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Research

Population Biology/Genetics

ReMOT Control Delivery of CRISPR-Cas9 Ribonucleoprotein Complex to Induce Germline Mutagenesis in the Disease Vector Mosquitoes *Culex pipiens pallens* (Diptera: Culicidae)

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Abstract

The wide distribution of *Culex* (*Cx.*) *pipiens* complex mosquitoes makes it difficult to prevent the transmission of mosquito-borne diseases in humans. Gene editing using CRISPR/Cas9 is an effective technique with the potential to solve the growing problem of mosquito-borne diseases. This study uses the ReMOT Control technique in *Culex pipiens pallens* (L.) to produce genetically modified mosquitoes. A microinjection system was established by injecting 60 adult female mosquitoes—14 µl injection mixture was required, and no precipitation occurred with ≤1 µl of endosomal release reagents (chloroquine or saponin). The efficiency of delivery of the P2C-enhanced green fluorescent protein-Cas9 (P2C-EGFP-Cas9) ribonucleoprotein complex into the ovary was 100% when injected at 24 h post-bloodmeal (the peak of vitellogenesis). Using this method for *KMO* knockout, we found that gene editing in the ovary could also occur when P2C-Cas9 RNP complex was injected into the hemolymph of adult *Cx. pipiens pallens* by ReMOT Control. In the chloroquine group, of the 2,251 G_0 progeny screened, 9 individuals showed with white and mosaic eye phenotypes. In the saponin group, of the 2,462 G_0 progeny screened, 8 mutant individuals were observed. Sequencing results showed 13 bp deletions, further confirming the fact that gene editing occurred. In conclusion, the successful application of ReMOT Control in *Cx. pipiens pallens* not only provides the basic parameters (injection parameters and injection time) for this method but also facilitates the study of mosquito biology and control.

Key words: CRISPR/Cas9, gene editing, ReMOT control, *Culex pipiens pallens*

Mosquitoes within the *Culex* (*Cx.*) *pipiens* complex are widespread and abundant and can act as vectors of viruses that cause several important diseases, including Japanese encephalitis virus, West Nile virus, Sindbis virus, and Usutu virus (Karthika et al. 2018, Leggewie et al. 2018). The lack of vaccines and increasing resistance of pathogens and disease vectors necessitate the use of alternative strategies to control disease vectors (Lucas et al. 2015). Nowadays, the development of transgenic mosquito strains with beneficial phenotypes is coupled with conventional control methods for mosquito-borne diseases such as malaria and dengue (Basu et al. 2015, Buchman et al. 2020). The CRISPR/Cas9 system is useful in mosquitoes for genetic manipulations that enables research on mosquito biology and pathogen transmission, and it is used to develop efficient gene drive systems for mosquito and pathogen control (Gantz et al. 2015, Hammond et al. 2016, Kyrou et al. 2018). The classical CRISPR/Cas9 system relies on embryo microinjection; it requires specialized equipment and training, which limits the application of this technique in mosquitoes, especially in *Culex* mosquitoes, because the freshly laid eggs by *Cx. pipiens pallens* are fragile and in the form of an egg raft with small volume. Embryo microinjection is not only difficult to perform but also destroys the original structure of the egg raft, resulting in an extremely high mortality rate (Itokawa et al. 2016, Grigoraki et al. 2017, Anderson et al. 2019). The limited number of offspring available for screening greatly compounds the difficulty of gene editing. ReMOT Control is a method based on fusion of Cas9 with a P2C ligand, which has been successfully used for the delivery of gene-editing moieties through the hemolymph to the ovaries in *Aedes* (*Ae.*) *aegypti* and *Anopheles* (*An.*) *stephensi* (Chaverra-Rodriguez et al. 2018, Macias et al. 2020). Therefore, these studies can be considered as proof-of-principle experiments

which demonstrated that heritable mutations can be achieved by injecting adults with these moieties by ReMOT Control instead of using embryo microinjection.

To date, the gene-editing method extended to *Culex* mosquitoes was not established. The ReMOT Control technique will help overcome the challenges in gene editing in disease vectors. In this study, we modified the injection conditions making the application of this method more convenient and improve the efficiency of delivery into the ovary. We used the ReMOT Control technique to efficiently edit the gene kynurenine monooxygenase (*KMO*) in the disease vector mosquitoes *Cx. pipiens pallens*.

Methods

Mosquitoes

Culex pipiens pallens were reared at 28–30°C, 75 ± 5% relative humidity, and a constant light/dark cycle (14 h: 10 h) in a walk-in environmental chamber. Larvae were raised in water and fed daily with ground mouse food pellets. Adults had ad libitum access to 5% sterilized sugar on a sponge wick. Before the injection experiments, 3 d post-eclosion (3 d PE) mated female mosquitoes were fasted for 24 h and then blood-fed on Institute of Cancer Research mice for approximately 3 h to initiate vitellogenesis. All laboratory blood-feeding procedures with mice were approved and monitored by The National Science and Technology of China and People's Government of Jiangsu Province Animal Care and Use Committee and Institutional Review Board (No. IACUC-1812047).

Adult Female Injection Mixture Preparation

The expression of the following proteins and determination of their concentration was performed by GenScript (Nanjing, China). P2C-Cas9 and P2C-enhanced green fluorescent protein-Cas9 (P2C-EGFP-Cas9) proteins were expressed using the plasmids pET28a-P2C-Cas9 and pET28a-P2C-EGFP-Cas9 (received from Prof. Rasgon, Pennsylvania State University), respectively (Chaverra-Rodriguez et al. 2018). Single-guide RNAs (sgRNAs) were designed and synthesized according to the protocol described by Kistler et al. (2015). sgRNAs were synthesized using a PCR template amplified using CRISPR-F primers and CRISPR-R primers designed for each *KMO* target sequence, which are listed in Table 1 (Anderson et al. 2019). sgRNAs were synthesized from the purified PCR template by in vitro transcription using MegaScript T7 kits with a T7 promoter; the minimal sequence requirement was TAATACGACTCACTATAGG (Ambion, USA). sgRNAs were purified using phenol: chloroform extraction and isopropanol precipitation according to the manufacturer's instructions of the MEGAscript Kit. In addition to P2C-Cas9 and sgRNAs, saponin (50 mg/L; Purity: >98%, MCE, USA) or chloroquine (2 mM, MCE, USA) was included as an endosomal release reagent (ERR). The ERR was freshly diluted in normal saline (NS) immediately before each injection. The P2C-Cas9/P2C-EGFP-Cas9 (6.0–6.35 μg/μl) complex with sgRNAs (>1.0 μg/μl) was in the same dialysis buffer (50 mM Tris–HCl, pH 8.0, 300 mM KCl, 0.1 mM EDTA, 0.5 mM phenylmethanesulfonyl fluoride) as that used in previous experiments. To avoid precipitation upon addition of ERR, a low volume (<1 μl) of a higher concentration of ERR was used (total volume = 14 μl).

Table 1. List of oligonucleotides used to generate sgRNAs

[a]sgRNA primer sequence 5′-3′	
sgRNA935 F	GAAAT**TAATACGACTCACTATAGG***ACAGTGCG GTCCGCAAGGG*TTTTAGAGCTAGAAATAGC
sgRNA936 F	GAAAT**TAATACGACTCACTATAGG***CCAGACGT ACATCGAGCA*GTTTTAGAGCTAGAAATAGC
*sgRNA938 F	GAAAT**TAATACGACTCACTATAGG***ATCATCATG AACTTGCCGG*TTTTAGAGCTAGAAATAGC
sgRNA R	AAAAGCACCGACTCGGTGCCACTTTTTCAAGT TGATAACGGACTAGCCTTATTTTAACTTGCT ATTTCTAGCTCTAAAAC

[a]T7 promoter sequence is indicated in bold; sgRNA target site is italics.
* indicates that sgRNA targets the reverse stand.

RNA Extraction, cDNA Synthesis, and Quantitative Real-Time PCR

Total RNA was extracted using RNAiso Plus reagent (TaKaRa, Dalian, China) from 3 d PE and 12, 24, 36, 48, 60, and 72 h post-bloodmeal (PBM) female mosquitoes. The cDNA was synthesized from total RNA using the PrimeScript Master Mix (TaKaRa, Dalian, China) according to the manufacturer's instructions.

The quantitative real-time PCR (qRT-PCR) was performed with a QuantStudio 5 Real-Time PCR instrument (384-well Block, Applied Biosystems, California) using EvaGreen 2×qPCR MasterMix-Low ROX (ABM, CAN). qRT-PCR primers specific for *vitellogenin-A1* (*Vg-A1*, *CpipJ_CPIJ010190*) and β-*actin* were designed using Primer Premier 6.0 software (Table 2). The PCR reactions were performed in a 384-well optical plate using the following thermal conditions: 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The raw threshold cycle (Ct) values were used to quantify the target gene expression for each sample. The relative expression levels were normalized to the internal control β-*actin* by using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). Three technical and biological replicates were performed for qRT-PCR.

Microinjections

Microinjections were performed using an aspirator tube assembly Nanoject III (Cat. No. 3-000-207, Drummond) fitted with a glass capillary needle (3.5", Drummond) using a needle puller (Sutter P-97). Before injection, 24 h PBM females from the same cage were evenly and randomly divided into the following experimental groups: In the experiment "Delivery efficiency of P2C-EGFP-Cas9 cargo into mosquito oocytes", Group 1: Wild-type (WT); Group 2: P2C-EGFP-Cas9 + sgRNAs + chloroquine; Group 3: P2C-EGFP-Cas9 + sgRNAs + saponin. In the experiment "*KMO* gene knockout by ReMOT Control", Group 1: WT; Group 2: P2C-Cas9 + sgRNAs + chloroquine; Group 3: P2C-Cas9 + sgRNAs + saponin. Adult females were immobilized by cold shock at −20°C for 3–5 min until motionless, then kept on ice, and transferred to a dry bath incubator at a constant temperature 0°C (OSE-100C, TIANGEN, China) during injection. Sixty adult female mosquitoes were ready to be injected. The injection mixture was preferably 14 μl (the volume of ERR ≤1 μl). Females with visible blood meals were intrathoracically injected with approximately 200 nl injection mixture per female

Table 2. Primers for qPCR and PCR in *Culex pipiens pallens*

primer sequence 5′-3′	
β-*actin* F	AGCGTGAAACTGACGGCTCTTG
β-*actin* R	ACTCGTCGTACTCCTGCTTGG
Vg-A1 F	ACCTTCACCGGTGTGTACAAGGTC
Vg-A1 R	TTCCGGGACAACGTTCACATCGTA
KMO F	TTGTTGAGTGAGTTTTCGCAATG
KMO R	GTAGAAGTTGGTGAACGGCAT

Table 3. Delivery efficiency of P2C-enhanced green fluorescent protein (EGFP)-Cas9 ribonucleoprotein with single-guide RNA and ERR

Culex pipiens pallens	G_{-1}								
	#Inj. Mix			Inj. time (PBM)	No. of inj. (♀)	No. of survived females (♀)	Survival rate (%)	Dissected time (PBM)	No. of dissections	No. of ovaries with EGFP (%)
	Protein	sgRNAs	ERR							
	C (μg/μl)	C (μg/μl)	C							
6 (7 μl)			Chl. 2 mM (1 μl)	24 h	16	9	56.3	72 h	9	100
		[a]1.23 [b]2.18								
6 (7 μl)		[c]3.10 (2 μl*3)	Sa. 50 mg/L (1 μl)	24 h	31	13	41.9	72 h	13	100

Chl., Chloroquine; Sa., Saponin; ERR, endosomal release reagent; PBM, post-bloodmeal.
#Injection mixture (Inj. Mix) = P2C-Cas9+sgRNAs+ERR (Chloroquine or Saponin).
[a]sgRNA935; [b]sgRNA936; [c]sgRNA938.

until visible distention of the abdomen, diuresis, or liquid emerging from the injection site was observed.

Adult Female Dissection and Imaging

We first prepared the glass slides with SlowFade Gold antifade agent (Invitrogen) in the center of permanent reinforcement labels (Office Depot). Female mosquitoes (72 h PBM) were immobilized by freezing at –20°C for 5 min and placed on a dry bath incubator (OSE-100C, TIANGEN, China) and dissected under an Olympus XZ61 microscope. Intact ovaries were immediately transferred to the prepared glass sides and covered with a cover glass. To visually confirm the expression of EGFP in the ovaries following microinjection was visually confirmed by imaging under an Olympus BX10 fluorescent microscope (exposure time = 333.3 ms; magnification 40×).

Screening

After mating, females (G_{-1}) were blood fed and, at 24 h PBM, were injected with Cas9 fusion protein-ribonucleoprotein (RNP) complexes and ERRs. In total, 58 and 50 females were injected with P2C-Cas9 + sgRNAs + chloroquine and P2C-Cas9 + sgRNAs + saponin, respectively. The resulting offspring (G_0) were screened for the presence of white or mosaic eyes (*KMO* gene-editing phenotypes). G_0 were screened at pupal and adult stages. Pupae and adults were immobilized by transferring to the dry bath incubator at a constant temperature 0°C (OSE-100C, TIANGEN, China), visualized with an Olympus BX4 microscope and imaged on an Olympus BX10 microscope.

Molecular Analysis of Mutation

Genomic DNA from identified G_0 mosquitoes was extracted individually using the Dneasy Blood & Tissue Kits (Qiagen, Germany) following the manufacturer's protocol (Step 1 referred to as 1a.). Genomic DNA was used as a template for PCR using the Q5 enzyme (NEB, USA) and primers against the region spanning the nucleotides 935, 936, and 938 of *KMO*. The primers used here are listed in Table 2 (Anderson et al. 2019). Real-time PCR was performed with a Veriti 96 Well Thermal Cycler PCR System (Applied Biosystems, Thermo Fisher Scientific, USA). The reaction conditions were as follows: denaturing at 98°C for 30 s; followed by 35 cycles at 98°C for 5 s, 55°C for 20 s, and 72°C for 15 s; with a final extension at 72°C for 2 min. The PCR product was run on a 2% agar-sugar gel

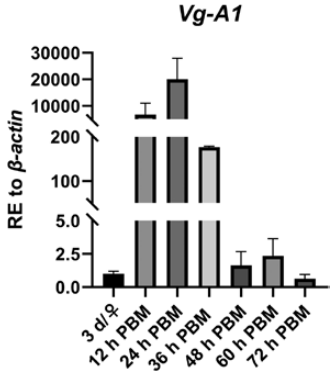

Fig. 1. The expression level of *vitellogenin A1* (*Vg-A1*) in mosquitoes at 3 d post-eclosion (3 d PE) and 12, 24, 36, 48, 60, and 72 h post-bloodmeal (PBM). Expression level of β-*actin* in the same replicate was considered as the background level. Results were expressed as mean ± standard error (SE) of three independent experiments.

at 150 V for 25 min. Diagnostic gel bands were extracted using the QIAquick Gel Extraction Kit (Qiagen, Germany) and cloned into a pClone007 blunt vector (TSINGKe, China). At least six clones per individual were randomly selected and sequenced by General Boil Company (China). Sequencing data were analyzed using SnapGene software.

Results and Discussion

Modifications of Injection Parameters and Injection Time

For the successful application of ReMOT Control in gene editing for mosquitoes, the injection parameters, including injection mixture and timing, needed to be modified. The injection mixture was composed of P2C-Cas9 or P2C-EGFP-Cas9 RNP complex, sgRNAs, and ERR. The difficulty in the preparation of injection mixture lies in solving the precipitation problem—the white flocculent precipitation (it may be the Cas9 RNP), which may affect the efficiency of gene editing. We calculated the delivery efficiency by estimating the proportion of eggs in the ovaries with visible EGFP protein. Adjustment of the ERR concentration in the gene-editing moieties could be a much faster and simpler method for the first-time user of ReMOT Control, which we addressed by using a low volume of ERR. Initially, we used two different concentrations of ERRs (chloroquine [2 mM and 4 mM]; Saponin [50 mg/liter or 100 mg/liter]). However, the higher concentration did not increase the efficiency of gene editing but increased the mortality rate. Therefore, we used 2 mM for chloroquine and 50 mg/liter for saponin as the final concentration. ERR can significantly improve the efficiency of gene editing. There is a need to identify more substances that can be used as protective agents, such as those used in cationic lipid-mediated delivery into eukaryotic cells (Shi et al. 2001). Based on the mechanistic understanding of gene delivery mediated by highly efficient multicomponent envelope-type nanoparticle systems, the physical state of ReMOT Control injection moieties can also be altered while ensuring activity, using methods such as nanotechnology, which makes the application of this method more convenient (Pozzi et al. 2013).

Injection time relative to bloodmeal has been reported to affect the genome modifications in *Ae. aegypti* and *An. stephensi*. The optimal timing lies between 24 h PBM and 48 h PBM. After 48 h PBM, the proportion of EGFP observed in the ovaries was greatly reduced likely because the peak of vitellogenesis was past (Chaverra-Rodriguez et al. 2018, Macias et al. 2020). To determine the best injection time, we measured the expression level of *vitellogenin* in *Cx. pipiens pallens*. We found that the expression level was the highest at 24 h PBM (Fig. 1). Therefore, *Cx. pipiens pallens* females were injected at 24 h PBM for gene editing to achieve a higher transport efficiency and thus a higher gene-editing efficiency and to ensure a balance between survival and egg laying. The survival rate was 56.3% (chloroquine: 9/16) and 41.9% (saponin: 13/31) in mosquitoes injected with P2C-EGFP-Cas9 RNP + sgRNA + ERR (Table 3), and the oviposition rate was 62.1% (chloroquine: 36/58) and 78% (saponin: 39/50) in mosquitoes injected with P2C-Cas9 RNP + sgRNA + ERR (Table 4). Because the number of survived females was lower than the number of injected females, the actual oviposition rate is the number of ovipositing females divided by the number of survived females, suggesting that the true oviposition rate is higher than the rate mentioned above (we did not record statistics on survived females). Our experimental modifications in *Cx. pipiens pallens* are valuable because the method can be applied to another species.

Table 4. ReMOT Control gene editing with optimized injection conditions for P2C-Cas9 ribonucleoprotein, single-guide RNA, and ERR at 24 h PBM

Culex pipiens pallens	G_{-1}						G_0			
	[#]Inj. Mix			Inj. time (PBM)	No. inj. (♀)	No. of ovipositing females (♀)	[e]Oviposition rate (%)	No. of larvae hatched	No. of offspring with mosaic eyes	No. of offspring with white eyes	EEF (%)	GEF (%)
	P2C-Cas9	[d]sgRNAs	ERR									
	C (μg/μl)	C (μg/μl)	C									
Adult injections: P2C-Cas9/sgRNA/ERR mix injections into wild-type females crossed with wild-type males												
	6.35 (7 μl)	[a]2.26	Chl. 2 mM (1 μl)	24	58	36	62.1	2,251	8	1	25	0.4
		[b]1.82										
	6.35 (7 μl)	[c]1.02	Sa. 50 mg/liter (1 μl)	24	50	39	78	2,462	7	1	20.5	0.32
		2 μl/*3										

Chl., chloroquine; Sa., saponin; ERR, endosomal release reagent; PBM, post-bloodmeal; EEF, effort efficiency; GEF, G_0 gene-editing efficiency.
[#]Injection mixture (Inj. Mix) = P2C-Cas9 + sgRNAs + ERR (chloroquine or saponin)
[a]sgRNA935; [b]sgRNA936; [c]sgRNA938.
[d]sgRNAs were mixed together to generate *KMO* knockout mosquitoes; the target mutation site was sgRNA935 in this experiment.
[e]Oviposition rate (%) = No. of ovipositing females/ No. of injected females; however, it is not exact because the number of surviving females is lower than the number of injected females. The exact oviposition rate is the number of ovipositing females divided by the number of surviving females, suggesting that the true oviposition rate is higher than the rate mentioned in Table 4.

Delivery Efficiency of P2C-EGFP-Cas9 cargo Into Mosquito Oocytes

To achieve heritable mutagenesis in *Cx. pipiens pallens* using the ReMOT Control technique, we first verified the delivery efficiency of P2C-EGFP-Cas9 cargo into mosquito oocytes. The injection mixture was injected into the thorax of females at 24 h PBM. Ovaries dissected at 72 h PBM were examined for visible EGFP fluorescence (Fig. 2A). An EGFP signal with strong intensity was observed in 100% of the *Cx. pipiens pallens* primary oocytes, regardless of the ERR used (chloroquine: 9/9; saponin: 13/13; Fig. 2B; Table 3). We found the delivery efficiency of P2C-EGFP-Cas9 cargo into mosquito oocytes was 100%, which was higher than that in *Ae. aegypti* and *An. stephensi*, in which EGFP was visualized in >88% of primary oocytes within 48 h PBM (Chaverra-Rodriguez et al. 2018, Macias et al. 2020). Therefore, the presence of EGFP in all oocytes suggested that 24 h PBM is the optimal time for injection.

KMO Gene Knockout by ReMOT Control

The target gene of the sgRNAs-*KMO*-used in *Cx. pipiens pallens* gene-editing experiments has already been validated in a highly homologous species, *Cx. quinquefasciatus*, by embryo microinjection, allowing a direct investigation of the efficiency of ReMOT Control in the present study (Anderson et al. 2019). After mating, 24 h PBM females (G_{-1}) were injected with P2C-Cas9 and ERRs (Fig. 3A). The females showed a sufficiently high oviposition rate (chloroquine: 62.1%; saponin: 78%) to allow the screening of an adequate number of G_0 progeny (Table 4). The G_0 progeny were positive for either white or mosaic eye phenotype or wild-type eye phenotype. The mutant phenotypes from successful editing are expected to be white and mosaic eyes. The efficiency of ReMOT Control-mediated gene editing was also calculated and compared using two measures introduced by Chaverra-Rodriguez et al. (2018): 1) effort efficiency (EEF; the ratios of the number of gene-edited G_0 progeny to the number of individuals injected); 2) G_0 gene-editing efficiency (GEF; the ratio of the number of gene-edited G_0 progeny to the total number of larvae hatched). We recovered white and mosaic G_0 individuals from the two experimental groups. In the chloroquine group, of the 2251 G_0 progeny screened, 9 individuals showed the white and mosaic phenotypes (number of injected females = 58; EEF = 25%; GEF = 0.4%). In the saponin group, of the 2462 G_0 progeny screened, eight mutant individuals were recovered (number of injected females = 50; EEF = 20.5%; GEF=0.3%; Fig. 3B; Table 4).

Our results showed that gene editing by ReMOT Control of *KMO* in the females results in a mutant progeny with the white or

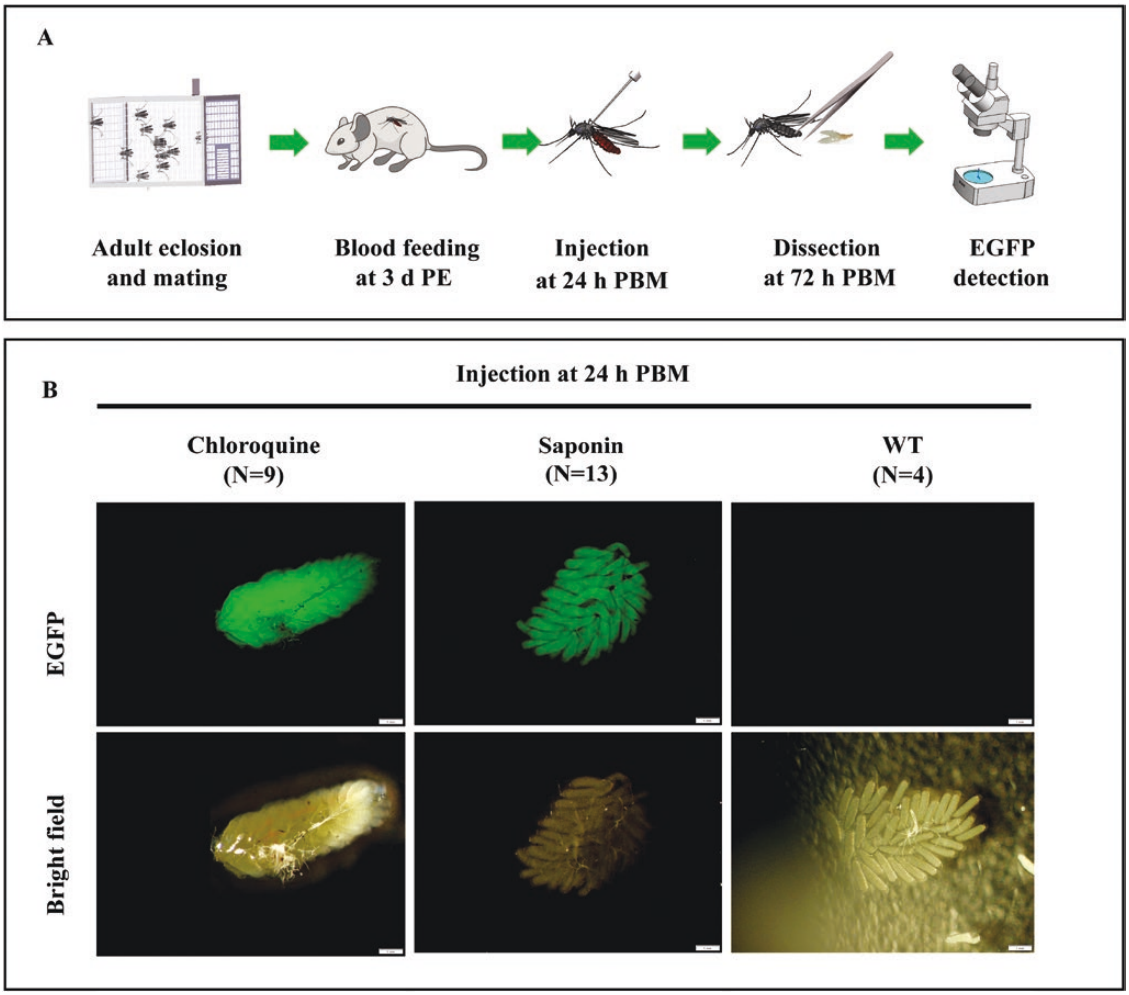

Fig. 2. Efficiency of delivery of P2C-EGFP-Cas9 cargo into mosquito oocytes. A: The procedure of EGFP detection; Mating female mosquitoes were blood-fed at 3 d post-eclosion (3 d PE), injected intrathoracically at 24 h post-bloodmeal (PBM) and dissected at 72 h PBM for enhanced green fluorescent protein (EGFP) detection. B: Transduction of P2C-EGFP-Cas9 RNP complex into *Culex pipiens pallens* ovaries. From left to right: P2C-EGFP-Cas9 + sgRNAs + chloroquine ($n = 9$); P2C-EGFP-Cas9 + sgRNAs + saponin ($n = 13$); Wild-type (WT; $n = 4$). Scale bar: 1 mm. RNP, ribonucleoprotein; sgRNAs, single-guide RNAs.

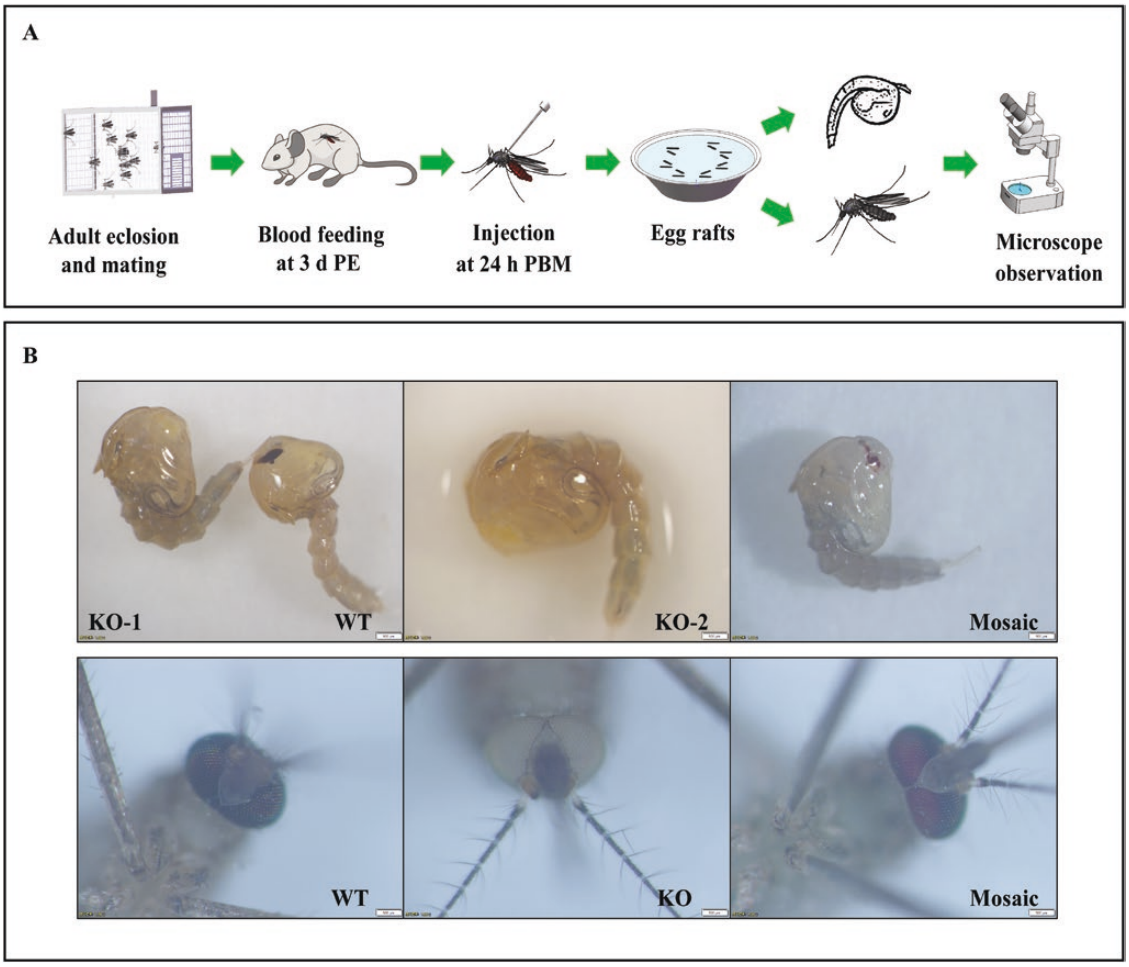

Fig. 3. Kynurenine monooxygenase (*KMO*) gene knockout by ReMOT Control. (A)The procedure of obtaining and observing G_0 mosquitoes; 24 h post-bloodmeal (PBM) female mosquitoes were intrathoracically injected with P2C-Cas9 + sgRNAs + chloroquine and P2C-Cas9 + sgRNAs + saponin and allowed to lay eggs; then, we observed the eye phenotypes in the pupal and adult stages of G_0. (B) Observation of G_0 pupal and adult phenotypes (WT, wide type; KO, knockout). From top to bottom:The G_0 phenotypes of pupae and adults obtained after injection of P2C-Cas9 + sgRNAs + chloroquine and P2C-Cas9 + sgRNAs + saponin into *Culex pipiens pallens* females. RNP, ribonucleoprotein; sgRNAs, single-guide RNAs.

mosaic eye phenotype. The EEF was higher in *Cx. pipiens pallens* than in *Ae. aegypti* and *An. stephensi* injected at 24 h PBM with ERR; however, the GEF values were lower (Chaverra-Rodriguez et al. 2018, Macias et al. 2020). The ReMOT Control technique used in *Culex* mosquitoes was similar to the technique used in *Ae. aegypti* and *An. stephensi* and showed a gene-editing efficiency similar to that of standard embryo microinjections (Anderson et al. 2019). Embryo microinjection is significantly challenging owing to the low integrity of the extremely small embryos and high mortality of injected eggs. Recently, this method was successfully used in the silverleaf whitefly *(Bemisia tabaci)*, with the only change being the use of BtKV instead of P2C as the ovary-targeting ligand (Heu et al. 2020). During vitellogenesis, the multiple powerful receptor-mediated yolk protein precursors (YPPs) uptake in the oocyte membrane machinery accumulated in endosomal vesicles, and sorted in yolk granules hold the potential for targeted gene delivery to developing oocytes (Sappington and Raikhel 1998). Therefore, the extensive background in receptor-mediated YPP endocytosis and the essential role of vitellogenin receptors in ovary development suggests that, with the correct ligand, transduction of Cas9 RNP into the ovaries of females using the ReMOT Control technique has the potential to be used in most species (Cong et al. 2015).

Molecular Detection of Mutation

A primer pair targeting a 476 bp segment spanning all three sgRNA target sites in *KMO* was used to characterize the modifications in *KMO*. PCR and further sequencing confirmed the presence of the lesion at the original locus; a 476 bp diagnostic band was observed and purified for cloning to identify the mutations in white-eyed mosquitoes (Fig. 4A). The sequencing results were validated as intact, indicating a deletion of 13 nucleotides of the sequence (Fig. 4B). These results suggest that deletions occurred commonly with the use of ReMOT Control in *Cx. pipiens pallens*.

We studied the phenotype of the G_0 offspring of *KMO* knockouts. There were two mutant phenotypes: white eyes, which represented a complete gene knockout due to the Cas9-induced DNA cleavage in the oocyte occurring before embryogenesis, and mosaic eyes, which was a heterozygous phenotype, indicating that targeted editing may occur both before and after embryogenesis.

In these applications, especially for genes that are not associated with visible phenotypes, PCR-based methods or homologous repair templates to insert fluorescent screening tags have to be used to identify the mutants. Sequencing results showed a consecutive 13 bp fragment deletion in knockout *Cx. pipiens pallens*, further confirming that this method could indeed produce genetically modified

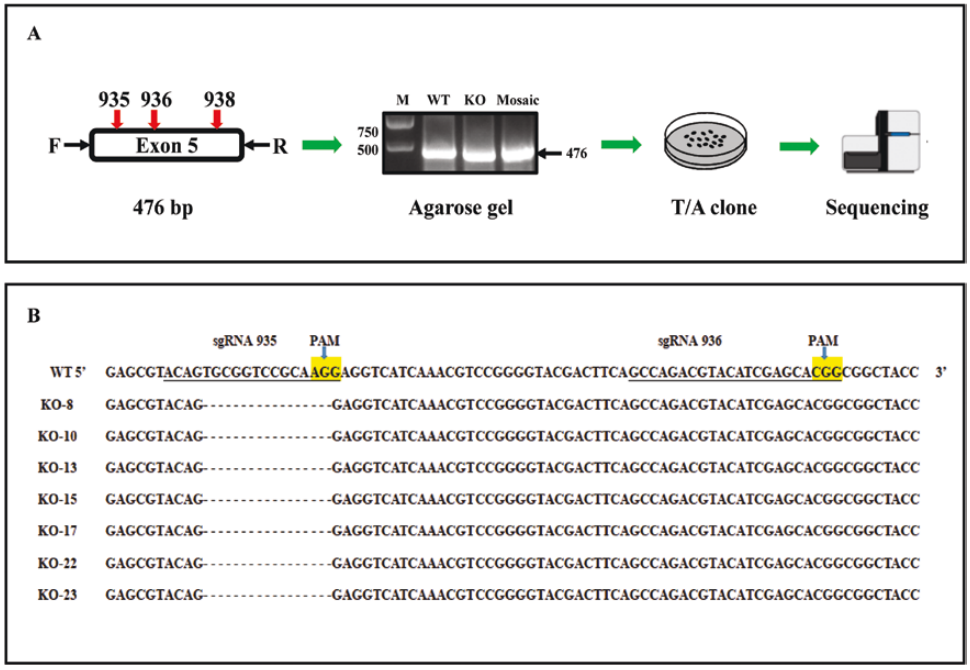

Fig. 4. Molecular detection of mutation. (A) The procedure for mutation site detection; the purified PCR product was used for cloning and sequencing. (B) Genotypes of the G_0 *KMO* knockout (KO) adults.

mosquitoes. This study provides some evidence for the efficiency of the ReMOT Control technique in producing genetically modified mosquitoes. Further research is needed for the ReMOT Control to become a commonly used tool for gene editing in *Culex* mosquitoes and other species.

Taken together, the findings suggest that the ReMOT Control technique eliminates these constraints, thus enabling any laboratory to apply CRISPR techniques. In our opinion, such developments, together with the simple set of modifications for use in *Cx. pipiens pallens* demonstrated herein, are significant in the molecular biology research in this organism and will lead to the use of P2C-Cas9 in genetic studies of disease vectors and other species currently recalcitrant to gene-editing techniques and the development of novel control strategies for this harmful arboviral host.

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Author Contributions

XL, YX, HZ and HY performed the experiments. XL and YX wrote the manuscript and prepared the figures. XL, DZ, YS, LM, BS, and CZ conceived the idea and coordinated the project. All authors read and approved the final manuscript.

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