Université Pierre et Marie Curie

ECOLE DOCTORALE 515 Complexité du Vivant

Unité Arbovirus et Insectes Vecteurs

Transgenic mosquitoes for controlling transmission of arboviruses

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Thèse de doctorat de Virologie et Entomologie Médicale

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Présentée et soutenue publiquement le 15 Décembre 2017

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“Life finds a way.”

-Dr. Ian Malcolm, Jurassic Park, June 1993

Acknowledgement

There are no proper words to convey my deep gratitude and respect for my thesis and research advisor, Dr. Anna-Bella Failloux. She has inspired me to become an independent researcher and helped me realize what a professional should be. She also demonstrated what a brilliant and hard-working scientist can accomplish. With her fully support, wise advice, and endless patience. I almost forgot how to survive in the professional world without her mothering.

I would like to thank the jury members: Dr. Dominique Higuet, Dr. Stephanie Becker, Dr. Frédéric Simard, Dr. Chun-Hong Chen, and Dr. Claire Valiente-Moro for examining and evaluating this work, and being part of this achievement.

My sincere thanks must also go to the members of my thesis advisory committee: Dr. Catherine Bourgouin, Dr. Eric Marois, and Dr. Christian Mitri. Thanks to your annual efforts that keep me on the right track, stay far from the possible traps on the way to publish.

I am most grateful to Dr. Anthony James, Dr. Alain Kohl, and Dr. Chun-Hong Chen, for lending me their expertise and intuition to my scientific and technical problems

Also, I am extremely grateful to the PPU office, who organized such a marvelous PhD program, has offered me this extraordinary opportunity to work, to learn, and to have fun with all the young dedicated souls.

I also have to express my appreciation to all the present and past labmates, as a foreigner, I was unfairly treated, unfairly good. Thank you all so very much for roping me in this unique, beautiful, delicious, and intoxicating (mostly red, but white as well) culture. I will never forget the life I spent here in UAIV.

Abstract

Mosquito-borne arboviruses cause some of the world's most devastating diseases and are responsible for recent dengue, chikungunya and Zika pandemics. The yellow-fever mosquito, *Aedes aegypti*, plays an important role in the transmission of all three viruses. The ineffectiveness of chemical control methods targeting *Ae. aegypti* makes urgent the need for novel vector-based approaches for controlling these diseases. Mosquitoes control arbovirus replication by triggering immune responses. RNAi machinery is the most significant pathway playing a role on antiviral immunity. Although the role of exogenous siRNA and piRNA pathways in mosquito antiviral immunity is increasingly better understood, there is still little knowledge regarding interactions between the mosquito cellular miRNA pathway and arboviruses. Thus further analysis of mechanisms by which miRNAs may regulate arbovirus replication in mosquitoes is pivotal.

In the first part of the thesis, we carried out genomic analysis to identify *Ae. aegypti* miRNAs that potentially interact with various lineages and genotypes of chikungunya (CHIKV), dengue (DENV) and Zika viruses. By using prediction tools with distinct algorithms, several miRNA binding sites were commonly found within different genotypes/and or lineages of each arbovirus. We further analyzed the miRNAs that could target more than one arbovirus and required a low energy threshold to form miRNA-vRNA (viral RNA) complexes and predicted potential RNA structures using RNAhybrid software. Thus, we predicted miRNA candidates that might participate in regulating arboviral replication in *Ae. aegypti*.

In the second part of the thesis, we developed a miRNA-based approach that results in a dual resistance phenotype in mosquitoes to dengue serotype 3 (DENV-3) and chikungunya (CHIKV) viruses for stopping arboviruses spreading within urban cycles. The target viruses

are from two distinct arboviral families and the antiviral mechanism is designed to function through the endogenous miRNA pathway in infected mosquitoes. Ten artificial antiviral miRNAs capable of targeting ~97% of all published strains were designed based on derived consensus sequences of CHIKV and DENV-3. The antiviral miRNA constructs were placed under control of either an *Aedes PolyUbiquitin* (*PUb*) or *Carboxypeptidase A* (*AeCPA*) gene promoter triggering respectively expression ubiquitously in the transgenic mosquitoes or more locally in the midgut epithelial cells following a blood meal. Challenge experiments using viruses added in blood meals showed subsequent reductions in viral transmission efficiency in the saliva of transgenic mosquitoes as a result of lowered infection rate and dissemination efficiency. Several components of mosquito fitness, including larval development time, larval/pupal mortality, adult lifespan, sex ratio, and male mating competitiveness, were examined: transgenic mosquitoes with the *PUb* promoter showed minor fitness costs at all developing stages whereas those based on *AeCPA* exhibited a high fitness cost. Further development of these strains with gene editing tools could make them candidates for releases in population replacement strategies for sustainable control of multiple arbovirus diseases.

Résumé

Les arbovirus (virus transmis par des arthropodes) sont à l'origine de maladies humaines telles que la dengue, le chikungunya ou encore le Zika. Le moustique *Aedes aegypti*, est le vecteur majeur de ces trois arbovirus. La faible efficacité des méthodes de contrôle des populations de moustiques, principalement réalisées au moyen d'insecticides chimiques ouvre un champ de développement de nouvelles approches en lutte antivectorielle. Le moustique, hôte vecteur, contrôle la réplication virale en limitant les réponses immunitaires antivirales. La machinerie RNA interférence (RNAi) est la voie jouant un rôle majeur dans l'immunité antivirale chez le moustique. Alors que le rôle des deux voies, siRNA (« small interfering RNA ») et piRNA (« piwi-interfering RNA »), est de mieux en mieux compris dans les réactions antivirales du vecteur, peu de connaissances sont disponibles à ce jour en ce qui concernent les interactions entre la voie miRNA (« micro RNA ») et les arbovirus. Ainsi, nous proposons une analyse détaillée des mécanismes par lesquels les miARN tentent de réguler la réplication virale chez le moustique.

Dans la première partie de la thèse, nous avons effectué une analyse génomique pour identifier les miRNAs pouvant interagir chez *Ae. aegypti* avec divers lignées/génotypes des virus chikungunya (CHIKV), de dengue (DENV) et de Zika. Avec l'aide d'outils de prédiction faisant appel à divers algorithmes, plusieurs sites de liaison de miARN avec différents lignées/génotypes de chaque arbovirus ont été identifiés. Nous avons ensuite sélectionné les miARN pouvant cibler plus d'un arbovirus et nécessitant un faible seuil d'énergie lors de la formation des complexes entre l'ARNm du moustique et l'ARN viral. Nous avons également prédit les structures des ARNs en utilisant le logiciel RNAhybrid. Ainsi, nous avons pu définir des candidats miARN pouvant participer à la régulation de la réplication virale chez le moustique *Ae. aegypti*.

Dans la deuxième partie de la thèse, nous avons développé une approche basée sur le miARN dans le but de produire un moustique présentant un phénotype de résistance pour la réplication virale de la dengue et du chikungunya. Les virus ciblés sont DENV-3 et CHIKV. Dix miARN antiviraux capables de cibler ~ 97% de souches virales ont été sélectionnés en se référant aux séquences consensus de DENV-3 et CHIKV. Les cassettes miARN ont été placées sous le contrôle du promoteur *Aedes PolyUbiquitin (PUb)* ou *Carboxypeptidase A (AeCPA)* qui déclenche respectivement une expression ubiquitaire de la réaction antivirale ou une expression localisée dans les cellules épithéliales de l'intestin moyen des moustiques après la prise d'un repas sanguin. Les infections expérimentales des moustiques par les virus proposés dans les repas sanguins ont montré une réduction significative de l'efficacité de la transmission virale avec peu ou pas de virus détectés dans la salive des moustiques transgéniques. Nous avons, par ailleurs, étudié l'impact de la transformation génétique sur les valeurs sélectives des moustiques en mesurant la durée du développement pré-imaginal, la mortalité larvaire/pupale, la durée de vie des adultes, le sexe ratio et la compétitivité des mâles. Nous avons ainsi mis en évidence que les moustiques transgéniques ayant le promoteur *PUb* étaient beaucoup moins compétitifs que les moustiques ayant le promoteur *AeCPA*. Le développement de ces souches de moustiques en ayant recours aux nouveaux outils d'édition de gènes pourrait améliorer les performances de ces candidats. Ces candidats pourraient être ainsi proposés dans les stratégies de remplacement des populations de moustiques vecteurs d'arbovirus.

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List of abbreviations

A.D.	Anno Domini
AaArg3	Aedes Aegypti Arginine Methyltransferase 3
AeAct-4	Aedes Actin-4
AeCPA	Aedes Carboxypeptidase A
AePUb	Aedes PolyUbiquitin
Ago-1	Argonaute-1
Ago-2	Argonaute-2
Ago-3	Argonaute-3
AGO-CLIP	Argonaute-Crosslinking Immunoprecipitation
AMP	Anti Microbial Peptides
ATP	Adenosine triphosphate
CFP	Cyan Fluorescent Protein
CHIKV	Chikungunya Virus
cHP	Capsid-coding Hairpin
DALY	Disability-Adjusted Life Year
DB	dumbbell
Dcr-1	Dicer-1
Dcr-2	Dicer-2
DDT	Dichloro-Diphenyl-Trichloroethane
DENV	Dengue virus
DF	Dengur fever
DHF	Dengue hemorrhagic fever
DOME	Domeless
Dpi	Day Post-Infection
DR	Dissemination Rate
Dsx	Doublesex
EA	Environmental Assessment
ECSA	East/Central/South African
EEEV	Eastern Equine Encephalitis virus
EIP	Extrinsic Incubation Period
ER	Endoplasmic Reticulum
FBS	Fetal Bovine Serum
FFA	Focus Forming Assay
Ffu	focus forming unit
FONSI	Finding Of No Significant Impact
Fru	Fruitless
fsRIDL	Female-specific RIDL
GESS	Genome-wide Enrichment of Seed Sequence matches
GR	Gustatory Receptor
GSD	Glutamate Semi-aldehyde Dehydrogenase
HCV	Hepatitis C virus
HDR	Homology-Directed Repair
IFA	Immunofluorescence Assay

INF	Interferon
IOL	Indian Ocean Lineage
IR	Ionotropic Receptor
IR	Infection Rate
ITD	Immune To Drive
JAK-STAT	Janus kinase/signal transducers and activators of transcription
JHA15	Juvenile Hormone regulated serine protease
Kb	Kilo Base
ldsRNA	Long Double Strand RNA
MEB	Midgut Escape Barrier
MFE	Minimum Free Energy
MIB	Midgut Infection Barrier
miRISC	micro RNA-Induced Silencing Complex
miRNA	micro RNA
NGS	Next Generation Sequencing
Nm	Nano Meter
NNIs	Neonicotinoid Insecticides
Npylr	Neuropeptide y-like receptor
nsP	Non-Structural Protein
ONNV	O'nyong-nyong virus
OR	Ordant Receptor
Orco	Ordant Co-Receptor
ORF	Open Reading Frame
PAMP	Pathogen Associated Molecular Pattern
PBM	Post Blood Meal
piRNA	Piwi-interfering RNA
pri-miRNA	Precursor miRNA
prM	Precursor Membrane protein
RC	Replication Complex
RdRp	RNA dependent RNA polymerase
RIDL	Releasing of Insects carrying a Dominant Lethal
RISC	RNA-Induced Silencing Complex
RNA	Ribonucleic Acid
RNAi	RNA interference
SFV	Semliki Forest virus
SGEB	Salivary Gland Escape Barrier
SGIB	Salivary Gland Infection Barrier
sHP-3'SL	small hairpin 3'stem-loop
siRNA	small interfering RNA
SIT	Sterile Insect Technique
SL	Stem-Loop
SLA	Stem Loop-A
TE	Transmission Efficiency
tetO	Tetracycline Operator
TLR	Toll-like Receptor
TNF	Tumor Necrosis Factor

TRAF	TNF receptor-associated Factors
tTA	Tetracycline Transactivator
UAR	Upstream AUG Region
USD	United States Dollar
UTR	Untranslated Region
vRNA	viral RNA
vsiRNA	viral small interfering RNA
WA	West African
WNV	West Nile Virus
WWII	World War II
YFV	Yellow Fever virus
ZFN	Zinc-Finger Nuclease
ZIKV	Zika virus

GENERAL INTRODUCTION

The global threat of arbovirus diseases

Unlike other human infectious diseases, the control of arbovirus diseases needs to consider three partners: the vertebrate host, mainly humans, the insect vector (e.g; mosquito) and the pathogen (e.g. the arbovirus). Because efficient vaccines and specific treatments are still lacking, main efforts should be focused on developing innovative control strategies against the vector.

Vaccines can induce virus-cross-reactivity which is proven to drive antibody-dependent enhancement of infection with other arboviruses limiting vaccines wide use (de Alwis et al., 2014; Dejnirattisai et al., 2016). Thus, increasing human antiviral immunity is not considered as a promising solution for controlling arboviral diseases spreading.

In addition, owing to climate change, intensification of international commerce and travel, the distribution of arboviral diseases is no longer restricted to historical regions and conventional host populations (Rezza, 2014). More and more arboviral diseases are reported in temperate countries, accumulating evidence of adaptation between arbovirus and invasive mosquitoes in Europe (Medlock et al., 2012; Vega-Rua et al., 2013). Although arboviral diseases are mostly considered as non-lethal diseases, the tremendous societal costs and loss of productivity caused by these arboviral diseases represent significant health and economic burden for countries in financial difficulties (Stanaway et al., 2016).

Considering all the factors described above, several conditions should be taken into consideration for a more comprehensive arboviral diseases control, notably, as the recent Zika outbreaks and the Yellow fever reemerging in South America have highlighted the limits of conventional vector control strategies: inefficient, costly, and unpredictable ecological impacts (McGraw and O'Neill, 2013). Therefore, seeking for an effective and environmental

friendly vector control strategy has become urgent. Many genetically-engineered or microbial-mediated alternatives were proposed to reduce the risk of disease transmission in recent years, including the reduction of insect populations by *Wolbachia*-harboring (McGraw and O'Neill, 2013) and genetically-modified mosquitoes (Alphey, 2014) or replacing the wild population by a genetically-modified refractory strain (Champer et al., 2016). Other approaches which consider the manipulation of the mosquito host-seeking behavior have also been developed to reduce the contacts between mosquitoes and hosts (DeGennaro et al., 2013; Liesch et al., 2013; McMeniman et al., 2014).

1. Dengue virus

Dengue fever (DF)/dengue hemorrhagic fever (DHF) is one of the most important arbovirus disease circulating in tropical countries (Murray et al., 2012). It is believed to be an ancient disease and could be traced back to A.D. 992 in China (Gubler, 2006). Yet, until 1635 and 1699, dengue-like illnesses were reported in French West Indies and Panama, respectively. One hundred years later, several DF-like cases were periodically reported in Batavia, Cairo, Philadelphia, Cadiz and Seville, and Spain during 1779-1788 (Vasilakis and Weaver, 2008). DF became a global epidemic from 1823 to 1916, as the consequence of international commerce and slave trade (Halstead, 2008). In the mid 20th century, the World War II (WWII) expedited the spreading of DF, as the importation of mosquito vectors and military troops deployed overseas increased the frequency of mosquito-human contacts and consequently, lead to DF outbreaks in East Africa and the Caribbean region. The entire Pacific became the theater of DF epidemics, from Australia to Hawaii and from Guinea to Japan (Gibbons et al., 2012). Since then, the number of countries with reported DF cases exponentially increased, although the DF epidemic could be controlled by successful

mosquito vector eradication program in America (Gubler, 1997). However, recolonizations by mosquitoes and re-emergence of associated arboviral diseases have pronounced the failure of the program in the early 1970s as the consequence of program's ending.

1.1. The burden of dengue

Although only 9 countries had suffered this disease in early 1970s, today, more than 125 countries are endemic with dengue, approximately 50-100 million infections and 20,000 deaths annually (Bhatt et al., 2013). The four serotypes of DENV are currently circulating worldwide except the Antarctica (Huang et al., 2014; Messina et al., 2014). Among these countries, several areas had reported more than three serotypes of DENV, especially in Latin America and the Caribbean islands, Southeast Asia, the Indian subcontinent, Indonesia, Australia, and several neighboring areas had reported sequentially each serotype since 2000 (Messina et al., 2014).

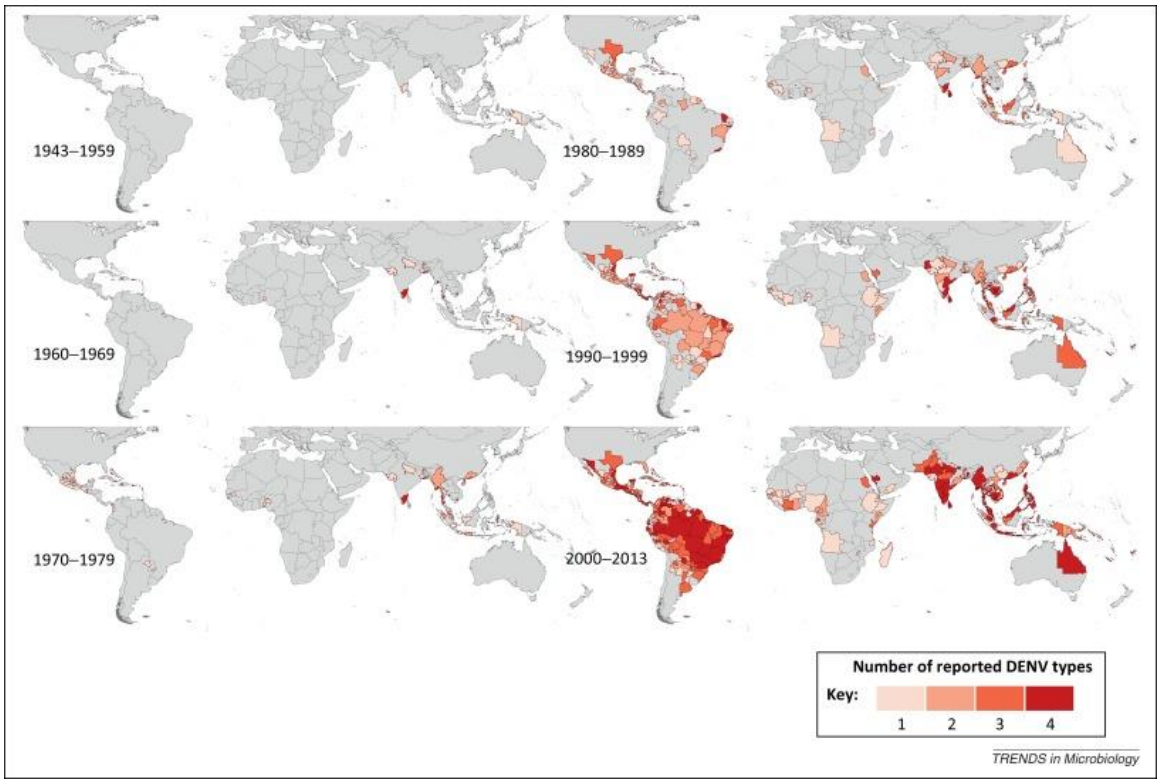


Figure 1. Global distribution of DENV. Cumulative number of DENV serotypes reported by decade since 1943. Adapted from (Messina et al., 2014).

More than the number of DENV epidemic countries, the incidence of dengue has also increased greatly in the past two decades; the estimated dengue infections have increased from 8.3 million in 1993 to 58.4 million in 2013 (Stanaway et al., 2016). Although the mortality rate of dengue was decreased from 1.64 to 1.27 (95% UI; per million), the impacts of dengue on population health were increasing since 1993, especially for Latin America and Caribbean regions; the disability-adjusted life-years (DALYs) has approximately doubled in 2013 since 1990, from 0.72 million (95% UI; 0.43-0.95 million) to 1.14 million (95% UI; 0.73-1.98 million) (Stanaway et al., 2016). Furthermore, an estimation of 18% of symptomatic infections in 2013 were hospitalized whereas 48% were medically treated without hospitalization, 8% were not taking any medical treatment, and less than 1% were fatal cases (Shepard et al., 2016). Thus, a total of 8.9 billion USD was drained for dengue related medication treatments in 2013. In addition, it is remarkable that more than 58% of the

annual budget were used for the hospitalized or fatal cases which therefore caused a important economic burden on DENV-endemic countries.

1.2. Dengue epidemiology

The repetitive DF outbreaks during WWII have alerted the scientific community. Main attention has been focused on dengue viruses with remarkable progress on understanding the biology of this virus. Four serotypes of dengue virus (DENV) were identified: DENV-1, -2, -3, -4. The DENV-1 Mochizuki strain was first isolated in 1943 (Hotta, 1952). One year after, DENV-2 New Guinea C strain was identified (Sabin, 1952). The identification of DENV-3 H87 strain and DENV-4 H241 strain were done in Philippine during late 1950s (Hammon et al., 1960a; Hammon et al., 1960b). The four serotypes of DENV share distinct phylogenic and antigenic features with only 60-80% in sequence homology (Green and Rothman, 2006). Long-term immunity could be induced when infected successively with the same serotype of DENV. However, severe DHF symptoms can be developed when people are infected with different dengue serotypes, even different subtypes within the same serotype (Nisalak et al., 2003; Rico-Hesse, 2003).

1.3. DENV characteristics

DENV belongs to the genus *Flavivirus* in the Family Flaviviridae. Similar to other flaviviruses, DENV is a 500 Å in diameter, spherical shape virus enveloped with lipids (Kuhn et al., 2002). When including the highly structured 5' and 3' untranslated regions (UTR) (Friebe and Harris, 2010; Gebhard et al., 2011; Ng et al., 2017), the RNA genome is approximately 10,700 nucleotides in length; it encodes a single open reading frame (ORF), which is translated as a polyprotein (Perera and Kuhn, 2008; Screaton et al., 2015). There are

3 structural proteins and 7 non-structural proteins organized as follows: NH_2-C-prM-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5-COOH, each protein can be cleaved by viral proteases NS3 except the structural proteins whose cleavage is carried out by a host protease signal peptidase (Arias et al., 1993). In addition, the viral particle maturation is processed by furin, a cellular serine protease that cleaves the precursor membrane protein (prM) (Stiasny and Heinz, 2006).

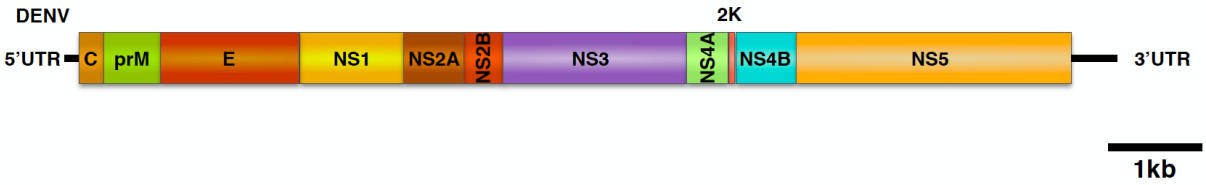


Figure 2. The genome structure of dengue virus.

Three structural proteins are the major components that form the capsid and the viral membrane during assembly whereas the 7 non-structural proteins are responsible for viral RNA replication. These latter are organized in a replication complex that facilitates viral replication. Among them, the hydrophilic NS1 protein participates in different steps during viral replication as a dimer that embedded in ER membrane. Along with other non-structural proteins and viral RNA, the NS1 protein anchors the viral replicase proteins to ER membrane and form a replication complex (Scaturro et al., 2015). The NS3 protein is composed by a N-terminal serine protease and a C-terminal helicase domains (Li et al., 1999; Luo et al., 2008a). NS3 is responsible for viral polyprotein cleavage at the early stage of viral replication, with a cofactor, NS2B, the protease domain of NS3 protein which cleaves the viral polyprotein between NS proteins (Yusof et al., 2000). During viral RNA replication, the helicase and 5'-RNA triphosphatase activity of NS3 helicase domain are required for unwinding the positive-negative strands RNA intermediate duplex and 5'-RNA cap formation respectively (Luo et al., 2008b; Xu et al., 2005). NS5 is a widely studied and largest DENV non-structural protein,

which consists of a N-terminal methyltransferase (MTase) and a C-terminal RNA-dependent RNA polymerase (RdRp) domains (Zhou et al., 2007). The MTase domain of NS5 consists of a RNA guanylyltransferase and methyltransferase activities which are essential for 5'-RNA capping and cap methylation (Egloff et al., 2002; Issur et al., 2009), whereas the RdRp domain of NS5 carries out the positive and negative RNA synthesis in the replication complex. As the NS1 protein, the membrane protein NS2A by interacting with NS4A-NS4B forms the viral replication complex (Scaturro et al., 2015). In addition, several non-structural proteins of DENV such as NS2A, NS4A, NS4B, also regulate the host immunity by inhibiting the interferon (INF) signaling. The replication of INF-sensitive virus could be enhanced by the expression of dengue NS2A, NS4A, and NS4B proteins (Munoz-Jordan et al., 2003). Moreover, the INF-α signaling could be also inhibited by ectopic expression of NS5 in human cells (Mazzon et al., 2009).

Except the viral proteins, DENV has several functional RNA elements at the 5' and 3'UTR of viral genome, involved in viral replication (Friebe and Harris, 2010; Gebhard et al., 2011; Ng et al., 2017). The initiation of viral RNA replication starts with the engagement of NS5 protein mediated 5'-3'UTR interaction, which circularized the viral genome and primes the negative strand RNA synthesis (Alvarez et al., 2005; Friebe and Harris, 2010). The circularization is initiated by the highly structured 5'UTR containing a 5' upstream AUG region (UAR) which is complementary to a 3' UAR in 3'UTR (Friebe and Harris, 2010; Villordo and Gamarnik, 2009). There are two other functional RNA elements called stem-loop A (SLA) and capsid-coding region hairpin (cHP) in the 5'UTR of dengue viral RNA (Gebhard et al., 2011). SLA with its stable stem-loop structure is involved as a promoter that interacts with NS5 protein for viral RNA synthesis (Filomatori et al., 2006; Lodeiro et al., 2009). cHP structure acts as a barrier between the first and second start codon that facilitates

the recognition of RdRp to the first start codon (Clyde and Harris, 2006). In contrast, the 3'UTR of DENV has more complex RNA structures and could be divided into three regions: stem-loop region (SL), dumbbell region (DB), and a terminal structure small hairpin 3' stem-loop (sHP-3'SL) (Villordo et al., 2016). The structures in the 3'UTR of DENV is essential for viral RNA replication but also for protein translation. The deletion of each structure in the 3'UTR results in abolishing viral RNA replication (Alvarez et al., 2005). Moreover, the sHP-3'SL in the end of viral 3'UTR is essential for DENV replication in mosquito and mammalian cells, although some mutations in the stem or loop region are tolerable for mammalian but not mosquito cells (Villordo and Gamarnik, 2013). Although the mechanisms of functional structure regulating viral RNA replication in the 3'UTR are not clear, it is believed that the pseudoknot interactions in SL and DE structures play an important part in viral replication (Sztuba-Solinska et al., 2013).

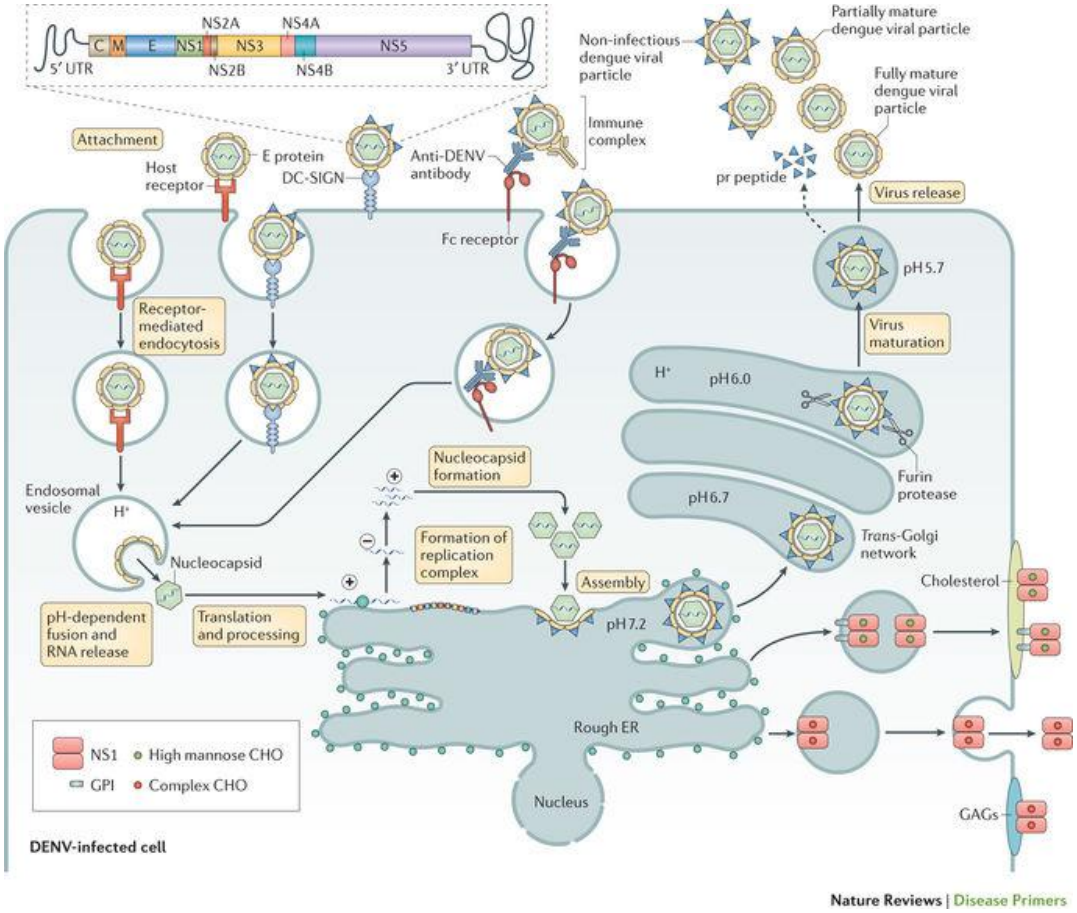


Figure 3. Dengue life cycle. DENV infects an eukaryotic cell through receptor-mediated endocytosis mechanism. The viral RNA is released from the endosomal vesicle in the cytoplasm in response to pH change. The replication-required viral proteins are translated from the exposed viral RNA, and form replication complex in ER membrane for viral RNA synthesis. The newly synthesized viral RNA is then transported out of the replication complex and packed by viral proteins. After several modifications, the mature virion is released from the cell membrane. Adapted from (Guzman et al., 2016).

Upon DENV infection, the virus fuses with the endosomal membrane and releases the viral RNA in the cytoplasm. The exposed viral RNA is translated into a polyprotein within the endoplasmic reticulum (ER) membrane, and remains associated with ER on the cytoplamic side or in the lumen after the polyprotein is cleaved into individual viral proteins (Mukhopadhyay et al., 2005; Screaton et al., 2015). For viral RNA synthesis, the membrane structure of ER is changed by the newly synthesized NS1 and forms a viral replication complex (RC), which comprised viral RNA, replication proteins, and certain cellular proteins

in a double–membrane vesicle, to avoid the viral RNA and replication proteins from triggering host immune responses (Klema et al., 2015; Welsch et al., 2009). In the replication complex, the negative and positive strand genomic RNA are synthesized by the RdRp activity of NS5. Newly synthesized positive strand of viral RNA is subsequently modified (capped and methylated) respectively with the helicase and MTase activity of NS3 and NS5 in the replication complex (Klema et al., 2015). After, the capped and methylated RNA genome is released from the necks open to the cytoplasm, the newly synthesized RNA genome then attaches to the Capsid protein on the cytoplasmic side of the ER, forms a Capsid-RNA complex (Welsch et al., 2009). On the contrary, the viral RNA that was not actively synthesized in replication complex is not encapsidated (Khromykh et al., 2001). The Capsid-RNA complex is then incorporated into the budding particle and acquires the lipid bilayer, E, and prM proteins at the ER membrane (Byk and Gamarnik, 2016), followed by virus assembly with structural proteins to form an immature virus. The final step before release is the transport of immature particles to the Golgi apparatus for surface protein glycosylation and modification generating mature virons (Yap et al., 2017).

2. Chikungunya virus

Chikungunya is currently the fastest expanding arbovirus disease worldwide, and the geographical distribution of chikungunya has reached a global distribution in the past decade, causing millions of cases over 50 countries (Nsoesie et al., 2016). Although chikungunya was not considered as a fatal disease, the long-term disabilities caused by the disease has largely increased the economic and health burdens. Therefore, chikungunya has become a major arboviral threat that transmitted by mosquitoes (Gerardin et al., 2011; Labeaud et al., 2011).

Chikungunya virus (CHIKV) is believed to originate in Africa, and has diverged into several genotypes and lineages in the subsequent years (Volk et al., 2010; Weaver et al., 2012). Although the outbreaks were misdiagnosed and attributed to DENV as they cause very similar clinical symptoms, the earliest records of chikungunya epidemic could be traced during 1779-1785, started from Cairo, then followed by several outbreaks in Arabia, India, and Southeast Asia regions (Christie, 1881). However, the first recognized CHIKV was reported in 1952 in Tanzania (Ross, 1956). The genotypes of current circulating CHIKV strains had already diverged from the original lineage. The first branching was dated 500 years ago while West African (WA) and East/Central/South African (ECSA) lineages were established in two distinct branches,. WA strains were mainly circulating in enzootic cycles and responsible for few small focal outbreaks in Western Africa (Powers and Logue, 2007). In contrast, the ECSA strains were mainly circulating outside of Africa in an urban cycle, and arrived in Asia 70 to 150 years ago; it subsequently evolved into the Asian genotype (Volk et al., 2010).

2.1. Chikungunya epidemiology

The ECSA genotype has contributed to a major outbreak in 2004, enlarging the epidemic areas (Staples et al., 2009). The virus expanded to islands in the Indian Ocean region, India, and parts of Southeast Asia. Over 6 million cases of chikungunya could be estimated during the epidemic (Powers and Logue, 2007; Schwartz and Albert, 2010; Staples et al., 2009; Thiberville et al., 2013). An adaptive mutation (E1-A226V) was detected in strains of the ECSA genotype which gave the newly diverged Indian Ocean Lineage (IOL) (Volk et al., 2010). The E1-A226V mutation in CHIKV regulates the lipid and pH sensing, which alters the pH requirement and causes a higher dependence for cholesterol for infection and fusion (Kuo et al., 2012; Tsetsarkin et al., 2007; Tsetsarkin and Weaver, 2011).

Therefore, the mutation increases viral fitness in *Aedes albopictus* and consequently, facilitates the spreading of CHIKV (Tsetsarkin et al., 2007; Vazeille et al., 2007; Vega-Rua et al., 2014). The growth of international travels has greatly contributed to increase the geographic distribution of CHIKV (Tatem et al., 2012). Viremic travelers arriving in Europe became the source of contamination leading to detect autochthonous CHIKV cases in Italy (Rezza et al., 2007) and France (Grandadam et al., 2011).

2.2. The burden of chikungunya

Although chikungunya is not considered as a fatal disease and the possibility of chikungunya associated lethality is still under investigation, the severe acute and chronic phases of infection still caused a significant societal burden (Dupuis-Maguiraga et al., 2012; Javelle et al., 2017; Yactayo et al., 2016). In 2014, chikungunya has caused an average of 26.88 (95% UI; 25.45-28.31) DALYs per 100,000 population in Latin America, but the value varies greatly by region. The DALYs in Dominican Republic was 962.07 (UI; 911.04-1013.10) per 100,000 population in the same year whereas it was 397.86 (UI; 376.78-418.99) in El Salvador, and 156.53 (UI; 148.23-164.83) in Puerto Rico (Cardona-Ospina et al., 2015). Nevertheless, data simulation indicated that more than 90% of DALYs and 95% of costs were attributed to chronic inflammatory rheumatism (Bloch, 2016), causing a important economic and society burden even though chikungunya incidence corresponded to only 20% of dengue incidence in Americas from 2012 to 2016 (Rodriguez-Morales et al., 2016).

2.3. Characteristics of Chikungunya virus

CHIKV is an approximately 70-nm in diameter, enveloped virus which belongs to the genus *Alphaviruses* in the Family Togaviridae. The 12kb genome of CHIKV is consisting of a

single-stranded, positive-sense RNA which contains a 5' cap and a 3' polyadenylation. Unlike DENV, CHIKV genome could be divided into 49S genomic RNA and 26S subgenomic RNA, which are synthesized at different stages of infection. The non-structural viral proteins are encoded in the 49S genomic RNA, which could be translated into two polyproteins as follows: NH_2-nsP1-nsP2-nsP3-COOH, or NH_2-nsP1-nsP2-nsP3-nsP4-COOH as a result of translational readthrough (Li and Rice, 1993; Strauss et al., 1983). The cleavage of non-structural polyprotein is carried out by the protease activity of viral nsP2, which acts in *cis* and *trans* to process a non-structural viral polyprotein into individual proteins (Karpe et al., 2011; Pastorino et al., 2008). The production of subgenomic RNA is primed at the late infection stage by a RNA subgenomic promoter on the 5' of subgenomic RNA (Strauss and Strauss, 1994). The structural proteins of CHIKV are encoded in the subgenomic RNA which could be translated as: NH_2-CP-E3-E2-6k-E1-COOH, or NH_2-CP-E3-E2-TF-COOH as a result of ribosomal frameshifting (Firth et al., 2008; Snyder et al., 2013). However, the cleavage of structural proteins is more complicated, except the CP-E3 cleavage which is carried out by the *cis*-acting protease activity of viral Capsid protein. The other proteins are cleaved by other host factors, for instance, a furin-like protease is responsible for E3-E2 cleavage whereas E2-6k and 6k-E1 are cleaved by a Signal Peptidase (Leung et al., 2011).

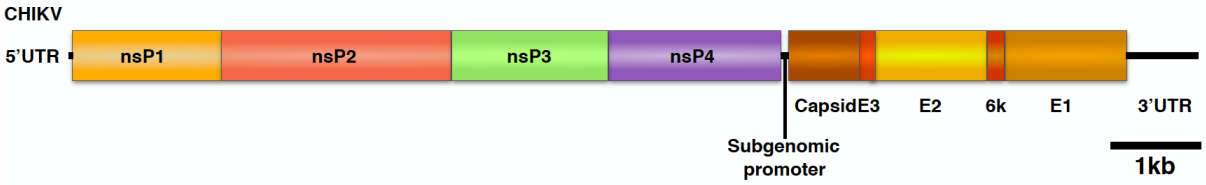


Figure 4. The genome structure of chikungunya virus.

Like DENV, CHIKV enters mosquito cells *via* receptor-mediated endocytosis. By sensing the acidic environment of the endosome, the E1 protein containing viral envelop undergoes an irreversible conformational change, resulting in the fusion of virus-cell

membranes and subsequently, the release of the viral genome into the cytoplasm (Kielian and Rey, 2006; Marsh and Helenius, 2006). With the host translational machinery, the non-structural proteins are translated and processed into proteins that are responsible for viral RNA synthesis. Presumably with host proteins, spherules containing viral RNA and replicase (nsP4) are formed on plasma membrane (Spuul et al., 2011; Thaa et al., 2015). In the early stage of infection, a negative strand of viral RNA is synthesized together with nsP4 and uncleaved nsP123 in the spherules (Kallio et al., 2015; Shirako and Strauss, 1994; Utt et al., 2015). At the late infection stage, the non-structural proteins in the spherules are further processed into individual nsPs which transform the minus-strand replication complex into a stable positive-strand genomic and subgenomic RNA replication complex (Lemm et al., 1998; Raju and Huang, 1991). The newly synthesized subgenomic RNAs are translated into structural proteins by ribosomes. Before transporting the structural polyprotein to the ER, the self-cleaved viral capsids are formed, allowing the interaction with newly synthesized genome from spherules for oligomerization (Jose et al., 2017). The structural polyproteins E3-E2-6k-E1 and E3-E2-TF are further processed in ER that undergo palmitoylation, N-linked glyscosylation, and followed by the release of E3 with furin modification (Jose et al., 2009). E1 and E2 are transported to plasma membrane to form mature spikes on plasma membrane. Viral assembly is initiated with the binding of the viral nucleocapsid to the viral RNA and the membrane-associated envelop glycoproteins (Jose et al., 2017). The mature viral particles acquire E1/E2 protein dimers by budding out of cell surface that is covered with viral spikes (Schwartz and Albert, 2010).

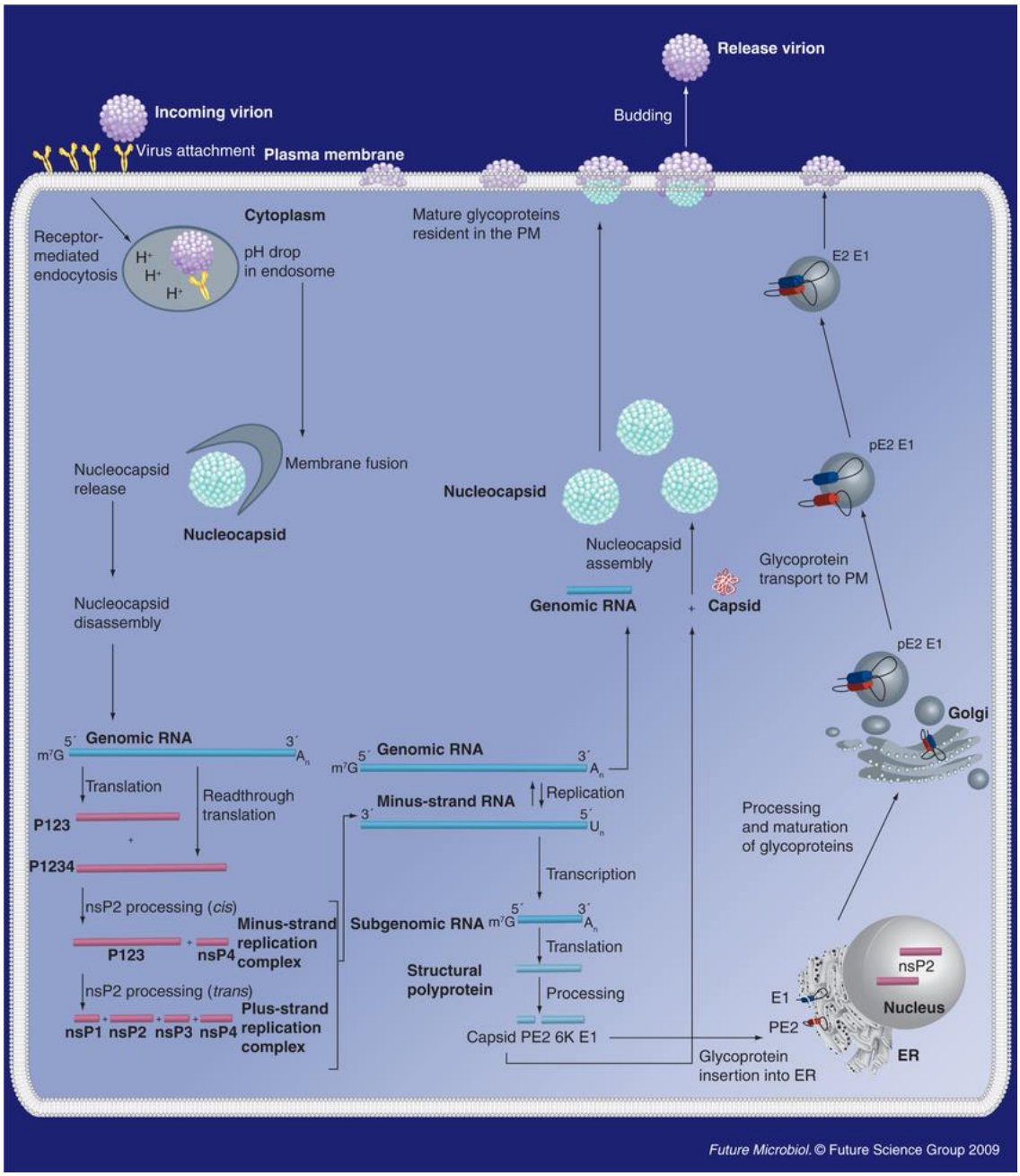


Figure 5. Alphavirus life cycle. CHIKV infects an eukaryotic cell through receptor-mediated endocytosis mechanism. The viral RNA is released from the endosomal vesicle in response to the change of pH in the cytoplasm. Non-structural proteins are first translated from the 49S genomic RNA. As DENV, the nsPs form a spherule for negative strand RNA synthesis without triggering host immune responses. At the late stage of infection, the nsPs were cleaved into individual nsPs and the spherule is then switched into a positive strand synthesizing complex, which is responsible for positive strand RNA synthesis. Including the 49S genomic RNA and 26S subgenomic RNA, the newly synthesized viral RNA is transported out of the spherule, and the viral structure proteins are then translated from the 26S subgenomic RNA and subsequently, transported onto the cell membrane after modifications in Golgi. The newly synthesized 49S genomic RNA is then packed by viral proteins. The nucleocapsid acquires a mature glycoprotein envelop upon budding out of the membrane as a mature virion.

3. Co-infection of DENV and CHIKV

As DENV and CHIKV cover the same geographical regions and are both transmitted by the same vectors *Aedes* spp., mosquitoes in the epidemic areas are potentially able to carry and transmit multiple viruses. Co-infection of DENV and CHIKV were frequently reported in Africa (Baba et al., 2013; Caron et al., 2012; Leroy et al., 2009), Southeast Asia (Neeraja et al., 2013; Schilling et al., 2009), Eastern Mediterranean (Rezza, 2012), and Western Pacific regions (Chang et al., 2010). Until now, *Aedes* mosquitoes were present in 154 countries, and local transmission of DENV and CHIKV were reported in 98 countries, among them, 13 countries have reported DENV-CHKV co-infection (Furuya-Kanamori et al., 2016b). The first co-infection of DENV-CHIKV was reported in Thailand in 1962, and few co-infected cases were reported in the following years until 1964 (Nimmannitya et al., 1969). Since then, DENV-CHIKV co-infections were reported in several Africa and South-East Asia countries, causing a major epidemic between 2006 to 2012 (Furuya-Kanamori et al., 2016b). Especially in Madagascar (Ratsitorahina et al., 2008), Nigeria (Baba et al., 2013), and India (Taraphdar et al., 2012), high prevalence of DENV and CHIKV co-infections was reported in each country between 2006 to 2010, causing dozens of co-infected cases during the epidemic. Although chikungunya was not considered as a lethal disease, and only 0.5-3.5% mortality could be observed for dengue hemorrhagic fever (Guzman and Kouri, 2002; Suaya et al., 2009), accumulating evidence have suggested the co-infection of CHIKV and DENV was associated to more severe clinical symptoms leading to death (Gandhi et al., 2015; Mercado et al., 2016), however, the mechanism that caused higher mortality is still not understood, as the mono-and co-infected patients were sharing very similar median age and gender distribution

(Gandhi et al., 2015). Therefore, eliminating both viruses from circulation is pivotal for reducing the burdens caused by DENV and CHIKV co-circulation.

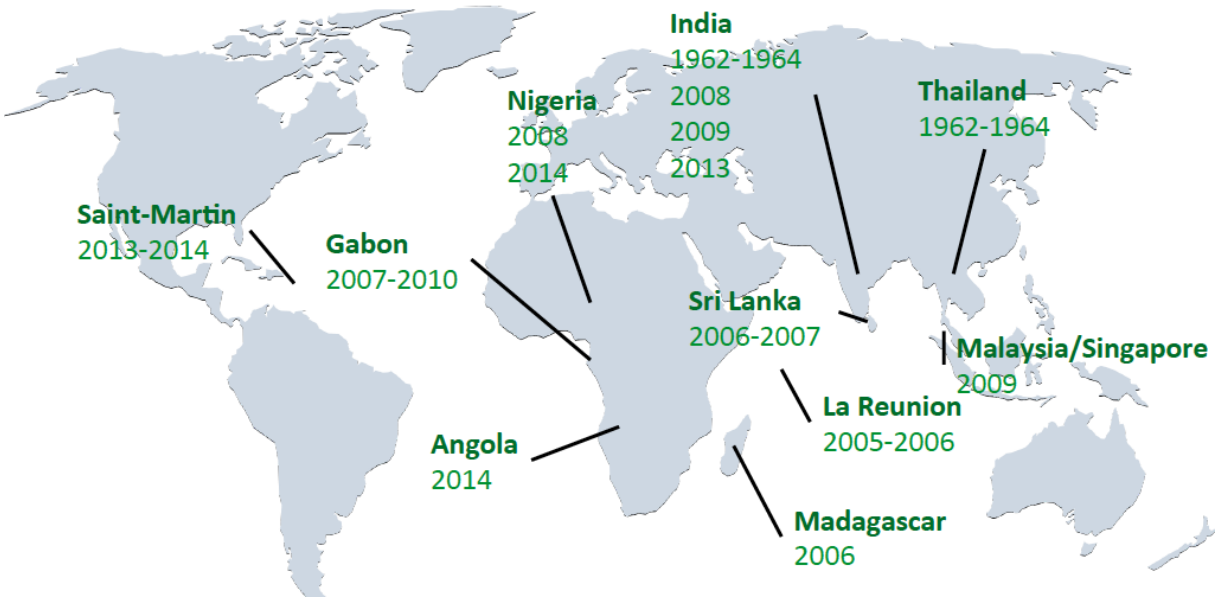


Figure 6. Co-infection of CHIKV and DENV

Chapter 1: AEDES MOSQUITOES AND ANTIVIRAL IMMUNITY

Arboviruses are acquired by mosquitoes through a viremic blood meal from infected hosts. Through the foregut, the virus enters into the midgut and infects the epithelial cells, followed by virus replication before it escapes from the midgut barrier and disseminates to secondary tissues through tracheal or muscles (Girard et al., 2004; Romoser et al., 2004; Salazar et al., 2007). For *Aedes* mosquitoes, fat body, hemocytes, and nervous system are potential tissues for harboring the disseminated virus. Moreover, the infected hemocytes that circulate in the hemolymph could act as a media for salivary glands infection (Parikh et al., 2009). The virus could enter the salivary glands in the distal lateral lobes that potentially contain receptors for medicating viral endocytosis (Salazar et al., 2007). The interval between the acquisition of a virus from a blood meal and the ability to transmit to other hosts by the mosquito vector is also known as the extrinsic incubation period (EIP), which can vary widely depending on mosquitoes and viruses. Several anatomical barriers have been identified affecting the EIP: the midgut infection barrier (MIB), midgut escape barrier (MEB), salivary gland infection barrier (SGIB), and salivary gland escape barrier (SGEB) (Franz et al., 2015). The viral pathogenesis could be obstructed by each barrier in a physical or immunological manner, for instance, the basal laminar that covers the midgut and the salivary glands can physically prevent the midgut dissemination and salivary gland infection. On the contrary, the antiviral immunity of midgut epithelia cells, hemocytes, and salivary gland cells is also critical for the midgut infection, the dissemination from midgut, and the transmission from salivary glands (Franz et al., 2015).

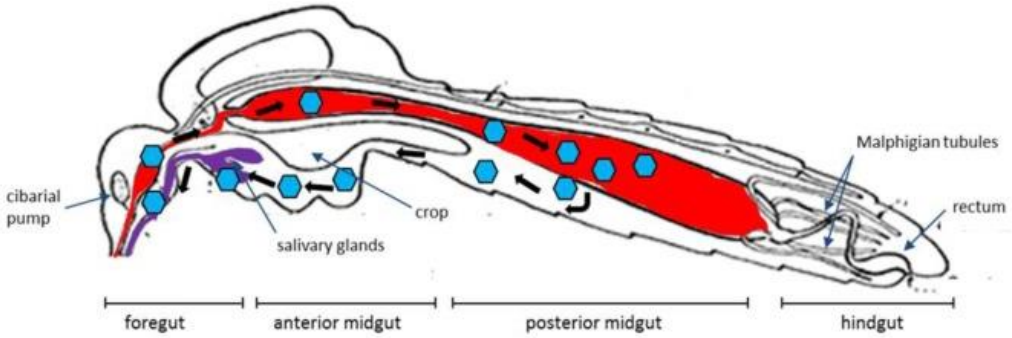


Figure 7. Schematic representation of arbovirus tropism in a mosquito vector. Virions are represented by blue hexagons. Mosquitoes acquire virus through a viremic blood meal from infected hosts. The virus enters the midgut and replicates before dissemination. The virus breakthrough the midgut barrier is able to infect the other organs such as salivary glands. Only the infected salivary glands are able to transmit the virus.

1. *Aedes* mosquitoes

Aedes aegypti and *Aedes albopictus*, are the primary vectors of the most important human arboviruses in the world. The wide range of temperature tolerance and the egg desiccation resistance features of *Aedes* mosquitoes have increased the ability of these species to survive in extreme conditions and long-distance transportation (Brady et al., 2013; Kobayashi et al., 2002), which also helped to establish a new population in non-native habitats (Juliano and Lounibos, 2005). With the transportation of a variety of goods, including used tires and lucky bamboo (Medlock et al., 2012; Scholte et al., 2010), *Ae. albopictus* were introduced in Europe from its original cradle, Southeast Asia (Paupy et al., 2009). In contrast, *Ae. aegypti* have a more complex origin, which includes two subspecies, *Ae. aegypti aegypti* and *Ae. aegypti formosus*. The forest form, *formosus*, is considered as the ancestor of the domestic form of *Ae. aegypti*, and both still exist in sub-Sahara Africa (Brown et al., 2014; Powell and Tabachnick, 2013). The forest form is darker and preferring non-human blood. It is still unclear whether the higher viral susceptibility of domestic form (Vazeille-Falcoz et al., 1999), dated before it came to the New World, or became domesticated upon arrival (Powell

and Tabachnick, 2013). Later on, *Ae. aegypti* was introduced into Europe through the route of triangular slave trade between Europe, Africa, and America (Brown et al., 2011). Although there is still a lack of evidence, it is believed that *Ae. aegypti* was introduced into Asia through the eastern migration and trade between India and East Africa (Powell and Tabachnick, 2013).

As an efficient vector of CHIKV, DENV, yellow fever virus (YFV), and Zika virus (ZIKV), the global distribution of these arboviral diseases are highly co-related with the distribution of *Aedes* mosquitoes (Charrel et al., 2014). Although the climate might influence mosquito development, mortality, behavior, and even the viral replication in *Aedes* mosquitoes (Morin et al., 2013), the rapid adaptation of arboviruses have caused unexpected autochthonous transmission in temperate areas, e.g. Italy, France, and Spain. Moreover, *Ae. albopictus* has already crossed the Channel extending its distribution to southern England, and it could potentially introduce some arboviral diseases (Medlock et al., 2017). Thus, knowing more on the mosquito antiviral responses against arboviruses is essential to better apprehend prevention and control of arboviral diseases.

2. Mosquito immune pathways: Toll, Imd, and JAK/STAT

Several immune pathways are involved in insect antiviral responses, including the Toll, Imd and Janus Kinase-signal transduction and activators of transcription (JAK-STAT) pathways (De Gregorio et al., 2002; Fragkoudis et al., 2009; Kingsolver et al., 2013). Toll and Imd pathways correspond to conserved innate immunity closely related to mammalian Toll-like receptor (TLR) and tumor necrosis factor (TNF) pathways. Both immune pathways are primarily induced by microbial infection *via* pathogen associated molecular patterns (PAMPs) recognition (Royet et al., 2005; Werner et al., 2000). Although the viral pattern recognition

receptors in mosquitoes are still unknown, growing evidence has proved the role of Imd and Toll pathways in *Ae. aegypti* defenses against DENV (Xi et al., 2008). Through an unknown mechanism, the Toll receptor might be activated by virus direct interaction or through the ligand Spätzle (Weber et al., 2003), the activated Toll receptor recruits Myd88 for activating a NF-kB-like transcription factor Rel1 to initiate Toll-related gene expression (Horng and Medzhitov, 2001). For the initiation of Imd pathway, an unknown receptor that could be activated by viral infection recruits the Imd and FADD proteins, and these two proteins participate to the maturation of another NF-kB-like transcription factor, Rel2, to initiate Imd-related gene expression (Valanne et al., 2011).

Both Toll and Imd pathways trigger the expression of multiple anti-microbial peptides (AMPs) that restrict the replication of pathogens within infected cells. On the contrary, a systematic immunity mediated by JAK-STAT pathway is triggered by the recognition of unpaired ligand Upd through its receptor Domeless (Dome), which triggers the downstream signaling and phosphorylates, the Janus kinase (Hop) and Dome on the cytoplasmic end, leading to the phosphorylation and dimerization of STAT. The activated STATs act as transcription factor and is translocated into the nucleus for initiating the downstream AMPs and Vir-1 expression.

In addition, the JAK-STAT pathway can also be activated by Vago as a result of double strand RNA (dsRNA) accumulation. The accumulated dsRNA (including viral RNA) is sensed by Dcr-2, which activates TNF receptor-associated factors (TRAF) and then triggers the maturation of Rel2, followed by induction of Vago expression (Cheng et al., 2016; Deddouche et al., 2008; Paradkar et al., 2014). This suggests that the JAK-STAT pathway can be also triggered by virus infection.

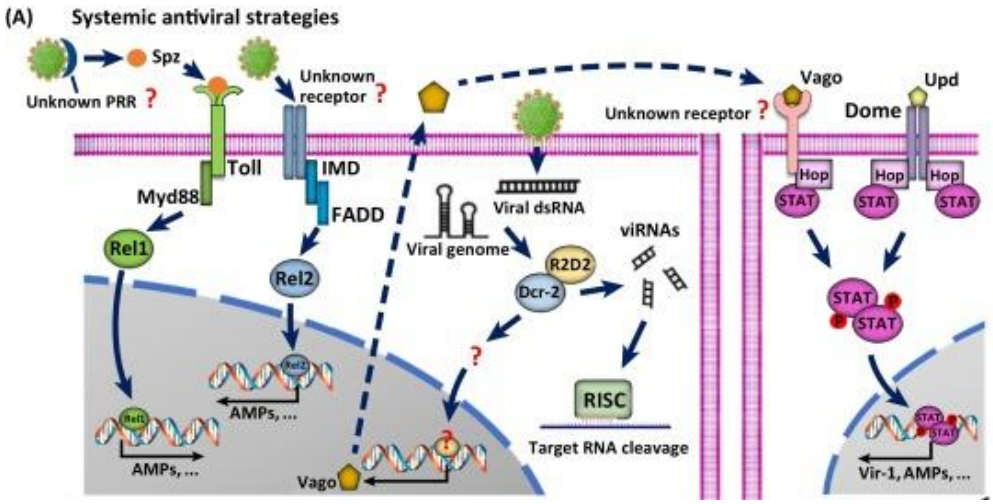


Figure 8. Mosquito antiviral mechanisms. Three major insect innate immunity pathways are described in mosquitoes for antiviral responses. Through different pattern recognition signaling, different immune pathways are triggered in response to microbial infection. The pattern recognition receptor for viruses in Toll- and Imd-mediated antiviral innunity is still unknown whereas it has been proven that the JAK-STAT signaling could be elicted by Vago, induced by sensing the viral dsRNA in the cytoplasm. (Cheng et al., 2016).

3. Part I: Assessing the potential role of miRNAs in the antiviral response of vector mosquitoes

In this chapter, we present an article submitted to the journal *mSphere*.

ABSTRACT

Although the role of exogenous siRNA and piRNA pathways in mosquito antiviral immunity is increasingly better understood, there is still little knowledge regarding interactions between the mosquito cellular miRNA pathway and arboviruses. Thus further analysis of mechanisms by which miRNAs may regulate arbovirus replication in mosquitoes is pivotal. Here, we carried out genomic analysis to identify *Aedes aegypti* miRNAs that potentially interact with various lineages and genotypes of chikungunya, dengue and Zika viruses. By using prediction tools with distinct algorithms, several miRNA binding sites were commonly found within different genotypes/and or lineages of each arbovirus. We further analyzed those miRNAs that could target more than one arbovirus and required a low energy threshold to form miRNA-vRNA (viral RNA) complexes and predicted potential RNA structures using RNAhybrid software. In summary, we predicted miRNA candidates that might participate in regulating arboviral replication in *Ae. aegypti*, and this study can shed further light on the role of miRNA in mosquito innate immunity and targets for future studies.

IMPORTANCE

The role of the siRNA and piRNA pathways in controlling virus infections in mosquitoes has been extensively studied and they are considered to be a major part of antiviral innate immune response. However, the potential role of miRNAs in mosquitoes antiviral immunity is still unclear. The cellular miRNAs of the mosquito *Aedes aegypti*, a vector for many arboviral diseases, may participate in regulating the replication of three major arboviruses: chikungunya, dengue, and Zika viruses. By using the miRanda and TargetSpy tools, several miRNAs were predicted to have potential binding sites that are common to multiple viral genotypes or lineages. Further analysis was carried out on miRNA-vRNA

interactions that required a low energy threshold to form a complex. This revealed a broad picture of possible interactions between mosquito cellular miRNAs and different genotypes/lineages of arboviruses, which could shed further light on the role of miRNA in mosquito innate immunity and targets for future studies.

KEYWORDS chikungunya, dengue, Zika, arboviruses, transmission, miRNA, *Aedes aegypti*

INTRODUCTION

Emerging and reemerging arthropod-borne viruses (arboviruses) are spreading globally (Powers and Waterman, 2017). Arboviruses usually have RNA genomes, including positive-strand RNA alphaviruses (genus *Alphavirus*, family *Togaviridae*), and flaviviruses (genus *Flavivirus*, family *Flaviviridae*) (Weaver and Reisen, 2010) (see Fig. S1 for genomic organization). The evolution of mosquito-borne RNA viruses and the complex interplay between the vector, host and virus can shape arboviral emergence and re-emergence (Grubaugh et al., 2016; Jones et al., 2008). Contrary to many other viruses, arboviruses replicate in two hosts: vertebrate and arthropod (including mosquito species). Infection of the arthropod midgut epithelial cells occurs following ingestion of a viremic blood meal. The arbovirus must disseminate through internal tissues and organs before reaching the salivary glands. Thus the virus has to overcome a series of tissue barriers before being secreted in mosquito saliva when it takes its next blood meal (Kramer and Ebel, 2003). Each barrier has different tissue-specific immune properties which, once triggered by viral infection, may affect the mosquitoes' overall vector competency (Franz et al., 2015). Immune responses to arboviruses are varied and involve different pathways but key roles are played by small RNA / RNA interference (RNAi) pathways, which are further described below (Blair and Olson, 2015; Merkling and van Rij, 2013; Olson and Blair, 2015; van Mierlo et al., 2011). The role of RNAi pathways as insect antiviral response mechanisms was initially characterized in *Drosophila melanogaster* (Galiana-Arnoux et al., 2006; van Rij et al., 2006; Wang et al., 2006; Zambon et al., 2006). RNAi has been shown to be a major innate antiviral immune response in mosquitoes (and other vectors) against arboviruses of all families (Donald et al., 2012; Miesen et al., 2016b). Mosquitoes have three major types of small RNA pathways and associated molecules: the small interfering RNA (siRNA), PIWI-interacting RNA (piRNA),

and microRNA (miRNA) pathways (Blair and Olson, 2015; Donald et al., 2012; Fragkoudis et al., 2009; Miesen et al., 2016b).

The exogenous siRNA pathway in mosquitoes is triggered by exogenous long double-stranded RNA (dsRNA) molecules, which are produced during viral replication. By analogy to *D. melanogaster* (Campbell et al., 2008), these dsRNAs are recognized by the RNAse III enzyme Dicer 2 (Dcr-2) and cleaved in mosquitoes into predominantly 21 nucleotides (nt) viral siRNAs (vsiRNA). These vsiRNAs are loaded into the multi-protein RNA-Induced Silencing Complex (RISC), which contains the endonuclease Argonaute-2 (Ago-2), a member of the Argonaute family. Ago-2 unwinds the siRNAs and retains one strand as a guide strand to target RNAs with complementary sequence (mRNAs, viral genomes, antigenomes), which triggers cleavage of the complementary RNA by Ago-2. These results have stressed the role of exogenous RNAi pathway in controlling viral replication (Campbell et al., 2008; Carissimo et al., 2015; Dietrich et al., 2017; Keene et al., 2004; Sanchez-Vargas et al., 2009).

Accumulating data suggest that the Piwi-interacting RNA (piRNA) pathway is also involved in mosquito RNAi antiviral immunity (Brackney et al., 2010; Hess et al., 2011; Miesen et al., 2016a; Miesen et al., 2016b; Morazzani et al., 2012; Schnettler et al., 2013). However, the effector and effector mechanism are unclear and the Piwi4 protein has been identified as a non-canonical effector (Varjak et al., 2017). piRNAs are 24-30 nt in size and produced in a Dicer-independent manner. piRNA molecules interact with Argonaute-3 (Ago-3) and Piwi proteins in a so-called “ping-pong” mechanism which amplifies these small RNAs. During this amplification, a typical U1/A10 positional bias in the piRNA molecules is observed (Brennecke et al., 2007). Several studies have identified and characterized viral piRNA (vpiRNA) or piRNA-like small RNAs in mosquitoes or mosquito cells (Brackney et al., 2010; Hess et al., 2011; Miesen et al., 2016b; Morazzani et al., 2012; Schnettler et al., 2013). Nonetheless, it remains unclear if vpiRNAs exert any antiviral activity.

Finally, the miRNA pathway is a major endogenous gene expression regulation mechanism. Again it is presumed that the pathway in mosquitoes largely resembles that of *D. melanogaster* given close relationships of effector proteins (Campbell et al., 2008). This pathway shares some similarities with the siRNA pathway, but with important differences (Blair, 2011; Blair and Olson, 2015; Donald et al., 2012), such as it has both nuclear and cytoplasmic phases as the precursor RNAs are produced in the nucleus. From miRNA precursors to mature miRNAs, the biogenesis of miRNAs is a process involving several steps carried out by different proteins. The miRNA precursors (pri-miRNAs) originate from independent miRNA genes or mirtrons which are encoded as introns within RNA transcripts. The hairpin-structured pri-miRNAs are processed by the RNase III type endonuclease Drosha into ~70bp hairpins, which are then cleaved into ~20bp miRNA duplexes by Dicer-1 after being exported from the nucleus into the cytoplasm by Exportin5 (Ha and Kim, 2014; Kim et al., 2009; Yi et al., 2003). The miRNA duplexes in the cytoplasm are loaded into Ago-1 or Ago-2 proteins in miRNA-induced silencing complexes (miRISCs) according to their different structures (Forstemann et al., 2007; Ghildiyal et al., 2010; Yang et al., 2014). Mainly using residues 2-8 at the 5' end of mature miRNA (seed region), the miRISC uses the guide strand to find complementary RNA sequences which leads to RNA degradation (carried out by Ago-2), translational inhibition or both (mediated by other Ago proteins, not Ago-2) (Li and Rana, 2014; Wilczynska and Bushell, 2015). Commonly, animal miRNAs binding sites are mainly in the 3'UTR (Pillai et al., 2007); however miRNA binding sites in the 5'UTR or coding regions of mRNAs were also reported and are involved in post-transcriptional gene regulation (Brummer and Hausser, 2014; Hausser et al., 2013; Lee et al., 2009).

Only a few studies have shown direct evidence that mosquito miRNAs interfere with virus replication *via* complementarity with viral RNA genome or regulation of the immune genes in mosquitoes. A miRNA from *Ae. aegypti*, aae-miR-2940, was reported as a mosquito-

specific antiviral miRNA which inhibits the replication of DENV and West Nile virus (WNV) in *Ae. aegypti* or *Ae. albopictus* cell lines respectively (Slonchak et al., 2014; Zhang et al., 2013). The antiviral mechanism of aae-miR-2940 may involve a complex immune network, and could be regulated by endosymbionts or virus infection. One target of aae-miR-2940 in *Aedes* spp. is a metalloprotease, which is required by *Wolbachia* but also essential for WNV and DENV replication. However, by up-regulating aae-miR-2940 expression (which facilitates *Wolbachia* proliferation), the replication of WNV and DENV was consequently inhibited (Hussain et al., 2011; Zhang et al., 2013). Moreover, the same mechanism was observed in *Ae. albopictus* derived C6/36 cells, although instead of inducing metalloprotease expression, it was down-regulated by WNV infection thus limiting viral replication (Slonchak et al., 2014). Bioinformatic approaches suggest that the target site of aae-miR-2940 is in the 3‘UTR of WNV; although, it has been experimentally demonstrated that this miRNA has no significant effect on viral replication (Slonchak et al., 2014). In addition, aal-miR-2940-5p and aal-miR-2940-3p were presumed to be involved in CHIKV replication in *Ae. albopictus* (Shrinet et al., 2014). Furthermore, genomic RNA levels of DENV and WNV were increased significantly in either Drosha, Ago-1 or Ago-2 knock-down *Ae. aegypti* derived Aag2 cells (Kakumani et al., 2013), and an increase in DENV titer could also be observed in either Dcr-1, Dcr-2, Ago-1, or Ago-2 deficient *Drosophila* S2 cells (Mukherjee and Hanley, 2010). Although silencing of Ago-1 in *An. gambiae* mosquitoes or Aag2 cells had no effect on either o’nyong-nyong virus (ONNV), Semliki Forest virus (SFV) or CHIKV (Keene et al., 2004; McFarlane et al., 2014b; Schnettler et al., 2013), some studies have shown that the miRNA could be loaded into Ago-2 under certain conditions (Fu et al., 2017; Hussain et al., 2013); thus the effects of miRNAs on the replication of alphaviruses needs to be carefully examined. Complementarity within the seed region is critical for initiating interactions with potential targets. However, mismatches within this region could be compensated by pairing to the 3’

region of miRNA in metazoans (Shin et al., 2010). In addition, an increasing number of studies have demonstrated that thermodynamic considerations in the seed region play an important role for seed region-dependent silencing (Hibio et al., 2012; Ui-Tei et al., 2008; Ui-Tei et al., 2012). The relatively flexible thermodynamic rules of miRNA binding sites can potentially lead to the multiple targets of a single miRNA. Whereas the targets might also be regulated by multiple miRNAs, interactions between miRNAs and viral RNA could also contribute to antiviral innate immunity in the host.

In mammals, more evidence is available on the role of virus replication regulated by direct miRNA-vRNA interaction (Scheel et al., 2016; Trobaugh and Klimstra, 2017). Host miRNAs may bind to the cytoplasmic viral RNA genome as regular mRNA in a seed region-dependent manner. However, binding does not necessarily lead to the inhibition of viral replication. For instance, the enhancement of viral replication has been demonstrated with the human liver specific miR-122 which targets the 5‘UTR of HCV and stabilizes the viral RNA, facilitating viral replication (Conrad et al., 2013; Jopling et al., 2005). In any case, the direct interaction between cellular miRNAs and RNA virus genomes could play a very important role in replication. In vertebrates, several studies have underlined the regulation of virus replication following the direct binding of miRNAs to the viral RNA genome. In addition to HCV, several other viral vertebrate pathogens have been shown to be regulated in this manner. These include, amongst others, Eastern equine encephalitis virus (EEEV) (Bai and Nicot, 2015; Huang et al., 2007; Lecellier et al., 2005; Nathans et al., 2009; Trobaugh et al., 2014; Wen et al., 2013; Zheng et al., 2013).

Importantly, EEEV is a mosquito-borne virus (closely related to CHIKV) and infections result in high mortality rates in humans and long-term neurological damage in surviving patients (Deresiewicz et al., 1997). Replication of the North American EEEV strain, FL93-939, was reduced by the human haematopoietic cell specific miR-142-3p that has four

binding sites in the 3‘UTR. Moreover, the replication of an EEEV strain depleted of miR-142-3p binding sites was nearly 1,000-fold higher than wild-type EEEV (Trobaugh et al., 2014). This indicates viral replication is mediated by cellular miRNAs in humans and suggests a similar mechanism might be found in the mosquito vectors.

There is still a remarkable lack of information about the interactions between mosquito miRNAs and arboviruses. However, miRNAs were identified by next generation sequencing (NGS) techniques in numerous studies and are now available in databases, while improved bioinformatics tools have been developed. To identify potential mosquito miRNA binding sites in the viral RNA, we compared published small RNA sequencing data from *Ae. aegypti* to virus sequence data from major arboviruses, e.g. CHIKV, DENV (serotypes 1-4), and Zika virus (ZIKV). We predicted and analyzed the potential target sites on each virus genome to reveal practicable miRNA-vRNA interactions by combining thermodynamics and miRNA expression profiles. This study can underpin future work on the role of miRNAs in regulating arbovirus replication in mosquito cells.

RESULTS

miRNA binding sites in the CHIKV genome

CHIKV belongs to the family of *Togaviridae* and the genus *Alphavirus*, with three genotypes circulating worldwide: East/Central/South African (ECSA), West African (WA), and Asian. The CHIKV Indian Ocean lineage (IOL) emerged in 2004-5 from the ECSA phylogroup and has spread throughout many tropical regions (Powers & Logue, 2007). It is believed that the WA and ECSA lineages diverged from the ancestor lineage 500 years ago, and the Asian genotype evolved from the ECSA genotype an estimated 70 to 150 years ago (Volk et al., 2010). The IOL lineage predominates in regions where the vector *Ae. albopictus*

is present/dominant, in part due to selection of an *Ae. albopictus*-adaptive substitution in the CHIKV E1 envelope glycoprotein (E1-A226V). This substitution confers efficient infection and dissemination in *Ae. albopictus* for IOL of CHIKV (Tsetsarkin et al., 2007; Vazeille et al., 2007).

Four CHIKV genotypes were selected for this study: Asian (EU703762), ECSA (HM045811), IOL (AM258992), and WA (HM045816). Two analytical tools,miRanda and TargetSpy, were used to identify a total of 20, 25, 26, and 22 miRNAs binding sites that were commonly found for CHIKV of different genotypes and lineages (Asian, ECSA, IOL, and WA) (Fig. 1).

When comparing the binding sites previously identified among all four genotypes, the highest number of shared binding sites was between ECSA and IOL genotypes (N=18), whereas WA and Asian genotypes were relatively independent to each other with no common binding sites (Fig. 1). Therefore, for our analysis, WA and Asian sequences were considered in two separate groups comparing each to ECSA and IOL: (i) WA, ECSA, IOL, and (ii) Asian, ECSA, IOL. When comparing viruses of group (i) six miRNAs binding sites were common and could be targeted by aae-miR-263a-5p, aae-miR-279, aae-miR-305-5p, aae-miR-34-3p, and aae-miR-996 (Table 1). When examining group (ii) (WA, ECSA, IOL), three other miRNAs binding sites were detected, which potentially interacted with aae-miR-285, aae-miR-989, and aae-miR-iab-4-5p (Table 1).

The predicted miRNA binding sites shared between Asian, ECSA, and IOL viruses of group (i) were all located within non-structural protein coding regions. aae-miR-263a-5p is one of the most highly expressed miRNA in saliva of *Ae. aegypti* and could be detected throughout all mosquito developmental stages (Hu et al., 2015; Maharaj et al., 2015). miR-279 is related to olfactory regulation, and miR-279 in *D. melanogaster* is involved in the formation of the CO_2 sensory neuron in maxillary palps (Cayirlioglu et al., 2008). In *Ae.*

aegypti, aae-miR-279 was present in embryos and was found to be induced in the midgut following a blood meal (Li et al., 2009). aae-miR-305-5p was predicted to target the nsP2 region which encodes the RNA helicase, RNA triphosphatase and proteinase (Karpe et al., 2011; Rupp et al., 2015) and was reported to be abundant during the mosquito pupal stage and in *Ae. aegypti* Aag2 cells (Hu et al., 2015), and could be upregulated in mosquito salivary glands after CHIKV infection (Maharaj et al.). Interestingly, the expression of aae-miR-305-5p switched to aae-miR-305-3p in response to DENV-2 infection (Etebari et al., 2015). aae-miR-34-3p was predicted to target the nsP1 region involved in the synthesis of the negative strand of viral genomic RNA (Rupp et al., 2015). This miRNA could be detected at all mosquito developmental stages but the expression level was relatively low during the pupal stage. Moreover, the expression of aae-miR-34-3p correlated with the nutritional status of adults and can be suppressed in the midgut when they are starved (Ray, 2013). aae-miR-996 is the most abundant miRNA in embryos and was also found to be up-regulated in saliva during CHIKV infection (Akbari et al., 2013a; Maharaj et al., 2015).

There are three potential miRNA binding sites that are common between WA, ECSA, and IOL genotypes of the group (ii). Two of them might potentially be targeted by the miRNA aae-miR-285 and aae-miR-989 in the E1 and nsP4 coding regions respectively. While the expression of aae-miR-285 could be detected in both male and female adults, it was rarely observed in the larval stage of *Ae. aegypti* (Hu et al., 2015). It was also reported to be up-regulated in the cytoplasm of *Wolbachia*-transinfected Aag2 cells (*Wolbachia* induced miRNA). In contrast, aae-miR-989 was reported as an abundant miRNA expressed in *Ae. aegypti* females but relatively rare in male adult (Hu et al., 2015). The other potential miRNA binding site shared between WA, ECSA, and IOL genotypes may potentially be targeted by aae-miR-iab-4-5p in the 3'UTR. Notably, this miRNA might have additional binding sites on the 5'UTR of ECSA and IOL genotypes. Any activity of this miRNA might be increased by

multiple binding sites on the 5'- and 3' UTR of CHIKV involved in initiating viral RNA replication (Rupp et al., 2015).

miRNA binding sites in DENV genomes

DENV has evolved independently into four serotypes from distinct sylvatic progenitors and then into several genotypes (Vasilakis and Weaver, 2008). They only share 60-80% sequence similarity and need to be analyzed separately to find potential miRNA-vRNA interactions. Several conserved miRNA binding sites could be found in each serotype which might participate in viral regulation, as outlined below.

DENV-1

Five genotypes of DENV-1 were selected for analysis: genotype I (AF298808), genotype II (JQ922547), genotype III (DQ285562), genotype IV (EF025110), and genotype V (JX669462). A total of 28, 30, 34, 33, and 26 potential binding sites were found in genotypes I, II, III, IV, and V respectively (Fig. 2). Among them, only two potential miRNA binding sites were common to all five genotypes of DENV-1, which could be targeted respectively by aae-miR-1 and aae-miR-282-5p on the capsid and NS3 protein coding regions respectively (Table 2). aae-miR-1 is a conserved miRNA which is upregulated 24 hours post blood meal in *Ae. aegypti* (Bryant et al., 2010). It was also identified in *Wolbachia*-infected cells (Mayoral et al., 2014b). These features are shared by aae-miR-282-5p (Mayoral et al., 2014a). Furthermore, other potential miRNA binding sites were commonly found between four of the five genotypes (Table 2). The NS5 region of genotypes I, II, III and V could potentially be targeted by aae-miR-316, aae-miR-92a-3p, and aae-miR-92b-3p. aae-miR-316 has not been extensively studied miRNA in mosquitoes, however it is known that its expression was induced at 24 hours post blood meal (Li et al., 2009). In addition, this miRNA might have an

further potential binding site within the capsid region (genotypes I, III, and V). This may increase the probability of miRNA-vRNA interactions. Both aae-miR-92a-3p and aae-miR-92b-3p are present in the cytoplasm and nucleus of *Ae. aegypti* Aag-2 cells. A decrease in their expression levels was observed when cells were infected with *Wolbachia* (*Wolbachia*-suppressed miRNA) (Mayoral et al., 2014a). aae-miR-11-5p is the only miRNA with a common binding site in genotypes I, II, IV and V within NS4B (Table 2). In mosquitoes, the expression of aae-miR-11-5p was detected at all developmental stages and is induced in the midgut after blood meal (Hu et al., 2015; Li et al., 2009). Nevertheless, it is also reported as a *Wolbachia*-suppressed miRNA (Mayoral et al., 2014a). Two potential miRNA binding sites for aae-miR-263a-3p and aae-miR-998 were found conserved on the 3'UTR of genotypes I, III, and V (Table 2). However, although aae-miR-263a-3p is detectable in mosquito cells, the *in vivo* expression profile is still unclear. It has been shown that its byproduct, aae-miR-263a-5p, is increased in the saliva of CHIKV-infected mosquitoes at 10 days post infection (Maharaj et al., 2015). This suggests that virus infection could be involved in the regulation of aae-miR-263a-5p, at least in some tissues. In contrast, aae-miR-998 is a widely expressed miRNA which is abundant in embryos and decreases during development. Although its expression is lower in adults, it could be triggered in the adult midgut after a blood meal (Li et al., 2009). Interestingly, the location of aae-miR-998 is close to aae-miR-11 (-3p or -5p) in the mosquito genome (less than 300 bp apart) suggesting that their regulation might be correlated.

DENV-2

Five genotypes of DENV-2 were selected for our analysis: Asian I (DQ181799), Asian II (AJ968413), Asian American (DQ181801), American (AY702040) and Cosmopolitan (AB189122). We found a total of 28, 31, 21, 22, and 28 potential binding sites on each genotype respectively (Fig. 3). Among them, binding sites for aae-miR-2944a-3p, aae-miR-

316, and aae-miR-9c-5p were common to all five genotypes (Table 3).

aae-miR-316 has a potential binding site on the capsid region of viral RNA. It could be detected in both male and female adults and is induced after a blood meal (Hu et al., 2015; Li et al., 2009). Little is known about the expression profile of aae-miR-9c-5p, which was predicted to have a binding site within the NS5 region. It can be detected in *Ae. aegypti* embryos and at low levels in DENV-2 infected adults (Campbell et al., 2014). Furthermore, three other miRNA binding sites were shared between at least four genotypes. Two binding sites were common between Asian I, Asian II, Asian American, and Cosmopolitan genotypes: aae-miR-281-3p within NS2B, and aae-miR-998 within the 3'UTR region of DENV-2. aae-miR-281-3p was described in *Ae. aegypti* Aag2 cells (Mayoral et al., 2014a), and its expression in mosquito midgut was triggered by a blood meal (Li et al., 2009). Moreover, its byproduct, aae-miR-281-5p, was reported to be an abundant midgut-specific miRNA that enhances DENV-2 infection in *Ae. albopictus* (Zhou et al., 2014). The only miRNA predicted to target Asian I, Asian American and Cosmopolitan genotypes of DENV-2 is aae-miR-263a-3p which has a potential binding site on the 3'UTR. Its presence was detected in *Ae. aegypti* embryos and Aag2 cells (Hu et al., 2015; Hussain et al., 2011). Interestingly, the same miRNA binding site within the 3'UTR of DENV-2 was also found within that region of DENV-1. aae-miR-315-5p is abundant in embryos, but decreases during development, although it is reported to remain detectable in both male and female adult (Hu et al., 2015; Mayoral et al., 2014a). The possible target sites were predicted to be located in the 3'UTR of the *Ae. aegypti* JHA15 (juvenile hormone regulated serine protease) (Lucas et al., 2015) and AaArgM3 (arginine methyltransferase 3) (Zhang et al., 2014).

DENV-3

Four genotypes of DENV-3 were selected for our analysis: genotype I (AY744677), genotype

II (AY676352), genotype III (AY099336), and genotype V (AF317645). Again, 25, 38, 41 and 29 potential binding sites were identified in genotypes I, II, III, and V respectively (Fig. 4). Among these, five were shared between all four genotypes.

The capsid, prM, NS4A and 3'UTR regions of DENV-3 are potentially targeted by miRNAs aae-miR-124, aae-miR-281-3p, aae-miR-316, aae-miR-79-3p, and aae-miR-998, respectively (Table 4). The capsid region of DENV-3 has two potential binding sites that could interact with aae-miR-316 and aae-miR-79-3p. aae-miR-316, which also potentially interacts with DENV-1 and DENV-2, has been described above. The prM region of DENV-3 contains a potential binding site for aae-miR-281-3p, which is detected in the cytoplasm of *Ae. aegypti* Aag2 cells and is upregulated in response to *Wolbachia* infection (Hussain et al., 2011). aae-miR-124 potentially targets NS4B, and its expression levels peak during the larval stage (Hu et al., 2015). aae-miR-998 could potentially interact with the 3'UTR of DENV-3, but also has an additional binding site within the NS3 region, which is conserved between genotypes II, III, and V of DENV-3.

DENV-4

Three genotypes of DENV-4 were selected for our analysis: genotype I (AY618992), genotype II (FJ639737), and genotype III (AY618988). We identified 32, 37, and 21 potential miRNA binding sites for genotypes I, II, and III of DENV-4 respectively (Fig. 5). Among them, three potential miRNA binding sites were common to all three genotypes and may be targeted by aae-miR-1, aae-miR-219, and aae-miR-281-3p (Table 5).

Most of the miRNAs that were predicted to interact with DENV-4 sequences have been mentioned above. Among them, aae-miR-281-3p is predicted to target NS2A and aae-miR-1 and aae-miR-219 target a region of NS5. aae-miR-219 is a *Wolbachia*-induced miRNA in Aag2 cells and is expressed continuously from the larval to adult developmental stages (Hu

et al., 2015; Mayoral et al., 2014a).

miRNA targeting the ZIKV genome

ZIKV was first isolated from *Aedes africanus* mosquitoes in 1948 (Dick, 1952) although serological evidence has shown a broader geographic distribution of human infections including North/East Africa, and South/Southeast Asia (Dick, 1953; Hammon et al., 1958; Macnamara, 1954; Pond, 1963; Smithburn, 1952, 1954; Smithburn et al., 1954a; Smithburn et al., 1954b). Since the first human case reported in Nigeria in 1952 (Macnamara, 1954), only 13 cases of mild, febrile illness were reported until the outbreak in the State of Yap (Federated States of Micronesia) in 2007 (Fagbami, 1979; Moore et al., 1975; Olson et al., 1981; Simpson, 1964), where more than 70% of the population were infected (Duffy et al., 2009). Later, cases of ZIKV related Guillain-Barré syndrome were notified during the outbreak in French Polynesia in 2013-2014 (Cao-Lormeau et al., 2014; Oehler et al., 2014). The first ZIKV case reported in America was in 2015 in Bahia (Brazil) (Campos et al., 2015; Zanluca et al., 2015). ZIKV caused a total of 51473 suspected cases and more than 4300 cases of microcephaly in Brazil by March 2016 (Victora et al., 2016; WHO, 2016), and the virus spread to at least in 33 countries or areas in the Americas (Hennessey et al., 2016; WHO, 2016). Related to DENV, ZIKV belongs to the *Flaviviridae* family and the genus *Flavivirus*. The three distinct genotypes East Africa (EA), West Africa (WA), and Asian were likely to be originated in East Africa (Faye et al., 2014; Petersen et al., 2016). ZIKV has a conserved genome with less than 12% divergence at nucleotide level among all virus strains, and even with 99% nucleotide similarity for the strains from the Americas (Haddow et al., 2012). Thus, the interactions between mosquito miRNA and viral RNA could be more relevant than between highly divergent viruses, and might provide a new insight for evaluating the antiviral immunity of mosquitoes against newly emergent viruses. Three genotypes of ZIKV were

selected for analysis: EA (KF268949), WA (JU955592), and Asian (KU365778). According to our results, 30, 22 and 27 potential binding sites were found in EA, WA, and Asian genotypes respectively (Fig. 6).

Five potential miRNA binding sites within the 5'UTR, NS2A, NS5 and 3'UTR were common among all three genotypes (Table 6). aae-miR-286a has two potential binding sites within the 5'UTR and NS5 regions, while aae-miR-286b could potentially target the 5'UTR. Both miRNAs have been detected in mosquito embryos (Li et al., 2009), although were less abundant in adults (Hussain et al., 2011). aae-miR-new-8 is a novel miRNA that has been recently discovered. Its expression could be detected in the very early stages of embryo development and decreases during embryogenesis (Hu et al., 2015). No information is available for the other miRNAs. aae-miR-34-3p (mentioned previously) is predicted to target to the 3'UTR region and NS2A region (involves in virus assembly) of ZIKV respectively.

Selection of miRNAs and thermodynamic analysis

Several miRNAs were predicted to have a low minimum free energy (MFE) indicating a relatively high affinity to form miRNA-vRNA complexes. In *Ae. aegypti*, we set up a MFE cut-off with -20 kcal/mol for evaluating miRNA-vRNA affinity (Etebari et al., 2016; Hu et al., 2015; Hussain and Asgari, 2014; Su et al., 2017). According to this prediction data, we identified eight miRNAs - namely aae-miR-10, aae-miR-11-5p, aar-miR-278-3p, aae-miR-282-5p, aae-miR-286a, aae-miR-286b, aae-miR-316, and aae-miR-34-3p. These could potentially target more than one genotype of each virus species with a MFE below -20 kcal/mol for each miRNA-vRNA complex (Fig. S1). The possible structures formed by miRNA-vRNA interactions were predicted using RNAhybrid. We found that aae-miR11-5p and aae-miR-316 were forecasted to have binding sites within all the virus genomes we choose in this study. Thus, we suggest that these two miRNAs might play a critical role for

viral replication in *Ae. aegypti*.

DISCUSSION

miRanda and TargetSpy are powerful algorithms for predicting the miRNA potential binding sites on target sequences. miRanda is one of the most extensively used miRNA target prediction tools and was applied originally for identifying miRNA binding sites in *Drosophila*. The miRanda algorithm works in three phases. Firstly, the complementarity matches between the input miRNAs and RNA sequences were identified based on dynamic programming algorithm alignment. Secondly, a thermodynamic calculation was made to rule out the matches with a MFE value above the threshold. Finally, the remaining results were filtered by checking the sequence conservation with *D. pseudoobscura* and *An. gambiae*. In addition, miRanda can also weigh the matches between the 2nd to the 8th nucleotide from the 5' arm of miRNA (seed-region), to evaluate the potential for miRNA-vRNA interaction to form (Enright et al., 2003). For a more stringent selection, the same database was analyzed with another algorithm, TargetSpy. Unlike miRanda, TargetSpy is an algorithm based on machine learning and automatic feature selection with a broad spectrum of compositional, structural, and base pairing of each miRNA to the targeting sequence (Sturm et al., 2010). Using a combination of miRanda and TargetSpy, we obtained consensus results which should be more robust for detecting miRNAs involved in viral replication. Moreover, a more reliable miRNA-vRNA interaction was examined by comparing these results with the structure information predicted by RNAhybrid.

Several potential miRNA binding sites were found for all three arboviruses, with conservation within each genotypes/lineage or even serotype for DENV (Fig. 7). Although many of the miRNA-vRNA complexes can be identified based on the relatively low MFE value, it remains necessary to evaluate the role of each potential miRNA on virus replication,

and the subsequent reduction or facilitation of virus replication has to be demonstrated experimentally. Although there is still a lack of miRNA targetome information available for *Ae. aegypti,* the critical roles of cellular miRNAs in host viral immunity have received more attention elsewhere with conformation of mammalian host miRNA-vRNA interactions c(Scheel et al., 2016; Trobaugh and Klimstra, 2017). The miRNA-vRNA interactions could regulate virus replication through different effects and thus lead to distinct outcomes, such as an inhibition caused by miRNA mediated gene silencing, or through enhancing the virus replication as a consequence of miRNA mediated viral RNA stabilization. Inhibitor studies should shed light on individual miRNA-vRNA interactions. However, miRNA-vRNA interactions can also be proved by techniques such as Argonaute-crosslinking immunoprecipitation (AGO-CLIP) and mutagenic analysis of virus genomes is possible for many of the arboviruses investigated here. This study provides an analysis and rationale for selecting miRNAs and miRNA target sequences in genomes and should underpin such studies.

METHODS

Identification strategies for miRNA and v-RNA interactions

Key, human pathogenic arboviruses (flaviviruses, DENV1-4 and ZIKV, and the alphavirus, CHIKV) were chosen for analyzing the relationship between miRNAs and viral genomes (vRNA). The genome sequences for each virus were collected from the virus database Virus Pathogen Resource (ViPR, www.ViPRbrc.org) (Pickett et al., 2012a; Pickett et al., 2012b), while the miRNA sequences of *Ae. aegypti* were retrieved from the miRNA database, miRbase, and published results of small RNA sequencing (Hu et al., 2015). Predictions of miRNA-vRNA interactions were carried out mainly using miRanda software

(Enright et al., 2003) and coordination with TargetSpy (Sturm et al., 2010) via the online tool sRNAtoolbox (Rueda et al., 2015) with default settings (http://bioinfo5.ugr.es/srnatoolbox). The consensus binding sites predicted by both software were extracted by BEDtools (Quinlan and Hall, 2010). Only the prediction sites shared by the two prediction algorithms were chosen for further case study (see below) and evaluation of the affinity of each miRNA-vRNA complex. In addition, the structures of these complexes were predicted using the tool RNAhybrid (Kruger and Rehmsmeier, 2006) via BiBiServ2 (https://bibiserv2.cebitec.uni-bielefeld.de) (Fig. 8).

Flowchart validation

A mosquito endogenous mRNA-miRNA interaction was used to validate the workflow for predicting miRNA binding sites we have adopted in this study. The transcript of *Ae. aegypti* glutamate semialdehyde dehydrogenase (GSD, AAEL006834) was shown to be regulated by aae-miR-275-3p in the 3'UTR (Zhang et al., 2017). The GDS transcript and the miRNA database of *Ae. aegypti* were used as input data. Using the default setting, a total of two binding sites were predicted by the software miRanda at the position 362-381 and 2682-2700 in the coding sequence and 3'UTR, respectively. However, only one binding site on the 3'UTR was filtered by TargetSpy which matches with aae-miR-275-3p identified earlier (Zhang et al., 2017).

ACKNOWLEDGEMENTS

This study was partly supported by the European Union's Horizon 2020 Research and innovation program under "ZIKALLIANCE" Grant Agreement n° 734548) (ABF, AK), the French Government's Investissement d'Avenir program, Laboratoire d'Excellence "Integrative Biology of Emerging Infectious Diseases, IBEID" (grant n°ANR-10-LABX-62-IBEID)

(ABF), and the UK Medical Research Council [MC_UU_12014] (AK). PSY was supported by the Pasteur-Paris University (PPU) program. We thank Dr. Catherine DAUGA, Mr. Adrien PAIN, and Mr. Michele MARCONCINI for advice.

Table 1 List of the nine most common miRNA binding sites in the CHIKV genome.

Groups	Number of prediction binding sites	miRNAs	Predicted binding sites
(i) Asian ECSA IOL	6	aae-miR-263a-5p aae-miR-279 aae-miR-305-5p aae-miR-34-3p aae-miR-34-3p aae-miR-996	nsP4 E1 nsP3 nsP1 nsP1 E1
(ii) WA ECSA IOL	3	aae-miR-285 aae-miR-989 aae-miR-iab-4-5p	E1 capsid 3'UTR

Table 2 List of the nine most common miRNA binding sites in the DENV-1 genome.

Genotypes	Number of prediction binding sites	miRNAs	Predicted binding sites
I II III IV V	2	aae-miR-1 aae-miR-282-5p	NS3 Capsid
I II III V	3	aae-miR-316-2 aae-miR-92a-3p aae-miR-92b-3p	NS5 NS5 NS5
I II IV V	1	aae-miR-11-5p	NS4B
I III IV V	3	aae-miR-263a-3p aae-miR-316 aae-miR-998	3'UTR Capsid 3'UTR

Table 3 List of the six most common miRNA binding sites in then DENV-2 genome.

Genotypes	Number of prediction binding sites	miRNAs	Predicted binding sites

Asian-I Asian-II Asian American American Cosmopolitan	2	aae-miR-316 aae-miR-9c-5p	Capsid NS5
Asian-I Asian-II Asian American Cosmopolitan	2	aae-miR-281-3p aae-miR-998	NS2B 3’UTR
Asian-I Asian American American Cosmopolitan	1	aae-miR-263a-3p	3’UTR
Asian-II Asian American American Cosmopolitan	1	aae-miR-315-5p	NS5

Table 4 List of the five most common miRNA binding sites in the DENV-3 genome.

Genotypes	Number of prediction binding sites	miRNAs	Predicted binding sites
I II III V	5	aae-miR-124 aae-miR-281-3p aae-miR-316 aae-miR-79-3p aae-miR-998	NS4A prM capsid capsid 3’UTR

Table 5 List of the three most common miRNA binding sites in the DENV-4 genome.

Genotypes	Number of prediction binding sites	miRNAs	Predicted binding sites
I II III	3	aae-miR-1 aae-miR-281-3p aae-miR-219	NS5 NS2A NS5

Table 6 List of the five most common miRNA binding sites in ZIKV sequences.

Genotypes	Number of prediction binding sites	miRNAs	Predicted binding sites
East African West African Asian	5	aae-miR-286a aae-miR-286a aae-miR-286b aae-miR-34-3p aae-miR-new8	5'UTR NS5 5'UTR 3'UTR NS2A

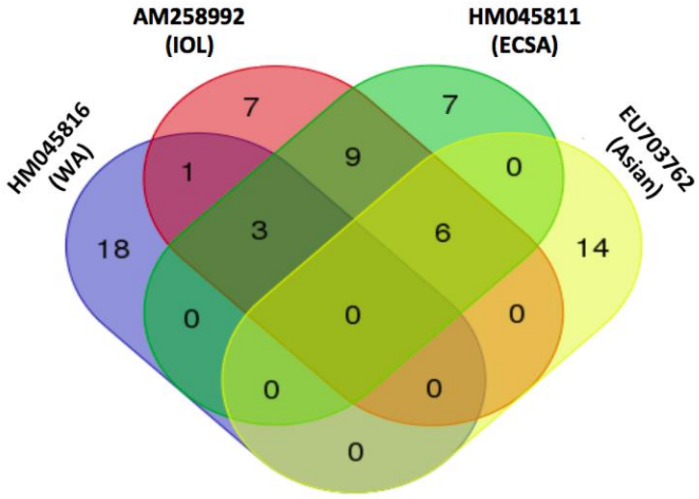


FIG 1 Venn-diagram presentation of common prediction binding sites on four genotypes of CHIKV for *Ae. aegypti* miRNAs. AM258992, Indian Ocean Lineage, IOL; HM045816, West African, WA; EU703762, Asian; HM045811, East/Central/South African, ECSA. The six miRNAs binding sites common to the group Asian/ECSA/ IOL and the group WA/ECSA/IOL were listed in Table 1.

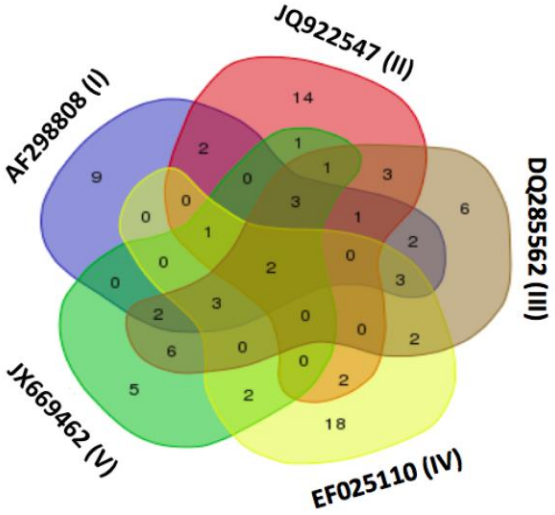


FIG 2 Venn-diagram of the predicted common binding sites on five genotypes of DENV-1 for *Ae. aegypti* miRNAs. AF298808, genotype I; JQ922547, genotype II; DQ285562, genotype III; EF025110, genotype IV; JX669462, genotype V. The nine miRNAs binding sites that are common to at least four genotypes of DENV-1 were listed in Table 2.

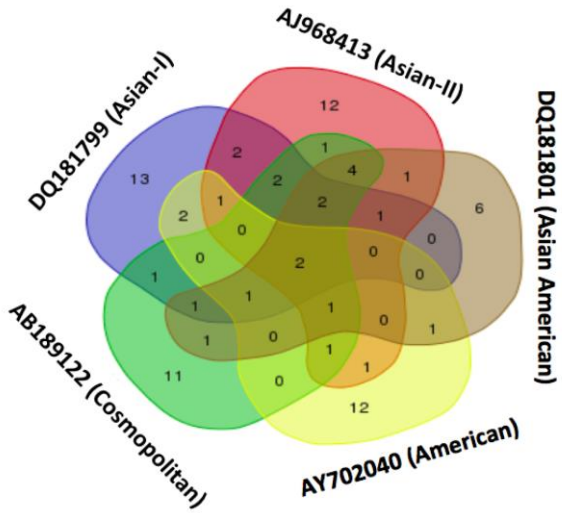


FIG 3 Venn-diagram of the predicted common binding sites for five genotypes of DENV-2 for *Ae. aegypti* miRNAs. DQ181799, Asian-I; AJ968413, Asian-II; DQ181801, Asian American; AY702040, American; AB189122, Cosmopolitan. The nine miRNAs binding sites common to at least four genotypes of DENV-2 were listed in Table 3.

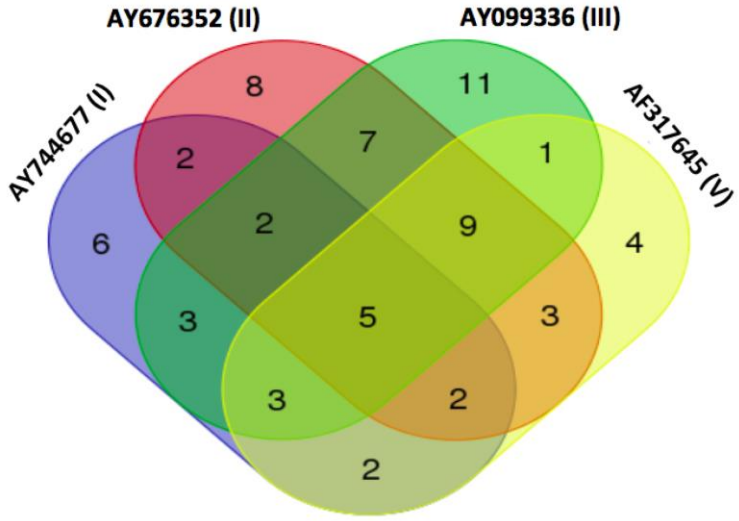


FIG 4 Venn-diagram of the predicted common binding sites in four genotypes of DENV-3 for *Ae. aegypti* miRNAs. AY744677, genotype I; AY676352, genotype II; AY099336, genotype III; AF317645, genotype V. The five miRNAs binding sites that are common to all four genotypes of DENV-3 were listed in Table 4.

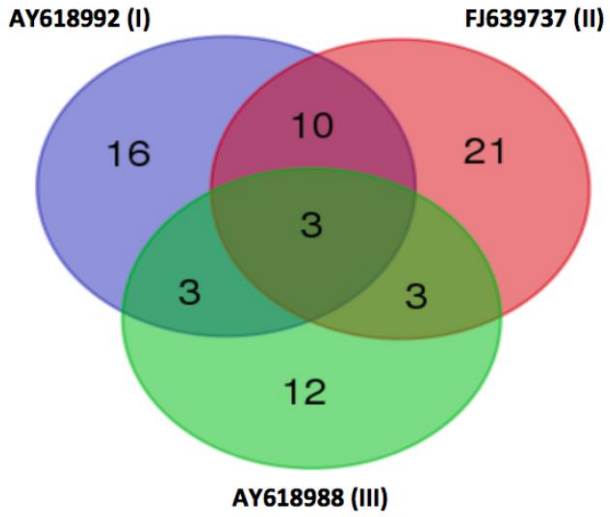


FIG 5 Venn-diagram of the predicted common binding sites in three genotypes of DENV-4 for *Ae. aegypti* miRNAs. AY618992, genotype I; FJ639737, genotype II; AY618988, genotype III. The five miRNAs binding sites that are common to all three genotypes of DENV-4 were listed in Table 5.

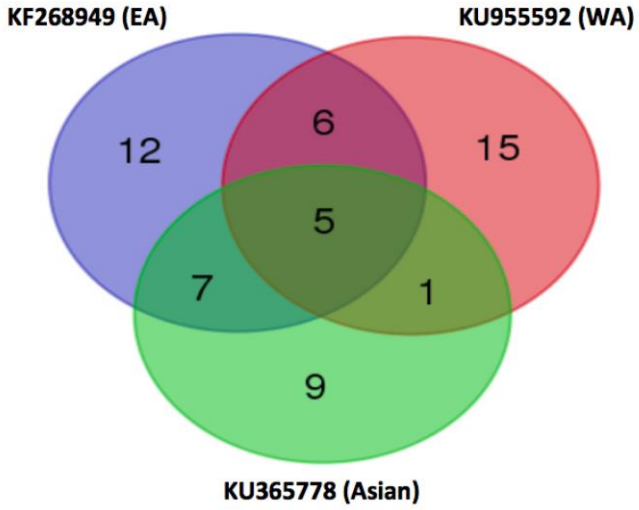


FIG 6 Venn-diagram of the predicted common binding sites in three genotypes of ZIKV for *Ae. aegypti* miRNAs. KF268949, East African, EA; KU955592, West African, WA; KU365778, Asian. The five miRNAs binding sites common to all genotypes of ZIKV were listed in Table 6.

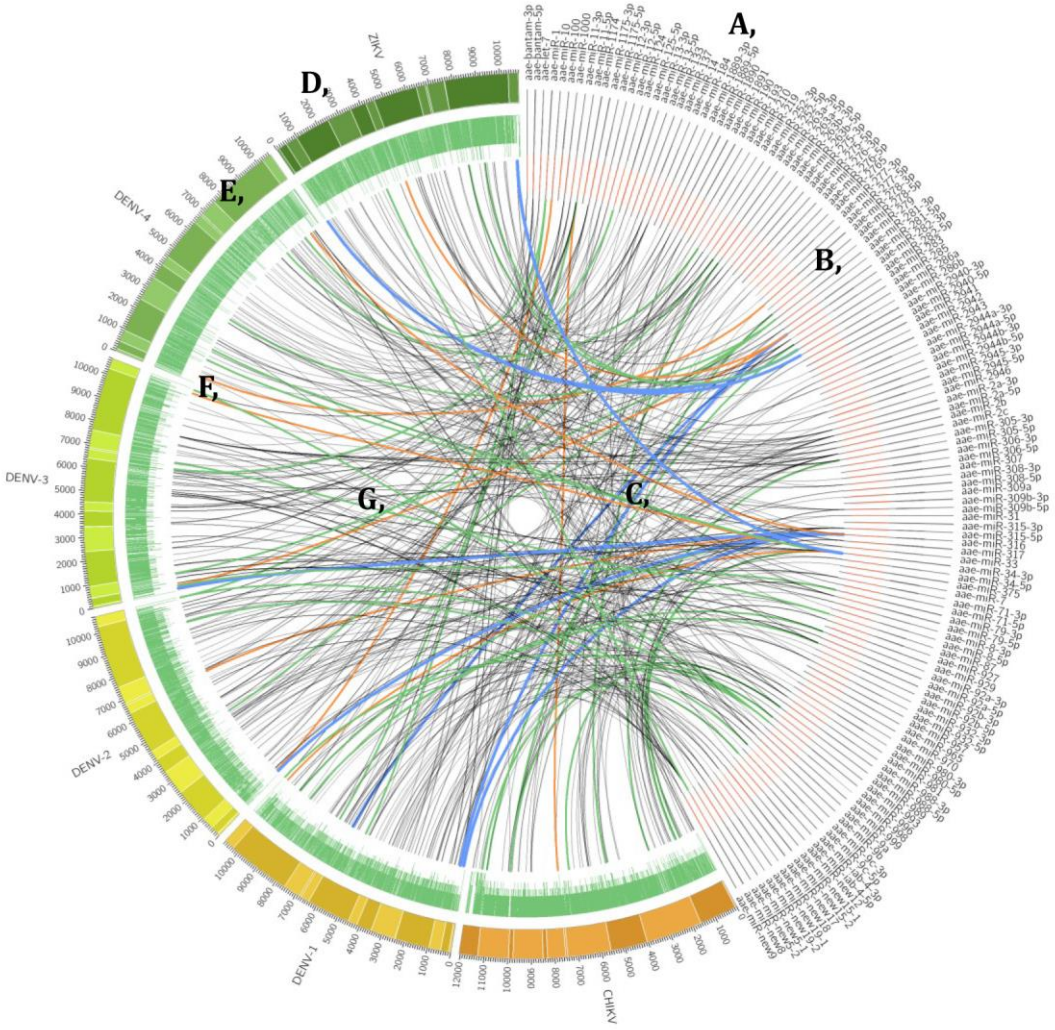


FIG 7 The interactome of *Ae. aegypti* miRNAs with DENV, ZIKV and CHIKV genomes as predicted by miRanda and TargetSpy. (A) miRNAs of *Ae. aegypti*; (**B)** miRNA and its seed region (seed region in red); (**C)**, links between miRNAs and potential binding regions on the viruses; (**D**) genomes of arboviruses; (**E)** dark and light areas represent the protein coding regions in each virus; **(F**) conservation scores of virus among all the genotypes; green links, the miRNA binding site that shared among most of genotypes discussed in this study; orange links, the miRNA-vRNA with low energy; blue links, the miRNA-vRNA with low energy and is shared among most arboviruses; black links, remaining miRNA-vRNA interactions.

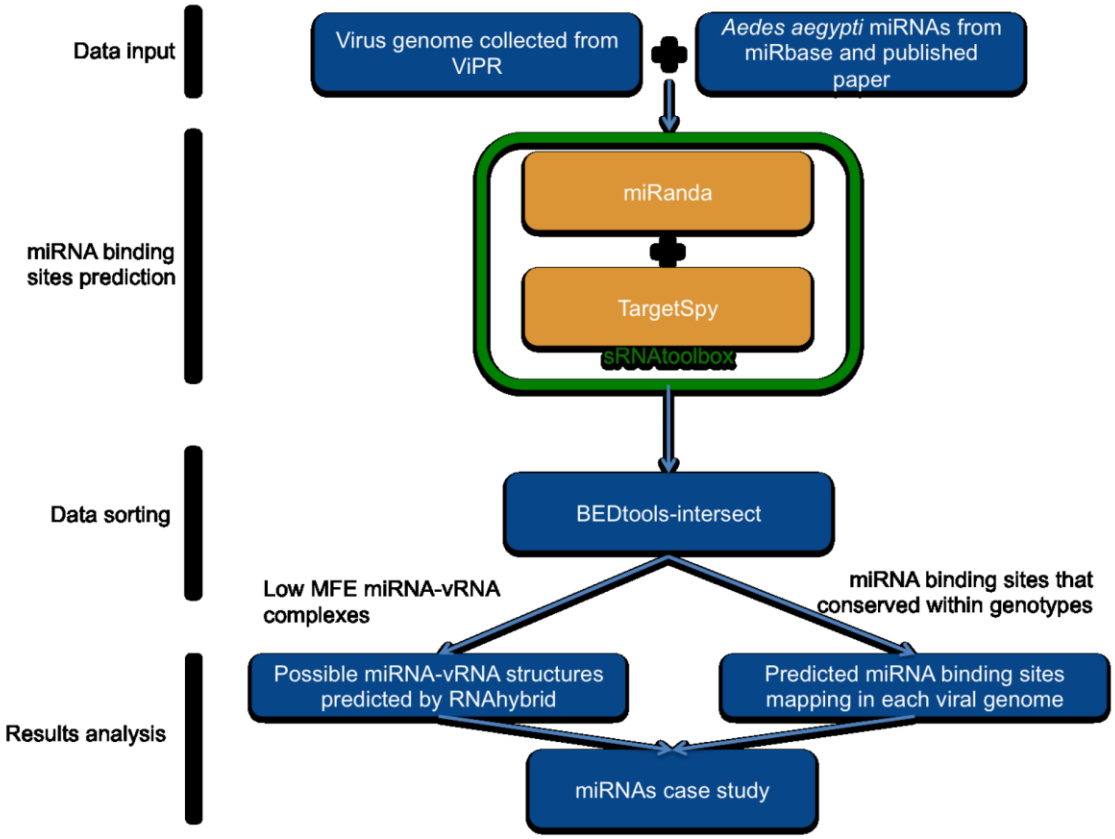


FIG 8 Flowchart for the detection of *Ae. aegypti* miRNA binding sites in arbovirus genomes. The flowchart comprises four major steps: data input, miRNA binding site prediction, data sorting, and result analysis.

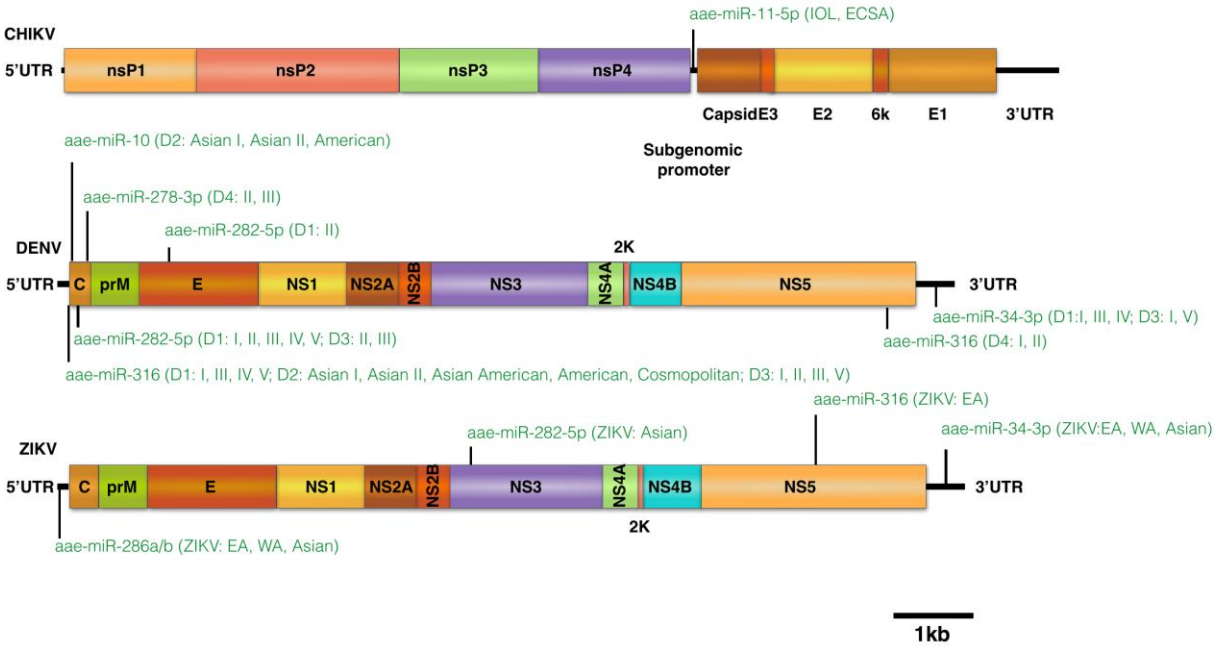


FIG S1 Potentially true miRNA-vRNA interaction predicted in this study. The scheme shows the most influence miRNA-vRNA interactions predicted in this study. Each miRNA could targets to multiple viruses and has a relatively low MFE (around -20 kcal/mol).

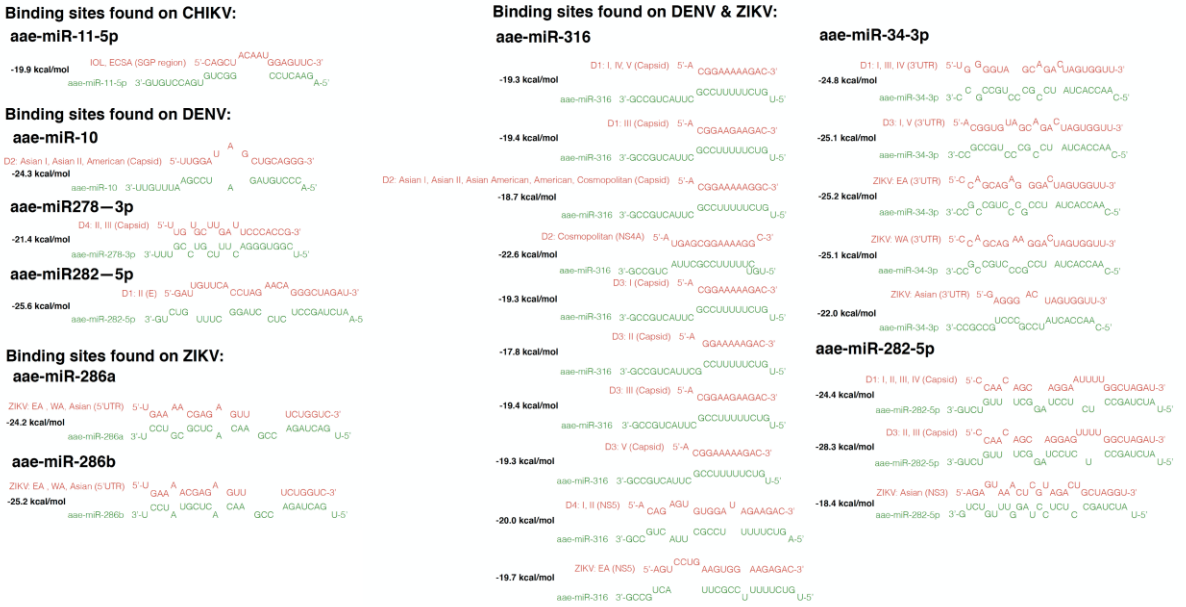


FIG S2 The possible structures of each low MFE miRNA-vRNA interaction that predicted by RNAhybrid. The structures of seven low MFE miRNAs of *Ae. aegypti* were potentially interact with CHIKV, DENV, and ZIKV. Among them, the possible binding sites for aae-miR-316 and aae-34-3p, and aae-miR-282-5p could be commonly found on both DENV and ZIKV.

CHAPTER 2: ALTERNATIVES FOR ARBOVIRAL DISEASES CONTROL

1. Arboviral diseases and burdens

As the consequence of climate change and growing human activities, the impacts caused by mosquito-borne arbovirus diseases have become a global issue. Newly and re-emergent arboviral diseases cause millions of infections and billions of costs annually for prevention and control (Fredericks and Fernandez-Sesma, 2014; Shepard et al., 2016). Consequently to the climate change and globalized exchanges (travelers and goods), the mosquito-borne diseases have been introduced into areas considered as non-endemic (Rezza, 2014), *e.g.* DENV and CHIKV in Europe (Tomasello and Schlagenhauf, 2013) and the recent Zika pandemic (Petersen et al., 2016), causing millions of infections and significant health and economic burden for countries in financial difficulties (Stanaway et al., 2016)

2. Using of insecticide for arboviral diseases control

Mosquitoes are the site where is constituted a collection of different viral variants (Coffey et al., 2014; Stapleford et al., 2014). Until now, no positive treatment or more broadly, effective vaccines are available. Thus, controlling the mosquito vector is essential for mosquito-borne diseases control. The most extensively used approach is the application of insecticides. The first widely used insecticide, dichlorodiphenyltrichloroethane (DDT) was synthesized in 1874, although the insecticide activity was only discovered in 1939 and commercialized shortly after in 1943 for agriculture and health usages. During WWII, soldiers started using DDT to prevent typhus fever and malaria (U.S.E.P.A., 1975). Meanwhile, a WHO leading malaria eradication program has achieved a great success by using DDT in 1955, thereby, DDT has become a major insecticide for pest control. However, the resurgences of malaria and the environmental damages caused by DDT were raised later in 1960s (Najera et al., 2011). The insecticide affects the avian calcium metabolism which causes thinning of the eggshell and high rates of breakage during development (Hamlin and

Guillette, 2010). Most organochlorine compounds were then banned after the 1970s. Since then, more and more compounds with insecticidal properties were synthesized. Until present, the major insecticides could be divided into several groups: organochlorines, organophosphates, carbamates, pyrethroid,s and neonicotinoid insecticides (Isman, 2008).

However, insecticide resistance still remains an issue; the increasing cost for control and decreasing efficiency as a consequence of insecticide resistance lead to a vicious cycle (Vontas et al., 2012). Besides, accumulating evidence showed the effects of insecticides on the non-target species leading to unpredictable ecological or even economical impacts. For instance, neonicotinoid insecticides (NNIs) were proved to be involved in honeybee population decline. Even though neonicotinoids are systemic insecticides for agricultural application, the pollen from non-target plants was suggested to be source of exposure to NNIs (Iwasa et al., 2004; Tsvetkov et al., 2017). Therefore, a controllable, species-specific and environmental friendly biological alternatives for mosquito vectors control has become an emergency.

3. Genetically modified-based control strategy-population reduction and replacement

With the increasing availability of mosquito genomic information, various genetic tools are available for mosquito genome manipulations. Many genetically-engineered mosquitoes have been developed in the past two decades. Genetic control strategies could be roughly divided into two categories: (i) reducing the population size of vector mosquitoes and (ii) replacing the wild mosquitoes by a refractory population to reduce the probably of

transmission. Both approaches are highly species-specific, relatively predictable, and environmental friendly compared to insecticides (Alphey, 2014).

3.1 Sterile Insect Technique

The sterile insect technique (SIT) was the first genetic control strategy which relies on the release of large amount of radio-sterilized male mosquitoes to reduce the reproductive ability of wild population through mating with wild females (Benedict and Robinson, 2003). However, attempts of large field releases for mosquito control revealed the drawbacks of SIT (Alphey et al., 2010). The poor mating performance of irradiated male mosquitoes (Oliva et al., 2012), the high mortality during transportation between the site of irradiated males production and the target areas for releasing (Bellini et al., 2013) and the high demands of human power for mosquito sexing to discard female mosquitoes, largely increase the cost of mosquito SIT program (Papathanos et al., 2009).

Mosquitoes sexing is a major issue for releasing sterile mosquitoes, although the sterile female mosquitoes would not significantly affect the reduction efficiency of the mosquito population size, the biting nature of female mosquitoes can potentially increase the risk of pathogen transmission. Thus, the genetic sexing of mosquitoes is developed with genetically engineering technique, for example, a selective lethality to eliminate female mosquitoes from the population or a sex-specific expression of an appropriate marker, e.g. fluorescent proteins or pigments, to distinguish the mosquito sex and facilitate sex separation (Smith et al., 2007).

3.2 Release of insects carrying a dominant lethal

Based on SIT, a genetically-engineered self-limiting approach, the releasing of insects carrying a dominant lethal (RIDL) was developed to overcome the disadvantages resulted by mosquitoes irradiation (Gabrieli et al., 2014; Phuc et al., 2007). Compared to SIT, RIDL strategy decreased the costs by reducing the damage and mortality during radiation (Alphey et al., 2010). The expression of a repressible lethal phenotype of RIDL mosquitoes reduces the population size after mating with wild-type mosquitoes (Thomas et al., 2000). The lethal gene is regulated under the tetracycline operator (tetO) system which initiates the expression of tetracycline-repressible transactivator fusion protein (tTA). Under regular conditions, the expression of tTA that also acts as a toxin is initiated by the tetO, and a positive feedback loop is formed as a result of tTA expression. As tTA accumulates, the progeny is killed due to tTA toxicity (Gabrieli et al., 2014). In laboratory conditions, the expression of tTA is neutralized by tetracycline provided, and subsequently abolishes tTA expression (Lewandoski, 2001). For mass rearing, the tetracycline could be added in the diet to maintain a normal metabolism and development (Heinrich and Scott, 2000). The supply of tetracycline is interrupted after the mosquitoes are released and the positive feedback loop is initiated leading to kill the progeny in the field.

The control mechanism of RIDL mosquitoes is very similar to traditional SIT with the progeny killing and the lethality of non-transmitting male mosquitoes that still able to pass the genetic traits to next generation. Therefore, multiple releases if RIDL mosquitoes are essential to reach population reduction.

To improve the population reduction efficiency of RIDL mosquitoes, a female-sterile RIDL (fsRIDL) system was developed by replacing the tetO with a female-specific promoter

for tTA expression. It results in female-specific lethality and consequently, reduces the cost of RIDL mosquitoes control strategy. Moreover, the fsRIDL traits could be passed to next generation by the male mosquitoes even without the presence of tetracycline (Alphey, 2014; Harris et al., 2012; Harris et al., 2011). The subsistence of fsRIDL male mosquitoes provide a notable advantage for mosquitoes population control.

A variant of fsRIDL, female flightless mosquitoes were also developed to ensure the inability of female mosquitoes for transmission. The flightless mosquito shares all the advantages of the RIDL system, but could be released at any life stages during development (Fu et al., 2010; Labbe et al., 2012). Combining with *AeAct-4* promoter (which activates in the indirect flight muscles) and a female-specific alternative splicing intron, a mature tTA is expressed in female muscle cells without tetracycline. The muscle cells are subsequently disrupted and lead to the loss of flight ability. This phenotype is potentially lethal, as flight is essential for host seeking and mating ability. Whereas male mosquitoes have no significant impact on flight ability due to the insufficient activity of female-specific alternative intron, the female flightless traits could be sustained among the target population (Wise de Valdez et al., 2011).

3.3 Field trials of RIDL mosquitoes

Caged and semi-field trials of RIDL mosquitoes have demonstrated the efficiency and potential of RIDL for controlling wild populations (Phuc et al., 2007; Wise de Valdez et al., 2011). Thereby, the RIDL mosquito strain, OX513A was further subjected to open field testing in the Cayman Island (Harris et al., 2012; Harris et al., 2011), Malaysia (Lacroix et al., 2012), Brazil (Carvalho et al., 2015), and Panama (Gorman et al., 2016). A permission for a

trial in Key West, Florida, US, was under reviewing by the Food and Drug Administration (FDA). Except the trial in Malaysia that released six thousands of OX513A males in two weeks and ended before any outcome could be determined (Lacroix et al., 2012), the other trials have shown a great success for suppressing *Ae. aegypti* population. In Cayman, a 82% of suppression was achieved after releasing of approximately 3.3 million OX513A males in 23 weeks whereas mosquito suppression rate was more than 90% in Brazil; a total of 17.4 millions of OX513A males were released in 17 months (Carvalho et al., 2015). More than 90% of mosquito population size was reduced in Panama with a total of 4.3 million of releases in 27 weeks (Gorman et al., 2016).

The RIDL mosquito control strategy was strongly encouraged by the successful trials undergone in countries or areas submitted to the threat of arboviral diseases transmitted by *Ae. aegypti* or *Ae. albopictus.* It also helps local communities in understanding this alternative arboviral diseases control strategy. Thus, following the dengue outbreak in Florida, US in 2010 (Graham et al., 2011; Radke et al., 2012), the local administration, Florida Keys Mosquito Control District has considered more carefully the RIDL mosquitoes, and obtained the approval after a final finding of no significant impact (FONSI) and a final environmental assessment (EA) on March 2017. According to the survey in June 2012 examining the community awareness and support for releasing the genetically-modified mosquitoes to suppress the wild population, more than half of the 400 residents in Monroe County (51.1%) have heard of the proposed releasing project before the survey. Among them, 57% were in favor, 25.1% neutral and only 17.9% tending to oppose (Ernst et al., 2015), indicating that the GM-based mosquitoes control strategy has become more acceptable after the successful trials obtained in other countries.

3.4 Others mosquito population reduction approaches

Except the RIDL-based population reduction strategy, a similar outcome could be obtained by modifying the sexual phenotype of mosquitoes that leads to a mechanical isolation and subsequently, to a reduction of target population size. For *Ae. aegypti*, the male sexual phenotype is controlled by a dominant male-determining factor (M factor) located on the Y chromosome (Glichrist and Haldane, 1947; Hall et al., 2014), which was proven to be a master factor in *Ae. aegypti* male determination, however the detailed mechanism was not clear. Nevertheless, a M factor related gene, *Nix*, was recently shown to exhibit a male determining activity in *Ae. aegypti* (Hall et al., 2015). By continual ectopic expression of *Nix* in female genital organs, females develop sexually dimorphic traits with male testes and accessory glands (Hall et al., 2015). Thus, the mating ability is abolished due to the mechanical isolation between the modified and the wild-type population, leading to a reduction of population size.

Moreover, the orthologue of *Drosophila* sex behavior genes, *Fruitless* (*fru*) and *Doublesex* (*dsx*) were also identified in *Ae. aegypti* (Salvemini et al., 2013; Salvemini et al., 2011). The female isoform of both genes could be detected in *Nix* knockout male mosquitoes although the *dsx* knockdown experiment has shown no blood feeding or mating defects. In *dsx*-deficient female mosquitoes, the reproduction abilities were decreased; the size of ovaries and ovarioles and the ovariole number were lower (Mysore et al., 2015). In addition, the olfactory system was also disrupted in *dsx*-deficient female mosquitoes which exhibited a reduced length and sensilla of female antenna and maxillary palps. This leads to an odorant receptor expression in the antenna (Mysore et al., 2015) without demonstrating any consequences on the host seeking ability of mosquitoes. Converting the female mosquitoes

into harmless males by *Nix* related genes in *Ae. aegypti* is considered a potential tool that could be applied for population reduction of mosquitoes.

4. Effector gene and population replacement

Although the population reduction is the most widely used strategy for mosquito control, a concern is raised by eliminating certain species from the environment. It can cause ecological imbalance (food chain), and subsequently; facilitate the secondary pest re-emergence (Beech et al., 2009). For instance, in nature, *Ae. aegypti* and *Ae. albopictus* share the same larval habitats, and their co-existence was frequently reported (Braks et al., 2004; Juliano et al., 2004; Simard et al., 2005). The oscillation of both population size in the same habitat have been observed in many countries after the introduction of *Ae. albopictus*. In some cases, *Ae. albopictus* has a better population growth rate which was likely to be the dominant species in competitive interactions with *Ae. aegypti* (Barrera, 1996; Braks et al., 2004; Juliano et al., 2004). This suggests that if one species is eliminated, the other might take over the ecological niche, and become a re-emerging secondary pest.

Besides, *Aedes spp.* are considered important pollinators for many subarctic plants (*Dryas integrifolia*) (Kevan, 1972), even the only pollinators for *Platanthera obtusata* (Foster, 1995). Thus, instead of eliminating the entire population, replacing the wild population by refractory mosquitoes could minimize the ecological impacts. It keeps the mosquito population in its ecological niche while reducing the risk of transmission. The population replacement strategy combining a gene drive system and a pathogen effector gene was rapidly developed in the past decade. Several gene drive systems, including medea, toxin-antidote underdominant, and homing based gene drive system were developed and showed high

efficiencies of population replacement. Whereas the development of effector genes against abroviruses were mainly focused on RNAi systems, few strategies were related to immune genes and antiviral ribozymes.

4.1 Gene drive systems

To facilitate the introduction of the desired traits into the target population, synthetic gene drive systems that increase the inheritance, were rapidly developed in the past decade (Champer et al., 2016; Sinkins and Gould, 2006). Gene drive systems are not following the Mendelian's rule and significantly, increase the inheritance probability of desired traits among the target population (Hammond et al., 2016). According to the outcomes of different gene drive systems, it could be classified into: (i) a modification drive that spreads the desired traits through target duplication or ii) a suppression drive which reduces or eliminates the target population (Champer et al., 2016). Medea, toxin-antidote underdominance, and homing-based gene drive system belong to the modification drive. Except the homing-based gene drive system, the modification drive is composed by an independent killing and rescuing system, the progeny without rescuing system will be killed, whereas the progeny that are able to produce the antidote could survive and pass the traits to the next generation. By using a maternal promoter to express the toxin, medea (maternal effect dominant embryonic arrest) kills the naïve progeny at embryo stage and rescues the progeny that inherited with the same medea element to replace the target population in few generations (Akbari et al., 2014; Akbari et al., 2013b; Chen et al., 2007). In contrast, the underdominance system uses two different alleles for toxin and antidote expression. The progeny is killed in the absence of the corresponding allele responsible for antidote expression and is able to spread the desired traits within the target population (Champer et al., 2016; Edgington and Alphey, 2017). The population replacement could be achieved by killing the progeny that has not inherited the

same gene drive element. Through the kill-and-rescue based population replacement approach, only a moderate rate of spread could be expected due to the population reduction phase caused by the killing at the initial step of replacement (Akbari et al., 2013b).

On the contrary, the homing-based gene drive system is able to spread the gene drive element without killing the progeny, through a CRISPR/Cas9-mediated cut-and-copy machinery (Champer et al., 2016; Gantz and Bier, 2015; Gantz et al., 2015; Hammond et al., 2016). The site-specific endonuclease activity and DNA homology-directed repair (HDR) mechanism have provided a novel approach to rapidly increase the gene frequency among the target population. By encoding the CRISPR/Cas9 system that target to the flanking sequence of the gene drive system in the chromosome, the flanking sequence-specific Cas9 endonuclease breaks the target chromosome upon initiation. The HDR DNA repairing mechanism is triggered in response to DNA damage which uses the gene drive element containing sequence as the template for DNA repairing. Thus, a repaired sequence containing the homing-based gene drive element is synthesized and the frequency of desired traits in the target population is therefore rapidly increased (Gantz and Bier, 2015; Hammond et al., 2016). Although concerns of off-target effect and Cas9 immune to drive (ITD) allele were raised (Cho et al., 2014; Drury et al., 2017), CRISPR/Cas9-based gene drive is currently the most effective gene drive system for population replacement pest control strategy.

4.2 Antiviral effector genes

Combined with gene drive system, the effector gene that triggers the mosquito antiviral immunity is another essential element to inhibit virus transmission among the target population. Several components involved in mosquito innate immunity were used as an anti-pathogen effector gene for suppressing arbovirus replication in genetically-engineered

mosquitoes (Jupatanakul et al., 2017; Kokoza et al., 2000; Kokoza et al., 2010). Under the control of *Aedes* vitellogenin promoter, two antimicrobial peptide (AMP) of the Toll pathway, Cecropin A and Defensin A have shown to exhibit an antimicrobial activity that suppresses the proliferation of Gram-positive bacteria *Staphylococcus aureus* and *Staphylococcus epidermidis*, Gram-negative bacteria *Pseudomonas aeruginosa*, and the parasite *Plasmodium* in genetically-engineered *Ae. aegypti* (Kokoza et al., 2010). Moreover, the antiviral activity of the two AMPs was demonstrated later in DENV-2 challenged mosquitoes; the viral load in the midgut and fat body of genetically-engineered mosquitoes were significant lower than in wild-type *Ae. aegypti* (Pan et al., 2012).

4.3 Genes that trigger anti-microbial immune responses

Except the components from Toll and Imd pathways, the components of JAK/STAT pathway in *Ae. aegypti* were also involved as an antiviral effector in genetically-engineered mosquitoes (Jupatanakul et al., 2017). Through the recognition of unpaired ligand Upd, the receptor of JAK/STAT Domeless (Dome) and the Janus kinase (Hop), activate the downstream signaling and expression of immune genes (Cheng et al., 2016). The two receptor-related components are used as the antiviral effector to reduce arbovirus replication in genetically-engineered mosquitoes. Under the regulation of a blood meal-inducible and fat body-specific vitellogenin in *Ae. aegypti*, the genetically-engineered mosquitoes have shown an increased resistance to DENV-2 and DENV-4 infection in the midgut and salivary glands (Jupatanakul et al., 2017).

The use of components in mosquito innate immune pathway to induce a systemic antiviral response, could potentially achieve a stronger or more extensively effective antiviral activity. However, not only immunity, several components involved in mosquito immune

pathways have proven to participate in maintaining mosquito homeostasis, cell proliferation, and development (Halfon and Keshishian, 1998; Hoffmann and Reichhart, 2002); the ectopic expression of immune related genes could be a fitness burden for mosquitoes. Besides, the enhanced innate immunity might also alter the mosquito microbiome that plays a critical role for antiviral immunity, especially in certain tissue, e.g. midgut and salivary glands (Ramirez et al., 2012).

4.4 Antiviral RNAi

To minimize the non-intended impacts on mosquito microbiome and biological processes while maintaining the high antiviral efficiency, the highly efficient and specific RNAi machinery has been applied as a reliable system against RNA arboviruses in genetically-engineered mosquitoes. Until present, several attempts in using long dsRNA (ldsRNA) as an effector to induce the antiviral responses were done with tissue specific or ubiquitious expression systems (Adelman et al., 2002b; Franz et al., 2006; Khoo et al., 2013; Mathur et al., 2010). The mosquitoes or drosophila Ubiquitious promoter and Carboxypeptidase promoter that respectively activate ubiquitiously or tissue-specifically, were applied to demonstrate the antiviral efficiency in genetically-engineered *Ae. aegypti*. Later on, a female salivary gland specific promoter was developed for expressing the antiviral ldsRNA, to suppress the virus replication in the salivary glands and attempt to reduce the risk of transmission (Mathur et al., 2010). Although only a weak antiviral phenotype was observed in the mosquitoes mentioned above, a genetically-engineered *Ae. aegypti* with a strong viral suppression phenotype was constructed in 2006. An anti-DENV-2 ldsRNA was driven by Carboxypeptidase A promoter, and the viral load in the midgut and salivary glands were significantly reduced in the genetically-engineered mosquitoes with subsequent blockage of transmission (Franz et al., 2006). However, a total suppression of viral infection in

mosquitoes has not been achieved yet. The highly efficient and relatively low fitness impact of RNAi-based genetically-engineered mosquitoes could serve as a-proof-of-concept for using the potential of mosquito RNAi machinery to increase resistance to arboviruses.

4.5 Antiviral ribozymes

In addition to RNAi-based control approaches, a ribozyme-based antiviral effector for reducing arbovirus virus replication was developed (Mishra et al., 2016; Nawtaisong et al., 2009). The catalytic activity of ribozymes is capable of guiding and cleaving target viral RNA spontaneously. Therefore, the viral replication could be reduced in mosquitoes. With a different silencing mechanism than antiviral siRNA, the antiviral ribozymes are able to target multiple sites which are short sequences (15-16 nt) necessary for RNA cleavage. No host factors are needed and non temperature sensitive features are requested making antiviral ribozyme a powerful effector against viruses. A hammerhead ribozyme has been demonstrated to inhibit CHIKV replication in genetically-engineered *Ae. aegypti*. The antiviral ribozymes were expressed under the *Ae. aegypti* t-RNA^{val} Pol III promoter. The ubiquitously expressing antiviral ribozymes significantly reduced infection, dissemination, and transmission efficiencies in genetically-engineered mosquitoes. Therefore it provides the proof that the antiviral hammerhead ribozymes are potential effectors for population replacement strategies.

4.6 Behavior alteration

Instead of enhancing the antiviral immunity, knocking-out mosquito genes which are related to the sensory system, can reduce the virus transmission. Until now, 14 genes that are strongly correlated with a preference for humans were identified by analyzing the

transcriptomes in the antennae between the domestic and forest forms of *Ae. aegypti* (McBride et al., 2014). By receiving the human odorous molecules, mosquito host seeking and blood feeding behavior were elicited through the activation of odorant receptors (ORs), gustatory receptors (GRs), and ionotropic receptors (IRs) (Sparks et al., 2015). *Ae. aegypti* ORs were found in the antenna, maxillary palps, proboscis, and even on tarsi. They sense various odorant molecules as ligand binding (Bohbot et al., 2007; Bohbot et al., 2014; Sparks et al., 2014), whereas GRs were found in the labella and tarsi of *Ae. aegypti*, involved in detecting the presence of CO_2 in the environment (Erdelyan et al., 2012; Sparks et al., 2013). Although there is still a lack of functional study on mosquito IRs, the presence of IRs were found on the lactic acid-sensitive cells (Melo et al., 2004), suggesting that the IRs of *Ae. aegypti* could detect acid and amine with similar functions than for *Drosophila* IRs (Abuin et al., 2011).

Several attempts have demonstrated that the knock-out of AaegGr3 (GR), Orco (OR), and npylr (IR), can interrupt the signal transduction in mosquito sensory neuron system, and subsequently, suppress the host-seeking behavior in female mosquitoes. AaegGr3 is a subunit of the CO_2 receptor of *Ae. aegypti* as its CO_2 sensitivity can be suppressed by knock-down experiments (Erdelyan et al., 2012). The zinc-finger nuclease (ZFN) mediated AaeGr3 knocking-out mosquitoes successfully inhibits CO_2 sensing ability. However, attraction to humans is reduced but not abolished in semi-field experiments (McMeniman et al., 2014), suggesting that a heat and odor dependent sensory can also participate in host sensing of *Ae. aegypti*. Thus, a multimodal integration approach is essential to reduce the mosquito attraction to human. The odorant receptor (*orco*) and neuropeptide Y-like receptors (*npylr1*) were knocked-out in *Ae. aegypti*, however, only a weak reduction of preference for humans was

observed in *orco* knocked-out mosquitoes (DeGennaro et al., 2013), whereas no effect for host-seeking inhibition was observed for *npylr1* mutant mosquitoes (Liesch et al., 2013).

Although the olfactory-deficient mosquitoes are not yet fully developed for arboviral diseases control due to the inefficient inhibition of female mosquito attraction to humans, many factors involved in mosquito blood-feeding behavior were gradually revealed in those studies. The ultimate goal of this mosquito control strategy is knocking-out the essential component that switches the mosquito attraction from human to other animal host, so that the mosquito population could be preserved in their natural habitat and stay at a relatively stable ecological balance. Combining with a gene drive system, the olfactory-deficient phenotype could be introduced into the target population; the risk of disease transmission will be therefore reduced while the mosquito natural ecological niche is not disturbed.

4.7 Part II: Synthetic miRNAs induce dual arboviral-resistance phenotypes in the vector mosquito, *Aedes aegypti*

In this chapter, we present an article was accepted by the journal *Communications Biology*.

Abstract

Mosquito-borne arboviruses are responsible for recent dengue, chikungunya and Zika pandemics. The yellow-fever mosquito, *Aedes aegypti*, plays an important role in the transmission of all three viruses. We developed a miRNA-based approach that results in a dual resistance phenotype in mosquitoes to dengue serotype 3 (DENV-3) and chikungunya (CHIKV) viruses. The target viruses are from two distinct arboviral families and the antiviral mechanism is designed to function through the endogenous miRNA pathway in infected mosquitoes. Challenge experiments showed reductions in viral transmission efficiency of transgenic mosquitoes. Several components of mosquito fitness were examined, and transgenic mosquitoes with the *PUb* promoter showed minor fitness costs at all developing stages. Further development of these strains with gene editing tools could make them candidates for releases in population replacement strategies for sustainable control of multiple arbovirus diseases.

Introduction

Dengue and chikungunya are two major arboviral diseases that have emerged as global threats in the past decades. Approximately 390 million people are infected annually with dengue and over 50% of the world's population live under the risk of infection, draining annually an estimated $40 billion for health-care spending and lost productivity in affected countries (Selck et al., 2014). Compared to dengue, chikungunya has a lesser impact on public health and had been a neglected tropical disease until the 2005 outbreak in La Réunion Island when one-third of the population was affected. Since then, there have been several chikungunya outbreaks worldwide including in Southeast and East Asia, Central Africa, South Pacific Islands, and lately in Latin America and the Caribbean (Zouache and Failloux, 2015). Many imported cases have been reported in Europe and North America raising the risk of local transmission. Autochthonous cases of dengue were recorded in Croatia (Gjenero-Margan et al., 2011), France (Marchand et al., 2013; Succo et al., 2016), and Madeira (Lourenco and Recker, 2014), while chikungunya has appeared in Italy (Angelini et al., 2007) and France (Delisle et al., 2015; Grandadam et al., 2011).

Dengue virus (DENV) and chikungunya virus (CHIKV) co-circulate in several tropical areas and co-infections in human are frequently reported (Chahar et al., 2009; Chang et al., 2010; Chipwaza et al., 2014; Hapuarachchi et al., 2008; Laoprasopwattana et al., 2012; Nayar et al., 2007; Omarjee et al., 2014; Parreira et al., 2014; Ratsitorahina et al., 2008; Raut et al., 2015; Rezza et al., 2014; Schilling et al., 2009; Tun et al., 2014). The viruses belong to two distinct families but share the same mosquito vectors, *Aedes* species. Mosquitoes can acquire DENV and CHIKV simultaneously after feeding on a co-infected patient or after two consecutive blood meals on viremic hosts (Furuya-Kanamori et al., 2016a). Co-infected mosquitoes can transmit concomitantly

DENV and CHIKV to subsequent hosts (Vazeille et al., 2010), and this is likely to cause more severe symptoms than mono-infections (Chahar et al., 2009; Mercado et al., 2016).

DENV-3 is the fastest spreading DENV serotype in the past two decades (Messina et al., 2014). Because a licensed tetravalent dengue vaccine is still not available (Capeding et al., 2014; Villar et al., 2015), novel vector control strategies are needed to prevent virus transmission between mosquitoes and hosts. Furthermore, while vaccination would greatly reduce urban transmission, enzootic circulation of arboviruses carries the risk of mutation accumulation and spillover infections that would not be impeded (Sun et al., 2006; Thiboutot et al., 2010; Wolfe et al., 2001). Eliminating both CHIKV and DENV-3 viruses in mosquito vectors would reduce the burden on population health, particularly for countries already under stress in their health-care system.

While most arboviruses can induce significant morbidity and/or mortality in some vertebrate hosts, infections of mosquito vectors are generally considered non-pathogenic (Martin et al., 2010). However, interactions between the replicating virus and the mosquito immune defense system may influence subsequent viral dissemination and transmission. Considerable progress has been achieved in understanding the innate defenses of the mosquito against arboviruses. Among them, RNA interference (RNAi) has been shown to be a major innate response of mosquitoes against arboviruses. Knock-down experiments targeting RNAi components such as Dcr2, R2D2 and Ago2 in *Aedes* show increased viral loads or decreased extrinsic incubation periods in mosquitoes (Sanchez-Vargas et al., 2009). Furthermore, virus replication is suppressible in cultured mosquito cell lines expressing long double-stranded RNA (dsRNA) molecules designed to target the viral genome (Adelman et al., 2002a). RNAi-based, virus-resistant mosquitoes were developed in which transgenes comprising long dsRNAs targeting DENV-2 under the control of a blood meal-inducible gene promoter were able to confer a strong serotype-specific, virus- resistance phenotype (Franz et al., 2006; Mathur et al., 2010; Travanty et al., 2004). According to the species-conserved miRNA

processing pathway, the miRNA precursors (pri-miRNA) were processed into ~70 nt hairpins by Drosha in the nucleus, which was followed by exporting into the cytoplasm by Exportin5. In the cytoplasm, the hairpins are cleaved into ~22 nt miRNA duplexes by Dicer-1, which are then loaded into Ago-1 or Ago-2 proteins in miRNA-induced silencing complexes (miRISCs) according to their different structure properties (Forstemann et al., 2007; Ghildiyal et al., 2010; Yang et al., 2014). By recognizing the complementary sequence of the target RNA, miRISCs are executing the silencing through RNA degradation, translational inhibition or both (Li and Rana, 2014; Wilczynska and Bushell, 2015).

Here we report the first miRNA-based genetically-engineered mosquitoes that are refractory to DENV-3 and CHIKV simultaneously. In addition, we show some fitness costs resulting from the transgenes, but anticipate that could be mitigated with additional modifications to the transgenes and their insertion sites.

Results

Constructing the artificial antiviral miRNA

Two consensus sequences of DENV-3 and CHIKV are defined from 356 and 32 isolates, respectively, of each virus (Supplementary Data 1). Four regions from DENV-3 and six from CHIKV are selected as the targets of antiviral miRNAs on the basis of their sequence coverage and targeted regions (Fig. 1). Corresponding miRNAs are designed and cloned in tandem to make compound anti-viral effector genes. The sequence coverages of the anti-DENV-3 miRNAs to the viruses used to generate the consensus sequence range from 96.6%-98.6%, and the targeted genes encode the non-structural proteins, NS2B, NS3, and NS5. The anti-CHIKV miRNAs have 96.9%-100% coverages to the viruses used to generate the consensus sequence, and the targeted genes encode the non-structural proteins, NSP1, NSP2, NSP3-4, NSP4, and the structural proteins E2, and E1 (Supplementary Table 1). In addition, to verify if any miRNA off-target effect might be caused

by the synthetic antiviral miRNAs, all the sequences of each antiviral miRNA are examined by the miRNA off-target effect prediction software Genome-wide Enrichment of Seed Sequence matches (GESS) (Sigoillot et al., 2012), including the passenger strands of each antiviral miRNA; no statistically significant interaction is reported against any transcript of *Ae. aegypti*. All antiviral miRNA clusters are constructed to place them under either the *Aedes PolyUbiquitin* or *Aedes Carboxypeptidase A* gene promoters to elicit constitutive or blood meal-inducible, midgut-specific expression of the effector molecules (Fig. 1).

Generation of transgenic mosquitoes

A Class II TE *mariner MosI* system (Lobo et al., 2006) is used to generate transgenic mosquito lines by microinjection in four separate experiments mixtures of the donor plasmids, pMosI_AePUb>4miR:D3 (4miR:D3), pMosI_AePUb>6miR:CHIKV (6miR:Chik), pMosI_AePUb>10miR:D3+CHIKV (AePUb>10miR), and pMosI_AeCPA>10miR:D3+CHIKV (AeCPA>10miR), with the transposase expressing helper plasmid pKhsp82MOS. A total of 432, 595, 310 and 355 embryos are injected with each donor plasmid, and of these, 151, 153, 141 and 62, developed into adults. Following outcrossing of G_1 adults, a total of 1, 5, 3 and 5 lines, respectively, are obtained from each crossing family (Supplementary Table 2). Homozygous lines are generated by screening inter-crossed families in which progeny are 100% reporter-positive for two generations. The copy number of transgenic cassettes in mosquito chromosomes is confirmed by Southern blot analyses using restriction enzymes that have no or only a single cutting site within the transgene and ^{32}P-labeled probes complementary to the 10 miRNA cluster region. The results indicate that both mosquito lines contain only a single copy of the transgene cassette in a different locus in the genome (Supplementary Figure 1).

Expression of artificial miRNAs

Signals confirming the expression of anti-CHIKV-4 and anti-DENV3-1 are detected by miRNA qPCR analyses of female midguts and carcasses prepared from tissues harvested 0 and 24 hours post blood meal (PBM; Fig. 2a, Supplementary Figure 2). The mature miRNAs are polyadenylated, followed by reverse transcription with poly(A)-adaptor primer for synthesizing an adaptor-linked miRNA complementary DNA (miRNA cDNA). With the miRNA-specific and adaptor primers (Supplementary Table 3), the mature miRNA can be detected by qPCR analysis. The antiviral miRNAs of AePUb>10miR mosquitoes are detectable in the midgut and carcass, and a slightly increased expression level can be observed 24hPBM. As the AeCPA promoter is reported to be active in the midgut and salivary glands (Edwards et al., 2000), the antiviral miRNAs are detected in the midgut and carcass of AeCPA>10miR mosquitoes, and the expression levels are increased 24hPBM. The two antiviral miRNAs can be also detected from the samples of female salivary glands at day 0, 1, and 6 after receiving a viremic blood meal; the results show that both antiviral miRNAs remain detectable in the salivary glands even at day 6 after virus challenge (Fig. 2b, Supplementary Figure 2). We interpret these data to indicate that the expression of the antiviral miRNAs in the midgut, carcass and salivary glands, remains inducible after receiving a blood meal.

Impacts of transgene on life-table parameters

A number of life-table parameters that might be expected to affect fitness are evaluated. These include larval development time, larval/pupal mortality, adult lifespan, sex ratio, and male mating competitiveness (Table 1).

In our rearing conditions, wild-type (Orlando) mosquitoes need an average of 6.43±0.03 (males) and 7.02±0.05 (females) days for development from first instar larvae to pupae, while AePUb>10miR mosquitoes have development times of 6.12±0.03 (males) and 6.29±0.03 (females) days, and AeCPA>10miR had 6.7±0.04 and 6.87±0.05 days for males and females, respectively (Kruskal-Wallis test: $p < 10^{-4}$ (males), $p < 10^{-4}$ (females)). The larval mortality rate is 4.40±0.86%

and 16.90±2.02% for AePUb>10miR and AeCPA>10miR mosquitoes, respectively, and these latter are significantly higher than wild-type Orlando mosquitoes at 1.88±0.56% (Fisher's exact test: $p < 10^{-4}$). As for the pupal mortality, AeCPA>10miR mosquitoes have a significantly higher (Fisher's exact test: $p < 10^{-4}$) pupal mortality rate, 7.75%, than AePUb>10miR and wild-type Orlando, 2.0 and 2.83%, respectively. The adult life spans are also analyzed, the mean survival times of AePUb>10miR male and female adults are respectively 36.9±11.75 and 41.78±14.91 days, which are not significantly different than 39.51±11.58 and 44.32±14.23 days for wild-type Orlando mosquitoes (Kruskal-Wallis test: $p = 0.07$). However, AeCPA>10miR mosquitoes have shorter survival times with means of 23.61±10.00 and 28.15±12.83 days for males and females, respectively, significantly shorter than wild-type Orlando mosquitoes (Kruskal-Wallis test: $p < 10^{-4}$). AeCPA>10miR mosquitoes have a significantly lower survival rate than the two other strains (log rank test: $p < 10^{-4}$ (males), $p < 10^{-4}$ (females)) (Fig. 3). Among these adult mosquitoes, the percent of female AeCPA>10miR mosquitoes is 40.35±2.91%, which is lower than 46.32±2.08 % of wild-type Orlando and 46.77±2.14% of AePUb>10miR mosquitoes, there is no significant difference among the three lines (Fisher's exact test: $p = 0.17$). We conclude that the high larval and pupal mortality rate of AeCPA>10miR mosquitoes is not sex-dependent. Male mating competitiveness of both transgenic lines is determined by mating competition with the same number of wild-type males. Results show that the mating competitiveness of AePUb>10miR male is 58.5±7.8%, indicating an advantage when compared with wild-type mosquitoes. For AeCPA>10miR mosquitoes, the proportion of reporter-positive mosquitoes is 26.3±7.2%, supporting the conclusion that they are less competitive in the presence of wild-type mosquitoes. Mating competitiveness of AePUb>10miR males is significantly higher compared to AeCPA>10miR males in the presence of wild-type males (Fisher's exact test: $p = 0.004$).

Virus suppression test of transgenic mosquitoes

The antiviral efficiency of artificial miRNAs for each virus is confirmed separately for AePUb>4miR:DENV3 and AePUb>6miR:CHIKV mosquitoes (Fig. 4, Supplementary Figure 3). These mosquitoes are less capable of transmitting CHIKV at 6 days post-infection (dpi) (Orlando (mean±SE): 27.08±6.4, AePUb>4miR:DENV3: 10.41±4.45, AePUb>6miR:CHIKV: 8.33±4.03) and DENV-3 at 21 dpi (Orlando: 27.08±6.48, AePUb>4miR:DENV3: 0, AePUb>6miR:CHIKV: 2.08±2.06).

Then we co-challenge two selected strains from AePUb>10miR and AeCPA>10miR mosquito lines with DENV-3 at 10^7 ffu/ml and CHIKV at 10^6 ffu/ml. Whole bodies, heads, and saliva are collected for analyzing viral titers. Among three groups of saliva collected from 24 AePUb>10miR and AeCPA>10miR mosquitoes, CHIKV transmission efficiency has respectively an average of 11.11% and 6.94% at 6 dpi, whereas the wild-type Orlando mosquitoes average 41.67% (Fig. 5, Supplementary Figure 4).

CHIKV infection and dissemination barriers are assayed by recovering virus particles from bodies and heads, and both AePUb>10miR and AeCPA>10miR mosquitoes show lower but not significant infection and dissemination rates (Fig. 5). The CHIKV transmission-reducing phenotypes of the transgenic mosquitoes also are confirmed by a salivary glands immunofluorescence assay. Salivary glands dissected at 6 dpi, reacted with antibodies and visualized under fluorescent microscope, show qualitatively lower signals in samples from each transgenic line than wild-type Orlando mosquitoes (Supplementary Figure 5). Anti-DENV-3 phenotypes tested at 21 dpi show that the infection rate, dissemination and transmission efficiencies of the transgenic lines are significantly lower than wild-type mosquitoes (Fisher's exact test: $P < 10^{-4}$) (Fig. 5).

Discussion

Against arboviruses, exogenous RNAi induced by long dsRNA molecules is an effective mechanism to interrupt viral infection and transmission (Frantz, 2006). Several studies have demonstrated the highly effective antiviral siRNAs in genetically-engineered mosquitoes, i.e. long dsRNAs (>500 bp in length) derived from DENV-2, were processed into several siRNAs targeting the viral genome and suppressing the viral replication (Franz et al., 2006; Mathur et al., 2010). The high coverage of the antiviral siRNAs on the viral genome provides a high level of resistance against DENV-2 and reduces the risk of generating siRNA escape variants. However, owing to the large diversity of antiviral siRNAs produced, it is difficult to predict regions targeted in the mosquito transcriptome (Joga et al., 2016). The effects of RNAi machinery employing siRNA to suppress viral replication can be transient as some viruses replicate so quickly that they overcome the RNAi response (McFarlane et al., 2014a). These limitations can be surmounted using hammerhead ribozymes. In cells and genetically-engineered mosquitoes experiments, the small catalytic hammerhead ribozymes mediate a 15-16 nt sequence-specific cleavage and are efficiently used as an antiviral effector against CHIKV, which increased the range of possible target sites (Mishra et al., 2016). However, the error-prone activities of RNA polymerase generate opportunities for arboviruses to escape from ribozyme catalysis, which is only triggered in high sequence specificity (Ohmichi and Kool, 2000; Scherer and Rossi, 2003). This deficiency could be overcome by using antiviral group-I introns (Carter et al., 2014; Carter et al., 2015) and by targeting the conserved DENV and CHIKV sequences, which then could lead to viral RNA trans-splicing and cell apoptosis. So the resistance to arboviruses could be triggered by incomplete viral RNA synthesis and cell death. Targeting the conserved viral sequences successfully increased the coverage of the four serotypes of DENV and CHIKV, without inducing significant fitness impacts in naïve C6/36 cells. The antiviral activities of group-I introns were initiated after recognition of several components mediating RNA splicing, including internal guide sequence (IGS), external guide sequence (EGS), and a helix forming sequences (P10) on both viruses. However, the unknown mismatch tolerance of antiviral group-I introns might favor escape variants due to the

quasispecies nature of viral populations. The cell-based experiments might not reflect the complexity of a mosquito organism, so the fitness impact needs to be examined carefully in mosquitoes. Besides, the antiviral apoptotic cell death that is triggered upon virus infection, might result in different outcomes depending on the virus; cell apoptosis can suppress DENV replication but not SINV in mosquitoes (Clem, 2016; Wang et al., 2012). Nevertheless, if a few mismatches between the guide sequence and the target virus could be tolerated, the antiviral group-I introns system in mosquitoes is potentially an applicable molecular effector against arboviruses.

In this study, we generated several miRNA-based genetically-engineered mosquito lines with resistance to DENV-3 and CHIKV triggered either ubiquitously or midgut specifically in responding to a blood meal. The synthetic miRNAs we used were 22-nt in length, which are capable of targeting broad range strains of virus. Additionally, the predictable off-target effect of antiviral miRNA provides tolerable features with mutant variants that reduce the risk for the virus to escape from miRNA-mediated silencing. Besides, the small sized synthetic miRNAs with distinct targets could be easily assembled and transcribed as a miRNA cluster, then processed into mature miRNAs through endogenous miRNA pathway, without eliciting the unintended silencing resulted by the siRNA that derived from long dsRNA.

Although the recipient mosquito strain Orlando was reported as a weakly susceptible to DENV-2 (Sim et al., 2013), its vector competence depends on the virus (Bonizzoni et al., 2012). Orlando mosquitoes are still susceptible to DENV-3 when provided at a viral titer of $1x10^7$ ffu/mL.

Insertions of transgenes and their subsequent expression may impose a load on the mosquitoes carrying them. This load could result in a fitness cost for the transgenic lines and may impair their ability to be used in control strategies. There are several previous reports of exogenous gene expression causing a variety of effects on transgenic mosquitoes (Franz et al., 2014; Irvin et

al., 2004). We observed a significant effect in AeCPA>10miR compared to wild-type mosquitoes at immature and adult stages: longer larval development time, higher larval and pupal mortalities, lower adult survival, lower proportion of females at emergence and lower male mating competitiveness. These effects may be caused by the strongly expressed reporter DsRed in the AeCPA>10miR mosquito line. Nevertheless, AePUb>10miR mosquitoes does not share the same effects. On the contrary, they had a shorter larval development time, lower larval and pupal mortalities, higher adult survival, higher proportion of females at emergence and higher male mating competitiveness, which could facilitate vector control (Irvin et al., 2004). Although a distinct result was observed between both mosquito lines, we are not able to conclude that the AePUb>10miR construct has a lower fitness impact to mosquitoes than AeCPA>10miR construct, as they are not sharing the same insertion site which caused some bias. The fitness tests in this study could only provide additional information for the two selected lines carrying their distinct antiviral construct.

For testing the viral reduction phenotype under AePUb and AeCPA induction strategies, we examined co-infected mosquitoes with CHIKV at 6 dpi and DENV-3 at 21 dpi during the plateau phase of viral replication in *Ae. aegypti* (Dubrulle et al., 2009). Saliva titers of DENV-3 and CHIKV were reduced in both transgenic mosquito lines, however, viral infection and dissemination were not impaired compared to wild-type mosquitoes. For CHIKV, although transmission efficiencies were reduced in both transgenic lines, AePUb>10miR and AeCPA>10miR mosquitoes showed lower but not significant difference in infection rate and dissemination efficiency suggesting that only salivary glands but not midgut behave as an efficient barrier to the release of the virus in saliva. In contrast, the transgenic mosquitoes showed more promising results on DENV-3 suppression than CHIKV when examining infection, even though only four regions on DENV-3 were targeted by our anti-DENV-3 miRNAs.

It is likely that the anti-DENV-3 miRNAs have higher silencing efficiency than anti-CHIKV miRNAs, or the expression levels of anti-CHIKV miRNAs were not sufficient to suppress CHIKV characterized by a shorter extrinsic incubation period (Arvey et al., 2010; Dubrulle et al., 2009; Ye et al., 2015). To overcome this issue, replacing the miRNA targeting regions or substituting the promoter with other high-activity promoters would be a solution for optimizing the miRNA-based mosquitoes (Chen et al., 2008; Lam et al., 2012). In addition, the different expression patterns of antiviral miRNAs might cause different viral reduction phenotype for each mosquito line. Although the characteristics of *Aedes PolyUbiquitin* and *Carboxypeptidase A* gene promoters are well studied (Anderson et al., 2010; Moreira et al., 2000), the position of transgene integration also could be important in determining antiviral potential (Wilson et al., 1990).

In summary, we successfully demonstrated the feasibility of using artificial antiviral miRNAs to reduce the transmission of two major arboviruses in transgenic *Ae. aegypti*. Although most of the genetically-engineered mosquito lines are still able to transmit DENV-3 and CHIKV, the DENV-3 transmission rates were reduced of 94.16% in AePUb>10miR mosquitoes (from 23.61% to 1.38%), and the CHIKV transmission rates were reduced of 77.33% (from 41.67% to 11.11%) and 83.35% (from 41.67% to 6.94%) in AePUb>10miR and AeCPA>10miR mosquitoes, respectively. These reductions would greatly limit the virus circulation. However, the effector has to be optimized to approach 100% of viral suppression at midgut infection level, eliminating the risk of virus dissemination. To apply these genes in a population replacement strategy, they should be combined with a gene-drive system, such as Cas9-mediated or toxin-antidote underdominance gene drive system, by introgressing the homozygous antiviral effector gene into target wild populations to reduce disease transmission (Champer et al., 2016; Gantz et al., 2015). Therefore, maintaining high viral suppression efficiency with low fitness impacts after combining with mosquito gene drive system is needed. Thus, the mosquitoes that we presented in this study, are not yet applicable in the field and viral suppression at infection level should be improved. Besides, for

the mosquitoes that are released in the field, a “localized” transgenic line for control program is needed. A local mosquito strain must be used for mosquito transgenesis, to reduce the alteration of the population’s gene pool. Therefore, the fitness issue of released mosquitoes should be analyzed again for determining the replacement efficiency in the target population. In this study, we have shown the potential of using synthetic antiviral miRNAs as effector genes to combat multiple arboviruses simultaneously. As the proof-of-concept has been validated, we can extend our strategy to other A*edes* mosquito-borne arboviruses such as YFV and ZIKV.

Materials and methods

Plasmid DNA constructions

All the plasmids in this study were generated based on the backbone of pMOS1_nanos-mimyd88_3xp3-CFP originated from Dr. Bruce A. Hay (Caltech, CA), and re-modified by replacing the *Bgl*II site upstream of tub 3' UTR with *Bam*HI/*Xho*I sites (underlined) using PCR primers tub-3'UTR_ BamHI/XhoI-F and SV40-3'UTR_ NotI-R (Supplementary Table 4), generating pMOS1_nanos-mimyd88_3xp3-CFP'. The anti-DENV-3 and anti-CHIKV miRNA stem-loop backbones containing 5'-EcoRI/BglII and 3'-XhoI/BamHI were generated by oligo synthesis, and subcloned into pMOS1_nanos-mimyd88_3xp3-CFP with *EcoR*I and *Xho*I sites, generating pMOS1_nanos-Den3-4miR_3xp3-CFP and pMOS1_nanos-CHIKV-6miR_3xp3-CFP. The AePUb promoter was amplified from *Ae. aegypti* genomic DNA by PCR primers pMOS1_fusion_FseI/PstIAePUb-pr-F and pMOS1_fusion_BglII/EcoRIAePUb-pr-R, and subcloned into *Fse*I and *EcoR*I double digested pMOS1_nanos-Den3-4miR_3xp3-CFP and pMOS1_nanos-CHIKV-6miR_3xp3-CFP with In-Fusion® HD Cloning technology (Clontech), generating pMOS1_AePUb-Den3-4miR_3xp3-eGFP (GenBank accession: MG603748)and pMOS1_AePUb-CHIKV-6miR_3xp3-eGFP (GenBank accession: MG603749).

DENV-4miR was extracted from *EcoR*I and *BamH*I double digested pMOS1_AePUb-Den3-4miR_3xp3-CFP, and CHIKV-6miR was extracted from *Bgl*II and *Xho*I double digested pMOS1_AePUb-CHIKV-6miR_3xp3-CFP. The two antiviral miRNA clusters were then subcloned into *Bgl*II and *Xho*II double digested pMOS1_AePUb-Den3-4miR_3xp3-CFP, generating pMOS1_AePUb-Den3-CHIKV-10miR_3xp3-eGFP (GenBank accession: MG603750). AeCPA promoter was amplified from *Ae. aegypti* genomic DNA by PCR primers pMOS1_fusion_FseI/PstIAeCPA-pr-F and pMOS1_fusion_BglII/EcoRIAeCPA-pr-R, and then

subcloned into *Fse*I and *EcoR*I double digested pMOS1_AePUb-Den3-CHIKV-10miR_3xp3-CFP, generating pMOS1_AeCPA-Den3-CHIKV-10miR_3xp3-GFP. This plasmid was then re-modified by replacing the reporter 3xp3-CFP with *Bgl*II and *Xho*I sites (bolded) disrupted Hr5IE1-DsRed which carried out by In-Fusion® HD Cloning technology with mutation primers Oxitec_#4573_BglII-mutate-F and Oxitec_#4573_XhoI-mutate-R. By using In-Fusion® HD Cloning technology with PCR primers pMOS1_fusion_Hr5IE1-DsRed_marker_NotI-F and pMOS1_fusion_Hr5IE1-DsRed_marker_XmaI-R, the Hr5IE1_DsRed was subcloned into *Not*I and *Xma*I double digested pMOS1_AeCPA-Den3-CHIKV-10miR_3xp3-CFP, generating pMOS1_AeCPA-Den3-CHIKV-10miR_Hr5IE1-DsRed (GenBank accession: MG603751).

miRNA off-target effect examination

The off-target effect of synthetic antiviral miRNAs was predicted by using GESS (version 1.2) with the input parameters as followed, 7 nt of siRNA seed sequence to test; Minimum 1 of seed matches to consider an siRNA Seed Matching; Guide and Passenger strands were used for analysis; P1C-seeds of active siRNAs as inactive siRNA seeds were used; All siRNA seed sequences were scrambled as GESS control; No siRNA exclusion was allowed; p-value 0.05 was set as significance threshold parameter; Benjamini & Hochberg False Discovery Rate was selected for testing correction.

Generation of transgenic mosquitoes

Ae. aegypti Orlando strain was used as the recipient for germ-line transformation; the preblastoderm embryos were injected with the mixture of donor and helper plasmids at a ratio of 300:500 ng/цL in injection buffer (5 mM KCl and 0.1mM NaH_2PO_4, pH 6.8). Mosquitoes were

reared at 28°C, 70% relative humidity, and a 12:12 light/dark regime and fed ad libitum with a 10% sucrose solution.

Embryo microinjection was carried out as described in (Lobo et al., 2006). Each surviving G_0 male adult was outcrossed with 3 wild-type females, G_0 females were pooled together and crossed with wild-type males at a male/female ratio 1:3. All the eggs collection and G_1 larvae screening were carried out individually. G_1 larvae were screening for reporter gene expression under a fluorescent microscope (LeicaMZ12.5, Wetzlar, Germany). The transgenic G_1 mosquitoes were then outcrossed with wild-type mosquitoes for one generation to confirm the Mendelian inheritance in progenies. To establish homozygous lines, the transgenic mosquitoes were inter-crossed individually and the homozygous candidates were screened for two generations.

Mosquito experimental infections

Seven-day-old female adults were fed on artificial infectious blood meal containing 1.4 mL of washed rabbit red blood cells and 0.7 mL of virus infected C6/36 cells suspension. The blood meal was supplemented with ATP as a phagostimulant at a final concentration of 1 mM and provided to mosquitoes using a Hemotek membrane feeding system. Virus titers of the artificial infectious blood meal were at 10^6 and 10^7 ffu/mL for CHIKV and DENV respectively. Engorged mosquitoes were transferred into cardboard containers and maintained with 10% sucrose under a photoperiod of 12:12, at 28°C. Mosquito saliva was collected using the forced salivation technique described in Dubrulle et al. (2009) (Dubrulle et al., 2009). After removing mosquito wings and legs, the proboscis was inserted into P20 tips filled with 5 µL of fetal bovine serum (FBS). After 30 min, saliva was expelled from the tip to 45 µL of L-15 medium. After salivation, mosquito head and body were collected and grounded individually in 300 µL of L-15 medium supplemented with 2% FBS. 200 µL of homogenates were collected for titration after centrifugation at 10,000 g for 5 min.

Mosquitoes were examined at 6 dpi when infected with CHIKV and 21 dpi with DENV-3. Infection rate (IR) refers to the proportion of mosquitoes with infected body among engorged mosquitoes. Dissemination efficiency (DE) corresponds to the proportion of mosquitoes with infected head among mosquitoes with infected body. Transmission efficiency (TE) represents the proportion of mosquitoes with infectious saliva among mosquitoes examined.

Mosquitoes screening test

To avoid the bias caused by the position effect of integration on the antiviral efficiency, mosquito screening test was conducted with three independent lines of AePUb>10miR and five independent lines of AeCPA>10miR mosquitoes (Supplementary Table 2), and the virus transmission efficiency was analyzed to select the mosquito lines that exhibit the strongest antiviral phenotype for each construct (Supplementary Figure 6). Mosquitoes were co-challenged with both DENV-3 and CHIKV as previously described, and the saliva were collected at 6 and 14 dpi for analyzing the virus transmission efficiency. The two selected lines for each antiviral construct were used for further analysis.

Southern blot analysis

20 µg of genomic DNA were produced and digested with restriction enzymes *Bgl*II or *Sca*I, followed by DNA separation on 0.8% agarose gel. The separated DNA were then transferred onto nylon membrane and hybridized with random-primed [α^{32}P] dCTP-labeled DNA probes complementary to the 10miRNA clusters at 42°C for 16h. No restriction enzyme site of *Sca*I was contained in the transgene and only one *Bgl*II site was in the upstream of miRNA cluster, which make the expected size of hybridization patterns>>6698 bp and >>7591 bp for *Sca*I; >>4927 bp and >>6079 bp for *Bgl*II digested AePUb>10miR and AeCPA>10miR mosquitoes, respectively.

Virus detection in salivary glands

The viral particles of CHIKV were detected in mosquito salivary glands by immunofluorescence assay. Salivary glands were dissected in PBS and fixed with 4% paraformadehyde at 6 and 21 dpi, followed by hybridization with anti-CHIKV antibodies respectively. After exposing to secondary antibodies, tissues were transferred on a slide with mounting solution (ProLong® Gold Antifade Mountant). The infection patterns were visualized under fluorescent microscope. DAPI were used for cell localization.

Life-table parameters of transgenic mosquitoes

Seven-day old eggs were vacuum hatched to synchronize the rearing process. Newly hatched larvae were counted and reared in daily renewed 1 L of water with 1 yeast tablet. Larvae were daily checked until pupation and adult emergence. 50 adults of each sex from the same batch of mosquitoes were pooled together and maintained on 10% sucrose for adult lifespan analysis. For mating competitiveness test, 20 virgin females of wild-type mosquitoes were grouped with 10 wild-type males and 10 transgenic males. Mosquitoes were fed on blood meal at 7 days after grouped up, and eggs were collected from each female on 4 days after blood meal. All mosquitoes were reared at 28°C and 70% in relative humidity with a photoperiod of 12:12.

Artificial miRNA expression analysis

Mosquito small RNA was extracted from the midguts and carcasses of sugar fed and 24hPBM. Tissues were lysed in Trizol solution and the total RNA were precipitated with 75% ethanol (v/v). Total RNA were applied for miRNA cDNA synthesis by using MystiCq™ microRNA cDNA Synthesis Mix (Sigma-Aldrich), whereas qPCRs were conducted using

MystiCq®microRNA®SYBR® Green qPCR Ready Mix™ (Sigma-Aldrich) on Applied Biosystems 7500 Fast. Primer sequences were included in Supplementary Table 3. aae-miR-1 is one of the most highly and relatively stable expressed miRNA in *Ae. aegypti* and was used as an internal control for detecting miRNAs expression in this study. Because artificial miRNAs were not expressed in wild-type mosquitoes, data were normalized twice to each aae-miR-1 and wild-type aae-miR-1 presenting the relative expression profile.

Statistical analysis

All statistical tests were conducted using the STATA software (StataCorp LP, Texas, USA). Proportions were compared using Fisher's exact test and sample distributions with the Kruskal-Wallis test. P-values>0.05 were considered non-significant.

Acknowledgements

This study was funded by the Institut Pasteur and the French Government's Investissement d'Avenir program, Laboratoire d'Excellence "Integrative Biology of Emerging Infectious Diseases" (grant n°ANR-10-LABX-62-IBEID). PY was supported by the Pasteur-Paris University (PPU) program. We are grateful Hervé Blanc and Carla Saleh for technical advice and, Marie Vazeille and Laurence Mousson for technical help.

Author Contributions

P-S.Y. designed, performed the research and wrote the paper. A.J. designed the research and wrote the paper. J-C.L. constructed the synthetic antiviral cassettes. C-H.C designed the research and provided reagents. A-B.F. designed, analyzed the research and wrote the paper.

Competing Interests

The author(s) declare no competing financial interests.

Data Availability

All data generated or analyzed during this study are included in this published article (and its supplementary information files). The accession codes of the plasmid sequences are MG603748, MG603749, MG603750 and MG603751.

Table 1. Life-table parameters of transgenic mosquitoes.[1]

Mosquito lines	Larval development time (days)		Larval mortality rate (%)	Pupal mortality rate (%)	Adult lifespan (days)		Sex ratio (%)	Male mating competitiveness (%)
	Male	Female			Male	Female		
Orlando	6.43±0.03 (307)	7.02±0.05 (265)	1.88±0.56 (583)	2.83±0.67 (600)	39.51±11.58 (150)	44.32±14.23 (150)	46.32±2.08 (572)	ND
AePUb>10miR	6.12±0.03 (289)	6.29±0.03 (254)	4.40±0.86 (568)	2.0±0.57 (600)	36.9±11.75 (150)	41.87±14.91 (150)	46.77±2.14 (543)	58.5±15.8 (41)
AeCPA>10miR	6.7±0.04 (170)	6.87±0.05 (115)	16.90±2.02 (343)	7.75±1.33 (400)	23.61±10.0 (150)	28.15±12.83 (150)	40.35±2.91 (285)	26.3±14.7 (38)

[1]Mosquito larval development time, larval/pupal mortality, adult lifespan analysis, sex ratio, and test of male mating competitiveness were conducted at 28℃. Larval developmental time was determined by the period from the first instar larva to pupal stage; Larval mortality corresponds to the number of emerged adults among analyzed larvae; Pupal mortality corresponds to the number of pupae among emerged adults; Adult life spans were recorded daily by counting the number of dead mosquitoes and separated by sex; Proportion of females was determined by the number of females among all adults; Male mating competitiveness was defined as the proportion of reporter positive individuals compared to negative individuals in the same experimental cage. In brackets, the number of mosquitoes tested is given.
ND: not determined.

Figures

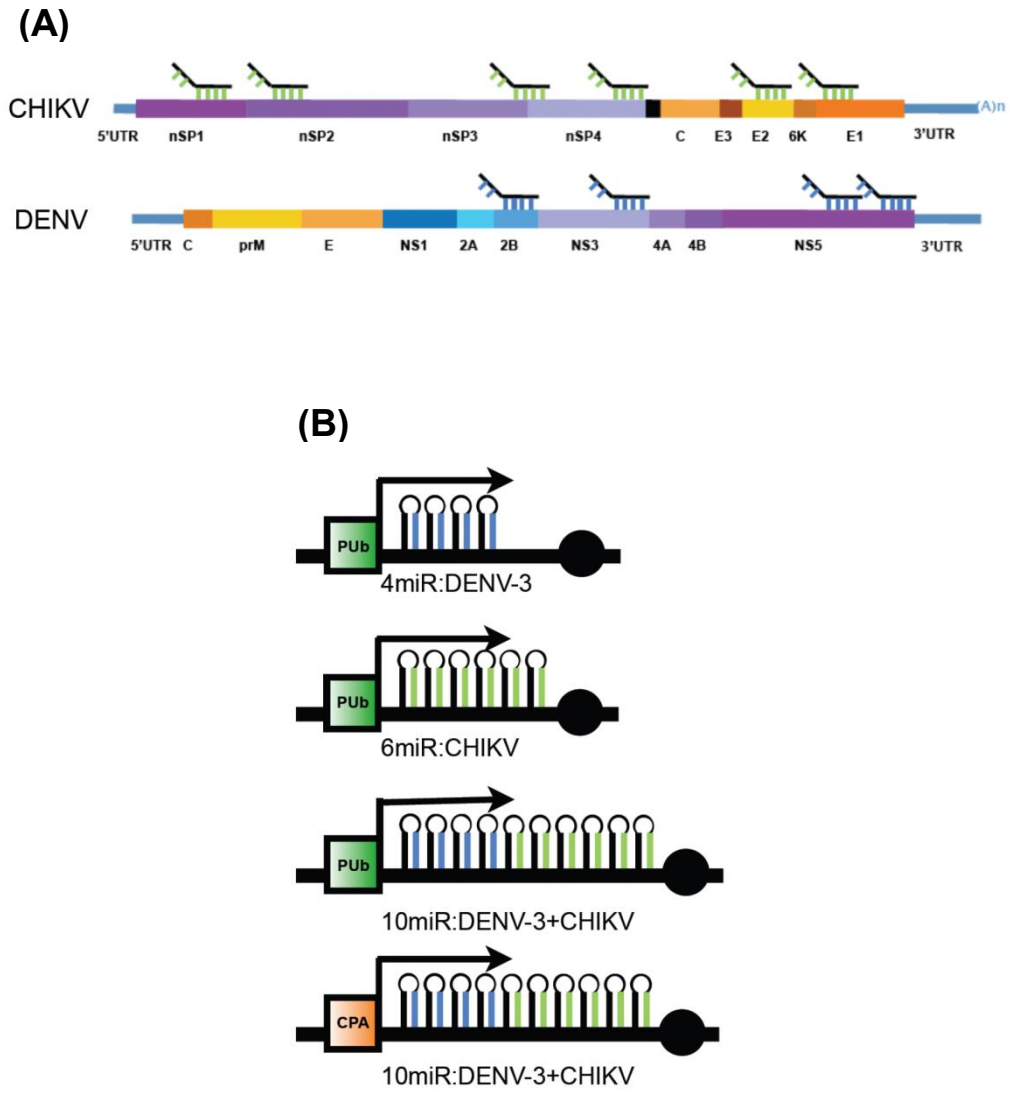


Figure 1. Scheme of the artificial antiviral miRNA. **a** target genes. **b** expression cassettes. Based on *mariner* transposon system, the ubiquitous and midgut-specific induction promoters were applied for expressing the downstream synthetic miRNAs. AeCPA/PUb promoter, *Ae. aegypti* carboxypeptidase A/PolyUbiquitin promoter; 4miR:DENV-3, anti DENV-3 miRNA cluster for four anti-DENV-3 miRNAs; 6miR:CHIKV, anti CHIKV miRNA cluster for six anti-CHIKV miRNAs; 10miR:DENV-3+CHIKV, anti DENV-3/CHIKV miRNA cluster for four for DENV-3 and six for CHIKV.

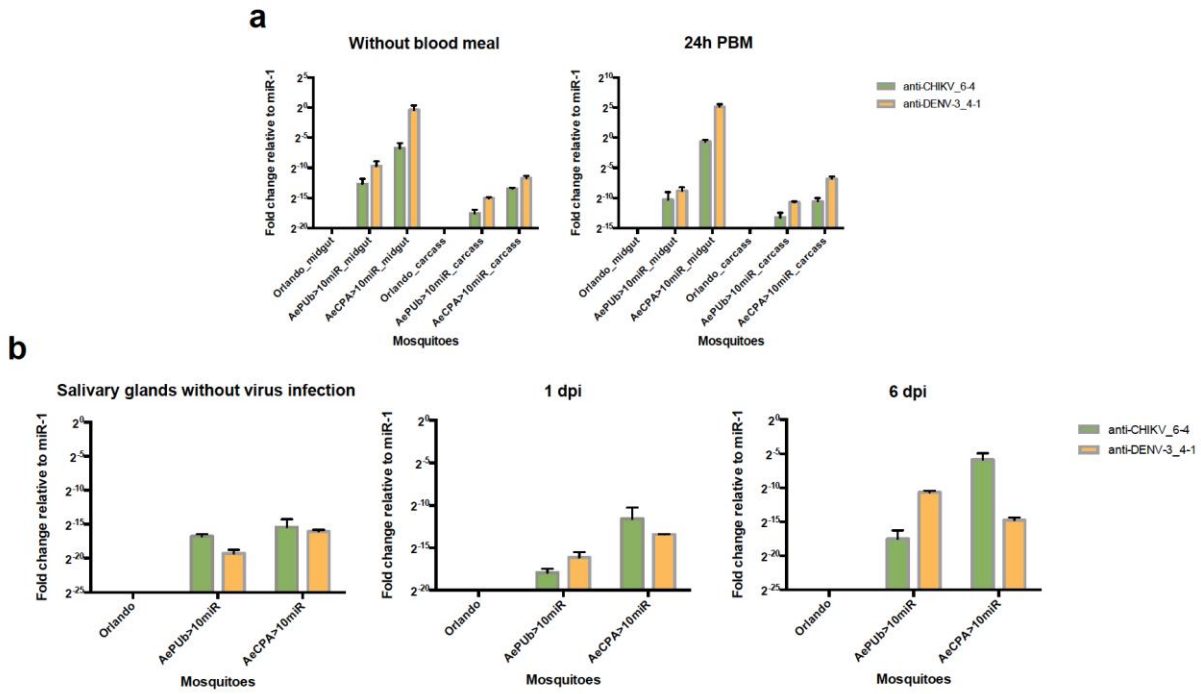


Figure 2. Detection of artificial antiviral miRNAs. **a** in midgut and carcass. **b** in salivary glands**.** Total RNA were isolated from mosquito midguts and carcasses dissected at 0 and 24h post blood meal, whereas the RNA of salivary glands were extracted from the mosquitoes co-challenged with CHIKV and DENV-3 at 0, 1, and 6 days after infection. Reverse transcription and qPCR were conducted as described in materials and methods. anti-CHIKV_6-4, the 4th anti-CHIKV miRNA; anti-DENV-3_4-1, the 1st anti-DENV-3 miRNA. Data were normalized to normalized values of aae-miR-1, and presented in relative expression levels to aae-miR-1. Each sample corresponds to 2 replicates (2x12 mosquitoes). The error bars correspond to the standard error of the mean.

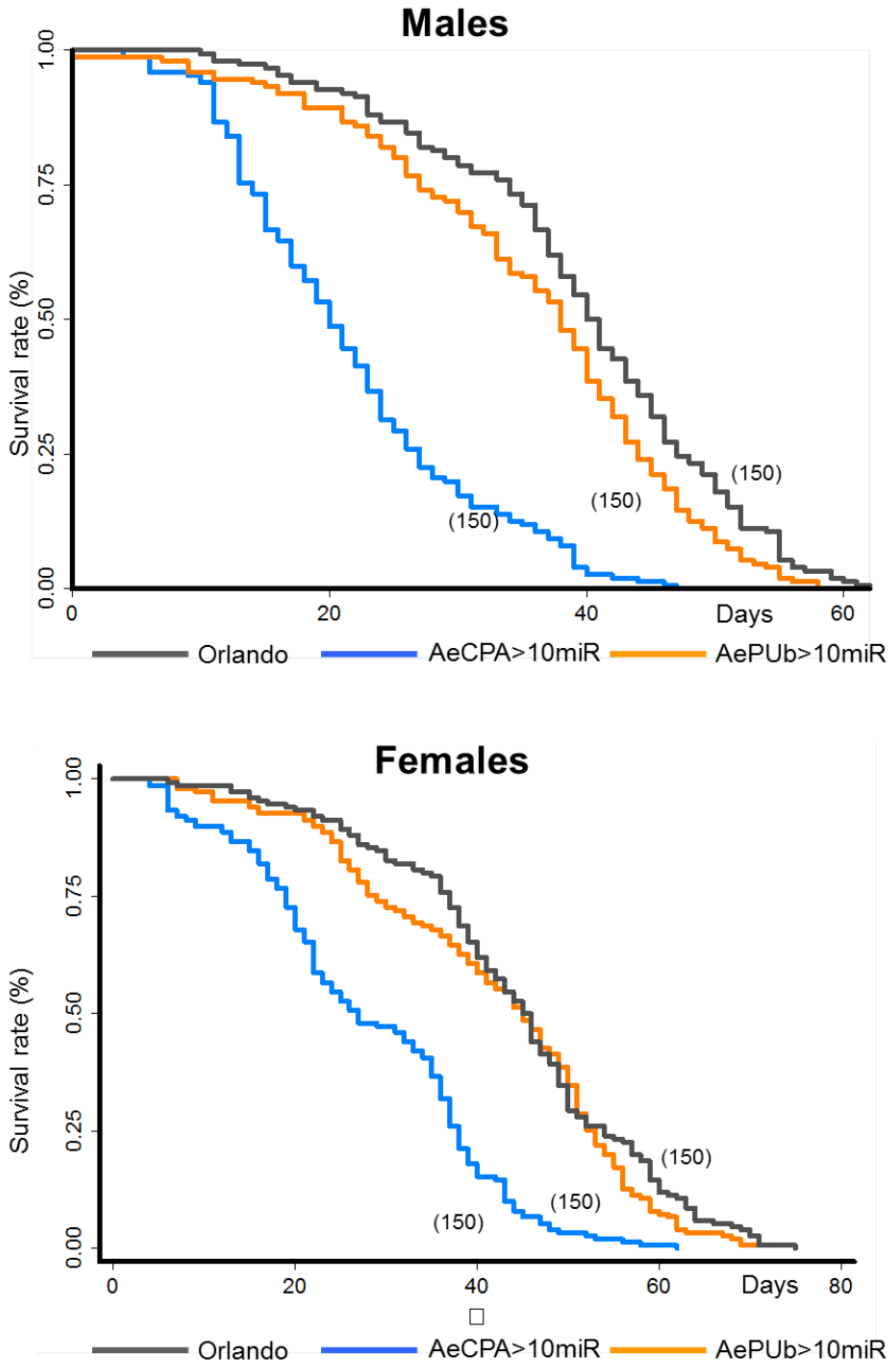


Figure 3. Survival of transgenic mosquitoes. **a** males. **b** females. Survival curves were compared between AePUb>10miR, AeCPA>10miR and wild-type Orlando mosquitoes. In brackets, the number of mosquitoes is given.

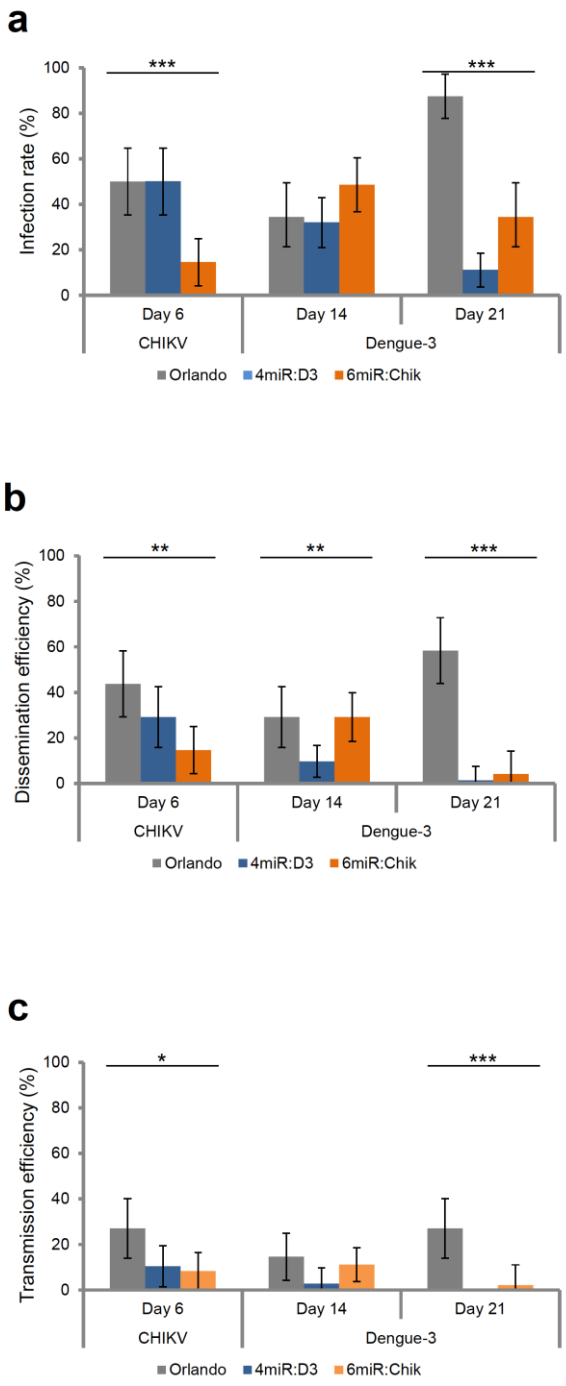


Figure 4. Anti-DENV-3/CHIKV phenotype of transgenic 4miR:D3 and 6miR:Chik mosquitoes. **a** infection rate. **b** dissemination rate. **c** transmission efficiency. Mosquitoes were co-challenged with DENV-3 Cambodia and CHIKV 0621 strain at titer 10^7 and 10^6 ffu/ml, respectively. Samples were collected and titrated at 6 and 21 dpi on C6/36 cells. The infection rate was defined as number of positive midgut samples of the total number tested; dissemination efficiency was defined as number of positive head samples of the total number

tested; transmission efficiency defined as number of positive saliva among number of tested. Saliva samples were collected via salivation by inserting the proboscis of leg- and wing-less mosquito into a P20 tip containing 5 microliter of FBS, then expelled into 45 microliter of L-15 media after 30 min for analysis. Each sample corresponds to 2 replicates (2x24 mosquitoes) or 3 replicates (3x24 mosquitoes). The error bars correspond to the confidence intervals (95%). Significant p values are indicated by an asterix: * $p<0.05$, **<0.01, *** $p<0.001$.

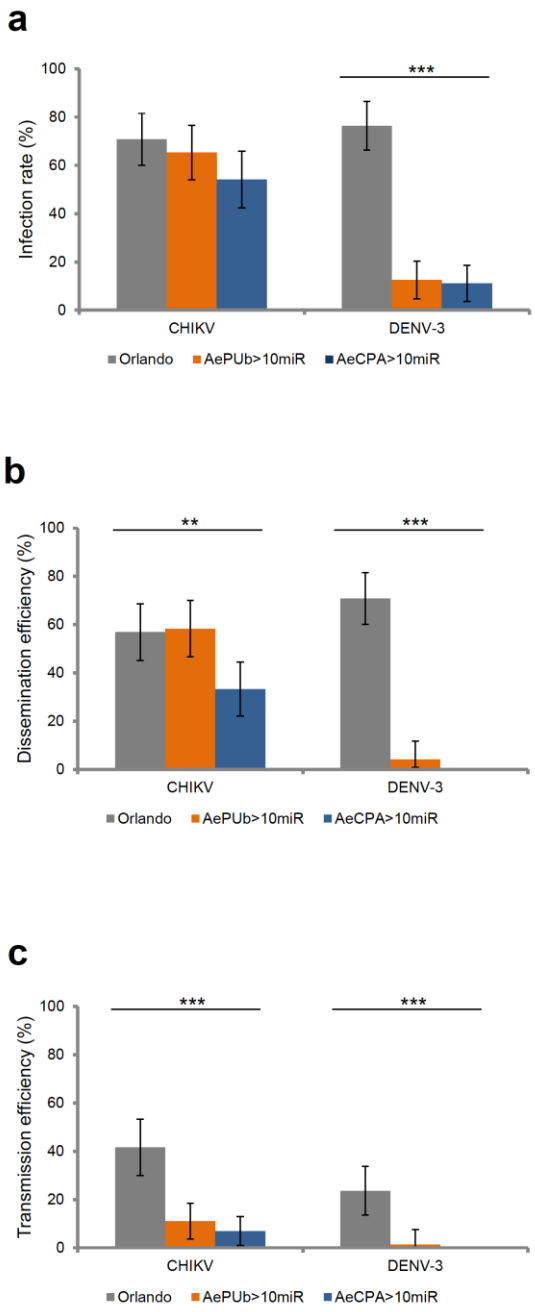


Figure 5. Anti-DENV-3/CHIKV phenotype of transgenic AePUb>10miR and AeCPA>10miR mosquitoes. **a** infection rate. **b** dissemination rate. **c** transmission efficiency. Mosquitoes were co-challenged with DENV-3 Cambodia (Supporting information) and CHIKV 06.21 (Vazeille et al., 2007) strains at titers of 10^7 and 10^6 ffu/mL, respectively. Samples were collected and titrated at 6 and 21 dpi on C6/36 cells. Infection rate was defined as number of positive body samples among tested ones; dissemination efficiency refers to the

number of positive head samples (i.e. successful viral dissemination after passing the midgut barrier) among tested ones; transmission efficiency was defined as the number of positive saliva (i.e. successful transmission) among tested ones. Saliva samples were collected after 30 min in a P20 tip containing 5 µL of FBS and then expelled into 45 µl of L-15 media for analysis. Each sample corresponds to 3 replicates (3x24 mosquitoes). The error bars correspond to the confidence intervals (95%). Significant p values are indicated by an asterix: **<0.01, *** p<0.001.

Supplementary Table 1. Details on artificial antiviral miRNAs

miRNA name	Sequence (5’ – 3’)	Targeting virus	Targeting region	Coverage (%)
Anti-CHIKV-1	AGTCAGTTCTGCTTCTCGTTCT	CHIKV	NSP1	96.9
Anti-CHIKV-2	ACTCATTCGTAGTGCGCATTTT		NSP2	96.9
Anti-CHIKV-3	TATATACCCACCTGCCCTGTCT		NSP3-NSP4	96.9
Anti-CHIKV-4	TCTATGATCTTCACTTCCATGT		NSP4	100
Anti-CHIKV-5	ACTCTTCTTGATAGTTTGGTTC		E2	96.9
Anti-CHIKV-6	GTTTTGCATGATTCGGACTTCT		E2	96.9
Anti-DENV3-1	TCTCATTGTTCCATCATCATCA	DENV-3	NS2B	96.6
Anti-DENV3-2	CCTGTGTGTTCAGATTTTGTTG		NS3	96.9
Anti-DENV3-3	AATATGACCAGCCTCCTCTTCC		NS3	98.6
Anti-DENV3-4	CATTTATCATGGAGGAGGCTGA		NS5	97.2

Supplementary Table 2. Performances of *Aedes aegypti* microinjections

Construct	Eggs injected	Survival to larval stage	G0 adults		Integration events
			Male	Female	
PUb>4miR:DENV-3	432	187	81	70	1
PUb>6miR:CHIKV	595	153	85	68	5
PUb>10miR	310	153	76	60	3
CPA>10miR	185	85	11	12	5

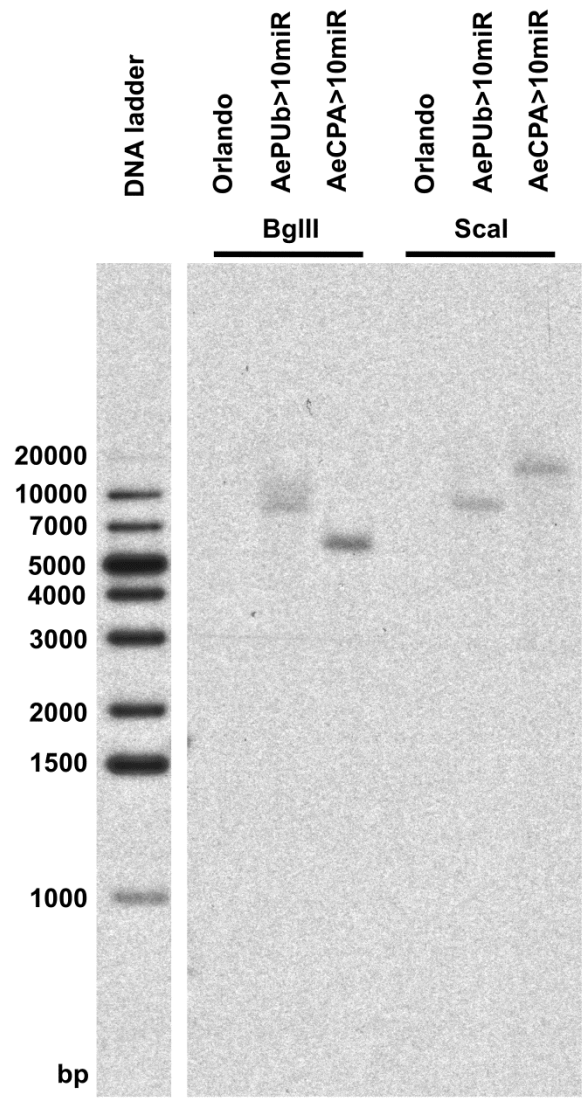
Supplementary Table 3. Primers used for miRNA qPCR in this study

aae-miR-1:	5’-TGGAATGTAAAGAAGTATGGAG-3’
10miR_DENV-3:	5’-TCTCATTGTTCCATCATCATCA-3’
10miR_CHIKV:	5’-TCTATGATCTTCACTTCCATGT-3’

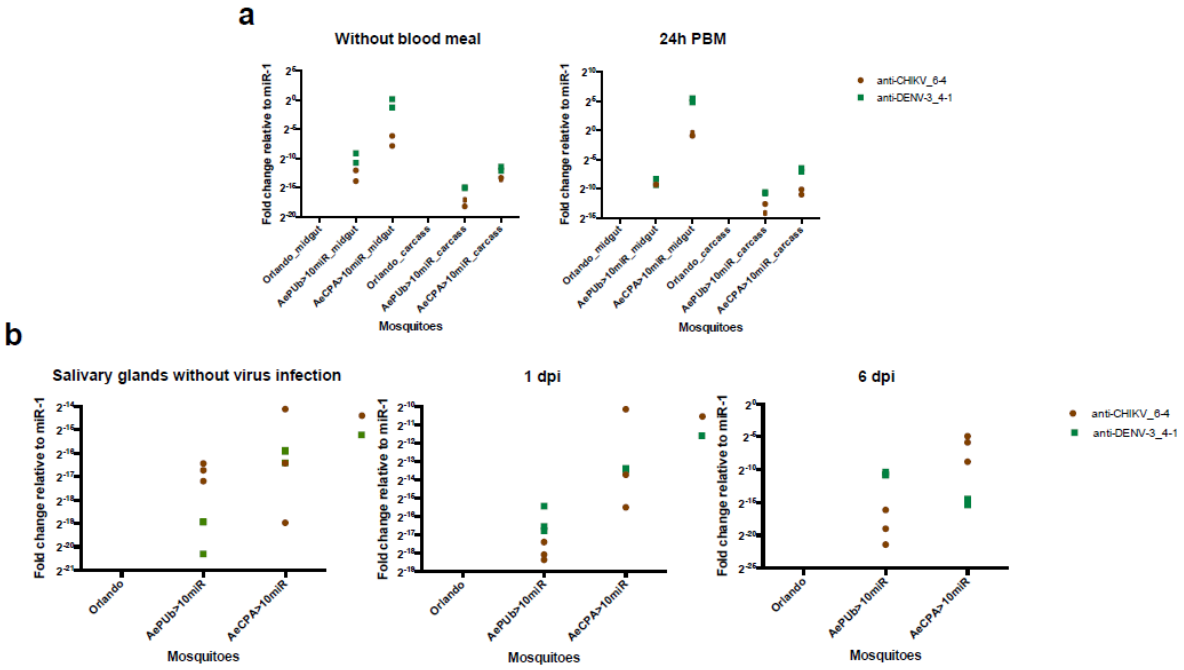
Supplementary Table 4. Details on primers used for constructing synthetic antiviral miRNA cassettes

Oligo name	Sequence (5' - 3')
tub-3'UTR_ BamHI/XhoI-F	GGCGGATCCCGCCCTCGAGATCCGTCACAAGCAATCTCACTA
SV40-3'UTR_ NotI-R	GGCCGCGGCCGCGACTCTAGATCATAATCAGCCATAC
pMOS1_fusion_FseI/PstIAePUb-pr-F	GACGAGATCGGCCGGCCCGCCCTGCAGTATCTTTACATGTAGCTTGTGCATTGAATCC
pMOS1_fusion_BglII/EcoRIAePUb-pr-R	AGATCTGGCCGAATTCGTTGAAATCTCTGTTGAGCAGAAAAAGAAACGAG
pMOS1_fusion_FseI/PstIAeCPA-pr-F	GACGAGATCGGCCGGCCCGCCCTGCAGATACATAAACTAGTTTTTGCACA
pMOS1_fusion_BglII/EcoRIAeCPA-pr-R	AGATCTGGCCGAATTCTCCAACTAACCGATACACACTAACCTGG
Oxitec_#4573_BglII-mutate-F	TCTTGGGTCGAGAGCGCAGGAACAGGTGGTGGCGGCCCTCGGTGCGC
Oxitec_#4573_XhoI-mutate-R	CAAACGGACGCCCGAGGTTGCACAACACTATTATCGATTTGC
pMOS1_fusion_Hr5IE1-DsRed_marker_NotI-F	TCTAGAGTCGCGGCCGCTCCGGTGGATCTTACGGGTCCTCCACCTTCCGC
pMOS1_fusion_Hr5IE1-DsRed_marker_XmaI-R	TTCGAGCTCGCCCGGGCATTGCTTGTCATTTATTAATTTGGATGATGTCA

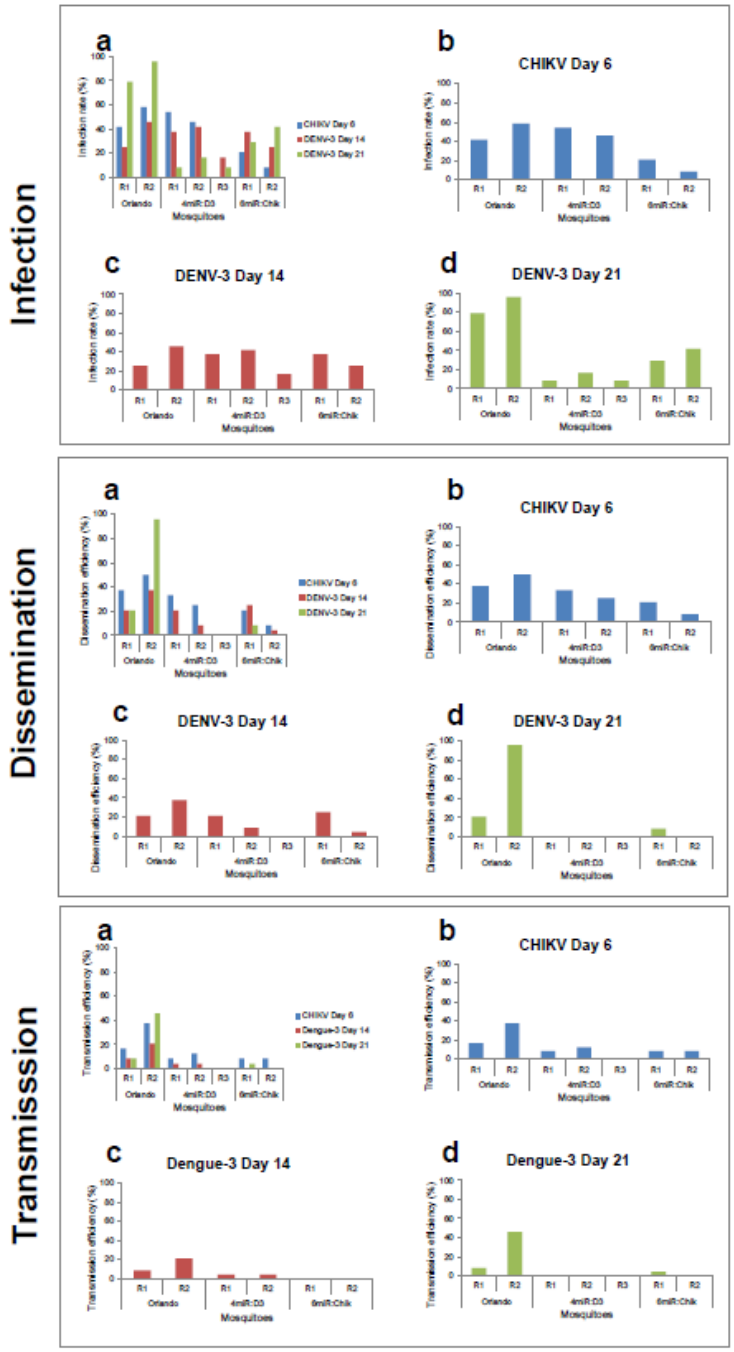
Supplementary Figure 1. Southern blot analyses of transgenic mosquitoes. Genomic DNAs were digested with restriction enzyme *Bgl*II or *Sca*I, and hybridized at 42°C with random primed alpha [^{32}P]-labeled DNA probes complementary to the sequence of the antiviral miRNA cluster.



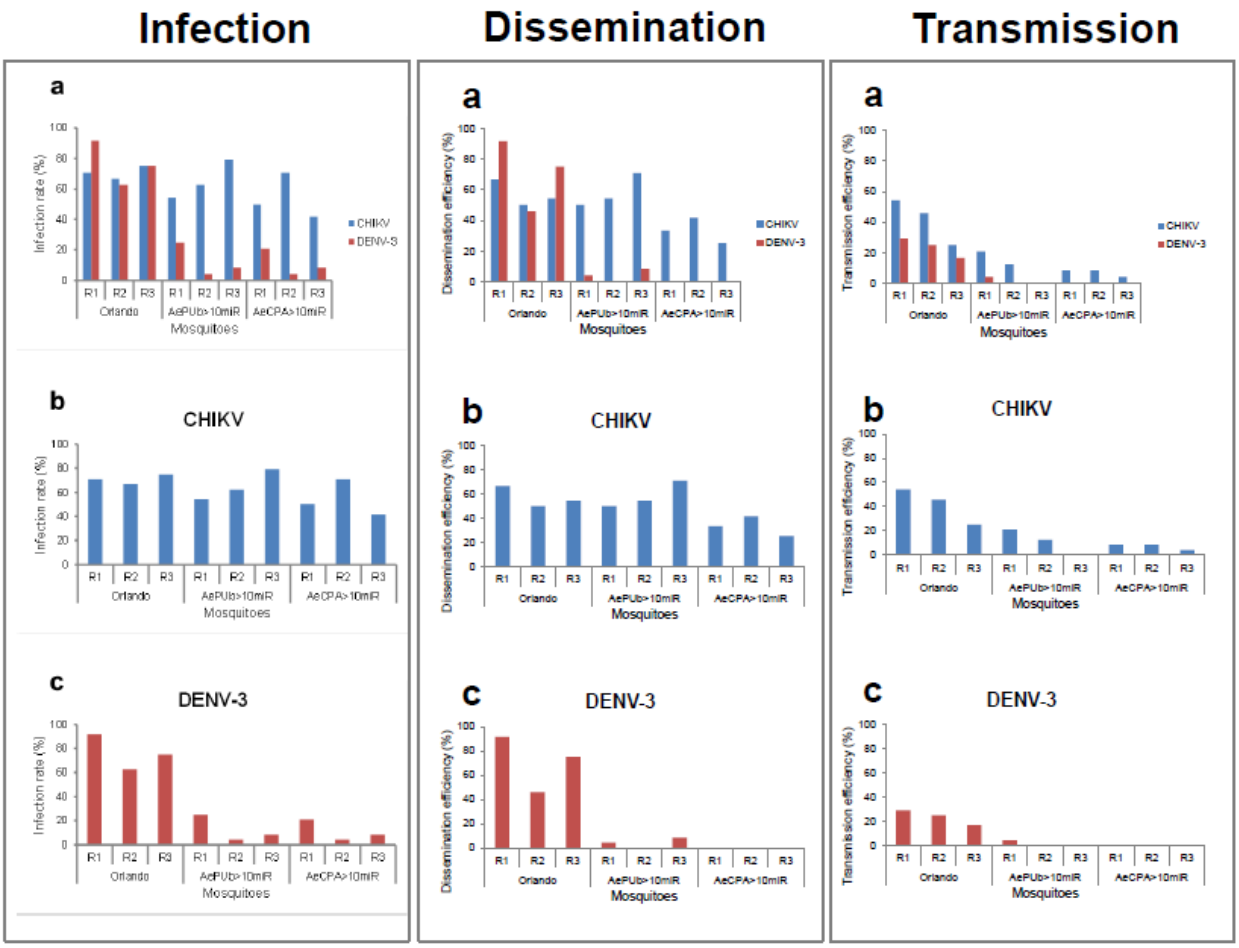
Supplementary Figure 2. Detection of artificial antiviral miRNAs. a in midgut and carcass. **b** in salivary glands. Information on replicates is provided and details are described in the legend of the figure 2.



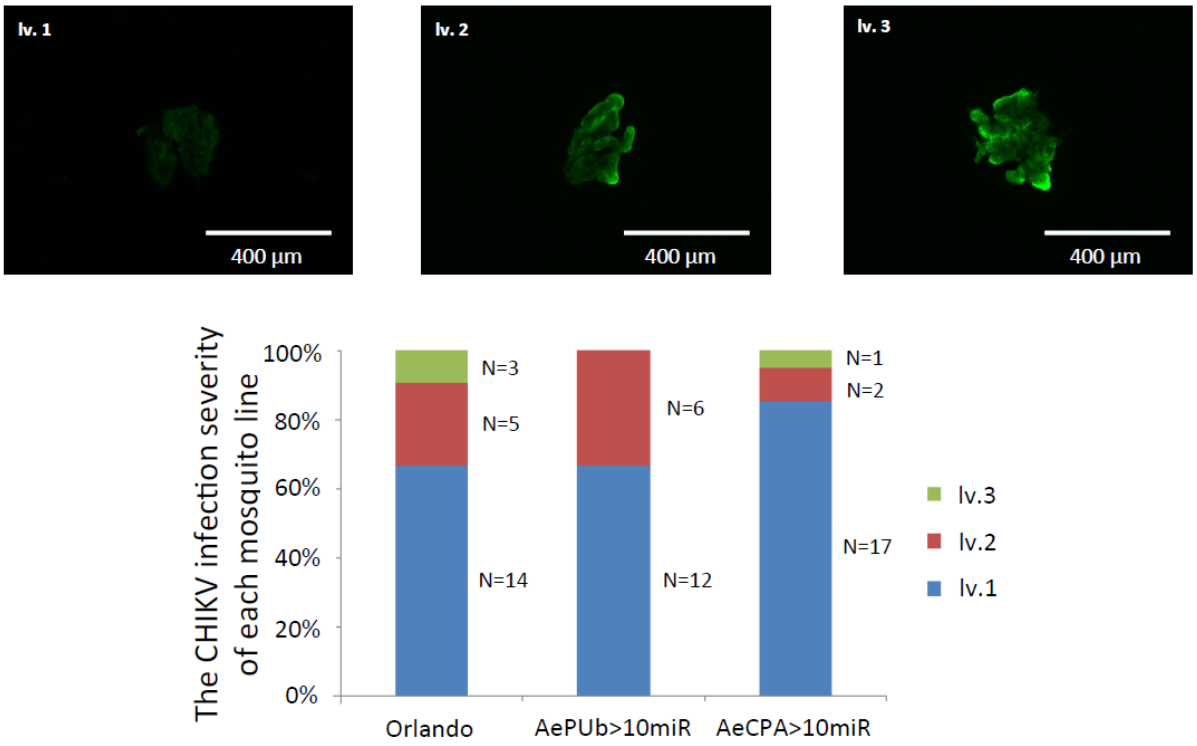
Supplementary Figure 3. Anti-DENV-3/CHIKV phenotype of transgenic 4miR:D3 and 6miR:Chik mosquitoes. a All samples. **b** Day 6 post CHIKV-infection. **c** Day 14 post DENV-3 infection. **d** Day 21 post DENV-3 infection. Information on replicates is provided and details are described in the legend of the figure 4. R1, replicate 1. R2, replicate 2



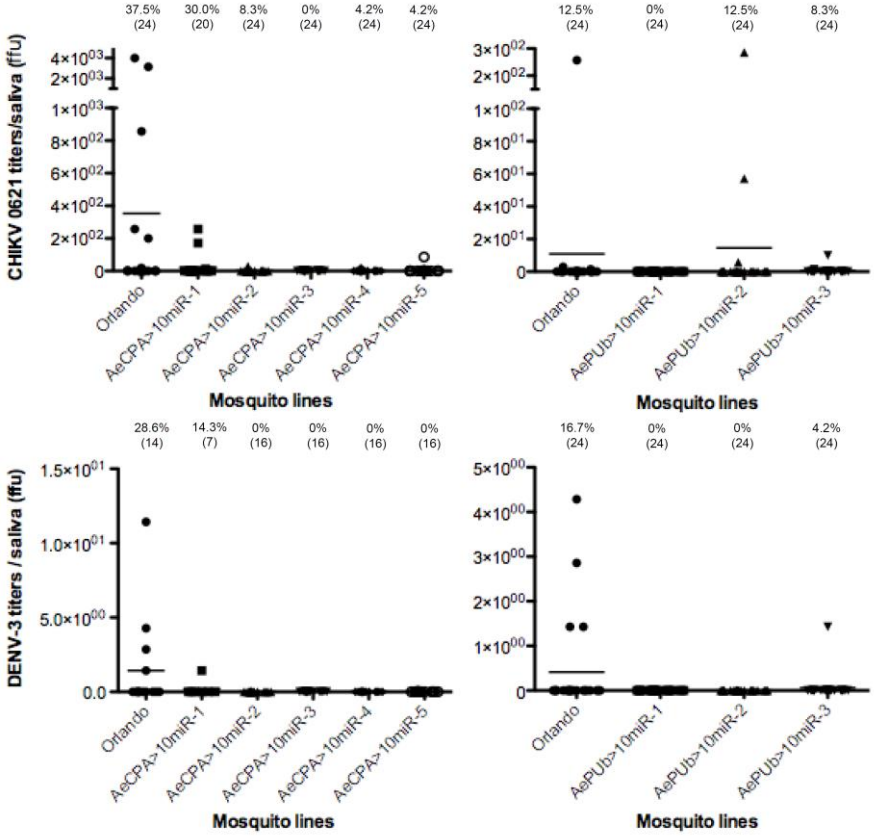
Supplementary Figure 4. Anti-DENV-3/CHIKV phenotype of transgenic AePUb>10miR and AeCPA>10miR mosquitoes. a All samples. **b** CHIKV. **c** DENV-3. Information on replicates is provided and details are described in the legend of the figure 5. R1, replicate 1. R2, replicate 2. R3, replicate 3.



Supplementary Figure 5. Antiviral phenotype in salivary glands. Mosquito salivary glands were dissected in PBS and fixed with 4% paraformadehyde at 6 days post-infection, followed by detection with anti-CHIKV antibody. The viral infection patterns were visualized under fluorescent microscopy.



Supplementary Figure 6. Antiviral phenotypic screenings for AePUb>10miR and AeCPA>10miR mosquito lines. The mosquito lines AePUb>10miR and AeCPA>10miR were co-challenged with DENV-3 at 10^7 ffu/ml and CHIKV at 10^6 ffu/ml. The viral suppression efficiency was determined by the transmission efficiency at 6 and 14 dpi for CHIKV and DENV-3 respectively. AeCPA>10miR-3 (AeCPA>10miR) and AePUb>10miR-1 (AePUb>10miR) were analyzed in this study. Numbers above are the transmission efficiency and sample size.



CONCLUSION AND DISCUSSION

Introducing an antiviral effector gene into target population with gene drive system in order to replace the naive wild population with a virus refractory strain for reducing the risk of arboviral diseases transmission, is an alternative that can be considered as a truly environment-friendly arboviral diseases control strategy. Compared to the current control with insecticides, the accumulating resistance of mosquitoes have increased the cost and reduced the control efficiency (van den Berg et al., 2012). Besides, the selective pressure of insecticides can potentially affect the vector competence, so the risk of diseases spreading as well (Beerntsen et al., 2000). The unpredictable outcomes of insecticide-based control strategy have raised the uncertain risk of newly re-emergence of mosquito-borne diseases.

A relatively environment-friendly mosquito control strategy based on genetically-engineering technology, RIDL has been proposed and become the most current applied control strategy (Alphey, 2014). By releasing the self-limiting gene carrying by male mosquitoes to reproduce with wild-type females, the size of target population is reduced due to the progeny lethality. The great success of trials in America (Carvalho et al., 2015; Gorman et al., 2016; Harris et al., 2012; Harris et al., 2011) with a drastic decrease of mosquito populations has gained better acceptance by people living under the risk of infection (Ernst et al., 2015). This RIDL-based mosquitoes control strategy is now under the consideration of other governmental authorities in America and Asia.

However, the total elimination of target species has increased the risk of ecological impacts and reemergence of secondary pests, especially *Ae. aegypti* who is sharing the same ecological niche than *Ae. albopictus* (Braks et al., 2004; Juliano et al., 2004; Simard et al., 2005), both being vectors of major arboviruses, *e.g.* CHIKV, DENV, YFV, and ZIKV.

As the synthetic gene drive systems have gained interest in recent years (Champer et al., 2016), the control strategy combining an antiviral effector gene with a gene drive system provides another solution for arboviral diseases control. It can reduce the risk of diseases transmission while keeping the mosquitoes at their natural ecological niche to avoid the reemergence of secondary pests. Homing-based gene drive systems were well developed and proved their replacing efficiency in mosquitoes (Gantz et al., 2015; Hammond et al., 2016). However, the appropriate effector genes able to block replication and transmission of arboviruses in mosquitoes are still a field to explore. An appropriate effector gene has to be efficient and broadly effective, to suppress the virus replication efficiently while remaining a low fitness cost to compete with wild population. Moreover, the specificity of effector gene should be also taken into consideration due to the possible unintended toxicity of effector released into the environment. Therefore, a RNAi-based approach is an ideal effector gene for controlling the arboviruses which are mostly single stranded RNA viruses.

In this thesis, we have investigated the potential antiviral ability of cellular miRNAs of *Ae. aegypti*, and demonstrated the possibility of using a set of synthetic miRNAs to induce an antiviral immunity to CHIKV and DENV-3.

Although the RNAi machinery is considered as a major antiviral innate immunity in mosquitoes, and the virus-induced siRNA and piRNA pathways are extensively studied (Blair, 2011; Miesen et al., 2016b), very limited information were reported regarding the potential antiviral activity of miRNAs. Even though a complete complementarity between genome sequences of arboviruses and known miRNAs of *Ae. aegypti* is not found, the seed region-dependent silencing feature of miRNAs has suggested a possible interaction between arboviruses and mosquito cellular miRNAs, proposing a new insight for miRNA antiviral immunity research.

According to the prediction results, several miRNAs binding sites were found in CHIKV, DENV, and ZIKV, which are common among different genotypes/lineages of each virus. However, these miRNA-virus interactions were associated with low expression level, which are less likely to form an effective miRNA-vRNA duplex and intervene in virus replication. On the other hand, the potential binding sites for miRNAs that are abundantly expressed in mosquitoes usually have a high minimum free energy. For example, aae-miR-1 is an abundant miRNA expressed ubiquitously in *Ae. aegypti*, which has a potential binding site in the Capsid region of DENV-1 with a MFE of -17.3 kcal/mol for genotype III and IV, whereas for genotype I, the MFE of the same binding site shifts to -17.2 kcal/mol with one nucleotide change, decreasing the probability of interaction. Interestingly, a simulation was proposed with a single nucleotide mutation within the same binding site, and the result showed a MFE reduced to -19.8 kcal/mol that largely increased the probability of miRNA-vRNA, and possibly participated in virus replication. A similar example could also be found for aae-miR-1 in the NS3 region of DENV-1; a conserved interaction with high MFE was predicted between miR-1 and NS3 region in genotype I-V of DENV-1. Few nucleotide mutations within the binding site might reduce the MFE and consequently, increase the probability of miRNA-vRNA interaction, suggesting that the cellular miRNAs might act as an important evolutionary force for shaping arbovirus-mosquito co-evolution.

On the other hand, a synthetic miRNA with low MFE when matched with the target virus might increase the antiviral efficiency. Synthetic antiviral miRNAs are considered as an ideal effector gene in this study due to its high specificity, capacity, and broad efficiency. Compared to the RNAi-based genetically-engineered mosquito control strategy (Franz et al., 2006), the siRNA that are generated from the viral genome encoded long dsRNA, has shown a very effective antiviral efficiency to DENV-2 at infection and transmission levels. The uncertain selection of siRNA sequence provides an extra advantage for reducing the virus

replication, however, the unpredictable off-target effects of siRNA can potentially increase the fitness impacts by unintended targeting the essential mRNAs in genetically-engineered mosquitoes. In contrast, the miRNA off-target effects are predictable with certain sequences of miRNA; the potential targets on mosquito mRNA could be avoided using various miRNA off-target prediction tools. Interestingly, the mismatch tolerance on target sequence of miRNA is an advantage for regulating arboviruses replication because the quasispecies of arboviruses generated by the error prone feature of viral RdRp, generate different sequences in the target sites of antiviral miRNAs in an infected mosquito (Vazeille et al., 2016). In addition, the very short sequence of each synthetic miRNA could increase the capacity of expression cassette, and express a miRNA cluster that could target multiple arboviruses after processing.

In this thesis, we have demonstrated the possibility of using synthetic miRNA to trigger mosquitoes antiviral RNAi immunity for reducing the transmission of CHIKV (*Alphavirus*) and DENV-3 (*Flavivirus*) simultaneously. Some fitness damages might have been caused in AeCPA>10miR mosquitoes, including a shorter life span, higher mortality before maturation, and lower male mating competitiveness compared to wild-type mosquitoes. In contrast, AePUb>10miR mosquitoes show a minor (if any) fitness cost for all parameters measured, and even have a shorter developmental period and higher male mating competitiveness.

Both genetically-engineered mosquitoes show a great reduction on viral transmission efficiency for CHIKV and DENV-3. However, both mosquito lines are still susceptible for CHIKV and DENV-3, especially for CHIKV; there were no significant differences at the infection and dissemination levels. This provides an opportunity for viruses to develop into a antiviral miRNAs insensitive variant escaping the anatomical barriers and to be transmitted.

Although a relatively better viral resistance phenotype could be observed for DENV-3 in both mosquito lines, the antiviral efficiency should be carefully analyzed due to the low efficiency for DENV reported for the Orlando mosquito strain used as the recipient strain (Sim et al., 2013).

Environmental temperature is also a major concern for using synthetic miRNA as an effector gene to suppress arboviral replication in genetically-engineered mosquitoes. As a poikilothermic animal, the body temperature of a mosquito varies with the ambient temperature, and potentially affects the stability of miRNA-vRNA interactions and the activity of miRNA components (Carmel et al., 2012). It is therefore difficult to estimate the antiviral efficiency of each miRNA in field conditions, particularly with the average temperatures in epidemic areas usually higher than 28 °C in summer, this temperature being used in our experiments. Although the probability of high temperature-induced inactivity is reduced with the highly complementarity of synthetic antiviral miRNAs increasing the strength for each miRNA-vRNA interaction (Carmel et al., 2012; Hibio et al., 2012), the antiviral miRNAs activity should be tested under semi-field conditions to test this hypothesis.

Although the miRNAs could be detected in salivary glands, the critical organ for viral transmission, even at day 6 post infection, however, the relatively low expression levels of each synthetic antiviral miRNA might be the reason for the insufficient resistance against the virus in the midgut in both AePUb>10miR and AeCPA>10miR mosquitoes.

With effector genes and population replacement control strategy, a 100% resistance against arboviruses is essential to reduce the probability of viruses to escape and develop into a resistance quasispecies within infected mosquitoes. Therefore, in a next step, a more

powerful or delicate promoter to initiate the expression of synthetic miRNA cluster should be envisaged to optimize the antiviral activity in genetically-engineered mosquitoes. Besides, the antiviral efficiency of each synthetic miRNA could be further optimized according to the results of prediction of miRNA-vRNA interactions discussed previously. Especially for the interaction involving an abundantly expressed miRNA and a conserved binding site that could be commonly found among each genotype, e.g. aae-miR-1 vs. Capsid or NS3 regions in DENV-1, a synthetic miRNA with a lower MFE (which is more likely to form) designed based on the viral sequences of these interactions, might provide a more promising and stronger resistance against arboviruses.

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