Silkworm genetic sexing through W chromosome-linked, targeted gene integration

Zhongjie Zhang[a,b,1], Baolong Niu[c,1], Dongfeng Ji[c], Muwang Li[d], Kai Li[b], Anthony A. James[e,f,2], Anjiang Tan[a,2], and Yongping Huang[a,2]

[a]Key Laboratory of Insect Developmental and Evolutionary Biology, Center for Excellence in Molecular Plant Sciences, Shanghai Institute of Plant Physiology and Ecology, Chinese Academy of Sciences, 200032 Shanghai, China; [b]School of Life Science, East China Normal University, 200062 Shanghai, China; [c]Sericultural Research Institute, Zhejiang Academy of Agricultural Sciences, 310021 Hangzhou, China; [d]Sericultural Research Institute, Jiangsu University of Science and Technology, 212018 Zhenjiang, China; [e]Department of Microbiology & Molecular Genetics, University of California, Irvine, CA 92697-3900; and [f]Department of Molecular Biology & Biochemistry, University of California, Irvine, CA 92697-3900

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Sex separation methods are critical for genetic sexing systems in commercial insect production and sterile insect techniques. Integration of selectable marker genes into a sex chromosome is particularly useful in insects with a heterogametic sex determination system. Here, we describe targeted gene integration of fluorescent marker expression cassettes into a randomly amplified polymorphic DNA (RAPD) marker region in the W chromosome of the lepidopteran model insect *Bombyx mori* using transcriptional activator-like effector nuclease (TALEN)–mediated genome editing. This silkworm strain shows ubiquitous female-specific red or green fluorescence from the embryonic to adult stages. Furthermore, we developed a binary, female-specific, embryonic lethality system combining the TALEN and the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) technology. This system includes one strain with TALEN-mediated, W-specific Cas9 expression driven by the silkworm germ cell-specific *nanos* (*nos*) promoter and another strain with U6-derived single-guide RNA (sgRNA) expression targeting *transformer 2* (*tra2*), an essential gene for silkworm embryonic development. Filial 1 (F1) hybrids exhibit complete female-specific lethality during embryonic stages. Our study provides a promising approach for *B. mori* genetic sexing and sheds light on developing sterile insect techniques in other insect species, especially in lepidopteran pests with WZ/ZZ sex chromosome systems.

Lepidoptera | transgenesis | W chromosome | female lethality

Identifying and adapting sex-specific traits for sex separation is an important goal in developing insect genetic sexing systems. Current insect sex separation methods are based mainly on classic genetic approaches (1–5) or transposon-based genetic transformation (6–8). Most insect genetic sexing strains originated from irradiation-induced translocations between the sex chromosome and an autosome (9, 10). However, this process is poorly controlled, and radiation-induced chromosome rearrangements often result in mutations at multiple loci, which inevitably decrease strain fitness. Transgenic systems using sex-specific promoters to drive expression of marker genes have been established, although with the disadvantage of late expression in development, which leads to difficulty in mass separation (11–14). In previous work, we developed a transgene-based, female-specific lethal system for genetic sexing of the silkworm, *Bombyx mori* (15). In this system, the female-specific alternative splicing form of the *doublesex* product combined with the tetracycline-suppressible system was used to drive expression of the tetracycline-controlled transactivator protein (tTA) gene as a lethal effector only in females, leading to repressible female-specific lethality during embryonic and early larval stages. Alternatives to introducing transgenes into the sex chromosome have been reported in *Ceratitis capitata* (8) and *B. mori* (16). These methods are dependent on transposon-based random insertions, which are costly in both the labor and time needed for prescreening a sex-linked transgene insertion.

Other challenges include the identification of sex chromosome-linked, sex-specific detectable phenotypes that are convenient for manipulation (5). Insect sex is determined by sex chromosome composition and shows a high degree of mechanistic diversity throughout the class (17–19). *B. mori* has the most common type of WZ/ZZ sex chromosome system found in lepidopteran insects (20), where sex determination is dependent primarily on the presence or absence of the W chromosome and the females are heterogametic (WZ). Although several silkworm strains with visible W-linked phenotypes have been established for genetic sexing (21), targeted integration of a marker gene into the W chromosome would be a significant improvement. The *B. mori* W chromosome is replete with repetitive DNA sequences, with only a single functional gene, *fem*, recently identified (22, 23). Therefore, we expect there to be few or no deleterious effects incurred by random transgene insertions into the chromosome and few insertion site effects influencing the expression characteristics of the integrated genes.

Recently developed genome editing tools, such as zinc finger nucleases, transcription activator-like effector nucleases

Significance

Insect sex is determined genetically and shows high diversity among different species. The silkworm, *Bombyx mori*, has a WZ/ZZ sex chromosome system seen in lepidopteran insects in which the females are heterogametic. Sex separation methods are critical for commercial aspects of rearing silkworms, and genetic-sexing systems also could serve as the basis for adaptation to sterile insect techniques for pest lepidopteran species. Here, we describe a W chromosome-based, genetic-sexing system combining transcriptional activator-like effector nucleases and CRISPR/Cas9 technologies in *B. mori*. Development of silkworm strains with ubiquitous female-specific fluorescence for convenient genetic sorting or complete female-specific embryonic lethality for male-only rearing provides a successful example of targeting an insect sex chromosome with genome editing tools, which should assist future sterile insect technique development for pest insects.

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[1]Z.Z. and B.N. contributed equally to this work.

[2]To whom correspondence may be addressed. Email: aajames@uci.edu, ajtan01@sibs.ac.cn, or yphuang@sibs.ac.cn.

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(TALENs), and clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) systems, have been applied in targeted gene mutagenesis analysis in many organisms, including several insect species (24–29). However, compared with targeted gene knockout analysis, success in targeted gene integration, especially large DNA fragments, is still limited. This is likely due to a relatively low frequency of homology-directed repair compared with nonhomologous end joining after induction of double-strand DNA breaks by customized nucleases (30, 31). In previous studies, we achieved targeted gene "knock in" at the *B. mori* ortholog of the human biogenesis of lysosome-related organelles complex 1, subunit 2 (*BmBlos2*) locus using TALENs (32); however, no further success has been reported with this method since then.

Here, we report targeted gene integration onto the W chromosome of *B. mori* using customized TALENs that are designed to target a W chromosome-specific sequence in a previously identified random amplified polymorphic DNA (RAPD) marker region. Different fluorescent markers or Cas9 expression cassettes were integrated into this W chromosome site. We then developed a female-specific, embryonic lethal system based on these transgenic silkworm strains. This genetic sexing system will greatly contribute to sex separation in male-only silkworm rearing for better silk production and the development of genetic control strategies in pest species with heterogametic sexes.

Results

TALEN Design and Donor Plasmid Construction. Although the *B. mori* W chromosome consists mostly of repetitive DNA sequences as well as the primary sex determination factor, *fem* PIWI (P-element induced wimpy testis)-interacting RNA (piRNA) (23), at least 12 W-specific RAPD markers have been identified (22), providing potential unique sequences for chromosome-specific, targeted gene integration. To generate female-specific, fluorescence-marked silkworm strains, we randomly selected one of the W-specific RAPD marker regions, Rikishi, as the site to perform targeted gene integration onto the W chromosome (Fig. 1*A*). To confirm that the Rikishi RAPD marker was female-specific in our experimental Nistari strain, we determined its presence or absence in three male and three female adults by gene amplification (PCR) with primers that specifically amplify the genomic region of the RAPD marker. The PCR-based analysis confirmed that the Rikishi RAPD marker was female-specific in Nistari (*SI Appendix*, Fig. S1*A*). CRISPR/Cas9-mediated targeted gene integration is still challenging in *B. mori*, so we performed TALEN-mediated homologous recombination (HR), which was demonstrated in our previous studies to be highly effective (32). Sequence-specific TALENs were synthesized, and their activity was validated using a single-strand annealing (SSA) recombination assay (*SI Appendix*, Fig. S1*B*).

A donor plasmid (donor A) was constructed that contained a baculovirus IE1 promoter-directed DsRed2 fluorescence protein expression cassette. The left and right homologous arms (HAs; L-Homo and R-Homo, respectively) DNA sequences were amplified from silkworm genomic DNA and cloned into the same plasmid with TALEN sites flanking the HAs (Fig. 1*B*). This donor plasmid could be digested by the corresponding endonuclease and release a linearized donor, which is predicted to be more efficient than a circular molecule for enhancing HR efficiency in vivo (32, 33). Another donor plasmid (donor B) containing an IE1 promoter-directed EGFP fluorescence protein expression cassette and a *B. mori nanos* (*Bmnos*) promoter-directed Cas9 expression cassette also was constructed (Fig. 1*C*). This plasmid contains the previous HA DNA sequences flanked by the TALEN sites.

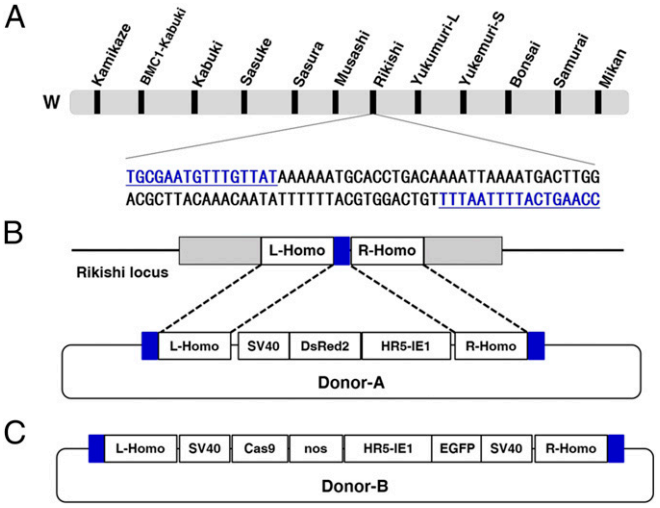

Fig. 1. Schematic representation of the *B. mori* W chromosome and donor plasmids. (*A*) Schematic representation of female-specific RAPD markers on the W chromosome (not to scale). The gray region represents the W chromosome. The black rectangles are the female-specific RAPD markers. A 50-bp fragment located in the Rikishi RAPD marker is the targeted site. The sequences in blue and underlined are the targets of the TALENs. A 17-bp sequence serves as a spacer between the two sites. (*B*) Schematic representation of the genomic target site and donor plasmid A. Donor A contains a cassette expressing *DsRed2* under the control of the *HR5-IE1* promoter and the SV40 polyadenylation site. Two homologous DNA fragments (L-homo and R-homo), each 1,000 bp in length at the 5′- and 3′-ends of the TALEN sites, were cloned separately into the left and right sides of the *DsRed2* cassette. The two homologous fragments were both flanked by two of the 50-bp TALEN sites (blue). (*C*) Donor B contains two cassettes expressing EGFP under the control of an *HR5-IE1* promoter and Cas9 driven by the embryo-specific *nos* promoter. Two homologous DNA fragments (L-homo and R-homo), each 1,000 bp in length at the 5′- and 3′-ends of the TALEN site, were cloned separately to the left of the Cas9 cassette and to the right of the *EGFP* cassette. All other descriptions are the same as in *B*.

Establishment of Female-Specific Fluorescent Silkworm Lines. A total of 640 preblastoderm Nistari eggs were injected with the mixed solution of TALEN mRNA and donor plasmid A. Of these, 226 (35.3%) eggs hatched and 153 (23.9%) animals survived to the adult stage. These moths were sib-mated or crossed to wild-type (WT) individuals generating 69 broods [generation 1 (G1)]. Six of the broods (8.7%) produced a total of 35 DsRed-positive individuals during screening of late embryos by fluorescence microscopy. In parallel, another set of 640 preblastoderm silkworm eggs was injected with the mixed solution of TALEN mRNA and donor plasmid B. Of these, a total of 325 eggs hatched (50.8%) and 128 (20%) animals survived to the adult stage. These moths were sib-mated or crossed to WT individuals, generating 58 broods (G1). Two positive broods yielded 18 EGFP-positive individuals following screening, for a transformation efficiency of 3.4%. Positive individuals in both silkworm lines showed ubiquitous red or green fluorescence throughout all developmental stages, from late embryos to adults (*SI Appendix*, Fig. S2).

Molecular Analysis of W Chromosome-Linked Integration. All fluorescence-positive individuals were reared to adults, and morphological observation confirmed that all were female. To verify site-specific insertion of the transgene cassette, we performed PCR-based analysis using primers spanning the HAs and the expression cassette. Genomic DNA was extracted from positive female moths of all positive broods (six broods in donor A and two broods in donor B). The fragments of the 5′- and 3′-end junctions were amplified separately and sequenced (*SI Appendix*,

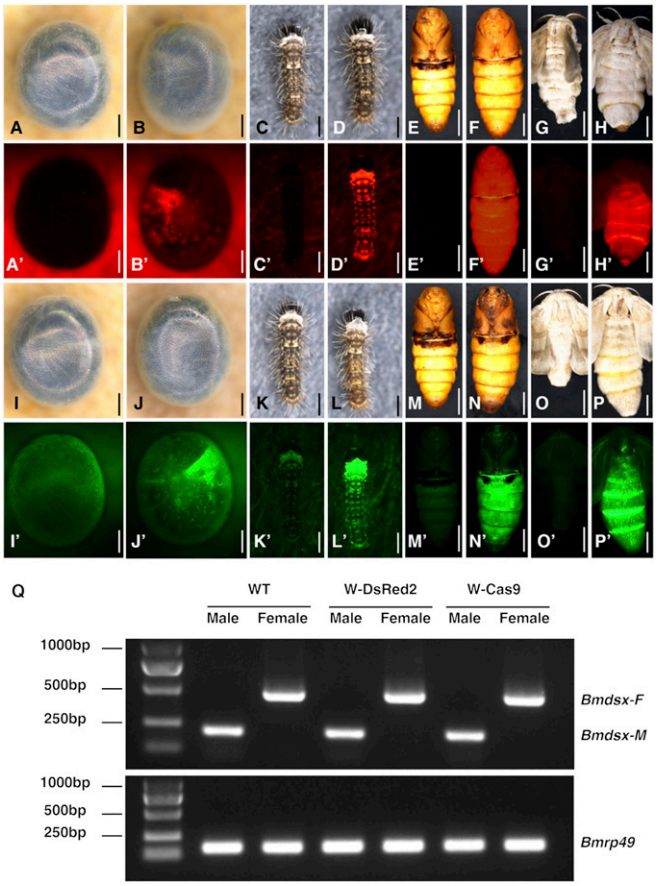

Fig. 2. Expression of W-linked fluorescence in W-DsRed2 and W-Cas9 transgenic silkworms. Insects were photographed with normal light (*A*–*P*) or under UV light with an RFP or GFP filter (*A′*–*P′*), respectively. G2 females with W-DsRed2 or W-Cas9 showed red or green fluorescence, respectively, at the embryonic (*B′* and *J′*), larval (*D′* and *L′*), pupal (*F′* and *N′*), and adult (*H′* and *P′*) stages. No fluorescence was detected in G2 males for W-DsRed2 or W-Cas9 at embryonic (*A′* and *I′*), larval (*C′* and *K′*), pupal (*E′* and *M′*), or adult (*G′* and *O′*) stages. (Scale bars: *A*, *A′*, *B*, *B′*, *I*, *I′*, *J*, and *J′*, 0.2 mm; *C*, *C′*, *D*, *D′*, *K*, *K′*, *L*, and *L′*, 0.4 mm; *E*–*H′*, *M*–*P′*, 40 mm). (*Q*) Sex-specific alternative splicing. Sex-specific splicing of *B. mori doublesex* (*Bmdsx*) was detected in WT, W-DsRed2, and W-Cas9 animals. The *Bmdsx*-specific sense and antisense primers bind to exons 2 and 5, respectively. A 207-bp amplicon was detected in males, and a 457-bp amplicon was detected in females. A 136-bp fragment of the *B. mori* ribosomal protein 49 (*Bmrp49*) gene was used as an internal control and was detected as expected in all animals.

Fig. S3). The transgene integration sites of all broods were identical and precisely located to the targeted sites (Dataset S1). These data support the conclusion that the transgene cassettes were integrated precisely into the targeted Rikishi RAPD marker region in the W chromosome through HR. To confirm heritable germline transformation, female G1 moths were mated with WT male moths. All G2 female animals showed red or green fluorescence in all developmental stages, while males did not (Fig. 2). These data support our conclusion that the W chromosome-linked, site-specific transgene integration was stable and heritable.

Establishment of a Female-Specific, Embryonic Lethal System. A binary, female-specific, embryonic lethal system was established in *B. mori* in which W-specific nanos (nos)-cas9 lines with EGFP fluorescence served as the activator. For the effector, we constructed transgenic lines expressing the single-guide RNA (sgRNA) targeting *B. mori transformer 2* (*Bmtra2*) driven by the silkworm U6 promoter (U6-sgRNA). *Bmtra2* is a gene in the sex determination pathway that is essential for embryonic development (34). Although the molecular mechanism remains to be identified, deletion of this gene induces complete embryonic lethality (34). Thus, it is an ideal target for constructing an embryonic lethal system. Three independent transgenic U6-sgRNA lines (*tra2-1*, *tra2-2*, and *tra2-3*) targeting *Bmtra2* were established, and the genomic localization of each of the transgenes was determined by inverse PCR and searches of the silkworm genome database at KAIKOBLAST (sgp.dna.affrc.go.jp/KAIKObase/) (*SI Appendix*, Fig. S4).

No deleterious phenotypes were evident in either W-Cas9 strains or U6-sgRNA transgenic lines, all of which hatched normally (Fig. 3 *A*–*C*). Furthermore, the sex ratios of W-Cas9 strains and transgenic U6-sgRNA lines did not differ significantly from those of WT moths (Fig. 3*E*). The three independent homozygous U6-sgRNA lines were crossed with the W-Cas9 line. Approximately 50% of the embryos in the filial 1 (F1) progeny failed to hatch (Fig. 3 *D* and *D′*). Scoring sex ratios revealed that all females died during the embryonic stage, whereas only males survived to pupal and adult stages (Fig. 3*E*). Genomic DNA was extracted from female embryos, and the fragments spanning both target sites were amplified and sequenced, confirming genomic mutagenesis at the *tra2* locus (*SI Appendix*, Fig. S5).

Discussion

This is a report of gene integration targeting insect W chromosome-specific sequences with genome editing tools. Although random,

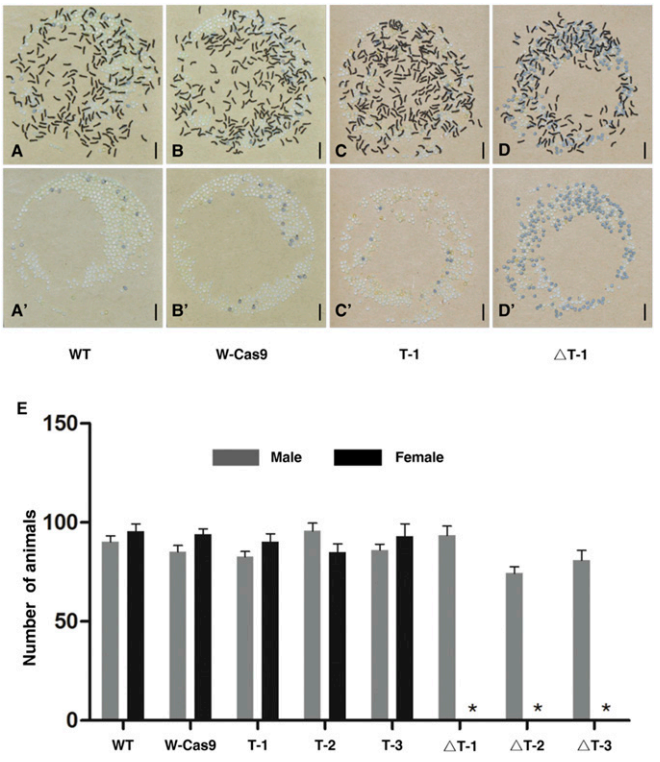

Fig. 3. Female-specific embryonic lethality with sex-specific deletion of *tra2*. Newly hatched first-instar larvae from WT (*A*), W-Cas9 (*B*), *tra2-1* (T-1) (*C*), or *tra2-1* crossed with W-Cas9 (ΔT-1) (*D*). Both W-Cas9 (*B′*) and T-1 (*C′*) showed similar hatching numbers in comparison to WT (*A′*) (dark round spheres are unhatched eggs). (*D′*) Approximately half of the eggs failed to hatch in ΔT-1. (Scale bars: *A*–*D′*, 20 mm). (*E*) W-Cas9, *tra2-1* (T-1), *tra2-2* (T-2), and *tra2-3* (T-3) larvae surviving to the pupal stage showed no sex ratio deviations compared with WT. In contrast, only males survived in the progeny of *tra2-1* (ΔT-1), *tra2-2* (ΔT-2), and *tra2-3* (ΔT-3) lines crossed with W-Cas9. The data shown are mean ± SEM ($n = 10$). The asterisks indicate significant differences with a two-tailed Student *t* test (*$P < 0.05$).

transposon-mediated integration of a transgene onto the Y chromosome in the mosquito (8) and onto the W chromosome in the silkworm (16) has been achieved, because of its highly repetitive nature, no previous success of directly targeted gene integration into a lepidopteran W chromosome has been reported. Additionally, establishing such strains by transposon-mediated random insertions requires screening and analysis of large numbers of Y- or W-specific transgenes, which is difficult and time-consuming. The current study thus provides a more efficient approach for targeting insect sex chromosomes.

The W chromosome of *B. mori* is full of repetitive sequences, including long terminal repeat (LTR) and non-LTR retrotransposons, which leads to difficulty in identifying its specific genomic sequences (35). Fortunately, partial *B. mori* W chromosome sequences have been released, and at least 12 W-specific RAPD markers have been identified (22). Of these, we targeted the Rikishi RAPD marker region, which was mapped genetically on an X-ray–induced fragment of the W chromosome associated with the classic female sex-determining factor *fem* (22) and recently identified as a female-specific, piRNA (23). We believe additional sequences on the W chromosome can be selected as candidate targeting sites and this technique could be expanded to other insect species with a repetitive sex chromosome (36).

Sex separation techniques are important in silkworm breeding to avoid sibling mating since F1 hybrids are needed for mass rearing. Sex separation is done manually during pupal stages based on sexual dimorphisms, but this is time- and labor-intensive and adds significantly to production costs. The development of automated approaches would be a major advance for the industry. For example, sex-specific fluorescence can be used with automated sorters for screening mosquitoes (12), and we anticipate similar equipment can be developed for sorting silkworm pupae. However, most sex-specific genes in *B. mori*, such as the female-specific vitellogenins (13) and male-specific tubulins or TSA2 (14), are expressed only in specific tissues or at late developmental stages, making them unsuitable for automation. In contrast, the robust fluorescence expression directed by the baculovirus IE1 early promoter is detected from late embryos to adults, and we anticipate that automated fluorescent sex sorting can be done during the embryonic stage, saving rearing costs as well.

In a previous study, we established a transgene-based, female-specific lethality system in *B. mori* (15). This strain showed partial embryonic lethality, and most females died during the early larval stages when removed from a conditional diet supplemented with tetracycline. In contrast, the binary system described here achieves complete embryonic lethality without the costly maintenance of the supporting strains. The TALEN-mediated integration technologies developed here are more convenient than previous efforts with transposon-mediated integration since no prescreening of sex chromosome-linked transgenes is needed. Furthermore, female-specific embryonic lethality is particularly useful for male-only rearing. This sex chromosome-specific genetic engineering system also can be applied to developing sterile insect techniques in other insect species (37).

Methods

Silkworm Strains. A multivoltine silkworm strain, Nistari, was used for all experiments. Larvae were reared on fresh mulberry leaves at 25 °C under standard conditions (38).

PCR Validation of the W-Rikishi RAPD Marker. Genomic DNA was extracted from three male and three female adults with DNA extraction buffer [1:1:2:2.5 ratio of 10% SDS, 5 mol of NaCl, 100 mmol of EDTA, and 500 mmol of Tris·HCl (pH = 8), respectively], incubated with proteinase K, and then purified via a standard phenol/chloroform wash and isopropanol precipitation extraction, followed by RNaseA treatment. Primer pairs, Rikishi-F and Rikishi-R (*SI Appendix*, Table S1), were used to amplify a partial DNA fragment of the W chromosome-specific Rikishi RAPD marker (22). The amplification conditions were as follows: 98 °C for 2 min and 35 cycles of 94 °C for 10 s, 55 °C for 30 s, and 68 °C for 2 min, followed by a final extension period of 72 °C for 10 min. The PCR products were cloned into pJET1.2 vectors (Fermentas) and sequenced directly.

TALEN Vector Construction and Luciferase SSA Assay. The pSW-peas-T-TALEN plasmids were provided by view-Solid Biotech (www.v-solid.com/) and contain sequences encoding the T7 promoter, TALEN repeats, FokI restriction endonuclease site, and SV40 transcription terminator. The activity of TALENs was validated with an SSA assay as described previously (32). For transcription in vitro of TALEN mRNA, TALEN vectors were linearized with NotI (Fermentas) and purified with phenol/chloroform/isoamylalcohol (25:24:1) treatment. The linearized vectors were used as templates in subsequent in vitro transcription experiments. TALEN mRNA was synthesized using an mMESSAGE mMACHINE T7 kit (Ambion). Purified TALEN mRNA was stored at −80 °C until use.

Donor Plasmid Construction. Donor plasmids were constructed as follows:

Donor A (pGEMT_L-Homo_HR5-IE1-DsRed2-SV40_R-Homo): An HR5-IE1-DsRed2-SV40 cassette was subcloned into the pGEMT-simple vector (Takara) to generate pGEMT_HR5-IE1-DsRed2-SV40. The HAs (1,000 bp) flanking TALEN sites were amplified using genomic DNA as the template and cloned into pGEMT_HR5-IE1-DsRed2-SV40 to generate donor A.

Donor B (pGEMT_L-Homo_HR5-IE1-EGFP-SV40_Nos-Cas9-SV40_R-Homo): An HR5-IE1-EGFP-SV40 cassette was subcloned into the pGEMT-simple vector (Takara) to generate pGEMT_HR5-IE1-EGFP-SV40. The same HAs as used in donor A were cloned into pGEMT_HR5-IE1-EGFP-SV40. The nos-Cas9-SV40 cassette was amplified using pBac[IE1-EGFP-nos-Cas9] (39) as the template. Subsequently, the nos-Cas9-SV40 cassette was subcloned into pGEMT_HR5-IE1-EGFP-SV40 to generate donor B. The pBac[IE1-DsRed2-U6-*Bmtra2*-gRNAs] (U6-sgRNA), which expresses *tra2*-specific sgRNA driven by a U6 promoter, was described previously (34). The primers used for plasmid construction are listed in *SI Appendix*, Table S1.

Genetic Transformation. A mixed solution of TALEN mRNA (200 ng/μL for each) and each DNA (donor A or B, 200 ng/μL) was coinjected separately into preblastoderm embryos. Injected embryos were incubated in a humidified chamber for 10–12 d until larval hatching. Larvae were reared with fresh mulberry leaves at 25 °C under standard conditions. G0 moths were sibmated or crossed to WT moths, and G1 progeny were scored for the presence of the fluorescent marker gene using fluorescence microscopy (Nikon AZ100).

Transgene Integration and Mutagenesis Analysis. Targeted amplification of genomic DNA, followed by sequencing, was carried out to investigate site-specific integration in the positive G1 adults. Genomic DNA was extracted from positive G1 adults with the methods described above. The 5′- and 3′-end junction fragments flanking the integration sites were amplified and sequenced. Genomic mutagenesis in *Bmtra2* mutant animals also was investigated. PCR conditions were as follows: 94 °C for 2 min and 30 cycles of 94 °C for 10 s, 55 °C for 30 s, and 68 °C for 2 min, followed by a final extension period at 68 °C for 10 min. The primers used for transgene integration and mutagenesis analysis are listed in *SI Appendix*, Table S1.

Inverse PCR. Inverse PCR was performed as previously described (15) to locate the insertion sites of the U6-sgRNA transgenic silkworm lines. The genomic DNA was extracted from G1 transgenic moths, digested with BfuCI (New England Biolabs), and circularized by T4 ligase (Fermentas) overnight at 16 °C. PCR was performed using the circularized fragments as templates with primers designed from the 5′-end arm of the *piggyBac* vector: 5′-F1 and 5′-R1 (reverse primer 1) for the first PCR assay and 5′-F2 and 5′-R2 for the second PCR assay (*SI Appendix*, Table S1). The PCR conditions were as follows: 94 °C for 2 min and 30 cycles of 94 °C for 10 s, 55 °C for 30 s, and 68 °C for 2 min, followed by a final extension period 68 °C for 10 min. Amplified products were gel-purified and sequenced directly.

RT-PCR. Total RNA was extracted from three whole bodies of larvae using TRIzol Reagent (Invitrogen), followed by DNase treatment to remove genomic DNA contamination. The cDNA was prepared using a RevertAid First Strand cDNA Synthesis Kit (Fermentas). RT-PCR was performed using gene-specific primers (*SI Appendix*, Table S1). Another primer pair, RP49-F and RP49-R, which amplifies a 136-bp fragment from the *B. mori* ribosomal protein 49, was used as an internal control (15). The PCR conditions were the same as those used for inverse PCR.

Phenotypic Investigation. The G1 moths (W-DsRed2 or W-Cas9) were crossed to WT moths of the opposite sex. The G2 embryos, larvae, pupae, and adults were investigated under fluorescence microscopy (Nikon AZ100) with appropriate filter sets for the detection of DsRed2 and EGFP fluorescence. In the binary, female-specific embryonic lethality system, female moths of W-Cas9 lines were crossed with U6-sgRNA lines and the hatching and sex ratios in the F1 hybrid progeny were scored.

Statistical Analysis. All experiments in this study were performed with at least three replicates. All data are expressed as the mean ± SEM. The differences between groups were examined by either a two-tailed Student *t* test or two-way ANOVA. Statistically significant differences are indicated by asterisks in Fig. 3.

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