Germline transformation of the silkworm *Bombyx mori* L. using a *piggyBac* transposon-derived vector

Tamura Toshiki[1], Thibert Chantal[2], Royer Corinne[3], Kanda Toshio[1], Abraham Eappen[1], Kamba Mari[1], Kômoto Natuo[1], Thomas Jean-Luc[3], Mauchamp Bernard[3], Chavancy Gérard[3], Shirk Paul[4], Fraser Malcolm[5], Prudhomme Jean-Claude[2], and Couble Pierre[2]*

*[1]National Institute of Sericultural and Entomological Science, Tsukuba, Ibaraki 305-8634, Japan. [2]Centre de Génétique Moléculaire et Cellulaire, CNRS, 69622 Villeurbanne, France. [3]Unité Nationale Séricicole, INRA, 69150 La Mulatière, France. [4]USDA ARS CMAVE, Gainesville, FL. [5]Department of Biological Science, University of Notre Dame, Notre Dame, IN. *Corresponding author (couble@univ-lyon1.fr).*

Received 26 July 1999; accepted 24 October 1999

We have developed a system for stable germline transformation in the silkworm *Bombyx mori* L. using *piggyBac,* a transposon discovered in the lepidopteran *Trichoplusia ni*. The transformation constructs consist of the *piggyBac* inverted terminal repeats flanking a fusion of the *B. mori* cytoplasmic actin gene *BmA3* promoter and the green fluorescent protein (GFP). A nonautonomous helper plasmid encodes the *piggyBac* transposase. The reporter gene construct was coinjected into preblastoderm eggs of two strains of *B. mori*. Approximately 2% of the individuals in the G1 broods expressed 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. The presence of the inverted terminal repeats of *piggyBac* and the characteristic TTAA sequence at the borders of all the analyzed inserts confirmed that transformation resulted from precise transposition events. This efficient method of stable gene transfer in a lepidopteran insect opens the way for promising basic research and biotechnological applications.

Keywords: *Bombyx*, transgenesis, *piggyBac*, transposon

P-element based gene vectors for stable transgenesis of *Drosophila melanogaster*[1] do not function in insects from other genera[2]. Recently, several other dipterans, including *Ceratitis capitata*[3,4], *Aedes aegypti*[5,6], and *Drosophila virilis*[7–9] have been successfully transformed with the transposable elements *hermes*, *hobo*, *mariner, minos*, and *piggyBac*. However despite numerous attempts, an efficient, stable germline transformation protocol for nondipteran insects has not been achieved. Most of the tested transposons originated from dipteran insects, and their ability to function in insects of other orders is questionable.

Transgenesis would obviously benefit research of the domestic silkworm *Bombyx mori*, a lepidopteran insect of great scientific and economic importance[10]. Recently, gene targeting was achieved in *B. mori* using a baculovirus-derived vector[11]; however, the extremely low frequency of the homologous recombination events precludes routine use of this methodology for transferring genes into this species. Here we have tested *piggyBac*, a transposon discovered in the lepidopteran *Trichoplusia ni*[12], for its ability to transform the silkworm. We found that it mediates stable germline transformation of the lepidopteran insect *B. mori* and possibly a wide range of other insect species.

Results

Transformation experiments. *PiggyBac*-mediated germline transformation of pnd-w1 and Nistari strains of *B. mori* was tested using a vector marked with a green fluorescent protein (GFP)-encoding gene and a nonautonomous helper that carried the transposase gene under the control of the cytoplasmic *B. mori* actin gene *BmA3* promoter. Preblastodermic eggs were injected with a mixture of vector and helper DNA. From 1,058 pnd-w1– and 1,440 Nistari-injected eggs, 424 and 230 fertile moths were recovered, respectively. After sibling mating or backcrossing, three pnd-wl and nine Nistari broods with GFP-positive larvae were identified (Table 1). The yield of G0 adults with transformed gametes was thus 1.8%. In the GFP-positive G1 broods, 1.6–26.7% of the larvae displayed fluorescence (Table 2 and Fig. 1). Green fluorescent protein expression was visible in all larval, pupal, and adult tissues but, unexpectedly, was not detectable in embryos.

Insertion analysis. The presence of vector DNA in the genome of transformed insects was shown by Southern blot analysis of DNA isolated from G1 pnd-w1 individuals, using a part of the GFP-encoding sequence as a probe (Fig. 2). DNA extracted from four adults from each of the three positive broods was digested with *Xho*I, which cuts the vector only once. All 12 DNA samples contained pPIGA3GFP hybridizing sequences, while DNA from noninjected controls did not. Six of the insects carried a single insertion, five had two insertions, and one had three insertions (Fig. 3A). The same DNA samples digested with *Eco*RV and *Xmn*I showed that all 12 individuals contain the predicted 3.5 kbp vector fragment showing that no detectable modification of the inserted DNA had occurred (Fig. 3B).

Two of the G1 moths carrying a single insertion were backcrossed to the parental line, giving rise to 55 (among 118) and 109 (among 209) GFP-positive G2 larvae, respectively. Silk gland DNA from GFP-positive larvae was digested with *Xho*I and analyzed with the GFP probe. Figure 3C shows that the inserted *piggyBac* DNA was transmitted to the next generation without modification.

Sibling matings between the other G1 transformants were made, and several G2 larvae were analyzed using the same hybridization

Figure 1. G1 transgenic silkworms positive for GFP. Transformed larvae are fluorescent, as compared with nontransformed controls.

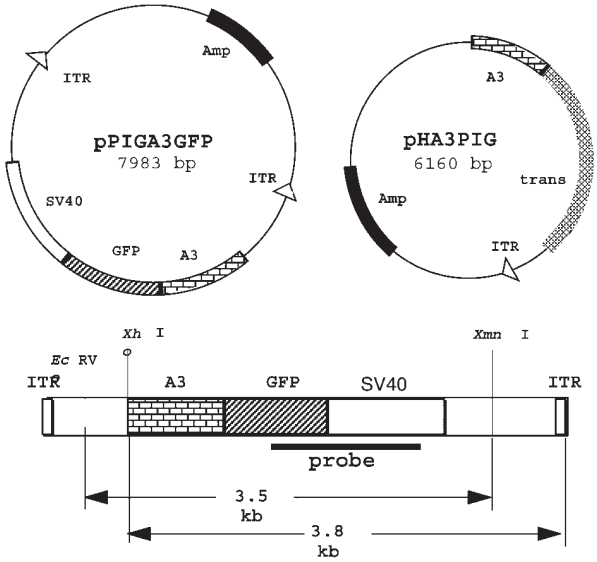

Figure 2. Organization of the *piggyBac*-derived constructs. The marker, pPIGA3GFP, contains the *B. mori* A3 cytoplasmic actin gene promoter fused to the GFP coding sequence and SV40 3′ untranslated sequences. The nonautonomous helper, pHA3PIG, is a source of the *piggyBac* transposase (trans). ITR, inverted terminal repeats of *piggyBac*; Amp, ampicillin-resistance gene of the plasmid. Positions of the probe used for Southern blot analysis and of *Eco*RV, *Xho*I, and *Xmn*1 restriction sites used for digesting the genomic DNA are shown on the enlarged view of the pPIGA3GFP insert.

protocol. The GFP-negative larvae had no insert (data not shown) whereas the GFP-positive ones had one or several inserts. Figure 3D shows that the inserts were normally transmitted as independent loci.

Insertion site determination. To verify that the insertion of the transgenes was due to transposition events, we examined the genomic sequence at the insertion sites. Inverse PCR was performed on silk gland DNA from eight pnd-w1 G1 GFP-positive larvae. For all insertions, the sequence of the *piggyBac* inverted terminal repeats was recovered and found to be bordered by the characteristic TTAA sequence (Fig. 4), the known target site of *piggyBac*[13]. The insertions were flanked by unknown sequences, except that of the no. 5/16 insect in which surrounding genomic DNA was 100% identical to sequence in the *B. mori mariner 1* transposon[14]. One insert from insect 5/13 was identical to one of no. 5/17, in agreement with the Southern blot result (Fig. 3A).

Discussion

Our experiments show that *piggyBac* is capable of mediating stable germline transformation in two distinct strains of *B. mori*. From 2,498 injected embryos, 654 moths were recovered and crossed, leading to 12 broods with GFP-positive individuals.

The expression of GFP in all tissues of the transformed larvae is in agreement with the ubiquitous activity of the driving actin *BmA3* promoter[15]. Genetic analysis of G2 families demonstrated that the acquired GFP-dependent fluorescence was inheritable and segregated as a Mendelian factor, confirming that the transformation events resulted from chromosomal insertions.

Southern blot analyses of the DNA of transformed G1 insects showed that one to three different inserts were present in a single animal and that larvae from the same progeny had different insertions. These insertions were inherited independently at the G2 generation, showing that they were dispersed within the chromosomes. All the insertion sites that were recovered and sequenced had the TTAA motif flanking the inverted repeats, which is consistent with the known transposition properties of *piggyBac*[13]. The finding that one insertion occurred in a *mariner* transposon further confirms that the insertion was chromosomal in nature.

The presence of multiple independent inserts in many G1 larvae indicates that a single gamete from the G0 parents can harbor several insertions and that different gametes can have different insertions. Eighteen insertions were observed in 12 G1 individuals issued from three transformed parents. It is likely that this result underestimates the total number of insertion events that occurred in the G0 moths.

If these multiple insertions were present in the same gonocyte in the G0 individuals, the frequency distribution of inserts in the gametes would lead to a high average number of inserts in each G1 insect, which was not observed. Our data suggest that, before meiosis, the gonocytes contain only a few transgenes and that they are different from one another. Either the integration events in the germ line occurred late during development, or successive rounds of transposition took place after an initial insertion event. Such a hypothesis would explain why—despite the low frequency of insertion in the parental population—the number of inserts is high in the transformed insects. Successive transpositions could be the result of the presence of some endogenous activity or of the stability of the transposase encoded by the helper vector. However, because the inserts are stably transmitted to the next generations, we can rule out the first possibility. A similar situation was also observed in transgenic *C. capitata*, and it was also attributed to secondary mobilizations of an initial single insert[4]. This property makes the *piggyBac* system highly efficient, as several independent lines can be derived from a few initially transformed individuals.

Table 1. Injection of *piggyBac*-derived vectors in silkworm embryos of the strains pnd-wl and Nistari.

Silkworm strain	Number of injected embryos	Number of hatched embryos	Number offertile moths	Number of single-pair matings	Number of backcrosses	Number of broods with GFP-positive larvae	Percentage of G0 transformed moths
pnd-w1	1,058	695	230	204	16	3	0.7
Nistari	1,440	469	424	86	58	9	3.9

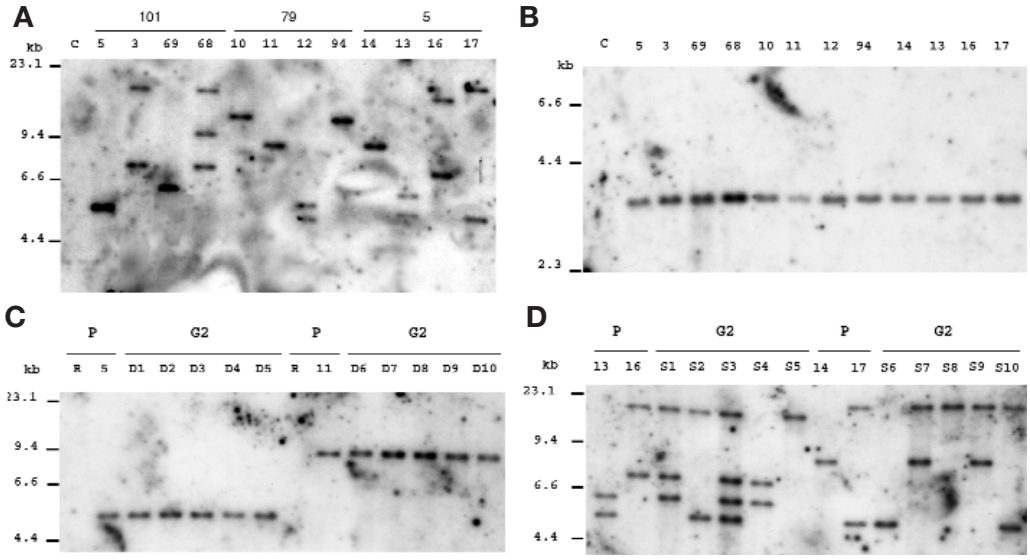

Figure 3. Southern blot hybridization of DNA from transformed and control animals. (A) Hybridization of *Xho*I-cut genomic DNA to the probe described in Figure 1 in four G1 GFP-positive individuals respectively from broods 101, 79, 5, and control (C). Note the presence of single, double, or triple insertions among the analyzed individuals. (B) Same samples as in (A) after *Eco*RV and *Xmn*I cutting of the DNA, with the 3.5 kbp vector fragment appearing in all samples. (C) Hybridization of the DNA from the progeny of two backcrosses between G1 parents (101/5 and 79/11) that carry a single insert and the parental strain (R). (D) Hybridization of the DNA of five G2 individuals from two crosses between transformed G1 parents (5/13 × 16 and 5/14 × 17). Note the independent segregation of the different *piggyBac* insertions in the G2 progeny.

Our data show that a *piggyBac*-derived vector is able to transpose into *B. mori* chromosomes at a frequency that allows the established protocol to be used routinely. Stable germline transformation of *B. mori* will be of considerable help in deciphering fundamental mechanisms of gene regulation and in improving commercial sericultural strains. Moreover, the high proteosynthesis that occurs in the silk gland cells could be exploited to produce proteins of pharmaceutical or veterinary interest that could be harvested directly from the cocoon. The textile industry could also benefit from novel fibers made by silkworms transformed with various genes encoding fibrous proteins.

Experimental protocol

Silkworm strains. The *B. mori* polyvoltine strains, pnd-w1 and Nistari, were obtained from the National Institute of Sericultural and Entomological Science (Tsukuba, Ibaraki, Japan) and the Unité Nationale Séricicole (La Mulatière, France), respectively. After DNA injection, the embryos were maintained at 25°C in moist petri dishes until hatching. First-instar larvae were transferred to an artificial larval diet and reared in groups under standard conditions. G1 adults were mated within the same family or backcrossed to moths of the recipient strain.

Table 2. Study of G1 GFP-positive transgenic animals.

G0 lines	Number of G1 examined larvae	Number (%) of GFP-positive G1 larvae	Number of independent insertions
pnd101	156	41 (26.7)	>5
pnd79	157	15 (9.6)	>4
pnd5	167	12 (7.2)	>6
N1	133	34 (25.6)	ND[a]
N2	416	49 (11.8)	ND
N3	378	12 (3.2)	ND
N4	214	20 (9.3)	ND
N5	210	21 (10.0)	ND
N6	134	7 (5.2)	ND
N7	366	19 (5.2)	ND
N8	245	4 (1.6)	ND
N9	29	2 (6.9)	ND

[a]ND, not determined.

Plasmid construction. The marked integrative vector pPIGA3GFP was derived from the wild-type *piggyBac* element present in p3E1.2 (ref. 12), in which the central part of the transposase gene was replaced by a transgene comprising the GFP coding sequence and the promoter of the *B. mori* cytoplasmic actin gene *BmA3*. The *BmA3* sequences from -130 to +762 in the plasmid pA3LacZ (ref. 16) were inserted into the polylinker of the pEGFP-N1 protein fusion vector (Clontech, Palo Alto, CA), giving rise to pA3GFP. Then, the 2.7 kbp *Bgl*II/*Stu*1 fragment of pA3GFP was inserted in place of the 0.75 kbp *Bgl*II/*Hpa*1 fragment of p3E1.2 (ref. 12). The construct pPIGA3GFP (Fig. 2) carries the *BmA3* 5′ flanking region (devoid of the negative regulatory sequence RA3) and the transcribed sequence up to codon 54 fused in phase with the GFP coding sequence and the 3′ SV40-derived sequences present in pEGFP-N1.

The transposase-producing plasmid pHA3PIG carries a modified *piggyBac* element devoid of the left-hand inverted repeat, the 5′ flanking, and the leader sequences of the transposase gene. These were replaced by the 5′ flanking and leader sequences of *BmA3* (Fig. 2). For this, the *Ava*1/*Bgl*II fragment of p3E1.2 was replaced with a hybrid *BmA3*/*piggyBac* transposase sequence that was constructed by PCR amplification as follows. First, a fragment from pA3GFP was amplified using a reverse oligonucleotide primer containing the *Ava*1 site of the pUC polylinker (5′-GGACTCAGATCCCGGGCTCAAGCTTGATGCGCG-3′) and a second primer complementary in its 5′ end to the sequence flanking the ATG initiation codon of the A3 actin coding sequence and in its 3′ end to the first codons of the *piggyBac* transposase (5′-AACGTCTGATTAAGTTCTAC-CCTACAAGAAATCTGC-3′). Then, a second fragment was amplified from p3E1.2, which contained the wild-type *piggyBac* sequence downstream of the ATG initiation codon, by using a reverse primer bearing the first codons of the transposase (5′-GGGATGTTCTTTAGACGATGAG-3′) and a second primer containing a *Bgl*II site (5′-CCAGGCTGCGCATACACGGCGT-3′).

```
79/11   TGTTTAGGTTATTTGTTTAA---piggyBac---TTAATAGAATTGGACGCGTG

79/12   AAACTATCCACATGTCTTAA---piggyBac---TTAATGGTTAAGTGGTGAA
        CTGCCCTTAGAGTTTATTAA---piggyBac---TTAAAGAAACTTCTAGAGAA

79/94   AAATACTACGCATTCATTAA---piggyBac---TTAAGTTCATTTTCATCAAA


5/13    CCTTTATATTCTATCATTAA---piggyBac---TTAAAGTTCCCTTTGCTTTT
        TACTGTGAATTGCAGATTAA---piggyBac---TTAAGTATATGTATATATTT

5/14    TCAAACACATAGGTACTTAA---piggyBac---TTAATGAATAAACAAAATCA

5/16    AAAAGACTTAAATATGTTAA---piggyBac---TTAAAAACAAATGATATGAT
        TAACTGGCCTCAACGTTTAA---piggyBac---TTAAAGGACTGTATTGCAGC
        ||||||||||||||||||.....||||||||||||||||
        taactggcctcaacgttaa----Bmmar1--------aggactgtattgcagc

5/17    CCTTTATATTCTATCATTAA---piggyBac---TTAAAGTTCCCTTTGCTTTT
        .....piggyBac---TTAAGCATTTTATTAAGCAT
```

Figure 4. Genomic DNA sequences surrounding *piggyBac* insertions, with the TTAA duplicated sequence appearing at all 5′ and 3′ insert boundaries. The *B. mori mariner* 1 transposon (*Bmmar1*)[14] has 100% identity with the genomic DNA flanking the *piggyBac* insertion in insect 5/16. One insert from insect 5/13 is identical to one from no. 5/17.

The two resulting overlapping fragments were annealed and used to synthesize the composite *Ava*1/*Bgl*II fragment, the nucleotide sequence of which was verified.

Embryo injection and screening of transformed animals. Eggs were collected between 1 and 2 h after egg laying at the syncytial preblastoderm stage. About 15–20 nl of a 1:1 mixture of vector and helper plasmids (0.4 µg/µl total DNA concentration) in 0.5 mM phosphate buffer (pH 7.0), 5 mM KCl, were injected into each egg, as previously described[17]. The injection opening was sealed with glue (Borden, Columbus, OH) and the embryos were allowed to develop at 25°C.

Green fluorescent protein fluorescence was observed under a microscope equipped for epifluorescence detection (Olympus, Tokyo, Japan). Filters passing light between 507 and 510 nm and between 450 and 480 nm were used for detection and excitation, respectively.

Southern blot analysis. Total DNA was extracted from G1 moths or from silk glands of G2 fifth instar larvae previously frozen in liquid nitrogen and stored at -80°C. DNA was purified by standard SDS-phenol treatment after incubation with proteinase K, digested with the indicated restriction enzymes, and separated on 0.8% agarose gels. DNA was stained with ethidium bromide and blotted onto nylon filters (Hybond N+, Amersham, Buckinghamshire, England) under vacuum. The labeled probe was prepared as follows. The pPIGA3GFP was amplified using the primer pair 5′-ACGACGGCAACTACAAGACC-3′ and 5′-GGCGGAGAATGGGCGGAACT-3′. The amplified product was cloned into pGEM-T vector (Promega Corporation, Madison, WI) and sequenced. The probe DNA (nucleotides 4,259–5,601 of pPIGA3GFP), which also carries 25 bp of pGEM-T, was released from the pGEM-T vector by double digestion with *Sal*1 and *Sph*1 and labeled by random labeling (Takara Biomedicals, Gennevilliers, France) with α^{32}P-dCTP.

Hybridization was carried out at 65°C in 5× SSC (0.015 NaCl, 0.015 M sodium citrate), 5× Denhardt's solution and 0.5% SDS buffer. Washing was done at room temperature in 2× SSC and 0.2×% SDS, followed by a 15 min wash at 65°C in the same solution, 15 min at 65°C in 1× SSC and 0.1% SDS, and two 10 min washes at 65°C in 0.1× SSC and 0.1% SDS.

Inverse PCR analysis. Genomic DNA extracted as above was further treated with RNase and purified. DNA (3µg) was then digested with *Sau*IIIA1 and circularized by overnight ligation at 16°C using T4 DNA ligase. The ligated DNA (50–100 ng) was amplified using Taq polymerase under standard conditions with primers designed from the left- and right-hand regions of the vector. The left primer pair was 5′-ATCAGTGACACTTACCGCATTGACA-3′ and 5′-TGACGAGCTTGTTGGTGAGGATTCT-3′. Amplification was carried out with a 2 min denaturing cycle at 96°C followed by 40 cycles of 1 min at 96°C, 30 sec at 65°C, 4 min at 72°C, and a final extension at 72°C for 10 min. Amplification using the right primer pair (5′-TACGCATGATTATCTTTAACGTA-3′ and 5′-GGGGTCCGTCAAAACAAAACATC-3′) was carried out under the same conditions except that annealing was performed at 58°C.

Amplified products were sequenced directly or after cloning in pGEM-T. DNA sequences were determined on an ABI Prism 373 DNA Sequencer using BigDye termination DNA sequencing kit containing AmpliTaq FS polymerase (Perkin Elmer Cetus,, Foster City, CA). Sequences were analyzed using the Sequence Navigator version 1.0 (Applied Biosystems, Warrington, UK).

Acknowledgments

*We thank Marie-Andrée Guérin, Valérie Carraro, Seo Kazuko, and Yamazaki Hiroko for technical assistance, and Hervé Bossin and Yukuhiro Kenji for their help. We also thank Paul Brey and Charlie Roth for editorial help. The group in Tsukuba was supported by the Japanese MAFF. The group in Villeurbanne was supported by the CNRS and the French MENRT and the group in La Mulatière by the INRA; both also received support from the Europe Union (Contract n° CI1*CT94-0092) and the DRET (Contract n° 95/099).*

1. Rubin, G.M. & Spradling, A.C. Genetic transformation of *Drosophila* with transposable element vectors. *Science* **218**, 348–353 (1982).
2. O'Brochta, D.A. & Atkinson, P.W. Transposable elements and gene transformation in non-drosophilid insects. *Insect Biochem. Mol. Biol*. **26**, 739–753 (1996).
3. Loukeris, T.G., Livadaras, I., Arca, B., Zabalou, S. & Savakis C. Gene transfer into the medfly, *Ceratitis capitata*, with a *Drosophila hydei* transposable element. *Science* **270**, 2002–2005 (1995).
4. Handler, A.M., MacCombs, S.D., Fraser, M.J. & Saul, S.H. The lepidopteran transposon vector, *piggyBac*, mediates germ-line transformation in the mediterranean fruit fly. *Proc. Natl. Acad. Sci. USA*. **95**, 7520–7525 (1998).
5. Coates, C.J., Jasinskiene, N., Miyashiro, L. & James, A.A. Mariner transposition and transformation of the yellow fever mosquito, *Aedes aegypti. Proc. Natl. Acad. Sci. USA* **95**, 3748–3751 (1998).
6. Jasinskiene, N. et al. Stable transformation of the yellow fever mosquito *Aedes aegypti,* with the *hermes* element from the housefly. *Proc. Natl. Acad. Sci*. *USA* **95**, 3743–3747 (1998).
7. Lozovskaya, E.R., Nurminsky, D.I., Hartl, D.L. & Sullivan, D.T. Germline transformation of *Drosophila virilis* mediated by the transposable element hobo. *Genetics* **142**, 173–177 (1995).
8. Lohe, A.R. & Hartl, D.L. Germline transformation of *Drosophila virilis* with the transposable element *mariner*. *Genetics* **143**, 365–374 (1996).
9. Gomez, S.P. & Handler, A.M. A *Drosophila melanogaster* hobo-white (+) vector mediates low frequency gene transfer in *D. virilis* with full interspecific white (+) complementation. *Insect Mol. Biol*. **6**, 165–171 (1997).
10. Nagaraju, J., Klymenko, V. & Couble, P. In *Encyclopedia of genetics* (ed. Reeves, E.) (Fitzroy Dearborn, London, UK; In press).
11. Yamao, M. et al. Gene targeting in the silkworm by use of a baculovirus. *Genes Dev*. **13**, 511–516 (1999).
12. Cary, L.C. et al. Transposon mutagenesis of baculoviruses: analysis of *Trichoplusia ni* transposon IFP2 insertions within the FP-locus of nuclear polyhedrosis viruses. *Virology* **172**, 156–69 (1989).
13. Wang, H.H. & Fraser, M.J. TTAA serves as the target site for the TFP3 lepidopteran transposon insertions in both nuclear polyhedrosis virus and *Trichoplusia ni* genomes. *Insect Mol Biol*. **1**, 109–116 (1993).
14. Robertson, H.M. & Asplund, M.L. *Bmmar1*: a basal lineage of the *mariner* family of transposable elements in the silkworm moth *Bombyx mori*. *Insect Biochem. Mol. Biol*. **26**, 945–954 (1996).
15. Mounier, N. & Prudhomme, J.C. Differential expression of muscle and cytoplasmic actin genes during development of *Bombyx mori*. *Insect Biochem*. **21**, 523–533 (1991).
16. Mangé, A., Julien, E., Prudhomme, J.C. & Couble, P. A strong inhibitory element down-regulates SRE-stimulated transcription of the A3 cytoplasmic actin gene of *Bombyx mori*. *J. Mol. Biol*. **265**, 266–274 (1997).
17. Kanda, T. & Tamura, T. Microinjection system into the early embryo of the silkworm, *Bombyx mori*, by using air pressure. *Bulliten of the National Institute of Sericultural and Entomological Science* **2**, 32–46 (1991).