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Evidence of green fluorescent protein and growth hormone expression in red abalone (*Haliotis rufescens*) larvae

DOI 10.1515/opag-2017-0024
Received November 21, 2016; accepted February 24, 2017

Abstract: The red abalone *Haliotis rufescens* is a highly appreciated mollusk in the national and international markets. Due to its natural over-exploitation and low growth rate, several genetic improvements were made, however special efforts are needed to increase its production. This study presents transgenic abalone's larvae expressing green fluorescent protein (GFP) fused to Cobia (*Rachycentron canadum*) Growth Hormone (GH) using sperm media transgenesis technique (SMT), pAcGFP1-N vector under the control of cytomegalovirus (CMV) promoter. Sperms were exposed to three voltages (0.5, 0.75 and 1.0 Kv) using a micropulser electroporator (Bio-Rad®). The highest GFP-GH expression average (40%) was obtained in abalone larvae at 0.75 v. GFP and GH transgenes were positively detected by PCR, western blot and confocal microscope, respectively.

Keywords: mollusk, GFP, transfection, CMV promoter

1 Introduction

Haliotis rufescens is a mollusk highly appreciated as a gourmet food. It is one of the largest abalones in the world; however, its natural population has decreased considerably. In Baja California, Mexico, its production depends mainly on aquaculture. Commercial farms grow abalones over a period of 4 to 5 years until they reach the parameters that represent a high production cost: 7- 8 cm of length and 80 g of weight (Searcy et al. 2010). Genetic engineering has been successfully applied for decades in animal farms, however research on marine invertebrates is still poorly documented (Rasmussen and Morrisey 2007; Niemann et al. 2011). Gene transfer comprises different techniques, such as microinjection in spermatophore, testis (Li and Tsai 2000; Chen et al. 2006), pronuclei of zygotes and embryos (Niemann and Kues 2007) and electroporation of sperm and embryos from vertebrates and invertebrates (Wall 2002). Sperm electroporation has been of great utility and has demonstrated to be successful gene vehicle, compared with egg and larvae electroporation. Although there are other modern techniques such as lipofection and microencapsulation, among others, they are not standardized for marine invertebrates cell (Rasmussen and Morrisey 2007). Few reported cases are available about mollusks using sperm as a media for transgenesis (SMT), including mussels (*Mytilus galloprovincialis* and *M. chilensis*), clam (*Chamelea gallina*) (Kuznetsov et al. 2001; Guerra et al. 2005), and other marine invertebrates such as sea urchin (*Strongylocentratus purpuratus*) (Arezzo 1989) and amphioxus (B*ranchiostoma belcheri*) (Yu and Zhang, 2005). In the case of abalone, only one study report is available (Tsai 2000; Jeong et al. 2012), in which used Japanese abalone *H. diversicolor* sperms to express chloramphenicol acetyl transferase gene (CAT) under the control of antifreeze protein promoter (AFP). They demonstrated the feasibility of incorporating the transgene into the abalone genome as well the sperm viability through different voltages using electroporation equipment. These methodologies are improving, however, the standardization in marine invertebrate's cells is still scarce. Electroporation is a very simple technique to introduce exogenous DNA into cells; however, it is a method that should be optimized for *H. rufescens*. The objective of this study was to standardize spermatozoa transfection using a plasmid vector that contains a fish growth hormone (*Rachycentron canadum*), and the green fluorescent protein marker under the control of cytomegalovirus promoter (CMV), a technique never used before in this species.

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2 Materials and Methods

2.1 Abalone gametes

Abalone gametes were obtained from Abulones Cultivados, S.R.L. de C.V. and Productores Marinos Baja, S.A. de C.V. located at Eréndira, Baja California, México. Liberation of gametes was induced by adding 2 M TRIS solution under darkness. Then, 0.66 mL per liter of 30% hydrogen peroxide was added and gametes were released after 2 to 3 hours. Immediately, after the liberation of gametes, the organisms were rinsed with filtered sea water (FSW to 0.45 µm) and placed into individual containers that had ~50 mL of FSW to complete the liberation of gametes. A sperm sample was fixed in formaldehyde and stained with methylene blue to be quantified in a hematocytometer, and then the concentration was adjusted to 5 x 10^6 cel. mL^{-1} with FSW. A sample of oocytes was quantified in a chamber Sedgewick Rafter and then adjusted to 1000 cells.mL^{-1}.

2.2 Plasmid DNA preparation

Plasmid pACGFP1-N ready vector (CLONTECH®) containing CMV promoter and GFP gene as a marker was used. The synthetic sequence of GH gene (GenScript, USA Inc.; genetic sequence database at the National Center for Biotechnical Information, GenBank ID: GQ861507.1) was attached to the vector via ligation-independent cloning (LIC) (Jeong et al. 2012); GH gene was amplified by PCR using the primers suggested by the commercial company (forward 5' AAG GCC TCT GTC GAC ATG GCT NCA GRC TCT CGG AC 3' and reverse 5' AGA ATT CGC AAG CTT GAA GGC ACA GCT GCT TTC CA 3'). GFP was expressed fused to the N-terminal of GH. LIC reaction was carried out including: vector 1 µg, T4 polimerase 2U (NEB ®), purified product of PCR 6 µL (0.5 µg.µL-1) in a total volume of 10 µL. The reaction was incubated 5 min at room temperature, then 10 min to 75°C, followed by ice. Electrocompetent *E. coli* cells (DH5α, Life Technologies®) were transformed and cultured in kanamycin LB agar (75 µg mL-1) at 37°C. Positive clones were corroborated by PCR, using purified plasmid DNA (QIAprep miniprep, QIAGEN®) with CMV primers (forward: 5 ' CGC AAA TGG GCG GTA GGC GTG 3' and reverse GFP: 5' CTT GTA CAG CTC ATC GC CAT'). Each reaction mixture contained 1X green PCR master mix (PROMEGA®), 10 pmol of each primer (Allele Biotech, San Diego CA. USA) and 30 ng of template DNA, final concentration. Cycling conditions were 94˚C for 3 min, 30 cycles; 94˚C for 60 sec; 62˚C for 60 sec and 72˚C for 60 sec. The final extension was 10 min at 72°C. Positive clones were analyzed by plasmid DNA restriction analysis using *Not*I and *Sa*lI enzymes. PCR products and enzymatic digestion were observed in agarose gel electrophoresis stained with Ethidium Bromide and UV light. Three clones were sequenced to confirm the correct open reading frame (ORF), using CMV primer.

2.3 Electroporation and fecundation

Sperm was electropored with FSW in a MicroPulser™ electroporator (Bio-Rad®) with voltages of 0.5, 0.75 and 1.0 Kv, two pulses of 0.5 ms, into 0.4 cm cuvettes, in a total volume of 1 mL. Five hundred ng of circular form of plasmid DNA was added, except to the control, and experiments were done in triplicate. The viable sperms were identified as those that had vigorous and continuous movement: high (70-100%), medium (40-70%) and low (10-40%) and finally without viability (0%). Fertilization took place in a proportion of one oocyte for a hundred sperm. After that, they were incubated with constant aeration at room temperature (18-20°C). Samples were collected after 2 h of being fertilized to assess the fertilization rate and 48 h after to evaluate *in vivo* expression. Hundred cells from each replica were evaluated through an optical microscope. Samples were frozen to –20°C for PCR and Western blot assays.

2.4 Transfection analysis by fluorescence

Growth hormone fused to green fluorescent protein expression was observed and registered in a confocal microscope (Olympus IX81). Hundred cells of three replicas were counted under an epifluorescence microscope (Olympus BH-2).

2.5 Presence of transgene DNA

Fifty microliters of PBS prewashed pool of larvae were incubated in 10 volumes lysis buffer (10 mM Tris-HCl, 50 mM KCl, 0.2% Triton X-100, 150 µg Proteinase K ml^{-1}) at 55°C for 3 h, followed by a 10 min incubation period at 100°C, then centrifuged 1 min to max velocity (14,000 rpm).

(Collares et al. 2010). One microliter of lysate was used for PCR. PCR reactions of GH and GFP were carried out separately and conditions were as previously mentioned (Cobia GH Forward: 5' AAG GCC TCT GTC GAC ATG GA 3'

and Cobia GH reverse: 5' AGA ATT CGC AAG CTT CAG GGT 3'; Ac GFP 15F: 5' GAG CAA GGG CGC CGA GC 3', AcGFP 10 R: 5' CTT GTA CAG CTC ATC CAT GC 3').

2.6 Protein expression analysis

Western blot was done with 5 µL of defrosted larvae pellet from each treatment and negative control. Then 25 µL of 2X sample buffer (SGB 2.5 mL, glycerol 1 mL, 2-ME 0.5 mL, 10% SDS 3.2 ml, 0.001% Bromophenol blue) was added to each sample and boiled for 5 min. 10 µL of each sample was loaded into 14% acrylamide gel (Laemmli 1970), Following electrophoresis, proteins were electro transferred to PVDF membranes (Osmonics, Inc.). GH was detected with affinity-purified polyclonal anti-GH. A donkey anti-rabbit IgG peroxidase conjugate (Thermo Sci®., both antibodies diluted to 1:000) and Tetramethylbenzidine (TMB) stabilized substrate for HRP (PROMEGA®) were added. Pre-stained protein ladder was from BIORAD.

2.7 Data Analysis

Fertilization rate and fluorescent cell data were analyzed by Kruskal-Wallis test and a multiple comparison Z' test was used *posteriori* to identify differences in groups. A $p<0.05$ was chosen as the significance level. Tests were performed with STATISTICA 7.1 software (StatSoft, Inc. 1984-2005).

3 Results

To select the conditions for electroporation using Micropulser (Bio-rad) device, we performed different tests using 0.2 and 0.4 cm gene pulser® cuvettes, as well as variable volumes of diluted sperm in FSW and three voltages (0.5, 0.75 and 1.0 Kv). It was found that in cuvettes of 0.2 cm and volumes of more than 100 µL, the sperm completely lost mobility but not in 0.4 cm. Therefore, the experiment was carried out using these cuvettes (0.4 cm) in volumes of 1 mL and two pulses of 0.5 ms, since the sperm showed high mobility response. The average fertilization rates were of 75 - 80% the three treatments and control. Eggs fertilized with electropored sperm from three voltages demonstrated no significant difference as compared to the control ($p<0.05$).

Transgene expression analysis measured by fluorescent larvae average was 9.7% for 0.5 Kv, 40% for 0.75 Kv, and 27.7% for 1.0 Kv (Figure 1, a-f). Fluorescence was observed evenly in the cells of larvae, whereas controls did not show any fluorescence (Figure 1, g-h). Fluorescent larvae between treatments showed significant statistical differences.

The expression of GH was analyzed by Western blot produced an intense band (~ 26.6 KDa; GH+GFP) in each of the three treatments obtained from the total larvae protein, while it was negative from the control (Figure 2).

The transgenesis evaluated by PCR from larvae of each treatment resulted in a positive band of 642 bp for GH (Figure 3) and a band of 711 bp for GFP (data not shown).

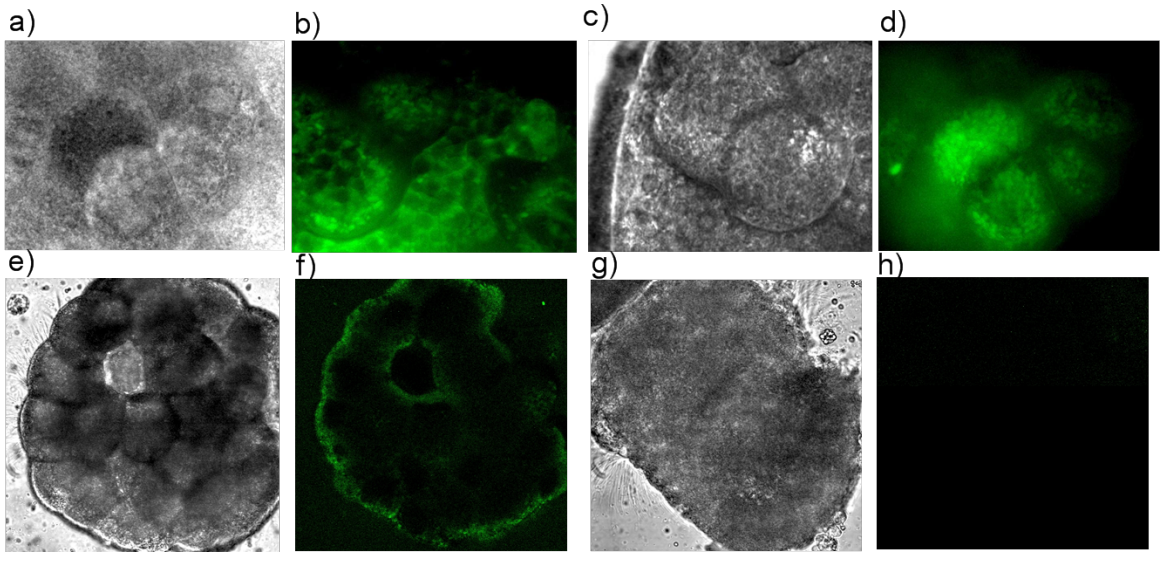

Figure 1: Images of GH-GFP expression in abalone larvae 48 h after fertilization with electropored sperm; a) - b), c) - d), e) - f), the same larvae without fluorescence excitation and with fluorescence excitation. g) - h) control negative embryos, without and with fluorescence excitation, respectively

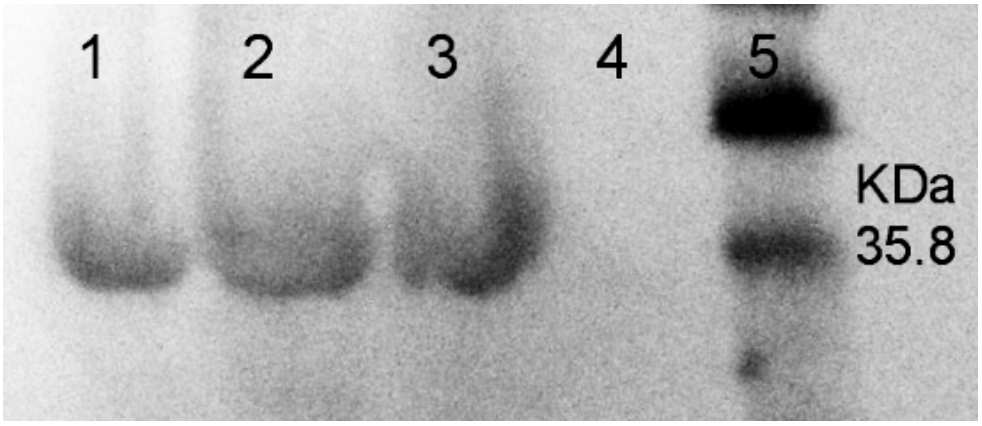

Figure 2: GH+GFP protein detection by Western blot from the different treatments showing positive reaction in larvae pool protein 48 h after fertilization: 1) 0.5 Kv, 2) 0.75 Kv, 3) 1.0 Kv and 4) negative control, 5) protein ladder

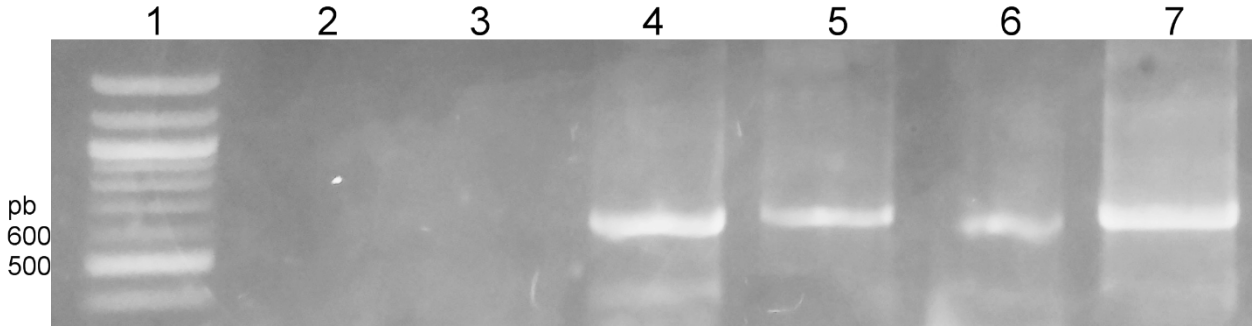

Figure 3: PCR used to detect GH in genomic DNA larvae from 48 h after fertilization with electropored sperm from different treatments. 1) DNA ladder, 2) Eggs without fertilization, 3) PCR mix without template, 4) 0.5 v, 5) 0.75 v, 6) 1.0 v, 7) plasmid with GFP-GH as a positive control

4 Discussion

Sperm media transgenesis (SMT) was performed for the first time in 1989 (Lavitrano et al 1989) using a mouse. Since then, greater advances have been made (Lavitrano et al. 2013; Gandolfi 2000; Robl et al. 2007). However, one of the characteristics of this technique is that it must be standardized for each species and equipment used; therefore, we were motivated to standardize for *H. rufescens*, an important aquaculture marine animal, using the Micropulser-Biorad electroporator currently available in market. Previous studies with marine invertebrates using SMT reported more efficiency when electroporation was used instead of only exposing the sperm to vector DNA (sperm-naked DNA). However, there have been controversies in obtaining 58 to 70% transfection by naked-DNA in mollusks (Guerra et al. 2005). 5% and 25% transfection was reported with catfish sperm (*Rhamdia quelen*). The use of the electroporator device to incorporate plasmid DNA to the sperm increased the transfection efficiency considerably. Although, the sperm has proven to be resistant to different voltages, when this feature is coupled with the intermolecular properties between the cell and the DNA, the process can be highly improved (Lavitrano et al. 2013). Among the reported cases successfully using electroporation in black footed abalone (*H. iris*) sperm; DNA uptake increased up to 40% when voltage of 1 Kv and two pulses of 27 ms was used (Sin et al. 1995). It was found the transgene in 65% of Japanese abalone larvae (*H. diversicolor*) by using 10 Kv and 27 pulses per six cycles. It should be noted that in our work, abalone sperm treated with voltages above 1Kv completely lost their mobility and they were not capable of fertilizing oocytes. Perhaps the difference found in this work lies mainly in the electroporation equipment and promoter type (AFP). However, 70% of fertilization rate was reported from mussels' sperm electroporated by using 0.2 Kv (Kuznetsov et al. 2001) and 45% positive expression was found in sea bream using 0.6 - 2 Kv (Lu et al. 2002), which are similar to the 40% using 0.75 Kv

obtained in this work. Also, similar results were found using salmon sperm and 0.6 to 1 Kv (Symonds et al. 1994; Walker et al. 1995; Sin et al. 2000). Voltages between 0.2 to 2 Kv are more consistent with our results than others reports (Tsai et al. 1997; Tsai 2000).

Promoters to control gene expression tested in abalone are B-actin (from medaka fish (Tsai et al. 1997) or fruit fly (Taylor et al. 1996), SV40 (Simian Vacuolating virus 40) (Tsai et al. 1997) and opAFP (ocean pout) (Tsai 2000). CMV promoter is considered a stronger promoter in vertebrates; however, it has never been tested in abalones. We obtained a successful expression (40%) as others reports in abalones who obtained 25% of CAT activity under AFP promoter (Tsai et al. 1997).

Transgene detection use PCR, Southern blot and enzyme activity. However, few studies in invertebrates use live expression marker and sperm media transgenesis as it was with *Amphioxus* where GFP was expressed in 15% larvae when sperm was electroporated using 0.5 Kv (Yu and Zhang, 2005), which is lower than the value obtained in our study. We did not follow the abalone growth rate to corroborate the GH effect since previous studies reported a positive effect using GH from bovines or fishes (Tsai 2000; Taylor et al. 1996).

We can conclude that the success of electroporation using sperm as a vehicle is variable among the species already demonstrated. The first recommendation is to standardize the voltage, the type of solution, the number of pulses and the device to be used.

We could not observe mosaicism since fluorescence was difficult to see due to the larvae shell thickness. The transgenesis rate obtained in this study using CMV promoter and 0.75 Kv is higher than other reports of aquatic invertebrates (abalone, 25%; amphioxus 15%) and quite similar to fishes (38%) (Tsai et al., 1997; Yu and Zhang, 2005; Li et al., 1996).

Acknowledgements: We would like to thank to Abulones Cultivados, S.R.L. de C.V. and Productores Marinos Baja, S.A. de C.V. for providing the gametes. The present study was financed by the academic group: Biotecnología integral from SEP-UABC.

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