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Current Opinion in Insect Science

Genetic technologies for disease vectors

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The first genetic technologies for insect vectors of disease were introduced 20 years ago. As of today there are 12 classes of genetic technologies used as functional genomic tools for insect vectors of important diseases. Although the applications of genetic technologies in insect disease vectors have been conducted primarily in mosquitoes, other insect systems could benefit from current technologies. While the various technological platforms are likely to function in diverse arthropods, the delivery of these technologies to cells and tissues of interest is the major technical constraint that limits their widespread adoption. Increased community resources of various types would enhance the adoption of these technologies and potentially eliminate technical limitations.

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Current Opinion in Insect Science 2015, **10**:90–97

This review comes from a themed issue on **Vectors and medical and veterinary entomology**

Edited by **Nora J Besansky**

For a complete overview see the Issue and the Editorial

Available online 12th May 2015

http://dx.doi.org/10.1016/j.cois.2015.04.012

Introduction

Technological advances in DNA sequencing technologies and bioinformatics are leading to a deep understanding of the biology, genetics and evolution of insect vectors of human parasites and pathogens. Genome sequence data are now available for some 20 species of mosquitoes and many of the major non-mosquito vectors of human parasites and pathogens, for example, tsetse fly, lice, triatomines, sand fly, and ticks [1]. Although there have been great advances in insect disease vector genomics over the past 20 years, we have just scratched the surface of these systems and to go deeper researchers will need genetic technologies that will allow them to manipulate the genomes of these insects, enabling them to more fully explore the functions of genes, genetic networks and physiological systems. Powerful genetic technologies that enable the functional genomic analysis of model plant and animal systems are evolving rapidly and many have been used successfully in some insect vectors of disease. Here we review briefly genetic technologies that are currently available with an emphasis on those that have been applied to insect vectors of disease. We also note technologies that could be used in insects and discuss the factors that limit the adoption of genetic technologies in some cases and how those limitations might be eliminated.

Technologies for moving genetic technologies into genomes

Some genetic technologies are useful even when they are delivered to only somatic cells and this usually involves injecting DNA, RNA and proteins directly into the hemocoel of the insect either with or without a transfecting agent to promote cellular uptake. Injecting large quantities of double-stranded RNA into the hemocoel of insects for the purposes of silencing gene expression has been widely effective in insect vectors of disease although this approach is not without limitations [2]. Cornel *et al.* reported transfection of somatic cells of *Aedes aegypti* by simply injecting transgene-containing plasmids into adults and Isoe *et al.* reported regulated expression of plasmid borne transgenes up to four weeks after injection [3,4]. Peng *et al.* reported similar results but included a transfecting reagent in the injection cocktail that resulted in sufficient uptake of DNA and RNA by developing oocytes of *Ae. aegypti* to alter gene expression in the larvae and adults subsequently developing from those oocytes [5•]. Transgenerational effects following *in vivo* transfection have also been reported in ticks [6•]. Feeding dsRNA to larvae or adults has also been a successful delivery strategy in some cases [7•].

Delivery of genetic technologies to germ cells to create heritable changes in genomes is much more challenging and the technical options available are currently limited to direct micro-injection of early-stage insect embryos. Injection of early-stage embryos allows DNA, RNA and protein-based technologies to be incorporated into embryonic cells that will go on to form the germ line, resulting in some cases

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in heritable changes in the genome. Alternatives to microinjection of early-stage embryos such as biolistics and electroporation have been reported and while somatic delivery using these methods could be confirmed, germline modifications have not been reported in vector insects following these modes of delivery [8,9].

Integrating DNA into insect chromosomes can be accomplished with the use transposons, site-specific recombinases and homologous recombination. Other than mosquitoes, no other insect vectors have been genetically transformed using these technologies. A robust and versatile collection of transposon-based gene-vectors is available to insect scientists with *piggyBac*-based gene-vectors being very popular and effective, and *Hermes*-, *Mos1*-, *Minos*-and *Tn5*-based gene-vectors proving to be effective in a few species of mosquito. For example, *Mos1* and *Hermes* are effective gene-vectors in *Ae. aegypti* [10,11••], but there are no reports of their successful use in *Anopheles*. *Hermes* was used successfully as a gene-vector in *Culex quinquefasciatus* [12], and *Minos* is functional as a germ line transformation vector in *Anopheles stephensi* [13] but apparently not in *Ae. aegypti* or *Anopheles gambiae* (see Figure 2 for transposase usage). Regardless of these species-specific limitations, insect scientists are no longer limited by the availability of sufficiently active transposon-based gene vectors for introducing transgenes into the genomes of insect vectors of disease.

Site-specific recombination systems including the ΦC31, Flp/FRT and Cre/Lox systems are powerful systems for integrating and removing transgenes from genomes as well as for creating chromosomal deletions and rearrangements. The ΦC31 system has been used successfully in disease vectors and a potential advantage of this system over transposon-based gene-vectors is the invariant position into which transgenes are integrated [14,15,16••]. If recombination target sites or landing sites are in appropriate genomic positions variance in transgene expression arising from regional differences in chromatin structure and local enhancers that are encountered by randomly inserting transposons can be avoided. However, transposon-based gene-vectors are essential in using the ΦC31 system, for example, because appropriate recombination target sequences must first be integrated into the host's genome prior to their use as transgene integration sites. Other strategies for reducing integration site-dependent variance in transgene expression include flanking transgenes with insulator sequences prior to their introduction into genomes by either transposon-based gene vectors or site-specific recombination [15,16••,17].

Transgenes can also be integrated into chromosomes by homologous recombination that is stimulated by double-stranded breaks in chromosomal DNA. Meganucleases (homing endonucleases) such as *Y2-I-AniI*, *I-CreI* and *I-SceI* can be used to create double-stranded breaks in chromosomal DNA at prescribed sites however the target sites of these endonucleases must be introduced into the genome using transposon-based gene vectors or site-specific recombination. Double-stranded DNA breaks at prescribed sites can stimulate homologous recombination with transgene DNA that is flanked by sequences homologous to the prescribed target sites. The *I-SceI* system has been successfully used in *An. gambiae* [18] while the *Y2-I-AniI*, *I-CreI* and *I-SceI* systems are known to function in *Ae. aegypti* [19]. Tunable endonucleases such as zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and clustered regularly interspaced short palindromic repeats (CRISPRs)/Cas9 can be used to created double-stranded breaks at almost any position in a genome allowing any transgene or DNA sequence to be seamlessly incorporated into a genome. The CRISPR/Cas9 system has been used to integrate transgenes into the genome of *Ae. aegypti* by homologous recombination [20•].

Technologies for modifying genes in genomes

Modifying genes within the genomes of disease vectors with varying degrees of precision is now possible. The *piggyBac* transposon can be used to mutagenize the genome of *An. stephensi* following its remobilization from an initial genomic location resulting in some cases in loss-of-function mutations [21]. Highly precise modifications of insect genomes are now possible using robust gene editing technologies whose essential biochemistries are completely host independent (ZFNs, TALENs, CRISPRs). Although their functionality in diverse species is not in doubt, there have been few reported applications of these technologies in vectors of disease. ZFNs have been used to create a null allele of the gene encoding a subunit of the heteromeric CO_2 receptor in *Ae. aegypti* (*AaegGr3*), resulting in mosquitoes lacking the ability to sense and respond to CO_2 [22•]. TALENs have been used to create mutant alleles of *TEP1* in *An. gambiae* [23] and resulted in mosquitoes with compromised immune responses to *Plasmodium* infections, and were also used to create eye pigmentation mutants in *Ae. aegypti* [24]. More recently, the CRISPR/Cas9 gene editing system has been used to modify genes in *Ae. aegypti* [20•].

Technologies for regulating gene expression

Controlling transgene expression is essential in genetically modified insects and for disease vectors there are a number of options that have been successfully developed and applied. The most popular and widely used technology in arthropods is RNA interference-based gene silencing. Injection of large amounts of double-stranded RNA (~500 bp) identical to the target mRNA into the hemocoel of immature-stage or adult-stage insects can trigger the specific destruction of targeted messages-gene silencing. This technology has been successfully employed in larval-stage, pupal-stage and adult-stage