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Mutation of the seminal protease gene, *serine protease 2*, results in male sterility in diverse lepidopterans

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ABSTRACT

Sterile insect technology (SIT) is an environmentally friendly method for pest control. As part of our efforts to develop a strategy that results in engineered male-sterile strains with minimum effects on viability and mating competition, we used CRISPR/Cas9 technology to disrupt *Ser2*, which encodes a seminal fluid protein, in the model lepidopteran insect, *Bombyx mori*, and an important agricultural pest, *Plutella xylostella*. Disruption of *Ser2* resulted in dominant heritable male sterility. Wild-type females mated with *Ser2*-deficient males laid eggs normally, but the eggs did not hatch. We detected no differences in other reproductive behaviors in the mutant males. These results support the conclusion that *Ser2* gene is necessary for male reproductive success in diverse lepidopterans. Targeting *Ser2* gene has the potential to form the basis for a new strategy for pest control.

1. Introduction

Pest species endanger human health and cause harm to economically important crops, therefore insect population control is vital. Traditional pest control relies on the use of toxic chemicals, but these methods have significant negative impacts on the environment and other species including humans. Sterile insect technology (SIT) is an environmentally safe approach to pest control because it targets a single and specific species.

In the past six decades, three major types of SIT have been developed: radiation-based sterile technology (rSIT), microbe-mediated sterile technology (mSIT), and genetic-based inheritable sterile technology (gSIT). The earliest forms of SIT relied on radiation to create dominant lethal mutations that resulted in sterile males (Bushland et al., 1955). The assumption was made that irradiated males released into the environment mate with wild-type females, and subsequent offspring fail to develop. However, rSIT has three major shortcomings: the males are often not competent to mate, mass rearing must be conducted, and release must be repeated from generation to generation (Baumhover et al., 1955; Black et al., 2011; Smith, 1963). Most microbe-mediated infertility techniques, such as males infected with cytoplasmic incompatibility-inducing *Wolbachia*, modify their sperm so that it can no longer successfully fertilize uninfected egg, while infected eggs can be fertilized by sperm from any male (Panagiotis and Bourtzis, 2010; Sinkins, 2004; Zhang et al., 2015a, 2015b). gSIT has been demonstrated by modeling the release of *Drosophila melanogaster* carrying a dominant lethal mutation, which introduces a lethal gene into insect genome and expresses it conditionally to achieve population suppression (Thomas et al., 2000). Another gSIT approach implemented in *D. melanogaster* involves the ectopic expression of a hyperactive pro-apoptotic gene that causes highly efficient female-specific lethality and male sterility when driven by the tetracycline-controlled transactivator (Horn and Wimmer, 2003). A tetracycline repressible self-limiting mutant strain was developed that causes over 95% of the offspring of homozygous males to die before becoming adults (Alphey, 2014; Harris et al., 2012; Phuc et al., 2007). Furthermore, high-level expression of

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the tetracycline-repressible transactivator in females using transgenic technologies with sex-specific alternative splicing results in female-specific lethality (Tan et al., 2013). These gSIT technologies overcome some disadvantages of radiation, such as weak individual competitiveness and poor survival ability. This type of transgenic technology has been implemented in a few insect species. CRISPR/Cas9 transgenic technology can be used to edit the genomes of many insect pests (Basu et al., 2015; Bi et al., 2016; Chen et al., 2019; Collins, 2018; Hammond et al., 2016; ; Kyrou et al., 2018; Smagghe et al., 2019; Wilkins et al., 2018) and has the potential to enable development of environmentally friendly pest control methods.

The ideal SIT is to achieve sterility without affecting the viability and competitiveness of individuals and then to achieve pest population control. Therefore, we reasoned that we should develop females carrying a factor that causes males to produce sterile offspring. When mutant females are released to the field, they mate with wild-type males as normal pheromone attraction is not impaired. In the succeeding generation, mutant females can copulate with wild-type males and produce offspring, and mutant males copulate with wild-type females, but no offspring are produced. In this way, females carrying the mutation that results in male sterility spread, and the overall population decreases due to male sterility.

We therefore sought to identify a factor that did not affect basic growth and development of the insect but did affect male fertility. We screened many genes from the sexual determination pathway to sperm formation as well as some genes involved in copulation. The genes encoding male seminal fluid proteins (SFPs) are essential for reproduction (Avila et al., 2011; Findlay et al., 2008; Nagaoka et al., 2012; Poiani, 2006; Sirot et al., 2008) but could be deleted with little effect on insect growth. SFPs promote fertility in diverse ways including facilitating the storage of sperm and enhancing female spawning (Singh et al., 2018; Sloan et al., 2018; Wigby et al., 2009). In *Drosophila* mated females, the levels of both ovulin and sex peptide decline with time after mating, but Acp36DE is essential for the initial storage of sperm (Avila and Wolfner, 2017; Liu and Kubli, 2003; Rubinstein and Wolfner, 2013; Wigby et al., 2009). SPF genes are associated with increased sperm viability in the cricket, *Teleogryllus oceanicus* (Neubaum and Wolfner, 1999; Simmons and Lovegrove, 2017; Sirot et al., 2009; Sloan et al., 2018; Wigby et al., 2009).

SFPs of the serine protease family are detected in male gonads, and these proteins affect male reproductive success in *Drosophila melanogaster* (LaFlamme et al., 2012). Inhibition of expression of a predicted serine protease, seminase, using RNA interference (RNAi) resulted in a decrease of egg production in females following mating with ablated males, and mutant females were unable to store other essential SFPs. Seminase initiates a protease cascade signaling pathway by hydrolyzing accessory gland proteins (Acps), thus participating in an early regulatory stage of the post-mating process (Laflamme and Wolfner, 2013). Serine proteases also are energy resources for sperm motility, and their absence affects the fertilization success (Nagaoka et al., 2012). *Serine protease 2* (*Ser2*) is the *seminase* orthologue in the silkworm, *Bombyx mori*. This gene is expressed specifically in male reproductive organs and is necessary for the reproductive success of males (Nagaoka et al., 2012).

Here we show that mutagenesis of *BmSer2* using CRISPR/Cas9 resulted in male sterility, but females with a null mutation in this gene were normal. In addition, we obtained the same mutant phenotype by knocking out the *Ser2* orthologue in the distantly related lepidopteran pest. *Plutella xylostella*. Therefore, our strategy for producing mutant females can be used for future applications of gSIT for the selective biological control of lepidopteran pests and possibly other insect pests from other orders with minimal harm to the environment.

2. Materials and methods

2.1. Insect strain and rearing

The multivoltine, nondiapausing silkworm strain, Nistari, was used for germline transformation and subsequent experiments. Larvae were reared on fresh mulberry leaves at 25 °C under standard conditions (Tan et al., 2005).

A laboratory strain of diamondback moth, *P. xylostella*, was obtained from the Institute of Zoology, Chinese Academy of Science (Beijing). Larvae were reared in an incubator at 26 ± 1 °C, 65 ± 5% relative humidity, a photoperiod of 16:8 h (light:dark) and fed with an artificial diet as described previously (Huang et al., 2017). Adult moths were fed with a 10% honey solution.

2.2. Phylogenetic analysis and sequence comparisons

The evolutionary history of the Ser2 was inferred using the neighbor-joining method (Saitou and Nei, 1987). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) was determined (Felsenstein, 1985). The evolutionary distances were analyzed using the Poisson correction method (Zuckerkandl and Pauling, 1965). Evolutionary analyses were conducted in MEGA7 (Kumar et al., 2016). The alignment of the protein sequences from *B. mori, P. xylostella*, *Agrius convolvuli*, *Samia ricini*, *Pieris rapae*, *Helicoverpa armigera*, and *Papilio machaon* was created with the ClustalX2 software and GENEDOC program.

2.3. Qualitative and quantitative real-time PCR

Total RNA was extracted from insect male accessory glands or other tissues using the TRIzol® reagent (Invitrogen). The extract was treated subsequently with DNase I (Invitrogen) to remove genomic DNA. cDNA was synthesized from 1 μg of total RNA using the RevertAid™ First Strand cDNA Synthesis Kit (Fermentas). qRT-PCR analysis for *Ser2* was performed using a SYBR Green Realtime PCR Master Mix (Thermo Fisher Scientific) on an Eppendorf Real-time PCR System. The PCR conditions were as follows: initial incubation at 95 °C for 5 min, 35 cycles at 95 °C for 15 s and 60 °C for 1 min. *Bombyx mori Bmrp49* and *P. xylostella Pxactin* were used as internal controls (Tan et al., 2013). A relative quantitative method ($^{\triangle\triangle}$Ct) was used to evaluate quantitative variation. All qRT-PCR samples were determined in triplicate. The gene-specific primers used for qRT-PCR are listed in Table S2.

2.4. Immunoblot analysis

For protein analyses, samples were homogenized, dissolved in phosphate buffered saline (PBS: 135 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, and 8 mM K_2HPO_4 in distilled water, pH 7.2) and quantified using a BCA kit (Thermo Fisher Scientific). Extracted proteins were separated by 10% SDS/PAGE and transferred to a nitrocellulose membrane (GE Healthcare). The polyclonal rabbit anti-BmSer2 primary antibody was used for BmSer2 detection (1:1000 dilution; Youke). β-Actin was used as the control and detected using the rabbit anti-β-actin primary antibody (1:5000 dilution; Vazyme Biotech). Horseradish peroxidase-conjugated anti-rabbit IgG (1:5000 dilution; Beyotime) was used as a secondary antibody. Signal detection was achieved using the ECL Plus Western Blotting Detection Kit (GE Healthcare). The ImageJ software (US National Institutes of Health) was used to quantify the protein.

2.5. Plasmid construction and silkworm germline transformation

BmSer2 is located on chromosome 6 and comprises 8 exons and 7 introns (Figure S1A). The open reading frames (ORFs) of *BmSer2* were screened to identify single-guide RNA (sgRNA) target sites using the

GGN19GG rule (Hwang et al., 2013). Two 23-base pair (bp) sgRNA target sites in exon 4 and exon 5 (TS1 and TS2, respectively) were identified; the fragment spanning the two sites is 2143 bp in length (Figure S1A). We chose as the activator line the strain *pBac[IE1-EGFP-Nos-Cas9]* (*Nos-Cas9*), in which Cas9 is driven by the *nanos* promoter and also expresses the enhanced green fluorescent protein (EGFP) marker (Figure S1B) (Xu et al., 2018). The plasmid pBac*[IE1-DsRed2-U6-sgRNA]* (*U6-BmSer2 sgRNA*) was constructed to express the sgRNA under control of the silkworm *U6* promoter; this plasmid also carries the DsRed fluorescence marker gene under control of the *IE1* promoter. The primers used for plasmid construction are listed in Table S2.

Silkworm germline transformation was performed by microinjection of a mixed solution of transformation and helper plasmids into pre-blastoderm Nistari strain embryos. Embryos were incubated in a humidified chamber at 24.5 °C until hatching. Larvae were reared to moths and sib-mated or backcrossed with wild-type (WT) moths. G1 progeny were scored for the presence of the marker gene during the embryonic stage under a fluorescence microscope (Nikon AZ100). The *Nos-Cas9* and *U6-sgRNAs* lines were previously shown by our laboratory to have no effect on growth and development of silkworm, and the *Nos-Cas9* line has been used in several studies (Chen et al., 2017; Liu et al., 2017; Xu et al., 2017, 2018). We obtained three *U6-sgRNAs* lines of *BmSer2* in this experiment. These three lines were crossed with *Nos-Cas9* line, and all were used in experiments. There were four lines in the heterozygous F1 progeny; individuals with double-fluorescence were used in subsequent experiments.

2.6. Transcription of sgRNA and diamondback moth embryonic microinjection

The putative *PxSer2* gene was identified from the genome posted in DBM-DB (http://iae.fafu.edu.cn/DBM/index.php). The *PxSer2* open-reading frame was PCR amplified with KOD FX (TOYOBO). The amplified products were confirmed by sequencing after sub-cloning into the vector, PMD-18T (Takara). The sequencing results were compared with *PxSer2* genomic sequence to identify single-nucleotide polymorphisms. A 23-bp sgRNA target site was selected in exon 9 of *PxSer2* that met the 5′-GGN19GG-3′ criterion (Figure S2A). The sgRNAs were synthesized with a MAXIscript® T7 Kit (Ambion). The *Cas9* mRNA was synthesized using an mMESSAGE T7 Kit (Ambion) as described previously (Hwang et al., 2013).

Plutella xylostella embryos were collected within 1 h of oviposition and injected as described previously (Huang et al., 2016). The *Cas9* mRNA and sgRNA were mixed at final concentrations of 300 ng/μL and 150 ng/μL, respectively, and microinjected into eggs. Eggs were incubated in at 25 ± 1 °C and 65 ± 5% relative humidity until hatching.

2.7. Mutagenesis analysis

To confirm the presence of mutations in hybrid double-fluorescence silkworm lines, genomic DNA was purified from first-instar larvae using a standard extraction buffer (1:1:2:2.5 ratio of 10% SDS to 5 M NaCl to 100 mM EDTA to 500 mM Tris–HCl, pH 8) followed by incubation with proteinase K, then purified further by phenol:chloroform extraction and isopropanol precipitation, followed by RNaseA treatment. *P. xylostella* genomic DNA was extracted from injected eggs (n = 20) at 48 h after microinjection. The genomic PCR conditions were as follows: 94 °C for 2 min, 35 cycles at 94 °C for 15 s, 55 °C for 30 s, and 72 °C for 1 min, followed by a final extension period at 72 °C for 10 min. The PCR products were cloned into pJET1.2 vectors (Fermentas) and sequenced directly. The primers used to detect mutations at target sites are listed in Table S2.

2.8. Silkworm female attraction and male competitiveness assay

The assay was performed in a plastic container ($30 \times 18 \times 4.5\ cm^3$). One newly emerged WT male moth was placed in the center of the container. WT and *ΔBmSer2* female moths were placed on either side, leaving a distance of 10 cm to the center. Male moths that reached one side and mated with a female were considered responsive, and the number of responsive moths was recorded. The response index was calculated as the number of responsive moths divided by the total number of test moths multiplied by 100. The male competitive test method was the same as the female attraction assay except that a newly emerged WT female moth was placed in the center of the container and WT and *ΔBmSer2* male moths were placed on either side, and the numbers of responsive moths were recorded.

2.9. Diamondback moth mating behavior and hatchability assay

To further characterize the sterility of the *Ser2* knockout and verify the silkworm results, adult mating behavior and offspring hatchability were compared for diamondback adults from G0-injected moths. In brief, newly emerged moths were released into a mesh cage ($25 \times 25 \times 25\ cm^3$). In order to observe mating behavior and count offspring hatchability, each pair of mating males and females were placed alone in a tube. We recorded the mating duration of each pair of moths and subjected them to additional behavioral investigation using a stereo microscope (Nikon SW-2B/22). A total of 65 pairs of moths successfully mated and laid eggs.

2.10. Phalloidin and Hoechst staining of silkworm sperm

Testes from unmated male adults were dissected on an anatomical plate and placed in PBS. Each testis was incubated in dye fixing solution (Beyotime) for 10 min, then washed three times with PBS for 5 min per wash. Phalloidin (dilution ratio, 1:100) (Yeasen) was used to stain actin in a 30-min incubation followed by three washes with PBS for 5 min each. Subsequently, nuclei were stained with Hoechst dye (dilution ratio, 1:100) (Beyotime) for 15 min, followed by washing 3 times with PBS for 5 min each. Tissues were transferred in droplets to a slide and covered with a cover glass. We used the same method to stain the sperm in mated bursa copulatrix (BC). Images were captured using a fluorescence microscope (Olympus BX51).

2.11. Toluidine blue O (TBO) staining of silkworm eggs

Postpartum eggs at 0, 5, and 10 h after deposition were washed three times with 95% ethanol and then placed in 70% ethanol. The egg shells were removed with forceps, and the eggs stained with TBO dye (0.5% v/v in PBS) (Leagene) for 10 min. The dye solution was removed, and the eggs were decolorized by sequential incubation in 10%, 50%, 70%, 80%, 90%, and 95% ethanol for 5 min each followed by incubations in 99% and 100% ethanol for 10 min each solution. The eggs were soaked in transparency buffer (3:1 ratio of absolute ethanol to 98% methyl salicylate) for 10 min, and then transferred to methyl salicylate, followed by transfer to a slide. The tissue was then fixed with neutral balsam (Leagene), covered with a cover glass, and photographed under a microscope (Nikon AZ100, Japan).

2.12. RNA-seq analysis

The glandula prostatica was dissected from virgin WT and *ΔBmSer2* adults, and the samples placed in freeze-storage tubes in dry ice. Transcriptome sequencing analysis was performed by Shanghai OE Biotech.

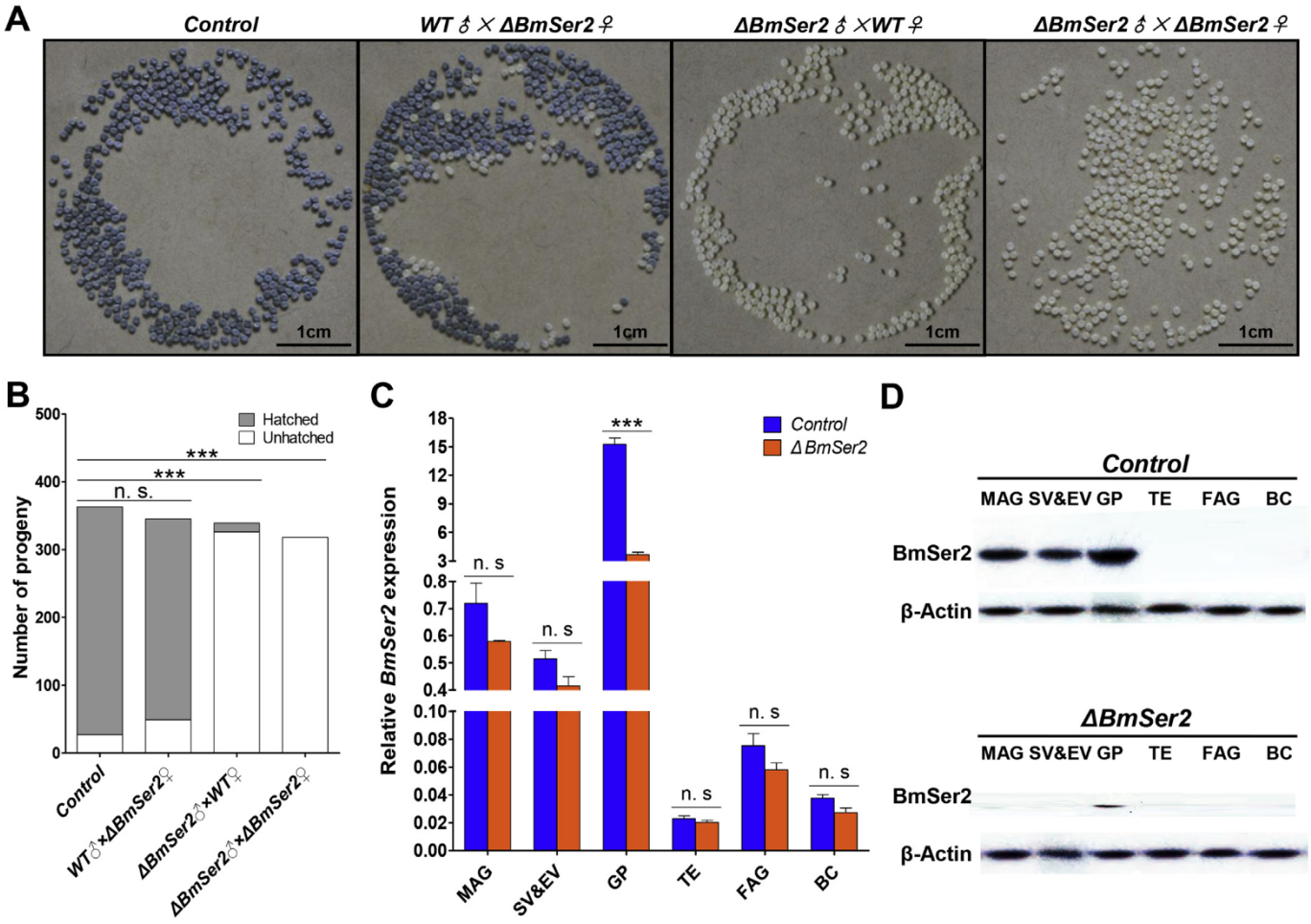

Fig. 1. Loss of *BmSer2* function results in male sterility. (A) Male and female *ΔBmSer2* adults were mated with each other or with WT moths; WT female and WT males were mated as controls. Eggs were photographed 8 days after spawning, 2 days before hatching. Developing eggs are dark and undeveloped embryos are light. (B) The number of eggs resulting from the crosses performed as described in panel A. (C) Relative *BmSer2* mRNA expression in six reproductive tissues of virgin adults. *ΔBmSer2* mutant is in orange and WT is in blue. Abbreviations: male accessory gland (MAG), seminal vesicle and ejaculatory vesicle (SV&EV), glandula prostatica (GP), testis (TE), female accessory gland (FAG), and bursa copulatrix (BC). The mRNA expression was normalized to *Bmrp49*. The data shown are means ± S.E.M. Asterisks indicate significant differences with a two-tailed *t*-test: *P < 0.05; **P < 0.01; ***P < 0.001; n. s. P > 0.05. (D) Expression of BmSer2 in six reproductive tissues of virgin adults. Protein expression was normalized to levels of β-actin. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

2.13. Germline mutation transmission assay

Silkworm lines were produced by hybridizing the *Nos-Cas9* line with the *U6-sgRNA* line with double-fluorescence, the *Nos-Cas9* line with green fluorescence, the *U6-sgRNA* line with red fluorescence, and a non-mutant line without fluorescence. The inheritance of double-fluorescence was confirmed in each subsequent generation. Random hybridization of these four lines produced only these four types of individuals. To investigate whether the mutation induced by CRISPR/Cas9 was transmitted into the next generation in the diamondback moth, the offspring from seven female mutant G0 moths were intercrossed. After oviposition, we examined the hatchability of G2 eggs and sequenced the *PxSer2* gene in male G1 moths.

2.14. Statistical analysis

A two-tailed Student's t-test was used to analyze differences between WT and mutant individuals. Three independent replicates were used for each treatment and error bars are means ± SEM.

3. Results

3.1. Phylogenetic identification of BmSer2 and PxSer2

Ser2 gene sequences from 16 different animal species were analyzed to evaluate evolutionary conservation. The species included examples from Lepidoptera (*B. mori*, *P. xylostella*, *Agrius convolvuli*, *Samia ricini*, *Pieris rapae*, *Helicoverpa armigera*, and *Papilio machaon*), Hymenoptera (*Trachymyrmex cornetzi* and *Athalia rosae*), Orthoptera (*Gryllus bimaculatus*), Hemiptera (*Halyomorpha halys* and *Cimex lectularius*), Diptera (*D. melanogaster*), Rodentia (*Mus musculus*), Artiodactyla (*Sus scrofa*), and Primate (*Homo sapiens*). Phylogenetic analysis of the protein sequences showed conservation consistent with previous placements of the various species (Figure S3A). Lepidopteran species grouped into a single branch, and the branches containing *B. mori* and *P. xylostella* were relatively closely related.

In addition, we aligned the Ser2 amino acid sequences of the seven Lepidoptera species and found that they were highly conserved. The amino acid sequence of BmSer2 protein had 63% identity with PxSer2, 67% with PmSer2, 70% with PrSer2, 66% with AcSer2, 70% with HaSer2, and 65% with SrSer2 (Figure S3B). These data support the conclusion that *Ser2* genes could be a general target for SIT pest control.

3.2. Targeted mutagenesis using the CRISPR/Cas9 system

Large deletions of 2.1–2.6 kilobase pairs in length were observed in five of the first-generation offspring of the *ΔBmSer2* individuals from crosses of two microinjected transgenic silkworm lines carrying double-fluorescence markers (Figure S1). In parallel experiments, following sequencing of randomly selected samples of amplified DNA from the diamondback moth, mutations were shown to be both insertions and deletions (indels) and nucleotide differences of between 4 and 30 bases as expected by the design of the knock-out protocol (Figure S2). The numbers of injected eggs and resulting larvae, pupae, and mating moths are listed in Table S1.

3.3. Ser2 mutations induce male sterility

We counted the number of eggs produced in broods produced by male and female *ΔBmSer2* adults, one set sib-mated and the other with WT, and compared them to WT males mated with WT females (control). The number of eggs laid by the four groups was approximately the same: control, 363; WT males mated with mutant females, 345; male mutants mated with WT females, 339; mutant males mated with mutant females, 318 (n = 30 per group). Furthermore, almost all control eggs (~93%, 336/363) hatched. Most of the eggs (~86%, 296/345) of female mutants mated with WT also hatched, but few of the eggs (~2%, 6/318) of *ΔBmSer2* females mated with male mutants hatched (Fig. 1A and Table S3). The number of progeny produced by female mutants mated with WT was not significantly different ($P = 0.09399$) from the control, whereas the number of progeny produced by male mutants mated with WT or *ΔBmSer2* females was significantly less ($P = 0.00004$) than the control (Fig. 1B and Table S3). Based on these phenotypes, we tested the expression of *BmSer2* mRNA in six major reproductive tissues of virgin WT and *ΔBmSer2* males including male accessory gland, seminal vesicle and ejaculatory vesicle, glandula prostatica, testis, female accessory gland, and bursa copulatrix. *BmSer2* was significantly down-regulated in the glandula prostatica of *ΔBmSer2* males compared with WT ($P = 0.00028$, n = 3) (Fig. 1C) as was expression at the protein level (Fig. 1D) ($P = 0.00019$, n = 3).

To investigate further the role of *PxSer2* in the diamondback moth, we examined the hatchability of eggs produced by CRISPR/Cas9-treated females mated with CRISPR/Cas9-treated males. We found no significant difference in the numbers of eggs produced by 65 single pairs of G0 moths that were successfully mated and laid eggs compared to 61 control pairs ($P = 0.55911$). However, we observed that 13 single pairs of mutant moths laid eggs that did not hatch, whereas the other 52 pairs of moths laid eggs that all hatched. In contrast, all 61 control pairs laid eggs that hatched (Fig. 2A and Table S1). Compared to controls, there was a significant difference ($P = 0.00001$) in the hatchability rate for eggs produced by WT females mated with CRISPR/Cas9-treated males, but there was no significant difference ($P = 0.10489$) in matings of WT males with CRISPR/Cas9-treated females (Fig. 2B).

Genomic DNA was extracted from all treatment-group moths, and PCR products from the sgRNA site region were sequenced. This showed that all male and two female CRISPR/Cas9-treated G0 moths in the 13 pairs that produced unhatched offspring had mutations in the *PxSer2* gene. Four males and seven female moths were mutant in the 52 pairs of moths that laid eggs that hatched completely (Table S1). We then tested the relative mRNA expression of *PxSer2* in WT and CRISPR/Cas9-treated moths. We found that *PxSer2* was significantly down-regulated ($P = 0.00093$ and $P = 0.00017$, n = 3) in CRISPR/Cas9-treated females and males compared with WT moths (Fig. 2C). These preliminary results support the conclusion that *Ser2* has a crucial role in male diamondback moth fertility. In summary, the diamondback moth results are consistent with results from experiments in silkworm results and indicate that mutation of *Ser2* results in male sterility in lepidopteran species.

3.4. Mating behavior of Ser2 mutants

We next evaluated the mating behaviors of *Ser2* mutant male and female moths. Behavior was considered normal if females and males could attract each other and copulate for at least 0.5 h (Liu et al., 2017). The response index was determined as the percentage of successful matings relative to the total trials in a group. There was no significant difference in response indices of the *B. mori* female mutant and the WT control (control, 51.85%; female mutant, 48.15%; $P = 0.28767$; n = 30 per group) (Fig. 3A). The *B. mori* mutant males also had a response index that was the same as that for the WT controls within experimental error (control, 50.76%; male mutant, 49.24%; $P = 0.80128$; n = 30 per group) (Fig. 3B).

P. xylostella mutant adults also mated normally (Fig. 4A). Mating durations of the four different groups of moths were not significantly different (n = 8 for each group). Control moths mated for 67 min; WT males mated with female mutants for 65 min ($P = 0.90034$); mutant males mated with WT females, 69 min ($P = 0.76186$); and mutant males mated with mutant females, 60 min ($P = 0.45079$) (Fig. 4B). Thus, the *B. mori* and *P. xylostella* moths with mutations in *Ser2* had normal mating behavior.

3.5. Mutant sperm morphology is normal but sperm do not enter eggs

Phalloidin and Hoechst staining of *BmSer2*-mutant and WT virgin testis showed no visible differences in morphology of eupyrene sperm or apyrene sperm bundles (Figure S4A-B). Furthermore, staining of sperm in the bursa copulatrix isolated after 5 h of mating with the same methods also showed no obvious differences between mutant and WT sperm (Figure S4C). These results support the conclusion that the formation and morphology of sperm lacking Ser2 are normal. In TBO-stained samples obtained after mating of WT controls, sperm and egg nuclei were observed immediately after egg deposition, and cleavage nuclei were visible at 5 and 10 h post-mating (Fig. 5). In contrast, when WT females were mated with mutant males, only nuclei from eggs were observed, and no cleavage nuclei were observed (Fig. 5). This indicates that the *BmSer2*-mutant sperm failed to enter the egg and fertilized nuclei did not form, and this could be the cause of male sterility.

3.6. CRISPR/Cas9-induced mutations are dominant and heritable

Binary transgenic CRISPR/Cas9 technology was used to construct silkworm germline transformants, and the mutations of the *BmSer2* mutant should thus be transmitted to progeny (Fig. 6). Four lines were produced by hybridization of the *Nos-Cas9* line with the *U6-sgRNAs* line, and random hybridization of the four lines also produced only these four types of individuals. Hatch rates of the F2 generation were consistent with expected inheritance of the mutation, and the male mutant remained sterile (Figure S4D). To analyze whether the CRISPR/Cas9 mediated mutations were transmitted to the progeny in *P. xylostella*, G1 moths from seven G0 female mutant moths were selected to produce G2. The 50 pairs of G1 moths successfully mated, and all females laid eggs (Table S1). Eggs laid by three pairs of moths did not hatch, whereas the other groups hatched normally (Figure S4E). DNA was isolated from the moths from the pairs that laid unhatched eggs, and sequencing demonstrated that the three male moths were mutant (Figure S5). These results demonstrate that male sterility induced by disruption of *Ser2* with CRISPR/Cas9 was transmitted into the next generation.

3.7. RNA-seq analysis in BmSer2 mutant adults

The potential molecular mechanism of male sterility was explored by RNA-seq analysis of *ΔBmSer2* male adults. Among 1511 differentially expressed genes (DEGs) identified in the glandula prostatica of *ΔBmSer2* adults, 628 and 883 genes were up- and down-regulated, respectively

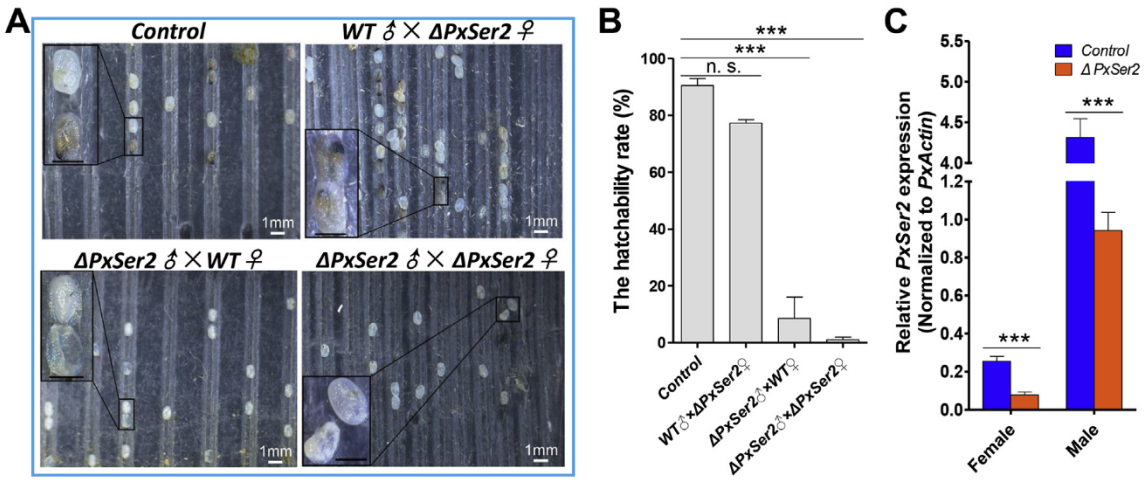

Fig. 2. Loss of *PxSer2* function results in male sterility. (A) Male and female G0 CRISPR/Cas9-treated *P. xylostella* adults, one set sib-mated and the other mated with WT. Male and female WT moths were sib-mated as controls. The hatchability is plotted. (B) The hatchability rate of G0 eggs laid by G0 CRISPR/Cas9-treated males mated with WT females and by G0 CRISPR/Cas9-treated females mated with WT females compared to WT male and WT female moths as controls. (C) Relative expression of *PxSer2* mRNA in virgin adults. *ΔPxSer2*, orange; WT, blue. The data shown are means ± S.E.M. Asterisks indicate significant differences with a two-tailed *t*-test: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; n. s. $P > 0.05$. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

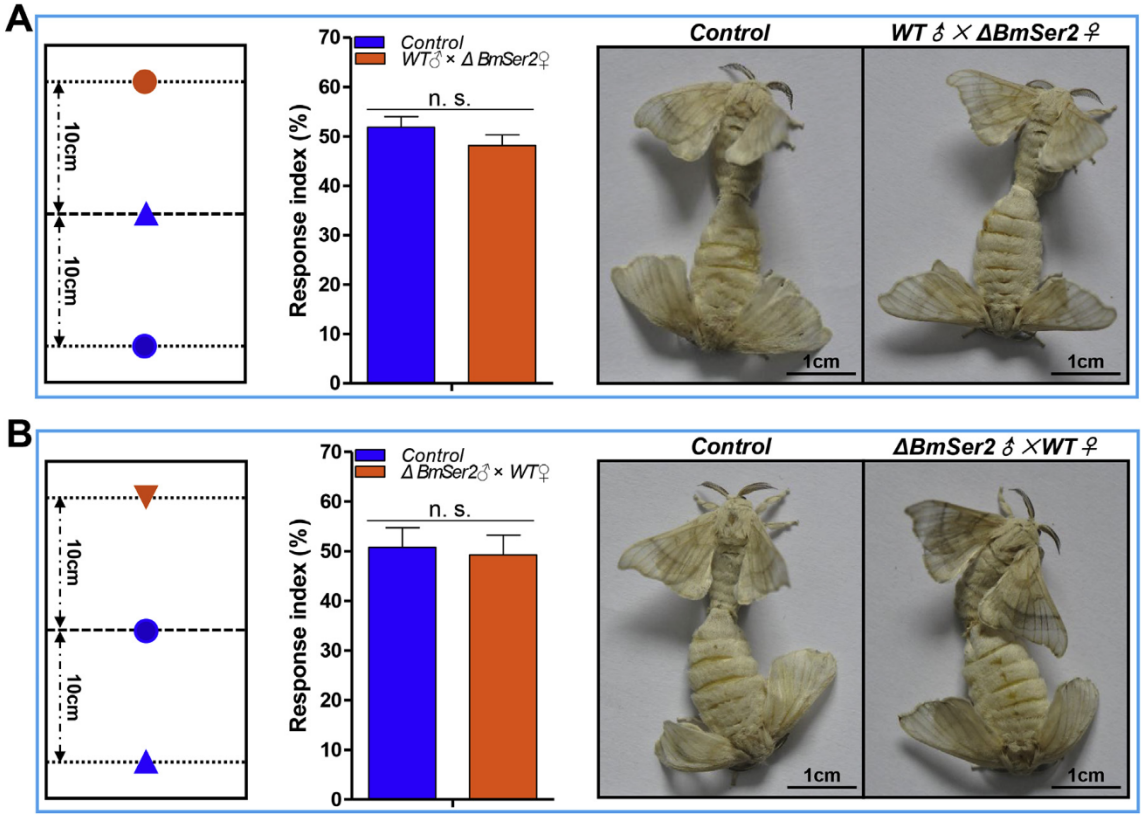

Fig. 3. Loss of Ser2 does not alter adult mating behavior in *B. mori*. (A) The female attraction assay. (B) The male competitiveness assay. *ΔBmSer2* is in orange and WT is in blue. Female, circle; male, triangle. The data shown are means ± S.E.M. (n = 60). Asterisks indicate significant differences with a two-tailed *t*-test: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; n. s. $P > 0.05$. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

(Figure S6A). Gene ontology analysis revealed that the DEGs were related mostly to the molecular functions of binding and catalytic activity (Figure S6B). Analysis using the Kyoto Encyclopedia of Genes and Genomes showed that the DEGs were mainly involved in signal transduction associated with environmental information processing (Figure S6C). Such biological processes are essential for sperm function and presumably were disrupted by the deletion of *BmSer2*, resulting in the failure of the sperm to enter the egg normally.

4. Discussion

Through target gene screening, gene expression analysis, and genome editing, we demonstrated the importance of *Ser2* in male reproductive success in two lepidopteran species, the silkworm and

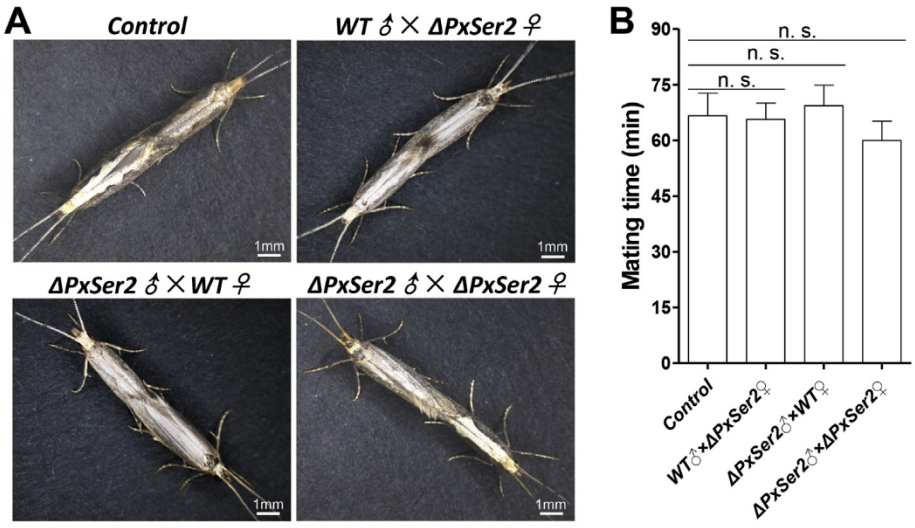

Fig. 4. Loss of Ser2 does not alter adult mating behavior in *P. xylostella*. (A) Analysis of mating behavior in adults. (B) Analysis of mating duration of *P. xylostella* WT pairs (control), WT males mated with CRISPR/Cas9-treated females; CRISPR/Cas9-treated males mated with WT females, and mutant males mated with mutant females (n = 8 pairs for each genotype). The data shown are means ± S.E.M. Asterisks indicate significant differences with a two-tailed *t*-test: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; n. s. $P > 0.05$.

diamondback moth, using CRISPR/Cas9 technology. Ser2 is a member of the SFP family, which includes a number of factors essential for male reproductive processes (Avila et al., 2011; Laflamme and Wolfner, 2013; Nagaoka et al., 2012). Insects such as *Drosophila* and *Teleogryllus oceanicus* require SFPs for reproductive success (Laflamme and Wolfner, 2013; Simmons and Lovegrove, 2017; Sirot et al., 2009; Sloan et al., 2018; Wigby et al., 2009). We successfully obtained *Ser2* mutants using transgenic knockout in silkworm. Because this technology is not available in the diamondback moth, we directly injected the sgRNA targeting *PxSer2* and *Cas9* mRNA into diamondback moth embryos, and successfully obtained mutants demonstrating that this method is feasible in pests.

The male silkworm and diamondback moths were sterile. However, females carrying the ablated genes were fertile. Furthermore, there were no significant differences in mating behaviors of either males or females deficient in Ser2. Moreover, the CRISPR/Cas9-induced mutations were dominant and heritable. These features bode well for the development of SIT technologies that target this gene. Released mutant females should be able to mate with wild-type males and reproduce effectively. Mutant males of the next generation will be sterile, although able to copulate. Mutant females will copulate with wild-type males, eventually suppressing the population. Thus, a heritable *Ser2*-mutation-induced male sterility gSIT system should, in theory, enable control of moth pests.

We demonstrated the importance of *Ser2* in male reproductive success in the silkworm and diamondback moth. Results from analysis of the mutant silkworms suggest that sterility results from the inability of mutant sperm to successfully enter the egg. Although formation and morphology of the sperm from mutant males were normal, RNA-seq analysis showed significant differences from wild-type *B. mori* males in gene expression profiles related to signal transduction in the glandula prostatica. These observations are consistent with studies that previously reported that sperm motility factors are essential for fertilization, and the most critical factors are binding and signal transduction proteins (Degner and Harrington, 2016; Findlay et al., 2008; Lu, 2013; Sirot et al., 2014; Soulavie et al., 2014; Swanson et al., 2001). Furthermore, previous studies reported that *Ser2* is vital to sperm motility and essential for activity of silkworm spermatozoa *in vitro* (Nagaoka et al., 2012).

Our phylogenetic sequence analysis indicated that *Ser2* orthologues are present in all insect studied and in other distantly related species. If functional conservation is also demonstrated for *Ser2* genes among

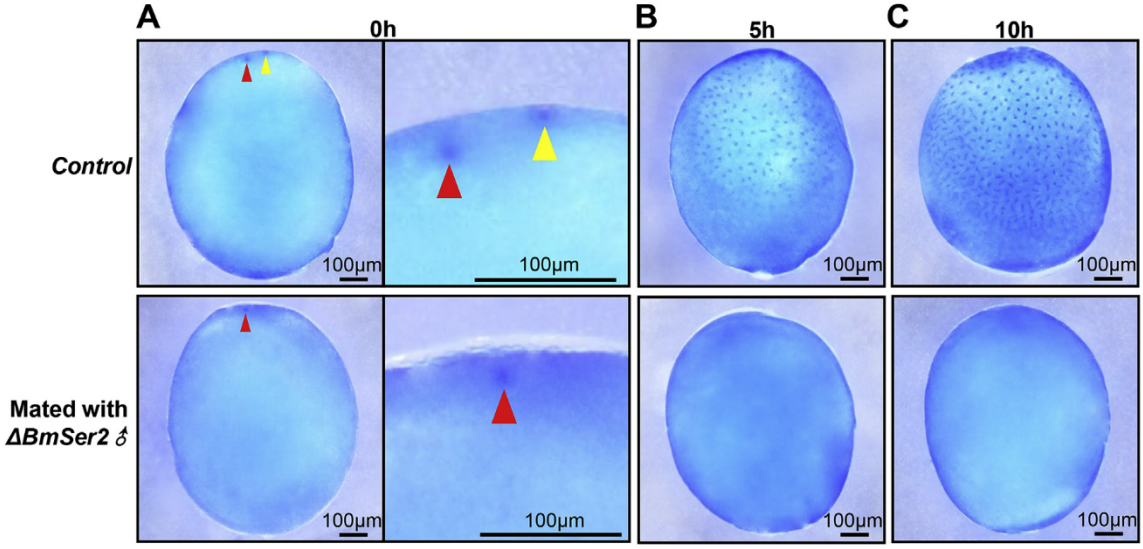

Fig. 5. Sperm deficient in Ser2 do not enter eggs. Images of eggs from *B. mori* females mated with mutant or WT at (A) 0, (B) 5, and (C) 10 h post deposition. The red arrowhead indicates the nucleus of the egg; yellow arrowhead indicates the nucleus of the sperm. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

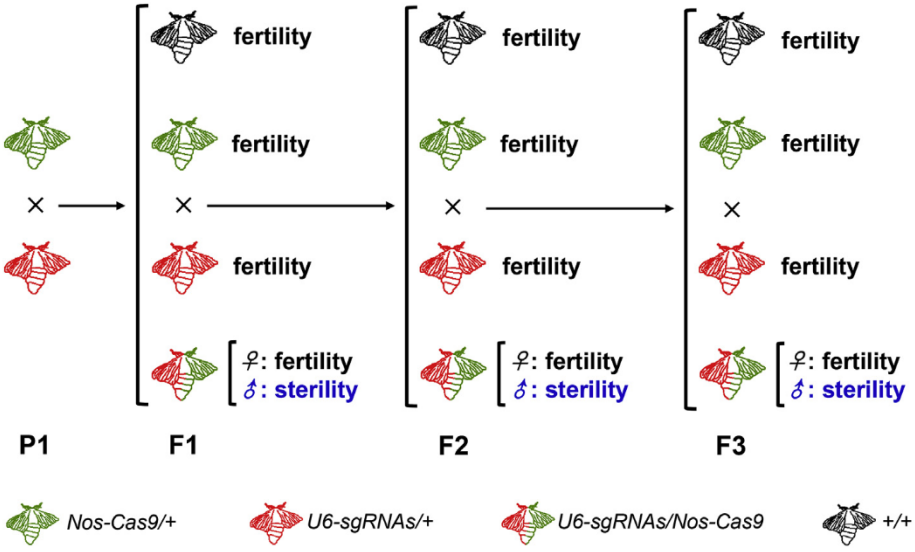

Fig. 6. Mutation of *Ser2* results in stable inheritance of male sterility. Schematic of crosses done to demonstrate heritably of the male sterility phenotype in *B. mori*. The activator line is represented as a green moth, the effector line is in red, the positive line is in half green and half red, and the negative line is in black. Four lines are produced by the hybridization of the *Nos-Cas9* line with the *U6-sgRNAs* line in silkworm in F1 progeny, and free hybridization of individuals of the four lines (meaning that adults were free to choose a mate from any of the four types) also produces only these four types of individuals. The percentage of four lines in each generation was 25%, and the percentage of mutant male sterility was 12.5%. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

insects, this would support the hypothesis that the gSIT strategy tested here could be applied to a number of pest and vector insect species. Given the accessibility and ease with which CRISPR/Cas9 technology can now be applied, successful implementation of this approach offers a new direction for biological control of pests in an environmentally responsible manner.

Conflicts of interest

We declare that we have no competing interests.

Authors' contributions

Y.H., K.L., L.H., and X.X. conceived and coordinated the study; X.X. and Y.W. performed and analyzed the experiments; H.B., J.X., and Z.L. participated in the data analysis; X.X. and Y.W. wrote the draft manuscript. A.A.J., L.H., K.L., and Y.H. revised the manuscript. All authors approved the manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ibmb.2019.103243.

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