

Gene Editing in *Aedes aegypti*

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Abstract

Aedes aegypti (*Ae. aegypti*) is one of the most important vectors of dengue, chikungunya and yellow fever viruses. The use of chemical control strategies such as insecticides is associated with problems including the development of insecticide resistance, side effects on animal and human health, and environmental concerns. Because current methods have not proven sufficient to control these diseases, developing novel, genetics-based, control strategies to limit the transmission of disease is urgently needed. Increased knowledge about mosquito-pathogen relationships and the molecular biology of mosquitoes now makes it possible to generate transgenic mosquito strains that are unable to transmit various parasites or viruses.

Ae. aegypti genetic experiments are enabled, and limited by, the catalog of promoter elements available to drive transgene expression. To find a promoter able to drive robust expression of firefly (FF) luciferase in *Ae. aegypti* embryos, an experiment was designed to compare *Ae. aegypti* endogenous and exogenous promoters. The *PUB* promoter was found to be extremely robust in expression of FF luciferase in different stages of embryonic development from 2-72 hours after injection. In subsequent experiments, transformation frequency was calculated using four different promoters (*IE1*, *Ub_{L40}*, *hsp82* and *PUB*) to express the *Mos1* transposase open reading frame in *Mos1*-mediated transgenesis. Germline transformation efficiency and size of transgenic cluster were not significantly

different when using endogenous *Ae. aegypti PUB* or the commonly used exogenous *Drosophila hsp82* promoter to express *Mos1* transposase.

This study also describes the development of new tools for gene editing in the *Ae. aegypti* mosquito genome and the use of these tools to design an efficient gene drive system in this mosquito.

Homing endonucleases (HEs) are selfish elements which catalyze double-stranded DNA (dsDNA) breaks in a sequence-specific manner. The activities of four HEs (Y2-I-*AniI*, I-*CreI*, I-*PpoI*, and I-*SceI*) were investigated for their ability to catalyze the excision of genomic segments from the *Ae. aegypti* genome. All four enzymes were found to be active in *Ae. aegypti*; however, the activity of Y2-I-*AniI* was higher compared to the other three enzymes. Single-strand annealing (SSA) and non-homologous end-joining (NHEJ) pathways were identified as mechanisms to repair HE-induced dsDNA breaks.

TALE nucleases (TALENs) are a group of artificial enzymes capable of generating site-specific DNA lesions. To examine the ability of TALENs for gene editing in *Ae. aegypti*, a pair of TALENs targeted to the *kmo* gene were expressed from a plasmid following embryonic injection. Twenty to forty percent of fertile G₀ produced white-eyed progeny which resulted from disruption of the *kmo* gene. Most of these individuals produced more than 20% white-eyed progeny, with some producing up to 75%. A small deletion of one to seven bp occurred at the TALEN recognition site.

These results show that TALEN and HEs are highly active in the *Ae. aegypti* germline and can be used for gene editing and gene drive strategies in *Ae. aegypti*.

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Attribution

Several colleague aided in the writing and research behind two of my chepters presented as part of this dissertation. A brief description of their contribution is included here:

Chapter 3: Catalyzing double-stranded DNA breaks and excision of transgenes in *Aedes aegypti* germline by homing endonucleases

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Chapter 1. Introduction

1.1 Public health impact

Arthropod-borne pathogens sicken and kill millions annually around the world with mosquitoes responsible for the spread of many of these diseases (Breman *et al.*, 2001). *Aedes aegypti* (*Ae. aegypti*) mosquitoes are vectors of several viruses, such as the yellow fever virus and the dengue viruses (Severson *et al.*, 2004). More than 2.5 billion people, mostly in tropical and subtropical regions, are affected by dengue which is increasing in public health importance (Guzman *et al.*, 2010). A global increase in *Ae. aegypti* distribution and dengue virus epidemics has been observed in the last 25 years (Mackenzie *et al.*, 2004). Dengue hemorrhagic fever, the more severe form of dengue, is also increasing (Gratz, 1999, Sutherst, 2004).

For successful viral transmission from invertebrate to vertebrate hosts, viral particles must first infect the cells lining the midgut and then eventually move to the salivary glands to be passed on to a new host during bloodfeeding (Blair *et al.*, 2000). Compared to other mosquito species, *Ae. aegypti* feeds on humans (anthropophilic) during the day and may feed on multiple individuals during a single gonotrophic cycle (Scott *et al.*, 1993). Humidity, temperature, rainfall, photoperiod and wind velocity all have a direct effect on mosquito survival and ecology; however, these variables cannot be considered independently for the transmission of viruses (Patz, 2003). Some other factors, such as the complexity of the vector-pathogen-host system, must be considered (Jansen and Beebe, 2010).

1.2 Vector control strategies

The goal of vector control strategies is to reduce the number of infectious vectors to below a certain threshold, such that the probability of disease transmission is low enough to interrupt the pathogen's lifecycle. Current vector control strategies include the use of chemical pesticides, biological control, and environmental management (Opiyo *et al.*, 2007).

Insecticides/larvicides have been used as a vector control approach (Halstead, 1984, Monath, 1994). Because of economic factors, most insecticides have been developed for use on arthropods of agricultural importance (Marquardt and Kondratieff, 2005). Insect behavior and ecological differences should be considered when applying any insecticide treatments. Resting behavior of the vector is another important element in determining the appropriate use of control methods (Paaijmans and Thomas, 2011). However, at this time, insecticide control strategies can result in increasing insecticide-resistant mosquito populations (Ranson *et al.*, 2000, Christophides, 2005, Vontas *et al.*, 2012).

Two classes of insecticides, organophosphates and pyrethroids, have been used to control *Ae. aegypti* populations. To kill mosquito larvae, treating water supplies with insecticide have been used (WHO, 2006). Space spraying using pyrethroids or organophosphates have been used to reduce the density of adult mosquitoes (WHO, 1997). Use of different insecticide classes for dengue vectors, especially *Ae. aegypti* has probably in the selection for insecticide resistance in this species (Brown, 1986). Resistance to both pyrethroids and organophosphates, has been reported in *Ae. aegypti* in South-East Asia (Jirakanjanakit *et al.*, 2007a, Jirakanjanakit *et al.*, 2007b), South America and the Caribbean (Rawlins *et al.*, 1998, Marcombe *et al.*, 2009). This information demonstrates the need to develop

new tools and strategies for maintaining an effective control of *Ae. aegypti* mosquito populations.

1.3 Genetic control of *Ae. aegypti*

Unlike dengue, there is an effective and affordable yellow fever vaccine; however, providing this vaccine in developing countries is often difficult due to expense and logistics (Harrington *et al.*, 2001, Severson *et al.*, 2004). The high levels of human morbidity and mortality caused by mosquitoes and the failure of current strategies in eradication and control programs makes the use of refractory transgenes an attractive alternative. Genetic manipulation as a vector control method has been discussed since the 1960s (Curtis, 1968). However, most of the experiments associated with genetic technologies have been done in the last 10-15 years (Atkinson *et al.*, 2001).

Increasing knowledge about vectors at a genetic level may help provide valuable information for population control by fueling an understanding of insecticide response, innate immunity, genome evolution, and genetic manipulation of mosquitoes (Blair *et al.*, 2000, Severson *et al.*, 2004).

Population reduction and population replacement are two genetic control strategies that have been proposed. Population reduction aims to decrease the number of mosquitoes to lower the probability of contact between mosquitoes and their human hosts (Terenius *et al.*, 2008). In contrast, population replacement aims to develop a modified strain of vector mosquito that is incapable of disease transmission and then releasing this strain into the target population (Collins and Besansky, 1994, Curtis, 1994, Olson *et al.*, 1996, Olson *et al.*, 2002).

In the 1950's, the United States Department of Agriculture (USDA) implemented a genetic control strategy known as sterile insect technique (SIT) to eradicate the screwworm fly (Gould and Schliekelman, 2004). SIT consists of releasing sterile males to compete with wild-type males for wild-type females, which become refractory after mating. Thus, this technique reduces the reproductive potential of the population with the goal of causing the population to crash. In the case of the screwworm fly, eradication was successful for all of North America and much of Central America (Wyss, 2000).

For mosquitoes, the story is different, with variable successes and problems {reviewed by (Wilke and Marrelli, 2012)}. Since 1970, there have been several field trials to demonstrate that SIT could be used against mosquitoes (Benedict and Robinson, 2003, Dame et al., 2009). SIT has been used to control *Anopheles albimanus* (Lofgren et al., 1974), *Aedes albopictus* (Oliva et al., 2012) and *Ae. aegypti* (Lacroix et al., 2012b). Although several other trials have been described using genetically sterile *Ae. aegypti* (Dame et al., 2009), attempts to use SIT for mosquito population reduction have been less successful because of high density and the frequency of immigration/emigration in mosquito populations. The technique's efficacy is also reduced due to the loss of competitive mating ability relative to wild type (Wood, 2005, Alphey, 2002, Marrelli et al., 2006). Overall, as a possible major tool for vector management, SIT technologies required further optimization for sufficient efficacy to reduce the number of mosquito-borne diseases (Yakob et al., 2008, Alphey and Andreasen, 2002).

To improve the SIT system, the RIDL (Release of Insects Carrying a Dominant Lethal Gene) system was proposed (Thomas et al., 2000). In this

system, a lethal dominant gene is introduced which is under control of a female specific promoter. Expression of this gene could be inactivated by treatment with tetracycline, allowing a colony to be maintained. By removing tetracycline from the system, all of the females will die and RIDL males are released to mate with wild female. The released males are not sterile, but all of the female progeny will carry the dominant lethal gene, and will die; therefore the number of females in the population will be reduced causing the overall decrease in population numbers. {reviewed by (Black Iv et al., 2011). However, the theoretical RIDL strains under the control of the female promoter are not the ones being used in field releases. In the current strategy, males and females are separated manually (pupae sorter) and both males and females would pass on to any progeny a dominant marker resulting in death during the late instar stages of the next generation (Lacroix et al., 2012a). RIDL has been used in *Ae. aegypti* (Alphey and Andreasen, 2002, Alphey, 2002). This technique has been tested in laboratory and/or semi field in Brazil, Malaysia, Mexico and Cayman Islands (Beech CJ, 2011).

One example of a population replacement strategy involves the use of *Wolbachia*, an intracellular bacterium to introduce a target gene into a susceptible population. This bacterium is found in many arthropods and rapidly spreads itself in a population via a mechanism known as cytoplasmic incompatibility (CI). CI is a form of induced sterility within or between populations, whereby *Wolbachia*-infected males can successfully mate with wild-type females; however, their progeny is unviable (McGraw et al., 2001). Releasing *Wolbachia*-infected females, results in an increased percentage of infected individuals in subsequent generations. *Wolbachia*-infected females can mate with both infected and uninfected males resulting in a selective reproductive advantage over uninfected

females (Hoffmann AA, 1997). The eggs produced by infected females can develop normally due to the rescue function (Sinkins and Gould, 2006).

Wolbachia infection also generates pathogen resistance, and there are field trials of this strategy underway. Significant inhibition of dengue virus replication and dissemination resulting in either complete or partial block of viral transmission was observed by artificially introducing one of three types of *Wolbachia* (*wAlbB*, *wMelPop-CLA*, and *wMel*) into *Ae. aegypti* (Walker et al., 2011, Bian et al., 2010, Moreira et al., 2009).

Wolbachia infection also generates an *Ae. polynesiensis* strain which is resistant to dengue virus serotype 2 with a reduced viral infection in the mosquito whole body, midgut, head, and saliva. This strain may also be used in *Wolbachia*-based strategies to control dengue (Bian *et al.*, 2013).

1.4 Transposable elements and insect transformation

Transposable elements, or jumping genes, are DNA sequences that have the ability to change their genomic location (Lisch and Bennetzen, 2011). These elements comprise 15% of the *Drosophila* genome, 45% of the human genome, and 50% of the *Ae. aegypti* genome (Nene *et al.*, 2007, Kidwell, 2002). In 1950, McClintock described these jumping elements in maize (McClintock, 1950). She discovered that genetic elements could move from one place to another within the genome, a process that resulted in the generation of chromosomal breaks. TEs are mobile and capable of rapidly spreading through entire populations. About 30 years ago, spread of the P-element in the world's natural populations of *D. melanogaster* was discovered (Kidwell, 1983).

Jumping genes can be classified into two groups, Class I and Class II. This classification is based on the presence of a DNA/RNA intermediate during transposition. RNA-mediated transposable elements require a reverse transcription step and thus are called Class I or retrotransposons. Class II transposable elements are also called DNA transposable elements, and have a DNA intermediate. They function through a cut-and-paste mechanism, while Class I elements work by a copy-and-paste system (Finnegan, 1992, Eickbush T, 2002). The transposase protein is necessary for catalyzing the excision of the transposon from one site and insertion into another site of the genome. Active TEs can be inherited in higher frequency than the Mendelian rate, because these elements can move and increase in copy number following homologous recombination-based repair. Thus, TEs provide a very powerful potential tool as a gene drive mechanism. However, the use of transposable elements for mosquito transformation/gene drive has some real limitations due to their limited carrying capacity, difficulty in remobilization, low transformation efficiency and random integration mechanism that can cause mutations in the target organism (Lorenzen *et al.*, 2002, O'Brochta *et al.*, 2003). Most known Class II TEs prefer a target site like TTAA or TA and these sites are abundant in the genome. A transposable element may function efficiently in one species but inefficiently or not at all in another species (O'Brochta *et al.*, 1996).

The random integration of class II of TEs results in loci that demonstrate position effects (Nimmo *et al.*, 2006), for example integration in heterochromatic region of the genome has different pattern of transgene expression compare to integration close by repressors and/or enhancers of endogenous genes (Wallrath and Elgin, 1995, Sarkar *et al.*, 2006, Anderson *et al.*, 2010).

The first transgenic *Drosophila melanogaster* were generated in the early 1980s (Rubin and Spradling, 1982) and have helped researchers discover and characterize many genes, as well as interactions and regulatory mechanisms at the DNA, RNA and protein levels (Coates, 2005). While the use of P-elements was highly efficient in *Drosophila* genetic transformation, in non-Drosophilid species these elements were not active (Handler *et al.*, 1993). In non-Drosophilids, the low efficiency of P-element-based transgene integration motivated a search for other types of Class II transposable elements. At least four transposable elements have been used to create a transgenic insect: *Mos1* (*mariner*), *Minos*, *Hermes* and *piggyBac* (Atkinson, 2001).

Mos1 (*mariner*) remobilizes efficiently in *Drosophila mauritiana*, however it is inefficient in *D. melanogaster* (Bryan *et al.*, 1987, Lidholm *et al.*, 1993). In 1998, *Mos1* was used for germline transformation in *Ae. aegypti* (Labbe *et al.*, 2010). *Mos1* recognizes a TA recognition site and has a high level of stability (O'Brochta *et al.*, 2003, Wilson *et al.*, 2003a).

Transformation efficiency using *Hermes* transposable element is more than 50% in *Drosophila melanogaster* and can be remobilized in this species; whereas efficiency is less than 10% in *Ae. aegypti*, and remobilization is limited to the soma of this mosquito (O'Brochta *et al.*, 1996, Jasinskiene *et al.*, 1998).

PiggyBac transposable elements are found in the genomes of almost all eukaryotes (Sarkar *et al.*, 2003) and have also been successfully used for germline transformation in *Ae. aegypti* (Kokoza *et al.*, 2001), *Ae. albopictus* (Labbe *et al.*, 2010, Lobo *et al.*, 2001) and *Anopheles albimanus*; however the activity of this TE is less in *Anopheles gambiae* (Grossman *et al.*, 2001). Remobilization using

piggyBac is very efficient in *An. stephensi* (O'Brochta *et al.*, 2011), but rarely occurs in *Ae. aegypti* (O'Brochta *et al.*, 2003).

Minos behavior is less well characterized, however it seems to be similar to the other elements, with evidence for remobilization in *D. melanogaster* but not in mosquitoes (O'Brochta *et al.*, 2003). *Minos* has been used to generate transgenic *An. stephensi* (Catteruccia *et al.*, 2000) but has been used less than other transformation vectors. *Minos* activity in *Anopheles* cells is characterized by unusual functionality of the transposon (Scali *et al.*, 2007)

The technique of generating transgenic mosquitoes typically involves the microinjection of two plasmids, a “donor” and a “helper,” into the posterior end of preblastoderm embryos where the germ cells are initially formed. The donor plasmid carries inverted terminal repeats (ITRs) of the transposon and a marker gene for identifying transgenics, as well as any genes of interest. The helper plasmid consists of a promoter that drives expression of a transposase open reading frame. The expressed transposase protein cuts and moves the ITRs and internal sequences from the donor plasmid and integrates them into target sites within the genome. Successful integration of the target sequence into the germ cells of a parental line results in the inheritance of the transgene in the next generation. Note that since the helper plasmid does not contain ITRs, it therefore cannot integrate into the genome. Thus, transgene integration is only stable if integration occurs in the germline, as the activity of transposase will be lost in subsequent generations (O'Brochta *et al.*, 2011, O'Brochta *et al.*, 2003).

Other methods of introducing transgenes into *Ae. aegypti* include the *Streptomyces* phiC31 recombinase. This system enables integration of inserts larger than the 42.4 kb *Streptomyces* phage genome (Venken *et al.*, 2006). In this

site-specific integration technique, phiC31 integrase mediates recombination between the bacterial attachment site *attB* and the phage attachment site *attP* (Thorpe H. M., 1998). Random transposable element-based integration is required for introducing of *attP* site in the genome of organism. A plasmid containing an *attB* site is coinjected with integrase in to the embryos. These two sites share a 3 bp central region, where the crossover occurs. The phiC31 integrase catalyses integration (*attP/attB* recombination) but not excision (*attL/attR*), therefore the phiC3-based integration technique is unidirectional (Thorpe *et al.*, 2000). One advantage of using this technique is high site specificity (Venken *et al.*, 2006).

The phiC31 system has been used to transform *Ae. albopictus* (Labbe *et al.*, 2010) and *Ae. aegypti* mosquitoes (Nimmo *et al.*, 2006, Franz *et al.*, 2011). Other gene drive tools that may be used include Zinc-Finger-Nucleases (ZFN), Homing Endonucleases (HEs), and Transcription Activator-Like Effector Nucleases (TALENs).

1.5 Promoter driven transgene expression

Drosophila heat inducible promoters are commonly used to drive transposase expression for mosquito transgenesis (Atkinson and Michel, 2002) despite a reduced efficiency in mosquitoes (Morris *et al.*, 1991).

Transformation frequency could be increased by the use of more robust promoters that drive both targeted and spatially limited gene expression (Adelman *et al.*, 2002). Several strong tissue-specific promoters have been identified in *Ae. aegypti* that allow transgene expression in salivary glands, midgut, fat body, or germline tissue (Mathur *et al.*, 2010, Edwards *et al.*, 2000, Kokoza *et al.*, 2000,

Moreira *et al.*, 2000, Adelman *et al.*, 2007). Anderson and colleagues discovered and characterized two sequences upstream of the *Ae. aegypti* ubiquitin genes *Ub_{LA0}* and *PUB*. The *PUB* promoter expressed the transgene in all developmental life stages as well as in the adult female midgut, while *Ub_{LA0}* was highly active in early larvae and ovaries (Anderson *et al.*, 2010). In 2009, Gross *et al.* identified and characterized the expression pattern of *Ae. aegypti hsp70* genes (Gross *et al.*, 2009), and determined the ability of these sequences to drive transient luciferase expression in cell and embryo assays as well as *Ae. aegypti* chromosomal insertions via the transposon *Mos1* (Carpenetti *et al.*, 2011).

1.6 Homing endonucleases

The ability to induce site-specific double-strand DNA (dsDNA) breaks would allow for the study of gene function through targeted gene disruption as well as mechanisms of dsDNA break repair.

Homing endonucleases (HEs) are DNA meganucleases that can recognize specific DNA motifs (14-40bp) and catalyze the formation of a dsDNA break (Marcaida *et al.*, 2008, Hafez and Hausner, 2012). Using the homologous recombination repair system, the chromosome containing the homing endonuclease gene is used as a template to convert the chromosome lacking the HE gene into a chromosome that includes this gene (Burt and Koufopanou, 2004b). This process increases the frequency of HE in subsequent generations to greater than the expected Mendelian frequency of 50%. Though mostly found in bacteria and phages, HE has also been found in eukaryotic organisms such as fungi, plants, and cnidarians (Wessler, 2005, Goddard *et al.*, 2006).

HE-mediated gene conversion could drive an anti-pathogen gene into a neutral site; successful drive of anti-pathogen genes may cause population conversion. Alternatively, successful HE-mediated gene drive into a critical gene may cause population reduction/elimination (Deredec *et al.*, 2011).

There are at least five families of HEs, classified based on structural similarity: LAGLIDADG, His-Cys Box, GIY-YIG, PD-(D/E)xK and HNH (Taylor *et al.*, 2011). Phage, bacterial, and archaea/eukaryotic hosts produce three of these families (GIY-YIG, PD-(D/E)xK, and LAGLIDADG) of homing endonucleases (Jurica and Stoddard, 1999, Stoddard, 2011).

I-*SceI*, I-*CreI* and I-*AniI* are members of the LAGLIDADG-type HEs (LHEs) which are best characterized in terms of structural information, biochemical redesign, and sequence diversity. I-*SceI* is one of the best characterized HEs of those that are encoded by introns. The intron encoding I-*SceI* is present in the mitochondria of *Saccharomyces cerevisiae* (Hafez and Hausner, 2012). I-*CreI* is encoded by a mobile intron in the chloroplast genome of *Chlamydomonas reinhardtii*, a species of unicellular green algae (Li *et al.*, 2012). I-*PpoI*, encoded by the slime mold *Physarum polycephalum*, has a conserved recognition site in the 28S rDNA repeat region of virtually all eukaryotes. A high mortality rate was observed by expressing I-*PpoI* in the *Anopheles gambiae* male germline (Windbichler *et al.*, 2007) and *Ae. aegypti* somatic cells (Traver *et al.*, 2009). Biochemically-modified HEs designed to target new sequences may also be an option to drive transgenes into native populations (Burt, 2003b). By targeting a highly conserved area of the mosquito genome, HEs designed to carry an anti-pathogen gene should be able to invade a population when introduced at low frequencies (Sinkins and Gould, 2006). In *Anopheles gambiae*, I-*SceI* and I-

PpoI can induce dsDNA breaks and recombination in cells and embryos (Windbichler *et al.*, 2007). I-*PpoI* expressed in the testes induces embryonic lethality by shredding the X-chromosome specific rDNA repeats (Windbichler *et al.*, 2008). Transgenic I-*PpoI* expressing strains can suppress mosquito populations in large cage trials (Klein *et al.*, 2012). In malaria vector mosquitoes, I-*SceI* can spread rapidly through cage populations by homing via dsDNA break induction followed by gene conversion (Windbichler *et al.*, 2011). Additionally, both I-*AniI* and I-*CreI* were biochemically redesigned to target endogenous genomic loci (Windbichler *et al.*, 2011). In the dengue vector, *Ae. aegypti*, I-*Ani*, I-*SceI*, I-*PpoI* and I-*CreI* can generate dsDNA breaks in the soma, and I-*PpoI* also cleaves rDNA repeats (Traver *et al.*, 2009).

HEs could be used as a tool for targeted gene disruption as well as to study dsDNA break repair mechanisms. Using HEs can provide useful information about genes of interest for manipulation of the genome as well as information about the physiological context of how and when genes are used (Porteus and Carroll, 2005). This approach is very important because at the initiation of this project there was no way to generate gene knock-out transgenic mosquitoes.

1.7 Double-strand DNA repair

DNA damage can happen frequently during different stages of a cell's lifecycle. Failure to successfully repair this damage can result in genomic rearrangements, cell death, and carcinogenesis (Rich *et al.*, 2000). Double-stranded DNA (dsDNA) breaks (Sunder *et al.*, 2012), can be triggered by ultraviolet light, mutagenic chemicals, and reactive oxygen species due to ionizing radiation (Jackson, 2002).

The two main repair mechanisms known to process dsDNA breaks are homologous recombination (HR) and non-homologous end joining (NHEJ). The NHEJ pathway is preferred in the G₀/G₁ phase because there is no homologous template. Using short homologies of one to six base pairs results in the repair of dsDNA breaks and usually loss of the coding information (O'Driscoll and Jeggo, 2006). This phenomenon occurs because free ends are repaired through base pairing between single stranded DNA; therefore, this pathway is known as the more error-prone pathway, because of the high frequency of small insertions or deletions (Moore and Haber, 1996, Wilson *et al.*, 1997).

Homologous recombination is a universal mechanism in different organisms and especially eukaryotic cells. Gene conversion happens when cells use homologous recombination as a repair mechanism (Seoighe *et al.*, 2000).

In the HR pathway, two broken DSB ends are not simply rejoined, as in the NHEJ pathway. Instead, the corresponding sequence on the another chromosome (sister chromatid, homologous chromosome or even a homologous template at an independent location in the genome) is used as a template for DNA repair (Seoighe *et al.*, 2000). Unlike the NHEJ pathway, some forms of HR are considered error-free mechanisms. HR repairs dsDNA break before the cell enters mitosis (M phase). It occurs during and shortly after DNA replication, in the S and G₂ phases of the cell cycle, when sister chromatids are available.

Traditionally, there are two major models for HR in eukaryotic cells: DSBR (Double-Strand Break Repair or Szostak Model) and SDSA (Synthesis-dependent Strand Annealing).

When a double-strand break is formed in DNA, the protein complex of MRX binds to DNA on the break sites and starts forming short 3' overhangs in

single-strand DNAs. On these 3' single strand DNAs, a nucleoprotein filament is responsible for homology searching. Afterwards the process of strand invasion will happen during which the single-stranded 3' DNA invades the sister chromatid or homologous chromosome. During this step a D-loop is formed (displacement loop) (Helleday *et al.*, 2007, Khanna and Jackson, 2001). After that, the 3' single-strand DNA is extended through DNA synthesis by a DNA polymerase. (Helleday *et al.*, 2007, Khanna and Jackson, 2001).

In the SDSA pathway, the extended 3' DNA strand is released as a Holliday junction via branch immigration (Helleday *et al.*, 2007, Khanna and Jackson, 2001). In the DSBR pathway, both 3' DNA strands will form Holliday junctions (Double Holliday Junctions) and then this double Holliday junction is resolved. (Helleday *et al.*, 2007, Khanna and Jackson, 2001).

More recently, several other types of HR-based repair have been described, such as the single-strand annealing (SSA) pathway and break-induced replication (BIR) pathway (Helleday *et al.*, 2007, Khanna and Jackson, 2001).

When two repeated sequences are oriented in the same direction, SSA-directed repair occurs between these two sequences. In this repair mechanism, two direct repeat sequences are located next to dsDNA break points. In this case, 3' DNA strands will find homology with each other anneal and form flaps. RPA and RAD52 control this process. RAD52 binds to repeat sequences and controls the alignment process. (Helleday *et al.*, 2007). These flaps will be removed by a group of nucleases called RAD1/RAD10 (Helleday *et al.*, 2007, Lyndaker and Alani, 2009b, Mimitou and Symington, 2009). By collapsing these two sequences, all of the information between these sequences will be deleted; therefore, this mechanism is always mutagenic. Some gene products are necessary

for SSA pathway including RAD52, RAD59 and RAP (Fernandes *et al.*, 2009, Helleday *et al.*, 2007, Cramer *et al.*, 2008, Krejci *et al.*, 2012).

BIR happens when double-strand breaks are formed at replication forks when DNA helicase unzips the template strand. The exact molecular mechanisms controlling BIR is poorly understood (McEachern and Haber, 2006).

1.8 Zinc Finger Nucleases

Like Homing Endonucleases, Zinc Finger Nucleases (ZFN) are capable of recognizing and cleaving large recognition sites (15-30 bp) within the genome of an organism (Joung and Sander, 2012a, Stoddard, 2011). ZFNs are composed of a modified DNA binding domain fused to a non-specific *FokI* nuclease domain (Kim *et al.*, 1996). To cut the dsDNA, dimerization of the *FokI* cleavage domain is necessary (Bitinaite *et al.*, 1998, Smith *et al.*, 2000). Each DNA binding domain binds to a three-base-pair target site to increase the binding specificity. Also, each nucleotide binds to a single amino acid and therefore each amino acid residue changes the specificity of the individual fingers (Porteus and Carroll, 2005). ZFNs can induce double-stranded DNA breaks at high frequencies in somatic and germline cells of humans (Porteus and Baltimore, 2003, Kim *et al.*, 2009, Perez *et al.*, 2008, Moehle *et al.*, 2007), fruit flies (Beumer *et al.*, 2006, Bibikova *et al.*, 2002), zebrafish (Meng *et al.*, 2008), mice (Meyer *et al.*, 2010, Carbery *et al.*, 2010) and tobacco (Wright *et al.*, 2005, Townsend *et al.*, 2009); therefore engineered ZFN could be used to study gene function. ZFNs are able to modify their recognition sites and therefore can cut any targeted position in the genome. Gene disruption happens by introducing an error during DNA repair [reviewed by (Urnov *et al.*, 2010)].

One limitation to using ZFN for gene editing is locating the target site for specific ZFN. This sequence cannot be easily predicted based on the known binding site for the individual finger modules when two independent ZFN modules are assembled into a new array; therefore, reassembling/reengineering of these molecules is difficult (Sanjana et al., 2012a).

1.9 TALEN

To date, the most promising method in genome editing is the use of another custom-designed nucleases known as Transcription Activator-Like Effector Nucleases (TALENs). TALENs are produced by fusion of the high-specificity DNA binding domain to the restriction enzyme *FokI* endonuclease domain (Sanjana et al., 2012a, Gurlebeck et al., 2006).

Each DNA binding domain derives from TALE proteins produced in various strains of *Xanthomonas* species by Type III secretion mechanisms and are translocated into host cells; therefore, they have been called Type III effectors (White *et al.*, 2009). These domains are the most important domains in these molecules as they recognize specific DNA sequences and also determine the specificity of each effector. Each DNA binding domain contains a central repetitive region consisting of varying numbers of repeat units of typically 33-35 amino acids, and are responsible for specific DNA sequence recognition [reviewed in (Gaj *et al.*, 2013)]. Except for two variable amino acids at positions 12 and 13, which are called repeat variable di-residues (RVD), each repeat is virtually identical (Moscou and Bogdanove, 2009).

These enzymes have been successfully used to modify the *Drosophila* genome by generating small insertions and deletions that result in endogenous

DNA repair of double-strand breaks in the genome at specific target DNA sequences (Liu *et al.*, 2012). The same result was observed by using TALEN for gene disruption in the silkworm *Bombyx mori* (Sajwan *et al.*, 2012, Ma *et al.*, 2012) and also in the cricket *Gryllus bimaculatus* (Watanabe *et al.*, 2012).

Dimerization is required for *FokI* cleavage domain to work; therefore using TALEN, no significant off target effects have been observed (Hockemeyer *et al.*, 2011).

In this study we were interested in comparing the activity of the endogenous *Ae. aegypti* promoters (*Ub_{LA0}* and *PUB*) with the exogenous promoters *IE1* and *hsp82* for mosquito transgenesis. We also determined whether homing endonucleases and TALEN can induce dsDNA breaks at their recognition site in the *Ae. aegypti* genome at high frequency. We found that these HEs and TALEN can edit the *Ae. aegypti* genome at useful frequencies. They could be used for targeted gene disruption as a mean to study dsDNA break repair, gene function and to develop a possible mechanism for gene drive.

Chapter 2. Development of an improved helper system in the yellow fever mosquito, *Aedes aegypti*

2.1 Abstract

The efficiency of germline transformation is substantially lower in *Aedes aegypti* (*Ae. aegypti*) than *Drosophila melanogaster*. Increasing transformation frequency could be possible by improving the helper system currently used for *Ae. aegypti* transgenic experiments. A good candidate promoter would drive strong expression for early embryos with the eventual goal of using it to improve the helper system. Polyubiquitin (*PUB*) and ubiquitin (*Ub_{LA0}*) are previously characterized endogenous promoters that drive strong expression of a marker gene in most life stages and tissues of *Ae. aegypti*. Our results show that the *PUB* promoter is more effective in *Mos1* mediated transgenesis than *IE1* and *Ub_{LA0}*. Following *Mos1*-transformation, minimum germline transformation efficiency was measured as the minimum number of independent integration events that occur per fertile adult. Frequencies similar to the *hsp82* helper plasmid were observed when co-injecting *PUB* helper plasmids with a set of donor plasmids containing marker genes *Mos1* with a *PUB* driver produced also similar G₁ transgenic cluster sizes than *Mos1* with an *hsp82* driver. We conclude that the amount of transposase produced during Helper-guided transgenesis experiments is not a limiting factor in the generation of new germline transformants.

2.2 Introduction

Aedes aegypti (*Ae. aegypti*) is an important mosquito vector, capable of transmitting different viral diseases such as dengue fever, dengue haemorrhagic fever and yellow fever (Halstead, 2007). So far, control of this vector using

insecticides and habitat removal has not been successful (Fu *et al.*, 2010). As an alternative, novel genetics-based control strategies are currently being developed, aiming to limit the transmission of diseases by suppression or replacement of mosquito populations. Some possible uses of these techniques may include sterile insect technique (Harris *et al.*, 2012b, Harris *et al.*, 2011), synthesis and expression of antipathogen effector genes in mosquitoes (Olson *et al.*, 1996, Yoshida *et al.*, 1999) or over expression of immune molecules (James, 2005).

Transposable element (TE) mediated transgenesis is a well established technique in *Ae. aegypti* and its efficiency generally varies {reviewed by (Fraser, 2012, Shin *et al.*, 2003)}. TEs serve as tools for transformation of somatic and germ cells (Garza *et al.*, 1991), however transposition requires the presence of transposase which can be obtained from a helper plasmid or *in vitro* transcribed mRNA (Li *et al.*, 2001). In helper plasmid mediated transformation, the transposase encoded by a helper plasmid interacts with the inverted terminal repeats (ITRs) of the transposon on a donor plasmid to catalyze the excision and insertion into a target site in the chromosomes (Maragathavally *et al.*, 2006).

Mos1 (*mariner*), *Minos*, *Hermes* and *piggyBac* are class II transposable elements that have been used in insect transformation (Atkinson, 2001). *Mos1* has been used to generate transgenic mosquitoes with the transformation efficiency less than 5% (Atkinson *et al.*, 2001). *Hermes* transposable element with the efficiency of more than 50% is very functional in *Drosophila melanogaster* compared to *Ae. aegypti* with the less than 10% efficiency (O'Brochta *et al.*, 1996, Jasinskiene *et al.*, 1998). *PiggyBac* has also been successfully used for germline transformation in *Ae. aegypti* (Kokoza *et al.*, 2001).

Increasing the transformation frequency is a key point in generating transgenic mosquitoes. Potentially, this could be accomplished by using a helper plasmid based on a stronger/earlier promoter.

Mohammed and Coates (2004) tested five different exogenous promoters to express *piggyBac* transposon in potato tuber moth (*Phthorimaea operculella*), and found that the highest level of transposition was observed when using the *IE1* promoter (Mohammed and Coates, 2004). In *Drosophila melanogaster*, the highest rate of transposition of the *Minos* transposon was observed when using synthesized mRNA as a source of transposase (Kapetanaki *et al.*, 2002). Three endogenous promoters and *in vitro* transcribed mRNA were tested in *Drosophila melanogaster* to induce transposition of the *piggyBac* transposon. The highest germline transformation rates were found when using the $\alpha 1$ -tub promoter (Li *et al.*, 2001).

Our lab recently described two *Ae. aegypti* endogenous *heat shock protein 70 (hsp70)* promoters that can induce expression of a reporter in both transient and germline transformation (Carpenetti *et al.*, 2011). We also found that *Ae. aegypti* endogenous *PUB* and *Ub_{LA0}* promoters can drive strong expression of luciferase in cultured mosquito cells. *Ub_{LA0}* was also able to drive expression of fluorescent markers in early larvae and ovaries, while the *PUB* promoter induced robust EGFP expression in all developmental stages, including constitutive expression throughout the midgut (Anderson *et al.*, 2010).

In this report we tested and compared the activity of two *Ae. aegypti* endogenous promoters, namely *PUB* and *Ub_{LA0}*, with the baculovirus immediate-early1 (*IE1*) promoter (Pfeifer *et al.*, 1997) and the *hsp82* promoter from *Drosophila pseudoobscura* (Coates *et al.*, 1996). We hypothesized that

transposition in *Ae. aegypti* is limited by timing and strength of the expression of transposase gene in helper plasmid and transformation frequency in *Ae. aegypti* is increased when using a helper plasmid based on a promoter, that drives stronger/earlier gene expression.

We determined the minimum transformation frequency when *PUB*, *Ub_{LA0}* or *IE1* were used to drive *Mos1* transposase expression from the helper plasmid. Surprisingly, the transformation frequency was similar when using *hsp82* or *PUB* to drive the *Mos1* transposase, despite substantial differences in expression levels from these promoters. There was also no significant difference between cluster sizes using *hsp82* and *PUB* promoters. We conclude that the amount of *Mos1* transposase produced following embryonic injection of *Ae. aegypti* is not the limiting factor in the successful integration of donor transposon cassettes into the mosquito germline.

2.3 Materials and Methods

2.3.1 Mosquito rearing

Ae. aegypti (Liverpool strain) were reared at 28°C and 50-60% humidity with a photoperiod of 8 hours dark and 16 hours light (Adelman *et al.*, 2008). Larvae were fed with pulverized fish food (Tetra, Madison, WI) in 4 liters of (RO) purified water until pupation. Larvae were hatched and reared at a density of approximately 300 larvae per pan. During larval rearing, the pan water was changed as necessary; food was provided *ad libitum*. Pupae were picked and placed in a colony cage (Bioquip, CA). Pupae emerged into adult mosquitoes in 24-48 hours. Adult mosquitoes were maintained on 10% sucrose and blood-fed

using artificial membrane feeders and defibrinated sheep blood (Colorado Serum Company, Denver, CO).

2.3.2 Manufacture and beveling of the needles

For embryo microinjection, microcapillaries (Kwik-Fil, Sarasota, FL) were manufactured using a Sutter P-2000 Micropipette puller (Novato, CA, USA) (Heat=270, FIL=3, VEL=37, DEL=250, PUL=140). The Sutter BV-10 Microelectrode Beveller was used to bevel the needles. The needles were beveled at a ~20° angle. After beveling, each needle was examined to ensure the opening of the needle was suitable.

2.3.3 Microinjection of embryos

Three to four days after blood feeding, 20-22 female mosquitoes were placed in a 50 ml tube that had been prepared with a piece of water-saturated cotton at the bottom of tube that had a damp piece of filter paper covering the cotton. A flash light aspirator (Bioquip, CA) was used to transfer mosquitoes, and then the stoppered tube was left in the dark. After one hour, the mosquitoes were removed and the filter paper was extracted.

Approximately 100 to 120 gray to darkish-gray embryos were transferred to a new piece of filter paper using tweezers (Dumont #5 Inox 11cm). Embryos were arranged in a line on damp filter paper (3MM Whatman, PA) under a dissecting microscope. All embryos were orientated in the same direction to allow injection of the posterior pole. To facilitate efficient embryo transfer to a coverslip (Thermo Fisher, MA), the filter paper was dried using dry filter paper. Transfer of the embryos involved inverting a coverslip that had been prepared

with double-sided tape (Scotch, St. Paul, MN) and gently pressing it onto the embryos. The posterior ends of the embryos were very close to the edge of the double sided tape.

The embryos were desiccated at room temperature and observed during this stage to assess the 'dimpling' that indicated the appropriate level of dessication (time varies, but roughly 20-60 sec). Embryos were covered with halocarbon oil (Sigma, St. Louis, MO) to prevent overdesiccation. All microinjections were performed using a Leica (Buffalo Grove, IL, USA) micromanipulator and a FemtoJet microinjector (Eppendorf, Westbury, NY, USA).

2.3.4 Luciferase assay and *Mos1*-mediated transformation of *Ae. aegypti*

Plasmids pGL3-*IE1*, pGL3-*Ub_{L40}* and pGL3-*PUB* were described previously (Anderson *et al.*, 2010). To generate pGL3-*hsp82*, a 992-bp region containing the *Drosophila pseudoobscura hsp82* promoter was amplified by polymerase chain reaction (PCR) from pK*hsp82* (Coates *et al.*, 1996) using the primers 5'-TTTTCCATGGGTTTTAATTTAACAGCAGAG-3' and 5' - TTTTAAGCTTATGGATTTTTACCATATTATTA-3'. All PCR reactions were performed using Platinum Pfx (Invitrogen, Carlsbad, CA) as follows: 94°C for 2mins, 94°C for 15s, 65°C for 30s, 68°C for 70s, 35 cycles, 68°C for 10mins. Amplicons were digested with HindIII and NcoI (NEB, Ipswich, MA), purified, and ligated into the corresponding sites of pGL3-Basic (Promega, Madison, WI). The normalization control plasmid pRL-CMV-Renilla was purchased from Promega.

For luciferase experiments, the experimental plasmid (pGL3-*PUB*, pGL3-*Ub_{L40}*, pGL3-*IE1*, pGL3-*hsp82* or pGL3 Basic) and normalization control plasmid (pRL-*CMV*) were co-injected at 0.3 µg/µl each into *Aedes aegypti* Liverpool strain embryos (G₀). Three replicates of 100-120 embryos each were injected with each promoter construct. Injected embryos were snap-frozen in liquid nitrogen at 2, 4, 12, 24, 48 and 72 hours post injection. Embryos were homogenized in the lysis buffer provided by the manufacturer (Promega, Madison, WI). Activity of both firefly luciferase (FF-luc) and Renilla luciferase (R-luc) were determined by using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI) with a GloMax 20/20 instrument according to the manufacturer's protocol.

For *Mos1*-mediated transgenesis, the pMos-3xP3DsRed-5HE-*Ub_{L40}*-EGFP-*attP* (referred to as UUGFP), *AaHsp70Bb*-2447-FFLuc and pMos-*PUB*DsRed-5HE-MCS-5HE plasmids were used as donors. Construction of these plasmids was described previously (Anderson *et al.*, 2010, Carpenetti *et al.*, 2011).

Each donor plasmid (pMos-3xP3DsRed-5HE-*Ub_{L40}*-EGFP-*attP*, pMos-*PUB*DsRed -5HE-MCS-5HE and *AaHsp70Bb*-1456) was co- injected at 0.5 µg/µl separately with one of the helper plasmids (pGL3-*IE1Mos1*, pGL3-*PUBMos1* and pGL3-*Ub_{L40}Mos1*) at 0.3 µg/µl in 1X injection buffer. Donor/Helper plasmids were injected into one hour old Liverpool strain embryos (Labbe *et al.*, 2010). Surviving G₀ females were merged into pools of 20-25 individuals and mated to males of the recipient strain. G₀ males were mated individually to 5 recipient strain females and pooled prior to blood feeding and egg collection. G₁ larvae were screened for DsRed⁺ eyes/bodies and/or GFP⁺ bodies. Positive G₁

individuals were crossed with the parental strain to ensure all transgene cassettes were stably inherited to the G₂ generation germline.

2.3.5 Southern analysis

Genomic DNA was isolated from six females or 10 males as described previously (Adelman *et al.*, 2008). Genomic DNA was digested overnight with *NsiI*, *Sall*, *EcoRI*, *PstI*, *NdeI* or *SacII* followed by size- fractionation on a 0.8% agarose gel. The DNA was capillary transferred to a nylon membrane using 10X SSC. A probe corresponding to *HindIII* restriction fragments of the *MosI* construct was randomly primed and labeled with [α -³²P]-dATP using the Amersham Megaprime DNA labeling system (GE Healthcare, Little Chalfont, UK), and purified using an Illustra NICK column (GE Healthcare). Following hybridization overnight at 65°C, membranes were washed two times in 2X SSC, 0.1% SDS and in 0.22X SSC, 0.1% SDS at 65°C. Hybridization signals were detected by exposure to Kodak BioMax maximum sensitivity film at -80°C.

2.3.6 Inverse PCR analysis

Total DNA was extracted from six females or 10 males and digested overnight with *EcoRI*, *SacI*, *NsiI* and *PstI*. After ligation under control of dilute DNA concentration with excess T4 ligase, gene amplification was performed using the primers 5' -AACGTGTGAACGGTGGTTTCAACGCTTC 3'- and 5' ATGGTGGTTCGACAGTCAAGGTTG- 3' as primary set of primers, and 5'- CGAACCGACATTCCCTACTTGTACACC -3' and 5'- CCAGTTGGGGCACTACATAACTTCGTATAATG -3' as a set of nested

primers. The reaction performed under the following amplification conditions: 95°C for 2mins, 95°C for 30s, 63°C for 1m, 68°C for 5m, 29 cycles, 68°C for 10mins. Amplification products were cloned into pGEM-T (Promega) and the results of sequencing were analyzed by Seqman (DNASTAR, Madison, Wisconsin, USA).

2.4 Results

2.4.1 Embryo injections

Two novel endogenous *Ae. aegypti* promoters, *PUB* and *Ub_{L40}*, and an exogenous promoter, *IE1*, were compared to the *hsp82* promoter to determine the relative ability of these promoters to drive gene expression in early embryos. A promoter-less pGL3-Basic plasmid was used to measure background expression. To normalize the experiments, REN luciferase under control of the *cytomegalovirus* (*CMV*) regulatory sequences was co-injected into all embryos. The quantities of embryos injected with each construct are presented in Table 2.1.

Table 2. 1. Number of embryos injected with different constructs over time.

Five construct were injected in the biological replicates. Each replicate involved injection of approximately 100 embryos over six time points. *CMV* promoter was used to drive REN luciferase.

Construct	No. embryos injected (hours post injection)					
	2	4	12	24	48	72
pGL3-basic/pRL- <i>CMV</i>	273	275	274	307	293	300
pGL3- <i>PUB</i> /pRL- <i>CMV</i>	384	316	308	290	275	298
pGL3- <i>Ub_{L40}</i> /pRL- <i>CMV</i>	300	283	286	311	592	504
pGL3- <i>IE1</i> /pRL- <i>CMV</i>	290	307	296	268	342	210
pGL3- <i>hsp82</i> /pRL- <i>CMV</i>	295	318	280	291	317	300

2.4.2 Promoter activity

Luciferase and REN luciferase raw data are represented in Fig.2.1. REN expression was always under the control of the *CMV* promoter. Four experimental promoters were used to express FF luciferase, therefore the majority of variation was found in FF luciferase expression, as opposed to in REN luciferase expression. Using the raw data generated from the FF and REN values, a ratio was calculated by dividing the FF luciferase expression by REN luciferase expression.

Using a dual luciferase reporter assay, we examined the ability of three additional promoters to drive gene expression at various times following injection into pre-blastoderm embryos; the baculovirus *IE1* promoter and the *Ae. aegypti polyubiquitin (PUB)* and *Ub_{L40}* promoters (Anderson *et al.*, 2010). The result of the activity assays are presented in page 58, shown as the ratio of experimental promoter-FF luciferase activity to *CMV*-REN luciferase activity (see section 3.4.1 for result).

2.4.3 Control plasmid variability

The result of the luciferase assay showed that *PUB* promoter had very early robust expression compared to *IE1*, *Ub_{L40}*, and *hsp82*. We considered that the data may be influenced by competition between the promoters of the control plasmid and experimental plasmid, respectively, including competitive acquisition of transcriptional factors.

To confirm there was no competition between the *PUB* promoter in the experimental plasmid and the *CMV* promoter in the control plasmid, another experiment was designed. In this set of experiments pkhsp82-Renilla was used as

a control plasmid. pGL3-*hsp82* was injected into the embryos to generate a background reading and compared to pGL3- *PUB*. All samples were co-injected with pKhsp82-REN to normalize for differences between the ratios of gene expression in each replicate. The number of injected embryos, with the construct using the *hsp82* to express REN luciferase as a control plasmid, are represented in Table 2.2.

The results of the activity assays of *PUB* and *hsp82* promoters are represented in Fig.2.2, shown as the ratio of experimental promoter-FF luciferase activity to *hsp82*- REN luciferase activity. The expression profile of *PUB* with pKhsp82-REN as a control plasmid was very similar to the expression profile of *PUB* with pRL-CMV as a control plasmid for all of the time points. The raw values of FF luciferase with two different control plasmids are represented in Fig.2.3.

Unexpectedly, the ratio of FF luciferase to REN activity was less than 1 when both FF and REN luciferase were expressed by the same promoter, *hsp82*. We considered that plasmid backbones may have an effect on gene expression, thus leading to higher expression of REN than FF under control of the same promoter, *hsp82*.

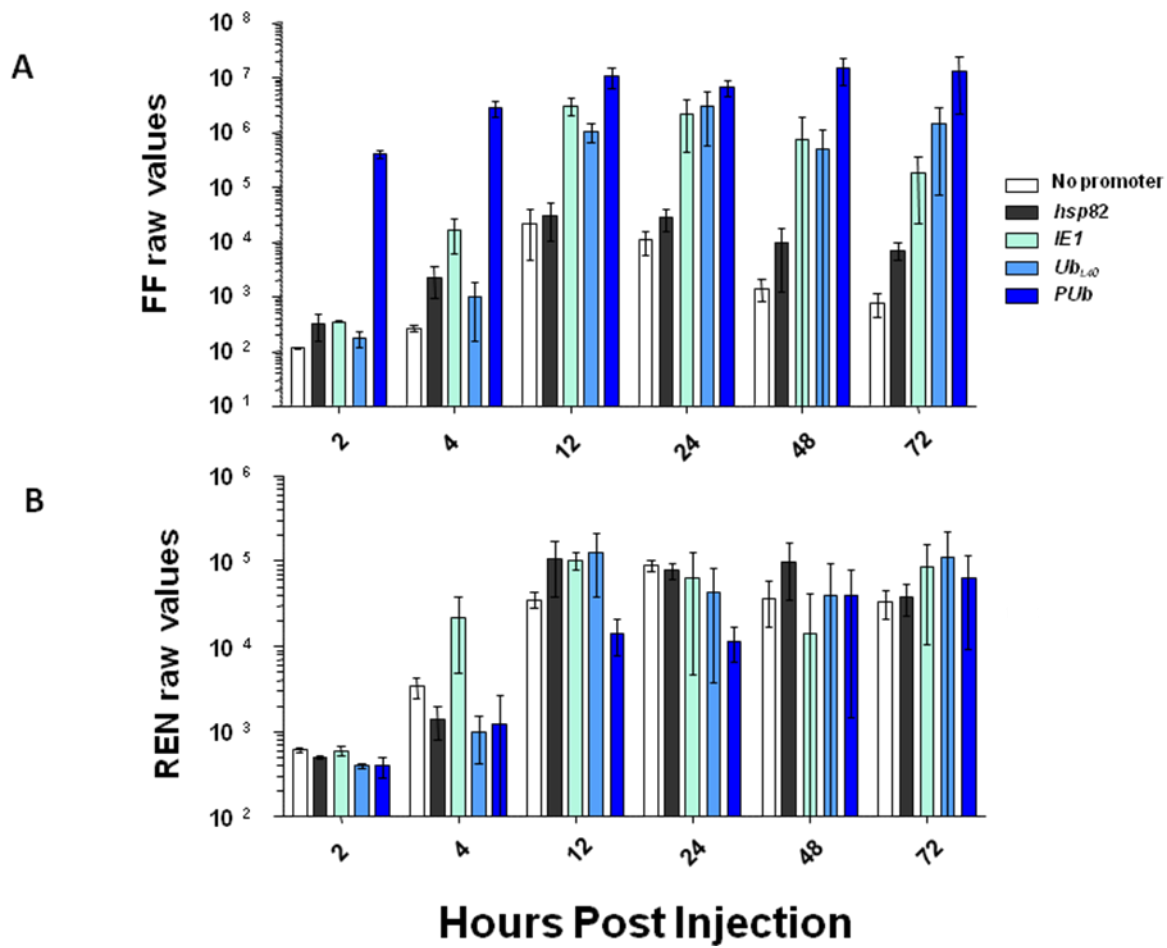


Figure 2. 1. Variation in FF and REN raw values over time

(A) Raw for FF values for the five promoters over six different timepoints. (B) REN raw value over time. REN under control of *CMV* was used to normalize FF luciferase to control for variability due to amount of DNA injected and embryo survival.

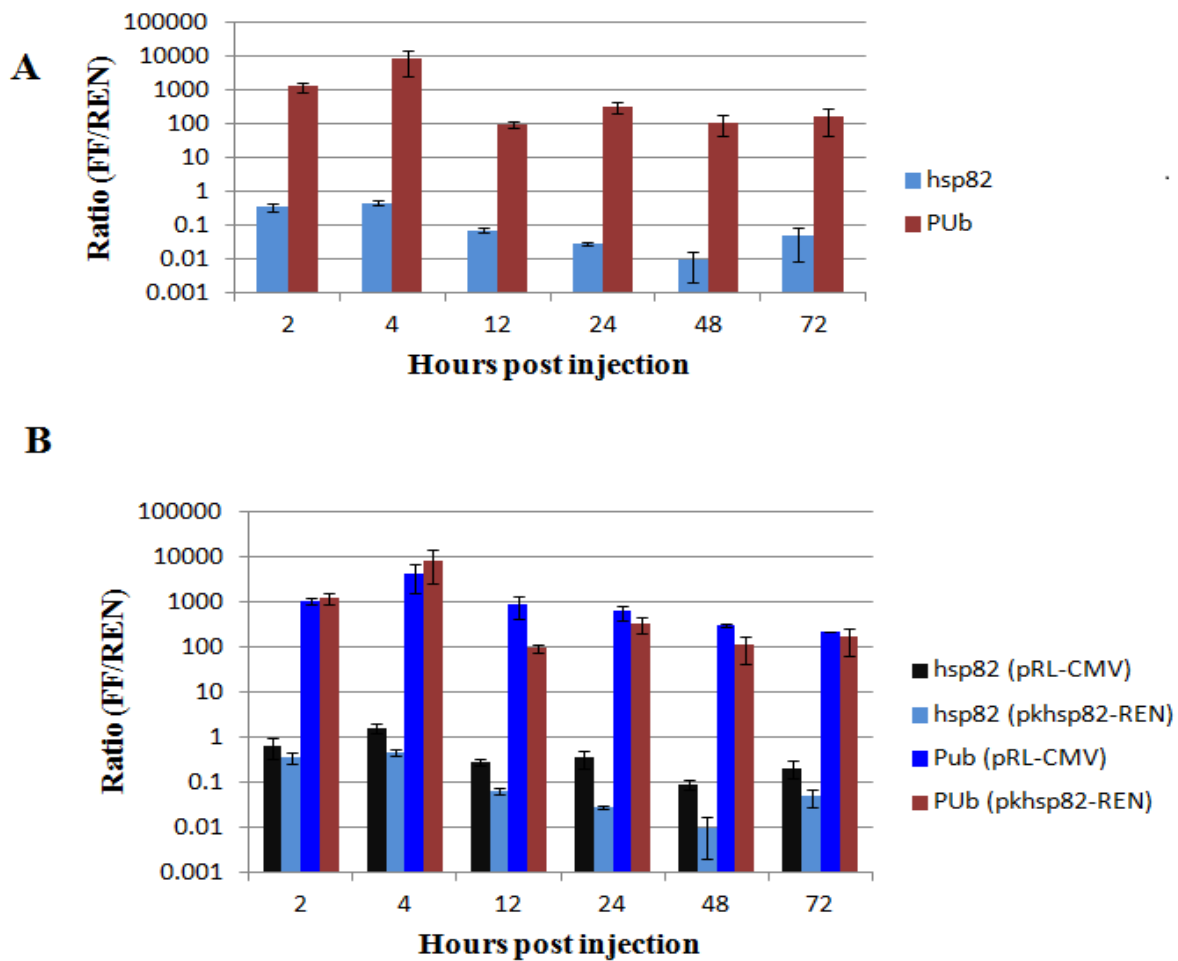


Figure 2. 2. Comparison of promoter activity to express REN luciferase in control plasmids.

Ratio of relative FF luciferase activity driven by *PUb* and *hsp82* promoters to REN luciferase activity driven by *hsp82* over time. (B) Comparison of FF/REN ratio with two different control plasmids (pRL-CMV and pKhsp82-REN).

Table 2. 2. Number of embryos injected with two constructs over six different time-points.

Construct	No. embryos injected (hours post injection)					
	2	4	12	24	48	72
pGL3-hsp82/pkhsp82-REN	305	428	297	322	331	339
pGL3-PUB/pkhsp82-REN	302	309	266	321	636	608

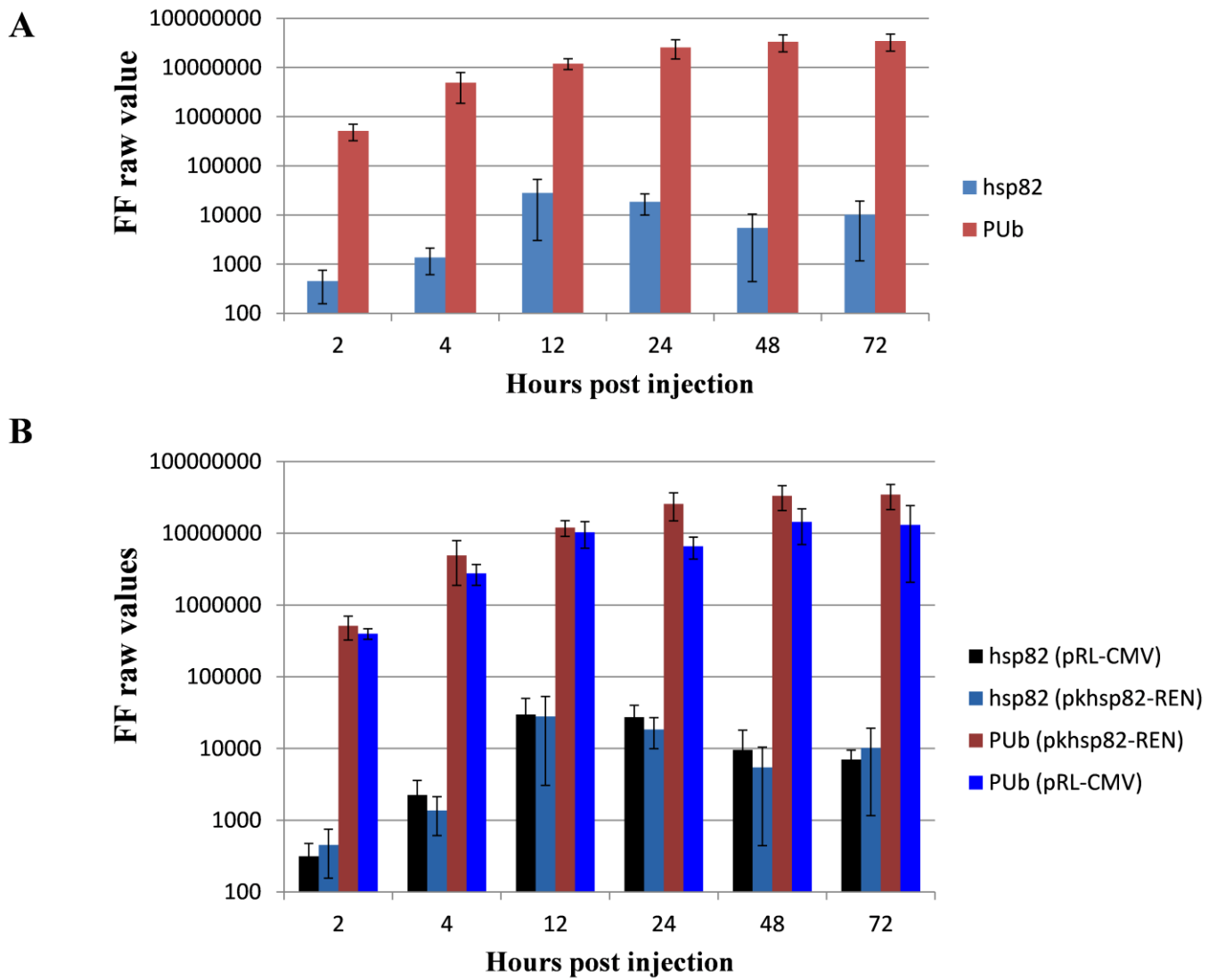


Figure 2. 3. Variation in FF raw values over time using different control plasmids

(A) raw values of FF luciferase with pKhs82-REN as a control plasmid. (B) Comparison of FF raw values with two different control plasmids (pRL-CMV, pKhs82-REN). There was no measurable competition between promoters in experimental and control plasmids.

2.4.4 Plasmid backbones switch

A ratio of FF to REN luciferase close to 1 had been expected when both genes were under control of the same promoter. However, this ratio was less than 1 for all time points. To determine whether the plasmid backbone also had an effect on gene expression, another experiment was designed. Two plasmid backbones, pGL3 and pkhsp82 were switched, and pkhsp82-FF and pGL3-*hsp82*-REN were generated. Both plasmids were injected into the *Ae. aegypti* embryos.

Results of the activity assays are presented in Fig.2.4, shown as the ratio of FF luciferase to REN luciferase activity. *hsp82*-REN provided a 10-fold stronger signal than *hsp82*-FF. Thus, we conclude that the observed effects are independent of plasmid backbone.

2.4.5 Generation of transgenic *Aedes aegypti*

As the *PUB* promoter showed a very high level of expression of FF luciferase in early embryos and based on the results of other experiments (Mohammed and Coates, 2004, Kapetanaki et al., 2002), we hypothesized that the transformation efficiency would increase by using the *PUB* promoter to express *Mos1* transposase in the helper system in *Ae. aegypti*. Three *Mos1* based donor constructs: pMos-3xP3DsRed-5HE-*Ub_{L40}*-EGFP-*attP* (referred to as UUGFP, Fig. 2.5A), pMos-*PUB*DsRed -5HE-MCS-5HE (Fig. 2.5B) and *AaHsp70Bb*-1456 (Fig. 2.5C) were injected into *Ae. aegypti* embryos (Liverpool) with three different helper plasmids (pGL3-*IE1Mos1*, pGL3-*PUBMos1* and pGL3-*Ub_{L40}Mos1*). When G₁ positive individuals were mated with the parental strain, we observed that approximately 50% of the progeny inherited the transgene, indicating stable germline transformation (Table 2.3A, B, C).

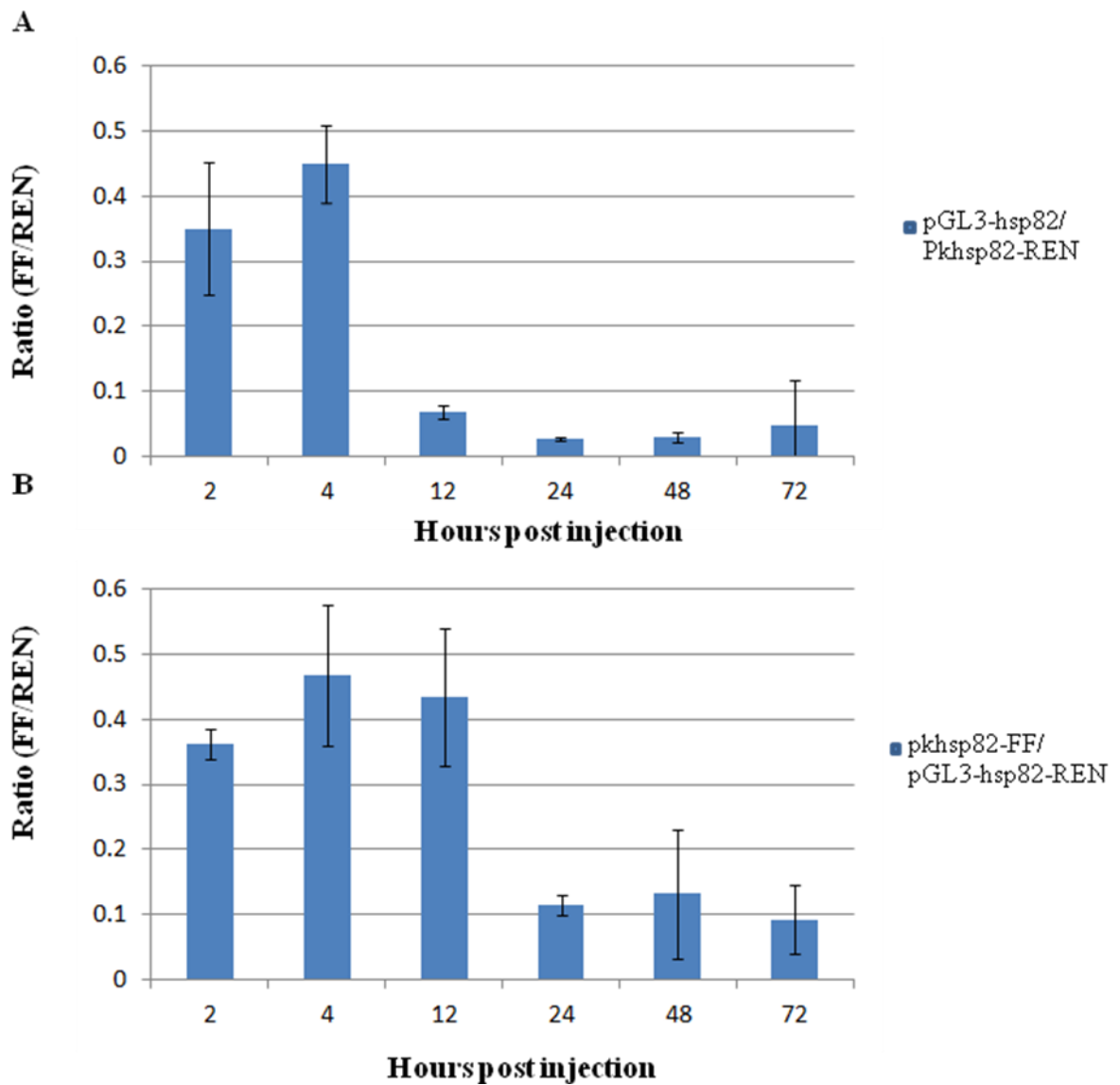


Figure 2. 4. Plasmid backbone effect over gene expression.

(A) Ratio of FF to REN luciferase activity before switching the plasmid backbones. The ratio was found to be less than 1 for all time-points. (B) Ratio of FF to REN luciferase activity after switching the plasmid backgrounds. This ratio also was less than 1..

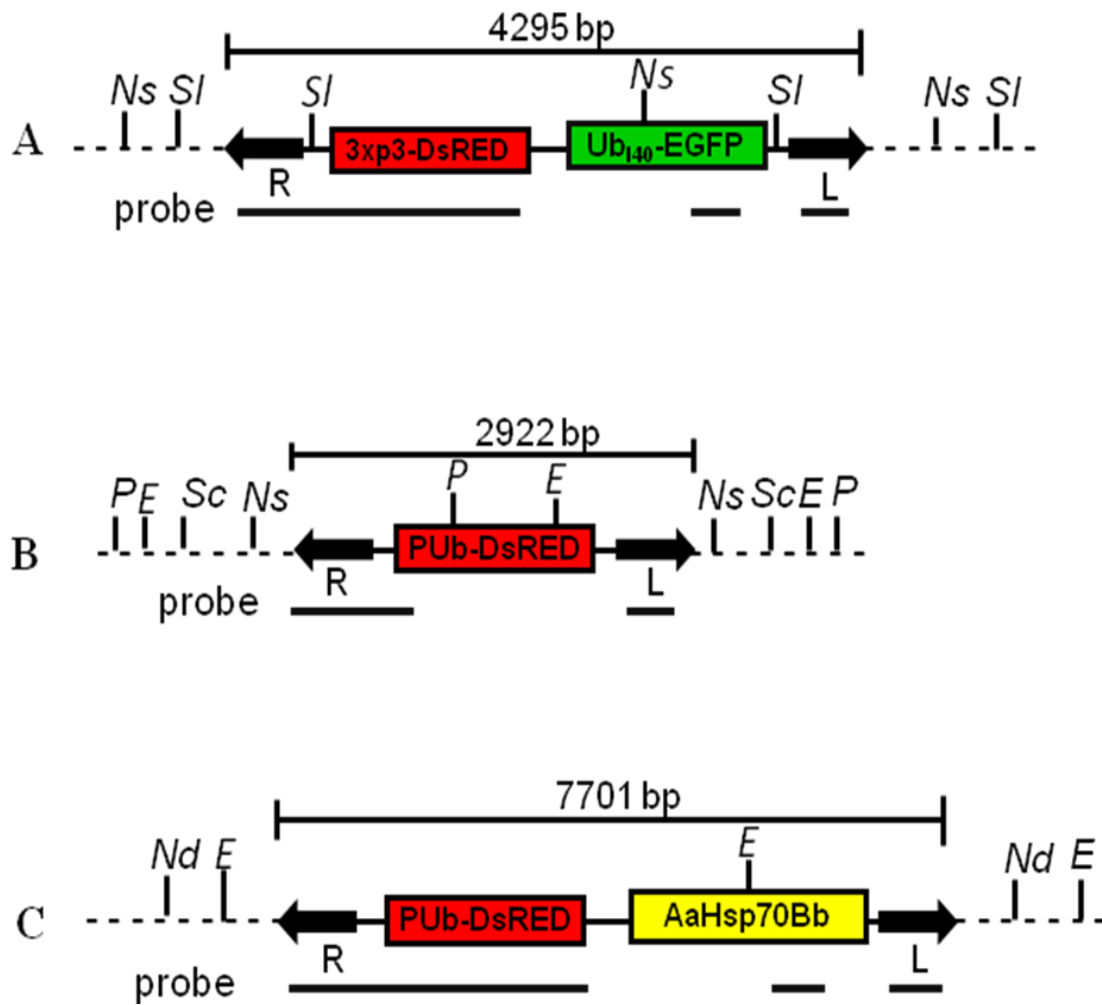


Figure 2. 5 Schematic representation of hypothetical transgene insertion.

for (A) *3xp3DsRed-5HE-Ub_{L40}-EGFP-attP*, (B) *pMos-PUbDsRed -5HE-MCS-5HE* and (C) *AaHsp70Bb-1456*. Block arrows represent the right (R) and left (L) hand of the *MosI* transposable element. The bar indicates the size of entire insertion and the dashed line represents mosquito genome DNA. Restriction enzyme sites *NsiI* (Ns), *SalI* (Sl), *EcoRI* (E), *PstI* (P), *NdeI* (Nd) and *SacII* (Sc) are indicated. Southern blot probes are depicted below each schematic.

Table 2. 3. *Mos1*- mediated transformation of *Ae. aegypti* with (A) *Ub_{L40}*, (B) *PUB* and (C) *Hsp70* gene cassette.

A	Donor plasmid	Mos1 helper	# embryos injected	# G ₀ survivors (%)	# pools	# G ₁ progeny screened	Pools with	G ₂ ⁺ /Total (%+)	G ₃ ⁺ /Total (%+)
							DsRed ⁺ progeny (#)		
	pMos-3xP3DsRed-5HE- <i>Ub_{L40}</i> -EGFP- <i>attP</i>	pGL3- <i>PUB</i> Mos1	1106	90 (8%)	5	~10380	# 1 (32 ⁺)	102 ⁺ /312 (33%)	73 ⁺ /149 (49%)
							# 3 (29 ⁺)	93 ⁺ /240 (39%)	126 ⁺ /245 (52%)
							# 5 (40 ⁺)	102 ⁺ /200 (51%)	9 ⁺ ♀/800 ⁺ ♂ (45%)
	pMos-3xP3DsRed-5HE- <i>Ub_{L40}</i> -EGFP- <i>attP</i>	pGL3- <i>Ub_{L40}</i> Mos1	1100	215 (19.6%)	9	~33810			
	pMos-3xP3DsRed-5HE- <i>Ub_{L40}</i> -EGFP- <i>attP</i>	pGL3- <i>IE1</i> Mos1	1209	156 (13%)	11	~27350			

B	Donor plasmid	Mos1 helper	# embryos injected	# G ₀ survivors (%)	# pools	# G ₁ progeny screened	Pools with	G ₂ ⁺ /Total (%+)
							DsRed ⁺ progeny (#)	
	pMos- <i>PUB</i> DsRed -5HE-MCS-5HE	pGL3- <i>PUB</i> Mos1	1160	230 (18.8%)	11	~18630	# 6 (18 ⁺)	195 ⁺ /380 (51%)
							# 7 (28 ⁺)	117 ⁺ /240 (49%)
	pMos- <i>PUB</i> DsRed -5HE-MCS-5HE	pGL3- <i>Ub_{L40}</i> Mos1	1253	246 (19.6%)	9	~16700		
	pMos- <i>PUB</i> DsRed -5HE-MCS-5HE	pGL3- <i>IE1</i> Mos1	1006	84 (8.4%)	3	~6000		

C	Donor plasmid	Mos1 helper	# embryos injected	# G ₀ survivors (%)	# pools	# G ₁ progeny screened	Pools with	G ₂ ⁺ /Total (%+)
							DsRed ⁺ progeny (#)	
	<i>AaHsp70Bb</i> -1456	pGL3- <i>PUB</i> Mos1	1315	242 (18.4%)	10	~11700	#1 (5 ⁺)	160 ⁺ /290 (55%)
							#6 (30 ⁺)	160 ⁺ /310 (51%)
							#7 (7 ⁺)	92 ⁺ /200 (46%)
	<i>AaHsp70Bb</i> -1456	pGL3- <i>Ub_{L40}</i> Mos1	1209	197 (16.2%)	7	~7600	# 3 (19 ⁺)	215 ⁺ /450 (47.7%)
	<i>AaHsp70Bb</i> -1456	pGL3- <i>IE1</i> Mos1	1208	162 (13.3%)	6	~9300		

Putative transgenic individuals were identified in three pMos-3xP3DsRed-5HE-*Ub_{L40}*-EGFP-*attP* pools (p1, p3 and p5), two pMos-*PUB*DsRed-5HE-MCS-5HE pools (p6 and p7) and four *AaHsp70Bb*-1456 pools (p1, p3, p6 and p7).

To determine and compare the activity of *PUB* and *hsp82* for *Mos1* mediated transgenesis we calculated the number of positive larvae and the minimum transformation efficiency when using *PUB* and *hsp82* to drive *Mos1* transposase in helper plasmid, including both published studies and unpublished historical data from our lab (see Appendix 1 for a list of experiments and results). There was no statistical significant difference between the number of positive larvae using *PUB* and *hsp82* to drive *Mos1* transposase ($p=0.27$) (Fig. 2.6A). Comparison the minimum transformation efficiency also showed that there was no statistical significant difference between the minimum transformation efficiency using *PUB* and *hsp82* to drive *Mos1* transposase ($p=0.97$) (Fig. 2.6B).

2.4.6 Southern blot and Inverse PCR analyses

Southern analysis was performed for all pools to verify insertion of the *Mos1* construct into the mosquito genome (Fig. 2.7). As no expression of EGFP was observed in pMos-3xP3DsRed-5HE-*Ub_{L40}*-EGFP-*attP* pools, we also sought to verify the integrity of each transgene (Anderson *et al.*, 2010). Common ~3 kb and ~4 kb fragments were observed in lines p1, p3 and p5, along with a junction fragment expected to vary with the insertion site (Fig.2.7A). This confirmed there the *Ub_{L40}*-EGFP cassette gene was integrated intact into the mosquito genome for all *Ub_{L40}*-EGFP transgenic lines. Genomic DNA from each putative transgenic

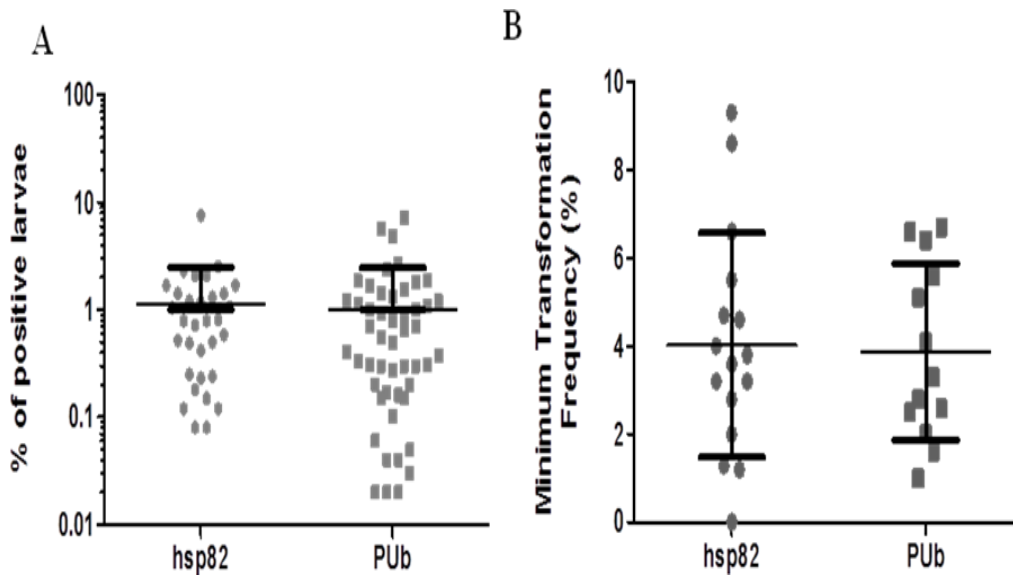


Figure 2. 6. Comparison of *hsp82* and *PUb* promoters for *Mos1* mediated transgenesis.

(A) There is no statistical significant difference between the number of positive larvae using *PUb* and *hsp82* to drive *Mos1* transposase ($p=0.27$). (B) There is no statistical significant difference between the minimum transformation efficiency using *PUb* and *hsp82* to drive *Mos1* transposase ($p=0.97$).

line of the pMos-*PUBDsRed* experiment was digested with *NsiI* or *SacII* with no target recognition site or *EcoRI* and *PstI* with one recognition site within the transgene. One insertion was observed in lines p6 and p7 (Fig. 2.7B). Genomic DNA from each putative transgenic line of the *AaHsp70Bb*-1456 experiment was digested with *NdeI* with no target recognition site and *EcoRI* with one site within the transgene. All transgenic lines appear to contain only a single insertion, with the exception of line p3.

In this case, the *EcoRI* digest produced two strong hybridization fragments, whereas the *NdeI* digest only produced one. It was not clear if there are one or two insertions in this line. We performed another southern with genomic DNA from six transgenic mosquitoes which were digested individually with *EcoRI* with one target recognition site within the transgene (Fig. 2.8). These results suggest that there was either more than one G₁ transgenic mosquito in p3 which produced offspring with a different genotype, or there was incomplete genomic DNA digestion.

To determine the 5' and 3' integration site junction, 2-4 independent clones originating from inverse PCR (iPCR) products were sequenced for each transgenic line. A comparison of insertion site junction sequences of the *MosI* transformation vectors with the genomic integration sites in *Ae. aegypti* transgenic lines is shown in Table 2.4. Results of iPCR showed that all of the *MosI* insertions are intergenic except p6 from the *AaHsp70Bb*-1456 experiment. The iPCR results show this is a genic integration. The transgene was integrated inside intron 2 of gene AAEL004189 which is a member of family G protein-coupled receptor. All insertion site junctions have putative TA duplications immediately adjacent to the *MosI* inverted terminal repeats.

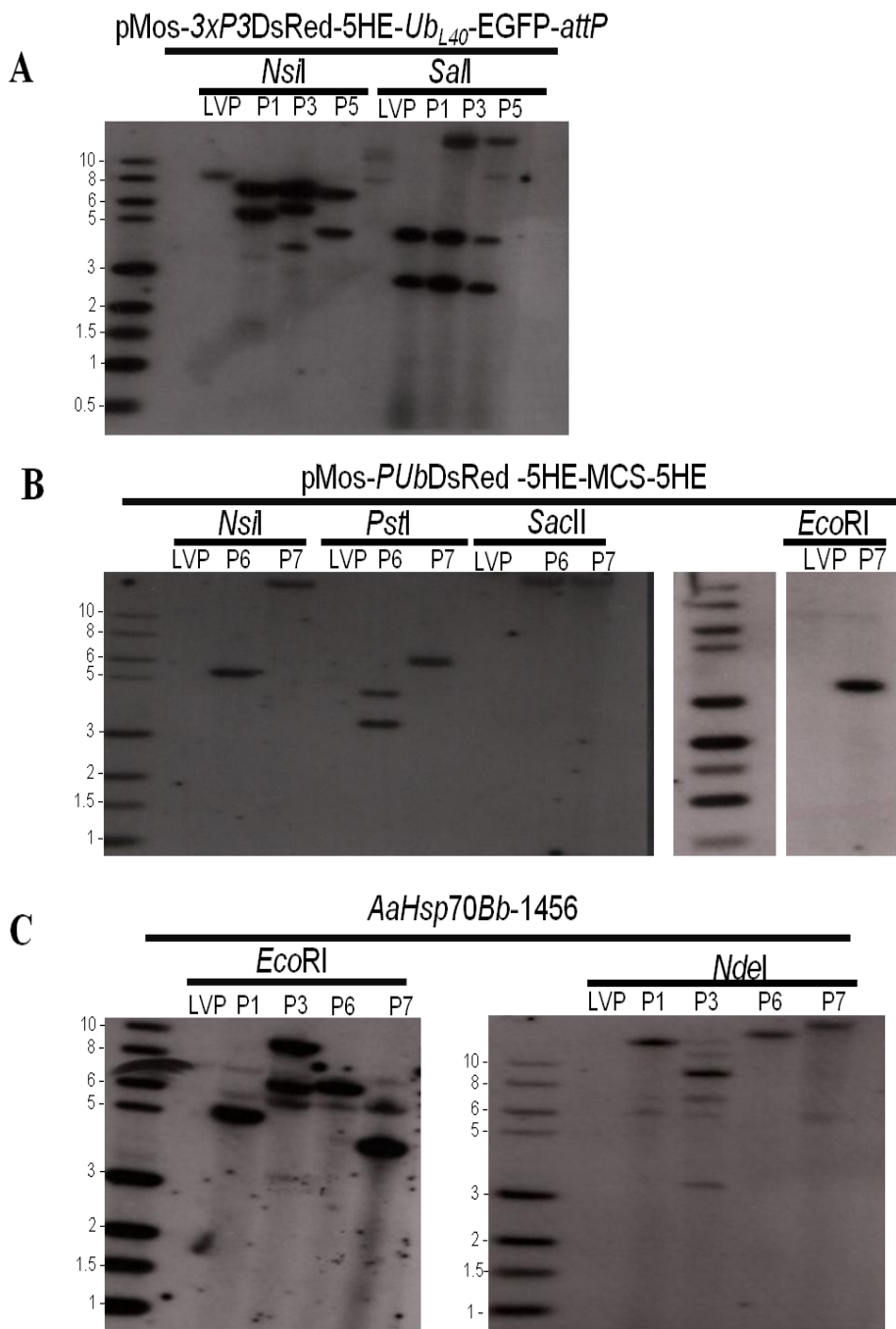


Figure 2. 7. Southern analysis

of (A) *Ub_{L40}*-EGFP, (B) *PUB*-DsRed and (C) *AaHsp70Bb*-1456 to detect the number of independent transposition events in *Mos1*-transformed *Ae. aegypti*. Genomic DNA from each of the families identified as DsRED⁺ was digested with the indicated enzymes and hybridized with a probe corresponding to the *Mos1* arm as well as the DsRED-SV40 gene cassette. The recipient strain Lvp is included as a negative control for all hybridizations. Molecular weight markers are shown on the left (kbp).

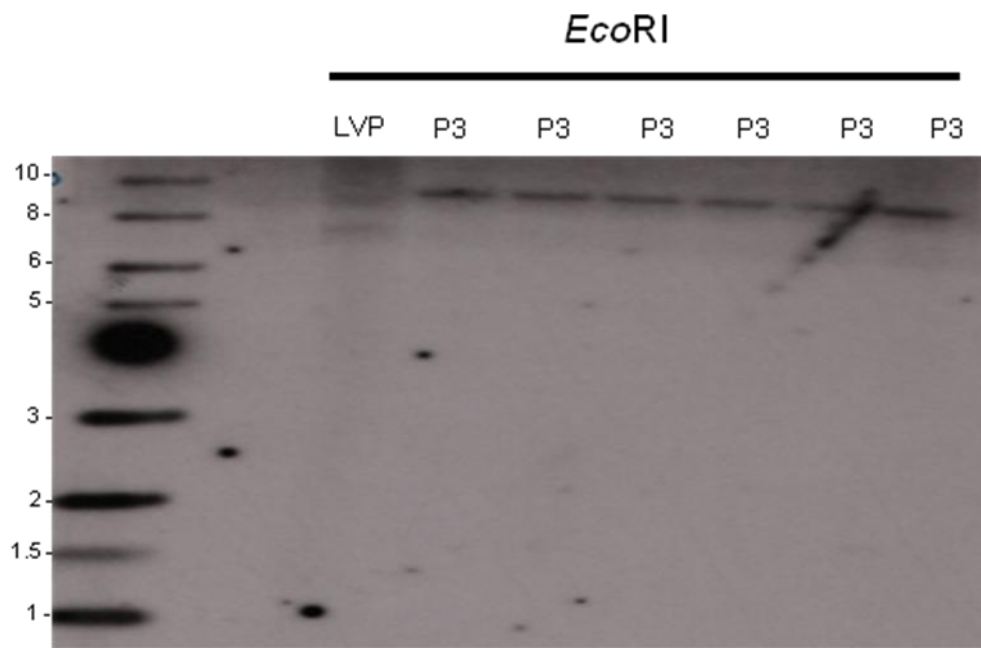


Figure 2. 8. Southern analysis of AaHsp70Bb-1456 genome.

Genomic DNA was extracted from six individual P3 female mosquitoes and digested with *EcoRI*, which has one target recognition site within the transgene. The recipient strain Lvp is included as a negative control.

Table 2. 4. Insertion site junction sequences of the Mos1 transformation vectors within the genome of Ae.aegypti transgenic lines.

Donor plasmid	Pools with DsRed ⁺ Progeny (#)	Supercontig	Position of insertion	Nearest gene	Nearest gene distance (kb)
pMos-3xP3DsRed-5HE-Ub _{L40} -EGFP-attP	#1	1.62: 2,875,624 – 2,900,623	Intergenic	CYP9J31, Cytochrome P450 (Metabolism)	~11
	#3	1.92: 1,254,739 – 1,304,738	Intergenic	AAEL016985	~61
	#5	1.6: 2,217,349 – 2,267,348	Intergenic	AAEL000328	~12
pMos-PUbDsRed -5HE-MCS-5HE	#6	1.371: 668,298 – 693,297	Intergenic	CYP6Z6, Cytochrome P450 (Metabolism)	~62
	#7	1.443: 938,590 – 963,589	Intergenic	AAEL009980	~2.5
AaHsp70Bb-1456	#6	1.109: 2,420,318 – 2,508,478	Genic	AAEL004189, Family G protein-coupled receptor (Regulation)	inside intron #2

2.5 Discussion

We have compared the activity of the *Ae. aegypti* endogenous promoters *PUB* and *Ub_{L40}* with exogenous promoters *IE1* and *hsp82* to express FF luciferase in *Ae. aegypti* early embryos at different time-points. In the promoter activity assay, the use of the endogenous *polyubiquitin* promoter resulted in a higher level of firefly luciferase activity than *Ub_{L40}*, *hsp82* and *IE1* within *Ae. aegypti* early embryos. *PUB*-driven expression was found to be robust in all embryo life stages, especially in early embryos (two to four hours post injection). We have also recently shown that the *Ub_{L40}*-driven expression was strongest in early larvae and in ovaries, and weaker in other tissues and life stages. *PUB*-driven expression was robust in most tissues at all life stages, with strong expression observed in the midgut (Anderson *et al.*, 2010).

By changing the control plasmid, no measurable competition between the promoters in experimental (*PUB*) and control plasmids (*hsp82* and *CMV*) was observed. Switching the plasmid backbone also did not have any obvious effect on gene expression. The results of this experiment showed that the promoters play the most important role in gene expression. As we expected, different levels of gene expression were due to changes in the promoter, while different plasmid backbones had no measurable effect over gene expression.

FF and REN luciferase activities were compared under control of the same *hsp82* promoter. This ratio was less than 1 for all of the time-points, which was consistent with previous studies (Willard *et al.*, 1999, Behre *et al.*, 1999). Our data suggests a high level of REN expression compared to FF expression under control of the same promoter, *hsp82*. One possible reason for this outcome could be explained by translation efficiency. Although using the same promoter (*hsp82*)

has shown similar transcription efficiency when expressing different genes, translation efficiency may be higher for REN than for FF. To the contrary, a different group of investigators found that the ratio of FF to REN luciferase was close to 1 when both FF and REN were expressed by *hsp82* promoter in potato tuber moth (Mohammed and Coates, 2004).

The results of the promoter activity assay allowed us to further hypothesize that the *Mos1* transformation efficiency in *Ae. aegypti* might be increased when using a helper plasmid based on an endogenous *PUB* promoter, which drives stronger and earlier gene expression. However, our data suggests that the amount of transposase produced during Helper-guided transgenesis experiments is not a limiting factor in the generation of new germline transformants.

There are other possibilities that might limit *Mos1* transformation. The length of transposon, the DNA sequence (in term of GC content) and the super-helicity of the transposon have important roles on the transposition efficiency (Sinzelle *et al.*, 2008). Recent studies show that Piwi-interacting RNAs (piRNAs) has an important role for transposon silencing during germline development in *Drosophila* {reviewed by (Khurana and Theurkauf, 2010)}. This system could be important system against TEs in Mosquitoes. *Mos1*-based transgenesis in *Ae. aegypti* also has a very high degree of stability, and thus is not a good candidate for genetic drive, enhancer trap, or transposon tagging systems in this species (Wilson *et al.*, 2003b). *Mos1* transposition in vitro can occur when *Mos1* donor elements, target DNA, purified *Mos1* transposase and Mg²⁺ are present (Lampe *et al.*, 1996). In vivo, even in the presence of functional *Mos1* transposase, the chance of *Mos1* to be lost by excision or to transpose to new locations in the genome is very low. On the other hand, if a high level of post-integration stability

is preferred then *Mos1* is a good candidate for transformation, therefore, these elements essentially become dysfunctional upon integration (Wilson *et al.*, 2003b).

Chapter 3. Catalyzing double-stranded DNA breaks and excision of transgenes in *Aedes aegypti* germline by homing endonucleases

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3.1 Abstract:

Aedes (Ae.) aegypti is the primary vector for dengue viruses (serotypes 1-4) and chikungunya virus. The inability to control this vector, and thus the disease agents it transmits, has prompted the development of novel genetic-based control strategies. Homing endonucleases (HEs) are ancient selfish elements that catalyze double-stranded DNA breaks (DSB) in a highly specific manner, making them a powerful tool for targeted gene disruption and gene drive. In this report, we show that the HEs Y2-I-*AniI*, I-*CreI* and I-*SceI* are all capable of catalyzing the excision of genomic segments from the *Ae. aegypti* genome in a highly specific manner. Of these, Y2-I-*AniI* demonstrated the highest efficiency at two independent genomic targets, with 20-40% of Y2-I-*AniI*-treated embryos giving rise to offspring that had lost the target transgene. HE-induced DSBs were found to be repaired via the single-strand annealing (SSA) and non-homologous end-joining (NHEJ) pathways in a manner dependent on the availability of direct repeat sequences in the transgene. Interestingly, direct repeats as short as 18-20 base pairs were sufficient to direct the SSA response. These results support the development of HE-based gene editing and gene drive strategies in *Ae. aegypti*, as

well as confirm the utility of HEs in the manipulation and modification of transgenes in this important vector.

3.2 Introduction

Aedes (Ae.) aegypti is the most important vector of arboviruses worldwide, due to its central role the transmission of dengue viruses, yellow fever virus and chikungunya virus to human hosts (Halstead, 2007). To augment current control efforts such as source reduction and insecticide application, considerable effort has been put into the development of genetics-based strategies such as population replacement and reduction. Of these, population reduction programs using genetically sterilized mosquitoes have showed great promise (Harris et al., 2012a, Harris et al., 2011). In contrast, population replacement strategies have been limited to the non-transgenic introduction of beneficial *Wolbachia* endosymbionts (Hoffmann et al., 2011). One of the primary limitations in this regard has been the lack of experimentally validated gene drive mechanisms for *Ae. aegypti*, despite some dramatic success stories in both *Drosophila* (Chan et al., 2011, Chen et al., 2007) and *Anopheles (An.) gambiae* (Windbichler et al., 2011).

Homing endonucleases (HEs), zinc-finger nuclease (ZFNs) and transcription activator-like effector nucleases (TALENs), the so-called meganucleases, are able to recognize and cleave rare occurring (15-30 bp) double-stranded DNA sequences, allowing precise editing of large, complex genomes [reviewed in (Joung and Sander, 2012a, Stoddard, 2011)]. Whereas HEs are naturally occurring selfish elements, both ZFNs and TALENs are artificial hybrids of tailored DNA binding domains and a non-specific nuclease domain. HEs can be divided into four distinct families based on their structure and mechanism of DNA binding and restriction [reviewed in (Stoddard, 2011)]. Of these, the LAGLIDADG-type HEs (LHEs) such as I-SceI, I-CreI and I-AniI are

by far the best characterized in terms of structural information, biochemical redesign, and sequence diversity.

In most organisms, the repair of double-stranded DNA breaks (DSB) occurs through either non-homologous end-joining (NHEJ) or one of several forms of homologous recombination, such as single-strand annealing (SSA), synthesis-dependent strand invasion and gene conversion [reviewed in (Huertas, 2010)]. These pathways are often in competition with each other, with the choice of repair mechanism influenced by the cell cycle stage (Longhese 2010), developmental stage (Chan *et al.*, 2011), and the presence and proximity of homologous sequences (Agmon *et al.*, 2009, Chung *et al.*, 2010, Kappeler *et al.*, 2008, Mansour *et al.*, 2008, Preston *et al.*, 2002). NHEJ-based repair may conservatively restore the parent sequence, but often results in the insertion or deletion of base pairs around the break site. SSA uses homology between two direct repeat sequences that flank the DSB to guide the repair process, with the result being a loss of all genetic information located between the repeats. In contrast, gene conversion-based repair using the sister chromatid or homologous chromosome is the most conservative, typically restoring the damaged region without error. Where repair during meiosis uses the homologous chromosome, the result is a loss of heterozygosity; though undesirable from the host organism's perspective, from the perspective of a selfish element this loss of heterozygosity represents super-Mendelian inheritance of the copied sequence.

Based on their natural tendencies to invade populations through DSB induction followed by gene conversion-based repair, HEs have been suggested as a potential gene drive system for vector-borne disease control (Burt, 2003a, Burt and Koufopanou, 2004a). Current models suggest that significant impacts on

public health could be observed following the release of just a few HEs in relatively short timeframe [2-3 years ; (Deredec *et al.*, 2011)]. Most excitingly, several groups have reported the successful establishment of LHE-based gene drive systems in *D. melanogaster* (Chan *et al.*, 2011) and *An. gambiae* (Windbichler *et al.*, 2011) using I-SceI. An unrelated HE, I-PpoI, has been shown to induce shredding of ribosomal DNA in *An. gambiae*, leading to extensive sterility (Windbichler *et al.*, 2008) and population crashes in large cage trials (Klein *et al.*, 2012).

We have previously shown that the HEs I-SceI, I-CreI, I-AniI and I-PpoI can induce DSBs in the *Ae. aegypti* soma when appropriate target sites are present in the mosquito chromosome, and that I-PpoI targets the *Ae. aegypti* rDNA repeats (Traver *et al.*, 2009). In this report we show that I-CreI, I-SceI, Y2-I-AniI can induce DSBs at their specific target sites in *Ae. aegypti* germline using two independent transgenic strains bearing HE target sites. Of these, the efficiency of transgene excision was substantially higher for Y2-I-AniI in both cases, with 20-40% of injected survivors giving rise to progeny that had lost the target transgene. Both NHEJ- and SSA-type repair were observed, with the choice of repair associated with the presence of direct repeats in the transgene sequence. We conclude that these LHEs can edit the *Ae. aegypti* genome at useful frequencies, and are suitable scaffolds for targeted redesign efforts and the development of HE-based gene drive systems.

3.3 Materials and Methods

3.3.1 Plasmid construction and Luciferase assays

Plasmids pGL3-*IE1*, pGL3-*Ub^{L40}* and pGL3-*PUB* were described previously (Anderson *et al.*, 2010). To generate pGL3-*hsp82*, a 992-bp region containing the *Drosophila pseudoobscura hsp82* promoter was amplified by polymerase chain reaction (PCR) from p*Khsp82* (Coates *et al.*, 1996) using the primers 5'-TTTTCCATGGGTTTTAATTTAACAGCAGAG-3' and 5' - TTTTAAGCTTATGGATTTTACCATATTATTA-3'. All PCR reactions were performed using Platinum Pfx (Invitrogen, Carlsbad, CA) as follows: 94°C for 2mins, 94°C for 15s, 65°C for 30s, 68°C for 70s, 35 cycles, 68°C for 10mins. Amplicons were digested with HindIII and NcoI (NEB, Ipswich, MA), purified, and ligated into the corresponding sites of pGL3-Basic (Promega, Madison, WI). The normalization control plasmid pRL-CMV-Renilla was purchased from Promega.

The *PUB*-HE (I-*CreI*, I-*SceI* and I-*PpoI*) expression vectors were generated using p*Khsp82*-HE plasmids (Traver *et al.*, 2009) as a template and primers 5'-tttccatggTTAAATTTAAAACACGGATCCATGC-3' and R 5'-tttgcggccgcGATCTTGATCTTCATGGTCGACG-3 in order to add *NcoI* and *NotI* restriction sites (underlined bases). Plasmid pSLfa-*PUB*-EGFP-SV40 (Anderson *et al.*, 2010) was digested with *NcoI* and *NotI* to remove the EGFP coding region; all three homing endonuclease genes were ligated into these sites to generate pSLfa/*PUB*-I-*CreI*, pSLfa/*PUB*-I-*SceI*, pSLfa/*PUB*-I-*AniI*, and pSLfa/*PUB*-I-*PpoI*. pSLfa/*PUB*-Y2-I-*AniI* was produced using the QuikChange II-E Site-Directed Mutagenesis Kit (Stratagene) to introduce the F13Y and S111Y mutations described by Takeuchi *et al* (2009).

For luciferase experiments, the experimental plasmid (pGL3-*PUB*, pGL3-*Ub_{L40}*, pGL3-*IE1*, pGL3-*hsp82* or pGL3 Basic) and normalization control plasmid (pRL-*CMV*) were co-injected at 0.3 µg/µl each into *Aedes aegypti* Liverpool strain embryos (G₀). Three replicates of 100-120 embryos each were injected with each promoter construct. Injected embryos were snap-frozen in liquid nitrogen at 2, 4, 12, 24, 48 and 72 hours post injection. Embryos were homogenized in the lysis buffer provided by the manufacturer (Promega, Madison, WI). Activity of both Firefly luciferase (FF-luc) and Renilla luciferase (R-luc) were determined by using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI) with a GloMax 20/20 instrument according to the manufacturer's protocol.

3.3.2 Mosquito strains and microinjections

Ae. aegypti kh^w, *PUB*-EGFP line #P5 (Anderson *et al.*, 2010) and transgenic line P11A (Adelman *et al.*, submitted) were maintained as previously described (Adelman *et al.*, 2008). For embryonic microinjections, 0.3 µg/µl of pSLfa/*PUB*-Y2-I-*AniI*, pSLfa/*PUB*-I-*SceI*, pSLfa/*PUB*-I-*CreI* or pSLfa/*PUB*-I-*PpoI* in 1X injection buffer (Coates *et al.*, 1998) was injected separately into 1 hour old embryos of transgenic lines *PUB*-EGFP line #P5 or #P11A. Eggs were hatched 5 days post injection and surviving G₀ females were merged into pools of 20-25 individuals and mated to *kh^w* strain males. G₀ males were mated individually to 5 *kh^w* females for 2-3 days and pooled prior to blood feeding and egg collection. G₁ larvae were screened using a fluorescent Leica MZ16F microscope for presence/absence of marker genes (DsRed or GFP).

3.3.3 Analysis of DSB repair events

To identify cleavage events in the transgenes from *PUB*-EGFP line #P5, primers 5'-CGCCACCACCTGTTCTGTA-3' and 5'-CTCTCAGTGCAGTCAACATGTCGAG-3' were used to amplify the target region from genomic DNA for DsRED positive, EGFP negative individuals (G^-R^+). PCR conditions were: 94°C for 2mins, 94°C for 30s, 60°C for 30s, 68°C for 3.5min, 35 cycles, 68°C for 10min using Platinum *Pfx* (Invitrogen, Carlsbad, CA). For G_1 individuals scored as EGFP positive, DsRED negative (G^+R^-), primers 5'-CTTACTTCCAAGCTGCTCTGCGA-3' and 5'-CTTTGTTCACTCTGAAATTTTCTCCTCTGGC-3' were used with the same PCR conditions. To identify cleavage events in line 3xP3-P11A, primers 5'-AAGTGGTGATTTTGACGTCGACGAGATCGG-3' and 5'-TACCACCAAGCTGTCAGTTCCAAC-3' (G^-R^+ ; 94°C for 2min, 94°C for 30s, 67°C for 30s, 68°C for 2min, 35 cycles, 68°C for 10min) or 5'-TTGCCGGTGGTGCAGATGAACTTCAGG-3' and 5'-CTTACTTCCAAGCTGCTCTGCGA-3' (G^+R^- ; 94°C for 2min, 94°C for 30s, 60°C for 30s, 68°C for 2min, 35 cycles, 68°C for 10min) were used. All amplicons were gel purified and sequenced directly.

3.4 Results

3.4.1 Development of a plasmid-based assay for HE function in *Ae.*

aegypti embryos.

Initially, we designed a series of HE-expression constructs based on the *D. pseudoobscura hsp82* promoter (Coates et al., 1996), used previously to successfully drive the expression of *Mos1* transposase in *Ae. aegypti* embryos

(Coates *et al.*, 1998). With the exception of I-*PpoI*, where significant mortality was observed, none of the other homing endonucleases had detectable activity (defined as excision of the EGFP reporter gene) in this system (data not shown). Therefore, we sought to identify alternative, more active promoters for controlling HE expression in the early embryo. Using a dual luciferase reporter assay, we examined the ability of three additional promoters to drive gene expression at various times following injection into pre-blastoderm embryos; the baculovirus *IE1* promoter and the *Ae. aegypti polyubiquitin (PUB)* and *Ub_{L40}* promoters (Anderson *et al.*, 2010). At all times examined, expression from the *PUB* promoter substantially exceeded all the others by several orders of magnitude (Fig. 3.1). Maximum expression from the *PUB* promoter was achieved just 2-4 hr after injection, while with the *IE-1* and *Ub_{L40}* promoters expression did not peak until 12 hr. Therefore we placed each HE ORF downstream of the *Ae. aegypti PUB* promoter to analyze embryonic and germline endonuclease activity.

To test the ability of each HE to introduce double-stranded DNA breaks in early stage mosquito embryos, we co-injected each *PUB*-HE expression construct with a single-stranded annealing (SSA)-dependent luciferase reporter, wherein the first 300 bp of the firefly luciferase ORF was duplicated (Fig. 3.2A). A series of stop codons, along with the recognition sequence for each HE, was placed in the intervening spacer region. In all three cases (Y2-I-*AniI*, I-*SceI*, I-*CreI*), injection of a *PUB*-HE expression construct resulted in a significant increase in firefly luciferase expression compared to embryos injected with a non-specific control construct, *PUB*-EGFP (Fig. 3.2B). Expression of Y2-I-*AniI* induced significantly more luciferase expression than either I-*SceI* or I-*CreI*, which did not differ from each other. We conclude that all three HE are active in

the early embryos of *Ae. aegypti*, and thus may be capable of catalyzing DSBs in the germline of this mosquito.

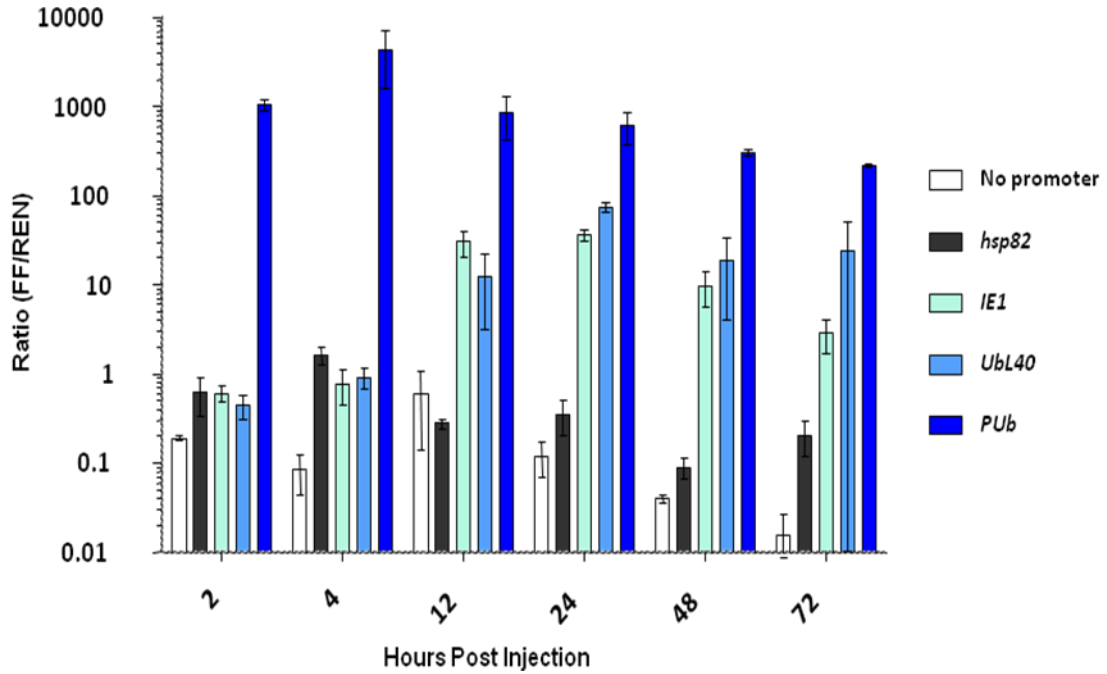


Figure 3. 1. Transcriptional activity of *IE1*, *Ub_{L40}*, *PUb* and *hsp82* promoters in *Ae. aegypti* embryos.

The ratio of Firefly (FL) to Renilla (RL) luciferase activity for each experimental promoter was compared with no-promoter control plasmid (pGL3) at six different time-points post-injection into *Ae. aegypti* embryos. Error bars indicate the standard deviation amongst 9 biological replicates, with each replicate consisting of approximately 100 injected embryo

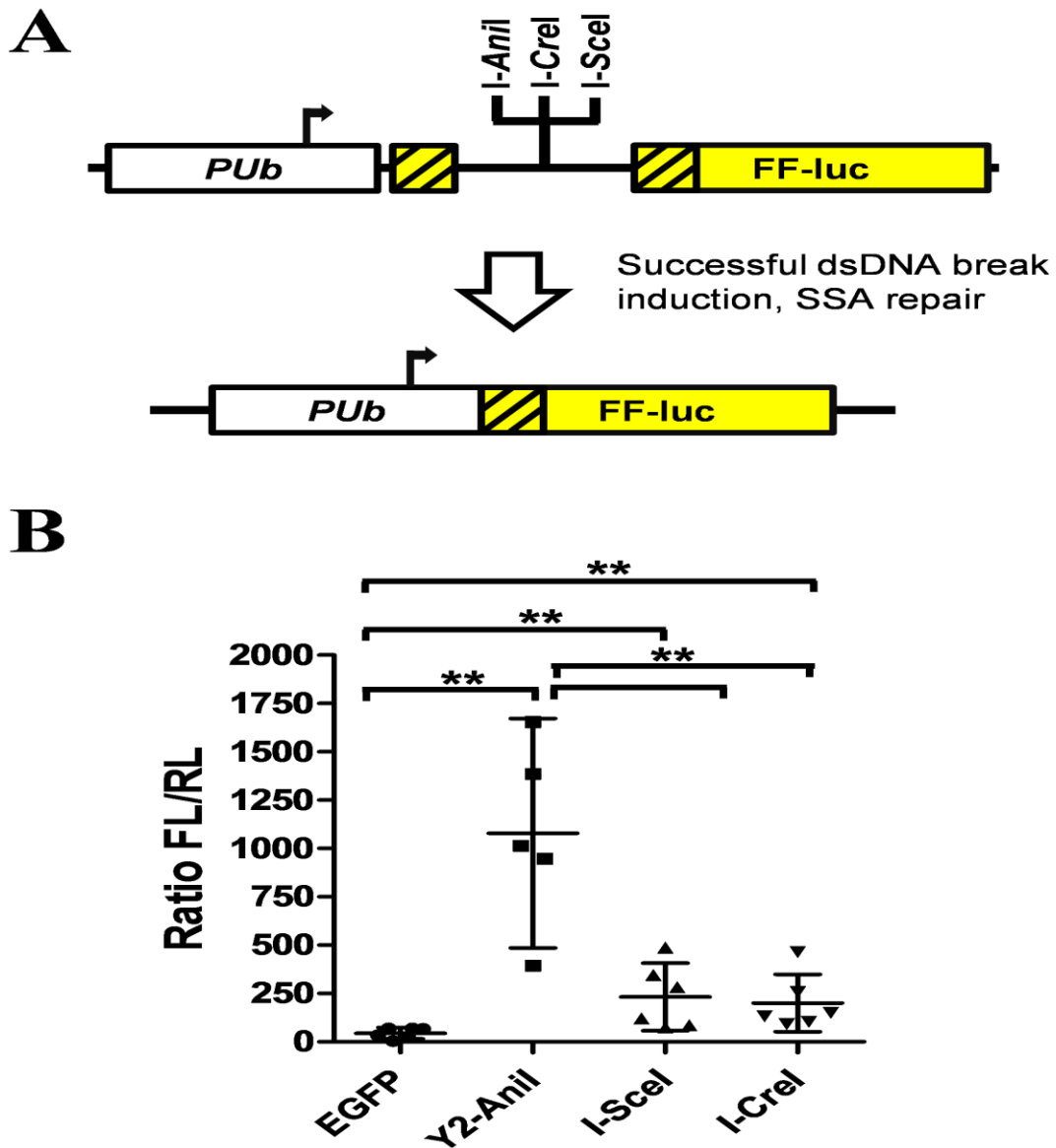


Figure 3. 2. Embryo-based single-strand annealing (SSA) assay.

(A) Schematic representation of the SSA test construct injected into *Ae. aegypti* embryos along with the specified *PUb*-HE expression vector; successful cleavage of the HE target site followed by SSA-based repair restores the FF-luc ORF. (B) Ratio of Firefly (FL) to Renilla (RL) luciferase (24hr or 48 hr?) following injection into pre-blastoderm embryos. Error bars indicate the standard deviation; each point represents a group of ~100 injected embryos. Statistical significance between pairs was determined using the Mann-Whitney test; ** indicates $P < 0.01$

3.4.2 Germline excision by homing endonuclease in line PUB-P5

To determine whether HEs were able to catalyze the excision of *Ae. aegypti* genomic segments in a heritable manner, HE expression constructs were injected individually into the embryos of a transgenic strain (P5) containing a *PUB*-EGFP cassette flanked by the recognition sequences for each HE (Anderson *et al.*, 2010), as well as a 3xP3-DsRED cassette (Fig. 3.3A). Surviving individuals were pooled, mated to an unmarked strain (*kh^w*) and offered a bloodmeal; progeny were screened as larvae for the presence/absence of each fluorescent marker (Table 3.1). Progeny that had lost expression of the EGFP marker were identified at varying frequencies for HEs I-*CreI*, I-*SceI* and Y2-I-*AniI* (Table 3.1). EGFP⁻ progeny were detected in all four Y2-I-*AniI* pools, and consisted of 2-4% of the total progeny; while the excision of EGFP by I-*SceI* (4/8 pools) and I-*CreI* (1/6 pools) appeared to be less efficient. No evidence for transgene excision was seen from the few survivors of I-*PpoI* injection.

Table 3. 1. HEs catalyze germline excision of genome segments in PUB-EGFP line #P5.

HE	# embryos injected	# G ₀ survivors (%)	Pool #	G ₁ ⁺ /total(% ⁺)	# of G ₁ individuals	
					G ⁻ /R ⁺	G ⁺ /R ⁻
I-CreI	1,100	149 (13.5%)	4	2/2,800 (0.07%)	1	1
			1-3;5-6	0/11,800	0	0
Y2-I-AniI	1,010	100 (9.9%)	1	46/1,200 (3.8%)	46	0
			2	66/1,500 (4.4%)	66	0
			3	70/2,900 (2.4%)	70	0
			4	4/800 (0.5%)	4	0
I-SceI	1,170	199 (17.0%)	1	2/1,500 (0.4%)	0	2
			2	1/3,000 (0.1%)	1	0
			6	38/3,300 (2%)	38	0
			7	5/3,600 (0.25%)	5	0
			3-5;8	0/10,700	0	0
I-PpoI	1,050	13 (1.2%)	1-2	0/2,300	0	0

Sequencing of the remaining transgene sequence from EGFP⁺ progeny confirmed the complete loss of the *PUB*-EGFP gene cassette and revealed that both NHEJ and SSA were used as repair mechanisms to different extents (Fig. 3.2). For Y2-I-*AniI*, we obtained 47 sequences corresponding to at least nine independent events that appeared to result from NHEJ following Y2-I-*AniI*-induced DSBs at both the upstream and downstream recognition sites (Fig. 3.3A, 3.3B). Of these, 14 sequences (3 events) corresponded to perfect repair that restored a single I-*Ani* recognition site, with the remaining events characterized by small deletions or insertions (Fig. 3.3B). In these instances, independent events were defined as having a distinct modified transgene sequence, or as being derived from independent G₀ pools. The introduction of a single DSB could also be followed by SSA-based repair, resulting in the collapse of direct repeat sequences. In line P5, the largest direct repeat sequence consisted of the 240 bp SV40 3'UTR which followed each of the EGFP and DsRED ORFs. All EGFP⁺ progeny from the I-*Scel* experiment were found to have resulted from the collapsing of these two repeats (Fig. 3.3C). SSA-based repair collapsing the SV40 repeats was also commonly observed for Y2-I-*AniI*. In this case, however, most sequenced mosquitoes contained small deletions or insertions at the remaining I-*AniI* site. We interpret this to be a result of SSA-based repair, followed by further cleavage by Y2-I-*AniI* and subsequent NHEJ (Fig. 3.3C). While most SSA-repair utilized the SV40 repeats, we recovered at least four instances where other HE recognition sites were used to direct the SSA response (while the order of these sites is scrambled in each cluster, they remain organized as direct repeats). Thus, even sequences as short as 18-20bp can direct the SSA repair response. While almost all progeny recovered that displayed a phenotypic change were found to be

DsRED⁺/EGFP⁻, two instances where the DsRED marker was lost were identified. Of these, a single I-*CreI* larvae died early in development and we were unable to determine the nature of the excised region. For both individuals derived from the I-*SceI* experiment, a large deletion (and small insertion) spanning the entire 3xP3-DsRED gene cassette was identified (Fig. 3.3D). As there is no homologous sequence with the transgenic construct at this location, we classify this as a NHEJ event.

3.4.3 Germline excision by homing endonuclease in line 3xP3-RG-P11A

Local chromosomal structure may influence the accessibility of HEs to their target sites, and thus may affect the rate of DSB formation. Therefore, we repeated our experiments using a second recipient line (P11A), containing another double-marked transgene (Fig 3.4A). Inverse PCR revealed that the P11A insertion mapped to a different genomic scaffold than line P5, though both were found to be incorporated into the large intronic regions of protein-coding genes (Table 3.S1). Progeny found to have lost the expression of at least one of the two markers were recovered following injection with Y2-I-*AniI* and I-*CreI*, but not from I-*SceI* or I-*PpoI* (Table 3.2). Interestingly, of those showing a loss of marker gene expression, injection of I-*CreI* resulted in mostly G⁺/R⁻ progeny, while Y2-I-*AniI* produced mostly G⁻/R⁺ progeny. A class of progeny that had lost both marker genes was recovered only following Y2-I-*AniI*. Once again, survival of I-*PpoI* mosquitoes was extremely low.

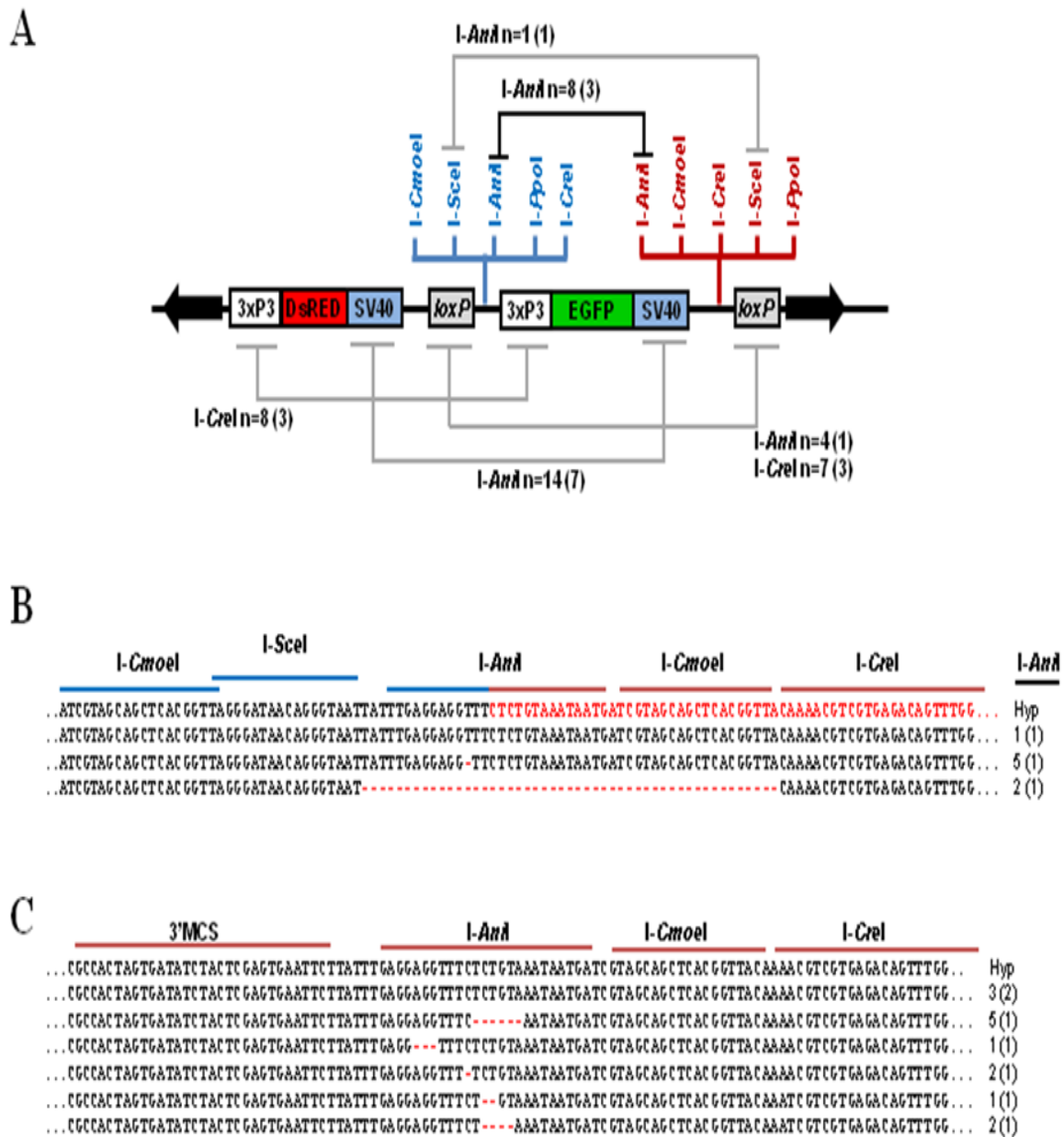


Figure 3.4. HE-catalyzed germline excision in *Aedes aegypti* transgenic line P11A.

(A) Transgenic construct for line P11A. Connectors indicate NHEJ (black) or SSA (gray) based repair following deletion of the intervening segment. Sequences obtained following NHEJ (B) or SSA (C) -based repair. For each, the number of sequenced mosquitoes (n) along with the minimum number of unique occurrences (in parentheses) are indicated.

Table 3. 2. Germline excision of genome segments in transgenic line P11A

HE	# embryos injected	# G ₀ survivors (%)	Pool #	G ₁ ⁺ /total (%) [†]	# of G ₁ individuals		
					G ⁻ R ⁺	G ⁺ R ⁻	G ⁻ R ⁻
I-CreI	1010	105 (10.6%)	1	50/1250 (4.0%)	9	41	0
			2	10/1400 (0.7%)	10	0	0
			3	7/2150 (0.3%)	3	4	0
			4	77/2550 (3.1%)	0	77	0
Y2-I-AniI	1090	110 (10.1%)	1	26/1350 (1.9%)	20	1	5
			2	50/1000 (5.0%)	43	4	3
			3	49/1900 (2.5%)	34	0	15
			4	79/1650 (4.7%)	76	0	3
			5	15/1700 (0.8%)	15	0	0
I-SceI	1000	110(11.0%)	1-4	0/6400	0	0	0
I-PpoI	1100	14 (1.2%)	1-2	0/2650	0	0	0

Consistent with the previous data from line P5, we observed both NHEJ (Fig 3.4B) and SSA type repair (Fig. 3.4C) following the complete excision of transgene segments following injection with Y2-I-*AniI*. Once again, when SSA-directed repair was used, most sequenced individuals contained small deletions at the remaining I-*AniI* recognition site, indicating additional cutting by Y2-I-*AniI* following the initial repair event. For Y2-I-*AniI*, the SSA pathway primarily utilized the SV40 direct repeats (7 events) flanking the upstream I-*AniI* site to direct the repair process. Two additional SSA repair events were found based on the *loxP* and I-*SceI* repeats present in the transgene construct. In contrast, all I-*CreI*-mediated events were found to be the result of SSA-repair resulting in the collapse of the ~260 bp 3xP3 promoter (3 events) or the 35 bp *loxP* sites (3 events), with no evidence for NHEJ (Fig. 3.3A). Attempts to identify the genetic basis for the R-/G- phenotypes seen in the Y2-I-*AniI* experiment were unsuccessful, as it appeared that the entire transposon construct, and an unknown quantity of the surrounding chromosomal DNA had been lost. As the G₁ individuals were only hemizygous for the transgene insertion, PCR using primers outside of the transgene was confounded by the presence of the alternate, wild-type allele. The lack of such R-/G- individuals from the other three experiments argues against a more mundane explanation such as incomplete homozygosity in the parental strain with respect to the transgene insertion (all injections were performed from a single parental cage), and suggests HEs may also trigger larger scale deletions of chromosomal segments.

For both the P5 and P11A experiments, we calculated the minimum HE excision frequency, defined as the number of independent excision events per fertile G₀ individual (Table 3.3). Y2-I-*AniI* proved the most effective in both

genetic backgrounds, with 20-40% of fertile G₀ individuals producing at least one offspring bearing an excision event (Table 3.3). While both I-*SceI* and I-*CreI* were also able to catalyze transgene excision, they did so at a reduced rate and in a manner that appeared to be more dependent on the transgene insertion site.

Table 3. 3. Minimum HEs excision frequency and number of independent repair mechanisms in two transgenic lines.

HE	P5				P11A			
	Min. efficiency*	# of independent events			Min. efficiency*	# of independent events		
		SSA	NHEJ	SSA+NHEJ		SSA	NHEJ	SSA+NHEJ
Y2-I- <i>Anil</i>	40%	6	9	5	22%	4	3	5
I- <i>Scel</i>	4%	3	1	0	<1.8%	0	0	0
I- <i>Crel</i>	2.7%	0-2	0-2	0-2	11.4%	6	0	0

* defined as the ratio of independent events per fertile G₀ survivor.

Table 3S. 1. Genomic locations of the *Mos1* insertions used in this study.

Transgenic line	Coordinates (TA dinucleotide)	Description	5' Flanking Sequence...TA... 3' Flanking Sequence
P11A	supercont1.102: 2,266,658- 2,266,659	Genic: AAEL003959 (within intron 4-5)	tgattttataaacattttacatagcgcgatccatgaaaattta TA atgctcaattagtttgaccttttctaacggcttagtgc
P5	supercont1.450: 904,391-904,392	Genic: AAEL010064 (within intron 3-4)	gtagataacttctcttgattctaaatctagatctaaagttc TA cattttcaacaagtaatgaaaaattgataaacattc

3.5 Discussion

Two different transgenic lines were used to determine the ability of four HEs to perform site-specific excision of gene segments in the *Ae. aegypti* germline. Of these, Y2-I-*AniI* gave the most useful frequencies of transgene excision, followed by both I-*CreI* and I-*SceI*. Both of the target transgenes in our study were found to have integrated into the large intronic space of a gene. While HE-based editing at both of these locations was highly effective, additional target sites within tightly wound heterochromatic space would be required to determine if such local structure poses a substantial barrier to HE-based targeting. However, for most downstream applications HE-based gene targeting efforts are likely to focus on euchromatic regions.

Embryonic survival following I-*PpoI* injection was substantially lower (~1%) than that of the other HEs used in this study. This was not unexpected, given that I-*PpoI* has a substantial number of recognition sites within the 28S rDNA repeats of *Ae. aegypti* (Traver *et al.*, 2009) and has been shown to shred the rDNA of *An. gambiae* leading to early embryonic lethality (Windbichler *et al.*, 2007, Windbichler *et al.*, 2008, Klein *et al.*, 2012). We reasoned that the few survivors from these injections may still produce excision events due to receiving a sub-lethal dose, but this was not observed. This may be due to the fact that I-*PpoI* sites within the rDNA (n>300) vastly outnumber those in the transgene (n=2). Embryonic survival following injection with Y2-I-*AniI*, I-*CreI* and I-*SceI* ranged from 10-17%, similar to what we typically observe following injection of transposon-based DNA constructs (Adelman *et al.*, 2008, Anderson *et al.*, 2010, Carpenetti *et al.*, 2011), suggesting that these HEs have recognize few (if any) cryptic off-target sites in the *Ae. aegypti* genome.

Sequencing of the transgene following HE-induced DSBs revealed the footprint of both NHEJ and SSA-based repair processes. We note that gene conversion-based repair would result in the restoration of the lost transgene sequence, and would be phenotypically invisible in our assay; thus all excision frequencies we report likely underestimates the magnitude of HE-induced DSB formation. The method of DNA repair we observed varied based on both the HE and the structure of the target transgene. It is well-established that these two repair methods are in competition, and that the presence and proximity of direct repeats can influence the decision for repair to proceed via NHEJ or SSA [reviewed in (Huertas, 2010)]. Both transgenes contained a number of direct repeats that varied in length from 18-260bp, and a single DSB introduced at either cluster would leave at least one viable SSA-based repair option. For example, following *I-SceI* injection into line P5, we only observed SSA-based repair at the SV40 direct repeats. This suggests that only a single DSB occurred in the upstream cluster (a similar break at the downstream cluster would leave both SV40 repeats upstream of the break). Simultaneous induction of DSBs at both the upstream and downstream clusters would eliminate one of the repeats, and thus might favor NHEJ as observed for Y2-*I-AniI*. Similarly, all *I-CreI* induced excision events were associated with SSA repair centered on the 3xP3 or *loxP* repeats, consistent with the induction of a single DSB at either the upstream or downstream clusters, respectively. Interestingly, *I-CreI* induced DSBs in the upstream cluster of the P11A transgene were only associated with collapse of the 3xP3 repeats, whereas this was not observed for Y2-*I-AniI*, whose action instead led primarily to the choice of SV40 repeats. One possible explanation is that the *I-CreI* recognition site in the upstream cluster was immediately adjacent to the second 3xP3 repeat.

Thus the speed of resection and proximity of each repeat to the end of the DSB may dictate the ultimate choice of homologous sequence used for repair (Agmon *et al.*, 2009, Chung *et al.*, 2010). Further exploration of this using the HE-based system we have developed would be an interesting platform to explore the mechanisms of DSB repair in *Ae. aegypti*, whose genome is rich in short repetitive elements.

The LHEs Y2-I-*AniI*, I-*CreI* and I-*SceI* have all been successfully redesigned through a variety of approaches to recognize new target sequences (Smith *et al.*, 2006, Arnould *et al.*, 2006, Arnould *et al.*, 2007, Rosen *et al.*, 2006, Redondo *et al.*, 2008, Chen and Zhao, 2005, Doyon *et al.*, 2006, Windbichler *et al.*, 2011). In particular, Y2-I-*AniI* and I-*CreI* were successfully altered to recognize sequences in the *An. gambiae* genome (Windbichler *et al.*, 2011). Our data indicate that the Y2-I-*AniI* scaffold [or its close relatives (Jacoby *et al.*, 2012)] may be the best suited for targeted redesign experiments involving *Ae. aegypti*. Extensive sequencing and structural analysis has revealed many more active LHE members (Takeuchi *et al.*, 2011), providing additional scaffolds as potential starting material for redesign efforts. While testing a large number of variant LHEs or a large pool of newly described scaffolds in germline-based experiments is likely not feasible, we note that data obtained from the transient SSA assay were highly predictive of success in the more time-consuming germline experiments. Thus, we anticipate that candidate HEs based on the Y2-I-*AniI* or other scaffolds validated in simple yeast-based assays (Chames *et al.*, 2005) can subsequently be tested for the potential to edit the mosquito genome in a more medium to high-throughput manner (1-3 test constructs per day) compared to what is possible with germline-based experiments (2-3 months per test construct).

The recent success of TALENs in editing a wide range of genomic targets at high efficiency, combined with the almost complete modularity of TALE sequence recognition is already leading to their adoption over other technologies such as ZFNs and LHEs (Mussolino and Cathomen, 2012, Joung and Sander, 2012a). However, there are clear situations where LHEs remain the preferred choice due to their small size and extreme target specificity (Stoddard, 2011). One such application may be the development of genetics-based control strategies for vector-borne disease agents such as dengue viruses. Such strategies depend on the ability to achieve super-Mendelian inheritance of one or more transgene sequences (James, 2005). This gene drive may be coupled to an anti-pathogen gene(s), resulting in the conversion of a competent vector population to an incompetent one, or used alone to inactivate one or more essential genes, resulting in population crash (Deredec *et al.*, 2011). Effective laboratory-based gene drive systems using maternal lethality/embryonic rescue (Chen *et al.*, 2007) and LHEs (Chan *et al.*, 2011) have been successfully demonstrated in model systems such as *Drosophila* and in the malaria mosquito (Windbichler *et al.*, 2011), yet this has not been achieved in *Ae. aegypti*. We conclude that HEs such as Y2-I-AniI (or its variants) should be considered as strong candidates for evaluation in gene drive experiments in this important disease vector species.

Chapter 4. TALEN-based gene disruption in the dengue vector *Aedes aegypti*

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4.1 Abstract

In addition to its role as the primary vector for dengue viruses, *Aedes aegypti* has a long history as a genetic model organism for other bloodfeeding mosquitoes, due to its ease of colonization, maintenance and reproductive productivity. Though its genome has been sequenced, functional characterization of many *Ae. aegypti* genes, pathways and behaviors has been slow. TALE nucleases (TALENs) have been used with great success in a number of organisms to generate site-specific DNA lesions. We evaluated the ability of a TALEN pair to target the *Ae. aegypti kmo* gene, whose protein product is essential in the production of eye pigmentation. Following injection into pre-blastoderm embryos, 20-40% of fertile survivors produced *kmo* alleles that failed to complement an existing *kh^w* mutation. Most of these individuals produced more than 20% white-eyed progeny, with some producing up to 75%. Mutant alleles were associated with lesions of 1-7 bp specifically at the selected target site. White-eyed individuals could also be recovered following a blind intercross of G₁ progeny, yielding several new white-eyed strains in the genetic background of the sequenced Liverpool strain. We conclude that TALENs are highly active in the *Ae. aegypti* germline, and have the potential to transform how reverse genetic experiments are performed in this important disease vector.

4.2 Introduction

Vector-borne diseases such as malaria and dengue fever remain large public health burdens, and novel interventions are still needed. The development of new methods of vector control would be aided substantially by a more detailed genetic and biochemical understanding of many critical behaviors such as development, host seeking, bloodfeeding and vector competence. Though the genomes of several disease vector mosquitoes have been sequenced, many mosquito-specific genes remain without any functional annotation, and there is much still to be learned with regards to understanding the genetic basis for these key behaviors. Of the disease vector mosquitoes that have a sequenced genome, *Aedes aegypti*, the primary vector for dengue viruses, is probably the most tractable due to the ease of adapting new strains to the laboratory environment and the ability to delay the hatching of developed embryos for months at a time. Progress in the field of site-specific gene editing with meganucleases indicates that these tools are sufficiently mature as to provide a novel means of performing reverse genetic experiments in a range of non-traditional organisms, including *Ae. aegypti*.

Though other meganucleases such as homing endonucleases and zinc finger nucleases have been used to perform custom editing of various genomes (reviewed in (Joung and Sander, 2012b, Stoddard, 2011)), their adoption by the research community has been limited at best. Limitations with these systems relate to the difficulty of assembling/reengineering these molecules to recognize new target sites due to the strong context-dependence of their DNA-binding regions. In contrast, transcription activator-like elements (TALEs) from the plant pathogenic bacteria *Xanthomonas* contain a simple, context independent DNA

binding region (Moscou and Bogdanove, 2009, Boch *et al.*, 2009). In these molecules, DNA binding is conferred by a series of 34 amino acid repeats, differing only at two positions (the repeat variable diresidue, or RVD), where each RVD specifies a given target nucleotide (Moscou and Bogdanove, 2009, Boch *et al.*, 2009). Fusion of TALE repeat domains to the *FokI* nuclease domain confers extreme site specificity and has allowed the editing of a number of diverse genomes (reviewed in (Joung and Sander, 2012b, Mussolino and Cathomen, 2012)), including the insects *Drosophila melanogaster* (Liu *et al.*, 2012), *Bombyx mori* (Sajwan *et al.*, 2012, Ma *et al.*, 2012) and *Gryllus bimaculatus* (Watanabe *et al.*, 2012). However, at present there are no reports of TALE nuclease editing in any disease vector species.

To examine the possibility of using TALE-based nucleases to edit the *Ae. aegypti* genome, we sought to take advantage of a known physical mutant with a clearly defined and easily recognizable phenotype (Sajwan *et al.*, 2012, Ma *et al.*, 2012, Liu *et al.*, 2012). While many physical mutants for this mosquito have been described (reviewed in (Craig and Hickey, 1967)), few have been associated with a specific gene product. A white-eyed mutant strain (Bhalla, 1968) was hypothesized to be orthologous to the *Drosophila cinnabar (cn)* mutant; later work confirmed that eye pigmentation in this strain could indeed be complemented by the *Drosophila cn+* gene both transiently and through stable germline transformation (Cornel *et al.*, 1997, Jasinskiene *et al.*, 1998, Coates *et al.*, 1998). This strain, first identified as *w*, but now known as *kh^w* (Cornel *et al.*, 1997), is used routinely in our lab as a convenient recipient for transgene insertions (Adelman *et al.*, 2008, Anderson *et al.*, 2010) as the lack of eye pigment facilitates screening using the eye-specific 3xP3 synthetic promoter

(Berghammer *et al.*, 1999). *kh^w* strain mosquitoes are deficient in kynurenine 3-monooxygenase (KMO) activity, and thus fail to produce ommochromes from tryptophan precursors (Bhalla, 1968, Cornel *et al.*, 1997, Han *et al.*, 2003).

We found that TALEN-based targeting of the *Ae. aegypti kmo+* allele was a highly efficient process, with 20-40% of fertile G₀ females producing new *kmo* mutant alleles in a complementation assay with the *kh^w* strain. Mutation rates were sufficiently robust that blind G₁ intercrosses resulted in several new white-eyed strains (Lvp^{*kmo*}) developed entirely within the genetic background of the sequenced Liverpool (Lvp) strain of *Ae. aegypti*. These results suggest that TALE-based applications are poised to revolutionize the study of *Ae. aegypti* genetics and allow the development of new genetic methods to disrupt disease transmission by this important mosquito vector.

4.3 Materials and Methods

4.3.1 Plasmid construction

To generate the SSA reporter, a synthetic fragment encoding the first 298bp of the Firefly luciferase gene and an additional 354 bp spacer region was inserted in between the *PUB* promoter and FF-luc ORF of pGL3Basic/*PUB*-FFluc (Anderson *et al.*, 2010). The spacer region included a portion of the *Ae. aegypti kmo* gene containing the target site. TALEN constructs were obtained from Collectis Bioresearch (Paris, France). Each TALEN-encoding sequence was placed downstream of the *Ae. aegypti polyubiquitin* promoter through standard cloning procedures. DNA for each of the *PUB*-TALEN plasmids was prepared using the Qiagen Endo-free Maxi-prep kit (Experiment #1) or the Machery-Nagel

endo-free midi kit (Experiment #2) as directed by the manufacturer prior to injection into mosquito embryos.

4.3.2 Mosquito rearing, crosses, and embryonic injections

Ae. aegypti mosquitoes (Lvp and *kh^w* strains) were maintained in an insectary at 28°C and 60-70% humidity, with a 14/10 h day/night light cycle. Embryonic injections were performed as described previously (Adelman *et al.*, 2008). For the transient assay, an injection mix containing the SSA test construct, *PUB*-TALEN and a normalization control in injection buffer (Coates *et al.*, 1998) were introduced into ~1 hr old pre-blastoderm embryos. All plasmids were present at 0.2 µg/µl, for a total DNA concentration of 0.8 µg/µl. Embryos were snap-frozen in liquid nitrogen at 24 hours post injection and lysate prepared for dual luciferase assay (Promega, Madison, WI). Luciferase activity was determined using the Dual-Luciferase Reporter Assay System with a GloMax-Multi Detection System instrument according to the manufacturer's instructions (Promega, Madison, WI). For germline experiments, *PUB*-TALEN constructs (0.3 µg/µl of each) were similarly introduced into developing embryos. G₀ survivorship counts were based on the number of individuals emerging as adults. For mating, G₀ survivors were separated into single vials as pupae; emergent adults were collected each day and transferred into male-only or female-only cages. G₀ males were anesthetized under CO₂ and mated individually to 5 virgin *kh^w* or Lvp strain females for two to three days, at which point they were either directly offered a bloodmeal (for Lvp experiments) or combined into families. Groups of G₀ females were combined with 15-20 males of the appropriate parental strain prior to bloodfeeding and egg collection.

4.3.3 PCR and mutational analysis.

Primers 5'-TCAACATAATTATACATGGCCAGATCGCAG-3' and 5'-TCTGATTGGTCGTGAGCGGTTGGTTAAGGA-3' were used to amplify the region containing the *kmo* target site from wild-type individuals or from TALEN-injected progeny. PCR was performed using the Phire Animal Tissue Direct PCR kit (Thermo Scientific, Lafayette, CO) using either a portion of the larval body in dilution buffer or an adult leg placed directly in the master mix as described by the manufacturer. Amplification conditions were: 98°C for 5 min, 98°C for 5 s, 70°C for 5 s, 72°C for 20 s, 39 cycles, 72°C for 1 min. Where amplification was unsuccessful, a second set of primers was used under the same conditions (5'-TCCAACGACGAAGGAATCTACTC-3' and 5'-CAAAACGACCGCATACAAAAC-3'). All amplicons were purified and sequenced directly in both directions using the same primers used during the PCR step.

4.4 Results

4.4.1 Selection of TALEN target site and transient embryo assay

Full-length cDNAs for both the wt and *kh^w* (*kmo*) gene (AAEL008879) have been characterized, with an in-frame deletion of 162 bp implicated as the causative mutation in the *kh^w* strain (Han *et al.*, 2003). The KMO protein is predicted to contain transmembrane domains near both the N and C termini, with the majority of the protein located on the cytoplasmic face of the membrane (Fig. 4.1A). Alignment of the *kmo* cDNA described by Han *et al* (2003) to the *Ae. aegypti* genome assembly revealed a structure consisting of seven exons (Fig. 4.1A). Interestingly, the proposed 162 bp deletion corresponded precisely to exon

6, suggesting that the *kh^w* phenotype may in fact be due to the failure to correctly splice in this exon. Indeed, sequencing of genomic DNA from this region from both *kh^w* and Lvp strain mosquitoes revealed an 11 bp deletion in the splice acceptor site of exon 6 only in the *kh^w* strain (Fig. 4S.1). As the loss of exon 6 was sufficient to eliminate KMO activity, we designed our TALEN pair to cleave the region just upstream of the exon 5-6 junction. A frameshift mutation at this location would be expected to result in the loss of coding information present in both exons 6 and 7, including the C-terminal membrane spanning domain.

To screen our TALEN pair for activity in *Ae. aegypti* embryos, we inserted the ~50bp TALEN target site from the *Ae. aegypti kmo* gene into a firefly luciferase-based reporter construct containing a tandem duplication of the first ~300 bp of the luciferase open reading frame (Fig. 4.1B). Successful TALEN-based cleavage at the target site, followed by single-strand annealing (SSA) repair is expected to result in the collapse of the two direct repeats and thus translation of the full length luciferase protein (reviewed in (Lyndaker and Alani, 2009a)). Indeed, following injection into pre-blastoderm embryos, we observed strong activation of firefly luciferase activity (Fig. 4.4.). We conclude that TALE-based nucleases are active in the early embryo of *Ae. aegypti* mosquitoes.

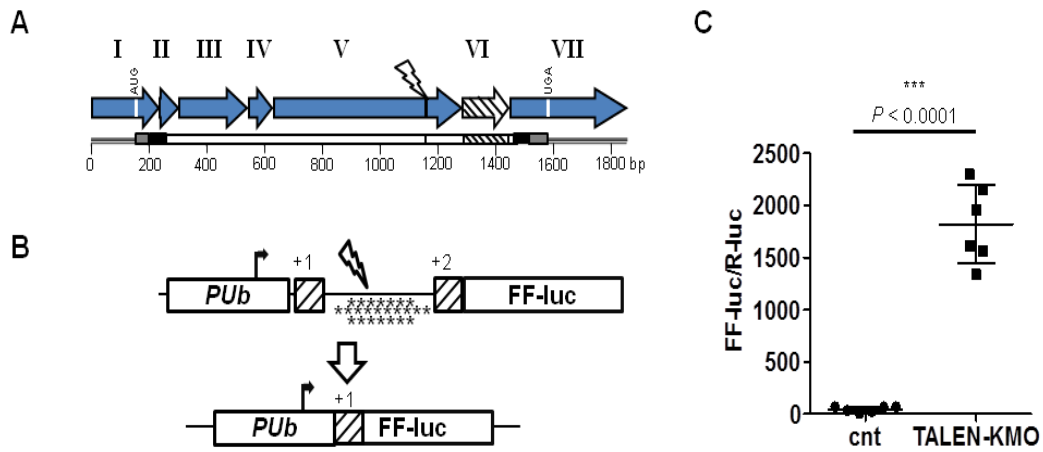


Figure 4. 1. Plasmid-based SSA assay for TALEN activity in *Ae. aegypti* embryos.

(A) cDNA structure of the *Ae. aegypti kmo* gene (AAEL008879). Exons (roman numerals), initiation and termination (white vertical bars) codons, and TALEN recognition site (black vertical bar) are indicated. The exon skipped in *kh^w* strain is indicated (white, cross-hatched arrow). The KMO ORF, with predicted extracellular (grey), transmembrane (black) and intracellular (white) domains are indicated below. (B) Schematic representation of the SSA test plasmid. TALEN recognition sites for *Ae. aegypti kmo* were located between two direct repeats (cross-hatched boxes) of the initial 298bp of the Firefly luciferase (FF-luc) coding region. Stop codons (denoted by *) in the +1 (7), +2 (10) and +3 (7) reading frames in the spacer are indicated. Transcription from the *polyubiquitin (PUB)* promoter is expected to lead to translation in the +1 ORF at the FF-luc AUG in the first repeat, resulting in a truncated protein. Fourteen additional AUG codons are present prior to the full-length +2 frame FF-luc ORF to minimize read-through translation. Double-stranded DNA break induction by the introduced TALEN pair (lightning shape) followed by SSA-mediated repair restores the FF-luc ORF. (C) Relative levels of FF-luc activity in the presence or absence of the KMO-targeted TALEN pair 24 hours following injection into *Ae. aegypti* embryos. Statistical significance following the Mann-Whitney test is indicated.

4.4.2 Identification of new TALEN-generated *kmo* alleles through lack of complementation with *kh^w*

To detect heritable gene editing, we injected the *kmo*-targeting TALEN pair into pre-blastoderm embryos of the black-eyed Liverpool (Lvp, *kmo*⁺/*kmo*⁺) strain and screened the progeny of the surviving individuals for white eyes. As the *kh^w* phenotype is completely recessive, injected survivors were mated to *kh^w* (*kmo^w*/*kmo^w*) mosquitoes in order to detect new mutant alleles. A test cross between untreated Lvp and *kh^w* strains demonstrated that 100% of progeny retained wild-type eye color (Table 4.1), confirming that our Lvp strain was free from rare *kmo* mutant alleles that might otherwise go undetected. In contrast, following injection of the TALEN constructs, white-eyed progeny were identified in seven of nine pools in experiment 1, and all three pools in experiment 2 (Table 4.1).

Since most of the pools produced white-eyed progeny, it seemed likely that by pooling G₀ individuals (a strategy common in *Ae. aegypti* transgenic experiments, due to the low rate of transposon-based transformation) we may have been underestimating the rate of TALEN-based editing. All six female pools were given a second bloodmeal, after which fed female mosquitoes were transferred to single rearing tubes and allowed to deposit eggs individually. From 65 fertile G₀ females, we obtained 23 that produced white-eyed progeny, an editing rate of ~35% (Table 4.2). This is an order of magnitude greater than transposable-element transformation in this species and confirms that our initial pooling strategy underestimated the amount of editing by a factor of four. Individual females produced an average of 38% white-eyed progeny, with some females producing up to 75% (Table 4.3).

Table 4. 1. Generation of new mutant kmo alleles from pooled G₀ populations.

Exp.	# embryos		G ₀		Phenotype*		
	injected	# G ₀ (%)	gender	Pool ID	wt	we	%we
Neg.	n.a.	n.a.	n.a.	n.a.	8970	0	0
control							
#1	1020	187 (18.3%)	♀	P1	1200	64	5.3%
			♀	P2	1350	70	5.2%
			♀	P3	250	24	9.6%
			♂	P4	1700	56	3.3%
			♂	P5	900	23	2.6%
			♂	P6	1100	11	1.0%
			♂	P7	2400	0	0%
			♂	P8	2700	0	0%
			♂	P9	1400	56	4.0%
#2	1010	195 (19.3%)	♀	B1	1800	130	7.2%
			♀	B2	200	31	15.5%
			♀	B3	36	3	8.3%

* wt, wild-type; we, white-eyed.

Table 4. 2. Frequency of TALEN-generated *kmo* alleles per fertile G₀ female.

	G₀ ♀	G₀ ♀ (fertile)	# G₀ ♀ producing	TALEN
	(total)		<i>kmo</i> progeny	frequency
P1	30	12 (40%)	3	25%
P2	30	18 (60%)	6	33%
P3	25	6 (24%)	1	17%
Exp1 total	85	36 (42%)	10	28%
B1	35	12 (34%)	6	50%
B2	30	11 (37%)	5	45%
B3	27	6 (22%)	2	33%
Exp2 total	92	29 (32%)	13	45%

Table 4. 3. Generation of new mutant kmo alleles from single G₀ females*

G ₀ ♀**	P1			P2			P3			B1			B2			B3		
	wt	we	%	wt	we	%	wt	we	%	wt	we	%	wt	we	%	wt	we	%
1	7	14	67%	15	28	39%	52	3	6%	43	28	39%	9	13	59%	36	18	33%
2	16	3	17%	17	5	13%				35	5	13%	46	1	2%	20	8	29%
3	8	24	75%	39	7	20%				29	7	20%	6	5	46%			
4				46	27	59%				19	27	59%	18	6	25%			
5				34	9	23%				30	9	23%	36	8	18%			
6				27	9	41%				13	9	41%						

* wt, wild-type; we, white-eyed.

** Each row represents the 1st, 2nd, 3rd, etc... female in each pool that produced one or more kmo mutant progeny.

Sequencing of the *kmo* target site from each of these families confirmed the existence of deleted bases (1-7 bp) in 21 of 23 cases (91%) (Fig. 4.2A). The remaining two cases may represent larger deletions that spanned at least one of the PCR primers, allowing amplification of only the *kh^w* allele. While most (18/21, 86%) of the deletions recovered represented frame-shift mutations, three in-frame deletions were also found: ΔThr^{337} , $\Delta\text{ThrVal}^{337-8}$, and $\Delta\text{CysThr}^{336-7}$, suggesting a potential critical role for these residues in KMO activity or stability. Based on these data, we conclude that TALEN-based gene editing is a highly efficient process in *Ae. aegypti*.

4.4.3 Identification of new *kmo* alleles in a complete *Lvp* genetic background

The identification of new *kmo* mutant alleles in the above experiments was simplified through the use of an existing mutant strain that failed to provide complementation. However, such a luxury would not be found in most circumstances, where investigations will focus on targeting new genes in order to identify novel phenotypes. Likewise, gene editing experiments will likely need to be performed entirely within the strain of study, without the introgression of confounding genetic material from unrelated and highly inbred strains. To determine if we could identify novel *kmo* mutations without the assistance of the *kh^w* complementation assay, we injected the *kmo*-targeting TALEN pair into *Lvp* embryos, and this time backcrossed the surviving individuals to *Lvp* strain mosquitoes. Offspring from this cross were 100% black-eyed; siblings within each family were intercrossed to obtain G_2 progeny. From just 10 fertile G_0 founders, we identified three that produced white-eyed progeny in the G_2 generation (Table 4.4). The frequency of white-eyed individuals in the G_2

A*supercont1.354*

1099815 bp

1099907 bp

	RH	<i>15 bp spacer</i>	LH		<u>exp1</u>	<u>exp2</u>
CTATCGACGTCAGTCCATGTTGATTGAACAACACTCGGTCAACACAGTACAATCCTCGAATCCGGCATTTCATCCCTGCCCGTAGAAGGGAACCGTGG					wt	
CTATCGACGTCAGTCCATGTTGATTGAACAACACTCGGTCAACACAGTACAATCCTCGAATCCGGCATTTCATCCCTGCCCGTAGAAGGGAACCGTGG					0/10	2/13
CTATCGACGTCAGTCCATGTTGATTGAACAACACTCGGTCAACA--AGTACAATCCTCGAATCCGGCATTTCATCCCTGCCCGTAGAAGGGAACCGTGG					1/10	0/13
*CTATCGACGTCAGTCCATGTTGATTGAACAACACTCGGTCAACACA---CAATCCTCGAATCCGGCATTTCATCCCTGCCCGTAGAAGGGAACCGTGG					1/10	0/13
CTATCGACGTCAGTCCATGTTGATTGAACAACACTCGGTCAACA----ACAATCCTCGAATCCGGCATTTCATCCCTGCCCGTAGAAGGGAACCGTGG					1/10	3/13
CTATCGACGTCAGTCCATGTTGATTGAACAACACTCGGTCAAC----TACAATCCTCGAATCCGGCATTTCATCCCTGCCCGTAGAAGGGAACCGTGG					1/10	0/13
CTATCGACGTCAGTCCATGTTGATTGAACAACACTCGGTCAACAC----CAATCCTCGAATCCGGCATTTCATCCCTGCCCGTAGAAGGGAACCGTGG					0/10	1/13
CTATCGACGTCAGTCCATGTTGATTGAACAACACTCGGTCAACACA-----ATCCTCGAATCCGGCATTTCATCCCTGCCCGTAGAAGGGAACCGTGG					5/10	3/13
*CTATCGACGTCAGTCCATGTTGATTGAACAACACTCGGTCAA-----ACAATCCTCGAATCCGGCATTTCATCCCTGCCCGTAGAAGGGAACCGTGG					1/10	0/13
*CTATCGACGTCAGTCCATGTTGATTGAACAACACTCGGTCAACACA-----TCCTCGAATCCGGCATTTCATCCCTGCCCGTAGAAGGGAACCGTGG					0/10	1/13
CTATCGACGTCAGTCCATGTTGATTGAACAACACTCGGTCAACA-----ATCCTCGAATCCGGCATTTCATCCCTGCCCGTAGAAGGGAACCGTGG					0/10	3/13

B

	RH	LH	
CTATCGACGTCAGTCCATGTTGATTGAACAACACTCGGTCAACACAGTACAATCCTCGAATCCGGCATTTCATCCCTGCCCGTAGAAGGGAACCGTGG			wt
CTATCGACGTCAGTCCATGTTGATTGAACAACACTCGGTCAAC----ACAATCCTCGAATCCGGCATTTCATCCCTGCCCGTAGAAGGGAACCGTGG			59
CTATCGACGTCAGTCCATGTTGATTGAACAACACTCGGTCAACA-----TCCTCGAATCCGGCATTTCATCCCTGCCCGTAGAAGGGAACCGTGG			2
CTATCGACGTCAGTCCATGTTGATTGAACAACACTCGGTCAACA----ACAATCCTCGAATCCGGCATTTCATCCCTGCCCGTAGAAGGGAACCGTGG			3a
CTATCGACGTCAGTCCATGTTGATTGAACAACACTCGGTCA-----ACAATCCTCGAATCCGGCATTTCATCCCTGCCCGTAGAAGGGAACCGTGG			3b

Figure 4. 2. TALEN-induced deletions in AAEL008879, the *Ae. aegypti* *kmo* gene.

Sequenced amplicons obtained from white-eyed individuals were aligned and compared to the wt *kmo* sequence in the *Lvp/kh^w* hybrid genetic background (A) or the *Lvp* background alone (B). The DNA-binding regions of the right (RH) and left (LH) TALENs are indicated. The three in-frame deletions are indicated (*).

Table 4. 4. Identification of new kmo mutant alleles in the Lvp genetic background.

ID#	G ₀ gender	G ₂ eye phenotype		
		wt	we	%we
1	♀	1700	0	
2	♀	1500	105	6.5%
3	♂	950	110	10.4%
5	♂	650	0	
9	♂	1050	0	
12	♂	750	0	
16	♂	450	0	
36	♂	650	0	
50	♂	800	0	
59	♂	750	36	4.6%

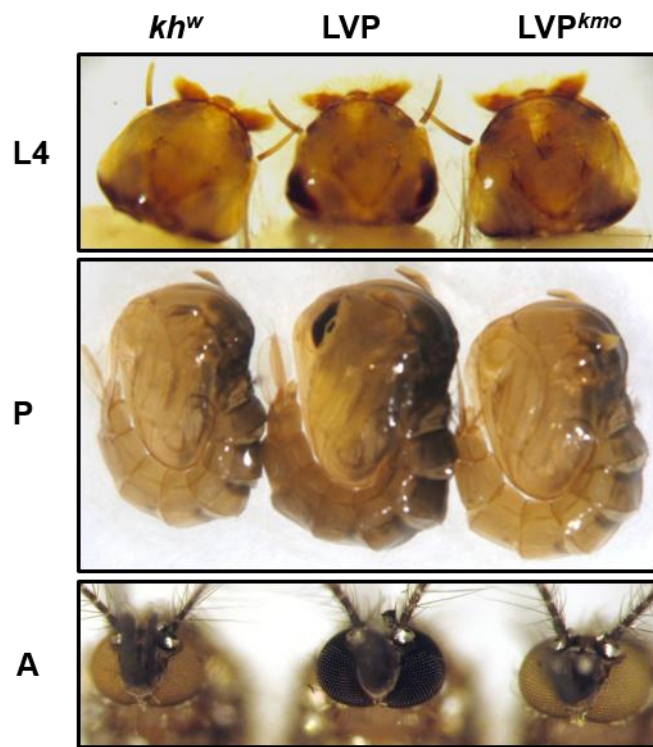


Figure 4. 3. TALEN-generated kmo alleles phenocopy kh^w strain mosquitoes.
Lvp, kh^w and LvpP^{kmo} mosquitoes imaged as larvae (L4), pupae (P) and adults (A).

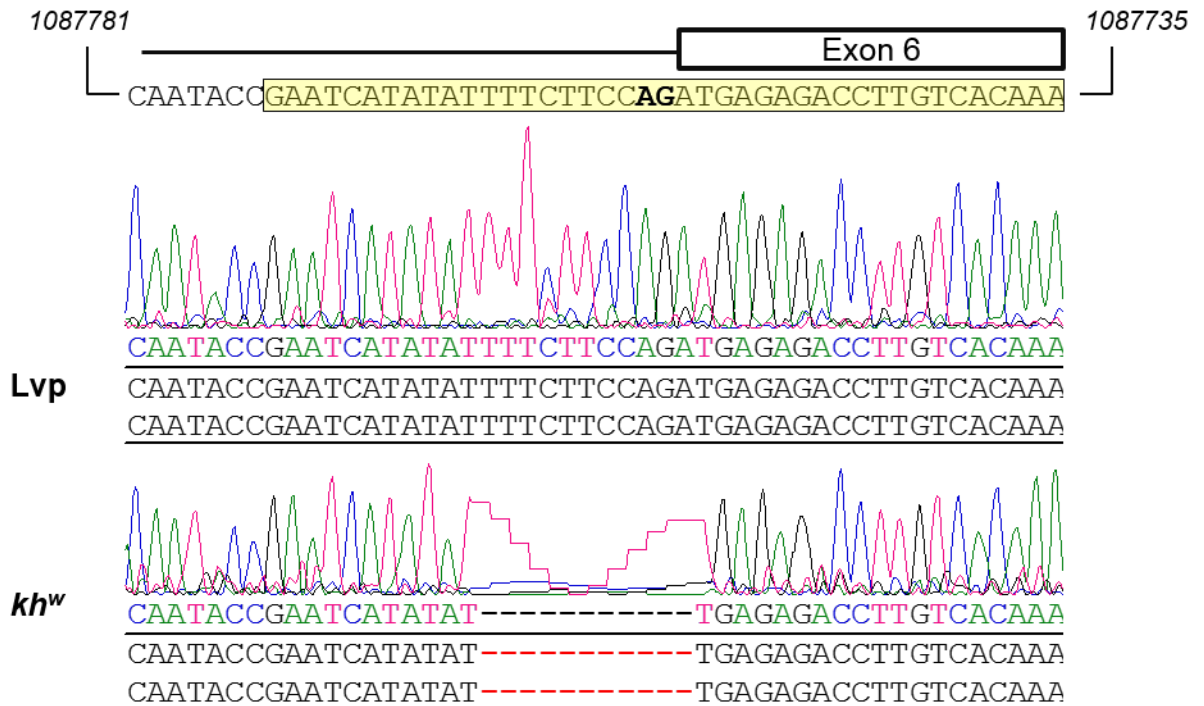


Figure 4S. 1. The kh^w phenotype is due to exon skipping.

Sequences obtained following PCR of the intron 5-6/exon 6 genomic interval of gene AAEL008879. Coordinates on supercontig1.354 are given. The splice acceptor site is highlighted in yellow; the final AG of the intron is indicated in bold.

generation ranged from 4.6-10.4%. This is consistent with an initial mutant allele frequency of 21-32% in the G₁ generation, similar to our prior experiments (Table 4.3). Sequencing of the TALEN target site in white-eyed G₂ individuals revealed genetic lesions consistent with a loss of function phenotype in all cases (Fig. 4.2B). In fact, we recovered four independent lesions from these three founders, suggesting that a single individual male produced multiple sperm with independent deletion events. Phenotypically, *Lvp^{kmo}* individuals were indistinguishable from *kh^w* strain mosquitoes at all life stages (Fig. 4.3). Thus, we conclude that TALENs can be used to edit the *Ae. aegypti* genome in a strain-independent manner at high efficiency, and that individuals homozygous for an expected mutation can be recovered at the G₂ stage at useful frequencies, even in the absence of any screening at the G₁ (hemizygous) state.

4.5 Discussion

Research efforts using model organisms such as *D. melanogaster*, *S. cerevisiae*, *C. elegans* and *A. thaliana* have benefitted tremendously from the availability of genetic stock centers housing large collections of mutant strains; whereas reverse genetic experiments in non-model organisms have been more limited. While the development of RNAi technology has enabled some such experiments to move forward, this technology is limited by low penetrance of injected double-stranded RNA into some tissues (Boisson *et al.*, 2006), gene by gene variation in the degree and timing of knockdown (Adelman, unpublished observations), and off-target effects resulting from the large pool of siRNAs generated from the introduced precursor molecules (Mohr *et al.*, 2010). In contrast, the ability to directly and specifically disrupt a gene of interest offers the possibility to perform intricate reverse genetic experiments on any gene, in any organism. We confirm that TALEN-based gene disruption can be a highly efficient process in *Ae. aegypti*, with editing rates between 20-40%. This is an order of magnitude greater than both traditional transposon-based transformation (Adelman *et al.*, 2002) and phiC31-based recombination (Franz *et al.*, 2011), and offers up the possibility that TALE-based experiments will be much more amenable to moderate or higher throughput applications than what has been achieved over the past decade with these less robust genetic systems.

In our experiments, we only examined a single TALEN pair. Thus, it is possible that not every such pair will achieve the same or similar activity. However, the success rates we observed are similar to those described in many other organisms, including several other insects (Watanabe *et al.*, 2012, Ma *et al.*, 2012, Liu *et al.*, 2012). Given the success of others in large scale TALEN pairs

screens (Reyon *et al.*, 2012, Carlson *et al.*, 2012, Lei *et al.*, 2012, Schmid-Burgk *et al.*, 2012, Cade *et al.*, 2012), the rate of TALEN failure appears to be acceptably low (<20%). The primary difficulty with developing new TALEN pairs to target genes of interest is the time and effort required to assemble the numerous TAL repeat constructs. However, the recent availability of many new assembly methods have substantially decreased the time required for developing new TALEN pairs, with full assembly decreasing from 6-8 weeks to 3-24 hrs (Reyon *et al.*, 2012, Briggs *et al.*, 2012, Sanjana *et al.*, 2012b, Schmid-Burgk *et al.*, 2012). We anticipate that if need be groups of TALEN pairs can be screened initially using the SSA assay we described in pre-blastoderm embryos. Others have demonstrated that in vitro SSA results are highly correlated with germline editing activity (Zhang *et al.*, 2012, Sajwan *et al.*, 2012); we have made similar observations with homing endonucleases in *Ae. aegypti* (Adelman, unpublished). Thus, germline editing experiments can be restricted to those TALEN pairs which perform well in this assay.

TALEN-based editing was associated with small deletions ranging in size from 1-7 bp. This is similar to results obtained in other insects such as the vinegar fly *D. melanogaster* (Liu *et al.*, 2012), the silkworm *B. mori* (Sajwan *et al.*, 2012, Ma *et al.*, 2012) and the cricket *G. bimaculatus* (Watanabe *et al.*, 2012). This limited deletion size has several favorable consequences; the most significant in our opinion is that we were able to recover essentially the same set of deletions in two independent experiments, where identical 4-bp and 5-bp deletions were recovered in both instances. This indicates that there may be no substantial burden for the long-term maintenance of TALEN-modified mosquito strains. Thus, there is no need for large (expensive) stock centers to house an ever-growing collection

of TALEN-modified strains. As long as the TALE-binding sites are made available (or the TALEN constructs themselves), the disruption in question could be re-generated at any point in the future, in the most useful genetic background at the time. In the same vein, identical deletions obtained from separate founders could be mixed into a single population, substantially eliminating the influence of any off-target effects possibly occurring within a single founder.

The modularity of TALE-binding domains lends them to applications beyond the generation of double-stranded DNA breaks. Though not addressed directly in our experiments, our data indicate that TALE fusions to other active domains, such as transcriptional activators/repressors (Mahfouz *et al.*, 2012) or recombinases (Mercer *et al.*, 2012), are certainly worth pursuing in *Ae. aegypti*. Likewise, experiments involving the knock-in of a transgene (Zhang *et al.*, 2012) or single-stranded oligonucleotide (Bedell *et al.*, 2012, Briggs *et al.*, 2012) through homologous recombination may further increase the ever growing utility of TALE-based enzymes in specifically editing the genome of this mosquito.

Chapter 5. Summary

5.1 General review

The goal of this study was to describe valuable tools for targeted gene disruption in the mosquito *Ae. aegypti*. Despite complete sequencing of the *Ae. aegypti* genome, the functions of most of the encoded genes remain unknown. Improving techniques for mosquito transgenesis facilitates this task. To generate stably transformed insects incapable of transmitting diseases, we must increase the transformation frequency; and for targeted gene disruption we need useful tools such as TALENs and HEs, with which we have had success. The ability to directly and specifically disrupt a gene of interest will play a vital role in the future of gene and genome characterization in the mosquitoes or any gene in any organism.

5.2 Review of chapter 2

The strength of four different promoters (*hsp82*, *IE1*, *Ubl40*, *PUB*) was determined in early *Ae. aegypti* embryos using the dual-luciferase assay system. Early and robust FF luciferase gene expression was observed using the *PUB* promoter compared to the *hsp82* promoter in *Ae. aegypti* embryos. The expression of luciferase driven by the *PUB* promoter was comparably higher for all time points. By switching the CMV promoter to *hsp82* for expression of REN luciferase in a helper plasmid, no measurable competition between promoter in experimental (*PUB*) and control plasmids (*hsp82* and CMV) was observed. Switching plasmid backbones between control and experimental plasmids resulted in no measurable effect on gene expression.

It was proposed that utilizing endogenous promoters to drive transposase expression would greatly increase the frequency of transgene integration; therefore, the minimum transformation frequency in *Ae. aegypti* was calculated when using a helper plasmid based on a promoter that drives stronger/earlier gene expression. The *PUB* promoter was more effective in *Mos1*-mediated transgenesis as compared to *IE1* and *Ub_{L40}* promoters. There was no significant difference between the minimum transformation efficiency with pGL3-*PUB Mos1* and p*Khsp82 Mos1* as a helper plasmid. There was no significant difference between the size of the clusters of transgenic mosquitoes using pGL3-*PUB Mos1* and p*Khsp82 Mos1*.

5.3 Review of chapter 3

We found that I-*CreI*, I-*SceI*, I-*PpoI* and Y2-I-*AniI* can induce double-stranded DNA breaks at their specific target sites in the *Ae. aegypti* germline. The ratio of survival calculated for each enzyme was consistent with the results from a previous study (Traver *et al.*, 2009). I-*PpoI* recognition sites are present in the 28S rDNA of *Ae. aegypti*, while 10-18% of survival after injecting I-*CreI*, I-*SceI* and Y2-I-*AniI* showed that these enzymes have fewer off-target effect. Results from the SSA assay were predictive of success with the germline excision experiments. This should simplify the examination of new HE scaffolds as they are described.

Based on the analyzing of the remaining transgene sequences, we hypothesized that SSA, NHEJ and SSA followed by NHEJ were used as repair mechanisms in the germline of *Ae. aegypti*. However we could not prove that these are the only repair mechanisms. NHEJ followed by SSA (for example, using *loxP* to guide the

repair process) was also possible; however evidence for this order of events would have been removed during the repair, leaving a footprint indistinguishable from SSA alone. This contrasts with our previous results (Traver *et al.*, 2009) in which only NHEJ was observed in somatic cells. The choice of direct repeats used by the SSA repair pathway varied based on the HE used, even within the same target strain, as I-*CreI* led to collapse of the *loxP* repeats while I-*AniI* cleavage was followed by collapse of the SV40 direct repeats. Of the HEs tested, Y2-I-*AniI* displayed the greatest ability to generate dsDNA breaks in *Ae. aegypti*. Excision progeny were recovered in all pools at two different target sites at a rate of 3-4% of the total progeny; with SSA-repaired events showing a large amount of re-cutting followed by NHEJ.

5.4 Review of chapter 4

We concluded that TALEN-based nucleases are active in the early embryo of *Ae. aegypti* mosquitoes. Following injection into pre-blastoderm embryos, we observed strong activation of firefly luciferase activity and confirmed that TALEN-based gene disruption can be a highly efficient process in *Ae. aegypti*, with editing rates between 20-40% which is greater than both traditional transposon-based transformation and phiC31-based recombination. TALEN-based editing was associated with small deletions ranging in size from one to seven bp.

Currently only few number of tools are available to study of vector biology and genetics. Our result shows that HEs and TALENs could be used for targeted gene disruption and mutagenesis and they might be useful to study dsDNA repair mechanisms. HEs and TALENs are good candidates for genetic control strategies and developing in gene drive investigations for dengue vector,

Ae. aegypti. A next step could be using TALEN-based and HE-based enzymes for editing specific genes by knocking in of transgene or introducing single stranded oligonucleotide through homologous recombination.

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Appendix

hsp82 promoter has been used to drive *Mos1* transposase gene in helper plasmid.

donor plasmid	embryos injected	# G ₀ survivors (%)	# of pools	pool with+ progeny	# of G ₁ ⁺ for each pool	G ₁ ⁺ /total for each pool (%)	# G ₁ ⁺ min transformation frequency (%)	References
pM[cn]	1625	231 (14.2%)					38 (3.8%)	Coats <i>et al.</i> , 1998
pM[cn]	1407	224 (16%)					5 (1.2%)	Coats <i>et al.</i> , 2000
pM[cn]	702	115 (16%)					121 (8.6%)	Coats <i>et al.</i> , 2000
pBCMhspRedcn							1 (0.01%)	Wilson <i>et al.</i> , 2003
pMos-3xp3 sensor	1478	142 (9.6%)					2.80%	Adelman <i>et al.</i> , 2008
PUb-GFP	1902	218 (11.5%)	7	P2	34		113 (5.5%)	Anderson <i>et al.</i> , 2010
PUb-GFP	1902	218 (11.5%)	7	P3	38		113 (5.5%)	Anderson <i>et al.</i> , 2010
PUb-GFP	1902	218 (11.5%)	7	P4	13		113 (5.5%)	Anderson <i>et al.</i> , 2010
PUb-GFP	1902	218 (11.5%)	7	P5	13		113 (5.5%)	Anderson <i>et al.</i> , 2010
PUb-GFP	1902	218 (11.5%)	7	P6	10		113 (5.5%)	Anderson <i>et al.</i> , 2010
PUb-GFP	1902	218 (11.5%)	7	P7	18		113 (5.5%)	Anderson <i>et al.</i> , 2010
UbL40 -GFP (exp1)	1347	126 (9.4%)	24	P18	3		5 (3.2%)	Anderson <i>et al.</i> , 2010
UbL40 -GFP (exp1)	1347	126 (9.4%)	24	P19	2		5 (3.2%)	Anderson <i>et al.</i> , 2010
UbL40 -GFP (exp2)	1825	248 (13.6%)	17	P10	3		68(3.2%)	Anderson <i>et al.</i> , 2010
UbL40 -GFP (exp2)	1825	248 (13.6%)	17	P13A	53		68(3.2%)	Anderson <i>et al.</i> , 2010
UbL40 -GFP (exp2)	1825	248 (13.6%)	17	P17A	7		68(3.2%)	Anderson <i>et al.</i> , 2010
UbL40 -GFP (exp2)	1825	248 (13.6%)	17	P17B	5		68(3.2%)	Anderson <i>et al.</i> , 2010
nos- <i>Mos1</i>	1676	440 (26%)					3.60%	Adelman <i>et al.</i> , 2007
W2	1629	292 (17.9%)	5	P1	14	1.30%	17 (1.3%)	Adelman, unpublished
W2	1629	292 (17.9%)	5	P2	5	0.79%	17 (1.3%)	Adelman, unpublished
W1	1642	136 (8.2%)	3	P1	17	2.30%	28 (4.7%)	Adelman, unpublished
W1	1642	136 (8.2%)	3	P2	13	2.10%	28 (4.7%)	Adelman, unpublished
W1	1642	136 (8.2%)	3	P3	7	0.81%	28 (4.7%)	Adelman, unpublished
Elav-KMO	1949	203 (10.4%)	4	P1	20	0.72%	4 (2%)	Adelman, unpublished
Elav-KMO	1949	203 (10.4%)	4	P2	1	0.08%	4 (2%)	Adelman, unpublished

hsp82 promoter has been used to drive *Mos1* transposase gene in helper plasmid.

donor plasmid	embryos injected	# G ₀ survivors (%)	# of pools	pool with+ progeny	# of G ₁ ⁺ for each pool	G ₁ ⁺ /total for each pool (%)	# G ₁ ⁺ min transformation frequency (%)	References
Nrv2L	782	86 (10.9%)	??	P1	1	0.49%	4 (2%)	Adelman, unpublished
Nrv2L	782	86 (10.9%)	??	#6	3	0.12%	3 (4.6%)	Adelman, unpublished
Nrv2L	782	86 (10.9%)	??	#10	1	0.08%	3 (4.6%)	Adelman, unpublished
Nrv2s	932	96 (10.3%)	??	P2	3	0.25%	2 (2%)	Adelman, unpublished
VMP	805	100 (12.4%)	??	#12	10	1.20%	47 (4%)	Adelman, unpublished
VMP	805	100 (12.4%)	??	P1	12	1.18%	47 (4%)	Adelman, unpublished
VMP	805	100 (12.4%)	??	P2	25	1.68%	47 (4%)	Adelman, unpublished
3xp3RG	1708	197 (11.5%)	14	P8	3	0.42%	25 (4%)	Adelman, unpublished
3xp3RG	1708	197 (11.5%)	14	P9	2	0.23%	25 (4%)	Adelman, unpublished
3xp3RG	1708	197 (11.5%)	14	P10	11	1.70%	25 (4%)	Adelman, unpublished
3xp3RG	1708	197 (11.5%)	14	P11	9	1.04%	25 (4%)	Adelman, unpublished
3xp3RG	1708	197 (11.5%)	14	P13	1	0.12%	25 (4%)	Adelman, unpublished
3xp3GFP-AeCPA								
GFP	1140	193 (16.9%)					9.30%	Franz <i>et al.</i> , 2011
AgCP	1579	19 (1.2%)					6.60%	Moreira <i>et al.</i> , 2000

***Pub* promoter has been used to drive *Mos1* transposase gene in helper plasmid.**

donor plasmid	embryos injected	# G ₀ survivors (%)	# of pools	pool with+ progeny	# of G ₁ ⁺ for each pool	G ₁ ⁺ /total for each pool (%)	# G ₁ ⁺ min transformation frequency (%)	References
G1AseLuc	1508	656 (36.87%)	11	P2	31	1.80%	67 (1.6%)	Gross <i>et al.</i> , 2011
G1AseLuc	1508	656 (36.87%)	11	P4	1	0.03%	67 (1.6%)	Gross <i>et al.</i> , 2011
G1AseLuc	1508	656 (36.87%)	11	P5	29	1.01%	67 (1.6%)	Gross <i>et al.</i> , 2011
G1AseLuc	1508	656 (36.87%)	11	P10	6	0.30%	67 (1.6%)	Gross <i>et al.</i> , 2011
AGO3ir	869	152 (17.5%)	6	K3	23	2.36%	24 (2.6%)	Adelman, unpublished
AGO3ir	869	152 (17.5%)	6	K6	1	0.17%	24 (2.6%)	Adelman, unpublished
DCR2ir	700	60 (8.5%)	4	L3	35	2.70%	32 (3.3%)	Adelman, unpublished
miRW DCR2	663	71 (10.7%)	11	W3	2	0.33%	8 (2.8%)	Adelman, unpublished
miRW DCR2	663	71 (10.7%)	11	W4	1	0.15%	8 (2.8%)	Adelman, unpublished
miRW DCR2	663	71 (10.7%)	11	W6	7	0.80%	8 (2.8%)	Adelman, unpublished
miRW DCR2	663	71 (10.7%)	11	W8	5	0.30%	8 (2.8%)	Adelman, unpublished
miRW DCR2	663	71 (10.7%)	11	W9	5	0.20%	8 (2.8%)	Adelman, unpublished
miRW DCR2	663	71 (10.7%)	11	W10	1	0.04%	8 (2.8%)	Adelman, unpublished
Pub-GFP	1437	142 (19%)	10	D1	41	1.86%	136 (5.6%)	Adelman, unpublished
Pub-GFP	1437	142 (19%)	10	D2	56	1%	136 (5.6%)	Adelman, unpublished
Pub-GFP	1437	142 (19%)	10	D4	1	0.02%	136 (5.6%)	Adelman, unpublished
Pub-GFP	1437	142 (19%)	10	D7	37	7.12%	136 (5.6%)	Adelman, unpublished
Pub-GFP	1437	142 (19%)	10	D8	1	0.02%	136 (5.6%)	Adelman, unpublished
miR DCR2	552	156 (28.2%)	11	H2	9	0.27%	14 (6.4%)	Adelman, unpublished
miR DCR2	552	156 (28.2%)	11	H3	5	0.06%	14 (6.4%)	Adelman, unpublished
miR DCR2	552	156 (28.2%)	11	H8	1	0.02%	14 (6.4%)	Adelman, unpublished
miR DCR2	552	156 (28.2%)	11	H9	2	0.05%	14 (6.4%)	Adelman, unpublished
miR DCR2	552	156 (28.2%)	11	H11	5	0.37%	14 (6.4%)	Adelman, unpublished
Pub-B2	1460	16 (1.1%)	2	B1	1	0.10%	7 (25%)	Adelman, unpublished
Pub-B2	1460	16 (1.1%)	2	B2	6	0.30%	7 (25%)	Adelman, unpublished
3xp3miR-DCR2	1010	177 (17.5%)	8	P2	4	0.64%	137 (6.7%)	Adelman, unpublished
3xp3miR-DCR2	1010	177 (17.5%)	8	P3	28	1.30%	137 (6.7%)	Adelman, unpublished
3xp3miR-DCR2	1010	177 (17.5%)	8	P4	21	1.44%	137 (6.7%)	Adelman, unpublished

***PUB* promoter has been used to drive *Mos1* transposes gene in helper plasmid.**

donor plasmid	embryos injected	# G₀ survivors (%)	# of pools	pool with+ progeny	# of G₁⁺ for each pool	G₁⁺/total for each pool (%)	# G₁⁺ min transformation frequency (%)	References
3xp3miR-DCR2	1010	177 (17.5%)	8	P5	33	1.20%	137 (6.7%)	Adelman, unpublished
3xp3miR-DCR2	1010	177 (17.5%)	8	P7	46	4.80%	137 (6.7%)	Adelman, unpublished
3xp3miR-DCR2	1010	177 (17.5%)	8	P8	5	0.16%	137 (6.7%)	Adelman, unpublished
PubmiR-W	1010	232 (23%)	11	P3	2	0.31%	21 (5.1%)	Adelman, unpublished
PubmiR-W	1010	232 (23%)	11	P4	1	0.15%	21 (5.1%)	Adelman, unpublished
PubmiR-W	1010	232 (23%)	11	P6	7	0.80%	21 (5.1%)	Adelman, unpublished
PubmiR-W	1010	232 (23%)	11	P8	5	0.31%	21 (5.1%)	Adelman, unpublished
PubmiR-W	1010	232 (23%)	11	P9	5	0.20%	21 (5.1%)	Adelman, unpublished
PubmiR-W	1010	232 (23%)	11	P10	1	0.04%	21 (5.1%)	Adelman, unpublished
UbL40-EGFP	1106	90 (8%)	5	P1	32	1.57%	101 (6.6%)	Adelman, unpublished
UbL40-EGFP	1106	90 (8%)	5	P3	29	1.67%	101 (6.6%)	Adelman, unpublished
UbL40-EGFP	1106	90 (8%)	5	P5	40	1.20%	101 (6.6%)	Adelman, unpublished
PUB-DsRed	1160	230 (18.8%)	11	P6	18	0.70%	46 (2.0%)	Adelman, unpublished
PUB-DsRed	1160	230 (18.8%)	11	P7	28	1.90%	46 (2.0%)	Adelman, unpublished
AaHsp70Bb-1456	1315	242 (18.4%)	10	P1	5	0.50%	42 (2.5%)	Adelman, unpublished
AaHsp70Bb-1456	1315	242 (18.4%)	10	P6	30	1.14%	42 (2.5%)	Adelman, unpublished
AaHsp70Bb-1456	1315	242 (18.4%)	10	P7	7	0.40%	42 (2.5%)	Adelman, unpublished
AaHsp70Bb-1456	1315	197 (16.2%)	7	P3	19	1.10%	19 (1.0%)	Adelman, unpublished

Attribution

Several colleagues aided in the writing and research behind two of my chapters presented as part of this dissertation. A brief description of their contribution is included here:

Chapter 3: Catalyzing double-stranded DNA breaks and excision of transgenes in *Aedes aegypti* germline by homing endonucleases
Chapter 3 was submitted to the Scientific Reports, April 2013

Chapter 4: TALEN-based gene disruption in the dengue vector *Aedes aegypti*
Chapter 4 was submitted to the PlosOne, March 2013

For both chapters 3 and 4:

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