An efficient strategy for producing a stable, replaceable, highly efficient transgene expression system in silkworm, *Bombyx mori*

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We developed an efficient strategy that combines a method for the post-integration elimination of all transposon sequences, a site-specific recombination system, and an optimized fibroin H-chain expression system to produce a stable, replaceable, highly efficient transgene expression system in the silkworm (*Bombyx mori*) that overcomes the disadvantages of random insertion and post-integration instability of transposons. Here, we generated four different transgenic silkworm strains, and of one the transgenic strains, designated TS1-RgG2, with up to 16% (w/w) of the target protein in the cocoons, was selected. The subsequent elimination of all the transposon sequences from TS1-RgG2 was completed by the heat-shock-induced expression of the transposase in vivo. The resulting transgenic silkworm strain was designated TS3-g2 and contained only the attP-flanked optimized fibroin H-chain expression cassette in its genome. A phiC31/att-system-based recombinase-mediated cassette exchange (RMCE) method could be used to integrate other genes of interest into the same genome locus between the attP sites in TS3-g2. Controlling for position effects with phiC31-mediated RMCE will also allow the optimization of exogenous protein expression and fine gene function analyses in the silkworm. The strategy developed here is also applicable to other lepidopteran insects, to improve the ecological safety of transgenic strains in biocontrol programs.

Since the first report of insect transformation with the *P* element in *Drosophila melanogaster*1, transposon-based germline transformation systems had been widely used for both basic and applied studies in insects and other invertebrates over the past 30 years2,3. At present, four transposon vector systems have been used relatively extensively for the transformation of insect species. These include the *mariner* family transposon *Mos1* isolated from *D. mauritiana*4 and *Minos* isolated from *D. hydei*5, the *hAT*-related transposon *Hermes* isolated from *Musca domestica*6, and the TTAA-specific family transposon *piggyBac* derived from *Trichoplusia ni*7. *piggyBac* is a distinct class II transposon, encoding a 594-amino-acid transposase that mediates its excision and reinsertion8. The transposition of *piggyBac* occurs in a precise cut-and-paste fashion and targets TTAA sites in the host genome7,9. *piggyBac* is currently the most widely used transposon vector and has been successfully used for transgenic engineering in more than 30 insect species, including *D. melanogaster*10, *Ceratitis capitata*11, *Tribolium castaneum*12, *Bombyx mori*13, *Aedes aegypti*14, and *Apis mellifera*15. It facilitates the extension of enhancer-trapping strategies, allowing the identification and functional analysis of genes, the expression of foreign proteins, the biological control of pest species, and other genetic engineering operations3.

By far the most frequently published application of nondrosophilid insect transgenesis has been the transformation of the silkworm, *B. mori*, using *piggyBac*. This useful model lepidopteran and economically important insect has been used as an effective protein bioreactor for more than 5000 years in the production of silk16. At present, the silk glands of silkworms have been developed as fine bioreactors for the production of recombinant proteins from transgenic expression vectors, all based on *piggyBac*-mediated germline transformation, using the various promoters of the sericin 1 (*Ser1*), fibroin heavy chain (H-chain; *FibH*), fibroin light chain (L-chain; *FibL*), and fibrohexamers (*fhx*)17–19. We previously constructed an optimized fibroin H-chain expression system to express a recombinant fusion protein in the silk glands of silkworms, and the recombinant fusion protein...
constituted up to 15% (w/w) of the transgenic silkworm cocoon. To our knowledge, this is still the most efficient silkworm silk gland expression system to date. Methods using piggyBac to introduce exogenous DNA into the silkworm genome are characterized by random integration, which could be used to screen for favorable insertion loci for the highly efficient expression of exogenous proteins in transgenic silkworms. It would thus contribute to the utilization of silkworm bioreactors for the commercial production of exogenous proteins. However, position effects and insertional mutagenesis during piggyBac-mediated random integration can have several side effects, including unpredictable variations in gene expression, disruption of the gene structure of the host, and the reduced fitness of the transgenic strain. It is also almost impossible to repeatedly introduce several different exogenous genes into a specific target locus using only piggyBac or other transposons.

Site-specific recombinase (SSR) systems have been developed using recombinases that catalyze the exchange of DNA strands between two target recombination sites, in contrast to the random insertion of genes by transposons. SSR systems provide many advantages for transgenic engineering that are lacking in transposon-based systems. The capacity for reproducible insertion into genetically active loci can be useful in defining and utilizing chromosomal sites with low silencing potential. Currently, the most commonly used SSR systems are FLP/FRT from the 2 μm plasmid of Saccharomyces cerevisiae, Cre/loxP from Escherichia coli phage P1, and phiC31/att from the Streptomyces phage phiC31. These systems have been successfully used for the site-specific integration of transgenes in the genomes of mosquitioes, D. melanogaster, and the fruit fly C. capitata. Recently, the recombination activity of these three recombinases has also been used to manipulate the silkworm genome. Duan et al. demonstrated that the Cre/loxP system can be used to control the activation and expression of marker genes in the middle silk gland cells of transgenic silkworms. We previously used the FLP/FRT system to site-specifically excise a target gene at predefined chromosomal sites in the silkworm. We also used the phiC31/att system to produce heritable site-specific transgene integration into predetermined chromosomal sites in the transgenic silkworm genome with phiC31-mediated cassette exchange (phiC31-mediated recombinase-mediated cassette exchange [RMCE]) reaction. Although these SSR systems have been developed as effective targeted recombination systems in a variety of insect species, they have one drawback: naturally occurring inte-grase loci are extremely rare in the genomes of insects. Occasionally, there may be functional pseudointegration sites, but these sites are generally not ideal and cannot be targeted by inte-grases with the frequency of native recombinase sites. Therefore, it is necessary in many insect species, including D. melanogaster, C. capitata, mosquitoes, and silkworms, to first introduce a canonical site (such as FRT, loxP, attP, or attB) into their genomes using transposon-mediated germline transformation.

One potential concern relating to the field use of transgenic insects is that using transposons as gene vectors may lead to postinsertion instability of the transgene. It has been reported that integrated piggyBac can be remobilized in the genomes of D. melanogaster, C. capitata, Anastrepha ludens, T. castaneum, Anopheles stephensi, Harmonia axyridis, and B. mori. We have also observed the phenomenon of piggyBac remobilization during the large-scale rearing of commercial strains of transgenic silkworms. This phenomenon can be caused by the unintended presence of a mobilizing transposase, which may have been undetected in endogenous piggyBac-like transposons or subsequently entered the host species by horizontal gene transfer. Bombyx mori is a domesticated organism, completely dependent on humans for its survival and reproduction, so its exposure to exogenous transposases by horizontal gene transfer is unlikely. However, according to some previous reports, at least 100 piggyBac-like sequences (BmPBLE1–98, yabu-same-1, and yabu-same-W) are present in the silkworm genome, and some of these transposons might encode potential transposase activity. Therefore, endogenous piggyBac-like transposase activity cannot be avoided in the silkworm. In contrast, the piggyBac transposase acts via an internally encoded transposase acting on the flanking 5′ and 3′ terminal inverted repeat (TIR) sequences and adjacent DNA, which may include subterminal inverted repeat sequences. In principle, piggyBac-based vectors can be stabilized by the deletion of one or both TIRs after their genomic integration. Indeed, all piggyBac-derived sequences can be eliminated with this method, including the selectable marker genes used for the initial germline transformation. Until now, this method has been successfully used for the post-integration stabilization of transgenes in the genomes of D. melanogaster, C. capitata, Anastrepha ludens, and more recently, H. axyridis. However, this strategy has not yet to be used in B. mori or any other lepidopteran species. To overcome the disadvantages of the position effects and potential insertional mutagenesis incurred by piggyBac-mediated random integration and to provide a stable, replaceable, and highly efficient expression system, a combination of the piggyBac- and SSR-based systems was developed, which had not previously been established in B. mori or any other species in vivo.

Based on the considerations discussed above, we developed an efficient strategy for producing a stable, replaceable, and highly efficient transgene expression system in the silkworm. First, we used piggyBac-mediated germline transformation to generate a transgenic silkworm strain that produces exogenous proteins with high efficiency in the silk gland and characterized the strain. The subsequent elimination of all transposon sequences, including the marker genes used for the initial germline transformation, resulted in the post-integration stabilization of the target gene expression cassette of interest in the transgenic silkworm genome. Because this expression cassette was flanked by two attP sites, a phiC31-mediated RMCE system can be used to repetitively integrate other gene expression cassettes into the same genomic locus. Our strategy offers a novel way to establish stable and replaceable transgenic silkworm strains for use as protein bioreactors and for fine gene function analyses. It will facilitate the development of lepidopteran species carrying stabilized transgenic insertions for both basic and applied purposes, including the comparative analysis of true transgenic alleles and the biological control of pest species. Our study also provides insight into the further improvement of various genetic manipulation systems.

**Results**

**Plasmid and experimental design.** Figure 1A shows the structure of the piggyBac-derived target plasmid (PB-TP) vector. Details of the construction procedure are described in the Methods section. The PB-TP vector containing an FibH-EGFP-LBS expression cassette (FibH, 5′-flanking sequence of FibH gene; EGFP, enhanced green fluorescent protein; LBS, L-chain binding site) was placed between two phiC31 integrase recognition sites (attP), and flanked by two short piggyBac arms L2 and R2; a 3×P3-promoter-driven DsRed gene expression cassette, 3×P3-DsRed-SV40 (SV40, 38, and more recently, H. axyridis signal sequence (polyA)), was placed between piggyBac arms R1 and L1, and a 3×P3-promoter-driven EGFP gene expression cassette, 3×P3-EGFP-SV40, was placed between piggyBac arms R2 and L1; a Drosophila heat shock protein 70 (hsp70)-promoter-driven piggyBac transposase (PBase) gene expression cassette, Hsp70-PBase-SV40, which was used to express PBase in vivo with heat shock treatment (HST), was placed behind the 3×P3-EGFP-SV40 expression cassette and also between R2 and L1. Thus, PB-TP structurally combines four different transposons (R1–L1, R1–L2, R2–L1, and L2–R2) that can potentially be expressed from this type of construct, and each transposase can be identified by a different combination of the 3×P3-DsRed (R), 3×P3-EGFP (G), and FibH-EGFP (F) fluorescent markers (Figure 1A). Supplementary
Figure S1 shows the constructs and the sequences of the *piggyBac* arms used in this study.

As described in the Methods section of this study, “G1 transgenic strain (TS)” is abbreviated as “TS1”, and subsequent TS generations are thus referred to as TS2, TS3, etc. TsN individuals with different fluorescent phenotypes, such as 3×P3-DsRed (R), FibH-EGFP (g), or 3×P3-EGFP (G), are abbreviated to TSN-R, TSN-G, or TSN-g, respectively (n = generations). For example, the transgenic individuals from G1 generations displaying three kinds of fluorescence were designated “TS1-RgG”. To select for high-efficiency transgene expression and the post-integration stability of the transgene by eliminating all the transposon sequences from the silkworm, we proceeded as follows steps (illustrated in Figure 1).

1. The PB-TP vector was integrated into the *B. mori* G0 strain with the *piggyBac*-mediated germline transformation of diapause silkworm strains48, and TS1-RgG individuals containing a single copy of the R1–L1 construct in their genomes were identified. The flanking transposons (R1–L2 and R2–L1) can then be eliminated in TS2-RgG germ-cell genome (D) by reexposure to transposase, mediated by heat shock treatment, resulting in the removal of one flanking transposon (R2–L1 or R1–L2) or both flanking transposons from the TS3-Rg, TS3-gG, or TS3-g genome (E–G).

2. Remobilized TS3 individuals were identified by their fluorescent marker phenotypes. (iv) TS3-gG individuals in which only the R1–L2 transposon was deleted from the genome (F) were also used for a second round of excision, completely eliminating the transposons (as above), leaving only the FibH-EGFP expression cassette flanked by two 39-bp *attP* sites (triangles with diagonal lines) in the same orientation in the TS4-g genome (H).

3. 3×P3, 3×P3 promoter; DsRed, *DsRed* gene (red box); SV40, SV40 polyadenylation signal sequence; FibH, 5′-flanking sequence of the silkworm fibroin heavy chain gene (~2.3-kb); EGFP, *EGFP* gene (green box); LBS, light chain binding site sequence of the silkworm fibroin heavy chain gene; Hsp70, *D. melanogaster* hsp70 promoter; A3, silkworm cytoplasmic actin 3 promoter; PBase, *piggyBac* transposase gene (blue box).

### Figure 1 | Strategy for the selection of high-efficiency transgene expression and the post-integration stabilization of the transgene using PB-TP vector in silkworms.

The initial transformation uses the PB-TP vector (A) carrying a composite transposon comprising two pairs of opposed transposon arms. The PB-TP structurally combines four different transposons (R1–L1, R1–L2, R2–L1, and R2–L2). The desired transposon R1–L1 was inserted into the TTA4 site of the G0 silkworm germ cell genome to produce the TS1-RgG individuals (C) mediated by *piggyBac* transposase derived from plasmid pHa3PIG (B). TS1-RgG individuals containing a single copy of the R1–L1 construct in their genomes (selected by the 3×P3-DsRed, FibH-EGFP and 3×P3-EGFP markers) and expressing high-level EGFP in their cocoons were identified. The flanking transposons (R1–L2 and R2–L1) can then be eliminated in TS2-RgG germ-cell genome (D) by reexposure to transposase, mediated by heat shock treatment, resulting in the removal of one flanking transposon (R2–L1 or R1–L2) or both flanking transposons from the TS3-Rg, TS3-gG, or TS3-g genome (E–G). TS3-gG individuals in which only the R1–L2 transposon was deleted from the genome (F) were also used for a second round of excision, completely eliminating the transposons (as above), leaving only the FibH-EGFP expression cassette flanked by two 39-bp *attP* sites (triangles with diagonal lines) in the same orientation in the TS4-g genome (H).
four potential transposons, as described above, the TS1 larvae display
different fluorescence phenotypes with the insertion of different
transposons. The analysis of the different fluorescence phenotypes
of the TS1 individuals from the positive G1 broods is shown in
Table 2. Supplementary Figure S2 shows fluorescent images of TS1
silkworms in the early larval stage. It is noteworthy that the four
screened TS1-RG larvae from one positive G1 brood (Table 2) not
only had the L2–R2 insertion, but also the simultaneous insertion
of both R1–L2 and R2–L1 into the genomes of TS1 individuals
(Supplementary Figure S2D). However, as described in
Supplementary Figure S3, the polymerase chain reaction (PCR)
results confirmed that each TS1-RG individual in this positive G1
brood contained only the L2–R2 construct in its genome, and the
simultaneous insertion of both R1–L2 and R2–L1 was not detected.

As shown in Table 2, four TS1-RgG individuals were obtained from
four of the 11 positive G1 broods for subsequent experiments and
designated TS1-RgG1–TS1-RgG4. Because all the TS1-RgG
individuals contained the FbI1-EGFP-LBS expression cassette in
their genomes (Figure 2A), the cocoons from the TS1-RgG1–TS1-
RgG4 silkworms displayed strong green fluorescence, indicating a
large amount of recombinant EGFP was spun into their cocoons
(Figure 2B). The cocoon from TS1-RgG2 displayed the strongest
fluorescence among the four cocoons at the same exposure time
and excitation light intensity (Figure 2B). The cocoon silk proteins
from the TS1-RgG and wild-type 871 silkworms were analyzed with
SDS-PAGE and immunoblotting with an anti-GFP antibody
(Figure 2C and D). The concentrations of the cocoon proteins
extracted from the different TS1-RgG individuals ranged from
511.9 to 862.7 ng/mL (Figure 2E). The results of SDS-PAGE sug-
gested that the EGFP/H-chain fusion proteins derived from each
TS1-RgG individual were single proteins of about 57 kDa
(Figure 2C, arrowhead). Based on the results of immunoblotting
(Figure 2D), we calculated that the contents of pure EGFP in the
TS1-RgG1–TS1-RgG4 silkworm cocoons were 14.6%, 16.5%, 7.1%,
and 9.9% (w/w), respectively (Figure 2E), which is consistent with the
fluorescence stereomicroscopic observations.

Southern blotting and inverse PCR were used to determine the
copy numbers and insertion positions of the transgene construct in
the TS1-RgG1 and TS1-RgG2 individuals (Figure 2A). Southern
blotting showed that both the TS1-RgG1 and TS1-RgG2 adults con-
tained only one copy of the R1–L1 transgene construct (Figure 2F).
The 20-bp silkworm genomic sequences flanking the piggyBac arms
are shown in Table 3. Both TS1-RgG1 and TS1-RgG2 carried the
transgene in a heterozygous state. The R1–L1 inserts in the genomes
of TS1-RgG1 and TS1-RgG2 were located on chromosomes 24 and
18, respectively. Thus, we had established two heterozygous G1 TSSs,
TS1-RgG1 and TS1-RgG2, containing a single copy of the R1–L1
transgene construct in their genomes. Because the TS1-RgG2 indi-
vidual more efficiently expressed the recombinant EGFP protein in
its cocoon, it was selected for subsequent experiments.

Table 1 | Injection of PB-TP vector in G0 silkworm embryos of the strain 871

| Experiment No. | Injected eggs | Hatched eggs (%) | G1 broods | Positive G1 broods | Percent of positive G1 broods, % |
|---------------|---------------|-----------------|-----------|-------------------|-------------------------------|
| 1             | 268           | 93 (34.70)      | 22        | 3                 | 13.6                          |
| 2             | 358           | 186 (51.96)     | 78        | 8                 | 10.3                          |
| Total         | 626           | 279 (44.57)     | 100       | 11                | 11.0                          |

Table 2 | Analysis of different fluorescence phenotypes of TS1 individuals from positive G1 broods

| Fluorescence phenotype of TS1 individuals (broods) | TS1-RgG | TS1-Rg | TS1-gG | TS1-RG | TS1-R | TS1-g | TS1-G | Total number of TS1 individuals in one experiment (broods) |
|--------------------------------------------------|--------|-------|--------|--------|-------|-------|-------|-------------------------------------------------------------|
| Experiment No.                                   | 1      | 2     | 1      | 2      | 1     | 2     | 1     | 15 (3)                                                     |
| TS1-RgG                                         | 1 (1)  | 3 (3) | 0      | 0      | 4 (1) | 23 (6) | 0     | 32 (8)                                                     |
| TS1-Rg                                          | 0      | 0     | 0      | 4 (1)  | 23 (6) | 0     | 11 (4) |                                                            |
| TS1-gG                                          | 0      | 0     | 0      | 0      | 4 (1)  | 28 (7) | 0     | 47 (11)                                                    |

TS1 individuals were identified from positive G1 broods by fluorescence phenotypes, that is, presence of which of the three markers they expressed. 3×P3-DsRed (R), FbI1-EGFP (g) and/or 3×P3-EGFP (G). Numbers of TS1 individuals containing different fluorescence phenotypes are shown, with the number of broods containing at least one transgenic larva (designated the “positive brood”).
Figure 2 | Analysis of the expression of recombinant EGFP in the cocoons from TS1-RgG silkworms and insertion copy number of R1–L1 transgene construct in TS1-RgG silkworms. (A) Schematic map of the R1–L1 transgene construct in genome of TS1-RgG individual. PLF/PLR and PRF/PRR are the piggyBac left arm 1 (L1) and right arm 1 (R1) primer pairs, respectively. H, HaeIII; X, XhoI; S, SpeI. (B) The cocoons from wild-type 871 and TS1-RgG1–TS1-RgG4 silkworms were observed under white light (exposure time 1/800 sec) and GFP-fluorescence (exposure time 1/10 sec). White scale bar represents 1 cm. (C) Cocoon silk protein samples from wild-type 871 (WT), TS1-RgG1–TS1-RgG4 silkworms were subjected to SDS-12% PAGE with 2-ME treatment. Arrowheads indicate recombinant EGFP (~57-kDa), and triangle indicates the GFP standard (27-kDa). M is molecular mass markers. Sizes are indicated on the left of the panels. (D) Immunoblotting analysis of proteins in the gel as in (C) with an anti-GFP antibody. Arrowheads indicate recombinant EGFP, triangle indicates the GFP standard (25 ng, 50 ng and 100 ng). Sizes are indicated on the left of the panels. (E) Comparison of the content of recombinant proteins in the cocoons from different TS1-RgG individuals. The ratio of pure EGFPs to the cocoon silk proteins within the membrane lanes were quantified by densitometry with Image-J software and the GFP standard used as a control. (F) Southern blotting analysis of XhoI- or SpeI-digested genomic DNA using DsRed probe. The individual DNA hybridization patterns of the XhoI- or SpeI-digested TS1-RgG1 and TS1-RgG2 lanes are shown. Sizes are indicated on the left of panel.
The transgene structures in the TS3 individuals were also confirmed with a PCR analysis using primers complementary to the genomic DNA and internal vector DNA (Supplementary Table S1), which yielded product sizes consistent with the deletion of R2–L1 from the TS3-Rg2 individuals, the deletion of R1–L2 from the TS3-g2 individuals, and the deletion of both R1–L1 and R1–L2 from the TS3-g2 individuals (Figure 4A and C). Because the TS3-g2 individuals were heterozygous for the transgene insertion, the PCR products were a 3893-bp DNA fragment that spanned the app-flanked FibH-EGFP-LBS expression cassette sequence and a 358-bp DNA fragment from the wild-type B. mori genome for the TS3-g2 individuals when the primer pair pBm2902-3′/pBm2902-5′ was used. The PCR product from the wild-type 871 individuals was only a 358-bp DNA fragment when the same primer pair was used. The PCR products from all TS3-g2 individuals were sequenced, and no structural changes were detected in either the cassette itself or the transgenic genomic DNA, indicating that piggyBac was excised without leaving a footprint at the excision-site TTAA element, as we expected. Supplementary Figure S5 shows the sequencing results for the 3′ and 5′ sequences of the app-flanked FibH-EGFP-LBS expression cassette in the genomes of the TS3-g2 individuals and for the wild-type genomic sequence at the same site in the wild-type 871 individuals.

A few TS2-R2 and TS2-g2 individuals were also screened from each group of this G2 brood (Table 4). The inverse PCR results confirmed that the R1–L2 transgene construct was located at an identical site on chromosome 6 in the genomes of all the TS2-R2 individuals, and the transgene construct in the genomes of the TS2-Rg2 and TS2-g2 individuals was located on chromosome 18, as in the TS1-Rg2 male (Supplementary Table S2). These results suggest that the R1–L2 remobilization event occurred in the spermatocytes of the TS1-Rg2 male, and also confirm the background expression activity of the hsp70 promoter in the silkworm.

Characterization of the optimal HST strategy for producing transposon-free transgenic silkworms. To identify the optimal HST strategy for producing transposon-free transgenic silkworms, as illustrated and described in Figure S6, one heterozygous TS3-gG2 male was backcrossed with three different wild-type 871 females (a, b, and c) to produce three G4 broods, and the G4 individuals from the three groups (1′, 2′, and 3′) of each G4 brood were treated with or without HST, as described in Supplementary Figure S4B. Finally, the fluorescent phenotypes of the larvae from 50 G5 broods of each group (G5 a, b and c broods) were analyzed (Supplementary Figure S6). The results suggest that the frequency of g-positive broods in the G5 broods of group 1′ (without HST), group 2′ (HST in the embryonic stage), and group 3′ (HST in the larval stage) were 0%–2%, 70%–80%, and 10%–14%, respectively (Supplementary Table S3). Thus, the frequency of the removal of R2–L1 by HST was significantly higher in the embryonic stage than in the larval stage or without HST, and the frequency of the removal of R2–L1 by HST was also significantly higher in the larval stage than without HST (Figure 5A).

### Table 3 | Identification of genomic insertion sites of R1–L1 transgene construct in TS1-RgG individuals by inverse PCR

| Strain | Scaffold | Chromosome | 5′-Genomic sequence | 3′-Genomic sequence |
|--------|---------|------------|---------------------|---------------------|
| TS1-RgG1 | nsscanf2891 | 24 | AGTCCTACGTCCACCTGTTAAGTTAA | TTAAGTGCTATTGCGGACCCATAG |
| TS1-RgG2 | nssca2902 | 18 | AGTCAGTCACTGACACAAATTAAGTTAA | TTAAGTATATTGTAATTTATAT |
| TS1-RgG3 | scaffold16066d | – | TGAAGAGTACCGCTAAAGTTAAGTTAA | TTAATGCTATTTGCGGTATCT |
| TS1-RgG4 | Not identified | |

| Strain | Scaffold | Chromosome | 5′-Genomic sequence | 3′-Genomic sequence |
|--------|---------|------------|---------------------|---------------------|
| TS1-RgG1 | nsscanf2891 | 24 | AGTCCTACGTCCACCTGTTAAGTTAA | TTAAGTGCTATTGCGGACCCATAG |
| TS1-RgG2 | nssca2902 | 18 | AGTCAGTCACTGACACAAATTAAGTTAA | TTAAGTATATTGTAATTTATAT |
| TS1-RgG3 | scaffold16066d | – | TGAAGAGTACCGCTAAAGTTAAGTTAA | TTAATGCTATTTGCGGTATCT |
| TS1-RgG4 | Not identified | |

### Table 4 | Analysis of different fluorescence phenotypes of TS2 individuals obtained by crossing a heterozygous TS1-RgG2 male with a wild-type 871 female

| Group | G2 eggs | Hatched eggs (%) | Total G2 fertile moths (%) | Total TS2 fertile moths |
|-------|--------|------------------|---------------------------|------------------------|
| 1#    | 140    | 138 (98.57)      | 121 (87.68)               | 59                     |
| 2#    | 137    | 108 (78.83)      | 90 (63.33)                | 47                     |
| 3#    | 138    | 133 (96.38)      | 98 (73.68)                | 54                     |

| Fluorescence phenotype of TS2 individuals |
|------------------------------------------|
| TS2-RgG2 | TS2-Rg2 | TS2-gG2 | TS2-R2 | TS2-g2 | TS2-G2 |
| 54 | 0 | 3 | 2 | 0 | 0 |
| 41 | 0 | 3 | 2 | 0 | 0 |
| 51 | 0 | 2 | 1 | 0 | 0 |

*aPercentage of (Number of hatched eggs)/(Number of G2 eggs).*

*bPercentage of (Number of total G2 fertile moths)/(Number of hatched eggs).*
exactly 50% (Figure 5B and Supplementary Table S3), which was consistent with the theoretical value. The results confirmed that the FibH-EGFP expression cassette cannot be remobilized in the genomes of TS4-g2 individuals when PBase is present \textit{in vivo}.

In addition, EGFP expression was detected in TS7-g2 individuals and their cocoons. As shown in Figure 6A, EGFP was highly efficiently and specifically expressed in the silk glands of the TS7-g2 silkworms and was spun into their cocoons. To further confirm the stability of transgene integration in the TS3 offspring whether PBase is present or absence \textit{in vivo}, PCR was performed on the genomic DNA from TS3-g2 (1/2), TS4-g2 (1/2), TS4-g2 (1/1), and TS7-g2 (1/1) individuals using primer pairs pBm2902-3'/pBm2902-5' and pBm2902-3'/FibH-MR. As shown in Figure 6B, the PCR products were a 3893-bp DNA fragment for TS3-g2 (1/2), TS4-g2 (1/2), TS4-g2 (1/1), and TS7-g2 (1/1) individuals using the primer pair pBm2902-3'/pBm2902-5', a 358-bp DNA fragment for wild-type 871 (WT, 2/2), indicates a nontransgenic), TS3-g2 (1/2), and TS4-g2 (1/2) individuals using the primer pair pBm2902-3'/pBm2902-5', and a 612-bp DNA fragment for TS3-g2 (1/2), TS4-g2 (1/2), TS3-g2 (1/1), and TS7-g2 (1/1) individuals using the primer pair pBm2902-3'/FibH-MR, which is consistent with the expected pattern. All the above results confirmed that the integration of FibH-EGFP expression cassette and the expression of EGFP were stable in the TS3 offspring whether PBase is present or absence \textit{in vivo}.

Table 5 | Post-integration removal of the flanking \textit{piggyBac} transposons in TS3 individuals obtained by crossing TS2-RgG2 moths with wild-type 871 moths

| Group | TS2-RgG2 fertile moths | G3 broods | Rg-positive broods | gG-positive broods | g-positive broods | Total [%]a |
|-------|------------------------|-----------|-------------------|-------------------|-----------------|-----------|
| 1#    | 54                     | 48        | 0                 | 2 (4.17)          | 0               | 2 (4.17)  |
| 2#    | 41                     | 37        | 7 (18.92)         | 18 (48.65)        | 6 (16.22)       | 23 (62.16) |
| 3#    | 51                     | 45        | 3 (6.67)          | 6 (13.33)         | 1 (2.22)        | 7 (15.56) |

Group 1#, 2#, and 3# represent the individuals of G2 broods without HST, HST in the embryonic stage and HST in the larval stage, respectively (as described in Supplementary Figure S4B and Supplementary Methods). Rg-, gG- and g-positive broods represent the G3 broods containing at least one TS3-Rg2, TS3-gG2 and TS3-g2 larva, respectively.

aTotal number of G3 broods containing at least one TS3-Rg2, TS3-gG2 or TS3-g2 larva.

Discussion

To allow fine functional research into unknown genes and the establishment of a stable and efficient \textit{B. mori} silk gland bioreactor, the disadvantages of the position effects and insertional mutagenesis caused by \textit{piggyBac}-mediated random integration must be overcome. Therefore, we used a combination of different genomic manipulation techniques and the optimized fibroin H-chain expression system to develop a generic and efficient strategy for establishing a stable, replaceable, and highly efficient transgene expression system in the silkworm. To develop this strategy, we first inserted into the genomes of silkworms an exogenous target gene expression cassette that efficiently and selectively expresses the target protein in the silk glands of the TS7-g2 silkworms, and was spun into their cocoons. To further confirm the stability of transgene integration in the TS3-offspring, PCR was performed on the genomic DNA from TS3-g2 (+/−), TS4-g2 (+/−), TS4-g2 (+/+) and TS7-g2 (+/+) individuals using primer pairs pBm2902-3'/pBm2902-5' and pBm2902-3'/FibH-MR. As shown in Figure 6B, the PCR products were a 3893-bp DNA fragment for TS3-g2 (+/−), TS4-g2 (+/−), TS4-g2 (+/+) and TS7-g2 (+/+) individuals using the primer pair pBm2902-3'/pBm2902-5', a 358-bp DNA fragment for wild-type 871 (WT, −/−, indicates a nontransgenic), TS3-g2 (+/−), and TS4-g2 (+/+) individuals using the primer pair pBm2902-3'/pBm2902-5', and a 612-bp DNA fragment for TS3-g2 (+/−), TS4-g2 (+/−), TS4-g2 (+/+) and TS7-g2 (+/+) individuals using the primer pair pBm2902-3'/FibH-MR, which is consistent with the expected pattern. All the above results confirmed that the integration of FibH-EGFP expression cassette and the expression of EGFP were stable in the TS3 offspring whether PBase is present or absence \textit{in vivo}.
Figure 4 | Molecular confirmation of the deletion of the flanking transposons in the TS3 individuals by piggyBac remobilization. (A) Schematic of four different transgene constructs in genomes of TS3 silkworms and the same genome site of wild-type 871 silkworms. The relative primer positions were indicated above the diagram of different transgene constructs, and the suitable primer pairs were used for PCR analysis of genomic DNA. Primers pBm2902-3' and pBm2902-5' were each designed from the 3' and 5' piggyBac-flanking genomic sequences of TS1-RgG2 genome based on the results of inverse PCR. Primers FibH-MR and LBS-MF were each designed from the H-chain gene promoter and L-chain binding site sequence of the *B. mori* fibroin H-chain gene (Supplementary Table S1). Red and green lines indicate the region homologous to the DsRed probe and EGFP probe, respectively. The Xhol restriction enzyme sites for the transgene constructs are shown. (B) Southern blotting analysis of Xhol-digested genomic DNA using EGFP probe (left) and DsRed probe (right), respectively. The individual DNA hybridization patterns of the wild-type 871 (WT), TS3-RgG2, TS3-Rg2, TS3-gG2 and TS3-g2 lanes are shown. Sizes are indicated on the left of panel. (C) PCR confirmation of genomic DNA using suitable primer pairs. The left panel shows the PCR products of genomic DNA from individuals of TS3-RgG2, TS3-g2 and wild-type 871 (WT), and the right panel shows the PCR products of genomic DNA from individuals of TS3-Rg2 and TS3-gG2. Lane M, Trans2K Plus DNA Marker. Sizes of the PCR products are shown below each lane.
transformation. The structure of the PB-TP vector has been described above. It is noteworthy that this composite vector, with two full-length wild-type piggyBac arms R1 (1050 bp) and L1 (678 bp) and two shortened piggyBac arms L2 (309 bp) and R2 (238 bp), encodes four potential transposons (Supplementary Figure S1). It has been reported that the short piggyBac arm constructs can be used to improve the mobilization efficiency of piggyBac in the genomes of D. melanogaster and B. mori. Therefore, L2 and R2 were developed to improve the efficiency of R1–L2 and R2–L1 remobilization in B. mori in vivo, thereby improving the deletion efficiency of R1–L2 and R2–L1 from the initial chromosomal insertion locus. Several previous studies have also demonstrated that not only the TIRs but also the flanking sequences of piggyBac, especially the TTAA sites, are required for its successful transposition. Therefore, the TTAA sites were constructed at the 3’ end of the L2 and R2 sequences in the composite vector (shown in the primer sequences in Supplementary Table S1). To achieve the precise integration of other exogenous genes at the same genomic locus as the target gene with phiC31-mediated RMCE, two attP sites were introduced, flanking the target gene expression cassette, in the same orientation.

In this study, we established four different transgenic strains, TS1-RgG1–TS1-RgG4, with the random insertion of transposon R1–L1 into the silkworm genome, and the cocoons of the different TS1-RgG strains displayed pure EGFP contents ranging from 0.9% to 16% (w/w). This result indicates that a transgene inserted at different chromosomal loci greatly affects the expression of the exogenous protein in the silkworm. To our knowledge, TS1-RgG2 is so far the most efficient transgenic silkworm strain producing exogenous protein in the silk gland. The subsequent elimination of all transposon sequences containing the PBase gene expression cassette and all the marker genes for TS2-RgG2 was completed with the heat-shock-induced expression of the transposase in vivo, generating TS3-g2, which contains only the attP-flanked optimized fibroin H-chain expression cassette in its genome. This method not only prevents the remobilization of the target gene, but also eliminates the adverse effects of the selectable marker genes in any future application of the transgenic insect, which may affect the expression of the target genes, the growth and development of the transgenic individuals, horizontal gene transfer, or any of the other potential ecological security problems associated with transgenic insects. The sequencing results showed that there was no footprint or any structural transformation.
changes, such as deletions, inversions, or rearrangements, at the excision-site TTAA elements or attP sites in the genomes of the TS3-g2 individuals. Further results confirmed the genetic stability of the integration and the expression of the EGFP gene in the TS3-g2 offspring. In a future study, different genes of interest will be placed precisely at the same genomic locus of the TS3-g2 silkworm using a phiC31-mediated RMCE reaction. This will achieve the highly efficient, stable expression of different exogenous proteins in the transgenic silkworms, mediated by the fibroin H-chain promoter. Because the phiC31-integrase-mediated recombination between the attP and attB sites is unidirectional, it ensures the stability of the transgenes after the RMCE reaction. The TS3-g2 silkworms established in this study can be used to create a highly efficient transgenic silkworm silk gland bioreactor for the production of exogenous proteins. Importantly, they can also be used to improve the natural cocoon silks and produce novel silk fibers with high tensile strength, high adhesion, and other excellent properties with the silk-gland-specific expression of structurally related proteins (such as the spider dragline protein). Actually, other silk-gland-specific promoters (such as the FibL, fhx, and Ser1 promoters) or tissue-specific promoters (such as fat-body, midgut, and hemocyte-specific promoters) can also be used to establish stable, replaceable, and highly efficient transgene expression systems in the silkworm with this generic strategy (Figure 1). In addition, the FLP/FRT and Cre/loxP systems have been successful used for RMCE reactions in D. melanogaster. Furthermore, Schetelig and Handler recently described a Cre-mediated RMCE system that is highly efficient in D. melanogaster, and for the first time in a non-drosophilid, the tephritid fly, Anastrepha suspensa. Compared with the phiC31-mediated RMCE system, FLP- and Cre-mediated RMCE have the...
main advantage that allowed for multiple insertion/deletion events of transgenes at a single locus. In the future work, FLP- and Cre-mediated RMCE systems also could be combined with different genomic manipulation techniques described above, and introduced as a powerful tool for functional genomic comparisons and to develop the most advanced transgenic silkworm strains for applied use.

In this study, a mixture of the PB-TP vector and the helper plasmid pHA3PIG was injected into G0 eggs to create TS1 individuals with initial germline transformation. The transgenic individuals could also be generated by the injection of the PB-TP vector alone, without the helper plasmid, because the Drosophila hsp70 promoter in the PB-TP vector induces highly efficient transient protein expression in the embryos of several different insect species, including D. melanogaster, Anopheles stephensi, and B. mori. The structural combination of four different transposons is encoded in the composite PB-TP vector, but the germline transformation of only the R1–L1 construct was expected during the initial transformation. Therefore, a silkworm cytoplasmic A3-promoter-driven Pbase gene expression vector, pHA3PIG, was used as the helper plasmid for the production of PBase, which will increase the efficiency of the initial expected transformation, thereby enhancing the probability of producing TS1-RgG individuals.

Here, we also used the hsp70 promoter to induce Pbase gene expression in the TS2-RgG2 individuals in vivo with HST. The HST method is simpler than the direct injection method, and
main disadvantage of the sexual hybridization method is that the PBase gene sequence is introduced into the genome of the hybrid offspring, allowing the persistent expression of PBase, which can reduce the deletion efficiency of R1–L2 and R2–L1. Previous studies have shown that the hsp70 promoter best induces the expression of downstream genes when silkworms are exposed to continuous and repeated HST at 42°C in their developmental stages.68,69. The embryonic silkworm develops from a single-celled zygote to a larva, and the fourth instar larval stage of the silkworm is a critical period in the formation of its secondary spermatocytes or the development of its primary oocytes. Therefore, continuous and repeated HST at 42°C was applied to treat the transgenic silkworms at the embryonic stage or fourth instar larval stage in this study. The results suggest that HST in the embryonic or larval stage affects the normal growth of the transgenic silkworms, and that HST in the larval stage causes the highest mortality rate (Table 4). Compared with the offspring of individuals in the non-HST control groups, the deletion efficiencies of R1–L2 and/or R2–L1 were significantly higher in the offspring of TS2-Rg2 individuals in the HST groups, especially in the offspring of groups with HST at the embryonic stage (Table 5). The deletion efficiency of R2–L1 was up to 80% in the offspring of TS4-gG-2 individuals with HST applied in the embryonic stage (Supplementary Table S3 and Figure 5A). All these results suggest that continuous and repeated HST at 42°C in the embryonic stage of transgenic silkworms is the most effective way to delete the transposons from their offspring.

However, a few G3 g-G-positive broods from TS2-Rg2 individuals and G5 g-positive broods from TS4-gG individuals were also observed in the non-HST groups, which is attributable to the background expression of PBase under the control of the hsp70 promoter, even though this background activity was very low in the transgenic silkworms in vivo (Table 5 and Supplementary Table S3). These results are consistent with those of a previous study that reported the background expression of a Bombbyx nuclear receptor Ftz-F1 gene (BmFtz-F1) under the control of the Drosophila hsp70 promoter in transgenic silkworms.68,69. Although the basal activity of the hsp70 promoter was low at 25°C, our study confirms that the Drosophila hsp70 promoter is a very effective inducible promoter for regulating the expression of exogenous genes in transgenic silkworms.

In recent years, genome-editing methods, such as zinc finger nucleases (ZFN), transcription-activator-like effector nucleases (TALEN), and clustered regularly interspersed short palindromic repeats (CRISPR) RNA-guided Cas9 nucleases, have been successfully used to target and cleave genes in the silkworm.71–73. However, the length of the DNA fragment integrated into the silkworm genome by TALEN-mediated gene editing using single-stranded DNA oligonucleotides is very limited, and the only reported efficient GFP expression cassette (A3-GFP-SV40T) knock-in was still very limited in the silkworm when mediated by ZFN (just 0.008%, 1/11770). Figure 7 shows an efficient method for modifying previously inserted transgenes and for the integration of large DNA fragments into the silkworm genome using a combination of the piggyBac-based transposon-free method, the phiC31-mediated RMCE system, and the FLP/FRT system. The phiC31/att system has been shown allow the integration of DNA of up to 100 kb into specific recipient sites in D. melanogaster,74 so this method could be used to overcome the disadvantages of these genome-editing systems. Furthermore, a combination of the SSR system and the genome-editing methods described above should overcome both the random insertion of transposons and the problems associated with the integration of large DNA fragments using genome-editing systems.

In conclusion, we have developed an efficient and generic strategy for producing a stable, replaceable, and highly efficient transgene expression system in B. mori. Our strategy effectively eliminates the remobilization of piggyBac-mediated integrated transgenes in the silkworm. It is also applicable to other lepidopteran insects to improve the ecological safety of transgenic strains intended for release in biocontrol programs. Because silkworms are a commercially important insect and are widely used as an experimental model of lepidopteran insects, the transgenic strains established in this study can be used not only to optimize exogenous protein expression and to improve the properties of natural cocon silk, but also in functional genomic research, such as investigating the functions of different genes at the same locations in the silkworm genome. The use of the piggyBac-based transposon-free method combined with a phiC31-mediated RMCE system in B. mori or any other species has not been reported until now. In a future study, we will combine our strategy with the genome-editing systems described above to establish functional genomic research technologies in the silkworm and other lepidopteran species.

Methods

Experimental animals. The Chinese lineage diapause B. mori strain 871 (white cocoon, commercial strain) is maintained at the Gene Resource Bank of Domesticated Silkworms, Southwest University, Chongqing, China. The 15°C-CMES germline transformation strategy developed by us68 was used to change the diapause character of the 871 eggs before DNA preblastoderm microinjection. After they were injected, the eggs were maintained at 25°C in a moist chamber (85%–90% relative humidity) until hatching. The larvae were reared at 25°C (75%–80% relative humidity) and fed mulberry leaves.

Plasmid construction. The piggyBac-derived target plasmid vector pBac[R1–3]-DsRed-SV40-L2-attP-FibH-EGFP-LBS-attP-R2–3]-P3-EGFP-SV40-Hsp70-PBase-SV40-L1) (designated “PB-TP”; Figure 1A) was constructed as described in the Supplementary Materials. The sequences of the primers used in the plasmid construction are shown in Supplementary Table S1. Briefly, (i) the attP-pBacR2 fragment was PCR amplified from pBac[1–3]-P3-DsRed)72 and cloned into the plasmid pSL[3–P3-EGFP-SV40]77 to generate pSL[attP-2–3]-P3-EGFP-SV40; (ii) the Drosophila hsp70 promoter, the PBase gene, and the SV40 polyA were PCR amplified from the plasmids pBac[1–3]-P3-DsRed,72 and pBac[1–3]-P3-DsRed)72, respectively, and the three amplified fragments were inserted into the plasmid pSLFA180a72 to generate pSL[Hsp70-PBase-SV40]; (iii) the Hsp70-PBase-SV40 fragment was Spbl-digested from pSL[Hsp70-PBase-SV40] and cloned into the plasmid pSL[attP-2–3]-P3-EGFP-SV4077 to generate pSL[attP-2–3]-P3-EGFP-SV40-Hsp70-PBase-SV40; (iv) the pBacL2-attP fragment was PCR amplified from pBac[3–P3-DsRed)72 and inserted into the plasmid pBac[3–P3-DsRed)72 to generate pBac[R1–3]-P3-DsRed-SV40-L2-attP-FibH-EGFP-LBS-1); (v) the attP-P3-3–P3-EGFP-SV40-Hsp70-PBase-SV40 fragment was Fsel-digested from pSL[attP-2–3]-P3-EGFP-SV40-Hsp70-PBase-SV40 and cloned into the plasmid pBac[R1–3]-P3-DsRed-SV40-L2-attP-FibH-EGFP-LBS-L1) to generate the PB-TP vector.

Germline transformation and marker detection. The germline of B. mori was transformed with a piggyBac vector, as previously described.8–10, G0 nondiapause eggs from strain 871 were collected for microinjection within 2 h of oviposition. A 1:1 mixture of 450 ng/pL PB-TP vector and 400 ng/pL pHA3PIG in superpure water was injected into each egg with a FemtoJet 5247 (volume ratio) mixture of 450 ng/pL PB-TP vector and 400 ng/pL pHA3PIG in superpure water was injected into each egg with a FemtoJet 5247 microinjection machine (Eppendorf, Hamburg, Germany). The G0 embryos were allowed to develop at 25°C. The fertile G0 adults were backcrossed with wild-type 871 adults to produce G1 offspring. The expression of 3×P3-DsRed and 3×P3-EGFP in the eyes and nervous systems of the G1 embryos, larvae, pupae, and adults was detected with an Olympus MacroView MVX10-AUTO fluorescence stereomicroscope (Olympus, Tokyo, Japan) with a red fluorescent protein (RFP) or green fluorescent protein (GFP) filter, respectively. The expression of the EGFP/H-chain fusion protein (FibH-EGFP) in the silk gland of the G1 larvae and in the cocoon silk from G1 individuals was detected with the same fluorescence stereomicroscope and a GFP filter. Filters passing light at 510–550 nm for DsRed and at 460–490 nm for EGFP were used for excitation. Positive G1 larvae from different broods were reared with each brood considered a unit, and the transgenic strains (TSs) were then produced.

SDS-PAGE and immunoblotting analysis. SDS-PAGE and immunoblotting analysis of EGFP in the silkworm cocoon were performed as described in our previous report.11 Briefly, about 25 mg of each silkworm cocoon was dissolved in 1 mL of 60% (w/v) lithium thiocyanate (LiSCN), and 1 μg/pL GFP standard (Abcam, Cambridge, UK) was dissolved in LiSCN containing 1 μM of Plasmid DsRed (1.5 μL) and the GFP standard were subjected to SDS-PAGE (12% [w/v] polyacrylamide slab gel) by dissolving them in equal volumes of sample loading buffer with 2% (v/v) β-mercaptoethanol (2-ME), and boiling them for 5 min. After electrophoresis, the gel was stained with Coomassie Brilliant Blue R-250. An aliquot (0.5 μL) of each sample and the GFP standard were subjected to SDS-PAGE with a 60% (w/v) lithium thiocyanate (LiSCN) solution, incubated at room temperature for 1 h with TBST containing 2500-fold diluted anti-GFP antibody (Beyotime, Jiangsu, China). The membrane was then incubated at...
room temperature for 1 h in TBST containing horseradish-peroxidase-labeled anti-rabbit IgG secondary antibody diluted 10,000-fold (Beysinte). The immuno-reactive bands were visualized with the ECL Plus Western Blotting Detection Reagents, according to the manufacturer’s instructions (Beysinte), and a chemiluminescence imaging system (Clinite ChemilumiScope series, Shanghai, China). The relative intensities of the bands were calculated with the ImageJ software and compared with that of the GFP standard used as the control.

Southern blotting analysis. Genomic DNA was prepared from the silkworms using the procedure described by Zhao et al. About 25 μg of genomic DNA was digested with the indicated restriction enzymes and blotted onto a Hybond-N+ nylon filter (Amersham Bioscience, Piscataway, NJ, USA) after agarose gel electrophoresis. A 678-bp DsRed gene fragment was amplified from pBac[3×P3-DsReda] with primers pDsRed-f and pDsRed-r, and a 720-bp EGFP gene fragment was amplified from pBac[3×P3-EGFPa] with primers pEGFP-f and pEGFP-r (Supplementary Table S1). The sequencing results were analyzed with the Silkworm Genome Database (SILKDB http://www.silkdb.org/silkdb/). Localization of the silkworm genomic insertion sites of the transgene constructs was completed using the SilkMap software (www.silkdb.org/silksoft/silkmap.html).

piggyBac remobilization. The flanking transposons in the TSs individuals were remobilized with heat shock treatments (HSTs), as described in detail in the Supplementary Material. Briefly, (i) TS1-RGg adults were backcrossed with wild-type 871 adults to produce G2 eggs, and these eggs were treated with HCl solution to break the diapause; (ii) three-day-old G2 nondiapause eggs were heat shocked at 42 °C for 60 min three times a day at 6 h intervals for five days, or day 0 fourth instar G2 larvae were heat shocked at 42 °C (for 60 min three times a day at 6 h intervals for three days); (iii) after heat shock, the G2 eggs or larvae were maintained at 25 °C. TS2-RGg adults from these G2 individuals were selected and backcrossed to wild-type 871 adults. TS3-g individuals from newly hatched G3 larvae were screened (for both L1–L2 and L2–L1 deletion) under a fluorescence stereomicroscope, as described above, and these TS3-g individuals were reared to adulthood and sibling-mated or backcrossed with wild-type 871 adults to generate offspring.

PCR analysis. Extracted genomic DNA from the TSs and wild-type 871 silkworms was used as the templates for PCR. The primer sequences used in the PCR analysis are shown in Supplementary Table S1. The purified PCR fragments were cloned into the plasmid pMD19-T Simple (Takara, Dalian, China) and sequenced.

8. Fraser, M. J., Ciszczon, T., Elick, T. & Bauser, C. Precise excision of TTAA-specific Inverse PCR analysis

9. Elick, T. A., Bauser, C. A., Principe, N. M. & Fraser, M. J., Jr. PCR analysis of genomic DNA was digested with HaeIII overnight at 37 °C and circularized by ligation overnight at 16 °C. The ligated product was PCR amplified with the transposon-specific primer pairs PLF/PLR (for piggybac left arm 1, L1) and PRF/PRR (for piggybac right arm 1, R1) (Supplementary Table S1). The sequencing results were analyzed with the Silkworm Genome Database (SILKDB http://www.silkdb.org/silkdb/). Localization of the silkworm genomic insertion sites of the transgene constructs was completed using the SilkMap software (www.silkdb.org/silksoft/silkmap.html).

In reverse PCR analysis, the chromosomal insertion sites of the transgene constructs were determined with inverse PCR, as previously described42–44. About 10 μg of genomic DNA was digested with HaeIII overnight at 37 °C and ligated by circularization by ligation overnight at 16 °C. The ligated product was PCR amplified with the transposon-specific primer pairs PLF/PLR (for piggybac left arm 1, L1) and PRF/PRR (for piggybac right arm 1, R1) (Supplementary Table S1). The sequencing results were analyzed with the Silkworm Genome Database (SILKDB http://www.silkdb.org/silkdb/). Localization of the silkworm genomic insertion sites of the transgene constructs was completed using the SilkMap software (www.silkdb.org/silksoft/silkmap.html).

In this study, we have demonstrated that the piggyBac transposon provides an efficient method for the generation of transgenic silkworms, which is likely to have wide applications in areas of pest control, disease vectors, and production of recombinant proteins.
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Author contributions

A.C.Z., D.P.L. and Z.H.X. conceived and designed the study. D.P.L., W.J.L., Y.L.Z. and L.H.B. performed and analyzed all the experiments. D.P.L. and A.C.Z. wrote the paper. All authors reviewed the manuscript.

Additional information

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