FINAL PROJECT REPORT **YEAR**: 3 of 3

Project Title: Engineering transgenic *Drosophila suzukii* for wild population suppression & eradication

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Objectives: Spotted wing Drosophila, *D. suzukii*, is a major worldwide crop pest of various soft-skinned fruits(Walsh et al. 2011). A highly promising approach to *D. suzkuii* control that could complement existing control methods is genetic pest management, which includes strategies such as gene drive and precision-guided sterile insect technique (pgSIT)(Kandul et al. 2018; Ant et al. 2012). SIT has been a successful technology for insect population suppression, which is achieved by introducing large numbers of sterile males into a target population. While the classic irradiation-based SIT presents an environment-friendly method of local population suppression, it is not technically feasible or scalable for the control of most insects. PgSIT, on the other hand, is a simplified way to generate sterile males and should be less expensive and labor intensive than irradiation-based SIT even at scale.

We also propose engineering *D. suzukii* gene drive strains, which can be utilized to more rapidly spread desirable genes (e.g., susceptibility to a novel bio-friendly pesticide) throughout, or to entirely suppress/eradicate, wild *D. suzukii* populations. Such an approach is catalytic, with release of only modest numbers of engineered insects required to spread desirable genes or achieve population suppression. Additionally, since such a system relies on only a few releases of transgenic insects to do all of the work on an ongoing basis, it is affordable as compared to the use of insecticides, which need to be applied regularly. Finally, such an approach is environmentally friendly and entirely insect-specific and would have no effect on crops or on beneficial organisms.

Our objective is to therefore engineer *D. suzukii* gene drive strains that could be utilized as part of current integrated pest management programs to control wild *D. suzukii* populations. Specifically, out of the multiple types of gene drive systems that can be utilized in a genetic pest management program(Champer, Buchman, and Akbari 2016; Scott et al. 2018). We aim to develop a pgSIT system in *D. suzukii* using the design principles we have optimized in *D. melanogaster(Kandul et al. 2018)*. We also aim to develop synthetic *Medea* elements that can be used to suppress wild *D. suzukii* populations. Ultimately, our goal is to develop a product (a genetically modified *D. suzukii*) that can be mass-reared and deployed into the wild to catalytically suppress, and completely eliminate, the wild populations of this significant pest.

Objective A - Refinement of a *Medea* drive system for *D. suzukii* population suppression. We have developed a synthetic *Medea* gene drive system for population suppression(Buchman et al. 2018). Engineered *Medea* systems rely on a *Medea* element consisting of a toxin-antidote combination. The toxin consists of a miRNA that is expressed during oogenesis in *Medea*-bearing females, disrupting an embryonic essential gene. A linked antidote is expressed early during embryogenesis and consists of a recoded version of the target gene that is resistant to the miRNA. This combination results in the survival of half of the embryos originating from a *Medea*-bearing heterozygous female, as those that do not inherit the *Medea* element perish. If a heterozygous *Medea* female has mated with a heterozygous

Medea male, the antidote from the male will also take effect in the embryo, resulting in 3/4 of the embryos surviving. Therefore, *Medea* will rapidly spread through a population, carrying any linked genes with it.

We have already engineered a first-generation *Medea* system in *D. suzukii* (Buchman et al. 2018), which is the first functional gene drive developed in this pest. We had rigorously tested it in laboratory cage populations, and had characterized it in different genetic backgrounds to determine effectiveness and fecundity. We found that this first-generation *Medea* system was capable of biasing Mendelian inheritance rates with up to 100% efficiency and could maintain itself at high frequencies in a wild population; however, drive resistance, resulting from naturally occurring genetic variation and associated fitness costs, was present and could hinder the spread of such a drive. Therefore, since mathematical modeling indicates that our *Medea* drive system could spread to fixation if resistance was reduced(Buchman et al. 2018), we need to engineer a second-generation *Medea* system that should obviate the specific resistance that we observed. To safeguard, reduce risk, and mitigate the spread of the *D. suzukii Medea* system into wild populations, we also aim to develop a reversal *Medea* (RM) system that can be used to replace the original *Medea* in case a recall is necessary. Reversing the drive of a *Medea* system has been theorized; however, it has never been experimentally demonstrated. Finally, in order to use *Medea* to bring about population suppression, we need to link it to a cargo gene capable of killing *D. suzukii* under specific conditions to bring about a population crash. We have already identified several promising putative cargo genes and are testing them in *D. melanogaster*, a closely related species to *D. suzukii* that is easier to work with and provides a useful testing platform for transgenes. However, we will still need to build and test them in *D. suzukii*. Successful completion of the above objectives would lead to the development of a genetically modified *D. suzukii* strain (carrying a synthetic *Medea* element) that can be mass-reared and deployed into the wild to catalytically suppress, and completely eliminate, wild populations of *D. suzukii*.

Objective B: Precision guided sterile insect technique (pgSIT) for *D. suzukii* population suppression. The Sterile Insect Technique (SIT) is an alternative, proven pest management approach that could complement existing control methods(Nikolouli et al. 2018; Alphey and Bonsall 2018; Lees et al. 2015). SIT involves the mass-production and release of sterile males, and has historically been used to control, and eradicate, insect pest populations dating back to the mid-1930s(Knipling 1955; Bushland, Lindquist, and Knipling 1955; Klassen and Curtis 2005; Vanderplank 1944; Dyck, Hendrichs, and Robinson 2005). Traditional SIT methodologies have relied on DNA-damaging agents for sterilization, substantially reducing overall fitness and mating competitiveness of released males. A next-generation highly-efficient technology that can be used for biocontrol of *D. suzukii* is precision guided SIT (pgSIT). PgSIT functions by exploiting the precision and accuracy of CRISPR to simultaneously disrupt genes essential for either female viability or male fertility. It utilizes a simple breeding scheme requiring two homozygous strains - one expressing Cas9 and the other expressing double guide RNAs (dgRNAs). A single mating between these strains mechanistically results in synchronous RNA-guided dominant biallelic knockouts of both target genes throughout development, resulting in the complete penetrance of desired phenotypes in all progeny. We have previously built pgSIT in *Drosophila melanogaster*, a model organism that is closely related to *D. suzukii*, and shown that it is extremely robust at genetically sexing and simultaneously sterilizing resulting progeny reproducibly with 100% efficiency, and that pgSIT sterile males are fit and can compete for mates(Kandul et al. 2018). We therefore aim to develop pgSIT technology in *D. suzukii* (Objective B). Successful development of this technology would produce a genetic-based sterile insect strain that can be mass-reared and released to reduce populations of *D. suzukii* in a straightforward manner with respect to regulations.

Significant Findings:

Objective A:

● We have developed a modified version of our original *Medea* system that is designed to reduce resistance to the drive.
● We have developed a second-generation "reversal" *Medea* system that should be more robust in the face of genetic diversity in general and could be used to replace the original *Medea* in case a recall is necessary.
● We have identified several promising putative cargo genes that could be spread with the *Medea* gene drive to cause population suppression.

Objective B:
● We developed a pgSIT system that is competitive, fit and consistently produces sterile males.
● Plans for a confined field trial of the pgSIT technology is planned at a USDA facility in Corvallis, Oregon.

Results and Discussion:

Objective A - Refinement of a *Medea* drive system for *D. suzukii* population suppression.

Second generation *Medea* drives: We have developed the first proof of concept *Medea* drive in *D. suzukii(Buchman et al. 2018)*. Given our observations regarding resistance and its effect on *Medea* function, we have engineered improved *Medea* systems that could reduce the chances of resistance acting as an impediment to spread. We first performed some sequencing-based characterization of naturally occurring genetic variation in various geographically distinct target populations to help guide selection of target sites that are well conserved across all populations in which the drive is intended to function. We then designed a modified version of the original *Medea* system that targeted different, conserved sequences (still in the 5'UTR of the *myd88* target gene), reasoning that such a *Medea* element should function very similarly to the original element but not be impeded by the resistance we previously observed. We obtained transgenic lines for this improved *Medea* element. Preliminary data indicated that it work better than the original *Medea*, producing 100% inheritance bias, but due to COVID19 related work delays we are still continuing to rigorously test this second-generation *Medea* element to characterize its function and ability to bias inheritance 100% in geographically distinct populations. Due to the higher likelihood that the pgSIT will be approved for field use before a *Medea* drive, since pgSIT is simply a new and improved version of the long used sterile insect technique (SIT), when forced to work at a lower capacity to adhere to the state of California and University of California COVID19 safety guidelines, we prioritize work on pgSIT.

Reversal *Medea* systems: Additionally, we hypothesized that to reduce resistance, miRNA target site selection could be limited to the coding DNA sequence regions of a genome, which tend to be strongly conserved, as opposed to regions such as the 5'UTR, which canonically have higher tolerance for sequence variation. We have therefore also developed a second-generation "reversal" *Medea* system in *D. suzukii* that should be more robust in the face of genetic diversity in general (because it targets coding DNA regions as opposed to the 5'UTR) and could be used to replace the original *Medea* in case a recall is necessary. Specifically, to reduce risk and mitigate the spread of the *D. suzukii Medea* system into wild populations, it is important to develop a reversal *Medea* (RM) system and demonstrate that it can function as predicted. Reversing the drive of a *Medea* system has been theorized; however, it has never been experimentally demonstrated. We finished designing and building a reversal *Medea* system capable of spreading on its own and of replacing the first *Medea* described above, but due to COVID19 related work delays we did not have time to develop transgenic lines for this construct. We focused our efforts instead on completing the pgSIT work, which should have a faster and more predictable path to approval for field implementation.

Identification of putative "cargo" genes: For *D. suzukii*, elimination of the pest populations is ultimately the goal. An engineered *Medea* system could achieve this by spreading a "cargo" gene proffering susceptibility to a particular pesticide, or a conditional lethal gene that would be activated by some substance or environmental cue such as high temperature or diapause. One promising type of candidate "cargo" gene is a thermally activated TRPA1 cation channel(Castillo et al. 2018).

Specifically, TRPA1 is an ion channel located on the plasma membrane of many human and animal cells, and is finely tuned to detect specific temperatures ranging from extreme cold to noxious heat(Castillo et al. 2018). Upon exposure to a critical "threshold" temperature, this cation channel can "open" and modulate Ca2+ and Mg2+ entry into the cell(Guimaraes and Jordt 2011); when TRPA1 is overexpressed in an exogenous tissue (such as the fly brain, for example), this "opening" can lead to total fly paralysis and death. We therefore have started to engineer *D. suzukii* to express a specific TRPA1 channel in the brain, so that exposure of the engineered individuals to a threshold temperature (determined by the specific TRPA1 channel used) would paralyze/kill the flies. We have designed these lines, but due to COVID19 related work delays, we were unable to test whether these transgenic lines are able to spread this temperature-activated "cargo" gene through wild populations. The goal would have been to couple this with a *Medea* system whereby at cooler temperatures the cargo is inactive, but at higher temperatures the TRPA1 and achieve population suppression when the TRPA1 gene is activated at warmer temperatures.

Objective B: Precision guided sterile insect technique (pgSIT) for *D. suzukii* population suppression.

Precision guided SIT: In order to construct a pgSIT system, we need functional Cas9 tools (including gRNA lines that target genes essential for female viability and male sterility and Cas9 expressing lines (**Fig. 1-2**) in *D. suzukii*. We developed multiple transgenic lines that express Cas9 (*bicC-cas9, vasa-cas9, nanos-cas9, ubiq-cas9*)(Kandul et al., n.d.). To robustly express and import Cas9 into nuclei, 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 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 63E* (*ubiq*). 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) or strong translation of Cas9, and a red (Opie2-dsRed) transgenesis marker. We also built two alternative piggyBac constructs that contain either the *vas* or *nos* promoters driving expression of the Cas9-NLS terminated with *vas* or *nos* 3'UTR's from *D. melanogaster*, with a green (ubiq-ZsGreen) transgenesis marker (**Fig. 1A**). In total, six Cas9 constructs were engineered that were used to generate 8 homozygous transgenic strains (at least one homozygous transgenic strain per construct), two of which were X-linked (**Fig. 1B**). Initially, the efficiency of these promoters was tested by genetically crossing two gRNA lines targeting the *white* and *yellow* phenotypic genes (**Fig. 1C, 2**). To assess the functionally of the lines 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**). This cross was performed to explore the rates of mutagenesis in the F_1 somatic tissues, which is augmented by maternal deposition of Cas9. High percentages (61.1%-100%) of F_1 trans-heterozygous progeny generated by dsRed+ Cas9 females crossed to *yellow* targeting gRNA lines (gRNAy) males had visible yellow, instead of brown, body coloration indicating robust somatic *yellow* gene disruption (y– phenotype) (**Fig. 2B,C**).

Also, essential to building a pgSIT system are guide RNA (gRNA) lines that target genes essential for female viability and male fertility. We have previously identified genes essential for female viability or male fertility in *D. melanogaster* and have shown that disrupting these genes via CRISPR/Cas9 produces the desired results (e.g., female death or conversion of females into sterile intersex individuals for the former group, male sterility for the latter(Kandul et al. 2019). Since *D. melanogaster* is closely related to *D. suzukii*, we reasoned that disruption of these same genes would have a similar effect in *D. suzukii*. Specifically, to disrupt female viability in *D. suzukii*, we targeted several sex-specifically alternatively spliced sex-determination genes including *sex lethal* (*sxl*), *transformer* (*tra*), and *doublesex* (*dsxF*), as well as *zero population growth* (*zpg*), a germline-specific gap junction gene. We identified *D. suzukii* homologues of all of these genes and have carefully selected two gRNA target sites in each gene that are highly conserved and thus unlikely to have high sequence

variation in the wild. We generated multiple transgenic lines for each gRNA target and crossed to the Cas9 strains to see whether the combinations of Cas9+gRNA will produce female lethality and male sterility. To track transgenes, gRNAdsx, gRNAtra, and gRNAβTub plasmids also harbored a green fluorescent tag, Opie2-mVenus, while the gRNAsxl plasmid carried Ubiq-dsRed (**Fig. 3**). Each gRNA plasmid was injected into embryos harboring hsp-pBac intergrase, and one or two gRNA transgenic lines were generated for each construct. Then, virgin transgenic flies from each lines were repeatedly backcrossed to establish homozygous stocks of gRNAsxl.L1, gRNAsxl.L2, gRNAdsx, gRNAtra.L1, gRNAtra.L2, gRNAβTub.L1, and gRNAβTub.L2 lines. The *vas* and *BicC* Cas9 lines had the highest and most consistent gene knockout efficiencies with the least fitness costs, so we used these lines to evaluate the targeting efficiency of each gRNA line (**Fig. 4)**. This evaluation was done by genetically crossing each gRNA strain to both vasCas9 and BicC.Cas9 strains, scorring sex frequency and fertility of F1 trans-heterozygous progeny, and then sequencing target loci.

The lines that most efficiently killed or masculinized females, targeted *sxl*, which is a sex determination gene essential for female-specific development in *D. melanogaster* and other insect species and disruption of this gene has been shown to cause female lethality or masculinization in other studies. (Kandul et al. 2019). Two lines that target *βTub* showed the high rate of male sterility. which arrests spermatid elongation and sperm mobility resulting in the male-specific sterility *(*Kandul et al. 2019; Li et al. 2021). We then built multiple lines with, which targeted both *sxl* and *βTub* (**Fig. 3**). The most efficient and healthy lines ($gRNA^{sxl,\beta Tub\#4.L1}$, $gRNA^{sxl,\beta Tub\#4.L2}$, and $gRNA^{sxl,\beta Tub\#5.L1}$) were chosen for further characterization and to ensure these results were reproducible over many generations. When crossed to vasCas9 we demonstrated complete female-specific lethality and nearly complete male sterility in the F_1 trans-heterozygous progeny (**Fig. 5**). To further quantify the fertility of pgSIT males, we crossed batches of 5 pgSIT ♂ to 20 *wt* virgin ♀ and scored numbers of laid and hatched eggs. Not a single egg out of 952 and 321 eggs laid by females mated with $gRNA^{sxl,\beta Tub\#4}.L1$/+; *vasCas9*/+ and $gRNA^{sxl,\beta Tub\#5}.L1$/+; *vasCas9*/+ males respectively, hatched (Table 1). Five eggs hatched out of 518 eggs laid by females mated with $gRNA^{sxl,\beta Tub\#4}.L2$/+; *vasCas9*/+ males (0.9% egg hatching rate, Table 1). Therefore, these results indicate that genetic crosses of the *vasCas9* strain to any of three autosomal $gRNA^{sxl,\beta Tub}$ strains result in complete lethality or masculinization of females into sterile intersexes while the generated pgSIT males are nearly 100% sterile. The induced pgSIT phenotypes, i.e. female lethality / masculinization and male sterility, correlated with mutagenesis at *slx* and *βTub* target loci induced by Cas9/gRNA in trans-heterozygous flies (**Fig. 6**). Then we chose one line to be rigorously tested in male competition and fitness studies as well as laboratory population cages to ensure the sterile males are fit to compete in field conditions (**Fig. 7-8**). pgSIT males were able to compete for mates with wildtype males (**Fig. 7**) and were able to successfully suppress small laboratory populations of *D. suzukii*.

Developing a field-ready strain: We have developed pgSIT strain ready for use in the field for *D. suzukii* biocontrol. We are currently using this data to inform mathematical models to predict the introduction frequencies we will need to use to achieve suppression and this strain has been transferred to Agragene, a local biotechnology company for future confined field assessments at a USDA facility in Corvallis, Oregon. Our collaborator has received a field cage study permit from USDA-APHIS BRS/PPQ. APHIS is responsible for issuing permits for the import, transit and release of regulated animals, animal products, veterinary biologics, plants, plant products, pests, organisms, soil, and genetically engineered organisms. They have also applied for a BRS 2000 (Application for Permit or Courtesy Permit for Movement or Release of Genetically Engineered Organisms). These permits have been successfully issued for the release of transgenic insects in the USA. For example, in 2009 the USDA approved the integration of genetically engineered pest insects (including pink bollworm moth (*P. gossypiella*), Mediterranean fruit fly (*Ceratitis capitata*), Mexican fruit fly (*Anastrepha ludens*), and oriental fruit fly (*Bactrocera dorsalis*)) into ongoing SIT programs(Reeves et al. 2012). Some key advantages of the pgSIT approach will be that only males will need to be released (so crops will not be damaged); that it is very species-specific, since the released males will be sterile and not capable of mating with wild *D. suzukii* or any other species; and that the approach is self-limiting, which makes it a safer alternative than self-sustaining approaches and thus more likely to win public and regulatory

approval. Therefore, the key point here is that obtaining regulatory approval for releasing transgenic insects in the USA, that are engineered to reduce wild populations and prevent crop damage, has been achieved in the past, and therefore we do not envision it to be a limitation with our approach.

Table 1: Fertility of pgSIT (trans-heterozygous) males measured by the hatching rate of sired eggs.

gRNA strain	Groups of 5 ♂ and 20 virgin ♀	Total number of laid eggs	Total number of hatched eggs	Hatching rate
gRgRNA$^{sxl,\beta Tub\#4}$.L1	5	952	0	0%
gRgRNA$^{sxl,\beta Tub\#4}$.L2	3	518	5	0.9%
gRgRNA$^{sxl,\beta Tub\#5}$.L1	3	321	0	0%

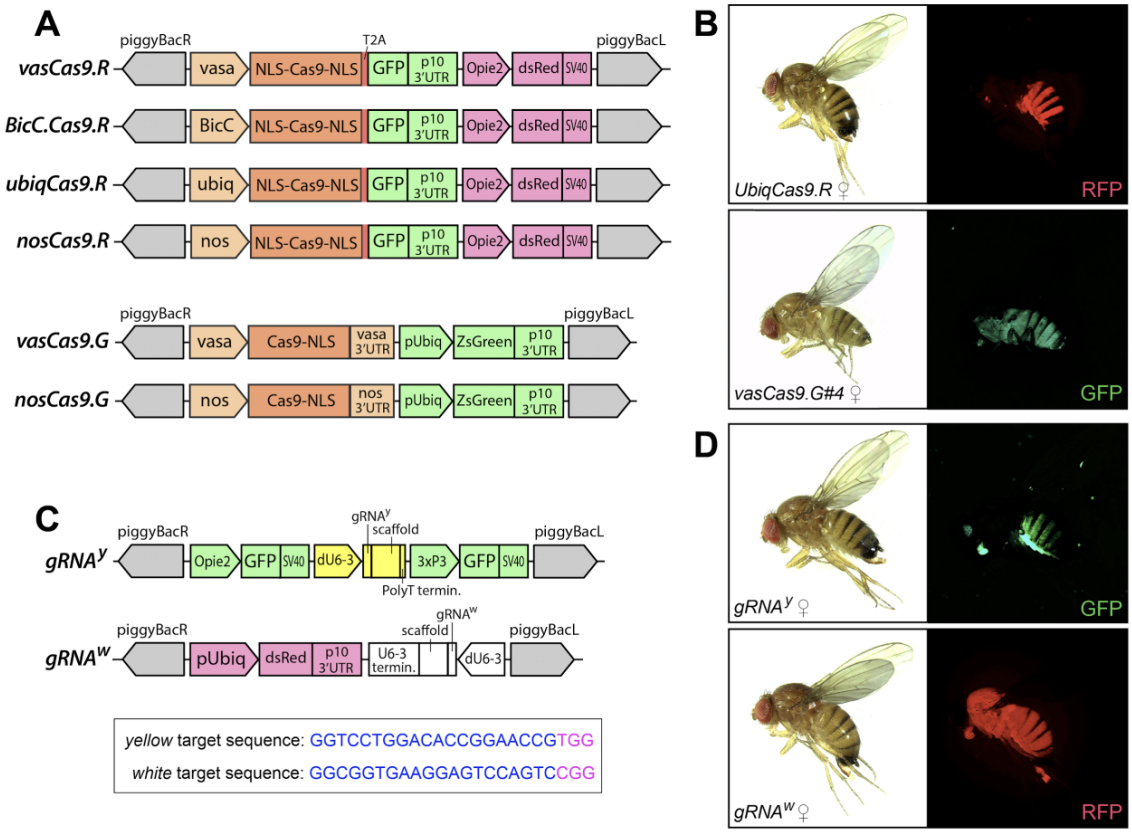

Figure 1. Schematic maps of genetic constructs and images of transgenic SWD. (A) Schematic maps of two sets of Cas9 constructs. The first four Cas9 constructs harbor a human-codon-optimized SpCas9 (Cas9) (61) coding sequence (CDS) surrounded by two nuclear localization sequences (NLS-Cas9-NLS), linked 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). 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 SWD Cas9 ♀ 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 terminates the pU6-3 terminator sequence, and a red transgenesis marker, Ubiq-dsRed. (D) Images of homozygous SWD gRNAy and gRNAw ♀. Both sets of RGB images for each ♀ fly were taken under the white light and corresponding fluorescent light illumination.

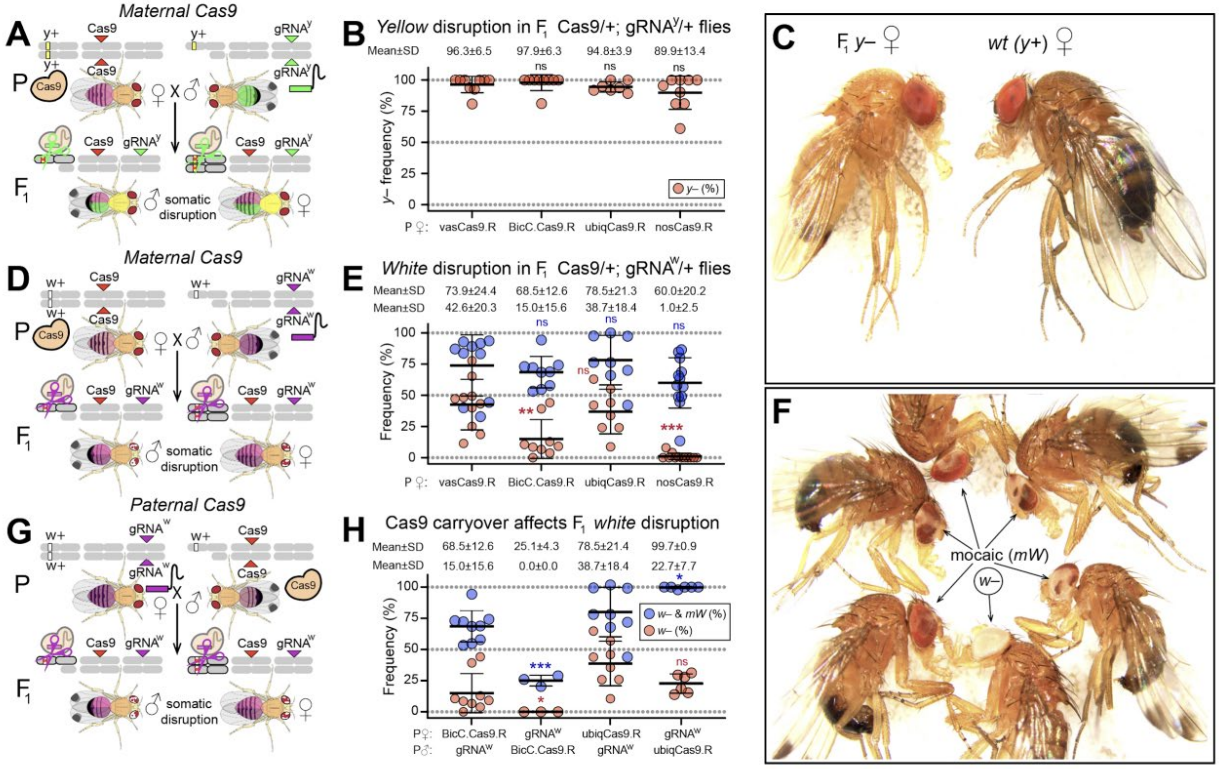

Figure 2. Disruption of *yellow* and *white* loci in F1 trans-heterozygous flies. (A) The schematic of a genetic cross between Cas9 and gRNAyellow(y) flies. To generate F_1 trans-heterozygous flies, homozygous Cas9 ♀ (red marker) crossed to homozygous gRNAy ♂ (green marker). The targeted wildtype (wt) *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/+; gRNAy /+) progeny using the Cas9.R strains inherited maternally. (C) Images of F_1 y– trans-heterozygous and wt y+ ♀. (D) The schematic of a genetic cross with maternal homozygous Cas9 (red marker) and paternal homozygous gRNAw (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/+; gRNAw/+) 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 gRNAw flies. To generate F1 trans-heterozygous flies, homozygous Cas9 ♂ (red marker) crossed to homozygous gRNAw ♀ (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 transheterozygous (Cas9/+; gRNAy /+) progeny using the Cas9.R strains inherited paternally. Plots show the mean ± SD over at least three biological replicates. Statistical significance was estimated using a two-sided Student's t test with unequal variance. ($p \geq 0.05$ns, $p < 0.05$*, $p < 0.01$**, and $p < 0.001$***).

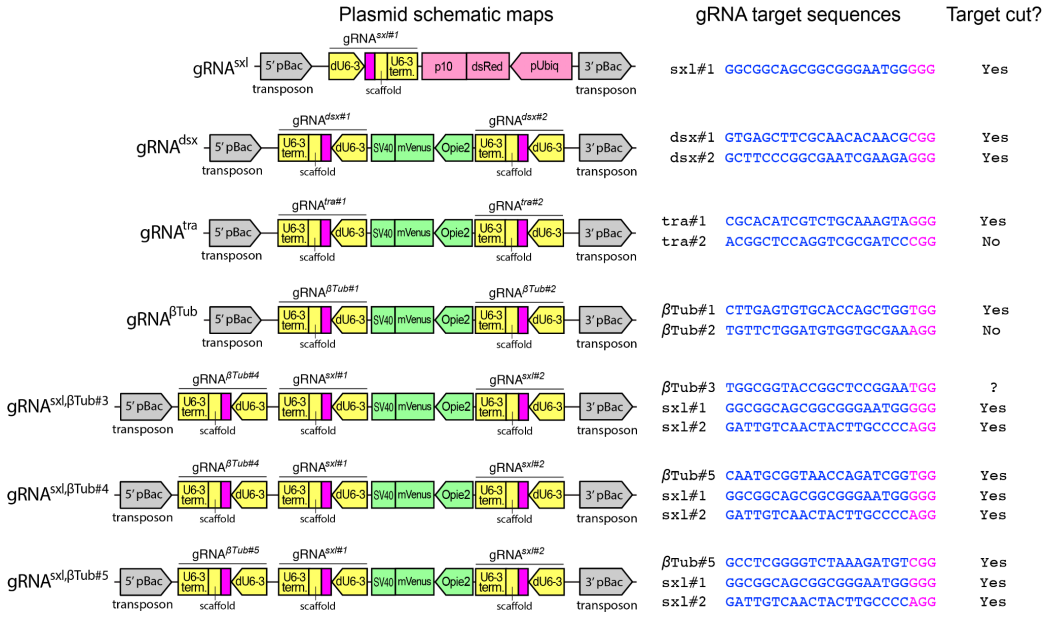

Figure 3. Schematic maps of pgSIT plasmids and gRNA target sequences. Different gRNA lines expressing one or two gRNA targeting *sex lethal* (*sxl*), *double sex* (*dsx*), *transformer* (*tra*), or *βTubuline 85D* (*βTub*). To track transgenes, the gRNAdsx, gRNAtra, and gRNAβTub plasmids also harbored a green fluorescent tag, Opie2-mVenus, while the gRNAsxl plasmid carried Ubiq-dsRed. The gRNA was driven by the constitutively expressed *D. melanogaster small nuclear RNA U6-3* promoter.

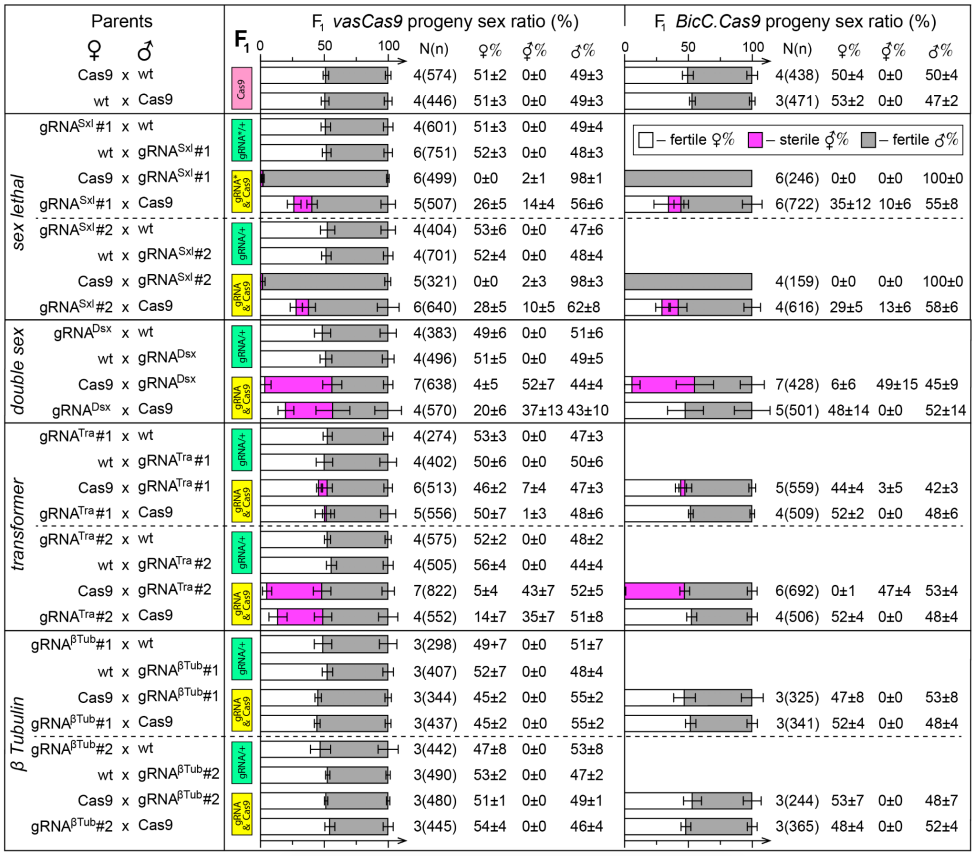

Figure 4. Assessment of Cas9/gRNA-mediated disruption of single-gene. Cas9/gRNA-mediated knockout causes lethality and/or masculinization of F_1 trans-heterozygous females. Different gRNA lines expressing one or two gRNA targeting *sex lethal* (*sxl*), d*ouble sex* (*dsx*), *transformer* (*tra*), or *βTubuline 85D* (*βTub*) were assessed in combination *vasCas9* and *BicC Cas9* lines.

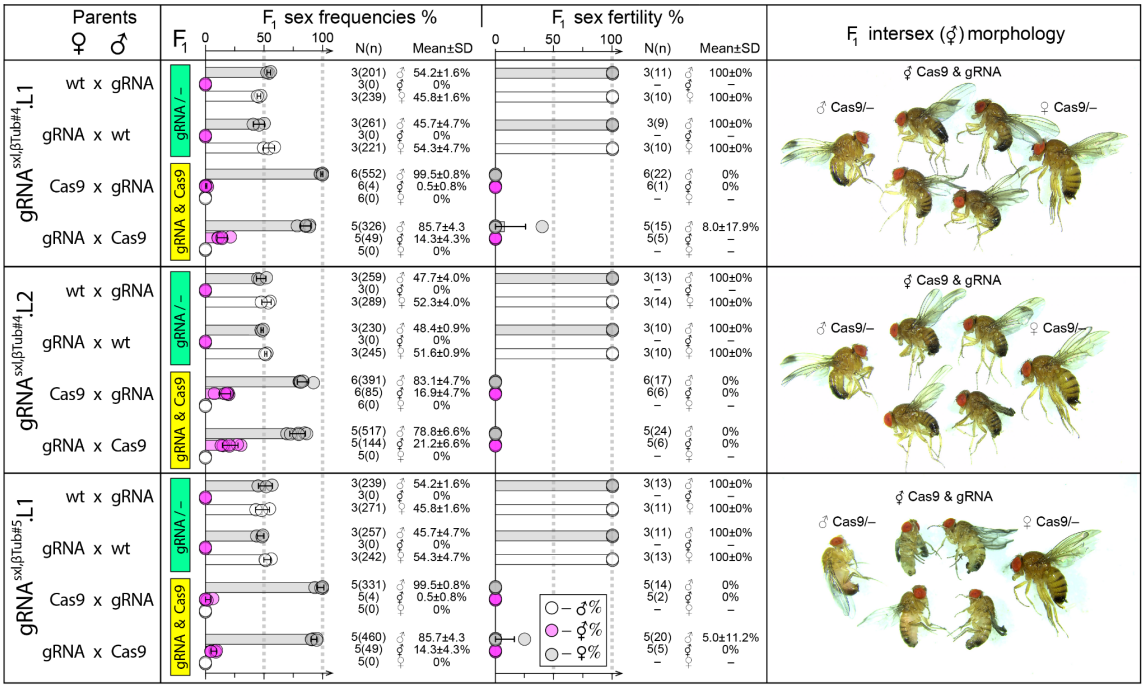

Figure 5. Three active *D. suzukii* pgSIT systems. Cas9/gRNA-mediated knockout causes lethality and/or masculinization of F_1 trans-heterozygous females. Different gRNA lines expressing one or two gRNA targeting *sex lethal* (*sxl*), d*ouble sex* (*dsx*).

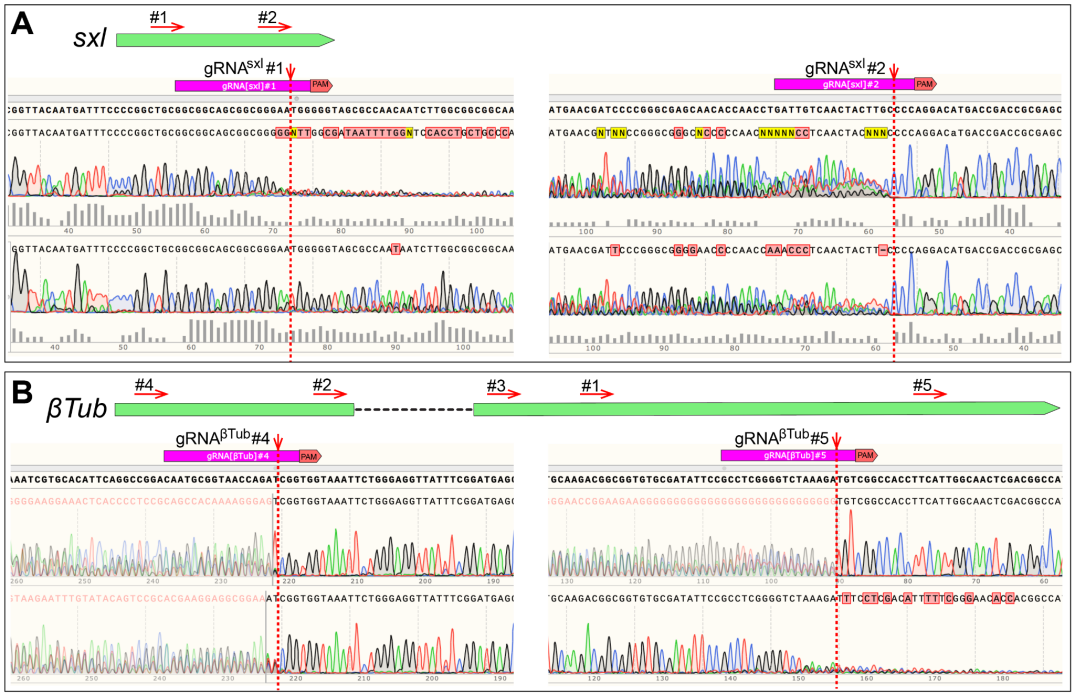

Figure 6. Double-gene KO Sanger Sequence reads of targets in *Sxl* **(A)** and *βTub* **(B)**.

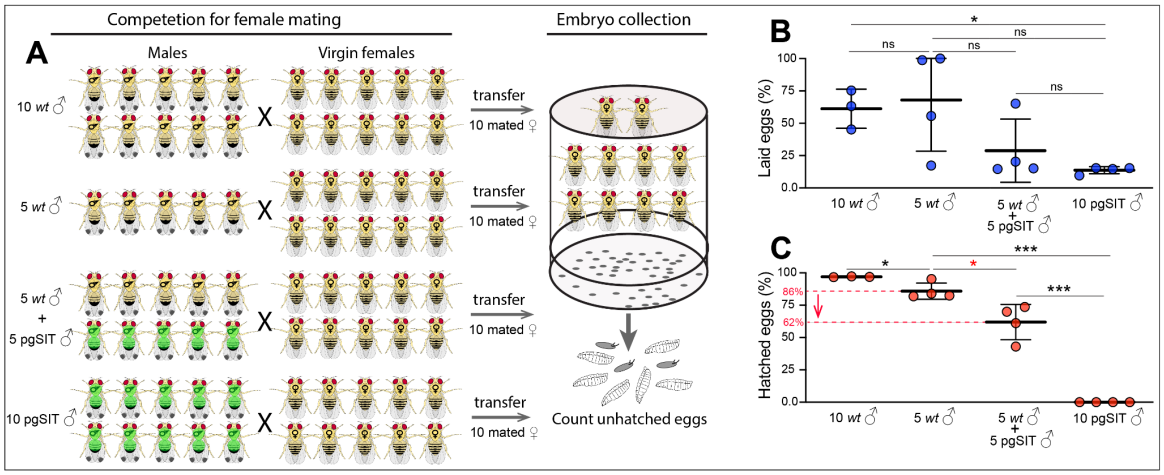

Figure 7. pgSIT males are competitive with wildtype males. In small population cage studies, male pgSIT flies (green flies) were released in 1:2 or 1;1 male to female ratios. All wildtype and all pgSIT male controls were also evaluated. As expected, there was no difference in the number of eggs laid in any of the groups containing wildtype males. However, in experimental groups with pgSIT there were significantly less eggs hatched and in the pgSIT only control group, few eggs were laid and none hatched.

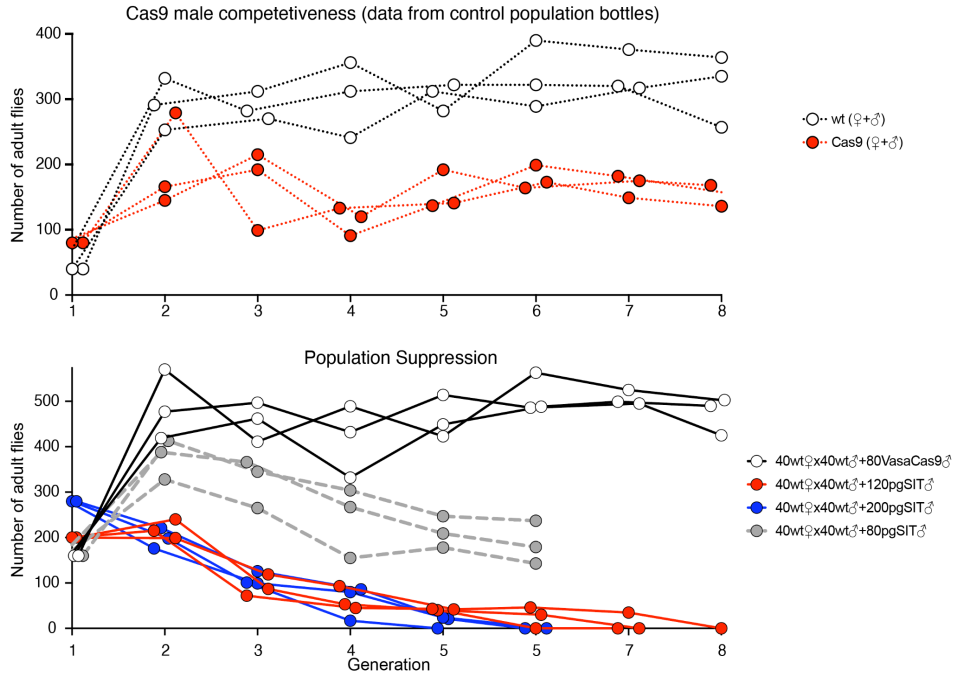

Figure 8. pgSIT males suppress discrete populations. To establish the fixed size populations of *D. suzukiii*, were seeded groups of forty 4-5-day-old virgin wt females in 0.3 L plastic bottles (VWR Drosophila Bottle 75813–110). The mixture of 4-5-day-old 40 wt and 80 vasCas9 (1:2 ratio) males was added to control bottles (white circles). For test bottles, we added 40 wt and 80 pgSIT (1:2 ratio- gray circles) or 40 wt and 120 pgSIT (1:3 ratio- rec circles) or 40 wt and 200 pgSIT (1:5 ratio-blue circles) males per bottle. After 4 days at 21˚C/70˚F, parent flies were transferred into fresh bottles and the first bottles were discarded. In 5-7 days, depending on the food condition, the parent flies were transferred again into the fresh bottles before being removed permanently in another 5 days. After a total of 18-25 days, progeny adults that emerged from the second and third bottles were collected, sexed, sorted for the presence or absence of the fluorescent protein marker, and then counted. The control bottles were set with the same number of flies, 40 wt pairs and 80 vasCas9 males, per bottle each generation. For the test bottles, the number of virgin wt females per bottle was based on the average number of females produced in the previous generation in the text bottles normalized that in the control bottles. The number of added females was calculated according to the formula N = 40 × (T/C), where N is the number of females added to the current generation of test bottles, T is the average number of females produced from the test bottles in the previous generation and C is the average number of females pro- duced from the control bottles in the previous generation. To keep the release ratio of pgSIT males constant,

N wt males mixed with 2N or 3N or 5N pgSIT males were added to the corresponding test bottles. The experiment was continued until no females were produced from the test bottles with the 1:3 pgSIT male release ratio.

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Executive Summary

Project Title: Engineering transgenic *Drosophila suzukii* for wild population suppression & eradication

Keywords: *Drosophila suzukii*, gene drive, precision guided sterile insect technique

Abstract: The spotted wing Drosophila (SWD) is a major worldwide crop pest of soft-skinned fruits. Current methods to control the SWD rely considerably on the use of expensive, broad-spectrum insecticides (e.g., malathion), which have variable efficacy, are difficult to use due to timing of fruit infestation, and face the risk of *SWD* evolving resistance. Additionally, use of broad-spectrum insecticides has led to disruption of integrated pest management systems developed for crops such as cherries and berries, and has had a serious impact on beneficial arthropods. However, there are no effective alternatives to managing the SWD infestation, and it is likely that, unless more effective control measures are developed, this pest will continue to spread.

Gene drives can be utilized to more rapidly spread desirable genes (e.g., susceptibility to a novel bio-friendly pesticide) to suppress/eradicate wild SWD populations. Such an approach is catalytic, with release of only modest numbers of engineered insects required to spread desirable genes or achieve population suppression. Additionally, since such a system relies on only a few releases of transgenic insects to do all of the work on an ongoing basis, it is affordable as compared to the use of insecticides, which need to be applied regularly. Finally, such an approach is environmentally friendly and entirely insect-specific and would have no effect on crops or on beneficial organisms. We initially built a *Medea* drive to support the rapid modification of SWD populations and in this project we made improvements to this drive system. Engineered *Medea* systems rely on a *Medea* element consisting of a toxin-antidote combination. The toxin consists of a miRNA that is expressed during oogenesis in *Medea*-bearing females, disrupting an embryonic essential gene. A linked antidote is expressed early during embryogenesis and consists of a recoded version of the target gene that is resistant to the miRNA. This combination results in the survival of half of the embryos originating from a *Medea*-bearing heterozygous female, as those that do not inherit the *Medea* element perish. If a heterozygous *Medea* female has mated with a heterozygous *Medea* male, the antidote from the male will also take effect in the embryo, resulting in 3/4 of the embryos surviving. Therefore, *Medea* will rapidly spread through a population, carrying any linked genes with it. However, since there is much uncertainty about whether gene drives will be acceptable for field use, or even needed, we also developed alternative technologies to control SWD, but will have a higher likelihood of gaining public and regulatory approval.

An alternative, highly promising approach that could complement existing control methods is genetic pest management, which includes strategies such as gene drive and transgenic-based precision-guided sterile insect Technique (pgSIT). SIT has been a successful technology for insect population suppression, which is achieved by introducing large numbers of sterile males into a target population. Over time, repeated mass releases of sterile males suppresses and can even eliminate the target population. This technique has been used to control other insect pests, so it has a clear regulatory pathway, but it uses irradiation-based methods, which are not technically feasible or scalable for the control of many insects. PgSIT, on the other hand, is a simplified way to generate sterile males and should be less expensive and labor intensive than irradiation based SIT even at scale. We therefore engineered pgSIT and synthetic *Medea* gene drives that can be used to suppress wild SWD populations. We developed multiple pgSIT strains that were able to efficiently generate 100% sterile male SWDs that were fit, competitive to wildtype males. When released into small laboratory populations of SWD, the pgSIT technology was able to eradicate SWD populations in only a few generations. Plans are now underway to test the pgSIT technology in confined field trials and if successful we hope this technology can be evaluated in the field in the next few years.