UTTAR PRADESH JOURNAL OF ZOOLOGY

41(22): 31-37, 2020
ISSN: 0256-971X (P)

SPERM MEDIATED GENE TRANSFER IN LABEO ROHITA (HAMILTON 1822)

S. SHEELA[1*]

[1]Department of Zoology, TKMM College, Nangiarkulangara, Harippad, Alappuzha, Kerala, India.

AUTHOR'S CONTRIBUTION

The sole author herself designed the study, performed the statistical analysis, wrote the protocol and the first draft of the manuscript and managed the analyses of the study and the literature searches. The author read and approved the final manuscript.

Article Information

Editor(s):
(1) Dr. Telat Yanik, Professor, Atatürk University, Turkey.
Reviewers:
(1) Carlos Scotto, Universidad Nacional Federico Villarreal, Perú.
(2) Belem-Costa, Andrea, Federal University of Amazonas, Brazil.

Received: 10 September 2020
Accepted: 11 November 2020
Published: 16 December 2020

Original Research Article

ABSTRACT

'Transgenesis' may be defined as the introduction of exogenous DNA by different methods such as microinjection, sperm mediation electroporation etc. into the host genome resulting in its stable integration, expression and transmission. In the present study sperm-mediated gene transfer was carried out in ROHU *(Labeo rohita*) using melanin concentrating hormone gene, (p-CMV-MCH). Milt was soaked in different concentrations of plasmid DNA (50-200 µg / ml) for 10 minutes and it was used to fertilize the eggs. Slot blot analysis was carried out to study the integration of transgene. It showed integration of foreign genes which indicates that the carp sperm do pick up DNA in about 10 minutes. Moreover the MCH gene causes contraction of melanophores in the body, caudal region and on the eyeball resulting in brightness of the transgenic fish. Therefore this study clearly indicates that MCH gene construct can be used as a marker gene to identify transgenesis and to study the molecular basis of gene integration expression and transmission.

Keywords: Transgenesis; sperm mediated gene transfer; melanin concentrating hormone gene; marker gene; slot blot analysis.

1. INTRODUCTION

The production of transgenic individuals is one of the important areas of biotechnology of farm animals. 'Transgenesis' may be defined as the introduction of exogenous DNA into the host genome resulting in its stable integration, expression and transmission [1]. Fishes are excellent models for transgenesis than mammals by virtue of their higher fecundity, external fertilization and development, and transparency of embryos in many species, in addition to the easy manipulation of sex, ploidy and production of haploid gynogenetic and androgenetic individuals. The gene construct has to be introduced into the single-cell embryos for the transgene to be integrated stably into the genome of every cell. Techniques such as direct

**Corresponding author: Email: sheelaachyuth@gmail.com;*

microinjection [2], electroporation [3], sperm-mediated gene transfer [4], retrovirus infection [3] particle gun bombardment [5] and electrotransfection [6] have been widely used to introduce foreign DNA into animal cells, plant cells, as well as germ lines of mammals and other vertebrates. Sperm cells can be used as carriers for introducing foreign DNA into eggs [7,8,9]. This was done by simply incubating the milt in a solution of foreign DNA and then using these sperm to fertilize the ova in vitro.

One of the problems in transgenic research is that it is not possible to identify transgenic fish without sacrificing the animal. To overcome this problem MCH marker gene was selected to study its integration and expression. Transgenic fish can be identified by the distribution of melanin pigments. The main objective of the present study is to produce transgenic carp by sperm mediated transfer of pCMV-MCH and to check the possibility of using MCH gene as a marker gene.

2. MATERIALS & METHODS

Live brood fish (*Labeo rohita*) were collected from a local fish hatchery. They were maintained in outdoor cement tanks. It is a seasonal breeder. For induced breeding Ovaprim ((Syndel Laboratories, Canada)) was used. It contain 20 mg of gonadotropin releasing hormone along with 10 mg of donsperidone per ml. The plasmid CMV-MCH was obtained from Dr. Maseto Kinoshita, Japan. It contains melanin concentrating hormone gene driven by cytomegalo virus. The size of the plasmid is 7.46 Kb and its restriction map is shown in Fig. 1. p - CMV-MCH was digested with Pvu 1 Restriction Enzyme for 2 - hours at 37°C in order to linearize the plasmid. Matured female (Fig. 2a) and male fishes (Fig. 2b) of 1-2 years of age having a weight of 1 to 2.5 kg were kept separately and the temperature was kept at the range of 28-30°C. To accelerate induced breeding Ovaprim injection of 0.5 ml /kg and 0.3 ml/kg were given to female and male fish respectively and they were released together in a specially maintained breeding tank. Milt from adult fish were obtained by stripping (Fig. 3) and the motility was checked under a phase contrast microscope. Milt was incubated in linearized p CMV-MCH at concentrations of 50, 100,150 and 200 µg / ml for 10 minutes, and then used to fertilize eggs stripped from a gravid female. The embryos were reared and their genomic DNA was extracted at different stages of development such as gastrula, 1-day after fertilization, 3 days old fry and 3 months old fish for slot blot analysis. The expression of p CMV-MCH was assessed by the pattern of melanin distribution in the body.

3. RESULTS

Survival of rohu, a large sized edible fish, after sperm-mediated transfer of p CMV-MCH was studied. Before fertilization sperm was soaked in various concentrations of linearized p-CMV-MCH at concentrations from 50 µg/ml to 200 µg / ml and the hatching rate was observed (Table 1). Maximum hatching rate was obtained at a concentration of 150 µg/ml. Therefore this concentration was used for further experiments. When the DNA from treated fish were analyzed by slot blot hybridization technique, the integration of transferred genes varied from 0 to 50% (Table 2). As shown in Fig. 4, genomic DNA from gastrula, 1 day old fry, 3 days old fry and 3 months old fish hybridized with the probe. MCH gene integrated fish could be identified externally by their melanin distribution. They appeared pale in color (Fig. 5). Absence of pigmentation in the eye was prominent in some fish. The black spot near the caudal fin disappeared in a few number of transgenic fishes (Fig. 6).

Table 1. Effect of different concentration of linearized p CMV-MCH

DNA concentration (µg/ml)	Total No of eggs treated	Hatching (%)
50	1800	51±9.5
100	1700	50±10.2
150	1700	52±10.1
200	1750	49± 8.1

Table 2. Integration of linearized p CMV-MCH in Rohu after sperm mediated gene transfer

Batch No.	No. of eggs treated	Survival %		
		Hatching	Feeding	
1	1800	50 ±20.2	45±15.2	25
2	1850	60±15	47±7.3	0
3	1800	66.2±9.3	52±10	13
4	1800	55.3±19	40±15.1	50
5	1750	54±14.2	42 14	0

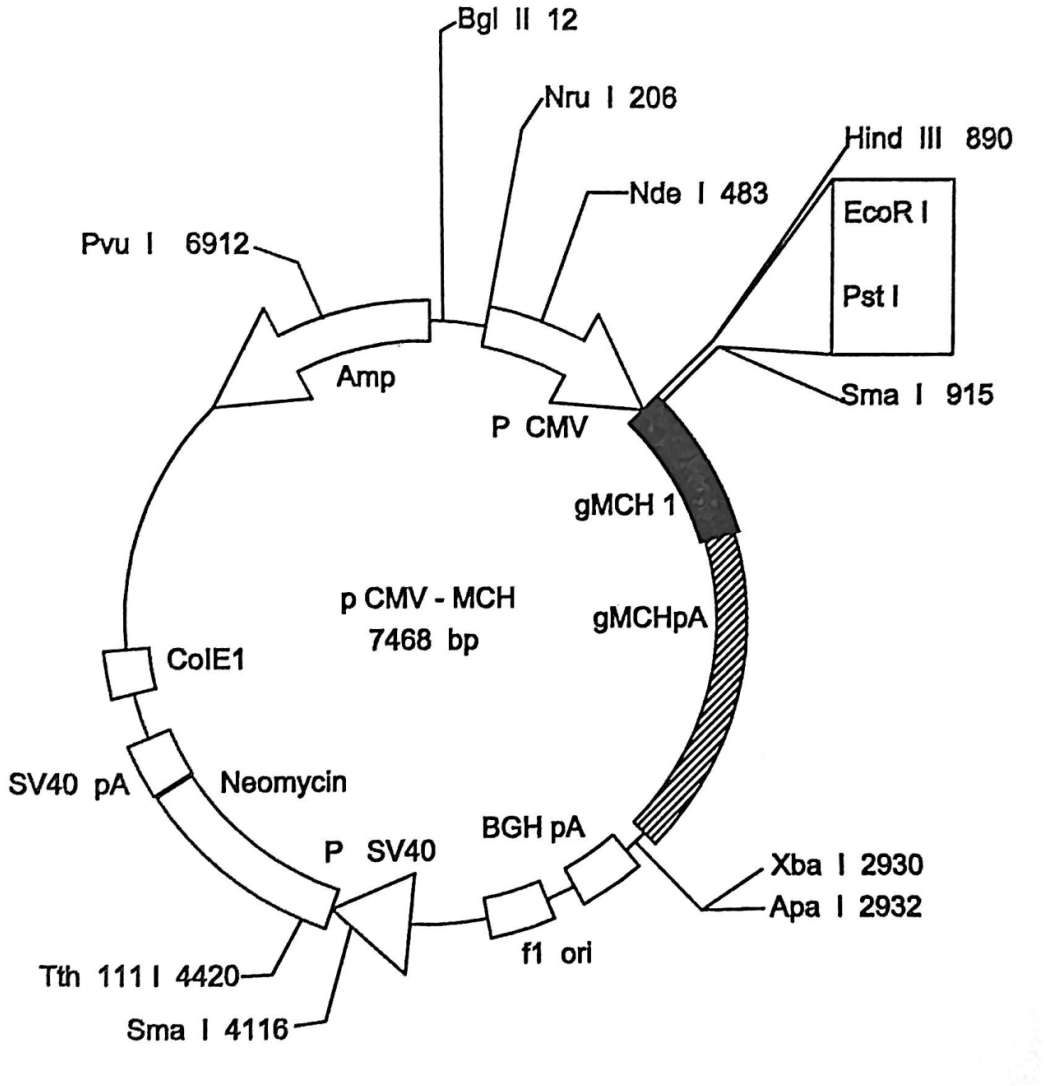

Fig. 1. Restriction map of p- CMV-MCH which carries CMV promoter fused to melanin concentrating hormone gene

Fig. 2a. Female *Labeo rohita*

Fig. 2b. Male *Labeo rohita*

Fig. 3. Artificial fertilization by stripping eggs and milt

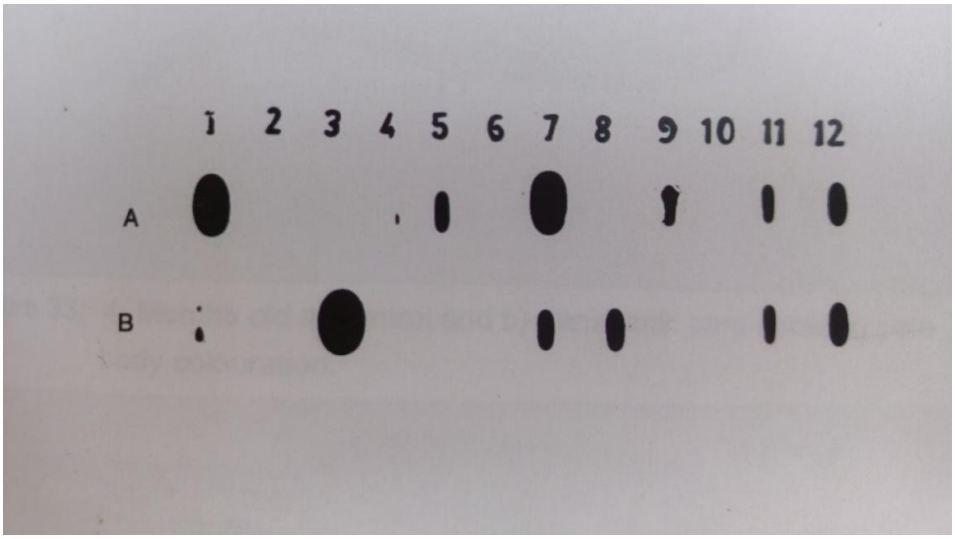

Fig. 4. Slot - blot analysis of genomic DNA of carp after sperm-mediated gene transfer. Lane Al: plasmid DNA: A2: genomic DNA from control fish; A3-A12: genomic DNA from gastrula; B1-B6: genomic DNA from 1 - day old fish B7- B8: genomic DNA from 3 days-old fry, B9-B1 2, genomic DNA from 3 months old fish

Fig. 5. 4 months old control carp fish showing black spots in the caudal region a) In transgenic fish pigmentation in the eye and head region is lacking. b) black spot in caudal region is absent in certain transgenic fish

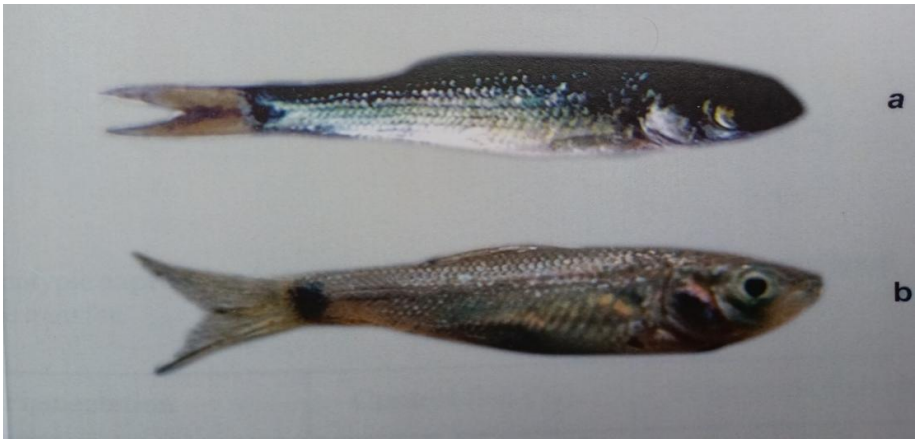

Fig. 6. 4 months old a) control and b) transgenic carp showing pale body color

4. DISCUSSION

The sperm-mediated gene transfer affords many potential advantages over other methods such as microinjection and electroporation. The rate of survival is greatly increased, an elaborate apparatus is not needed and does not necessitate a high level of skill. Furthermore the process is simpler in both concept and procedure and may be considered as a mass production method, being far more rapid than the microinjection of a single egg. Numerous work has been carried out in this field with various modifications [10]. These factors combined with the ability to carry out in vitro fertilization in oviparous fishes afford great advantage for the introduction of novel genes into fish genomes. Slot blot analysis of DNA extracted from gastrula, at 1-day, 3-days and 3-months old fish showed that integration rate ranged

from 0 to 50%. The idea of using sperm for exogenous gene transfer in fish was conceived by Khoo and the study that helped to suggest that spermatozoa is a possible vehicle for introducing exogenous genes into the fish embryo was that by Mulcahy and Pascho [11]. If sperm could be used, gene transfer would become dramatically simpler [12]. It's drawback, however is the low efficiency [13] of gene transfer such that DNA incorporation rates vary from 2.6% [14] to 50% [15].

In normal carp, in early stages a black spot is visible in the caudal region. Such a spot was absent in 18% of transgenic fishes. Similarly pigmentation in the head region especially in the eyeball was absent in 15% of transgenic fishes. This showed that Carp sperm do pick up DNA in about 10 minutes. Absence of integration and expression in some fishes indicated that some sperm were unable to pick up DNA. In sperm-mediated gene transfer the sperm was simply incubated with plasmid DNA and the chance of integration was very low. Khoo [16] incubated p GEM lac plasmid DNA for 10 and 30 minute and showed that zebrafish sperm do pickup DNA in less than 10 minutes. Khoo [17] incubated zebrafish sperm with p MTL plasmid for 30 minutes and showed that some sperm cells were unable to pick up DNA, and in others it was internalized into the sperm head. They observed plasmid DNA in sperm mid piece but none was observed in the tail. Lavitrano [18] did experiments with p SV2 CAT plasmid to which washed epididymal mouse sperm cells were exposed for 30 minutes at $37^{\circ C}$ at a concentration of 0.2 to 2µg /106 sperm cells and indicated the presence of the transgene in approximate 30% of the animals and the transgene was detected in the F_1 progeny. Salmon sperm failed to take foreign DNA without electroporation [19]. Mendelian inheritance or evidence of genomic integration was not obtained by Khoo [4] in zebrafish when sperm mediation was used for the transfer of genes. DNA transfer into embryos after its incubation with sperm was not successful in rainbow trout also [20]. But tip type electroporation enhances the internalization of exogenous DNA into zebrafish sperm cells with minimal harmful effects to sperm cells [21].

5. CONCLUSION

The present study was carried out to check the efficiency of using, p-CMV-MCH gene as a marker gene. Sperm mediated gene transfer was carried out in an edible fish *LABEO ROHITA*. The rate of integration was analyzed by slot blot analysis and their expression was observed through the distribution of melanophores in the body. Since the study clearly showed the integration and expression of the transgene the p-CMV-MCH gene construct can be used as a marker gene to identify transgenesis.

COMPETING INTERESTS

Author has declared that no competing interests exist.

REFERENCES

1. Khoo HW. Transgenesis and its applications in aquaculture. Asian Fisher. Sci. 1995;1:24-48.
2. Nottle MB, 1˙laskard KA, Verma P1, Du ZT, Grupen CG. Effect of DNA concentration on transgenesis rates in mice and pigs. Transgen. Res. 2001;6:523-531.
3. Baer A, Schubeler D, Bodei. Transcriptional properties of genomic transgene integration sites marked by electroporation or retroviral infection. Biochemistry. 2000;24:7041-7049.
4. Khoo HW. Sperm-mediated gene transfer studies on zebrafish in Singapore. Mol. Reprod. Dev. 2000;2:278-280.
5. Tucker C, Endo M, Hirono I, Aoki 1. Assessment of DNA vaccine potential for juvenile Japanese flounder Paralichthys olivaeus, through the introduction of reporter genes by particle bombardment and histopathology. Vaccine. 2000;19:801s-809.
6. Cervia LD, Yuan F. Current progress in electro transfection as a non-viral method for gene delivery. Mol. Pharm. 2018;15:3617–3624.
7. Collares T, Campos Vf, Seixas FK, Cavalcangi PV, Dellagostin OA, Morea HL, Deschamps JC. Transgene transmission in South American catfish (Rhamia quelen) larvae by sperm-medoated gene transfer. J. Biosci. 2010;35(1):39-47.
8. Marialuisa L., Roberto Giarannoni and Maria Cerrito. Methods in Molecular Biology. 2012;927:519-529.
9. Chaparian S, Abdulahnejad A, Rashidi F, Toghyani M, Gheisari A and Eghbalsaied S. Is passive transmission of non viral vectors through artificial insemination of sperm- DNA mixtures sufficient for chicken tragenesis? J. Reprod. Dev. 2016;17;62(3):265-70.
10. Konoval O, Korol P, Tabaka P et al. Generation of transgenic ducks by crispr/CAS9-mediated gene inser-tion combined with the sperm-mediated gene transfer (SMGT). Biopolym Cell. 2019;35:427–436.
11. Mulcahy D, Pascho Ri. Adsorption to fish sperm of vertically transmitted fish viruses. Science 1984;325:333-335.
12. Archibald AL. Molecular biological approaches and their possible applications. In:

Owen J.B. and Word R.F.E. (eds), CAB Breeding of disease resistance in farm animals, International, U.K. 1991;100-122.
13. Kang J, Yoshizaki G, Homma 0, Strussman A, Takashima F. Effect of an osmotic differential on the efficiency of gene transfer by electroporation of fish spermatozoa. Aquaculture. 1999;173:297-307.
14. Muller F, Ivics Z, Erdelyi F, Papp T, Varadi L, Horvath L, Maclean N and Orban L. Introducing foreign genes into fish eggs by electroporated sperm as a carrier. Mol. Mar. Biol. Biotech. 1992;1:276-281.
15. Tsai HJ, Tseng FS, Liao IC. Electroporation of sperm to introduce foreign DNA into the genome of loach (*Misgurnus anguillicaudatus*). Can J. Fish. Aquat. Sci. 1995;52:776-787.
16. Khoo HW, Patil J, Chen ES. Sperm mediated gene transfer: a review. Seameo Jasper Fellowship Monograph; 1999.
17. Khoo HW, Ang LH, Lim HB, Wong KV. Sperm cells as vectors for introducing foreign DNA into zebrafish. Aquaculture. 1992;107:1-1-9.
18. Lavitrano M, Camaion A, Fazio VM. Sperm cells as vectors for introducing foreign DNA into eggs-genetic transformation of mice. Cell. 1989;57:717-723.
19. Sin FYI, Bartley L, Symonds JE, Walker SR Sin L, Hawke Land Hopkins CL. Electroporation of salmon sperm for gene transfer. The Third Asian Fisheries Forum. 1994;599-602.
20. Chourrout D, Perrot E. No transgenic rainbow trout produced with sperm incubated with linear DNA. Mol. Mar. Biol. Biotech. 1992;1:282-285.
21. Izani B, Acosta, Leandro S, Nunes1, Eduardo B, Blodorn, William B, Domingues, Amanda WS, Martins, Eduardo N. Dellagostin, Gabriela T. Rassier1, Carine D. Corcini, Charles N. Fróes, Eliza R. Komninou, Antônio S. Varela Jr,Vinicius F. Efficiency and cell viability implications using tip type electroporation in zebrafish sperm cells Molecular Biology Reports. 2020;47:5879–5887.