FLP Recombinase-Mediated Site-Specific Recombination in Silkworm, Bombyx mori

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Abstract

A comprehensive understanding of gene function and the production of site-specific genetically modified mutants are two major goals of genetic engineering in the post-genomic era. Although site-specific recombination systems have been powerful tools for genome manipulation of many organisms, they have not yet been established for use in the manipulation of the silkworm Bombyx mori genome. In this study, we achieved site-specific excision of a target gene at predefined chromosomal sites in the silkworm using a FLP/FRT site-specific recombination system. We first constructed two stable transgenic target silkworm strains that both contain a single copy of the transgene construct comprising a target gene expression cassette flanked by FRT sites. Using pre-blastoderm microinjection of a FLP recombinase helper expression vector, 32 G3 site-specific recombinant transgenic individuals were isolated from five of 143 broods. The average frequency of FLP recombinase-mediated site-specific excision in the two target strains genome was approximately 3.5%. This study shows that it is feasible to achieve site-specific recombination in silkworms using the FLP/FRT system. We conclude that the FLP/FRT system is a useful tool for genome manipulation in the silkworm. Furthermore, this is the first reported use of the FLP/FRT system for the genetic manipulation of a lepidopteran genome and thus provides a useful reference for the establishment of genome manipulation technologies in other lepidopteran species.

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Introduction

Site-specific recombinase (SSR) technology is an important molecular biotechnology that was developed during the 1980s. Through the genetic manipulation of the eukaryotic genome and exogenous DNA by SSR-mediated recombination between two recombination target sites (RTs), SSR can induce the replacement, inversion and tissue-specific knockout of target genes [1]. SSR can overcome the disadvantages of other types of recombination technology, such as homologous recombination and transposon-mediated recombination; such disadvantages include low efficiency and random integration without targeting. As a result, this technology has gradually been widely applied to many areas of transgenic organism research, particularly for the genetic engineering of higher eukaryotic organisms [2–6].

Currently, the most commonly used SSR systems are Cre/loxP from Escherichia coli phage P1 [7], FLP/FRT from the 2-m plasmid of Saccharomyces cerevisiae [8] and ΦC31/att from the Streptomyces phage ΦC31 [9]. Since the first report of the application of the Cre/loxP system for generating a tissue-specific knockout mice model [10], this system has been widely used to study gene function in mammalian cells and to construct transgenic mouse models of disease [2,11]. Because the recombination catalyzed by ΦC31 integrase (Int-ΦC31) between the heterotypic sites attP [34 base pairs (bp) long] and attB (39-bp long) is directional and irreversible [12], Gao et al. developed an efficient site-specific integrase-mediated repeated targeting (SIRT) method to target a single locus repeatedly and to facilitate targeted mutagenesis in Drosophila [3]. The ΦC31/att system has also had an important role in the integration of transgenes into the mammalian genome and for the development of gene therapy [13,14,15]. As a member of the integrase or tyrosine-based family of SSR technologies [16], the FLP/FRT system has also emerged as a powerful tool to manipulate genomes of transgenic plants, mammals, insects and other higher eukaryotic model organisms. In recent years, the FLP/FRT system has been widely used in Arabidopsis thaliana [17], rice (Oryza sativa) [4], mouse (Mus musculus) [5], Drosophila melanogaster [18], Caenorhabditis elegans [6] and other higher eukaryotic organisms, to achieve gene knockouts, gene knock-ins, point mutations, deletion mutations, genomic large fragment deletions and other genetic engineering operations.

FLP recombinase can identify specifically FRT sites (FLP recombination target site) and mediate site-specific recombination reactions between two identical FRT sites [19,20]. The position and relative orientation (i.e. same or opposite direction) of the two FRT sites determine the outcome (i.e. insertion, excision, inversion or reciprocal translocation) of the FLP recombinase-mediated recombination reaction [21]. In plants,
Materials and Methods

Experimental Animals

The Chinese lineage B. mori bivoltine inbred strain Dazao has been maintained in our laboratory. It was necessary to change the diapause character of Dazao eggs for DNA pre-blastoderm microinjection. The 15°C-IMES germline transformation strategy used for the Dazao strain was based on the report of Zhao et al. [38]. After being 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 with mulberry leaves.

Construction of Vectors

Recombinase-mediated site-specific recombination target construct. The piggyBac-derived vector pBac3×3-DsRed; FRT-3×3P-EGFP-SV40-FRT} (3×3P, 3×3P promoter; DsRed, red fluorescent protein; EGFP, enhanced green fluorescent protein; SV40, SV40 polyadenylation signal sequence) (Figure 1A) was constructed as described below. A 1.3-kb 3×3P-EGFP-SV40 fragment was amplified by PCR from pBac3×3P-EGFPaf) [39] with the primer pair 3×3P-F-XhoI (5’-tatactcgagATCCTCCCCAACATGTTAATCTG-3’) and SV40-R-SphI (5’-cgagctcTACGCGTATCGATAAGCTTAAAG-3’). The amplified fragment was double-digested with XhoI and SphI, and inserted into the XhoI/SphI site of the plasmid pSLs11800fa [39] to generate pSL{3×3P-EGFP-SV40}. Two 48-bp FRT fragments were obtained by annealing the following sequences of the oligonucleotides: FRT-SpeI/XhoI-F (5’-ctagGAAGTTCC-TAATCCGAATGTTACCTATCCTAGAAATATGAGAAGCTTAAAG-3’) and FRT-SpeI/XhoI-R (5’-ctagGAAGTTCC-TATACCTTCTAGAGAAATGAGAAGCTTAAAG-3’); FRT-SphI/BglII-F (5’-cgaAGTTCTTACATGTTACATCCTATCTTTGAAAGCCAGATGAGAAGCTTAAAG-3’) and FRT-SphI/BglII-R (5’-tgatctcctct concatctctttctagagaaaaagccagatgagaaagctttcgcgtc-3’). The annealing conditions were as follows: initial denaturation at 94°C for 5 min; reduced by 1°C per 90 sec until 25°C; 25°C for 5 min; and then stored at 4°C.

The two 48-bp FRT fragments were inserted between the XhoI and SphI/BglII sites of the plasmid pSLs{3×3P-EGFP-SV40}. The plasmid pSLs{FRT-3×3P-EGFP-SV40-FRT} was generated. pBac{3×3P-DsRed; FRT-3×3P-EGFP-SV40-FRT} was then constructed by cloning a 1.3-kb AscI fragment from pSLs{FRT-3×3P-EGFP-SV40-FRT} into AscI cut pBac{3×3P-DsRedaf). The FLP recombinase expression construct. The FLP recombinase-expressing helper vector pSLA3-FLP (Figure 1D) was constructed as described below. A 0.65-kb silkworm cytoplasmic actin 3 gene promoter (A3 promoter) fragment was amplified by PCR from pH33Pig (Figure 1B) [41] with the primer pair A3-F-SacI (5’-tatatgacagTCGCGGTACCTAGATATGTTG-3’) and A3-R-KpnI (5’-ctatgacagCTCGAGTTCTCTCTGACGAAAGAGGAC-3’). The amplified fragment was digested with SacI and KpnI, and inserted into the plasmid pSLs11800fa to generate pSL-A3. The 0.37-kb silkworm A3 polyadenylation signal sequence (A3 polyA) was PCR-amplified from Dazao genome with an A3 polyA-F-SphI (5’-ctagCGGTTTAAATTCCTCTGCTGACGAAAGAGGACGTTCC-3’) and A3 polyA-R-BamHI (5’-gtatagTGCGTTATCTGACGAAAGAGGACGTTCC-3’) primer pair. The PCR product was digested with SphI and BamHI, and cloned into the plasmid pSL-A3 to generate pSL-A3-A3 polyA. The FLP recombinase gene was amplified by PCR from the plasmid pKhsd02-FLP [28] with the following primer pair: FLP-F-KpnI (5’-ctatgacagGCTCGAGTTCTCTGCTGACGAAAGAGGACGTTCC-3’) and FLP-R-BamHI (5’-gtatagTGCGTTATCTGACGAAAGAGGACGTTCC-3’).
The 1.28-kb PCR product was inserted into the KpnI/SphI site of pSL-A3-A3 polyA to generate pSLA3-FLP. The above sequences that are underlined show the restriction enzyme cutting sites. The sequences of the PCR products and resulting recombinant plasmids were confirmed by sequencing.

Production of the Target Transgenic Silkworm Strain

Plasmid DNA for pre-blastoderm microinjection was purified using a QIAGEN Plasmid Midi Kit (Qiagen, Hong Kong, China), and the prepared DNA solution was stored at −20°C until being used. pHA3PIG was used as the helper plasmid for the production of piggyBac transposase. According to the 15°C-IMES germline transformation strategy [38], we collected the G0 non-diapause eggs from strain Dazao within 2 h following oviposition for microinjection. A 1:1 (volume ratio) mixture of the 450 ng/μL pBac[3×P3-DsRed; FRT-3×P3-EGFP-SV40-FRT] vector and 400 ng/μL helper plasmid pHA3PIG in super-pure water were injected into each egg with a FemtoJet 5247 microinjector system (Eppendorf, Hamburg, Germany), and each egg was injected with 5–10 nL of the mixture. The injection hole was sealed with nontoxic glue (Instant Strong GlueMini, Japan) and the G0 embryos were allowed to develop at 25°C. G0 adults were mated with each other or backcrossed with the wild-type Dazao strain.

The expression of the red fluorescent protein (DsRed) and enhanced green fluorescent protein (EGFP) in G1 embryos, larvae, pupae and adults was detected using an Olympus MacroViewMVX10-AUTO fluorescent stereomicroscope (Olympus, Tokyo, Japan) with a RFP or GFP filter, respectively. Filters passing light between 510 and 550 nm for DsRed, and between 460 and 490 nm for EGFP were used for excitation. The individuals with DsRed- and GFP-positive G1 offspring were identified as germline-positive transgenic silkworms. G1-positive larvae from different broods were reared (with each brood being a unit), and the FLP/FRT system transgenic target strains (TTSs) were then produced.

Determination of the Insertion Position and Copy Number of TTS Transgene Constructs

Genomic DNAs were extracted from G1 TTS moths and wild-type moths (as controls). DNA was purified using an improved

Figure 1. Strategy for FLP recombinase-mediated site-specific recombination in silkworms. The piggyBac-derived vector pBac[3×P3-DsRed; FRT-3×P3-EGFP-SV40-FRT] (A) was inserted into the TAA site of the G0 silkworm germ cell genome to produce a stable G1 transgenic target strain (TTS) (C) mediated by piggyBac transposase derived from plasmid pHA3PIG (B). The TTS was transgenic for a 3×P3 promoter-driving DsRed gene (red box) expression cassette and a cassette that was flanked by two 48-bp FRT sites (black triangles) in the same orientation. A 3×P3 promoter-driving EGFP gene (green box) was placed internally to the two FRT sites. Site-specific recombination between the two FRT sites of G2 TTS germ cell genome (E), mediated by FLP recombinase derived from helper vector pSLA3-FLP (D), result in the deletion of the 3×P3-EGFP expression cassette from the genome of G3-positive site-specific recombination strain (SSRS) individuals (F). 3×P3, 3×P3 promoter; SV40, SV40 polyadenylation signal sequence; A3, silkworm cytoplasmic actin 3 promoter; A3 polyA, polyadenylation signal sequence of silkworm A3 gene; pBacL, left arm of piggyBac transposon; pBacR, right arm of piggyBac transposon. doi:10.1371/journal.pone.0040150.g001
phenol/chloroform method after proteinase K treatment [42]. Genomic DNA (approximately 10 µg) was digested with 
HaeIII and circularized by overnight ligation at 16°C using T4 DNA ligase (Promega, USA). The ligated DNA was treated with

phenol/chloroform and then precipitated with ethanol. Approximately 50–100 ng ligated DNA was used as a template for

PCR. Primers were used to recover the flanking sequence of the

piggyBac transposon as described by Ding et al. [43]. For the 5’

junction (piggyBac left arm), the forward primer PLF (5’-

CTTGACCTTGCCACAGAGGACTATTAGAGG-3’) and reverse

primer PRR (5’-CTCTGATATACAGCAGTAAACACATGC-3’) were

used. For the 3’ junction (piggyBac right arm), the forward primer PRF (5’-CTCTGATATACAGCAGTAAACACATGC-3’) and reverse primer PRR (5’-CTCTGATATACAGCAGTAAACACATGC-3’) were used. PCR was performed as follows: initial denaturation at 95°C for 10 min, then 35 cycles of 95°C for 30 sec, 63°C for 45 sec and 72°C for 3 min, followed by 72°C for 10 min.

PCR fragments were separated by electrophoresis in a 0.8% (w/v) agarose gel. Each single band was picked up from the gel and purified using a gel extraction kit (Omega, USA). The purified fragments were cloned into the plasmid pMD19-T simple and sequenced with the M13F/R primer to identify the boundary sequence of the insertion site.

Sequencing results were analyzed using NCBI BLAST searches (www.ncbi.nlm.nih.gov) and the silkworm genome database

SilkDB (http://www.silkdb.org/silkdb/). Localization of the silkworm genomic insertion sites of the piggyBac-derived vector was completed using the SilkMap application (www.silkdb.org/silksoft/silkmap.html).

Injection of the pSLA3-FLP Vector into Embryos and

Detection of Positive Site-specific Recombinant

Silkworms

pSLA3-FLP was used as the helper plasmid for the production of

FLP recombinase. Heterozygous G1 FLP/FRT system TTS adult males were backcrossed with the female adults of the wild-type Dazao strain (treated with 15°C-IMES [38]) to produce a G2 line of non-diapause embryos, heterozygous for the transgene, for microinjection. Microinjection of the pSLA3-FLP helper plasmid (325 ng/µL) was completed following the procedure described above. G2 larvae were reared at 25°C and fed with mulberry leaves. The G2 adults with DsRed- and GFP-positive phenotypes were selected and backcrossed to adults from the wild-type Dazao strain. Six-day or seven-day-old G3 embryos were screened for DsRed and EGFP expression in the larval nervous system and ocelli using the fluorescent stereomicroscope equipped with appropriate filters. Only DsRed-positive recombinant G3 individuals lacking EGFP expression were reared to adulthood and mated to generate offspring. Finally, the FLP recombinase-mediated site-specific recombination strains (SSRSs) of transgenic silkworm were established.

Analysis of Positive Site-specific Recombinant Silkworms

PCR analysis. The primer pairs P-F (5’-TACGGGCGCG-

CAAGGTTAACGTGCGA-3’) and P-R (5’-ATTGCGAACGT-

CATGGGACGTCGA-3’) were used to confirm individuals from

FLP recombinase-mediated SSRSs of silkworms. The extracted genomic DNA from G1 TTS adults, G3 SSRS adults and wild-type adults were used as the template for PCR. The purified PCR fragments were cloned into the plasmid pMD19-T simple and sequenced with the M13F/R primer to identify the sequence of the FLP recombinase-mediated excision site.

Southern blotting analysis. 25 µg genomic DNAs (G1 TTS adults, G3 SSRS adults and wild-type adults) were fully digested with XhoI and EcoRI, and separated by electrophoresis in 0.8% (w/v) agarose gel. DNAs were transferred directly onto nylon filters (Hybond N+, Amersham Bioscience) and immobilized by incubation for 30 min at 120°C. The probes were prepared as follows: a 720-bp EGFP was amplified by PCR from pBac(3’×3P-EGFPaf) with the primer pair pEGFP-f (5’-ATGGTGCGCTCCTCCAAGAACGT-3’) and pEGFP-r (5’-CTACCTTGTA-

CAGGTCGCCATGGCGC-3’). A 678-bp DsRed fragment was amplified by PCR from pBac(3’×3P-3DsrDel) with the primer pair pDsRed-f (5’-ATGGTGCGCTCCTCCAAGAACGT-3’) and pDsRed-r (5’-CAGGAAACAGGTTGGCAGGGG-3’).

These two PCR products were subjected to electrophoresis and recovered from the gel. Both fragments were labeled with DIG-High Prime reagent from the DIG High Prime DNA Labeling and Detection Starter Kit II (Roche, Mannheim, Germany). The DNA samples on the membrane were prehybridized for 1 h at 68°C, and hybridized overnight with the DIG-labeled EGFP probe and DsRed probe. The membrane was washed twice in 2×SSC containing 0.1% SDS for 15 min, and then washed twice at 65°C in 0.1×SSC containing 0.1% SDS for 15 min each time.

The detection of hybridized DNA was done using a chemiluminescent method with ready-to-use CSPD (Roche, Mannheim, Germany) according to the manufacturer’s instructions. The blotting results were observed using a chemiluminescence imaging system (Clinix ChemiScope3400 Mini, Shanghai, China).

Results

Experimental Design

The method of deleting the target gene using the FLP/FRT site-
specific recombination system in silkworm involves the following steps, as illustrated in Figure 1: (1) Genomic loci were tagged by

piggyBac-mediated germline transformation of diapause silkworm strains [38], resulting in the production of stable G1 TTSs

| Table 1. Injection of piggyBac-derived vectors in G0 silkworm embryos of the strain Dazao. |

| Injected vector | Number of injected eggs | Number of hatched eggs (%) | Number of fertile moths | Number of G1 broods | Number of broods with DsRed and GFP-positive larvae | Number of DsRed and GFP-positive G1 larvae in the broods | Percent of G1 broods with DsRed and GFP-positive larvae (%) |
|----------------|------------------------|---------------------------|------------------------|-------------------|-----------------------------------------------|--------------------------------------------------|--------------------------------------------------|
| pBac(3’×3P-3DsrDel; FRT-3’×3P-EGFP-SV40-FRT)+pHA3Pig | 330 | 119 (36.06%) | 40 | 28 | 2 | 28 | 7.14 |

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The FLP recombinase-expressing helper vector pSLA3-FLP (Figure 1D) was microinjected into heterozygous G2 TTS embryos (Figure 1E). Site-specific recombination between two FRT sites of the TTS genome was mediated by the FLP recombinase expressed by the helper vector pSLA3-FLP, resulting in the deletion of the 3×P3-EGFP expression cassette in G3 SSRSs (Figure 1F). On the basis of the different fluorescence phenotypes for either site-specific recombinant or non-site-specific recombinant silkworms, site-specific recombinant-positive individuals were screened using fluorescence microscopy. The TTAs contained a 3×P3-DsRed expression cassette and a FRT-flanking 3×P3-EGFP expression cassette, whereas the SSRSs should have only the 3×P3-DsRed expression cassette after the site-specific deletion of 3×P3-EGFP between the two FRT sites in the TTSs genome. Therefore, recombination of the two FRT sites would result in loss of green fluorescence but retention of red fluorescence within the eyes and nervous system of the silkworms.

Production of TTSs for Silkworm FLP Recombinase-mediated Site-specific Recombination

To create stable silkworm TTSs containing FRT-flanked 3×P3-EGFP expression cassettes, 330 G0 non-diapause eggs from the wild-type B. mori strain Dazao were microinjected with the pBac{3×P3-DsRed; FRT-3×P3-EGFP-SV40-FRT} vector and helper plasmid pHA3PIG mixture. G0 adults were mated with each other or backcrossed with the wild-type Dazao strain. In total, we obtained 28 G1 broods, including two broods that had at least one DsRed- and one GFP-positive larva (Table 1). The percentage of G1 broods with DsRed- and GFP-positive larvae was 7.14%. G1-positive individuals from the two broods were reared (each brood was a unit), and 28 G1 DsRed- and GFP-positive individuals were obtained. The fluorescence images of a positive individual are shown in Figure 2. Finally, we established two stable G1 FLP/FRT system TTSs, which were named TTS-1 and TTS-2.

Genomic DNA was extracted from TTS adults, and inverse PCR analyses were performed to determine the insertion position and copy number of the transgene construct in individuals from TTS-1 and TTS-2. The inverse PCR results showed that each TTS adult contained only one copy of the transgene construct (data not shown). The silkworm genomic sequences flanking the piggyBac arms are shown in Table 2. The comparison of these sequences in the SilkDB showed that all of them fully matched contig sequences in the database. Two TTSs carried the transgene vector pBac{3×P3-DsRed; FRT-3×P3-EGFP-SV40-FRT}.

| Strain | Scaffold | Chromosome | 5’-Genomic sequence | 3’-Genomic sequence |
|--------|----------|------------|---------------------|---------------------|
| TTS-1  | nscaf3026| 23         | CTTAATATCTTCTTTCTTAA| TTAATAAGCTTTGACATCTGATA |
| TTS-2  | nscaf2902| 18         | TGAATGTCAGAAAAACATGC| TTAATGCGACAGAAGGTCACAA |

The flanking genomic sequences obtained with insertion site TTA on the piggyBac left arm and piggyBac right arm are shown separately as 5’- and 3’-Genomic sequence. Localization of the silkworm genomic insertion sites of the pBac(FRT-3×P3-EGFP-SV40-FRT) vector was completed using the SilkMap application (www.silkdb.org/silksoft/silkmap.html).

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in a heterozygous state. The inserts of piggyBac in the genome of TTS-1 and TTS-2 were located on chromosome 23 and 18, respectively.

### Production and Analysis of Site-specific Recombinants

To explore the feasibility and efficiency of FLP recombinase-mediated site-specific recombination in silkworm, heterozygous G1 male adults from each of the two TTSs were backcrossed to wild-type female adults to produce G2 non-diapause embryos for helper plasmid pSLA3-FLP microinjection. In total, 987 G2 embryos were injected, and 174 G2 DsRed- and GFP-positive adults were obtained. To screen for the individuals with germline site-specific recombination, 174 G2 DsRed- and GFP-positive adults were backcrossed with those from the wild-type Dazao strain and the 143 G3 broods obtained were analyzed for fluorescence phenotypes (Table 3). Finally, 32 G3-positive recombinant embryos with only RFP fluorescence were obtained from five broods among the 143 G3 DsRed- and GFP-positive broods (Figure 3). The piggyBac transposon-derived construct in the genome of two TTSs were both single copies; the average frequency of FLP recombinase-mediated site-specific excision in two TTSs was 3.49%. Figure 4 shows the expression of the DsRed and EGFP genes in larvae and adults of TTS-1 and SSRS-1 silkworms.

To confirm FLP recombinase-mediated site-specific excision of positive recombinant individuals, PCR was performed on genomic DNAs from G1 TTS adults, G3 SSRS adults and wild-type adults

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**Table 3. Injection of FLP recombinase expression vector into silkworm embryos obtained by crossing heterozygous G1 TTSs males with wild-type females.**

| Crossing (♂ x ♀) | Injected vector (ng/μL) | Number of Injected eggs | Number of hatched eggs (%) | Number of total G2 fertile moths | Number of DsRed and GFP-positive G2 fertile moths | Number of total G3 broods with DsRed and GFP-positive larvae | Number of G3 broods with contains only DsRed-positive larvae | Recombination frequency %* |
|------------------|-------------------------|--------------------------|---------------------------|---------------------------------|-----------------------------------------------|-------------------------------------------------|-----------------------------|---------------------------|
| TTS-1 ♀ x wild-type ♂ | pSLA3-FLP (325 ng/μL)  | 524                      | 246 (46.95%)              | 186                             | 98                                            | 85                                             | 3                           | 18                        | 3.53                     |
| TTS-2 ♀ x wild-type ♂ | pSLA3-FLP (325 ng/μL)  | 463                      | 187 (40.39%)              | 147                             | 76                                            | 58                                             | 2                           | 14                        | 3.45                     |
| Total            |                         | 987                      | 433 (43.87%)              | 333                             | 174                                           | 143                                            | 5                           | 32                        | 3.5                      |

*Percentage of (Number of G3 broods with contains only DsRed-positive larvae)/(Number of total G3 broods with DsRed- and GFP-positive larvae).

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Figure 3. Expression of the DsRed and EGFP genes detected at different developmental stages of G3 silkworm individuals. (A–C) show white light (A), RFP-fluorescent (B) and GFP-fluorescent (C) images of 6-day-old G3 silkworm embryos. (D–F) show white light (D), RFP-fluorescent (E) and GFP-fluorescent (F) images of the 7-day-old G3 silkworm embryos. The DsRed- and GFP-positive non-site-specific recombinant transgenic embryos are highlighted with an arrowhead; DsRed-positive site-specific recombinant transgenic embryos are highlighted with a triangle, and wild-type embryos are indicated with an asterisk.

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Figure 4. Expression of the DsRed and EGFP genes in larvae and adults from TTS and SSRS silkworms. (A) The newly hatched larvae of wild-type strain (a–c), TTS-1 (d–f) and SSRS-1 (g–i) showing white light (a,d,g), RFP fluorescence (b,e,h) and GFP fluorescence (c,f,i) in the developing larval ocelli. (B) The adults of the wild-type strain (a–c), TTS-1 (d–f) and SSRS-1 (g–i) showing white light (a,d,g), RFP fluorescence (b,e,h) and GFP fluorescence (c,f,i) in the compound eye.

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piggyBac was used to make templates for inverse PCR. PLF/PLR and PRF/PRR are the Bgl probes. The EGFP probe hybridized fragment size calculated before (B) PCR confirmation of FLP recombinase-mediated site-specific excision in transgenic silkworms. Two FRT sites (black triangles) before (top) and after (bottom) recombination are flanked by different restriction sites. The SpeI, Xhol and BgIIl are shown, as are the recognition sites of P–F/P–R primer pair used for PCR analysis. A 1514-bp amplicon was detected from the G1 TTS genome (Figure 5A). Genomic DNAs were obtained from G1 TTS adults, G3 SSRS adults and wild-type adults and fully digested with XhoI and BgIll (Figure 5A). The blotting results presented two bands in the samples from G1 TTS individuals, which is consistent with the expected pattern of a 1.3-kb band blotted by the EGFP probe and another band blotted by the DsRed probe. The samples from G3 SSRS individuals all showed only one band blotted by the DsRed probe, and those derived from the same TTS had the same size of band blotted by the DsRed probe. Thus, the Southern blotting results not only showed that the blotting band pattern is identical to the expected band pattern, but also confirmed that individuals from each TTS contained a single copy of the transgene construct as described above.

Discussion

The results reported herein provide the first demonstration of the use of the FLP/FRT system in the genetic manipulation of the silkworm genome. Pre-blastoderm microinjection of an FLP helper vector resulted in the deletion of the FRT-flanked target gene in genome of TTS offspring. The average frequency of FLP recombinase-mediated site-specific excision in two TTSs was approximately 3.5%. The recombination efficiency was lower compared with reported numbers from other higher eukaryotes (Table 4). One possible reason is the high efficiency of the transient expression of the FLP gene in silkworm eggs at the pre-blastoderm stage, which can increase the recombination efficiency by FLP recombinase-mediated expression in the silkworm. In our experiment, a 0.65-kb truncated silkworm A3 promoter [41] was used to regulate the transient expression of the FLP gene in the pre-blastoderm of eggs from TTSs. It has been reported that the truncated silkworm A3 promoter had an approximately 20-fold increase in promoter activity in transient expression assays compared with the wild-type silkworm A3 promoter [44]. Another possible reason for the low recombination efficiency is that only a small amount of mature and activated FLP proteins derived from helper vector pSLA3-FLP is produced in silkworm eggs at the pre-blastoderm stage. The union of male and female silkworm gametes...
forms a zygote approximately 2 h after oviposition, and the zygote splits to form the blastoderm 13 h after fertilization [45]. During the embryonic development of silkworm, karyokinesis occurs first, then followed by cytokinesis [45]. The germ cells of the adult silkworm are derived from primordial germ cells during early embryo development stages [45]. Thus, the stable SSRS individuals might have been produced only when these site-specific recombination events had occurred in the primordial germ cells. In this experiment, a large amount of mature and activated FLP proteins derived from pSLA3-FLP might have been produced after blastoderm formation, resulting in a low frequency of positive site-specific recombinant offspring. To accelerate FLP protein aggregation in the pre-blastoderm of silkworm eggs, FLP mRNA can be injected into the embryos of TTSs to direct FLP recombinase synthesis. At present, this method has only been reported in some higher model organisms such as zebrafish (Danio rerio) [46] and Caenorhabditis elegans [47], showing a high recombination efficiency in somatic cells of transgenic zebrafish (Table 4). Although a low efficiency of FLP/FRT system-mediated site-specific gene excision was obtained in the current study, the recombination efficiency was similar to the piggyBac-mediated transgenic germline transformation of the silkworm [38,48], which is the most conventional transgenic methodology for this species. These data also suggest that the FLP/FRT system is a potentially useful tool for the site-specific integration or knockout of transgenes in the silkworm.

In our study, a direct injection method rather than sexual hybridization method was used to introduce and express the FLP gene in the pre-blastoderm of eggs from TTSs. The main disadvantage of the sexual hybridization method is that the FLP gene sequence would be introduced to the genome of the hybrid offspring, and unless it is crossed out, persistent FLP expression could negatively affect the presence or function of the target or donor genes. Although there have been no reports of FLP toxicity in vivo, the risk of FLP toxicity still cannot be completely ruled out from the persistent expression of the FLP gene in hybrid offspring. Moreover, the FLP recombinase-mediated site-specific excision reaction between two FRT sites is reversible [21]; thus, the persistent expression of the FLP gene might affect the recombination efficiency and the stability of the target site. The injected FLP helper vector is gradually degraded during embryonic development, thereby effectively avoiding occurrence of the above problems.

Table 4. Comparison of the recombination efficiency mediated by the FLP/FRT system in other higher eukaryotes.

| Species               | Recombinase type | Method for introducing FLP-expression | Target gene/Target sequence | Use of FRT site | Recombination efficiency | Reference |
|-----------------------|------------------|---------------------------------------|----------------------------|----------------|--------------------------|-----------|
| Arabidopsis thaliana  | FLP              | Cross                                 | l-glucuronidase (GUS)      | Gene inversion | 20%                      | Sonti et al. [17] |
| Oryza sativa          | FLP              | Cross                                 | Neomycin phosphotransferase II (NPTII) | Gene excision | ~25.6%                  | Hu et al. [4] |
| Zea mays              | FLP              | Cross                                 | Acetolactate synthase (ALS) | Gene excision | 40.7%                    | Li et al. [23] |
| Nicotiana tabacum     | FLP              | Chemo-Uly-duced                       | Hygromycin phosphotransferase (HPT); FLP | Gene excision | 13–41%                  | Woo et al. [22] |
| Drosophila melanogaster| FLP              | FLP expression plasmid injection       | EYFP; ECFP                 | FLP-RMCE<sup>3</sup> | 22–31%<sup>5</sup>       | Horn et al. [28] |
| Danio rerio           | FLPe<sup>1</sup> | FLPe mRNA injection                   | myZ22-EGFP                 | Gene excision | ~84%                     | Wong et al. [46] |
| Caenorhabditis elegans| FLP              | FLP expression plasmid injection       | unc-119p:unc-119           | Gene excision | Not given                | Vázquez-Manrique et al. [6] |
| Xenopus laevis        | FLPe            | FLPe cRNA<sup>2</sup> injection       | CarAct-eGFP; Rhodopsin-mCherry; FLP-RMCE | ~25%          | Zuber et al. [47]       |

<sup>1</sup>FLPe, a thermostable FLP mutant.  
<sup>2</sup>cRNA, complementary RNA.  
<sup>3</sup>FLP-RMCE, FLP recombinase-mediated cassette exchange.  
<sup>4</sup>Efficiency of recombinant individuals in F1 transgenic plants or G0 injected transgenic animals somatic cells except Drosophila melanogaster.  
<sup>5</sup>Percentage of (F1 crosses with at least one recombinant offspring)/(fertile F1 crosses).

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that integrated piggyBac elements could be remobilized in the genomes of D. melanogaster, the beetle Tribolium castaneum, the mosquito Anopheles stephensi and B. mori [53–56]. During the large-scale rearing of the commercial transgenic silkworm strains, we also observed the phenomenon of piggyBac transposon remobilization (data not shown). Based on the results of our study, after the specific acceptor sites (FRT sites) have been generated by piggyBac-mediated transgenesis and suitable loci have been identified in silkworm, the introduction of transgenes by FLP recombinase-mediated site-specific recombination will be easy to generate and examine. By removing one or both of the terminal sequences of the piggyBac transposon after integration, the insertion can be stabilized [57,58]. This strategy can not only eliminate undesirable transgene expression that results from piggyBac-mediated random insertions into the silkworm genome, but also minimize the position effect in silkworm functional genomics research by means of creating and selecting appropriate TTS for subsequent germine transformation. Thus, once an appropriate and stable TTS with or no one of two terminal sequences of the piggyBac transposon after integration is established, this system would be a good candidate for site-specific transformation of B. mori. Furthermore, controlling for position effects by FLP/FRT system-based genomic targeting will also enable optimization of heterologous protein expression in B. mori for use as a protein bioreactor [40,59,60]. In the future work, FLP-RMCE will be introduced as a powerful tool for site-specific gene targeting in silkworm.

In conclusion, this study is the first to demonstrate the feasibility of FLP recombinase-mediated site-specific recombination for B. mori genome manipulation. Our experiments have a huge improvement for B. mori genome manipulation using the FLP/FRT system since our experiments first obtained stable germine transformation in the individual level rather than the cell and tissue levels reported by Tomita et al, which only obtained the extrachromosomal (plasmid-based) site-specific excision [31]. Our results are likely to accelerate the practical application of the FLP/FRT system in the genomic manipulation of silkworm and promote the establishment of a FLP/FRT system-based research platform for the functional analysis of unknown genes in silkworm. Moreover, the significance of this work is not confined to studies of silkworm functional genomics, but would also be relevant for the practical utilization of silkworm transgenic lines in sericulture, silkworm bioreactors and silkworm modeling.

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Author Contributions
Conceived and designed the experiments: A-CZ. Performed the experiments: D-PL X-JC YZ. Analyzed the data: D-PL W-JL QG A-CZ. Contributed reagents/materials/analysis tools: A-CZ AMH Z-HX. Wrote the paper: D-PL A-CZ.

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