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High efficiency site-specific genetic engineering of the mosquito genome

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Abstract

Current techniques for the genetic engineering of insect genomes utilize transposable genetic elements, which are inefficient, have limited carrying capacity and give rise to position effects and insertional mutagenesis. As an alternative, we investigated two site-specific integration mechanisms in the yellow fever mosquito, *Aedes aegypti*. One was a modified CRE/lox system from phage P1 and the other a viral integrase system from *Streptomyces* phage phi C31. The modified CRE/lox system consistently failed to produce stable germ-line transformants but the phi C31 system was highly successful, increasing integration efficiency by up to 7.9-fold. The ability to efficiently target transgenes to specific chromosomal locations and the potential to integrate very large transgenes has broad applicability to research on many medically and economically important species.

Keywords

mosquito; *Aedes aegypti*; transformation; transgene; site-specific

Introduction

At least four transposable elements have been developed as vectors for genetic modification of insects and have been used in dozens of species across at least four orders (Handler, 2001; O'Brochta et al., 2003). Despite this, apart from the model insect *Drosophila melanogaster*, transformation efficiencies are low and compounded by the fact that many non-drosophilid species are more technically demanding to transform and maintain. In the yellow fever mosquito, *Aedes aegypti*, the vector of choice is *piggyBac* but typical transformation efficiencies of only 8% make transgenesis a laborious and time consuming task (Handler, 2002; Kokoza et al., 2001; Lobo et al., 2002). Moreover, by virtue of their natural biology, transposable elements have limited transgene carrying capacity and their essentially random integration can potentially lead to insertional mutagenesis and position effects on transgene expression (Handler & Harrell, 1999; Lorenzen et al., 2002).

Site-specific transgene integration systems such as FLP-*FRT* from the 2 micron plasmid of *Saccharomyces cerevisiae* (O'Gorman et al., 1991), CRE-lox from bacteriophage P1 (Sauer & Henderson, 1988) and phi C31 from a *Streptomyces* bacteriophage (Thorpe & Smith, 1998; Thyagarajan et al., 2001) present an alternative to transposable elements and have been used in mice (Araki et al., 2002), human cells (Thyagarajan et al., 2001), plants (Albert

et al., 1995; Day et al., 2000) and *Drosophila* (Gong & Golic, 2003; Groth et al., 2004). However, integration reactions for CRE-*lox* and FLP-*FRT* are reversible, making stable site-specific integration inefficient (Thyagarajan et al., 2001). Mutant *lox* sites designed to favour integration have been used in plants (Albert et al., 1995), mice (Araki et al., 2002) and mammalian cells (Araki et al., 1997) but not insects. One advantage of phi C31 integrase is that recombination is unidirectional, with interaction between *attB* and *attP* sites creating *attL* and *attR* junctions, that are no longer recognized by integrase, making integration both stable and efficient (Thorpe & Smith, 1998; Thyagarajan et al., 2001).

Site-specific integration precludes position effects on transgene expression, as demonstrated in plants (Srivastava et al., 2004) and mammalian cells (Feng et al., 1999), facilitating *in vivo* promoter and transgene comparisons. It also offers the potential to deliver large and complex constructs such as those required for combinatorial transgene strategies. The natural biology of phi C31 results in integration of the entire 42.4 kb phage genome (Chater et al., 1981) and it has been suggested that there may be no upper size limit on genomic integration by this mechanism (Olivares & Calos, 2003). This contrasts with transposable elements, where the maximum carrying capacity is around 10–13 kb and transformation efficiency decreases logarithmically with insert size (Geurts et al., 2003).

Here, we report the first use of site-specific transgene integration systems in the technically demanding environment of non-drosophilid insects. Specifically, we report on the use of the phage phi C31 system (Thyagarajan et al., 2001) and a modified CRE-*lox* mechanism (Araki et al., 1997) in the yellow fever mosquito, *A. aegypti*.

Results

Creation and characterization of targeting strains

Five targeting strains for each of CRE/*lox* and phi C31 were created by genomic integration of the relevant target sites using *piggyBac*. For the modified CRE/*lox* system, 2271 embryos were injected with pBac[3 × P3-EGFPaf]-*lox71* of which 327 hatched and 117 (36%) survived to adulthood (G_0). For the phi C31 system, 3242 embryos were injected with pBac[3 × P3-ECFPaf]-*attP* of which 754 hatched and 408 (54%) survived to adulthood (G_0). Males were back-crossed to wild-type in pools of 1–3 and females in pools of 5–10. G_1 transformants were identified by eye-specific expression of either EGFP (enhanced green fluorescent protein) or ECFP (enhanced cyan fluorescent protein). As a percentage of fertile G_0 males, *lox71* transformation efficiency varied from 10 to 17.6% and that for *attP* from 4.7 to 12.5% (average 11%). We established five *lox71* strains each with a single copy of the target site (data not shown) and five *attP* strains with between one and four target sites (Fig. 1a). Genomic integration sites were characterized by inverse PCR and all showed the canonical *piggyBac* TTAA sequence duplication (Fig. 1b). Flanking sequences for the four target sites in strain 17 could not be resolved by inverse PCR but they also seem likely to represent canonical integrations. Strains 12 and 13 were subsequently crossed to *A. aegypti* (*kh^w*) to generate additional strains (12 W and 13 W) with identical *attP* target sites but with a white-eyed phenotypic background.

Site-specific integration mediated by CRE recombinase

Homozygotes of the five *lox71* targeting strains were tested for site-specific integration by injecting embryos with p*AttB*[3 × P3-DsRed2nls]*lox66* in the presence of purified CRE recombinase. Between 1500 and 3000 embryos from each line were injected, with an average hatch rate of 13%. Survival of hatched embryos to adulthood averaged 84%. Transient expression of 3 × P3-DsRed2nls was evident in approximately 30–50% of individuals. Surviving males were backcrossed to the host strain in pools of 1–3 and females in pools of 5–10, with G_1 progeny screened for eye-specific expression of DsRed2. None of

the 1253 backcrosses produced any progeny with DsRed2 expression indicating an absence of site-specific transgene integration. In separate experiments, we also failed to generate *Drosophila melanogaster* transformants using this system (data not shown). Functionality of *lox71-lox66* was confirmed by PCR identification of recombination products in interplasmid assays both *in vitro* and following recovery from injected embryos (data not shown).

Site-specific integration mediated by phi C31 integrase

Four independent *attP* strains were tested for site-specific integration by injecting heterozygous embryos with pBattB[3 × P3-DsRed2nls]/*lox66* in the presence of phi C31 integrase mRNA. Between 689 and 1195 embryos for each strain were injected with an average hatch rate of 20% and average survival to adulthood of 85%. Transient expression of DsRed2 was evident in 30–50% of G₀ individuals. Males were backcrossed to the host strain individually and females in pools of 10, with G₁ progeny screened for eye-specific expression of DsRed2 following *attB-attP* integration (Fig. 2a,b; Table 1). Transformation efficiency based on individual males ranged from 16.7 to 31.8% and averaged 23.0% and, for all strains, pooled females also gave rise to transformed progeny. Phenotypic identification of transformants using 3 × P3-DsRed2nls was unambiguous in larvae (regardless of the eye colour phenotype) and DsRed2 localization mirrored exactly that of the ECFP marker that defined the targeting site (Fig. 3). Some strains showed additional tissue-specific fluorescence outside of the eye that was helpful in identification. For example, strain 11 showed anal papillae expression of both ECFP and DsRed2 (Fig. 3e,f) thus supporting the co-localization of markers at the target site.

Nonspecific integration at pseudo-attP sites mediated by phi C31 integrase

Previous work had shown that phi C31 was occasionally able to integrate *attB* into pseudo-*attP* sites in mammalian cells (Thyagarajan et al., 2001). Conversely, no pseudo-*attP* sites were identified in the *Drosophila* genome (Groth et al., 2004). We investigated the presence and efficiency of pseudo-*attP* sites in *A. aegypti* by injecting 698 wild-type embryos with pBattB[3 × P3-DsRed2nls]/*lox66* in the presence of phi C31 integrase mRNA. The hatch rate was 26%, survival to adulthood 93%, with typical levels of transient DsRed2 expression. Males were backcrossed to the host strain individually and females in pools of 10, with G₁ progeny screened for eye-specific expression of DsRed2 following integration at a pseudo-*attP* site. Only one of 28 males produced transformed G₁ progeny, giving a maximum transformation efficiency by this route of 3.6% (Table 1). Since no transformants were found in the progeny from a comparable number of pooled females, the transformation efficiency mediated by pseudo-*attP* sites is best estimated as 1.8%.

Characterization of phi C31-mediated site-specific integration events

For molecular confirmation of site-specific integration at the *attP* target sites, we used both Southern blotting and PCR. By digesting genomic DNA with *Xba*I and using an ECFP probe we identified a 1527 bp fragment characteristic of the target site, which increased in size to 2698 bp following site-specific integration of pBattB[3 × P3DsRed2nls]/*lox66* (Fig. 2c). Using PCR primers specific to *attP* we identified a 391 bp product characteristic of the empty target site, whilst primers specific to *attL* and *attR* detected 301 bp and 224 bp products that were characteristic of site-specific integration (Fig. 2d). Sequencing of these PCR products on both strands revealed a perfect match to the expected sequences (data not shown).

Discussion

Transgenic insect technology has the potential to combat a broad range of vector-borne diseases and insect pests but requires considerable technical improvement. Here, we show

that phi C31-mediated site-specific integration provides significant efficiency gains for germline transformation in *A. aegypti*. In our experiments, the efficiency of *piggyBac* integration ranged from 4.7 to 17.6% and averaged 11%. This slightly elevated efficiency is probably due to the small size of the inserts used (3692 bp for *lox71* and 3883 bp for *attP*) compared to published results elsewhere (5.8 kb transgene – Kokoza et al., 2001; 7.2 kb transgene – Lobo et al., 2002). Overall, these data are consistent with a decline in efficiency as insert size grows and this is an accepted limitation of transposon vectors, typically under 3 kb in their natural state. In contrast, the efficiency of site-specific transgene integration mediated by phi C31 ranged from 17–32% and averaged 23%. The plasmid inserted into the target site in these experiments was 4612 bp but the natural biology of bacteriophage phi C31 suggests that integrations of over 40 kb should be just as efficient and it has been speculated that there may be no upper size limit to integration by this mechanism (Olivares & Calos, 2003). Thus, this procedure may facilitate the insertion of multi-transgene complexes, such as those that may be required for combinatorial control strategies.

Importantly, significant efficiency gains also resulted from a doubling of larval survival to adulthood (average 85%) in comparison to that for *piggyBac* (average 45%). We speculate that this is because developing larvae are not subjected further to the mutagenic effects of *piggyBac* transposition, which might be expected to cause significant mutation in somatic cells as well as targeting the desired germ line cells. The impact of this increased survival is most apparent when comparing transformation efficiency as a percentage of eggs injected. For phi C31 this gives an average of 0.61% compared to 0.13% for *piggyBac* (an efficiency increase of 469%) whilst the efficiency for the best strain (1.02%; strain 17) represents an improvement of 785% in comparison to *piggyBac*. Such efficiency gains make mosquito transformation a much more practical proposition and offer real possibilities in relation to other technically demanding species.

An unexpected outcome of the work presented here was the variable performance of alternative recombinase/integrase systems in insects. It was previously shown that CRE recombinase could give rise to wild-type *loxP* excision reactions with high efficiency in both *Drosophila* (Siegal & Hartl, 1996) and *A. aegypti* (Jasinskiene et al., 2003). In other experiments, excision reactions of wild-type *FRT* sites, mediated by FLP recombinase, had been demonstrated in *Drosophila* (Golic et al., 1997; Gong & Golic, 2003; Groth et al., 2004) but not in *Aedes* (Jasinskiene et al., 2003). However, neither of these systems proved capable of producing stable germline transformation in any insect species. For this reason, we chose to investigate a modified version of the CRE/*lox* system in both *A. aegypti* and *D. melanogaster*, where mutant *lox66* and *lox71* sites prevent excision reactions and therefore bias the dynamics in favour of integration (Albert et al., 1995). In mammalian cells, this system gives an integration efficiency of 2–16% (Araki et al., 1997), which is comparable to that of phi C31 integrase in mammalian cells (4.8–17% – Thyagarajan et al., 2001). However, we were unable to generate any *A. aegypti* or *D. melanogaster* germline transformants with this system, despite confirming recombination between *lox66* and *lox71* sites in interplasmid assays both *in vitro* and in plasmids recovered from injected embryos. The contrast between this and phi C31-mediated transformation is stark and leads us to conclude that even the modified CRE/*lox* system is not efficient enough to target the limited number of available pole cells in the developing insect germ line.

Another unexpected outcome of phi C31-mediated transformation is the lack of symmetry of the integration reaction. In natural situations, the *attP* site of the phage targets an *attB* site in the bacterial genome. However, evidence from transformation of mammalian cells indicates that targeting of *attP* by *attB* is much more efficient (Thyagarajan et al., 2001). We have tested this hypothesis in insects by *piggyBac*-mediated transformation of *D. melanogaster* to create a transgenic strain carrying a single copy *attB* target site. In subsequent rounds of

transformation, we attempted to integrate an *attP* plasmid (carrying a DsRed2 reporter gene) at the target site, emulating the natural biology of the phage. From 1336 injected embryos and 221 backcrosses of surviving fertile adults, no transformed individuals expressing DsRed2 were identified (data not shown). This strongly suggests that a similar asymmetry is evident in insect, as well as mammalian genomes.

It has been demonstrated that phi C31 integrase can occasionally insert *attB* into pseudo-*attP* sites within the genome of mammalian cells (Thyagarajan et al., 2001), although no such sites were found in the *D. melanogaster* genome (Groth et al., 2004). Where they have been identified, pseudo-*attP* sites have variable sequences, often only having around 25% homology to the wild-type consensus (Thyagarajan et al., 2001). If such interactions are efficient, or many pseudo-*attP* sites exist in a given genome, this may complicate isolation of the desired site-specific events. Alternatively, high efficiency pseudo-*attP* sites may negate the need to insert an *attP* target site into the genome. In our experiments, we identified one pseudo-*attP* site in the *A. aegypti* genome and this yielded an integration efficiency around one twelfth that of wild-type *attP*. Integration of the DsRed2 reporter gene at the pseudo-*attP* site generated a red fluorescence phenotype that was distinct from the ECFP fluorescence phenotype that characterized the targeting strain. This phenotypic difference made it possible to screen out the pseudo-*attP* integration events at an early stage and therefore focus on the desired site-specific integration events. We were unable to isolate and sequence this pseudo-*attP* site by inverse PCR due to the absence of suitable flanking restriction sites. In separate experiments, we also detected a pseudo-*attP* integration event in *D. melanogaster*, suggesting that such sites may exist in all genomes. The *D. melanogaster* pseudo-*attP* site was sequenced (5'-GACTTGCAGCGAAAGCTCTTGTCAACGATCGTTTCGTTTT-3') and revealed 28% nucleotide identity to wild-type *attP* (underlined nucleotides) in addition to the core TTG motif (bold nucleotides). This low level of nucleotide identity is consistent with pseudo-*attP* sites identified elsewhere (Groth et al., 2004). Our data, as well as that available elsewhere, suggest that pseudo-*attP* sites are much less efficient at mediating transgene integration than wild-type *attP*.

In the future, regulatory concerns over the deployment of transgenic insects in biocontrol programmes will require demonstrable transgene stability. Transposable elements raise the possibility of instability through re-mobilization by an identical or closely related endogenous transposase, perhaps causing altered transgene expression or horizontal transfer to non-target species (Handler & McCombs, 2000; Handler et al., 2004). One solution to this problem has been tested with *piggyBac* and involves the prevention of remobilization by deletion of one terminal inverted repeat (TIR) sequence after genomic integration has occurred (Handler et al., 2004). Transgenes flanked by only one TIR do not present a substrate for transposase and are therefore inherently stable. This procedure, however, requires that each new transgene must be separately stabilized. We propose that *piggyBac*-mediated integration of an *attP* target site followed by postintegration stabilization would generate a flexible targeting strain capable of phi C31-mediated integration of any desired transgene but incapable of remobilization. This may come to represent the paradigm for next-generation transgenic technology in insects of medical or economic importance.

Experimental procedures

Plasmid constructs

pBac[3 × P3-ECFPaf]-*attP* was created by cloning the *SpeI attP* fragment from pBCPB + (Groth et al., 2004) into the *SpeI* site of pBluescript II SK + (Stratagene, La Jolla, CA), transferring the *NotI/EcoRV attP* fragment into the *NotI/EcoRV* sites of pSLfa1180fa (Horn

& Wimmer, 2000) and then cloning the *PsII attP* fragment into the unique *PsII* site of pBac[3 × P3-ECFPaf] (Handler & Harrell, 1999).

pBac-*lox71*[3 × P3-EGFPaf] was created by inserting a *lox71* site into pBac[3 × P3-EGFPaf] (Handler & Harrell, 1999). The *lox71* sequence was initially synthesized as oligonucleotides with flanking *PsII* sites and cloned into pBluescript-SK + (Stratagene). The 77 bp *PsII lox71* fragment was then cloned into the unique *PsII* site of pBac[3 × P3-EGFPaf].

To create pBattB[3 × P3-DsRed2nls-SV40]*lox66*, the DsRed2 *Bam*HI/*Bgl*II fragment from pLA643 (Alphey, unpublished) was cloned into the *Bam*HI site of pBluescript II SK + to make pBDsRed2. The 3 × P3 promoter from pB3 × P3-DsRed2 (Eggleston, unpublished) was cloned into pBDsRed2 using *Xho*I/*Eco*RI. The 3 × P3-DsRed2 *Xho*I/*Xba*I fragment was inserted into the *Xho*I/*Nhe*I sites of pActin-SV (Huynh & Zieler, 1999) to create pB3 × P3-DsRed2-SV40. The *lox66* sequence was synthesized as oligonucleotides and cloned into pBluescript SK- prior to transferring the *Xho*I/*Hinc*II *lox66* fragment into the *Xho*I/*Pme*I sites of pB[3 × P3-DsRed2-SV40]. The DsRed2 sequence lacked a nuclear localization signal (nls) so it was added by digesting pLA643 with *Bam*HI, blunting and then digesting with *Hinc*II to isolate the DsRed2nls and SV40 fragment. This was cloned into the *Hinc*II sites of pB[3 × P3-DsRed2-SV40]*lox66*. The *attB* site was released from pBCPB + (Groth et al., 2004) by *Apa*I digestion and ligated into the *Apa*I site of pB[3 × P3-DsRed2nls-SV40]*lox66*.

Recombinase and Integrase

Purified CRE recombinase (New England BioLabs, Ipswich, MA) was used at 0.1 U/μl for all *lox66-lox71* reactions. For *attB-attP* interactions we used phi C31 integrase mRNA at 500–900 ng/μl. This was transcribed from pET11phiC31poly(A) (Groth et al., 2004) using Message Machine (Ambion, Austin, TX), DNase treated, purified with MegaClear (Ambion), precipitated and re-suspended in 10–15 μl of nuclease-free water.

Microinjection of *Aedes aegypti*

Microinjection was performed essentially as described (Morris, 1997) with a modification after injection to increase throughput. The cover slip supporting the injected embryos was supported vertically in water so that the eggs were covered and the halocarbon oil was able to run off. Embryos were left for up to 5 h before removing on to damp filter paper.

To introduce the target sites, preblastoderm embryos of the wild-type strain *A. aegypti* (Bangkok) were coinjected with either pBac[3 × P3-ECFPaf]-*attP* or pBac[3 × P3-EGFPaf]-*lox71* at 500 ng/μl and the *piggyBac* transposase helper phsp-pBac (Handler & Harrell, 1999) at 300 ng/μl in injection buffer. Injected embryos were heat shocked 14–20 h postinjection at 42 °C in a covered water bath for 1 h to optimize transposase expression.

For site-specific transformation, targeting strain embryos were coinjected with pBattB[3 × P3-DsRed2nls-SV40]*lox66* at 150 ng/μl and either CRE recombinase or phi C31 mRNA. Putatively transformed larvae were screened for fluorescence using a Leica MZ FLIII microscope with the appropriate filter sets from Chroma Technology (Rockingham, VT) (EGFP: exciter HQ470/40x; emitter HQ525/50 m; ECFP: exciter D436/20x; emitter D480/40 m; DsRed2: exciter HQ545/30x; emitter HQ620/60 m).

Insect strains

A. aegypti (Bangkok) is a wild-type strain and *A. aegypti* (*kh^w*) is a white-eyed strain carrying a mutation of the *kynurenine hydroxylase* gene (Bhalla, 1968). Both these and the

derived transgenic strains were reared under standard insectary conditions at 27 °C and 80% RH, with larvae fed on fish food and adults on 0.2 µm filtered 10% glucose with 14 U/ml penicillin/14 µg/ml streptomycin. Three to five day old previtellogenic females were blood-fed and preblastoderm embryos collected for microinjection 72–96 h post-bloodmeal.

Inverse PCR

Inverse PCR was performed essentially as described (Handler et al., 1998). For *piggyBac* integrations, *TaqI*, *HaeIII* or *MspI* were used to isolate 5' junctions and *DpnII*, *MspI* or *HaeIII* to isolate 3' junctions. Initial difficulties in isolating and cloning the 3' junctions were solved by designing two new inverse PCR primers; 3'FORnew (5'-CATTTGCCTTTTCGCCTTATTTTAGA-3') and 3'REVnew (5'-AAACCTCGATATACTGACCGATAAAAACAC-3').

For integrations into pseudo-*attP*, *SacI*, *Clal* or *HindIII* were used together with 5' junction primers DsRed2-5F (5'-ATGTAGGTCACGGTCTCGAAG-3') and DsRed2-5R (5'-GCAATTAACCCTCACTAAAGGGA-3') or 3' junction primers DsRed2-3F (5'-ATGATGGACCAGATGGGTGA-3') and DsRed2-3R (5'-CAGCGTCAGCGGGTTCTC-3'). Inverse PCR products were cloned into pCRII-TOPO (Invitrogen, Paisley, UK) and sequenced on both strands.

PCR analysis of site-specific integration

Occupied target sites were identified using primers designed against *attL* (*attL*-forward 5'-GAGGTCGACGATGTAGGTCAC-3'; *attL*-reverse 5'-ACCTTTTCTCCCTTGCTACTGAC-3') and *attR* (*attR*-forward 5'-TCAAATAAGGCGGAGTGG-3'; *attR*-reverse 5'-GATGGGTGAGGTGGAGTACG-3'). Unoccupied *attP* target sites were identified using the primers No-*att* forward (5'-CAAATGTGTTCTGTGATGACCTG-3') and No-*att* reverse (5'-CTCCCTTGCTACTGACATTATGG-3').

200–400 ng of genomic DNA template was amplified with the relevant primers (2 µM) using New England BioLabs *Taq* DNA polymerase and ThermoPol buffer. Cycling parameters (MJ Research PTC-100) were 95 °C for 10 min then 30 cycles (95 °C, 30 s; 57 °C, 30 s; 72 °C, 30 s) followed by a final extension at 72 °C for 10 min. PCR was inhibited by eye pigment following genomic DNA extraction therefore all genomic DNA was isolated from headless mosquitoes.

Southern blotting

Genomic DNA was isolated using GenElute™ (Sigma-Aldrich, Gillingham, UK) from approximately 20–30 headless mosquitoes, crushed using Pellet Pestles (Anachem, Luton, UK) in lysis buffer and proteinase-K. The preparation was incubated at 55 °C for 1–3 h and extraction continued following the manufacturer's guidelines. Approximately 10 µg of genomic DNA was digested, separated on 1% agarose and blotted on to Hybond-N+ (Amersham Biosciences, Chalfont St. Giles, UK). To detect *piggyBac* integrations, genomic DNA was digested with *EcoRV* and the probe was made by digesting pBac[3 × P3-ECFPaf]-*attP* with *EcoRV* and *PstI* and isolating the 2195 bp band containing 3 × P3-ECFP and a segment of the *piggyBac* left terminus. To detect site-specific phi C31-mediated integrations, genomic DNA was digested with *XbaI* and the ECFP probe was produced by digesting pBac[3 × P3-ECFPaf]-*attP* with *NoI*, *SaI* and *Clal* prior to isolating the 751 bp ECFP fragment.

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Figure 1.

Genomic analysis of *attP* target sites in five transgenic strains (17, 13 W, 13, 12, 11 and 2) of *Aedes aegypti* generated by *piggyBac*-mediated germline transformation. The suffix 'W' in strain 13 W indicates a white-eyed phenotypic background. Strain 13 W carries the same *attP* target site as strain 13 and was generated by crossing strain 13 (wild-type eye colour) to the *kh^w* white-eyed mutant strain, followed by selection of individuals homozygous for the *white* mutation. (a) Southern blot of genomic DNA (10 µg) digested with *EcoRV* and probed at high stringency with the fragment indicated in Fig. 2(a). Three of the strains (2, 12, and 13/13 W) have a single *attP* site, strain 11 has two *attP* sites and strain 17 has four *attP* sites. The wild-type host (WT) is devoid of *attP* sites. (b) Inverse PCR analysis of *attP* locations showing mosquito genomic sequence at the 5'- and 3' flanks of the *piggyBac* integrations. All insertion sites are unique and all have the characteristic TTAA sequence duplication. The four integration sites in strain 17 could not be resolved by inverse PCR. The 3' flanking sequence obtained for strain 13 by inverse PCR on *DpnII* restricted DNA was foreshortened by the presence of a *DpnII* restriction site seven nucleotides downstream of the TTAA motif.

Figure 2.

phi C31-mediated site-specific transgene integration in *Aedes aegypti*.

(a) chromosomal organization of the targeting strains (not to scale) showing an unoccupied *attP* target site inserted by *piggyBac* and marked by ECFP. pB-L and pB-R are the left and right *piggyBac* terminal inverted repeats and the black double arrow is the probe used in Fig. 1(a) to identify *attP* sites. Short arrows show the location of PCR primers that amplify a 391 bp fragment specific for *attP*. (b) chromosomal organization at the occupied target site (not to scale) following site-specific integration of pBattB[3 × P3-DsRed2nls-SV40]/ox66. The black double arrow is the probe used to detect site-specific integration in Fig. 2(c). Short arrows show the location of PCR primers that amplify 224 bp and 301 bp fragments specific to *attR* and *attL*, respectively. Relevant restriction sites are *Xba*I (X), *Eco*RV (E), *Pst*I (P), *No*I (N) and *Sal*I (S). (c) Southern blot analysis of three targeting strains (17, 12 W and 11) both before and after (*) site-specific transgene integration. Genomic DNA (10 µg) was digested with *Xba*I and probed at high stringency with the fragment identified in Fig. 2(b). In all strains, a 1527 bp band that defines the empty *attP* target site increases to 2698 bp following site-specific integration of pBattB[3 × P3-DsRed2nls-sv40]/ox66. (d) PCR amplification with primers specific for *attP* identifies a 391 bp product that defines the unoccupied target site. Following site-specific integration, primers specific for *attL* and *attR* identify characteristic products of 301 bp and 224 bp, respectively.

Figure 3.

Fluorescence profiles of transgenic *Aedes aegypti* showing phi C31-mediated site-specific transgene integration. Site-specific integration in strain 12 W (white-eyed background) is evident from colocalization of the ECFP (a) and DsRed2 (b) fluorophores in the eyes, optic nerves and antennae. Strain 11 (wild-type eye background) (c,d,e,f) exhibits fluorescence in the anal papillae as well as in the eyes and optic nerves and this emphasizes the colocalization of fluorophores following site-specific integration. Note that the ECFP (cyan) fluorescence profile (c, e) is mirrored exactly by that of DsRed2 (d, f). Note also that DsRed2 expression is restricted to the cell nuclei by a nuclear localization signal.

Table 1

phi C31-mediated site-specific transgene integration efficiencies

Strain	<i>attP</i> sites.	Total♂	Fertile♂	Transformed♂	Efficiency
2	1	126	59	11	18.6%
11	2	20	6	1	16.7%
12	1	23	8	2	25.0%
17	4	52	22	7	31.8%
WT *	0	90	28	1	3.6% (1.8%)

* No positives in grouped females – estimated transformation efficiency is halved.