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RESEARCH ARTICLE

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

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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 pest[1–3] 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

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males and females,[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 *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.[22,23] While IIT can be a promising application, accidental release of infected females 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.[24] Further, both naturally occurring and trans-infected *Wolbachia* strains do not provide 100% CI in *D. suzukii*, which is required for an effective IIT.[25] The combination of SIT and IIT was recently assessed to improve the sterility of *Wolbachia* infected *D. suzukii*. However, the mating competitiveness of released *D. suzukii* males was not assessed.[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 *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 *D. suzukii* biology and to innovate alternative environmentally friendly control methods. For instance, transgenesis has been achieved by several groups,[27–31] a high-quality reference genome has been assembled,[32] a male-only strain has been developed and evaluated,[33] and the versatile RNA-guided CRISPR-Cas9 nuclease has recently been used for gene editing.[30,34,35] For gene editing in *D. suzukii*, site-specific mutations were initially made through microinjection of embryos with Cas9/gRNA plasmid DNAs.[35] The efficiency of mutagenesis was later improved by microinjection with recombinant Cas9 protein and synthetic gRNA rather than plasmid DNAs,[34] or by injecting gRNAs into embryos derived from a Cas9 expressing strain.[30] The genome editing efficiency could be further improved by generating transgenic strains that encode Cas9/gRNA, as done in *Drosophila melanogaster*.[36–39]

Transgenic strains that express Cas9 are also required for the development of strains for efficient genetic suppression of *D. suzukii*. For example, both the precision-guided SIT (pgSIT) method[40,41] and homing gene drive systems[42,43] utilize Cas9 for the gRNA-directed cleavage of genes essential for female development.[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_1 progeny. To develop pgSIT for *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 *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 *D. suzukii*. In total, we generated eight homozygous Cas9 expressing strains that were driven by four separate promoters (*nos*, *vasa*, *BicC*, and *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 *yellow* bodies and *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 *piggyBac* plasmids harboring coding sequences of the *Cas9-T2A-eGFP* under different promoters and the *Opie2-dsRed* marker[40,46,47] to generate *D. suzukii Cas9.R* strains: *vasCas9.R* (874Z plasmid, Addgene #112687), *BicC.Cas9.R* (874R plasmid, Addgene #168295), *UbiqCas9.R* (874W plasmid, Addgene #112686), and *nosCas9.R* (874Z1 plasmid, Addgene #112685). The Gibson enzymatic assembly method[48] or standard recombinant DNA methods were used to build the *piggyBac* transformation plasmids that carried *vasCas9.G* (Addgene #169012), *nosCas9.G* (Addgene #169011), $gRNA^{y}$ (982G.2 plasmid; Addgene #168294), and $gRNA^{w}$ (Addgene #169010) constructs. To assemble *vasCas9.G* and *nosCas9.G* driven by the *D. melanogaster vasa* and *nos* promoters, the vasa5′-Cas9-vasa3′ and nos5′-Cas9-nos3′ fragments were excised from *vasa-Cas9* plasmid DNA (Drosophila Genomics Resource Center #1340)[49] and pBFv-nosp-Cas9 plasmid[36] (NIG-Fly, Japan), respectively, and then ligated into a *piggyBac* vector that was cut with HpaI and PspOMI. The *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 MS1419[50] and replacement with ZsGreen from pB[Lchsp83-ZsGreen].[51] We utilized previously described *yellow*[47] and *white*[35] gRNA sequences to build the *gRNA*^{y} and *gRNA*^{w} *piggyBac* plasmids (Fig. 1C). To assemble *gRNA*^{y}, 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

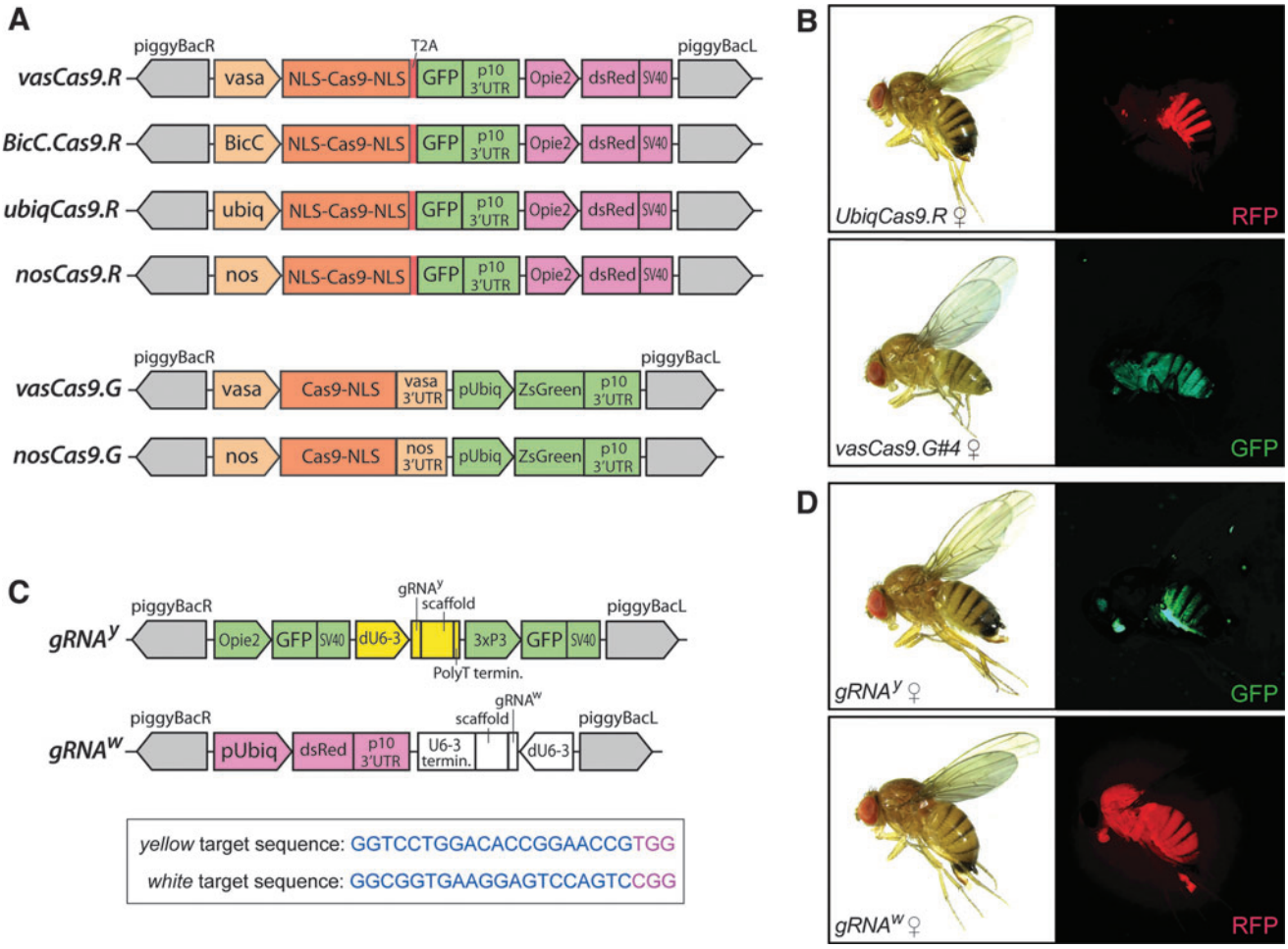

FIG. 1. Schematic maps of genetic constructs and images of transgenic *Drosophila suzukii*. **(A)** Schematic maps of two sets of Cas9 constructs. The first four Cas9 constructs harbor a human-codon-optimized *SpCas9* (*Cas9*)[68] coding sequence (CDS) surrounded by two nuclear localization sequences (*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 Cas9* 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 *gRNA*^{y} construct harbors the *yellow* gRNA (*gRNA*^{y}) 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 *gRNA*^{w} construct harbors the *white* gRNA (*gRNA*^{w}) 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 gRNA*^{y} and *gRNA*^{w} 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[53,54] with the poly(T)$_6$ termination on the other side as previously described,[40] 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^y*. To build *gRNA^w*, the *U6:3-ex2* plasmid that contains *white* gRNA targeting *D. suzukii*, *white* exon#2 (DS10_00006062)[52] and *D. melanogaster* U6:3 promoter and terminator[35] was digested with BglII, and the excised U6:3p-gRNA-U6:3t fragment was ligated with the *piggyBac* vector MS1425-p10 that contains a *ubiq-dsRed* marker. MS1425-p10 was derived from MS1425[50] 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 Corvallis, Oregon,[27] and North Carolina.[35] 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 Hsp70Bb-piggyBacTransposase[31] or the North Carolina wild-type strain with *piggyBac* helper plasmid.[55] G_0 adults emerging from injected embryos were outcrossed to *D. suzukii wt* flies, and their G_1 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 F_1 progeny by maternal Cas9 deposition.[40,47] 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 F_1 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 F_1 trans-heterozygous *Cas9.R/+; gRNA^w/+* flies of both sexes. This was recorded as a *mW* phenotype. We also performed genetic crosses with the paternal Cas9 for *BicC.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 F_1 trans-heterozygous virgin females were crossed to 10 *wt* males, and *y–* or *w–* phenotype was scored in the F_2 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, *w^{R2}*) and functional in-frame resistant (R1, *w^{R2}*) alleles. We polymerase chain reaction (PCR) amplified a genomic region containing the target site for *gRNA^y* or *gRNA^w* (Fig. 1C) using single-fly genomic DNA preps[40,47] from individual F_2 males, which were scored for *yellow* or *white* phenotype. The 397 bp PCR fragment of *yellow* target (exon #2) was amplified with 5′-GAATTCCAGCCACTCTGACTTATATCAATATGG-3′ (982G.s10F) and 5′-CAGGAGTAGGCAATTAAACCATAGCCC-3′ (982G.s11R); and 5′-GTGCCAGCACACGATCATCGGAGTGC-3′ (982A.s11R) and 5′-TGAGAAGAAGTCGACGGCTTCGCTGG-3′ (982A.s10F) primers were used to amplify the 322 bp PCR fragment of *white*

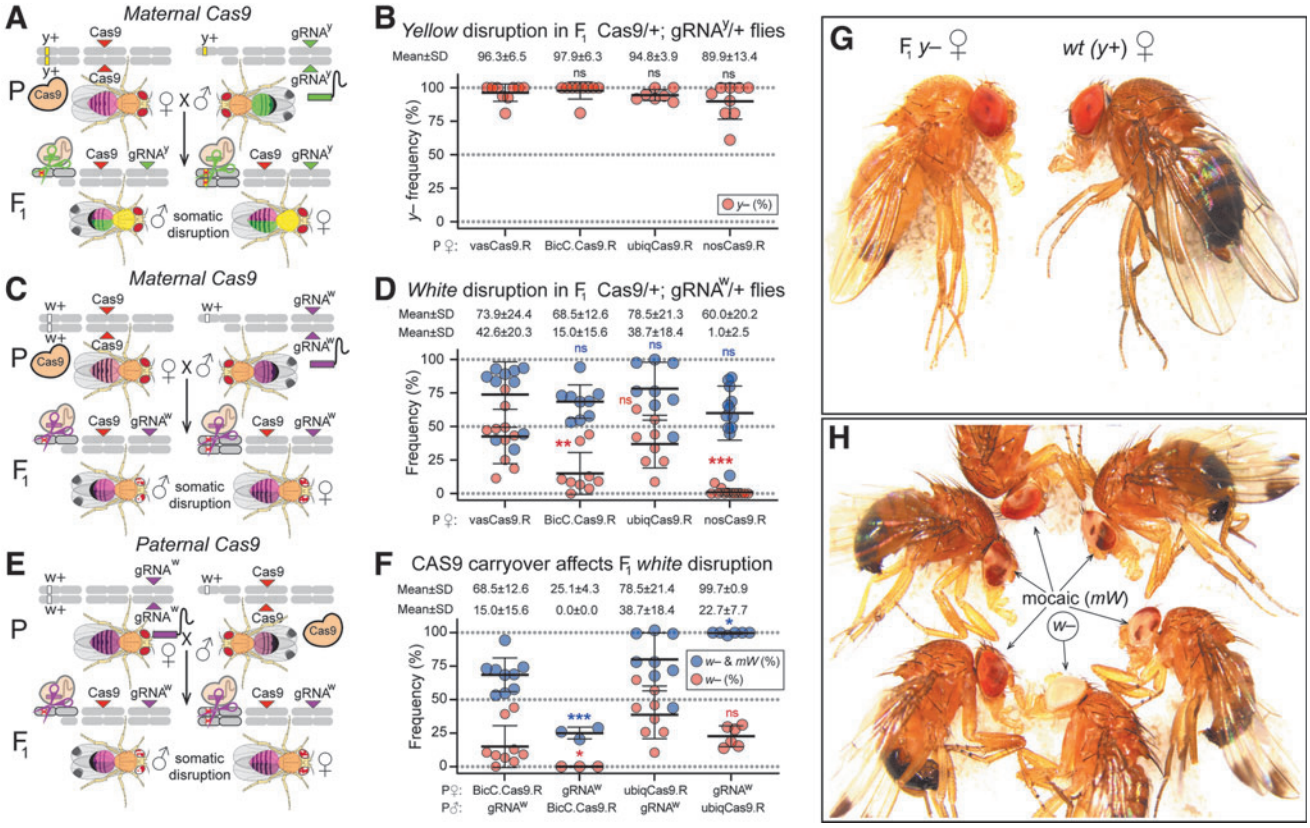

FIG. 2. Disruption of *yellow* and *white* loci in F_1 trans-heterozygous flies. **(A)** The schematic of a genetic cross between *Cas9* and *gRNA*^{y} flies. To generate F_1 trans-heterozygous flies, homozygous *Cas9* females (*red marker*) crossed to homozygous *gRNA*^{y} males (*green marker*). The targeted wild-type (*wt*) *yellow* gene (*y+* alleles, *yellow stripes* on X chromosome) is on the X chromosome. *Yellow-colored* thoraxes in F_1 flies indicate disruption of the *yellow* gene (*y–*). **(B)** Dot plot depicting the results of disruption of the *yellow* gene in somatic cells from F_1 trans-heterozygous (*Cas9/+; gRNA*^{y}*/+*) progeny using the Cas9.R strains inherited maternally. **(C)** Images of F_1 *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 F_1 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 F_1 trans-heterozygous (*Cas9/+; gRNA*^{w}*/+*) progeny using the Cas9.R strains inherited maternally. **(F)** Images of F_1 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 F_1 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 F_1 trans-heterozygous (*Cas9/+; gRNA*^{y}*/+*) 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. ($p \geq 0.05$ (n.s.); *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$). n.s., not statistically significant.

target (exon #2). To explore the pre-existent 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.

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*)[36] or on both terminals (*NLS-Cas9-NLS*; Fig. 1A).[46] To visualize and confirm expression for the *NLS-Cas9-NLS*, we incorporated a C-end self-cleaving T2A-*eGFP*,[56,57] which would generate two separate proteins (i.e., *NLS-Cas9-NLS* and *eGFP)* via ribosomal skipping.[56] To drive expression of Cas9, we used *D. melanogaster* promoters expressed in either early germ cells, *vasa* (*vas*)[58] or *nanos* (*nos*),[59] or in late germ cells, *Bicaudal C* (*BicC*),[47] or in both germ and somatic cells, *polyubiquitin 63E* (*ubiq*).[60] Using each promoter, we built four *piggyBac* constructs that express *NLS-Cas9-NLS* terminated by a p10 3′-UTR derived from the *Autographa californica* nucleopolyhedrovirus (AcNPV)[61,62] 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 *vas* or *nos* promoters driving expression of the *Cas9-NLS* terminated with *vas* 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 homozygous viable X-linked genes with recessive phenotypes: *yellow* (*y*) and *white* (*w*; Fig. 1C).[34,35] Each construct encoded one gRNA driven by the *D. melanogaster* small nuclear RNA *U6-3* promoter providing constitutive expression.[53] The *gRNA^{y}* construct harbors two green transgenesis markers (*Opie2-eGFP* and *3xP3-eGFP*), and the *gRNA^{w}* construct contains one red marker (*ubiq-dsRed*). Both *gRNA^{y}* and *gRNA^{w}* constructs were assembled in *piggyBac* (Fig. 1C), and homozygous *gRNA^{y}* and *gRNA^{w}* strains were generated (Fig. 1D and Table 1).

CRISPR-mediated mosaicism in F_1 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 F_1 progeny (Fig. 2A–F and Supplementary Tables S1 and S2). This cross was performed to explore the rates of mutagenesis in the F_1 somatic tissues, which is augmented by maternal deposition of Cas9.[47] High percentages (61.1–100%) of F_1 trans-heterozygotes generated by *Cas9.R* females crossed to *gRNA^{y}* 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.R* females were crossed to *gRNA^{w}*

Table 1. *Drosophila suzukii* Transgenic Strains Generated in the Study

Strain	*Construct ID*	*Strain marker*	*Sequence reference*	*Strain availability*	*Additional information*
vasCas9.R	vasCas9.R (874Z)	Opie2-dsRed	AddGene #112687	NDSSC #4023-0311.13	Cas9 expressed in somatic and germ cells
BicC.Cas9.R	BicC.Cas9.R (874R)	Opie2-dsRed	AddGene #168295	NDSSC #4023-0311.15	Cas9 expressed in late germ cells
ubiqCas9.R	ubiqCas9.R (874W)	Opie2-dsRed	AddGene #112686	NDSSC #4023-0311.14	X-linked Cas9 expressed in somatic and germ cells
nosCas9.R	nosCas9.R (874Z1)	Opie2-dsRed	AddGene #112685	O. Akbari at UCSD	Cas9 expressed in somatic and germ cells
vasCas9.G#4	vasCas9.G	ubiq-ZsGreen	Addgene #169012	M. Scott at NCSU	Cas9 expressed in germ cells
vasCas9.G#6	vasCas9.G	ubiq-ZsGreen	Addgene #169012	M. Scott at NCSU	Cas9 expressed in germ cells
nosCas9.G#14	nosCas9.G	ubiq-ZsGreen	Addgene #169011	M. Scott at NCSU	Cas9 expressed in germ cells
nosCas9.G#36	nosCas9.G	ubiq-ZsGreen	Addgene #169011	M. Scott at NCSU	X-linked Cas9 expressed in germ cells
gRNA^{y}	gRNA^{y} (982G.2)	Opie2-eGFP, 3xP3-eGFP	AddGene #168294	NDSSC #4023-0311.17	gRNA targets *yellow* exon #2
gRNA^{w}	gRNA^{w}	ubiq-dsRed	Addgene #169010	NDSSC #4023-0311.16	gRNA targets *white* exon #2

Five transgenic strains are deposited at the National Drosophila Species Stock Center (NDSSC) at the Cornell College of Agriculture and Life Sciences and available for distribution.

males (Fig. 2D), we observed much higher variability (0–100%) for somatic *white* disruption in resulting F_1 progeny (Supplementary Table S2). While both sexes were affected, the majority of F_1 trans-heterozygotes manifested highly variable mosaic eye coloration (*mW*), and a smaller fraction of F_1 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* (*wt w*+) allele or possibly a functional resistant (w^{R1}) allele. The extensive somatic disruption of *yellow* and *white* was induced by all *Cas9.R* strains. However, *Cas9.G* crossed to $gRNA^{y}$ and $gRNA^{w}$ strains did not appear to induce somatic mutations in the resulting F_1 progeny (Supplementary Tables S1 and S2).

Maternal deposition and F_1 mosaicism

To determine whether maternal *Cas9* deposition is essential to achieve high rates of F_1 mosaicism, we compared the rates of *white* mosaicism in F_1 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 F_1 *BicC-Cas9.R/+; $gRNA^{w}$/+* 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/+; $gRNA^{w}$/+* 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 F_1 trans-heterozygous progeny.

Cas9/gRNA-mediated heritable mutations

To estimate the frequency of heritable germline mutations, we mated F_1 trans-heterozygous females harboring either maternal or paternal *Cas9* to *wt* males, and assessed mutagenesis rates by scoring phenotypes in hemizygous F_2 males progeny (Fig. 3A). Both *yellow* and *white* are X-linked. Therefore, F_2 males generated from F_1 trans-heterozygous females inherit their mothers' X chromosome. If the F_1 females encode germline mutations in either *yellow* or *white*, then F_2 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 F_2 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 $gRNA^{y}$, the *Cas9.R* strains induced significantly higher rates of *y*– 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 F_1 *ubiqCas9.R/+; $gRNA^{y}$/+* females harboring maternal *Cas9* induced significantly higher levels of *y*– 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 F_1 *vasCas9.G#6/+; $gRNA^{y}$/+* 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 F_1 *BicC-Cas9.R/+; $gRNA^{w}$/+* or *vasCas9.G#4/+; $gRNA^{w}$/+* females generated F_2 males with loss of function (LOF) *w*– alleles in 92.7% and 1.1% 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 F_2 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 F_1 and F_2 mutagenesis of *white* as compared to *yellow* (Figs. 2B and E and 3B and D). We hypothesized that the F_2 *w*+ males may harbor either uncut *wt w*+ alleles or functional repaired alleles that were resistant to Cas9/$gRNA^{w}$ cleavage (termed w^{R1} alleles; Fig. 4A). To explore this hypothesis, we genotyped F_2 *w*+ males. Interestingly, we did not identify *wt w*+ alleles among 37 F_2 males generated from multiple independent crosses. Instead, each genotyped F_2 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 *wt* 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 F_2 *w*– males and identified diverse LOF resistant alleles (termed w^{R2}) induced by Cas9/$gRNA^{w}$ (Fig. 4C). Each genotyped F_2 *w*– males harbored one LOF w^{R1}

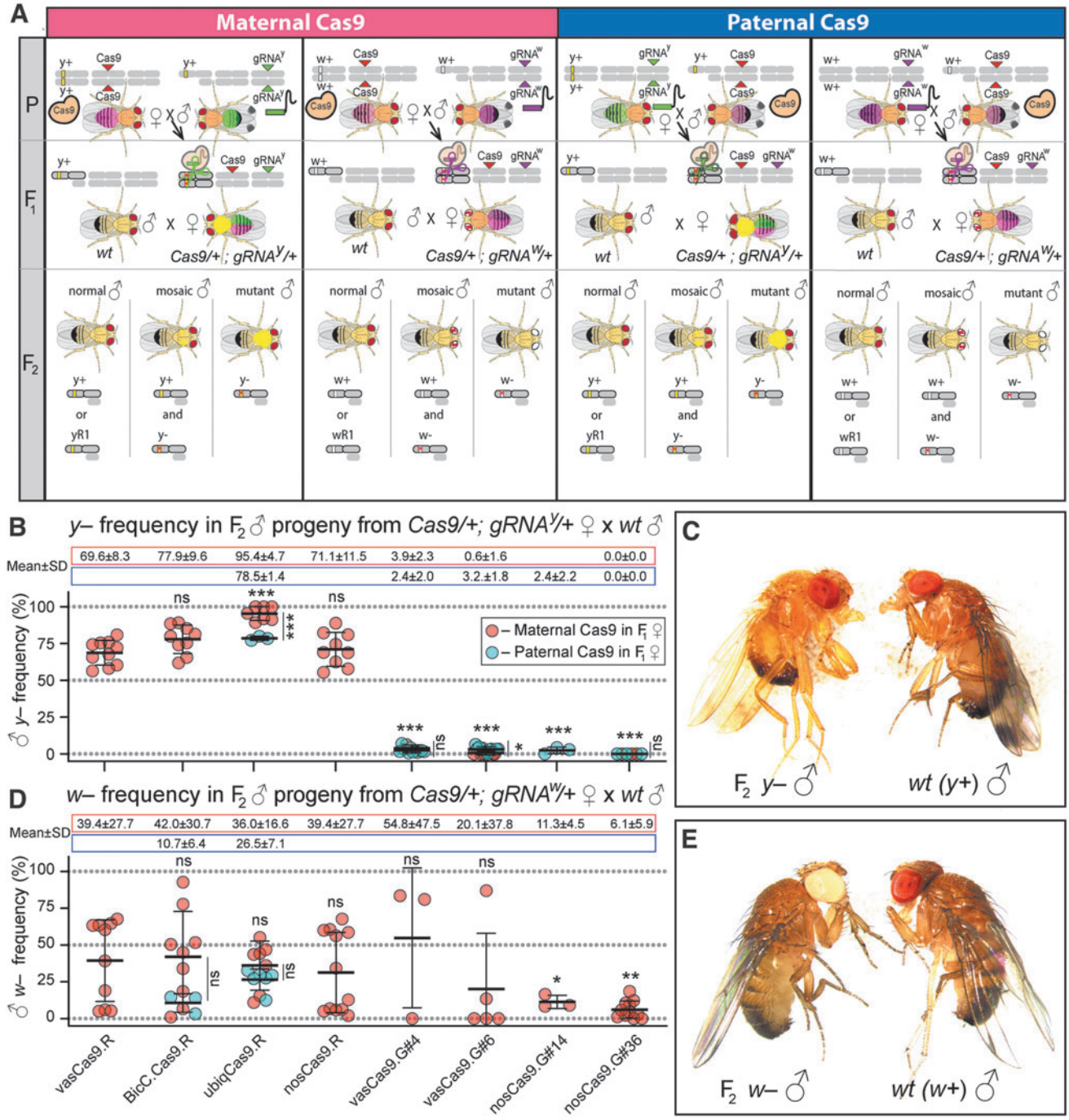

FIG. 3. The efficiency of heritable *yellow* and *white* mutations. **(A)** To assess for heritable *yellow* or *white* mutations in germ cells, F_1 trans-heterozygous *Cas9/+; gRNA/+* females were crossed to *wt* males, and *yellow* or *white* phenotype was scored in the generated F_2 males that inherited their mother's X chromosome. Two types of trans-heterozygous females were used: the F_1 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 F_2 males derived from crosses with the eight homozygous Cas9 strains generated in this study. **(C)** Images of the generated F_2 *y*– and *wt* (*y*+) males. **(D)** *Dot plot* depicting the *w*– frequency in the F_2 males derived from crosses with the eight homozygous Cas9 strains generated in this study. **(E)** Images of the F_2 *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. ($p \geq 0.05$ [n.s.]; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

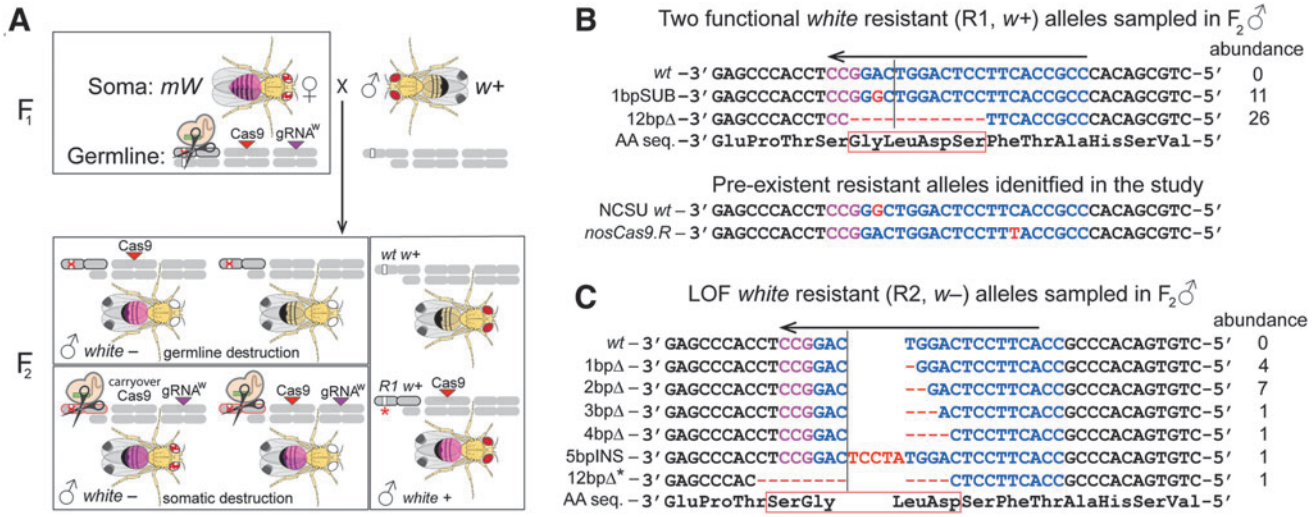

FIG. 4. Functional and loss of function (LOF) resistant alleles at the *white* target loci. **(A)** Schematic depicting potential males progeny from the genetic cross of F_1 trans-heterozygous *Cas9/+; gRNA^w/+* females and *wt* (*w+*) males. The F_1 females has mosaic eye coloration (*mW*), indicating disruption of the *white* gene in its somatic tissues. Some *w+* alleles can be mutated in its germ cells resulting in F_2 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 F_2 males that inherited the *gRNA^w* 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 F_2 males. The F_2 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/gRNA^w 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/gRNA^w. 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 F_2 *w+* males. **(B)** Each genotyped males from 37 F_2 *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 *w^{R1}* alleles persisted for two generations of cutting by Cas9/gRNA^w and maintained *wt* function of the *white* gene. No *wt w+* alleles were identified in any sampled F_2 *w+* males. After screening *D. suzukiii wt* stocks and all transgenic strains established in the study, we found one *w^{R1}* 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 F_2 *w^{R1}* males at UCSD has not identified any chromatogram peak ambiguity at base #5. **(C)** LOS R2 allele sampled in 15 F_2 *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.[52] The 20 bases of gRNA^w (in *blue*) and its protospacer adjacent motif (PAM; *purple*) are depicted over the *white* target sequence. *Arrows* point the direction of the gRNA^w target. Mutated bases (*red letters*) and/or their absence (*red dashes*) are indicated relative to the *wt* sequence.

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 *w^{R1}* 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 *w^{R1}* 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/gRNA^w because it is more distant from the cut site, and it was not sampled in our sequencing analysis

of F_2 *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 F_2 progeny (Fig. 3), and intercrossed individual F_2 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).

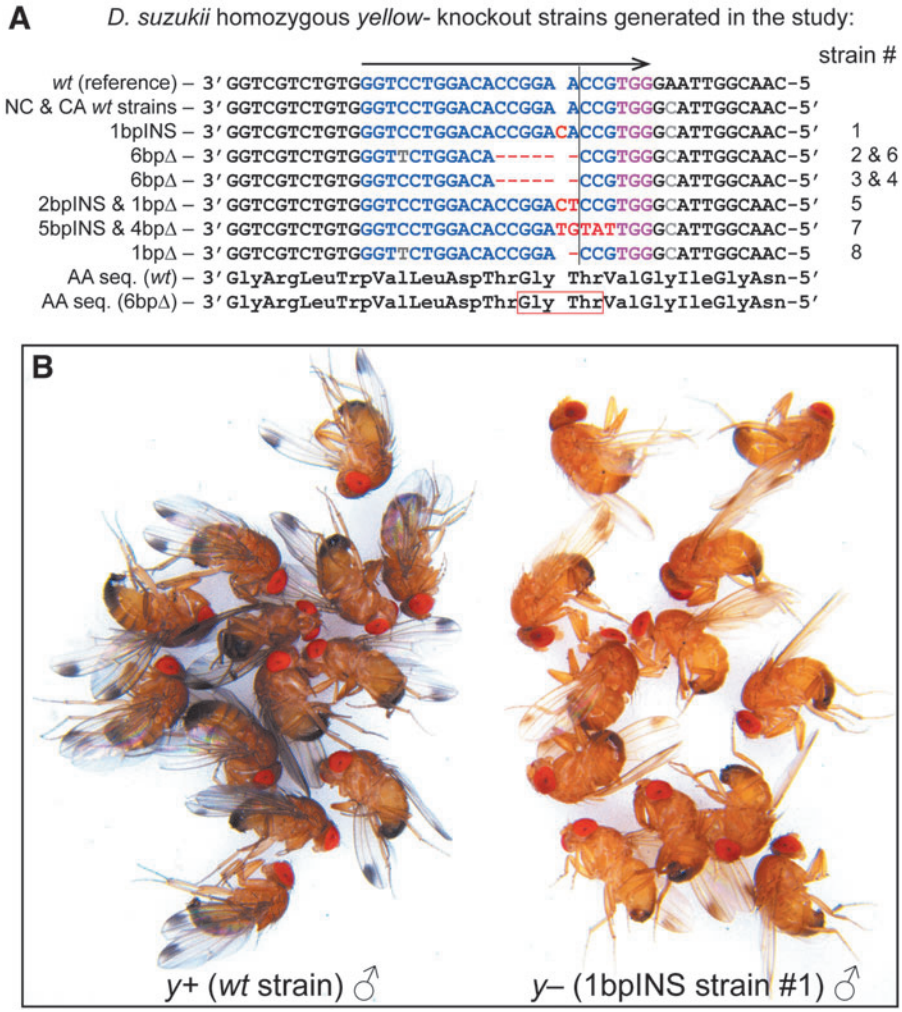

FIG. 5. *D. suzukii yellow* knockout (LOF) strains. We established eight independent homozygous viable *y*– knockout strains. **(A)** The sequence alignment of induced LOF alleles against the *wt* reference *yellow* sequence.[52] The 20 bases of gRNA are in *blue*, while the PAM sequence is in *purple*. 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 $gRNA^y$ 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.[61,62] In addition, a 7 aa-long SV40 NLS is shared between both *Cas9.R* and *Cas9.G* types, while *Cas9.R* constructs also contain a 16 aa bipartite nucleoplasmin 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.[63] 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 *nos.Cas9.G* did not induce visible F_1 somatic mutagenesis of *yellow* or *white* in the presence of the corresponding *gRNA*. Instead, they caused the *yellow* or *white* mutagenesis in the F_2 progeny, indicating a possible germline-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 F_2 *w+* males and found that each genotyped *w+* males harbored one of the two functional w^{R1} alleles. Our results indicate that the Cas9/gRNA^{w} cleavage of *white* was efficient. We did not sample *wt w+* alleles in F_2 males. Instead, they each harbored w^{R1} and w^{R2} resistant alleles. Both identified w^{R1} alleles, 1bpSUB and 12bpΔ, may have been induced by Cas9/gRNA^{w} 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) w^{R1} 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 F_2 *w+* males. The fact that the 1bpSUB w^{R1} allele was sampled in each cross with four *Cas9.R* strains and occurred naturally in the *wt* stock at NCSU suggests that this w^{R1} might not be generated, but instead could be selected out from pre-existent alleles, by Cas9/gRNA^{w}. The 12bpΔ w^{R1} 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/gRNA^{w} (see Results; Fig. 4B) than the 1bpSUB (A > G at base #5) w^{R1} allele because it is located farther away from the cut site (base #6). Therefore, our data suggest that the 1bpSUB w^{R1} allele pre-existed in *D. suzukii* genetic background and might not be completely resistant to the Cas9/gRNA^{w}, while the 12bpΔ allele was induced by Cas9/gRNA^{w} 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.[64] Previous studies demonstrated that unlike *D. melanogaster*, *D. suzukii* males harboring LOF *w–* alleles were sterile,[34,35] 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 know-how 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 pgSIT[40,41] and other Cas9-mediated genetic systems, such as homing split gene drives,[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

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