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Stability and Loss of a Virus Resistance Phenotype Over Time in Transgenic Mosquitoes harboring an antiviral effector gene

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Abstract

Transgenic *Aedes aegypti* were engineered to express a virus-derived, inverted repeat (IR) RNA in the mosquito midgut to trigger RNA interference (RNAi) and generate resistance to dengue virus type 2 (DENV2) in the vector. Here we characterize genotypic and phenotypic stabilities of one line, Carb77, between generations G₉ and G₁₇. The anti-DENV2 transgene was integrated at a single site within a non-coding region of the mosquito genome. The virus resistance phenotype was strong until G₁₃ and suppressed replication of different DENV2 genotypes. From G₁₄–G₁₇ the resistance phenotype to DENV2 became weaker and eventually was lost. Although the sequence of the transgene was not mutated, expression of the IR effector RNA was not detected and the Carb77 G₁₇ mosquitoes lost their ability to silence the DENV2 genome.

Keywords

mosquito; dengue virus; RNA interference; transgene integration site; resistance phenotype

Introduction

Dengue viruses (species: *dengue virus*, serotypes 1–4 [DENV1–4], genus: *Flavivirus*, family: *Flaviviridae*) are the most important mosquito-borne viral pathogens that cause morbidity and mortality in humans (CDC, 2005). DENVs are spherical, enveloped viruses with an RNA genome enclosed by 180 identical capsid proteins (Chambers, 1990; Kuhn *et al.*, 2002). The nucleocapsid is surrounded by a lipid bilayer containing the envelope and membrane proteins. The single-stranded, positive-sense RNA genome, ~10,700 nt in size, comprises one major open reading frame. The 5'-end encodes three structural genes, C, prM, E and these are followed by seven non-structural genes, NS1, NS2a, NS2b, NS3, NS4a, NS4b, and NS5. The principal DENV vector is the mosquito *Aedes aegypti*, which becomes infected persistently with the viruses. The typical, urban disease cycle involves only humans

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and mosquitoes. DENVs have been hyperendemic over several decades in Southeast Asia, Central and South America, and in the Caribbean (Gubler & Trent, 1994; Gubler, 1997; Lorono-Pino *et al.*, 1999). The prevalence of dengue hemorrhagic fever or dengue shock syndrome has increased in these regions due to the emergence of virulent virus strains and sequential infections of people with different DENV serotypes (Lorono-Pino *et al.*, 1999; 2004). To date, no vaccine or drugs are available to treat or prevent DENV infections (Hombach *et al.*, 2005). Targeting the vectors may be a viable alternative to break the DENV disease cycle. A novel proposed strategy involves mosquito population replacement in which DENV-competent wild-type mosquitoes would be replaced in an endemic region with engineered, refractory mosquitoes (Curtis & Graves, 1988; James, 2005). In several studies DENV2 has been shown to trigger and be a target of the innate RNAi pathway in the mosquito (Adelman *et al.*, 2001, 2002; Sanchez-Vargas *et al.*, 2004, 2009). However, dsRNA replicative intermediates are thought to form in intracellular membranes during replication of the viral genome allowing limited protection and possibly inefficient triggering of the RNAi pathway (Uchil and Satchidanandam, 2003). We hypothesized that expression of viral-derived dsRNA in the mosquito midgut would induce an antiviral state in this tissue at the time of virus entry creating resistance to DENV2 infection.

We developed a transgenic mosquito line, Carb77, that expressed a virus-derived inverted repeat (IR) effector RNA in the midgut of a female 27–48h after ingesting a bloodmeal (Franz *et al.*, 2006). Transcription of the IR effector generated a dsRNA molecule with sequence identity to a portion of the prM gene of DENV2 triggering the endogenous RNAi pathway of the mosquito. Based on the principle of homology dependence, the RNAi machinery of the mosquito not only targets and digests the dsRNA derived from the IR effector of the transgene but also degrades DENV2 genomic RNA having high sequence homology with the dsRNA trigger. The Carb77 mosquitoes were designed to be refractory only to DENV2 instead of all DENV serotypes and they lack a gene drive system to which the effector gene is linked (Sinkins & Gould, 2006; Chen *et al.*, 2007).

In a previous study, we tested the vector competence of intercrossed Carb77 mosquitoes at generations G₅–G₈ and showed that the resistance phenotype to DENV2 was strong (Franz *et al.*, 2006). Here we evaluate the phenotypic and genotypic stability of Carb77 mosquitoes at later generations (G₉–G₁₇). We characterize the integration pattern of the transgene. We also show that the RNAi-based resistance mechanism in Carb77 mosquitoes was effective against several DENV2 genotypes. The resistance phenotype of Carb77 was strongly maintained in the genetic background of the laboratory-derived Higgs white eye strain (HWE, Wendell *et al.*, 2000) for 13 generations. However, from G₁₄ onwards it became weaker and eventually was lost in G₁₇. We investigate and discuss possible reasons for this phenomenon.

Results

Identification of the transgene integration site in Carb77 mosquitoes

Amplification of genomic DNA from GenomeWalker libraries yielded two products using primers corresponding to the adaptor DNA and to the left and right arms of the *MosI* *mariner* transposable element (TE) (Fig. 1A). The sequences of genomic mosquito DNA

adjacent to the left and right inverted terminal repeats (ITRs) of the TE were determined to be contiguous following alignment comparisons with the results of whole-genome sequencing from the EMBL *Ae. aegypti* Whole Genome Shotgun and VectorBase databases. The integration site is at nucleotide position 22,643 within a non-coding region of contig AGGE02013121 (69 kilo base-pairs [kbp] in length) which is part of supercontig 1.278. The distance between the transgene and protein encoding sequence motifs in neighboring contigs is ~100 kbp. The transgene integration site is located within a non-repetitive sequence motif; however, two highly repetitive motifs, 156 bp (Fig. 1B) and 412 bp in length (not shown) are in close proximity. We evaluated whether the transgene integration site could be used as a reliable molecular marker to allow easy identification of the Carb77 insertion genotype. Primers were designed to amplify the regions of the genomic DNA flanking the left and right arms of the TE (Table 1). The primer combination 13121 FWD/maRight REV yielded an amplification product of 515 bp (288 bp genomic DNA + 227 bp DNA corresponding to the TE right arm; lane 1), whereas the maLeft FWD/13121 REV combination resulted in a product of 525 bp (374 bp genomic DNA + 150 bp DNA corresponding to the TE left arm; lane 3) (Fig. 1C). No amplification products were obtained using the same primer combinations in reactions that contained template DNA from the HWE (control) recipient (Fig. 1C, lanes 2 and 4). It was not possible to amplify the entire integrated TE from genomic Carb77 DNA using primers 13121 FWD/13121 REV. Using the three primers 13121 FWD/13121 REV/maLeft FWD in combination enabled us to differentiate between wild-type (no transgene insertions), homozygous and hemizygous Carb77 mosquitoes in a standard gene amplification reaction. A single 622 bp amplification product was diagnostic for wild-type mosquitoes (Fig. 1C, lane 5), whereas a single 525 bp product showed the presence of the transgene (lane 6). The presence of both amplification products in lane 7 indicated a hemizygous Carb77 mosquito. When 12 male and 12 female individuals were selected randomly from the Carb77 colony (G₁₁), three males and three females (25%) were determined to be homozygous.

Stability of the Carb77 resistance phenotype over several generations

Carb77 mosquitoes of generations G₁₂, G₁₃, G₁₄, and G₁₅ were challenged orally with DENV2 Jamaica 1409 and tested by plaque assays at 7 and 14 days post bloodmeal (pbm) for presence of infectious virus (Fig. 2). The virus titers of the bloodmeals ranged from 5×10^6 pfu/ml for G₁₄ to 2.8×10^7 pfu/ml for G₁₅. Seven days after challenge with DENV2 significantly fewer Carb77 females of G₁₂, G₁₃, and G₁₄ were infected with the virus than the HWE controls (Fig. 2A). However, the difference for G₁₅ was not significant. Between 70% (G₁₅) and >90% (G₁₂) of the transgenic mosquitoes were completely refractory to the virus, whereas >45% of the control females were susceptible. A significantly lower mean titer was observed for Carb77 G₁₂ infected females in comparison to HWE. Titers were not significantly lower than the control for the other Carb77 generations. However, only 3/37 (8%) G₁₃, 2/20 (10%) G₁₄ and 1/20 (5%) G₁₅ Carb77 females had virus titers exceeding 1000 pfu/ml and average titers ranged from 165 pfu/ml (G₁₂) to 3048 pfu/ml (G₁₄). In contrast, ~67% of the infected HWE control mosquitoes had DENV2 titers above 1000 pfu/ml.

Fourteen days pbm significantly fewer Carb77 G₁₂ and G₁₃ females (20–21 %) were DENV2 infected as compared to the control (46.7–55 %), and transgenic females of G₁₄ followed this trend. In contrast, the proportion of Carb77 G₁₅ females that were virus-infected was similar to that of the HWE control. There was no significant difference between the virus titers of Carb77 mosquitoes and HWE. Average titers among Carb77 females were between 3020 pfu/ml (G₁₃) and 12,760 pfu/ml (G₁₄), whereas those of the control mosquitoes reached 8-fold higher levels. Our data show that the resistance phenotype of Carb77 G₁₂–G₁₅ mosquitoes to the DENV2 Jamaica 1409 (high passage) strain was based on a lower proportion of females that became infected after oral challenge rather than the level of virus titers in the infected animals. In this experiment Carb77 mosquitoes did not show significant levels of resistance to the virus at 7 and/or 14 days pbm from generation G₁₄ onwards, indicating that in advanced generations of the transgenic line the resistance phenotype to DENV2 became weaker.

Resistance of Carb77 mosquitoes to three different genotypes of DENV2

We investigated whether the resistance phenotype of Carb77 is effective for DENV2 genotypes other than the virus strain from which the sequence of the Mnp IR effector was derived (Fig. 3). G₉ and G₁₃ Carb77 females were fed artificial bloodmeals containing different DENV2 genotypes: Indonesia 1051 (Cosmopolitan genotype), Puerto Rico 159 (American genotype), or Jamaica 1409 ‘low passage’ (American-Asian genotype). Virus titers in the artificial bloodmeals ranged from 1.35×10^6 pfu/ml (Jamaica 1409 ‘low passage’) to 5×10^7 pfu/ml (Puerto Rico 159). Virus titers in whole body females were analyzed 7, 12 or 14 days pbm by plaque assays in LLC-MK2 cells and compared to the titers of control mosquitoes. In those experiments in which Carb77 mosquitoes were challenged with the Indonesian or Puerto Rican strains of DENV2, titers of infected females were significantly lower than those of the controls at 7, 12, or 14 days pbm (Fig. 3A, B). Seven and 12 days after oral challenge with DENV2 Indonesia 1051 only 3/40 (7.5%) females had virus titers exceeding 200 pfu/ml while the average titers were less than 200 pfu/ml. In contrast ~40% of all control females produced virus titers higher than 600 pfu/ml at the same time points with the highest titers exceeding 50,000 pfu/ml at 12 days pbm. The bloodmeal titer of the Puerto Rican 159 strain (5×10^7 pfu/ml) was the highest of the three virus strains tested in this experiment. Previously, DENV2 strains of the American genotype have been observed to produce relatively weak infections in many *Ae. aegypti* strains (Armstrong and Rico-Hesse, 2003). However, the virus readily infected control mosquitoes in this experiment with average titers of 4287 pfu/ml at 7 days pbm (Fig. 3B). In Carb77, average titers of infected females were ~100 pfu/ml. Seven days later the average titers in DENV2 infected Carb77 females remained significantly lower than those in HWE with >50% of the former having virus titers below 600 pfu/ml. In contrast, more than 30% of the controls reached titers exceeding 10,000 pfu/ml. Transgenic mosquitoes were not significantly different from the control when comparing the prevalence of DENV2 Indonesia 1051 or Puerto Rico 159 infections, but the mean intensities of infection were significantly different. This can be explained by the fact that instead of being completely refractory to the two virus strains 25 to 50 % of the Carb77 females supported low levels of virus replication (<100 pfu/ml) after oral challenge. Challenging Carb77 mosquitoes with the low passage strain of DENV2 Jamaica 1409 resulted in significantly fewer infected mosquitoes compared

to the HWE control (Fig. 3C). This was in accordance with the high passage strain of the virus (Fig. 2) which usually produces higher titers in cell culture and mosquitoes than the low passage strain. Fifteen of 20 (75%) females were completely refractory to the virus at 7 days pbm and only 3/17 (17.6%) Carb77 mosquitoes had virus titers at 14 days pbm with a mean of 4048 pfu/ml. Average virus titers of infected controls were significantly higher at 7 but not at 14 days pbm with ~50% of them having virus titers >3000 pfu/ml. Taken together, the results of this experiment show that the effector gene in Carb77 mosquitoes is able to efficiently silence the replication of heterologous DENV2 strains.

Stability of the mechanism underlying the resistance phenotype of G₁₅ Carb77 mosquitoes

We evaluated if the RNAi-based resistance mechanism to DENV2 was still functional in Carb77 G₁₅ mosquitoes, which appeared to be more susceptible to the virus than earlier generations. Carb77 G₁₅ females were challenged seven days after eclosion with a DENV2 Jamaica 1409-containing bloodmeal with a titer of 1×10^7 pfu/ml. (Fig. 4) When tested by plaque assays at 7 days pbm, 15/19 (~79%) Carb77 females appeared to be refractory to the virus (Fig. 4A) indicating the presence of a DENV2-specific resistance phenotype. The HWE control was susceptible to the virus since mean titers of the infected individuals were at least three-fold higher than those of the transgenic ones. However, this difference was not statistically significant. In Carb77 mosquitoes, resistance to the virus was attenuated significantly when they were injected intrathoracically with dsRNAs targeting the RNAi pathway gene *Aa-dcr2* three days before they acquired DENV2. As shown previously, intrathoracic injection of dsRNA-*Aa-dcr2* leads to a transient down-regulation of the *Ae. aegypti* RNAi pathway (Sanchez-Vargas *et al.*, 2009). This became apparent when comparing the proportion of infected females that were injected before with dsRNA-*Aa-dcr2* with that of individuals that either did not receive any dsRNA or were injected with the non-target control dsRNA- β gal, derived from the *Escherichia coli* β gal gene. Following intrathoracic injection of dsRNA- β gal and oral challenge with DENV2 80% of Carb77 females remained refractory to the virus in comparison to only ~28 % of those that were injected with dsRNA-*Aa-dcr2*. Northern blot analysis confirmed a reduced accumulation of *dcr2* mRNA in individual Carb77 mosquitoes that were injected with dsRNA-*Aa-dcr2*, whereas intrathoracic injection of dsRNA- β gal had no effect on *dcr2* mRNA accumulation (Fig. 4B). Carb77 mosquitoes injected with PBS had significantly lower mean titers when compared to those injected with dsRNA-*Aa-dcr2* (Fig. 4A). Mean titers of the former were ~1300 pfu/ml with only 3/18 individuals reaching titers above 1000 pfu/ml. In dsRNA-*Aa-dcr2*-injected transgenic females, mean titers were ~16-fold higher with a maximum titer exceeding 60000 pfu/ml. The mean DENV2 titer of infected Carb77 females injected with dsRNA-*Aa-dcr2* resembled that of similarly treated HWE mosquitoes. In summary, the experiment demonstrated that in Carb77 G₁₅ females the resistance phenotype to DENV2 was based on the transgene mediated RNAi response.

Loss of the resistance phenotype to DENV2 in Carb77 G₁₇ mosquitoes

When we evaluated Carb77 G₁₇ mosquitoes in DENV2 challenge experiments they appeared to be highly susceptible to the high passage strain of DENV2 Jamaica 1409 (Fig. 5A) consistent with the interpretation that the transgene-mediated resistance mechanism was no longer functional. This could have been caused by mutations in the transgene itself or by

inhibition of its expression. The proportion of Carb77 G₁₇ mosquitoes expressing the eye specific selection marker was ~82 % in our colony which slightly lower than the average of 88% over eight generations. In gene amplification assays, in which total DNA of 14 single females was tested, we confirmed the presence of the principal components the transgene (Fig. 5B). The following regions were amplified: fragments of the EGFP selection marker as well as the left and right arms of the mariner TE, the full length carboxypeptidase promoter of the transgene, as well as the complete sense and anti-sense fragments of the Mnp IR effector (see Fig. 1A and Table 1). Components of the transgene were only detectable in EGFP-expressing mosquitoes of the Carb77 G₁₇ colony, indicating that those animals of the colony that did not express the marker gene in their eyes were not transgenic. Each component of the transgene was readily detected by gene amplification in 9/9 or at least 8/9 EGFP expressing females. If an amplicon could not be obtained from a DNA sample in the initial assay, it was repeated under modified conditions, i.e. increasing the amount of template DNA and/or increasing the concentration of MgCl₂ in the reaction. Usually the fragment was then detectable. We sub-cloned multiple amplicons from total DNA of three different females and sequenced as many as four plasmid DNA of each amplicon. We did not detect any point mutations, inversions, deletions or insertions within the sequenced regions of the transgene. This supports the conclusion that mutation of the Carb77 transgene might not be the reason for the loss of the resistance phenotype of Carb77 G₁₇ mosquitoes. Northern blot analysis was performed to investigate whether the resistance phenotype of Carb77 G₁₇ mosquitoes was lost because transcription of the (intact) IR effector was inhibited. Using a probe corresponding to the Mnp fragment of the IR effector no signal was detected among total RNA extracted from midguts of females that had received a bloodmeal 24, 48, or 72 h before (Fig. 5C). As a positive control another transgenic mosquito line, Vg40, was chosen which expresses the Mnp IR effector in fat body tissue under control of the *vitellogenin 1* promoter. A strong signal corresponding to an RNA moiety of ~1200 nt was visible at 24 h post bloodmeal, which coincides with the expression profile of this promoter. The 1200 nt size of the RNA band is consistent with the size of the Mnp+/i/Mnp-transcript of the anti-DENV2 effector. The lack of expression of the Carb77 transgene in G₁₇ females was confirmed by quantitative real time RT-PCR using primers that were complementary to the Mnp IR effector (data not shown). In summary, these results indicate that expression of the Carb77 transgene in G₁₇ mosquitoes was not functional whereas the transgene itself did not appear to be mutated.

Discussion

A prerequisite for the successful implementation of a population replacement to replace DENV susceptible mosquito populations by genetically-modified, refractory populations, is the phenotypic and genotypic stability of the effector gene over an extended number of generations (James, 2005; Huang *et al.*, 2007). Previously, we reported a strong resistance phenotype to DENV2 among Carb77 mosquitoes of G₅ to G₈ (Franz *et al.*, 2006). Here we monitored the genotypic and phenotypic stability of Carb77 mosquitoes between G₉ and G₁₇. G₉ through G₁₃ Carb77 mosquitoes were significantly refractory to DENV2. Nevertheless, a few individual Carb77 mosquitoes in each challenge assay did not appear to be refractory to DENV2, instead the virus reached relatively high titers in these mosquitoes.

It is possible that the artificial feeding of high titers of DENV2 mixed with defibrinated blood may promote infection of the insects. Perhaps this feeding system allows the virus to leak through the midgut epithelium in a few mosquitoes in high enough quantities to by-pass the RNAi-based resistance mechanism. Weaver and colleagues (1993) observed that Western Equine Encephalomyelitis virus acquired by *Culex tarsalis* through artificial bloodmeals had a different infection pattern in the alimentary tract of the insects as compared to virus that was acquired through viremic blood. We showed that the underlying mechanism of the resistance phenotype in Carb77 G₁₅ mosquitoes was based on RNAi and thus had not changed since Carb77 G₆ females had been analyzed (Franz *et al.*, 2006). However, the resistance phenotype of Carb77 mosquitoes became progressively weaker from G₁₄ onwards until it appeared to be completely lost in G₁₇. The loss of the resistance phenotype in G₁₇ mosquitoes could be attributed to several phenomena: (i) selection pressure caused by the DENV2 targeting effector has generated virus mutants that enabled them to escape the resistance mechanism, (ii) the transgene has lost its function through mutation, or (iii) the expression of the transgene was inhibited. As for the first possibility, preliminary data have shown so far that none of the DENV2 viruses that were isolated from Carb77 infected mosquitoes showed mutations in their prM gene substantial enough to overcome recognition by the RNAi trigger of the transgene (Katie Poole-Smith, Ken E. Olson, and Carol D. Blair, unpublished). Partial sequencing of the transgene of Carb77 G₁₇ mosquitoes revealed no mutations, strongly suggesting that the RNAi effector against DENV2 was still intact. However, as shown by Northern blot analysis and quantitative real time RT-PCR, expression of the IR effector gene was not detectable in Carb77 G₁₇ mosquitoes, even though eye-specific selection marker expression appeared to be undisturbed. In eukaryotic organisms such as mosquitoes, loss or erratic expression levels of a transgene have been generally attributed to strong fitness loads caused by the transgene itself or by its integration site within the genome of the host (Irvin *et al.*, 2004; Marelli *et al.*, 2006; Catteruccia *et al.*, 2003). Unfortunately, we do not have comprehensive data concerning the fitness of Carb77 mosquitoes available. However, we do not anticipate the transgene itself having strong toxic effects on the mosquito organism because it does not encode a protein other than the selection marker, and the IR effector RNA is expressed only temporarily in the midgut. We identified a single integration site for the Carb77 transgene that was located in a non-protein encoding region of the *Ae. aegypti* genome. In view of stable transgene expression levels, a single integration event might constitute the least harmful scenario. Multiple integration events, amounting to several hundred transgene copies have been shown to jeopardize predictable gene expression patterns in subsequent generations of an *Ae. aegypti* strain (Adelman *et al.*, 2004).

The resistance phenotype of Carb77 mosquitoes was strong for at least 13 generations and then after became weak indicating that the transgene did impose an initial fitness load. We speculate that the loss of effector gene expression could have been caused by chromatin/heterochromatin re-arrangements which might have enabled regulatory elements of neighboring or even distant genes to silence the effector (Sabl and Henikoff, 1996). Unfortunately, there are no genetic tools currently available for *Ae. aegypti* to thoroughly investigate this possibility.

The IR-DNA effector derived from the DENV2 genome tolerated a limited number of mismatches with the viral RNA of the target present in different isolates without compromising the ability to trigger an effective RNAi response. This is consistent with observations made when targeting poliovirus using RNAi (Gitlin *et al.*, 2005). The Puerto Rican 159 and the Indonesian 1051 strains of DENV2 share nucleotide sequence similarities in their prM genes of 90% and 92%, respectively, with the Jamaican 1409 strain from which the IR effector in Carb77 mosquitoes was derived. Carb77 mosquitoes targeted and silenced both DENV2 strains in midgut tissue. This ‘cross protection’ phenomenon can be best explained by the presence of several regions in the ~580 nt prM encoding RNA sequence, which are identical among these virus strains and at least 21 nucleotides in length. In view of the mosquito population replacement strategy it is important that genetically engineered, DENV-resistant mosquitoes be capable of silencing efficiently non-homologous DENV2 strains. The dynamic ecology of DENVs makes it likely that several virus strains of the same serotype are prevalent in a given region, and these might emerge or disappear over time (Anderson and Rico-Hesse, 2006). Therefore, it would not be feasible to develop transgenic mosquitoes harboring anti-DENV effector genes that match precisely every virus strain present.

The observation of the loss of effector gene expression among Carb77 G₁₇ mosquitoes could be seen as a singular event which is caused by the specific genetic background of this particular mosquito line and/or its inbred maintenance under laboratory conditions. We do not currently have another mosquito family with the same transgene available for comparison. Nevertheless, if the observation of loss of transgene expression is regarded as a potential risk among transgenic mosquitoes - especially in conjunction with a population replacement strategy in the field - some considerations regarding transgene design should be taken into account. The use of chromatin insulators has been proposed before to shield a transgene from interference of neighboring regulatory elements that could affect its expression (Sarkar *et al.*, 2006). Furthermore, when considering a gene drive system it might be useful to design it in such a way that it enforces not only inheritance of the transgene throughout a population but also its expression. Such a gene drive system could be based on a killer-rescue system in which the expression of the antidote (rescuer) is dependent on the expression of the effector gene (Chen *et al.*, 2007; Gould *et al.*, 2008).

Experimental Procedures

Mosquito strains and their maintenance

Carb77 and HWE mosquitoes were maintained using insectary conditions as described previously (Franz *et al.*, 2006). Briefly, mosquitoes were kept under a temperature regime of ~28 °C, ~78–82% humidity and a 12 h/12 h light/darkness cycle. Adults were fed on raisins or sucrose and provided with water. Mice were provided as blood source for routine colony maintenance. Carb77 mosquitoes were maintained as an inbred cage colony for eight generations. Then twenty-five single crossings between intercrossed transgenic males (G₈) and females (G₈) were set up in an attempt to generate a homozygous line. The F₁ larvae of these crossings were screened for eye-specific EGFP expression. Four of the 25 crosses produced progeny (F₁) that were all transgenic. Wild-type individuals were present in the F₂

of these four crosses indicating that only one of the grandparents was homozygous for the transgene whereas the other one was still hemizygous. Since we could not identify a purely homozygous family, we decided to select the F₁ of one of those four crosses as founders for a new Carb77 mosquito colony. In eight subsequent generations (G₉–G₁₆) the average ratio of EGFP expressing : non-expressing larvae was 88:12.

Identification of the transgene integration site and detection of the transgene by gene amplification

The TE integration site of Carb77 mosquitoes was identified by genome walking. Total genomic DNA was extracted from transgenic males and females (Adelman *et al.*, 2004). Using the GenomeWalker Universal Kit (BD Biosciences, Palo Alto, CA) purified DNA was digested with either *Dra* I, *Eco*R V, *Pvu* II, or *Stu* I followed by ligation to the adaptor molecule provided with the kit. Amplification reactions were conducted using the outer and nested primers complementary to the mariner left and right arms, respectively (Table 1) as well as the outer and nested adaptor primers of the kit. Amplification products were generated using the Advantage 2 polymerase (BD Biosciences) according to the manufacturer's recommendations, inserted into the TOPO-TA cloning vector (Invitrogen, Carlsberg, CA) and sequenced. Using the primer pairs 13121 FWD/ maRight REV and 13121 REV/ maLeft FWD (Table 1) TE integration and its orientation within the mosquito genome were confirmed.

Total DNA for the detection of the transgene in individual mosquitoes was extracted from single mosquitoes using the DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA). For each reaction 500 ng of template DNA was used. Gene amplification was performed using Taq polymerase following a standard protocol.

Transient down-regulation of the RNAi pathway in Carb77 mosquitoes and challenge with DENV2

dsRNA that targeted the ribonuclease domain encoding region of *Aa-dcr2* was synthesized by *in vitro* transcription from purified DNA templates using the T7 MegaScript Kit (Applied Biosystems, Foster City, CA). The DNA template (~500 bp in size) was generated by conventional gene amplification using primers that contained the T7 promoter encoding sequence at their 5'ends (Table 1). Four to five day-old females were injected intrathoracically with 1 µg of dsRNA each. Mosquitoes received an artificial bloodmeal three days later containing 10⁶–10⁷ pfu/ml DENV2 (Jamaica 1409). Virus titers of whole body mosquitoes were assessed as described (Franz *et al.*, 2006) at seven and 12–14 days pbm. Twelve to 14 days prior to mosquito challenge experiments C6/36 cells (~80% confluent) were infected with DENV2 at a MOI of 0.01. Cells and supernatants of cultures that showed moderate cytopathic effects were harvested and used in artificial bloodmeals. The Puerto Rican 159 (NCBI accession number M19197), Indonesian 1051 (L10044), and Jamaican 1409 (M20558) 'low passage' strains of DENV2 were propagated in C6/36 cells for two to three passages to increase their titers before using them in the feeding experiments.

Northern blot analysis

Degradation of *Aa-dcr2* was confirmed by Northern blot analysis. Total RNA was extracted from individual female mosquitoes three days after intrathoracic injection with dsRNAs using TRIzol (Invitrogen, Carlsbad, CA) as described before (Sanchez-Vargas *et al.*, 2009). Approximately 3–5 µg of RNA of each individual mosquito was separated electrophoretically on a 1.2% agarose gel and blotted onto a positively charged nylon membrane (Applied Biosystems). Blots were hybridized with random primed ³²P-dCTP labeled DNA probes (Megaprime DNA Labelling Kit, Amersham Biosciences, NJ) which were specific to *Aa-dcr2* or *βgal*. For the detection of the transcribed IR effector in Carb77 mosquitoes total RNA was extracted from midguts of 15–20 females that had received a sugarmeal or a bloodmeal 24h, 48 h, or 72 h before. For the detection of the Mnp IR effector transcript among Vg40 mosquitoes RNA was extracted from whole body females that had received sugar- and bloodmeals before at similar time points. Northern blots were performed as described above. The probe was a ~500 bp ³²P-dCTP random labeled DNA fragment corresponding to the Mnp IR effector of the Carb77 or Vg40 transgene.

Statistical analysis

Statistical analysis was performed using the FREQ and GLIMMIX procedures of the SAS software package (Cary, NC, USA). Proportions of infected mosquitoes were analyzed using Fisher's exact test ($\alpha = 0.05$) whereas virus titers of infected mosquitoes were analyzed in one-way ANOVA ($\alpha = 0.05$) following log transformation and Tukey's error adjustment. To compare the proportions of infected mosquitoes among multiple groups, pair wise comparisons were conducted following logit transformation and Tukey's error adjustment.

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Figure 1.

Detection of the Carb77 transgene and its integration site. (A) Schematic representation of the *Mos1 mariner* TE containing an IR construct targeting the prM gene of DENV2 and the eye-specific selection marker. The black line represents genomic DNA flanking the transgene. Numbers below the diagram of the TE indicate DNA fragment sizes in base pairs (bp). Abbreviations: ma. left, ma. right = left, right arms of the *Mos1 mariner* TE; *AeCPA* promoter = promoter region of the *Ae. aegypti carboxypeptidase A* gene; Mnp+, Mnp- = cDNA fragments corresponding to the prM encoding region of DENV2 in sense (+) and anti-sense (-) orientations; i = minor intron of the *Ae. aegypti sialokinin I* gene; svA = transcription termination signal derived from the SV40 virus; EGFP = green fluorescent protein marker; 3xP3 = eye tissue specific promoter. Black bars below the TE diagram indicate regions that have been amplified from total mosquito DNA. Numbers indicate the sizes of amplicons in bp and letters in front of the bars are referring to the primer pairs used (Table 1); a: 13121 FWD/maRight REV; b: EGFP FWD/REV; c: Mnp2 FWD/SV40A REV; d: Mnp1 FWD/REV; e: Carb FWD/Mnp2 REV; f: maLeft REV/Carb REV; g: maLeft FWD/13121 REV. (B) Partial sequence of the *Ae. aegypti* genomic DNA of contig AAGE02013121. A highly-repetitive sequence motif is underlined. Annealing sites of primers 13121 FWD and REV are highlighted in red. In capital letters and highlighted in green: right arm of the mariner TE; highlighted in blue: left arm of the TE. The duplicated endogenous TA target sites are shown in capital letters and bold. (C) Detection of the integrated TE and the flanking genomic DNA by gene amplification among total DNA extracted from single HWE or Carb77 females. Lane 1: Carb77, primers 13121 FWD/maRight REV; lane 2: HWE, the same primers; lane 3: Carb77, primers maLeft FWD/13121 REV; lane 4: HWE, same primers; lane 5: HWE, primers maLeft FWD/13121 FWD/13121 REV; lane 6: homozygous Carb77, same primers; lane 7: hemizygous Carb77, same primers. Sizes of the amplicons in bp are shown below the gel image.

Figure 2. Resistance of Carb77 females (G₁₂–G₁₅) to DENV2. One week-old females received artificial, DENV2 containing bloodmeals. Virus titers ranged from 5×10⁶ pfu/ml (G) to 2.8×10⁷ 14 pfu/ml (G₁₅). Titters of single females were analyzed by plaque assays at 7 days pbm (A) and 14 days pbm (B) using LLC-MK2 cells. Raw data and parameters of the statistical analysis are presented based on the proportion of infected vs. non-infected females (Fisher’s exact test, α = 0.05) and mean virus titers of infected females (ANOVA, α = 0.05).

Figure 3.

Resistance of Carb77 females (G_9 and) to three different genotypes of DENV2. One-week old females were challenged orally with the DENV2 strains Indonesia 1051 (A), Puerto Rico 159 (B), or Jamaica 1409 ‘low passage’ (C) representing the genotypes ‘Cosmopolitan’, ‘American’, and ‘American-Asian’, respectively. Virus titers in the artificial bloodmeals were 2.1×10^7 pfu/ml (Indonesia 1051), 5×10^7 pfu/ml (Puerto Rico 159), and 1.35×10^6 (Jamaica 1409 ‘low passage’). Virus titers of females were assessed 7, 12 or 14 days pbm in LLC-MK2 cells. Raw data and parameters of the statistical analysis are presented based on the proportion of infected vs. non-infected females (Fisher’s exact test, $\alpha = 0.05$) and mean virus titers of infected females (ANOVA, $\alpha = 0.05$).

Figure 4.

Evaluation of the resistance mechanism to DENV2 in Carb77 G_{15} mosquitoes. (A) Virus titers of single females infected orally with DENV2 or injected intrathoracically with PBS (injection control), dsRNA targeting β gal (non-template control), or dsRNA targeting *Aa-dcr2* mRNA three days before receiving a DENV2 containing bloodmeal (titer: 1×10^7 pfu/ml). Virus titers were assessed at 7 days pbm by plaque assays in LLC-MK2 cells. Raw data and parameters of the statistical analysis are presented based on the proportion of infected vs. non-infected females (Fisher's exact test, $\alpha = 0.05$) and mean virus titers of infected females (ANOVA, $\beta = 0.05$). P-values that resulted from pair-wise comparisons and were <0.05 are shown. (B) Northern blot analysis to confirm degradation of the *Aa-dcr2* mRNA in Carb77 females that were injected intrathoracically with dsRNAs three days before. The templates for the random labeled 32 P-dCTP DNA probes were ~500 bp fragments corresponding to the *E. coli* β gal and *Aa-dcr2* genes, respectively. Hybridization was performed overnight at 55°C. The ethidium-stained rRNA is shown in each lane of the gel as loading control.

Figure 5.

Loss of the resistance phenotype to DENV2 among Carb G₁₇ mosquitoes. (A) One week-old Carb77 (G₁₇) and HWE mosquitoes received artificial, DENV2 containing bloodmeals (titer in the bloodmeal: 8×10^6 pfu/ml). Virus titers of females were assessed 7 and 14 days pbm in LLC-MK2 cells. Raw data and parameters of the statistical analysis are presented based on the proportion of infected vs. non-infected females (Fisher's exact test, $\alpha = 0.05$) and mean virus titers of infected females (ANOVA, $\beta = 0.05$). (B) Detection of the Carb77 transgene in individual females by gene amplification. Total DNA was extracted from single females of the Carb77 colony that did not express EGFP in their eyes (lanes 1–5) or that were EGFP positive (lanes 6–14). Primers for the DNA amplification: a. 13121 FWD/maRight REV; b. EGFP FWD/REV; c. Mnp2 FWD/SV40A REV; d. Mnp1 FWD/REV; e. Carb FWD/Mnp2 REV; f. maLeft REV/Carb REV; g. maLeft FWD/13121 REV. (C) Northern blot analysis to assess the transcription of the Mnp IR effector gene in the midguts of bloodfed Carb77 females (G₁₇) at 24–72 h pbm. As a positive control for the Northern blot another transgenic mosquito line (Vg40) was used that expresses the identical effector in the fatbody of bloodfed females at 24 h pbm. The template for the random labeled 32 P-dCTP DNA probe was a 500 bp fragment corresponding to the Mnp IR effector. Blots were hybridized over night at 55°C.

Table 1

PCR primers for the molecular analysis of Carb77 mosquitoes.

Purpose	Primer Name	Forward Primer, 5'-3'	Reverse Primer, 5'-3'
Transgene insertion site	maRight_nested REV		GACGATGAGTTCTACTGGCGTGGAATCC
	maLeft_nested FWD	GTGGTTCGACAGTCAAGGTTGACACTTC	
	maRight REV		GAGCAGCGCTTCGATTCTTACGAAAGTGTG
	maLeft FWD	CAATTATGACGCTCAATTCGCGCCAAAC	
	13121 FWD / REV	TTGAAGCGAACCTCTGCAGCGTAATG	CAAGTTTTTCCACTTTGTTGAACTACAGATC
Transgene sequencing	maLeft REV		CGAATGCTTGTGAAGCCTTTGGC
	Carb FWD / REV	TAGAATACCTGCTGTGAACCTATCC	GTTACAGCAGGTAATCTAATTTAAAC
	EGFP FWD / REV	TGACCCTGAACCTCATCTGCACCAC	CTCCAGCAGGACCATGTGATCGCG
	Mnp1 FWD / REV	GCAGGCGTGATTATATGTTGATTCCAACAG	AGTCTCTATTTGATATTCCTATGCAACGCATTG
	Mnp2 FWD / REV	ATGTTCAGAGAATTGAAACCTGGATCTTG	CTCTTCTGTGTTCTCCTGTGGTGGCAC
dsRNA generation	SV40A REV		GCATCACAAATTCACAAATAAAGC
	Aa- <i>dcr2</i> FWD / REV	GGCATTGACGACGAAATCATCGTCCGATG	ACCATGGCATCCGCCGGTGTCTTGTCC
	β <i>gal</i> FWD / REV	GGTCGCCAGCGCACCGCGCCTTC	GCCGGTAGCCAGCGCGGATCATCGG
	T7 promoter sequence	TAATACGACTCACTATAGG	