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Using chimeric *piggyBac* transposase to achieve directed interplasmid transposition in silkworm *Bombyx mori* and fruit fly *Drosophila* cells*

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Abstract: The *piggyBac* transposon has been long used to integrate foreign DNA into insect genomes. However, undesirable transgene expression can result from random insertions into the genome. In this study, the efficiency of chimeric Gal4-*piggyBac* transposase in directing integration onto a DNA target plasmid was evaluated in cultured silkworm *Bombyx mori* Bm-12 and fruit fly *Drosophila* Schneider 2 (S2) cells. The Gal4-*piggyBac* transposase has a Gal4 DNA-binding domain (DBD), and the target plasmid has upstream activating sequences (UAS) to which the Gal4 DBD can bind with high affinity. The results indicate that, in the Bm-12 and S2 cells, transpositional activity of Gal4-*piggyBac* transposase was increased by 4.0 and 7.5 times, respectively, compared to controls, where Gal4-UAS interaction was absent. Moreover, the Gal4-*piggyBac* transposase had the ability of directing *piggyBac* element integration to certain sites of the target plasmid, although the target-directing specificity was not as high as expected. The chimeric *piggyBac* transposase has the potential for use in site-directed transgenesis and gene function research in *B. mori.*

Key words: *Bombyx mori*, *piggyBac*, Gal4-upstream activating sequences (UAS), Transposition assay, Transgenesis
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1 Introduction

DNA transposons have been used successfully to genetically transform insect species. However, a number of problems exist despite this success, including low integration efficiency, unpredictable gene-expression patterns, mutation of important genes due to the lack of insertion site specificity, and so on. Several approaches can be used to increase site-selectivity of transposases, such as site-directed mutagenesis, random mutagenesis or exchange, or the addition of a heterologous DNA-binding domain (DBD) (Coates *et al*., 2005).

It was suggested that, when a transposase is fused with an exogenous DBD, the transposon-based integration can be directed to definite target sites of the genome (Kaminski *et al*., 2002). As the DBD of a transcriptional activator allows the activator to bind to a target site, the DBD-fused transposases can be expected to bring the transposon-transposase complex to the vicinity of a DBD-specific site, and thereby increase the likelihood of transgene integration. As an example, in a recent study reported by Maragathavally *et al*. (2006), the DBD of the yeast transcriptional activator Gal4 was fused to the NH_2 terminus of the *Mos1* and *piggyBac* transposases, and a target plasmid was added with upstream activating sequences (UAS) to which the Gal4 DBD can bind with high affinity. In embryos of the yellow fever mosquito, *Aedes aegypti* (L.), the transpositional activity of the Gal4-*piggyBac* transposase was found to be 11.6 times higher compared to controls, where the Gal4-UAS interaction was absent, and 67% of the

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integrations occurred at a single TTAA site. The Gal4-*piggyBac* transposase has also been demonstrated to be active in vertebrates, such as in human embryonic kidney cells (Wu *et al*., 2006). Therefore, the use of DBD-transposase fusions can be expected to increase the efficiency of transposition and to target the integration of transgene to selected genomic sites (Dematteі *et al*., 2010).

The Gal4-UAS system has been used to study gene function in several insects, such as the fruit fly *Drosophila* (Phelps and Brand, 1998; Klueg *et al*., 2002; Robertson *et al*., 2002; Nicholson *et al*., 2008; Zhong and Yedvobnick, 2009) and the domesticated silkworm *Bombyx mori* L. (Imamura *et al*., 2003; Dai *et al*., 2008; Yamagata *et al*., 2008). However, currently there is only one report in insects regarding the use of chimeric transposases for site-specific integration, as described above in *A. aegypti* (Maragathavally *et al*., 2006). In this study, we used the Gal4-*piggyBac* transposase with the aim of realizing site-directed integration in *B. mori* and *Drosophila* cells. *B. mori* has been long utilized as an important economic insect, and is now serving as an insect model for genetic, biochemical, and physiological researches, taking advantage of the draft genome sequences (Mita *et al*., 2004; Xia *et al*., 2004) as well as the functional analyses of some important genes (The International Silkworm Genome Consortium, 2008). Genetic transformation work with *B. mori* has been active for over a decade, and a number of transgenic germlines have been produced by using the *piggyBac* vector since Tamura *et al.* (2000). However, the research progress targeting site-specific gene integration has been slow in this insect. Recently, Nakayama *et al*. (2006) demonstrated that the integrase from the *Streptomyces* phage φC31 is active in cultured silkworm cells (*Bm*N4), and thus can be used to guide site-specific gene integration into a specific chromosome.

In this study, a *B. mori* cell line (Bm-12) and a *Drosophila* cell line (Schneider 2 (S2)) were transfected together with a helper plasmid expressing the chimeric *piggyBac* transposase, a donor vector containing a kanamycin resistance gene, and a target plasmid carrying UAS. The transposition assay demonstrated that the chimeric Gal4-*piggyBac* transposase is capable of increasing transposition efficiency in the two cell lines tested, and also has the potential of directing the integration to definite sites, although the target-directing specificity was not as high as expected. Hence, the Gal4-*piggyBac* transposase would be useful for the functional analysis of target genes in *B. mori*.

2 Materials and methods

2.1 Insect cell lines

The Bm-12 cell line derived from *B. mori* (Khurad *et al*., 2009) was maintained in TNM-FH insect medium (Sigma Chemical Co., St. Louis, MO, USA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS; Gibco, USA). The *Drosophila* S2 cells were maintained in Schneider's *Drosophila* medium (Invitrogen, Carlsbad, CA, USA) containing 10% (v/v) heat-inactivated FBS. Both cell lines were maintained in a T-25 culture flask (Corning, NY, USA) at 28 °C.

2.2 Plasmids

The helper plasmid pIE1-Gal4-*pB*, donor plasmid pB[KOα], and target plasmids pGDV1 and pGDV1-UAS were donated by Dr. Craig COATES at the Department of Entomology, Texas A & M University, USA. Details of these plasmids and the schematic of interplasmid transposition assay can be found in Maragathavally *et al*. (2006).

The helper plasmid pIE1-Gal4-*pB* is designed to express *piggyBac* transposase under the control of a Hr5-IE1 enhancer-promoter. A nuclear localization signal (TPPKKKRKVED) is incorporated upstream of the Gal4 DBD, and a flexible linker sequence (KLGGGAPAVGGGPK) is present between the Gal4 DBD and transposase.

The donor plasmid pB[KOα] has an *Escherichia coli* origin of replication (*ori*) and a kanamycin (Kan) resistance gene (*kan*), and has been used previously by Thibault *et al*. (1999) and Maragathavally *et al*. (2006). The *E. coli ori* and *kan* gene are transposed to the target plasmid as interplasmid transposition takes place.

pGDV1 and pGDV1-UAS are target plasmids. pGDV1 is derived from *Bacillus subtilis* and cannot replicate in *E. coli* (Thibault *et al*., 1999): it lacks the UAS target site and bears a chloramphenicol (Cam) resistance marker. pGDV1-UAS has the UAS target site and an *E. coli ori*. Prior to use, it was modified

according to Maragathavally *et al*. (2006). Briefly, the *E. coli ori* was removed from pGDV1-UAS by restriction digestion with *Sph*I and *Xba*I, and after blunt-ending and self-ligation the plasmid was transformed into chemical-competent cells of *B. subtilis* (strain BS 1700, Institute of Microbiology, Chinese Academy of Sciences), which were prepared according to Xue *et al*. (1999).

2.3 Transposition assay

The DNA of each plasmid used for transposition assays was prepared using an EndoFree plasmid kit (Qiagen, USA). Transposition assays were performed in *B. mori* Bm-12 and *Drosophila* S2 cells according to the protocols described by Wu *et al*. (2007) with some modifications. Briefly, for the transfection in each independent assay (three assays done for each cell line), a mixture of the helper plasmid pIE1-Gal4-*pB* (1 μg), the donor plasmid pB[KOα] (1 μg), and the target plasmid pGDV1-UAS (2 μg) was used; for control transposition assays, a mixture of the plasmids pIE1-Gal4-*pB* (1 μg), pB[KOα] (1 μg), and pGDV1 (2 μg) was used. Plasmids were transfected into the cells in six-well dishes using Lipofection 2000 (Invitrogen), following the manufacturer's protocols.

At 72 h for Bm-12 and 48 h for S2 cell post-transfection at 28 °C, plasmid DNA was recovered from the cells according to the protocols (Wu *et al*., 2007), and was introduced into *E. coli* DH10B cells (Invitrogen) by electroporation (Gene Pulser Xcell™ Electroporation System, Bio-Rad, USA). Electroporation was performed using 1 μl of plasmid rescue DNA at the parameters 2.5 kV and 200 Ω with a 2-mm cuvette gap. The electroporated cells were transferred into 1 ml of SOC medium and maintained at 37 °C for 1 h. Then, 5 μl of the media were plated onto an LB plate containing ampicillin (50 μg/ml) to determine the recovery of donor plasmids. The remaining cells were pelleted and resuspended in 100 μl of remaining SOC media and plated onto LB plates containing Cam (10 μg/ml), Kan (25 μg/ml), and X-gal (25 μg/ml) to recover transposition products.

Blue colonies surviving selection on the LB medium containing Cam and Kan were subjected to polymerase chain reaction (PCR) using the primers, LTR (5′-CGGATTCGCGCTATTTAG-3′) and RTR (5′-ACCTCGATATACAGACCG-3′), which were designed to bind near the terminal of *piggyBac*. Colonies that appeared to be products of transposition events, i.e., having a PCR product of approximately 2.6 kb, were selected for DNA sequencing using the primer RTR. Sequences were analyzed using the Vector NTI 8.0 Suite software (InforMax, North Bethesda, MD, USA) to determine the sites of *piggyBac* insertions on the pGDV1 or pGDV1-UAS.

Transposition frequency is calculated as the number of transpositions recovered per donor plasmid recovered. The times of increase in transposition frequency due to the Gal4-UAS system are calculated by dividing the transposition frequency when using the pGDV1-UAS target plasmid by that when using the pGDV1 target plasmid.

2.4 Statistical analyses

Chi-square test was used to test the relations of *piggyBac*-element integration sites with the type of target plasmid. To identify the possible sites to be preferentially targeted for integration, chi-square test was used to compare the frequencies (percentages) of insertions at a given site between the treatment (using pGDV1-UAS as a target plasmid) and control (using pGDV1 as a target plasmid) experiments. All analyses were done at the significance levels P=0.05 and P=0.01 (SPSS Inc., 1999).

3 Results

3.1 Transposition assays in *B. mori* Bm-12 cells

We performed three independent transposition assays in Bm-12 cells. After the screening of 7.76×10^5 donor plasmids in the pGDV1-UAS experiments and 2.10×10^6 donor plasmids in the pGDV1 (control) experiments, a total of 56 and 40 transposition products were discovered, respectively. Sequencing indicated that all of the *piggyBac* element insertions occurred at TTAA sites and duplicated these. Due to the Gal4-UAS interaction, the transpositional activity was increased by an average of 4.0 times over the control, where the UAS target site is absent (Table 1).

The insertion sites of *piggyBac* elements into the pGDV1-UAS and pGDV1 sequences are shown in Fig. 1a. Statistical analysis indicates that the distribution of insertion sites was significantly related with the type of target plasmid (χ^2=43.989, df=14,

Table 1 Results of *piggyBac*-based transposition assays in *B. mori* Bm-12 and *Drosophila* S2 cells using the chimeric Gal4-*piggyBac* transposase

Insect cell line	Target plasmid	Exp.	No. of recovered donor plasmids	No. of transposition events	Transposition frequency	Times of transpositional activity increase	Average times of activity increase
Bm-12	pGDV1-UAS	1	2.65×10^5	17	6.42×10^{-5}	3.36	4.0±0.9
		2	2.57×10^5	20	7.78×10^{-5}	5.64	
		3	2.54×10^5	19	7.48×10^{-5}	2.85	
	pGDV1	1	1.10×10^6	21	1.91×10^{-5}		
		2	5.80×10^5	8	1.38×10^{-5}		
		3	4.20×10^5	11	2.62×10^{-5}		
S2	pGDV1-UAS	1	1.35×10^5	25	1.85×10^{-4}	16.67	7.5±4.6
		2	9.80×10^4	10	1.02×10^{-4}	2.17	
		3	1.14×10^5	17	1.49×10^{-4}	3.71	
	pGDV1	1	1.80×10^5	2	1.11×10^{-5}		
		2	1.91×10^5	9	4.71×10^{-5}		
		3	1.74×10^5	7	4.02×10^{-5}		

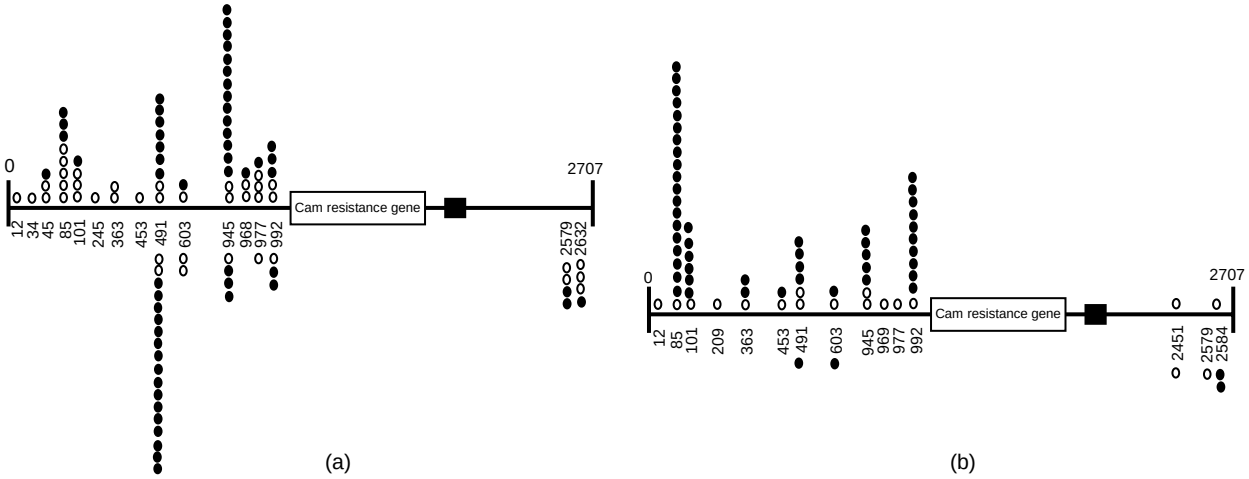

Fig. 1 Insertion sites of *piggyBac* donor elements in pGDV1-UAS and pGDV1 (control, lacking UAS target site) target plasmids in the transposition assays performed in *Bombyx mori* Bm-12 cells (a) and *Drosophila* S2 cells (b)
Solid and open circles represent the insertions into the pGDV1-UAS and pGDV1 targets, respectively. The circles below the line represent the insertions occurring in an opposite direction relative to the Cam gene. The numbers represent the nucleotide locations on the pGDV1 plasmid. The solid box stands for the UAS site on the pGDV1-UAS plasmid

P<0.001). When pGDV1 was utilized, the *piggyBac* element was inserted randomly at multiple TTAA points. However, in the presence of pGDV1-UAS, insertions occurred primarily at positions of 491 and 945. The position 491 was about 1050 bp to the 3′ terminal of the inserted UAS, and the position 945 was also about 1050 bp to the 5′ terminal of the UAS, indicating that the TTAA sites about 1 kb from the UAS were the hot insertion sites. Insertions at these two sites accounted for 41.1% and 30.4% (71.5% in total) of all of the insertions, respectively. Both of these two insertion percentages were significantly higher than those (10.0% and 7.5%) obtained in the control (Table 2).

Table 2 Comparison on the percentages of *piggyBac*-element insertions at a given position between the assays using different target plasmids[#]

Insect cell line	Position on the pGDV1 target plasmid	Chi-square test			
		χ^2	*df*	*P*	Sig.
Bm-12	491	11.144	1	0.001	**
	945	7.391	1	0.007	**
S2	85	6.290	1	0.012	*
	101	0.532	1	0.466	NS
	491	0.033	1	0.855	NS
	945	0.033	1	0.855	NS
	992	1.888	1	0.169	NS

[#] The details of assays are shown in Table 1. Sig.: significance. * and ** stand for significant differences at the levels *P*=0.05 and *P*=0.01, respectively; NS: not significant

3.2 Transposition assays in *Drosophila* S2 cells

Three independent transposition assays were performed in this cell line. A total of 52 transposition events were found after the screening of 3.47×10^5 donor plasmids in the pGDV1-UAS experiments, and 18 transposition events were found after the screening of 5.45×10^5 donor plasmids in the control. The transpositional activity was observed to be 7.5 times higher in the presence of UAS target as compared with the control (Table 1). All of the *piggyBac* element insertions occurred at TTAA sites.

The distribution of insertion sites was significantly related with the type of target plasmid (χ^2= 32.495, *df*=13, *P*=0.002). In the assays using pGDV1-UAS target plasmid, the position 85 (about 650 bp from 3′ terminal of the UAS) was preferentially targeted (Fig. 1b). The insertions at this position accounted for 36.5% of all of the insertions, which was significantly higher than that (5.6%) in the control. In addition, the positions 101, 491, 945, and 992 also appeared to be preferentially targeted, but statistically none of the percentages of these insertions were significantly different from that in the control (Table 2).

4 Discussion

Our results indicate that the Gal4-*piggyBac* transposase can increase transpositional activity (by 4.0 times) in the *B. mori* Bm-12 cells. By comparison, this fusion transposase can bring a greater increase of transpositional activity (by 7.5 times) in the *Drosophila* S2 cells. In an earlier study conducted in *A. aegypti* embryos, the Gal4-*piggyBac* transposase could elevate the transpositional activity to a much greater level (by 11.6 times) (Maragathavally *et al*., 2006). This suggests that fusing a Gal4 DBD to *piggyBac* transposase might be a promising approach for increasing transposition efficiency in insects.

The transpositional activity of a transposase fused with DBD can be affected by several factors, such as efficiency of the transposase, reciprocal compatibility of the DBD and transposase selected, and accuracy of the linker between DBD and transposase (Dematteі *et al.*, 2010). In addition, it can also be affected by ratios of donor and helper plasmids, as demonstrated by Wu *et al.* (2006) who found that the activity of Gal4-*piggyBac* transposition was dosage-sensitive with increasing helper levels at a fixed donor amount. In other words, Gal4-*piggyBac* has the property of overproduction inhibition. In the present study, we used 1 μg helper, 1 μg donor, and 2 μg target plasmids for cell transfection as recommended by the manufacturer's protocols of Lipofection 2000 (Invitrogen), although whether this amount/ratio is most suitable for the transposition in Bm-12 and S2 cells has not been determined. At the same time, *piggyBac* was also reported as lacking the property of overproduction inhibition (Wilson *et al*., 2007). Therefore, the relationship of Gal4-*piggyBac* activity with plasmid amount/ratio appeared to be unclear.

In the presence of the chimeric *piggyBac* transposase, in the *B. mori* Bm-12 cells, 41.1% and 30.4% of transpositions occurred at the positions 491 and 945, respectively, and in the *Drosophila* S2 cells 36.5% of transpositions occurred at the position 85. As each of these percentages was significantly higher than that in the control, these positions are presumably preferred for integration to other ones, which suggests the potential of Gal4-*piggyBac* transposase in achieving site-specific gene integration in insect cells.

By using Gal4-*piggyBac* and Gal4-*Mos1* transposases in *A. aegypti* embryos, Maragathavally *et al*. (2006) identified a hot insertion spot about 1 kb from the UAS site. This insertion preference was also found in our work reported here using *B. mori* cells, but not found in the work using *Drosophila* cells where the position about 650 bp from the UAS was much preferred. In terms of mechanisms, which positions are more targeted for insertion depends on: (1) the relative position of *cat* antibiotic resistance gene to the UAS; (2) the relative position of the transposase-transposon complex to the UAS (the molecular complex is likely to be located at some distance from the UAS due to the presence of the linker peptide); and, (3) the possibly increased affinity of transposase for some specific TTAA sites due to the addition of Gal4 DBD (Maragathavally *et al*., 2006; Dematteі *et al*., 2010).

In the embryo of *A. aegypti*, by using Gal4-*piggyBac*, 67% of transpositions occurred at the 1103 site of the target plasmid. In comparison, in our assays performed in *B. mori* Bm-12 and *Drosophila* S2 cells, the site specificity was much lower, with only 30%–41% of insertions occurring at each preferred

site (positions 85, 491, and 945). It is not known at the time what caused the great difference of preferred integration sites among organisms. Maragathavally *et al*. (2006) speculated that interaction with host cell factors might influence target site section for certain transposases.

Insect cell culture can provide a rapid means to quantify the transpositional activities and insertional preferences of modified transposons, utilizing plasmid-to-plasmid or plasmid-to-chromosome mobilization assays. The main advantages of the plasmid-to-plasmid assay are that they can be performed rapidly and importantly, and that they can also be utilized to confirm DBD-target sequence interactions without a need to have the specific target already located on a chromosome (Wu *et al*., 2007). Based upon our results, we suggest that the interplasmid transposition assay can be used to compare different silkworm strains or cell lines for their potential use for targeted transgenesis or gene expression research.

5 Conclusions

The Gal4-*piggyBac* transposase could increase the transpositional activity in both *B. mori* Bm-12 and *Drosophila* S2 cells. This chimeric *piggyBac* could also direct the integration of the *piggyBac* element to several specific sites of the target plasmid, although the target-directing specificity was lower than that previously reported in *A. aegypti*. Therefore, we conclude that the Gal4-*piggyBac* transposase has the potential for use in site-directed transgenesis and for investigating gene function in *B. mori*.

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