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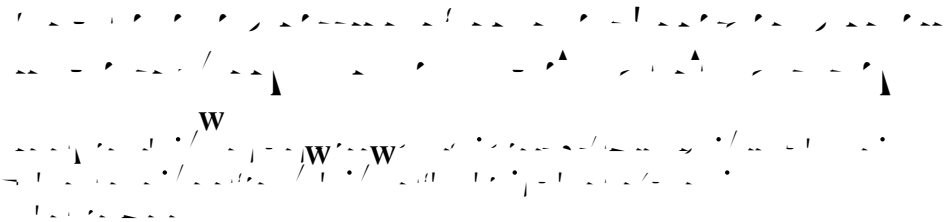

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This novel orange uorescent protein (OFP) emits brilliant orange uorescent light. OFP has high uorescence quantum ield, fast maturation rate, and stabilit , hich impl this protein should be the most favorable biotechnological tools used to investigate the function of target gene b visua ing, monitoring, and quantif ing in living cells. *B. mori*, silk orm has been used as an important bioreactor for the production of recombinant proteins through baculovirus e pression s stem (BES). In this paper, e used infection technique hich introduced the baculovirus DNA into silk orms using a cationic lipofectin reagent instead of directl injecting the virus, and demonstrated a high-level e pression of the orange uorescent protein (OFP) gene in the *Bombyx mori*, silk orm larvae. When recombinant rBacmid/BmNPV/OFP DNA ranging from 50 100 ng/larval as injected, a suf cient OFP e pression in hemol mph as harvested. The recombinant viruses could be obtained from the hemol mph of infected larvae and stored as seed hich could be used for the large-scale e pression. This procedure omitted the costl and labor-consumed insect cell culture. Further investigation of OFP should provide us ith more insight in unlocking the m ster of the mechanisms of autocatal tic bioluminescence and its uti- ation in biotechnolog .

Silk orm (*Bombyx mori* L.) · E pression · Orange uorescent protein · Bac-to-Bac s stem

The baculovirus e pression s stem (BES) has been e tensivel used since its inception 24 ears ago to e press a large variet of the recombinant proteins in cultured insect cells or the insect larvae. Recentl , a bacmid (a baculovirus shuttle vector) s stem has been developed for BmNPV, hich can be replicated in *Escherichia coli* as a large plasmid, generates recombinant virus DNA b site-speci c transposition, and remains infectious in insect cells. Because this method eliminates the need for multiple rounds of puri cation and ampli cation of viruses, it markedl decreases the technical dif cult and the time required to select and purif recombinant viruses [1, 2].

During the last de en ears, uorescent proteins have become one of the most favorable biotechnological tools scientists use to investigate the function of their genes of interest b visua ing, monitoring, and quantif ing protein e pression directl in living cells.

GFP as the rst uorescent protein puri ed from the bioluminescent jell sh *Aequorea Victoria* [3 5], and has started to nd idespread applications and acceptance in man branches of biological science [6]. GFP mutants that can emit blue, c an, or ello uorescence ere reported but further e tension of the emission into the red spectral region (ith an emission avelength greater than 550 nm) as not achieved [7 10]. A red emitter as desirable because this spectrum of light can penetrate further into

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

Diluting 6 μg of puri ed recombinant rBacmid/BmNPV/OFP DNA in 300 μl of unsuplemented Grace s Insect Cell Medium. Mi x cellfectin reagent thoroughl before use b 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 ith the diluted cellfectin Reagent (total volume 600 μl). Mi x gentl and incubate for 45 min at room temperature.

The rst da of fth instar larvae ere used. 10 μl of above DNA mi ture as injected into dorsal of the silk orm larvae b a s ringe. Screening the orange uorescent proteins using uorescent illuminator in complete darkness ever da of post-infection, picking out the silk orm larvae hich had e pressed the OFP 7 8 da s of post-injection, e tracting the hemol mph and centrifuging at 500*g* for 5 min to remove the impurit , the virus as obtained from the clari ed supernatant, Stored at 4 C, protected from light. For long-term storage, stored an aliquot of the viral stock at −80 C.

E pression and e amination of the target gene

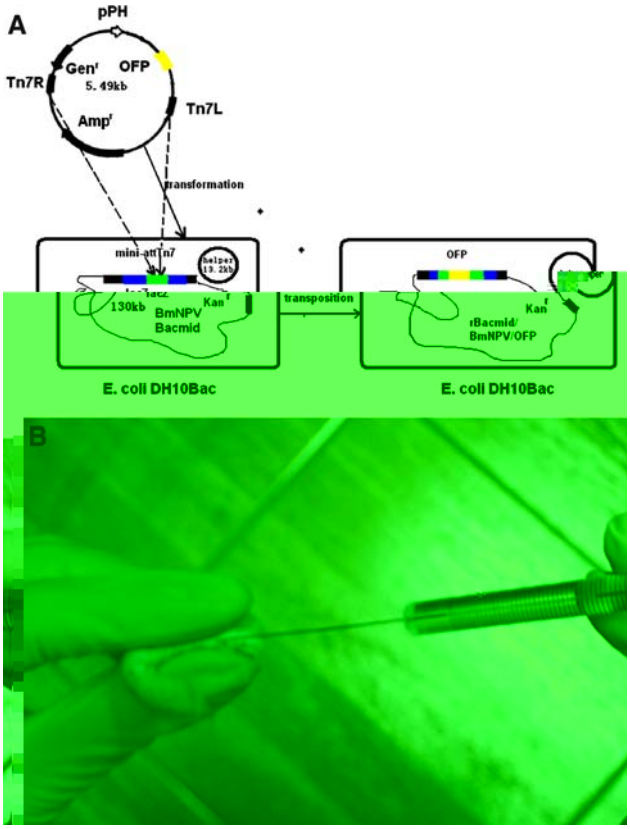
Centrifuging the hemol mph at 500*g* for 5 min to remove the impurit and obtain the virus. Diluting the virus in unsuplemented Grace's Insect Cell Medium. Then 10 μl of this virus mi ture as injected into dorsal of the silk orm larvae b a s ringe. The hemol mph as e tracted dail to e amine the OFP b SDS-PAGE.

After SDS-PAGE, the proteins ere transfer onto a PVDF membrane under 2 mA/cm^2 for 1 h, and subsequentl blocked ith TBST (100 mM Tris-HCl, pH 7.5, 0.9%NaCl, 0.1% T een 20) containing 2% of non-fat dried milk for 1 h at room temperature. After three times ashing ith TBST, the membrane as incubated in TBST containing 2% of non-fat dried milk 1000× diluted OFP-antibod for overnight at 4 C. The membrane as ashed three times (each for 10 min) ith the same TBST follo ed b 5 h incubation of horseradish pero idase labeled Goat anti Rabbit IgG antibod at room temperature. After ashing ith TBST, the antibod as detected ith Konica immunostaining HRP-1000 kit (Konica Minolta, Tok o, Japan).

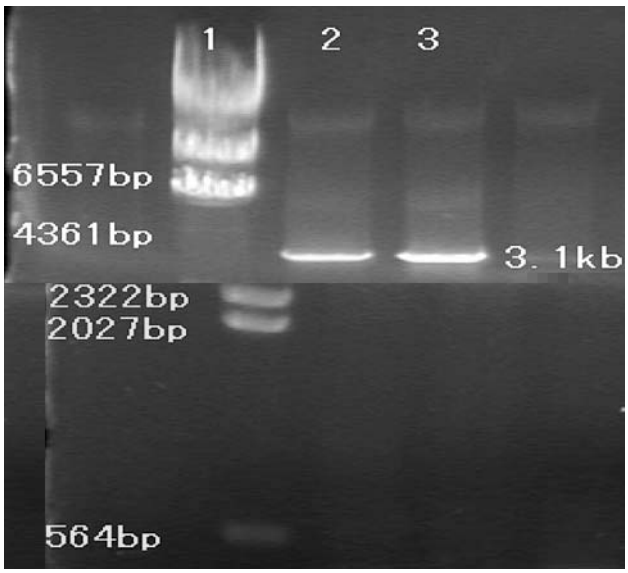
Obtainment of the rBacmid/BmNPV/OFP DNA and recombinant BmNPV virus

Transforming 1 ng of puri ed pFastBac^{TM}HTb-OFP recombinant plasmid into the *E.coli* Bm DH10 Bac competent cell. Transposition occurs bet een 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 b the helper plasmid. The hite colonies ere selected for anal sis from the LB agar plates containing 50 μg/ml kanam cin, 7 μg/ml gentamicin, 10 μg/ml tetrac cline, 100 μg/ml X-gal, and 40 μg/ml IPTG. Picking the hite colonies and restreak them again on fresh LB agar plates containing 50 μg/ml kanam cin, 7 μg/ml gentamicin, 10 μg/ml tetrac cline, 100 μg/ml X-gal, and 40 μg/ml IPTG. Incubate the plates overnight at 37 C (Fig. 1a).

From a single colon con rmed to have a hite phenot pe on re-streaked plates containing X-gal and IPTG, inoculate a lipid culture containing 50 μg/ml kanam cin, 7 μg/ml gentamicin and 10μg/ml tetrac cline. Isolate the recombinant bacmid DNA and anal ing it b PCR using the M13 For ard (−40) and M13 Reverse primer. Electrophoresis to e amine the si e of the PCR product is 3.1 KB, demonstrate the correct results (Fig. 2).



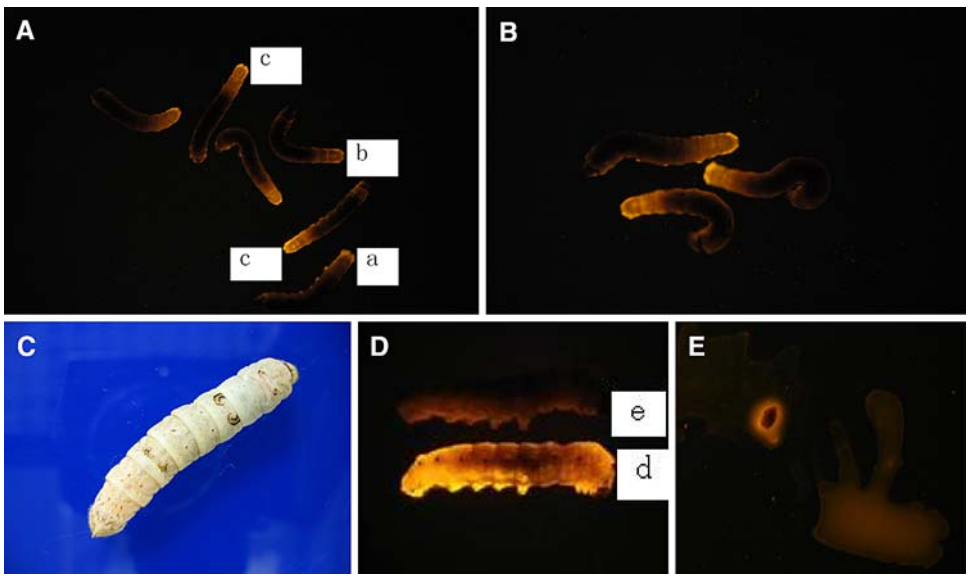
( ) The construction of recombinant rBacmid/BmNPV/OFP DNA b the Bac-To-Bac s stem. The donor plasmid pFastBac^{TM}HTb-OFP including the OFP gene as transformed into *E.coli* Bm DH10Bac competent cell for the transposition, and the recombinant DNA obtained as designated rBacmid/BmNPV/OFP. ( ) The rBacmid/BmNPV/OFP DNAs or its recombinant virus ere injected to silk orm larvae b a s ringe



¬ . - E amine the PCR product b electrophoresis. Band 1 is the marker, band 2 and 3 is the PCR product

Large-scale e pression of the OFP in silk orm larvae

The rst da of fth instars larvae ere used. 10 µl of DNA: Cellfectin Reagent mi ture as injected into dorsal of the silk orm larvae b s ringe (Fig. 1b). To e amine the orange uorescent proteins using uorescent illuminator in complete darkness ever da of postinfection, found that the e pression of the OFP ill begin 4 da s of postinjection (Fig. 3A-a), be get the highest level 8 da s of postinfection (Fig. 3D). E tract the hemol mph 7 8 da s of postinjection, centrifuge at 500*g* for 5 min to remove the impurit , the virus as obtained from the clari ed supernatant. The optimum amount of rBacmid/BmNPV/OFP virus injection as determined to be 160 pfu/larval. When rBacmid/BmNPV/OFP virus ith 800 pfu/larval as injected into the larvae, the are markedl eak 3 da s postinjection, and one-half of the larvae ere dead 4 da s postinjection. When rBacmid/BmNPV/OFP virus ranging from 160 pfu/larval to 400 pfu/larval as injected, a suf- cient amount of hemol mph as harvested. For the recombinant bacmid, the optimum rBacmid/BmNPV/OFP DNA as determined to be in the range of 50 100 ng/larva b a preliminar e periment. So dilute the supernatant in unsuplemented Grace s Insect Cell Medium. Then 10µl of the virus as injected into dorsal of the silk orm larvae b s ringe. The optimal harvest time of the hemol mph as 4 or 5 da s postinjection. On the other hand, hen the recombinant bacmid DNA as injected into the larvae, the e pression of the recombinant protein began 4 da s postinjection and reached ma imum level of e pression 8 da s postinjection. 9 da s postinjection, the recombinant protein as degraded, possibl o ing to signal peptides might be inef cientl recogn ed b the protein translocation machiner in insect cell. 8 da s postinjection, the larvae ere subsequentl dissected out, and the tissues ere



¬ . - OFP e pression in *B.mori* larvae. *B.mori* larvae ere infected b direct s ringe injection of rBacmid/BmNPV/OFP DNAs ( ), and rBacmid/BmNPV/OFP virus ( ). ( ) The photograph (a-c) of the larvae as taken at 4 6 da s of postinjection time using uorescent illuminator in complete darkness respectivel . The photograph ( ) of the larvae as taken at 4 da s of postinjection time using uorescent illuminator in complete darkness. The photograph ( ) of the larvae as taken at 8 da s of postinjection using light illuminator in normal light. The photograph ( ) of the larvae as taken at 8 da s of postinjection using uorescent illuminator in complete darkness. And the (e) is the control. ( ) The OFP e press in the fat bod and hemol mph of the larvae, revealing the OFP e pression. The photograph as taken under the uorescent illuminator in complete darkness

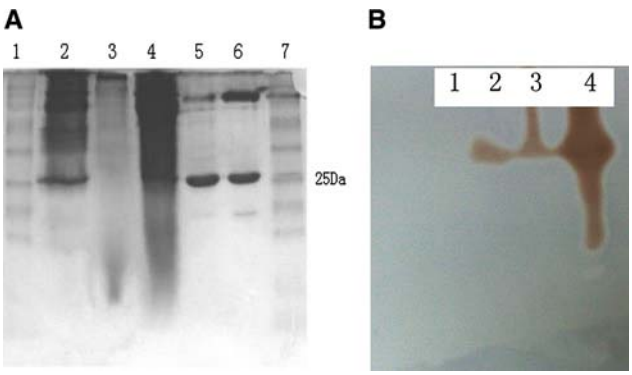
vie ed under a uorescence illuminator (Fig. 3E). Found that the color of fat bod is orange, and hemol mph is turbid. While the color control is hite, hemol mph is clear. The possible reason for this is that the silk orm larva has an open circulator s stem and the OFP from the fat bod readil leach out and, in the late stage of virus infection, the fat bod undergoes l sis, leading to the release of proteins into the hemol mph.

SDS-PAGE and PAGE anal sis

To detect the OFP in the hemol mph of the silk orm larvae, the supernatants and the total hemol mph ere subjected to SDS-PAGE and PAGE on a 12% pol acr l-amide gel using the pipette, respectivel . Ten microliters of supernatant and hemol mph as mi ed ith the same amount of sample buffer respectivel , and the applied to a 12% pol acr lamide gel. For the detection of OFP on the PAGE gel, the samples ere onl mi ed ith the sample buffer ithout boiling and orange uorescent bands ere then directl observed using a uorescence illuminator in complete darkness. The target protein bands ere located at the 25 Da (Fig. 4a).

Western blot anal sis

The Western blot anal sis as performed b using the e tracts of the hemol mph and its supernatant respectivel . Ten microliters of supernatant and hemol mph as mi ed ith the same amount of sample buffer respectivel , and the applied to a 12% SDS-PAGE gel and Western blot anal sis. Fig. 4b clearl demonstrated that the OFP e pression level b injecting recombinant bacmid DNA directl is as high as injecting recombinant virus.



- ( ) SDS-PAGE anal sis of the OFP e pressed in silk orm larvae hich

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With the development of biotechnolog , *B. mori* has been used as an important bioreactor for the production of recombinant proteins through baculovirus e pression s s-tem (BES) [18, 19]. Recentl , e established the practical BmNPV bacmid s stem to e press the foreign proteins. This method eliminates the need for multiple rounds of puri cation and ampli cation of viruses, it markedl decreases the technical dif cult and the time required to select and purif recombinant viruses.

The levels of protein using the silk orm larvae is 10 100 fold higher than that using *B.mori* cells, indicating that the silk orm larvae is an optimal s stem for the mass production of recombinant proteins [2]. Using this s stem, e demonstrated a high-level e pression of the orange uorescent protein (OFP) gene in the *B. mori* silk orm larvae b directl injecting recombinant bac-mid DNA. Ho ever comple proteins are generall not ell e pressed as biologicall functional proteins. The reason for this in unkno n, but it is possible that there is not an signal peptide in this s stem. Some papers reported that insect cell infected ith a baculovirus recombined ith the gene encoding propapain fused to the hone bee melittin signal peptide secreted more than ve-fold the amount of the papain precursor than those infected the gene encoding a plant signal peptide. We could not e press some of the functional human en me using Bac-To-Bac s stem ithout encoding a signal sequence, but could e press them in a functionall active form using Bac-To-Bac s stem ith encoding the hon-e bee melittin signal peptide. In addition, the signal sequence from a silk orm *Bombyx mori* and a silk orm prophenolo idase-activating en me are designate *bx* and *ppa* respetivel have been demonstrated that the signal peptide is important for the e pression of active protein [20 23].

The optimum amount of rBacmid/BmNPV/OFP virus injection as determined to be 160 pfu/larval. When rBacmid/BmNPV/OFP virus of 800 pfu/larval as injected into the larvae, the e pression as markedl eak 3 da s postinjection, and one-half of the larvae ere dead 4 da s postinjection. When rBacmid/BmNPV/OFP virus ranging from160 pfu/larval to 400 pfu/larval as injected, a suf - cient amount of hemol mph as harvested. For the recombinant bacmid, the optimum rBacmid/BmNPV/OFP

DNA as determined to be in the range of 50 100 ng/ larval b a preliminar e periment.

The optimal harvest time of the hemol mph as 4 or 5 da s postinjection. On the other hand, hen the recombinant bacmid as injected into the larvae, the e pression of the recombinant protein began 4 da s postinjection and reached ma imum level of e pression 7 da s postinjection. In 8 da s postinjection, the recombinant protein as degraded, possibl o ing to signal peptides might be inef cientl recogni ed b the protein translocation machiner in cells. In 7 da s postinjection, the larvae ere subsequentl dissected out, and the tissues ere vie ed under a uorescence illuminator, nding that the color of fat bod is orange, and hemol mph is turbid. While the hemol mph of the control is clear. The possible reason for this is that the silk orm larval has an open circulator s stem and the OFP from the fat bod readil leach out and, in the late stage of virus infection, the fat bod undergoes l sis, leading to the release of proteins into the hemol mph.

Up to no , several proteins have been produced using *B.mori* silk orm larvae. Maeda et al. rst reported the production of α-interferon in silk orm using BmNPV baculovirus vector. On our stud , the OFP e pression as further investigated in silk orm larvae b both direct infection of recombinant Bacmid DNA and infection virus using a s ringe. In this case the orange uorescence as screened ithin 4 da s, hich as slo er than that of the virus infection. On the other hand, the recombinant virus infection using a s ringe, the larvae appeared orange in 3 da s postinjection and the uorescence intensit further increased.

The estern blot anal sis demonstrated that the OFP e pression level b injecting recombinant bacmid DNA directl is as high as injecting recombinant virus into silk orm larvae.

Our ork sho ed great advantages of the Bac-To-Bac s stem, such as high e pression levels of the protein. Furthermore, b direct injection of the recombinant bacmid DNA into silk orms using a cationic lipofectin reagent instead of directl injecting the virus, the orange uorescence as also identi ed in the *Bombyx mori*, silk orm larvae. The recombinant viruses could be obtained from the hemol mph of infected larvae and stored as seed hich could be used for the large-scale e pression. This procedure omitted the costl and labor-consumed insect cell culture.

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