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SHORT COMMUNICATION

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# Targeted somatic mutagenesis through CRISPR/Cas9 ribonucleoprotein complexes in the olive fruit fly, *Bactrocera oleae*

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## Abstract

The olive fruit fly, *Bactrocera oleae* (Diptera: Tephritidae), is the most destructive insect pest of olive cultivation, causing significant economic and production losses. Here, we present the establishment of the clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 methodology for gene disruption in this species. We performed targeted mutagenesis of the autosomal gene *white* (*Bo-we*), by injecting into early embryos in vitro preassembled and solubilized Cas9 ribonucleoprotein complexes loaded with two gene-specific single-guide RNAs. Gene disruption of *Bo-we* led to somatic mosaicism of the adult eye color. Large eye patches or even an entire eye lost the iridescent reddish color, indicating the successful biallelic mutagenesis in somatic cells. Cas9 induced either indels in each of the two simultaneously targeted *Bo-we* sites or a large deletion of the intervening region. This study demonstrates the first efficient implementation of the CRISPR/Cas9 technology in the olive fly, providing new opportunities towards the development of novel genetic tools for its control.

**KEYWORDS**

CRISPR/Cas9 ribonucleoprotein complexes, genome engineering, insect pest control, Tephritidae, *white* gene

  

## 1 | INTRODUCTION

The olive fruit fly, *Bactrocera oleae* (Diptera: Tephritidae), is a monophagous species that infests olive fruits, causing serious damages in olive production, and resulting in significant economic losses (Daane & Johnson, 2010). The major research focus of such insect pests is directed towards the development of novel strategies for their effective control (Ant et al., 2012). Although the olive fly research has entered the realm of genomics (Pavlidi et al., 2017; Sagri et al., 2017, 2014) reliable tools for reverse genetics, functional analyses, and applied research are still under development, hampering the progress towards the establishment of genetic control technologies. The availability of clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 technology and its ability to induce gene-specific mutations is expected to revolutionize the genome engineering toolbox also in the olive fly. Indeed, the CRISPR/Cas9 system has been successfully used for gene knockout in several insect pests (Taning, Van Eynde, Yu, Ma, & Smagghe, 2017). An ideal genetic target to test CRISPR/Cas9 mutagenesis in *Drosophila melanogaster* was the *white* gene (Bassett, Tibbit, Ponting, & Liu, 2013) because mutations on this X-linked gene that controls the red eye color are easily screened by visual observation in males (Green, 2010). The *white* gene was previously found structurally and functionally conserved in *Ceratitis capitata* as an autosomal locus (Loukeris, Livadaras, Arcà, Zabalou, & Savakis, 1995; Zwiebel et al., 1995) and was successfully targeted by CRISPR/Cas9 in this (Meccariello et al., 2017) and other Tephritidae species (see references in Table 1).

The main initiative of this study was to transfer the CRISPR/Cas9 editing technology into the olive fruit fly. To this end, we identified by sequence similarity the genomic locus of the eye pigmentation gene *white* (*Bo-we*) in *B. oleae*, which showed high homology with its orthologue in *C. capitata*. Unlike in *Drosophila* and as in *Ceratitis capitata*, *Bo-we* is located in an autosomal region (Drosopoulou, Nakou, & Mavragani-Tsipidou, 2014). Therefore, we expected to observe phenotypic effects on the eye colour only in case of biallelic mutations induced by Cas9, as previously observed in *C. capitata* (Meccariello et al., 2017).

This is the first report of successful CRISPR-based mutagenesis in the olive fruit fly. We anticipate that this technology will contribute to the generation of new molecular tools that will improve both our understanding of the olive fruit fly biology and the manipulation of its reproductive capacity via genetic control strategies for pest management.

**TABLE 1** List of CRISPR/Cas9-based applications in Tephritidae

| Insect | Delivery | Gene | Mutation | Reference |
|---|---|---|---|---|
| ***Bactrocera oleae*** | Protein | *white* | Knockout | Present study |
| ***Bactrocera tryoni*** | Protein | *white* | Knockout | Choo, Crisp, Saint, O'Keefe, and Baxter (2018) |
| ***Bactrocera dorsalis*** | Plasmid | *Bdmew* | Knockout | Zheng, Li, Sun, Ali, and Zhang (2019) |
| | Protein | *white* | Knockout | Bai et al. (2019); Sim, Kauwe, Ruano, Rendon, and Geib (2019) |
| | mRNA | *transformer* | Knockout | Zhao et al. (2019) |
| ***Ceratitis capitata*** | Protein | *white, paired MoY* | Knockout | Meccariello et al. (2017, 2019) |
| ***Anastrepha ludens*** | Protein | *white* | Knockout | Sim et al. (2019) |
| ***Anastrepha suspensa*** | Protein | *transformer-2* | Knockout | Li and Handler (2019) |

Abbreviations: CRISPR, clustered regularly interspaced short palindromic repeats; mRNA, messenger RNA.

## 2 | MATERIALS AND METHODS

### 2.1 | Insect rearing

The experiments were carried out using the *B. oleae* "Demokritos" laboratory strain provided by Kostas Bourtzis, Carlos Caceres, and Ahmad Sohel (Insect Pest Control Laboratory, Seibersdorf, Austria) to Prof Saccone's Laboratory (Naples). Flies were maintained under constant laboratory rearing conditions at 25 ± 2°C, 65 ± 5% RH, and a photoperiod of L14:D10. Paraffin cones were used as oviposition substrates and laid eggs were collected by washing them with water, maintained on filter paper soaked in 0.3% propionic acid, and then transferred to artificial larval diet (Tzanakakis, 1989). Pupae were then collected and stored in petri dishes until adult emergence.

### 2.2 | *B. oleae white* gene identification

The *Bo-we* gene was identified by Basic Local Alignment Search Tool (BLAST) homology searches with the *C. capitata* orthologous gene. The gene structure was further curated manually at the *B. oleae* genomic database (https://i5k.nal.usda.gov/Bactrocera_oleae) on the Apollo platform of the i5k Workspace@NAL (Poelchau et al., 2014). Multiple sequence alignments of the complete amino acid sequences were performed with Clustal Omega (Madeira et al., 2019).

### 2.3 | Suitable single-guide RNAs synthesis and ribonucleoprotein complex preparation

Suitable single-guide RNAs (sgRNAs) were identified using the CHOPCHOP software (Labun, Montague, Gagnon, Thyme, & Valen, 2016). Two 20-bp sgRNAs (*Bo-white*_g1 and *Bo-white*_g2) were selected for double-sequence targeting of *Bo-we* gene, showing 53.04% and 73.79% predicted efficiencies, respectively. Each sgRNA including the protospacer adjacent motif (PAM) sequence was used as a query against *B. oleae* genome in Blastn searches to identify putative off-targets. Templates for sgRNA in vitro transcription were generated by annealing two complementary oligonucleotides (polyacrylamide gel electrophoresis-purified, Life Technologies) as previously described (Bassett et al., 2013), using:

(1) a specific forward primer for each sgRNA containing both the T7 promoter sequence and the respective *white* targeting sequence (underlined):
   (*Bo-white*_g1:GAAATTAATACGACTCACTATAGAGTTGTTTCCAGCCCGAACCgttttagagctagaaatagc)
   (*Bo-white*_g2:GAAATTAATACGACTCACTATAGAGAGTAGGTGAGATTATCCGgttttagagctagaaatagc).
(2) a common reverse primer encoding the remainder of the sgRNA sequence (AAAAGCACCGACTCGGTGC CACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAAC).

The template-free polymerase chain reaction (PCR) for primer extension and the sgRNA in vitro transcription was performed as described by Meccariello et al. (2017). The ribonucleoprotein (RNP) complex mix contained 1.8 μg/μl of purified Cas9 protein with 0.2 μg/μl of sgRNA in injection buffer solution (20 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid, 300 mM KCl, 1 mM dithiothreitol, 10% glycerol, and pH 7.5). The preassembly of the RNP for each sgRNA was performed by incubating the freshly prepared solution at 37°C for 10 min, and equal quantities of those generated RNPs were mixed before use and kept on ice upon injections.

## 2.4 | Embryo microinjection

Embryo microinjections were carried out at 22°C according to standard procedures with minor modifications. Eggs were collected within 30 min after egg laying (AEL) and aligned on a slide with double-stick tape. Before the injections, eggs were dehydrated for 20 min by placing the slide in a petri dish containing calcium chloride granules and covered after with Halocarbon oil 700 (Sigma-Aldrich, Munich, Germany).

Preblastoderm embryos were injected within 90 min AEL into the posterior pole through the chorion using commercially available open-ended needles filled with 1 μl of preloaded sgRNA-Cas9 mix for both targets. Injections were performed under an inverted compound microscope (Leica DM-IRB), using a Leica mechanical micromanipulator at injection pressures of 25–28 psi. After injection completion, excess oil was wiped off and embryos were placed on tomato juice agar plates and incubated at 26°C until larvae hatching. The surviving larvae were then transferred to artificial diet under the standard rearing conditions. For the phenotypic mutation analysis, individual $G_0$ flies were visually screened for red-white eye mosaicism under a Leica MZ10F stereomicroscope. Images were obtained using the camera Leica DFC295.

## 2.5 | Cloning and mutation analysis

Single fly genomic DNA was extracted using the NucleoSpin® Tissue kit (MACHEREY-NAGEL) according to the manufacturer's instructions and used as a template for genotyping. PCR of the target sequence that spans both the sgRNA sites was carried out using the primers Bo-white-F: CAATGAGCAGTCCTACGAGC and Bo-white-R: AGGCAATCGCATTCAGAAGTG and Q5®High-Fidelity DNA Polymerase (New England Biolabs) under cycling conditions of 98°C 3 min, 35 cycles of 98°C 15 s, 58°C 20 s and 72°C 25 s, and 72°C 2 min.

PCR products were visualized on 1% agarose gel and all amplicons were purified separately using the NucleoSpin® Gel and PCR Clean-up kit (MACHEREY-NAGEL) and subcloned in the pJET 1.2 vector using the Thermo Scientific™ CloneJET™ PCR Cloning Kit (Thermo Fisher Scientific) following the manufacturer's protocols. Clones were subsequently sequenced using Sanger method (CeMIA SA) and the results were analyzed with Geneious® 8.0.5 (Biomatters Ltd.).

# 3 | RESULTS AND DISCUSSION

To explore the feasibility of CRISPR/Cas9 system as a gene-specific mutagenesis method in the *B. oleae* genome, we chose to disrupt the *white* gene through embryo injections of RNP complexes. Initially, we identified the *B. oleae white* (*Bo-we*) gene based on BLASTp homology searches with the *C. capitata* orthologue (94% amino acid identity; XP_014094495.1). The identified NCBI GenBank gene sequence (XM_014239020.1) was partial, missing the first exon and the long first intron (16,025 bp). The intact gene structure (JAMg_model_4806.1) was identified by manual curation at the i5k Workspace@NAL based on gene models and supporting data on our newest *B. oleae* genome assembly (GCA_001188975.3). *Bo-we* spans a 18,503 bp genomic locus and consists of seven exons (2,040 bp total transcript length) that code for a 679 aa protein (Figure 1a). A multiple alignment analysis of Bo-we protein with relative species revealed its high similarity with *B. tryoni* (98%), *B. dorsalis* (97.9%), but also *D. melanogaster* (84%). The characterization of the *Bo-we* structure facilitated the identification of the exonic target regions for producing a loss-of-function phenotype. We applied a simple protocol of CRISPR mutagenesis, by designing specific sgRNAs to direct the Cas9 RNPs, after injections into embryos, to the desired gene regions, encoding the amino-terminal portion of the Bo-we protein.

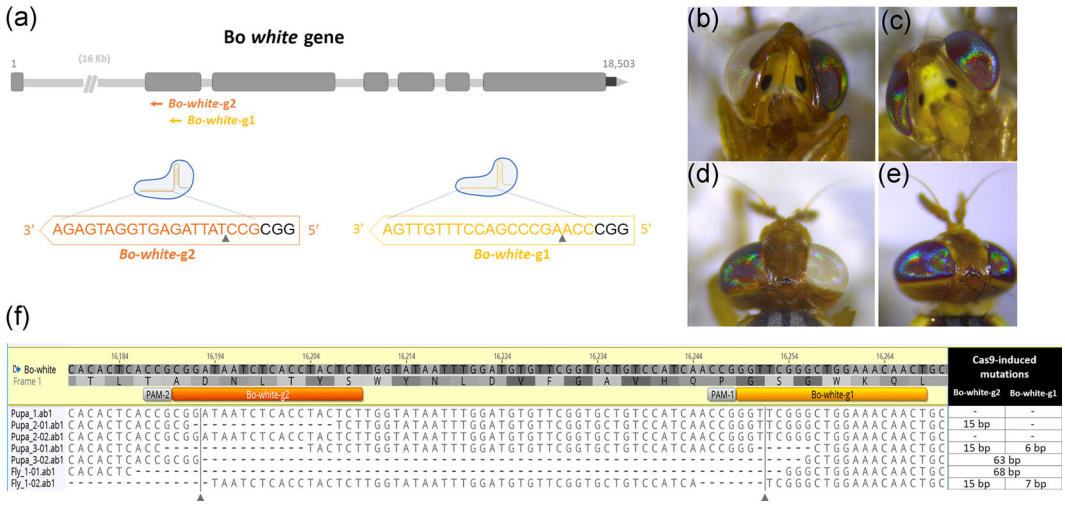


**FIGURE 1** CRISPR/Cas9-mediated *Bo-we* gene editing. (a) A schematic representation of the *Bo-we* gene structure and the respective target sites of the sgRNAs *Bo-white*-g1 (yellow) and *Bo-white*-g2 (orange). The 20 nt sequence of each sgRNA is indicated in the respective boxes; PAM sequences are shown in black; vertical arrowheads below the sequence indicate the cleavage site. (b and d) Induced white eye somatic mutant and (c and e) wild-type eyes. (f) Sequencing results of *Bo-we* at the targeted sites. Pupa_1 was genotyped as a wild-type (only one 410-bp fragment was amplified in PCR). Presence or absence of indels in the cloned amplicons were found in Pupa_2, Pupa_3 and the emerged female adult (Fly_1). All sequences were aligned on the wild-type *Bo-we* genomic sequence. Dashes indicate gaps automatically introduced to show the Cas9 deletions. The right column shows the size of the induced deletions for both sgRNAs; vertical arrowheads indicate the expected cleavage site after Cas9 activity. CRISPR, clustered regularly interspaced short palindromic repeats; PAM, protospacer adjacent motif, PCR, polymerase chain reaction; sgRNA, single-guide RNA

Potential Cas9 target sequences were identified using the bioinformatics online software CHOPCHOP, using the coding sequence as an input. Two target sites were selected to induce either one or dual cleavage, leading to indels, frameshift mutations, and possibly also deletion of the intervening region (Figure 1a). The distance between the two target sites and their associated PAM sites is 62 bp within the *Bo-we* exon 2. A total of 900 preblastoderm *B. oleae* embryos were injected with the preassembled RNP complexes. A total of 170 larvae hatched, showing a hatching rate of about 18%, whereas the observed egg-to-pupa survival rate was very low (4/900). Among the four surviving pupae, only one emerged to a female adult, with an egg-to-adult survival rate of 1 of 900. Such low survival rates ranging from 0.8% to 6.5% have also been reported in previous genetic transformation studies that involved *B. oleae* embryo microinjections (Genc, Schetelig, Nirmala, & Handler, 2016; Koukidou et al., 2006). Moreover, "Demokritos" laboratory populations are highly inbred, a characteristic that even under standard rearing conditions negatively affects the egg-to-adult survival rate which ranges between 30% and 50%. Therefore, the observed very low survival rate is the likely result of a combination of causes. Technical parameters associated with the microinjection procedure itself could affect the survival efficiency. However, these were not solely associated with the low number of survived flies, since a satisfying hatching rate from egg-to-larva (18%) was observed. However, a high mortality rate was seen during larval development. This observation highlights the necessity for improvement of the synthetic larval diet used in the artificial rearing of the olive fly. Although several aspects of the artificial rearing of *B. oleae* have been improved through the years (Rempoulakis, Dimou, Chrysargyris, & Economopoulos, 2014), there are still many drawbacks that restrict the effective and consistent production of large number of flies mainly associated with larval development. Larval development possibly could be improved by introducing new ingredients and/or nutrients into the artificial synthetic diet.

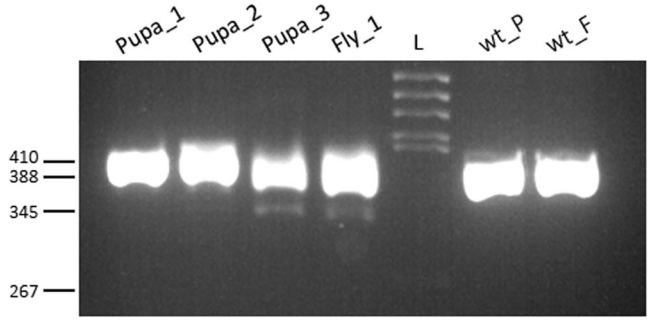


**FIGURE 2** PCR amplification of the targeted *Bo-we* genomic region. The genomic DNA of the four $G_0$ individuals (three pupae (Pupa_1-3) and one female adult (Fly_1)) were used as templates in each PCR reaction, respectively. The resulting PCR products were analyzed by electrophoresis through 3% (wt/vol) agarose gel. Different size amplicon bands indicate putative deletion mutations. Each amplicon band was purified and cloned for subsequent sequencing. As positive controls (410 bp) were used DNA from wild-type single pupa (wt_P) and single female adult (wt_F), respectively; L: pBR322 DNA/BsuRI (*Hae*III) Marker (Thermo Fisher Scientific™). PCR, polymerase chain reaction

The CRISPR/Cas9 mediated disruption of the *Bo-we* gene, by indels and/or by deletion, was expected to induce red-white eye color mosaic phenotypes. However, only in the case of biallelic mutations, recognizable red-white color changes should be produced in the somatic clones, derived from a cell in which these mutational events were induced on the two homologous chromosomes. The phenotypic analysis of the *B. oleae* adult showed that one of the two eyes was completely white (Figures 1b and 1d). We extracted genomic DNA from this adult, which died within a day after eclosion, and from three pupae. We PCR-amplified genomic DNA fragments from the targeted *Bo-we* genomic region. Gel electrophoresis analysis showed that the PCR products contained, not only an expected 410-bp-long DNA fragments, but also DNA products having shorter length. This analysis suggested the presence of deletions of the intervening sequence between the two targeted sites. These amplicons were subcloned and sequenced (Figure 2). The genotyped-mutant individuals revealed heterogeneity in the generated indel mutations (Figure 1f), which is consistent with the random repair mechanism followed by the nonhomologous-end-joining (NHEJ) mechanism. Comparison of sequencing data to the reference wild-type sequence showed diverse indels and deletion patterns in proximity to the cleavage positions of both target sites, including large deletions between the sgRNA target sites. The identified NHEJ-induced indels ranged from 7 to 15 bp, whereas two longer 63-and 68-bp identified gaps resulted from the deletion of the whole region that spans the two sgRNA targets (Figure 1f). In the case of Pupa_2, the indel was generated only by the *Bo-white*-g2 sgRNA. This result could be explained considering the lower predicted efficiency of *Bo-white*-g1 compared to *Bo-white*-g2. Although somatic knockout of the *Bo-we* gene was clearly shown in this study, the efficacy of Cas9-induced germ-line disruption could not be determined, because the emerged $G_0$ fly died and no crosses could be performed. Future experiments should address whether the *white* mutations also occurred in primordial germ cells and could be inherited in successive generations. However, the molecularly identified somatic mutations in 75% of $G_0$ individuals (including pupae and one adult) demonstrate the effectiveness of the CRISPR/Cas9 system in inducing indel mutations and a large deletion using two sgRNAs.

## 4 | CONCLUSION

In conclusion, this study is the first application of in vitro assembled RNP complexes—formed by synthetic sgRNA components and a cost-effective lab-purified Cas9 nuclease—to induce a gene disruption in the olive

fruit fly. Diverse mutations in somatic cells were observed in the different individuals (indel mutations or large deletions) that survived the embryo injections, as a result of multiple Cas9 targeting. The next challenge will be to understand and/or reduce the high lethality observed at the later stages of development, which, however, could not be attributed solely to the experimental procedure but also to the low survival rate of this species in standard rearing. The development of the CRISPR/Cas9-mediated targeted mutagenesis in this insect is of fundamental importance for both basic and applied research. At a basic level, it enables the generation of desirable mutations in any physiological system that governs vital life traits. At an applied level, the ability to generate mutations in genes that control early embryogenesis or sex determination of Tephritidae (such as *MoY*, *tra*, or *dsx*) (Meccariello et al., 2019; Saccone, Salvemini, & Polito, 2011; Saccone, Salvemini, Pane, & Polito, 2002) acquires eminent importance, since such processes play a critical role in genetic sexing in mass rearing facilities performing the sterile insect technique. This provides also the means of future genome modifications via homology-directed repair insertions as successfully shown recently in *C. capitata* (Aumann, Schetelig, & Häcker, 2018).

Thus, such CRISPR-based strategies could provide powerful means to accelerate the development of alternative species-specific genetic control methods, which are still hindered in *B. oleae* by the unavailability of genetic sexing strains. This study suggests that the implementation of innovative, simple, and accurate genetic methods in the olive fruit fly is not far from being deployed, once the appropriate molecular tools will be generated.

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## AUTHOR CONTRIBUTIONS

G. S. and K. D. M. initiated the project. K. T. T collected the embryos and prepared them for the injections, performed the molecular genotyping analysis, and drafted the manuscript; and wrote the manuscript with inputs from all authors. A. M. prepared the gRNAs and injected Cas9-gRNA mix into olive fly embryos. A. G. and P. P. contributed to the rearing and genetic crosses of the olive flies for embryo production. M. B. purified the recombinant Cas9 protein. All authors reviewed and approved the final version of the manuscript.

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