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Stable and heritable gene silencing in the malaria vector *Anopheles stephensi*

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ABSTRACT

Heritable RNA interference (RNAi), triggered from stably expressed transgenes with an inverted repeat (IR) configuration, is an important tool for reverse genetic studies. Here we report on the development of stable RNAi in *Anopheles stephensi* mosquitoes, the major vector of human malaria in Asia. Transgenic mosquitoes stably expressing a RNAi transgene, designed to produce intron-spliced double-stranded RNA (dsRNA) targeting the green fluorescent protein *EGFP* gene, were crossed to an *EGFP*-expressing target line. *EGFP* expression was dramatically reduced at both the protein and RNA levels. The levels of gene silencing depended upon the RNAi gene copy number and its site of integration. These results demonstrate that specific RNAi-mediated knockdown of gene function can be achieved with high efficiency in *Anopheles*. This will be invaluable to systematically unravel the function of *Anopheles* genes determining the vectorial capacity of the malaria parasite.

INTRODUCTION

Stable and heritable RNA interference (RNAi), a form of post-transcriptional gene silencing that results in the sequence-specific degradation of mRNA by homologous dsRNA molecules (1,2), has been utilized in numerous organisms including *Caenorhabditis elegans* (3), trypanosomes (4,5), *Drosophila* (6–8), plants (9) and, recently, by expressing short-interfering RNAs in mammalian cells (10) to investigate the function of target genes. Because of its flexibility, RNAi technology has become a powerful tool for reverse genetic studies in organisms where generating loss-of-function phenotypes by the direct manipulation of target genes has proven difficult or impossible. In *Anopheles* mosquitoes, the vectors of human malaria, genetic transformation is routinely achieved through a transposon-mediated integration process which occurs at random locations in the genome (11,12), thus preventing the targeted disruption or manipulation of any gene of choice. As an alternative to gene targeting by conventional methods, RNAi in *Anopheles* has been employed in the form of injection of *in vitro* synthesized dsRNA molecules into adult *Anopheles gambiae* mosquitoes (13). However, experimental evidence indicates that the silencing effects of injected dsRNA are generally limited in time and space (14,15). The development of a heritable and robust RNAi technology for anopheline mosquitoes would provide the opportunity to exploit the information generated by the recent completion of the *A.gambiae* genome sequencing project (16). The application of this RNAi technology for reverse genetic screens could be used to assess the role of putative genes involved in interactions with *Plasmodium* parasites, as well as expanding the knowledge of important biological processes, such as embryogenesis, sex determination and behavior.

To validate heritable RNAi technology in *Anopheles*, we targeted an *EGFP* gene integrated in the genome of transgenic *Anopheles stephensi* mosquitoes (17). We anticipated that this approach would facilitate the assessment of the efficiency of the technology without the bias introduced by the loss of function of endogenous genes. For this purpose *A.stephensi* lines were developed using a red fluorescent protein DsRed-marked transposon, which contained an RNAi gene (18) designed to encode an intron-spliced dsRNA targeting the green fluorescent protein *EGFP* gene. Transgenic lines were crossed to *EGFP*-expressing mosquitoes, and the progeny of the crosses analyzed for *EGFP* expression at both the protein and the RNA levels. Various degrees of inhibition were observed, ranging from marginal to high level silencing. The reduction in *EGFP* expression was dependent upon the site of integration of the inverted repeat (IR) transgene and its copy number.

MATERIALS AND METHODS

Plasmid construction

Plasmid pIR-EGFP was developed as described previously (19). Briefly, *EGFP* IR were produced by duplicating, in the opposite direction, the *EGFP* gene (Clontech) by directionally inserting a 465 bp (from position 219 to 683) PCR product into the plasmid pCR2.1v (Invitrogen). To link the two arms of the inverted repeat, a 67 bp PCR product corresponding to intron 1 from the *A.gambiae* lysozyme gene (GenBank accession no. U28809) was amplified from *A.gambiae* genomic DNA and inserted between the *EGFP*-IR (Fig. 1a), as previously described (19). This IR-*EGFP* cassette was then inserted between the *Actin*5C promoter and *Hsp*70 terminator elements from *Drosophila melanogaster* and cloned as a NotI cassette

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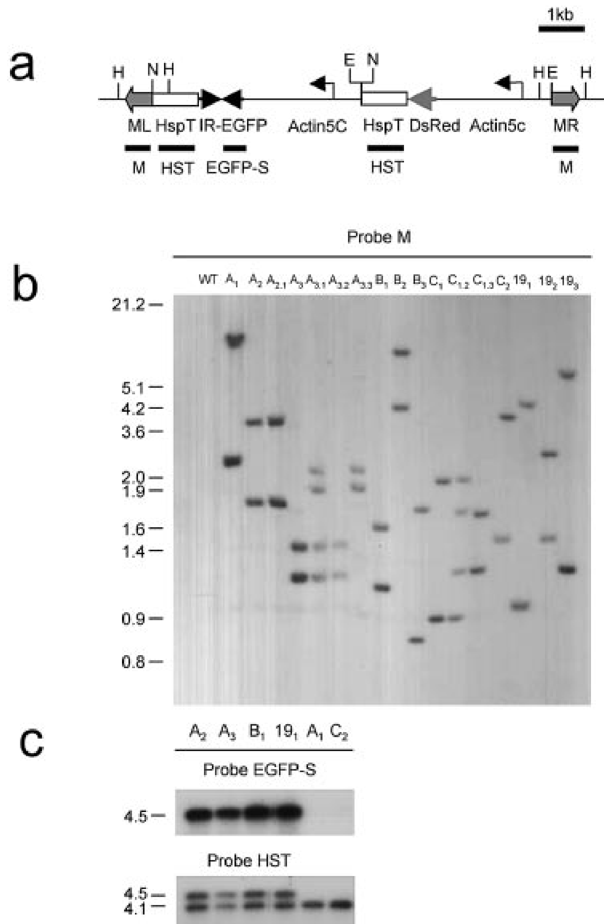

Figure 1. Plasmid pIR-EGFP and Southern blot analysis of transgenic lines. (**a**) Schematic representation of transformation vector pIR-EGFP. Actin5C, *D.melanogaster Actin*5C promoter; HspT, *D.melanogaster Hsp*70 terminator sequence; ML, *minos* left arm; MR, *minos* right arm; IR-EGFP, *EGFP* inverted repeat; DsRed, *DsRed* selectable marker. The restriction sites (H, HincII; E, EcoRI; N, NotI) and the probes (M, EGFP-S and HST, represented by black bars) used in the Southern blot analyses are indicated. A 1 kb scale marker is shown. (**b**) Southern blot analyses of genomic DNA from 17 transgenic lines digested with HincII and hybridized with probe M. (**c**) Southern blot analyses of genomic DNA from selected lines eIR-A_1, eIR-A_2, eIR-A_3, eIR-B_1, eIR-C_2 and eIR-19_1, digested with EcoRI/NotI and hybridized with probe EGFP-S (upper) or probe HST (lower). The size marker is in kilobases (kb). A_2, eIR-A_2; A_3, eIR-A_3; B_1, eIR-B_1; 19_1, e-IR-19_1; A_1, eIR-A_1; C_2, eIR-C_2.

into a *minos*-based plasmid that contained the *DsRed* marker gene, also cloned under the control of the *Actin*5C promoter (Fig. 1a).

Development of transgenic RNAi lines

Wild-type *A.stephensi* embryos (strain sd 500) were injected, essentially as described previously (11), with a mixture of helper plasmid pHSS6hsILMi20 (100 µg/ml) (20) and pIR-EGFP (400 µg/ml) (19). Homozygous lines were developed as described previously (11).

Southern blot analyses of transgenic lines

Genomic DNA from transformed adult mosquitoes (G_3 and G_9 generations) was digested with either EcoRI and NotI or HincII restriction endonucleases. Digested genomic DNA (~4 µg/lane) was separated on a 0.8% (w/v) agarose gel and transferred onto a nylon membrane. Membranes were hybridized overnight at 65°C with the following ^{32}P-labeled probes. Probe M: a PCR product encompassing the terminal inverted repeats of the left and right *minos* arms, as described previously (11). Probe EGFP-S: a 0.65 kb PCR product complementary to the sense arm of IR-*EGFP*, amplified using the primer combination pEGFP-$S_{465}$for (5′-CCCGGA-TCCCCGCTACCCCGACCA-3′) and pEGFP-S_{465}com (5′-CCCGATATCGCGGCGGTCACGAAC-3′). Probe HST: a 0.9 kb PCR product corresponding to the *D.melanogaster Hsp*70T gene, amplified using the primer combination pHSTfor (5′-CCCCTCTAGATGTACGAACACGCATTT-ATC-3′) and pHSTcom (5′-CCCCGCGGCCGCCACACG-GGCGAGCGACCA-3′). Between different hybridizations, the filter was washed with a 1.5 M NaCl, 0.5 N NaOH solution for 10 min to remove the hybridized probe.

Quantification of EGFP protein expression

Digital images of larvae were captured on a Nikon inverted microscope using an attached Nikon DXM1200 digital camera. Fluorescent gene expression was quantified using the Lucia G image processing and analysis software (version 4.61; Nikon UK) as previously described (21). EGFP expression was calculated for 20 individual F_1 and $F_{1\ Backcross}$ larvae per experiment, 5 days post-hatching. For statistical analyses, unpaired *t*-tests were performed; the null hypothesis was rejected at $P \geqslant 0.05$. χ^2 tests were performed to demonstrate that the observed level of gene expression in the $F_{1\ Backcross}$ crosses did not statistically deviate from the expected (50%) level of EGFP expression in F_1 progeny when a second copy of the RNAi gene was supplied.

RNA extraction and cDNA synthesis

Total RNA was extracted from 10 fourth instar larvae from the $F_{1\ Backcross}$ crosses. Briefly, larvae were homogenized in RNA extraction buffer (1% SDS, 1 mg/ml proteinase K, 100 mM EDTA, 125 mM NaCl, 50 mM Tris–HCl, pH 7.6) and incubated at 50°C for 1 h. Homogenates were phenol–chloroform extracted and RNA selectively precipitated from solution by three successive 8 M lithium chloride precipitations. Samples were treated with DNase RQ1 (0.2 U/µg total RNA) (Promega) and used for cDNA synthesis. An aliquot of 1 µg of DNase-treated total RNA was reverse transcribed using oligo$(dT)_{12–18}$ and SuperScript™ II (Invitrogen) according to the manufacturer's instructions.

Quantitative real-time PCR

EGFP mRNA quantification was performed on an ABI 7700 PRISM™ Sequence Detector (Applied Biosystems). EGFP MGB-TaqMan™ primers and probes (ABI) were designed using Primer Express software (ABI) in the 5′ coding sequence upstream of the IR-*EGFP*, to amplify a 69 bp PCR product (nucleotides 66–133). The sequences of the primers were as follows: EGFP-F, 5′-CGTAAACGGCCACAAGTT-CAG-3′; EGFP-R, 5′-GGGTCAGCTTGCCGTAGGT-3′. The probe (EGFP-P, 5′-CCCTCGCCCTCGC-3′, nucleotides 95–108) was labeled with the reporter dye 6-carboxyfluorescein (6′-FAM) at the 5′ end and with the quencher dye 6-carboxytetramethylrodamine (TAMRA) at the 3′ end. *EGFP* expression values were normalized to expression levels of the

A.stephensi ribosomal *S7* gene. Primers and a FAM/TAMRA probe were designed from the orthologous *A.gambiae S7* gene (22) to amplify an 84 bp product: S7-F, 5′-GGTGCACCTG-GATAAGAACCA-3′; S7-R, 5′-CGGCCAGTCAGCTTCTT-GTAC-3′; S7-P, 5′-AGACCACCATCGAACACAAAGT-TGACACGT-3′. cDNA prepared from F_1 progeny of the control cross between line V_B and wild-type mosquitoes was used to develop standard curves for each gene and to determine the relative *EGFP* expression in the experimental crosses. Measurements of gene expression were taken in quadruplicate from two separate experiments and their mean used for further analyses. In each run a negative control (wild-type mosquito cDNA) and an RNA sample without a reverse transcriptase step (to determine genomic DNA contamination) was included.

RESULTS

Development of transgenic RNAi lines

To assess the feasibility of targeted gene silencing through the *in vivo* expression of dsRNA from stably integrated RNAi genes in *Anopheles* mosquitoes, we employed *EGFP* as the target gene (17). We reasoned this fluorescent marker gene would provide a simple and impartial model for assessing the efficacy of gene silencing by allowing analysis at the protein level both visually and quantitatively using image processing software. Transgenic mosquito lines were engineered to stably express an *EGFP*-targeting RNAi gene. A total of 610 *A.stephensi* embryos were injected with transformation construct pIR-EGFP (19), which contained the *EGFP* RNAi gene under the transcriptional control of the *Actin*5C promoter from *D.melanogaster* and a *DsRed* selectable marker expression unit (Fig. 1a). The progeny of the 60 surviving adults showed the presence of 17 fluorescent phenotypes derived from a minimum of four G_0 individuals. Southern blot analyses on HincII-digested genomic DNA (G_3 generation) were performed and hybridized with probe M, spanning the left and right inverted repeats of *minos* (Fig. 1b). A total of 13 individual transgenic lines were detected (Fig. 1b), representing a minimum integration frequency of 6.7%. In these lines, two bands of variable size hybridized with probe M in each case (Fig. 1b), suggesting the occurrence of a single *minos*-mediated transformation rather than random integration of the whole plasmid. Six independent lines (lines eIR-A_1, eIR-A_2, eIR-A_3, eIR-B_1, eIR-C_2 and eIR-19_1) were selected for further studies. To determine the structural stability of the IR-*EGFP* integrated transgene, EcoRI/NotI-digested genomic DNA was also hybridized with probe EGFP-S, corresponding to the sense arm of IR-*EGFP*. On the basis of the specific restriction pattern of pIR-EGFP (Fig. 1a), a single internal band of 4.5 kb was expected. Surprisingly, no band was detected in two (lines eIR-A_1 and eIR-C_2) of the six transgenic lines (Fig. 1c, upper panel), indicating a deletion within the IR-*EGFP* cassette. Hybridization with probe HST, spanning the terminator sequence present in both the *DsRed* and IR-*EGFP* cassettes, gave two bands of the expected sizes in four out of the six lines. However, only one band, corresponding to the *Hsp*70T element of the *DsRed* expression unit, was obtained in lines eIR-A_1 and eIR-C_2 (Fig. 1c, lower panel), demonstrating that the deletion involved the entire RNAi gene cassette. Southern blot analysis was also performed on the same six transgenic lines at generation G_9 using the same three probes. Hybridizations identical to those obtained in generation G_3 were observed (data not shown).

Analysis of *EGFP* silencing

To estimate the silencing efficiency of the integrated RNAi gene, homozygous male mosquitoes from the six selected transgenic lines (eIR-A_1, eIR-A_2, eIR-A_3, eIR-B_1, eIR-C_2 and eIR-19_1) were crossed to virgin females of transgenic line V_B, homozygous for *EGFP* (11). These six RNAi lines were selected as they exhibited various phenotypes in terms of the spatial distribution of the marker and intensity of its fluorescence and carried a single copy insertion of the transgene. Genetic crosses had proven that these IR-*EGFP* lines contained the RNAi gene integrated into a chromosome different from that carrying the *EGFP* gene of target line V_B (data not shown), to ensure two copies of the RNAi transgene and one copy of the *EGFP* target gene could be obtained in backcross experiments. Quantitative analysis of EGFP protein levels in the F_1 progeny of the crosses, heterozygous for both the RNAi gene and *EGFP* target gene, was performed using image processing and analysis software (21). Among the four lines that Southern blot analysis had shown to contain the RNAi gene, eIR-B_1 and eIR-19_1 mediated a significant inhibition of gene expression, reducing EGFP levels by 43 and 37%, respectively, as compared to heterozygous line V_B larvae (Fig. 2a). As expected, no inhibition was observed in lines eIR-A_1 and eIR-C_2, where the RNAi gene had been deleted. Heterozygous adult females from each cross were then backcrossed to males homozygous for the RNAi gene, and individuals possessing two copies of the RNAi gene and one copy of the *EGFP* target gene were analyzed for EGFP levels ($F_{1\ Backcross}$). Lines eIR-B_1 and eIR-19_1 consistently mediated a highly significant reduction in EGFP expression, averaging 73 and 65%, respectively (Fig. 2a). Remarkably, EGFP levels in these crosses were approximately halved with respect to those obtained in the F_1 cross ($\chi^2 = 0.7$). EGFP expression in lines eIR-A_1 and eIR-C_2, which did not contain the RNAi unit, was again unaffected. Finally, lines eIR-A_2 and eIR-A_3 did not show a significant reduction in EGFP levels in either the F_1 or $F_{1\ Backcross}$ progeny (Fig. 2a). Interestingly, in these lines the spatial pattern of DsRed expression did not co-localize perfectly with EGFP expression from line V_B (Fig. 3), thus suggesting that the RNAi gene and its target were not transcribed in the same cells. As previously demonstrated (19), *DsRed* expression did not affect EGFP levels, as determined by EGFP intensity in the crosses between line V_B and lines MinRED1, eIR-A_1 and eIR-C_2, which contained the *DsRed* marker cassette but not the RNAi unit.

To determine the levels of *EGFP* transcripts, a random sample of 10 $F_{1\ Backcross}$ larvae from each cross was used for RNA extraction and cDNA synthesis. The abundance of *EGFP* mRNA in each cross was determined by real-time PCR, using a probe and primers to amplify a 69 bp region of the *EGFP* gene not common to the IR-*EGFP* sequence. Results were normalized to the *A.stephensi* ribosomal *S7* gene (22) and relative *EGFP* mRNA levels calculated.

In accordance with the analysis of EGFP protein expression, $F_{1\ Backcross}$ larvae derived from crosses involving lines eIR-B_1 and eIR-19_1 exhibited a significant reduction in *EGFP*

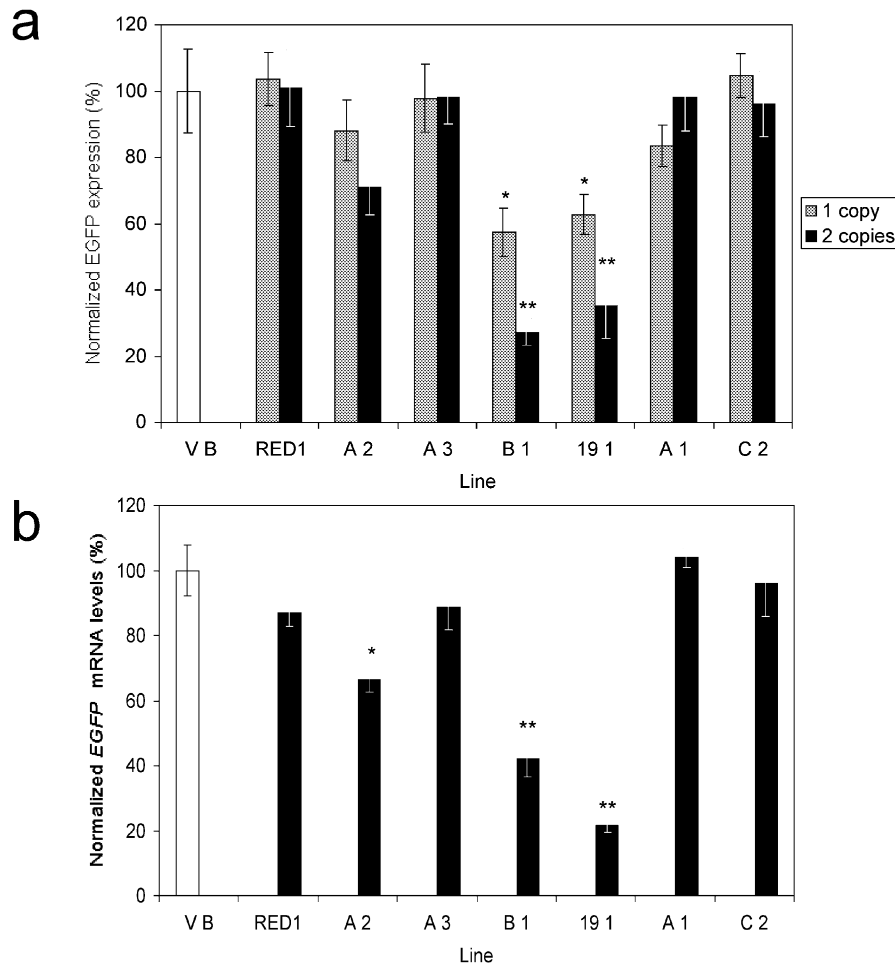

Figure 2. Quantification of RNAi in *A.stephensi* larvae. (**a**) The intensity of EGFP protein expression in the F_1 and $F_{1\ Backcross}$ progeny of the crosses between the eIR lines and line V_B was calculated using the Lucia G image processing and analysis software. Gray bars indicate normalized EGFP expression levels in F_1 progeny (one transgene copy); black bars indicate normalized EGFP expression in $F_{1\ Backcross}$ progeny (two transgene copies). (**b**) Relative *EGFP* mRNA levels in $F_{1\ Backcross}$ progeny, as determined by real-time PCR. Black bars indicate normalized *EGFP* mRNA levels. EGFP protein and RNA expression levels were normalized to control larvae derived from a cross between line V_B and wild-type mosquitoes (V_B) (white bars). The plotted data show mean values ± SE. RED1, MinRED1; A 2, eIR-A_2; A 3, eIR-A_3; B 1, eIR-B_1; 19 1, e-IR-19_1; A 1, eIR-A_1; C 2, eIR-C_2. Asterisks indicate the significance value of the result. *Significant, $P < 0.05$; **highly significant, $P < 0.01$.

Figure 3. Phenotype of line V_B, eIR-19_1 and eIR-A_3. *EGFP*-expressing *A.stephensi* larvae from transgenic line V_B (left) and F_1 larvae derived from the cross between line V_B and eIR-A_3 (center) or eIR-19_1 (right). In the center and right panels, DsRed fields are superimposed upon EGFP fields to show co-localization (yellow). Line eIR-A_2 exhibited a phenotype similar to that of line eIR-A_3 (data not shown). Larvae were photographed at 10× magnification 5 days post-hatching.

transcripts, averaging 58 and 78%, respectively, as compared to heterozygous line V_B control larvae (Fig. 2b). Line eIR-A_2 also mediated a significant reduction in *EGFP* mRNA levels (34%). In agreement with the protein data, no significant reduction in *EGFP* transcripts was observed in crosses with lines eIR-A_3, eIR-A_1 and eIR-C_2.

DISCUSSION

In order to investigate the feasibility of establishing a stable and heritable RNAi technology in mosquitoes, *EGFP* from a transgenic *A.stephensi* line was selected as the target gene. It was reasoned that *EGFP* could provide an impartial model to determine the feasibility of stable RNAi in *Anopheles*. The rationale for this was based on the consideration that silencing of the *EGFP* transgene would produce a neutral phenotype for the mosquito, avoiding the risks of selection bias associated with high silencing activity against important or vital endogenous genes.

The RNAi gene was designed to produce intron-spliced dsRNA, which our previous studies had demonstrated could mediate transient RNAi with high efficiency against *EGFP* (19). In that study, *EGFP* silencing achieved in transient transfection experiments of *Anopheles* cells and larvae averaged 93 and 69%, respectively. When stably expressed, this RNAi gene suppressed *EGFP* expression by up to 73% at

the protein level and 78% at the RNA level. The intron-containing RNAi gene was utilized here on the assumption that it would be less susceptible to the genomic rearrangements reported to occur in constructs containing uninterrupted repeats (23). Our analysis, however, revealed the complete absence of the RNAi unit in two of the transgenic lines studied. This finding could be explained by recombination events that took place either within the plasmid itself or genomically. Recombination may have been triggered by the presence of the symmetrically parallel RNAi and *DsRed* transcription units (Fig. 1a), sharing identical promoter and terminator sequences, rather than as a direct consequence of the presence of the IR in the RNAi gene itself. Importantly, the integrated constructs showed genomic stability beyond nine generations, which argues for construct instability at the time of injection.

Our data clearly indicate that silencing effects were improved by doubling the RNAi gene copy number, in accordance with similar studies using IR transgenes in *Drosophila* (7,8). Levels of EGFP protein expression were approximately halved when a second copy of the IR transgene was provided in two of the crosses. Furthermore, silencing efficiency appeared to be dependent upon the site of integration of the RNAi gene. Silencing was only observed in those lines where the expression pattern of the *EGFP* target gene and the RNAi gene, as determined by *DsRed* expression, overlapped. This is in agreement with studies of transgene-mediated RNAi in *Drosophila* (23,24), indicating that RNAi is not systemic and does not spread across mosquito cells. Whether this suggests a lack of dsRNA amplification in Diptera, common to *Neurospora* (25), plants (26) and *C.elegans* (27), is yet to be established, but it may simply reflect the postulated inefficiency of dsRNA formation from transgenes, possibly because nuclear RNA-binding proteins prevent dsRNA annealing (28).

While it is impossible to extrapolate the results of this study to endogenous mosquito genes, the level of gene silencing achieved here is encouraging despite the fact that we were unable to completely abolish the EGFP phenotype. Since RNAi only knocks down target gene expression, it could offer an advantage in performing functional genomic studies on vital genes and produce a range of useful phenotypes that are missed in classical knockout studies. Because *minos* reliably creates multiple genetic lines, an array of useful hypomorph phenotypes is likely to be generated for most studies.

The results reported here represent the first example of stable RNAi in *Anopheles*. This approach can now be applied to study mosquito molecules that have been postulated to function as receptors for malaria parasite development. Furthermore, stable RNAi could greatly contribute to the understanding of important mosquito physiological functions, including chemoreception, locomotion, osmoregulation and homeostasis, taking advantage of the recently published *A.gambiae* genome sequence (16).

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