

Engineering RNA interference-based resistance to dengue virus type 2 in genetically modified *Aedes aegypti*

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Mosquitoes (*Aedes aegypti*) were genetically modified to exhibit impaired vector competence for dengue type 2 viruses (DENV-2). We exploited the natural antiviral RNA interference (RNAi) pathway in the mosquito midgut by constructing an effector gene that expresses an inverted-repeat (IR) RNA derived from the premembrane protein coding region of the DENV-2 RNA genome. The *A. aegypti* carboxypeptidase A promoter was used to express the IR RNA in midgut epithelial cells after ingestion of a bloodmeal. The promoter and effector gene were inserted into the genome of a white-eye Puerto Rico Rexville D (Higgs' white eye) strain by using the nonautonomous *mariner Mos1* transformation system. A transgenic family, Carb77, expressed IR RNA in the midgut after a bloodmeal. Carb77 mosquitoes ingesting an artificial bloodmeal containing DENV-2 exhibited marked reduction of viral envelope antigen in midguts and salivary glands after infection. DENV-2 titration of individual mosquitoes showed that most Carb77 mosquitoes poorly supported virus replication. Transmission *in vitro* of virus from the Carb77 line was significantly diminished when compared to control mosquitoes. The presence of DENV-2-derived siRNAs in RNA extracts from midguts of Carb77 and the loss of the resistance phenotype when the RNAi pathway was interrupted proved that DENV-2 resistance was caused by a RNAi response. Engineering of transgenic *A. aegypti* that show a high level of resistance against DENV-2 provides a powerful tool for developing population replacement strategies to control transmission of dengue viruses.

RNA silencing | transgenesis | genetic control | mosquito | dengue disease

Dengue viruses (DENV) [Flaviviridae; *Flavivirus*; DENV types 1–4 (DENV-1–4)] threaten public health in >100 countries and infect an estimated 50 million people annually (1, 2). The mosquito, *Aedes aegypti*, is the principal vector for epidemic dengue disease (3). The urban DENV transmission cycle involves only humans and mosquitoes. No DENV vaccines are currently available, and vector control strategies that minimize human–mosquito contact have largely failed (4, 5). New control strategies are needed. One possible strategy is to replace vector populations competent to transmit DENVs with pathogen-incompetent vectors (6). The essential features of this genetic control strategy are to identify genes that express antiviral molecules in the vector, link this gene (or genes) to a genetic drive system [transposable elements (TE), meiotic drive, or homing endonuclease genes] and introgress the gene(s) into field populations (7–9). A key step in developing this control strategy is to identify effector genes that, when expressed in the vector, inhibit DENV replication. Proof of principle for RNA interference (RNAi)-like disruption of DENV-2 vector competence was previously demonstrated by using a nonheritable alphavirus expression system (10). Applying the principle of heritable gene silencing in transgenic *Drosophila melanogaster*, *Caenorhabditis*

elegans, and *A. aegypti* (11–13), we genetically manipulated *A. aegypti* mosquitoes to express inverted-repeat (IR) sequences derived from DENV-2 genomic RNA (see Fig. 5, which is published as supporting information on the PNAS web site). IR-RNAs form dsRNA that intracellularly trigger the RNAi response. Expressing the IR-RNA in the midgut of female mosquitoes soon after ingestion of viremic blood ensures that the dsRNA forms when the virus is in its most vulnerable state at the onset of replication and before the establishment of infection foci inside the mosquito. We demonstrated previously that IR-RNAs derived from the DENV RNA genome are triggers for RNAi in mosquito cell culture (14, 15). Here we report the development of genetically engineered mosquitoes that are resistant to DENV-2 by expressing virus-derived IR-RNAs to trigger RNAi in the midgut epithelium.

Results

Transgenic Mosquito Families. Transgenic mosquito lines were generated by microinjecting preblastoderm embryos with a nonautonomous Class II TE based on *mariner Mos1* (16). The donor plasmid, Mos-carb/Mnp+/i/Mnp-/svA, contained a 578-bp IR sequence (Mnp+/-) derived from the prM region of the DENV-2 Jamaica 1409 genome (Fig. 1A). A total of 1,160 preblastoderm-stage embryos were coinjected with the Mos-carb/Mnp+/i/Mnp-/svA donor plasmid and helper plasmid. Of the 157 surviving embryos, 148 G₀ developed into adult mosquitoes, which became founders of 91 families. We selected one family, Carb77, displaying strong eye-specific EGFP expression from the eye-specific 3xP3 promoter (17) for evaluating resistance to DENV-2 infection. When G₁ mosquitoes of family Carb77 were outcrossed to Higgs' white eye (HWE), ~50% of the progeny larvae had EGFP-expressing eyes (see Fig. 6, which is published as supporting information on the PNAS web site). Outcrosses continued until G₅, and then transgenic Carb77 mosquitoes were intercrossed for an additional five generations. Approximately 90% of the mosquitoes analyzed at G₁₀ were transgenic with eye-specific EGFP expression. These observations were consistent with the Mendelian inheritance of a single integration of the transgene into the genomic DNA and EGFP functioning as dominant trait. Genomic DNA of Carb77 mosquitoes (G₁₀) and HWE were analyzed by Southern blot hybridization (Fig. 1A and B). All Southern blot data were consistent

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Abbreviations: RNAi, RNA interference; RISC, RNAi-induced silencing complex; DENV, dengue virus; DENV-*n*, DENV type *n*; IR, inverted repeat; *AeCPA*, *A. aegypti* carboxypeptidase A; pbm, postbloodmeal; IFA, immunofluorescence assay; pfu, plaque-forming units; *ago2*, *A. aegypti* argonaute 2; HWE, Higgs' white eye.

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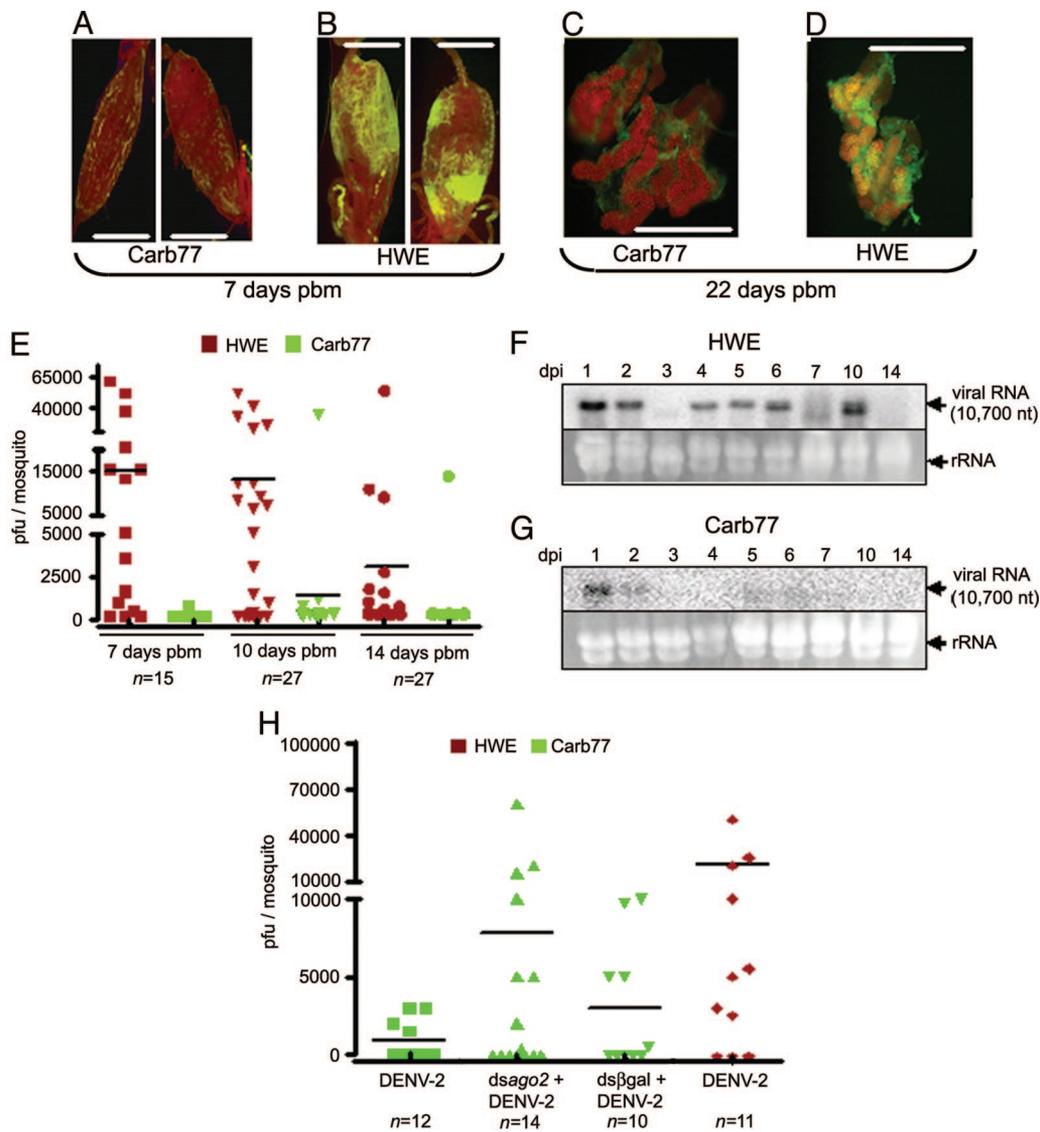


Fig. 3. Characterization of the Carb77 DENV-2 resistance phenotype. Detection of DENV-2 antigen in midguts (scale bars, 0.5 mm) (A and B) and in salivary glands (three salivary glands/image; scale bars, 0.5 mm) (C and D) of Carb77 and HWE by IFA by using mAb 3H5 recognizing an epitope of DENV-2. E. Images represent typical infection patterns. (E) DENV-2 plaque titrations of single whole body Carb77 and HWE mosquitoes 7, 10, and 14 days pbm (bars indicate mean values of titers). Plaque assays were performed in LLC-MK2 monkey kidney cells at 10-fold dilutions. (F and G) Detection of DENV-2 viral RNA in midguts of HWE (F) and Carb77 (G) 1–14 days pbm by Northern analysis by using “Mnp+” probe. Ribosomal RNAs are shown (Lower) to indicate amounts of RNA loaded per lane. (H) Transient silencing of *A. aegypti ago2* in Carb77 and HWE followed by challenge with a DENV-2-containing bloodmeal. Four days before bloodfeeding, 1 μ g dsRNA was injected into 15 3-day-old females. β -gal dsRNA was used as a control. Virus titers of single mosquitoes were assessed 7 days pbm by plaque titration in LLC-MK2 cells (bars indicate mean values of titers).

HWE at the same time point (Fig. 3D). Because the *AeCPA* promoter activates gene expression in the midgut epithelium (18, 19), reduced DENV-2 dissemination in Carb77 could be due to the inability of the virus to reach a concentration threshold in the midgut tissue. Thus, virus amounts escaping the midgut were insufficient to cause infections at later times in salivary glands. Indeed, when Carb77 mosquitoes were injected intrathoracically with 10^4 pfu DENV-2, thus bypassing midgut infection, all mosquitoes showed the presence of viral antigen in head tissues and salivary glands comparable to similarly injected HWE mosquitoes (data not shown). Oral challenge of Carb77 and HWE with DENV-4 (Philippines H241) resulted in similar midgut and disseminated infection profiles as detected by IFA (see Fig. 7A and B), confirming that Carb77 mosquitoes were not resistant to DENV-4. Thus, the effects of the inserted transgene were specific to the targeted virus serotype.

As further evidence of engineered resistance, DENV-2 titers were determined for Carb77 (outcross G₅) and HWE mosquitoes 7, 10, and 14 days pbm. Carb77 mosquitoes were much less susceptible to infection with the virus and, when infected, much less permissive to virus replication (Fig. 3E). Virus was detectable in only 1/15 Carb77 females at 7 days pbm, and during the entire time course of 7–14 days pbm, only 2/69 transgenic mosquitoes exceeded titers of 2,000 pfu per mosquito. In contrast, >50% of the HWE controls developed titers above 2,500 pfu/mosquito 7–10 days pbm.

Viral RNA was abundantly detected by Northern blot analysis in midguts of HWE mosquitoes for 1–10 days pbm with the exception of day 3 (Fig. 3F). This observation was repeated, and we conjectured that day 3 pbm represents the onset of virus replication after its eclipse phase. Viral RNA detected at days 1 and 2 originated from the input virus in the bloodmeal, and RNA

of *A. aegypti*. In this study, the temporal expression pattern of the IR construct appeared to coincide with the DENV eclipse phase followed by a slow onset of viral replication within newly infected midgut tissue. Thus, DENV replication in the midgut and further infection of secondary tissues were generally reduced in Carb77 and in many transgenic mosquitoes were blocked completely.

The aim of this study was to demonstrate the feasibility of developing transgenic *A. aegypti* that exhibit a reduced vector competence for DENV-2 by genetically triggering their RNAi pathway. Here we successfully showed the proof of principle. Potential applications of similarly engineered mosquitoes would be mosquito population replacements in DENV-endemic regions to profoundly reduce vector competence in a population and reduce DENV spread. For this purpose, engineered mosquitoes might need to approach 100% levels of resistance against the virus analogous to predictions from a model developed for the control of malaria if a similar strategy is pursued (29). Thus, in a next step, we need to further optimize anti-DENV effector constructs targeting all four DENV serotypes and evaluate other tissue-specific *A. aegypti* promoters such as apyrase (30), glutamine synthetase (31), ferritin heavy-chain (32), and vitellogenin (33). Using them in combination could block DENV in multiple tissues of the mosquito and thus further reduce the vector competence for the virus.

Materials and Methods

Plasmid DNA Constructions. The construction of the Mos-carb/Mnp+/i/Mnp-/svA donor plasmid is described in detail in *Supporting Text*.

Germ-Line Transformation of *A. aegypti* and Establishment of Transgenic Families. Eye-pigment-deficient HWE *A. aegypti*, a variant of the Rexville D strain, was used as the recipient for germ-line transformations (34, 35). Mosquitoes were reared at 28°C, 82% humidity, under a 12-h darkness/12-h light regime. Adults were maintained on sucrose.

Germ-line transformations were carried out as described (34, 36). Each surviving G₀ male was outcrossed to 20 HWE females. Female G₀ mosquitoes in pools of five were outcrossed to one HWE male. Progeny larvae of these crosses (G₁) were screened for EGFP expression in their eyes under a fluorescence microscope [Olympus (Melville, NY) SZX12] equipped with an EGFP-specific filter set. Transgenic G₁ mosquitoes were outcrossed as described above, and their progeny (G₂) were analyzed for expression of the IR construct. To establish homozygous lines, we set up 25 individual crosses between transgenic males and females. Up to 300 progeny larvae of the two following generations were screened for eye-specific EGFP expression.

Total DNA Extraction and Southern Blot Analysis. DNA extractions and Southern blot analyses followed the procedures described (34, 36). About 20 µg of extracted total DNA was digested either with EcoRI, KpnI, or PstI and column-purified (QIAquick PCR Purification Kit, Qiagen, Valencia, CA) followed by electrophoresis in a 0.8% agarose gel and blotting onto a positively charged nylon membrane (Ambion, Austin, TX). Random-primed DNA probes were labeled with [α -³²P]dCTP/ml (3,000 Ci/mmol; 1 Ci= 37 GBq) by using the Megaprime labeling kit (Amersham Pharmacia Biosciences). DNA probes were derived from the 354-bp left arm ("mariner left") and the 1,189-bp right arm/3xP3 fragment ("mariner right/3xP3") of MosI as well as from the Mnp portion ("Mnp+") of the IR DNA construct. Hybridizations were carried out at 45°C.

Northern Blot Analysis. Northern blot analyses were performed as described (14). Briefly, 10–15 µg of total RNA from midguts of bloodfed and nonfed female mosquitoes were electrophoresed in a 1.2% agarose gel and blotted onto a

positively charged nylon membrane (Ambion). Blots were hybridized with antisense ³²P-UTP-labeled RNA probes that were transcribed *in vitro* from linearized pBluescript II SK (Stratagene) containing a 290-bp cDNA fragment derived from the prM sequence of DENV-2 RNA. Alternatively, random-primed ³²P-dCTP-labeled DNA probes were generated from the same template by using the Megaprime DNA Labeling Kit (Amersham Pharmacia Biosciences).

Detection of siRNAs Derived from DENV-2 in Midgut Tissue of Female Mosquitoes. siRNAs were enriched from total midgut RNA extracted from female mosquitoes 24–48 h pbm according to a previously described protocol (37). High-molecular-weight RNA was precipitated in presence of 5% (wt/vol) polyethylene glycol (*M_r* 8,000) and 0.5 M NaCl. Supernatants containing siRNAs were analyzed further in a ribonuclease protection assay by using the *mirVana* miRNA Detection Kit (Ambion). For nuclease protection, siRNAs were hybridized with sense or antisense RNA probes 30 nt in length containing 22 nt of sequence complementary to the DENV-2 (Jamaica 1409) prM-encoding region. RNA probes were end-labeled with [γ -³²P] ATP (4,500 Ci/mmol) by using the *mirVana* Probe & Marker Kit (Ambion). Hybridizations were performed at 42°C. After RNase digestion, hybridized RNA samples were electrophoretically separated on a 16% polyacrylamide gel containing 7 M urea.

DENV-2 and -4 Challenge Experiments. Approximately 2,000 mosquitoes were reared for each DENV challenge experiment. Using UV light-emitting goggles (Biological Laboratory Equipment, Budapest), transgenic females were selected for eye-specific EGFP expression originating from the transgene. Selected 7- to 8-day-old females were challenged with an artificial infectious bloodmeal (38) consisting of defibrinated sheep blood (40% vol/vol), DENV-2 Jamaica 1409- or DENV-4 Philippines H241-infected C6/36 cell suspension (60% vol/vol), and 1 mM ATP. DENV titers in the cell suspensions ranged from 5 × 10⁶ to 1.5 × 10⁷ pfu/ml. Infected mosquitoes were kept under BSL3 insectary conditions.

DENV Antigen Detection in Mosquito Tissue. DENV antigen was detected in mosquito tissue by indirect IFA (39). Dissected midguts and salivary glands were fixed in 4% (vol/vol) paraformaldehyde in PBS. IFAs were performed by using either the DENV-2 E-specific mAb 3H5 or the DENV1–4 E-specific mAb 4G2 (40, 41).

Dengue-2 Virus Transmission Assay. HWE and Carb77 (G₁₁) mosquitoes were infected with a bloodmeal containing 10⁷ pfu/ml of DENV-2 and maintained for 14 days. Groups of 15 females each were then allowed to probe and feed on 350 µl of a feeding solution [50% FBS/164 mM NaCl/100 mM NaHCO₃/0.2 mM ATP/≈50 µg sucrose/phenol red, pH 7.0] that was placed between two parafilm membranes stretched over a glass feeder. After probing, mosquitoes and feeding solutions were collected and subjected to plaque assays (42).

Transient Silencing of *ago2* in *A. aegypti*. dsRNA (540 bp) derived from the genomic DNA region encoding *ago2* of *A. aegypti* (K. M. Keene, personal communication) was synthesized according to the method used before (43). Four-day-old females were intrathoracically injected with 1 µg of dsRNA each and 3 days later given an infectious bloodmeal containing 1.25 × 10⁷ pfu/ml DENV-2. Virus titers were assessed 1 week postinfectious bloodmeal.

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