Expression of Red Fluorescent Protein (RFP) in Transgenic Angelfish (*Pterophyllum scalare* Schultze, 1823)

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ABSTRACT

The freshwater angelfish (*Pterophyllum scalare* Schultze, 1823) is a common aquarium species. Similar to many other cichlids, angelfish provide parental care for their embryos and larvae. In contrast to zebrafish and medaka, it is difficult to raise angelfish eggs in the lab. Therefore, obtaining angelfish embryos for transgenic purposes will also be challenging. Our aim in this study was to employ microinjection techniques to develop transgenic angelfish expressing red fluorescent protein (RFP). Using the zebrafish myosin light chain 2 (mylz2) promoter, the expression patterns of the RFP founder transgenic angelfish were analyzed. An open loop of plasmid pDsred2-1 was cloned with the 1999-bp Mylz2 promoter fragment at the *Sac*I and *Age*I sites to generate the pMylz2-RFP transgenic construct. After angelfish laid their eggs on the substrate, the embryos were carefully collected with a plastic pipette in preparation for the microinjection procedure. A single-cell-stage angelfish embryo was microinjected with pMylz2-RFP. Sixteen of 524 pMylz2-RFP microinjected embryos survived to five days post fertilization (5 dpf), with twelve displaying red fluorescence. Only two RFP-positive larvae survived to adulthood. In adult angelfish, red LED illumination clearly displayed RFP expression in trunk muscles, indicating successful transmission of the transgene. The pMylz2-RFP transgene, however, was not passed on to offspring, indicating that it was not incorporated into the germline.

Keywords: Angelfish, Mylz2, *Pterophyllum scalare*, Red fluorescent protein, RFP

INTRODUCTION

Transgenic ornamental fish have become the first transgenic pets available on the market (Debode *et al*., 2020). Zebrafish (*Danio rerio*) (Gong *et al*., 2003), medaka (*Oryzias latipes*) (Chou *et al*., 2001), medaka (*O. dancena*) (Cho *et al*., 2011; Vu *et al*., 2014) and white skirt tetra (*Gymnocorymbus ternetzi*) (Pan *et al*., 2008) are the main species that have been studied to create fluorescent glowing-protein transgenic fish. Zebrafish, the first GloFish, became commercially available in 2003. Since then, GloFish varieties have been created for the black tetra (*G. ternetzi*), tiger barb (*Puntigrus tetrazona*), betta (*Betta splendens*) and rainbow shark (*Epalzeorhynchos frenatum*). Although a fluorescent transgenic angelfish has been mentioned (Chen *et al*., 2015), the details of the technique used to generate this transgenic angelfish are still not well understood.

Freshwater angelfish comprise three species (*Pterophyllum altum*; *P. leopoldi*; and *P. scalare*). In aquariums, *P. scalare* is the species that is most frequently kept. *Pterophyllum* spp. are found throughout the Amazon, Orinoco, and Essequibo River drainage basins, forming the epicenter of neotropical cichlid biodiversity (Çelik

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et al., 2014). Here, we chose albino red eye pearlscale angelfish (*P. scalare*) as the model of study because its body is uniformly white (easy to observe the fluorescent protein expression) and has been successfully studied for reproduction (tankfacts.com/fish/freshwater/angel-fish/albino-angelfish_301). Thus, it may be a suitable model for genetic transfer research and to create new fluorescent protein transgenic angelfish.

Selection of appropriate promoters is one of the important factors for transgenic research (Cho *et al*., 2011; Thuy *et al*., 2021). To date, five types of promoters have been studied and used in gene transfer to create luminous transgenic fish: α-actin (Chou *et al*., 2001; Bertola *et al*., 2008; Thuy *et al*., 2021), β-actin (Hamada *et al*., 1998; Cho *et al*., 2011; Ge *et al*., 2012; Lee *et al*., 2013), myosin light chain 2 (Ju *et al*., 2003; Pan *et al*., 2008), myosin light chain 3 (Lin *et al*., 2014), and muscle-type creatine kinase b (CKMb) (Chen *et al*., 2015). However, the genome of this angelfish has not yet been reported; therefore, it is challenging to use this fish to perform successful luminous transgenic analysis. Importantly, the myosin light chain 2 promotor from zebrafish (mylz2) has been shown to work effectively in several species, such as white skirt tetra (Pan *et al*., 2008), minnow (*Pimephales notatus*) (Aivaz *et al*., 2020), medaka (Zeng *et al*., 2005) and itself (Xu *et al*., 1999; Gong *et al*., 2003; Ju *et al*., 2003).

The mylz2 promoter has been used to generate different transgenic fish that derived the expression of genes for green fluorescent protein (GFP) (Amsterdam *et al*., 1995), cyan fluorescent protein (CFP) (Vu *et al*., 2014) and red fluorescent protein (RFP) (Wan *et al*., 2002; Gong *et al*., 2003; Pan *et al*., 2008; Jian *et al*., 2012; Cho *et al*., 2013). Among these, RFP transgenic fish can easily be seen with the naked eye. Therefore, the purpose of this study was to describe the process of producing red fluorescent angelfish under the control of the mylz2 promoter using microinjection technology.

MATERIALS AND METHODS

Breeding and collecting angelfish embryos

Albino red eye pearlscale angelfish (*Pterophyllum scalare*) were obtained from a domestic ornamental aquarium shop in Ho Chi Minh City and maintained at the Fisheries Laboratory at the Biotechnology Center of Ho Chi Minh City. The angelfish were raised in 50 L tanks. During the larval stage, angelfish were fed twice daily with artemia (Century, USA) and commercial feed NRD2/3 (INVE, Thailand) until they reached a size of 2-3 cm in length. As fish reached a size of 4 cm, beef heart meat and worms were provided. Fifty percent of the water was exchanged weekly. The water temperature in the fish tank was maintained at 28±1 °C and at pH 7.0-7.5.

Female and male fish usually cleaned the artificial substrate provided by the aquarium (Figure 1a) for approximately one to two hours before mating. Spawning occurred between 12 p.m. and 3 p.m. (Thilakarathne *et al*., 2021). The angelfish male spread his mucus over the substrate before spawning, and the angelfish female laid her eggs on the substrate (Figure 1b). Using a 3 mL plastic straw, eggs were aspirated into a petri dish ready for injection of transgenic construct after 5-15 min of spawning.

Angelfish took approximately 45 to 60 min for the male to fertilize the eggs after the female began laying eggs (Thilakarathne *et al*., 2021). As a result, during angelfish spawning, eggs could be taken from the substrate for microinjection 4-6 times. For each collection, approximately 30-50 embryos were obtained.

Angelfish embryos were kept at room temperature (28±1 °C) in petri dishes until microinjection was performed. Fifty microinjected embryos and non-microinjected embryos (a control) were incubated at 28±1 °C and pH 7-7.5, in a 1 L tank. The hatching and survival of embryos were measured.

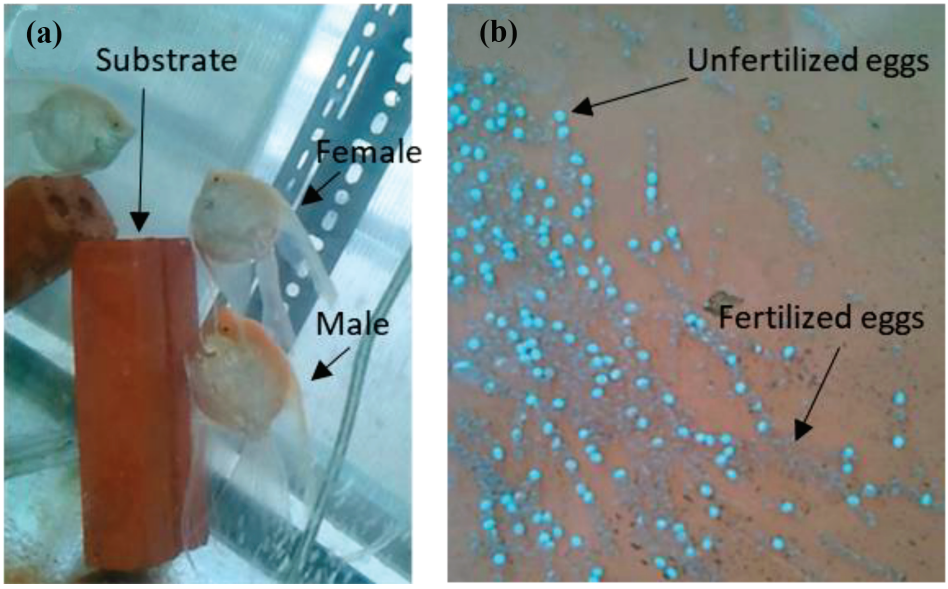


Figure 1. Spawning of *Pterophyllum scalare*: (a) A female begins laying eggs, and the male follows and fertilizes them. (b) An unfertilized egg is white (arrow), while a fertilized egg is grayish (arrow).

The experiments were carried out following the "Guideline for the Care and Use of Laboratory Animals" (approved by the Biotechnology Center of Ho Chi Minh City in January 2013 with decision No. 02/QĐ-CNSH).

Isolation of the mylz2 promoter

Genomic DNA (gDNA) was isolated from zebrafish muscles using a phenol/chloroform/isoamyl alcohol method. Approximately 50 mg of fish muscle was added to a microcentrifuge tube containing 750 µL TNES buffer (1 M Tris-HCl, pH 8.2; 5 M NaCl; 0.5 mM EDTA; and 10% SDS) and homogenized, and 5 µL of 20 $mg \cdot mL^{-1}$ proteinase K was added and vortexed for 30 s. The homogenate was incubated at 55 °C for 4 h. An equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added and well mixed. After centrifugation (5 min at 13,000 rpm), the top aqueous phase (approximately 700 µL) was collected, and the extraction was repeated with chloroform:isoamyl alcohol (24:1). DNA precipitation was performed by adding an equal volume of isopropanol andgently mixing for 15 min before incubation at 4 °C for 5 min. Precipitated DNA was collected by centrifugation (13,000 rpm for 2 min), washed with 70% ethanol and air-dried. Extracted genomic DNA was resuspended in 20 µL of 1X TE-RNase A (100 µg per 250 µL solution) and further incubated at 37 °C for 40 min. The quality and quantity of genomic DNA were estimated by 1.0% agarose gel electrophoresis and spectrophotometry. DNA was kept at -20 °C until further use.

To generate a blunt-end Mylz2 promoter, PCR was performed with Mylz2-*Sac*I-F and Mylz2-*Age*I-R primers (containing *Sal*I and *Age*I restriction sites; GenBank accession no. NC007114.6, Table 1) using *Pfu* DNA polymerase (Promega, USA). The reaction was composed of 1X buffer, 0.2 mM dNTP, 0.5 µM each of Mylz2-*Sac*I-F and Mylz2-*Age*I-R, 1.25 $U \cdot 50\ \mu L^{-1}$ *Pfu* polymerase and 100 ng DNA template. Thermal cycling was 95 °C for 2 min followed by 35 cycles at 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 4 min. The final extension was performed at 72 °C for 7 min. The resulting product was electrophoretically analyzed by 1.0% agarose gel electrophoresis.

Table 1. Primer sequences used to isolate the promoter and PCR procedures.

Primer	Sequence	Product size	Purpose
Mylz2-*Sac*I-Fv	5′-AGGGAGCTCAATTCGCCACAGAGGAATGAGC-3′	1999 bp	Isolation of
Mylz2-*Age*I-Rv	5′-AGGACCGGTTGTCGAGACGGTATGTGTGAAGTCT-3′		Mylz2 promoter
Mylz2-Fv2	5′-GCTATTTTGGTCACCACAGCTGTTC-3′	1199 bp	Ligation test of
DsRed-Rv	5′-TGGAACTGGGGGGACAGG-3′		pMylz2-RFP
pJET1.2-F	5′-CGACTCACTATAGGGAGAGCGGC-3′	2004 bp	Ligation test of
pJET1.2-R	5′-AAGAACATCGATTTTCCATGGCAG-3 ′		pJET1.2-Mylz2

Generation of pMylz2-RFP expression vectors

To generate the transgene construct *pMylz2-RFP*, we first created plasmid pJET1.2-Mylz2. First, the Mylz2 gene fragment was amplified by PCR, and 1 µL of PCR product was ligated to the cloning vector pJET1.2/blunt using a CloneJET PCR Cloning Kit (Thermo Scientific, USA) following the procedures recommended by the manufacturer. The ligation product (5 µL) was transformed into *Escherichia coli* DH5α, and the transformants were cultured and selected on an LB plate containing 50 $\mu g \cdot mL^{-1}$ ampicillin as described in Sambrook and Russell (1989).

Colony PCR of ten clones was performed in a 25 µL reaction mixture containing 1X Dream Taq PCR Master Mix (Thermo Scientific, USA), 0.4 µM each of pJET1.2-F and pJET1.2-R (Table 1) and half of each colony. The other half of the same colony was spread on the LB plate. PCR was performed with the thermal cycling of predenaturation at 95 °C for 5 min followed by 30 cycles of 95 °C for 30 s, 60 °C for 30 s and 72 °C for 2 min.

Recombinant plasmid was extracted using an ISOLATE II Plasmid Mini Kit (BIOLINE, USA) and digested with *Sac*I and *Age*I for preliminary confirmation of the insert. The purified plasmid was then sequenced and analyzed using an ABI 3130 DNA Sequencer (Applied Biosystems, USA) with the pJET1.2-F or pJET1.2-R primers (Table 1).

After verification, a Mylz2 promoter and an open-loop plasmid were obtained from plasmids pJET1.2-Mylz2 and pDsRed-1 (Clontech Laboratories, US) by digestion with *Sac*I-HF and *Age*I-HF (NEB, USA). For each plasmid, 50 µL of double digestion of restriction enzymes was simultaneously performed. The reaction was composed of 1 µg DNA, 1X CutSmart Buffer, and 10 U each of *Age*I-HF and *Sac*I-HF. The samples were incubated at 37 °C for 1 h. The digestion products were electrophoresed through a 1.0% agarose gel and eluted using a MEGAquick-spin™ plus Total Fragment DNA Purification Kit (iNtRON, Korea). The purified Mylz2 fragment was cloned into the open-loop pDsred2-C1 vector to generate the pMylz2-RFP plasmid and plated on LB plates containing 50 $\mu g \cdot mL^{-1}$ kanamycin.

The successful generation of pMylz2-RFP was confirmed by PCR with the Mylz2-F2 and DsRed-R primers (Table 1) as described previously. A representation map for the pJET1.2-Mylz2 and pMylz2-RFP plasmids was created using Snapgene software version 5.3.2.

Establishing transgenic angelfish by microinjection

After quality checking, the plasmid was diluted with 1X TE to a final concentration of 100 $\mu g \cdot mL^{-1}$ and aliquoted to 5 µL each.

The microinjection procedures were as follows: 100 pg pMylz2-RFP plasmid was directly injected into each one-cell stage of angelfish embryos using a Carl Zeiss micromanipulator (Gottingen, Germany). During injection of the transgene construct, embryos were held using curved tweezers.

Positive RFP signals of microinjected embryos (1-5 dpf) were observed using an RFP G-2A filter (EX 510-560 nm; DM 575 nm; and BA 590 nm of a Nikon ECLIPSE TS100 fluorescence microscope). The expression of RFP in founder transgene angelfish at the adult stage was observed under blue LED light (wavelength of 400-500 nm).

To evaluate the capacity of plasmid transmission from founders to offspring, RFP angelfish were crossbred with wild-type fish. F1 fry were tested under a fluorescence microscope for RFP-positive signals.

RESULTS

Zebrafish Mylz2 promoter isolation and pMylz2-RFP construction

PCR was used to amplify the 1999-bp Mylz2 promoter region (Figure 2a) using the Mylz2-*Sac*I-F and Mylz2-*Age*I-R primers (Table 1). The Mylz2 promoter was cloned into the pJET1.2/blunt cloning vector to create pJET1.2-Mylz2 (Figure 2b) for future generation of the transgene construct. The Mylz2 promoter was sequenced to identify TATA boxes, E boxes, and MEF2 boxes (Figure 2c).

pMylz2-RFP transgenes were produced by inserting the Mylz2 fragment from pJET1.2-Mylz2 into pDsRed2-1. A Mylz2 promoter band with a size of 1999 bp and a 3911-bp open-loop pDsRed2-1 plasmid are shown in Figure 3a (lanes 1 and 2). A pMylz2-RFP transgene (Figure 3b) was generated by subcloning the Mylz2 fragment into the open loop pDsRed2-1. The successful ligation of pMylz2-RFP into *Escherichia coli* DH5α was verified by colony PCR, and 1199-bp PCR products were obtained (Figure 3c), suggesting that the pMylz2-RFP plasmid was successfully constructed.

The Mylz2 promoter drives RFP expression in angelfish founders

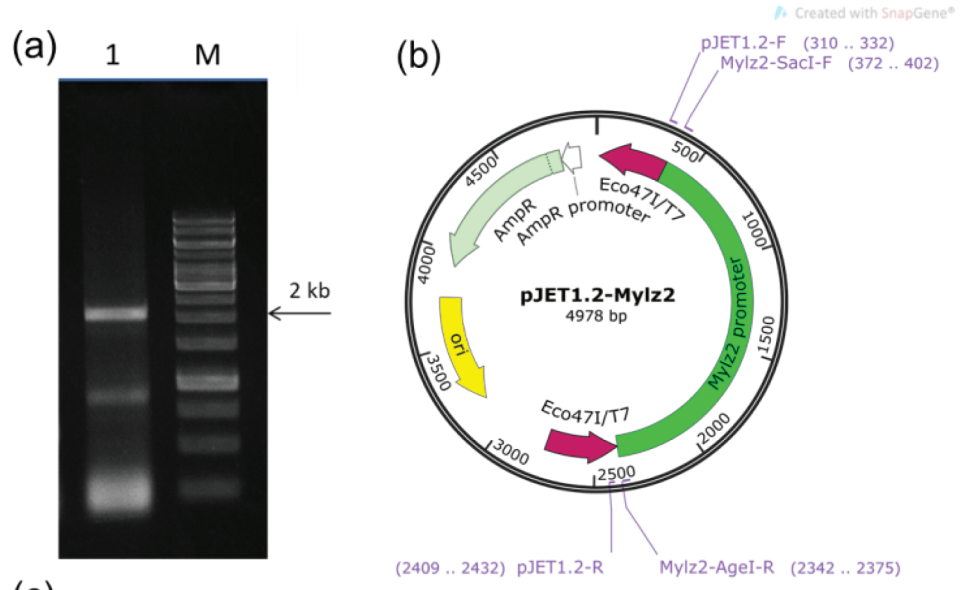
Approximately 1 µL of 100 $\mu g \cdot mL^{-1}$ pMylz2-RFP plasmids could be microinjected into 1,000 angelfish embryos. As a result, sixteen embryos out of 521 microinjected embryos showed normal development (Table 2). However, only twelve of sixteen surviving embryos exhibited red fluorescence signals (Table 2).

In contrast to non-microinjected eggs (56 %), microinjected eggs had a hatching rate of 3.07 % (Table 2). Embodiments microinjected with pMylz2-RFP demonstrated diverse morphologies and levels of RFP expression (under RFP filter light) in the trunk muscle area at 2 dpf (Figures 4e-4h). This expression was not observed under white light (Figure 4a-d).

Characterization of transgenic founder angelfish

At 5 dpf, the trunk muscles of angelfish larvae exhibited transparent (Figure 5a-5c) and red fluorescence (Figure 5d-5f) expression under white light and RFP filter light, respectively. Interestingly, at one month after fertilization, RFP expression was visible in the trunk region (Figures 6a-6c). RFP expression was widely visible over the entire angelfish body at two months of age under blue LED illumination (Figure 6d).

As part of the research, two out of twelve RFP-positive angelfish larvae survived to adulthood and were crossed with wild-type fish to evaluate germline transmission. Approximately 700 F1 embryos from each cross were tested for RFP expression (using an RFP G-2A filter [EX 510-560 nm; DM 575 nm; and BA 590 nm] of Nikon ECLIPSE TS100 fluorescence microscope) for up to 5 dpf. It should be noted, however, that none of the founders in the trial produced embryos capable of expressing this gene, and that even when pooled F1 embryos from a few founders were analyzed, PCR analysis of these embryos demonstrated no evidence of this DNA sequence inserted within the embryos.



(c)

Mylz2-SacI-Fv

AGGGAGCTCAATTCGCCACAGAGGAATGAGCCACCAACTCATCCAGTGTATTTTTTATGCAGCGGATGCC
CATCCAGTTGCAACCCTACACTGGGAAACACCCAAATCTGTCTTTTAAATTAATATTTTTAATAGGAAGC
TATGTTATATTTGTGCATATACATTAGATTAGTCAGTACACTCTCAGAAATAAATGGTCCGCAAGCTGTC
ACTGCGGTGGTACCTTTTCAAAAGGTACATATTTGTACTTAAAGGGTCCATATTGATACCTCAAAAGTAT
ATATTAGTGCCTAAAAATTTTAAGAGAAACACTTTTGTACTTTTTAGGTACTAATATATACCCTTGAGGT
TTTATTATGGACCTTTAAGGTACAAATTTTTATCATTTGGAAAGGTACCACCCCAGTGACAGCTCACGCC
CTTTATTTCTGAGAGTGAAGCTAAATCTGGAGCTTATTTAACAAAATAACTTATGATAACGGTCCAAAAA
CTACTACACCCATATTTATATGTTATTGAAAAATATTAAATACAAATTTATAAACAGGGGAAAATCAAGA
GAAGCAAAACAATGGAAAAAAATTAGTTCAAAATTTGTAGGTTGTAATTTTTTTGTTGCAATATTTAGCT
TTAAATTGTTTTATCTTTCAATTTTGTGACTAAAATATTATTTTAATAAATATAGCTGTTTAATAAATCT
GTTTTGTTTAAATGCACCAAAATACATTGCCTATATTCACTGAGAGATGGAGAAAAATATTCATTTTCAA
AATGGGCTGTACTCAATTACTCTAAGCACTGTAATTATCTGTTTTTCTAGGTACTGTAAAATAATGCTAA
TCTTAAACAATTTTGGAACAAGCAGGAGCAAAACAAGCCGACAGACTCACAGGATATCAAATTAGTCTAA
AGAAAGAAGGGAATGCACAACAAACTCAAGGGGGGCAAAACAGTGACTGATGAATTAGACAAGAAAAAGA
GAGCAAGGAGCGCTCAAGATTGTTTAGCTATTTTGGTCACCACAGCTGTTCCTTATGCCTGCCTTCCCAA
AAAAAAACTGTCTTAAGCCTCAAATTTCTCTTCATGAGGGTCCAACATCAACCACTCAGAGGGCTGTAGT
GTGCTGACCATCTAAAAACTGGGAAAAAGGGGTAATTACGTGCTTGTCCACAGGGCAGCTTCCCACAAAT
GGCACCTCACAGTCACTGAAGTGACCGGGTGAGGTCATAGGTCGATCGGCAGAGAGAGAGAGAGAGAG
AGAGAGAGAGTGCTGAATATGGGCATGGCCATGTGGACGGGTGTGGTGGGGGCACTTGAACCGAAATCTT
ACAGCATCACTATACTAGGAAAAGCATTAAAACCTATTTTCGATTAGGGCTGATTTGAAATAAGGGTTAA
GACACCAGAACGTCCTCTTATATATCAGCCAGGGCGTGAACAAATATGAACAACATAATCATTGGCTCAA
AAATCTCTGGATTGAAATCCATCAGGATCTATCACTGCAACCCTCCCCATCCAACAAGTAATCCTGCAAG
ATAAGCCAGTATTGATCTGCTGCTAATCTAACTTTAGGGGAGGGAGTTGCGTCCCCTTTAGACTCAGTGG
CTACAGCTCATTCATTTCAAATTGAGTTATGTGATTGTATGAAGCTCAAACAGTCCCTTACGTCCCCATG
TCCTTATTAGACAACGCGAGACATGCAGGCCGCTGCCATCAGTATCAGATTCATCCCATTCCAAGACTCC
AATAGCTATTTCTGAGCACTGTAAGATGATAGTACATCCCAGCCGGTGTCCCTCCATCACTTTCCCCCTA
CCTCATAGTTTTTCCTCTTTCTCTCTCGGTCTGCTATTTCCCAAACCTCACTTAAGGTTGGGTCTATAAT
TAGAAGGGGCCTTCGTCAGTATATAAGCCCCTCAAGTACAGGACACTACGCGGCTTCAGACTTCTCTTCT
TGATCTTCTTAGACTTCACACATACCGTCTCGACAACCGGTCCT

Mylz2-AgeI-Rv

Figure 2. Structure of the pMylz2-RFP construct using the zebrafish Mylz2 promoter: (a) The Mylz2 promoter was isolated by PCR and yielded a product of approximately 2 kb in size; (b) pJET1.2-Mylz2 is shown using Snapgene 5.3.2; (c) Characterization of the 1999-bp Mylz2 promoter region. The MEF2 box, E-box, and TATA box are indicated in red, gray, and green, respectively. Primer sequences are highlighted in yellow.

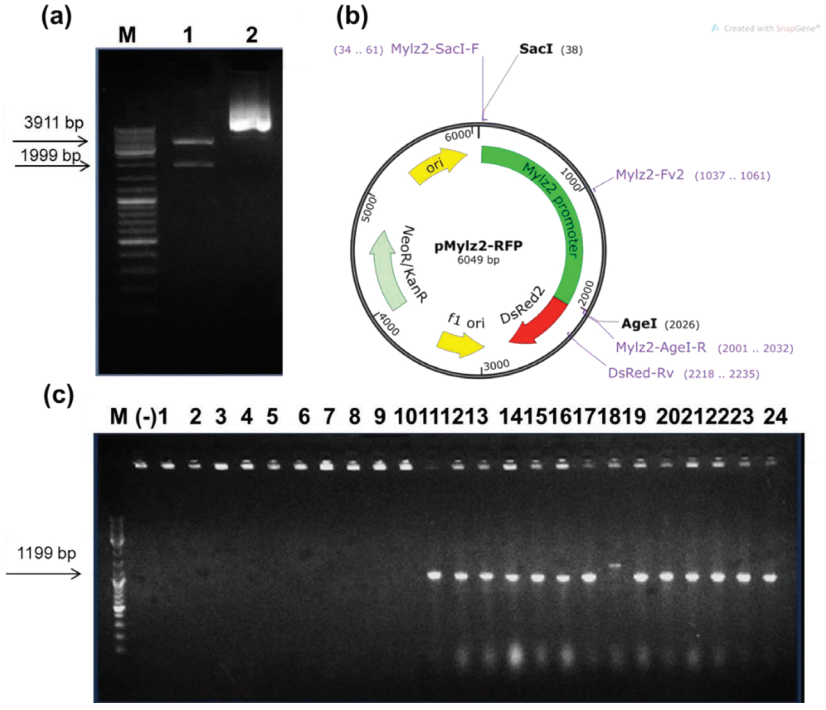


Figure 3. Generation of the pMYlz2-RFP transgene: (a) On the purified and presumably purified plasmid pMylz2-RFP, we verified that the Mylz2 promoter was ligated to the open-loop pDsRed2-1 plasmid to produce pMylz2-RFP, a transgenic plasmid. The *Sac*I and *Age*I enzymes were used to verify the ligation of the pMylz2-RFP plasmid; (b) A representation map for the pMylz2-RFP plasmid was created using Snapgene software (version 5.3.2); (c) Colony PCR was performed to verify the successful ligation and transformation of the pMylz2-RFP plasmid. M is a GeneRuler^{TM} 1 kb DNA ladder. Lanes 1-10 indicate unsuccessful transformation of the pMylz2-RFP plasmid colony, lanes 11-17 and 19-24 indicate unsuccessful transformation of the pMylz2-RFP plasmid colony, and lane 18 is an unidentified transformation colony.

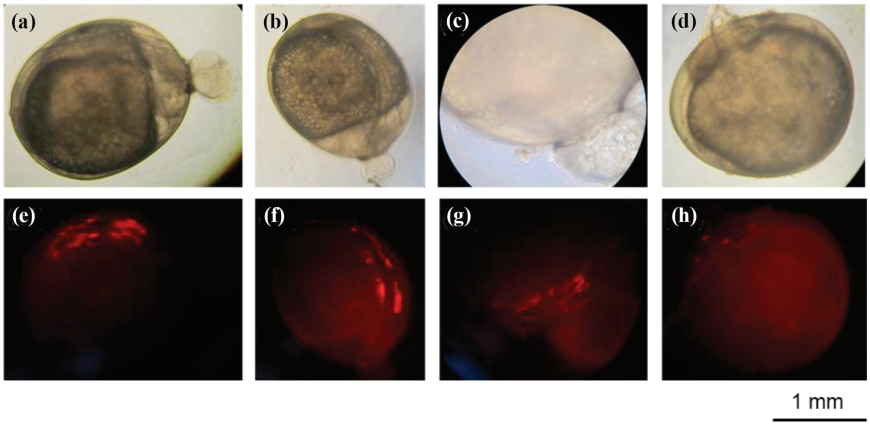


Figure 4. RFP signal expression in 2-dpf angelfish embryos: (a), (b), (c), and (d) Micrographs of embryos examined under bright-field illumination using fluorescence microscopy; (e), (f), (g), and (h) Images obtained using a RFP G-2A filter (BA: 590 nm; DM: 575 nm; and EX: 510-560 nm).

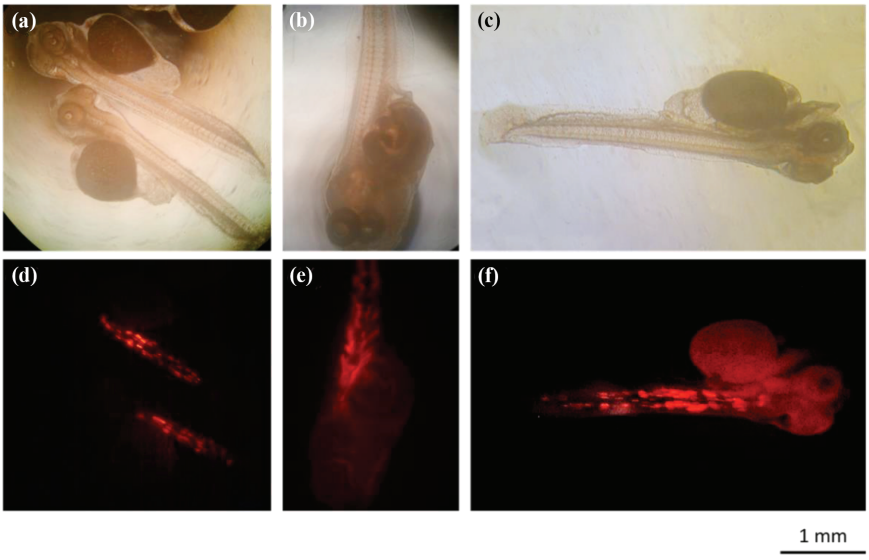


Figure 5. Red fluorescent protein expression observed in 5-dpf RFP-positive angelfish larvae: (a), (b), and (c) Bright-field microscopes (without a G-2A filter); (d), (e) and (f): Fluorescence microscopy with a G-2A filter.

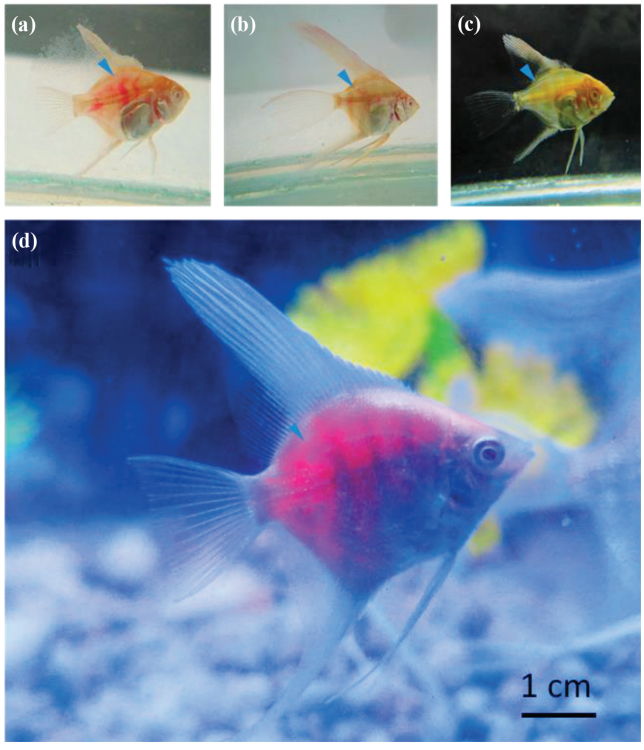


Figure 6. The founder transgenic angelfish with red fluorescent protein expression: (a-c) The expression of RFP at one month post fertilization under normal lighting (arrows); (d) The expression of RFP in founder transgene angelfish two months after fertilization under blue LED light (wavelength 400-500 nm).

Table 2. pMylz2-RFP microinjection frequency of founder embryos.

Microinjection assay	No. of microinjected eggs	Hatching rate	Embryos and larvae with RFP signals
1	167	3.04 % (5/164)	1.82 % (3/164)
2	185	3.78 % (7/185)	2.7 % (5/185)
3	172	2.33 % (4/172)	2.33 % (4/172)
Total of hatching and RFP signal larvae		3.07 % 16/521	2.30 % (12/521)
Control (No microinjected embryos)	200	56 % (112/200)	0 % (0/200)

DISCUSSION

Here, we report the successful transformation of pMylz2-RFP into one-cell angelfish embryos. However, the low survival rate of angelfish embryos after micromanipulation raises concerns and should be addressed. The hatching rate of pMylz-RFP microinjection of white skirt tetra fish (69.7 %) (Pan *et al*., 2008) was much higher than that of the microinjected angelfish from this study (2.33-3.78 %). This difference in hatching rate results could be attributed to the fact that skirt tetras (Pan *et al*., 2008) lay non-adhesive eggs, while angelfish (Thilakarathne *et al*., 2021) lay adhesive eggs. Because adhesive eggs are attached to the substrate by filaments, removing the eggs from the substrate may have effect on the cell membrane of the eggs and ultimately increase the likelihood that the embryos may be damaged before receiving the transgene.

One of the most likely reasons for the low angelfish larval survival rate in this study is that we used the angelfish albino strain. Albino fish have abnormal eye defects (red eyes) and skin pigmentation (white color). They frequently have poor light perception, making foraging difficult throughout their lifespan. Furthermore, because albino angelfish have a different skin pigmentation structure, their bodies are less resistant to harsh environmental conditions than non-albino angelfish (Slavík *et al*., 2016). Therefore, the failure to detect germline transfer may partly relate to the insufficient number of the produced angelfish founders (Pan *et al*., 2008).

There is no doubt that microinjection remains the most effective and widespread method for the delivery of DNA to fish embryos (Tanaka and Kinoshita, 2001; Kinoshita, 2004). The microinjected DNA molecules are incorporated directly into the genomes of animals. It is more successful due to its ease of integration into the genome of a cell and its ability to pass through during cell division (Penman *et al*., 1990; Dunham and Winn, 2014). It has been found that angelfish embryos remain in the single-cell stage for approximately 90 min (Thilakarathne *et al*., 2021). During this time, a substantial amount of plasmid can be transferred into the embryos. Because of this, even though embryos survived poorly following microinjection, there was a high level of red fluorescence protein expression. Consequently, we were able to generate up to 12 out of 16 surviving larvae that expressed RFP signals (Table 2).

As shown in Figures 4-6, RFP expression patterns were similar in angelfish muscles driven by the Mylz2 promoter to those in medaka (Vu *et al*., 2014) and white skirt tetra (Pan *et al*., 2008). As a result of this finding, it is apparent that the microinjected angelfish are able to successfully integrate the Mylz2 promoter and produce red fluorescence proteins.

CONCLUSION

We have described how to create angelfish founders that express red fluorescence protein. The fact that we were unable to produce transgenic germ lines in the subsequent generation suggests that there was not a sufficient number of founder angelfish to produce transgenic germ lines. As a result of the strong expression of RFP under zebrafish mylz2 promoter in angelfish founders, it is possible to produce other useful fluorescent transgenic ornamental fish using the zebrafish promoter. The present study presents the first detailed description of the transfer of a gene from an angelfish model, and should enhance the confidence in the application of transgenic technology to a wide range of ornamental fish species in the future.

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