FINAL PROJECT REPORT

Project Title: *D.suzukii* transgenic, population replacement, eradication, suppression

PI: Omar Akbari
Organization: University of California, San Diego
Telephone: 858-246-0640
Email: oakbari@ucsd.edu
Address: 9500 Gilman Drive
City/State/Zip: La Jolla, CA 92093

Cooperators: Bruce Hay, Caltech

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Budget History:

Item	Year 1:	Year 2:	Year 3:
WTFRC expenses			
Salaries			
Benefits			
Wages			
Benefits			
Equipment			
Supplies	$25,000		
Travel			
Plot Fees			
Miscellaneous			
Total	$25,000		

ORIGINAL OBJECTIVES

Drosophila suzukii is a major invasive pest of ripening small fruit including raspberries, blueberries, strawberries, and cherries [1,2]. It has caused significant worldwide economic losses including significant damage in the berry- and cherry-growing industries of western North America [2–5]. Achieving effective control of *D. suzukii* has been difficult in a number of crop systems including cherries [6,7], and control measures have largely relied on prophylactic application of expensive broad spectrum insecticides [6–8]. This is problematic, as the repeated 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, resulting, for example, in an increased use of miticides [4]. Additionally, broad use of insecticides makes it inevitable that resistance will become a major problem in the foreseeable future [8], increases the risk of residues on fruits [8], and arouses public concern [6]. However, there are no effective alternatives to managing *D. suzukii* infestation, and it is likely that, unless more effective control measures are developed, this pest will continue to spread [8].

An alternative, highly promising approach that could complement existing control methods is genetic pest management [9], which includes strategies such as gene drive [10,11] and transgenic-based sterile insect technique (SIT) [12,13]. In particular, engineered *D. suzukii* gene drive strains can be utilized to 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 the all of the work on an ongoing basis, it is cheap as compared to the use of insecticides, which need to be applied regularly. Finally, a major appeal of this approach is that it is environmentally friendly and entirely insect-specific, and would have no effect on crops or on beneficial organisms.

Our objective over the last year, therefore, was to make progress towards engineering gene drive systems in *D. suzukii*. Specifically, out of the multiple types of gene drive systems that can be utilized in a genetic pest management program [11,14], we decided to focus our efforts on developing *Medea* and Cas9-mediated systems. Our goals were to evaluate the feasibility of engineering each strategy in *D. suzukii*, and to take concrete steps towards developing 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.

SIGNIFICANT FINDINGS

I. Objective A - Development of CRISPR/Cas9-based drive systems in *D. suzukii*
    A. Achieved an efficient means of transgenesis (required to test any gene drive components)
    B. Developed and characterized multiple Cas9 transgenes in *D. suzukii* that are highly functional and enable efficient Cas9-mediated mutagenesis
    C. Developed several ways to efficiently express gRNAs from the *D. suzukii* genome
    D. Developed/optimized several components needed to build Y-gene drive
        1. Identified *D. suzukii* X and Y chromosome regions
        2. Identified putative X chromosome specific target sites
        3. Efficiently engineered the Y chromosome of flies
    E. Developed/optimized several components needed to build Cas9-based suppression gene drive
        1. Identified promising suppression drive candidate target genes
        2. Identified *D. suzukii* homologues of target genes and selected suitable gRNA target sites within these genes
        3. Designed gRNA-expressing transgenes to test ability to target these genes
        4. Built a proof of principle Cas9-based homing system in the *white* gene to test its ability to self-replicate

II. <u>Objective B - Development of a *D. suzukii Medea*-based drive system</u>

A. Finished characterizing and testing previously developed *D. suzukii Medea* drive system

   1. Characterized resistance to this drive system, which could hinder the spread of such a drive

B. Developed a modified version of this same system that should obviate the observed resistance

   1. Currently testing this system; preliminary evidence suggests that it does, as expected, function better that the original *Medea*

C. 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

   1. Currently testing this system

D. Identified several promising putative cargo genes that could be spread with the *Medea* gene drive to cause population suppression

   1. Currently testing these in *D. melanogaster* as proof of principle

RESULTS & DISCUSSION

(A) Development of CRISPR/Cas9-based drive systems

Summary

CRISPR/Cas9 technology has great applicability to the development of genetic pest management approaches, and can be used to build various gene drives - including Y-chromosome drive and Cas9-mediated homing-based drive - that can be employed to suppress and eliminate pest populations. We have made significant progress in developing the tools needed to engineer both of these types of gene drives in *D. suzukii*. Specifically, we have developed and characterized multiple Cas9 transgenes in *D. suzukii* that are highly functional and enable efficient Cas9-mediated mutagenesis in this pest. We have also developed several ways to efficiently express gRNAs from the *D. suzukii* genome. Together, these tools enable efficient CRISPR/Cas9-based manipulations of the *D. suzukii* genome, and provide the basis for building Cas9-based gene drives. Furthermore, we have developed/optimized several components needed to build Y-gene drive, including identifying *D. suzukii* X and Y chromosome regions, identifying putative X chromosome specific target sites, and efficiently engineering the Y chromosome of flies. Additionally, we have also taken steps towards engineering Cas9-based suppression gene drive, including: identifying promising candidate genes to be targeted by this drive; finding *D. suzukii* homologues of, and selected suitable gRNA target sites within, these genes; designing gRNA-expressing transgenes to test our ability to target these genes; and building a proof of principle Cas9-based homing system in the white gene to test its ability to self-replicate. We can now begin putting these components together to generate functional suppression gene drives in *D.suzukii*.

Background

The arrival of CRISPR technologies heralded a new era for traditional genome manipulation and site-specific transgenesis [15,16], and for advanced engineering of target genomes including the construction of gene drives [14,17]. Out of all the types of gene drives that have been proposed, drives based on the CRISPR/Cas9 gene-editing system may be the simplest to build (especially given CRISPR's functionality in many insects [18–26]) and the most effective [11]. Most CRISPR technologies used in insects utilize a simplified two-component system consisting of a *S. pyogenes* Cas9 endonuclease (SpCas9) and a single chimeric guide RNA (gRNA) [27] that can generate DNA double-strand breaks (DSB) in a location of one's choosing. These breaks can them be repaired either randomly (via non-homologous end-joining, NHEJ) or based off a template (via homology-directed repair, HDR) [27,28]. The functionalities of CRISPR/Cas9 systems can be exploited to bring about gene drive-based population suppression.

For example, distortion of the sex ratio in favor of males can lead to a gradual population reduction and eventual elimination of a target population [29–32], and natural so-called meiotic driving Y-chromosomes have been described [33–35]. A system for sex-ratio distortion can also be engineered by designing CRISPR-based transgenes that target the X-chromosome during spermatogenesis [36,37](Figure 1). This Y-gene drive approach would depend on the destruction of X-bearing sperm to produce males that only give rise to male progeny [14,38], and would require the ability to meiotically express an X-chromosome targeting element from the Y-chromosome [36,39]. Importantly, CRISPR/Cas9 technology could straightforwardly be utilized to engineer Y-gene drive elements by designing gRNAs that target only the X chromosome [36,37]. Such a system has already been developed in one species of mosquito [36,40,41], and should be portable to *D. suzukii*.

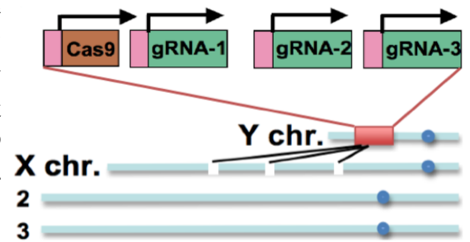

Fig. 1

Another way CRISPR/Cas9 can be utilized to bring about population suppression is via Cas9-mediated homing-based gene drive [14]. This concept is based on the idea of using homing endonuclease genes (HEGs) to manipulate populations [42]. These genes are extraordinarily selfish, and this property can be exploited for both population suppression and replacement. HEGs have the ability "cheat" during meiosis by converting their corresponding allele on the opposite chromosome into an exact copy of themselves, by encoding a sequence-specific endonuclease that severs and disrupts their competing chromosomal allele, which can force the call to use the HEG as a template for homology-directed repair (HDR), resulting in the HEG copying itself (i.e., homing) into its competing allele. If the latter repair option occurs in the germline, or early embryo, then the proportion of offspring that receive the HEG will be above that expected with normal Mendelian transmission (i.e., 50%), allowing for rapid invasion of the HEG into a target population [43]. A HEG can be used to spread a payload gene (replacement drive) or for population suppression and possibly eradication by homing into a target gene, the disruption of which leads to recessive lethality or sterility (Figure 2). In such a suppression approach, homing must be confined to the germline during gamete formation, leading to sterility/non-viability only in homozygotes that receive the HEG allele from both parents. Consequently the HEG can rapidly spread, and once a large fraction of the population is heterozygous, it can cause a population crash as heterozygote pairings will produce sterile/non-viable offspring.

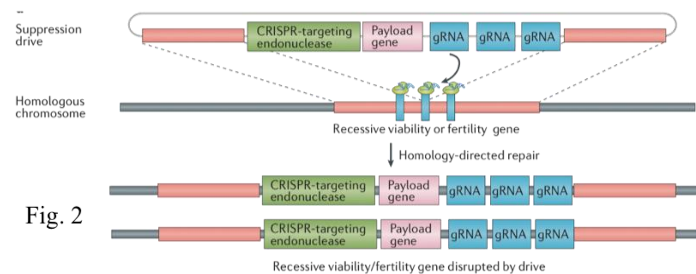

Fig. 2

Although several proof-of-principle studies have shown the utility of HEGs as gene drives prior to the advent of CRISPR/Cas9 (e.g., [44]), this powerful system is enabling the efficient design of homing-based drive systems in many contexts [17]. Several replacement Cas9-mediated homing-based gene drives have been developed [18,45,46]; additionally, several Cas9-based suppression drive systems have recently been engineered in fruit flies [47,48] and one species of mosquito [20,49], and should also be possible to transfer to *D. suzukii*. However, neither this approach nor Y-gene drive have been developed in this pest species.

Results and Future Directions

Efficient Transgenesis in *D. suzukii*

In order to engineer any type of gene drive system in *D. suzukii*, we first have to be able to efficiently generate transgenic flies. Although transgenesis in *D. suzukii* has been previously established [50], it is not very efficient [51], and we had previously struggled with obtaining *D. suzukii* transgenic fly lines. However, a recent work [52] described the generation of a "jumpstarter" *D. suzukii* strain that carries the *transposase* gene necessary for *piggyBac* transposition, and reported that performing germline transformation in this strain dramatically increased transgenesis rates (in some cases 40- to nearly 60-

fold [52]). Since increased rates of transgenesis would help us accelerate our gene drive development efforts, this past year we obtained the USDA/APHIS permits necessary to acquire this transgenic strain from the researchers that developed it, have expanded the obtained stocks into a large colony, and are carrying out all microinjections for transgenesis into this strain. This has been greatly helpful, as we are now able to obtain transgenic lines with much greater efficiency.

Development of Cas9 Tools in *D. suzukii*

The development of both Y-gene drive and Cas9-mediated suppression drive in *D. suzukii* requires functional CRISPR/Cas9 tools in this fly. Although Cas9-mediated genome editing had been previously demonstrated in *D. suzukii* [53], it was carried out by microinjection of gRNAs and Cas9 protein into embryos. Conversely, the building of a gene drive requires a germline source of Cas9 and gRNAs driven by an effective promoter, typically a PolIII promoter such as U6.

Leveraging our experience in designing and optimizing CRISPR/Cas9 tools in *D. melanogaster*, we have generated both of these components. Specifically, we have generated four distinct functional transgenic Cas9 lines, where expression of Cas9 is driven by either strong female germline specific promoters (*BicC* and *Dhd*) or by male and female germline specific promoters (*vasa* and *nanos*) that have been previously validated in *D. melanogaster* [12,46]. We have tested these Cas9 lines, and have shown that all four work, with up to 100% mutagenesis efficiency (for *vasa*-Cas9). We have also generated several functional gRNA-expressing transgenes by targeting the *white* gene, which gives flies a red eye color, as a proof of principle. Specifically, after several failed attempts, we have demonstrated that a genomically encoded, PolIII U6:3 promoter-driven gRNA targeting *white* produces up to 100% mutated (white and mosaic-eyed) progeny when crossed to a Cas9 expressing line (Figure 3). We have also shown that a genomically encoded tRNA-gRNA expression cassette [54], driven by a PolII germline specific promoter, also functions to produce mutated progeny (albeit at a more modest frequency of ~15-30%).

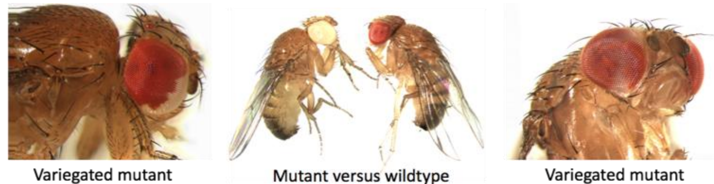

Fig. 3

The development of these tools lays the foundation for the ability to engineer Cas9-based gene drives in *D. suzukii*.

Engineering a Y-gene Drive System

Assuming that efficient CRISPR/Cas9 tools are available, the ability to build a Y-gene drive requires three further components: the ability to identify X and Y chromosomes in *D. suzukii*; the ability to insert large transgenes on the Y-chromosome; and the ability to target and cut sequences only present on the X-chromosome.

Identifying, and inserting genes on, the Y chromosome in D. suzukii

The current genome annotation of *D. suzukii* (http://spottedwingflybase.org) is divided into over 29, 000 contigs (independent fragments that have not been brought together to make a clear linear sequence map of each chromosome), and it is not entirely clear which of these contigs comes from the *D. suzukii* Y and X chromosomes. Therefore, we have used a bioinformatic approach to try to identify fragments of these chromosomes. To do this, we took the entire *D. melanogaster* Y chromosome sequence and carried out a search for related sequences (a BLAST homology search) among the *D. suzukii* contigs; essentially, we looked for regions of *D. suzukii* that were nearly identical to those from the melanogaster Y chromosome, as these are likely to represent *D. suzukii* Y chromosome sequence. We identified a total of 134 contigs that had extremely high homology (E-value = 0) to the *D. melanogaster* Y chromosome. Given this high homology, we are confident that these contigs are pieces of the *D. suzukii* Y chromosome. From this data we have identified several regions of the putative *D.*

suzukii Y chromosome that should be ideal locations for integrating an X chromosome targeting Cas9/gRNA cassette (outside of any known transcribed regions, in unique, non-repetitive DNA).

In order to assay whether we could use CRISPR/Cas9 to dock transgenes on the Y chromosome, we first set out to develop a CRISPR/Cas9-based technique for site-specific engineering of the *D. melanogaster* Y chromosome as a proof of principle [55], as it is much easier and faster to test and troubleshoot components in this species before porting them to *D. suzukii*. To do this, we engineered a vector comprising a fluorescent marker (tdTomato) driven by the eye-specific 3xP3 promoter and flanked by the gypsy and CTCF insulators, with unique restriction sites upstream and downstream for cloning specific homology arms (Figure 4). We then selected ten distinct intergenic regions spanning the Y chromosome for targeting, identified a suitable sgRNA target site in each region, and cloned in homology arms, corresponding to ~800-1,000 base pairs of sequence 5' and 3' of each selected target site, upstream and downstream of the insulator-flanked 3xP3-tdTomato element to generate ten unique Y chromosome targeting transgenes. Each transgene was then injected, along with the appropriate in vitro transcribed sgRNA and Cas9 protein, into a transgenic line expressing a germline source of Cas9 using standard procedures, and G1 progeny were screened for presence of the transgene marker. Two of the injected transgenes inserted in the correct positions on the Y chromosome, demonstrating that we can use the above approach to insert, and detect expression from, a fluorescently marked transgenic cassette at specific locations on the Y-chromosome in *D. melanogaster* using CRISPR/Cas9-mediated HDR.

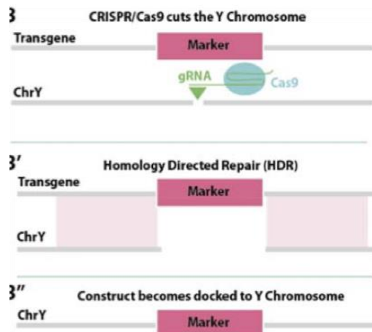

Fig. 4

We are now testing whether we can insert, and detect expression from, Cas9-containing transgenes at these same Y chromosome locations, as we will need to be able to express Cas9 cassettes from the Y in order for the Y gene drive approach to work. Once these experiments are complete, we plan to port this approach to *D. suzukii*.

Identifying and cutting the X chromosome in D. suzukii

We performed a similar bioinformatic analysis to the one described above identify the X chromosome of *D. suzukii*, and identified 388 contigs from *D. suzukii* as being X-linked. Then, to identify potential gRNA sequences specific to the *D. suzukii* X chromosome, and present in multiple copies, we first developed a program to predict all possible Cas9 cleavage sites on the X-chromosome by searching for the PAM motif (XGG in the target sequence N(21)XGG). Once potential X-chromosome cleavage sites were identified, they were aligned to the rest of the genome (all the other non-X contigs) and those that showed a sequence match to these contigs were eliminated. The final output of this program was a conservative list of X chromosome specific Cas9 cleavage sites. From all of this, we conservatively predicted several potential target sequences repeated exclusively on the X chromosome in up to ten locations, making them ideal for the development of guide RNAs to cleave the *D. suzukii* X chromosome. However, our initial attempts at testing these gRNAs for their ability to cut the X did not succeed because, as discussed above, our initial gRNA-expression configuration were not functional. However, now that we have a highly functional gRNA expression configuration, we can proceed to clone X chromosome-targeting gRNAs into our gRNA expression cassettes and test them.

<u>Engineering a Cas9-mediated Suppression Drive System</u>

To engineer a Cas9-mediated suppression homing drive, we need to introduce the coding sequence for Cas9 and gRNA into the genomic site targeted by the Cas9/gRNAs [11] to generate a self-replicating transgene that could continuously mutate a target gene every generation and/or carry a transgene into the population. This self-replicating (i.e., homing) Cas9-based transgene would need to

be placed within a gene necessary for female fertility, so that eventually all of the females in a target population would become sterile and the population would collapse [49].

As described above, we now have working Cas9 and gRNA transgenes that we can utilize as the basis for such a gene drive. After analyzing recent efforts to develop such suppression drive systems in fruit flies [47,48] and mosquitoes [49], we have also identified several promising candidate target genes, including *dsx*, *tra*, *sxl*, and *zpg*, which are conserved in *D. suzukii*. After analyzing the sequences of the *D. suzukii* homologues of these genes to find regions that are highly conserved and thus unlikely to contain sequence variation, we have selected two gRNA target sites within each gene, and have engineered separate U6-driven gRNA transgenes targeting each gene to test whether the selected gRNA sequences will work to efficiently cut the selected targets. (We are currently working on obtaining transgenic lines for these transgenes.) After we verify that the gRNAs work, we will proceed to construct full Cas9-based suppression drive cassettes targeting the most promising candidates (based on gRNA function). In parallel, we are also testing a split Cas9-based gene drive cassette [56] targeting the *white* eye color gene as a proof of principle, to determine whether we can: a). dock transgenes in a site-specific location using CRISPR/Cas9 in *D. suzukii*; and b). observe the efficiency of self-replication/homing of this Cas9-based transgene in *D. suzukii*.

(B) Development of a *D. suzukii Medea*-based drive system

Summary

Previously, we had developed the first *D. suzukii* functional replacement gene drive system termed *Medea*, had rigorously tested it in laboratory cage populations, and had characterized it in different genetic backgrounds to determine effectiveness and fecundity (our results on this project were published in *PNAS* this year [57]). 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 indicated that our *Medea* drive system could spread to fixation if either its fitness costs or toxin resistance were reduced [57], we have developed a modified version of this same system that should obviate the specific resistance that we observed, and have preliminary evidence to suggest that it does, in fact, function better that the original *Medea* we tested. We have also developed a second-generation *Medea* system in *D. suzukii* 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. Finally, we have identified several promising putative cargo genes that could be spread with the *Medea* gene drive to cause population suppression, and are moving forward with testing them in *D. suzukii*.

Background

Medea was first discovered in the flour beetle [58], and multiple versions were later reverse engineered from scratch and shown to act as robust gene drives in the laboratory fruit fly, Drosophila melanogaster [59,60]. Such engineered *Medea* systems rely on a *Medea* element consisting of a toxin-antidote combination (Figure 5). 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.

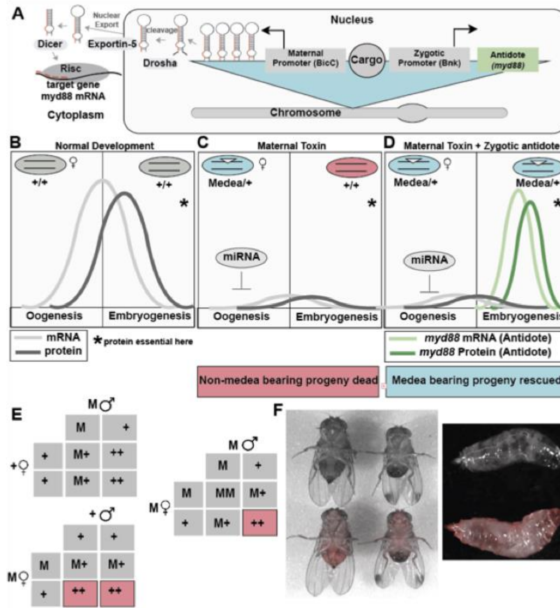

Fig. 5

In the case of *D. suzukii*, since elimination of the pest population is ultimately the goal, an engineered *Medea* system could spread a 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 - a state that allows insects survive periods of adverse conditions such as cold [61]. For example, a *Medea* element can be used to spread a gene conferring sensitivity to a particular chemical that is normally innocuous, rendering such a chemical capable of being used as an environmentally-friendly, species-specific pesticide. Trigger-inducible transcription control elements – ones that turn on expression in the presence of a chemical such as tetracycline or vanillic acid [62,63] – can be engineered to drive expression of an insect-specific toxin (e.g., [64]). A *Medea* element can also be used to spread a gene under the control of a diapause-induced promoter that will splice to produce a toxin in females only, so that, upon the onset of the diapause-inducing environmental cue, all of the females will perish, causing a population crash [59]. Furthermore, a *Medea* element can be utilized to spread a thermally activated TRPA1 cation channel [65] that, upon exposure to a specific threshold temperature, renders flies paralyzed or dead. However, although transgenesis of *D. suzukii* has been established [50], no effective suppression gene drive systems in this major pest have yet been engineered.

Results and Future Directions

Generation of Synthetic *Medea* Gene Drive

To create a synthetic *Medea* gene drive in *D. suzukii*, we engineered a *piggyBac* vector comprising a miRNA toxin coupled with a toxin-resistant antidote, inspired by the architectures used to generate previous *Medea* systems in *D. melanogaster* [66,67]. We designed synthetic miRNAs to target *D. suzukii* myd88, a highly conserved gene shown to be maternally deposited and required for dorsal-ventral patterning in the early embryo in *D. melanogaster* [68]. We used the predicted *D. suzukii* female germline-specific bicoid (BicC) promoter to drive expression of a "toxin" consisting of a polycistronic array of four synthetic microRNAs (miRNAs) each designed to target the 5' untranslated region (UTR) of *D. suzukii* myd88 (Figure 5). Importantly, to ensure these miRNAs could target the desired sequence, we performed genomic DNA sequencing of the myd88 5'UTR target region in our reference *D. suzukii* strain (collected from Corvallis, Oregon) and designed the miRNAs against this sequence. This *Medea* drive also contained an "antidote" consisting of the *D. suzukii* myd88 coding region, insensitive to the miRNAs as it did not contain the miRNA-targeted 5'UTR, driven by the predicted *D. suzukii* early embryo-specific bottleneck (bnk) promoter, and two separate transformation markers – eGFP driven by the eye-specific 3xP3 promoter [69], and dsRed driven by the ubiquitous hr5-IE1 promoter [70].

Characterization of *Medea* Genetic Behavior

Following microinjection of the *Medea* transgene into *D. suzukii* embryos, a single G_1 transformant male was recovered, as identified by ubiquitous hr5-IE1 driven expression of dsRed (Figure 5), and weak eye-specific 3xP3-driven eGFP. When outcrossed to several wildtype (non-*Medea* bearing; +/+) females, this male produced roughly ~50% *Medea*-bearing and ~50% wildtype offspring, as would be expected from standard Mendelian segregation without biased inheritance. Resulting heterozygous G_2 *Medea*-bearing progeny were individually outcrossed to wildtype individuals of the opposite sex to determine inheritance patterns, and these individual outcrosses were continued for six

generations (Table 1). Remarkably, until the G_5 generation, all heterozygous *Medea*/+ mothers (n = 91) produced 100% *Medea*-bearing progeny (n = 1028), while heterozygous *Medea*/+ fathers (n = 16) produced ~50% *Medea*-bearing progeny (n = 268). While the majority of heterozygous *Medea*/+ G_5 (23/31) and G_6 (16/25) generation females also produced 100% *Medea*-bearing progeny, some heterozygous G_5 (8/31), and G_6 (9/25) females unexpectedly produced a small yet notable number (52/1219) of wildtype offspring. Although the exact reason for the difference is unclear, later analysis suggested that resistance to the miRNA toxin might explain this unexpected observation. Notwithstanding, individually these G_5 and G_6 heterozygous *Medea*/+ females displayed significantly biased biased inheritance rates ranging from 76%-96%, with an average rate of 86.4%. Overall, in six generations of individual female outcrosses, the percentage of *Medea*-bearing progeny borne by single heterozygous *Medea*/+ mothers (n = 147) was 97.7% (2195/2247) as opposed to the 50% that would be expected with standard Mendelian segregation, indicating that the *Medea* drive is extremely functional at biasing inheritance.

To further characterize the genetics behind the highly biased inheritance patterns described above, additional crosses between individuals of various *Medea* genotypes were performed, and confirmed that *Medea* exhibits maternal-effect lethality and zygotic rescue. For example, matings between heterozygous *Medea*/+ mothers and wildtype fathers resulted in 55.63±0.76% total embryo survival with 94.20±1.33% of the progeny being *Medea*-bearing, while matings between heterozygous *Medea/*+ mothers and heterozygous *Medea/*+ fathers yielded 79.11±3.95% total embryo survival with 94.12±0.67% of the progeny being *Medea*-bearing. The higher-than-expected embryo survival is consistent with the observation that not all heterozygous *Medea*/+ mothers give rise to 100% *Medea*-bearing progeny, indicating that not all wildtype progeny from a heterozygous *Medea*/+ mother perish.

<u>*Medea* Functionality in Geographically Distinct Populations</u>

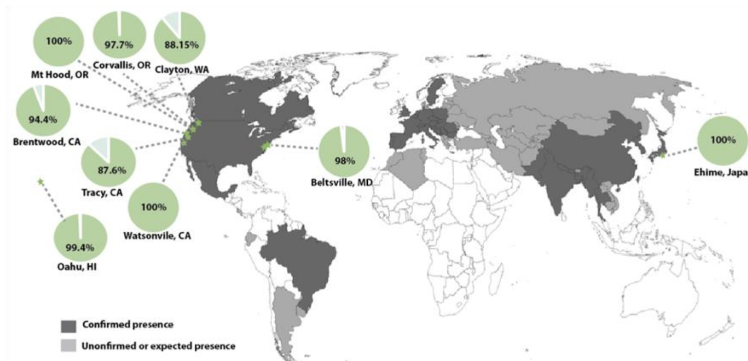

Fig. 6

To assess whether the *D. suzukii Medea* could function in geographically distinct populations that possibly harbor genetic variability in regions that canonically have less conservation such as the 5'UTR, heterozygous *Medea*/+ flies were tested in eight additional *D. suzukii* strain backgrounds. These strains were collected from various locations around the world, including: Mt. Hood, OR; Clayton, WA; Brentwood, CA; Tracy, CA; Watsonville, CA; Oahu, HI; Beltsville, MD; and Ehime, Japan. Interestingly, for 3/8 strains, the *Medea* inheritance rate from heterozygous *Medea*/+ mothers was 100%, while from 5/9 strains the inheritance rate ranged from 87.6% to 99.4%, with an overall transmission rate of 94.2% (Figure 6). These results strongly demonstrate that the *Medea* drive described here can dominantly bias transmission in diverse *D. suzukii* populations.

<u>Long Term Population Cage Experiments</u>

The above observations suggested that *D. suzukii Medea* should be able to drive robust population replacement. To test this prediction, we performed several long term multi-generational population cage experiments specifically challenging the *Medea* drive with a wildtype strain that harbored pre-existing resistance (Corvallis, OR). We set up these population cage studies after maintaining this population for approximately ten generations, we mated *Medea*-bearing fathers to wildtype Corvallis, OR, strain mothers at three distinct introduction (G_0) frequencies: low frequency (25 heterozygous *Medea*/+ and 25 wildtype +/+ males mated to 50 wildtype +/+ virgins, *Medea* allele

frequency of ~12.5% and genotype frequency of ~25%); medium frequency (50 heterozygous *Medea*/+ males mated to 50 wildtype +/+ virgins, *Medea* allele frequency of ~25% and genotype frequency of ~50%); and high frequency (50 homozygous *Medea*/*Medea* males mated to 50 wildtype +/+ virgins, *Medea* allele frequency of ~50% and genotype frequency of ~50%). These experiments were conducted in separate bottles in biological triplicate for the low and medium threshold and quadruplicate for the high threshold drives, producing ten distinct populations with G_1 *Medea* allele frequencies ranging from ~12.5-50% and genotype frequencies ranging from ~25-100%. Altogether, these population cage experiments were followed for 9 generations (for lower allele frequency populations, as the *Medea* allele disappeared from the population by that time) or 19 generations (for higher allele frequency populations), counting the number of *Medea*-bearing adults each generation to determine the genotype frequency, as described previously [60,67]. Interestingly, the observed changes in *Medea* frequency over time indicated that, for release proportions (defined as the genotype frequency in the G_1 population) of 50% or smaller, the *D. suzukii Medea* drive was unable to drive into the wildtype population, likely because of selected drive resistance combined with high fitness costs outweighing the effect of drive. However, at higher release proportions of >90%, similar to classical chromosomal rearrangement thresholds [71], the drive largely compensated for the fitness cost, allowing the gene drive to remain in the population at high frequencies for the duration of the experiment (19 generations). Although unintended, the self-limiting dynamics of the generated *Medea* system may be useful in achieving a transient population transformation of the type associated with other proposed gene drives (e.g.,[72]).

Mathematical Modeling

To characterize the population dynamics observed in the above cage experiments, we fitted a mathematical model to the observed data in which the *Medea* drive had an associated fitness cost in heterozygotes and homozygotes and there was a *Medea*-resistant allele present in the population that reduced toxin efficiency. For the fitted model, the *Medea* drive was estimated to have a toxin efficiency of 93% in individuals homozygous for the resistant allele (95% credible interval (CrI): 90-95%) and was assumed to have a toxin efficiency of 100% in individuals lacking the resistant allele. The *Medea* drive was estimated to confer a large fitness cost on its host - 28% in heterozygotes (95% CrI: 27-30%) and 65% in homozygotes (95% CrI: 62-67%) - and the resistant allele was estimated to have an initial allele frequency of 78% in the population (95% CrI: 57-97%).

Predictive mathematical modeling based on these parameter estimates suggests that the *Medea* drive would spread to fixation in the absence of toxin resistance if released above a threshold frequency of 79%. Spread to fixation would also be expected if the fitness costs of the generated *Medea* drive were halved, even if all individuals in the population were homozygous for the *Medea*-resistant allele, provided the drive was released above a threshold frequency of ~25-27%. Consistent with the experimental results, a *Medea* drive with a large fitness cost in a *Medea*-resistant population is expected to be maintained at high frequencies through its drive; however, its eventual elimination is inevitable unless supplemental releases are carried out. However, for high release frequencies (90-95%), the drive may be maintained at high frequencies (>75%) for ~20 generations, which likely exceeds the duration required for agricultural impact.

Improved *Medea* Construct and Reversal *Medea*

Given our observations regarding resistance and its effect on *Medea* function, we set out to engineer improved *Medea* systems that could reduce the chances of resistance acting as an impediment to spread. Specifically, we 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 have obtained transgenic lines for this improved *Medea* element, and preliminary data indicates that it works better than the original *Medea*, producing 100% inheritance bias. We will continue rigorously testing this second-generation *Medea* element in the coming year.

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 *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. Therefore, this should be of high impact and relevance when it comes to regulators assessing the risk associated with gene drives. We have finished designing and building a Reversal *Medea* system capable of spreading on its own and of replacing the first *Medea* described above, and are in the process of obtaining transgenic *D. suzukii* individuals containing this *Medea* and of rigorously characterizing this system.

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 [65]. 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 [65]. Upon exposure to a critical "threshold" temperature, this cation channel can "open" and modulate Ca2+ and Mg2+ entry into the cell [73]; 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 would like 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 should then be able to spread this temperature-activated "cargo" gene through wild populations by using our *Medea* system during cooler months, and achieve population suppression when the TRPA1 gene is activated in warmer months.

To achieve this, we are working to leverage data from the Montell lab (UCSD), which is developing this technology for mosquito control. The Montell lab is currently testing several TRPA1 channels with different activation temperatures (including rattlesnake TRPA1, python snake TRPA1, boa snake TRPA1 and fruit fly TRPA1) in *D. melanogaster* as a proof of principle, and has preliminary data indicating that at least some of the tested TRPA1 channels, when expressed in the fly brain, work as expected. Once we know which TRPA1 channel appears most promising, we will insert it into our best *Medea* element and begin testing this approach in *D. suzukii*.

EXECUTIVE SUMMARY

Drosophila suzukii is a major invasive pest of many small fruits, and has caused significant damage in agricultural industries of western North America. Control measures have largely relied on prophylactic application of broad spectrum insecticides, which is problematic, as repeated use of insecticides is expensive, has had a serious impact on beneficial arthropods, and makes it inevitable that resistance will arise in the foreseeable future. However, there are no effective alternatives to managing *D. suzukii* infestation, and it is likely that this pest will continue to spread.

An alternative, highly promising approach that could complement existing control methods is genetic pest management, which includes strategies such as gene drive. In particular, engineered *D. suzukii* gene drive strains can be utilized to 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, and can be cheap, since it relies on only a few releases of transgenic insects. A major appeal of this approach is that it is environmentally friendly and entirely insect-specific, and would have no effect on crops or on beneficial organisms. Our objective over the last year, therefore, was to make progress towards engineering *Medea* and Cas9-mediated gene drive systems in *D. suzukii*.

We had previously developed the first *D. suzukii* functional replacement gene drive system termed *Medea*, had rigorously tested it in laboratory cage populations, and had characterized it in different genetic backgrounds to determine effectiveness and fecundity (our results on this project were published in *PNAS* this year). 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 indicated that our *Medea* drive system could spread to fixation if either its fitness costs or toxin resistance were reduced, we have developed a modified version of this same system that should obviate the specific resistance that we observed, and have preliminary evidence to suggest that it does, in fact, function better that the original *Medea* we tested. We have also developed a second-generation *Medea* system in *D. suzukii* 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. Finally, we have identified several promising putative cargo genes that could be spread with the *Medea* gene drive to cause population suppression, and are moving forward with testing them in *D. suzukii*.

We have also made significant progress in developing the tools needed to engineer CRISPR/Cas9 based gene drives (including Y-chromosome drive and Cas9-mediated homing-based drive) in *D. suzukii*. Specifically, we have developed and characterized multiple Cas9 transgenes in *D. suzukii* that are highly functional and enable efficient Cas9-mediated mutagenesis in this pest. We have also developed several ways to efficiently express gRNAs from the *D. suzukii* genome. Together, these tools enable efficient CRISPR/Cas9-based manipulations of the *D. suzukii* genome, and provide the basis for building Cas9-based gene drives. Furthermore, we have developed/optimized several components needed to build Y-gene drive, including identifying *D. suzukii* X and Y chromosome regions, identifying putative X chromosome specific target sites, and efficiently engineering the Y chromosome of flies. Additionally, we have also taken steps towards engineering Cas9-based suppression gene drive, including identifying promising candidate genes to be targeted by this drive, finding *D. suzukii* homologues of and identifying suitable gRNA target sites within these genes, designing gRNA-expressing transgenes to test our ability to target these genes, and building a proof of principle Cas9-based homing system in the white gene to test its ability to self-replicate. We can now begin putting these components together to generate functional suppression gene drives in *D.suzukii*.