

Germ-line transformation of the South American malaria vector, *Anopheles albimanus*, with a *piggyBac*/EGFP transposon vector is routine and highly efficient

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Abstract

Stable and efficient germ-line transformation was achieved in the South American malaria vector, *Anopheles albimanus*, using a *piggyBac* vector marked with an enhanced green fluorescent protein gene regulated by the *Drosophila melanogaster polyubiquitin* promoter. Transgenic mosquitoes were identified from four independent experiments at frequencies ranging from 20 to 43% per fertile G₀. Fluorescence was observable throughout the body of larvae and pupae, and abdominal segments of adults. Transgenic lines analysed by Southern hybridization had one to six germ-line integrations, with most lines having three or more integrations. Hybridized transposon vector fragments and insertion site sequences were consistent with precise *piggyBac*-mediated integrations, although this was not verified for all lines. The *piggyBac*PUBnlEGFP vector appears to be a robust transformation system for this anopheline species, in contrast to the use of a *piggyBac* vector in *An. gambiae*. Further tests are needed to determine if differences in anopheline transformation efficiency are due to the marker systems or to organismal or cellular factors specific to the species.

Keywords: *piggyBac* vector, germ-line transformation, green fluorescent protein, *Anopheles albimanus*, anopheline mosquitoes, malaria vector.

Introduction

Germ-line transformation of nondrosophilid insects has expanded to species in three orders using four transposon-

based vector systems (see Handler, 2001; Atkinson *et al.*, 2001). Most of these insects are within the Diptera, and include several mosquito species of medical importance. The most important of these in terms of disease transmission are the vectors of human malaria within the genus *Anopheles*. Stable germ-line transformation of these species and other mosquitoes has been a high priority because this methodology should allow practical applications that will aid in controlling their population size or their ability to transmit disease (see James, 2000). Routine transformation of mosquitoes, especially with multiple vector systems, will be critical to functional genomic studies that will identify genes by insertional mutagenesis and analyse sequences for structure–function relationships. Rapid progress in sequencing the genome of *Anopheles gambiae*, indeed, heightens the need for routine gene transfer methods in this and related species.

Some of the first mosquito transformation experiments succeeded in the yellow fever vector, *Aedes aegypti* with the *mariner* (*Mos1*) (Coates *et al.*, 1998) and *Hermes* (Jasinskiene *et al.*, 1998) vectors, with later transformations succeeding with *piggyBac* (Kokoza *et al.*, 2001; Lobo *et al.*, 2002). Recent experimentation in this species already holds promise for salivary gland expression of plasmodium-resistant genes (James *et al.*, 1999) and fat body expression of antipathogenic defensin genes (Kokoza *et al.*, 2000). Similar strategies using transgenic mosquitoes should be possible in malaria vectors, and germ-line transformation of two anopheline species with two different vectors has been recently reported. *Anopheles stephensi* was transformed with a *Minos* vector marked with EGFP regulated by the *Drosophila melanogaster actin5C* promoter, resulting in an approximate transformation frequency of 7% (Catteruccia *et al.*, 2000). Next was transformation of *An. gambiae* with a *piggyBac* vector marked with EGFP regulated by the baculovirus *hr5-ie1* promoter (Grossman *et al.*, 2001). Although transformation of this highly important species is quite significant, only two transformant individuals arising from the same single germ-line integration were selected from this experiment, and it remains to be determined if *piggyBac* can be used for routine transformation of *Anopheles*. In an effort to expand the number of anopheline

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species amenable to germ-line transformation, and the vector-marker systems available for this methodology, we tested *piggyBac*-mediated transformation of the South American malaria vector, *Anopheles albimanus*. This mosquito has a neotropical distribution and is one of the five major vectors of malaria in South and Central America including Mexico, and is found in the southernmost regions of Texas and Florida. Here we report the routine and highly efficient transformation of *An. albimanus* with *piggyBac* marked with a *polyubiquitin*-regulated EGFP marker.

Results

Transformation experiments

Germ-line transformation was tested in a wild strain of *Anopheles albimanus* with the *piggyBac* vector, pB[PUBnlEGFP], and a *hsp70*-regulated transposase helper in four independent experiments using a vector and transposase helper plasmid mixture of 300 µg/ml vector and 200 µg/ml helper. For experiment AA1, 485 eggs were injected from which forty-four adults survived and these were mated by backcrossing G₀ males and females in groups to wild mosquitoes (see Table 1). A total of twenty-two G₁ flies expressing EGFP emerged from these matings. G₀

fertility could not be determined for this experiment, but a survey of transgenic sublines by Southern hybridization indicated that at least nine germ-lines were independently transformed (see below), yielding a transformation frequency of at least 20% if all G₀ flies were fertile. In experiment AA2, 695 eggs were injected from which sixty adults survived. In this and the following experiments, G₀ males were backcrossed individually while females were mated in groups but egged separately to determine fertility. Ten G₀ flies were fertile of which two G₁ adults expressed EGFP yielding a transformation frequency of 20%. In experiment AA3, 584 eggs were injected from which forty-one adults survived. Nine G₀ flies were fertile of which three independent families yielded G₁ adults expressing EGFP resulting in a transformation frequency of 33% per fertile G₀. In experiment AA4, injection of 436 embryos resulted in thirty-one surviving G₀ adults. This experiment yielded sixteen fertile lines of mosquitoes, of which seven families expressed EGFP resulting in a transformation frequency of 43%. Transgenic lines from the four experiments have remained stable based on marker phenotype for eight to fourteen generations.

Expression of EGFP was most evident throughout the body in larvae and pupae, with expression most evident from abdominal segments in adults (Fig. 1). Variations in

Table 1. Transformation experiments

Experiment	Embryos injected	Larvae (% hatch)	Eclosed adults	Fertile families	Transgenic (GFP) families	Transformation frequency
AA1	485	104 (21%)	44	nd*	22	> 20%
AA2	695	202 (29%)	60	10	2	20%
AA3	584	85 (15%)	41	9	3	33%
AA4	436	72 (17%)	31	16	7	43%

*Fertility not determined due to grouped backcrosses of G₀ adults. Transformation frequency for AA1 is a minimal estimate.

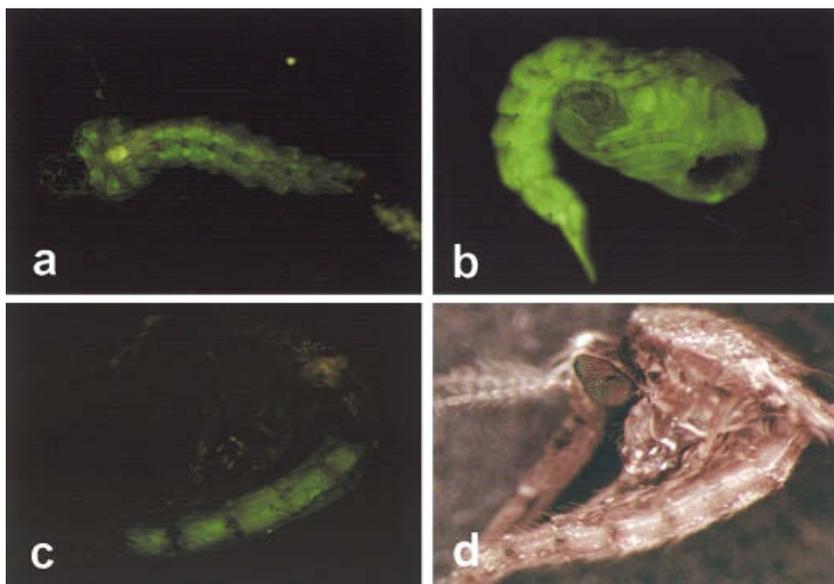


Figure 1. GFP fluorescence at different developmental stages in *Anopheles albimanus* individuals transformed with the pB[PUBnlEGFP] vector including the larva (a), pupal (b), and adult (c) stages, and the adult mosquito under brightfield (d).

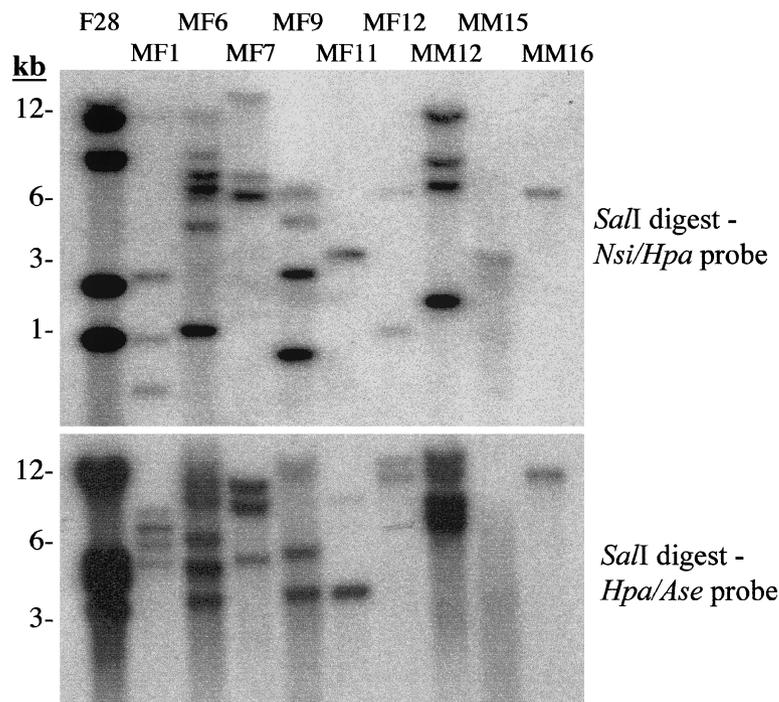
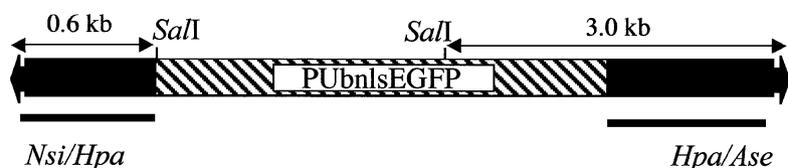


Figure 2. Southern DNA hybridization analysis of *Anopheles albimanus* transformant sublines. On top is a schematic (not to scale) of the pB[PUBnlsEGFP] vector showing the *SalI* restriction sites used to digest genomic DNA, with sequence distances to the termini given above. The *piggyBac* vector is in black and the PUBnlsEGFP marker construct in white and black diagonal lines. Below are the probes (bars) and their designations used for hybridization. DNA size markers are shown to the left of the autoradiograms with lane designations given for the transformed G₁ sublines. See Experimental procedures for details.

tissue-specificity and levels of expression among different lines were noted, although it could not be determined if this was an effect of integration number or genomic position effects.

Southern DNA hybridization

Transposition of the *piggyBac* vector into the *An. albimanus* genome was verified by Southern hybridization to DNA from nine G₁ sublines from experiment AA1 and one line from AA2 (F28) (Fig. 2). For all hybridizations genomic DNA, from G₄ to G₇ adults, was digested with *SalI*, which cuts the vector at the 5' junction of *piggyBac* DNA and the PUBnlsEGFP marker, and also cuts the vector at the junction between the *polyubiquitin* promoter and EGFP. Hybridizations were performed to the 5' vector arm using the *piggyBac* *NsiI/HpaI* fragment as probe that should result in vector/

genomic fragments of at least 600 bp from precisely integrated vector. Hybridizations to the 3' vector arm used the *piggyBac* *HpaI/AseI* fragment as probe that should result in vector/genomic fragments from precisely integrated vector of at least 3.0 kb.

Of the ten lines analysed, most were consistent in terms of the number of integrations based on hybridization to each vector arm, and all fragments had sizes consistent with precise integrations. Three lines had single integrations (MF11, MM15 and MM16), one line had two integrations (MF12), one line had three integrations (MF7), four lines had four integrations (F28, MF1, MF9 and MM12), and one line had six integrations (MF6). There is some variation in fragment intensity within the same lines that we attribute to DNA samples from a mixed population of transformants having a varying number of unlinked transgene

Table 2. Insertion site junction sequences of *piggyBac* in the pB[PUBnlsEGFP] vector and genomic integrations in transgenic lines

DNA	5' junction	3' junction
pB[PUBnlsEGFP]	GCGCAAATCTTTTAA- <i>piggyBac</i> -TTAAATAATAGTTTCT	TGTAACGATAAATTAA- <i>piggyBac</i> -TTAAGTAAGGCATAGT
line F28	TGTAACGATAAATTAA- <i>piggyBac</i> -TTAAGTAAGGCATAGT	AAAGTGTGGTTTAA- <i>piggyBac</i> -
line MF6	AAAGTGTGGTTTAA- <i>piggyBac</i> -	

alleles. This results from backcrossing G_1 individuals and inbreeding subsequent generations allowing some alleles to be crossed out.

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, the entire vector was circularized by digesting at a restriction site (*SpeI*) absent from the vector sequence, and generating PCR fragments across the genomic sequence by using a long template PCR protocol and reagents. Both the 5' and 3' insertion site sequences were determined for one of the four integrations in line F28, and these show the duplicated TTAA insertion site indicative of a *piggyBac*-mediated transposition (Table 2). The 5' junction sequence was also determined for one of the integrations in line MF6. Direct alignments and BLAST analysis (Altschul *et al.*, 1997) did not reveal further significant commonalities among the insertion sites or to other sequences in the database.

Discussion

Here we report the efficient and routine germ-line transformation of the South American malaria vector, *Anopheles albimanus*, with a *piggyBac* transposon vector marked with *polyubiquitin*-regulated EGFP. In four independent experiments, transformation occurred at relatively high frequencies between 20% and 43%, with an average frequency of 34% from three experiments where fertility could be determined (experiments AA2, AA3 and AA4). This is among the highest frequencies reported for *piggyBac* transformation, and is in the upper range of transposon-mediated transformation for all insects (see Atkinson *et al.*, 2001). Notably, an *hsp70*-regulated helper was used but injected embryos were not subjected to heat shock to limit mortality. Similar to previous attempts at transformation of *Anopheles* species, survival to adulthood after embryo injection was approximately 10%, with a relatively low level of fertility in surviving G_0 adults (although this could not be determined accurately in group-mated G_0 flies). The *An. albimanus* experiments reported include all our attempts at *piggyBac/EGFP* transformation in this species (i.e. successful experiments were not preceded by preliminary studies), and *piggyBac* appears to be a robust vector system for this mosquito. As with other transformation experiments, improvements in DNA delivery and embryo survival may help increase transformant numbers, but for this species there was no apparent relationship between the relatively low yield of fertile adults from injected embryos and transformation frequency, as has been suggested for other anopheline transformations (Grossman *et al.*, 2001). The possibility remains that detection of transformants in *An. gambiae*

may have been hindered by the marker construct used, and it should be useful to determine whether *piggyBac* transformation can be improved in this species with more widely used GFP markers under *polyubiquitin*, *actin5C* or *Pax-6* regulation (see Horn *et al.*, 2002). In particular, use of the pB[PUb]nsEGFP vector in several dipterans has established its patterns of EGFP expression and the influence of genomic position effects on the polyubiquitin promoter. If routine transformation is not possible with one of these markers in *An. gambiae*, then the *piggyBac* vector or species-specific genomic restrictions on vector transposition or transformant survival could be considered as possible limitations.

In addition to a high transformation frequency, the number of integrations per germ-line in *An. albimanus* was higher than in previous *piggyBac* transformations, and for most other vectors. Most of the *An. albimanus* transformant lines tested by hybridization had three or more integrations with one line having six. Three or four integrations were detected in a few germ-lines in the Caribbean fruit fly, *Anastrepha suspensa* (Handler & Harrell, 2001) and the sheep blowfly, *Lucilia cuprina* (Heinrich *et al.*, 2002), while most other insect transformations with *piggyBac* have yielded only one or two integrations per germ-line (see Handler, 2001). A single germ-line integration was also determined for the *piggyBac* transformation of *An. gambiae* (Grossman *et al.*, 2001). The only reported transformation resulting from more germ-line integrations occurred in a *Hermes* transformation of *D. melanogaster*, where ten integrations were detected in one line and clustered integrations were noted (O'Brochta *et al.*, 1996). As in *An. albimanus*, transformation frequencies were relatively high, but it is difficult to determine if there is a statistically significant relationship between transformation frequency and multiple or clustered integrations. The transformation frequencies in *A. suspensa* and *L. cuprina* were relatively low, while routine transformations with a variety of *piggyBac* vectors in *D. melanogaster* and *C. capitata*, at frequencies of 20% or above, have most often yielded single integrations, with no more than three detected in a germ-line (Handler *et al.*, 1998; Handler & Harrell, 1999; A.M.H., unpublished data). At present, the highest frequencies of multiple *piggyBac* germ-line integrations may depend on high transformation frequencies, but multiple integrations are not necessarily a consequence of strong vector mobility. Multiple integrations can be useful for enhanced transgene expression, although it does increase the potential for lethal or semilethal mutations decreasing the viability of transgenic lines, and their breakdown after inbreeding. The *An. gambiae* transformant was associated with a recessive lethal (Grossman *et al.*, 2001), and we have observed sex-linked lethality in *Drosophila* transformants and the loss of transgenics upon inbreeding of some strains of *An. albimanus* and other species. Multiple integrations may also complicate insertional mutagenesis analysis and applied uses of transgenic strains.

A caveat for transposon-mediated transformation in mosquitoes is the possibility of integrations occurring by unusual recombination events. While DNA integration by recombination is typical in some organisms, and may occur as low frequency events with vector plasmid in many insects, it is notable that most verified recombinant transposon integrations in insects have occurred in mosquitoes. A nonhomologous recombination resulted in the first P vector integration reported for *An. gambiae* (Miller *et al.*, 1987), and similar events were observed subsequently in *Aedes* species (McGrane *et al.*, 1988; Morris *et al.*, 1989). Replicative-recombination events were suggested for the unusual *Hermes* integrations in *Aedes aegypti* (Jasinskiene *et al.*, 1998) and *Culex quinquefasciatus* (Allen *et al.*, 2001), and it has been theorized that these resulted from interactions with genomic elements related to *Hermes* (Jasinskiene *et al.*, 2000). Such integrations may be used for basic and applied studies, but precise cut-and-paste integrations are generally preferred for maintaining the integrity of the integrated vector and for predictable and controllable vector mobility (i.e. remobilization for insertional mutagenesis studies). Precise *Hermes* integrations have been demonstrated in an *An. gambiae* cell line genome (Zhao & Eggleston, 1998), but until anopheline germ-line transformation is tested, *Minos* and *piggyBac* may be preferable for mosquito transformations. We showed precise *piggyBac*-mediated integrations into TTAA sites in *An. albimanus*, as was shown for *An. gambiae* transformation (Grossman *et al.*, 2001) and plasmid transpositions (Grossman *et al.*, 2000). TTAA integrations were also observed in *Ae. aegypti* transformations (Kokoza *et al.*, 2001; Lobo *et al.*, 2002), and in transposition assays in this and other *Aedes* species (Lobo *et al.*, 1999; Lobo *et al.*, 2001). Given the large number of total integrations recovered in *An. albimanus*, we have not excluded the possibility that some integrations occurred by a recombination event, although hybridization patterns are consistent with individual nonrearranged transposon integrations. Further characterization of *piggyBac* vector integrations should clarify this possibility.

From our initial experiments, it appears that *piggyBac* can be used for routine transformation of at least one anopheline species, yielding high frequencies despite low levels of survival to adulthood and fertility. Further experimentation is needed to determine if this success with the pB[PUBnlsEGFP] vector can be extended to other important malaria vectors, or whether differences in transformation efficiency can be attributed to species-specific factors.

Experimental procedures

Insect strains and rearing

Anopheles albimanus were obtained from a laboratory colony that was originally established from mosquitoes collected from the east

coast of El Salvador in 1972. Approximately 100 mosquito larvae were reared in enamel pans with 500 ml water at 27 °C. Larvae were fed with a 1 ml slurry of Tetramin Baby-E fish food and yeast (2 : 1 mixture). Pupae were collected and placed in plastic cups until adult emergence, with adults placed in half-pint paper cups with fine mesh lids and a 10% sugar solution. Females were fed with warm bovine blood (42 °C) placed in sausage casings. Female and male mosquitoes emerging from injected eggs (G_0) were placed in separate cages and backcrossed to wild mosquitoes in groups for experiment AA1. For other experiments individual G_0 males were mated to three wild-type females for approximately 4 days. Females were mated in groups in mesh cages with about three wild-type males per female with eggs collected from individual females.

Embryo preparation and injections

Eggs were collected in 8 oz. black cups lined with 3MM filter paper and filled with water to a level of 1 cm from the bottom to soak the filter paper. Cups were placed inside population cages for 5–10 min after which the 3MM paper with oviposited eggs was removed and placed in a square Petri dish for 15–20 min. Eggs beginning to turn grey were lined up in horizontal orientation, with their narrow posterior ends to one side, on 1 cm² squares of black filter paper. The eggs were placed on coverslips with double stick tape by lowering the coverslip on to the eggs from above with forceps. Excess water around the eggs was blotted with filter paper and the eggs were allowed to desiccate for 2–3 min and then covered with halocarbon oil. Preblastoderm embryos were microinjected with a 300 µg/ml vector and 200 µg/ml helper DNA mixture in injection buffer (5 mM KCl; 0.1 M sodium phosphate pH 6.8) under oil in their posterior ends. The coverslips were then held on damp filter paper for 20–30 min at which time the taped strips were removed from the coverslip with the oil blotted off, and the strips were placed on damp filter paper within Petri dishes and kept under oxygen at 27 °C. Eggs were brushed off the strip the following day and hatched larvae were collected after 3 days. Eggs were not subjected to heat shock because preliminary tests indicated increased mortality, and the phspBac transposase helper includes the transposase promoter in addition to the *hsp70* promoter.

Plasmids

The *piggyBac* helper plasmid, phspBac (originally phsp-pBac), having the transposase gene under *hsp70* regulation, as well as the PUBnlsEGFP construct have been described previously (Handler & Harrell, 1999). The PUBnlsEGFP marker includes an enhanced GFP gene from EGFP-1 (Clontech) linked in-frame to the nuclear localizing sequence of the SV40 T-antigen, and placed under *D. melanogaster polyubiquitin* regulation (Davis *et al.*, 1995). The *polyubiquitin*-nls cassette was ligated into the 5' multiple cloning site of EGFP-1, with the PUBnlsEGFP marker isolated as a 4.1 kb *Bgl*II-*Stu*I fragment and ligated into the *Bgl*II-*Hpa*I site of *piggyBac* within p3E1.2 (Cary *et al.*, 1989) to create pB[PUBnlsEGFP].

Southern hybridization

Approximately 5 µg of genomic DNA was digested with the *Sal*I restriction enzyme, separated on 0.8% agarose gels, blotted to nylon filters and immobilized by ultraviolet irradiation. Hybridization probes were generated from indicated *piggyBac* restriction fragments that were isolated from p3E1.2 by agarose gel-elution. Probe DNA was radiolabelled with [32P]-dCTP by random priming

(Gibco BRL) according to the manufacturer's specifications. Hybridizations were performed in phosphate buffer pH 7.5; 1% BSA; 7% SDS at 65 °C with an initial wash in 2X SSC; 0.2% SDS at room temperature and two washes in 1X SSC; 0.1% SDS at 55 °C for 30 min. The filter was first hybridized to the 5'-*Nsi*/*Hpa* probe, with radiolabelled probe removed by washing in boiling 2X SSC, 0.01% SDS, and subsequent hybridization to the 3' *Hpa*/*Ase* *piggyBac* probe. Autoradiography was performed by exposure on Kodak X-Omat film at -70 °C.

Inverse PCR

Inverse PCR was performed as described previously (Handler & Harrell, 2001) by initial digestion of transformant genomic DNA with *Spe*I, which does not cut within the vector. Digested DNA was purified by phenol-chloroform extraction and circularized by ligation at 12 °C overnight. PCR was performed on the circularized fragments using *piggyBac* primer sequences in outward facing orientation. For the 5'-terminus the reverse primer (159R) 5'-ATCAGTGACACTTACCGCATGACA-3' was used, and for the 3' terminus the forward primer (2388F) 5'-CCTCGATATACAGACGATAAAAACACATG-3' was used. PCR was performed using the Expand Long Template PCR System (Roche Applied Science) using cycling conditions of initial denaturation at 94 °C for 2 min, and ten cycles of 15 s denaturation at 94 °C, 30 s annealing at 63 °C, and 8 min elongation at 68 °C, with an additional twenty cycles having elongation times extended for an additional 20 s at each cycle with a final elongation for 8 min. PCR products were subcloned directly into the p-GEM T-Easy vector (Promega) with vector termini and insertion sites sequenced 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).

GFP analysis

EGFP was observed at all developmental stages under a Leica MZ FLIII stereozoom microscope using a mercury lamp and a FITC/RSGFP longpass wavelength filter set #HQ 41012 (Chroma Technology). Digital photographic documentation used a SPOT-1 cooled CCD camera (Diagnostic Instruments) with digital images processed with Adobe Photoshop 6.0 software (Adobe Systems).

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