Development of Transgenic Sterile Insect Technique Strains for the Invasive Fruit Pest *Drosophila suzukii*

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DECLARATION

I hereby declare that the doctoral thesis entitled "Development of Transgenic Sterile Insect Technique Strains for the Invasive Fruit Pest *Drosophila suzukii*" has been composed by myself with no other resources and aid than quoted. I cofirm that the submitted thesis is my own, except where co-authored publications have been included. My contribution and contribution of the other co-authors has been appropriately and explicitly indicated.

Dedication

To

My Mother, My Father, My Siblings

&

My beloved Wife

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1 Summary

Globalization has contributed enormously to emergence and expansion of invasive pests. A recent example is the invasion in Europe and the USA by the cherry vinegar fly *Drosophila suzukii*. The pest has high potential to invade and establish itself in different climatic zones, the fact that is made clear by looking at the current global distribution map of the fly. The use of pesticide chemicals is so far the first line of defense against it due to lack of reliable alternatives. The use of pesticides against this fly has major problems including possible emergence of insecticide resistance due to the short generation time and number of generations per year. Secondly, the fly lays eggs inside the fruit which make the egg and larvae not exposed to insecticide. Thirdly and most importantly is the time of invasion with regard to ripening and harvest of the fruits. This makes the pesticide choice a very hard one. To be able to fight this onerous pest, the Sterile Insect Technique (SIT) offers an effective, environment friendly genetic pest management that does not interfere with the natural enemies and pollinators.

Here, I propose the use of biotechnological approaches to develop transgenic strains suitable for SIT against *D. suzukii*. In the first part of this study, the use of a CRISPR/Cas9 genome editing system to engineer a sex conversion suppression gene drive in *Drosophila melanogaster* is presented. We found that current designs of homing-based gene drive induce development of resistance against them. We proposed possible solutions to avoid rapid development of resistance and the application of such drive in Tephritid flies where targeting the sex determination gene *transformer* leads to fertile XX males. Second, the hyperactive version of the *piggyBac* transposase was successfully used to improve insect transgenesis with demonstration in three species belonging to two different orders, the genetic model *Drosophila melanogaster*, the global fruit pest *Ceratitis capitat*a and the new beetle model for development and evolution *Tribolium castaneum*. In the third chapter improvement in CRISPR/Cas9 genome editing of the invasive fruit pest *Drosophila suzukii* is presented. Different endogenous regulatory elements were isolated and used to drive the expression of Cas9 and the guide RNAs. In addition, the application of CRISPR/Cas9 to engineer the first sperm marking strain for *D. suzukii* is presented, which represents an important contribution to the establishment of the SIT for this pest. In the fourth part, the φC31 integrase based site-specific germline transformation of the fruit pest *D. suzukii* was established both by integration in one *attP* landing site and by recombinase mediated cassette exchange (RMCE). This provides a platform for testing several enhancers and/or complete system in the same genome context. We also present the development of embryonic and spermatogenesis specific driver lines that can be used for different biotechnological improvements of SIT against *D. suzukii*. The last three chapters present a perspective paper describing a new reproductive sterility

system and the first steps toward its development. It is based on CRISPR/Cas9 chromosome shredding during spermatogenesis that should lead to the same results commonly achieved by ionizing radiation but with no deleterious effect on the males' fitness. This should culminate in embryonic lethality due to chromosomal aberration causing aneuploidy. We first generated several spermatogenesis specific driver lines and Cas9 responder lines to test the suitability of the *tet-off* binary system to control gene expression during spermatogenesis. Suitable genomic targets for chromosome shredding were identified and *gRNAs* to target them were designed.

Genome editing and insect transgenesis tools developed in this study will facilitate further biotechnological improvements of the SIT and its transfer to the invasive agricultural pest *D. suzukii*.

2 Introduction

2.1 The growing population of the world

According to the World Population Prospects 2019 published by the Department of Economic and Social Affairs of the United Nations, the world population has increased by 2 billion since 1994 having reached 7.7 billion in mid-2019. Despite the fact that the growth rate declines, the world population is expected to continue growing to reach 8.5 billion in 2030, 9.7 billion in 2050 and 10.9 billion in 2100 (1). Concomitantly with this growth, the world is faced by global warming, which has among its impacts the increase in the number of insect pests and the severity of infestation (2). One of the major problems that faces mankind at the moment is the production of enough food to feed the growing population of the world (3). Insect pests are said to be responsible for loss of one fifth of the annual total world production of crops. Exotic or invasive pests present higher threat to food production than endogenous ones due to several factors including lack of natural enemies (4,5). A prominent example is the introduction of the Asian spotted stemborer *Chilo partellus* into Africa (6). In a study by Groote et al. (2003) reviewed in (7) they found that the average loss in maize production in Africa oscillates between 20-40%. Maize is considered the staple food in most of Africa. More severe damages were reported in east Africa in Ethiopia where loss in maize production reaches 100% (7) Another classical example of exotic pests though not an insect but worth mentioning is the cassava green mite. It was introduced into Africa in 1971 (8) and by the year 1985 it has spread all over the cassava belt in Africa (9). Crop loss due to infestation reach up to 80% (10). Recently an Asian fruit pest has invaded Europe and the USA and in short time become a global problem for fruits industry (11). It is obvious that global trade and transport play a major role in insect pests' invasion which necessitate strict legislation and quarantine.

2.2 The invasive fruit pest *Drosophila suzukii*

2.2.1 Description and biology

The dipteran fruit pest *Drosophila suzukii* was collected for the first time in Japan in 1916 and described later on by Shounen Matsumura as *Drosophila suzukii* Matsumura (12). The fly belongs to the family Drosophilidae and is commonly known as the cherry vinegar fly and in the USA termed Spotted Wing Drosophila (SWD) due to the fact that the male flies have two dark spots, one on each wing at the distal edge (13). The adult fly is 2-3 mm long with characteristic red eyes, a pale to yellowish brown thorax and black traverse stripes on the abdomen. The female has a prominent serrated ovipositor, which enables the fly to infest intact ripening fruits. The eggs are milky white with two dorsal appendages at the anterior. The larvae are maggot-like, white transparent with black mouthparts. There are three larval instars and the fully-grown larvae can reach up to 3.5 mm long. The pupae are reddish brown and have two stalks at the anterior end with finger-shaped projections. One complete cycle

from egg to adult stage takes from a bit more than a week up to two weeks depending on the temperature (13). In California it has been found that the fly can produce up to 10 generation per year (13). During its life, a single fly has the potential to lay between 200-600 eggs with 1 to 6 eggs a day (13,14)

2.2.2 Invasion and distribution

The fruit pest *D. suzukii* invaded the Hawaiian Islands in 1980 (Kaneshiro 1983) but it took until 2008 that it has been reported in three states of the mainland USA (12,13,15). In the same year, it was detected in Europe, in Italy (12) and Spain (16). Owing to its high fecundity, short generation time, and lack of regulations regarding Drosophila, the fruit pest *D. suzukii* has managed to expand its territories and invade more countries including France, Switzerland (17), Austria (18), the Netherlands (19), Germany (18), Croatia (19), the UK (20), Hungary (21,22), and Turkey (23). In the Americas, the situation is not better than Europe. The fly has been detected in 41 states in the USA just four years after the first invasion in California. It has also been reported in Canada in (13). In South America the fly has established itself in Brazil (24) and recently in Argentina in four localities (25). The fly has also been reported in the middle east in Iran (26), which clearly shows the high potential of the fly to adapt to different climates.

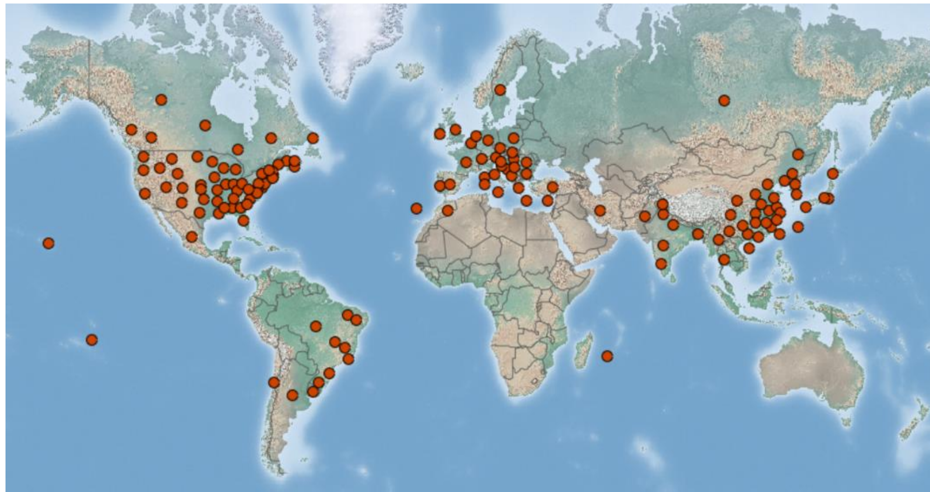

Figure. 1. World Distribution of *D. suzukii* **Source: CABI/EPPO**

2.2.3 Damage and Economic importance

Unlike other *Drosophila* species, the cherry vinegar fly is armed with a prominent serrated ovipositor that enables it to lay its eggs inside intact ripening fruits at the stage of colour changing (27). The hatching larvae cause the main damage by eating the fruits from inside and rendering them unmarketable. The wounds caused by the ovipositor provide entry points for bacteria and fungi that lead to fruit rotting (28,29) Fig. 2.

The fruit pest *D. suzukii* is polyphagous with a broad host range spanning wild fruit plants as well as cultivated crops and non-crop ornamental plants (30,31) which exacerbate the

situation by serving as refuge for the fly during the off-season. Among the affected crops are, blueberry, strawberry, currants, raspberry, blackberry, plums, grapes, as well as stone fruits such as cherries and apricots [lee et al 2015, 19–25] (30,31). A study conducted by Bolda et al (2010) (15) to assess the magnitude of the economic impact of *D. suzukii* infestation in strawberry, blueberry, blackberry, raspberry, and cherry in three US states (California, Oregon and Washington) revealed revenue loss of more than 500 million USD (15). This study however, didn't consider price adjustment due to reduced supply of fruits. The impact of *D. suzukii* in small fruit production in Trentino (Italy) in term of revenue with regard to the five fruit crops strawberry, raspberry, blueberry, blackberry, and cherry was more than 3.3 million Euro (32).

Figure. 2. Consequences of *D. suzukii* infestation on cherry
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2.2.4 Control methods

Insect pests can be controlled by different strategies such as cultural control, chemical control, biological control, or genetic control and a combination thereof applied in the integrated pest management (IPM) strategy. To control the invasive fruit pest *D. suzukii*, growers have tried different strategies. Cost-benefit studies and estimations of revenue losses due to infestations by *D. suzukii* in selected fruit crops gave strong indication that the benefit gained from management overweigh the costs of crop loss when no control measures are deployed (33,34). The use of nets around the crop to prevent infestation of the fruits by the fly proved to be very effective but has high initial cost and is suitable only for small areas (34). Currently most efficient method for the control of the invasive pest *D. suzukii* are pesticide chemicals. Among the groups of insecticides that are tested against the cherry vinegar fly are spinosyns,

organophosphates, pyrethroids, and neonicotinoids (35,36). In a broad screen for effective insecticides, Bruck et al. (2011) (35) tested insecticides belonging to different groups. Effective control against adult *D. suzukii* was obtained by direct application of pyrethroids (bifenthrin, beta-cyfluthrin, permethrin, zeta-cypermethrin), organophosphates (malathion, diazinon), and spinosyns (Spinosad, spinetoram). Spinosad efficiency was later on confirmed in another screen by Cuthbertson et al. (2014) (37).

2.3 The sterile insect technique

The recent years have witnessed increase in the awareness of the adverse effect of chemical control of insect to the environment and the human health. The fact that urged the need for alternative pest control approaches that are environment friendly and ideally species specific. One approach to address this need is the sterile insect technique (SIT), the first genetic control strategy in which reproductively sterile males of the target pest are released to introduce reproductive sterility on the wild population by competition with the wild type fertile males over the wild type fertile females leading to infertile mating and thus population suppression (38). The idea of releasing pest insects to supress the pest population in the field by introduction of reproductive sterility was conceived in the 1930s – 1940s by three independent scientist. Knippling based on his observations that the New World Screwworm mates only once (monogamous), he proposed, if large numbers of the insect can be produced and if the males can be rendered sterile and released in the target area, the local population of the pest will be suppressed (38). He also proposed that, if the treatment area is isolated, successive releases of the sterile insects can result in complete eradication of the pest. Bushland (1960) reviewed in (39) added that even polygamous insect pests can be controlled by means of SIT providing that the sperm produced by the sterile males is used to fertilize the egg (40).

The SIT on practice today encompasses mass production of the target pest or vector, removal of females, sterilization of the males by ionizing radiation, marking, and sustained inundative release of the sterile males in the target area to compete with their wild type counterparts over the wild type females leading to infertile mating and thus population suppression (38,40). Now the SIT is considered an important component of Area-Wide Integrated Pest Management Programs (41). The first and most successful example of integration of SIT into AW_IPM programs was launched in the 1950s against the New World Screwworm in the south eastern USA, where decade long efforts were crowned by the eradication of the insect from USA, Mexico, and Central American including Panama (42). The same approach has also been deployed to eradicate the new world screw warm from North Africa, Libya (43)

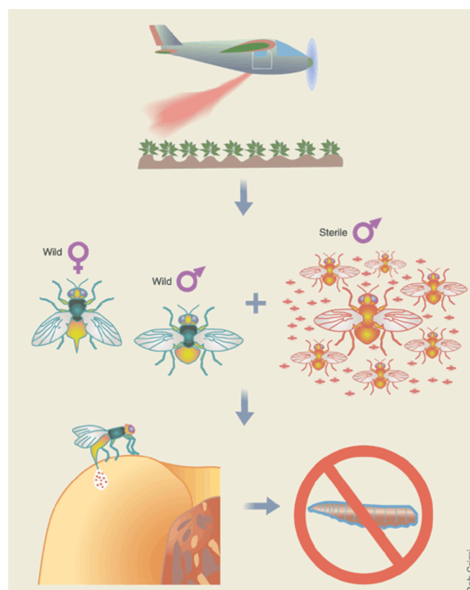

Figure. 3. Illustration of the SIT
Source (Wimmer, 2005)

To be able to establish an SIT program against a particular insect pest, a method of mass rearing should be available. Importantly a method for sex separation (sexing) that ensures production of fit males only is required. Release of female insects even if they are sterile is not accepted especially for insect vectors of disease because sterile females can still transmit the disease (44). Also release of females of some agricultural pests is not accepted, e.g. Tephritid fruit flies have ovipositors by which they pierce and lay their eggs inside intact fruits and damage them (44). Furthermore, release of big numbers of females along with the sterile males compromise the efficiency of the system since the released females will act as competitive mates for the sterile males (45).

So far, the best and most efficient sexing strain develop and deployed worldwide in operational Area wide IPM programs is the so-called Vienna 8 genetic sexing strain (GSS) strain of the Mediterranean fruit fly, *Ceratitis capitata* (46). The strain is based on temperature sensitive lethal (*tsl*) allele and white pupa marker linked together on an autosome and a translocation of the wild type genomic region onto the Y chromosome (47). This allows removal of females during embryonic development by exposing the eggs to 34°C for 24 h (48,49). The stability of the strain and the efficiency of sexing can be regularly checked, since the females develop white pupae whereas males produce wild type brown pupae. Unfortunately, such strains cannot be directly copied in other insect pests and the whole procedure used for generation of Vienna 8 has to be repeated for each new pest species in a hope that a *tsl* allele can be obtained, a wild type copy be translocated to the male sex chromosome, and a linked visible marker be identified for ease of checking. A generic approach for generation of sexing strains so far developed is the transgenic female-specific embryonic lethality which has so far been developed for several insects (50–52). Sexing has also been achieved based on natural variation

between males and females for example in the size of the pupae (53,54) or based on biology such as the difference of the time of emergence of the males and females (Tse-tse flies) (55).

So far, sterilization of males to be released is achieved by ionizing radiation of the pupae. Irradiation induces chromosomal breaks, which lead to reproductive sterility due to chromosomal aberrations causing aneuploidy in the progeny (56). One important feature of ionizing radiation is redundancy in the cause of sterility, which decreases the probability of resistance development (56). However, the use of radiation affects the fitness of the males compared to their wildtype counterparts and therefore males have to be released in big numbers to compete with the wild types for the females. One of the biotechnological improvements of the SIT with regard to sterilization is the conditional transgenic embryonic lethality system which has first been tested in *Drosophila melanogaster* and later on developed for several agricultural pests (57,58). The conditionality of the system is achieved by using the *tet-off* binary system (59,60), whereby an early embryonic promoter is used to drive the expression of a heterologous transcription factor the tetracycline-controlled transactivator (tTA) (57,58), which in turn activates an effector such as a proapoptotic gene that is placed downstream of the *tTA responsive element* (*TRE)*. A double homozygous strain of the two transgenes must be reared in food supplemented with tetracycline (tet) or an analogue to suppress the lethality when tet binds tTA to prevent its binding to the *TRE* and the effector is off. Reproductive sterility has also been induced by the development of a system based on conditional establishment of a positive feedback loop of expression of the tTA leading to a cytotoxic effect. The system is referred to as the release of insects carrying a dominant lethal (RIDL). (61)

It is beneficial to mark the males before release to be able to track and monitor the dynamic and success of the SIT program. So far this has been done using fluorescent dusts to dust the pupae before release and when the males emerge, they get some of that dust on their bodies. This however, beside its bad impact on the health of the facility workers it is also not very efficient. Improvement in this regard was also been done using a transgenic approach, in which a spermatogenesis-specific promoter is used to drive the expression of a fluorescent protein such as the green fluorescent protein EGFP or the red fluorescent protein DsRed (62–65). The system has the advantage over the classical way, that it allows also tracking of the success of the program by random collection of females and examination of their spermatheca (sperm storage organ) for the presence of marked sperm transferred from the released sterile males.

2.4 Insect Transgenesis

The term transgenesis refers to the introduction of foreign DNA into the genome of an organism (66). The ability to generate transgenic animals has contributed tremendously to our understanding of the mechanisms of development and gene regulatory networks (67–69). Transgenic animals have been used over the years in biomedical research to study human

diseases, disease associated alleles, and cancer as well as in basic research to understand diverse biological processes.

To be able to generate transgenic animals, three components have to be secured, first a suitable gene vector to allow integration of the gene of interest in the genome of the organism, secondly, a method to deliver the vector to the primordial germline, and thirdly, a marker to facilitate screening for the transformants (66). Different vectors have been developed to suit the particular need including virus and transposon-based vectors, site specific recombinases, and endonucleases. Insect germline transformation is routinely done using transposon-based vectors with the first demonstration in the genetics model *Drosophila melanogaster* using the *P-element* (68)

At that time transformation markers were based on rescue of visible but viable recessive mutant phenotypes such as the use of mini-*white* gene to rescue the white eye phenotype in the flies (70). The discovery of fluorescence proteins enabled the development of different transformation markers even for non-model insects. The *Drosophila* promoter of the *Ubiquitin* gene fused to *EGFP* was found to drive strong ubiquitous expression in *Drosophila* (71). It has also been used in the medfly *Ceratitis capitata.* The viral promoter of the baculovirus immediate early gene *ie1* has also been used alone and in combination of the baculovirus enhancer sequence *hr1* to drive strong expression of fluorescent proteins (72,73). The best example of a versatile transformation marker is based on the synthetic eye-specific promoter 3XP3 based on the Pax-6 binding site and the basal promoter of the *Dm hsp70* gene (67). It has been used to drive the expression of the fluorescence protein in the eyes of many different insect species (74–77)

2.4.1 Random transposon-mediated germline transformation

Transposable elements or jumping genes were described originally in the late 1940s by McClintock, when she was studying chromosomal breakage in maize and her findings were published in 1950 in a PNAS article entitled 'The origin and behaviour of mutable loci in maize' (78). The discovery of the potential of using transposable elements as gene vectors to stably integrate a transgene into the chromosome of the target organism has revolutionized the field of genetics and allowed detailed studies in functional genetics and genome structure including gene disruption, deletions, translocations etc. Type II DNA transposons are the most commonly used mobile genetic elements in genetic engineering (66). They move between host chromosomes through a 'cut and paste' mechanism facilitated by an enzyme called transposase encoded within the transposable element (79). To use a transposon as a gene vector, plasmids are engineered in a way that the gene of interest is flanked by the transposon terminal inverted repeats (TIRs) and the transposase necessary for transposition is provided in trans commonly from a helper plasmid, on which the transposase coding sequence is fused to a constitutive or inducible promoter such as the heat shock promoter (66).

The *Drosophila* specific *P-element*, is unfortunately limited in functionality to *Drosophila* and closely related species due to the requirement of host-specific factors (80). The most commonly used transposons in insect germline transformation belong to three super-families, the Tc/mariner, the *piggyBac*, and the hAT superfamily. Members of Tc/*mariner* are the most abundant transposons and distributed among different taxa from plants to mammals (81). *Mos1* was discovered in *D. mauritiana* and was used as gene vector in several systems (82,83). Another example of Tc/*mariner* is the *Minos* transposon from *D. hydei*. The hAT superfamily is represented by the *Hermes* transposon from the house fly *Musca domestica*. The best characterized and widely used transposon in genetic engineering is the *piggyBac* vector from the *piggyBac* superfamily. The element was originally identified from a mutant baculovirus strain in a cell culture of the cabbage looper *Trichoplusia ni* (84). Vectors based on *piggyBac* were used to transform many model and non-model organisms. The efficiency of *piggyBac* germline transformation can be improved by the use of mutant hyperactive variants of the transposase enzyme (85).

2.4.2 Site-specific germline transformation

Besides transgenesis by random transposon-based integration, scientists have developed site-specific strategies that allow integration of the transgene into a predefined and characterized docking site. This facilitates testing different transgenes or enhancers at the same chromosomal environment and thus avoid variation in expression due to position effects. The most commonly used systems are the site-specific recombinase systems such as Cre/*lox*, flp/*FRT* and the φC31 integrase system (86–88). In all cases, recombination takes place between a sequence in the genome usually introduced by random transgenesis and a sequence in a donor plasmid along with the transgene to be integrated. The Cre (causes recombination) or cyclization recombinase is a tyrosine site-specific recombinase of 38 KD discovered in the bacteriophage P1 (89). The enzyme recognizes short DNA sequences (*loxP*) and mediates recombination between two identical lox sites (89). Based on the orientation of the two sites, the recombination event results in deletion, inversion or translocation (90–92). The system has been widely used to manipulate transgenic mice, to drive tissue specific ectopic expression, or tissue specific knockdown. In insects, it has been used as site-specific germline transformation tool, as well as for functional genetic studies by ectopic tissue specific expression of genes or by tissue specific knockout. Another example of a tyrosine site-specific recombinase is the Flp/*FRT* system of the 2μ plasmid which is similar to Cre/*lox*, as the flipase enzyme mediates recombination between two identical flipase recognition targets (*FRT*). The system has been extensively used in insects. Especially in *Drosophila* (87,93,94) it has also been used as a tool for site-specific germline transformation only by RMCE due to reversibility of the recombination reaction and the kinetic barrier that favour the excision, the two system Flp/FRT and Cre/lox function only by RMCE (95).

A third but distinct example of site-specific recombinases is the φC31 integrase which is derived from the bacteriophage φC31(96). It is a serine site-specific recombinase that mediates recombination between two different DNA sequences called attachment sites (*att*) one in the bacteriophage genome (*attP*) that facilitates its integration into the bacterial chromosome by recombination with the bacterial attachment site (*attB*) (97). The advantage of this system over the Cre/*lox* and the Flp/*FRT* systems is that the recombination is unidirectional and therefore the integration is stable (98). This system was used in many model and non-model insects as a tool for site-specific germline transformation by simple integration or by recombinase mediated cassette exchange (99). In the later, case two reciprocal recombination events occur between two *attP* sites integrated in the genome and two *attB* sites in the donor plasmid.

2.4.3 Genome editing

The ability to manipulate the genome of organisms is of paramount importance both in basic as well as in applied research. especially in the field of gene therapy and biotechnology. Traditionally, chemical and transposon-mediated random mutagenesis was used in forward genetics to induce mutations in the genome followed by intensive screening for phenotypes of interest and more work to identify the molecular basis of the phenotype (100–103). These approaches, despite being time consuming and labour intensive, have served the community for many years in the pre-genomic era. One of the drawbacks of these methods is the lack of specificity which is often accompanied by undesired changes in the genome. In recent years, with next generation sequencing being affordable and the genome of many non-model organisms being sequenced, huge genomic data are being generated and made available to researchers through public databases. To study the function of these genomic information, tools for reverse genetics are required especially for targeted gene mutagenesis. Homologous recombination-based gene targeting has been used, however, with very low efficiency (104). Two discoveries have prompted the development of site-specific genome editing tools: (i) The discovery that a double strand break (DSB) in the targeted gene increases homologous recombination by at least 3 orders of magnitude (105,106); and (ii) that in the absence of a homologous repair template, the DSB is repaired by the error-prone Non-Homologous End Joining (NHEJ) mechanism (107).

In functional genetic analysis, the induction of a site specific DSB in the gene of interest (GOI) is enough to obtain *loss-of-function* mutations, since the repair by NHEJ leads to insertions or deletions (indels) of a few nucleotides, which result in frameshifts and/or premature stop codons (107). It is important in this approach to target close to the translation start codon, however, not too close, since an alternative translation start may be used. Alternatively, a repair template may be provided that has the modification of interest flanked by homology arms to facilitate homology dependent repair (HDR) of the DSB (108). The later approach allows precise modification to be introduced down to the resolution of a single nucleotide

exchange. Among the endonucleases that were used until recently are the rear cutting restriction endonucleases such as the yeast meganuclease *I-SceI* (109). More recently, synthetic zinc finger nucleases have gained popularity among scientists and were adopted for many systems including many insects (110). In 2011, it has been chosen by Nature Methods as the ''Method of the Year''. It is a programable endonuclease generated by fusion of Zinc-Finger DNA binding domains to the catalytic domain of the restriction enzyme *Fok*I (110). The TALENs, are another programable endonucleases based on the transcription activator-like effector TALE, a protein that is secreted by the plant pathogenic bacteria of the genus *Xanthomonas* during infection (111). They are engineered by fusion of the TAL effector DNA binding domain to the cleave domain of the restriction enzyme *Fok*I (111). One of the advantages of TALENs over ZFNs is that it can be programmed to target any part of the genome (112). The discovery of the bacterial adaptive immune system (113,114) called CRISPR/Cas9 and its adoption as a tool for genome engineering has opened many doors that were not possible otherwise (115). The main advantage of this system over TALENs and ZFN, is the very low cost and the ease of programming of Cas9 to target any part of the genome (115). The system consists of the Cas9 endonuclease and two small RNAs called *CRISPR-RNA* (*crRNA*) which dictates the specificity of Cas9 by means of 17-20 nucleotides, and *trans-activator RNA* (*tracrRNA*) which is involved in processing and maturation of crRNA and facilitates the interaction of crRNA with Cas9 (116). Scientists have engineering the two small RNAs into one chimeric guide RNA (*gRNA*) to simplify the delivery (117). A very important feature of Cas9 target sites is the PAM sequence, an acronym for Protospacer Adjacent Motif (118). This sequence varies for different Cas9 enzymes. However, for the most commonly used version, *SpCas9*, which was isolated from the bacteria *Streptococcus pyogenes*, the PAM is NGG where N is any of the four different nucleotides (118). The crystal structure of the Cas9 complex with *gRNA* and a PAM-containing DNA target revealed that the PAM sequence remains in a base-paired duplex. The PAM GG in the target strand (non-complementary) is scanned via the major groove by a conserved arginine residue in the PAM interacting domain of Cas9 at the C-terminus. Interaction of the PAM-Domain with the PAM through the minor groove put the target DNA strand in the right orientation for base pairing. This recognition and interaction lead to local strand destabilization and un-winding. This clearly explains the importance of the PAM sequence (118).

The CRISPR/Cas9 system has been rapidly adopted and established for many organisms from yeast to human as a tool for genome editing, functional genetic analysis by gene knockout and targeted mutagenesis (117,119–121) or by activation and repression of gene expression (122–125) and even modification of epigenetic imprinting by methylation or demethylation (126,127)

To achieve all the mentioned functions, the Cas9 molecule itself has been engineered. For example, a dead Cas9 (dCas9) that can still recognize and bind the genomic target was

engineered by two amino acids substitutions D10A and H840A inactivating the two nuclease domains RuvC and HNH, respectively (122,125). This dCas9 was then utilized to create heterologous transcription factors (TF) by fusion of activation domains from natural transcription factors to the N or C-terminus of dCas9 and thereby creating a programable TF that can be targeted to enhancers of genes of interest (122,124,125). It has also been engineered by fusion of enzymatic effector domains to dCas9 to induce targeted epigenetic reprograming for example methylation, demethylation, or histone acetylation (128). Furthermore, a nickase Cas9 (nCas9) was generated with only one nuclease domain inactivated. This has been exploited for genome editing by homology directed repair, targeted mutagenesis, and importantly as a scaffold to fuse more functional domains (129–131). With first demonstration in yeast, cultured mammalian cells, and plants, Cas9-deaminase was generated by fusion of cytidine deaminases such as APOBEC1 or AID to nCas9 or dCas9 (132–136) and it has been shown to efficiently substitute a C>T at the target site.

In applied research using CRISPR/Cas in gene therapy, the search for more smaller and highly precise versions of Cas9 is ongoing. Another CRISPR/Cas system that is gaining popularity is Cas12a due to its slightly smaller size, different PAM requirement and the ability to process an array of *gRNAs* from a single transcript without the need for extra factors (137,137). In applied insect biotechnology the system has been used or proposed to be used to develop new pest control strategies such as gene drive (138–140), and X chromosome shredder (141).

2.5 Gene Drive

Some naturally occurring genes tend to be inherited in a super-mendelian fashion, which means more often than is predicted by Mendelian segregation (142,143). Such genes are referred to collectively as selfish genetic elements. Homing Endonuclease Genes (HEG) are selfish genetic elements that reside in the chromosome of their host organism and encode an enzyme, which recognizes and cleaves a sequence in the sister chromosome and thus get themselves copied by the cell DNA repair machinery, namely by homology dependent repair in a process called homing (144). The idea has incited scientist to attempt to engineer these genes into 'gene drive' to manipulate natural population of insect pests and diseases vectors by introducing desired traits or fitness costs. Austin Burt suggested three criteria to be met for an engineered HEG to be used for pest control: (i) HEGs should be engineered in a way that they recognize and cut a sequence in an essential gene and get copied in the middle of its recognition sequence and thus disrupt the function of the gene. (ii) The knockout of the target gene should be recessive with minimal phenotypic effect in the heterozygous situation and deleterious in the homozygous individuals. (iii) The engineered HEG is driven by a meiosis-specific promoter to ensure normal development of the heterozygous zygote that will transmit the HEG in a biased fashion to its gametes (145).

The discovery of the CRISPR/Cas9 system as a programmable tool to introduce site-specific DSBs in the genome opened the door for scientists to realize the long-standing dream of engineering complete natural populations of insects of agricultural and medical importance. The advantage of this system over natural HEG is the ease of programming Cas9 to target any part of the genome simply by designing 20bp of the *gRNA* (115). The repertoire of genomic targets is increasing by the discovery of new *Cas9* systems such as *Cpf1* (137,146) and by the engineering of the old ones (147,148). The first demonstration that CRISPR/Cas9 can be used to transform heterozygous mutations into homozygous with high efficiency in what is called the mutagenic chain reaction (MCR) has put the cornerstone for the first CRISPR/Cas9-based gene drive (140). The simplest CRISPR/Cas-based gene drive cassette consists of the *Cas9* endonuclease coding sequence under a meiosis-specific promoter, and the custom *gRNA* driven by a promoter of an *RNA polIII* gene such as the *U6 small nuclear RNA* gene flanked by flaking sequences of the *gRNA*-targeted sequence A few months after the MCR was published, the same group has reported on the use of CRISPRCas9 to engineer gene drive for manipulation of the malaria mosquitoes *Anopheles stephensi* (138). Their results showed that for such a gene drive to function its activity has to be restricted to the germline otherwise, many mutant alleles lacking the drive can form. A second study demonstrated the feasibility of using CRISPRCas9 to build suppression gene drive by targeting three female fertility genes with homozygous sterile phenotype (139). Those two studies were then followed by several attempts to address issue such as containment of the drive and improvement of CRISPR/Cas9 design. A simple homing-based gene drive that doesn't impose fitness cost on the carrier should presumably be able to take over the whole population over many generations Fig. 4.

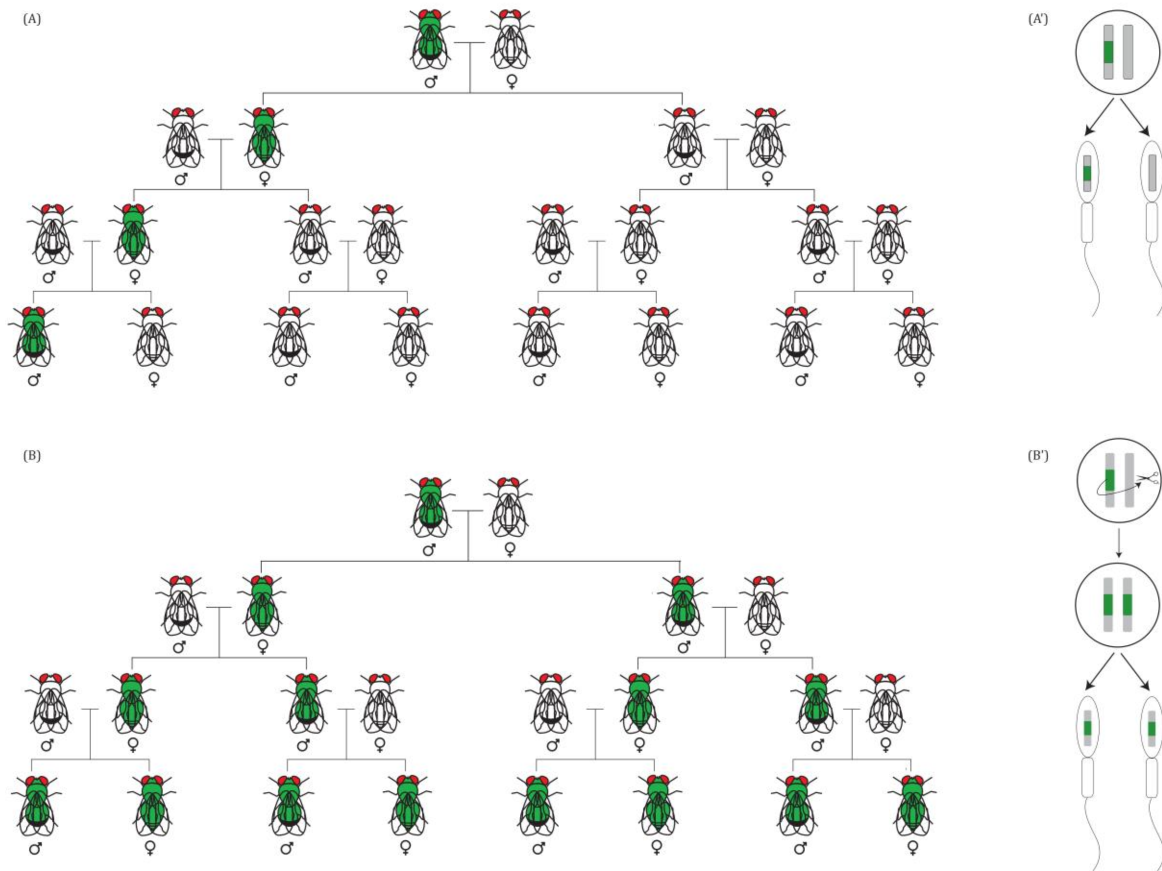

Figure. 4. Mendelian inheritance versus hypothetical gene drive. (A) Show Mendelian inheritance of a single dominant allele of a gene that produce green flies, abbreviated hereinafter as '*gr*'. Starting with a heterozygous male for *gr*, and assuming no advantage or fitness cost associated with this allele, half of the progeny will inherit *gr*. If only a few alleles of *gr* are introduced into the population, over the time the allele will be diluted and lost. (A') A simplified depiction of the cytogenetic basis of the observed ratio. During spermatogenesis, half of the sperm produced should carry the *gr* allele whereas the other half not. (B) if *gr* is linked to a homing-based gene drive, and no fitness cost is associated with the drive cassette, all the progeny from a heterozygous male will develop into green flies and the process will continue until the drive takes over the population. (B´) explains the homing process, the drive cassette contains a homing endonuclease gene driven by a male meiosis promoter and is placed in the middle of its target sequence. Starting with a heterozygous male, during meiosis, the HEG will target the homologous chromosome, induce DSB and the cell copies it to that chromosome creating a homozygous situation. This should theoretically lead to 100% of the sperm producedcarry the *gr* allele.

2.6 The main aims of the study

I. Explore the possibility of using Cas9-based gene drive for population suppression and possible challenges and pitfalls.
II. Establish and/or improve transgenesis and genome editing tools for *D. suzukii*.
III. Develop new reproductive sterility systems to establish the SIT against *D. suzukii*

2.7 Specific objectives:

The emergence of CRISPR/Cas9 as a genome editing tool has provoked scientists to build Cas9-based synthetic gene drives as pests' and vectors' control approach. However, we still don't know the suitability of Cas9 to build such systems and the problems that might arise. To address that we set the following specific objectives forth:

i. Generation of Cas9-based gene drive targeting *D. melanogaster transformer* (*tra*) gene
ii. Study the dynamic of the drive over several generations.
iii. Study the effect of emergence of resistance alleles on the drive efficiency.

The second aim of the study can be broken down into the following specific objectives:

i. Improvement in *piggyBac* germline transformation.
ii. Improvement of CRISPR/Cas9 genome editing system in *D suzukii*.
iii. Establishment of φC31 site-specific recombination and RMCE in *D suzukii*.
iv. Generation of sperm-marking system for *D. suzukii*.

The third aim is to develop conditional reproductive sterility systems based on CRISPR/Cas9 or CRISPR/Cas9 and RNA interference. The first system relies on Cas9 and RNAi to target and knockout/down spermatogenesis-specific paternal effect genes involved in fertilization, which should lead to production of viable males that produce sperm and transfer it successfully to the females, and the sperm should be able to enter the egg but should fail to initiate embryogenesis. Specifically, the following will be done:

i. Identification and validation of CRISPR targets in the paternal effect gene *sneaky* (*snky*)
ii. Design of *shRNAs* to target and knockdown *Dm snky*.

The second system relies on Cas9 targeting abundant sequences in the genome of the fly leading to chromosome shredding during spermatogenesis. This should lead to embryonic lethality due to aneuploidy in the embryo. This is similar to the action of radiation but specific to spermatogenesis. As a proof-of-concept we aim to establish the system in *D. melanogaster*.

i. Identification of abundant CRISPR-targets in *D. melanogaster* transposable elements
ii. Use of the *tet-off* system during spermatogenesis.

3 Results

Results are presented in seven chapters 3.1 – 3.7. Each chapter is a manuscript which is either published, submitted or work is on going. Each of which is preceded by a one-page describing the following:

- The main objective of the manuscript in the context of the whole thesis.
- Contribution of the authors in the practical work.
- The status of the manuscript.

References cited in each manuscript are included at the end of the respective chapter.

References cited in the general introduction and discussion are at the end of the thesis in chapter 5

3.1 Consequences of resistance evolution in a Cas9-based sex conversion-suppression gene drive for insect pest management

This chapter discusses the use of a Cas9-based synthetic gene drive for insect population suppression and the observed rapid emergence of resistance. The vinegar fly *D. melanogaster* offers a safe model.to study the emergence and dynamics of resistance development against a sex conversion gene drive, since sex conversion in *Drosophila* by disruption of the female sex determination pathway leads to the production of sterile intersexes due to the discordance between the entity of the soma and the karyotype of the germline. This therefore presents a barrier against an unintentional introgression of the gene drive cassette into a wild population. We further go to discuss and provide a model for the use of such a system in the cosmopolitan fruit pest *Ceratitis capitata* in which concordance between the somatic cell identity and that of the germline is not necessary. Therefore, gene drive targeting the *transformer* gene, which is responsible for the establishment of the program to produce females, leads to production of viable and fertile XX males. This chapter thus presents an important advancement in understanding CRISPR/Cas9-based homing element gene drives as well as their problems in the application for the control of agricultural pests and proposes a solution to avoid development of resistance.

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Authors contributions as stated in the published paper:
M.K., H.M.S.C., S.D., J.M.M., and E.A.W. designed research; M.K., K.N.E., **H.M.M.A.**, H.M.S.C., and J.M.M. performed research; M.K., K.N.E., **H.M.M.A.**, H.M.S.C., S.D., J.M.M., and E.A.W. analyzed data; M.K., K.N.E., J.M.M., and E.A.W. wrote the paper; and H.M.S.C. and J.M.M. designed and analyzed the population dynamic models.

My specific contributions were particularly to Fig. S1D, Fig. S3, and Fig. S5 leading to main Fig. 2.

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Consequences of resistance evolution in a Cas9–based sex conversion–suppression gene drive for insect pest management

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The use of a site-specific homing-based gene drive for insect pest control has long been discussed, but the easy design of such systems has become possible only with the recent establishment of CRISPR/Cas9 technology. In this respect, novel targets for insect pest management are provided by new discoveries regarding sex determination. Here, we present a model for a suppression gene drive designed to cause an all-male population collapse in an agricultural pest insect. To evaluate the molecular details of such a sex conversion-based suppression gene drive experimentally, we implemented this strategy in *Drosophila melanogaster* to serve as a safe model organism. We generated a Cas9-based homing gene-drive element targeting the *transformer* gene and showed its high efficiency for sex conversion from females to males. However, non-homologous end joining increased the rate of mutagenesis at the target site, which resulted in the emergence of drive-resistant alleles and therefore curbed the gene drive. This confirms previous studies that simple homing CRISPR/Cas9 gene-drive designs will be ineffective. Nevertheless, by performing population dynamics simulations using the parameters we obtained in *D. melanogaster* and by adjusting the model for the agricultural pest *Ceratitis capitata*, we were able to identify adequate modifications that could be successfully applied for the management of wild Mediterranean fruit fly populations using our proposed sex conversion-based suppressiongene-drive strategy.

Background

The use of CRISPR-Cas9 systems as a homing-based gene-drive tool to alter the genotype of insect populations has theoretically (1–5) and practically (6–8) been shown to be feasible. These systems can potentially allow the spread of any desired trait in a wild population of target species even if the desired phenotype imposes a fitness cost (2, 4, 5, 8). Therefore, the spread of lethality or sterility traits that could result in suppression and eventually collapse of the target population should be possible. This has recently attracted special attention in pest and disease vector control (1,3,6–8). However, the effort had focused mainly on disease-vector mosquitoes such as Anopheles (7, 8). In homing CRISPR/Cas9 gene-drive (HCGD) systems, a CRISPR/Cas9 homing element (CHE) composed of at least the Cas9 endonuclease-coding sequence and a guide RNA (gRNA) is integrated in the host genome at the gRNA target site. In the heterozygous state, Cas9 introduces an RNA-guided double-strand break in the wild-type allele (similar to homing endonucleases) which then will be repaired either by homology-directed repair (HDR) or error-prone mechanisms such as non-homologous end joining (NHEJ). In the former case, the CHE allele serves as the repair template and is copied into the homologous chromosome. Directing this process to the germline will result in super-Mendelian inheritance driving the CHE and any accompanying genes into the population. Therefore, the highly customizable nature of CRISPR/Cas9 allows simple design of HCGDs to drive any desired trait, even those resulting in sterility, into wild populations as long as the cost of this phenotype does not surpass a certain threshold (1, 4).

In a recent study, Hammond et al. (8) identified a set of genes whose knockout resulted in female-specific sterility in Anopheles. However, they found that only one of these genes could be used as a target for HCGDs to achieve an efficient drive of female-specific sterility into the population. The remaining sterility genes imposed a very strong cost on the carriers that eventually resulted in the elimination of the drive allele from the population. As predicted by mathematical population genetics models, the spread of female-specific sterility traits in a population using HCGDs should eventually result in a population collapse and local or global elimination of the target species (1, 8). Another proposed strategy to achieve this goal is to design drive elements that alter the population's sex ratio toward males. Surprisingly, such gene-drive elements have naturally been observed in some organisms. In *Aedes aegypti*, for example, a type of drive element known as a "Killer-Y chromosome" is able to shatter the X chromosome during spermatogenesis, and therefore all offspring of mosquitoes carrying such a chromosome will be male. To replicate this phenomenon, Galizi et al. (9) employed a specific homing endonuclease, I-PpoI, to specifically shatter the X chromosome during spermatogenesis of *Anopheles gambiae*. By generating transgenic males carrying an engineered version of such a homing endonuclease gene (HEG) on somatic chromosomes, they have shown that at high initial load frequencies these flies will result in population

collapse in cage experiments. They proposed that integration of such a HEG on the Y chromosome could be an effective gene-drive strategy for population control of *An. gambiae*. The distortion of the sex ratio using an X chromosome-specific CRISPR/Cas9 system has also been shown to be successful in *An. gambiae* (10).

Here, we propose an independent approach that converts female individuals into fertile males by disturbing the developmental sex-determination pathways, which distorts the sex ratio without adverse effects on the reproductive success of carrier males. A prime target gene to achieve this goal is *transformer* (*tra*). *Tra* plays a pivotal role in female sex determination in different insect orders, including Diptera (11). In a devastating agricultural fruit pest, the Mediterranean fruit fly, *Ceratitis capitata* ("medfly"), *tra*-knockdown XY males develop normally, while XX individuals develop as fertile males (12). Therefore, *C. capitata* XX males carrying a CHE-targeted *tra* locus could further spread the CHE to all their progeny (Fig. 1A), resulting in an effective gene drive without any direct effect on the fecundity of individuals carrying the drive element. This in theory could lead to an all-male population collapse that can be used for controlling the wild population of this aggressive pest.

Because of the strict guidelines on gene-drive experiments and to adhere to recommendations of scientific communities (13–15), we decided to test this gene-drive strategy first using *Drosophila melanogaster* as a model organism. In *D. melanogaster*, *tra*-mutant XX individuals develop into infertile pseudomales (16), not giving rise to further progeny (Fig. 1B). Since the cost of this infertility is significantly higher than the threshold tolerated by gene-drive systems (1, 4), a CHE targeting the tra locus in *D. melanogaster*, despite its ability to show super-Mendelian inheritance in individual crosses, is not able to drive into a population (Fig. 1C and D). This biological confinement allows us to employ *D. melanogaster* as a safe model organism for studying the limitations of our suggested suppression gene-drive systems at the molecular level in the laboratory and thereby experimentally identify parameters that might need to be adjusted to achieve an efficient suppression gene-drive system in *C. capitata*.

In our study, we found that targeting *tra* works as an efficient means of sex conversion in *D. melanogaster*. However, the early onset of the formation of in-frame drive-resistant alleles com-promises drive efficiency. Based on our observations, we simulated the use of a *tra*-based suppression gene-drive system for control of *C. capitata* populations and showed that HCGD systems employing multiple gRNAs that target the *tra* locus can serve as an effective pest-control strategy for *C. capitata*.

Results

Design of a *tra*-Based Sex Conversion-Suppression Gene-Drive System.

The proposed CHE is composed of an *spCas9*-coding sequence under the control of a suitable promoter, as explained below, a gRNA targeting the first exon of *tra* under the control of a

PolIII promoter, and a fluorescent marker to identify the genomic integration (SI Appendix, Fig. S1C). The activity of this CHE unit will be similar to that of homing endonucleases and would be able to perform homing into the wild-type *tra* allele. For our *tra*-targeting CHE to drive in a population, it is essential that Cas9 is expressed in the germ cells to promote homing into the wild-type *tra* allele by HDR. To achieve sex conversion, however, *tra* needs to be inactivated in the somatic cells of XX individuals. Thus, two scenarios in XX individuals heterozygous for the drive allele are plausible: (i) Cas9 protein is expressed only in a fraction of the cells, and its activity results in the development of mosaic intersex individuals or (ii) Cas9 is expressed in all somatic cells and uniformly destroys the wild-type *tra* allele, resulting in the development of XX males. In *C. capitata*, the latter will result in development of fertile XX males (12), which can further spread the drive allele into the population (Fig. 1A). It is important to note that it is irrelevant whether the mutation of the wild-type tra allele in the somatic cells is based on HDR or NHEJ as long as the mutation disrupts the function of *tra* and thereby causes sex conversion.

Therefore, the combination of germline homing at the tra locus (which results in the spread of the drive allele) and somatic targeting of the wild-type tra allele (which results in sex conversion) is needed to enable our proposed suppression gene-drive strategy to be effective. To achieve this, different types of promoters or combinations thereof could be used. (i) A germline-specific pro-moter could be combined with an early zygotic promoter from a cellularization gene for high and ubiquitous blastoderm expression (17). Such cellularization promoters have already been success-fully applied for transgenic approaches in *C. capitata* (18). It is important to note that these early cellularization genes are not expressed in the primordial germ cell (PGC) nuclei (19, 20), which are therefore not exposed to NHEJ-based mutation in the early embryo (21, 22). In *D. melanogaster*, one of these cellularization genes, *Sry-α*, is in fact expressed both in a somatically limited way in the blastoderm and in the PGCs at later developmental stages (23, 24), and its promoter therefore might be sufficient for both germline homing and somatic sex conversion. (ii) Since PolII-dependent transcription is actively suppressed in the PGCs (25), a ubiquitous cell cycle-specific promoter, such as the *DNApol-α180* promoter (26), could result in uniform targeting of all cells during development except early-stage PGCs. The paternal-only transmission of our proposed gene-drive strategy is likely to help overcome the problem of DNA cleavage at early embryonic stages when HDR is unlikely to occur (21, 22) and therefore is expected to result in both uniform sex conversion and germline homing. (iii) Since the target gene *tra* is expressed in the somatic cells at very early embryonic stages, the genomic context might mediate suitable amounts of expression independently of the introduced promoter. Thus, the introduction of a germline-specific promoter, such as the *Rcd-1r* promoter, which had previously been shown to result in efficient homing-based gene drive in *D. melanogaster* (27), might by itself be sufficient to drive Cas9 expression for both purposes.

D. melanogaster as a Safe Model System for Evaluation of a *tra*-Based Suppression Gene Drive.

In our experiments, we followed the recommended physical containment procedures (15) (SI Ap-pendix, SI Materials and Methods). Moreover, since in *D. melanogaster* XX males are always sterile, the somatic sex conversion imposes a strong fitness cost on the XX individuals carrying the drive allele, which impedes the spread of the drive allele in the population (1, 4), rendering *D. melanogastera* safe model system to study this suppression gene-drive strategy at the molecular level (Fig. 1B). Nevertheless, to ensure that the use of a CHE against the *tra* locus in *D. melanogaster* is indeed biologically confined in case of an unlikely accidental escape, a deterministic model for an ideal scenario (homing efficiency of 90% and assuming that one-third of NHEJ events result in the formation of in-frame indels) based on predicted phenotypic outcomes of the drive in *D. melanogaster* was used. The modeling graphs demonstrate that, because of its high fitness cost, even at 90% initial frequency a CHE targeting the *tra* locus not only is unable to drive into a population but also is actively eliminated from the population (Fig. 1C). In this example, the presence of the drive allele at high frequencies may result in the generation of cleavage-resistant alleles, which theoretically could alter the genetic makeup of the population at the targeted locus (28). However, our results indicate that at the low release frequencies (<1%) that are expected in case of an accidental release, the drive allele becomes eliminated from the population at very early stages without any significant effect on the wild population (Fig. 1D). Therefore, it is safe to assume that such a drive system is biologically confined in D. melanogaster and thus meets the recommendations for gene-drive experiments (13–15, 28).

Implementation of the *tra*-Based Suppression Gene-Drive System in *D. melanogaster*.

Since our sex conversion-based gene-drive system requires both somatic and germline Cas9 activity, we tested three different promoters (SI Appendix, Fig. S1): (i) the *Sry-α* promoter, (ii) the *DNApol-α180* promoter, and (iii) the *Rcd-1r* promoter. We also included the 3′UTR of the *β2 Tubulin* (*βTub85D*) gene at the 3′end of the Cas9 transcript, as it had been shown to increase the homing efficiency in *D. melanogaster* (27). Moreover, the first intron of *αTub84B* was inserted upstream of the *Cas9* coding sequence to further enhance *Cas9* expression (29).

To allow the simple generation of various strains in an isogenic background for these promoters, we used a transgenesis approach similar to that demonstrated in *Anopheles* (SI Appendix, Fig. S2) (8). First, a *tra*^{nDOCK} strain was established by site-specific integration of a recombinase-mediated cassette exchange (RMCE) docking site into the first exon of the *tra* gene using an efficient *gRNA* (SI Appendix, Fig. S1 A and B). Second, togenerate the homing strains for each of the promoters, RMCE was performed in *tra*^{nDOCK} embryos using φC31

integrase. All individuals that carried the CHE allele (*tra*^{nCHE}) were found either to be males or to show a mosaic intersex phenotype, indicating that targeting the *tra* locus is indeed an efficient sex-conversion strategy in *D. melanogaster* (SI Appendix, Fig. S3A).

To assess the efficiency of each promoter in performing gene drive as well as inducing somatic sex conversion, 10 males from each of the *tra*^{nCHE} strains and the *tra*^{nDOCK} strain were individually crossed with *w*–virgins, and the ratio of females in the F1 generation from each single cross was determined (SI Ap-pendix, Fig. S1D). The results show that all three promoters can block female development of heterozygous (*tra*$^{nCHE/+}$) XX in-dividuals (somatic sex conversion) and drive into the next generation (germline activity). However, since HDR in the germline is of key importance for the molecular study of gene drive, we continued our experiments with the *Rcd1r* strain.

To evaluate the drive efficiency of this CHE and the rate at which the *tra* locus is targeted to cause sex conversion, 12 heterozygous (*tra*$^{nCHE/+}$) males were crossed individually with virgin *w*–flies. Screening the F1 progeny revealed that up to 92% of theindividuals carried the *DsRed* eye marker (on average 78%, corresponding to a homing efficiency of 56%), and up to 96% (on average 89%) were males/intersexes (SI Appendix, Fig. S3B and C). These results further confirmed that our proposed suppression gene-drive strategy is indeed able to perform super-Mendelian inheritance, similar to findings in another recent study in *D. melanogaster* (21).

Evolution of Cleavage Drive-Resistant *tra* Alleles.

While we found our system to be highly efficient for sex conversion in *D. melanogaster*, we noticed during routine screening of the stocks the appearance of female flies with the *DsRed* eye marker phenotype. This was contrary to our previous observation and expectations that all heterozygous (*tra*$^{nCHE/+)}$ XX flies should develop at least an intersex phenotype. Two scenarios could explain the presence of females with the DsRed eye marker: (i) an aborted or imperfect HDR, during which the DsRed eye marker is copied faithfully while an essential part of the drive element was lost or mutated, which would result in a dead CHE allele (*tra*^{nD}), or (ii) the presence of an in-frame mutation in the tra allele, which abolishes the recognition site of the gRNA without affecting the function of the tra-encoded protein (*tra*^{Rst}). Such mutations are likely to emerge from in-frame indel mutations as a result of NHEJ events induced by the CHE itself.

To check these hypotheses, virgin females with the DsRed eye marker were isolated and individually crossed with *w*–males. One of these crosses did not show any signs of an active drive system, with about 50% of the offspring showing the DsRed eyemarker. Molecular analysis of the mother and some female offspring from this cross revealed a large deletion in the CHE as the result of an aborted HDR event (SI Appendix, Fig. S4A). The other crosses, however, showed an efficient super-Mendelian inheritance, indicating the presence of an

active CHE in the mother, which could be a sign of the presence of a *tra*^{Rst} allele in the mother. Sequencing the tra locus of these mothers confirmed the presence of in-frame indel mutations in the recognition site of the gRNA (similar to sequences in SI Appendix, Fig. S4B). By crossing such females carrying an active CHE with *tra*^{nCHE} males, we were able to obtain homozygous *tra*^{nCHE}/*tra*^{nCHE} males. When these homozygous males were crossed with *w*–virgins, all offspring were either male or intersex (SI Appendix, Fig.S3A), which further confirmed the high sex-conversion efficiency of this tra-targeting CHE.

To further evaluate the drive-resistant allele hypothesis and to estimate the rate at which resistant alleles may emerge from NHEJ events, we crossed heterozygous virgins carrying the docking-null allele (*tra*$^{nDOCK/+}$) with heterozygous (*tra*$^{nCHE/+}$) driver males (Fig.2). By looking at the progeny that carry the *tra*^{nDOCK} allele (marked by ECFP fluorescence) but lack a *tra*^{nCHE} allele (DsRed fluorescence), we confined our analysis to situations of non-HDR at the paternal wildtype tra allele. Sequencing the tra allele in non-DsRed, ECFP females of the first generation resulted in the discovery of various independent in-frame indel mutations (SI Appendix, Fig.S5A). This suggests that drive-resistant alleles, *tra*^{Rst}, are readily created as a result of NHEJ in heterozygous males that carry the CHE allele. To determine the frequency at which these *tra*^{Rst} alleles are generated, we crossed four heterozygous (*tra*$^{nCHE/+}$) males individually to *tra*$^{nDOCK/+}$virgins and sequenced all progeny that showed only an ECFP fluorescence. We identified in-frame indels (*tra*^{Rst}) in up to 10% of all progeny, representing about one-third of all NHEJ events. The relative high emergence rate of such *tra*^{Rst} alleles in the F1 progeny demonstrates the rapid evolution of resistance as a direct consequence of an active homing CHE (SI Appendix, Fig.S5B) and confirms similar results from other groups (21, 30).

Resistant Allele Dynamics and Spread.

To estimate the dynamics of resistance allele emergence and spread in a population, we crossed (in five replicates each) w–virgins with four different ratios of heterozygous *tra*$^{nCHE/+}$ males to *w*– males and followed the progeny for up to 15 generations. Thereby we documented the sex ratios as well as the spread of the DsRed-marked *tra*^{nCHE} allele, whose presence in females indicates potential drive-resistant *tra*^{Rst} alleles (Fig. 3 and SI Appendix, Figs.S6–S8). The ratio of such DsRed-positive females increased progressively over the generations, corresponding to the expected selective increase of resistance allele frequency. To characterize the molecular basis of the resistance to HCGD, we sequenced the tra locus from DsRed-fluorescent females from all the experimental settings at generation F6 and observed a diverse set of in-frame mutations representing drive-resistant *tra*^{Rst} alleles (SI Appendix, Fig. S4B). We also selected one setting for a molecular time-course analysis (setting D, replicate 4, at generations F1, F2, F6, and F13) and found that such mutations were heritable (SI Ap-pendix, Fig.S5C). The diversity of these in-frame indels across experimental settings and generations shows that these

tra^{Rst} alleles are constantly created, independently of each other, at the site of cleavage. Interestingly, we already had observed DsRed-fluorescent females at the F1 generation of this replicate. These possessed a wild-type tra allele (SI Appendix, Fig.S5C) but contained a large deletion in the Cas9 gene of the *tra^{nCHE}* allele, which was likely the result of a rare, aborted HDR event (similar to that in SI Appendix, Fig.S4A). Following the populations to generation F15, we found an almost regular 1:1 ratio of males to females in all the replicates (Fig. 3 and SI Appendix, Fig.S8), independent of the original frequency of *tra^{nCHE}* allele inoculation (settings B and D).

A *tra*-Based Suppression Gene-Drive System for *C. capitata*.

Having shown the capability of our proposed CHE in inducing sex conversion in *D. melanogaster* and after identifying potential weaknesses of the system due to resistance evolution, we simulated the outcome of using our proposed method as a pest-control strategy in *C. capitata* (Fig. 4). Our population dynamics simulation results indicate that the evolution of in-frame drive-resistant alleles at rates that we observed in *D. melanogaster* would indeed impede a population collapse in *C. capitata* (Fig.4A), even if multiple releases were implemented in quick succession (Fig. 4C). To tackle the issue of in-frame drive-resistant alleles (these are problematic because they prevent homing while still allowing tra expression and hence are not removed due to a selective advantage), we considered the use of multiple *gRNAs* to target the tra gene to reduce the proportion of resistant alleles that are in-frame. Using multiple gRNAs may not have a drastic effect on the overall NHEJ rate but will reduce the in-frame resistant allele formation rate exponentially with each additional gRNA, as each new target site would have to obtain an in-frame mutation that does not affect the function of the protein (22, 31). Our simulation study predicts that by using multiple *gRNAs*, and thereby reducing the generation rate of in-frame resistance alleles by at least two orders of magnitude, the effectiveness of the system is greatly improved. A single release is still not sufficient to achieve a population collapse in *C. capitata* (Fig. 4B); however, three releases in quick succession are sufficient (Fig. 4D).

The above-mentioned simulations are for the scenario in which *$tra^{nCHE/+}$*; XX individuals are infertile intersexes; however, we also explored the case in which these individuals are fertile males (Fig. 4E and F). In D. melanogaster, we observed that heterozygous (*$tra^{nCHE/+}$*) XX individuals develop into mosaic intersex individuals (SI Appendix, Fig.S3A). This is likely because the ectopic expression of *Cas9* under the control of the Rcd-1r promoter in only a proportion of the cells results in a mosaic phenotype. Since the intersex-based infertility of *tra^{nCHE}* XX individuals places a fitness load on the system and reduces the drive (by preventing its occurrence in XX individuals), we propose the use of an early embryonic promoter, such as *Sry-α*, for the expression of Cas9.Expression from Sry-α in germ cells will allow gene drive to occur,and the early blastoderm expression guarantees a uniform destruction of the wild-

type tra allele in all cells of the embryo at avery early stage; therefore heterozygous (*$tra^{nCHE/+}$*) XX individuals could develop into fertile medfly males. This in turn reduces the fitness load associated with the drive allele and increases the drive (by allowing super-Mendelian inheritance of the drive allele to occur in *$tra^{nCHE/+}$*; XX individuals). Our simulation shows that enabling the fertility of heterozygous (*$tra^{nCHE/+}$*) XX individuals does enhance the effectiveness of the system in collapsing a *C. capitata* population following a single release, provided that the in-frame drive-resistant allele generation rate is reduced by using multiple gRNAs (Fig. 4E and F). The tolerable generation rate of in-frame resistant alleles depends on the size of the targeted medfly population. Our simulations predict the extent by which this rate must be reduced to achieve a population collapse as a function of population size (SI Appendix, Fig.S9).

Discussion

Our mathematical modeling has shown that two main factors—the formation rate of the in-frame resistance allele and the fitness of heterozygous (*$tra^{nCHE/+}$*) sex-converted XX individuals—can havea significant effect on the expected outcome of a release in the wild. When heterozygous XX individuals are infertile, our model predicts that a population collapse can be achieved only if multiple inundative releases of the driver males are performed (Fig. 4D). While, this limitation could potentially be overcome by using a nearly embryonic stage promoter such as *Sry-α* (Fig. 4F), this may not be desirable to ensure the local treatment of an insect pest population without the potential concern about the elimination of an entire species. Similar considerations have also been brought forward by Prowse et al. (32) with respect to fighting invasive vertebrate species.

In addition, we have shown that in our system it is the generation rate of in-frame drive-resistant alleles, rather than the overall NHEJ rate, that has a significant impact on the outcomeof release scenarios. This is of significant importance for species such as *D. melanogaster* (and possibly for related pest species such as *Drosophila suzuki*) that might seem semirefractory toward homing-based gene-drive strategies (27), as it indicates that future designs may not necessarily require an extremely high homing rate but that only lowering the formation rate of the in-frame resistance allele and thus employing multiple *gRNAs* (22,31) might be sufficient for an efficient suppression gene-drivestrategy in such species.

Our results support the idea that using a CHE to target genes that are essential for female-specific development in insects, such as *tra*, can effectively result in a gender-biased population, finally resulting in a population collapse. This provides a basis for further development of similar suppression gene-drive strategies to introduce a gender bias in wild populations of insect pest ssuch as the medfly or disease vectors. If such a gender bias can be sustained long enough, species-specific elimination of the target species can be achieved. If the HCGDs explored here were applied to efficient pest-control management, the strategy in

which *tra*$^{nCHE/+}$; XX individuals are infertile intersexes is safer, because it requires multiple releases to achieve population collapse and hence will cause a population collapse only where these releases are carried out.Overall, we provide here an example, an implementation strategy, and the mathematical modeling required for the design and optimization of a homing-based sex conversion-suppression gene-drive approach for local or global species-specific elimination of insect pest or disease vector species. Moreover, we show that only lowering the formation rate of in-frame drive-resistant alleles by employing multiple *gRNAs* may be sufficient to achieve an effective suppression gene-drive outcome, which has important implications for the design of such systems in species that exhibit a low homing rate in their germ cells.

Materials and Methods

Detailed methods on cloning, transgenesis, screening, molecular analysis, stock keeping of *D. melanogaster* strains, population modeling, and simulations can be found in SI Appendix, SI Materials and Methods.

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Figures

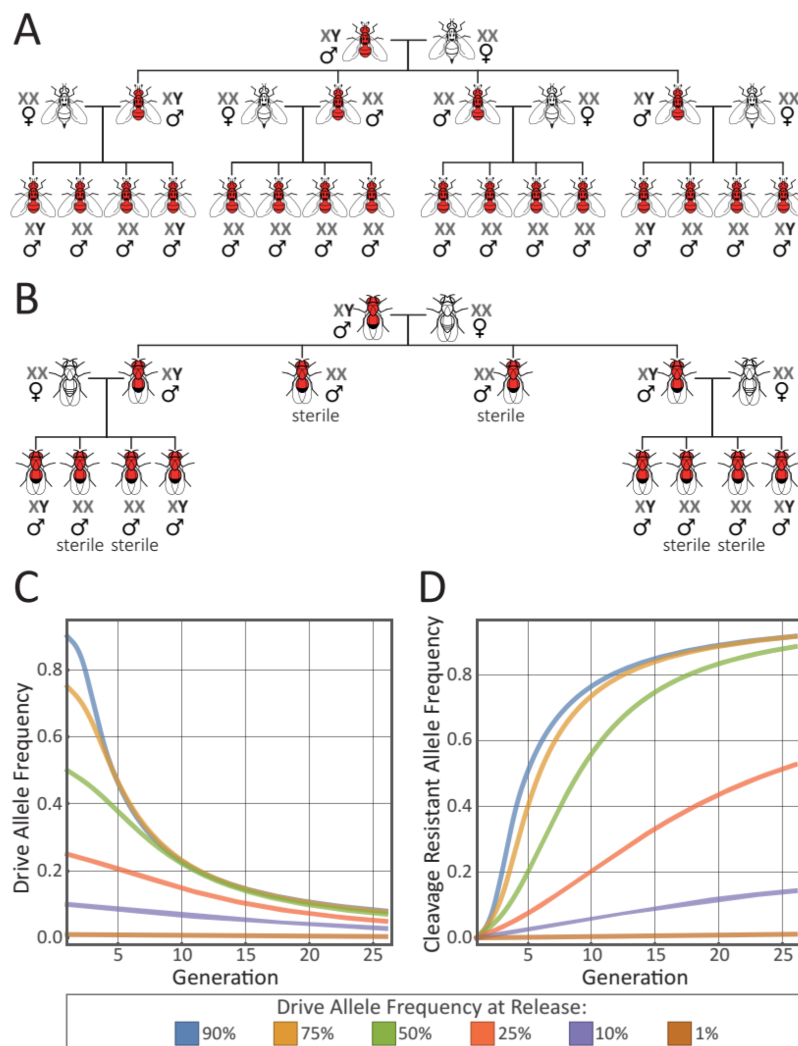

Fig. 1. Insect suppression gene drive based on forced all male offspring. (A) In *C. capitata*, a *tra* targeting Cas9 Homing Element (CHE) with both germline and somatic expression will cause super-Mendelian inheritance of the red fluorescence-marked CHE null allele, but also results in transformation of XX individuals into males and in theory lead to a subsequent collapse of the population. (B) In *D. melanogaster*, homing into *tra,* in somatic cells transforms XX individuals into sterile pseudo-males, which halts the spread of the selfish element, but allowed us to safely study the dynamics and molecular consequences of using CHEs in a suppression gene drive system. (C) Predicted transience of a Cas9-based homing construct targeting the *tra* locus in *D. melanogaster*. Predictions are based on an introduction of $tra^{nCHE}/+$ males at frequencies of 1-90% into a population of otherwise wild type males and females in equal proportion. We assume a Cas9-mediated cleavage efficiency of 100%, a probability of accurate homology-directed repair (HDR) following cleavage of 90%, $\frac{1}{3}$ of drive-resistant alleles (NHEJ products) being in-frame indels, and with no fitness cost associated with in-frame drive-resistant alleles. A construct having these parameter values and released in the form of $tra^{nCHE}/+$; XY males is expected to be eliminated from the population within ~25 generations regardless of the introduction frequency. (D) Although at high release frequencies, presence of the drive allele results in generation and establishment of cleavage resistant alleles in the population. At low release frequencies, which may occur because of accidental escapes, the drive allele will be eliminated early, and as a consequence the cleavage resistance alleles will only appear at negligible frequencies. This indicates that *D. melanogaster* is a safe model organism for the evaluation of a *tra*-based suppression gene drive causing sex conversion.

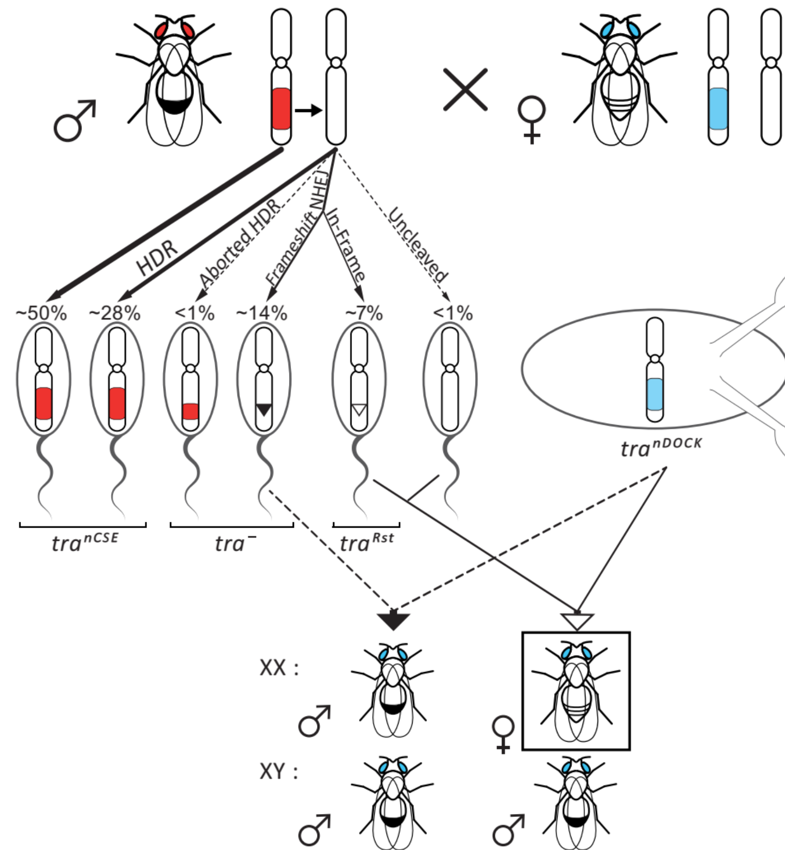

Fig. 2. CHE-targeting of the homologous gene locus. By analyzing the ECFP/non-DsRed progeny of *tra*^{nCHE}/+ males and *tra*^{nDOCK}/+ virgins, we focused on the non-HDR targeting events at a single *tra* locus. Values above each sperm indicate an estimation of each genotype based on the observed efficiency values. Molecular analysis of the CHE target site in F_1 female progeny (boxed) identified independent NHEJ events causing various in-frame indels that resulted in drive-resistant functional alleles (*SI Appendix*, Fig. S5*A*).

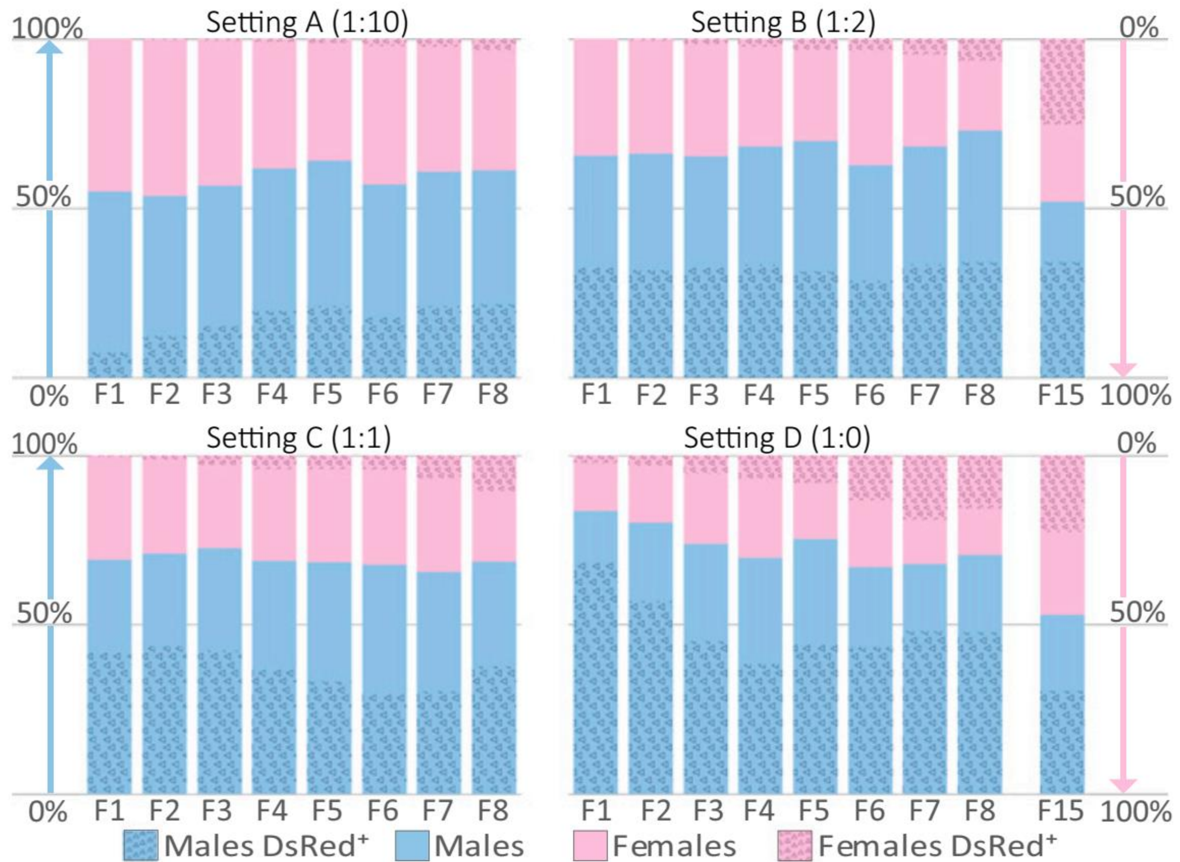

Fig. 3. Dynamics of sex ratio and indicated resistance allele spread in population experiments. *w⁻* virgins were crossed with various ratios of CHE (*tra*^{nCHE}/+) and wild-type (*w⁻*) males (settings A–D). For each setting five replicates were carried out (*SI Appendix*, Fig. S6). Progenies were screened for sex and presence of the DsRed eye marker for up to 15 generations. In setting D, where only *tra*^{nCHE} males were used, a sex ratio of over 80% males was achieved within one generation, which indicates the collapse potential of this forced male-only offspring system. In XX embryos, *tra*^{nCHE} attacks the wild type *tra* locus resulting in inter-sex individuals (*SI Appendix*, Fig. S2). Thus, only females carrying non-functional defective *tra*^{nCHE} or drive-resistant functional *tra*^{Rst} alleles can show the DsRed marker. Therefore, the DsRed marker serves as an indicator for the *tra*^{Rst} allele presence in females and the rise in the percentage of DsRed females indicates the resistance spread in the population. Screening the F_{15} progenies in settings B and D showed that the populations adopted to the presence of the *tra*^{nCHE} homing allele with the female sex ratio reaching back to about 50% (*SI Appendix*, Fig. S6).

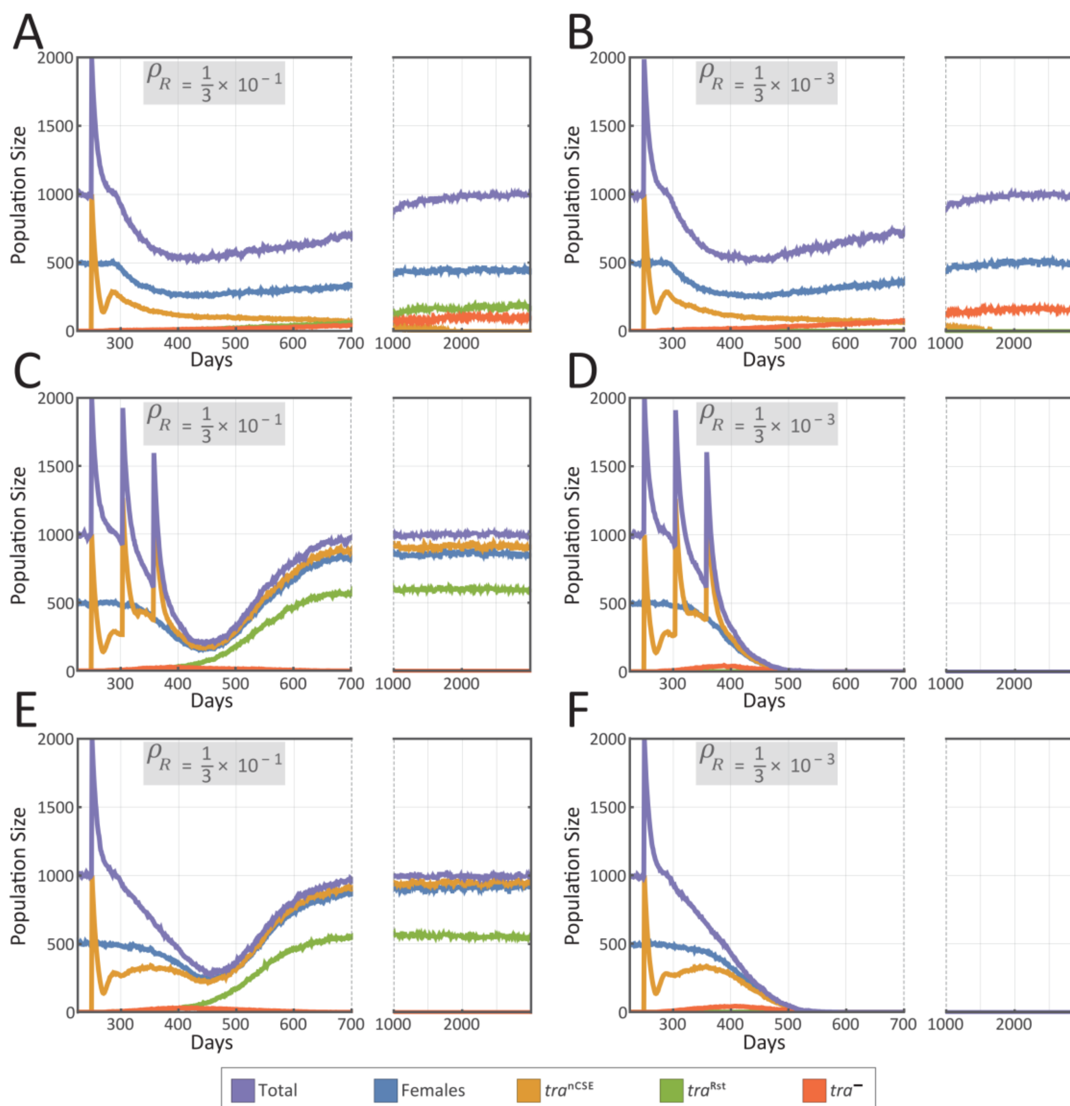

Fig. 4. Predicted dynamics of a Cas9-based homing system targeting the *tra* locus in *C. capitata*. Predictions are based on the population genetic model depicted in *SI Appendix*, Fig. S10 combined with the population dynamic model depicted in *SI Appendix*, Fig. S11 in which the life cycle of *C. capitata* is divided into four stages – egg, larva, pupa and adult – with density-dependent mortality occurring at the larval stage (*SI Appendix*, Table S1). Homing only occurs in *tra*^{nCHE}/+ heterozygotes, where + represents the wild type allele and *tra*^{nCHE} represents the intact drive allele. We assume a Cas9-mediated cleavage efficiency of 100% and a probability of accurate homology-directed repair following cleavage of 90% (NHEJ rate (δ)=0.1). By default, in-frame drive-resistant alleles (*tra*^{Rst}), account for $\frac{1}{3}$ of generated resistant alleles, although this proportion may be reduced through gRNA multiplexing. The remaining cleavage resistant alleles, are out-of-frame or other mutations that result in a *tra*$^{-}$ null allele. The equilibrium population size of *C. capitata* is 1,000. Releases consist of 1,000 *tra*^{nCHE}; XY males once or at intervals. In panels A-D, the scenario in which *tra*^{nCHE}; XX individuals are infertile intersex is considered. (A) For a homing efficiency of 90% and an in-frame resistant allele generation rate (ρ_R=$\delta\theta$, where δ is the NHEJ rate and θ is the fraction of NHEJs that produce in-frame indels) of $\frac{1}{3}$ of 10%, a single release of 1,000 *tra*^{nCHE}; XY males results in temporary population suppression, halving the adult population size, with the population rebounding over a period of several years. (B) Decreasing the in-frame drive-resistant allele generation rate, ρ_R, by two orders of magnitude to $\frac{1}{300}$ of 10%, and hence increasing the out-of-frame resistant allele generation rate, ρ_B=$\delta(1-\theta)$, to ~10%, the population suppression is still only moderate and transient. (C) If three releases of 1,000 *tra*^{nCHE}; XY males are carried out in succession, the extent of population suppression is much greater (>75% suppression); however, at a ρ_R of $\frac{1}{3}$ of 10%, the population still rebounds over a period of several years with an increase in the frequency of *tra*^{Rst} alleles. (D) Nevertheless, if three consecutive releases are carried out for a construct with the decreased in-frame drive-resistant allele generation rate, population

elimination can be achieved within ~1 year after the last release. (E) In panels E-F, the scenario in which *tra*^{nCHE}; XX individuals are fertile males is considered. For a homing efficiency of 90% and an in-frame resistant allele generation rate, ρ_R, of $\frac{1}{3}$ of 10%, a single release of 1,000 *tra*^{nCHE}; XY males results in temporary population suppression, as in-frame drive-resistant alleles become prevalent, preventing population elimination. (F) However, if the in-frame resistant allele generation rate, ρ_R, is reduced by two orders of magnitude to $\frac{1}{300}$ of 10%, the emergence of in-frame drive-resistant alleles is unlikely, and the population can be eliminated following a single release of 1,000 *tra*^{nCHE}; XY males.

SUPPLEMENTARY INFORMATION (SI) APPENDIX:

Consequences of instant induction of resistance evolution on a sex conversion–based suppression gene drive for insect pest management

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Materials and Methods

Computational modeling of gene drive in *D. melanogaster*. Equation B4 from the model by Unkless et. al. for resistance evolution in gene drive (147) was expanded as below to include all the important parameters of our study, where x_w, x_d, x_r and x_b are allele frequencies for wild-type, driver, in-frame resistance, and frameshift alleles, superscripts represent the sex. Parameters c, δ, and θ represent cleavage rate, NHEJ rate, and fraction of NHEJs that produce in-frame indels, respectively. ω_{ab} shows the fitness of an individual with genotype ab.

$$\overline{\omega}(t) = \left(x_w^m x_d^f + x_d^m x_w^f\right)[(1-c)\omega_{dw} + c(1-\delta)\omega_{dd} + c\delta(1-\theta)\omega_{db} + c\delta\theta\omega_{dr}] + \left(x_w^m x_r^f + x_r^m x_w^f\right)\omega_{rw} + \left(x_w^m x_b^f + x_b^m x_w^f\right)\omega_{bw} + \left(x_d^m x_r^f + x_r^m x_d^f\right)\omega_{dr} + \left(x_d^m x_b^f + x_b^m x_d^f\right)\omega_{db} + \left(x_b^m x_r^f + x_r^m x_b^f\right)\omega_{rb} + \sum_i x_i^m x_i^f \omega_{ii} \quad \text{(E1)}$$

For the specific case of *D. melanogaster* where ω_{dw}, ω_{dd}, ω_{db}, and ω_{bb} are 0 for females and all other fitness values are considered 1, the following equations can be derived from equation E1 to recursively calculate the allele frequencies at each generation for females:

$$x'^f_d = \frac{\left(x_w^m x_d^f + x_d^m x_w^f\right)c\delta\theta + \left(x_d^m x_r^f + x_r^m x_d^f\right)}{2\overline{\omega}_{Dm}(t)} \quad \text{(E2)}$$

$$x'^f_r = \frac{\left(x_w^m x_d^f + x_d^m x_w^f\right)c\delta\theta + \left(x_w^m x_r^f + x_r^m x_w^f\right) + \left(x_d^m x_r^f + x_r^m x_d^f\right) + \left(x_b^m x_r^f + x_r^m x_b^f\right) + 2x_r^m x_r^f}{2\overline{\omega}_{Dm}(t)} \quad \text{(E3)}$$

$$x'^f_b = \frac{\left(x_w^m x_b^f + x_b^m x_w^f\right) + \left(x_b^m x_r^f + x_r^m x_b^f\right)}{2\overline{\omega}_{Dm}(t)} \quad \text{(E4)}$$

$$x'^f_w = \frac{\left(x_w^m x_r^f + x_r^m x_w^f\right) + \left(x_w^m x_b^f + x_b^m x_w^f\right) + 2x_w^m x_w^f}{2\overline{\omega}_{Dm}(t)} \quad \text{(E5)}$$

and for males:

$$x'^m_d = \frac{\left(x_w^m x_d^f + x_d^m x_w^f\right)[c(1-\delta)+1] + \left(x_d^m x_r^f + x_r^m x_d^f\right) + \left(x_d^m x_b^f + x_b^m x_d^f\right) + 2x_d^m x_d^f}{2\overline{\omega}_{Dm}(t)} \quad \text{(E6)}$$

$$x'^m_r = \frac{\left(x_w^m x_d^f + x_d^m x_w^f\right)c\delta\theta + \left(x_w^m x_r^f + x_r^m x_w^f\right) + \left(x_d^m x_r^f + x_r^m x_d^f\right) + \left(x_b^m x_r^f + x_r^m x_b^f\right) + 2x_r^m x_r^f}{2\overline{\omega}_{Dm}(t)} \quad \text{(E7)}$$

$$x'^m_b = \frac{\left(x_w^m x_d^f + x_d^m x_w^f\right)[c\delta(1-\theta)] + \left(x_w^m x_b^f + x_b^m x_w^f\right) + \left(x_d^m x_b^f + x_b^m x_d^f\right) + \left(x_b^m x_r^f + x_r^m x_b^f\right) + 2x_b^m x_b^f}{2\overline{\omega}_{Dm}(t)} \quad \text{(E8)}$$

$$x'^m_w = \frac{\left(x_w^m x_d^f + x_d^m x_w^f\right)(1-c) + \left(x_w^m x_r^f + x_r^m x_w^f\right) + \left(x_w^m x_b^f + x_b^m x_w^f\right) + 2x_w^m x_w^f}{2\overline{\omega}_{Dm}(t)} \quad \text{(E9)}$$

where

$$\bar{\omega}_{Dm}(t) = \left(x_w^m x_d^f + x_d^m x_w^f\right)(1 + c\delta\theta) + 2\left(x_w^m x_r^f + x_r^m x_w^f + x_w^m x_b^f + x_b^m x_w^f + x_d^m x_r^f + x_r^m x_d^f + x_b^m x_r^f + x_r^m x_b^f + x_w^m x_w^f + x_r^m x_r^f\right) + x_d^m x_b^f + x_b^m x_d^f + x_d^m x_d^f + x_b^m x_b^f \quad \text{(E10)}$$

Guide RNA selection and HRMA. Several gRNAs targeting the first exon of the *D. melanogaster tra* locus were selected using CRISPR DESIGN online tool. Selected guide sequences (Fig. S1*B*) were cloned into the pCFD2 plasmid (148) (Addgene 49409, gift from S. Bullock) using annealed oligonucleotides for each gRNA (Fig. S1*A*). One hour old embryos from the act5-cas9 *D. melanogaster* strain (148) (Bloomington 54590) were injected using the construct for each gRNA. After 24 hours, individual embryos were homogenized in 50 µl of smashing buffer (10 mM Tris-HCl, pH 8.2, 25 mM NaCl, 1mM EDTA, 0.2% Triton x100 and 200 µg/ml Proteinase K) (149) using small tips. Samples were kept at 55 °C for 1 hour followed by 5 minutes incubation at 95°C to heat inactivate Proteinase K. 1 µl was used for PCR using MK078 and MK079 primers (Table S1) with EvaGreen qPCR master mix and High Resolution Melt curves (150) were obtained in a BioRad CFX96 real-time C1000 thermal cycler at 0.2°C steps. Relative fluorescent at 75°C and 85°C were normalized to 1 and 0, respectively, and the control graph was subtracted from all the guide RNA graphs. The guide that showed the largest difference (g4) was selected as the most efficient guide RNA. To make sure that off-target effects are minimal (150), three of the top similar sequence hits from the CRISPR DESIGN online tool (151) were checked for the most efficient guide (g4) using High Resolution Melting Analysis (HRMA) employing MK155/MK156, MK157/MK158 and MK159/MK160 primer pairs (Table S1).

Constructs. To generate the SG022 plasmid (pCRII-tra4R-attP-3xP3CFP-attP-tra4L, GeneBank KY171964), In-Fusion assembly (Clontech, USA) was performed on HindIII/ApaI digested pCRII (ThermoFisher, USA) backbone and PCR products of (i) MK024/MK122 primers on genomic DNA from the *D. melanogaster* strain Oregon-R for left homologous arm, (ii) MK117/MK116 primers on pBac{3xP3-ECFPaf} (72) for 3xP3-ECFP-SV40pA, and (iii) MK123/MK025 primers on genomic DNA from the Oregon-R strain for right homologous arm. MK116 and MK117 primers (Table S1) introduce *attP* sites (152) at both ends of the ECFP marker to generate a recombinase-mediated exchange cassette. The SG012 plasmid (pCFD3-g4) was generated by cloning annealed MK083 and MK044 oligonucleotides into the pCFD3 plasmid (148) (Addgene 49410, gift from S. Bullock) using the depositor's suggested protocol (153). To generate the SG011 plasmid (pCRII-attBSmaI), the pCRII vector was first digested with XbaI/HindIII followed by ligation of annealed MK060 and MK061 oligonucleotides (Table S1). Then the NsiI cut site in the vector was destroyed by first digesting the plasmid with NsiI followed by T4 DNA polymerase treatment and religation using T4 DNA Ligase. In-Fusion assembly was performed on the BamHI/NotI digested pIE4 plasmid (154) and PCR products of (i) MK072/MK075 primers (Table S1) on genomic DNA from Oregon-R strain for first intron of *alpha-tub84B* and (ii) MK076/MK077 primers on pBS-Hsp70-Cas9 (Addgene 46294, gift from M. Harrison, K. O'Connor-Giles, J. Wildonger) for *D. melanogaster* codon optimized SpCas9 coding sequence, to generate the SG020 plasmid (pIE4-aTubIGT1-Cas9). MK134 and MK135 primers (Table S1) were used to amplify the aTubI1-Cas9 fragment from SG020 plasmid. The

fragment was directly ligated to SmaI digested SG011 to generate the SG023 plasmid (pCRII-attB-aTubCas9-attB). For the SG024 plasmid (pCRII-attB-aTubCas9bTub-U63g4-DsRed-attB, GeneBank KY171962), in-Fusion assembly was performed on AscI digested SG023 plasmid and PCR products of (i) MK144/MK145 primers on genomic DNA from Oregon-R strain for the 3' UTR of beta-tub85D (155), (ii) MK147/MK146 primers on SG012 for U6:3-g4, and (iii) MK149/MK148 primers on pBac{3xP3-DsRedaf} (156) for 3xP3-DsRed-SV40pA. To amplify each of the promoters Oregon-R genomic DNA was used as template and (i) MK140/MK141 primer pairs (Table S1) were used to amplify a 900 bp fragment of the Rcd-1r promoter (155), (ii) MK142/MK143 primer pairs (Table S1) were used to amplify a 550 bp fragment of the *Sry-α* promoter (157), and (iii) MK138/MK139 primer pairs (Table S1) were used to amplify a 500 bp fragment of the *DNApol-α180* promoter (158). The PCR products were then digested using XbaI (or AvrII) and XhoI restriction enzymes and the products were ligated to AvrII/XhoI digested SG024 plasmid to generate SG039 (pCRII-attB-Rcd1rp-aTubCas9bTub-U63g4-DsRed-attB, GeneBank KY171963), SG040 (pCRII-attB-sryap-aTubCas9bTub-U63g4-DsRed-attB), and SG037 (pCRII-attB-DPol180p-aTubCas9bTub-U63g4-DsRed-attB) plasmids respectively. MK153 and MK154 primers (Table S1) were used to amplify the phiC31 coding sequence from the plasmid pcDNA3.1-phiC31 (159) (Addgene 68310, gift from K. Basler). The PCR product was then digested with BsaI and NotI restriction enzymes and was ligated to NcoI/NotI digested pSL[faHSfa] plasmid (160) to generate the SG042 helper plasmid (pSL-DmHsp70-phiC31-Hsp70).

Generation of the docking (*tra*$^{\mathbf{nDOCK}}$) strain. To generate the docking strain (Fig. S2*A*), *tra*$^{\mathrm{nDOCK}}$, 30 minutes old de-chorionated embryos from the act5-Cas9 *D. melanogaster* strain (148) were covered with hydrocarbon oil (Voltalef 10S) and injected with an injection mix containing 500 ng/µl of SG022 (pCRII-tra4R-attP-3xP3CFP-attP-tra4L) HDR donor plasmid and 300 ng/µl of SG012 (pCFD3-g4) gRNA-producing plasmid. Embryos were kept humid at 25°C for 24 hours on an apple agar plate. Newly hatched larvae were gently collected from the apple agar plate using a size 00 brush and placed on *D. melanogaster* food supplemented with dried yeast in a small vial. Larvae were kept at 25°C until eclosion and only male offspring were individually crossed with w^- virgins. F_1 third instar larvae from individual vials were collected from the food by applying CO_2 to the media. The larvae were aligned on a cold microscope slide and screened under a Zeiss fluorescent binocular for the ECFP eye marker. Positive larvae from each cross were placed into new vials until eclosion. Individual male flies with the ECFP eye marker were then crossed with virgins from the $\left[\frac{w^-}{w^-};\frac{CyO\ (Cy)}{Sp};\frac{TM3(Sb)}{Dr}\right]$ balancer strain. F_2 flies carrying the ECFP eye marker, *Cy* (*Curly wings*) and *Sb* (*Stubble*) phenotypes were self-crossed. F_3 flies carrying the ECFP eye marker and the *Sb* phenotype without any of the other balancer phenotypes were kept to establish the strain. Molecular analysis was performed to verify the proper genome editing at the third chromosomal *tra* locus that generated *tra*$^{\mathrm{nDOCK}}$.

Molecular Characterization of *tra*^{nDOCK} strain. To confirm the correct integration of the docking cassette into the *tra* locus, MK126/MK128 and MK127/MK129 primer pairs (Table S1) were used to amplify products of ~3 kbp from the *tra*^{nDOCK} genomic DNA. Templates were prepared by homogenizing the head of individual flies in 50 µl of smashing buffer (149) using small tips. MK126 and MK127 primer binds to regions upstream and downstream of the Left and Right Homologous Arms used for the HDR, respectively. MK128 and MK129 primers, however, bind to 3' end and 5'

end of the ECFP CDS, respectively. Therefore, a product of around 3 kbp from each of these primer pairs can only form if integration at the correct locus has occurred. Off-target integration does not result into an amplification product with these primer pairs because MK126 and MK127 primers bind outside the homologous arms regions. One of the fly strains that passed all of the quality control criteria was then kept as a stock.

Generation of the homing *tra*^{nCHE} strain. To generate the homing *tra*^{nCHE} strains (Fig. S2*B*), 30 minutes old embryos de-chorionated from the docking strain (*tra*^{nDOCK}) were covered with hydrocarbon oil and injected with an injection mix containing 500 ng/µl of each of the donor plasmids for respective promoters (SG039, SG040, and SG037) and 300 ng/µl of SG042 (pSL-DmHsp70-phiC31-Hsp70) helper plasmid to perform a Recombinase-Mediated Cassette Exchange (RMCE) (161). Embryos were kept humid at 25 °C for 24 hours on an apple agar plate. Newly hatched larvae were gently collected from the apple agar plate using a size 00 brush and placed on *D. melanogaster* food supplemented with dried yeast in a small vial. Larvae were kept at 25°C until eclosion. Newly eclosed flies were individually crossed with *w*$^-$ flies. For each of the promoters F_1 offspring was screened for the absence of ECFP and the presence of DsRed eye marker. Individual males with only the DsRed eye marker were then crossed with *w*$^-$ virgins. Positive F_2 individuals (consisting of males/intersexes only) from each single cross were then pooled together and *w*$^-$ virgins were added to the pool. Few of the F_2 males/intersexes were used for DNA extraction for molecular characterization and verification of RMCE. To sustain the strain over generations, a few *w*$^-$ virgins were added to the vials every two weeks.

Molecular Characterization of Rcd-1r *tra*^{nCHE} strains. To confirm the fidelity of the RMCE and also to determine the orientation of the integration, MK073/MK078 and MK073/MK079 primer pairs (Table S1) were used to amplify a ~2 kbp region from the *tra*^{nCHE} genomic DNA. Templates were prepared by homogenizing the head of individual flies in 50 µl of smashing buffer (149) using small tips. MK073 binds to the upstream of the SpCas9 coding sequence within the CHE. MK078 and MK079 bind to upstream and downstream of the g4 target site, respectively. A 2 kbp amplification product with MK073/MK078 primer pairs indicates a sense integration event (endogenous *tra* and SpCas9 CDSs on the same strand). However, a 2 kbp amplification product with MK073/MK079 primer pairs indicates an anti-sense integration event (endogenous *tra* and SpCas9 CDSs on opposite strands). One of the fly strains with an anti-sense integration that passed all the quality control criteria was kept as a stock and was used for all further experiments.

Stock keeping of *tra*^{nCHE} strains. Despite the fact that the high cost of drive in our *tra*^{nCHE} *D. melanogaster* strains does not allow for spread of the drive allele in any population and thus serves as a very effective biological confinement strategy (Fig. 1 C and D), we carried out stock keeping of this homing *tra*^{nCHE} strain as well as all experiments generating and using this strain with utmost care to not have potential gene drive individuals escape the laboratory. All experiments were performed in our well-equipped safety level one (S1) laboratory, but only in windowless internal rooms, with ventilation in- and outlets covered with tight mesh and doors supplied with brushes. Flies were always anesthetized before opening containers as well as analyzed and sorted under constant anesthetization. Fly traps were installed in the rooms and in the neighboring corridors. For transport between rooms, flies were put in double-walled containers. Before discarding, flies were finally deep frozen for more

than twelve hours. Despite the design and commencement of our study in 2013, it already met the criteria for safeguarding gene drive experiments in the laboratory as published in Science in 2015 (162) since two stringent confinement strategies were employed: (i) biological confinement based on the high cost of this specific gene drive in *D. melanogaster* and (ii) organizational as well as physical containment based on the rules for handling this strain in our S1 laboratory. In 2016, we were informed of the new guidelines on handling gene drive experiments in Germany by the German Central Commission for Biological Safety (ZKBS) that suggested to perform such experiments only in safety level two (S2) laboratories. Therefore, we stopped our experiments and moved our tra^{nCHE} strain temporarily to a neighboring S2 laboratory for stock maintenance only. At the same time, we applied for a single case evaluation of our experiments with the ZKBS that approved of further handling the tra^{nCHE} strain in our S1 laboratory with the above-mentioned measures (file reference: ZKBS 45110.1933). After publication of this study, we will discard the strain that can easily be re-generated by RMCE from the docking line tra^{nDOCK}.

Estimating the sex conversion and homing efficiencies. The sex conversion efficiency of the tra^{nCHE} allele was evaluated by crossing twelve homozygous tra^{nCHE}/tra^{nCHE} males individually with three w^- virgins each. The offspring was then screened for sex and kept to inbreed to check for any sign of fertility of potential not-recognized F_1 females. All inspected F_1 progeny was male or intersex and no F_2 progeny was observed. To estimate the homing efficiency of the tra^{nCHE}, 12 heterozygous $tra^{nCHE}/+$ males were individually crossed with three w^- virgins each. The offspring was then screened for sex and the presence of DsRed fluorescent eye marker under a Zeiss fluorescent binocular (Fig. S3 *B* and *C*). The homing efficiency was calculated as the ratio of targetable alleles that had been successfully homed by the tra^{nCHE} allele, by equation E11, assuming an equal segregation of chromosomes in male gametes.

$$\frac{DsRed^{+}-(Total/2)}{Total/2} \quad \text{(E11)}$$

Estimating the targeting efficiency. To estimate the targeting efficiency of our CHE, heterozygous $tra^{nCHE}/+$ males were crossed with heterozygous $tra^{nDOCK}/+$ virgins (Fig. 2) – both in batch and in single crosses with individual tra^{nCHE} males. The F_1 offspring was then screened for eye marker and only individuals carrying the ECFP eye marker but not the DsRed eye marker were selected for molecular analysis. MK058/MK059 primer pairs (Table S1) were used to amplify a ~750 bp region which was used for Sanger sequencing using the MK058 primer. Sequencing results were analyzed using Geneious software (Biomatters, New Zealand). Templates were prepared by homogenizing the head of individual flies in 50 µl of smashing buffer (149) using small tips.

Population experiments. Population experiments were performed in four settings A–D with five replicates for each of the settings (Fig. S6*A*). To avoid any bias in mating, we made sure all flies were of the same age and before putting them together in the same vial all flies were kept anesthetized using CO_2. After 72 hours of mating time, a 3–6 hours egg lay collection was obtained, after which all parents were removed and were kept frozen at -80 °C. For each following generation, all progeny of the 3–6 hour egg collection was allowed to eclose and then used as parents to set up the next generation. Again after a 3–6 hours egg lay, these parents were removed and used for screening their sex and the presence

of the DsRed eye marker prior to freezing and storage at -80 °C. The 3-6 hour egg collection was based on the amount of time that is necessary for the population of parents to lay enough eggs to generate a progeny size which fills but not over-populates a medium size fly food vial (Diameter: 36 mm; Height: 82 mm). At each generation, enough time was given to all the pupae to eclose followed by 72 hours of mating to allow for a representative population in the next generation. For each egg collection, all parents as well as all the respective progeny were screened.

Molecular analysis of *tra* loci and aborted HDR *tra*$^-$ alleles. MK058 and MK059 primers (Table S1) were used to PCR-amplify a ~750 bp fragment surrounding the g4 target site. Templates were prepared by homogenizing the head of individual flies in 50 µl of smashing buffer (149) using small tips. Samples were kept at 55°C for 1 hour followed by 5 minutes incubation at 95°C to heat inactivate the Proteinase K. 5 µl of samples were used for PCR using Phusion DNA polymerase in 50 µl by 2 minutes incubation at 96°C followed by 40 cycles of 96°C for 20", 70°C for 30", and 72°C for 40" with 5 minutes final extension at 72°C. PCR products were then sent for Sanger sequencing using primer MK058. Sequencing results were analyzed using Geneious software (Biomatters, New Zealand). MK146 and MK134 primers (Table S1) were used to PCR amplify a ~6 kbp fragment consisting of the U6:3-g4 and SpCas9 CDS from DsRed female's genomic DNA. PCR products were analyzed by agarose gel electrophoresis and fragment sizes were compared with the PCR product of same primer pairs on genomic DNA of a male individual from the stock as reference. The reduction in size of the fragments served as an indication of an aborted HDR (Fig. S4*A*). To confirm the results at the molecular level, PCR products were then used for Sanger sequencing using MK085, MK086, MK087, and MK088 primers (Table S1).

Population genetic model for homing system targeting the *tra* locus in *C. capitate*. To model the potential application of the homing-based gene drive system targeting the *tra* locus to suppress populations of the medfly, *C. capitata*, we combined a population genetic model describing the inheritance pattern of the homing system (Fig. S10) with a population dynamic model described previously (163) adapted for the *C. capitata* (the original model describes the population dynamics of the main African malaria vector, *An. gambiae*) (Fig. S11).

In the population genetic model (Fig. S10), we describe the offspring genotype distribution in *C. capitata* for each combination of maternal and paternal genotypes for a homing-based gene drive system targeting the *tra* locus. We denote the wild-type *tra* allele by “W”, the drive allele, *tra*$^{\mathrm{nCHE}}$, by “D”, a drive-resistant allele with an in-frame indel at the *tra* locus, *tra*$^{\mathrm{Rst}}$, by “R”, and a drive-resistant null allele, *tra*$^-$, by “B”. Homing is only manifest in WD heterozygotes, whereby WD individuals produce D gametes in the germline at a frequency equal to $(1+e)/2$, where e denotes the “homing efficiency,” which represents the proportion of W gametes that are converted into D gametes through the act of homing. Homing efficiency, e, is equal to the product of the cleavage rate, c, and the probability of accurate homology-directed repair (HDR) given that cleavage has occurred, P_{HDR}, i.e. $e=cP_{HDR}$. Wild-type alleles may also be produced by WD individuals at a rate equal to $(1-c)/2$; however, it should be noted that the rate at which this occurs has been shown to be negligible in a recent analysis (164) and hence this possibility is not depicted in the crosses in Fig. S10. Homing-resistant alleles, which result from W alleles that are cleaved but for which accurate HDR does not occur, are produced at a frequency equal to $c\delta/2$, where $\delta=(1-P_{HDR})$. A proportion, θ, of these resistant

alleles are in-frame indels (R), for which expression of functional *tra* gene is retained, and the remainder, (1-θ), are out-of-frame or other mutations (B), for which the function of *tra* gene is disrupted. The proportion of W gametes that are converted to R or B gametes is therefore given by $\rho_R = c\delta\theta$ and $\rho_B = c\delta(1-\theta)$, respectively. The proportion of generated resistant alleles that are in-frame indels is expected to be ~1/3, although this proportion can be reduced through multiplexing. All other individuals produce gametes at standard Mendelian frequencies. In this system, there are six possible fertile female genotypes – DR/XX, RR/XX, RB/XX, WR/XX, WB/XX and WW/XX – and 14 possible fertile male genotypes – DD/XY, WD/XY, DR/XY, DB/XY, RR/XY, RB/XY, BB/XY, WR/XY, WB/XY, WW/XY, DD/XX, WD/XX, DB/XX and BB/XX (Fig. S10).

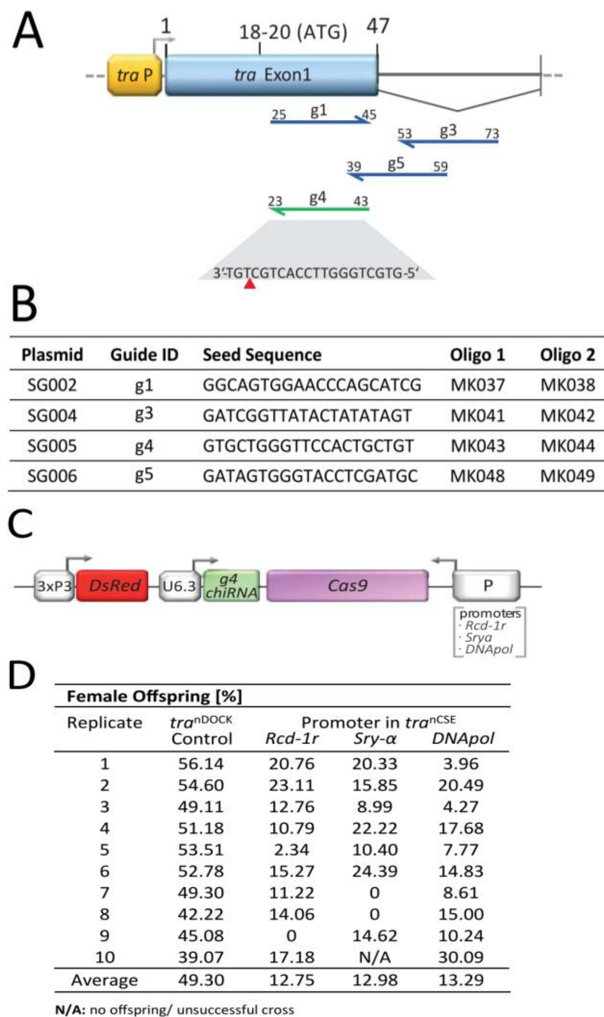
Population dynamic model for *C. capitate*. In the adapted population dynamics model (Fig. S11), the medfly life cycle is divided into four life stages – egg, larva, pupa and adult (both male and female adults are modeled) (Fig. S10). The durations of the juvenile stages differ (Table S2) but their daily, density-independent mortality rates are assumed to be identical and are chosen for consistency with the population growth rate in the absence of density-dependent mortality. The intrinsic population growth rate of medfly populations in the absence of density-dependent mortality, r_M, was calculated from average monthly trap catch data across four sites in Western Cape Province, South Africa between the months of December 2009 and March 2010 and shown to be consistent with a population growth rate of r_M=1.031 per day (165). Additional density-dependent mortality occurs at the larval stage, and we used a density-dependent equation of the form, $F(L) = \sqrt[T_L]{a(a+L)}$, where L is the number of larvae, T_L is the duration of the larval stage, and α is a parameter influencing the strength of density-dependence. Adult males mate throughout their lifetime, while adult females mate only once, soon after that they mature. For these simulations, we assumed fecundity rates to be consistent between genotypes, with fertile females laying β eggs per day. Initial estimates for these and other parameter values are provided in Table S2 and the equations describing the equivalent implementation of this model for *An. gambiae* are included in Supplementary File S1 of (163).

We used a stochastic implementation of this model to capture random effects at low population sizes, for instance when the CRISPR-based homing system is causing significant population suppression. We assume the number of eggs produced per day by females follows a Poisson distribution, the number of eggs having each genotype follows a multinomial distribution according to the parental genotypes and inheritance pattern, and all survival/death events follow a Bernoulli distribution. Finally, female mate choice follows a binomial distribution with probabilities given by the relative frequency of each male genotype in the population.

Experimental design and statistics. All individual data points are displayed in supplementary figures with mean and s.d., and sample size for all experiments are mentioned in the main text and figures as appropriate. No power calculations were performed to estimate the sample size. No randomization or blinding was performed, for each cross all progeny was screened. Replicate numbers for estimation of efficiencies are consistent with other similar gene drive studies in insects. A normal probability plot was generated for obtained homing efficiencies of each replicate to ensure the normal distribution of data points. In crosses with individual heterozygous *tra*^{nCHE} flies, replicates that did not result in progenies were excluded. These were most likely a result of a cross between an infertile intersex fly

which are often indistinguishable from true males. In crosses between individual heterozygous *tra*^{nCHE} males with heterozygous *tra*^{nDOCK} virgins, two of the replicates that showed a 1:1 ratio of DsRed^{+}:DsRed^{-} offspring were not used for molecular analysis and sequencing. All statistical analyses were performed on Microsoft Excel 2007. Plots were generated using GraphPad Prism 7 and R.

SI Figures



B

Plasmid	Guide ID	Seed Sequence	Oligo 1	Oligo 2
SG002	g1	GGCAGTGGAACCCAGCATCG	MK037	MK038
SG004	g3	GATCGGTTATACTATATAGT	MK041	MK042
SG005	g4	GTGCTGGGTTCCACTGCTGT	MK043	MK044
SG006	g5	GATAGTGGGTACCTCGATGC	MK048	MK049

D

Female Offspring [%]

Replicate	tra^{nDOCK} Control	Promoter in tra^{nCSE} *Rcd-1r*	*Sry-α*	*DNApol*
1	56.14	20.76	20.33	3.96
2	54.60	23.11	15.85	20.49
3	49.11	12.76	8.99	4.27
4	51.18	10.79	22.22	17.68
5	53.51	2.34	10.40	7.77
6	52.78	15.27	24.39	14.83
7	49.30	11.22	0	8.61
8	42.22	14.06	0	15.00
9	45.08	0	14.62	10.24
10	39.07	17.18	N/A	30.09
Average	49.30	12.75	12.98	13.29

N/A: no offspring/ unsuccessful cross

Fig. S1. Generation of a homing CHE for the *D. melanogaster tra* locus I. (A) Four gRNAs targeting the first exon of the *D. melanogaster tra* locus with g4 (green) identified as the most efficient gRNA using HRMA (numbers indicate bases after transcription start site). (B) Oligos (Table S1) used for the generation of gRNA plasmids. (C) Structure of the CHE used in this study. Human codon optimized *SpCas9* coding sequence is placed under the control of each of the three promoters under study. *Rcd-1r* germline specific promoter, *Sry-α* early zygotic promoter, and *DNApol-α180* cell cycle dependent promoter. g4 guide RNA is expressed by *U6:3* promoter. A 3xP3 driven DsRed eye marker is used to allow for screening and identification of strains. (D) To evaluate the somatic sex conversion efficiency of each of the promoters, individual tra^{nCHE} males from each of the three CHE variants (*Rcd-1r*-CHE, *Sry-α*-CHE, *DNApol-α180*-CHE) were crossed with *w-* virgins and offspring were screened for their sex. tra^{nDOCK} strain was used as control. The very low frequency of female offspring clearly indicates that all three of these promoters are capable of inducing somatic sex conversion in D. melanogaster. Assuming an equal distribution of X and Y chromosomes and a sex conversion rate of 100% in XX embryos receiving a tra^{n}CHE allele from their father, a homing rate of ~49% can be calculated for each of these promoters from the below equation which indicates all three of these promoters had equally well been able to successfully perform homing in the germline of the heterozygous tra^{n}CHE males and drive into the next generation.

$$\text{Homing efficiency} = \frac{E_f' - O_f}{E_f'}$$

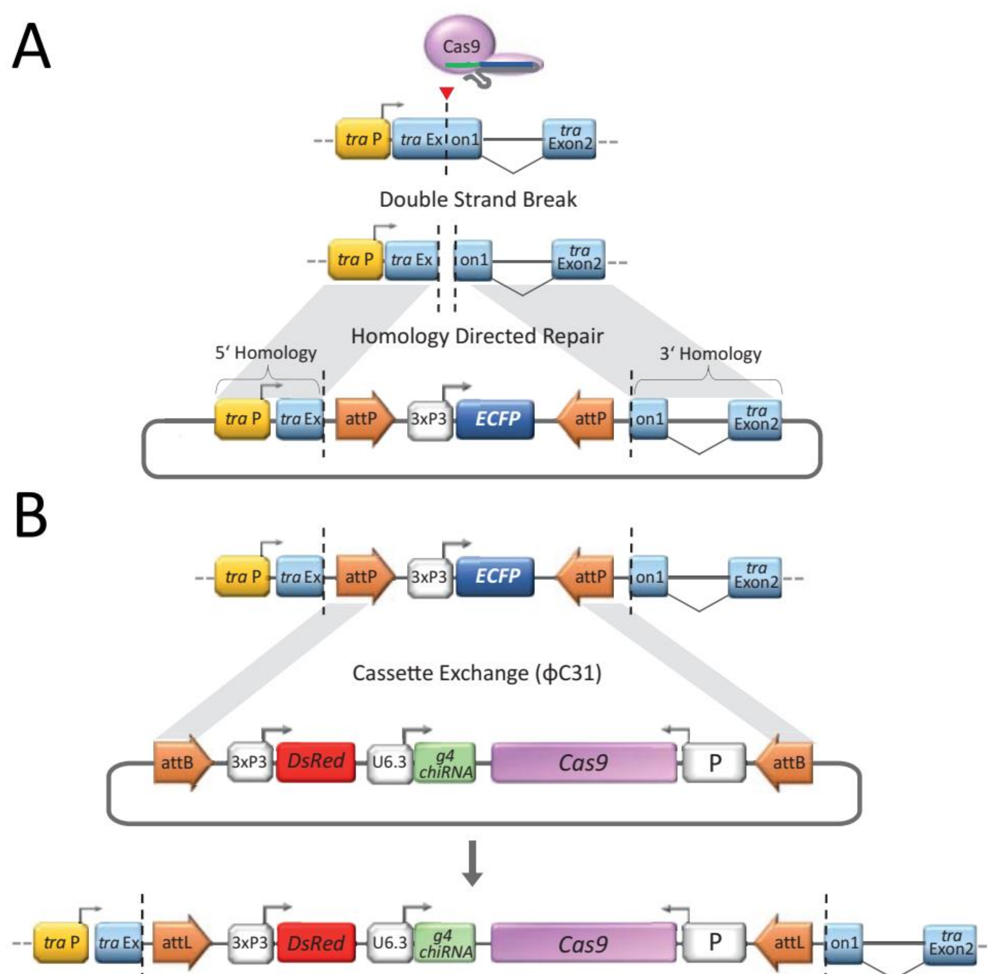
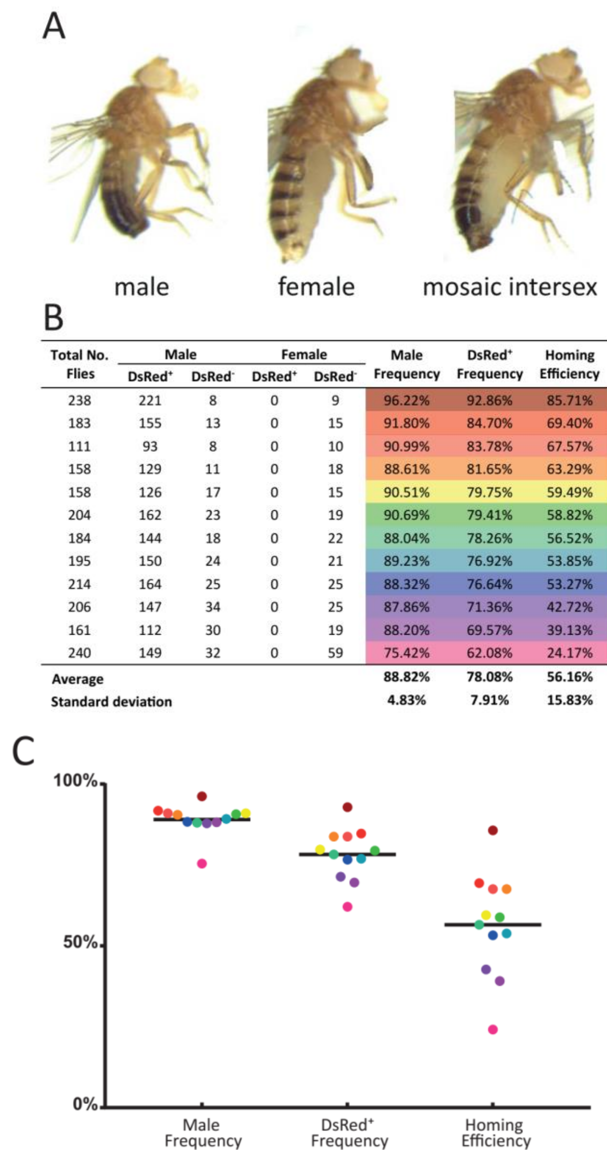


Fig. S2. Generation of a homing CHE for the *D. melanogaster tra* locus II. (A) Using the g4 gRNA and Cas9, a ΦC31 RMCE docking cassette containing an eye-specifically driven (3xP3) cyan fluorescent marker (ECFP) flanked by *attP* sites was integrated into the 1st exon of the *tra* locus to generate the docking null allele tra^{nDOCK}. (B) A cassette – containing the coding sequence of SpCas9 endonuclease (Cas9, under the control of *Rcd-1r* germline specific promoter), the *U6.3* driven chimeric gRNA (*g4 chiRNA*), and a red fluorescent eye marker (DsRed) – was used to replace the ECFP marker by ΦC31-mediated RMCE to generate the homing CHE null allele tra^{nCHE}.



Total No. Flies	Male DsRed+	Male DsRed-	Female DsRed+	Female DsRed-	Male Frequency	DsRed+ Frequency	Homing Efficiency
238	221	8	0	9	96.22%	92.86%	85.71%
183	155	13	0	15	91.80%	84.70%	69.40%
111	93	8	0	10	90.99%	83.78%	67.57%
158	129	11	0	18	88.61%	81.65%	63.29%
158	126	17	0	15	90.51%	79.75%	59.49%
204	162	23	0	19	90.69%	79.41%	58.82%
184	144	18	0	22	88.04%	78.26%	56.52%
195	150	24	0	21	89.23%	76.92%	53.85%
214	164	25	0	25	88.32%	76.64%	53.27%
206	147	34	0	25	87.86%	71.36%	42.72%
161	112	30	0	19	88.20%	69.57%	39.13%
240	149	32	0	59	75.42%	62.08%	24.17%
Average					88.82%	78.08%	56.16%
Standard deviation					4.83%	7.91%	15.83%

Fig. S3. Sex Conversion and Homing Efficiency. (A) Mosaic intersex fly (right). Because of the high efficiency of Cas9 at cleaving wild-type *tra* alleles also in somatic cells, heterozygous $tra^{nCHE}/+$ XX individuals develop into infertile mosaic intersex individuals. These intersex individuals, often lack sex combs on one or both of their front legs, show a larger size, show a malformed ovipositor and have an inconsistent yellow and black pattern on their abdomen. (B) Estimation of the homing efficiency at the *tra* locus. Twelve $tra^{nCHE}/+$ males were crossed individually with virgin w^{-} flies and progenies were screened for sex and presence of the DsRed eye marker. On average about 78% of the offspring carried the DsRed marker as opposed to the expected 50% based on Mendelian inheritance. The homing efficiency was calculated using the formula $\frac{DsRed^{+}-(Total/2)}{Total/2}$ and determined to be around 56%. (C) Dot plots showing the distribution of male frequency, DsRed-frequency, and homing efficiency values of the twelve replicates (colors represent the different replicates as indicated in panel B).

Fig. S4. Example of aborted HDR and CHE-resistant *tra* alleles in generation F_6 of the population experiments. (A) Aborted HDR: In one of the crosses of females with the DsRed eye marker no signs of an active drive system was observed. Amplification of the Cas9 expression cassette using MK134/MK146 primer pair should result into a 6 Kb DNA fragment (Third lane from right; tra^{nCHE}). However, PCR on the genomic DNA of the mother and its female offspring that

carried the DsRed eye marker with these primers resulted in a truncated ~2.5 Kb product, indicating a large deletion in the CHE as a result of an aborted HDR event. (B) Molecular analysis of the CHE target site sequences in DsRed-marked females derived from all replicates (first number) of all four setting A–D (letter). The last number indicates different sequences from the same replicate. Few of the sequences of Setting A were obtained from F_{13}, as DsRed females were absent in F_6 of some replicates. The unchanged wild type *tra* allele was identified in six occasions (indel size: $0^{\#}$), but those individuals had defective *tra*^{nCHE} alleles with large deletions in the *Cas9* coding sequence (not shown) as the result of an aborted HDR (panel A). In all other DsRed females, *tra*^{Rst} mutations were found that represent in-frame indels, which have, at least, either the seed sequence of the target site or the CRISPR protospacer adjacent motif (PAM) abolished. The size of the indels were multiples of three keeping the *tra* coding sequence in-frame but destroying the g4 gRNA's recognition sequence at the cleavage site (indicated by a red triangle). The diversity of the indels indicates frequent independent events that can result in the emergence of resistant alleles. A three-base pair AGC deletion at the site of cleavage was identified in eight independent occasions (-3^{*}).

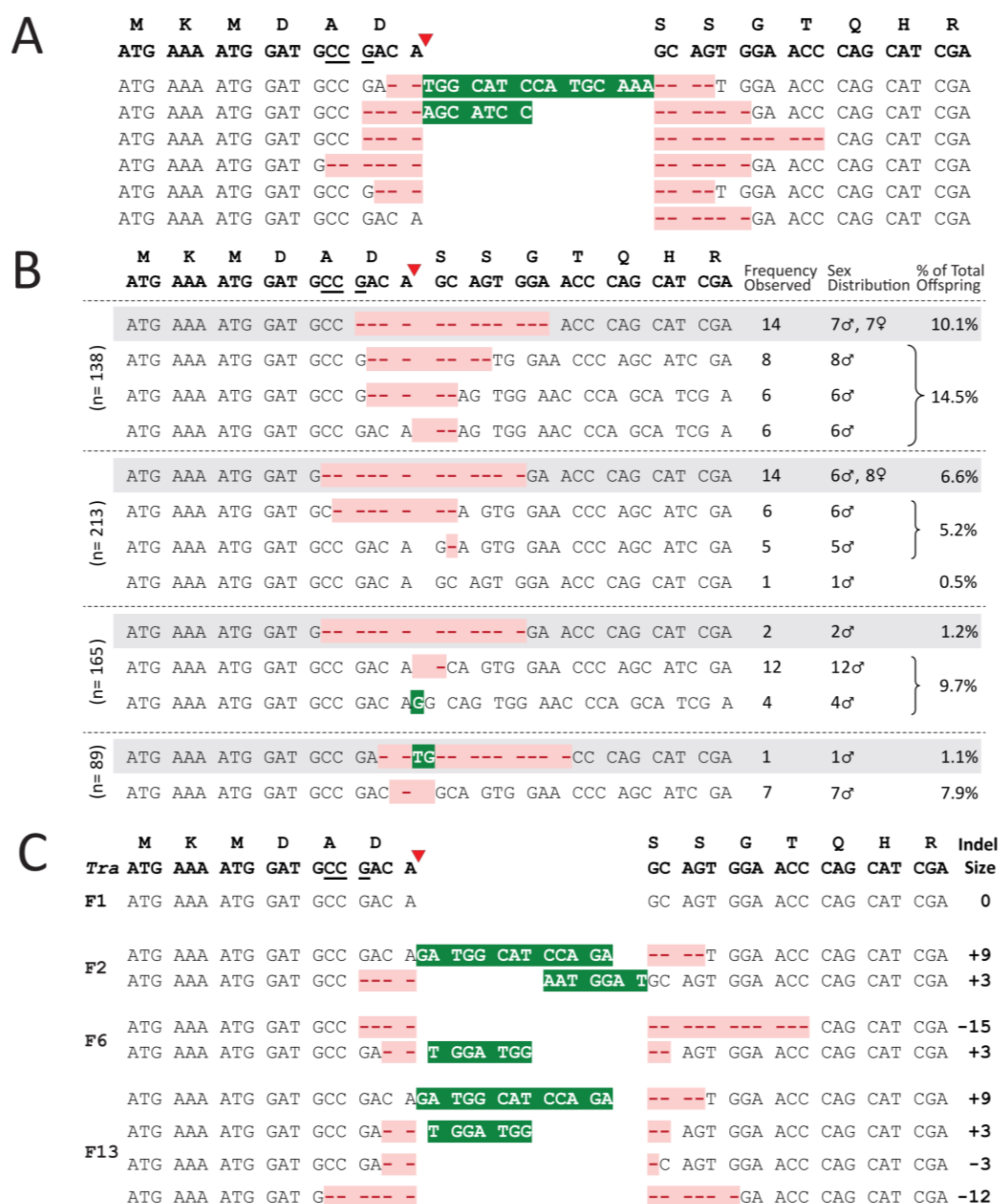
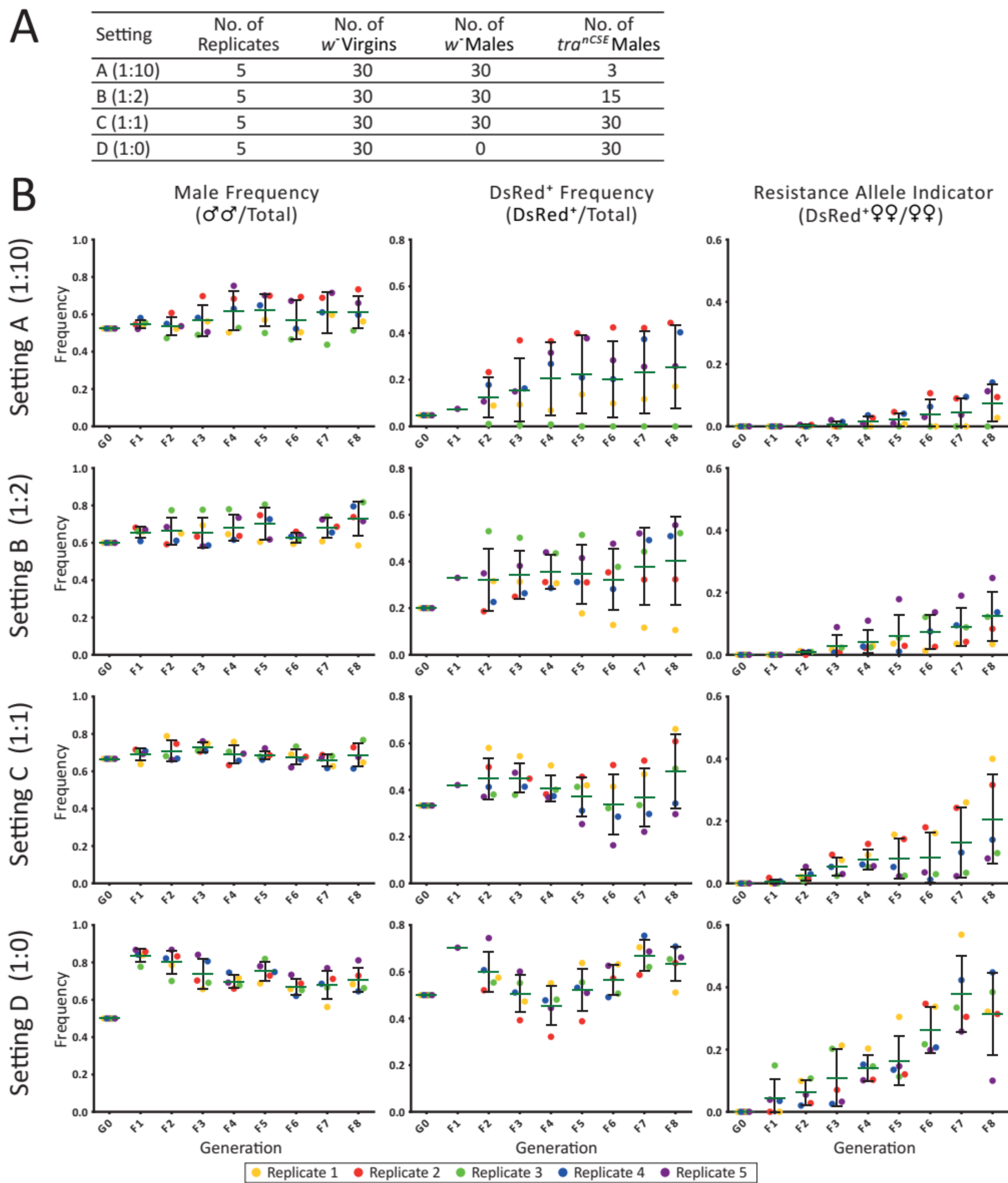


Fig. S5. **Molecular analysis of non-HDR events at the site of cleavage**. (**A**) Molecular analysis of the CHE target site (red triangle indicates cleavage site) in F_1 female progeny of a cross between *tra*^{nCHE}/+ males and *tra*^{nDOCK}/+ virgins (Fig. 2) identified independent NHEJ events causing various in-frame indels (red/green) that resulted in resistant alleles. (**B**) Analysis of all ECFP/non-DsRed progeny derived from four individually crossed *tra*^{nCHE}/+ males and *tra*^{nDOCK}/+ virgins (separated by dashed lines) indicates the very efficient targeting of the *tra* locus in the presence of an active homing CHE (only one wild type allele in a progeny of 605). NHEJ-derived alleles were identified in 9–24% of all progeny causing frameshift mutations (*tra*$^{-}$) in about two thirds of these cases and in-frame indels (*tra*^{Rst}, shaded in grey) in about one third, whereby *tra*$^{-}$ alleles were expectedly only observed in males or intersexes. The limited number of different NHEJ-derived alleles per single male cross indicates an activity at very early stages of primordial germ cell development. (**C**) Molecular analysis of the CHE target site (red triangle indicates cleavage position) in the *tra* locus of population experiment-derived DsRed-marked females. All sequences are taken from flies of the fourth replicate of setting D (SI Appendix, Fig. S4) across different generations. DsRed-marked females from F_1 carried a wild type tra allele but defective tra^{nCHE} alleles with large deletions in the Cas9 coding sequence as a result of an aborted HDR (Fig. 2). In F_2 and F_6, various in-frame indel mutations were identified indicating independent emergences of these alleles. Some alleles already observed in F_2 and F_6 were also isolated in F_{13}, which implies the spread and fixation of these resistant alleles in the population.



A

Setting	No. of Replicates	No. of w^- Virgins	No. of w^- Males	No. of tra^{nCSE} Males
A (1:10)	5	30	30	3
B (1:2)	5	30	30	15
C (1:1)	5	30	30	30
D (1:0)	5	30	0	30

Fig. S6. **Population experiments**. (A) Set-ups of the different cage experiments (settings A–D). Values in parentheses show the ratio of carrier *tra*^{nCHE} males to w^- males used in each setting. (B) Dot plots representing the results of all five replicates (each indicated by different colors) for each setting of the population experiments. The increase in the DsRed females over the generations is an indicator for the emergence and spread of resistant alleles. Data for the DsRed frequency in F_1 is from replicate five only (purple dots). In setting A, replicate three (green dots) lost its *tra*^{nCHE} allele from F_2 onwards.

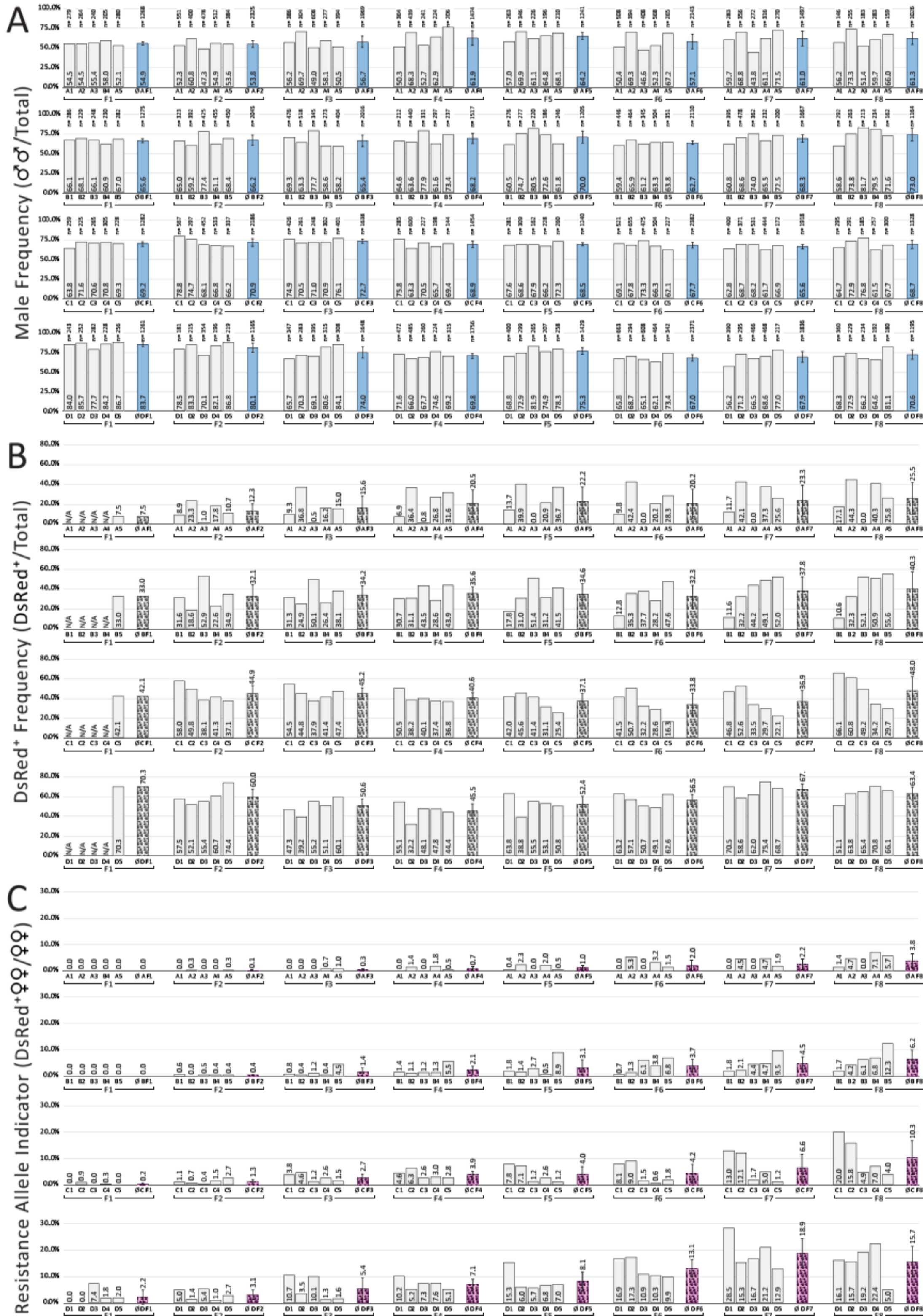
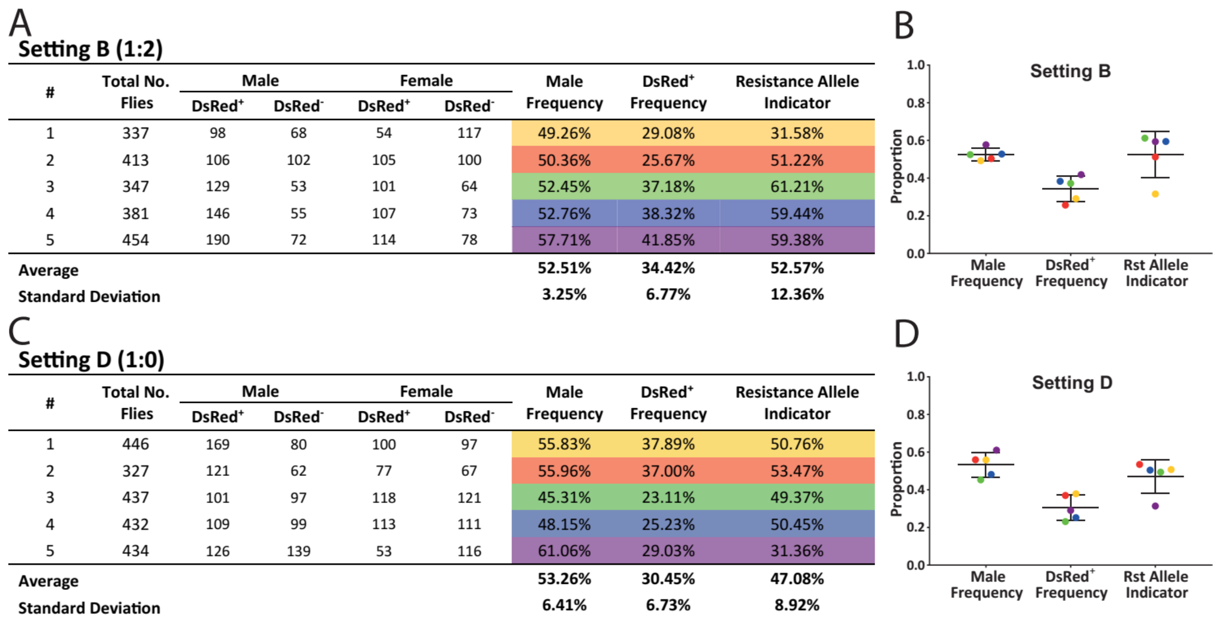


Fig. S7. Original data of the population experiments. Five replicates for each of the four settings A–D (Fig. S6A) were monitored over eight generations (F_1–F_8). (A) The frequency of males in the respective population. (B) The DsRed frequency depicting the percentage of flies carrying the DsRed eye marker (in F_1 data only available for replicate five). (C) The resistance allele indicator represented by the proportion of females that carry the DsRed eye marker. Color/pattern-marked columns represent mean and standard deviation for each setting and generation. These means were taken to produce Fig. 3*A* (please note, for the DsRed frequency in F_1, only replicate five was counted and that value taken as representative).



A

Setting B (1:2)

#	Total No. Flies	Male DsRed⁺	Male DsRed⁻	Female DsRed⁺	Female DsRed⁻	Male Frequency	DsRed⁺ Frequency	Resistance Allele Indicator
1	337	98	68	54	117	49.26%	29.08%	31.58%
2	413	106	102	105	100	50.36%	25.67%	51.22%
3	347	129	53	101	64	52.45%	37.18%	61.21%
4	381	146	55	107	73	52.76%	38.32%	59.44%
5	454	190	72	114	78	57.71%	41.85%	59.38%
Average						52.51%	34.42%	52.57%
Standard Deviation						3.25%	6.77%	12.36%

C

Setting D (1:0)

#	Total No. Flies	Male DsRed⁺	Male DsRed⁻	Female DsRed⁺	Female DsRed⁻	Male Frequency	DsRed⁺ Frequency	Resistance Allele Indicator
1	446	169	80	100	97	55.83%	37.89%	50.76%
2	327	121	62	77	67	55.96%	37.00%	53.47%
3	437	101	97	118	121	45.31%	23.11%	49.37%
4	432	109	99	113	111	48.15%	25.23%	50.45%
5	434	126	139	53	116	61.06%	29.03%	31.36%
Average						53.26%	30.45%	47.08%
Standard Deviation						6.41%	6.73%	8.92%

Fig. S8. Population collapse experiment data for generation F_{15}. (A, B) Setting B and (C, D) Setting D populations recovered from the masculinizing effect of the tra^{nCHE} homing allele as the sex ratios are back to around normal 50%. The DsRed eye marker was present in about one third of the population but in almost half of the females serving as resistance allele indicator. Original data (A, C) and dot plot representation (B, D) of all five replicates (each indicated by a different color). The averages were taken to produce Fig. 3*A*.

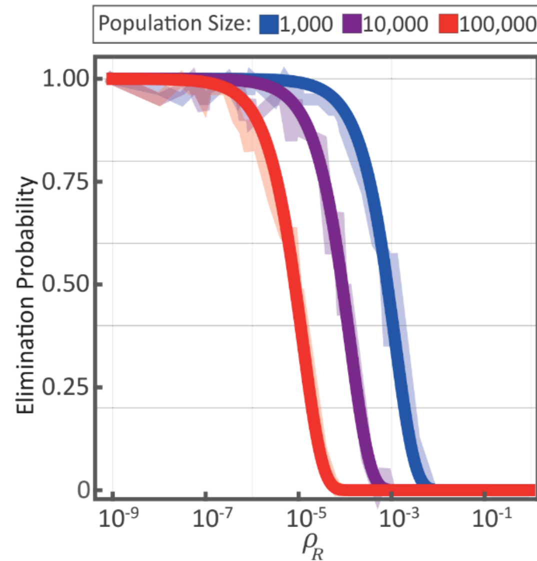


Fig. S9. Elimination probability as a function of in-frame resistant allele generation rate, ρ_R, for a range of population sizes, *N*, between 1,000 and 100,000. We consider the scenario in which tra^{nCHE}; XX individuals are fertile males, and assume a 1:1 release to wild ratio, that CRISPR-mediated cleavage efficiency is 100%, that the probability of accurate homology-directed repair following cleavage is 90%, that the in-frame resistant allele generation rate is as specified above, that in-frame resistant alleles have no associated fitness cost, and that the remainder of resistant alleles are out-of-frame or other mutations. Sigmoidal curves are fitted to data points covering 30 in-frame resistant allele generation rates sampled logarithmically between 10^{-1} and 10^{-9}. Faint lines represent interpolation between simulated data points while solid lines represent fitted sigmoidal relationships. For an adult population size of 1,000, an in-frame resistant allele generation rate of less than 10^{-4} is required to achieve likely elimination, while for an adult population size of 100,000, an in-frame resistant allele generation rate of less than 10^{-6} is required to achieve likely elimination.

Male \ Female	DR/XX	RR/XX	RB/XX	WR/XX	WB/XX	WW/XX
DD/XY	(1/2) DR (1/2) DD	(1) DR	(1/2) DR (1/2) DB	(1/2) WD (1/2) DR	(1/2) WD (1/2) DB	(1) WD
WD/XY	((1+e)/4) DD ($(1+e+\rho_R)/4$) DR ($\rho_R/4$) RR ($\rho_B/4$) DB ($\rho_B/4$) RB	((1+e)/2) DR ($\rho_R/2$) RR ($\rho_B/2$) RB	((1+e)/4) DR ((1+e)/4) DB ($\rho_R/4$) RR ($(\rho_R+\rho_B)/4$) RB ($\rho_B/4$) BB	((1+e)/4) WD ((1+e)/4) DR ($\rho_R/4$) WR ($\rho_R/4$) RR ($\rho_B/4$) WB ($\rho_B/4$) RB	((1+e)/4) WD ((1+e)/4) DB ($\rho_R/4$) WR ($\rho_R/4$) RB ($\rho_B/4$) WB ($\rho_B/4$) BB	((1+e)/2) WD ($\rho_R/2$) WR ($\rho_B/2$) WB
DR/XY	(1/4) DD (1/2) DR (1/4) RR	(1/2) DR (1/2) RR	(1/4) DR (1/4) DB (1/4) RR (1/4) RB	(1/4) WD (1/4) WR (1/4) DR (1/4) RR	(1/4) WD (1/4) WR (1/4) DB (1/4) RB	(1/2) WD (1/2) WR
DB/XY	(1/4) DD (1/4) DR (1/4) DB (1/4) RB	(1/2) DR (1/2) BR	(1/4) DR (1/4) DB (1/4) RB (1/4) BB	(1/4) WD (1/4) WB (1/4) DR (1/4) RB	(1/4) WD (1/4) WB (1/4) DB (1/4) BB	(1/2) WD (1/2) WB
RR/XY	(1/2) DR (1/2) RR	(1) RR	(1/2) RB (1/2) RR	(1/2) WR (1/2) RR	(1/2) WR (1/2) RB	(1) WR
RB/XY	(1/4) DR (1/4) RR (1/4) DB (1/4) RB	(1/2) RR (1/2) RB	(1/4) RR (1/2) RB (1/4) BB	(1/4) WR (1/4) WB (1/4) RR (1/4) RB	(1/4) WR (1/4) WB (1/4) RB (1/4) BB	(1/2) WR (1/2) WB
BB/XY	(1/2) DB (1/2) RB	(1) RB	(1/2) RB (1/2) BB	(1/2) WB (1/2) RB	(1/2) WB (1/2) BB	(1) WB
WR/XY	(1/4) WD (1/4) WR (1/4) DR (1/4) RR	(1/2) WR (1/2) RR	(1/4) WR (1/4) WB (1/4) RR (1/4) RB	(1/4) WW (1/2) WR (1/4) RR	(1/4) WW (1/4) WR (1/4) RB (1/4) WB	(1/2) WW (1/2) WR
WB/XY	(1/4) WD (1/4) WR (1/4) DB (1/4) RB	(1/2) WR (1/2) RB	(1/4) WR (1/4) WB (1/4) RB (1/4) BB	(1/4) WW (1/4) WR (1/4) WB (1/4) RB	(1/4) WW (1/2) WB (1/4) BB	(1/2) WW (1/2) WB
WW/XY	(1/2) WD (1/2) WR	(1) WR	(1/2) WR (1/2) WB	(1/2) WR (1/2) WW	(1/2) WB (1/2) WW	(1) WW
DD/XX	(1/2) DR/XX (1/2) DD/XX	(1) DR/XX	(1/2) DR/XX (1/2) DB/XX	(1/2) WD/XX (1/2) DR/XX	(1/2) WD/XX (1/2) DB/XX	(1) WD/XX
WD/XX	((1+e)/4) DD/XX ($(1+e+\rho_R)/4$) DR/XX ($\rho_R/4$) RR/XX ($\rho_B/4$) DB/XX ($\rho_B/4$) RB/XX	((1+e)/2) DR/XX ($\rho_R/2$) RR/XX ($\rho_B/2$) RB/XX	((1+e)/4) DR/XX ((1+e)/4) DB/XX ($\rho_R/4$) RR/XX ($(\rho_R+\rho_B)/4$) RB/XX ($\rho_B/4$) BB/XX	((1+e)/4) WD/XX ((1+e)/4) DR/XX ($\rho_R/4$) WR/XX ($\rho_R/4$) RR/XX ($\rho_B/4$) WB/XX ($\rho_B/4$) RB/XX	((1+e)/4) WD/XX ((1+e)/4) DB/XX ($\rho_R/4$) WR/XX ($\rho_R/4$) RB/XX ($\rho_B/4$) WB/XX ($\rho_B/4$) BB/XX	((1+e)/2) WD/XX ($\rho_R/2$) WR/XX ($\rho_B/2$) WB/XX
DB/XX	(1/4) DD/XX (1/4) DR/XX (1/4) DB/XX (1/4) RB/XX	(1/2) DR/XX (1/2) BR/XX	(1/4) DR/XX (1/4) DB/XX (1/4) RB/XX (1/4) BB/XX	(1/4) WD/XX (1/4) WB/XX (1/4) DR/XX (1/4) RB/XX	(1/4) WD/XX (1/4) WB/XX (1/4) DB/XX (1/4) BB/XX	(1/2) WD/XX (1/2) WB/XX
BB/XX	(1/2) DB/XX (1/2) RB/XX	(1) RB/XX	(1/2) RB/XX (1/2) BB/XX	(1/2) WB/XX (1/2) RB/XX	(1/2) WB/XX (1/2) BB/XX	(1) WB/XX

Fig. S10. **Crosses representing the inheritance pattern of a CRISPR-based homing system targeting the *tra* locus in *C. capitata*.** "D" denotes the drive allele, *tra*^{nCHE}, "W" denotes the wild-type tra allele, "R" denotes a drive-resistant allele with an in-frame internal deletion at the *tra* locus, *tra*^{Rst} and "B" denotes a drive-resistant null allele *tra*$^{-}$. *C. capitata* is an XY species in which female development requires presence of the tra allele, hence XY individuals are fertile males, XX individuals with a functioning *tra* allele (i.e. having the genotypes WW, WR, WB, RR, DR and RB) are fertile females, and XX individuals without a functioning *tra* allele (i.e. having the genotypes DD, DB and BB) are fertile males. The only exception is WD/XX individuals, which are infertile intersex individuals, unless a uniform somatic destruction of the *tra* locus in all cells is guaranteed by using multiple guide RNAs and an early embryogenic promoter such as *Sry-α* promoter. Homing is only manifest in WD heterozygotes, whereby WD individuals produce D gametes in the germline at a frequency equal to $(1+e)/2$, where e denotes the proportion of W gametes that are converted into D gametes through the act of homing. Homing-resistant alleles may be generated during the process of DNA cleavage and repair whereby WD individuals produce resistant alleles that are in-frame indels, R, at a rate equal to $\rho_R/2$, and produce cleavage resistant alleles that are out-of-frame or other mutations, B, at a rate equal to $\rho_B/2$. Crosses involving WD/XX males are shaded out as WD/XX individuals may be rendered either infertile intersex or fertile males as described above. Offspring are half XX and half XY. The inheritance pattern of the homing and resistant alleles depicted here is incorporated into the population dynamic model described above and in Fig. S11.

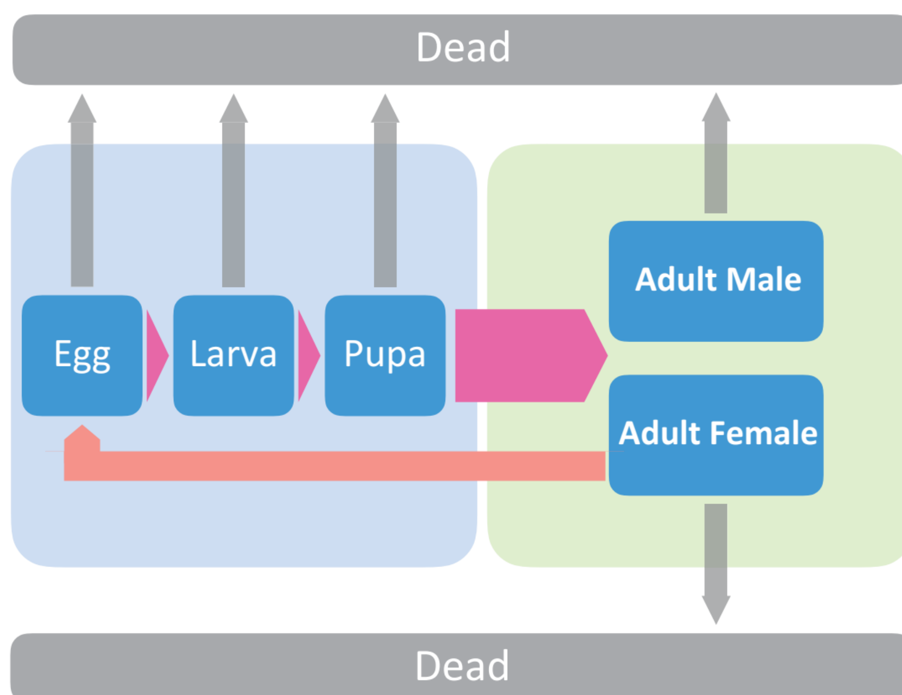


Fig. S11. Population dynamic model of *C. capitata* Eggs develop into larvae which develop into pupae, some of which develop into adult males and some into adult females. Death can occur at any life stage, and adult females lay eggs following fertilization. Additional density-dependent mortality occurs at the larval stage. Parameter values are provided in Table S2 and the equations describing the equivalent implementation of this model for *An. gambiae* are included in Supplementary File S1 of Marshall *et al.*, 2017 (163).

Table S1. Parameter values for population genetic/ dynamic model for *C. capitata*.

Symbol:	**Parameter**:	**Value**:	**References**:
Primary parameters:			
β	Egg production per adult female	20 /day	Diamantidis *et al.*, 2011
T_E	Duration of egg stage	2 days	Diamantidis *et al.*, 2011
T_L	Duration of larval stage	6 days	Diamantidis *et al.*, 2011
T_P	Duration of pupal stage	10 days	Diamantidis *et al.*, 2011
m_M	Death rate of adult stage	0.1 /day	Carey *et al.*, 2005 (167)
r_M	Population growth rate (in absence of density-dependent mortality)	1.031 /day	Nyamukondiwa *et al.*, 1980 (165)
c	Probability of CRISPR-mediated cleavage in WD heterozygote	1.0	Champer *et al.*, 2017 (163)
P_{HDR}	Probability of accurate homology-directed repair given cleavage	0.90	This paper
Variable parameters:			
θ	Proportion of resistant alleles that are in-frame internal deletions	[1/3, (1/3) x 10^{-4}]	This paper
N	Equilibrium adult medfly population size (male and female)	[10^3, 10^6]	Diamantidis *et al.*, 2011 (166)

Table S2. List of primers used in this study

ID	Name	Sequence
MK024	DTra_F	CGGCGACAAGCTTGAGGTACCCACTATATAGTATAAC
MK025	DTra_R	CTATAGGGCGAATTGTGTAGCCAAATCGCGGAACTC
MK037	G1-S	CTTCAGCAGTGGAACCCAGCATCG
MK038	G1-AS	AAACCGATGCTGGGTTCCACTGCT
MK041	G3-S	CTTCGATCGGTTATACTATATAGT
MK042	G3-AS	AAACACTATATAGTATAACCGATC
MK043	G4-S	CTTCGTGCTGGGTTCCACTGCTGT
MK044	G4-AS	AAACACAGCAGTGGAACCCAGCAC
MK048	G5-S	CTTCTATAGTGGGTACCTCGATGC
MK049	G5-AS	AAACGCATCGAGGTACCCACTATA
MK058	Tra_T7endo_F	CCTGCTAATTCTGCTTTCCCTATGTTTGTG
MK059	Tra_T7endo_R	CCTCGTCTGCAAAGTACGGAATCTTGTG
MK060	attB-SmaI-S	CTAGCCGCGGTGCGGGTGCCAGGGCGTGCCCTTGGGCTCCCCGGGGAGCCCAA GGGCACGCCCTGGCACCCGCACCGCGG
MK061	attB-SmaI-AS	AGCTCCGCGGTGCGGGTGCCAGGGCGTGCCCTTGGGCTCCCCGGGGAGCCCAA GGGCACGCCCTGGCACCCGCACCGCGG
MK072	aTubE1_F	CCAAGTGACCGCGGATCTTCATATTCGTTTTACGTTTGTCAAGCCTC
MK073	aTubE1_R	TCGTGGTCCTTATAGTCCATATTGAGTTTTTATTGGAAGTGTTTCAC
MK075	aTubI1GT_R	TCGTGGTCCTTATAGTCCTCAACCTGTGGATGAGGAGGAAGGGAAAACGGATG
MK076	Cas9DYK_F	GACTATAAGGACCACGACGGAGACTACAAGGATCATG
MK077	Cas9_R	GATCTAGATCTGCGGCCGATCACTAGATTACTTTTTCTTTTTTGCCTG
MK078	HMA_F1	CGGTCACACTGAGGAAAGTG
MK079	HMA_R1.1	CAACAAAAAGATGGCACTGG
MK085	Cas9_SeqR1	TGGTGCTCGTCGTATCTC
MK086	Cas9_SeqR2	TTGATAATTTTCAGCAGATCGTG
MK087	Cas9_SeqR3	CTTGTTGTCGATGGAGTC
MK088	Cas9_SeqR4	CAGCACAGAATAGGCCAC
MK116	3xP3attP_F	ACTGGGGTAACCTTTGAGTTCTCTCAGTTGGGGGCGTAGGGGGGATTATTCATT AGAGAC
MK117	SV40attP_R	GGGGTAACCTTTGAGTTCTCTCAGTTGGGGGCGTAGGGATGATGAGTTTGGACA AACCAC
MK122	Transformer4_UR	CAAAGGTTACCCCAGTTGGGGCACTACTCTGTCGGCATCCATTTTCATC
MK123	Transformer4_DF	CTCAAAGGTTACCCCAGTTGGGGCACTACTGCAGTGGAACCCAGCATCGAG
MK126	Tra_HRCheck_F	CCGACCGAATCGTGAGGACTTGAAG
MK127	Tra_HRCheck_R	GAATTAAGTAACTTCCACTTCCTAACTCGTGTGAC
MK128	XFPct_F	AACGAGAAGCGCGATCACATGGTC
MK129	XFPnt_R	ACGCTGAACTTGTGGCCGTTTACGTC
MK134	attBaTubE1_F	GGGCGCGTACTCCACCTCACCTAGGTGACTCGAGTTCATATTCGTTTTACGTTT GTC
MK135	Cas_attBSmaI_AscIR	GGGCGCGTACTCCACCTCACGGCGCGCCATTACTTTTTCTTTTTTGCCTG
MK138	DPa180_AvrIIF	AATAACCTAGGTGGTGATCATTGTTCTTTCTTACTTGGTG
MK139	DPa180_XhoIR	TAATCCTCGAGTAATAATTTCCCCGTGTTGTGCTG
MK140	Rcd1r_XbaIF	AATAATCTAGACACGGCCAAATCGATGCAGAC
MK141	Rcd1r_XhoIR	TAATCCTCGAGGTTAGCTTGCAAAGATCTAGTAG
MK142	Srya_AvrIIF	AATAACCTAGGGCCACCAGCAGTTCAAGACCAAG
MK143	Srya_XhoIR	TAATCCTCGAGTATCAGATGTGCTCCGGGAAACAG
MK144	bTub3UTR_F	AAAAGTAATGGCGCGATTAACTTCCCACTCAAGATCAC
MK145	bTub3UTR_R	CGCTTAATGCGTATGGTTTAGGTTTATGCAATGCCT
MK146	U63P_F	CTGTTTTGCTCACCTGTGATTGCTCCTACTC
MK147	U63DS_R	CATACGCATTAAGCGAACATTAAAAAGATG
MK148	3xP3attB_F	CCACCTCACGGCGCGGGGGATTATTCATTAGAGAC
MK149	SV40toU63_R	AGGTGAGCAAAACAGGATGATGAGTTTGGACAAACCAC
MK153	PhiC31_BsaI_F	ATGGTCTCACATGGACACGTACGCGGGTGCTTACGAC

MK154	PhiC31_NotI_R	GTGTATGCGGCCGCTTACTAGGCAGCTACGTCTTC
MK155	HRMA_OT1F	GGACCAGGAGCGTTATCTG
MK156	HRMA_OT1R	GGCAAATTGATGTCGAGCAC
MK157	HRMA_OT2F	CCATATCCGACCTGACCAC
MK158	HRMA_OT2R	CGGTTGCTGTTCCGTTTC
MK159	HRMA_OT3F	CAGCTTGTTGTCCTCGATG
MK160	HRMA_OT3R	GTGGCAGACCGAATCCAG

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3.2 Hyperactive *piggyBac* transposase improves transformation efficiency in diverse insect species

The use of transposon-based transformation vectors remains to be the first strategy to try when starting transgenesis work in a new insect organism. The versatile *piggyBac* transformation is widely used in many biological systems. This chapter presents tremendous improvement in *piggyBac* germline transformation in three different insect species from two different orders by using a mutated hyperactive *piggyBac* transposase. The important contribution of this work is correcting the previous perception that the hyperactive *piggyBac* transposase does not improve the efficiency of germline transformation. This is particularly relevant to those who are trying to develop biotechnological pest control strategies based on transgenesis.

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Authors contributions as stated in the published paper:
E.A.W., K.N.E., and S.D. conceived and designed the research; S.D., K.N.E., and M.K. designed the helper plasmids; K.N.E., M.K., P.K. and C.E.O. constructed the plasmids; K.N.E., M.K., and **H.M.M.A**. designed the experiments and performed the injections; K.N.E, M.K., **H.M.M.A**., and M.D.I performed the crossings and screenings; K.N.E., S.D., and **H.M.M.A**. performed the inverse PCR and K.N.E. analyzed the sequence data; K.N.E, S.D., and E.A.W. wrote the manuscript; K.N.E prepared the figures and tables; All authors edited and approved the final version of the manuscript.

My specific contributions were particularly to Table 1 and Fig. 1 with a minor contribution to Table 4.

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Hyperactive *piggyBac* transposase improves transformation efficiency in diverse insect species

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Abstract

Even in times of advanced site-specific genome editing tools, the improvement of DNA transposases is still on high demand in the field of transgenesis: especially in emerging model systems where evaluated integrase landing sites have not yet been created and more importantly in non-model organisms such as agricultural pests and disease vectors, in which reliable sequence information and genome annotations are still pending. In fact, random insertional mutagenesis is essential to identify new genomic locations that are not influenced by position effects and thus can serve as future stable transgene integration sites. In this respect, a hyperactive version of the most widely used piggyBac transposase (PBase) has been engineered. The hyperactive version (hyPBase) is currently available with the original insect codon-based coding sequence (*ⁱhyPBase*) as well as in a mammalian codon-optimized (*ᵐhyPBase*) version. Both facilitate significantly higher rates of transposition when expressed in mammalian *in vitro* and *in vivo* systems compared to the classical PBase at similar protein levels. Here we demonstrate that the usage of helper plasmids encoding the hyPBase - irrespective of the codon-usage - also strikingly increases the rate of successful germline transformation in the Mediterranean fruit fly (Medfly) *Ceratitis capitata*, the red flour beetle *Tribolium castaneum,* and the vinegar fly *Drosophila melanogaster*. hyPBase-encoding helpers are therefore highly suitable for the generation of transgenic strains of diverse insect

orders. Depending on the species, we achieved up to 15-fold higher germline transformation rates compared to PBase and generated hard to obtain transgenic *T. castaneum* strains that express constructs affecting fitness and viability. Moreover, previously reported high sterility rates supposedly caused by hyPBase (iPB7), encoded by *ihyPBase,* could not be confirmed by our study. Therefore, we value hyPBase as an effective genetic engineering tool that we highly recommend for insect transgenesis.

- Keywords: Coleoptera; molecular entomology; Tephritid fruit flies; transgenics; transposon.

Introduction

Class II DNA transposases are enzymes that are utilized as genetic tools based on their ability to translocate DNA fragments by a "cut-and-paste-like" mechanism. The *piggyBac* transposase (PBase), isolated from a mutant Baculovirus strain in the cabbage looper moth *Trichoplusia ni* (Cary et al., 1989; Fraser et al., 1995; Handler and Harrell, 1999), is widely recognized for its broad range of targetable species and its ability to integrate large DNA cargo (Ding et al., 2005; Kahlig et al., 2010; Li et al., 2011). *piggyBac*-based elements can be excised without leaving a footprint, thus restoring the genomic locus to its pre-transposition state of the original TTAA target sequence (Elick et al., 1996). These features opened new possibilities for successful manipulation of various mammalian somatic and stem cell lines (Saridey et al., 2009; Wang et al., 2008; Yusa et al., 2009) and successful germline transformation in a vast variety of species including the mouse (Ding et al., 2005) as well as species of holometabolous and hemimetabolous insects (Berghammer et al., 1999; Handler et al., 1998; Nakamura et al., 2010; Tamura et al., 2000). This versatility led to its use in a wide range of scientific fields such as insect pest and disease vector control (Fu et al., 2010; Schetelig et al., 2009), gene or enhancer trap experiments (Bonin and Mann, 2004; Horn et al., 2003), the induction of pluripotent stem cells (Woltjen et al., 2009), or gene therapy (Wilson et al., 2007). Another distinguishing quality of PBase is the possibility to generate chimeric fusion constructs with e.g. DNA-binding domains to target specific genomic loci without hampering the transposition efficiency (Owens et al., 2012).

Despite the fact that there have been substantial variations in the reported relative transposition efficiencies and germline transformation rates of PBase throughout diverse target species and cell lines, direct comparisons of PBase with other transposases – including the hyperactive versions SB11 and SB12 of the previously widely used transposase Sleeping Beauty (SB) – affirmed PBase to be the most effective (Wilson et al., 2007; Wu et al., 2006). Moreover, applications in mammalian systems highly benefited from the adjustment of the codon-usage from the original – by default insect codon-based – *iPBase* to a mammalian codon-optimized *mPBase* version of the *piggyBac* coding sequence (CDS). This elevated the transposition

efficiency up to twenty times due to increased levels of PBase protein (Cadiñanos and Bradley, 2007). Furthermore, Yusa et al. (2011) generated a hyperactive version of PBase termed hyPBase*,* which carries seven amino acid substitutions that were implemented into the ^{m}*PBase* CDS background (^{m}*hyPBase*). Expression of ^{m}*hyPBase* in mouse embryonic stem cells demonstrated an additional tenfold increase in the transposition rate when compared to ^{m}*PBase* (Yusa et al., 2011). Besides, examination of ^{m}*hyPBase,* and the insect codon-based equivalent ^{i}*hyPBase,* confirmed the hyperactivity when compared to their wild-type counterparts for several human cell lines *in vitro* and mouse liver cells *in vivo* (Burnight et al., 2012; Doherty et al., 2012).

First results in our laboratory using ^{m}*hyPBase* driven by the *Drosophila melanogaster (Dm) heat-shock protein 70* regulatory region (*Dm-hsp70*) (Lis et al., 1983) for germline transformation in the agricultural pest *Ceratitis capitata* (*Cc*) and the storage pest and emerging coleopteran model organism *Tribolium castaneum* (*Tc*)*,* supported the literature and gave rise to approximately fourfold elevated transformation rates. This effect was even more prominent when utilizing the *T. castaneum* endogenous *heat-shock protein 68* upstream region (*Tc-hsp68*) (Schinko et al., 2012) for germline transformation in *T. castaneum* (Dippel, 2016). Consequently, hyPBase has been established in our laboratory as a standard tool. To our surprise, the publication by Wright et al. (2013) reported low transformation efficiency and high sterility rates when co-injecting the ^{i}*hyPBase* helper for germline transformation in *Drosophila melanogaster* and *Aedes aegypti*.

Because of these disparate experiences to deploy hyPBase for transgenesis in insects, we decided to conduct a systematic comparison and analysis of the performance of expressed ^{i}*PBase*, ^{i}*hyPBase,* and ^{m}*hyPBase* for germline transformation in *C. capitata*, *T. castaneum,* and *D. melanogaster*. Our data from seven large-scale injection-sets with various donor plasmids confirmed our initial observations of substantially increased transformation efficiencies and could not detect any correlations between the use of hyPBase and elevated sterility rates.

We decided to use the indices $^{\mathbf{i}}$ or $^{\mathbf{m}}$, respectively, to ease the discrimination of mammalian and insect codon-optimized coding sequences of the wild-type (PBase) or hyperactive (hyPBase) transposase protein. Various nomenclatures and abbreviations have been used in the published *piggyBac* research, which are summarized in the materials and methods

Materials and methods

Nomenclature of wild-type and hyperactive *piggyBac* transposases and respective coding sequences

The wild-type ^{i}*PBase* CDS was isolated from the cabbage looper moth and is therefore by default insect codon-based and was previously also abbreviated as *pBac* (Handler and Harrell, 1999), *PB, PBase* (Ding et al., 2005), *iPB* (Cadiñanos and Bradley, 2007), *iPBase* (Yusa et al., 2011), *pB* and *ipB* (Doherty et al., 2012).

^{m}*PBase* is the mammalian codon-optimized version of ^{i}*PBase* and both encode the same wild-type PBase protein. ^{m}*PBase* was previously also abbreviated as *mPB* (Cadiñanos and Bradley, 2007) *mpB* (Doherty et al., 2012) and *mPBase* (Yusa et al., 2009, 2011).
^{i}*hyPBase* stands for the insect codon-based CDS of the hyperactive transposase hyPBase and was previously also abbreviated as *i7pB, i7piggyBac* (Doherty et al., 2012) and *iPB7* (Burnight et al., 2012).
^{m}*hyPBase* stands for the mammalian codon-optimized CDS of the hyperactive transposase hyPBase and was previously also abbreviated as *hyPBase* (Yusa et al., 2011), *m7pB, m7piggyBac* (Doherty et al., 2012) and *hypPB* (Burnight et al., 2012). Again, both ^{i}*hyPBase* and ^{m}*hyPBase* encode the same hyPBase transposase.

Plasmid construction

Helper plasmids

The *NC-iPB7* plasmid containing the ^{i}*hyPBase* CDS (Doherty et al., 2012; Burnight et al., 2012) was purchased from Transposagen Biopharmaceuticals, Inc. (Lexington, U.S.A.). Sequencing of the received plasmid revealed an undesired "eighth" mutation (V336A) that we re-mutated by site directed mutagenesis of whole plasmids as previously described (Laible and Boonrod, 2009), using the primers *iPB-7_CtoT_F* and *iPB-7_CtoT_R*, prior to further usage of the CDS for subsequent cloning steps. Primer sequences are listed in Supplementary Table 1.
The helper plasmid *Tc-^{m}hyPBase (pSL-fa_Tc-hsp_5'UTR_^{m}hyPBase_3'UTR_fa)* was generated by cloning the ^{m}*hyPBase* CDS from *pCMV-hyPBase* (Yusa et al., 2011) with *Kpn*I/*Xho*I into *pSL-fa_Tc-hsp68_5'_3'UTR_fa* (Schinko et al., 2012) between the *T. castaneum heat-shock protein 68* upstream region including the 5' UTR and the 3'UTR. Furthermore, an undesired *Nco*I-site in the backbone of *Tc-^{m}hyPBase* was removed by *Bst*BI/*Sma*I digest, blunting with T4 DNA polymerase, and re-ligation with T4 DNA ligase. To create *Tc-^{i}hyPBase* and *Tc-^{i}PBase* corresponding transposase CDS was amplified with primers *piggyBac-NcoiFor*/ *piggyBac-NotIrev* from templates *remutated_NC-iPB7* and *phsp-pBac* (Handler and Harrell, 1999), respectively, and was subsequently cloned with *Nco*I/*Not*I into *Tc-^{m}hyPBase,* replacing the ^{m}*hyPBase* CDS.
The helper plasmid *Dm-^{m}hyPBase* was created by cloning the ^{m}*hyPBase* CDS with *Eco*RI/*Not*I into the plasmid *pSLfaHSfa* (Ramos et al., 2006) between the upstream region plus 5' and 3' UTR of the *D. melanogaster heat-shock protein 70.* Helper plasmids *Dm-^{i}hyPBase* and *Dm-^{i}PBase* were cloned analogously to *Tc-^{i}hyPBase* and *Tc-^{i}PBase.*

Donor plasmids

The donor plasmid *PK01 (pBac{3xP3-gTcv;Tc'aTub1P-Tc'H2Av-EGFP}af)* was used without further modifications (Kitzmann, 2016). To generate the donor plasmid *PK13 (pBac{3xP3-gTcv;alphaTubP-GAP43-mcherry}af)*, the chimeric CDS of the *growth associated protein-43* fused to *mCherry* was amplified via PCR from plasmid *pCS2+{GAP43-mCherry}* (a kind gift from Jubin Kashef, University Medical Center of Goettingen), using the primers *PK124_FseIGAP43Fw* and *PK122_mCherryAscIRv*. The PCR product was subsequently cloned with *Asc*I/*Fse*I into *PK01*, between the *Tc-α-tubulin1* promoter (5') and the *SV40PolyA* site (3').

Plasmids *pMK007* and *KNE006* were assembled with the *In-Fusion® HD Cloning System* (Takara Bio Europe/ Clontech., Stain-Germain-en-Laye, France) according to the manufacturer's protocol. To create plasmid *pMK007 (pBac{3xP3-DsRed_5xQUAS-tGFP}),* the *QUAS* and *tGFP* CDS were amplified from *pQUAST* (Potter et al., 2010) and *pSL-fa_UAS_Tc-bhsp_tGFP* (Schinko et al., 2010) with primers *QUAS_F*/ *QUAS_R* and *Hsp68_F*/ *tGFP_R*, respectively. The two fragments were then cloned simultaneously into the *Avr*II-linearized plasmid *#707 pBac{3xP3-DsRed>af>}* (Horn et al., 2003) via the *In-Fusion®* reaction. *pQUAST* was a gift from Liqun Luo (Addgene plasmid # 24349).

To generate plasmid *KNE006* (*pBac{attP_TREhs43-mCherry_PUbEGFP}*), the *TREhs43* and *mCherry_SV40* fragments were amplified from *KNE008 pJFRC_20xUAS_Actin5c_mCherry_SV40* and *#1402 pBac{fa_attP_TREhs43-ATGCctra-hidAla5_PUb-nls-EGFP}* (Ogaugwu et al., 2013) with primers *IF_TRE_hs43_F*/ *IF_TRE_ hs43_KS_R* and *IF_Cherry_SV40_F/ IF_Cherry_SV40_R,* respectively. The two fragments were then cloned simultaneously into the *Asc*I-linearized plasmid *#1402* via the *In-Fusion®* reaction. To generate *KNE008* the *Actin5C* regulatory region and *mCherry* fragment were amplified from *pAC-GAL4* (Potter et al., 2010) and *pcDNA3.1/hChR2(H134R)-mCherry* (Zhang et al., 2007) with primers *IC102/ IC83* and *IC86/ IC91*, respectively. The two fragments were then cloned simultaneously into the *Aat*II/ *Bam*HI linearized plasmid *pJFRC7-20XUAS-IVS-mCD8::GFP* (Pfeiffer et al., 2010) via the *In-Fusion®* reaction. Plasmids *pAC-GAL4, pcDNA3.1/hChR2(H134R)-mCherry* and *pJFRC7-20XUAS-IVS-mCD8::GFP* were gifts from Liqun Luo (Addgene plasmid # 24344), Karl Deisseroth (Addgene plasmid # 20938), and Gerald Rubin (Addgene plasmid # 26220), repectively.

To create plasmid *KNE007 (pXLII{Dmß2tubulin-tTA_PUb-DsRed_attP}),* the *D. melanogaster ß2-tubulin* upstream region plus 5'UTR was PCR amplified from *pCRII[ß2-tubulinP]* (Michiels et al., 1989) with primers *B2Tub-F*/ *B2Tub-R,* which added an *Avr*II and *Xba*I cut site to the 5' and 3' of the amplicon, respectively. The *AvrII_Dmß2tubulin_XbaI* fragment and *#437 pXLII_attP_PUb-DsRedT3_Ccvas-tTA* (Schetelig and Handler, 2013) plasmid were restriction digested with *Avr*II/ *Xba*I and

subsequently ligated. *#437* was a gift from Marc Schetelig (Justus-Liebig-University Giessen, Germany).

For *KNE017 (pXLII{ACP70A-tTA; PUb-DsRed_attP})*, the *ACP70A* upstream region plus 5'UTR was amplified from genomic DNA with primers *AP70AgeIF*/ *AP70NheIIR* which added an *Age*I and *Nhe*I cut-site to the 5' and 3' of the amplicon, respectively. The *AgeI_Dm-ACP70A_NheI* fragment and *KNE007* plasmid were restriction digested with *Age*I and *Nhe*I and subsequently ligated.

To generate *#1413 (pBac{Ccß2tubulin-tTA_PUb-DsRed}),* the *Ccß2tubulin-tTA* fragment was excised with *Bgl*II/*Asc*I from *#1412 pSL_Ccß2tubulin-tTA* and subsequently ligated into *Bgl*II/*Asc*I cut *#1200 pBac{fa_PUb-DsRed}* (Scolari et al., 2008). To create *#1412,* the *Ccß2tubulin* upstream region plus 5' UTR was PCR amplified from *#1228 pSLaf_Ccß2t-tGFP-SV40_af* (Scolari et al., 2008) with primers *co109*/ *co110,* which added an *Nco*I and *Xba*I cut-site to the 5' and 3' of the amplicon, respectively. The *NcoI_Cc-ß2-tubulin_XbaI* fragment was then cloned with *Nco*I/*Xba*I into the plasmid *#1225 pSLaf_sryα2-tTA-SV40_af* (Schetelig, 2008).

DNA preparation

Helper and donor plasmids were precipitated individually. To 90 µl of an aqueous plasmid solution, containing 50 µg DNA, 10 µl 3M NaAc and 900 µl ice-cold EtOH were added and incubated over night at -80°C. Following centrifugation (16000 rcf, 4°C, 30 min) the DNA pellet was washed with 70% ice-cold EtOH and again centrifuged (16000 rcf, 4°C, 15 min), before being dried and redesolved in de-ionized H_2O. Helper and donor plasmids were mixed in a ratio of 300 ng/µl to 500 ng/µl, respectively, in 1x injection buffer (5 mM KCl, 0.1 mM NaH_2PO_4, ph 6.8, 5% phenol red) (Bachmann and Knust, 2008) and filtered using Millex®-HV 0.45 µm (Merck Millipore, Billerica, U.S.A.).

Insect strains

Experiments in *C. capitata* were conducted with the *Egypt-II (EgII)* wild-type strain which was obtained from the FAO/IAEA Agriculture and Biotechnology Laboratory (Entomology Unit, Seibersdorf, Austria). For experiments in *T. castaneum,* the white-eyed Tc-*vermillion*^{white} *(*v^w*)* strain was used (Lorenzen et al., 2002), whereas experiments regarding *D. melanogaster* were performed in the *Oregon-R* wild-type strain. All strains of the different species were maintained under their respective standard relaxed artificial rearing conditions (Brown et al., 2009; Roberts, 1998; Saul, 1982).

Germline transformation

All experiments – injection, screening, and recording of the data – were performed double-blind regarding which helper plasmid was used in the injection subsets. To reduce the impact of technical errors, which could induce additional lethality and sterility, we decided to inject only moderate volumes into the embryos. This is in contrast to experiments that do not aim

for a systematic comparison but only seek for the highest possible transformation rate with the minimum effort for subsequent crossing and screening.

Germline transformation in Ceratitis capitata and Drosophila melanogaster

Germline transformation in *C. capitata* and *D. melanogaster* was carried out based on the principles of the previously described procedures (Bachmann and Knust, 2008; Handler et al., 1998; Spradling and Rubin, 1982) unless stated otherwise. Embryos were collected in a time interval of 30 minutes and were subsequently de-chorionated for 3 minutes in a 50% Klorix solution containing 2.5% sodium hypochlorite (DanKlorix, CP GABA GmbH, Hamburg, Germany), briefly washed in washing buffer (100 mM NaCl, 0.02% Triton X-100), washed thoroughly with double-deionized H_2O and left for 5-10 min in double-deionized H_2O to increase the internal pressure, before being fixed on double sided tape (Scotch™ Brand/ 3M, St. Paul, USA) and covered with a thin layer of Voltalef 10S oil (Lehmann & Voss & Co., Hamburg, Germany). Microinjections were performed using a FemtoJet® Microinjector (Eppendorf, Hamburg, Germany) and needles made from 10 mm x 1 mm quartz capillaries (Sutter Instrument, Novato, U.S.A.) using a P-2000 micropipette puller (Sutter Instrument, Novato, U.S.A.) applying the following settings: Heat= 750, Fil= 4, Vel= 50, Del= 125, PUL=175. Needles were opened and sharpened using a microelectrode beveler (Bachofer GmbH, Reutlingen, Germany). All steps post embryo collection were done at 18°C. After injection, the oil was partially drained and *D. melanogaster* embryos were kept until hatching at 25°C and *C. capitata* embryos at 18°C. A subset of *D. melanogaster* embryos were heat-shocked twenty hours after injection at 37°C for 1 hour. Hatched larvae were transferred into the respective larval diet at the respective temperature according to standard laboratory rearing conditions.

Germline transformation in *Tribolium castaneum*

Germline transformation in *T. castaneum* was carried out based on the principles of the previously described procedure (Berghammer et al., 1999). Embryos were collected in a time interval of 1 hour and kept for one more hour at RT. The up to two-hours-old embryos were washed twice for 30 seconds in a 1% Klorix solution (equivalent to 0.05% sodium hypochlorite) (DanKlorix, CP GABA GmbH, Hamburg, Germany), and let dry for 5 minutes after aligning them into a row. Microinjection was performed using a FemtoJet® Microinjector (Eppendorf, Hamburg, Germany) and needles made from 10 mm x 1 mm borosilicate capillaries (Hilgenberg GmbH, Malsfeld, Germany) using a P-2000 micropipette puller (Sutter Instrument, Novato, U.S.A.) applying the following settings: Heat= 350, Fil= 4, Vel= 50, Del= 225, PUL=150. Needles were opened and sharpened using a microelectrode beveler (Bachofer GmbH, Reutlingen, Germany). All steps post embryo collection were done at RT. After injection, the embryos were placed onto an apple agar plate in a sealed box at 30°C for 72 hours. Hatched larvae were transferred into vials with whole-wheat flour at 30°C according to standard laboratory rearing conditions.

Crossings and screening

Irrespective of the species, G_0 animals were backcrossed individually to three virgin animals of the opposite sex. Crosses were monitored regularly to differentiate between sterile G_0 animals and those that have died. F_1 offspring were anesthetized with CO_2 and screened under a Leica M205 FA fluorescent stereo microscope (Leica, Wetzlar, Germany).

Inverse PCR and sequence analysis

To determine the genomic location of the *piggyBac* insertions and investigate for possible multiple insertions, inverse PCR (iPCR) was in principle carried out as described (Horn et al., 2003; Huang et al., 2000). The following modifications were introduced: genomic DNA was purified from approx. 10 adult *D. melanogaster* flies of each tested line, using the NucleoSpin® DNA Insect Kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany); isolated genomic DNA was restriction digested with *Msp*I or *Mbo*I for the 5' junction and *Bst*I or *Hind*III for the 3' junction, respectively (Supplementary Table 2). Resulting DNA fragments were self-ligated and circularized DNA was used as template for the first PCR with primers *iPCR_5pBac_F/ iPCR_5pBac_R* for the 5' junction and primers *iPCR_3pBac_F/ iPCR_3pBac_R* for the 3' junction. An aliquot of this first PCR reaction served as direct template for subsequently conducted nested PCRs, carried out with primers *iPCR_5pBac_F_nested/ iPCR_5pBac_R_nested* and primers *iPCR_3pBac_F_nested/ iPCR_3pBac_R_nested*, respectively (Supplementary Table 1). Distinct DNA bands were obtained by electrophoresis and DNA was purified from cut gel slices using the NucleoSpin® Gel and PCR Clean-up Kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany). The isolated DNA was Sanger-sequenced with either primer *5'-PB-SEQ* for the 5' junction or *3'-PB-SEQ* for the 3' junction (Supplementary Table 1). Sequences were used for BLAST search against the *D. melanogaster* genome (genome version: dmel_r6.20_FB2018_01; http://flybase.org/blast/).

Results

For a systematic comparative analysis of expressed *^{i}PBase*, *^{i}hyPBase* and *^{m}hyPBase* in germline transformation, we generated two independent sets of the three helper plasmids for co-injection along with *piggyBac* donor plasmids in either *C. capitata* and *D. melanogaster* or *T. castaneum*. To be able to offer an objective comparison, helper plasmids within one set are identical except for the respective transposase CDS. For embryonic injections in *Ceratitis* and *Drosophila* we used the same set of helper plasmids which all have the *Dm-hsp70* upstream region driving the expression of the transposase, whereas the *Tribolium* helper possess the endogenous *Tc-hsp68* upstream region. To enable reliable screening for transgenic F_1 offspring, all donor plasmids contained a fluorescent eye or body marker.

Performance of the hyperactive *piggyBac* transposase variants in *C. capitata*

The analysis of the germline transformation experiments conducted with the three different helper plasmids *Dm-^{i}PBase*, *Dm-^{i}hyPBase*, and *Dm-^{m}hyPBase* in combination with either of the different donor plasmids (#1413 and KNE006) in *C. capitata* (Table 1) revealed a substantially increased transformation rate (Figure 1A) for the experiments with both helper plasmids encoding the hyPBase (12.5% to 16%) compared to the wild-type PBase (0%). The codon usage of the hyperactive *piggyBac* helper plasmids had no significant influence on its performance. Furthermore, in all experiments about two-thirds (62.5% to 72.4%) of the flies reaching adulthood produced offspring, with no obvious differences between the different helper plasmids (Figure 1B, n=228), which indicates no influence of the hyperactive *piggyBac* on the fertility rate.

The activity of the hyperactive *piggyBac* variants in *T. castaneum*

The systematic analysis of the germline transformation experiments in *T. castaneum* Table 2), which were performed using the helpers *Tc-^{i}PBase*, *Tc-^{i}hyPBase*, and *Tc-^{m}hyPBase* in combination with either of the three different donor plasmids (*PK13, PK01*, and *pMK007*), showed a substantial and at least doubled increase in the transformation rate by the hyPBase helpers (17.6% to 39.4%) compared to the wild-type *PBase* (0% to 15%), and therefore confirmed our preliminary observations as well as the results from *C. capitata*. Interestingly, we found that both hyperactive helpers remarkably improved the transformation rate of constructs *PK13* and *PK01*, which were previously difficult to transform in our lab. Also in our initial experiment, we failed to generate transgenic beetles using the wild-type helper (*Tc-^{i}PBase*). Only by repeating one injection set (Figure 2A, *PK13*, second column) in a much larger scale, we eventually achieved a transformation rate of 1.6%. In comparison to the over 20% transformation rate obtained with both hyperactive helpers, this clearly indicates an enormous improvement and offers the possibility to circumvent problems with constructs that are difficult to transform.

In *T. castaneum* we observed in contrast to *C. capitata* a slightly better performance using the helper with the insect codon-based hyperactive transposase CDS (*Tc-^{i}hyPBase*).

Besides the much better performance of the hyPBase, we could not observe a negative effect on the survival or fertility rate in comparison to the wild-type *PBase* of the injected beetles (Figure 2B).

The performance of the hyperactive *piggyBac* variants in D*. melanogaster*

The systematic analysis of the germline transformation experiments conducted with the three different helper plasmids *Dm-^{i}PBase*, *Dm-^{i}hyPBase*, and *Dm-^{m}hyPBas* in combination with either of the two donor plasmids (*KNE007* and *KNE017*) and with or without heat-shock in *D. melanogaster* (Table 3) confirmed our previous results from the germline transformation experiments in *T. castaneum* and *C. capitata*. Under all conditions the two hyperactive helper

variants outperformed the wild-type helper (Figure 3A), with a 3 to 11-fold increased transformation rate compared to the wild-type helper. The codon usage of the *hyPBase* vectors had no consistent influence on the transformation rate. Heat-shock treatment however, consistently led to better performance of all tested helpers. As in the other species, also in *D. melanogaster* the fertility rate was not affected by the type of helper plasmid used (Figure 3B).

hyPBase does not cause an increased rate of multiple insertions

During the regular *Drosophila* crossings to determine the chromosomal localization of the diverse *piggyBac* insertions, we did not find any indication for multiple insertions on different chromosomes for either of the three helper plasmids. To further investigate whether the enhanced germline transformation rate of hyPBase might result in an increased number of multiple insertion events on the same chromosome, we performed iPCR on ten lines generated by using the three different helper plasmids. In all cases, the 5' insertion sequence matches the 3' sequence (Table 4), which argues for single insertions. Therefore, we have no implication of an increased rate of multiple insertions for hyPBase compared to PBase that already had been shown to generate rare multiple insertions (Handler and Harrell, 1999).

Discussion

Our results clearly show that the hyperactive *piggyBac* transposase hyPBase, regardless of the codon-usage, decidedly increases the rate of successful germline transformation compared to the wild-type *piggyBac* transposase PBase in all three tested insect species. In *C. capitata,* we were not able to produce any transgenic offspring using *Dm-^{i}PBase* at the scale of our experimental setup but reached transformation rates of up to 14.3% or 16.0% deploying *Dm-^{m}hyPBase* or *Dm-^{i}hyPBase,* respectively. In *D. melanogaster,* we achieved a 3- to 11-fold increase in germline transformation when using *Dm-^{i}hyPBase* and 5- to 8-times higher transformation efficiencies when co-injecting the *Dm-^{m}hyPBase* helper plasmid than with *Dm-^{i}*PBase. However, in *C. capitata* and *D. melanogaster* we could not observe a constant trend towards a better performance for either of the codon-usages (Figure 1 and 3), indicating similarly effective translation of both codon-variants. Only in *T. castaneum,* the *^{i}hyPBase* helper showed consistently a slightly higher transformation efficiency than *^{m}hyPBase*, where with 39.4% (*Tc-^{i}hyPBase*) and 36.4% (*Tc-^{m}hyPBase*) also the highest transformation rates were obtained in our study (Figure 2). Actually, our results – especially the higher transformation rates of heat-shocked compared to not heat-shocked *D. melanogaster* embryos (Figure 3) – are consistent with the current opinion that *piggyBac* transposases lack the phenomenon of overproduction inhibition (Burnight et al., 2012; Cadiñanos and Bradley, 2007; Wilson et al., 2007).

Considering that *C. capitata*, *D. melanogaster* and *T. castaneum* belong to two different insect orders and three different families, it is reasonable to speculate that similar improvements for

germline transformation could also be achieved applying hyPBase to a variety of other insect species. This might be of particular interest when targeting non-model organisms, since their more elaborated handling during and after the injection procedure is often very labor intensive due to the species-specific biology, sensitivity to micro-manipulation and laborious artificial rearing conditions. In *C. capitata* for instance, successful individual backcrossing is one of the most restrictive steps since their polyandrous and complex mating behavior favors mating in larger populations (Bertin et al., 2010; Bonizzoni et al., 2006), which is directly represented in the lower average fertility rate in *C. capitata* of 67.4% compared to 96% in *T. castaneum* and 88% in *D. melanogaster*.

Furthermore, cytoplasmic localization of morphogenetic determinants at the posterior pole of the developing embryo are essential for germline formation and consequently for the fertility of the adult insect. Injuries at the posterior pole can result in the loss of germ cell development, followed by a decreased percentage of fertile survivors (Swanson and Poodry, 1980). In contrast to the injection directly into the tip of posterior pole in embryos of *C. capitata* and *D. melanogaster,* the injection from the lateral site at the posterior end in *T. castaneum* embryos seems less invasive and thus poses an additional factor for the observed high average fertility rates of the adult beetles. Therefore, we assume that sterility is mainly a consequence of the injection procedure itself, the injected volume, the degree of experience of the injecting person to prevent leakage, the mating behavior of the organism and a species-specific natural variation in fertility, rather than being a direct effect of the transposase as it has been previously hypothesized for the *^{i}hyPBase* helper (Wright et al., 2013).

In ordinary germline transformation experiments in our laboratory, we would usually inject higher volumes at the expense of lower survival rates and higher sterility rates. This can be convenient as it simultaneously reduces the workload on backcrossing and screening and increases the chance that survivors of this procedure will give rise to transgenic offspring. However, to be able to accurately evaluate the possible effects of the different transposases on survival and fertility rates in this systematic comparison, we decided to not exhaust the maximally injectable volume, allowing the speculation that even higher transformation rates could be achieved.

In conclusion, the main aim of our study was to systematically test the performance of the hyperactive *piggyBac* transposase compared to its wild-type version and further elucidate whether the codon usage could make a difference for germline transformation of insects. We found that the hyperactive variant indeed functions hyperactively in all respects but could not see a general strong favor in insects for either of the insect-based or mammalian codon-optimized version. Therefore, based on our findings, we encourage molecular entomologists to consider hyPBase for future germline transformation experiments.

Author Contributions

E.A.W., K.N.E., and S.D. conceived and designed the research; S.D., K.N.E. and M.K. designed the helper plasmids; K.N.E., M.K., P.K. and C.E.O. constructed the plasmids; K.N.E., M.K. and H.M.M.A. designed the experiments; K.N.E, M.K., and H.M.M.A. performed the injections; K.N.E, M.K., H.M.M.A. and M.D.I performed the crossings and screenings; K.N.E, S.D. and E.A.W., wrote the manuscript; K.N.E prepared the figures and tables; All authors edited and approved the final version of the manuscript.

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Figures:

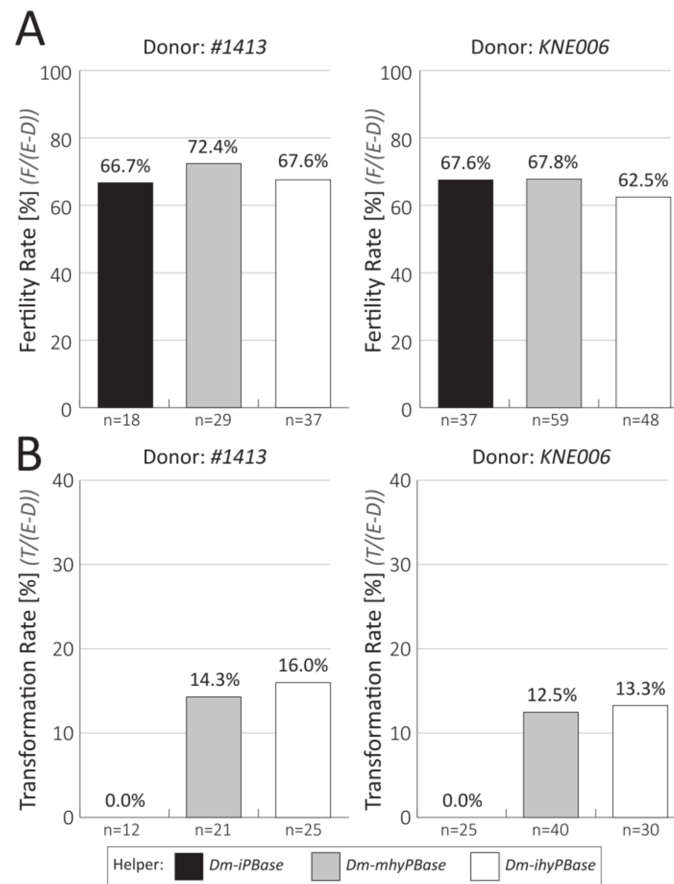

Figure 1 Transformation rate (A) and fertility rate (B) obtained from co-injection of three different helper plasmids (*Dm-^{i}PBase* (black), *Dm-^{i}hyPBase* (grey), and *Dm-^{m}hyPBase* (white)) in combination with two different donor plasmids (#1413 and KNE006) in *C. capitata*. D, death; E, eclosed flies; F, fertile crosses; T, producing transgenic offspring).

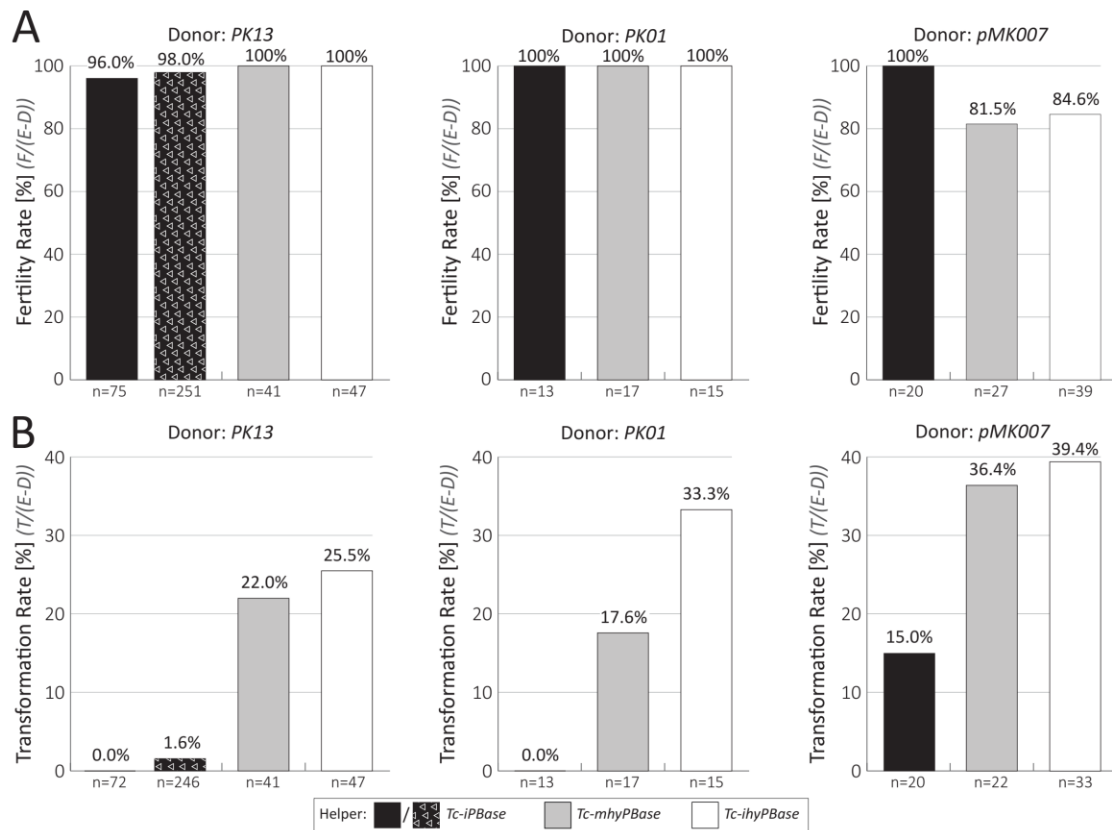

Figure 2 Transformation rate (A) and fertility rate (B) obtained from co-injection of three different helper plasmids (*Tc-ⁱPBase* (black), *Tc-ⁱhyPBase* (grey), and *Tc-ᵐhyPBase* (white)) in combination with three different donor plasmids *PK13*, *PK01*, and *pMK007*) in *T.castaneum*. D, death; E, eclosed beetles; F, fertile crosses; T, producing transgenic offspring).

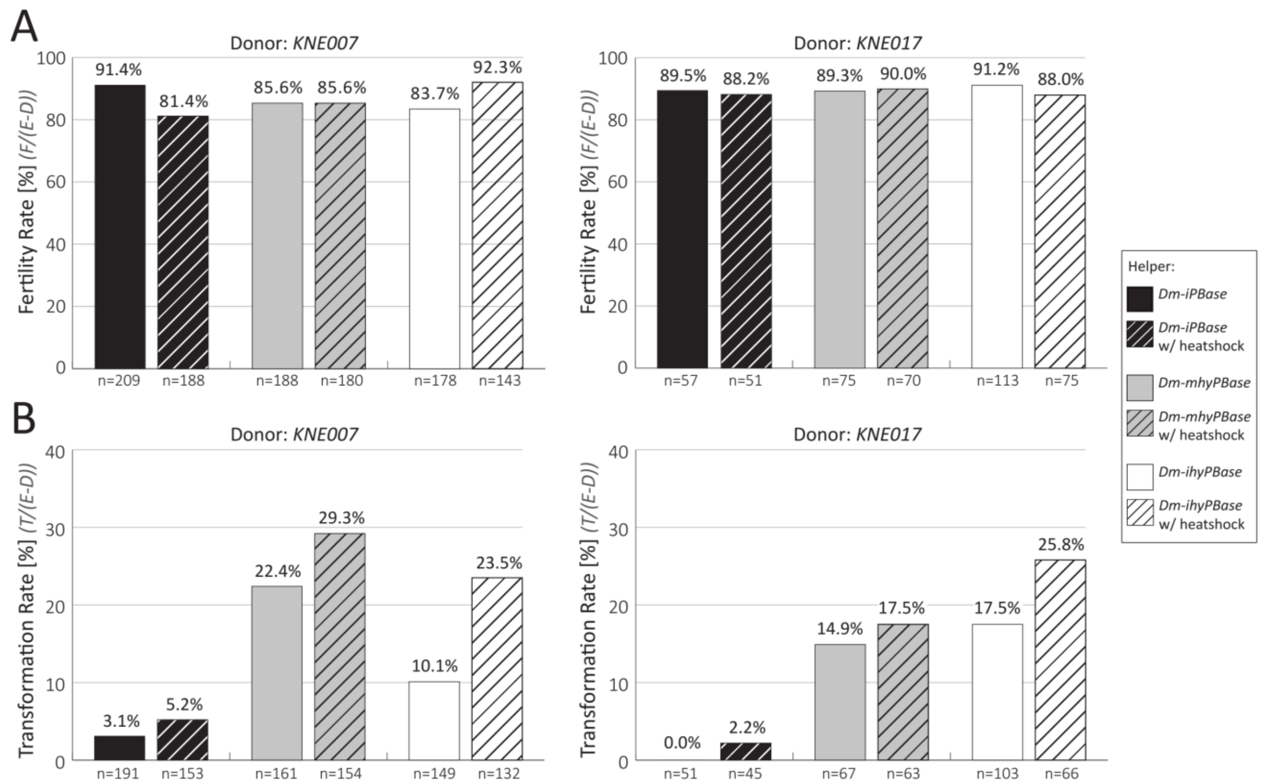

Figure 3 Transformation rate (A) and fertility rate (B) obtained from co-injection of three different helper plasmids (*Dm-ⁱPBase* (black), *Dm-ⁱhyPBase* (grey), and *Dm-ᵐhyPBase* (white)) in combination with two different donor plasmids *(KNE007* and *KNE017*) as well as without and with heat-shock (striped columns) in *D. melanogaster*. D, death; E, eclosed flies; F, fertile crosses; T, producing transgenic offspring).

Table 1 Numbers of injected embryos, survivors and fertile crosses, and their respective rates, using the helpers *Dm-*^{i}*PBase*, *Dm-*^{i}*hyPBase*, and *Dm-*^{m}*hyPBase* in *C. capitata*.

Donor / Size	Helper	# Injected Embryos [Em]	# Hatched Larvae [L]	# Pupae [P]	# Eclosed Flies [E]	# Dead [D]	# Sterile [S]	# Fertile Crosses [F] =E-(D+S)	# Transgenic [T]	Hatch Rate =L/Em	Pupation Rate =P/L	Eclosion Rate (to Larvae) =E/L	Eclosion Rate (to Pupae) =E/P	Sterility Rate =S/(E-D)
Donor: **1413** Size: **8kb [11.5kb]**	*Dm-*^{i}*PBase*	224	79	41	27	9	6	12	**0**	35.3%	51.9%	34.2%	65.9%	33.3%
	Dm-^{m}*hyPBase*	229	109	74	47	18	8	21	**3**	47.6%	67.9%	43.1%	63.5%	27.6%
	Dm-^{i}*hyPBase*	240	102	65	60	23	12	25	**4**	42.5%	63.7%	58.8%	92.3%	32.4%
Donor: **KNE006** Size: **8kb [11.5kb]**	*Dm-*^{i}*PBase*	226	132	60	41	4	12	25	**0**	58.4%	45.5%	31.1%	68.3%	32.4%
	Dm-^{m}*hyPBase*	163	151	89	63	4	19	40	**5**	57.4%	58.9%	41.7%	70.8%	32.2%
	Dm-^{i}*hyPBase*	211	145	81	58	10	18	30	**4**	68.7%	55.9%	40.0%	71.6%	37.5%

Table 2 Numbers of injected embryos, survivors, fertile crosses, and their respecitve rates, using the helpers *Tc-*^{i}*PBase*, *Tc-*^{i}*hyPBase*, and *Tc-*^{m}*hyPBase* in *T. castaneum*.

Donor	Helper	# Injected Embryos [Em]	# Hatched Larvae [L]	# Pupae [P]	# Eclosed Beetles [E]	# Dead [D]	# Sterile [S]	# Fertile Crosses [F] =E-(D+S)	# Transgenic [T]	Hatch Rate =L/Em	Pupation Rate =P/L	Eclosion Rate (to Larvae) =E/L	Eclosion Rate (to Pupae) =E/P	Sterility Rate =S/(E-D)
Donor: **PK13** Size: **5.2kb [8.7kb]**	*Tc-*^{i}*PBase*	450	144	75	75	0	3	72	**0**	32.0%	52.1%	52.1%	100%	4.0%
		2550	625	251	251	0	5	246	**4**	24.5%	40.2%	40.2%	100%	2.0%
	Tc-^{m}*hyPBase*	450	94	41	41	0	0	41	**9**	20.9%	43.6%	43.6%	100%	0%
	Tc-^{i}*hyPBase*	450	119	47	47	0	0	47	**12**	26.4%	39.5%	39.5%	100%	0%
Donor: **PK01** Size: **5.2kb [8.7kb]**	*Tc-*^{i}*PBase*	450	39	13	13	0	0	13	**0**	8.7%	33.3%	33.3%	100%	0%
	Tc-^{m}*hyPBase*	450	35	17	17	0	0	17	**3**	7.8%	48.6%	48.6%	100%	0%
	Tc-^{i}*hyPBase*	450	33	15	15	0	0	15	**5**	7.3%	45.5%	45.5%	100%	0%
Donor: **pMK007** Size: **6.6kb [10kb]**	*Tc-*^{i}*PBase*	300	36	20	20	0	0	20	**3**	12.0%	55.6%	55.6%	100%	0%
	Tc-^{m}*hyPBase*	300	54	27	27	0	5	22	**8**	18.0%	50.0%	50.0%	100%	18.5%
	Tc-^{i}*hyPBase*	300	76	39	39	0	6	33	**13**	25.2%	51.3%	51.3%	100%	15.4%

Table 3 Numbers of injected embryos, survivors, fertile crosses, and their respective rates, using the helpers *Dm-*^{i}*PBase*, *Dm-*^{i}*hyPBase*, and *Dm-*^{m}*hyPBase* in *D. melanogaster* (greyscale: heat-shock).

Donor	Helper	# Injected Embryos [Em]	# Hatched Larvae [L]	# Eclosed Flies [E]	# Dead [D]	# Sterile [S]	# Fertile Crosses [F] =E-(D+S)	# Transgenic [P]	Hatch Rate =L/Em	Eclosion Rate =P/L	Sterility Rate =S/(E-D)
Donor: **KNE007** Size: **6kb [9.5kb]**	*Dm-*^{i}*PBase*	401	258	222	13	18	191	**6**	64.3%	86.1%	8.6%
		392	207	194	6	35	153	**8**	52.8%	93.7%	18.6%
	Dm-^{m}*hyPBase*	390	243	203	15	27	161	**36**	62.3%	83.5%	14.5%
		390	236	198	18	26	154	**45**	60.5%	83.9%	14.4%
	Dm-^{i}*hyPBase*	422	212	181	3	29	149	**15**	50.2%	85.4%	16.3%
		337	170	146	3	11	132	**31**	50.5%	85.9%	7.7%
Donor: **KNE017** Size: **6.2kb [9.7kb]**	*Dm-*^{i}*PBase*	209	115	63	6	6	51	**0**	55.0%	54.8%	10.53%
		209	107	52	1	6	45	**1**	51.2%	48.6%	11.8%
	Dm-^{m}*hyPBase*	197	117	84	9	8	67	**10**	59.4%	71.8%	10.7%
		208	119	73	3	7	63	**11**	57.2%	61.3%	10.0%
	Dm-^{i}*hyPBase*	402	221	129	16	10	103	**18**	55.0%	58.4%	8.9%
		406	209	92	17	9	66	**17**	51.5%	44.0%	12.0%

Table 4 Insertion-site sequences isolated by inverse PCR from sets of *D. melanogaster* lines which were generated by either of the three helper plasmids (*Dm-^{i}PBase*, *Dm-^{i}hyPBase* or *Dm-^{m}hyPBase*). In all ten examined lines only a single chromosomal transposition could be detected.

Donor	Helper	Line	Genomic 5' and 3' Sequence of *piggyBac* Insertion	Genomic Locus of Insertion
KNE007	*Dm-^{i}PBase*	007.2	5'-tgaaaaggtaatttcacgcactttt**TTAA-*piggyBac*-TTAA**ataacgtttcatatcgatttggttt-3'	2R:11605849
		007.4	5'-agctccgtgctaacgttgtcatcgt**TTAA-*piggyBac*-TTAA**tctattatttttcacccaaggtaag-3'	X:10070165
		007.7	5'-tctttaagggtgagtgacttcattg**TTAA-*piggyBac*-TTAA**aggctttaccacatacactctgtga-3'	3L:10473387
	Dm-^{m}hyPBase	007.3	5'-ttccagcatgctcacataattaact**TTAA-*piggyBac*-TTAA**ataaaaacagttgtaaactatatat-3'	2R:17563360
		007.6	5'-cttgcattgcggttcaaggacatgg**TTAA-*piggyBac*-TTAA**tggtcttccattgtgggaaagggta-3'	2R:23868999
		007.8	5'-aaatgcatgcatttcacaacaggct**TTAA-*piggyBac*-TTAA**agttgagcaatagatcgccagccat-3'	3L:16708553
KNE017	Dm-*^{i}hyPBase*	017.1	5'-ataggtacctgtcattcaaataaca**TTAA-*piggyBac*-TTAA**agctttgcaaaaaagtagctacatt-3'	2R:11639211
		017.2	5'-aataggaccgaccaccgggtattct**TTAA-*piggyBac*-TTAA**atattgtttacgttgcacttataac-3'	3R:9592260
		017.5	5'-atgatttaataaatatactaaataa**TTAA-*piggyBac*-TTAA**atatgataactgtttattgcaaaag-3'	2R:8566663
		017.7	5'-aattctcacacttcttctttcaatt**TTAA-*piggyBac*-TTAA**aaagggggcattgtaaaattaaaaa-3'	4:1156850

Supplementary Table 1. Primers used in this study

Primer name	Sequence
AP70AgeIF	AGCATACCGGTCAGGAATAAGGTTGGCTGCTGC
AP70NheIIR	AGCATGCTAGCTTTTACACCGACATTCAAGCTAATCGGC
B2Tub-F	AATAACCTAGGACCGGTCATTGTAGGAGCCAGAGCCAATG
B2Tub-R	CTAATCTAGACATTTTGCTAGCAAAGTTAGGGCCCCTCTTTCAC
co109	ATTCGAATGGCCATGGGACG
co110	TTTTATCTAATCTAGACATCTTTTAAATTATCTACCGATTTAATTAC
Hsp68_F	CGTTTCATATATAAGCGCGGTCTCGCGGCGCGTTGTC
IC102	GCGGAGACTCTAGCGGAAGTACACTCTTCATGGCGATA
IC83	GCCTTTGCTCACCATGGTGTCTCTGGATTAGACGACT
IC86	ATGGTGAGCAAAGGCGAAGAAG
IC91	CTTCACAAAGATCCTCTAGATTATTTATACAGTTCAT CCATGCCG
IF_Cherry_SV40_F	TCGAATTCCAAAATGGTGAGCAAGGGCGAGGA
IF_Cherry_SV40_R	CGAGATCTAGGCGCGCCGGCCAGATCGATCCAGAC
IF_TRE_ hs43_KS_R	CATTTTGGAATTCGATATCAAGCTTATCGATGGATTG
IF_TRE_hs43_F	GCCGGCCTTGGCGCGCCAAG
iPB-7_CtoT_F	GTTATCAAAGCCTGTGCACGGTAGTTGTCG
iPB-7_CtoT_R	CGACAACTACCGTGCACAGGCTTTGATAAC
piggyBac-NcoiFor	ATATCCATGGGTAGTTCTTTAGACGATGAGCATATC
piggyBac-NotIrev	TCGAGCGGCCGCTCATCAGAAACAACTTTGGCACATATCA
PK124_FseIGAP43Fw	GTGACTGGCCGGCCATGACGTCAATGGGAGGGCAATG
PK124_FseIGAP43Fw	GTCAGTGGCGCGCCCTACTTGTACAGCTCGTCCATGC
QUAS_F	GATCGGCCGGCCTAGGCTAGCGCAAAGCTTGGCTGCATC
QUAS_R	CTTATATATGAAACGGCTCGAGCAATTCGATATCAAG
tGFP_R	GTACGGCGCGCCTAGCTTTATTCTTCACCGGCATCTG

3.3 Improvement and Use of CRISPR/Cas9 to Engineer a Sperm-marking Strain for the Invasive Fruit Pest *Drosophila suzukii*

In this chapter improvement in CRISPR/Cas9 genome editing system of the invasive pest is described. It gives details on isoaltion and use of endogenous promoters of *Ds heat shock protein 70* and the *small nuclear RNA* genes *U6* to drive the expressoin of Cas9 and guide RNAs for genome engineering and provide comparison and improvement in CRISPR/Cas9 mediated homolgoy directed repair (HDR). In addition, the generation of the first *D. Suzukii* embryonic driver line is described which can be used to develop conditional embryonic or conditional female specific embryonic lethality as a first step to etablish the sterile insect technique (SIT) for this onerous fruit pest. Furthemore, the isolation, characterization of *D. suzukii β-2-tubulin* gene (*β2t*) and the use of its promoter to generate the first sperm-marking strains for this pest. This strain is another addition towards the establishment of the SIT for *D. Suzukii*. This work is considered the first record of using *D. suzukii* own regulatroy elemets to drive the expression of genes from an episome or as trangene and present an important contribution to the development of biotechnological pest control strategies.

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Authors contributions

Hassan M. M. Ahmed: All experiments besides the ones performed by LH. Writing of first draft of manuscript and composition of all figures.

Luisa Hildebrand: Testes whole mount in situ hybridizations.

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Improvement and Use of CRISPR/Cas9 to Engineer a Sperm-marking Strain for the Invasive Fruit Pest *Drosophila suzukii*

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Abstract

Background: The invasive fruit pest *Drosophila suzukii* was reported for the first time in Europe and the USA in 2008 and has spread since then. The adoption of type II clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) as a tool for genome manipulation provides new ways to develop novel biotechnologically-based pest control approaches. Stage or tissue-specifically expressed genes are of particular importance in the field of insect biotechnology. The enhancer/promoter of the spermatogenesis-specific *beta-2-tubulin* (*β2t*) gene was used to drive the expression of fluorescent proteins or effector molecules in testes of agricultural pests and diseases vectors for sexing, monitoring, and reproductive biology studies. Here, we demonstrate an improvement to CRISPR/Cas-based genome editing in *D. suzukii* and establish a sperm-marking system.

Results: To improve genome editing, we isolated and tested the *D. suzukii* endogenous promoters of the small nuclear RNA gene *U6* to drive the expression of a guide RNA and the *Ds heat shock protein 70* promoter to express *Cas9*. For comparison, we used recombinant Cas9 protein and *in vitro* transcribed gRNA as a preformed ribonucleoprotein. We demonstrate the homology-dependent repair (HDR)-based genome editing efficiency by applying a previously established transgenic line that expresses *DsRed* ubiquitously as a target platform. In addition, we isolated the *Ds_β2t* gene and used its promoter to drive the expression of a red fluorescence protein in the sperm. A transgenic sperm-marking strain was then established by the improved HDR-based genome editing.

Conclusion: The deployment of the endogenous promoters of the *D. suzukii U6* and *hsp70* genes to drive the expression of *gRNA* and *Cas9*, respectively, enabled the effective application of helper plasmid co-injections instead of preformed ribonucleoproteins used in previous reports for HDR-based genome editing. The sperm-marking system should help to monitor the success of pest control campaigns in the context of the Sterile Insect Technique and provides a tool for basic research in reproductive biology of this invasive pest.

Furthermore, the promoter of the *β2t* gene can be used in developing novel transgenic pest control approaches. The CRISPR/Cas9 system can be used as an additional tool for the modification of previously established transgenes.

Keywords: Cherry vinegar fly, insect transgenesis, molecular entomology, pest management, Spotted Wing *Drosophila*,

Background

Native to East Asia (1), the cherry vinegar fly *D. suzukii*, also known as the Spotted Wing *Drosophila* "SWD" was reported for the first time in Europe, Spain, and Italy, and the mainland USA in California in 2008 (1–3). The pest has since then expanded its geographic distribution to include all of Europe as reported by the European Plant Protection Organization (2). In the USA, the situation is as severe as in Europe. Four years after its first invasion in California, the SWD has been reported in more than 41 states (4). By now, this invasive insect pest has also been reported further down in South America: for the first time between the years 2012 and 2013 in Brazil (5) and more recently also in Argentina in four localities (6).

The devastating fruit pest *D. suzukii* infests mainly soft-skinned as well as stone fruits with a wide host range spanning cultivated and wild plants (7). In contrast to other *Drosophila* spp., the SWD is armoured with a sharp serrated ovipositor, which allows it to infest ripening and not only overripe or rotten fruits (8). Earlier studies have shown that economic impact due to the infestation is in the order of millions of US dollar (9,10). Current control efforts mainly rely on heavy application of insecticides (11,12), which is on the one hand not compatible with organic farming and prone to rapid emergence of insecticide resistance owning to the short generation time of this fly. And on the other hand, it is not safe, as the time between onset of infestation and harvest is very short and does not allow for a sufficiently long period post pesticide application. Other control strategies include the use of natural enemies such as parasitoids, predators, or pathogens (13), netting to cover the plants (14), and good cultural practices to minimise the source of infestation (15). The sterile Insect technique (SIT) presents itself as an additional safe and effective pest management strategy. It provides a species-specific, environmentally sound pest control approach (16) and is compatible with other pest control strategies in integrated pest management (IPM) programs. The system has been proposed more than half a century ago and was used to successfully eradicate the tsetse fly from Zanzibar as well as the screw worm from Libya and the USA (17,18). It encompasses mass production of the target insect, removal of the females, and sterilization of males by ionizing radiation prior to release (16). Using transposon-based germline transformation, many transgenic strategies have been developed to overcome some of the drawbacks of classical SIT. A transgene-based embryonic lethality system was developed for several dipterans including the model *D. melanogaster* and the cosmopolitan fruit pest *Ceratitis capitata* (19,20). The system relies on

the ectopic expression of a pro-apoptotic gene during early embryonic stages, which leads to cell death and hence reproductive sterility (19). The same system has also been used for sexing, when the embryonic lethality was rendered female-specific by making use of the sex-specifically spliced intron of the *transformer* gene, which allows for elimination of females at the embryonic stage (20–22). Furthermore, for monitoring the competitiveness of released males, sperm-marking systems were developed for a number of pest insects and diseases vectors by driving the expression of fluorescent protein during spermatogenesis (23–26).

Recently, a revolution in genome engineering was started by the application of the CRISPR/Cas system, which stands for type II clustered regularly interspaced short palindromic repeats CRISPR/CRISPR-associated. Respective sequences were first observed in bacterial genomes in 1987 (27). Two decades later, researchers found an association between these repeated sequences and resistance of bacteria to bacteriophages (28) and showed that the bacteria use this system as an adaptive defence mechanism against invading DNA elements (29). The system consists of the Cas9 effector endonuclease, the CRISPR RNA (*crRNA*), which confers specificity to Cas9, and the transactivating crRNA (*tracrRNA*), which facilitates maturation of *crRNAs* and the interaction with Cas9 protein for forming active RNP complexes (30,31). The *crRNA* and *tracrRNA* were fused together to generate a single chimeric gRNA that facilitated the use of the system (32). The Cas9 endonuclease can easily be programmed to target and induce DNA double strands break (DSB) by replacing the 20 nucleotides (spacer) at the 5' of the *crRNA* with 17-20 nucleotides (nt) complementary to the target of interest. The prerequisite for the RNP complex to unwind, bind, and induce DSB in the target DNA is a proto-spacer adjacent motif (PAM) immediately downstream of the 20 nt target sequence, which is NGG in the case of the most commonly used *Sp_Cas9* from *Streptococcus pyogenes* (31). Similar to other programmable endonucleases such as Zinc finger nucleases (ZFNs) and Transcription activators like nucleases (TALENs), the role of Cas9 as a genome editing tool ends with the induction of a DSB. Repairing the genome - by either homology directed repair (HDR) or by non-homologous end joining (NHEJ) - is a function of the cell own DSB repair machinery, the stage of the cell at which the DSB is induced, and the availability of homologous DNA (32). The system has rapidly been adopted as a genome engineering tool for many model and non-model organisms including zebrafish (33), mouse (34,35), *Drosophila* (36), mosquitoes (37,38), and human cell lines. The CRISPR/Cas9 system has also been used to induce chromosomal translocations in embryonic stem cells (39), and to engineer new balancer chromosomes in the nematode model *Caenorhabditis elegans* (40).

In the genetics power horse *D. melanogaster*, CRISPR/Cas9 has been used and delivered in different forms: as helper plasmids, mRNA and gRNA, as well as a ribonucleoprotein complex. Several promoters have been used to drive the expression of *Cas9* including germline-specific promoters of genes such as *nanos* and *vasa*, inducible promoters such as *heat*

shock protein 70 (*hsp70*), and promoters of ubiquitously expressed genes such as *Actin5C*. Systematic analysis of the three different promoters of the *small nuclear RNA (U6)* genes in *D. melanogaster* has shown that the *U6:3* promoter drives the strongest expression measured by gene editing events (41,42).

In *Drosophila suzukii*, the CRISPR/Cas9 system has been used albeit with low efficiency to mutate the genes *white* (*w*) and *Sex lethal* (*Sxl*) using *D. melanogaster* promoters to drive the expression of *gRNA* and *Cas9* (43). Another study reported on the use of pre-assembled a ribonucleoprotein complex (RNP) to induce mutations in the *white* gene (44). The introduction of the mutations was in both studies based on NHEJ. The system has also been used to engineer by HDR a temperature sensitive mutation in the *Ds_transformer-2* gene (*Ds_tra-2*) that leads to sex conversion. In this study a RNP complex in combination with RNA interference against the *Ds_lig4* gene was used and an HDR frequency of 7.3% was reported (45). Furthermore, a RNP complex has also been used in a behavioural study of *D. suzukii* to knockout the gene that encodes the odorant receptor co-receptor (Orco) by HDR-mediated mutagenesis (46).

In applied insect biotechnology, CRISPR/Cas9 has become very popular particularly in the development of insect control strategies. One possible application for the system in SIT is the development of a reproductive sterility system that targets Cas9 to induce many DSBs at defined loci during spermatogenesis. This could mimic the desired effect of ionizing radiation in generating redundant sterility and at the same time overcome the random action of radiation affecting all organs, which reduces the overall fitness of the sterile males (47).

To restrict Cas9 activity to spermatogenesis, the isolation of a tissue-specific promoter is essential. The *Drosophila β2t* gene has been shown to code for a β-tubulin, which is expressed in a tissue-specific manner during spermatogenesis (48). Its testes-specific expression makes it a good candidate for developmental studies related to reproductive biology and male germline development as well as pest control strategies. *Dm_β2t* is a TATA-less gene, which relies on an initiator element (Inr) as a core promoter with the testes-specific expression conferred by a 14bp activator element called *β2 Upstream Element 1* (*β2UE1*) (49). Further elements required for the expression level are *β2UE2* at position -25 and *β2DE1* at position +60 (50). Homologs of *Dm_β2t* were identified in a number of insects including *Anopheles stephensi, Aedes aegypti, Ceratitis capitata, Anstrepha suspensa, Anastrepha ludens*, and *Bacterocera dorsalis* (23–26). The upstream regulatory sequence has been used to drive the expression of fluorescent protein in the testes, which serves as a strategy for sex separation as well as for monitoring released males in SIT. In the major malaria vector *Anopheles gambiae*, the promoter of the *β2t* gene was used to drive the expression of the homing endonuclease *I-PpoI* during spermatogenesis. *I-PpoI* is a highly specific Homing Endonuclease Gene (HEG), which targets and cuts a conserved sequence within the *rDNA* on the X chromosome and

thereby leads to X-chromosome shredding leaving mostly Y-chromosome bearing sperm functional, which results in sex-ratio distortion (51).

In this study, we present an improved CRISPR/Cas9-based genome engineering system for the invasive fruit pest *D. suzukii* and its application to edit a transgenic line generated using *piggyBac* germline transformation. Moreover, we report on the use of this editing system to generate a *D. suzukii* sperm marking line based on the *Ds_β2t* promoter driving the expression of *DsRed* in the testes.

Results

Improvement on CRISPR/Cas9 genome editing in *Drosophila suzukii*

In order to improve on the HDR-mediated genome editing based on CRISPR/Cas9-induced DSBs, we isolated endogenous polymerase II (*hsp70* gene) and polymerase III promoters (*U6* genes) from *D. suzukii* to drive *Cas9* or *gRNAs*, respectively. Searching for homologs of the *D. melanogaster heat shock protein 70* (*hsp70*) gene, we identified the *D. suzukii Ds_hsp70* gene, cloned and sequenced 500bp upstream of the ATG translation start codon and used this upstream sequence to drive the expression of *Cas9*.

First attempts using PCR to isolate the *U6* genes based on *D. suzukii* genome database sequences were not successful. The presence of three tandem copies obviously rendered the assembly inaccurate. Since *D. suzukii* is a close relative to *D. melanogaster*, we then tried to isolate the *U6* locus based on synteny cloning: we amplified and sequenced a 3.7 kbp fragment encompassing the *U6* locus. We identified three *U6* genes and refer to them in 5' to 3' direction as *U6a*, *U6b*, and *U6c* (Figure 1A) to distinguish them from their *D. melanogaster* equivalents.

To test the efficiency of the endogenous *hsp70* and *U6* promoters in order to drive the expression of *Cas9* and *gRNA*, respectively, for mediating HDR-based genome editing, we used the embryonic line 06_F5M2 generated by *piggyBac* germline transformation as a target platform (Figure 1B). This driver line can be used to express the heterologous tetracycline-controlled transactivator *tTA* gene specifically at early embryonic stages due to the use of the enhancer/promoter element of the cellularization gene *Ds_sryα*. Such lines can be employed to establish conditional embryonic lethality for reproductive sterility (19,20) or conditional female-specific embryonic lethality (21,22,52). As a transgenic marker, this line expresses *DsRed* under the *D. melanogaster* promoter of the *polyubiquitin* (*PUb*) gene. Based on a T7EndoI assay, a functional guide targeting upstream of the *DsRed* translation start codon was identified (Figure 1B). In a first attempt, in which donor (HMMA134), Cas9 (HMMA 056), and gRNA (HMMA104; *U6c*) plasmids were injected at concentrations of 350, 400, and 150 ng/µl, respectively, we obtained 9.5% homology directed repair (HDR) knock-in events, which we scored based on the change of the body marker from *DsRed* to *EGFP* (Figure 1C-

E). Sequencing of the knock-in junctions revealed faithful scar-less HDR events. The HDR was facilitated by the 1989bp left homology arm (*PUb* promoter) and the 672bp right homology arm (*DsRed*).

To compare the three promoters of the *DsU6* genes, we injected in a second attempt donor (HMMA134), *Cas9*(HMMA056), and either of the three gRNA plasmids HMMA102 (*U6a*), HMMA103 (*U6b*), or HMMA104 (*U6c*) at a concentration of 400, 400 and 250 ng/µl, respectively. This resulted in HDR events of 12.5%, 2%, and 15.5% for *U6a*, *U6b*, and *U6c*, respectively (Figure 1F). Injection of a RNP complex resulted in 33% HDR events (Figure 1F). This indicates, that at slightly higher concentrations of donor template and gRNA plasmids, we were able to obtain 15.5% knock-in events using the *U6c* promoter. The *U6b* showed the lowest performance with only 2% knock-in events, and *U6a* was intermediate with 12.5% efficiency (Figure 1F). Interestingly, the tendency observed for the strength of the different promoters is in line with their *D. melanogaster* counterparts. The high HDR-rates of above 10% indicate that the use of the endogenous promoters allows for effective application of helper plamids instead of RNPs to induce HDR-dependent knock-ins, which represents an improvement for CRIPR/Cas9-based genome editing in *D. suzukii*.

Isolation of the *ß2 tubulin* gene from *Drosophila suzukii*

To be able to drive sperm-specific gene expression, we identified the *Ds_β2t* gene by homology search in the *D. suzukii* genome database (www.spottedwingflybase.org) using the *Dm_β2t* sequence as query. The open reading frame of the *Ds_β2t* gene from the translation start codon to the stop codon is 1341bp, which is interrupted by a 215 bp intron. The gene has a 5'UTR of 196 bp, which demarcates the transcription start site (Figure 2A). Conceptual translation of the *Ds_β2t* coding sequence gives rise to a protein of 446 amino acids.

To validate the testes-specific gene expression of the isolated *Ds_β2t* gene, we performed whole mount *in situ* hybridization on the complete reproductive tract of 3-5 days old males using DIG-labelled antisense and sense RNA probes against the *Ds_β2t* 5'UTR and exon I. These *in situ* hybridizations detected expression only in the testes with no expression at the apical part that consists of stem cells (Figure 2B). No transcription was detected in the rest of the reproductive tract (Figure 2B) or with sense RNA probe as negative control (Figure 2C).

Generation of a sperm-marking line of *Drosophila suzukii*

To identify the necessary upstream and downstream regulatory elements driving sperm-specific gene expression, we compared the *D. suzukii β2t* sequence with the characterized counterpart in *D. melanogster*. The 14bp upstream activator element *β2tUE1* that confers testes specificity to the *β2t* gene was found at the exact position -51 to -38 relative to the transcription start site with a C>G exchange at position -41 and a T>A exchange at position -39 (Figure 3A). A second upstream regulatory element, *β2tUE2*, which is not involved in

specificity but its overall activity, was identified at position -32 to -25 with a G>T exchange at position -32 and an A>C exchange at position -28. Another element that functions as a TATAAA-box in TATA-less promoter is the 7bp initiator sequence encompassing the transcription start, which was identified -3 to +4 with the first and last nucleotide differing from *D. melanogaster* (Figure 3A). A further element involved in *β2t* promoter function is the *β2tDE1* element that is highly conserved and lies relative to the transcription start site at position +51 to +68 (Figure 3A).

To examine whether the 51 bp upstream regulatory element plus 196 bp 5'UTR (-51 to +196) drives strong testes-specific gene expression, we fused this 247bp enhancer/promoter fragment of the *Ds_β2t* gene to *DsRed.T3* (Figure 3B) and performed an HDR-based knock-in into the *D. suzukii* embryonic *piggyBac* line 06_F5M2, which we had used before as target platform (Figure 3B). The repair template consisted in this case of *EGFP* fused to the *PUb* promoter followed by *SV40* 3'UTR and the 247 bp *Ds_β2t* promoter fused to *DsRed.T3* (Figure 3B). The HDR-based knock-in resulted with 13.3% efficiency. One of the resulting *D. suzukii* lines, 134M16M2, showing a ubiquitous green fluorescence and testes-specific red fluorescence (Figure 3C-H), was moleculary characterized to confirm the proper HDR event. In this line, red fluorescent sperm could be detected in the testes (Figure 3I-L) and males of this line transferred red fluorescent sperm to the female spermatheca (Figure 3M-P). This line 134M16M2 thus serves as a sperm-marking line for this invasive pest insect.

Discussion

The programmable genome editing system CRISPR/Cas9 has enabled a series of new strategies of biotechnological engineering in model and non-model organisms. Based on the objective of the study, financial resources, and availability of functional promoters, researchers can choose the best strategy for delivery of CRISPR/Cas9 components. From published literature, it can be concluded that the most efficient strategy is germline-specific transgenic expression of Cas9, followed by application of RNP-complexes, then mRNA and gRNA co-injection, and with the least efficiency helper plasmids co-injection (42,53). The latter, however, is the most convenient even though it requires the identification and characterization of suitable promoters.

CRISPR/Cas9 holds big promises in the field of insect biotechnology especially for the development of novel pest control strategies, such as reproductive sterility systems based on chromosome shredding (47). To be able to engineer such strategies in *D. suzukii*, promoters that drive strong expression of gRNAs and other components are of particular importance. Inducible promoters of heat shock genes such as *D. melanogaster hsp70* and *Tribolium*

castaneum Tc_hsp68 have been used for a long time to conditionally express genes both transiently from a plasmid and as transgenes (54,55).

Due to their defined transcription start site and transcription termination, the RNA *polIII* promoters of the small nuclear RNA genes (snRNA) *U6* have been widely used to express short hairpins to induce an RNA interference effect. With the development of the CRISPR/Cas9 genome editing system, such promoters gained even more popularity and have intensively been used to drive the expression of the chimeric gRNAs transiently and as transgene components from mammals to plants. *D. melanogaster* has three copies in tandem on the right arm of chromosome 3 and have the cytological map location 96A, based on which they were termed *U6:96Aa*, *U6:96Ab*, and *U6:96Ac*. The promoters of the three genes were systematically tested and the promoter of the *U6:96Ac* gene (referred to also as *U6:3*) outperforms the other two, which made it the promoter of choice among Drosophilists. Our results are consistent in this respect, as also the *Ds_U6c* promoter has the highest effectivity (Figure 1F).

Previous reports demonstrated the functionality of the promoters of *Dm-U6:3* and *vasa* genes to drive expression of *gRNA* and *Cas9*, respectively, to target and mutate *D. suzukii w* and *Sxl* by NHEJ but with low frequency. The authors argued that this low efficiency might be attributed to the use of plasmids to drive the expression of *Cas9* and *gRNA* or their bulk crossing scheme (43). Another study demonstrated the feasibility of using RNP-complexes to induce mutations in *D. suzukii w* by NHEJ (44). In a more recent study, researchers used RNP-complexes to induce DSBs and were able to knock-in by HDR a mutated temperature-sensitive version of *Ds-tra2* along with a transformation marker cassette. They reported on 7.3% HDR events even though they tried to shift the cell DSB repair machinery towards HDR by co-injection of dsRNA against the *Ds_lig4* gene (45). In our hands, using RNP complex resulted in a four times higher rate of HDR-based knock-ins. However, no direct comparison with the previous studies is possible since the target itself is different. Anyway, also our helper plasmid co-injections yielded a two times higher rate of HDR-based knock-ins, which indicates that the isolated endogenous promoters allow for an efficient application of the CRISPR/Cas system with the more convenient use of plasmid helpers. However, if the objective is to manipulate the genome and recombinant Cas9 is available, the RNP approach is probably the best option, if no transgenic lines expressing *Cas9* in the germline are available. Studies in *D. melanogaster* and mosquitoes also showed that the use of RNP-complexes always leads to better editing results compared to injection of plasmids or mRNA and *in vitro* transcribed gRNA.

The use of the regulatory elements (enhancer/promoter) of sex-, tissue-, or stage-specifically expressed genes to drive effector molecules in a particular sex or developmental stage is not only useful in basic research to elucidate gene function, but also in applied insect

biotechnology to develop transgene-based pest control strategies. The gene *β2t* has been identified in a number of insects to be testes-specific with its activity starting at the late larval instar. The gene in *D. melanogaster* is known to code for a 446aa protein. Here, we identified the *D. suzukii* homolog that shows at the amino acid level 100% identity but not at the nucleotide level. Interestingly, the transcript structure of the *Ds_β2t* gene revealed the presence of a 215 bp intron (Figure 2A) compared to a highly conserved intron of 57 bp in *Aedes egypti* (24), 58 bp in *Anastrepha ludens*, 59 bp in *D. melanogaster*, 60 bp *Anstrepha suspensa*, and 67 bp in *Bacterocera dorsalis* (25). Testes whole mount *in situ* hybridization identified a similar expression pattern as previously obtained in *D. melanogaster* with the apical part of the testes that contains the stem cells not expressing the gene. The testes specificity of the gene is conferred by a 14bp activator element upstream of the transcription start site called upstream element *1 β2tUE1*, which is not only contextually conserved but also spatially relative to the transcription start site and other regulatory elements. This activator element was also identified in *D. suzukii*, which shares high similarity to its *Dm_β2t* counterpart. The other elements that are quantitatively contributing to the expression of *β2t* were also identified in exactly the same positions as in *D. melanogaster* relative to each other and to the transcription start site.

The promoter of the *β2t* gene has been used to drive the expression of a fluorescent protein in mosquitoes and tephritid fruit flies (23,24,26), which serve as a sexing system to automate separation of males from females and also as a monitoring system for released males in the context of SIT programs. The generated sperm marking strain of *D. suzukii* proved that the 247 bp regulatory sequence made of 51 bp upstream sequence plus 196 bp leader immediately upstream of the translation start codon has the necessary elements to drive expression of effector molecules specifically in the sperm. The fluorescent sperm can also be identified stored in the spermathecae of wild type females mated to the transgenic sperm marked strain, which facilitates monitoring and allows assessment of the competitiveness of released sterile males compared to their wild type counterparts. The sperm marking system can also help in conducting reproductive biology studies that will enrich our understanding of the biology of this pest and allow us to better design pest control strategies. For example, the promoter of the *β2t* gene in *Anopheles* was used to drive the expression of an HEG that targets and shreds the X chromosome in the mosquito during spermatogenesis leading towards a Y sperm bias and as a consequence to sex ratio distortion, which eventually can lead to a population collapse (51).

Conclusion

We obtained improved usability of the CRISPR/Cas9 gene editing in *D. suzukii* compared to previous reports (43–45) by the employment of helper plasmids that contain endogenous promoters of the *U6* and *hsp70* genes to drive the expression of *gRNA* and *Cas9*, respectively.

Moreover, we show that the CRISPR/Cas9 system can be used as an additional tool for the modification of previously established transgenes. The identification and cloning of the *β2t* promoter enabled us to generate a sperm-marking system in *D. suzukii*, which provides a tool for basic research in reproductive biology and should help to monitor the success of pest control campaigns in the context of SIT (23–26). In addition, the *β2t* promoter can be used in developing novel transgenic pest control approaches (47) for this invasive pest insect.

Methods

Unless otherwise specified, all PCR amplifications were performed using Phusion DNA polymerase and Phusion-HF buffer (New England Biolabs GmbH, D-65926 Frankfurt am Main). Routine plasmid min-preps and PCR products were purified using NucleoSpin® Plasmid and NucleoSpin® Gel and PCR Clean-up kits (Macherey-Nagel GmbH & Co., 52355 Dueren, Germany), respectively. Plasmid vectors for microinjections were prepared using NucleoSpin® Plasmid Transfection-grade (Macherey-Nagel) or QIAGEN Plasmid Plus Midi Kit (QIAGEN GmbH, 40724 Hilden, Germany). Primers used are listed in Additional File 1: Supplementary Table 1.

Fly strain and husbandry

All fly experiments were performed in our well-equipped safety level one (S1) laboratory, which is certified for generating and using genetically modified insects. Wild type *D. suzukii* from Italy (kindly provided by Prof. Marc F. Schetelig) as well as generated transgenic lines were reared on standard *Drosophila* food supplemented with baker yeast and kept at 25°C throughout this study. For germline transformation, flies were transferred to *Drosophila* egg laying cages and allowed to lay eggs on apple juice agar plates with some yeast on top to increase egg laying.

Nucleic acid isolation

Genomic DNA was isolated from a mix of adult males and females of *D. suzukii* (Italian strain) using NucleoSpin® DNA Insect (Macherey-Nagel) according to the manufacturer instructions. To generate a testes-specific cDNA library, testes of 100 males (3-4 days old) were dissected in ice cold 1X PBS and used for total RNA preparation using ZR Tissue & Insect RNA MicroPrep (Zymo Research Europe, 79110 Freiburg) according to manufacturer instructions.

Isolation of *DsU6* and *hsp70* genes

Based on synteny we identified *D. suzukii* the homologs of *D. melanogaster* genes *Esyt2* and *REPTOR* bordering the *U6* locus. Primer pair HM#137/138 was designed on the conserved

parts of these genes and used to PCR amplify the sequence between them supposedly containing the *Ds_U6* locus, (initial denaturation temperature 98°C 3min followed by 35 cycles of 98°C 30sec, 72°C 2 min 30 sec). A 3.7 kbp fragment was obtained and sequenced.

To identify the *D. suzukii heat shock protein 70* (*Dshsp70*) gene, we BLASTed *D. melanogaster hsp70*Aa in the *D. suzukii* genome data base (www.spottedwingflybase.org) and compared the amino acid sequence as well as the corresponding DNA sequence individually to their *D. melanogaster* counterparts using the geneious program version 10.2.6 (Auckland, 1010, New Zealand).

Isolation of *Dsβ2t* gene and its 5'UTR

To isolate the spermatogenesis specific *beta-2-tubulin* (*β2t*) gene of *D. suzukii*, we searched in the www.spottedwingflybase.org with the *D. melanogaster Dm_β2t* gene. A putative *Ds_β2t* gene sharing high homology to *Dm_β2t* was PCR amplified from genomic DNA using primer pair HM#25/26 and the PCR program 98°C for 3 min followed by 35 cycles of 98°C 30 sec, 72°C 1 min 40 sec, and 7 min final elongation at 72°C. The amplified fragment was purified, blunt cloned into pJet1.2 vector (Thermo Fisher Scientific, 64293 Darmstadt, Germany), and sequenced using standard primers pJet1.2_fwd and pJet1.2_rev.

Since the 5'UTR of *β2t* has some regulatory elements, whose position relative to the transcription start site and the upstream regulatory elements is highly conserved and important for correct tissue specific expression, it was imperative to isolate the 5'UTR and to identify the transcription start site. To do so, 1.7 µg of testes total RNA were used to generate a 5' RACE-ready cDNA library using the SMARTer™ RACE cDNA amplification kit (Takara Bio Europe SAS, 78100 Saint-Germain-en-Laye, France) according to manufacturer instructions. The 5'UTR was recovered by RACE PCR using gene specific primer HM#33 and universal primer (UPM) provided with the kit using Advantage2 DNA polymerase (Takara) with the following program: 94°C 2 min, (94°C 30 sec, 72°C 3 min) 5X, (94°C 30 sec, 70°C 30 sec, 72°C 3 min) 5X, (94°C 30 sec, 68°C 30 sec, 72°C 3 min) 30X. A single prominent band was recovered, purified, cloned into pCRII (Thermo Fisher Scientific) to generate pCRII_Dsb2t_5'UTR (HMMA24), and sequenced using a standard M13 primer.

Testes whole mount *in situ* hybridization

To generate DIG-labelled sense and antisense RNA probes of *Ds_β2t*, we prepared DNA templates for *in vitro* transcription by PCR amplification of the 5'RACE-fragment including the Sp6 or T7 promoters from pCRII_Ds *β*2t_5'UTR (HMMA24). Primer pairs HM#33/128and HM#41/127 were used respectively with the following PCR conditions: initial denaturation at 98°C 3 min, followed by 35 cycles of 98°C 30 sec, 72°C 50 sec with a final elongation step of 7 min. RNA probes were synthesized using DIG-labelling kit (Thermo Fisher Scientific) according to manufacturer instructions using 200ng of DNA as

template in a total reaction mix of 10µl. The reaction was allowed to proceed for 2h at 37°C followed by Turbo DNaseI treatment (Thermo Fisher Scientific) for 15 min to remove template DNA. 2µl of 0.2M EDTA was used to inactivate the reaction. Sense and antisense probes were precipitate and resuspended in 100µl RNA resuspension buffer (5:3:2 H2O: 20X SSC: formaldehyde) and stored at -80°C.

Testes of 3-5 days old males were dissected in ice cold 1X Phosphate buffered saline (PBS) and fixed in PBF-tween (4% formaldehyde and 0.1% tween 20 in 1X PBS) for 20 min at room temperature. *In situ* hybridization was performed according to an established protocol (56) with inclusion of dehydration steps according to Zimmerman et al. (57).

Plasmid construction

To generate plasmid HMMA006, 300 bp upstream of *Ds_sryα* plus 50bp 5'UTR sequence were PCR amplified using primer pair HM#23/24 introducing *AgeI/NheI* cut sites respectively and cloned into *AgeI/NheI* cut site of KNE007 (58) upstream of *tTA* CDS replacing the *Dm_β2t* promoter. Description of the *Ds_sryα* gene and its cloning will be described elsewhere (Ahmed et al.)

To generate pSLaf_*T7-BbsI-BbsI-ChiRNA*_af (HMMA034) for *in vitro* transcription of gRNAs, annealed oligos HM#55/56 generating T7 promoter and 2X *Bbs*I restriction sites were cloned into *Bbs*I/*Hind*III digested plasmid p*U6-chiRNA* (Addgene: #45946) giving rise to HMMA033. Next, the *Hind*III/*Sac*I *T7-BbsI-BbsI-chiRNA* fragment from HMMA033 was cloned into pSLaf1180af (59) *Hind*III/*Sac*I cut sites.

To generate plasmids *pDsU6a-BbsI-BbsI-chiRNA-DSE* (HMMA091), *pDsU6b-BbsI-BbsI-ChiRNA DSE* (HMMA092), and *pDsU6c-BbsI-BbsI-chiRNA-DSE* (HMMA093) for transient expression of gRNAs, primer pairs HM#358/159, HM#104/158, and HM#360/160 were used to amplify the promoters of *snRNA* genes *U6a*, *U6b*, and *U6c*, respectively, with PCR condition 98°C 3 min followed by 5 cycles of 98°C 30 sec, 66°C 40 sec, and 72°C 1 min then 30 cycles of 98°C 30 sec, 72°C 1 min 40 sec with a final elongation 72°C for 7 min. The promoters were then cloned into HMMA034 by megaprimer PCR cloning (60) using 30ng of plasmid HMMA034 and 200ng of the promoter as megaprimer in a 25µl reaction with PCR (98°C 3 min, [98°C 30 sec, 72°C 2min 30 sec] 30X, 72°C 7min) generating plasmids HMMA088, HMMA089, and HMMA090. Finally, 250 bp of the sequence downstream of the *U6c* termination sequence was PCR amplified from genomic DNA using primer pair HM#186/187 with PCR (98°C 3 min, [98°C 30 sec, 68°C 30 sec, 72°C 20 sec] 35X with a final elongation of 7 min at 72°C). The amplified fragment was then cloned into HMMA088, HMMA089, and HMMA090 by megaprimer cloning as described above with annealing temperature at 68°C.

For Cas9 recombinant protein expression, the plasmid *pET-T7-3XFlag-nls-Cas9-nls-6XHisTag* (HMMA101) was generated. The sumo part of the pET-SUMO expression vector was removed using *XhoI/NdeI* and the annealed oligos HM#152/153 were cloned introducing 2X *BsaI* sites giving rise to HMMA080. The 4.3Kb *Bbs*I/*Xba*I *3XFlag-nls-Cas9-nls* fragment was excised from HMMA066 and cloned into *BsaI* linearized HMMA080 to give rise to HMMA099. Finally, annealed oligos HM#180/181 introducing a *6XHisTag* were cloned into *FseI/BasI* digested plasmid HMMA099. Plasmid HMMA066 was generated by cloning *ClaI/HpaI* fragment *3XFlag-nls-Cas9-nls* from HMMA039 into *ClaI/HpaI* cut #1215 (20) giving rise to HMMA065 followed by cloning of annealed self-complementary oligo HM#102 into the *ClaI* site of HMMA065 to introduce 2X *BbsI* restriction sites. Cas9 protein was expressed and purified according to Paix *et al.* (61), and frozen at -20°C until needed.

The plasmid *pSLaf_Dshsp70P-Cas9-SV40_af* (HMMA056) to express Cas9 transiently was generated by cloning of the 4.2Kb *ClaI/XbaI* fragment containing insect codon optimized *Cas9* CDS with N and C terminal nuclear localization signals from plasmid #46294 (Addgene) into *ClaI/XbaI* digested pCS2-Sp6-Cas9-SV40 (Addgene: #47322) replacing the mammalian codon optimized *Cas9* CDS giving rise to HMMA039. The *Ds_hsp70* promoter was PCR amplified from genomic DNA using primer pair HM#73/75 with PCR using the following condition: 98°C 3 min [(98C°C 30 sec, 66°C 40 sec, 72°C 1min) 5X, (98°C 30 sec, 72°C 1 min 40 sec) 35X with a final elongation step of 7 min at 72°C. The fragment was purified and cloned into *EcoRI/ClaI* cut #1215 (20) to give rise to HMMA052. Finally, *Cas9-SV40* was excised from HMMA039 by *ClaI/HpaI* and cloned into *ClaI/HpaI* cut HMMA052 generating HMMA056.

To generate donor plasmid HMMA134, a 3.2Kb fragment containing *PUb-nls-EGFP-SV40* was excised from #1254 (20) using *SacI/AflII* and cloned into *SacI/AflII* cut *pSLaf1108af* (59) giving rise to plasmid HMMA094. *DsRed* CDS was PCR amplified from plasmid KNE007 (58) using primer pair (HM#37/167) with PCR (98°C 3 min followed by 35 cycles of 98°C 30 sec, 72°C 1 min and a final elongation of 7 min at 72°C). The fragment was phosphorylated and ligated into blunted *AflII* cut HMMA095 generating HMMA096. To change the target PAM sequence in front of *EGFP* from TGG to TGA in the repair template (Figure 1B), PCR mutagenesis using primer pair HM#221/222 was performed (98°C 3 min followed by 30 cycles of 98°C 30 sec, 72°C 4 min and final elongation of 7 min at 72°C) to give rise to HMMA097, which results in changing the second amino acid of the EGFP from valine to methionine. Finally, the 247 bp *Ds_β2t* regulatory sequence spanning -51 to +196 was PCR amplified using primer pair HM#285/252 with PCR conditions 98°C 3 min [(98°C 30 sec, 60°C 30 sec, 72°C 20 sec) 5X, (98°C 30 sec, 72°C 1 min) 30X with a final elongation step of 7 min at 72°C. The promoter was then cloned upstream of *DsRed* in HMMA097 by megaprimer PCR cloning as described previously with annealing at 61°C.

Guide RNAs design, cloning, and validation

Guide RNAs were identified using the online target finder tool built by Wisconsin University (http://targetfinder.flycrispr.neuro.brown.edu/). Identified potential targets were checked against *D. suzukii* database to exclude those with off-target sites. For each potential target, two oligos, a forward and reverse, were designed and the respective overhangs were added. Oligos were ordered as normal primers without phosphorylation. The two oligos for each target were annealed at a concentration of 10 μM in a total volume of 100 μl in a heat block. The gRNAs were validated using a T7EndoI assay (62,63). Each *gRNA* plasmid was mixed with *Cas9* plasmid HMMA056 at a concentration of 400/500 ng/μl, respectively, and injected into 50 pre-blastoderm embryos. 10 – 15 hatching larvae were collected in 1.5 ml Eppendorf tubes and crushed by using a pipette tip against the tube wall. 200μl of squishing buffer (19) was added and mixed well. The tubes were then incubated at 55°C for 1 h with occasional vortexing. Tubes were then centrifuged, and 5μl of the supernatant was used as a template in 50 μl PCR reactions using primers HM#192/69. PCR products were gel purified, quantified, and 400ng were mixed in 1X NEB 2.1 buffer in a total volume of 19 μl. DNA was denatured, rehybridized, 0.75 μl of T7 EndoI (NEB) were added, and incubated at 37°C for 20 minutes. The reactions were stopped using 2 μl of 0.25M EDTA and run in a 1.5% agarose gel. Only one guide showed obvious digest by T7 EndoI. Wild type un-injected larvae were used as control. To generate the plasmids expressing the functional guide RNA against the identified target upstream of *DsRed* (Figure 1B), annealed oligos HM#161/162 and HM#169/162 were cloned by golden gate (64,65) into gRNA vectors HMMA091, HMMA092, and HMMA093 to generate p*U6a_Red1chic* HMMA102, p*U6b_Red1chi* HMMA103, and p*U6c_Red1chi* HMMA104, respectively.

***In vitro* transcription of the gRNA**

The functional gRNA was cloned by ligation of annealed oligos HM#162/215 into *BbsI* cut plasmid HMMA035, which was then used to generate the template for *in vitro* transcription by PCR using primer pair HM#84/128. *In vitro* transcription of *gRNA* was performed using MEGAscript® (Ambion) according to the manufacturer protocol. The reaction was allowed to proceed for 2h at 37°C followed by DNA template removal using 1 μl DNase I for 30 minutes. *gRNA* was purified using RNA clean and concentrator (Zymo Research) and the concentration was determined by nano-drop (Thermo Fisher Scientific) and stored at -80°C.

Germline transformation

All embryonic injections were performed using transfection grade plasmid preparations without further precipitation steps. To generate the embryonic driver line 06_F5M2 by random *piggyBac* integration, the transformation vector HMMA006 and the helper plasmid MK006 (58) were mixed at a final concentration of 400 and 200 ng/μl respectively. To validate

that the transgene represents a single integration even, we performed inversePCR as described (58) using *XhoI and EcoRI* restriction enzymes. For both the 5 and 3' junctions, we each obtained only a single fragment, whose sequences confirmed a single integration site in the second intron of a gene referred to as *Suppressor of Under Replication* (Additional File 2: *piggyBac* insertion in *D. suzukii* line 06_F5M2).

For the transgene editing experiments using CRISPR/Cas9, DNA was mixed at a concentration of 400, 150, and 350 ng/µl for *Cas9* (HMMA056), *gRNA* (HMMA102, HMMA103, or HMMA104), and donor plasmid HMMA097, respectively. Higher concentration was used at 400, 250, and 400 ng/µl, respectively. All DNA injection mixes were prepared in 1X injection buffer (5mM KCl, 0.1 mM NaH_2PO_4, pH 6.8). For RNP injection, recombinant Cas9 endonuclease, gRNA, and donor plasmid HMMA097 were mixed together at a final concentration of 300 ng/µl, 150 ng/µl, and 400 ng/µl respectively, incubated at 37°C for 10 minutes for the RNP-complex formation, and injected into 90 pre-blastoderm embryos.

Injection needles were prepared as previously described (58). To inject in *D.suzukii* embryos, the eggs have to be squeezed out of the apple agar plates individually using home-made closed-tip glass pipettes. Embryos were then de-chorionated for 3 minutes using generic Clorox (DanKlorix, CP GABA GmbH, Hamburg, Germany) containing 2.5% sodium hypochlorite at final concentration of 1.25% sodium hypochlorite and washed in washing buffer (100mM NaCl, 0.02% Triton X-100) followed by thorough wash with desalted water. Embryos were then aligned on apple agar blocks and transferred to double sticky tape on a coverslip and covered by Voltalef 10S oil (VWR International, Darmstadt, Germany). Injections were performed using a Femtojet (Eppendorf, Hamburg, Germany) and a manual micromanipulator. Excessive oil was drained and the injected embryos were incubated on apple agar plates at the room temperature until hatching. Larvae were manually transferred to fly food vials. Each emerging G_0 fly was out-crossed to 3-4 wild type individuals of the opposite sex.

Microscopy

Screening for transgenic flies and fluorescence imaging were performed using a Leica M205 FA fluorescence stereomicroscope equipped with camera Q imaging Micropublisher 5.0 RTV (Leica Mikrosysteme Vertrieb Gmb, Wetzlar, 35578 Germany). Transgenic flies were screened using filter sets RFP (excitation: ET546/10x, emission: ET605/70m) or GFP-LP (excitation: ET480/40, emission: ET510 LP), respectively, and imaged using cold light (Figure 1C) or filter sets: RFP (Figures 1D; 3F-H), EYFP (excitation: ET500/20, emission: ET535/30) for Figure 1E, or GFP-LP (Figure 3C-3E).

Epifluorescence microscopy was performed using a Zeiss Imager.Z2 equipped with two cameras, Axiocam 506 mono and Axiocam 305 colour (Zeiss, 73447 Oberkochen, Germany). The testes or the spermathecae were dissected in ice-cold PBS, fixed for 10 minutes in 4% formaldehyde prepared in 0.1% PBS-tween 20, permeabilized for 10 minutes using 1% Triton X-100 in PBS, and nuclei were stained for 10 minutes using DAPI (4',6-Diamidino-2-Phenylindole, Dihydrochloride) at a concentration of 1 μg/ml. Samples were mounted in 70% glycerol and the spermathecae were broken open using dissection needles. The tissues were imaged under bright field and to observe cell nuclei and expression of DsRed, images were taken with filters for DAPI (excitation: 335-383, emission: 420-470) or DsRed (excitation: 533-558, emission: 570-640), and composed in ZEN Blue (Zeiss).

Competing interests:

The authors declare that they have no competing interests.

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Authors' Contributions:

E.A.W and H.M.M.A. conceived and designed the study; H.M.M.A. isolated the genes, designed the constructs and generated the transgenic lines; L.H. performed *in situ* hybridizations; E.A.W and H.M.M.A. wrote the manuscript; H.M.M.A. prepared the figures; all authors read and approved of the final manuscript.

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Figures

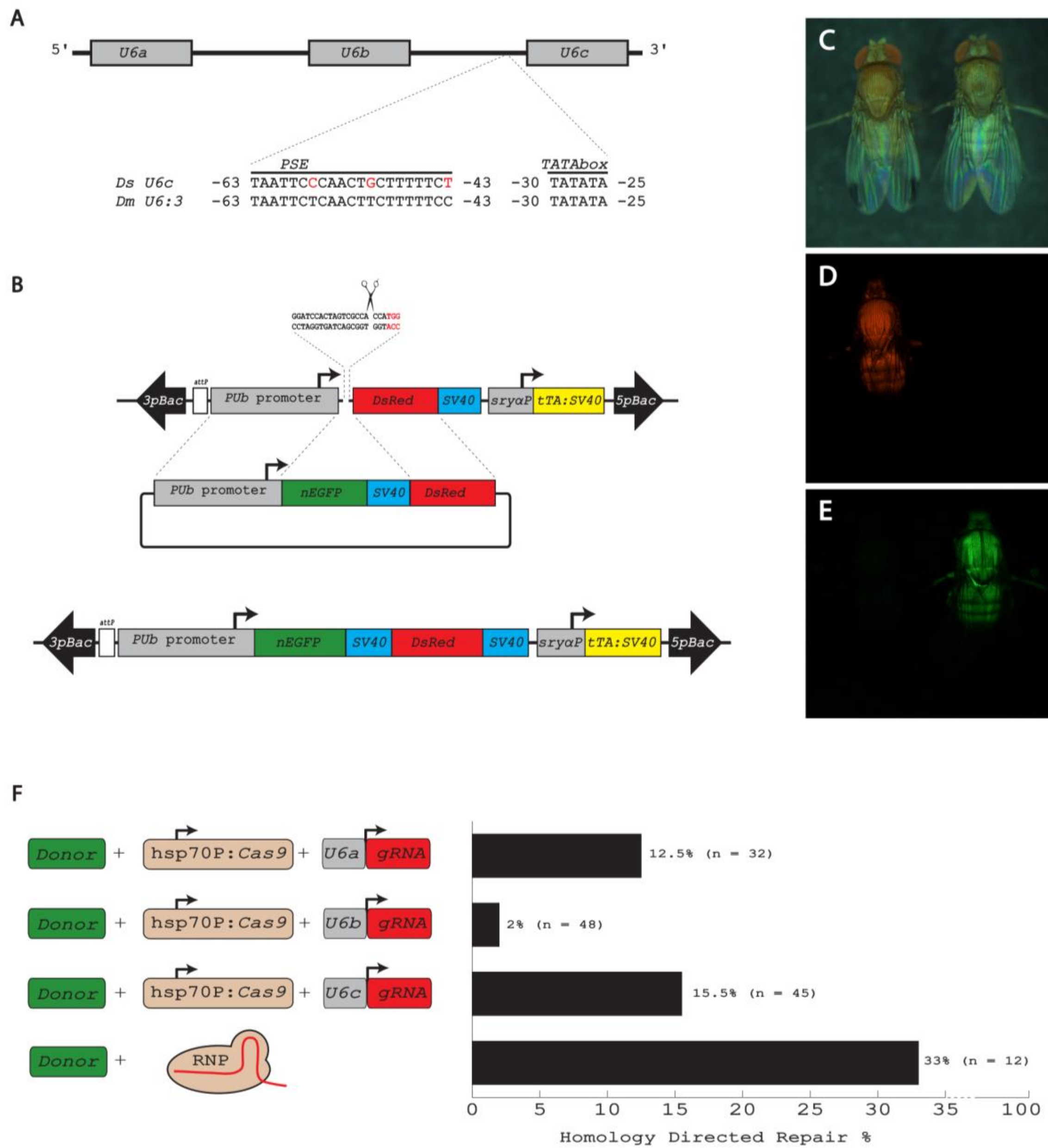
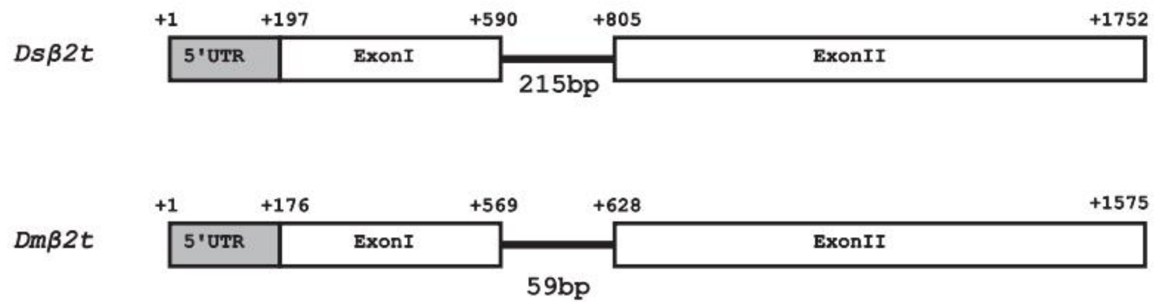


Fig. 1 Improvement of genome editing in *D. suzukii*. (**A**) Three copies of the *snRNA* gene *U6* in the genome of *D. suzkuii*. The transcription from *U6* genes by *RNA pol III* is directed by the proximal sequence element *PSE* which is highly conserved between *D. suzukii* and *D. melanogaster*. (**B**) Scheme for HDR-based genome editing at a transgenic target platform. Sequence of the target site in the transgenic strain showing the PAM sequence in red. The scissors indicate where Cas9 induces the DSB three nucleotides upstream of the PAM. (**C–E**) Fluorescent marker change as the result of the HDR knock-in: images of two male flies taken with cold light (**C**), RFP fliter (**D**), and EYFP filter (**E**). (**F**) Comparison of *Ds U6a, U6b, U6c* promoters as well as RNP in their efficiency to promote HDR-mediated knock-ins.

(A)



(B)

(C)

Fig. **2** ***D**. **suzukii β2t*** **gene and its expression**. (**A**) *Dsβ2t* gene has two exons and one intron similar to *D. melanogaster*. The gene is slightly longer in *D. suzukii* due to increase in the size of the 5'UTR and the intron. The numbers indicate the first nucleotide of the respective feature relative to the first transcribed nucleotide. (**B**) Testes whole mount *in situ* hybridization using DIG labeled RNA antisense probe against *Dsβ2t* 5'UTR and exon I detects strong and testes-specific expression. The gene is not expressed at the tip of the testes (black triangle) where stem cells reside. (**C**) Negative control using DIG labeled sense probe shows no signs of staining. The abbreviations Tt and Ag refer to testes or the accessory glands, respectively.

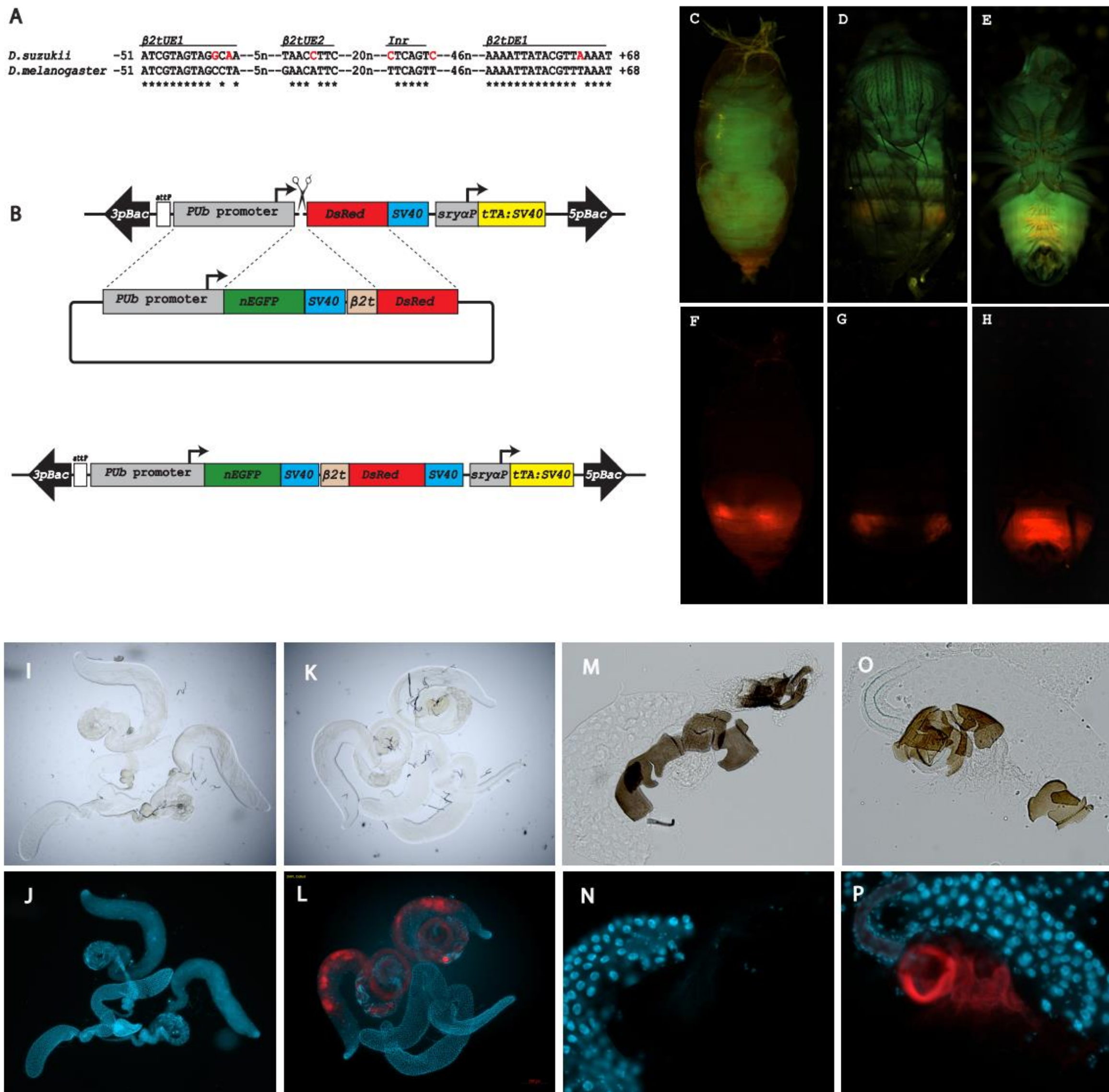


Fig. 3 Generation of a sperm marking strain. (**A**) *Drosophila β2t* genes have a very short and highly conserved promoter/enhancer region with a 14bp upstream element (*β2tUE1*) that confers testes-specific expression while the other indicated elements play quantitative roles. (**B**) Scheme for HDR knock-in of the repair template having *EGFP:SV40* and *β2t* promoter fused to *DsRed.* (**C–H**) Result of the HDR knock-in: images of Pupae (**C**, **F**) as well as adult males in dorsal (**D**, **G**) or ventral view (**E**, **H**) taken with GFP-LP (**C–E**) or RFP (**F–H**) filters, respectively. Compared to wild type (**I**, **J**), the testes of the knock-in males show strong expression of *DsRed* under control of the *β2t* promoter (**K**, **L**). In contrast to wild type females mated to wild type males (**M**, **N**), the fluorescent sperm can also be detected in the storage organ (spermatheca) of wild type females mated to the transgenic sperm-marked strain (**O**, **P**). I, K, M, O images were taken under bright field, and J, L, N, P are composites of images made of the same objects using a DAPI and a DsRed filter.

Additional File 1: Supplementary Table 1: List of primers used.

Code	Name	Sequence 5'-3'
HM#23	AgeI_srya_F4	GAACCGGTGTGCACAAGCGAGTCCACCAG
HM#24	NheI_Srya_R	CCGCTAGCCCTAGGAGCTCTATAAGATGTGCT
HM#25	Ds_B2t_F1	AAGATGCGTGAAATCGTGCACATTCAG
HM#26	Ds_B2t_R1	TTATTCATCGCCGCCACCCTCTTC
HM#33	Ds_B2t_GSP	GTCCGGCCTGAATGTGCACGATTTCACGC
HM#37	Hma-44F	ACTCATCGATATGGCCTCCTCCGAGGACGTCATC
HM#41	B2t5UTR-F1	AGTCCACCCTAGTATCAGCTAGCAAGCACACG
HM#55	HindIII-T7-BbsI-F	AGCTTGATGTGAATTGTAATACGACTCACTATAGGGTCTTCGAGAAGACCT
HM#56	BbsI-T7-HindIII-R	AAACAGGTCTTCTCGAAGACCCTATAGTGAGTCGTATTACAATTCACATCA
HM#69	DsRed-mega-R	GGAAGGACAGCTTCTTGTAGTCGGGG
HM#73	hspEcoRIF2	GCGAATTCTCCAGAAACTCAAACAGAAACC
HM#75	Dshsp-ClaIR	GCATATCGATTTGTGTGTTTGTGTTTGTGGATGCAGTTG
HM#84	chiRNA_R	AAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTG
HM#104	DsU6MegaF	CGAGGTCGACGGTATCGATAAGCTTGTTCCTTGCTAACAATATCTTTTTATGTC ATTTCC
HM#127	HM_pSL_F	CGAAAGGGGGATGTGCTGCAAGGCGATTAAG
HM#128	HM_pSL_R	CCGGCTCGTATGTTGTGTGGAATTGTG
HM#137	U6_neuF2	CGTTTGGGAGCAGGGCTTCACCTTCCTGG
HM#138	U6_neuR2	GCACTCGTATCACCCACAGCGATGAACACG
HM#152	pET_BsaI_BsaIF	TATGGAGACCGTCGACCTAGGGAGACC
HM#153	pET_BsaI_BsaIR	TCGAGGTCTCCCTAGGTCGACGGTCTCCA
HM#158	DsU6MegaR2	CTCTAAAACAGGTCTTCTCGAAGACCCGAAGTTCAAGTGAGATTCTTCCCTATT TATATTG
HM#159	U6a_Mega_R2	CTCTAAAACAGGTCTTCTCGAAGACCCGAAGTTCAAGTGGGTTTCTTCCCTATT TATACTGC
HM#160	U6c_Mega_R2	CTCTAAAACAGGTCTTCTCGAAGACCCGATTTCGTATTGAAAAATGTCGTATAT ATACTACC
HM#161	Red_T1F	CTTCGGATCCACTAGTCGCCACCA
HM#162	Red_T1R	AAACTGGTGGCGACTAGTGGATCC
HM#169	Red_T1_CF	AATCGGATCCACTAGTCGCCACCA
HM#167	Red_T5F	CTTCGAACAGGTGGTGGCGGCCCT
HM#180	NdeI_Histag_F	TATGCATCATCATCATCACCA
HM#181	NdeI_Histag_R	TATGGTGATGATGATGATGCA
HM#186	U6_Down_MegaF	CTTGAAAAAGTGGCACCGAGTCGGTGCTTTTTTTGCAAACCTGAGGGTGAATAT TCAAAC
HM#187	U6_Down_MegaR	GTGGATCCCCCGGGCTGCAGGAATTCGATATGTATGCGTTAAGCAAAAATTGAA AACATAAC
HM#192	PUbT7	CGAACGCACTCGAGCATTTGTGTGCATG
HM#215	T7_Red1F	TATAGGATCCACTAGTCGCCACCA
HM#221	Red1_mutG2AF	CATGATGAGCAAGGGCGAGGAGCTGTTC
HM#222	Red1_mutG2AR	GCTCATCATGGTGGCGACCGGTGGATC
HM#252	B2t-DsRedR	GATGACGTCCTCGGAGGAGGCCATCTTAACCGACTGTCAAGGAAC
HM#285	SV40-B2tF	CATCAATGTATCTTAAACTCATCGATATCGTAGTAGGCAAGCTAATAACC
HM#358	U6a_Mega_F	CGAGGTCGACGGTATCGATAAGCTTTCCGCCGAGTCGCCCATCAGCGAGGAGG
HM#360	U6c_Mega_F	CGAGGTCGACGGTATCGATAAGCTTGTTCCTTAAACACTCAATATTTTTATAAT CTGC

Additional File 2: *piggyBac* insertion in *D. suzukii* line 06_F5M2

piggyBac insertion in the second intron of a gene referred to as *Suppressor of Under-Replication* (*SuUR*). Underlined are the restriction sites for *Eco*RI and *Xho*I, respectively. The bold ***TTAA*** sequence represents the site of *piggyBac* integration, which was duplicated.

TTCGCGAATTCTGCATCCTGAACGATGAGAGTGGCCTGGGTAAACTGGCCACGGTGGCGGCACTTC
TCAGTGCCCTAGATCCCGCCAAGAAAACTCTCATTGTGCTGCAGAACGACGAGCAACTGCTCGCTG
GTTGGCGGTTCCATCTGGACACACTCACGAACCTGCAGGTGTACACCATTCAAGGAGTCCAAGGTA
ATGCTCTGCGTTTAATGATATTCTTGGATTTCTATGTGGATTAGAATTTGGAAAACACCATGTTCT
GATATTTTTATACTTCTCCTAAATCAACATTCCTTGATAGTTAGTTTCGAAGAATGAATCTCWCTG
CAGCGTAAAATGMYTATATTTTGAAAACCACAAAATTGATCAGTTTTTTATTTTGTGTGTACAATT
TCGCAATTAAACGAAAGTAGTGGGTTCCATTTAAGGGATCTGTTAAAATTTGATTTTACCAACTTT
ATTTTGCTATACGGTCTGAACCATTTTAAATAATTTTTTATTATAGTTTAGTTTATAGTTTTATTT
ACAAAATAAATAATGAATATTCTTTAAACTGCTAAAACAATTTGAAAAATTAACATTATTTCCATA
TAATCAATTTTTAAAACGAAAACTTGT***TTAA_5′piggyBac3′_TTAA***TTAAGTCTTGTATAAAC
CTTATCAAAGAACTATATATTTTTATTCGGAAACTAACAGTTTGTTTTACCTCCTTTACAGACACC
ACAGACTCCCCGCACAACGTTTACCTGGCAAAGTGGAGCCAGTTACGCAGCATTGGAGATCTCAGT
CGCCTCAAGTTCGACTACGTTCTGGTAGACAATCGGGGCCACACGCTGAACAACAGCTTCTGCACA
TCAATGCTTCTCAAGCATTTTGAGGGAAGGGTAAACATTCTTATCTCCAGTGTTGACATTACGGTG
AGGCAATGCACCCATAAGTGGTTCACATGGAGCTTTATTTAGTTTGTTTCCCTATCTGCAGTCAGA
CGTAAGGTTGCTGTTCATGTTTTGCGGTTGGGCGGGCGCCTGGAGCATCAGTATCGGAGCTTCCAG
AGCTTCGACCGCAAGTTCCATTTGCCAGATCCAAAGGAGGTCTTTAGCAAGCGTATAGATCTCGAG
AT

3.4 Improvement on the genetic engineering of an invasive agricultural pest insect, the cherry vinegar fly, *Drosophila suzukii*

To be able to develop any transgene-based improvement of the sterile insect technique (SIT) as a pest control strategy, it is necessary to have at least one relaible and efficient tool for germline transformation. In this part, we present an improvement on *piggyBac* germline tranformation with regard to the suitability of different *D. suzukii* strains and identified the AM strain as the most suitable. In addition, we present for the first time the use of site-specific germline transformation based on the phage φC31 integrase system that will facilitate testing different trangenes in the same genomic environment and can be used to stabilize transgenes generated by transposon-based vectors. We also showed for the first time that φC31 RMCE works in *D. suzukii.* Moreover, the *tet-off* binary system was tested.

This chapter thus provides a set of genome manipulation tools for the fruit pest *D. suzukii* that should enable development of transgene-based pest control strategies necessary for the improvement of the SIT.

Hassan M. M. Ahmed, Fabienne Heese, & Ernst A. Wimmer

Authors contributions:

Hassan M. M. Ahmed: Isolation of genes, *in situ* hybridization, generation of constructs, generation of the transgenic *attP* self-docking lines, generation of *tTA* driver lines, testing of the tet-Off binary system, RMCE analysis. Writing of first draft of manuscript and composition of all figures.

Fabienne Heese: Generation of transgenic *attP* docking lines for RMCE and Cas9 responder line.

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Improvement on the genetic engineering of an invasive agricultural pest insect, the cherry vinegar fly, *Drosophila suzukii*

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Abstract

Background: The invasive fly *Drosophila suzukii* has become an established fruit pest in Europe, the USA, and South America with no effective and safe pest management. Genetic engineering enables the development of transgene-based novel genetic control strategies against insect pests and disease vectors. This, however, requires the establishment of reliable germline transformation techniques. Previous studies have shown that *D. suzukii* is amenable to transgenesis using the transposon-based vectors *piggyBac* and *Minos*, site-specific recombination (*lox*/Cre), and CRISPR/Cas9 genome editing.

Results: We experienced differences in the usability of *piggyBac*-based germline transformation in different strains of *D. suzukii*: we obtained no transgenic lines in a US strain, a single rare transgenic line in an Italian strain, but observed a reliable transformation rate of 2.5 to 11% in a strain from the French Alps. This difference in efficiency was confirmed by comparative examination of these three strains. In addition, we used an *attP* landing site line to successfully established φC31-integrase-mediated plasmid integration at a rate of 10% and generated landing site lines with two *attP* sequences to effectively perform φC31-Recombinase Mediated Cassette Exchange (φC31-RMCE) with 11% efficiency. Moreover, we isolated and used the endogenous regulatory regions of *Ds nanos* to express φC31 integrase maternally to generate self-docking lines for φC31-RMCE. Besides, we isolated the promoter/enhancer of *Ds serendipity ▯* to drive the heterologous *tetracycline-controlled transactivator (tTA*) during early embryonic development and generated a testes-specific tTA driver line using the endogenous *beta-2-tubulin* (*β2t*) promoter/enhancer.

Conclusion: Our results provide evidence that the *D. suzukii* strain AM derived from the French Alps is more suitable for *piggyBac* germline transformation than other strains. We demonstrated the feasibility of using φC31-RMCE in the cherry vinegar fly and generated a set of lines that can be used for highly efficient integration of larger constructs. The φC31-based integration will facilitate modification and stabilization of previously generated

transgenic lines that carry at least one *attP* site in the transgene construction. An early embryo-specific and a spermatogenesis-specific driver line were generated for future use of the binary expression system *tet-off* to engineer tissue- and stage-specific effector gene expression for genetic pest control strategies.

Keywords: Binary expression system, enhancer/promoter, insect transgenesis, molecular entomology, pest management, Spotted Wing *Drosophila*, Sterile Insect Technique.

Background

The invasive pest *Drosophila suzukii* commonly referred to as the cherry vinegar fly or the spotted wing *Drosophila* (SWD) originated from East Asia [1, 2]. It was described for the first time in Japan in 1916. In 2008, the fly has concomitantly been reported in Europe (Spain and Italy) and the USA (California), where the SWD presents a major threat to the soft and stone fruit industry [1–3]. The fly is armed with a prominent serrated ovipositor that enables it to lay eggs inside ripening intact fruits. The larvae eat and develop inside the fruits and lead to a crop loss of up to 100% [4]. Several insecticides have been used to control the fly with limited degrees of success [5, 6]. A genetic control method, the Sterile Insect Technique (SIT), might provide the most promising pest management strategy. SIT was proposed more than 75 years ago as biological control method to fight agricultural pests and diseases vectors. It is a species-specific birth control strategy, which makes it safe for pollinators and natural enemies and is thus environmentally friendly [7]. The SIT consists of mass rearing of the target pest in large numbers, sexing, sterilization of the males and successive inundative release in the target area. Genetic engineering offers different approaches for improvement of SIT [8–13]. For example, a transgene-based conditional embryonic lethality system was developed as a way to induce reproductive sterility, which can replace the need for ionizing radiation and ensure production of competent males [9, 10]. A transgenic female-specific embryonic lethality system developed for several dipterans, notably tephritid fruit flies, serves a method to eliminate females during early embryonic development and facilitates the production of only males for SIT releases [11–14].

The ability to genetically manipulate biological systems from mammalian and insect cell lines to insects and mouse has been revolutionized by the discovery and utilization of the most versatile transposon, *piggyBac* [15–17]. It belongs to the class II DNA transposons, which work by a cut and paste mechanisms [18]. *piggyBac*-based vectors were generated to insert cargo sequences at a *TTAA* recognition sequence in the genome of the target species. *piggyBac*-based germline transformation has been successfully established for many model and no-model organisms including *Drosophila melanogaster* [19–21], *Ceratitis captitata* [22, 23], *Anastrepha suspensa* [24], *Drosophila suzukii* [25], *Anopheles gambiae* [26], *Aedes aegypti* [27], *Musca domestica* [28], among others. The increase in the efficiency of germline transformation due to the use of a hyperactive version of the *piggyBac* transposase was

demonstrated in several insects [23]. An inherent characteristic of transposon vectors using *piggyBac* is the random integration in the genome which makes them a useful tool for mutagenesis screens, enhancer traps, and exon traps [19, 29–31]. Also, in cases, when no clear target sequence can be identified, the random integration might result in a set of insertions, from which to choose the most suitable ones. However, this randomness is considered a drawback, when different transgenes were to be compared in the same genomic context [32, 33].

Site-specific recombinases (SSR) offer a more precise approach for genetic engineering of biological systems [34, 35]. In the presence of the respective recombinase, recombination takes place between two identical sequences in case of Flp/*FRT* and Cre/*lox* [36, 37] or non-identical sequences in case of φC31 *attP*/*attB* [38]. The use of SSR necessitates the generation of landing site lines by integrating at least a single landing site (*FRT*, *lox* or *attP*) into the genome of the target species. This is routinely done by including the sequence within a transposon vector and integrate it randomly in the genome. Once generated, these landing sites can be used repeatedly to integrate different transgenes. In case a single landing site is integrated, the transgene of interest has to be delivered in a plasmid vector that has the respective recombinase recognition sequence which leads to integration of the whole plasmid including the antibiotic resistance gene. To avoid this, two landing sites can be placed close to each other into the genome ideally separated by a marker. The transgene to be inserted has to be flanked by two recombinase recognition sequences, which facilitate double recombination events leading to a recombinase mediated cassette exchange (RMCE). The φC31-based integration and RMCE have been established in many insects for either modification and or stabilization of previously generated transgenes [39] or for site-specific germline transformation, which allows examination of different transgenes in the same genomic context [40]. Furthermore, the use of the φC31 system allows for large transgenes to be integrated. In fact, BAC constructs of up to 133 kb were integrated using this system [41]. Moreover, in *Drosophila* and mosquitoes the φC31 system has been used to generate self-docking strains that expresses the integrase from the enhancer/promoter of the maternal effect gene *nanos*. This has remarkably improved the efficiency of site-directed germline transformation [42, 43].

To generate transgene-based reproductive sterility or sexing strains, food supplement-controlled binary expression systems have widely been used for conditional and tissue- or stage-specific gene expression [8–14]. The UAS/Gal4 system has intensively been used in *D. melanogaster* to drive tissue-specific expression of dsRNA to knockdown genes and study their function [44, 45]. The tet system has initially been developed to be used in human cell culture and has since been engineered into *tet-off* and *tet-on* systems [46–48]. In insect biotechnology, the *tet-off* system was used e.g. to control the expression of effector molecules such as the proapoptotic gene *head involution defective* (*hid*), which leads to apoptotic cell

death [8]. To drive the heterologous transactivator of such a binary expression system to cause effective reproductive sterility [9, 10] or female-specific killing [11, 13, 14, 49] based on early embryonic lethality, the promoter/enhancers of cellularization-specific genes need to be identified and isolated. Moreover, to direct sperm-specific expression for transgenic marking [50–52] or the development of multifactorial reproductive sterility [53], the use of promoters/enhancers active during spermatogenesis are of interest.

Here we show that *D. suzukii* strains originated from different locations can be transformed using *piggyBac* germline transformation with varying efficiency. In addition, we demonstrate the successful use of φC31-based site-specific germline transformation both by integration in one *attP* site or by RMCE. Moreover, we provide a set of *D. suzukii* self-docking lines expressing φC31 integrase maternally during oogenesis. Furthermore, we provide an early embryo-specific and a spermatogenesis-specific driver line for using the *tet-off* binary expression system to drive tissue-specific expression of effector genes.

Results

Comparison of *piggyBac* germline transformation in different *D. suzukii* strains

Transposon-based vectors have been intensively used for genetic manipulation from cell culture to mouse. The vector *piggyBac* has gained particular attention due to its versatility and usability in different systems. When we started to use *piggyBac* for germline transformation of an Italian strain of *D. suzukii*, we had only poor success and retrieved a rare transgenic line (06_F5M2) carrying construct HMMA006 [52], which mediates early embryonic expression of *tTA* (Fig. 1), with a transformation rate of 1.6% (300 embryos injected, 200 survived, 60 fertile, 1 transgenic line). However, several previous attempts with the same construct and additional attempts with five other constructs were unsuccessful. Changing to a US strain did not improve our approach, since trying the same five different constructs in this strain did not yield any transgenic lines. Only once we changed to the strain Alpes Maritimes (AM) isolated from the French Alps [54], we started to get reliable *piggyBac* germline transformation to work. In this strain, we regularly obtained transgenic lines for three different constructs with transformation rates between 2.5 and 11% (Additional file 1): The testes-specific driver construct HMMA389, which is designed to be also useable for φC31-mediated RMCE and mediates spermatogenesis-specific expression of *tTA* (Fig. 2); the DsRed-marked construct HMMA185 containing two *attP* sites for φC31-mediated RMCE (Fig. 3); as well as the construct HMMA223 to generate self-docking lines for φC31-mediated RMCE (Fig. 4). Additional file 2 provides a list of the obtained lines.

To examine the suitability of the three different strains for *piggyBac* germline transformation in a truly comparative manner, we injected construct HMMA223 to generate more self-docking lines for φC31-mediated RMCE (Fig. 2C) into similar amounts of embryos on the

same day and with the same injection needle to minimize variations in the injection procedure. Table 1 shows that no transgenic lines were obtained with the US or Italian strains, but were successfully obtained with the French AM strain with a transformation rate of 4.2%. This demonstrates the higher usability of the AM strain for *piggyBac* germline transformation.

Isolation of an enhancer/promoter region active during early embryonic development

To direct gene expression specifically at early embryonic development, we identified the *serendipity α* (*sry α*) gene by homology search in the *D. suzukii* genome database (www.spottedwingflybase.org) using the *Dm_sry α* sequence as query. The open reading frame of the *Ds_sry α* gene from the translation start codon to the stop codon is 1593 bp without introns. The gene has a 5'UTR of 49 bp, which demarcates the transcription start site (Fig. 1A). The *Ds_sry α* coding sequence encodes a putative protein of 530 amino acids, which shares 86% identity to Dm_Sry α protein.

To validate the cellularization-specific expression of the isolated *Ds_sry α* gene, we performed whole mount *in situ* hybridization on different stage wild type embryos using a DIG-labelled antisense probe against the whole *Ds_sry α* ORF plus the 5' UTR. These *in situ* hybridizations detected expression only during blastoderm cellularization with no expression at earlier or later embryonic stages (Fig. 1C-E).

To identify the necessary upstream and downstream regulatory elements driving cellularization-specific gene expression, we compared the *Ds_sry α* sequence with the characterized counterpart in *D. melanogaster* [9]. To examine, whether the 300 bp upstream regulatory element plus the 49 bp 5'UTR drive cellularization-specific gene expression, we fused this 349 bp enhancer/promoter fragment of the *Ds_sry α* gene to *tTA* (Fig. 1B) and generated *D. suzukii* line 06_F5M2 [52] by *piggyBac*-based germline transformation. Embryos from this line were then tested by whole mount *in situ* hybridization for expression of *tTA*, which revealed the respective cellularization-specific expression pattern of *Ds_sry α* (Fig. 1F-H) indicating that the isolated promoter/enhancer element is suitable for stage-specific gene expression during early embryonic development.

Spermatogenesis-specific driver for binary *tet-off* expression system

Since direct expression of effector molecules potentially causing harm obstructs the generation of transgenic lines, we aim to establish the *tet-off* binary system in *D. suzukii* to develop transgenic improvements for SIT approaches. To examine this binary expression system, we used the *Ds_β2t* enhancer/ promoter [52] to generate construct HMMA389 (Fig. 2A). By *piggyBac*-based germline transformation, we obtained the spermatogenesis-specific driver line 389_F25M1 that expresses *tTA* in the testes. The spermatogenesis-specific expression was

confirmed by *in situ* hybridization and compared to the endogenous expression of *Ds_β2t* (Fig. 2B-D).

φC31-mediated site-specific germline transformation

Modification and/or stabilization of transgenes generated by transposon-based vectors by site-specific recombination have been demonstrated in *D. melanogaster* and *Ceratitis capitata* [39, 55]. To establish φC31-based site-specific germline transformation by integration of a transgene construct into a single *attP* site, we injected donor plasmid HMMA182 carrying an *EGFP* transformation marker and the bacterial attachment sequence *attB* along with helper plasmid HMMA098 expressing φC31 integrase under the promoter of the *Ds-hsp70* gene into pre-blastoderm embryos of the DsRed-marked transgenic embryonic driver line 06_F5M2 (attP#1). This line was generated with construct HMMA006 [52], which harbours in addition to the early embryonic tTA-driver also an *attP* site (Figs. 1B, 3A). Out of 250 injected embryos 110 hatched and 40 fertile G_0 crosses gave rise to four independent integrations (Additional File 2), which were identified by showing both red and green fluorescent markers (Fig. 3A'-A'''), resulting in a site-specific transformation efficiency of 10%.

φC31-mediated recombination mediated cassette exchange

To examine a docking line with two *attP* sites in opposite orientation of a DsRed-based transformation marker for establishment of RMCE in *D. suzukii*, we used the docking line 185_F3F1 (RMCE#1), which resulted from *piggyBac*-mediated integration of vector HMMA185 (Fig. 3B) into the AM strain (Additional files 1 and 2). In this line, we confirmed the presence of the two *attP* sites by sequencing. To see whether the φC31-based RMCE works in *D. suzukii*, we co-injected into this line plasmid HMMA336 having two *attB* recombination sites in opposite orientation flanking an EGFP-based transformation marker and the transgene of interest (an effector to drive *Cas9* expression under the control of the binary expression system *tet-off*) along with the helper plasmid HMMA098 (Fig.3B). We obtained 71 G_0 fertile crosses, of which eight gave rise to F_1 progeny that showed EGFP and absence of DsRed fluorescence (Fig. 3B'-B''') indicating an RMCE rate of 11,3%. RMCE line 336_F3F2 was then used to verify the faithful double recombination event by PCR and sequencing of the resulting hybrid *attL* and *attR* sites (Fig. 3B).

Isolation of an enhancer/promoter region active during oogenesis and in the germline to generate self-docking lines for φC31-mediated RMCE

To improve φC31-mediated RMCE further, we wanted to establish self-docking lines (Fig. 4) that express φC31 integrase maternally in addition to carrying two *attP* recombination sites. In this respect, we identified the *Ds_nanos* gene by homology search in the *D. suzukii* genome database (www.spottedwingflybase.org) using the *Dm_nanos* sequence as query. The open

reading frame of the *Ds_nanos* gene from the translation start codon to the stop codon is 2433 bp, which is interrupted by three introns. The gene has a 5'UTR of 236 bp, which demarcates the transcription start site and a 3' UTR of 878 bp (Fig. 4A). To validate the oogenesis- and germline-specific gene expression of the isolated *Ds_nanos* gene, we performed whole mount *in situ* hybridization on ovaries using DIG-labelled antisense probes against the *Ds_nanos* 3'UTR and 103 bp of exon IV. These *in situ* hybridizations detected expression in ovarian nurse cells (Fig. 4B).

To identify the necessary upstream and downstream regulatory elements driving oogenesis-specific gene expression, we compared the *Ds_nanos* sequence with the characterized counterpart in *D. melanogaster*. To examine, whether the 2 Kb enhancer/promoter region including the 5'UTR drives oogenesis-specific gene expression, we fused this 2 Kb enhancer/promoter fragment of the *Ds_nanos* gene to the coding region of *φC31 integrase* (Fig. 4C) and generated *D. suzukii* lines 223_F7M1 and 223_M3M2 by *piggyBac*-based germline transformation of the AM strain (Additional files 1 and 2). In addition, two more self-docking lines were generated in the comparative approach to evaluate the different *D. suzukii* strains (Table 1). Ovaries from line 223_M3M2 were then tested by whole mount *in situ* hybridization for expression of *φC31 integrase*, which revealed the respective nurse cell-specific expression in the ovaries (Fig. 4D) resembling *Ds_nanos* expression, which indicates that the isolated promoter/enhancer element is suitable for maternal gene expression.

Discussion

The discovery that exogenous DNA can be stably introduced into the germline of living organisms which can then be stably inherited by the offspring has tremendously contributed to the advancement of biological and biomedical research and in particular functional genetic studies [15, 19, 30, 31]. The road for insect genetic engineering has been well paved by geneticists working with the model organism *D. melanogaster*. Genetic screens in *D. melanogaster* using *P-element* based transformation vectors to perform insertional mutagenesis, enhancer- and gene-traps, as well as ectopic or overexpression studies provided an enormous contribution to our understanding of gene function [56–58]. Unfortunately, the *P-element* is not functional in other organisms due to the requirement of host-specific factors [59]. Transformation vectors based on the lepidopteran transposable element *piggyBac* have been used to engineer many insects [20, 22, 25, 26] and encouraged the establishment of new insect model systems such as *Tribolium castaneum* [60, 61].

The invasive fruit pest, *D. suzukii*, had been successfully transformed using *piggyBac*-based vectors [25, 54, 62]. We have used three different lab strains of *D. suzukii* from Italy, USA, and France. After many attempts to generate transgenic *D suzukii* using *piggyBac* germline transformation with different constructs by microinjection into the Italian strain, we obtained only one transgenic line, 06_F5M2, with a low transformation efficiency of 1.6% and failed

to obtain any transgenic flies from the US strain. When we obtained the French strain AM (which was kindly provided to us by N. Gompel, Munich), we managed to get reliably transgenics with varying efficiency. Based on these observations and the comparative examination of these three strains (Table 1), we recommend the AM strain for *piggyBac* germline transformation.

Due to the random integration of transposon-based transformation vectors and the limited size of cargo they can carry, we decided to extend the toolkit for *D. suzukii* transformation by firmly establishing a site-specific transformation technology. Recombinase-based site-specific germline transformation such as (Cre/*lox*, flp/*FRT* and φC31 *attP*/*attB*) had been established in many model and non-model insects and shown to overcome the shortcomings of transposon-based germline transformation [40]. The Cre/*lox* Recombinase Mediated Cassette Exchange has recently been established for the cherry vinegar fly *D. suzukii* [63]. In this study, we demonstrate the feasibility of using the *φC31* integrase system to integrate a construct in a single *attP* landing site. This approach had previously been established for *D. melanogaster* and the Mediterranean fruit fly *Ceratitis capitata*, where it was used to modify transgenic lines generated by random transposon-based vectors and to stabilize the transgene by subsequent deletion of one of the inverted repeats required for transposition [39, 55]. In addition, we have generated a docking line with two *attP* sites in opposite orientations and show that φC31-mediated RMCE works in *D suzukii*. The use of an endogenous source of φC31 integrase by expression from a germline specific enhancer/promoter had been shown to increase the efficiency of φC31-mediated integration and RMCE [42, 43]. In this regard, we set to generate self-docking lines that express *φC31 integrase* maternally. We isolated the endogenous *Ds-nanos* gene (Fig. 4) in order to use the upstream enhancer/promoter and the downstream 3'UTR for directing the expression of *φC31 integrase* to the nurse cells for maternal contribution to the early embryo. By random *piggyBac* germline transformation, we generated four transgenic lines with a *DsRed* body marker and the *φC31 integrase* cassette flanked by *attP* sites.

To be able to conditionally drive expression of effector genes in a tissue- or stage-specific manner, a suppressible or inducible binary expression system is required. This has been successfully exploited to develop biotechnological pest control strategies such as early embryonic lethality or female-specific embryonic lethality [9–14]. To develop such transgenic pest control strategies for the invasive pest *D. suzukii*, we identified a gene that is active during early embryonic stages (*Ds_sry* α) and a gene that is spermatogenesis-specific (*Ds_*β*2t*) [52]. 350 bp upstream regulatory sequence of the *Ds_sry* α gene were identified to be sufficient to drive the expression of *tTA* specifically during cellularization similar to the endogenous gene. This driver line will be usable to generate reproductive sterility or sexing lines by driving expression of pro-apoptotic genes as previously described for several tephritid fruit pests [10–12, 14]. Such systems will be very important to establish SIT programs for the control of this

invasive pest species. In addition, we were able to generate a spermatogenesis specific driver line using the promoter of the *Ds_β2t* gene described previously [52].

Conclusion

By comparing different *D. suzukii* strains for their usability for *piggyBac*-based germline transformation, we could clearly identify the AM strain derived from the French Alps as the most suitable one. In addition, we demonstrated that φC31-based site-specific integration and RMCE can be used routinely in the cherry vinegar fly, *D. suzukii*, and generated four self-docking lines for RMCE. The φC31-based integration will facilitate efficient integration of larger transgenic constructs and allow for the modification and stabilization of previously generated transgenic lines that carry at least one *attP* site in the transgene construction.

Methods

***Drosophila suzukii* strains**

All fly experiments were performed in our well-equipped safety level one (S1) laboratory, which is certified for generating and using genetically modified insects. Wild type *D. suzukii* from Italy, USA (both kindly provided by Prof. Marc F. Schetelig), and French Alps (Prof. Dr. Nicolas Gompel) as well as the generated transgenic flies were reared on standard *Drosophila* food and kept at 25°C throughout this study.

Nucleic acid isolation

Genomic DNA isolation was done from a mix of adult males and females using NucleoSpin® DNA Insect (Macherey-Nagel) according to the manufacturer instructions. Total RNA was isolated from 0-24 hours embryos enriched for 0-4 hours stages using ZR Tissue & Insect RNA MicroPrep (Zymo Research Europe, 79110 Freiburg) according to manufacturer instructions.

All PCR amplifications during the course of this study were performed using Phusion DNA polymerase and Phusion-HF buffer (New England Biolabs GmbH, D-65926 Frankfurt am Main). A list of the used primers is provided in Additional file 3. Plasmid min-preps and PCR products were purified using NucleoSpin® Plasmid and NucleoSpin® Gel and PCR Clean-up kits (Macherey-Nagel GmbH & Co., 52355 Dueren, Germany), respectively. NucleoSpin® Plasmid Transfection-grade (Macherey-Nagel) or QIAGEN Plasmid Plus Midi Kit (QIAGEN GmbH, 40724 Hilden, Germany) were used to prepare plasmids for germline transformation.

Amplification of cDNA ends

To isolate the 5'UTR and the 3'UTR of the early embryonic gene *Ds_sryα* and the maternal effect gene *Ds_nanos*, total RNA from 0-24 h old (enriched for 0-4h) *D. suzukii* embryos

was isolated and 1.3 μg were used to generate 5' RACE-ready cDNA or 3'RACE-ready cDNA using SMARTer™ RACE cDNA amplification kit (Takara Bio Europe SAS, 78100 Saint-Germain-en-Laye, France) according to manufacturer instructions.

The 5'UTR of *Ds_sryα* and *Ds_nanos* were recovered by RACE PCR using gene specific primers HM#34 and HM#76, respectively, along with the universal primer (UPM) provided with the kit using Advantage2 DNA polymerase (Takara) with the following program: 94°C 2 min, (94°C 30 sec, 72°C 3 min) 5X, (94°C 30 sec, 70°C 30 sec, 72°C 3 min) 5X, (94°C 30 sec, 68°C 30 sec, 72°C 3 min) 30X. A single prominent band for each gene was recovered, purified, cloned into pCRII (Thermo Fisher Scientific) to generate pCRII_sryα_5'UTR (HMMA001) and pCRII_nos_5UTR (HMMA012), and sequenced using standard M13 primers.

To recover the 3'UTR of *Ds_sryα* and *Ds_nanos,* the gene specific primers HM#42 and HM#77, respectively, along with UPM provided with the kit using Advantage2 DNA polymerase (Takara) were used with the following program: 94°C 2 min, (94°C 30 sec, 72°C 3 min) 5X, (94°C 30 sec, 70°C 30 sec, 72°C 3 min) 5X, (94°C 30 sec, 68°C 30 sec, 72°C 3 min) 30X. A single prominent band for each gene was recovered, purified, cloned into pCRII (Thermo Fisher Scientific) to generate pCRII_sryα_3UTR (HMMA002) and pCRII_nos_3UTR (HMMA013), and sequenced using standard M13 primer.

Plasmids construction

The plasmid HMMA020 was generated by PCR amplification of the coding sequence of *D. suzukii sryα* gene plus the 5'UTR using primer pair HM#16/HM#17 and advantage 2 DNA polymerase (Invitrogene) with program 98 °C 3' followed by [98 °C 30'', 55 °C 30'',72 °C 2']35X and cloned into the pCRII vector (Invitrogene).

To generate plasmid HMMA021 for *in vitro* synthesis of RNA probes, the *tTA* coding sequence was excised from mfs#1215[10] using *Eco*RV/*Bam*HI and cloning into pCRII vector digested by the same enzymes.

To generate plasmid HMMA339 for *in vitro* synthesis of RNA probe against *φC31* integrase mRNA, 800 bp of the coding sequence was digested out from plasmid HMMA98 using *Sma*I/*Not*I and cloned into pCRII plasmid digested by *Eco*RV/*Not*I.

The plasmid FCMH01 was generated by PCR amplification of 800 bp of *Cas9* coding sequence using primers pair HM#560/HM#561 with program 98°C 3' followed by [98 °C 30'', 64 °C 30'', 72 °C 30''] 5X [98 °C 30'', 72 °C 1'] 35X, digested by and cloned into *Xho*I/*Bam*HI sites of pCRII vector.

To generate *piggyBac* transformation vector HMMA185 and HMMA186, first plasmid HMMA006 [52] was digested by *Asc*I to remove *sryα-tTA*, and the backbone was ligated to

give rise to HMMA007. *attP220* was PCR amplified from HMMA007 using primer pair HM#368/HM369 and program 98 °C 3'followed by [98 °C 30'', 58 °C 30'', 72 °C 20''] 5X [98 °C 30'',72°C 1'] 35X and cloned into *Eco*RV cut site of HMMA007 to give rise to HMMA185. To generate HMMA186 the *Eco*RI/*Hpa*I fragment *PUb::nlsEGFP* from mfs#1213 [51] was cloned into the *Eco*RI/*Hpa*I sites of HMMA185.

For the generation of *piggyBac* transformation vectors HMMA330 and HMMA331, first Gibson assembly was performed to clone *EGFPSV40* and the *3XP3* promoter into the *piggyBac* backbone of HMMA007 digested by *Eco*RI to give rise to HMMA227, in which the *EGFP* gene was then replaced by *DsRed.T3* from HMMA007 by *Age*I/*Not*I to give rise to HMMA228. Then the *attP220* was PCR amplified from HMMA007 using primer pair HM#131/HM#117 with PCR program 98 °C 3'followed by [98 °C 30'', 60 °C 30'', 72 °C 20''] 35X and cloned into *Eco*RI site of HMMA227 and HMMA228 giving rise to HMMA304 and HMMA305, respectively. Finally, the *Asc*I/*Age*I fragments from mfs#1213 and mfs#1214 [51] containing the *PUb* promoter were cloned into *Asc*I/*Age*I sites of HMMA304 and HMMA305 to give rise to HMMA330 and HMMA331, respectively.

To generate the spermatogenesis specific driver construct HMMA389, 1 kb upstream region of *D. suzukii Ds-β2t* gene including the 5'UTR was PCR amplified from genomic DNA of the wild type Italian strain using primer pair HM#35/HM#36 with program 98 °C 3' [98 °C 30'', 61 °C 30'', 72 °C 30''] 5X [98 °C 30'',67 °C 30''72 °C 30''] 35X and cloned in *Nco*I/*Xba*I sites of mfs#1215 [10] giving rise to HMMA015. The *Dm-β2t* 3UTR was then PCR amplified from gDNA of wild type *D. melanogaster* strain OreR using primer pair HM#706/HM#707 with program 98 °C 3' [98 °C 30'', 63 °C 30'', 72 °C 20''] 5X [98 °C 30'',70 °C 30''72 °C 20''] 35X and cloned into HMMA015 to give rise to HMMA253. Finally, the *Asc*I fragment from HMMA253 was cloned into the *Asc*I site of the transformation vector HMMA331.

To generate *attB* integration vector HMMA182 which can be used to integrate a plasmid into single *attP* site, the *5-piggyBac* region was PCR amplified from plasmid HMMA006 using primer pair T7/mfs#370, with program 98 °C 3' [98 °C 30'', 51 °C 30'', 72 °C 20''] 40X digested by *Eco*RV and cloned into the blunted *BamH*I site of HMMA172, giving rise to HMMA181. Then the *Eco*RI/*Apa*I fragment containing the *PUb::nlsEGFP* was excised mfs#1213 [51] and cloned into *Eco*RI/*Apa*I of HMMA181.

To generate the helper plasmid HMMA098, the coding sequence of φC31 was PCR amplified from plasmid mfs#1289 [39] using primers pair MK153/HM#123 with program 98 °C 3' [98 °C 30'', 72 °C 1' 20''] 35X. The reverse primer introduces the *SV40 nuclear localization sequence* at the C-terminus, which can improve the efficiency of φC31 integrase [64]. A second round of PCR using primer pair MK153/HM#203 was used to amplify *φC31nls* using 1 µl of the first PCR reaction as a template with program 98 °C 3' [98 °C 30'',

67 °C 30'', 72 °C 1'] 5X [98 °C 30'',72 °C 1' 20''] 35X and clone into HMMA051 *Nco*I/*Not*I replacing the *piggyBac* transposase coding sequence and giving rise to HMMA098. The *piggyBac* helper HMMA051 was generated by cloning the *SV40* 3'UTR digested from CH#705 by *Hind*III/*Not*I into HMMA050 *Hind*III/*Not*I. sites. The latter was made by PCR amplification of *Ds-hsp70* promoter [52] from gDNA using primer pair HM73/HM#74 and program 98 °C 3'followed by [98 °C 30'', 58 °C 30'', 72 °C 30''] 5X [98 °C 30'', 66 °C 30'', 72 °C 30'] 35X and cloning into *Eco*RI site of HMMA049,which was generated by cloning the *piggyBac* transposase coding sequence excised from MK004 [23] by *Eco*RI/*Not*I into the shuttle vector pSLaf1180af [65].

To generate φC31 integrase based RMCE donor plasmids, HMMA253 and HMMA254, the annealed oligos HM#101/HM#337 generating the bacterial attachment site *attB* were cloned into *Spe*I site of pCRII vector (Invitrogene) giving rise to HMMA172. The *gypsy* insulators were digested out using *Spe*I/*Eco*RI from a fragment amplified from mfs#1213[51] using primer pair HM#469/HM#470 with program 98 °C 3'followed by [98 °C 30'', 70 °C 30'', 72 °C 2'] 35X and cloned into the cut site of HMMA172 to give rise to HMMA189. The *Eco*RI/*Not*I fragments *PUb::nlsEGFP* and *PUb::DsRed.T3* were excised from HMMA186 and HMMA185, respectively, and cloned into HMMA189 to give rise to HMMA190 and HMMA191, respectively. Finally, *SV40* was PCR amplified from HMMA007 using primer pair HM#179/HM#124 and program 98 °C 3'followed by [98 °C 30'', 62 °C 30'', 72 °C 20''] 5X [98 °C 30'', 68 °C 30'', 72 °C 20''] 35X and cloned along with annealed oligos HM#101/HM#108 into HMMA190 and HMMA191 *Not*I/*Xba*I-blunted.

To generate HMMA336, for φC31-RMCE, the tetracycline responsive element TRE along with the *P-element* basal promoter was PCR amplified from CH 727 [9] using primers pair HM#584/ CH6R [9] with PCR program 98 °C 3'followed by [98 °C 30'', 69 °C 30'', 72 °C 30''] 35X and cloned into *Eco*RI/*Cla*I sites of HMMA56 [52] replacing the *hsp70* promoter giving rise to HMMA317 then the *Asc*I fragment containing *Cas9* fused to the *TREp* and the *SV40* 3'UTR was clone into *Asc*I site of HMMA253

To generate self-docking transformation plasmid HMMA223 the *Asc*I fragment containing nosE/P-φC31-nos was excised from the shuttle vector HMMA221 and cloned into *Asc*I site of HMMA185. HMMA221 was generated by replacement of *Cas9* coding sequence in plasmid HMMA167 by *φC31 integrase* CDS. To make HMMA167, first the 3UTR of *Ds-nanos* was PCR amplified from HMMA013 using primer pair HM#94/HM95 with program 98 °C 3'followed by [98 °C 30'', 66 °C 30'', 72 °C 30] 5X [98 °C 30'', 72 °C 1'] 35X and cloned into the shuttle vector pSLaf1180af [65] *Xba*I/*Afl*II sites giving rise to HMMA062. Then *Cas9* CDS was excised from HMMA056 [52] and cloned into *Cla*I/*Xba*I sites of HMMA062 giving rise to HMMA165. Then the palindromic (self-complementary) oligo HM#102 was annealed to itself to introduce the 2X *Bbs*I recognition site and cloned into the

*Cla*I site of HMMA165 to give rise to HMMA166. Finally, a 2 Kb upstream regulatory region of *Ds-nanos* gene including the 5'UTR was PCR amplified from gDNA using primer pair HM#345/HM#113 and program 98 °C 3'followed by [98 °C 30'', 72 °C 1' 30''] 35X and cloned into HMMA166 *Bbs*I site by golden gate resulting in HMMA167.

Germline transformation

All *piggyBac* germline transformation experiments were performed using transformation vector and helper plasmid MK006 [23] at a final concentration of 500 ng/µL and 200 ng/µL respectively. For φC31-mediated site-specific transformation and φC31-mediated RMCE, the donor vectors were injected along with the helper plasmid HMMA098 at a concentration of 500 ng/µL and 300 ng/µL, respectively. The materials and the procedure of germline transformation were as described previously [23, 52]. Emerged G_0 flies were crossed individually to three wild type flies of the opposite sex.

Generation of RNA probes

To generate DIG-labelled antisense RNA probes for *in situ* hybridization against *Ds_sryα, Ds_nanos, tTA*, *Cas9*, or *φC31 integrase*, DNA templates for *in vitro* transcription were prepared by restriction enzyme linearization of pCRII vectors containing either the whole gene pCRII_*Ds-sryα* (HMMA020), the 3'RACE fragment pCRII_*Ds-nos_3UTR* (HMMA013), the coding sequence pCRII_*tTA* (HMMA021), or 800 bp of the coding sequence of in case of pCRII_*Cas9* (FCMH01) and pCRII_φC31 (HMMA399) using *Xho*I*, Bam*HI*, Not*I, *Not*I, or *EcoR*I, respectively. The antisense RNA labelling reaction was done using the DIG-labelling kit (Thermo Fisher Scientific) according to manufacturer instructions using 1 µg of DNA as template in a total reaction mix of 20 µL. The reaction was allowed to proceed for 3h at 37°C followed by Turbo DNaseI treatment (Thermo Fisher Scientific) for 30 min to remove template DNA. 2 µL of 0.2 M EDTA were used to inactivate the reaction. The probes were then ethanol precipitated and resuspended in 100 µL RNA resuspension buffer (5:3:2 H_2O: 20X SSC: formaldehyde) and stored at -80°C.

Testes, ovary, and embryo whole mount *in situ* hybridization

Testes from 3-5 days old males from wild type *D. suzukii*, spermatogenesis specific driver line 389_M25M1, or progeny of the cross of the driver 389_M25M1 to the responder line 366_F3F1 were dissected in ice cold 1X phosphate buffered saline (PBS). Fixation and *in situ* hybridization were performed according to protocol by Lecuyer [66]. Anti-sense DIG labelled RNA probe against *tTA* was used to detect the expression driven by the *Ds-β2t E/P*. The *Cas9* anti-sense RNA probe was used to detect the expression of *Cas9* in the progenies arising from the cross testing the *tet-off* system. Anti-sense and sense probes previously described [52] were used as control.

To confirm the expression of the isolated *Ds-nanos* gene and the *φC31 integrase* driven by the regulatory regions of *Ds-nanos* in the ovaries of *D. suzukii* wild type flies and the transgenic self-docking line 223_F7M1, respectively, we collected 3-5 days old female flies and dissected the ovaries in ice-cold 1X PBS. The fixation and the *in situ* hybridization were performed as described [66].

To confirm the endogenous cellularization-specific expression of *Ds_sryα* in wild type embryos. and whether the 349 bp of its upstream regulatory region including the 5'UTR are enough to drive expression of *tTA* in the transgenic driver line 06_F5M2 in a similar pattern, we performed embryo whole mount *in situ* hybridization using respective anti-sense DIG-labelled RNA probes in 0-24 h old embryos. Fixation and *in situ* hybridization were performed according to Lecuyer [66].

Microscopy

To observe and image testes, ovaries, and embryos, Zeiss Imager.Z2 equipped with two cameras, Axiocam 506 mono and Axiocam 305 colour (Zeiss, 73447 Oberkochen, Germany) was used. Images were taken using Axiocam 305 with bright field or DIC settings.

Screening for transgenic flies and fluorescence imaging were performed using Leica M205 FA fluorescence stereomicroscope equipped with camera Q imaging Micropublisher 5.0 RTV (Leica Mikrosysteme Vertrieb Gmb, Wetzlar, 35578 Germany). Transgenic flies were screened using filter sets RFP (excitation: ET546/10, emission: ET605/70) or GFP-LP (excitation: ET480/40, emission: ET510 LP), respectively, and imaged using cold light (Fig. 3A', B'), filter sets RFP (Fig. 3A'', B''), or EYFP (excitation: ET500/20, emission: ET535/30; Fig. 3A''', B''').

Declarations:

Ethics approval and consent to participate: Not applicable.

Consent to publish: Not applicable.

Availability of data and materials:

All data generated or analysed during this study are included in this published article and its supplementary information files.

Competing interests:

The authors declare that they have no competing interests.

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Authors' Contributions:

E.A.W and H.M.M.A. conceived and designed the study; H.M.M.A. isolated the genes, designed the constructs, and performed *in situ* hybridizations; H.M.M.A. and F.H. generated the transgenic lines; E.A.W and H.M.M.A. wrote the manuscript; H.M.M.A. prepared the figures; all authors read and approved the final manuscript.

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Table 1: Comparative *piggyBac* transformation efficiency in different *D. suzukii* strains

Origin of *D. suzukii* strain	No. of injected embryos	Hatched larvae	Fertile crosses	No. of transgenics	Transformation rate in %
Italy	400	190	35	0	–
France (AM)	450	210	47	2	4.2
USA	430	240	50	0	–

Figures:

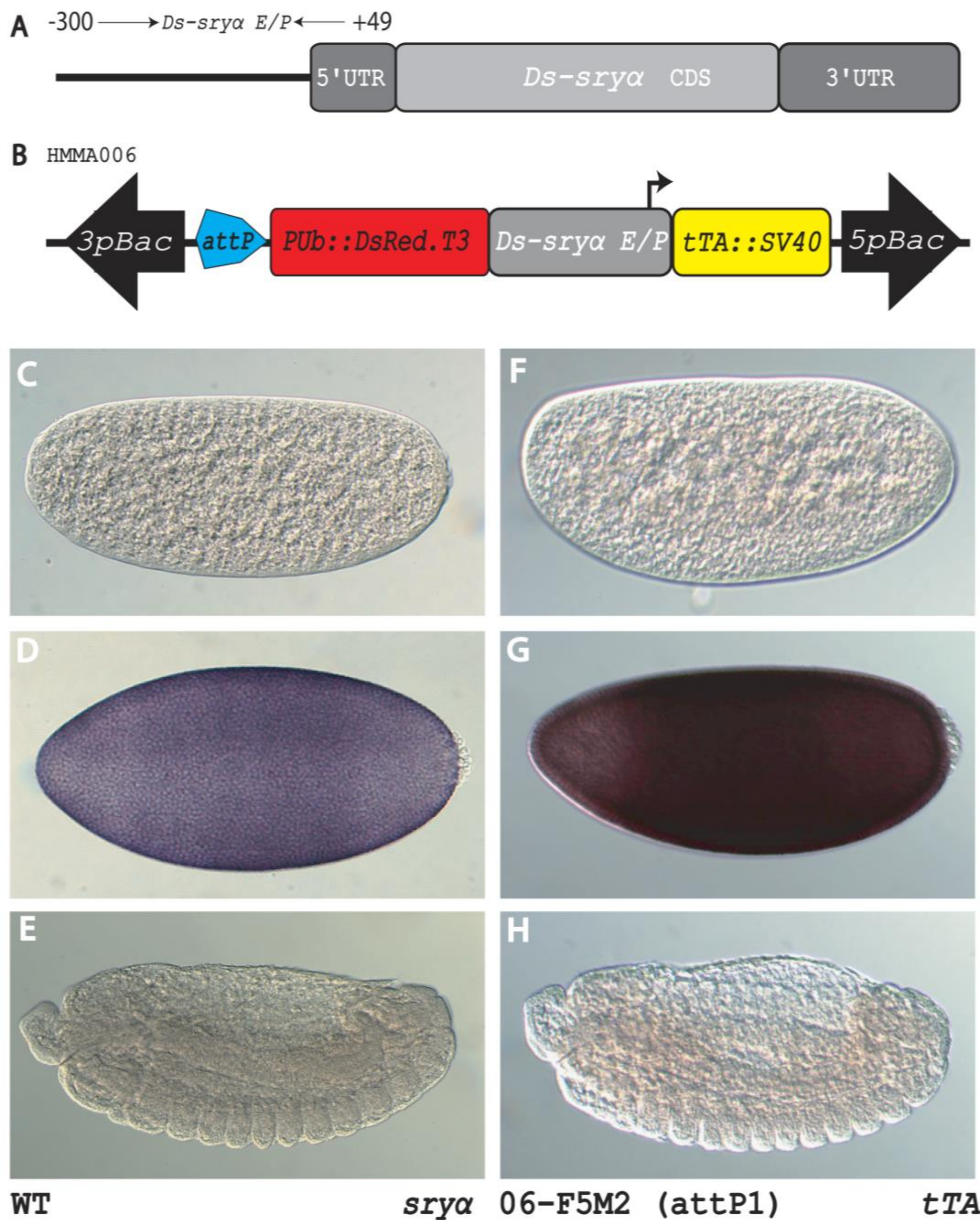

Fig. 1. *D. suzukii serendipity α* and the use of its promoter/enhancer for directed expression. (**A**) Schematic representation of the *D. suzukii* gene *serendipity α*. (**B**) *piggyBac*-based transgenic construct HMMA006 [52] to drive *tTA* during early embryonic development. (**C–E**) Whole mount *in situ* hybridisation to detect *Ds_sry α* expression in wildtype *D. suzukii* embryos. (**F–H**) Whole mount *in situ* hybridisation to detect *tTA* expression in transgenic *D. suzukii* embryos of line 06_F5M2 (attP#1) carrying construct HMMA006. (**C**, **F**) Syncytial blastoderm embryos before start of cellularization. (**D**, **G**) Syncytial blastoderm embryos during cellularization show expression of *sry α* or *tTA*, respectively. (**E**, **H**) Germ band retracting embryos.

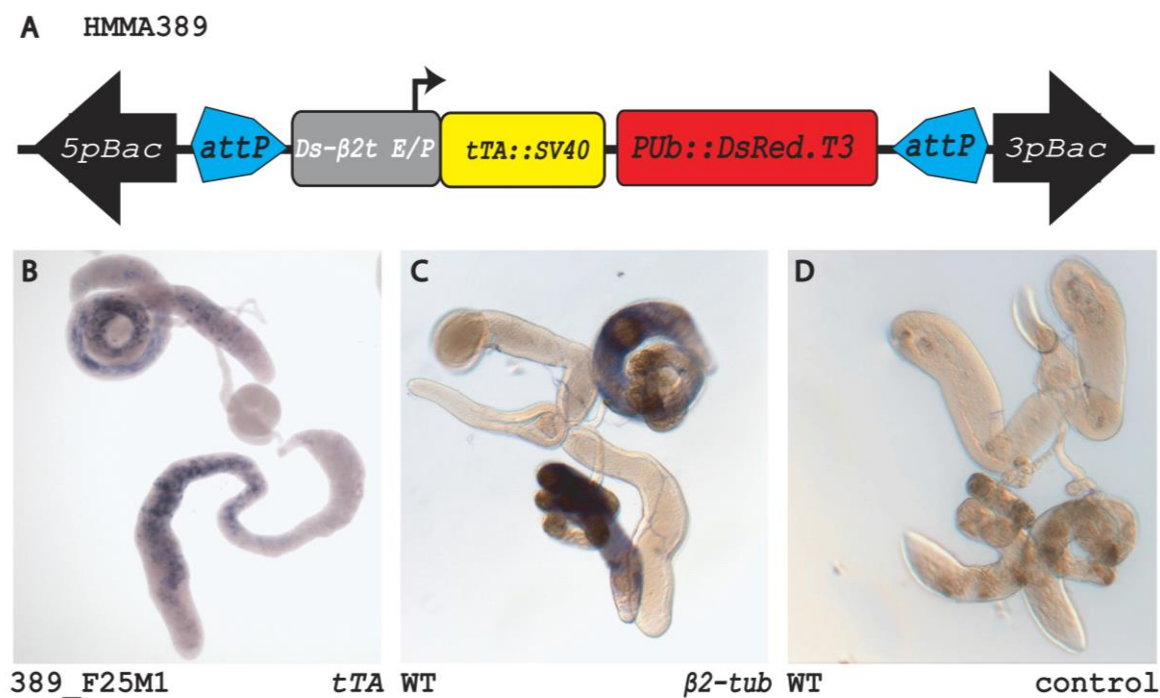

Fig. 2. Spermatogenesis-specific driver for binary *tet-off* expression system. (**A**) *piggyBac*-based transgenic construct HMMA389 to generate a testes-specific driver line carrying the *β2t* promoter [52] fused to *tTA*. (**B–D**) Whole mount *in situ* hybridisation to detect gene expression in *D. suzukii* male reproductive organs. (**B**) Testes-specific *tTA* expression driven by the *Ds_β2t* promoter in line 389_F25M1. (**C**) *Ds_β2t* expression in wildtype testes detected by an antisense probe. (**D**) Negative control using a *Ds_β2t* sense probe on wildtype testes.

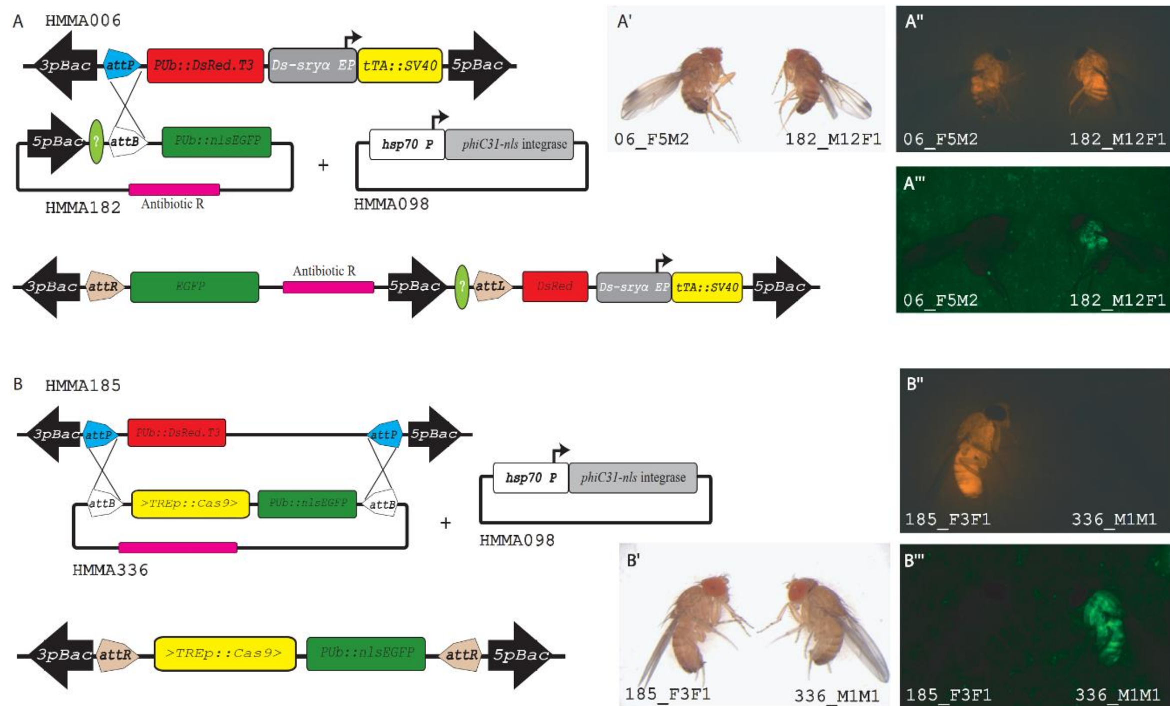

Fig. 3. φC31-mediated site-specific integration and RMCE. (**A**) Scheme for site-specific germline transformation. *D. suzukii* line 06_F5M2 [52] carries construct HMMA006 that contains an *attP* recombination target sequence, which – in the presence of a helper plasmid providing φC31 integrase (HMMA098) – is targeted by construct HMMA182 carrying the corresponding *attB* recombination site to integrate the complete plasmid. The integration leads to a modification of the transgenic insert, which can be used for additional integration of transgenes (light green "?") as well as transgene stabilization by removing part of the transgenic composition by *piggyBac* excision [39]. (**A'–A'''**) Integration can be detected by the addition of the EGFP marker. (**B**) RMCE to generate diverse transgenes at the same genomic position. *D. suzukii* line carrying construct HMMA185 is targeted by construct HMMA336 in the presence of a helper plasmid (HMMA098) providing φC31 integrase to exchange marker genes and integrate a specific cargo gene (*TRE-Cas9*). (**B'–B'''**) RMCE can be detected by the replacement of the DsRed marker with the EGFP marker. Images of a male fly of each indicated line are taken with cold light (**A'**, **B'**), RFP filter (**A''**, **B''**), or EYFP filter (**A'''**, **B'''**).

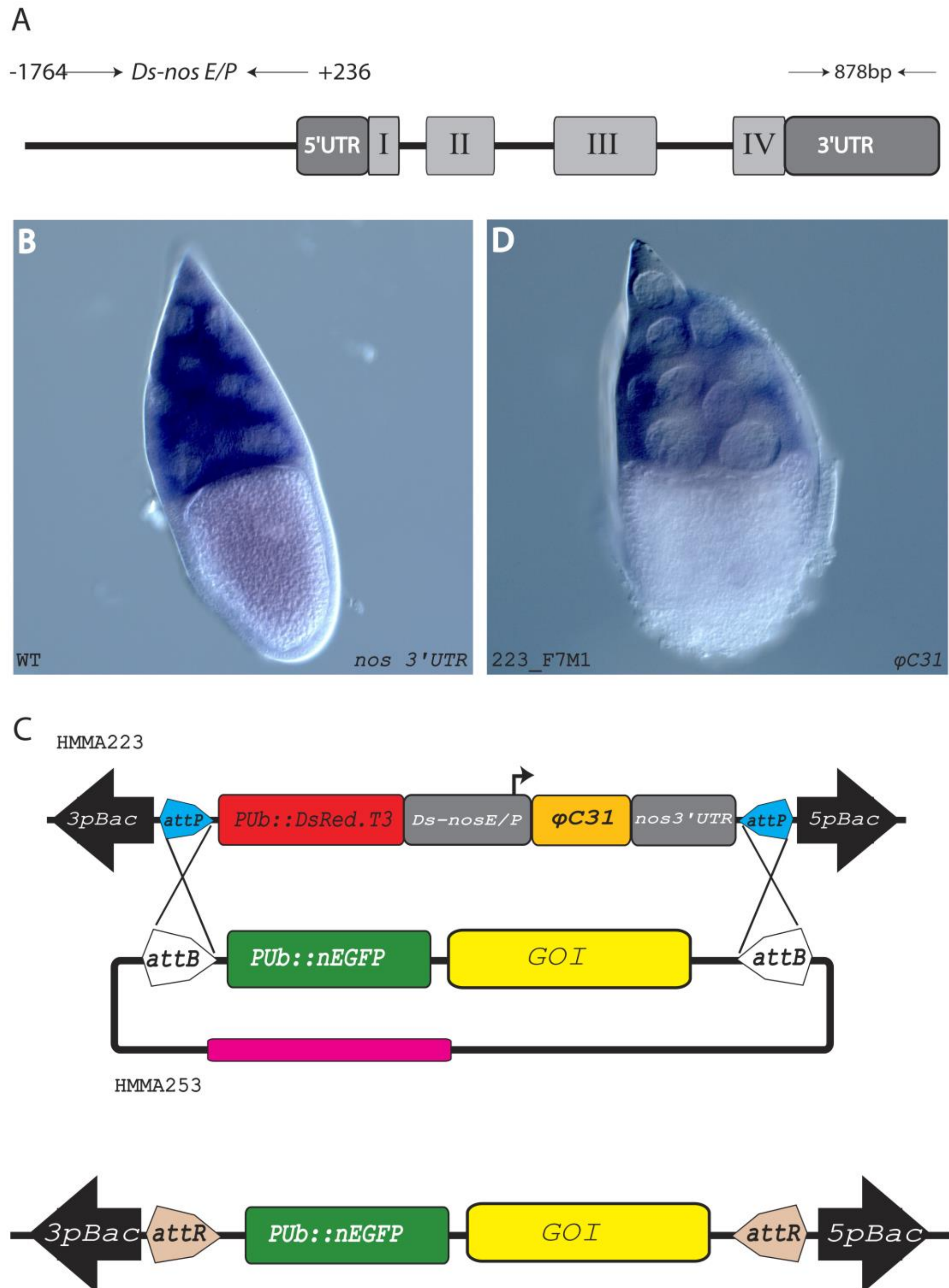

Fig. 4. *D. suzukii nanos* and the use of its promoter/enhancer for directed expression. (**A**) Schematic representation of the *D. suzukii* gene *nanos*. (**B**) Whole mount *in situ* hybridisation to detect *nanos* expression in wildtype *D. suzukii* ovaries. (**C**) *piggyBac*-based transgenic construct HMMA223 to generate φC31 integrase RMCE self-docking lines. RMCE in a self-docking line, which provides both the recombination target sequences *attP* as well as the φC31 integrase driven by the *nanos* promoter/enhancer providing maternal expression, will result in marker exchange as well as cargo gene (*GOI*) integration and removal of the integrase source. (**D**) Whole mount *in situ* hybridisation to detect *φC31 integrase* expression in transgenic *D. suzukii* ovaries carrying construct HMMA223. Expression of *nanos* or *φC31 integrase*, respectively, is detected in the nurse cells of the ovaries.

Additional File 1

Supplementary Table 1: ***piggyBac* transformation rates in *D. suzukii* AM strain**

Construct	No injected embryos	Hatched larvae	Fertile crosses	No. transgenics	Transformation rate in %
HMMA389	350	185	40	1	2.5
HMMA185	475	181	45	2*	4.4
HMMA223	290	150	18	2	11

* For one transgenic F1, no line could be established.

Additional File 2.

Supplementary Table 2: **List of transgenic lines**

Strategy	Construct	Transgenic lines
piggyBac	HMMA006 (embryonic driver, *attP*), Figs. 1B; 3A	06_F5M2 (attP#1)
	HMMA389 (spermatogenesis driver), Fig. 2A	389_F25M1
	HMMA185 (φC31 RMCE docking), Fig. 3B	185_F3F1 (RMCE#1)
	HMMA223 (φC31 RMCE self-docking), Fig. 4C	223_M3M2 (RMCE-sd#1) 223_F7M1 (RMCE-sd#2) 223_F5F1 (RMCE-sd#3) 223_M10F1 (RMCE-sd#4)
***φC31*-int**	HMMA182 (single *attB* donor), Fig. 3A Injected into 06_F5M2 (attP#1)	attP#1_182_M12F1 attP#1_182_F8M1 attP#1_182_F15F1 attP#1_182_F25M1
***φC31*-RMCE**	HMMA336 (RMCE donor *TREp:Cas9*), Fig. 3B Injected into 185_F3F1 (RMCE#1)	RMCE#1_336_M1M1 RMCE#1_336_M17F1 RMCE#1_336_M21M1 RMCE#1_336_M32M1 RMCE#1_336_M33F1 RMCE#1_336_M34M3 RMCE#1_336_F12F1 RMCE#1_336_F3F2

Additional File 3

Supplementary Table 3: List of primers used

Code	Name	Sequence 5'- 3'
HM#16	sryaR4	TTGTGTGTCATGGATGTTCAATCTAATC
HM#17	Srya5UTR_F1	GTACTTAGTTGAAAAGTTCAGCTTTACCCG
HM#34	Ds_srya_GSP3	GGCATCCAGGCTAATGGTCCGCTCCAAGTG
HM#35	B2t_NcoI_F1	GCAACCATGGGATGCCAAGAGAGATGAGCAGG
HM#36	B2t_XbaI_R3	CGATTCTAGACATCTTAACCGACTGTCAAGGATC
HM#42	srya_GSP_F	GCCTCTCTGGCTCCGATTCCCCCTAATG
HM#73	hspEcoRIF2	GCGAATTCTCCAGAAACTCAAACAGAAACC
HM#74	hspEcoRIR	GCGAATTCTTGTGTGTTTGTGTTTGTGGATGCAG
HM#76	nos_GSPR	GAGTCCTCCTCTTGCGTGGAATGCCG
HM#77	nos_GSPF	GTACTGTCCCAAGAAGCCGATTATCAC
HM#94	nos3UTRXbaIF	GGTCTAGAGAACACATCCGGCAGGAGC
HM#95	nos3UTRAflIIR	ATACTTAAGACTGAGCTCCAAGCAGTGGTATCAACGCAGAG
HM#101	SpeI-atBF	CTAGTGTGAGGTGGAGTACGCGCCCGGGGAGCCCAAGGGCACGCCCTGGCACCCGCAC
HM#102	BbsI-BbsI	CGGTCTTCGCGAAGAC
HM#113	BbsI_nosR	GCGAAGACCCATATGGCGAAAGTCCGGCTCGAAAGTTACC
HM#117	HM_Pub_R	CATTGGAATCTCTGTCGCTGCGTTCCG
HM#123	phi_nls_R	CTAGACCTTCCGCTTCTTCTTTGGGGCCGCCGCTACGTCTTCCGTGCCGTCCTG
HM#124	SV40_SpeI_R	CACACTAGTGATACATTGATGAGTTTGGACAAACCACAAC
HM#131	PLF2	GTCAAAATGACGCATGATTATCTTTTACG
HM#179	DsRed_End	CGAGGGCCGCCACCACCTGTTCCTG
HM#203	NotI_phiC31_R	TCGCGGCCGCCTAGACCTTCCGCTTCTTCTTTGG
HM#337	SpeI_attB_R	CTAGGTGCGGGTGCCAGGGCGTGCCCTTGGGCTCCCCGGGCGCGTACTCCACCTCACA
HM#345	nosP_Bbsi_F	CGGAAGACCGCGATTCCTTCAGTATCTCCAAATCGCCCCGGAC
HM#368	attP220_Fwd	TCATCAATGTATCACTAGTACTGACGGACACACC
HM#369	attP220_Rev	CTGGCTGGGGAATCTGTACTAGTCGCGCTCG
HM#469	SpeI_gypsy_F	GCTTACTAGTGATGGTCTCAAGCTTGTCAGATCGGC
HM#470	ApaI_SV40_R	TTAGGGCCCCGCCTTAAGATACATTGATGAGTTTGG
HM#560	FH_Cas9_HidIII_F	CATCAAGCTTACAAGTTCATCAAGCCCATCCTGG
HM#561	FH_Cas9_XhoI_R	CATGCTCGAGATAGGTTTTCAGCCGTTCCTCGATC
HM#584	HM_EcoRI_TRE_F2	TACGAATTCGGCGCGCCTAGGCCGGCCGAATTTC
HM#706	HM_b2t_3UTR_F	CGAGGATCCTAGGATTAACTTCCCACTCAAGATCACACATG
HM#707	HM_b2t_3UTR_R	GCCAAGCTTGTCTGCTTATAAATCAACATTTATTCGTAACCC
mfs#370	AflII-5pBac_F	AACTTAAGTTAACCCTAGAAAGATAGTCTGC
MK153	PhiC31_BsaI_F	ATGGTCTCACATGGACACGTACGCGGGTGCTTACGAC
T7	T7	TAATACGACTCACTATAGGG
CH6R	CH_3'PIClaI_2	CCATCGATGGAATGAACAGGACCTAACGC

Additional File 2

Supplementary Table 2: List of primers used

Code	Name	Sequence 5'- 3'
HM#16	sryaR4	TTGTGTGTCATGGATGTTCAATCTAATC
HM#17	Srya5UTR_F1	GTACTTAGTTGAAAAGTTCAGCTTTACCCG
HM#34	Ds_srya_GSP3	GGCATCCAGGCTAATGGTCCGCTCCAAGTG
HM#35	B2t_NcoI_F1	GCAACCATGGGATGCCAAGAGAGATGAGCAGG
HM#36	B2t_XbaI_R3	CGATTCTAGACATCTTAACCGACTGTCAAGGATC
HM#42	srya_GSP_F	GCCTCTCTGGCTCCGATTCCCCCTAATG
HM#73	hspEcoRIF2	GCGAATTCTCCAGAAACTCAAACAGAAACC
HM#74	hspEcoRIR	GCGAATTCTTGTGTGTTTGTGTTTGTGGATGCAG
HM#76	nos_GSPR	GAGTCCTCCTCTTGCGTGGAATGCCG
HM#77	nos_GSPF	GTACTGTCCCAAGAAGCCGATTATCAC
HM#94	nos3UTRXbaIF	GGTCTAGAGAACACATCCGGCAGGAGC
HM#95	nos3UTRAflIIR	ATACTTAAGACTGAGCTCCAAGCAGTGGTATCAACGCAGAG
HM#101	SpeI-atBF	CTAGTGTGAGGTGGAGTACGCGCCCGGGGAGCCCAAGGGCACGCCCTGGCACCCGCAC
HM#102	BbsI-BbsI	CGGTCTTCGCGAAGAC
HM#113	BbsI_nosR	GCGAAGACCCATATGGCGAAAGTCCGGCTCGAAAGTTACC
HM#117	HM_Pub_R	CATTGGAATCTCTGTCGCTGCGTTCCG
HM#123	phi_nls_R	CTAGACCTTCCGCTTCTTCTTTGGGGCCGCCGCTACGTCTTCCGTGCCGTCCTG
HM#124	SV40_SpeI_R	CACACTAGTGATACATTGATGAGTTTGGACAAACCACAAC
HM#131	PLF2	GTCAAAATGACGCATGATTATCTTTTACG
HM#179	DsRed_End	CGAGGGCCGCCACCACCTGTTCCTG
HM#203	NotI_phiC31_R	TCGCGGCCGCCTAGACCTTCCGCTTCTTCTTTGG
HM#337	SpeI_attB_R	CTAGGTGCGGGTGCCAGGGCGTGCCCTTGGGCTCCCCGGGCGCGTACTCCACCTCACA
HM#345	nosP_Bbsi_F	CGGAAGACCGCGATTCCTTCAGTATCTCCAAATCGCCCCGGAC
HM#368	attP220_Fwd	TCATCAATGTATCACTAGTACTGACGGACACACC
HM#369	attP220_Rev	CTGGCTGGGGAATCTGTACTAGTCGCGCTCG
HM#469	SpeI_gypsy_F	GCTTACTAGTGATGGTCTCAAGCTTGTCAGATCGGC
HM#470	ApaI_SV40_R	TTAGGGCCCCGCCTTAAGATACATTGATGAGTTTGG
HM#560	FH_Cas9_HidIII_F	CATCAAGCTTACAAGTTCATCAAGCCCATCCTGG
HM#561	FH_Cas9_XhoI_R	CATGCTCGAGATAGGTTTTCAGCCGTTCCTCGATC
HM#584	HM_EcoRI_TRE_F2	TACGAATTCGGCGCGCCTAGGCCGGCCGAATTTC
HM#706	HM_b2t_3UTR_F	CGAGGATCCTAGGATTAACTTCCCACTCAAGATCACACATG
HM#707	HM_b2t_3UTR_R	GCCAAGCTTGTCTGCTTATAAATCAACATTTATTCGTAACCC
mfs#370	AflII-5pBac_F	AACTTAAGTTAACCCTAGAAAGATAGTCTGC
MK153	PhiC31_BsaI_F	ATGGTCTCACATGGACACGTACGCGGGTGCTTACGAC
T7	T7	TAATACGACTCACTATAGGG
CH6R	CH_3'PIClaI_2	CCATCGATGGAATGAACAGGACCTAACGC

3.5 Reproductive Sterility System for *Drosophila suzukii* control based on knock–out or knock–down of specific male fertility genes

This chapter describes the first steps towards the development of a reproductive sterility system for the control of the invasive fruit pest *D. suzukii*. The system is based on the conditional knockout or knockdown of the paternal effect lethal (PEL) gene *sneaky* (*snky*), which is important for sperm plasma membrane breakdown (PMBD) after fertilization. Failure of this process leads to embryonic lethality. We want to utilize the conditional *tet-off* system and CRISPR/Cas9 to knockout *snky* during spermatogenesis and thereby introduce reproductive sterility. We also describe an approach for conditional expression of short hairpin RNAs to knockdown *snky* during spermatogenesis as a second mechanistically independent method to induce reproductive sterility. The described strategy to target PEL genes, which do not affect male fitness, sperm production or transfer, but exert the effect only after sperm use and entrance into the egg during zygote formation, present a novel approach for improvement of the SIT. If we succeed, we generate a new foundation for the control of *D. suzukii* using the genetic pest strategy SIT.

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Authors contribution to the practical work:

Hassan M. M. Ahmed: All experiments.

Status: Work in progress.

Reproductive Sterility System for *Drosophila suzukii* control based on knock-out or knock-down of specific male fertility genes

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Background

The sterile insect technique (SIT) is considered an important component of area wide integrated pest management (AW-IPM) (1). The system is based on mass rearing and inundative releases of sterile males of the target species to compete with the wild type males for the wild type females leading to infertile mating and hence reduction of the population (2). The sterility is so far introduced by exposing the pupae to ionizing radiation which leads to chromosome breaks resulting in different chromosomal aberrations causing aneuploidy in the sired embryos, which is the basis of the reproductive sterility (2,3). However, irradiation has negative impact on the fitness of the sterile males to be released. To overcome this, scientists exploited the tools of molecular biology to engineer transgenic conditional embryonic lethality systems that preclude the need for ionizing radiation. The system is controlled by the *tet-off* binary system and an enhancer/promoter of an early embryonic stage to express a proapoptotic gene (4,5). Another approach that was engineered to induce reproductive sterility is the release of insects carrying a dominant lethal RIDL (6). This system also relies on the use of the *tet-off* system but in this case the effector is a heterologous transcription factor, the tetracycline-controlled transactivator (tTA), which is placed downstream of the tet operator and a weak basal promoter (6). In the absence of tetracycline, it leads to establishment of positive feedback loop of tTA which results in cytotoxicity.

The emergence of the bacterial adaptive immune system CRISPR/Cas, an acronym of Clustered Regularly Interspaced Short Palindromic Repeat and the Associated protein, as tool for genome editing, opened new doors for biotechnological improvements of the SIT. Eckermann et al. (7) proposed the use of the system to develop multifactorial reproductive sterility system by expression of *Cas9* during spermatogenesis and targeting it to repetitive elements in the genome and thereby induce many chromosomal breaks, which eventually leads to chromosomal aberrations and reproductive sterility. Beside the system proposed earlier (Eckermann et al., (7), the CRISPR/Cas system can also be used in various other ways to introduce reproductive sterility. However, when targeting male fertility genes, it needs to

be emphasized that the sterile males should still produce competitive sperm keeping the ability of sperm transfer to females. Furthermore, the transferred sperm should be able to compete with wild type sperm, since in many of the target pests the females mate more than once (polyandry). Very few genes causing paternal effect embryonic lethality (PEEL) when mutated were identified, which don't interfere with spermatogenesis, but the downstream steps involved in fertilization or gamete fusion. An example of such a mutation that affect sperm-egg recognition is *casanova* (*csn*) (8), which causes the sperm failing to enter the egg. The failure is attributed to a lack of β-N-acetylglucosaminidase on the plasma membrane covering the acrosome. Another gene causing PEEL is *sneaky* (*snky*), which is expressed explicitly in the testes of *Drosophila* during spermatogenesis and codes for an acrosomal protein involved in sperm plasma membrane breakdown (PMBD) during fertilization (9,10). Knockout of this gene does not affect male fitness or sperm competence (9). Sperm is normally produced, transferred and utilized to fertilize the egg, however, the sperm fails to form a functional male pronucleus. The *Drosophila* gene *misfire* (*mfr*) is expressed in the ovaries and testes and encodes a membrane protein, which is also required for PMBD and activation of the sperm during fertilization and a null mutant is therefore male sterile (11)

Originally identified in plants (12–14) and the nematode *Caenorhabditis elegans* (15) RNA interference pathway has been found in all studied organisms so far. It is a mechanism of post transcription regulation of gene expression as well as a defence mechanism against parasitic genetic elements such as viruses and transposable elements (16). The RNAi pathway is activated by dsRNA molecule which is processed to short 21-27 dsRNAs fragments by an enzyme called Dicer (17). The small duplexes are then unwound into ssRNA molecules, the targeting strand is then incorporated into the RNA-silencing complex (RISC) and guided the complex by complementary based pairing to target and degrade mRNA. (17)

Since its discovery, the system has been intensively used in reverse genetics to study the function of gene by knocking down the gene and studying the resulting phenotype (18). This has been exploited to perform systematic large-scale screen in *D. melanogaster* (19–21), and *Tribolium castaneum* (22–24). The RNAi pathway can be triggered by direct injection of the dsRNA into the embryos, or also in other stages in case of systemic effect (25). It can also be triggered by expression of dsRNA or short hairpin RNA from a transgene using either inducible promoters or binary expression system such as *Gal4/UAS* (26). The RNAi beside its broad applications in functional genetic studies and its potentials in gene therapy (27), has also been explored for its applicability to engineer transgenic strains suitable for the SIT. Knock-down of the sex determination gene *transformer* or *transformer2* in the tephritid flies and possibly in some other insect leads to female to male conversion (28,29).

Development of a reproductive sterility system by targeting paternal effect genes for knockout or down necessitates that the effector molecule (e.g. Cas9 or a dsRNA) be restricted to

spermatogenesis, which makes identification of suitable regulatory elements a prerequisite. The *Drosophila β Tub85D* (*β2t*) gene is known to be spermatogenesis-specific and is active in all the stages of sperm production starting at the third larval instar (30). The enhancer/promoter of this gene has been used to drive strong expression of reporter genes such as lacZ and fluorescent proteins such as *EGFP* that facilitate basic research in male reproductive biology (30). The *E/P* of the *β2t* gene has also been used to generate sperm-marking strains to help monitoring released males in SIT programs (31–34). Furthermore, it has been used to drive the expression of a homing endonuclease gene (HEG), the *I-PpoI*, in the malaria mosquito in a biotechnological vector control approach, where the HEG targets conserved sequences within the ribosomal DNA in the X chromosome of *Anopheles gambiae* and shred it leading to Y chromosome biased sperm and thus sex ratio distortion (35). The *Drosophila* genes *don juan* (*dj*) and *don juan like* (*djl*) are testes-specifically expressed with a translation repression element in their 5' UTR that delay translation of the mRNA to post-meiotic stage of spermatogenesis (36). Since those elements are well characterized, their deletion can allow the use of the respective promoters also for early spermatogenesis expression.

Here, we have chosen to target the PEEL gene *snky* which is involved in sperm plasma membrane breakdown during fertilization (10). Homozygous null mutants of this gene display complete male sterility. We proposed the use of CRISPR/Cas9 or RNA interference to conditionally knock-out or knock-down, respectively, this gene individually or in combination for production of reproductively sterile males to establish the sterile insect technique for the control of the invasive fruit pest *D. suzukii* with first proof-of-concept in *D. melanogaster*.

Results

Identification and validation of suitable CRISPR targets

Before identifying suitable CRISPR target sites in *D. melanogaster* PEEL gene *snky*, Fig. 1A, the gene was PCR-amplified and sequenced to avoid variations that may affect target recognition or lowers the efficiency of targeting. Using the online target finder tool developed by the university of Wisconsin (https://flycrispr.org/protocols/), we identified and designed four *gRNA*s with zero off target sites in *D. melanogaster* genome (37), *Dm_snky_g3*, *Dm_snky_g13*, *Dm_snky_g38*, and *Dm_snky_g41* (Fig. 1A and 1B). The four *gRNAs* were subsequently cloned into vector HMMA332 to express the *gRNAs* from Dm *U6:3* promoter, injected along with Cas9 plasmid HMMA056 into *D. melanogaster* embryos and their efficiency was evaluated by T7 EndoI assay. Based on the results, the three guides, *Dm_snky_g13*, *Dm_snky_g38*, and *Dm_snky_g41*, are chosen to develop the system (Fig. 1C).

Spermatogenesis-specific gRNAs-driver constructs and transgenic lines.

To achieve conditional knockout or knockdown of *snky* by CRISPR/Cas9 or by expression of short hairpin RNAs (*shRNA*) respectively, we used the *tet-off* binary expression system and the *enhancer/ promoter* (*E/P*) of *D. melanogaster* spermatogenesis specific genes *β2t* and *Don juan (dj)*. We fused the *E/P* of *Dm-β2t* or *Dm-dj* (without the *dj TSE* to allow premeiotic translation of tTA) upstream of the heterologous transcription factor *tetracycline transactivator* (*tTA*). The spermatogenesis-specific driver construct HMMA389 for *D. suzukii* is described elsewhere (chapter 3.4).

To be able to conditionally and simultaneously express two *gRNAs*, we made use of the *tet-off* binary expression system and the *tRNA* processing system for *gRNA* multiplexing (38–40). and generated vectors HMMA324 and HMMA325 by fusion of a synthetic multiplexing cassette consisting of two *gRNA* scaffolds interspaced and flanked by three *tRNA* genes to the *TREp* and *TREhs43* promoters respectively. This will allow the release of two mature *gRNAs* after processing of the *tRNA* genes by the highly conserved ribonucleases P and Z (41) (Fig. 2B and 2C). The vector HMMA324 was used to eventually generate the four driver constructs HMMA371, HMMA372, HMMA373, and HMMA374 that in addition to mediate *tTA* expression also express *gRNAs* targeting *Dm-snky* (Fig. 2A and 2B). The construct HMMA372 that expresses *tTA* under *Dm-β2t E/P* and *gRNAs Dm_snky_g13*, and *Dm_snky_g41* was used to generate spermatogenesis-specific *gRNA*-driver lines. Interestingly, the majority of the F_1 males were sterile. These driver lines will be crossed to *Cas9* responder lines (see below) to transactive expression of Cas9 during spermatogenesis and knockout the *Dm-snky* gene

In addition, we generated the spermatogenesis-specific driver constructs HMMA425 (*Ds-β2t E/P*), HMMA426(*Dm-β2t* E/P) and HMMA427 (*Dm-dj* E/P) by cloning the coding sequence of the *turbo green fluorescent protein* gene (*tGFP*) in frame with *tTA* and separated by the picornavirus *P2A* peptide sequence. The *tGFP* gene serves as a reporter for the tissue specific expression of the *tTA* and should later facilitate analysis of the functionality of the system by following the ability of the males to transfer viable sperm to the females, the entrance of the sperm in the egg and the failure of PMB during fertilization.

Moreover, we generated vectors HMMA349 and HMMA410 that allow constitutive expression of three *gRNAs* simultaneously based on the *tRNA* processing system and the promoter of the *RNA pol III small nuclear* genes *U6:3* and *U6c* from *D. melanogaster* and *D. suzukii*, respectively. We then engineered spermatogenesis specific gRNA-driver constructs HMMA433 and HMMA434 that express *tTA::2A::tGFP* from *E/P* of *Dm-β2t* or *Dm-dj* genes, respectively, plus *gRNAs Dm_snky_g13*, *Dm_snky_g38*, and *Dm_snky_g41* from *U6:3* promoter (Fig. 4A).

CRISPR/Cas9 responder constructs and transgenic lines

The second part of the proposed CRISPR/Cas9 reproductive sterility system consist of *Cas9* coding sequence fused to the *tTA* responsive element (*TRE*) and a basal promoter (Fig. 3B). We generated the responder constructs HMMA338 by fusion of the insect codon optimized *Cas9* coding sequence (with N and C-termini *nuclear localization signal* (*nls*) and N-terminus 3XFlag tag) downstream of the *TRE* and the *P-element* basal promoter. This should allow pre-meiotic translation of *Cas9* mRNA. The construct was used to generate several responder lines.

Cross of gRNA-driver lines to Cas9 responder lines

To generate a reproductive sterility strain (RSS), all the components of the reproductive sterility system must be combined in one strain, to do so we crossed the gRNA-driver lines 372_F26F1 and 372_M14M1 separately to the responder lines 338_F6M1, 338_F13F1 and 338_M10M2 (Fig. 3A and 3B). Supplementing the flies' food with tetracycline should supress the expression of the effector molecules Cas9 and the *gRNAs* by preventing the heterologous transcription factor tTA from binding to the *TRE* promoter (Fig. 3A). In the absence of tetracycline, the tTA is free to bind to *TRE* and thereby activates the expression of Cas9 and the *gRNAs* (Fig. 3B). The *gRNAs-tRNAs* transcript should then be processed by the ribonucleases P and Z and free the two *gRNAs Dm_snky_g13* and *Dm_snky_g41* to form the Cas9-gRNA Ribonucleoprotein complex (RNP) which should then target *Dm-snky*. (Fig. 4C)

Activation of the system in double homozygous males for the gRNAs-driver and responder transgene after withdrawal of the tetracycline from the food leads to expression of Cas9 and the *gRNAs* starting at the 3rd larval instar in the gonads, which then should target and induce double strand breaks to knockout the male fertility gene *snky*. This should result in the production of competent males that produce viable sperm, which effectively enters the egg but embryonic development will not occur due to failure of the formation of a functional male pronucleus.

Testes specific expression of *Cas9*

A preliminary reverse transcriptase PCR (RT-PCR) experiment was performed to investigate, whether Cas9 is expressed in the testes of the RSSs. A pair of primers was used to PCR amplify a 460bp fragment of Cas9, and another pair was used for a positive control amplifying the 3'UTR of *Dm-β2t*. As negative controls, we performed RT-PCR using total RNA from the respective responder lines 338_F6M1, 338_F13F1, and 338_M10M2 not crossed to driver lines as well as no RT PCR for all the different strains. Cas9 transcript was detected in all three RSSs, interestingly it has also been detected in the negative control

responders not crossed to driver lines but not in the no RT PCR (Fig. 3C). This might be due to leakiness in the expression of Cas9 from the basal *P-element* promoter.

Over expression of *tTA* may cause male sterility

Out of 14 independent F_1 transgenic gRNA-driver lines generated, 5 were females and 9 were males of which 7 were sterile males that didn't sire offspring. It is well known that overexpression of *tTA* can cause cytotoxicity (6). Due to the way the driver construct HMMA372 was generated, with *TREp* upstream of the *β2t* promoter, a positive self-sustaining loop may have formed that leads to over-expression of *tTA* This might well be a reason of cell toxicity resulting in male sterility. Besides, the 3'UTR of the *Dm-β2t* gene fused downstream of *tTA* is also known to lead to overexpression (42) (Fig. 2B and 3A).

Conditional Knockdown of *sneaky*

Another approach to generate RSSs is by knocking down the expression of the male fertility genes such as *snky* by expression of *shRNA* or double strand RNA (*dsRNA*) during spermatogenesis which will then induce RNA interference depletion of *snky* mRNA. To this end, we utilized the *tet-off* binary expression system and the *tRNA* processing system to engineer responder construct HMMA309 and HMMA310 that allow expression of two *shRNAs* or *dsRNAs*. We designed two 21bp long *shRNAs* (*shRNA_13* and *shRNA_41*) with a 9bp loop driven from *D. melanogaster mir14* and generated the responder construct HMMA445 (Fig. 4A). This construct is being used to generate transgenic responder lines. This system, if proved to be effective in inducing male sterility, can be used as stand-alone or in combination with CRISPR/Cas9 reproductive sterility system to generate a double hit reproductive sterility system to avoid resistance development (Fig 4.B).

Identification of *D. suzukii* orthologue of the gene *sneaky*

To be able to eventually transfer the system to the invasive fruit pest *D. suzukii*, it is necessary to identify suitable paternal effect genes. Searching in *D. suzukii* database (http://spottedwingflybase.org/) with *D. melanogaster* sequence of the gene *snky* as query, we were able to identify homologous sequences that shares 76% and 89% similarity at the nucleotide and amino acid levels respectively to *D. melanogaster* counterparts. Part of the gene including exon I was PCR amplified from genomic DNA and sequenced.

Discussion

In this study we describe the first steps towards the development of a new reproductive sterility system for the fight of the invasive fruit pest *D. suzukii*. The idea is to use the CRISPR/Cas9 system or the RNA-interference approach to target and knockout (knock-down) the paternal effect lethal gene *snky* during spermatogenesis. This gene encodes an acrosomal protein which is expressed exclusively during spermatogenesis but is not necessary for the successful completion of this biological process (10). Homozygous mutant males are sterile in the sense

that they don't sire progeny despite the fact that they produce morphologically functional sperm that can be efficiently transferred to females during copulation and can enter the egg but does not form a functional male pro-nucleus due to failure of PMBD (10). These characteristics of *snky* mutants are very important for developing the reproductive sterility system.
The CRISPR/Cas9 system was used previously to knock out *Dm-β2t* gene which results in male sterility (43) however, those males don't produce functional sperm. In such a case, the females will search for other males, which suggest that *Dm-β2t* is not a suitable target to induce sterility for application in the SIT programs. Our proposed system is controlled both by the tissue specific promoter of *Dm-β2t* gene and the *tet-off* binary expression system to allow conditional activation when desired. We used the construct HMMA372 consisting of the *E/P* of *Dm-β2t* gene to drive the expression of *tTA* and a gRNA cassette (g13, g41) under the control of *TRE* to generate transgenic gRNA-driver lines (Fig. 2A and 2B). We observed male sterility in 7 out of 9 independent F_1 transgenic males and managed to establish 7 transgenic lines, only two of them were F_1 fertile males and the other 5 were established from F_1 females. This sterility might be attributed to either of two factors or a combination thereof. First, the use of the *TRE* upstream of the *Dm-b2t* promoter can lead to enhancement of expression of *tTA* by establishment of a positive feed-back loop (6) (Fig. 2B). Accumulation of tTA can be toxic to the cells but the mechanism of this toxicity is so far unresolved. In fact, such a construct has been made intentionally to develop a reproductive sterility system known as RIDL, an acronym of release of insects carrying a dominant lethal, which results in cytotoxicity and death (6). Secondly, the 3'UTR of *Dm-b2t* is known to control the level of expression (42) and gives high expression but with slight delay in translation (44). In our construct HMMA372, we have fused the 3'UTR from *Dm-b2t* gene to the transcription factor encoding gene *tTA*. So, the simplest explanation is that the two factors might be involved in the toxicity and can explain the observed male sterility. This can be overcome by either using an *RNA polIII*-based strategy for expression of *gRNAs* (Fig. 2C) or place the *gRNAs* cassette along with the *Cas9* responder construct and not with the driver construct. Whether or not the 3'UTR alone can lead to overexpression of *tTA* to levels that are toxic needs to be determined empirically.

To investigate the functionality of the gRNA-driver lines we crossed two lines to three different Cas9 responder lines and performed a preliminary RT-PCR. We observed Cas9 transcript in all 6 crosses plus the negative controls (uncrossed *Cas9* responder lines) (Fig. 3C). It is obvious that this is not genomic DNA contamination due to the fact that the respective negative control (no RT PCR) didn't show amplification for all the crosses and the Cas9 responder negative controls (Fig. 3C). Some level of expression from the basal promoter is expected and might not interfere with the functionality of the strain but that has to be determined. This basal expression can be minimized by flanking the transgene with insulators

such as the chicken hypersensitive site 4 (HS4) from the chicken *β-globin* gene to avoid activation by surrounding enhancer elements in the site of integration or in the vector itself. Especially as the construct has the *E/P* of the *D. melanogaster polyubiquitin* gene.

The utilization of the RNA interference pathway to knockdown *snky* provides another approach for generation of the reproductive sterility system. In this respect we developed responder constructs based on the *tet-off* and the *tRNA* processing systems to conditionally drive the expression of two *shRNAs* or *dsRNA* (Fig 4B). Two 21bp *shRNAs* targeting *D. melanogaster snky* were generated with a 9bp loop derived from *D. melanogaster mir14*, which proved suitable in Schneider cells (45). After testing this system, it can be combined with the CRISPR/Cas9 system described above to have two mechanisms for induction of sterility (Fig. 4B). Each system acts as a surveillance mechanism for the other to eliminate any escapers and ensure 100% reproductive sterility (Fig. 4C and 4E).

It has been suggested that the *β2t* gene like many other genes involved in spermatogenesis probably has a translation delay element within the 5' UTR (44). Deletion of this element from the *P/E* of the medfly *Ceratitis capitata* permitted early transcription and translation of genes fused to it (46). Early expression of tTA ensures early expression of the downstream effector molecule (Cas9 and/or shRNA) before shut-down of transcription in spermatogenesis. Removal of the translation repression element should also be possible in *D. melanogaster*, since its regulatory elements are well characterized. Moreover, as an alternative to *Dm-b2t E/P*, we can use the *P/E* of the germline specific genes *nanos* or *Rcd-1r* to drive the expression of *tTA* particularly since *snky* is not required and not expressed in the females and is not required for males. Another promoter that could be used instead is the one from the *bag of marbels* gene (*bam*), which is expressed in early testes primordial germ cells early enough for the GAL4 system to be used in spermatogenesis studies in *D. melanogaster* (47)

The two approaches described for the generation of a reproductive sterility system in *D. melanogaster*, if proven successful can easily be developed for the fruit pest *D. suzukii*, since we have already identified the *Ds-snky* gene, and sequenced it. This system along with the sperm-marking already developed (34) would be a giant leap forward towards the establishment of the sterile insect technique against *D. suzukii*.

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Figures:

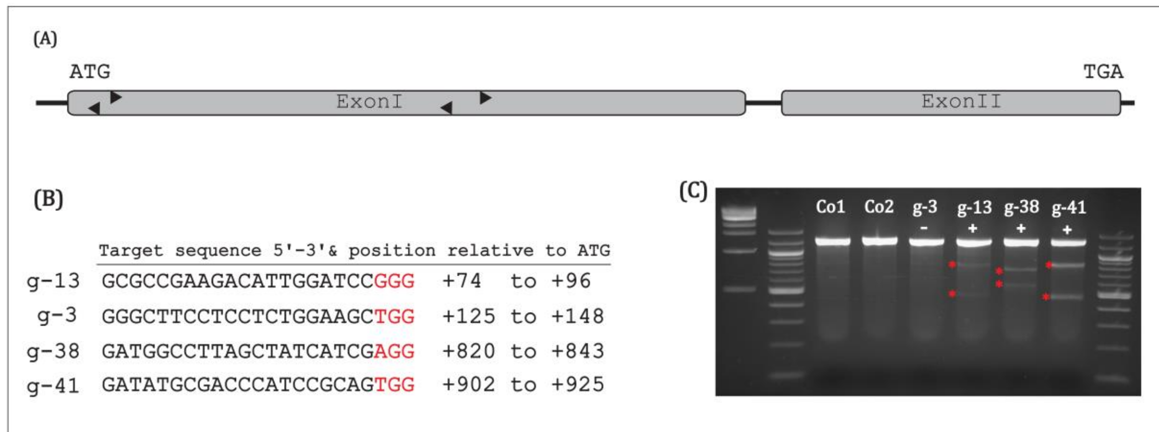

Figure. **1**. **Identification and validation of guide RNAs targeting *Dm sneaky* gene**. (**A**) the structure of *Dm snky* has two exons and one intron. Four *gRNAs* were identified. Solid black triangles indicate the relative position and the strand for each gRNA relative to each other and to the translation start codon. (**B**) sequence of each target with the PAM shown in red. The relative position relative to the first nucleotide of the translation start codon is shown. (**C**) agarose gel picture showing the result of T7 Endo assay. g-13, g-38 and g-41 are positive whereas g-3 was not digested by T7Endo and therefore considered negative. Co1 is T7 Endo-less negative control for g-3 and g13 while Co2 control for g-38 and g-41

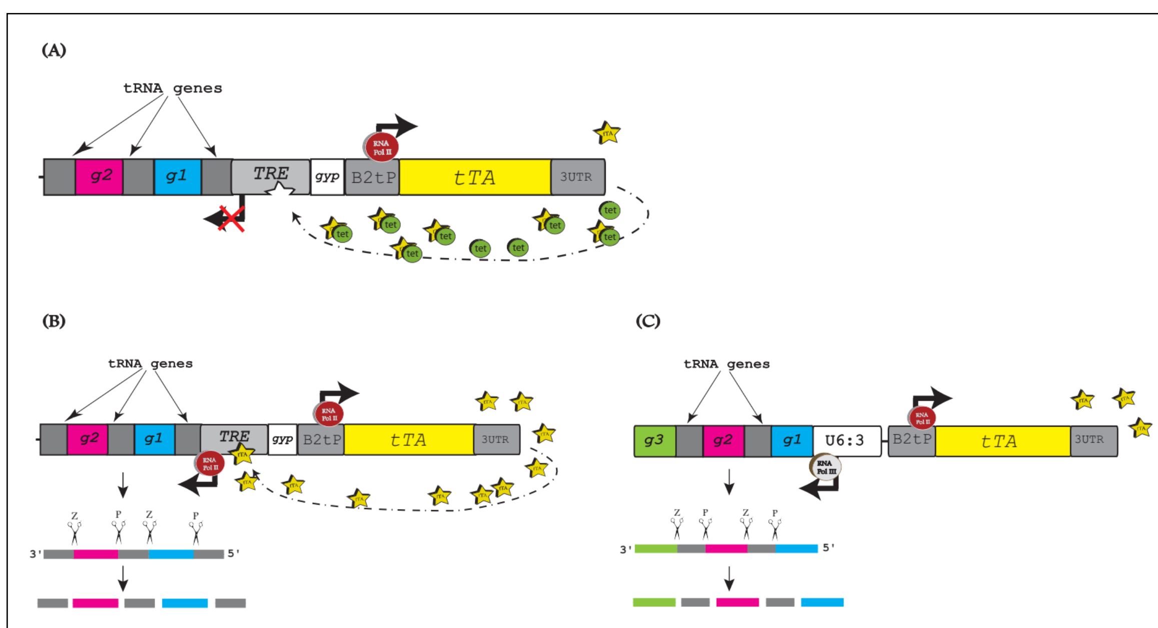

Figure. 2. Illustration of the gRNA-driver constructs. (**A**) shows the different components of the gRNA-driver construct in which the *tRNA* processing strategy was adopted to express two *gRNAs* simultaneously. The *gRNAs* are flanked and interspaced by *tRNA* which should facilitate correct processing of *gRNAs*. The second part of the construct is the *tTA* under the control of E/P of *D. melanogaster β2t* gene. In the presence of tetracycline in the fly food, the tet bind the tTA and prevent it from binding to the tTA responsive element (*TRE*) and as a result the *gRNA* cassette will not be expressed. (**B**) in the absence of tet in the food, the tTA is free to bind to the *TRE* and direct the expression of the *gRNAs* cassette in the testes. The transcript will then be processed to individual *gRNA* by the cell ribonuclease P and Z. having the *TRE* in the same construct close to driver construct can lead to overexpression of *tTA* by a positive autoregulatory loop which may lead to unintended cytotoxicity (**C**) As a second strategy for constitutive expression of *gRNAs* we used the promoter of *D. melanogaster small nuclear RNA* gene *U6:3* to drive three *gRNAs* by utilizing the *tRNA* processing system described in B.

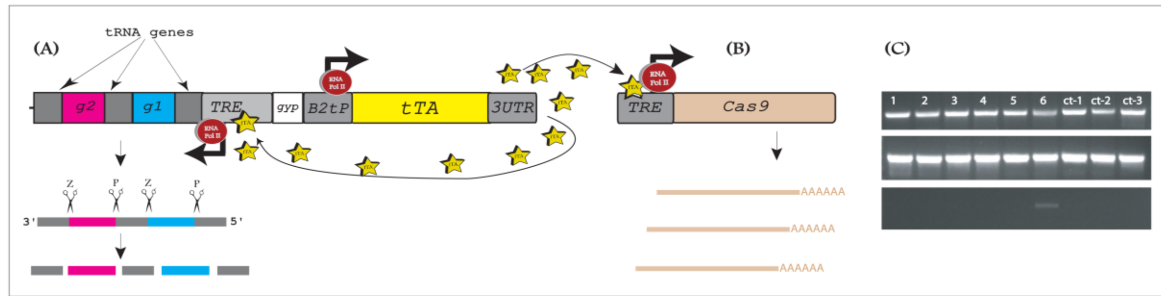

Figure. **3**. **Transactivation of Cas9 and gRNAs expression during spermatogenesis** (**A**) Illustration of the two components gRNA-driver construct in which the expression of two *gRNA* from *RNA polII* promoter is controlled by the tTA and *β2t* promoter. In the absence of tetracycline, tTA binds and activates the expression of two gRNAs interspaced and flanked by three tRNAs genes to facilitate correct processing into individual gRNAs by the action of the cell ribonuclease P and Z. despite incorporation of the *gypsy* insulator between the gRNAs cassette and the spermatogenesis-specific driver construct, the tTA can potentially overexpress itself as a result of a positive feedback loop due to its binding to the *tTA responsive element* (*TRE*). (**B**) shows the expression of Cas9 under the control of *TRE* directed by spermatogenesis-specific expression of the *tTA*. (**C**) the upper panel of the gel picture shows that Cas9 is expressed in the testes of double heterozygous *D. melanogaster* strain harboring the two components of the paternal effect embryonic lethality system PEEL shown in A and B. each of the chosen three responder lines was crossed individually to two gRNA-driver line. Each two consecutive numbers represent one responder line crossed to driver lines 372_M14_M1 and 372_F26_F1 respectively. The last three lanes are RT-PCR negative control using the respective responder line not crossed to any driver. Ct-1 controls for 1 and 2, ct-2 controls for 3 and 4, and ct3 controls for 5 and 6. Ideally the negative controls should not express Cas9. However, basal promoters can drive expression at a basal level depends on the position of integration. The middle lane is the positive control for the RT-PCR procedure using primer pair to amplify from the same cDNA pool the 3'UTR of the spermatogenesis-specific *β2t* gene. All of the lanes gave strong bands which reflect the reliability of the tests, especially when looking at the result on the light of the lower lane which represent the RT-less PCR following the same procedure and the same amount of the initial RNA but without addition of the reverse transcriptase. Cas9 expression in the negative non-crossed control in the upper doesn't not necessarily means that the responder lines are not functional due to leakiness, but the PCR is highly sensitive and can detect low levels of expression from very few molecules.

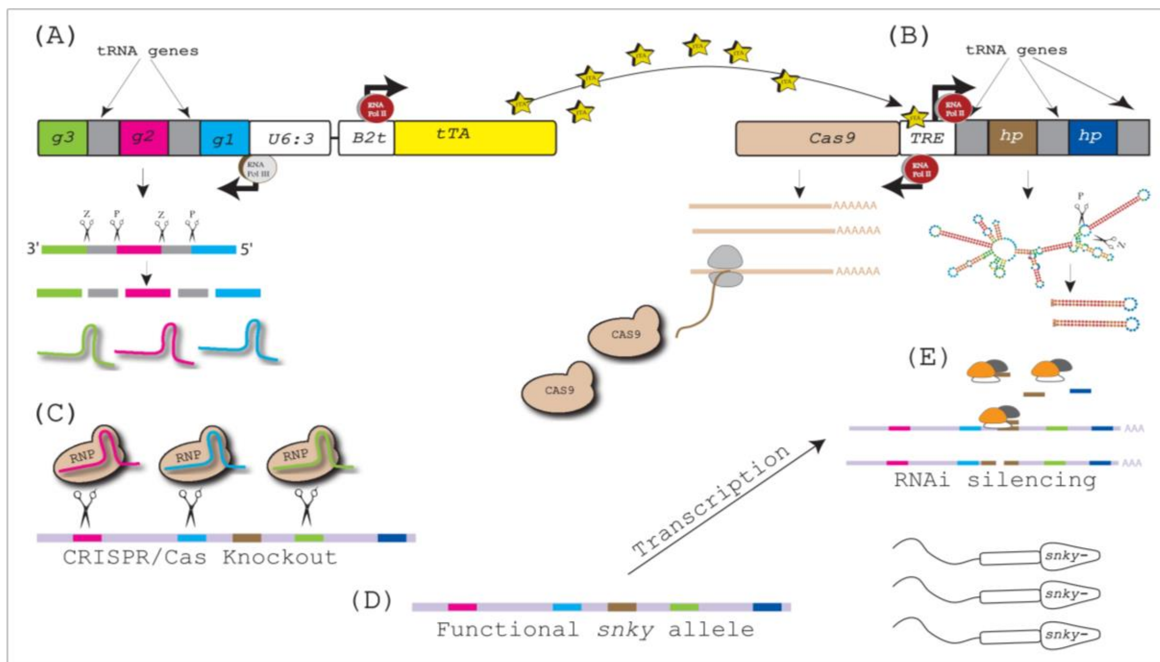

Figure.4. Two conditional mechanisms for paternal effect embryonic lethality system. (**A**) Depiction of a two components gRNA-driver construct where the RNA polIII transcribes from the promoter of the *snRNA* gene *U6:3* three gRNAs *g1*, *g2* and *g3* shown in blue, lila and green respectively, targeting the PEEL gene *snky*. The *gRNAs* are interspaced by two *tRNA* genes to ensure correct processing by the cell ribonucleases P and Z (shown as scissors) to individual *gRNAs*. The second part of the construct shows the expression of the heterologous transcription factor tTA driven by the promoter of the gene *β2t* during spermatogenesis. (**B**) Illustrates the conditional expression of the two effectors, Cas9 and shRNA (*hp*) against the PEEL gene *snky*. In the absence of tetracycline in the fly's food, the tTA is allowed to bind to its responsive element (TRE) placed between the two effector cassettes and since the TRE is bidirectional it should allow expression of the two effectors at the same time. Cas9 is expressed and translated early before meiosis. The second effector transcribes two hairpins (*hp1* and *hp2* depicted in brown and blue respectively) interspaced and flanked by three *tRNA* genes to facilitates correct processing of the hairpins by the ribonucleases P and Z. The targets of the two hairpins in *snky* mRNA are different from CRISPR genomic targets (**C**) Cas9 forms RNP complexes with the constitutively expressed *gRNAs* against *snky*, and therefore will be guided to the respective target and induce simultaneously three DSBs which should lead to efficient knockout of the gene. (**D**) Despite early knockout of the gene, some *snky* mRNA might have already been transcribed, or some resistant alleles are naturally available or formed as a result of NHEJ repair of the DSBs that can still be functional. (**E**) illustrates triggering of the RNAi pathway by the two hairpin RNAs to ensure depletion of *snky* mRNA if available. This double action effect should provide a tight filter system to produce 100% sterile males with competent sperm that are *snky*$^{-}$ and thus should fail to form functional male pronucleus during fertilization and as a consequence embryonic development will be arrested.

Materials and Methods

Drosophila strains

All fly experiments were performed in our well-equipped safety level one (S1) laboratory, which is certified for generating and using genetically modified insects.

Wild type *D. melanogaster* Oregon R strain as well as the transgenic flies generated during this study were reared on standard *Drosophila* food and kept at 25°C throughout this study.

Nucleic acid isolation

Genomic DNA was isolated from adult males and females using NucleoSpin® DNA Insect (Macherey-Nagel) according to the manufacturer instructions. Total RNA was isolated from testes of 4-5 days old males using ZR Tissue & Insect RNA MicroPrep (Zymo Research Europe, 79110 Freiburg) according to manufacturer instructions.

Unless otherwise indicated all PCR amplifications during this study were performed using Phusion DNA polymerase and Phusion-HF buffer (New England Biolabs GmbH, D-65926 Frankfurt am Main). Primers used are available in supplementary Table 1. Plasmid isolation and PCR purification were performed using NucleoSpin® Plasmid and NucleoSpin® Gel and PCR Clean-up kits (Macherey-Nagel GmbH & Co., 52355 Dueren, Germany), respectively. NucleoSpin® Plasmid Transfection-grade (Macherey-Nagel) or QIAGEN Plasmid Plus Midi Kit (QIAGEN GmbH, 40724 Hilden, Germany) were used to prepare plasmids for germline transformation.

Sequencing of *D. melanogaster* and *D. suzukii* snky gene

About 2.k Kb fragment of *D. melanogaster snky* gene spanning exon I was PCR amplification OreR and w- using primers pair HM#154 /HM#156. And program [98°C3'' (98°C 30'' 68°C 30'' 72°C 1') 35X with a final elongation step of 7' at 72°C]. The amplified DNA fragments were resolved in 1% agarose gel, documented, purified using PCR and Gel purification kit and sequenced.

To identify *D. suzukii* homologue we used the sequence of *D. melanogaster* as a query to search in *D. suzukii* genome data base http://spottedwingflybase.org/. primer pair HM#610/HM#611 were designed and used to amplify 1.7Kb spanning exon I of the potential *Ds-snky* gene with PCR program [98°C3' (98°C 30'' 68°C 30'' 72°C 1') 35X with a final elongation step of 7' at 72°C]. The amplified DNA fragment was resolved in 1% agarose gel, documented, purified and sequenced

Design of gRNAs targeting *Dm-snky*

Based on the obtained sequence for *D. melanogaster* OreR and w- strains, we used 1Kb sequence starting at the ATG translation start codon and searched for gRNA using online

target finder tool developed by Wisconsin university https://flycrispr.org/.. The following parameters were chosen for the search, target length 20 nucleotides, CRISPR targets with at least one G at the 5', PAM sequence NGG. The obtained targets were evaluated for off-targets based on the latest *D. melanogaster* release r-6 (37) with high stringency. Four targets with zero-off targets were chosen, two of which were close to the ATG translation start codon (*Dm_snky_g13 and Dm_snky_g3*) and two about 800 to 900bp downstream of the ATG (*Dm_snky_g38*, and *Dm_snky_g41*), table S1.

Constructs:

To generate plasmids HMMA324 and HMMA325 to conditionally express two *gRNAs*, first a synthetic 312bp dsDNA fragment containing *D. melanogaster Ala:Val:gly* was purchased from Integrated DNA Technologies (Integrated DNA Technologies, BVBA, B-3001 Leuven, Belgium) and cloned into pJet1.2 giving rise to HMMA289 which is confirmed by sequencing. The fragment was then digested from HMMA289 using *EcoR*I/*BamH*I and cloned into HMMA265 and HMMA266 respectively giving rise to HMMA190 and HMMA191. The *Asc*I fragment containing *TREp-Ala:Val:gly:SV40* from HMMA190 and HMMA191 was cloned into HMMA307 Giving rise to HMMA309 and HMMA310 respectively. the CRISPR *gRNA* scaffold was then PCR amplified from HMMA093 (3.3) using primers pairs HM#633/#634HM and HM#635/#636HM and cloned into the *Bsa*I and *Sap*I sites respectively of HMMA309 and HMMA310 resulting in HMMA324 and HMMA325 respectively.

To generate plasmid HMMA349 and HMMA410 to constitutively express three gRNA from *Dm U6:3* or *Ds U6c* promoter, the fragment containing the *tRNA* genes and *gRNA* scaffolds was PCR amplified from HMMA324 using primer pair HM#757/HM#758 and HM#759/HM#758 and subsequently cloned by Gibson assembly into *Bbs*I site of HMMA308 and HMMA332 respectively.

To generate gRNA-Driver constructs HMMA371, HMMA372, HMMA373 and HMMA374 expressing tTA and *gRNAs* targeting *Dm-snky*, first annealed oligos generating *Dm_snky_g13* (HM#688/HM#651) and *Dm_snky_g38* (HM#689/HM#655) were cloned into *Bbs*I and *Bsa*I sites of HMMA324 respectively giving rise to HMMA347, and *Dm_snky_g13* (HM#688/HM#651) and *Dm_snky_g41* (HM#690/HM#657) were cloned together into *Bbs*I and *Bsa*I sites of HMMA324 to give rise to HMMA348, and subsequently the *gRNA* cassette was then PCR amplified from HMMA347 and HMMA348 using primers pair HM#704/HM#705, and program (98°C 3' 98°C 30'' 62°C 30'' 72°C 30'') 5x followed by 35X (98°C 30'' 72°C 1') and final elongation of 7' at 72°C. The fragments were then digested by *Bsa*I and cloned into *EcoR*I site of HMMA355 and HMMA356 giving rise to HMMA367, HMMA368, HMMA369 and HMMA370. Finally, the *Asc*I fragment from

HMMA367, HMMA368, HMMA369 and HMMA370 containing the gRNA and driver cassette were cloned into the *Asc*I site of HMMA331 (chapter 3.4).

To generate plasmids HMMA355 and HMMA356, the *gypsy* insulator was PCR amplified from mfs#1221 using primer pair HM#702/HM703, digested by *Bsa*I/*Bbs*I and cloned into *EcoR*I site of HMMA352 and HMMA353

To generate the dual sperm-marking drive construct HMMA425, HMMA426 and HMMA427, the picornavirus self-cleaving peptide *P2A* was cloned into HMMA131 *EcoR*I/*Nco*I sites to give rise to HMMA382. The *P2A::DsRed* was PCR amplified using primers pair HM#128/HM#782 and digested by *Bsa*I. The *tTA* was amplified using primers HM#780/HM781 and program [98°C 3' (98°C 30''63°C 30'' 72°C 30'') 35X with final elongation of 7' at 72°C] and digested by *Bsa*I/*Xba*I. The two fragments were then cloned together into the *Xba*I/*Bam*HI sites of HMMA351, HMMA352 and HMMA353. To give rise to HMMA404, HMMA405 and HMMA406. The *tTA::P2A* was PCR amplified from HMMA404 using primer pair HM#780/HM#69 and program [98°C 3'(98°C 30'' 63°C 30'' 72°C 30'') 35X and a final elongation of 7' at 72°C] and digested by *Xba*I/*Nco*I. The *tGFP* was PCR amplified from mfs#1256 (32) using primer pair HM#792/HM#571, digested by *Bbs*I/*BamH*I and cloned together with *tTA::2A* into *Xba*I/*BamH*I sites of HMMA352, HMMA352 and HMMA353 giving rise to HMMA407, HMMA408 and HMMA409 respectively. And finally, the *Asc*I fragment from HMMA407, HMMA408 and HMMA409 was cloned into *Asc*I site of HMMA331 (chapter 3.4).

To generate plasmid HMMA350 pSL{af_*DmU6:3_g13_g38_g41*_af} expressing *Dm_snky_g13*, *Dm_snky_g38*, and *Dm_snky_g41*simultaneously from *U6:3* promoter, the fragment containing the *Dm_snky_g13*, *Dm_snky_g41* was PCR amplified from plasmid HMMA348 using primer pair HM#683/HM#684 and cloned along with annealed oligos HM#807/HM#808 introducing *Dm_snky_g38* into *Bbs*I site of HMMA332

To finally generate the transformation plasmid HMMA433 and HMMA434, the *gRNA* cassette under the *U6:3* promoter was PCR amplified from HMMA350 using primers pair HM#704/HM#705, digested by *Bsa*I (generates *EcoR*I compatible ends) and cloned in the *EcoR*I site of HMMA408 and HMMA409 giving rise to HMMA431 and HMMA432. Eventually the *Asc*I fragment from HMMA431 and HMMA432 was cloned into HMMA331.

To generate *Cas9* responder construct HMMA338, the *Asc*I fragment *TREp:3XFag:nls:Cas9:nls:SV40* from HMMA327 was cloned into the *piggyBac* transformation vector HMMA331. HMMA327 is generated by replacement of *attP-TREp* in HMMA326 by *EcoR*I/*Age*I *TREp* from HMMA265. HMMA326 was generated by cloning of annealed oligo HM#102 into *Cla*I site of HMMA313 which is generated by removal of *Dj* TSE and the ATG between the 3XFlag tag and the nuclear localization signal at the N-terminal of *Cas9* from HMMA295 using *Bgl*II (pJet intermediate and mutagenesis

PCR in supp). HMMA295 was generated by cloning of *EcoR*I/*XbaI* fragment form HMMA249 into *EcoR*I/*Xba*I sites of HMMA51 (chapter 3.4). HMMA249 was made by cloning of *Dj* TSE into *EcoR*I site of HMMA194 which is generated by replacement of *Ds-hsp70* promoter in HMMA056 (chapter 3.3) by *attP-TREp EcoR*I/*Cla*I.

All final construct mentioned in this chapter are listed in supplementary table S2.

Generation of shRNA responder construct

To express two *shRNA* targeting *Dm snky* at the same time under the control of *tTA*, we cloned annealed oligos HM#772/HM#773 (T13) and HM#774/HM#775 (T41) into HMMA309 golden gate to give rise to HMMA430

Germline transformation

All injections for *piggyBac* germline transformation were performed using the hyperactive helper plasmid MK006 along with the respective transformation vector at a final concentration of a 500 ng/μL and 300 ng/μL respectively. Emerged G_0 flies were crossed individually to three wildtype flies of the opposite sex.

Identification of F_1 transgenic flies was based on the respective fluorescent body marker. Screening for transgenic flies was performed using Leica M205 FA fluorescence stereomicroscope equipped with camera Q imaging Micropublisher 5.0 RTV (Leica Mikrosysteme Vertrieb Gmb, Wetzlar, 35578 Germany). Transgenic flies were screened using filter sets RFP (excitation: ET546/10x, emission: ET605/70m) or GFP-LP (excitation: ET480/40, emission: ET510 LP). F_1 transgenic flies were outcrossed to WT flies of the opposite sex.**Fly crosses**

To test for sterility or partial sterility of the recovered gRNA-driver transgenic lines, 1 males of each strain was individually crossed to 2-3 virgin wild type females.

About 10 gRNA-driver males from each strain were crossed to 10-15 female Cas9 responder females to test whether the driver lines are functional and can lead to sterility. 15 F_1 males of each cross were used for RNA isolation.

RT-PCR

To investigate whether Cas9 is driven in the testes by the heterologous transcription factor tTA, we performed RT-PCR. Total RNA, was isolated from testes of 15 individual 4-5 days old adult males double heterozygous for the driver and responder transgenes. The males were dissected in ice cold PBS 1X and testes were transferred to bashing tube with beads and fixed on a vortex. Homogenization was allowed to proceed for 10 minutes. And RNA was isolated according to the instructions of the manufacturer. In column DNase treatment was preformed according to the manufacturer instructions.

In tube DNase treatment was performed using dsDNase provided with the kit. RNA was 1μg was used. First strand cDNA synthesis was done using Maxima First Strand cDNA Synthesis Kit for RT-qPCR, with dsDNase (thermofisher). 2μL cDNA was used for each PCR reaction in a total volume of 25μL. to check the expression of cas9, we used primer pair HM#619/HM#135 to amplify 460 bp of Cas9. As a negative control the respective Cas9 responder not crossed to driver were used. As a positive control, a pair of primers (HM#706/HM#707) that amplify the 3'UTR of b2t was used which give 250bp. The PCR was performed using 2μL cDNA, 1.25μL forward and 1.25 μl reverse primer (10μM), 2μL dNTPs mix and Phusion polymerase in a total reaction of 25μL

15μL of the PCR product was run in 1.5% agarose gel and documented using UV lamp and a camera attached to a printer.

Table S1. Primers sequences

Code	Name	Sequence 5'-3'
HM#69	DsRed-mega-R	GGAAGGACAGCTTCTTGTAGTCGGGG
HM#128	HM_pSL_R	CCGGCTCGTATGTTGTGTGGAATTGTG
HM#135	cas9-R	GTAGATGGTGGGGTACTTCTCGTGG
HM#154	Snky_F	GGGATGCAATCAAGGCCACCACTTCTCC
HM#156	Snky_R	CCGTAGTGGACTCGTGATGGGTTCGAC
HM#571	HM_tGFP_BamHI_R	CGCTGGATCCTTATTCTTCACCGGCATCTGCATCC
HM'610	HM_Dssnky_F	ATGTTCTCCTTTCTGACGCTGCCATGTC
HM#611	HM_Dssnky_R	TTATTTCTCTTTCCTGTAGGCGTACACCTC
HM#619	HM_Cas9_ATG_F	GACGATGACGATAAGGCCCCAAAGAAGAAGCGGAAGGTC
HM#633	HM_BsaI_Scaf_BbsI_F	CATGGTCTCCTCCAGGGTCTTCGAGAAGACCTG
HM#634	HM_BsaI_Scaf_BbsI_R	ACGGGTCTCGAAACGCACCGACTCGGTGCCACTTTTTC
HM#635	HM_SapI_Scaf_BsaI_F	GGGGCTCTTCAACAGAGAGACCGAGAGAGGGTCTCAG
HM#636	HM_SapI_Scaf_BsaI_R	CCGGCTCTTCACGCGCACCGACTCGGTGCCACTTTTTC
HM#650	HM_Dmsnkyg13_F	CGTCGCGCCGAAGACATTGGATCC
HM#651	HM_Dmsnkyg13_R	AAACGGATCCAATGTCTTCGGCGC
HM#652	HM_Dmsnkyg3_F	CGTCGGGCTTCCTCCTCTGGAAGC
HM#653	HM_Dmsnkyg3_R	AAACGCTTCCAGAGGAGGAAGCCC
HM#654	HM_Dmsnkyg38_F	CGTCGATGGCCTTAGCTATCATCG
HM#655	HM_Dmsnkyg38_R	AAACCGATGATAGCTAAGGCCATC
HM#656	HM_Dmsnkyg41_F	CGTCGATATGCGACCCATCCGCAG
HM#657	HM_Dmsnkyg41_R	AAACCTGCGGATGGGTCGCATATC
HM#683	HM_gBlk_U6:3_F	GCGAGAAGACTACGTCGGGGATGTAGCTCAGATGGTAGAG
HM#684	HM_gBlk_U6:3_R	CAAGAAGACCTTGCGTCGGCCGGGAATCG
HM#688	HM_Dmsnkyg13_F2	TCCAGCGCCGAAGACATTGGATCC
HM#689	HM_Dmsnkyg38_F2	AACAGATGGCCTTAGCTATCATCG
HM#690	HM_Dmsnkyg41_F2	GCAGATATGCGACCCATCCGCAG
HM#702	HM_EcoRI_gypsy_F	GGCGAAGACCGAATTCGATCCGGCTAAATGGTATGGCAAGAAAAG
HM#703	HM_EcoRI_gypsy_R	CGAGAAGACCGAATTAGGCCCGGTACCCTATTCGCAAAAACATTG
HM#704	HM_Bsa_EcoR_TRE_F	TAGGTCTCGAATTCGCCGGCCGAATTTCGAGTTTACC
HM#705	HM_EcoRI_SV40_F	CGCGGTCTCGAATTCAAGCTTGATACATTGATGAGTTTGGAC
HM#706	HM_b2t_3UTR_F	CGAGGATCCTAGGATTAACTTCCCACTCAAGATCACACATG
HM#707	HM_b2t_3UTR_R	GCCAAGCTTGTCTGCTTATAAATCAACATTTATTCGTAACCC
HM#757	HM_U6c_gib_F	ATATACGACATTTTTCAATACGAAATCGGGGATGTAGCTCAGATGGTAGA GCGCTC
HM#758	HM_U6c_gib_R	ACTTGCTATTTCTAGCTCTAAAACAGAAGAGCACGCTCTTCCTGCGTCGG CCGGGAATCGAACC
HM#759	HM_U6:3_gib_F	TAGACCTATTTTCAATTTAACGTCGGGGATGTAGCTCAGATGGTAGAGCG CTC
HM#772	HM_DmT13sh_F	TCCACGGATTTAATGTCTTCGGTGCCGAATAATTCGGATCCAATGTCTTC GGCGC
HM#773	HM_DmT13sh_R	AAACGCGCCGAAGACATTGGATCCGAATTATTCGGCACCGAAGACATTAA ATCCG
HM#774	HM_DmT41sh_F	ACAGATATGTGACTCATCTGTAGTCGAATAATTACTGCGGATGGGTCGCA TATC
HM#775	HM_DmT41sh_R	CGCGATATGCGACCCATCCGCAGTAATTATTCGACTACAGATGAGTCACA TATC

HM#780	HM_XbaI_tTA_F	AAGATGTCTAGATTAGATAAAAGTAAAGTGATTAACAGC
HM#781	HM_BsaI_tTA_F	CCGGTCTCCACCCCCCACCGTACTCGTCAATTCCAAG
HM#782	HM_BsaI_DsRed_R	GCCGGTCTCGGATCCCTACAGGAACAGGTGGTGGCGGCC
HM#792	HM_tGFP_F	GCGAAGACATCATGGAGAGCGACGAGAGCGG
HM#807	HM_Dmsnkyg38_F3	GCAGATGGCCTTAGCTATCATCG
HM#808	HM_Dmsnkyg38_R3	AACCGATGATAGCTAAGGCCATC

Table S2. List of vectors

Code	**components of the construct**
HMMA324	pSL{*af_TREp_Ala::chiRNA::Val::chiRNA::Gly::SV40_af*}
HMMA325	pSL{*af_TREhs43_Ala::chiRNA::Val::chiRNA::Gly::SV40_af*}
HMMA349	pSL{*af_U6:3_Ala::chiRNA::Val:chiRNA::Gly::chiRNA::DSE_af*}
HMMA410	pSL{*af_U6c_Ala::chiRNA::Val::chiRNA::Gly::chiRNA::DSE_af*}
HMMA371	pBXLII{*attP220_TREp:Ala:snkyg13:Val:g41:gly:>_β2t:tTA:5UTR_PUb:DsRed_attP220*}
HMMA372	pBXLII{*attP220_TREp:Ala:snkyg13:Val:g38:gly:>_β2t:tTA:5UTR_PUb:DsRed_attP220*}
HMMA373	pBXLII{*attP220_TREp:Ala:snkyg13:Val:g41:gly:>_DjE/P:tTA:5UTR_PUb:DsRed_attP220*}
HMMA374	pBXLII{*attP220_TREp:Ala:snkyg13:Val:g38:gly:>_DjE/P:tTA:5UTR_PUb:DsRed_attP220*}
HMMA430	pSL{*af_TREp_Ala::snkyshRNAT13::Val::snkyshRNAT41::Gly::SV40_af*}
HMMA433	pBXLII{*attP220_U6:3:snkyg13:g41:g38_β2t:tTA:P2A:tGFP:5UTR_PUb:DsRed_attP220*}
HMMA434	pBXLII{*attP220_U6:3:snkyg13:g41:g38_β2t:tTA:P2A:tGFP:5UTR_PUb:DsRed_attP220*}
HMMA338	pBXLII{*attP220_TREp:Cas9_PUb:EGFP_attP220*}
HMMA425	pBXLII{*attP220_Dsβ2tE/P:tTA:P2A:tGFP:5UTR_PUb:DsRed:SV40_attP220*}
HMMA426	pBXLII{*attP220_Dmβ2tE/P:tTA:P2A:tGFP:5UTR_PUb:DsRed:SV40_attP220*}
HMMA427	pBXLII{*attP220_DmDjE/P:tTA:P2A:tGFP:5UTR_PUb:DsRed:SV40_attP220*}

3.6 Perspective on the combined use of an independent transgenic sexing and a multifactorial reproductive sterility system to avoid resistance development against transgenic Sterile Insect Technique approaches

This chapter presents and discusses a new perspective for the use of genetic engineering to develop pest control strategies. The idea is inspired by the mechanism of sterility induced by ionizing radiation, when multiple chromosomal breaks cause aneuploidy in the sperm or the sired progeny, which leads to embryonic lethality and thus to reproductive sterility. The proposed system of using CRISPR/Cas9 to mimic the action of radiation by induction of many double strand breaks overcomes the deleterious effect on male fitness associated with ionizing radiation. Furthermore, the chapter discusses the use of two independent binary expression systems, the *tet-off* and the *Q-system*, to enable combing the female-specific embryonic lethality established previously for a number of insect pests as a sexing mechanism with the proposed reproductive sterility system. This should provide then a complete set of independent SIT tools in one system.

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Authors contributions:

All authors contributed to the manuscript by discussion of the ideas and by revising the text. EAW wrote the first draft and KNE prepared all Figures.

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Perspective on the combined use of an independent transgenic sexing and a multifactorial reproductive sterility system to avoid resistance development against transgenic Sterile Insect Technique approaches

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Keywords

CRISPR; *cas9*; Genetically modified insect; genetically modified organism (GMO); insect control; insecticide resistance; insect pest management; molecular entomology; quinic acid

Abstract

Background

The Sterile Insect Technique (SIT) is an accepted species-specific genetic control approach that acts as an insect birth control measure that can be improved by biotechnological engineering to facilitate its use and widen its applicability. First transgenic insects carrying a single killing system have already been released in small scale trials. However, to evade resistance development to such transgenic approaches, completely independent ways of transgenic killing should be established and combined.

Perspective

Most established transgenic sexing and reproductive sterility systems are based on the binary tTA expression system that can be suppressed by adding tetracycline to the food. However, to create 'redundant killing' an additional independent conditional expression system is required. Here we present a perspective on the use of a second food-controllable binary expression system – the inducible Q system – that could be used in combination with site-specific recombinases to generate independent transgenic killing systems. We propose the combination of an already established transgenic embryonic sexing system to meet the SIT requirement of male-only releases based on the repressible tTA system together with a redundant male-specific reproductive sterility system, which is activated by Q-system controlled site-specific recombination and is based on a spermatogenesis-specifically expressed endonuclease acting on several species-specific target sites leading to chromosome shredding.

Conclusions

A combination of a completely independent transgenic sexing and a redundant reproductive male sterility system, which do not share any active components and mediate the induced lethality by completely independent processes, would meet the 'redundant killing' criteria for suppression of resistance development and could therefore be employed in large scale long-term suppression programs using biotechnologically enhanced SIT.

Background

Many insects heavily damage agriculture and forestry or transmit deadly diseases to animals and humans. Current control efforts still mostly rely on the use of insecticides, but chemical control is not always harmless and the costs of developing new chemical compounds to overcome the world-wide threat of insecticide resistance are escalating [1]. Moreover, to protect biodiversity the establishment of pest-specific management methods is desirable. The Sterile Insect Technique (SIT) is a species-specific genetic control approach that acts as an insect birth control measure, which relies on the mass rearing, sterilization and field release of large numbers of insects. The competition between released sterile and resident males for mating with wild females leads to the reduction of the reproductive potential. If continued releases of high-quality sterile males in inundating numbers over several consecutive generations are performed, a progressive reduction of the population size and eventually the total eradication of the pest population will occur [2,3]. SIT is now an accepted component of various integrated approaches to control, suppress, prevent, or even eradicate invasive insect pest species from islands, large fruit production areas, or even complete continents [4]. Classically, both male and female insects were released, particularly because the distinction between male and female pupae is hardly manageable or requires the development of genetic sexing strains [5]. Released females, however, although sterile, sting fruits with their ovipositors or keep blood feeding and potentially transmit diseases as well as compete against wild females for mating with the sterile males [5]. In addition, sterilization is classically achieved by irradiation, a procedure that often renders insects very weak and unfit to compete with the wild mates [6]. Such drawbacks and several years of experience have put forward several key requirements for an efficient SIT application: intensive rearing of large numbers of insects for mass release, the availability of efficient sex-separation methods, sterilization techniques able to produce large numbers of insects with minimal effects on fitness and competitiveness, effective release methods, and efficient marking systems to identify released individuals.

Biotechnological engineering of insects makes novel approaches possible to efficiently mark insects as well as selectively produce vigorous and potent sterile males, which are generated by conditional male reproductive sterility in combination with conditional female lethality. This will improve efficacy and widen applicability to further insect pest species [7,8]. To minimize

the concerns coupled with the release of transgenic organisms, SIT programs are actually ideal, as the sterility of the released males will serve as a biological safety mechanism for containment as it impedes the spread of transgenes and allows for a safe deployment [9,10].

In accordance to this hope for novel successful genetic pest management strategies, the first biotechnologically engineered designer insects have already been released in small scale trials: pink bollworm moths in Arizona, USA [11], as well as yellow fever mosquitoes in the Grand Cayman Islands [12], Malaysia [13], with a currently ongoing release in Brazil [14,15]. For the release in the Grand Cayman Islands, it has been shown that the sustained release of transgenic mosquitos carrying a dominant lethal gene could successfully suppress a field population [16] demonstrating the great potential of transgenic SIT approaches. Envisioning the beneficial future use of genetically modified insects, the European Food Safety Authority has recently published a scientific opinion on the guidance on the environmental risk assessment of genetically modified animals including insects [17]. Since reproductive sterility based on lethality systems serves as an intrinsic containment against vertical transmission of transgenes in biotechnologically engineered SIT, its application does not present real concerns in respect to humans and the environment [18].

Nonetheless, the use of transgenic SIT approaches is still at initial stages and an ongoing large-scale use somewhat premature, as potential resistance development might pose a significant threat to the further use of this technology [19]. In the currently released transgenic mosquitoes, the dominant lethality is mediated by the overexpression of a synthetic transcription factor that is deleterious to cells at very high levels reached by auto-activation in a positive feedback loop [20]. This presents just one single killing system based on an unclear mechanism. Since most pest insects produce large numbers of offspring, they have a high propensity to evolve resistance to control measures. Actually, classic SIT based on sterilization by radiation is an exception in the resistance development context, as the radiation-induced breaks of the chromosomes are random and vary among all individuals thus providing built-in redundancy [21]. However, transgenic SIT approaches will have defined killing systems that are in principle susceptible to resistance development. Thereby, we assume that the released insects still contain functional transgenes and are themselves susceptible to the dominant lethality [22]. The potential break-down of transgenic characters during mass rearing is an important but different issue for quality control before release. In respect to resistance development the heterogeneous genomes of the field populations are important [21], which might contain genotypes that lead to suppression or partial suppression of the lethality traits. For the avoidance of behavioural resistance, where wild type insects reject mass-reared insects as mating partners, regular introgression of wild type genetic material into the mass rearing strains has been successful [2]. However, there is also the possibility of biochemical resistance to biotechnologically engineered lethality. Due to the inundation of the population with susceptible alleles by the release of the sterile insects during an ongoing

SIT program, only strong resistance-mediating alleles acting dominant and having only low fitness costs propose a threat to SIT programs and are so far only hypothetical [22].

Nevertheless, insects have successfully developed resistance to synthetic chemicals as well as to microbial agents [23], and are also likely to develop resistance to transgenic SIT approaches when employed in long-term suppression programs [24]. One strategy to significantly impede or at least delay resistance development could be based on the principle of 'redundant killing' [25,26]. Therefore, transgenic SIT strains with effective and necessary sterility or lethality traits should only be considered in large scale long-term suppression programs, once completely independent toxicity systems have been combined. Since actually two traits are favourably introduced by transgenesis – female lethality for male only releases as well as reproductive sterility by dominant lethal transgenes – one task is to identify two completely independent ways of mediating them.

Perspective

Combination of two independent systems: male reproductive sterility and female lethality

A sterile insect in the sense of SIT is defined as "an insect that, as a result of a specific treatment, is unable to reproduce" [27]. A first approach to cause such reproductive sterility by biotechnological engineering was successfully demonstrated in the non-pest insect *D. melanogaster* [28]. The system is based on the transmission of a transgene combination that causes conditional embryo-specific lethality in the progeny without larval hatching and has successfully been transferred to tephritid fruit flies [29,30]. This prevents larval damage to fruits and the introgression of transgenes into wild type fruit fly populations. Furthermore, for tephritid fruit flies and mosquitoes, transgenic strains were produced using an autocidal overexpression loop of the protein tTA, which leads to dominant lethality when transgenic males were mated to wild type females [20, 31]. Additional transgenic reproductive sterility systems [32,33] might be based on species-specific homing endonucleases [34].

To generate transgenic sexing systems, female lethality was first developed and tested in *D. melanogaster* and based on the female-specific expression of conditional lethal genes [35,36]. More recently transgenic sexing systems for tephritid fruit flies have been generated using a female-specifically spliced intron from the *transformer* gene. First it was used in an autocidal expression loop with the female lethality occurring at late larval stages in the Medfly *Ceratitis capitata* [37]. This system has successfully been transferred to other Tephritids such as the olive fly *Bactrocera oleae* [38] and also to blowflies [39] – devastating pests of livestock – as well as to lepidopterans [40]. Furthermore, embryonic transgenic sexing systems have combined the use of such a female-specifically spliced intron with an early embryonic expression mediated by *cis*-regulatory elements from early acting cellularization genes that indirectly and

controllably drive the expression of a hyper-active pro-apoptotic gene (Figure 1) [41,42]. An even better understanding of the sex differentiation pathway in insects will provide us with additional strategies for synthetic genetic-based tools for large scale sex separation in SIT applications [43] based on either female killing or actual female sex-reversal [44,45].

tTA: the commonly used conditionally repressible expression system

The conditionality of the so far established transgenic sexing and reproductive sterility systems is based on a binary expression system, which can be suppressed by supplementing the food with tetracycline (Figure 1). The *tetracycline-controlled transactivator* (*tTA*) consists of a bacterial-viral fusion protein [46] that activates gene expression after binding to a *tTA-response element* (*TRE*). The major advantage of this binary expression system is that a food supplement can suppress the activation providing an additional control to the directed gene expression [47]. tTA complexed with tetracycline is prevented from binding to its response element and the downstream gene is not activated. The expression system is thus switched off by supplementing the food with tetracycline which allows for an additional control on top of the tissue-specific promoter driving *tTA* expression. Since only small amounts of tetracycline are needed to suppress the expression, this system has become the most favorable expression system to develop transgenic SIT approaches. However, to create a situation of 'redundant killing' based on two completely independent mechanisms to mediate sterility or female lethality, an additional conditional expression system is necessary.

Second food-controllable expression system: Q system

Recently a second food-additive controllable expression system – the Q system – has been shown to work *ex vivo* in mammalian cells as well as *in vivo* in the vinegar fly *D. melanogaster* [48,49]. The broad applicability of this system is also demonstrated by its functionality in the nematode worm *Caenorhabditis elegans* [50]. The Q system is based on the regulatory genes of the gene cluster *qa* from the bread mold *Neurospora crassa*, which allows the fungus to utilize quinic acid as a carbon source [51]. Quinic acid can be found in high concentrations both in herbaceous plants as well as conifers [52] and at especially high levels in unripe fruits [53]. Several molds are able to use quinic acid as carbon source and have specific gene clusters for the catabolic pathway [54]. The regulatory genes of the cluster ensure that the catabolic enzymes are only expressed at the presence of quinic acid: one gene, *qa-1F* (*QF*), acts as DNA-binding transcriptional activator of all cluster genes, whereas another regulatory gene, *qa-1S* (QS), acts as a repressor that does not bind DNA itself but inactivates the activator QF by complex formation [54]. Quinic acid acts as an inducer by hindering the repressor QS from complexing QF, which then can activate its target genes (Figure 2). Therefore, the Q system is actually an inducible binary expression system with the food additive, quinic acid, leading to the activation of controlled gene expression. This and the fact that quinic acid is found widespread in nature [52] do not allow us to use this system in an analogous way to the tTA

system. However, it offers a completely independent expression system that should be utilized for novel transgenic SIT approaches.

Render inducible system suitable for transgenic SIT approaches

An inducible system would usually require that the inducer is constantly present to have the system activated. But as this cannot be warranted for a food-additive after release, a temporary induction of the system needs to be stabilized into a continuous expression. For this purpose, site-specific recombination systems [55] can be utilized to stabilize an inducer pulse into a persistent activation. For the *flp* recombinase (FLP) it was demonstrated in *D. melanogaster* that a region-specific promoter can be separated from the downstream coding region by a *flp*-out cassette that contains a transcriptional terminator and is flanked by *flp* recombinant target sites (FRTs) [56,57]. The transcriptional terminator prohibits the directed expression mediated by the tissue-specific promoter until FLP removes the *flp*-out cassette by site-specific recombination of the FRTs that are in direct orientation (Figure 2). The left over single FRT in the 5'UTR does not interfere with effective transcription and translation of the downstream coding sequences [56,57]. On this basis, the Q binary system can be combined with the FLP mediated transcriptional activation system to stably activate the expression of a gene after a pulse induction with an inducer (Figure 2).

To reduce the number of constructs necessary for such a complex inducible Q and immediate targeted gene expression system, actually the regulatory components of the Q system can be placed into the *flp*-out cassette (Figure 2) which will also place the Q system components under the same control as the later expressed effector gene [57]. To actually place both regulator genes – QF and QS – into the same construct, the two coding regions can be separated by an internal ribosome entry site (IRES) to allow for a bi-cistronic transcript. Depending on the translational start efficiency of the insect virus IRES compared to the actual capped mRNA [58], the QS and QF coding sequences should be placed accordingly to make sure that repressor QS will be in surplus to the activator QF.

In *D. melanogaster* it has been shown that FLP expression driven by the *β2 tubulin* (*β2 tub*) promoter is highly efficient to cause cassette flip-out during spermatogenesis leading to the transmission of the activated effector construct into the next generation [56,57]. Since the *β2 tub* promoter would also enable the generation of reproductive sterility systems [7], this promoter would be very suitable for such a complex system. Respective promoters have already been cloned from a number of different tephritid and mosquito species and functionally used for sperm marking purposes [59,60,61].

To cause reproductive sterility, finally an effector needs to be activated that either causes male sterility by sperm depletion, e.g. by expression of a cell death gene or a cell-specific toxin that is active in the cytoplasm only and has no trans-membrane movement abilities to protect

adjacent tissue or predatory organisms [7,61]. However, as such sterile males would not transfer sperm to females, such females would continue to search further for sperm-providing wild type males. Therefore, an effector that would kill the progeny but not the sperm would thus be much more suitable. This will allow for sperm development and transfer and therefore renders the females at least temporarily refractory to subsequent mating with wild type males. Such an effector could be a homing endonuclease (Figure 2) that does not affect spermatogenesis – thus producing functional sperm – but attacks the genome of the zygote or prevents the fusion of the male and female pro-nuclei [34]. This would serve as the best reproductive sterility mechanism as it would cause a dominant early embryonic lethality without affecting the sperm itself by stopping the development of the progeny at the very beginning. Moreover, a homing endonuclease would also be independent in its function from the proposed hyperactive pro-apoptotic gene suggested for the sexing system (Figure 1). However, it should be noted that for an applicable transgenic reproductive sterility system, 100% male sterility needs to be reached, which requires efficient *flp* recombinase repression in the absence of quinic acid and its effective induction in the presence of quinic acid as well as strong expression of a highly active homing endonuclease.

Partial redundancy of the female lethality and reproductive sterility systems.

The described female lethality and reproductive sterility systems will in fact not be fully redundant, as only the female progeny of the released males will indeed have both lethality systems working. In the male progeny only the reproductive sterility providing the homing endonuclease will be active. Thus, rare strong resistance-mediating alleles might be selected in such male progeny and potentially lead to the accumulation of both the resistance allele and the transgenic lethality allele [22]. However, in case of direct linkage between the two lethality systems, which can be achieved by transgene modification based on site-specific recombination [62], the female lethality in the following generation would severely reduce the chance of accumulation of the lethality allele and thus reduce also the selection of the resistance allele. Since only resistant males would survive, they would be immediately outcompeted by the released SIT males [22].

Redundancy in reproductive sterility by an endonuclease causing chromosome shredding.

Ideally the reproductive sterility system itself should be highly redundant to cause many different lethal mutations similar to the built-in redundancy of radiation-induced sterility [21]. To achieve this, it would be great to have a number of diverse endonucleases or endonuclease target sites causing chromosome shredding [63]. For this, we propose the employment of an endonuclease from the adaptive bacterial immune system using as essential component clustered regularly interspaced short palindromic repeats (CRISPR) [64,65], which allows bacteria to defend themselves against viruses they encountered before by recognizing and

cutting the viral DNA sequences. For the human pathogen *Streptococcus pyogenes*, it could be shown that a single endonuclease – CRISPR-associated nuclease 9 (Cas9) is sufficient to cleave the target DNA [66]. Since it was shown that Cas9 can be directed to any 'protospacer' sequence followed by a protospacer-adjacent motif (PAM) that has only two required bases (NGG) [67] by using short guide RNAs (gRNAs) [68], this CRIPSR/Cas9 system has been successfully employed in many model and non-model organisms to generate gene knock-outs and genome editing [69]. Recently a feature article on this emerging technology has discussed possible uses of the CRIPSR/Cas9 system in gene drives to alter wild populations [70].

By transgenic expression of several gRNAs using RNA polymerase III-dependent promoters, such as the *U6* snRNA promoter, it has been shown that the Cas9 endonuclease can actually be targeted to several diverse targets, which can lead to a mutagenesis rate of up to 100% [71,72]. By our proposed use of the *2 tub* promoter, Cas9 will be highly expressed during spermatogenesis and the mRNA still be highly translated during spermiogenesis [73] to expose the sperm chromosomes to high amounts of the endonuclease (Figure 3). To cause chromosome shredding, several guide RNAs can be employed to direct the CRISPR/Cas9 endonuclease to para-centromeric, sub-telomeric, and microsatellite sequences. The induced double strand breaks will lead to large chromosomal aberrations causing aneuploidies that will mediate multifactorial reproductive sterility.

In fact, one of the caveats of the Cas9 technology – the potential lack of specificity leading to off-target effects [74] – can serve as an additional advantage in the proposed use here, since it might lead to pleiotropic effects harming further genomic loci. Targeting many chromosomal locations will thus provide the intended redundancy bringing the transgene-induced reproductive sterility a step closer to the built-in redundancy of radiation-induced sterility [21].

Conclusions

The combination of a transgenic sexing system to meet the SIT requirement of male-only releases based on the repressible tTA directed expression system to create female-specific embryonic lethality using a sex-specifically spliced intron and a hyperactive pro-apoptotic gene (Figure 1) together with a reproductive sterility system based on a sperm-specifically expressed endonuclease controlled by the inducible Q-system in combination with site-specific recombination (Figure 2) seems a promising approach. These two systems would not share any active components and the lethality would be mediated by completely independent processes. Therefore, cross-resistance to both lethality-mediating processes is extremely unlikely and resistance development would require at least two independent gene loci with the likelihood of co-existence and selection being significantly reduced [25]. It should be noted, however, that this redundancy is only partial as only the female progeny of respective released males will have both lethality systems at work. While this will still reduce the

likelihood of accumulating transgenic lethal alleles and resistance alleles, we propose an additional level of redundancy for the reproductive sterility system using the CRISPR/Cas9 endonuclease system targeting several chromosomal locations to induce chromosome shredding in the sperm (Figure 3).

The insect strains carrying the combined transgenic female lethality and reproductive male sterility systems would be reared on tetracycline containing food to suppress the female-specific lethality. The male reproductive sterility would not be activated yet, since the repressor QS would keep the system in an OFF state (Figure 4A). The adult flies of the pre-release generation would then be aged on tetracycline-free food (Figure 4B) in order to stop the suppression of the embryonic female-specific lethality in the next generation [29,41,42]. The release generation should then be grown also on tetracycline-free larval food in order to keep the embryonic sexing system on to produce males only: in the absence of tetracycline, the synthetic transactivator tTA would activate a hyper-active pro-apoptotic gene that would lead to programmed cell death in the female embryos, as only the female-specific splicing of the *transformer* intron in this transcript results in the production of an mRNA capable of translating the functional hyper-active pro-apoptotic protein (Figure 4C). The larval food for the release generation would, however, need to contain quinic acid to inactivate the repressor QS, which would then allow the activator QF to induce the expression of the *flp* recombinase gene, which then in turn would remove the Q system regulators and activate the expression of the heterologous endonuclease Cas9 during spermatogenesis (Figure 4C). Released males (Figure 4D) would produce sperm with shredded chromosomes leading to lethal aneuploidy in the next generation similar to radiation-induced reproductive sterility without suffering of somatic damages that causes reduced fitness.A transgenic SIT approach using independent lethality systems would meet the 'redundant killing' criteria for suppression of resistance development and could therefore be employed in large scale long-term suppression programs.

Competing interests

EAW holds a patent on 'Universal Markers of Transgenesis' (United States Patent No. 6,518,481 B1).

Authors' contributions

EAW designed the study and wrote the first draft of the manuscript. KNE created the figures. All authors contributed to the conception of the study as well as critically revised and approved of the manuscript.

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Figures

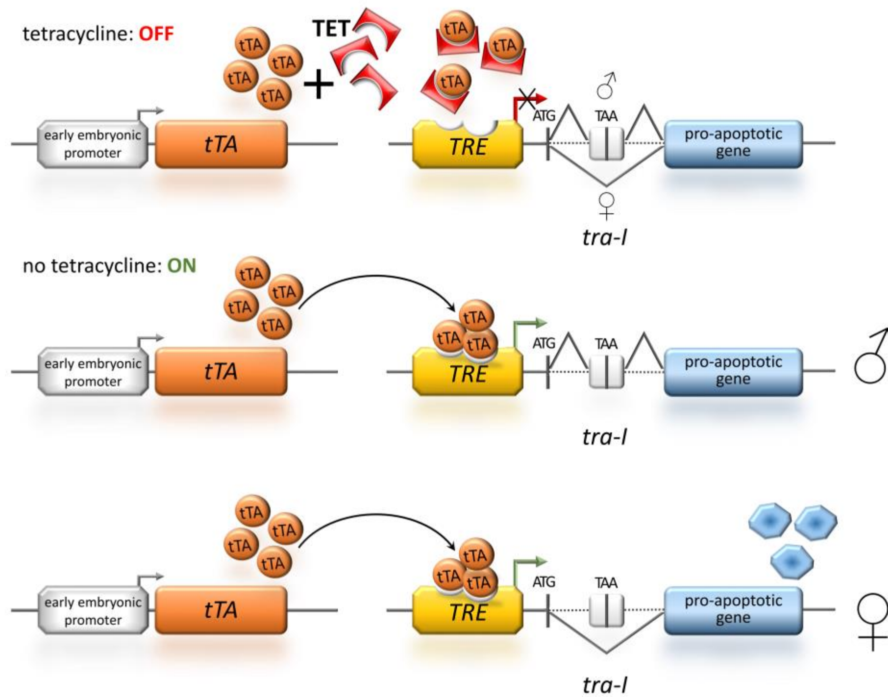

Figure – 1 Sexing using female-specific splicing under the control of the repressible tTA-system.
The depicted transgenic sexing system [41,42] uses a sex-specifically spliced intron and a hyperactive pro-apoptotic gene to generate female-specific lethality under the regulation of the tetracycline-controlled transactivator (tTA). To cause early embryonic lethality and thus avoidance of larval survival, the tTA is under the control of an early embryonic promoter. During rearing of such strains, addition of tetracycline (TET) to the food keeps the system in the OFF state, as tetracycline blocks the binding of tTA to its response element (*TRE*). For the release generation, tetracycline is absent in the food and therefore the sexing system is ON: in males, the male specific splicing of the transformer intron (*tra-I*) – placed after the translation start codon (ATG) of the effector gene – includes a small exon containing a TAA stop codon between the start codon and the rest of the effector gene and therefore prevents the production of the functional pro-apoptotic effector protein allowing the males to survive; whereas in the females the female specific splicing of the *tra-I* produces a functional effector and the embryonic cells are driven into apoptosis, which leads to female-specific embryonic lethality.

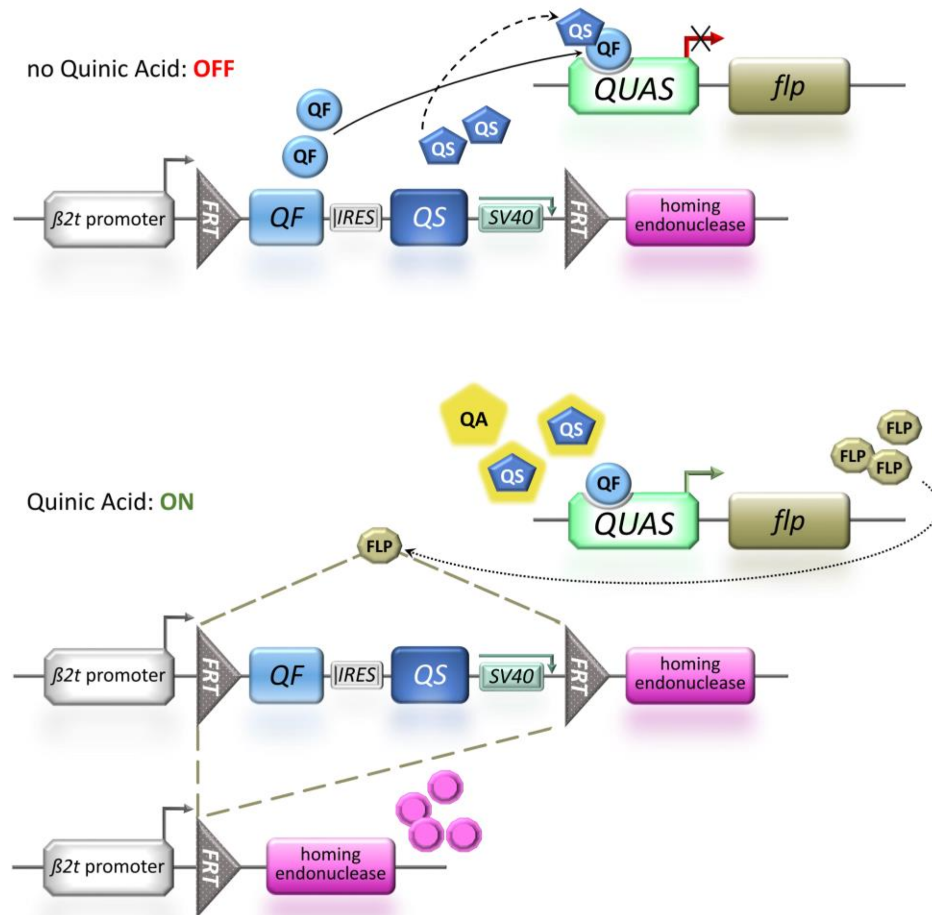

Figure 2 – Reproductive sterility using homing endonucleases controlled by the inducible Q-system in combination with site-specific recombination. The proposed reproductive sterility system is based on the inducible binary expression system Q [48], in which quinic acid (QA) acts as an inducer that hinders the repressor QS from complexing the transcriptional activator QF that can activate its target genes by binding to a Q upstream activation sequence (*QUAS*). To generate male reproductive sterility systems the spermatogenesis-specific promoter of the *β2 tubulin* (*β2t*) gene can be suitably used to affect either the sperm itself or the progeny sired by the sperm. The Q system can be combined with a recombinase mediated transcription regulation system to render the induction of an effector gene expression permanent and independent of the presence of the inducer QA. In this dual system, QF drives the expression of a site-specific recombinase (FLP) that can in turn remove a *flp*-out cassette [57], which contains a transcriptional terminator (*SV40*) and is flanked by *flp* recombinant target sites (*FRTs*) in direct orientation. After the removal of the transcriptional terminator, the directed expression of an effector gene is mediated by the tissue-specific promoter 5' to the FRT. Since the Q system components are superfluous after the activation of the effector gene, they can also be placed into the *flp*-out cassette. To make sure that both components of the Q system are translated in a bi-cistronic messenger RNA, they will be separated by an internal ribosome entry site (IRES). A homing endonuclease targeting the progeny genome can be employed as an effector that would kill the progeny but not the sperm itself [34]. During regular rearing this male reproductive sterility would be kept in an OFF state, as at the absence of QA the repressor QS will mask QF and block its activation potential. Only after the addition of QA to the food in the release generation, QS will be inactivated and QF thereby allowed to activate the expression of the *flp* recombinase (FLP), which in turn would remove the Q system regulators and at the same time activate the expression of the homing endonuclease that could block development of the next generation and thus cause male reproductive sterility.

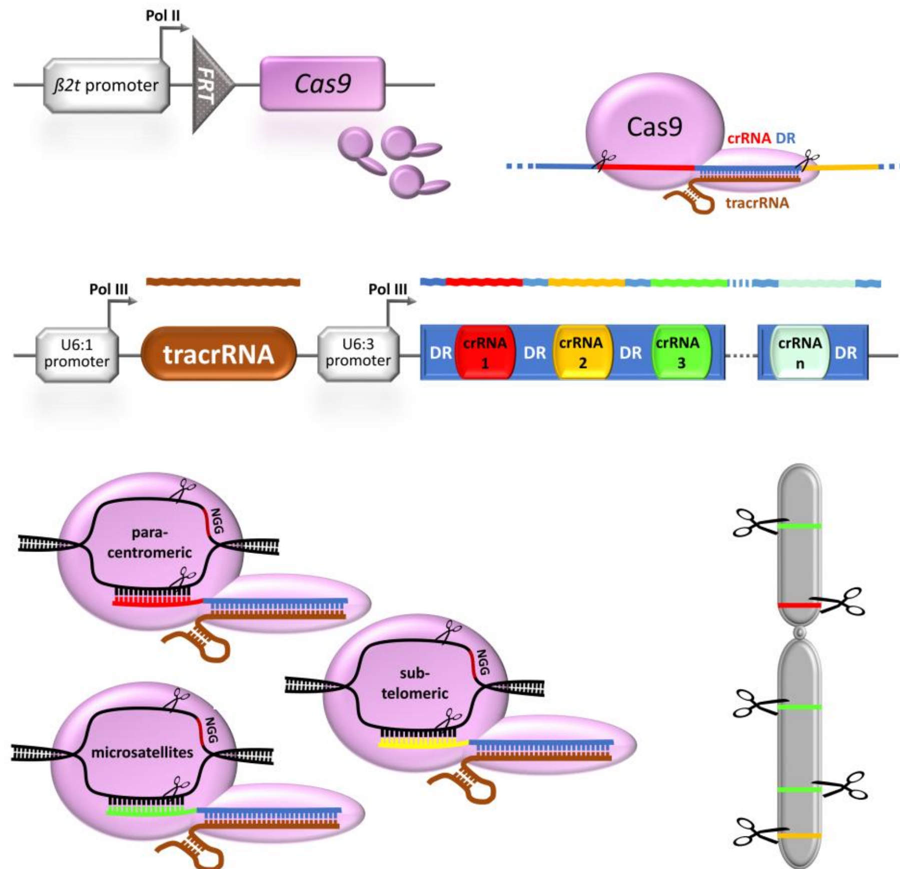

Figure 3– Redundant reproductive sterility based on the CRISPR/Cas9 system. The bacterial derived Cas9 endonuclease will be expressed under the control of the *β2 tubulin* (*β2t*) promoter. Cas9 will be targeted to para-centromeric, sub-telomeric, and diverse macrosatellite sequences by guide RNAs, which are encoded by a CRISPR RNA (crRNA) array. This crRNA array as well as the *trans*-acting crRNA (tracrRNA) will be expressed under diverse RNA polymerase III promoters such as from the snRNA *U6* (U6:1, U6:3). In the crRNA array the diverse crRNAs are separated by direct repeat sequences (DR) derived from the *Streptococcus pyogenes* CRISPR. The expressed Cas9 is loaded with tracrRNA and subsequently binds the crRNA array based on complementarity between tracrRNA and the DR sequences, thereby randomly selecting one of the crRNAs as a guide to produce a functional CRISPR/Cas9 endonuclease targeting the respective genomic loci [75].

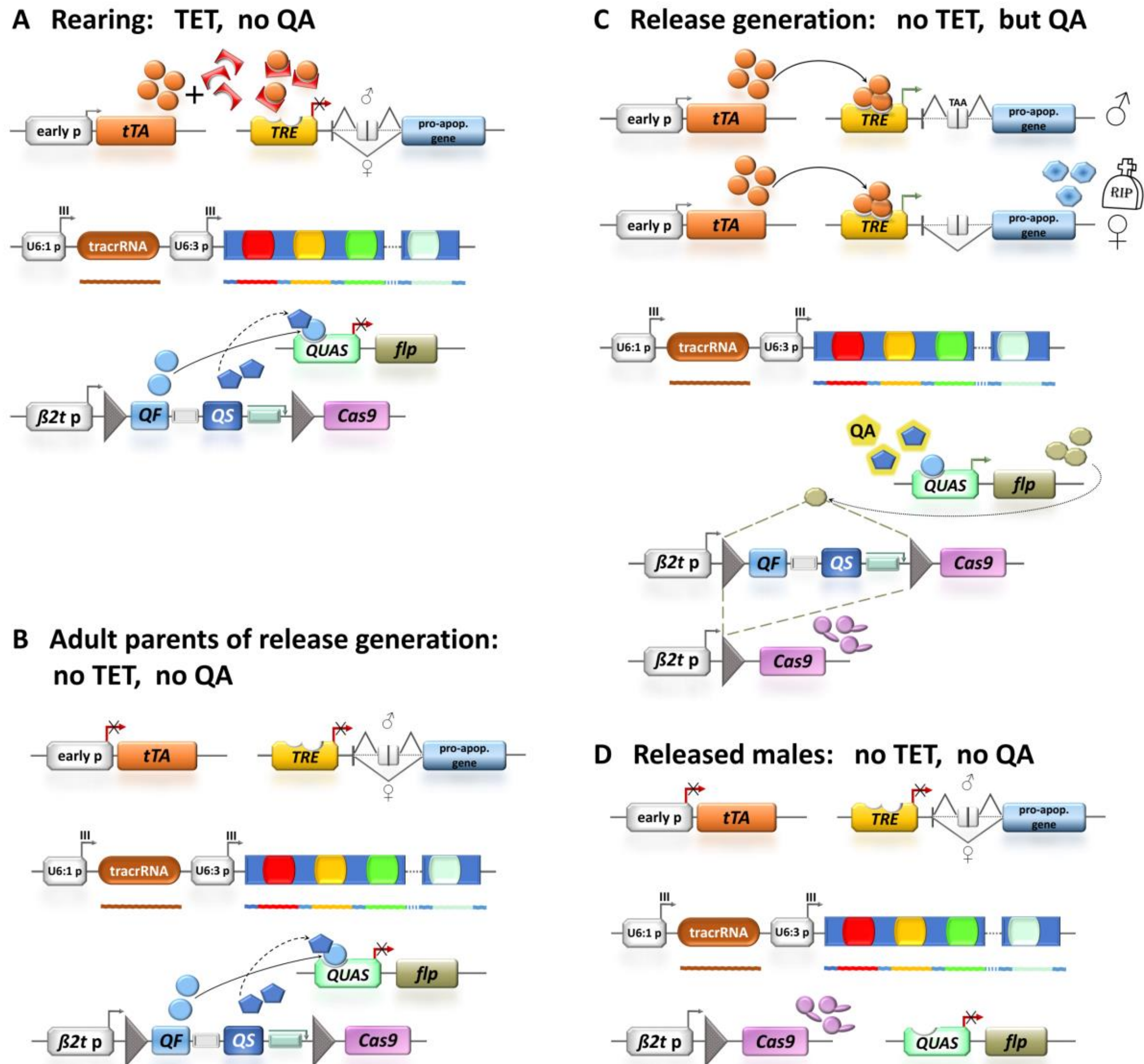

Figure 4 – Rearing scheme for combined female lethality and reproductive sterility systems.

A Under regular rearing conditions, tetracycline (TET) is added to the food to repress the female lethality, quinic acid (QA) is not required for rearing. **B** The adult parents of the release generation will be changed to food without TET, still also without QA. This is necessary to avoid suppression of the early embryonic lethality in the next generation by maternally transferred TET to the oocyte. The female lethality system is still off, since the early embryonic promoter is not driving tTA at adult stages. **C** The release generation is then reared on food without TET but with added QA. Due to the lack of TET the female lethality system is switched on and the females die during early development. The QA leads to the activation of the Q system that leads to the expression of a site-specific recombinase, which in turn mediates the spermatogenesis-specific expression of the Cas9 endonuclease by removing a recombination-site flanked spacer cassette. **D** The released males (no TET, no further QA) express high levels of the endonuclease Cas9 and multiple guide RNAs during spermatogenesis causing shredded chromosomes that will lead to lethal aneuploidy in the next generation.

3.7 Development of a CRISPR/Cas9-induced multifactorial reproductive sterility system based on sperm-specific chromosome shredding

In this chapter, we present the first steps toward the development of a reproductive sterility system with proof of concept in *D. melanogaster*. The idea was initially described earlier (see chapter 3.6). We present a set of transgenic driver and Cas9 responder lines and their use to evaluate the suitability of the E/P of the spermatogenesis-specific gene *β2t*, the *tet-off* system, and the basal promoters for the proposed system. Importantly, we present the identification of suitable CRISPR targets and the design of multiplexing constructs harboring two to three guide RNAs to increase the number of DSBs. We also present the first attempt to transfer the female-specific embryonic lethality system to the fruit pest *D. suzukii* and present possible solutions to failure regarding the generation of transgenic effector lines. All together this chapter presents a new strategy for the development of the SIT. If this system works as predicted, it can be combined with sexing and sperm-marking systems to have an all in one SIT package to fight agricultural pests and diseases vectors.

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Authors contributions to practical work:

Hassan M. M. Ahmed:	All experiments besides the ones performed by FH. This also includes target identification and genetic construct design.
Fabienne Heese:	Generation of transgenic lines.
Status:	Work in progress

Development of a CRISPR/Cas9-induced multifactorial reproductive sterility system based on sperm-specific chromosome shredding

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Background

Resistance development is a major concern when developing a new system for pest management, especially because insects have short generation time and high fecundity, which makes emergence of resistance against the control system very likely (1). We have proposed the use of CRISPR/Cas9 to develop a new reproductive sterility system that is characterized by redundancy, which means the source of sterility is not based on one particular gene locus and should therefore overcome the possibility of resistance development (2). Three prerequisites need to be fulfilled to develop such a system: (1) abundant CRISPR targets, (2) enhancer/promoter that is spermatogenesis specific, and (3) a suitable binary expression system to facilitate conditional activation of the reproductive sterility system. Targeting Cas9 to multiple genomic loci such as para-centromeric, sub-telomeric, and microsatellites to induce simultaneously many double strand breaks (DSBs) will mimic the action of ionizing radiation specifically during spermatogenesis and does not damage necessary genes (2). This will lead to production of competent males that are better suited to compete with the wild type males. Multiplexing of CRISPR target sites using the transfer RNA (*tRNA*) processing system was demonstrated in plants and in *Drosophila* (3–5). CRISPR/Cas9 driven by the enhancer/promoter of spermatogenesis-specific genes has proven to be a suitable effector to induce chromosome shredding, which was demonstrated in the mosquito *Anopheles gambiae* by targeting it to the rDNA on the X chromosome to allow for production of only Y chromosome bearing sperm (6). In the same study, they used the enhancer/promoter of the gene *β2t* to confine the expression of *Cas9* to spermatogenesis. In fact, *β2t* E/P was successfully used in previous study to direct the expression of the X-shredder homing endonuclease gene (HEG) *I-PpoI* to spermatogenesis and generated a sex ratio distortion system (6,7). The enhancer/ promoter of the *Drosophila β2t* gene is a great candidate to drive strong expression of effector molecules during spermatogenesis in a conditional manner. Three binary expression systems (*tet-on*, *tet-off*, and the Q-system) (8–12) are of relevance and can be used to control and confer conditionality to the system. In fact, to realize the ultimate goal of this

study by combining the reproductive sterility and the sexing systems, two independent binary system are needed (2). Other interesting genes that are spermatogenesis specific and are also regulated at the level of translation by translation arrest are *Don juan* (*dj*) and *Don juan like* (*djl*) in *D. melanogaster* (13). Interestingly the sister genes are next to each other and both are regulated post-transcriptionally by a translation suppression element in their 5'UTR. This sequence element leads to translation delay of the mRNA until post meiotic stages, when the genes are needed and translated. In this study we aim to develop a redundant male sterility system by targeting Cas9 to transposable elements during spermiogenesis, to avoid interfering with production of sperm and the targeting of somatic chromosomes. To this end, we will test the suitability of the *tet-off* system to control gene expression during spermatogenesis. Furthermore, we will exploit the translation delay element of the *Dm dj* gene to delay the translation of Cas9 to post-meiotic stages of spermatogenesis.
To eventually be able to transfer these systems to the invasive pest *D. suzukii* and to combine the reproductive sterility system with the well establish transgenic female-specific embryonic lethality system (FSEL), we have also taken the first steps in establishing FSEL in *D. suzukii*.

Results

Identification of CRISPR targets for chromosome shredding

To be able to use the CRISPR/Cas9 system to induce reproductive sterility equivalent to the action of ionizing radiation, we need to induce as many double strands breaks as possible. To achieve that we searched for euchromatin transposable elements in the genome of *D. melanogaster*. We based our search on Kaminker et al. (14), since heterochromatin might not be accessible for Cas9. Based on their overall representation in the genome and the distribution across the chromosomes, we have chosen the three transposable element families, *roo*, *jockey*, and *1360*, representing three classes: long terminal repeat LTR, LINE-like, and inverted terminal repeat ITR. *Roo* is represented by 146 elements distributed on all chromosomes except chromosome 4. *jockey* is identified 69 times with distribution on all chromosomes including two times on chromosome 4. The family *1360* appears 105 times with distribution on all chromosomes including 30 times on chromosome 4.
Three guide RNAs for each element were designed based on the latest *D. melanogaster* genome release, r-6 (15). Each gRNA is shown in Table S2 with the number of exact matches in the genome of *D. melanogaster*.

Design of constructs and generation of transgenic lines

To drive conditional and testes specific expression of Cas9 and *gRNAs* targeting transposable elements, we used the *tet-off* binary expression system and *E/P* of the spermatogenesis specific genes *Dm-β2t* and *Dm-dj*. We generated driver construct HMMA334 by fusion of *Dm-β2t E/P* upstream of the heterologous transcription factor *tetracycline transactivator* (*tTA*). The construct was use to generate 24 independent transgenic lines. We also used the vector HMMA324 (chapter 3.5) to conditionally and simultaneously express two *gRNAs* targeting

either of the three transposable elements *roo*, *jockey*, and *1360*. In addition, we used vector HMMA349 (chapter 3.5) to generate *gRNA*-driver constructs HMMA446, HMMA447, and HMMA448 to constitutively and simultaneously express three *gRNAs* targeting the same TEs, and construct HMMA449 to express one target for each of the three TEs.
Unlike in the case of using *Cas9* to target paternal effect genes, which do not interfere with male fitness or spermatogenesis, we aim to delay the effect in the proposed CRISPR/Cas9 chromosome shredding system to post-meiosis stages of spermatogenesis to avoid interfering with spermatogenesis. To achieve that we utilized the translation suppression element from the 5' UTR of the *Dm-dj* gene or the 5'UTR of the *Dm-β2t* gene. This sequence, when fused upstream of a gene, is supposed to delay the translation of that gene to the post-meiotic stage (Fig. 1D). We generated three different *Cas9* responder constructs HMMA365, HMMA366, and HMMA203 by fusion of the insect codon optimized *Cas9* coding sequence (with N and C-terminal *nuclear localization signal* and an N-terminal Flag tag) downstream of the *TRE* with the basal promoter of either *the P-element* or *Dm-dj* and both have *dj TSE* just upstream of the Cas9 translation start codon. The third construct HMMA203 has the *P-element* basal promoter and the 5' UTR from *D. suzukii β2t* gene. The responder constructs HMMA203 was used to generate 10 independent transgenic lines, HMMA365 (29 independent lines), and HMMA366 (12 independent lines).

Testes specific expression of Cas9

To check whether tTA can drive testes specific expression of Cas9 under the control of the *TRE* with the *P-element* or *dj*-basal promoter, we crossed driver lines 334_F1F4 and 334_F48M1 separately to responder lines 365_M7_F1, 366_M7F1, and 204_M1F1. We then performed RT-PCR on total RNA isolated from the testes of flies carrying both constructs (Fig. 2C). The results show that Cas9 is expressed from responders with *P-element* basal promoter with (365_M7_F1) and without the TSE (204_M1F1) and weak expression was detected from responder with *dj*-basal promoter (366_M7F1), however, this is most likely specific to this particular line and not a construct problem. Worth noting is detection of *Cas9* expression in the negative control (Cas9 responder not crossed to driver). However, this can be due to high sensitivity of the assay and or leakiness of the basal promoter, which needs to be evaluated.

Discussion

The SIT offers a clean pest control approach since it is species-specific and does not harm natural enemies and pollinators (16). Sterility in the released males is induced by ionizing radiation, which is very effective in this regard and resistance development against it is unlikely due to random chromosome breakdown causing reproductive sterility (17). However, the males' fitness is also affected (17,18) and therefore the area targeted for control with SIT must be flooded with a large amount of such sterile males to compete with the wild type males in numbers rather than in individual fitness. To overcome the fitness cost due to irradiation, we

proposed to develop a reproductive sterility system similar to ionizing radiation with regard to redundancy, and therefore a reduced chance of resistance development, but superior to it in the sense that the fitness of the sterile males is not compromised (2). The system relies on the use of the CRISPR/Cas9 system to induce many double strand breaks in the sperm chromosomes during spermatogenesis, not affecting the males themselves or the process of sperm production. Therefore, males should transfer sperm with multiple chromosomal aberrations due to incorrect repair of the DSBs (2). Such sperm should be uncapable of forming a functional zygote and as a result embryonic development should not start. To achieve that, we searched for *D. melanogaster* transposable elements (TEs) and we have chosen three families, *roo*, *jockey*, and *1360* for proof of concept in *D. melanogaster*. Three gRNAs targeting each TE were designed and used to generate gRNA-driver constructs using the same multiplexing strategies described in chapter 3.5. The total number of genomic targets for construct HMMA448 with three gRNAs against 1360 are 874, for construct HMMA447 targeting *jockey* are 212, and for HMMA446 targeting *roo* are 379. The combination of *1360_gRNA2*, *roo_gRNA26*, and *jockey_gRNA10* together in construct HMMA449 should target and induce 661 DSBs. These overwhelming numbers of target sites combined with abundant expression of Cas9 and gRNAs should with lead to many chromosomal aberrations culminating in embryonic lethality and thus provide a suitable way to cause reproductive sterility. This, however, should be induced post meiotically to avoid interfering with meiosis and spermatogenesis. To induce such chromosomal abnormalities at the post meiotic stage of spermatogenesis, fine coordination of transcription and translation of the effector molecule Cas9 is required. The tissue specificity and conditionality are supposed to be achieved by the use of the P/E of the spermatogenesis-specific gene *Dm-β2t* and the *tet-off* binary expression system. It is important that enough *Cas9* transcript is produced during the mitotic amplification divisions before transcription shutdown in the maturing primary spermatocytes. However, the translation of these transcripts should not start until after meiosis has completed and spermiogenesis started. To arrange for that, we fused the translation repression element of the *D. melanogaster dj* gene (19) in front of the *Cas9* coding sequence. This shouldn't interfere with *Cas9* transcription but the mRNA should undergo translational arrest until the end of meiosis and start of spermiogenesis (Fig. 1D).

Crossing spermatogenesis specific driver lines to Cas9 responder lines should allow investigation of whether the *β2t E/P* is suitable to drive the *tTA* and whether the later can activate *Cas9* expression. Our attempt to test the expression of *Cas9* during spermatogenesis by means of RT-PCR was not conclusive since we also detected *Cas9* transcripts in the negative, driver-less control (Fig. 2C). It is likely that the RT-PCR is very sensitive and can detect low basal expression from the minimal promoters fused to the *tTA* responsive element *TRE* (Fig. 2C). *In situ* hybridization and antibody staining against *Cas9* mRNA and protein, respectively, will be more informative and can also give information about the stage of

spermatogenesis at which cas9 is transcribed and whether the translational repression element is functional to delay translation of *Cas9* mRNA to post meiotic stages. The basal expression due to leakiness of promoters used might be harmful for the fertility of the males during rearing. Thus, the use of insulators might reduce the likeliness of expression due to position effects caused by nearby enhancers (20–22). However, identification and evaluation of a tight basal promoter, preferably from genes involved in spermatogenesis might provide a better solution.

To prepare all the components necessary for a fully equipped transgenic SIT approach against *D. suzukii*, we started to transfer the female-specific embryonic lethality system as a sexing system to remove females during early embryonic development and thus produce male-only progenies (23–25). The embryonic driver line 06_F5M2 described in Ahmed et al., (26) as well as the embryonic driver line 319_F11F1, which are the first components. Our attempt to generate transgenic effector lines expressing *hid*^{Ala} and having the sex-specifically spliced intron of the gene transformer either from *Ceratitis capitata* HMMA423 with no insulator or *D. suzukii* HMMA108 was not successful. The constructs HMMA108 have the effector gene flanked by *gypsy* insulators, which, however, might not provide enough protection. In *D. melanogaster*, using similar constructs without insulators didn't result in transgenic lines since basal expression of the effector gene from the minimal promoter was considered high enough to induce cell death (27). Only when the HS4 insulator was used, it was possible to generate functional strains (27). In fact, using construct HMMA322 with HS4 insulators, we managed to generate one transgenic line but could not establish a strain, since the transgenic F_1 fly was lost, before we could cross it. Another possible explanation can be that the *P-element* basal promoter is too leaky in *D. suzukii*. With this regard, testing different basal promoters, preferentially endogenous ones, for their suitability is necessary.

Development of a CRISPR/Cas9-induced reproductive sterility system in *D. melanogaster* should provide a first proof of concept and pave the way for the transfer to the destructive fruit pest *D. suzukii*. This system, if successful, would represent a milestone in the fight against the cherry vinegar fly. Ultimately, combining this system with the female specific embryonic lethality system as described in (2) and the sperm-marking system described in Ahmed et al., (26) results in an all in one system to launch an SIT campaign against *D. suzukii*. This system can as well be combined with the aforementioned reproductive sterility system based on targeting PEEL genes to have less chances of resistance against the SIT.

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Figures:

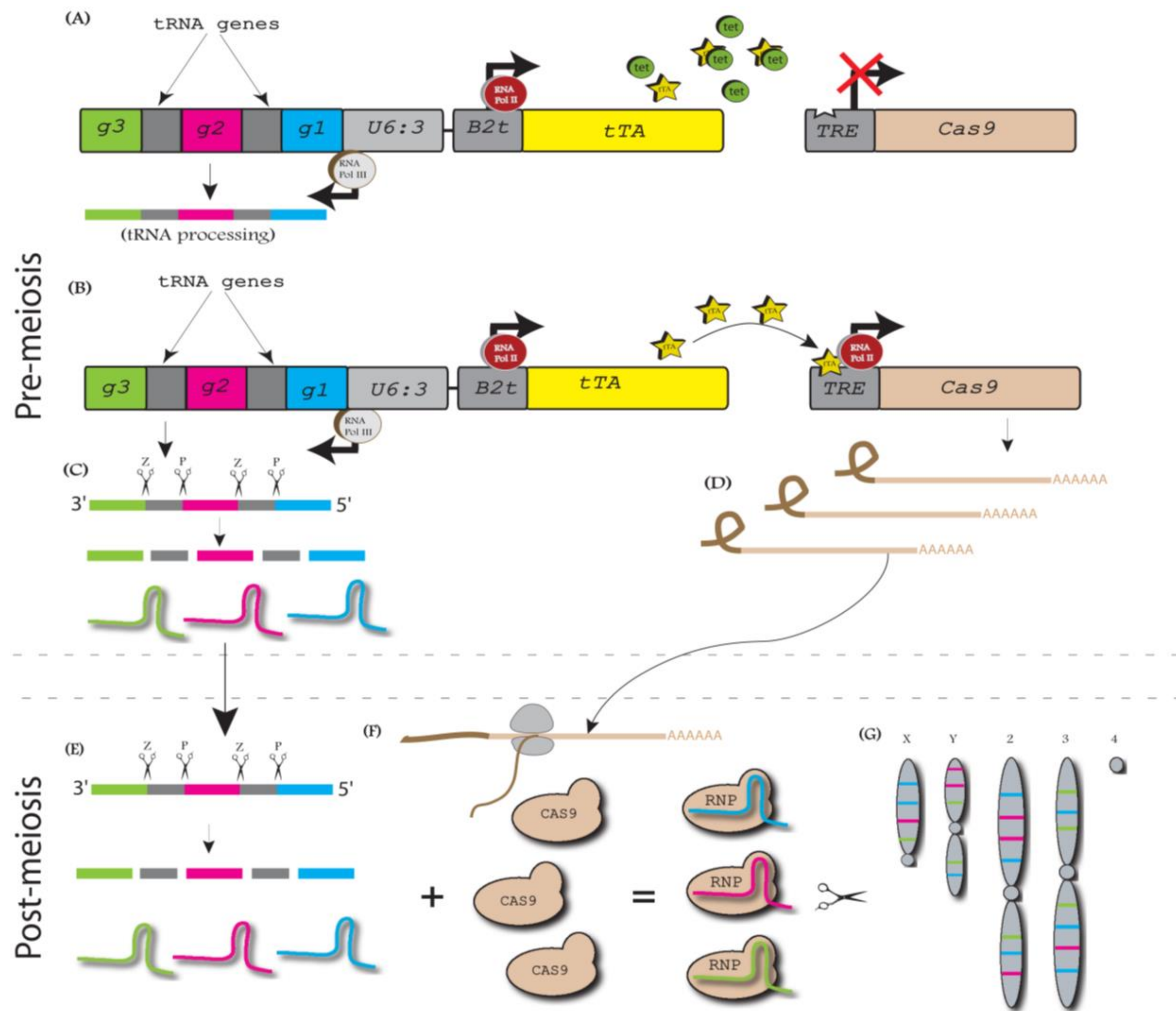

Figure. 1. Schematic illustration of the CRISPR/Cas9-based chromosome shredding system. (**A**) the system consists of a dual gRNA-driver construct and a *Cas9* responder construct controlled by the *tet-off* binary expression system. In the first part, heterologous transcription factor tTA is expressed during spermatogenesis under the control of *E/P* of *D. melanogaster* spermatogenesis-specific gene *β2t* and the *guide RNAs* (*gRNAs*) are constitutively transcribed by RNA polIII from the promoter of *D. melanogaster small nuclear RNA* gene *U6:3*. In the presence of tetracycline in the fly food, the tTA is bound by tet and can not bind to the *tTA responsive element* (*TRE*) and therefore Cas9 is not produced and the system is off. This should be the situation during establishment, maintenance and mass-production of the strain. (**B**) In the absence of tet in the fly food, tTA is free to bind the *TRE* and drive the transcription of *Cas9* during pre-meiosis stages of spermatogenesis. (**D**) However, Cas9 mRNA should undergo translational arrest until post meiosis due to fusion of the translational repression element from *Dm dj* gene upstream of Cas9 coding sequence. (**C**) Three gRNAs are interspaced by tRNA genes which facilitate their processing into individual *gRNA* by the *tRNA* processing endonucleases P and Z. (**E**) during post meiosis *gRNAs* should still be available. (**F**) Cas9 arrest should be relieved by factors available during spermiogenesis and will then be translated into Cas9 protein and complex with the *gRNAs* forming the ribonucleoprotein complex. (**G**) Cas9 loaded with gRNAs should target the chromosomes and induce multiple double strand breaks. This provide many sticky ends that leads to chromosome rearrangements among other aberration leading at the end to embryonic lethality.

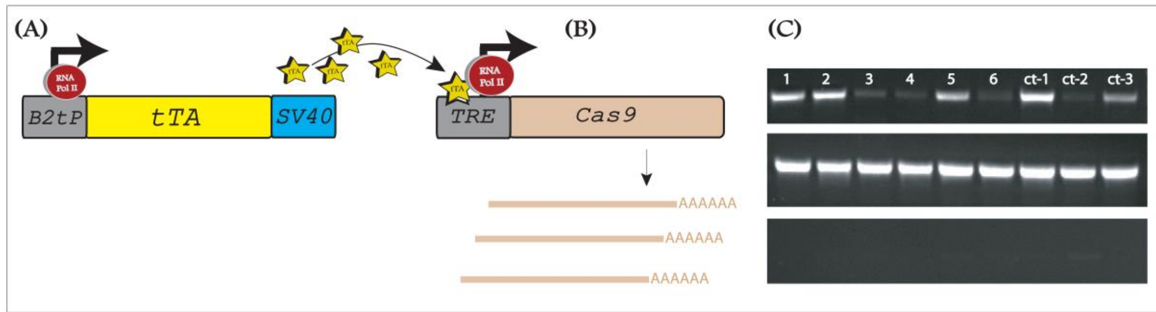

Figure. 2. Cas9 expression during spermatogenesis (**A**) the heterologous transcription factor tetracycline controlled transactivator tTA is driven by the E/P of *D. melanogaster* spermatogenesis specific gene *β2t* gene (**B**) shows the expression of Cas9 under the control of *TRE* directed by spermatogenesis-specific expression of the *tTA*. (**C**) the upper panel of the gel picture shows that Cas9 is expressed in the testes of double heterozygous *D. melanogaster* strain harboring the two components shown in (**A**) and (**B**). each of the chosen three responder lines was crossed individually to two gRNA-driver line. Each two consecutive numbers represent one responder line crossed to driver lines 372_M14_M1 and 372_F26_F1 respectively. The last three lanes are RT-PCR negative control using the respective responder line not crossed to any driver. Ct-1 controls for 1 and 2, ct-2 controls for 3 and 4, and ct3 controls for 5 and 6. Ideally the negative controls should not express Cas9. However, basal promoters can drive expression at a basal level depends on the position of integration. The middle lane is the positive control for the RT-PCR procedure using primer pair to amplify from the same cDNA pool the 3'UTR of the spermatogenesis-specific *β2t* gene. All of the lanes gave strong bands which reflect the reliability of the tests, especially when looking at the result on the light of the lower lane which represent the RT-less PCR following the same procedure and the same amount of the initial RNA but without addition of the reverse transcriptase. Cas9 expression in the negative non-crossed control in the upper doesn't not necessarily means that the responder lines are not functional due to leakiness, but the PCR is highly sensitive and can detect low levels of expression from very few molecules.

Materials and Methods

***Drosophila suzukii* strains**

All fly experiments were performed in our well-equipped safety level one (S1) laboratory, which is certified for generating and using genetically modified insects.

Wild type D. melanogaster Oregon R strain as well as the transgenic flies generated during this study were reared on standard *Drosophila* food and kept at 25°C throughout this study.

Nucleic acid isolation

Genomic DNA was isolated from adult males and females using NucleoSpin® DNA Insect (Macherey-Nagel) according to the manufacturer instructions. Total RNA was isolated from testes of 4-5 days old males using ZR Tissue & Insect RNA MicroPrep (Zymo Research Europe, 79110 Freiburg) according to manufacturer instructions.

PCR amplifications during this study were performed using Phusion DNA polymerase and Phusion-HF buffer (New England Biolabs GmbH, D-65926 Frankfurt am Main). Plasmid isolation and PCR purification were performed using NucleoSpin® Plasmid and NucleoSpin® Gel and PCR Clean-up kits (Macherey-Nagel GmbH & Co., 52355 Dueren, Germany), respectively. NucleoSpin® Plasmid Transfection-grade (Macherey-Nagel) or QIAGEN Plasmid Plus Midi Kit (QIAGEN GmbH, 40724 Hilden, Germany) were used to prepare plasmids for germline transformation.

Identification of abundant CRISPR targets

We have chosen three families of *D. melanogaster* euchromatin transposable elements, *roo*, *jockey* and *1360* on the ground that they have higher chance to be accessed by Cas9. We retrieve the sequence from https://www.fruitfly.org/p_disrupt/TE.html natural transposable elements project. Natural transposable elements dataset Maintained by Michael Ashburner was downloaded and the sequence of the chosen TE was manually retrieved and examined using program version 10.2.6 (Auckland, 1010, New Zealand). The sequences were then blasted in https://flybase.org/ against *D. melanogaster* genome r-6 and the part of the sequence that showed the most coverage possibly over all the chromosomes was used to search for potential CRISPR targets. To identify abundant targets the online tool maintained by Wisconsin university was used. About 300bp of the chosen sequenced was use to find targets with the following parameters: target length 20bp, all CRISPR targets, PAM sequence NGG. The obtained targets were evaluated based on the latest *D. melanogaster* release r-6 and the targets with the most abundant representation but also exact matches were chosen.

Generation of constructs

To generate the spermatogenesis-specific driver construct HMM334, first the 700bp enhancer/promoter region of *Dm-β2t* was PCR amplified from gDNA of *D. melanogaster*

wild type strain OreR using primers pair HM#351/HM#352 and program (98°C 3' 57°C 30'' 73°C 30'')5X followed by 35X of (98°C 30'' 63°C 30'' 72°C 30'') and final elongation of 7 min at 72°C, digested by *Nco*I/*Xba*I and cloned into msf#1215 *Nco*I/*Xba*I sites giving rise to HMMA175. The 450 bp *SV40 3UT'R* was replaced by the shorter version (240bp) which is amplified from HMMA006 using primers pair HM#593/HM#38 with program (98°C 3' 98°C 30'' 59°C 30'' 72°C 30'')5X followed by 35X (98°C 30'' 72°C 1)' with final elongation of 7' at 72°C. The resulting fragment was digested by *BamHI*/*Hind*III and cloned into HMMA175 giving rise to HMMA251. Finally, the *Asc*I fragment *Dm-β2t :tTA:SV40* from HMMA251 was cloned into the *piggyBac* transformation vector HMMA331 (chapter 3.4).

To generate spermatogenesis driver construct HMMA335, first 300bp enhancer/promoter region of *Dm-dj* without the 60bp translation repression element was PCR amplified from gDNA of *D. melanogaster* wild type strain OreR using primers pair HM#582/HM#583 and program 98°C 3' 54°C 30'' 72°C 30'' 5X followed by 35X of 98°C 30'' 63°C 30'' 72°C 30'' and final elongation of 7' at 72°C. digested by *EcoR*I/*Xba*I and cloned into mfs#1215 *EcoR*I/*Xba*I sites giving rise to HMMA248. The 450 bp *SV40 3UT'R* was replaced by the shorter version (240bp) which is amplified from HMMA006 using primers pair HM#593/HM#38 with program 98°C 3' 98°C 30'' 59°C 30'' 72°C 30'' 5X followed by 35X 98°C 30'' 72°C 1' with final elongation of 7' at 72°C. The resulting fragment was digested by *BamHI*/*Hind*III and cloned into HMMA248 giving rise to HMMA252. Finally, the *Asc*I fragment *Dm-dj:tTA:SV40*from HMMA252 was then cloned into the p*iggyBac* transformation vector HMMA331 (chapter 3.4).

To generate the *Cas9* responder construct HMMA203, the *Asc*I fragment from HMMA193 containing *Cas9* fused to *TRE*p and the *attP* site was cloned into the transformation vector mfs 1201 (Scolari et al 2008). HMMA193 was generated by cloning of *Ds-b2t 5'UTR* amplified from gDNA of *D. suzukii* Italian strain using primer pair HM#36/HM#401and cloned into *Cla*I site of HMMA076 which was generated by cloning of the *attP-TREp* fragment amplified from mfs1262 into *EcoR*I/*Cla*I site of HMMA056 replacing *Ds-hsp70* promoter (26).

To generate *Cas9* responder construct HMMA356 the *Dm Dj* translation suppression element was PCR amplified from gDNA of OreR strain of *D. melanogaster* using primer pair HM#691/HM#692 and cloned into *Bbs*I site of HMMA327 (described in chapter 3.5) HMMA366 was generated by cloning of two pairs of annealed oligos HM#694/HM#695 and HM#692/HM#693 to generate the basal promoter and translation repression element of *Dm dj* gene respectively into the *Bbs*I/*Kpn*I sites of HMMA327.

To generate plasmid HMMA446 to express *gRNAs* against *D. melanogaster* transposable element *roo,* the annealed oligos HM#830/HM#831, HM#832/HM#833 and HM#834/HM#835 were cloned into plasmid HMMA349 into *Bbs*I, *Bsa*I and *Sap*I sites respectively.

To generate HMMA447 to express *gRNAs* against *D. melanogaster* TE *jockey*, the annealed oligos HM#836/HM#837, HM#838/HM#839 and HM#840/HM#841 were cloned into plasmid HMMA349 into *Bbs*I, *Bsa*I and *Sap*I sites respectively.
To generate HMMA448 to express *gRNAs* against *D. melanogaster* TE *1360*, the annealed oligos HM#842/HM#843, HM#844/HM#845 and HM#846/HM#847 were cloned into plasmid HMMA349 into *Bbs*I, *Bsa*I and *Sap*I sites respectively.
To generate HMMA449 to express g*RNAs* against *D. melanogaster* TEs *jockey*, *1360* and *roo* at the same time, the annealed oligos HM#836/HM#837, HM#844/HM#845 and HM#834/HM#835 were cloned into plasmid HMMA349 into *Bbs*I, *Bsa*I and *Sap*I sites respectively
The total number of genomic targets for construct HMMA448 with three *gRNAs* against 1360 are 874, for construct HMMA447 targeting *jockey* are 212, and for HMMA446 targeting *roo* are 379. The combination of *1360_gRNA2*, *roo_gRNA26*, and *jockey_gRNA1* together in construct HMMA449 should target and induce 659 DSBs
All final construct generated during this study are shown in Table S3.

Germline transformation

To generate transgenic driver and Cas9 responder lines, piggyBac germline transformation was used. Microinjection of transformation and helper vector at a final concentration of 500 and 300 ng/µL respectively.

All injections for *piggyBac* germline transformation were performed using the hyperactive helper plasmid MK006 along with the respective transformation vector at a final concentration of a 500 ng/µL and 300 ng/µL respectively. Each G_0 fly was crossed individually to three wildtype flies of the opposite sex.

The F_1 transgenic flies were identified by presence of the respective fluorescent body marker. Screening was performed using Leica M205 FA fluorescence stereomicroscope equipped with camera Q imaging Micropublisher 5.0 RTV (Leica Mikrosysteme Vertrieb Gmb, Wetzlar, 35578 Germany). Transgenic flies were screened using filter sets RFP (excitation: ET546/10x, emission: ET605/70m) or GFP-LP (excitation: ET480/40, emission: ET510 LP). F_1 transgenic flies were outcrossed to WT flies of the opposite sex.

RT-PCR

To investigate whether Cas9 expression can be steered by the use of the tet-off binary system, each of the three randomly selected Cas9 responder lines was crossed to different driver lines. Transcription of Cas9 in the testes was detected by RT-PCR. Total RNA, was isolated from testes of 15 individual 4-5 days old adult carrying the driver and responder construct in a heterozygous situation. Dissection was performed in ice cold PBS 1X and testes were transferred to bashing tube with beads and fixed on a vortex and homogenized for .10 minutes.

RNA was isolated using ZR Tissue & Insect RNA MicroPrep (Zymo research) according to the manufacturer instructions. Genomic DNA carryover was removed by in-column digestion using turbo-DNase I for 30 minutes at 30°C.

Second DNase treatment was performed using dsDNase provided with the kit cDNA synthesis kit, first strand cDNA synthesis was done using Maxima First Strand cDNA Synthesis Kit for RT-qPCR, with dsDNase (thermofisher). 1µg RNA was used to make first strand cDNA and 2µL of the cDNA was used for each PCR reaction n a total volume of 25µL. to check the expression of cas9, primer pair HM#619/HM#135 were used to amplify 460 bp of Cas9. As a negative control the respective Cas9 responder not crossed to driver were used. As a positive control, a pair of primers (HM#706/HM#707) that amplify the 3'UTR of *β2t* was used which give 250bp. The PCR was performed using 2µL cDNA, 1.25µL forward and 1.25 µl reverse primer (10µM), 2µL dNTPs mix and Phusion polymerase in a total reaction of 25µL

15µL of the PCR product was run in 1.5% agarose gel and documented using UV lamp and a camera attached to a printer.

Table S1. Primers sequences

Code	Name	Sequence 5'–3'
HM#36	B2t_XbaI_R3	CGATTCTAGACATCTTAACCGACTGTCAAGGATC
HM#38	Hma-45R	TAAGAAGCTTGATACATTGATGAGTTTGGACAAACCAC
HM#135	cas9-R	GTAGATGGTGGGGTACTTCTCGTGG
HM#351	Dm_b2t_F	TACCCATGGATTGTAGGAGCCAGAGCCAATGGATC
HM#352	XbaI_Dm_B2tR	TAATCTAGACATTTTGATAGTAAAGTTAGGGCC
HM#401	ClaI_Dsb2t_F	CCTCATCGATAGTCCACCCTAGTATCAGCTAGCAAGC
HM#582	HM_EcoRI_DmDj_F	GCCGAATTCCCTTTAAATATTCTAGTAAAATTCTTTAAG
HM#583	HM_XbaI_DmDj_R	TAATCTAGACATAAGAATTTTGAAAAAACCACAGC
HM#593	HM_BamHI_SV40_F	CTAGGATCCGCGGCCGCGACAGATCATAATCAGCCATAC
HM#619	HM_Cas9_ATG_F	GACGATGACGATAAGGCCCCAAAGAAGAAGCGGAAGGTC
HM#691	HM_DJTSE_F	CCATGCTGTGGTTTTTTCAAAATTCTTTGTAAAACTTTTGGTACAA AATTTAAAAATTTTTCTC
HM#692	HM_DJTSE_R	ATATGAGAAAAATTTTTAAATTTTGTACCAAAAGTTTTACAAAGAAT TTTGAAAAAACCACAGC
HM#693	HM_DJTSE_F2	GATCGCTGTGGTTTTTTCAAAATTCTTTGTAAAACTTTTGGTACAA AATTTAAAAATTTTTCTC
HM#694	HM_DJ_Pro_F	CTAAACTTGTATAGTTTTGGGGGCAGGTTA
HM#695	HM_DJ_Pro_R	GATCTAACCTGCCCCCAAAACTATACAAGTTTAGGTAC
HM#706	HM_b2t_3UTR_F	CGAGGATCCTAGGATTAACTTCCCACTCAAGATCACACATG
HM#707	HM_b2t_3UTR_R	GCCAAGCTTGTCTGCTTATAAATCAACATTTATTCGTAACCC

Table S2. Guide RNAs sequences

Code	Name	Sequence 5'–3'
HM#830	HM_roo_g2_F	TCCAAGGATGGTTGGCACCAGTCA
HM#831	HM_roo_g2_R	AAACTGACTGGTGCCAACCATCCT
HM#832	HM_roo_g12_F	AACATTAACCACTGTGGAGGACAC
HM#833	HM_roo_g12_R	AAACGTGTCCTCCACAGTGGTTAA
HM#834	HM_roo_g26_F	GCATGCAATATCTACCAGAACCC
HM#835	HM_roo_g26_R	AACGGGTTCTGGTAGATATTGCA
HM#836	HM_jockey_g1_F	TCCAGGTTAGGGAGGTCATGAGGG
HM#837	HM_jockey_g1_R	AAACCCCTCATGACCTCCCTAACC
HM#838	HM_jockey_g2_F	AACACCCTCATGACCTCCCTAACC
HM#839	HM_jockey_g2_R	AAACGGTTAGGGAGGTCATGAGGG
HM#840	HM_jockey_g10_F	GCATCAACGCACTGTTACCCATG
HM#841	HM_jockey_g10_R	AACCATGGGTAACAGTGCGTTGA
HM#842	HM_1360_g1_F	TCCAATGTTCTCAGCGTGAGCGAG
HM#843	HM_1360_g1_R	AAACCTCGCTCACGCTGAGAACAT
HM#844	HM_1360_g2_F	AACAGTGGCTCTAGAGGTGGCTCC
HM#845	HM_1360_g2_R	AAACGGAGCCACCTCTAGAGCCAC
HM#846	HM_1360_g11_F	GCAATATCTTGAGGCACGAAGTG
HM#847	HM_1360_g11_R	AACCACTTCGTGCCTCAAGATAT

Table S3. List of vectors

Code	**components of the construct**
HMMA334	pBXLII{*attP220_β2tE/P:tTA:SV40_PUb:DsRed:SV40_attP220*}
HMMA345	pBXLII{*attP220_Dj:tTA:SV40_PUb:DsRed:SV40_attP220*}
HMMA446	pSL{*af_U6:3:roo2:12:26_β2t:tTA:P2A:tGFP:5UTR_af*}
HMMA447	pSL{*af_U6:3:jockey1:2:10_β2t:tTA:P2A:tGFP:5UTR_af*}
HMMA448	pSL{*af_U6:3:1360g1:2:11_ β2t:tTA:P2A:tGFP:5UTR_af*}
HMMA449	pSL{*af_U6:3:jocky1:1360g2:roo26_β2t:tTA:P2A:tGFP:5UTR_af*}
HMMA203	pBac{*attP_TREpβ2t5UTR:Cas9_PUb:nEGFP:SV40*}
HMMA366	pBXLII{*attP220_TREp:DjTSE:Cas9_PUb:EGFP:SV40_attP220*}
HMMA365	pBXLII{*attP220_TREDjP:DjTSE:Cas9_PUb:EGFP:SV40_attP220*}

4 Discussion

4.1 Gene drive

Homing-based gene drive refers to selfish genetic elements that have the ability to target the sister chromosome that lacks them and allow the cell repair mechanism to copy them by homology directed repair in a process called homing (145). Such selfish genetic elements are either natural or synthetically engineered, do not obey the Mendelian law of inheritance, and are therefore passed on to the off-spring more often than can be explained by Mendelian inheritance. Homing endonuclease genes (HEG) are an example of naturally occurring selfish genetic elements that have been proposed for engineering of wild populations of diseases vectors more than 15 years ago (145). The hope was to use them to introduce or delete genes to affect the fertility or to impair the ability to transmit diseases, leading to population suppression or replacement, respectively. Unfortunately, it was not possible to engineer HEG to target sequences other than their natural targets and therefore it was not possible to use them for a custom-made gene drive. The HEG *I-PpoI* is by chance highly specific to clusters of rDNA that reside in the X chromosome of the malaria vector *Anopheles gambiae* (145,149,150). It therefore offered a good model to test the feasibility of using HEG to engineer a meiotic sex bias gene drive by targeting and destroying the X chromosome during spermatogenesis and thereby bias the sex ratio toward males (150,151). Use of ZFNs and TALENs to engineer gene drive was not successful due to the repetitive nature of the sequence coding for the endonuclease and therefore instability during homing (152). Only after the recent genome editing revolution exploded by the discovery of the potential of using an RNA-guided programable endonuclease to manipulate the genomes of organisms, have scientists had the chance to custom-make and explore the possibilities of gene drive (138,139,153,154). Despite the fact that they may have unpredicted even catastrophic ecological implication, CRISPR/Cas9-based gene drives hold big promises for ecological engineering including conservation of biodiversity, control of invasive species, and of course in pest and vector control (155).

Several synthetic gene drives based on CRISPR/Cas9 system were designed and tested for their functionality. They fall in one of two general groups: suppression gene drive, intended to decrease the population of the target insect and maybe eventually eradicate it completely from the eco-system, or replacement gene drive to render a population unable to transmit a certain disease (156). For the suppression drives, eradication might sound great but it can have unforeseen ecological consequences, especially when targeting endogenous species that are part of the ecological balance (157). In this case, if the gene drive was very efficient to the point of eradication of a species, a vacuum in the system would be created that would have to be filled again. As a consequence, another species that was not a major vector might fill this

gap and our fight might have to start again from scratch. This is obvious already with insecticides and the emergence of new major pests. However, when targeting invasive species that are still not established in the new ecosystem, this species can be eradicated without serious consequences. And here it should be emphasized that what is meant by eradication is a localized and not global eradication

The second group of gene drives, the replacement drives, was initially proposed to replace populations of diseases' vectors by introducing into the population a gene drive that targets genes necessary for pathogen transmission to reduce or completely abolish their ability to transmit that disease (145). In this case, if the drive does not impose a fitness cost, it should reach fixation in the target population over many generations (145). Replacement gene drives have also been proposed to reverse insecticide resistance by including within the drive cassette the wild type version of the gene that renders the insect susceptible for a give insecticide or groups of insecticides with the same mode of action. For example, point mutations in the voltage-gated sodium channel gene render insects resistant to pyrethroid insecticides due to target site insensitivity (158). This mutation is referred to as knockdown resistance (*kdr*) (159) and can be reversed by including within the cassette a wild type version of the gene to restore the sensitivity to pyrethroids. Some scientists have even thought about using gene drive to restore susceptibility of weeds to herbicides (160), however, due to cross pollination, the susceptibility could be introgressed into crop plants and as a result the respective group of herbicides could not be used any longer.

To investigate the molecular events and the dynamic of a CRISPR/Cas9-based gene drives we built a sex conversion suppression gene drive targeting *D. melanogaster* female sex determination gene *transformer* (*tra*) (161). The main finding was that CRISPR/Cas9-based gene drive creates a hotspot for resistance evolution against the drive itself. Induction of DSB is catastrophic for the cell and has to be immediately repaired. The cell repairs such damage by either of two major pathways, the error prone NHEJ or the more precise HDR (162). When targeting an important gene such as *tra*, which when knocked out in *Drosophila* leads to sterile intersexes, the drive exerts high fitness cost and therefore evolution favours selection for resistant alleles. We observed resistance at the very first generation and after 15 generation the resistance reached fixation in the laboratory population of *Drosophila*. One possible solution to overcome rapid emergence of resistance against gene drive is by using multiple gRNAs. It is very unlikely that several DSBs in the same gene be repaired in a way that keep a functional gene. However, this is not the only way resistance can evolve. Failure to copy the whole drive cassette during homing is another source for emergence of resistance that we have observed. It can also appear as a result of a mutation in the Cas9 or the gRNA cassette that leads to loss of activity.

Such sex conversion suppression gene drives could be used in the Tephritid fruit flies, where targeting of *tra* leads to fertile XX males (163). Over the time this should lead to population collapse due to lack of males. Despite all the promises, gene drives with the current designs do not seem to fulfil the requirements to be implemented into operational vector and pest control programs, yet. Several issues need to be addressed such as resistance development, unintended migration of insects carrying drive elements into neighbouring populations, the need to release vast numbers of insects carrying the drive element particularly in the case of threshold-dependent gene drive (164). In addition, and despite several new designs that have taken into consideration gene drive containment, reliable methods for reversal of the drive, in case of unexpected outcomes are still needed.

4.2 The tools of the trade

Recent advancement in insect genome manipulation tools offers the opportunity for development of transgene-based insect pest control strategies such as gene drive mentioned in 4.1 above as well as improvements to the SIT, which will be discussed in 4.3 below. The use of transposon-based germline transformation has so far been the main tool in insect genetic engineering, whether in basic research or applied biotechnology. Different transposon systems were used to introduce transgenes into the genome of insects including the *P-element* (68), *Tc1/mariner*, *Minos* (165), *Hermes* (166), and *piggyBac* (84). Moreover, such vectors were also intensively used for functional genetic screens by insertional mutagenesis, enhancer and gene traps (74,167). The most commonly used vectors so far are based on the lepidopteran transposon *piggyBac*, which has been shown to function in many different species. The generation of a mutant hyperactive version of the *piggyBac* transposase with the optimized codons for mammalian cells (85), inspired entomologists to test it in different insects (168). Improvement in *piggyBac* germline transformation by the use of the hyperactive transposase is a major contribution to researchers in the field of insect biotechnology, especially those working with non-model insects such as agricultural pests or diseases vectors. Using classical transposase, researchers had to spend time and resources with less success to obtain transgenic lines or with low efficiency. We compared the efficiency of three versions of the *piggyBac* transposases, the same person injected the same construct in the same strains of *D. melanogaster,* the flour beetle *Tribolium castaneum*, and the Medfly *C. capitata* and we observed significant increase in transgenesis compared to the wildtype version. This is particularly important in the case of Medfly where improvements in the SIT are desired. Importantly, this increase in efficiency is not accompanied by multiple integrations of the vector. Our results of using the hyperactive transposase disagree with previous work (168), which concluded that this transposase not only does not increase the efficiency of germline transformation, but increases the rate of sterility among injected G_0 flies. In the case of the invasive fruit pest *D. suzukii, piggyBac* germline transformation showed varying efficiency based on published studies (169) and our own experience. This variation can be attributed to

differences in the genetic background of the different strains used. We compared three WT strains of *D. suzukii* derived from different geographical areas namely, USA, France, and Italy. The results gave a clear indication that the strain AM derived from the French Alps (170) is more suitable for genetic manipulation using *piggyBac* vectors compared to the other two strains.

In some cases, especially when different enhancers, or different systems are to be tested and compared, the random transposon-based germline transformation is not suitable. Fortunately, the toolbox for insect genetic manipulation offers as an alternative, the use of site-specific recombinases such as Flp/*FRT*, Cre/*Lox* and the φC31 integrase system (86). Unlike the use of transposon-based vectors, site specific recombinases (SSR) lead as the name suggests to integration of the transgene in a pre-defined genomic site. One successfully SSR that has been used in insect biotechnology is derived from the bacteriophage φC31 which mediates recombination between two non-identical recognition sequences (98). We have established this system for the invasive fruit pest *D. suzukii* as a way to introduce new transgenes into an established transgenic line that harbours one *attP* recombination site. This should allow for modification of characterized functional transgenic lines for example by integrating sperm-marking transgenes into the embryonic driver lines 06_F5M2 (171) and crossing the combined line to a sexing responder line will allow establishment of a transgenic sexing system and marking system in one strain. Another important application of this system - particularly for transgenic strains to be used in operational SIT - is the stabilization of transgenes. This has already been demonstrated in the Medfly and the vinegar fly by removal of one of the TIRs necessary for transposition of the vector in question (172,173). It is also possible - once a good genomic locus is identified and providing that the transgene contains at least one *attP* site - to use the φC31 system to integrate a new transgene and in a second step to remove the old one along with either of the two ITRs and thus end up with only the new transgene, which is at the same time stabilized. In fact, with the use of the programable genome editing system CRISPR/Cas9 already established for many insect pests including *D. suzukii* (171) and diseases vectors (174), it is possible to introduce by HDR an *attP* site in a transgenic line that doesn't have it but has been identified to be at a suitable genomic site. This approach however, leads to the integration of the whole plasmid including the antibiotic resistance gene and therefore always requires a second step for removal of the plasmid backbone.

Another way of using site-specific recombinases is cassette exchange in which reciprocal recombination happens between two sites integrated in the genome by transgenesis and two sites in the donor plasmid flanking the transgene of interest (172). In the case of φC31 RMCE, reciprocal recombination takes place between two inverted *attP* sites in the genome, ideally flanking a marker gene and two inverted *attB* sites in a donor plasmid flanking the transgene of interest plus a different marker in the presence of φC31integrase. The advantage of this system over integration in one site is that, only the desired construct is integrated. We

generated a set of *piggyBac* vectors suitable for generation of docking lines, and successfully managed to establish the φC31 RMCE (chapter 3.4). We furthermore developed self-docking lines that expresses φC31 from the *E/P* of the maternal effect gene *nanos* (chapter 3.4). Those lines can be evaluated and categorized according to their suitability to allow expression of transgenes at different stages and in different tissues. That will then reduce the efforts required to test different system by choosing the right set of docking lines according to when and in which tissue the transgene should be expressed. One important advantage of using the φC31 system is the possibility of integration of large genetic constructs which is not the case with transposon-based transformation vectors.

The recent revolution in genome editing ignited by the discovery that the bacterial adaptive immune system CRISPR/Cas can be used to manipulate genes has led to adoption of the system to many insect pests and diseases vectors in the hope to use it for engineering biotechnological pest control strategies. Previous studies in the invasive pest *D. suzukii* used either plasmids derived from *D. melanogaster* to drive expression of *Cas9* and *gRNA* or they used Cas9 protein along with *in vitro* transcribed *gRNA* (73,170,175,176). To use the CRISPR/Cas9 to develop transgenic strains for pest control application, it is important to identify and use endogenous regulatory elements as they are supposed to drive a more reliable expression compared to exogenous ones. In this respect, our work presents a new set of regulatory elements that can be used in genome editing or development of pest control strategies based on CRISPR/Cas9.

One Possible application of the CRISPR/Cas9 system is the design of synthetic gene drive systems (chapter 3.1). However, it can also be used in SIT context to generate reproductive sterility systems (chapter 3.5 -3.7) or sexing systems (chapter 4.3.2).

4.3 Biotechnological improvements of the SIT

The SIT as defined by the International Plant Protection Convention 'is a method of pest control using area-wide inundative releases of sterile insects to reduce fertility of a field population of the same species (39). It involves mass rearing of the target pest, removal of females by any of different strategies, sterilization using gamma radiation and inundative successive release to suppress wild populations of the pest. It is obvious that SIT is intended for the control of sexually reproducing insects, however, there are other questions to be answered before taking a decision of using the SIT against a particular pest or disease vector. The question of sexing or precisely the removal of females is of paramount importance. This applies particularly to female mosquitoes which even if sterile can still bite and transmit diseases, and fruit flies such as *C. capitata* that use their ovipositor to sting the fruits. This constrain can be alleviated by the development of a method for sex separation to allow removal of the females before release of the males, which we will discussed in section 4.3.1 below.

Does the insect in question tolerate high doses of radiation to render them sterile without strong effect in their competence? If radiation affects the ability of sterile males to search, find, and compete with wild type males in mating with wild type females and or the ability of the sperm to compete with the wild type sperm in fertilizing the egg, then it is imperative to find different strategy to induce reproductive sterility. Here, biotechnological approaches offer alternatives to radiation to induce sterility that ensure production of competent males, which I will discuss in section 4.3.2 below. Another aspect of the SIT that can be biotechnologically improved is the marking system, which facilitates monitoring of the released males (section 4.3.3). This has been traditionally achieved by the use of some kind of fluorescent dust.

4.3.1 Sexing systems

One of the most critical steps in the establishment of SIT for a particular insect pest or disease vector is to develop a method for sex separation. Ideally the system should act as early as possible to allow removal of females during embryonic development and thereby reduce the cost incurred and space required for raising double the number of insects, when only the males are the actual sex to be released. Historically different approaches were exploited to sex insects including temperature sensitive lethal phenotypes induced by chemical and radiation mutagenesis, as well as physiological and morphological characteristics. These approaches, however, lack universality and are either species specific (pupal size difference and time of eclosion) or requires to be generated *de novo* for each species (*tsl*), which demands decades of efforts and resources.

In this regard genetic engineering offers the tools necessary for the development of more generic approaches for sexing. For example, a transgene-based female specific embryonic lethality system (FSEL) has been developed (50–52) based on the knowledge about insect sex determination which mainly relies on sex-specific splicing. The requirements of this system are enhancer/promoters (*E/P*) of a gene active during early embryonic development, the *tet-off* binary expression system, a pro-apoptotic gene, and a female specifically spliced intron. Those components together allow the conditional activation of transcription of the proapoptotic effector gene at embryonic stages and the correct splicing only in female embryos and as a consequence female lethality before hatching. Being generic, this system has thus far been transferred to several agricultural pests. Such transgenic sexing system namely strain #32 developed for the Medfly *C. capitata* (51) has shown comparable performance to the famous genetic sexing strain Vienna8 (49). However, the transgenic strain should be more competent since it has aside from the transgene insertion a clean wild type background compared to the genetic sexing strain generated by random chemical or radiation mutagenesis and translocations causing partial aneuploidy in some progeny

We have prepared the first components to transfer such a FSEL system to the invasive fruit pest *D. suzukii*. We developed two transgenic embryonic driver lines using the *E/P* of an early

embryonic gene to drive the expression of the heterologous transcription factor *tTA*. Those lines can be crossed to a *hid* sexing responder line to generate a female-specific embryonic lethality system for removal of females.

As mentioned earlier, the genetic sexing strain Vienna 8 is the best strain that is currently used in operational area wide SIT. If the molecular basis underlying the *tsl* and white pupa phenotypes are resolved and providing orthologues of those genes are found in other pests that are suitable for SIT, it should be possible using the CRISPR/Cas9 system to engineer such mutations and translocate rescue alleles to the male-specifying chromosome. However, based on published *tsl* alleles in *D. melanogaster* (177), it should be possible to engineer these mutations and test them at suitable permissive and non-permissive temperatures. This approach has already been used to engineer a temperature-sensitive allele in the *D. suzukii transformer-2* gene, which at a non-permissive temperature of 29°C leads to conversion of females to infertile intersexes (73). This mutation, if engineered in Tephritid flies, should lead to generation of an elegant sexing system in which the XX female are converted to fertile XX males at the non-permissive temperature, and as a consequence, reduction of the production cost since all the produced biomass is eventually used for release. However, special emphasis should be put on the temperature at which the sex-conversion takes place.

In fact, *tsl* alleles can be engineered *de novo* based on the amino acid sequence (178). One approach to achieve that is by targeting predicted buried amino acids in the protein of interest and confirm the burial by replacement of that aa residue with an Asp residue, which is known to inactivate the protein. Then three to four buried aa residues should be replaced for Lys, Ser, Ala, and Trp (178). Once a functional *tsl* is generated, a wild type rescue allele of the gene should be transferred to the Y chromosome or its equivalent using CRISPR/Cas9.

4.3.2 Reproductive sterility

The beating heart of the SIT is sterilization. If no suitable method to induce reproductive sterility for the target insect is identified, SIT cannot be used. Historically it has been achieved using ionizing radiation (38,56). This has advantages and disadvantages. Among the advantages are the redundancy of the cause of sterility, which is random chromosomal breaks that leaves almost no chance for the development of resistance against SIT. On the other hand, ionizing radiation hits not only the sperm cells but also the somatic cells and therefore adversely affects the fitness of the sterile males to be released (179). Another disadvantage is that not all insects can be made 100% sterile by radiation, especially lepidopteran pest harbouring holocentric chromosomes (180). Besides, working with radiation can lead to serious health problems. To overcome these disadvantages, scientists exploited molecular biology tools to engineer reproductive sterility systems such as the RIDL based on a tTA positive feed-back loop (61) and transgene-based conditional embryonic lethality systems (57,58), which neither affects the competence of the sterile males nor does it pose health problems to the workers. If a

combination of FSEL (50–52) with a transgenic reproductive sterility system is to be generated, then a new system for reproductive sterility has to be developed since the conditional embryonic lethality and the FSEL cannot be combined, when based on the same lethality principle. In this regard we proposed the development of a new reproductive sterility system by destroying the function of a specific paternal effect gene (*snky*) (chapter 3.5). This gene when knocked-out or knocked-down should lead to embryonic lethality due to failure of sperm plasma membrane breakdown and as a consequence failure of preparation of functional male pronucleus (181,182). This system is superior compared to RIDL in that the embryonic development does not continue and larvae are not produced, which represent the most destructive stage in agricultural pests. In the so far published RIDL, the lethality takes place only at the larval or even pupal stages. We proposed two different mechanisms, CRISPR/Cas9 knockout with designed gRNAs to target the gene at a time during spermatogenesis, or RNA interference using a construct that expresses two s*hRNA*s against the gene. These two mechanisms, if combined together in one transgene, should provide a tight reproductive sterility system that should not allow any escapers. Eventually, this reproductive sterility system should be combined with a sexing system (FSEL) (50–52) as well as a marking system (sperm-marking) (62–65) and thereby provide an all in one SIT system for control of the invasive fruit pest *D. suzukii*. This system should be transferable to other insects especially that the gene *snky* is conserved even in mammals. However, with the genome of many of the major agricultural pest and disease vectors sequenced, it is possible to search for the orthologs in the genome of the target insect. Furthermore, knowledge about the reproductive biology and fertilization is necessary to develop such a system.

Another approach to the generation of a reproductive sterility system is by using CRISPR/Cas9 to induce multiple double strand breaks (DSBs) in the chromosomes of the target insect during spermatogenesis by targeting repetitive sequences such as transposable elements among others as described in (chapter 3.6 and 3.7). The requirements to develop such a system are, *E/P* of spermatogenesis-specific gene to drive the expression of *tTA*, Cas9 under the control of *TRE* and a *gRNA* cassette driving the expression of multiple *gRNAs*. To realize such a system in *D. melanogaster*, we have chosen euchromatin TEs namely (*roo, jockey* and *1360* families) as our targets to affect chromosomal breakage. To allow normal sperm production before chromosome shredding by Cas9 we exploited the translational repression element of the *D. melanogaster dj* gene (183,184) to delay the translation of Cas9 mRNA. As for many other genes involved in late spermatogenesis or spermiogenesis, the transcription takes place before the maturation of the spermatocyte at which transcription cessation happens and their mRNA remains arrested (by the action of a translation arrest element mainly residing at the 5'UTR of the genes) until post meiosis.

This approach for inducing reproductive sterility should overcome the deleterious effect of radiation on male fitness. However, the end result should be the same, embryonic aneuploidy

leading to abortion of embryonic development and thus lethality before hatching. Another important point to mention is that ionizing radiation makes the sterile males disposable, once they are empty of sperm produced before pupal exposure to radiation they are useless, since radiation destroys all spermatogonia. Our proposed system, however, ensures continuous production of sperm since the stage of action is post meiosis and therefore spermatogonia continue to form and differentiate. Therefore, as long as the released males are alive, they should potentially be able to produce sperm (with chromosomal breaks). This system should be easily developed for other insects of agricultural and health relevance since repetitive elements including transposable elements form the bulk of eukaryotic genomes. With next generation sequencing becoming cheaper and the genomes of many insect pests and diseases vectors have already been sequenced, it takes some bioinformatics analysis of the sequences to identify suitable abundant targets. This has already been demonstrated in the major malaria vector *Anopheles gambiae* where bioinformatics tools were used to identify abundant targets on the X chromosome and used CRISPR/Cas9 during spermatogenesis to develop X shredder leading to sex ratio distortion (141,185). The redkmer pipeline for identification of abundant and X -specific CRISPR targets can in principle be used to identify abundant targets that are distributed genome-wide (185). Therefore, the proposed chromosome shredding system offers an alternative to ionizing radiation and should have broad applicability in the establishment of the SIT for the control of many insects.

4.3.3 Marking

The sterile males (precisely the pupae) to be release are first dusted with some sort of fluorescent powder to facilitate monitoring and evaluating the success of the control program. (186,187) This is, however, not ideal, since the dye is on the surface and might be washed off by rain, or transferred to wild type males when insects come into contact and also presents health hazards for the facility workers. Again, biotechnology came with an elegant solution that has many applications. The sperm-marking present another biotechnological improvement in the SIT. The idea is to use the *E/P* of a spermatogenesis-specific gene to drive the expression of a fluorescent protein such as EGFP and DsRed (62–65). Such systems have already been established for some insects by using the *E/P* of the spermatogenesis specific gene *β2t* (62–65). This allows monitoring of the competence of sterile males and their success for mating with wild type females simply by capturing random females from the wild, open the sperm storage organ (spermatheca), and examine it for the presence of marked sperm. This is more accurate and informative than the original dusting approach in practice. It also helps to understand the reproductive biology of the insect to be able to develop more specific pest control strategies. For this purpose, we isolated the spermatogenesis specific gene *β2t* of the fruit pest *D. suzkii* and used its *E/P* to generate a sperm-marking strain that can be used in the SIT against the fly. This strain can also help us to understand more about the reproductive biology, mating behaviour, and post mating response. The system can also be combined with

other biotechnological improvements such as the sexing system to build strains more suitable for operational SIT. The gene *β2t* is spermatogenesis-specific and highly conserved and therefore its *E/P* presents a good candidate for a generic sperm-marking system based on the isolation of the endogenous *E/P* in the respective species.

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6 Curriculum Vitae

M.Sc. Genetics & Molecular Biology
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Education

01/10/2014–20.12.2019 — **Dr. rer.nat. ''Biology''** Georg-August-Universität Göttingen, Göttingen (Germany). Topic: Development of Transgenic Sterile Insect Technique Strains for the Invasive Fruit pest *Drosophila suzukii*.

2008–2011 — **M.Sc. Genetics & Molecular Biology**, Department of Zoology - University of Khartoum, Khartoum (Sudan).
Compulsary: Genetics, Molecular Biology, Biochemistry, Physiology, Bacteriology, Virology, Parasitology, Statistics, Techniques and Instrumentations. Elective: Human Immunology, Human Genetics and Human infectious diseases

2000–2005 — **B.Sc. (Honours) Agric. Entomology "First Class"**, Faculty of Agriculture - University of Khartoum, Khartoum North (Sudan) Compulsary: five semesters covering all aspects of biological sciences, chemistry, physical chemistry, biochemistry, physics, Mathematics, English, Arabic.
Elective: five semesters covering the field of Entomology, pest control, plant pathology, and pesticide science

1999- 2000 — Sudan high school certificate, choice "Biology" grade 83.4%

Additional skills:

Languages

- Arabic (mother tongue)
- English second language (fluent)
- German third language (fairly good)

Computer skills

- Microsoft package
- Basic linux
- Adobe Ilustrator

Awards & Grants

- Khartoum University prize for the best performace in Crop protection
- DAAD scholarship for PhD studies

Conferences & Meetings

- The 1st FAO/IAEA Research Coordination Meeting (RCM) of the Coordinated Research Project (CRP) on "Generic approach for the development of genetic sexing strains for SIT applications" 7-11 October 2019
- Third FAO/IAEA Research Coordination Meeting (RCM) on "Comparing Rearing Efficiency and Competitiveness of Sterile Male Strains Produced by Genetic, Transgenic or Symbiont-based Technologies" Bangkok, Thailand 18-22 June 2018
- Göttingen Biotech Symposium, German Primate Center DPZ, Göttingen, Germany, 26 September 2017 ''Horizons in Molecular Biology Symposium. Max Plank Institute for biophysical Chemistry, Göttingen, Germany, 11-14 September 2017
- Third FAO–IAEA International Conference on Area-wide Management of Insect Pests: Integrating the Sterile Insect and Related Nuclear and Other Techniques. Vienna, Austria 22–26 May 2017

Courses

- RNAseq Data Analysis, organized by The Transcriptome and Genome Analysis Laboratory TAL and the Medical Biometry and Statistical Bioinformatics, Institut für Medizinische Statistik, Universitätsmedizin Göttingen, Göttingen, Germany, 14-15 September 2017
- EMBO Practical Course, Mouse genome engineering, 25 August – 06 September 2019 | Dresden, Germany

Publications

- Max S. Farnworth, Kolja N. Eckermann, **Hassan M.M. Ahmed**, Dominik S. Mühlen, Bicheng He, and Gregor Bucher. The Red Flour Beetle as Model for Comparative Neural Development: Genome Editing to Mark Neural Cells in Tribolium Brain Development. Brain Development: Methods and Protocols 2019
- KaramiNejadRanjbar M, Eckermann KN, **Ahmed HMM**, Sánchez C. HM, Dippel S, Marshall JM, et al. Consequences of resistance evolution in a Cas9-based sex conversion-suppression gene drive for insect pest management. Proc Natl Acad Sci USA. 2018 Jun 12;115(24):6189–94.
- Eckermann KN, **Ahmed HMM**, KaramiNejadRanjbar M, Dippel S, Ogaugwu CE, Kitzmann P, et al. Hyperactive piggyBac transposase improves transformation efficiency in diverse insect species. Insect Biochem Mol Biol. 2018 Jul 1; 98:16–24
- Eckermann KN, Dippel S, KaramiNejadRanjbar M, **Ahmed HM**, Curril IM, Wimmer EA. Perspective on the combined use of an independent transgenic sexing and a multifactorial reproductive sterility system to avoid resistance development against transgenic Sterile Insect Technique approaches. BMC Genet. 2014;15(Suppl 2): S17

Signature