV bacmid/*Δv-cath*:*ΔchiA* were sufficient to directly infect *A. pernyi* pupae for expression. The recombinant huEGF in our study was found to have a higher molecular weight than what was expected; this was probably due to posttranslational modifications in insects, as shown in other studies (Gomez-Sebastian et al. 2012, Dudognon et al. 2014, Targovnik et al. 2016). EGF is a small peptide of 53 amino acids and contains 6 cysteine residues that form three intramolecular disulfide bonds, which are required for its proper tertiary structure and biological activity. It was considered to be nonglycosylated (Boonstra et al. 1995). However, EGF-like repeats found in functionally diverse proteins can be modified by some of posttranslational modifications, including several forms of *O*-linked glycan modifications in *Drosophila* and mammals (Harris and Spellman 1993, Haltom and Jafar-Nejad 2015). It is unknown whether the recombinant huEGF AnpeNPV expressed in *A. pernyi* pupae could be modified with *O*-linked glycans. The genes *v-cath* and *chiA* encode enzymes involved in the liquefaction of infected insects (Rohrmann 2019). Deletion of these viral genes improves the stability and yield of the secreted recombinant proteins (Hitchman et al. 2010, Lemaitre et al. 2019). In the future, we will further evaluate the expression level, posttranslational modifications and functions of recombinant proteins in *A. pernyi* pupae with the AnpeNPV bacmid/*Δv-cath*:*ΔchiA*.

In summary, the AnpeNPV bacmid based on the pSMART BAC vector was successfully constructed and could be propagated in *E. coli*. The AnpeNPV Bac-to-Bac expression system was developed, and it can be used to generate recombinant AnpeNPVs by site-specific transposition for protein expression in *A. pernyi* pupae. The AnpeNPV bacmid/λ Red recombination system was established and it provided a powerful tool for studying the AnpeNPV gene function.

Supplementary Data

Supplementary data are available at *Journal of Insect Science* online.

Acknowledgments

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Author Contributions

BY, ZZ, and DY carried out the experimental procedures. LW performed the methods for insect cell culture. PL and BZ analyzed the data. BY and QF wrote the manuscript. QF designed and coordinated the study. All authors have read and approved the final version of the manuscript.

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