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Transgenesis and paratransgenesis to control insect-borne diseases: Current status and future challenges

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Abstract

Insect-borne diseases cause significant human morbidity and mortality. Current control and preventive methods against vector-borne diseases rely mainly on insecticides. The emergence of insecticide resistance in many disease vectors highlights the necessity to develop new strategies to control these insects. Vector transgenesis and paratransgenesis are novel strategies that aim at reducing insect vectorial capacity, or seek to eliminate transmission of pathogens such as *Plasmodium sp.*, *Trypanosoma sp.*, and Dengue virus currently being developed. Vector transgenesis relies on direct genetic manipulation of disease vectors making them incapable of functioning as vectors of a given pathogen. Paratransgenesis focuses on utilizing genetically modified insect symbionts to express molecules within the vector that are deleterious to pathogens they transmit. Despite the many successes achieved in developing such techniques in the last several years, many significant barriers remain and need to be overcome prior to any of these approaches become a reality. Here, we highlight the current status of these strategies, pointing out advantages and constraints, and also explore issues that need to be resolved before the establishment of transgenesis and paratransgenesis as tools to prevent vector-borne diseases.

Keywords

Transgenesis; Paratransgenesis; Gene drive systems; Transposons; Symbionts; Vector-borne disease control

1. Introduction

Insect-borne diseases are responsible for severely affecting human life around the world, causing significant morbidity and mortality. Malaria alone is responsible for 1-2 million deaths annually, and approximately 300 million are at risk of becoming infected. The lack of effective vaccines to control the spread of a significant number of vector-borne diseases makes control mainly dependent on insecticides. However, the appearance of insecticide resistance requires the development of new strategies to reduce pathogen transmission in the field [1]. Novel

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approaches against vector borne diseases include transgenesis and paratransgenesis to reduce vector competence.

For vector transgenesis, the goal is to transform vectors with a gene (or genes) whose protein (s) impair pathogen development. A few stably transformed insect germ lines using transposons are available [2], and mosquitoes such as *Aedes* [3-6], *Anopheles* [7-16], and *Culex* [17] were genetically altered or transformed (Table 1). For some transformed mosquitoes, proteins targeting development of *Plasmodium sp.* and Dengue virus were successfully expressed [12,13,18-20].

Paratransgenesis aims at reducing vector competence by genetically manipulating symbionts. Transformed symbionts are spread maternally or via coprophagy across an insect population [21]. Symbionts currently targeted in paratransgenesis include bacteria found within triatomine [21-23], tsetse fly [24], and mosquito [25,26] tissues, and densoviruses infecting *An. gambiae* and *Ae. aegypti* mosquitoes [27,28].

This review explores technical issues, future challenges and potential applications of transgenesis and paratransgenesis against vector-transmitted pathogens.

2. Transgenesis in insect disease vectors

The main objective of vector transgenesis is interruption of pathogen transmission via the introduction of exogenous DNA fragment (i.e., gene) into an insect genome, followed by expression of the gene to inhibit pathogen development within the vector. Several mosquitoes species vectors of different parasites and viruses have been transformed (Table 1). Some of the transformed mosquitoes were shown capable of blocking pathogen development via tissue-specific expression of molecules that impair pathogen attachment to the midgut [12], or activate biochemical pathways detrimental to pathogen survival [18]. However, vector transgenesis is still a somewhat complex approach, highlighted by the fact that insect germ line transformation technique is only successfully performed by a handful of laboratories. Various issues associated with development of transgenic vectors including stability of the transgene in the genome and fitness of the transformed insect in the field are significant hurdles to be overcome before transgenic insects can be applied against insect-borne diseases. Some of the issues involved in development of transgenic vectors, how interference or blockage of parasite is achieved, and what may lie ahead regarding challenges and perspectives for vector transgenesis are discussed below.

2.1. Germ line transformation

Stable genetic transformation of insects is accomplished by inserting an exogenous gene into the insect genome via the inoculation of plasmids containing the transgene (donor plasmids) into insect eggs (Figure 1). Donor plasmids are constructed to carry an engineered transposon element lacking the gene that encodes the transposase, the enzyme that mediates transposon activity by a cut and paste mechanism. *Hermes*, *Mariner* (*Mos1*), *minus*, and *piggyBac*, for the most part, have been the transposable elements of choice. Transposases are then supplied *in trans* (expressed in a separate plasmid) by the co-inoculation of a transposase-encoding helper plasmid along with the donor plasmid. Transposase expression is usually driven by a heat shock protein promoter that is activated upon raising the temperature, as it is frequently performed with injected mosquito eggs[2].

Transformed offspring can be identified by a specific phenotype alteration, mediated by the expression of a reporter protein encoded by the donor vector, such as eye color [2]. For example, the *Ae. aegypti* kh^W strain displays white eyes; upon transformation with the transposable element *Hermes* or *piggyBac* expressing the *Drosophila melanogaster* *cinnabar* gene (*cn*⁺),

mosquito eye color (brown) was rescued [3,29,30]. However, this strategy is restricted to insect species displaying polymorphic eye phenotypes, such as *Ae. aegypti*.

Alternatively, phenotypic markers such as firefly luciferase, or green (EGFP), red (dsRED) and cyan fluorescent (CFP) proteins can be used as transformation markers [8,9,31-33]. Luciferase as reporter gene was specifically expressed in midguts of adult *Ae. aegypti* controlled by a carboxypeptidase promoter [31], while *Anopheles albimanus* displayed green fluorescence in whole body of larvae and pupae, and in the abdomen of adults following expression of GFP via *piggyBac* [8]. *Anopheles stephensi* also were modified to express dsRED and cyan fluorescent protein either second and third-instar larvae and in adult female midguts (dsRED) [9], or in fat bodies (cyan) [33].

2.2. Tissue-specific transgene expression

Within the insect vector, a pathogen may interact with specific tissues, such as the midgut (as is the case for *Trypanosoma cruzi* and *Leishmania sp.*), or midgut, hemocoel and salivary glands (as is the case for *Plasmodium sp.*). Frequently, molecules that can block pathogen development within its vector can be expressed in a tissue-specific manner to increase effectiveness. Tissue-specific expression of a transgene is accomplished by the use of a tissue-specific promoter (Table 1). Most promoters used in vector transgenesis drive protein expression specifically within the midgut, the hemocoel, or the salivary glands, as these are sites where pathogens are commonly found within an infected vector [10,19,30,31,34-36].

In mosquitoes, promoters for carboxypeptidase and peritrophin have been widely used to drive midgut-specific expression of several transgenes. In *Ae. aegypti*, expression of luciferase under the control of the carboxypeptidase promoter was similar to the expression profile detected for the native carboxypeptidase: expressed in the midgut, after a blood-meal, and only in females [31]. Similar results were obtained with an *An. gambiae* peritrophin promoter driving expression of a transgenic protein in *An. stephensi* and *Aedes fluviatilis* [10,19].

In *Ae. aegypti*, a vitellogenin promoter specifically drives expression of transgenes in the fat body. This promoter was used to express innate immune defense-related genes [30,32,37,38], as well as dsRNA targeting *REL1* transcripts [37]. The same promoter was used to express CFP in *An. stephensi* [33]. The robustness of the vitellogenin promoter was confirmed by its capacity to function following multiple gonotrophic cycles in transgenic *An. stephensi* [35].

Salivary gland-specific promoters also have been used in germ-line transformation of mosquitoes. *D7* and *apyrase* promoters from *An. gambiae* and *antiplatelet* from *An. stephensi* were used to transform *An. stephensi* [34,36]. *Ae. aegypti* also was transformed and successfully expressed luciferase within salivary glands using *maltase-like 1* and *apyrase* promoters [39].

2.3. Transgenes targeting *Plasmodium* development

In spite of the many mosquito species successfully transformed (Table 1), only a handful has been transformed with molecules that impair pathogen development [4,10,12-14,18]. A list of genetically modified mosquitoes obtained to date, including the transposon and reporter genes used, tissue-expression specificity and target pathogens, among others is seen on Table 1.

In one example of a transgene targeting *Plasmodium*, expression of phospholipase-2 (PLA2) in *An. stephensi* [13] led to an 87% reduction of *P. berghei* oocyst intensity compared to non-PLA2-expressing controls. Moreover, parasite transmission to naïve mice was completely inhibited in three out of four experiments [13]. When a peritrophin promoter was used to drive the expression of PLA2 in *An. stephensi*, inhibition of *P. berghei* oocyst intensity ranged from 74% to 94% [10]. Nevertheless, expression of PLA2 in the mosquito midgut did not exert a

direct effect on the parasite, but rather led to structural damage of the midgut epithelium [10, 13]. When an inactive form of PLA2 (mPLA2) was used to transform *Ae. fluviatilis* using *piggyBac* under the control of a peritrophin promoter from *An. gambiae*, reduction in *P. gallinaceum* oocyst intensity ranged from 17.5% to 68.5% [19]. Here, despite the fact that inhibition of *P. gallinaceum* oocysts was not as accentuated as the inhibition detected for *P. berghei* oocysts using the active PLA2, the mPLA2 transgenic *Ae. fluviatilis* displayed greater survival rate than wild type mosquitoes [19].

Synthetic peptides that block *Plasmodium* development in mosquitoes also have been identified. Using a phage display library Gosh et al [40] identified SM1, a peptide that blocked *P. berghei* invasion of *An. stephensi* midgut and salivary glands. In *An. stephensi* transformed with *piggyBac* expressing a four tandem repeat of SM1 under a carboxypeptidase promoter, *P. berghei* intensity was inhibited by 81.6%. Interestingly, these transgenic mosquitoes even with sporozoites in their salivary glands were unable to transmit *P. berghei* to mice [12].

Besides PLA2 and SM1, mosquitoes also were transformed with antimicrobial peptides [14, 30,37]. *An. gambiae* transformed with a *cecropin A* driven by an *Ae. aegypti* carboxypeptidase promoter inhibited *P. berghei* intensity by 61% on average when compared with non-transformed controls [14]. However, this study failed to demonstrate expression of cecropin A within the midguts of transgenic mosquitoes [14]. Similarly, *Ae. aegypti* transformed with *piggyBac* expressing *defensin A* under a peritrophin promoter, inhibited *P. gallinaceum* oocyst intensity by 65% to 70% [20].

2.4. Transgenes inducing gene silencing and targeting viral transmission

Stable transformation of mosquitoes with two inverted repeats of the same gene to induce assembly of double-strand RNAs (dsRNAs) and activation of the RNAi pathway has also been obtained [11,37]. This strategy takes advantage of the RNAi mechanism to block expression of insect molecules associated with vectorial competence [37], or it can directly target viral replication within insect tissues [18]. In spite of the fact that both approaches are technically feasible, only the latter has led to substantial reduction in the development of any human pathogen [18].

Gene silencing via dsRNA was first demonstrated with *An. stephensi* expressing sense and anti-sense RNAs targeting EGFP [11]. EGFP dsRNA-expressing mosquitoes were crossed with a transgenic mosquito line that expresses EGFP. The double transgenic offspring displayed lower level of EGFP expression than the parental line expressing it, indicating the effect of the RNAi machinery reducing the expression of the EGFP transgene [11]. *Ae. aegypti* expressing dsRNA targeting *RELI*, a gene involved in innate immune response, also inhibited expression of *RELI* via RNAi [37].

As indicated, the activation of the RNAi pathway is intended to affect the replication of infecting RNA viruses transmitted by mosquitoes. *Ae. aegypti* expressing DEN2 sense and antisense RNAs reduced viral load by fivefold, confirming the effectiveness of the RNAi in controlling virus replication in disease vectors [18].

2.5. Challenges and perspectives on vector transgenesis

Despite advances in the development of stable lines of genetically modified disease vectors [8,12,29,31], many challenges exist to the application of transgenesis to control vector-borne diseases outside the laboratory. Beyond issues dealing with social and environmental impact (s) that are inherent to the potential use of genetically modified organisms (not the scope of this review), of significance also is the fact that, for example, blockage of *Plasmodium* development by transgenic mosquitoes was achieved only for non-human parasites. One such

case involved a non-natural mosquito-parasite pair (*An. stephensi*-*P. berghei*) [12,13], while other were obtained using the avian malaria *P. gallinaceum* transmitted by *Ae. aegypti* or *Ae. fluviatilis* [19,20]. Strains of transgenic mosquitoes blocking development of human malaria parasites (such as *P. falciparum* and *P. vivax*) have yet to be developed [41]. The only exception of a transgenic insect robustly impairing development of a human pathogen is the transgenic strain of *Ae. aegypti* capable of inhibiting DEN virus development [18].

Fitness of transgenic mosquitoes in natural habitats is also an important issue. Laboratory tests demonstrated that in four lines of transgenic *An. stephensi* the frequency of transgenic individuals declined over time [42]. Although SM1-transgenic hemizygous *An. stephensi*, carrying a single transgene copy in the genome, exhibited higher fitness than wild type when fed on infected mice [43], transgenic homozygous *An. stephensi* (harboring two transgene copies), possibly advantageous for field releases, exhibited lower fitness than non-transformed mosquitoes [44]. Transgenic lines of *Ae. aegypti* expressing either EGFP or a transposase also exhibited lower fitness than wild type [45].

Although fitness in natural habitats is one of the main constraints of transgenic disease vectors, mathematical models suggest that a highly efficient transposon can spread through natural populations if it affects fitness by less than 50% [46,47]. Nevertheless, pathogen refractoriness needs to be at or very close to 100% to substantially decrease disease prevalence in high endemic areas [48]. Future studies mimicking field conditions likely will uncover the importance of fitness to the establishment of transgenic mosquitoes in natural habitats.

Problems also are associated with transposons as genetic drive systems for transgenes. Transposons can remobilize in somatic tissues possibly causing damage in regions of the genome [49]. A mechanism to drive transposase expression and restrict gene drive system activity to germ-line tissues was created using the regulatory sequence of *nanos*, a gene involved in early embryonic development [50]. Interestingly, none of the transposable elements (*Hermes*, *Mos1*, *minos*, and *piggyBac*) appears to remobilize in *Ae. aegypti* germ line, possibly reflecting a resistance mechanism, since the same elements can remobilize in *Drosophila* germ line tissues [51].

Further issues regarding the design and potential field release of transgenic disease vectors include: i) non-canonical transposition reactions, such as transgene insertion by a mechanism other than cut-and-paste, resulting in integration of donor plasmid fragments into the insect genome, as observed in transposition events accomplished by the transposons *Hermes*, *Mos1*, and *piggyBac* in *Ae. aegypti* [51]; and ii) transgene size influencing transposon activity, as shown for *Mariner* [52]. Another issue is the possibility of horizontal transfer of the transgene between mosquito sibling species, as proposed for the introgression of the *P*-element between *Drosophila* lines [53]. Horizontal transfer also can be virus mediated, such as the case of *piggyBac*, initially identified in a *Tricoplusia ni* virus [54]. Technology to prevent the potential horizontal transposon transfer by viruses and to inhibit transposition activity mediated by endogenous transposases still needs to be developed.

Other gene-drive mechanisms have been developed to assist with problems associated with transposon elements in insect germ-line transformation [55]. Recently, a driving mechanism known as *Medea* (maternal-effect dominant embryonic arrest) was shown capable of driving population replacement in *Drosophila* without an apparent fitness cost [56]. This gene drive system consists of a DNA segment encoding a protein lethal to insects and an antidote that neutralize the lethal protein. A heterozygous female (*Medea*⁺) expresses the toxin within all oocytes, killing all the ^{+/+} offspring as they do not express the antidote to neutralize the maternal toxin. *Medea* can be designed to restrict transgene activity to the host species through the utilization of siRNAs-encoding genes as toxin genes [56]. Although *Medea* has been postulated

to function in *An. gambiae* population replacement, it has yet to be developed for mosquitoes [57].

Transgenic insects also can be developed to express female dominant-lethal genes to reduce the number of females in an insect population [58,59]. RIDL, or release of insects carrying a dominant lethal, was originally designed to overcome issues associated with SIT (sterile insect technique). Although SIT was successfully applied against the screwworm fly *Cochliomyia hominivorax* [60], the fruit fly *Ceratitis capitata* [61], and the tsetse fly *G. austeni* [62], drawbacks such as reduced sterile male fitness and sterile female contamination, were detected [59]. RIDL consists of release of transgenic male insects expressing the female dominant-lethal genes, causing a reduction on the numbers of females in the following generations [61]. Robust transgenic vectors approaches could also be used with RIDL [59]. In fact, a transgenic mosquito sexing-system has already been developed [15]; however, RIDL requires a reliable genetic transformation system not yet available for mosquitoes.

2.6. Future directions on vector transgenesis

In our view, two hurdles to the establishment of an efficient transgenic vector approach are the lack of a transgene(s) that effectively reduce pathogen load, and the inefficiency of transposons as gene-drive mechanism(s). Further studies utilizing QTL mapping, reverse genetics, gene knockdown, or other techniques to identify traits associated with vector competence can unveil candidate genes that, when targeted, may effectively block pathogen development and transmission. The availability of gene drive mechanisms to overcome issues associated with the use of transposons, such as remobilization, fitness load, and the potential to introgress to closely related species, also is of interest. *Medea* was suggested as a gene drive that could overcome such issues, but it is yet to be developed for insect vectors. As for RIDL, the current absence of a gene drive mechanism also prevents its application against insect vectors.

Clearly, much work remains before genetically modified insect vectors are released into natural habitats. When realized transgenesis may provide a significant tool in the fight against vector-borne diseases.

3. Paratransgenesis to reduce vector competence

Paratransgenesis refers to the use of genetically modified symbiotic organisms expressing molecules that can block pathogen development or transmission by vectors. Bacteria symbionts of blood sucking bugs [21-23], tsetse flies [24], and mosquitoes [25,26], and symbiotic viruses of *An. gambiae* [27] and *Ae. aegypti* [28,63,64], have been used (Figure 2). Current data indicate that symbionts expressing molecules targeting pathogen development have the potential to reduce transmission in endemic regions, and appear unrelated to any fitness load [21,24]. As with transgenesis, spread of transformed symbionts also would benefit from the availability of a gene drive system to replace non-transformed symbionts present in natural vector populations.

3.1. Transformation of reduviids, tsetse, and mosquitoes with bacterial symbionts

Paratransgenesis in disease vectors was demonstrated through the expression of cecropin A by *Rhodococcus rhodni* within the midgut of the kissing bug (reduviid) *Rhodnius prolixus* [21]. A 99% reduction in the intensity of *Trypanosoma cruzi* infection in the hindgut of *R. prolixus* was observed without interfering with insect fitness. Additionally, transformed symbionts were shown to be horizontally transmitted to *R. prolixus* carrying non-transformed symbionts via reduviid coprophagic habits [21]. Subsequently, functionally active antibody fragments also were successfully expressed in the guts of *R. prolixus* [22] and *Triatoma*

infestans [23] utilizing symbionts. Transformed symbionts were stably maintained within the gut of the insects without need for antibiotic selection [21-23].

Paratransgenesis also appears to be a promising strategy to reduce African trypanosomes transmission by tsetse flies. Genetically transformed *Sodalis*, a symbiont of tsetse flies commonly found in the midgut and hemolymph of *Glossina m. morsitans*, *Glossina p. palpalis*, *Glossina austeni*, and *Glossina brevipalpis*, and the salivary glands of *Gl. p. palpalis*, is transmitted vertically via the female milk glands [24,65,66]. Vertical transmission was demonstrated with GFP-transformed (*recSodalis*) that was detected in 9 out of 12 F₁ offspring and eight out of 12 F₂ descendents, indicating the ability of the transformed symbiont to be spread across tsetse populations [24]. In addition, when *Sodalis* originally isolated from *Gl. m. morsitans* and *Gl. fuscipes* was transformed with GFP, the *recSodalis* obtained colonized septic non-native tsetse host species at a density similar to a native colonization and without reducing host fitness [66].

Recently, symbiotic bacteria also were isolated from *An. stephensi* and their application in paratransgenesis was suggested [25,67]. In one case, *Asaia* sp. bacteria were successfully transformed with GFP-expressing plasmids and used to re-infect adult mosquitoes through sugar or blood meals mixed with the transformed bacteria [25]. Larval stages also were able to pick up the transformed bacteria from their aquatic environment in the laboratory. Transformed *Asaia* were found in mosquito organs which are sites for pathogen development, such as midgut and salivary gland, as well as in male and female reproductive tracts, thus *Asaia* may represent a candidate for malaria control [25]. Further, Damiani et al [68] demonstrated that *Asaia* are transmitted from male to female *An. stephensi* during mating (venereal transmission), and the bacteria acquired are vertically transmitted to offspring. This transmission route was confirmed by the use of *Asaia* carrying either a DsRed or GFP fluorescent protein gene inserted into the bacteria genome. GFP and Ds-Red-labeled *Asaia* were fed (mixed with the sugar meal) to male mosquitoes that were later transferred to a cage with virgin females. After mating, the females were transferred to a new cage; blood fed and allowed to oviposit. Resulting male and females (F₁) were later investigated and found to carry the DsRed (63%) or GFP (27%) labeled bacteria. Besides the fact that this study unequivocally demonstrated paternal transmission of symbionts in *An. stephensi*, it also lends significant support to the possibility of using paratransgenesis with male, non-biting mosquitoes to spread anti-pathogen gene-expressing symbionts to female mosquitoes [68].

In a separate study, bacteria were tested for their ability to express anti-plasmodium molecules in mosquito midgut [67]. Firstly, *Escherichia coli* expressing the anti-plasmodium molecules SM1 and phospholipase-A (PLA2) were successfully produced and able to inhibit *P. berghei* development in *An. stephensi* midguts. However, survival of the transformed bacteria was poor, and did not last more than three days after a blood meal. According to the same report [67], *Enterobacter agglomerans*, a bacteria associated with *An. stephensi*, was isolated and selected for survival in the mosquito midgut by multiple passages, increasing their survival from 2 days to two weeks [67]. As non-pathogenic, wide spread bacteria, *A. agglomerans* can potentially be applied in paratransgenesis approaches against *Plasmodium*.

3.2. Transformation of mosquito viral symbionts

Symbiotic densovirus also can be genetically manipulated to express molecules to reduce vector competence. Densoviruses are linear single-stranded DNA viruses with the genome packaged in a non-enveloped particle. These viruses are suitable vectors for expression of foreign genes in mosquitoes because they are highly specific, environmentally stable, kill mosquito larvae in a dose-dependent manner, decrease lifespan of surviving adults, and are transmitted vertically [63,64]. In *Ae. aegypti*, densoviruses can spread to fat body, muscles, and nerves [28] following infection through the anal papillae. Densoviruses infecting *Ae.*

aegypti (AeDNV) and *An. gambiae* (AgDNV) were isolated and modified to express GFP [27,28]. The green phenotype obtained by the expression of GFP in recombinant AgDNV-infecting *An. gambiae* was observed in 20% of F2 and F3 generations, suggesting that transformed densoviruses may be used to express molecules targeting pathogen development in mosquitoes [27].

3.3. Challenges and perspectives on vector paratransgenesis

Although transformation of species-specific bacteria symbionts can overcome the potential problems associated with transgene introgression and horizontal transgene transfer, only symbionts of obligatory blood-sucking insects, such as tsetse and triatomine bugs, have been transformed [21,24]. In spite of the low number of bacteria symbionts transformed to date, a potential advantage of this approach over transgenesis is lack of fitness load [21,66]. Species-specificity and fitness load associated with mosquito bacterial symbionts have yet to be assessed [25,26].

Alternatively to the transformation of bacterial symbionts, genetically modified symbiotic viruses can be used against pathogen development. Densoviruses efficiently express heterologous proteins in *An. gambiae* and *Ae. aegypti* and are transmitted vertically [27,63]. As demonstrated for bacterial symbionts [22,23], viral symbionts can be engineered to express single chain antibodies (scFv), blocking pathogen development [69]. Sindbis virus expressing a scFv targeting the *P. gallinaceum* circumsporozoite (SZ) protein reduced *Ae. aegypti* salivary gland infection by 96.8% [69].

Recombinant Sindbis expressing transcripts from an infecting virus genome can reduce viral load (of the infecting virus) in mosquitoes [70,71]. The results from Sindbis expressing transcripts from LaCrosse (LAC), dengue (DEN), or yellow fever (YF) viruses indicated a substantial interference with the replication of these viruses in *Aedes triseriatus* (LAC) and in *Ae. aegypti* (DEN and YF) [70,72-74].

Although the use of Sindbis may not be feasible in paratransgenesis due to its reduced specificity (Sindbis is capable of infecting a broad range of insect and vertebrate species) [64,75], scFVs or transcripts from infecting viruses can be used to transform species-specific viruses, such as densovirus.

Viral paratransgenesis also can be used to target endogenous genes involved in pathogen development, as demonstrated by *Armigeres subalbatus* infected with Sindbis virus transiently expressing RNA complementary to the mosquito *phenoloxidase* gene [76]. Phenoloxidase is one of the enzymes of the melanization cascade [77] and is upregulated upon infection. In *A. subalbatus*, upregulation of phenoloxidase kills infecting *Dirofilaria immitis* [76]. In contrast, inhibition of *phenoloxydase* expression by Sindbis virus expressing *phenoloxydase* complementary RNA, allowed for the development of *D. immitis* in these mosquitoes [76].

3.4. Future directions on vector paratransgenesis

Despite early successes with transformation of insect vector symbionts, it is not known if transformed symbionts can replace non-transformed in natural insect populations, and potentially affect pathogen development and transmission in natural habitats. Symbionts seem to have no fitness load on insect hosts and are capable of being transmitted vertically (via trans-ovarian transmission) or laterally (due to feeding habits). Thus, a strong gene drive system can potentiate the effectiveness of paratransgenesis. *Wolbachia* endosymbionts have been proposed as such gene drive system [65].

Wolbachia are intracellular, maternally inherited bacteria that manipulate reproduction of insects via cytoplasmic incompatibility (CI) [55]. Due to the effects of CI, a *Wolbachia*-

uninfected female will not breed with infected males, reducing the frequency of uninfected individuals and increasing the frequency of *Wolbachia*-infected insects in a population [55]. Thus, other maternally inherited transformed symbionts would be spread within an insect population in association with *Wolbachia* [65], increasing the frequency of the transformed symbiont. This mechanism has been observed in *Ae. aegypti*, *Aedes albopictus*, and *Culex quinquefasciatus* [55], representing a potential manner to spread transformed symbionts, such as densovirus, in natural populations of mosquitoes.

Recently, a life-shortening strain of *Wolbachia* (*wMelPop*) identified in *D. melanogaster* was used in *Ae. aegypti* and *An. gambiae* [26,78]. Beyond promoting the spread of the transformed symbiont across the mosquito population (i.e., acting as a gene drive mechanism), this strain of *Wolbachia* would also reduce the time frame available (i.e., mosquito life span) for pathogen development within the mosquito (known as the extrinsic incubation period or EIP) [78]. However, a potential application of *wMelPop* to eliminate disease vectors may be faced with a significant drawback: this approach might be associated with the ability of the pathogen to overcome the reduced vector life span for its own development, thus adapting a shorter developmental time [79]. In contrast to this suggestion, *Wolbachia*, as well as densovirus, potentially target older mosquitoes over younger ones, and are considered evolution-proof mosquitoicidal biocontrols agents [80]. For *Anopheles*, due to high mortality in natural conditions (from 20% to 40% per gonotrophic cycle, [81]), a selective pressure on pathogen developmental time already exists, especially in the case of *Plasmodium*-infected mosquitoes [80]. Thus, such selective pressure from the additional of *Wolbachia* is likely not strong enough to shorten the parasite life cycle within the vector.

Reduction of vector survival interferes exponentially with vectorial capacity [82,83] as the time for pathogen development within the vector is significantly shortened. An ideal approach should reduce vector competence (linear parameter), and vector survivorship (exponential parameter). These two effects combined may significantly reduce vectorial capacity and disease burden in endemic areas. A symbiont, such as *wMelPop*, in combination with a genetically modified densovirus capable of inhibiting pathogen development may provide just the double-whammy needed to prevent transmission.

4. Conclusion

Here we described the current status of two molecular strategies to control vector borne diseases and highlighted many challenges facing these approaches. Several studies still need to be performed before transgenesis and paratransgenesis can be considered against vector-borne diseases. Regardless of the approach, it is clear to many investigators that new technologies must be added to current applications if we are to be successful in preventing or significantly reducing the burden of vector-borne diseases.

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Transgenesis

Figure 1. Transgenesis

The procedure to generate transgenic insects by germ line transformation is depicted. **(I)** Insect eggs are microinjected with a donor plasmid, expressing a transgene (in orange) and a reporter gene (in green), and with a helper plasmid, expressing a transposase (purple). **(II)** After inoculation into the eggs, plasmids are taken up by some (or all) of the germ line cells. **(III)** Transformed larvae can be distinguished from non-transformed due to the expression of the reporter gene (green eye phenotype), which can be controlled by an eye specific promoter. **(IV)** Transformed insects are crossed with wild type specimens so as to confirm that the transposon-carrying transgene was inserted into the insect chromosome. **(V)** Transgenic adult insect expressing the transgene (e.g., orange proteins in the insect midgut) is depicted.

Paratransgenesis

Figure 2. Paratransgenesis

(B) Paratransgenesis. The procedure of insect transformation via transgenic symbionts is depicted. Here two orders are represented: reduviids (Hemiptera) on the left panel and mosquitoes (Diptera) on the right panel. **(I)** Bacteria or viruses symbionts can be genetically modified to express a gene blocking parasite development in vectors' tissues. **(II)** Symbiotic bacteria are transformed with plasmids (blue) expressing a gene (green) to inhibit parasite development in insect gut. Alternatively, viral genome (red) is inserted into a plasmid (blue) and manipulated to express a transgene (green). Viral particles can be generated by expression of such a plasmid in insect cells. **(III)** The transformed symbionts are acquired by insect hosts through larvae or nymph feeding, or through thoracic injection. **(IV)** Once insects acquire the

transformed symbionts, these microorganisms can express proteins to inhibit pathogen development.

Table 1

Germ line transformed mosquitoes

Insect species	Transposon	Promoter	Reporter gene	Transgene	Targeted pathogen	Reference
<i>Ae. aegypti</i>	<i>Hermes</i>	<i>D. melanogaster</i> Cinnabar	<i>D. melanogaster</i> Cinnabar	Cinnabar	-	[3]
	<i>Mos1</i>	<i>D. melanogaster</i> Cinnabar	<i>D. melanogaster</i> Cinnabar	Cinnabar	-	[5]
	<i>Hermes</i>	<i>Ae. aegypti</i> maltese-like 1 / apyrase	Firefly luciferase	Firefly luciferase	-	[37]
	<i>Hermes</i>	<i>D. melanogaster</i> Actin 5C	EGFP	EGFP	-	[6]
	<i>Hermes</i>	<i>Ae. aegypti</i> vitellogenin	<i>D. melanogaster</i> Cinnabar	Defensin A	<i>Micrococcus luteus</i>	[28]
	<i>Hermes</i>	<i>Ae. aegypti</i> carboxypeptidase	Firefly luciferase	Firefly luciferase	-	[29]
	<i>Mos1</i>	<i>An. gambiae</i> carboxypeptidase	Firefly luciferase	Firefly luciferase	-	[29]
	<i>piggyBac</i>	<i>Ae. aegypti</i> vitellogenin	EGFP	Defensin A	<i>Enterobacter cloacae</i> / <i>P. gallinaceum</i>	[20,30]
	<i>Minos</i>	<i>D. melanogaster</i> Actin 5C	dsRED	dsRED	-	[9]
	<i>piggyBac</i>	<i>D. melanogaster</i> Actin 5C	dsRED	dsRED	-	[9]
	<i>piggyBac</i>	<i>D. melanogaster</i> Cinnabar	<i>D. melanogaster</i> Cinnabar	Cinnabar	-	[27]
	<i>Mos1</i>	<i>D. melanogaster</i> Cinnabar/hsp70	<i>D. melanogaster</i> Cinnabar/dsRED	Cinnabar/dsRED	-	[80]
	<i>piggyBac</i>	<i>D. pseudoobscura</i> hsp82	EGFP	<i>Mos1</i> transposase	-	[80]
	<i>piggyBac</i>	<i>Ae. aegypti</i> vitellogenin	EGFP	Δ REL ^a	-	[36]
	<i>piggyBac</i>	<i>Ae. aegypti</i> vitellogenin	EGFP	Cecropin A	<i>Enterobacter cloacae</i>	[36]
	<i>piggyBac</i>	<i>Ae. aegypti</i> vitellogenin	EGFP	Δ REL1-A ^b	-	[35]
	<i>piggyBac</i>	<i>Ae. aegypti</i> vitellogenin	EGFP	dsRNA against REL1 transcripts	-	[35]

Insect species	Transposon	Promoter	Reporter gene	Transgene	Targeted pathogen	Reference
<i>Ae. aegypti</i>	<i>Mos1</i>	<i>An. gambiae</i> carboxypeptidase	EGFP	dsRNA against <i>prM</i> of DENV-2	<i>Dengue-2 virus</i>	[18]
	<i>Mos1</i>	<i>Ae. aegypti</i> <i>nos</i>	dsRED	<i>Mos1</i> transposase	-	[48]
	<i>Mos1</i>	<i>Ae. aegypti</i> <i>D7</i>	EGFP	<i>Gal4</i>	-	
<i>Ae. fluviatilis</i>	<i>piggyBac</i>	<i>An. gambiae</i> <i>peritrophin</i>	EGFP	<i>mPLA2</i>	<i>P. gallinaceum</i>	[19]
<i>An. albimanus</i>	<i>piggyBac</i>	<i>D. melanogaster</i> <i>polyubiquitin</i>	EGFP	EGFP	-	[8]
<i>An. gambiae</i>	<i>piggyBac</i>	<i>Baculovirus</i> <i>hr5-ie1</i>	EGFP	EGFP	-	[7]
	<i>piggyBac</i>	<i>Ae. aegypti</i> carboxypeptidase	EGFP	<i>Cecropin A</i>	<i>P. berghei</i>	[14]
<i>An. stephensi</i>	<i>minos</i>	<i>D. melanogaster</i> <i>actin 5C</i>	EGFP	EGFP	-	[16]
	<i>piggyBac</i>	<i>D. melanogaster</i>	dsRED	dsRED	-	[9]
	<i>piggyBac</i>	<i>An. gambiae</i> carboxypeptidase	EGFP	<i>PLA2</i>	<i>P. berghei</i>	[13]
	<i>piggyBac</i>	<i>An. gambiae</i> carboxypeptidase	EGFP	<i>[SM1]4</i>	<i>P. berghei</i>	[12]
	<i>minos</i>	<i>D. melanogaster</i> <i>actin 5C</i>	dsRED	dsRNA against EGFP	-	[11]
	<i>minos</i>	<i>An. gambiae</i> <i>apyrase</i> <i>An. gambiae D7r4</i>	EGFP	<i>LacZ</i>	-	[34]
	<i>piggyBac</i>	<i>An. gambiae</i> <i>peritrophin</i>	EGFP	<i>PLA2</i>	<i>P. berghei</i>	[10]
	<i>piggyBac</i>	<i>D. melanogaster</i> β 2-tubulin	dsRED	EGFP	-	[15]
	<i>piggyBac</i>	<i>An. stephensi</i> vitellogenin	EGFP	<i>CFP</i>	-	[31]
<i>An. stephensi</i>	<i>minos</i>	<i>An. stephensi</i> <i>antiplaetel</i>	EGFP	dsRED	-	[32]
	<i>piggyBac</i>	<i>An. gambiae</i> vitellogenin	dsRED	EGFP	-	[33]
	<i>piggyBac</i>	<i>D. melanogaster</i> <i>hsp70</i>	dsRED	<i>minos</i> transposase	-	[81]

Insect species	Transposon	Promoter	Reporter gene	Transgene	Targeted pathogen	Reference
	<i>minos</i>	<i>D. melanogaster</i> <i>actin 5C</i>	<i>EGFP</i>	<i>EGFP</i>	-	
<i>Cx. quinquefasciatus</i>	<i>Hermes</i>	<i>D. melanogaster</i> <i>actin 5C</i>	<i>EGFP</i>	<i>EGFP</i>	-	[17]

^a*Drosophila* Relish-related gene lacking the transactivator domain.

^bREL1-A lacking a C-terminal domain.