Engineered Promoter System Enables High-efficiency Transgenic CRISPR Editing in Malaria Transmitting Mosquito *Anopheles sinensis*

Jun-Feng Hong[1#], Qi-Li Zou[1#], Xin-Yuan Xie[1#], Yan-Ping Jiang[1], Si-Yi Wang[1], Xia Ling[1], Cao Zhou[1], Wei Sun[2], Xi Cai[1], Yong-Xin Yang[1], Yu Chen[1], Bin Chen[1*], Liang Qiao[1*]

[1]Chongqing Key Laboratory of Vector Control and Utilization, Institute of Entomology and Molecular Biology, College of Life Sciences, Chongqing Normal University, Chongqing, China; [2]Laboratory of Evolutionary and Functional Genomics, School of Life Sciences, Chongqing University, Chongqing, China.

∗ Corresponding authors. E-mail addresses: qiaoliangswu@163.com (L. Qiao), bin.chen@cqnu.edu.cn (B. Chen).

[#]These authors contributed equally to this work.

Running title: Transgenic CRISPR editing of *Anopheles sinensis*

Abstract: The CRISPR/Cas9 system deployed through crosses of transgenic lines expressing *Cas9* and *gRNA* facilitates efficient mutagenesis. However, its application in non-model insects remains limited, primarily due to a lack of well-characterized promoters capable of driving robust and stable expression of *Cas9* and *gRNA*. In the malaria mosquito *Anopheles sinensis*, we evaluated several ovary-biased promoters—*Asvasa2*, *Aszpg*, and *Asnanos*—for driving *Cas9* expression. Notably, the *Asvasa2* promoter mediated mutagenesis in nearly 60% of G_0 individuals following microinjection of $gRNA^{Aswhite}$. Among four RNA polymerase III promoters derived from *AsU6* genes, *AsU6*-1 yielded the highest *gRNA* transcriptional output, enabling 62% editing efficiency in G_0 offspring. In addition, hybrid crosses between established transgenic lines demonstrated that the *Asvasa2-Cas9* and *AsU6*-1-*gRNA* combination enabled complete germline editing penetrance, where all F_2 progeny inherited the intended mutations. This work provides a essential genetic toolkit for synthetic biology applications in *Anopheles* mosquitoes and a scalable framework for engineering other non-model insects.

Keywords: Promoter engineering; Transgenic system; CRISPR/Cas9 genome editing; Genetic modification; *Anopheles* mosquitoes

1. Introduction

Mosquitoes are major vectors of severe infectious diseases, including malaria and dengue fever, representing a persistent public health challenge worldwide[1]. Beyond their medical significance, mosquitoes serve as important systems for fundamental biological research, contributing to studies on chemosensation, swarming mating dynamics, host–pathogen interactions, and innovative genetic control strategies[2-4]. The CRISPR/Cas9 genome editing system has emerged as a pivotal tool for dissecting molecular mechanisms, evolutionary adaptations, and for developing novel genetic interventions in mosquitoes[5]. However, the conventional approach of microinjecting Cas9-*gRNA* ribonucleoprotein (RNP) complexes into

embryos suffers from the absence of selectable markers, making the screening of edits inefficient in the absence of visible phenotypes. Moreover, this method is hampered by labor-intensive procedures and high embryonic mortality[6, 7]. Additionally, RNP-based editing lacks the spatiotemporal precision necessary for stage- or tissue-specific genetic modification.

In established model mosquitoes such as *Anopheles gambiae* and *Aedes aegypti*, these limitations have been overcome through the development of stable transgenic lines that express *Cas9* and *gRNA* endogenously, often coupled with dual visible markers to facilitate identification of edited progeny[8, 9]. This strategy depends critically on the availability of germline-biased promoters for *Cas9* and RNA polymerase III promoters for *gRNA* expression. The selection of species-specific promoters with high transcriptional activity is therefore essential for robust *in vivo* expression of CRISPR components[10, 11]. Nevertheless, such well-characterized regulatory elements remain scarce for many non-model yet medically important mosquito species, including *Anopheles sinensis*, significantly impeding progress in functional genetics and targeted control strategies in these vectors.

Here, we developed and characterized germline-biased promoters and *U6* promoters capable of driving *Cas9* and *gRNA* expression in *Anopheles sinensis*. Using these regulatory elements, we established transgenic lines harboring stably integrated *Cas9* and *gRNA* expression cassettes and demonstrated that crosses between these lines achieve 100% germline editing efficiency, providing a robust and heritable genome editing platform for this neglected malaria vector.

2. MATERIALS and METHODS

2.1 Mosquito strains

The *Anopheles sinensis* LS-WX strain, inbred for ~50 generations, was maintained at the Chongqing Key Laboratory of Vector Control and Utilization. Mosquitoes were reared at 27 ± 1 ℃, 75 ± 5% relative humidity, and a 12:12 h light-dark cycle.. Larvae were fed powdered fish food in distilled water. Pupae were transferred to mesh-covered containers for emergence. Newly emerged adults were provided with 10% glucose solution, and blood meals were administered via mice on the third day post-eclosion.

2.2 Expression pattern analysis of germline-biased genes

Asvasa2, *Aszpg*, and *Asnanos* were selected as candidate germline-active genes in *Anopheles sinensis*. To characterize their expression patterns, samples from multiple developmental stages and tissues of the LS-WX strain were collected, including: eggs at 0, 24, and 48 h post-oviposition (from females 3 days after blood feeding); first to fourth instar larvae (feeding and molting stages of the fourth instar); male and female pupae at 0, 12, 24, and 36 h; virgin females at 0, 24, 48, and 72 h post-emergence; blood-fed virgin females at 0, 6, 12, 24, 36, and 48 h post-feeding; and unmated males at 0, 24, 48, 72, 73, 79, 85, 97, 109, and 121 h post-emergence. Additional samples included ovaries and carcasses from blood-fed females(48 h) and testes and carcasses from 3-day-old males. Total RNA was extracted using TRIzol reagent (Invitrogen, USA), and cDNA was synthesized with PrimeScript RT reagent Kit with gDNA Erase (RR047, Takara, Japan). Gene expression was quantified in three biological replicates, with *AsRPL49* was used as the internal control. Primer sequences are provided in Table S14.

2.3 Gene identification and regulatory region selection

Homologs of *Agvasa2* (*AGAP008578*), *Agzpg* (*AGAP006241*), and *Agnanos* (*AGAP006098*) in *Anopheles gambiae* were identified in *Anopheles sinensis* as *Asvasa2*, *Aszpg*, and *Asnanos*, respectively. For each gene, the upstream regulatory region was defined as at least the sequence from its start codon to the transcription start site of the nearest upstream gene. (Fig. S1). The 3′ untranslated regions (3′ UTRs) were obtained by using GeneRacer Advanced RACE Kit (L150201, Thermo Fisher Scientific, USA), and sequences containing at least the complete 3′ UTR were used as terminators. Primers are listed in Table S14.

AsU6 genes were identified by homology to *AgU6*. A 600 bp upstream region was screened for cis-regulatory elements, and sequences containing both a proximal sequence element and a *TATA* box were selected as candidate promoters(Gonzalez et al., 2025).

2.4 Plasmid construction

To generate *Cas9* expression plasmids, promoters and corresponding terminators of *Asvasa2*, *Aszpg*, and *Asnanos* were amplified from *Anopheles sinensis* genomic DNA using PrimeSTAR high-fidelity DNA polymerase (R045A, Takara, Japan), based on the selected regulatory regions.. The *Cas9-T2A* fragment was amplified from *pAc-sgRNA-Cas9* (Addgene No: 49330), and the *DsRed* reporter gene from *pBac-3×P3*::*DsRed-SV40*. The backbone vector *pBac-3×P3*::*EGFP-SV40* was digested with *BglII* and assembled with the *Asvasa2* promoter, *Cas9-T2A*, *DsRed*, and *Asvasa2* terminator using the In-Fusion cloning kit (No. 638947, Takara, Japan), generating *pBac-3×P3*::*EGFP-SV40-Asvasa2*:: *Cas9-T2A-DsRed*-terminator. Using this plasmid as a template, *Cas9-T2A-DsRed* fragments were amplified and similarly cloned with the *Aszpg* or *Asnanos* promoters and terminators to produce *pBac-3×P3*::*EGFP-SV40-Aszpg*::*Cas9-T2A-DsRed*-terminator and *pBac-3×P3*::*EGFP-SV40-Asnanos*::*Cas9-T2A-DsRed*-terminator.

To construct *gRNA* plasmids targeting the kynurenine 3-monooxygenase (*Aswhite*) gene. 600 bp upstream regions of four *AsU6* genes, containing both a proximal sequence element and *TATA* box, were amplified from *Anopheles sinensis* genomic DNA, The *gRNA* expression unit targeting the validated *Aswhite* site was amplified from a synthesized *gRNA* scaffold template (Yang et al., 2024). The *pBac-3×P3*::*EGFP-SV40* vector was linearized with *BglII*, and the *AsU6* promoter and *gRNA* fragment were inserted via In-Fusion cloning to generate *pBac-3×P3*::*EGFP-SV40-AsU6s*::$gRNA^{Aswhite}$.

Following confirmation of editing efficiency mediated by germline-biased and *AsU6* promoters, an additional validated *gRNA* target site within the *Aswhite* (Yang et al., 2024) to construct dual-*gRNA* transgenic plasmids for further evaluation of the promoter-driven transgenic system. Fragments corresponding to *AsU6*-1::$gRNA^{Aswhite1}$ and *AsU6*-1::$gRNA^{Aswhite2}$ fragments were amplified from *pBac-3×P3*::*EGFP-SV40-AsU6*-1::$gRNA^{Aswhite}$ plasmid, while *AsU6* -2::$gRNA^{Aswhite1}$ and *AsU6*-2::$gRNA^{Aswhite2}$ were amplified from the *pBac-3×P3*::*EGFP-SV40-AsU6*-2::$gRNA^{Aswhite}$ plasmid. Both the *pBac-3×P3*::*DsRed-SV40-AsU6*-1::$gRNA^{Aswhite1}$-*AsU6*-1::$gRNA^{Aswhite2}$ and *pBac-3×P3*::*DsRed-SV40-AsU6* -2::$gRNA^{Aswhite1}$-*AsU6*-2::$gRNA^{Aswhite2}$ plasmids were constructed using the Golden Gate assembly kit (E1601S, NEB, USA), following the manufacturer's instructions. The assembly was performed using p*Bac-3×P3::DsRed* (*BsaI*)-*SV40* and the aforementioned amplified fragments as genetic elements.

All constructs were verified by Sanger sequencing (Sangon, China). Primer sequences are

listed in Table S14.

2.5 *In vitro* transcription of *sgRNA*

Templates for sgRNA synthesis were generated by annealing and extending primers encompassing the *T7* promoter, target *gRNA* sequence, and partial scaffold. Following purification, *sgRNAs* were transcribed in vitro using the *T7* RiboMAX™ Express RNAi System (P1700, Promega, USA) according to the manufacturer's instructions. Primer sequences are provided in Table S14.

2.6 Embryo microinjection

Three days after blood feeding, *Anopheles sinensis* females were allowed to oviposit. Embryos were aligned within 30 minutes post-oviposition and injected using a WPI PV830 microinjection system (WPI, USA).For *Cas9* transgenic line generation, the injection mix contained 150 ng/μL of the *pBac-3×P3*::*EGFP-SV40*- promoter::*Cas9-T2A-DsRed*-terminator plasmid and 300 ng/μL of a *helper* plasmid expressing piggyBac transposase. To establish the *AsU6*-1-*gRNA*^{Aswhite} and *AsU6*-2-*gRNA*^{Aswhite} lines, embryos were injected with 150 ng/μL of the donor plasmid *pBac-3×P3*::*DsRed-SV40 -AsU6*::*gRNA*^{Aswhite1}-*AsU6*::*gRNA*^{Aswhite}2 and 300 ng/μL of the *helper* plasmid.

For experiments assessing *Cas9* line efficiency, we accounted for the *X*-linked haploinsufficiency of the *Aswhite* gene (Yang et al., 2024), whereby female mosquitoes require mutations on both *X* chromosomes to display the white-eyed phenotype. To avoid phenotype masking (e.g., $X^{+}X^{W}$ females) and improve statistical accuracy, injections were performed in embryos from *Cas9* females × lab-reared *Aswhite* mutant males, using 1000 ng/μL *sgRNA*^{Aswhite1}. As a reference for comparison, embryos from wild-type females × lab-reared *Aswhite* mutant males were injected with Cas9-*sgRNA*^{Aswhite1} RNPs (1000 ng/μL *sgRNA*^{Aswhite1} + 300 ng/μL commercial Cas9 protein (GenScript Biotech, USA)).

To evaluate the transient expression efficiency of *AsU6-gRNA* plasmids, injections were performed in hybrid embryos derived from crosses between females of three *Cas9* transgenic lines (*Asvasa2-Cas9*, *Aszpg-Cas9*, and *Asnanos-Cas9*) and lab-reared *Aswhite* mutant males. The injection mix contained 400 ng/μL donor plasmid *pBac-3×P3*::*EGFP-SV40-AsU6*::*gRNA*^{Aswhite1}.

2.7 Phenotypic observation and genotyping analysis

A dual-fluorescence marker system was employed to screen positive *Cas9* transgenic individuals. GFP driven by the *3×P3* promoter served as a transgenesis marker, while *Cas9* expression under germline-biased promoters was linked to DsRed via a T2A self-cleaving peptide, allowing DsRed fluorescence to report *Cas9* expression. Transgene insertion sites were determined using inverse PCR (Li et al., 2017).

For *Cas9* transcript analysis, ovaries were dissected from mated females at 72 h post-mating and 70 h post-blood feeding, when eggs remained unfertilized. Fluorescence distribution was examined in these ovaries and in 0 h eggs laid by females at 48 h post-blood feeding. Additionally, ovaries (excluding accessory glands) from females of the three *Cas9* lines and testes (excluding accessory glands) from 2-day-old unmated males were collected for quantitative analysis of *Cas9* transcript levels.

To assess editing efficiency of *Cas9* lines or transient *AsU6-gRNA* plasmid expression

targeting *Aswhite*, compound eye pigmentation was examined at the pupal stage, when eye color is most distinct in *Anopheles sinensis*. Pigmented areas were quantified using the 'measure' function in ImageJ. PCR amplification and sequencing of partially pigmented mutants were performed using primers flanking the *Aswhite* target site.

Primers for inverse PCR, qPCR, and mutation site detection are listed in Table S14.

2.8 Germline transmission analysis of *Aswhite* mutations

To evaluate whether *Aswhite* mutations induced by *Cas9* transgenic lines or *AsU6-gRNA* plasmids occur at the germline level, G_0 females—potentially carrying exogenously introduced *Aswhite* mutations — were excluded to avoid confounding effects. G_0 males, either mosaic (potentially harboring new mutations) or, in the absence of visible phenotypes, wild type (black-eyed), were crossed with lab-reared *Aswhite* mutant females at a 1:2 ratio. Only female progeny were analyzed for mutation frequency, as all male offspring inherently display the white-eyed phenotype due to the maternal mutant *X* chromosome and were thus excluded from statistical evaluation.

To assess germline transmission in F_1 offspring derived from crosses between homozygous *Cas9* and *AsU6-gRNA* homozygous lines, F_1 females (dual-fluorescent eye phenotypes) were selected. Ovaries (excluding accessory glands) were dissected at 70 h post-blood feeding, and the targeted regions of the *Aswhite* locus in ovarian genomic DNA were analyzed by deep sequencing (PE300, Sangon, China). The background error rate (~0.01%), determined from *Cas9* lines amplicon sequencing, was used to calculate the corrected editing efficiency according to the formula: (observed mutation rate-0.01%)/(1-0.01%). To further avoid potential masking of phenotypes from heterozygous mutations, subsets of dual-fluorescent females with distinct compound eye phenotypes (white-eyed, mosaic, or wild-type) were crossed with lab-reared *Aswhite* mutant males at a 1:2 ratio, and the proportions of white-eyed individuals in F_2 offspring were recorded. Primer sequences used for high-throughput sequencing are listed in Table S14.

2.9 Statistical analysis

Statistical analyses were performed using one-way ANOVA and Student's t-tests, as appropriate. Data are presented as the mean ± standard deviation (SD). All analyses were conducted using GraphPad Prism version 9.5.

3. Results and Discussion

Spatiotemporal expression profiling confirmed predominant transcription of *Asvasa2*, *Aszpg*, and *Asnanos* in female gonads (Figure 1A), consistent with the germline-biased patterns of their orthologs in other mosquito species[12, 13]. Using regulatory sequences from these genes, we generated transgenic constructs and established corresponding *Cas9*-expressing lines (Figure 1B, Figure S1, Table S1). The *Asvasa2-Cas9* line exhibited strong fluorescence in nurse cells by 48 h and throughout developing eggs at 70 h post-blood feeding, while signal intensity was remarkably reduced in *Aszpg-Cas9* and nearly absent in *Asnanos-Cas9* lines (Figure 1B, Figure S2). qPCR analysis further confirmed that the *Cas9* mRNA levels were substantially higher in the ovaries of *Asvasa2-Cas9* mosquitoes relative to the other two lines (Figure 1B). Notably, *Asvasa2-Cas9* also drove stronger *Cas9* expression in male testes relative to *Aszpg-Cas9* and *Asnanos-Cas9* (Figure S3).

To determine editing efficacy driven by female germline-biased promoters, we observed that the *Asvasa2-Cas9* line induced mosaic phenotypes in 59.9% of G_0 individuals—markedly higher than rates observed in WT (32.8%), *Aszpg-Cas9* (8.4%), and *Asnanos-Cas9* (0%) lines (Figure 1C, Table S2). Germline transmission was confirmed in G_1 progeny, with 35.9% of females from the *Asvasa2-Cas9* group and 27.1% from WT microinjection group exhibiting the white-eyed phenotype (Figure 1D, Table S3, File S1). In contrast, no heritable edits were detected in G_1 offspring derived from *Aszpg-Cas9* or *Asnanos-Cas9* G_0 males (Figure 1D, Table S3). These results demonstrate that endogenous *Cas9* expression under germline-biased promoters provides a robust alternative to direct protein injection. The pronounced differences in efficiency among orthologous promoters underscores the importance of developing functional regulatory elements tailored to each target organism.

To identify functional Pol III promoters, we screened the *Anopheles sinensis* genome and identified six putative *AsU6* genes, four of which contained essential promoter elements—the proximal sequence element and TATA box (Figure 1E). Editing efficiency mediated by these promoters varied substantially: *AsU6*-2 yielded the lowest activity (31.1%), while *AsU6*-1 achieved the highest (62.1%), slightly exceeding *AsU6*-3 (60.2%) and *AsU6*-4 (59.9%) (Figure 1F, Table S4). All four *AsU6* promoters supported germline transmission, as evidenced by white-eyed G_1 females following crosses of mosaic G_0 males with lab-reared *Aswhite* mutant females (Figure 1G, File S2). Furthermore, when delivering the *AsU6*-1-*gRNA* construct was delivered into other two *Cas9* backgrounds, the *Aszpg-Cas9* line showed a mosaic editing rate of 10.3%, while no edits were detected in the *Asnanos-Cas9* background (Figure S4, Table S5). Although *AsU6*-3 and *AsU6*-4 exhibited activities comparable to *AsU6*-1, their suitability for germline editing requires further validation to enable diversified regulatory toolkits for gene drive applications and mitigate risks associated with repeated use of identical promoters[14].

Leveraging the validated promoters as a foundation, we next established a promoter-driven transgenic system to assess heritable editing efficiency. Direct crosses between *Asvasa2-Cas9* females and *AsU6*-1-$gRNA^{Aswhite}$ males produced 100% white-eyed F_1 progeny. In the reciprocal cross, 57.4% of F_1 offspring were fully white-eyed, while the remaining 42.6% exhibited mosaic eyes with ~92.6% unpigmented area (Figure 1H, S5, S6; Table S1, S6). The complete absence of mosaic phenotype in the direct cross suggests that maternal Cas9 deposition from oocytes enhanced editing efficacy in early embryos[15]. Ovarian editing frequencies exceeded 99.6% in F_1 females from both cross schemes (Figure 1H, Table S7, File S3). All F_2 offspring from subsequent crosses of F_1 females to lab-reared *Aswhite* mutant males displayed the white-eyed phenotype (Figure 1I, Table S8), demonstrating 100% germline transmission using the *Asvasa2* and *AsU6*-1 promoters.

Crosses between *Aszpg-Cas9* females and *AsU6-1-gRNA*^{Aswhite} males produced 42.6% F_1 offspring with mosaic eyes (~19.3% unpigmented area), while the reciprocal cross yielded 32.0% mosaics (~8.3% unpigmented area) (Figure 1H, S5, S6; Table S6). Ovarian editing rates in F_1 females ranged from 30.3% to 36.7% (Figure 1H, Table S7, File S3), and subsequent F_2 crosses (obtained from from crosses of mosaic or wild-type F_1 females with white-eyed males) produced white-eyed progeny at frequencies of 39.4–46.8% (Figure 1I, Table S9). These results indicate that the *Aszpg* promoter supports moderate germline editing, consistent with its utility in *Anopheles gambiae* gene-drive systems[13]. In contrast, crosses involving *Asnanos-Cas9* and *AsU6*-1-*gRNA* lines yielded no detectable eye phenotypes in F_1 (Figure 1H, S5, S6; Table S6), low

ovarian editing rates (~15%; Figure 1H, Table S7, File S3), and minimal white-eyed F_2 offspring (< 0.2%; Figure 1I, Table S10). Collectively, these data demonstrate that the *Asvasa2* promoter drives *Cas9* expression with superior efficiency and stability in the germline compared to *Aszpg* and *Asnanos*.

We also evaluated the *AsU6*-2 promoter in combination with *Asvasa2-Cas9*. Direct crosses produced 36.8% white-eyed and 63.2% mosaic F_1 progeny (~88.2% unpigmentation) (Figure S6, Figure S7, Table S11). No white-eyed individuals occurred in the reciprocal cross, and mosaic eyes showed reduced unpigmentation (53.8%) (Figure S6–S8; Table S11). Ovarian editing rates ranged from 63.3% to 68.2% (Figure S9, Table S12, File S3), and white-eyed F_2 frequencies were ≤ 62.5% (Figure S10, Table S13), and significantly lower than those with *AsU6*-1, confirming that *AsU6*-2 drives functional but less efficient editing.

In summary, we have established a promoter-driven transgenic system that enables highly efficient gene editing in *Anopheles sinensis*, a major malaria vector (Figure 1J). Our findings greatly expands the genetic toolkit available for synthetic biology, such as gene drive-based population suppression, in non-model mosquitoes, as well as providing a transferable conceptual framework for genetic modification in other recalcitrant insect species.

Figure legends

Figure 1. An engineered promoter system enables highly efficient transgenic-based genome editing in the malaria mosquito *Anopheles sinensis*. A. Spatiotemporal transcription profiles of *Asvasa2*, *Aszpg*, and *Asnanos*. Blue and green shading denote sexually undifferentiated and differentiated stages, respectively (n = 3; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$). B. *Cas9* expression and fluorescence in ovaries of transgenic lines under control of germline-biased promoters. White and green arrows indicate green and red fluorescence, respectively (n = 3; $****P < 0.0001$; scale bar: 1 mm). C. Editing efficiency in G_0 progeny from crosses between *Cas9* transgenic females and lab-reared *Aswhite* mutant males, compared with conventional WT females (crossed with lab-reared *Aswhite* mutant males) following injection of Cas9-$sgRNA^{Aswhite}$ complexes (M: male; F: female; red arrows: mosaic eyes; n = 3; $****P < 0.0001$). D. Germline transmission assay via G_0 mosaic males crossed to lab-reared *Aswhite* mutant females. G_1 mutations were verified by sequencing (n = 3; $****P < 0.0001$). E. Genomic features of *AsU6* promoter regions and gRNA expression cassette architecture. F. *Aswhite* editing efficiency mediated by four *AsU6-gRNA* plasmids in G_0 individuals (Different letters indicate significant differences; n = 3; $P < 0.0001$). G. Qualitative assessment of germline transmission edits driven by four *AsU6* promoters. G_1 mutations were sequence-verified. H. Phenotypic distribution (n = 3) and editing frequencies (n ≥ 2) at dual $gRNA^{Aswhite}$ sites in F_1 offspring from crosses between *Cas9* and *AsU6*-1-*gRNA* lines. $** P < 0.01$, $***P < 0.001$. I. Phenotypic distribution of F2 progeny from crosses of F_1 females (derived from *Cas9* × *AsU6*-1 crosses) to lab-reared *Aswhite* mutant males (n = 3; $****P < 0.0001$). J. Schematic overview of the promoter-driven editing system development and implementation.

Conflict of interest

The authors declare that they have no conflict of interest.

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Author contributions

Jun-Feng Hong: Writing - Original draft, Writing - Review & Editing, Visualization, Methodology, Investigation, Data Curation. **Qi-Li Zou**: Writing - Review & Editing, Methodology, Validation, Investigation, Data curation. **Xin-Yuan Xie**: Methodology, Validation, Resources, Investigation, Data curation. **Yan-Ping Jiang**: Methodology, Validation, Investigation, Data curation. **Xia Ling**: Methodology, Investigation. **Cao Zhou**: Data Curation. **Wei Sun**: Data curation. **Si-Yi Wang**: Resources. **Xi Cai**: Resources. **Yong-Xin Yang**: Resources. **Bin Chen**: Writing - Review & Editing, Supervision, Funding acquisition, Data curation, Conceptualization. **Liang Qiao**: Writing - Original draft, Writing - Review & Editing, Methodology, Validation, Supervision, Funding acquisition, Resources, Data curation, Conceptualization.

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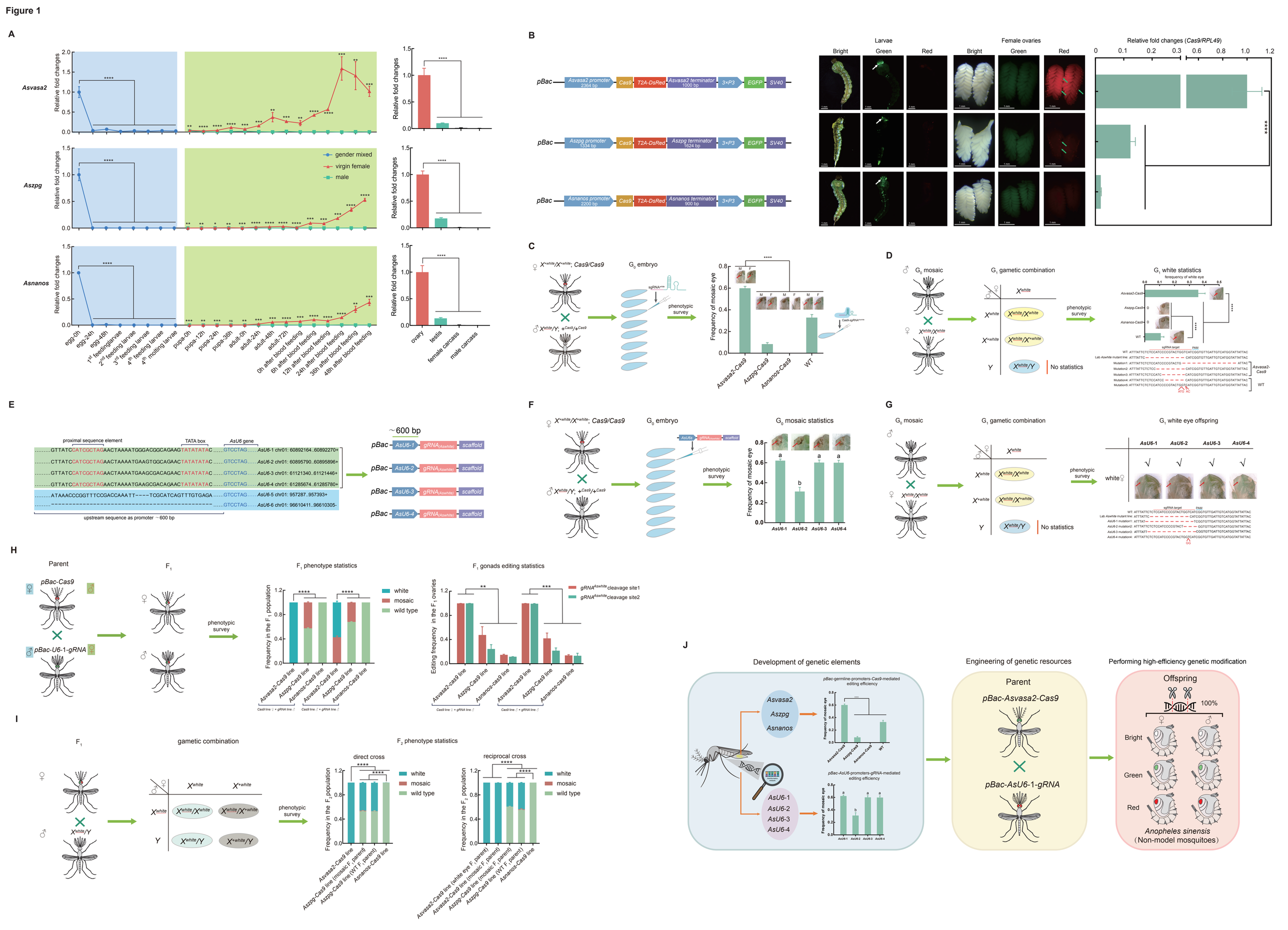
Figure 1


A
Asvasa2
Aszpg
Asnanos
Relative fold changes
gender mixed
virgin female
male
egg-0h
egg-24h
egg-48h
1st feeding larvae
2nd feeding larvae
3rd feeding larvae
4th feeding larvae
4th molting larvae
pupa-0h
pupa-12h
pupa-24h
pupa-36h
adult-0h
adult-24h
adult-48h
adult-72h
0h after blood feeding
6h after blood feeding
12h after blood feeding
24h after blood feeding
36h after blood feeding
48h after blood feeding
ovary
testis
female carcass
male carcass
B
pBac
Asvasa2 promoter 2364 bp
Aszpg promoter 1334 bp
Asnanos promoter 2200 bp
Cas9
T2A-DsRed
Asvasa2 terminator 1000 bp
Aszpg terminator 1624 bp
Asnanos terminator 900 bp
3×P3
EGFP
SV40
Larvae
Bright
Green
Red
Female ovaries
Relative fold changes (Cas9/RPL49)
C
♀ X+white/X+white; Cas9/Cas9
♂ Xwhite/Y; +Cas9/+Cas9
G0 embryo
sgRNAAswhite
phenotypic survey
Cas9-sgRNAAswhite
Frequency of mosaic eye
Asvasa2-Cas9
Aszpg-Cas9
Asnanos-Cas9
WT
D
♂ G0 mosaic
♀ Xwhite/Xwhite
G1 gametic combination
Xwhite
X+white
Y
Xwhite/Xwhite
Xwhite/X+white
Xwhite/Y
No statistics
phenotypic survey
G1 white statistics
ferequency of white eye
Asvasa2-Cas9
Aszpg-Cas9
Asnanos-Cas9
WT
sgRNA target
PAM
WT: ATTTATTCTCTCCATCCCCGTACTGGTCATCGGTGTTGATTGTCATGGTATTATTAC
Lab Aswhite mutant line: ATTTATTC CATCGGTGTTGATTGTCATGGTATTATTAC
Mutation1: ATTTATTCTCTCCATCCCCGTACTG ATTAC
Mutation2: ATTTATTCTCTCC CATCGGTGTTGATTGTCATGGTATTATTAC
Mutation3: ATTTATTCTCTCCATC CATCGGTGTTGATTGTCATGGTATTATTAC
Mutation4: ATTTATTCTCTCCATCC CATCGGTGTTGATTGTCATGGTATTATTAC
Mutation5: ATTTATTCTCTCCATCCCCGTACTGGTCATCGGTGTTGATTGTCATGGTATTATTAC
Asvasa2-Cas9
WT
E
proximal sequence element
TATA box
AsU6 gene
GTTATCCATCGCTAGAACTAAAATGGGACGGCAGAAGTATATATACGTCCTAG AsU6-1 chr01: 60892164..60892270+
CTTATCCATCGCTAGAACTAAAATGAAGTGGCAGAACTATATATACGTCCTAG AsU6-2 chr01: 60895790..60895896+
GTTATCCATCGCTAGAACTAAAATGAAGCGACAGAACTATATATACGTCCTAG AsU6-3 chr01: 61121340..61121446+
GTTATCCATCGCTAGAACTAAAATGAAGCGACAGAACTATATATACGTCCTAG AsU6-4 chr01: 61285674..61285780+
ATAAACCCGGTTTCCGACCAAATT----TCGCATCAGTTTGTGAGAGTCCTAG AsU6-5 chr01: 957287..957393+
GTCCTAG AsU6-6 chr01: 96610411..96610305-
upstream sequence as promoter ~600 bp
~600 bp
pBac
AsU6-1
AsU6-2
AsU6-3
AsU6-4
gRNA(Aswhite)
scaffold
F
♀ X+white/X+white; Cas9/Cas9
♂ Xwhite/Y; +Cas9/+Cas9
G0 embryo
AsU6s
gRNAAswhite
scaffold
phenotypic survey
G0 mosaic statistics
Frequency of mosaic eye
AsU6-1
AsU6-2
AsU6-3
AsU6-4
G
G0 mosaic
♂
♀ Xwhite/Xwhite
G1 gametic combination
Xwhite
X+white
Y
Xwhite/Xwhite
Xwhite/X+white
Xwhite/Y
No statistics
phenotypic survey
G1 white eye offspring
AsU6-1
AsU6-2
AsU6-3
AsU6-4
white♀
√
sgRNA target
PAM
WT: ATTTATTCTCTCCATCCCCGTACTGGTCATCGGTGTTGATTGTCATGGTATTATTAC
Lab Aswhite mutant line: ATTTATTC CATCGGTGTTGATTGTCATGGTATTATTAC
AsU6-1 mutation1: ATTTAT TCGGTGTTGATTGTCATGGTATTATTAC
AsU6-2 mutation2: ATTTATTCTCTCCATCCCCGTACT GGTGTTGATTGTCATGGTATTATTAC
AsU6-3 mutation3: ATTTATT CGGTGTTGATTGTCATGGTATTATTAC
AsU6-4 mutation4: ATTTATTCTCTCCATCCCCGTACTGGTCATCGGTGTTGATTGTCATGGTATTATTAC
G2
H
Parent
pBac-Cas9
pBac-U6-1-gRNA
F1
phenotypic survey
F1 phenotype statistics
Frequency in the F1 population
white
mosaic
wild type
Asvasa2-Cas9 line
Aszpg-Cas9 line
Asnanos-Cas9 line
Cas9 line ♀ × gRNA line ♂
Cas9 line ♂ × gRNA line ♂
F1 gonads editing statistics
Editing frequency in the F1 ovaries
gRNAAswhite cleavage site1
gRNAAswhite cleavage site2
I
F1
♀
♂ Xwhite/Y
gametic combination
Xwhite
X+white
Y
Xwhite/Xwhite
Xwhite/X+white
Xwhite/Y
X+white/Y
phenotypic survey
F2 phenotype statistics
direct cross
reciprocal cross
Frequency in the F2 population
white
mosaic
wild type
Asvasa2-Cas9 line
Aszpg-Cas9 line (mosaic F1 parent)
Aszpg-Cas9 line (WT F1 parent)
Asnanos-Cas9 line
Asvasa2-Cas9 line (white eye F1 parent)
Asvasa2-Cas9 line (mosaic F1 parent)
Aszpg-Cas9 line (mosaic F1 parent)
Aszpg-Cas9 line (WT F1 parent)
J
Development of genetic elements
Asvasa2
Aszpg
Asnanos
AsU6-1
AsU6-2
AsU6-3
AsU6-4
pBac-germline-promoters-Cas9-mediated editing efficiency
pBac-AsU6-promoters-gRNA-mediated editing efficiency
Frequency of mosaic eye
Engineering of genetic resources
Parent
pBac-Asvasa2-Cas9
pBac-AsU6-1-gRNA
Performing high-efficiency genetic modification
Offspring
100%
Bright
Green
Red
Anopheles sinensis
(Non-model mosquitoes)