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Novel Genetic and Molecular Tools for the Investigation and Control of Dengue Virus Transmission by Mosquitoes

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Abstract

Aedes aegypti is the principal vector of dengue virus (DENV) throughout the tropical world. This anthropophilic mosquito species needs to be persistently infected with DENV before it can transmit the virus through its saliva to a new vertebrate host. In the mosquito, DENV is confronted with several innate immune pathways, among which RNA interference is considered the most important. The *Ae. aegypti* genome project opened the doors for advanced molecular studies on pathogen-vector interactions including genetic manipulation of the vector for basic research and vector control purposes. Thus, *Ae. aegypti* has become the primary model for studying vector competence for arboviruses at the molecular level. Here, we present recent findings regarding DENV-mosquito interactions, emphasizing how innate immune responses modulate DENV infections in *Ae. aegypti*. We also describe the latest advancements in genetic manipulation of *Ae. aegypti* and discuss how this technology can be used to investigate vector transmission of DENV at the molecular level and to control transmission of the virus in the field.

Keywords

dengue virus; mosquito; *Aedes aegypti*; *Aedes albopictus*; virus transmission; innate immunity; RNA interference; Toll; JAK-STAT; apoptosis; transgenesis; transposon; site-specific recombination; promoter; gene-knockout; gene expression; homing endonuclease; TALEN; zinc finger nuclease; *Wolbachia*; RIDL; population replacement; effector gene; viral tropical medicine

Introduction

The global epidemiology of dengue virus (*Flaviviridae; Flavivirus*; dengue virus 1–4; [DENV1–4]) depends on the presence of two mosquito vectors, *Aedes* (*Stegomyia*) *aegypti* (L.) and *Aedes* (*Stegomyia*) *albopictus* (Skuse). *Ae. aegypti* is the principal DENV vector in urban environments of tropical countries [1]. This mosquito species is of African origin and breeds in tropical regions of Africa, Asia, Australia, South-Pacific, the Middle East, and the

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Human and Animal Rights and Informed Consent

This article does not contain any studies with human or animal subjects performed by any of the authors.

Compliance with Ethics Guidelines

Conflict of Interest

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Americas [2]. *Ae. aegypti* exemplifies a peridomestic, anthropophilic day biter. In contrast, *Ae. albopictus* is a zoophilic, catholic biter, which easily adapts to peridomestic environments in temperate regions [3,4]. During the last few decades, *Ae. albopictus* has undergone a dramatic expansion in its geographic distribution [5]. However, due to lower viral infection and transmission rates, *Ae. albopictus* is considered to be a less important vector for DENV than *Ae. aegypti*. The DENV disease cycle between the mosquito vector and the human host requires persistent infection of the mosquito [6]. DENV typically causes disease symptoms in the human host but there is no apparent pathology associated with the infection of the mosquito [7]. DENV is acquired by female mosquitoes while orally ingesting a viremic bloodmeal from a human host. A correlation exists between disease severity of the host, high DENV titer in the blood and the level of infection of the mosquito vector [8]. The ingested bloodmeal enters the midgut lumen to be digested, and virions enter midgut epithelial cells and replicate [9]. Virus then spreads cell-to-cell to form infection foci in the midgut epithelium [10]. At 4–7 days post-infection, DENV starts disseminating from the midgut to secondary tissues such as muscle, nerve, fat body, ovary, hemocytes, and eventually salivary glands, which typically become infected between 10–14 days post-infectious bloodmeal. Once the salivary glands are infected, DENV is transmitted to a new human host through release of virion-containing mosquito saliva during feeding. The extrinsic incubation period (EIP) is defined as the time period between initial infection of the vector and appearance of virus in the saliva [11]. The EIP can vary according to virus strain, mosquito strain, and virus titer in the mosquito. The midgut and salivary glands constitute physical barriers, which DENV needs to overcome before it can be transmitted [12]. A midgut infection barrier (MIB) can prevent the virus from infecting the mosquito midgut, while a midgut escape barrier (MEB) allows DENV to productively infect midgut epithelial cells but prevents the virus from disseminating from the midgut. The presence of MIB and MEB depend on specific virus strain-mosquito strain combinations [13,14]. Although higher midgut infection rates have been observed for DENV in *Ae. albopictus*, dissemination rates were significantly lower in this mosquito species compared to *Ae. aegypti* [5].

Vertical transmission of DENV by *Ae. aegypti* and *Ae. albopictus* through a transovarial route has been demonstrated in several studies [15,16]. Transovarial transmission rates (percentage of infected females transmitting virus to their progeny) of up to 13 % for *Ae. aegypti* and 11–41 % for *Ae. albopictus* have been reported [17,18]. Vertical transmission of DENV strongly affects its etiology because it allows the virus to be maintained among mosquitoes during inter-epidemic periods, i.e. during dry seasons or winter seasons, when there is only little active horizontal DENV transmission.

Molecular interactions between DENV and innate immune pathways of *Ae. aegypti*

During infection of a mosquito, DENV is confronted with several innate immune pathways such as RNA interference (RNAi), Toll, and JAK-STAT, which modulate DENV infection. As with MIB and MEB, the effectiveness of these pathways in inhibiting DENV replication likely varies depending on the virus and mosquito strains in question.

RNAi—RNAi has been considered the most important antiviral innate immune pathway in *Ae. aegypti* [7]. The RNAi pathway in mosquitoes follows the same principle as described in great detail for *Drosophila* [19–21]. The genome of *Ae. aegypti* encodes key gene homologs of the three small RNA (miRNA, siRNA, and piRNA) regulatory pathways [22,23]. Initial studies showed that the siRNA pathway in *Ae. aegypti* could be triggered to silence DENV replication. Mosquitoes infected with recombinant Sindbis virus (*Togaviridae; Alphavirus*; [SINV]) expressing ~300 nt anti-sense cDNAs complementary to the genomes of DENV1, 2, 3, or 4 silenced DENV replication in serotype-specific manner [24]. Dicer2 of the siRNAi

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pathway senses long double-stranded (ds)RNA formed during replication of RNA viruses and cleaves it into 21 base-pair (bp) duplexes, the hall-mark of active RNAi [25,26]. In DENV-infected mosquito tissue, DENV-derived siRNAs are readily detectable, indicating RNAi-mediated degradation of viral genomes. Nevertheless, DENV is still able to persistently infect the mosquito, indicating that the virus is counteracting the mosquito's RNAi response. A recent study suggests that a flavivirus-specific small RNA (subgenomic flavivirus RNA) originating from the 3'UTR of the virus has the ability to inhibit cleavage of dsRNA by Dicer [27].

Impairment of the RNAi pathway in *Ae. aegypti* by transiently silencing key genes, *dcr2*, *r2d2*, or *ago2* significantly increased DENV2 replication in the mosquito up to 10-fold and shortened the EIP by three days (Fig. 1a); [26]. Likewise, transgenic over-expression of an RNAi suppressor, B2 of Flockhouse virus (*Nodaviridae; Alphanodavirus*) blocked dsRNA processing by dicer2 and caused a significant increase in DENV2 titers in midgut tissue of *Ae. aegypti* (Fig. 1b); [28]. These observations indicate that the RNAi pathway acts as a gate-keeper in *Ae. aegypti*, modulating DENV2 replication to protect the mosquito from virus-induced pathogenic effects when exceeding a tolerable concentration in the infected cell.

Toll—In invertebrates, the Toll pathway is activated by gram-positive bacteria, fungi and viruses through pattern recognition receptors [29]. DENV infection leads to Toll pathway activation in midgut tissue of *Ae. aegypti* [30,31]. As a consequence, the negative regulator of the pathway, Cactus, is degraded resulting in the nuclear translocation of various transcription factors such as Relish (Rel1), and release of antimicrobial peptides. Transient silencing of Cactus spontaneously activates the Toll pathway. In DENV2-infected *Ae. aegypti*, silencing of Cactus resulted in a ~4-fold reduction of DENV2 infection levels in midgut tissue [30]. Impairment of the Toll pathway by transient silencing of the adapter protein-encoding MYD88 gene resulted in a 2.7-fold viral load increase of DENV2. In contrast to Toll, the Immune Deficiency (Imd) pathway, typically responsive to gram-negative bacteria in invertebrates, has not been shown to be activated in DENV2-infected *Ae. aegypti*.

JAK-STAT—The Janus kinase and signal transducer and activator of transcription (JAK-STAT) pathway is a cytokine signaling pathway in mammals. In *Drosophila*, extracellular binding of the ligand Unpaired (Upd) to the receptor Domeless (Dome) initiates the JAK-STAT pathway [32]. As a result, Dome undergoes a conformational change, leading to self-phosphorylation of the associated Janus kinase, Hop, which then phosphorylates Dome. This results in the formation of docking sites for the cytoplasmic STATs. The recruitment of STATs by the Dome/Hop complex induces phosphorylation of STATs, leading to their translocation into the nucleus and transcription activation of specific target genes. Two negative regulators, PIAS (protein inhibitor of activated STAT) and SOCS (suppressor of cytokine signaling) can suppress the JAK-STAT pathway. In *Ae. aegypti* the JAK-STAT pathway is activated upon DENV2 infection of the midgut [33]. Impairment of the JAK-STAT pathway by transient silencing of Hop and Dome significantly increased DENV2 infection levels by up to 3-fold. Silencing of PIAS, however, reduced DENV2 infection by up to 5-fold.

In summary, RNAi is the major antiviral pathway specifically targeting actively replicating DENV in the cytoplasm of *Ae. aegypti* cells [7]. Toll and JAK-STAT pathways both contribute to the control of DENV infection. However, it is not clear to what extent either pathway specifically responds to the presence of DENV in *Ae. aegypti*. So far, viral determinants that would trigger Toll and JAK-STAT pathway activation have not been elucidated.

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Apoptosis—There have been recent efforts to elucidate the role of apoptosis in arbovirus infection of *Ae. aegypti*. The *Ae. aegypti* genome encodes numerous genes with homology to apoptosis-related genes identified in *Drosophila*, including eleven caspases, three inhibitor of apoptosis (IAP) proteins, two IAP antagonists, and orthologs of Ark, Dnr1, and Fadd [34]. Several of the *Ae. aegypti* apoptosis-related genes have been shown to function in apoptosis including the initiator caspase AeDronc, the effector caspases CASPS7 and CASPS8, the caspase inhibitors AeIAP1 and AeDnr1, and the IAP antagonists IMP and AeMx [34–37].

So far, effects of apoptosis on arbovirus infection of *Ae. aegypti* have been investigated in two experiments using SINV as a model virus. The first experiment showed that transient silencing of *IAP1* in *Ae. aegypti* induced spontaneous apoptosis in midgut tissue of females, leading to a 60–70% increase in mortality [36]. Surprisingly, this also led to increased SINV replication and dissemination rates, while silencing of *AeDronc* reduced SINV replication and dissemination. It was speculated that inducing widespread apoptosis by silencing of *AeIAP1* may have weakened other innate defenses of the mosquitoes, resulting in higher levels of virus replication. In the second approach, expression of the apoptosis-inducing *Drosophila reaper* gene from a recombinant SINV also induced apoptosis in the mosquito midgut but resulted in reduced SINV titers at early stages of infection [38]. Furthermore, examining SINV progeny from these mosquitoes revealed a strong selective advantage for viruses that had lost their *reaper* insert, indicating that induction of apoptosis by SINV was deleterious for the virus. Thus, although apoptosis is usually not observed in naturally occurring virus-vector combinations, it may occur in non-compatible combinations, involving specific arbovirus and/or mosquito strains. A current research effort aims at elucidating the role of apoptosis in DENV infection of *Ae. aegypti*.

Genetic manipulation of *Ae. aegypti* to investigate and control DENV transmission

Genetic manipulation of *Ae. aegypti* has become a well-established procedure, which has greatly advanced our ability to investigate mosquito gene – pathogen gene interactions. Transgenic *Ae. aegypti* have been generated for three major purposes: i) vector and/or pathogen control, ii) study of gene function, iii) tool development to improve genetic manipulation. Further development and refinement of molecular-genetic tools has laid the foundation for innovative approaches to investigate the molecular basis of vector competence for DENV in *Ae. aegypti* and to develop novel strategies for mosquito/DENV control in the field.

Tools for the genetic manipulation of *Ae. aegypti*

Transposable elements: Since the initial report of successful germline transformation of *Ae. aegypti* [39], considerable progress regarding the genetic manipulation of this mosquito species has been made. As insertion vectors for germline transformation, DNA-based Class II transposable elements (TE) such as (Tc1) *mariner Mos1* (originating from *D. mauritiana*), *piggyBac* (originating from *Trichoplusia ni*), or *Hermes* of the hAT superfamily (origin: *Musca domestica*) have been used [40]. These TE have poor mobility in the mosquito germline. In numerous germline transformation experiments of *Ae. aegypti*, *mariner Mos1* has shown predictable integration patterns, thus making it an ideal choice among TE insertion vectors [41]. Research groups also use *piggyBac* routinely for the transformation of this mosquito species as well as for the genetic manipulation of *Ae. albopictus* [42,43].

Site-specific integration: A major disadvantage associated with the use of Class II TE is their quasi-random integration pattern, depending only on short recognition motifs such as TA for *mariner Mos1* or TTAA for *piggyBac* [40].

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Since the TE integration site is random, transgene expression is often affected by position effect variegation [44]. To ensure predictable and stable transgene expression, alternative transformation systems such as the *ΦC31* site-directed integration system have been successfully used in *Ae. aegypti* (Fig. 2a); [28,42,45,46]. The underlying principle is a recombination event mediated by the *ΦC31* integrase between a short recognition (=docking) sequence (*attP*), which has been inserted into the genome of the target organism and a corresponding recognition sequence (*attB*), which is linked to the donor encoding the gene-of-interest. Since the *attP* site containing docking strain needs to be generated using a TE as insertion vector, a challenge of the *ΦC31* system is to obtain docking strains, which contain the *attP* site in an optimal genome locus supporting strong gene-of-interest expression levels [46].

UAS-Gal4 binary expression system: Another important tool for improved gene-of-interest expression in *Ae. aegypti* was the development of the binary UAS-Gal4 expression system [47]. The system is based on the production of two independent transgenic lines: (1) the driver line containing the yeast Gal4 activator gene under control of a promoter-of-choice and (2) the responder line containing the binding sites (UAS=upstream activating sequences) for the Gal4 protein upstream of the gene-of-interest expression cassette (Fig. 2b). To use in *Ae. aegypti*, the essential DNA-binding and activation domains of Gal4 were fused directly, resulting in the chimeric Gal4Δ activator [48]. In a proof-of-principle experiment, Gal4Δ of the transgenic driver line was placed under control of the bloodmeal-inducible fat body-specific *Vg* promoter. The responder line contained of the EGFP reporter fused to UAS. Crossing the two transgenic lines resulted in hybrids strongly expressing EGFP in fatbody of bloodfed females, confirming the functionality of the system. An important advantage of the system is that no expression of the gene of interest takes place when the trans-activator is absent. This allows transgenic overexpression of genes, which have potentially harmful effects for the organism.

Promoters: A range of promoters has been isolated from *Ae. aegypti*, allowing predictable gene-of-interest expression in various tissues. *AeCPA* and *Vg* are female-specific, bloodmeal-inducible promoters that drive gene expression in midgut epithelial cells and fat body, respectively [49–51]. Both promoters activate gene expression for relatively short periods of time, 4–32 h post-bloodmeal (pbm) (*AeCPA*) and 2–24 h pbm (*Vg*). The *Ae. aegypti* 30K promoter is a strong constitutive promoter for gene expression in the distal-lateral lobes of salivary glands [52]. Recently, promoters of two *Ae. aegypti* ubiquitin genes, *Ub(L40)* and *PUb*, have been characterized and tested in transgenic mosquitoes [53]. The *Ub(L40)* promoter drives gene expression predominantly in larvae and ovaries, whereas the *PUb* promoter drives gene expression in embryos, larvae, pupae, males, and constitutively in the female midgut. Two heat-shock protein 70 (hsp70)-like promoters of *Ae. aegypti*, *AaHsp70Aa*, and *AaHsp70Bb* were recently described, which significantly activated transcription following a 1 h exposure at 39°C in various mosquito tissues, such as salivary glands, midguts, and ovaries [54]. For female germline expression and for male-only expression the *nanos* and testes-specific *b2 tubulin* promoters, respectively, have been isolated and characterized [55,56].

Investigating gene function in *Ae. aegypti* by genome editing—Genome editing is an innovative approach to investigate gene function in *Ae. aegypti* in relation to DENV infection. Several research efforts have been aimed at developing tools to edit the genome of *Ae. aegypti* via targeted gene-knockout. So far, three different approaches have been successfully applied for targeted gene disruption in *Ae. aegypti*: homing endonucleases (HEG) [57], zinc finger nucleases (ZFN) [58–60], and transcription activator-like effector nucleases (TALEN) [61–64].

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HEG are selfish elements catalyzing specific dsRNA breaks in the genome of their target organism [57].

Proof-of-concept experiments showed that HEG such as I-*Ppo*I, I-*Sce*I, I-*Cre*I, and Y2-I-*Ani*I are active in the soma and germline of *Ae. aegypti* [65,66]. HEG expression in a transgenic mosquito line resulted in target site-specific dsDNA breaks followed by DNA gap repair via single-strand annealing and non-homologous end-joining. As a consequence, a reporter gene was excised, which was flanked by HEG-specific recognition sequences in the engineered *Ae. aegypti* line. Despite these successful demonstrations, a major task remains to re-engineer HEG for targeted gene-of-interest knockout in *Ae. aegypti*. Nevertheless, HEG appear to be promising candidates for a gene drive system, due to their ability to invade populations through dsDNA break induction.

Gene-of-interest-specific genome editing in *Ae. aegypti* has been recently accomplished when using TALEN and ZFN. TALEN have been engineered to disrupt via the germline the *Ae. aegypti kmo* gene encoding a protein essential for eye pigmentation [67]. Up to 40% of surviving mosquito individuals had disrupted *kmo* alleles in which 1–7 bp of the CDS were deleted, resulting in a lack of eye pigmentation. This observation indicates that TALEN are highly active in the *Ae. aegypti* germline.

Using ZFN the coding sequence of *orco*, encoding the co-receptor of odorant receptors, was disrupted in the germline of *Ae. aegypti*, which caused 1 to >20 nucleotide deletions in the target sequence among the different mutant lines [68]. Resulting *orco* gene-knockout mosquitoes produced a phenotype, which did not respond to human scent in the absence of CO_2. Together with genome editing, whole-transcriptome analysis using NextGen sequencing technology can be used to reveal how loss-of-gene function affects other pathways in the mosquito. Combining these two powerful techniques may lead to the discovery of novel gene candidates that antagonize DENV in the mosquito vector.

Transgene-mediated manipulation of gene expression levels in *Ae. aegypti*— Stable, inheritable disruption of an endogenous gene could be limited by the fact that the resulting loss-of-function genotype would be non-viable. Furthermore, engineering of a nuclease to specifically target a gene-of-interest in the genome is still technically challenging and/or costly. Thus, transgene-mediated, inducible and tissue-specific knockdown of endogenous genes is an alternative technique to investigate endogenous gene-function in *Ae. aegypti*. The approach is based on temporal and/or spatial expression of a long inverted-repeat (IR) RNA from a transgene as a trigger for gene-of-interest-specific RNAi. This can result in strong phenotypes and is technically simple to achieve [69,70]. As an example, midgut-specific, bloodmeal-inducible expression of an IR effector targeting the RNAi pathway gene *dcr2* resulted in about 50% silencing efficiency for ~28 h pbm [70]. This level of RNAi impairment was sufficient to significantly increase SINV replication in those mosquitoes.

Genetic pest management strategies to control *Ae. aegypti* and DENV transmission in the field

The on-going spread of multi-level insecticide resistance among *Ae. aegypti* populations in DENV endemic regions of the world demands the development of alternative *Ae. aegypti*/DENV control strategies, which are largely based on genetic pest management [71]. Three major novel control strategies that avoid the use of insecticides are currently being developed.

Wolbachia—Moreira and colleagues [72] discovered that strains of *Wolbachia* (Rickettsiales), a maternally inherited Gram-negative endosymbiotic bacterium, are capable

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of blocking DENV replication in *Ae. aegypti. Wolbachia* occurs naturally in ~65% of all insect species [73]. In nature, numerous mosquito species including *Ae. albopictus* and *Culex pipiens* but not *Ae. aegypti* are *Wolbachia*-infected [74]. The bacterium can cause cytoplasmic incompatibility in the male sperm leading to embryo lethality when infected males mate with uninfected females. However, mating between infected males and infected females leads to the production of offspring, thereby favoring infected females over non-infected ones. Thus, *Wolbachia* has the ability to drive itself into populations [75]. Two *Wolbachia* strains (*wMel* and *wMelPop*-CLA) naturally occurring in *Drosophila* have been successfully trans-infected into *Ae. aegypti* [76,77]. Both *Wolbachia* strains limited DENV2 infection in the mosquito by blocking the virus' ability to disseminate from the midgut into mosquito saliva and affected mosquito fitness [75,77]. The mechanism behind the *Wolbachia*-induced resistance to DENV2 in *Ae. aegypti* is not fully understood so far [77–82]. A major open field trial was conducted in which ~300,000 *Wolbachia wMel*-infected *Ae. aegypti* raised under laboratory conditions were deliberately released in 2011 at two locations near Cairns, Australia [83]. The frequency of *Wolbachia*-infected *Ae. aegypti* initially increased to more than 15% in both locations at 2-weeks post-release. After additional releases, frequencies increased to >60% and reached near fixation levels five weeks after releases were terminated. These observations suggest that *Wolbachia* could potentially become a powerful bio-control agent to suppress DENV transmission by *Ae. aegypti* in endemic areas.

RIDL—RIDL (Release of Insects carrying Dominant Lethals), is a novel genetic female-killing (FK) control strategy aimed at mosquito population reduction [84]. The technique involves the generation of transgenic mosquitoes in which lethal genes are sex-specifically and conditionally over-expressed [85]. An example would be to place a pro-apoptotic gene under control of a female-specific promoter. Using the TET on/off transcriptional activation system, expression of the pro-apoptotic gene is repressed in the presence of tetracycline, which is added as a food supplement during mosquito rearing (Fig. 2c). This allows both sexes to be viable for breeding purposes. When releasing RIDL mosquitoes into the environment, tetracycline is absent resulting in female-specific lethal gene expression. Inheritance of the dominant gene construct by wild-type mosquitoes causes only male offspring to survive, which will lead to collapse of the mosquito population over time. Several variants of the RIDL system have been developed for *Ae. aegypti* over the years, including single or two-component systems, bisex lethals, flightless females and non-sex specific late-acting lethal systems [43,86].

In limited cage experiments, RIDL mosquito populations were unable to reproduce in the absence of tetracycline. Repeated introductions of homozygous RIDL males into cage populations of wild-type mosquitoes at initial ratios of 8.5–10 : 1 (RIDL : wild-type) eliminated the target populations by 10–20 weeks after initial release [86]. This indicates that the RIDL-based population control strategy can be highly successful in the field.

Transgenic expression of anti-DENV effectors

Gene drive system: Another novel concept of DENV control in the field is based on population replacement. Here, DENV-susceptible mosquito populations are replaced by genetically-modified refractory strains [87]. The underlying principle of this genetic pest management strategy involves the design of strong anti-DENV effector genes and a highly species-specific genetic drive system to which the anti-DENV effector needs to be tightly linked [88]. A genetic drive system circumvents Mendelian inheritance patterns and thereby allows the anti-DENV effector, which potentially carries a fitness load, to be driven through a wild-type mosquito population at an accelerated rate until fixation.

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Until now, there is no gene drive system readily available for *Ae. aegypti*. Current attempts to develop gene drivers for *Ae. aegypti* include the design of HEG and a killer-rescue system based on a synthetic *Medea* (maternal effect dominant embryonic arrest) variant [89–92]. *Medea* is a selfish gene element when present in females; all offspring will die if they do not inherit a maternal or paternal copy of the element. This feature enables *Medea* to drive itself through populations.

Anti-DENV2 effectors: As described above, priming the innate RNAi pathway against DENV is a highly effective approach to target and silence the virus in *Ae. aegypti*. As a further proof-of-principle for this approach, transgenic mosquitoes have been generated expressing an (578 bp) IR RNA derived from the prM-M encoding sequence of DENV2 under control of the *AeCPA* promoter [93]. Female mosquitoes of line Carb77 expressed the IR effector between 4–48 h pbm in midgut tissue. The 578 bp dsRNA of the effector was recognized by the endogenous RNAi machinery of the mosquitoes and processed into siRNAs. Carb77 mosquitoes were highly resistant to different strains of orally acquired DENV2 but not to other DENV serotypes or other arboviruses. However, after 17 generations in laboratory culture Carb77 mosquitoes lost their anti-DENV2 resistance phenotype, even though the transgene appeared to be intact [94]. It was speculated that expression of the anti-DENV2 effector was silenced in these mosquitoes. Meanwhile, another transgenic line, Carb109, has been established harboring the same transgene as line Carb77. Current studies show that Carb109 are completely refractory to DENV2 after more than 33 generations in laboratory culture (Fig. 1c); [A.W.E. Franz, I. Sanchez-Vargas, R.R. Raban, W.C. Black, A.A. James, K.E. Olson, unpublished data]. Mathur and colleagues [52] generated transgenic *Ae. aegypti* in which a similar anti-DENV2 IR effector was placed under control of the constitutive salivary gland-specific 30K promoter. DENV2 titers and infection rates were significantly reduced in salivary glands of the transgenic mosquitoes and the virus was absent in their saliva. However, to further develop the concept of RNAi-mediated transgenic resistance to DENV, an effector molecule needs to be generated that causes resistance to all four DENV serotypes in *Ae. aegypti*.

Recent mathematical models revealed that “Replace” and “Reduce” (R&R) could be a powerful alternative to sole population replacement [95]. R&R is a combination of population replacement and population reduction (i.e., FK), which could be achieved by inserting an anti-DENV effector transgene into RIDL mosquito strains. The R&R approach promises to be more effective that FK alone, even if the anti-DENV effector would be associated with substantial fitness costs.

Conclusions

The availability of genomic sequences of *Ae. aegypti* has facilitated detailed studies on the molecular basis of vector competence for DENV. One important outcome of these studies was the discovery of RNAi as the major anti-viral immune pathway modulating DENV replication in the vector. Several studies showed that the RNAi machinery in *Ae. aegypti* is able to completely silence DENV replication.

Over the past decade, significant advances have been made regarding the genetic manipulation of *Ae. aegypti*. Novel, tissue-specific promoters have been described and tested, and site-specific integration and binary gene expression systems have been developed to manipulate endogenous gene expression with greater efficiency. Importantly, gene editing tools such as HEG, TALEN, and ZFN have been successfully applied to knockout reporters or endogenous genes in *Ae. aegypti*.

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Several innovative genetic pest management-based control strategies are under development to control *Ae. aegypti* populations and/or DENV transmission in the field. One of these involves trans-infection with *Wolbachia*, which can spread through a wild-type population and causes *Ae. aegypti* to become refractory to DENV. RIDL, as a transgenic alternative to classical sterile insect technology has the potential to eliminate *Ae. aegypti* populations in the field. Another concept is based on population replacement in which DENV-competent mosquito populations are replaced in the field by mosquitoes, which have been genetically-modified to be refractory to the virus. As a proof-of-concept, inheritable RNAi-based resistance to DENV2 has been successfully engineered in genetically modified *Ae. aegypti*.

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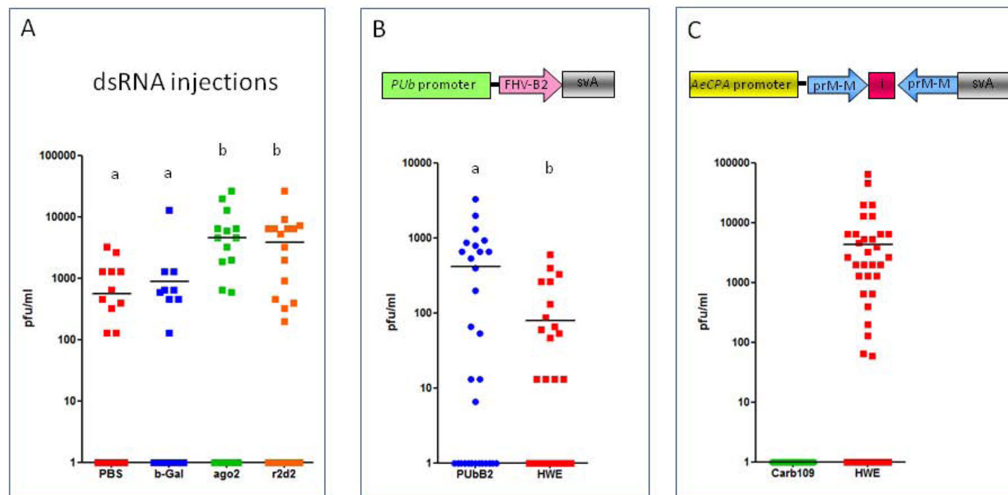

Fig. 1.

DENV2 is targeted by the innate RNAi pathway in *Aedes aegypti* and responds to RNAi pathway manipulation in mosquitoes. (**a**) Intrathoracic injection of dsRNAs derived from sequences of the RNAi pathway genes *ago2* and *r2d2* into mosquitoes of the Higgs White Eye (HWE) strain triggers RNAi and leads to transient silencing of these genes. In presence of the impaired RNAi pathway, DENV2 titers are significantly increased at 7 days post-infectious bloodmeal (pbm). Controls: (HWE) mosquitoes injected with PBS or dsRNA derived from the β-Gal gene (non-target control). Each data point represents the virus titer of an individual female. (**b**) Transgene-mediated, constitutive overexpression of a potent RNAi suppressor (FHV-B2) in midgut tissue of PUbB2 mosquitoes impairs the RNAi pathway by inhibiting dsRNA processing. This leads to a significant increase of DENV2 titers in midguts at 7 days pbm in comparison to the HWE control. Each data point represents the virus titer of an individual female midgut. (**c**) Transgene-mediated expression of an IR effector complementary to the prM-M gene of DENV2 triggers RNAi against the virus in midguts of bloodfed females. Transgenic mosquitoes of line Carb109 are completely refractory to the virus. In contrast, HWE control mosquitoes are highly susceptible to DENV2 at 7 days pbm. DENV2 bloodmeal titers: 10^6–10^7 plaque forming units (pfu)/ml. Each data point represents the virus titer of an individual female. Virus titers were assessed by plaque assays in LLC-MK2 monkey kidney cells. Letters [a], [b] next to numbers represent statistically significant groupings (p-value:<0.05; Tukey-Kramer Test).

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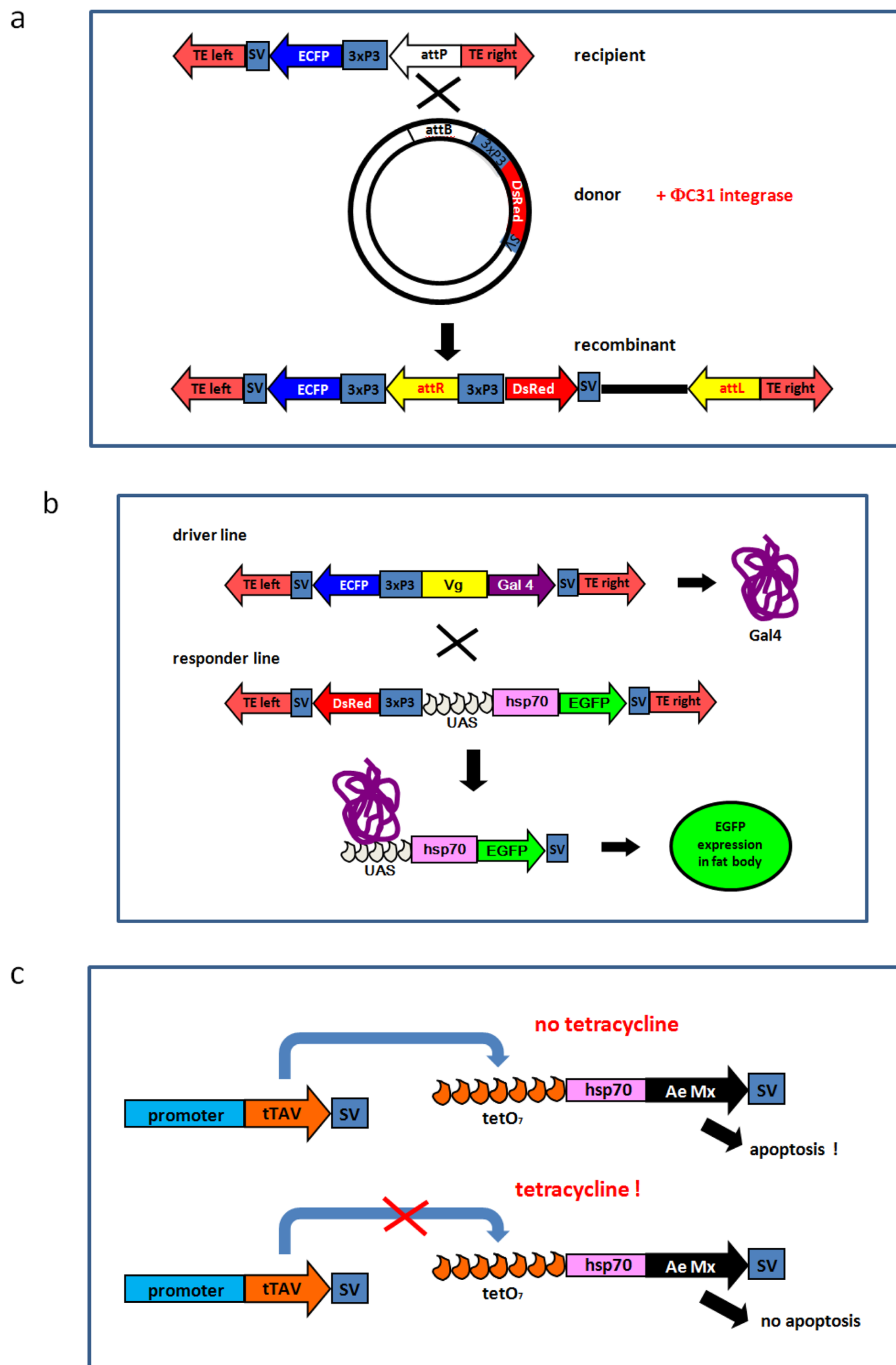

Fig. 2.

Tools for the genetic manipulation and control of *Aedes aegypti*. (**a**) Principle of the *ΦC31* site-directed recombination system. With the help of a transposable element (TE) the phage attachment site *attp* is anchored in the genome of the docking strain. The docking strain is then ‘super-transformed’ with the *attB* site containing donor plasmid, which also contains

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the gene-of-interest. Co-injecting the donor and *in vitro* transcribed *ΦC31* integrase into embryos of the docking strain leads to a recombination event between *attP* and *attB*. As a consequence, the entire donor plasmid is integrated at the *attP* site; *attP* and *attB* are converted into *attL* and *attR*. Using the *ΦC31* site-directed recombination system position effects can be avoided because the transgene is integrated at a defined locus. (**b**) Principle of the Gal4-UAS binary expression system. A transgenic driver line is generated to express the yeast Gal4 activator from a promoter-of-choice. The responder line contains the Gal4 binding site (UAS) and the gene-of-interest (i.e, the EGFP reporter) to be expressed under control of a minimal promoter such as *hsp70*. Crossing the two transgenic mosquito lines will result in progeny in which the Gal4 protein is binding to the UAS sequences. As a result, the EGFP reporter is expressed from the promoter-of-choice. The advantage of this system is that tissue-specific promoters can be tested with genes-of-interest in various combinations by setting up simple crossings between different driver and responder lines. (**c**) Principle of the RIDL system for population reduction of *Ae. aegypti*. The tetracycline repressible system shown here consists of two components. One component encodes the tetracycline-repressible transcriptional activator, tTAV, which is under control of a promoter-of-choice, i.e., a female-specific promoter (to achieve female-specific killing). The other component contains the tTAV binding site $tetO_7$ linked to a pro-apoptotic gene such as *michelob x* (*AeMx*), which is under control of a minimal promoter (*hsp70*). In absence of tetracycline, tTAV will bind to $tetO_7$, resulting in expression of *AeMx*. Addition of tetracycline as a food supplement will result in binding of tTAV to tetracycline. Tetracycline-bound tTAV shows an altered structure, which is no longer complementary to $tetO_7$ and consequently, *AeMx* is not expressed.

Abbreviations: TE left and right: lest, right terminal inverted-repeats of the transposable element; 3xP3: eye tissue-specific promoter; EGFP, ECFP, DsRed: fluorescent reporters; SV: transcription termination signal of Simian virus 40; Vg: promoter of the *Ae. aegypti vitellogenin 1* gene; hsp70: heat-shock promoter 70 of *Drosophila; AeMx: michelob x* gene of *Ae. aegypti*.