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Short communication

Rapid non-invasive monitoring of baculovirus infection for insect larvae using green fluorescent protein reporter under early-to-late promoter and a GFP-specific optical probe

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Abstract

We investigated monitoring for baculovirus infection of insect *Trichoplusia ni* larvae by combining the green fluorescent protein (GFP) reporter, the early-to-late (ETL) baculoviral promoter, and a GFP-specific optical probe. Because GFP is under the ETL promoter control, it facilitated rapid monitoring of recombinant baculovirus infection on insect larva system. Employment of GFP-specific optical probe also enabled non-invasive and accurate GFP monitoring with ~10 h prior to invasive and GFP Western blot assay. This combination may assist in monitoring protein production runs, determining optimal infection and harvest timing, and in general, help to increase the yield of recombinant proteins expressed in larvae.

Keywords: Baculovirus infection; Insect larvae; Early-to-late promoter; Green fluorescent protein; GFP-specific optical probe; Non-invasive monitoring

1. Introduction

Advances in recombinant DNA technology have made it possible to produce recombinant proteins that may not have been unachievable or unfeasible. Further, a variety of host and expression systems can be employed to produce the desired recombinant protein. To facilitate the expression of recombinant proteins in eukaryotes, virus systems have been gaining much attention. The baculovirus, *Autographa califonica* nuclear polyhedrosis virus (AcNPV), in particular, has been of important academic and industrial interest because of the advantages the system offers. These include correct functionality of the foreign protein [1,2], high expression levels [1,3], the possibility of post-translational modifications [4,5], capacity for large DNA inserts, capacity for the expression of unspliced genes, simplicity, and simultaneous expressions of multiple genes.

In general, the recombinant baculovirus is added to the insect cell culture during mid to late exponential phase, and the recombinant protein is expressed in high quantities, sometimes as great as 50% of the total cellular protein [6]. Because of the cost and bioreactor complexities, we have been studying the insect larvae/baculovirus expression system as a cost-effective alternative to the insect cell/baculovirus expression system [7–11]. Larvae have been used successfully for the production of a variety of foreign proteins at high levels [9,12–15]. Currently, the system entails synchronous infection with recombinant virus, either by feeding per os (pre-absorbed in diet) or by injection into the cuticle [16]. As the larvae grow, the virus replicates and eventually, the larvae melanize (a polyphenol oxidase-mediated process that results in a discoloration (browning) of the cuticle), and die. This results in the loss of recombinant protein [8]. The synchronous infection and synchronous harvesting is a key to obtain the maximum recombinant protein.

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Our previous works have demonstrated the use of a fusion protein construct that contains the green fluorescent protein (GFP) fused to the recombinant protein of interest could be used as an effective indicator of product level [8,9,11] and facilitate purification [10] in insect larvae expression system. Larvae that produced GFP were easily distinguished from uninfected individuals by simply looking for fluorescence under UV light [8,9]. Further, the quantity of GFP directly related to the amount of recombinant protein [8,9,11] and facilitated the selection of elution fractions, which contain the recombinant product [10]. We also showed that a GFP-specific optical probe [17] can be successfully employed to quantitatively detect GFP level in insect larvae system [11]. By using a baculovirus early-to-late (ETL) promoter that is activated early in the infection cycle and is of medium strength [18] instead of a conventional very late polyhedrin (Polh) promoter that is of strong strength, we demonstrated that early monitoring of baculovirus infection for insect *Sf*-9 cell system is possible [19]. In the present work, we investigated non-invasive and rapid monitoring of baculovirus infection for insect *Trichoplusia ni* larvae system using the GFP-specific optical probe and the GFP reporter under the ETL promoter.

2. Materials and methods

2.1. Construction of recombinant baculovirus

Recombinant baculovirus, $v\mathrm{P_{ETL}}$–GFPuv, was obtained by co-transfection of pBBH(Δ*lacZ*)GFPuv transfer vector [19] (Fig. 1) with wild type AcNPV DNA (Bac-N-Blue™ baculovirus DNA, Invitrogen, USA) into *Sf*-9 insect cells. Successful co-transfection was observed through green fluorescence using a fluorescence microscope (Olympus, Japan). Recombinant virus stocks were propagated in *Sf*-9 cells in tissue culture flasks at 27 °C. Recombinant virus titer expressed as pfu (plaque forming units)/ml was obtained by the end-point dilution assay and calculated using the standard method (50% tissue-culture infectious dose; $\mathrm{TCID_{50}}$) with the relationship pfu = $\mathrm{TCID_{50}} \times 0.69$ [20].

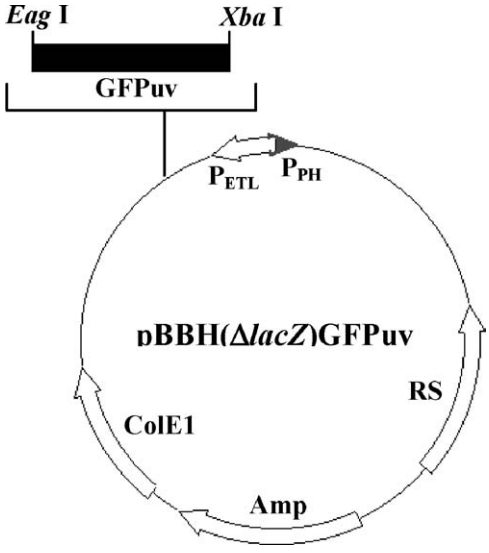

Fig. 1. Gene map of recombinant transfer vector pBBH(Δ*lacZ*)GFPuv. $\mathrm{P_{PH}}$: polyhedrin promoter, $\mathrm{P_{ETL}}$: early-to-late promoter, ColE1: *Escherichia coli* replication origin, Amp: ampicillin resistant gene, RS: recombination site, GFPuv: UV-optimized *gfp* gene.

2.2. Larval system and infection

The cabbage looper, *T. ni*, insect larvae were hatched from eggs (Entopath, USA) in Styrofoam cups containing solid food (Entopath) at 30 °C. The larvae were monitored during the hatching process and each hour all the larvae that had hatched were placed in food cups and labeled as to the time of hatching. Fourth instar larvae were used for infection experiments. At 120–124 h after hatching, the larvae were infected with 5 μl of virus diluted so as to deliver 2×10^4 pfu per larvae. A 50 μl Hamilton syringe (Hamilton, USA) with a 27-gauge needle (Hamilton) was used for injection. The needle was inserted into the larvae at the front of the fourth set of prolegs just under the skin (∼0.1–0.25 cm deep).

2.3. Sample preparation and storage

Three larvae were collected every 6 h post-infection (hpi) until the control pupated or the larvae became too liquefied to manipulate. Three identical experimental set-ups were performed in tandem. For the 0 hpi collection, six larvae were harvested from the control cups because no recombinant protein is produced immediately upon infection. Six larvae were also collected for the 6 and 12 hpi time points as larvae size is too small to yield enough homogenized sample for assays. Starting at 18 hpi, three larvae were harvested for each time point.

Collected larvae were homogenized in 2.5 volumes of homogenization buffer (phosphate buffered saline (PBS) pH 7.0 containing, 6 mM dithiothreitol (DTT) and 0.5% Triton X-100), based on the assumption that the density is that of water, so that a 100 mg larva would be homogenized in 250 μl of buffer. The larvae were homogenized using a Tissue Tearor™ (Biospec Products Inc., USA). Homogenates were divided into two fractions; a 500 μl homogenate sample was centrifuged at $12{,}000 \times g$ for 10 min at 4 °C. The supernatant was collected and aliquoted into 50 μl for protein assay and homogenized fluorescence readings, 100 μl for Western blot analysis, and 50 μl for further analysis if necessary. The aliquots and left over pellets and supernatants were stored at −80 °C until needed.

2.4. Analytical assays

Each larva at the time of collection was individually weighed and then measured using the GFP-specific optical probe [17] using the experimental set-up (Fig. 2) [11]. A Spectro Max Gemini Plate Reader with SoftMax Pro 3.1 software (Molecular Devices, USA) was used for GFP measurements of homogenized larvae. A 96-well assay plate (Costar, USA) was used to mix and measure samples. For each sample, 5 μl of homogenized larvae was added

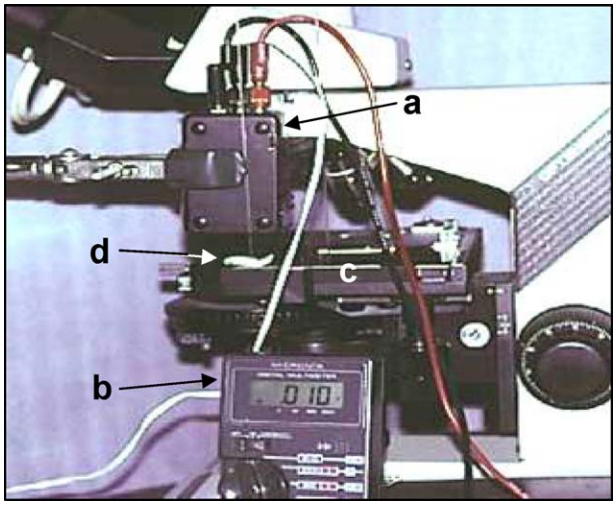

Fig. 2. GFP-specific optical probe. The probe (a) was constructed on the basis of the GFP sensor described in Kostov et al. [17]. The signal is registered as volts using a digital multimeter (b). The probe was mounted onto a laboratory microscope with a movable stage (c) enabling incremental adjustment of the gap between the larva (d) and sensor [11].