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ORIGINAL ARTICLE

Efficient DIPA-CRISPR-mediated knockout of an eye pigment gene in the white-backed planthopper, *Sogatella furcifera*

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Abstract Although CRISPR/Cas9 has been widely used in insect gene editing, the need for the microinjection of preblastoderm embryos can preclude the technique being used in insect species with eggs that are small, have hard shells, and/or are difficult to collect and maintain outside of their normal environment. Such is the case with *Sogatella furcifera*, the white-backed planthopper (WBPH), a significant pest of *Oryza sativa* (rice) that oviposits inside rice stems. Egg extraction from the stem runs the risk of mechanical damage and hatching is heavily influenced by the micro-environment of the rice stem. To bypass these issues, we targeted embryos prior to oviposition via direct parental (DIPA)-CRISPR, in which Cas9 and single-guide RNAs (sgRNAs) for the WBPH eye pigment gene *tryptophan 2,3-dioxygenase* were injected into the hemocoel of adult females. Females at varying numbers of days posteclosion were evaluated to determine at what stage their oocyte might be most capable of taking up the gene-editing components. An evaluation of the offspring indicated that the highest G0 gene-edited efficacy (56.7%) occurred in females injected 2 d posteclosion, and that those mutations were heritably transmitted to the G1 generation. This study demonstrates the potential utility of DIPA-CRISPR for future gene-editing studies in non-model insect species and can facilitate the development of novel pest management applications.

Key words CRISPR/Cas9; DIPA-CRISPR; gene editing; *tryptophan 2,3-dioxygenase*; WBPH

Introduction

CRISPR (clustered regularly interspaced short palindromic repeats)-based genome editing technologies that modify the genetic code of living organisms have been rapidly developed in recent years (Mojica *et al.*, 2009). The most extensively adopted editing approach couples Cas9 with single-guide RNAs (sgRNAs) to induce

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double-strand breaks (DSBs) at specific sites in the DNA sequence that are flanked by a protospacer adjacent motif (PAM). The induced DSBs can be repaired by either nonhomologous end joining, which results in indel mutations, or by homologous recombination when paired with donor template DNA during the repair process (Taning *et al.*, 2017). For insects, embryonic microinjection of the Cas9/sgRNA complex has resulted in successful CRISPR-based genome editing in a diverse number of species, including *Aedes aegypti* (yellow fever mosquito) (Li *et al.*, 2021a), *Apis mellifera* (honeybee) (Hu *et al.*, 2019), *Helicoverpa armigera* (cotton bollworm) (Ai *et al.*, 2021), *Nilaparvata lugens* (brown planthopper) (Xue *et al.*, 2018), and *Phlebotomus papatasi* (sandfly) (Martin-Martin *et al.*, 2018). Despite these successes, a number of extrinsic and intrinsic factors have limited the more pervasive use of gene-editing technology in insects. The costs associated with the sophisticated equipment and training typically required for embryonic microinjection can be prohibitive (Albadri *et al.*, 2017; Bui *et al.*, 2020; Sieber *et al.*, 2021). For some species, the dependence of egg viability on the milieu of the oviposition site can hamper embryonic injections. For instance, physical attachment to host plants via chorionic extrusions may be necessary to maintain egg hydration, as is the case for rice planthopper eggs that depend on the *Oryza sativa* (rice) stem microenvironment for hatching (Xue *et al.*, 2018). During ootheca formation in cockroaches, the eggs are deposited within the oocyst, a specialized structure formed and released through the coordinated activity of the accessory glands that provides essential materials for egg development and that poses significant challenges for glass capillary needle penetrance (Lawson, 1951; Roth & Willis, 1955; Roth & Stay, 1959; Gao *et al.*, 2019b). To overcome these limitations, a number of alternative delivery methods have been developed. Receptor-mediated ovary transduction of cargo (ReMOT Control), which relies on the targeted ovarian uptake of Cas9 ribonucleoprotein (RNP) complexes following direct injection into the abdomens of adult females, has been successfully used to induce guide mutations, albeit with varying degrees of target gene editing efficiency (GEF), in diverse species, including *A. aegypti* (0.7%) (Chaverra-Rodriguez *et al.*, 2018b), *Anopheles stephensi* (3.7%) (Macias *et al.*, 2020), *Bemisia tabaci* (0.1%–12.7%) (silverleaf whitefly) (Heu *et al.*, 2020), *Culex pipiens* (0.4%) (Li *et al.*, 2021b), *Homalodisca vitripennis* (0.75%) (Chaverra-Rodriguez *et al.*, 2020; Dalla Benetta *et al.*, 2020), and *Ixodes scapularis* (4.1%) (Sharma *et al.*, 2022). However, the method typically has relatively low target GEF and is complicated by the need for plasmid construction and the inclusion of a small ovary-targeting peptide to facilitate RNP uptake. Nanomaterials such as branched amphiphilic peptide capsules (BAPCs) have likewise been used to enhance RNP uptake by female ovaries; however, they too have had low GEFs (Avila *et al.*, 2018; Dalla Benetta *et al.*, 2020). In contrast, the direct parental (DIPA) approach, which is based on injecting high concentrations of Cas9 and sgRNA into appropriately staged adult females, can achieve relatively high embryonic gene knockout efficiencies (21.8% in the German cockroach, *Blattella germanica*, and >50% in the red flour beetle, *Tribolium castaneum*) (Shirai *et al.*, 2022) by temporally coupling the uptake of hemocoel RNPs with yolk protein precursors into developing ovaries. The extension of the method to more intractable species, such as rice planthoppers, which are poor candidates for embryonic microinjection, has yet to be established.

Target gene selection is critical for evaluating the utility of new CRISPR/Cas9 methods. Genes that yield visible phenotypes but that have minimal developmental effects when disrupted have proven to be especially useful for assessing knockout efficiency. Among the most effective genes are those associated with pigmentation pathways, particularly eye coloration (Adrianos *et al.*, 2018). The CRISPR/Cas9-mediated disruption of genes in ommochrome biosynthesis and uptake have yielded white eye phenotypes in *A. aegypti* (Li *et al.*, 2017), *Bactrocera dorsalis* (Bai *et al.*, 2019), *N. lugens* (Xue *et al.*, 2018), *Oncopeltus fasciatus* (Reding & Pick, 2020), and *T. castaneum* (Adrianos *et al.*, 2018), or a yellow eye phenotype in *Helicoverpa zea* (Perera *et al.*, 2018), that have facilitated the tracking of stable gene manipulation across multiple generations. Current knowledge of hemipteran eye pigmentation is limited to just a few species (Shamim *et al.*, 2014; Xue *et al.*, 2018; Bai *et al.*, 2019; Brent & Hull, 2019; Reding & Pick, 2020; Berni *et al.*, 2022; de Souza Pacheco *et al.*, 2022; Heu *et al.*, 2022). RNAi-based approaches targeting a suite of potential pigmentation genes have provided clear, albeit subtle, insights into gene roles in *Lygus hesperus* (Heu *et al.*, 2022) and *Rhodnius prolixus* (Berni *et al.*, 2022). In contrast, CRISPR modification of *white*, which encodes an ABC transporter critical for ommochrome pigment uptake, yielded clearly visible white eye phenotypes in *H. vitripennis* (de Souza Pacheco *et al.*, 2022), *N. lugens* (Xue *et al.*, 2018), and *O. fasciatus* (Reding & Pick, 2020).

As vectors of the southern rice black streak dwarf virus (SRBSDV), *Sogatella furcifera* (white-backed planthopper, WBPH) poses a significant economic threat to regional rice production. The WBPH life cycle consists of an embryonic stage of about 8 d, followed by 5 nymphal stages, and then by an adult stage that lasts approximately 10 d; each female is capable of only a single mating event and typically has 10–40 eggs. In recent years, their pest status has been exacerbated by reports of pesticide

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resistance (Zhou *et al.*, 2013; Ali *et al.*, 2019). Alternative pest management strategies are thus required. Although gene editing approaches have potential, the small size and the hardness of WBPH eggs precludes microinjection-based genetic manipulation (Bogolyubov, 2007; Beasley & Dowse, 2016; Paulo *et al.*, 2022). To address this limitation, a DIPA-CRISPR method was established in WBPH to efficiently knock out the ommochrome biosynthetic pathway gene *tryptophan 2,3-dioxygenase* (*TDO*). This study expands the scope of DIPA-CRISPR application and lays a foundation not only for functional genomics approaches in WBPH but also for CRISPR-based pest management.

Materials and methods

Insect collection and rearing

The laboratory strain of WBPH was collected from rice fields in Nanjing (Jiangsu, China) in 2012 and reared on rice seedlings (*O. sativa* Nangeng 918) in an artificial climate incubator held at 26 ± 1°C, 80% ± 10% relative humidity, and with a 14 : 10 h (light/dark) photoperiod.

Gene identification and preparation of the Cas9/sgRNAs mixture

BLASTp was used to identify Sfur00632-PA (the protein ID used in the genome annotation file) as the *S. furcifera TDO* (*Sf-TDO*) sequence in the WBPH genome (Ma *et al.*, 2021). Target gene cloning and sequencing were performed using the lab strain to avoid any single-nucleotide polymorphisms hampering sgRNA effects. Four 20-nt sgRNAs with the essential 5′-GGN and 3′-NGG Cas9 PAM features were designed with high specificity and predicted cutting efficiency for the 5th and 6th exons using CRISPOR (http://crispor.tefor.net) (Concordet & Haeussler, 2018). The sgRNAs were synthesized by GenScript (EasyEdit, Nanjing, Jiangsu, China).

Microinjection

Prior to microinjection, a 10-μL RNP mixture consisting of recombinant Cas9 with a double nuclear localization signal (NLS) (3.3 μg/μL; CP02; PNA Bio, Newbury Park, CA, USA), the 4 sgRNAs (1.3 μg/μL in total), and 2.54 μL of nuclease-free water was prepared. The RNP mixture was gently vortexed for 15 s, briefly centrifuged, incubated at room temperature (20–25°C) for 20 min, and then placed on ice prior to microinjection. Fifth-instar WBPH nymphs were randomly collected from cages and maintained in mesh-covered glass jars with the fresh rice seedlings at the 2-leaf stage. Newly eclosed female adults were collected daily and reared separately. At 1–4 d posteclosion, the collected females (more than 30 females for each day were selected) were anaesthetized under CO_2 and placed on 2% agarose gel. A pointed soft brush was used to orient the WBPHs on the gel for abdominal injections, which consisted of 200 nL of the RNP mixture per female, with injections performed using a Nanoject III microinjector (Drummond Scientific Company, Broomall, PA, USA). The injection volume for each female was normalized to approximately 0.66 μg of Cas9 and 0.26 μg of sgRNA (0.065 μg for each sgRNA). After injection, the WBPHs were immediately transferred to disposable plastic cups with fresh rice seedlings and placed in an artificial climate incubator. Injected females were then individually paired with 2 wild-type (WT) males, with the resulting fertilized eggs defined as the G0 line.

The hatching of the G0 eggs was determined under a stereomicroscope, with mortality determined daily throughout development. Posteclosion G0 adults were assessed for atypical eye color phenotypes and the GEF was determined based on the proportion of G0 adults exhibiting the phenotype relative to the total adult eclosion. G0 genomic DNA was extracted from individual insects using a MicroElute Genomic DNA Kit (Omega Bio-Tek, Guangzhou, China) according to the manufacturer's protocol. Fragments of the target gene, which included the potential cut sites, were amplified using a 2× Phanta Flash Master Mix (Vazyme, Nanjing, China) in a 25-μL reaction containing 100 ng of genomic DNA template, 12.5 μL of 2× Phanta Flash Super-Fidelity DNA Polymerase Master Mix, 2 μL of the forward and reverse primers (10 μmol/L; the *Sf-TDO*-1 forward primer sequence for mutation detection is 5′-TGGATCAAATAACAATTCTGGAAAC-3′, and the *Sf-TDO*-1 reverse primer sequence is 5′-CTCACTCGCCTCCACCTTG-3′). The thermocycler program was: 30 s at 98 °C, followed by 10 cycles at 98 °C for 10 s, 65 °C for 5 s, 72 °C for 30 s, then 30 cycles at 98 °C for 10 s, 60 °C for 5 s, 72 °C for 30 s, and finally 72 °C for 10 min. The remaining crude PCR products were purified using a FastPure Gel DNA Extraction Mini Kit (Vazyme, Nanjing, China), according to the manufacturer's instructions, after electrophoresis on 1% agarose gels. CRISPR/Cas9-mediated mutagenesis was confirmed using genomic DNA isolated from randomly selected red-eyed adults and an *in vitro* T7 Endonuclease I assay (Vazyme, Nanjing, China) that cleaves imperfectly matched DNA. The reaction mixtures were electrophoresed on 2% agarose gels to verify

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the PCR products. PCR products were subcloned into a TOPO-Blunt cloning kit (Vazyme, Nanjing, China) and transformed into DH5α competent cells (Protein Interaction, Wuhan, China). Transformed *Escherichia coli* was cultured in Luria-Bertani culture medium and plasmid DNA isolated using the Plasmid Mini Kit II (Omega Bio-Tek, Guangzhou, China). Single clones were sequence validated by General Biology Co., Ltd (Chuzhou, Anhui, China).

Germ-line mutagenesis analysis

Red-eyed WBPH (G0) were crossed with each other to acquire G1 offspring. The number of G1 WBPH containing compound red eyes was determined by visual examination. Genomic DNA extraction and subsequent cloning of red-eyed WBPH were performed as described as above. Thirty clones from 3 mosaic and 3 dark red-eyed individuals were sequence validated.

Results

Gene identification

To identify a putative *Sf-TDO* gene, BLASTp was used to search the WBPH genome (Ma *et al.*, 2021) for homologs of the *S. frugiperda* TDO sequence (Zhu *et al.*, 2020). A single gene (WBPH genome ID Sfur00632, http://www.insect-genome.com) was identified. The 2 protein sequences shared 58.01% identity. The Sf-TDO protein shared relatively higher identities with hemipteran orthologs: the top 2 hits were with *N. lugens* (89.9%, ALQ52682.1) and *Macrosteles quadrilineatus* (63.9%, XP_054262119.1). The *Sf-TDO* genomic sequence consisted of 9 exons and 10 introns, with the 1164-bp mRNA transcript predicted to encode a 388-amino-acid protein.

Post-eclosion efficiency of DIPA-CRISPR in WBPH

A high GEF (70%) for the *TDO* gene was reported in the fall armyworm (*Spodoptera frugiperda*) following co-injection of multiple sgRNAs (Zhu *et al.*, 2020). The application of multiple sgRNAs to drive higher gene knockout efficiency (4%−81%) than that achieved with a single sgRNA alone (0%−67%) has also been reported elsewhere (Bi *et al.*, 2016; Zhu *et al.*, 2019). Thus, in this study, we designed sgRNAs targeting 4 sites in exons 5 and 6 of *Sf-TDO* (Fig. 1A) with sgRNA1 located in exon 5 and the other 3 sgRNAs (sgRNA2, -3, and -4) in exon 6. To determine how the pre-oviposition age of adult female WBPH impacts RNP uptake, and by extension the GEF of the target gene, females were injected with RNPs at 1, 2, 3, and 4 d posteclosion and the effects on oviposition and offspring eye coloration were determined (Fig. 1A, 1B).

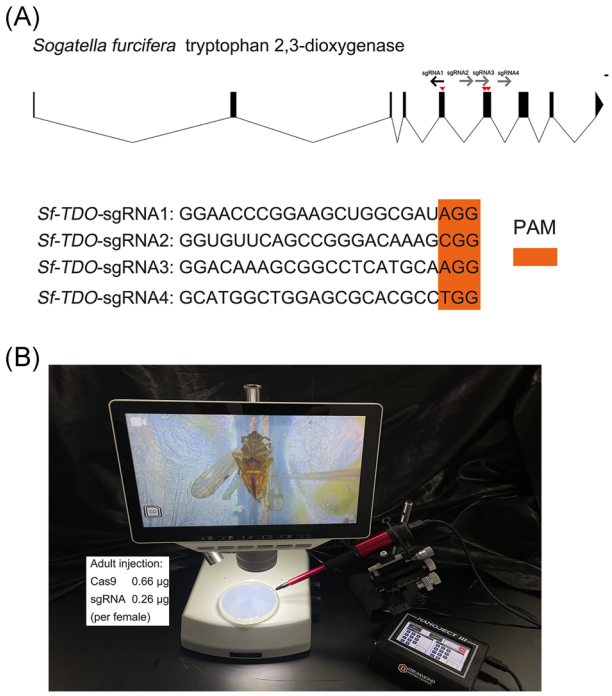

Fig. 1 DIPA-CRISPR in WBPH. (A) DIPA-CRISPR target sites in the *Sf-TDO* gene (gene ID: Sfur00632). The forward target positions are shown by the gray arrows, whereas the reverse target point is indicated by the black arrow. The red arrowheads indicate the single-guide RNA (sgRNA) sites, and the highlighted orange area indicates the protospacer adjacent motif (PAM) sequences. (B) Enlarged image of an injected WBPH.

The percentage of surviving females that exhibited oviposition was comparable among the day-2, -3, and -4 groups (53.1%−57.6%), but was significantly lower (approx. 40%) in the day-1 group (Table 1). Rates of G0 emergence ranged from 76.3% in the day-2 group to 97.5% in the day-4 group.

Phenotypic effects

The WT WBPH eyes are typically olive colored (Figs. 2A–C and 3A–C). In contrast, eyes in the CRISPR *Sf-TDO* G0 WBPH exhibited either a red mosaic phenotype (Figs. 2D–F and 3D–F) or a homogenous dark-red phenotype (Figs. 2G–I and 3G–I). G0 adults derived from the 4 posteclosion/pre-oviposition injection

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Table 1 DIPA-CRISPR efficiency in the white-backed planthopper *Sogatella furcifera*.

	Days posteclosion			
Statistical items	1	2	3	4
Females injected (*n*)	40	34	32	33
Females survived/laying G0 (*n*)	16	19	17	19
Oviposition	40.0%	55.9%	53.1%	57.6%
Percentage of nymphs that eclosed	80.4% (45/56)	76.3% (74/97)	78.8% (67/85)	97.5% (39/40)
Red-eyed G0 (*n*)	6	55	16	10
GEF	13.3%	56.7%	23.9%	25.6%
Number of G0 adults crossed	–	9♂/9♀	–	–
Adult G0 with mosaic red eye phenotype	–	37.11% (36/97)	–	–
Adult G0 with dark red eye phenotype	–	19.59% (19/97)	–	–
Adult G1 with mosaic red eye phenotype	–	32.75% (56/171)	–	–
Adult G1 with dark red eye phenotype	–	7.02% (12/171)	–	–

Note: GEF, gene editing efficiency = number of red-eyed G0/number of adult G0 screened.

groups had differing phenotypic frequencies (Table 1), with the highest frequency (56.7%) observed in those derived from the day-2 group. In contrast, the day-1 progeny had the lowest frequency of the red eye phenotype. Taken together, the data suggest that RNP uptake and CRISPR-mediated gene editing is most effective when injected into females at 2 d posteclosion.

In this study, mutants were specifically screened based on the red eye WBPH phenotype, which encompassed both mosaic and dark-red phenotypes. In day-2 G0 adults, a total of 97 WBPHs were screened, with 36 (i.e., 37.11%) found to exhibit the mosaic phenotype (Table 1), whereas 19 WBPHs (19.59%) displayed the dark-red phenotype.

To determine whether germ-line mutations occurred, 9 red-eyed G0 females were mated with G0 males (1 : 1 pairing) to obtain G1 progeny (Table 1). After G1 adult emergence, a cohort of 171 WBPHs was screened (Table 1), with 39.8% exhibiting either mosaic red eyes (56/171, 32.75%; Fig. 2J–H) or dark-red eyes (12/171, 7.02%; Fig. 2M–O), in contrast to the olive eyes typical of WT adults (Fig. 2A–C). From these results, it can be inferred that the prevalence of the mosaic eye phenotype was more prevalent than the dark-red eye phenotype in both G0 and G1 progeny derived from females at 2 d posteclosion.

Sequence verification

To verify the gene-editing events, the sequence flanking both target sites was amplified from the genomic DNA of WBPHs with the red eye phenotype. *Sf-TDO* DNA was cleaved *in vitro* using T7 endonuclease I as an indicator of imperfectly paired DNA following CRISPR-mediated gene editing. Multiple bands were observed in samples derived from the red-eyed WBPH, as compared with a single band in the nonedited WT DNA (Fig. 4A, lower panel), suggesting that gene editing had occurred. Consistent with these results, PCR amplification across the target genomic DNA region yielded bands of varying sizes (Fig. 4A, upper panel). Amplification products from both mosaic- and dark-red-eyed individuals were sequenced. Single clone sequencing of the PCR products revealed indel mutations at all 4 sgRNA sites (Fig. 4B). Two clones had relatively large 88-bp deletions between the sgRNA1 and sgRNA4 sites. The deletions, however, affected differing parts of the DNA, with 1 deletion spanning the region −1193 in the mosaic-eyed WBPH and with 1 deletion spanning the region −1222 in the dark-red-eyed WBPH, respectively. This latter deletion was also accompanied by a 7-bp insertion. Additional mutations identified in the sequence data included a single 5-bp deletion and a 21-bp deletion coupled with a 5-bp insertion in 2 mosaic-eyed WBPHs. Another mutation affecting the sgRNA2 and sgRNA4 sites included an 84-bp deletion and a 9-bp insertion in dark-eyed individuals. Furthermore, the phenotypic expression in G1 individuals of *Sf-TDO* knockout show some variability. Some G1 individuals exhibit the dark-eyed phenotype, similar to the G0 parent (with mutations in 2 alleles), whereas others have the mosaic-eyed phenotype (with 1 allele containing a mutation and the other allele retaining the WT sequence). A small fraction of G1 individuals exhibiting the WT phenotype probably indicates the nonpenetrance of the mutation.

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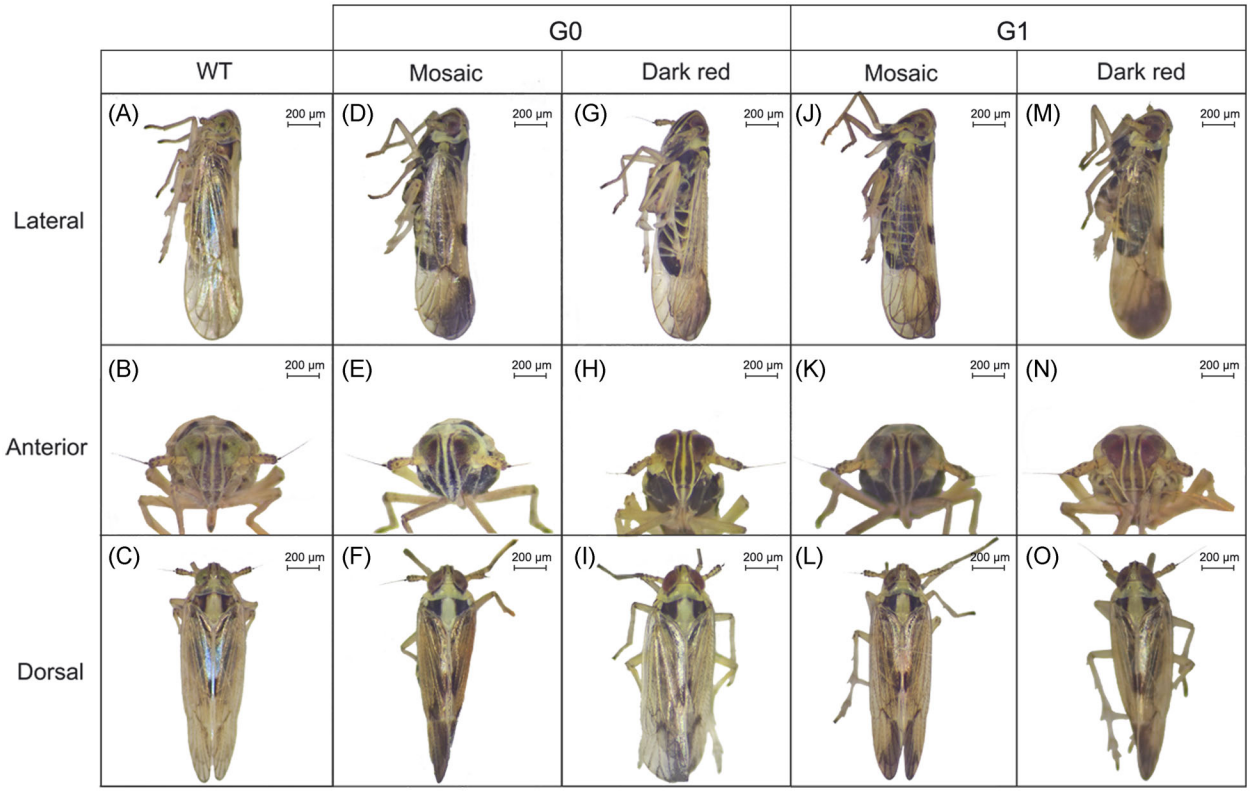

Fig. 2 Red eye phenotypes in G0 and G1 *Sogatella furcifera* (the white-backed planthopper, WBPH). Wild-type WBPH with olive-colored compound eyes (A−C). In G0 mutants, eye coloration is distinct from the wild type and is characterized by either mosaic red compound eyes (D−F) or dark-red compound eyes (G−I). In G1 individuals derived from G0 mutant crosses, the eyes likewise exhibit the mosaic red phenotype (J−L) and the uniformly dark-red phenotype (M−O). Scale bars: 200 μm.

Discussion

In the present study, higher concentrations of Cas9 and sgRNAs (0.66 μg of Cas9 and 0.26 μg of sgRNA for each insect) than those used in conventional embryonic injections (approx. 0.1−0.3 ng of Cas9 or sgRNA for each embryo) were delivered into the hemolymph for direct uptake by ovaries at different stages of development in virgin WBPH females. Although phenotypic effects were observed in all 4 experimental groups, the gene-editing activities in the developing oocyte were most pronounced when virgin females were injected at 2 d posteclosion. These effects declined with increasing female age. This suggests a higher incidence of genetic mutations during the early stages of development, which gradually decreases as the females age. This time frame coincides with WBPH vitellogenesis and is consistent with the vitellogenin-associated uptake reported for DIPA-CRISPR in *B. germanica* and *T. castaneum* (Shirai *et al.*, 2022), which had highest efficiencies at 4 d post-ootheca drop in the cockroach and in 4-d-old female red flour beetles. The 56.7% GEF achieved in the WBPH is significantly higher than that reported for *B. germanica* (21.8%), but is lower than that in *T. castaneum* (71.4%) (Shirai *et al.*, 2022). These species-specific differences in the efficacy of the DIPA-CRISPR approach may be linked to the mechanisms associated with delivering nutrients to the ovarioles. However, it is important to note that the relationship between nutrient delivery mechanisms and gene-editing efficiency in DIPA-CRISPR is purely speculative at this stage and other factors could indirectly influence the success of the technique. The reproductive cycle of the insect is one such factor to take into consideration. Vitellogenesis is a crucial process in the female reproductive cycle that could differentially impact the efficacy of gene editing depending on the point in time that the injections occurred, similar to that observed in *B. germanica* and *T. castaneum* (Shirai *et al.*, 2022). Optimal nutrient availability and transport mechanisms may also be necessary for the successful development and maturation of eggs in the ovaries (Chaverra-Rodriguez *et al.*, 2018a). Further, WBPH and

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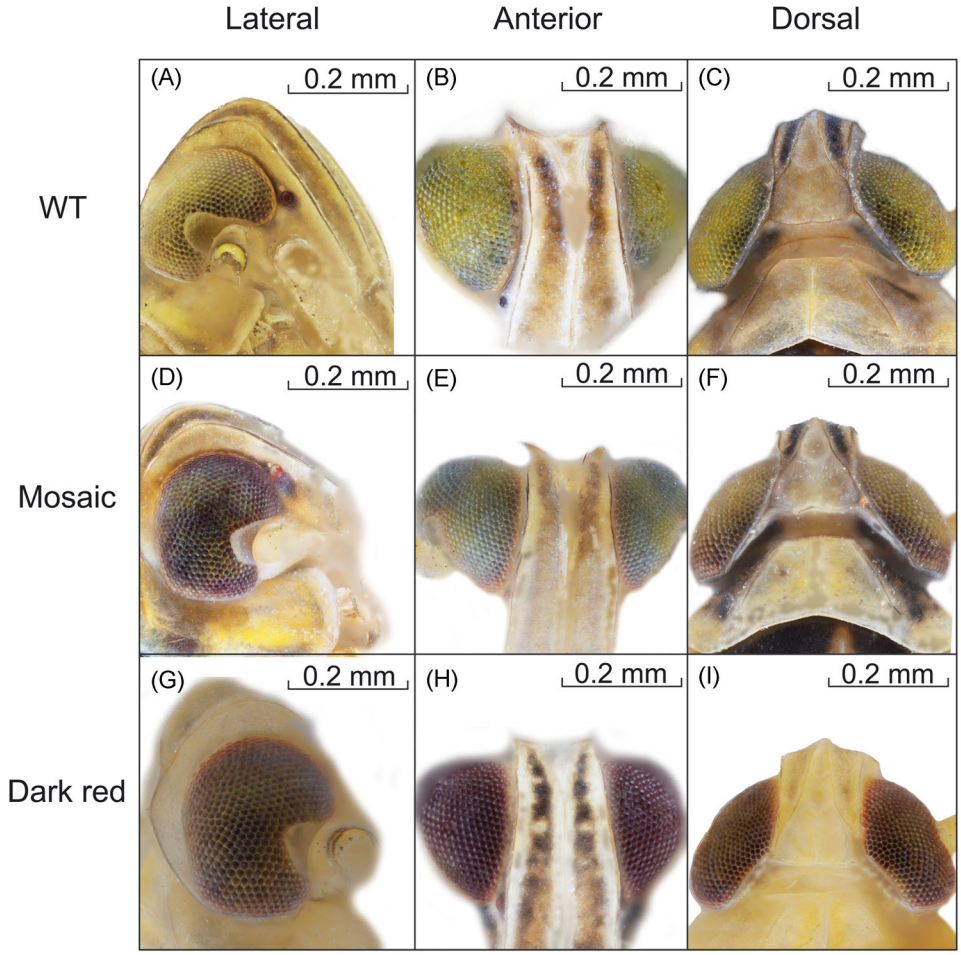

Fig. 3 Enlarged images of the G0 red eye phenotypes in wild-type (WT) and mutant *Sogatella furcifera*: (A–C) wild type; (D−F) mosaic red; and (G−I) complete dark-red pigmentation. Scale bars: 0.2 mm.

T. castaneum have telotrophic ovarioles that are supported by nurse cells localized in the germarium (Shirai *et al.*, 2022). Although the changes in gene expression that take place during the execution of long-term developmental programs in these growing ovarioles are an active area of research, the processes and mechanisms involved remain to be fully elucidated (Orikasa *et al.*, 1993; Swevers & Iatrou, 2003; Tanaka *et al.*, 2022).

The application of 2 different parental-based injections to induce gene editing in developing oocytes has been reported previously. Although the ReMOT Control approach has been successfully applied to a number of species (Chaverra-Rodriguez *et al.*, 2018b; Chaverra-Rodriguez *et al.*, 2020; Heu *et al.*, 2020; Macias *et al.*, 2020; Shirai & Daimon, 2020; Li *et al.*, 2021b; Sharma *et al.*, 2022), the method is complicated by requirements for plasmid construction and the need to potentially identify a species-specific ovary-targeting peptide to facilitate ovarian uptake of the RNP. Furthermore, the GEF of the ReMOT Control-based approach is often less than that achieved by DIPA-CRISPR.

An alternative parental injection method utilizes BAPC nanoparticles (Barros *et al.*, 2017). BAPC-assisted delivery of CRISPR/Cas9 components into nymph and adults successfully induced indel mutations in the Asian citrus psyllid, *Diaphorina citri* (Wayne *et al.*, 2018). Furthermore, BAPC facilitated a greater than 10-fold increase in CRISPR gene editing (0.8%) in *N. vitripennis* eggs, relative to the ReMOT Control (0.0%) method (Chaverra-Rodriguez *et al.*, 2020). This GEF, however, is still much lower than that of the DIPA-CRISPR method, and whether BAPC can be combined with DIPA-CRIPSR to further enhance the efficiency remains to be further researched.

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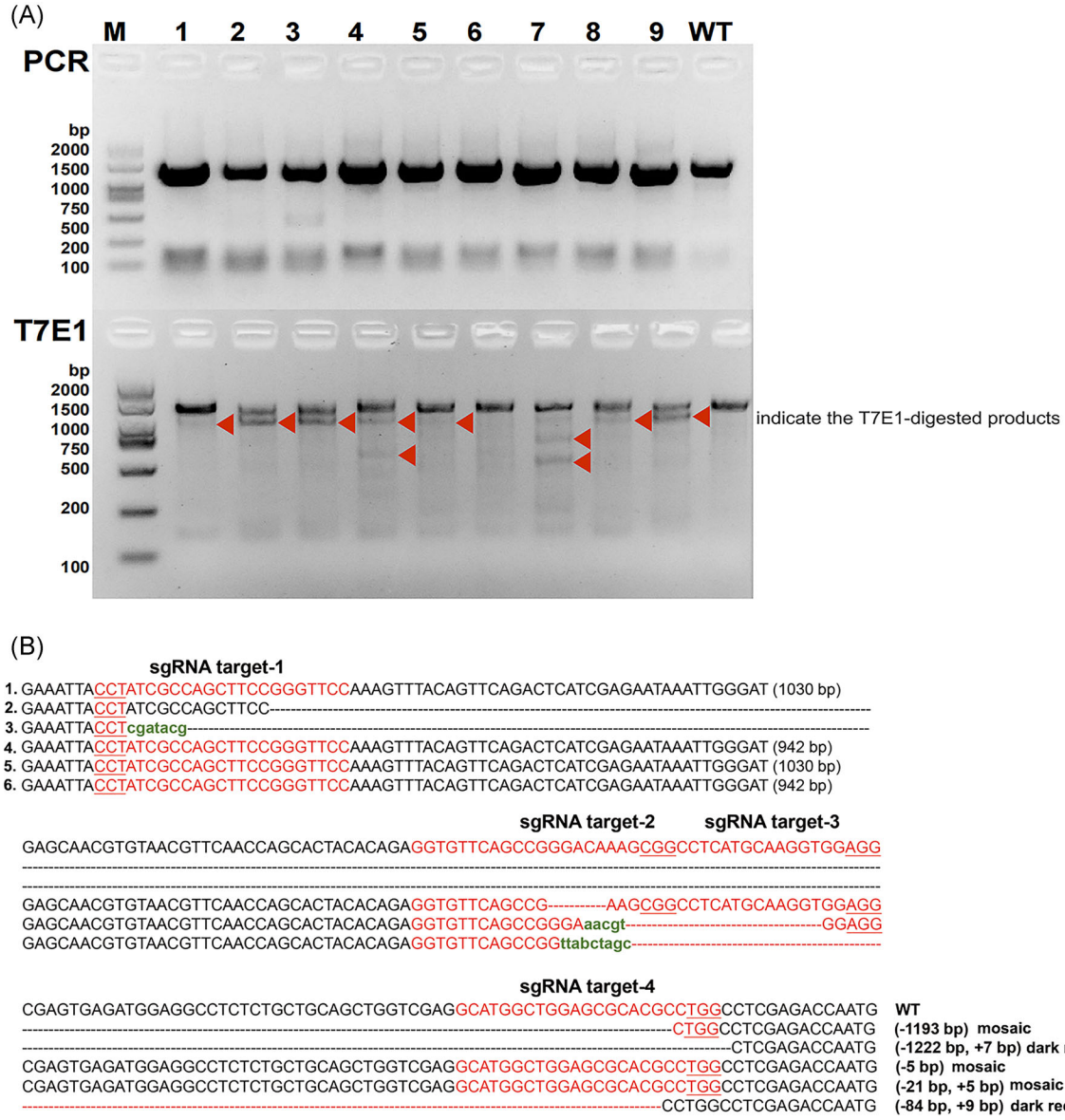

Fig. 4 Mutant G1 genotypes. (A) PCR amplification across the target region (upper panel) and associated *in vitro* T7E1 cleavage (lower panel). The band from the wild-type (WT) allele is shown on the right. The DNA marker is shown on the left. (B) DNA sequences of mutant alleles identified in multiple red-eyed mutants. The red font indicates the single-guide RNAs (sgRNA) target sites and the protospacer adjacent motif (PAM) sites are underlined. The dashed lines indicate deletions and the green font indicates base-pair insertions. The values indicate the length of the detection region in *Sf-TDO*.

Although embryonic CRISPR-Cas9 injection has been successfully used for knockout or knockin events in rice planthoppers (Xue *et al.*, 2018; Zhao *et al.*, 2019; Gao *et al.*, 2019a; Chen *et al.*, 2021; Wang *et al.*, 2022; Liu *et al.*, 2023; Zhang *et al.*, 2023), the embryo hatching rates remain quite low, probably as a result of damage to the chorionic shell. The GEFs for *cinnabar* and *white* in G0 *N. lugens* were 48.8% and 3.2%, respectively, whereas the corresponding hatching rates were less than 3.5% (Xue *et al.*, 2018). The application of an alternative embryo incubation method in *N. lugens* yielded a 9.5% GEF and a 5.8% hatch rate (Chen *et al.*, 2021). An agarose-based egg-laying method was used to obtain a 32% GEF and a 28% hatch rate in *Peregrinus maidis* (corn planthopper) (Klobasa *et al.*, 2021). In *Laodelphax striatellus* (small brown planthopper), researchers were

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able to obtain a relatively high mutation rate of 75%, but at the cost of a 4% hatch rate (Wang *et al.*, 2022). For the DIPA-CRISPR method, noticeable impacts of female *B. germanica* age at injection (e.g., 4- and 5-d-old females) on survival, which ranged from 80% to 100%, were reported (Shirai *et al.*, 2022). In this study, we successfully adapted DIPA-CRISPR to introduce heritable indel mutations in WBPHs with high efficiency and determined that 2 d posteclosion was the optimal age for introducing CRISPR-based mutations. This relatively simple approach, which can yield high-efficiency gene editing in WBPHs, can be a powerful tool for functional gene research in nonmodel insect species, and may represent a new approach for more difficult gene editing manipulation techniques, like gene knock in.

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Disclosure

The mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply a recommendation or endorsement by the US Department of Agriculture (USDA). The USDA is an equal opportunity provider and employer.

Data Availability Statement

The datasets used or analyzed during the current study are available from the corresponding author, upon reasonable request.

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