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Germline Transformation of the Silkworm Bombyx mori L. by Sperm-Mediated Gene Transfer1

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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 (NeoR) 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 NeoR 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 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 hydrochloridization. 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 (NeoR) 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 NeoR 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


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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 pH3P1G (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'-GCCGAATTCAGACATGA-3'.

The sequence is deposited in GenBank (ID: AY616665). NeoR and its downstream 325-bp SV40 3' untranslated sequence were amplified by PCR with the forward Neo-F primer (Neo-F) 5'-ATGATTGAA and the reverse Neo-R primer (Neo-R) 5'-GATTTGCAGTTCGGGAC-3'.

Transgenic Procedures

The plasmid DNA was purified using the EndoFree Plasmid Kit (Qiagen). The gene transfer vector pPIGA3GFP-IE-NEO and the helper plasmid pH3P1G 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 mixed DNA of gene transfer vectors was drawn into a glass needle (diameter, 20–40 μm; Narishige) and injected into the posterior silk gland of each G0 generation and used as template to amplify the gfp and NeoR expression cassettes. PCR primers were designed as: forward gfp primer (GFP-1), 5'-CGCGATCCAGACATGA-3', and reverse gfp primer (GFP-2), 5'-GATTTGCAGTTCGGGAC-3'.

PCR Identification of 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.
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-NeoR-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 NeoR 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.

<table>
<thead>
<tr>
<th>Egg batch</th>
<th>No. of eggs</th>
<th>No. of surviving larvae at fifth instar</th>
<th>No. of pupae</th>
<th>No. of fluorescent pupae</th>
<th>No. of adult moths</th>
<th>No. of PCR positive adult moths</th>
<th>PCR-positive rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TS-1</td>
<td>418</td>
<td>7</td>
<td>5</td>
<td></td>
<td>3</td>
<td>0</td>
<td>0.24</td>
</tr>
<tr>
<td>TS-2</td>
<td>326</td>
<td>15</td>
<td>6</td>
<td></td>
<td>6</td>
<td>2</td>
<td>2.00</td>
</tr>
<tr>
<td>TS-3</td>
<td>343</td>
<td>12</td>
<td>3</td>
<td></td>
<td>3</td>
<td>1</td>
<td>0.15</td>
</tr>
<tr>
<td>TS-4</td>
<td>404</td>
<td>13</td>
<td>2</td>
<td></td>
<td>2</td>
<td>0</td>
<td>0.15</td>
</tr>
<tr>
<td>TS-5</td>
<td>357</td>
<td>9</td>
<td>3</td>
<td></td>
<td>3</td>
<td>1</td>
<td>0.87</td>
</tr>
<tr>
<td>TS-6</td>
<td>394</td>
<td>17</td>
<td>0</td>
<td></td>
<td>0</td>
<td>0</td>
<td>0.25</td>
</tr>
<tr>
<td>TS-7</td>
<td>412</td>
<td>8</td>
<td>4</td>
<td></td>
<td>3</td>
<td>1</td>
<td>0.75</td>
</tr>
<tr>
<td>TS-8</td>
<td>297</td>
<td>21</td>
<td>2</td>
<td></td>
<td>2</td>
<td>0</td>
<td>0.00</td>
</tr>
<tr>
<td>Total</td>
<td>2951</td>
<td>102</td>
<td>25</td>
<td></td>
<td>22</td>
<td>5</td>
<td>0.47</td>
</tr>
</tbody>
</table>
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<sup>R</sup> 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<sup>R</sup> in screening transgenic silkworms [17,18] and demonstrated that the introduction of dual reporters of Neo<sup>R</sup> 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<sup>R</sup> 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.

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