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2 **Direct parental CRISPR gene editing in the predatory bug *Orius***
3 ***strigicollis*, a biocontrol agent against small arthropods**
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5 Short running title: DIPA-CRISPR gene editing in the predatory bug *Orius strigicollis*
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Abstract

BACKGROUND

The predatory flower bug *Orius strigicollis* serves as a valuable biocontrol agent against small arthropods; however, its effectiveness can vary, especially when population establishment fails due to low prey/pest densities. A promising approach to improve the efficacy of *O. strigicollis* as a biocontrol agent is through gene editing. However, as females lay their eggs in plant tissue, the conventional embryo injection approach is challenging in this species.

RESULTS

In this study, we aimed to develop an efficient and practical gene editing technique for *O. strigicollis* using direct parental CRISPR (DIPA-CRISPR). Female bugs at various postemergence stages received Cas9 ribonucleoprotein injections, with subsequent genotyping of their offspring (G₀) using PCR and a heteroduplex mobility assay. We targeted the *kynurenine 3-monooxygenase* gene (*cinnabar*), pivotal for insect ommochrome pigment biosynthesis. Through experimental optimization, we achieved a peak gene editing efficiency of 52%, i.e., 52% of G₀ progeny carried gene-edited alleles when injecting 1 day postemergence. Notably, some gene-edited G₀ adults exhibited a red-eye mosaic phenotype, in contrast to the black-eyed wild type. Crossing experiments confirmed the heritability of the introduced mutations in the subsequent generation (G₁), enabling the establishment of a *cinnabar*-knockout line with bright red eyes.

CONCLUSION

We demonstrate that our DIPA-CRISPR gene editing method tailored for *O. strigicollis* is efficient and practical. Our findings highlight the potency of DIPA-CRISPR as a tool for *O. strigicollis* genetic engineering and suggest broader applications for enhancing other biocontrol agents.

Keywords: *cinnabar*, flower bug, integrated pest management, natural enemy, ommochrome

1. Introduction

Biological control using natural enemies is a key component of integrated pest management, reducing reliance on chemical pesticides. However, the efficacy of biocontrol agents is often hindered by the various physiological, behavioral, and ecological characteristics of natural enemies. For example, a high dispersal propensity of predators or parasitoids can diminish the efficacy of biocontrol programs¹⁻³. Addressing this issue has prompted advocacy for the genetic enhancement of biocontrol agents^{4,5}. Although the traditional method of selective breeding has been attempted in several insect species⁶, it relies on existing genetic variation in the wild and requires numerous generation cycles to achieve desired trait evolution. In contrast, genome editing can generate novel alleles not found in nature, requiring only a few generations to establish a genetically engineered line.

Recent advances in CRISPR/Cas9 gene editing have facilitated targeted modifications in the genomes of various insect species^{7,8}. However, despite its versatility, gene editing of biocontrol insects has been documented in only a few species, such as the ladybird beetle *Harmonia axyridis*^{9,10} and the ectoparasitic wasp

*Habrobracon hebetor*¹¹, natural enemies of aphids and lepidopteran caterpillars, respectively. Advancing gene editing techniques for natural enemies will not only deepen our understanding of gene function but also improve the insects' efficacy as biocontrol agents, although restrictions on the release of gene-edited organisms persist in some countries^{12,13}.

Predatory flower bugs, belonging to the genus *Orius* Wolff (Hemiptera: Anthocoridae), are among the most valuable natural enemies targeting diverse minute insect pests, such as thrips¹⁴. In Japan, *Orius strigicollis* (Poppius) is commercially available and widely used as an effective biocontrol agent in greenhouses¹⁵. However, the species' effectiveness in pest control can be inconsistent; for instance, the bugs may migrate out of greenhouses due to their high dispersal activity, thereby failing to establish populations when prey/pest densities are too low¹⁶. Although gene editing has emerged as a potential approach to confer beneficial traits in biocontrol agents, conventional embryo injection is unfeasible in *Orius* species, as females lay their eggs in plant tissues (Fig. 1(A)). This complicates newly laid egg extraction from plants, which must be achieved without causing mechanical damage and with maintenance of the requisite microenvironment for normal embryonic development postinjection.

Alternative methods circumventing the need for embryo injection have emerged in insects. Cas9 ribonucleoproteins (RNPs) injected into the hemolymph of adult females can be incorporated into developing oocytes alongside yolk protein precursors, introducing heritable mutations. The mechanism, termed Receptor-Mediated Ovary Transduction of Cargo (ReMOT), has been successfully employed in various insect species^{17–19}. ReMOT uses engineered Cas9 proteins fused with peptide ligands, facilitating ovarian uptake of Cas9 RNPs. However, its application entails time-

consuming processes, such as ligand development tailored to the target species and recombinant Cas9 protein production. Conversely, the direct parental CRISPR (DIPA-CRISPR) method employs commercially available nontagged Cas9 protein for adult injection. Notably, the use of DIPA-CRISPR has yielded successful gene editing in several insect species, including the red flour beetle *Tribolium castaneum*²⁰, the German cockroach *Blattella germanica*²⁰, the mosquito *Aedes aegypti*²¹, the Asian citrus psyllid *Diaphorina citri*²², and the white-backed planthopper *Sogatella furcifera*²³. Given its efficiency and feasibility, DIPA-CRISPR holds promise for application in biocontrol agents that are difficult to transform, such as *O. strigicollis*.

In the present study, we aimed to establish a DIPA-CRISPR gene editing method tailored for *O. strigicollis*. We targeted the *cinnabar* gene, which encodes kynurenine 3-monooxygenase involved in ommochrome pigment biosynthesis^{24,25}. Knockout of *cinnabar* results in red-eye phenotypes across diverse insect species, including hemipterans^{26–28}. Since the injection timing is the critical parameter for successful gene editing by DIPA-CRISPR^{20,21,23}, we initially examined the optimal injection timing in *O. strigicollis*. Through experimental optimization, we successfully introduced heritable mutations in the *cinnabar* gene of *O. strigicollis*, establishing red-eye *cinnabar*-knockout lines, thereby paving the way for further genetic engineering of *O. strigicollis*. Our tailored DIPA-CRISPR approach for *O. strigicollis* holds promise for extension to other biocontrol agents.

2. Materials and methods

2.1. Insects

An *O. strigicollis* colony, purchased as a biocontrol agent from Agrisect (Ibaraki,

Japan), was reared in a plastic mass-rearing container (150 × 103 mm; 47 mm deep) under a 14:10 h light:dark cycle at 24°C ± 1°C. Pieces of a Post-it note (Cat#658R, 3M), to which Mediterranean flour moth, *Ephestia kuehniella* Zeller (Agrisect), eggs were attached, served as their food source while twigs of the Mexican stonecrop, *Sedum mexicanum* Britton, provided both an oviposition substrate and a moisture/water source. Newly emerged adults were collected daily and transferred to new containers for staging.

2.2. Preparation of Cas9-sgRNA RNPs

To identify the putative *O. strigicollis cinnabar* gene, a tblastn search was performed against an in-house *O. strigicollis* transcriptome provided by Prof. Yukio Ishikawa (Setsunan University). The amino acid sequence of the cinnabar protein from the common bedbug *Cimex lectularius* served as the query (accession: XP_014255663.1). Three sgRNAs (sgRNA #1–#3) targeting the identified *O. strigicollis cinnabar* gene were designed using CRISPRdirect²⁹ and synthesized by Integrated DNA Technologies (IDT).

2.3. Adult injections

Adult females were carefully staged based on the day after emergence (AE), where zero days AE represented the day of emergence. Injections were performed using a glass capillary needle, made from a glass capillary (Cat#BF100-50-10, Sutter) using a PC-100 needle puller (Narishige) set at heater level 60.0, and attached to a Femtojet 4i microinjector (Eppendorf). Females were anesthetized on ice and mounted on a double-sided adhesive sheet (17 × 17 mm; Cat#859JN-10, 3M) on a glass slide. Cas9 Nuclease

V3 (Cat#1081058, IDT) and two sgRNAs targeting *cinnabar* (sgRNA #1 and #2 or sgRNA #2 and #3; refer to Table S1 for nucleotide sequences) were mixed at an approximate molar ratio of 1:2²⁰ and incubated for 10 min at room temperature to facilitate Cas9 RNP formation. Approximately 300 nL of the Cas9 RNP solution containing 3.33 µg/µL Cas9 protein and 0.63 µg/µL each of the two sgRNAs was injected into the intersegmental membrane region between the thorax and abdomen (Fig. 1). In our previous DIPA-CRISPR experiments involving *T. castaneum*, optimal gene editing efficiency was achieved when injections were administered to young females at the onset of vitellogenesis²⁰. Given that *O. strigicollis* females typically begin oviposition approximately 3 days postemergence under our rearing conditions, injections were administered at 0, 1, 2, or 3 days AE (Fig. 2(A)). For each injection time point, 1–4 independent experiments were conducted. Injected females were reintroduced into the mass-rearing container alongside sibling males. Stonecrop twigs hosting injected females' eggs were collected and replaced daily until 4–5 days postinjection to investigate the relationship between oviposition day and gene editing efficiency (GEF; the proportion of gene-edited G₀ individuals out of the total G₀ individuals). Twigs with laid eggs were individually stored in plastic vials for fruit flies (25 mm in diameter and 90 mm deep; Cat#AS519, Fisher Scientific) containing a Post-it note (Cat#715RP-K, 3M), to which *E. kuehniella* eggs were attached, until hatching.

2.4. Genotyping

Gene editing efficiency was calculated by extracting genomic DNA individually from the whole bodies of generation zero (G₀) hatchlings. For mutation rate calculation in G₀ gametes, genomic DNA was extracted individually from a middle leg of each G₁ adult.

Genomic DNA extraction followed a previously established method³⁰. Briefly, hatchlings were homogenized in 50 mM NaOH, heated at 95°C for 10 min, and neutralized using 200 mM Tris-HCl (pH 7.5). Genomic PCR was performed using KOD FX Neo (Cat#KFX-201, TOYOBO). The PCR amplification temperatures were as follows: initial denaturation at 94°C for 2 min; 38 cycles at 98°C for 10 sec, 65°C for 30 sec, and 68°C for 30 sec; final denaturation at 94°C for 5 min. Then the temperature was decreased to 16°C with a rate of 0.5°C/5 sec for final annealing and heteroduplex formation. Mutations were screened by analyzing the PCR products through a heteroduplex mobility assay (HMA) using the MultiNA Microchip Electrophoresis System (MCE-202, Shimadzu). In the HMA, heteroduplexes were identified as bands with reduced mobility³¹. Primers for the HMA (sequences: 5'-TCCATAAATCTCGCGCTCTC-3' and 5'-TTTGTAAACGGGATCGTATGG-3') were designed to amplify a 156 bp fragment containing the target sites of sgRNAs using Primer-BLAST³². Only bands within the 156–242 bp range were considered heteroduplexes owing to the presence of nonspecific PCR products in some individuals.

2.5. Phenotypic screening and amplicon sequencing

Adult females at the optimized stage (i.e., 1 day postemergence) were injected with Cas9 RNP, as described above, and the effects of *cinnabar*-knockout on adult phenotypes were examined in G₀ individuals. Two independent injection experiments were performed. Fourth or fifth instar G₀ nymphs were individually transferred from the mass-rearing container into a fly vial. Subsequently, eye colors of G₀ adults were examined under a stereomicroscope SMZ645 (Nikon). To verify whether G₀ individuals with external phenotypes harbored edited alleles and to characterize these alleles,

mosaic-eyed or red-eyed G₀ adults underwent deep sequencing of PCR products using the MiSeq system (Illumina). Genomic DNA was extracted from whole adult bodies, amplified using Ex Taq HS (TaKaRa), and purified using AMPure XP beads (Beckman Coulter). PCR amplification, library preparation, and sequencing (2 × 250 bp reads) were performed by Fasmac. CRISPResso2³³ was employed to analyze FASTQ sequencing files and assess introduced mutations in the *O. strigicollis* genome.

2.6. Mutant line establishment

To assess the inheritance of edited alleles from G₀ individuals to the next generation, G₀ adults exhibiting eye color phenotypes were crossed with wild types. G₁ progeny then underwent mutation presence screening via HMA using MultiNA. For *cinnabar*-knockout line establishment, heterozygous G₁ mutants were crossed, and the resulting homozygous G₂ mutants were selected for desired eye color phenotypes.

3. Results

3.1. Target gene identification

A tblastn search identified a single putative cDNA sequence of the *O. strigicollis* *cinnabar* gene (1,309 bp) exhibiting 70.4% amino acid sequence identity with the bedbug *C. lectularius*. Analysis using the HMMER web server³⁴ revealed the presence of a FAD-binding domain in the *O. strigicollis* *cinnabar* protein, considered essential for the proteins' enzymatic activity (Fig. S1)^{26,27}. Within this FAD-binding domain, we designed three sgRNAs (sgRNA #1–#3) for subsequent use in gene editing experiments (refer to Table S1 for nucleotide sequences).

3.2. Optimization of DIPA-CRISPR experimental conditions

Cas9 RNPs targeting *cinnabar* were injected into adult females at selected days AE (0, 1, 2, or 3 days postemergence; Fig. 1). Out of 499 injected females, we obtained 3,393 G₀ progeny, all of which were genotyped individually using the HMA (Fig. 2(B)). We observed marked variation in GEF depending on the injection day and subsequent oviposition (Fig. 2(C)). Comparable efficiencies were observed for both sgRNA mixtures (sgRNA #1 and #2 or sgRNA #2 and #3).

In females injected on day 0 AE, gene-edited G₀ progeny were predominantly found in eggs laid on the first day of oviposition, occurring 3 days postinjection. A similar trend was observed in females injected on day 1 AE, where the highest GEF of 31.1% (range: 14.3%–52.0%) was achieved in eggs laid on the first day of oviposition. However, this trend was not evident in females injected on day 2 or 3 AE. In these cases, the highest GEF values were observed in eggs laid on the second or third day of oviposition, although the values were lower than those observed in females injected on day 1 AE.

Collectively, these findings indicate that the optimal time for Cas9 RNP injection in *O. strigicollis* is 1 day AE. Furthermore, this timing markedly reduces the screening effort required for mutants by targeting eggs laid 3–4 days AE.

Considering the efficacy of DIPA-CRISPR in females undergoing active vitellogenesis^{20,21} and the commencement of oviposition by *O. strigicollis* females 3 days AE, our results suggest that the optimal injection timing coincides with the onset of vitellogenesis. On the day of emergence, terminal (or primary) oocytes may be in the previtellogenic stage, making them less susceptible to injected Cas9 RNPs. Conversely, on the subsequent day, an optimal time for injection, terminal oocytes would be in the

vitellogenic stage, thereby facilitating targeting by injected Cas9 RNPs. This explains why the optimal time for adult injection is 1 day AE and why gene-edited G₀ progeny are enriched in eggs laid on the first day of oviposition.

3.3. Phenotypes and mutant alleles in G₀ edited individuals

To assess the presence of G₀ somatic mosaic phenotypes in *O. strigicollis*, we performed the DIPa-CRISPR experiment under optimized conditions. For this experiment, Cas9 RNPs targeting *cinnabar* were injected into 123 females 1 day AE, and eggs oviposited 3–4 days AE were collected. The hatched G₀ nymphs were reared, and eye color phenotypes were examined at the adult stage (Fig. 3(A)). Out of 729 G₀ individuals reaching adulthood, 9 (1.2%) exhibited red or mosaic eyes, contrasting with the black-eyed wild type (Fig. 3(B)). This value is underestimated compared to that of HMA-based screening because phenotypic screening cannot detect monoallelic mutations.

To characterize the introduced mutations, the nucleotide sequence of *cinnabar* was analyzed in two selected G₀ individuals displaying eye color phenotypes (i.e., G₀ individuals #1 and #2; Fig. 3(B)). Deep sequencing of PCR products using the MiSeq system revealed that both individuals carried *cinnabar* mutant alleles with insertions and/or deletions (indels) close to the predicted Cas9 cleavage sites (Fig. 3(C–E)). Multiple edited alleles were observed in these G₀ individuals, likely due to mosaicism. Notably, the total indel frequency in the red-eyed G₀ individual (G₀ #2) was 99.3%, indicating widespread mutation presence in a homozygous or transheterozygous manner, resulting in the knockout phenotype in the eyes. However, the mosaic-eyed G₀ individual (G₀ #1) exhibited an indel frequency of 16.6%. These findings suggest a

possible correlation between phenotype severity and mutant allele frequency in G_0 individuals.

3.4. Inheritance of *cinnabar* mutant alleles

To determine the heritability of introduced *cinnabar* mutations, five G_0 individuals exhibiting red or mosaic eyes were crossed with wild types. If *cinnabar* is an autosomal gene and edited alleles in G_0 are transmitted to G_1 , resulting G_1 individuals would be expected to be heterozygous. HMA-based genotyping experiments involving G_1 adults revealed that three out of five G_0 adults produced G_1 progeny carrying *cinnabar* mutant alleles (Fig. 4(A)). The germline mutation rate of such G_0 adults was 5%–70%. Our HMA analysis demonstrated the inheritance of a variety of edited alleles from G_0 to G_1 individuals (Fig. 4(B)), consistent with deep sequencing results for G_0 adults (Fig. 3(C)). All resulting G_1 adults had black eyes, akin to the wild type, indicating the autosomal recessive nature of *cinnabar*. This suggests that the somatic mosaic phenotypes observed in G_0 (Fig. 3) arose from disruption of both maternal and paternal alleles before and/or after fertilization.

To establish a *cinnabar*-knockout line, we subjected G_1 heterozygous adults, which were progeny of the red-eyed G_0 #2 (Fig. 3(B)) and confirmed heterozygotes based on HMA results, to sibling mating (Fig. 4(C)). For this experiment, 10 G_1 females and 3 G_1 males were mated in a group. Among the resulting 398 G_2 individuals, 97 (24.4%) had red eyes, consistent with the expected segregation ratio of 3:1 via Mendelian inheritance (Chi-squared test, $P = 0.77$; Fig. 4(D)). Interestingly, one G_2 individual had white eyes (Fig. S2), unlike the red or black eyes of *cinnabar*-knockout or wild type adults. This occurrence could be due to a rare off-target mutation or a pre-

existing, exceedingly rare, mutation in the original commercial colony, given that only 1 out of 398 G₂ individuals displayed such a phenotype. Apart from eye color, no discernible abnormalities were observed in *cinnabar*-knockout *O. strigicollis*. Their body color, growth, development, and fertility appeared normal, allowing us to maintain the *cinnabar*-knockout line as a homozygous stock.

4. Discussion

In the present study, we pioneered and optimized the DIPA-CRISPR gene editing approach in the predatory bug *O. strigicollis*, an important biocontrol agent in agriculture. We achieved a remarkable gene editing efficiency of up to 52% when injecting Cas9 RNPs 1 day AE and collecting eggs laid 3–4 days AE. The optimal injection timing is relatively early in *O. strigicollis* compared to that reported in *S. furcifera* (2 days AE), *T. castaneum* (4–5 days AE), and *B. germanica* (4 days after ootheca drop), which may reflect differences in the timing of the onset of vitellogenesis, an important parameter for successful DIPA-CRISPR experiments. Our technique holds promise for both gene function investigations and further genetic engineering of *O. strigicollis*. Enhancing the performance of this species as a biocontrol agent could involve editing genes and genomic regions associated with dispersal activity, locomotion, feeding behavior and preference, and temperature tolerance.

To the best of our knowledge, this study marks the first successful gene editing in the superfamily Cimicoidea, including not only pirate bugs, such as *O. strigicollis*, but also medically important pests, such as the bedbug *C. lectularius*³⁵. Considering the notably high gene editing efficiency observed in *O. strigicollis* using DIPA-CRISPR, this species holds potential as a valuable model organism for Cimicoidea-related

research, especially considering its multiple advantages, i.e., a short life cycle of approximately 14 days³⁶, ease of rearing, and an established mass-production system tailored for agricultural use. Moreover, our DIPA-CRISPR methodology could provide a deeper understanding of the molecular mechanisms underlying the physiology and behavior of *O. strigicollis*, with the potential for extension to the other Cimicoidea insects.

The *cinnabar*-knockout line of *O. strigicollis* exhibited a red-eye phenotype, implicating *cinnabar* in ommochrome pigment synthesis in the eyes, consistent with findings in other hemipterans^{26–28}. However, we observe no discernible changes in body coloration, suggesting that ommochrome pigments may not influence body color in *O. strigicollis*. Interestingly, spontaneous red-eyed mutants have been identified in *O. strigicollis* and *Orius sauteri*, with crossing experiments indicating that these mutants emerge due to autosomal recessive mutations³⁷, similar to our *cinnabar*-knockout alleles. Additionally, a spontaneous mutant line with an orange body has been established in *Orius laevigatus*³⁸. However, the genes responsible for these spontaneous mutations, including those associated with the white eye mutation observed in the present study (Fig. S2), remain unidentified; thus, further studies are warranted to elucidate the molecular mechanisms governing body and eye coloration in *Orius*. These visible marker genes would be invaluable in extending DIPA-CRISPR to the co-CRISPR approach^{39–41}, in which the recovery of edited alleles for target genes with unknown or externally invisible phenotypes is facilitated by existing knowledge of codisrupted visible marker gene phenotypes.

Our DIPA-CRISPR approach offers several advantages over conventional selective breeding methods. Previous attempts at selective breeding in *Orius* species

necessitated numerous generations to artificially evolve desired traits. For example, in *O. strigicollis*, it took 45 generations to reduce locomotor activity and enhance biocontrol efficacy in greenhouses⁴². Similarly, in *O. laevigatus*, 30–47 generations were required to augment body size and predation rates⁴³ as well as pesticide resistance^{44,45}. In contrast, our gene editing approach requires only three generations (G₀–G₂) to establish a gene-edited line. Given that the egg-to-adult cycle of *O. strigicollis* completes in approximately 14 days³⁶, our gene editing approach will markedly reduce breeding timelines, potentially saving over a year during the breeding process. Therefore, the adult injection approach promises to substantially expedite genetic manipulation in *O. strigicollis*.

The successful establishment of the DIPA-CRISPR technique tailored to *O. strigicollis* opens avenues for further genetic engineering in this species and other biocontrol agents. Although CRISPR/Cas9 gene editing has been accomplished via conventional embryo injection in various insect species, its application has remained limited to only a few natural enemies^{9–11}. Given the comparative ease of the DIPA-CRISPR method over the embryo injection approach, we anticipate it becoming a valuable tool for advancing current biocontrol research. Importantly, many biocontrol agents pose challenges for conventional embryo injections. Examples include the mirid bug *Nesidiocoris tenuis*, a predator of whiteflies and thrips that lays eggs in plant tissue, and the endoparasitic wasps *Aphidius colemani* and *Aphidius ervi*, known for laying a single egg in aphid nymphs. Furthermore, DIPA-CRISPR promises to facilitate the generation of novel biocontrol agents by providing predesigned physiological and/or ecological traits to natural enemies previously unexploited as biocontrol agents.

Since the development of ReMOT Control and DIPA-CRISPR, gene editing via

adult injection has rapidly expanded to include a growing number of insect species. Notably, these techniques are now being extended beyond insects to noninsect arthropods, such as ticks and mites ^{46,47}, and even nonarthropod ecdysozoans, including tardigrades (water bears) ⁴⁸. We envisage that our study will contribute to the continued advancement of sophisticated genetic engineering systems across a broad spectrum of animal diversity.

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Conflict of interest statement

The authors declare that they have no competing interests.

Data availability statement

Data are available upon request by contact with the corresponding author.

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Figures

Figure 1

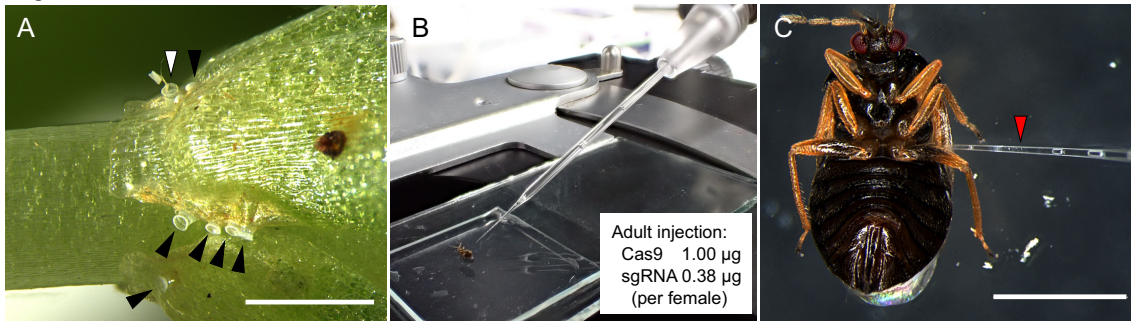


Figure 1. Adult injection in *Orius strigicollis*.

(A) Eggs of *O. strigicollis* laid in a *Sedum mexicanum* twig. Black and white arrowheads indicate unhatched and hatched eggs, respectively. Scale bar: 1 mm.

(B) Adult female *O. strigicollis* being injected.

(C) Magnified image of the injection. A glass capillary needle (red arrowhead) was inserted into the thoracic–abdominal intersegmental membrane. Scale bar: 1 mm.

Figure 2

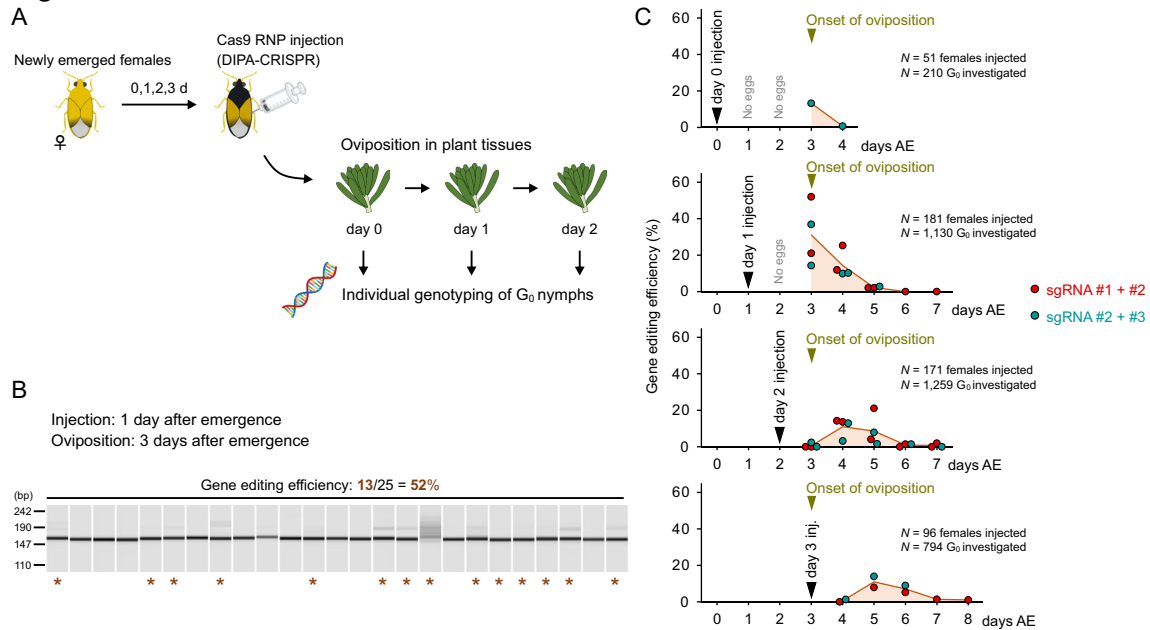


Figure 2. Optimization of DIPA-CRISPR in *Orius strigicollis*.

(A) Schematic of the DIPA-CRISPR experiment.

(B) Representative results of genotyping via the heteroduplex mobility assay. Asterisks indicate G₀ individuals with edited alleles.

(C) Relationship between gene editing efficiency and injection and oviposition timing.

Points indicate the results of independent experiments consisting of 6–178 G₀ hatchlings (refer to Table S2 for detailed results). Lines represent the means of pooled results from experiments involving two sgRNAs mixtures: sgRNA #1 and #2 (red) or sgRNA #2 and #3 (teal). Notably, females began oviposition 3 days after emergence (AE).

Figure 3

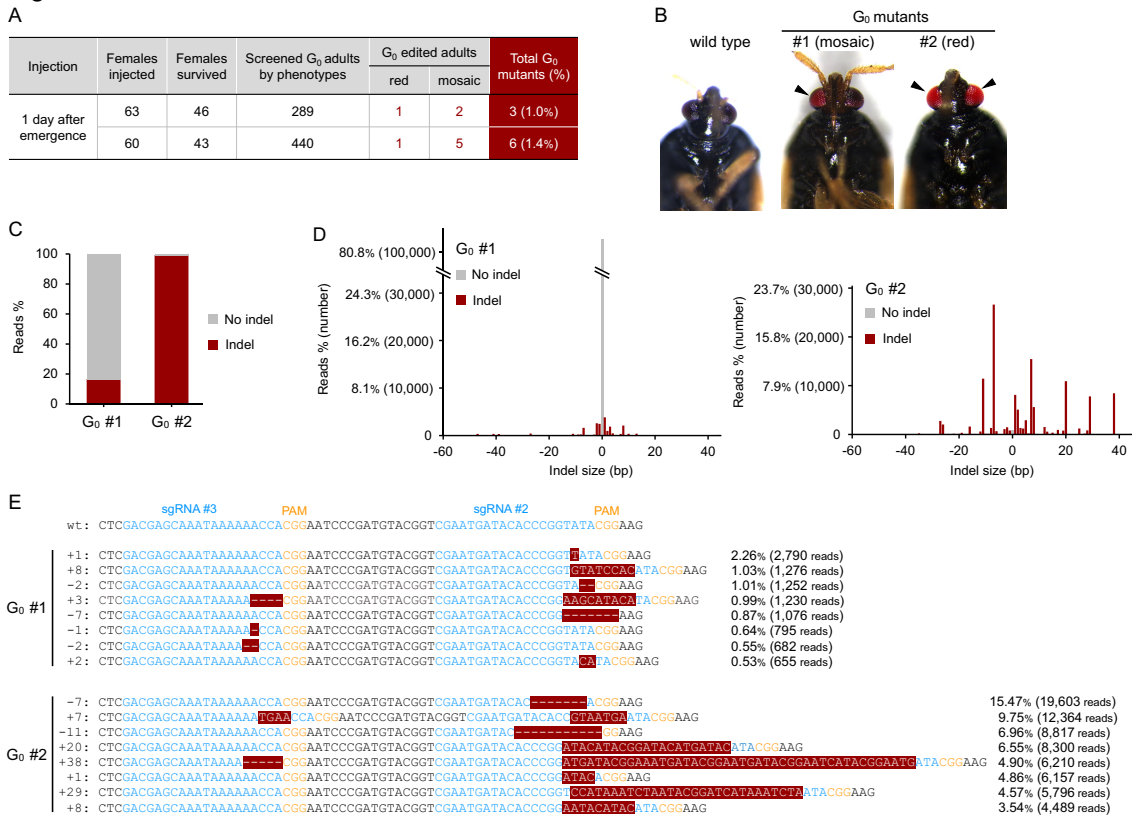


Figure 3. Phenotypes and genotypes of G₀ individuals.

(A) Results of G₀ screening based on phenotypes. Eggs were collected 3–4 days after emergence.

(B) G₀ adults with eye color phenotypes. Arrowheads indicate the absence of black pigment in the eyes.

(C) Percentage of indels in the two G₀ individuals shown in (B) (i.e., individuals #1 and #2).

(D) Size distribution of indels in the two G₀ individuals #1 and #2.

(E) Nucleotide sequences of indels in the two G₀ individuals #1 and #2. Nucleotide sequences of the eight alleles with the highest frequencies in each adult are shown. Allele frequencies are provided (righthand side).

Figure 4

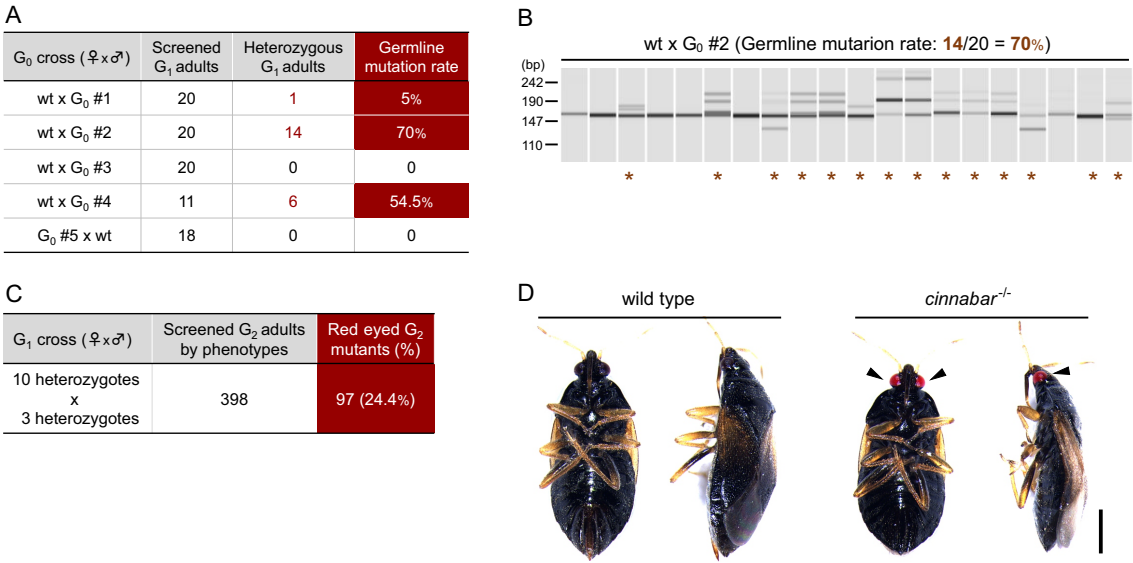


Figure 4. Inheritance of edited *cinnabar* alleles.

(A) Genotypes in G₁ animals. Numbers (#) correspond to those shown in Fig. 3.

(B) Representative results of the heteroduplex mobility assay involving G₁ individuals. Asterisks indicate heterozygous G₁ individuals.

(C) Phenotypes in G₂ animals. Heterozygous G₁ individuals derived from the wild type × G₀ #2 cross were mated to produce homozygous G₂ individuals.

(D) Wild type and *cinnabar*-knockout adults. Arrowheads indicate red eyes. Scale bar: 0.5 mm.