Recombinant *Bombyx mori* Nucleopolyhedrovirus Harboring Green Fluorescent Protein

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ABSTRACT

*The gene encoding the green fluorescent protein (*gfp*) under the control of the highly expressed* Autographa californica *nucleopolyhedrovirus (AcMNPV)-polyhedrin promoter has been introduced into the* polyhedrin *(*polh*) locus of* Bombyx mori *nucleopolyhedrovirus (BmNPV) by homologous recombination. The insect host larvae and the cultured cells infected with this recombinant virus (*vBmGFP*) showed high levels of expression of* gfp*. The larval tissues permissive to virus multiplication could be readily visualized using the tagged recombinant virus, thus providing a direct approach to study the progress of virus infection or its control in the animal host. The highly expressed recombinant protein, GFP, could be easily solubilized from fat bodies. Thus, the caterpillar-based expression could serve as an economic alternative method for the large-scale production of recombinant proteins, even when they are nonsecretory in nature. Further, if the recombinant vBmGFP is used as a parent in generating other recombinants, conversion of the fluorescent plaques to colorless plaques serves as an easy means for screening recombinants. Such a method is especially helpful for BmNPV-recombinant selections in the absence of the other simplified techniques as are available for the prototype baculovirus AcMNPV system.*

INTRODUCTION

Baculovirus-based expression of cloned foreign genes in insect cell lines has been extensively exploited to synthesize large quantities of proteins of basic biological interest or of biomedical importance (11,13–16,20). The advantages of the baculovirus system for recombinant protein expression over the bacterial and yeast systems include the capacity to splice multiple introns from the recombinant gene [although the splicing efficiency itself is limited (28)] and posttranslational modification of the protein products, such as signal peptide cleavage, phosphorylation, glycosylation and fatty acylation (14,15, 20). A large collection of transfer vectors for optimized expression and possessing purification tags for the easy isolation of the synthesized proteins and improved and user-friendly methods for recombinant virus selection are currently available for the prototype baculovirus *Autographa californica* nucleopolyhedrovirus (AcMNPV)-based expression system (11,24).

The use of insect larvae in place of cultured cell lines as hosts has been a fascinating economic alternative for the large-scale synthesis of commercially important biomolecules (16,22,28). The

recombinant protein production through the larval caterpillar is particularly appealing in the case of *Bombyx mori* nucleopolyhedrovirus (BmNPV)-based expression system, because the host larvae possess a high level of protein synthesizing capacity (6,17,22). Also, the fact that the silkworms can be readily reared in large numbers with minimal sophistication, as compared to cell culture techniques, makes the exploitation of the larval system for expression an attractive proposition. A major difficulty however, in the BmNPV-based expression system has been the relative lack of optimized transfer vectors and the absence of simplified recombinant virus selection procedures. We had partially overcome the former problem by demonstrating that homeologous recombinations can be successfully performed between the AcMNPV polyhedrin (*polh*) or *p10* sequences and the BmNPV genome to generate recombinants, and that these promoters are equally functional in both systems (27). However, the recombinant selection is still cumbersome in BmNPV because one has to resort to the traditional occlusion-negative plaque screening or the isotopically labeled probe screening techniques. Here, we have generated a recombinant BmNPV tagged with the reporter gene *gfp*, which on infection results in the emanation of green fluorescence. The *gfp*-tagged virus, if used as a parent in recombination, provides an easy way for scoring the recombinants by directly screening for the absence of fluorescence. Further, the recombinant BmNPV harboring *gfp* could be exploited to study the path and progression of viral infection in silkworm larvae, a problem of economic significance in the commercial rearing of *B. mori* for silk production.

MATERIALS AND METHODS

Insect Cell Lines and Virus Growth

The silkworm *B. mori* (strain NB-4D2) was routinely reared on mulberry leaves. The *B. mori*-derived BmN cells were grown at 27°C in TC-100 Insect Medium supplemented with 10% fetal calf serum (FCS) (Life Technologies, Gaithersburg, MD, USA) and 50 µg/mL gentamycin (Sigma Chemical, St. Louis, MO, USA). BmNPV-BGL, the local isolate of BmNPV (21), was propagated in BmN cells in culture or through larval infection. The purified virions from cell cultures or the polyhedra-derived virus (isolated from the hemolymph of virus-infected larvae) were used for the generation of recombinant virus.

Recombinant Plasmid and Virus Constructions

The gene encoding green fluorescent protein (*gfp*) (modified cDNA harboring the S65T mutation to enhance the fluorescence) (7,25) was cloned in the baculovirus transfer vector pVL1393 at the *Eco*RI-*Pst*I sites to generate pVL1393-GFP. This construct harboring *gfp* under the control of AcMNPV *polh* promoter was used to generate BmNPV recombinants by homeologous recombination (27).

Generation of the Recombinant Virus, vBmGFP

pVL1393-GFP DNA (5 µg) was transfected into 1×10^6 BmN cells in incomplete TC-100 medium (without serum) using LIPOFECTIN® (Life Technologies) (22). After 8 h, the cells were infected with wild-type (WT) BmNPV [multiplicity of infection (MOI) 10] and transferred to the complete medium. At 48 h post-infection, the cells were pelleted at 2000× *g* and serial dilutions (10^{-4} and 10^{-5}) of the supernatant were used to infect BmN cells in a 96-well plate (2×10^4 cells/well). Four days post-infection, the cells were examined under Model BX60 Fluorescence Microscope (Olympus, Tokyo, Japan). The supernatant from the wells showing a maximum number of fluorescing cells was used to purify the recombinant virus vBmGFP, using two rounds of plaque purification by selecting for occlusion-negative green fluorescence positive phenotypes. When the recombination frequencies are low, screening by the 96-well infection assay can be repeated before plaque purification.

Infection of BmN cells with vBmGFP resulted in the generation of green fluorescence, confirming the authenticity of the recombinant virus. The recombinant vBmGFP was amplified, and a high-titer stock [10^8 plaque-forming units (pfu)/mL] was used for infecting the cells and larvae.

Infection of *B. mori* Larvae

Fifty microliters of vBmGFP (10^8 pfu/mL) were directly injected into hemocoel at the second abdominal spiracle of the silkworm larvae on the first day of fifth instar. The larvae were fed on mulberry leaves for 4–5 days until the signs of infection appeared. The hemolymph was then collected by cutting at one of the legs. The larvae were subsequently

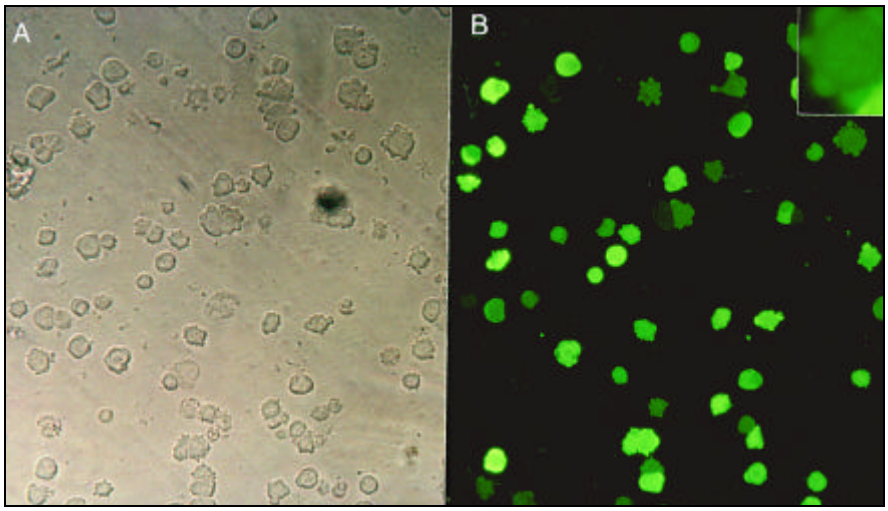

Figure 1. Expression of *gfp* in BmN cells. BmN cells were infected with the recombinant virus, vBmGFP (MOI 10), and the cells were seen at 60 h post-viral infection in (A) bright-field or (B) under fluorescence. A magnified view of the blebbing fluorescent bodies from a virus-infected cell is shown as an inset (B).

dissected out, and the tissues were viewed under fluorescence microscope.

Protein Extraction and Characterization

The fat bodies frozen under liquid nitrogen were crushed to a powder and sonicated 3× for 30 s each in the extraction buffer [0.1% sodium dodecyl sulfate (SDS), 36% urea, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 10 mM Tris-HCl, pH 9.0]. After incubation on ice for 30 min, the homogenate was centrifuged at 10 000× *g* for 10 min at 4°C, and the supernatant was used for protein analysis.

RESULTS AND DISCUSSION

gfp Expression in BmN Cells

A recombinant virus, vBmGFP, was generated using recombination between WT BmNPV and the plasmid transfer vector pVL1393-GFP DNA. Although the AcMNPV-*polh* flanking sequences present in the transfer vector located upstream to the reporter gene *gfp* are distinctly different from the *polh* upstream sequences of BmNPV, homeologous recombination between these sequences was feasible (27). Insertion of *gfp* replaced the *polh*, resulting in occlusion-negative plaques showing significant levels of green fluorescence. The location of *gfp* at the *polh* locus was also confirmed by Southern blots (data not shown). The AcMNPV-*polh* promoter was fully functional in BmNPV (Reference 27; our unpublished observations). The cells infected with recombinant virus showed complete absence of polyhedra under the Model JEM-100 CXII Electron Microscope (JEOL Ltd., Tokyo, Japan). The phenotypic selection of recombinant plaques under the fluorescence microscope based on green fluorescence provided an easy means for identifying the recombinant.

Upon infection with the recombi-

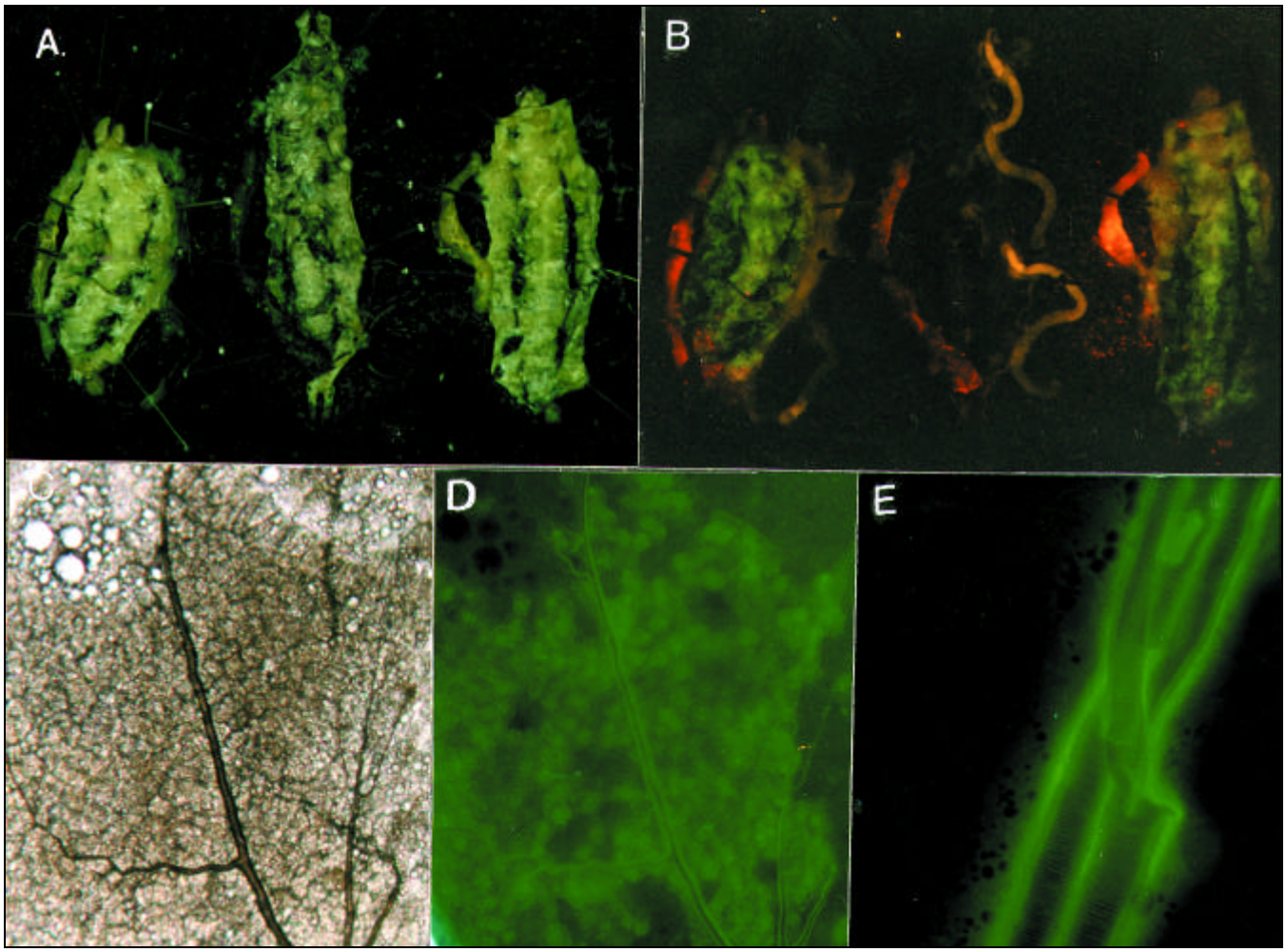

Figure 2. *gfp* expression in *Bombyx* larvae. *B. mori* larvae were infected with vBmGFP (5×10^6 pfu/larva) by direct injection into hemocoel. After 4 days of infection, the larvae appeared fluorescent green under long-range UV. The dissected whole larvae seen under (A) visible and (B) UV lights. The larvae in the middle was infected with the WT BmNPV, and the larvae on both sides were infected with recombinant vBmGFP. The endogenous fluorescence (red or yellow) of certain larval tissues such as the gut or occasionally the silk glands was distinct from the fluorescence due to the expression of *gfp* seen in fat bodies and trachea. The dissected tissues: (C and D) fat body (seen under visible and UV light, respectively) and (E) trachea (seen under UV light from vBmGFP-infected larvae). The corresponding tissues from the uninfected or WT virus-infected larvae did not show any green fluorescence.

nant virus vBmGFP, the BmN cells showed high levels of green fluorescence (Figure 1). Blebbing fluorescent bodies pinching out of the infected cells (Figure 1B, inset), presumably due to apoptotic process, were present in more than 75% of the cells in about 60 h post-infection. Such apoptotic blebbing of BmN cells has been reported previously following infection with a mutant BmNPV harboring mutation in the anti-apoptotic gene *p35*, though not in WT virus infection (12). We do not know the status of the *p35* in the virus strain BmNPV-BGL used in the present study or how much it differs from the virus strain T3 used by those authors.

gfp Expression in Silkworms

The silkworm larvae infected with vBmGFP showed high levels of *gfp* expression. The larvae appeared greenish in 3–4 days following infection, and upon exposure to long range UV, emitted bright green fluorescence.

Viral infection was extensively localized in the fat bodies that showed the highest level of fluorescence (Figure 2, C and D). The trachea also showed significant levels of green fluorescence (Figure 2E), but no fluorescence was detected in the gut, silk glands, malphigian tubules and the cuticle, indicating the absence of viral multiplication in these tissues (Figure 2, A and B). The hemolymph and hemocytes from the infected silkworms also showed green fluorescence. Although GFP is not a secretory protein, it was present in the hemolymph because the insects have an open circulatory system, and the proteins from the fat bodies readily leach out as they are suspended in the hemolymph. Also, in late stages of virus infection, the fat bodies undergo lysis, leading to the release of proteins. The release of proteins due to the lysis of hemocytes also contributes to their presence in the hemolymph.

The gene encoding *gfp* from the jelly fish (the parental and modified versions of the gene) (10,25,30) compliments the other well-exploited reporters such as β-galactosidase (β-gal), alkaline phosphatase (AP), chloramphenicol acetyltransferase (CAT), β-glucoronidase and luciferase (1,3,19,22). Non-invasive, color-based detection, without addition of exogenous substrates or activators, makes *gfp* a reporter of choice. It has also been successfully used in mammalian cells (18), *Drosophila* (31), zebrafish (2), *Caenorhadbditis* (4), plants (8) and mice (9). Here, we have used the modified *gfp* (S65T) as the reporter to overexpress and examine the localization of viral infection in the silkworm. Four days post-infection, the whole larvae emitted green fluorescence because of the presence of large quantities of the expressed recombinant

protein in the tissues, predominantly in the fat bodies. Viral infection, especially due to NPVs, is a major problem of economic concern in the commercial rearing of silkworms for silk production. Therefore, the control and prevention of NPV infections attain great commercial significance, and the availability of a *gfp*-tagged virus facilitates the pathological investigations on BmNPV. Contrary to the long drawn belief that the primary site for viral replication is the gut tissue of the infected larvae, our results clearly establish that the fat bodies were the main targets, and the gut or the other tissues, such as the silk glands or malphigian tubules, appeared to be free of viral infection. However, in the present studies, the virus was directly introduced into the hemolymph, although the same pattern of viral multiplication was seen even after oral administration of the virions as polyhedral particles, as in the natural infection process (our unpublished results).

The GFP present in the fat bodies of vBmGFP-infected larvae was readily solubilized. The levels of expression being very high, the protein was directly visible as fluorescent bands on electrophoretic gels without need for UV or protein staining. The functional expression of *gfp* has been previously reported in cultured *Sf*21 cells (26) and in the larvae of the Diamondsack moth, an insect pest (5).

Use of vBmGFP for the Selection of Other Recombinants

vBmGFP could be used as the parent to select recombinants carrying the genes of choice by scoring for the loss of fluorescence consequent to the generation of recombinants. In the early rounds of plaque screening, a mixed population of parental type (fluorescent plaques) and recombinant (nonfluorescent plaques) is generated. By repeating two rounds of plaque selection, a single purified recombinant virus could be readily isolated. The selection of recombinants using the vBmGFP as parent provided a convenient and more sensitive method for the BmNPV system, which does not have the large collection of commercial optimized vectors or easy recombinant se-

lection methodologies (11,23) as are available for the prototype baculovirus AcMNPV. This approach avoids the long conventional end-point dilution method of repeated screening for the selection of recombinant virus and is quick, nonradioactive and eliminates the need for mutagenized BmNPV genome for selecting the recombinant virus. A somewhat similar approach that uses *gfp* as a positive selection marker instead of β-gal for recombinant selection in AcMNPV, has been reported recently (29).

REFERENCES

1. **Alam, J. and J.L. Cook.** 1990. Reporter genes: applications to the study of mammalian gene transcription. Anal. Biochem. *188*:245-254.
2. **Amsterdam, A., S. Liu and N. Hopkins.** 1995. The *Aequorea victoria* green fluorescent protein can be used as reporter in live zebrafish embryos. Dev. Biol. *171*:123-129.
3. **Bronstein, I., J. Fortin, P.E. Stanley, G.S.A.B. Steward and K. Li.** 1994. Chemiluminiscent and bioluminiscent reporter gene assays. Anal. Biochem. *219*:169-181.
4. **Chalfie, M., Y. Tu, G. Euskirchen, W.W. Ward and D.C. Prasher.** 1994. Green Fluorescent Protein as a marker for gene expression. Science *263*:802-805.
5. **Chao, Y., S. Chen and C. Li.** 1996. Pest control by fluorescence. Nature *380*:396-397.
6. **Gopinathan, K.P.** 1992. Biotechnology in sericulture. Curr. Sci. *62*:283-287.
7. **Heim, R. and R.Y. Tsien.** 1996. Engineering green fluorescent protein for improved brightness, longer wavelengths and fluorescence resonance energy transfer. Curr. Biol. *6*:178-182.
8. **Heinlein, M., B.L. Epel, H.S. Padgett and R.N. Beachy.** 1995. Interaction of tobamovirus movement proteins with the plant cytoskeleton. Science *270*:1983-1985.
9. **Ikawa, M., K. Kominami, Y. Yoshimura, K. Tanaka, Y. Nishimune and M. Okabe.** 1995. A rapid and non-invasive selection of transgenic embryos before implantation using green fluorescent protein. FEBS Lett. *375*:125-128.
10. **Inouye, S. and F.I. Tsuji.** 1994. *Aequorea* green fluorescent protein: expression of the gene and fluorescence characteristics of recombinant protein. FEBS Lett. *341*:227-280.
11. **Jones, I. and Y. Morikawa.** 1996. Baculovirus vectors for expression in insect cells. Curr. Opin. Biotechnol. *7*:512-516.
12. **Kamita, S.G., K. Majima and S. Maeda.** 1993. Identification and characterization of the p35 gene of *Bombyx mori* nuclear polyhedrosis virus that prevents virus-induced apoptosis. J. Virol. *67*:455-463.
13. **King, L.A. and R.D. Possee.** 1992. The Baculovirus Expression System. Chapman & Hall, London.
14. **Luckow, V.A.** 1991. Cloning and expression of heterologous genes in insect cells with baculovirus vectors, p. 97-152. *In* A. Prokop, R.K. Bajpai and C. Ho (Eds.), Recombinant DNA Technology and Applications. McGraw-Hill, New York.
15. **Luckow, V.A.** 1996. Insect cell expression technology, p. 183-218. *In* J.L. Cleland and C.S. Craik (Eds.), Protein Engineering: Principles and Practice. Wiley-Liss, New York.
16. **Maeda, S.** 1989. Expression of foreign genes in insects using baculovirus vectors. Annu. Rev. Entomol. *34*:351-372.
17. **Maeda, S., T. Kawai, M. Obinata, H. Fujiwara, T. Horiuchi, Y. Saeki, Y. Sato and M. Furusawa.** 1985. Production of human alpha interferon in silkworm using baculovirus vector. Nature *315*:592-594.
18. **Marshall, J., R. Molloy, G.W. Moss, J.R. Hoine and T.E. Hughes.** 1995. The jellyfish green fluorescent protein: a new tool for studying ion channel expression and function. Neuron *14*:211-215.
19. **Martin, C.S., P.A. Wight, A. Dobretsova and I. Bronstein.** 1996. Dual luminiscence-based reporter gene assay for luciferase and β-galactosidase. BioTechniques *21*:520-524.
20. **Miller, L.K.** 1988. Baculoviruses as gene expression vectors. Annu. Rev. Microbiol. *42*:177-199.
21. **Palhan, V.B. and K.P. Gopinathan.** 1996. Characterization of a local isolate of *Bombyx mori* nuclear polyhedrosis virus. Curr. Sci. *70*:147-153.
22. **Palhan, V.B., S. Sumathy and K.P. Gopinathan.** 1995. Baculovirus mediated high-level expression of luciferase in silkworm cells and larvae. BioTechniques *19*:97-104.
23. **Phillips, G.N., Jr.** 1997. Structure and dynamics of green fluorescent protein. Curr. Opin. Struct. Biol. *7*:821-827.
24. **Possee, R.D.** 1997. Baculoviruses as expression vectors. Curr. Opin. Biotechnol. *8*:569-572.
25. **Prasher, D.C., V.K. Eckenrode, W.W. Ward, F.G. Prendergast and M.J. Cormier.** 1992. Primary structure of the *Aequorea victoria* green fluorescent protein. Gene *111*:229-233.
26. **Reilander, H., W. Haase and G. Maul.** 1996. Functional expression of the *Aequorea victoria* green fluorescent protein in insect cells using the baculovirus expression system. Biochem. Biophys. Res. Commun. *219*:14-20.
27. **Sriram, S., V.B. Palhan and K.P. Gopinathan.** 1997. Heterologous promoter recognition leading to high-level expression of cloned foreign genes in *Bombyx mori* cell lines and larvae. Gene *161*:181-189.
28. **Sumathy, S., V.B. Palhan and K.P. Gopinathan.** 1996. Expression of human growth hormone in silkworm larvae through recombinant *Bombyx mori* nuclear polyhedrosis virus. Protein Expr. Purif. *7*:262-268.
29. **Wilson, L.E., N. Wilkinson, S.A. Marlow, R.D. Possee and L.A. King.** 1997. Identification of recombinant baculoviruses using green fluorescent protein as a selectable marker. BioTechniques *22*:674-681.
30. **Wood, K.V.** 1995. Marker proteins for gene expression. Curr. Opin. Biotechnol. *6*:50-58.
31. **Yeh, E., K. Gustafson and G.L. Boulianne.** 1995. Green fluorescent protein as a vital marker and reporter of gene expression in Drosophila. Proc. Natl. Acad. Sci. USA *92*:7036-7040.

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