Formosan Entomologist
Journal Homepage: entsocjournal.yabee.com.tw

Germline Transformation of the Oriental Fruit Fly Bactrocera dorsalis (Hendel) Using a piggyBac-derived Vector【Research report】

利用 piggyBac 轉基因子進行東方果實蠅之生殖細胞系基因轉型【研究報告】

Shiu-Ling Chen1, Cheng Chang2, Shaw-Yhi Hwang1, and Kuang-Hui Lu1*
陳秀玲1、張誠2、黃紹毅1、路光暉1*

*通訊作者E-mail : khlu@dragon.nchu.edu.tw

Received: 2009/11/12 Accepted: 2009/12/27 Available online: 2010/03/01

Abstract

The oriental fruit fly, Bactrocera dorsalis (Hendel), is a serious pest of fruit crops in most Asian countries including Taiwan. In this study, we have developed a stable germline transformation system with the ultimate goal of using transgenic technology to control this insect pest. A non-drosophilid transposable element, i.e. piggyBac, which contains the red fluorescent reporter gene, DsRed, under control of the Drosophila polyubiquitin promoter, was used to transform B. dorsalis. The average transformation frequency with different ratios of reporter and helper vectors was 5.3%, which is comparable to those reported in other piggyBac-transformed flies. The red fluorescence of DsRed is expressed in all developmental stages of the transgenic B. dorsalis. Transposition assays show that the exogenous fragment is integrated into TTAA sites. In addition, Southern blot analyses verify that the exogenous genes are stably inherited over 65 generations.

摘要

東方果實蠅 (Bactrocera dorsalis (Hendel)) 為亞洲國家及台灣的重要果樹害蟲。本研究，我們利用生殖細胞系轉型 (germline transformation) 技術建立轉基因東方果實蠅 (transgenic orient fruit fly)，期以進一步發展利用昆蟲轉基因技術 (insect transgenic technology) 建立害蟲防治的方法。進行基因轉殖所使用的轉基因子 (transposon) 為 piggyBac；將帶有果蠅泛素啟動子 (Drosophila polyubiquitin promoter) 調控紅色螢光蛋白基因 (DsRed gene) 之 piggyBac 轉基因子載體，以微注射 (microinjection) 注入胚胎內進行細胞轉型作用，使外來基因嵌入果實蠅基因組 (genome) 上。試驗結果顯示轉基因之平均成功率為 5.3%，此與其他同樣以 piggyBac 轉基因子轉殖雙翅目昆蟲之結果相近。轉基因東方果實蠅個體均可於各發育期穩定表現紅色螢光蛋白 (DsRed)。轉位分析 (transposition assay) 證實標的片段嵌入基因組的 TTAA 位置，符合 piggyBac轉因子之作用特性；而南方墨點法 (Southern blot) 則顯示該標的片段可穩定遺傳至子代，目前已超過 65 代。

Key words: germline transformation, transgenesis, piggyBac, microinjection, Bactrocera dorsalis

關鍵詞: 生殖細胞系轉型、基因轉殖、piggyBac 轉基因子、微注射、東方果實蠅。

Full Text: PDF(2.13 MB)

下載其它卷期全文 Browse all articles in archive: http://entsocjournal.yabee.com.tw

台灣昆蟲 30: 27-40 (2010)
Formosan Entomol. 30: 27-40 (2010)

Germline Transformation of the Oriental Fruit Fly *Bactrocera dorsalis* (Hendel) Using a *piggyBac*-derived Vector

Shiu-Ling Chen[1], Cheng Chang[2], Shaw-Yhi Hwang[1], and Kuang-Hui Lu[1]*

[1] Department of Entomology, National Chung Hsing University, Taichung City 40227, Taiwan
[2] Biotechnology Center, National Chung Hsing University, Taichung City 40227, Taiwan

ABSTRACT

The oriental fruit fly, *Bactrocera dorsalis* (Hendel), is a serious pest of fruit crops in most Asian countries including Taiwan. In this study, we have developed a stable germline transformation system with the ultimate goal of using transgenic technology to control this insect pest. A non-drosophilid transposable element, *i.e. piggyBac*, which contains the red fluorescent reporter gene, *DsRed*, under control of the *Drosophila* polyubiquitin promoter, was used to transform *B. dorsalis*. The average transformation frequency with different ratios of reporter and helper vectors was 5.3%, which is comparable to those reported in other *piggyBac*-transformed flies. The red fluorescence of DsRed is expressed in all developmental stages of the transgenic *B. dorsalis*. Transposition assays show that the exogenous fragment is integrated into TTAA sites. In addition, Southern blot analyses verify that the exogenous genes are stably inherited over 65 generations.

Key words: germline transformation, transgenesis, *piggyBac*, microinjection, *Bactrocera dorsalis*

Introduction

Transposable elements (or transposons), short moveable DNA sequences that can independently replicate themselves and then insert that copy into a new position in either the same or another chromosome, provide useful molecular tools for studying gene functions and genetics. Since Rubin and Spradling (1982) first successfully introduced an exogenous gene into the *Drosophila* germline using a *P* element, transposable elements have contributed greatly to our understanding of the biology of the fruit fly, uncovering hundreds of genes involved in insect development, immunity, tissue modeling, embryogenesis, and so on.

Up till now, a number of transposable elements, such as *P*, *Minos*, *piggyBac*, and *Hermes*, have been discovered and successfully used to integrate exogenous

Corresponding email: khlu@dragon.nchu.edu.tw

DNAs into fruit fly and other insect genomes (Handler, 2001; Raphael *et al*., 2004). The *piggyBac* transposon, originally discovered in the cabbage lopper moth *Trichoplusia ni* (Cary *et al*., 1989), has been widely used to transform non-drosophilid insects including insects in Diptera, Lepidoptera, Coleoptera and Hymenoptera (reviewed in Robinson *et al*., 2004). It is mainly used to study different issues, such as germline transformation efficiency, eye color marker gene expression and transformation vector characteristics of various flies (Handler *et al*., 1998; Handler and McCombs, 2000; Hediger *et al*., 2001; Handler and Harrell, 2001b; Heinrich *et al*., 2002; Allen *et al*., 2004) and mosquitoes (reviewed in Handler, 2002). In addition, it has been successfully used on other insects, including the sawfly *Athalia rosae* (Sumitani *et al*., 2003), silkworm *Bombyx mori* (Tamura *et al*., 2000; Thomas *et al*., 2002), pink bollworm *Pectinophora gossypiella* (Peloquin *et al*., 2000) and the red flour beetle *Tribolium castaneum* (Lorenzen *et al*., 2003). In addition to its application in basic research, *piggyBac*- mediated transgenesis is also used to establish insect bioreactors for the production of any proteins of interest (Royer *et al*., 2005; Kurihara *et al*., 2007; Maraki *et al*., 2007; Ogawa *et al*., 2007). Recently, *piggyBac* has been considered as a tool for developing the method for gene therapy (Feschotte, 2006; Shinohara *et al*., 2007; Wilson *et al*., 2007).

Lately, transgenic techniques are being applied to develop strategies for the control of destructive insects and disease vectors. Thomas *et al.* (2000) reported a strategy, named release of insects carrying a dominant lethal gene (RIDL), which applies the transgenic technique of manipulating insect pests to carry a conditional lethal gene in their genome and to then mass release them so that they will mate with wild females and eventually suppress the pest population in the field. They claim that this system can species-specifically control insect pests and improve the efficiency of the conventional sterile insect technique (SIT).

The oriental fruit fly, *Bactrocera dorsalis* (Hendel), infests flowers, sprouts and fruits and causes serious damage to numerous fruits in Taiwan. For many years, chemical insecticides have been used to control *B. dorsalis*; however, this over-dependence on insecticides has resulted in this fly becoming highly insecticide resistant. In order to seek an alternative method for controlling *B. dorsalis* by molecular genetic manipulation, we attempted to develop a germline transformation technique for this fly using *piggyBac*. In this study, we set up the method for *B. dorsalis* transgenesis and established and maintained a stable transgenic line for more than 65 generations. In addition, the integration of the exogenous gene frame with the *B. dorsalis* genome was confirmed by the transposition and Southern blot assays.

Materials and Methods

Fly colony

Oriental fruit flies, *B. dorsalis*, were maintained in the laboratory as described in our previous paper (Chen *et al*., 2008).

Transformation plasmids

The plasmids used for the germline transformation were gifts from Dr. Alfred M. Handler (the USDA-ARS at Gainesville, FL, USA). There are two different constructs: (1) the transposon vector, pBac (PUb-DsRed), consisting of the *Drosophila* polyubiquitin promoter-controlled reporter gene, *i.e. DsRed*, flanked by the inverted terminal repeats of the *piggyBac* (Handler and Harrell, 2001a); and (2) the helper vector, phspBac, containing the *piggyBac* transposase controlled by the *Drosophila* heat shock 70 promoter (Handler and Harrell, 1999).

Embryonic microinjection

For microinjection, both vectors were prepared using the QIAGEN Plasmid Mini Kit (QIAGEN, Valencia, CA, USA) and dissolved in sterile distilled water. The injection mixtures were prepared by *i.e.* mixing 500 ng/μL of the transposon vector with 125, 167, 250, 300, and 500 ng/μL of helper vector, respectively, in the injection buffer (5 mM KCl, 0.1 mM sodium phosphate, pH 6.8). Control flies received equal volumes of injection buffer.

The method for embryonic injection was modified from the standard *Drosophila* procedures (Kiehart *et al.*, 2000). In short, newly laid eggs were collected, washed thoroughly in 0.02% Triton X-100, placed on a glass slide covered with double-sided tape, and desiccated for 8-10 min. Afterwards, the eggs were de-chorionated with sharp-tipped forceps, transferred and lined up using a fine brush to another glass slide covered with double-sided tape, then desiccated further for a few minutes, and then covered with Halocarbon 700 oil (Sigma-Aldrich Co., St. Louis, MO, USA).

The egg microinjection was processed under a stereomicroscope (Leica MZ FLIII, Leica Microsystems GmbH., Wetzlar, Germany) with a glass needle pulled out with a needle puller (World Precision Instruments, Inc. Sarasota. USA). After injection, the eggs were placed in a humidified chamber and heat-shocked at 37°C for 1 h, and then maintained at 28°C for development. The hatched larvae were collected and fed on a regular diet. The G_0 and G_1 adults were each crossed with three wild-type flies, and then the eggs of G_1 were collected and reared under normal conditions.

Isolation of total RNA and RT-PCR

Total RNA (5 μg of each sample) isolated from *B. dorsalis* was used for the first strand cDNA synthesis by SuperScript III (Invitrogen, Carlsbad, CA, USA). The specific PCR primers of *DsRed*, for sense (DsRed-1S): 5'-TGCGCATGGAG GGCACCGTGAAC-3' and for antisense (DsRed-1A): 5'-TCTCGCCCTTCAGCACG CCGTC-3', were designed based on the *DsRed* sequence of pBac(PUb-DsRed). PCR was performed at 94°C for 2 min; followed by 30 cycles of 94°C for 30 sec, 62°C for 30 sec, and 72°C for 50 sec, and a final extension at 72°C for 7 min. The PCR products were subjected to analysis on 1% agarose gels.

DNA cloning and sequence analysis

All PCR products were subcloned into pGEM®-T Easy Vector (Promega, Madison, WI, USA), and the DNA were sequenced with a BigDye Terminator Cycle Sequencing Kit (Applied Biosystems Inc., Foster City, CA, USA) using an ABI Prism® 373 DNA Sequencer (Applied Biosystems). DNA sequences were analyzed with the software of Vector NTI Advance™ 10 (Invitrogen).

Fluorescent protein analysis

DsRed protein was examined under a fluorescent stereo microscope (Leica MZ FLIII) equipped with a Texas Red plus filter set (Exciter: HQ560/55; Emitter: HQ645/75, Leica). Images were collected with a 3.3 MegaPixel CCD camera and adjusted by Soft Imaging System analySIS® (Color View I, Olympus Soft Imaging Solutions PTE Ltd., Japan).

Genomic DNA extraction and transposition assay

To extract genomic DNA, flies were ground up in liquid nitrogen, and the homogenate was digested with proteinase K (20 mg/mL) in the grinding buffer (0.1 M NaCl, 0.1 M Tris-HCl, 0.1 M EDTA, 0.05% SDS, and 0.2 M sucrose) at 55°C for 30 min; and then isolated using the standard phenol/chloroform method.

For the transposition assay, the insertion sites of the target fragment were analyzed with inverse polymerase chain reaction (inverse PCR) based on Sambrook and Russell (2001) with a slight modification. In brief, 5 μg of *B. dorsalis*

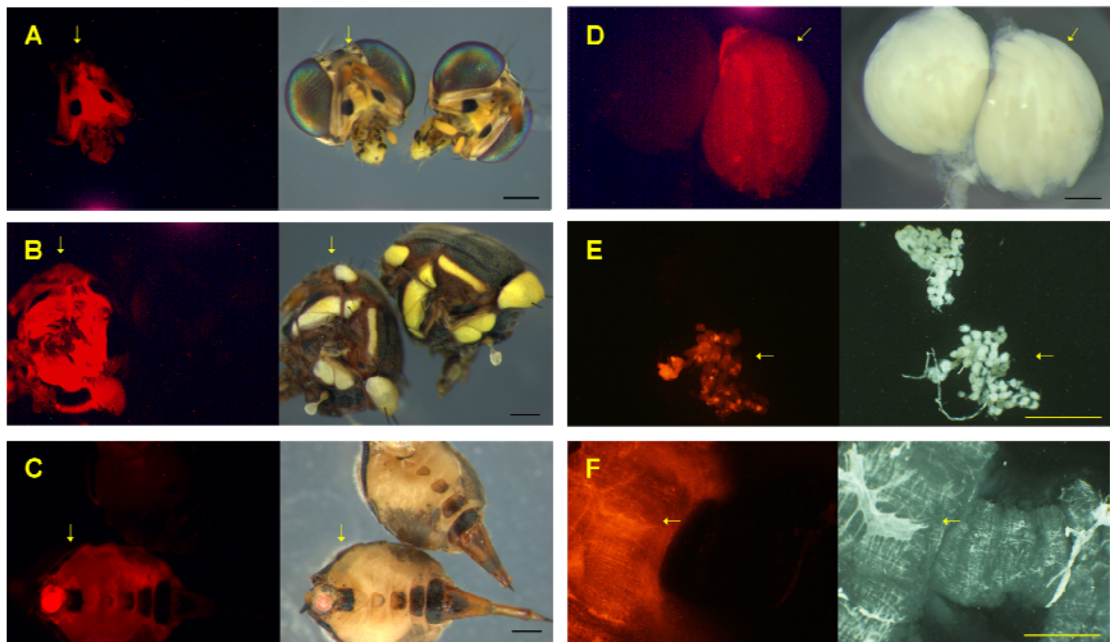


Fig. 1. Expression of the DsRed gene in transgenic *B. dorsalis*. A: head; B: thorax; C: abdomen; D: ovaries; E: fat bodies; F: thoracic muscles. Left panel of each pair was observed under UV light; and right panel was observed under bright field. Arrows indicate the transgenic individuals and the corresponding wild type (unmarked one) body parts and tissues are used as the control. Scale bars = 0.5 mm.

genomic DNA was digested overnight with *Sma*I and purified with phenol/ chloroform. Subsequently, 100 ng of the restriction fragment was self-ligated to form circular genomic DNA. Then sequential PCRs were performed on the circularized fragments using three sets of specific primers as shown in Table 1 and Fig. 3A. The final PCR products were separated in 1% agarose gel; and the expected products were subcloned and sequenced.

Southern blot hybridization

For Southern blot hybridization, the probe, named PD, covering parts of the polyubiquitin promoter and the DsRed gene (Fig. 4A) was obtained by the digestion of pBac(PUb-DsRed) with the restriction enzymes *Xba*I and *Hpa*I, purified with phenol/chloroform, and labeled using DIG-High Prime (Roche Diagnostics GmbH, Mannheim, Germany).

The circularized fragments described above were amplified by PCR with the specific primers, DsRed-1S (the primer for RT-PCR) and anti 5'endsite-1 (Table 1), performed at 94°C for 5 min, followed by 5 cycles of 94°C for 30 sec, 65°C for 15 sec, and 68°C for 3 min, and 35 cycles of 94°C for 30 sec, 60°C for 15 sec, and 68°C for 3 min. The PCR products were subjected to 0.8% agarose gel electrophoresis and blotted onto the Hybond-N^+ nylon membranes (Amersham Biosciences, Chalfont St. Giles, UK). Hybridizations were performed in 0.25 M sodium phosphate buffer (pH 7.5; 1% BSA, and 7% SDS) at 60°C over-night; and then washes with low-stringency buffer (2X SSC, 0.2% SDS) twice at room temperature for 20 min followed by two washes in a high-stringency buffer (1X SSC, 0.1% SDS) at 60°C for 30 min. The result was detected using a DIG Nucleic Acid

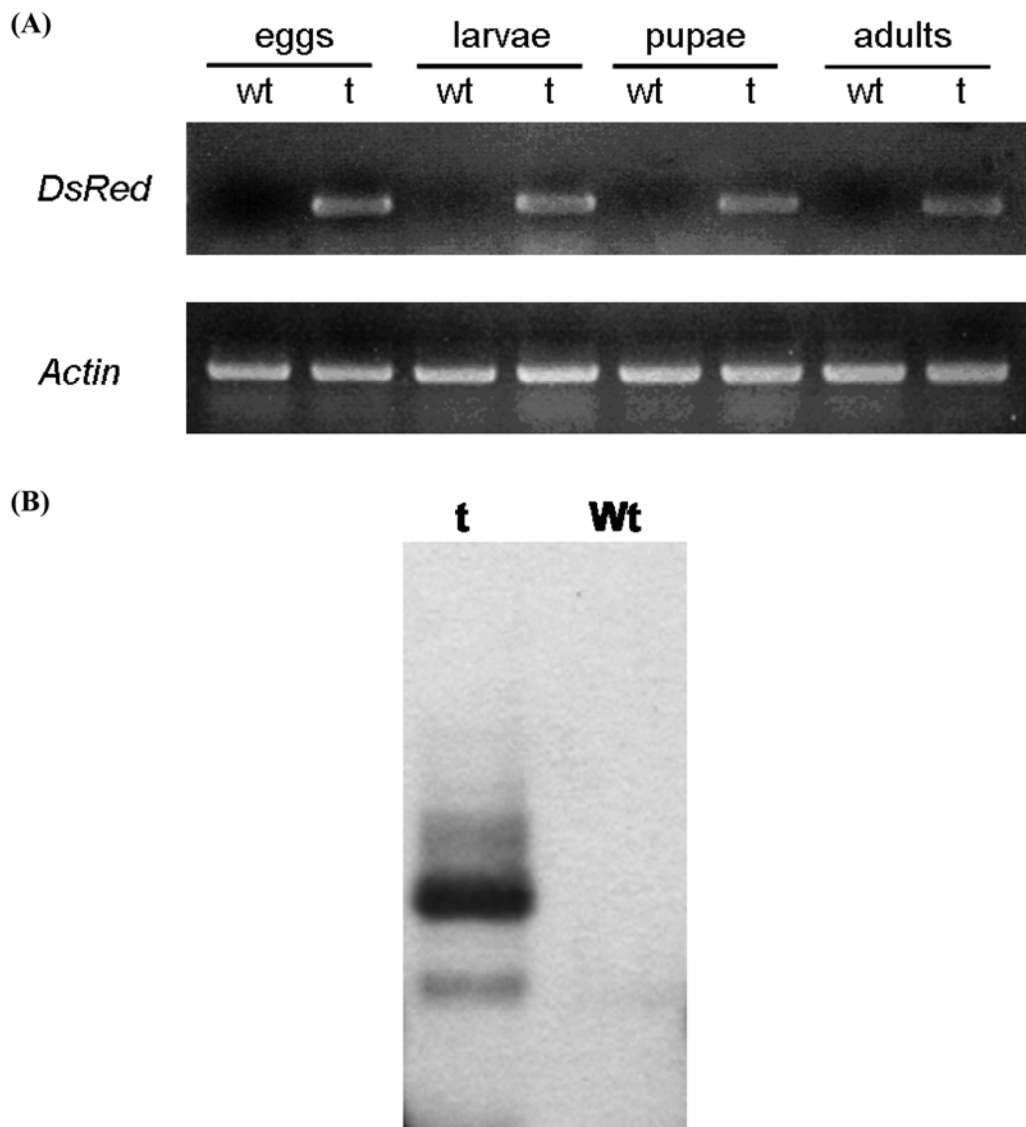


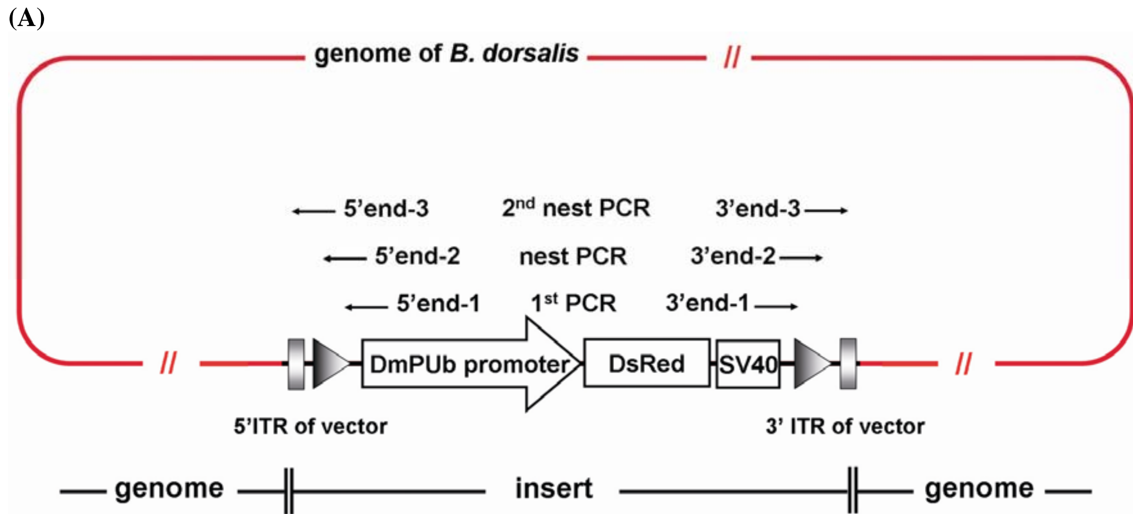
Fig. 2. The DsRed gene expression profile in different stages of transgenic *B. dorsalis*. (A) Total RNA was isolated from eggs, larvae, pupae and adults of wild type (wt) and transgenic *B. dorsalis* (t), respectively. The β-actin gene was used as the internal control. (B) The Southern blot profile. The DNA were extracted the respectively from generation 2 (t) of line B7-8 transgenic *B. dorsalis* and wild type individual (wt), digested by restriction enzyme *Sma*I and detected by the probe of partial DsRed gene.

Detection Kit (Roche Diagnostics) and exposure on X-ray film (Kodak).

Results

Germline transformation using *piggyBac* vector

(A)



(B)

	Sequence of the junction of the *piggyBac* insert and the host genome		
	5' end		3' end
pBac(PUb-DsRed)	taaagcgcaatcttttTTAA		TTAAataatagtttctaattt
t- 8	cctttaagacattttacTTAA		TTCAAtttagtaaatggtgct
t-11	ccaacattaaattttgtTTAA		TTAAatctttacattatactt
t-16	cctttaagacattttacTTAA	insert	TTAAatctttacattatactt
t-18	cctttaagacattttacTTAA		TTAAatctttacattatactt
t-19	cctttaagacattttacTTAA		TCAAtttagtaaatggtgcta
t-20	cctttaagacattttacTTAA		TTAAatctttacattatcatt
t-33	cctttaagacattttacTTAA		TTAAatctttacattatcatt

Fig. 3. Inverse PCR used to identify the sequences of the *piggyBac* insertion sites in the progeny of transgenic strain B7 of *B. dorsalis*. (A) Schematic diagram shows the insertion structure, PCR amplified region, primers sites, and opposite junctions between insert and genomic DNA of *B. dorsalis*; (B) The 5'- and 3'-end flanking sequences of the transgenic vector, pBac(PUb-DsRed) and various transgenic individuals, *i.e.* t-8, t-11, t-16, t-18, t-19, t-20, and t-33. The capital letters are the terminal repeats of the inserted *piggyBac*; and the lower case letters are the *B. dorsalis* genomic DNA sequences.

As shown in Table 2, among nearly 2500 pre-blastodermal embryo injections with the pBac(PUb-DsRed) vector mixed with different concentrations of the phspBac helper vector, a total of 513 larvae hatched, and 32 individuals survived and grew up to adults. These adults were individually crossed with wild-type flies, and finally 19 reproductive individuals were obtained; however, only one of the transformed *B. dorsalis*, namely B7, produced DsRed-expressing progeny. Therefore, the average transformation frequency is about 5.3% (1/19).

In this study, the G_1 progeny of the line B7 was used to analyze the integration of the exogenous gene; and one of them, B7-8, was continuously cultured to observe the transformation stability.

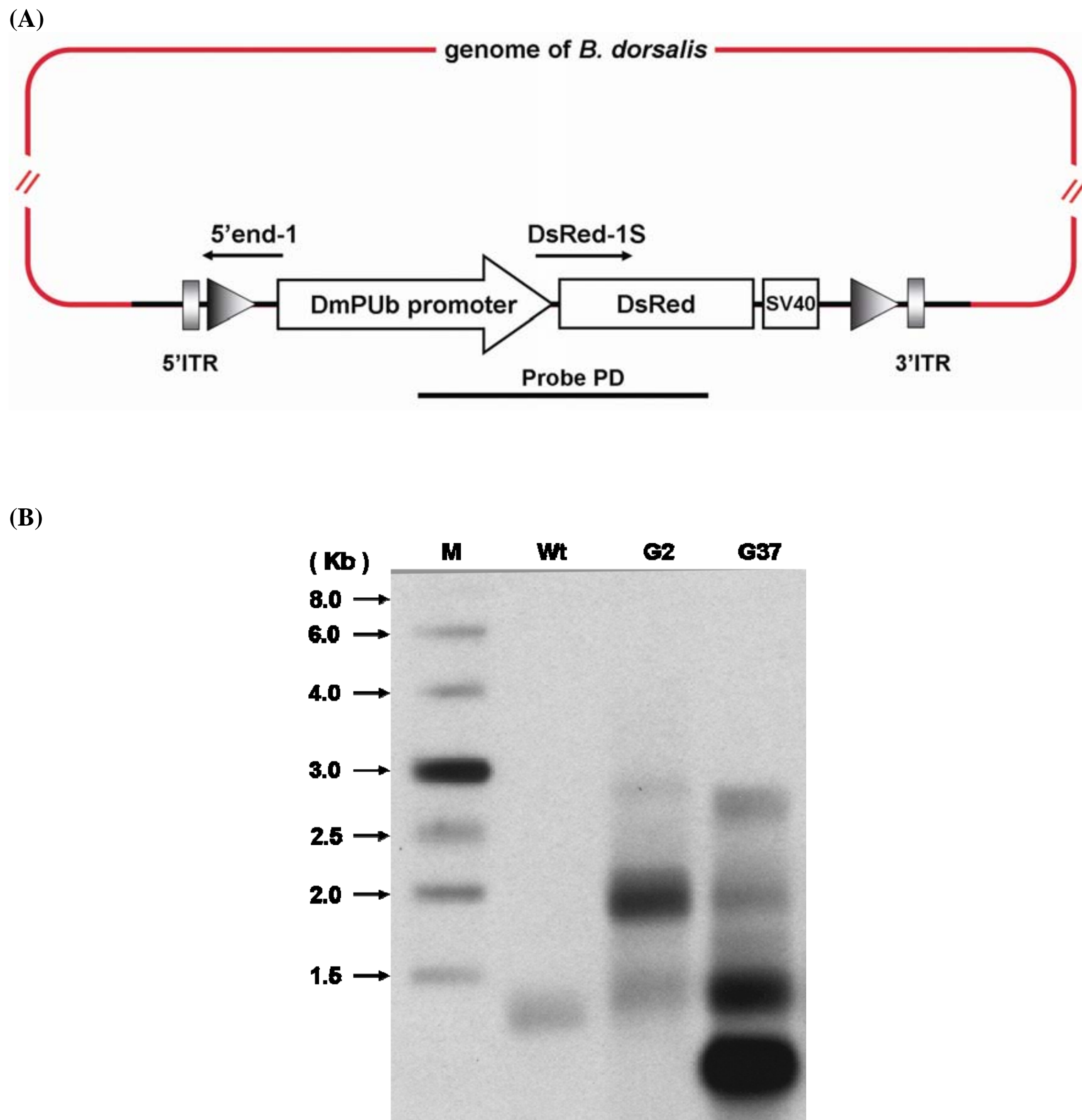


Fig. 4. Southern hybridization analyses of the exogenous fragment in the transgenic *B. dorsalis*, line B7-8. (A) The schematic diagram shows the insertion structure of the transformation construct of *piggyBac*, PCR amplified region, primer sites, and probe region. The PD probe for hybridization contains partial sequences of the DsRed and PUb promoter regions. (B) Southern blot profile. The DNA were extracted respectively from generations 2 and 37 (G2 and G37) of transgenic *B. dorsalis* of line B7-8 and wild type individual (wt), digested by restriction enzyme *Sma*I and detected by the PD probe. M: DNA marker.

Table 1. Primers used for the inverse PCR analysis of the transposition of *piggyBac* in the *B. dorsalis* genome

Primer	Sequence	Position on pBac(PUb-DsRed)
5'end-1	5' GGCGACAAGATCGGGTACCGTC 3'	3146-3167
5'end-2	5' CGCTTTGCAGAAGAGCAGAGAGGATATG 3'	2792-2819
5'end-3	5' CCAAGCGGCGACTGAGATGTCC 3'	2533-2554
3'end-1	5' AGCTATTCCAGAAGTAGTGAGGAGGC 3'	7001-7026
3'end-2	5' CTCCAGGCCAGTGGGAACATCG 3'	7101-7122
3'end-3	5' CCCCTCTAAAATAAGGCGAAAGGCA 3'	7629-7653

Table 2. The outcome of the germline transformation experiments in *B. dorsalis* eggs injected with different ratios of vector-helper mixtures

Plasmid DNA conc. (ng/μL) Vector : Helper	No. of eggs injected	No. of larvae (Hatching rate, %)	No. of G_0 adults (Eclosion rate, %)	No. of reproductive G_0	No. of Transformed G_0*
500 : 125	548	128 (23.36)	6 (1.64)	6	1
500 : 167	475	117 (24,63)	13 (2.11)	6	-
500 : 250	490	101 (20.61)	11 (2.24)	6	-
500 : 300	438	105 (23.93)	2 (0.46)	1	-
500 : 500	507	62 (12.23)	0 (0.00)	-	-
Total No.	2488	513	32	19	1
Saline (control)	307	108 (35.18)	58 (18.89)	-	-

* Transformation frequency = the total numbers of transformed G_0/the total numbers of fertile G_0.

DsRed expression in the transgenic *B. dorsalis*

With the transposon successfully integrated into the *B. dorsalis* genome of line B7, their progeny displayed red fluorescence from DsRed expression in almost all body parts, such as the head, thorax, and abdomen (Fig. 1A-C). The uneven distribution of red fluorescence was probably caused by the variable degree of gene expression in different tissues and/or by shielding of the pigmentation and melanization of the cuticle. The internal tissues, especially muscle, fat body and ovary, were the main places of DsRed expression (Fig. 1D-E).

To date, the transgenic line has been maintained for over 65 generations. The *DsRed* expression appears to be stable, and RT-PCR confirmed strong *DsRed* expression in the embryos, larvae, pupae and adults (Fig. 2). These results indicate that transgenic *B. dorsalis* can stably inherit the marker (or target gene).

Evidence for the integration of the exogenous gene cassette in *B. dorsalis*

The transposition assay for the insertion sites of B7 progeny (G_1, shown with a t-number in Fig. 3B) with inverse PCR revealed that the *piggyBac*-mediated target fragment was indeed integrated into the *B. dorsalis* genome. The conserved sequence of the integrated site of *piggyBac*, *i.e.* TTAA, was identified at the junctions of the inverted terminal repeats (ITRs) of *piggyBac* and the host genomic DNAs in all seven transgenic *B. dorsalis* lines (Fig. 3). Among them, t-16, t-18, t-20, and t-33 possessed the same adjacent genomic DNA sequences at both the 5'- and 3'-ends; however, the 3'-terminal sequences of t-8 and t-19 and the 5'-terminal sequences of t-11 were different from other siblings (Fig.

3), indicating that they had different insertion sites.

Southern blot data showed that the integrated fragments could be detected (Fig. 4), indicating that the exogenous frame was integrated into the genomic DNA of *B. dorsalis*. Distinct patterns were observed between generations 2 and 37 of the line B7-8 digested with *Sma*I (Fig. 4). In the samples of generation 2 (G2), at least 3 bands with different sizes were labeled, while at least 4 bands were labeled in the sample of generation 37 (G37). Moreover, there was a faint band visible in the wild type sample (wt).

Discussion

Low transformation frequency in transgenesis

Several methods, including microinjection, electroporation (Moto *et al*., 1999), biolistics, and transfection reagents, have been used for insect transgenesis (Kiehart *et al*., 2000). For microinjection of insects, the most difficult step is how to soften or strip the eggshell. In *Drosophila* or *Ceratitis capitata*, dechorionation with diluted hypochlorite solution (Handler, 2000) has been commonly used, but this method was not successful for processing *B. dorsalis* eggs. Therefore, we developed a method for removing *B. dorsalis* eggshells with sharp-tipped forceps. This method worked well for our system.

The final average transformation frequency in this fly was 5.3% (Table 2), which is comparable to other dipteran species transformed with the *piggyBac* vector (Handler *et al*., 1998; O'Brochta *et al*., 2000). Low transformation frequencies of germline transformation were also noted in other insects (Catteruccia *et al*., 2000; O'Brochta *et al*., 2000; Peloquin *et al*., 2000). There is still no firm evidence to explain the cause of the low efficiency, although some possible reasons have been proposed. Handler and Harrell (1999) reported that the increasing vector size causes a decrease of the transformation frequency; Sarkar *et al*. (1997) suggested that the presence of endogenous transposable elements might affect the transformation frequency; and Li *et al*. (2001) and Kapetanaki *et al*. (2002) pointed out that the low frequency might be due to the low expression level of the transposase.

Marker selection in germline transformation

In order to establish a transgenic line and determine its stability, an appropriate selection marker is very critical. In recent researches on insect transgenesis, the green fluorescent protein (GFP) or its enhanced derivative (EGFP) has been widely used as the selection marker in dipteran and lepidopteran insects (Handler and Harrell, 1999; Tamura *et al*., 2000; Peloquin *et al*., 2000; Pinkerton *et al*., 2000). However, it is not suitable for *B. dorsalis* transgenesis because of a significant autofluorescence of *B. dorsalis* tissues under UV illumination. This drawback is readily solved by replacing EGFP with DsRed. Up to now, the expression of *DsRed* under the control of a polyubiquitin promoter in transgenic *B. dorsalis* has been strong, ubiquitous and constitutive throughout the entire development and for almost all tissues, which is quite similar to that of *GFP* in the Caribbean fruit fly (Handler and Harrell, 2001b). These expression characteristics are also similar to those of the *polyubiquitin* in *Drosophila* (Lee *et al*., 1988).

Re-translocation of ectopic insertions may be caused by potential endogenous transposase in transgenic *B. dorsalis*

As shown in our results, the integration of an exogenous gene into the *B. dorsalis* genome has been confirmed by Southern blot and transposition assay. However, we were unable to ascertain

whether the transgenic status is stable in the fly. Handler and McComb (2000) indicated that there are approximately 10-20 transposon-like elements in two strains of *B. dorsalis*. Furthermore, *piggyBac*-like sequences were reported as well in other dipterans and lepidopterans, including *D. melanogaster* (Sarkar *et al*., 2003), *Bactrocera* spp. (Handler and McComb, 2000; Bonizzoni *et al*., 2007) and some moths (Wang *et al*., 2006; Xu *et al*., 2006; Zimowska and Handler, 2006; Sun *et al*., 2008). In the present study, the Southern blot results showed that the integrated sites appear to have changed after several generations (Fig. 4B). This might be because it is possible that endogenous *piggyBac*-like elements exist in our *B. dorsalis*. In addition, the faint labeling shown in the wild type (Wt) as detected by the PD probe (Fig. 4B) indicates that the homologous sequences of the promoter of the ubiquitin gene or its promoter also exist in the *B. dorsalis* genome.

In conclusion, although the phenomenon of re-translocation remains to be confirmed, we have successfully established a germline transformation in *B. dorsalis*, and the transgenic marker, DsRed, remains stably expressed after more than 65 generations. These results indicate that using the RIDL concept to control *B. dorsalis* is feasible although the release of transgenic insects into the field to control pests is impermissible at the moment. Moreover, using transgenic *B. dorsalis* as a bioreactor to produce valuable exogenous proteins is also possible.

Acknowledgments

We like to thank Dr. Alfred M. Handler (the USDA-ARS at Gainesville, FL, USA) for providing the plasmids pBac (PUb-DsRed) and phspBac. We also like to thank Dr. Chih-Ning Sun for her critical reading and valuable discussion of this article. This work was supported by grants 92AS-1.8.1-BQ-B3 and 93AS-1.8.1-BQ-B3 from the Bureau of Animal and Plant Health Inspection and Quarantine (Council of Agriculture, ROC). We also like to acknowledge the support from the ATU plan of the Ministry of Education, ROC, for the publication of this paper.

References

Allen, M. L., A. M. Handler, D. R. Berkebile, and S. R. Skoda. 2004. *piggyBac* transformation of the New World screwworm, *Cochliomyia hominivorax*, produces multiple distinct mutant strains. Med. Vet. Entomol. 18: 1-9.

Bonizzoni, M., L. M. Gomulski, A. R. Malacrida, P. Capy, and G. Gasperi. 2007. Highly similar *piggyBac* transposase-like sequences from various *Bactrocera* (Diptera, Tephritidae) species. Insect Mol. Biol. 16: 645-650.

Cary, L. C., M. Goebel, B. G. Corsaro, H. G. Wang, E. Rosen, and M. J. Fraser. 1989. Transposon mutagenesis of baculoviruses: analysis of *Trichoplusia ni* transposon IFP2 insertions within the FP-locus of nuclear polyhedrosis viruses. Virology 172: 156-169.

Catteruccia, F., T. Nolan, T. G. Loukeris, C. Blass, C. Savakis, F. C. Kafatos, and A. Crisanti. 2000. Stable germline transformation of the malaria mosquito *Anopheles stephensi*. Nature 405: 959-962.

Chen, S. L., S. M. Dai, K. H. Lu, and C. Chang. 2008. Female-specific *doublesex* dsRNA interrupts yolk protein gene expression and reproductive ability in oriental fruit fly, *Bactrocera dorsalis* (Hendel). Insect Biochem. Mol. Biol. 38: 155-165.

Feschotte, C. 2006. The *piggyBac* transposon holds promise for human gene therapy. Proc. Natl. Acad. Sci. USA 103: 14981-14982.

Handler, A. M. 2000. An introduction to the history and methodology of insect gene transfer. pp. 3-26. *In*: A. M.

Handler, and A. A. James, eds. Insect Transgenesis: Methods and Applications. CRC Press, New York.

Handler, A. M. 2001. A current perspective on insect gene transfer. Insect Biochem. Mol. Biol. 31: 111-128.

Handler, A. M. 2002. Use of the *piggyBac* transposon for germ-line transformation of insects. Insect Biochem. Mol. Biol. 32: 1211-1220.

Handler, A. M., and R. A. Harrell. 1999. Germline transformation of *Drosophila melanogaster* with the *piggyBac* transposon vector. Insect Mol. Biol. 8: 449-457.

Handler, A. M., and R. A. Harrell. 2001a. Polyubiquitin-regulated DsRed marker for transgenic insects. BioTechniques 31: 820-828.

Handler, A. M., and R. A. Harrell. 2001b. Transformation of the Caribbean fruit fly with a *piggyBac* transposon vector marked with polyubiquitin-regulated GFP. Insect Biochem. Mol. Biol. 31: 199-205.

Handler, A. M., and S. D. McCombs. 2000. The *piggyBac* transposon mediates germ-line transformation in the oriental fruit fly and closely related elements exist in its genome. Insect Mol. Biol. 9: 605-612.

Handler, A. M., S. D. McCombs, M. J. Fraser, and S. H. Saul. 1998. The lepidopteran transposon vector, *piggyBac*, mediates germ-line transformation in the Mediterranean fruit fly. Proc. Natl. Acad. Sci. USA 95: 7520-7525.

Hediger, M., M. Niessen, E. A. Wimmer, A. Dubendorfer, and D. Bopp. 2001. Genetic transformation of the housefly *Musca domestica* with the lepidopteran derived transposon *piggyBac*. Insect Mol. Biol. 10: 113-119.

Heinrich, J. C., X. Li, R. A. Henry, N. Haack, L. Stringfellow, A. C. G. Heath, and M. J. Scott. 2002. Germ-line transformation of the Australian sheep blowfly *Lucilia cuprina*. Insect Mol. Biol. 11: 1-10.

Kapetanaki, M. G., T. G. Loukeris, I. Livadaras, and C. Savakis. 2002. High frequencies of *Minos* transposon mobilization are obtained in insects by using *in vitro* synthesized mRNA as a source of transposase. Nucleic Acids Res. 30: 3333-3340.

Kiehart, D. P., J. M. Crawford, and R. A. Montague. 2000. Quantitative microinjection of *Drosophila* embryos. pp. 345-359. *In*: W. Sullivan, M. Ashburner, and R. S. Hawley, eds. Drosophila Protocol. Cold Spring Harbor Laboratory Press, New York.

Kurihara, H., H. Sezutsu, T. Tamura, and K. Yamada. 2007. Production of an active feline interferon in the cocoon of transgenic silkworms using the fibroin H-chain expression system. Biochem. Biophys. Res. Commun. 355: 976-980.

Lee, H. S., J. A. Simon, and J. T. Lis. 1988. Structure and expression of ubiquitin genes of *Drosophila melanogaster*. Mol. Cell. Biol. 8: 4727-4735.

Li, X., J. C. Heinrich, and M. J. Scott. 2001. *piggyBac*-mediated transposition in *Drosophila melanogaster*: an evaluation of the use of constitutive promoters to control transposase gene expression. Insect Mol. Biol. 10: 447-455.

Lorenzen, M. D., A. J. Berghammer, S. J. Brown, R. E. Denell, M. Klingler, and R. W. Beeman. 2003. *piggyBac*-mediated germline transformation in the beetle *Tribolium castaneum*. Insect Mol. Biol. 12: 433-440.

Markaki, M., D. Drabek, I. Livadaras, R. K. Craig, F. Grosveld, and C. Savakis. 2007. Stable expression of human growth hormone over 50 generations in transgenic insect larvae. Transgenic Res. 16: 99-107.

Moto, K., S. E. A. Salam, S. Sakurai, and M. Iwami. 1999. Gene transfer into insect brain and cell-specific expression of *bombyxin* gene. Dev. Genes Evol.

209: 447-450.

O'Brochta, D. A., P. W. Atkinson, and M. L. Lehane. 2000. Transformation of *Stomoxys calcitrans* with a *Hermes* gene vector. Insect Mol. Biol. 9: 531-538.

Ogawa, S., M. Tomita, K. Shimizu, and K. Yoshizato. 2007. Generation of a transgenic silkworm that secretes recombinant proteins in the sericin layer of cocoon: production of recombinant human serum albumin. J. Biotechnol. 128: 531-544.

Peloquin, J. J., S. T. Thibault, R. Staten, and T. A. Miller. 2000. Germ-line transformation of pink bollworm (Lepidoptera: Gelechiidae) mediated by the *piggyBac* transposable element. Insect Mol. Biol. 9: 323-333.

Pinkerton, A. C., K. Michel, D. A. O'Brochta, and P. W. Atkinson. 2000. Green fluorescent protein as a genetic marker in transgenic *Aedes aegypti*. Insect Mol. Biol. 9: 1-10.

Raphael, K. A., S. Whyard, D. Shearman, X. An, and M. Frommer. 2004 *Bactrocera tryoni* and closely related pest tephritids-molecular analysis and prospects for transgenic control strategies. Insect Biochem. Mol. Biol. 34: 167-176.

Robinson, A. S., G. Franz, and P. W. Atkinson. 2004. Insect transgenesis and its potential role in agriculture and human health. Insect Biochem. Mol. Biol. 34: 113-120.

Royer, C., A. Jalabert, M. Da Rocha, A. M. Grenier, B. Mauchamp, P. Couble, and G. Chavancy. 2005. Biosynthesis and cocoon-export of a recombinant globular protein in transgenic silkworms. Transgenic Res. 14: 463-472.

Rubin, G. M., and A. C. Spradling. 1982. Genetic transformation of *Drosophila* with transposable element vectors. Science 218: 348-353.

Sambrook, J., and D. W. Russell. 2001. In vitro amplification of DNA by the polymerase chain reaction. pp. 8.1-8.113. *In*: J. Sambrook, and D. W. Russell, eds. Molecular Cloning, A Laboratory Manual. Cold Spring Harbor Laboratory Press, New York.

Sarkar, A., C. J. Coates, S. Whyard, U. Willhoeft, P. W. Atkinson, and D. A. O'Brochta. 1997. The *Hermes* element from *Musca domestica* can transpose in four families of cyclorrhaphan flies. Genetica 99: 15-29.

Sarkar, A., C. Sim, Y. S. Hong, J. R. Hogan, M. J. Fraser, H. M. Robertson, and F. H. Collins. 2003. Molecular evolutionary analysis of the widespread *piggyBac* transposon family and related "domesticated" sequences. Mol. Gen. Genomics 270: 173-180.

Shinohara, E. T., J. M. Kaminski, D. J. Segal, P. Pelczar, R. Kolhe, T. Ryan, C. J. Coates, M. J. Fraser, A. M. Handler, R. Yanagimachi, and S. Moisyadi. 2007. Active integration: new strategies for transgenesis. Transgenic Res. 16: 333-339.

Sumitani, M., D. S. Yamamoto, K. Oishi, J. M. Lee, and M. Hatakeyama. 2003. Germline transformation of the sawfly, *Athalia rosae* (Hymenoptera: Symphyta), mediated by a *piggyBac*-derived vector. Insect Biochem. Mol. Biol. 33: 449-458.

Sun, Z. C., M. Wu, T. A. Miller, and Z. J. Han. 2008. *piggyBac*-like elements in cotton bollworm, *Helicoverpa armigera* (Hübner). Insect Mol. Biol. 17: 9-18.

Tamura, T., C. Thibert, C. Royer, T. Kanda, E. Abraham, M. Kamba, N. Kômoto, J. L. Thomas, B. Mauchamp, G. Chavancy, P. Shirk, M. Fraser, J. C. Prudhomme, and P. Couble. 2000. Germline transformation of the silkworm *Bombyx mori* L. using a *piggyBac* transposon-derived vector. Nat. Biotechnol. 18: 81-84.

Thomas, D. D., C. A. Donnelly, R. J. Wood, and L. S. Alphey. 2000. Insect population control using a dominant, repressible, lethal genetic system. Science 287: 2474-2476.

Thomas, J. L., M. Da Rocha, A. Besse, B. Mauchamp, and G. Chavancy. 2002. 3xP3-EGFP marker facilitates screening for transgenic *Bombyx mori* L. from the embryonic stage onwards. Insect Biochem. Mol. Biol. 32: 247-253.

Wang J., X. Ren, T. A. Miller, and Y. Park. 2006. *piggyBac*-like elements in the tobacco budworm, *Heliothis virescens* (Fabricius). Insect Mol. Biol. 15: 435-443.

Wilson, M. H., C. J. Coates, and A. L. George Jr. 2007. *PiggyBac* transposon-mediated gene transfer in human cells. Mol. Ther. 15: 139-145.

Xu, H. F., Q. Y. Xia, C. Liu, T. C. Cheng, P. Zhao, J. Duan, X. F. Zha, and S. P. Liu. 2006. Identification and characterization of *piggyBac*-like elements in the genome of domesticated silkworm, *Bombyx mori*. Mol. Gen. Genomics 276: 31-40.

Zimowska, G. J., and A. M. Handler. 2006. Highly conserved *piggyBac* elements in noctuid species of Lepidoptera. Insect Biochem. Mol. Biol. 36: 421-428.

Received: November 12, 2009
Accepted: December 27, 2009

利用 ***piggyBac*** 轉基因子進行東方果實蠅之生殖細胞系基因轉型

陳秀玲[1]、張誠[2]、黃紹毅[1]、路光暉[1*]

[1] 國立中興大學昆蟲學系 40227 台中市南區國光路 250 號
[2] 國立中興大學生物科技發展中心 40227 台中市南區國光路 250 號

摘　　要

東方果實蠅 (*Bactrocera dorsalis* (Hendel)) 為亞洲國家及台灣的重要果樹害蟲。本研究，我們利用生殖細胞系轉型 (germline transformation) 技術建立轉基因東方果實蠅 (transgenic orient fruit fly)，期以進一步發展利用昆蟲轉基因技術 (insect transgenic technology) 建立害蟲防治的方法。進行基因轉殖所使用的轉基因子 (transposon) 為 *piggyBac*；將帶有果蠅泛素啟動子 (*Drosophila* polyubiquitin promoter) 調控紅色螢光蛋白基因 (DsRed gene) 之 *piggyBac* 轉基因子載體，以微注射 (microinjection) 注入胚胎內進行細胞轉型作用，使外來基因嵌入果實蠅基因組 (genome) 上。試驗結果顯示轉基因之平均成功率為 5.3%，此與其他同樣以 *piggyBac* 轉基因子轉殖雙翅目昆蟲之結果相近。轉基因東方果實蠅個體均可於各發育期穩定表現紅色螢光蛋白 (DsRed)。轉位分析 (transposition assay) 證實標的片段嵌入基因組的 TTAA 位置，符合 *piggyBac* 轉因子之作用特性；而南方墨點法 (Southern blot) 則顯示該標的片段可穩定遺傳至子代，目前已超過 65 代。

關鍵詞：生殖細胞系轉型、基因轉殖、*piggyBac* 轉基因子、微注射、東方果實蠅。

*論文聯繫人
Corresponding email: khlu@dragon.nchu.edu.tw