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Transcription activator-like effector nuclease (TALEN)-mediated female-specific sterility in the silkworm, *Bombyx mori*

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Abstract

Engineering sex-specific sterility is critical for developing transgene-based sterile insect technology. Targeted genome engineering achieved by customized zinc-finger nuclease, transcription activator-like effector nuclease (TALEN) or clustered, regularly interspaced, short palindromic repeats/Cas9 systems has been exploited extensively in a variety of model organisms; however, screening mutated individuals without a detectable phenotype is still challenging. In addition, genetically recessive mutations only detectable in homozygotes make the experiments time-consuming. In the present study, we model a novel genetic system in the silkworm, *Bombyx mori*, that results in female-specific sterility by combining transgenesis with TALEN technologies. This system induces sex-specific sterility at a high efficiency by targeting the female-specific exon of the *B. mori doublesex (Bmdsx)* gene, which has sex-specific splicing isoforms regulating somatic sexual development. Transgenic animals co-expressing TALEN left and right arms targeting the female-specific *Bmdsx* exon resulted in somatic mutations and female mutants lost fecundity because of lack of egg storage and abnormal external genitalia. The wild-type sexual dimorphism of abdominal segment was not evident in mutant females. In contrast, there were no deleterious effects in mutant male moths. The current somatic TALEN technologies provide a promising approach for future insect functional genetics, thus providing the basis for the development of attractive genetic alternatives for insect population management.

Keywords: *doublesex*, gene targeting, genitalia, morphology.

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Introduction

The domesticated silkworm, *Bombyx mori*, is one of the most economically important insects and is the foundation of sericulture. A transgene-based genetic sexing system was established recently in this species, extending sterile insect technology (SIT) into a non-pest insect in which sex separation is valuable (Tan *et al.*, 2013). This female-specific lethality system is dependent on targeting the sex-specific alternative splicing modules of *doublesex* (*dsx*), a gene that controls somatic sex determination and differentiation in the final steps of the insect 'sex-determination cascade' (Baker, 1989; Steinmann-Zwicky *et al.*, 1990). The hierarchy of sex determination gene function in lepidopteran insects is poorly understood when compared with the model dipteran insect, *Drosophila melanogaster* (Harrison, 2007). Defining insect sex determination pathways, including functional analysis of key regulators such as *dsx*, is critical for developing SIT in lepidopteran insects.

Emerging genome engineering tools such as customized zinc-finger nucleases (ZFNs), TALENs or clustered,

regularly interspaced, short palindromic repeats (CRISPR)/Cas9 endonuclease-mediated systems have been applied extensively in a wide range of model organisms (Urnov *et al*., 2005; Hockemeyer *et al*., 2011; Hwang *et al*., 2013). These tools provide the basis for developing new strategies for SIT; however, screening *in vivo* mutant alleles in genes with no a *priori* visible phenotypes is challenging for the targeted genome editing mediated by current customized nucleases systems. Furthermore, directing expression of these nucleases in tissue-, stage- and sex-specific manners is not trivial. Gene targeting technologies in *B. mori* have been established recently using ZFNs, TALENs and CRISPR/Cas9 systems (Takasu *et al*., 2010; Sajwan *et al*., 2013; Wang *et al*., 2013). Also, germline transformation technologies mediated by the *piggybac* transposon are well established and numerous *cis*-regulatory elements have been identified (Tamura *et al*., 2000). Integrating genome engineering technologies with transgenic approaches may contribute to novel SIT approaches.

In the present study, we report the establishment of a *piggyBac*-based binary transgenic system in *B. mori* in which custom designed TALEN left and right arm constructs are expressed separately. Sequence-specific somatic mutagenesis is induced in the offspring after crossing of the two lines. We show that this method is highly efficient in inducing somatic mutagenesis when targeting the female-specific exon of *Bmdsx*. The results show this transgenic TALENs system has great potential in insect functional genetics.

Results

Design the transgene-based TALENs system for Bombyx mori

We established a *piggybac*-based, transgenic TALENs system in *B. mori* to exploit *dsx* gene function and its potential application in SIT. We designed TALENs targeting the sequence in *Bmdsx* exon 3, present only in the female-specific splicing isoforms (*Bmdsx*^{F}; Fig. 1A).

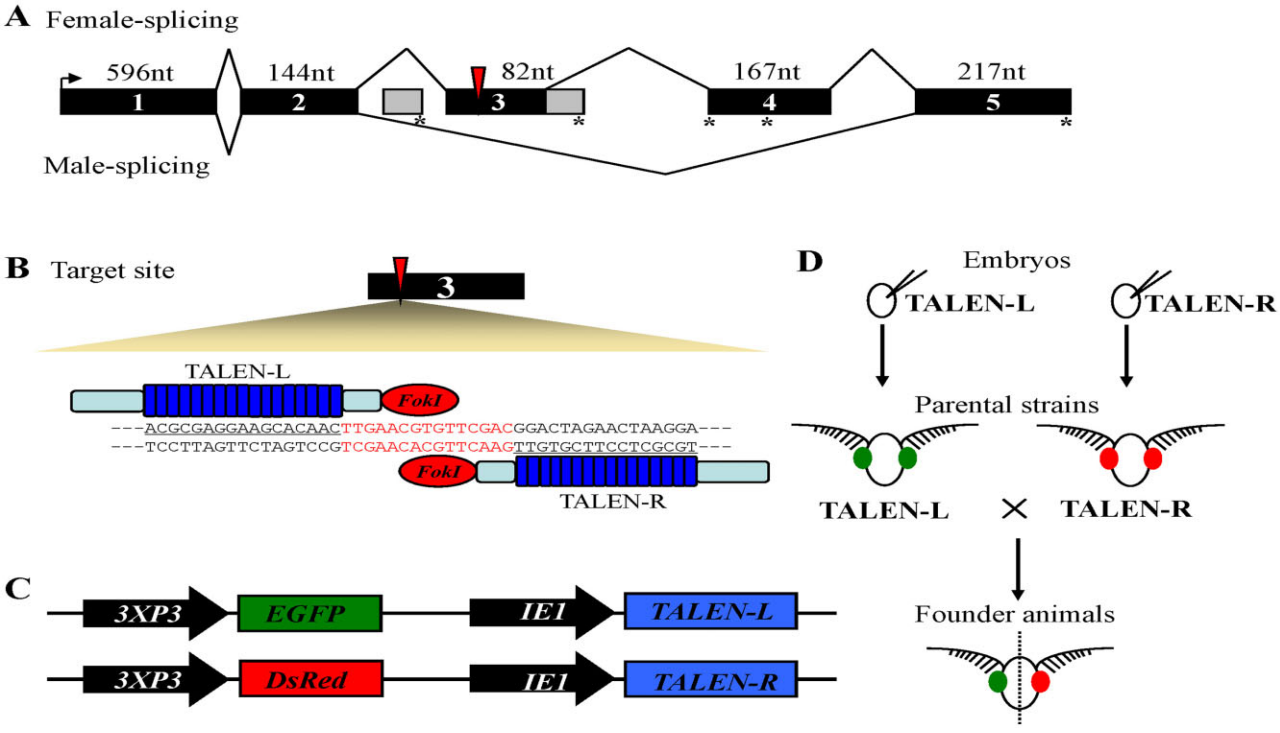

Figure 1. Sex-specific gene targeting using a transgenic transcription activator-like effector nuclease (TALEN) system. (A) Alternative splicing of the *Bombyx mori doublesex* (*Bmdsx*) gene generates both female- and male-specific isoform. Black boxes, previously reported canonical exons (15); numerals, length in nucleotides of canonical exons; grey boxes, newly-identified alternative exons in females; lines, introns; red arrows, TALEN target site; horizontal arrow, start codons; asterisks, stop codons. (B) TALENs and their DNA targets in exon 3 of the *Bmdsx* female-specific splice form. TALENs bind and cleave as dimers on a target DNA site. TALEN pairs were engineered to have 17 TALEN repeats in left arm and 16 TALEN repeats in right arm (dark blue boxes). The target sequences are underlined in black. Cleavage sequences are highlighted in red lettering. (C) The transgenic vectors TALEN-L and TALEN-R contain the full open reading frame of the TALENs protein driven by *IE1* promoter, also with reporter genes *EGFP* or *DsRed2*, respectively, under the control of *3*× *P3* promoter. (D) Transgenic strains expressing TALEN left (L) or TALEN right (R) arms are established as parental strains. Somatic mutations are induced in F_1 founder animals following crosses of the left and right TALEN strains. Red and green fluorescence in the eyes confirm the presence of the appropriate transgenes construct.

TALEN activity was validated *in vitro* using the luciferase SSA assay in 293T cells (Table S1). Both TALEN left-arm (TALEN-L) and TALEN right-arm (TALEN-R) constructs had sequences encoding the *Fok*I DNA endonuclease and a sequence-specific DNA recognition repeat domain, which targets the female-specific *Bmdsx* exon 3, and these were cloned into *piggyBac*-based transgenic vectors (Fig. 1B). An IE1 promoter was introduced to direct expression of TALENs in a ubiquitous manner, and genes encoding fluorescent marker proteins were also integrated into the transgene vectors (enhanced green fluorescent protein [EGFP] for TALEN-L and the red fluorescent protein [DsRed2] for TALEN-R). Both EGFP and DsRed2 expression were under the control of the eye-specific promoter, 3 × P3 (Fig. 1C). TALEN-L or TALEN-R plasmids were microinjected separately with helper plasmids into pre-blastoderm eggs, and transgenic lines were established (Figs 1d, S1, S2). The resulting transgenic lines were viable and fertile, supporting the conclusion that the TALEN-L- or TALEN-R alone were not functional.

Targeted mutagenesis in Bmdsx loci

The TALEN-L and TALEN-R containing transgenic lines were crossed with each other and the F_1 founder animals were subjected to somatic mutagenesis analysis. Individual moths were sexed using *B. mori* W chromosome-specific primers, genomic DNA of F_1 founder animals as templates and gene amplification analyses (Fig. S3). Remarkably, all (18/18) male and female animals analysed had mutations at the target site caused by non-homologous end joining-induced indels (Fig. 2).

$BmDSX^F$ mutation induced female genital abnormality and egg-free females

All F_1 founder animals of either sex developed to adults without any apparent distortion of the sex ratio, despite the high mutagenesis efficiency; however, one of the conserved biological functions of insect *dsx* genes is the regulation of genital disc development (Vincent *et al.*, 2001), and we therefore investigated the genital morphology of the transgenic females. Distinct morphological defects were seen in the external genitalia of transgenic females. The dorsal chitin plate was absent and the genital papilla exhibited severe morphological abnormalities (Figs 3A, S4). Furthermore, the gross overall morphology of transgenic females resembled that of wild-type males. Specifically, their abdomens were not enlarged and appeared to be free of developed ovaries. Dissection of these females confirmed that no or only few eggs were present in the ovaries (Fig. 3B). Importantly, transgenic F_1 females were sterile and did not lay eggs (Fig. 4A). We also observed that the wild-type sexually-dimorphic abdominal segment number, seven, was absent in mutant females, and they had eight segments (Figs 4A, S5); however, there was no difference between mutant and wild-type males, which all had eight abdominal segments (Figs 4B, S4).

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AAGATGATCGTCGACGAGTACGCGAGGAAGCACAACTTGAACGTGTTCGACGGACTAGAACTAAGGAACTCGACACGCCA   WT

AAGATGATCGTCGACGAGTACGCGAGGAAGCACAACTTGAACGT---------//----------ACTAACATAATTAAT  △107   M1-1
AAGATGATCGTCGACGAGTACGCGAGGAAGCACAACTTGAACGT---------//----------CGCGACAGATACATG  △80    M1-2
AAGATGATCGTCGACGAGTACGCGAGGAAGCACAACTTGAACGTACGC-ACGGACTAGAACTAAGGAACTCGACACGCCA  △1+3   M1-3

AAGATGATCGTCGACGAGTACGCGAGGAAGCACAACTTGAA------------//------------AATTAATCAGTAA  △118   M2-1
AAGATGATCGTCGACGAGTACGCGAGGAAGCACAACTTGAA------------//------------ATAAATTACGTGT  △202   M2-2
AAGATGATCGTCGACGAGTACGCGAGGAAGCACAACTTGAACG------ACGGACTAGAACTAAGGAACTCGACACGCCA  △6     M2-3

AAGATGATCGTCGACGAGTACGCGAGGAAGCACAACTTGAACTGGCGTGTC-----//---------AAGTTTTCCTTCA  △183+9 M3-1
AAGATGATCGTCGACGAGTACGCGAGGAAGCACAACTTGAAC-TGTTCGACGGACTAGAACTAAGGAACTCGACACGCCA  △1     M3-2
AAGATGATCGTCGACGAGTACGCGAGGAAGCACAACTTGAAAAC-----------//----------ACAAAGTTTTCCT  △181+3 M3-3

AGTTTCCTGAAAT-----------------------------------//------------------ACTAACATAATTA  △95    F1-1
CAGTGTTGCCAGT--------------//-----------------TCGACGGACTAGAACTAAGGAACTCGACACGCCA  △41    F1-2
AAGATGATCGTCGACGAGTACGCGAGGAAGCACAACTTGAAC-----------//------------AACATAATTAATC  △112   F1-3

TTCCTATGTTAGA-----------------------------------//------------------ACAAAGTTTTCCT  △237   F2-1
AAGATGATCGTCGACGAGTACGCGAGGAAGCACAACTTGAAC---TTCGACGGACTAGAACTAAGGAACTCGACACGCCA  △3     F2-2
AAGATGATCGTCGACGAGTACGCGAGGAAGCACAACTTGAAC----------//-------ACATGCATCACATCGAGAC  △92    F2-3

AAGATGATCGTCGACGAGTACGCGAGGAAGCACAACTTGAACGTGTT----//-------------GTACAAACAAAGTT  △165   F3-1
AAGATGATCGTCGACGAGTACGCGAGGAAGCAC---------------------------------ACTCGACACGCCA  △35    F3-2
ATTACTAATTAAC-----------------------------------//------------------AACACACGCGACA  △276   F3-3
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Figure 2. Sequence of *Bmdsx* gene mutations in exon 3 induced by transcription activator-like effector nucleases (TALENs). TALEN-binding sequences are underlined in the wild-type (WT) gene sequence. Deletions and insertions are indicated by red dashes and red letters, respectively. Sex and number of detection individuals marked with M1, 2, 3 or F1, 2, 3. The length of nucleotide deletions next to the delta character is listed for each individual mutant gene.

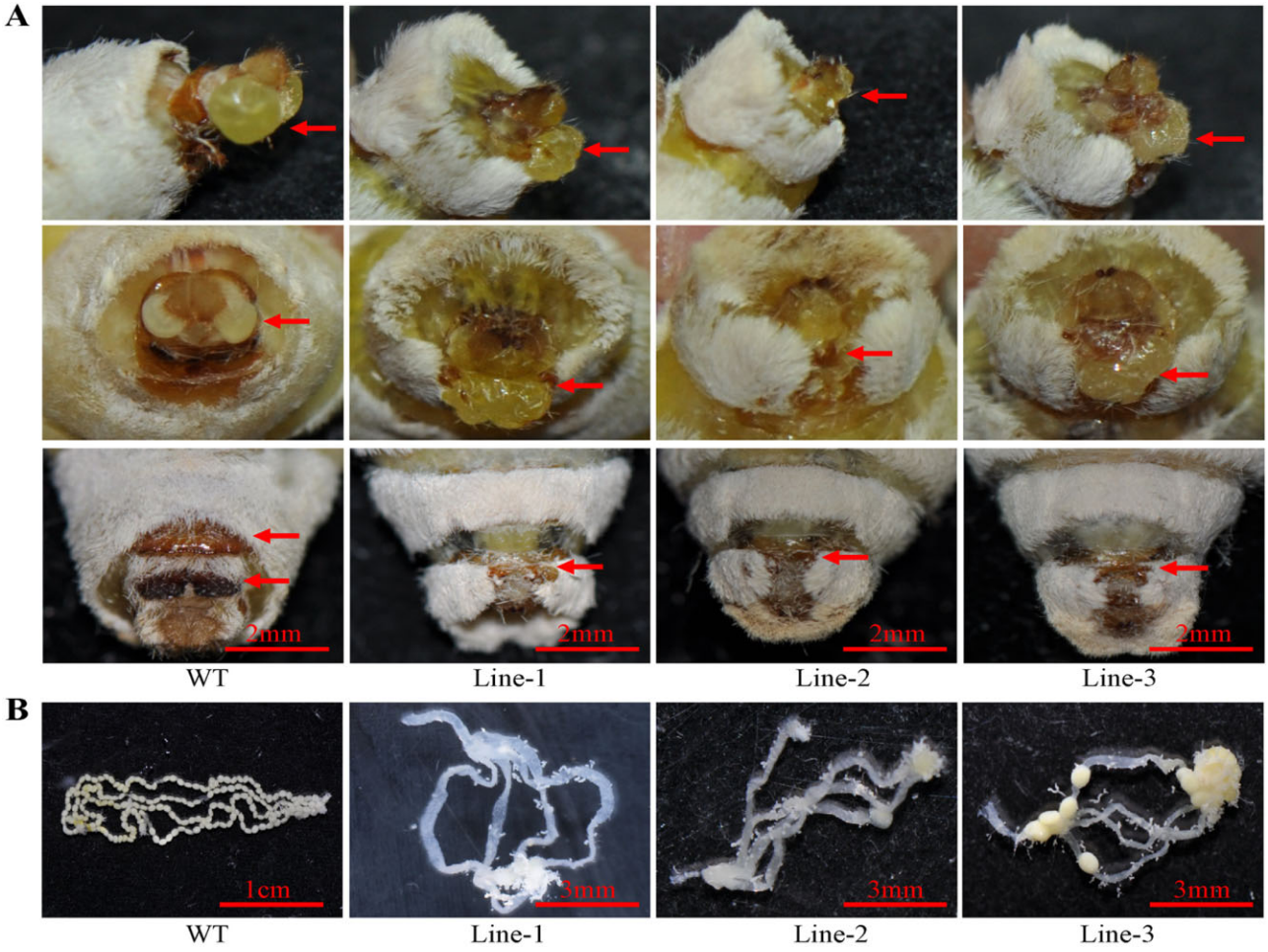

Figure 3. Photographs of external genitalia and ovipositors of mutant females. (A) External genitalia of mutant females: lateral view (upper panel) and front view (middle panel) show the genital morphology of wild-type (WT) and mutant line 1–3 individuals. Red arrows indicate morphology of genital papilla. The lower panel shows that the female chitin plate is absent in the mutant individuals. Red arrows indicate morphology of chitin plate. (B) Mutant females have no (lines -1 and -2) or few (line-3) eggs in their ovarioles.

BmDSX^F mutation blocked female mating behaviour but not courtship

We investigated transcription levels of genes with sex-specific expression that are expected to be regulated by *dsx*. The expression level in mutant females of the *vitellogenin* (*Vg*) gene, which encodes a protein essential for oogenesis, was only 25% of that seen in wild-type females (Fig. 4C). In contrast, *Vg* expression levels in mutant males were tenfold higher than that seen in wild-type males. This finding supports the conclusion that female-specific isoform, Bmdsx^F, contributes to *Vg* suppression in males. The expression level of a male-specific pheromone-binding protein (BmPBP) was high in transgenic females but not males, supporting the conclusion that Bmdsx^F suppresses PBP in females (Fig. 4D).

We crossed egg-free F_1 females with wild-type or transgenic males to investigate whether courtship behaviour was also affected. Mutant females could attract mutant or wild-type males and the time taken by the male to recognize the female and begin courtship was similar to that in wild-type animals (Movies S1–4). The results support the conclusion that female infertility most likely results from copulation failure caused by the abnormal external genitalia.

Discussion

This transgene-based, somatic mutagenesis technology provides a fast and robust gene analysis tool. Distinct from other transgenic TALEN or CRISPR/CAS systems in which germ-cell-specific promoters were used (Kondo & Ueda, 2013; Treen *et al.*, 2014), our system allows somatic mutagenesis analyses to be performed in F_1 animals. Importantly, the observed 100% efficiency in generating mutations is useful, particularly for analysis of those genes without visible phenotypes. This system is anticipated to contribute to functional gene analyses and

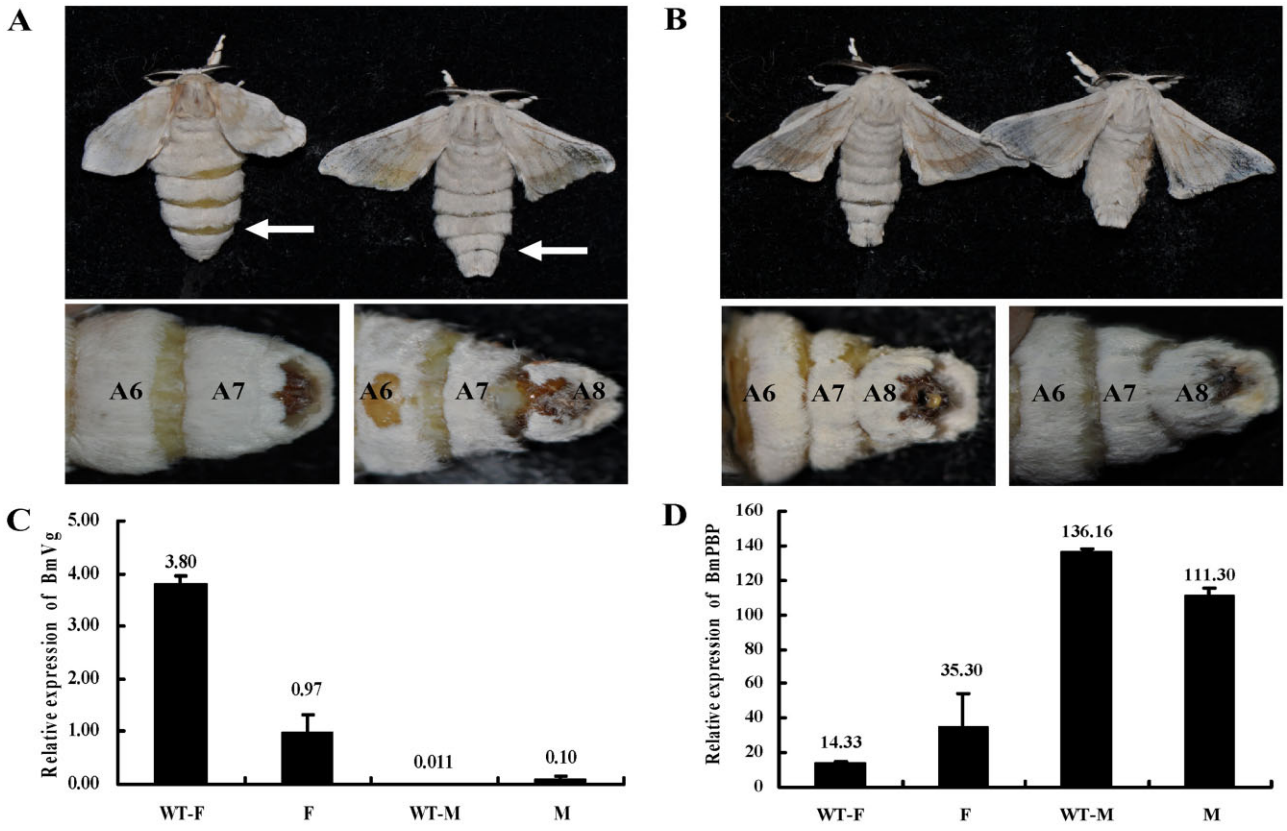

Figure 4. Gross morphology of segment of wild-type (WT) and transgenic F_1 founder moths and expression analysis of downstream target genes. (A) A WT female moth with a large abdomen filled with mature eggs (left); transgenic F_1 female moth with a small abdomen without eggs (right). Numbers in the lower panel show the abdominal segments. The abdomens are indicated by white arrows. (B) WT moth and transgenic F_1 male moths. (C, D). Relative mRNA expression of vitellogenin (*BmVg*) and pheromone-binding protein (*BmPBP*) encoding genes in WT animals and transcription activator-like effector nuclease-induced mutants. The mean is an average value of mRNA measurements from three individuals.

provide the basis for generating novel applications in SIT for agricultural and medical insect pest management.

The present study provides the first evidence that transgenic TALENs efficiently induced female-specific sterility in insects through targeting *Bmdsx*. Insect *dsx* is a well characterized double-switch gene that produces sex-specific transcription factors via alternative splicing of its transcripts, which function at the final step in the sex-determination cascade. Genetic null mutants of *dsx* in *Drosophila* affect sex differentiation and induce intersexual phenotypes in both males and females (Waterbury *et al*., 1999). Transgenic analysis showed that female *D. menalogaster* Dsx protein (Dsx^F) functions as a positive regulator of female differentiation and a negative regulator of male differentiation (Waterbury *et al*., 1999). Transgenic expression of $Bmdsx^F$ in *B. mori* males induces *Vg* that is expressed specifically in wild-type females. It also represses the pheromone-binding protein (*BmPBP*) gene that is expressed dominantly in males, although transgenic animals display normal morphological characteristics (Suzuki *et al*., 2003).

Ectopic expression of $Bmdsx^M$ in females resulted in abnormal differentiation of female-specific genital organs and caused partial male differentiation in female genitalia. This latter phenotype is in contrast to what is seen with ectopic expression of Dsx^F (Suzuki *et al*., 2003; Duan *et al*., 2014). These reports support the conclusion that the products of *Bmdsx* play an important sex-specific role in sex determination and differentiation. Furthermore, our somatic knockout analysis showed that the chitin plate structure completely disappeared in the Dsx^F mutant females, not an intermediate phenotype reported before (Duan *et al*., 2014). This result indicated that loss-of-function analysis is needed to fully exploit the mechanism of the *Bmdsx* mode of action and the transgenic TALEN technologies will greatly contribute to this achievement.

Mutation of $Bmdsx^F$ by somatic TALEN technologies caused severely deleterious defects in oogenesis development. Transgenic females have normal oviducts with no or a few eggs and this is correlated with significantly decreased *Vg* expression. In contrast, although transgenic females with $Bmdsx^F$ deletion had abnormal

external genitalia and copulation failure, they still showed normal sexual behavior, indicating that *Bmdsx* was not involved in sexual behaviour regulation. Sexual orientation and courtship behaviour in *D. melanogaster* are controlled by *fruitless* (*fru*), the first gene in a branch of the sex-determination hierarchy functioning specifically in the central nervous system (Kimura *et al*., 2008; Kohatsu *et al*., 2011). The phenotypes of loss-of-function *fru* mutants encompassed nearly all aspects of male sexual behaviour (Ito *et al*., 1996; Anand *et al*., 2001). Other genes such as *intersex* (*ix*) and *hermaphrodite* (*her*) also act independently or dependently to regulate some aspects of sexual differentiation in *D. melanogaster* (Waterbury *et al*., 1999; Garrett-Engele *et al*., 2002; Ito *et al*., 2012). Whether these homologous genes regulate *B. mori* sexual behaviour needs further investigation.

The regulation of customized nucleases expression in sex-, tissue- or stage-specific manners is still challenging, despite its wide application in genome editing. The screening of gene knockout mutants is dependent largely on visible phenotypes, or large-scale sequencing of many putative mutant insects, which is costly in labour and resources (Li *et al*., 2012; Katsuyama *et al*., 2013; Sajwan *et al*., 2013). Limited success was reported for gene knock-in strategies based on homologous recombination of donor templates (Auer *et al*., 2014). Genetic transformation technologies mediated by *piggybac* have been established in many insect species including *B. mori*. Thus, it would be a fast and efficient strategy to apply somatic nucleases technologies, including the ZFN, CRISPR/Cas (Kondo & Ueda, 2013; Ren *et al*., 2013) system and current TALEN technologies in insect genome editing. These technologies will be useful particularly for dissecting genes without visible phenotypes when mutated. In addition, by using different promoters to regulate TALEN expression, it will be easy to dissect gene function with sex, stage and tissue specificity. The somatic TALEN technologies established in the present study will not only provide a powerful tool for dissecting the sex-specific regulatory mechanism of *Bmdsx*, but will also greatly facilitate future insect functional gene analysis.

Experimental procedures

Silkworm strains

The *B. mori* strain used in the present study was a multivoltine, nondiapausing silkworm strain, Nistari. Larvae were reared on fresh mulberry leaves under standard conditions.

Plasmid construction

Plasmids of Psw-peas-T-TALENs containing the cassettes of TALEN Repeat, *Fok*I, and SV40 polyA were provided by View Solid Biotech (http://www.v-solid.com/). The TALEN cassette was moved to the transgenic plasmid PXL-BacII (kind gift from Prof. Malcolm Fraser at the University of Notre Dame) by digestion and ligation through *Not*I and *Hin*dIII restriction sites to generate intermediate plasmids PXLBacII-TALEN-L and PXLB*ac*II-TALEN-R. Subsequently, the IE1 promoter was inserted into the *Hin*dIII site in the upstream region of TALEN repeat sequence to generate PXLBacII-IE1-TALEN-L-arm and PXLBacII-IE1-TALEN-R-arm. 3 × P3-DsRed and 3 × P3-EGFP were amplified from the plasmid pBac[3 × P3/DsRed] and pBac[3 × P3/EGFP] using primers F: 5′-TTATCGAATTCCTGCAGCCCGTACGCGTATCGATAAGCTT-3′ and R: 5′-GAGGTTTTTTAATTCGCTTCCCACAATGGTTAATTCG-3′ and inserted into *Not*I and *Sma*I site in multiple clone sites of the PXLBacII-IE1-TALEN-L-arm and PXLBacII-IE1-TALEN-R-arm, respectively, to generate PXLBacII-3 × P3-DsRed-IE1-TALEN-R (pBac-DsxR) and PXLBacII-3 × P3-EGFP-IE1-TALEN-L (pBac-DsxL).

Germ line transformation

DNA solutions containing pBac-DsxL or pBac-DsxR mixed with helper plasmids were microinjected into preblastoderm G_0 embryos that then were incubated at 25°C in a humidified chamber for 10–12 days until larval hatching (Tan *et al*., 2013). Larvae were reared on fresh mulberry leaves and putative transgenic G_0 adults were mated with wild-type moths, and G_1 progeny were scored for the presence of the marker gene using fluorescence microscopy (Nikon AZ100).

Mutagenesis analysis

Genomic DNA was extracted from *B. mori* larvae by using standard sodium dodecyl sulphate lysis-phenol treatment after incubation with proteinase K, followed by RNase treatment and purification. PCR amplification was carried out using 50ng genomic DNA as the template. Primers used for amplification of the target region were forward primer, 5′-GGAGACTGCACTATTTCAATGTT-3′ and reverse primer, 5′-CGTACGACGTGTCTATATTGCAT-3′, which were used to amplify a region of 608 base pairs (bp) in length that encompassed the target sites. PCR products were sub-cloned into pJET-1.2 vector (Fermentas, Burlington, ON, USA) and sequenced.

mRNA detection of Vg and PBP genes

For real-time-PCR analysis, total RNA was extracted from silkworm larvae or cultured cells using Trizol reagent (Invitrogen, Carlsbad, CA, USA) and treated with RNase-free DNAse I (Ambion, Austin, TX, USA) according to the manufacturer's protocol. cDNAs were synthesized using the Omniscript Reverse transcriptase kit (Qiagen, Hilden, Germany), in a 20-μl reaction mixture containing 1 ug total RNA, followed the manufacturer's instruction. Reverse-transcription PCR reactions were carried out using gene-specific primers (forward, 5′-GCCTCGATTTTCCAACTTCA -3′, reverse, 5′- CCATTCTGAAGCAACAGGAG -3′) for amplifying a 218-bp fragment of the *BmVg* gene; (forward, 5′-CATGGAGCCGATGAGACGAT-3′, reverse, 5′- TCATCGTTAGCTGGAGTGGACTT -3′) for amplifying an 80-bp fragment of the *BmPBP* gene. Another primer pair set (forward, 5′-TCAATCGGATCGCTATGACA-3′, reverse, 5′-ATGACGGGTCTTCTTGTTGG-3′) amplifies a 136-bp fragment from the *B. mori* ribosomal protein 49 (*Bmrp49*) as an internal control.

Courtship behaviour analysis

Individual virgin females and males were separated at the pupal stage for morphological observation and courtship assays. Gross morphology of external genitals was investigated as reported previously (Suzuki *et al.*, 2005). For courtship assays, males and females were collected at late pupal stage and aged individually for 3 days. Behavioural assays were performed at 25°C, 60% relative humidity under normal ambient light.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1. Eye-specific expression of selecting fluorescent protein markers in transgenic silkworms.

Figure S2. Genomic insertion of the IE1-TALEN-L and IE1-TALEN-R construct.

Figure S3. Results of PCR analyses with primers to amplify a region of 608 bp revealed deletion mutation events in G0 mutants.

Figure S4. Schematic drawings of external genitalia of the female moth.

Figure S5. Sexually dimorphic segment number in wild type and mutant *Bombyx mori*.

Table S1. Relative luciferase activity (firefly/renilla) of TALENs designed for targeting female-specific exon3 of *Bmdsx*. TALENs activity was measured *in vitro* with the luciferase SSA assay in 293T cells and was thirty-fivefold higher than in control.

Movie S1. A transgenic male failed copulate with a transgenic female.

Movie S2. A transgenic male successfully copulates with a wild-type female.

Movie S3. A wild-type male fails to copulate with a transgenic female.

Movie S4. A wild-type male successfully copulates with a wild-type female.