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This novel orange fluorescent protein (OFP) emits brilliant orange fluorescent light. OFP has high fluorescence quantum yield, fast maturation rate, and stability, which implies 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, the 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-consuming 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.

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

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 as 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

J.-m. Liu · X.-h. Li · G.-l. Li · X.-f. Wu · W.-f. Yue · Y.-g. Miao (✉)
Institute of Sericulture and Apiculture, College of Animal Sciences, Zhejiang University, Hangzhou 310029, P.R. China
e-mail: miaog@ju.edu.cn

W. C. C. David · D. T.-M. Ip
Department of Biochemistry, The Chinese University of Hong Kong, Hong Kong, P.R. China

C.-. Zhang
Institute of Insect Sciences, Zhejiang University, Hangzhou 310029, P.R. China

Method of injection the silk worm larvae and the obtain of rBmNPV/OFP virus

Diluting 6 μg of purified recombinant rBacmid/BmNPV/OFP DNA in 300 μl of unsupplemented Grace's Insect Cell Medium. Microcellfectin reagent thoroughly before use by inverting the tube 5–10 times. Removing 30 μl of cellfectin Reagent and dilute in 270 μl of unsupplemented 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 silk worm larvae by a syringe. Screening the orange fluorescent proteins using fluorescent illuminator in complete darkness every day of post-infection, picking out the silk worm larvae which had expressed the GFP 7–8 days of post-injection, extracting the hemolymph and centrifuging at 500g for 5 min to remove the impurities, the virus was obtained from the clarified supernatant. Stored at 4 $^{\circ}\text{C}$, 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 500g for 5 min to remove the impurities and obtain the virus. Diluting the virus in unsupplemented Grace's Insect Cell Medium. Then 10 μl of this virus mixture was injected into dorsal of the silk worm larvae by a syringe. The hemolymph was extracted daily to examine the GFP by SDS-PAGE.

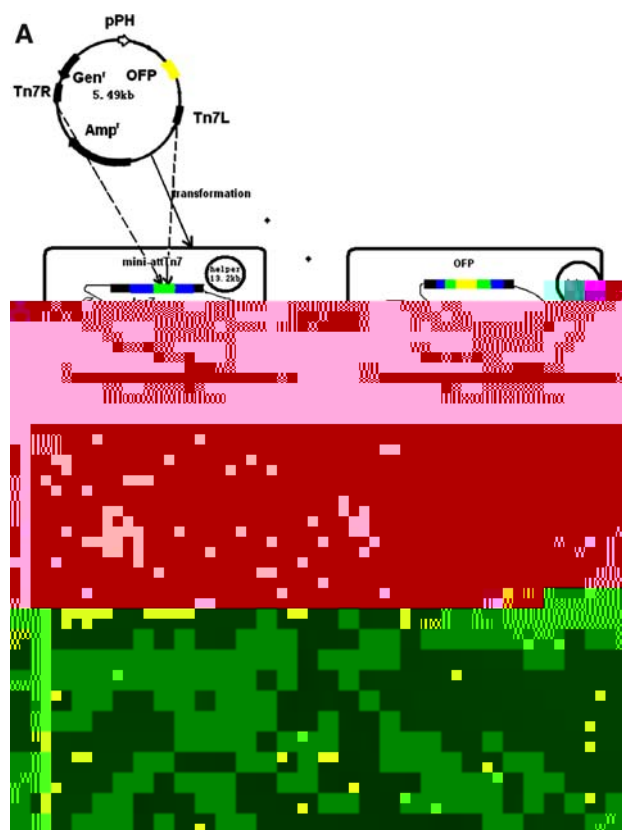
After SDS-PAGE, the proteins were transferred onto a PVDF membrane under 2 mA/cm^2 for 1 h, and subsequently blocked with TBST (100 mM Tris-HCl, pH 7.5, 0.9% NaCl, 0.1% Tween 20) containing 2% of non-fat dried milk for 1 h at room temperature. After three times washing with TBST, the membrane was incubated in TBST containing 2% of non-fat dried milk 1000 \times diluted GFP-antibody for overnight at 4 $^{\circ}\text{C}$. 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).

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

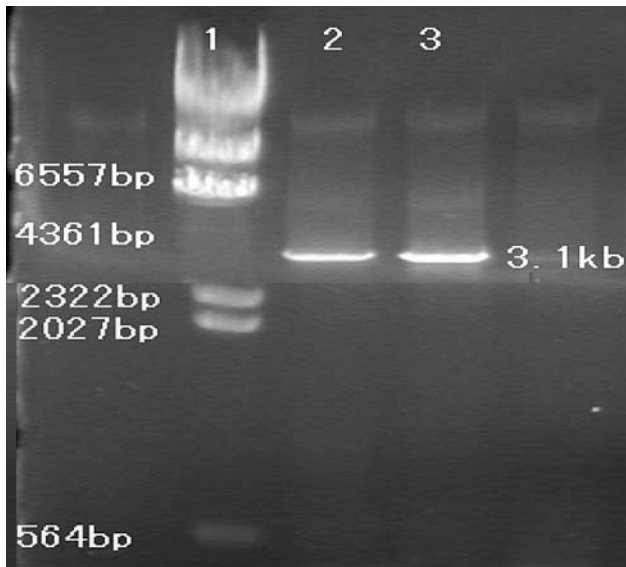
Transforming 1 ng of purified pFastBacTMHTb-OFP recombinant plasmid into the *E. coli* Bm DH10 Bac

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 $\mu\text{g/ml}$ kanamycin, 7 $\mu\text{g/ml}$ gentamicin, 10 $\mu\text{g/ml}$ tetracycline, 100 $\mu\text{g/ml}$ X-gal, and 40 $\mu\text{g/ml}$ IPTG. Picking the white colonies and restreak them again on fresh LB agar plates containing 50 $\mu\text{g/ml}$ kanamycin, 7 $\mu\text{g/ml}$ gentamicin, 10 $\mu\text{g/ml}$ tetracycline, 100 $\mu\text{g/ml}$ X-gal, and 40 $\mu\text{g/ml}$ IPTG. Incubate the plates overnight at 37 $^{\circ}\text{C}$ (Fig. 1a).

From a single colony confirmed to have a white phenotype on re-streaked plates containing X-gal and IPTG, inoculate a liquid culture containing 50 $\mu\text{g/ml}$ kanamycin, 7 $\mu\text{g/ml}$ gentamicin and 10 $\mu\text{g/ml}$ tetracycline. Isolate the recombinant bacmid DNA and analyze 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).



(A) The construction of recombinant rBacmid/BmNPV/OFP DNA by the Bac-To-Bac system. The donor plasmid pFastBacTMHTb-OFP including the GFP gene was transformed into *E. coli* Bm DH10Bac competent cell for the transposition, and the recombinant DNA obtained as designated rBacmid/BmNPV/OFP. (B) The rBacmid/BmNPV/OFP DNAs or its recombinant virus were injected to silk worm larvae by a syringe

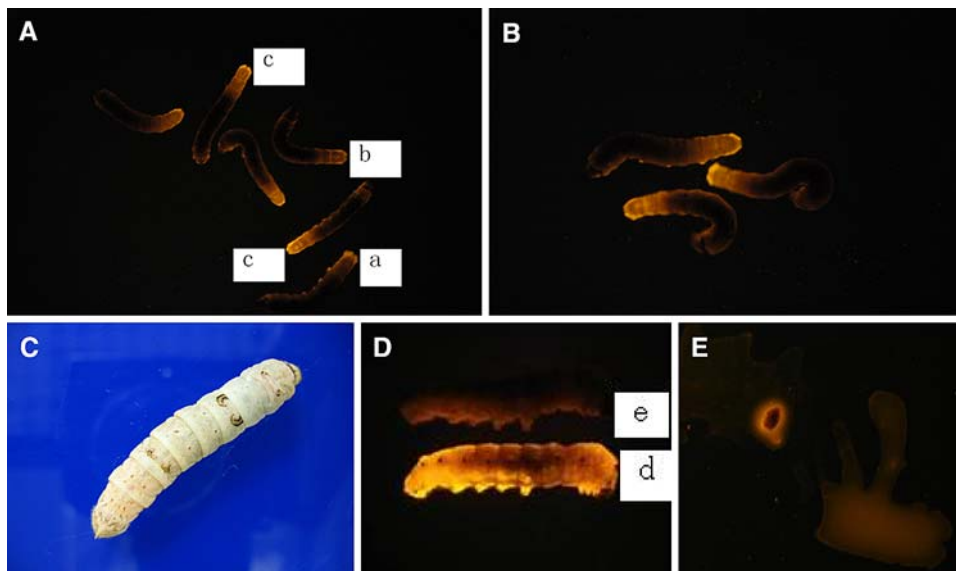


Examine the PCR product by electrophoresis. Band 1 is the marker, band 2 and 3 is the PCR product

Large-scale expression of the OFP in silk worm larvae

The first day of fifth instars larvae were used. 10 μ l of DNA: Cellfectin Reagent mixture was injected into dorsal of the silk worm larvae by syringe (Fig. 1b). To examine the orange fluorescent proteins using fluorescent illuminator in complete darkness ever day of postinfection, found

that the expression of the OFP will begin 4 days of post-injection (Fig. 3A-a), be get the highest level 8 days of postinfection (Fig. 3D). Extract the hemolymph 7–8 days of postinjection, centrifuge at 500g for 5 min to remove the impurities, 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 were 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 unsupplemented Grace's Insect Cell Medium. Then 10 μ l of the virus was injected into dorsal of the silk worm 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



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). (c) 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 (d) of the larvae was taken at 4 days of postinjection time using fluorescent illuminator in complete darkness. The photograph (e) 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. (f) The OFP expression in the fat body and hemolymph of the larvae, revealing the OFP expression. The photograph was taken under the fluorescent illuminator in complete darkness

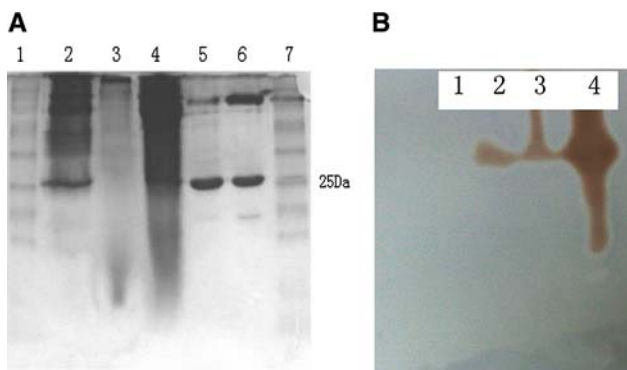
viewed under a fluorescence illuminator (Fig. 3E). Found that the color of fat body is orange, and hemolymph is turbid. While the color control is white, hemolymph is clear. The possible reason for this is that the silk worm larva has an open circulatory system and the OFP from the fat body readily leach 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 silk worm 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 as 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 as mixed



4 () SDS-PAGE analysis of the OFP expressed in silk worm larvae which were 7 days post-injection. The data are shown in Table 1 and 7 (polaris) 56905led

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.

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 silk worm larvae is 10-100 fold higher than that using *B. mori* cells, indicating that the silk worm 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* silk worm larvae by directly injecting recombinant bacmid DNA. However, complete proteins are generally not well expressed as biologically functional proteins. The reason for this is unknown, but it is possible that there is not an signal peptide in this system. Some papers reported that insect cell infected with a baculovirus recombined with the gene encoding propapain fused to the honey bee 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 honey bee melittin signal peptide. In addition, the signal sequence from a silk worm *Bombyx mori* and a silk worm prophenolase-activating enzyme are designated *bx* and *ppa* respectively 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 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 as determined to be in the range of 50–100 ng/larval body 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 silk worm 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* silk worm larvae. Maeda et al. first reported the production of α -interferon in silk worm using BmNPV baculovirus vector. On our study, the OFP expression was further investigated in silk worm 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 silk worm 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 silk worms using a cationic lipofectin reagent instead of directly injecting the virus, the orange fluorescence was also identified in the *Bombyx mori*, silk worm 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-consuming insect cell culture.

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