DsRed2 transient expression in *Culex quinquefasciatus* mosquitoes

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Culex quinquefasciatus *mosquitoes have been successfully genetically modified only once, despite the efforts of several laboratories to transform and establish a stable strain. We have developed a transient gene expression method, in* Culex*, that delivers plasmid DNA directly to the mosquito haemolymph and additional tissues. We were able to express* DsRed2 *fluorescent protein in adult* Cx. quinquefasciatus *mosquitoes by injecting plasmids directly into their thorax. The expression of* DsRed2 *in adult* Cx. quinquefasciatus *mosquitoes is an important stepping stone to genetic transformation and the potential use of new control strategies and genetic interactions.*

Key words: *Culex quinquefasciatus* - transient expression - *DsRed2*

Mosquitoes are responsible for the transmission of major human disease agents (Moreira et al. 2000, Cheng et al. 2011). *Anopheles, Culex* and *Aedes* genera include vectors for the three major groups of human pathogens: parasites of the *Plasmodium* genus, which cause malaria, filarids of the *Wuchereria* and *Brugia* genera, which cause filariasis, and a variety of arboviruses, including dengue, yellow fever and West Nile (Atkinson & Michel 2002, Wilke et al. 2009b).

Given the failure of current methods to control the spread of many of these diseases, alternative methods of control are desperately needed, so considerable effort has gone into novel genetic mosquito control strategies (Atkinson et al. 2007). Substantial progress has been made over the last decade towards generating transgenic mosquitoes (Wilke et al. 2009a).

Two broad classes of genetic control strategies have been proposed. "Population suppression" strategies aim to reduce the number of vector mosquitoes in the target area. "Population replacement" strategies aim to make the vector population less effective at transmitting relevant pathogens, without necessarily reducing the number of mosquitoes. Population suppression strategies require the expression of effector molecules that reduce the fitness (e.g. viability, fertility) of affected mosquitoes. Population replacement requires the expression of effector molecules that affect the ability of the mosquito to transmit the pathogen ("refractoriness genes"); additionally "gene drive" systems may be required to spread such genes through the target population. In each case, there is a need to identify specific DNA sequences which will impart the necessary phenotype when present in the mosquito genome. The traditional way to test candidate effector sequences is by constructing stable transgenic lines in which the candidate sequence is inserted into the mosquito genome. However, germline transformation is a time-consuming process and routine in only a few mosquito species. In particular, there has been only two reports of transformation of *Culex* mosquito by a same group (Allen et al. 2001, Allen & Christensen 2004), despite the efforts of several laboratories.

While germline transformation is likely to remain essential for many purposes, it would be highly desirable to have a faster method for initial screening of candidate effector molecules and plasmid functionalities. Based on the success of adult RNAi microinjection in *Aedes* and *Anopheles* mosquito species (Blandin et al. 2002, Hansen et al. 2004) and the observation that plasmid DNA is transcribed in various mammalian tissues following injection of purified DNA (Zhang et al. 2003, Danialou et al. 2005), we have developed a mosquito transient gene expression method, based on the delivery of plasmid DNA directly to the mosquito haemolymph and additional tissues.

The development of a well-established adult microinjection system in *Culex* mosquitoes is crucial to the implementation of new technologies such as paratransgenesis and interactions between bacteria and mosquito (Kambris et al. 2010), as well as the study of gene promoters and refractory genes (Ren et al. 2008, Coutinho-Abreu et al. 2010, Fang et al. 2011, Rasgon 2011). We were able to express *DsRed2* fluorescent molecular marker in adult *Culex quinquefasciatus* mosquitoes by micro-injecting plasmids directly into the thorax.

To be able to test if the target mosquito is capable of expressing the gene of interest, in such a way, is highly advantageous since it is a very arduous process to transform and maintain transgenic mosquito strains. *Cx. quinquefasciatus* mosquitoes were injected in the thorax with a *DsRed2* marker comprising *DsRed2* coding sequence under the control of a baculovirus promoter (Hr5IE1) which has previously been shown to give visible ubiquitous red fluorescence in transgenic *Aedes aegypti* (Dafa'alla et al. 2006). This marker gene was flanked by the ends of a *piggyBac* element potentially

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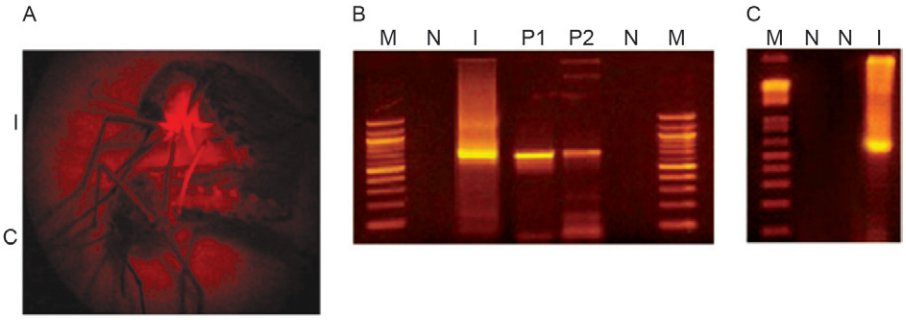

A: fluorescence micrograph showing transient expression of *DsRed2* molecular marker in a *Culex quinquefasciatus* mosquito injected with a *DsRed2* plasmid (I) and a control group mosquito (C); B: ethidium bromide stained 1% agarose gel showing the amplification of the *DsRed2* gene by polymerase chain reaction [M: DNA marker New England Biolabs (100 bp); N: no template control; I: gDNA from *DsRed2* expressing individual; P: *piggyBac Hr5IE1-DsRed2* plasmid (675 bp)]; C: reverse transcription-polymerase chain reaction of *Cx. quinquefasciatus* injected mosquito (I) utilising specific *DsRed2* primers confirming the presence of cDNA M, marker as above.

suitable for germline transformation. Newly born female mosquitoes were divided into groups of 50 mosquitoes each and injected in the thorax with plasmid injection mix (n = 200) or phosphate buffered saline for the control group (n = 50). After injection mosquitoes were fed on sugar and after three-five days screened for red fluorescence under a microscope. Mosquitoes that survived after the thorax injection (60-80%) were screened by fluorescence microscopy and fluorescence was observed in 2% of the injected mosquitoes (A in Figure).

To test that this fluorescence did correspond to *DsRed2* and not autofluorescence, oligonucleotides were designed to amplify a 675 bp *DsRed2* fragment (*DsRed2*-for: 5'ATGGCCTCCTCCGAGAACGT, *DsRed2*-rev: 5'CAGGAACAGGTGGTGGCGGC3'). The polymerase chain reaction (PCR) amplification showed a product of the expected size indicating the presence of *DsRed2* sequences (B in Figure).

The only samples that were amplified in the PCR reaction were from mosquitoes with phenotypic expression; all other samples did not show any sign of plasmid in the PCR reaction (data not showed). To confirm that *DsRed2* mRNA was being expressed, we performed reverse transcription-PCR using the same primers (C in Figure).

Several mosquito species have been successfully transformed and maintained as transgenic strains, such as: *Ae. aegypti, Aedes albopictus*, *Aedes fluviatilis, Anopheles gambiae, Anopheles stephensi* (Miller et al. 1987, Jasinskiene et al. 1998, Catteruccia et al. 2000, Rodrigues et al. 2006, Labbé et al. 2010). However, *Culex* mosquitoes are especially difficult to transform by the conventional method of injecting eggs and manipulating embryos because females lay their eggs in "rafts" so one is required to split the raft into individual eggs to perform microinjection and it is not possible to re-assemble them afterwards. This is not a problem for *Aedes* and *Anopheles* mosquitoes, which have been more easily genetically modified, as these mosquitoes lay their eggs individually, and in some cases *Aedes* eggs can resist desiccation for several months in a state of diapause, so it is possible for the egg to heal before embryogenesis starts. *Culex* mosquitos' embryogenesis cannot be delayed, so these two factors represent major limitations in *Culex* survival rate and transformation success.

The transient expression of *DsRed2* in *Cx. quinquefasciatus* described here, demonstrates the mosquito's capability of expressing an effector gene driven by *Hr5IE1* baculovirus promoter. The fact that RNA was transcribed from this plasmid, by the mosquito, demonstrates its capacity to express foreign effector genes and molecular markers. *Piggybac* transformation results in fairly random insertion of the transgene into the genome (with a recognition sequence of TTAA), so that expression can be heavily influenced by the surrounding DNA, resulting in a range of phenotypes (Nolan et al. 2002). Site-specific integration methods have recently been developed (Nimmo et al. 2006), but they still require an initial transposon based transformation. An innovative gene insertion method involving insertion of transgenes into the genome of adult mosquitoes via sterol carriers offers new prospects for transformation (Peng et al. 2011). This technique is especially interesting for mosquitoes such as *Cx. quinquefasciatus*, where other approaches have proved difficult. A transient expression system for rapid testing of candidate effector molecules would facilitate the development of genetic control strains in vector species, especially those where germline transformation is difficult.

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