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Research

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CRISPR/Cas9-induced Mutation of *Sex Peptide Receptor* Gene *Bdspr* Affects Ovary, Egg Laying, and Female Fecundity in *Bactrocera dorsalis* (Hendel) (Diptera: Tephritidae)

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Abstract

The oriental fruit fly, *Bactrocera dorsalis* (Hendel) (Diptera: Tephritidae), is an invasive and polyphagous pest of horticultural crops, and it can cause huge economic losses in agricultural production. The rapid development of CRISPR/Cas9 gene editing technology has provided new opportunities for the scientific control of agricultural pests. Here, we explore the applicability of the *B. dorsalis* sex peptide receptor (*Bdspr*) as a target gene for the CRISPR/Cas9-based sterile insect technique (SIT) in *B. dorsalis*. We screened two high-efficient single guide RNAs (sgRNAs) for gene editing. The results showed that both mutation efficiency and germline transmission rate were 100% in the surviving G_0 females (8/8) from injected embryos, and that 75% of mosaically mutated G_0 females (6/8) were sterile. The 50% of heterozygous G_1 females (4/8) could not lay eggs; 100% of eggs laid by them could not survive; and 62.5% of individual females (5/8) had abnormal ovaries. These results indicate that *Bdspr* plays an important role in regulating fertility, egg viability, and ovary development in female *B. dorsalis*, suggesting that the *spr* gene can be used for CRISPR/Cas9-based SIT in *B. dorsalis*.

Key words: *Bactrocera dorsalis*, sterile insect technique, CRISPR/Cas9, *spr* gene, reproduction

The oriental fruit fly, *Bactrocera dorsalis* (Hendel) (Diptera: Tephritidae), is a major pest throughout Southeast Asia and numerous Pacific Islands (Stephens et al. 2007). This insect pest is highly polyphagous and damages more than 250 host plant vegetable species worldwide (Zhang 2012). The traditional methods for controlling *B. dorsalis* mainly include bait-luring and chemical control (Jaime et al. 2009).

Sterile insect technique (SIT) is a target-specific, nondisruptive pest control method among the biologically-based approaches (Orankanok et al. 2007), and SIT is widely used against dipteranpests such as *Aedes aegypti* (Thomé et al. 2009), *Ceratitis capitata* (Walther et al. 2015), *Cydia pomonella* (Thistlewood and Judd 2019), and *Aedes albopictus* (Zheng et al. 2019b). Conventionally, SIT usually requires releasing a large number of sterile male flies to mate with wildtype females (Zheng et al. 2019a). Gene drive has a great potential in the genetic control of pests and the application of SIT (Sinkins and Gould 2006). Gene drive characterized by non-Mendelian inheritance promotes the mutations to spread more quickly in target populations than by natural selection (Rode et al. 2019). Recently, *Anopheles gambiae* and *Drosophila melanogaster* have been successfully engineered through CRISPR-based SIT (Esvelt et al. 2014, Galizi et al. 2016, Champer et al. 2018, Simoni et al. 2020). If insects carrying a sterile gene are released into the environment, the population can be significantly reduced (Kyrou et al. 2018). However, few reports on the sterility-related target genes used for CRISPR/Cas9-based SIT in *B. dorsalis* are available. The sex peptide receptor (*spr*) was first discovered in *D. melanogaster* in 2008 (Yapici et al. 2008). During *Drosophila* mating, the sex peptide (SP) is transferred along with the sperm cells of the male to the ovarian tract of the female, and it then interacts with the nervous and reproductive systems of the female, inducing the post-mating response (PMR) and reproductive cost of mating (Ottiger et al. 2000, Kubli 2003). Previous study

has shown that silencing the *spr* gene affects the hatching-rate of *B. dorsalis* by continuously feeding with ds-*spr* (Zheng et al. 2015). Subsequently, homologous sequences of *spr* have been found in *Helicoverpa armigera*, *Bombyx mori*, *Tribolium castaneum*, *Lygus hesperus*, and *Anopheles gambiae* (Kim et al. 2010, Yamanaka et al. 2010, Hanin et al. 2011, Hull and Brent 2014). *Spr* functions as a prothoracicostatic peptide receptor in *B. mori* (Yamanaka et al. 2010). SPR protein is a typical GPCR (G protein-coupled receptor) family protein, and it participates in many physiological reactions and plays an important role in signal transduction (Kim et al. 2010). *Spr* in *D. melanogaster* has been reported to be mainly expressed in the central nervous system (CNS), and *Spr* might have a role in regulating the access of sex peptide to neurons. Thus, using *spr* as a target gene might affect the reproductive behavior of host insects (Haussmann et al. 2013). Whether *Spr*can be used as a potential target gene for gene drive in *B. dorsalis* depends on its influence on reproductive behavior and fertility. Therefore, it is necessary to determine the influence of *Spr* on reproduction and fertility in *B. dorsalis*.

The purpose of this study was to reveal the effect of the *spr* gene on reproduction and to explore whether the *Bdspr* gene is suitable for CRISPR/Cas9-based SIT in *B. dorsalis*. The heterozygous mutants of *Bdspr* were obtained through gene editing based on the CRISPR/Cas9 system. Our results indicate that *Bdspr* plays an important role in female fertility and ovarian development, and that *spr* may be used as a potential target for CRISPR/Cas9-based SIT in *B. dorsalis*.

Materials and Methods

Insect Rearing

B. dorsalis were reared at 28°C, 70–80% RH, and a photoperiod of 12:12 (L:D) h at the Institute of Horticultural and Urban Entomoloy, Huazhong Agricultural University, China. The hatched larvae were maintained on bananas, and pupae were transferred into wet vermiculite after pupation. Adult flies were reared on artificial diet containing yeast/sugar powder (1:3) (Li et al. 2011).

Gene Cloning and Sequence Analysis

B. dorsalis genomic DNA was extracted using E.Z.N.A. Insect DNA Kit (Omega, USA). *Bdspr* sequences (*NW_011876269.1, XM_011200889.1*) were downloaded from NCBI database. Specific primers were designed by Primer Premier 6 software for PCR amplification. *Bdspr* exon1 was PCR amplified using the Prime-STAR Max (Takara, Japan). The primer sequences used for amplification were shown in Supp Table S3 [online only]. PCR amplification was performed as follows: denaturation at 95°C for 3 min, 30 cycles of denaturation at 95°C for 10 s, annealing at 55°C for 10 s, and extension at 72°C for 10 s, followed by a final extension at 72°C for 3 min. Genomic DNA from *B. dorsalis* adults was used as the template of PCR amplification, and then PCR products were purified using a E.Z.N.A. Gel Extraction Kit (Omega, USA). Phylogenetic trees were conducted by neighbor-joining method using MEGA 6.0 software (Tamura et al. 2013). The multiple alignment analysis of protein sequences was performed using online tools COBALT (https://www.ncbi.nlm.nih.gov/tools/cobalt/) and DNAman software.

Quantitative Real-time PCR (qRT-PCR)

Total RNA was extracted from *B. dorsalis* in different development stages: 0 h after egg laying (AEL), 3 h AEL, 6 h AEL, 12 h AEL, 24 h AEL, 45 h AEL, first-instar larvae, second-instar larvae, third-instar larvae, prepupae, mature female, and male adult, and from different tissues of adults: head, thorax, fat body, gut, ovary, testis, malpighian tube (MT) with RNAiso Plus reagent (TaKaRa, Kyoto, Japan). Three independent biological replicates for each sample were carried out with 3 technical replicates per biological replicates. The first-strand cDNA (1,000 ng RNA as template) of each pool was synthesized for quantitative reverse transcription PCR (qRT-PCR) using a PrimeScript RT reagent Kit with gDNA Eraser (TaKaRa, Kyoto, Japan). Reverse transcription was conducted at 37°C for 15 min and 85°C for 5 min. qPCR was performed as follows: 95°C for 10 min, 40 cycles of 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s with a SYBR Premix ExTaq kit (TaKaRa) using a real-time thermal cycler (Bio-Rad, Hercules, CA), according to previously reported method (Peng et al. 2015, 2019). Specific primers were designed by Primer Premier 6 software, and the relative expression of target gene was normalized with rpl49 expression as internal control (Peng et al. 2015). Specific gene primers were shown in Supp Table S3 [online only]. The qRT-PCR results were analyzed by $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001).

Target Designs and In Vitro Synthesis of sgRNA and Cas9 mRNA

Five guide RNAs (gRNA) were designed for each gene using the CRISPR design online tool (Gratz et al. 2014, Doench et al. 2016) CRISPR Optimal Target Finder (http://targetfinder.flycrispr.neuro.brown.edu/) and sgRNA designer CRISPRko (https://portals.broadinstitute.org/gpp/public/analysis-tools/sgrna-design) by observing the following principles: The design site was supposed to contain a protospacer adjacent motif (PAM) region with nucleotide sequence NGG; The GC content of sgRNA should be within the range of 35–50%; The target site was located on the first exon region; The distance between targets was more than 100 bp. DNA templates for sgRNA in vitro transcription were synthesized using two complementary fragments (Supp Table S3 [online only]). PCR conditions were as follows: predenaturation at 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 30 s, ending with a final extension at 72°C for 3 min. The templates of Cas9 mRNA in vitro transcription were amplified with plasmid pmlM3613 (addgene, USA). The primer sequences used for amplification were shown in Supp Table S3 [online only], and mRNA amplification conditions were as follows: predenaturation at 95°C for 3 min, followed by 38 cycles of denaturation at 95°C for 10 s, annealing at 55°C for 15 s, and extension at 72°C for 50 s, ending with a final extension at 72°C for 3 min. sgRNAs and Cas9 mRNA were synthesized using the Megascript T7 kit (Ambion) with 1 μg of template and a 5′ flanking T7 promoter as starting materials according to manufacturers' instructions. After RNA synthesis, the template was removed by incubating with TurboDNase (Ambion, USA) at 37°C for 15 min. The sgRNA was purified with MEGAclear-Kit (Ambion, USA). The product quality was detected by gel electrophoresis.

In vitro Activity Test of sgRNA

Bdspr exon1 was PCR amplified with the Prime-STAR Max (Takara, Japan). The primer sequences used for amplification were shown in Supp Table S3 [online only], and PCR amplification conditions were as follows: predenaturation at 95°C for 3 min, followed by 30 cycles of denaturation at 95°C for 10 s, annealing at 55°C for 10 s, and extension at 72°C for 10 s, ending with a final extension at 72°C for 3 min. PCR products were purified with E.Z.N.A. Cycle Pure Kit (Omega Bio-Tek, USA) for sgRNA in vitro activity test. The quality and concentration of DNA and RNA were analyzed by electrophoresis using a NanoDrop2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA). Five sgRNAs (200 ng) were mixed with 200 ng purified PCR product and 1U Cas9 endonuclease (ViewSolid

Biotech, Beijing, China) in a 20 µL volume at 37°C for 2 h, respectively. The sgRNA activity was detected by agarose gel electrophoresis. Gel-pro Analyzer V4.0 was used to detect the maximum OD value (optical density) of the electrophoresis bands, and sgRNA efficiency was calculated as the average value of three different tests.

Embryo Microinjection

The embryo microinjection was performed by previously reported method (Handler et al. 1998). *B. dorsalis* eggs were collected into sealed cups with holes on the side wall. Banana was put inside the cup to attract female adults to lay eggs. Each oviposition time lasted for no more than 20 min. The eggs were collected by rinsing the cup inside wall with water. The collected eggs were immersed in 1.2% sodium hypochlorite solution for 1 min to remove the egg chorion and rinsed with 0.02% TritonX-100. The obtained 40 eggs were fixed on slides with double-sided tape for microinjection (Bai et al. 2019). Two sgRNAs with maximum activity were selected from 5 sgRNAs (200 ng/µl, mentioned in Section 4.5) and mixed with 1 U/µl Cas9 protein to obtain RPNs. The resultant RPNs were microinjected into the dechorionated embryos within 1 h using the InjectMan NI2 (Eppendorf, Hamburg, Germany) equipped with the FemtoJet (Eppendorf) microinjection system. After injection, embryo was covered with halocarbon oil to prevent the eggs from being exposed to air. The injected embryos were placed into a transparent box filled with oxygen, and incubated in a constant temperature incubator at 28℃ for 48 h until hatching with oxygen added every 6 h. After hatching, the larvae were transferred into banana to pupate, thus the G_0 generation adults were obtained.

Screening of Mutants

To screen germline mutation, all the G_0 adult flies from injected embryos were crossed randomly to obtain G_1. Each of 8 randomly chosen G_1 females was reared with 3 wildtype males in independent box to obtain the G_2 offspring (Supp Fig. S1 [online only]). DNA from the G_1 females was extracted as a template for PCR amplification of the *Bdspr* exon1 containing the selected sgRNA target sites. With the specific primer pair (F1 and R4, Supp Table S3 [online only]), PCR amplification was conducted as follows: predenaturation at 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s, ending with a final extension at 72°C for 10 min. To determine the mutation types of G_1 flies, the amplified *spr* exon1 fragments containing s1 and s5 target sites were purified and cloned into the PMD-19 vector (TransGen Biotech, Beijing, China) according to the manufacturer's instructions. The plasmid DNA was transformed into trans-5α competent cells (TransGene). The positive clones were then sequenced.

Off-target Analysis

The whole genome sequence of *B. dorsalis* was obtained from NCBI database (ASM78921 v2) to search for potential off-target sites using software CasOT 1.0 (Xiao et al. 2014). The potential off-target sites were PCR amplified with G_0 1 genome DNA as a template. PCR conditions were as follows: predenaturation at 95°C for 3 min, followed by 30 cycles of denaturation at 95°C for 10 s, annealing at 55°C for 10 s, and extension at 72°C for 15 s, ending with a final extension at 72°C for 3 min. PCR products were sequenced with specific primers designed to flank potential off-target sites with the primers shown in Supp Table S3 [online only].

Analysis of Fertility and Mating Latency

All the G_0 female adults from injected embryos were crossed to obtain G_1 offspring. G_1 females were raised in boxes alone, and the phenotypes of 8 randomly chosen G_1 females were observed. G_1 females were allowed to lay eggs on the sealed paper cup with holes on side wall. On the second day after successfully mating with wildtype males, the number of eggs laid by each female individual was counted. Counting was carried out every 2 d with a total span of 16 days. All the eggs were collected from the paper cup and transferred to petri dishes containing wet filter paper. The petri dishes were cultured in an incubator in the same culture environment with the adults, and water was sprayed regularly to keep the filter paper moist all the time. The hatched egg number was counted at hour 48 post oviposition to calculate egg-hatching rate. The adult mutants were dissected to observe the development of their reproductive system and the phenotypic characteristics.

Mating latency was calculated as the time from putting one mutant adult and three wildtype heterosexual adults into the box to mating.

Statistical Analysis

The relative gene expression was analyzed using the $2^{-\Delta\Delta CT}$ method, as described by Livak and Schmittgen (2001). The data were presented as the mean ± SD of three independent biological replicates. GraphPad Prism 7.0 software was used to plot and analyze the data. Student's *t*-test and one-way ANOVA were performed to reveal the difference between groups. $P < 0.05$ was considered as statistically significant.

Results

Evolutionary Conservation of *Bdspr* Across Insects

The full-length *Bdspr* open reading framework (ORF) sequence was cloned, and the sequencing results showed that there were 1,440 bases and 479 amino acids in this ORF region (Fig. 1A). The neighbor-joining tree of *spr* sequences from 13 species showed that *spr* from *B. dorsalis* was clustered with that from *C. capitata*, *Bactrocera latifrons*, *Bactrocera oleae*, *D. melanogaster*, and *Zeugodacus cucurbitae*, and *B. dorsalis* exhibited the closest relationship to *B. latifrons* (Fig. 1B). *B. mori* and *Papilio xuthus* were clustered together, whereas *T. castaneum* fell into a separate cluster. The *spr* amino acid alignment results showed that the identity between *B. dorsalis* and *B. latifrons*, *B. oleae*, *C. capitate*, or *D. melanogaster* was 96.19%, 91.43%, 75.51%, and 60.80%, respectively, (Fig. 1C). These data indicated that *Bdspr* was evolutionarily conserved across insects.

Expression Profiles of *Bdspr*

We investigated the expression profiles of *Bdspr* from various adult tissues and from *B. dorsalis* in different life stages. The qRT-PCR results indicated that the *Bdspr* gene was widely expressed in all the tested life stages. The expression level of *Bdspr* was significantly higher in the adult stage than in the egg, larval, and pupal stages, and in female adults than in male adults (Fig. 2A). Additionally, *Bdspr* was expressed in all the tested tissues in spite of its low expression level in fat body. Its expression was significantly higher in the head, thorax, and gut than in the Malpighian tubules, testes, ovaries, and fat body ($P < 0.05$, Fig. 2B).

Determination of *Bdspr* gene editing efficiency by single guide RNAs (sgRNAs)

Sequencing results showed that *Bdspr* gene had 4 exons and 3 intros (Fig. 3A). Five sgRNAs were searched from *Bdspr* exon 1regionusing CRISPR Optimal Target Finder and sgRNA Designer online tools for

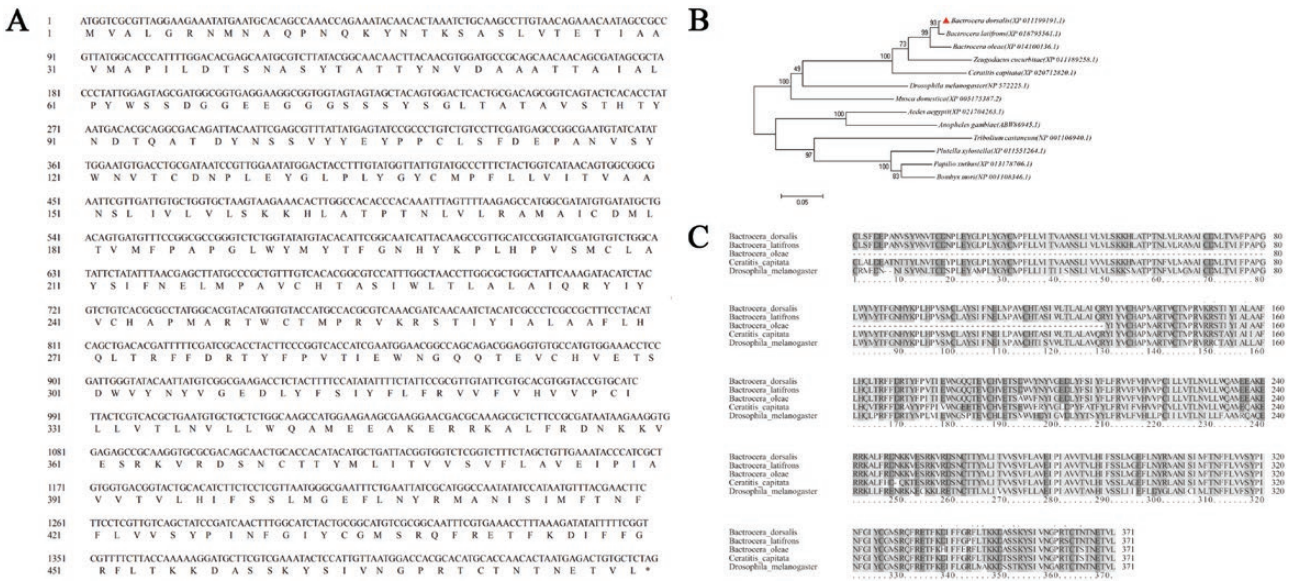

Fig. 1. Sequence and protein alignment of *Bdspr* gene. (A) Amino acid sequence in ORF region of *Bdspr*. The ORF region of *Bdspr* gene had 1,440 bases and 479 amino acids. (B) Phylogenetic tree of *Bdspr* gene. The numbers at branch nodes indicate bootstrap support from 1,000 replicates. The lower left corner represents the genetic distance scale, and in parentheses is amino acid sequence accession number. (C) Amino acid sequence alignment of the sex peptide receptor protein in various species. Black background indicates identical amino acids, and gray background denotes similar amino acids/conservative changes.

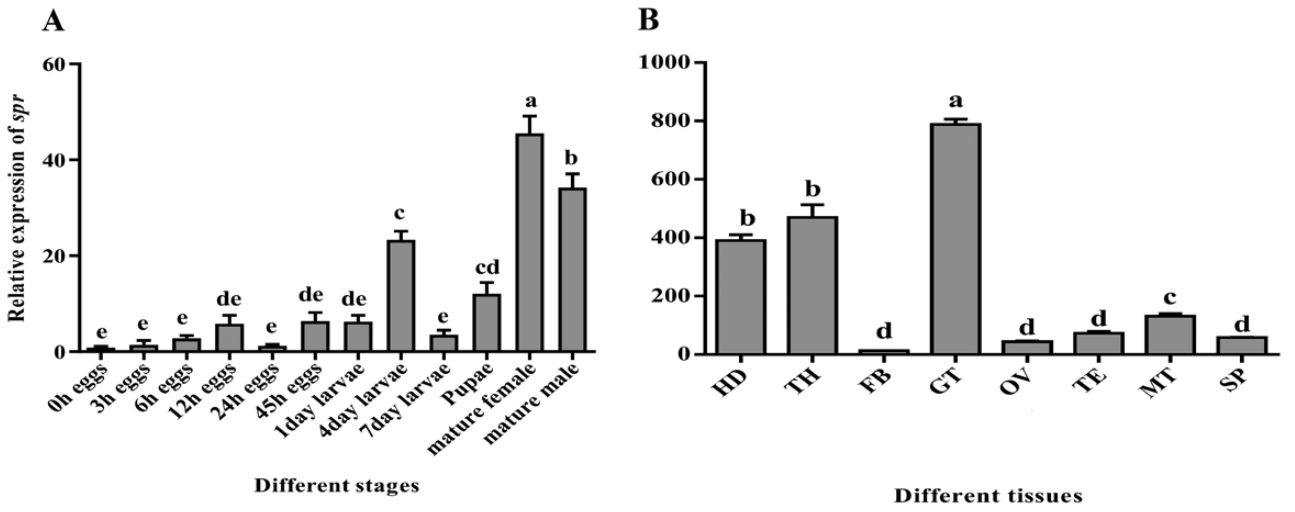

Fig. 2. Expression profiles of *Bdspr* gene determined by quantitative PCR in various developmental stages (A) and adult tissues (B). All the experiments were conducted in triplicates. The standard error is represented by the error bar, and different lower-case letters above each bar denote significant differences ($P < 0.05$) according to one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. MT, Malpighian tubules.

genomic editing (Fig. 3A and B). The Cas9 endonuclease cleavage experiment showed that all the tested sgRNAs induced the cleavage of the PCR-amplified fragments to different degrees (Fig. 3C), resulting in the generation of multiple peaks of various length fragments (Supp Fig. S2 [online only]). The Cas9 endonuclease cleavage efficiency induced by the five candidate sgRNAs (s1, s2, s3, s4, and s5) were 60.7%, 9.2%, 27.9%, 38.3%, and 47.8%, respectively (Supp Table S1 [online only]). Among the five sgRNAs, s1 and s5 showed the highest activity in vitro, thus they were selected for editing the *Bdspr* gene.

Bdspr Mutation at a Specific Site Based on CRISPR/Cas9

In *Bdspr* group, a total of 122 dechorionated embryos were injected with the mixture of Cas9 protein and 2 *Bdspr* sgRNAs (s1 and s5). In control group, 137 embryos were injected with Cas9 protein and EGFP-sgRNA. The 19.7% and 20.4% injected embryos in *Bdspr* group and control group were hatched into larvae, respectively. The 62.5% and 85.7% larvae in *Bdspr* group and control group survived to adulthood, respectively (Table 1). Overall, 15 G_0 flies (7 males and 8 females) were developed from *B. dorsalis* embryos injected with 2 spr-sgRNAs and Cas9 protein. G_0 females were then randomly crossed with G_0 males to obtain a homozygous mutant. In the *Bdspr* group, 2 out of 8 G_0 females were fertile and produce G_1 progeny, whereas the other 6 females were sterile. In contrast, all the females in control group were fertile. When nonmated heterozygous G_1 females crossed with wildtype males, we observed the significant reductions in the number of laid eggs by (*Bdspr* group vs control group, 30.4 ± 5.1 vs 59.4 ± 3.7, n = 48), the number of hatched larvae (0.9 ± 0.3 vs

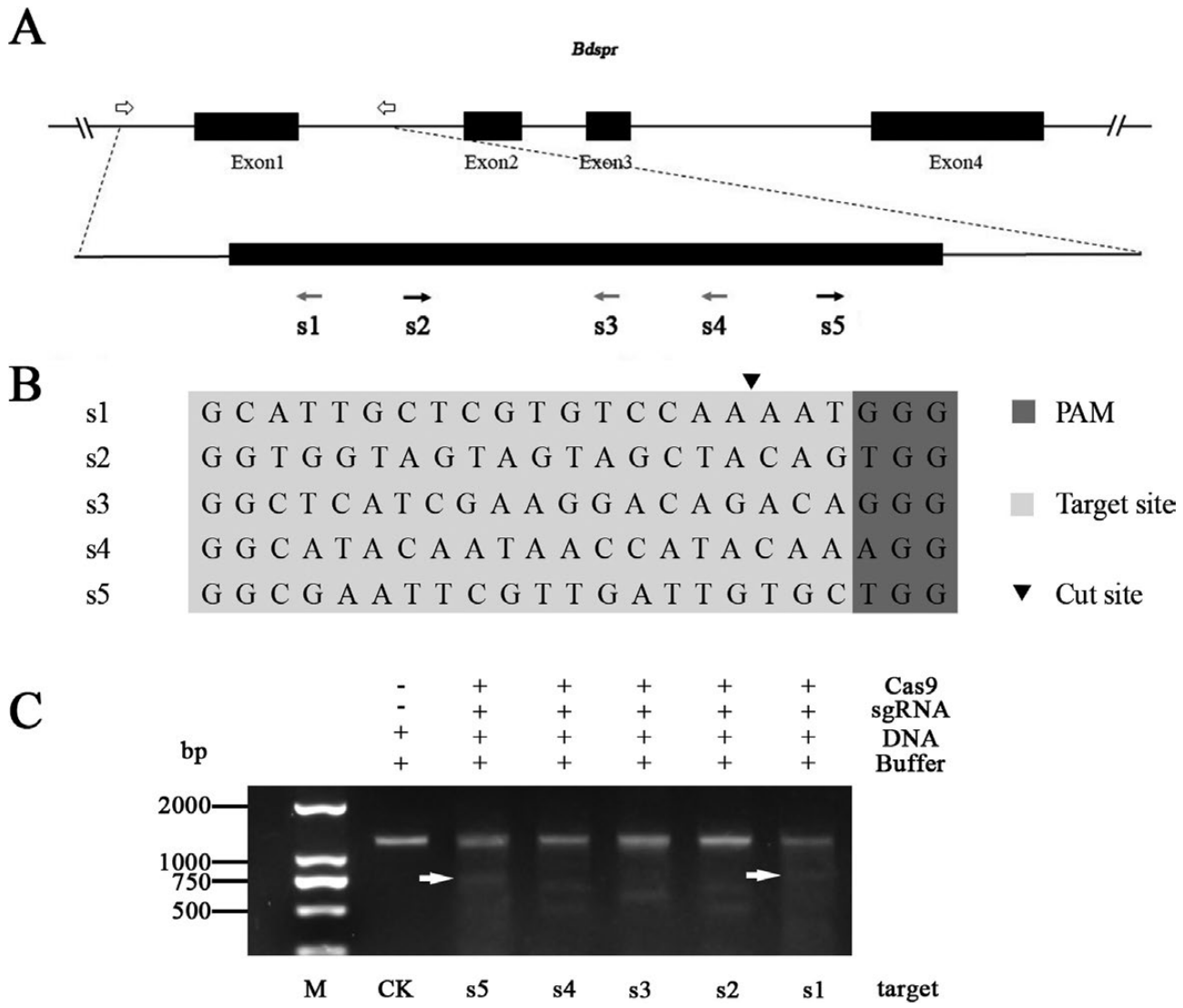

Fig. 3. Activity of sgRNAs for editing *Bdspr* gene. (A) Schematic of *Bdspr* gene. Black blocks indicate exons and lines between black blocks denote introns. Dotted line region indicates the amplified target fragment region. The hollow arrow represents specific primers for PCR detection, and the black arrow indicates the target site on the sense strand, and the gray arrow shows the target site on the anti-sense strand. (B) Sequences of the sgRNA target sites. The target sites were highlighted in dark gray, and the protospacer adjacent motif (PAM) sequence were in light gray. Black triangle indicates cleavage site. (C) sgRNA activity detected by agarosegel electrophoresis. The white arrow points to the cut electrophoresis bands.

Table 1. Injection and survival of G_0

Groups	Injection mixture	# of Injected embryos	# (%) Of larvae	# (%) of Larvae to adults	# Of females	# Of males	# (%) Of sterile females
Bdspr	s1, s5, Cas9 protein	122	24 (19.7%)	15 (62.5%)	8	7	6 (75%)
control	EGFP-sgRNA, Cas9 protein	137	28 (20.4%)	24 (85.7%)	13	11	0

#indicates number, and % denotes percentage.

45.4 ± 3.4, n = 48), and the percentage of viable eggs per female (1.4% ± 0.01 vs 76.0% ± 0.02, n = 48) (Fig. 4A–C). Although mut1, mut2, mut4, and mut6 could lay eggs, no adult was obtained since the larvae could not develop normally (Fig. 4D and E, Supp Table S2 [online only]). The dissection showed that the sterile females (mut3, mut5, mut7, and mut8) exhibited smaller and underdeveloped ovaries compared with control group (Fig. 4F and G). Notably, no G_2 was generated from G_1 sib-crosses since the mutational phenotypes had a serious effect on their egg viability and fecundity (Table 2).

Targeted Mutagenesis at *Bdspr* Locus

To determine whether the phenotypic defects described above resulted from genomic mutagenesis, all the G_0 female DNA were extracted, and target fragments were amplified with specific primers. Sequencing results of amplicons showed that 100% females (8/8) had multiple peaks, suggesting the mutations were present in all the G_0 females (Fig. 5A). Furthermore, the *Bdspr* gene target site in G_1 adults from injected embryos was analyzed by PCR amplification, TA cloning, and sequencing. Gel electrophoresis results

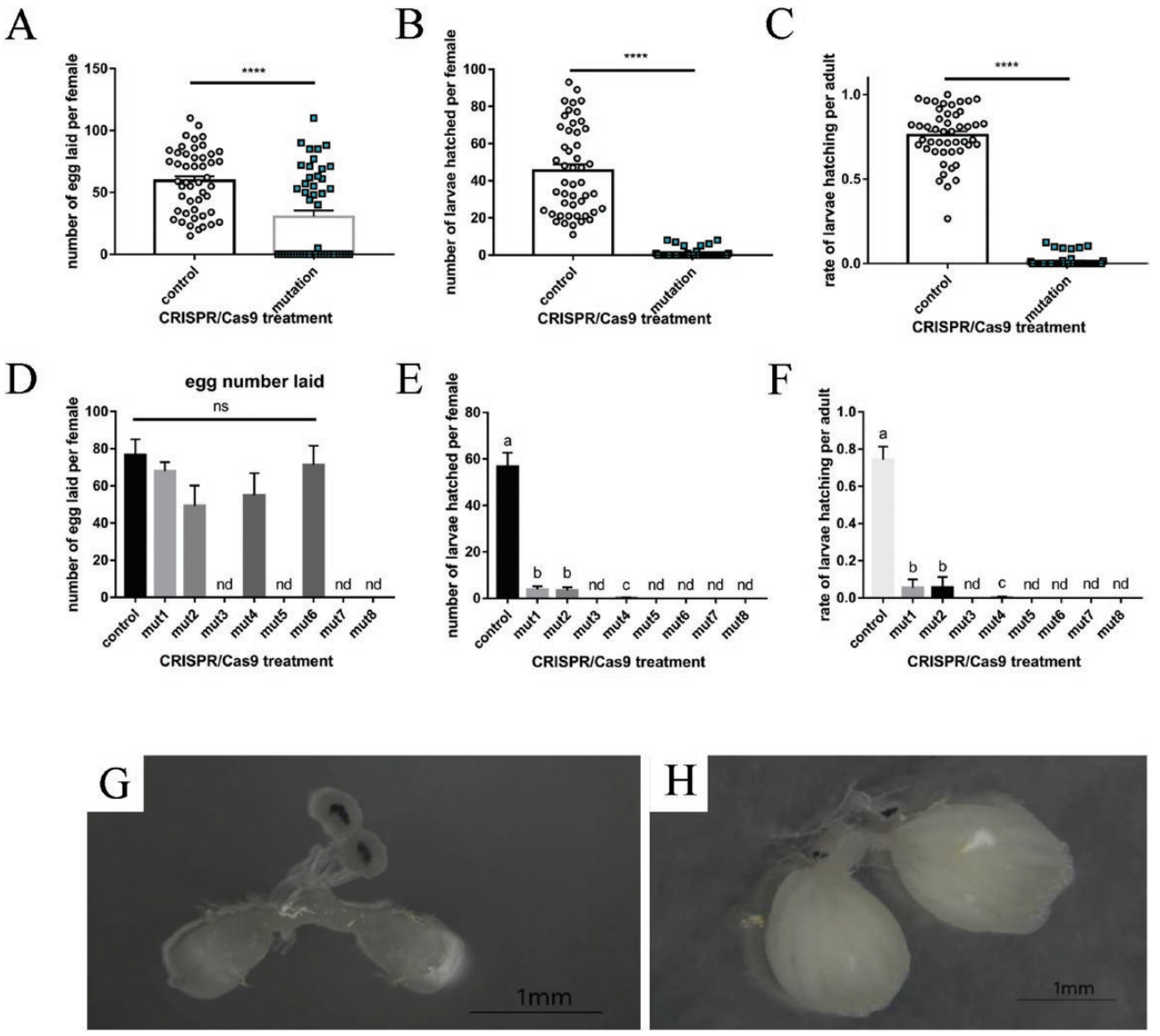

Fig. 4. Effect of knockout of *Bdspr* on fecundity and ovary of *B. dorsalis* based on CRISPR/Cas9 system. (A) Number of eggs($P < 0.0001$), (B) Number of hatched larvae ($P < 0.0001$), and (C) Larval hatching rate ($P < 0.0001$) from G_1 females. (D) Number of laid eggs per female. (E) Number of larvae hatched per female. (E) Larval hatching rate per female. (G) Abnormal ovaries of G_1 *B. dorsalis*. (H) Normal ovaries of control group. Data are shown as mean ± SEM (each point represents an independent replicate value). Different lower-case letters above bars denote significant differences ($P < 0.05$). nd, not detected.

Table 2. Influence of knockouting *Bdspr* on fecundity and egg viability

	Normal females		Reduction in egg viability		Inability to lay eggs		Reduction in size of ovaries	
Groups	*n*	Rate (%)	*n*	Rate (%)	*n*	Rate (%)	*n*	Rate (%)
Bdspr	0	0	8	100	4	50.0	5	62.5
control	8	100	0	0	0	0	0	0

Bdspr group, heterozygous G_1female; Control group, females from EGFP-sgRNA injected eggs.

showed two distinct bands in all the mutants except mut6 and mut8 (Fig. 5B). Sequencing results showed that mut3, mut5, mut6, mut7, and mut8 individuals produced insertion and/or deletion mutations in both the sgRNA targets sites (s1 and s5), and that mut1, mut2, and mut4 individuals produced insertion and/or deletion mutations at one of the sgRNA target sites (Fig. 5C). In addition, long deletion mutation from s1 to s5 (−358, +141) was found in mut3, mut5, and mut7 individuals. These results suggested that the mutations were heritable, and that the G_1 females were heterozygous mutant with the mutation transmission rate of G_0 females of 100% (8/8).

High Specificity of CRISPR/Cas9 Technique Based on Off-target Effect Analysis

Potential off-target effects might result in unexpected indel mutations, especially in the case of pest genetic control, since uncertain mutations in the genome will increase the evolution speed of resistance. To test the off-target effect of CRISPR/Cas9 technique, we screened the potential off-target sequences using the

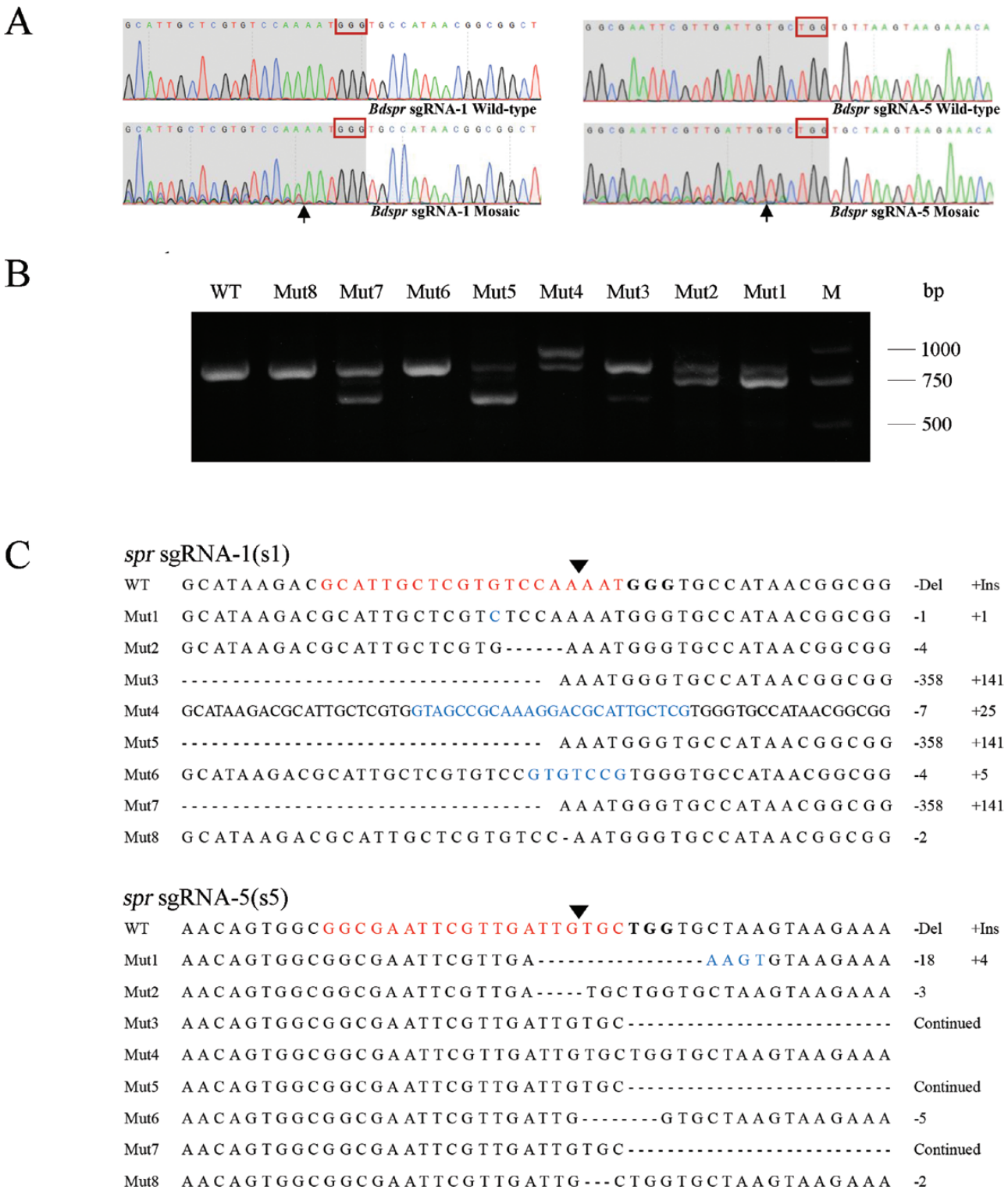

Fig. 5. Genomic DNA sequences of mutations induced by Cas9 cleavage at two *Bdspr* sgRNA target sites. (A) PCR amplification of DNA fragments from genomic DNA of G_1 females. Two distinct bands were from wildtype male parent and mutant female parent, respectively. Two bands were undetectable in mut6 and mut8 due to the small difference (<4 bp) between mutant band and wildtype band. M; marker; WT, wildtype. (B) Sequencing of *spr* from the G_0 females. The multiple peaks suggest the presence of the indel mutation. The arrow points to the site of the multiple peaks. The red box indicates the protospacer adjacent motif (PAM) sequence. (C) Sequencing of *spr* from G_1 females with heterozygous mutation. Red indicates sequence of target site; bold indicates PAM sequence; and arrow points to Cas9 cleavage site.

CasOT 1.0 software (Xiao et al. 2014). A total of 13 potential off-target sites were screened from the genome of *B. dorsalis*. The two sites with highest similarity to each sgRNA target site of the *Bdspr* gene (NW_011876124.1 and NW_011876329.1 for s1 and NW_011876234.1 and NW_011875955.1 for s5, respectively) were selected for off-target effect analysis. Sequencing results showed that

no multi-peaks were found in these four potential off-target sites, suggesting the high specificity of CRISPR/Cas9 technique in *B. dorsalis* (Fig. 6).

Discussion

The CRISPR/Cas9 system has been used as a highly efficient gene editing tool in insect models for gene function research and organism modification (Gratz et al. 2013; Wang et al. 2013a, 2014). The mutation rate of gene editing based on injection-introduced CRISPR/Cas9 system is sometimes low due to multiple factors such as the injury caused by microinjection, sgRNA sequence selection, and the variable concentration of sgRNA and Cas 9 (Tanihara et al. 2019). It has been reported that in mammalian cell lines, sgRNAs with medium GC content have a higher mutagenesis efficiency than those with low or high GC content in the case of the targeting sequence length of 20-nt (Wang et al. 2013b), which is consistent with our enzyme cleavage results. No correlation has been found between mutagenesis efficiency and 20-nt GC content in *Drosophila* (Ren et al. 2014), and these nonsignificant effects might be due to an insufficient concentration of sgRNA (Ren et al. 2013). In the present study, we injected a mixture of Cas9 protein and 2 sgRNAs, resulting in 100% editing mutagenesis and 100% germline transmission rate of the *Bdspr* locus. Our high mutation efficiency of *Bdspr* gene is consistent with that of *white* gene in *B. dorsalis* (Bai et al. 2019), and it is higher than that of most genes in other insects such as *Agrotis ipsilon*, *Bactrocera tryoni*, and *Bactrocera oleae* (Chen et al. 2018, Choo et al. 2018, Sim et al. 2019, Koidou et al. 2020). The reason for our high mutation efficiency might be attributed to rapid injection (within 1 h post oviposition) with the Cas9 protein rather than the Cas9 mRNA and two high-activity sgRNAs screened in vitro. The dual-target Cas9 system showed high specificity and low off-target effects, suggesting that this Cas9 system was suitable for *B. dorsalis*.

Expression pattern analysis showed that *Bdspr* was highly expressed in the tissues including the head, thorax, and gut, which is consistent with *spr* expression in *D. melanogaster* (Yapici et al. 2008) and *B. mori* (Yamanaka et al. 2010). In *Spodoptera litura*, the *spr* gene is expressed both in males and females, but males usually exhibit higher expression levels than females (Li et al. 2014), which is in contrast with our results. This might be due to a more important role that the *spr* gene might play in *B. dorsalis* females. In Drosophila, *spr* can regulate sex peptide (SP) targets (Haussmann et al. 2013). SP is the main regulator of the postmating response in females and is transferred along with sperm and accessory gland fluid in males during mating (Chen et al. 1988, Aigaki et al. 1991, Chapman et al. 2003, Liu and Kubli 2003). In spite of no reports of SP in *B. dorsalis*, we speculated that *spr* might play a role in neuro regulation of *B. dorsalis* by its expression profiles.

Our results showed that knocking out the *spr* gene caused a significant decrease in the fertility of *B. dorsalis* females, which is consistent with previous report on *H. armigera* (Hanin et al. 2011). Feeding *B. dorsalis* with ds-*spr* resulted in a significant reduction in the number of laid eggs, but it did not affect the egg laying period (Zheng et al. 2015). The impairment of neuronal sex peptide signaling can decrease in female germline stem cell (GSC) proliferation in *D. melanogaster* (Ameku and Niwa 2016). Our data indicated that the eggs laid by some G_0 females of *B. dorsalis* showed lower hatchability, or even no hatching, which could be due to a decrease in germ cells in females. In applying CRISPR/Cas9gene editing technique, the delayed microinjection with Cas9 mRNA or enzyme into the cell (after the first replication of the *B. dorsalis* genome) may induce mosaicism (Burkard et al. 2017). In *B. dorsalis*, different degrees of white eye occurred in the mosaic G_0 flies (Bai et al. 2019). Mosaicism may lead to a decrease in hatchability to various degrees, compared with the wildtype, but such a mosaicism still allows G_0 mosaic females to produce offspring that can develop into adults. Our data showed that heterozygous G_1 females with mutation at both 2 sgRNA target sites (s1 and s5) were unable to lay eggs, and their ovaries were undeveloped, whereas heterozygous G_1 females with mutation at only one sgRNA target site could lay low-viability eggs, but these eggs cannot survive to adult. The reason for the complete absence of egg-laying ability might lie in that the mutations at two target sites severely disrupted protein structure. Our results indicated that *Bdspr* gene might play a role in GSC of *B.dorsalis*, ultimately resulting in significant reductions in the percentage of viable eggs and the size of ovaries.

Recently, SIT has been successfully applied in the biological control of *A. gambiae*, *A. aegypti*, *C. capitata*, and *B. oleae* (Schetelig et al. 2009, Thomé et al. 2009, Ant et al. 2012, Oliva et al. 2012). The *spr* gene was evolutionarily highly conserved across insects, making *spr* a promising target for genetic control of insect pests. Since sterile lines are generally difficult to maintain, new breakthrough are needed in rearing the lines. In this study, although heterozygous females with *Bdspr* mutation were unable to lay

Fig. 6. Detection results of potential off-target sites. The first row of letters represents the *spr* sgRNA target sequence. The second line of letters is the sequence of potential off-target sites. Red letters denote mismatched bases, and the sequencing results are below. The blue letters are PAM sequences.

viable eggs, *Bdspr* mutation had no significant effect on the fertility of G_1 males originated from injected eggs (Supp Fig. S3 [online only]), which made it possible to for heterozygous mutant males to compete for mating. In one of our other injections, G_2 females generated from heterozygous G_1 males also showed a low egg viability and fecundity (Supp Fig. S4 and Supp Table S4 [online only]). This dominant female sterile phenotype can be passed through the male germline, which would be necessary for practical application. The application of gene drive is an alternative and potential tool to control populations of agricultural pests (Romeis et al. 2020), and it provides new developmental directions for SIT. Employing *Bdspr* gene as gene drive target will greatly improve the efficiency of male transmission of mutant gene in the population. Collectively, *spr* exhibits a high application potential in the genetic modification and pest management of *B. dorsalis* and other pests. In addition, the CRISPR/Cas9-based agricultural pest modification offers a potential tool for pest control.

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Author Contributions

HC: Data curation; Formal analysis; Investigation; Validation; Visualization; Writing – original draft. HS: Methodology; Investigation; Writing – original draft. JFX: Methodology; Writing – review & editing. ZCY: Methodology; Writing – review & editing. WPZ: Methodology; Software; Writing – review & editing. ZNL: Investigation; Validation. ZRD: Investigation. XXL: Writing – review & editing. HYZ: Conceptualization; Funding acquisition; Methodology; Project administration; Resources; Supervision; Writing – review & editing.

Supplementary Data

Supplementary data are available at *Journal of Insect Science* online.

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