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Review Article

Genetically Modified Mosquitoes: A New Strategy to Control Mosquito Borne Diseases

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Abstract

Mosquito borne diseases remain a major human and animal health concern in many areas of the world. Chemical control strategy faces a number of problems including the development of insecticide resistance in mosquitoes, the impact of insecticides on human and animal health and the environment and the constant inability to control mosquitoes effectively. Advances in the knowledge about mosquito-pathogen relationships and the molecular biology of mosquitoes make it now possible to produce mosquito strains that are unable to transmit various parasites or viruses. Strategies of using the genetically modified mosquitoes to control mosquito borne diseases are discussed here.

Keywords : Mosquito, genetically modified mosquito, transgenic mosquito, transposon, mosquito borne disease.

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บทคัดย่อ

ยุงดัดแปลงพันธุกรรม: กลยุทธ์ใหม่ในการควบคุมโรคที่นำโดยยุง

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โรคที่นำโดยยุงยังคงเป็นปัญหาทางสุขภาพทั้งในคนและสัตว์ในหลายพื้นที่ของโลก วิธีการควบคุมยุงโดยใช้สารเคมีประสบปัญหาหลายอย่างรวมทั้งการดื้อสารเคมีกำจัดแมลง ผลกระทบต่อสารเคมีกำจัดแมลงเหล่านี้ต่อคนสัตว์และสิ่งแวดล้อม ทำให้การควบคุมยุงไม่มีประสิทธิภาพ ความก้าวหน้าในความรู้เรื่องความสัมพันธ์ระหว่างยุงกับเชื้อก่อโรครวมทั้งความก้าวหน้าในด้านอณูชีววิทยาทำให้มีความเป็นไปได้ที่จะสามารถสร้างยุงสายพันธุ์ที่ไม่มีความสามารถในการถ่ายทอดโรคที่เกิดจากปรสิตและไวรัสได้ บทความนี้เกี่ยวข้องกับกลยุทธ์การใช้ยุงดัดแปลงพันธุกรรมในการควบคุมโรคที่นำโดยยุง

คำสำคัญ: ยุง, ยุงดัดแปลงพันธุกรรม, transgenic mosquito, transposons, โรคที่นำโดยยุง

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Introduction

Mosquito-borne diseases are still a major human and animal health problem in many countries. Diseases transmitted by mosquitoes include malaria, filariasis, yellow fever, Japanese encephalitis and dengue fever. The presence of multi-drug resistant strains of the pathogen has led to difficulty in controlling the diseases. The development of novel vaccines also faces the problem of antigenic variation in the pathogens. Chemical control used to be the primary strategy for controlling mosquito-borne diseases, but concerns about the impact of the available compounds on human and animal health and the environment, together with the development of insecticide resistance in mosquitoes, has limited the usefulness of this approach (Taksinvarajarn et al., 2004; Thavara et al., 2006). Through advances in our knowledge of mosquito-pathogen relationships and the molecular biology of the mosquito, it is possible to produce mosquito strains that are incapable of transmitting various parasites or viruses (Collins and James, 1996; Beerntsen et al., 2000). There are a number of molecules which, when expressed or introduced into mosquitoes, are able to block the transmission of pathogens. For example, monoclonal antibodies directed against a circumsporozoite protein of the avian parasite, *Plasmodium gallinaceum*, blocks sporozoites from entering the salivary glands of the mosquito, *Aedes (Ae.) aegypti*, when injected into the hemolymph (Warburg et al., 1992). Expression of a 12 amino-acid peptide (SM1) that binds specifically to the midgut lumen and distal salivary gland lobes of mosquitoes or a bee venom phospholipase (PLA2) in *Anopheles (An.) stephensi* mosquitoes strongly reduces the number of the developing rodent parasite, *Plasmodium berghei*, in

the midgut of these transgenic mosquitoes (Ito et al., 2002; Moreira et al., 2002). More recently, expression of RNA interference against the dengue virus type 2 in the midgut of genetically modified *Ae. aegypti* mosquitoes interrupted the dengue type 2 replication in the transformed mosquitoes (Franz et al., 2006). Expression of similar molecules in genetically transformed mosquitoes could lead to the production of strains that can be used in the control of transmission of pathogens (Coates et al., 1999; James, 2002).

Requirements for creating genetically modified mosquitoes

Genetic transformation is defined as the uptake and expression of exogenous DNA by cultured cells or whole organisms. Gene transfer may be transient, reflecting the episomal state of the introduced DNA, or stable following the integration of DNA into the chromosome (Besansky et al., 1992). Genetic manipulation depends on the successful solution to four separate but independent problems: (1) the delivery of DNA into the mosquito embryo, (2) the efficient and stable integration of DNA into chromosome, (3) a promoter to control expression of the exogenous gene and (4) a suitable marker gene that encodes a dominant phenotypic trait.

DNA delivery

The problem of introducing DNA into mosquito embryos has been overcome by the development of microinjection techniques. The technique for microinjection of mosquito embryos was based on that used to introduce DNA into the developing embryo of *Drosophila (D.) melanogaster* (Rubin and Spardling, 1982). The microinjection technique has been carried out using *An. gambiae* (Miller et al., 1987), *Ae. triseriatus* (McGrane et al., 1988), *Ae. aegypti* (Morris et al., 1989), *An. stephensi* (Catteruccia et al., 2000) and *Culex (Cu.) quinquefasciatus* (Allen et al., 2001).

To facilitate germline transformation, the exogenous DNA must be introduced into the developing embryo prior to pole cell formation (approximately 90-120 minutes after mosquito eggs have been laid, Fig. 1). The introduced plasmid will be incorporated into the developing pole cells and will integrate into the chromosomal DNA. The integrated DNA sequences may then be expressed throughout the somatic tissue of subsequent generations.

DNA integration

Transposable elements (transposons) are DNA segments that can insert themselves in a genome. They can be maintained in a variety of related genomes, serve as an agent of chromosomal insertion, deletion or rearrangement, and provide the basis for the transformation of somatic or germ cells (Garza et al., 1991). Class II transposable element or DNA element, a group which consists of elements that transpose DNA to DNA directly are widely used for genetic transformation including mosquito transformation. The class II transposable elements are characterized by their small inverted terminal repeats of less than 100 base pairs bordering an internal transposase-encoding sequence which functions in a DNA-only mechanism of transposition (McDonald, 1993).

Exogenous DNA has been successfully introduced into the mosquito germ line using the P element transposon and some integration events have been stable over multiple generations (Miller et al., 1987; McGrane et al., 1988; Morris et al., 1989). However, in all cases, the efficiency of integration has been at least 10-fold lower than expected from *Drosophila* even though survival rates following injection were comparable. It is now accepted that the P element is not a functional transformation vector in non-drosophilid insects in that it is phylogenetically restricted (Handler et al., 1993). To date, the elements

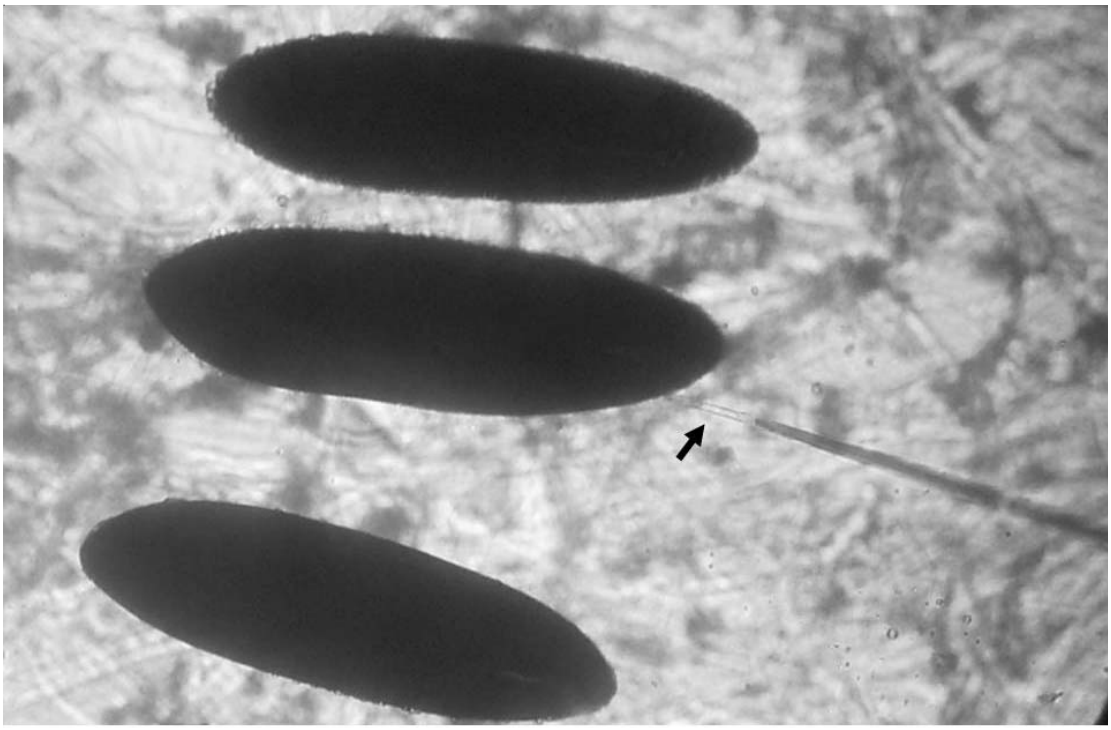

Figure 1 Microinjection of *Aedes aegypti* embryos: The embryos were oriented with their posterior aligned. DNA solution was injected into the embryos via a capillary needle (arrow).

which have been used effectively to transform mosquitoes are the *Mos1 (mariner), Hermes, Minos and piggyBac* (O'Brochta et al., 2003).

Promoters

The promoter is crucial to express the introduced gene (transgene) in cooperation with the transcriptional apparatus of the host. The application of species-specific promoters could provide the benefits of increase levels and control of gene expression. Numerous groups have cloned a variety of both constitutively expressed (the promoters are those active in all cells at all times) and tissue, sex and stage-specific genes from the mosquitoes *An. gambiae* and *Ae. aegypti,* including those for vitellogenin (Romans et al., 1995), α glucosidase, α amylase, the female-enriched protein D7, and the *Apyrase (Apy)* gene from female *Ae. aegypti* salivary gland (James et al., 1989;1991; Smart et al.,1995). The *Maltase-like I (Mal I)* and the *Apy* gene promoters have been successfully used to express the reporter gene luciferase *(luc)* in both cultured cells and in *Ae. aegypti* embryos (Coates et al., 1999). More recently the hexamerin promoter from the mosquito *Ochlerotatus atropalpus* has been used to drive expression of the *luc* reporter gene in *D. melanogaster* (Jinwal et al., 2006) and it seems to be a sex-, stage- and tissue-specific promoter.

Reporter genes and Selectable markers

Marker genes are one of the most important factors in genetic transformation. The choice of suitable marker genes remains critical for detection transformants. A dominant genetic marker or a

recessive marker and a suitable mutant recipient are essential. The marker must be suitable for the detection of a relatively rare event. If no suitable mutant strains are available, then the choice is usually limited to a marker that confers resistance to a toxic chemical or to one that, when expressed, confers an easily scored visible phenotype (Ashburner et al., 1998).

The use of an antibiotic or insecticide resistance strategy to select transgenic insects can lead to a generation of false positives due to, for example, the breakdown of the insecticide or antibiotic. Furthermore, genuine transformants can be lost if the level of expression of the transgene is not sufficient to confer antibiotic or insecticide resistance to the transgenic individual (Pinkerton et al., 2000).

Eye color marker genes appear to be the most useful markers to select transformants for several reasons; they generally produce unequivocal phenotypes, the transformants are easily identifiable and are viable under normal laboratory conditions. The genes involved in eye color biosynthesis fall into three classes: genes required for ommochrome biosynthesis, genes required for pteridine biosynthesis and genes required for both pathways. Typically, the last class gives an unpigmented (white) eye. The *white* genes of *Drosophila, An. gambiae* (Kaiser et al., 1982; Besansky et al., 1995), *Ceritatis capitata* (Zweibel et al., 1995), *Lucilia cuprina* (Garcia et al., 1996), *Batrocera tryoni* (Bennett and Frommer, 1997) and *Ae. aegypti* (Coates et al., 1997) are members of this class. In some cases a similar phenotype may also be caused by a mutation in the ommochrome or pteridine pathways as in *An. gambiae* (Mukabayire et al., 1996) or *Ae. aegypti* (Bhalla, 1968), where color metabolites of only one pigment class are present. Mutations in early biochemical steps of ommochrome or pteridine biosynthesis can be especially valuable as they may well be non-autonomous at the cellular level. This means that neither tissue-specific transgene expression nor expression at a high level is required for complementation (Ashburner et al., 1998). This is the case, for example, with the *rosy* (xanthine dehydrogenase), *cinnabar* (kyneurenine 3-hydroxylase) and *vermilion* (tryptophan oxidase) mutants of *Drosophila.* The *cinnabar* (cn^+) gene from *D. melanogaster* has been cloned (Warren et al., 1996) and has been successfully used to complement the white-eye phenotype of the *kynurenine hydroxylase-white* (kh^w) strain of *Ae. aegypti* (Cornel et al., 1997; Coates et al., 1998; Jasinskiene et al., 1998; Siriyasatien, 2000; Sethuraman and O'Brochta, 2005).

The Enhanced Green Fluorescent Protein (EGFP) gene from the jelly fish *(Aequorea victoria)* has been used as a marker in both cultured mosquito cells (Zhao and Eggleston, 1999) and in mosquitoes *Ae. aegypti* (Pinkerton et al., 2000) and *An. stephensi* (Catteruccia et al., 2000). Recently, the Ds Red gene from the coral Discosoma has been cloned (Fradkov et al., 2000) and introduced into transgenic An. stephensi mosquitoes (Catteruccia et al., 2003). The EGFP and the Ds Red genes appear to be stably integrated into the genome as they have been inherited over at thirty generations without detectable loss of the fluorescent marker or evidence of transposon mobilization (Catteruccia et al., 2003). The EGFP and Ds Red genes are now said to be the universal markers for transgenic insects. The benefit of these genes is that microinjection can be performed using wild type mosquitoes and it is easy to identify the transformants by examination under a fluorescence microscope (Berghammer et al., 1999).

Strategies for using genetically modified mosquitoes to control mosquito borne diseases

With the microinjection technique now developed and the availability of a number of transposable element based vector systems, the tissue

specific promoters and the universal marker genes, it is possible routinely to introduce exogenous DNA into mosquitoes. If the gene encoding for anti-pathogens can be introduced and expressed in mosquitoes as a part of a transposable element-based expression construct, it may be possible to block the development of pathogens in mosquitoes. Several studies have reported the success of creating transgenic mosquito strains which are resistant to pathogens. Expression of an SM1 or a bee venom phospholipase PLA2 in *An. stephensi* mosquitoes in a midgut specific manner strongly reduced the number of the developing rodent parasite, *Plasmodium berghei*, in the midgut of these transgenic mosquitoes (Ito et al., 2002; Moreira et al., 2002). Allen and Christensen (2004) demonstrated the expression of the GFP under the control of the flight muscle specific *act88F* gene in *Cu. quinquefasciatus mosquitoes*. The GFP was expressed specifically in the flight muscle of the transformed mosquitoes, and this is the part of the strategy to engineer genetically modified mosquitoes refractory to filarial parasite development. More recently, expression of RNA interference against the dengue virus type 2 under the control of a bloodmeal specific promoter, in the midgut of genetically modified *Ae. aegypti* mosquitoes was able to interrupt dengue virus type 2 replication in the transformed mosquitoes (Franz et al., 2006).

Genetically modified mosquitoes in natural populations

Once transgenic mosquitoes have been created, it is necessary to consider the problems likely to be faced in applying the technology in experimental and natural populations. There are several issues that must be addressed.

Fitness cost

Fitness cost is defined as the relative success with which a genotype transmits its genes to the next generation (Marrelli et al., 2006). There are two major components of the fitness cost, survival and reproduction, which can be evaluated by analyzing several parameters, such as fecundity, fertility, larval biomass productivity, developmental rate, adult emergence, male ratio and mating competitiveness. Fitness of genetically modified mosquitoes can be reduced from the negative effect of transgene products such as fluorescent markers and anti-pathogen proteins or from insertional mutagenesis after a transposition event (Catteruccia et al., 2003; Marrelli et al., 2006). Catteruccia et al. (2003) demonstrated that the reductions of fitness of the transgenic *An. stephensi* mosquitoes is caused by the expression of an exogenous gene and the mutations from the insertion of the transgene. The fitness cost of transgenic *Ae. aegypti* mosquitoes has been examined by Irvin et al. (2004), the results show that fitness of transgenic mosquitoes were reduced significantly compared to not-transgenic mosquitoes. The fitness of the genetically modified mosquitoes should therefore be evaluated under laboratory conditions for planning release strategies.

Stability

Stability of the integrated gene is a crucial issue for application, in either the mass-rearing factory or in the field. The problems posed by these two environments differ greatly, since in the former there will be control over the genotype while in the field it will not be the case except in particular cases of the field release of sterile transformants. In the field, the release of fertile transformants may lead to potential interactions between the transgene and other genes or transposable elements that will affect stability (Asburner et al., 1998). The stability of transformants should therefore be tested under experimental conditions before any release can be completed.

Safety

There are other concerns that will dictate the use of transgenes in mass-rearing factory conditions. Transgenes that might be considered safe under

laboratory conditions will have to face severe regulatory tests before being cleared for use in the factory or in the field. The problem of containment needs to be addressed. Transgenes requiring selection with a toxic agent or conferring pesticide resistance should not be used.

Field Objective

The objectives of field release will differ among target organisms but will include eradication, population suppression and population replacement. However, there are some general principles. Firstly, the need for markers for field-release organisms, enabling the unambiguous distinction between transformants and the endemic population. Secondly, a consideration of the consequences of a successful field release of another transgenic strain of the same species. Some designs may be essentially "one-shot", for example, any release of a mobile transgene that leads to the evolution of a transposition suppression may prevent any subsequent release of a transgene carried by the same vector (Ashburner et al., 1998).

For target species where population replacement is an objective there is an urgent need for research on how this may be achieved. One possible mechanism is meiotic drive. This describes any event occurring during meiosis, by which one particular chromosome is recovered preferentially over its homologue. Such a mechanism has been demonstrated in *Ae. aegypti* when linkage to the M^D locus was used to drive the marker gene, *re* (red eye) into a caged population (Wood et al., 1977). Transposable elements, the possible basis of a gene transfer system, also have potential as drive mechanisms. The efficiency of a transposon-mediated drive mechanism is such that it is theoretically possible to spread even and unfavorable traits through a population, before natural selection can act on it, in spite of the sometime deleterious consequences of transposition itself (Ribiero and Kidwell, 1994). The rapid spread of a transposable elements through a natural population has already been demonstrated by the P element, which has invaded populations of *D. melanogaster* worldwide within the past fifty years (Anxolabehere et al., 1988), having invaded the species from the distantly related *D. willistoni*. Another advantage of transposable elements is that some have shown themselves capable of transferring across species boundaries (Houck et al., 1991). However, the impact of releasing fertile transgenic mosquitoes in the gene pool of the natural population needs to be fully assessed.

Risk evaluation and public acceptance

The release of any transgenic organism into the field will be governed by local and national regulatory agencies. Any proposal to release will generate concern among both the general public and pressure groups. Both the must be addressed earlier, rather than later, if field release is the intended end-point of technology development (Hoy, 2003).

Conclusion

Advances in our knowledge of the molecular biology of mosquito vectors and mosquito-pathogen relationships, allow us to create transgenic mosquitoes that are refractory to the pathogens. Although the stability of these transgenic mosquitoes in a caged population can be made, the fitness of the transgenic mosquitoes is diminished compared to wild type mosquitoes. Once the problem of the fitness of transgenic mosquitoes has been overcome, the safety and effect of releasing these transgenic mosquitoes into environment needs to be fully assessed. Before contemplating release of transgenic mosquitoes containing active transposable elements, one must be aware of the possibility of horizontal transmission of the transgene to non-target species. Public acceptance of the release of transgenic mosquitoes must also be considered.

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