Fundam. appl. Nematol., 1997, **20** (4), 323-327

GFP: green fluorescent protein a versatile gene marker for entomopathogenic nematodes

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Accepted for publication 19 July 1996.

Summary - Gene expression and protein distribution within cells may be monitored using reporters, such as, β-galactosidase, firefly luciferase, and bacterial luciferase. These reporter systems require exogenously added substrates and cofactors which kill the cells or organism. The jellyfish (*Aequorea victoria*) green fluorescent protein (*gfp*) gene has been used as a marker for gene expression in *Caenorhabditis elegans*. Because exogenous substrates or cofactors are not required for the fluorescence, *gfp* can be used to monitor gene expression in living organisms. Natural autofluorescence in nematodes however, can, hinder the use of such markers. We studied autofluorescence in entomopathogenic nematodes to explore the use of *gfp* as a marker for gene expression. All stages of *Heterorhabditis* and *Steinernema* spp. autofluorescent ranged from green to light yellow, the intensity of autofluorescence increased with the age of nematodes. We used *gfp* that was under the control of *mec-4* promoter from *C. elegans* and injected the plasmid (pGFP/mec-4) into the gonad of *Heterorhabditis bacteriophora* using microinjection. *Gfp* was expressed in *H. bacteriophora* and was easily identified in the tail region of the nematode. Expression of *mec-4* indicates the presence of touch receptors neurons in *H. bacteriophora* at the same position as in *C. elegans*. We conclude that *gfp* is an efficient marker for gene expression in entomopathogenic nematodes.

Résumé - ***GFP, protéine à fluorescence verte, un marqueur de gènes commode pour les nématodes entomopathogènes*** - L'expression des gènes et la répartition des protéines dans les cellules peuvent être révélées à l'aide de gènes rapporteurs, tels que la β-galactosidase, la luciférase du vert luisant et la luciférase bactérienne. Ces systèmes rapporteurs nécessitent des substrats et des cofacteurs exogènes qui tuent les cellules ou les organismes. Le gène de la protéine fluorescente verte (*gfp*) de la méduse *Aequoria victoria* est utilisé comme marqueur de l'expression de gènes chez *Caenorhabditis elegans*. Les substrats ou cofacteurs exogènes n'étant pas nécessaires, la *gfp* peut servir à suivre l'expression de gènes dans les organismes vivants. Cependant l'autofluorescence naturelle des nématodes peut gêner l'utilisation de ces marqueurs. Nous avons examiné l'autofluorescence chez les nématodes entomophages pour établir l'utilité de la *gfp* comme marqueur de l'expression de gènes. Des espèces d'*Heterorhabditis* et de *Steinernema* de tous stades montraient une autofluorescence allant du vert au jaune pâle, son intensité s'accroissant avec l'âge des nématodes. La *gfp* utilisée a été sous le contrôle du promoteur *mec-4* de *C. elegans*. Le plasmide (pGFP/mec-4) a été injecté par microinjection dans la gonade de *Heterorhabditis bacteriophora*. La *gfp* a été exprimée dans *H. bacteriophora* et a pu être facilement repérée dans la région caudale du nématode. L'expression par *mec-4* indique la présence de neurones récepteurs tactiles chez *H. bacteriophora* au même endroit que chez *C. elegans*. Nous concluons que la *gfp* est un marqueur efficace de l'expression de gènes chez les nématodes entomopathogènes.

Key-words: autofluorescence, green fluorescent protein, gene expression, nematodes, transformation.

Significant attention in recent years has focused on biological control of insect pests using entomopathogenic nematodes of the genera *Steinernema* and *Heterorhabditis*. This interest reflects their wide spectrum of insecticidal activity, ability to kill most hosts within 48 h, efficient mass rearing, and an exemption from government registration in most countries (Gaugler & Kaya, 1990). Nevertheless, these insecticidal nematodes are perceived as viable alternatives to chemicals in only a few niche markets (*e.g.*, cranberries, turf, etc.).

Nematode sensitivity to environmental extremes including temperature, solar radiation, and desiccation are important factors restricting efforts to extend their use (Gaugler & Bousch, 1979*a, b*; Molyneux, 1985; Klein, 1990; Georgis & Gaugler, 1991). One potential solution is to develop genetically engineered strains with increased tolerance to environmental stress. Our goal is to develop methods resulting in enhanced nematode biocontrol potential through incorporation of genes expressing useful traits. Moreover, genetic transformation techniques will be useful to investigate aspects of the molecular biology of entomopathogenic nematodes. Protocols for the successful DNA transformation of *Caenorhabditis elegans* have been developed (Fire, 1986; Mello *et al.*, 1991), and

these protocols have been adapted for use with the entomopathogenic nematode, *Heterorhabditis bacteriophora* HP88 (Hashmi *et al.*, 1995*a*).

In gene transfer studies, markers are needed to monitor gene expression and protein distribution within cells. The *Escherichia coli lacZ* encoded enzyme β-galactosidase (β-Gal) has been widely used as a reporter molecule in a variety of prokaryotic and eukaryotic systems (Sihavy & Beckwith, 1985). β-galactosidase and other marker genes such as, β-glucorinidase, firefly luciferase (*luc*) (Ow *et al.*, 1987) and bacterial luciferase (*lux*) (Koncz *et al.*, 1987) require a lengthy staining procedure which kill the cell or organism (Sihavy & Beckwith, 1985).

Chalfie *et al.* (1994) used green fluorescent protein gene (*gfp*) as a marker for gene expression in *C. elegans*. The jellyfish, *Aequorea victoria gfp*, a protein of 238 amino acids (Prasher *et al.*, 1992) absorbs blue light at l_{max} = 395 nm and emits green light at l_{max} = 509 nm (Morise *et al.*, 1974; Ward *et al.*, 1980). A complementary DNA for *A. victoria gfp* produces a fluorescent product when expressed in prokaryotes (*E. coli*) and eukaryotes (*C. elegans*) and is capable of producing a strong green fluorescence when excited by blue light (Chalfie *et al.*, 1994). A characteristic feature of *gfp* is that it does not require other proteins or cofactors for the green fluorescence, so genes can be expressed and monitored in virtually any living cell as a fully fluorescent product.

Because nematodes accumulate the fluorescent compound lipofuscin (Davis *et al.*, 1982), they are autofluorescent. Use of *gfp* as a reporter for gene expression requires the ability to distinguish *gfp* fluorescent from the target nematode's autofluorescence. Clokey and Jacobson (1986) identified autofluorescent globules in the intestinal cells of *C. elegans*. We examined whether nematode autofluorescence would interfere with the observation of *gfp* expression, thereby precluding our use of this marker. We used *gfp* that was under the control of *mec-4* promoter from *C. elegans*. *Mec-4* encodes a protein express exclusively in the touch receptor neurons. We selected *mec-4* because of its site of expression in the tail region where no autofluorescence occurred in the nematode. Our objective was to achieve effective expression of *gfp* in *H. bacteriophora* so that it can be used as gene marker to accelerate genetic engineering effort with entomopathogenic nematodes.

Materials and methods

Nematodes were grown in the dark at 25°C on lipid agar media pre-seeded with their respective symbiotic bacteria (*Photorhabdus luminescens* for *H. bacteriophora*, *Xenorhabdus* sp. for *S. riobrave*, and *X. nematophilus* for *S. carpocapsae*). All stages of nematodes were collected from lipid agar plate in sterile M9 buffer (KH_2PO_4; Na_2HPO_4; NaCl; 1M $MgSO_4$; H_2O) and washed three times. Nematodes were fixed in a glutaraldehyde fixative (M9 buffer, 1% glutaraldehyde [v/v], 0.5% [v/v] glycerol), and washed twice.

DNA TRANSFORMATION

Because of our previous success with genetic transformation of *H. bacteriophora* with *C. elegans* genes (Hashmi *et al.*, 1995*a, b*) we next wished to use a *mec-4* promoter from *C. elegans* with *gfp*. *Mec-4* expresses in the touch receptor neurons in the tail region of *C. elegans*, and we expected it would express similarly in *H. bacteriophora*. Plasmid DNA (pGFP/mec-4) was provided by Dr. Monica Driscoll (Rutgers University); pGFP/mec-4 contained the *A. victoria gfp* under the control of the *C. elegans mec-4* promoter. Plasmid DNA was purified for injection using the miniprep system (Zhou *et al.*, 1990); DNA concentration was determined spectrophotometrically. DNA for injection was prepared in 1X TE buffer (10mM Tris, pH 7.5, and 1 mM EDTA). This solution flowed effectively through injection needles.

We used the microinjection procedures described by Fire (1986) and Mello *et al.* (1991) to generate a transgenic *H. bacteriophora* HP88 line carrying a *mec-4/gfp* fusion. Microinjection needles were pulled on a Narishige PN-3 horizontal micropipette puller (Narishige, NY, USA). Tip size was 1 μm diameter. The needle was filled with DNA, and held in a needle holder that was connected to a micromanipulator (Narishige Model # MN-151). The micromanipulator was loaded into microINJECTOR™ (Tritech Research Inc., CA, USA) that served to attach the injection needle to a pressure source using silicon tubing. A pressure regulator and a valve on the nitrogen tank allowed pressure to be maintained at 30 PSI during the injection process. Agarose pads for immobilizing nematodes were prepared as described by Fire (1986). Prior to injection, the agarose pad was spread with halocarbon oil (series 700, Halocarbon Products Corp., Augusta, SC, USA). Adult hermaphrodite nematodes containing four to six eggs were transferred to a partially desiccated agar plate without bacteria to remove excess water so that when they were transferred to an oil-covered agarose pad, they stuck well to the pad.

Nematodes (usually five at once) on the agarose pad were transferred to the inverted microscope for microinjection and observed at 400 × mag. The injection needle filled with 150 μg/ml of DNA was aligned and inserted into the nematode gonad (Mello *et al.*, 1991). After injection, a drop of M9 buffer was placed onto the nematode, and the nematode was transferred to a fresh agar plate seeded with *P. luminescens*. Injected nematodes were incubated in a group of five nematodes per plate with a single transfer of three to five

nematodes to a fresh bacterial plate after 24 h. Plates were incubated at 25 °C until injected nematodes produced progeny. Several uninjected nematodes of the same age group were transferred to fresh agar plates to serve as controls. Both live and fixed preparations of the F1 generation of the injected worms were examined under fluorescent microscope. We selected a long pass emission filter to study the expression of *mec-4/gfp* in *H. bacteriophora* HP88.

AUTOFLUORESCENCE

We examined a thousand nematodes of each species and isolate. Nematodes were mounted on slides and examined with 20 × objectives under a microscope equipped with epifluorescent illumination. Both live and fixed preparations of *H. bacteriophora*, *S. carpocapsae*, and *S. riobrave*, were examined. The light source and the filter system consisted of a 50-watt

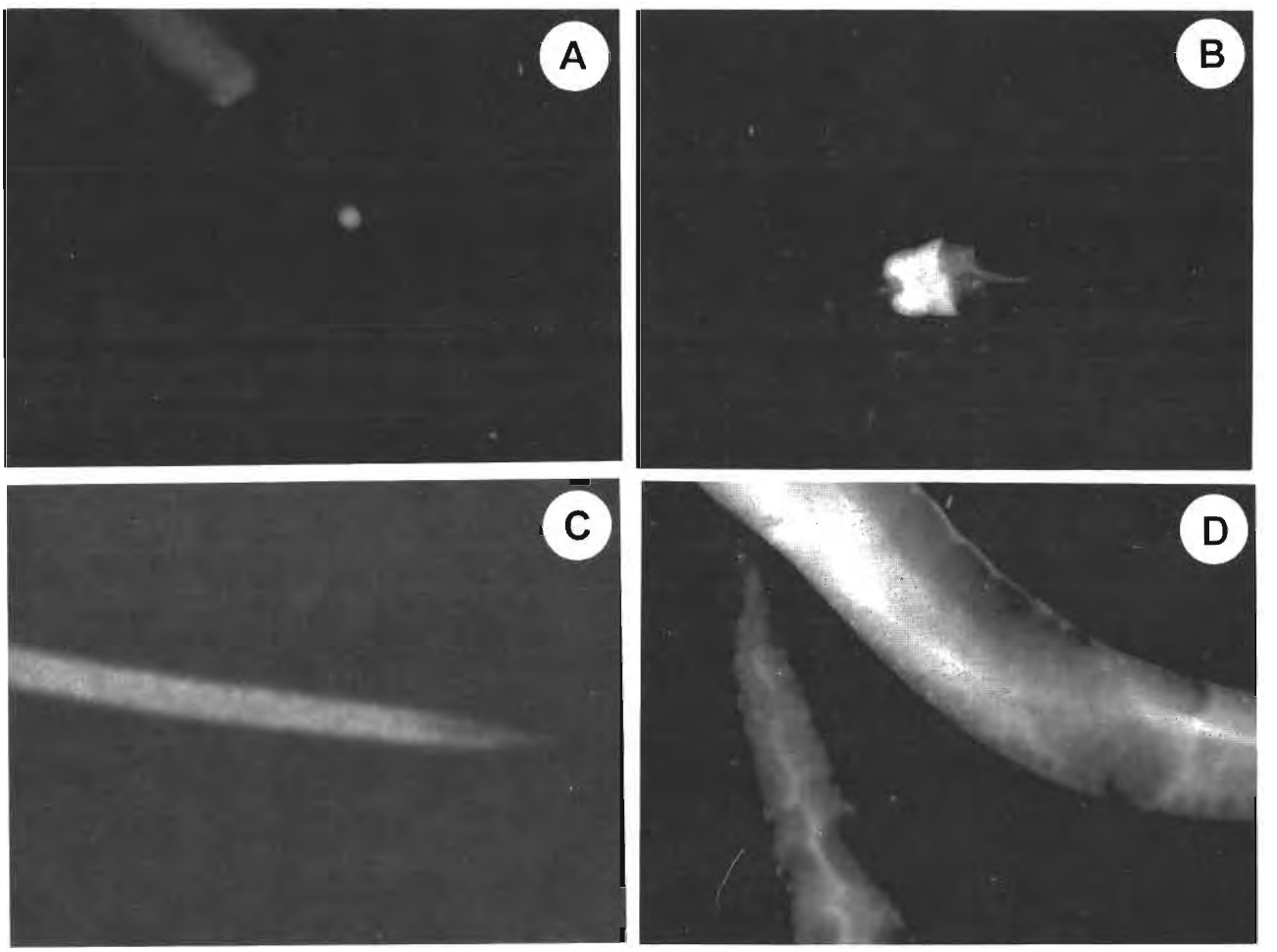

Fig. 1. *A wild type strain of* Heterorhabditis bacteriophora *was transformed with plasmid pPGFP/mec-4 as described in materials and methods and the progeny obtained from the injected animals were prepared for fluorescence microscopy. A similar preparation was made of untransformed wild type nematodes. A: Expression of* gfp *in young adult* H. bacteriophora. *Touch receptor neuron labeled in the unbranched fluorescing cell bodies in the tail region of nematode; B: Green autofluorescence in a wild-type young adult* H. bacteriophora. *Fluorescence are dispersed throughout the nematode's body but are absent in the tail region of nematode. (Photographs were taken with a 20 × objective). Autofluorescence in entomopathogenic nematodes. Wild type strain of* Steinernema *spp. were prepared for fluorescent microscopy as described in materials and methods and more than a thousand nematodes from each species were observed under fluorescent microscope at 20 ×. C: Green autofluorescence dispersed throughout the nematode's body except in the tail region of nematode in a wild type* S. carpocapsae*; D: Yellowish green autofluorescence dispersed throughout the nematode's body except in the tail region of nematode in a wild type* S. riobrave.

mercury vapor bulb, long-pass emission filter (range 460-485 nm) and FITC chromatic splitter.

Results

DNA TRANSFORMATION

Transformation of *H. bacteriophora* produced a readily detected green fluorescence that was not observed in wild type nematodes (Fig. 1 A, B). Because of the color and high intensity signal of *gfp* fluorescent it was easily distinguished from nematode's autofluorescent in the transformed nematodes. Since the expression of *gfp* was under the control of a promoter for the *mec-4* gene, the fluorescent cell bodies were identified as touch receptor cells present in the tail region of young adult *H. bacteriophora*. Fluorescence was not detected in second- or third-stage juveniles, probably because the process from these cells were more difficult to detect at these stages in *H. bacteriophora*. The strongest fluorescence was seen in the unbranched cell bodies of the touch receptor cells in 2% of the young adult progeny of the injected nematode. Autofluorescence was not detected in the tail region of several thousand uninjected control or untransformed nematodes examined. Regardless of how samples were prepared for microscopic examination, the fixative did not affect *gfp* fluorescent in *H. bacteriophora*; both live and fixed preparations exhibited similar patterns of *gfp* expression.

AUTOFLUORESCENCE

All stages of *H. bacteriophora* and *Steinernema* spp. exhibited autofluorescence in the form of globules in the intestinal and esophageal regions of live nematodes and dispersed throughout the nematode's body in fixed preparations (Fig. 1 C, D). Autofluorescence was not detected in the tail region of either live or fixed preparations of *H. bacteriophora* or *Steinernema* species. The color, intensity, and quality of nematode's autofluorescence differed slightly in color from green to light yellow between two genera as well as between species within a genus. Autofluorescent globules were faint in all juvenile stages and their intensity increased with nematode age.

Discussion

At long-pass emission filter, the color and persistence of fluorescent *gfp* in *H. bacteriophora* match those described in *C. elegans* (Chalfie *et al.*, 1994). Our selection of *mec-4* was a good choice to study *gfp* expression in *H. bacteriophora* because of its site of expression where no autofluorescent occurred in the nematode. *Mec-4* has been proposed to be a subunit of a mechanosensory ion channel that plays a key role in touch transduction and is likely to be cell specific (Chalfie *et al.*, 1988). A key feature of *mec-4* expression in *H. bacteriophora* is that touch receptor neurons in these nematodes might have a morphology and position similar to that of *C. elegans*.

We used a DNA concentration of 150 µg/ml for injection and recorded 2% transformed progeny at this concentration. The frequency of transformation was lower than our earlier reports of 6-8% using the other *C. elegans* genes (Hashmi *et al.*, 1995*a*, *b*). Mello *et al.* (1991) showed a four fold increase in percentage heritable transformation in *C. elegans* as the DNA concentration was doubled from 50 to 100 µg/ml. We found that at a concentration of 100 µg/ml or above the frequency of transformation remained the same (Hashmi *et al.*, 1995*a*). Because the expression of *mec-4* occurs in the touch cells in the tail region of nematode, it is often difficult to locate the transformants with the *mec-4* gene at least in *H. bacteriophora*. In future studies a promoter with a broader spectrum of expression should be used with *gfp* so that the transformants can be selected easily. For instance, a highly conserved 70 kDa heat-shock protein gene (*hsp-70*) expresses in all cell types and a *hsp-70* gene from *C. elegans* has been transformed successfully into *H. bacteriophora* (Gaugler & Hashmi, 1996). So, using *hsp-70* gene with *gfp* should show a long-range of expression in *H. bacteriophora*. Such attempts are in progress in our laboratory.

Like other nematode genera and species, entomopathogenic nematodes are autofluorescent and the pattern of autofluorescence in fixed differs from that of live worms: in the form of globules in the live worms and dispersed throughout the body in the fixed or dead worms. It is likely that in the fixed preparation the breakdown of lysosomal membranes occurs which allows the lipofuscin to disperse throughout the body. Autofluorescence is faint in the juvenile stages of all nematodes. Accumulation of a fluorescent compound, lipofuscin, is known to occur in many cell types throughout the metazoa (Davis *et al.*, 1982; Clokey & Jacobson, 1986) and therefore, the organism are autofluorescent. The accumulation of lipofuscin is also related to age (Davis *et al.*, 1982; Clokey & Jacobson, 1986), which may explain why the juvenile stages do not autofluoresce as intensely as older stages of the same species. Furthermore, the absence of autofluorescence in the tail region of *H. bacteriophora*, *S. carpocapsae* and *S. riobrave* were simply maybe due to absence of lipofuscin in the tail region of nematode.

The central problem dealing with *gfp* as a reporter is nematode's autofluorescence. Therefore, attention must be given while preparing and observing the nematode samples for *gfp* expression under fluorescent microscope. We used a long-pass emission filter. The maximum excitation wavelength for this filter set was 475 nm (range 460-485 nm). With this filter the

nematodes' green autofluorescence is less intense and does not interfere with the *gfp* expression. Also, the thickness of the older adult nematodes may obscure the *gfp* fluorescence in cells. Therefore, infective juveniles, young adult and early fourth stage nematodes should be used for observation of *gfp* expression.

The successful introduction of *gfp* in entomopathogenic nematodes provides a powerful genetic tool with multiple implications. *Gfp* will allow us to isolate pure lines of live transformed nematodes. This marker will also be useful to create recognizable populations of nematodes (nematode tagging) for diverse experiments including competition assays for survival, infectivity, and reproduction. Until now, there has been no method for entomopathogenic nematode tagging. *Gfp* does not appear to interfere with cell growth and function. Therefore, *gfp* can be used as an indication of transformation and would provide unique opportunities to assess the functions of particular gene products in cells.

Acknowledgments

We thank Dr. Monica Driscoll for providing microinjection facility, Dr. Ghazala Hashmi for discussion and helpful suggestions during this research, Dr. Robin Stuart for comments on the manuscript, and Ms. Anna Monroy for technical assistance. This work was supported by State funds, U. S. Hatch Act, and U. S. Department of Agriculture National Agricultural Research Project (No. 58-319R-3-001). This is New Jersey Agricultural Research Station Publication No. D-08255-18-95.

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