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ORIGINAL PAPER

PhiC31 integrase-mediated cassette exchange in silkworm embryos

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Abstract To construct an effective site-specific integration system in the silkworm, we examined if phiC31 integrase works in silkworm embryos. As an assay system, we constructed an extrachromosomal cassette exchange reaction system between two *attP* sites of an acceptor plasmid and two *attB* sites of a donor plasmid. To evaluate the activity, integrase mRNAs synthesized from three different plasmids were used. We injected a mixture of the acceptor and donor plasmids with the mRNA synthesized in vitro from one of the three plasmids into silkworm embryos at 4–6 h after oviposition and recovered plasmid DNAs from the embryos 3 days after injection. The resultant plasmids were transformed into *Escherichia coli* and spread on selection medium plates containing the appropriate antibiotics. A colony-forming assay and restriction enzyme digestion of the plasmids purified from the colonies showed that the phiC31 integrase worked very efficiently in the silkworm embryos. Notably, a phiC31 integrase mRNA synthesized from two of the plasmids produced cassette exchange plasmids at a high frequency, suggesting that the mRNA can be used to construct a targeted integration system in silkworms.

Keywords *Bombyx mori* · PhiC31 integrase · Cassette exchange · Targeting · Transgenic

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Introduction

Transgenic organisms are very useful tools to analyze gene function. Methods have been developed for germ-line transformation in the domesticated silkworm *Bombyx mori* using the transposons *piggyBac* or *minos* as vectors (Tamura et al. 2000; Uchino et al. 2007). Using these methods, we have developed a targeted GAL4/UAS binary gene expression system (Imamura et al. 2003), a transposon *piggyBac*-based enhancer trap system (Uchino et al. 2008); these methods are useful for post-genomic studies in the functional analysis of silkworm genes (Tan et al. 2005; Quan et al. 2007; Sakudoh et al. 2007). In addition, we have shown that transgenic silkworms can be used as bioreactors for the production of recombinant proteins (Tomita et al. 2003, 2007; Inoue et al. 2005; Tatematsu et al. 2010). However, current methods using transposons as vectors result in random integration of the transgene into the genome. Consequently, expression of the inserted gene is often unstable due to the strong position effect in the silkworm (Uchino et al. 2006, 2008). Therefore, evaluating the effects of the transgene in silkworm is problematic, which limits the use of transgenic silkworms in post-genomic studies.

To overcome this limitation, developing a gene targeting system that can introduce the transgene at a specific position in the genome is necessary. Two systems for site-specific integration have been studied in the silkworm. One, which

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is based on the FLP recombinase from yeast, has been successfully applied to silkworm cell lines and embryos (Tomita et al. 1999). The FLP system is highly efficient for site-specific integration of a transgene into the *Drosophila* genome (Horn and Handler 2005). However, our attempt to construct a targeting system based on the FLP recombinase was unsuccessful for site-specific integration in the silkworm (T. Tamura, personal communication). The other candidate for site-specific gene targeting is an integration system based on an integrase from phiC31 phage. We previously reported that site-specific integration occurs in cells cultured from silkworms, but the efficiency was not high (Nakayama et al. 2006). The phiC31 integrase system is thought to be more efficient than the FLP recombinase for the construction of transgenic insects because it has been successfully applied to diverse organisms, such as humans, flies, and mosquitoes (Groth et al. 2000, 2004; Bateman et al. 2006; Nimmo et al. 2006; Bischof et al. 2007). In addition, the phiC31 integrase system allows for the integration of DNA fragments exceeding 150 kb (Venken et al. 2006). This is another advantage of using the phiC31 integrase system for germ-line transformation; in contrast, methods that use transposons have lower limits for the size of the gene to be integrated.

In this study, we examined whether the phiC31 integrase works in the silkworm embryo. To measure phiC31 activity, we constructed a plasmid-based recombinase-mediated cassette exchange (RMCE) reaction system in which the translational product of an integrase mRNA synthesized from three different plasmids catalyzes the integration. Evaluation of the integrase activity of the in vitro synthesized mRNA showed that RMCE occurred at a very high frequency in the embryos. This result strongly suggests that phiC31 integrase works well in the silkworm embryo and can be used for the targeted integration of transgenes into the silkworm genome.

Materials and methods

Silkworm strain

We used a non-diapausing silkworm strain, w1-pnd, maintained at the Transgenic Silkworm Research Unit, Genetically Modified Organism Research Center at the National Institute of Agrobiological Sciences (Tsukuba, Ibaraki, Japan). The larvae were reared on an artificial diet (Nihon Nosan Co., Yokohama, Japan) at 25 °C.

Plasmids

Physical maps of the plasmids used in the experiments are shown in Fig. 1, including the acceptor plasmid (p*Bac3xP3DsRed2attP*) and the donor plasmid (p*ZErOEM7ZeoGFPattB*). The acceptor plasmid possessed an ampicillin resistance gene (Amp^R, for colony selection) and the DsRed2 gene under control of the 3xP3 promoter. The phiC31 integrase targeting site *attP* flanked the DsRed2 gene. As the

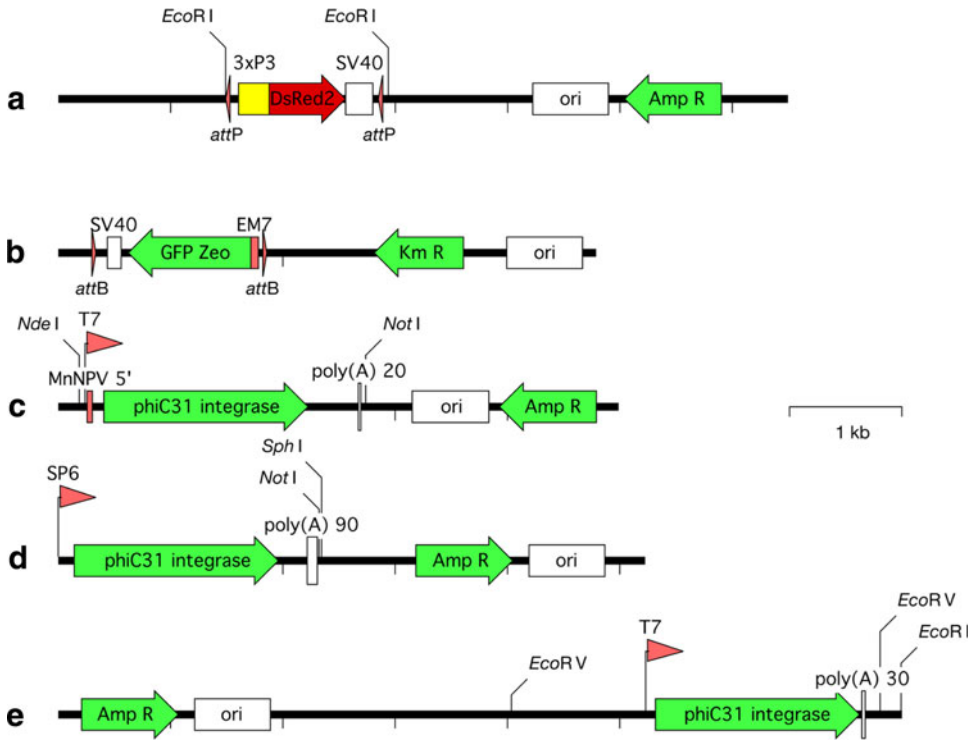

Fig. 1 Physical plasmid maps to examine phiC31 activity in the silkworm. **a** p*Bac3xP3DsRed2attP*, acceptor plasmid containing the ampicillin resistance gene and two *attP* sites flanking the 3xP3DsRed gene. **b** p*ZErO2EM7GFPattB*, donor plasmid containing the kanamycin resistance gene and two *attB* sites flanking the zeocin–GFP fusion gene under control of the EM7 promoter. **c** p*TD1-phiC31Int2*, plasmid for the synthesis of uncapped phiC31 integrase mRNA under control of the T7 promoter. **d** p*GEMe-phiC31Int-p(A)90*, plasmid for the synthesis of capped phiC31 integrase mRNA under control of the SP6 promoter. **e** p*ET11phiC31-polyA*, plasmid for the synthesis of capped phiC31 integrase mRNA under control of the T7 promoter

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3xP3 promoter does not function in bacteria, the DsRed2 gene was not expressed. The donor plasmid possessed a GFP/zeocin fusion resistance gene under control of the bacterial promoter, EM7. Fusion gene expression was monitored by the detection of GFP expression, while zeocin was used for colony selection. Two plasmids to synthesize phiC31 integrase (p*TD1-phiC31Int2* and p*GEMe-phiC31Int-p(A)90*) were constructed for the extrachromosomal cassette exchange assay. The nucleotide sequences of these plasmids are available at GenBank (Accession Nos.: AB713995-AB713998). The plasmid p*ET11phiC31-polyA* (Groth et al. 2004) was obtained from Dr. M. P. Calos (Department of Genetics, Stanford University School of Medicine, Stanford, CA, USA). The plasmid DNA for injection was first extracted from 50 ml of *Escherichia coli* cultures with a HiSpeed Plasmid Midi Kit (Qiagen, Hilden, Germany). To avoid RNase contamination, DNA was extracted with phenol/chloroform, treated once with chloroform, precipitated by adding 1/10 volume of 3 M potassium acetate and 2.5 volumes of ethanol, dissolved in distilled water (DW) and stored at −20 °C.

In vitro synthesis of phiC31 integrase mRNA

The phiC31 integrase mRNA was synthesized from the three template plasmids. The plasmid p*TD1-phiC31Int2* was linearized by double digestion with *Not*I/*Nde*I, and the digested plasmid DNA was treated with proteinase K. DNA was extracted twice with phenol/chloroform, treated once with chloroform, precipitated with 3 volumes of ethanol, and dissolved in RNase-free water. Uncapped mRNA was synthesized using a MEGAscript RNAi Kit (Ambion, Austin, TX, USA). The plasmids p*GEMe-phiC31Int-p(A)90* and p*ET11phiC31-polyA* were linearized by double digestion with *Not*I/*Sph*I and *Eco*RV/*Bam*HI, respectively. The digested DNAs were then treated with the same procedures as p*TD1-phiC31Int2*. The capped mRNAs of these two plasmids were synthesized using an mMessage m-MACHINE Kit (Ambion) and stored at −80 °C.

Injection into the embryos

Mixtures of acceptor and donor plasmids along with the in vitro synthesized phiC31 integrase mRNA were injected into the eggs 4–6 h after oviposition, as described by Tamura et al. (2007). The concentrations of each plasmid and the mRNA were adjusted to 0.2 μg/μl for the injection. The injected eggs were maintained at 25 °C with moisture.

Purification of plasmid from the embryos and transformation into *E. coli*

The purification of the plasmid DNA was performed from the embryos 3 days after injection. The plasmids were purified from 20–30 embryos using previously described methods (Shimizu et al. 2000). When an unknown organic layer appeared after the addition of ethanol in the final purification step, a further purification step using a DNA Clean & Concentrator™ Kit (Zymo Research, Irvine, CA, USA) was performed. The purified plasmid DNA was dissolved in 10 μl of DW, and 2 μl of the DNA solution was used for transformation into *E. coli*. The transformation was performed using ElectroMAX™ DH5α-E™ Competent Cells (Invitrogen, Carlsbad, CA, USA) and an *E. coli* Pulser™ Transformation Apparatus (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The transformed cells were suspended in 1 ml of SOC medium, spread on Luria–Bertani (LB) plates containing ampicillin (100 μg/ml) or LB plates containing ampicillin (100 μg/ml) and zeocin (50 μg/ml), and incubated at 37 °C.

Detection of colonies expressing GFP and analysis of purified plasmids

The colonies that appeared on the plates were observed using a fluorescence microscope equipped with a GFP filter set (Leica, Wetzlar, Germany). Purification of the plasmid from a colony was performed using the automated DNA isolation system PI-200 (Kurabo, Osaka, Japan). The purified plasmid was digested with *Eco*R1 and analyzed by agarose gel electrophoresis.

Results

Assay system

Our assay system to evaluate the activity of phiC31 integrase in the *B. mori* embryo is illustrated in Fig. 2. In this system, the acceptor plasmid p*Bac3xP3DsRed2attP* (Fig. 1a) possessed an ampicillin resistance gene and 3xP3DsRed2 sequences between two *attP* sites. The donor plasmid p*ZErO2EM7GFPattB* (Fig. 1b) contained a kanamycin resistance gene and a fusion of zeocin resistance and EGFP genes between two *attB* sites. When the mixture of the acceptor and donor plasmids was injected into fertilized silkworm eggs with one of the three phiC31 integrase mRNAs synthesized in vitro, RMCE should occur between the two *attP* sites of the acceptor plasmid and the two *attB* sites of the donor plasmid if the integrase was translated from the mRNA and functioned in the silkworm embryo. If recombination occurred between the two *attP* and *attB* sites, the 3xP3DsRed region of the acceptor plasmid would be eliminated and replaced by the EGFP–Zeo fusion gene (Fig. 2; Type A). If the activity of the integrase was not sufficient to catalyze this reaction and recombination occurred only at one *attP* and *attB* site, a plasmid with the

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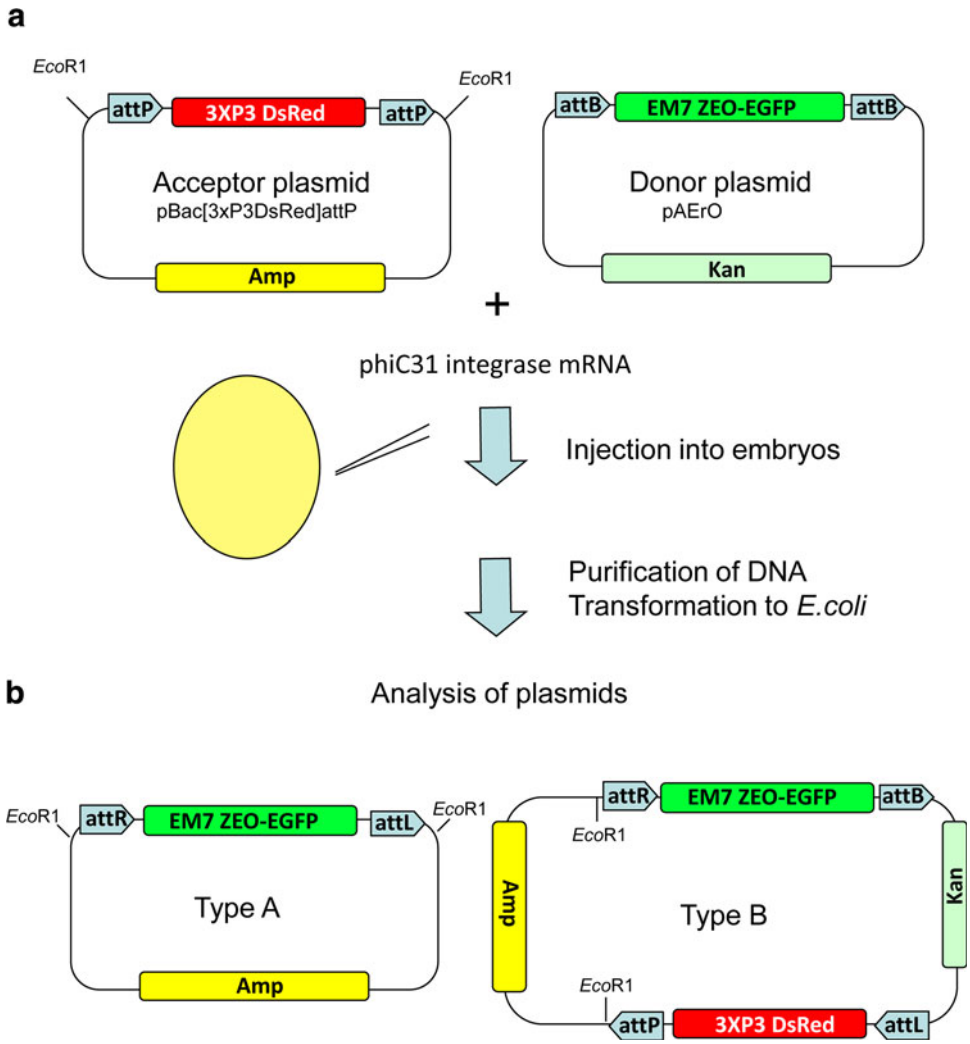

Fig. 2 The procedure to examine the phiC31 integrase activity in the silkworm embryo (**a**) and illustrated structure of representative resulting plasmids by recombination between the acceptor and donor plasmids (**b**). Type A, the plasmid produced by cassette exchange between 3xP3DsRed and EGFP–Zeo fusion genes. The plasmid by cassette exchange between 3xP3DsRed and Kan^R also occurred (see Fig. 4). Type B, the plasmid generated by recombination at only one site of *attP* of the acceptor and *attB* of donor. Four different types of plasmid can be produced when only one recombination occurs between the donor and acceptor plasmids. However, these plasmids cannot be distinguished, as the fragments produced by *Eco*RI digestion are the same in number and of the same sizes

combined size of the acceptor and the donor plasmids would be produced (Fig. 2; Type B). Colonies with the acceptor plasmid with and without recombination would grow on ampicillin plates. However, only plasmids that underwent RMCE (Fig. 2; Type A) as well as the acceptor and donor plasmid fusions (Fig. 2; Type B) expressed EGFP and grew on the ampicillin plus zeocin plates. Therefore, the plasmids produced by phiC31 integrase-mediated recombination could be detected based on the GFP expression (Fig. 3) and growth on ampicillin plus zeocin plates. As the loss of the DsRed marker cannot be scored in *E. coli*, restriction analysis was performed to distinguish between the two forms (Fig. 4).

Activity of phiC31 integrase in silkworm embryos

The three variants of integrase mRNA were synthesized in vitro using three different plasmids, i.e., p*TD1-phiC31Int2*, p*GEMe-phiC31Int-p(A)90*, and p*ET11phiC31-polyA*, and the integrase activities of the three mRNAs were tested in silkworm embryos. The integrase mRNA synthesized from the plasmid p*TD1-phiC31Int2* did not possess the 5′ cap structure but started from the 5′ UTR sequence of the *Malacosoma neustria* NPV polyhedrin gene and had an additional 20-bp poly A sequence. The mRNAs from p*GEMe-phiC31Int-p(A)90* and p*ET11phiC31-polyA* possessed the cap structure on the 5′ end as well as an additional 90- and 30-bp poly A sequence, respectively. As shown in Table 1, transformation of *E. coli* with plasmid DNA purified from the injected eggs yielded a large number of colonies on ampicillin or ampicillin plus zeocin plates. The frequency of colonies with EGFP (expressed as a percentage of the total number of colonies growing on the plate) in the control was 1.2 %, while the frequencies in the plasmids p*TD1-phiC31Int2*, p*GEMe-phiC31Int-p(A)90*, and p*ET11phiC31-polyA* were 6.9, 13.2, and 87.5 %, respectively. The frequency of colonies resistant to ampicillin and zeocin was 5.9 % for the control without phiC31 integrase mRNA. The frequency increased to 20.7, 16.2, and 85.4 % when the mRNAs from p*TD1-phiC31Int2*, p*GEMe-phiC31Int-p(A)90*, and p*ET11phiC31-polyA* were present, respectively. The number of EGFP-positive

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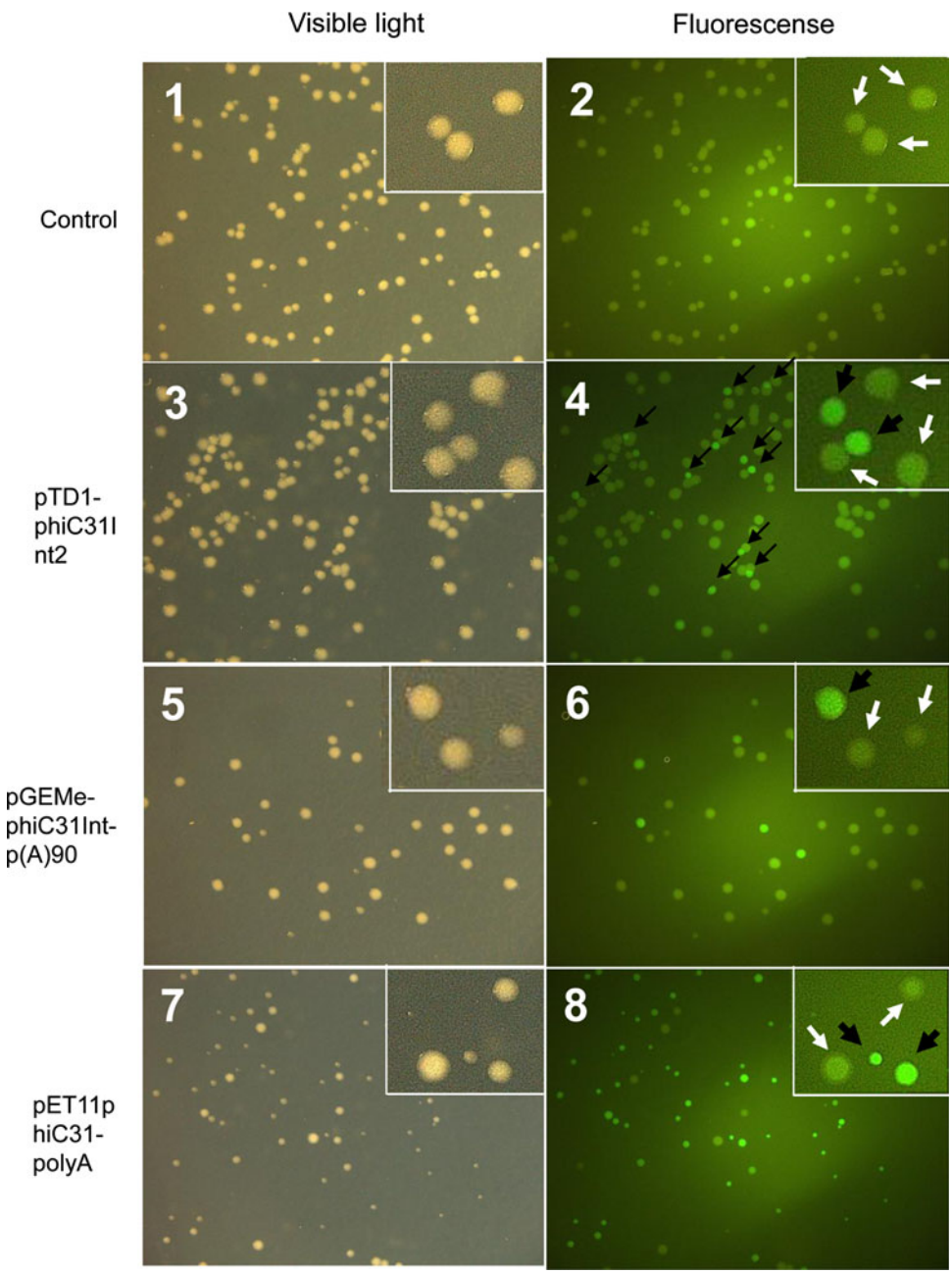

Fig. 3 Frequency of the appearance of colonies expressing EGFP on ampicillin plates. Plasmid DNA extracted from the embryos 3 days after injecting the mixture of donor and acceptor plasmids with each phiC31 integrase mRNA was used for transformation. The control indicates the plasmid from the embryos injected with only donor and acceptor plasmids. The inset shows an enlarged photograph of the colonies; the *filled* and *white arrows* in the *insets* indicate colonies with and without EGFP, respectively

colonies on the ampicillin plates was markedly different from the mRNA variants, whereas the total number of colonies per 1 ml of transformed cells did not differ significantly (Table 1). These results suggested that the amounts of plasmid DNA recovered from the eggs and the efficiency of the *E. coli* transformation were similar for each experiment. Unexpectedly, when the bacteria were transformed with the plasmid purified from the eggs in the control, which were injected with the acceptor and donor plasmids but without the phiC31 integrase mRNA, a few colonies with EGFP fluorescence and a few colonies with ampicillin and zeocin resistance were present (Table 1). These colonies yielded no plasmids derived by recombination of phiC31 integrase. Therefore, these results indicated that the assay system gave false positives at a low frequency. The frequency of false-positive colonies after selection with ampicillin and zeocin was higher than that of GFP selection on ampicillin plates in the control (Table 1). Despite the presence of false-positive colonies, the frequencies of EGFP-positive colonies or colonies with both ampicillin and zeocin resistance were much higher when phiC31 integrase mRNA was added to the injection mixture. In conclusion, we could reliably determine the activity of different phiC31 mRNAs based on the appearance of GFP-positive colonies or colonies resistant to ampicillin and zeocin. The results in Table 1 show that the phiC31

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Table 1 Efficiency of the integration of the host plasmid obtained by the selection markers and EGFP

Source of mRNA	Exp. no.	Plate medium	Amount of plated cells (ml)	No. of colonies that appeared	No. of colonies with EGFP (%)	No. of total colonies in 1 ml of transformed cells[a] (%[b])
Control	1	Amp	0.23	6,210	12 (1.2)	2.7×10^4
	1	Amp + Zeo	0.47	732	64 (8.7)	1.6×10^3 (5.9)
pTD1-phiC31Int2	1	Amp	0.13	3,600	248 (6.9)	2.8×10^4
	1	Amp + Zeo	0.47	2,710	2,124 (78.4)	5.8×10^3 (20.7)
pGEMe-phiC31Int-p(A)90	1	Amp	0.23	4,760	630 (13.2)	2.1×10^4
	1	Amp + Zeo	0.47	1,614	1,562 (96.8)	3.4×10^3 (16.2)
pET11phiC31-polyA	1	Amp	0.32	2,872	2,512 (87.5)	8.9×10^3
	1	Amp + Zeo	0.32	2,428	2,376 (97.9)	7.6×10^3 (85.4)

The plasmid DNA from 4–6 embryos was used for the transformation, and the cells were suspended with 1 ml of SOC medium. Control indicates that colonies appeared after the transformation of *E. coli* using the DNA purified from silkworm embryos that were injected with only acceptor and donor plasmids without phiC31 integrase mRNA

[a] The total numbers of colonies per 1 ml of the transformed cells was calculated from the amount of cells used to obtain the colonies examined

[b] The numbers in parentheses indicate the frequency of the appearance of colonies resistant to ampicillin (Amp) and zeocin (Zeo) in comparison to the total number of colonies resistant to ampicillin

integrase works well in the embryonic cells of the silkworm. The mRNAs synthesized from p*ET11phiC31-polyA* plasmid gave the highest activity, followed by mRNAs from the plasmid p*GEMe-phiC31Int-p(A)90*. The lowest level of activity was observed from the plasmid p*TD1-phiC31Int2*.

Analysis of plasmids produced by the phiC31 integrase reaction

As shown in Fig. 2, many different types of plasmid can be generated by recombination between the two *attP* sites of the acceptor plasmid and the two *attB* sites of the host plasmid in the embryos. However, some of these plasmids could not be distinguished based on the GFP expression and antibiotic resistance alone. Therefore, a plasmid from each colony was purified and analyzed by restriction digestion. Figure 4 shows the restriction fragment patterns of the plasmids obtained from the colonies in Table 1. The structures of the purified plasmids were determined based on the restriction fragment patterns (Fig. 2b) and inferred the results shown in Table 2. For control experiments, we analyzed 27 plasmids (23 GFP-negative and 4 GFP-positive) from the ampicillin plates and 9 plasmids (5 GFP-negative and 4 GFP-positive) from ampicillin plus zeocin plates. These plasmids showed the same restriction fragment pattern as the acceptor plasmid, indicating that the colonies possessed only the intact acceptor plasmid. When mRNA synthesized from p*TD1-phiC31lin2* was used as an integrase source, the frequency of GFP-positive or ampicillin- and zeocin-resistant colonies was low, and most of the recombinant plasmids obtained from the ampicillin plus

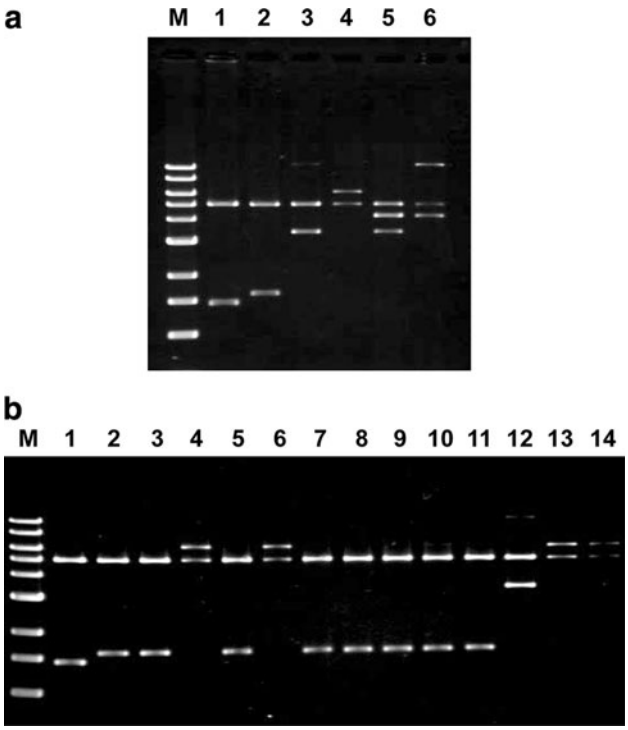

Fig. 4 Restriction digest analysis of the plasmids obtained from transformed *E. coli* colonies. **a** Agarose gel electrophoresis of DNA fragments of the purified plasmids after digestion by *Eco*RI. 1, intact acceptor plasmid; 2, type A in Figs. 2b, 3, acceptor plasmid in which the 3xP3 region was substituted for the donor plasmid sequence including the Kan^R gene and ori between the two *attB* sites by cassette exchange reaction; 4, type B in Fig. 2b or the plasmid produced by recombination between one acceptor *attP* site and one donor *attB* site; 5 and 6, unidentified plasmids (see Fig. 2b). **b** Restriction digest analysis of the plasmids purified from colonies on the ampicillin plus zeocin LB plate. The integrase mRNA synthesized from p*GEMe-phiC31Int-p(A)90* was used for the injection

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Table 2 Numbers of plasmids analyzed by restriction enzyme digestion and agarose gel electrophoresis

Source of mRNA	Exp. no.	Plate medium	No. of plasmids examined	No. of plasmids produced by one integration	No. of plasmids produced by expected cassette exchange (%)	No. of plasmids produced by opposite cassette exchange (%)	No. of plasmids produced by other types of integration (%)	No. of accepter plasmids (%)
Control	1	Amp	27	0	0	0	0	27 (100)
	1	Amp + Zeo	9	0	0	0	0	9 (100)
p*TD1-phiC31Int2*	1	Amp + Zeo	39	29 (74.4)	0	0	0	11 (29)
	2	Amp	50	1 (2.0)	0	0	0	49 (98)
	2	Amp + Zeo	48	31 (64.9)	0	0	3 (6.3)	14 (29)
p*GEMe-phiC31Int-p(A)90*	1	Amp + Zeo	28	6 (21.4)	17 (60.7)	1 (3.6)	4 (14.3)	0 (0)
p*ET11phiC31-polyA*	1	Amp + Zeo	31	0	23 (74.2)	0	8 (25.8)	0 (0)
	2	Amp	50	1 (2.0)	26 (52.0)	7 (14.0)	4 (8.0)	12 (24)
	2	Amp + Zeo	45	8 (17.8)	33 (73.3)	0	4 (8.9)	0 (0)

zeocin plates were the result of recombination at one target site (Fig. 2, Type B). No RMCE occurred with this mRNA. In addition, the plasmids selected with both antibiotics using mRNA synthesized from p*TD1-phiC31lin2* included many false positives (Table 1). When mRNA from p*GEMc-phiC31Int-p(A)90* was used, a much higher ratio of RMCE type plasmids appeared than in p*TD1-phiC31lin2*. In addition, type B plasmid (Fig. 2) appeared at a lower frequency than the RMCE type (Fig. 2 Type A). When phiC31 integrase mRNA from the plasmid p*ET11phiC31-polyA* was used, the frequency of the recombination type was high, even from colonies on ampicillin plates, and most of the plasmids were RMCE type. Similar results were obtained by analyzing plasmids from ampicillin plus zeocin plates.

Discussion

The phiC31 integrase catalyzes the recombination reaction between the two nonhomologous recognition sites, *attP* and *attB*, which are approximately 50 bp in length (Groth et al. 2000). The reaction generates two new sequences, *attR* and *attL*. Unlike the Cre enzyme from P1 phage and the FLP recombinase from yeast, phiC31 integrase catalyzes a unidirectional reaction. Therefore, excision or translocation of the inserted sequences will not occur. For transgenic applications in eukaryotic cells, the foreign DNA with an *attB* sequence is usually introduced into the *attP* site of the genome because the efficiency of *attB* integration into chromosomal *attP* is greater than in the opposite direction (Thyagarajan et al. 2001; Groth et al. 2004). RMCE between tandem *attP* sites introduced into the genome and tandem *attB* sites carried on a donor plasmid is an excellent strategy as it incorporates the gene of interest, flanked by tandem *attB* sites, into the genome, but does not incorporate the plasmid backbone. RMCE with phiC31 integrase was demonstrated to occur in vivo in *Drosophila* (Bateman et al. 2006). Here, we developed an assay system for RMCE in the silkworm embryo; we successfully demonstrated RMCE between the tandem *attP* sites that flanked the 3xP3DsRed of the acceptor plasmid and the tandem *attB* sites that flanked an EGFP–Zeo fusion gene of the donor plasmid.

As a source of the integrase, a helper plasmid encoding the phiC31 integrase was used to perform the integration between the *attP*-carrying plasmid and the *attB*-carrying genome in the BmN4 cell line (Nakayama et al. 2006). In contrast, in vitro transcribed mRNA from the plasmid p*ET11phiC31poly(A)* has been used in *Drosophila melanogaster* and *Anopheles gambiae* embryos (Bateman et al. 2006; Meredith et al. 2011). In our experiment, we chose mRNA as the source of integrase because mRNA has been shown to be more efficient for construction of transgenic silkworms (Uchino et al. 2007). The injection of the acceptor and donor plasmids into the eggs demonstrated that the silkworm embryonic cells have no endogenous integrase activity because recombination did not occur without the integrase mRNA. In contrast, the mixture of the two plasmids with the mRNAs synthesized in vitro produced a high ratio of the recombinant type plasmid, indicating that the mRNA served as the source of the phiC31 integrase in the early embryonic stage of *B. mori*. In addition, uncapped mRNA transcribed in vitro from the p*TD1-phiC31Int2* construct also showed activity in *B. mori*. The observation that three different types of phiC31 mRNA synthesized from different constructs showed different efficiencies for RMCE suggests that the phiC31 integrase system can be adjusted (at least partially) using sequences of the 5′ or 3′ UTRs and/or codon usage relevant to *B. mori*.

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The efficiency of the recombination observed in the silkworm embryos is high when mRNA synthesized from the plasmid p*GEMe-phiC31Int-p(A)90* or p*ET11phiC31-polyA* was used. These values were comparable to the results in other organisms in which similar integration systems with phiC31 integrase were used (Groth et al. 2000). In human cells and *Drosophila* embryos, the intramolecular excision assay was used to measure integrase activity. The frequency of the reaction between *attP* and *attB* sites in the plasmids was about 50 % in human cells and 100 % in *Drosophila* embryos (Groth et al. 2000, 2004). In our system, more than 50 % of the RMCE was detected when the mRNA was synthesized from the plasmid p*ET11phiC31poly(A)*.

The integration of foreign genes into the genome using phiC31 integrase has been reported in many organisms, including human cells (Ishikawa et al. 2006), *Drosophila* (Groth et al. 2004; Bischof et al. 2007; Fish et al. 2007), Mediterranean fruit flies (Schetelig et al. 2009), and mosquitoes (Nimmo et al. 2006; Labbe et al. 2011; Meredith et al. 2011). The frequency of obtaining foreign gene integration in insects varies between species. The highest rate was observed in *Drosophila*, with a value of more than 50 %, comparable to the methods using the transposon P element (Fish et al. 2007). However, the rates were less than 5 % in mosquitoes and Mediterranean fruit flies (Nimmo et al. 2006; Meredith et al. 2011). Therefore, the construction of a targeted transgene integration system in an insect other than *Drosophila* was considered difficult without careful vector construction and the development of a method to supply the active phiC31 integrase. Although the efficiency of genome targeting by the phiC31 integrase in the silkworm remains unknown, the extrachromosomal RMCE reported here may be used to construct a phiC31 integrase-mediated targeted integration system for the silkworm genome. Currently, the construction of such a system is underway in our laboratory.

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References

Bateman JR, Lee AM, Wu CT (2006) Site-specific transformation of Drosophila via phiC31 integrase-mediated cassette exchange. Genetics 173:769–777

Bischof J, Maeda RK, Hediger M, Karch F, Basler K (2007) An optimized transgenesis system for *Drosophila* using germ-line-specific phiC31 integrases. Proc Natl Acad Sci USA 104:3312–3317

Fish MP, Groth AC, Calos MP, Nusse R (2007) Creating transgenic *Drosophila* by microinjecting the site-specific phiC31 integrase mRNA, a transgene-containing donor plasmid. Nat Protoc 2:2325–2331

Groth AC, Olivares EC, Thyagarajan B, Calos MP (2000) A phage integrase directs efficient site-specific integration in human cells. Proc Natl Acad Sci USA 97:5995–6000

Groth AC, Fish M, Nusse R, Calos MP (2004) Construction of transgenic *Drosophila* by using the site-specific integrase from phage phiC31. Genetics 166:1775–1782

Horn C, Handler AM (2005) Site-specific genomic targeting in *Drosophila*. Proc Natl Acad Sci USA 102:12483–12488

Imamura M, Nakai J, Inoue S, Quan GX, Kanda T, Tamura T (2003) Targeted gene expression using the GAL4/UAS system in the silkworm *Bombyx mori*. Genetics 165:1329–1340

Inoue S, Kanda T, Imamura M, Quan GX, Kojima K, Tanaka H, Tomita M, Hino R, Yoshizato K, Mizuno S, Tamura T (2005) A fibroin secretion-deficient silkworm mutant, Nd-sD, provides an efficient system for producing recombinant proteins. Insect Biochem Mol Biol 35:51–59

Ishikawa Y, Tanaka N, Murakami K, Uchiyama T, Kumaki S, Tsuchiya S, Kugoh H, Oshimura M, Calos MP, Sugamura K (2006) Phage phiC31 integrase-mediated genomic integration of the common cytokine receptor gamma chain in human T-cell lines. J Gene Med 8:646–653

Labbe GM, Nimmo DD, Alphey L (2011) piggybac- and PhiC31-mediated genetic transformation of the Asian tiger mosquito, *Aedes albopictus* (Skuse). PLoS Negl Trop Dis 4:e788

Meredith JM, Basu S, Nimmo DD, Larget-Thiery I, Warr EL, Underhill A, McArthur CC, Carter V, Hurd H, Bourgouin C, Eggleston P (2011) Site-specific integration and expression of an anti-malarial gene in transgenic *Anopheles gambiae* significantly reduces Plasmodium infections. PLoS One 6:e14587

Nakayama G, Kawaguchi Y, Koga K, Kusakabe T (2006) Site-specific gene integration in cultured silkworm cells mediated by phiC31 integrase. Mol Genet Genomics 275:1–8

Nimmo DD, Alphey L, Meredith JM, Eggleston P (2006) High efficiency site-specific genetic engineering of the mosquito genome. Insect Mol Biol 15:129–136

Quan GX, Kobayashi I, Kojima K, Uchino K, Kanda T, Sezutsu H, Shimada T, Tamura T (2007) Rescue of *white egg 1* mutant by introduction of the wild-type *Bombyx* kynurenine 3-monooxygenase gene. Insect Sci 14:85–92

Sakudoh T, Sezutsu H, Nakashima T, Kobayashi I, Fujimoto H, Uchino K, Banno Y, Iwano H, Maekawa H, Tamura T, Kataoka H, Tsuchida K (2007) Carotenoid silk coloration is controlled by a carotenoid-binding protein, a product of the Yellow blood gene. Proc Natl Acad Sci USA 104:8941–8946

Schetelig MF, Scolari F, Handler AM, Kittelmann S, Gasperi G, Wimmer EA (2009) Site-specific recombination for the modification of transgenic strains of the Mediterranean fruit fly *Ceratitis capitata*. Proc Natl Acad Sci USA 106:18171–18176

Shimizu K, Kamba M, Sonobe H, Kanda T, Klinakis AG, Savakis C, Tamura T (2000) Extrachromosomal transposition of the transposable element Minos occurs in embryos of the silkworm *Bombyx mori*. Insect Mol Biol 9:277–281

Tamura T, Thibert C, Royer C, Kanda T, Abraham E, Kamba M, Komoto N, Thomas JL, Mauchamp B, Chavancy G, Shirk P, Fraser M, Prudhomme JC, Couble P (2000) Germline transformation of the silkworm *Bombyx mori* L. using a piggyBac transposon-derived vector. Nat Biotechnol 18:81–84

Tamura T, Kuwabara K, Uchino K, Kobayashi I, Kanda T (2007) An improved DNA injection method for silkworm eggs drastically increases the efficiency of producing transgenic silkworms. J Insect Biotechnol Sericol 76:155–159

Tan A, Tanaka H, Tamura T, Shiotsuki T (2005) Precocious metamorphosis in transgenic silkworms overexpressing juvenile hormone esterase. Proc Natl Acad Sci USA 102:11751–11756

Tatematsu K, Kobayashi I, Uchino K, Sezutsu H, Iizuka T, Yonemura N, Tamura T (2010) Construction of a binary transgenic gene

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expression system for recombinant protein production in the middle silk gland of the silkworm *Bombyx mori*. Transgenic Res 19:473–487

Thyagarajan B, Olivares EC, Hollis RP, Ginsburg DS, Calos MP (2001) Site-specific genomic integration in mammalian cells mediated by phage phiC31 integrase. Mol Cell Biol 21:3926–3934

Tomita S, Kanda T, Imanishi S, Tamura T (1999) Yeast FLP recombinase-mediated excision in cultured cells and embryos of the silkworm, *Bombyx mori* (Lepidoptera: Bombycidae). Appl Entomol Zool 34:371–377

Tomita M, Munetsuna H, Sato T, Adachi T, Hino R, Hayashi M, Shimizu K, Nakamura N, Tamura T, Yoshizato K (2003) Transgenic silkworms produce recombinant human type III procollagen in cocoons. Nat Biotechnol 21:52–56

Tomita M, Hino R, Ogawa S, Iizuka M, Adachi T, Shimizu K, Sotoshiro H, Yoshizato K (2007) A germline transgenic silkworm that secretes recombinant proteins in the sericin layer of cocoon. Transgenic Res 16:449–465

Uchino K, Imamura M, Sezutsu H, Kobayashi I, Kojima K, Kanda T, Tamura T (2006) Evaluating promoter sequences for trapping an enhancer activity in the silkworm *Bombyx mori*. J Insect Biotechnol Sericol 75:89–97

Uchino K, Imamura M, Shimizu K, Kanda T, Tamura T (2007) Germ line transformation of the silkworm, *Bombyx mori*, using the transposable element Minos. Mol Genet Genomics 277:213–220

Uchino K, Sezutsu H, Imamura M, Kobayashi I, Tatematsu K, Iizuka T, Yonemura N, Mita K, Tamura T (2008) Construction of a piggyBac-based enhancer trap system for the analysis of gene function in silkworm *Bombyx mori*. Insect Biochem Mol Biol 38:1165–1173

Venken KJ, He Y, Hoskins RA, Bellen HJ (2006) P[acman]: a BAC transgenic platform for targeted insertion of large DNA fragments in *D. melanogaster*. Science 314:1747–1751