Ectopic Expression of a Cecropin Transgene in the Human Malaria Vector Mosquito *Anopheles gambiae* (Diptera: Culicidae): Effects on Susceptibility to *Plasmodium*

WON KIM,[1, 2] HYEYOUNG KOO,[2, 3] ADAM M. RICHMAN,[4] DOUGLAS SEELEY, JACOPO VIZIOLI,[5, 6] ANDREW D. KLOCKO, AND DAVID A. O'BROCHTA[7]

Center for Biosystems Research, University of Maryland Biotechnology Institute, College Park, MD 20742

J. Med. Entomol. 41(3): 447–455 (2004)

ABSTRACT Genetically altering the disease vector status of insects using recombinant DNA technologies is being considered as an alternative to eradication efforts. Manipulating the endogenous immune response of mosquitoes such as the temporal and special expression of antimicrobial peptides like cecropin may result in a refractory phenotype. Using transgenic technology a unique pattern of expression of cecropin A (cecA) in *Anopheles gambiae* was created such that cecA was expressed beginning 24 h after a blood meal in the posterior midgut. Two independent lines of transgenic *An. gambiae* were created using a *piggyBac* gene vector containing the *An. gambiae cecA* cDNA under the regulatory control of the *Aedes aegypti carboxypeptidase* promoter. Infection with *Plasmodium berghei* resulted in a 60% reduction in the number of oocysts in transgenic mosquitoes compared with nontransgenic mosquitoes. Manipulating the innate immune system of mosquitoes can negatively affect their capacity to serve as hosts for the development of disease-causing microbes.

KEY WORDS *Anopheles gambiae*, *Plasmodium*, cecropin, malaria, transgenic insects

MALARIA RESULTS FROM INFECTION with *Plasmodium*, a protozoan parasite transmitted (vectored) by mosquitoes of the genus *Anopheles*. The disease imposes an enormous burden on the health and socioeconomic well-being of a large fraction of the earth's population. An estimated 300–500 million clinical cases and 2–3 million deaths from malaria occur each year. More than 40% of the world's inhabitants are at risk of infection. This reservoir of potential disease victims is rendered increasingly vulnerable in the face of drug-resistant parasites, insecticide-resistant vector mosquitoes, and absent or degraded public health infrastructures (Greenwood and Mutabingwa 2002).

The contemporary and future challenges of controlling malaria call for new approaches and tools. This is particularly true in sub-Saharan Africa, where the toll from malaria is highest and where a multi-faceted approach may be required to address the convergent biological, environmental, and sociological factors enhancing disease severity. Efforts to produce an effective and practical malaria vaccine are underway, but it is not yet evident that this approach will succeed. Anti-malarial drug development is limited, and the evolution of drug-resistant parasites will continue to pose a problem. By contrast, strategies designed to limit contact with infective mosquitoes continue to represent a mainstay of successful vector-borne disease control, including malaria.

Advances in insect biotechnology, in particular the development of germ-line transformation, has led to a renewed interest in genetic insect control strategies (Handler and James 2000). Altering the vector or pest status of insects using recombinant DNA technologies is being considered as a potential solution to certain medical and agricultural insect problems that have proven difficult to solve using more conventional chemical and cultural practices aimed at control or eradication. *An. gambiae*, the major vector of human malaria in Africa, is seen by some as a potential target for this new form of genetic insect control (Curtis and Graves 1988, Collins 1994). In this case, the mosquito's susceptibility to malaria parasites would be genetically altered. Mosquitoes expressing the new genotype would be created and introduced in such way as to lead to the ultimate replacement of the native, susceptible vector population with a parasite-resistant (refractory) population, thereby limiting human con-

[1] School of Biological Sciences, Seoul National University, Seoul 151–742, Korea.
[2] These authors contributed equally to this work.
[3] Department of Biological Sciences, Sangji University, Wonju 220–702, Korea.
[4] Medical Research Service, Department of Veterans Affairs, Washington, DC 20005; and Department of Entomology, University of Maryland, College Park, MD 20742.
[5] Institut de Biologie Moléculaire et Cellulaire, 15, rue René Descartes, 67084 Strasbourg Cedex, France.
[6] Current address: Laboratoire de Neuroimmunologie des Annélides, UMR 8017 CNRS, SN3, Université des Sciences et Technologies de Lille, 59655 Villeneuve d'Ascq, France.
[7] E-mail: obrochta@umbi.umd.edu.

0022-2585/04/0447–0455$04.00/0

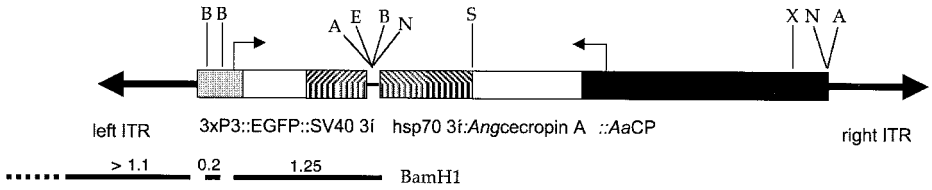

Fig. 1. Map of the transformation vector pPBMG-CEC (not to scale). 3xP3::EGFP::SV40 3′ is the transformation marker resulting in brain specific expression of enhanced green fluorescent protein. AaCP::Ang.CECA::hsp70 3′ is the effecter gene cassette consisting of the *carboxypeptidase A* promoter from *Ae. aegypti*, *cecropin A* from *An. gambiae*, and the 3′ region of the *D. melanogaster hsp70* gene containing a polyadenylation signal. The thin arrows indicate the direction of transcription from the functional promoters within the vector. The thick arrows represent the terminal inverted repeats and subterminal sequences of the *piggyBac* transposable element. The arrows at the end of the construct represent the *piggyBac* inverted terminal repeats (ITR) and subterminal sequences. Expected fragments hybridizing to a left end-specific probe on a Southern blot are shown along with their expected sizes in kilobase pairs. Restriction enzyme sites: B, *Bam*HI; E, *Eco*RI; A, *Asc*I; N, *Not*I; S, *Sal*I; X, *Xho*I.

tact with infective mosquitoes and reducing malaria transmission. The first step in exploring the feasibility of this novel method of malaria control is the creation of mosquitoes with appropriate genotypes and phenotypes.

For a mosquito to serve as a vector of malaria, it must provide a permissive environment for the multistage development and growth of *Plasmodium* parasites. Parasite gametes are ingested by the mosquito while feeding on the blood of an infected vertebrate host. Within the mosquito midgut gametocytes fuse to form zygotes, which rapidly differentiate into motile ookinetes that pass through the gut epithelium. Once through the gut, the ookinetes cease further movements, adhere to the basal surface of gut epithelium, and further differentiate into an oocyst. Oocyst growth and development results in the formation of large numbers of haploid, motile sporozoites that enter the hemolymph (circulatory system) and ultimately invade and colonize the insect's salivary glands. Parasite transmission to a new vertebrate host ensues in the course of subsequent blood feeding. Successful exploitation of the host insect by the parasite requires invasion and colonization of multiple tissue environments. These features of vector–parasite interactions might be exploited and has led to the conceptualization of distinct and potentially complementary approaches to creating refractory mosquitoes. For example, genes might be introduced into mosquitoes that kill the parasites or merely block their interactions with the host thereby preventing further parasite development. Furthermore, the genes responsible for conferring these phenotypes might be native to the host insect or they may be exotic, i.e., synthetic or from heterologous species.

Recently the feasibility of expressing exotic genes that result in blocking critical *Plasmodium*–mosquito interactions with the gut and salivary glands of *An. stephensi* was reported (Ito et al. 2002, Moreira et al. 2003). While representing an important advance, it is only one approach to creating refractory mosquitoes, namely, the introduction of foreign genes with antiparasitic activity. Other approaches are possible, including the manipulation of the mosquito's endogenous immune system. There is evidence from a variety of sources that indicate that the ingestion and subsequent development of *Plasmodium* stimulates an immune response in mosquitoes (Dimopoulos et al. 1997, Richman et al. 1997, Dimopoulos et al. 1998, Vizioli et al. 2000). Furthermore, there is evidence that some of the immune peptides expressed in response to *Plasmodium* infection have anti-*Plasmodium* activity (Gwadz et al. 1989, Shahabuddin et al. 1998). The limited ability of these endogenous immune responses to block *Plasmodium* development is due in part to the parasite's ability to invade tissues where these antiparasitic peptides are not synthesized. Thus, creating mosquitoes with altered temporal and spatial patterns of immune-peptide expression represents a possible means of producing insects refractory to *Plasmodium* infection. Here we test this hypothesis directly by measuring the effects of altered patterns of *cecA* expression in *An. gambiae* on the early stages of *P. berghei* development.

Materials and Methods

Germ-Line Transformation Vector. The effector-gene cassette was assembled in the shuttle vector pSLfa1180fa (Horn and Wimmer 2000). The *An. gambiae cecropin A* (*AngCecA*) cDNA was cloned as a 250-bp polymerase chain reaction (PCR) fragment downstream of a 1181-bp PCR fragment containing the 5′ regulatory sequences from the *Aedes aegypti carboxypeptidase A* (CP) promoter (Edwards et al. 2000). The 3′ region of the *Drosophila melanogaster hsp70* gene containing a polyadenylation signal was added (Knipple and Marsella-Herrick 1988). The effector-gene cassette was inserted as an *Asc*I fragment into the pBac(3xP3-EGFPafm) transformation vector containing the synthetic, eye-specific promoter (3xP3) regulating the expression of the enhanced green fluorescent protein gene (EGFP) flanked by the essential terminal sequences of the *piggyBac* transposable element (Horn and Wimmer 2000). The resulting vector was referred to as pPBMG-CEC (for *piggyBac* midgut cecropin; Fig. 1).

***Anopheles gambiae* Transformation.** The transformation vector pPBMG-CEC (300 μg/ml) was co-injected with the *piggyBac* transposase-encoding helper plasmid phsp-pBac (Handler and Harrell 1999) (150 μg/ml) into *An. gambiae* embryos of the strain G3 essentially as described previously (Grossman et al. 2001). Eggs were collected from blood-fed females 72–120 h after a blood meal over a period of ≈30 min. Eggs were permitted to age ≈30 min until they were pale gray. Aged eggs were collected, aligned, and fixed to a cover slip using a strip of double-sided tape. The eggs were desiccated slightly and covered with Halocarbon oil (Series 27; Sigma, St. Louis, MO). The oil was removed immediately after injection, and the cover slip with the injected eggs was immersed in a beaker containing deionized water and incubated at 27°C until hatching. Hatched larvae were pooled and reared in conventional mosquito larvae-rearing trays using standard practices. Emerged adults were sorted by sex and used to establish founder families. Each founder family consisted of ≈20 adult mosquitoes originating from injected embryos (G_0) and mated with 60–100 wild-type mosquitoes of the opposite sex. Progeny of these families (G_1) were screened as young larvae for the presence of tissue expressing the green fluorescence protein. At each generation during this experiment, mosquitoes were propagated by crossing transgenic males with virgin nontransgenic G_3 females.

Southern Hybridization. Fifteen micrograms of genomic DNA were digested to completion with *Bam*HI according to the manufacturer's recommendations (New England Biolabs, Beverly, MA). The digested DNA was size-fractionated on a 1% agarose gel, transferred to a nylon filter by capillary action, hybridized with a ^{32}P-labeled probe specific for the left end of *piggyBac*, and prepared using a random priming method according to the manufacturer's recommendations (Prime-It II; Stratagene, La Jolla, CA). Filters were prehybridized and hybridized in QuickHyb (Stratagene) at 60°C and washed under high stringency conditions. Hybridization was detected using a Storm 860 phosphoimager (Amersham Biosciences, Piscataway, NJ).

Transposable Element Display. Transposable element (TE) display is a DNA fingerprinting technique similar to amplified fragment length polymorphism (AFLP) analysis (Vos et al. 1996) but results in only genomic fragments containing specific transposable elements being detected as determined by the specific PCR primers used. TE display was performed essentially as described previously (Casa et al. 2000). Genomic DNA from individual adult mosquitoes was isolated and digested with *Msp*I. Adapters consisting of a duplex of oligonucleotides MspIa (5′-GACGATGAGTCCTGAG) and MspIb (5′-CGCTCAGGACTCAT) were ligated, and semi-nested PCR reactions were performed. The initial preselective PCR reaction was conducted with the primers MspIa and the *piggyBac*-specific primers piggyL1 (5′-TATGAGTTAAATCTTAAAACTCACG) for analysis of the left end and piggyR1 (5′-GTGAATTTATTATTAGTATGTAAGTG) for analysis of the right end. Preselective reactions were performed in 2.5 mM $MgCl_2$ using the following cycle conditions: 95°C × 3min + 25(95°C × 15 s + 54°C × 30 s + 72°C × 1 min) + 72°C × 5 min. These reactions were followed by a round of selective PCR using primers *Msp*Ia and the Cy5-labeled primers piggyL2Cy5 (5′-Cy5-CAGTGACACTTACCGCATTGACAAGC) for analysis of the left end and piggyR2Cy5 (5′-Cy5-ATATACAGACCGATAAAAACACATGCG) for analysis of the right end. Selective PCR reactions were performed in 2.5 mM $MgCl_2$ with the following cycle conditions: 95°C × 3 min + 5(95°C × 15 s + 59°C – 1°C/cycle × 30 s + 72°C × 1 min) + 25(95°C × 15 s + 54°C × 30 s + 71°C × 1 min) + 72°C × 5 min. Reaction products were fractionated on an 8% denaturing polyacrylamide DNA sequencing gel, blotted onto 3MM paper, dried, and scanned on a Storm 860 phosphoimager (Molecular Dynamics). Reaction products of interest were cut from the gel, reamplified using the selective PCR conditions described above with unlabeled primers, and sequenced.

Reverse Transcriptase-PCR. Total RNA was isolated from adult females using the RNeasy procedure according to the manufacturer's specifications (Qiagen, Valencia, CA). cDNA synthesis and subsequent PCR were performed essentially as described previously (Richman et al. 1997). To detect actin transcripts, the primers actinf (5′-ATTAAGGAGAAGCTGTGCTACGTC) and actinr (5′-CATACGATCAGCAATACCTGGG) were used. To detect cecropin transcripts, the primers CECf (5′-AAAGCTTAACAACAATGAACTTCTCC) and CECr (5′-CGCCGACGCTCTAACCGAG) were used. To detect only the transgenic cecropin transcript, the primers tCECf (5′-TTGGAAAAGCTTAACAACAATG), which spans the junction between the *carboxypeptidase A* untranslated leader and the 5′ end of the cecropin transgene, and tCECr (5′-TATTTGGCTTTAGTCGAGGTCG), which spans the junction between the 3′ end of cecropin transgene and the *D. melanogaster hsp 70* sequences containing a polyadenylation signal, were used.

Immunofluorescence. Midguts were dissected in cold Grace's media from sugar- and blood-fed females (24 ± 2 h after blood feeding). The contents of the guts were removed, and the guts were thoroughly washed with fresh Grace's media. Tissue was fixed in 200 μl of a 1:1 mixture of 4% paraformaldehyde and heptane in a 96-well plate and shaken at 250 rpm for 20 min. Fixative (lower phase) was removed, 100 μl of methanol was added, and the tissue was shaken for 1 min. Both phases were removed, and tissue was rinsed in 200 μl of methanol three times before treating with a mixture of 180 μl methanol and 20 μl of 30% H_2O_2 for 15 min at room temperature. The tissue was washed three times (20 min each) in 200 μl of a 1:1 mixture of methanol and phosphate-buffered saline with 0.1% Triton × 100 (PBST). The tissue was washed five times (15 min each) in 200 μl PBST with 1% bovine serum albumin (PBSBT). Blocking was performed in PBSBT for 1 h at room temperature. The primary antibody was a rat polyclonal antibody (URANO) raised against *An. gambiae cecropin A* and

with cross-reactivity to the synthetic amidated and acid forms of the protein (J. V., unpublished data). Primary antibody (1:1,000 in PBST) was added to the fixed and blocked tissue and allowed to incubate at 4°C overnight. The primary antibody was removed, and the tissue was washed in PBSBT (3 × 5 min; 5 × 15 min). The secondary antibody was Oregon Green–labeled goat anti-rat IgG (Molecular Probes) diluted 1:200 in PBST. Secondary antibody binding was performed in the dark at room temperature for 2 h. The tissue was washed in PBSBT (3 × 5 min; 5 × 15 min) and mounted on a glass slide in Vecta-Shield (Vector Laboratories, Burlingame, CA) and visualized using a Zeiss M^2Bio fluorescence microscope with EGFP filters (Carl Zeiss, Thorn Wood, NY). The URANO antibody was effective at detecting cecA in tissue preparations but was inefficient at detecting cecropin peptides on Western blots. Therefore, experiments to detect expressed cecA protein relied on immunofluorescence and not Western blotting.

Plasmodium Infection. Mosquitoes (3–5 d old) were fed on mice (Balb c) infected with *P. berghei* ANKA 2.34 with 10–15% parasitemia and 1–1.5% gametocytemia. Blood-fed mosquitoes were kept at 19°C, and the number of oocysts per midgut was counted between days 12 and 14 after feeding following dissection and staining with mercurochrome.

Results

Transgenesis. Of the 3,452 embryos injected with pPMG-CEC and the helper plasmid phsp-pBac, 381 hatched (11%), resulting in 163 (4.7%, 87 male and 76 female) adults (G_0). G_0 adults were used to establish seven families that yielded a total of 9,626 G_1 larvae. Two families produced transgenic progeny for an estimated transformation frequency of 1.2%. Physical evidence for the presence of the vector in the host's genome came from three sources. First, individuals from both lines have strong expression of EGFP in the brain and ventral nerve cord of larvae, which is characteristic for the 3xP3 promoter (Fig. 2). Line 1 also has strong EGFP expression in the larval salivary glands and anal papillae. Adults from both lines had detectable EGFP expression in the brain (Fig. 2). Second, hybridization analysis of total genomic DNA using the method of Southern revealed the presence of a single hybridizing "junction fragment" (a fragment containing the inverted terminal repeat [ITR] of the element and genomic DNA consisting of the target site and flanking DNA). Line 1 had a unique 3.5-kb *Bam*HI junction fragment, whereas line 2 had two junction fragments, which were 3.0 and 6.0 kb, respectively (Fig. 3). Third, the AFLP-like DNA-fingerprinting method, TE display, was used to detect, quantitate, and isolate junction fragments from each line. Line 1 yielded a single junction fragment containing the right ITR, whereas line 2 yielded two fragments containing the right ITR. In both lines, only those sequences precisely flanked and including the inverted terminal repeats of the *piggyBac* vector found originally in the donor plasmid were present in the genome of the transgenic mosquitoes. In all cases, the vector integrated into a TTAA target site as is typical of *piggyBac* elements (Fig. 3). Flanking genomic DNA sequences determined by TE display analysis were used in a BLAST search and showed that one insertion site in transgenic line 2 occurred in the third chromosome and the other insertion occurred in a segment of the genome that has not yet been linked to any of the chromosomes of *An. gambiae* (Altschul et al. 1990). BLAST searches of existing DNA sequence databases did not reveal any significant similarities to the integration site found in transgenic line 1 (searches performed June 2003).

Genetic evidence for the integrative transformation of *An. gambiae* using pPMG-CEC consists of 18 mo (as of June 2003) of continuous culture of lines 1 and 2, and both are currently maintained as homozygotes.

Cecropin Transgene Transcription. The *piggyBac* vector pPMG-CEC contains a copy of the *An. gambiae cecA* (*AngCEC*) cDNA under the regulatory control of the *Ae. aegypti carboxypeptidase* (*AeCPA*) promoter. The *carboxypeptidase* promoter is blood-meal inducible and gut-specific in *Ae. aegypti* and was expected to result in the production and accumulation of transgene transcripts beginning ≈24 h after blood feeding. The temporal and spatial patterns of *AeCPA::AngCecA* transgene transcription were investigated using reverse transcriptase (RT)-PCR. Using transgene-specific primers, we detected transgene transcripts only in the midguts of blood-fed females from lines 1 and 2. Transgene transcripts were not detected in the carcasses of transgenic insects from which the guts had been removed. No transgene transcripts were detected in the midguts of unfed females or in their carcasses after removal of the midgut (Fig. 4). The highest levels of transcripts were observed ≈24 h after blood feeding (data not shown), and this is consistent with the temporal pattern of expression displayed by the promoter of the *carboxypeptidase A* gene in *Ae. aegypti* (Edwards et al. 2000, Moreira et al. 2000). Furthermore, the transgenic insects had a new spatial pattern of cecropin transcription. In nontransgenic *An. gambiae*, endogenous *AngCecA* transcription does not occur in the posterior midgut, but transcripts are found in the anterior midgut as well as other tissues (Vizioli et al. 2000). In both transgenic lines, however, *AngCecA* transcripts were detected in the posterior midgut, the sole site of ookinete invasion (Fig. 4).

Cecropin Synthesis. Using immunofluorescence methods on whole mounts of midguts, the pattern and levels of cecA was determined in nontransgenic and transgenic insects. Midguts from unfed transgenic and nontransgenic female mosquitoes had clear evidence of anti-cecA antibody binding in the cardia and in the first three quarters of the anterior midgut. The posterior quarter of the anterior midgut and posterior midgut had no evidence of cecA antibody binding. Blood-fed insects 24 h after feeding had a similar pattern of anti-cecA antibody binding. There was abundant anti-cecA antibody binding in the cardia and

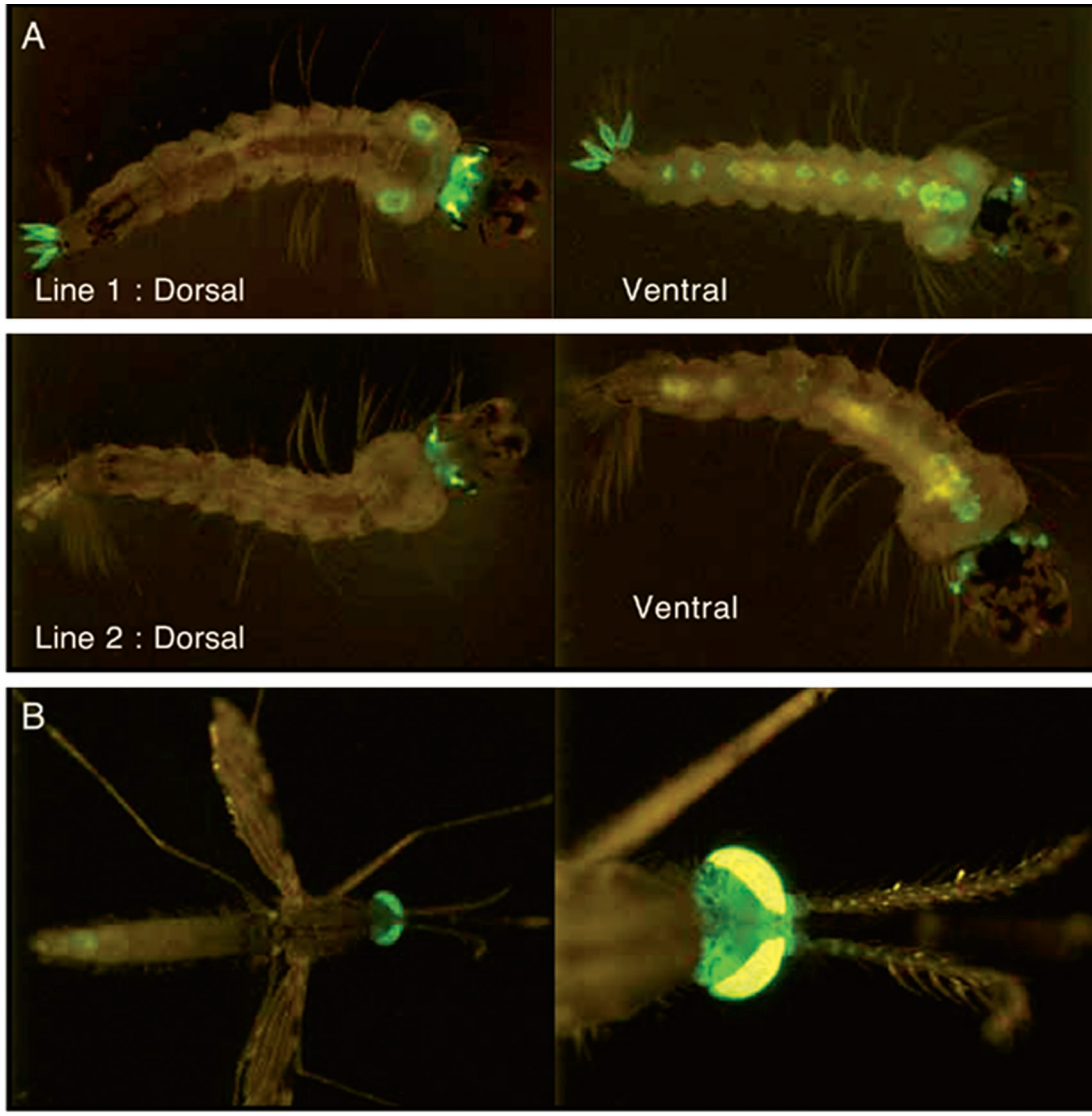

Fig. 2. Fluorescence photomicrographs of transgenic *An. gambiae* larvae and adults. (A) Top: dorsal and ventral view of line 1 larvae showing EGFP expression in the brain, ventral nerve cord, anal papillae, and salivary glands; bottom: dorsal and ventral view of line 2 larvae showing EGFP expression in the brain and ventral nerve cord. B, newly-emerged transgenic adult of line 1.

anterior three-fourths of the anterior midgut but not in the posterior midgut of either transgenic or nontransgenic insects.

Oocyst Development. Targeted expression of the *AngCecA* immune peptide to the posterior midgut resulted in significant reductions in oocyst development in the two transgenic lines of *An. gambiae.* Parasite development in both lines, as measured by counting the number of oocysts on the midgut approximately 2 wk after infection, was consistently and significantly impaired in transgenic mosquitoes (Table 1; Fig. 5). During the analysis of line 1, the number of oocysts observed in nontransgenic control mosquitoes was 26.6 ± 3.3 (mean ± SE) compared with 12.9 ± 2.1 in transgenic individuals. For experiments involving line 2, which were not conducted at the same time as studies involving line 1, the number of oocysts in nontransgenic and transgenic mosquitoes was 13.7 ± 2.2 and 6.1 ± 0.9, respectively. In both studies, the mean oocyst number in transgenic insects was significantly different from that in nontransgenic control insects ($P \leq 0.0.003$; *t*-test). On average we observed an ≈61% inhibition of oocyst formation by expressing *AngCecA* in the posterior midgut at the appropriate time. No notable effects on prevalence of infected mosquitoes was detected (Table 1).

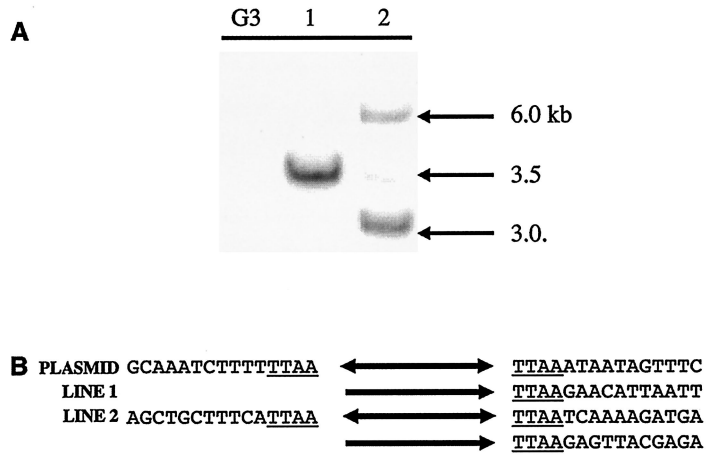

Fig. 3. Physical evidence of integrated gene vectors. (A) Southern blot of total genomic DNA digested with *Bam*HI. G3 refers to nontransgenic controls; 1 and 2 refer to lines 1 and 2, respectively. In addition to the bands shown, lines 1 and 2 had a common 1.25-kb hybridizing band as predicted from the map of the vector. (B) Results of cloning and sequencing junction fragments obtained from TE display. The TTAA canonical target site is shown. The dark arrows represent the *piggyBac* vector and the sequences are flanking genomic DNA. The sequence flanking the vector in the original donor plasmid is shown.

Discussion

This is the first report of genetically engineered *Plasmodium* refractoriness in *An. gambiae*, the most important vector of human malaria in sub-Saharan Africa, it and demonstrates the feasibility of modulating innate defense mechanisms to confer resistance or partial resistance to a human parasite in an insect vector. An earlier study, involving transgenic expression of a defensin peptide in the yellow fever mosquito *Ae. aegypti*, while demonstrating tissue-specific expression resulting in secretion of defensin into the hemolymph, did not describe effects on parasite development (Kokoza et al. 2000). Cecropin synthesis from transgenic *Rhodococcus rhodnii* in the hindgut of reduviid vectors of *Trypanosoma cruzi* has previously been shown to reduce the number of *T. cruzi* parasites in the insect host (Durvasula et al. 1997). Gwadz et al. (1989) injected cecA peptide into the hemolymph of

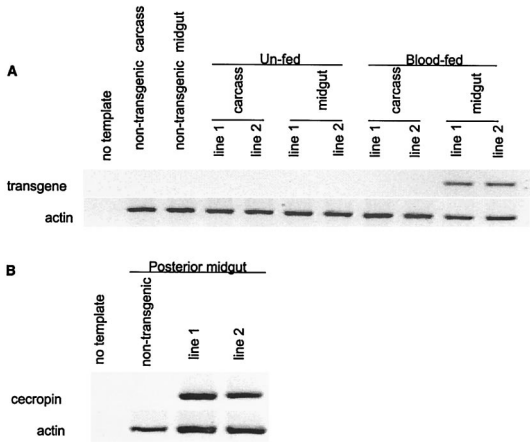

Fig. 4. Pattern of expression of native and transgenic cecropin. (A) RT-PCR analysis of cecropin transgene expression in adult females. Analysis was performed using primers specific for the transgene. (B) RT-PCR analysis of cecropin expression in the posterior midgut of blood-fed females 24 h after feeding. Analysis was performed using primers that will recognize native and transgenic cecropin.

Table 1. Comparison of susceptibility of transgenic and nontransgenic lines to *P. berghei* infection

Experiment		*n*	Prevalance (%)	Intensity (mean ± SD)	Inhibition (%)
I	Line 1	18	55.6	5.4 ± 5.8	70.2[a]
	Control	20	85.0	18.2 ± 24.9	
II	Line 1	20	80.0	12.7 ± 15.3	43.1
	Control	20	95.0	22.3 ± 10.7	
III	Line 1	20	95.0	17.5 ± 7.8	36.4
	Control	20	85.0	27.5 ± 41.2	
IV	Line 1	6	83.3	7.2 ± 8.8	70.3[a]
	Control	20	95.5	24.1 ± 23.0	
V	Line 1	14	78.6	9.6 ± 9.7	76.4[a]
	Control	20	85.0	40.9 ± 47.0	
VI	Line 2	20	85.0	13.0 ± 10.1	32.5
	Control	11	45.0	19.3 ± 33.4	
VII	Line 2	20	50.0	1.7 ± 3.1	83.1[a]
	Control	20	60.0	10.1 ± 12.7	
VIII	Line 2	20	45.0	4.4 ± 9.4	63.3[a]
	Control	20	75.0	11.9 ± 17.3	
IX	Line 2	20	80.0	7.1 ± 12.5	55.6[a]
	Control	20	95.0	16 ± 13.7	
X	Line 2	20	65.0	4.3 ± 5.9	82.5[a]
	Control	20	85.0	24.4 ± 22.7	

Lines 1 and 2 refer to the transgenic lines (represented by a mixture of hetero- and homozygous siblings). Control refers to nontransgenic mosquitoes that were fed on the same infected mouse as transgenic siblings. Prevalence is the percentage of mosquitoes that became infected. Intensity is the mean number of oocysts per gut. The SD of the mean number of oocysts per gut is shown. Inhibition is 100 [(control intensity − transgenic intensity)/control intensity].

[a] Statistically significant difference in mean intensities using a *t*-test. $P < 0.005$.

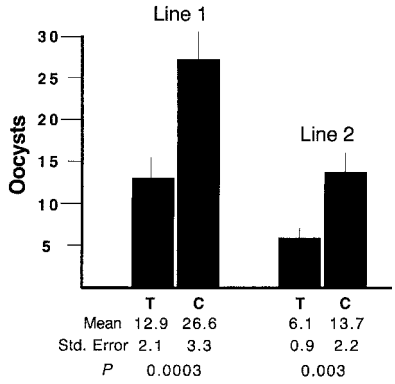

Fig. 5. Inhibition of oocyst development in transgenic mosquitoes. Pooled data from experiments involving lines 1 and 2 compared with nontransgenic controls examined at the same time. T, transgenic; C, nontransgenic. Shown are means ± SE and *P* values after analysis with a *t*-test.

An. gambiae and reported a reduction in the number of *Plasmodium* sporozoites, and we have shown that this peptide has anti-*Plasmodium* activity in vitro (A.M.R., unpublished data). These earlier studies have led directly to the hypothesis being tested in this study, namely, mosquitoes with altered temporal and spatial patterns of immune peptide expression have altered susceptibilities to *Plasmodium.*

The successful creation of transgenic insects in this study confirm the initial report of Grossman et al. (2001) of the ability of the *piggyBac* transposable element to serve as a gene vector in *An. gambiae.* Transposition-mediated integration occurred in this study at a frequency of ≈1.2% and is similar to the frequency reported by Grossman et al. (2001). In this study, the creation of transgenic *An. gambiae* posed a significant technical challenge. Although key parameters for transformation were not systematically analyzed in this study, we felt that the quality of the eggs used during the microinjection process was of great importance. Great care was taken to create egg-donor females under ideal laboratory conditions, resulting in large, healthy insects. Egg-donor females were fed at the earliest possible time postemergence, and only eggs laid early during the first gonotrophic cycle were used for injections. Larvae hatching from injected eggs were also reared under ideal laboratory conditions, as were their progeny. *An. gambiae* germ-line transformation remains a challenge, although it is clear that the *piggyBac* vector is functional, although inefficient, in this species.

The results of this study also demonstrate the functionality of the *3xP3* and *Ae. aegypti carboxypeptidase* promoters in *An. gambiae.* The *3xP3* promoter has been used in a wide variety of insects from four orders (Berghammer et al. 1999, Thomas et al. 2002, Sumitani et al. 2003), and its ability to yield clear tissue-specific expression of EGFP in *An. gambiae* was not unexpected. The pattern of expression in line 1 was similar to that described by others in *An. stephensi* (Ito et al. 2002) and included the salivary glands, as well as the brain, ventral ganglion, and anal papillae. The *Ae. aegypti carboxypeptidase* promoter functions in *An. gambiae* and is expressed in the posterior midgut beginning ≈24 h after blood feeding. A detailed timecourse of promoter induction in the transgenic insects used in this study was not performed; however, expression (as reflected by the presence of transcripts) was detectable at 24 h after feeding. Whereas we were able to detect transcripts of the cecA transgene, we were unable to obtain evidence for the peptide based on immunofluorescence detection methods. The pattern of cecA peptide observed by immunofluorescence was the same in transgenic and nontransgenic as well as sugar- and blood-fed females. The pattern observed was consistent with the description of the distribution of cecA in nontransgenic *An. gambiae* (C. Lowenberger and J. Vizioli, personal communication). Although transgenic cecA peptide could not be physically detected in the posterior midgut, and because transgenic cecA expression did correlate with a significant biological phenotype (partial refractoriness), we suggest that the negative results from the immunofluorescence experiment were caused by either low steady-state protein levels or rapid protein turnover.

We observed a significant reduction in the number of oocysts present on the gut walls of infect guts of transgenic insects compared with nontransgenic controls. While significant cecropin-dependent refractoriness to *P. berghei* was observed, complete elimination of infection did not occur under these laboratory conditions, and there are a number of possible explanations for this. First, cecA may not be a potent enough to eliminate all parasites in vivo. Second, the levels of cecA peptide may not have been high enough to result in a complete elimination of the parasite. The inability to detect cecA peptide by immunofluorescence suggests that the peptide may be at very low levels in the posterior midgut. Efforts to increase the levels of cecA either by manipulating transcription levels or protein turnover rates might result in an increase in antiparasitic activity. Third, the strategy used to create refractory mosquitoes in this experiment depended on temporally coordinated expression of the antiparasitic protein. Here that was done by using the promoter from the *carboxypeptidase A* gene from *Ae. aegypti* and while blood-meal inducible expression was observed at the appropriate time, if the parasites were beginning to penetrate the gut somewhat before expression was initiated they may escape the antiparasitic effects of cecA. Therefore, a strategy that does not depend on the precise coordination of cecA expression in the posterior midgut with the biology of the parasite to be effective may permit the antiparasitic potential of cecA in vivo to be more directly assessed. Finally, it is important to note that laboratory infection conditions used here are optimized to yield maximum oocyst numbers. By contrast, under conditions designed to approximate natural infection, midgut oocyst loads of approximately two per midgut are characteristic, and only 10–20% of challenged mosquitoes are usually infected (Boudin et al.

1993, Tchuinkam et al. 1993). Furthermore, examination of the midguts of wild-caught *An. gambiae* revealed very low mean ookinete rates, usually of <5 (Beier et al. 1992). It is speculated, in fact, that the effectiveness of *An. gambiae* as a vector of *P. falciparum* is due in large measure to the relatively high efficiency with which ookinete to oocyst differentiation succeeds in this mosquito species. As such, modification of the midgut environment to an "immune active" state, as reported here, may effectively eliminate oocysts from the mosquito gut under natural infection conditions. Laboratory infections yielding abnormally large numbers of ookinetes may simply "titrate out" available, steady-state concentrations of transgene-derived cecropin peptide. Further studies are required to determine how the effectiveness of various antiparasitic peptides such as cecA varies as a function of the intensity of infection. The implicit assumption by those interested in creating these types of insects is that the introduced antiparasite activity is independent of infection intensity. More experimentation in that area is needed. Nevertheless, the insects created in this study are still likely to be effective transmitters of malaria, and we are not proposing that the insects created here represent candidates for future releases. We are proposing that, based on the results of these findings, continued interest in manipulating the endogenous innate immune system for the purposes of developing refractory phenotypes is warranted.

The innate immune system of insects with its suite of genes encoding small peptides with a variety of antimicrobial activities is one means by which insects defend themselves from pathogens and parasites. Normal spatial and temporal patterns of expression of the innate immunity genes, however, can limit their effectiveness. The significance of the work reported here is that it demonstrates the feasibility of manipulating an insect's endogenous immune system in such a way as to alter its ability to serve as a host for a human pathogen. Although a number of strategies for altering the vector status of malaria-transmitting mosquitoes need to be explored before an effective genetic control strategy involving population replacement can be designed, the strategy demonstrated here has a number of features that may make it attractive and potentially useful in future efforts. First, the creation of parasite resistant mosquitoes can be expected to impose unique selection pressures on parasite populations. The emergence of parasites resistant to any transgenic antiparasite strategy is something that will need to be thoroughly explored. Certain effecter genes and resistance strategies may be more difficult for the parasites to circumvent then others. Because cecropin works by disrupting lipid membranes, it may be less likely that parasite resistance will develop quickly, although, clearly, any refractory mechanism poses a strong selection pressure that will be a strong force in driving the evolution of the parasite. Second, transgenic organisms that are to be released into the environment are scrutinized and evaluated as part of an effort to ensure that they will not pose a threat to human and environmental safety. The introduction of exotic genes, either synthetic or from heterologous species, tends to complicate risk assessment efforts. The strategy described here has relied on manipulating the expression of an endogenous mosquito gene and has tended to minimize the amount of foreign DNA being introduced into this species. This strategy may facilitate any subsequent risk assessment efforts. Furthermore, cecropin has been shown to be active against the metazoan parasite *Brugia pahangi*, which is also vectored by mosquitoes (Chalk et al. 1995). Thus, use of a broadly active transgenic resistance determinant such as cecropin may have additional beneficial effects on human health compared with an "exotic" construct specifically designed to block development of a specific pathogen. Clearly, the feasibility of manipulating the susceptibility of the major human malaria vector, *An. gambiae*, using transgenic technologies has been demonstrated. The challenge for the future will be to find effecter genes or combinations of effecter genes that produce robust phenotypes that cannot be readily circumvented by the parasites. Furthermore, means by which these genotypes can be introduced into natural populations in such a way as to result in their rapid distribution remain to be identified.

Acknowledgments

We thank M. Jacobs-Lorena for the plasmid pBac3xP3-EGFPafm and information about *carboxypeptidase A* of *Aedes aegypti*, J. Orsetti for technical advice and assistance, and D. Hawthorne for advice and assistance with data analysis. The first two authors conducted this study during their sabbatical leave within the Department of Entomology, University of Maryland, College Park. The research was supported by the Department of Entomology, University of Maryland, College Park (W. K., H. K., A.M.R.), Grant KRF-DP0467 (W. K.), Sangji University 2001–2002 Grant (H. K.), and National Institutes of Health Grants GM48102 and GM20075 (D. S., D.A.O'B.).

References Cited

Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman. 1990. Basic local alignment search tool. J. Mol. Biol. 215: 403–410.

Beier, J. C., R. S. Copeland, R. Mtalib, and J. A. Vaughan. 1992. Ookinete rates in Afrotropical anopheline mosquitoes as a measure of human malaria infectiousness. Am. J. Trop. Med. Hyg. 47: 41–46.

Berghammer, A. J., M. Klingler, and E. A. Wimmer. 1999. A universal marker for transgenic insects. Nature (Lond.). 402: 370.

Boudin, C., M. Olivier, J.F.C. Molez, J. P., and P. Ambroise-Thomas. 1993. High human malarial infectivity to laboratory-bred *Anopheles gambiae* in Burkino Faso. Am. J. Trop. Med. Hyg. 48: 700–706.

Casa, A. M., C. Brouwer, A. Nagel, L. Wang, Q. Zhang, S. Kresovich, and S. R. Wessler. 2000. The MITE family *Heartbreaker* (*Hbr*): molecular markers in maize. Proc. Natl. Acad. Sci. USA. 97: 10083–10089.

Chalk, R., H. Townson, and P. J. Ham. 1995. *Brugia pahangi:* The effects of cecropins on microfilariae in vitro and in *Aedes aegypti.* Exper. Parasitol. 80: 401–406.

Collins, F. H. 1994. Prospects for malaria control through the genetic manipulation of its vectors. Parasitology Today. 10: 370–371.

Curtis, C. F., and P. M. Graves. 1988. Methods for replacement of malaria vector populations. J. Trop. Med. Hyg. 91: 43–48.

Dimopoulos, G., A. M. Richman, H. M. Muller, and F. C. Kafatos. 1997. Molecular immune resonses of the mosquito *Anopheles gambiae* to bacteria and malaria parasites. Proc. Natl. Acad. Sci. USA. 94: 11508–11513.

Dimopoulos, G., D. Seeley, A. Wolf, and F. C. Kafatos. 1998. Malaria infection of the mosquito *Anopheles gambiae* activates immune-responsive genes during critical transition stages of the parasite life cycle. EMBO J. 17: 6115–6123.

Durvasula, R. V., A. Gumbs, A. Panackal, O. Kruglov, S. Aksoy, R. B. Merrifield, F. F. Richards, and C. B. Beard. 1997. Prevention of insect-borne disease: An approach using transgenic symbiotic bacteria. Proc. Natl. Acad. Sci. USA. 94: 3274–3278.

Edwards, M. J., L. A. Moskalyk, M. Donelly-Doman, M. Vlaskova, R. G. Noriega, V. K. Walker, and M. Jacobs-Lorena. 2000. Characterization of a carboxypeptidase A gene from the mosquito, *Aedes aegypti.* Insect Molec. Biol. 9: 33–38.

Greenwood, B., and T. Mutabingwa. 2002. Malaria in 2002. Nature (Lond.). 415: 670–672.

Grossman, G. L., C. S. Rafferty, J. R. Clayton, T. K. Stevens, O. Mukabayire, and M. Q. Benedict. 2001. Germline transformation of the malaria vector, Anopheles gambiae, with the *piggyBac* transposable element. Insect Molec. Biol. 10: 597–604.

Gwadz, R. W., K. Kaslow, J. Y. Lee, W. L. Maloy, M. Zasloff, and L. H. Miller. 1989. Effects of magainins and cecropins on the sporogonic development of malaria parasites in mosquitoes. Infect. Immunol. 57: 2628–2633.

Handler, A. M., and R. A. Harrell. 1999. Germline transformation of *Drosophila melanogaster* with the *piggyBac* transposon vector. Insect Molec. Biol. 4: 449–458.

Handler, A. M., and A. A. James (eds.). 2000. Insect transgenesis. CRC, Boca Raton, FL.

Horn, C., and E. A. Wimmer. 2000. A versatile vector set for animal transgenesis. Dev. Genes Evol. 210: 630–637.

Ito, J., A. Ghosh, L. A. Moreira, E. A. Wimmer, and M. Jacobs-Lorena. 2002. Transgenic anopheline mosquitoes impaired in transmission of a malaria parasite. Nature (Lond.). 417: 452–455.

Knipple, D. C., and P. Marsella-Herrick. 1988. Versatile plasmid vectors for the construction, analysis and heat-inducible expression of hybrid genes in eukaryotic cells. Nucleic Acids Res. 16: 7748.

Kokoza, V., A. Ahmed, W. L. Cho, N. Jasinskiene, A. A. James, and A. Raikhel. 2000. Engineering blood meal-activated systemic immunity in the yellow fever mosquito, *Aedes aegypti.* Proc. Natl. Acad. Sci. USA. 97: 9144–9149.

Moreira, L. A., M. J. Edwards, F. Adhami, N. Jasinskiene, A. A. James, and M. Jacobs-Lorena. 2000. Robust gut-specific gene expression in transgenic *Aedes aegypti* mosquitoes. Proc. Natl. Acad. Sci. USA. 97: 10895–10898.

Moreira, L. A., J. Ito, A. Ghosh, M. Devenport, H. Zieler, E. G. Abraham, A. Crisanti, T. Nolan, F. Catteruccia, and M. Jacobs-Lorena. 2002. Bee venom phospholipase inhibits malaria parasite development in transgenic mosquitoes. J. Biol. Chem. 279: 40,839–40,843.

Richman, A. M., D. G., D. Seeley, and F. C. Kafatos. 1997. Plasmodium activates the innate immune response of *Anopheles gambiae* mosquitoes. EMBO J. 16: 6114–6119.

Shahabuddin, M., I. Fields, P. Bulet, J. A. Hoffman, and L. H. Miller. 1998. *Plasmodium gallinaceum:* differential killing of some mosquito stages of the parasite by insect defensin. Exp. Parasitol. 89: 103–112.

Sumitani, M., D. S. Yamamoto, K. Oishi, J. M. Lee, and M. Hatakeyama. 2003. Germline transformation of the sawfly, *Athalia rosae* (Hymenoptera: Symphyta), mediated by a *piggyBac*-derived vector. Insect Biochem. Mol. Biol. 33: 449–458.

Tchuinkam, T., B. Mulder, K. Dechering, H. Stoffels, J. P. Verhave, M. Cot, P. Carnevale, J. H. Meuwissen, and V. Robert. 1993. Experimental infections of *Anopheles gambiae* with *Plasmodium falciparum* of naturally infected gametocyte carriers in Cameroon: factors influencing the infectivity to mosquitoes. Trop. Med. Parasitol. 44: 271–276.

Thomas, J. L., D. M., A. Besse, B. Mauchamp, and G. Chavancy. 2002. 3xP3-EGFP marker facilitates screening for transgenic silkworm *Bombyx mori* L. from the embryonic stage onwards. Insect Biochem. Mol. Biol. 32: 247–253.

Vizioli J., P. Bulet, M. Charlet, C. Lowenberger, C. Blass, H. M. Muller, G. Dimopoulos, J. Hoffmann, and R. A. Kafatos. 2000. Cloning and analysis of a cecropin gene from the malaria vector mosquito, *Anopheles gambiae.* Insect Molec. Biol. 9: 75–84.

Vos, J. C., I. DeBaere, and R.H.A. Plasterk. 1996. Transposase is the only nematode protein required for in vitro transposition of *Tc1.* Genes Develop. 10: 755–761.

Received for publication 24 June 2003; accepted 30 October 2003.