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Transgene-mediated suppression of the RNA interference pathway in *Aedes aegypti* interferes with gene silencing and enhances Sindbis virus and dengue virus type 2 replication

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Abstract

RNA interference (RNAi) is the major innate antiviral pathway in *Aedes aegypti* that responds to replicating arboviruses such as DENV and SINV. The mosquito's RNAi machinery is capable of completely eliminating DENV2 from *Ae. aegypti*. On the other hand, transient silencing of key genes of the RNAi pathway increases replication of SINV and DENV2, allowing the viruses to temporally overcome dose-dependent midgut infection and –escape barriers at higher rates. Here we expressed FHV-B2 from the poly-ubiquitin (*PUB*) promoter in *Ae. aegypti* using the $\Phi C31$ site-directed recombination system to investigate the impact of transgene-mediated RNAi pathway suppression on infections with SINV-TR339eGFP and DENV2-QR94, the latter of which has been shown to be confronted with a strong midgut escape barrier (MEB) in *Ae. aegypti*. FHV-B2 was constitutively expressed in midguts of sugar- and bloodfed mosquitoes of transgenic line PUBB2 P61. B2 over-expression suppressed RNA silencing of *carboxypeptidase A-1* (*AeCPA-1*) in midgut tissue of PUBB2 P61 mosquitoes. Following oral challenge with SINV-TR339eGFP or DENV2-QR94, mean titers in midguts of PUBB2 P61 females were significantly higher at 7 days post-bloodmeal (pbm) than in those of non-transgenic control mosquitoes. At 14 days pbm, infection rates of carcasses were significantly increased in PUBB2 P61 mosquitoes infected with SINV-TR339eGFP. Following infection with DENV2-QR94, midgut infection rates were significantly increased in the B2-expressing mosquitoes at 14 days pbm. However, B2 expression in PUBB2 P61 did not increase the DENV2-QR94 dissemination rate, indicating that the infection phenotype was not primarily controlled by RNAi.

Keywords

Aedes aegypti; mosquito; transgenic; $\Phi C31$ recombination; dengue virus; Sindbis virus; FHV-B2; RNA interference; RNAi suppression

Introduction

The yellow fever mosquito, *Aedes aegypti*, is a vector of medically important arboviruses including the flaviviruses (*Flaviviridae*; *Flavivirus*) dengue virus type 1–4 (DENV1–4) and yellow fever virus (YFV) and alphaviruses (*Togaviridae*; *Alphavirus*) such as chikungunya virus (CHIKV) and under laboratory conditions Sindbis virus (SINV) (Sanchez-Vargas *et al.*, 2004; Weaver and Reisen, 2010). Arboviruses often cause severe pathology in their

vertebrate hosts but are usually not pathogenic to their invertebrate vectors (Blair, 2011). In the mosquito, arboviruses are confronted with several innate immune pathways, such as RNA interference (RNAi), Toll, immune deficiency (Imd), and Janus kinase-signal transducer and activator of transcription (JAK-STAT) (Sanchez-Vargas *et al.*, 2004; Blair, 2011; Xi *et al.*, 2008; Souza-Neto *et al.*, 2009; Ramirez and Dimopoulos, 2010). Of these, RNAi is the major antiviral innate immune pathway in *Ae. aegypti* (Franz *et al.*, 2006; Campbell *et al.*, 2008; Sanchez-Vargas *et al.*, 2009; Khoo *et al.*, 2010; Mathur *et al.*, 2010; Scott *et al.*, 2010). RNA silencing is divided into three distinct, highly conserved small RNA pathways, micro RNA (miRNA), small interfering RNA (siRNA), and piwi-interacting RNA (piRNA) (Ghildiyal and Zamore, 2009). Based on the origin of the RNA substrate the siRNA pathway is further divided into endogenous small RNA (endo-RNAi) and exogenous small RNA (exo)-RNAi, the latter of which has a major impact on virus infections in mosquito vectors. RNAi can eliminate DENV2 from transgenic mosquitoes expressing an inverted-repeat (IR) RNA to trigger the RNAi pathway against the virus (Franz *et al.*, 2006; Mathur *et al.*, 2010). However, arboviruses are able to persistently infect vectors despite being targeted by the RNAi machinery as shown by the presence of 21 nt virus-derived small interfering RNAs (viRNAs) in arbovirus-infected, transmission-competent mosquito vectors (Scott *et al.*, 2010; Hess *et al.*, 2011). Moreover, temporally induced silencing of the RNAi machinery in *Ae. aegypti* led to significantly increased SINV and DENV2 titers combined with increased midgut infection and dissemination rates and a shortened extrinsic incubation period (EIP) without compromising insect survival (Campbell *et al.*, 2008; Sanchez-Vargas *et al.*, 2009; Khoo *et al.*, 2010). Arboviruses must have evolved strategies to cope with the mosquito's RNAi pathway. One such strategy could be a nucleic acid-based decoy mechanism (Siu *et al.*, 2011). Plant viruses and several animal viruses encode proteins that suppress RNAi to protect their viral RNA from homology-dependent degradation (Burgyn and Havelda, 2011; Song *et al.*, 2011). Effects of RNAi suppression in *Ae. aegypti* by a recombinant SINV have been recently investigated (Myles *et al.*, 2008; Cirimotich *et al.*, 2009). A recombinant SINV strain was engineered to express the Flock house virus (*Nodaviridae*; [FHV]) B2 protein, a potent suppressor of RNAi. The 12kDa B2 protein is essential for the replication of FHV (Chao *et al.*, 2005; Wang *et al.*, 2006; Li and Ding, 2005). B2 protein is a homodimer and indiscriminately binds to dsRNA molecules independent of their nucleotide sequences and sizes such as siRNAs duplexes and long dsRNAs, thereby protecting dsRNA from being accessed and processed by dicer2 of the RNAi machinery. When mosquitoes were orally infected with SINV-B2, virus titers, midgut infection and escape rates were significantly increased compared to the control virus. Strikingly, SINV-B2 caused high mortality among the mosquitoes at 4–6 days post-infection, suggesting that RNAi has the potential to protect the vector from pathogenic effects of replicating arboviruses (Myles *et al.*, 2008; Cirimotich *et al.*, 2009).

In previous studies key genes of the RNAi machinery in *Ae. aegypti* were temporarily and/or tissue specifically silenced. As a consequence, the effects on arbovirus replication were transient and did not affect mosquito fitness. The aim of this study was to generate transgenic mosquitoes expressing FHV-B2 as tools to study effects of constitutive RNAi suppression *in trans* on unrelated arboviruses such as SINV (recombinant strain: TR339eGFP) and DENV2 (strain: QR94). The QR94 strain belongs to the American genotype of DENV2 and has been shown to inefficiently disseminate from the mosquito midgut following oral acquisition (Salazar *et al.*, 2010). SINV-TR339eGFP has been previously shown to encounter a dose-dependent MEB in *Ae. aegypti*, which was diminished when the virus titer in the midgut was increased (Kramer *et al.*, 1981; Khoo *et al.*, 2010). In this study, we wanted to assess whether both viruses would respond similarly to B2-mediated RNAi suppression in *Ae. aegypti* by increasing their replication efficiencies, eventually reaching concentrations high enough to become detrimental for the mosquito. We also wanted to observe whether stable RNAi suppression would allow SINV-TR339eGFP

and DENV2-QR94 to disseminate more efficiently from the mosquito midgut, which would be an indication that the viruses are confronted with a dose-dependent MEB (Kramer *et al.*, 1981).

Here we describe the generation of transgenic *Ae. aegypti*, which constitutively express FHV-B2 under control of the *Ae. aegypti* poly-ubiquitin (*PUB*) promoter (Anderson *et al.*, 2010). To obtain a predictable transgene expression phenotype transgenic mosquitoes were generated using the $\Phi C31$ site-directed integration system, which has recently been adapted for this purpose (Franz *et al.*, 2011). SINV-TR339eGFP and DENV2-QR94 infection patterns in the RNAi impaired mosquitoes were evaluated and compared with those in non-transgenic control mosquitoes.

Results

Generation of $\Phi C31$ recombinants of *Aedes aegypti* expressing FHV-B2 from the PUB promoter

As shown in Fig. 1A, FHV-B2 expression was under control of the constitutive *Ae. aegypti* *PUB* promoter (Anderson *et al.*, 2010). The gene-of-interest expression cassette was inserted into the *attB* site containing donor for site-specific integration into docking strain attP26 as described before (Franz *et al.*, 2011). Following co-injection of 1828 preblastoderm embryos with *in vitro* transcribed $\Phi C31$ integrase mRNA (600 ng/ μ l) and the modified *attB* donor plasmid (300 ng/ μ l) 196 G₀ individuals (100 females, 96 males) survived. After outcrossing to the HWE recipient strain, 32 male and four female pools were established. One pool, P61, consisting of 29 G₀ females produced offspring that showed both enhanced cyan fluorescent protein (ECFP) and DsRed eye marker expression indicating recombination between the *attP* site of the docking strain and the *attB* site of the donor plasmid (Fig. 1A). The estimated transformation frequency was 1%.

Molecular characterization of line PUBB2 P61

To confirm the $\Phi C31$ -based recombination event among PUBB2 P61 mosquitoes at the molecular level, we PCR amplified genomic DNA of regions encoding the recombination sites, which were expected to be converted from *attP* and *attB* into *attR* and *attL*. Previously, we reported that docking strain attP26 contains two *attP* site integrations (Franz *et al.*, 2011). As shown in Fig. 1B, PCR data revealed that only one of the two *attP* sites in PUBB2 P61 mosquitoes was converted into *attR* and *attL* by recombination with the *attB* site of the donor whereas the other *attP* site remained intact. This indicates that there was a single donor integration event in PUBB2 P61 mosquitoes even though these mosquitoes could have potentially accommodated two $\Phi C31$ -mediated donor integrations.

FHV-B2 expression in PUBB2 P61 mosquitoes

v5-tagged B2 protein was readily detected by Western blots in midguts of sugarfed PUBB2 P61 females at 5–7 days post-eclosion but not in the HWE (negative) control (Fig. 2A). In carcasses, however, B2 protein was only faintly detected at similar time points. Similarly, B2 was readily detected in midguts but not in carcasses of mosquitoes that had received a bloomeal before (data not shown). Presence of B2 antigen in midguts of sugar- or bloodfed females was confirmed in immunofluorescence assays (IFA). As shown in Fig. 2B, v5-tagged B2 antigen was detected in the entire midgut epithelium at different time points following sugarfeeding, bloodfeeding, or DENV2 infection. These results confirm that FHV-B2 was present in midguts of PUBB2 P61 mosquitoes regardless of sugar- or bloodfeeding, whereas in carcasses B2 was barely detectable.

FHV-B2 expression in PUBB2 P61 mosquitoes suppresses induced silencing of *AeCPA-1* in midguts of bloodfed females

The ability of ν_5 B2 to suppress RNAi in midguts of PUBB2 P61 females was analyzed in a functional assay in which induced silencing of the midgut-specific (*Ae. aegypti*) *AeCPA-1* gene was monitored over time. Females were intrathoracically injected with 1 μ g dsRNA targeting *AeCPA-1* (Isoe *et al.*, 2009). Two days later, females received a non-infectious bloodmeal to induce *AeCPA-1* transcription. Abundance of *AeCPA-1* transcripts in midguts was monitored between 7–48 h post-bloodmeal (pbm). Within 24 h pbm, *AeCPA-1* transcript copy numbers per 100 ng of total midgut RNA increased four-fold in PBS-injected or non-injected HWE and PUBB2 P61 females (Fig. 3). The highest *AeCPA-1* transcript copy number (7.0×10^6) was detected at 24 h pbm among midgut RNA of non-injected HWE. After 24 h pbm, *AeCPA-1* transcription levels steadily declined until reaching a base level of <10,000 copies by 48 h pbm. Following dsRNA injection, *AeCPA-1* transcript copy numbers in midguts of HWE mosquitoes decreased at least 10-fold during the 48 h observation period, indicating robust silencing of the gene (Fig. 3). In midguts of similarly treated PUBB2 P61 females, *AeCPA-1* transcription was reduced less than 2-fold at 24 h pbm, which indicates RNAi suppression through functional activity of B2.

The results show that in midguts of PUBB2 P61 females, expression of ν_5 B2 suppressed silencing of an endogenous gene with an efficiency of >50% during the observation period. This is a strong indication that in principle, B2 in midguts of PUBB2 P61 females was functional, which was confirmed in a second independent experiment (data not shown). We speculate that the inability of B2 to completely suppress silencing of an endogenous gene in PUBB2 P61 mosquitoes is an effect of B2 expression in these mosquitoes rather than being caused by the intrinsic nature of the B2 protein. It is possible that slight imbalances in the dsRNA : B2 protein ratio could affect the efficiency of gene silencing or silencing suppression.

Challenging of PUBB2 P61 mosquitoes with SINV and DENV2

After oral challenge, SINV-TR339eGFP mean titers were significantly increased at 7 days pbm in midguts of PUBB2 P61 females (p-value: 0.0457; ANOVA, Tukey-Kramer test) as compared to the HWE control (Fig. 4A). Infection rates and mean virus titers of carcasses did not differ significantly at 7 days pbm. At 14 days pbm, viral infection rates were significantly increased in carcasses (p-value: 0.0471; chi-square test) but not in midguts (Fig. 4B), indicating that B2-mediated suppression allowed SINV-TR339eGFP to overcome the dose-dependent MEB more efficiently.

We then challenged PUBB2 P61 females with the QR94 strain of DENV2, which belongs to the American genotype of DENV2 (Lorono-Pino *et al.*, 2004). DENV2 strains of the American genotype have been shown to disseminate inefficiently from the midgut in *Ae. aegypti* (Armstrong and Rico-Hesse, 2003; Anderson and Rico-Hesse, 2006; Salazar *et al.*, 2010). RNAi suppression in PUBB2 P61 females did not enable DENV2-QR94 to overcome the MEB since mean virus titers and infection rates in carcasses remained low for the transgenic and (HWE) control mosquitoes at both time points (Fig. 4A, 4B). However, similar to SINV-TR339eGFP, mean titers of DENV2-QR94 were significantly increased at 7 days pbm (p-value: 0.0172; ANOVA, Tukey-Kramer test) in midguts of PUBB2 P61 females when compared to the HWE control, even though mean DENV2-QR94 titers in midgut tissue of PUBB2 P61 mosquitoes remained 1–2 logs pfu/ml lower at both time points than mean titers of SINV-TR339eGFP. At 14 days pbm, DENV2-QR94 midgut infection rates were significantly increased in the transgenic mosquitoes (p-value: 0.0321; chi-square test). In summary, FHV-B2 expression in line PUBB2 P61 potentially increased the vector competence for SINV-TR339eGFP but not for DENV2-QR94. SINV-TR339eGFP mean

titers were significantly increased in midgut tissue at 7 days pbm, which then led to increased disseminated infection rates at 14 days. In contrast, significantly increased DENV2-QR94 mean titers in mosquito midguts at 7 days pbm did not affect virus dissemination rates at 14 days pbm.

Discussion

The underlying hypothesis of this study was that FHV-B2-mediated suppression of the RNAi pathway in *Ae. aegypti* would strongly increase arboviruses titers and infection rates. As a result the viruses would become pathogenic to the vector if not kept in check by the antiviral immune pathway. Previously, we had shown that transgene-mediated silencing of *Aa-dcr2* in the midgut of *Ae. aegypti* led to statistically significant higher SINV-TR339eGFP titers as well as midgut infection and dissemination rates (Khoo *et al.*, 2010). However, due to the promoter used to drive effector gene expression in these transgenic mosquitoes, the RNAi impairment was temporal and tissue-specific. As a consequence, arboviruses did not produce titers in the mosquitoes high enough to affect overall vector fitness. We optimized our strategy regarding transgene design and expression in three ways to increase transgene-mediated suppression of RNAi in *Ae. aegypti*. 1) We chose to over-express a strong RNAi suppressor, FHV-B2, which had been shown before to inhibit siRNA biogenesis in *Ae. aegypti* (Myles *et al.*, 2008; Cirimotich *et al.*, 2009); 2) we used the *Ae. aegypti PUb* promoter to constitutively over-express B2 in mosquito midguts and secondary tissue; 3) we generated transgenic mosquitoes using the $\Phi C31$ site-directed integration system to ensure predictable transgene expression in each mosquito generation. Constitutive over-expression of B2 in PUBB2 P61 mosquitoes suppressed the RNAi pathway in the mosquitoes as shown by the reduced efficiency of dsRNA targeting *AeCPA-1* to silence its mRNA in female midguts after bloodfeeding. Though its original function is to render the replication intermediate (dsRNA) of FHV inaccessible to dicer2, the B2 protein is able to inhibit the activity of any dsRNA species present in the cytoplasm of an invertebrate cell (Lingel *et al.*, 2005). Since the *PUB* promoter drives constitutive gene expression in midguts and to a lesser extent in secondary tissues as shown earlier and also in this study, B2-mediated suppression of RNA silencing of midgut-specific *AeCPA-1* was not surprising (Anderson *et al.*, 2010). Furthermore, we showed that two unrelated arboviruses, SINV-TR339eGFP and DENV2-QR94, responded similarly to RNAi suppression in midgut tissue of PUBB2 P61 mosquitoes by producing significantly increased mean virus titers at 7 days pbm. At 14 days pbm, PUBB2 P61 females infected with DENV2-QR94 had significantly increased midgut infection rates, whereas carcass infection rates of this virus were not affected. At the same time point, however, SINV-TR339eGFP had significantly increased carcass infection rates in the B2 expressing mosquitoes, indicating that the virus overcame the dose-dependent MEB more readily. It is possible that DENV2-QR94 failed to overcome the MEB in PUBB2 P61 mosquitoes because after all the overall virus dose in midgut tissue of the transgenic mosquitoes was still low by not exceeding 420 pfu/ml at 7 days pbm, which was ~5-fold higher than in the HWE control. Thus, the relatively low virus titer DENV2-QR94 generated in *Ae. aegypti* appeared to be a phenomenon, which was primarily not controlled by the mosquito's RNAi response.

In conjunction with earlier observations described below the results of this study suggest that the efficiency of RNAi suppression depends on the concentration of B2 molecules being expressed in the cell. Following this scenario, the ratio between dicer2, B2 and viral RNA molecules in a cell would dictate how virus replication becomes affected. In an earlier germline transformation experiment we generated a transgenic mosquito line, Carb/B2-133, which over-expressed FHV-B2 from the midgut-specific, bloodmeal-inducible *AeCPA* promoter (Edwards *et al.*, 2000; Moreira *et al.*, 2000). Since this line was produced by using a TE instead of the $\Phi C31$ site-directed recombination system, we had no control over the

number of transgene integration events per cell. In early generations Carb/B2-133 showed an unusually strong eye marker expression level and when challenged with SINV-TR339eGFP, the virus produced significantly higher titers and infection rates in the transgenic line than in the HWE control (see Fig. S1). However, following three generations of outcrossing to the recipient strain, mosquitoes of this line displayed weak eye-marker expression levels and diminished effects on SINV-TR339eGFP titers and infection rates. We speculate that line Carb/B2-133 originally contained multiple transgene copies, which substantially enhanced B2 expression levels and as a consequence strongly increased virus infection rates and titers. However, the multi-copy transgene integration pattern was unstable in line Carb/B2-133 and therefore lost after three generations.

Previous reports showed that FHV-B2 expression from recombinant SINV-TE3'2J caused a dramatic increase in virus titers associated with significant mortality of infected HWE mosquitoes (Myles *et al.*, 2008; Cirimotich *et al.*, 2009). The authors showed that complete RNAi impairment due to *in cis* expression of the RNAi suppressor caused the recombinant arbovirus to become pathogenic for the mosquito. When we infected PUBB2 P61 mosquitoes with SINV or DENV2, we did not observe any increased levels of mortality among the mosquitoes (see also Fig. S2). The mode of B2 production in the two systems could account for the observed differences. B2 expression from a recombinant virus leads to RNAi pathway suppression in each virus-infected cell, since each viral RNA generating cell also produces equivalent quantities of B2 (Myles *et al.*, 2008; Cirimotich *et al.*, 2009). However, when expressing B2 *in trans* using PUBB2 P61 mosquitoes it is possible that not every cell, which is infected by a virus also produces sufficient concentrations of B2 to completely suppress RNAi, thereby enhancing virus replication.

The only other example of transgene-mediated FHV-B2 expression in insects is that of *Drosophila*, in which B2 was expressed from the *Daughterless*, *actin5C*, or *hsp* promoter (van Rij *et al.*, 2006; Chou *et al.*, 2007; Berry *et al.*, 2009). Injection of the transgenic flies with *Drosophila C virus* (DCV; *Dicistroviridae*) lowered the 50% lethal dose of the virus by ~280-fold as compared to the similarly infected, non-transgenic control. The virus is lethal when injected into flies but not when orally acquired (Lautie-Harivel, 1992). In addition, DCV encodes a potent suppressor of RNAi, DCV-1A at the N-terminus of ORF 1 (van Rij *et al.*, 2006). Thus, the presence of both virus-encoded and heterologous RNAi suppressors in the insect cell could have increased the pathogenic potential of DCV. Previous investigations suggest that arboviruses do not encode strong suppressors of RNAi (Myles *et al.*, 2008; Cirimotich *et al.*, 2009). Thus, effects of RNAi suppression in the *Drosophila*-DCV system might not be directly comparable to those in the mosquito-arbovirus system.

Experimental procedures

Transgene construction and generation of transgenic mosquitoes

The template of the v5-tagged FHV-B2 cDNA originated from recombinant SINV-TE3'2J_{v5}B2 (Cirimotich *et al.*, 2009). In plasmid p*SLfa1180fa-AeCPA/EGFP/svA* (Franz *et al.*, 2011), the *AeCPA* promoter and EGFP encoding fragments were exchanged for *PUB* and v5B2. The resulting PUB/v5B2/svA expression cassette was then transferred into modified *pattB*-DsRed2 (Nimmo *et al.*, 2006) using the *AscI* sites to generate plasmid *pattB*-DsRed2/PUB/v5B2/svA. Transgenic *Ae. aegypti* were generated using the Φ C31 site-directed integration system and the Higgs White Eye (HWE)-based docking strain attP26 as described before (Franz *et al.*, 2011). The transgenic *Ae. aegypti* line was named PUBB2 P61.

Molecular characterization

Site-directed donor integration by recombination between *attB* of the donor plasmid and *attP* of docking strain attP26 was confirmed in a conventional PCR assay. Total DNA was extracted from single females of lines attP26 and PUBB2 P61 using the DNeasy Blood & Tissue kit (Qiagen, Valencia, CA). For each PCR reaction 500 ng of total DNA were used. The (non-recombined) *attP* site was detected using primers attP FWD:

5'CAAATGTGTTCTGTGATGACCTG3' and attP REV:

5'CTCCCTTGCTACTGACATTATGG3'. Recombined *attL* and *attR* sites were detected using primer pairs attL FWD 5'GAGGTCGACGATGTAGGTCAC3', attL REV

5'ACCTTTTCTCCCTTGCTACTGAC3' and attR FWD

5'TCAAATAAGGCGGAGTGG3', attR REV 5'GATGGGTGAGGTGGAGTACG3', respectively (Nimmo *et al.*, 2006).

Transient silencing of AeCPA-1

Total RNA was extracted from 10 *Ae. aegypti* females using TRIzol Reagent (Invitrogen, Carlsbad, CA). A 490 bp cDNA template of the coding region of the *AeCPA-1* gene (VectorBase accession # AAEL010782) was amplified from total RNA by RT-PCR using primers CPA FWD (5'GACTTATGGAAACGAAGCCGCT3') and CPA REV (5'ACAAATATGCCTGGATTTCCTGTTTGG3') and the SuperScript One-Step RT-PCR kit with Platinum Taq (Invitrogen, Carlsbad, CA). The amplicon was cloned into pCR II-TOPO (Invitrogen). cDNA obtained from this cloning step was PCR amplified using primers that contained T7 promoter sequences at their 5' ends (T7-CPA FWD: 5'-TAA TAC GAC TCA CTA TAG GGG ACT TAT GGA AAC G-3' and T7-CPA REV: 5'-TAA TAC GAC TCA CTA TAG GGA CAA ATA TGC CTG G-3'; T7 sequences are underlined). The resulting amplicon was *in vitro* transcribed using the Ambion MEGAscript Kit (Invitrogen) to produce *AeCPA*-derived dsRNA. Seven day-old HWE and PUBB2 P61 females were intrathoracically injected with 1 µl of 1 µg/µl *AeCPA-1* dsRNA each. Controls were either not injected or injected with Phosphate Buffered Saline (PBS). Mosquito midguts were dissected at 7, 12, 24, 36, and 48 h post-injection. Total RNA was extracted from pools of 20 midguts using TRIzol Reagent and used for quantitative RT-PCR (qRT-PCR).

Quantitative RT-PCR

Using the *AeCPA-1*-specific primers CPA qRT FWD (5'CAT ACC GGT GAG CAC GCC TC3') and CPA qRT REV (5'GTG TAG GCA ATC TTT ACG TCC AAA G3'), a 185 bp cDNA fragment of the gene was PCR-amplified from total RNA of *Ae. aegypti*, which differed from the cDNA fragment used as template for dsRNA production. Following pCR II-TOPO cloning of the amplicon, the resulting plasmid was serially diluted and used as the standard curve to calculate transcript copy number equivalents per ng of total RNA. Using the QuantiFast SYBR Green RT-PCR Kit (Qiagen, Valencia, CA), qRT-PCR reactions contained the above primers at 10 µM concentration and 100 ng of total RNA from mosquito midguts. qRT-PCR reactions were conducted in a IQ5 Real-Time PCR Detection system (Bio-Rad, Hercules, CA) according to the following protocol: 10 min at 50°C, 5 min at 95°C and 35 cycles each of 10 s at 95°C and 30 s at 60°C.

Immunofluorescence assay

Midguts of PUBB2 P61 and HWE mosquitoes were dissected and stored in 4% formaldehyde (Vector, Burlingame, CA) for 3 days at 4°C. Fixed midguts were washed in PBS and permeabilized with PBT (PBS with 1% 10X BSA and 2% Triton X-100) for 1 h at room temperature. The v5B2 protein was detected by incubating midguts with the mouse anti-v5 IgG antibody (Invitrogen Carlsbad, CA) at a dilution of 1:200 for 1.5 h at 37°C. Midguts were subsequently incubated with a biotin-labeled anti-mouse secondary antibody

(Sigma-Aldrich, Carlsbad, CA) at a dilution of 1:200 for 1.5h at 37°C. Midguts were incubated with streptavidin-fluorescein at a dilution of 1:100 in PBS before being washed in PBS and mounted onto glass slides.

Immunoblot analysis

Immunoblot analysis was conducted according to Cirimotich *et al.* (2009). Briefly, midguts (n=5) of PUBB2 P61 and HWE mosquitoes were dissected and homogenized in 10 µl of sample buffer [NuPAGE® LDS Sample Buffer with NuPAGE Reducing Agent (Invitrogen, Carlsbad, CA)] using a handheld tissue grinder with pestle. Ten microliters of the total protein (~180 ng/lane) was electrophoretically separated in a 10% SDS-polyacrylamide (SDS-PAGE) gel (Invitrogen) and transferred onto a nitrocellulose membrane at 30 volts for 1 h. Membranes were blocked for 1 h at room temperature in blocking buffer (PBS with 0.05% Tween 20 and 5% nonfat dry milk) and incubated at 4°C overnight with a mouse anti-v5 IgG antibody at a dilution of 1:5,000 in blocking buffer. Membranes were then incubated with a horseradish peroxidase-conjugated goat anti-mouse IgG secondary antibody (KPL Inc., Gaithersburg, MD) diluted 1:1,000 in blocking buffer for 1 h at room temperature. The Vector VIP substrate Kit for Peroxidase (Vector, Burlingame, CA) was used to develop the membrane.

Virus propagation

SINV-TR339eGFP stocks were generated according to Khoo *et al.* (2010). SINV was grown in Vero cells at 0.01 m.o.i and harvested 36–38 h post infection by removing the cell culture medium. DENV2-QR94 is an American genotype DENV2 strain, which was collected in 1994 from a patient in Quintana Roo State, Mexico (Lorono-Pino *et al.*, 2004). Stocks of the virus were generated in *Ae. albopictus* (C6/36) cells by infecting cells at 0.01 m.o.i., changing the cell culture medium 6 days post infection and maintaining the cells until 12 days post infection. Virus was harvested by removing the cell culture medium and scraping off the cells.

Oral virus infection of mosquitoes

Until bloodfeeding, adult mosquitoes were reared on raisins and water. Each 2.5 L carton contained 100 females and five males. Raisins and water were removed from cartons 36 h and 5 h, respectively prior to bloodfeeding. To infect one week-old females with SINV-TR339eGFP or DENV2-QR94, defibrinated sheep blood was mixed at a 1:1 ratio with freshly harvested virus. The SINV titer in bloodmeal was 2.0×10^7 pfu/ml and that of DENV2 was 1.0×10^6 pfu/ml. Female mosquitoes were fed for 1 h with 2 ml of bloodmeal at 37°C using one glass feeder per carton. After bloodfeeding, only those females that were three quarters or fully engorged were selected. These females were reared in 470 ml cartons and fed with sucrose and water until further analysis.

Determination of virus titers by plaque assay

Virus titers from individual midguts and carcasses were determined by plaque assay at 7 and 14 days pbm. SINV-TR339eGFP- and DENV2-QR94-infected samples were homogenized in 0.5 ml 7% FBS-complemented MEM and DMEM medium, respectively. Homogenized samples were filtered with Acrodisc HT Tuffryn 0.2 µm syringe filters (Pall Life Sciences, East Hills, NY). Vero cells (for SINV) or LLC-MK2 cells (for DENV2) were seeded into 24-well plates and left for three days to achieve confluence. Cells were infected with 10-fold serial dilutions of individual midgut or carcass homogenates. Cells were incubated for 1 h at 37°C before overlaid with an agarose nutrient mixture [1x Medium 199 (Sigma-Aldrich, St. Louis, MO), 10% FBS, 4% NaHCO₃, 0.5% MEM vitamins, 0.5% MEM amino acids (Mediatech Inc., Manassas, VA); 1% Hanks-DMEM medium (only used for DENV2)]. For

SINV, plates were incubated at 37°C for 4 days and for DENV2, plates were incubated at 37°C for 12 days. Cells were then stained with MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) (Sigma-Aldrich, St. Louis, MO), incubated at 37°C for 24 h and the number of plaques was counted for each sample. Virus titers of individual mosquitoes were calculated as pfu/ml.

Statistical analysis

Statistical analyses were performed using SAS Statistical Analysis Software (SAS Institute Inc., Cary, NC). The MIXED procedure was used for restricted maximum likelihood parameter estimation with incomplete data followed by the Tukey-Kramer test. SINV-TR339eGFP and DENV2-QR94 infection levels were normalized by log₁₀ transformation. Infection rates were analyzed with the Chi-square test.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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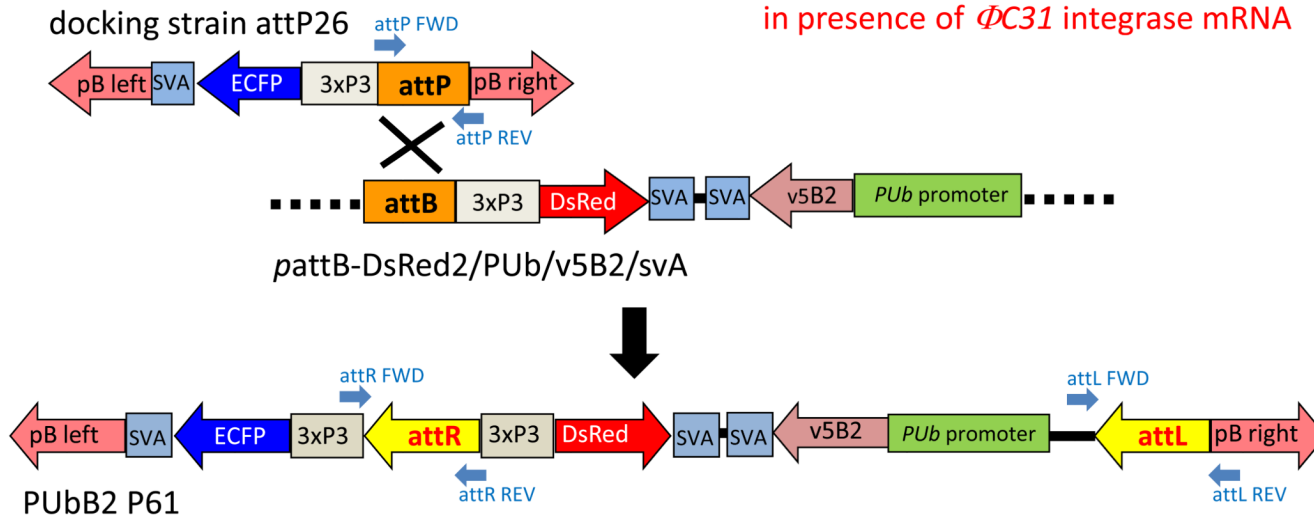
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A



B

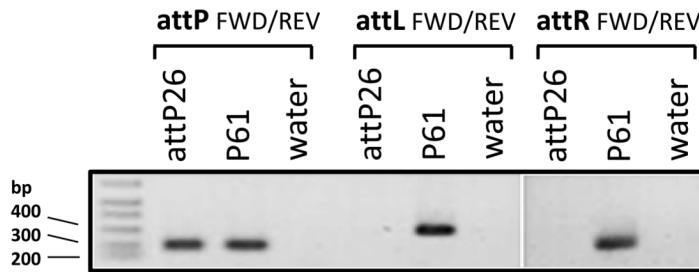
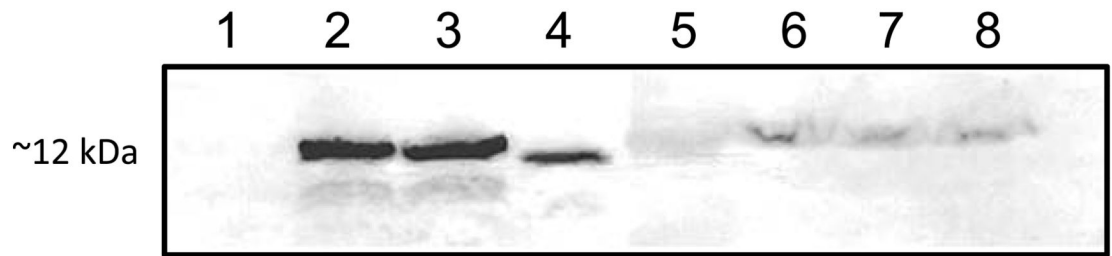
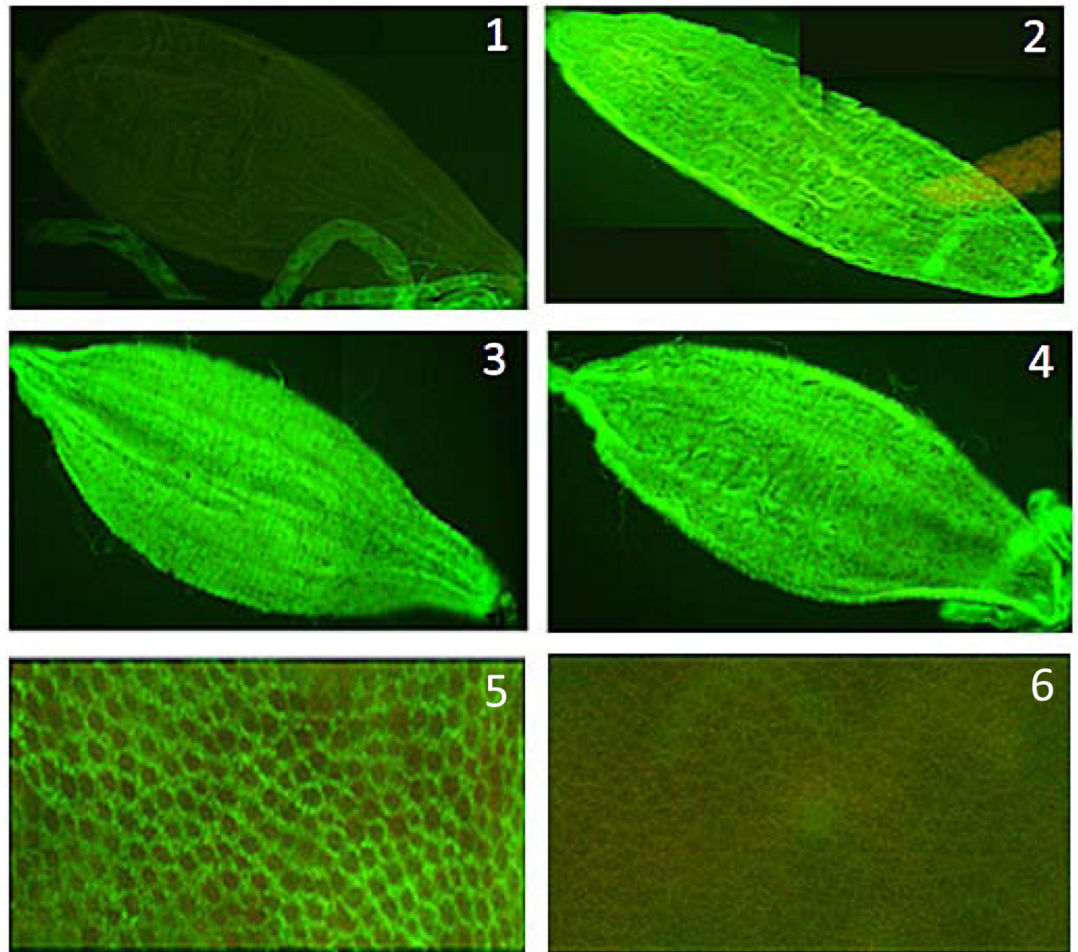


Figure 1. Molecular characterization of $\Phi C31$ -generated recombinants of *Ae. aegypti* over-expressing FHV-B2 from the constitutive *PUB* promoter. (A) Diagram of the recombination event between the *attP* site of docking strain attP26 and the *attB* site of the donor *pattB-DsRed2/PUB/v5B2/svA*. (B) Confirmation of site-directed donor integration in PUBB2 P61 mosquitoes by conventional PCR. Annealing sites of PCR primers are shown in the diagram.

A



B

**Figure 2.**

FHV-B2 over-expression in midguts of transgenic mosquitoes of line PubB2 P61. (A) Western blot analysis of PubB2 P61 females to detect FHV-B2 expression. Total protein extracted from five midguts or carcasses of sugarfed PubB2 P61 and HWE (negative control) mosquitoes was electrophoretically separated in 10% SDS-PAGE. B2 over-expression was detected with an IgG antibody specific for the v5 epitope tag. Total protein of SINV-TE3'2J-B2 infected cell culture medium was used as positive control. **Lane 1:** HWE midguts, 5 days post-eclosion; **lane 2:** PubB2 P61 midguts, 5 days post-eclosion; **lane 3:** PubB2 P61 midguts, 7 days post-eclosion; **lane 4:** SINV-TE3'2J-B2 culture supernatant, 36 h post-infection; **lane 5:** molecular size marker; **lane 6:** PubB2 P61 carcasses, 1 day post-eclosion; **lane 7:** PubB2 P61 carcasses, 5 days post-eclosion; **lane 8:** PubB2 P61

carcasses, 7 days post-eclosion. (B) Detection of FHV-B2 protein in midguts by Immunofluorescence assay using anti-v5 IgG as detection antibody. **(1)** sugarfed HWE (negative control); **(2)** sugarfed PUbB2 P61 female; **(3)** PUbB2 P61 female at 5 days pbm; **(4)** DENV2-QR94 infected female at 5 days pbm; **(5)** close-up image (400×) of midgut epithelium over-expressing FHV-B2 in a sugarfed PUbB2 P61 female at 7 days post-eclosion; **(6)** close-up image (400×) of midgut epithelium of a sugarfed HWE female at 7 days post-eclosion.

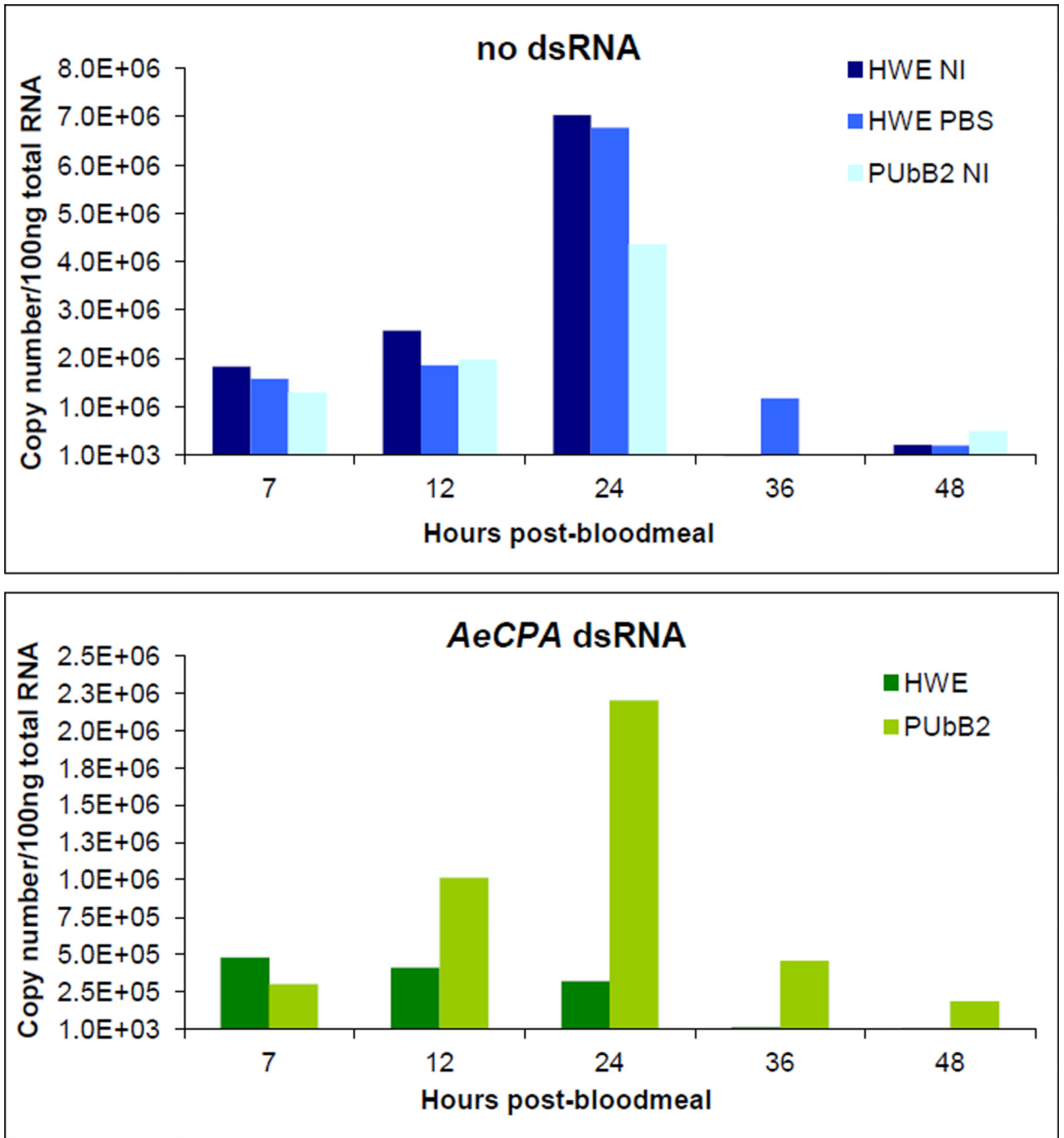


Figure 3. Functional assay to confirm suppression of induced silencing of the endogenous *AeCPA-1* gene in midguts of PUBB2 P61 females. HWE and PUBB2 P61 females were intrathoracically injected with 1 μ l of 1 μ g/ μ l dsRNA targeting *AeCPA-1* or with PBS (control). Twenty-four hours post-injection mosquitoes were given a bloodmeal. Following total RNA extraction from pools of 20 midguts at 7–48 h pbm, *AeCPA-1* transcript copy number equivalents were quantified by qRT-PCR. NI = non-injected.

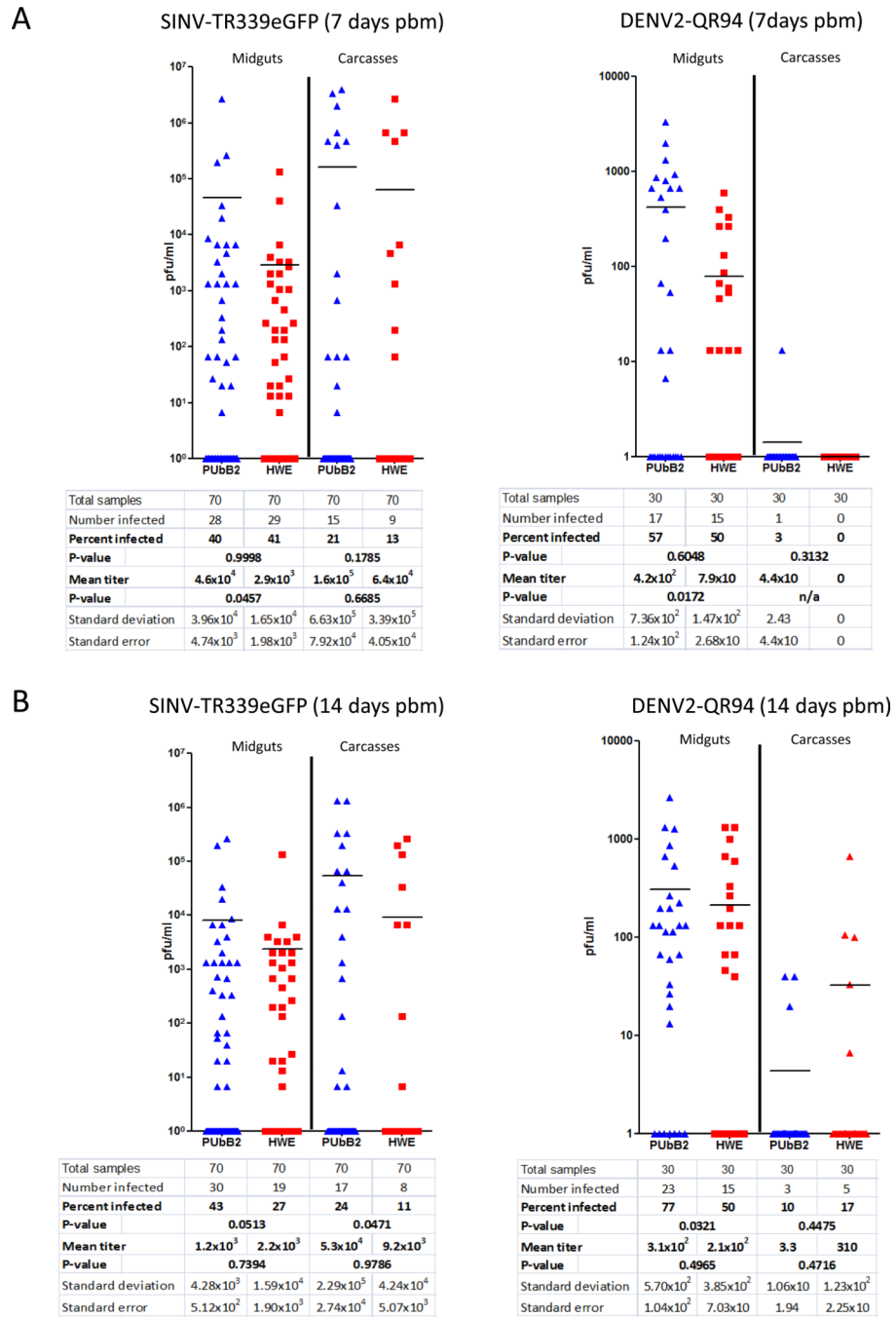


Figure 4. Intensity of SINV-TR339eGFP and DENV2-QR94 infections in PUBB2 P61 and HWE mosquitoes. (A) virus titers and infection rates at 7 days pbm; (B) virus titers and infection rates at 14 days pbm. One-week post-eclosion, females were orally challenged with SINV-TR339eGFP (titer in the bloodmeal: 2.0×10^7 pfu/ml) or DENV2-QR94 (titer in the bloodmeal: 1.0×10^6 pfu/ml). Each data point represents the virus titer (pfu/ml) in an individual midgut or carcass as analyzed by plaque assays. *P*-values ($\alpha=0.05$) for infection rates and mean virus titers are shown in the table.