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Effect of Transgenic Genotype on Transgene Expression in Mud Loach (*Misgurnus mizolepis*): I. Copy Number-Dependent Expression in Gynogenetically Derived Homozygous Transgenics

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To examine the effect of copy number-dependent transgenic genotype on the expression of foreign gene, stable hemizygous and homozygous transgenic breeding line was established using artificial parthenogenesis. For this purpose, induced diploid gynogenetic transgenesis was optimized in mud loach (*Misgurnus mizolepis*) using UV-irradiated cyprinid loach (*M. anguillicaudatus*) sperm and thermal shocks. Optimum UV range for inactivation of cyprinid loach sperm was between 3,150 to 4,050 ergs/mm^2. The UV-irradiated sperm were inseminated into eggs from recessive color strain (yellow) or heterozygous transgenic mud loach containing CAT gene. Cold shock at 2℃ for 60 min, 5 min post fertilization successfully restored the diploidy of eggs inseminated with UV-irradiated sperm. Restoration to diploidy was confirmed by flow cytometry and gynogenetic status was verified by examining maternal exclusive inheritance of multi-locus DNA fingerprints, body color and transgenic marker. Putative isogenic transgenic fish clearly showed homozygous status at transgene locus based on Southern blot hybridization and progeny testing. Further, such homozygous gynogenetic diploids revealed the increased levels of transgene expression, when compared to those of heterozygous (hemizygous) transgenic fish.

Key words: Transgenic, Induced gynogenesis, Mud loach, Meiotic gynogenesis

Introduction

Transgenic technique is a potential tool to generate novel aquatic animals that may play an important role in aquaculture with particular emphasis on the enhancement of production efficiency (Devlin et al., 1994; 1995). More importantly, it may also offer new possibility to address variety of biological questions by providing unique pheno- and genotypic markers (Iyengar et al., 1996).

Gynogenesis (all-maternal inheritance) is a form of parthenogenesis in which activation of zygotic development is induced by genetically inactivated sperm. It allows the maternal haploid set of chromosomes to be doubled without any contribution of paternal genome. This technique has been given much attention as a potential tool for rapid establishment of highly inbred broodstock of fish (Komen et al., 1991; Palti et al., 1997). Such inbred strains can be used as experimental systems for a lot of biological studies including immunology, endocrinology and genetics (Sarder et al., 1999). By combining these two techniques (transgenesis and gynogenesis), transgene may serve as a novel genetic marker for rapid verification of gynogenetic status. On the other hand, gynogenesis may help to generate inbred transgenic lines exhibiting not only transgenic homozygosity but also high degree of isogenecity.

Mud loach (*Misgurnus mizolepis*) is an important freshwater species in Korea, and its domestic market expands rapidly in recent years. This species has also many attractive advantages as a model system for genetic studies, such as (1) small body size (10 g

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in adult), (2) transparent and fast embryonic development, (3) short generation time (3~5 months), (4) year round spawning under controlled condition and (5) high fecundity (more than thousands eggs from a female). Based on these merits, various genetically improved strains have been generated especially with respect to chromosome-set manipulations (Kim et al., 1994; 1995; Nam et al., 2001) and transgenesis (Nam et al., 1998; 1999; 2000).

The objectives of this study were (1) to optimize conditions for heterospecific gynogenesis, (2) to verify the isogenic status of gynogenetic diploids using phenotypic color and transgenic markers, and (3) to examine the effectiveness of induced gynogenesis on transgene expression and germ line transmission.

Materials and Methods

Fish and gamete collection

Normal female and male broodstocks of mud loach (*Misgurnus mizolepis*) and cyprinid loach (*M. anguillicaudatus*) were 12-year-old individuals that have been maintained in Fish Genetic Manipulation Laboratory, Pukyong National University. Albino-like recessive strain (yellow color) of mud loach is 3-year-old fish. Transgenic mud loach is F3 generation, containing chloramphenicol acetyl transferase (CAT) gene. Detailed characteristics of these transgenic fish with regard to Mendelian transmission and stable expression can be referred to Nam et al. (1999). To obtain gametes, induced spawning was performed using intraperitoneal (IP) injections of HCG (6~8 IU/g body weight for mud loach females and 2 IU/g body weight cyprinid loach males) as described previously (Kim et al., 1995).

Inactivation of the spermatozoan DNA by ultraviolet ray

The sperm from cyprinid loach (*M. anguillicaudatus*) was diluted 1:10 with ice-cold 0.85% NaCl solution, spread uniformly onto a glass petridish and irradiated with various dosages of UV irradiation (0 to 9,000 ergs/mm^2). An aliquot of the inactivated sperm (200 $\mu\ell$) was added to each lot of 330 eggs, and activated by adding water. The moment of water addition was taken as the insemination (fertilization) time. The optimal range of UV dose was determined by examining both the incidence of haploid syndrome and percent survival of embryo at 24 hours post insemination (just before hatching).

Meiotic diploid gynogenesis

Based on the results from UV irradiation experiments, cyprinid loach sperm were treated with the amount of UV (3,870 ergs/mm^2) and inseminated with eggs from albino-like strain with recessive yellow color or transgenic mud loach females. The inseminated eggs were cold-shocked at 2℃ for 60 min, 5 min post insemination in order to prevent the second polar body extrusion (Kim et al., 1994; 1995). The number of eggs inseminated was 2,500±100 for each replicate. Fertilization, hatching and early survival were monitored with 330 eggs per each replicate. The fertilization and hatching success were evaluated as a percentage of eggs inseminated. Early survival up to the 1st feeding (3 days after hatching) was also assessed as a percentage of hatched larvae. The 3 replicated experiments were performed. The body colors of presumptive gynogenetic diploid fish (termed as meiogyne) that had been developed from eggs of recessive strain with yellow color were examined at the age of 1st feeding. On the other hand, the gynogenetic fish developed from transgenic eggs were subjected to transgenic analyses.

Flow cytometry

Successful restoration of diploidy was checked using WinBryte HS flow cytometer (BioRad, USA). The cell suspensions (from blood cells or larval cells) of presumptive gynogenetic individuals were prepared according to protocol provided by manufacturer. For blood samples, 3~5 $\mu\ell$ of heparinized whole blood (about 1.5×10^6 cells) were directly added to reconstituted Kinesis-50 kit (BioRad) containing 50 $\mu\ell$/mℓ propidium iodide (PI), and incubated for 1 hour at 4℃ in the dark. For larval cells, the samples were homogenized in PBS (pH 7.8), followed by passages through G26 gauge needle and 15 mm mesh, and then stained with PI as described above. Relative DNA amount was estimated using the human white blood cell as an internal control.

Multi-locus DNA fingerprinting

Quality control and verification of induced gynogenesis were performed based on comparing multilocus DNA fingerprint profiles of parents and randomly

taken gynogenetic progenies. Genomic DNA was isolated from whole blood using conventional SDS /proteinase K method as described by Nam et al. (1999). Five μg of purified DNA was digested with HinfI (10 unit) for 12 hours and separated on 0.9% agarose gel. The gel was de-purinated by 0.2 N HCl for 10 min, denaturated by 1.5 M NaCl and 1 N NaOH for 45 mins, and then neutralized by 1 M Tris pH 8.0 and 1.5 M NaCl for 45 min. The DNA in gel was transferred to a positively charged nylon membrane (Roche Molecular Biochemicals, Germany) using a capillary method under 20X SSC, and fixed with UV cross linker. The membrane was probed with $(GACA)_4$ oligonucleotide that had been labeled with digoxygenine-11-dUTP (Synthetic Genetics Inc., USA). Prehybridization, hybridization and stringent washes were also carried out according to the procedures as described by Gross et al. (1994). Detection of hybridized signals was performed with a Non-Isotopic Labeling and Detection Kit according to the manufacturers recommendation (Roche, Germany).

Transgene analysis

To confirm the successful induction of homozygosity at transgenic locus, the transgenic meiotic gynogens were identified using PCR and subjected to Southern blot hybridization for examining the transgene homozygosity. Genomic DNA was isolated from blood or fin tissues (Nam et al., 1999). PCR analysis was made using two oligonucleotide primers (FCAT-1 and FCAT-2) to amplify the internal CAT gene fragment. Information on transgenic vector and primer sequences can be referred to Nam et al. (1999). Blot hybridizations were carried out using with a Non-Isotopic Labeling and Detection Kit according to instruction manual provided by supplier (Roche, Germany).

Progeny testing

The putative transgenic homozygous meiogynes were crossed with non-transgenic fish to examine whether they could transmit transgene to next generation at 100% frequency. The gametes from homozygous or heterozygous transgenic fish were crossed with those from non-transgenic controls. Randomly selected 27 progeny from each cross were subjected to PCR analysis.

Expression assay of transgenic fish

Transgenic expression in hetero- and homozygous transgenic siblings was examined using a CAT-enzyme-linked assay (ELISA). Tissues were surgically removed and the samples were prepared according to the method described by Nam et al. (2000). All subsequent steps were followed by the protocol of a kit provided by manufacturer (Roche, Germany).

Statistics

The differences in fertilization, survival of embryo, hatching success, early survival of hatched larvae and yields of gynogens were assessed by ANOVA test. Raw data of percent reading were subjected to arcsine transformation prior to tests. The difference was considered to be significant at $P<0.05$.

Results

Effect of UV irradiation on survival and incidence of haploidy

The embryos developed from mud loach eggs inseminated with UV-irradiated cyprinid loach sperm displayed quite different percent survival depending on the amount of UV irradiated. Typical Hertwig effect was also observed in optimum UV range between 3,150 to 4,050 ergs/mm^2 (Fig. 1). Incidence of haploidy was up to near 100% with more than 50% survival just prior to hatching. Many haploid fish could also hatch but died before the 1st feeding

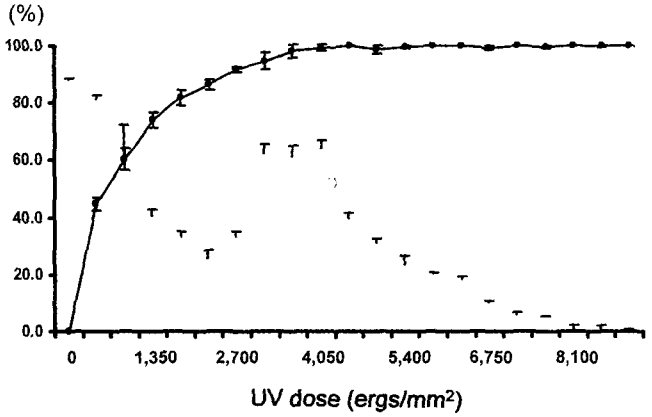

Fig. 1. Mean (±SD) percent survival at 24 hours post insemination (histograms) circles) and haploid incidence (line with closed circles) of mud loach eggs inseminated with UV-irradiated cyprinid loach sperm.

(3 days post hatching). Although exceptionally few individuals retained their viability until 3 days of age, none of them survived within 5 days after hatching (Table 1). Haploid fish showed typical haploid syndrome such as distorted body and short underdeveloped tails (Fig. 2).

Blocking the 2nd meiosis by cold shock for generation meiogynes

Cold shock treatment (2℃ for 60 min with 5 min post insemination) was applied based on our previous studies on auto- and allotriploidy induction (Kim et al., 1994; 1995). The treatment successfully produced diploid mud loach individuals from eggs inseminated with UV-irradiated cyprinid loach sperm. However mean hatching success (53%) and early survival rate (72%) of the meiotic gynogenetic group were significantly lower than those of normal crosses between mud loach females and males, and also than those of hybrid crosses between mud loach female and cyprinid loach male ($P<0.05$) (Table 1). The overall yield for viable meiogynes at the 1st feeding was about 37%. Most gynogenetic progeny (98.5% in average) of which mother was recessive color strain (yellow) displayed exclusive maternal inheritance of the color (i.e. yellow) (Table 1).

Ploidy estimation by flow cytometry

Ploidy levels of presumptive gynogenetic haploid and diploid individuals were verified by flow cytometry. When the human white blood cells (WBC) were considered as 7.0 pg/cell, haploids and diploids

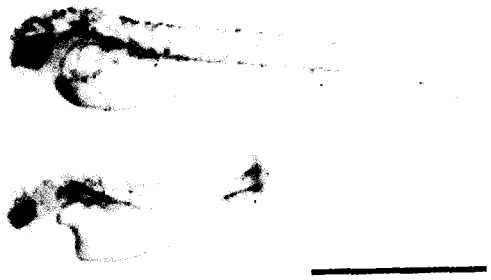

Fig. 2. External morphologies of diploid (upper) and haploid (lower) mud loach. Bar indicates 1.0 mm.

revealed expected DNA contents, as $N=1.4$ pg/cell and $2N=2.8$ pg/cell, respectively (Blacklidge, 1997) (Fig. 3).

Multi-locus DNA fingerprint profiles of gynogens

Multi-locus DNA fingerprinting detected a number of loci using synthetic oligonucleotide $(GACA)_4$ probe in combination with the restriction enzyme HinfI. Generally concordance was found in DNA fingerprint profiles between the mother and her gynogenetic progenies. Most identifiable bands in progenies were observed in the mother. Any notable indication of paternal contribution couldn't be found in gynogenetic progeny (Fig. 4).

Verification of homozygosity at transgenic locus in gynogenetic fish

Transgenic gynogens were identified using PCR of DNA from 1-month-old gynogenetic fish (Fig. 5). Of

Table 1. Mean (±s.d.) fertilization, hatching, early survival, yield of viable diploid and percent pigmentation of meiotic gynogenetic groups and control crosses, based on 3 replicated groups.

Exp. cross	Fertilization (%)	Hatching (%)	Early survival (%)	Yield of viable 2N (%)	Pigmentation[1] (%)
MM♀×MM♂	93.1±4.3[a]	83.8±7.3[a]	94.9±4.7[a]	79.3±4.6[a]	100
MM♀×AA♂	93.3±4.2[a]	82.6±4.5[a]	95.6±3.9[a]	78.9±4.4[a]	100
MM♀×UV-AA♂	89.9±6.6[a]	67.2±3.7[b]	0.2±0.2[c,2]	0.0±0.0[c]	–
MM♀ (albino)[3]× UV-AA♂ +CS	91.0±3.7[a]	54.7±7.3[c]	73.6±3.8[b]	39.6±3.7[b]	1.5
MM♀ (transgenic)[4]× UV-AA♂ +CS	91.0±2.5[a]	50.7±7.9[c]	68.9±8.9[b]	34.2±2.0[b]	100

Means within a column with different superscript are significantly different ($P<0.05$).
Abbreviations: MM, mud loach; AA, cyprinid loach; UV, ultraviolet irradiation (3,870 ergs/mm^2); CS, cold shock treatment (2℃ for 60 mins) with 5 mins post insemination.
[1]Determined at 4 days after hatching.
[2]All of fish died within 5 days post hatching.
[3]Yellow phenotype loach (homozygous for a recessive demelanogenesis gene).
[4]Heterozygous transgenic containing CAT gene (Nam et al., 1999).

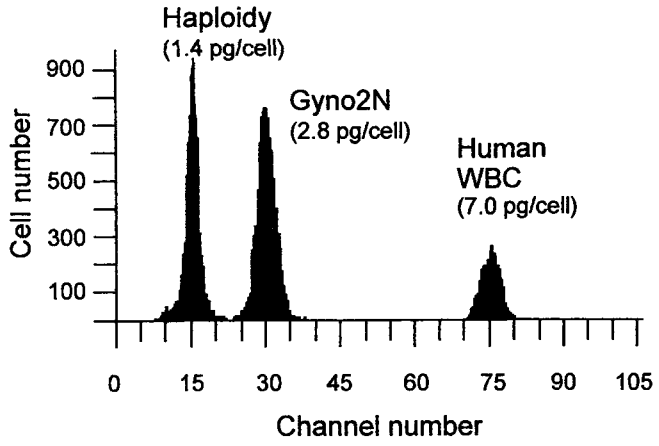

Fig. 3. Histograms showing the DNA content profiles of gynogenetic haploid, diploid and human white blood cell control, as assessed by flow cytometry.

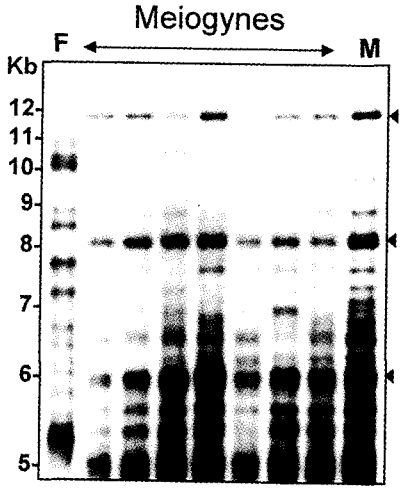

Fig. 4. Representative gel showing multi-locus DNA fingerprint profiles of randomly cho-sen gynogenetic progeny and their parents (F, father cyprinid loach and M, mother mud loach). Arrow heads indicate the putative diagnostic bands.

41 PCR-positive fish tested, only 3 individuals turned out to be homozygous at transgenic locus, when determined by Southern hybridizations (Fig. 6). These transgenic homozygous fish exhibited twice intensity of each hybridized signal, as compared to heterozygous fish, based on scanning densitometry (data not shown).

Germ-line transmission of transgenic homozygous gynogenetic fish

In two independent crosses with non-transgenic fish, the homozygous fish (identified by Southern blot) transmitted the transgene to their offspring (F5) at 100% frequency, indicating they are true homozygous transgenic. On the other hand, both traditional transgenic heterozygous controls and heterozygous transgenic meiogynes revealed consistently 50% frequency of germ-line transmission (Table 2).

Expression of homozygous transgenic meiogynes

The transgenic line in the present study expressed CAT transgene in liver and spleen (see Nam et al., 2000). Increased level of transgene expression was found in homozygous transgenics, based on CAT-ELISA. In liver, heterozygous transgenics (whether or not they were gynogens), the expressed CAT was ranged from 2.31 to 2.46 ng/mg protein, while homozygous fish showed the range from 4.05 to 4.82 ng CAT/mg protein. Consequently the average increase in homozygous fish was 1.8 fold. On the other hand, expression in spleen was 1.6-fold higher in transgenic homozygous fish than in heterozygous fish (Table 3).

Discussion

Meiotic gynogenesis in mud loach was optimized using heterospecific insemination. Ultraviolet (UV)

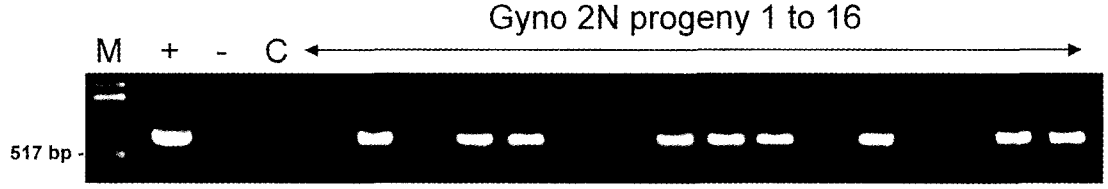

Fig. 5. PCR detection of transgenic gynogens. M, 1 kb ladder (Gibco BRL); +, positive amplification of plasmid; −, negative blank; C, non-transgenic control. Expected size of PCR product is 562 bp.

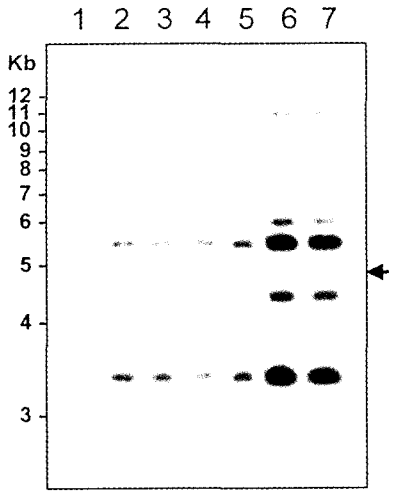

Fig. 6. Southern blot hybridizations of heterozygous (lanes 1 to 5) and homozygous (lanes 6 & 7) CAT-transgenic gynogenetic fish. DNA from blood tissue were digested with DraII, separated onto 0.8% gel, transferred to nylon membrane, and probed with dig-labeled CAT gene segment. Arrow in-dicates the expected size of fragment (4.7 Kb) produced positive digestion of pFV4CAT with DraII.

Table 2. Progeny testing of putative transgenic homozygous meiogynes along with their heterozygous and non-transgenic siblings

Exp. fish	Cross I	Cross II	Average (%)
Non-transgenic fish			
1	0(0.0)	0(0.0)	0
2	0(0.0)	0(0.0)	0
Heterozygous transgenic			
1	13(48.1)	15(55.6)	51.9
2	13(48.1)	14(51.9)	50.0
Heterozygous transgenic meiogyne			
1	14(51.9)	14(51.9)	51.9
2	13(48.1)	16(59.3)	53.7
Homozygous transgenic meiogyne			
1	27(100.0)	27(100.0)	100.0
2	27(100.0)	27(100.0)	100.0
3	27(100.0)	27(100.0)	100.0

Number of transgenic progeny (%) out of 27 fish subjected to PCR.

Table 3. CAT-ELISA of homozygous and heterozygous transgenic fish. Expressed CAT was represented as ng/mg total protein, based on the standard curve made by BSA.

Exp. fish	Liver	Spleen
Heterozygous transgenic		
1	2.30	2.31
2	2.34	2.44
3	2.46	2.22
4	2.45	2.33
5	2.33	2.30
6	2.56	2.45
Mean±SD	2.41±0.10[a]	2.34±0.09[a]
Homozygous transgenic		
1	4.05	3.88
2	4.82	3.71
3	4.45	3.32
Mean±SD	4.44±0.39[b]	3.64±0.29[b]
Relative fold to heterozygote	1.8	1.6

Means with different superscripts within a column are significantly different based on t-test ($P< 0.05$).

Microplate was read at 405 nm using microwell plate reader (BioRad, USA) and concentrations of CAT were calculated from the standard curve using standard CAT control enzyme provided in a Kit (Roche).

irradiation successfully inactivated genetic materials in cyprinid loach sperm with Hertwig effect around optimal UV range from 3,150 to 4,050 ergs/mm^2. The resultant gynogenetic haploid mud loach that have been developed from eggs activated by the UV-irradiated sperm showed haploid syndrome, which was similar with those observed in other previous haploid loaches (Suzuki et al., 1985; Arai et al., 1993).

The thermal shocks efficiently produced viable gynogenetic diploids. Cold shock treatment to produce meiogyne confirms our previous data on induction of auto- and allotriploid mud loach (Kim et al., 1994; 1995). Although the present treatment was reliable for producing viable diploid gynogens of mud loach, the cold-shocked eggs suffered from significantly lower hatchability than non-treated eggs ($P< 0.05$).

Mother-specific inheritance of color (recessive yellow) was observed in most gynogenetic fish, suggesting there was no significant contribution of paternal genetic materials to gynogenetic offspring.

However, few individuals (about 1.5%) revealed indeed the wild type color, suggesting that some of spermatozoa might avoid the UV-ray (i.e. they could be hybrid rather than real gynogens), although we haven't yet clarified the reason for this phenomenon. The present results were well coincided with previous gynogenetic studies concerning body color as a phenotypic marker (Suzuki et al., 1985). The gynogenetic status diploid progeny was also successfully verified with multi-locus DNA fingerprinting. Several potential diagnostic bands effectively proved the gynogenetic status. The evaluation of the success of gynogenesis using DNA fingerprinting has been reported in tilapia (Carter et al., 1991) and also in African catfish (Volckaert et al., 1994). However, with our present DNA fingerprint profiles, the complete homozygosity couldn't be proven yet, as similarly in report for gynogenetic tilapia (Carter et al., 1991). Further studies to develop new genetic marker or probe allowing more clear verification of homozygous nature should be needed. Development of microsatellite DNA marker can be one of good strategies for such experiments.

From this study, it is clear that transgene integrated into host chromosome can be served as a novel genetic marker for verification of induced gynogenesis. Of 41 transgenic gynogens tested, only 3 individuals were homozygous at transgenic locus, even though all of them were believed to be actual gynogens rather than hybrids, evidenced by fingerprinting (data not shown). However, we were not surprised because there have been numerous reports on heterozygous characters of meiotically developed gynogenetic fish due to recombination process during the 2nd meiosis (Thorgaard et al., 1983; Thompson and Scott, 1984; Mair, 1993). The most plausible explanation for this phenomenon (extremely low frequency of homozygous fish at transgenic locus) is that transgene was integrated into chromosomal site which was far from its centromere, and recombination between transgene locus and centromere has occurred during the second meiosis. Performing the meiotic gynogenesis from various transgenic lines with different transgenic genotypes might provide a good system for studying the mapping genes relative to its centromere. Further study should be needed to carry out breeding experiment including progeny testing in order to evaluate the performance of inbred lines.

By performing gynogenesis from transgenic heterozygous fish, homozygous transgenics could be induced in a single generation, suggesting the haploid maternal set was successfully combined with its second polar body. Such transgenic homozygous line may facilitate the maintenance of a line by obviating the need to screen transgene in each individual. Furthermore, improved transgene expression was achieved by increased copy number(s) of transgene per cell. Further production of various isogenic transgenic lines (and subsequently cloned transgenics) with different transgenic genotypes will be carried out. In addition, an intraspecific hybridization between isogenic (or cloned) transgenic lines would be a good strategy, which may offer a new possibility to generate even better strain by combining desirable traits from two different transgenic lines.

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