Development of *piggyBac* transposon-derived gene vectors and their utilization for transgenic insects

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Introduction

The biotechnology necessary for the genetic transformation of insects is central to furthering the understanding of the genetic and biochemical basis of insect biology. It will also facilitate the design of efficient, species specific and environmentally benign strategies to control the population and behavior of beneficial and pest insects. The technology will also permit the utilization of insects for pharmaceutical and agricultural activities in ways that have never been possible. All of these applications are predicated on the ability to introduce foreign genes stably and efficiently into the genome of the host insect. The original report of insect transformation with the P-element transposon in *Drosophila melanogaster* (Rubin & Spradling, 1982) stimulated experiments to apply P-element vectors directly in a wide variety of insect species. Although highly successful in *Drosophila*, P-element ultimately could not be applied to transformation of non-drosophilid insects most likely because species-specific host cell factors are required for P-element transposition (Rio, 1990; Handler and Gomez, 1995). However, efforts made during the past six years have led to the development of additional genetic-transformation technologies that are applicable to broad groups of insects (for reviews see Handler and James, 2000; Atkinson et al., 2001; Handler, 2000, 2001).

The transposon-based gene vectors for insects are from four different families of eukaryotic class II transposable elements and have been used to genetically transform species within three orders of insects. Class II transposons have short terminal repeats surrounding a single open reading frame (ORF) that encodes for a protein that supplies the transposase enzyme activity important to the transposition of the DNA sequence from one site to another within the host genome. The four gene vector systems for insects are transposon derivatives of *Hermes* isolated from the house fly *Musca domestica* (Warren et al., 1994), *Minos* isolated from *Drosophila hydei* (Franz and Savakis, 1991), *Mos1,* which is a derivative of *mariner,* isolated from *Drosophila mauritiana* (Medhora et al., 1991) and *piggyBac* isolated from the cabbage looper, *Trichoplusia ni* (Cary et al., 1989).

The four transposon-based gene vector systems are based on the P-element paradigm that separates the two functional components of the transposon, i.e., the inverted terminal repeats and the transposase gene, into two separate plasmids that are co-injected into syncytial embryos (Rubin & Spradling, 1982). The transposase gene is cloned into a plasmid without the terminal repeats and supplies the transposase enzymatic activity. A gene expression cassette flanked by the transposon inverted terminal repeats is cloned into a second plasmid and provides the mobile sequence of DNA. Co-injection of vector and helper into syncytial embryos permits the mobilization of the vector sequence from the plasmid into the host genome under the action of the transposase from the helper plasmid. The vector insertion in the host genome is stable over time because the helper plasmid is not propagated through subsequent cell divisions.

The first successful transformation of a non-drosophilid insect was achieved in the

medfly, *Ceratitis capitata*, with the *Tc*1-like element *Minos* (Loukeris et al., 1995b). *Minos* was used to transform *D. melanogaster* (Loukeris et al., 1995a) and *Anopheles stephensi* (Catteruccia et al., 2000) as well and has demonstrated transposition activity in several different *Anopheles gambiae* and *Spodoptera fugiperda* cell lines. The *Hermes* transposon has been used to generate stable transgenic lines from six insect species: *D. melanogaster* (O'Brochta and Atkinson, 1996; Pinkerton et al, 2000), *Aedes aegypti* (Jasinskiene et al, 1998; Pinkerton et al, 2000), *C. capitata* (Michel et al., 2001), *Stomoxys calcitrans* (O'Brochta et al., 2000), and *Tribolium castaneum* (Berghammer et al., 1999). The *Mos1* element was used to transform *D. melanogaster* (Lohe and Hartl, 1996) and *Ae. aegypti* (Coates et al., 1998). The *piggyBac* transposon was used to transform the broadest range of insect species from Diptera, Coleoptera and Lepidoptera orders: *C. capitata* (Handler et al., 1998), *D. melanogaster* (Handler and Harrell, 1999), *Bactrocera dorsalis* (Handler and McCombs, 2000), *Anastrepha suspensa* (Handler and Harrell, 2001), *M. domestica* (Hediger et al., 2001), *Ae. aegypti* (Fraser, unpublished), *Anopheles albimanus* (Perera and Handler, unpublished), *T. castaneum* (Berghammer et al., 1999), *Bombyx mori* (Tamura et al., 2000), and *Pectinophora gossypiella* (Peloquin et al., 2000).

The *piggyBac* transposon was isolated as a DNA insert into the *Autographa californica* multiple nuclear polyhedrosis virus (AcMNPV) genome. Spontaneous FP (few polyhedra) mutants of nuclear polyhedrosis viruses can accumulate following serial propagation of the virus in insect cell cultures (for review see Fraser, 2000). The *piggyBac* transposon (formerly IFP2) was repeatedly isolated as an insertion within the FP locus of mutant AcMNPV or GmMNPV and is a member of the group that has an insertion site preference for TTAA sequences within the viral FP-locus (Cary et al., 1989; Wang et al., 1989; Fraser et al., 1996) as well as other regions of the viral genome, (Wang and Fraser, 1993; Fraser et al., 1995) and within the host cell genome (Wang and Fraser, 1993; Elick, et al., 1996).

The DNA sequence for the *piggyBac* transposon includes the TTAA insertion site at both ends followed by a 13 bp inverted terminal repeat plus additional internal 19 bp inverted repeats located asymmetrically with respect to the ends (Cary et al., 1989). The *piggyBac* ORF encodes for a single unspliced transcript of approximately 2.1 kb in length (Elick et al., 1996). A CAAT box consensus sequence is located at 233 bp from the beginning of the element and consensus AP1 enhancer sequences (Lee et al., 1987) that most likely direct transcription are located both 5' and 3' of the ORF (Bossin and Shirk, unpublished). Computer assisted comparisons of *piggyBac* sequences with other eukaryotic transposons do not reveal significant similarities with other known transposase sequences.

The *piggyBac* gene vector system has been used successfully to genetically transform ten insect species from three different insect orders and is being applied to others. It is the only gene vector that has been successfully applied to Lepidoptera. In an effort to explore some of the parameters that should be considered when utilizing this gene vector system, we have explored methods to optimize the production of transformants using the *piggyBac* gene vector system.

piggyBac Transformation

Varying concentrations of vector and helper were tested in *Drosophila melanogaster* w^m strain in order to determine their effect on the transformation rate. The *piggyBac* vector pB[3xP3EGFPaf] (Berghammer et al., 1999), which contains the Pax 6 artificial binding site that drives expression of the green fluorescent protein (EGFP) in neural and eye tissue, was used because the phenotype was readily scored in larvae and adults. pB[3xP3EGFPaf] was used in combination with the phsp-pBac helper (Handler and Harrell, 1999) which has demonstrated activity in *Drosophila.* The mixture of vector/helper was microinjected into syncytial embryos less than 15 minutes after egg laying at a time when there were fewer than

32 nuclei. Following injection, the eggs were sealed with Krazy Glue®. G0 adults were mated and the frequency of genetic transformation recorded. The expression of GFP in the G1 larvae and adults was determined by examining the larvae and flies with an EGFP filter set (Chroma Technology). The concentration of the phsp-pBac helper ranged from 10 ng/μl to 400 ng/μl (a forty fold increase) with no effect on the rate of transformation (Table 1). From the single pair matings of G0 adults, 16 to 30 % of the strains had G1 progeny with *piggyBac* transformants. The efficiency of the transformation events was exceptionally high in several of the G1transformant lines where 100 % of the progeny were transformed. This suggests that multiple *piggyBac* integrations occurred within both chromosomes of at least one pair of the chromosomes of the G0 parent in these lines. Likewise, the concentration of the pB[3xP3EGFPaf] vector ranged from 150 ng/μl to 600 ng/μl without effect on the transformation rate. By increasing the size of the mobilized element from 3.7 kb to 6.7 kb, the overall rate of transformation was not significantly changed either. These conditions indicate that *piggyBac* transformation can be supported over a wide range of conditions that permits a high efficiency of recovering germline transformants in *Drosophila* and potentially other insects.

Table 1. Efficiency of genetic transformation of *Drosophila melanogaster* w^m with *piggyBac* vectors and helpers.

Vector/Helper	Concentration of V/H (ng/μl)	Number eggs injected	G0 Adults Mated	% Matings GFP Positive	% G1 Progeny GFP Positive
pB[3xP3EGFPaf] (3.7 kb) /phsp-pBac	600:400	720	23 (3%)	17	ND*
	600:400	376	32 (9%)	16	63-97 ($\bar{x}$=80%)
	600:100	1072	59 (6%)	18	ND
	600: 10	1876	56 (3%)	30	3-100 ($\bar{x}$=31%)
	300:200	637	18 (3%)	16	ND
	300:200	913	88 (10%)	63	3-100 ($\bar{x}$=42%)
	300:200	633	27 (4%)	19	ND
	300:200	978	67 (7%)	33	ND
	300:200	928	161 (17%)	19	ND
pPIGA3GFP (4.5 kb) /phsp-pBac	300:200	1123	113 (10%)	31	ND
pB[3xP3EGFP,6A2x1A1] (6.7 kb)/phsp-pBac	300:200	1065	23 (2%)	9	ND
	300:200	1054	37 (3.5%)	8	ND
pB[PUbnlsEGFP] (5.9 kb) /phsp-pBac	150:100	1405	62 (4%)	18	2-24

* Not Determined

Conclusions

The *piggyBac* gene vector can be used to achieve high rates of germ line transformation in insects. By adjusting the timing of the microinjection to a period of embryogenesis when there are very few nuclei to transform, we have established protocols

with consistently high transformation rates. In addition, we have demonstrated that the transposition of *piggyBac* is supported by a very wide range of transposase activity and is not subject to feedback inhibition at high concentrations over a forty fold range of helper. This observation suggests that the *piggyBac* gene vector can be used in most insects to achieve genetic transformation with a high probability of success because the transposition of the gene vector is not restricted by the mechanics of the transposase activity or host factors.

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