Mol Biol Rep (2009) 36:329–335
DOI 10.1007/s11033-007-9183-2

High-level expression of orange fluorescent protein in the silkworm larvae by the Bac-to-Bac system

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Received: 8 October 2007 / Accepted: 12 November 2007 / Published online: 23 November 2007

Abstract This novel orange fluorescent protein (OFP) emits brilliant orange fluorescent light. OFP has high fluorescence quantum yield, fast maturation rate, and stability, which imply this protein should be the most favorable biotechnological tools used to investigate the function of target gene by visualizing, monitoring, and quantifying in living cells. *B. mori*, silkworm has been used as an important bioreactor for the production of recombinant proteins through baculovirus expression system (BES). In this paper, we used infection technique which introduced the baculovirus DNA into silkworms using a cationic lipofectin reagent instead of directly injecting the virus, and demonstrated a high-level expression of the orange fluorescent protein (OFP) gene in the *Bombyx mori*, silkworm larvae. When recombinant rBacmid/BmNPV/OFP DNA ranging from 50–100 ng/larval was injected, a sufficient OFP expression in hemolymph was harvested. The recombinant viruses could be obtained from the hemolymph of infected larvae and stored as seed which could be used for the large-scale expression. This procedure omitted the costly and labor-consumed insect cell culture. Further investigation of OFP should provide us with more insight in unlocking the mystery of the mechanisms of autocatalytic bioluminescence and its utilization in biotechnology.

Keywords Silkworm (*Bombyx mori* L.) · Expression · Orange fluorescent protein · Bac-to-Bac system

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Introduction

The baculovirus expression system (BES) has been extensively used since its inception 24 years ago to express a large variety of the recombinant proteins in cultured insect cells or the insect larvae. Recently, a bacmid (a baculovirus shuttle vector) system has been developed for BmNPV, which can be replicated in *Escherichia coli* as a large plasmid, generates recombinant virus DNA by site-specific transposition, and remains infectious in insect cells. Because this method eliminates the need for multiple rounds of purification and amplification of viruses, it markedly decreases the technical difficulty and the time required to select and purify recombinant viruses [1, 2].

During the last dozen years, fluorescent proteins have become one of the most favorable biotechnological tools scientists use to investigate the function of their genes of interest by visualizing, monitoring, and quantifying protein expression directly in living cells.

GFP was the first fluorescent protein purified from the bioluminescent jellyfish *Aequorea Victoria* [3–5], and has started to find widespread applications and acceptance in many branches of biological science [6]. GFP mutants that can emit blue, cyan, or yellow fluorescence were reported but further extension of the emission into the red spectral region (with an emission wavelength greater than 550 nm) was not achieved [7–10]. A red emitter was desirable because this spectrum of light can penetrate further into

cellular tissue and avoid crosstalk owing to the natural auto-green fluorescence of living cells. In 1999, the first naturally existing red fluorescent protein, DsRed, was cloned from a nonbioluminescent *Anthozoan Discosoma* coral [11], which has opened up a new era in fluorescent proteins used in biotechnological applications [12]. Nevertheless, subsequent studies indicated that DsRed has significant drawbacks in photophysical characteristics such as slow and incomplete maturation of the chromophore, protein oligomerization, and the tendency to form aggregates at high protein concentrations, all of which hinder its effectiveness as a molecular tag or protein fusion partner [13, 14]. Since then, many attempts have been made to overcome such drawbacks. In 2004, the Tsien group came up with several monomeric color mutants of DsRed with improved performance via qualities such as high maturation rate, better N-terminal fusion, and more photostability [15]. David group report the cloning and characterization of a novel GFP-like protein isolated from the tentacles of Cnidarian tube *anemone Cerianthus sp*. This novel orange fluorescent protein (OFP) has high fluorescence quantum yield, fast maturation rate, and excellent protein expression at 37°C. On excitation, it emits brilliant orange fluorescent light. OFP is also a very robust and stable protein, which is conserved with purified OFP sample stored at room temperature for years in which no protease inhibitors or antibiotics were added [16]. Further investigation of OFP should provide us with more insight in unlocking the mystery of the mechanisms of autocatalytic bioluminescence and its utilization in biotechnology.

In this paper, we demonstrate a high-level expression of the orange fluorescent protein (OFP) gene in the *Bombyx mori*, silkworm larvae by directly injecting recombinant bacmid DNA. The levels of protein using the silkworm larvae is 10–100 fold higher than that using *B. mori* cells, indicating that the silkworm larvae is an optimal system for the mass production of recombinant proteins [19]. From this point of view, BmNPV BES using *B. mori* silkworm larvae is the most suitable combination for the large-scale production of eukaryotic proteins such as orange fluorescent protein.

Materials and methods

Materials

Hybrid strain larvae of silkworm (commercial name: Qingsong Haoyue) were reared in this study for recombinant protein expression. The larvae were fed with artificial diet at 23–25°C under a 16 h light, 8 h dark photoperiod. After the fourth ecdysis, larvae were divided into groups and used for injection of recombinant virus DNA.

pFasBacHTb and cellfectin reagent (transfection reagent) were the product of Roche Applied Science, USA. The E. coli DH10Bac/BmNPV was constructed and supplied by Prof. E. Y. Park and Prof. K. Maenaka [17].

Construction of recombinant baculovirus transfer vector and recombinant rBacmid/BmNPV/OFP DNA

The pFastBac^{TM}HTb-OFP donor plasmid is supplied by the Chinese University of Hongkong. The size of this recombinant donor plasmid is about 5.49 kb. The expression cassette of this donor plasmid is flanked by the left and right arms of Tn7, and also contain a gentamicin resistance gene and an SV40 polyadenylation signal to form a mini-Tn7.

The *Escherichia coli* DH5α is used for the replication of donor plasmid. The *E. coli* DH10Bac strain is used as the host for the donor plasmid. The *E. coli* DH10Bac competent cells contain a baculovirus shuttle vector (bacmid) with a mini-attTn7 target site and a helper plasmid. Once the donor plasmid is transformed into DH10Bac cells, transposition occurs between the mini-Tn7 element on the donor plasmid and the mini-attTn7 target site on the bacmid to generate a recombinant bacmid. This transposition reaction occurs in the presence of transposition proteins supplied by the helper plasmid. The *E. coli* DH10Bac containing recombinant bacmid baculoviruses was propagated on LB medium containing antibiotics (50 ug/ml kanamycin, 7 ug/ml gentamicin, and 10 ug/ml tetracycline), 100 ug/ml X-gal and the inducer, 40 ug/ml IPTG. The white (LacZ–) colonies were selected for further amplification. The large recombinant bacmid baculoviruses (rBacmid/BmNPV/OFP) was isolated from the DH10Bac/BmNPV using the FlexiPrep kit (Amersham).

Analyzing recombinant bacmid baculoviruses (rBacmid/BmNPV/OFP) DNA

The large recombinant bacmid baculoviruses (rBacmid/BmNPV/OFP) DNA was isolated from the DH10Bac/BmNPV using the FlexiPrep kit (Amersham Pharmacia Biotech) and analyzed by PCR with the M13 forward and M13 reverse primers, because restriction analysis is difficult to perform with DNA of this size. The PCR was set on the following conditions: pre-denature at 94°C for 10 min, 35 cycles incubation at 94°C for 45 s, 55°C for 45 s, 72°C for 5 min, and final incubation at 72°C for 7 min.

Method of injection the silkworm larvae and the obtain of rBmNPV/OFP virus

Diluting 6 μg of purified recombinant rBacmid/BmNPV/OFP DNA in 300 μl of unsuplemented Grace's Insect Cell Medium. Mix cellfectin reagent thoroughly before use by inverting the tube 5–10 times. Removing 30 μl of cellfectin Reagent and dilute in 270 μl of unsuplemented Grace's Insect Cell Medium, then combining the diluted DNA with the diluted cellfectin Reagent (total volume 600 μl). Mix gently and incubate for 45 min at room temperature.

The first day of fifth instar larvae were used. 10 μl of above DNA mixture was injected into dorsal of the silkworm larvae by a syringe. Screening the orange fluorescent proteins using fluorescent illuminator in complete darkness everyday of post-infection, picking out the silkworm larvae which had expressed the OFP 7–8 days of post-injection, extracting the hemolymph and centrifuging at 500*g* for 5 min to remove the impurity, the virus was obtained from the clarified supernatant, Stored at 4°C, protected from light. For long-term storage, stored an aliquot of the viral stock at −80°C.

Expression and examination of the target gene

Centrifuging the hemolymph at 500*g* for 5 min to remove the impurity and obtain the virus. Diluting the virus in unsuplemented Grace's Insect Cell Medium. Then 10 μl of this virus mixture was injected into dorsal of the silkworm larvae by a syringe. The hemolymph was extracted daily to examine the OFP by SDS-PAGE.

After SDS-PAGE, the proteins were transfer onto a PVDF membrane under 2 mA/cm^2 for 1 h, and subsequently blocked with TBST (100 mM Tris-HCl, pH 7.5, 0.9%NaCl, 0.1% Tween 20) containing 2% of non-fat dried milk for 1 h at room temperature. After three times washing with TBST, the membrane was incubated in TBST containing 2% of non-fat dried milk 1000× diluted OFP-antibody for overnight at 4°C. The membrane was washed three times (each for 10 min) with the same TBST followed by 5 h incubation of horseradish peroxidase labeled Goat anti Rabbit IgG antibody at room temperature. After washing with TBST, the antibody was detected with Konica immunostaining HRP-1000 kit (Konica Minolta, Tokyo, Japan).

Result

Obtainment of the rBacmid/BmNPV/OFP DNA and recombinant BmNPV virus

Transforming 1 ng of purified pFastBac^{TM}HTb-OFP recombinant plasmid into the *E.coli* Bm DH10 Bac competent cell. Transposition occurs between the mini-Tn7 element on the donor plasmid and the mini-attTn7 target site on the bacmid to generate a recombinant bacmid. This transposition reaction occurs in the presence of transposase supplied by the helper plasmid. The white colonies were selected for analysis from the LB agar plates containing 50 μg/ml kanamycin, 7 μg/ml gentamicin, 10 μg/ml tetracycline, 100 μg/ml X-gal, and 40 μg/ml IPTG. Picking the white colonies and restreak them again on fresh LB agar plates containing 50 μg/ml kanamycin, 7 μg/ml gentamicin, 10 μg/ml tetracycline, 100 μg/ml X-gal, and 40 μg/ml IPTG. Incubate the plates overnight at 37°C (Fig. 1a).

From a single colony confirmed to have a white phenotype on re-streaked plates containing X-gal and IPTG, inoculate a lipid culture containing 50 μg/ml kanamycin, 7 μg/ml gentamicin and 10μg/ml tetracycline. Isolate the recombinant bacmid DNA and analyzing it by PCR using the M13 Forward (−40) and M13 Reverse primer. Electrophoresis to examine the size of the PCR product is 3.1 KB, demonstrate the correct results (Fig. 2).

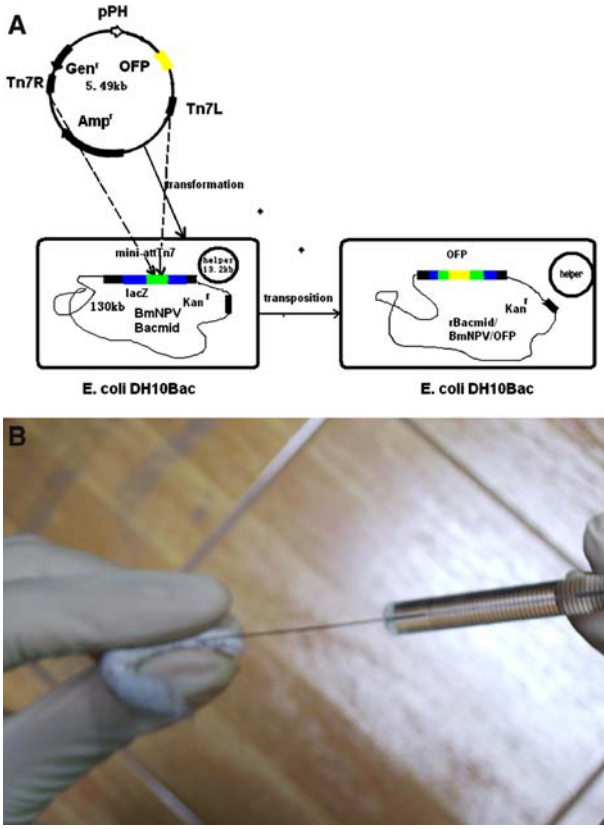

Fig. 1 (**a**) The construction of recombinant rBacmid/BmNPV/OFP DNA by the Bac-To-Bac system. The donor plasmid pFastBac^{TM}HTb-OFP including the OFP gene was transformed into *E.coli* Bm DH10Bac competent cell for the transposition, and the recombinant DNA obtained was designated rBacmid/BmNPV/OFP. (**b**) The rBacmid/BmNPV/OFP DNAs or its recombinant virus were injected to silkworm larvae by a syringe

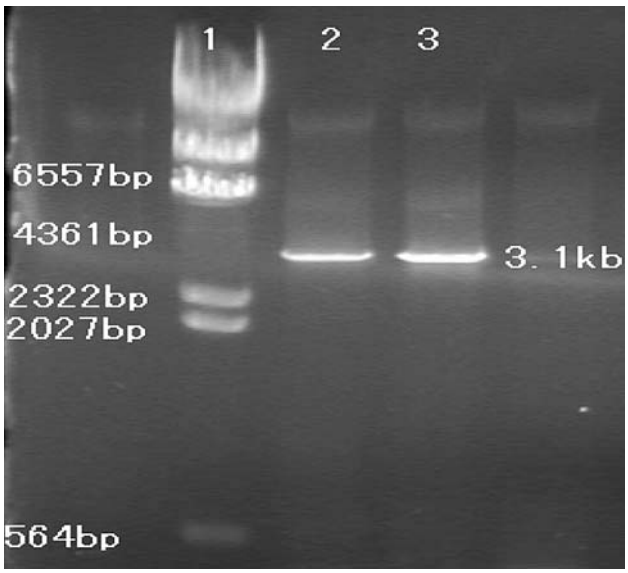

Fig. 2 Examine the PCR product by electrophoresis. Band 1 is the marker, band 2 and 3 is the PCR product

Large-scale expression of the OFP in silkworm larvae

The first day of fifth instars larvae were used. 10 μl of DNA: Cellfectin Reagent mixture was injected into dorsal of the silkworm larvae by syringe (Fig. 1b). To examine the orange fluorescent proteins using fluorescent illuminator in complete darkness everyday of postinfection, found that the expression of the OFP will begin 4 days of postinjection (Fig. 3A-a), be get the highest level 8 days of postinfection (Fig. 3D). Extract the hemolymph 7–8 days of postinjection, centrifuge at 500*g* for 5 min to remove the impurity, the virus was obtained from the clarified supernatant. The optimum amount of rBacmid/BmNPV/OFP virus injection was determined to be 160 pfu/larval. When rBacmid/BmNPV/OFP virus with 800 pfu/larval was injected into the larvae, they are markedly weak 3 days postinjection, and one-half of the larvae were dead 4 days postinjection. When rBacmid/BmNPV/OFP virus ranging from 160 pfu/larval to 400 pfu/larval was injected, a sufficient amount of hemolymph was harvested. For the recombinant bacmid, the optimum rBacmid/BmNPV/OFP DNA was determined to be in the range of 50–100 ng/larva by a preliminary experiment. So dilute the supernatant in unsuplemented Grace's Insect Cell Medium. Then 10μl of the virus was injected into dorsal of the silkworm larvae by syringe. The optimal harvest time of the hemolymph was 4 or 5 days postinjection. On the other hand, when the recombinant bacmid DNA was injected into the larvae, the expression of the recombinant protein began 4 days postinjection and reached maximum level of expression 8 days postinjection. 9 days postinjection, the recombinant protein was degraded, possibly owing to signal peptides might be inefficiently recognized by the protein translocation machinery in insect cell. 8 days postinjection, the larvae were subsequently dissected out, and the tissues were

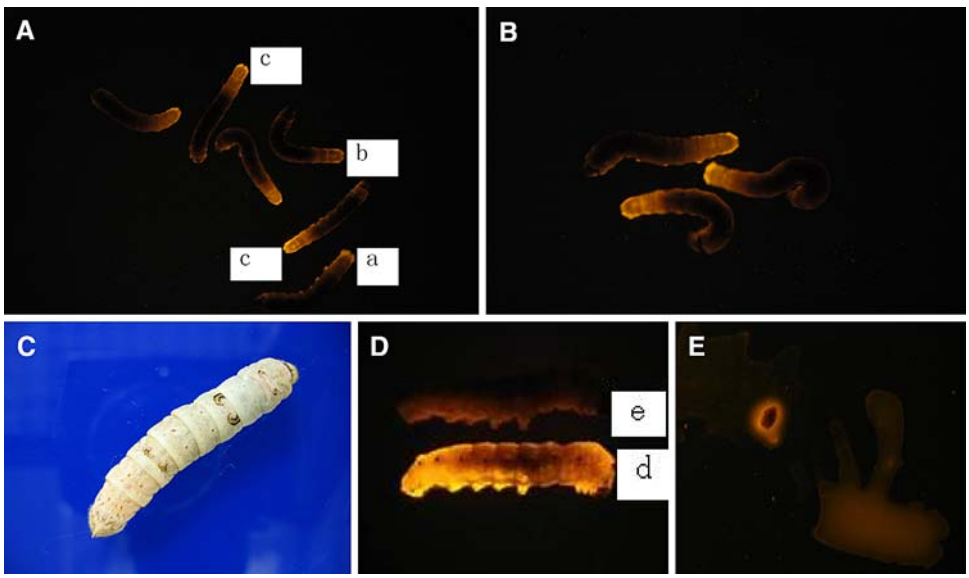

Fig. 3 OFP expression in *B.mori* larvae. *B.mori* larvae were infected by direct syringe injection of rBacmid/BmNPV/OFP DNAs (**A**), and rBacmid/BmNPV/OFP virus (**B**). (**A**) The photograph (a-c) of the larvae was taken at 4–6 days of postinjection time using fluorescent illuminator in complete darkness respectively. The photograph (**B**) of the larvae was taken at 4 days of postinjection time using fluorescent illuminator in complete darkness. The photograph (**C**) of the larvae was taken at 8 days of postinjection using light illuminator in normal light. The photograph (**D**) of the larvae was taken at 8 days of postinjection using fluorescent illuminator in complete darkness. And the (e) is the control. (**E**) The OFP express in the fat body and hemolymph of the larvae, revealing the OFP expression. The photograph was taken under the fluorescent illuminator in complete darkness

viewed under a fluorescence illuminator (Fig. 3E). Found that the color of fat body is orange, and hemolymph is turbid. While the color control is white, hemolymph is clear. The possible reason for this is that the silkworm larva has an open circulatory system and the OFP from the fat body readily leach out and, in the late stage of virus infection, the fat body undergoes lysis, leading to the release of proteins into the hemolymph.

SDS-PAGE and PAGE analysis

To detect the OFP in the hemolymph of the silkworm larvae, the supernatants and the total hemolymph were subjected to SDS-PAGE and PAGE on a 12% polyacrylamide gel using the pipette, respectively. Ten microliters of supernatant and hemolymph was mixed with the same amount of sample buffer respectively, and the applied to a 12% polyacrylamide gel. For the detection of OFP on the PAGE gel, the samples were only mixed with the sample buffer without boiling and orange fluorescent bands were then directly observed using a fluorescence illuminator in complete darkness. The target protein bands were located at the 25 Da (Fig. 4a).

Western blot analysis

The Western blot analysis was performed by using the extracts of the hemolymph and its supernatant respectively. Ten microliters of supernatant and hemolymph was mixed with the same amount of sample buffer respectively, and the applied to a 12% SDS-PAGE gel and Western blot analysis. Fig. 4b clearly demonstrated that the OFP expression level by injecting recombinant bacmid DNA directly is as high as injecting recombinant virus.

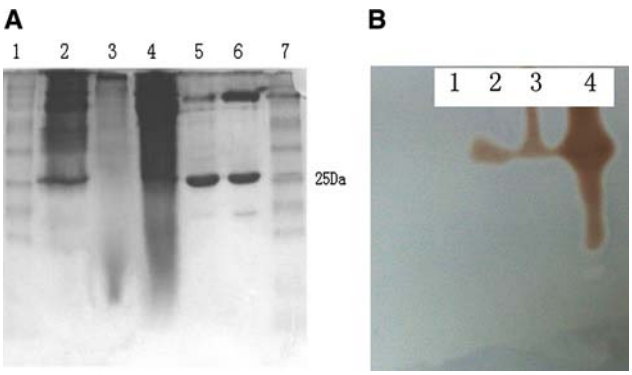

Fig. 4 (**a**) SDS-PAGE analysis of the OFP expressed in silkworm larvae which were 7 days postinjection. The band 1 and 7 is the protein standard; 2 is the supernatant of hemolymph; 3 is the supernatant of negative control; 4 is the total hemolymph; 5 and 6 are positive control. (**b**) Western blot analysis of OFP expression in hemolymph of *B. mori* larvae injected by rBacmid/BmNPV/OFP DNAs and rBacmid/BmNPV/OFP virus. Band 1 is the supernatant of negative control; 2 and 3 is the supernatant of hemolymph which injected by rBacmid/BmNPV/OFP DNAs and rBacmid/BmNPV/OFP virus, respectively; 4 is the total hemolymph which injected by rBacmid/BmNPV/OFP DNAs

Discussion

With the development of biotechnology, *B. mori* has been used as an important bioreactor for the production of recombinant proteins through baculovirus expression system (BES) [18, 19]. Recently, we established the practical BmNPV bacmid system to express the foreign proteins. This method eliminates the need for multiple rounds of purification and amplification of viruses, it markedly decreases the technical difficulty and the time required to select and purify recombinant viruses.

The levels of protein using the silkworm larvae is 10–100 fold higher than that using *B.mori* cells, indicating that the silkworm larvae is an optimal system for the mass production of recombinant proteins [2]. Using this system, we demonstrated a high-level expression of the orange fluorescent protein (OFP) gene in the *B. mori* silkworm larvae by directly injecting recombinant bacmid DNA. However complex proteins are generally not well expressed as biologically functional proteins. The reason for this in unknown, but it is possible that there is not any signal peptide in this system. Some papers reported that insect cell infected with a baculovirus recombined with the gene encoding propapain fused to the honeybee melittin signal peptide secreted more than five-fold the amount of the papain precursor than those infected the gene encoding a plant signal peptide. We could not express some of the functional human enzyme using Bac-To-Bac system without encoding a signal sequence, but could express them in a functionally active form using Bac-To-Bac system with encoding the honeybee melittin signal peptide. In addition, the signal sequence from a silkworm *Bombyx mori* and a silkworm prophenoloxidase-activating enzyme are designate *bx* and *ppa* respetively have been demonstrated that the signal peptide is important for the expression of active protein [20–23].

The optimum amount of rBacmid/BmNPV/OFP virus injection was determined to be 160 pfu/larval. When rBacmid/BmNPV/OFP virus of 800 pfu/larval was injected into the larvae, the expression was markedly weak 3 days postinjection, and one-half of the larvae were dead 4 days postinjection. When rBacmid/BmNPV/OFP virus ranging from160 pfu/larval to 400 pfu/larval was injected, a sufficient amount of hemolymph was harvested. For the recombinant bacmid, the optimum rBacmid/BmNPV/OFP

DNA was determined to be in the range of 50–100 ng/larval by a preliminary experiment.

The optimal harvest time of the hemolymph was 4 or 5 days postinjection. On the other hand, when the recombinant bacmid was injected into the larvae, the expression of the recombinant protein began 4 days postinjection and reached maximum level of expression 7 days postinjection. In 8 days postinjection, the recombinant protein was degraded, possibly owing to signal peptides might be inefficiently recognized by the protein translocation machinery in cells. In 7 days postinjection, the larvae were subsequently dissected out, and the tissues were viewed under a fluorescence illuminator, finding that the color of fat body is orange, and hemolymph is turbid. While the hemolymph of the control is clear. The possible reason for this is that the silkworm larval has an open circulatory system and the OFP from the fat body readily leach out and, in the late stage of virus infection, the fat body undergoes lysis, leading to the release of proteins into the hemolymph.

Up to now, several proteins have been produced using *B.mori* silkworm larvae. Maeda et al. first reported the production of α-interferon in silkworm using BmNPV baculovirus vector. On our study, the OFP expression was further investigated in silkworm larvae by both direct infection of recombinant Bacmid DNA and infection virus using a syringe. In this case the orange fluorescence was screened within 4 days, which was slower than that of the virus infection. On the other hand, the recombinant virus infection using a syringe, the larvae appeared orange in 3 days postinjection and the fluorescence intensity further increased.

The western blot analysis demonstrated that the OFP expression level by injecting recombinant bacmid DNA directly is as high as injecting recombinant virus into silkworm larvae.

Our work showed great advantages of the Bac-To-Bac system, such as high expression levels of the protein. Furthermore, by direct injection of the recombinant bacmid DNA into silkworms using a cationic lipofectin reagent instead of directly injecting the virus, the orange fluorescence was also identified in the *Bombyx mori*, silkworm larvae. The recombinant viruses could be obtained from the hemolymph of infected larvae and stored as seed which could be used for the large-scale expression. This procedure omitted the costly and labor-consumed insect cell culture.

Acknowledgments We thank Prof. Wan Chi Cheong David, Lin huangquan, Denis Tsz-Ming Ip, Li xinghua and Li shuo for suggestions and helps in these experiments. The work was supported by the National Basic Research Program of China under grant no. 2005CB121003 and by the Hi-Tech Research and Development Program of China (No. 2006AA10A119).

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