Published in final edited form as:

Insect Mol Biol. 2008 April ; 17(2): 175–183. doi:10.1111/j.1365-2583.2008.00791.x.

Expression of a mutated phospholipase A₂ in transgenic *Aedes fluviatilis* mosquitoes impacts *Plasmodium gallinaceum* development

F. G. Rodrigues^{*}, M. N. Santos^{*}, T. X. T. de Carvalho^{*}, B. C. Rocha^{*}, M. A. Riehle[†], P. F. P. Pimenta[‡], E. G. Abraham[§], M. Jacobs-Lorena[§], C. F. Alves de Brito^{*}, and L. A. Moreira^{*}

^{*}Laboratório de Malária, Instituto René Rachou, Fundação Oswaldo Cruz, Belo Horizonte-MG, Brazil

[†]Department of Entomology, University of Arizona, Tucson, AZ, USA

[‡]Laboratório de Entomologia Médica, Instituto René Rachou, Fundação Oswaldo Cruz, Belo Horizonte-MG, Brazil

[§]Department of Molecular Microbiology and Immunology, Johns Hopkins Bloomberg School of Public Health, Malaria Research Institute, Baltimore, MD, USA

Abstract

The genetic manipulation of mosquito vectors is an alternative strategy in the fight against malaria. It was previously shown that bee venom phospholipase A₂ (PLA₂) inhibits ookinete invasion of the mosquito midgut although mosquito fitness was reduced. To maintain the PLA₂ blocking ability without compromising mosquito biology, we mutated the protein-coding sequence to inactivate the enzyme while maintaining the protein's structure. DNA encoding the mutated PLA₂ (mPLA₂) was placed downstream of a mosquito midgut-specific promoter (*Anopheles gambiae* peritrophin protein 1 promoter, AgPer1) and this construct used to transform *Aedes fluviatilis* mosquitoes. Four different transgenic lines were obtained and characterized and all lines significantly inhibited *Plasmodium gallinaceum* oocyst development (up to 68% fewer oocysts). No fitness cost was observed when this mosquito species expressed the mPLA₂.

Keywords

transformation; bee venom phospholipase A₂; malaria; *piggyBac*; fitness

© 2008 The Authors Journal compilation © 2008 The Royal Entomological Society

Correspondence: Luciano A. Moreira, Laboratório de Malária, Instituto René Rachou, Fundação Oswaldo Cruz, Av. Augusto de Lima, 1715, Belo Horizonte-MG, Brazil, CEP 30190-002. Tel.: +55 31 3349 7772; fax: +55 31 3295 3115; luciano@cpqrr.fiocruz.br.

Supplementary material

The following supplementary material is available for this article:

This material is available as part of the online article from: <http://www.blackwell-synergy.com/doi/full/10.1111/j.1365-2583.2008.00791.x>.

Introduction

Malaria remains a major global problem, causing an unacceptable toll on the health and economic welfare of the world's poorest communities. An estimated 350–500 million clinical malaria episodes occur annually; most of these caused by infection with *Plasmodium falciparum* and *Plasmodium vivax*. *P. falciparum* malaria causes more than one million deaths a year, mainly in sub-Saharan Africa. It also contributes indirectly to many additional deaths, mainly in young children, through synergy with other infections and illnesses (WHO, 2005).

Problems related to parasite drug resistance, mosquito resistance to insecticides and the lack of an effective vaccine increase the need for alternative strategies for malaria control. By genetically manipulating the mosquito vector it is possible to insert effector molecules that block *Plasmodium* development in the insect (Ito *et al.*, 2002; Kim *et al.*, 2004). Various candidates, including the anticircumsporozoite protein antibody (de Lara Capurro *et al.*, 2000), a dodecapeptide SM1 (Ghosh *et al.*, 2001), cecropin-like peptides (Arrighi *et al.*, 2002), gomesin (Moreira *et al.*, 2007) and the bee venom phospholipase A₂ (PLA₂) (Zieler *et al.*, 2001), have been studied.

Phospholipase A₂ enzymes belong to a protein family which catalyses the hydrolysis of the two-acyl ester bond of glycerophospholipids, leading to the production of free fatty acids and lysophospholipids. Secreted phospholipases A₂ (sPLA₂) have been identified in venoms, but are also distributed in mammalian tissues, fluids, and secretions (Kudo & Murakami, 2002), plants (Lee *et al.*, 2003), bacteria (Sugiyama *et al.*, 2002) and viruses (Canaan *et al.*, 2004; Nagiec *et al.*, 2004). Venom PLA₂s cause diverse pharmacological effects such as neurotoxic, myotoxic or cardiotoxic reactions, or have anticoagulant properties, and some sPLA₂s have been shown to display antibacterial (Buckland & Wilton, 2000; Koduri *et al.*, 2002), antiviral (HIV) (Fenard *et al.*, 1999) or anti*Plasmodium* activities (Deregnacourt & Scrével, 2000; Guillaume *et al.*, 2004).

PLA₂s from snake, scorpion and bee venoms are potent *in vitro* inhibitors of the intraerythrocytic development of *P. falciparum*: their anti*Plasmodium* activity is more dependent on the presence of exogenous phospholipids in serum than on the hydrolysis of the erythrocyte membrane (Guillaume *et al.*, 2004, 2006). When administered via blood feeding, venom PLA₂s inhibit the *P. falciparum* and *P. gallinaceum* sporogonic cycle by associating with the mosquito midgut epithelium and blocking ookinete adhesion and invasion. This mechanism is independent of PLA₂ catalytic activity (Zieler *et al.*, 2001).

Transgenic *Anopheles stephensi* mosquitoes expressing the bee venom PLA₂ blocked both the development of *Plasmodium berghei* oocysts and transmission to naïve mice (Moreira *et al.*, 2002). However, the effector protein reduced female fertility, probably because of damage to midgut epithelial cells (Abraham *et al.*, 2005) that reduced uptake of blood nutrients, and consequently, egg production (Moreira *et al.*, 2004). Fitness of transgenic vectors is a crucial feature when one considers their use in disease control.

Previous studies have shown that histidine at position 48 of pancreatic PLA₂ is crucial for enzymatic activity. Substitution of this residue to asparagine does not affect the enzyme's structural integrity although it reduces its activity fivefold (Janseen *et al.*, 1999).

To maintain the *Plasmodium* blocking ability of the bee venom phospholipase A₂ without causing any fitness-related problem to mosquitoes, we generated point mutations, changing the conserved histidine in its active site (H67), and consequently inactivating the enzyme. We expressed this new protein (mPLA₂) in transgenic mosquitoes and evaluated its effect on the progression of the *P. gallinaceum* cycle.

Results

Using site-directed mutagenesis, we generated two point mutations in the bee venom phospholipase A₂ coding sequence, which resulted in amino acid changes and enzyme inactivation (Riehle *et al.*, 2007). This approach was pursued because Zieler *et al.* (2001) demonstrated that even inactive venom PLA₂ is able to block *Plasmodium* spp.

The recombinant protein was expressed in *Escherichia coli*, purified and dialysed in order to decrease the concentration of urea used for its purification. Its effect on *P. gallinaceum* was tested in comparison with the commercial bee venom PLA₂. Recombinant mPLA₂ inhibited *P. gallinaceum* oocyst formation up to 80% when compared with the control (buffer) ($P < 0.05$), while the commercial PLA₂ inhibited parasite development by 91 to 99% when compared to the control ($P < 0.01$). Additionally, the prevalence of infected mosquitoes was always lower when either protein was present in comparison to the control (Table 1).

We used a mosquito midgut-specific promoter [*Anopheles gambiae* peritrophin protein 1 promoter (AgPer1); Abraham *et al.*, 2005] to drive the expression of the mPLA₂ in four independent transgenic lines. Expression of the 3 × P3-EGFP (enhanced green fluorescent protein) marker gene was detected by fluorescence microscopy by screening the G1 larvae and had the same pattern as previously observed in other mosquito species (Kokoza *et al.*, 2001; Ito *et al.*, 2002; Kim *et al.*, 2004; Rodrigues *et al.*, 2006). By PCR, it was possible to amplify both marker (EGFP: 645 bp) and effector (mPLA₂: 480 bp) genes in transgenic lines, but not in control lines. The *Aedes aegypti* actin control (Krebs *et al.*, 2002) was amplified in both transgenic and control mosquitoes (Fig. 1A). The number of transgene copies that integrated was assessed by Southern blot analysis. Genomic DNA was digested with the *Bgl*III enzyme, blotted and a probe comprising the left arm of the *piggyBac* transposable element was used. We observed different transposition events in our four transgenic lines, confirming the integration of the foreign genes in the germline of *Ae. fluviatilis*. As expected, there was no hybridization of the radioactive probe in the wild-type mosquitoes' DNA (Fig. 1B).

To check whether the AgPer1 promoter could drive the expression of the mPLA₂ in mosquito midguts, we performed RT-PCR using RNA prepared from midgut samples collected prior to (sugar-fed midguts) and after blood feeding (8 and 48 h post bloodmeal). The AgPer1 promoter drove the production of mPLA₂ in mosquito midguts of all four transgenic lines (Fig. 2). Devenport *et al.* (2004) reported that there were no significant

changes in Ag-Aper1 mRNA abundance after a bloodmeal. Our RT-PCR data on expression of AgPer-mPLA₂, although not strictly quantitative, are consistent with similar behaviour for this recombinant construct in *Ae. fluviatilis* (Supplementary Material S1). In other mosquito tissues no mPLA₂ mRNA was detected (Supplementary Material Fig. S2).

The presence of mPLA₂ protein was investigated by immunostaining of mosquito midguts followed by confocal microscopy. The mutated PLA₂ was detected on epithelial cells of midguts from blood-fed mosquitoes using a commercial polyclonal antivenom PLA₂. A fluorescent signal was detected towards the lumenal side of midgut epithelial cells, where the cell secretory vesicles are also localized. There, one could expect to find AgPer1 in equivalent cells from *An. gambiae*. In nontransgenic mosquito midguts a light green background signal was detected (Fig. 3). The specificity of the polyclonal antibody was verified by Western blots using commercial bee venom PLA₂ in which our recombinant mPLA₂ protein was easily recognized (data not shown).

The effect of the mPLA₂ expression on the development of *P. gallinaceum* in the mosquito was assessed by simultaneously feeding transgenic and nontransgenic female mosquitoes on the same infected chickens and by counting the number of *Plasmodium* oocysts in mosquito midguts 7 days after infection. At least three independent experiments were performed for each transgenic line. All transgenic lines significantly reduced oocyst numbers compared to nontransgenic siblings (17.5 to 68.5% inhibition), although the level of inhibition was not as high as when recombinant protein was added to the bloodmeal (Table 2). When infected mosquitoes were maintained for 15 days and had their salivary glands removed to check for the presence of sporozoites, transgenic mosquitoes had significantly fewer sporozoites than nontransgenic adult females (Supplementary Material Table S1).

To check whether the expression of mPLA₂ had any influence on mosquito overall fitness, we performed some experiments to assess the stability of the transgene through generations and measured biological parameters such as fertility and survivorship. The proportion of transgenic mosquitoes from two different lines was compared with nontransgenic counterparts and, as shown in Supplementary Material Fig. S3, the number of transgenic individuals was significantly higher than nontransgenic mosquitoes for several generations. In addition, there was no difference in egg production between nontransgenic and transgenic mosquitoes; survival was significantly longer in most of the lines for both male and female mosquitoes (Supplementary Material Table S2).

Discussion

This is the first report on the generation of transgenic *Ae. fluviatilis* mosquitoes bearing a parasite-blocking molecule. The idea of expressing the inactive bee venom PLA₂ was based on our previous observations that transgenic *An. stephensi* mosquitoes expressing the active enzyme significantly reduced their egg production (Moreira *et al.*, 2004), probably because of midgut epithelia damage (Abraham *et al.*, 2005) which may have compromised nutrient absorption. That effect was not observed by Zieler *et al.* (2001), who were the first to report the *Plasmodium* blocking feature of venom PLA₂ in mosquitoes. When those authors chemically inactivated the enzyme, mixed with a parasite-enriched bloodmeal and fed

female mosquitoes, no fitness-related problems were detected (Zieler *et al.*, 2001). We presume that damage occurs when the wildtype enzyme is expressed intracellularly.

We expressed the mutated bee venom PLA₂ (mPLA₂) in bacteria as a recombinant protein. This protein had similar *in vitro* parasite-blocking ability when compared to our control, the commercial bee venom PLA₂, which encouraged us to express this molecule in mosquitoes. The idea of using midgut-specific promoters to express potential parasite effector molecules has been pursued in different studies (Ito *et al.*, 2002; Moreira *et al.*, 2002; Kim *et al.*, 2004; Abraham *et al.*, 2005; Franz *et al.*, 2006). As the parasite's early sporogonic stages develop within the mosquito midgut environment, the expression of a blocking molecule in that tissue could target gametocytes, gametes, zygotes and ookinetes (Baton & Ranford-Cartwright, 2005).

The AgPer1 protein is a major constituent of the peritrophic matrix, which protects the midgut during a bloodmeal (Shen *et al.*, 1998). In anopheline mosquitoes, peritrophic matrix proteins are stored in vesicles within the mosquito midgut epithelial cells (Devenport *et al.*, 2004) and are immediately released upon blood feeding. In culicine mosquitoes (*Aedes* spp.), epithelial cells store peritrophin mRNA and protein translation is initiated upon blood feeding (Devenport *et al.*, 2006). Although we used an anopheline promoter to express a transgene in *Aedes*, which last shared a common ancestor at least 140 million years ago (Service, 1993), the *Anopheles* regulatory sequences was recognized by *Ae. fluviatilis*. Previously, another gut-specific promoter from an anopheline mosquito (*An. gambiae* carboxypeptidase) was also able to drive a transgene expression in *Ae. aegypti* (Moreira *et al.*, 2000).

The effect of phospholipase A₂ towards *Plasmodium* development has been extensively studied (Deregnacourt & Scrével, 2000; Zieler *et al.*, 2001; Guillaume *et al.*, 2004), although the exact mechanism of action is not completely understood. In culture, PLA₂s have an indirect effect by degrading lipoproteins present in serum, which in turn produce components (eg arachidonic, linoleic and docosahex-aenoic acid) toxic to the parasite (Guillaume *et al.*, 2006).

Confocal microscopy was used to localize the transgenic protein in blood-fed mosquito midguts presenting a homogeneous distribution throughout the tissue towards the luminal side of the epithelial cells. The localization is similar to that of the peritrophic matrix protein (Ag-Aper1) in *An. gambiae* (Devenport *et al.*, 2004), which demonstrates that the transgenic protein is being properly processed within the mosquito midgut.

Inhibition of oocyst development varied among different transgenic lines and even within lines, in different experiments. Those differences can be a result of various factors including degree of mosquito infection, chicken parasitemia upon infection and effector protein expression in different lines (Coates *et al.*, 1998; Perera *et al.*, 2002; Marcus *et al.*, 2004). Furthermore, the location of transgene insertion can have a strong influence on the gene expression, whether lying on a euchromatic or heterochromatic region (Boulesteix & Biémont, 2005). It is important to note that in all experiments we worked with heterozygous transgenic lines, as we have been crossing transgenic mosquitoes with colony nontransgenic

adults for several generations in order to reduce the possibility of associating the transgene with deleterious alleles that might become fixed when one establishes homozygous lines early in the process (Catteruccia *et al.*, 2003; Marrelli *et al.*, 2006). When working with homozygous lines, which should have higher levels of the effector molecule, we would expect a higher degree of inhibition but that has yet to be tested.

This effector molecule is a strong candidate to be tested in human malaria vectors as Zieler *et al.* (2001) have already demonstrated the effect of PLA₂ towards *P. falciparum* infecting *An. stephensi* and *An. gambiae* mosquitoes, and also because we have shown here that mosquito fitness was not compromised with the expression of mPLA₂.

We are aware that transgenic mosquitoes bearing only one effector molecule will not be sufficient to block parasite transmission. For *P. gallinaceum* it has been experimentally demonstrated that mosquitoes bearing only 20 sporozoites can infect a naïve chick (Jasinskiene *et al.*, 2007). Other blocking molecules must be studied in order to generate a mosquito that, for instance, would block parasites within their midguts (by using midgut-specific promoters) and also when traversing the salivary glands (molecules driven by salivary gland-specific promoters). In that way, the possibility of plasmodia overcoming those barriers would be reduced.

The genetic modification of mosquito vectors is important not only for generating parasite-blocking mosquitoes for the control of malaria, but also as a tool for studying parasite-mosquito interactions.

Experimental procedures

Mutated phospholipase A₂ (mPLA₂) and protein expression

Point mutations relative to the original PLA₂ cDNA sequence (Moreira *et al.*, 2002) were obtained by PCR site-directed mutagenesis (Riehle *et al.*, 2007). The histidine (codon CAC) residue in the active site was modified to asparagine (AAC) in order to inactivate the bee venom phospholipase A₂ without altering its conformation (mPLA₂). An extra point mutation was made on position 62 [codon GCA (A) was changed to ACA (T)].

The mPLA₂ coding sequence was cloned into the pET32a expression vector. Recombinant protein was expressed in *E. coli* BL21(DE3)pLysS strain and induced with 1 mM IPTG for 4 h. After induction, cells were pelleted and resuspended in 250 ml chilled lysis buffer (50 mM Tris-HCl pH 7.5, 2 mM EDTA, 1 mM DTT, 2 mg/ml lysozyme), frozen in dry ice/ethanol bath and then sonicated. Lysed cells were centrifuged (10 000 g for 16 min, at 4 °C) and after washing, the pellet was resuspended in milli-Q water (50 ml). Protein purification was performed by affinity chromatography in a column packed with 1.0 ml nickel-nitrilotriacetic acid (Ni-NTA; Qiagen, Valencia, CA, USA) resin and eluted according to the manufacturer's protocol. Purified fractions were collected and dialysed (12 kDa cutoff) to decrease urea concentration and analysed by SDS-PAGE.

Construction for mosquito transgenesis

The promoter region, 5' UTR and 3' UTR sequences from *An. gambiae* peritrophic matrix protein 1 (AgAper1) were cloned in pUC19 (Abraham *et al.*, 2005). The mutated bee venom PLA₂ replaced the original PLA₂ by restriction digesting the pUC19 (AgPer1/PLA₂) plasmid with *KpnI* and *BamHI*. The mPLA₂ was inserted into *KpnI BamHI* sites, between the 5' and 3' fragments of AgAper1, in pUC19. A *PstI* site 610 bp downstream from the 5' end of the AgAper1 cloned promoter and pUC19 multiple cloning site *PstI* were used to release the fragment carrying 2570 bp 5'-AgAper1 + 480 bp mPLA₂ sequence + 907 bp AgAper1 3' sequence. This *PstI* fragment was subcloned into the *PstI* site of the pSLfa1180fa shuttle vector, which has unique *FseI* and *AscI* sites. The plasmid was digested with both enzymes and the fragment was inserted into *FseI/AscI* sites of *piggyBac*[3 × P3-EGFPpafm] to generate *piggyBac*[3 × P3-EGFP(AgAper1PLA₂ m)]. The final plasmid was sequenced to verify the inserts before microinjection.

Mosquitoes

Ae. fluviatilis were reared at 27 °C and 80% humidity under a 12 h light/dark cycle, and adults were fed on 10% sucrose solution *ad libitum*. For egg production female mosquitoes were blood fed on anaesthetized Swiss Webster mice.

Microinjection and mosquito rearing

Ae. fluviatilis embryos were injected, as previously described (Rodrigues *et al.*, 2006), with a mixture of *piggyBac*[3' P3-EGFP(AgPerPLA₂m)] plasmid (0.3 µg/µl) and *piggyBac* helper plasmid (0.2 µg/µl), which encodes the pBac transposase (Handler & Harrell, 1999). Transformants were selected by screening the G1 larvae for the presence of green fluorescent protein (GFP)-positive individuals using a fluorescent stereomicroscope. Individually transformed adult mosquitoes were mated with nontransgenic mosquitoes to establish stable transgenic lines. Transgenic mosquito lines have been maintained for several generations as heterozygotes.

Detection of the transgene

Larval genomic DNA was isolated by an adapted protocol (Black & Munstermann, 1996). Briefly, fourth instar larvae from all transgenic lines or nontransgenic siblings were frozen in dry ice: ethanol bath, homogenized in grinding buffer (0.1 M NaCl; 0.2 M sucrose; 0.1 M Tris-HCl pH 9.1; 0.05 M EDTA; SDS 0.05%) and incubated at 65 °C for 30 min. Following proteinase K digestion for 3 h at 52 °C, the DNA was extracted with phenol and phenol:chloroform:isoamyl alcohol. After air drying, the DNA pellet was solubilized in 10 mM Tris-HCl, pH 8.5. For PCR analysis, primers specific to EGFP marker (EGFPF: 5'-GGTGCCCATCCTGGTCGAGC-3' and EGFP R: 5'-GCGGTCACGAACTCCAGCAGG-3'); mPLA₂ (PLAMF: 5'-GGTACCTGGCAAATCAGGGATAGG-3' and PLAMR: 5'-GGATCCCTATCAGGCGTAGTCGG-3') and actin (ActinF: 5'-AAGGTGTGATGGTCGGTA-3' and actinR: 5'-TCATTGCCCCACCAGAAC-3'; Krebs *et al.*, 2002) genes were used.

For Southern blot analysis, 10 µg of genomic DNA was digested with 10 U of *Bgl*III (Promega, Madison, WI, USA), separated on a 0.7% agarose gel and the blot hybridized with a ³²P-labelled *piggyBac* left arm probe (0.8 kb *Sal*I fragment). Blots were exposed to x-ray film for 1 week, at –80 °C.

Transgenic PLA₂ mRNA expression

Mosquito midguts were dissected from transgenic and wildtype mosquitoes at several time-points after blood feeding. All tissues were immediately frozen in an ethanol/dry ice bath and stored at –80 °C. Total RNA was extracted with Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. First-strand cDNA synthesis was performed with SuperScript II reverse transcriptase (Invitrogen) according to the manufacturer's protocol with 0.5 µg total RNA from each sample as a template for the reactions and oligo-dT primer. To avoid genomic DNA carry over, total RNA was treated with RQ1 RNase-free DNase (Promega). After cDNA synthesis, oligonucleotides specific to the mPLA₂ fragment [AGPERF–5'-GTGTGGTGCTACTGTTGGC-3'; PLAMR (see above)] were used to amplify the mPLA₂ gene by PCR (95 °C for 2 min and 30 cycles of 95 °C for 1 min; 55 °C for 1 min; 72 °C for 1 min). As a control, we used a ribosomal specific gene (Gentile *et al.*, 2005) (RP49 F: 5'-GTGAAGAAGCGGACGAAGAAGTT-3' and RP49 R: 5'-TGCATCATCAGCACCTCCAGC-3') (95 °C for 2 min and 30 cycles of 95 °C for 1 min; 61 °C for 1 min; 72 °C for 1 min).

Midgut immunostaining

Midguts from transgenic and nontransgenic mosquitoes fed on naïve mice were dissected in phosphate-buffered saline (PBS; 130 mM NaCl, 7 mM Na₂HPO₄, 3 mM NaH₂PO₄·H₂O, pH 7.4) and fixed for 2 h in 4% paraformaldehyde. Fixed tissues were washed with PBS three times for 5 min and blocked in RPMI (Roswell Park Memorial Institute) medium for 2 h at room temperature. In between each of these steps, midguts were washed twice for 5 min with PBS, following incubation with PBT/BSA (1% bovine serum albumin, 0.2% Triton in PBS pH 7.5) for 30 min. The antirabbit PLA₂ (Nordic, The Netherlands) was diluted in blocking solution AC Dako (1 : 2000) (Dakocytomation, Denmark) and incubated with the midguts at 4 °C overnight. Secondary antibody conjugated to Alexa 488 (Molecular Probes, Eugene, OR, USA) was used at a 1 : 500 dilution in Ac Dako for 2 h at room temperature. After each antibody incubation, the tissue was washed three times for 5 min with PBT. The tissues were then incubated with rhodamine/phalloidin (diluted in PBT) for 2 h following another washing step. Immunostainings were analysed with a confocal microscope (LSM510, Zeiss, Jena, Germany) at a magnification of 400×, using a 505–530 nm filter.

Plasmodium gallinaceum blocking experiments

The effect of the recombinant protein (mPLA₂) on the development of the bird malaria parasite was tested, *in vitro*, in three independent experiments. mPLA₂ was added to *P. gallinaceum*-infected chicken blood (0.1 µmol/l) and offered to *Ae. fluviatilis* female mosquitoes through a membrane feeding apparatus. Buffer alone (100 mM NaH₂PO₄, 10 mM Tris, 0.5 M urea, pH 6.9) and native PLA₂ (0.1 µmol/l, Sigma, St Louis, MO, USA)

[able to inhibit the development of the parasite as demonstrated by Zieler *et al.* (2001)], were used as controls.

The effect of the mutated protein towards parasite development was also tested in transgenic mosquitoes. *P. gallinaceum* 8A parasite strain was inoculated intra-peritoneally into 2-day-old chicks and parasitemia checked daily via Giemsa-stained blood smears. Transgenic and nontransgenic mosquitoes (3–5 days old) maintained with sugar supplemented with 0.5 g/l p-aminobenzoic acid plus 0.025% gentamicin, were allowed to feed on gametocytepositive chicks (10–15% parasitemia). Fully engorged mosquitoes were separated after 24 h and kept at 27 °C with 10% sugar solution. After 7 days, midguts were dissected, stained with mercurochrome (2% solution) and the number of oocysts per midgut was determined by microscopy (DIC-100×). The head was separated from the thorax in order to screen, on a fluorescence stereoscope, which midguts belonged to each transgenic or nontransgenic mosquitoes. The procedure was carried out by another person in a double blind assay to avoid any bias with oocyst counting.

To check for sporozoites, transgenic and nontransgenic sibling mosquitoes were infected with *P. gallinaceum* and after 7 days some mosquitoes were dissected to check for oocysts. Fifteen days postinfection the thoraces were separated from the abdomens. Twelve mosquito thoraces and heads were directly transferred to a 0.5 ml tube (previously perforated with a 25 × 7 mm needle and containing glass wool on the bottom) that was then placed inside a 1.5 ml microcentrifuge tube. One hundred microlitres of RPMI 1640 was added to each tube and centrifuged at 3000 *g* for 5 min at 4 °C. The bottom tube was replaced and this last step was repeated twice, after which all the collected flow-through was mixed and centrifuged at 3000 *g* for 15 min. The supernatant was removed, leaving about 10 µL inside each tube. Forty microlitres of RPMI 1640 were added and after resuspension 10 µL was transferred to a hemacytometer and counted using a DIC-microscope at 400× magnification.

Fitness of transgenic mosquitoes

Fifty heterozygous virgin transgenic female mosquitoes were crossed with 50 nontransgenic male mosquitoes. One hundred larvae of each progeny were randomly selected in order to check the proportion of transgenic individuals. Subsequent crosses were made, with the same number of randomly chosen male and female individuals. The proportion of male and female adults was recorded. These experiments were followed for five generations.

For egg production, 3-day-old females from nontransgenic and transgenic lines were blood fed and after 3 days their wings were removed and the insects confined in 4 cm Petri dishes containing wet filter paper to collect the eggs. These experiments were repeated four times. To check mosquito survivorship, 1-day-old male and female mosquitoes were grouped in cages, offered sucrose *ad libitum* and mortality was observed every day until all had died. Three independent experiments using female mosquitoes and two with male individuals were performed.

Statistical analysis

The blocking ability of both the recombinant protein and the transgenic mosquitoes expressing the mPLA₂ towards *P. gallinaceum* was analysed with the nonparametric Mann–Whitney *U* test. Biological parameters were analysed with Kruskal–Wallis and Mann–Whitney *U* tests (Dawson & Trapp, 2001) and chi-square analysis was performed (5% probability) to check whether the population reached the Hardy–Weinberg equilibrium after the second generation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We are in debt to Tatiana A. da Silva for technical support on confocal analysis, Fernanda O. Rezende on sporozoite assays, Ana Paula M. Rebouças for statistical analysis and Paula M. Nogueira on fitness experiments. F.G.R., T.X.T.C., B.C.R. and L.A.M. received fellowships from CNPq. This work was partially funded by TDR/WHO and FAPEMIG.

References

- Abraham EG, Donnelly-Doman M, Fujioka H, Ghosh A, Moreira LA, Jacobs-Lorena M. Driving midgut-specific expression and secretion of a foreign protein in transgenic mosquitoes with AgPer1 regulatory elements. *Insect Mol Biol.* 2005; 14:271–279. [PubMed: 15926896]
- Arrighi RB, Nakamura C, Miyake J, Hurd H, Burgess JG. Design and activity of antimicrobial peptides against sporogonic-stage parasites causing murine malaras. *Antimicrob Agents Chemother.* 2002; 46:2104–2110. [PubMed: 12069961]
- Baton LA, Ranford-Cartwright LC. Spreading the seeds of million-murdering death: metamorphoses of malaria in the mosquito. *Trends Parasitol.* 2005; 21:573–580. [PubMed: 16236552]
- Black, WC.; Munstermann, LE. Molecular taxonomy and systematics of arthropod vectors. In: Beaty, BJ.; Marquardt, WC., editors. *The Biology of Disease Vectors*. University Press of Colorado; Niwot: 1996. p. 438–470.
- Boulesteix M, Biémont C. Transposable elements in mosquitoes. *Cytogenet Genome Res.* 2005; 110:500–509. [PubMed: 16093703]
- Buckland AG, Wilton DC. The antibacterial properties of secreted phospholipases A₂. *Biochim Biophys Acta.* 2000; 1488:71–82. [PubMed: 11080678]
- Canaan S, Zadori Z, Ghomashchi F, Bollinger J, Sadilek M, Moreau ME, et al. Interfacial enzymology of parvovirus phospholipases A₂. *J Biol Chem.* 2004; 279:14502–14508. [PubMed: 14726513]
- Catteruccia F, Godfray HC, Crisanti A. Impact of genetic manipulation on the fitness of *Anopheles stephensi* mosquitoes. *Science.* 2003; 299:1225–1227. [PubMed: 12595691]
- Coates CJ, Jasinskiene N, Miyashiro L, James AA. Mariner transposition and transformation of the yellow fever mosquito, *Aedes aegypti*. *Proc Natl Acad Sci USA.* 1998; 95:3748–3751. [PubMed: 9520438]
- Dawson, B.; Trapp, RG. *Basic & Clinical Biostatistics*. 3rd edn.. New York; Lange Medical Books/McGraw-Hill: 2001.
- De Lara Capurro M, Coleman J, Beerntsen BT, Myles KM, Olson KE, Rocha E, et al. Virus-expressed, recombinant single-chain antibody blocks sporozoite infection of salivary glands in *Plasmodium gallinaceum*-infected *Aedes aegypti*. *Am J Trop Med Hyg.* 2000; 62:427–433. [PubMed: 11220756]
- Deregnacourt C, Schrével J. Bee venom phospholipase A2 induces stage-specific growth arrest of the intraerythrocytic *Plasmodium falciparum* via modifications of human serum components. *J Biol Chem.* 2000; 275:39973–39980. [PubMed: 10988294]

- Devenport M, Fujioka H, Jacobs-Lorena M. Storage and secretion of the peritrophic matrix protein Ag-Aper1 and trypsin in the midgut of *Anopheles gambiae*. *Insect Mol Biol*. 2004; 13:349–358. [PubMed: 15271206]
- Devenport M, Alvarenga PH, Shao L, Fujioka H, Bianconi ML, Oliveira PL, et al. Identification of the *Aedes aegypti* peritrophic matrix protein AeIMUC1 as a heme-binding protein. *Biochemistry*. 2006; 45:9540–9549. [PubMed: 16878988]
- Fenard D, Lambeau G, Valentin E, Lefebvre JC, Lazdunski M, Doglio A. Secreted phospholipase A(2), a new class of HIV inhibitors that block virus entry into host cells. *J Clin Invest*. 1999; 104:611–618. [PubMed: 10487775]
- Franz AW, Sanchez-Vargas I, Adelman ZN, Blair CD, Beaty BJ, James AA, et al. Engineering RNA interference-based resistance to dengue virus type 2 in genetically modified *Aedes aegypti*. *Proc Natl Acad Sci*. 2006; 103:4198–4203. [PubMed: 16537508]
- Gentile C, Lima JB, Peixoto AA. Isolation of a fragment homologous to the rp49 constitutive gene of *Drosophila* in the Neotropical malaria vector *Anopheles aquasalis* (Diptera: Culicidae). *Mem Inst Oswaldo Cruz*. 2005; 100:545–547. [PubMed: 16302065]
- Ghosh AK, Ribolla PE, Jacobs-Lorena M. Targeting *Plasmodium* ligands on mosquito salivary glands and midgut with a phage display peptide library. *Proc Natl Acad Sci USA*. 2001; 98:13278–13281. [PubMed: 11687659]
- Guillaume C, Deregnancourt C, Clavey V, Schrével J. Anti-*Plasmodium* properties of group IA, IB, IIA, and III secreted phospholipases A₂ are serum-dependent. *Toxicon*. 2004; 43:311–318. [PubMed: 15033330]
- Guillaume C, Calzada C, Lagarde M, Schrével J, Deregnancourt C. Interplay between lipoproteins and bee venom phospholipase A₂ in relation to their anti-*Plasmodium* toxicity. *J Lipid Res*. 2006; 47:1493–1506. [PubMed: 16607035]
- Handler AM, Harrell RA 2nd. Germline transformation of *Drosophila melanogaster* with the piggyBac transposon vector. *Insect Mol Biol*. 1999; 8:449–457. [PubMed: 10634970]
- Ito J, Ghosh A, Moreira LA, Wimmer EA, Jacobs-Lorena M. Transgenic anopheline mosquitoes impaired in transmission of a malaria parasite. *Nature*. 2002; 417:452–455. [PubMed: 12024215]
- Janseen MJW, Van de Wiel WA, Beiboer SH, Van Kampen MD, Verheij HM, Slotboom AJ, et al. Catalytic role of the active site histidine of porcine pancreatic phospholipase A₂ probed by the variants H48Q, H48N and H48K. *Protein Eng*. 1999; 12:497–503. [PubMed: 10388847]
- Jasinskiene N, Coleman J, Ashikyan A, Salamessy M, Marinotti O, James AA. Genetic control of malaria parasite transmission: threshold levels for infection in an avian model system. *Am J Trop Med Hyg*. 2007; 76:1072–1078. [PubMed: 17556613]
- Kim W, Koo H, Richman AM, Seeley D, Vizioli J, Klocko AD, et al. Ectopic expression of a cecropin transgene in the human malaria vector mosquito *Anopheles gambiae* (Diptera: Culicidae): Effects on susceptibility to *Plasmodium*. *J Med Entomol*. 2004; 41:447–455. [PubMed: 15185949]
- Koduri RS, Gronroos JO, Laine VJ, Le Calvez C, Lambeau G, Nevalainen TJ, et al. Bactericidal properties of human and murine group I, II, V, X, and XII secreted phospholipases A₂. *J Biol Chem*. 2002; 277:5849–5857. [PubMed: 11694541]
- Kokoza V, Ahmed A, Wimmer EA, Raikhel AS. Efficient transformation of the yellow fever mosquito *Aedes aegypti* using the piggyBac transposable element vector pBac[3'P3-EGFPafm]. *Insect Biochem Mol Biol*. 2001; 31:1137–1143. [PubMed: 11583926]
- Krebs KC, Brzozza KL, Lan Q. Use of subtracted libraries and macroarray to isolate developmentally specific genes from the mosquito, *Aedes aegypti*. *Insect Biochem Mol Biol*. 2002; 32:1757–1767. [PubMed: 12429127]
- Kudo I, Murakami M. Phospholipase A₂ enzymes. *Prostaglandins Other Lipids Mediat*. 2002; 3–58.
- Lee HY, Bahn SC, Kang YM, Lee KH, Kim HJ, Nohn EK, et al. Secretory low molecular weight phospholipase A₂ plays important roles in cell elongation and shoot gravitropism in *Arabidopsis*. *Plant Cell*. 2003; 15:1990–2002. [PubMed: 12953106]
- Marcus JM, Ramos DM, Monteiro A. Germline transformation of the butterfly *Bicyclus anynana*. *Proc Biol Sci*. 2004; 271:263–265.
- Marrelli MT, Moreira CK, Kelly D, Alphey L, Jacobs-Lorena M. Mosquito transgenesis: what is the fitness cost? *Trends Parasitol*. 2006; 22:197–202. [PubMed: 16564223]

- Moreira CK, Rodrigues FG, Ghosh A, Varotti FP, Miranda A, Daffre S, et al. Broad effect of the antimicrobial peptide gomesin against different life stages of *Plasmodium* spp. *Exp Parasitol*. 2007; 116:346–353. [PubMed: 17376436]
- Moreira LA, Edwards MJ, Adhami F, Jasinskiene N, James AA, Jacobs-Lorena M. Robust gut-specific gene expression in transgenic *Aedes aegypti* mosquitoes. *Proc Natl Acad Sci USA*. 2000; 97:10895–10898. [PubMed: 11005862]
- Moreira LA, Ito J, Ghosh A, Devenport M, Zieler H, Eappen GA, et al. Bee venom phospholipase inhibits malaria parasite development in transgenic mosquitoes. *J Biol Chem*. 2002; 277:40839–40843. [PubMed: 12167627]
- Moreira LA, Wang J, Collins FH, Jacobs-Lorena M. Fitness of anopheline mosquitoes expressing transgenes that inhibit *Plasmodium* development. *Genetics*. 2004; 166:1337–1341. [PubMed: 15082552]
- Nagiec MJ, Lei B, Parker SK, Vasil ML, Matsumoto M, Ireland RM, et al. Analysis of a novel prophage-encoded group A *Streptococcus* extracellular phospholipase A₂. *J Biol Chem*. 2004; 279:45909–45918. [PubMed: 15304506]
- Perera OP, Harrell RA II, Handler AM. Germ-line transformation of the South American malaria vector, *Anopheles albimanus*, with a piggyBac/EGFP transposon vector is routine and highly efficient. *Insect Mol Biol*. 2002; 11:291–297. [PubMed: 12144693]
- Riehle MA, Moreira CK, Lampe D, Lauzon C, Jacobs-Lorena M. Using bacteria to express and display anti-*Plasmodium* molecules in the mosquito midgut. *Int J Parasitol*. 2007; 37:595–603. [PubMed: 17224154]
- Rodrigues FG, Oliveira SB, Rocha BC, Moreira LA. Germline transformation of *Aedes fluviatilis* (Diptera: Culicidae) with the *piggyBac* transposable element. *Mem Inst Oswaldo Cruz*. 2006; 101:755–757. [PubMed: 17160283]
- Service, MW. Mosquitoes (Culicidae). In: Lane, RP.; Crosskey, RW., editors. *Medical Insects and Arachnids*. Chapman & Hall; London: 1993. p. 120-240.
- Shen Z, Jacobs-Lorena M. A type I peritrophic matrix protein from the malaria vector *Anopheles gambiae* binds to chitin. *J Biol Chem*. 1998; 273:17665–17670. [PubMed: 9651363]
- Sugiyama MK, Ohtani K, Izuhara M, Koike T, Susuki K, Imamura S, et al. A novel prokaryotic phospholipase A₂. Characterization, gene cloning, and solution structure. *J Biol Chem*. 2002; 277:20051–20058. [PubMed: 11897786]
- WHO (World Health Organization). World malaria report 2005. Geneva Switzerland: 2005. Online
- Zieler H, Keister DB, Dvorak JA, Ribeiro JM. A snake venom phospholipase A(2) blocks malaria parasite development in the mosquito midgut by inhibiting ookinete association with the midgut surface. *J Exp Biol*. 2001; 204:4157–4167. [PubMed: 11809789]

Figure 1.

PCR amplification of the EGFP, mutated phospholipase A₂ (mPLA₂) and Actin genes from genomic DNA isolated from nontransgenic and transgenic *Aedes fluviatilis* larvae. (A) Genomic DNA from nontransgenic (NT) and transgenic larvae from 4T, 8T, 9T and 10T lines were amplified by PCR with primers specific to each target gene (*EGFP*; *mPLA₂*; *Actin*). (B) About 10 µg of transgenic and wild-type mosquito genomic DNA was digested with *Bgl*III and transferred to a nylon membrane. The blot was hybridized with a ³²P-labelled *piggyBac* left arm probe (0.8 kb *Sal*I fragment). M, 100 bp DNA marker; c(-), no DNA; 4T, 8T, 9T and 10T, transgenic lines; c(+), pBacEGFP, pBacmPLA₂ (EGFP and mPLA₂) and *Ae. aegypti* genomic DNA (*Actin*).

Figure 2.

Mutated phospholipase A₂ (mPLA₂) mRNA expression in *Aedes fluviatilis* transgenic lines. Total RNA samples isolated from nontransgenic (NT) and transgenic mosquito (4T, 8T, 9T and 10T) midguts (before and after a bloodmeal) were subjected to RT-PCR analysis using primers specific to the mPLA₂ gene (A), to the RP49 gene (ribosomal protein) (B) or as a control, without the reverse transcriptase enzyme (C). M1, 100 bp DNA marker; M2, 1 kb plus DNA marker; c(-), no cDNA; c(+), pBacEGFPagPermPLA₂ (in A).

Figure 3.

Immunofluorescence staining of transgenic mosquitoes midguts by antimutated phospholipase A₂ (antimPLA₂). Midgut from nontransgenic mosquito (NT), 4T and 10T transgenic mosquito lines 24 h postbloodmeal treated with antirabbit PLA₂ and Alexa 488 secondary antibody (green) plus rhodamine phalloidin (red), to label the muscle cells (400× magnification). Lu, midgut lumenal side; H, haemocoel.

Table 1

Effect of recombinant mutated phospholipase A₂ (mPLA₂) or commercial bee venom PLA₂ on *Plasmodium gallinaceum* oocyst formation[†]

Exp.	Samples	Infection prevalence [‡]	Oocyst number (range)	Mean oocyst number	Inhibition [§]
1	Buffer	74% (20/27)	(0–122)	32.4	–
	mPLA ₂	67% (12/28)	(0–72)	10.7	70%*
	PLA ₂	40% (8/20)	(0–21)	2.8	91%**
2	Buffer	90% (18/20)	(0–120)	21.3	–
	mPLA ₂	40% (8/20)	(0–34)	4.5	76.8%*
	PLA ₂	15% (3/20)	(0–2)	0.2	93%**
3	Buffer	70% (17/27)	(0–277)	33.7	–
	mPLA ₂	33% (8/24)	(0–45)	6.6	80.4%*
	PLA ₂	25% (6/24)	(0–5)	0.4	98.8%**

[†] Infected blood was mixed with recombinant mPLA₂ or commercial PLA₂ (0.1 μmol/l) or buffer alone (100 mM NaH₂PO₄, 10 mM Tris, 0.5 M urea, pH 6.9) prior to feeding. The midguts were dissected 7 days after the bloodmeal and checked for oocysts in order to measure prevalence and parasite intensity. Mean oocyst number was significantly different from mosquitoes fed on buffer, according to Mann–Whitney U test (**P* < 0.05) (***P* < 0.01). Data are from three independent experiments.

[‡] Percentage of infected mosquitoes (number of infected mosquitoes/total number of mosquitoes).

[§] $100 - [(\text{mean oocyst number per midgut in experimental mosquitoes} / \text{mean oocyst number per midgut in control mosquitoes}) \times 100]$.

Table 2

Effect of expression of mutated phospholipase A₂ (mPLA₂) in transgenic *Aedes fluviatilis* on *Plasmodium gallinaceum* oocyst formation

Experiment	Mosquitoes	Prevalence [†]	Oocyst [‡]	Inhibition [§]
1	Control	96% (43/45)	51.9 (0–170)	–
	4T	86.2% (25/29)	26 (0–63)	49.9%*
2	Control	96.4% (27/28)	41.9 (0–148)	–
	4T	75% (21/28)	13.4 (0–136)	68%*
3	Control	90% (29/32)	83.6 (0–287)	–
	4T	86% (19/22)	40.1 (0–168)	52%*
4	Control	87.5% (35/40)	104.75 (0–240)	–
	4T	71% (16/21)	65.6 (0–259)	37.3%*
5	Control	80.6% (25/31)	26.4 (0–136)	–
	8T	75.6% (31/41)	19.4 (0–120)	26.5%*
6	Control	74% (20/27)	12.7 (0–50)	–
	8T	59% (13/22)	4 (0–44)	68.5%*
7	Control	85.2% (41/49)	48 (0–213)	–
	8T	75% (23/30)	34 (0–200)	21.9%*
8	Control	83.3% (30/36)	34.8 (0–160)	–
	9T	71.4% (15/21)	20.5 (0–64)	41.2%*
9	Control	100% (39/39)	49.8 (1–172)	–
	9T	80.9% (17/21)	41.2 (0–184)	17.5%*
10	Control	78.3% (18/23)	11.9 (0–71)	–
	9T	65% (13/20)	8.6 (0–40)	27.7%*
11	Control	87.5% (21/24)	7.5 (0–56)	–
	10T	81.8% (9/11)	3.8 (0–9)	49.3%*
12	Control	93% (38/40)	63.8 (0–212)	–
	10T	50% (10/20)	27.1 (0–124)	57.5%*
13	Control	100% (32/32)	120.4 (12–291)	–
	10T	85.7% (19/21)	84.4 (0–198)	30%*

[†]Percentage of infected (actual numbers in parentheses).

[‡]Arithmetic mean of oocyst number per mosquito gut. The range of observed values is indicated in parentheses.

[§]100 – [(mean oocyst number in transgenic mosquitoes/mean oocyst number in control mosquitoes) × 100].

*Reduction of oocyst number/gut was significant (P-value < 0.05) for all lines, as calculated by the Mann–Whitney U test.