

# Transfer of Foreign Gene to Giant Freshwater Prawn (*Macrobrachium rosenbergii*) by Spermatophore-Microinjection

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**ABSTRACT** We developed a spermatophore-microinjection (SMI) technique that allows exogenous DNA fragments to be transferred easily into the giant freshwater prawn (*Macrobrachium rosenbergii*), an important aquacultural shellfish and aquatic invertebrate model. From 28 to 1,000 ng of the circular plasmid pGL, in a total volume of 1  $\mu$ l, were directly microinjected into spermatophores. Fertilization and hatching of prawns created with SMI were completed in vivo. Fertilization and hatching rates in the SMI treatments did not differ from those of the untreated control group. The genomes of free swimming, SMI-created larvae (21 days after fertilization) were analyzed using PCR and Southern blot analyses. A product with a molecular mass of 680 bp was amplified. It corresponded to amplifications of pGL, and Southern blot analysis revealed that the amplified band was positive. The gene transfer rate was primarily dependent on the concentration of DNA during SMI. The higher the concentration of pGL, the higher the rate of gene transfer. PCR and Southern blot analyses detected the existence of foreign DNA in 16 of 23 samples (70%) of genomic DNA isolated from hatched larvae in the 750 ng pGL SMI treatment. SMI, described here for the first time, is the simplest and most efficient method for mass producing transgenic giant freshwater prawns. *Mol. Reprod. Dev.* 56:149–154, 2000 © 2000 Wiley-Liss, Inc.

**Key Words:** gene transfer; microinjection; prawn; spermatophore

## INTRODUCTION

Transgenic animals provide a powerful system for in vivo study of gene regulation, expression, and function, and make possible the production of transgenic varieties having special genetic traits. Many approaches have been developed to introduce foreign DNA molecules into zygotes. In fish, methods for gene transfer include microinjection of foreign DNA into oocyte nuclei (Ozato et al., 1986; Tsai et al., 1995b), the cytoplasm of developing embryos (Chourrout et al., 1986; Dunham et al., 1987), and fertilized eggs (Fletcher et al., 1988; Dunham et al., 1992; Lu et al., 1992). Electroporation of exogenous DNA fragments into fertilized eggs (Inoue et al., 1990; Powers et al., 1992; Tsai and Tseng, 1994) is a popular technique because, compared with the tradi-

tional microinjection technique, electroporation is simple, convenient, and efficient. However, the efficacy of gene transfer by electroporation of fertilized eggs is still not high enough to treat the tremendously large number of eggs spawned within a very short time by cultured finfish and shellfish species.

Thus, for aquatic animals, interest in sperm-mediated gene transfer has increased because this technique has several advantages over other methods of gene transfer applied to finfish and shellfish. First, sperm-mediated gene transfer can be applied to huge numbers of oocytes. Second, sperm-mediated gene transfer overcomes some of the disadvantages of conventional gene transfer systems resulting from egg characteristics such as opaqueness, stickiness, buoyancy, invisible pronuclei, and a tough chorion. Third, foreign DNA carried by sperm is transferred into the nucleus; with electroporation, the probability that DNA fragments will be transferred into the blastodisc, the volume of which is extremely small in a fertilized egg, is very low. Fourth, finfish and shellfish sperm can be activated by simply adding water, and they can be cryopreserved. Thus, treated sperm is ready for use at any time.

A number of researchers have attempted to use sperm as a vector for introducing foreign DNA and producing transgenic finfish and shellfish. Chourrout and Perrot (1992) reported failure using a sperm-incubation technique with rainbow trout. However, some success has been achieved using sperm-incubation with zebrafish (Khoo et al., 1992), and sperm-electroporation with common carp, catfish, tilapia (Muller et al., 1992), salmon (Symonds et al., 1994), loach (Tsai et al., 1995a), and abalone (Tsai et al., 1997).

There have been few attempts to apply gene transfer techniques to an important group of shellfish: crustaceans. Gendreau and colleagues (1995) successfully used a ballistic technique to transfer genes into brine shrimp (*Artemia*). However, the facilities and DNA

Grant sponsor: National Science Council; Grant sponsor: Council of Agriculture, Republic of China.

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Received 15 November 1999; Accepted 20 January 2000

preparations required for ballistic transfer are extremely expensive, and the work is tedious and unsuitable for field use. Bensheg and Khoo (1997) used microinjection to transfer a reporter gene into the fertilized eggs of freshwater shrimp, *Macrobrachium lanchesteri*. Again, traditional microinjection is laborious, time-consuming, and can only treat a limited number of eggs. In this communication, we describe a simple and effective way to transfer foreign DNA fragments into giant freshwater prawn on a massive scale using spermatophore-microinjection (SMI). SMI may be quite useful for studying gene regulation and for creating new crustacean strains having special traits.

## MATERIALS AND METHODS

### Experimental Animals

Nine- to twelve-month-old, aquacultured, giant freshwater prawns were purchased locally. They averaged  $16.6 \pm 4.8$  cm (males) and  $11.5 \pm 2.9$  cm (females) in length,  $3.7 \pm 0.6$  cm (males) and  $2.5 \pm 0.4$  cm (females) in width, and  $61.4 \pm 11.7$  g (males) and  $33.6 \pm 7.4$  g (females) in weight. Males and females were maintained in separate aquarium tanks at a density of 1 (males) or 2 (females) per 10 liters at 28°C on a natural light cycle.

### Spermatophore Extrusion and In Vitro Fertilization

Spermatophores were extruded from male prawns using electric shock (36–10 V, DC, model PS-304, Shin-Ray Electronic, Taiwan). The positive pole was attached to the fifth swimming pod and the negative pole was placed on the ventral ganglia. Thirteen mature males were used to examine the degree of spermatophore extrusion caused by voltages ranging from 0 to 10 V at 10 A.

Foreign DNA, at concentrations of 28, 250, 400, 750, and 1,000 ng/ $\mu$ l in a total volume of 1  $\mu$ l, was directly microinjected into the extruded spermatophore. For the mock-treated control group, we microinjected 1  $\mu$ l of double-distilled water into the extruded spermatophore. For in vitro fertilization, a treated spermatophore was placed for 2–3 min in the spermatheca, located on the thorax between the third and fifth swimming pods, of a female prawn that had just finished molting and had well-developed ovaries. In general, treated females began to fertilize their eggs in vivo 2–6 hr after spermatophores were attached. When their eggs were close to hatching, these females were moved to a 100 liter tank containing 12 ppt salt water.

### DNA Preparation

pGreen Latern-1 (pGL, Gibco BRL), with a molecular mass of 5 kb, consists of an immediate early-gene promoter and enhancer of cytomegalovirus, fused with the cDNA of a green fluorescence protein (GFP) and a 3'-untranslated region of SV40. They were prepared by the cesium chloride-ethidium bromide ultra-centrifugation method (Sambrook et al., 1989). The circular form

of pGL was resuspended in double-distilled water (sterilized) at concentrations of 28, 250, 400, 750, and 1,000 ng per  $\mu$ l.

### DNase Digestion

In order to ensure PCR detection of foreign DNA molecules in embryos, we had to avoid contamination of the DNA fragments that remained outside the embryos. First, we determined the conditions that allowed DNase I (Sigma Chemical Co., St. Louis, MO) to completely digest the greatest amount of DNA employed in transgenesis. pGL of 1,000 ng was resuspended in 100  $\mu$ l of digestion buffer (10 mM MgCl<sub>2</sub> and 20 mM TrisCl, pH 8), then 0.5, 0.75, 1, or 10  $\mu$ g of DNase I was added, and the solution was incubated at 37°C for 1 hr. Each treatment was duplicated. One-hundredth of the reaction volume was analyzed by PCR to determine the concentrations of DNase capable of completely digesting DNA molecules.

### Genomic DNA Extraction

Five embryos that had developed to the zoea-I-stage were pooled and collected in an eppendorf tube, washed three times with double-distilled water, and resuspended in a 500  $\mu$ l of digestion buffer. Then, DNase I was added until a final concentration of 100  $\mu$ g/ml was achieved. The solution was incubated at 37°C for 1 hr. After the reaction, the embryos were centrifuged at 76g for 3 min. The supernatant was decanted, and samples were boiled for 10 min to halt DNase activity. A volume of 400  $\mu$ l lysis solution (6 M guanidine hydrochloride and 0.1 M sodium acetate, pH 5.5) was added to the embryos, which were briefly crushed and then incubated at 37°C for 1 hr in a roller shaker. Then the genomic DNA of embryos was obtained by phenol-chloroform extraction and ethanol precipitation.

Twenty-three free-swimming larvae, randomly selected from the experimental and control groups, were examined for foreign DNA. Genomic DNA was extracted from each larva and prepared as described above for embryo DNA, except that DNase was not added. Each larva was washed three times in double-distilled water, resuspended in 400  $\mu$ l lysis buffer, crushed slightly, and incubated at 37°C for 1 hr in a roller shaker. The genomic DNA was obtained by phenol-chloroform extraction and ethanol precipitation.

### PCR Analysis

Two oligonucleotide primers, EGFP-F and GFP-R, were synthesized for detection of GFP cDNA by PCR analysis. The nucleotide sequences for forward and reverse primers were ATGGTGAGCAAGGGCGAGGA (EGFP-F) and CAGCTCGTCCATGCCATGTG (GFP-R), respectively. The primers for detection of the endogenous  $\beta$ -tubulin gene, which served as an internal control, were CCCTTCCCTCGTCTCCAC (forward, tub-f6) and GCCAGTGTACCAGTGAAGGGA (reverse, tub-r7). Each PCR sample consisted of 20  $\mu$ l of solution containing 10–20 ng of templates, 5–10 pmol of each

primer, 25  $\mu$ M of each dNTP, 20  $\mu$ g of bovine serum albumin (Giambbernardi et al., 1998), and five units of ProZyme II (Protech) in a 1 $\times$  PCR buffer (Protech). Amplification was performed with a DNA Thermal Cycler (Perkin-Elmer Cetus, Norwalk, CT). PCR consisted of 25 cycles of denaturing at 94°C for 1 min, annealing at 60°C for 1 min and extension at 72°C for 2 min, followed by 10 min extension at 72°C. Each PCR sample (10  $\mu$ l) was subjected to electrophoresis on a 3% NuSieve GTG agarose gel (FMC BioProducts, Rockland, ME).

**Probe Preparation**

The 0.6 kb fragment containing GFP cDNA was recovered from the agarose gel using Jetsorb Gel Extraction (Genomed, Inc., Research Triangle Park, NC) after plasmid pGL was restricted by NotI and run on the gel. Three micrograms of the 0.6 kb DNA fragment were DIG-labeled by the random priming method at 37°C for 20 hr, according to the manufacturer’s recommended procedures (Boehringer Mannheim).

**Southern Blot Analysis**

PCR products were analyzed on an agarose gel and transferred onto a nitrocellulose membrane (Amersham, Pharmacia Biotech, Uppsala, Sweden). After air drying, the DNA was cross-linked to the membrane by UV irradiation and then hybridized to a probe. Hybridization was carried out overnight, at 42°C, in a standard buffer solution (Boehringer Mannheim) containing 50% formamide and 10 ng/ml of denatured probe. After the membranes were washed, colorimetric detection was carried out with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate reagents, as recommended by the manufacturer (Boehringer Mannheim).

**Fertilization Rate, Hatching Rate, and Gene Transfer Rate**

The fertilization rate was calculated as the proportion of 40 randomly selected eggs that developed one day after spawning. The hatching rate was calculated as the proportion of 40 randomly selected eggs that hatched three weeks after spawning. The gene transfer rate was the number of positive PCR and Southern blot analyses divided by the total number of hatched larvae examined.

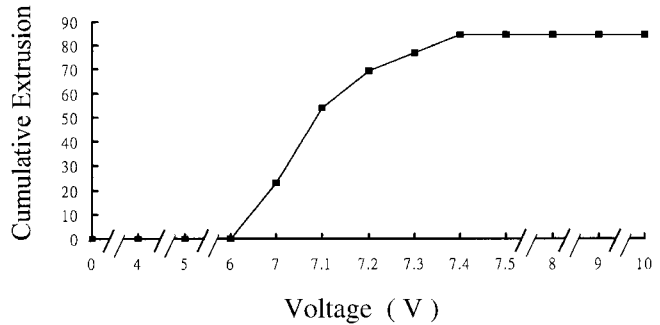
**Green Fluorescence Detection**

To detect green fluorescence, hatched larvae were examined under a microscope (Olympus BH-2, Tokyo, Japan) using an excitation filter (BP 490 nm) and a dichron mirror (DM 500 nm).

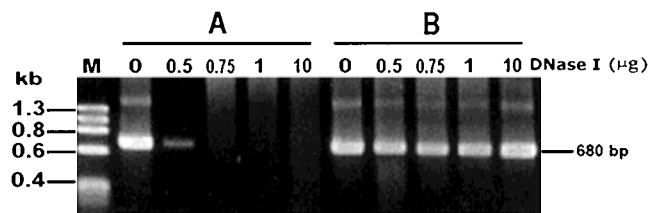
**RESULTS**

**Effect of Voltage on Spermatophore Extrusion In Vitro**

Eleven of 13 (85%) male giant freshwater prawns extruded their spermatophore in vitro when shocked with 7.4 V (Fig. 1). No spermatophores were extruded



**Fig. 1.** Effect of voltage (4–10 V at 10 A) on spermatophore extrusion. The cumulative percentage was the cumulative number of males, out of 13, whose spermatophore was extruded at or below a given voltage.



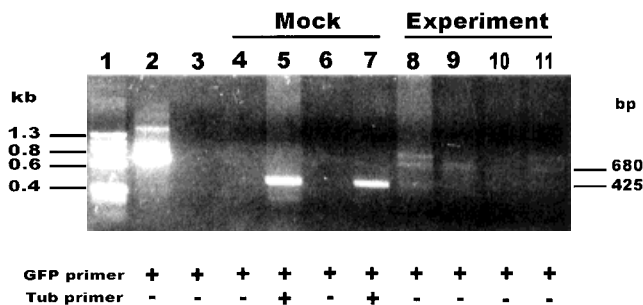
**Fig. 2.** Effect of DNase concentration on pGL digestion. An aliquot of 1  $\mu$ g pGL was incubated with 0.5, 0.75, 1, or 10  $\mu$ g DNase I in a 100  $\mu$ l solution at 37°C for 1 hr. After the DNase was denatured by boiling, samples were analyzed by PCR without (A) or with (B) an additional 1  $\mu$ g of pGL. M, molecular marker of phage  $\phi$ X174 digested with *Hae*III. The arrow indicates the 680 bp PCR-product.

at voltages less than 7 V, and two male prawns failed to extrude spermatophores even at voltages above 7.4 V.

**Presence of Foreign DNA in Embryos**

Before we could ascertain whether foreign DNA was present in the embryos in the SMI treatments, we had to determine the conditions under which PCR sensitivity was sufficient to detect DNA molecules that had not been completely digested by DNase I. In the control and 0.5  $\mu$ g DNase I treatments, a 680-bp band appeared on the gel, but this band did not form in treatments using 0.75  $\mu$ g, or more, DNase I (Fig. 2). In the next experiment, we determined whether the transgene was present in prawn embryos under a more stringent condition: embryos were treated with 10  $\mu$ g DNase I.

After DNase I digestion, genomic DNA was extracted from four samples, each consisting of five, pooled, zoea-I-stage embryos from the 400 ng/ $\mu$ l pGL SMI treatment. Three of the four samples of embryonic genomes yielded a PCR product with a molecular mass of 680 bp (Fig. 3, lanes 8, 9, and 11). The positive control group, for which pGL was used as a template, also generated a PCR product with the same size (Fig. 3, lane 3). Positive signal occurrence was at least 15%, that is, at least 3 of the 20 larvae in the four samples tested positive.



**Fig. 3.** Detection of pGL in giant freshwater prawn embryos using PCR analysis. Genomic DNAs were isolated from five pooled, zoea-I-stage embryos developed from SMI treatment using 400 ng pGL. All embryos were incubated with 10 µg DNase at 37°C for 1 hr before their genomes were extracted and analyzed. Lane 1, molecular markers, *Hae*III-digested  $\phi$ X-174-RF DNA; lane 2, 0.5 ng pGL as a template (positive control); lane 3, no template; lanes 4–7, from an SMI treatment using a solution lacking pGL (mock-treatment group); lanes 8–11, from an SMI treatment using a solution containing pGL (experimental group). Primers added to amplify a 680-bp fragment from transgenic GFP cDNA by using GFP primer, and a 425-bp from endogenous  $\beta$ -tubulin gene by using Tub primer, were indicated.

**Analysis of Transgenic Larva by PCR and Southern Blotting**

Genomic DNA was extracted from each hatched larva from the 750 ng pGL SMI treatment. Larval genomes were analyzed by PCR and Southern blot analyses. A single, positive band, with a molecular mass of 680 bp, was observed exclusively in the experimental group (lanes 5–14 of Fig. 4). Based on positive signal appearance in PCR and Southern blot analyses, the SMI gene transfer success rate for prawns was about 70% (16 of 23 larvae were positive).

**Effect of SMI on Fertility, Hatching, and Transgenesis**

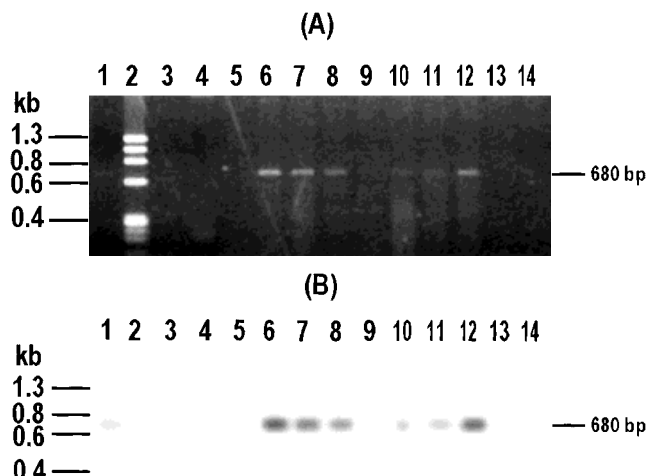
Egg fertilization and embryo hatching rates for the untreated group and the four SMI treatments (28, 250, 750, and 1,000 ng of pGL) did not differ (Fig. 5). However, transgenesis increased as the concentration of pGL used in SMI was increased, with the greatest increase occurring from 250 to 750 ng pGL.

**Green Fluorescence**

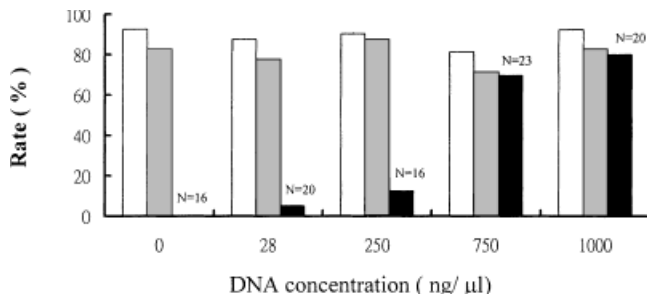
Green fluorescence was used to detect the expression of the GFP reporter gene in hatched larvae. Unexpectedly, green fluorescence was not seen in any larvae from the SMI with plasmid (experimental) treatments, nor in larvae from the SMI with water (mock-treated control) treatment.

**DISCUSSION**

Giant freshwater prawns are one of the most valuable aquacultural crustaceans, and they are also suitable for indoor, laboratory-scale culture. In addition, because embryogenesis can be achieved in vitro under



**Fig. 4.** PCR (A) and Southern blot (B) analyses of genomic DNA isolated from hatched larvae using GFP cDNA as a probe. Lane 1, 0.1 ng pGL as a template (positive control); lane 2, molecular markers, *Hae*III-digested  $\phi$ X-174-RF DNA; lane 3, no template added (negative control); lane 4, larva from an SMI treatment using a solution lacking pGL (mock-treatment group); lanes 5–14, larvae from an SMI treatment using a solution containing 750 ng pGL (experimental group). A 680-bp fragment amplified from GFP cDNA was indicated.



**Fig. 5.** Effect of the amount of microinjected DNA on the rates of fertilization, hatching, and transgenesis of giant freshwater prawns. Plasmid DNA prepared at the concentrations of 28, 250, 750, and 1,000 ng per µl of water were respectively microinjected in a volume of 1 µl into each spermatophore prior to in vivo fertilization. The number of genomes from hatched larvae that were examined to determine the gene transfer rate is indicated above each solid bar.

controlled conditions, this valuable decapod is used as an invertebrate model. Unfortunately, it is difficult to transfer genes into the fertilized eggs of *M. rosenbergii* using electroporation because they are retained in a clump on the abdomen and cannot be detached until 16 hr postfertilization (Caceci et al., 1996). Separating individual eggs from the clump and placing each fertilized egg in a container for electroporation is very tedious work. Consequently, only a small number of fertilized prawn eggs can be treated. In contrast, SMI provides a fast, efficient, and easy method for transferring genes to fertilized, giant freshwater prawn eggs.

We demonstrated that at least 1 µg of foreign DNA fragments (pGL) was completely digested by 0.75 µg DNase kept at 37°C for at least 1 hr. However, PCR

analysis of genomic DNA extracted from zoea-I-stage embryos in the 10 µg DNase SMI treatment still found a 680-bp band. Moreover, the oligonucleotide primers chosen for PCR amplification of the GFP cDNA (transgene) and β-tubulin gene (internal control gene) generated 680 bp and 425 bp DNA fragments, respectively. A 680-bp PCR product was observed when pGL fragments were present in transgenic, zoea-I-stage embryos and hatched larvae. Only nontransgenic, SMI-treated prawns and prawns from the control group contained a 425-bp PCR product. The 680-bp PCR product from the SMI treated samples was positive for Southern blot hybridization using a labeled, GFP cDNA probe. All this evidence indicates that the exogenous plasmid DNA in embryos and larvae were introduced by SMI. Our findings concur with those of Shamila and Mathavan (1998), who demonstrated that a foreign plasmid could be introduced into V instar silkworm larvae by testes microinjection.

Although we have not yet analyzed all the experimental larvae, results from the positive samples of PCR and Southern blot analyses from hatched larvae indicate that SMI achieved a gene transfer rate of about 70%. This success rate is far superior to that reported for microinjection of finfish, which ranged from 16% to 27% for medaka (Ozato et al., 1986; Inoue et al., 1990; Lu et al., 1992) and 0% to 35% for zebrafish, common carp, and channel catfish (Powers et al., 1992). It is also much better than the success rates for electroporation of fertilized finfish eggs, which ranged from 4% to 20% for medaka (Inoue et al., 1990; Lu et al., 1992), and electroporation of finfish sperm, which were only 2.6% to 4.2% for carp, tilapia, and catfish (Muller et al., 1992). The SMI gene transfer rate for giant prawns is comparable to that obtained by electroporation of zebrafish eggs (35% to 75%, Powers et al., 1992) and the electroporation of loach (50%, Tsai et al., 1995a) and abalone sperm (65%, Tsai et al., 1997). SMI probably achieved a high rate of gene transfer in giant freshwater prawns because the microinjected plasmids are retained in the spermatophore for 2–6 hr before *in vivo* fertilization begins. The longer the foreign plasmids remain in the spermatophore, the greater the probability they will enter sperm.

Green fluorescence, which was presumptively expressed by the transgenic GFP cDNA, was not detectable in hatched larvae. This may be attributed to transient expression of the GFP cDNA and to the functionality of the CMV promoter. It is also possible that the exoskeleton somehow blocks visual detection of GFP gene expression. Gendreau and colleagues (1995) reported that the transient expression of a luciferase gene transferred by ballistics into brine shrimp was detectable only at 12 hr after bombardment. Problems detecting expression of the GFP gene could be overcome in future experiments by using prawn-specific promoter fused with GFP cDNA.

Gendreau and colleagues (1995) reported that ballistic shots decreased survival of treated brine shrimp.

Bensheng and Khoo (1997) reported that the early developmental stage of freshwater shrimp embryos is extremely vulnerable to microinjection, and recommended that the intermediate stage be used for microinjection. However, when a gene is transferred to the intermediate stage, the transgene will have a mosaic distribution. In this study, we demonstrated several advantages of SMI gene transfer system, including: (1) a high gene transfer rate; (2) no effect on fertility, survival, and hatching rates; and (3) easy introduction of foreign DNA into oocytes, which definitely helps to minimize transgenic mosaicism. In our opinion, SMI is the most promising technology for prawn transgenesis.

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