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Development of transgenic fish for ornamental and bioreactor by strong expression of fluorescent proteins in the skeletal muscle

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Abstract

In the present study, new applications of the transgenic technology in developing novel varieties of ornamental fish and bioreactor fish were explored in a model fish, the zebrafish (*Danio rerio*). Three "living color" fluorescent proteins, green fluorescent protein (GFP), yellow fluorescent protein (YFP), and red fluorescent protein (RFP or dsRed), were expressed under a strong muscle-specific *mylz2* promoter in stable lines of transgenic zebrafish. These transgenic zebrafish display vivid fluorescent colors (green, red, yellow, or orange) visible to unaided eyes under both daylight and ultraviolet light in the dark. The level of foreign protein expression is estimated between 3% and 17% of total muscle proteins, equivalent to 4.8–27.2 mg/g wet muscle tissue. Thus, the fish muscle may be explored as another useful bioreactor system for production of recombinant proteins. In spite of the high level of foreign protein expression, the expression of endogenous *mylz2* mRNAs was not negatively affected. Furthermore, compared to the wild-type fish, these fluorescent transgenic fish have no advantage in survival and reproduction.

Keywords: Zebrafish; Generically modified organism; Green fluorescent protein; Red fluorescent protein; dsRed; Yellow fluorescent protein

The green fluorescent protein (GFP) originally isolated from the jellyfish (*Aequorea victoria*) is intrinsically fluorescent, allowing direct visualization without the need of substrate for chemical reaction. The cDNA for this protein has been cloned and modified by site-directed mutagenesis for different emission spectra and thus several artificial fluorescent color proteins become available, including yellow fluorescent protein (YFP), blue fluorescent protein (BFP), and cyan fluorescent protein (CFP) [1]. More recently, a new fluorescent protein cDNA encoding a red fluorescent protein (RFP or dsRed) has been cloned from the IndoPacific sea anemone relative (*Discosoma sp*) [2]. Due to the fact that these fluorescent proteins can be observed in live biological samples for labeling cells and subcellular organelles, these fluorescent proteins have been aptly termed "living colors" by Clontech.

The transgenic technology is widely used in biotechnology, from generation of genetically modified (GM) foods to production of pharmaceutical proteins. Inspired by the success of generation of super-mice using exogenously introduced *growth hormone* gene [3], this technology has been successfully used to develop fast-growing super-fish stocks for aquaculture. So far, fast-growing fish by transferring a growth hormone gene have been developed for several aquacultural species [4–11]; however, marketing of these transgenic food fish remains a controversial issue due to ecological and food safety concerns [12]. In this study, by taking advantages of "living color" fluorescent proteins, the feasibility of using the transgenic technology to develop novel varieties of ornamental fish was explored in zebrafish. Several stable lines of transgenic zebrafish expressing GFP, RFP, or YFP under a strong muscle-specific *mylz2* promoter were developed. These transgenic zebrafish display vivid fluorescent colors that are readily visible to unaided eyes. Meanwhile, we demonstrated that the fish muscle have a high capacity of expressing recombinant proteins without negative effect on the expression of the endogenous *mylz2* mRNA and thus may become another transgenic bioreactor system.

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Materials and methods

Production of transgenic zebrafish. Three transgenic DNA constructs used in the present studies: pMYLZ2-EGFP, pMYLZ2-RFP, and pMYLZ2-YFP were constructed by insertion of a 2-kb *mylz2* promoter into pEGFP-1, pEYFP-1, and pdsRed-1 (Clontech), respectively. *Mylz2*, short from *myosin light polypeptide 2*, is a fast skeletal muscle-specific gene [13] and was previously named *MLC2f* [14]. Transgenic zebrafish were generated by microinjection of plasmid DNAs in linearized form into embryos at 1- or 2-cell stage [15]. Transgenic lines were screened by direct observation of fluorescent protein expression in the offspring. Stable transgenic lines were established by standard breeding and confirmed by typical Mendelian inheritance ratios. These stable transgenic lines containing pMYLZ2-EGFP, pMYLZ2-RFP, and pMYLZ2-YFP are called *gfp*, *rfp*, and *yfp* transgenic fish, respectively, in this study.

Expression analysis. Estimation of the level of protein expression was carried out by SDS–polyacrylamide gel electrophoresis and quantified by the Gel-Pro Analyzer program (Media Cybernetics, USA). The content of total muscle protein was estimated by dissolving isolated muscle tissue to the SDS sample buffer (2% SDS; 7.5% glycerol; 5% 2-mercaptoethanol; and 80 mM Tris, pH 6.8) and protein concentration was determined by the method of Esen [16] using purified BSA (bovine serum albumin) (Merck) as standards. Transgenic and endogenous RNA expression was analyzed by Northern blot hybridization. Total RNAs were prepared from individual fish using Trizol reagent (Invitrogen) and autoradiograms were quantified by the Gel-Pro Analyzer program.

Survival rates. For comparison of survival rates (Table 1), eggs were collected from crosses between two hemizygous *gfp* and *rfp* transgenic zebrafish. About 25% of individuals with each of the four genotypes were obtained: wild type, *gfp*, *rfp*, and *gfp*/*rfp*. The genotypes/phenotypes were determined in fry of 3–4 day postfertilization when both RFP and GFP expression can be easily detected. These offspring from the same pair of parents were cultured in the same tank and counted again at the adult stage.

Reproduction success. Reproduction success was measured by counting the numbers of embryos produced from a pool of equal number of the following four types of spawning fish of similar size: wild-type males, wild-type females, *gfp* transgenic males, and *gfp* transgenic females. Each fish selected was pre-tested individually to ensure that it spawned actively. Eggs were collected for 7–8 consecutive days and the embryos were examined under a fluorescent microscope. The penetrance of phenotype is always 100%, as confirmed by PCR analysis.

Results

Expression of vivid fluorescent colors in stable lines of transgenic zebrafish

To generate fluorescent transgenic zebrafish, the three transgenic DNA constructs, pMYLZ2-EGFP, pMYLZ2-RFP, and pMYLZ2-YFP, were injected separately into zebrafish embryos at 1–2 cell stage. Stable transgenic lines were obtained from all of the three recombinant DNA constructs. In all transgenic lines, fluorescent proteins were highly expressed in the skeletal muscle, in a pattern faithfully mimicking the endogenous *mylz2* expression [15,17]. Due to the extremely high level of expression in the skeletal muscle and the large mass of muscle tissue, green, yellow, and red fluorescent colors were readily visualized even under normal day light (Figs. 1A and C). Under an ultraviolet light in the dark, these transgenic zebrafish displayed vivid fluorescent colors to unaided eyes (Figs. 1B and D). The transgenic fluorescent colors became visible at about four weeks postfertilization and intensified in the following weeks. Since the generation of the first *gfp* transgenic line in 1999, the *gfp* line is currently at its eighth generation, the *yfp* line at its fourth, and the *rfp* line at its sixth. In all these generations, the same intensity of fluorescence was observed in a total of a few thousand offspring produced in our laboratory, suggesting that the transgenes and fluorescent phenotypes have been stably inherited.

To investigate the possibility of creating more "rainbow" colors, double transgenic fish were obtained by crossing a *gfp* fish with an *rfp* fish. The double transgenic fish (*gfp*/*rfp*) displayed an orange color both under daylight and ultraviolet light (Fig. 1). To test the feasibility of generating more intermediate colors, muscle extracts from *gfp* fish and *rfp* fish were mixed at different proportions; a series of intermediate colors were obtained (Fig. 1E). Based on our previous observations by transient and stable transgenic assays, the level of GFP expression was related to the length of *mylz2* promoter and a weaker expression was obtained by a shorter promoter [17]. Thus, the level and spectrum of transgenic expression under the *mylz2* promoter could be further manipulated for more transgenic colors.

High level of muscle expression of foreign proteins in transgenic fish

The levels of GFP and RFP expression in *gfp* and *rfp* transgenic zebrafish were estimated by SDS–polyacrylamide gel electrophoresis. Total muscle proteins were extracted and analyzed. Both GFP and RFP were easily

Table 1
Survival rates of offspring of different genotypes between crosses of *gfp* and *rfp* transgenic zebrafish

Genotypes	*gfp*	*rfp*	*gfp*/*rfp*	wild type
Batch 1	53.1% (17/32)	64.0% (16/25)	67.9% (19/28)	66.7% (16/24)
Batch 2	68.9% (20/29)	100% (26/26)	86.9% (20/23)	86.2% (25/29)
Batch 3	43.5% (20/46)	57.4% (27/47)	54.8% (23/42)	64.3% (27/42)
Average	55.2%	73.8%	69.9%	72.4%

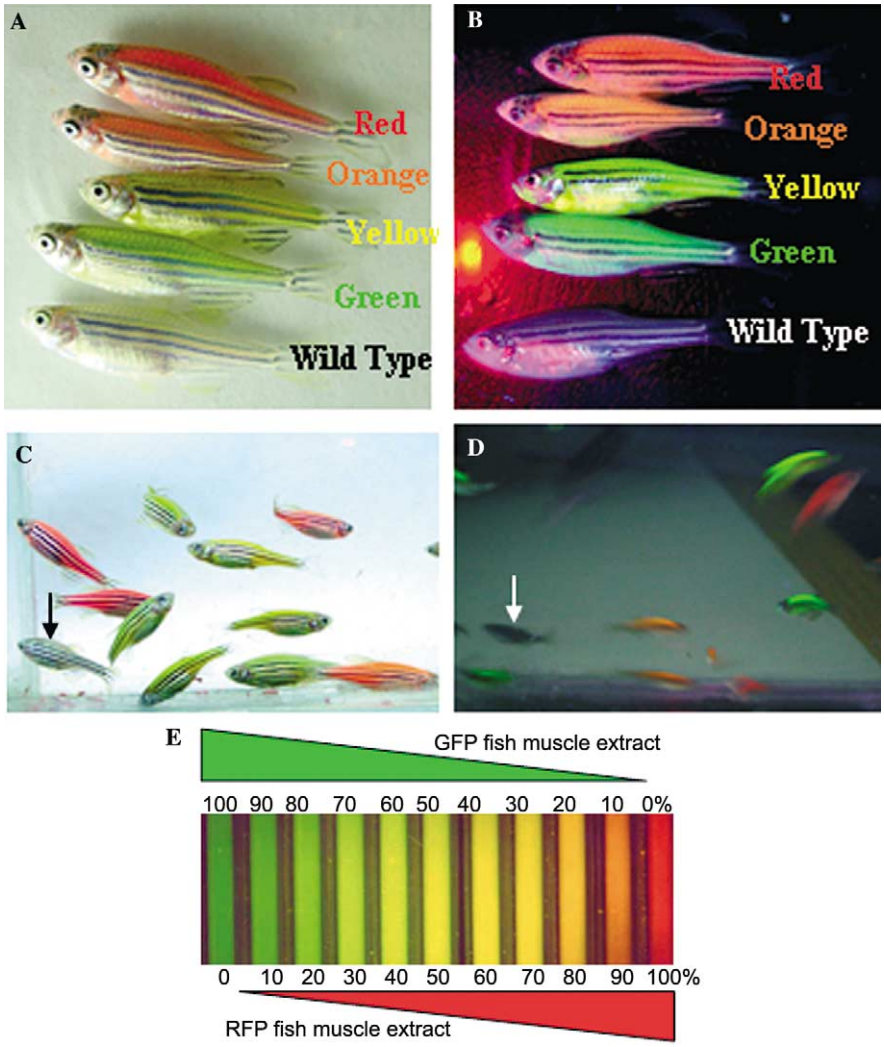

Fig. 1. Fluorescent transgenic zebrafish. (A,B) Fluorescent transgenic zebrafish in a rainbow array (top to bottom) under daylight (A) and 385 nm ultraviolet light (B). Red, *rfp* fish; orange, *rfp*/*gfp* fish; yellow, *yfp* fish; green, *gfp* fish; and wild-type fish. These fish were anesthetized in 0.1% phenoxyethanol and photographed with a digital camera. (C,D) Living color fluorescent transgenic zebrafish in swimming under daylight (C) and 385 nm ultraviolet light (D). Arrows in (C) and (D) indicate wild-type fish. (E) Intermediate colors by blending of GFP and RFP. Muscle extracts in phosphate buffered saline were prepared from *gfp* and *rfp* transgenic fish and mixed in different ratios. These mixtures were then loaded into capillary tubes and photographed under a microscope using a GFP plus filter (Leica). The proportions of GFP and RFP muscle extracts used are indicated at both the top and bottom of each tube.

identified as they appeared in the transgenic samples as additional protein bands with expected sizes (Fig. 2A). As analyzed by the Gel-Pro Analyzer program, we estimated that GFP and RFP were expressed at the level of ~3% and ~5% of total muscle proteins in *gfp* and *rfp* transgenic zebrafish (hemizygotes), respectively (Fig. 2A). In *gfp*/*rfp* double transgenic zebrafish, both levels remained the same. In homozygotes, the level of expression appeared to be doubled (Fig. 2B). Using purified bovine serum albumin as standards, we estimated that a homozygous *rfp* fish synthesizes the recombinant protein as high as 17% of total muscle proteins. We also measured the amount of protein per gram of wet muscle tissue to be about 160 mg. Thus, the transgenic fish muscle system could generate 4.8–27.2 mg of single recombinant protein in one gram of wet muscle tissue.

To examine whether endogenous *mylz2* mRNA expression is affected by such high level of transgene expression, Northern blot hybridization was carried out. As shown in Fig. 3, the endogenous *mylz2* mRNA expression was not inhibited in either male or female *gfp* transgenic zebrafish. Identical results were also obtained from the *rfp* transgenic line (data not shown). Real-time PCR analysis indicates that the *gfp* transgenic line contained only about two copies of *mylz2-gfp* transgene in a haploid genome, but the expression of *gfp* mRNA in the hemizygous transgenic fish is at least 10-fold higher than that of endogenous *mylz2* mRNA in the control fish (Fig. 3C). Therefore, these experiments demon-

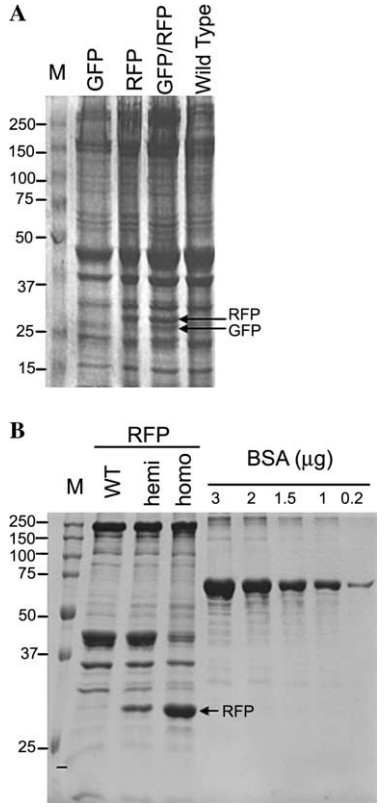

Fig. 2. Expression of GFP and RFP in transgenic zebrafish. Total muscle proteins were separated by SDS gel electrophoresis and stained with Coomassie Blue-G-250. Prestained protein standards (lane M) were used and molecular weights (Bio-Rad) are shown on the left of each panel. (A) Expression of GFP and RFP in *gfp*, *rfp*, and *gfp*/*rfp* transgenic zebrafish. GFP and RFP are indicated by arrows. (B) Quantification of the level of RFP expression in *rfp* transgenic zebrafish. 10 μg of protein each from wild type (WT), hemizygous (hemi), and homozygous (homo) *rfp* transgenic zebrafish was loaded on each lane. Different amounts of purified BSA, as indicated at the top of each lane, were loaded for a standard curve. The gels were analyzed by Gel-Pro Analyzer program. The amount of recombinant RFP was estimated at ~0.5 μg for the hemizygous sample and 1.7 μg for the homozygous sample.

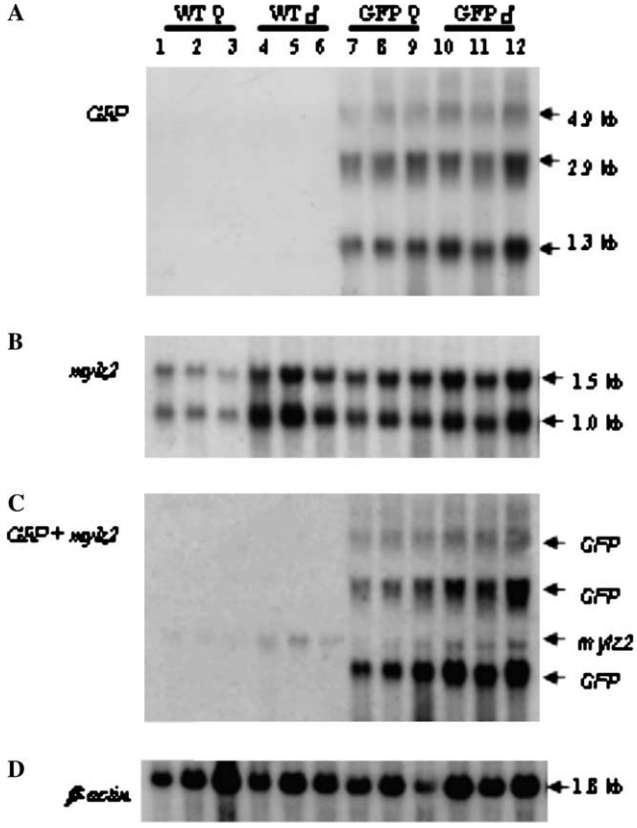

Fig. 3. No reduction of endogenous *mylz2* mRNA expression by high level of transgenic expression. Total RNAs were prepared from whole fish of three each of wild-type female, wild-type male, *gfp* transgenic female, and *gfp* transgenic male. Northern blot analysis was performed on the same RNA blot that was hybridized with *gfp* (A), *mylz2* (B), *gfp*/*mylz2* mix (C), and β-*actin* (D) probes, respectively, after the blot was repeatedly striped. Two *mylz2* transcripts were detected due to differential polyadenylation sites (B). To compare the relative expression levels of *mylz2* and *gfp* mRNAs, the same length of probes for *mylz2* (657 bp) and *gfp* (657 bp) was generated by PCR and labeled to the same specific activity in the same reaction tube with equal quantity of DNA templates. The autoradiogram is shown in (C) and the relative intensities should represent the relative levels of *mylz2* and *gfp* mRNA expression. The *mylz2* probe used in (C) was designed in the 3′UTR and only the 1.5-kb transcript was hybridized. The level of the three major *gfp* mRNAs is at least 10-fold higher than that of endogenous *mylz2* mRNA as estimated by the Gel-Pro Analyzer. The phenotype and sex of each fish are indicated at the top of each lane, the probes used are indicated on the left, and the size and names of the transcripts on the right.

strated that high level of muscle expression of a foreign gene can be achieved without any compromise of endogenous gene expression. Consistent with this, we did not notice any abnormal swimming behaviour and morphology associated with the transgenic fish.

Lack of evidence for enhanced fitness in fluorescent transgenic fish

Previously, Muir and Howard [18] proposed a Trojan gene hypothesis on large-sized *growth hormone* transgenic fish based on a mathematic model. According to this hypothesis, if the larger *growth hormone* transgenic fish have a lower viability and a favored mating behavior, accidental release of such transgenic fish will cause the extinction of the wild-type population in about 40 generations. To investigate whether the transgenesis will affect survival and reproduction of our fluorescent transgenic fish, both survival rates and the number of eggs produced were compared between transgenic and wild-type fish. The survival rates of transgenic fish were assessed from the offspring of crosses between a hemizygous *gfp* fish and a hemizygous *rfp* fish under our laboratory condition. These crosses produced four genotypes/phenotypes at a ratio of 1:1:1:1, wild type, *gfp* (green), *rfp* (red), and *gfp*/*rfp* (orange). As shown in Table 1, with the exception that *gfp* fish appeared to have a lower survival rate, there was no apparent

difference in survival rates among the other three phenotypes. To evaluate the reproduction success, a population of equal numbers of wild type and hemizygous *gfp* transgenic fish was maintained for breeding for a week (see Materials and methods). The expected ratio (43.75%) of transgenic offspring based on Mendelian genetics was observed (43.60%, 1158 out of 2656). Similar result was obtained from a second experiment (40.60%, 2121 out of 5224). Thus, there is no indication that the fluorescent transgenic fish would have any reproductive advantage. Overall, these experiments provided no evidence that the acquirement of fluorescent colors could bring about a better fitness for these transgenic fish.

Discussion

In the present study, the zebrafish was explored as a model to demonstrate the feasibility of using transgenic technology to generate new varieties of ornamental fish. It is obvious that this technology can be applied to other more exotic ornamental fish species, such as goldfish, Japanese koi (carp), etc. Using the same zebrafish promoter construct, pMYLZ2-EGFP, we recently generated a transgenic medaka line that also displayed visible green fluorescent color like *gfp* transgenic zebrafish (Z. Zeng and Z. Gong, unpublished). In the ornamental fish industry, the color pattern is also important. By using different tissue-specific promoters, these color proteins could be targeted into different tissues or body parts. Particularly, different variety of color patterns can be achieved from different combinations of tissue-specific promoters and color proteins. As only a limited number of new varieties of ornamental fish can be produced currently by classical breeding, the transgenic approach will be a new avenue for rapid production of novel ornamental fish. Furthermore, once the fluorescent transgenic fish is available for one strain, it is easy to transfer the transgene to other useful strains within the same species by standard genetic breeding for more varieties of fluorescent transgenic fish. Currently more and more fluorescent protein genes have been cloned from reef coral species [19] and the choice of original colors widens. New color can be further blended genetically as shown in this study (Fig. 1E). Thus, the application of transgenic technology in ornamental fish is promising.

The success of generating transgenic zebrafish with visible fluorescent colors is due to an unusually high level of expression of these fluorescent proteins in the muscle tissues. The promoter we used in the present study is derived from the muscle-specific *mylz2* gene. Previously, by analysis of transcript profile using an EST approach, we have shown that *mylz2* clone is the most abundant one in both zebrafish embryos and adults [20]. Thus, the *mylz2* promoter is likely the strongest muscle-specific promoter. So far, *gfp* transgenic zebrafish have been produced using many different tissue-specific promoters (e.g. [21–28]), but none of these transgenic lines display fluorescent color visible to unaided eyes. Thus, one key to success in the generation of colorful transgenic ornamental fish is in the strength of the promoter. Another factor is the selection of tissue; the muscle constitutes majority of the body and thus synthesizes more and visible color proteins. In contrast, transgenic GFP expression in only a single layer of skin cells cannot be visualized without using a fluorescent microscope [27].

At present, marketing of transgenic food fish is facing two major challenges, environmental concerns and food safety [12]. In contrast, the ornamental fish aquaculture is generally practiced in small and well-contained environments, and most ornamental fish species cannot survive in the wild after a long period of domestic selection and breeding. Our current study indicated that the fluorescent transgenic fish has no advantages in survival and reproduction. A preliminary study on mate choice between wild type and fluorescent transgenic zebrafish, based on a dichotomous choice test on guppies [29], also indicated the lack of mating advantage of transgenic zebrafish (W.K. Seah, D. Li, M. Chen, and Z. Gong, unpublished). Thus, the culturing of GM ornamental fish should be less concerned with regard to environmental and ecological issues. Moreover, there is no food safety issue for ornamental fish. Therefore, marketing of transgenic ornamental fish is a more viable concept.

Another important finding of this study is the extremely high level of muscle expression of foreign proteins in the transgenic fish. We have estimated that the fish muscle has a capacity of producing up to 27 mg of foreign protein per gram of wet tissue. This is comparable to or greater than the level of recombinant protein expression (~10 mg/ml) in the popular mammary gland system in transgenic farm animals [30] and is significantly higher than that (~1 μg/ml) in the egg white of transgenic hen [31]. Hence, the fish muscle could be developed to another transgenic bioreactor system. The advantages of the transgenic fish bioreactor include the speed of generation of transgenic fish, low cost, and lack of risk of transferring mammalian viruses and prions, etc. Furthermore, the fish muscle is edible and raw meat may be directly consumed for better preservation of bioactivity; thus, nutritional and pharmaceutical/therapeutical supplements could be expressed in the fish muscle system. To create a more successful fish bioreactor system, fast-growing and large size farm fish such as carp, tilapia, catfish, salmon, and rainbow trout may be used. For all these species, the transgenic technology is well developed [3–11].

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References

[1] M. Chalfie, S. Kain, Green fluorescent protein: properties, applications, and protocols, Wiley-Liss, New York, 1998.
[2] M.V. Matz, A.F. Fradkov, Y.A. Labas, A.P. Savitsky, A.G. Zaraisky, M.L. Markelov, S.A. Lukyanov, Fluorescent proteins from nonbioluminescent *Anthozoa* species, Nat. Biotechnol. 17 (1999) 969–973.
[3] R.D. Palmiter, R.L. Brinster, R.E. Hammer, M.E. Trumbauer, M.G. Rosenfeld, N.C. Birnberg, R.M. Evans, Dramatic growth of mice that develop from eggs microinjected with metallothionein-growth hormone fusion genes, Nature 300 (1982) 611–615.
[4] S.J. Du, Z. Gong, F.L. Fletcher, M.A. Shears, M.J. King, D.R. Ider, C.L. Hew, Growth enhancement in transgenic Atlantic salmon by the use of an "all fish" chimeric growth hormone gene construct, Bio/Technology 10 (1992) 176–180.
[5] R.H. Devlin, T.Y. Yesaki, C.A. Blagl, E.M. Donaldson, P. Swanson, W.K. Chan, Extraordinary salmon growth, Nature 371 (1994) 209–210.
[6] R.H. Devlin, T.Y. Yesaki, E.M. Donaldson, S.J. Du, C.L. Hew, Production of germline transgenic Pacific salmonids with dramatically increased growth performance, Can. J. Fish. Aqua. 52 (1995) 1376–1384.
[7] R. Martinez, M.P. Estrada, J. Berlanga, I. Guillen, O. Hernandez, E. Cabrera, R. Pimentel, R. Morales, F. Herrera, A. Morales, J.C. Pina, Z. Abad, V. Sanchez, P. Melamed, R. Lleonart, J. de la Fuente, Growth enhancement in transgenic tilapia by ectopic expression of tilapia growth hormone, Mol. Mar. Biol. Biotechnol. 5 (1996) 62–70.
[8] M.A. Rahman, R. Mak, H. Ayad, A. Smith, N. Maclean, Expression of a novel piscine growth hormone gene results in growth enhancement in transgenic tilapia (*Oreochromis niloticus*), Transgenic Res. 7 (1998) 357–369.
[9] R.A. Dunham, C. Chitmanat, A. Nichols, B. Argue, D.A. Powers, T.T. Chen, Predator avoidance of transgenic channel catfish containing salmonid growth hormone gene, Mar. Biotech. 1 (1999) 545–551.
[10] C.H. Fu, Y.B. Cui, S.S.O. Hung, Z. Zhu, Whole-body amino acid pattern of F-4 human growth hormone gene-transgenic red common carp (*Cyprinus carpio*) fed diets with different protein levels, Aquaculture 189 (2000) 287–292.
[11] R.H. Devlin, C.A. Biagi, T.Y. Yesaki, D.E. Smailus, J.C. Byatt, Growth of domesticated transgenic fish, Nature 409 (2001) 781–782.
[12] E. Stokstad, Engineered fish: friend or foe of the environment? Science 297 (2002) 1797–1798.
[13] Y. Xu, J. He, X. Wang, T.M. Lim, Z. Gong, Asynchronous activation of 10 muscle specific protein (MSP) genes during zebrafish somitogenesis, Dev. Dyn. 219 (2000) 201–215.
[14] Y. Xu, J. He, H.L. Tian, C.H. Chan, J. Liao, T. Yan, T.J. Lam, Z. Gong, 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 Cell Biol. 18 (1999) 85–95.
[15] B. Ju, Y. Xu, J. He, J. Liao, T. Yan, C.L. Hew, T.J. Lam, Z. Gong, Faithful expression of Green Fluorescent Protein (GFP) in transgenic zebrafish embryos under control of zebrafish gene promoters, Dev. Genet. 25 (1999) 158–167.
[16] A. Esen, A simple method for quantitative, semiquantitative, and qualitative assay of protein, Anal. Biochem. 89 (1978) 264–273.
[17] B. Ju, S.W. Chong, J. He, X. Wang, Y. Xu, H. Wan, Y. Tong, T. Yan, V. Korzh, Z. Gong, Recapitulation of fast skeletal muscle development in zebrafish by transgenic expression of GFP under the mylz2 promoter, Dev. Dyn. 227 (2003) 14–26.
[18] M.W. Muir, R.D. Horward, Possible ecological risks of transgenic organism release when transgenes affect mating success: sexual selection and the Trojan gene hypothesis, Proc. Natl. Acad. Sci. USA 96 (1999) 13853–13856.
[19] Y.A. Labas, N.G. Gurskaya, Y.G. Yanushevich, A.F. Fradkov, K.A. Lukyanov, S.A. Lukyanov, M.V. Matz, Diversity and evolution of the green fluorescent protein family, Proc. Natl. Acad. Sci. USA 99 (2002) 4256–4261.
[20] Z. Gong, T. Yan, J. Liao, S.E. Lee, J. He, C.L. Hew, Rapid identification and isolation of zebrafish cDNA clones, Gene 201 (1997) 87–98.
[21] Q. Long, A. Meng, H. Wang, J.R. Jenssen, M.J. Farell, S. Lin, GATA-1 expression pattern can be recapitulated in living transgenic zebrafish using GFP reporter gene, Development 124 (1997) 4105–4111.
[22] S. Higashijima, H. Okamoto, N. Ueno, Y. Hotta, G. Eguchi, High-frequency generation of transgenic zebrafish which reliably express GFP in whole muscles or the whole body by using promoters of zebrafish origin, Dev. Biol. 192 (1997) 289–299.
[23] B.N. Kennedy, T.S. Vihtelic, L. Checkley, K.T. Vaughan, D.R. Hyde, Isolation of a zebrafish rod opsin promoter to generate a transgenic zebrafish line expressing EGFP in rod photoreceptors, J. Biol. Chem. 276 (2001) 14037–14043.
[24] H. Huang, S.S. Vogel, N. Liu, D.A. Melton, S. Lin, Analysis of pancreatic development in living transgenic zebrafish embryos, Mol. Cell. Endocrinol. 177 (2001) 117–124.
[25] S.J. Du, M. Dienhart, Zebrafish tiggy-winkle hedgehog promoter directs notochord and floor plate green fluorescence protein expression in transgenic zebrafish embryos, Dev. Dyn. 222 (2001) 655–666.
[26] D. Goldman, M. Hankin, Z. Li, X. Dai, J. Ding, Transgenic zebrafish for studying nervous system development and regeneration, Transgenic Res. 10 (2001) 21–33.
[27] Z. Gong, B. Ju, X. Wang, J. He, H. Wan, P.M. Sudha, T. Yan, Green fluorescent protein expression in germ-line transmitted transgenic zebrafish under a stratified epithelial promoter from *keratin8*, Dev. Dyn. 223 (2002) 204–215.
[28] G.M. Her, C.-C. Chiang, W.-Y. Chen, J.-L. Wu, In vivo studies of liver-type fatty acid binding protein (L-FABP) gene expression in liver of transgenic zebrafish (*Danio rerio*), FEBS Lett. 538 (2003) 125–133.
[29] A.E. Houde, Sex, color, and mate choice in guppies, Princeton University Press, Princeton, NJ, 1997.
[30] C.A. Ziomek, Commercialization of proteins produced in the mammary gland, Theriogenology 49 (1998) 139–144.
[31] A.J. Harvey, G. Speksnijder, L.R. Baugh, J.A. Morris, R. Ivarie, Expression of exogenous protein in the egg white of transgenic chickens, Nat. Biotechnol. 19 (2002) 396–399.