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Enhancement of EPA and DHA biosynthesis by over-expression of masu salmon Δ6-desaturase-like gene in zebrafish

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Abstract

The *n* − 3 polyunsaturated fatty acids, especially eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) have important nutritional benefits in humans. Farmed fish could serve as promising sources of EPA/DHA, but they need these fatty acids or their precursors in their diets. Here we transferred masu salmon Δ6-desaturase-like gene in zebrafish to increase its ability for synthesizing EPA and DHA. Expression of this gene in transgenic fish elevated their EPA content by 1.4-fold and DHA by 2.1-fold. On the other hand, the α-linolenic acid (ALA) content decreased, it being a substrate of Δ6-desaturase, while the total lipid remained constant. This achievement demonstrates that fatty acid metabolic pathway in fish can be modified by the transgenic technique, and perhaps this could be applied to tailor farmed fish as even better sources of valuable human food.

Introduction

The *n* − 3 polyunsaturated fatty acids, especially eicosapentaenoic acid (EPA, C20:5*n* − 3) and docosahexaenoic acid (DHA, C22:6*n* − 3) have important nutritional benefits in humans (Simopolous, 1991; Lauritzen et al., 2001). They are predominantly derived from fish, but the decline in the stocks of major marine capture fisheries could result in these fatty acids being consumed less (Sargent and Tacon, 1999). Farmed fish could serve as promising sources of EPA and DHA, but they need these fatty acids or their precursors in their diets. Δ6-desaturase is a fatty acid metabolic enzyme that is involved in EPA and DHA biosynthesis. This enzyme uses α-linolenic acid (ALA, C18:3*n* − 3) as a substrate and allows the insertion of a double bond to produce octadecatetraenoic acid (OTA, C18:4*n* − 3). The OTA is converted by elongase to produce eicosatetraenoic acid (ETA, C20:4*n* − 3), a substrate of Δ5-desaturation in the EPA synthesis. In human (de Antueno et al., 2001) and mouse (D'Andrea et al., 2002), the same Δ6-desaturase also metabolizes the fatty acid C24:5*n* − 3 to C24:6*n* − 3, which serves as substrate for *β*-oxidation that produces DHA (Sprecher, 2000). In this study, we designed a gene construct, designated as pActD6 comprising of the masu salmon *Oncorhynchus masou* Δ6-desaturase-like gene (OmΔ6FAD) driven by medaka *Oryzias latipes* *β*-actin gene promoter. The gene construct was microinjected into one-cell embryos of zebrafish and their expression pattern and fatty acids composition in the resulting transgenic fish was analyzed.

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Materials and methods

Zebrafish maintenance and diets

Fish were spawned and cultured as described previously (Westerfield, 1995), with some modifications.

Non-transgenic and transgenic F_2 generation were reared in the same fish tank and fed on a commercial diet 'Otohime' (Nisshin Co., Tokyo, Japan) and *Artemia* (Salt Creek Inc., UT, USA) nauplii once daily, respectively.

Transgene construction

The transgene was provided as an 8.5 kb plasmid pActD6. Briefly, the bovine growth hormone polyadenilation (BGH poly (A)) sequence was removed from the pRc/RSV (Invitrogen, San Diego, CA) by digestion with *Xho*I and ligated into pBluescript SK (+/−) (Stratagene, Tokyo, Japan). The 3.7 kb of medaka β-Actin (Act) promoter was modified by PCR from pOBA-109 (Takagi et al., 1994) to provide an *Eco*RI site for ligation into pBluescript SK (+/−) containing BGH poly (A). The 1.5 kb of masu salmon Δ6-desaturase-like gene (OmΔ6FAD, GenBank Accession Number: AB070444) was amplified by PCR with two oligonucleotides primers: the forward primer '*Sal*I-desF3' (5′-TTGTCGACGGTGTGAGTGGAGCAGAG AGAA-3′) contained a *Sal*I recognition site (underlined), and reverse primer 'des-OmaR' (5′-ATCCAGGAAATGTCCTCTCTGTTCGCA-3′). The amplified products were cloned into the pGEM-T Easy vector (Promega, Madison, USA), digested with *Sal*I and then the Δ6-desaturase-like gene fragment was inserted between the Act and BGH poly (A) sequence to produce the pActD6 construct.

Production of stable transgenic fish

Transgenic zebrafish carrying the pActD6 construct was generated by microinjecting 1-cell embryos, with a construct concentration of 30 μg/ml in 0.1 M KCl/0.125% tetramethyl-rhodamine dextran (Meng et al., 1999). Transgenic individuals F_0 were identified by PCR with DNA template that had been extracted from caudal fins. Isolation of genomic DNA was performed using a DNA Isolation Kit (Puregene, Minneapolis, USA) according to the manufacturer's instructions. PCR analysis was performed in 20 μl of 10 × *Ex Taq* Buffer, 200 μM of dNTPs, 0.125 U of *Ex Taq* polymerase (Takara, Shiga, Japan), 2 μl of DNA as template and 1 pmol of each primer. The primer sequences for OmΔ6-FAD and β-actin gene were as follows: 'Fw-Omar' Forward (5′-AGGACTGGCTCACCATG CAGTTGAGT-3′) and 'des-Omar' Reverse (5′-ATCCAGGAAATGTCCTCTCTGTTCGCA-3′); β-actin 'actinZF-F2' Forward (5′-CCTTCCA-CCATGAAGATCAAGATCAT-3′), β-actin 'actinZF-R2' Reverse (5′-TCGTACTC-CTGCTT GCTGATCCAC-3′). A total of 32 cycles of denaturation for 30 s at 94 °C, annealing (at 62 °C for desaturase-like gene; at 58 °C for β-actin gene) for 30 s, and extension at 72 °C for 30 s were performed. The β-actin gene was used as an internal control for loading the genomic DNA. Two micro litre from the reaction was electrophoretically separated using 2% agarose gel, stained with ethidium bromide, and photographed under UV light. The matured DNA-positive F_0 was crossed with a non-transgenic zebrafish, and at least twenty 2-day-old larvae from the cross were pooled and used for genomic DNA isolation to identify the germ line transmitter fish. The germ lines-positive F_0 fish were then used to produce F_1 and F_2 generations. Production and screening procedures for F_1 and F_2 were the same as employed for F_0.

RNA isolation and real-time PCR

The levels of transgene mRNA in transgenic fish were quantitatively analyzed using real-time (RT)-PCR. Total RNA was extracted from different kinds of freshly collected tissues. RNA extraction and cDNA synthesis were performed as described previously (Boonanuntanasarn et al., 2002). Amplification of cDNA samples was performed using the iQ SYBR Green Supermix RT-PCR Kit (Bio-Rad Laboratories, Hercules, CA) in an iCycler Real-Time Detection System (Bio-Rad Laboratories) according to the manufacturer's instructions and under the following conditions: 95 °C for 3 min, followed by 40 cycles of 20 s at 95 °C, 20 s at 62 °C (at 59 °C for β-actin), and 20 s at 72 °C. The primer sequence for OmΔ6FAD gene was 'Fw-Omar-2' Forward (5′-AGAAAGGCATGACTGATGTTG TCA-3′) and 'des-Omar' Reverse (5′-ATCCA-GGAAATG TCCTCTCTGTTCGCA-3′). The β-actin gene expression was analyzed as an internal control of equal loading RNA with a set primer 'actinZF-F2' Forward and 'actinZF-R2' Reverse as mentioned above. Data were analyzed using

the comparative cycle threshold (CT) method (Pfaffl, 2001), where CT is defined as the cycle number at which fluorescence reaches a set threshold value.

Fatty acid analysis

Total lipid was extracted from a pool of 3–4 adults in triplicates for each non-transgenic and F_2 transgenic fish using the chloroform–methanol (2:1) method as described by Folch et al. (1957). Lipids were saponified by using 1 ml of 50% KOH in 15 ml ethanol and heated for 40 min at 80 °C. The saponifiable matter was then esterified using 6.7% of BF_3 in methanol and heated for 20 min at 80 °C (Morrison & Smith, 1964). Fatty acid methyl esters (FAME) was diluted in hexane (20 mg ml^{-1}) and analyzed by gas liquid chromatography (GLC) (GC-14B; Shimadzu, Kyoto, Japan) equipped with a hydrogen flame ionization detector and a silica capillary column (30 m × 0.32 mm × 0.25 μm film thickness, SUPELCOWAX^{TM}-10 FUSED SILICA, Supelco, Bellefonte, USA). Helium was used as the carrier gas and the pressure was adjusted to 100 kPa. Temperature in the column, injection port, and detector was adjusted to 205, 250 and 250°C, respectively. Fatty acids methyl ester peaks were identified by comparison of their retention times with an appropriate FAME standard (PUFA-3, Supelco, Bellefonte, USA).

Statistical analysis

Statistical analyses were conducted by using the SPSS 11.0 microcomputer software package (SPSS, Chicago, IL, USA). Analysis of variance was performed by a one-way ANOVA, followed by Duncan's test. Level of significance was set at $p = 0.05$.

Results and discussion

A total of 109 fish survived to adult from among the 400 injected embryos. The transgenic founders F_0 were screened from among the surviving fish by PCR amplification of genomic DNA extracted from their fins using primers specific for OmΔ6FAD gene. As a result, we obtained 16 fish carrying masu salmon Δ6-desaturase-like gene in their genome, and 4 of them carried the pActD6 gene construct in their germ lines (Figure 1). These germ line transmitters were then used to produce F_1 and F_2 generations. The transmission rate of transgene into F_1 generation varied between 4.2% and 44.1%, confirming that the transgenic F_0 were mosaic. The survival rates of all transgenic strains did not show any significant difference (data not shown). Transgene expression in F_1 generation was examined by reverse transcriptase PCR amplification with cDNA synthesized from RNA that had been extracted from fin. Transgenic F_1 having high mRNA transcription levels analyzed by semi-quantitative reverse transcription PCR (data not shown) were selected and used to produce F_2 generation. The transmission of transgene into F_2 generation followed the Mendelian segregation pattern. Here, we reported the expression levels of transgene from two F_2 transgenic strains, i.e. pActD6 10-1 and 15-2 strain. As shown in Figure 2, transgene was expressed in all the examined tissues, the surprising exceptions being the liver and intestine in the 15−2 strain. This is

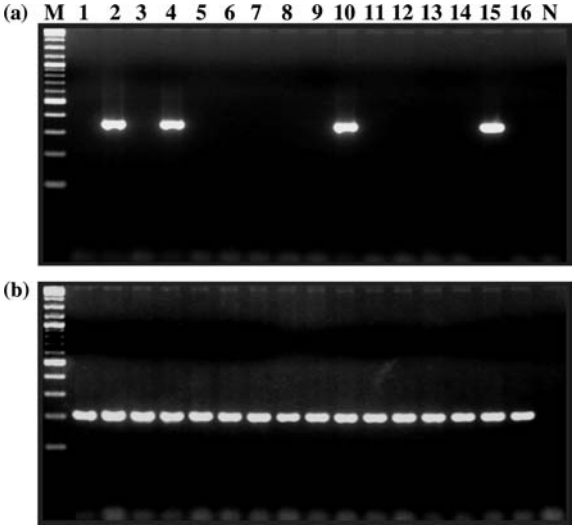

Figure 1. Result of F_0 germ line transmitter screening by PCR analysis with DNA template extracted from pooled-larvae. DNA was extracted from about twenty 2-day-old larvae obtained by crossing the DNA-positive F_0 with non-transgenic individuals. (a) PCR product amplified using genomic DNA with OmΔ6FAD gene specific primers. Lanes 1 through 16, PCR product amplified using genomic DNA samples from transgenic F_0 individuals; lane N, a sample PCR product without DNA template. M is 2-log ladder DNA marker (BioLabs, Inc., New England). The amplified fragment is 354-bp in size. (b) PCR product with β-actin gene primers [200-bp].

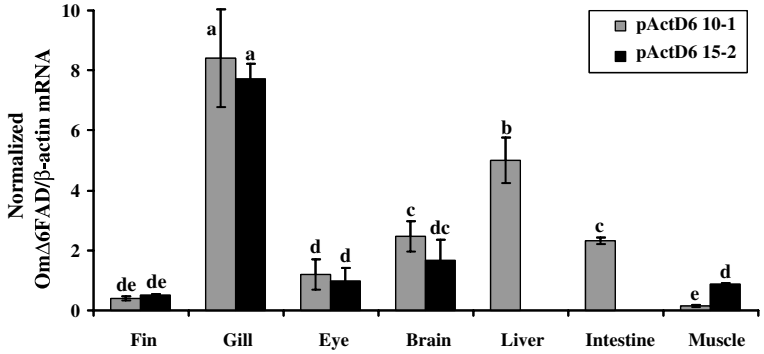

Figure 2. Transgene mRNA level in different tissues of the two transgenic strains was determined by real-time PCR as described in the Materials and methods. The amount of transgene mRNA was normalized to β-actin mRNA. The PCR template for β-actin gene was diluted 100×. Data represented are means ± SD from two individual transgenic fish performed in triplicate PCR. Values with different superscript letters are statistically significant at ($p < 0.05$) as determined by one-way ANOVA followed by Duncan's test.

probably because the transgene may have been integrated into the chromosomal site that suppresses its expression in these tissues due to the positional effect (Dobie et al., 1996).

In order to ascertain the benefit of the successful expression of the transgene, we examined the fatty acid composition of both non-transgenic and transgenic fish after they were fasted for 36 h. Fatty acid methyl esters from total lipid extracted from the fish were analyzed by GLC. Total lipid content and $n-3$ fatty acids compositions of the experimental fish are shown in Table 1. There was no significant difference ($p < 0.05$) in the amount of total lipid in non-transgenic and transgenic fish. The EPA content in whole body of transgenic fish having high transgene expression level (fish strain pActD6 15-2) was 1.4-fold (1.86 versus 1.32 mg/g), DPA was 2.6 fold (0.66 versus 0.25 mg/g), and that of DHA was 2.1 fold (4.62 versus 2.22 mg/g) higher ($p < 0.05$) than those in non-transgenic fish. About 75% (1.40 mg/g), 86% (0.57 mg/g), and 79% (3.63 mg/g) of EPA, DPA and DHA was distributed in muscle, respectively. There was a difference in the amount of EPA, DPA and DHA produced between the transgenic lines (pActD6 10-1 and pActD6 15-2) and it needs to be verified if such variations would prevail among new lines too. The transgenic fish appear to be normal, healthy and indistinguishable from non-transgenic zebrafish at least in their external morphology.

While the EPA and DHA contents of transgenic fish carrying the OmΔ6FAD gene dramatically rose, there was a decrease in the ALA content, particularly in pActD6 15-2 strain. This suggests that the transgene has been active in the conversion of ALA, resulting in greater amounts of the highly unsaturated fatty acids. A high expression of foreign Δ6-desaturase-like in transgenic fish may also stimulate the endogenous gene expression in the subsequent pathways, thereby increasing the levels of the end products. In fact, it has been reported that the activity of an enzyme involved earlier in the desaturation/elongation pathway of the fatty acid metabolism in turbot cell line affected the apparent activity of a subsequent enzyme (Ghioni et al., 1999). In addition, we have also isolated another form of desaturase-like gene from masu salmon (GenBank Accession Number: AB074149) that is phylogenetically clustered together with Δ6-desaturase-like gene from rainbow trout and Δ5-desaturase gene from Atlantic salmon (Zheng et al., 2004). Production of transgenic zebrafish carrying this masu salmon desaturase-like gene form-2 is ongoing in our laboratory. Comparison of fatty acid contents between the two transgenic zebrafish with different form of desaturase-like gene from masu salmon may clarify the specific activity of those desaturase-like genes. Further, whether the OmΔ6FAD used in this study has a Δ5-desaturase activity such as zebrafish desaturase (Hasting et al., 2001) remains to be elucidated.

Transgenic fish capable of producing high levels of EPA and DHA in muscle has implications for effective strategies to create a 'nutritionally-richer' fish for human consumption. The two

Table 1. Total lipid and $n-3$ fatty acid compositions (mg/g wet weight) in whole body of non-transgenic and transgenic zebrafish carrying pActD6 construct

Fish strain	Total lipid	$n-3$ fatty acid composition					
		ALA	OTA	ETA	EPA	DPA	DHA
Non-transgenic	56.43 ± 2.97[a]	5.30 ± 0.29[a]	0.54 ± 0.02[a]	0.30 ± 0.00[a]	1.32 ± 0.05[a]	0.25 ± 0.00[a]	2.22 ± 0.08[a]
		(9.39 ± 0.03)	(0.96 ± 0.01)	(0.54 ± 0.03)	(2.34 ± 0.05)	(0.44 ± 0.02)	(3.95 ± 0.14)
pActD6 10-1	61.67 ± 2.20[b]	5.91 ± 0.25[b]	0.63 ± 0.03[b]	0.44 ± 0.04[b]	1.70 ± 0.25[b]	0.40 ± 0.01[b]	3.01 ± 0.09[b]
		(9.58 ± 0.09)	(1.02 ± 0.02)	(0.71 ± 0.05)	(2.74 ± 0.31)	(0.65 ± 0.03)	(4.88 ± 0.16)
pActD6 15-2	55.33 ± 1.25[a]	4.30 ± 0.15[c]	0.43 ± 0.02[c]	0.42 ± 0.01[b]	1.86 ± 0.03[b]	0.66 ± 0.04[c]	4.62 ± 0.22[c]
		(7.77 ± 0.17)	(0.78 ± 0.03)	(0.75 ± 0.01)	(3.37 ± 0.06)	(1.19 ± 0.06)	(8.36 ± 0.42)

Transgenic (pActD6 10-1 and pActD6 15-2 are independent strains) and non-transgenic fish were maintained in the same aquaria and offered one and the same commercial feed. If the fish were possessing similar fatty acid metabolic capabilities, the body fatty acid profiles may have been alike. However, this is not the case, proving the success of the transgenic manipulation. The total fatty acids were extracted from the whole fish and the fatty acid methyl esters were analyzed and quantified by gas liquid chromatography. *Abbreviations*: ALA – α-linolenic acid (C18:3$n-3$); OTA – octadecatetraenoic acid (C18:4$n-3$); ETA – eicosatetraenoic acid (C20:4$n-3$); EPA – eicosapentaenoic acid (C20:5$n-3$); DPA – docosapentaenoic acid (C22:5$n-3$); DHA – docosahexaenoic acid (C22:6$n-3$). The lipid and fatty acid contents are given as means ± SD of triplicate samples, each sample constituted by 3–4 fish. SD = 0.0 implies an SD < 0.005. Values within parentheses are the contents of the respective fatty acid expressed as percentage of the total fatty acids. Different superscript letters in the same column indicate significant differences ($p < 0.05$).

fatty acids are important components of the human diet because they contribute to several aspects of health, including the development of the infant brain, the function of the eye and the prevention of cardiovascular disease (Simopolous, 1991; Lauritzen et al., 2001).

Diets for farmed fish generally contain fish oil. However, the rapid decline in harvests of 'low-value' pelagic fish used for oil extraction such as sardines, anchovy, and sand eels (Sargent & Tacon, 1999), has made fish oil a scarce commodity and consequently plant oils are increasingly employed in fish diets. Dietary lipids are easily profiled in the tissue fatty acid and the inclusion of high amounts of plant oils has reduced the quality of flesh in terms of EPA and DHA contents (Tocher et al., 2000; Bell et al., 2001, 2002). Currently, a subsequent 'wash out' step is being recommended to retain the flesh quality of fish fed on plant oil containing diets (Bell et al., 2003a). Our findings demonstrate a possible new strategy to reduce or may eliminate fish oils in fish diets, besides doing away with the necessity for pre-marketing altered dietary regimes. This also allows the growing aquaculture industry to depend less on ocean fisheries stocks as ingredients for aquatic diets. Furthermore, when transgenic technique of the kind developed here is adopted for farmed fish, diet costs could substantially be kept lower since cheaper plants sources rich in ALA would be nutritionally adequate for them.

The transgenic technological break-through achieved here may also have a major impact on larviculture. Currently, seed production of marine fish is almost entirely dependent on live food organisms, principally rotifer *Brachionus plicatilis* and *Artemia*. However, they are naturally poor sources of EPA and DHA (Takeuchi, 2001; Bell et al., 2003b), though the two fatty acids are required for normal growth and development of marine fish larvae. These live foods are fortified with these essential fatty acids to make them nutritionally adequate for fish larvae. However, this is time-consuming, laborious and costly. Further, the rotifers can become weak when they are enriched with $n-3$ highly unsaturated fatty acids methyl ester (Takeuchi, 2001) or in the case of *Artemia*, it is very difficult to achieve an ideal enrichment level (Takeuchi, 2001), because these organisms rapidly retro-convert DHA into EPA

(Navarro et al., 1999). On the other hand, both these live foods contain a relatively high level of ALA, which can serve as a substrate for Δ6-desaturase. Thus, when the transgenic fish with the altered capabilities of synthesizing EPA and DHA are employed, the process of enriching rotifer and *Artemia* with these fatty acids could become a redundant technique in the marine seed production process.

A high level of EPA and DHA content in transgenic fish may probably be of physiological advantage for fish. The inclusion of the two fatty acids in fish diets are known to improve the quality of broodstocks, and the growth and normal development of larvae (Watanabe, 1983; Furuita et al., 2000; Copeman et al., 2002; Mazorra et al., 2003; Watanabe & Vassallo-Agius, 2003). Moreover, the highly unsaturated fatty acids have been shown to promote tolerance to handling stress (Koven et al., 2001), increase cellular (Wu et al., 2003) and humoral (Kiron et al., 1995) defense responses and offer disease resistance (Kiron et al., 1995). These fatty acids also enhanced the schooling behavior of the larval yellowtail (Ishizaki et al., 2001). Therefore, it is conceivable that transgenic fish expressing high levels of Δ6-desaturase could probably become an ideal material for aquaculture in terms of their fecundity, growth, stress tolerance, disease resistance and survival.

We have successfully completed the first step in engineering the production of EPA and DHA in fish for human consumption by demonstrating the elevation of the two nutraceutical fatty acids in the edible portion. To our knowledge, this is the pioneering study that has achieved the modification of fatty acid composition by transgenic technology in teleostean fish. In addition the biotechnological approach described in this work could be applied for cost-effective marine seed production, besides reducing the dependence on wild stocks as sources of fish oil contained in aquatic diets. Transgenic fish can also function as a valuable model for studying the basic mechanism of fatty acids synthesis, and the biological functions of $n-3$ fatty acids not only in fish, but also in higher vertebrates including humans. As an extension of the research described here, the cloning of Δ5-desaturase and elongase genes are under progress in our laboratory. Generation of transgenic marine fish carrying various fatty acid metabolic enzymes holds exciting possibilities for the future of aquaculture.

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