Germline Transformation of the Silkworm Bombyx mori L. by Sperm-Mediated Gene Transfer 1

Authors: Zhou, Wenlin, Gong, Chengliang, Xue, Renyu, Cao, Guangli, Cao, Jinru, et al.

Source: Biology of Reproduction, 87(6)

Published By: Society for the Study of Reproduction

URL: https://doi.org/10.1095/biolreprod.112.102822

BIOLOGY OF REPRODUCTION (2012) **87**(6):144, 1–5
Published online before print 24 October 2012.
DOI 10.1095/biolreprod.112.102822

Germline Transformation of the Silkworm *Bombyx mori* L. by Sperm-Mediated Gene Transfer[1]

Wenlin Zhou,[3] Chengliang Gong,[4,5] Renyu Xue,[4,5] Guangli Cao,[4,5] Jinru Cao,[2,3] Aihong Ye,[3] Hongbiao Weng,[3] and Yongqiang Wang[3]

[3]*Sericulture Research Institute, Zhejiang Academy of Agricultural Sciences, Hangzhou, China*
[4]*Preclinical Medical and Biological Science College, Soochow University, Suzhou, China*
[5]*National Engineering Laboratory for Modern Silk, Suzhou, China*

ABSTRACT

The domesticated silkworm *Bombyx mori* L. has important roles in basic biological research and applied science. To explore the practical use of transgenic technology in agricultural silkworm varieties, we fused the neomycin-resistance gene (*Neo*^{R}) and the green fluorescent protein gene (*gfp*) into the piggyBac-based transposon vector and transduced it into silkworms by sperm-mediated gene transfer (SMGT). Fluorescence observation indicated the positive rate of G0 egg-batches is 72.7%. After screening against the antibiotic G418, development of individual larvae in the same brood showed significant size differences. PCR detection indicated the existence of *gfp* and *Neo*^{R} and confirmed the positive rate of transgenesis as 0.47%. Southern blot analysis confirmed the presence of the exogenous genes in the genome of G7 larvae. These results show that our strategy is practical and markedly improves the efficiency of SMGT.

Bombyx mori *L., piggyBac transposon, sperm-mediated gene transfer, transgenesis*

INTRODUCTION

Transfer of exogenous genes into the genome is an important technology in modern life sciences and has great potential to change the genetic traits of an organism for both basic and applied research. The technology of animal transgenesis is one of the most significant advances in experimental and applied biology in the past 20 years [1]. Commonly used methods for obtaining transgenic animals include microinjection of fertilized eggs in the prokaryotic stage, somatic or embryonic stem cell transplantation, retroviral infection, and so on [2].

The domesticated silkworm *Bombyx mori* L. undergoes four developmental stages during its short life cycle: egg, larva, pupa, and adult moth. *B. mori*, which has been an important economic insect for silk production for approximately 5000 years [3], has become a useful model of the Lepidoptera, with an increasingly important role in basic biological research [3, 4]. Completion of the framework of the *B. mori* genomic map in 2004 [5, 6] marked the start of the era of *B. mori* functional genomics and transgenic technology, which has received widespread attention. The major breakthrough in *B. mori* transgenic technology came when Tamura et al. [7] microinjected the piggyBac-derived transposon vector into the early stage of fertilized silkworm eggs and obtained the transgenic silkworm. This technology is now commonly used in silkworm transgenic studies; however, unlike the mammalian oocyte, the silkworm egg has a hard shell composed mainly of proteins and hydrocarbons [8], which is a physical hindrance to microinjection. Usually, an egg can be penetrated first by a fine tungsten needle, and then the exogenous DNA can be microinjected. However, at the moment of breaking the shell, pressure inside the egg often causes outflow of the egg contents, leading to embryonic death and, hence, a very low survival rate of microinjected eggs. In addition, unlike mammals and organisms in other classes, *B. mori* L. is in diapause during the egg stage. In sericulture, this diapause is usually broken by treatment with hydrochloric acid (hydrochlorization) as described previously [9]. Clearly, microinjection causes injury to the eggs, which cannot be repaired by hydrochlorization. Therefore, gene transfer via microinjection into the fertilized silkworm egg is successful in only a few cases, greatly hampering silkworm transgenic research.

Sperm-mediated gene transfer (SMGT) is based on the ability of sperm cells to bind, internalize, and transport exogenous DNA into an oocyte during fertilization [10]. To date, successful SMGT has been reported for a variety of animal classes, including mammals, birds, fish, and insects [11–13]. SMGT is a promising technology for the creation of transgenic animals, and reports have appeared of its successful use in silkworm transgenic studies [14–17]. However, increasing the present low level of efficiency of screening for positive transgenic silkworms is a new challenge.

The present study used the piggyBac transposon to construct a gene transfer vector containing both the green fluorescent protein gene (*gfp*) and neomycin-resistance gene (*Neo*^{R}) expression cassettes. We used the SMGT method for this transgenic study in *B. mori*, and the efficiency of screening was increased significantly by the introduction of *Neo*^{R} and G418 selection.

MATERIALS AND METHODS

Silkworm Culture and Succession of Generation

Bivoltine stock seed of *B. mori* Haoyue was supplied by the Sericulture Research Institute of the Zhejiang Academy of Agricultural Sciences. The larvae were fed leaves of the white mulberry tree (*Morus alba*) and kept at

[1]Supported by grants from Zhejiang Public Welfare Technologies Research Projects (2010C32028), Zhejiang Provincial Major Sci-Tech Projects (2011C312008), and Zhejiang Sericultural Sci-Tech Innovation Team (2011R50028).
[2]Correspondence: Jinru Cao, Sericulture Research Institute of Zhejiang Academy of Agricultural Sciences, 198 Shiqiao Road, Hangzhou 310021, China. E-mail: caojr@mail.zaas.ac.cn

Received: 15 June 2012.
First decision: 12 July 2012.
Accepted: 22 October 2012.

eISSN: 1529-7268 http://www.biolreprod.org
ISSN: 0006-3363

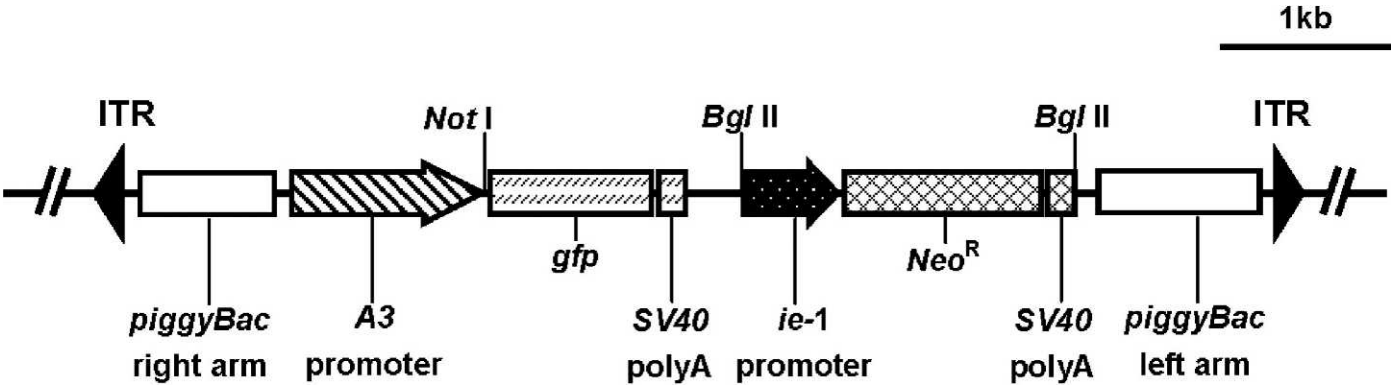

FIG. 1. Construction of the transgenic vector pPIGA3GFP-IE-NEO. *A3*, *B. mori* cytoplasmic actin 3 gene; ITR, inverted terminal repeats of the piggyBac transposon; SV40 polyA, the SV40 3′ untranslated sequence.

25°C. Typically, the silkworm egg enters a diapause, which can be broken by hydrochlorization immediately after egg laying (~20 h).

Transgenic Vector Construction

The piggyBac-based pPIGA3GFP transposon vector (7983 bp) contains the *B. mori* A3 promoter-driven *gfp* coding sequence. The piggyBac transposase-encoding nonautonomous helper plasmid pHA3PIG (6160 bp) has been described previously [7]. The 631-bp promoter region of the immediate early gene (*ie*-1) of *B. mori* nucleopolyhedrovirus (BmNPV) was cloned from BmNPV genomic DNA by PCR using forward primer (IE-F) 5′-TTCGAATTCGATTTGCAGTTCGGGAC-3′ and the reverse primer (IE-R) 5′-GCGGAATCAGTCGTTTGGTTGTTCA-3′.

The sequence is deposited in GenBank (ID: AY616665). Neo^R and its downstream 325-bp SV40 3′ untranslated sequence (total, 1120 bp) were amplified by PCR with the forward Neo^R primer (Neo-F) 5′-ATGATTGAA CAAGATGGATTGC-3′ and the reverse Neo^R primer (Neo-R) 5′-GCTA GAGGTCGACGGTATAC-3′ using plasmid pcDNA3.1 (Invitrogen) as the template. The *ie*-1 promoter-driven Neo^R expression cassette was constructed by homologous recombination (GenScrip USA, Inc.). Thus, the piggyBac-derived transposon vector pPIGA3GFP was constructed as pPIGA3GFP-IE-NEO (Fig. 1).

Transgenic Procedures

The plasmid DNA was purified using the EndoFree Plasmid Kit (Qiagen). The gene transfer vector pPIGA3GFP-IE-NEO and the helper plasmid pHA3PIG were mixed at a 1:1 molar ratio to a final DNA concentration of 2.0 μg/μl, mixed with Lipofectamine 2000 (Invitrogen) at a 1:3 mass ratio, and incubated at room temperature for 30 min. Unlike mammals, sperm from male silkworm moths are stored in the female's bursa copulatrix after mating, and eggs are fertilized only after egg laying [8, 9]. Therefore, the incubated mixture of gene transfer vectors was drawn into a glass needle (diameter, 20–40 μm; Narishige) and injected (3.0 μl/moth) through the vagina (copulatory aperture) (Fig. 2B) into the bursa copulatrix (Fig. 2C) of a newly hatched, virgin female moth. Injected female moths were allowed to mate normally. Copulating moths were separated after 3 h, and the females were allowed to spawn naturally in darkness.

Screening Transgenic Silkworms

The sperm-injected female moths were allowed to mate and lay eggs normally, and the brood from each female was stuck to an egg card to form an egg batch. To break the embryonic diapause, eggs were hydrochlorized (HCl, 4.565 M) for 5 min at 46°C at 20 h after egg-laying, then incubated at 25°C for 11 days [9]. At the pigmentation stage, green fluorescence-labeled G0 eggs were selected under a fluorescence stereomicroscope (SZX12; Olympus) for single-batch breeding. After hatching, the silkworms were kept at 25°C and fed on mulberry leaves. After the first molt of the larvae, mulberry leaves were cut into 1- × 1-cm pieces, and the surface was wiped with absorbent cotton soaked with 10 mg/ml of G418 so that all areas were moist but not dripping. Newly molted silkworms were fed on freshly G418-covered mulberry leaf pieces to ensure a sufficient supply of G418. After 24 h, the G418-coated mulberry leaf pieces were replaced with untreated leaves. During subsequent breeding, developmentally retarded larvae were removed, and silkworms in the pupa stage were examined for green fluorescence under the microscope. Male and female G0 transgenic moths in the same brood were mated to obtain the G1 generation. Genomic DNA was extracted from the G0 generation and used as PCR template to amplify the *gfp* and Neo^R expression cassettes. PCR primers were designed as: forward *gfp* primer (GFP-1), 5′-CGCGTTACCATA TATGGTGACA-3′; reverse *gfp* primer (GFP-2), 5′-GCGATCCAGACATGA TAAGAT-3′; forward Neo^R primer (Neo-1), 5′-GATTTGCAGTTCGG GACAT-3′; and reverse Neo^R (Neo-2), 5′-GCTAGAGGTCGACGGTAT-3′.

After the G1 transgenic silkworms had spawned, the populations were maintained and screening was done by the same GFP and G418 double selection. Standard thremmatology methods were used to breed the new transgenic silkworm strains [4, 8].

Southern Blot Analysis

Genomic DNA was extracted from the posterior silk gland of each transgenic silkworm larva using the Gentra Puregene Tissue Kit (Qiagen) and digested with *Not*I (TaKaRa). The Prime-a-Gene Labeling System (Promega) was used for *gfp* probe preparation, and the primer sequence was 5′-TCTGCTTGTCGGCCATGATA-3′. The [α-^{32}P]dCTP was purchased from Dupont. Kodak x-ray film (Kodak X-Omat) was used for signal exposure. Southern blot analysis was done as described previously [7].

RESULTS

Screening Transgenic Silkworms

The mixture of transgenic vector pPIGA3GFP-IE-NEO, helper plasmid, and Lipofectamine 2000 was injected into the bursa copulatrix of 11 virgin female moths. After mating and spawning, egg batches were selected by examination for green fluorescence. At the pigmentation stage, different numbers of eggs from eight batches were labeled with GFP (Table 1 and Fig. 3A), and the rate of positive batches was 72.7%. After further selection for G418, symptoms of poisoning and severe developmental retardation of larval development were observed; very few larvae developed normally. The difference in size of individual silkworms between these two groups became significant (Fig. 3B). More than 90% of individuals gradually died by the fifth instar: Less than 10% survived, and less than 1% of individuals completed pupation and developed into adult moths. The final positive rate was 0.47% (Table 1). The pupae were examined for green fluorescence (Fig. 3, C and D), and GFP-positive individuals were used for breeding.

PCR Identification of Transgenic Silkworms

The adult moths developed from the selected G0-positive transgenic silkworms were mated within the same brood. After spawning and hatching, the G1 generation was obtained; genomic DNA was extracted from the corresponding parental moths and used as template to amplify the *gfp* cassette by PCR.

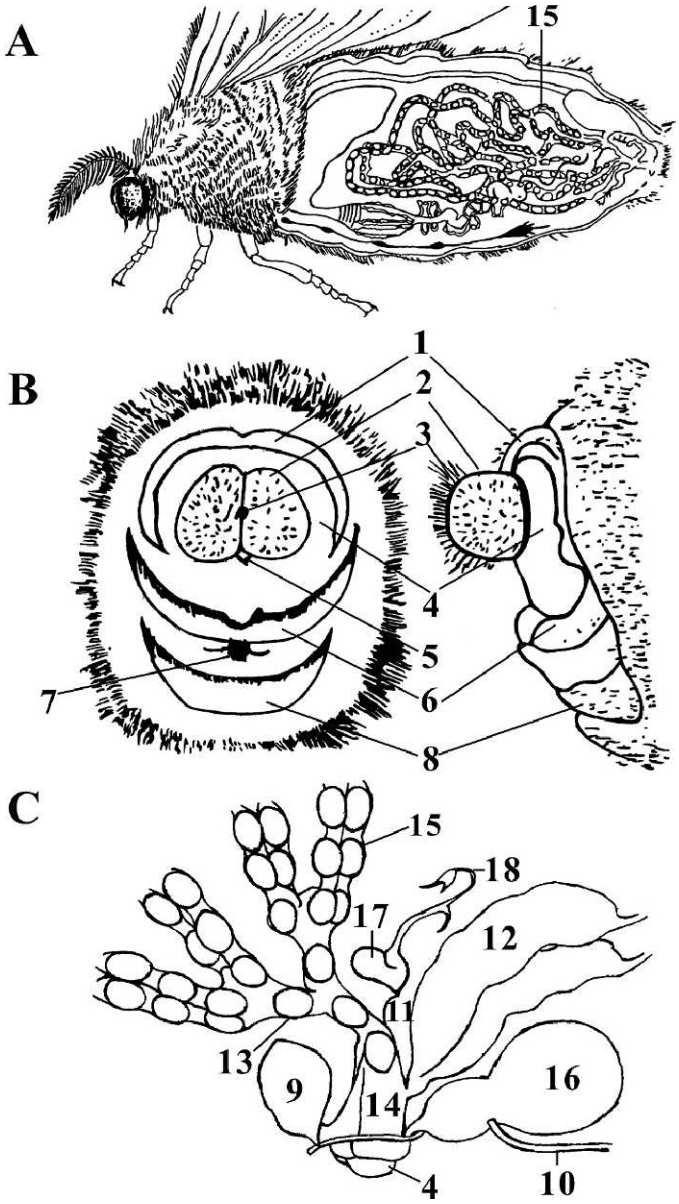

FIG. 2. Reproductive system of the female silkworm moth. **A**) Internal organs. **B**) External genitalia. **C**) Internal genitalia: 1, dorsal chitinous plate; 2, papilla genitals; 3, anus; 4, saccule lateralis (alluring gland); 5, oviparous orifice; 6, ventral chitinous plate; 7, vagina (copulatory aperture); 8, abdominal plate; 9, bursa copulatrix; 10, rectum; 11, tortuosus ductus; 12, mucous gland; 13, oviduct; 14, ovipositor; 15, ovariole; 16, rectal sac; 17, spermatheca; 18, spermathecal accessory gland.

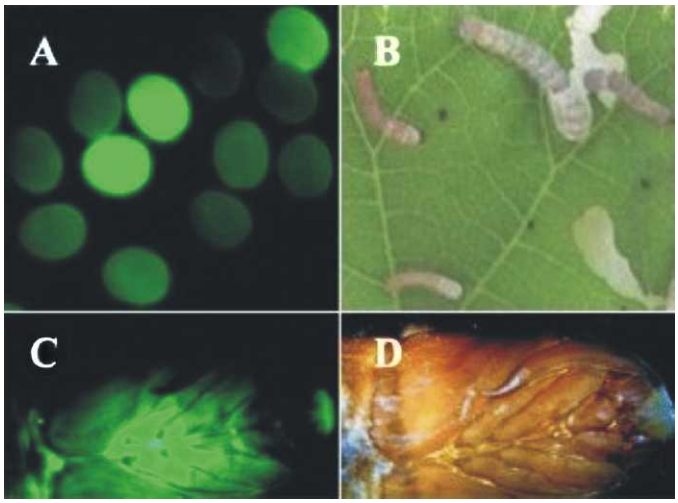

FIG. 3. Screening transgenic silkworms. **A**) Observation of the egg batch in the pigmentation stage showed that some eggs emitted green fluorescence. **B**) The sizes of larvae changed during development after addition of the antibiotic G418. **C**) Green fluorescence in transgenic pupae was confirmed by observation under the stereomicroscope. **D**) The bright field corresponding to **C**. Original magnification ×10.

Electrophoresis in agarose gels with size markers showed an approximately 1.8-kb DNA fragment, which is consistent with the expected size of *A3-gfp-SV40*. Another approximately 1.8-kb fragment amplified with the primer pair Neo-1 and Neo-2 matches the expected size of the *ie-1-Neo*^{R}*-SV40* cassette. These PCR products were cloned into the pMD19-T vector (TaKaRa) and sequenced, further verifying the correctness of the DNA sequences and indicating that genomic DNA from the G1 generation contained both the *gfp* (Fig. 4A) and the *Neo*^{R} gene expression cassettes (Fig. 4B). Each generation was confirmed by PCR.

Confirmation of Transgenic Silkworm Strains by Southern Blot Analysis

Transgenic silkworm strains were bred using standard thremmatology methods. Each generation was subjected to double selection for GFP and G418 and further confirmed by PCR. The transgenic strains were basically established until the G6 and G7 generations. The posterior silk glands of randomly selected, Day 3, fifth-instar larvae from two different strains (TS-2-2 and TS-7) were separated and examined by Southern blot analysis. The results showed that transgenic silkworm strains were bred successfully (Fig. 4C). Our data indicate that an exogenous gene was integrated successfully into the

TABLE 1. Survey of G0 transgenic silkworms.

Egg batch	No. of eggs	No. of surviving larvae at fifth instar	No. of pupae	No. of fluorescent pupae	No. of adult moths		No. of PCR positive adult moths	PCR-positive rate (%)
					Female	Male		
TS-1	418	7	5	3	0	1	1	0.24
TS-2	326	15	6	6	2	3	5	0.15
TS-3	343	12	3	3	1	2	3	0.87
TS-4	404	13	2	2	0	1	1	0.25
TS-5	357	9	3	3	1	2	3	0.84
TS-6	394	17	0	0	0	0	0	0.00
TS-7	412	8	4	3	1	2	1	0.24
TS-8	297	21	2	2	0	2	0	0.00
Total	2951	102	25	22	5	13	14	0.47

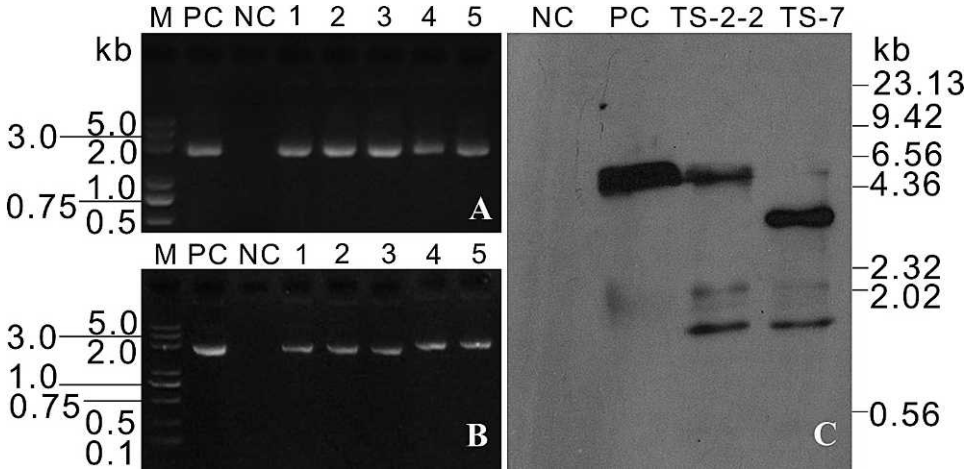

FIG. 4. Identification of the transgenic silkworms by PCR and Southern blot analysis. **A**) PCR amplification of the *gfp* expression cassette (*A3-gfp-SV40* fragment) using primers GFP-1 and GFP-2. **B**) PCR amplification of the Neo^R expression cassette (*ie-1-*Neo^R*-SV40* fragment) using primers Neo-1 and Neo-2. **C**) Southern blot hybridization of DNA from G7 generation transformed silkworms and controls with [α-^{32}P]-labeled *gfp* probe. M, DNA marker; NC, negative control (using the genomic DNA from control silkworms); PC, positive control (using plasmid pPIGA3GFP-IE-NEO-DNA); 1–5, transgenic silkworms (1, TS-2-1; 2, TS-2-2; 3, TS-3; 4, TS-5; 5, TS-7).

silkworm genome and transferred stably through seven generations.

DISCUSSION

Fertilization of a silkworm egg involves penetration by a number of sperm [9], which helps to increase the entry of exogenous DNA into the egg and improves the efficiency of SMGT. An adult silkworm moth produces hundreds of eggs, and the injection of sperm into the bursa copulatrix can be completed very quickly. In theory, it is possible to transfer a copy of an exogenous gene into many silkworm eggs to obtain multiple transgenic individuals, which is an important advantage of this technique over other gene transfer methods [15]. In the present study, 11 virgin female moths were injected with sperm carrying an exogenous gene, and fluorescence microscopy revealed that eight egg batches contained different numbers of fluorescent eggs (Table 1 and Fig. 3A), demonstrating that sperm can carry exogenous DNA into the egg. In the incubation stage, a large number of silkworm eggs emit green fluorescence (Fig. 3A), but less than 0.1% display the fluorescence in the late stage (Table 1). This implies the vast majority of exogenous DNA entering the egg does not become integrated into the silkworm genome and is gradually degraded during development of the individual silkworm.

Transposon piggyBac-mediated gene transfer is the most mature transgenic strategy for the silkworm, but the level of gene transfer efficiency remains low [7]. Although use of the *gfp* reporter can help in the screening of positive transgenic silkworms, the workload remains significant. In transgenic studies, antibiotic screening is more commonly used in cell culture systems and is seen only rarely in the screening of individual organisms, although attempts have been made in the field of silkworm transgenic studies [17, 18]. The present study introduced the Neo^R expression cassette into the transgenic vector and resolved this problem. Neomycin-sensitive silkworms gradually show attenuated development and eventually die after the addition of G418. The surviving individuals were selected on the basis of size: The developmentally retarded and drug-sensitive individuals gradually died, whereas large surviving individuals were used for breeding. Positive transgenic silkworms were finally selected via stereomicroscopic observation of fluorescence (Table 1 and Fig. 3). Our results confirmed the effectiveness of Neo^R in screening transgenic silkworms [17, 18] and demonstrated that the introduction of dual reporters of Neo^R and *gfp* and the subsequent screening against G418 in combination with fluorescence microscopy greatly reduced the workload and improved the level of screening efficiency (Table 1). Moreover, reports of the loss of an exogenous gene in the offspring of transgenic silkworms have appeared [19]. In the present study, introduction of Neo^R and screening against G418 in each generation actively and effectively prevented loss of the exogenous gene; the corresponding transgenic silkworm strains were established successfully after six generations of selection. Integration of the exogenous genes into the silkworm genome and their subsequent stability were confirmed by Southern blot analysis (Fig. 4C).

In summary, we constructed a piggyBac transposon-based gene transfer vector, established the SMGT system, and obtained transgenic silkworms by the SMGT method, which can promote the integration of exogenous target genes into the genome in the sperm or the early embryonic stage and can introduce the exogenous DNA into the fertilized silkworm egg through natural fertilization. This avoids any adverse effect on the egg-laying ability of female silkworm moths as well as damage to fertilized eggs, which facilitates subsequent hydrochlorization. In this sense, SMGT represents a transgenic technology that can be applied more practically to various productive silkworm varieties.

To date, the piggyBac transposon-based silkworm transgenic technology has been reported to have been successful in functional genomics studies [20–24], silkworm bioreactors [16, 17, 25–29], and biomaterials [30, 31] and in improving disease resistance [32]. The present results directly confirm these earlier results and establish a practical transgenic technology that can be applied directly to the productive sericulture of silkworm varieties. This resolves the bottleneck in the application of transgenic technology to sericulture production and is expected to facilitate sericulture production and basic biological studies.

ACKNOWLEDGMENT

We are grateful for help from the Zhejiang Provincial "12·5" Mulberry & Silkworm Breeding Research Collaboration Team.

REFERENCES

1. Celebi C, Guillaudeux T, Auvray P, Vallet-Erdtmann V, Jegou B. The making of ''transgenic spermatozoa.'' Biol Reprod 2003; 68:1477–1483.
2. Kues WA, Niemann H. The contribution of farm animals to human health. Trends Biotechnol 2004; 22:286–294.
3. The International Silkworm Genome Consortium. The genome of a lepidopteran model insect, the silkworm *Bombyx mori*. Insect Biochem Mol Biol 2008; 38:1036–1045.
4. Xiang ZH, Huang JT, Xia JG, Lu C. Biology of Sericulture. Beijing: China Forestry Publishing House; 2005:1–5.
5. Mita K, Kasahara M, Sasaki S, Nagayasu Y, Yamada T, Kanamori H, Namiki N, Kitagawa M, Yamashita H, Yasukochi Y, Kadono-Okuda K, Yamamoto K, et al. The genome sequence of silkworm, *Bombyx mori*. DNA Res 2004; 11:27–35.
6. Xia Q, Zhou Y, Lu C, Cheng D, Dai F, Li B, Zhao P, Zha X, Cheng T, Chai, C, Pan G, Xu J, et al. A draft sequence for the genome of the domesticated silkworm (*Bombyx mori*). Science 2004; 306:1937–1940.
7. Tamura T, Thibert C, Royer C, Kanda T, Abraham E, Kamba M, Kômoto N, Thomas JL, Mauchamp B, Chavancy G, Shirk P, Fraser M, et al. A piggyBac-derived vector efficiently promotes germ-line transformation in the silkworm *Bombyx mori* L. Nat Biotechnol 2000; 18:81–84.
8. Huang JT, Zhu WM, Xia JG, Xiang ZH. Complete Works of Sericultural Technology in China. Chengdu: Sichuan Publishing House of Science & Technology; 1996:488–556.
9. Feng JX. Silkworm Egg Production. Beijing: China Agriculture Press; 1993:8–23, 37–52.
10. García-Vázquez FA, Ruiz S, Grullón LA, Ondiz AD, Gutiérrez-Adán A, Gadea J. Factors affecting porcine sperm mediated gene transfer. Res Vet Sci 2011; 91:446–453.
11. Smith K, Spadafora C. Sperm-mediated gene transfer: applications and implications. Bioessays 2005; 27:551–562.
12. Lanes CF, Sampaio LA, Marins LF. Evaluation of DNase activity in seminal plasma and uptake of exogenous DNA by spermatozoa of the Brazilian flounder *Paralichthys orbignyanus*. Theriogenology 2009; 71: 525–533.
13. Ma HM, Quan FS, Chen DM, Zheng YL, Zhang BW, Wang YS, Zhang Y. Protective function of alpha-lipoic acid on sperm motility and mitochondrial function during goat sperm-mediated gene transfer. Small Ruminant Res 2011; 99:191–198.
14. Dai FY, Lu C, Xiang ZH, Chen ZY, Chen YL. Genetical research of BT924—a new line in white egg mutants of silkworm. Hereditas 2000; 22: 229–232.
15. Guo XY, Zhou ZY, Feng LC, Wang L, Lu C, Xiang ZH. Sperm-mediated gene transformation of silkworm. Prog Biochem Biophys 2001; 28: 423–425.
16. Xue RY, Chen HM, Cui LL, Cao GL, Zhou WL, Zheng XJ, Gong CL. Expression of hGM-CSF in silk glands of transgenic silkworms using gene targeting vector. Transgenic Res 2012; 21:101–111.
17. Liang C, Cao GL, Xue RY, Pan ZH, Zheng XJ, Zhou WL, Gong CL. Reducing blood glucose level in TIDM mice by orally administering the silk glands of transgenic hIGF-I silkworms. Biochem Biophys Res Commun 2011; 410:721–725.
18. Chen X, Zhao Y, Zhang F, Peng WP, Feng XL, Huang JT, Lu CD. Transformation of neomycin resistance gene (neo^R) into silkworm (*Bombyx mori* L.). Sheng Wu Hua Xue Yu Sheng Wu Wu Li Xue Bao (Shanghai) 1999; 31:90–92.
19. Wu XF, Cao CP. Targeting of human aFGF gene into silkworm, *Bombyx mori* L. through homologous recombination. J Zhejiang Univ Sci A 2004; 5:644–650.
20. Suzuki MG, Funaguma S, Kanda T, Tamura T, Shimada T. Analysis of the biological functions of a *doublesex* homologue in *Bombyx mori*. Dev Genes Evol 2003; 213:345–354.
21. Isobe R, Kojima K, Matsuyama T, Quan GX, Kanda T, Tamura T, Sahara K, Asano SI, Bando H. A fibroin secretion-deficient silkworm mutant, $Nd\text{-}s^D$, provides an efficient system for producing recombinant proteins. Insect Biochem Mol Biol 2005; 35:51–59.
22. Tan A, Tanaka H, Tamura T, Shiotsuki T. Precocious metamorphosis in transgenic silkworms overexpressing juvenile hormone esterase. Proc Natl Acad Sci U S A 2005; 102:11751–11756.
23. Uchino K, Sezutsu H, Imamura M, Kobayashi I, Tatematsu KI, Iizuka T, Yonemura N, Mita K, Tamura T. Construction of a piggyBac-based enhancer trap system for the analysis of gene function in silkworm *Bombyx mori*. Insect Biochem Mol Biol 2008; 38:1165–1173.
24. Zhang X, Xue RY, Cao GL, Hu XL, Wang XJ, Pan ZH, Xie M, Yu XH, Gong CL. Effects of *egt* gene transfer on the development of *Bombyx mori*. Gene 2012; 491:272–277.
25. Adachi T, Tomita M, Shimizu K, Ogawa S, Yoshizato K. Generation of hybrid transgenic silkworms that express *Bombyx mori* prolyl-hydroxylase α-subunits and human collagens in posterior silk glands: production of cocoons that contained collagens with hydroxylated praline residues. J Biotech 2006; 126:205–219.
26. Tomita M, Munetsuna H, Sato T, Adachi T, Hino R, Hayashi M, Shimizu K, Nakamura N, Tamura T, Yoshizato K. Transgenic silkworms produce recombinant human type III procollagen in cocoons. Nat Biotechnol 2003; 21:52–56.
27. Kurihara H, Sezutsu H, Tamura T, Yamada K. Production of an active feline interferon in the cocoon of transgenic silkworms using the fibroin H-chain expression system. Biochem Biophys Res Commun 2007; 355: 976–980.
28. Ogawa S, Tomita M, Shimizu K, Yoshizato K. Generation of transgenic silkworm that secretes recombinant proteins in the sericin layer of cocoon: production of recombinant human serum albumin. J Biotechnol 2007; 128: 531–544.
29. Li YM, Cao GL, Chen HM, Jia HF, Xue RY, Gong CL. Expression of the hGM-CSF in the silk glands of germline of gene-targeted silkworm. Biochem Biophys Res Commun 2010; 391:1427–1431.
30. Teuléa F, Miao YG, Sohn BH, KimYS, Hull JJ, Fraser MJ Jr, Lewis RV, Jarvis DL. Silkworms transformed with chimeric silkworm/spider silk genes spin composite silk fibers with improved mechanical properties. Proc Natl Acad Sci U S A 2012; 109:923–928.
31. Nagano A, Tanioka Y, Sakurai N, Sezutsu H, Kuboyama N, Kiba H, Tanimoto Y, Nishiyama N, Asakura T. Regeneration of the femoral epicondyle on calcium-binding silk scaffolds developed using transgenic silk fibroin produced by transgenic silkworm. Acta Biomater 2011; 7: 1192–1201.
32. Isobe R, Kojima K, Matsuyama T, Quan GX, Kanda T, Tamura T, Sahara K, Asano SI, Bando H. Use of RNAi technology to confer enhanced resistance to BmNPV on transgenic silkworms. Arch Virol 2004; 149: 1931–1940.