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Transformation of the Caribbean fruit fly, *Anastrepha suspensa*, with a *piggyBac* vector marked with polyubiquitin-regulated GFP

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Abstract

Germ-line transformation was achieved in the Caribbean fruit fly, *Anastrepha suspensa*, using a *piggyBac* vector marked with an enhanced green fluorescent protein gene regulated by the *Drosophila melanogaster* polyubiquitin promoter. Four transgenic G₀ lines were selected exhibiting unambiguous GFP expression. Southern hybridization indicated the presence of one to four integrations in each of the transgenic lines with two integrations verified as *piggyBac*-mediated by sequencing their insertion sites. Fluorescence was detectable throughout development, and in adults was most intense from the thoracic flight muscle. Although adult cuticle quenched fluorescence, GFP was routinely detectable in the thorax. A quantitative spectrofluorometric assay was developed for GFP fluorescence that indicated differing levels of fluorescence among the transgenic lines, suggesting some level of position effect variegation/suppression. These results are encouraging for the use of this marker system in insect species not amenable to mutation-based visible markers. Together with the *piggyBac* vector, a transformation system is presented that has the potential to be universally applicable in insect species. Published by Elsevier Science Ltd.

Keywords: *piggyBac* vector; Germ-line transformation; Green fluorescent protein; Position effect suppression; *Anastrepha suspensa*; Tephritidae

1. Introduction

Several recent studies have reported germ-line transformation of nondrosophilid insect species including the Mediterranean fruit fly, *Ceratitis capitata* (Loukeris et al., 1995; Handler et al., 1998) and the yellow fever mosquito, *Aedes aegypti* (Jasinskiene et al., 1998; Coates et al., 1998). Similar to typical transformation of *Drosophila melanogaster*, these transformations utilized marker genes that allowed transformant selection by their complementation of a visible mutation in the host strain, also known as mutant-rescue selection. In all cases the marker included the wild type allele for a eye color mutation in the host, that was inserted within the vector. The mosquito host strain was the *kynurenine hydroxylase*-white mutant (Cornel et al., 1997) that was complemented by the wild type *cinnabar* gene from *D.*

melanogaster, and the medfly host was a *white eye* strain that was complemented by the medfly wild type *white* cDNA (Zwiebel et al., 1995).

Transformation of these particular species obviously benefitted from the existence of eye color mutations and the availability of cloned wild type genes for the mutation. However, most insect systems have neither of these, and thus transformant selection is more challenging. In an effort to develop new markers that are generally useful for a wide array of species, several groups have begun testing green fluorescent protein (GFP) markers (Prasher et al., 1992; Chalfie et al., 1994) for transformant selection in *D. melanogaster* and nondrosophilid species. We tested the *piggyBac* vector marked with an enhanced GFP regulated by a polyubiquitin promoter (PUbnlsEGFP) in *D. melanogaster* (Handler and Harrell, 1999). GFP has also been tested recently with regulation by an artificial rhodopsin gene promoter (3×P3) in *D. melanogaster* and *Tribolium castaneum* (Berghammer et al., 1999), a *Drosophila* actin 5C promoter in *Aedes aegypti* (Pinkerton et al., 2000), and a *Bombyx mori* actin A3 promoter in the same species (Tamura et al., 2000).

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The *piggyBac*-marker construct tested in *Drosophila* had an enhanced GFP gene (EGFP-1; Clontech) regulated by the *Drosophila* polyubiquitin promoter (Lee et al., 1988) that was linked in-frame to the nuclear localizing sequences (NLS) of the SV40 large T-antigen (Lanford et al., 1986), and was additionally marked with the *Drosophila* mini-*white* marker. Polyubiquitin is a highly conserved gene found in most organisms and is active in all cells, and the NLS polypeptide is recognized by nuclear envelope receptors thought to exist in all eukaryotes, which facilitate its transport into the nucleus. Consistent with earlier versions of this construct (Davis et al., 1995) GFP expression occurred in all cells and nuclei of transgenic *Drosophila* throughout development. While GFP constructs such as this and others are routinely used as reporters for gene expression in *Drosophila*, use of GFP as a primary marker for transgenic selection is not typical. Notably, we found its expression to be significantly more efficient than the *white* marker to which it was linked. Less than half of the G₁ transformants selected by GFP expressed detectable eye pigmentation, and we theorized that this must have resulted from more pronounced position effect suppression of *white*⁺ than the polyubiquitin-regulated GFP. Since GFP has the potential for expression in all organisms, this result suggested that it would be highly effective for transformant selection in many other insect species as well, though it was unknown whether the *Drosophila* polyubiquitin promoter would be similarly functional.

To test the ability of the PUBnlsEGFP marker to allow transformant selection in a nondrosophilid species, as well as to test the *piggyBac* vector in another dipteran species, we used *piggyBac* marked solely with PUBnlsEGFP for germ-line transformation in the Caribbean fruit fly, *Anastrepha suspensa*. The caribfly is an appropriate test species for the marker since it is not amenable to any other mutation-based visible marking system. GFP also has considerable potential as a genetic marker for the field detection of released flies, especially for control programs such as the sterile insect technique for which GFP-marked caribflies could serve as a model. Since *piggyBac* had already successfully transformed the medfly and *D. melanogaster*, with efficient gene transfer in the latter species using an *hsp70*-regulated helper transposase, we were optimistic that the vector would be similarly effective in other tephritid flies.

2. Materials and methods

2.1. Insect strains and rearing

A wild *A. suspensa* strain was collected in south Florida and maintained under laboratory conditions for 2 years. Wild host strain and transformant larvae were maintained on a wheat germ–yeast–glucose diet at 27°C

with wandering larvae placed on moist vermiculite for pupation until adult emergence. Adults were maintained on a yeast–sucrose diet at 25–27°C.

2.2. Plasmids

The *piggyBac* helper plasmid, phspBac (originally phsp-pBac), having the transposase gene under *hsp70* regulation, as well as the PUBnlsEGFP construct were described previously (Handler and Harrell, 1999). Briefly, PUBnlsEGFP includes an enhanced GFP gene from EGFP-1 (Clontech; Cormack et al., 1996; Yang et al., 1996) linked in-frame to the nuclear localizing sequence of the SV40 T-antigen (Lanford et al., 1986), and placed under regulation by the *D. melanogaster* polyubiquitin promoter (Lee et al., 1988). The pB[PUBnlsEGFP] vector was created by ligating the PUBnlsEGFP cassette as a 4.1 kb *Bgl*II–*Sma*I fragment into the *Bgl*II–*Hpa*I site of *piggyBac* within its original host plasmid, p3E1.2 (Cary et al., 1989). Expression of GFP from a similar vector (but with an additional *white*⁺ marker) was shown previously in *D. melanogaster* transgenic flies (Handler and Harrell, 1999).

2.3. Injections

Embryo injections used procedures developed for the Mediterranean fruit fly (Handler et al., 1998), that were modified from standard *Drosophila* procedures (Rubin and Spradling, 1982). Eggs were dechorionated in 1.6% hypochlorite solution followed by several washes in 0.02% Triton-X 100. Eggs were placed on double-stick tape, desiccated in room-air and injected under Halocarbon 700 oil. DNA mixtures had vector:helper concentrations of 600:400 µg/ml in injection buffer (5 mM KCl; 0.1 sodium phosphate pH 6.8). Injected eggs were placed in an oxygenated and humidified tissue culture chamber at 22–23°C and heat shocked at 37°C for 1 h at no less than 5 h after injection, and in some cases overnight for injections performed late in the day. Eclosed G₀ adults were intermated in groups of three males to five females. G₁ eggs were collected for two weeks and reared under standard conditions.

2.4. Southern hybridization

Five to ten micrograms of genomic DNA was digested with indicated restriction enzymes and separated on 0.8% agarose gels. DNA was stained with ethidium bromide, blotted to nylon filters and immobilized by ultraviolet irradiation. Hybridization probes were generated from indicated *piggyBac* restriction fragments that were isolated from p3E1.2, or the entire EGFP gene from pEGFP-1 (Clontech), by agarose gel-elution. Probe DNA was radiolabeled with [³²P]dCTP by random priming (Gibco BRL) according to the manufacturer's speci-

fications. Hybridizations were performed in phosphate buffer pH 7.5; 1% BSA; 7% SDS at 65°C with an initial wash in 2× SSC; 0.2% SDS at room temperature and two washes in 1× SSC; 0.1% SDS at 55°C for 30 min. Autoradiography was performed by exposure on Kodak X-Omat film at −70°C.

2.5. Inverse PCR

Inverse PCR was performed by initial digestion of 1–3 µg of transformant genomic DNA with *SpeI* which does not cut within the vector. After 4 h digestions, restriction fragments were purified by phenol–chloroform extraction and ethanol precipitation and circularized by ligation at 12°C for 16 h. PCR was performed on the circularized fragments using primer sequences proximal to the *piggyBac* vector termini and in opposite orientation. For the 5′ terminus the reverse primer (159R) 5′-ATCAGTGACACTTACCGCATTGACA-3′ was used, and for the 3′ terminus the forward primer (2388F) 5′-CCTCGATATACAGACCGATAAAAACACATG-3′ was used. PCR was performed using the Expand Long Template PCR System (Boehringer–Mannheim) using cycling conditions of initial denaturation at 94°C for 2 min, and 10 cycles of 15 s denaturation at 94°C, 30 s annealing at 63°C, and 8 min elongation at 68°C, with an additional 20 cycles having elongation times extended for an additional 20 s at each cycle with a final elongation for 8 min. PCR products were analyzed by agarose gel electrophoresis and directly subcloned into the p-GEM T-Easy vector (Promega). The vector termini and adjacent insertion sites were sequenced from the subclones using M13 forward and reverse primers. Sequence analysis was performed using GeneWorks 2.5 software (Oxford Molecular Group) and BLASTN analysis (Altschul et al., 1997).

2.6. GFP analysis

GFP was observed at all developmental stages under a Leica MZ-12 stereozoom microscope using a mercury lamp and a FITC longpass wavelength filter set (Kramer). Digital photographic documentation used a SPOT-1 cooled CCD camera (Diagnostic Instruments) with digital images processed with Adobe Photoshop 4.0 software (Adobe Systems).

Spectrofluorometric analysis of GFP fluorescence was performed by collecting 50–60 mg of decapitated flies (to remove eye pigments that cause quenching) and either freezing them at −70°C or homogenizing them immediately. Tissue was homogenized in 500 µl fluorometric homogenization (FH) buffer (10 mM Tris pH 7.4, 1 mM EDTA, 400 mM KCl) in a glass tissue grinder, and washed in another 500 µl buffer. Homogenate was transferred to a 1.5 ml tube and centrifuged at 16,000g for 10 min at 4°C. The supernatant was diluted

to 2 ml and fluorescence determined by spectrofluorometry (SpectraMax 250; Molecular Devices) at 488 nm excitation/507 nm emission. Mean values of fluorescence for each transgenic line were derived from four to ten replicate samples and are given in arbitrary units. Background fluorescence was determined in non-transgenic wild type host flies, with this value subtracted from the GFP fluorescence in experimental samples.

3. Results

3.1. Transformation experiments

Germ-line transformation was tested in a wild strain of *A. suspensa* with the *piggyBac* vector, pB[PUBnlsEGFP], and a *hsp70*-regulated transposase helper. Expression of GFP from the vector construct was tested in preliminary studies by transient expression in caribfly cell lines and embryos (Harrell and Handler, unpublished). The vector was mixed with the *phspBac* helper at concentrations of 600 µg/ml vector and 400 µg/ml helper and injected into 1681 eggs from which 1089 larvae hatched. Of these, 569 larvae survived to adulthood, including 260 G₀ males and 301 G₀ females. Eight adults died before mating. The G₀ progeny were intermated in sixty small groups with three G₀ males mated to five G₀ females. Sixty remaining males were mass-mated to 50 wild females.

All of the groups yielded viable G₁ progeny that were screened for GFP expression as two to three day old embryos or larvae [Fig. 1 (a,b)], and rescreened as adults

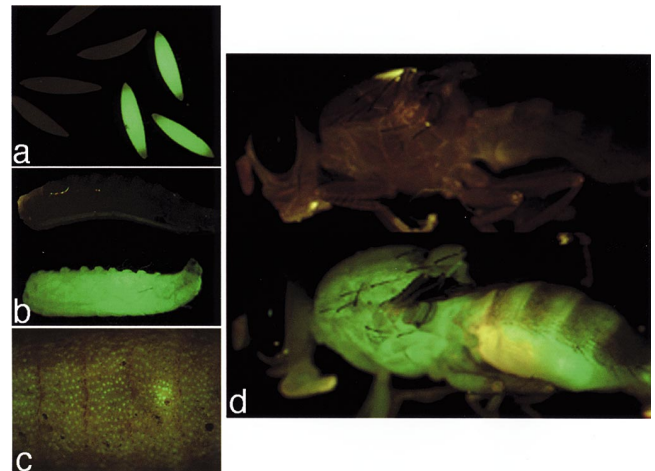


Fig. 1. Expression of GFP detected under UV at different developmental stages in *A. suspensa* individuals transformed with the pB[PUBnlsEGFP] vector, compared to non-transformed wild type individuals. These include (a) 2 day old embryos (wild type on the left), (b) second instar larvae (wild type on top), (c) a transformed pupa (close-up of cuticle showing epidermal nuclear expression), and (d) adults (wild type on top). Under UV, transformants exhibit bright green fluorescence, with wild type non-transformants exhibiting muted yellow autofluorescence.

[Fig. 1(d)]. Three of the small mating groups (lines 12, 36, and 51) yielded a total of 29 G₁ fluorescent progeny (Table 1), of which 24 were outcrossed individually to wild caribflies. A fourth mating group (line 60) yielded 28 fluorescent G₁ flies, of which 21 were interbred in three groups (60gp1, 2 and 3) to maximize the number of integrations in their offspring. Only GFP expressing flies were intermated in succeeding generations.

No fluorescent progeny were detected from the mass mating of G₀ males, which suggests that this is not an efficient method to select transformant progeny. A possible drawback of mass mating males is that a few robust males may mate with most of the females, prohibiting a comprehensive screen of the rest.

3.2. Southern DNA hybridization

Transposition of the *piggyBac* vector into the caribfly genome was verified by Southern DNA hybridization (Fig. 2). The basic strategy was to perform hybridizations to the 5' vector arm using the *piggyBac* *NsiI/HpaI* fragment as probe, and to internal fragments and the 3' vector arm using the EGFP-1 gene and the *piggyBac* *HpaI/NsiI* fragment as probe. Digestion with *XbaI* and hybridization with the *HpaI/NsiI* and EGFP probe indicated the presence of the 766 bp internal *XbaI* fragment in all transformant lines [Fig. 2(A)]. Hybridization to the vector arms indicated three to four integrations in the group 12 sublines and a single integration in groups 36 and 51. Individual integrations in the group 60 interbred lines were clarified by *SalI* digestion and hybridization to *NsiI/HpaI* probe which indicated four integrations in line 60gp1 and three integrations in line 60gp2 [Fig. 2(B)]. This digestion also reaffirmed the single integration in the group 51 sublines. Since the G₁ transformants from line 60 were interbred, the 60gp1 and 60gp2 integrations did not necessarily come from the same G₀ germ-line, and the differing fragment sizes indicate that the seven integrations occurred independent of one another. *NsiI* digestion of transformant DNA and hybridization to the *NsiI/HpaI* and *HpaI/NsiI* probes shows two (of the three) internal *NsiI* fragments of 2381 bp and 799 bp for all the integrations [Fig. 2(C)].

Table 1
Transgenic lines and integrations

G ₀ mating group	G ₁ GFP flies	G ₁ integrations ^a
12	20	3–4
36	4	1
51	5	1
60gp	28	3–4

^a Determined from selected lines (see Fig. 2).

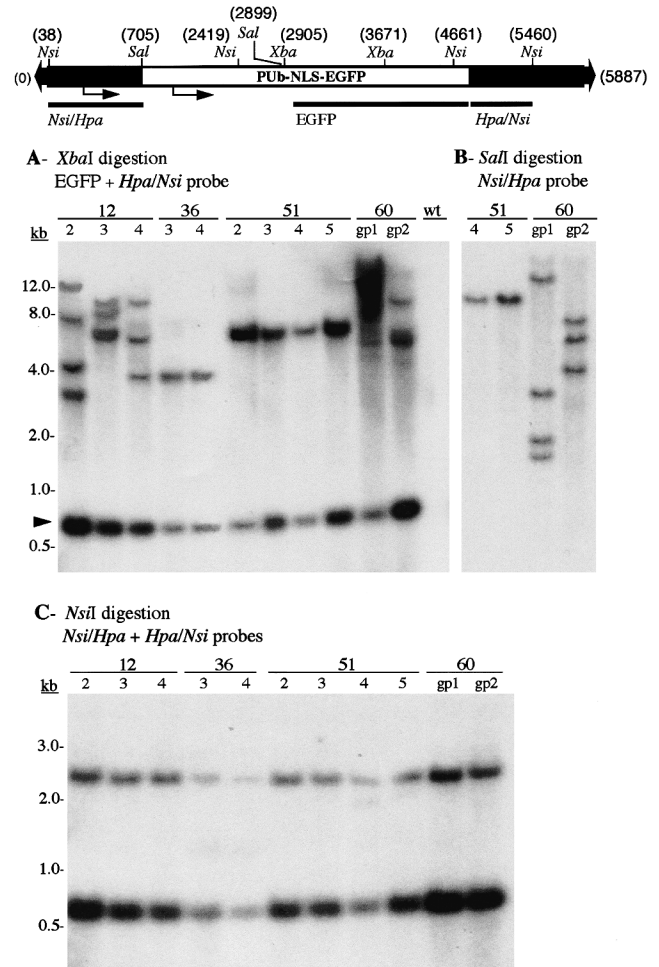


Fig. 2. Southern DNA hybridization analysis of *A. suspensa* transformant sublines and wild type host strain control samples. On top is a schematic (not to scale) of the pB[PUBnlsEGFP] vector showing the *NsiI*, *SalI*, and *XbaI* restriction sites used to digest genomic DNA, with nucleotide positions given above. The *piggyBac* vector is in black and the PUBnlsEGFP marker construct in white. Below are the probes (bars) and their designations used for hybridization. For each blot the restriction enzyme used for digestion and hybridization probe are indicated. DNA size markers are shown to the left of the autoradiograms with lane designations given as wild type (wt) and transformed G₀ lines with G₁ sublines below. The arrow in blot A refers to the internal *XbaI* vector fragment. See Section 2 for details.

3.3. Insertion site sequences

To verify that *piggyBac*-mediated integrations occurred, insertion sites were isolated by inverse PCR, subcloned and sequenced. To sequence *piggyBac* insertion sites from lines having multiple integrations we circularized the entire vector by digesting at a restriction site (*SpeI*) absent from the vector sequence, and generated PCR fragments across the genomic sequence by using a long template PCR protocol and reagents (see Materials and methods). Insertion sites containing 4 and 6 kb of adjacent genomic DNA were isolated from the 60gp1 and 12-4 lines, respectively. Both insertions

occurred at canonical *piggyBac* TTAA target sites that were duplicated, and having adjacent sequence different from that in the vector (Table 2). Direct alignments and BLAST analysis did not reveal further significant commonalities among the insertion sites or to other sequence in the database.

3.4. GFP expression

As with the previous transformation of *D. melanogaster* with the PUBnlsEGFP marker, detectable GFP expression was unambiguous in all the transformants, though subtle differences in expression between some of the lines could be detected by visual inspection. The line 12 and line 60 groups appeared to have stronger expression than the others, and this is generally consistent with these lines having three to four vector integrations, compared to a single integration in the lines 36 and 51 (though the amount of homozygosity was undetermined for the multiple integration lines). GFP was detectable by external observation throughout development (Fig. 1) starting with two day old embryos, and in all larval stages, pupae, and adults. Fluorescence was most obvious from the epidermal cell nuclei in pupae [Fig. 1(c)], and from the thoracic region in adults. GFP was only detectable from the thorax in the weaker expressing lines (line 36), but was detected in internal tissues after dissection. Expression was most intense from the thoracic flight muscle (data not shown).

If lines are to be developed for optimal GFP expression so that they may be used for the field detection of released flies (and transgenics in particular), then an objective quantitative measurement of GFP is required. To achieve this we developed a spectrofluorometric assay for GFP solubilized from tissue homogenates. The measured fluorescence levels shown in Fig. 3 were generally consistent with the observed relative fluorescence in the individual lines. The highest levels were also observed in transformants with three to four integrations (lines 12-2, 12-4, 60gp1, and 60gp2), with the lowest levels found in those with single integrations (lines 51-4 and 36-3). The difference in fluorescence expressed from lines with single integrations, or between the lines 12-3 and 12-4 having three integrations each, may be a result of position effect suppression.

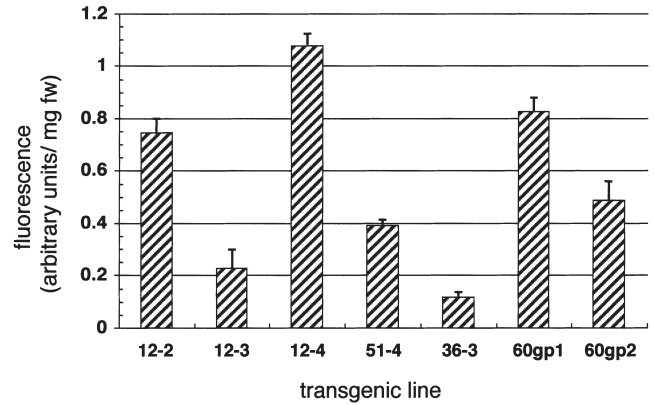


Fig. 3. Histogram showing relative levels of GFP fluorescence from *A. suspensa* transgenic lines. Mean values (\pm SEM) are derived from spectrofluorometric readings and are presented in arbitrary units as a function of fresh weight (fw) of the homogenized samples. See Section 2 for details.

4. Discussion

Transformation of the Caribbean fruit fly was achieved for the first time with a *piggyBac* vector marked solely with GFP. The *piggyBac* vector was derived from a transposon originally isolated from the cabbage looper moth, and since the Mediterranean fruit fly and *D. melanogaster* were previously transformed with *piggyBac*, its ability to transpose in another dipteran was not unexpected. Nevertheless, this does serve to widen the range of non-drosophilid species amenable to relatively efficient gene transfer. One to four integrations occurred in individual germ-lines, and the insertion site sequences of two of the integrations indicated *piggyBac*-mediated events.

Of particular interest was the use of a GFP marker for transgenic selection in a species that presently is not amenable to mutant-rescue based selections. The caribfly does not have appropriate visible mutations or cloned DNA for their wild type alleles, and indeed, this represents the current status for most insects that might use transposons or other vectors for germ-line transformation. The effective use of this particular marker construct in the Caribbean fruit fly is thus encouraging for its use in a broad range of species.

The marker construct had an enhanced GFP gene linked in frame to a nuclear localizing sequence, that

Table 2

Insertion site junction sequences of *piggyBac* in the pB[PUBnlsEGFP] vector compared to genomic integrations in transgenic lines

DNA	5' junction	3' junction
pB[PUBnlsEGFP]	GCGCAAATCTTTTAA- <i>piggyBac</i> -TTAAATAATAGTTTCT	
line 60gp1	AACGCTCTCAATTAA- <i>piggyBac</i> -TTAAGAACAAACGAGC	
line 12-4	TCATTTGGATAATTAA- <i>piggyBac</i> -TTAATATCGATCTTAA	

was placed under promoter regulation from the *D. melanogaster* polyubiquitin gene. This construct was previously tested in *D. melanogaster* (see Davis et al., 1995; Handler and Harrell, 1999), and given the high level of conservation of the ubiquitin gene from yeast to humans, we were optimistic that its promoter function would be maintained in other insects. Consistent with the unambiguous expression of GFP in *Drosophila* transformants, the marker was efficiently detected in caribfly transformants. Importantly, polyubiquitin is active in all cell types throughout development which makes it versatile in terms of GFP detection, and it was easily observed externally from all tissues in 1–2-day old transformant embryos and all larval stages. Embryonic detection is especially useful for transgenic selection since it eliminates the need to rear an entire G₁ population, as well as the time needed for G₁ individuals to reach adulthood when most visible phenotypes are scored. For many insect species generation times can be long and the effort needed for rearing many thousands of organisms can be formidable. Markers such as polyubiquitin-regulated GFP may be a deciding factor in whether transformation with *piggyBac*, or other vectors, is even considered for some species.

GFP was also detected in adults, and while expression was observed in all internal tissues examined, external detection varied owing to variable gene expression and quenching by adult cuticle and melanization. On inspection, strongest expression occurred in thoracic flight muscles and this may be a result of the way GFP accumulates in this tissue. Accordingly, GFP was most easily detected externally from the thorax, and only detected in this region from lines having weaker expression. The more strongly expressing lines showed GFP in the abdomen and legs, but this differed from *Drosophila* transformants where GFP was easily detectable in all body regions regardless of the general level of expression (Handler and Harrell, 1999). The use of GFP as a marker may, therefore, be influenced by the timing and extent of cuticle formation and melanization. For example, species whose embryos melanize may have to be screened at larval stages, and other GFP constructs are being developed that may be more useful for selections in some species. One of these has GFP regulated by the artificial 3×P3 promoter derived from the rhodopsin gene that expresses strongly from the eyes and ocelli, and should be functional in most insects (Berghammer et al., 1999). A caveat for the use of any GFP marker for G₁ transgenic selection is that GFP in embryos less than a day old may not be detectable given the time needed for zygotic expression and internal cyclization and oxidation necessary for fluorescence (see Davis et al. 1995). Conversely, subsequent generations can be selected as early embryos owing to maternal GFP expression, however care must be taken to re-select since

GFP may be detected in non-transgenic offspring arising from mothers heterozygous for the transgene.

A comparison of marker gene expression from PUBnlsEGFP and the *white*⁺ gene in *Drosophila* clearly showed that GFP was more reliable as a transgenic selection since a majority of transformants were detected by fluorescence alone. This was presumed to be the result of a more pronounced, if not complete position effect suppression of *white*⁺ resulting in a white eye phenotype in many of the G₁ sublines. In the caribfly transgenics, GFP expression was unambiguous, though differences in fluorescence could be detected among the lines based on visual inspection. To more fully explore these differences and to make comparisons in GFP expression for applied purposes, a simple quantitative spectrofluorometric assay was developed. This allows direct comparisons based on fluorescence, and extrapolations are possible allowing determinations of GFP concentration. In general, fluorescence was greatest in lines having three to four vector integrations, with weakest fluorescence from lines having a single integration. This is consistent with a gene dosage effect, though this was difficult to assess since we could not determine if all the transgenes were homozygous in the absence of marked chromosomes (though the lines were inbred for at least eight generations with all flies expressing GFP). Notably, of the lines with one integration (and thus likely to be homozygous), 51-4 had a mean value of fluorescence more than three-fold higher than that in 36-3. A five-fold difference in fluorescence was observed between lines 12-3 and 12-4 having three integrations. These differences may be due to position effects on the polyubiquitin-regulated *gfp* gene, and this will be more fully explored as more transgenic lines are tested.

GFP detection in adults is important to identifying transformants for simple rearing, but also presents the possibility of using it as a genetic marker for field detection of released insects. Many of the practical applications for transgenic insects will involve their release, and a common problem for release programs is the necessity to distinguish released from indigenous insects to determine program effectiveness. Release of transgenic insects will present the additional challenge of monitoring their dispersal and perdurance in the environment for risk assessment analysis. An unambiguous genetic marker with a unique visible and molecular phenotype, yet having minimal negative effects on viability, presents several advantages that should be explored in other insect species.

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References

- Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, J., Zhang, Z., Miller, W., Lipman, D.J., 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucl. Acids Res.* 25, 3389–3402.
- Berghammer, A.J., Klingler, M., Wimmer, E.A., 1999. A universal marker for transgenic insects. *Nature* 402, 370–371.
- Cary, L.C., Goebel, M., Corsaro, H.H., Wang, H.H., Rosen, E., Fraser, M.J., 1989. Transposon mutagenesis of baculoviruses: analysis of *Trichoplusia ni* transposon IFP2 inserions within the FP-Locus of nuclear polyhedrosis viruses. *Virology* 161, 8–17.
- Chalfie, M., Tu, Y., Euskirchen, G., Ward, W.W., Prasher, D.C., 1994. Green fluorescent protein as a marker for gene expression. *Science* 263, 802–805.
- Coates, C.J., Jasinskiene, N., Miyashiro, L., James, A.A., 1998. *Mari-ner* transposition and transformation of the yellow fever mosquito, *Aedes aegypti*. *Proc. Natl. Acad. Sci. USA* 95, 3742–3751.
- Cormack, B.P., Valdivia, R.H., Falkow, S., 1996. FACS-optimized mutants of the green fluorescent protein (GFP). *Gene* 173, 33–38.
- Cornel, A.J., Benedict, M.Q., Rafferty, C.S., Howells, A.J., Collins, F.H., 1997. Transient expression of the *Drosophila melanogaster cinnabar* gene rescues eye color in the white eye (WE) strain of *Aedes aegypti*. *Insect Biochem. Mol. Biol.* 27, 993–997.
- Davis, I., Girdham, C.H., O'Farrell, P.H., 1995. A nuclear GFP that marks nuclei in living *Drosophila* embryos; maternal supply overcomes a delay in the appearance of zygotic fluorescence. *Devel. Biol.* 170, 726–729.
- Handler, A.M., McCombs, S.D., Fraser, M.J., Saul, S.H., 1998. The lepidopteran transposon vector, *piggyBac*, mediates germline transformation in the Mediterranean fruitfly. *Proc. Natl. Acad. Sci. USA* 95, 7520–7525.
- Handler, A.M., Harrell, R.A., 1999. Germline transformation of *Drosophila melanogaster* with the *piggyBac* transposon vector. *Insect Mol. Biol.* 8, 449–458.
- Jasinskiene, N., Coates, C.J., Benedict, M.Q., Cornel, A.J., Rafferty, C.S., James, A.A., Collins, F.H., 1998. Stable, transposon mediated transformation of the yellow fever mosquito, *Aedes aegypti*, using the *Hermes* element from the housefly. *Proc. Natl. Acad. Sci. USA* 95, 3743–3747.
- Lanford, R.E., Kanda, P., Kennedy, R.C., 1986. Induction of nuclear transport with a synthetic peptide homologous to the SV40 T antigen transport signal. *Mol. Cell. Biol.* 8, 2722–2729.
- Lee, H., Simon, J.A., Lis, J.T., 1988. Structure and expression of ubiquitin genes of *Drosophila melanogaster*. *Mol. Cell. Biol.* 8, 4727–4735.
- Loukeris, T.G., Livadaras, I., Arca, B., Zabalou, S., Savakis, C., 1995. Gene transfer into the Medfly, *Ceratitis capitata*, with a *Drosophila hydei* transposable element. *Science* 270, 2002–2005.
- Pinkerton, A.C., Michel, K., O'Brochta, D.A., Atkinson, P.W., 2000. Green fluorescent protein as a genetic marker in transgenic *Aedes aegypti*. *Insect Mol. Biol.* 9, 1–10.
- Prasher, D.C., Eckenrode, V.K., Ward, W.W., Prendergast, F.G., Cormier, M.J., 1992. Primary structure of the *Aequorea victoria* green fluorescent protein. *Gene* 111, 229–233.
- Rubin, G.M., Spradling, A.C., 1982. Genetic transformation of *Drosophila* with transposable element vectors. *Science* 218, 348–353.
- Tamura, T., Thibert, T., Royer, C., Kanda, T., Eappen, A., Kamba, M., Kōmoto, N., Thomas, J.-L., Mauchamp, B., Chavancy, G., Shirk, P., Fraser, M., Prudhomme, J.-C., Couble, P., 2000. A *piggybac* element-derived vector efficiently promotes germ-line transformation in the silkworm *Bombyx mori* L. *Nature Biotechnol.* 18, 81–84.
- Yang, T.T., Cheng, L., Kain, S.R., 1996. Optimized codon usage and chromophore mutations provide enhanced sensitivity with the green fluorescent protein. *Nucleic Acids Res.* 24, 4592–4593.
- Zwiebel, L.J., Saccone, G., Zacharapoulou, A., Besansky, N.J., Favia, G., Collins, F.H., Louis, C., Kafatos, F.C., 1995. The *white* gene of *Ceratitis capitata*: a phenotypic marker for germline transformation. *Science* 270, 2005–2008.