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phiC31-integrase-mediated, site-specific integration of transgenes in the silkworm, *Bombyx mori* (Lepidoptera: Bombycidae)

Naoyuki Yonemura · Toshiki Tamura · Keiro Uchino · Isao Kobayashi · Ken-ichiro Tatematsu · Tetsuya Iizuka · Takuya Tsubota · Hideki Sezutsu · Mayandi Muthulakshmi · Javaregowda Nagaraju · Takahiro Kusakabe

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Abstract Transgenic silkworms can be useful for investigating the functions of genes in the post-genomic era. However, the common method of using a transposon as an insertion tool may result in the random integration of a foreign gene into the genome and suffer from a strong position effect. To overcome these problems, it is necessary to develop a site-specific integration system. It is known that phiC31 integrase has the capacity to mediate recombination between the target sequences attP and attB. To test the availability of site-specific integration in the silkworm, we first examined the efficiency of recombination between the target sites of the two plasmids in silkworm embryos and found that the frequency of recombination was very high. Then we constructed a host strain that possessed the target sequence attP using the common method. We injected the donor plasmid together with the phiC31 integrase mRNA into the embryos of the host strain and obtained positive lines. Structural analysis of the lines showed that site-specific integration occurred by recombination between the genomic attP site and the attB site of the donor plasmid. We can conclude from the results that phiC31 integrase has the ability to mediate the site-specific integration of transgenes into the silkworm chromosome.

Keywords Silkworm · *Bombyx* · Transgenic · phiC31 integrase · Site-specific integration

N. Yonemura · T. Tamura (✉) · K. Uchino · I. Kobayashi · K. Tatematsu · T. Iizuka · T. Tsubota · H. Sezutsu
Genetically Modified Organism Research Center, National Institute of Agrobiological Sciences, Tsukuba, Ibaraki 305-8634, Japan
e-mail: ttamura@affrc.go.jp

M. Muthulakshmi · J. Nagaraju
Laboratory of Molecular Genetics, Centre for DNA Fingerprinting and Diagnostics, Tuljaguda, Nampally, Hyderabad, India

T. Kusakabe
Laboratory of Silkworm Science, Graduate School of Bioresource and Bioenvironmental Sciences, Kyushu University, 6-10-1 Hakozaki, Fukuoka 812-8581, Japan

Introduction

The development of post-genomic tools using insects is an important subject of study in applied entomology. The silkworm is a model insect representing the order Lepidoptera, and a genome database including ESTs and whole genome sequences has been established (ISG Consortium 2008). Transgenic silkworms can be very useful for understanding the functions of genes of interest predicted from genome sequences (Tatemastu et al. 2012). Indeed, the genes responsible for juvenile hormone titer, silk coloration, and resistance to *Densovirus* and *Bacillus thuringiensis* have been identified using transgenic silkworms (Tan et al. 2005; Sakudoh et al. 2007; Ito et al. 2008; Atsumi et al. 2012). Furthermore, transgenic silkworms have been used as "bioreactors" for the production of recombinant proteins. Genetically modified silks have been shown to be applicable as biomaterials in the medical field. In addition, many different mammalian proteins can be produced using transgenic silkworms (Tatemastu et al. 2012); these include human collagen, human albumin, and mouse antibody (Tomita 2011). However, transgenic silkworms are often produced using the DNA-type transposons *piggyBac* or *minos* as vectors to carry the transgene into the chromosome (Tamura et al. 2000; Uchino et al. 2007). In such cases, the integration of transgenes occurs randomly

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at different chromosomal positions, and their expression levels differ according to the insertion position (Uchino et al. 2008), probably due to the influence of the adjacent chromatin structure. Therefore, the expression level of the inserted gene is unstable and much weaker than that of the endogenous gene (Tatematsu et al. 2010).

A site-specific integration system that inserts the transgene into a specific site of the genome would overcome these problems. Such systems have been attempted in fruit flies and other insects as well as in mammals using FLP recombinase from yeast and phiC31 integrase from phages (Horn and Handler 2005; Bischof et al. 2007; Nimmo et al. 2006; Meredith et al. 2011; Schetelig et al. 2009; Fish et al. 2007; Ishikawa et al. 2006). However, in our experiences of using FLP recombinase, we were not able to successfully establish an integration system in the silkworm, although it worked well in silkworm cell lines and embryos (Tomita et al. 1999). phiC31 integrase catalyzes the integration of foreign DNA with an approximately 50-bp attB target sequence into the attP site (about 50 bp in size), and the DNA can be integrated into the position of the attP site in the genomes of host organisms. We showed in our previous paper that phiC31 integrase works very efficiently in silkworm embryos using an extra chromosomal cassette exchange reaction system; recombination between the attP and attB sites of the plasmid occurred with a high frequency in the silkworm embryos when phiC31-integrase mRNA was supplied by injection and no site-specific integration of transgenes into the silkworm genome was performed (Yonemura et al. 2012).

In the present study, we investigated whether phiC31 integrase can be used to establish a site-specific gene integration system into the genome of the silkworm. We constructed a transposon vector possessing the attP target sequence, and a marker gene between the right and left arms of the *piggyBac* transposon that caused red fluorescent protein expression in the eye. We also constructed a donor plasmid with the attB target sequence and an eye marker gene expressing green fluorescent protein. We first examined the frequency of integration of the donor plasmid into the target site attP of the vector plasmid in silkworm embryos, and found that it was very high (based on an extrachromosomal assay). Next, we constructed a host strain by injecting the vector plasmid with the attP site into the embryos. Then we injected the donor plasmid with the attB sequence into the embryos of the host strain together with the phiC31-integrase mRNA. The result clearly demonstrated that the donor plasmid DNA was efficiently integrated into the attP site of the host strain. Our results indicate that a phiC31-integrase-mediated, site-specific integration system was established in the silkworm.

Materials and methods

Silkworm strains

A non-diapausing silkworm strain (w1-pnd) and a diapausing strain (w-1) were used in this study. The strains were maintained at the Transgenic Silkworm Research Unit, Genetically Modified Organism Research Center, at the National Institute of Agrobiological Sciences (Tsukuba, Ibaraki, Japan). The silkworms were reared on an artificial diet (Nihon Nosan, Yokohama, Japan) at 25 °C.

Construction of vector and donor plasmids

To construct the vector and donor plasmids, we first synthesized 223-bp attP and 287 bp attB target sequences with AscI and FseI sites at both ends (Genscript, Piscataway, NJ, USA). The vector plasmid pBac[3xP3DsRedattP] was constructed by inserting the synthesized attP sequence into the AscI–FseI site of the plasmid pBac[3xP3DsRed afm] (Horn et al. 2002). The resulting plasmid possessed the phiC31-integrase target site attP and a marker gene, 3xP3DsRed, between the arms of *piggyBac* that led to the expression of red fluorescent protein in the stemmata of embryos and the eyes of adults (Fig. 1a). The donor plasmid pBac[3xP3EGFPattB] (Fig. 1b) was designed to study the integration of the whole plasmid through recombination with the attP site inserted into the host silkworm genome. To construct the plasmid, the synthesized attB sequence was inserted into the AscI–FseI site of pBac[3xP3EGFPafm] (Horn et al. 2002). The nucleotide sequences of these plasmids are available at GenBank (accession nos.: vector plasmid, AB779766; donor plasmid, AB779767). The plasmid pET11phiC31-polyA (Fig. 1c) (Groth et al. 2004) for the synthesis of phiC31-integrase mRNA was obtained from Dr. M. P. Calos (Department of Genetics, Stanford University School of Medicine, Stanford, CA, USA).

Purification of plasmid DNA and synthesis of mRNA

The plasmid DNAs for injection into the silkworm embryos and the synthesis of mRNA were purified using a HiSpeed Plasmid Midi Kit (Qiagen, Hilden, Germany). To avoid RNaseA contamination, the purified plasmid was treated with proteinase K and extracted with phenol/chloroform. The vector and donor plasmids were then extensively washed with 70 % ethanol, vacuum-dried, and dissolved in injection buffer (5 mM KCl/0.5 mM phosphate buffer at pH 7.0). The plasmid pET11phiC31-polyA for synthesis of mRNA was dissolved in distilled water. The DNA was linearized by double digestion of *Eco*RV and *Bam*HI, and the digested plasmid DNA was treated with proteinase K, extracted with phenol/chloroform and chloroform,

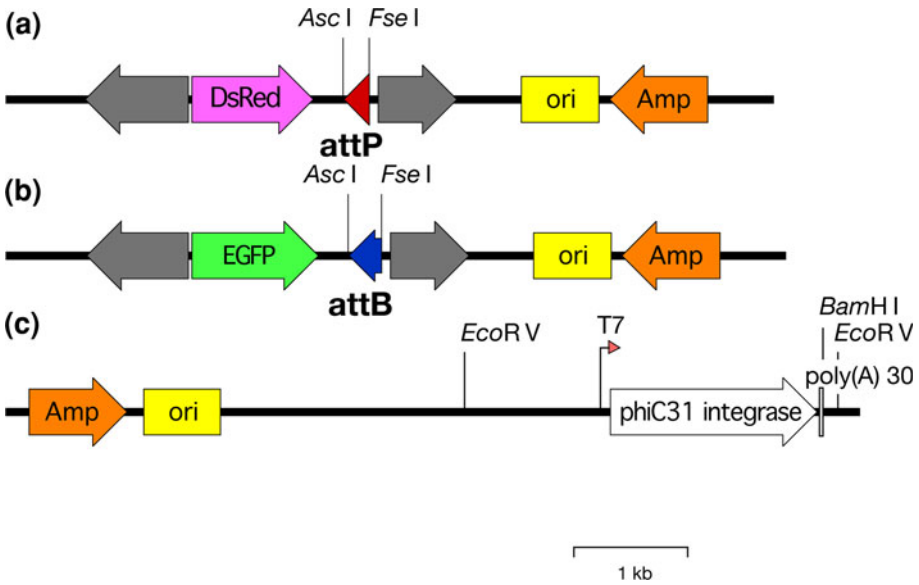

Fig. 1 Physical maps of the vector plasmid pBac[3xP3DsRedattP] (**a**), donor plasmid pBac[3xP3EGFPattB] (**b**), and template plasmid pET11phiC31-polyA (**c**) for the synthesis of phiC31-integrase mRNA. *Filled gray arrow* arm of the transposon *piggyBac*; *DsRed*: *DsRed* gene under the control of eye-specific promoter 3xP3; *EGFP*: *EGFP* gene under the control of eye-specific promoter 3xP3; *white arrow* phiC31 integrase with poly(A) signal; *red triangle* 223-bp attP sequence; *blue arrow* 287-bp attB sequence; *ori* origin of replication; *Amp*: ampicillin-resistant gene

precipitated with three volumes of ethanol, dissolved in distilled water, and used as a template. The mRNA was synthesized by an mMESSAGE mMACHINE T7 Kit (Ambion, Austin, TX, USA) and stored at −80 °C.

Extrachromosomal assay and injection of DNA into the embryos

The extrachromosomal assay was performed using a previously reported method (Yonemura et al. 2012). Briefly, the two plasmids pBac[3xP3DsRedattP] and pBac [3xP3EGFPattB], each at a concentration of 0.2 μg/μL, were mixed, and the phiC31-integrase mRNA was added at the same concentration. The mixed solution of plasmids and mRNA was injected into the embryos 4–6 h after egg-laying, and the eggs were incubated for 3 days at 25 °C. Then plasmid DNA was purified from the eggs and transformed into *Escherichia coli*. The plasmid DNA was purified from the colony grown on an LB-ampicillin plate, and the restriction pattern was analyzed by agarose gel electrophoresis after digestion with PstI.

Generation of the host strain

To create the host strain, we used the ordinary method to generate transgenic silkworms using the transposon *piggyBac* as a vector (Tamura et al. 2000, 2007): the vector plasmid pBac[3xP3DsRedattP] and helper plasmid were injected into the embryos in the preblastodermal stage, and G1 embryos expressing DsRed in stemmata were selected as transgenic individuals. The silkworms expressing DsRed were pooled and mated with each other. The progenies of the matings were used as hosts for site-specific integration by phiC31 integrase.

Site-specific integration of plasmid DNA into the attP site of the silkworm genome

To perform site-specific integration, we prepared an injection solution by dissolving pBac[3xP3EGFPattB] and phiC31 integrase mRNA, each at a concentration of 0.2 μg/μL, in the injection buffer. We injected the solution into the host strain embryos using the same method described above and incubated the embryos at 25 °C until hatching. The hatched silkworms were raised, and the G1 generation was obtained by sib mating. We identified the G1 embryos expressing both DsRed and EGFP as the silkworms resulting from site-specific integration. The identified silkworms were mated with the w-1 strain to create the integration lines.

Structural analysis of the integrated DNA

The genomic DNA of the silkworm was purified from the posterior silk gland on the third day of the fifth instar using a genomic-tip 20/G column (Qiagen). The primers used for the analysis are shown in Table 1. For inverse PCR, purified genomic DNA was triple-digested by Nhe I, Spe I, and XbaI and self-ligated using T4 DNA Ligase (New England Biolabs Inc., Ipswich, MA, USA). The PCR reaction mixture contained a pair of primers (phDReR18 and phDReF17), the self-ligated genome DNA, each dNTP, and KOD FX Neo (Toyobo, Osaka, Japan). The resulting PCR fragments were separated on 1 % agarose gels and purified using a Zymoclean Gel DNA Recovery Kit (Zymo Research, Irvine, CA, USA). The integration site was sequenced, and the obtained sequence was searched for in the KAIKObase (http://sgp.dna.affrc.go.jp/KAIKObase/index.html).

Table 1 List of primers used in the experiment

Primer name	Sequence
phDReF15	5′-CAGCCAACGTCAAGCGGTAGTGA-3′
phDReF17	5′-GCACCTTGAAGCGCATGAACTC-3′
phDReR18	5′-AGTTCCAGTACGGCTCCAAGGTGTA-3′
pUCoriR01	5′-GCACCGCCTACATACCTCGCTCTGCTAATC-3′
eGFPF0	5′-GCACAAGCTGGAGTACAACTACAACAG-3′
Y14-1F11	5′-CGGCGCATCAAGAACTTCGGCTACC-3′
Y14-1F23	5′-ACCTAAGGGAACCAAAGATATTAACCCT-3′
Y14-1R11	5′-CGAGACATGCCATTTCAGTTACGAT-3′
Y14-1R24	5′-GACTTTTACCCTTCTTTAACCCTAGAAAGA-3′
Y16-1F01	5′-CCACGTGCGCTTGCAGCTGAGTAGAC-3′
Y16-1R00	5′-TCGTCGCCCCGTTGCTCTTTTCA-3′
Y16-1R01	5′-CAGATATTGCTAACTAAACCACGACAGACA-3′

To analyze the recombination site attL of line 1, a 4.9-kb DNA fragment was amplified using a pair of primers (Y14-1F11 and pUCoriR01) from the genomic DNA and purified using the Zymoclean Gel DNA Recovery Kit after agarose gel electrophoresis. For the sequence of the attR site in line 1, a 3.3-kb fragment was amplified using the primers Y14-1R11 and eGFPF01. For analyses of the attL and attR sites in line 2, 4.3- and 1.8-kb fragments were amplified using the primers Y16-1F01 and PUCoriR01 and using the primers Y16-1R01 and eGFPF0 from the genome DNA, respectively. The sequences of the recombination sites (attL and attR) of the purified fragments were determined using the sequence primer phDReF15. The entire region of the transgene was amplified by long-distance PCR. The primers Y14-1F23 and Y14-1R24 for line 1 and Y16-1F01 and Y16-1R00 for line 2 were used to amplify the entire region inserted into lines 1 and 2, respectively. The resulting PCR fragment was separated by 1 % agarose gel electrophoresis and purified using the Zymoclean Gel DNA Recovery Kit. The purified DNA fragment was digested by EcoR I or EcoR V and analyzed by agarose gel electrophoresis.

Results

Measurement of the efficiency of phiC31-integrase-mediated recombination between the attP site of the vector plasmid and the attB site of the donor plasmid

To gauge how efficiently the phiC31-integrase-mediated site-specific integration occurs between the attP site of the vector plasmid pBac[3xP3DsRedattP] and the attB site of the donor plasmid pBac[3xP3EGFPattB], we measured the frequency of recombination between the two plasmids in silkworm embryos. We used a previously reported extrachromosomal assay method (Yonemura et al. 2012) to measure efficiency; we injected the vector and donor plasmids together with in-vitro-synthesized phiC31-integrase mRNA into embryos in the preblastodermal stage and recovered the plasmids 3 days after injection. The recovered plasmids were transformed into *E. coli* and colonies were selected from LB plates containing ampicillin. The plasmid was purified from each colony and subjected to restriction pattern analyses. We performed two independent experiments, and in both cases the majority of the plasmids investigated were positive for the integration (Table 2). In the first and second experiments, 22 out of 29 plasmids and 39 out of 43 plasmids, respectively, were found to be the recombination products. The recombination frequencies were calculated to be 78 and 91 %, respectively (Table 2). In a previous study using a similar plasmid-based recombination-mediated cassette exchange (Yonemura et al. 2012), the highest frequency was about 80 %. In the present study, the frequency of recombination between the vector and donor plasmids was comparable to the highest frequency in that previous study, suggesting that the enzyme synthesized from the injected mRNA efficiently mediates the recombination between the attP and attB sites of the newly constructed vector and donor plasmids in silkworm embryos.

Table 2 Frequency of integration between the two plasmids pBac[3xP3DsRedattP] and pBac[3xP3EGFPattB] in the extrachromosomal assay in the silkworm embryos

Experiment no.	Number of plasmids examined	Number of plasmids without integration	Number of plasmids produced by integration (%)	Number of plasmids not identified
1	29	7	22 (78)	0
2	43	4	39 (91)	0

Generation of host strain and site-specific integration mediated by phiC31 integrase

In general, the integration of transgenes mediated by phiC31 integrase is designed to occur between the pre-inserted genomic attP and attB sites of the donor plasmid. It has been reported that the frequency of integration is high when the genome possesses the attP site and the attB sequence is used as the donor plasmid compared to that between the genomic attB and attP in the donor (Thyagarajan et al. 2001; Groth et al. 2004). Therefore, we generated a host strain with the attP site using the ordinal transposon-mediated germline transformation method.

Table 3 Generation of host strain for site-specific integration of transgenes using phiC31 integrase and the germline transformation method using the transposon *piggyBac*

Host strain	Number of injected embryos	Number of hatched embryos	Number of total G1 broods	No. of G1 broods with DsRed-positive embryos (%)
w1-pnd	564	420	108	36 (33)

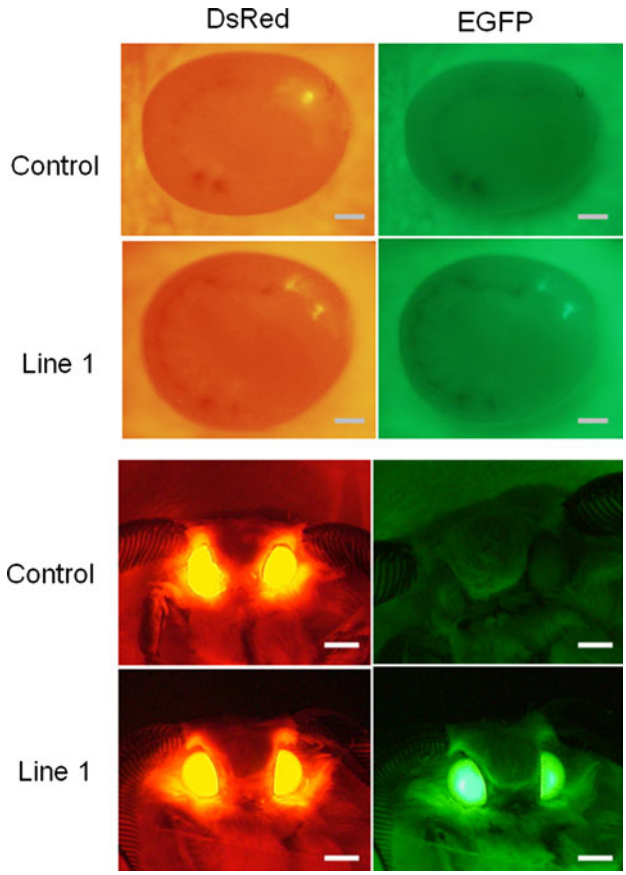

Fig. 2 Embryo stemmata and adult compound eyes of the host strain that possessed only 3xP3DsRed (control), and recombinant line 1 in which the whole sequence of pBac[3xP3EGFPattB] was integrated into the attP site of the host strain. The photo was taken under a fluorescent microscope equipped with DsRed and EGFP filters. *Gray* and *white bars* indicate 1 mm and 1 cm, respectively

Injection of the vector plasmid together with the helper plasmid pA3PIG (Tamura et al. 2000) into the embryos generated many transgenic silkworms with an attP sequence marked with 3xP3DsRed (Table 3; Fig. 2, control). We pooled these transgenic silkworms and used them as the host strain. Then we injected the mixed solution of the donor plasmid and phiC31-integrase mRNA into the embryos of the host strain and selected embryos expressing DsRed and EGFP in the next generation. Most G1 embryos were DsRed-positive and EGFP-negative. However, we

Table 4 Frequency of appearance of GFP-positive G1 silkworms following the injection of vector plasmid and phiC31 integrase mRNA into eggs

Experiment no.	Number of injected embryos	Number of hatched embryos	Number of total G1 broods	No. of G1 broods with EGFP-positive embryos (%)
1	600	N	72	1 (1.4)
2	575	228	58	3 (5.2)

N not counted

Table 5 Numbers of embryos expressing EGFP in the positive G1 brood

Positive G1 brood number	Number of total eggs	Number of EGFP-positive embryos (%)
1-1	310	5 (1.6)
2-1	295	65 (22)
2-2	286	45 (16)
2-3	382	25 (6.5)

obtained one brood that produced embryos expressing DsRed and EGFP in the first experiment, and obtained three positive broods in the second injection (Table 4; Fig. 2). The frequency of the appearance of the EGFP-positive broods when using phiC31 integrase was much lower (1.4 or 5.2 % after the first or second injection, respectively; Table 4) than that generated using the transposon (see Table 3). The number of embryos expressing EGFP in each positive brood is shown in Table 5, and is not much different to the number seen in an experiment to generate the transgenic silkworm using the transposon as a vector (Tamura et al. 2000; Uchino et al. 2007).

To establish integration lines, we backcrossed G1-positive moths obtained from the experiments with the adults of the w-1 strain more than three times and constructed four lines expressing DsRed and EGFP. We maintained one line from each positive brood and designated them lines 1–4. To examine the stability of the integration in successive generations, moths expressing DsRed and EGFP were mated with moths of the w-1 strain, and the numbers of embryos expressing DsRed and EGFP were counted in the next generation (Table 6). The constructed lines segregated the embryos expressing both DsRed and EGPF at a ratio of 1:1. No exceptional embryos appeared in the crosses, suggesting that DsRed and EGFP are linked on the silkworm chromosome.

Analysis of nucleotide sequences at the integration sites

When phiC31-mediated site-specific integration occurs at the chromosomal attP site of the host strain, the whole

Table 6 Segregation of the silkworms expressing only 3xP3-DsRed and both DsRed and EGFP in the backcross with the host strain

Line no.	Number of embryos examined	Number of embryos not expressing both DsRed and EGFP	Number of embryos expressing only DsRed (%)	Number of embryos expressing only 3xP3 EGFP (%)	Number of embryos expressing both DsRed and EGFP (%)
1	126	61	0	0	65
2	196	99	0	0	97
3	109	56	0	0	53
4	77	39	0	0	38

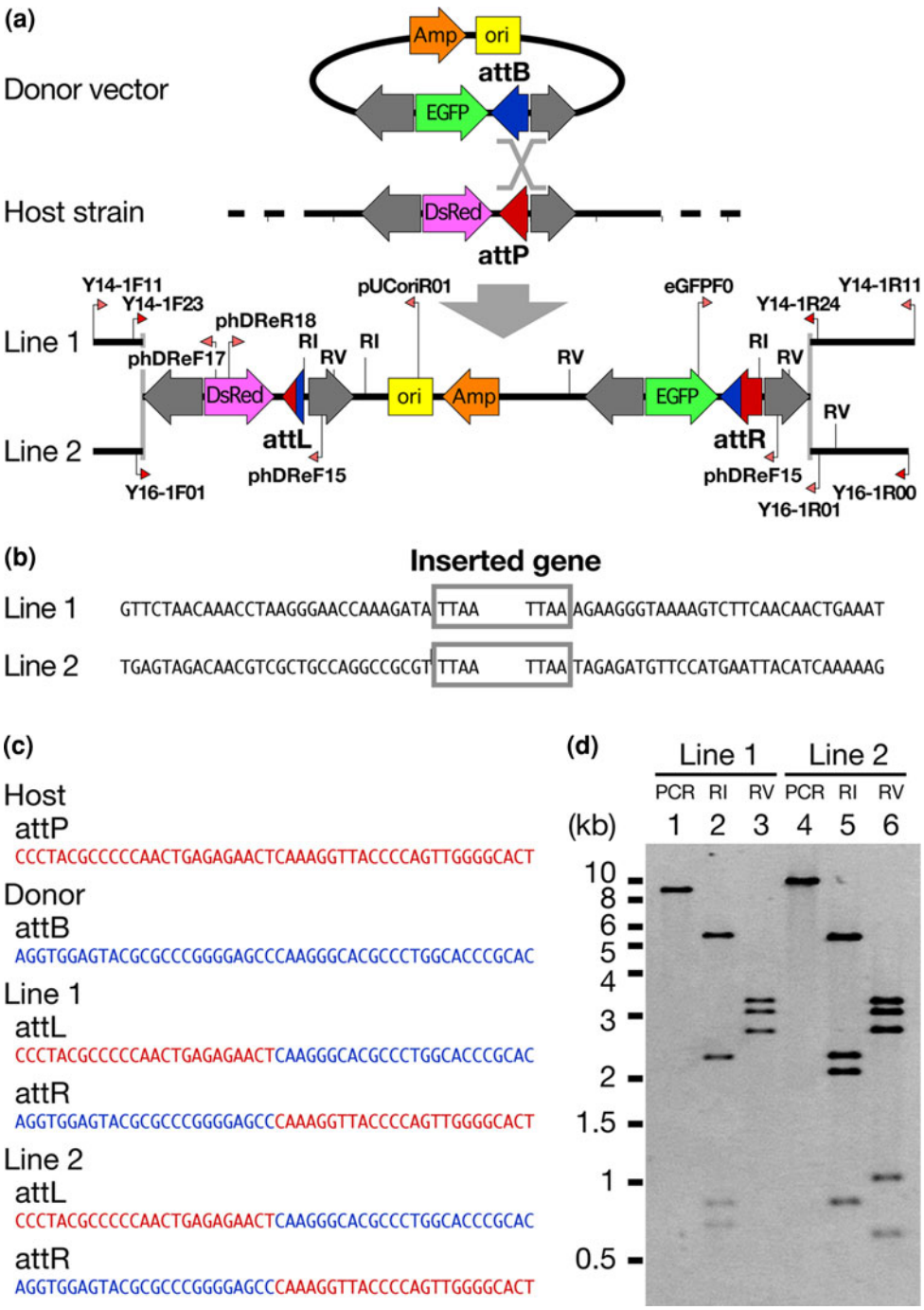

Fig. 3 Illustration of the site-specific integration of donor plasmid pBac[3xP3EGFP-attP] into the attP site of the host strain (**a**), sequences of the flanking region of the gene inserted into the chromosome (**b**), sequences of the attL and attR sites of the lines (**c**), and analysis of the PCR fragments (**d**) of the two integration lines 1 and 2. The *arrows with a red triangle* indicate the locations of the primers. RI and RV show restriction sites of EcoRI and EcoRV, respectively. The *red* and *blue letters* represent attP and attB sequences, respectively. *Lanes 1–6* indicate the analysis of the integration sites of lines 1 and 2 by agarose gel electrophoresis. *Lane 1* the amplified fragment of the entire region of the transgene in line 1; *lane 2* DNA fragments appeared following EcoRI digestion of the lane 1 fragment; *lane 3* DNA fragments appeared following EcoRV digestion of the lane 1 fragment; *lane 4* the amplified fragment of the entire region of the transgene in line 2, *lane 5* DNA fragments appeared following EcoR1 digestion of the lane 4 fragment, *Lane 6* DNA fragments appeared following EcoRV digestion of the lane 4 fragment

sequence of the donor plasmid containing the attB site is inserted into the genome (Fig. 3a). To determine how such a reaction occurred in the established lines, we first performed inverse PCR and determined the flanking sequences. We failed to determine the sequence for line 4, and the site of line 3 belonged to repetitious regions; therefore, we could not determine the chromosomal locations for lines 3 and 4. For lines 1 and 2, we determined that the insertion sites were in chromosomes 15 and 22, respectively (Fig. 3b). To examine whether the recombination occurred precisely at the target site, we amplified the left and right regions of the transgene using the primers designed from the genomic sequence near the integration sites and internal sequence of the transgene, and then determined their nucleotide sequences. As shown in Fig. 3c, the results from sequencing indicated that the recombination occurs precisely between the attP site of the host genome and the attB site of the donor plasmid, forming attR and attL sites. Then we amplified the entire region of the transgene to analyze the structure of the integrated gene. An approximately 10-kb fragment was amplified from line 1 (Fig. 3d, lane 1). The size of the amplified fragment agreed with that predicted in Fig. 3a because the fragment includes the 35-bp flanking sequence, and the total size of the predicted transgene is 10,152 bp. We also analyzed the internal structure by studying the restriction pattern of the amplified fragment. The amplified fragment was predicted to possess three EcoRI and three EcoRV restriction sites, respectively (Fig. 3a). As expected, 6.0-, 2.5-, 0.9-, and 0.8-kb DNA fragments were detected in the gel resolving the EcoRI digest (Fig. 3d, lane 2). Similarly, the EcoRV digest generated 3.6-, 3.3-, and 2.9-kb fragments, although the 0.3-kb fragment was not detectable in the gel (lane 2). The analysis of line 2 showed exactly the same results observed for line 1 (lanes 4–6). We concluded from these results that the site-specific integration of phiC31 integrase occurs precisely as shown in Fig. 3a. No structural changes in the transgene occur during the process of integration, and the integrated gene is stably transferred to the progeny. We concluded that the site-specific integration method was established in the silkworm and that the established method would be a useful tool for the functional analysis of lepidopteran insect genes.

Discussion

We successfully developed a silkworm site-specific transgene integration system using phiC31 integrase. This is the first success in relation to site-specific transgene integration in lepidopteran insects, including the silkworm. The transgene was integrated into the target sequence attP of phiC31 integrase, which was pre-inserted into the genome of the silkworm by a *piggyBac* transposon. To generate the integration lines, we injected a mixture of a donor plasmid with the attB sequence and synthesized phiC31-integrase mRNA into the embryos. The integrated silkworms were identified by screening the embryos in the next generation. The frequency of the integration was lower than that of germline transformation using the transposon *piggyBac* as a vector, indicating that the method developed in this experiment requires further improvement.

In the fruit fly, *Drosophila melanogaster*, the construction of an integration line using phC31 integrase was initially performed using in-vitro-synthesized mRNA as the source of the enzyme (Groth et al. 2004; Fish et al. 2007). Then the line expressing the enzyme in the germline cells was constructed and used for most experiments (Bischof et al. 2007). Site-specific integration using phiC31 integrase has also been reported in other insects, such as mosquito and medfly (Nimmo et al. 2006; Schetelig et al. 2009; Labbe et al. 2010; Meredith et al. 2011). However, the efficiency of integration differs between *Drosophila* and other insects. In *Drosophila*, more than half of the female and male pairs produced integrated progenies (Groth et al. 2004; Fish et al. 2007; Bischof et al. 2007). The frequency in other insects is reportedly less than 5 %, which is comparable to the results we obtained using the silkworm. Therefore, our results appear to be reasonable for the first successful application of a phiC31-integrase system for site-specific integration into the silkworm. To increase the efficiency, as in *Drosophila,* it may be useful to construct silkworm lines that express phiC31 integrase in germline cells. In addition, the integrase gene used in the present study originated from a phage; therefore, the codon of the integrated gene may not be adapted for translation in the silkworm. Furthermore, minor changes to the primary structure of the integrase might be effective. In a previous study, mutational changes in the integrase gene increased the efficiency of integration in *Drosophila* (Keravala et al. 2009).

Although the development of an integration system enables the placement of a foreign gene exactly at a specific site, the genome of the host organism must have a target-site attP that is about 50 bp. The silkworm does not have such a sequence according to our BLAST search of the silkworm genome database. Therefore, insertion of the target sequence at the desired position in the silkworm can be used for the host strain without interference from similar endogenous sequences. In terms of how to insert the attP sequence into the specific site of the silkworm genome, a genome-editing method such as ZFN or TALEN would be useful. Such methods have already been successfully applied to the silkworm (Takasu et al. 2010; Sajwan et al. 2013; Ma et al. 2012).

One attractive application of this system is recombinase-mediated cassette exchange (Bateman et al. 2006). We

previously reported that cassette exchange occurred between two plasmids injected into silkworm embryos (Yonemura et al. 2012). The success of the site-specific integration of the present study suggests that recombinase-mediated cassette exchange is also possible between the silkworm genome and a plasmid. For further application, it will be important to develop the integration method for larger genes. A method for constructing a large vector of more than 100 kb was recently developed. The ordinal method using a transposon as a vector cannot introduce such a large DNA fragment. However, it has been reported that the method using phiC31 integrase can easily introduce such a large gene (Venken et al. 2006). The development of these tools using phiC31 integrase would be useful for the functional analysis of insect genes in the post-genomic era.

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