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Targeting of human aFGF gene into silkworm, *Bombyx mori* L. through homologous recombination*

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Abstract: The long-arm and short-arm genes of fibroin light chain (L-chain) of silkworm, *Bombyx Mori* L., and the gene of human acidic fibroblast growth factor were cloned respectively and subsequently inserted into a transfer vector pVL1392 used as a tool to target the L-chain region of the silkworm genome. Genomic DNA from their offsprings was extracted and the expected targeting was detected using polymerase chain reaction and DNA sequencing, as well as protein analysis. The results showed that positive events occurred and that the FGF gene was integrated into the L-chain locus through homologous recombination.

Key words: Gene targeting, Recombinant vector, *Bombyx mori* L.
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INTRODUCTION

Transgenic technology developed over the 20 years, has become a common tool widely applied to livestock species because it is technically quite simple, requiring only the injection of 'naked' DNA into the nucleus of a fertilized eggs (Hammer *et al.*, 1985; Suraokar and Bradley, 2000). However, this technique has limitations in that the injected DNA integrates randomly into the genome and thus could not be expressed in the desired tissue or at the appropriate level. More importantly, with this technique, it is not possible to specifically modify the genes of the species itself, although it can add new genetic information. In order to overcome this shortcoming, great effort was exerted to produce gene-targeted animal in which the aim-gene was inserted correctly into the desired locus on the genome. Definitely, this approach has great significance and might bring numerous biomedical benefits: for example, ablation of xenoreactive transplantation antigens, inactivation of genes responsible for neuropathogenic disease and precise placement of transgenes designed to produce proteins for human therapy. Gene-targeting was successful firstly in mouse (Thompson *et al.*, 1989) but has not been achieved in other mammals because functional embryonic stem (ES) cells like those in mouse have not been derived until the latest report (Suraokar and Bradley, 2000) of a breakthrough in this field: gene-targeted sheep was successfully produced by nuclear transfer from cultured somatic cells which were treated by transfection and drug selection in advance (McCreath *et al.*, 2000).

Relatively, gene-targeting in insects lags behind. Early studies indicated that the plasmid DNAs injected into silkworm eggs were rapidly degraded

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(Nataraju *et al.*, 1996). For this reason, other researchers' use of a recombinant AcNPV as vehicle to target the intrinsic genome proved to be effective (Yamao *et al.*, 1999). Besides, stable germ line transformation in the silkworm was achieved by using a piggyBac transposon-derived vector (Toshiki *et al.*, 2000). A gene-targeting (in *Drosophila*) experiment reportedly applied the technique of using the organism's endogenous machinery of DNA repair and recombination to substitute one allele for another at a targeted gene or to integrate DNA at a target locus, as directed by DNA sequence homology (Rong *et al*., 2000). However, these techniques were relatively complicated and time-consuming. Although AcNPV cannot infect the silkworm so serious as to cause death, the growth and development of silkworm were seriously inhibited due to the expression of the ecdysteroid UDP-glucosyltransferase (EGT) gene of AcNPV (Oreilly and Miller, 1990; Flipsen *et al.*, 1995), causing difficulties in the following experiments.

Baculovirus expression vector system (BEVS) is the famous workhorse for large-scale production of heterologous proteins required for research, industry and medical application. The combination of BES and silkworm is very powerful due to its high-level expression and low-cost. Human aFGF and bFGF were already successfully produced in silkworm larvae in our previous studies (Wu *et al.*, 2001; 2003; 2004); but the expression is transient and finally causes the larvae to die due to the virus infection. For this reason, we would like to produce a new silkworm species which carries human aFGF gene and thus can express the FGF permanently. As fibroin is a natural protein and has many excellent properties, it is being exploited currently for applications in medicine, for example as sewing thread in surgical operation. Moreover, because the fibroin, which consists of heavy (H) and light (L) chains, is one major protein of silk and the most abundant protein produced by the silkworm, the fibroin promoter would be a useful promoter for the production of large quantities of heterologous proteins. We have an inspiration to utilize this promoter to produce the fibroin-FGF fusion protein and hope it can be directly applied in medicine. As the fibroin is secreted through spinning, thus the fibroin-FGF is easily available without the complicated operation of protein purification. We report here a simpler way to target human aFGF gene into silkworm by using a transfer vector.

MATERIALS AND METHODS

Materials

The transfer vector pVL1392 was a product of Pharmingen (San Diego, CA, USA). Lipofectin reagent was purchased from Life Technologies (Gaitherburg, MD, USA). DNA Extraction Kit was purchased from Stratagene (La Jolla, California, USA). PCR amplification kit, enzymes for DNA manipulation and proteinase K were obtained from Takara Biomedicals (Kyoto, Japan). The TA cloning kit was a product of Invitrogen Corp. (San Diego, CA, USA). A DNA Sequencing kit was obtained from PE Applied Biosystems (Foster City, CA, USA). Nonidet P40 was a product of Sigma (St. Louis, Missouri, USA). Other chemicals were from Wako Pure Chemicals (Osaka, Japan). The serum against human aFGF was prepared in our lab.

Two silkworm strains (commercial name: *Koishimaru* and *Jingsong.Haoyue*) were used in this experiment. The larvae were reared with artificial diet through all larval stages. The rearing conditions were: 29 °C during 1st–3rd instar and 25 °C for 4th–5th instar, natural humidity.

Cloning of the fibroin L-chain and human acidic fibroblast growth factor gene

Silkworm genome was extracted from the silkgland of the 5th instar larvae according to the instruction manual of DNA Extraction Kit (*Stratagene*). The long-arm (L-arm) gene spanning exon 2 and part of exon 7 (5 kb), and short-arm (S-arm) gene spanning the remaining part of exon 7 (0.53 kb) of the fibroin L-chain (Fig.1a) were cloned by polymerase chain reaction (PCR) using the following synthesized primers respectively:

Primer 1:5'-AGCTGCAGGCGTACCTGGT CGCATTAAC-3' (*Pst* I);

Primer 2: 5'-TTTGAGCGGCCGCAACTAAGCCGGTCG-3' (*Not* I);

Primer 3: 5'-GGCCATATGGCTAATGCTCAAAG-3' (*Nde* I);

Primer 4: 5'-ATCTAGACAACAGTACCGAAATCC-3'(*Xba* I).

Primers 1 and 2 were designed for long-arm and primers 3 and 4 were designed for short-arm gene. The PCR reaction mixture contained 1.0 μl of the silkworm genome DNA (0.1 μg) as template, 2.0 μl of 2.5 mmol/L dNTP, 1.0 μl (20×10^{-12} mol/μl) of each primer, 0.5 unit of Taq DNA polymerase (Takara Ex Taq, Takara Biomedicals, Japan), and 2.5 μl of 10-fold PCR buffer (100 mmol/L Tris-HCl, pH 8.3 containing 500 mmol/L KCl and 15 mmol/L $MgCl_2$) in a final volume of 25 μl. The reaction was run for 30 cycles using a thermal cycler (Gene Amp PCR System 2400, PE Applied Biosytems). The reaction conditions for long-arm gene were: denaturing at 95 °C for 50 sec, annealing at 64 °C for 50 sec, and extending the reaction at 72 °C for 8 min. The extension step in the last cycle was run for 10 min, and for short-arm gene were: denaturing at 95 °C for 50 sec, annealing at 57 °C for 30 sec, and extending reaction at 72 °C for 1 min. Human aFGF gene was cloned using primers 5 and 6:

Primer 5: 5'-GCGGCCGCTTTTAATCTGCCTCC-3' (*Not* I);

Primer 6: 5'-CATATGATCAGAAGAGGCAGG-3' (*Nde* I).

The template was the pAcGP67b-aFGF constructed in our previous work (Wu *et al.*, 2001). The reaction conditions were: denaturing at 95 °C for 50 sec, annealing at 55 °C for 30 sec, and extending reaction at 70 °C for 1 min. The PCR products were analyzed by electrophoresis on a 1% agarose (Nacalai Tesque, Kyoto, Japan) gel (Fig.1b). Thereafter, the products were cut and purified from 0.8% agarose (Sea Plaque GTG Agarose, FMC, Rockland, ME, USA) gel by degradation with agarose (FMC) and precipitation with ethanol.

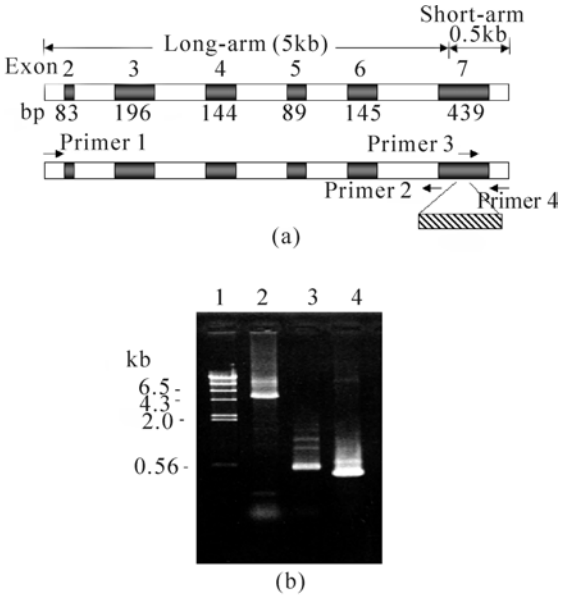

Fig.1 (a) Demonstration of genome structure of silkworm fibroin L-chain; (b) Amplified fragments of long-arm, short-arm and aFGF gene using polymerase chain reaction

Lane 1: λDNA/HindIII marker (size in kb); Lane 2: long-arm gene (5 kb); Lane 3: short-arm gene (0.53 kb); Lane 4: human aFGF gene

Construction of gene-targeting vector

The flow-chart for constructing the gene-targeting vector is shown in Figs.2 and 3. The long-arm of the fibroin L-chain was inserted into the sites of *Pst* I and *Not* I in transfer vector pVL1392 to obtain pVL1392.long-arm and subsequently treated with *Not* I and *Xba* I. The PCR product of aFGF was directly cloned into plasmid pCR2.1 vector and subsequently treated with *Nde* I and *Xba* I where the short-arm of the fibroin L-chain was inserted. Then the aFGF-short-arm fra-

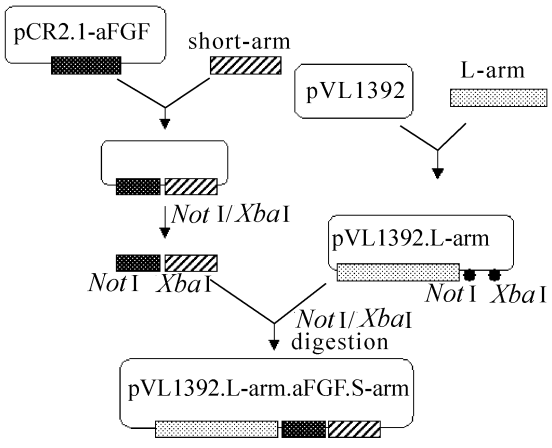

Fig.2 Strategy for construction of the recombinant transfer vector

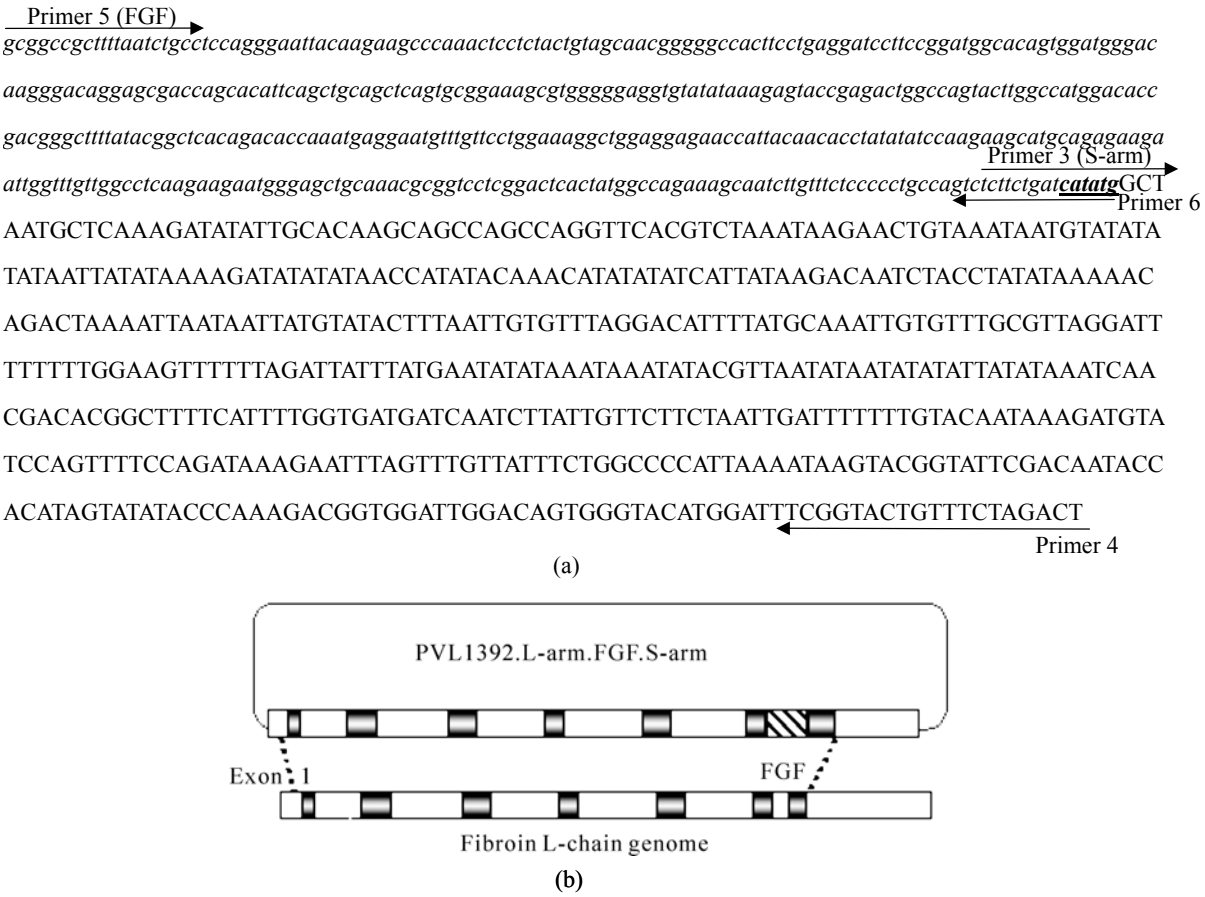

Fig.3 (a) Junction nucleotide sequence of aFGF and S-arm of L-chain (The aFGF sequence is shown in italics. A *Nde* I site between aFGF and S-arm is underlined. The primers used to detect the targeted individuals are indicated by an arrow); (b) Predicted homologous recombination between the constructed vector and fibroin genome

gment was excised with *Not* I and *Xba* I and recollected from agarose gel, and was inserted into the above *Not* I and *Xba* I sites of pVL1392.long-arm. The constructed pVL1392.L-arm.aFGF.S-arm vector was amplified and dissolved in distilled sterile water.

Treatment of the gene-targeting vector

The vector pVL1392.L-arm.aFGF.S-arm was mixed with cationic lipofectin reagent as follows: 50 μl of vector DNA (0.85 μg/μl), 160 μl of lipofectin reagent (1.0 μg/μl) and 2.8 ml of distilled sterile water. Gentle mixing and incubation at room temperature for 10–15 min before injection into the silkworm larvae.

Injection of vector into the silkworm larvae

The 1st-day female larvae of the 3rd instar were used for experimental treatment. Fifty microliters of mixture solution of vector and lipofectin reagent was injected subcutaneously into the abdominal segment where the ovary was located. The female moths were mated with normal males and arranged to lay eggs individually.

Genome extract and PCR

The genome of F1 eggs was extracted from one-fourth of the eggs of single female moth (shown in Fig.4a). Larval individual genome was prepared from their hematocytes. Hemolymph (100–200 μl) was collected on ice from one larva by stabbing the leg with a needle and the hematocytes were quickly precipitated by low-speed centrifugation. After washing with PBS, the cells were dissolved in 50 μl of PCR lysis buffer (50 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 10 mmol/L Tris-HCl pH

8.5, 0.5% Nonidet P40, 0.5% Tween and 400 μg/ml proteinase K) and kept at 65 °C for 30 min. Proteinase K was inactivated at 95 °C for 10 min. The supernatant was used as genome for template. The PCR reaction was conducted for 35 cycles, with reaction conditions described above when using primers 5 and 6. In the case of PCR with primers 5 and 4, the following conditions were applied: 95 °C for 50 sec, 57 °C for 30 sec, and 72 °C for 90 sec.

Nucleic acid analysis

DNA sequencing was performed with a nucleotide sequencer (Model 377, PE Applied Biosystems). The reaction conditions of PCR for sequencing were: 96 °C for 10 sec, 50 °C for 5 sec, and 60 °C for 240 sec with 25 cycles. The PCR reaction mixture contained 1.0 μl of template DNA (0.5 μg/μl), 4.0 ml of DNA sequencing kit (ABI PRISMTM, Dye Terminator Cycle Sequencing Ready Reaction) and 0.5 ml (3.2×10^{-12} mol/μl) of universal and reverse primers in final volume of 10 μl.

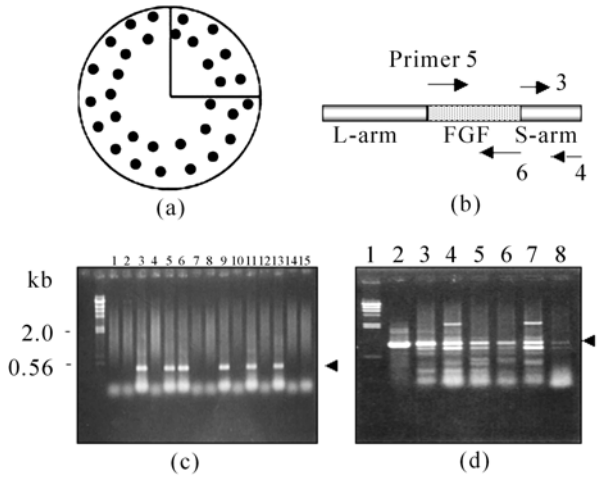

Fig.4 (a) One-fourth of eggs laid by one female moth was used to extract the genome; (b) The primers 5 and 6 were primarily used to amplify the aFGF gene, and in the positive case primers 5 and 4 were subsequently used to confirm the aFGF-S-arm fusion gene; (c) The detection of aFGF gene in the offspring. No. 3, 5, 6, 9, 11 and 13 showed a positive band; (d) Further detection of aFGF-S-arm fusion gene using primers 5 and 4 from the above 6 positive samples

Lane 1: λDNA/HindIII marker; Lane 2: control PCR using the constructed vector as template; Lane 3−8: PCR products of primers No. 3, 5, 6, 9, 11 and 13

SDS-PAGE and Western blotting analysis

The cocoon layer was cut into small pieces and dissolved into 70% LiSCN and subsequently dissloved by SDS-PAGE using 12% gel. After the electrophoresis, the protein was transferred to nitrocellulose membrane. The nitrocellulose blot was treated with 2% BSA for blocking and allowed to bind with rabbit anti-aFGF antibody overnight. The blots were then treated with goat anti-rabbit IgG labeled with horseradish peroxidase (*Bio-Rad*) and Konica Immunostaining HRP-1000 (Konica, Tokyo, Japan) as a substrate of horseradish peroxidase used to signal detection.

RESULTS AND DISCUSSIONS

Using genome extracted from one-fourth of the eggs of a single female moth as template, the PCR results using primers 5 and 6 showed that 6 samples among 78 F1 female moths have positive band (7.69%) with a size of about 430 bp, coinciding with human aFGF gene (Fig.4c). In order to confirm the integration of human aFGF gene into fibroin L-chain of silkworm, another PCR using primers 5 and 4, spanning the junction of aFGF and short-arm gene was also carried out. It was shown that all the above 6 samples had a band at the same position as the control band with a size of 960 bp (using targeting vector as template), although there were also some other nonspecific bands (Fig.4d). Moreover, these bands were cut and recollected from the agarose gel and subsequently cloned into PCR 2.1 vector respectively for sequencing analysis. DNA sequencing data indicated that the PCR products of primer 5 and 6 shared sequence completely identical to the predicted sequence of aFGF gene; and that the nucleotide sequences of primers 5 and 4 PCR product had aFGF sequence at 5' and short-arm sequence at 3'. Thus, the homologous recombination between the targeting vector and L-chain gene of silkworm was considered to be possibly generated and the human aFGF gene might have been integrated into the genome of fibroin L-chain.

The remaining three-fourth eggs of the above

positive samples were incubated into larvae. On the 3rd day of the 5th instar, individual genomes were extracted from larval haemocytes. PCR analyses detected that 5 among 270 larvae (1.85%) showed positive events. The 5 larvae were arranged to produce cocoon. The silk protein of the cocoon layer was analyzed using SDS-PAGE and Western blotting. The results showed that two individuals of the five positive had additional band with molecular weight of about 41 kDa (Fig.5a). This band is considered to be perhaps the fusion form of aFGF (16.0 kDa) and fibroin L-chain (25.0 kDa). For further confirmation, Western blotting analysis was performed using anti-aFGF antibody to detect the hybridization between aFGF and its antibody. As shown in Fig.5b, the additional band showed immunoreactions against aFGF antibody. However, in the control group, the 25 kDa band was also detected when the polyclonal antibody was applied in this experiment.

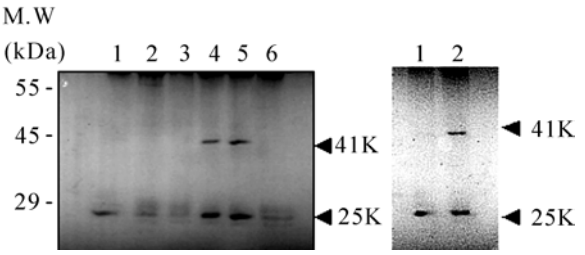

Fig.5 Fibroin prepared from the cocoon produced by the positive insect and analyzed with SDS-PAGE on 12 % gel. (a) Molecular weight marker is indicated at the left. Lane 1–5: protein from the 5 positive samples; Lane 6: control. Western blotting analysis; (b) indicated the additional band was immunoreactive against anti-aFGF antibody. Lane 1: control; Lane 2: positive sample

This study revealed it is possible to target the exogenous gene into silkworm using a recombinant vector in combination with lipofectin reagent. Lipofectin is suitable and usually used for transfection of DNA into tissue culture cells. It interacts spontaneously with DNA to form a lipid-DNA complex. The fusion of the complex with tissue culture cells results in the efficient uptake and expression of the DNA. Compared to transfection methods employing calcium phosphate or DEAE-dextran, a protocol using lipofectin reagent has been shown to be 5 to 100 times more efficient, depending on the cell type. Lipofectin reagent had been used to successfully transfect DNA (Felgner *et al.*, 1987), RNA (Malone *et al.*, 1989) and oligonucleotides into a variety of tissue culture cells and DNA into plant protoplasts (Sporlein and Koop, 1991). Most important for successful transfection is the careful optimization of transfection conditions, such as the optimal amount of lipofectin reagent, DNA concentration, and incubation time of lipofectin reagent-DNA complexes. In our experiment, we tested various conditions (data not shown) and obtained the optimal combination of conditions as described above; 600–700 ng of the DNA was found to be optimal for one larva.

Silkworm has an open haemolymph circulation system in which all organs including ovary, are located. It was reported that successful tansfection with lipofectin reagent may be also achieved in the presence of serum (Brunette *et al.*, 1992). Thus we considered that the in vivo condition of silkworm larva is similar to that of in vitro cell culture with serum. Actually it was quickly confirmed by the authors' previous experiment that exogenous DNA could be effectively transfected into silkworm cells in the presence of lipofectin reagent. The authors' previous experiment showed that 100% of larvae had typical NPV symptom and that polyhedrin was clearly observed after injection of the genome DNA of a hybrid baculovirus between AcNPV and BmNPV into the 5th instar larvae with addition of the lipofectin reagent; with which while no polyhedrin could be observed in the control group. This suggested that lipofectin reagent plays a key role in the DNA transfection so this phenomenon was exploited for gene targeting.

The stability of the foreign gene in transgenic silkworm is very important. This experiment revealed that aFGF gene targeted into silkworm silk L-chain was stable through 3 generations; but in the following generations, the DNA and protein signal detected became gradually weaker, implying the targeted gene was possibly expelled or lost from the targeted locus. To improve the stability of the in-

tergrated gene in the transgenic silkworm is becoming the most urgent task we are facing. Stable germ line transformation in the silkworm was reported to be developed by using a piggyBac, a transposon discovered in the lepidopteran Trichoplusia ni. (Toshiki *et al*., 2000). Approximately 2% of the individuals in the G1 broods expressed the reporter gene GFP. DNA analyses of GFP-positive G1 silkworms revealed that multiple independent insertions occurred frequently. The transgene was stably transferred to the next generation through normal Mendelian inheritance. This efficient method of stable gene transfer in a lepidopteran insect opens the way for promising basic research and biotechnological applications.

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