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https://doi.org/10.5109/16121

出版情報：九州大学大学院農学研究院紀要. 54 (2), pp.397-400, 2009-10-29. Faculty of Agriculture, Kyushu University
バージョン：
権利関係：

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J. Fac. Agr., Kyushu Univ., **54** (2), 397–400 (2009)

Construction of *piggyBac*–based Vectors Using Visible and Drug–resistance Marker for Introducing Foreign Genes into Silkworm Cultured Cells

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(*Received June 29, 2009 and accepted July 13, 2009*)

Transgenic organisms have been indispensable for modern genetic analysis, such as over expression or knocked–down of the genes of interest. Transposon is one of the most efficient tools for introducing foreign DNA sequences into host genome. DNA transposon, such as *piggyBac*, *Hermes*, *Minos*, *hobo*, and *mariner*, have been identified in insects and have been used successfully as vectors for germline tarsnformation in various insect species. *piggyBac*–based transformation vectors have been broadly used in generating transgenic silkworm. However, there are few studies reporting vectors for transformation of cultured *B. mori* cells. In this study, we constructed new *piggyBac*–based vectors pPigGate, which have visible and drug selectable marker, PuroDsRed or GFPZeo in cultured cells. In order to access the utility of these vectors, we introduced BmHop2 and BmMnd1, which are meiosis specific recombination proteins, into cultured *B. mori* cells using the pPigGate.

INTRODUCTION

Transgenic organisms including cultured cell lines are one of powerful tool for analyzing the functions of specific genes of interest. At present, very efficient gene transfer methods such as lipofection, electroporation and the virus–mediated transformation are available (Carbonell *et al*., 1985; Han 1996; Keith *et al*., 2000; Brunner *et al*., 2001; Myles *et al*., 2001). However, it is impossible to introduce foreign DNAs into all of the target cells, if any, using the effective methods. Nearly perfect introduction of foreign genes can be required in some cases of analyses. Combination of transposon mediated transformation into host chromosome and subsequent selection by appropriate marker is one of the effective method in introducing foreign genes into all cells. As for insects, most gene transfer studies were reported using fruit fly, *Drosophila melanogaster*, owing to the application of *P* element transposon for germline transformation (Handler *et al*., 1993; Rubin and Spradling, 1982). During a long history of germline transformation in *D. melanogaster*, several fluorescent transformation markers are proved to be effective in insect species (Horn *et al*., 2002). Recently, several different transposons, such as *piggyBac*, *Hermes*, *Minos*, *hobo*, and *mariner*, have been identified in insects and have been used successfully as vectors for germline tarsnformation in various insect species (Handler, 2001).

Domestic silkworm, *Bombyx mori*, is the model organism of lepidopteran insects.

B. mori is a useful model for genetic, biochemical, and physiological studies. Methods for transformation of the silkworm or silkworm cells have been developed which use vectors based on transposable element *piggyBac* (Tamura *et al*., 2000), *Minos* (Uchino *et al*., 2007) and ϕC31 (Nakayama *et al*., 2006). The stable germline transformation of *B. mori* was achieved by using *piggyBac* and fluorescent transformation markers (Tamura *et al*., 2000; Yamamoto *et al*., 2004; Tomita *et al*., 2003). However, there are few studies for introducing transgenes into chromosomes of cultured *B. mori* cells.

piggyBac is a class Ⅱ transposable element that was originally discovered as the IFP2 element from the lepidopteran insect cell line, TN–368 (Fraser *et al*., 1983). The *piggyBac* element transpose into a unique sequence, TTAA using accurate cut and paste mechanism. The *piggyBac* has also two terminal inverted repeat domains on both end of their sequence.

In this study, we construced new *piggyBac*–based vectors, pPigGate, which have minimal *piggyBac* terminal sequences and fluorescence and drug selectable marker (Li *et al*., 2005; Pfeifer *et al*., 1997; Tatsuke *et al*., in preparation).

MATERIALS AND METHODS

Plasmid construction

The *piggyBac* based constructs (named pPigGate, abbreviated to pPG) used in this study is summarized in Fig. 1 and Tab. 1. The plasmid pPigGate1.1 (Fig. 1A, abbreviated to pPG11), which contains *piggyBac* transposase recognition sequences (*piggyBac* L and R frag-

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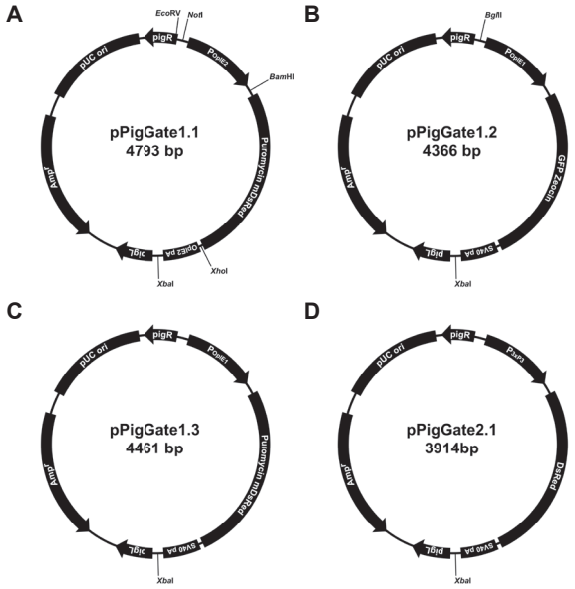

Fig. 1. Molecular structure of the *piggyBac*–based transformation plasmids, pPigGate.

ment) and PuroDsRed maker that is synthetic puromycin N–acetyltransferase and monomeric DsRed fusion gene, was used as a basic plasmid for the construction of pPG vectors.

To construct the pPG11, pPig1.4PuroDsRed (Tatsuke *et al*., in preparation) was digested with *Sac*Ⅱ, blunt–ended, and ligated into a *Apo*I/*Afl*Ⅲ blunt–ended site of pZErO–2 (Invitrogen), yielding pPigPuroGm/Km. The pPigPuroGm/Km was digested with *Avr*Ⅱ and *Cla*I, blut–ended, and ligated into a *Apo*I/*Afl*Ⅲ blunted site of pBluscriptⅡSK+ (pPigPuro–BSⅡ). The pPigPuro–BSⅡ was digested with *Xba*I and *Swa*I, blunt–ended, and self–ligated. The resulting plasmid was named pPG11.

OpIE1–GFPZeo marker cassette was amplified from pIZT/V5–His (Invitrogen) by PCR with the primer Z–GFP–ie1–5'Bg 5'–TAAGAGATCTTGGTCATGCGAAACACGCA C–3' and SV40polyA 3'–Xba 5'–CCCTCTAGAGATCCAG ACATGATAAGATACATTG–3'. The PCR product was digested with *Xba*I, and cloned into an *Eco*RV/*Xba*I site of the pPG11 (pPG12). *Xho*I and blunt–ended–*Eco*RI flagment of pPG11 was cloned into *Sal*I and blunt–ended *Pst*I site of the pPG12 (pPG13). 3xP3–DsRed flagment amplified from pBac[3xP3–DsRed] by PCR with the primer P3pro.5'–XmnI 5'–GGGGAACCAATTCGTTCCC ACAATGGTTAATTCGAGCT–3' and P3–DsRed3' 5'–TAC GCCTTAAGATACATTGATGAGTTTGGA–3', and cloned into a *Eco*RV/*Msc*I site of the pPG11 (pPG21).

To generate the Gateway based expression cassette, the destination plasmid pie2FW and pie2HW (Yamashita *et al*., in preparation), which contains the IE–2 promoter from the *Orgyia pseudotsugata* nuclear polyhedrosis virus (OpNPV; the promoter is abbreviated as OpIE2), 3xFlag or HA peptide tag N–terminal fused *att*R1–*ccdB*–*att*R2 cassettes, were used as forward oriented DEST cassettes. 3xFlag or HA peptide tag fused expression cassette were amplified from pie2FW or pie2HW plasmid by PCR with the primer pBend23A 5'–TGACGATGAG CGCATTGTTAGATTTCATAC–3' and OplE2pA–3' 5'–CA CGCGCTTGAAAGGAGTGTGTAAATGGAC–3', and introduced into blunt–ended *Xba*I site of the pPG12 or pPG13. The resulting plasmids were named pPG122, pPG131 and pPG132 (Tab. 1). All 3 destination vectors bear the OpIE2 promoter and the polyadenylation signal, as well as the Gateway DEST cassette containing the *att*R1, *ccdB* gene, and *att*R2.

Thus formed construct (pPGs) was subjected to the Gateway® LR reaction with entry vectors, using LR Clonase™ Enzyme Mix (Invitrogen) according to the protocols recommended in manufacturer's manual. To construct reporter plasmids, BmHop2–pENTR11 and BmMnd1–pENTR11, which were reported previously (Tobata *et al*., 2007), were subjected to LR reaction with the pPG vectors. The final form of the above–described vectors will be called pPG122[BmHop2] and pPG131 [BmMnd1].

Cell culture

B. mori BmN4 cells (from a stock of laboratory of Insect Pathology and Microbial Control, Faculty of Agriculture, Kyushu University Graduate School) were maintained in IPL–41 insect medium (Sigma Chemical) supplemented with 10% fetal bovine serum (GIBCO Invitrogen) at 27℃. Before use for transfection, the medium was replaced by Sf–900 SFMⅡ serum free culture medium (Invitrogen).

Transformation of cells

To select the transformed cells constitutively express-

Table 1. List of pPigGate plasmids

Plasmid	Abbreviation	Marker		Expression cassette
		E. coli	Cell or Silkworm	
pPigGate1.1	pPG11	Amp^r	OpIE2–PuroDsRed	–
pPigGate1.2	pPG12	Amp^r	OpIE1–GFPZeo	–
pPigGatge1.3	pPG13	Amp^r	OpIE1–PuroDsRed	–
pPigGate2.1	pPG21	Amp^r	3xP3–DsRed	–
pPigGate1.2.2	pPG122	Amp^r	OpIE1–GFPZeo	OpIE2–3xFlag–DEST
pPigGate1.3.1	pPG131	Amp^r	OpIE1–PuroDsRed	OpIE2–HA–DEST
pPigGate1.3.2	pPG132	Amp^r	OpIE1–PuroDsRed	OpIE2–3xFlag–DEST

ing HA–Mnd1 and Flag–Hop2, *B. mori* cultured cells (5.0×10^4 per well) were seeded and co–transfected with 0.5 μg of the transformation plasmids pPG122[BMHop2], pPG131[BmMnd1] and 0.5 μg of transposase expressing *piggyBac*–helper plasmid pA3helper (Cary *et al*., 1989), using PDD111 solution (Maeda *et al*., 2005). After 1 week of transfection, the cells selected for Puromycin–resistance by adding, to the medium, 10 μg puromycin per *ml* and 400 *ng* zeocin per *ml*. Resulting transformed cells were observed by using a KEYENCE BZ–8000.

RESULTS AND DISCUSSION

Construction of pPigGate

piggyBac based vector pPigGate have been constructed by modifying the pPig1.4PuroDsRed vector as described under MATERIALS AND METHODS and summarized in Fig. 1 and Tab. 1. The pPGs have PuroDsRed or GFPZeo marker cassette, the 3xFlag or HA peptide tag N–terminal fused Gateway DEST cassette containing *att*R1, *ccdB* gene, and *att*R2, and minimal *piggyBac* sequence (Li *et al*., 2005). The PuroDsRed is used as a marker gene, which permits screening of transformed cells with puromycin, and the efficiency of selection is possible to be monitored by the fluorescence of mDsRed (Tatsuke *et al*., in preparetion). The GFPZeo consists of zeocin resistant domain and GFP for selection and monitoring, respectively (Pfeifer *et al*., 1997). The Gateway DEST cassette is used for efficient and easy subcloning of genes in entry vectors containing the *att*L1 and *att*L2 sequences by lambda transposase–mediated recombination. 3xFlag and HA peptide tag allow us to detect foreign gene expression introduced into cultured cells by commercially available anti–peotide antibodies. The minimal *piggyBac* sequence is previously reported to be sufficient for movement of *piggyBac* vectored sequences from plasmids into the insect genome (Li *et al*., 2005).

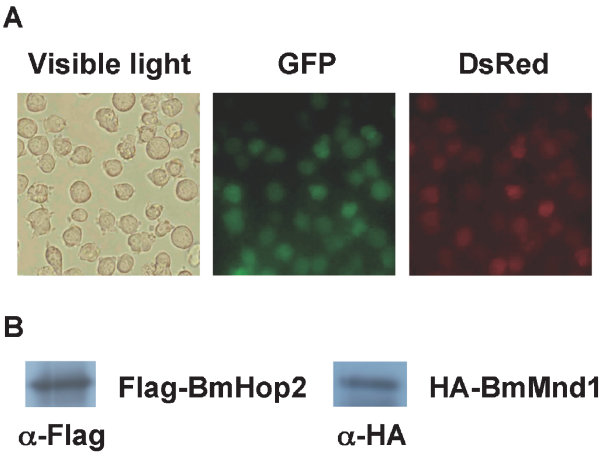

Fig. 2. The BmN4 cells stably expressing Flag–Hop2 and HA–Mnd1 introduced by pPigGate plasmids. (A) Expression of visible and drug–resistance markers, GFPZeo and PuroDsRed, in doble stable BmN4 cells. (B) Western blotting was performed on lysate of double stable BmN4 cells using antibodies against Flag or HA (1:2000 dilution).

Establishment of the BmN4 cell stably expressing Flag–Hop2 and HA–Mnd1

In order to verify the applicability of the pPGs, we subcloned cDNA encoding *B. mori* Hop2 and Mnd1 as a meiotic–specific recombinase protein, into pPG122 and pPG131, respectively (Tobata *et al*., 2007). The resulting plasmids, pPG122[Hop2] and pPG131[Mnd1] were transfected into BmN4 cells and stably transformed cells were selected by puromycin or zeocin as described in MATERIALS AND METHODS.

Microscopic observation of the BmN4 cells transformed with pPG122[Hop2] and pPG131[Mnd1] exhibited the expression of fluorescent maker genes, GFP or DsRed (Fig. 2A). The transformed cells were, then, subjected to Western blot analysis to confirm the expression of target genes, Flag–Hop2 and HA–Mnd1. (Fig. 2). As expected, anti–Flag or anti–HA antibodies detected the Hop2 and Mnd1 expression in the BmN4 cells. There results demonstrated that the pPGs constructed the present study are useful to introduce the genes of interest into cultured insect cells.

ACKNOWLEDGEMENTS

This work was supported in part by the Program for Promotion of Basic Research Activities for Innovative Biosciences (PROBRAIN) and by a grant from the Ministry of Agriculture, Forestry and Fisheries of Japan (Integrated research project for plant, insect and animal using genome technology INSECT–1201).

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