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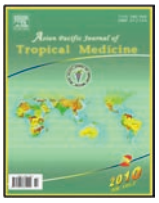

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Heterologous expression in transgenic mosquitoes

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ABSTRACT

Arthropod-borne diseases such as malaria and dengue virus afflict billions of people worldwide imposing major economic and social burdens. Control of such pathogens is mainly performed by vector management and treatment of affected individuals with drugs. The failure of these conventional approaches due to emergence of insecticide-resistant insects and drug-resistant parasites demonstrate the need of novel and efficacious control strategies to combat these diseases. Genetic modification (GM) of mosquito vectors to impair their ability to be infected and transmit pathogens has emerged as a new strategy to reduce transmission of many vector-borne diseases and deliver public health gains. Several advances in developing transgenic mosquitoes unable to transmit pathogens have gained support, some of them attempt to manipulate the naturally occurring endogenous refractory mechanisms, while others initiate the identification of an exogenous foreign gene which disrupt the pathogen development in insect vectors. Heterologous expression of transgenes under a native or heterologous promoter is important for the screening and effecting of the transgenic mosquitoes. The effect of the transgene on mosquito fitness is a crucial parameter influencing the success of this transgenic approach. This review examines these two aspects and describes the basic research work that has been accomplished towards understanding the complex relation between the parasite and its vector and focuses on recent advances and perspectives towards construction of transgenic mosquitoes refractory to vector-borne disease transmission.

1. Introduction

Mosquitoes are vectors of serious human infectious diseases, such as malaria, dengue and yellow fever. With an increased concern about insecticide resistance, genetic modification of mosquitoes has emerged as a new strategy to control mosquito-borne diseases. Three interconnected research objectives must be achieved before a disease control strategy involving the release of transgenic vectors could be attempted. Firstly, genes which encode traits that render the vector refractory to a particular pathogen must be identified. Secondly, methods to introduce and express these genes in insects in a stable, heritable fashion must be developed. Thirdly, a means for spreading these genes to high frequency in natural vector populations must be accomplished. There are two major lines of research being pursued in the search for genes which will render an individual vector refractory to a particular pathogen. Some researchers are attempting to identify and manipulate naturally occurring refractoriness mechanisms, while others are seeking to identify foreign genes which encode proteins that will disrupt the development of the pathogen in the insect host.

In recent years, the prospect of using transgenic mosquitoes has rapidly gained strength[1,2]. This advance in application of transgenic mosquitoes is attributed to the identification of transposable elements for mosquito germ line transformation, the finding of suitable transformation markers such as fluorescent proteins[3], the standardization of microinjection techniques[4,5], the characterization of promoters that can drive the expression of foreign genes in a tissue- and stage-specific manner[6-9] and the identification and characterization of effector molecules that can interfere with the development of parasites in the invertebrate host[10-13] .

One of the central issues in the development of transgenic mosquitoes is the heterologous expression of transgenes. Heterologous expression is referred to as the foreign or synthesized genes, such as enhanced green fluorescent protein (EGFP), salivary gland- and Midgut-binding peptide1 (SM1) and single chain antibody fragments (scFv), expressed in transgenic mosquitoes under a native or heterologous promoter (Vitellogenin promoter or 3× P3), or an endogenous gene, such as Cecropin A (CecA) or Defensin A (DefA), expressed ectopically under a

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heterologous promoter in a manner different from its native gene. Heterologous expression is crucial for the screening and effecting of the transgenic mosquitoes. Here, we reviewed the relevant progress in the field of heterologous expression in transgenic mosquitoes and proposed some points need to be attended in the future study.

2. Transgenic vectors

Generally, transgenic vectors consist of three functional parts, i.e. selective cassette, effective cassette and transposable element (Figure 1). Studies on these three aspects have gained much progress in the past decade and reliable techniques have been developed. At present, the most often used selective markers are EGFP and DsRed, commonly driven by a tissue specific promoter like 3× P3, the eye-specific promoter. After transformation, the positive transgenic mosquitoes can be easily screened under the fluorescent microscope by observing the specific fluorescence in specific area. Effector molecules include natural (Cecropin A, Defensin A) or synthetic antimicrobial peptides (SM1), antibodies against parasite or mosquito midgut proteins with toxic or inhibitory effect (1C3, Phospholipase A2). Those genes are usually driven by a promoter to be expressed in a specific manner, conferring tissue-, stage- or sex- specificity. The promoters under intensive study include carboxypeptidase (CP), adult peritrophic matrix protein 1 (Aper1), vitellogenin (VG) or antiplatelet protein (Aapp), which direct heterologous expression in midgut, fat body (both Aper1 and VG) and salivary gland, respectively.

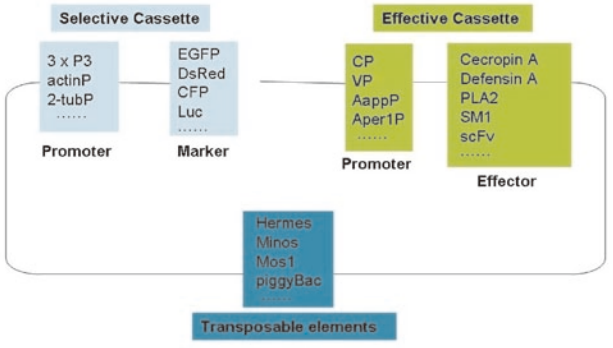

Figure 1. Transgenic Vectors.
3×P3: *Drosophila melanogaster* Pax-6 eye-specific enhancer-promoter; actinP: actin5C promoter from *Drosophila melanogaster*; beta2-tubP: beta2-tubulin promoter; EGFP: enhanced green fluorescent protein gene; DsRed: red fluorescent protein gene; CFP: cyan fluorescent protein gene; Luc: luciferase gene from Photinus pyralis; CP: carboxypeptidase promoter; VP: vitellogenin promoter; AappP: anopheline antiplatelet protein; Aper1P: adult peritrophic matrix protein promoter; PLA2: phospholipase A2; SM1: salivary gland- and midgut-binding peptide 1; scFv: single chain antibody fragment.

The transposable elements such as Hermes, Minos and piggyBac have been used successfully for germ line transformation in more than a dozen species of insects. However, integration sites are randomly distributed in the genome and transgene expression may be site-specific. Most recently, it was reported that chimeric Mos1 and piggyback transposases resulted in site-directed integration[14]. This technology has the potential to minimize non-targeted integration events for development of genetically modified mosquitoes. Another site-specific integration system based on a viral integrase streptomyces phage phi C31 has been proven to increase integration efficiency by up to 7.9-fold[15]. The ability to efficiently target transgenes to specific chromosomal locations and the potential to integrate very large transgenes has broaden research on many medical and economically important species. The integrations driven by nonautonomous transposable elements are stable in the absence of suitable transposase, however, such absence cannot always be guaranteed. This problem could be solved by a method of post-integration elimination of all transposon sequences which was carried out in the pest insect Medfly, Ceratitis capitata[16]. The resulting insertions lack transposon sequences and are therefore impervious to transposase activity.

3. Expression characteristics

The heterologous expression in transgenic mosquitoes can be evaluated at transcriptional and translational levels. Heterologous genes or endogenous genes are transcribed to mRNA under the regulation of varied promoter; this process can be analyzed by northern blot, RT-PCR or quantitative real-time RT-PCR (qRT-PCR). Further analysis includes hybridization in-situ to examine RNA distribution and accumulation in specific tissues, gene amplification methods for detecting the presence and abundance of transcription products, the sequencing analysis of genomic DNA and cDNAs for intron identification and processing of primary transcripts. More over the complete genome sequencing of both the major vectors and parasites helped to have an access at genome-wide transcription profiles. The translated peptide or protein can be analyzed by western blot, ELISA or phenotypic expression (Fluorescence, refractoriness). Heterologous expression in transgenic mosquitoes could be characterized as following:

3.1. Heterologous or endogenous gene expression driven by an endogenous promoter

A reporter gene driven by the endogenous promoter can be transformed into mosquitoes. By analyzing the expression of the reporter gene, the regulatory function of the promoter could be evaluated. Most of promoters, such as AgCP, AsVG, AaVgR, etc, are identified by such strategy (Table 1). For example, the genomic DNA fragments containing cis-acting promoter elements from the Maltase-like I (MalI) and Apyrase (Apy) genes were cloned as to direct the expression of the reporter gene, luciferase (luc)[8]. Analysis of transformed mosquito lines demonstrated that both the MalI and Apy promoter regions were capable of directing the expression of a reporter gene in the correct developmental-sex- and tissue-specific manner as observed for the respective endogenous genes. The result implies that those DNA sequences contain all of the necessary regulatory and control elements for a successful expression of molecules against parasite development or viral replication in the yellow fever mosquito, *Aedes aegypti*(*Ae. aegypti*) . In another study, an 800 bp fragment of AgApy directed expression

of a reporter gene in the salivary glands of transgenic *An. stephensi*[17]. Although the reporter gene product accumulated ectopically in the lobes of female salivary glands, the expression levels of these reporter genes were at low levels. The regulatory region of the vitellogenin (Vg) gene of *Ae. aegypti* were evaluated for its ability to express potential anti-pathogen factor, endogenous defensin, which acts as one of the major insect immune factor, in transgenic mosquitoes[18]. The result showed that high levels of transgenic defensin were ectopically expressed in the hemolymph of blood-fed female mosquitoes under the control of Vg promoter, persisting for 20-22 days after a single blood feeding. This represented the ability to engineer the genetically stable transgenic mosquito with an element of systemic immunity which is activated through the blood meal-triggered cascade rather than by infection.

3.2. Heterologous or endogenous gene expression driven by a heterologous promoter

Most of the selective molecules, like EGFP or DsRed driven by 3×P3 promoter, and effector molecules, like SM1 or PLA2 driven by CP promoter, are expressed in this mode (Table 1). Heterologous promoters cloned from different species but in same genus, or from different genus, or even from different families, have been proved to be capable of driving the heterologous or endogenous gene expression in transgenic mosquitoes.

Owing to the technical difficulty, transformation of *An. gambiae*, the principal vector of malaria in sub-Saharan Africa, is not routine. *An. stephensi*, a vector of urban malaria in southern Asia, is often used to validate the function of *An. gambiae* cis-acting control DNA. The promoter of β_2-tubulin, a gene transcribed selectively in the male gonads, isolated from *An. gambiae*, is used to drive EGFP expression in *An. stephensi*[9]. EGFP fluorescence was observed in the male gonads of the transgenic lines but not in adult female. In a similar study conducted in *Ae. aegypti* with the β_2 orthologous gene, expression of a fluorescent reporter gene marked sperms were detected in spermathecae of inseminated females[19]. The observation that the β_2-tubulin promoter of *An. gambiae* has a tight sex-specific expression pattern in *An. stephensi* argues that β_2-tubulin driven EGFP expression may serve as a robust and common genetic system to generate sexing strains in different mosquito species, in particular those belonging to the *An. gambiae* complex, but also other important disease vectors such as Aedes and Culex mosquitoes. In a recent study, the nanos (nos) gene was expressed in females and is localized in the oocytes which were responsible for determining the anterior-posterior axis in developing embryo. The nos orthologous gene of *Ae. aegypti* was able to control sex-and tissue-specific expression of exogenously derived MosI transposase encoding DNA[20]. Transgenic mosquitoes expressed transposase mRNA in abundance and exclusively in the female germ cells and in addition the transgene mRNA was deposited in developing oocytes and localized and maintained at the posterior pole during early embryonic development. *Ae. aegypti* and *An. gambiae* bear only a distant evolutionary relationship. Thus, it was important to predict whether the AgCP promoter, which does not contain any apparent sequence similarity to the AeCP promoter, would drive gut-specific expression in the *Ae. aegypti* gut. However, both promoters were able to drive robust expression of the reporter gene Luciferase, its mRNA and protein in a blood-inducible manner[6]. One significant difference was the induction of the AgCP promoter by a blood meal is rapid (3 h) in *An. gambiae*, but much slower (24 h) in *Ae. aegypti*. The *Ae. aegypti* carboxypeptidase promoter has also been proved to function well in *An. gambiae* and drive the CecA expression in the posterior midgut beginning 24 h after blood feeding[10].

A GFP marker gene under the control of the promoter region of the *Drosophila melanogaster* act88F gene, a flight muscle-specific promoter, was inserted into the genome of *Culex quinquefasciatus*[21]. GFP expression in the transgenic mosquitoes was restricted to the flight muscle. The result indicated that *Drosophila melanogaster* promoter region can be used in mosquitoes, and other heterologous insects.

3.3. Expression level

Expression levels of heterologous genes are crucial to their function. Many factors may affect the expression, such as the promoter, the coding sequence, the copy number and the insertion place. Robust salivary gland-specific transgene expression was shown in *An. stephensi* mosquito[22]. By comparison with purified DsRed protein commercially available, which was used as standard for quantification, the amount of DsRed protein was calculated to be 25 ng per pair of salivary glands. This represents approximately 5% of total salivary gland protein level, and over 1 000-fold higher level of expression than salivary gland-specific transgene expression reported by others[8,17]. Moreira *et al* reported a robust gut-specific gene expression in transgenic *Ae. aegypti* mosquitoes[6]. Based on the comparison of the signal of the transgenic luciferase protein on Western blots with that of a reference enzyme, it can be calculated that approximately 2 ng of luciferase protein accumulated per gut at 24 h after a blood meal. It is therefore feasible that a protein expressed from the carboxypeptidase promoter will accumulate at sufficiently high concentration in the midgut lumen to act as an effective inhibitor of *Plasmodium* development.

However, the levels of heterologous expression in many reports are not high even very low, so that they cannot be detected at protein level. Kim *et al* reported the ectopic expression of a cecropin transgene in *An. gambiae*[10]. The inability to detect cecA peptide by immunofluorescence suggests that the peptide may be absent or present at very low levels in the posterior midgut. The author´s laboratory had successfully transformed a scFv anti-Pfs25 gene into *An. stephensi*. The RT-PCR and northern blot showed that the transgene transcribed well but western blot did not detect the protein (Unpublished data). Efforts to increase the levels of effectors either by manipulating transcription levels or protein turnover rates might result in an increase in antiparasitic activity.

3.4.Stability of mRNA or protein

Although the expression pattern of heterologous genes is generally consistent with that of the endogenous, some differences have also been noticed, for example, the stability of the expressed mRNA or protein. Yoshida and Watanabe showed that promoters of anopheline antiplatelet protein (AAPP) could drive the Discosoma sp. red fluorescent protein (DsRed) for expressing abundantly in salivary gland[22].

However, unlike the endogenous aapp mRNAs, expression of the DsRed mRNA reached a high level at as early as 24h after a blood meal and kept the expression level for next 24h. One explanation of this abundance of the DsRed mRNA may be due to a high stability of its mRNA. High levels of transgenic defensin were accumulated in the hemolymph of blood fed female mosquitoes, persisting for 20–22 days after a single blood feeding[18]. This stability of the transgene product may make it an effective means of controlling pathogen infection throughout of the adult life of a mosquito. The mechanism of the longer stability still remains unclear.

3.5. Expression specificity

The specificity of heterologous expression includes spatial specificity and temporal specificity; both are determined by the function of promoters. Various promoters exhibit different characteristics of expressions (Table 1). For example, the promoters of carboxypeptidase and adult peritrophic matrix protein 1 can drive heterologous expression in midgut of female mosquito after a blood meal. Vitellogenin promoter can drive heterologous expression in fat bodies of female mosquito after a blood meal. The promoters of anopheline antiplatelet protein (AAPP) and maltase-like I can efficiently drive heterologous expression in salivary glands of female mosquitoes after blood meal[14]. Compared with the carboxypeptidase (midgut) and vitellogenin (fat body) promoters that are activated immediately (<3 h) and around 24 h, respectively, after a blood meal[7,23], some of salivary gland specific promoters may be activated as late as around 48 h after a blood meal to stock the saliva for the next blood feeding. Recently, the promoters driven heterologous expression specific in gonad[9] and ovary [24] have been identified, providing useful tools for the implementation of transgenic strategy.

Table 1
Heterologous genes expressed in mosquitoes.

Transformation Vector			Expression Specificity				Function		
Genes	Promoter	TE	Species	Sex	Tissue	Stage	Marker	Effector	Fitness
EGFP[1]	actinP	Minos	*An. stephensi*	Both	Muscle	Constitutive	Yes	No	NA
EGFP[2]	actinP	Minos	*An. stephensi*	Both	Muscle	Constitutive	Yes	No	Yes
EGFP[3]	β_2-tubP	piggyBac	*An. stephensi*	Male	Gonads	Constitutive	Yes	No	NA
EGFP[4]	3xP3	phi C31	*Ae. aegypti*	Both	Eyes	Constitutive	Yes	No	NA
EGFP[5]	act88FP	Hermes	*Cx. quinque*	Both	Muscle	Constitutive	Yes	No	NA
EGFP[6]	actinP	Hermes	*Cx. quinque*	Both	Muscle	Constitutive	Yes	No	NA
DsRed[7]	aappP	Minos	*An. stephensi*	Female	SG	Vitellogenic	Yes	No	NA
DsRed[3]	3xP3	piggyBac	*An. stephensi*	Both	Eyes	Constitutive	Yes	No	NA
DsRed[4]	3xP3	phi C31	*Ae. aegypti*	Both	Eyes	Constitutive	Yes	No	NA
DsRed[8]	AaVgRP	piggyBac	*Ae. aegypti*	Female	Ovary	Vitellogenic	Yes	No	NA
Luciferase[9]	AeCP	Hermes	*Ae. aegypti*	Female	Midgut	Vitellogenic	Yes	No	NA
Luciferase[9]	AgCP	Mos1	*Ae. aegypti*	Female	Midgut	Vitellogenic	Yes	No	NA
Luciferase[9]	MalIP	Hermes	*Ae. aegypti*	Female	SG	PBM	Yes	No	NA
Luciferase[9]	ApyP	Hermes	*Ae. aegypti*	Female	SG	PBM	Yes	No	NA
CFP[10]	AsVg1P	piggyBac	*An. stephensi*	Female	Fat body	Vitellogenic	Yes	No	NA
SM1[11]	AgCP	piggyBac	*An. stephensi*	Female	Midgut	PBM	No	Yes	No
PLA2[11]	AgCP	piggyBac	*An. stephensi*	Female	Midgut	PBM	No	Yes	NA
PLA2[12]	AgAper1P	piggyBac	*An. stephensi*	Female	Midgut	PBM	No	Yes	Yes
DefA[13]	AeVg	Hermes	*Ae. aegypti*	Female	Fat body	PBM	Yes	Yes	NA
DefA[13]	AeVg	piggyBac	*Ae. aegypti*	Female	Fat body	PBM	No	NA	NA
CecA[14]	AeCP	piggyBac	*An. gambiae*	Female	Midgut	PBM	No	Yes	NA

1: Reference 5; 2: Reference 32; 3: Reference 9; 4: Reference 15; 5: Reference 21; 6: Reference 36; 7:Reference 22; 8: Reference 24; 9: Reference 6; 10: Reference 8; 11: Reference 25; 12: Reference 26; 13: Reference 28; 14: Reference 7; 15: Reference 10.

4. Effect of the expression

The recent generation of genetically transformed mosquitoes has raised hopes for the production of mosquito strains that are unable to transmit various parasites or viruses. There are a number of examples of exogenous molecules that, when expressed or introduced into mosquitoes, are able to block the transmission of pathogens. Endogenously derived, tissue specific promoters are used to drive the expression of anti-parasitic effector molecules in mosquitoes to minimize potential fitness costs and to maximize parasite blocking.

4.1. Selective marker

The expression products of fluorescent genes, such as EGFP, DsRed, CFP and Luciferease, are often used as the selective marker to screen the transgenic mosquitoes. In 2000, Catteruccia *et al* reported the first reliable system of germ line transformation of the malaria vector using EGFP. Their results showed that EGFP was as a reliable marker for *An. stephensi*, as for other insect species. The coding sequences of fluorescent genes are usually driven by a tissue specific promoter like 3xP3, so that the transgenic mosquitoes can be easily identified if the fluorescence is observed in specific tissue (eyes). The 3xP3 promoter has been used in a wide variety of insects ranging four orders.

The expression of fluorescent genes can be used to analyze the function of promoter. The appearance of fluorescence at different times and tissues indicates the regulatory functions of the promoters. The functional characterization of AsVg1, the promoter of the vitellogenin gene of *An. stephensi* was observed by the expression of cyan fluorescent protein (CFP)[25]. The results showed that CFP expressed specifically in fat bodies of female mosquitoes after a blood meal, indicating that the AsVg1 promoter could serve as a candidate for controlling the expression of anti-pathogen effector molecules in *An. stephensi*. The following study confirmed that AsVg1 could direct a scFv gene expression in a sex-, stage- and tissue-specific manner in transgenic *An. stephensi* (unpublished data).

The disadvantage for fluorescence as a selective marker is the existence of plausible autofluorescence in some insects. Green and red antofluorescence was detected in the accessory glands of both transgenic and wild-type males. A weak green autofluorescence was also seen in the thorax of the wild-type mosquitoes[22]. Given the confounding autofluorescence, a specific detection of the fluorescent expression, such as RT-PCR or western blot, may be needed to verify the engineered expression.

4.2. Anti-pathogen

The identification of an "effector gene" able to interfere with the development of the parasite in the mosquito is an essential prerequisite for the generation of a refractory mosquito. Ideally, the effector gene expression should kill the parasite or block its further development without imposing a fitness load on mosquito. Several attempts have been made to identify such an effector gene. The first experiment of blocking malaria parasite transmission using transgenic approaches was reported in 2002[26] based on a 12-amino-acid peptide, termed SM1 for salivary gland and midgut binding was selected from a bacteriophages displaying library[13]. A synthetic gene [termed AgCP(SM1)$_4$] consisting of four SM1 units joined by 4-amino-acid linkers attached to the CP signal sequence and driven by the gut-specific and blood-inducible *An. gambiae* carboxypeptidase (AgCP) promoter was constructed and then inserted into a piggyback vector and transformed into the germ line of the mosquito *An. stephensi*[26]. The results showed that SM1 expressed specifically in female transgenic mosquito midgut after blood meal induction and the expressed SM1 tetramer binds to the luminal surface of the midgut, inhibiting parasite-epithelium interactions and midgut invasion.

In the same year, transgenic *An. stephensi* mosquitoes that expressed the bee venom phospholipase A2 (PLA2) gene with AgCP as the promoter were reported[27]. Northern blot analysis indicated that the PLA2 mRNA was specifically expressed in the guts of transgenic mosquitoes with peak expression at 4 h after blood ingestion. Western blot and immunofluorescence analysis detected PLA2 protein in the midgut epithelia of transgenic mosquitoes from 8 to 24 h after a blood meal. Importantly, transgene expression reduced *Plasmodium berghei* oocyst formation by 87% on average and greatly impaired transmission of the parasite to naive mice. The results indicate that PLA2 may be used as an additional effector gene to block the development of the malaria parasite in mosquitoes. In 2005, the function of phospholipase A2 (PLA2) was further analyzed with the regulatory gene of *An. gambiae* adult peritrophic matrix protein 1 (AgAper1) promoter[28]. The data displayed that AgAper1 regulatory elements were sufficient to promote the accumulation of PLA2 in midgut epithelial cells before a blood meal and its release into the lumen upon blood ingestion. *Plasmodium berghei* oocyst formation was reduced by 80% in transgenic mosquitoes.

Single-chain antibody fragments (scFv) directed against parasite ligands and expressed as a single gene have shown high efficacy in transmission blockage. An scFv against the circumsporozoite protein (N2 scFV), which is abundant on the surface of sporozoites and essential for gliding motility and the invasion of salivary glands, was expressed in *Ae. aegypti* through engineered Sindbis viruses[12]. After infection, the numbers of *P. gallinaceum* sporozoites detected in the salivary glands were reduced by 99%. ScFvs targeting parasite ligands expressed at earlier sporogonic stages (e.g. the ookinete surface proteins P28 and P25) are additional candidates for transmission blocking. Some have proven efficacy in reducing parasite numbers when provided to mosquitoes through the blood meal.

Immunity effector genes encoding antimicrobial peptides (AMPs) have been shown to be effective against *Plasmodium* in *in vitro* assays[29]. Transgenic mosquitoes carrying either Vg-DefA or Vg-CecA transgenes exhibited resistance to the gram-negative bacterium *Enterobacter cloacae* that was nearly twice as high as that of the wild-type mosquito. Further studies showed that two independent transgenic *Ae. aegypti* strains over-expressing Defensin A inhibited oocyst growth of *P. gallinaceum* by 65%-70%. Similarly, transgenic over-expression of endogenous Cecropin A or Defensin A in the fat body of adult *Ae. aegypti* mosquitoes have led to strong inhibition of *P. gallinaceum* sporozoite development. Scorpine, a 75 amino acid peptide isolated from *Pandinus imperator* venom, whose structure resembles a hybrid between a defensin and a secropin exhibits antibacterial activity and inhibits the sporogonic development of murine malaria parasite. The recombinantly

expressed scorpine (RScp) in *An. gambiae* cells under the control of *An. gambiae* serpin promoter showed antibacterial activity against *Bacillus subtilis* and *Klebsiella pneumoniae* and 98% mortality in sexual stages of *Plasmodium berghei* and 100% reduction in *P. falciparum* parasitemia[30]. Two independent lines of transgenic *An. gambiae* were created using a piggyback gene vector containing the *An. gambiae* cecA cDNA driven by the CP promoter originated from *Ae. aegypti*[10]. Infection with *Plasmodium berghei* resulted in a 60% reduction in the number of oocysts in the transgenic mosquitoes compared with non-transgenic mosquitoes. Manipulating the innate immune system of mosquitoes can negatively affect their capacity to serve as hosts for the development of disease-causing microbes. This was the first report of genetically engineered *Plasmodium* refractoriness in *An. gambiae*.

4.3. Fitness load

It has been proposed that transgenic organisms are less fit because they are evolutionary novelties with reduced viability[31]. Transgenic mosquitoes typically express multiple heterologous genes; for example, a fluorescent marker and an anti-pathogen effector protein. The accumulation of foreign proteins might be toxic to the cells in which they are expressed. Proteins expressed in a restricted cell type are less likely to have an impact on fitness than ubiquitously expressed proteins. For instance, fluorescent protein expressed in the eye does not appear to affect fitness. However, in another study[32], factors influencing fitness were investigated in cage experiments with four lines of transgenic *An. stephensi*, in which EGFP was expressed ubiquitously under the control of actin5C promoter of *Drosophila melanogaster* (actinP). The results indicate direct costs of the introduced transgenic in at least three out of four lines, as well as an apparent cost of the inbreeding involved in making transgenic homozygotes. The author suggested that it is highly desirable to construct engineered lines that are substantially out bred.

Catteruccia *et al*[32] have shown that homozygous transgenic *An. stephensi* has lower fitness than wild type. Similar studies have been characterized in transgenic *Ae. aegypti* also. Reduced fitness observed in the transgenic population may be caused due to the presence of recessive genes near transgene insertion point. Other possible explanations for this lower fitness suggests the homozygous expression (hitchhiking effect), over-expression of a foreign protein (eg: GFP) in somatic tissues and insertional mutagenesis[33].

The nature of the expressed protein itself is a crucial factor for fitness[33]. For example, although no effect on fitness was observed for mosquitoes expressing the 12 amino acid peptide SM1[26], mosquitoes expressing the phospholipase A2 (PLA2) were clearly less fit and less fertile than wild type[28]. In a recent study conducted by Chaoyang Li *et al*[34] compared the fitness of hemizygous transgenic mosquitoes which secrete into the midgut either of two effector proteins, SM1 and phospholipase A2 under the control of the blood inducible CP promoter. They showed that heterozygous SM1 expressing transgenic mosquitoes exhibited no detectable fitness load, whereas PLA2 expressing mosquitoes showed reduced fitness and midgut damage presumably as a result of phospholipase enzymatic activity. The SM1 transgenic mosquitoes when fed on mice infected with gametocyte producing *Plasmodium berghei* exhibited a fitness advantage over sibling non-transgenic mosquitoes, and showed higher fecundity and lower mortality in them[35]. At the same time this fitness load was not visible when fed on non infected mice. These suggest the existence of a strong selective advantage between the transgenic and non-transgenic lines.

The measure of fitness of these transgenic mosquitoes were studied recently[34] and the fitness load of three independently derived homozygous transgenic lines were attributed to lower mating success, possibly, derived from hitchhiking effect or insertional mutagenesis. Several advantages of using transgenic lines for future genetic approach to control most of the vector-born diseases are enlighted. These include stronger and more effective gene expression, ease in mass rearing and eventual field release and more efficient transgene introgression in the field. However, the use of homozygous mosquitoes encountered a basic problem of showing hitchhiking effects of potential recessive deleterious mutations near the site of transgene insertion. Hence, these findings highlights the need to use effector genes that minimize mosquito fitness costs and select lines with integration events that do not reside near loci which confer high fitness costs when homozygous. This fitness advantage has important implications for devising malaria control strategies by means of genetic modification of mosquitoes.

5. Conclusion

Mosquito transmitted diseases including malaria prevalence in the developing world is increasing, and there is an immediate need to develop novel strategies for disease control. Based upon the precedents of controlling mainly agricultural pests, and the potential of recent biotechnological advances, the concept of genetic control of mosquito populations is attractive. The research on heterologous expression in transgenic mosquitoes has progressed much in recent years. The expression of fluorescent proteins facilitates the screening of transformants and functional analysis of the regulatory regions. The identification of a growing number of effector molecules brings us closer to the promise of a genetic strategy to control the transmission of mosquito-borne diseases. We identified several critical gaps in our knowledge and technology. Firstly, the expression levels of heterologous genes in transgenic mosquitoes are usually low, which might affect their ability to block transmission. Secondly, there is yet no complete inhibition of transmission in transgenic mosquitoes. Novel effector molecules with more effective anti-pathogen function need to be developed. Thirdly, multi-effector molecules need to be expressed in the same transgenic mosquitoes, which might increase the effect of anti-pathogen as well as decrease the possibility of mosquito resistance to the effectors.

Conflict of interest statement

We declare that we have no conflict of interest.

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