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Mutation of *doublesex* induces sex-specific sterility of the diamondback moth *Plutella xylostella*

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ABSTRACT

Doublesex (dsx): the downstream gene in the insect sex determination pathway, plays a critical role in sexual differentiation and development. The functions of *dsx* have been characterized in several model insect species. However, the molecular mechanism and functions of sex determination of *dsx* in *Plutella xylostella*, an agricultural pest, are still unknown. In present study, we identified a male-specific and three female-specific *Pxdsx* transcripts in *P. xylostella*. Phylogenetic analyses and multiple sequence alignment revealed that *Pxdsx* is highly conserved in lepidopterans. The CRISPR/Cas9 technology was used to induce mutations in the male-specific isoform, the female-specific isoform, and common regions of *Pxdsx*. Disruptions of *Pxdsx* sex-specific isoforms caused sex-specific defects in external genitals and partial sexual reversal. In addition, we found that female specific transcripts were detected in $Pxdsx^{M}$ male mutants and male-specific transcripts were detected in $Pxdsx^{F}$ female mutants. Mutations also caused changes in expression of several sex-biased genes and induced sex-specific sterility. This study demonstrates that *Pxdsx* plays a key role in sex determination of *P. xylostella* and suggests novel genetic control approaches for the management of *P. xylostella*.

1. Introduction

The diamondback moth (DBM), *Plutella xylostella* (L.), is one of the most destructive agricultural pests of cruciferous crops and causes huge losses worldwide (Furlong et al., 2013; Talekar and Shelton, 1993). Currently, the management of DBM is mainly by pesticides that have negative effects on the environment and human health (Leftwich et al., 2016). The short life span of the moth and widely overlapping generations have resulted in rapid development of resistance to all major classes of commonly used insecticides (Tabashnik et al., 1990). Therefore, novel genetic pest management approaches are urgently needed. Genetic manipulation of the sex ratio of population may contribute to novel strategy for this pest control. Thus, it is important to understand the general mechanism of sex determination in *P. xylostella*.

The approaches about sex determination and sexual regulation on silkworm offer very good examples to other lepidopteran insects. In the recent years, studies of sex determination have focused mainly on the lepidopteran model species *Bombyx mori* (Fujii and Shimada, 2007; Traut et al., 2007). In *B. mori*, femaleness is predominantly determined by the presence of the W chromosome and males are homomorphic (Kiuchi et al., 2014). A recent report shown that gene integration targeting silkworm W chromosome specific sequences with genome editing tools, which development of silkworm strains with complete female-specific embryonic lethality for male-only rearing (Zhang et al., 2018). In female silkworms, the *Masculinizer* (*BmMasc*) gene is transcribed from the Z chromosome and responsible for sex determination and dosage compensation (Katsuma et al., 2015; Kiuchi et al., 2019). Transgenic expression of the *Masc-R* (piRNA-resistant *Masculinizer*) induced ovaries in females exhibited testis-like structures that contained sperm bundles and induced partial male differentiation in female genitalia (Sakai et al., 2016). In addition, *BmMasc* controls sex-specific splicing of *doublesex* (*dsx*) and the expression of IGF-II mRNA-binding protein (Imp) (Katsuma et al., 2015; Kiuchi et al., 2019). Among *dsx* have been well characterized in *B. mori*. RNAi-mediated knockdown

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studies showed that two female DSX proteins are required in the female sexual differentiation of silkmoths (Shukla and Nagaraju, 2010b). Ectopic expression sex-specific *Bmdsx* proved that DSX regulates the expression of *vitellogenin* (*Vg*), *olfactory receptor* (*OR*), *pheromone binding protein 1* (*PBP1*), and *storage protein* (*SP*) in *B. mori* (Suzuki et al., 2003, 2005). Furthermore, the depletion of sex-specific isoforms of *Bmdsx* gene in *B. mori* using the binary transgenic TALENs system induce sex-specific sterility (Xu et al., 2014, 2017b). Taken together, these sex determination related genes have been well identified and characterized in *B. mori* through gene editing techniques. These reports provide insight into potential for sex determination genes targeting in genetic control of *P. xylostella*.

The molecular mechanisms of sex determination are diverse (Gempe and Beye, 2011; Salz, 2011). In *Caenorhabditis elegans* and *Drosophila melanogaster*, the primary signal in sex determination is the ratio of X chromosomes to autosomes (Bridges, 1921, 1925; Hodgkin, 1999; Kuwabara, 1999). However, the functions of some genes are similar in diverse species. Amongst these are genes encoding DM domain-containing transcription factors that regulate expression of genes involved in sex determination (Matson and Zarkower, 2012). In *D. melanogaster*, the pre-mRNA of *doublesex* (*Dsx*) is sex-specifically spliced to produce female-specific and male-specific isoforms (Burtis and Baker, 1989). Subsequently, *dsx* homologues that are sex-specifically spliced have been reported in a number of insect species including, *Musca domestica* (Hediger et al., 2004), *Anopheles gambiae* (Scali et al., 2005), *Apis mellifera* (Cho et al., 2007), *Lucilia cuprina* (Concha et al., 2010), *Aedes aegypti* (Salvemini et al., 2011), *Tribolium castaneum* (Shukla and Palli, 2012), and *Culex pipiens* (Price et al., 2015). These reports suggest that the *dsx* gene function is highly conserved among different insect species, and thus may be targeted in the biological control program of *P. xylostella*. Therefore, in this study, we firstly identified and characterized the *Pxdsx* gene and employed the CRISPR/Cas9 system to disrupt *Pxdsx*. Our results demonstrate that sex-specific *Pxdsx* isoforms regulate sexual differentiation in *P. xylostella* and provide insight into potential genetic regulation of *P. xylostella*.

2. Materials and methods

2.1. Insect strain and rearing

A diamondback moth strain was obtained from the Institute of Zoology, Chinese Academy of Science (Beijing). The larvae were reared in an incubator at 25 ± 1 °C, 65 ± 5% relative humidity and photoperiod of 14 h:10 h (L:D) and fed with an artificial diet (Huang et al., 2017). Pupae were sexually separated and kept in plastic boxes. Adult moths were reared in a mesh cage (0.2 × 0.2 × 0.2 m^3) and fed with 10% honey solution.

2.2. Cloning and analysis of Pxdsx

Total RNA was extracted from female and male pupae, adult, or other tissues using the Trizol reagent (Invitrogen) following the manufacturer's protocol. The RNA quality was checked with a spectrophotometer and then treated with RQ1 RNase-free DNase at 37 °C for 30 min (Promega). First-strand cDNA was synthesized with RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific) using 1 μg total RNA. The putative *Pxdsx* gene was identified from the genomic database of DBM (http://iae.fafu.edu.cn/DBM/index.php). The open reading frame of *Pxdsx* was PCR amplified with KOD FX (TOYOBO) under the following conditions: initial denaturation at 98 °C for 2 min, 35 cycles of 98 °C for 30 s, 59 °C for 30 s, and 68 °C for 1 min, and an elongation step at 68 °C for 10 min. The amplified products were confirmed by Sanger sequencing after sub-cloning into pJET1.2 vector (Thermo Fisher Scientific). The sequencing results were compared with *Pxdsx* genomic sequence to identify the exon-intron boundaries.

2.3. Sequence comparison and phylogenetic relationships

The alignment of the DNA-binding oligomerization domain protein sequences from *P. xylostella*, *B. mori, Helicoverpa armigera*, *and Ostrinia furnacalis* was created with Clustal X2 software and the GENEDOC program. The amino acid sequence of *dsx* gene was aligned using Clustal W implemented in the MEGA5.1 program. Molecular phylogenetic analyses were conducted using the maximum-likelihood method, and the reliability of the tree was tested by bootstrap analysis with 1000 replications (Tamura et al., 2011).

2.4. Target design and in vitro transcription of Cas9 and sgRNA

Single guide RNAs (sgRNAs) were designed to target exon 2 of the *Pxdsx* region common to transcripts expressed by both males and females ($Pxdsx^C$), exon 3 of *Pxdsx* female-specific splicing isoform ($Pxdsx^F$), and exon 5 of *Pxdsx* male-specific splicing isoform ($Pxdsx^M$). Three sgRNAs were designed per target according to the 5′-GG-$(N)_{19}$-GG-3′ rule. sgRNAs were synthesized *in vitro* using the MEGAscript T7 kit (Ambion) following manufacturer's protocol. The *Cas9* mRNA was synthesized *in vitro* using the mMESSAGE mMACHINE Kit (Ambion) as previously described (Wang et al., 2013). sgRNAs and *Cas9* mRNA were purified with phenol:chloroform:isoamylol (25:24:1) and stored at −80 °C.

2.5. Microinjection of embryos

The sgRNAs and *Cas9* mRNA were mixed at final concentrations of 150 ng/mL and 300 ng/mL, respectively. Fertilized eggs were collected within 30 min after oviposition. Microinjection manipulation (IM300 Narishige) was accomplished within the next hour as described (Huang et al., 2016). The injected eggs were incubated at 26 ± 1 °C and 60 ± 5% relative humidity until they hatched.

2.6. Phenotypic observation and mutation analysis

Hatching rate, live pupae, and adults were counted. Phenotypes of pupae and adults were observed with a stereo microscope (Nikon SW-2B/22). To confirm the mutagenesis at *Pxdsx* locus, genomic DNA was extracted from adults with mutant phenotypes. The genomic DNA was then used as a PCR template to amplify the region flanking the target site. The amplified products were ligated into the pJET1.2 vector and sequenced.

2.7. Hatchability assay

In order to explore whether *Pxdsx* mutations result in sterility, hatchability assays were performed. In brief, wild-type virgin females were allowed to mate with wild-type, $Pxdsx^F$ mutant, $Pxdsx^C$ mutant, or $Pxdsx^M$ mutant males. Similarly, wild-type virgin males were allowed to mate with wild-type, $Pxdsx^F$ mutant, $Pxdsx^C$ mutant, or $Pxdsx^M$ mutant females. Females in all groups were allowed to lay eggs for two days. The hatching rates were analyzed. Each group had five pairs of moths, and the experiments were performed three times.

2.8. Qualitative and quantitative real-time PCR analysis

Total RNA extracted from the antennae of mutant and wild-type moths was used to investigate the expression of olfactory receptors and pheromone binding protein. To test the expression of *Vg*, total RNA was extracted from fat bodies of 2-day-old virgin mutant and wild-type moths. qRT-PCR was carried out using SYBR Green Real-time PCR Master Mix (Thermo Fisher Scientific) following the manufacturer's protocol and using an Eppendorf Real-time PCR System Mastercycler RealPlex. The ΔΔCt was used to evaluate the quantitative variation. All qRT-PCR experiments were performed in three independent biological

replications. RT-PCR was performed using KOD plus (TOYOBO). Ribosomal protein gene *S64* was used as a reference gene. All the primers are listed in Table S1.

2.9. Statistical analysis

Statistical analysis was performed using SPSS 22.0 software with an independent Student's *t*-test.

The data are presented as means ± SEM, and statistical significance was assumed for $p < 0.05$.

3. Results

3.1. Identification and characterization of Pxdsx

The *B. mori dsx* gene was used as a query to identify the putative coding sequence of *Pxdsx* from the *P. xylostella* genome (You et al., 2013). One male-specific and three female-specific *dsx* transcripts were identified in *P. xylostella*. These transcripts were designated as *Pxdsx-m*, *Pxdsx-f1*, *Pxdsx-f2*, and *Pxdsx-f3*. The *Pxdsx-m* and *Pxdsx-f1* transcript have three exons and two introns. *Pxdsx-f2* and *Pxdsx-f3* have four exons and three introns. The coding sequences of *Pxdsx-m*, *Pxdsx-f2*, and *Pxdsx-f3* are 795 base pairs (bp) in length, and *Pxdsx-f1* has a 738-bp coding sequence (Fig. 1A). These sex-specific *dsx* transcript sequences were shown in supporting information.

DSX amino acid sequences from seven insect species were used to construct a phylogenetic tree. *Pxdsx* genes are tightly clustered in the Lepidoptera clade (Fig. 1B). A comparison of DSX proteins from four lepidopteran species showed that both male and female PxDSX proteins had OD1 and OD2 domains with high sequence identity to DSX proteins from other lepidopteran insects (Fig. S1).

3.2. Abnormal phenotypes are induced by disruption of sex-specific Pxdsx transcripts

The CRISPR/Cas9 system was employed to investigate the function of the female- and male-specific *Pxdsx* transcripts *in vivo*. Three sgRNA sites were identified in exon 2 of *Pxdsx* common to male- and female-specific transcripts ($Pxdsx^C$), in exon 3 of the *Pxdsx* female-specific isoform ($Pxdsx^F$), and in exon 5 of the male-specific isoform ($Pxdsx^M$) (Fig. 2). We then injected 489, 431, and 518 fresh eggs with *Cas9* mRNA and the sgRNAs designed to disrupt $Pxdsx^C$, $Pxdsx^F$, and $Pxdsx^M$, respectively. Eggs from the control group (102 fresh eggs) were injected with doubly distilled H_2O. The hatching rates were 64.4% (315/489) in $Pxdsx^C$ group, 58.5% (252/431) in $Pxdsx^F$ group, 56.0% (290/518) in $Pxdsx^M$ group, and 73.8% (75/102) in control group (Table 1). We compared the phenotypes of animals in control group with three mutant groups. Wild-type female and male pupae have different abdominal morphologies. Female pupae have an X-shaped line, whereas male pupae develop two salient points at the abdominal tail end (Fig. 3). In the $Pxdsx^C$ mutants, all female and male pupae had abnormal abdominal phenotypes. Interestingly, male pupae of $Pxdsx^F$ mutants and female pupae of $Pxdsx^M$ mutants had normal phenotypes; however, female and male pupae in the corresponding sex-specific *Pxdsx* mutant groups all had the same abnormal phenotypes as $Pxdsx^C$ mutants (Fig. 3).

In the adults, sex-specific external genitalia are essential for a successful copulation. Wild-type males possess an aedeagus and a pair of harpago that are protected by an extended abdominal segment. Females have a genital papilla and a ventral plate. We found that $Pxdsx^M$ female mutants and $Pxdsx^F$ male mutants have normal external genitalia (Fig. 4). In $Pxdsx^F$ female mutants, the genital papillae were absent, however, and a tumor-like structure was observed (Fig. 4). Severe structural defects were also observed in $Pxdsx^M$ male mutants (Fig. 4). The harpago and extended abdominal segments were shorter than those in wild-type males, and the aedeagus was absent in $Pxdsx^M$ male mutants. As expected, in the $Pxdsx^C$ mutants, both male and female mutants showed severe malformations (Fig. 4). Furthermore, we compared the phenotypes of internal genitalia in control group with three mutant groups. Wild-type female adults possess a pair of end-merged ovaries, each harboring six oviducts (Fig. 5). The $Pxdsx^M$ mutant ovaries present normal phenotypes. The $Pxdsx^F$ and $Pxdsx^C$ mutant ovaries have abnormal phenotypes, including: 1) the mutant ovaries only have four oviducts; 2) The mutant ovaries exhibited degenerated ovaries with less eggs in the oviducts. (Fig. 5). In *P. xylostella*, the wild-type male adult showed one fused testis shaped like a sphere (Fig. 5). The $Pxdsx^F$ mutant testis present normal phenotypes. However, The $Pxdsx^M$ and $Pxdsx^C$ mutant testes significantly smaller than those of wild-type were detected. Also, mutant testes showed an irregular sphere suggesting incomplete testis fusion (Fig. 5).

3.3. CRISPR/Cas9-mediated mutagenesis of Pxdsx is observed in adults with abnormal phenotypes

In order to confirm that phenotypic defects were caused by the depletion of *Pxdsx*, we selected adult moths with obvious defects and extracted and sequenced relevant regions of genomic DNA. The results showed that deletions, substitutions, and insertions occurred at three

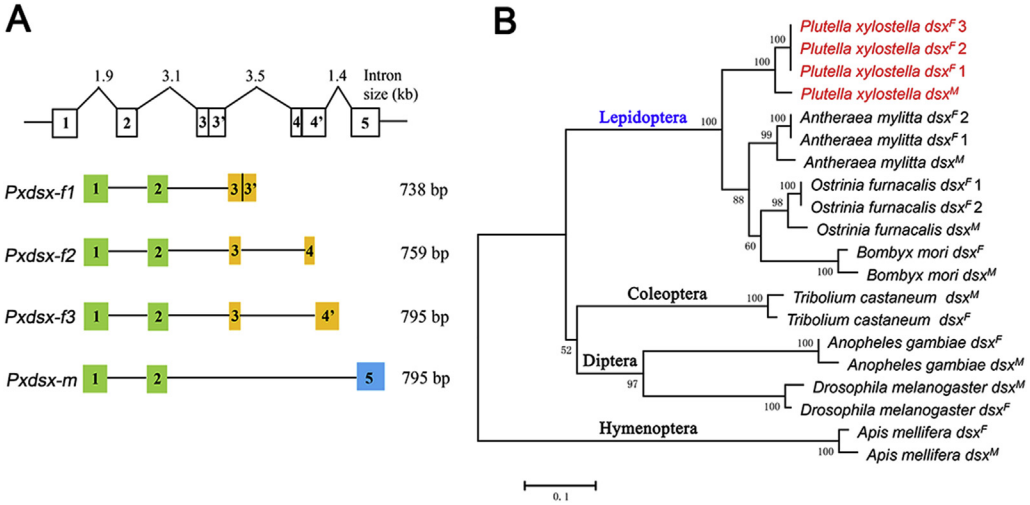

Fig. 1. Characterization of *P. xylostella dsx* gene. (B) Phylogenetic analysis of DSX based on the alignment of amino acid sequences of eight insect species. (A) Genomic structure of *Pxdsx*. The four different sex-specific transcripts in males and females are shown. Exons are indicated by boxes and introns by lines.

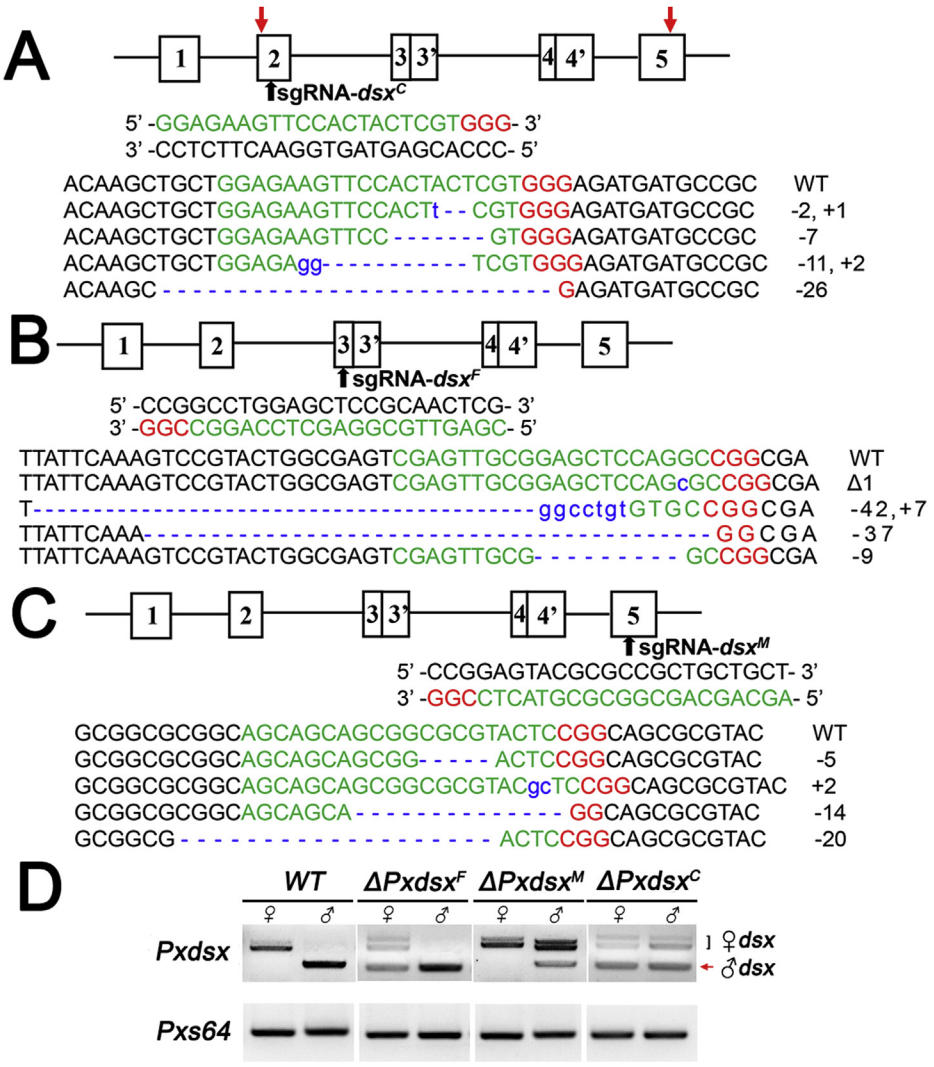

Fig. 2. Targeted mutation of *Pxdsx* induced by CRISPR/Cas9. (A, B, and C) Schematic diagram of gene sequences and three sgRNA target sites. The target sequences and PAM sequences are highlighted in green and red, respectively. $Pxdsx^{C}$, $Pxdsx^{F}$, and $Pxdsx^{M}$ mutant sequences were confirmed by cloning and sequencing. Dashed lines represent the deleted bases, and inserted bases are displayed in lower case. The net change in length is marked at the right of each sequence (−, deletion; +, insertion; Δ, substitution). (D) The splicing patterns of *Pxdsx* were examined by RT-PCR in wild-type and *Pxdsx* mutant insects. The red arrows and black brackets indicate male- and female-type splicing of *Pxdsx*, respectively. The related primer sites for splicing patterns of *Pxdsx* were indicated with red arrows in (A). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 1
Efficiency of CRISPR/Cas9-mediated mutagenesis.

	sgRNA/Cas9 concentration (ng/μL)	Injected embryos	Hatching rate (%)	Live pupae (F/M)	Mutation phenotype of pupae F/M (Rate %)	Mutation phenotype of adults F/M (Rate %)
$Pxdsx^{C}$	150/300	489	64.4	151 (72/79)	26/31 (36.1%/39.2%)	18/20 (25.0%/25.3%)
$Pxdsx^{F}$	150/300	431	58.5	126 (59/67)	27/- (21.4%/-)	16/- (27.1%/-)
$Pxdsx^{M}$	150/300	518	56.0	107 (53/54)	-/29 (-/27.1%)	-/17 (-/31.4%)
ddH_2O	–	102	73.8	49 (26/23)	–	–

targeted sites in the *Pxdsx* gene (Fig. 2A–C). We then examined the *Pxdsx* sex-specific transcripts in different mutants by RT-PCR. In $Pxdsx^{F}$ mutant group, the male *Pxdsx* transcript was expressed at normal levels, but female-specific *Pxdsx* transcripts were observed at lower levels in female moths than in the wild-type females, and the male *Pxdsx* transcript was also observed in mutant female moths. In the $Pxdsx^{M}$ mutant group, the female *Pxdsx* transcript was present at normal expression levels in female moths, whereas expression of male *Pxdsx* transcript was decreased in male moths, and female *Pxdsx* transcripts were detected in male moths. In the $Pxdsx^{C}$ mutants group, all *Pxdsx* transcripts were detected in both male and female moths, but transcripts were expressed at abnormally low levels (Fig. 2D). These results confirmed that CRISPR/Cas9 induced effective mutagenesis of *Pxdsx*.

3.4. Sex-biased gene expression is altered in Pxdsx mutants

To investigate whether the disruption of sex-specific *Pxdsx* transcripts influences the expression of known sex-biased genes in *P. xylostella*, we examined the female-biased *Vg* gene, which encodes a protein essential for oogenesis, and three previously reported male-biased genes *OR1*, *OR3*, and *PBP1* (Suzuki et al., 2005; Xu et al., 2017a). Compared with the wild-type females, the relative expression level of *Vg* was significantly down-regulated in $Pxdsx^{F}$ mutant females (Fig. 6A), whereas in $Pxdsx^{M}$ and $Pxdsx^{C}$ mutant males the levels of *Vg* was significantly increased (Fig. 6A). The relative expression levels of *PBP1*, *OR1*, and *OR3* were significantly increased in $Pxdsx^{F}$ and $Pxdsx^{C}$ female mutants but were significantly down-regulated in $Pxdsx^{M}$ and $Pxdsx^{C}$ male mutants (Fig. 6B–D). These results are consistent with a previous report that indicated the *Vg*, *PBP1*, *OR1*, and *OR3* genes are direct or an indirect targets of *dsx* in *P. xylostella* (Suzuki et al., 2005; Xu

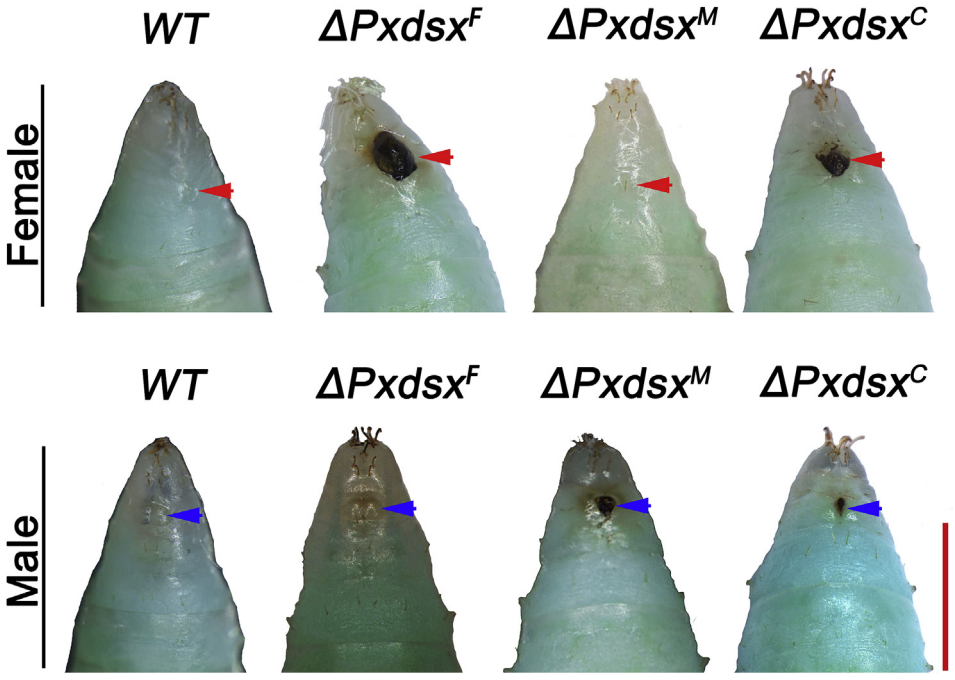

Fig. 3. The morphologies of wild-type and *Pxdsx* mutant pupae. Wild-type male pupae have two salient points (indicated by blue arrows) at the gonopore of the ninth abdominal segment. The two salient points were abnormal in $Pxdsx^{C}$ and $Pxdsx^{M}$ male mutant pupae. Wild-type female pupae have an X-shaped line (indicated by red arrows) at the abdominal gonopore. The X-shaped lines were not present in $Pxdsx^{C}$ and $Pxdsx^{F}$ mutant female pupae. Scale bar: 1 mm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

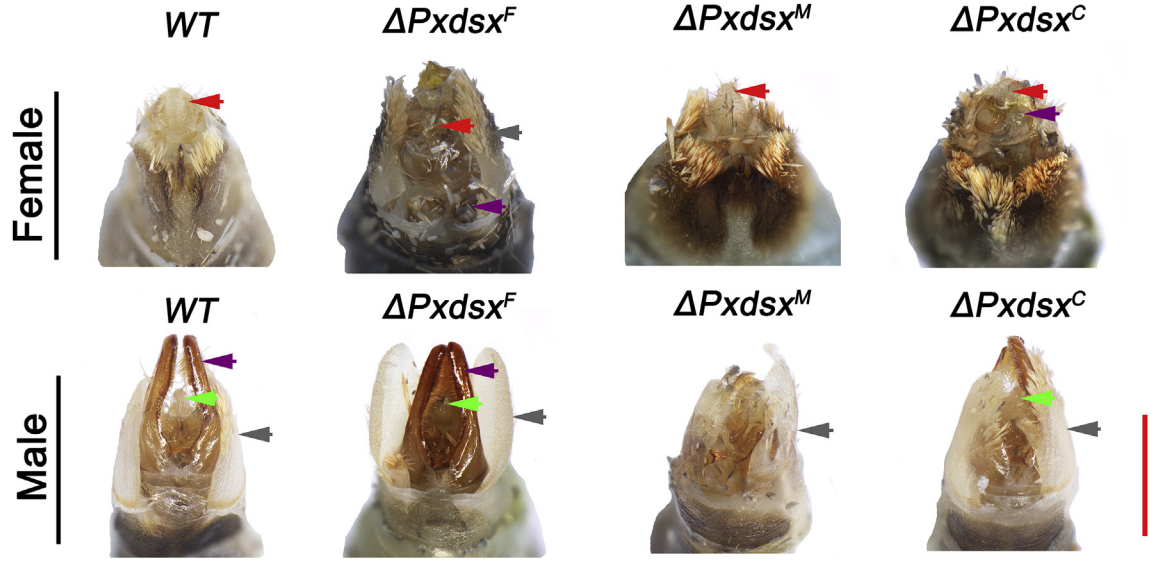

Fig. 4. The external genitalia morphology of the wild-type and *Pxdsx* mutant adults. Wild-type adult male external genitalia mainly consist of harpago (indicated by purple arrows) and aedeagus (indicated by green arrows). Wild-type female external genitalia mainly consist of a genital papilla (red arrows) and ventral plate. The male-specific external genitalia in $Pxdsx^{C}$ and $Pxdsx^{M}$ mutant males exhibited severe structural defects, and the genital papilla and ventral plate were not present in $Pxdsx^{C}$ and $Pxdsx^{F}$ mutant females. The gray arrows indicate the genital segment. Scale bar: 1 mm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

et al., 2017a).

3.5. *Pxdsx* mutations induce sterility in diamondback moths

To explore if the disruption of sex-specific *Pxdsx* transcripts induces sterility, the number of eggs and hatching rates were estimated. There were no significant differences in the numbers of eggs laid by wild-type females mated with wild-type males or $Pxdsx^{F}$, $Pxdsx^{C}$, or $Pxdsx^{M}$ male mutants (Fig. 7A). However, $Pxdsx^{C}$ or $Pxdsx^{F}$ female mutants that mated with wild-type males laid significantly fewer eggs compared with $Pxdsx^{M}$ female mutants and wild-type females mated with wild-type males (Fig. 7B). Hatching rates were estimated as a measure of fertility. Our results showed that eggs laid by wild-type female that mated with $Pxdsx^{M}$ or $Pxdsx^{C}$ male mutants failed to hatch (Fig. 7C). Similarly, eggs laid by $Pxdsx^{F}$ or $Pxdsx^{C}$ female mutants mated with wild-type males did not hatch (Fig. 7D). However, eggs laid by wild-type females that mated with $Pxdsx^{F}$ male mutants and those laid by $Pxdsx^{M}$ female mutants that mated with wild-type males hatched at rates that were not significantly different from those of the control eggs produced by wild-type females mated with wild-type males (Fig. 6C and D).

4. Discussion

The *doublesex* gene, which acts at the end of the sex determination cascade in insects (Salz, 2011; Shukla and Nagaraju, 2010a), plays a crucial role in sexually dimorphic morphology, physiology, and

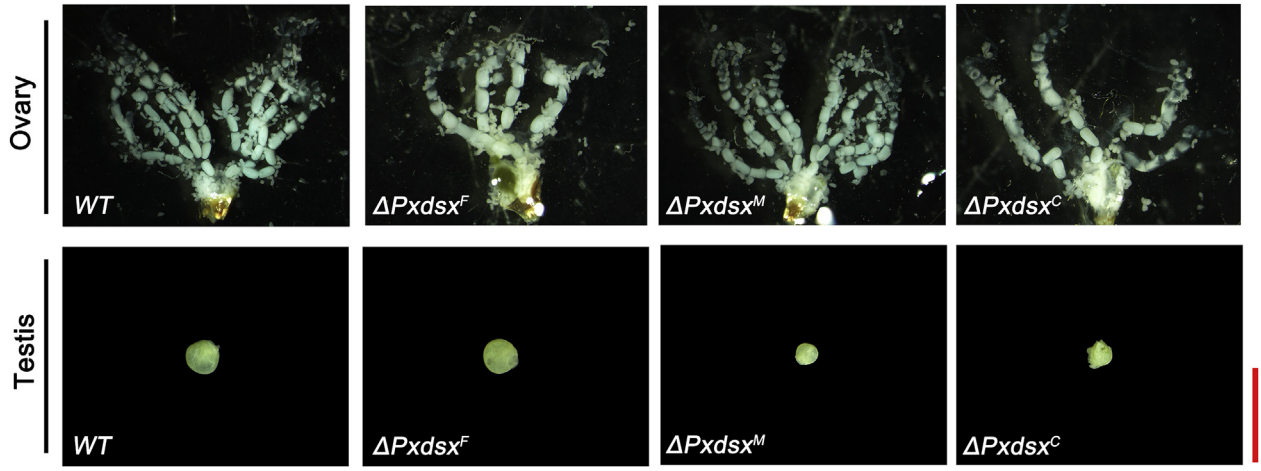

Fig. 5. Morphology of internal genitalia dissected from wild-type and *Pxdsx* mutant adults. The ovaries of wild-type, *Pxdsx*^{F}, *Pxdsx*^{M} and *Pxdsx*^{C} mutant females were dissected on the third day after eclosion. The testes of wild-type, *Pxdsx*^{F}, *Pxdsx*^{M} and *Pxdsx*^{C} mutant males were dissected on the third day after eclosion. Scale bars: 2 mm.

behavior (Kijimoto et al., 2012; Kimura et al., 2008). The *dsx* gene has been characterized in different insect species including dipterans *D. melanogaster* (Burtis and Baker, 1989), *A. gambiae* (Scali et al., 2005), and *Bactrocera dorsalis* (Chen et al., 2008), beetles (*T. castaneum* and *Onthophagus taurus* (Kijimoto et al., 2012), and lepidopterans (*B. mori* (Ohbayashi et al., 2001), *O. furnacalis* (Wang et al., 2014), and *Spodoptera litura* (Du et al., 2018). Here we characterized the gene expression and function of *dsx* in *P. xylostella*, a worldwide insect pest. *Pxdsx* consists of five exons; exons 3 and 4 are female-specific and are not incorporated into mature mRNA in males. The *Pxdsx* sex-specific

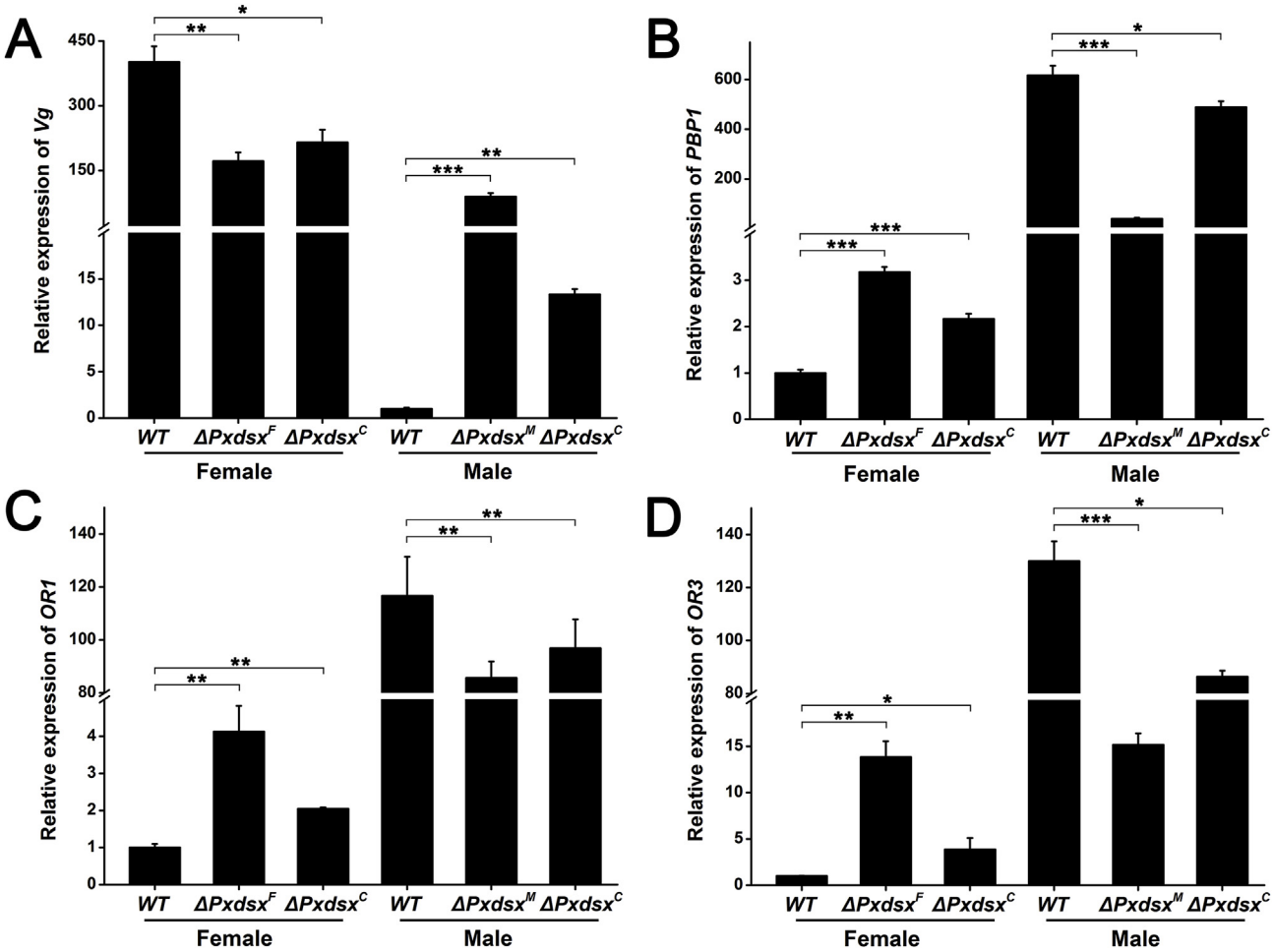

Fig. 6. Relative expressions of the putative *dsx* target genes in the *Pxdsx* mutants. Relative mRNA expression levels of (A, B) *PxVg*, (C, D) *PxPBP1*, (E, F) *PxOR1*, and (G, H) *PxOR3* in the *Pxdsx*^{C} and *Pxdsx*^{F} mutant females and in the *Pxdsx*^{C} and *Pxdsx*^{M} mutant males. Error bars: SEM; $^*P < 0.01$, $^{**}P < 0.01$, $^{***}P < 0.001$, two-tailed Student's *t*-test.

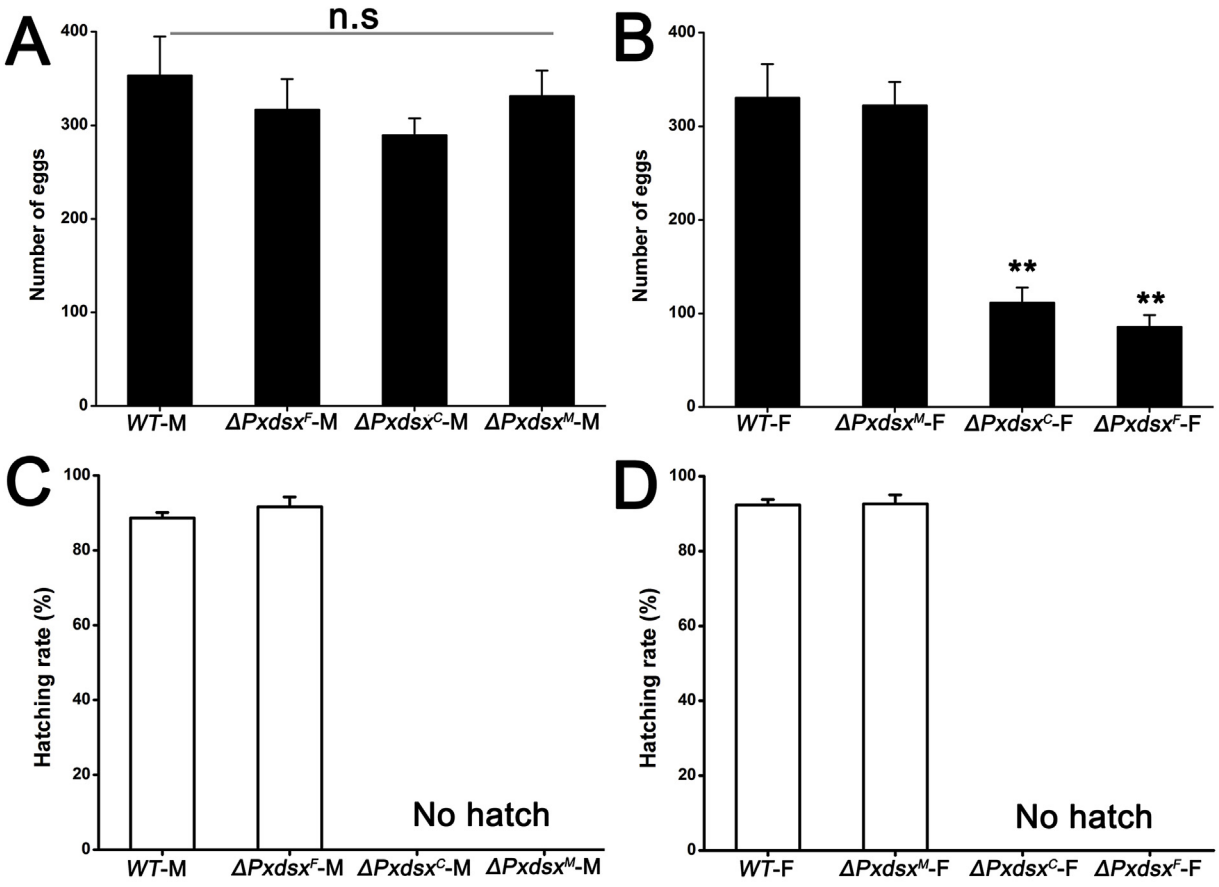

Fig. 7. Fertility of *Pxdsx* mutant insects. (A and B) Number of eggs laid by A) wild-type females mated to wild-type males, $Pxdsx^F$, $Pxdsx^C$, or $Pxdsx^M$ mutant males and B) $Pxdsx^F$, $Pxdsx^C$ or $Pxdsx^M$ mutant females mated to wild-type males. (C and D) Hatching rates of eggs laid by C) wild-type females mated to wild-type males, $Pxdsx^F$, $Pxdsx^C$, or $Pxdsx^M$ mutant males and D) $Pxdsx^F$, $Pxdsx^C$ or $Pxdsx^M$ mutant females mated to wild-type males. $^{**}P < 0.01$, two-tailed Student's *t*-test.

alternative splicing is similar to that in the model lepidopteran species *B. mori* (Ohbayashi et al., 2001; Shukla et al., 2011). In contrast, in *D. melanogaster* there is sex-specific use of alternative 3' splice sites (Burtis and Baker, 1989). Multiple sequence alignment revealed that there is a high level of similarity in DSX proteins of lepidopteran insects. These results preliminarily predicted that the function of *Pxdsx* is consistent with that of *B. mori* (Xu et al., 2014, 2017b).

In *D. melanogaster*, sex-specific transcription factors $Dmdsx^F$ and $Dmdsx^M$ regulate sexual dimorphism traits such as abdominal pigmentation and formation of the sex segments and sex comb. In the coleopteran model species *T. castaneum*, $Tcdsx^F$ and $Tcdsx^M$ affect development of ovary and testis, respectively (Shukla and Palli, 2012). We found that depletion of all *Pxdsx* transcripts resulted in an intersexual phenotype. $Pxdsx^F$ knockout transformed females into pseudomales characterized by deformed harpagones and genital segments. Similarly, $Pxdsx^M$ knockout transformed males into pseudofemales. Interestingly, we found that female specific transcripts were detected in $Pxdsx^M$ male mutants and male-specific transcripts were detected in $Pxdsx^F$ female mutants, indicating that genotype is consistent with phenotype. In *B. mori*, however, the precisely targeted disruption of *Bmdsx* expressed by one sex did not result in expression of transcripts typical of the other (Xu et al., 2017b). The difference between in silkworm and diamondback moth might be due to different levels of mutagenesis. In the silkworm, a high degree of mutagenesis likely occurred due to use of binary transgenic TALENs and CRISPR/Cas9 systems, whereas in diamondback moth mutants in this study there was a relatively low level of mutagenesis. The binary transgenic CRISPR/Cas9 system may be a better choice to elucidate detailed functions of alternatively spliced genes.

DSX targets transcription factors and signaling pathway components to directly and indirectly regulate the expression of sex-biased genes. In *D. melanogaster* and *T. castaneum*, some direct and indirect targets of DSX have been identified (Clough et al., 2014; Shukla and Palli, 2012). The knockout of sex-specific *Pxdsx* transcripts altered the expression of *Vg*, *PBP*, *OR1*, and *OR3*, the sex-biased genes tested. Our results are consistent with previously reported in *B. mori* (Xu et al., 2017b).

In *B. mori*, $Bmdsx^F$ and $Bmdsx^M$ mutant moths exhibit severely deformed external genitalia and are sterile (Xu et al., 2014, 2017b). A recent study revealed that knockdown of the male-specific *dsx* transcript in *Nilaparvata lugens* also affects the development of external genitalia and leads to male sterility (Zhuo et al., 2018). $Pxdsx^F$ and $Pxdsx^M$ mutants also exhibited severe structural defects that resulted in failure of courtship behavior (data not shown). The sterile insect technique (SIT) is an environmentally friendly pest management technique that operates by the mass release of sterile males resulting in a disruption in reproduction (Knipling, 1955; Wimmer, 2005). This technique has been used to manage different insect pests including screwworms and tephritids (Calla et al., 2014; Wyss, 2000). Traditional SIT needs an appropriate irradiation source and the treated pests are genetically unstable, thus limiting global application. In recent years, TALENs and CRISPR/Cas9 have been used to improve SIT in mosquitoes (Alphey, 2016; Gantz et al., 2015; Hammond et al., 2016). Furthermore, a recent report revealed that using CRISPR/Cas9 to target *dsx* gene caused complete population suppression in *A. gambiae* (Kyrou et al., 2018). In this study, three sex-specific target sites were identified in different transcripts of *P. xylostella*, and these three target sequences are species-specific to diamondback moth. Therefore, our work providing for a genetic basis for CRISPR/Cas9 gene drive targeting

doublesex causes complete population suppression. In conclusion, our study provides genetic evidence of the crucial roles of *Pxdsx* in sex determination and suggests potential for *Pxdsx* targeting in genetic control of *P. xylostella*.

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Appendix A. Supplementary data

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