Genetically Encoded CRISPR Components Yield Efficient Gene Editing in the Invasive Pest *Drosophila suzukii*

Nikolay P. Kandul,1, Esther J. Belikoff,2, Junru Liu,1 Anna Buchman,1 Fang Li,2 Akihiko Yamamoto,2 Ting Yang,1 Isaiah Shriner,1 Maxwell J. Scott,2,* and Omar S. Akbari1,*

**Abstract**

Originally from Asia, *Drosophila suzukii* Matsumura is a global pest of economically important soft-skinned fruits. Also commonly known as spotted wing drosophila, it is largely controlled through repeated applications of broad-spectrum insecticides by which resistance has been observed in the field. There is a pressing need for a better understanding of *D. suzukii* biology and for developing alternative environmentally friendly methods of control. The RNA-guided Cas9 nuclease has revolutionized functional genomics and is an integral component of several recently developed genetic strategies for population control of insects. Here, we describe genetically modified strains that encode three different terminators and four different promoters to express Cas9 robustly in both the soma and/or germline of *D. suzukii*. The Cas9 strains were rigorously evaluated through genetic crossing to transgenic strains that encode single-guide RNAs targeting the conserved X-linked yellow body and white eye genes. We find that several Cas9/gRNA strains display remarkably high editing capacity. Going forward, these tools will be instrumental for evaluating gene function in *D. suzukii* and may even provide tools useful for the development of new genetic strategies for control of this invasive species.

**Introduction**

*Drosophila suzukii* (Matsumura, 1931, Diptera: *Drosophilidae*), commonly known as spotted wing drosophila, is a significant crop pest1–3 of many soft-skinned fruits that has recently invaded much of the world.4–6 Unlike other *Drosophila* fly species that infest overripe or rotting fruits, *D. suzukii* targets ripening fruits.4,6,7 *D. suzukii* females use a serrated ovipositor to pierce the fruit skin, and deposit progeny inside to consume the fruit.4,8 The external wounds generated by oviposition alone leave the fruit vulnerable to secondary infections caused by pathogens, including bacteria, yeasts, and fungi. The short generation time of *D. suzukii* contributes to its rapid spread, resulting in significant revenue losses.1–3 Endemic to East Asia, *D. suzukii* has become established around much of Europe, North America, and South America since 2008,2,3,9,10 with modeling predicting even further spread.11 Given the invasiveness of *D. suzukii* and the significant crop damages it imposes, there exists a pressing need to generate molecular tools that can be used for both gaining a better understanding of *D. suzukii* biology and to innovate alternative environmentally friendly control methodologies.5,6,12,13

*D. suzukii* is largely controlled through the use of insecticides.14–17 However, insecticide applications can provide limited protection, indiscriminately affect beneficial species,18 and insecticide resistance has emerged.13 Environmentally friendly species-specific methods of insect pest control, such as the sterile insect technique (SIT) and the incompatible insect technique (IIT), are being developed for *D. suzukii*. In SIT applications, flies are mass reared and exposed to high doses of ionizing radiation for sterilization. Then, excess sterilized males are repeatedly released to mate with wild females, which in turn lay unfertilized eggs.19 While radiation conditions for *D. suzukii* have been identified that produce both sterile...
males and females,\textsuperscript{20,21} further testing is necessary to determine if these males are competitive enough to suppress populations. In IIT applications, flies are instead infected with the endosymbiont \textit{Wolbachia}, which has been shown to induce cytoplasmic incompatibility (CI) when infected males mate with non-infected females. Here, the paternal sperm is no longer recognized by the egg, leaving the egg unfertilized.\textsuperscript{22,23} While IIT can be a promising application, accidental release of infected males can lead to unintended replacement of a wild population with an infected/resistant one, as CI does not occur when infected females mate with either infected or non-infected males.\textsuperscript{24} Further, both naturally occurring and trans-infected \textit{Wolbachia} strains do not provide 100% CI in \textit{D. suzukii}, which is required for an effective IIT.\textsuperscript{25} The combination of SIT and IIT was recently assessed to improve the sterility of \textit{Wolbachia} infected \textit{D. suzukii}. However, the mating competitiveness of released \textit{D. suzukii} males was not assessed.\textsuperscript{26} Thus, while progress has been made, it remains to be determined if SIT and/or IIT can provide an alternative, scalable, economical means for control of \textit{D. suzukii}.

Over the past several years, there has been significant progress in developing molecular genetic tools that can be used for both gaining a better understanding of \textit{D. suzukii} biology and to innovate alternative environmentally friendly control methods. For instance, transgenesis has been achieved by several groups,\textsuperscript{27–31} a high-quality reference genome has been assembled,\textsuperscript{32} and the versatile RNA-guided CRISPR-Cas9 nuclease has recently been used for gene editing.\textsuperscript{33} For gene editing in \textit{D. suzukii}, site-specific mutations were initially made through microinjection of embryos with Cas9/gRNA plasmid DNAs.\textsuperscript{35} The efficiency of mutagenesis was later improved by microinjection with recombinant Cas9 protein and synthetic gRNA rather than plasmid DNAs,\textsuperscript{34} or by injecting gRNAs into embryos derived from a Cas9 expressing strain.\textsuperscript{30} The genome editing efficiency could be further improved by generating transgenic strains that encode Cas9/gRNA, as done in \textit{Drosophila melanogaster}.

Transgenic strains that express Cas9 are also required for the development of strains for efficient genetic suppression of \textit{D. suzukii}. For example, both the precision-guided SIT (pgSIT) method\textsuperscript{40,41} and homing gene drive systems\textsuperscript{42,43} utilize Cas9 for the gRNA-directed cleavage of genes essential for female development.\textsuperscript{44,45} The pgSIT approach relies on the dominant action of Cas9 and multiple gRNAs brought together by a genetic cross that enables simultaneous sex sorting and sterilization of the F\textsubscript{1} progeny. To develop pgSIT for \textit{D. suzukii} would require generating strains that express Cas9 and strains that express multiple guide RNAs (gRNAs) that target genes essential for female development and male fertility. The gene drive suppression approach relies on homing and disrupting a female-specific gene with super-Mendelian inheritance in males, resulting in gradual population collapse. To develop a homing gene drive system in \textit{D. suzukii} would require strains that express Cas9 in the germline and strains that express gRNAs that target a gene essential for female development flanked with sequences homologous to the cut site.

Given the many advantages of generating transgenic strains encoding Cas9/gRNAs, here we report the development and evaluation of such strains in \textit{D. suzukii}. In total, we generated eight homozygous Cas9 expressing strains that were driven by four separate promoters (\textit{nos}, \textit{vasa}, \textit{BicC}, and \textit{ubiq}) using three different terminators to provide robust expression in both the soma and/or germline. To evaluate the efficacy of these strains, we also generated strains encoding single guide RNAs that target two conserved X-linked recessive genes known to produce visible phenotypes when disrupted including \textit{yellow} bodies and \textit{white} eyes. By crossing the Cas9 and gRNA strains together, we found that several strains display remarkably high rates of editing capacity. Going forward, these tools will be invaluable for characterization of gene function and may also prove useful for engineering novel control strategies for this invasive crop pest.

**Methods**

**Molecular construct design and assembly**

We used the previously described \textit{piggyBac} plasmids harboring coding sequences of the Cas9-T2A-eGFP under different promoters and the \textit{Opie2-dsRed} marker\textsuperscript{40,46,47} to generate \textit{D. suzukii} Cas9.R strains: \textit{vasCas9.R} (874Z plasmid, Addgene #112687), \textit{BicCCas9.R} (874R plasmid, Addgene #168295), \textit{UbigCas9.R} (874W plasmid, Addgene #112686), and \textit{nosCas9.R} (874Z1 plasmid, Addgene #112685). The Gibson enzymatic assembly method\textsuperscript{48} or standard recombinant DNA methods were used to build the \textit{piggyBac} transformation plasmids that carried \textit{vasCas9.G} (Addgene #169012), \textit{nosCas9.G} (Addgene #169011), gRNA\textsuperscript{3} (982G.2 plasmid; Addgene #168294), and gRNA\textsuperscript{w} (Addgene #169010) constructs.

To assemble \textit{vasCas9.G} and \textit{nosCas9.G} driven by the \textit{D. melanogaster} \textit{vasa} and \textit{nos} promoters, the vasa\textsuperscript{5金融科技}-Cas9-vasa\textsuperscript{3} and nos\textsuperscript{5金融科技}-Cas9-nos\textsuperscript{3} fragments were excised from \textit{vasa-Cas9} plasmid DNA (\textit{Drosophila Genomics Resource Center #1340})\textsuperscript{49} and \textit{pBFv-nosp-Cas9} plasmid\textsuperscript{16} (NIG-Fly, Japan), respectively, and then ligated into a \textit{piggyBac} vector that was cut with \textit{HpaI} and \textit{PspOMI}. The \textit{piggyBac} vector contains a
ZsGreen fluorescent protein marker expressed by the *D. melanogaster* polyubiquitin gene promoter (ubiq-ZsGreen). This was made by excision of eGFP from MS141950 and replacement with ZsGreen from pB[Lchsp83-ZsGreen].51 We utilized previously described yellow47 and white35 gRNA sequences to build the gRNAy and gRNAw piggyBac plasmids (Fig. 1C). To assemble gRNAy, the yellow gRNA targeting an identical sequence in *D. melanogaster*47 and *D. suzukii*, yellow exon#2 (DS10_00005318)52 was encoded into overlapping primers.

**FIG. 1.** Schematic maps of genetic constructs and images of transgenic *Drosophila suzukii*. (A) Schematic maps of two sets of Cas9 constructs. The first four Cas9 constructs harbor a human-codon-optimized SpCas9 (Cas9)68 coding sequence (CDS) surrounded by two nuclear localization sequences (NLS-Cas9-NLS), linked to the eGFP CDS at its C end via a self-cleaving T2A sequence, and terminated by the p10 3’-UTR from the Autographa californica nucleopolyhedrovirus (AcNPV).62 The SpCas9 is expressed in early germ cells under *vasa* (vas) and *nanos* (nos) promoters, in late germ cells with *Bicaudal C* (BicC), and in both germ and somatic cells with *Ubiquitin 63E* (ubiq) promoter. These constructs also contain a red transgenesis marker (*Opie2-dsRed*). The second group of Cas9 constructs carry a human-codon-optimized SpCas9 expressed under the *vas* or *nos* promoter and terminated with a single NLS (Cas9-NLS) and the corresponding *vas* and *nos* 3’UTR, as well as a green transgenesis marker (*Ubiq-ZsGreen*). (B) Images of homozygous transgenic *D. suzukii* Cas9R females flies generated with *UbiqCas9.R* and *VasCas9.G*. (C) Schematic maps of two gRNA constructs, and the targeted sequences in both yellow and white loci. The gRNAy construct harbors the yellow gRNA (gRNAy) with a scaffold expressed with the Dmel pU6-3 promoter and terminated by a PolyT terminator, and two green transgenesis markers, *Opie2-GFP* and 3Xp3-GFP. The gRNAw construct harbors the white gRNA (gRNAw) with a scaffold expressed with the same Dmel pU6-3 promoter and terminated the pU6-3 terminator sequence, and a red transgenesis marker, *Ubiq-dsRed*. (D) Images of homozygous *D. suzukii* gRNAy and gRNAw females. Both sets of RGB images for each female fly were taken under the white light and corresponding fluorescent light illumination.
that amplified the *D. melanogaster* U6-3 promoter on one side and the single chimeric gRNA scaffold with the poly(T) terminator on the other side as previously described, and cloned into a piggyBac plasmid harboring the Opie2-eGFP-SV40 marker. Then, the additional 3xP3-eGFP-SV40 marker was added upstream from U6-3-gRNA. To build gRNA, the U6-3-ex2 plasmid that contains white gRNA targeting *D. suzukii*, white exon2 (DS10_00006062) and *D. melanogaster* U6:3 promoter and terminator was digested with BglII, and the excised U6:3-p-gRNA-U6:3t fragment was ligated with the piggyBac vector MS1425-p10 that contains a ubiq-dsRed marker. MS1425-p10 was derived from MS1425 by excision of the SV40 pA sequence and replacement with p10 pA.

Rearing and transgenesis

*D. suzukii* were maintained on a cornmeal-yeast-agar diet at 21°C with a 12 h/12 h light/dark cycle at UCSD and/or NCSU. All test crosses were performed under these conditions except for those with the *vasCas9.G* and *nosCas9.G* strains and the U6-3-ex2 white gRNA strains that were performed at 25°C. In both facilities, *D. suzukii* flies were kept in an institutional biosafety committee-approved ACL1 (NCSU) and ACL2 (UCSD) insectaries and handled by limited expert investigators to prevent any unintended release of flies. The *D. suzukii* wild-type (wt) strains used in the study originated from Corvalis, Oregon, and North Carolina. Embryo injections were carried out at Rainbow Transgenic Flies, Inc. (www.rainbowgene.com) or at the NC State University insect transgenesis facility. Plasmids diluted in water to 200–300 µg/µL were injected into freshly collected embryos of the *D. suzukii* harboring Hsp70BBpiggyBacTransposase or the North Carolina wild-type strain with piggyBac helper plasmid. G0 adults emerging from injected embryos were collected to *D. suzukii* wt flies, and their G1 progeny were screened for the expression of specific fluorescent markers with a Leica M165FC fluorescent stereomicroscope.

Genetics

To establish stable transgenic strains that can be maintained over multiple generations, *D. suzukii* expressing an independent insertion of a particular construct were repeatedly intercrossed over multiple generations to generate a homozygous stock. The homozygosity of generated stocks was confirmed by test crosses using homozygous transgenic strains crossed to *wt* flies and scoring specific transgenic markers in their progeny. To our surprise, we observed that freshly eclosed *D. suzukii* females would frequently mate with older males; and, unlike *D. melanogaster*, the freshly eclosed phenotype by itself is not a sufficient indicator of the female virginity. Therefore, to ensure that only virgin females were used for genetic crosses, vials with eclosing flies were cleared up multiple times daily to remove old males.

We set the majority of genetic crosses in one direction. *Cas9* virgin females were mated to gRNA males to stimulate the gene disruption in somatic tissues of the F1 progeny by maternal Cas9 deposition. Seven to ten homozygous Cas9 virgin females were crossed to 7–10 homozygous gRNA males in a fly vial, which were flipped into a fresh vial every 4–5 days, and the somatic disruption of *yellow* or *white* was scored in the F1 progeny emerging from vials seeded by the same parent flies (i.e., an independent biological replicate; Fig. 2A–F). Each genetic cross was set up at least three times using different parent flies (rows in Supplementary Tables S1–S3). In addition to the complete absence of eye pigmentation (white eyes, w– phenotype), mosaic eye coloration was frequently observed in F1 trans-heterozygous Cas9/R+/gRNA/+ flies of both sexes. This was recorded as a mW phenotype. We also performed genetic crosses with the paternal Cas9 for *Bic.Cas9.R* and *ubiqCas9.R* to assess the role of maternal Cas9 carryover on somatic gene disruption and to ascertain the insertion of *ubiqCas9.R* on the X chromosome, respectively (Fig. 2G and H). Both *yellow* and *white* genes are located on the X chromosome, and *D. suzukii* males have only one X chromosome (i.e., hemizygous), which they inherit from their mothers. Therefore, to assess the mutation frequency in germ cells, 7–10 F1 trans-heterozygous virgin females were crossed to 10 *wt* males, and w– phenotype was scored in the F2 male progeny. Flies were scored and imaged on a Leica M165FC fluorescent stereomicroscope equipped with a Leica DMC2900, View4K, or Leica DFC500 camera.

Genotyping the yellow and white target loci

To explore the molecular changes that caused resistant (R2, wR2) and functional in-frame resistant (R1, wR2) alleles. We polymerase chain reaction (PCR) amplified a genomic region containing the target site for gRNA or gRNA (Fig. 1C) using single-fly genomic DNA preps from individual F2 males, which were scored for *yellow* or *white* phenotype. The 397 bp PCR fragment of *yellow* target (exon #2) was amplified with 5′-GAAATTCCAGCCACTCTGACTTATATCAATATGG-3′ (982G.s10F) and 5′-CAAGGATGAACATTATACCATGGCC-3′ (982G.s11R) and 5′-GGAGGATCAAGCCGATGTGC-3′ (982A.s11R) and 5′-TGAGAAG AACGTCGACGGCTTCGCTGG-3′ (982A.s10F) primers were used to amplify the 322 bp PCR fragment of *white*...
target (exon #2). To explore the pre-existing diversity of white resistant alleles, we made multi-fly genomic DNA preps from 25–30 flies for each wt stocks kept in both labs at UCSD and NCSU as well as transgenic strains used in the study. PCR amplicons were purified using QIAquick PCR purification kit (Qiagen), and they were sequenced in both directions with the Sanger method at Genewiz, Inc. To characterize molecular changes at the targeted sites, sequence AB1 files were aligned against the corresponding reference sequences, and chromatogram peaks were examined in SnapGene 4.

**FIG. 2.** Disruption of yellow and white loci in F1 trans-heterozygous flies. (A) The schematic of a genetic cross between Cas9 and gRNA^y^ flies. To generate F1 trans-heterozygous flies, homozygous Cas9 females (red marker) crossed to homozygous gRNA^y^ males (green marker). The targeted wild-type (wt) yellow gene (y+ alleles, yellow stripes on X chromosome) is on the X chromosome. Yellow-colored thoraxes in F1 flies indicate disruption of the yellow gene (y–). (B) Dot plot depicting the results of disruption of the yellow gene in somatic cells from F1 trans-heterozygous (Cas9+/; gRNA^y^/+)) progeny using the Cas9.R strains inherited maternally. (C) Images of F1 y– trans-heterozygous and wild-type (wt) y+ females. (D) Schematic of a genetic cross with maternal homozygous Cas9 (red marker) and paternal homozygous gRNA^w^ (purple marker). The white gene is on the X chromosome (w+ alleles, white stripes on X chromosome). Red and white eye coloration in the F1 flies indicate somatic disruption of the white gene (mW). (E) Dot plot depicting the results of disruption of the white gene in somatic cells from F1 trans-heterozygous (Cas9+/; gRNA^w^/+)) progeny using the Cas9.R strains inherited maternally. (F) Images of F1 trans-heterozygous mosaic white disruption (mW) and complete disruption (w–) phenotypes. (G) The schematic of a genetic cross between paternal Cas9 and maternal gRNA^w^ flies. To generate F1 trans-heterozygous flies, homozygous Cas9 males (red marker) crossed to homozygous gRNA^w^ females (purple marker). The white gene is on the X chromosome (w+ alleles, white stripes). (H) Dot plot depicting the results of disruption of the white gene in somatic cells from F1 trans-heterozygous (Cas9+/; gRNA^w^/+)) progeny using the Cas9.R strains inherited paternally. Plots show the mean±standard deviation (SD) over at least three biological replicates. Statistical significance was estimated using a two-sided Student’s t-test with unequal variance. (p≥0.05 (n.s.); *p<0.05; **p<0.01; ***p<0.001). n.s., not statistically significant.
Statistical analysis
Statistical analysis was performed in JMP v8.0.2 by SAS Institute, Inc. At least three biological replicates were used to generate statistical means for comparisons. p-Values were calculated for a two-sample Student’s t-test with equal or unequal variance. O’Brien’s test was used to assess that the variance was equal. All plots were constructed using Prism v9 for macOS by GraphPad Software, LLC.

Results
Development of Cas9 and gRNA encoding strains
The aim of the study was to develop a versatile toolbox for gene editing and genome engineering in D. suzukii by generating transgenic strains encoding CRISPR components (i.e., Cas9 and gRNAs). To express and import Cas9 into nuclei robustly, we used Streptococcus pyogenes Cas9 (Cas9) with a nuclear localization sequence (NLS) on either the C-terminal end only (Cas9-NLS) or on both terminals (NLS-Cas9-NLS; Fig. 1A). To visualize and confirm expression for the NLS-Cas9-NLS, we incorporated a C-end self-cleaving T2A-eGFP, which would generate two separate proteins (i.e., NLS-Cas9-NLS and eGFP) via ribosomal skipping. To drive expression of Cas9, we used D. melanogaster promoters expressed in either early germ cells, vasa (vas) or nanos (nos), or in late germ cells, Bicaudal C (BicC), or in both germ and somatic cells, polyubiquitin (ubiq). Using each promoter, we built four piggyBac constructs that express NLS-Cas9-NLS terminated with NLS-Cas9-NLS and eGFP via ribosomal skipping. To encode expression of Cas9, we used D. melanogaster promoters expressed in either early germ cells, vasa (vas) or nanos (nos), or in late germ cells, Bicaudal C (BicC), or in both germ and somatic cells, polyubiquitin (ubiq). Using each promoter, we built four piggyBac constructs that express NLS-Cas9-NLS terminated with a p10 3’-UTR derived from the Autographa californica nucleopolyhedrovirus (AcNPV), for strong translation of Cas9, and a strong red (Opie2-dsRed) transgenesis marker (referred as Cas9.R). We also engineered two alternative piggyBac constructs that contain either the vasa or nos promoters driving expression of the Cas9-NLS terminated with vasa or nos 3’UTRs from D. melanogaster, with a green (ubiQ-ZsGreen) transgenesis marker (referred as Cas9.G; Fig. 1A). In total, six Cas9 constructs were engineered that were used to generate eight homozygous transgenic strains (at least one homozygous transgenic strain per construct), two of which were X-linked (Fig. 1B and Table 1).

To encode the gRNAs in D. suzukii genetically, we engineered two separate constructs encoding gRNAs targeting homologous viable X-linked genes with recessive phenotypes: yellow (y) and white (w; Fig. 1C). Each construct encoded one gRNA driven by the D. melanogaster small nuclear RNA U6-3 promoter providing constitutive expression. The gRNA* construct harbors two green transgenesis markers (Opie2-eGFP and 3xP3-eGFP), and the gRNAw construct contains one red marker (ubiQ-dsRed). Both gRNA* and gRNAw constructs were assembled in piggyBac (Fig. 1C), and homozygous gRNA* and gRNAw strains were generated (Fig. 1D and Table 1).

CRISPR-mediated mosaicism in F1 progeny
To assess the functionally of the strains produced, we genetically crossed homozygous Cas9 females to homozygous gRNA males and examined expected eye and/or body coloration phenotypes in the resulting F1 progeny (Fig. 2A–F and Supplementary Tables S1 and S2). This cross was performed to explore the rates of mutagenesis in the F1 somatic tissues, which is augmented by maternal deposition of Cas9. High percentages (61.1–100%) of F1 trans-heterozygotes generated by Cas9.R females crossed to gRNA* males had visible yellow instead of brown body coloration, indicating robust somatic yellow gene disruption (y– phenotype; Fig. 2B and C and Supplementary Table S1). Notably, when the Cas9.9 females were crossed to gRNA*
males (Fig. 2D), we observed much higher variabilty (0–100%) for somatic white disruption in resulting F1 progeny (Supplementary Table S2). While both sexes were affected, the majority of F1 trans-heterozygotes manifested highly variable mosaic eye coloration (mW), and a smaller fraction of F1 progeny had complete white eye color ($w^–$; Fig. 2E and F). These results suggest that some somatic cells in mosaic flies harbored at least one wild-type white ($w^+$) allele or possibly a functional resistant ($w^{R1}$) allele. The extensive somatic disruption of yellow and white was induced by all Cas9 strains. However, Cas9.G crossed to gRNAy and gRNAw strains did not appear to induce somatic mosaicism in the resulting F1 progeny (Supplementary Tables S1 and S2).

**Maternal deposition and F1 mosaicism**

To determine whether maternal Cas9 deposition is essential to achieve high rates of F1 mosaicism, we compared the rates of white mosaicism in F1 trans-heterozygotes inheriting Cas9 either maternally or paternally (Fig. 2G and H). We tested two Cas9 strains, including BicC-Cas9.R and ubiqCas9.R, which gave high rates of white mosaicism when Cas9 was provided maternally. The F1 BicC-Cas9.R+/+; gRNAy/+ with maternal Cas9 showed significantly higher rates of white mosaicism than those with paternal Cas9 (68.5 ± 12.6% vs. 25.1 ± 4.3% of $w^–$ and mW, respectively; $p<0.0002$ t-test with equal variance; Fig. 2H and Supplementary Table S3). Comparatively, the ubiqCas9.R+/+; gRNAy/+ with maternal Cas9 showed significantly lower rates of white mosaicism than those with paternal Cas9 (78.5 ± 21.4% vs. 99.7 ± 0.9% of $w^–$ and mW, respectively; $p<0.035$ t-test with unequal variance; Fig. 2H and Supplementary Table S3). Taken together, these data indicate that regardless of whether Cas9 was inherited maternally or paternally, high rates of mosaicism were observed in F1 trans-heterozygous progeny.

**Cas9/gRNA-mediated heritable mutations**

To estimate the frequency of heritable germline mutations, we mated F1 trans-heterozygous females harboring either maternal or paternal Cas9 to wt males, and assessed mutagenesis rates by scoring phenotypes in hemizygous F2 males progeny (Fig. 3A). Both yellow and white are X-linked. Therefore, F2 males generated from F1 trans-heterozygous females inherit their mothers’ X chromosome. If the F1 females encode germline mutations in either yellow or white, then F2 males will inherit those X-linked mutations and display visible phenotypes (i.e., non-mosaic complete white eyes or yellow body). Of note, we specifically scored complete yellow and white phenotypes in F2 males progeny here to differentiate heritable germline mutations from somatic mosaic mutations resulting from either maternal deposition or zygotic expression of transgenes. In combination with gRNAy, the Cas9.R strains induced significantly higher rates of $w^–$ alleles than those induced by the Cas9.G strains (77.6 ± 12.5% and 2.0 ± 2.1%, respectively; $p<0.0001$ t-test with unequal variance; Fig. 3B and C and Supplementary Table S1). The F1 ubiqCas9.R+/+; gRNAy/+ females harboring maternal Cas9 induced significantly higher levels of $w^–$ alleles than those carrying paternal Cas9 (95.4 ± 4.7% and 78.5 ± 1.4%, respectively; $p<0.0003$ t-test with equal variance), while the opposite was observed for F1 vasCas9.G#6/+; gRNAy/+ females (0.6 ± 1.6% and 3.2 ± 1.8%, respectively; $p<0.0163$ t-test with equal variance; Fig. 3B). Interestingly, we observed higher variability of heritable $w^–$ mutations among replicates for the majority of Cas9 strains (Fig. 3D). For example, independent groups of F1 BicC-Cas9.R+/+; gRNAy/+ or vasCas9.G#4/+; gRNAy/+ females generated F2 males with loss of function (LOF) $w^–$ alleles in 92.7% and 11.1% of progenies, respectively, or those with $w^–$ in 83.5% and 0%, respectively (Fig. 3D and Supplementary Table S2). Note that we did not find the mosaic eye coloration (mW phenotype; Fig. 2F) in F2 males. They had either $w^–$ or $w^+$ eye phenotype, indicating the absence of somatic mosaicism (Fig. 3E).

**Functional resistant alleles at the white locus**

We observed higher variations in both F1 and F2 mutagenesis of white as compared to yellow (Figs. 2B and E and 3B and D). We hypothesized that the F2 $w^+$ males may harbor either uncut $w^+$ alleles or functional repaired alleles that were resistant to Cas9/gRNAw cleavage (termed $w^{R1}$ alleles; Fig. 4A). To explore this hypothesis, we genotyped F2 $w^+$ males. Interestingly, we did not identify $w^+$ alleles among 37 F2 males generated from multiple independent crosses. Instead, each genotyped F2 males harbored either a one-base substitution (1bpSUB) or a 12-base deletion (12bpΔ) directly at the white target sequence (Fig. 4B). Both in-frame $w^{R1}$ alleles preserved $w^+$ function of the white gene. The A-to-G substitution at base #5 in the 1bpSUB allele did not change the amino acid sequence (i.e., silent mutation), while the 12bpΔ allele contains a deletion of 4 aa (Fig. 4B). Notably, the 12bpΔ allele was sampled more frequently than the 1bpSUB allele, though both $w^{R1}$ alleles were found in crosses with each Cas9.R strain. We also genotyped a few F2 $w^–$ males and identified diverse LOF resistant alleles (termed $w^{R2}$) induced by Cas9/gRNAw (Fig. 4C). Each genotyped F2 $w^–$ males harbored one LOF $w^{R1}$
FIG. 3. The efficiency of heritable yellow and white mutations. (A) To assess for heritable yellow or white mutations in germ cells, F1 trans-heterozygous Cas9/+; gRNA/+ females were crossed to wt males, and yellow or white phenotype was scored in the generated F2 males that inherited their mother’s X chromosome. Two types of trans-heterozygous females were used: the F1 females harboring maternal copy of Cas9 (red points), and the F1 females harboring paternal copy of Cas9 [blue points in (B) and (D)]. Maternal inheritance of Cas9 results in Cas9 protein carryover, aka. maternal Cas9 deposition, into embryos. (B) Dot plot depicting the y− frequency in the F2 males derived from crosses with the eight homozygous Cas9 strains generated in this study. (C) Images of the generated F2 y− and wt (y+) males. (D) Dot plot depicting the w− frequency in the F2 males derived from crosses with the eight homozygous Cas9 strains generated in this study. (E) Images of the F2 w− and wt (w+) males. Plots show the mean ± SD over at least three biological replicates. Statistical significance was estimated using a two-sided Student’s t-test with equal variance. (p ≥ 0.05 [n.s.]; *p < 0.05; **p < 0.01; ***p < 0.001).
allele. Notably, we identified two in-frame LOF deletion alleles (3bpΔ and 12bpΔ*) that could not rescue the wt white function. To explore the possibility that the 1bpSUB and 12bpΔ wR1 alleles pre-existed, we sequenced genomic DNA for each wt stock and transgenic strain used in the study and looked for any evidence of polymorphisms at the white target sequence. We did indeed identify the 1bpSUB wR1 allele as a polymorphism in the wt stock maintained at NCSU (Fig. 4B). In addition, one novel allele was found in the nosCas9.R genetic background (Fig. 4B). However, this C-to-T silent substitution at base #17 may not be resistant to Cas9/gRNAw because it is more distant from the cut site, and it was not sampled in our sequencing analysis.

FIG. 4. Functional and loss of function (LOF) resistant alleles at the white target loci. (A) Schematic depicting potential males progeny from the genetic cross of F1 trans-heterozygous Cas9/+; gRNAw/+ females and wt (w+) males. The F1 females has mosaic eye coloration (mW), indicating disruption of the white gene in its somatic tissues. Some w+ alleles can be mutated in its germ cells resulting in F2 males harboring the LOF w– allele (a crossed-out white bar on the X chromosome with a black outline). Some w+ alleles can be mutated in the somatic tissues of the F2 males that inherited the gRNAw transgene and have the carryover Cas9 protein (maternally deposited) or Cas9 transgene (a crossed-out white bar on the X chromosome with a red outline) also resulting in a LOF w– phenotype in F2 males. The F2 males with normal eye coloration harbors the wt w+ allele that escaped cleavage or the functional resistant alleles (R1, w+) that were mutated and became resistant to cutting by Cas9/gRNAw but preserved the function of the white gene (a white bar with a red star on the X chromosome with a black outline). Note that LOF w– alleles contain insertions or deletions at the ligated cut site and are also likely resistant to Cas9/gRNAw. Therefore, they are referred to as R2 alleles. To explore the course of high variabilities in the white knockout, we sequenced the white target in F2 w+ males. (B) Each genotyped males from 37 F2 w+ males sampled from multiple independent crosses with each Cas9.R strains had either one-base substitution (1spSUB at base #5) or 12-base deletion (12bpΔ) directly at the white target sequence. The two wR1 alleles persisted for two generations of cutting by Cas9/gRNAw and maintained wt function of the white gene. No wt w+ alleles were identified in any sampled F2 w+ males. After screening D. suzukii wt stocks and all transgenic strains established in the study, we found one wR1 allele (1spSUB at base #5) in the wt stocks maintained in NCSU and one novel resistance allele (1spSUB at base #17) in nosCas9.R. Notably, Sanger sequencing of PCR amplicons pooled from many flies for each strain and wt stock used to generate the F2 wR1 males at UCSD has not identified any chromatogram peak ambiguity at base #5. (C) LOS R2 allele sampled in 15 F2 w– males. Note that the two sampled R2 alleles are in-frame (3bpΔ and 12bpΔ*), and yet they cannot restore the wt function. The sequence alignment of induced LOF alleles against the wt reference white sequence. The 20 bases of gRNAw (in blue) and its protospacer adjacent motif (PAM; purple) are depicted over the white target sequence. Arrows point the direction of the gRNAw target. Mutated bases (red letters) and/or their absence (red dashes) are indicated relative to the wt sequence.
of F2 w+ males. Taken together, these data suggest that functional resistant alleles (either induced or previously present) are likely contributing to the higher variations of observed white mutagenesis.

**Mutant y-strains were established**

Both yellow and white genes have many features that make them attractive targets and/or tools for genetic research. Therefore, we attempted to establish w– and y– strains to facilitate future genetic research in *D. suzukii*. To this end, we generated independent F2 progeny (Fig. 3), and intercrossed individual F2 females and males harboring y– or w– mutant alleles in the absence of both Cas9 and gRNA transgenes. We were able to establish and maintain eight homozygous y– strains. However, we could not establish w– stocks as LOF w– males were sterile.34,35 We genotyped each y– strain and identified five different insertion/deletion mutations at the yellow target sequence (Fig. 5). One six-base-deletion (6bpΔ) was induced independently in four yellow knockout strains (Fig. 5).

**FIG. 5.** *D. suzukii* yellow knockout (LOF) strains. We established eight independent homozygous viable y– knockout strains. (A) The sequence alignment of induced LOF alleles against the wt reference yellow sequence.52 The 20 bases of gRNA are in blue, while the PAM sequence is in purple. The arrow points the direction of gRNA target. We identified two silent SNPs (gray-colored base) in the *D. suzukii* NC and CA wt stains relative to the wt reference strain:52 one at the 4th gRNAy base and at the 2nd base after the PAM sequence. Mutated bases (red letters) and/or their absence (red dashes) are indicated relative to the wt sequence. The same six-base deletion (6bpΔ) was independently induced in four strains #2, #3, #4, and #6. The 6bpΔ deletes two amino acids (glycine and threonine) that results in the LOF y– phenotype (Figs. 2C and 3B). (B) The 1bpINS (y–) strain males have the classic yellow phenotype in comparison with wt (y+) males.
Discussion

Here, we describe the generation and assessment of an array of *D. suzukii* transgenic strains expressing Cas9 driven under four promoters from *D. melanogaster*. To evaluate these Cas9 strains, two gRNA strains targeting X-linked recessive genes were also established. Using Cas9 and gRNA strains, we demonstrate that the Cas9.R strains result in high rates of mutagenesis of targeted genes, while the Cas9.G strains had more limited efficacy.

Our results indicate that the Cas9.R constructs containing NLS-Cas9-NLS terminated with the p10 3’UTR resulted in significantly stronger disruption of *yellow* in both somatic and germ cells than that by the Cas9.G harboring Cas9-NLS and terminated with the vas or nos 3’UTR. Due to random genomic integration of constructs and possible differences in protein stability afforded by different marker fusions (i.e., T2A-eGFP), direct comparison of Cas9 strains is confounded. Nevertheless, the observed differences were consistent among each Cas9.R and Cas9.G strain. The p10 3’UTR from the *Autographa californica* nucleopolyhedrovirus (AcNPV) is known to increase efficiency of both polyadenylation and mRNA translation. In addition, a 7a-long SV40 NLS is shared between both Cas9.R and Cas9.G types, while Cas9.R constructs also contain a 16 aa bipartite nucleoplasm NLS (Fig. 1A). The inclusion of an additional NLS into Cas9 may provide increased nuclear localization. The vas and nos 3’UTRs used in the Cas9.G gene constructs would be expected to lead to localization of the Cas9 mRNA to the posterior end of the oocyte. In contrast, the p10 3’UTR is not predicted to result in such localization during oogenesis. Thus, our expectation was that the Cas9.G strains would be mostly active in germ cells that develop at the posterior end of the embryo, while the Cas9.R strains would have high activity in both somatic and germ cells. In support of this hypothesis, several generated Cas9.G strains harboring independent insertions of vasCas9.G or nosCas9.G did not induce visible *F1* somatic mutagenesis of *yellow* or *white* in the presence of the corresponding gRNA. Instead, they caused the *yellow* or *white* mutagenesis in the *F2* progeny, indicating a possible germine-restricted Cas9 expression pattern.

We observed a high inter-replicate variability in frequencies of the *white* disruption in both somatic and germ cells. To explore cause of this variability, we genotyped many *F2* *w*+ males and found that each genotyped *w*+ males harbored one of the two functional *w*RI alleles. Our results indicate that the Cas9/gRNAw cleavage of *white* was efficient. We did not sample *wt* *w*+ alleles in *F2* males. Instead, they each harbored *w*RI and *w*R2 resistant alleles. Both identified *w*RI alleles, 1bpSUB and 12bpΔ, may have been induced by Cas9/gRNAw or may have possibly existed in the strains. To explore the later hypothesis, we screened the *white* target using Sanger sequencing of PCR amplicons from multi-fly DNA preps of both *wt* stocks maintained at UCSD and NCSU, each transgenic strain generated in the study, and the pBac helper strain used for transgenesis. The 1bpSUB(A > G at base #5) wRI allele was identified in only the *wt* stocks at NCSU, and not in the *wt* stock and strains established at UCSD that were used to generate the genotyped *F2* *w*+ males. The fact that the 1bpSUB wRI allele was sampled in each cross with four Cas9.R strains and occurred naturally in the *wt* stock at NCSU suggests that this wRI might not be generated, but instead could be selected out from pre-existent alleles, by Cas9/gRNAw. The 12bpΔ wRI allele, which removes 4 aa and potentially affects the fitness of its carriers, was not identified in any genetic background used in the study, and yet it was sampled more frequently than the 1bpSUB allele (Fig. 4B). Moreover, we also found a novel 1bpSUB (C>T at base #17) allele pre-existent in the nosCas9.R strain. The novel allele is likely less resistant to the Cas9/gRNAw (see Results; Fig. 4B) than the 1bpSUB(A > G at base #5) wRI allele because it is located farther away from the cut site (base #6). Therefore, our data suggest that the 1bpSUB wRI allele pre-existent in *D. suzukii* genetic background and might not be completely resistant to the Cas9/gRNAw, while the 12bpΔ allele was induced by Cas9/gRNAw in our experiments.

More than a century ago, the establishment of *D. melanogaster* knockout strains of *white* and *yellow* provided valuable genetic background for seminal transgenic research. Previous studies demonstrated that unlike *D. melanogaster*, *D. suzukii* males harboring LOF *w* alleles were sterile, thus preventing the maintenance of the *D. suzukii* *w*– strain. Here, we describe the development of eight viable *D. suzukii* *y*– strains. Five unique *y*– alleles were characterized from eight *y*– strains (Fig. 5). These strains may be useful reagents for applications in which fluorescent markers of transgenesis cannot be used, for example when genes of interest (GOI) themselves are linked to fluorescent tags. Therefore, the generated *y*– strains are valuable resources for genetic studies of *D. suzukii*.

*D. suzukii* is an invasive species and a close relative of the *D. melanogaster*, the classic genetic model organism for which a vast amount of detailed biological knowledge and a diverse array of genetic tools are available. Given the close phylogenetic proximity, the majority of knowledge and genetic tools are easily portable across both *Drosophila* species and can be applied for the
development of genetic methods for effective population control of *D. suzukii*. The CRISPR-Cas9 technology has been used extensively for precise genome editing in diverse animal and plant species. Going forward, the *D. suzukii* tools strains described here should facilitate precise genetic engineering in this invasive pest species. For example, the establishment of multiple Cas9 and gRNA strains supporting robust expression is the first step toward the development of pgSTI\textsuperscript{40,41} and other Cas9-mediated genetic systems, such as homing split gene drives,\textsuperscript{65-67} to provide scalable population control of *D. suzukii*.

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**Author Disclosure Statement**

O.S.A is a founder of Agragene., Inc., and has equity interest. The terms of this arrangement have been reviewed and approved by the University of California, San Diego in accordance with its conflict-of-interest policies. All other authors declare no competing interests.

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**Supplementary Material**

Supplementary Table S1
Supplementary Table S2
Supplementary Table S3

**References**


