Construction of a Baculovirus-Silkworm Multigene Expression System and Its Application on Producing Virus-Like Particles

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Abstract

A new baculovirus-silkworm multigene expression system named Bombyx mori MultiBac is developed and described here, by which multiple expression cassettes can be introduced into the Bombyx mori nuclear polyhedrosis virus (BmNPV) genome efficiently. The system consists of three donor vectors (pCTdual, pRADM and pUCDMIG) and an invasive daminopimelate (DAP) auxotrophic recipient E. coli containing BmNPV-Bacmid (BmBacmid) with a homologous recombination region, an attTn7 site and a loxp site. Two genes carried by pCTdual are firstly inserted into BmBacmid by homologous recombination, while the other eight genes in pRADM and pUCDMIG are introduced into BmBacmid through Tn7 transposition and cre-loxp recombination. Then the invasive and DAP auxotrophic E. coli carrying recombinant BmBacmid is directly injected into silkworm for expressing heterologous genes in larvae or pupae. Three structural genes of rotavirus and three flavus genes have been simultaneously expressed in silkworm larvae using our new system, resulting in the formation of virus-like particles (VLPs) of rotavirus and the color change of larvae. The VLPs were purified from hemolymph by ultracentrifugation using CsCl gradients, with a yield of 12.7 μg per larva. For the great capacity of foreign genes and the low cost of feeding silkworm, this high efficient BmMultiBac expression system provides a suitable platform to produce VLPs or protein complexes.

Introduction

Baculovirus expression system is one of the most ideal systems for routine production of recombinant eukaryotic proteins in insect cells, larvae and mammalian cells, which is widely-used in developing virus-like particles (VLPs) vaccine, displaying heterologous peptides or proteins, and transducing genes into mammalian cells [1,2]. Besides the traditional Autographa californica multicapsid nucleopolyhedrovirus-SPodoptera frugiperda 9 (AcMNPV-Sf9) cell line system, another highly efficient baculovirus expression system, named Bombyx mori (silkworm) nucleopolyhedrovirus (BmNPV)-silkworm larvae/pupa system, has also been constructed to express heterologous genes. Compared with the AcMNPV-Sf9 system, the BmNPV-silkworm system provides enhanced expression level and pretty low cost in silkworm larvae or pupae, which shows promising industrialization future. Moreover, recent study has found that the N-acetyl glucosamine and galactose residues also exist in the N-glycan structures produced by silkworms, indicating silkworm larvae might be a useful host for producing human glycoproteins [3].

Until today, great efforts have been made for efficiently constructing recombinant BmNPV, including the BmNPV-based Bac-to-Bac system [4,5], the mating-assisted genetically integrated cloning (MAGIC) method [6] and a method based on zero-background Tn7-mediated transposition in E. coli [7]. Other improvements relating to the baculovirus expression system also have been presented, such as utilizing cysteine protease and chitinase-deficient Bacmid to improve recombinant protein production and keep its stability [8,9], as well as a transfectant-free method by directly infecting insect cells or injecting silkworm larva with invasive E. coli containing recombinant Bacmid [10,11].

Normally, a foreign DNA fragment as large as 50 kb can be accommodated into the 130 kb dsDNA genome of baculovirus, which means several expression cassettes can be integrated into recombinant baculovirus. Thus, a multiple genes baculovirus expression system (MultiBac) has been rationally brought into the baculovirus vector for simultaneously expressing heterologous proteins [8]. The MultiBac system provides a powerful tool for over-expressing the low abundance protein complexes within cell for functional study. Another interesting multigene expression method in AcMNPV was performed using repeated homologous recombination and cre-loxp recombination to express up to 8 foreign proteins from 8 loci [12]. As some improvements on the
Construction of recombinant BmNPV have been made by us [6,7,10,11], we are going to combine these different methods to establish another efficient MultiBac system based on BmNPV and silkworm. We have ever expressed rotavirus-like particles in cultured BmN cells by using the original vectors derived from MultiBac and the traditional method of recombinant baculovirus construction [13]. We question if this multigene system could work in silkworm larva/pupa that shows promising future for large-scale production of protein complexes. In the present study, we successfully develop a novel silkworm-based baculovirus multigene expression system, in which multiple proteins can be expressed simultaneously to produce protein complexes or VLPs efficiently.

Results

Construction of BmNPV–silkworm multigene expression system

Our BmNPV–silkworm multigene expression system consists of the modified recipient strain E. coli IBIDsw106MultiBmBac and three donor vectors including pCTdual, pRADM and pUCDMIG. All the donor plasmids contain the conditional replication R6K or origin, the polh and p10 double promoters, and antibiotic resistance genes (ZeoR, GmR and CmR) for screening, as well as some specific elements (homologous arms flanking I-Sce I sites, mini-Tn7 transposition arms and a P1-loxP site) for transferring expression cassettes into recipient MultiBmBacmid (Fig. 1).

E. coli IBIDsw106MultiBmBac is composed by the multiple functional host strain E. coli IBISW106Aasal and a modified BmBacmid named MultiBmBacmid. The invasive attTn7-blocked and DAP auxotrophic host strain contains the recombination genes red and gam under the control of a temperature sensitive repressor cI857, a tightly controlled arabinose-inducible cre gene and the I-Sce I expression cassette driven by pbAD promoter, as well as the Tn7 transposition helper plasmid pHelper (TetR) and the invasive plasmid pGB2Ωmv-hly (Sp8). The modified BmBacmid has an attTn7 transposition recipient arms with the lacZ α fragment which replaces the original polyhedrin gene, a loxP site substituting the original chitin and v-cath genes, and a GmR cassette flanking two I-Sce I sites which substitutes the original p10 and p74 genes (Fig. 1).

Resulting from the great capacity of foreign DNA fragment in BmNPV genome, up to ten heterologous genes can be easily incorporated into the BmBacmid and expressed at the same time with our multigene system. However, the first two genes in pCTdual must be integrated into MultiBmBacmid using homologous recombination to remove the GmR, whereas the other eight genes can be introduced into BmBacmid through cre-loxp and mini-Tn7 transposition methods simultaneously. The efficiency of transferring genes from pCTdual to BmBacmid is 99.8%, and it is sufficient to ensure the positive recombinant identification with almost 100% success when combining the Gm sensitive testing [6]. The background of transposition using pRADM and attTn7-blocked host is negligible, and the white colonies are sure to be positive at 100% efficacy when the blue-white screening is still preserved [7]. However, the pUCDM-derivative integration into Bacmid is relatively less efficient (about 93%) through cre-loxp site-specific recombination method [8]. To fix this weakness, we introduced the IRES-egfp fragment into pUCDM to construct a new donor vector pUCDMIG, which contains the 5'-UTR IRES sequence from Rhopalosiphum padi virus [14] and a positive GFP marker. As the translation efficiency of IRES is about 3-fold weaker than that of the cap-dependent translation, the target gene upstream IRES-EGFP controlled by the same polh promoter will be expressed more efficient than the EGFP, showing the small IRES-EGFP cassette (1.3 kb) is a ideal illumination marker for recombinant baculovirus identification.

Moreover, a transfectant-free method is also included in the multigene expression system, through which producing recombinant BmNPV in silkworm becomes simple and rapid by intrahemocoelic injection with invasive diaminopimelate auxotrophic E. coli containing MultiBmBacmid [11].

Production of infective recombinant BmNPV expressing multiple foreign genes in B. mori larvae

The invasive DAP auxotrophic E. coli, which contains recombinant MultiBmBacmids carrying six foreign genes including egfp, dsRed, eyfp, vp2, vp6 and vp7, was injected into silkworm larval hemocoel with 15 μl overnight cultures at a 10 fold dilution (OD600=2.0, total cell number=10⁶ per larva). Most of the injected larvae (90%) turned red in sunlight six days post injection, which also displayed red when observed with the gel imaging system (Fig. 2a). The hemocytes were found to be expressing GFP, DsRed and YFP simultaneously when observed under a laser confocal microscope (Fig. 2b). It indicated that infective recombinant BmNPV has been generated and the three fluorescence proteins were also expressed successfully in B. mori larvae. The injected silkworm larvae displaying red as their major color due to the tetramers formed by DsRed fluorescence proteins expressed in the B. mori larvae.

Production of rotavirus-VLPs in silkworm larvae

In addition to the three fluorescence proteins, the three structural proteins (VP2, VP6 and VP7) of rotavirus were also successfully expressed in silkworm larvae according to the western blotting experiment (Fig. 2c). Furthermore, the round virus like particles were found in the EM specimen, which revealed VP2, VP6 and VP7 were coexpressed in silkworm larvae and had self-assembled into VLPs (Fig. 2d). The results above indicated multiple genes were able to be co-expressed in silkworm simultaneously using our new BmBacmid expression system.

About 150 ml hemolymph was collected from 500 red larvae. The rotavirus VLPs were purified from the larval hemolymph by traditional ultracentrifugation using CsCl gradients. Two major bands were recovered for VLPs detection by TEM. One band was composed of baculoviral particles in rod shape, the other was made of round rotavirus VLPs. The result of SDS-PAGE proved the VLPs were constructed by three viral coat proteins including VP2, VP6 and VP7 as expected (Fig. 3). The total protein content in the purified VLPs from the hemolymph collected from 500 larvae was 6.35 mg, which meant the yield of VLPs was 12.7 μg per larval hemolymph. Obviously this BmNPV-silkworm multigene expression system provides an economic and efficient solution for producing antiviral vaccine derived from VLPs.

Discussion

Baculovirus expression system is always a popular tool for expressing recombinant proteins for its high expression level and the great convenience of cell culture. Recombinant baculoviral genome is able to receive 50 kb foreign DNA fragment, which enables the possibility to express different genes or multiple copies of one gene simultaneously. Berger and his colleagues have established a multigene expression system based on AcMNPV-Sf9 cell line and successfully expressed a transcript factor TFII complex [8]. Comparing to AcMNPV-Sf9 system, BmNPV–silkworm larvae expression system is more attractive because of the lower cost of breeding silkworm and higher expression level. Following this idea, we successfully constructed a BmNPV—
The target genes are cloned into the three donor vectors (pCTdual, pRADM, and pUCDMIG) using usual methods. The first two genes carried by pCTdual are inserted into BmBacmid through I-SceI linearization and red-gam homologous recombination. Four genes in pRADM and the other four genes in pUCDMIG are then introduced into BmBacmid via Tn7 transposition and cre-loxp recombination, respectively. As a result, ten foreign expression cassettes and three antibiotic screening markers, as well as a GFP illumination marker, are introduced into BmBacmid. The invasive and DAP auxotrophic E. coli carrying recombinant BmBacmid are injected into silkworm larvae at an appropriate dose. Consequently, recombinant BmNPV will be produced and multiple foreign genes will be expressed in green B. mori larvae or pupae.

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Baculovirus-Silkworm Multigene Expression System

Figure 2. Multiple genes expression and rotavirus-VLPs production in silkworm. (a) The larvae of six days post injection were observed using the fluorescence detection device. 1: the mock injected larvae; 2&3: the larvae injected with invasive and DAP auxotrophic E. coli carrying BmBacmid with six genes including egfp, dsRed, eyfp, vp2, vp6 and vp7 at a dose of $8.0 \times 10^8$ cells per larva. (b) The hemolymph from a red larva was observed using laser confocal microscope. The images of hemocytes were taken at the bright (trans) channel (I), GFP (515 nm) detection channel (II), DsRed (590 nm) channel (III) and YFP (530 nm) channel (IV). (c) Western blot analysis of the hemolymph from the red larvae using anti-VP2, anti-VP6 and anti-VP7 rabbit antisera. (d) The EM image of hemocytes collected from red larvae. R: the round Rotavirus-VLPs; B: the rod shape baculovirus particle, bar = 100 nm.

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Silkworm multigene expression system, in which six foreign genes are introduced into BmBacmid through homologous recombination, zero background Tn7 transposition and cre-loxP site-specific recombination. In the model shown in Fig. 1, four expression cassettes can be inserted into the donor vector pRADM or pUCDMIG using isocaudamer technique described Berger and his colleagues, as a result, up to ten genes can be introduced into the recombinant baculovirus.

Upon the power of its three donor vectors (pCTdual, pRADM and pUCDMIG), the efficiency of transferring multiple foreign genes into BmBacmid keeps at an extremely high level. A positive GFP marker is also introduced into the BmBacmid to monitor the protein expression using the donor vector pUCDMIG, which contains IRES-EGFP sequence [14]. The small IRES-GFP fragment has little influence on the capacity of vector and the expression level of the upstream gene, proving it is a good illumination marker [15].

Furthermore, a transfectant-free method is also presented in our multigene expression system, which immensely reduces the procedures and working time by intraheamocoeic injection with invasive diaminopimelate auxotrophic E. coli containing Multi-BmBacmid rather than usual transfection method.

We have used this new system to co-express three structural genes of rotavirus in silkworm for VLPs production, and the yield of purified VLPs from hemolymph was up to 12.7 µg per larva. The productivity will be increased if we try to co-express two copies of the viral structural genes or improve the VLPs purification method. As VLPs are not only assembled in hemolymph, but also in other larval tissues, it is necessary to develop an efficient method for purification VLPs from the whole larvae in the next study. Recently, Noad et al used a kind of high efficient repeated homologous recombination method to express eight genes at eight different loci in Bacmid, and all the target genes at different loci were expressed at high level [12]. In our system, two or four expression cassettes need to incorporate into one locus for expressing multiple genes simultaneously. According to our previous experience, there was no significant difference when expressing two genes in one locus or in two separated loci. However, it is necessary to study whether expression level is hampered by the incorporation of more than two genes in one locus in our future work.

In brief, up to ten heterologous genes or ten copies of one gene can be co-expressed in silkworm efficiently with our multigene expression system, which provides an economic and rapid platform for both recombinant multiprotein production and multigene transfer applications. We are going to express macromolecular complexes in silkworm to study their molecular structure and function using the system in the future.

Materials and Methods

Bacterial strains, plasmids, viral Bacmid, reagents and larvae

E. coli DH10B, BW23474 and TOP10 were used for the propagation of BmBacmid, Röky origin derived plasmids and other pUC derived plasmids, respectively. E. coli SW106 was provided by Prof. Copeland [16]. Plasmids pML291, pcp15 and pcp20 were gifts from Dr. Li [17], and spectinomycin (spe) resistance plasmid pGB2Ωiny-hly (containing both hly and inv genes) was provided by Prof. Courvalin [18]. Plasmids pBlock, pRCDM and pCTdual, as well as the modified BmBacmid with gentamycin resistance gene between two I-sce I sites were constructed at our previous study [6,7]. Plasmids pUCDM and pFBDM were from Prof. Richmond [8]. Plasmid pBac-IR-GFP containing the 5′-UTR internal ribosome entry site (IRES) sequence of Rhopalosiphum padi virus was provided by Prof. Wu [14].

PfuTag restriction enzymes and T4 DNA ligase were purchased from NEB (New England Biotechnologh, England), while DL-α-ε-Diaminopimelic acid (DAP) was bought from Sigma (cat. D1377, USA). Low salt (LS) medium (10 g of tryptone, 5 g of NaCl and 5 g of yeast extract in 1 liter of broth, pH7.5) was used for cloning and growing the plasmids containing zeocin resistance gene. Silkworm variety named Chinese Ming-zhu from Yunyang Silkworm Breeding Farm (Nanyang City, China) was fed with mulberry leaves.
The asd gene was deleted from SW106 genome by homologous recombination, which result in DAP auxotrophy and defective cell wall synthesis. The kanamycin cassette was removed from the kan/DAP plate were picked and identified by PCR. Then the transformed cells were spread on agar plate supplemented with 50 mg/ml kanamycin and 0.5 mM DAP. The ampicillin resistance gene (Amp R) was amplified from pPCP20. As a result, the I-Sce I expression cassette was introduced, while the attTn7 site of SW106 genome was blocked simultaneously. Plasmid pGB2Qm-hly which contains in and hly expression cassettes was transformed into the modified E. coli strain to generate the invasive, attTn7 blocked, DAP auxotrophic, I-Sce I homing endonuclease and cre recombinase expressing E. coli IBISW106Δasd.

Construction of the recipient MultiBmBacmid

BmBacmid-Gm, constructed previously by introducing a Gm<sup>R</sup> cassette flanking two I-Sce I sites into the p10 and p74 locus of the original BmNPV-Bacmid through homologous recombination, was transformed into E. coli SW106 [6]. After the induction of lambda red-gam recombinase at 42°C for 15 minutes, the electro-competent cells of SW106 containing BmBacmid-Gm were prepared and stored as previous description [16]. A 2.6 kb fragment containing both chita<sup>I</sup> (accession number NC001962; GeneID: 172489) and v-cath<sup>I</sup> (accession number NC001962; GeneID: 1724490) was amplified from BmBacmid using the primers chita<sup>I</sup> and v-cath<sup>I</sup> expression cassette was introduced, while the asd genome through Tn7 transposition. Briefly, the multiBmBacmid, which contains an attTn7 transposition site, a helper plasmid pHelper to block the attTn7 site in E. coli genome as described previously [7,10]. The kanamycin cassette (sptI) was removed by Flp-mediated excision in vivo using plasmid ppcp20. As a result, the I-Sce I expression cassette was introduced, while the attTn7 site of SW106 genome was blocked simultaneously. Plasmid pGB2Qm-hly which contains in and hly expression cassettes was transformed into the modified E. coli strain to generate the invasive, attTn7 blocked, DAP auxotrophic, I-Sce I homing endonuclease and cre recombinase expressing E. coli IBISW106Δasd.

Construction of donor vectors

The ampicillin resistance gene (Amp<sup>R</sup>) was amplified from pFBDM (accession number DQ115702) using the primers AmpF (AAATTCGAA TTTGAGGTGCGACTTTTCCG, Bst I underlined) and AmpR (AAATTCGGA TTTCTACGGGCTCT-
GACGCT). The 1.1 kb PCR product was first digested with Bst I, and then cloned into the same site of pBlock to replace the chloramphenicol resistance gene (CmR) for constructing pBlockA. The gentamicin resistance gene (GmR) and the two promoters (polh and p10) were obtained from Sac II/Ava II-digested pFBDM. The target Sac II/Ava II fragment was cloned into the same sites of pBlockA to replace the zeoFRT fragment, forming the Tin7 transposon donor vector pRADM (Fig. 1; accession number : JN596961). IRES fragment (accession number AX376819) was amplified from pEGFP-1 (Clontech; accession number : JN596960) containing the IRES-EGFP fragment (Fig. 1). The 580 bp PCR product was digested with Xho I and Bst I, and then cloned into the same sites of pUCDM (accession number DJ417503 ) to produce pUCDM-IRES. Efgp cDNA was amplified from pEGFP-1 (Clontech; accession number U55761) using the primers egfp5p (AAACTGTTAACAATGGTGAGACAAGGCGG, Spe I underlined) and egfp3p (AAACTGCGATTCTATGACAGTCGTC, Pst I underlined). The 0.7 kb PCR product was finally cloned into pUCDM-IRES via Xho I and Pst I to create pUCDMIG (accession number : JN596960) containing the IRES-EGFP fragment (Fig. 1).

**Introduction of multiple genes into BmBacmid**

Both vp2 and DsRed genes were introduced into MultiBmBacmid through homologous recombination according to previous study [5]. In practice, the plasmid pCTdual-Red-vp2 was transformed into the 42°C C. L-arabinose induced electrocompetent cells of E. coli BIIDsw106MultiBmBac. The colonies grown on Zeocin/Kan/Tet/spe/DAP were picked for further Gm susceptible detection. The positive zeocin resistant and Gm susceptible strain was named E. coli SW106BmMutBac-Red-vp2. The vp6 and egfp were introduced from pUCDM-YPF-vp6 and pUCDM-YPF-egfp into BmBacmid (BmMutBac-Red-vp2) by Tin7 transposition as described previously [6]. Furthermore, the vp7 and IERS-egfp were introduced from pUCDMIG-egfp into BmBacmid (BmMutBacRed-VP2-YPF-VP6) through cre-loxp site specific recombination according to previous report [8].

Finally, the target DAP auxotrophic E. coli containing the recombinant BmBacmid carrying six foreign genes (BmBacmid-gry267) and the invasive plasmid pGB2inv–hly was injected into 5th instar B. mori larvae at a dose of 10⁸ cells per larva according to our previous procedure [11]. The injected larvae were monitored until they turned green, red or yellow.

**Production of VP2, VP6 and VP7 antisera**

For preparation of antisera, truncated VP2, VP6 and VP7 proteins were expressed and purified from E. coli. Partial sequences of vp2 (from start codon ATG +1 -465), vp6 (+1 –510) and vp7 (+1 -496) were respectively amplified from the plasmids pCTdual-Red-vp2, pUCDM-YPF-vp6 and pUCDMIG-egfp using the following primers: SVP2F (GGCGAATTCTGAGGCTACAGAAGGCCG, EcoR I underlined), SVP2R (GGCGAATTCTGAGGCTACAGAAGGCCG, EcoR I underlined), SVP6F (GGCGAATTCTGAGGCTACAGAAGGCCG, EcoR I underlined), SVP6R (GGCGAATTCTGAGGCTACAGAAGGCCG, EcoR I underlined), SVP7F (GGCGAATTCTGAGGCTACAGAAGGCCG, EcoR I underlined), SVP7R (GGCGAATTCTGAGGCTACAGAAGGCCG, EcoR I underlined). The PCR products were respectively cloned into the EosR I and Xho I sites of pET32a (Novagen) in frame with the His tag at N-terminus to form three recombinant pET32a expression vectors, which were transformed into E. coli BL21 (DE3) competent cells later.

The three truncated VP2,VP6 and VP7 proteins were expressed, isolated and affinity-purified following the instructions in the His-tag fusion protein purification manual (FPLC of GE Health). New Zealand white rabbits were inoculated with the purified proteins to produce polyclonal antibodies using the standard procedures [21].

**Fluorescence microscopy**

The silkworm larvae injected with E. coli containing recombinant BmBacmid-gry267 were observed using Landun 652 visible light gel imaging system. In this system, the blue filter is used to generate blue light (about 490 nm wavelength) for excitation, and the orange filter is used to observe green, yellow and red fluorescence simultaneously. The hemolymph was collected from the larvae injected with E. coli, and observed under a Nikon laser scanning confocal microscope. The excitation wavelengths used to excite EGFP, EYFP and DsRed were 488 nm, 514 nm and 543 nm, respectively. Correspondingly, the 515 nm, 530 nm and 590 nm channel were used to detect the green, yellow and red fluorescent proteins, respectively.

**Western blot analysis and electron microscopy**

The hemolymph of the red silkworm larva was collected, into which 5 mM phenyl-thiourea was added to prevent melanization. The hemocytes were harvested by centrifugation, and then lysed by incubation with lysis buffer [1% Triton-X 100 in 10 mM Tris-Cl, pH 7.4] on ice for 30 minutes. The cell lysates were separated in 10% SDS-PAGE, followed by western blot analysis with anti-VP2, anti-VP6 and anti-VP7 rabbit antiseraums (diluted 2000 fold) as well as appropriate secondary antibody (alkaline phosphatase-conjugated goat anti-rabbit IgG, GE Health) according to the manufacturer’s protocol.

The harvested hemocytes pellets were fixed in 4°C PBS buffer (pH 7.2) containing 2.5% glutaraldehyde for 2 h. After rinsing with PBS buffer, the samples were fixed in 1% osmium, and dehydrated through a series of graded ethanol baths. Then the dehydrated samples were transferred to propylene oxide for 20 min, and embedded in Epon812 for ultrathin sectioning.
Ultrathin sections were stained with 2% uranyl acetate and 1% lead citrate, and then they were examined under a JEM-100SX transmission electron microscope (TEM). For purified VLPs observation, a drop (3 μl) of VLPs sample recovered from the band was applied onto carbon coated 400 mesh copper grid and negatively stained with 2% uranyl acetate. Finally, the dried grins band was applied onto carbon coated 400 mesh copper grid and 8% lead citrate, and then they were examined under a JEM-100SX transmission electron microscope (TEM).

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**Author Contributions**

Conceived and designed the experiments: LY JS. Performed the experiments: LY SW YS JH LP. Analyzed the data: LY SW JS. Contributed reagents/materials/analysis tools: SW NY SS JH. Wrote the paper: LY JH JS. Editing and approval of manuscript: LY JH JS.