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Expression of *Bombyx mori Cecropin B* in Silkworm Larvae using BmNPV/Bac-to-Bac Expression System

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Antimicrobial peptides (AMPs) are a family of small molecular weight proteins produced by living organisms of all types, and have a broad range of antimicrobial activity against bacteria, viruses, and fungi. Thus it is important to efficiently express AMPs using Bombyx mori (Bm) baculovirus expression system. In the present study, we cloned Bm *cecropin B* (*CecB2*) and *polyhedrin* (*polh*) genes into the pFastBac DUAL vector. Recombinant baculovirus polyhedrin was generated by transfecting the recombinant plasmid into BmN cells and used to orally inoculate the silkworm larvae. We found that CecB2 was successfully expressed in the silkworm larvae and confirmed to have antibacterial activity to *E. Coli*, and ELISA quantative assay showed average concentration to 0.115¼g/ml CecB2 was detected

Key words: Antimicrobial peptides, cecropin B, ploy hedrin, pFastBac DUAL, Bac-to-Bac expression system, antibacterial activity.

Antimicrobial peptides (AMPs) are a assorted group of small molecules produced by living organisms, and have potent antimicrobial activity to a broad range of microbes, including viruses, bacteria, protozoa, and fungi. More significantly, the ability of these peptides to kill multidrug-resistant pathogens has gained considerable attention. In 1972, Swedish scientists Boman *et al.,*[1] firstly isolated the efficient antimicrobial peptide cecropin from the immune hemolymph of *Hyalopbora cecropia* after *E. coli* induction. With the development of biotechnology, AMPs have been used as new antibiotics and drugs, and play important roles in many fields, including agriculture, medicine, food, etc. To date, thousands of AMPs have been isolated from natural organisms. Cecropins, specifically cecropins A, B, C, D and E[2], are a group of AMPs that are clearly studied and have the most significant effect. Cecropins have the characteristics of high resistant capacity to acid, heat and alkali and low resistance to drug[3], have been widely used in the microorganisms, animals and plants[4].

Insect AMPs are a group of small molecular bioactive peptides from insect hemolymph, which are lowly expressed in the body and easily degraded by in vitro proteinases. The difficulty in isolating these AMPs is the biggest obstacle to produce them in a large scale. Thus, it is of great important to study AMPs through genetic engineering technology. The techniques for AMPs studies usually include eukaryotic and prokaryotic expression systems[5]. In the prokaryotic expression system, AMPs are usually expressed as fusion proteins in *E. Coli*, which can change the configuration of AMPs to decrease the toxicity to host cells[6]. This expression system can express

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AMPs quickly at low cost, but the products are difficult to purify and the posttranslational modification is not complete, consequently, the bioactivity of expressed products is quite low. Therefore, the eukaryotic expression system has become the hot technique to study AMPs using genetic engineering. Recently, Bac-to-Bac baculovirus expression system has been widely used and provides a rapid and efficient method to generate recombinant baculoviruses. This method is based on site-specific transposition of an expression cassette into a baculovirus shuttle vector (bacmid) propagated in *E. coli.*[7], which helps to avoid the problem in recombinant virus selection by traditional tedious plaque analysis. However, in the commonly used baculovirus expression vector system, the *polyhedrin* (*polh*) gene is replaced by a foreign gene to produce a recombinant baculovirus in which the powerful *polh* promoter drives high expression of the foreign gene[8-11]. As a result, the recombinant baculoviruses, without the protection of the polyhedrin protein, are needed to inoculate silkworm larvae through subcutaneous injection, which results in low efficiency. Therefore, the expression of both *polh* gene and foreign gene in one expression system is necessary to generate high titer baculoviruses in a large scale[12].

In this study, we successfully expressed Bm *cecropin B* (*CecB2*) and *polh* genes in the pFastBac DUAL vector, the recombinant baculoviruses were generated by Bm cell line, BmN and used to orally inoculate silkworm larvae. The recombinant CecB2 was confirmed to have antimicrobial activity. Moreover, we also measured the concentration of recombinant CecB2 after infection using ELISA assay.

MATERIALS AND METHODS

Materials

Hybrid strain larvae of silkworm (Qingsong×Haoyue) were hatched from disease-free eggs and reared under standard condition (26±2°C, 70–85% relative humidity, 12 h: 12 h light/darkness, free access to mulberry leaves). After the fourth ecdysis, larvae were divided to four groups and used for infection.

pFastBac DUAL vector was purchased from Invitrogen (San Diego, CA). FuGENE 6 transfection reagent was purchased from Roche Applied Science (Indianapolis, IN). The *ELISA kit* was purchased from Jijin chemical technology Co., LTD. (Shanghai, CHN). The E. coli DH10Bac/BmNPV was preserved in our laboratory.

Bm cell line, BmN, was originally derived from ovary and supplied by Laboratory of Life Science, Jiangsu University and cultured at 27°C with TC-100 medium containing 10% fetal bovine serum (FBS).

Construction of dual expression vector

The Bm *polh* gene was amplified by PCR from the wild BmNPV template using the primers: *Polh*-F: 5'-CGCGGATCCATGCC GAATTATT CATACA-3', *Polh*-R: 5'-CCCAAGCTTTTAATA CGCCGGACCAGTGAACAG-3'. The PCR product was subcloned into the pFastBac Dual vector digested by *Bam*H I and *Hin*d III enzymes.

The Bm *CecB2* gene (NCBI login: NM_001043995.1) was amplified from the PET-32a-*Bm-CecB2* plasmid using the primers: *CecB2*-F: 5'-CATGCCATGGCGCCGGAACCGCGTTGGAAA-3', *CecB2*-R: 5'-CGGGGTACCTCATTATTTACCG ATGGCTTTCG-3', and inserted into the pFastBac DUAL-*polh* (pFD-*polh*) plasmid digested by Nco I and Kpn I. The map of constructed plasmid pFastBac DUAL-*CecB2*/*polh* (pFD-*CecB2*/*polh*) was shown in Fig.1 and transformed to *E. coli* DH10Bac/BmNPV.

Generation and isolation of recombinant bacmid baculoviruses

The *E. coli* DH10Bac containing recombinant bacmid was propagated in LB medium containing antibiotics (50µg/ml kanamycin, 7µg/ml gentamicin and 10µg/ml tetracycline), 100µg/ml X-gal and 40µg/ml IPTG. The white colonies were selected for further amplification. The pFD-*CecB2*/*polh* plasmids was isolated using the alkaline lysis method, and transfected into BmN cells using FuGENE 6.The recombinant P1 viral solution was collected from BmN cells 120 h post-transfection and stored at 4°C protected from light. The P1 viral stock was further used to infect BmN cells to generate high-titer P2 stock that was used to infect the silkworm larvae.

Production of recombinant *Bm CecB2* in silkworm larvae

The silkworm larvae on the first day of fifth instar were fed with 7µl P2 pFD-*CecB2*/*polh* baculovirus solution which concentration of

polyhedron to $2*10^7$ NPB/mL by licking, while the same amount of wild BmNPV solution or water was licked by the control silkworm larvae. The infection on silkworm larvae was monitored every day, and the blood of silkworm larvae was collected to purify the polyhedrins on days of 6 and 7 after infection. The DNA from polyherins was isolated to verify the expression of *CecB2* by PCR amplication.

Polyhedrin observation

The larval hemolymph was checked for the presence of polyhedrins under light microscope and electronic microscope at 96 h post-infection.

Measurement of BmCecB2 activity

The blood of silkworm larvae was purified by PBS buffer, and undergone ultrasonic disruption. The supernatant was collected after centrifugation and used for Tricine-SDS-PAGE analysis and the assay for antimicrobial activity.

The antimicrobial activity of recombinant BmCecB2 was detected by using radial diffusion assay against *E. coli*. Cells were cultured overnight in LB medium to OD_{600} of 0.3, and added at a ratio of 1:100 (v/v) into warm LB containing 1% agar. The mixture was then plated on 90 mm petri dish. For the radial diffusion assay, 200µl of larvae blood cell supernatant containing recombinant BmCecB2 was added into an oxford cup in the bacterial plate. In the same plate, 10µl ampicilin (100mg/ml) was used as a positive control, while 200µl larvae blood cell supernatant containing wild BmNPV and 200 µl water were used as negative controls. The plate was incubated at 37°C for 16 h.

Bioassay of recombinant BmCecB2

The silkworm larvae after infection were grinded and mixed with ice-cold protein extraction buffer (50mM Na_3PO_4ÿ0.5 M NaClÿpH 8.0ÿ1 mM DTT)[13]. The mixture was centrifuged at *14,000 × g for* 30 min at *4°C to collect the supernatant (silkworm crude extract). The content of BmCecB2 in silkworm blood and crude extract was measured by ELISA kit according to manufacture's instructions.* The OD values for all samples in triplicates and the standard were measured at 450nm. The standard curve was made according to the OD_{450} values and corresponding concentration of the standard and a linear regression equation was figured out from the standard curve. The concentration of all samples was calculated by mapping out the sample OD_{450} values to the linear regression equation of the standard. The sample concentration was represented as mean±SE from three independent experiments, and the statistical analysis was carried out by using One-way ANOVA plus Duncan multiple comparisons.

RESULTS

Construction of pFD-*CecB2*/*polh* plasmid

The fragments of Bm *CecB2* and *polh* genes were amplified from the plasmids PET-32a-Bm-*CecB2* and wild BmNPV, respectively. The size of PCR products was consistent with the length of Bm *CecB2* DNA (147bp, Fig. 2A) and *polh* DNA (756bp, Fig. 2B). The PCR products were purified and inserted into pFastBac DUAL vector to generate the pFD-*CecB2*/*polh* plasmid. The accuracy of pED-*CecB2*/*polh* was confirmed by two sets of double enzymes digestion, *Nco* I/*Kpn* I for *CecB2* (Fig. 2C) and *Bam*H I/*Hin*d III for *polh* (Fig. 2D). The digested fragments were purified and sequenced, and the result of sequencing showed the inserted fragments were consistent with the sequences of *CecB2* and *polh* genes.

Transfection of recombinant plasmids into BmN cells

The recombinant plasmids were transfected into BmN cells using lipofectamine. 72 h after transfection, BmN cells showed obvious symptom of infection, most of cells were in round

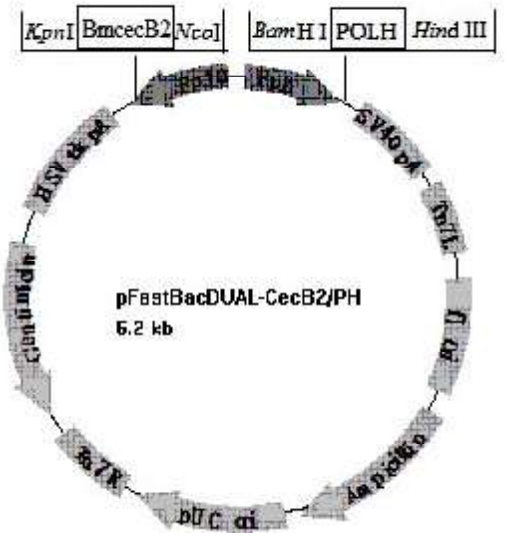


Fig. 1. Map of pFastBac DUAL-*CecB2*/*polh*

shape and lots of polyhedrins were observed under microscope (Fig. 3B), while normal control BmN cells showed polygonal morphology (Fig. 3A).

Expression and identification of recombinant BmCecB2 protein in silkworm larvae

To confirm the infectious ability of baculoviruses and expression of CecB2 in the body of silkworm larvae, we infected the silkworm larvae with baculovirus solution by licking. The silkworm larvae licked with baculovirus solution from wild BmNPV were set as positive control, while the

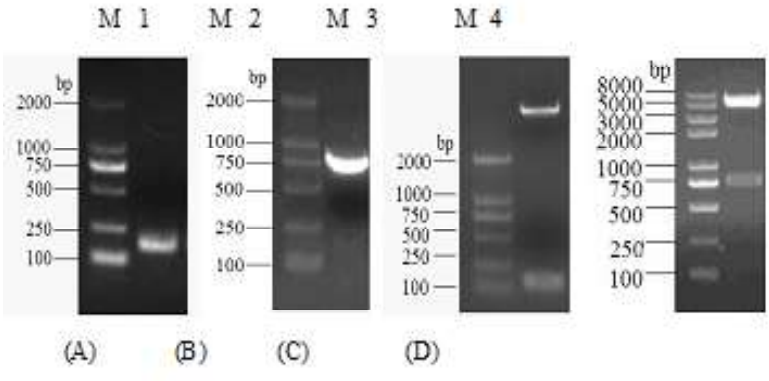
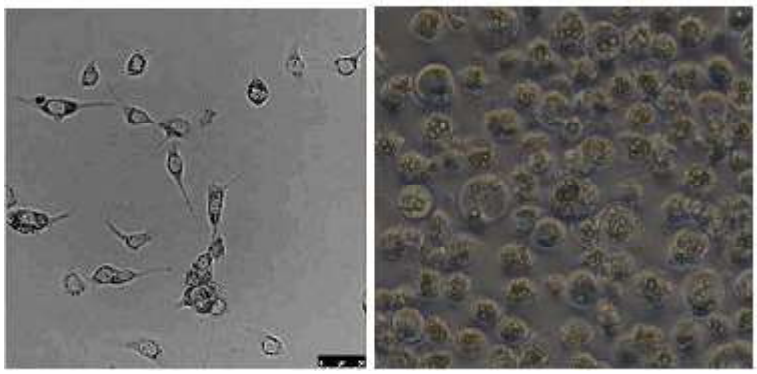


Fig. 2. Conctruction and identification of *CecB2* and *polh* DNA in the pFD-*CecB2*/*polh* plasmid. M: DL2000 DNA Marker (A, B, C), DL8000 DNA Marker (D); 1: PCR product of Bm *CecB2* gene; 2: PCR product of *polh* gene; 3: *Nco* I/*Kpn* I digestion of pFD-*CecB2*/*polh* plasmid; 4: *Bam*H I/*Hin*d III digestion of pFD-*CecB2*/*polh* plasmid



a) b)

Fig. 3. Cell morphology of normal BmN (A) and BmN cells infected by recombinant Bacmid (B)

a) b) c) d) e)

Fig. 4. Photos of polyhedrin, hemolymph and larvae after infection with recombinant baculoviruses. (A) Polyhedrins in larvae hemlymph under microscope; (B) The hemolymph from normal larvae (left) and infected larvae by wild BmNPV (middle) and pFD-*CecB2*/*polh* (right) baculoviruses; (C) Normal silkworm; (D) Larvae infected with pFD-*CecB2*/*polh* baculoviruses; (E) Larvae infected pFD-*polh* baculoviruses.

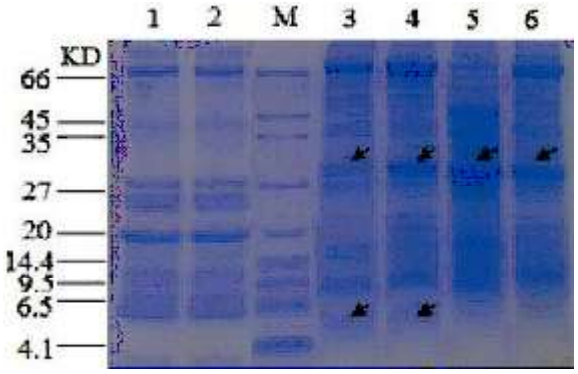


Fig. 5. SDS-PAGE analysis of BmCecB2 and polh expression. M: protein molecular mass standard; 1~2: Hemolymph from the 6th and 7th days of fifth intar silkworm larvae; 3~4: Hemolymph from the 6th and 7th days of fifth intar silkworm larvae after infection with pFD-*CecB2*/*polh* baculoviruses; 5~6: hemolymph from the 6th and 7th days of fifth intar silkworm larvae after infection with wild BmNPV. The arrows point out the polyhedrin protein (29KDa) and BmCecB2 protein (4.9KDa)

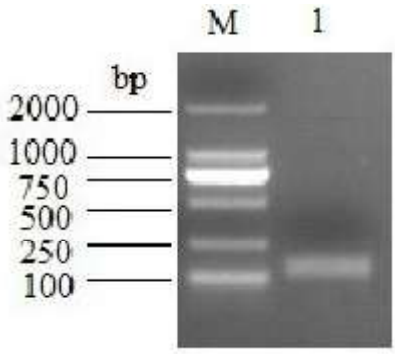


Fig. 6. Electrophoresis of Bm *CecB2* PCR product from pFD-CecB2/polh baculoviruses. M: DL2000 DNA Marker; 1: PCR product of Bm *CecB2* gene

Fig. 7. Antibacterial activity assay of BmCecB2. 1~2; supernatant of blood cells from the 6th and 7th days of fifth intar silkworm larvae after infection with pFD-*CecB2*/*polh* baculoviruses; 3: supernatant of blood cells from the 6th and 7th days of fifth intar silkworm larvae after infection with wild BmNPV; 4: supernatant of blood cells from normal silkworm; 5: ampicilin; 6: sterile water

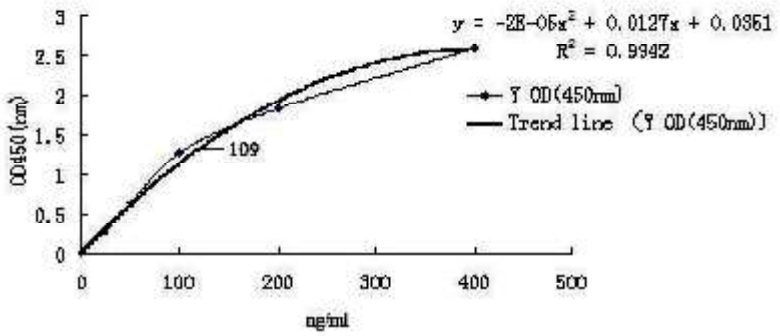


Fig. 8. The linear regression curve for the standard

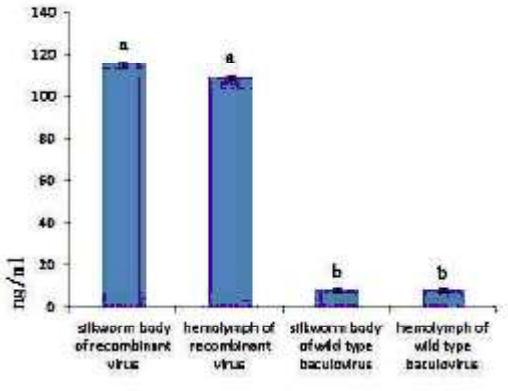


Fig. 9. Detection of BmCecB2 content in silkworm body and silkworm blood after fed orally with different baculoviruses. Different letters above bars represent significant difference at $P < 0.05$ level

normal silkworm larvae were used as negative control. 5 days (120 h) after infection, the silkworm larvae fed with baculovirus solution containing wild BmNPV and pFD-*CecB2*/*polh* showed obvious symptom of infection (Fig. 4C-E), and lots of polyhedrins were observed in the blood of silkworm larvae under microscope and the color of the blood (Fig. 4A and B). Moreover, we analyzed the expression of CecB2 and Polh proteins in the blood cells by SDS-PAGE. As shown in Fig. 5, the predicted band for polh protein (29KDa) was observed in the blood of silkworm larvae after infection with pFD-*CecB2*/*polh* and wild BmNPV baculoviruses (Fig. 5, as upper arrows shown), while the predicted band for CecB2 protein (4.9KDa) was only observed in the blood cells from pFD-*CecB2*/*polh* baculoviruses infected silkworm larvae (Fig. 5, as lower arrows shown). We further verified whether CecB2 gene was expressed in the recombinant baculoviruses from silkworm larvae blood after pFD-*CecB2*/*polh* baculoviruses infection by PCR amplification. A clean band at 150bp was observed (Fig. 6), thus we confirmed the baculoviruses were the Bm *CecB2* recombinant baculoviruses.

Detection of the antimicrobial activity of BmCecB2

The supernatant from silkworm larvae blood cells after ultrasonic disruption was used for the detection of antimicrobial activity. Our result showed the recombinant CecB2 in silkworm larvae blood cells had obvious antimicrobial activity to *E. Coli*, the strength of antimicrobial activity was up to 10mm in diameter (Fig. 7). Amplicilin showed strong antimicrobial activity (positive control), while the sterilized water and the blood cells supernatants from normal larvae and wild BmNPV infected larvae did not show any antimicrobial activity (Fig. 7).

Detection of BmCecB2 expression in silkworm larvae

To further detect the expression of recombinant BmCecB2 in each silkworm larva, the content of BmCecB2 in each larva was quantified by ELISA kit. The standard curve was made according to the actual concentration and OD_{450} values of the standard (Fig. 8). The concentration of samples was determined by the linear regression equation from the standard. Our results showed that the content of recombinant CecB2 in the silkworm larvae body and hemolypha after infection with pBF-*CecB2*/*polh* baculoviruses was significantly higher than those after wild BmNPV infection (Fig. 9, $P < 0.05$). According to the linear regression equation, the content of recombinant CecB2 after infection with pBF-*CecB2*/*polh* baculoviruses was detected concentration to 0.115¼g/ml in the body (crude extract) and 0.109¼g/ml in the hemolopha (blood), while the level of CecB2 in the silkworm larva after wild BmNPV infection was as low as the empty control (Fig. 9).

DISCUSSION

In the present study, we successfully constructed the Bm *CecB2* and *polh* genes into pFastBac DUAL vector and expressed the recombinant BmCecB2 protein in the silkworm larvae using Bac-to-Bac baculovirus expression system. The antimicrobial activity of recombinant BmCecB2 and its content in one silkworm larva were examined.

The p10 and ph promoters in pFastBac DUAL vector are very strong. Previous study reported that p10 promoter can induce higher gene expression than ph promoter[9]. Here we installed the Bm *CecB2* gene behind the p10 promoter and inserted the *polh* gene behind the Pph promoter and generated the recombinant baculoviruses packaged by the polh protein. We also infected the silkworm larvae with baculoviruses by licking because the strong alkaline environment in the

intestine of silkworm larvae can release the proteinases to digest polyhedrins[10]. Compare with subcutaneous injection, this technique can save time and labor, then increase the efficiency of production. In addition, the silkworm is good feed additive with high protein content. If we can highly express the AMPs in the silkworm and use it as feed additive without purification, the AMPs from silkworm feed additive will be hopeful to replace the antibiotics in the feed and have a good market prospect[8].

In this study, we did not attach any tag into the Bm *CecB2* gene. The main reason is that the molecular weight of AMPs is as small as 4 KDa and tag labeling might affect AMPs structure and their antimicrobial activity. However, without the assistance of tag, it is difficult to purify AMPs. In addition, although expression of recombinant CecB2 in silkworm has been reported in a recent study, they didn't detect and quantify the expression level of BmCecB2[11]. Here we employed a specifc BmCecB2 ELISA kit to quantify the content of recombinant BmCecB2 in one silkworm larva. In this kit, the coefficient R value between the linear regression of standard and expected concentration is above 0.99, and it is very sensitive to detect as low as 1.0ng/ml Cecropin B. Although other proteins are highly expressed in the silkworm, the expression of BmCecB2 is quite low in one silkworm larva. Thus we need to further investigate how to increase the expression level of recombinant BmCecB2 and purify the recombinant BmCecB2 with antimicrobial activity from silkworm. Some literatures reported that two strong promoters in one vector might compete and affect the expression of inserted genes, these data might help to explain low expression of recombinant BmCecB2 in the silkworm larvae.

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