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Comparison of transgene expression in *Aedes aegypti* generated by *mariner Mos1* transposition and $\Phi C31$ site-directed recombination

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Abstract

Transgenic mosquitoes generated by transposable elements (TE) often poorly express transgenes due to position effects. To avoid these effects, the $\Phi C31$ site-directed recombination system was used to insert transgenes into a locus favorable for gene expression in *Aedes aegypti*. We describe phenotypes of *mariner Mos1* TE and $\Phi C31$ transgenic mosquitoes expressing the EGFP reporter in midguts of bloodfed females. Mosquitoes of nine TE-generated lines (estimated transformation frequency (TF): 9.3%) clearly expressed the eye-specific selection marker but only 2/9 lines robustly expressed the EGFP reporter. The *piggyBac* TE-generated $\Phi C31$ docking strain attP26 supported recombination with *attB* site containing donors at an estimated TF of 1.7–4.9%. Using a codon-optimized $\Phi C31$ integrase mutant instead of the ‘wild-type’ enzyme did not affect TF. Site-directed recombination of line attP26 with an *attB*-containing donor expressing EGFP from the *Ae. aegypti* carboxypeptidase promoter produced one transgenic line with bloodfed females expressing the reporter in midgut tissue. Docking strain attP26 also supported robust expression of Flock House virus B2 from the *Ae. aegypti* poly-ubiquitin promoter. Our data confirm that eye-specific selection marker expression alone is not a reliable indicator for robust gene-of-interest expression in *Ae. aegypti* and that the $\Phi C31$ system can ensure predictable transgene expression in this mosquito species.

Keywords

Aedes aegypti; transposable element; $\Phi C31$; reporter gene; transgenic

Introduction

Genetically-modified mosquitoes are used to characterize gene regulation or innate immunity (Kokoza *et al.*, 2000; 2001; Shin *et al.*, 2003; Adelman *et al.* 2008; Papathanos *et al.*, 2009; Khoo *et al.*, 2010) and for developing vector-based, disease control strategies (Ito *et al.*, 2003; Franz *et al.*, 2006, 2009; Alphey *et al.*, 2008; Catteruccia *et al.*, 2009; Olson and

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Franz, 2009; Mathur *et al.* 2010)). The phenotype of genetically modified mosquitoes is determined by several factors including the regulatory elements that drive transgene expression, the choice and design of the expressed effectors, the transgene insertion site within the genomic DNA and associated fitness costs. The availability of the full-length genome sequence of *Ae. aegypti* allows for the improvement and optimization of transgene design. Researchers can now choose among strong, tissue-specific promoters that allow transgene expression in midgut, fat body, germline tissue, or salivary glands (Edwards, *et al.*, 2000; Kokoza *et al.*, 2000; Moreira *et al.*, 2000; Adelman *et al.*, 2007; Mathur *et al.*, 2010). In addition, two non-tissue specific, constitutive promoters of ubiquitin-encoding genes have been described (Anderson *et al.*, 2010). Fitness costs in mosquitoes vary among different insertions and most likely depend on the transgene design and integration site in the vector genome (Cateruccia *et al.*, 2003; Marrelli *et al.*, 2006; Amenia *et al.*, 2010).

Class II transposable elements (TE) such as *piggyBac* or *mariner Mos1* are used routinely for mosquito germline transformation. However, these TEs integrate quasi-randomly into the host genome because they recognize a short genome motif as a target site (TTAA or TA, respectively; [Jacobson *et al.*, 1986; Wang and Fraser, 1993; O'Brochta *et al.*, 2003]), and these sites are frequent and dispersed throughout the genome. One potential strategy to overcome transgene position effects is the use of chromatin domain insulators such as *scs* and *scs'* derived from of the *Drosophila gypsy* TE or the chicken β -globin 5'HS4 element (Kuhn and Geyer, 2003). Both insulators have been used successfully in *Drosophila* but so far not in mosquitoes (Sarkar *et al.*, 2006). Another approach exploits the site-directed recombination system of bacteriophage $\Phi C31$, in which two short recognition sequences, the phage attachment site *attP* and the bacterial attachment site, *attB*, recombine unidirectionally in the presence of the $\Phi C31$ integrase (Thorpe and Smith, 1998), to place a transgene in a characterized region of the genome. A large exogenous circular DNA molecule carrying the *attB* recognition site can be integrated in the host genome at an *attP* recognition site-containing locus (Groth *et al.*, 2000, 2004; Nimmo *et al.*, 2006; Labbe *et al.*, 2010). Although functional in *Drosophila* and mosquitoes, $\Phi C31$ -based recombination has not yet been widely used in mosquitoes (Nimmo *et al.*, 2006; Amenia *et al.*, 2010; Labbe *et al.*, 2010; Meredith *et al.*, 2011). In contrast to $\Phi C31$, other recombination systems such as Cre/Lox of bacteriophage P1 or FLP/FRT of yeast have not yet shown efficient recombination-mediated insertion of exogenous DNA into mosquitoes (Morris *et al.*, 1991; Jasinskiene *et al.*, 2003; Nimmo *et al.*, 2006).

The aim of this study was to validate the $\Phi C31$ system as a powerful tool to facilitate predictable transgene expression in a target tissue of *Ae. aegypti*. Here we compare phenotypes of transgenic mosquitoes generated by either TE insertion or $\Phi C31$ recombination that express enhanced green fluorescent protein (EGFP) in the female midgut. EGFP expression and transgene integration patterns are described for the transgenic lines. We also show that the $\Phi C31$ system facilitates predictable, robust gene expression of an immunity related gene under control of a constitutive promoter. Furthermore, we evaluate the effects of $\Phi C31$ integrase codon optimization on recombination efficiency.

Results

Generation and characterization of *mariner Mos1* transformed mosquitoes expressing a reporter gene in midguts of bloodfed females

The *mariner Mos1* TE contains an EGFP expression cassette under control of the bloodmeal-inducible, midgut-specific *Ae. aegypti carboxypeptidase A* (*AeCPA*) promoter and an eye-specific screenable marker using the same fluorescent protein (Fig. 1A). We co-injected this donor plasmid along with a helper plasmid expressing the *Mos1* transposase into 1140 Higgs White Eye (HWE) strain preblastoderm embryos (Table 1). When 193 G₀ mosquitoes were

out-crossed to HWE and G₁ progeny larvae were screened for eye-specific expression, we found EGFP positive larvae in nine crosses, Carb/egfp52, 54, 86, 105, 112, 157, 169, 188, and 191. This results in an estimated transformation frequency (TF) of 9.3% with the assumption that the average fertility rate for each male or female individual was 0.5 (Table 1; Adelman *et al.*, 2002). All nine crosses resulted in stable mosquito lines clearly expressing the EGFP selection marker in their eyes. However, ~90–95% of all transgenic (EGFP-positive) larvae in lines Carb/egfp86 and 105 developed into males. The sex-specific inheritance was observed in subsequent generations of Carb/egfp105 until G₆. From G₆ to G₁₉ the ratio of transgenic males to females shifted gradually to 57.7% (males) : 42.3% (females) by G₁₉ (n=324). The ratio between transgenic males and females was balanced (~50:50) from G₂ onwards in all other families. Outcrossing of transgenic heterozygous G₁ mosquitoes of the nine lines to HWE resulted in progeny of which approximately 50% expressed EGFP in the eyes. Female transgenic G₂ mosquitoes of all nine lines were analyzed visually for gene-of-interest expression. Based on the reported expression profile directed by the *AeCPA* promoter (Edwards *et al.*, 2000), EGFP expression in midgut tissue was monitored between 4–96 h post-bloodmeal (pbm). EGFP expression became visible in midguts of females that received a bloodmeal in three lines, Carb/egfp52, 105, and 112 (Fig. 1B; Table 2). In all other lines, reporter gene expression was never observed in the midgut. Carb/egfp105 exhibited the strongest level of EGFP expression among the positive mosquito lines. Reporter gene expression was apparent during the entire observation period, and at 24 h pbm, the entire midgut epithelium was expressing EGFP. EGFP expression was found to be slightly weaker in Carb/egfp52 than in Carb/egfp105, and started to become visible at 8 h pbm. Carb/egfp112 consistently showed only weak reporter gene expression in midgut tissue throughout the time course with only a few isolated cells of the epithelium expressing EGFP after 8 h pbm.

EGFP transcription profiles in midguts of G₄-G₅ Carb/egfp52, 54, 112, 157, 169, 188, 191, and G₁₉ Carb/egfp105 females were evaluated by Northern blot analysis to confirm visual reporter gene expression patterns in midgut tissue of the transgenic lines (Fig. 2). EGFP mRNAs were abundant in the female midguts of Carb/egfp52 and 105, but were low in Carb/egfp112. No signals were seen with RNA prepared from midguts of Carb/egfp 54, 157, 169, 188, and 191 mosquitoes. Midgut-specific EGFP expression was evaluated for line Carb/egfp105 G₁₉ females, since almost all transgenic individuals of this line were males in earlier generations. Carb/egfp52 and 105 females showed strongest transcriptional activity for EGFP in midgut tissue at 24 and 32 h pbm, respectively. EGFP transcripts were abundant until 56 h pbm in midguts of line Carb/egfp52 and 48 h pbm in line Carb/egfp105, although the latter showed far stronger marker gene expression *in situ*.

Southern blot analyses of the transgene integration patterns using *KpnI* digested genomic DNA of Carb/egfp52, 54, 86, 105, and 112 females hybridized to a probe comprising the ~940 bp right arm of the *MosI* TE showed signals corresponding to DNA fragments >1000 bp in length (Fig. 3A). Multiple TE integration events were detected in Carb/egfp105, single insertions in Carb/egfp52, 86, and 112, and no discrete signals were evident in Carb/egfp54. Thus, the stronger reporter gene expression levels in line Carb/egfp105 as compared to Carb/egfp52 could be caused by a dose effect due to high copy number integrations of the transgene.

Generation and characterization of *piggyBac* TE transformed mosquitoes containing the *attP* site for Φ C31-mediated recombination

Eight hundred embryos were co-injected with *pBac[3xP3-ECFPfa]attP* (Fig. 3B; Nimmo *et al.*, 2006) and the *piggyBac* helper plasmid to generate a HWE-based docking strain containing the *attP* landing site. The survival rate was 10%, and after out-crossing to the HWE recipient strain, 33 G₁ families and eight female pools were established. Progeny of

two families, attP26 and attP35, clearly expressed the enhanced cyan fluorescent protein (ECFP) marker in their eyes. Presence of the *attP* site in the genome of the lines was confirmed by gene amplification using primers complementary to the 171 bp *attP* site and flanking cloning sites (Fig. 3B). Both *attP* site-containing docking lines were intercrossed until G₁₄. Thereafter lines attP26 and attP35 were made homozygous. Southern blot analysis revealed two and four possible integration events in lines attP26 and attP35, respectively (Fig. 3C). Differences in signal intensity among the various hybridizing DNA fragments in the Southern blots could be due to independent assortments of the different insertions into the mosquito genome. It is likely that not every individual inherited each copy of the transgene in a homozygous state, resulting in potentially weaker hybridization signals.

We were unable to obtain DNA-hybridization patterns with higher resolution for line attP26 despite several attempts. This prompted us to perform Genome Walking on genomic DNA of this line to confirm the number of transgene integration events. Genome Walking revealed one TE integration event for line attP26 within supercontig 1.20 (contig 1407) at nt position 2,685,285 or 2,685,289. The *piggyBac* TE is inserted within the first intron of a gene, AAEL001004, whose function is unknown. This was the only integration site identified in repeated experiments using multiple restriction endonuclease-digested genomic DNA libraries in the Genome Walking procedure. The *piggyBac* integration pattern is unusual because it did not integrate at a TTAA motif in the host genome. Instead, insertion occurred at a 5'CTAG3' sequence motif (nt position 2,685,285–2,685,289) and upon integration the sequence motif was duplicated (Fig. 3D). This was confirmed in five independent gene amplification, cDNA cloning, and sequencing experiments. Curiously, both inverted terminal repeat (ITR) sequences of *piggyBac* contain the CTAG motif as well (Fig. 3D).

Comparing recombination efficiencies of 'wild-type' or codon-optimized $\Phi C31$ integrases

The $\Phi C31$ integrase mutant P3 (Keravala *et al.*, 2009) was codon-optimized for use in the mosquito *Anopheles stephensi* using the Codon Usage Database (<http://www.kazusa.or.jp/codon/>; Nakamura *et al.*, 2000). Base triplet sequences coding for certain amino acids were mutated as shown in Fig. 4A. Only two of these 10 mutations, AGG or CGA to CGC and ACA or ACT to TCG do not occur at highest frequencies in the *Ae. aegypti* genome. This prompted us to use the *An. stephensi* optimized $\Phi C31$ mutant for the experiments here. Females from the attP26 docking strain were transformed with the donor plasmid *pattB-DsRed2* (Nimmo *et al.*, 2006) using mRNA *in vitro* transcribed from either 'wild-type' or codon-optimized $\Phi C31$ integrase cDNA (Fig. 4B). Thirty percent (30%) of the 297 attP26 embryos injected with *pattB-DsRed2* and 'wild-type' integrase mRNA survived (Table 1). The survivors were grouped in three female pools (4–17 females crossed with 4–15 HWE males per pool) and 13 male pools (4–5 males crossed with 40 HWE females per family). Offspring that showed site-directed recombination with the donor plasmid based on eye-specific ECFP and DsRed expression were observed in one male and one female pool. A total of 557 attP26 embryos were injected with the codon-optimized $\Phi C31$ mRNA. Again, ~30% of the injected eggs survived and resulting adults were combined in five female pools (9–19 females crossed with 9–16 HWE males per pool) and 22 male pools (4–5 males crossed with 50 HWE females per family). Two female and two male pools produced recombinant offspring. Thus, 12.5% and 15% of the pools containing mosquitoes transformed with the 'wild-type' and codon-optimized $\Phi C31$ integrases respectively, had recombination events with the donor plasmid (Fig. 4B). Intensity of DsRed eye marker expression was very similar in all six attP26-attB/DsRed recombinant lines as would be expected after donor integration into the same *attP* site containing docking strain. Furthermore, the estimated TF were essentially the same for the transgenic lines generated with codon-optimized or 'wild-type' integrases, ranging from 4.4% to 4.5% (Table 1). This

indicates that the codon-optimized $\Phi C31$ integrase did not promote higher recombination rates in *Ae. aegypti* than the 'wild-type' enzyme in these experiments. For the following $\Phi C31$ site-directed recombination experiments we used the 'wild-type' integrase.

Generation and characterization of $\Phi C31$ recombinants expressing a reporter gene in midguts of bloodfed females and a viral suppressor of RNA interference

We co-injected 1340 embryos of the homozygous attP26 line with the donor plasmid *pattB-carb/EGFP/svA* and $\Phi C31$ integrase mRNA (Table 1). One hundred and twenty individuals survived and 22 male and five female pools were established. Each male pool contained three males and each female pool was made up of as many as 15 females originating from the injected embryos. One male pool, P14, contained mosquitoes that expressed strongly both ECFP and DsRed in their eyes when viewed under the respective fluorescent filter sets, indicating recombination between the *attP* and *attB* sites of the docking strain and the donor, respectively. Two experiments in which 725 and 1130 embryos of the attP35 docking strain were injected with the same donor plasmid did not result in any recombination-mediated integration (data not shown). This supports the conclusion that the *attP* landing sites in line attP35 are integrated at loci that do not promote sufficient recombination using the $\Phi C31$ system.

Recombinant P14 females expressed EGFP in their midguts between 8–72 h pbm (Table 2, Fig. 5A). The strongest reporter gene expression was observed at 32–56 h pbm, which was similar to the expression pattern of *Carb/egfp52*. In addition, P14 females, similar to *Carb/egfp52*, exhibited weaker EGFP expression in midgut tissue than line *Carb/egfp105*. Northern blot analysis showed abundant EGFP mRNA in P14 midgut tissue at 8–24 h pbm (Fig. 5B). From 24 h pbm onwards, EGFP mRNA levels declined steeply and were only weakly detectable at 48 h pbm. Thus, P14 recombinant females showed slightly weaker transcriptional activity of the EGFP gene in midgut tissue than the TE transformed lines *Carb/egfp52* and *Carb/egfp105* (Fig. 2). Gene amplification analysis using primers surrounding the recombination sites in P14 and subsequent DNA sequencing of the amplicons showed that the *attP* site of docking strain attP26 and the *attB* site of the donor plasmid had been recombined to become *attL* and *attR* sites (Fig. 5C). The $\Phi C31$ recombinants showed relatively balanced sex ratios similar to G₁₉ *Carb/egfp105* mosquitoes: 46.1 % females : 53.9% males (n=1786).

In another experiment we generated attP26 based $\Phi C31$ recombinants expressing the Flock House virus B2 protein, a viral suppressor of RNA interference, from the non-tissue specific, constitutive *Ae. aegypti* poly-ubiquitin (*PUB*) promoter (Fig. 6A; [Li *et al.*, 2002; Anderson *et al.*, 2010]). We obtained one pool, P61, in which larvae expressed both fluorescent eye markers, ECFP and DsRed originating from docking strain attP26 and the donor plasmid, respectively. Northern blot analysis showed the presence of ν_5 B2 transcripts in larvae, males, sugarfed, and bloodfed females of P61 mosquitoes (Fig. 6B). Thus, docking strain attP26 repeatedly facilitated predictable, robust gene-of-interest expression following recombination with two different donor plasmids. The ν_5 B2 expressing P61 mosquitoes will be further characterized and extensively analyzed in vector competence studies using a range of arboviruses. This work will be presented elsewhere.

Discussion

We show here that although several transformed lines of *Ae. aegypti* can be routinely obtained from a single experiment when using *mariner Mos1* as an insertion vector, only a fraction of them express the transgene in a manner consistent with the expression profile characteristic of the endogenous gene from which the regulatory DNA was derived. Only two of nine *mariner Mos1* generated transgenic lines, *Carb/egfp52* and *Carb/egfp105*,

expressed strongly the EGFP reporter gene in female midguts after induction by a bloodmeal. In Carb/egfp105 mosquitoes, the TE appeared to be integrated into the genome in high copy numbers, which resulted in a smear pattern in Southern blot analysis. A mass integration event into the genome of *Ae. aegypti* has been described before for the *piggyBac* TE but not for *mariner Mos1* (Adelman *et al.*, 2004). Whereas the *piggyBac* mass integration resulted in a highly unstable transgene expression pattern, the gene-of-interest was stably expressed in line Carb/egfp105 for at least 19 generations. The quasi-random integration patterns of *mariner Mos1* and *piggyBac* often lead to integrations at loci that exhibit position effects (Nimmo *et al.*, 2006; Labbe *et al.*, 2010). These position effects are caused in part by integration in heterochromatic regions of the genome or nearby repressors and/or enhancers of endogenous genes (Wallrath and Elgin, 1995; Sarkar *et al.*, 2006; Anderson *et al.*, 2010). However, expression of the eye-specific selection marker appears to be relatively robust despite position effects, since the marker was clearly visible among all nine transformants. Thus, the selection marker cannot be seen as a reliable indicator to predict gene-of-interest expression levels in transformed *Ae. aegypti*.

We tested the $\Phi C31$ site-directed recombination system as a strategy to overcome transgene position effects. An essential component of this system is an *attP* site-containing docking strain, which has the landing site integrated at a region of the genome that is permissive for strong gene-of-interest expression. Of two such lines established, attP26 and attP35, $\Phi C31$ -mediated recombinants could only be recovered from the former. This is notable because the Southern analysis showed at least four integrations of the docking site DNA in attP35. Apparently, none of these integrations was in a locus favorable for site-directed recombination. In contrast, at least one of the two sites in attP26 accepted integrase-mediated insertions at rates detectable with the number of embryos we injected. Comparisons of EGFP expression in Carb/egfp52 and 105 with attP26-attB/DsRed-Carb/egfp (P14) recombinants showed slightly weaker gene-of-interest expression levels for the latter. Position of the *attP* site within the mosquito genome and differences in insert copy number could account for these observations. Nevertheless, $\Phi C31$ -mediated recombinants based on line attP26 repeatedly facilitated robust gene-of-interest expression. This was shown by the abundance of ν_5B2 mRNA among samples of attP26-attB/DsRed-PUB/ ν_5B2 (P61) mosquitoes, which express the viral suppressor of RNA interference, B2, from the constitutive *PUB* promoter. Taken together this study supports the argument that any *attP* docking strain generated for the $\Phi C31$ system needs to be evaluated empirically for gene-of-interest expression after recombination. Similar to the observation with TE generated transformants, selection marker expression alone is not a reliable indicator for the gene-of-interest expression potential of $\Phi C31$ docking strains.

Several reports indicate that the use of the $\Phi C31$ system instead of a TE-based transformation system leads to substantially higher TF in *Drosophila* and mosquitoes (Groth *et al.*, 2004; Nimmo *et al.*, 2006). This study does not support these observations. Estimated transformation frequencies were similar or higher when using TEs instead of $\Phi C31$. One reason for this could be that the *Mos1* or *piggyBac* transposases in these experiments were generated from a helper plasmid whereas the $\Phi C31$ integrase was supplied as an *in vitro*-transcribed mRNA. Attempts to supply the integrase as a plasmid under control of the hsp70 promoter did not result in successful recombination events when co-injecting the attB/DsRed-carb/EGFP/svA donor plasmid into embryos (A.W.E. Franz, M.R. Smith, K.E. Olson, unpublished results). We attempted to increase recombination efficiency and specificity for the $\Phi C31$ system by optimizing the integrase coding sequence for expression in mosquitoes (Grosjean and Fiers, 1982). Since the natural environment for $\Phi C31$ integrase expression is the bacterium *Streptomyces ambifaciens*, the $\Phi C31$ recombinase-encoding sequence is optimized for translation by the tRNA spectrum of the bacterium, which differs from that present in mosquitoes. Codon-optimization has been shown previously to boost

gene expression in a specific organism by enabling the cell to use its major tRNAs for translation (Fletcher *et al.*, 2007; Liu, 2009). Codon-optimization was performed with the P3 mutant of $\Phi C31$, which arose from an unexpected recombination event (Keravala *et al.*, 2009). Mutant P3 contained nine engineered amino acid substitutions and an additional 33 amino acid sequence at the N-terminal portion of the protein. As a result, mutant P3 in human cells showed a 2.3-fold higher integration efficiency and a 7-fold higher specificity than the 'wild-type' integrase (Keravala *et al.*, 2009). When comparing the estimated recombination frequencies of the mosquito codon-optimized P3 mutant with that of the 'wild-type' integrase in *Ae. aegypti*, we did not observe a significant difference between the two enzyme variants. However, the codon-optimized P3 mutant has been shown to be functional for mosquito transgenesis, even though it might not substantially increase the efficiency of the $\Phi C31$ system in mosquitoes as we expected. More experiments need to be conducted to confirm this observation.

In early intercrossed generations (up to G₆) mosquitoes of line Carb/egfp105 exhibited a distorted sex ratio with ~ 95% of the insects being males. This sex ratio bias declined in subsequent generations and among G₁₉ mosquitoes the ratio was relatively balanced. Sex ratio distortions among transgenic mosquitoes have been reported previously (Irvin *et al.*, 2004). The most likely explanation for this observation is that it is a consequence of the genetics of sex-determination in *Ae. aegypti*. Sex in these mosquitoes is determined by a single autosomal gene on chromosome 1 with maleness being the dominant allele (McClelland, 1962). Males represent heterozygotes (*Mm*) at the sex locus, while females represent the homozygous, recessive (*mm*) condition. Integration of the transgene close to the *M* locus would link it to the male-determining factor. Meiotic recombination in males during inbreeding would move the transgene to the *m*-bearing chromosome and eventually increase the number of females carrying the transgene. Consistent with this hypothesis, we did not observe high mortalities among larvae, pupae or females of line Carb/egfp105 in early or late generations that might be indicative of a male-drive phenomenon. Despite repeated efforts we were unable to physically map the (multiple) TE integration sites in line Carb/egfp105, which would have been important to confirm linkage to the *M* locus.

Physical mapping of the *piggyBac* integration in line attP26 revealed that the TE did not integrate at a TTAA site in the genome as would be expected, but rather used the CTAG motif, which was duplicated upon TE integration. This unexpected result was confirmed by gene amplification using genome-specific primers in combination with primers complementary to the *piggyBac* left and right arms. The genomic sequence of HWE contains the CTAG motif at nt position 2,685,285, whereas the *Ae. aegypti* (strain: Liverpool) genome sequence in VectorBase shows the presence of a CTAA motif at the identical locus. The genomic sequences flanking the insertion site match the first intron of the predicted gene, AAEL001004. Almost all *piggyBac* integration patterns reported to date show insertion at the typical TTAA motif, which appears to be absent in the nearby region of genomic DNA in line attP26 where the TE was integrated. Since there is only one example in the literature for an atypical *piggyBac* integration at an ATAA site instead of a TTAA site, it is difficult to speculate about our observation (Elick *et al.*, 1996). However, it seems likely that the *piggyBac* transposase was able to utilize the genomic CTAG motif for TE integration.

This work shows potential advantages and disadvantages when using TE-based transformation or $\Phi C31$ -mediated recombination to generate gene-of-interest expressing *Ae. aegypti*. TE based transformation using a transposase expressing helper plasmid is technically simple and appears to result in higher TF compared to $\Phi C31$ -mediated recombination. However, position effects and variable insert copy number often lead to a range of different transgenic phenotypes that need to be analyzed before the optimal

phenotype can be chosen. Using the $\Phi C31$ system requires more effort in terms of preparing and supplying the integrase source. However, our study shows that we have identified an *attP* site-containing docking strain, attP26, that allows strong gene-of-interest expression following $\Phi C31$ mediated recombination with a donor. Furthermore, line attP26 is based on the well characterized, arbovirus competent HWE recipient strain (Franz *et al.*, 2006; Sanchez-Vargas *et al.*, 2009; Khoo *et al.*, 2010). This enables us to generate and use $\Phi C31$ recombinants for studies aimed at investigating mosquito-arbovirus interactions.

Experimental procedures

Construction of the donor plasmids

The EGFP expression cassette was constructed by inserting a cDNA fragment containing the EGFP coding sequence and the SV40A transcription termination signal (originating from pEGFP, Clontech, CA) into the *NotI/XbaI* digested p*SLfa1180fa* shuttle vector (Horn *et al.*, 2000). The 1120 bp *AeCPA* promoter cDNA was inserted into the *SacI* and *NotI* digested p*SLfa1180fa-EGFP/svA*. Finally, the *AeCPA/EGFP/svA* cassette was transferred from p*SLfa1180fa* into p*Mos1* or *pattB-DsRed2* using the *AscI* sites. An additional cloning site containing *FseI*, *AscI*, and *HindIII* restriction endonuclease sites was inserted into the *SpeI* and *NotI* sites of the original $\Phi C31$ donor plasmid *pattB-DsRed2* (Nimmo *et al.*, 2006).

mariner Mos1-based germline transformation of *Ae. aegypti* and establishment of transgenic mosquito lines

The eye-pigment-deficient Higgs White Eye strain (HWE), a variant of the *Ae. aegypti* Rexville D strain was used for germline transformation (Wendell *et al.*, 2000). Mosquitoes were reared at 28°C, 82% humidity under a 12 h darkness / 12 h light regime. Adults were maintained on sucrose. Germline transformations using the *mariner MosI* TE were performed as previously described (Jasinskiene *et al.*, 1998, 2007; Franz *et al.* 2006; Khoo *et al.*, 2010). Each surviving G₀ male was outcrossed to 20 female HWE. Five female G₀ mosquitoes were pooled and outcrossed to one HWE male. To produce offspring, females received bloodmeals from mice according to Institutional Animal Care and Use Committee (IACUC) protocol 09-1365A. Progeny larvae of these crosses (G₁) were screened for EGFP expression in their eyes using a fluorescence microscope (Olympus SZX12 Zoom) equipped with an EGFP-specific filter set. Transgenic G₁ mosquitoes were outcrossed to the HWE recipient strain and their progeny (G₂) were analyzed for gene-of-interest expression.

Generation of *attP* site containing docking strains and $\Phi C31$ based site-directed recombination

We used plasmid p*Bac[3xP3-ECFPfa]attP* encoding the left and right arms of the *piggyBac* TE, the *attP* recombination site and the eye tissue-specific ECFP selection marker to generate $\Phi C31$ docking strains (Nimmo *et al.*, 2006). Pre-blastoderm embryos of the HWE strain were co-injected with *piggyBac* plasmid DNA and a helper plasmid encoding the transposase under control of the *Drosophila* hsp70 heat shock promoter. Transgenic mosquitoes were identified and lines established as described above. The *attP* recombination site was detected in the transgenic mosquito lines by PCR using primers attP FWD: 5'caaatgttctgtgatgacctg3' and attP REV: 5'ctccccttgactactgacattatgg3'. Eventually, *attP* site containing lines were made homozygous by establishing sibling crosses between transgenic G₁₄ individuals over two generations.

For site-directed recombination the coding sequence of the $\Phi C31$ integrase was transcribed *in vitro* from plasmid p*ET11C31poly(A)* (Nimmo *et al.*, 2006) using the mMessage mMachine T7 Ultra Kit (Applied Biosystems). mRNA was purified using the MegaClear Kit (Applied Biosystems) followed by ethanol precipitation. Integrase mRNA and *attB-DsRed2*

donor plasmids were co-injected into homozygous embryos of the *attP* site containing docking strains at final concentrations of 960 ng/μl and 450 ng/μl, respectively.

Mutagenesis of the Φ C31 integrase gene

The 1903 bp coding sequence of the 'wild-type' Φ C31 integrase gene was codon-optimized for *An. stephensi* by commercial cDNA synthesis (Epoch Biolabs, Missouri City, TX) following the algorithm shown in Fig. 4A. Another 339 bp fragment representing the 5' portion of the cDNA of Φ C31 mutant P3 (Keravala *et al.*, 2009) was similarly codon-optimized. Using restriction sites *NdeI* and *BstBI*, a 243 bp fragment of the codon-optimized 'wild-type' Φ C31 was then replaced with the 339 bp fragment to generate codon-optimized mutant P3.

Northern blot analysis

Total RNA was extracted from pools of 25 midguts of bloodfed and sugarfed females using TRIzol reagent (Invitrogen, Carlsberg, CA). Approximately 15 μg of each RNA sample was electrophoresed in a 1.2 % agarose gel containing 1× MOPS and 0.66 M formaldehyde and blotted overnight onto a positively charged nylon membrane (Ambion). Using the T7 Maxiscript Kit (Ambion), a ~540 nt antisense RNA probe was transcribed *in vitro* from a plasmid containing the EGFP coding sequence. The probe was labeled with 20 mCi of [alpha-³²P]UTP/ml with an activity of 800 Ci/mmol. Alternatively, ³²P-dCTP labeled cDNA probes (3000 ci/mmol) containing a 500 bp fragment of the EGFP gene or a 368 bp fragment including the coding sequence of the Flock House virus B2 gene and the v5 epitope tag of Simian virus 5 were generated using the DECAprime II Kit (Ambion). Blots were hybridized at 58°C for ~16 h each with 20 μl ³²P-labeled probe in Ultrahyb hybridization buffer (Ambion). Hybridized blots then were washed twice for 20 min with 2× SSC, 0.1% SDS and twice for 20 min with 0.2× SSC, 0.1% SDS at 65°C. Phosphor storage screens were exposed to the hybridized blots for 20 min up to 3 days and then scanned on a Storm Phosphorimager (Molecular Dynamics Inc.).

Total DNA extraction and Southern blot analysis

For detection of the *mariner Mos1* TE, total DNA was extracted from pools of 10 whole-body female mosquitoes using the procedure described by Franz *et al.* (2006). Extracted DNAs were digested overnight with *KpnI*. DNAs were precipitated in 100 % ethanol and the resulting pellets resuspended in water. Seven to 15 μg digested DNA were electrophoresed in a 0.8 % agarose gel. The gel was denatured, neutralized and blotted onto a positively-charged nylon membrane using to standard procedures (Sambrook *et al.*, 2001). A DNA probe complementary to the right arm of the *mariner Mos1* TE was generated by random-prime labeling with 20 mCi of [alpha-³²P]CTP/ml (3000 Ci/mmol activity) using the Megaprime labeling kit (Amersham). Blots were hybridized with the probe at 45 or 48°C and washed at 50 or 55°C before being exposed to phosphor storage screens and scanned as described above. To detect the *piggyBac* TE in docking strains attP26 and 35, total DNA was extracted from three females of each line and digested overnight with *EcoRV*. The 2,200 bp [alpha-³²P]CTP labeled probe for hybridization was complementary to the left arm/3×P3/ECFP encoding region of the *piggyBac* TE. Hybridizations were carried out overnight at 48°C.

Genome walking and genotypic characterization of transgenic mosquitoes by gene amplification

Genome walking was performed to physically map the transgene integration locus in docking strain attP26. According to the protocol of the Genome Walker Universal Kit (BD Biosciences Palo Alto, CA), total mosquito DNA was digested with *DraI*, *EcoRV*, *PvuII*, or

StuI, followed by ligation to an adaptor molecule provided with the kit. For detection of the *piggyBac* TE integration site by PCR the following *piggyBac* specific primers were used in combination with the adaptor primers of the kit: Pleftarm REV: 5'gactcacgcggtcgttatagttcaaatcagt3', Pleftarm nested REV: 5'gacaagcagcctcagccgagctccaagcggegcac3', PBrightarm FWD: 5'acgtactactgtactactgcccctct3', PBrightarm nested FWD: 5'gtcagagacataatattgatatgtccaagt3'. Gene amplifications were performed with Advantage 2 polymerase (Clontech, CA). Resulting amplicons were cloned into the pCR4-TOPO TA cloning vector (Invitrogen, Carlsbad, CA) and then sequenced.

Transgene integration in line attP26 was confirmed from genomic DNA by gene amplification using the primer combinations contig1.2FWD: 5'ttccgtagatcttcattgaaggtttag3', PleftarmREV and PBrightarmFWD, contig1.2REV: 5'ctgcaaatcttccagagattgtccag3'. To amplify the corresponding genomic DNA region from HWE mosquitoes, primer combinations contig1.2FWD and contig1.2REV were used. Site-directed recombination of the *pattB-DsRed2-AeCPA/EGFP/svA* donor plasmid with the *attP* site of line attP26 was confirmed using primer combinations attlFWD: 5'gaggtcagcatgatgtaggtcac3', attlREV: 5'acctttctccttgctactgac3' and attrFWD: 5'tcaactaaggcggagtgg3', attrREV: gatgggtgaggtggagtacg3' (Nimmo *et al.*, 2006).

Acknowledgments

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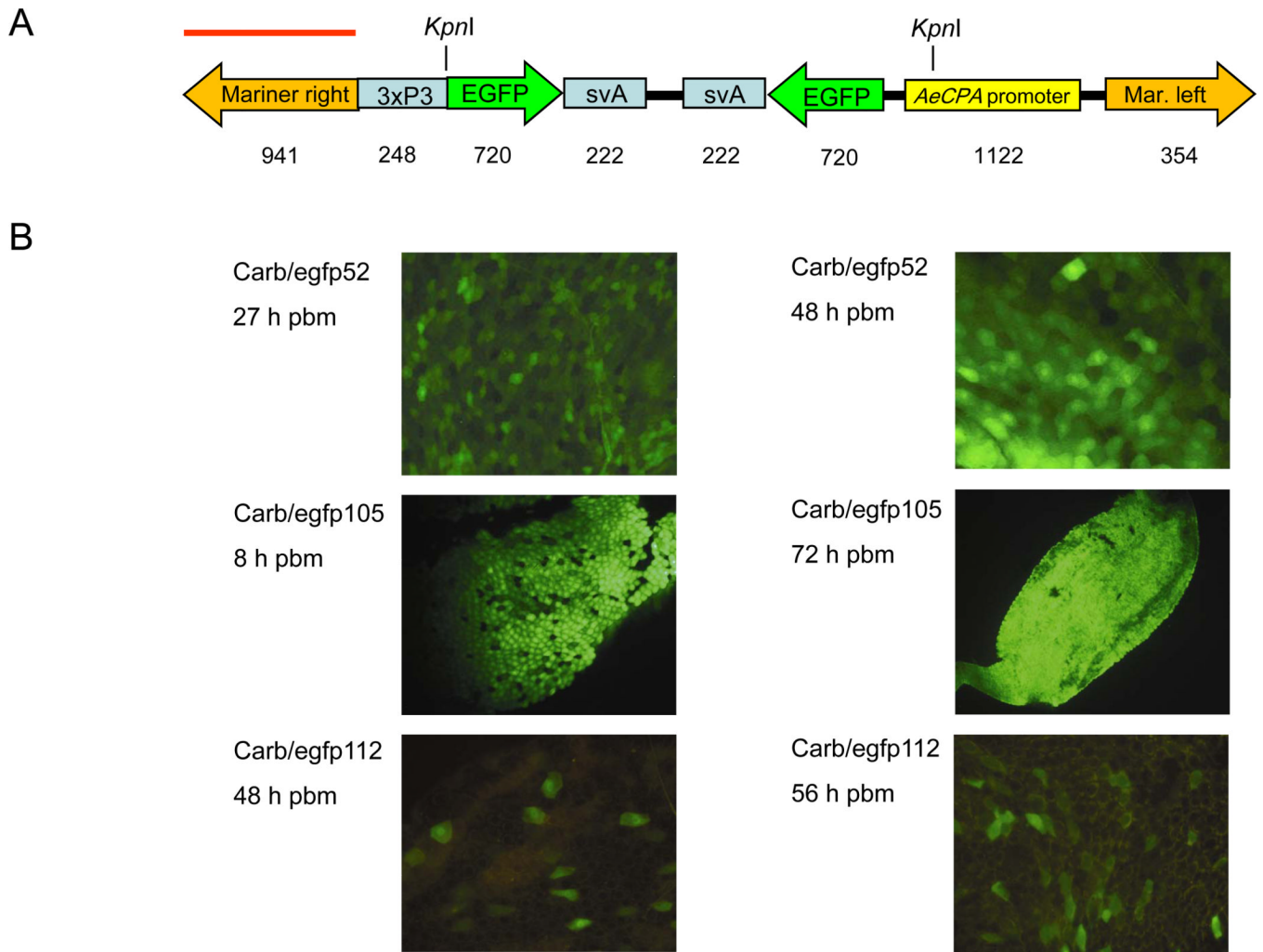


Figure 1. Midgut-specific reporter gene expression in *mariner Mos1* transformed *Aedes aegypti*. (A). Diagram of the *mariner Mos1* TE based transgene *pMos-carb/EGFP/svA*. Locations of *KpnI* sites and probe (red bar) for Southern analysis are indicated. Numbers below the diagram indicate DNA fragment sizes in base pairs (bp). (B). EGFP expression in female midguts of the *pMos-carb/EGFP/svA* transformed lines Carb/egfp52, 105, and 112 at 8–72 h post-bloodmeal (pbm). EGFP was viewed under a fluorescent microscope equipped with EGFP specific filter sets.

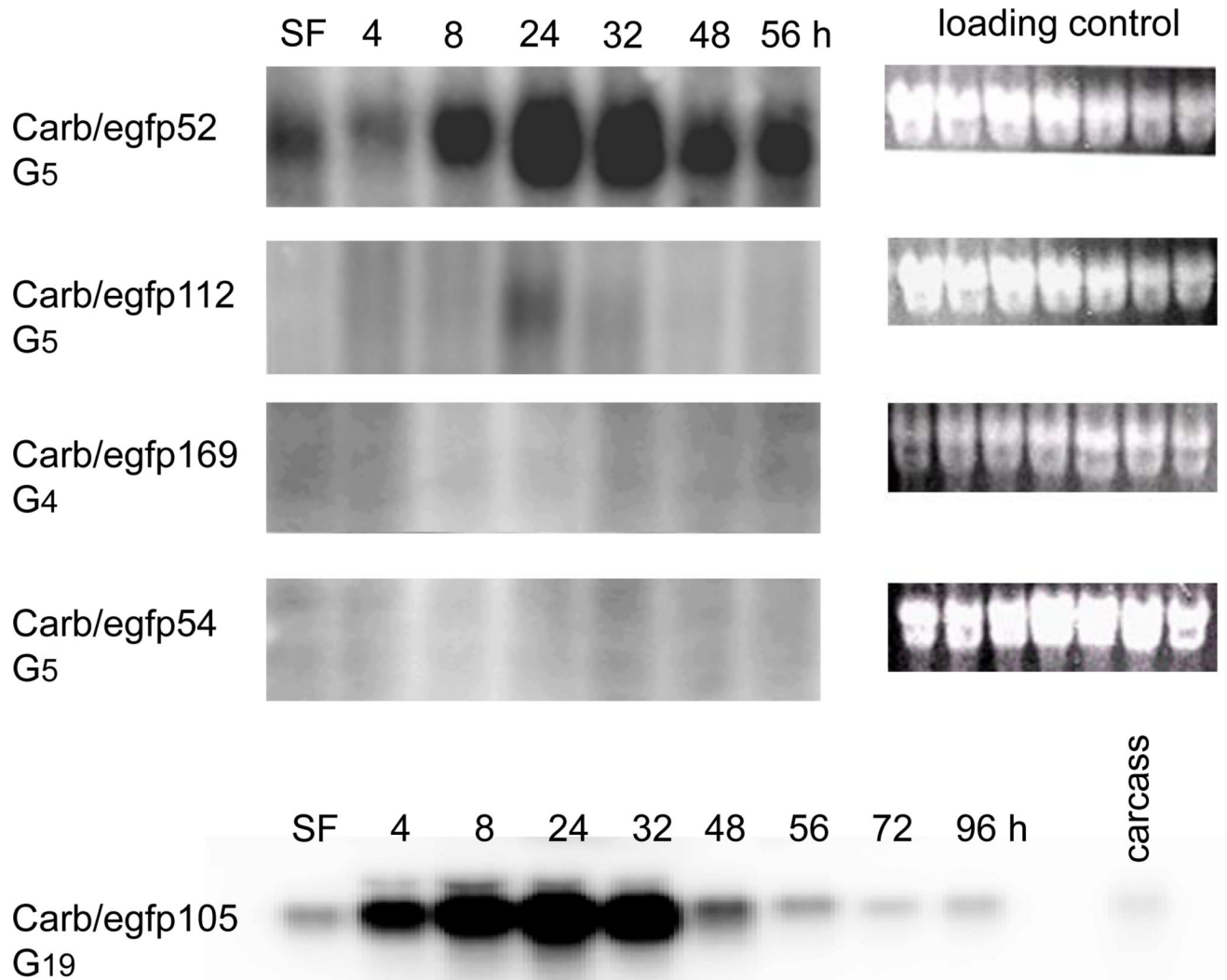


Figure 2.

Northern blot analyses of EGFP transcription in female midguts of the *mariner Mos1* transformed lines (G₄₋₅) Carb/egfp52, 112, 169, 54, and (G₁₉) 105. Samples were prepared from midguts harvested after sugarfeeding (SF) or 4–96 h pbm. The 10 carcasses of Carb/egfp105 mosquitoes were without heads. The hybridization pattern of Carb/egfp157, 188, and 191 (not shown) resembled those of Carb/egfp54 and 169. Total RNA was extracted from pools of 25 midguts per time point. Blots were hybridized with random primed ³²P-dCTP-labeled DNA probes or *in vitro*-transcribed ³²P-dUTP-labeled probes corresponding to the EGFP gene at 48°C. Ethidium bromide stained agarose gels are shown as loading controls.

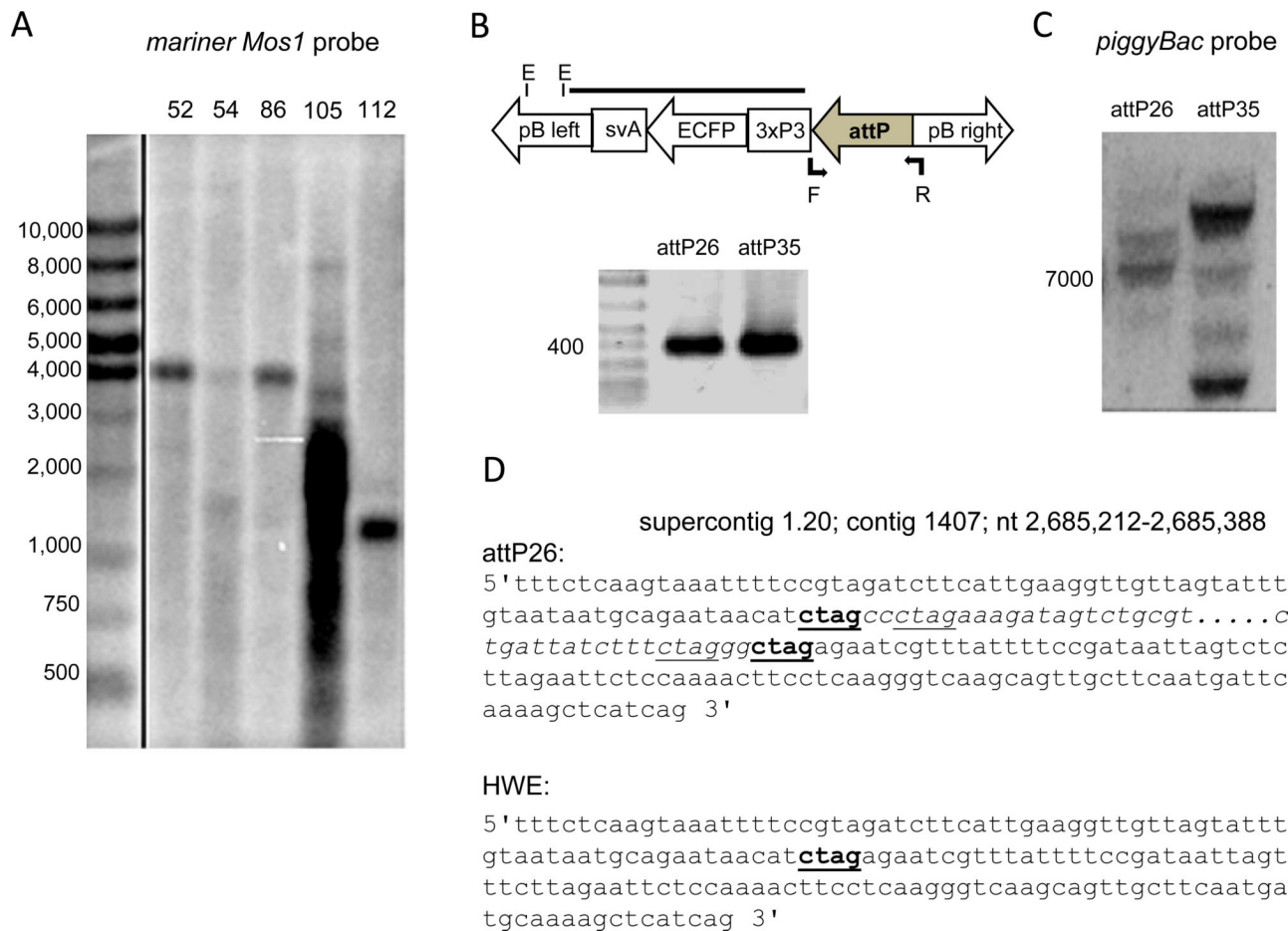


Figure 3.

Genotypic analysis of transgenic mosquito lines. (A). Southern blot analysis of *mariner Mos1* transformed lines Carb/egfp52, 54, 86, 105, and 112. Total DNA was extracted from 10 females per line, followed by digestion with *KpnI*. Blots were hybridized with a random-primed ^{32}P -dCTP-labeled DNA probe corresponding to the right arm of the *mariner Mos1* TE at 48°C. (B). Detection of the *attP* site-specific recombination site in transgenic mosquitoes of lines attP26 and attP35. Total DNA of five females was extracted and used as template for gene amplification using primers corresponding to the 171 bp *attP* site and 220 bp flanking cloning sites of plasmid *Bac[3xP3-ECFPfa]attP* shown above as a diagram (Nimmo *et al.*, 2006). (C). Southern blot analysis of *piggyBac* transformed lines attP26 and attP35. Total DNA was extracted from 3 females per line, followed by digestion with *EcoRV*. Blots were hybridized with a random primed ^{32}P -dCTP labeled DNA probe corresponding to the left arm/3xP3/ECFP encoding region of the *piggyBac* TE at 48°C. (D). Partial sequence (nt position 2,685,212–2,685,388) of the *Ae. aegypti* genomic DNA of supercontig 1.2, contig 1407. Highlighted in blue: partial sequence of *piggyBac* left arm; highlighted in green: partial sequence of *piggyBac* right arm. The CTAG sequence motif is underlined and/or in bold.

A

An. stephensi codon optimization changes:

Gly	GGG to GGT
Val	GTA to GTG
Arg	AGG and CGA to CGC
Ser	TCA and TCT to TCG
Ile	ATA to ATC
Thr	ACA and ACT to ACG
Cys	TGT to TGC
Tyr	TAT to TAC
Leu	CTT, CTA, and TTA to CTG
Pro	CCT to CCG

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B

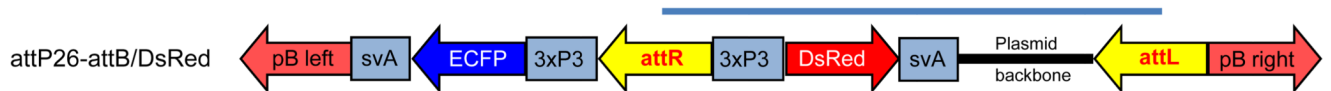
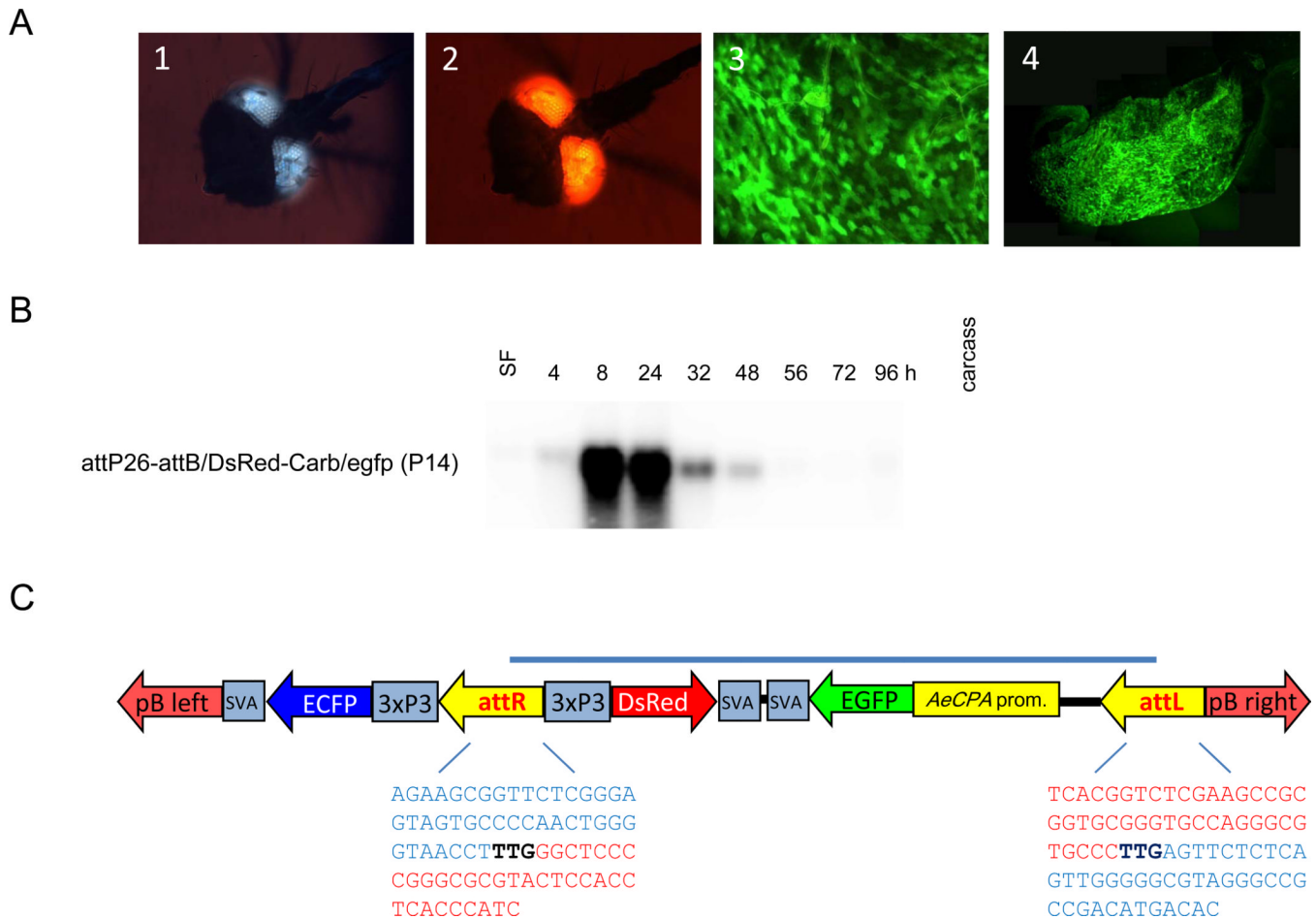
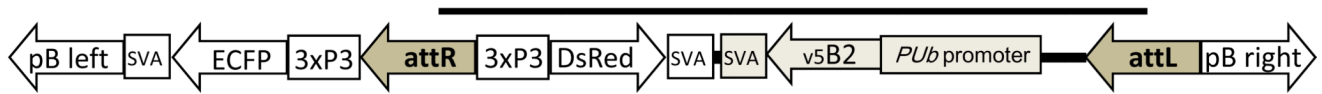


Figure 4. Codon optimization of the $\Phi C31$ integrase and $\Phi C31$ -mediated recombination of line attP26 with the *attB/DsRed2* donor plasmid. (A). Codon optimization was performed on the DNA sequence of $\Phi C31$ integrase P3 mutant (Keravala *et al.*, 2009), which contained an additional 33 amino acid sequence upstream of the ‘wild-type’ $\Phi C31$ start codon. The DNA sequence of the P3 mutant was codon optimized according to the most frequent codon usage in the *An. stephensi* genome, which is similar to that of *Ae. aegypti*. (B). Diagram of the *attB/DsRed2* donor plasmid integration into the genome of docking strain attP26 following recombination between *attB* and *attP* in presence of $\Phi C31$ (‘wild-type’) or codon optimized $\Phi C31$ P3 mutant integrases, respectively. As a consequence of recombination, *attP* and *attB* sites are converted into *attL* and *attR*. Grey bar indicates the part of the transgene that originates from the donor plasmid.

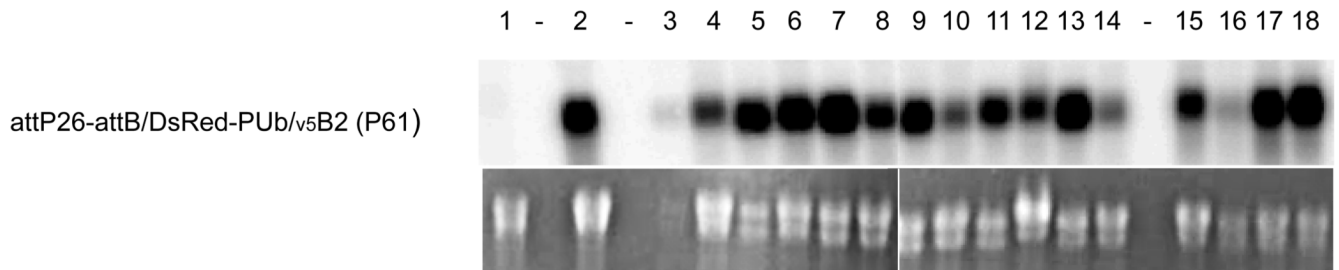
**Figure 5.**

ΦC31-mediated recombination of docking strain attP26 with the *attB/DsRed2-carb/EGFP/svA* donor plasmid. (A). Eye-specific marker gene expression in line P14. (1) ECFP expression originates from the selection marker of line attP26; (2) DsRed expression originates from the donor plasmid. (3) Midgut-specific EGFP expression represents the gene-of-interest of the donor and was visible at 48 h post-bloodmeal (pbm) in individual epithelial cells of (4) the entire midgut containing a bloodmeal. (B). Northern blot analysis for the detection of EGFP transcripts in female midguts of attP26-attB/DsRed-carb/egfp (P14) mosquitoes after sugarfeeding (SF) or 4–96 h pbm. Total RNA was extracted from 25 midguts per time point or 10 headless carcasses. Blots were hybridized with a random primed ³²P-dCTP labeled DNA probe corresponding to the EGFP gene at 48°C. (C). Diagram of the donor plasmid integration pattern following recombination between the *attP* site of line attP26 and the *attB* site of the donor. *attP* and *attB* sites are converted into *attL* and *attR* consisting of DNA sequence originating from *attP* (blue) and *attB* (red). In bold and black: crossover recognition motif. *attL* and *attR* sequences were confirmed by gene amplification and DNA sequencing of the amplicons. Grey bar indicates the part of the transgene that originates from the donor plasmid.

A



B

**Figure 6.**

$\Phi C31$ -mediated recombination of line attP26 with the *attB/DsRed2-PUB/v5B2/svA* donor plasmid to constitutively express the Flock House virus B2 suppressor of RNA interference in *Ae. aegypti*. (A) Diagram of the donor plasmid integration pattern following recombination between the *attP* site of line attP26 and the *attB* site of the donor. Grey bar indicates the part of the transgene that originates from the donor plasmid. (B) Northern blot showing v_5B2 expression over time in various tissues of recombinant P61 mosquitoes. Lanes: (1) docking strain attP26 (negative control); (2) P61 larvae; (3) P61 females - midguts, 1 day post-emergence (pe); (4) carcasses, 1 day pe; (5) midguts, 3 days pe; (6) carcasses, 3 days pe; (7) midguts, 5 days pe; (8) carcasses, 5 days pe; (9) midguts, 7 days pe; (10) carcasses, 7 days pe; (11) midguts of bloodfed females, 7 days post-bloodmeal (pbm); (12) carcasses of bloodfed females, 7 days pbm; (13) midguts of bloodfed females, 14 days pbm; (14) carcasses of bloodfed females, 14 days pbm; (15) P61 males - 1 day pe; (16) 3 days pe; (17) 5 day pe; (18) 7 days pe.

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Table 1

Transformation data of *Aedes aegypti* lines (HWE strain) using *mariner Mos1* or *piggyBac* TEs as insertion vectors or $\Phi C31$ for site-directed recombination

	Mos1-Carb/egfp	attP26-attB, 'WT' $\Phi C31$	attP26-attB, 'mut.' $\Phi C31$	piggyBac- attP site	attP26- attB/Carb/egfp
No. of embryos injected	1140	297	557	800	1340
No. of G ₀ survivors	193	89	181	82	120
No. of G ₁ families (f) or pools (p) ¹	116f	13f, 3p	22f, 5p	41f	27p
No. of G ₂ transgenic families or pools	9f	1f, 1p	2f, 2p	2f	1p
estimated TF or RF ²	9.3 %	4.5 %	4.4 %	4.9 %	1.7 %

¹ family: 1 transgenic male \times 20 HWE females or 4–5 transgenic males \times 40–50 HWE females; pool sizes ranged from 4 transgenic females \times 4 HWE males to 19 transgenic females \times 16 HWE males.

² estimated transformation or recombination frequency: number of independent integration events divided by number of surviving G₀ mosquitoes \times 0.5 (estimated average fertility rate per individual).

Table 2

EGFP expression patterns in midgut epithelium of bloodfed Mos 1-Carb/egfp and attP26-attB/DsRed-Carb/egfp (P14) mosquitoes

family	4 h pbm	8 h pbm	24 h pbm	32 h pbm	48 h pbm	56 h pbm	72 h pbm	96 h pbm
Carb/egfp52	-	+	++	+++	+++	++	+	+
54	-	-	-	-	-	-	-	-
86	-	-	-	-	-	-	-	-
105	++	+++	+++	+++	+++	+++	+++	+++
112	-	-	+	+	+	+	-	-
157	-	-	-	-	-	-	-	-
169	-	-	-	-	-	-	-	-
188	-	-	-	-	-	-	-	-
191	-	-	-	-	-	-	-	-
P14	-	+	++	+++	+++	+++	+	-

- = no midgut-specific EGFP expression; + weak EGFP expression; ++ intermediate EGFP expression; +++ very strong EGFP expression.