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Original Article

The relationship between internal domain sequences of *piggyBac* and its transposition efficiency in BmN cells and *Bombyx mori*

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The *piggyBac* transposon, which includes terminal inverted repeat sequences and internal domain (ID) sequences, is widely used as a tool for insect transformation. To optimize this system for transgenic research on *Bombyx mori*, we examined the effects of the amount of the transposase plasmid and its ID sequences on the expression of green fluorescent protein (GFP). Four kinds of transposon plasmids, pB[A3GFP]-1 with the full length of ID sequences, pB[A3GFP]-2 having only the 3′ ID sequence, pB[A3GFP]-3 without ID sequences, and pB[A3GFP]-4 containing 333 bp of the 5′ ID sequence, and 179 bp of the 3′ ID sequence were constructed with GFP as the marker. After transfecting these four plasmids into BmN cells, we analyzed the transfecting efficiency by comparing the GFP positive to negative cell ratio. Our results indicated that plasmid pB-4 got the highest ratio on the 22nd day. Moreover, the GFP positive to negative cell ratio increased with higher amount of transposase plasmid without overproduction inhibition. Furthermore, we injected three *piggyBac* transposon plasmids, pB[A3GFP]-1, pB[3×P3GFP]-3, and pB[3×P3RFP]-4 harboring different markers into preblastoderm stage eggs of *B. mori*, and found that the transformation efficiency of pB[3×P3RFP]-4 was 3.8 folds higher than pB[A3GFP]-1, whereas pB[3×P3GFP]-3 failed to produce transformants. Our results suggested that pB-4 may be one of the best *piggyBac* transposon plasmids currently available for germline transformation in *B. mori*.

Keywords *Bombyx mori*; BmN cell; *piggyBac*; transgenesis

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Introduction

The *piggyBac* transposon was originally isolated from the genome of the cabbage looper moth *Trichoplusia ni*. The mobility of *piggyBac* transposon in insects [1], mammalian cells including human cell lines [2], mice [3], and a number of other heterologous systems such as the human pathogen *Plasmodium falciparum* [4], has made it an attractive genetic tool for genome manipulation by insertional mutagenesis and transgenesis.

The primary *piggyBac* transposon is 2,472 bp in length, and has a complex configuration composed of two terminal inverted repeat (TIR) sequences, two internal domain (ID) sequences and a transposase-encoding domain. The TIR sequences are required for cutting out precisely sequences existing between them in transposon plasmids and pasting them exclusively at 5′-TTAA-3′ target sites [5–7]. For *in vivo* integration of foreign genes into a host genome, some ID sequences are required [8,9].

Great progress has been made based on the stable transformation of *piggyBac* transposon in *Bombyx mori* [10]. A transgenic strain of *B. mori* that could produce colored silk has been obtained using this system [11], and a series of *piggyBac* based silkworm-bioreactors have been constructed to express foreign proteins such as type III procollagen [12], juvenile hormone-specific esterase (JHE) [13], global protein [14], immune-inducible cecropin B-green fluorescent protein (GFP) reporter gene [15], human serum albumin [16] and human {micro}-opioid receptor [17]. *PiggyBac* has also been further developed as a gene tagging and enhancer trapping system [18,19].

All of these findings demonstrated that the *piggyBac* transposon is an effective vector for transgenesis and integration research in medical, agricultural and industrial fields. However, it would be desirable to further improve the efficiency of transposition.

To date, most of the researchers have utilized the native *piggyBac* transposon in *B. mori* [10–21]. Here we describe the use of *piggyBac* transposon vector derivatives in BmN cells and *B. mori* that allows *piggyBac*-based recombinant plasmids to increase its integration frequencies.

Materials and Methods

Silkworm strains

The polyvoltine strain of *B. mori*, Lan 10 (Chinese strain), was raised in the Laboratory of Germplasm Resource and Molecular Breeding of Silkworm, Zhejiang University (Hangzhou, China). The silkworm larvae were reared with fresh mulberry leaves under standard conditions. BmN cells derived from the ovarian tissue of *Bombyx mori* were maintained at the Shanghai Institutes for Biological Sciences (Shanghai, China).

Construction of *piggyBac* transposon-based plasmids

The *piggyBac* transposon plasmids pPIGA3GFP (pB[A3GFP]-1) and helper plasmids were kindly provided by Dr Toshiki Tamura (National Institute of Sericultural and Entomological Science, Tsukuba, Japan) and pPIG[3×P3GFP-P9DsRed] by Dr Paul D. Shirk (USDA ARS CMAVE, Gainesville, FL, USA). The helper plasmids (transposase plasmid) providing transposase sources for the transposons have been described [1].

The pB[A3GFP]-1 carries the original elements of *piggyBac* transposon, 35 bp of 5′ TIR and 63 bp of 3′ TIR and a cassette harboring the GFP coding sequence driven by the *B. mori* cytoplasmic actin 3 (A3) gene promoter (**Fig. 1**). Additionally, it contains 640 bp of 5′ ID sequences, of which 343 bp belongs to the transposase-encoding sequence and 987 bp of 3′ ID sequences, of which 686 bp belongs to the transposase-encoding sequence.

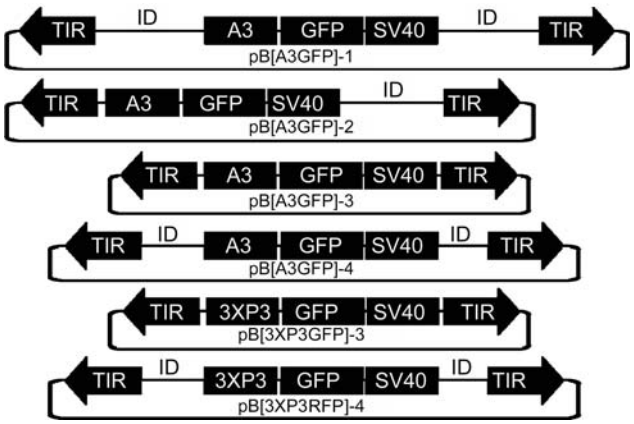

Figure 1 Schematic structure of the *piggyBac* transopon plasmids
The pPIGA3GFP (pB[A3GFP]-1) carries the original elements of *piggyBac* transposon and a cassette harboring the GFP coding gene driven by A3 promoter. The pB[A3GFP]-2 has no 5′ ID sequences. The pB[A3GFP]-3 has no ID sequences. pB[A3GFP]-4 contains some parts of 3′ ID sequence and 5′ ID sequence, respectively. These four plasmids harbor the GFP gene as the reporter gene controlled by A3 promoter. The GFP gene of pB[3×P3GFP]-3 is controlled by 3×P3 promoter. And the reporter gene of pB[3×P3RFP]-4 is RFP gene controlled by 3×P3 promoter.

First, a 640 bp fragment containing the 5′ ID sequences was completely deleted from pB[A3GFP]-1 with *Nsi*I and *Bgl*II digestion, giving rise to pB[A3GFP]-2. A 1019 bp PCR fragment containing the 3′ TIR sequence was amplified from pB[A3GFP]-1 using primers P1 and P2 (**Table 1**). The PCR product was digested with *Ngo*MIV and *Bcl*I, and then cloned into pB[A3GFP]-2, which was digested with the same enzymes to replace the 1800 bp fragment containing the 3′ ID and 3′ TIR sequence, generating pB[A3GFP]-3, which only retained the TIR sequences at both ends. Two PCR fragments, one of 468 bp containing a part of the 5′ ID sequence and the other of 1457 bp containing a part of the 3′ ID sequence were amplified from pB[A3GFP]-1 using primers P3–P4 and P5–P6, respectively (**Table 1**). These two fragments were digested with *Eco*RI and XhoI and *Aat*II and *Bgl*II, respectively, and were subsequently inserted into the corresponding sites of pB[A3GFP]-3, to generate pB[A3GFP]-4 (**Fig. 1**). It contains 333 bp of 5′ ID sequence and 179 bp of 3′ ID sequence, deprived of 343 bp of 5′ transposase-encoding sequence and 808 bp of 3′ ID sequences including 686 bp of 3′ transposase-encoding sequence.

A 2926 bp fragment was removed from pPIG[3×P3GFP-P9DsRed] with *Nco*I digest, generating pPIG[3×P3DsRed]. Subsequently a *Sac*I fragment comprising the 3×P3 promoter and the RFP coding gene was cut from pPIG[3×P3DsRed] and inserted into the same sites of pB[A3GFP]-4, generating pB[3×P3RFP]-4. Vector pB[3×P3GFP]-3 was obtained by inserting the 3×P3 promoter, which was isolated from pB[3×P3RFP]-4 with *Nco*I and *Bgl*II digest, into the same sites of plasmid pB[A3GFP]-3 (**Fig. 1**).

Transfection and screening of the GFP-positive cells

According to the plasmid transfection protocol, plasmids were transfected into BmN cells using Cellfectin reagent (Invitrogen, Carlsbad, CA, USA). For each transfection well, a total of 1.5 μg of plasmid DNA was added into 1 ml serum-free culture. Flow cytometry (FACS Aria, Becton Dickinson, NJ, USA) was used to quantify the cells expressing the GFP protein. The transfected cells were harvested in certain days and 1×10^4 of cells were counted in each assay.

Microinjection and screening of transgenic silkworms

Microinjection and screening of transgenic silkworms were done as described previously [22]. Approximately 15–20 nl of a 1:0.5 mixture of the transposon (donor) plasmids and transposase (helper) plasmids (total DNA concentration 0.3 μg/μl) were microinjected into each egg.

Table 1 Primers used for the amplification of the TRD and ID

Primer	Sequence (5′→3′)	Target positions	Size of product (bp)
P1	GCCGGCAGATCTATACAGACCGATA	3740–3752	1019
P2	GAGAGTGCACCATATGCGGTGTG	4747–4725	
P3	GATGAATTCCACACAGCTTG	7233–7242	468
P4	GACTCGAGCTGAGCAGAGAGGATAT	7696–7681	
P5	GCAGATCTAAAGTTTTGTT	3586–3597	1457
P6	TAAACAAATAGGGGTTCCGC	5035–5016	

P1 and P2 are for the pB[A3GFP]-3; P3 and P4, P5 and P6 are for the pB[A3GFP]-4.

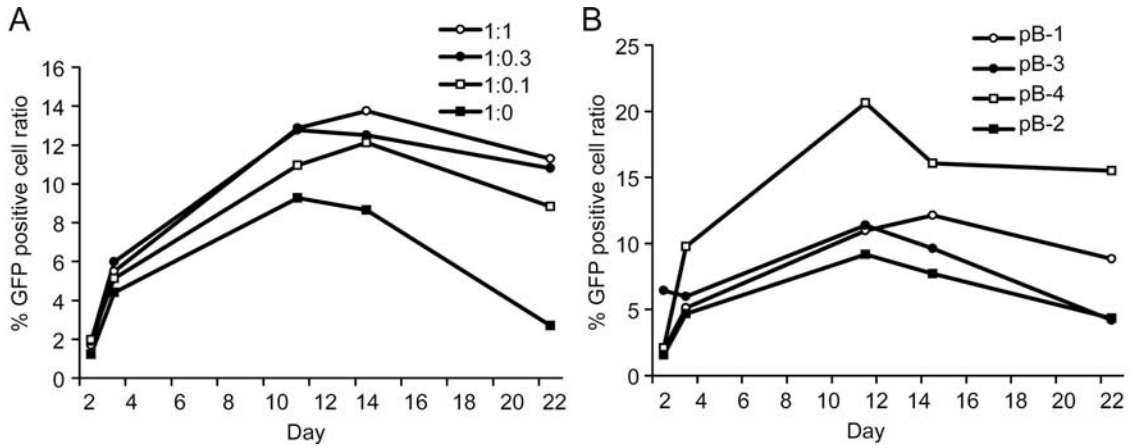

Figure 2 The relation of *piggyBac* transposon and GFP expression (A) The dose-effect of the transposase plasmid on the frequency of GFP-positive cell ratio. The ratios of transposon to transposase plasmids were 1:1, 1:0.3, and 1:0.1. The plasmid PUC19 was used to maintain the same amount of total transfected plasmids. BmN cells transfected with pB[A3GFP]-1 in the absence of transposase plasmids were used as controls. (B) The relation of ID sequences and GFP-positive cell ratio. The plasmids pB[A3GFP]-1, pB[A3GFP]-2, pB[A3GFP]-3, and pB[A3GFP]-4 were individually co-transfected with transposase plasmids into the BmN cells. The ratio of transposon to transposase plasmid was 1:0.5.

Results

The dose-effect of the transposase plasmid on the expression frequency of GFP

We tested the effects of different ratios, 1:1, 1:0.3, and 1:0.1, of transposon to transposase plasmids on the expression levels of GFP. To achieve these ratios, a constant amount of transposon plasmid pB[A3GFP]-1 was transfected into the cells and plasmid PUC19 was used to maintain the same amount of total transfected plasmids. BmN cells transfected with pB[A3GFP]-1 in the absence of transposase plasmids were used as controls. The transfected cells were harvested and screened for fluorescence by flow cytometry. The time course of GFP-positive cells for the different ratios is shown graphically in **Fig. 2(A)**. In the first 5 days, the GFP-positive ratios of the transfected cells with and without transposase plasmids were almost the same. Five days later, however, the difference between the two types of samples increased gradually. After 14 days, the numbers of GFP-positive cells decreased in all groups. However, the numbers of GFP-positive cells that were co-transfected with the transposase plasmids decreased much more slowly [**Fig. 2(A)**]. To evaluate the effects of different amounts of transposase plasmid on transposition, we examined in more detail the results of the 22nd day post-transfection. The group with the 1:1 ratio had the highest frequency of fluorescence, followed by the 1:0.3, 1:0.1, and 1:0.

Transposon efficiencies using plasmids with different ID sequences

To improve the *piggyBac* system for higher transposition efficiency, plasmids pB[A3GFP]-1, pB[A3GFP]-2, pB[A3GFP]-3, and pB[A3GFP]-4 were individually co-transfected with transposase plasmids into the BmN cells. The ratio of transposon to transposase plasmid was 1:0.5. The pB[A3GFP]-1 plasmid was used here as a relevant control. After transfection, the frequencies of GFP-positive cells in the four groups increased progressively, reached peak points on the 11th or 14th day, and then decreased with different slopes. All groups showed the same trend. However, the frequency of pB[A3GFP]-4

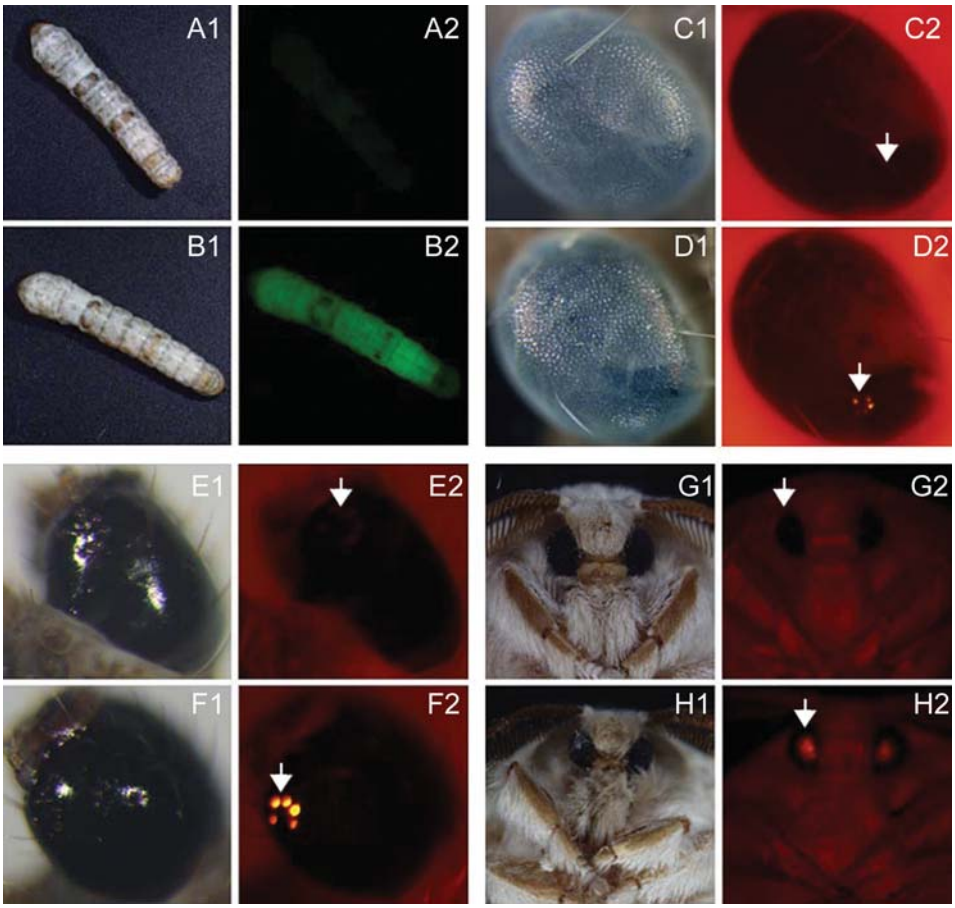

Figure 3 Expression patters in transgenic silkworm A1 and B1, C1 and D1, E1 and F1, G1 and H1 were in bright field in level, egg, new-hatched silkworm and moth respectively. A2, B2, C2, D2, E2, F2, and G2 were screened for green or red fluorescence. A, C, E, and G series were used as the controls. Arrows indicate the eyes.

group exhibited the fastest increase in fluorescent cell numbers and decreased at a slower rate compared with the other groups [**Fig. 2(B)**]. On the 22nd day, the pB[A3GFP]-4 group displayed the highest numbers of GFP-positive cells (15.5%), exhibiting ~2-fold increased frequency compared with the control plasmid pB[A3GFP]-1 (8.84%). Plasmids pB[A3GFP]-2 and pB[A3GFP]-3 displayed relatively lower frequencies.

Silkworm transformation

PiggyBac-mediated germline transformation was tested using the polyvoltine strain of *B. mori*, Lan 10. Three plasmids, pB[A3GFP]-1, pB[3×P3GFP]-3, and pB[3×P3RFP]-4 were individually co-injected with transposase plasmids into preblastoderm stage embryos of *B. mori*. The surviving adults were crossed and G1 adult progeny was screened based on fluorescence measurements. Positive pB[A3GFP]-1 transformants were expected to exhibit green bodies, whereas positive pB[3×P3GFP]-3 green eyes and positive pB[3×P3RFP]-4 red eyes. We have only obtained positive pB[A3GFP]-1 transformants and positive pB[3×P3RFP]-4 transformants (**Fig. 3**). Transformation frequencies from all injections are listed in **Table 2**. The pB[3×P3GFP]-3 plasmid failed to produce positive transformants. The pB[3×P3RFP]-4 plasmid yielded a relatively high transformation frequency of 18.5%, whereas the control plasmid pB[A3GFP]-1 yielded a frequency of 4.9%.

Discussion

PiggyBac is a DNA transposon capable of mediating gene insertion. However, the distinction between long-term expression of GFP due to stable transposition and integration into chromosomal loci or long-term maintenance of plasmid DNA in an extrachromosomal form has proven difficult to obtain. Once transfected, circular plasmids remained extrachromosomal for a period of time and then degraded. Meanwhile, a fraction of these plasmids was integrated into the genome. The GFP was generated both by transposon plasmids, which degraded gradually and the genomic DNA of transgenic cells, which increased along with the cell proliferation, so the expression profile of GFP reflected these two events in cells. Hence, in the advanced

Table 2 Results of G0 transgenesis after injection

Vector	Injected eggs	Hatched eggs	Fertile moths	Broods with GFP or RFP positive larvae	G0 transformed moths in fertile moths
pB[A3GFP]-1	5000	756	367	18	4.9%
pB[3×P3GFP]-3	2600	69	32	0	0
pB[3×P3RFP]-4	2400	151	27	5	18.5%

Percentage of G0 transformed moths is the number of the broods with GFP or RFP positive larvae divided by the number of fertile moths.

stage, the transposition events became dominant. To eliminate the survival extrachromosomal plasmids, Score *et al*. [23] used LoxP sites combined with 'Sleeping Beauty' transposon and Wilson *et al*. [24] referred to drug's resistance. All of these will lead to a much more complicated vector and less transformation frequency. As a result, we decided to scan for long-term expression of GFP in order to determine more specifically the levels of expression resulting from transposition, when using the simplest vector only with the necessary cassettes.

The 'Sleeping Beauty' transposon system was reported to cause overproduction inhibition [25–28]. In contrast, *piggyBac* did not demonstrate overproduction inhibition in human cells [24]. Our experiments in BmN cells also showed no inhibition. However, surplus transposase after the initial transposition could increase the probability of subsequent transposon excision and re-integration with associated risk of genotoxicity, which might lead to stable expression of transposase with destabilizing effects on the integrated transgene. Alternatively, we can supply the transposase-encoding molecules in the form of mRNA, because they can act as a transient source of transposase for gene insertion both *in vitro* and *in vivo* [29]. According to our results, the ratio of 1:0.3–1:1 of transposon plasmids to transposase plasmids may be sufficient to obtain a considerable number of transformants.

By comparison to the primary sequence of *piggyBac* transposon (accession No. J043642), we found the original ID sequences of *piggybac* transposon pB[A3GFP]-1 include two parts of transposase coding sequences flanking the cassette of A3GFP, whereas the pB-4 plasmid with 333 bp of 5′ ID sequence and 179 bp of 3′ ID sequence has eliminated any surplus transposase coding sequence. According to previous study, there contains a transposase transcriptional initiation region in the 333 bp of 5′ ID [5] and a cis-activating element in the 179 bp of 3′ ID sequence [30]. They may affect the expression level of the inserted genes, but how it works in the process of transgenesis is still unknown. It obtained a higher number of transformants whereas the pB-3 transposon that retains no ID sequences failed to produce transformants in *B. mori*. Our results are the same with Li's conclusions [8,9]. What is more, the pB-2 transposon with the full length of 3′ ID sequence but without any of the 5′ ID sequences was unable to obtain the full transposition capability in BmN cells. So we may conclude that these necessary ID sequences without any surplus transposase coding sequences may account for much for a better germline transformation frequency. We have also confirmed that this kind of germline transformation can be kept at least four generations.

The process of transfection of transposon plasmids into cultured cells, isolation of low-molecular-weight DNA from the transfected cells and analysis using restriction enzyme digestions to verify precise excision events [6], is fast and simple. However, it only analyzes excision and not stable integration events. Transposition assays by drug resistance [24] cannot be applied to *B. mori*. Injection into the eggs of *B. mori* employed in this experiment is difficult and time-consuming but reflects stable transposition. Because of the differences between transfection in BmN cells and injection into the eggs the efficient transposition of *piggyBac* vectors evidenced in BmN cells does not necessarily predict the properties of *piggyBac* transposon movement in germline transformants. Nevertheless, the results from the two methods have a similar trend. The transfected BmN cells therefore can be used as an indicator for transgenesis in *B. mori*.

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