Introduction of Foreign Genes into Silkworm Eggs by Electroporation and Its Application in Transgenic Vector Test

Xiu-Yang GUO#, Liang DONG#, Sheng-Peng WANG, Ting-Qing GUO, Jian-Yang WANG, and Chang-De LU*

Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, the Chinese Academy of Sciences, 320 Yueyang Road, Shanghai 200031, China

Abstract  Electroporation as a methodology to introduce foreign genes into silkworm eggs was systematically analyzed. The foreign gene in both the newly hatched and 3rd instar larva DNA can be detected by PCR. The amount of foreign gene in 3rd instar larva DNA was about 1/1000 of that in newly hatched larva DNA. The ratio of foreign gene entering into silkworm eggs was voltage dependent and showed significant difference between the tested silkworm strains. When the piggyBac transposon system was applied, the effect of nuclear localization signal (NLS) peptide and the in vitro transcribed transposase mRNA on the transposition rate has been measured. Results showed that the in vitro transcribed transposase mRNA facilitated transposition to take place earlier and NLS could result in higher transposition probability and earlier transposition as well. When linearized vectors containing varied length of flanking homologous sequences around a reporter gene were introduced into silkworm eggs by electroporation, the one with 2.6 kb total arm length gave higher G1 positive ratio than that with total arm length of 1.5 kb and 800 bp.

Key words  silkworm eggs; gene introduction; electroporation; transposon; homologous sequences

Up to now, the most practical method for introducing foreign gene into insect eggs is the microinjection as that was utilized in Drosophila melanogaster [1–3]. The discovery of transposable elements including: P, Minos, Hermes, mariner, hobo, piggyBac, etc., has supplied useful tools in gene transformation in various insects [1,4–10]. By the application of piggyBac transposable element and injection system, transgenic silkworm has also been successfully established by Tamura et al. [11–14]. To improve the transformation probability, the RNA transcript of transposase gene was used instead of the expression plasmid by Li et al. [15] in piggyBac system and Kapetanaki et al. [16] in Minos system respectively, but only the latter got positive results. Although most transgenic insect species were got by transposition, gene targeting in the Droso- phila and silkworm could also be achieved by homologous recombination [17–19].

Gene transformation using electroporation has been successfully applied in prokaryotic and eukaryotic cells. In our previous work, electroporation was used in introducing gfp gene, anti-BmNPV ribozyme gene and an artificial synthesized fibroin-gfp fusion gene, into silkworm eggs [20–22]. The advantage of the electroporation method is that large quantity of silkworm eggs can be transformed in a very short time. However, how to use it for introducing foreign gene into silkworm eggs needs systematicat investigation.

In this study, we reported that the efficiency of introducing foreign gene into silkworm eggs was voltage dependent and showed high difference between the tested silkworm strains. In virtue of the large quantity processing ability of the electroporation method, we used it for silkworm transgenic vector test. When piggyBac transposon system was used, it was found that in vitro transcribed transposase mRNA facilitated transposition to take place earlier and NLS could result in higher transposition probability and earlier transposition as well. In order to see the effect of length of homologous sequences on the recombination probability in silkworm, linearized vectors containing varied length of flanking homologous arms
around a reporter gene were transferred into silkworm eggs by electroporation. The one with 2.6 kb total arm length gave higher G1 positive ratio than the other two with total arm length of 1.5 kb and 800 bp respectively.

Materials and Methods

Silkworm strains

The Bombyx mori L. bivoltine strain 54A and polyvoltine strain S10 were obtained from Sericultural Research Institute, Chinese Academy of Agricultural Sciences (Zhenjiang, China). The 54A eggs were electroporated and incubated at 25 °C and 90% relative humidity, 18 h later were subjected to acid treatment to terminate the diapause, and then were incubated again till hatching. The S10 embryos were incubated at 22 °C and 90% relative humidity in culture chamber till hatching. Newly hatched larvae were transferred to mulberry leaves and raised under standard condition [23].

Plasmids

pUC18-gfp was constructed by inserting the green fluorescent protein gene into pUC18. It is about 3.6 kb long. Plasmid A3IFP2 carrying a PiggyBac transposase gene driven by BmA3 promoter was construct as following. Firstly, the BmA3 promoter was amplified by PCR from S10 genome DNA using primers 5'-AGCTGATATCGAAAGGCGTTGGTCGCTCATG-3' and 5'-GTCGACCTTGAATTTCGAGATGTCGTCGAGG-3', the latter complements with either the 3' end of BmA3 promoter or the 5' end of the PiggyBac transposase [24]. Another fragment containing initial codon ATG to polyA signal sequence of PiggyBac transposase gene was amplified by PCR with the primer pair 5'-CGGTCTGTATATCGAGG-3' from plasmid p3E1.2, a gift from Prof. JM Fraser [10]. Finally these two overlapping fragments were annealed to form the composite transposase expression box, which was amplified, cloned into pGEM-T vector (Promega Corporation, Madison, WI) to form A3IFP2. Plasmid A3IFP2 was verified by nucleotide sequencing and was used as DNA helper in this article. The piggyBac transposition vector pBac[3×P3-EGFPfami] carrying an egfp gene driven by promoter 3×P3 was generously provided by Horn et al. [25], and referred as donor plasmid in this article. The pPigT7, PiggyBac transposase in vitro transcription vector, was constructed by cloning PCR amplified fragment with T7 promoter containing primer 5'-GAATTCATATCAGACTCATAAGGCGCGCTTGGAGCTCGG-3' and polyT containing primer 5'-AAAGCTTCTTATTTTTTATTTTTTTTTTTTTGCGTGCTGATATCGAAAGGCGTTGGTCGCTCATG-3' from plasmid p3E1.2 into plasmid pUC19.

The plasmids for homologous recombination experiments were constructed by insertion of different length rDNA fragments as left and right flanking sequence into 3' and 5' end of IE-neo5 fragment of plasmid pIN, a neomycin resistance gene driven by BmNPV immediately early gene promoter which was previously constructed in our laboratory [26]. Plasmid pRinN with 2.6 kb total arm length has a 1.2 kb left arm amplified by using primer 5'-AAGCTGATATCGAAAGGCGTTGGTCGCTCATG-3' and 5'-GTCGACCTTGAATTTCGAGATGTCGTCGAGG-3', and a 0.8 kb right arm amplified by using primer 5'-CTTGGAGCTCGG-3 and 5'-GATATCGAGCTCTTGCGAGCATCGCGCCCTGA-3' from S10 genome. Plasmid pRinN with 1.5 kb total arm length has a 0.7 kb left arm amplified by using primer 5'-AAGCTTCTGATATCGAAAGGCGTTGGTCGCTCATG-3' and 5'-GTCGACATGCCGGTGCCTTGCCTGCGTGTCCTGC-3', and a 0.8 kb right arm amplified by using primer 5'-TCTAGAATATCGGATATTTGCCTGCGGCCGTCGTCCTGC-3' and 5'-GATATCGAGCTCTTGCGAGCATCGCGCCCTGA-3'. Plasmid pRsIN with 0.8 kb total arm length has a 0.35 kb left arm amplified by using primers: 5'-AAGCTTCTGATATCGAAAGGCGTTGGTCGCTCATG-3' and 5'-GTCGACATGCCGGTGCCTTGCCTGCGTGTCCTGC-3', and a 0.45 kb right arm amplified by using primer 5'-TCTAGAATATCGGATATTTGCCTGCGGCCGTCGTCCTGC-3' and 5'-GATATCGAGCTCTTGCGAGCATCGCGCCCTGA-3'. Plasmid pRsIN with 0.8 kb total arm length has a 0.35 kb left arm amplified by using primers: 5'-AAGCTTCTGATATCGAAAGGCGTTGGTCGCTCATG-3' and 5'-GTCGACATGCCGGTGCCTTGCCTGCGTGTCCTGC-3', and a 0.45 kb right arm amplified by using primer 5'-TCTAGAATATCGGATATTTGCCTGCGGCCGTCGTCCTGC-3' and 5'-GATATCGAGCTCTTGCGAGCATCGCGCCCTGA-3'.

RNA transcription in vitro

PiggyBac transposase mRNA, referred as RNA helper in this article, was synthesized applying Riboprobe system-T7 (Promega) with 10 µg HindIII linearized pPigT7 in 100 µl reaction solution using a modified transcription buffer [29]. After the synthesis, the DNA template was digested with 50 U RNase-free DNase I (Promega), and the transcripts were precipitated by adding 1/5 volume of 5 M NH4Ac and 2.5 volume of absolute ethanol. The transcript was verified by agarose gel electrophoresis and quantified by 260 nm UV absorbance.

Nuclear localization signal (NLS) peptide synthesis

Nuclear localization signal peptide (NLS) with sequence
of CGGPKKKRKVG-NH₂ was synthesized by using PSSM-8 polypeptide automatic synthesis system (Shimadzu).

Egg collection and electroporation

Female moths copulated for about 5 h, and laid eggs on plastic sheet with rough surface in dark. The eggs should be wiped off from plastic sheet with ice-cold water within two hours, collected and dried using gauze. Then they were put into ice-cold 0.4 cm electroporation cuvette containing electroporation solution. One cuvette can contain about 600–700 eggs. The electroporation was performed on Gene pulser II electroporation system (Bio-Rad) with varied voltage, constant resistance of 200 Ω and capacitance of 25 µF. After electroporation, the eggs were taken out immediately and air-dried at room temperature before incubation.

DNA extraction, PCR and Southern blot analysis

DNA was extracted either with proteinase K/RNase A method or by using DnAzol reagent following manufacturer’s instruction. Usually the DNA extracted with proteinase K/RNase A method was better, but if small amount of material was treated, e.g. one newly hatched silkworm, using DnAzol reagent was more expedient. In one comparison, all DNA samples were prepared by using the same method. In many experiments we detected the DNA mixture extracted from enough amount of samples, for the positive ratio assay, 50 or 60 samples at each development stage were detected separately.

DNA extracts were dissolved in TE buffer at a concentration of 0.5 µg/µl. All PCR reactions were run in Gene Amp 2400 (Applied Biosystems) in 25 µl, using 1 µl DNA extract or its different dilution as template. To avoid the influence of template DNA amount on the PCR amplification, when the DNA concentration was lower than 5×10⁻⁴ µg/µl, the dilution buffer would contain 5×10⁻⁵ µg/µl DNA extracted from normal silkworms.

egfp (or gfp) was amplified with primer 5'-ATGGTGAGCAAGGCGAGAGC-3' and 5'-TCTTCATGCAGAGTGATC-3'. neo8 was amplified with primer 5'-GCCAGGAACTCAGGATGATC-3' and 5'-CAAGAGCAAGGAATGAGGATCG-3'.

All of the PCR reactions were performed as follows: 94 °C for 5 min; 94 °C for 30 s, 60 °C for 1 min, and 72 °C for 30 s, 35 cycles.

In Southern blot analysis, the Dig DNA labeling and detection kit (Roche) was used following the manufacturer’s protocol. All probes for Southern blot hybridization were Dig labeled by PCR with the correspond-
also showed that there was positive signal with 5 pg newly hatched larva DNA mixture, for the 3rd-instar larva DNA, there were positive signals when no less than 500 pg DNA mixture was used as template (Fig. 1). There was no hybridization band detected using DNA from 5th-instar larva or pupa. By comparing band grey scale, the amount of foreign DNA in newly hatched larvae DNA is about \(1 \times 10^3\) times higher than that in the 3rd-instar larva DNA since the band intensity of lane 3 (IOD=33584) and lane 6 (IOD=35439) are similar while the difference in the amount of their template DNA is \(1 \times 10^3\) fold. To semi-quantify the degradation of the foreign DNA in each silkworm larva, the increased cellular DNA during the 10 days should be considered. The DNA extracted from each newly hatched larva and the third instar larva were on average of 63 ng and 570 ng respectively, so the foreign DNA in each silkworm larva was degraded about more than 100-fold during ten days (from newly hatched stage to the third instar).

The genome of silkworm is about \(4.5 \times 10^8\) base pairs, the insertion of one copy of 4 kb DNA fragment matches 1 pg of pUC18-GFP DNA mixed in 0.1 \(\mu\)g genomic DNA, and it is 100-fold of the detecting sensitivity by electrophoresis gel EB staining after PCR amplification. Even the integration of foreign DNA happened only in a small part of cells, it still could be easily detected.

We also clarified the least amount of template DNA needed when the PCR product to be detected by EB staining or Southern blot method. As shown in Fig. 2, when 0.01 pg of pUC18-GFP DNA was used as template for PCR, \(gfp\) band could be clearly detected by electrophoresis gel EB staining, while 0.01 fg of pUC18-GFP DNA could be clearly detected under the same condition using Southern blot method. The sensitivity of Southern blot is about a 1000-fold higher than EB staining. Although the assay sensitivity can be further improved by changing reaction condition of PCR, but for this experiment it is appropriate. The genome of silkworm is

Fig. 1 Detection of introduced foreign gene in silkworm of different developmental stages

Different amount of DNA extracted from silkworms of different developmental stages were used as template for PCR amplification of \(gfp\) and 5 \(\mu\)l of the PCR products were subjected to gel electrophoresis and Southern blot analysis. DNA templates for each PCR reaction were as follows: 1, 2, 3 and 4: 500 pg, 50 pg, 5 pg and 0.5 pg of newly hatched larvae DNA were used respectively; 5, 6 and 7: 50 ng, 5 ng and 0.5 ng of the third instar larvae DNA were used respectively; 8 and 9: 500 ng and 50 ng of fifth instar larvae DNA were used respectively; 10 and 11: 500 ng and 50 ng of pupal DNA were used respectively. IOD for each band were: 1, IOD=473960; 2, IOD=206160; 3, IOD=33584; 5, IOD=168490; 6, IOD=35439.

According to above results, to detect the entrance of foreign genes and calculate the positive ratio we used G0 newly hatched larvae. Considering the unevenness of the entry of foreign DNA into each silkworm egg, we applied both EB staining and Southern blotting assays. Whereas in detecting the transgenic events, G1 progeny silkworm was used. Since remaining un-integrated plasmid in 5th instar larvae of G0 silkworm was already below detectable level with this method, samples from 5th instar larva, pupa or moth of G0 silkworm can also be used if required.

Introduction of foreign gene into silkworm eggs by electroporation was voltage dependent and showed big difference between the tested silkworm strains.

The positive ratio of electroporated eggs was detected with newly hatched larvae. The \(piggyBac\) transposon system was applied in this test since it was proved effective in silkworm transgenesis [11,12]. Fig. 3 showed part results of gel electrophoresis of PCR product and its Southern blot counterpart. The interior control of 1 kb fragment amplified from \(ser1\) gene shown in each lanes. The visible 0.7 kb \(egfp\) band only showed in three lanes by EB staining (Fig. 3, A) but showed in 14 lanes in the Southern blot counterpart (Fig. 3, B). The three larvae with \(egfp\) gene
band as strong as the interior control may have some tissues with genomic integration of the introduced foreign gene or there was at least 0.1 pg residual \( p_{Bac}[3\times P3-EGFPafm] \) in the three newly hatched larvae inferring from the above results and the amount of the DNA template we used. The others with only hybridization positive signal had at least 0.1 fg residual \( p_{Bac}[3\times P3-EGFPafm] \).

It was found that the positive and survival ratio of electroporation for different strains of silkworms differed a lot. The positive ratio of a same strain was voltage dependent as expected when resistance and capacitance were fixed at 200 \( \Omega \) and 25 \( \mu F \) respectively. The survival ratio of 54A was 16% when voltage reached 700 V and related positive ratio in PCR product southern blot analysis was 23%. But for S10, there was still 16% survival rate when voltage reached 1700 V and the corresponding positive ratio was 78%. The results of electroporation were summarized in Table 1, from which we suggested that the electroporation condition should be optimized for each silkworm strain. For 54A transgenesis, 500–600 V was recommended, and for S10 transgenesis, 1500–1600 V was more suitable.

In vitro transcribed transposase mRNA facilitated transposition to take place earlier and NLS resulted in increase of transposition and earlier transposition compared with helper plasmid.

We tested the effect of the in vitro transcribed transposase mRNA and the synthesized NLS peptide on the excision and re-ligation rate of the donor plasmid \( p_{Bac}[3\times P3-EGFPafm] \). Three combinations of solutions were used for electroporation of S10 eggs: (1) RNA helper, 0.1 \( \mu g/\mu l \) PiggyBac transposase mRNA and 0.2 \( \mu g/\mu l \) donor plasmid \( p_{Bac}[3\times P3-EGFPafm] \) in RNase free water; (2) NLS+DNA helper, 0.2 \( \mu g/\mu l \) helper plasmid and donor plasmid, and 0.07 \( \mu g/\mu l \) NLS peptide at a plasmid:NLS molar ratio of 1:100 in ddH\(_2\)O; (3) DNA helper, 0.2 \( \mu g/\mu l \)

### Table 1 Survival ratio and positive ratio of 54A and S10 eggs electroporated at different voltages

<table>
<thead>
<tr>
<th>Voltage (V)</th>
<th>Survival ratio(^a)</th>
<th>Positive ratio(^b)</th>
<th>Survival ratio(^a)</th>
<th>Positive ratio(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>300</td>
<td>35</td>
<td>12</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>400</td>
<td>31</td>
<td>14</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>500</td>
<td>30</td>
<td>20</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>600</td>
<td>28</td>
<td>20</td>
<td>85</td>
<td>6</td>
</tr>
<tr>
<td>700</td>
<td>16</td>
<td>24</td>
<td>78</td>
<td>20</td>
</tr>
<tr>
<td>800</td>
<td>–</td>
<td>–</td>
<td>73</td>
<td>24</td>
</tr>
<tr>
<td>900</td>
<td>–</td>
<td>–</td>
<td>69</td>
<td>32</td>
</tr>
<tr>
<td>1000</td>
<td>–</td>
<td>–</td>
<td>62</td>
<td>42</td>
</tr>
<tr>
<td>1100</td>
<td>–</td>
<td>–</td>
<td>57</td>
<td>50</td>
</tr>
<tr>
<td>1300</td>
<td>–</td>
<td>–</td>
<td>33</td>
<td>60</td>
</tr>
<tr>
<td>1500</td>
<td>–</td>
<td>–</td>
<td>23</td>
<td>76</td>
</tr>
<tr>
<td>1700</td>
<td>–</td>
<td>–</td>
<td>16</td>
<td>78</td>
</tr>
</tbody>
</table>

\(^a\)The hatched silkworm to total electroporated eggs from one cuvette (about 600 per cuvette). They were the averages of three treatments; \(^b\)Positive newly-hatched larvae in total newly-hatched larvae. The number analyzed in each treatment was 60 larvae for strain 54A and 50 larvae for strain S10.
each of helper plasmid and donor plasmid in ddH₂O. For each combination about 5000 eggs were electroporated at 1500 V, 200 Ω and 25 µF, and incubated thereafter. 100 eggs of each combination were selected randomly every 4 h after electroporation for DNA extraction and PCR detection of the occurrence of the excision and re-ligation of donor plasmid.

PCR product electrophoresis gel from all three combinations showed no visible band, which indicated that the excision and re-ligation of donor plasmid was a very low probability event. So the hybridization analysis was further applied, and the bands of expected length were detected (Fig. 4). In RNA helper group, the band appeared 4–8 h after electroporation and was the earliest one in the three combinations, which meant that the transposase was translated sooner and caused excision and re-ligation of donor plasmid earlier than the two others. But the band lasted less than 24 h due to the rapid degradation of the RNA molecules. NLS+DNA helper group showed the band after 8–12 h and lasted for 40–44 h, whereas the band in DNA helper group existed from 12–16 to 32–36 h. It was indicated that NLS peptide could help more plasmid molecules entering the nuclear and more transposase to be expressed.

However, the longer band expected to be amplified by the same pair of primers from the intact donor plasmid was not detected in our experiment. The different amplifying efficiency may be the reason, which also existed in Minos transposable element system [16].

**Homologous recombinant vector with longer arm length gives higher G1 positive ratio when introduced into silkworm eggs by electroporation**

In gene targeting, the homologous recombinant is very important. To study the effect of the length of flanking homologous sequences on the homologous recombinant, three homologous recombinant vectors, pRbIN, pRmIN, and pRsIN, with different length of homologous sequences to silkworm rDNA gene were constructed as described. About 5000 eggs of S10 silkworm were electroporated with 0.2 µg/µl of pRbIN, pRmIN and pRsIN respectively. About 100 pairs from each group were copulated to give G1 progeny. All newly hatched G1 larvae were collected (about 30000 per group) and used for DNA extraction and PCR analysis. The gradient dilutions of each PCR product were subjected to electrophoresis and Southern blot analysis.

As shown in Fig. 5, the integrated optical density of pRmIN and pRsIN group were about the same. The pRbIN group gave about 10 fold higher intensity. Its third band (lane 3) was nearly equal to the second band of the two other (lane 5, and 8). This indicated that there was about ten times more foreign gene integration in pRbIN group than the two others. If the total length of the arms was less than 1.5 kb as pRmIN and pRsIN, the difference would be insignificant.

**Discussion**

Because it is hard to distinguish the plasmid in the embryo from on the eggshell, silkworm eggs were not used for detecting the existence of introduced foreign genes except detecting the excision and re-ligation of the donor plasmid.
The entrance of foreign gene by electroporation was found to be voltage dependent when other conditions were fixed. High difference also existed between the tested silkworm strains mainly originated from the egg morphology and physiology variations. The acid treatment of the electroporated 5A embryos might also be responsible for the low survival ratio and positive ratio. For the acid treatment might deteriorate the survival chance of the eggs that had been hurt somewhat by electroporation. Since silkworm eggs were easy to obtain, for getting higher transgenic ratio, relative lower survival ratio is acceptable. Furthermore, the electroporated eggs inclined to be affected by the hatching condition, especially the relative humidity, while the positive ratio did not affected by relative humidity.

The common strategy for insect transgenesis with transposon system is to introduce the donor plasmid and helper plasmid of the transposon system at the same time into fertilized eggs. Considering that there is no new transcription of mRNA at the early several proliferation cycles of embryo, the proteins required by embryo development are translated from those maternal mRNA stored in eggs. Thus the introduced transposase mRNA might be translated early and caused the earlier transposition. Our results showed that introduction of the in vitro transcribed transposase mRNA instead of DNA helper could make transposition happen earlier, which would certainly promote the whole transgenesis. When NLS exists, the excision and re-ligation of the donor plasmid happen earlier and last longer than DNA helper alone. This means that the occurrence of foreign gene expression cassette transposing into host chromosome will also increase. This indicates that NLS peptide facilitates the nuclear entrance of foreign gene to improve the expression of transposase. In zebrafish transgenesis, nuclear localization signal peptide has been shown effective in helping foreign gene to enter the nuclear, and elevate the transgenic ratio [31,32].

Our results showed that the vector with total arm length of 2.6 kb gave about 10 times more foreign gene integrations in G1 progeny than that of 1.5 kb and 800 bp. Kang et al. [33] have obtained similar results in injected embryos and in transfected cells when using SINEs as homologous arms.

This study showed that the electroporation could treat thousands of eggs in a very short time, and provided a good way for test transgenic vectors. It also could be a simple and useful routine method for foreign gene introduction in making transgenic silkworm or some other insects with hard shell eggs.

Acknowledgements

The authors thank Dr. EA Wimmer (University Bayreuth) for plasmid pBac[3×P3-EGFPafm] and Dr. MJ Fraser (University of Notre Dame) for plasmid p3E1.2. Thank Dr. Yun ZHAO for critical reading of the manuscript.

References

7. Warren WD, Atkinson PW, O’Brochta DA. The hermes transposable element from the house fly, Musca domestica, is a short inverted repeat-type element of the hobo, Ac, and Tam1(hAT) element family. Genet Res, 64(2): 87–97
15. Li X, Heinrich JC, Scott MJ. PiggyBac-mediated transposition in Dros-

Edited by
Zu-Xun GONG