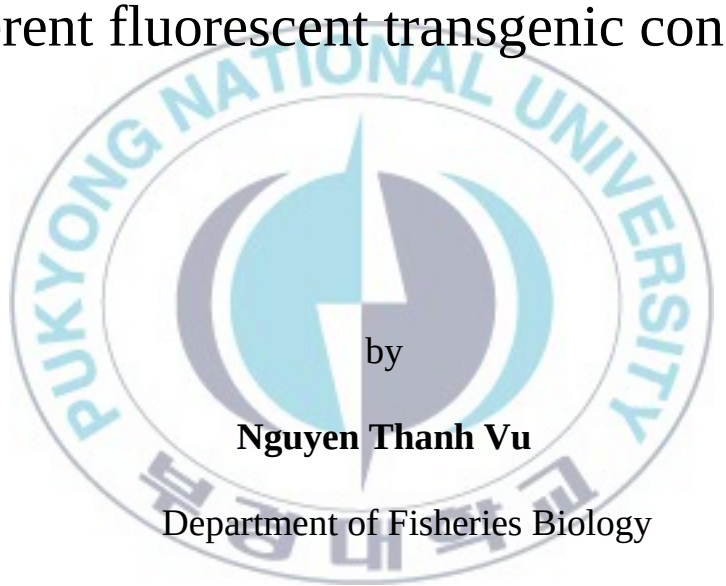
Thesis for the Degree of Master of Fisheries Science

Generation of various transgenic marine medaka (*Oryzias dancena*) strains harboring different fluorescent transgenic constructs



by

Nguyen Thanh Vu

Department of Fisheries Biology

The Graduate School

Pukyong National University

August 2012

Generation of various transgenic marine medaka (*Oryzias dancena*) strains harboring different fluorescent transgenic constructs

(다양한 형광발현벡터가 이식된 형질전환 바다송사리 (*Oryzias dancena*) 개발)

Advisor: Prof. **Kim Dong Soo**

by

Nguyen Thanh Vu

A thesis submitted in partial fulfillment of the requirements

for the degree of

Master of Fisheries Science

in Department of Fisheries Biology, The Graduate School,

Pukyong National University

August 2012

원탄부의 수산학석사 학위논문을 인준함

2012년 8월 25일

주 심 수산학박사 남 윤 권 (인)

위 원 이학박사 김 종 명 (인)

위 원 이학박사 김 동 수 (인)

Generation of various transgenic marine medaka (*Oryzias dancena*) strains harboring different fluorescent transgenic constructs

A dissertation

by

Nguyen Thanh Vu

Approved by:

Prof. Yoon Kwon Nam (Chairman)

Prof. Jong Myoung Kim (Member)

Prof. Dong Soo Kim (Member)

August, 2012

Generation of various transgenic marine medaka (*Oryzias dancena*) strains harboring different fluorescent transgenic constructs

August 2012 Nguyen Thanh Vu

Generation of various transgenic marine medaka (*Oryzias dancena*) strains harboring different fluorescent transgenic constructs

Nguyen Thanh Vu

Department of Fisheries Biology, The Graduate School,
Pukyong National University

Abstract

Transgenesis in small aquarium fish models can provide a number of advantageous merits as an experimental platform for various theoretical and practical researches. Marine medaka is one of novel candidates that could be subjected to ornamental application by using a transgenesis of fluorescent protein genes, due to its transparent body color and great osmoregulatory capacity. In this study, three tissue-specific promoters: two promoters derived from a muscle-specific gene encodes a myosin light chain 2 polypeptide (*mlc2*) and skeletal *α-actin* were used to express cyan fluorescent protein (CFP) and red fluorescent protein (RFP), respectively; and the another promoter, cytoskeletal *β-actin*, is a conserved housekeeping protein was also capable of directing endogenous GFP expression in transient transgenic marine medaka embryos and adults. Although transgenic founders exhibited mosaic expression, most of established transgenic germ-lines passed on the fluorescent transgene into their subsequent generations following the Mendelian single gene inheritance pattern. However, transgenic expression particularly in the sense of intensity of fluorescent signals could be largely governed by the kinds of promoters with differential strengths. CFP transgenic fish exhibited distinctive characteristics in terms of fluorescent intensity and hue saturation of the color, while the *α-actin*RFP displays tissue and developmental specificity in their expression during ontogenesis. On the other hand, the *β-actin* transgenic expression is the various strain-dependent or sex-related patterns of fluorescent signal during germ-line transmission from parental fish to their offspring. Data from this study could be a useful basis to select both the fluorescent protein genes and promoters for further development of various versions of different fluorescents and ornamental transgenic strains.

Table of contents

List of tables

List of figures

ACKNOWLEDGEMENTS

This work would never have been accomplished without the assistance and encouragement of past and current members of the Genetic Resources and Engineering Laboratory. Particularly, I would like to thank my professor, Prof. Dong Soo Kim and Prof. Yoon Kwon Nam for allowing me to grow as an independent thinker and scientist; Dr. Young Sun Cho, Dr. Sang Yoon Lee, Ji-eun Park and Byeong Soo Kim for assistance on molecular biology assays; Youn Kyoung Kim for her assistance foundation microinjected technique and on microscope imaging.

I gratefully acknowledge, Prof. Jong Myoung Kim for his critical evaluation, kind advises, suggestions, and comments to improve the scientific merit of this thesis. In addition, all professors in Fishereis Biology Department, Pukyong National University are greatly appreciated for their teaching, and advices throughout my study period.

Thank you to Dr. Yi Kyung Kim, Dr. Victor David, Ju Yeon Jung, Min Gyun Ko, Shim Bit Na ra, Vo Thi Thu Em, Kyunglim Kang, and Su Hee Choi for their supports, advices, friendships, company during living in Korea and the writing of my manuscripts thesis. This work was supported by Institute of Marine Living Modified Organisms (IMLMO), Pukyong National University.

I also would like to thank my director Dr. Truong Ha Phuong, Dr. Dao Van Tri for their accepting me to study in Korea, and Assoc. Prof. Hoang Tung for his recommendation to IMLMO.

Finally, I would like to thank my parents, my small family (my wife - Van Thi Quy Tho and son - Nguyen Van Thien Phuc) for their always being with me and motivational comments, unshakable confidence in me.

Chapter 1

Introduction

1. Introduction to transgenic marine medaka *Oryzias dancena* as a model for fish biology

Medaka (*Oryzias latipes)* has become one of the species studied for useful applications of transgenic fish (Ozato et al., 1992; Ozato and Wakamatsu, 1994), including studying of germ-cell environmental muragenesis (Shima and Shimada, 1994); observed in a live vertebrate germ cell carrying with fluorescent protein (Tanaka et al., 2001); organogenesis, human disease, evalution (Naruse and Takeda, 2011); rapid screening developmental neurotoxicity (Fan et al., 2011). Recently, the similar genus with *O.latipes*, marine medaka *O. dancena* has become one of the dominant species of advantageous merits and transgenic researches for ecotoxicogenomic (Chen et al., 2011) or potential in the field of ornamental fish application (Cho et al., 2011).

Marine medaka is a euryhaline species representing the easiness of laboratory rearing, transparent embryos and year-round multiple spawning under controlled conditions (Song et al. 2009). Further, the marine medaka represents higher fecundity and longer life-span than Japanese medaka, which is more desirable for the laboratory propagation and management of established strains. Moreover, this species displays a fairly transparent body color, which is advantageous for the in vivo visualization and imaging of fluorescently tagged protein(s) throughout nearly whole lifespan in a real-time fashion, which is often useful for various experimental transgenesis and heterologous expression assay

particularly in the field of ecotoxicology and developmental biology (reviews: Cho et al., 2011).

Based on these advances, herein I would like to emphasize in this regarding research on the use of this species, which is tagged the various fluorescent proteins. Hopefully, the novel ornamental fish species can be generated that they can survive in both fresh water and in sea water completely, to serve extensively for the aquarium trade.

2. Fluorescent transgenic fish as ornamental fish

In the past two decades, transgenic fish technology has been intensively employed to generate superior fish stocks with beneficial traits in aquaculture, such as producing transgenic fish with enhanced growth rates in common carp (Zhang et al., 1990), mud loach (Nam et al., 2001; Song & Kim, 2012), rainbow trout (Cavari et al., 1993), coho salmon (Devlin et al., 1995). In addition, transgenic fish is also potential application in freeze resistance and cold tolerance; salinity tolerance; disease resistance; sterility; metabolic modification; or fish-pharming (Maclean and Laight, 2000). Unfortunately, because of concerns in environmental issues and food safety, the application of transgenic fish in aquaculture remains in a painstaking process (Gong et al., 2001; Kapuscinski, 2005; 2007).

Brightly, since the green fluorescent (GFP) is the first discover in 1962 (Shimomura et al., 1962), combination with tissue-specific promoters have become an established technique in developmental analyzes and have brought many new promises to the transgenic fish studies, especially in the two model freshwater fish species, the zebrafish (*Danio rerio*) and the medaka (*Oryzias latipes*) (Gong et al., 2001; Kapuscinski, 2005). Fluorescent proteins have

properties which indicate that it is a neutral, non-toxic, universal marker when expressed in transgenic organisms. Fluorescent proteins do not confer any measurable ecological host costs when expressed in transgenic plants (Chalfie and Kain, 2005). In transgenic fish, the fluorescent proteins expression is directly observable; so it has become the idea to create transgenic fluorescent fish for aquarium purposes (Pan et al., 2008; Blake et al., 2010; Gong et al., 2003; 2008). Consequently, several fluorescent transgenic zebrafish (*Danio rerio*) and Japanese medaka (*Oryzias latipes*) strains have already been launched in the aquarium markets in US and Taiwan (Bratspies, 2005), respectively. Evidently, the most successful commercial ornamental transgenic organism to date is 'GloFish', marketed by Yorktown technologies (Krishna, 2009).

Moreover, success achieved in these pioneering works have been now encouraging the extension of fluorescent transgenic techniques to other fish species such as white skirt tetra (*Gymnocorymbus ternetzi*) (Pan et al., 2008). To date, the skeletal muscle-specific myosin light chain 2 (Wan et al., 2002; Gong et al., 2003; Zeng et al., 2005) and ubiquitous actin (Chou et al., 2001; Cho et al., 2011; Ge et al., 2012) promoters have been commonly used to drive fluorescent phenotypes in transgenic fish. Currently, although as at least five color proteins are so far available (Clontech), including *gfp*, *bfp*, *rfp*, *cfp* and *yfp* have been transgenically introduced to fish models, only the stable lines of RFP, GFP and YFP transgenic fish have successfully generated for ornamental fish purpose (Gong et al., 2003). Therefore, the introductions of successful new colors such as CFP or BFP on various species to cater for the diversification of species with odd colors are promising developments.

3. Microinjection – a suitable method for transgenesis fish

Several techniques are currently available for transgenic fish production which have been developed to increase the efficiency of transgene integration or to produce a large number of transformed individuals simultaneously (Figueiredo et al., 2007). There are many systems that deal with introducing foreign DNA fragments into conceived fertilized embryos or gametes of aquatic species, such as: microinjection, electroporation, particle bombardment, sperm-mediated gene transfer, retroviral infection, direct muscle injection, and insulator and transposon. Nuclear transplantation is also successfully developed by using embryonic- derived stem cells, cell-mediated gene transfer and zebrafish sperm (reviews: (Tsai, 2008). Although the degrees of success are variable among the reports, none of them has replaced completely the microinjection yet, particularly considering the yields of stably transformed embryos and resultant founder fish capable of germ-line transmission of the introduced transgene (Sin, 1997).

Microinjection directly transfers DNA into individual eggs or embryos. The optimal amount of DNA injected is varies with species and egg sizes. Importantly, the buffer used to suspend the DNA, the concentration of the DNA (Kinoshita 1995) and whether linear or circular DNA is used can affect the efficiency of gene transfer by microinjection and the survival of the embryos (Chourrout et al., 1986; Marini and Benbow 1991). In addition, the injected embryos have survival rates ranging from 16% in zebrafish (Stuart et al., 1988) to 85% in salmon (Devlin et al., 1995). However, when microinjection is used to produce transgenic fish it almost invariably produces mosaic fish due to delayed transgene integration, which occurs only after a few cycles of embryonic cell division (Figueiredo et al., 2007). So it may be the reason why the number of

transgenic founder fish produced varies with the laboratory and the species of fish, ranging from none in common carp, 5% in zebrafish, 6% in salmon, 13% in tilapia and 12% in carp to over 70% in rainbow trout (Reviews: Sin, 1997).

4. The focus of this thesis

The objective of this study was to test characteristic expression of distinctive promoters, fluorescent proteins, and evaluation of potential usefulness in ornamental applications, we generated various transgenic marine medaka strains harboring different fluorescent transgenic constructs. Based on this regard, the individual expression patterns of each gene are described in separate subject. Subject I: Characterization of cyan fluorescent protein gene (*cfp*)-transgenic marine medaka strains for ornamental application. Subject II: Development of stable line and expression of green fluorescent protein in transgenic marine medaka under a cytoskeletal beta-actin promoter. Subject III: Ontogenic expression of transgenic RFP under the control by alpha skeletal actin promoter in transgenic marine medaka.

Chapter 2

Characterization of cyan fluorescent protein gene (cfp)-transgenic marine medaka strains for ornamental application

1. Introduction

Generation of transgenic fish expressing living fluorescent protein reporter(s) under the regulation by tissue-specific promoters has become an established technique in the field of developmental genetics (Gong et al., 2001; Tsai, 2008; Chudakov et al., 2010). In addition to the theoretical research purpose, a certain fluorescent transgenic fish strain acquiring vivid and faithful fluorescent color in their external phenotype has been postulated as a novel ornamental, pet animal (Gong et al., 2003; Kinoshita, 2004; Zeng et al., 2005). Several fluorescent transgenic zebrafish (*Danio rerio*) and Japanese medaka (*Oryzias latipes*) strains have already been launched in the aquarium markets in US and Taiwan (Bratspies, 2005), respectively. Success achieved in these pioneering works have been now encouraging the extension of fluorescent transgenic techniques to other fish species (Pan et al., 2008; Cho et al., 2011). To date, the skeletal muscle-specific myosin light chain 2 (Wan et al., 2002; Gong et al., 2003; Zeng et al., 2005) and ubiquitous actin (Chou et al., 2001; Cho et al., 2011; Ge et al., 2012) promoters have been commonly used to drive fluorescent phenotypes in transgenic fish, although some other regulators have also been challenged (Gong et al., 2002; Burket et al., 2008). Not surprisingly, these previous studies above have shown that transgenic expression particularly in the sense of intensity of fluorescent signals could be largely governed by the kinds of promoters with differential strengths, although transgenic genotype-dependent

differences even within a given transgene construct should also be taken into consideration (Higashijima et al., 1997; Burket et al., 2008; Cho et al., 2011).

Besides the promoter dependency, our literature-based survey indicates that the expression properties of transgenic fish (*i.e.*, the visibility and brightness of fluorescence color in their external phenotypes) could also be considerably affected by the kinds of fluorescent protein genes used, even if they are under the control by the same promoter (Wan et al., 2002; Gong et al., 2003; Blake et al., 2010). Each fluorescent protein (e.g., GFP, RFP, CFP or YFP) has its own structural characteristics often including a targeted modification(s) to its wild-type progenitor, consequently leading a unique photophysical property in the maturation speed, extinction coefficient, quantum yield and photostability (Zhang et al., 2002; Chalfie and Kain, 2005; Shaner et al., 2005, 2007), all of which potentially have important impacts on the perceived brightness and ornamental quality of the fluorescent transgenic fishes.

To date, various fluorescent protein genes including *gfp*, *rfp*, *cfp* and *yfp* have been transgenically introduced to fish models particularly including the two common popular species zebrafish (*Danio rerio*) (Ju et al., 1999; Gong et al., 2002; Wan et al., 2002; Curado et al., 2007; Burket et al., 2008; Blake et al., 2010; Bian et al., 2011) and medaka (*Oryzias latipes*) (Hamada et al., 1998; Tanaka and Kinoshita, 2001; Tanaka et al., 2001; Kinoshita, 2004; Fan et al., 2011; Willems et al., 2012). However barring a few reports, many postmortem studies have claimed their results on the successful expression of fluorescent proteins in fish only based on the fluorescent microscopic examinations at a particular developmental stage(s) (Amsterdam et al., 1996; Gong et al., 2003; Huang et al., 2003; Zeng et al., 2005; Willems et al., 2012). On the other hand, the evaluation of stably persistent fluorescent characteristics over the body of transgenic fishes throughout their whole lifespan has been less explored,

although it may be one of the key factors that should be taken into account for the ornamental application of fluorescent transgenic fish. Currently, the fluorescent phenotypes of the transgenic fish harboring *gfp* and *rfp* have been relatively well characterized in terms of the ornamental purpose (Gong et al., 2003; Pan et al., 2008; Blake et al., 2010; Cho et al., 2011). However, apart from only a few studies (Finley et al., 2001; Morris et al., 2005; Curado et al., 2007; Joly, 2007; Ge et al., 2012; Willems et al., 2012), the characteristics of transgenic phenotypes developed using other fluorescent protein genes has not been yet extensively examined in different fish species. Moreover, recent explosion in the diversity of newly available fluorescent proteins with improved photostability, folding efficiency and brightness could offer novel opportunity to develop more qualified, ornamental transgenic fish strains displaying the better visibility of fluorescent color.

The AmCyan1, a variant of wild-type *Anemonia majano* cyan fluorescent protein (CFP), has a recent version of CFP reporter with improvements of brightness and photostability, as compared to the previous CFP, the enhanced CFP (ECFP; the cyan fluorescent variant of the *Aequorea victoria* GFP) (Fang et al., 2005; see also Clontech, Laboratories Inc., Mountain View, CA, USA; http://www.clontech.com). Owing to its improved properties, this recent CFP variant has been known to be able to overcome several drawbacks of earlier CFPs associated with the low wavelength of their signals (Fang et al., 2005). Consequently, the AmCyan1 has become now popular as a genetically and spectrally distinct alternative to other fluorescent proteins in a variety of *in vivo* protein localization and transgenic studies in plants (Wenck et al., 2003; Tang et al., 2006), mammals (Kawamata and Ochiya, 2010), insects (Sarkar et al., 2006) and algae (Mikami et al., 2011). However to date, the functionality of this improved CFP variant has not been challenged detail in ornamental transgenic

fish model. Only couples of previous studies using earlier version of CFP (*i.e.*, ECFP) gene have been reported in transgenic fish where the ornamental evaluation of cyan fluorescent phenotype was limited to founder generation (Gong et al., 2001); otherwise the ECFP-mediated transgenesis has been focused on only the monitoring of developmental events including the conditional cell ablations (Curado et al., 2007; Willems et al., 2012).

Marine medaka (*O. dancena*), a truly euryhaline teleost, possesses a number of advantageous merits as a candidate laboratory organism for various biological and ecotoxicological studies (Cho et al., 2010; Chen et al., 2011). Recently, we have shown that the transgenic expression of RFP reporter driven by ubiquitous *β-actin* promoter could be faithfully visualized in the whole body of marine medaka throughout its entire lifespan owing to the fairly transparent body color of this species (Cho et al., 2011). Finding from our previous work also strongly suggests that the fluorescent transgenic marine medaka holds promising potentials as novel ornamental fish that could be displayed in both marine and freshwater aquaria. In line with our long-term goal to develop various fluorescent marine medaka transgenic lines for novel ornamental varieties in future aquarium trade, the objective of this study was to establish and evaluate stable transgenic marine medaka germ-lines acquiring vivid cyan fluorescent phenotypes via experimental transgenesis of the AmCyan1 reporter gene under the control of marine medaka fast skeletal myosin light chain 2 (*mlc2*) promoter.

2. Materials and Methods

2.1. Fish maintenance and egg collection

Experimental marine medaka *O. dancena* specimens were maintained in the Institute of marine Living Modified Organisms (IMLMO), Pukyong National University, Busan, Korea. Salinity of water for general maintenance of both larvae and adults was 5 ppt and water temperature was kept at 26-27^{o}C with the dark/light cycle of 10/14 h. The fish were fed with commercial diet (Woosung Feed Co. Ltd., Korea) and *artemia nauplii*.

Fertilized eggs were collected from egg-laying females and immediately placed on an incubator at 15^{o}C until microinjected (around 2 hours). The detailed conditions for spawning, egg collection and embryonic development can be referred to previous reports (Song et al., 2009; Cho et al., 2010).

2.2. Plasmid preparation for microinjection

The plasmid pod*mlc2*AmCFP was constructed by insertion of a 2.96-kb *Oryzias dancena mlc2* promoter into the ATG initiation codon of CFP gene in the pAmCyan1-C1 plasmid (Clontech Laboratories, USA) (Fig. 1). The plasmid pod*mlc2*AmCFP was linearized by the digestion with *Cla*I (New England Biolabs, Ipswich, MA, USA), gel-purified and re-suspended in an injection buffer (10 mM Tris–Cl, 0.1 mM EDTA, pH 8.0) at the concentration 110 μg/ml.

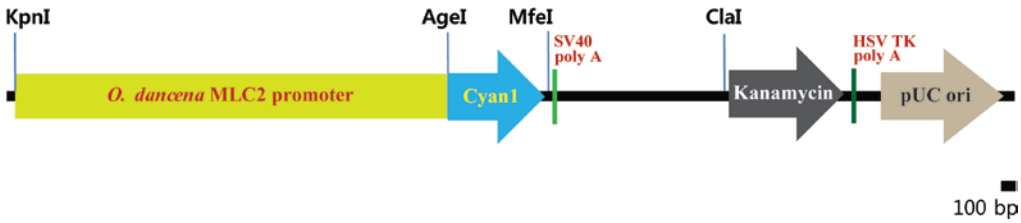


Figure 1. The *podmlc2*Am*CFP* vector consists of *O. dancena mlc2* promoter, AmCyan1 reporter gene, Kanamycin resistance gene and replication origin. *Kpn*I and *Age*I restriction sites were created at the *mlc2* promoter. The AmCyan1 fragment created *Age*I/*Mfe*I sites.

2.3. Microinjection and identification of positive injected embryos

The linearized podmlc2AmCFP construct (50 µg/ml) was injected into one-cell marine medaka embryos and transferred to an incubator at 26 ± 1°C until hatched. The salinity of the incubation water was 5 ppt. During the embryonic development, CFP-positive embryos were identified using the fluorescent microscopy at prehatching stage 28 (heart and spleen development stage. 8 days post fertilization).

The cyan fluorescent signal was analyzed with an AZ100 fluorescence microscope equipped with the NIS-Elements BR image analysis software (Nikon Corporation Instruments Company, Japan). The CFP expression was observed with the Nikon CFP filter (excitation filter wavelengths: 426-446 nm; dichromatic mirror cut-on wavelength: 455 nm; and barrier filter wavelengths: 460-500 nm), and the image was photographed using the digital camera (Nikon digital sight DS-Ri1) implemented in the AZ100 microscope.

2.4. Breeding of transgenic marine medaka

After hatching, the selected CFP-positive larvae were further grown up to sexual maturity (about 9 months old). At sexual maturity, the presumed transgenic founders with CFP signals were subjected to the test for germ-line transmission to F1 offspring. Each adult CFP-positive transgenic female founder was crossed with a wild-type, non-transgenic marine male fish in a 1:1 mating manner. On the other hand, transgenic male founder was crossed with four non-transgenic females. At least 100 embryos from each mating were examined for the CFP signals using fluorescence microscopy as described above. The F1 CFP-transgenic individuals from each transgenic line were mated with non-transgenic individuals in order to examine the transmission of fluorescent transgene to the F2 generation following the Mendelian single gene inheritance pattern. The fertilization, hatching rates and incidence of CFP positive were addressed.

2.5. Characterization of fluorescent phenotypes

The developmental and ontogenic expressions of the CFP signals were examined with F2 transgenic embryos and fry. Fertilized eggs collected from the three transgenic lines were incubated at 26 ℃ under 5 ppt of salinity condition. The fluorescent signals in embryos were examined with a 12-h interval using the fluorescent microscopy. After hatching, the transgenic CFP signals in larvae/fry were monitored also at 1, 15, 30 and 60 days post hatching (dph), in which each fish were examined under microscopy in dorsal, ventral and lateral views. After grown up to adults (around 9 months of age), external phenotype of CFP-transgenics were examined under different light illumination sources, including daylight (sunlight), normal white fluorescent room light (14W; ERI-Su1125-6007; China), UV light (Black light lamp; 352 nm; 15W; Sankyo Denki Co.,

Ltd., Tokyo, Japan), aquarium blue light (FHF 14STEX-D Blue lamp; 450-495 nm; 14W; Leedarson Lighting Co., Ltd. Fujian, China) and different colors of light-emitting diode (LED) lights. The adjustable LED illumination control system (LED-Lighting ZigBeeControl Program) was designated by LED-Marin Convergence Technology R&BD Center (Pukyong National University, Busan 608-739, Republic of Korea) LED-Marine Fusion Research Center (Pukyong National University, Busan, Korea) to emit blue (454 nm), green (517 nm) and red (628 nm) light in an independent or mixed fashion. The external RFP phenotype was photographed using a macro lens (EF 100 mm; 1:2.8L USM) connected to an EOD 5D Mark II digital camera (Canon, Tokyo, Japan).

3. Results and Discussion

3.1. Microinjection and stable of CFP-transgenic marine medaka lines

To generate the various strains for ornamental application, of 1650 embryos microinjected from the eight trials, 697 fish reached the sexual maturity and 62 fish possessed CFP signals in their external body as determined fluorescent microscopy. All the CFP-positive founder fish showed the mosaic distribution of the transgenic CFP signals in their body (Fig. 2), suggesting that their transgenic status would be mosaic as commonly found in founder generation of transgenic fish generated by microinjection (Nam et al., 1999; Figueiredo et al., 2007; Hartmann and Englert, 2012). Based on the intensity and expression areas of CFP signals observed in the external body, nineteen transgenic founders (eight females and eleven males) were selected for testing the germ-line transmission to F1 offspring. Of the nineteen founders tested, seven individuals (six males and one female) were proven to be able to pass on the fluorescent transgene to their F1 progeny. However, All founders transmitted their transgene to offspring with the germ-line transmission frequency lower than 50%, indicating they were mosaic also for their germ cells (Table 1). Despite severe mosaicism in founder generation, the transgenic hemizygous status was stabilized from the F1 generation in each transgenic line. All the tested F1 fish irrespective of transgenic lines passed on the fluorescent transgene to their F2 progeny at a frequency close to 50% (now up to F3) (Fig. 3).

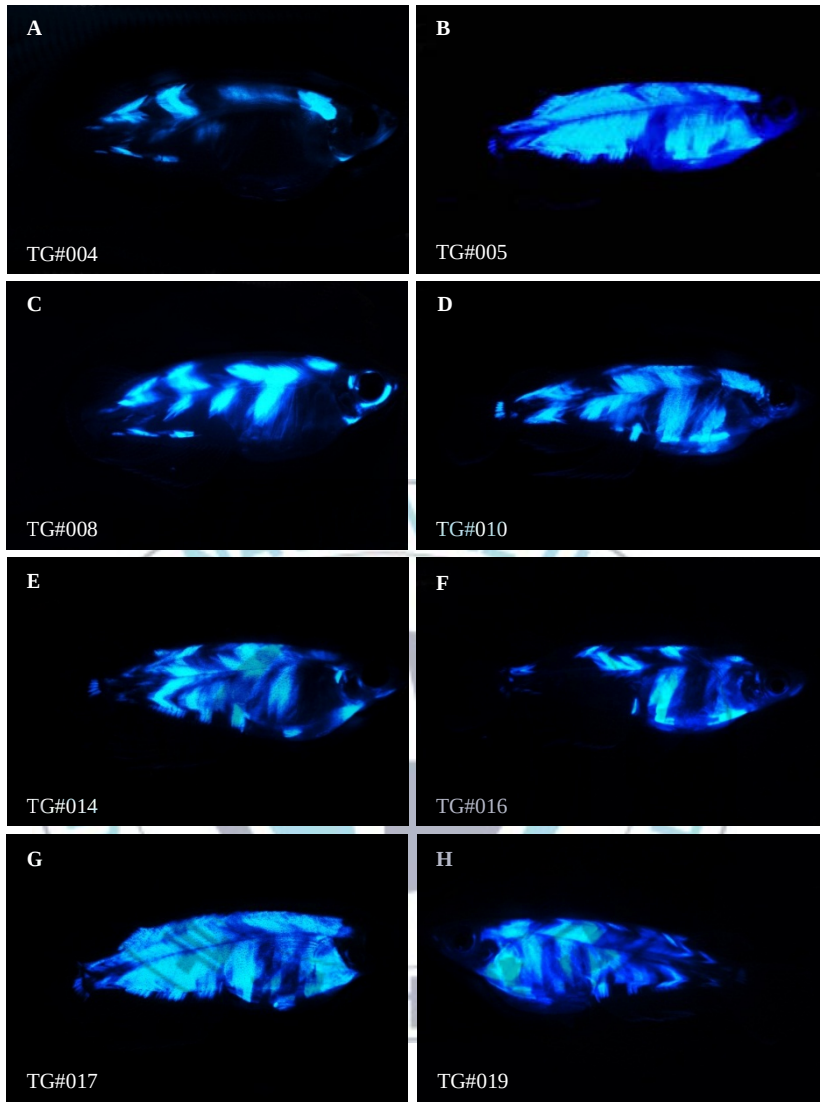


Figure 2. Mosaic distribution of the transgenic CFP-positive founder fishes. From A to H are the order of number of transgenic positive founders generation TG#004; TG#005; TG#008; TG#010; TG#014; TG#016; and TG#019, respectively.

Table 1. Frequency of germ-line transmission in F1 and Mendelian pattern of the inheritance of transgene construct podmlc2AmCFP in F2 generation

Transgenic line of founders	Sex of the founders	F1 positive fish		F2 positive fish	
		No. of incidence of CFP positive fish	Percentage of incidence of CFP positive	No. of incidence of CFP positive fish	Percentage of incidence of CFP positive
TG#004	Female	36/107	33.6	58/122	47.7
TG#005	Male	7/85	8.2	69/133	52.3
TG#008	Male	103/309	33.3	89/156	57.1
TG#010	Male	13/175	7.4	104/229	45.3
TG#014	Male	24/207	11.6	68/136	50.2
TG#016	Male	14/118	7.4	82/167	49.0
TG#019	Male	9/29	31	68/142	47.9

At least 100 embryos were obtained from each transgenic line to verify germ-line transmission. The incidence of CFP positive fish was estimated as the percentage of glowers out of total No. of incidence of CFP positive/hatched.

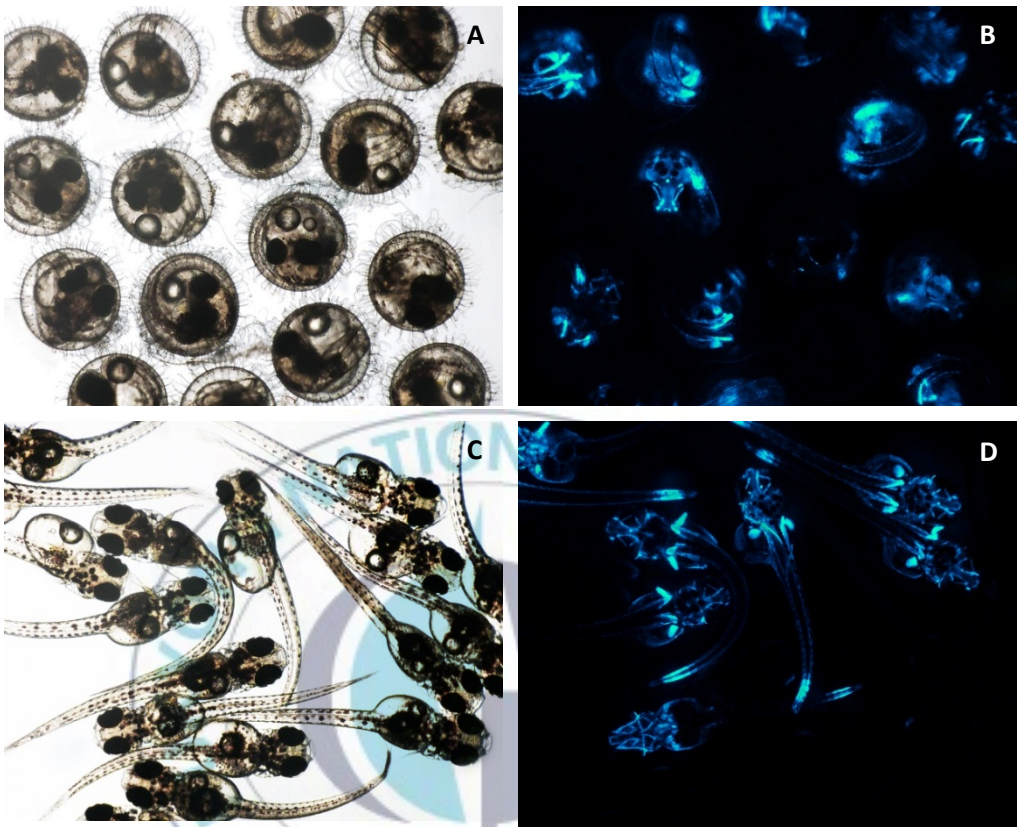


Figure 3. The transmission pattern following the Mendenlian single gene inheritance of fluorescent transgene podmlc2AmCFP in F2 generation. A and B are the group of bright and dark field images of CFP transgenic embryos at stage 27 (formation of visceral blood vessels stage – 6.5 dpf), respectively. C and D are the group of bright and dark field images of CFP transgenic embryos at stage 29 (Just hatched out larvae - 11 dpf), respectively.

3.2. Developmental and ontogenic expression patterns of transgenic CFP signals

During embryonic development, the onset of CFP signals was first detectable at stages 22 (12 somite, about 2.5 days post fertilization; dpf) from the completion of the somitogenesis to the formation of tubular heart, but the intensity was quite low in most transgenic lines examined (Fig. 4A1). The expression level was gradually increased as the development proceeded such that the CFP-positive embryos showed a strong CFP expression at the heart and spleen development stage 28 (8 dpf) (Fig. 4A3; 5A). In overall, the temporal patterns of CFP expression observed in transgenic marine medaka embryos is in accordance with not only the endogenous expression of mlc2f gene but also the patterns of fluorescent transgene previously examined in other transgenic strains (Xu et al., 1999; Gong et al., 2003; Ju et al., 2003). After hatching, the hatchlings at 1 dph displayed strong CFP expression in head region, pectoral fin-attaching muscles and caudal peduncle (Fig. 4B1-B3 and Fig. 5A1). The recapitulation of muscle development in head region was possible using the transgenic larvae, suggesting that the present mlc2f:cfp transgenics could be a useful tool for the real-time ontogenic monitoring in vivo of fast skeletal muscle development and differentiation in this marine medaka species (Gong et al., 2003; Ju et al., 2003). The CFP signals in both head and caudal regions became greater with age of the fish where the distribution of CFP expression was more spread over skeletal muscles and the strength of CFP signals were more intensified (Fig. 4 and Fig. 5).

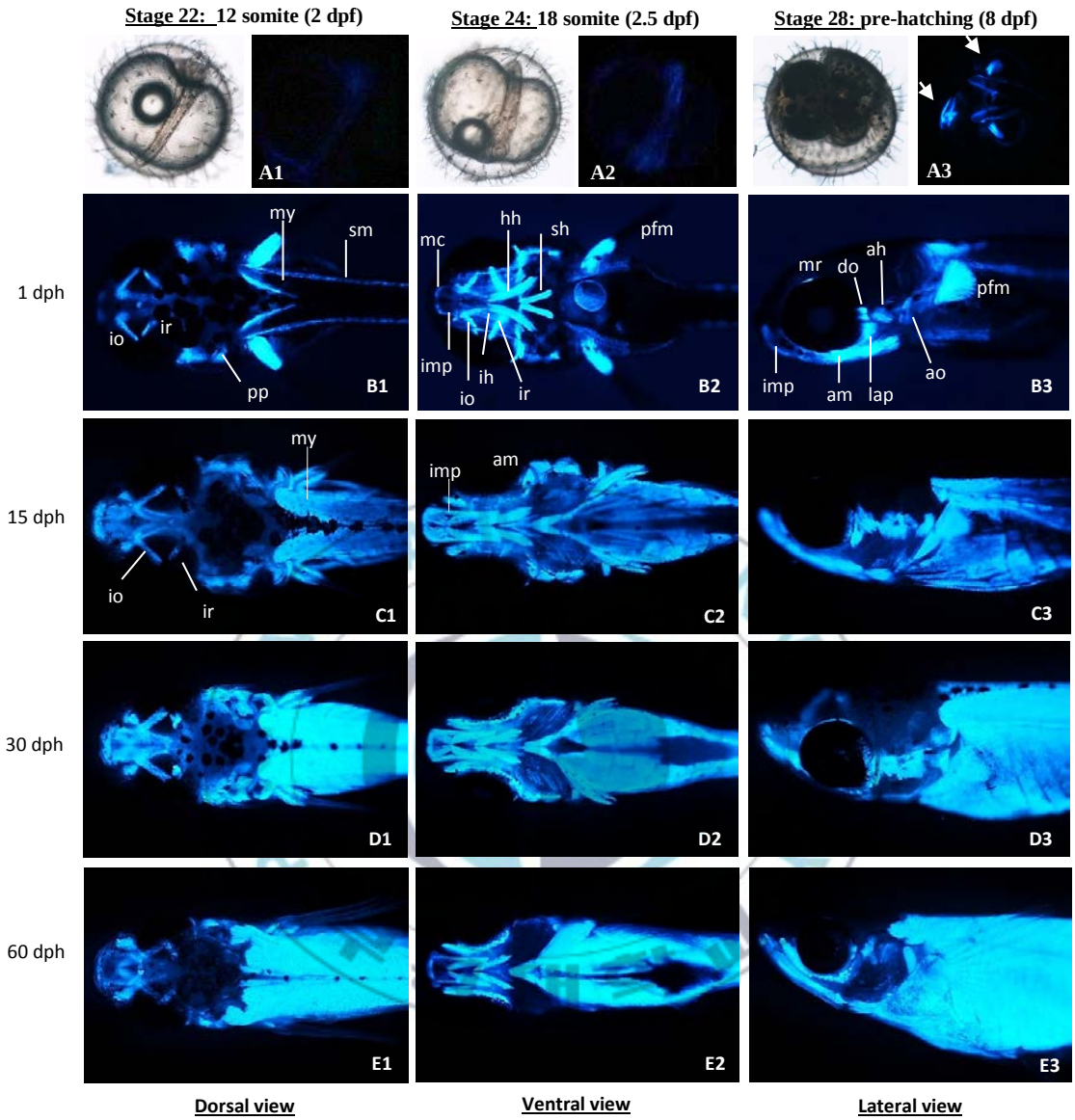


Figure 4. Expression of the cyan fluorescence CFP in F1 embryos and larvae. Initiation timing of a weakly expressed from stage 22 (12 somite) (A1), they gradually increased the level of expression to stage 24 (18 somite) (A2), and their strongly CFP expression was detected at stage 28 (pre-hatching, spleen development) (A3), stage 29 (newly hatched larvae; 1dph) (B1-B3) and the juvenile stage 15; 30; and 60 dph (C1-C3; D1-D3; and E1-E3, respectively).

Arrows in A3 indicated strong CFP expression in the fins bud and trunk of the embryo at stage 28. B1; C1; D1; and E1: Dorsal view of CFP expression at 1; 15; 30; and 60 dph. B2; C2; D2; and E2: Ventral view of CFP expression at 1; 15; 30; and 60 dph. B3; C3; D3; and E3: lateral view of CFP expression at 1; 15; 30; and 60 dph. The names of muscle are based on Ono et al. (2006) and Schilling & Kimmel (1997): ah: adductor hyoideus; am: adductor mandibulae; ao: adductor opercula; do: dilator opercula; hh: hyohyoideus; lap: levator arcus palatine; ih: interhyoideus; imp: intermandibularis posterior; io: inferior oblique; ir: inferior rectus; mc: meckel's cartilage; mr: medial rectus; my: myotome; pfm: pectoral finmuscle; pp: pterygoid process; sh: sternohyoideus.

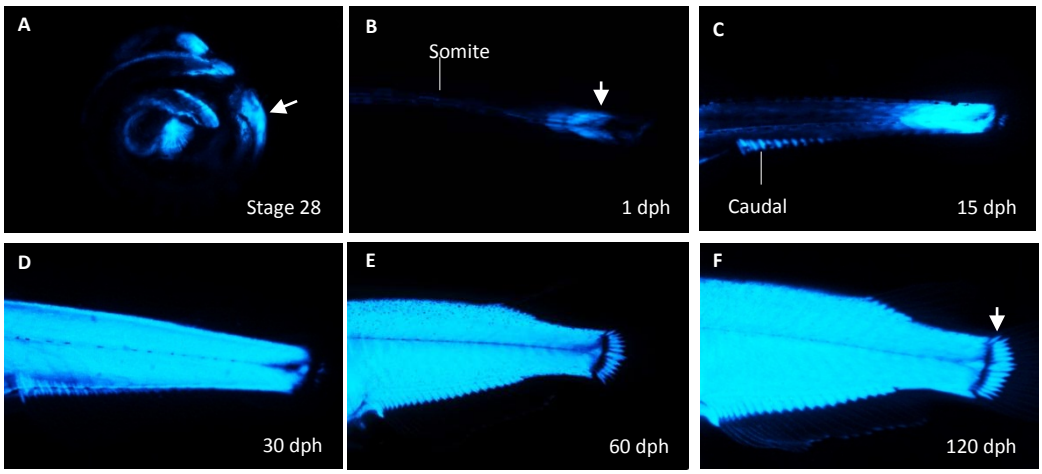


Figure 5. CFP expression in skeletal trunk muscle under control of *mlc*2 promoter in different stages of marine medaka development. Arrow in A indicated that CFP expressed strongly in the end of the myotome at stage 28 (8 dph). Arrow in B showed CFP signals were obtained at the somite and the end of the myotome at 1 dph. C; D; E; and F: CFP levels increased steadily during larvae developments at 10 dph; 3 dph; 60 dph; and 120 dph, respectively. Arow in F indicated that CFP expression at the proximal end of fin ray connected to hypural bones.

3.3. Phenotypic characteristics of CFP-transgenic fish under different light sources

Transgenic adult fish belonging to each transgenic line were easily distinguishable from non-transgenic individuals with unaided eye under normal daylight condition owing to the authentic fluorescent signals achieved in their external body. The distribution pattern of the transgenic CFP signal was the same for all the transgenic lines examined, although the intensity of the CFP signal was variable among the lines. Transgenic expression was highly dominant

in dorsal parts, caudal trunk and lower jaw borne muscles, and moderately in belly region. The fin buds especially including hypural bone-connected region was also clearly CFP-positive. On the other hand, in the external view, the CFP signal was not seen in the cranial region, eyes and fins (Fig. 4). This distribution pattern was in accordance with previous observations in other transgenic fish strains driven by mlc2f promoter (Xu et al., 1999; Ju et al., 2003).

However, the fluorescent colors were considerably affected by light irradiations (Fig. 6). Under normal daylight condition, the CFP-transgenic fish showed yellow-green phenotype. A similar greenish color was observable under the conventional white fluorescence room light. The green glow was highly intensified when the transgenic fish were irradiated to the shortwave UV light (352 nm), in which the very bright green fluorescence was visualized. Unlike UV illumination resulting in only the green fluorescence, the blue LED light (454 nm) made the CFP-transgenic fish bluer. The blue LED-shed CFP-transgenics exhibited more cyan-like as the bluish green phenotype, although the true cyan fluorescent glow that had been observed under fluorescent microscopy was not achieved. As expected, the CFP-transgenic fish responded to neither green (517 nm) nor red (628 nm) LED light (Fig. 6). The irradiation of mixed LED lights (e.g., blue together green or red) altered slightly the fluorescent color of the transgenic fish Mixture of equal amounts of blue and green LED lights resulted in the fluorescent phenotype, which was intermediate between those observed in UV- and blue LED-irradiated fish, while the blue LED mixed with red LED caused a slight shift to a turquoise blue/green owing to the magenta color by red LED light. On the other hand, mixing equal amounts of red-green-blue LED lights gave rise to the green CFP-transgenic fish, of which color was similar with that observed in the transgenics under sunlight or white room light conditions. Meanwhile, the CFP-transgenics shed by commercial aquarium blue

lamp (454 nm) exhibited the similar fluorescent phenotype to that irradiated with blue LED or blue-green LED lights (Fig. 6). As shown in the Fig. 6, the AmCyan1-transgenic marine medaka did not represent the true cyan-emitting phenotype under various light irradiations. Instead, the transgenics were basically green-glowing, although the transgenics represented a more blue-shift phenotype only in response to blue LED light. However, even under blue LED irradiation, the phenotype color of the transgenics was not true cyan color. Similar observation has been reported on the cestrum virus promoter-driven AmCyan1-transgenic rice (*Oryza sativa*) callus showing the slight green-yellow glow color without the use of fluorescence optics (Wenck et al., 2003). Some mechanisms of spectrophotometric tuning are currently being reported; however, most post-mortem studies have claimed their results based on the epifluorescence microscopy and the effects on the external phenotype of a whole organism in vivo should remain to be further challenged. Although, we haven't yet clarified details responsible for the basically green fluorescent phenotype of AmCyan1-transgenics, one possible, but untested, explanation for this finding might be the excitation (458 nm max.) and emission (489 nm max.) wavelengths, which are close to those of GFP and other GFP variants. Consequently, it may result in a similar sort of fluorescent look when they come to human eyes, although the specific filter sets in a fluorescent microscopy is able to distinguish one from another. Another related explanation is the similarity in the structure of chromophore-forming tripeptides (X-Y-G; X = hypervariable, Y = Trp and G = Gly) between AmCyan1 and GFPs. Although the AmCyan1 (a mutant version of amFP486) possesses Ala-165 (Ile in wild type GFP) and His-199 (Thr in wild type GFP) that are known to be the key residues contributing to the blue-shift (Henderson and Remington, 2005), the AmCyan1 still shares the conserved Tyr side chain in the chromophore-forming region with all GFPs in contrast to the

ECFP having Trp in the region. The importance of chromophore structure rather than its amino acid environments over the whole polypeptides for the photoconversion of fluorescent proteins has been reported (Kiseleva et al., 2008). In addition, one another possibility is the photoconversion-like effect (switching from cyan to green fluorescence). Although the mechanism behind the photoconversion of the AmCyan1 protein has not been empirically elucidated yet, the mutant CFP generated through the mutagenesis of Aequorea coerulescens GFP (by site-directed and then the random mutations) has been reported to be highly capable of photoconversion to a green fluorescent form in response to 405-nm UV light irradiation (termed photoswitchable CFP; PS-CFP) (Chudakov et al., 2004, 2010). The authors of the previous studies have suggested that the conversion process for PS-CFP is probably similar to that for wild type GFP including the decarboxylation of a certain key reside (i.e., Glu-222 in GFP) during red-shift photoconversion, leading to rearrangement of hydrogen-bonding network and chromophore deprotonation (see also van Thor et al., 2002). Our finding on the clear green-glowing phenotype of the AmCyan1-transgenic marine medaka in response to UV light irradiation might be, at least in part, in congruent with the previous observation on the attributes of the PS-CFP, although the initial color of our transgenics under daylight condition is already green-yellow. Further study should be extended in order to pinpoint effects of specific light wavelengths affecting the transgenic fluorescent phenotype in vivo in a finer manner possibly using LED-assisted fluorescent microscopy. Nevertheless, our data in this study strongly suggests that the ornamental characteristics of the AmCyan1-transgenic marine medaka could be differentially changed by the control of light irradiation sources, resulting in the fluorescence ranged from yellow green to turquoise/bluish color.

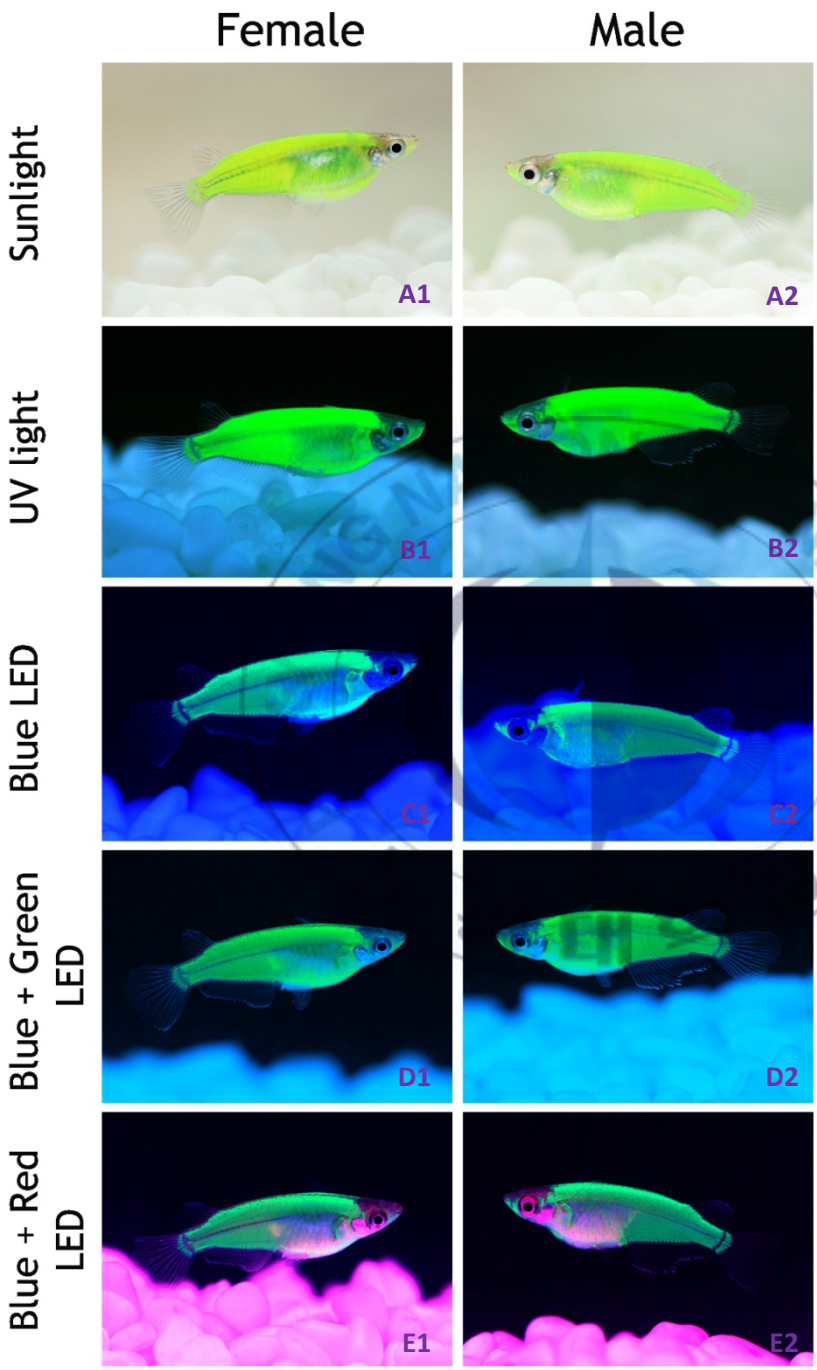


Figure 6. The phenotype characteristics of CFP transgenic marine medaka under different light irradiations. A1-A2; and B1-B2: CFP transgenic fish expressed under normal day light (sunlight) and UV light (352 nm)., respectively. Similarly, C1-C2; D1-D2; and E1-E2: CFP transgenic fish expressed under LED blue light (454 nm); Led blue (454 nm) and Green (517 nm) lights mixed, LED blue (454 nm) and red (628 nm) lights mixed, respectively.

4. Summary

Transgenic CFP marine medaka passed on successfully their fluorescent transgene into subsequent generations. Under different illumination sources, CFP transgenic fish exhibited distinctive characteristics in terms of fluorescent intensity and hue saturation of the color. Transgenics displayed the yellow-green color under normal daylight or white room light conditions, strongly green-glowing fluorescence under UV-light and cyan-like fluorescence under blue LED irradiations. Our results demonstrated that cyan fluorescent protein could be a useful candidate for ornamental applications in this species. Data from this study could be a useful basis to select both the fluorescent protein genes and aquarium light irradiation sources for further development of various versions of fluorescent, ornamental transgenic strains.

Chapter 3

Development of stable line and expression of green fluorescent protein in transgenic marine medaka (*Oryzias dancena*) by using a cytoskeletal *β-actin* promoter

1. Introduction

Transgenesis in small aquarium fish models can provide a number of advantageous merits as an experimental platform for variously theoretical and practical researches. Marine medaka (*O. dancena*) is one of a novel candidate that could be subjected to ornamental application by using a transgenesis of fluorescent protein genes. *O. dancena* is a euryhaline species possessing the great capability of hypo- and hyper-osmoregulation (Chen et al., 2009), such that they can survive, grow and reproduce in a wide range of salinities from complete freshwater to normal seawater (Kang et al., 2008; Song et al., 2009). Additionally, *O. dancena* exhibits a number of merits as a laboratory organism for transgenic experiments: small size; the easiness of laboratory rearing; the fairly transparent of the embryos or body color, which is advantageous for the in vivo visualization and imaging of fluorescently tagged proteins throughout nearly whole lifespan in a real-time fashion (Cho et al., 2011); many mutant collections and inbred strains are available; and year-round multiple spawning under controlled conditions with high fecundity and longer lifespan than medaka *O. latipes* (Song et al., 2009). Moreover, the development of pigment-free medaka by successive crossing of different color mutants enables us to investigate phenomena in living medaka using a suitable marker such as fluorescent protein (Wada et al., 1998). Based on those advantageous merits, the transgenic, marine medaka has not only useful for various experimental

transgenesis and heterologous expression assay particularly in the field of ecotoxicology and developmental biology but also great potential as a novel ornamental fish that could be displayed marine as well as freshwater aquarium.

Cytoskeletal *β-actin* is a structural gene with a role in supporting cell motility and structure, and has been also widely used as an internal control in gene expression studies due to a conserved housekeeping protein found in most animal cell types (Quitschke et al., 1989), because its expression is constitutively and ubiquitously high in all tissues (Lee, 2000). Furthermore, the *β-actin* promoter is powerful to drive ubiquitous expression of an ectopic gene in most cell types and useful basis for future application of living fluorescence color reporters to genomic and transgenic researches with the *O. dancena* species (Cho et al., 2011) or yellow catfish (Ge et al., 2012). Stable transgenesis using this promoter for driving the expression of desired transgenes continually from the embryonic development to adulthood is often valuable for tracing the cell lineage or cell migration in vivo, studying the mechanism of transgene silencing, visualizing the process of tissue regeneration, and identifying the sexual dimorphism or phenotypic sex in juveniles (Hsiao et al., 2001; Cho et al., 2011).

The living color, green fluorescent protein (GFP), was isolated from the jelly-fish Aqueous victoria, and have become a useful tool in investigation of various cellular processes (Blake et al., 2010). The GFP gene is also as a molecular marker for transgenic fish study was first reported in live zebrafish embryos by Amsterdam et al., (1995) and provides an excellent means for monitoring gene expression and protein localization in living organisms in transgenic medaka (Hamada et al., 1998). Following the initial this success, the GFP transgenic fish system is useful to investigate gene expression patterns and tissue/organ development, analysis of tissue-specific promoters/enhancers,

tracing cell lineage and migration, analysis of upstream regulatory genes, mutagenesis screening and characterization, cellular localization, chimeric embryos and nuclear transplantation, cell sorting, promoter/enhancer trap etc. (Gong et al., 2001). Moreover, GFP expression can be useful in generation of novel varieties of ornamental fish or bio-monitoring organisms for surveillance of environment pollution (Chou et al., 2001; Gong et al., 2001, 2003).

Based on a number of merits above and our long-time goals to develop an optimum combination between various fluorescent transgenic constructs, we evaluated the GFP as a genetically characteristic beside other used fluorescent markers such as CFP and RFP in our transgenic marine medaka. To evaluate the characteristic expression pattern green fluorescent protein reporter gene driven by *β-the* promoter in transgenic marine medaka, herein we report the various strain-dependent or sex-related patterns of green fluorescent signal during germ-line transmission from parental fish to their offspring.

2. Materials and Methods

2.1. Preparation of plasmid DNA for microinjection

The plasmid pod*β-actin*GFP was constructed by insertion of a 2.946-kb *O. dancena* beta-actin promoter into pEGFP-C1 (purchased from Clontech) in the front of the GFP reporter gene (10 mM Tris–Cl, 0.1 mM EDTA, pH 8.0) at the concentration 110 µg/ml.

Figure 7. Plasmid pod*β-actin*GFP constructs used for gene transfer into marine medaka *Oryzias dancena*. The pod*β-actin*GFP (7144 bp) expression vector was originated from pDsRed2-1 & pEGFP-C1 (Clontech, USA). *O. dancena β-actin* (expressed exclusively in fast skeletal muscles) promoter (2946 bp, gray) with *Kpn*I, *Age*I restriction sites were located in pDsRed 2-1 MCS. The DsRed2 fragment (803 bp) is changed EGFP fragment (911 bp) with *Age*I, *Mfe*I restriction sites.

2.2. Microinjection and embryonic development

Microinjection was carried out at the one-celled stage embryos. The DNA solution, the circular pod*β-actin*GFP, was injected into the interface between the yolk and blastodisc. The average amount of DNA introduced to each embryo was approximately 50 pg. After microinjection, the injected embryos were incubated at temperature 26-27^{o}C and salinity 5 ppt.

2.3. Germ-line test of transgenic transmission

Embryos injected with pod*β-actiin*GFP were raised to sexual maturity. To determine the inheritance of the transferred DNA fragments, the germ-line transmission was tested by crossing each adult GFP transgenic female with a wild-type male fish (1:1 mating). However, each cross GFP transgenic male with non-transgenic wild-type female was made by 1:4 mating. At least 150 offspring obtained from each pair were examined for GFP fluorescent expression. Fertilization, hatching rate and the incidence of GFP positive larvae (F1 and F2) were checked and scored under the fluorescence microscopy.

2.4. GFP signals observation

GFP signals in developing embryos, fries, and adults were monitored under the MetaVueTM Imaging System equipped in the AZ100 fluorescence microscopes (Nikon Corporation Instruments Company, Japan), and photographed using the digital camera (Nikon digital sight DS-Ri1). GFP expression was observed with the Nikon GFP–B-2A filter (Excitation Filter Wavelengths: 450-490 nanometers; Dichromatic Mirror Cut-on Wavelength: 500 nanometers; and Barrier Filter Wavelengths: 515 anometer cut-on).

3. Results

3.1. Transgenic expression of pod *β-actin*GFP injected embryos and larvae

To investigate the gene expression characteristics of *β-actin*, a 2.96-kb *β-actin* promoter region was isolated and characterized. A plasmid pod*β-actin*GFP circular form was injected into *O. dancena* embryos at one-celled stage, and GFP expression pattern was observed (Fig.8). At the results of microinjection, the viability of injected embryos was significantly lower than that of the controls. The mean percent hatching was 22.1%, which is about less than fourth of the average score that observed in the non-injected control groups (97.6%). After hatching, the larvae from the microinjected group experienced also the significantly higher incidence of abnormality (6.2%) than those from the non-injected control groups (1.3%). In a total, 687 larvae hatched out of 1602 embryos injected from nine microinjection trials. Approximately, 10.3% (71/687) of the GFP-positive fry (4.4% of 3106 injected embryos) showed uniform expression.

The microinjected embryos with the pod*β-actin*GFP circular construct appeared the successful expression of green fluorescence signals. Onset of GFP expression was detected from stage 18 (1 dpf, 2 somites) and it seemed difficult to detect or observe the incidence of GFP positive embryos from this stage due to GFP expression patterns was highly mosaic and extremely faint (Fig. 8A; B and C). However, the incidence of GFP positive embryo could be identified by observation of GFP signals in eye lens and in head regions (Fig. 8A; B), whereas GFP negative embryos showed GFP expression only in the yolk sac (Fig. 8A; C). Arrows in D, E and F indicated strong GFP expression in yolk sac of the larvae at stage 29 (0 dph), in the trunk and region at stage 30 (3dph, mean 4.55±0.23

mm in TL), respectively. In addition, the larvae displayed strong GFP expression in the entire trunk and head region from stage 29 (11 dpf), and showed highly mosaicism in this stage.

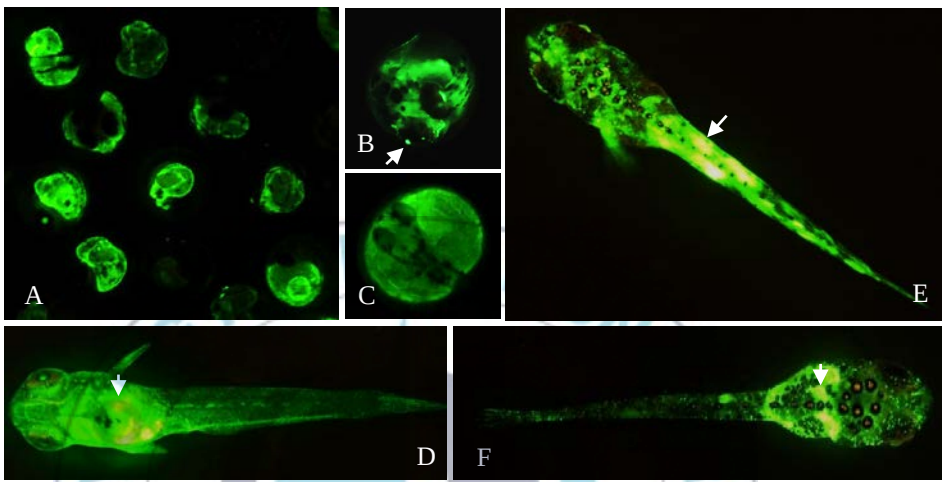


Figure 8. Cytoskeletal expression of GFP under the *β-actin* promoter in F_0 embryos and larvae. A: GFP strongly expression in yolk sac at stage 25 (4 dpf; -onset of retinal pigmentation stage). B and C: the incidence of GFP positive embryo was identified by observation of GFP signals in eye lens and head region, whereas GFP negative embryos showed GFP expression only in the yolk sac at stage 27 (six dpf, -formation of visceral blood vessels stages), respectively. D and E: Ventral and dorsal views of *β-actin*-GFP expression in the head and fin muscle at stage 29 (0 dph, newly hatched larvae), respectively. Arrows in D, E and F indicated strong GFP expression in yolk sac of the larvae at stage 29 (0 dph), in the trunk and region at stage 30 (3dph, mean 4.55±0.23 mm in TL), respectively.

3.2. Frequency of germ-line transmission of injected pod *β-actin*-GFP sequences

In total 71 GFP expressing fries, which have patched of GFP expression in fish body, were grown up to sexual maturity (6 months post hatching). Sixteen (8 females and 8 males each) out of the 71 presumed transgenic founders were examined the presence of strong GFP signal in their body surface to screen for germ-line transmitting founders (Fig. 9). All the founder transgenic fishes represented the chimerical distribution of green fluorescence color in their body, indicating that they were mosaic for the transgenic status. Among 16 founders fish that were screened by crossing with wild–type fish, thirteen (81.2%) of them were successfully produced GFP expressing offspring. However, the patterns of transgene transmission, hatched, fertilized rates were differences between two lines of *O. dancena* female (ODF) and male (ODM) founders. The frequency of germ-line transmission in the ODF, 32%, was nearly double higher than the 16.8% obtained in the ODM. The transmission rates vary from 2.4% to 56.6% among the ODF transgenic lines, and from 10.7% to 72.7% for the ODM transgenic lines, indicating mosaicism of germ cells in the founders. In addition, there was a noticeable here was that the mean of fertilized rates (97.1%) and hatched rates (92.9%) in the ODF was significantly higher than ODM's (81.1.4% and 83.85%, respectively). The fertilized, hatched rates; maternal expression and the frequency of appearance GFP of pod*β-actin*GFP in positive F1 generation marine medaka *O. dancena* fries is summarized in Table 2.

Table 2. Fertilized, hatched rates; maternal expression and incidence of GFP of circular pod*β-actin*GFP in positive F1 generation marine medaka *O. dancena* fries

Group	Founder fish lines	Fertilized rates (%)		Hatched rates (%)		Maternal expression (%)		Incidence of GFP positive fries (%)	
Female (ODF)	C1-FM	99.2	97.1% (814/837)	97.6	92.9% (756/814)	30.3	24.7% (187/756)	56.6	32% (242/756)
	C2-FM	95.0		100		49.1		47.4	
	C3-FM	98.1		95.5		37.8		36.5	
	C4-FM	96.2		97.0		16.5		35.1	
	C5-FM	97.8		97.1		15.2		22.0	
	C6-FM	95.8		91.3		0		4.8	
	C7-FM	95.6		97.7		2.4		2.4	
	C8-FM	96.7		67.8		1.3		0	
Male (ODM)	C 9-M	38.1	81.1% (1448/1786)	57.1	83.8% (1214/1448)	0.0	0	72.7	16.8% (204/1214)
	C10-M	71.0		56.8		0.0		29.3	
	C11-M	68.0		76.4		0.0		22.6	
	C12-M	98.8		93.4		0.0		22.5	
	C13-M	94.6		96.1		0.0		14.8	
	C14-M	98.6		88.4		0.0		10.7	
	C15-M	73.3		84.1		0.0		0.0	
	C16-M	85.9		82.5		0.0		0.0	

Sixteen (8 females and 8 males each) out of the 71 presumed transgenic founders were examined the presence of strong GFP signal in their body surface to generate stable transgenic lines. For each transgenic line, at least 100 embryos obtained from more than ten embryo batches were examined.

3.3. Transmission pattern of green fluorescent transgene from F1 to F2 offspring

Five lines out of the sixteen presumed transgenic F1 generation was selected to verify the germ-line transmission in F2 generation. The GFP in F1 transgenic individuals were successfully transmitted to F2 offspring (Table 3). Generally, the incidence of GFP positive progeny in F2 generation was clearly in accordance with an expected Mendenlian ratio (50%). However, the incidence of GFP positive larvae from two lines of the female groups was less than 50%. The percent incidence of GFP positive F2 larvae were 30.9% and 41.8% for transgenic C4-FM and C12-M, respectively. In addition, the patterns of hatched, fertilized rates were differences between two sex groups of F1 transgenic lines. The mean of fertilized rates was not a difference between two sex groups. The mean of fertilized rates was from 79.2% to 100% for female groups, and from 86.4% to 100% for male groups. On the other hand, the mean of hatched rates in the female groups (69.3%-96.9%) was significantly higher than in the male group (44.2%-66.5%) of F1 transgenic lines.

Table 3. Mean of fertilized, hatched rates; maternal expression and incidence of GFP of circular pod*β-actin*GFP in positive F2 generation marine medaka *O. dancena* fries

Transgenic lines (F1)	Sex groups	Fertilized rates (%)	Hatched rates (%)	Maternal expression (%)	GFP positive fries (%)
C4-FM	Female	79.2±20.3	69.3±6.3	8.4±4.7	30.9±15.8
C5-FM		100±0.0	96.9±2.1	45.2±2.3	48.4±1.7
C12-M		98.3±1.7	70.9±13.7	8.5±3.9	41.8±14.5
C13-M		98.4±1.6	95.9±2.2	7.6±1.5	53.6±6.4
C16-M		100±0.0	94.4±1.0	3.4±1.8	50.8±12.5
C4-FM	Male	99.7±0.3	44.2±9.5	0	45.8±4.6
C5-FM		98.7±1.3	57.9±20.2	0	49.3±1.2
C12-M		99.8±0.3	66.2±6.6	0	53.3±8.6
C13-M		86.4±12.7	59.8±9.5	0	51.8±1.6
C16-M		100±0.0	66.5±4.6	0	52.4±2.1

Five lines out of the sixteen presumed transgenic F1 generation was selected to verify the germ-line transmission in F2 generation. For each F1 transgenic line, six (three females and three males) transgenic individuals were participants for verification of germ-line transmission at F2 generation, and at least 600 embryos (100 embryos/individual) obtained from each line of strain were examined. The incidence of GFP positive offspring was estimated as the percentage of glowers out of total No. of incidence of GFP positive/hatched embryos.

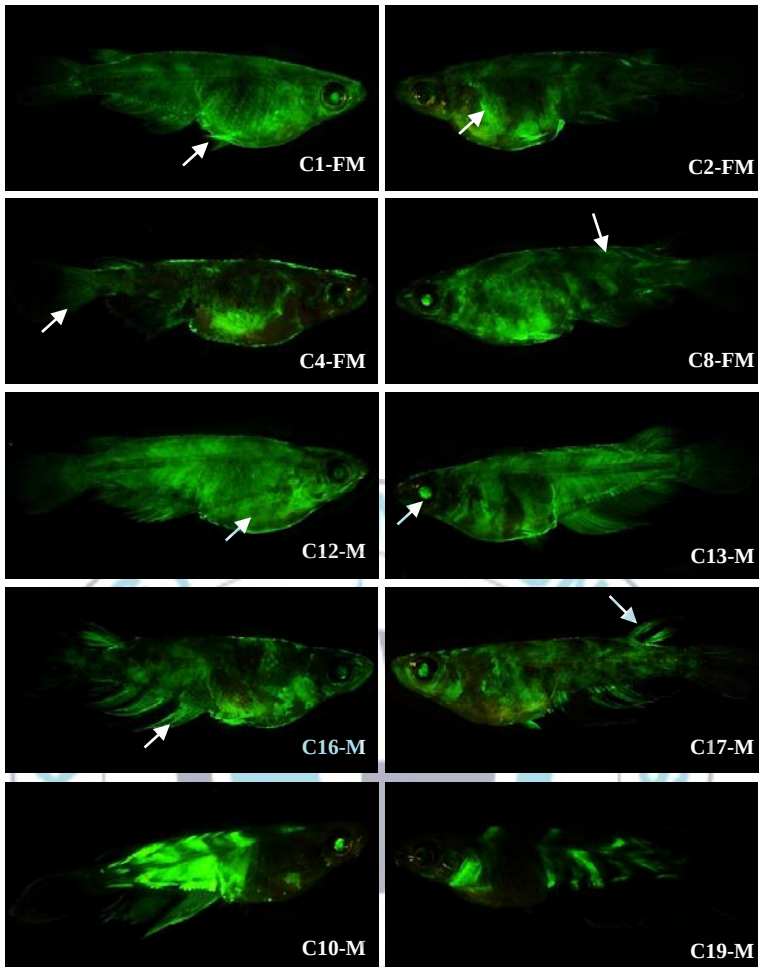


Figure 9. The circular pod*β-actin*GFP transgenic marine medaka *O. dacena* at 6 month post hatching. Lines C1-FM; C2-FM; C4-FM; C8-FM; C12-M; C13-M; C16-M; and C17-M shown are homozygous. Line C10-M and C19-M shown strong GFP expression in nearly all body surface throughout the trunk, opercle and eye, but they were the failure of generating germ-line transmission. Arrows in these transgenic founders: C1-FM; C2-FM; C4-FM; C8-FM; C12-M; C13-M; C16-M; and C17-M indicated green fluorescent protein (GFP) expression in pelvic fin; opercle; caudal fin; trunk region; belly; eye; anal fin; and adipose fin, respectively.

3.4. Expression of pod*β-actin*GFP fusion protein to F1 and F2 offspring

Expression of green fluorescence in F1 embryo and larvae was different from that observed in F0's. Moreover, the initiation timing of the transgene expression in F1 embryos was difference between the two groups of female (ODF) and male (ODM) founders (Fig. 10). In ODF group, the strong GFP expression was first observed at 1 cell-stage (formation of blastodisc) (Fig. 10a), and then they keep retaining the level of expression to pre-hatching and the juvenile stage (21dph). On the other hand, in ODM group, GFP expression was rarely first observed in pre-mid-gastrula at stage 13 (14 hpf). The expression level was very low at the beginning (Fig. 10c1), but gradually increased as embryonic development proceeded (Fig. 10d1, e1). However, in the both lines, GFP fluorescence in transgenic *O. dancena* embryos was gradually expanded to all somite and became more intensified by stage 28; 29; and 31. In addition, at 3 months post hatching of transgenic F1 and F2, GFP signals exhibited very strong whole body include head, trunk, region, eye, and fins of transgenic fish. However, an interesting difference of two groups was maternal protein of GFP, which was detected in the yolk of embryo (Fig. 10a-e) and pre-hatching larvae (Fig. 10f1) derived from transgenic females ODF, but not from transgenic males ODM (Fig. 10e1; f2). Arrows in Fig10f, f2 indicated strong GFP expression in the nearly whole body of the larvae at stage 29 (0 dph).

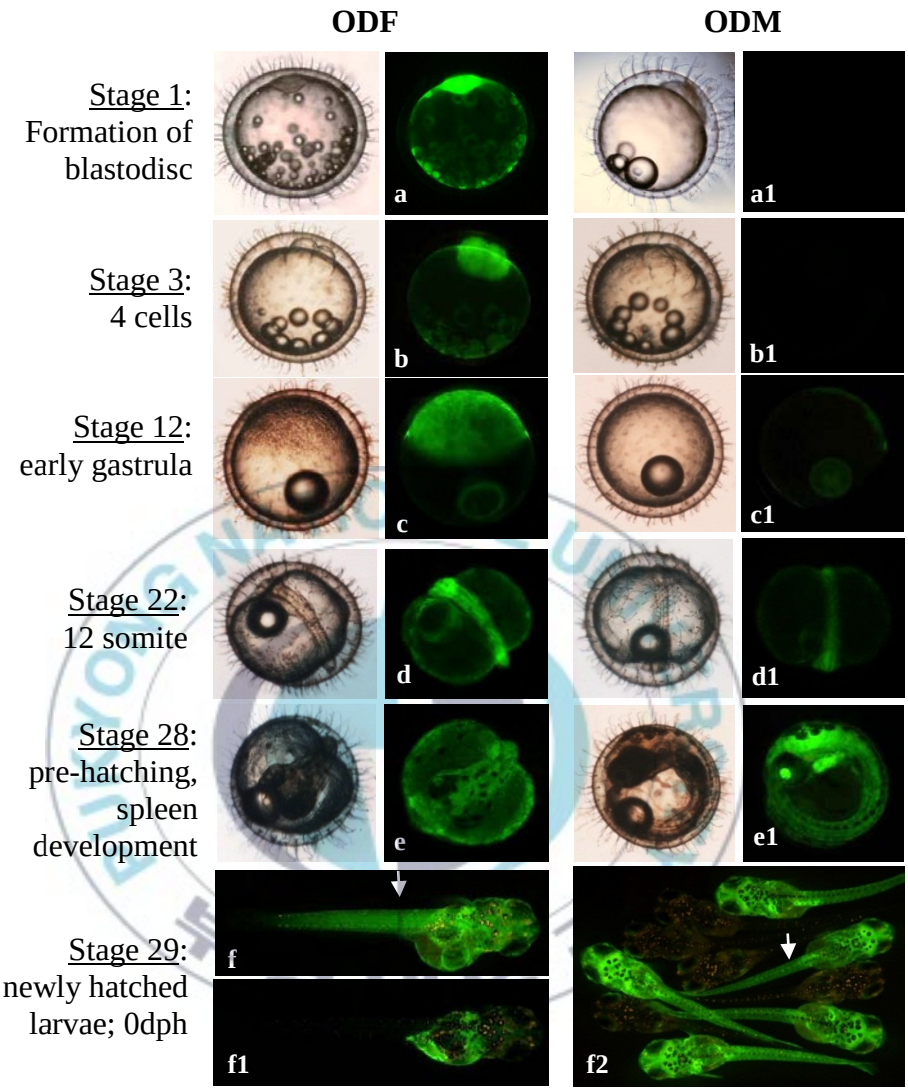


Figure 10. Expression of the green fluorescence GFP in F1 embryos and larvae. Different initiation timing of the transgene expression in F1 embryos was difference between the two groups of male and female founders. In ODF group, the strong expression of GFP signals was detected from stage 1 - formation of blastodisc; they retain the level of expression to pre-hatching and the juvenile stage (21dph). On the other hand, in ODM group, the starting point of a weakly expressed from stage 12 (early gastrula), they gradually increased the level of

expression to stage 22 (12 somite), and their faithful GFP expression was the same with ODM group at stage 28 (pre-hatching, spleen development), and stage 29 (newly hatched larvae; 0dph)

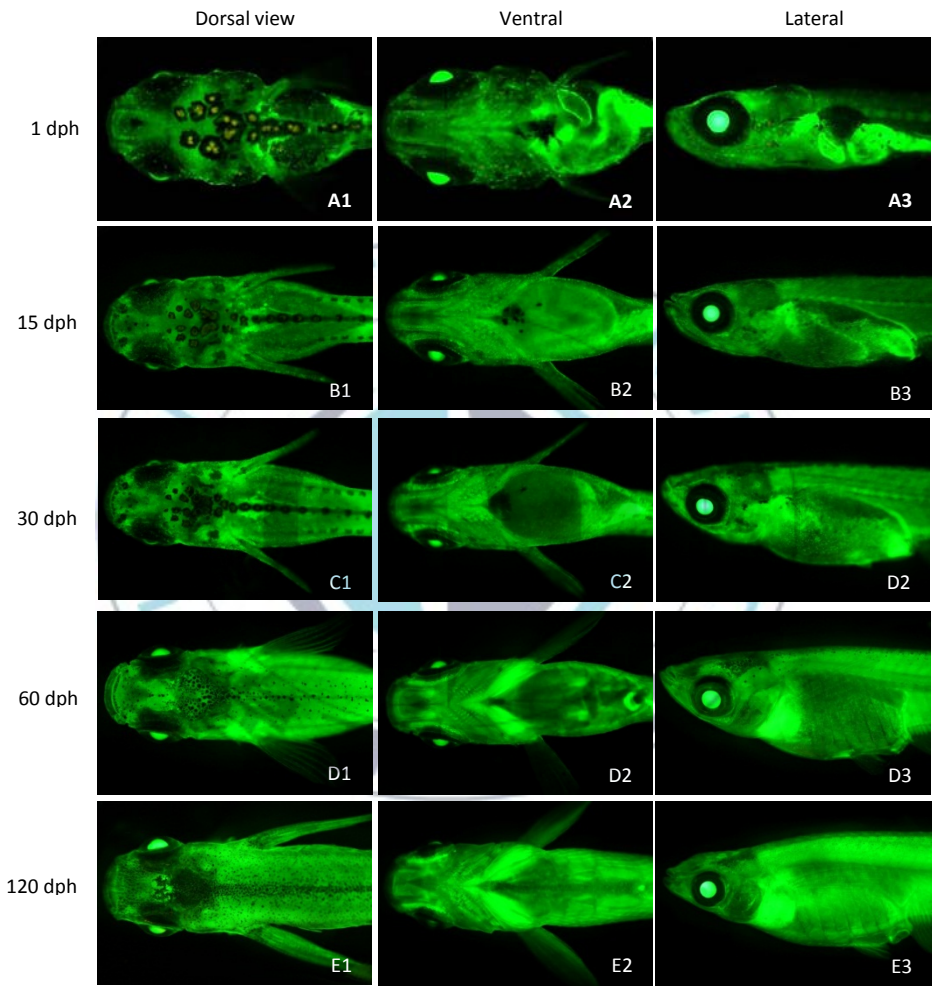


Figure 11. GFP expression in head regions under the control of β-actin promoter from 1 to 120 day(s) post hatching in F2 transgenic marine medaka. A1-E1; A2-E2; A3-E3 were order GFP expression under dorsal, ventral and lateral view, respectively.

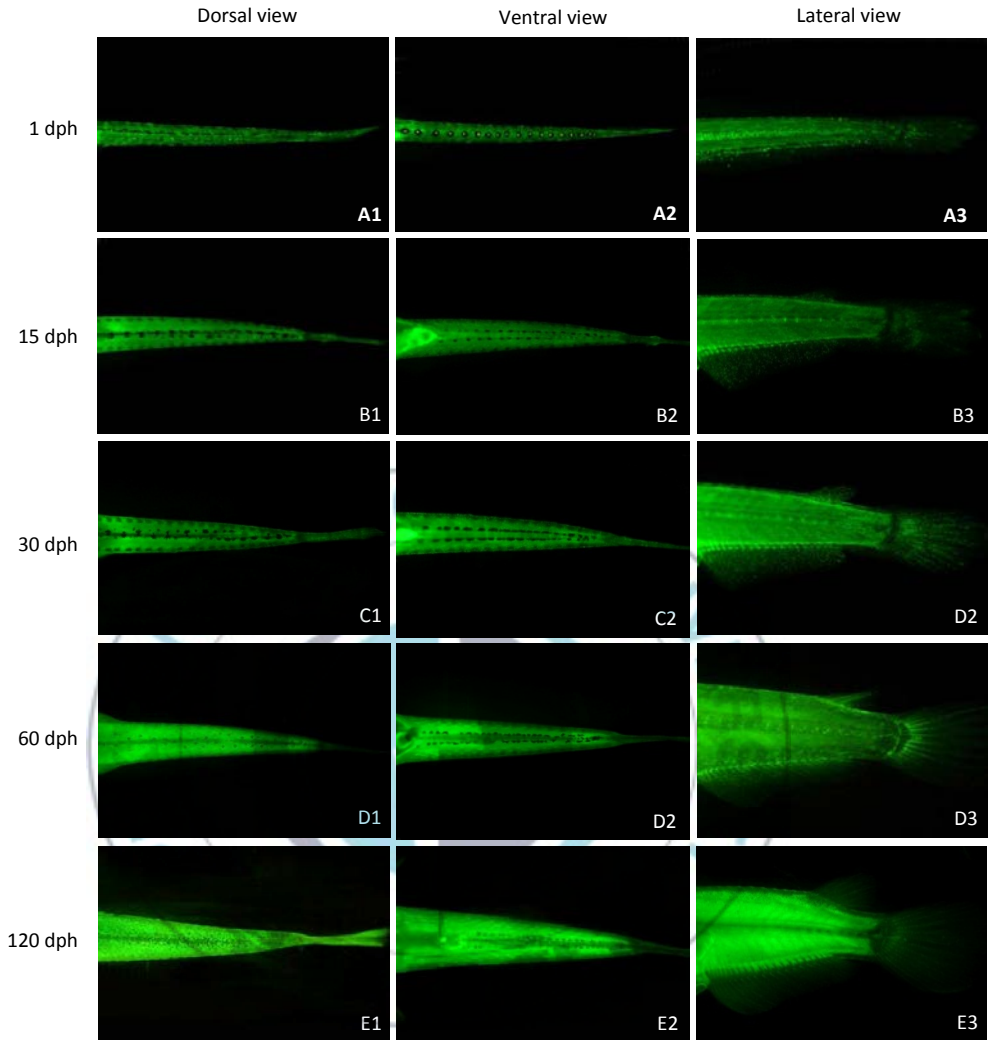


Figure 12. GFP expression in trunk regions under the control of *β-actin* promoter from 1 to 120 day(s) post hatching in F2 transgenic marine medaka. A1-E1; A2-E2; A3-E3 were order GFP expression under dorsal, ventral and lateral view, respectively.

To distinguish the difference between maternal protein and transgenic fish, the expression of GFP in the eye of embryo stages and eye or trunk region of the larvae stages were focused (Fig 13C; D; and E). At the embryo stages, the transgenic embryos expressed in whole embryos including eye lens, yolk sac and head region, whereas the maternal protein embryo expressions were only observed in the yolk sac (Fig. 13C). Similarly, the transgenic fish were selected by relying on the expression of GFP in the fish-eye lens and trunk region (Fig. 13E; F1 and G1), while GFP expression with maternal proteins was distinguished by observation of GFP expression only in the yolk sac (Fig. 13C). On the contrast, in transgenic ODM group, GFP signal expressions were only detected in the gastrula stage (Fig. 10c1) and trunk region (Fig. 10d1, e1, and f2). However, the maternal protein ratios were not similar between different lines. The maternal protein rates vary from 1.3% to 49.1% in F1 and 3.4%-45.2% in F2 generation among the ODF transgenic lines, and 0% for the ODM transgenic lines. The maternal protein expression would be disappearing when the yolk was absorbed about 5 or 10-day post hatching.

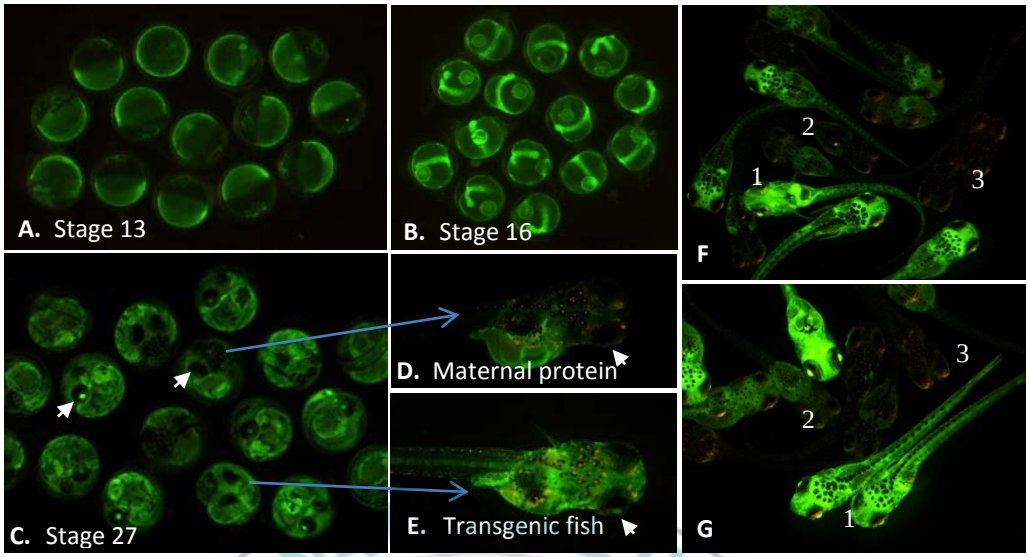


Figure 13. Maternal protein and GFP expression of ODF group F1 and F2 generation of embryos/larvae. A: 100% GFP signals expressed in F1 embryos at stage 13 (pre-mid-gastrula). B: 100% GFP signals expressed in F1 embryos at stage 16 (early neurula). C: embryos showed 100% GFP signals but only 47.4% embryo was the incidence of GFP positive embryos; the 49.1% remaining embryos were maternal protein. D and E: zoom out from the embryos which were showed maternal and transgenic fish, respectively. F and G showed the GFP signals of transgenic (1-expression in whole body), maternal protein (2-expression only in yolk sac) and non-transgenic (3-non-expression) fish expression at 1 day post hatching in F2 generation. Arrow in C; D; and E showed the expression of GFP in eye lens was the transgenic embryo or larvae, and non–expression of GFP in eye lens were maternal protein expression fish.

4. Discussion

4.1. Microinjection and frequency of germ-line transmission

In these studies, we have successfully generated a high frequency of germ-line transmissions of injected pod*β-actin*GFP. Interestingly, green fluorescence was observed in most transgenic lines in which the plasmid integrated stably into the fish genome and was inherited through the germ-line. Thirteen germ-line transmissions out of 16 founders, a success rate approximately 81.25%. The inherited plasmid DNA sequences are integrated into host chromosomes, by then they are passed to the F2 generation with Mendelian fashion (Fig. 13).

The explanation is the lower fertilizing ability of the transgenic sperm than non-transgenic sperm, in which the transgenic sperm might be less competitive than non-transgenic sperm in the fertilization event (Cho et al., 2011). When compares to previous studies of GFP injections into embryos of zebrafish (Ju et al., 2003), the viability of the microinjected marine medaka *O. dancena* embryos (22.1% survival rates) was significantly lower than that of zebrafish injected embryo (57%). This can be interpreted as the medaka blastodisc diameter and embryo is smaller than that zebrafish's (Wang et al., 2007). Thus, injecting through the medaka embryo chorion, the cell membrane and the yolk sac will be largely damages.

We have successfully generated high frequency germ-line transmission of injected circular pod*β-actin*-GFP sequence. The result of frequency of germ-line transmission in the ODF, 32%, was nearly double higher than the 16.8% obtained in the ODM. It was found that the greatest proportion was the C1-FM line, which made up 56.6% of incidence of GFP positive fry found in ODF group. Whereas the fertilization rate (38.1%), hatching rate (57.1%) of the C9-M

line in ODM group was not higher than the other lines, but the frequency of the highest germ-line transmission was up to 72.7% indicated that this transgenic fish was not mosaic expression. Despite the same experimental conditions, our results were in stark contrast with Cho et al., 2011 that by using the linearize pod*β-actin*RFP, the female founder passed on the fluorescent transgene to its offspring at quite a low frequency of 2.3% on average, while a male founder passed on the transgene at a rate of 13.3% of offspring. Ju et al., 2003 reported that the GFP transmission rate to F1 was approximately 10% of using the linearize *mlc2*-GFP in transient transgenic zebrafish founders. This suggests the use of genetic structure also affects the frequency of germ-line transmission (Penman et al., 1990; Haobin et al., 2000; Ekici et al., 2010).

4.2. Mosaic founders and GFP expression in F1 generation

The observation of the founder transgenic fish in our marine medaka experiments was highly mosaic due to result from integration of the transgene copies at some time after cleavage of the fertilized eggs (Alam et al., 1996) and common phenomenon in transgenic founders generated by cytoplasmic microinjection (Nam et al., 1999, 2001). This mosaicism results in scattered expression patterns of integrated reporter genes even when driven by ubiquitous promoters, frequent lack of transgenic status in germ line tissues, and even if such tissues are transgenic, non-uniform transgenic status of the gametes (Tsai, 2008).

Interestingly, green fluorescence was observed in most transgenic line in which the plasmid integrated stably into the fish genome and was inherited through the germ-line. However, expression of the green fluorescence in F1 embryos was different from that observed in F0 embryos. Moreover, the

initiation timing of the transgene expression in F1 embryos was also different between the two F1 groups of ODF and ODM. In the ODF F1 embryos, the strong expression began at the one-cell stage. The green fluorescence signals were uniformly distributed in the whole embryonic body throughout the embryonic development. Whereas the GFP expression in the F1 embryos belonging to ODM group was not detectable until pre-mid-gastrula at stage 13 (14hpf), and the green fluorescence signals in these embryos showing the levels of expression were much weaker than those observed in ODM F1 embryos. However, the same model *O. dancena* studying, by using pod*β-atc*RFP construct, the strong RFP expression began at early cleavages (stage 16, 24 hpf) and the RFP expression in the F1 embryos was not detectable until early neurula stage in the male lines F1 embryos (Cho et al., 2011). While Zeng et al., 2005 tested the zebrafish *mylz2* promoter in medaka embryos, linearized pMYLZ2-EGFP containing 1.9-kb of *mylz2* promoter was injected into medaka embryos at the stage of one to two cells, and in transgenic embryos, initial GFP fluorescence was detected in the first few anterior somites at stage 27 (58 hpf), when 24 somites were formed. In terms of muscle development, this stage is approximately the same stage when *mylz2: gfp* zebrafish embryos were first detected for GFP expression (20 hpf or 22-somite stage) (Ju et al., 2003). This unexpected difference haven't been yet clarified the mechanism behind in the onset timing of transgene expression between the two transgenic lines, although in general, the transgenic strain-specific pattern of transgene expression could result from different profiles of transgene integration (Nam et al., 1999). In addition, Cho et al., 2011 also explained that the female founder may contain a certain portion of un-integrated transgene copies in its gametes and the extrachromosomal copies have influenced the expression pattern of the transgene in the resultant embryos of this transgenic line. Relatively long lasting,

extrachromosomal transgene copies have already been reported previously in other transgenic lines.

One plausible, that this has been verified in our experiments, GFP signal was first detected at one cell-stage maybe due to maternal protein. The observed fluorescence in these embryos is highly mosaic, presumably due to unequal distribution of the injected DNA during the embryonic cell divisions. The observation of fluorescence so soon after the beginning of zygotic transcription indicates that the posttranslational modifications required making the fluorophore in GFP can take place in cells in the early embryo (Amsterdam et al., 1995). According to our results, the C2-FM line of ODF group embryos showed 100% GFP signals for each round of reproduction absolutely. The GFP expression level was highest at stage 16 (1dpf), then it decreased gradually until stage 27 (6dpf). In this stage, we can distinguish between maternal protein and incidence of GFP positive embryos that were shown in Fig. 13C; D and E. On the contrary, maternal protein had not occurred in ODM group. Previously, by using the linearized pod*mlc2*-GFP and pod*mlc2*-CFP, or circular of pod*α-actin*RFP we never observed maternal storage of GFP in the yolk (data not shown).

4.3. Contribution for ornamental fish development program

Stably fluorescent transgenic lines can be developed by breeding a GFP transgenic individual with a wild type fish or another transgenic fish for ornamental fish in the market (Gong et al., 2008). Moreover, the multiple color fluorescent marine medaka transgenic fish carrying different promoter have been generated by the same technique as blue fluorescent protein (BFP); yellow fluorescent protein (YFP); red fluorescent protein (RFP) and cyan fluorescent

protein (CFP) gene with myosin light chain 2, alpha actin or beta actin promoter (data not shown). Thus, a transgenic fish with GFP under an eye-specific promoter, BFP under a skin-specific promoter, and CFP/RFP under a muscle-specific promoter will show the following multiple fluorescent colors: green eyes, blue skin and cyan/red muscle. In addition, by inter-strain cross breeding between two transgenic line expression different colors with each other or by recombining different tissue specific promoters or expression of more than two different fluorescent proteins in the same tissue and fluorescent protein genes, more varieties of transgenic fish of different fluorescent color patterns will be generated.

5. Summary

In the present study, transgenic fish production technology by using circular pod*β-actin*GFP plasmid was established at the first time in marine medaka *O. dancena*. The technique significantly increases the frequency of positive founder fish in F0, thus improving transgenesis frequency. In addition, transmission pattern of transgene was stabilized in the pass-on from F1 to F2 in most transgenic strains examined, wherein almost transgenic families revealed the transmission pattern following the Mendenlian single gene inheritance, suggesting the integration of transgene copies into a closely related chromosomal site(s) in the marine medaka genome. On the other hand, a few transgenic strains represented the frequency that was not in accordance with Mendelian single gene inheritance pattern, including significantly lower or higher incidences of fluorescent signals in developing embryos. Further, several transgenic females would transfer the maternally expressed fluorescent protein into their gametes regardless the transgene presence in the gametes, and the fluorescent signals were fairly persistent during development of resultant offspring embryos.

Chapter 4

Characteristics of red fluorescent protein harboring the alpha skeletal actin promoter in transgenic marine medaka

1. Introduction

Actin is a cytoskeletal protein that is ubiquitously expressed as different isoforms (Vandekerckhove et al., 1986). Actin plays many importance roles in numerous cellular functions in muscle and non-muscle cells (Herman, 1993), in maintaining the cytoskeletal structure, cell motility, cell division, intracellular movements and contractile processes (Morita, 2000), part of the specialized contractile apparatus of skeletal muscle cells in higher vertebrates (Bulinski et al., 1983), modulation of a variety of membrane responses, translation of several mRNA species and modulation of enzyme activity and localization within the cell (Bertola et al., 2008). There are six primary actin isoforms have been identified in higher vertebrates, including α-skeletal, α-cardiac, α-smooth muscle, γ-smooth muscle, β-cytoplasmic and γ-cytoplasmic isoactin (Vandekerckhove and Weber, 1978). Although the actin gene family is expressed in all tissues, individual genes show tissue and developmental specificity in their expression (Gómez-Chiarri et al., 1999). For example, α-skeletal actin becomes the dominant isoform in the adult tissue, accounting for more than 95% of the total striated muscle actin isoforms (Gunning et al., 1983; Bertola et al., 2008), which was demonstrated the actin expressed in adult human muscle and 50% of the total actin mRNA in adult human heart muscle (Gunning et al., 1983), rat heart (Mayer et al., 1984; Schwartz et al., 1986), salamander *Pleurodeles waltlii* (Khrestchatisky and Fontes, 1987) muscle, in skeletal muscle and in the heart of

all five species (mouse, chicken, snake, salamander fish) and also many novel non-muscular expression domains including several in the central nervous system (Bertola et al., 2008).

Since, skeletal α-actin become an idea to study their characteristic in transgenic fish including zebrafish (Higashijima et al., 1997; Hsiao et al., 2001; Bertola et al., 2008), Japanese medaka *O. latipes* (Chou et al., 2001), and two species of rattail fish, *Coryphaenoides acrolepis* and *C. cinereus* (Morita, 2000). However, the results of these works described only in embryos and early larvae development (Higashijima et al., 1997; Chou et al., 2001; Bertola et al., 2008), but ontogenic profiling in transgenic fish has not been yet established.

Marine medaka (*Oryzias dancena*) is a euryhaline species possessing a number of advantageous merits for ecotoxicogenomic and transgenic researches (Chen et al., 2011). Based on our aim to develop a qualified means to visualize the regulatory patterns of α-actin gene, especially their ontogenic expression, we established the stable transgenic marine medaka germlines carrying RFP reporter driven by skeletal α-actin promoter. Herein, we present the expression characteristics of the transgenic RFP under the control by α-actin promoter during the early life of transgenic fish.

2. Materials and Methods

2.1. Preparation of plasmid DNA and microinjection

The plasmid pod*α-actin*RFP was constructed by insertion of a 2.466-kb *Oryzias dancena* skeletal *α-actin* promoter in the front of the pDsRed2-1 reporter gene (purchased from Clontech) (10 mM Tris–Cl, 0.1 mM EDTA, pH 8.0) at the concentration 110 µg/ml.

Microinjection was carried out at the one-celled stage embryos. The DNA solution, the circular pod*α-actin*RFP, was injected into the interface between the yolk and blastodisc. The average amount of DNA introduced to each embryo was 50 pg based on our preliminary experiments to optimize microinjection procedure with this species.

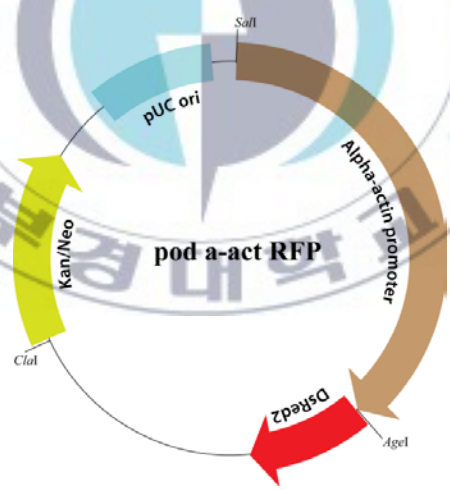


Figure 14. Plasmid, pod*α-actin*RFP constructs, used for gene transfer into marine medaka *Oryzias dancena*. The pod*α-actin*RFP (6551 bp) expression vector was originated from pDsRed2-1. *Oryzias dancena* skeletal *α-actin* (expressed exclusively in skeletal muscles) promoter (2466 bp, gray) with *Sal*I, *Age*I restriction sites were located in pDsRed 2-1 MCS.

2.2. Detection of skeletal *α-actinRFP transgenic fish*

By fluorescent microscopes

RFP signals and ontogenic expression in developing embryos were monitored under the MetaVueTM Imaging System equipped in the AZ100 fluorescence microscopes (Nikon Corporation Instruments Company, Japan), and photographed using the digital camera (Nikon digital sight DS-Ri1). RFP expression was observed with the Nikon TXRed filter (Excitation Filter Wavelengths: 540-580 nanometers; Dichromatic Mirror Cut-on Wavelength: 595 nanometers; Barrier Filter Wavelengths: 600-660 nanometers cut-on). The images of transgenic fish were observed in different direction include the ventral, dorsal, and lateral views.

By PCR analyses

Polymerase chain reaction (PCR) was used to identify individuals carrying the pod*α-actin*RFP transgene. Genomic DNA template was prepared from the caudal fin. PCR amplified with *AccuPower* HF PCR Premix (Bioneer, Daejon, Korea). The PCR amplification used primer pair: OD alpha-*actin* 1F (5'-GTTCTCTCCGAGACGCAG-3') and DsRed2-1R (5'-CATGGTCTTCTTCTGCATCA-3'). The PCR condition was one cycle of pre-denaturation at 94°C for 2 min, 30 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, elongation at 72°C for 30 s. The amplified products were separated on a 1.5 % agarose gel electrophoresis and visualized by ethidium bromide staining.

2.3. Verification of germ-line transmission

Embryos injected with pod*α-actinR*FP were raised to sexual maturity. To determine the inheritance of the transferred DNA fragments, the germ-line

transmission was tested by crossing each adult RFP transgenic female with a wild-type male fish (1:1 mating). However, each cross RFP transgenic male with non-transgenic wild-type female was made by 1:4 mating. At least 150 offspring obtained from each pair were examined for RFP fluorescent expression. Fertilization, hatching rate and the incidence of RFP positive larvae in F1 were checked and scored under the fluorescence microscopy.

3. Results and Discussion

3.1. Expression of *α-actin*RFP in injected embryos

To generate transgenic marine medaka *O. dancena* expressed red fluorescent protein (RFP) harboring the skeletal *α-actin* promoter, up to 1355 embryos were injected. However, only 578 eggs (42.6%) were hatched successfully. After hatching, the larvae would be examined under fluorescent microscopy to identify individuals with the expression of RFP. Consequently, 73/578 (12.63%) larvae were transmitted successfully. Nevertheless, only 35 of these larvae exhibited a strong and faithful expression of RFP, as to be indistinguishable to the naked eye. In contrast, the number of these remaining fish, they rather represented moderate or dark dim fluorescent signals that could not be observed under normal day light conditions.

A characteristic of *α-actin*RFP expression in injected embryos was described in Fig. 15. The initial RFP expressions of the injected embryos were detected in stage 26 (four days after injected; gill blood vessel of information stage), that were strongly expressed in the trunk muscle from the hindbrain regions stretching down to the tail of the fish (Fig. 15A1-A3). The micro-injected embryos would be hatched about 10-12 days. At 1 dph, the larvae represented a powerful expression in the trunk region (Fig. 15B2). The expression of RFP also increased steadily as to be visible in white light with the naked eye or clearly in a period for 10 or 30 days of age (Fig. 15C1; D1). In addition, they were expressed in the pectoral and pelvic fin which was shown by two arrows in Fig. 15B2 and C2, respectively.

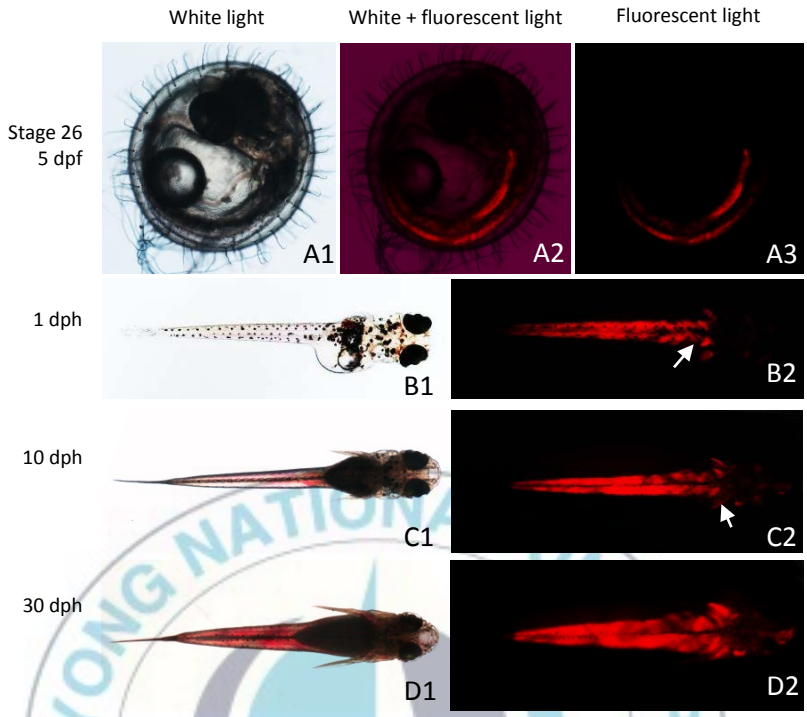


Figure 15. Expressions of RFP transgene construct of marine medaka skeletal α-actin promoter in injected embryos and larvae. A1, A2, A3: onset RFP expression in trunk region at stage 26 (Gill blood vessel information stage, 4 days 20 hours after fertilization) was checked under white light (A1) or white light combined with fluorescent light (A2) and only fluorescent light (A3). B1; C1; and D1: RFP expression under white light at 1; 10 and 30 days post hatching, respectively. B2; C2; and D2: RFP expression under fluorescent light at 1; 10 and 30 days post hatching, respectively. Arrow in B2 and C2 showed the RFP expression in pectoral and pelvic fins, respectively.

Aims to further understanding of the external phenotypic characteristic's expression of skeletal muscle α-actin in the incidence of RFP positive founders, whether or not related to the transmission in next generation, ten founders that completed through the verification of germ-line transmission would be fully checked by the parties on both sides of the fish under a fluorescent microscope (Fig. 16). Normally, the transgenic founders had a very strong and faithfull expression in a whole body of the fish (Fig. 16A1- E2). On the hand, in non-transgenic founders groups which have failed the germ-line transmission to the next generation, RFP signals were the partial body expressions (Fig. 16F1; G1; I1, and K1). However, there were exceptions such as in line α-C9, the RFP signals were weaker than other lines in the same group, but the frequency germ-line transmission of RFP harboring the skeletal muscle a-actin promoter was highest in transgenic marine medaka. In the contrast, although the RFP signals in line α-C7 of the non-transgenic group (Fig. 16H1; H2) showed powerful expression in the whole body, the fish failed germ-line transmitted for next generation. Remarkably, in both groups, the RFP fish represented a disproportionate expression in the front- or back-side of the same fish that they were observed from the lateral view. For example, as the line α-C2, in the front-side, the transgenic fish expressed RFP in the trunk and abdominal muscles' region (Fig. 16A1). However, in the back-side of the fish, the RFP signals were not detected in the abdominal area (Fig. 16A2). Similarly, in the front-side of a line α-C11 of the non-transgenic founder group, the RFP expression was observed in the gill, but not in the back-side (Fig. 16K1; K2). Therefore, all of these founders indicated that they were highly mosaic.

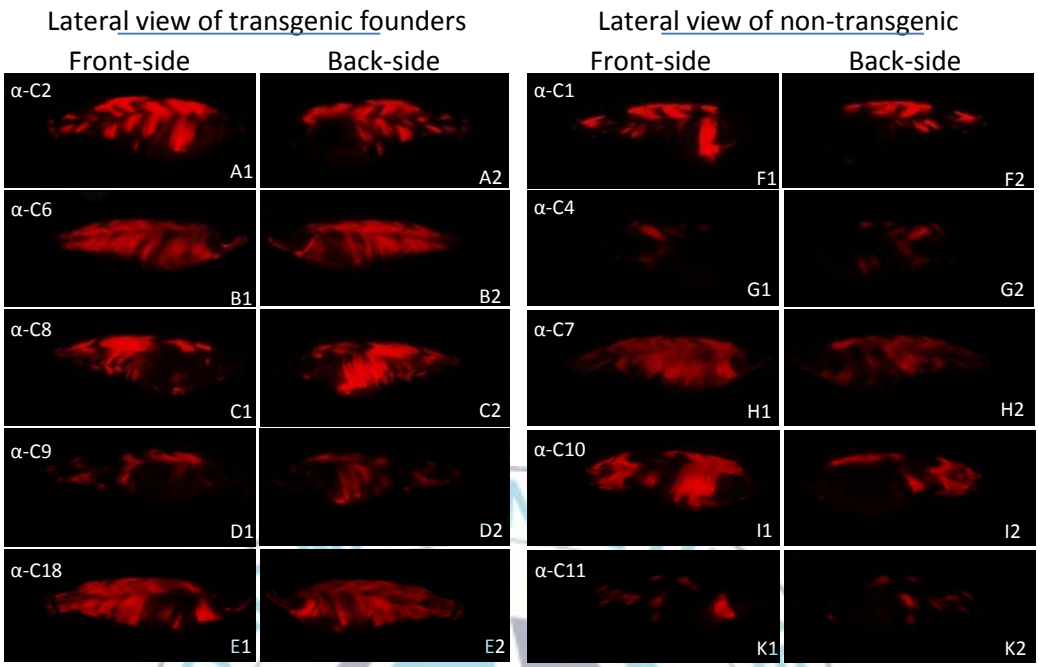


Figure 16. The external phenotype of pod*α-actin*RFP expression in transgenic and non-transgenic marine medaka founders (6 months of age). In lateral view of transgenic founder groups (A1-E1), each individual exhibited the faithful expression in the whole body of the fish, whereas they are only expressed on whether parts or whole body of the non-transgenic founder groups (F1-K1). However, in both cases, the red fluorescent fishes represented a disproportionate expression in the front- or back-side of the same fish that they were observed from the lateral view. Although F1; H1; and K1 showed strong expression and apparently the whole body of fish, they failed to transmit the germ-line to the next generation. On the other hand, D1 displayed weak expression, but frequency germ-line transmission of RFP in transgenic marine medaka *O. dancena* under the skeletal *α-actin*RFP promoter is highest in the transgenic founder groups.

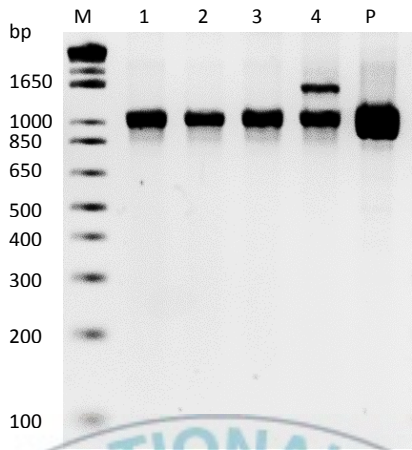


Figure 17. Polymerase chain reaction (PCR) detection of the transgene in marine medaka founder. PCR using the OD alpha-*actin* 1F and DsRed2-1R primers pair. The following DNAs were used as a template, lanes 1-3: DNA samples prepared from three transgenic founder females α-C6; α-C8; α-C9, respectively. Lanes 4: DNA sample prepared from transgenic founder male α-C18. Lane P: positive control. Lane M: DNA size markers.

3.2. Germ-line transmission pattern of pod*α-actin*RFP in F1 generation

To determine the expression pattern and verification germ-line transmission pattern of pod*α-actin*RFP in embryonic development and larvae at F1 generation, microinjected marine medaka embryos were raised to sexual maturityand mating with wild-type fish. Up to 28 in total 73 of incidence RFP positive founders that expressed the vivid red fluorescent were selected for verification of germ-line transmission in F1. Surprisingly, the efficient transgenesis in germ-line marine medaka was very high. More than 50% (16/28) was successful in the transmission of *α-actin*RFP for their progeny. However,

gender and expression characteristics of the founders have affected to the fertilizable, hatching rate and especially the frequency of transgenic offspring in subsequent generations (Table 4). The results showed that, although fertilization rates (94.6%) of the founder male group were higher compared with founder female (93.5%) group, but it's hatching rates (68.8%) less than when compared with founder males (75.4%). In addition, the frequency of germ-line transmission in F1 generation of a male group is uneven, ranging from 5.6-34.9%, and the frequency of transgenic line occurrence of α-C18; α-C19 was highest. In contrast, the difference frequency of germ-line transmission was very high among the female group. Unexpectedly, up to three lines, including α-C6; α-C8; and α-C26 had the lowest frequency germ-line transmission (0.6%), whereas only one line, α-C9, had the highest the efficient transgenesis in fish, 45%. It may indicate that most of the founder females were highly mosaicism, which usually found in microinjected embryos (Nam et al., 1999). The presence of the plasmid DNA pod*α-actin*RFP in transgenic founder α-C6; α-C8; α-C9; and α-C18 was confirmed by PCR (Fig. 17).

After establishing *α-actin*RFP transgenic in F1 generation, it is important to determine whether RFP expression is stable after passage through a germ-line. The exposure time at 4 ms was used for checking the intensity of the embryo RFP signal manifestation on the fluorescent microscope. We found that expressions of RFP were initial detected at stage 22 (12 somite; formation of tubular heart stage, 2dpf). At this stage, the faint signal of RFP appeared mainly on the middle part of the trunk muscle of the fish. However, the expression of RFP increased steadily and spread out in the entire trunk region by development time of embryos at stages 24, 26, in particular, the appearance of the RFP clearly at stage 27 (6dpf) and 28 (8dpf) (Fig. 18D2; FE and F2). Remarkably, strong expression of RFP was primarily in the trunk area of the fish, which was not

observed in the head region at 4 ms of exposure time. On the other hand, in zebrafish, alpha-actin expressed early as the 4cell stage in the germinal plate and there is clear expression at the lens (Bertola et al., 2008), which we did not obtain from marine medaka.

3.3. Ontogenic profiling of *α-actin*RFP transgenic marine medaka

Immediately, after the transgenic embryos hatched that were injected pod*a-actin*RFP, the expression of RFP was very strong in the trunk region that can be distinguished from non-transgenic larvae with the naked eye under white light (data not shown), whereas RFP expression only weakly in the head regions (Fig. 19). However, the RFP expression purely in some specific regions of the head as the hindbrain, auditory capsule, midcerebral vein, and especially the expression of RFP in the heart that is observed in lateral view. In addition, it also expressed in the posterior intermandibularis, interhyoideus, sternohyoideus, hyohyoideus, operculi adductor, pectoral fin in ventral view. Nevertheless, the RFP signals did not show in the midbrain and forebrain regions and eye or lens (Fig. 18; 19; 20).

As an observation of embryos and larvae expression, RFP signals displayed in the whole trunk muscle region (Fig. 18 and Fig. 20). Then, RFP intensity increased steadily during embryonic development that was easily observed under ventral, dorsal, and lateral views (Fig. 20). In addition, to promote in develop ornamental application, adults of transgenic fish were observed under blue light (454 nm) with faithful expression (Fig. 21).

Table 4. Fertilized, hatched rates; and incidence of RFP of circular *pod α-actin*RFP in positive F1 generation marine medaka *O. dancena* fries

TG founder lines	Sex	Fertilized rates (%)*	Hatched rates (%)**	RFP positive in F1 fries (%)***	
α-C2	M	98.3	94.1	41/332	(12.3)
α-C12	M	92.3	66.7	5/48	(10.4)
α-C13	M	98.3	60.1	17/170	(10.0)
α-C18	M	98.0	89.8	117/360	(32.5)
α-C19	M	92.3	46.1	29/83	(34.9)
α-C21	M	83.2	46.8	3/44	(6.8)
α-C25	M	100	78.3	1/18	(5.6)
Mean (Male)		**94.6**	**68.8**		**16.1**
α-C5	F	100	71.4	26/135	(19.3)
α-C6	F	79.6	66.3	1/106	(0.6)
α-C8	F	99.6	76.9	2/356	(0.6)
α-C9	F	99.3	69.0	134/298	(45.0)
α-C14	F	100	89.5	7/77	(9.1)
α-C15	F	98.2	65.5	2/146	(1.4)
α-C23	F	100	73.0	12/135	(8.9)
α-C24	F	68.4	97.4	2/38	(5.3)
α-C26	F	96.2	69.7	1/177	(0.6)
Mean (Female)		**93.5**	**75.4**		**10.1**

*: Number of fertilized eggs/number of spawned eggs

**: Number of hatched larvae/number of fertilized eggs

***: Number of *α-actin*RFP positive larvae /number of hatched larvae at 1dph

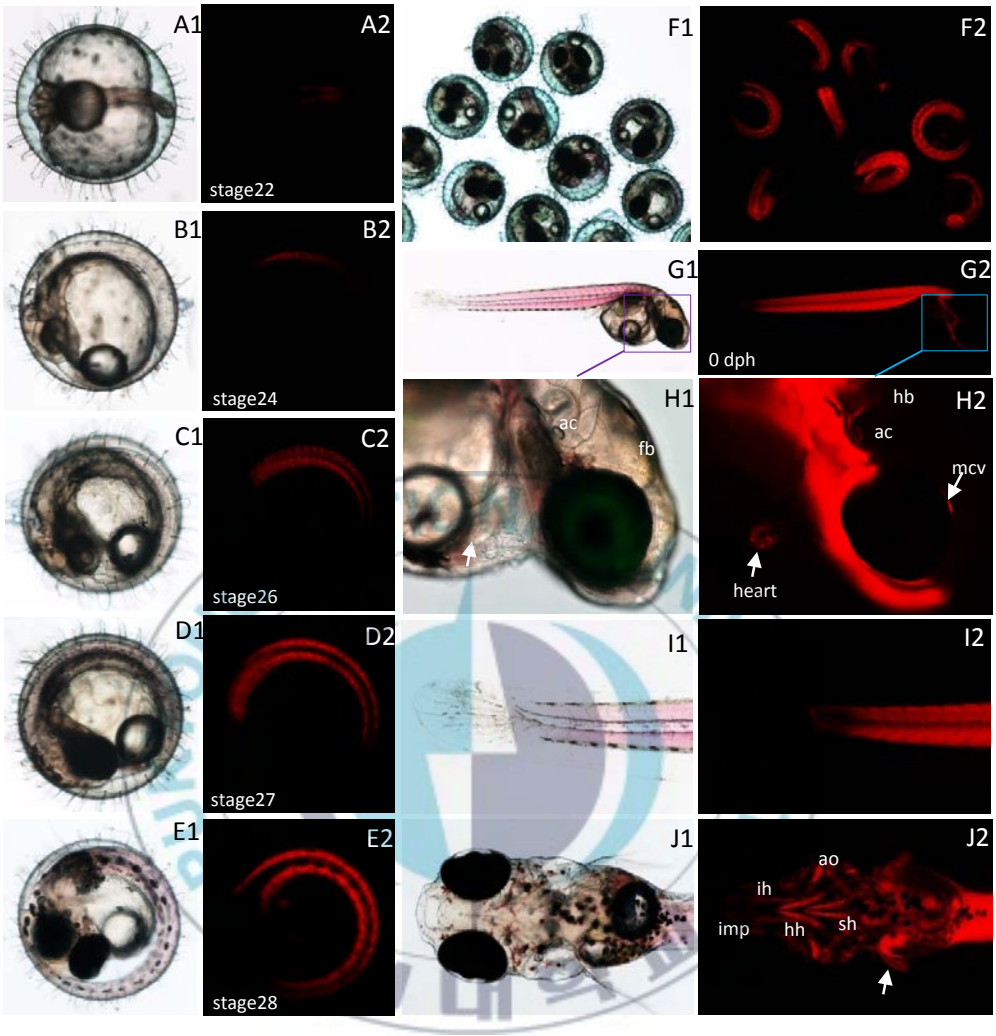


Figure 18. Transient expression of RFP gene driven by the skeletal *α-actin* promoter in a F1 generation of transgenic marine medaka during embryonic development and at 0 day post hatching (0 dph). A1, A2: initial timing of a weakly expressed from stage 22 (12 somite; formation of tubular heart stage – 2dpf). B1, B2: the level of RFP expression gradually increased to stage 24 (18 somite, onset of blood circulation stage – 3dpf). C2, D2, E2: strong RFP expression was detected at stage 26 (4d 20hr af), stage 27 (6 d 12hr) and 28

(heart and spleen development stage. 8-10 d af). H1, H2: Note the lateral view of RFP expression in heart. Arrows in H2 indicated RFP expression in the heart of marine medaka at 0dph. I1, I2: Enlargement of trunk region in G1; G2, respectively. Note the RFP strongly expressed in somatic muscle bibers. J1, J2: Ventral view of RFP expression in the head skeletal muscle in 0 dph. Arrow in J2 indicated RFP also expression in pectoral fin. The names of muscle are based on (Schilling and Kimmel, 1997; Ono et al., 2006): ac, auditory capsule; fb, forebrain; imp, intermandibularis posterior; ih, interhyoideus; sh, sternohyoideus; ao, adductor operculi; hh: hyohyoideus; hb: hindbrain; mcv: midcerebral vein.

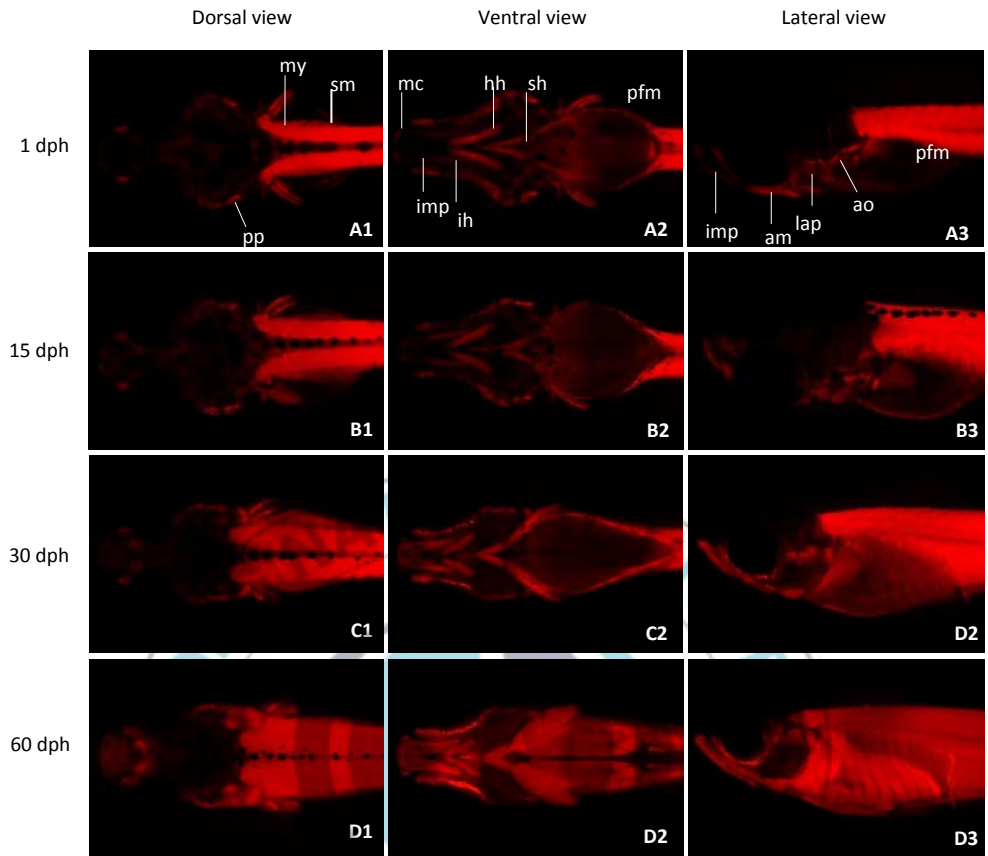


Figure 19. Ontogenic expression patterns of red fluorescence observed in head region during fish developments from 1 to 60 dph. A1; B1; C1 and D1: the RFP signals were observable under dorsal view at 1; 15; 30 and 60 day post hatching (dph), respectively. A2; B2; C2; D2: the RFP signals were observable under ventral view at 1; 15; 30 and 60 dph. A3; B3; C3; D3: the RFP signals were observable under lateral view at 1; 15; 30 and 60 dph. The names of muscle are based on (Schilling and Kimmel, 1997; Ono et al., 2006): am: adductor mandibulae; ao: adductor operculi; hh: hyohyoideus; ih: interhyoideus; imp: intermandibularis posterior; lap: levator arcus palatine; mc: meckel's cartilage; my: myotome; pfm: pectoral finmuscle; pp: pterygoid process; sh: sternohyoideus; sm: somite.

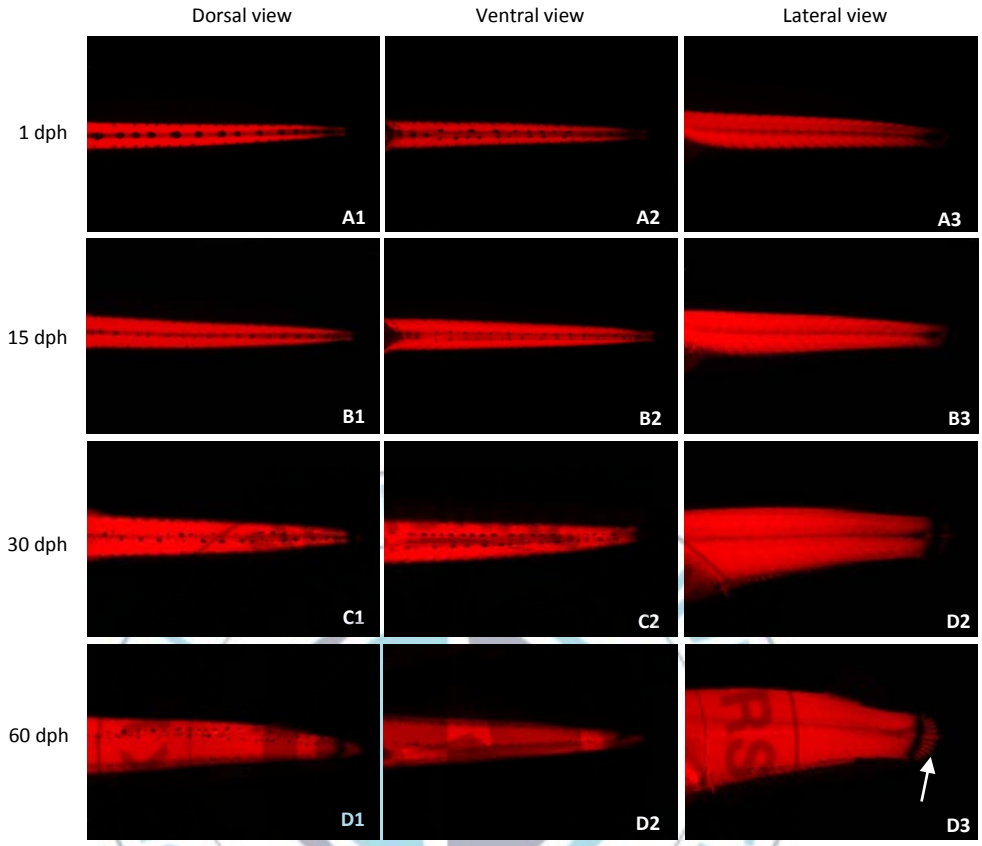


Figure 20. RFP expression pattern of skeletal α-actin in trunk regions in transgenic marine medaka from 1 to 60 dph. A1; B1; C1 and D1: the RFP signals were observable under dorsal view at 1; 15; 30 and 60 day post hatching (dph), respectively. A2; B2; C2; D2: the RFP signals were observable under ventral view at 1; 15; 30 and 60 dph. A3; B3; C3; D3: the RFP signals were observable under lateral view at 1; 15; 30 and 60 dph. Arrow in D3 indicated RFP expression in the proximal end of fin ray connected to hypural bones.

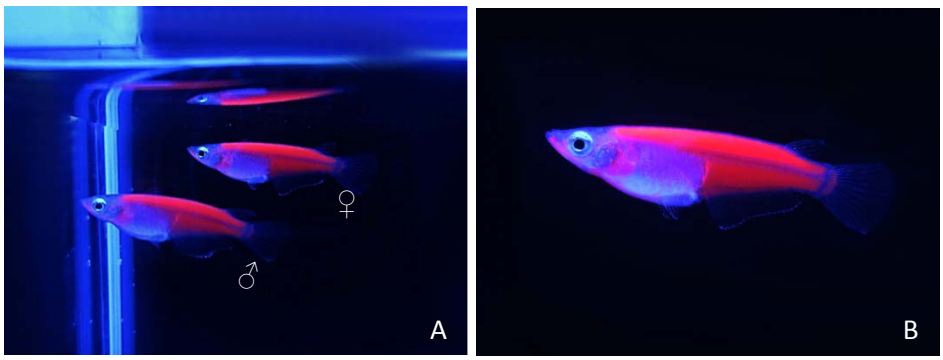


Figure 21. Expression of *α-actin*RFP F1 transgenic marine medaka in LED blue light (454 nm) aquarium. A: combination expression of male and female in an aquarium expression. B: transgenic expression of female at 90 day post hatching.

4. Summary

The results demonstrate that alpha-actin promoter is strong enough to control the expression of RFP during development from embryos to adult stage. Onset of RFP expression in transgenic marine medaka embryos was detectable at two days post fertilization (formation of tubular heart stage). The spatial pattern of transgene expression was observed in some specific regions in the head, including the hindbrain, auditory capsule and mid-cerebral vein. Heart muscles also expressed the transgenic RFP. Afterwards, the expression was spread over nearly whole skeletal muscles with the development and growth of the transgenic fish, such that authentic red fluorescence was visible with unaided eye under normal daylight. Most of established transgenic germlines passed on the fluorescent transgene into their subsequent generations following the Mendelian single gene inheritance pattern.

Chapter 5

CONCLUSION

This work consists of three studies designed to generate the stable transgenic germ lines, and develop highly qualified transgenic ornamental strains using marine medaka *Oryzias dancena* species.

The results show that depending on the transgenic genotypes, each strain showed the different efficiency or intensity of fluorescent color.

Different fluorescent proteins exhibited different characteristics in terms of intensity and degree of vividness when they transgenically expressed in the fish specific tissues.

Although transgenic expression in most strains resembled the pattern of endogenous gene, several exceptional transgenic lines resulted in exotic appearance of strong fluorescent phenotype in non-skeletal muscles.

Furthermore, the qualities of transgenically expressed fluorescence phenotypes are more desirable in transgenic strains carrying CFP or RFP than those harboring GFP.

Most transgenic founders are mosaic not only in the external phenotype but also in their germ cells. Interestingly, the frequency of germ line transmission from founder to F1 was highly variable among strains. However, transmission pattern of transgene was stabilized in the pass-on from F1 - F2 in most transgenic strains examined. Almost transgenic families revealed the transmission pattern following the Mendelian single gene inheritance.

The technique significantly increases the frequency of positive founder fish in F0, thus improving transgenesis frequency.

REFERENCES

Alam, S., Popplewell, A., Maclean, N., 1996. Germ line transmission and expression of alacZ containing transgene in tilapia (*Oreochromis niloticus*). Transgenic Research 5, 87–95.

Amsterdam, a, Lin, S., Hopkins, N., 1995. The Aequorea victoria green fluorescent protein can be used as a reporter in live zebrafish embryos. Developmental Biology 171, 123–9.

Amsterdam, a, Lin, S., Moss, L.G., Hopkins, N., 1996. Requirements for green fluorescent protein detection in transgenic zebrafish embryos. Gene 173, 99–103.

Bertola, L.D., Ott, E.B., Griepsma, S., Vonk, F.J., Bagowski, C.P., 2008. Developmental expression of the alpha-skeletal actin gene. BMC Evolutionary Biology 8, 166.

Bian, Y.-H., Xu, C., Li, J., Xu, J., Zhang, H., Du, S.J., 2011. Development of a transgenic zebrafish model expressing GFP in the notochord, somite and liver directed by the hfe2 gene promoter. Transgenic Research 20, 787–98.

Blake, A., Crockett, R., Essner, J., Hachett, P., Nasevicius, A., 2010. Recombinant constructs and transgenic fluorescent ornamental fish therefefrom. US 2010/0037331 A1. United States Patent Application Publication.

Bratspies, R.M., 2005. Glowing in the dark: how America's first transgenic animal escaped regulation. Minnesota Journal of Law, Science & Technology, Vol. 6, No. 2, p. 457.

Bulinski, J.C., Kumar, S., Titani, K., Hauschka, S.D., 1983. Peptide antibody specific for the amino terminus of skeletal muscle alpha-actin. Proceedings of the National Academy of Sciences of the United States of America 80, 1506–10.

Burket, C.T., Montgomery, J.E., Thummel, R., Kassen, S.C., LaFave, M.C., Langenau, D.M., Zon, L.I., Hyde, D.R., 2008. Generation and characterization of transgenic zebrafish lines using different ubiquitous promoters. Transgenic Research 17, 265–79.

Cavari, B., Hong, Y., Funkenstein, B., Moav, B., Schartl, M., 1993. All-fish gene constructs for growth hormone gene transfer in fish. Fish Physiology and Biochemistry 11, 345–352.

Chalfie, M., Kain, S.R., 2005. Green fluorescent protein: properties, applications, and protocols, Second. ed, Methods of Biochemical Analysis. John Wiley & Sons, Inc., Hoboken, NJ, USA.

Chen, X., Li, L., Cheng, J., Chan, L.L., Wang, D.-Z., Wang, K.-J., Baker, M.E., Hardiman, G., Schlenk, D., Cheng, S.H., 2011. Molecular staging of marine medaka: a model organism for marine ecotoxicity study. Marine Pollution Bulletin 63, 309–17.

Chen, X., Li, L., Wong, C.K.C., Cheng, S.H., 2009. Rapid adaptation of molecular resources from zebrafish and medaka to develop an estuarine/marine model. Comparative biochemistry and physiology. Toxicology & pharmacology : CBP 149, 647–55.

Cho, Y.S., Lee, S.Y., Kim, D.S., Nam, Y.K., 2010. Spawning performance, embryonic development and early viability under different salinity conditions in a Euryhaline Medaka Species, *Oryzias dancena*. Korean Journal of Ichthyology 22, 25–33.

Cho, Y.S., Lee, S.Y., Kim, Y.K., Kim, D.S., Nam, Y.K., 2011. Functional ability of cytoskeletal *β-actin* regulator to drive constitutive and ubiquitous expression of a fluorescent reporter throughout the life cycle of transgenic marine medaka *Oryzias dancena*. Transgenic Research 20, 1333–55.

Chou, C.Y., Horng, L.S., Tsai, H.J., 2001. Uniform GFP-expression in transgenic medaka (*Oryzias latipes*) at the F0 generation. Transgenic Research 10, 303–15.

Chourrout, D., Guyomard, R., Houdebine, L.-M., 1986. High efficiency gene transfer in rainbow trout (*Salmo gairdneri* Rich.) by microinjection into egg cytoplasm. Aquaculture 51, 143–150.

Chudakov, D.M., Matz, M.V., Lukyanov, S., Lukyanov, K.A., 2010. Fluorescent proteins and their applications in imaging living cells and tissues. Physiological Reviews 90, 1103–1163.

Chudakov, D.M., Verkhusha, V.V., Staroverov, D.B., Souslova, E. a, Lukyanov, S., Lukyanov, K. a, 2004. Photoswitchable cyan fluorescent protein for protein tracking. Nature Biotechnology 22, 1435–9.

Curado, S., Anderson, R.M., Jungblut, B., Mumm, J., Schroeter, E., Stainier, D.Y.R., 2007. Conditional targeted cell ablation in zebrafish: a new tool for regeneration studies. Developmental dynamics : an official publication of the American Association of Anatomists 236, 1025–35.

Devlin, R.H., Yesaki, T.Y., Donaldson, E.M., Hew, C.-L., 1995. Transmission and phenotypic effects of an antifreeze/GH gene construct in coho salmon (*Oncorhynchus kisutch*). Aquaculture 137, 161–169.

Ekici, A., Aktoprakli, D., Timur, M., Akkoc, T., Bagis, H., 2010. The Effect of Linear Versus Circular Vector on Enhanced Green Fluorescent Protein (EGFP) Expression in Transgenic Zebrafish (*Danio rerio*). Journal of Animal and Veterinary Advances 9, 1232–1236.

Fan, C.-Y., Simmons, S.O., Law, S.H.W., Jensen, K., Cowden, J., Hinton, D., Padilla, S., Ramabhadran, R., 2011. Generation and characterization of neurogenin1-GFP transgenic medaka with potential for rapid developmental neurotoxicity screening. Aquatic toxicology (Amsterdam, Netherlands) 105, 127–35.

Fang, Y., Déry, O., Haugwitz, M., Turpin, P., Kain, S.R., 2005. Practical considerations for use of reef coral fluorescent proteins in mammalian cells: applications in fluorescence microscopy and flow cytometry., in: Chalfie, M., Kain, S.R. (Eds.), Green Fluorescent Protein: Properties, Applications, and Protocols. John Wiley & Sons, Inc., Hoboken, NJ, USA, pp. 339–359.

Figueiredo, M. de A., Lanes, C.F.C., Almeida, D.V., Marins, L.F., 2007. Improving the production of transgenic fish germlines: in vivo evaluation of mosaicism in zebrafish (*Danio rerio*) using a green fluorescent protein (GFP) and growth hormone cDNA transgene co-injection strategy. Genetics and Molecular Biology 30, 31–36.

Finley, K.R., Davidson, A.E., Ekker, S.C., 2001. Three-color imaging using fluorescent proteins in living zebrafish embryos. BioTechniques 31, 66–70, 72.

Ge, J., Dong, Z., Li, J.J., Xu, Z., Song, W., Bao, J., Liang, D., Li, K., Jia, W., Zhao, M., Cai, Y., Yang, J., Pan, J., Zhao, Q., 2012. Isolation of yellow catfish *β-actin* promoter and generation of transgenic yellow catfish expressing enhanced yellow fluorescent protein. Transgenic Research.

Gong, Z., He, J., Ju, B., Lam, T.J., Xu, Y., Yan, T., 2008. Chimeric gene construct for generation of fluorescent transgenic ornamental fish. US 2008/0052787 A1 (Patent application).

Gong, Z., Ju, B., Wan, H., 2001. Green fluorescent protein (GFP) transgenic fish and their applications. Genetica 111, 213–25.

Gong, Z., Ju, B., Wang, X., He, J., Wan, H., Sudha, P.M., Yan, T., 2002. Green fluorescent protein expression in germ-line transmitted transgenic zebrafish under a stratified epithelial promoter from keratin8. Developmental dynamics : an official publication of the American Association of Anatomists 223, 204–15.

Gong, Z., Wan, H., Tay, T.L., Wang, H., Chen, M., Yan, T., 2003. Development of transgenic fish for ornamental and bioreactor by strong expression of fluorescent proteins in the skeletal muscle. Biochemical and Biophysical Research Communications 308, 58–63.

Gunning, P., Ponte, P., Blau, H., Kedes, L., 1983. alpha-skeletal and alpha-cardiac actin genes are coexpressed in adult human skeletal muscle and heart. Molecular and Cellular Biology 3, 1985–95.

Gómez-Chiarri, M., Kirby, V., Powers, D., 1999. Isolation and Characterization of an Actin Promoter from the Red Abalone (*Haliotis rufescens*). Marine Biotechnology (New York, N.Y.) 1, 269–278.

Hamada, K., Tamaki, K., Sasado, T., Watai, Y., Kani, S., Wakamatsu, Y., Ozato, K., Kinoshita, M., Kohno, R., Takagi, S., Kimura, M., 1998. Usefulness of the medaka beta-actin promoter investigated using a mutant GFP reporter gene in transgenic medaka (*Oryzias latipes*). Molecular Marine Biology and Biotechnology 7, 173–80.

Haobin, Z., Shangping, C., Yonghua, S.U.N., Yaping, W., Zuoyan, Z.H.U., 2000. Time course of foreign gene integration and expression in transgenic fish embryos. Chinese Science Bulletin 45, 734–739.

Hartmann, N., Englert, C., 2012. A microinjection protocol for the generation of transgenic killifish (Species: *Nothobranchius furzeri*). Developmental Dynamics 1133–1141.

Herman, I.M., 1993. Actin isoforms. Current Opinion in Cell Biology 5, 48–55.

Higashijima, S., Okamoto, H., Ueno, N., Hotta, Y., Eguchi, G., 1997. High-Frequency Generation of Transgenic Zebrafish Which Reliably Express GFP in Whole Muscles or the Whole Body by Using Promoters of Zebrafish Origin. Developmental Biology 192, 289–299.

Hsiao, C.D., Hsieh, F.J., Tsai, H.J., 2001. Enhanced expression and stable transmission of transgenes flanked by inverted terminal repeats from adeno-associated virus in zebrafish. Developmental Dynamics 220, 323–36.

Huang, C.-J., Tu, C.-T., Hsiao, C.-D., Hsieh, F.-J., Tsai, H.-J., 2003. Germ-line transmission of a myocardium-specific GFP transgene reveals critical regulatory elements in the cardiac myosin light chain 2 promoter of zebrafish. Developmental Dynamics 228, 30–40.

Joly, E., 2007. Optimising Blue Fluorescent Protein (BFP) for use as a mammalian reporter gene in parallel with Green Fluorescent Protein (GFP). Nature Precedings < http://hdl.handle.net/10101/npre.2007.1259.1>

Ju, B., Chong, S.W., He, J., Wang, X., Xu, Y., Wan, H., Tong, Y., Yan, T., Korzh, V., Gong, Z., 2003. Recapitulation of fast skeletal muscle development in zebrafish by transgenic expression of GFP under the mylz2 promoter. Developmental Dynamics 227, 14–26.

Ju, B., Xu, Y., He, J., Liao, J., Yan, T., Hew, C.L., Lam, T.J., Gong, Z., 1999. Faithful expression of green fluorescent protein (GFP) in transgenic zebrafish embryos under control of zebrafish gene promoters. Developmental Genetics 25, 158–67.

Kang, C.-K., Tsai, S.-C., Lee, T.-H., Hwang, P.-P., 2008. Differential expression of branchial Na+/K(+)-ATPase of two medaka species, *Oryzias latipes* and *Oryzias dancena*, with different salinity tolerances acclimated to fresh water, brackish water and seawater. Comparative biochemistry and physiology. Part A, Molecular & Integrative Physiology 151, 566–75.

Kapuscinski, a R., 2005. Current scientific understanding of the environmental biosafety of transgenic fish and shellfish. Revue Scientifique et Technique 24, 309–22.

Kapuscinski, A.R., Hayes, K.R.K., Li, S., Dana, G., Gong, Z., Maclean, N., Devlin, R.H., Martinez, R., Omitogun, O., Estrada, M.P., Hallerman, E.M., Schei, P.J., 2007. Environmental risk assessment of genetically modified organisms, Volume 3. Methodologies for transgenic fish, Conservation Biology. CABI, Wallingford.

Kawamata, M., Ochiya, T., 2010. Generation of genetically modified rats from embryonic stem cells. Proceedings of the National Academy of Sciences of the United States of America 107, 14223–8.

Khrestchatisky, M., Fontes, M., 1987. There is an alpha-actin skeletal muscle-specific gene in a salamander (*Pleurodeles waltlii*). J. Mol. Biol. 193, 409–412.

Kinoshita, M., 1995. Cytoplasmic microinjection of DNA into fertilized medaka (*Oryzias latipes*) eggs. The Fish Biology Journal Medaka 7, 59–64.

Kinoshita, M., 2004. Transgenic medaka with brilliant fluorescence in skeletal muscle under normal light. Fisheries Science 70, 645–649.

Kiseleva, Y.V., Mishin, a. S., Bogdanov, a. M., Labas, Y. a., Luk'yanov, K. a., 2008. The ability of green fluorescent proteins for photoconversion under oxygen-free conditions is determined by the chromophore structure rather than its amino acid environment. Russian Journal of Bioorganic Chemistry 34, 638–641.

Krishna, K., 2009. Evolving trends in biosciences: Multi-purpose proteins-GFP and GFP-like proteins. Current Science 97, 1022–1032.

Lee, J., 2000. The Internally Self-fertilizing Hermaphroditic Teleost Rivulus marmoratus (*Cyprinodontiformes*, Rivulidae) Beta-actin Gene: Amplification and Sequence Analysis with Conserved Primers. Marine Biotechnology (New York, N.Y.) 2, 161–166.

Maclean, N., Laight, R.J.J., 2000. Transgenic fish: an evaluation of benefits and risks. Fish and Fisheries 1, 146–172.

Marini, N.J., Benbow, R.M., 1991. Differential compartmentalization of plasmid DNA microinjected into *Xenopus laevis* embryos relates to replication efficiency. Molecular and Cellular Biology 11, 299–308.

Mayer Y., Czosnek H., Zeelon1 P.E., Y.D. and N.U., 1984. Expression of the genes coding for the skeletal muscle and cardiac actins in the heart. Nucleic Acids Research 12, 1087–1100.

Mikami, K., Hirata, R., Takahashi, M., Uji, T., 2011. Transient transformation of red algal cells: breakthrough toward genetic transformation of marine crop Porphyra species, in: Alvarez, M. (Ed.), Genetic Transformation. InTech, Open Access Publisher, Croatia, pp. 241–258.

Morita, T., 2000. Amino acid sequences of alpha-skeletal muscle actin isoforms in two species of rattail fish, *Coryphaenoides acrolepis* and *Coryphaenoides cinereus*. Fisheries Science 66, 1150–1157.

Morris, A.C., Schroeter, E.H., Bilotta, J., Wong, R.O.L., Fadool, J.M., 2005. Cone survival despite rod degeneration in XOPS-mCFP transgenic zebrafish. Investigative Ophthalmology & Visual Science 46, 4762–71.

Nam, Y.K., Noh, C.H., Kim, D.S., 1999. Transmission and expression of an integrated reporter construct in three generations of transgenic mud loach (*Misgurnus mizolepis*). Aquaculture 172, 229–245.

Nam, Y.K., Noh, J.K., Cho, Y.S., Cho, H.J., Cho, K.N., Kim, C.G., Kim, D.S., 2001. Dramatically accelerated growth and extraordinary gigantism of transgenic mud loach *Misgurnus mizolepis*. Transgenic Research 10, 353–62.

Naruse, K., Takeda, H., 2011. Medaka - A Model for Organogenesis, Human Disease, and Evolution, Science. Springer Japan, Tokyo.

Ono, Y., Liang, C., Ikeda, D., Watabe, S., 2006. cDNA cloning of myosin heavy chain genes from medaka *Oryzias latipes* embryos and larvae and their expression patterns during development. Developmental Dynamics 235, 3092–101.

Ozato, K., Wakamatsu, Y., 1994. Developmental Genetics of Medaka. Sciences-New York 36, 437–443.

Ozato, K., Wakamatsu, Y., Inoue, K., 1992. Medaka as a model of transgenic fish. Molecular Marine Biology and Biotechnology 1, 346–54.

Pan, X., Zhan, H., Gong, Z., 2008. Ornamental expression of red fluorescent protein in transgenic founders of white skirt tetra (*Gymnocorymbus ternetzi*). Marine Biotechnology 10, 497–501.

Penman, D., Beeching, A., Penn, S., Maclean, N., 1990. Factors affecting survival and integration following microinjection of novel DNA into rainbow trout eggs. Aquaculture 85, 35–50.

Quitschke, W.W., Lin, Z.Y., DePonti-Zilli, L., Paterson, B.M., 1989. The beta actin promoter. High levels of transcription depend upon a CCAAT binding factor. The Journal of Biological Chemistry 264, 9539–46.

Sarkar, A., Atapattu, A., Belikoff, E.J., Heinrich, J.C., Li, X., Horn, C., Wimmer, E. a, Scott, M.J., 2006. Insulated piggyBac vectors for insect transgenesis. BMC Biotechnol. 6, 27.

Schilling, T.F., Kimmel, C.B., 1997. Musculoskeletal patterning in the pharyngeal segments of the zebrafish embryo. Development (Cambridge, England) 124, 2945–60.

Schwartz, K., Bastie, D.D., Bouveret, P., Oliviro, P., Alonso, S., 1986. Alpha-skeletal muscle actin mRNA's accumulate in hypertrophied adult rat hearts. Circulation Research 551–555.

Shaner, N.C., Patterson, G.H., Davidson, M.W., 2007. Advances in fluorescent protein technology. Journal of Cell Science 120, 4247–60.

Shaner, N.C., Steinbach, P.A., Tsien, R.Y., 2005. A guide to choosing fluorescent proteins. Nature Methods 2, 905–9.

Shima, A., Shimada, A., 1994. The Japanese medaka, Oryzias latipes, as a new model organism for studying environmental germ-cell mutagenesis. Environmental Health Perspectives 102, 33–35.

Shimomura, O., Johnson, F.H., Saiga, Y., 1962. Extraction, purification and properties of aequorin, a bioluminescent protein from the luminous

hydromedusan, *Aequorea*. Journal Of Cellular And Comparative Physiology 59, 223–239.

Sin, F.Y.T., 1997. Transgenic fish. Reviews in Fish Biology and Fisheries. 7, 417–441.

Song, H.Y., Kim, D.S., 2012. Growth performance of transgenic mud loach misgurnus mizolepis carrying a gh transgene driven by mud loach c-type lectin regulator. Aquatic Sciences 15, 43–47.

Song, H.Y., Nam, Y.K., Bang, I.C., Kim, D.S., 2009. Embryogenesis and early ontogenesis of a marine medaka, *Oryzias dancena*. Korean Journal of Ichthyology 21, 227–238.

Stuart, G.W., McMurray, J.V., Westerfield, M., 1988. Replication, integration and stable germ-line transmission of foreign sequences injected into early zebrafish embryos. Development (Cambridge, England) 103, 403–12.

Tanaka, M., Kinoshita, M., 2001. Recent progress in the generation of transgenic medaka (*Oryzias latipes*). Zoological Science 18, 615–622.

Tanaka, M., Kinoshita, M., Kobayashi, D., Nagahama, Y., 2001. Establishment of medaka (*Oryzias latipes*) transgenic lines with the expression of green fluorescent protein fluorescence exclusively in germ cells: a useful model to monitor germ cells in a live vertebrate. Proceedings of the National Academy of Sciences of the United States of America 98, 2544–9.

Tang, W., Coughlan, S., Crane, E., Beatty, M., Duvick, J., 2006. The application of laser microdissection to in planta gene expression profiling of the maize anthracnose stalk rot fungus *Colletotrichum graminicola*. Molecular Plant-Microbe Interactions : MPMI 19, 1240–50.

van Thor, J.J., Gensch, T., Hellingwerf, K.J., Johnson, L.N., 2002. Phototransformation of green fluorescent protein with UV and visible light leads to decarboxylation of glutamate 222. Nature Structural Biology 9, 37–41.

Tsai, H., 2008. Use of Transgenic fish Possessing Special Genes as model Organisms and Potential Applications. Genetics and Molecular Biology 9, 22–38.

Vandekerckhove, J., Bugaiskyv, G., Buckingham, M., 1986. Simultaneous Expression of Skeletal Muscle and Heart Actin Proteins in Various Striated Muscle Tissues and Cells. Society 1838–1843.

Vandekerckhove, J., Weber, K., 1978. At least six different actins are expressed in a higher mammal: an analysis based on the amino acid sequence of the amino-terminal tryptic peptide. Journal of Molecular Biology 126, 783–802.

Wada, H., Shimada, a, Fukamachi, S., Naruse, K., Shima, a, 1998. Sex-linked inheritance of the lf locus in the medaka fish (*Oryzias latipes*). Zoological Science 15, 123–6.

Wan, H., He, J., Ju, B., Yan, T., Lam, T.J., Gong, Z., 2002. Generation of two-color transgenic zebrafish using the green and red fluorescent protein reporter genes gfp and rfp. Marine Biotechnology 4, 146–54.

Wang, W., Liu, X., Gelinas, D., Ciruna, B., Sun, Y., 2007. A fully automated robotic system for microinjection of zebrafish embryos. PloS one 2, e862.

Wenck, A., Pugieux, C., Turner, M., Dunn, M., Stacy, C., Tiozzo, A., Dunder, E., van Grinsven, E., Khan, R., Sigareva, M., Wang, W.C., Reed, J., Drayton, P., Oliver, D., Trafford, H., Legris, G., Rushton, H., Tayab, S., Launis, K., Chang, Y.-F., Chen, D.-F., Melchers, L., 2003. Reef-coral proteins as visual, non-destructive reporters for plant transformation. Plant Cell Reports 22, 244–51.

Willems, B., Büttner, A., Huysseune, A., Renn, J., Witten, P.E., Winkler, C., 2012. Conditional ablation of osteoblasts in medaka. Developmental Biology 364, 128–37.

Xu, Y., He, J., Tian, H.L., Chan, C.H., Liao, J., Yan, T., Lam, T.J., Gong, Z., 1999. Fast skeletal muscle-specific expression of a zebrafish myosin light chain 2 gene and characterization of its promoter by direct injection into skeletal muscle. DNA and Cell Biology 18, 85–95.

Zeng, Z., Liu, X., Seebah, S., Gong, Z., 2005. Faithful expression of living color reporter genes in transgenic medaka under two tissue-specific zebrafish promoters. Developmental Dynamics 234, 387–92.

Zhang, P.J., Hayat, M., Joyce, C., Gonzalez-Villaseñor, L.I., Lin, C.M., Dunham, R.A., Chen, T.T., Powers, D.A., 1990. Gene transfer, expression and inheritance of pRSV-rainbow trout-GH cDNA in the common carp, *Cyprinus carpio* (Linnaeus). Molecular reproduction and development 25, 3–13.

Zhang, Y.Q., Rodesch, C.K., Broadie, K., 2002. Living synaptic vesicle marker: synaptotagmin-GFP. Genesis (New York, N.Y. : 2000) 34, 142–5.