

Bee Venom Phospholipase Inhibits Malaria Parasite Development in Transgenic Mosquitoes*

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Malaria kills millions of people every year, and new control measures are urgently needed. The recent demonstration that (effector) genes can be introduced into the mosquito germ line to diminish their ability to transmit the malaria parasite offers new hope toward the fight of the disease (Ito, J., Ghosh, A., Moreira, L. A., Wimmer, E. A. & Jacobs-Lorena, M. (2002) *Nature*, 417, 452–455). Because of the high selection pressure that an effector gene imposes on the parasite population, development of resistant strains is likely to occur. In search of additional antiparasitic effector genes, we have generated transgenic *Anopheles stephensi* mosquitoes that express the bee venom phospholipase A2 (PLA2) gene from the gut-specific and blood-inducible *Anopheles gambiae* carboxypeptidase (AgCP) promoter. Northern blot analysis indicated that the PLA2 mRNA is specifically expressed in the guts of transgenic mosquitoes with peak expression at ~4 h after blood ingestion. Western blot and immunofluorescence analyses 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.

Worldwide mortality due to malaria has increased in the past decade mainly because of parasite and mosquito resistance to drugs and insecticides, respectively, and the lack of effective vaccines (1). Genetically engineering mosquito vectors for refractoriness to malaria parasites is a strategy for reducing disease transmission that should be explored. *Plasmodium*, the causative agent of malaria, has to complete a complex developmental program in the mosquito for transmission to occur. The first interactions between the parasite and the mosquito occur in the midgut lumen, where the parasite has to traverse two barriers, the peritrophic matrix, and the midgut epithe-

lium (2, 3). Because the gut is a closed compartment that limits diffusion, antimalarial compounds secreted into the midgut lumen are expected to efficiently target the initial stages of parasite development.

Previous studies have demonstrated that venom phospholipases A2 (PLA2s)¹ strongly inhibit oocyst formation when administered to mosquitoes with an infectious blood meal (4). Although the mechanism of inhibition has not been established, it is known that PLA2 does not kill ookinetes and does not interfere with their development *in vitro*. Furthermore, inhibition of oocyst formation did not depend on PLA2 enzymatic activity. It is possible that PLA2 inhibits ookinete invasion by modifying the properties of the midgut epithelial membranes that are invaded by the parasite.

The best candidates for driving the expression of foreign gene products to be secreted into the mosquito midgut are the promoters of bloodmeal-inducible midgut genes because of their strength, tissue specificity, and synchrony of expression with parasite ingestion by the mosquito. In this context, we have shown that the *Anopheles gambiae* and *Aedes aegypti* carboxypeptidase promoters can be used to drive strong expression of recombinant protein in the midgut of transgenic mosquitoes (5) and also that they can drive the expression of a parasite blocking peptide (6) in transgenic mosquitoes (7). Although the latter results indicate that genetic manipulation of mosquito vectors is a promising strategy for reducing malaria transmission, it is also important to consider that the use of a single effector gene is likely to lead to rapid selection of resistant parasites. Thus, we have searched for additional effector genes whose products interfere with parasite development by mechanisms different from the previously developed ones (6, 7). The results reported in this article suggest that bee venom PLA2 may be used as an alternate effector gene.

EXPERIMENTAL PROCEDURES

Cloning of a Full-length Bee Venom Phospholipase A2 cDNA—The full-length cDNA of the honeybee phospholipase A2 gene was cloned for the first time by relying on the previously published partial sequence (GenBank™ accession number X16709). Messenger RNA was isolated from 105 venom glands dissected out of newly emerged honeybees using the Micro Fast-Track mRNA isolation kit from Invitrogen. The mRNA was reverse-transcribed with Superscript II reverse transcriptase (Invitrogen) using the SMART cDNA synthesis kit (Clontech) in the presence of a primer specific to the previously published 3' end of the honeybee PLA2 cDNA (5'-cgccgtctagataaaataacgatctcgaagtgtactc-3'), as well as a custom-synthesized SMART primer (5'-agcagtgttttaaacg-

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF438408.

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¹ The abbreviations used are: PLA2, phospholipase A2; AgCP, *A. gambiae* carboxypeptidase; UTR, untranslated region; PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate; GFP, green fluorescent protein; EGFP, enhanced GFP; DAPI, 4',6-diamidino-2-phenylindole.

cagagtggccattatggccggg-3'). The first strand cDNA was PCR-amplified using the same two primers and Platinum-*Taq* high fidelity polymerase (Invitrogen), and the PCR product was cloned with the TOPO-TA cloning kit (Invitrogen). The resulting construct BV PLA2 5-7 consisted of the full-length bee venom PLA2 open reading frame flanked by 5'- and 3'-untranslated sequences inserted into the pCR-TOPO vector (Invitrogen). The PLA2 sequence thus obtained included the entire signal peptide, which was only partially present in previously published sequences, as well as a portion of the 5'-untranslated region (UTR). Inserts from several identical clones were sequenced, and the sequence was used to prepare PCR primers for the construction of the mosquito expression constructs.

Carboxypeptidase-PLA2 Construct—A 3.9-kb fragment from the *A. gambiae* carboxypeptidase gene (8) (*AgCP5'*; containing the promoter, 5'-UTR, and signal peptide) was amplified by PCR from a pBluescript *AgCP* genomic subclone using *AgCPKpn* (5'-GGTACCTCGGCCGCT-TCGACACT-3') and T7 (5'-GTAATACGACTCACTATAGGGC-3') primers and cloned into pGemT-easy. Bee venom PLA2 coding region (450 bp) was cloned into pGemT-easy (Promega) from a cDNA clone using primers PLA2K (5'-GGTACCTGGCAAATCAGGGAT-3') and PLA2B (5'-GGATCCTTATCAATACTTGCAGATC-3'). The *AgCP5'* in pGemT-easy was digested with *Kpn*I, and the resulting 1.8-kb fragment was ligated into the PLA2 plasmid (*Kpn*I-digested). *AgCP3'* (555 bp) (untranslated 3' region) was obtained by PCR amplification with primers *AgCP3BH* (5'-GGATCCTGAAGTCTCTCTACCGG-3') or *AgCP3SC* (5'-CCGCGGTAAGGCTAGCATTGCCA-3') on an *AgCP* pBluescript genomic subclone and cloned into pGemT-easy. Both the *AgCP/PLA2* and *AgCP* in pGemT were digested with *Bam*HI and *Sac*II and the *AgCP* 3'-UTR fragment ligated to *AgCP5/PLA2* (pGemT-easy). A *Not*I fragment containing *AgCP/PLA2/AgCP3* was cloned into *pSLfa1180fa* plasmid (9) that contains unique *Fse*I and *Asc*I sites. The plasmid was then cut with these enzymes and cloned into *piggyBac*[3xP3-EGFP α m] plasmid. The final transposon plasmid *pBac*[3xP3-EGFP(*AgCPPLA2*)] contains 1.7 kb of *AgCP* promoter, around 100 bp of *AgCP* coding region to provide the signal sequence, 0.45 kb of PLA2 coding region, and 0.58 kb of *AgCP*-3' UTR apart from the EGFP sequence (see Fig. 1).

Microinjection and Mosquito Rearing—*Anopheles stephensi* embryos were injected as described (7, 10). Embryos were injected with a mixture of the transposon construct (0.5 mg/ml) and of the *piggyBac* helper plasmid (11) (0.3 mg/ml), both purified on Qiagen midi-prep columns. No heat shock was performed. The surviving adults were pooled into families as follows. Adult injected males (three to five G_0 mosquitoes) were crossed with 5–10 virgin non-injected females. Between three and six G_1 virgin injected females were pooled and mated with three to five non-injected males. Females were allowed to feed on blood, and G_1 embryos were collected. Transformants were selected by screening cold-immobilized larvae for EGFP expression using a dissecting fluorescence microscope at a wavelength of 490 nm.

Southern Blot Analysis—Individual G_1 transgenic mosquitoes (males or females) from positive families were mated with non-transgenic mosquitoes. Progenies of these crosses were checked for integration of the transgene as follows. Total genomic DNA was isolated from transgenic and wild type mosquitoes as described (12) but with 100 μ g/ml proteinase K treatment at 52 °C for 3 h and followed by phenol:chloroform extractions and precipitation. DNA digested with *Bgl*II was separated on a 0.8% agarose gel, blotted, and hybridized with [α - ^{32} P]dCTP-labeled *piggyBac* probe originating from the *piggyBac* vector left arm (~0.8-kb *Sal*I fragment from *pBac* [3xP3-EGFP(*AgCPPLA2*)] (see Fig. 1, probe a)).

Northern Blot Analysis—Female mosquito guts and carcasses (whole body minus gut) and male guts were dissected from 4–5-day old adults. All tissues were immediately frozen in an ethanol/dry ice bath and stored at –80 °C. To determine the temporal profile of PLA2 expression in transgenic mosquitoes, guts were dissected at increasing time intervals following a blood meal. Total RNA was extracted with TRI-Reagent (Molecular Research Center). Around 2.5 μ g of total RNA was separated in each lane of a 1.5% agarose-formaldehyde gel and blotted by capillary action to a nylon membrane (Gene Screen). Hybridization was performed first with a PLA2 probe (450 bp of coding region amplified by PCR; see Fig. 1, probe b) and then with a mitochondrial rRNA probe (13) as a loading control.

Immunoblot Analysis—Guts were dissected in phosphate-buffered saline (PBS) at different times after a blood meal. The equivalent of 0.4 guts/lane was analyzed by electrophoresis on a 15% polyacrylamide/SDS gel followed by electrotransfer to a polyvinylidene fluoride membrane (Millipore). A prestained protein ladder (Benchmark, Invitrogen) was loaded on the same gel. The membrane was incubated with an

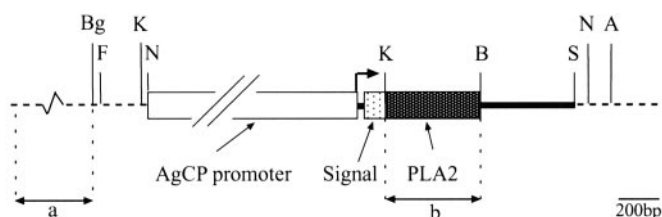


FIG. 1. Schematic diagram of the *AgCP-PLA2* gene inserted into the *A. stephensi* germ line. The construct consists of the *AgCP* promoter (open box) plus the 5'-UTR (thick line), the *AgCP* signal sequence (light stippled box), the coding sequence of bee PLA2 (dark stippled box), and the *AgCP* 3'-UTR (thick continuous line on the right). The bent arrow at the top localizes the transcription initiation site. Dotted horizontal lines represent vector sequences. The following restriction sites are indicated. A, *Asc*I; N, *Not*I; K, *Kpn*I; B, *Bam*HI; F, *Fse*I; S, *Sac*II; Bg, *Bgl*II. This construct was introduced into the *Asc*I-*Fse*I site of *pBac* [3xP3-EGFP α m] plasmid for germ line transformation of *A. stephensi*. a indicates the probe used for Southern analysis (see "Experimental Procedures" and the legend for Fig. 2). b indicates the probe used for Northern analysis.

anti-rabbit bee venom phospholipase A2 polyclonal antibody (Accurate; 1:2,000 dilution), and the bound antibody was detected with an anti-rabbit immunoglobulin, horseradish peroxidase-linked (New England Biolabs, 1:3,000 dilution) by exposing the blots to x-ray films.

Oocyst Formation Assays—About 2×10^7 *Plasmodium berghei* parasites (ANKA 2.34) were inoculated per naive mouse, and gametocyte-positive mice were used to feed a mixed population of non-transgenic and transgenic mosquitoes. Mosquitoes that blood-fed were separated after 24 h and kept at 21 °C with 10% sugar solution. On day 15, midguts were dissected, and the number of oocysts was determined.

Transmission Blocking Assays—Transgenic and sibling non-transgenic mosquitoes were mixed in the same container and allowed to feed on a single infected mouse. Mosquitoes that blood-fed were separated after 24 h and kept at 21 °C with 10% sugar solution. On day 25, individual female mosquitoes were separated into small feeding cups, and each mosquito was allowed to feed on a single naive mouse (Swiss Webster (CFW), Charles River Laboratory). After feeding, mosquitoes were cold-immobilized, salivary glands were dissected and homogenized in a small volume of PBS, and sporozoites were counted with a hemacytometer. The infection status of each mouse was followed by observing tail vein blood smears on days 5, 7, 9, 13, 17, 22, and 25.

Immunofluorescence Assays of Midgut Sheets—Female mosquito guts were dissected at different times after a blood meal in 50% ethanol and opened longitudinally to make a sheet. The gut sheets were treated with a methanol series to remove the autofluorescence as described (6), fixed overnight in 4% paraformaldehyde at 4 °C, washed six to seven times with PBS, and blocked for 6 h with PBS/4% bovine serum albumin/4% fetal calf serum at 4 °C. The guts were then incubated overnight at 4 °C with an anti-rabbit bee venom phospholipase A2 antibody (Accurate; 1:2,000 dilution). The sheets were washed seven times and incubated in the dark for 2 h with a fluorescein isothiocyanate-conjugated anti-rabbit IgG (Sigma; 1:600 dilution). The gut sheets were mounted onto glass slides, covered with a drop *Slowfade* Antifade (Molecular Probes), and examined by fluorescence microscopy.

Immunofluorescence Assays of Midgut Sections—Guts from blood-fed mosquitoes were dissected in 4% paraformaldehyde in PBS, fixed for 2 h at room temperature, and washed three times in PBS. After dehydration in a graded ethanol/PBS series, the guts were treated with xylenes and embedded in Paraplast (Oxford Labware), and 7–14- μ m sections were mounted onto glass slides. The slides were washed twice in xylenes at room temperature followed by rehydration in a graded ethanol/PBS series. To reduce autofluorescence, slides were dehydrated and rehydrated in a graded methanol/PBS series. The slides were blocked in 10% nonfat milk and 0.1% Triton X-100 in PBS for 2 h at room temperature and incubated overnight at room temperature with an anti-bee venom phospholipase A2 antibody (Accurate; 1:300 dilution in blocking solution). Slides were washed three times for 30 min with blocking solution and incubated for 2 h at room temperature with FITC-conjugated anti-rabbit antibodies (Sigma; 1:600 dilution). Nuclei were visualized by staining with 4',6-diamidino-2-phenylindole (DAPI; Molecular Probes) diluted with *Slowfade* Antifade solution. Immunostaining was observed by fluorescence microscopy at $\times 400$ magnification. DAPI staining was observed on the same sections by UV illumination and merged with the FITC-fluorescence images using Adobe Photoshop software (Adobe Systems Inc.).

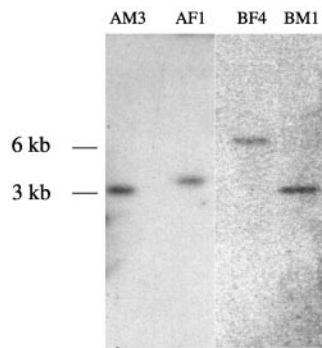


FIG. 2. Verification of transgene integration by Southern blot analysis. Genomic DNA from the transgenic AgCP-PLA2 mosquito lines was digested with *Bgl*II, blotted onto a nylon membrane, and hybridized with a 0.8-kb *piggyBac* probe (Fig. 1, a). Different size fragments were detected in the two lines that originated from each family (A and B), thus demonstrating four different integration events. The source of DNA is indicated at the top of each lane.

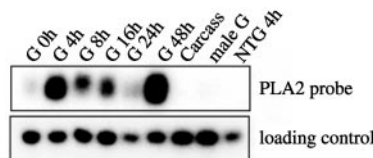


FIG. 3. Time course of phospholipase A2 mRNA accumulation after a blood meal. Total gut (or carcass) RNA was extracted from transgenic AgCP-PLA2 BF4 mosquitoes dissected at different times after a blood meal. The RNA was fractionated by gel electrophoresis and blotted to a nylon membrane. The membrane was first hybridized with a radioactive PLA2 probe and then with a mitochondrial rRNA probe, used as a loading control. Numbers above the lanes refer to the times after a blood meal when mosquitoes were dissected. 0h, guts from sugar-fed mosquitoes; G, guts; Carcass, non-gut tissues dissected at 4 h after a blood meal; NTG 4h, guts from non-transformed mosquitoes dissected 4 h after a blood meal.

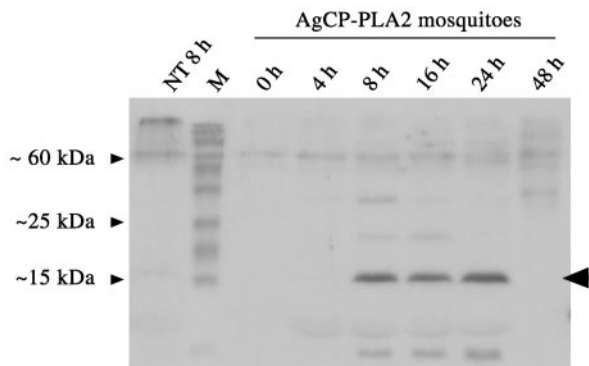


FIG. 4. Time course of recombinant PLA2 protein expression. Guts from the non-transformed recipient (NT) or from transgenic AgCP-PLA2 BF4 mosquitoes were dissected at different times after a blood meal (indicated at the top of the lanes). Protein equivalent of 0.4 gut was analyzed on a Western blot with an anti-bee venom PLA2 antibody. The arrow on the right points to a 17-kDa band corresponding to PLA2 detected at 8, 16, and 24 h. M, prestained molecular size marker (from top to bottom: 190, 120, 85, 60, 50, 40, 25, 20, 15, 10 kDa). The mobility of these markers is only approximately related to the molecular sizes (indicated by ~ symbol).

RESULTS AND DISCUSSION

A. stephensi Transformation—For the expression of PLA2 in the *A. stephensi* midgut, we constructed the AgCP-PLA2 gene that consists of the promoter, the 5'-UTR, and the signal peptide from the AgCP gene (8) linked to the coding sequence of the bee venom PLA2 gene and the AgCP 3'-UTR (Fig. 1). This gene was inserted into the *pSL1180fa* shuttle plasmid, which has unique restriction sites for cloning into the *3xP3-EGFP piggy-*

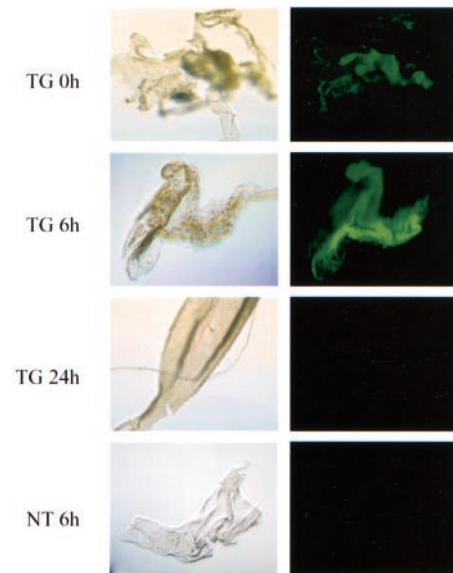


FIG. 5. Immunofluorescence of midgut sheets. Guts from the non-transformed recipient (NT) or from the transgenic AgCP-PLA2 mosquitoes (TG) were dissected at different times after a blood meal (indicated to the left of the panels) and opened longitudinally to make a sheet. The sheets were incubated with a rabbit antiserum to bee venom phospholipase A2 followed by an anti-rabbit FITC-conjugated antibody. The gut sheets were mounted on glass slides and examined by differential interference contrast (left panels) or fluorescence microscopy (right panels).

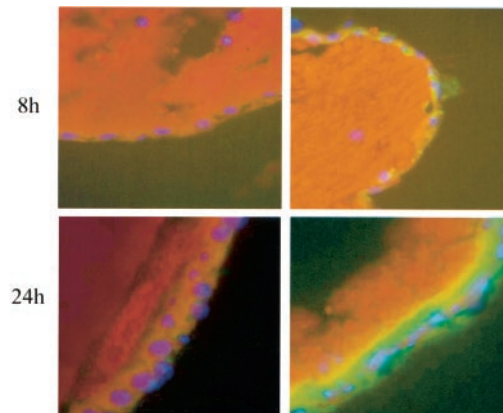


FIG. 6. Immunofluorescence of midgut sections. Fixed guts from non-transformed recipient or from transgenic AgCP-PLA2 BF4 mosquitoes, dissected at different times after a blood meal (indicated to the left of the panels), were sectioned and mounted onto glass slides. The slides were incubated with a rabbit antiserum to bee venom phospholipase A2 followed by a FITC-conjugated anti-rabbit antibody (green signal). The nuclei of the midgut epithelial cells (EC) are stained with DAPI (blue signal). The ingested blood meal (BM) is also visible.

Bac transformation vector (9). This vector allows convenient screening of transformed mosquitoes since fluorescence can easily be detected in the eyes of both larvae and adult mosquitoes. Importantly, the dark pigments of the adult eye do not interfere with detection of the GFP marker protein (7). Moreover, confined GFP expression in only a few tissues may be beneficial for mosquito fitness. *piggyBac* proved to be an efficient vector for *A. stephensi* transformation. Of 399 embryos injected, 58 adults were obtained and pooled into 18 families. Four different transgenic mosquito lines (AM3, AF1, BF4, and BM1) were established from two GFP-positive families (details in the following text). The minimum calculated transformation rate among the surviving adults was 6.9%. By comparison, transformation rates were 7% for *A. stephensi* with *Minos* (10)

TABLE I
Effect of PLA2 expression on *P. berghei* oocyst formation

Transgenic and non-transgenic (control) mosquitoes were fed on the same *P. berghei*-infected mouse. On day 15, transgenic and non-transgenic mosquitoes (positive and negative GFP eye fluorescence, respectively) were separated and after gut dissection and the number of oocysts per gut was determined.

Experiment	Mosquitoes	Prevalence ^a	No. oocysts/gut ^b	Inhibition ^c
1	Control	84% (31/37)	28.8 ± 44.8	98.8%
	AF1 line	14% (3/21)	0.3 ± 0.96	
2	Control	96% (28/29)	23.7 ± 22.1	76.5%
	AF1 line	44% (7/16)	5.6 ± 15.6	
3	Control	95% (19/20)	41.2 ± 37.2	76.9%
	BM1 line	63% (20/32)	9.5 ± 15.1	
4	Control	100% (27/27)	184.7 ± 137.8	95.7%
	AF1 line	10% (2/21)	7.9 ± 25.8	
5	Control	100% (10/10)	102.9 ± 45.0	84.7%
	BM1 line	56% (5/9)	15.8 ± 26.6	

^a Percentage of infected mosquitoes (actual numbers in parentheses).
^b Average number of oocysts per midgut and standard deviation.
^c 100 - [(average oocyst number per gut in transgenic mosquitoes/average oocyst number per gut in control mosquitoes) × 100]. Reduction of oocyst number/gut was significant (*p* < 0.001) for all experiments, as calculated by the Mann-Whitney test.

TABLE II
The vectorial capacity of transgenic mosquitoes is severely impaired

For each experiment, control non-transgenic and transgenic mosquitoes were fed on the same *P. berghei*-infected mouse. To measure transmission, single mosquitoes were fed on individual naive mice 25 days after the infectious blood meal. The salivary gland of each mosquito was dissected immediately after feeding on the mouse, and the number of sporozoites per salivary gland was determined (results reported in columns 2 and 3). The infection status of each mouse was established by examining a smear of tail vein blood on alternate days. Mice that had no parasites by day 25 were considered to be non-infected (results reported in column 4).

Experiment	% infected mosquitoes (number infected/total)	Mean sporozoite number ^a (range)	% infected mice (number infected/total)
1. Control	90 (10/11)	569 (0–1,800)	90 (10/11)
BF4 line	30 (3/10)	10 (0–50)	0 (0/10)
2. Control	100 (16/16)	1,976 (38–9,500)	88 (14/16)
BF4 Line	56 (5/9)	10 (0–38)	0 (0/9)
3. Control	88 (14/16)	884.5 (0–3,450)	44 (7/16)
AM3 line	50 (5/10)	6.5 (0–13)	0 (0/10)
4. Control	100 (12/12)	1,774 (50–6,800)	83 (10/12)
BF4 Line	80 (4/5)	39.2 (0–120)	20 (1/5)

^a This is a minimum estimate. Sporozoites from only an aliquot of the salivary gland homogenate were counted. The number of sporozoites was significantly lower (*p* < 0.01) in transgenic than in non-transgenic mosquitoes in all experiments, as calculated by the Mann-Whitney test.

and 4% for the following combinations: *A. stephensi* with *piggyBac* (14), *A. aegypti* with *Hermes* (5, 15, 16), and *A. aegypti* with *mariner* (5, 17).

Characterization of the Transgenic Lines—To confirm that the transgene was stably integrated and to determine the number of integrated transposons per genome, DNA from each mosquito line was analyzed by Southern blot hybridization. Mosquito genomic DNA was digested with *Bgl*II, fractionated, blotted, and hybridized with a probe from the *piggyBac* left arm (Fig. 1, probe *a*). As shown in Fig. 2, each mosquito line carried a single transgene integrated at a different position, indicating that independent integration events had occurred in each of the four families (note that AM3 and BM1 originated from different families). No signal was detected with DNA from non-transformed mosquitoes (data not shown).

Expression of the AgCP-PLA2 mRNA was investigated by Northern analysis (Fig. 3). A single band of the expected size (~800 bases) was detected (18). The AgCP-PLA2 mRNA was present in the midgut of sugar-fed mosquitoes and was strongly induced by blood ingestion (peak at ~4 h), consistent with the pattern of expression of the *A. gambiae* carboxypeptidase gene (8). Enhanced expression at 48 h was also observed, but the reasons for this are not clear. Robust mRNA expression as well as tissue and sex specificity together indicate that the 1.7-kb AgCP upstream sequence contains the necessary regulatory elements.

Immunoblot analysis showed that the anti-PLA2 antibody detected a protein that was produced in a blood-inducible manner in the midguts of transgenic mosquitoes (Fig. 4). The electrophoretic mobility of the protein (17 kDa) on acrylamide gels

was identical to that of an high pressure liquid chromatography-purified bee venom PLA2 protein (data not shown). Less than 0.5 gut equivalent of protein per lane was sufficient for PLA2 detection. Importantly, the recombinant protein showed a peak of expression between 8 and 24 h after a blood meal, coinciding with the time of ookinete invasion of the midgut (3). By 48 h, the protein was not detectable anymore, which contrasts with the Northern data.

Immunofluorescence assays detected the protein in whole-mount gut sheets (Fig. 5) and in gut sections (Fig. 6). In sheets, the strongest signal appeared in epithelia prepared from guts 6 h after a blood meal, whereas in sections, a much stronger signal was detected at 24 h. Presumably, by 24 h, the majority of the PLA2 had been secreted into the lumen and was lost during washing of the gut sheets (Fig. 5). Secreted PLA2 can be seen as a thick FITC signal between the epithelium and the blood meal in 24-h transgenic gut sections (Fig. 6), and this agrees with the Western data (Fig. 4).

Effect of PLA2 Expression on the Progression of Parasite Development in the Mosquito and on Mosquito Vectorial Capacity—To investigate the effect of recombinant PLA2 expression on *P. berghei* development, we fed both transgenic and non-transgenic mosquitoes on the same infected mouse and counted the number of oocysts that formed in each group of mosquitoes. As indicated in Table I, infection prevalence (column 3) and oocyst formation (column 4) were strongly reduced in transgenic mosquitoes. In five independent experiments, oocyst formation was inhibited from 77 to 99% (average inhibition 87%, column 5).

The effect of recombinant gene expression on the ability of

mosquitoes to transmit the parasite to uninfected animals (vectorial capacity) was assessed by letting single infected mosquitoes feed on individual naive mice and determining whether these mice became infected. As reported in columns 2 and 3 of Table II, in every experiment, fewer transgenic than non-transgenic mosquitoes became infected, and the number of sporozoites in salivary glands of transgenic mosquitoes was correspondingly lower. More importantly, the ability of transgenic mosquitoes to transmit the parasite to naive mice was strongly inhibited. In three out of four experiments, transmission of *P. berghei* parasites from infected transgenic mosquitoes to naive mice was completely blocked, and in a fourth experiment, the proportion of transgenic mosquitoes that transmitted the parasite was much lower than in control wild type mosquitoes (Table II, column 4). We considered the possibility that inhibition of parasite development was a consequence of the fortuitous disruption of a mosquito gene upon transposon insertion or of marker GFP expression. The following considerations strongly argue against these possibilities: 1) the same phenotype (inhibition of parasite development and transmission) was observed in different mosquito lines, in which the transposon integrated at different positions of the mosquito genome (Fig. 2 and Tables I and II); 2) the same phenotype was observed when PLA2 was provided exogenously to wild type mosquitoes (4); and 3) *P. berghei* developed equally well (oocyst and sporozoite numbers) in transgenic mosquitoes that express only GFP and hygromycin resistance gene (10) as in wild type mosquitoes.² The PLA2 protein secreted in the midgut lumen of transgenic mosquitoes is most likely responsible for inhibition of ookinete midgut invasion, as observed with the exogenously administered protein (4).

The binding of phospholipases to their substrates, such as aggregated phospholipids and membrane surfaces, is independent of their enzymatic activity (19). Indeed, it has been shown that PLA2 inhibited *Plasmodium* development even when its enzymatic activity was inhibited (4), suggesting that PLA2 acts primarily via its binding to exposed membrane lipids. Moreover, PLA2 had no effect on exflagellation and zygote formation and did not affect normal ookinete motility on glass slides, suggesting that this enzyme does not kill the parasite (4). These considerations and the lipophilicity of the enzyme support the hypothesis that PLA2 may be acting by interfering with the interactions between *Plasmodium* and the midgut cell surface. PLA2-expressing mosquitoes were normal in appearance and lived as long as the non-transformed counterparts, although an abnormal coloration of the blood meal was observed even 24 h after feeding (bright red instead of dark brown). Egg laying may also be affected. A detailed assessment of the fitness of transgenic as compared with wild type mosquitoes is under way.

In summary, these experiments demonstrate that expression of the bee PLA2 gene in the midgut of transgenic mosquitoes seriously compromises their ability to sustain *Plasmodium* development and to transmit the parasite to other vertebrate hosts. Since this PLA2 also interferes with development and transmission of *Plasmodium falciparum* in *A. gambiae* (4), we presume that PLA2 will be equally effective in curtailing transmission in this most important parasite-vector combination. Whereas reports of attempts to interfere with *Plasmodium*

transmission by expression of defensin (20) or single chain antibodies (21) in *A. aegypti* have appeared, their effectiveness in transgenic mosquitoes remains to be demonstrated. Separate work from this laboratory indicates that expression of SM1, a midgut and salivary gland binding peptide, also effectively inhibits development and transmission of the parasite (6, 7). The availability of multiple targets to inhibit *Plasmodium* development is crucial for future implementation of the transgenic mosquito approach to reduce malaria transmission in the field. This is because the *Plasmodium* genome is known for its plasticity, and the possibility of the emergence of resistant parasite strains needs to be avoided at all cost. Before one can consider actual release assays, much work remains to be done. Issues that need to be addressed include determination of wild mosquito population structures, an evaluation of the ability of gene(s) to spread through populations, considering the likelihood that the parasites will develop resistance to the foreign effector gene product, and concerns about horizontal transfer. The experiments reported here strongly suggest that genetic modification of mosquito vectorial capacity is feasible and represent a major step toward the goal of containing the spread of malaria. However, for maximum effectiveness, we will have to rely on a multipronged approach that may include drugs, insecticides, vaccines, and mosquito vectors expressing a combination of effector genes.

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² A. Crisanti, T. Nolan, and F. Catteruccia, unpublished observations.