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Engineered promoter system enables high-efficiency transgenic CRISPR editing in Malaria transmitting mosquito *Anopheles sinensis*

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ABSTRACT

The binary CRISPR/Cas9 system deployed through crosses of transgenic lines facilitates efficient mutagenesis, but its application in non-model insects remains limited by the scarcity of validated species-specific regulatory elements. In the malaria vector *Anopheles sinensis*, we screened three germline-biased promoters (*Asvasa2*, *Aszpg*, *Asnanos*) for Cas9 expression, and found that *Asvasa2* drove the highest editing efficiency with respect to target site mutagenesis. For *gRNA* transcription, comparative analysis identified *AsU6-1* as the most active of four endogenous *U6* promoters. Crossing stable transgenic lines harboring these components yielded F₁ progeny with complete germline editing penetrance at the *Aswhite* locus, a phenotype inherited in the F₂ generation. Quantitative sequencing of F₁ ovaries confirmed near-saturation (>99%) targeted mutagenesis using the optimal *Asvasa2/AsU6-1* combination, whereas alternative promoters showed markedly lower mutagenesis efficiency. Functional validation through knockout of *Asdsx-F*, a key sex differentiation regulator, efficiently induced complete female-to-male sexual reversal and sterility. This study provides a foundational genetic toolkit for genome engineering in this vector species, as well as an effective reference for binary transgenic manipulation in non-model insects.

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

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INTRODUCTION

Mosquitoes serve as primary vectors for devastating infectious diseases such as malaria and dengue fever, posing a persistent global public health threat (Cabezas-Cruz et al., 2025; Datta Mudi et al., 2024). Beyond their medical importance, mosquitoes represent invaluable model systems for fundamental biological inquiry, advancing studies on chemosensation, swarming mating behavior, host–pathogen interactions, and innovative genetic control strategies (Giraldo et al., 2023; Somers et al., 2022; Wang et al., 2022). The CRISPR/Cas9 genome editing system has emerged as a pivotal tool for dissecting molecular mechanisms, evolutionary adaptations, and developing novel genetic interventions in mosquitoes (Weng et al., 2024). However, conventional microinjection of Cas9-*gRNA* ribonucleoprotein (RNP) complexes into preblastoderm embryos lacks selectable markers, complicating the identification of edited individuals in the absence of visible phenotypes. In the absence of visual markers, the requisite blind sampling of somatic tissues for DNA extraction raises mortality risks, compromising the recovery of edited founders for subsequent breeding. More critically, this delivery method is less amenable to multiplex genome editing. Moreover, this method is hampered by labor-intensive procedures and high embryonic mortality (Bottino-Rojas & James, 2024; Cao et al., 2024).

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In established model mosquitoes including *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 (Li et al., 2024; Smidler et al., 2024). By characterizing multiple lines at different integration sites to exclude positional effects, the transgenic approach offers exceptional efficiency and convenience for genetic manipulation applications, including gene editing and the assessment of transcriptional activity of regulatory elements such as promoters via overexpression or heterologous expression (Dilani et al., 2022; Franz et al., 2011). 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 (Feng et al., 2021; Li et al., 2020). Nevertheless, such well-characterized regulatory elements remain scarce for many non-model yet medically important mosquito species, including *An. sinensis*.

From a broader perspective of insect genetic manipulation, the establishment of robust and stable transgenic systems constitutes a cornerstone for in-depth research in model insects such as *An. gambiae* and *Drosophila melanogaster* (Dong et al., 2018, 2022; Garrood et al., 2021; Port et al., 2014, 2020; Xu et al., 2019b). Such systems are equally essential for elucidating the molecular basis of intriguing biological phenomena and for achieving precise genetic manipulation in non-model insects. Therefore, developing similarly efficient and reliable binary transgenic editing systems in key vectors such as *An. sinensis* or in other non-model insects not only overcomes current genetic manipulation challenges, but also lays an indispensable technical foundation for future advances in functional genetics and the development of targeted genetic control strategies.

Here, we identified and characterized core regulatory elements (germline-biased promoters and *U6* promoters) capable of driving *Cas9* and *gRNA* expression in *An. sinensis*. Using these regulatory elements, we established a functional binary transgenic system harboring stably integrated *Cas9* and *gRNA* expression cassettes. Crosses between transgenic lines achieve complete germline editing penetrance for a visual marker, *Aswhite*, and a key downstream regulator of sex differentiation, *Asdsx-F*, providing a robust and heritable genome editing platform for this neglected malaria vector.

MATERIALS AND METHODS

Mosquitoes

The *An. 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°C±1°C, 75%±5% relative humidity, and a 12:12 h light-dark cycle. Larvae were fed powdered fish food in water. Pupae were transferred to mesh-covered containers for eclosion. Newly emerged adults were provided with 10% glucose solution, and blood meals were administered via mice on the third day post-eclosion.

Expression pattern analysis of germline-biased genes

Asvasa2, *Aszpg*, and *Asnanos* were selected as candidate

germline-active genes in *An. 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; first to fourth instar larvae (including the 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-eclosion; 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-eclosion. Additional samples included ovaries and female carcasses from blood-fed females (48 h), and testes and male 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 (Takara, Japan). Gene expression was quantified in three biological replicates, with *AsRPL49* used as the internal control. Primer sequences are provided in Supplementary Table S1.

Gene identification and regulatory region selection

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

AsU6 genes were identified by homology to *An. gambiae* *U6* genes. A 1 500 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).

Plasmid construction

To generate *Cas9* expression plasmids, promoters and corresponding terminators of *Asvasa2*, *Aszpg*, and *Asnanos* were amplified from *An. sinensis* genomic DNA using PrimeSTAR high-fidelity DNA polymerase (Takara, Japan), based on the selected regulatory regions. The *Cas9-T2A* fragment was amplified from *pAc-gRNA-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 (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, we first amplified the approximately 600 bp upstream regions of four *AsU6* genes from *An. sinensis* genomic DNA; each region contained both a proximal sequence element and a TATA box. Next, the *gRNA* expression unit targeting the validated *Aswhite* site was amplified from a synthesized *gRNA* scaffold template (Yang et al., 2024). The recipient vector, *pBac-3×P3::EGFP-SV40*, was linearized with *BglII*. Finally, the *AsU6* promoter and the

gRNA fragment were assembled into the linearized vector via In-Fusion cloning to generate the final construct, *pBac-3×P3::EGFP-SV40-AsU6s::gRNA^{Aswhite}*.

Following confirmation of the editing efficiency mediated by the germline-biased and *AsU6* promoters, we selected an additional validated *gRNA* target site within the *Aswhite* gene (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}* were amplified from *pBac-3×P3::EGFP-SV40-AsU6-1::gRNA^{Aswhite}* plasmid. Similarly, *AsU6-2::gRNA^{Aswhite1}* and *AsU6-2::gRNA^{Aswhite2}* were amplified from the *pBac-3×P3::EGFP-SV40-AsU6-2::gRNA^{Aswhite}* plasmid. For the construction of the *3×P3::DsRed-SV40-AsU6-1::gRNA^{Asdsx-F-1}-AsU6-1::gRNA^{Asdsx-F-2}* plasmid, both *gRNA* target sites were designed to lie within *Asdsx-F*-specific exons. The amplification strategy was the same as that used for the *AsU6-1::gRNA^{Aswhite}* cassette. All final constructs—namely *pBac-3×P3::DsRed-SV40-AsU6-1::gRNA^{Aswhite1}-AsU6-1::gRNA^{Aswhite2}*, *pBac-3×P3::DsRed-SV40-AsU6-2::gRNA^{Aswhite1}-AsU6-2::gRNA^{Aswhite2}* plasmids and *3×P3::DsRed-SV40-AsU6-2::gRNA^{Asdsx-F-1}-AsU6-2::gRNA^{Asdsx-F-2}*, were assembled using the Golden Gate assembly kit (NEB, USA) according to the manufacturer's instructions. The assembly was performed using linearized *pBac-3×P3::DsRed (BsaI)-SV40* plasmid as the backbone and the aforementioned amplified fragments as genetic elements. All constructs were verified by Sanger sequencing (Sangon, China). Primer sequences are listed in Supplementary Table S1.

In vitro transcription of gRNA

Templates for *gRNA* synthesis were generated by annealing and extending primers encompassing the *T7* promoter, target *gRNA* sequence, and a truncated *gRNA* scaffold sequence that partially overlaps the sense primer. Following purification, *gRNAs* were transcribed *in vitro* using the *T7* RiboMAX™ Express RNAi System (Promega, USA) according to the manufacturer's instructions. Primer sequences are provided in Supplementary Table S1.

Embryo microinjection

Three days after blood feeding, *An. sinensis* females were allowed to oviposit. Embryos were aligned within 15 min post-oviposition and injected using a WPI PV830 microinjection system (WPI, USA).

For the generation of transgenic lines (*Asvasa2-Cas9*, *Aszpg-Cas9*, *Asnanos-Cas9*, *AsU6-1-gRNA^{Aswhite}*, *AsU6-2-gRNA^{Aswhite}*, *AsU6-1-gRNA^{Asdsx-F}*), the injection mix contained 150 ng/μL of the donor plasmid and 300 ng/μL of a helper plasmid expressing *transposase*. For delivery of *gRNA* into embryos, a concentration of 1 000 ng/μL was used. For delivery of *Cas9-gRNA* ribonucleoprotein (RNP) complexes, the mixture consisted of 1 000 ng/μL *gRNA* and 300 ng/μL of commercial *Cas9* protein (GenScript Biotech, USA). For delivery of *AsU6-gRNA* plasmids into embryos of *Cas9* transgenic lines, the donor plasmid concentration was 400 ng/μL.

Establishment and characterization of Cas9 and U6 transgenic lines

A dual-fluorescence reporter system was used to identify and select positive *Cas9* transgenic lines. In this system, *GFP* expression driven by the *3×P3* promoter served as a visual

marker for successful transgenesis. Concurrently, *Cas9*, expressed under the control of germline-biased promoters, was translationally fused to *DsRed* via a *T2A* self-cleaving peptide, thereby linking Red fluorescence directly to *Cas9* protein expression. From the *G*₁ positive populations, individuals with comparable reporter fluorescence intensity were selected and crossed with wild-type individuals, and subjected to single-female oviposition. Subsequently, positive individuals were selected from the *G*₂ generation, crossed with wild-type individuals, and subjected to single-female oviposition to obtain the *G*₃ generation. Starting from the *G*₃ generation, for red fluorescence observation, ovaries were dissected from mated females of different lines at 70 h post-blood feeding (and a waterless environment was created to prevent oviposition). Fluorescence distribution was examined in these ovaries as well as in 0 h eggs laid by females. Additionally, ovaries (excluding accessory glands) from females of multiple *Cas9* transgenic lines, including two *Cas9* lines with distinct insertion sites for each promoter type, and testes (excluding accessory glands) from 3-day-old unmated males were collected for quantitative analysis of *Cas9* transcript levels. The fluorescence intensity of the reporter in the compound eyes of pupae (24 h after pupation) of the retained *Cas9* transgenic lines was measured in ImageJ (<https://www.imagej.com.cn/>). The fitness of transgenic lines established through single-female oviposition was assessed, and transgene insertion sites were mapped with inverse Polymerase Chain Reaction (Inverse PCR) (Li et al., 2017). The effects of transgene integration on the structural features of adjacent genomic regions were further analyzed. According to the results from these analyses, *Cas9* transgenic lines were selected for subsequent gene editing experiments. For the establishment and selection of *U6* transgenic lines, because direct detection of *gRNA* expression was not feasible, suitable individuals were retained for line establishment solely on the basis of strong reporter fluorescence intensity. Insertion site analysis, fitness assessment, and genotyping were performed following the same procedures as those for *Cas9* lines. The *Cas9* coding region and the *U6-gRNA* expression cassette in individuals of the transgenic lines used for gene editing were both amplified and verified through sequencing. Flanking sequences of the transgene insertion sites in the six transgenic lines used for gene editing experiments were amplified and sequenced. Primers for inverse PCR, genotyping, and real-time quantitative polymerase chain reaction (RT-qPCR) are listed in Supplementary Table S1.

Assessment of editing efficiency across Cas9 transgenic lines by delivering gRNA^{Aswhite}

For experiments assessing the efficiency of the *Cas9* line, we accounted for the X-linked recessive mutation locus *white* (Yang et al., 2024), which requires biallelic mutations in females for the white-eyed phenotype. To prevent phenotypic masking (e.g., *X^{+white}X^{white}* females) and improve statistical accuracy, embryos from crosses between *Cas9* transgenic females and lab-reared *Aswhite* mutant males were injected with *gRNA^{Aswhite1}*. As a reference control, embryos from crosses between wild-type females and lab-reared *Aswhite* mutant males were injected with *Cas9-gRNA^{Aswhite1}* RNPs (WT-*Cas9* RNP).

To assess the editing efficiency resulting from this delivery, *G*₀ individuals were screened while *G*₀ females that may carry exogenous *Aswhite* mutations were excluded to avoid

confounding effects. To quantify heritable mutations, G₀ males—either mosaic (potentially harboring new mutations) or wild-type if no visible phenotypes was observed—were crossed with lab-reared *Aswhite* mutant females at a 1:2 ratio. Only female progeny were analyzed for mutation frequency because all male offspring inherently display the white-eyed phenotype due to the maternal mutant X chromosome and were therefore excluded from statistical evaluation. Primers for mutation site detection are listed in Supplementary Table S1.

Comparative analysis of *U6* Promoter activity by delivering *AsU6-gRNA* plasmids into *Cas9* transgenic lines

To evaluate the transient expression efficiency of *AsU6-gRNA* plasmids, hybrid embryos from crosses between *Asvasa2-Cas9-Chr. 1₁₀₅₂₅₃₆₂₈* females and lab-reared *Aswhite* mutant males were injected with donor *AsU6-gRNA* plasmids. In parallel, the most effective *AsU6-1-gRNA* plasmid was also injected into hybrid embryos derived from crosses between females of the *Aszpg-Cas9-Chr. 1₁₄₅₀₇₈₇₅* line (as well as *Asnanos-Cas9-Chr. 2₃₆₅₆₈₆₈₁* line) and laboratory-reared *Aswhite* mutant males. This approach of delivering *U6-gRNA* plasmids into embryos of *Cas9* transgenic lines for rapid assessment of *U6* promoter activity has been validated in *Ae. aegypti* (Li et al., 2020). Subsequently, somatic editing efficiency in G₀ individuals and germline transmission in G₁ progeny of the induced *Aswhite* mutations were assessed following the assay detailed in the part 8 of M&M section. Primers for mutation site detection are listed in Supplementary Table S1.

Assessment of editing efficiency at *Aswhite* and *Asdsx-F* loci via binary transgenic crosses

We documented mutant phenotypes in F₁ progeny from independent crosses of homozygous (*Cas9*×*AsU6-gRNA^{white}*) and heterozygous (*Asvasa2-Cas9-Chr. 1₁₀₅₂₅₃₆₂₈*×*AsU6-1-gRNA^{dsx-F-Chr. 1₅₂₃₀₄₄₃₂}*) lines. For *Aswhite* locus, the percentage of pigmented area in the compound eyes of double-fluorescent individuals was quantified using ImageJ. For *Asdsx-F* locus, both external morphological traits and internal reproductive system features were examined in double-fluorescent and single red-fluorescent individuals. To precisely determine editing efficiency for the binary transgenic system, the *Aswhite* locus was selected as the test target. Ovaries (excluding accessory glands and any non-ovarian tissue) were dissected at 70 h post-blood feeding, and the targeted regions in ovarian genomic DNA were analyzed by deep sequencing (PE300, Sangon, China). The background error rates are 3.11% for *gRNA^{white}* cleavage site 1 and 2.95% for *gRNA^{white}* cleavage site 2. All mutation types were single-base substitutions (Supplementary File S1), as determined by amplicon sequencing of the *Cas9* lines. These sequencing data were then used to calculate the corrected editing efficiency using the formula: (observed mutation rate–background error rate)/(1–background error rate). The F₁ 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 F₂ offspring were recorded.

For genotyping analysis of *dsx-F* locus, genomic DNA was extracted from F₁ individuals including dual-fluorescence females (with intersex morphological features), males; and red-fluorescent intersex females (resulting from maternal *Cas9*

deposition), followed by sequencing analysis. Primer sequences used for high-throughput sequencing are listed in Supplementary Table S1.

Relative genomic quantitative polymerase chain reaction (genomic qPCR)

To determine the sex chromosome constitution of individuals in the F₁ progeny from crosses between *Asvasa2-Cas9-Chr. 1₁₀₅₂₅₃₆₂₈* females and *AsU6-1-gRNA^{Asdsx-F-Chr. 1₅₂₃₀₄₄₃₂}* males, we employed a genomic quantitative PCR assay. The *Aswhite* locus, a single-copy marker on the X chromosome, was used to assess the relative dosage of the X chromosome. The single-copy *AsRPL49* locus on an autosome served as the internal control. Genomic DNA was extracted from wild-type male, wild-type female, intersex individuals (dual-fluorescence and single red fluorescence individuals) and diluted to a uniform concentration of 10 ng/μL. The relative copy number of the X chromosome marker was calculated using the 2^{–ΔΔCT} method to deduce the individuals' sex chromosome constitution (XX/XY). The primer sequences used are listed in Supplementary Table S1.

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 v.9.5 (<https://www.graphpad-prism.cn/>).

RESULTS

Identification of female germline-biased promoters for *Cas9* expression

Spatiotemporal expression profiling confirmed the predominant transcription of *Asvasa2*, *Aszpg*, and *Asnanos* in female gonads (Figure 1), a pattern consistent with germline-biased expression in tissue transcriptome data (Supplementary Figure S1). The orthologs of *Asvasa2*, *Aszpg*, and *Asnanos* in other mosquito species also show similarly enriched transcription in female gonads (Hammond et al., 2021; Terradas et al., 2022). Using regulatory sequences from these genes, we generated transgenic constructs and established corresponding *Cas9*-expressing lines (Figure 2A; Supplementary Figure S2; Supplementary Table S2; Supplementary File S2). These transgenic lines showed no obvious fitness differences with respect to wild-type controls (Supplementary Table S3). The *Asvasa2-Cas9* line exhibited strong fluorescence in the nurse cells of ovarian tissue at 70 h post-blood feeding (Figure 2B; Supplementary Figure S3), with red fluorescence persisting throughout newly laid eggs (Supplementary Figure S4). These fluorescent patterns were similar to those reported in *Ae. aegypti* (Akbari et al., 2014). However, the fluorescence signal intensity was remarkably reduced in *Aszpg-Cas9* and nearly absent in *Asnanos-Cas9* lines (Figure 2B; Supplementary Figure S3). RT-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 2C; Supplementary Figure S5). Notably, *Asvasa2-Cas9* also drove stronger *Cas9* expression in male testes relative to *Aszpg-Cas9* and *Asnanos-Cas9* (Supplementary Figure S6). On the basis of criteria including *Cas9* expression efficiency, integration site architecture, transgenic line fitness, and

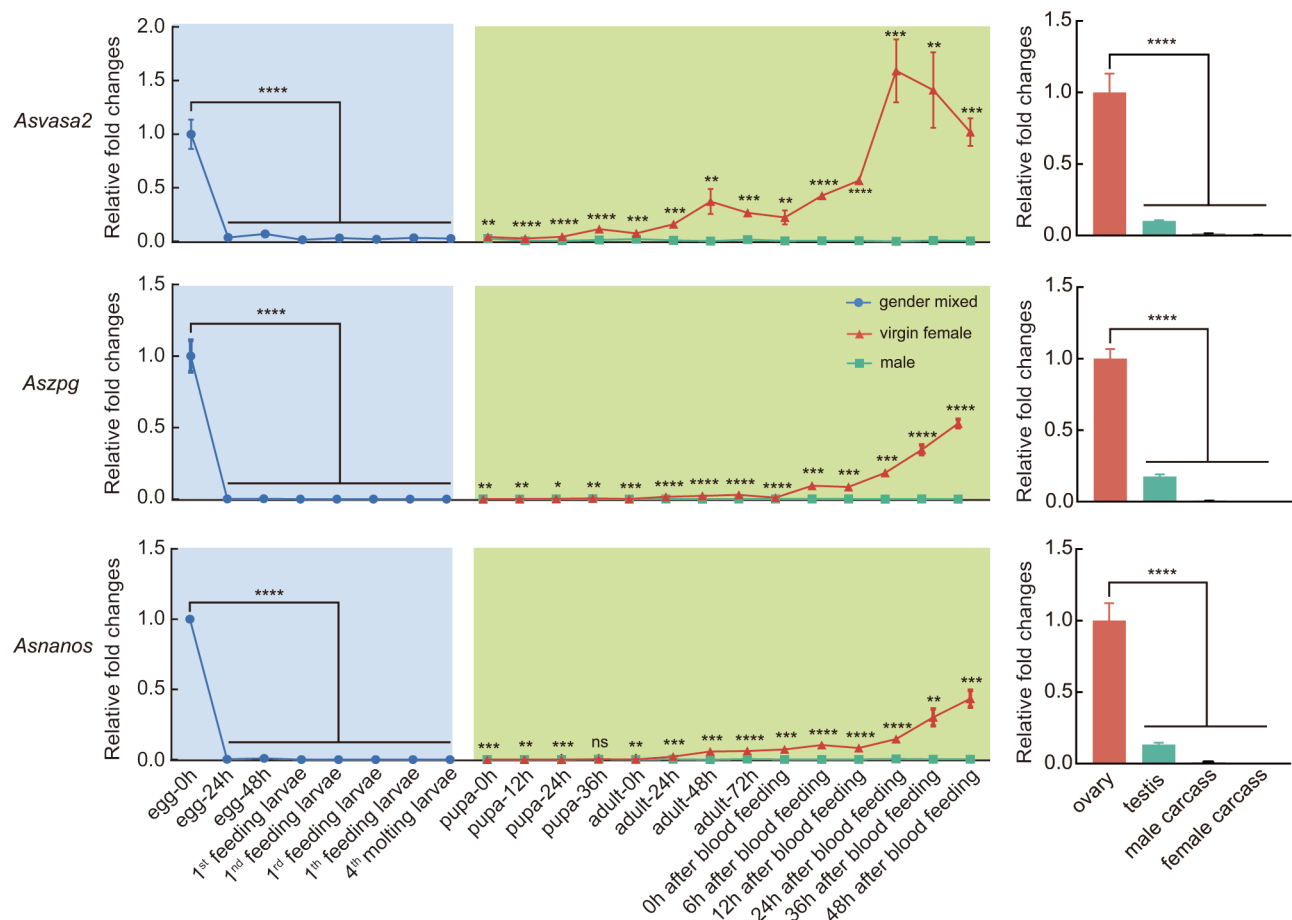


Figure 1 Spatiotemporal Expression Patterns of *Asvasa2*, *Asnanos*, and *Aszpg* in *An. sinensis*

Blue and green shading denote sexually undifferentiated and differentiated stages, respectively (*t*-test. *n*=3. ns: Not significant; *: *P*<0.05; **: *P*<0.01; ***: *P*<0.001; ****: *P*<0.0001. Data are presented as mean±SD.).

reporter fluorescence intensity (Figure 2; Supplementary Figures S5, S7 and Tables S2, S3), we selected one representative line from each of the three transgenic populations, namely *Asvasa2*-Cas9-Chr. 1₁₀₅₂₅₃₆₂₈, *Aszpg*-Cas9-Chr. 1₁₄₅₀₇₈₇₅, and *Asnanos*-Cas9-Chr. 2₃₆₅₆₈₆₈₁ for subsequent evaluation of gene-editing capability.

Mutagenesis via embryonic gRNA delivery in germline-biased Cas9 lines

To determine editing efficacy driven by female germline-biased promoters, we observed that the *Asvasa2*-Cas9-Chr. 1₁₀₅₂₅₃₆₂₈ line induced mosaic phenotypes in 59.9% of G₀ individuals—markedly higher than rates observed in WT-Cas9 RNP (Embryo microinjection with Cas9-gRNA^{Aswhite1} ribonucleoprotein complexes, 32.8%), *Aszpg*-Cas9-Chr. 1₁₄₅₀₇₈₇₅ (8.4%), and *Asnanos*-Cas9-Chr. 2₃₆₅₆₈₆₈₁ (0%) lines (Figure 3A; Supplementary Table S4). Germline transmission was confirmed in G₁ progeny, with 35.9% of females from the *Asvasa2*-Cas9-Chr. 1₁₀₅₂₅₃₆₂₈ group and 27.1% from WT microinjection group exhibiting the white-eyed phenotype (Figure 3B; Supplementary Table S5; Supplementary File S3). In contrast, no heritable edits were detected in G₁ offspring derived from *Aszpg*-Cas9-Chr. 1₁₄₅₀₇₈₇₅ or *Asnanos*-Cas9-Chr. 2₃₆₅₆₈₆₈₁ G₀ males (Figure 3B; Supplementary Table S5). Together, under the direct embryonic injection scheme, the *Asvasa2*-Cas9-Chr. 1₁₀₅₂₅₃₆₂₈ line mediated the highest editing and germline transmission efficiency compared to the other two Cas9 lines tested. These findings therefore confirm that

endogenous Cas9 expression under germline-biased promoters provides a robust alternative to injected commercial Cas9 protein for target site mutagenesis.

U6 promoter identification and transcriptional activity analysis via editing efficiency

To identify functional Pol III promoters, we screened the *An. sinensis* genome and identified six putative *AsU6* genes (Figure 4A). Analysis of their upstream regulatory regions revealed that four of these genes, *AsU6-1*, *AsU6-2*, *AsU6-3*, and *AsU6-4* contained the essential promoter elements: the proximal sequence element and TATA box (Figure 4A; Supplementary Figure S8). Among these, only *AsU6-1* possessed a distal regulatory element, the octamer motif (OCT), within its selected upstream regulatory regions (Supplementary Figure S8). Editing efficiency mediated by these promoters varied substantially, as assessed by microinjecting *AsU6*-gRNA plasmids into eggs produced from crosses between female *Asvasa2*-Cas9-Chr. 1₁₀₅₂₅₃₆₂₈ individuals and male laboratory-reared *Aswhite* individuals. *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 4B; Supplementary Table S6). All four *AsU6* promoters supported germline transmission, as evidenced by white-eyed G₁ females following crosses of mosaic G₀ males with lab-reared *Aswhite* mutant females (Figure 4C; Supplementary File S4). Furthermore, when the *AsU6-1*-gRNA construct was delivered into other two Cas9 backgrounds, the *Aszpg*-Cas9-Chr.

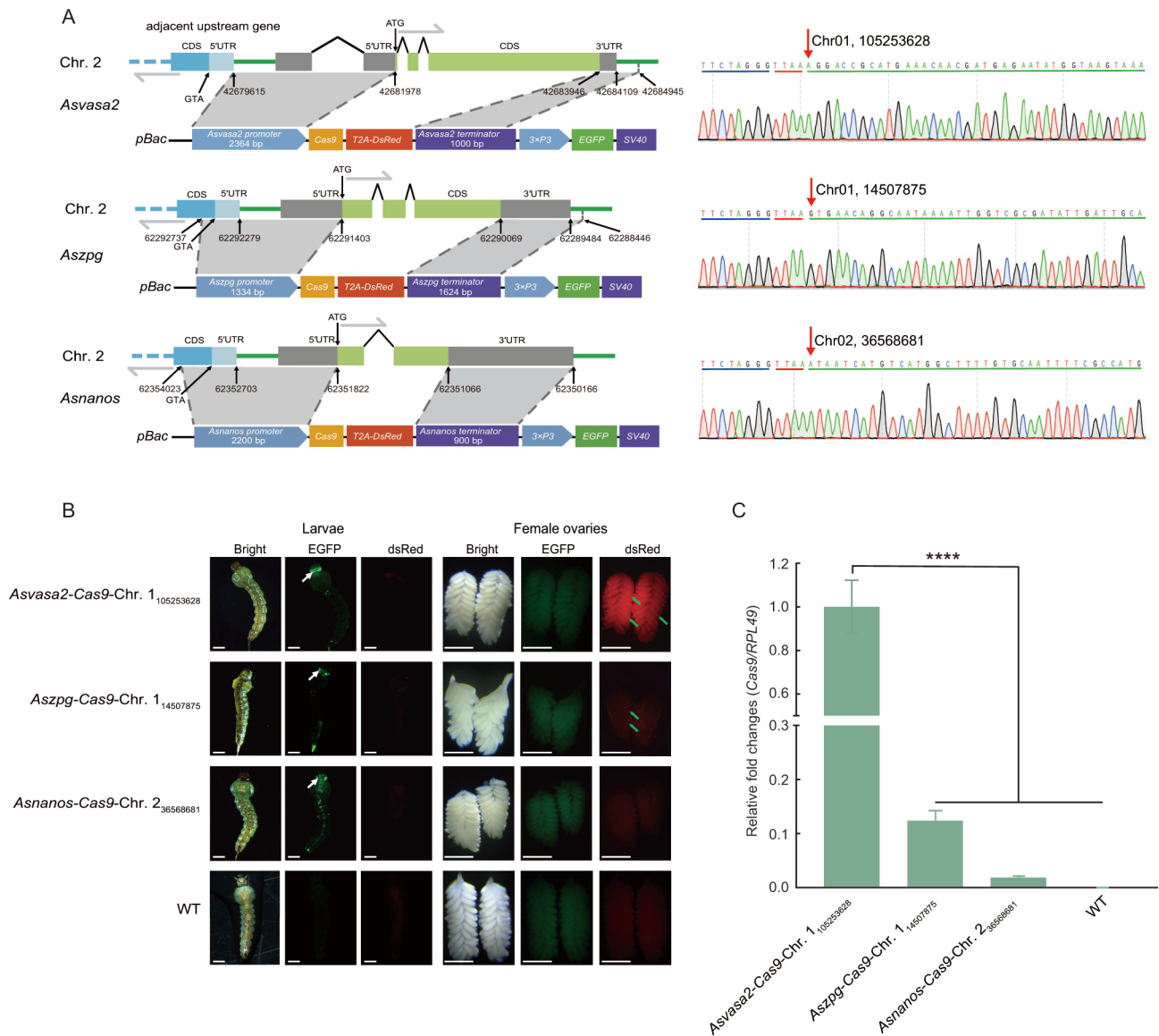


Figure 2 Construction and characterization of germline-biased promoter-driven Cas9 transgenic lines in *An. sinensis*

A: Schematic of the Cas9 expression plasmids constructs and inverse PCR results for the three Cas9 transgenic lines (Underline colors denote: blue, *piggyBac*-derived sequences; red, TTA insertion site; green, flanking genomic regions). **B:** Fluorescence validation in ovaries of Cas9 transgenic lines. White and green arrows indicate green and red fluorescence, respectively. Scale bar: 1 mm. **C:** Cas9 transcription levels in three transgenic lines (*t*-test. *n*=3. ****: *P*<0.0001).

1₁₄₅₀₇₈₇₅ line showed a mosaic editing rate of 10.3%, while no edits were detected in the *Asnanos*-Cas9-Chr. 2₃₆₅₆₈₆₈₁ background (Supplementary Figure S9; Supplementary Table S7). These results indicate that we have successfully screened three *AsU6* promoters (*AsU6-1*, *AsU6-3*, *AsU6-4*) with high transcriptional activity, and further confirmed that the editing efficiency of Cas9 driven by the *Aszpg* and *Asnanos* promoters is relatively limited with respect to mutagenesis.

Optimal cross between *Asvasa2*-Cas9-Chr. 1₁₀₅₂₅₃₆₂₈ and *AsU6-1*-Chr. 1₄₈₄₁₁₂₅₁ lines achieves complete editing efficiency

We next leveraged the validated promoters to construct the *AsU6-1-gRNA^{Aswhite}*-Chr. 1₄₈₄₁₁₂₅₁ line (Figure 5A; Supplementary Table S2), establishing a promoter-driven transgenic system for assessing heritable editing efficiency. Orthogonal crosses between *Asvasa2*-Cas9-Chr. 1₁₀₅₂₅₃₆₂₈ females and *AsU6-1-gRNA^{Aswhite}*-Chr. 1₄₈₄₁₁₂₅₁ males produced 100% white-eyed F₁ progeny, whereas in the

reciprocal cross, 57.4% of F₁ offspring were fully white-eyed (Figure 5B; Supplementary Table S8) and the remaining 42.6% exhibited mosaic eyes with ~92.6% unpigmented area (Supplementary Figures S1, S11). The complete absence of mosaic phenotype in the orthogonal cross suggests that maternal Cas9 deposition from oocytes enhanced editing efficacy in early embryos (Carballar-Lejarazú et al., 2022; Sakurai et al., 2020). Ovarian editing frequencies observed for *gRNA* target site 1 and site 2 exceeded 99.2% across both cross schemes (Figure 5C; Supplementary Table S9; Supplementary File S5). All F₂ offspring from subsequent crosses of F₁ females to lab-reared *Aswhite* mutant males displayed the white-eyed phenotype (Figure 5C; Supplementary Table S10), confirming complete germline transmission using the *Asvasa2* and *AsU6-1* promoters.

Crosses between *Aszpg*-Cas9-Chr. 1₁₄₅₀₇₈₇₅ females and *AsU6-1-gRNA^{Aswhite}*-Chr. 1₄₈₄₁₁₂₅₁ males produced 42.6% F₁ offspring with mosaic eyes (~19.3% unpigmented area), while

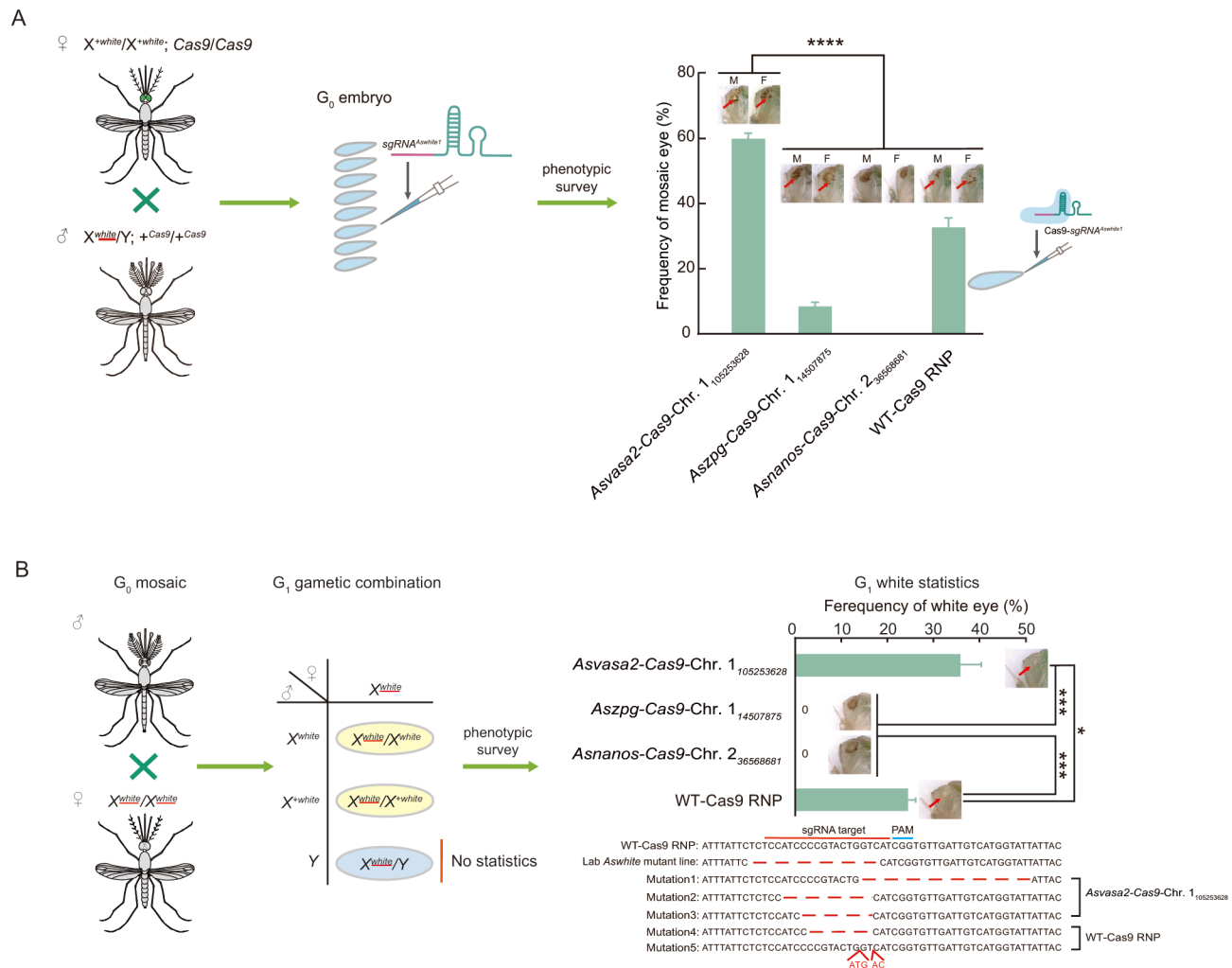


Figure 3 Assessment of somatic and heritable editing by *gRNA* delivery in *Cas9*-expressing lines

A: Editing efficiency in G_0 progeny was compared between two crosses: embryos from *Cas9* transgenic females $\times$ *Aswhite* mutant males injected with *gRNA*^{*Aswhite1*}, and embryos from WT females $\times$ *Aswhite* mutant males injected with *Cas9*-*gRNA*^{*Aswhite1*} complexes (*t*-test, $n=3$. M: male; F: female). B: Germline transmission assay via G_0 mosaic males crossed to lab-reared *Aswhite* mutant females (*t*-test, $n=3$). G_1 mutations were verified by Sanger sequencing. Red arrows point to mosaic eye phenotypes. *: $P<0.05$, ***: $P<0.001$, ****: $P<0.0001$

the reciprocal cross yielded 32.0% mosaics (~8.3% unpigmented area) (Figure 5B; Supplementary Figures S10, S11 and Table S8). In F_1 females, ovarian editing rates ranged from 38.1% to 45.0% at *gRNA* target site 1 and from 20.6% to 23.0% at target *gRNA* site 2 (Figure 5C; Supplementary Table S9 and File S5). Subsequent F_2 crosses (obtained 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 5D; Supplementary Table S11). These results indicate that the *Aszpg* promoter supports moderate germline editing with respect to mutagenesis. This form of editing differs from the utilization of the *zpg* promoter to facilitate efficient homology-directed repair (HDR) in *An. gambiae* (Hammond et al., 2021; Kyrou et al., 2018). In contrast, crosses involving *Asnanos*-*Cas9*-Chr. 2₃₆₅₆₈₆₈₁ and *AsU6-1-gRNA*^{*Aswhite*}-Chr. 1₄₈₄₁₁₂₅₁ lines yielded no detectable eye phenotypes in F_1 (Figure 5B; Supplementary Figures S10, S11 and Table S8), low ovarian editing rates ($\leq 12.2\%$ for *gRNA* target 1 and $\leq 10.2\%$ for *gRNA* target 2; Figure 5C; Supplementary Table S9 and File S5), and minimal white-eyed F_2 offspring ($\leq 0.2\%$; Figure 5D; Supplementary Table S12). Collectively, these data demonstrate that the *Asvasa2* promoter drives *Cas9*

expression resulting in a higher rate of target site mutagenesis.

Constitutive *gRNA* expression reveals higher activity of *AsU6-1* compared to *AsU6-2*

Following our initial screening of *An. sinensis* *U6* promoters via transient expression assays, which identified *AsU6-1* as the most active, we further validated their activities under stable, constitutive expression conditions. To directly compare the transcriptional efficiency of the constitutively expressed *AsU6-1* and *AsU6-2* promoters, we generated a stable *AsU6-2-gRNA*^{*Aswhite*}-Chr. 2₉₁₃₀₄₉₂₈ transgenic line and crossed it with the *Asvasa2-Cas9*-Chr. 1₁₀₅₂₅₃₆₂₈ line (Figure 6A, B; Supplementary Table S2). Orthogonal crosses produced 36.8% white-eyed and 63.2% mosaic F_1 progeny (~88.2% unpigmentation) (Figure 6B; Supplementary Figures S11, S12 and Table S13). No white-eyed individuals occurred in the reciprocal cross, and mosaic eyes showed reduced unpigmentation (~53.8% unpigmentation) (Figure 6B; Supplementary Figures S11, S12 and Table S13). In F_1 females, ovarian editing rates ranged from 68.8% to 90.1% at *gRNA* target site 1 and from 31.2% to 72.1% at *gRNA* target site 2 (Figure 6C; Supplementary Table S14 and File S5). The

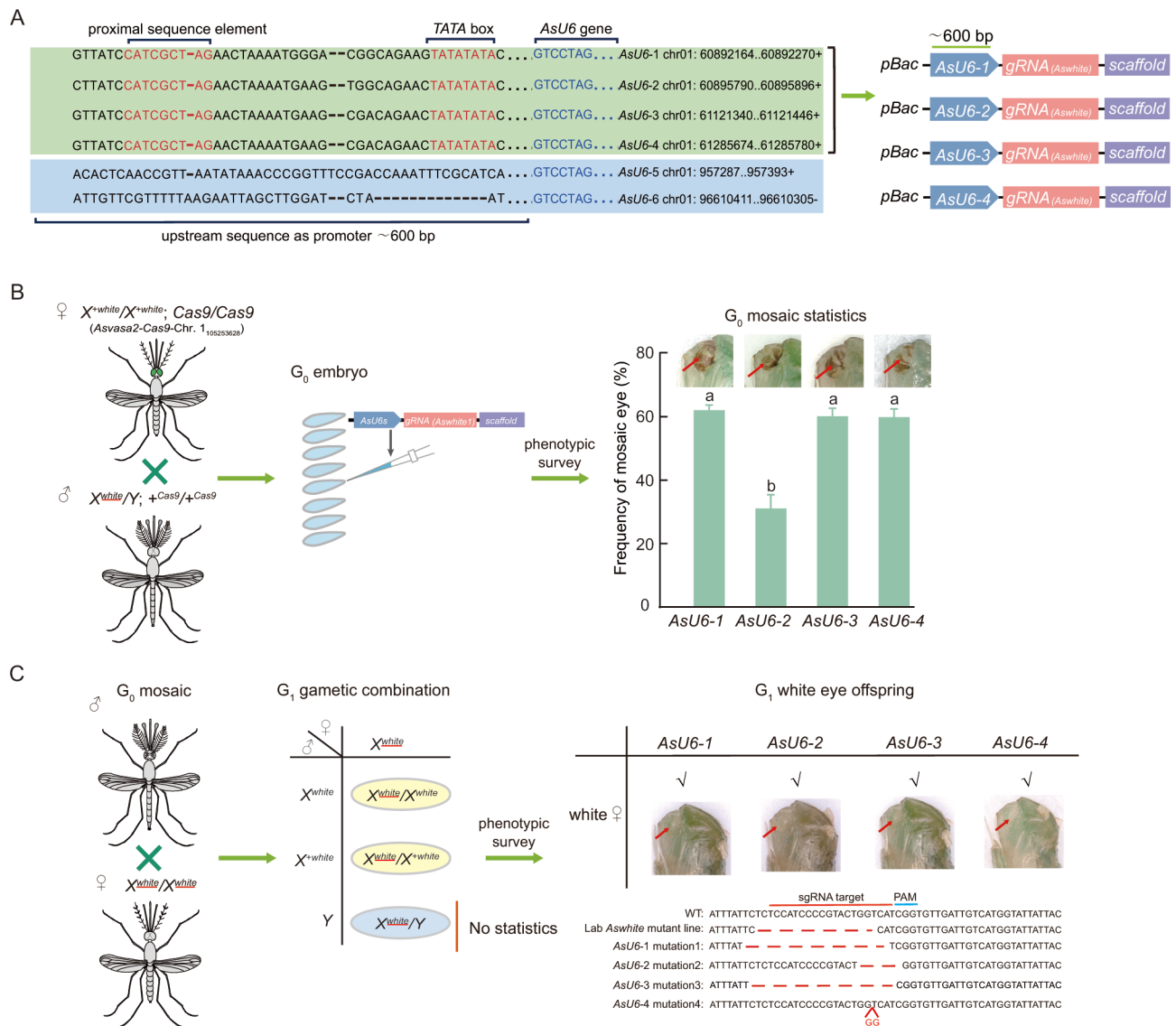


Figure 4 Evaluation of editing efficiency by *AsU6s*-gRNA plasmid injection in the *Asvasa2-Cas9-Chr. 1*₁₀₅₂₅₃₆₂₈ transgenic line

A: Genomic features of *AsU6* promoter regions and *gRNA* expression cassette architectures. **B:** *Aswhite* editing efficiency mediated by four *AsU6*-*gRNA* plasmids in G_0 individuals (One-way ANOVA analysis, $F=76.46$, different letters indicate significant differences, $n=3$). **C:** Qualitative assessment of germline transmission edits driven by four *AsU6* promoters. Verification of G_1 mutations was performed by Sanger sequencing. ✓ indicates heritable mutations with mutation patterns distinct from those of the pre-existing *Aswhite* mutant line maintained in the laboratory. Red arrows point to mosaic eye phenotypes.

white-eyed F_2 frequencies were $\leq 62.5\%$ (Figure 6D; Supplementary Table S15), which is appreciably lower than the rates achieved with *AsU6-1* promoter. This confirms that *AsU6-2* promoter drives functional but less efficient editing. Together, these data support the conclusion that under stable constitutive expression, the *AsU6-1* promoter drives more robust *gRNA* transcription than *AsU6-2* promoter, yielding superior editing penetrance and establishing its clear advantage for sustained CRISPR applications.

Female-to-male transformation by *dsx-F* disruption using binary transgenic editing system

We constructed the *AsU6-1-gRNA*^{*Asdsx-F*}-Chr.1₅₂₃₀₄₄₃₂ transgenic line (Figure 7A, B; Supplementary Table S2), where the transcribed *gRNA* targets both the intron 4-exon 5 boundary and an internal sequence within the female-specific exon 5 (Figure 7B). This strategy specifically disrupts functional *Asdsx-F* transcript formation while maintaining intact

Asdsx-M expression. Following crosses between *AsU6-1-gRNA*^{*Asdsx-F*}-Chr. 1₅₂₃₀₄₄₃₂ (+/-) males and *Asvasa2-Cas9-Chr. 1*₁₀₅₂₅₃₆₂₈ (+/-) females, within the double-fluorescent F_1 progeny (Supplementary Figure S13), we observed no individuals with typical female morphological features, and approximately 52.6% of them displayed both male and female morphological features (intersex phenotype) (Figure 7C; Supplementary Table S16). Specifically, during the pupal stage, these intersex individuals exhibited male-like genital lobes (Supplementary Table S16). Adult intersex individuals displayed male-like plumose antennae, and their female cerci were replaced by masculinized external genitalia with claspers that failed to undergo normal rotation within two days post-eclosion (Figure 7C). Dissection of internal reproductive organs revealed the presence of male accessory glands and the absence of ovaries and spermathecae (Figure 7C). All intersex phenotypic individuals showed efficient editing at the *dsx-F* locus (Figure 7D; Supplementary File S6). Furthermore,

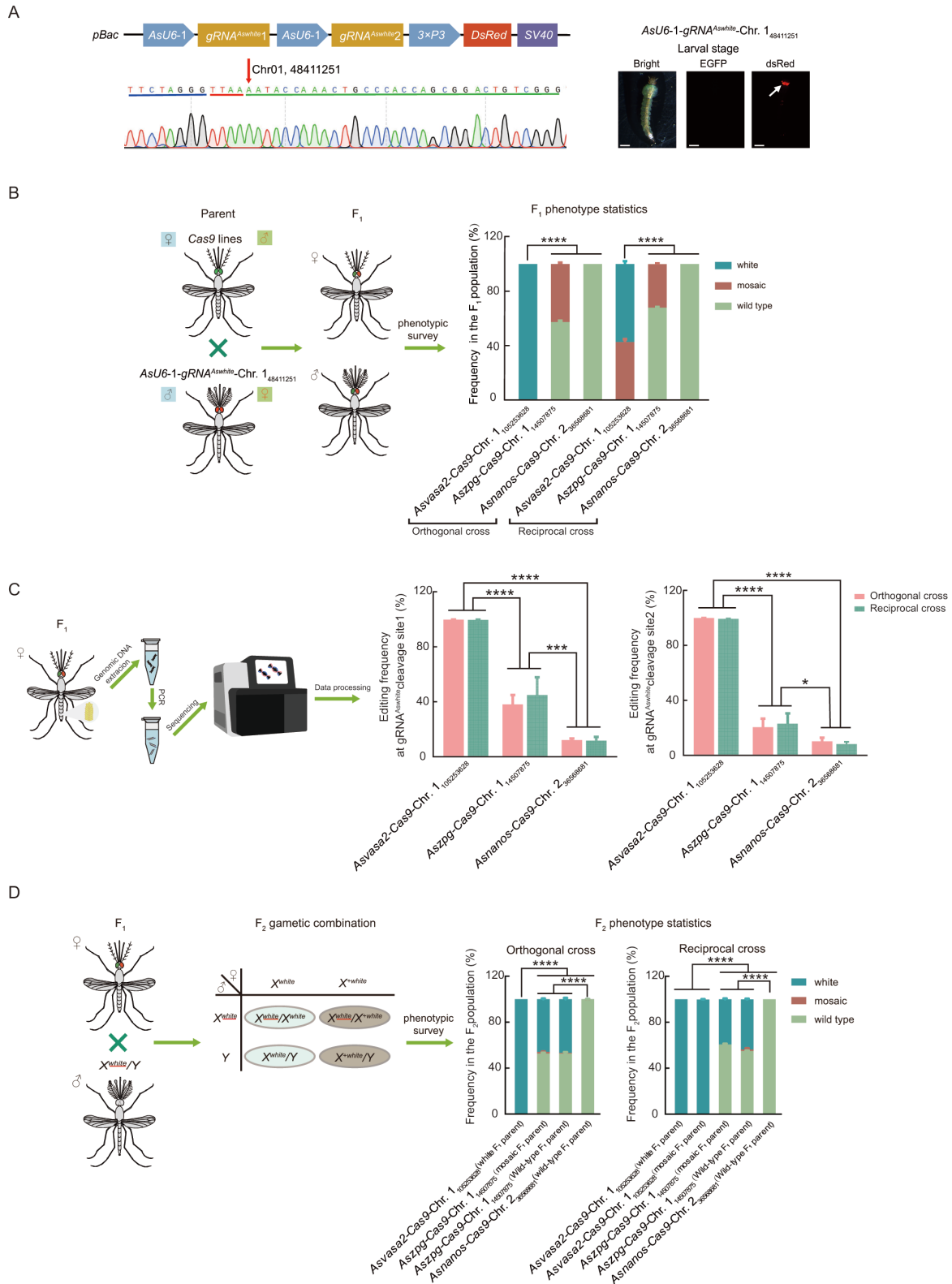


Figure 5 Quantification of editing efficiency across three *Cas9* transgenic lines using a binary transgenic system

A: Transgenic plasmid construct of *AsU6-1-gRNA^{Aswhite}*, fluorescence phenotype of its transgenic line, and inverse PCR mapping of the insertion site (Scale bar: 1mm; underline colors denote: blue, *piggyBac*-derived sequences; red, *TTAA* insertion site; green, flanking genomic regions). B: Phenotypic distribution, including the statistically significant proportions of white-eyed individuals, were determined for the F_1 offspring from crosses between *Cas9* and *AsU6-1-gRNA^{Aswhite}-Chr. 1₄₈₄₁₁₂₅₁* lines (t -test, $n=3$). C: Mutation frequency in F_1 ovaries determined by high-throughput sequencing (t -test, $n=3$). D: Phenotypic distribution, including the statistically significant proportions of white-eyed individuals, were determined for the F_2 progeny derived from crossing F_1 (*Cas9*×*AsU6-1-gRNA^{Aswhite}-Chr. 1₄₈₄₁₁₂₅₁*) females with *Aswhite* mutant males (t -test, $n=3$). Orthogonal cross: *Cas9* lines females×*AsU6-1-gRNA^{Aswhite}-Chr. 1₄₈₄₁₁₂₅₁* line males, reciprocal cross: *Cas9* lines males×*AsU6-1-gRNA^{Aswhite}-Chr. 1₄₈₄₁₁₂₅₁* line females. *: $P<0.05$; ***: $P<0.001$; ****: $P<0.0001$.

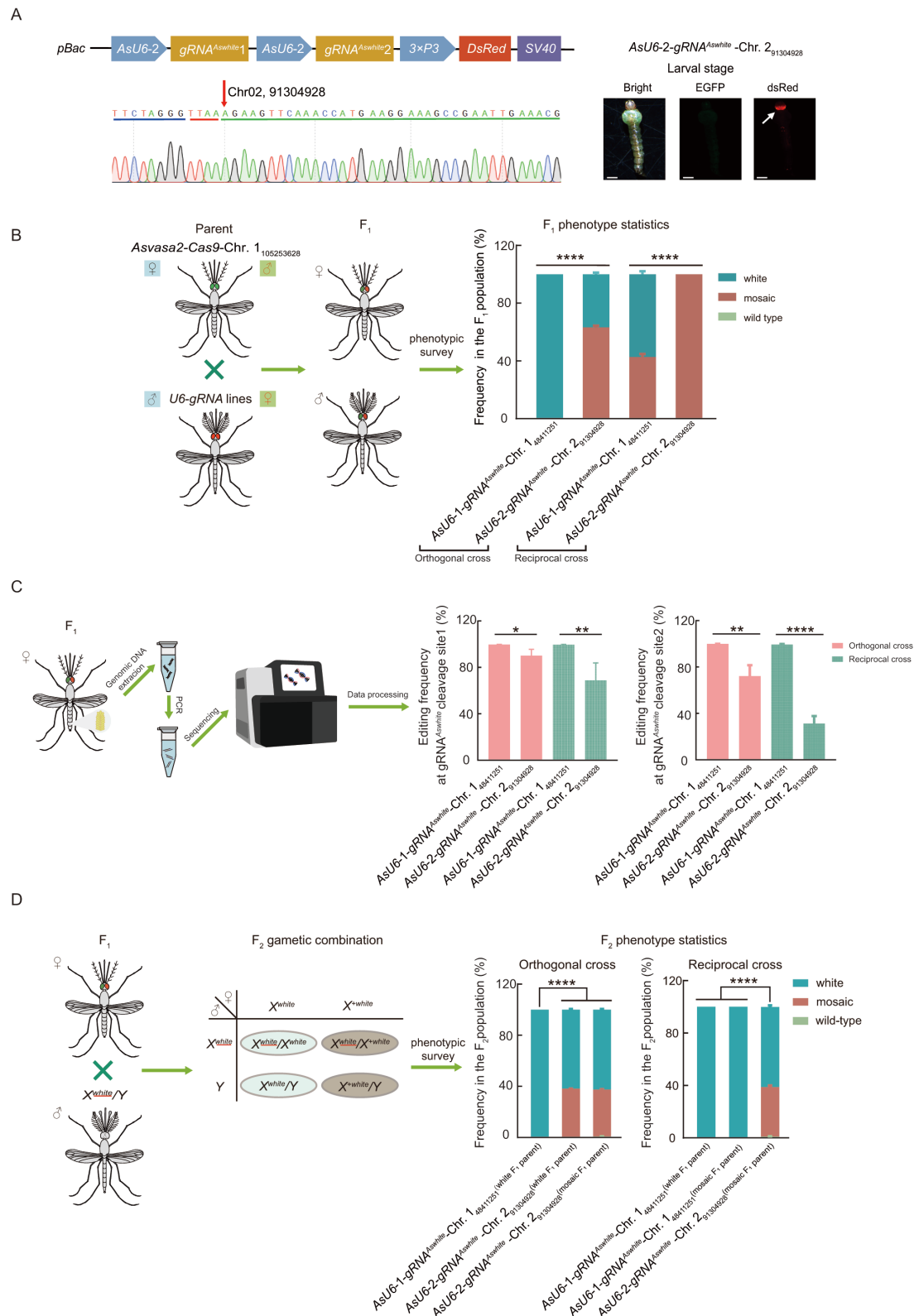


Figure 6 Comparison of editing efficiency mediated by *AsU6-1* versus *AsU6-2* promoters in the binary transgenic system

A: Transgenic plasmid construct of *AsU6-2-gRNA^{Aswhite}*, fluorescence phenotype of its transgenic line, and inverse PCR mapping of the insertion site (Scale bar: 1mm; underline colors denote: blue, *piggyBac*-derived sequences; red, *TTAA* insertion site; green, flanking genomic regions). **B:** Phenotypic distribution, including the statistically significant proportions of white-eyed individuals, were determined for the F_1 offspring from crosses between *Asvasa2-Cas9-Chr. 1₁₀₅₂₅₃₆₂₈* and *AsU6-gRNA* lines (t -test. $n=3$). **C:** Mutation frequency in F_1 ovaries determined by high-throughput sequencing (t -test. $n \geq 3$). **D:** Phenotypic distribution, including the statistically significant proportion of white-eyed individuals, was determined for the F_2 progeny derived from crossing F_1 (*Asvasa2-Cas9-Chr. 1₁₀₅₂₅₃₆₂₈* × *AsU6-gRNA^{Aswhite}*) females with *Aswhite* mutant males (t -test. $n=3$). Orthogonal cross: *Asvasa2-Cas9-Chr. 1₁₀₅₂₅₃₆₂₈* line females × *gRNA* lines males, reciprocal cross: *Asvasa2-Cas9-Chr. 1₁₀₅₂₅₃₆₂₈* line males × *gRNA* lines females. *: $P < 0.05$; **: $P < 0.01$; ****: $P < 0.0001$

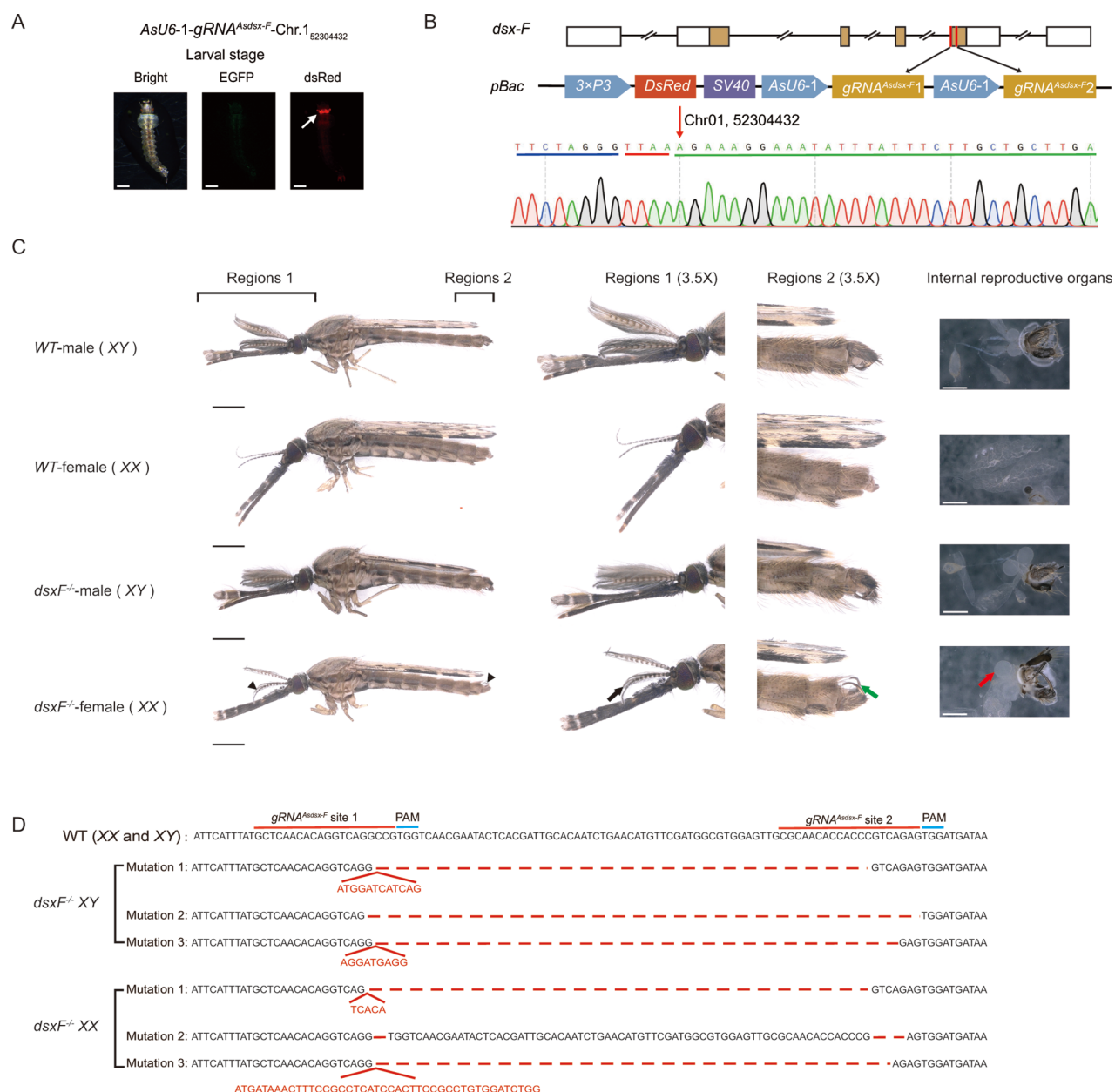


Figure 7 Sex reversal from *dsx-F* mutation via binary transgenic crosses

A: Fluorescence phenotype of the transgenic *AsU6-1-gRNA^{Asdsx-F}-Chr.1₅₂₃₀₄₄₃₂* line (Scale bar: 1mm). **B:** Schematic of female-specific *dsx-F* transcripts with *gRNA* target sites (Coding regions of exons (CDS) are shaded in khaki color, and non-coding regions (UTR) are in white color) and inverse PCR mapping of the insertion site (Underline colors denote: blue, *piggyBac*-derived sequences; red, *TAA* insertion site; green, flanking genomic regions). **C:** Morphological characteristics of the wild-type and *dsx-F* mutant individuals (Scale bar: 0.5 mm). Triangle symbols indicate the presence of aberrant structural features in *dsx-F* knockout females. Body regions 1 and 2 are magnified 3.5×. In *dsx-F* knockout females, black and green arrow represents the male-like plumose antennae and the non-rotated external genitalia with masculinized phenotype, respectively. The red arrow indicates abnormal tissue resembling the male accessory glands in *dsx-F* knockout females (Scale bar: 0.25 mm). **D:** *F*₁ mutations were verified by Sanger sequencing.

relative genomic qPCR targeting the X-linked marker gene *Aswhite* confirmed that intersex individuals carry twice the copy number compared to wild-type males, demonstrating that they are genetically female with an XX genotype (Supplementary Figure S14). The observed intersex phenotypes, which are associated with an XX genotype, are consistent with those reported in *An. gambiae* and *Anopheles stephensi* following *dsx-F* depletion in females (Kyrou et al., 2018; Xu et al., 2025). These sex-reversed females completely lost reproductive capacity compared to wild-type females (Supplementary Table S17). Together, results from

editing efficiency tests targeting *dsx-F* confirm the robustness and high efficiency of established binary transgenic editing system.

DISCUSSION

In this study, by assessing the phenotypes and editing efficiencies of the marker genes *Aswhite* and *Asdsx-F*, we found that a binary transgenic system combining the *Asvasa2* promoter, which drives predominant expression in female gonadal tissues, and the *AsU6-1* promoter enables complete and heritable genome editing in the malaria vector *An.*

sinensis. The binary transgenic system streamlines genome editing in multiple aspects. First, it enables rapid identification of potentially edited individuals in hybrid progeny through straightforward fluorescence-based selection (Li et al., 2025; Ursache et al., 2021; Zhu et al., 2021). Thus, even if the phenotypic trait of a target gene becomes apparent only at later developmental stages (e.g., adulthood), researchers can nondestructively identify edited individuals as early as the embryonic or larval phase, greatly simplifying the workflow. Particularly for genetic loci without obvious morphological phenotypes, this fluorescence-based screening avoids the physical harm and mortality risks associated with traditional genotyping methods that require tissue sampling (e.g., wing-clipping or leg removal) for DNA extraction, making it a highly efficient and specimen-friendly approach, especially for small-sized insects. Beyond this operational simplicity, the system is inherently modular: multiplexed editing is easily achieved by replacing the *U6*-driven *gRNA* sequences and adjusting the number of *U6* promoter-*gRNA* cassettes. More importantly, the spatiotemporal control of *Cas9* expression, governed by its promoter, establishes a foundation for future conditionally restricted editing systems. Such promoter-directed regulation is crucial for dissecting the roles of essential genes, such as probing the functions of embryonic lethal genes in specific tissues or at post-embryonic stages, thereby circumventing the early lethality that precludes their analysis in a constitutive knockout. Even without employing the binary crossing system, delivering *gRNA* into the *Asvasa2-Cas9-Chr. 1₁₀₅₂₅₃₆₂₈* line, an approach that is simpler, resulted in improved mutagenesis efficiency compared with delivering *Cas9-gRNA* RNP complexes into wild-type individuals (Figure 3A). It also reduces the cost of commercial *Cas9* protein and avoids technical issues such as needle clogging due to protein precipitation and denaturation during microinjection. Furthermore, in the context of genetic control for agricultural or medical pests, such as release strategies based on precision-guided sterile insect technique (pgSIT), crossing between binary transgenic lines constitutes an essential step to generate sterile individuals (Li et al., 2021; Smidler et al., 2023). Similarly, in split-gene drive systems, highly active germline and *U6* promoters lines likewise constitute core genetic components (Chennuri et al., 2024; Xu et al., 2025).

Notably, the random integration of transgenes in conventional transgenic systems raises the possibility that positional effects, rather than the promoter itself, influence the transcription of the expression cassette (Dilani et al., 2022). To investigate this possibility, we examined *Cas9* transcript levels among lines carrying the same expression cassette integrated at different genomic positions and found that *Vasa2*-driven *Cas9* expression was consistently higher than that driven by the *zpg* or *nanos* promoters (Supplementary Figure S5). This trend was corroborated by tissue transcriptome data, which showed significantly higher TPM for *Asvasa2* than for *zpg* and *nanos*. In addition, no notable differences in reporter fluorescence intensity were observed among the three lines selected for gene editing analysis (Supplementary Figure S7). These observations suggested that positional effects have a limited influence on promoter activity in the present experimental system. In future studies, we will achieve more precise comparisons of transcriptional activities among different promoters. This will be accomplished either by using site-specific integration, for example, by targeting the expression cassette to the cardinal

locus (Feng et al., 2021), or by first identifying suitable integration sites where positional effects are minimal through comparative analysis of the transcriptional activities of identical constructs inserted at multiple distinct genomic positions, and then using such sites to evaluate different promoters.

Additionally, although the promoter of *zpg* ortholog in *An. sinensis* mediated significantly lower levels of *Cas9* expression and editing efficiency with respect to target site mutagenesis compared to the *Asvasa2* promoter (Figures 2, 3, 5), the *zpg* promoter has been validated in *An. gambiae* as an efficient driver of *Cas9* transcription for gene knock-in in gene-drive applications (Hammond et al., 2021; Kyrou et al., 2018). This suggests that transgenes driven by the *zpg* promoter may better facilitate genetic modifications via homology-directed repair, and also indicates that different types of germline promoters may have distinct application priorities depending on the specific genetic manipulation scenario. Moreover, the *Asnanos* promoter failed to support detectable germline editing with respect to gene mutagenesis (Figures 3, 5), contrasting with the high efficiency reported for *nanos*-driven *Cas9* for targeted mutagenesis in *Bombyx mori* and *D. melanogaster* (Ren et al., 2013; Xu et al., 2019a). These results further underscore the necessity of developing species-specific germline promoters for reliable genetic applications in target insect species (Green et al., 2023). Our strategy for selecting germline-biased promoters included extending promoter boundaries into upstream untranslated or coding regions (Figure 2A). Nevertheless, whether promoters such as *Aszpg* or *Asnanos* depend on distal regulatory elements like enhancer or intron sequences remains unclear. Chromatin accessibility assays in future studies could clarify this possibility and guide optimization. The development of germline promoters with enhanced specificity and potency also warrants further investigation.

We observed mosaic eye pigmentation in progeny from the *Asvasa2-Cas9-Chr. 1₁₀₅₂₅₃₆₂₈* ♂ × *AsU6-1-gRNA^{Aswhite}-Chr. 1₄₈₄₁₁₂₅₁* ♀ cross, a phenotype absent in the *Asvasa2-Cas9-Chr. 1₁₀₅₂₅₃₆₂₈* ♀ × *AsU6-1-gRNA^{Aswhite}-Chr. 1₄₈₄₁₁₂₅₁* ♂ cross, where all offspring displayed uniform white eyes (Figure 5B; Supplementary Table S8). Separately, in *Asvasa2-Cas9-Chr. 1₁₀₅₂₅₃₆₂₈* ♀ × *AsU6-1-gRNA^{Asdsx-F}-Chr. 1₅₂₃₀₄₄₃₂* ♂ crosses, single red-fluorescent progeny, carrying only the *AsU6-1-gRNA* expression cassette without the *Cas9* expression cassette, included intersex individuals (Supplementary Figures S13–S15 and File S7). Collectively, these observations demonstrate maternal deposition of *Cas9* protein, a phenomenon previously documented in binary transgenic systems in *Culex quinquefasciatus*, *D. melanogaster*, and *Hermetia illucens* (Harvey-Samuel et al., 2023; Kou et al., 2024; Lin & Potter, 2016). When *Asvasa2-Cas9-Chr. 1₁₀₅₂₅₃₆₂₈* females serve as the maternal parent, germline-expressed *Cas9* protein is deposited into oocytes during oogenesis (Gokcezaade et al., 2014) (Figure 2B). Following fertilization, the maternally deposited *Cas9* protein binds to *gRNAs* that are transcribed from the paternally inherited *AsU6-1-gRNA^{Aswhite}* or *AsU6-1-gRNA^{Asdsx-F}* transgenic cassettes, initiating editing before zygotic genome activation. Consequently, this mechanistic cascade appears to be considered responsible for producing the aforementioned phenotypes. Maternal deposition of *Cas9* may facilitate drive resistance in gene-drive systems, thereby reducing drive efficiency (Champer et al., 2017, 2019; Du et al., 2024). To

address uncontrolled editing from maternal Cas9 deposition, employing spatiotemporally restricted promoters (e.g., zygotic or gonad-specific) for *gRNA* expression presents a promising solution. Identifying endogenous promoters active in specific developmental stages or tissues would enable precise spatiotemporal control of editing, enhancing safety and efficacy in gene drive applications. However, in contexts emphasizing editing outcomes, this maternal deposition ensures persistent Cas9 presence during development, supporting phenotypic manifestation (Kou et al., 2024; Lin & Potter, 2016). Therefore, we favor using *Asvasa2-Cas9-Chr. 1₁₀₅₂₅₃₆₂₈* females as the maternal parent for gene knockout experiments in the binary transgenic system.

Among the six predicted *U6* genes, the distal regulatory element, octamer motif *OCT*, was identified only in the upstream regulatory region of *AsU6-1* (Supplementary Figure S8). In studies focusing on the identification and functional characterization of Pol III promoters in mosquitoes, promoter regions are commonly defined according to the proximal *TATA* box and proximal sequence element (*PSE*) (Anderson et al., 2020; Gonzalez et al., 2025; Li et al., 2020). Similarly, in *Epinephelus coioides*, not all *U6* promoters were found to contain the *OCT* motif (Yan et al., 2023). In this study, the selected *AsU6* promoters were functional in gene editing assays, indicating that the current screening strategy is applicable in this species. We speculate that the *OCT* motif might not be a critical regulatory element for *U6* promoter activity in mosquito gene editing. On the basis of considerations of practical efficiency and ease of construct design, *U6* promoters of moderate length that contain the *PSE* and *TATA* box provide a practical option in mosquitoes.

In addition, *AsU6-3* and *AsU6-4* promoters also exhibit strong *gRNA* transcriptional activity comparable to *AsU6-1* promoter (Figure 4), enriching the *U6* promoter toolkit for mosquito genetic engineering (Xu et al., 2025). This expansion of *U6* promoters is particularly valuable for gene drive strategies, which typically require two or more *gRNA* modules within a single knock-in cassette (Vad-Nielsen et al., 2016; Zhu et al., 2021). To prevent deletion mutations from repetitive promoter sequences (Green et al., 2023; Kosicki et al., 2018), distinct RNA polymerase III promoters should drive individual *gRNAs*. The availability of multiple promoters such as *AsU6-1*, *AsU6-3*, *AsU6-4*, and potential candidates including *7SK* in *An. sinensis* enables efficient multiplexed *gRNA* expression while reducing resistance allele formation. Additionally, the binary transgenic system provides a versatile platform for validating candidate RNA polymerase III promoters and assessing their endogenous expression efficiency, supporting the development of effective genetic control strategies against mosquitoes.

The editing system developed in this study, which relies on crosses between transgenic tool lines, holds substantial potential for translation to other insect taxa. Successful adaptation requires several foundational conditions: (1) embryonic microinjection capability in the target insect; (2) basic omics resources, such as gonadal transcriptome data, for promoter screening; (3) access to fluorescence-equipped stereomicroscopy for phenotypic screening; and (4) availability of core vector backbones, including plasmids containing the *piggyBac* transposon. These requirements are relatively moderate and can often be satisfied in many insect systems. Therefore, following the genetic element screening and efficiency assessment pipeline established in this work, the

development of analogous binary editing platforms in other insects and the consequent expansion of their genetic toolkits should significantly deepen and broaden related research fields and promote the application of transgenic-based synthetic biology approaches in a wider range of species.

In summary, we have established a promoter-driven transgenic system that enables highly efficient gene editing in *An. sinensis*, a major malaria vector. Our findings greatly expand the genetic toolkit available for synthetic biology, such as gene drive-based population suppression in non-model mosquitoes, and provide a transferable conceptual framework for genetic manipulation in other recalcitrant insect species.

SUPPLEMENTARY DATA

Supplementary data to this article can be found online.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

J.F.H.: Writing-Original draft, Writing-Review & Editing, Visualization, Methodology, Investigation, Data Curation. Q.L.Z.: Writing-Review & Editing, Methodology, Validation, Investigation, Data curation. X.Y.X.: Methodology, Validation, Resources, Investigation, Data curation. Y.P.J.: Methodology, Validation, Investigation, Data curation. S.Y.W.: Investigation, Resources. X.L.: Methodology. C.Z.: Data Curation. X.C.: Resources. Y.X.Y.: Resources. Y.C.: Resources. W.S.: Data curation, Writing-Review & Editing, Conceptualization. B.C.: Writing-Review & Editing, Supervision, Funding acquisition, Data curation, Conceptualization. L.Q.: Writing-Original draft, Writing-Review & Editing, Methodology, Validation, Supervision, Funding acquisition, Resources, Data curation, Conceptualization. All authors read and approved the final version of the manuscript.

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