

## Genetic Transformation of *Drosophila willistoni* Using *piggyBac* Transposon and GFP

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### ABSTRACT

Studies were carried out on the use of *piggyBac* transposable element as vector and the green fluorescent protein (EGFP) from the jellyfish, *Aequorea victoria*, as a genetic marker for the transformation of *Drosophila willistoni*. Preblastoderm embryos of *D. willistoni* white mutant were microinjected with a plasmid containing the EGFP marker and the *piggyBac* ITRs, together with a helper plasmid containing the *piggyBac* transposase placed under the control of the *D. melanogaster hsp70* promoter. G<sub>0</sub> adults transformants were recovered at a frequency of approximately 67%. Expression of EGFP in larvae, pupae and adults was observed up to the third generation, suggesting that this transposon was not stable in *D. willistoni*. Transformed individuals displayed high levels of EGFP expression during larvae and adult stages in the eye, abdomen, thorax and legs, suggesting a wide expression pattern in this species than reported to other species of *Drosophilidae*.

**Key words:** Transposable elements, insect transgenesis, germ-line transformation, fluorescent protein marker, microinjection

### INTRODUCTION

Genetic manipulations of insects and other arthropods are an important tool for the study of the molecular basis of development and the evolutionary process (Horn and Wimmer, 2000; O'Brochta and Atkinson, 1998). The use of these methodologies is also being considered as a possible solution to certain medical and agricultural problems caused by some insects, through the cautious releasing in nature of insects with desirable genetic alterations (Kidwell and Wattam, 1998; Irvin et al., 2004). A widely method employed for insect germ-line transformation utilizes transposon-based vectors in

a binary vector/helper system. The vector contains the parts of a transposon necessary for the transposition; however it is unable to produce the transposase, the enzyme required for the transposition. Furthermore, it can carry exogenous DNA fragments of interest that can be implanted in the genome together with the transposable element. The helper plasmid codifies a transposase. Even though it does not contain the ITR's (inverted terminal repeats), the sequences present at the ends of the transposon are essential for transposition. In this way, the helper plasmid provides the enzyme to vector transposition but cannot insert itself in the host genome (Handler, 2001; Miller and Capy, 2004).

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The germ-line transformation was first demonstrated more than 20 years ago in *Drosophila melanogaster* by the use of the transposable *P* element (Rubin and Spradling, 1982). However, studies in many different species showed that this element was non-functional outside the *Drosophilidae*. The *P*-element system is dependant on the presence of several host factors specific to this species, or to closely related species. This has restricted the *P* mediated transformation to a few species of subgenus *Sophophora* of the genus *Drosophila* (Handler et al., 1993). The first transposon-mediated transformation of a non-drosophilid insect was registered 13 years later in the Mediterranean fruit fly, *Ceratitidis capitata*, using the *minos*-mediated transformation (Loukeris et al., 1995).

Alternative transposable elements as *minos*, *mariner*, *Hermes* and *piggyBac* have recently been used for transformation in more than 15 different species of insects, including some that are important for human health and agriculture (Robinson, et al., 2004). The properties of the bacterial transposon *TN5* have also been tested looking for improving insect transgenesis methods (Rowan et al., 2004).

The *piggyBac* element was originally identified by its association with a mutation in a baculovirus passed through the *Trichoplusia ni* cell line culture (Fraser et al., 1983; Cary et al., 1989). This transposon is naturally found in the genomes of some lepidopteran (Cary et al., 1989). The *piggyBac* element was first used to transform the medfly, *C. capitata* (Handler et al, 1998), and more recently it has been used for transformation of many other species (Horn and Wimmer, 2000; Handler and McColombs, 2000; Handler, 2001; Horn et al., 2002; Atkinson, 2002; Robinson et al., 2004; Irvin et al., 2004).

Other important component for the effective development of insect transformation technology is the use of genetic markers that allows recognition of the transformed individuals (O'Brochta and Atkinson, 1997). Eye color genes are quite often used as transformation markers in insects carrying eye color mutations allowing the identification of transformants by mutant-rescue selection. In these cases, the marker gene represents the wild-type allele of a gene that, when mutated, causes a recessive, visible but viable phenotype. However, the lack of suitable recipient mutant strains for most insects of medical and agricultural importance limits their application.

Initially, the search for dominant-acting selection markers that could act independently of pre-existing mutant strains focused on genes which confer chemical or drug resistance. These marker systems showed low reliability and efficiency (Handler, 2001; Horn et al., 2002).

Recently, an interesting marker system was developed based on the properties of fluorescent proteins like GFP (green fluorescent protein) and their variants (Horn et al., 2002). The GFP gene from the jellyfish *Aequorea victoria* (Prasher et al., 1992) is well suited, since it is easily detectable *in vivo* and has proved to be functional in different tissues of many heterologous systems (Tsien, 1998). It has been used in several organisms as insects, plants, fish, mice and cells of mammals, (Amsterdam et al., 1995; Plautz et al., 1996; Bagis and Keskinetepe, 2001). For the expression control of GFP several promoters have been used. Constitutive promoters active in all cells provide the advantage of allowing selection of transformants at all stages since GFP fluorescence can be scored in living embryos, larvae, and adults. The promoter *polyubiquitin* of *D. melanogaster* successfully employed by Handler and Harrel (1999) allowed the generation of a transformation marker and the identification of transgenic flies in *D. melanogaster*. Another commonly used constitutive promoter to drive EGFP is derived from the *D. melanogaster actin5C* gene tested in *Aedes aegypti*, *Anopheles stephensi* and others insects (Pinkerton et al., 2000; Catteruccia et al, 2000). For the stable germ-line transformation of lepidopteran species, the *Bombyx mori* A3 cytoplasmic actin gene promoter (*BmA3*) was chosen to drive EGFP in the silkworm *B. mori* (Tamura et al., 2000). At present, transformation markers based on constitutive promoters have only been applied to closely related species, and it is questionable if any such promoter can be functional across insect orders (Horn et al., 2002).

Other constructs already tested in *D. melanogaster* and *Tribolium castaneum* which have wide applicability usage as promoters are derived from the *rhodopsin* gene (*3xP3*) from *Drosophila* (Berghaman et al.1999). This is a transformation marker based on a single transcription factor-activated, artificial promoter, containing three tandem repeats of the P3 site in front of a TATA box to drive a strong, eye-specific expression of EGFP (Horn et al., 2002, 2003; Horn and Wimmer, 2000). An evolutionary conserved

genetic circuit governs eye development of all metazoan animals, which is under the control of the transcriptional activator *Pax-6/Eyeless* (Callaerts et al., 1997). This marker construction (*3xP3-EGFP*) expresses most strongly in the brain, eyes, and ocelli in adults, but can also be observed from several structures in pupae and larvae (Handler et al., 1998). The evolutionary conserved “master regulator” function of *Pax-6* in eye development of metazoan suggests that this marker should be applicable to all eye-bearing animals (Callaerts et al., 1997; Handler, 2001; Horn et al., 2002). Furthermore, the small size (1.3 kb) of the *3xP3-EGFP* marker provides an additional advantage, as it allows small transposon constructs resulting in high transformation rates (Horn et al., 2002). This transformation system has been applied to generate transgenic insects in different orders as Diptera, Hymenoptera, Lepidoptera and Coleoptera (Horn et al., 2002; Lorenzen et al., 2003; Robinson et al., 2004; Irvin et al., 2004).

Among the *Drosophila* species, *D. willistoni* stands out for possessing a great genetic variability and ecological versatility, being one of most studied of the family *Drosophilidae*. The genome of this species was chosen to be completely sequenced (<http://bugbane.bio.indiana.edu:7151/>). Despite the great variability and importance in evolutionary and genetic studies, *D. willistoni* has not been tested in experiments of genetic transformation. This work aimed to achieve the genetic transformation of a mutant *white* of *D. willistoni* using the binary system (vector/auxiliar) introducing a gene marker that codified the protein EGFP, through the microinjection of recombinant plasmids in pre-blastoderm embryos. The pattern of expression of the protein in the larva and adult, and the stability of the transformants were analyzed along six generations.

## MATERIALS AND METHODS

### *Drosophila* Stocks and Rearing Conditions

The *D. willistoni white* mutant strain EM1.00 described by Goñi et al. (2002) was used for genetic transformation. The strain were maintained in standard cornmeal medium (Klein et al., 1999) at constant temperature and humidity (20°C +/- 2°C; 60% r.h.).

### Plasmids

The vector pBac [3xP3-EGFP] (Horn and Wimmer, 2000) containing the transposon *piggyBac* inverted terminal sequences (ITRs) necessary for mobility as well as the marker gene EGFP were used. As helper plasmid was used pBf'Sac containing the *piggyBac* functional transposase gene which possesses a deletion in the 5' ITR enabling it to transpose (Handler and Harrel, 1999). The plasmids were prepared according to the PEG (polyethylene glycol) purification protocol (Fujioka et al., 2000). The solution for microinjection had a final concentration of 100 µg/ml of each plasmid in ultra-pure water.

### Microinjection and Cultural Treatments

The methodology used for microinjection and post-injection treatments is described in Deprá et al. (2004). The hatching larvae were maintained separately in banana medium consisting of 20% (w/v) banana and 1.5% (w/v) agar. After pupation, they were transferred to a standard cornmeal medium.

### Analysis of Transformants

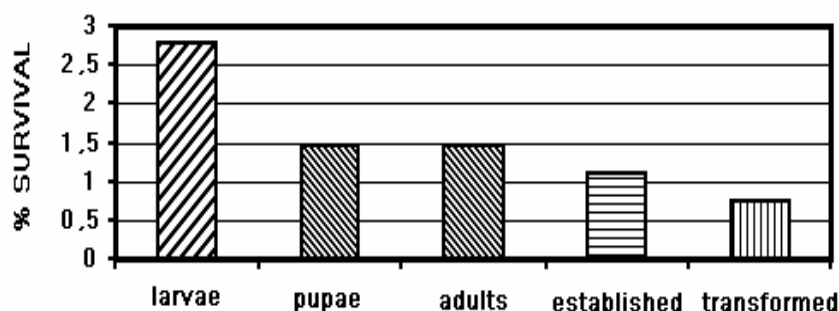
The adult flies outcoming from the microinjected eggs (Go) were backcrossed to *white* flies and the progeny and subsequent generations (G<sub>1</sub>-G<sub>5</sub>) were examined to identify the fluorescence expression. A diodo laser system was used for this analysis where a filter of emission of 510nm was adapted to stereomicroscopic lens. The observations were made in a dark room (Deprá et al. 2004). The stability of the transgenic lineages was analyzed by the transmission of the transgene to the next generation. During six generations, the individuals were routinely examined and the frequency of larvae and adults that manifested fluorescence was annotated.

## RESULTS AND DISCUSSION

A total of 539 embryos (Go) were injected with a mixture of vector and helper plasmids, obtaining 15 larvae and 8 adults. Go adults were backcrossed to *white* flies, six of them were fertile allowing to establish six isolines. Expression of the fluorescent protein EGFP could be observed in the progeny of four of the isolines established (Fig. 1). The rate of genetic transformation of the Go adults was of 66.7%. This rate is high when compared with that

obtained by other authors for other species. In a review about the use of different transposons and genetic markers, Handler and Harrel (1999)

describe transformation rates, to *piggyBac*-GFP varying from 6 to 7%.

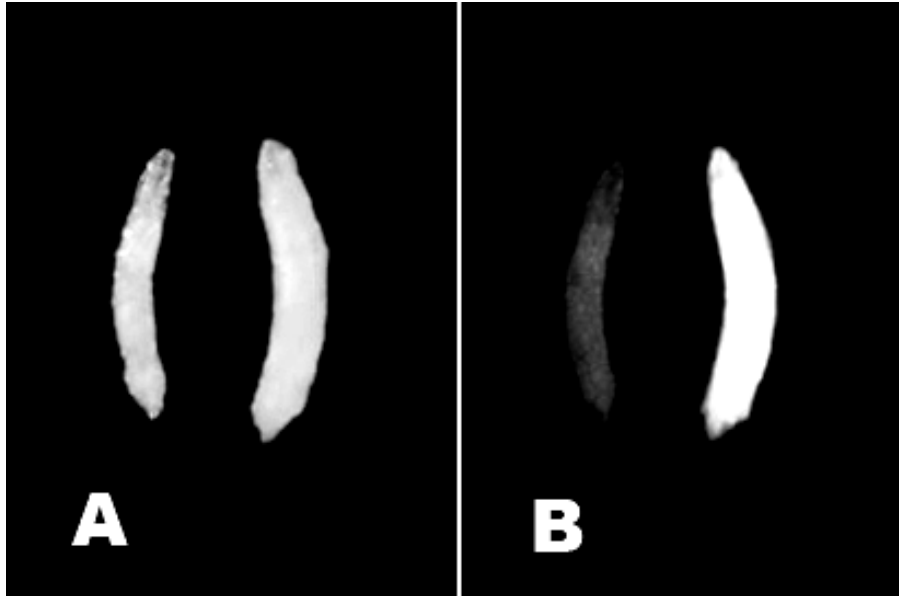


**Figure 1** - Survival percentages in larvae, pupae and adult, and percentage of established and transformed lineages

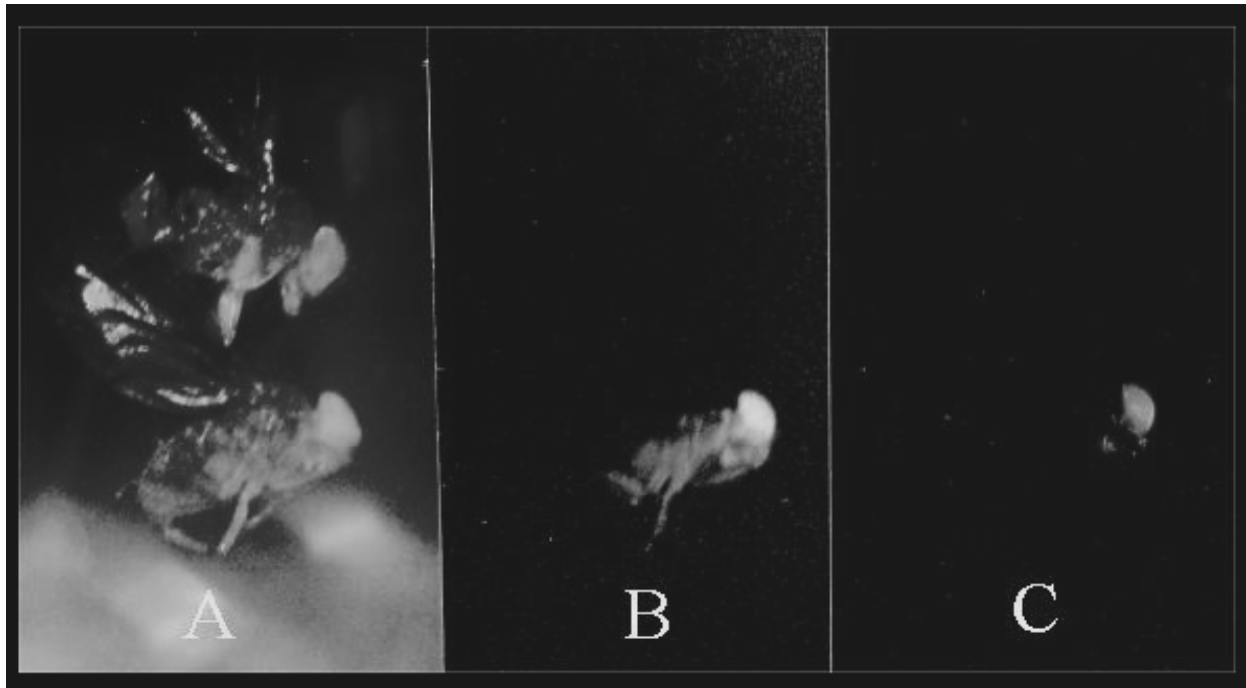
According to Horn et al. (2002), one of the obstacles for the use of fluorescent markers in insects is the auto fluorescence generated by many biological materials, as ingested foodstuff, malpighian tubules, the chitinous exoskeleton and necrotic tissue. Therefore, an appropriate emission filter was used to help distinguish the yellowish auto fluorescence of the fluorescence green resultant of the expression of EGFP in the flies. Using this method, it was possible to verify that all the transformed larvae expressed the protein GFP in the whole body, but they presented variation in the intensity among the lineages. The fluorescence of GFP easily could be differentiated of the auto fluorescence common in controls larvae (Fig.2). Although, for adults another pattern was observed, the expression was more intense in the eyes; however, it could also be observed in the abdomen, thorax and legs (Fig. 3).

The results demonstrated that the *piggyBac* transposable element was an efficient gene-transfer vector system for *Drosophila willistoni*. Actually, it was much more efficient to *D. willistoni* genetic transformation, producing a higher frequency of transformants than in other dipterans so far investigated, as *D. melanogaster* and *Ceratitis capitata* (Handler et al., 1998). The transposition rate for each transposon varied

amongst the tested species. The *piggyBac* transposon could be extremely active in *D. willistoni*. Other fact that could be an indicative of high transposition activity of *piggyBac* in this species was the observation that GFP expression was maintained for only three generations what could be explained by a lost, in the isolate, caused by mobilization of the transposon carrying the EGFP gene. While *piggyBac* was not present in the *D. willistoni* genome, some intrinsic transposase source present in the *D. willistoni* genome could be able to mobilize *piggyBac*. As illustration of this kind of cross-mobilization, was *hobo* transposable element from *D. melanogaster* that could be mobilized by intrinsic sources in a large number of *Drosophilidae* species (Handler and Gomez, 1995). Other hypothesis for the missing of EGFP expression in *D. willistoni* in the older generations could be the inactivation of the chromosomal region containing the transgene. Garcia (2004) demonstrated that *D. willistoni* presented a DNA methylation mechanism not present in *D. melanogaster*. This mechanism could be involved in gene inactivation, mainly in those regions with transposable elements.



**Figure 2** - Pattern of 3xP3-EGFP gene expression in *D. willistoni* transgenic larvae. (A) Control (left) and transformed (right) larva. (B) Yellowish auto fluorescence (left) and the green fluorescence expression of EGFP (right) in the whole larvae.



**Figure 3** - Pattern of 3xP3-EGFP gene expression in *D. willistoni* transgenic flies. (A) Control (top) and transformed (bottom) flies. (B) Pattern of expression in the eyes was the most intense, however, fluorescence could also be observed in the abdomen, thorax and legs. (C) Eyes fluorescence detected at low time of film exposition

A high mortality at the egg-larvae developmental stage, about 97.2% was observed, a similar rate was also described by several authors (Irvin et al., 2004). Usually, this high mortality was due to problems associated with the technique or to the specific stresses caused by manipulation. According to O'Brochta and Atkinson (2004), the preparation of embryos for injection, the insertion of needles in the eggs and the procedures post-injection were critical steps for the success in the development and maintenance of flies and it was characteristic for each species. Hanazono et al (1997) suggested that the high mortality at the beginning of the development could be associated with the possible toxicities of the GFP and its variants, when expressed in high levels.

According to Atkinson (2002), the transposable elements and the fluorescent proteins seemed to have general application in the production of transgenic insects. However, the use of appropriate promoters to command the expression of the reporter proteins, as well as the stability of the transgene, could depend, or be influenced by the species or the family of the insect to be transformed.

Horn and Wimmer (2000) and Horn et al. (2002, 2003) described the expression of the GFP under the tissue-specific artificial promoters control (*3xP3*) in several species of *Drosophila* and observed the reporters protein expression in the eyes and ocelli of adult flies. However, some of the transformants of *D. willistoni* showed a wider expression that was previously described in other species. As suggested by Atkinson (2002) this promoter could have a diverse expression in different species as it happened in *D. willistoni*. Lorenzen et al. (2003) also obtained the most obvious expression in the eyes and brain with *Tribolium castaneum*. However, in several additional insertions novel EGFP expression patterns appeared, suggesting that the EGFP marker was influenced by chromosomal enhancers sequences near the sites of *piggyBac* integration. This was not surprising, since previous reports have shown that the 3xP3 promoter acted as an enhancer detector (Horn et al., 2002, 2003). As seen in the Figs. 2 and 3, the EGFP expression was not restricted solely to the eyes in adults. This was an interesting observation considering the modest phylogenetic distance splitting *D. willistoni* and *D.*

*melanogaster*, and the fact that 3xP3 promoter worked precisely in the later species.

The results of the experiment presented here demonstrated that the transformation system composed by transposon *piggyBac* and marker EGFP was quite efficient to mediate the transformation in *D. willistoni*. Meanwhile, no stable isolines were obtained suggesting a high mobilization of the *piggyBac* in this species. The utilization of a concentration of a plasmid smaller than the one usually employed by other researchers (Handler et al., 1998) did not impede the successful genetic transformation of *D. willistoni*, being the species that showed the highest transformation rate using *piggyBac* as a vector.

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## RESUMO

Descrevemos neste trabalho a transformação genética de *Drosophila willistoni* empregando o elemento transponível *piggyBac* como vetor e o gene EGFP (*green fluorescent protein*) retirado da água-viva *Aequorea victoria*, como marcador de transformação. Embriões de *D. willistoni* em estágio pré-blastoderme, mutantes para o gene *white*, foram microinjetados com plasmídeo contendo o marcador EGFP e as regiões ITRs do transposon *piggyBac* concomitantemente com um plasmídeo auxiliar possuindo o gene da transposase de *piggyBac* sobre o controle do promotor do gene *hsp70* de *Drosophila melanogaster*. Adultos transformantes foram gerados em uma taxa de 67%. A expressão de GFP em larvas, pupas e adultos foi observada somente até a terceira geração, sugerindo que este transposon não é estável em *D. willistoni*. Os indivíduos transformados exigem um alto nível de expressão de EGFP durante os estágios de larva e, também em adultos o gene marcador é expresso

nos olhos, abdome, tórax e patas, mostrando um padrão de expressão mais amplo nesta espécie do que o registrado para outros drosofilídeos.

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