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Transgenic expression of salmon delta-5 and delta-6 desaturase in zebrafish muscle inhibits the growth of *Vibrio alginolyticus* and affects fish immunomodulatory activity

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A B S T R A C T

Marine fish are an important nutritional source for highly polyunsaturated fatty acids (PUFAs). PUFA biosynthesis requires the following key enzymes: delta-4 (Δ-4) desaturase, delta-5 (Δ-5) desaturase, delta-6 (Δ-6) desaturase, delta-5 (Δ-5) elongase, and delta-6 (Δ-6) elongase. The effect of overexpressing delta-5 desaturase and/or delta-6 desaturase in zebrafish muscle has not previously been reported. Herein, we investigated the effects of these proteins on antibacterial and immunomodulatory activity in transgenic zebrafish infected with *Vibrio alginolyticus*. Overexpression of delta-5 and delta-6 desaturase enhanced antibacterial activity at 4 and 12 h after injection of bacteria into muscle, as compared to controls. Furthermore, expression of immune-related genes (IL-1β, IL-22, and TNF-α) was observed to be altered in transgenic fish after 4 h of bacterial infection, resulting in a significant decrease in the inflammatory response, as compared to control fish. These results demonstrate that muscle-specific expression of transgenic desaturases in zebrafish not only enhance PUFA production, but also enhance antibacterial and anti-inflammatory activity. Overall, these results identify delta-5 and delta-6 desaturase as novel candidate genes for use in aquaculture, to enhance both disease resistance and fish oil production.

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1. Introduction

Dietary polyunsaturated fatty acids (PUFAs) affect both the immune response and the production of arachidonic acid (ALA, 20:4n-6), eicosapentaenoic acid (EPA, 20:5n-3), and docosahexaenoic acid (DHA, 22:6n-3) [1]. In addition, PUFAs modulate leukocyte function and the production of adhesion molecules and inflammatory cytokines [2]. PUFA biosynthesis requires delta-5 and delta-6 desaturases, and ALA and DHA are required for various physiological functions in mammals [3].

Previous studies have indicated that n-3 PUFAs exert anti-inflammatory effects through inducing expression of inflammatory genes via (i) nuclear factor kappa B (NF-κB) and (ii) peroxisome proliferator-activated receptors (PPARs) [4]. NF-κB is a transcription factor that plays an important role in various inflammatory signaling pathways: it regulates several cytokines (IL-1, IL-2, IL-6, IL-12, and TNF-α), as well as chemokines, adhesion molecules, and inducible effector enzymes, such as COX-2 and inducible nitric oxide synthase [5,6]. Degradation of the murine NF-κB inhibitory subunit, IκB, is blocked by EPA, thereby inhibiting NF-κB activity [7]. Furthermore, transgenic mice capable of endogenous biosynthesis of n-3 PUFAs exhibited decreased NF-κB activity, which protected the mice from colitis [8]. PPARs are ligand-activated nuclear transcription factors involved in inflammation, insulin sensitization, cellular differentiation, cancer, atherosclerosis, and several metabolic diseases [9,10]. Furthermore, EPA and DHA down-regulate lipopolysaccharide-induced activation of NF-κB through a PPAR-γ-dependent pathway in human kidney-2 cells [11].

In teleosts, inflammation arises from the release of cytokines and eicosanoids by injured cells. Eicosanoids, such as EPA, ARA, and DG-LIN, are converted into leukotrienes and prostanoids, which mediate inflammation [16]. Increasing nutritional intake of EPA can ameliorate inflammation and Atlantic salmon reovirus (ASRV) infection in Atlantic salmon [12]. Moreover, dietary immunomodulation can reduce the morbidity of heart and skeletal muscle

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Table 1
Primer sequences.

Primer	Gene name	Sequence
TNF-α-F	Tumor necrosis factor alpha	AAGGAGAGTTGCCTTTACCG
TNF-α-R		ATTGCCCTGGGTCTTATGG
IL-1β-F	Interleukin-1 beta	TGGACTTCGCAGCACAAAATG
IL-1β-R		CACTTCACGCTCTTGGATGA
IL-10-F	Interleukin-10	TCACGTCATGAACGAGATCC
IL-10-R		CCTCTTGCATTTCACCATATCC
TLR4-F	Toll like receptor 4	TGTCAAGATGCCACATCAGA
TLR4-R		TCCACAAGAACAAGCCTTTG
EF1α-F	Elongation factor 1 alpha	AACAGCTGATCGTTGGAGTCAA
EF1α-R		TTGATGTATGCGCTGACTTCCT
TLR1-F	Toll like receptor 1	CAGAGCGAATGGTGCCACTAT
TLR1-R		GTGGCAGAGGCTCCAGAAGA
TLR3-F	Toll like receptor 3	TGGAGCATCACAGGGATAAAGA
TLR3-R		TGATGCCCATGCCTGTAAGA
Lysozyme-F	Lysozyme	GATTTGAGGGATTCTCCATTGG
Lysozyme-R		CCGTAGTCCTTCCCCGTATCA
c3b-F	Complement component 3	CGTCTCCGTACACCATCCATT
c3b-R		GGCGTCTCATCAGGATTTGTTAC
IL-21-F	Interleukin-21	AATCATTCATCGTGGACAGTGTGT
IL-21-R		AACGTTCGGCTGTTGACCAT
IL-22-F	Interleukin-22	CATCGAGGAACAACGGTGTACA
IL-22-R		CACGAGCACAGCAAAGCAAT
NFκB-F	Nuclear factor kappa-light-chain-enhancer of activated B cells	AGAGAGCGCTTGCGTCCTT
NFκB-R		TTGCCTTTGGTTTTTCGGTAA
TRAM1-F	Translocation associated membrane Protein 1	AGAAGGCCAAGAAAGAAGACATTC
TRAM1-R		CCCAACCGGTTCAGATTCAG

inflammation (HSMI) in salmon [13]. In addition, EPA exerts significant bactericidal and bacteriostatic effects against *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Sardinella fimbriata*, and *Sardinella longiceps in vitro* [14,15].

Vibrio alginolyticus is a motile, straight rod, Gram-negative bacterial species, positive for oxidase and catalase; the *V. alginolyticus* strain S3y was isolated from an outbreak of vibriosis in grouper (*Epinephelus malabaricus*) larvae in Taiwan [17]. *V. alginolyticus* causes vibriosis with gastroenteritis, and is a common pathogen in marine fish and shrimp (such as *Penaeus monodon*) [18]. In this article, we demonstrate that overexpression of salmon delta-5 desaturase or delta-6 desaturase in transgenic zebrafish decreases the growth of *V. alginolyticus* and enhances fish immunomodulatory activity. These findings suggest that the genes encoding delta-5 and delta-6 desaturase may be potential candidates for enhancing disease resistance and PUFA production in transgenic fish, thereby improving the yield and nutritional quality of aquacultural products.

2. Materials and methods

2.1. Transgene constructs and generation of transgenic zebrafish

Salmon delta-5 desaturase and delta-6 desaturase primer sequences were designed based on sequences obtained from the NCBI Genbank database, with flanking *Xho*I and *Nhe*I restriction enzyme sites. Polymerase chain reaction (PCR) was performed using an Applied Biosystems 2700 (Applied Biosystems, USA), with 20 μl of 5× Phusion HF Buffer, 10 mM dNTPs, Phusion® High-Fidelity DNA Polymerase (5 units/μl; Thermo, USA), 100 ng template, and 50 μM primer pairs, to a total volume of 25 μl. The reaction conditions were as follows: 94 °C for 5 min; 35 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s; and final extension at 72 °C for 10 min. DNA fragments were electrophoresed on agarose gels, before being extracted and ligated into the pME-MCS vector. The gene encoding TCFP 11 (Taiwan *Acropora* coral pink fluorescent protein) or TCFP 13 (Cyan fluorescent protein) (constructs kindly provided by Dr. Hong-Yi Gong, National Taiwan Ocean University) was ligated into the delta-6 or delta-5 fatty acyl desaturase plasmid, respectively. Sequences were inserted into plasmids using the Gateway® recombinant system (Invitrogen, USA); the plasmid derived from the Tol2 kit [19] was used to generate the p5E-T.mlc3-p-4314-3882/-800-1-i1 plasmid [20], which also contains sequences from pME-MCS, p3E-IRES-EGFPpA, and pDestTol2pA2.

The synthesized plasmids were microinjected into zebrafish eggs at the one-cell stage, as described previously [21]. The eggs were placed in a 28 °C incubator after microinjection. The F2 generation of transgenic zebrafish was obtained by mating one transgenic zebrafish with a wild-type (WT) zebrafish, and following standard propagation procedures. F2 progeny were used in all experiments. To confirm germline transmission, transgenic zebrafish were examined under fluorescence microscopy using an FITC filter (IX71; Olympus).

2.2. Western blot and copy number estimation

In order to analyze protein expression and estimate bacterial copy numbers, total protein was extracted from 100 mg of zebrafish muscle tissue. Total protein was added to 1 ml of 2-D rehydration sample buffer and 10 μl of ready prep TBP reducing agent (ReadyPrep Protein Extraction Kit, Bio-Rad, USA) in a 2 ml microcentrifuge tube, and homogenized using Lyser II (Qiagen, USA). Samples were placed on ice for 30 min, before being centrifuged at 12,000 rpm for 30 min at 18 °C to pellet tissue debris. The supernatant was transferred to a clean tube, and kept at −80 °C for long-term storage. Protein concentrations were determined using the RC DC protein Assay (Bio-Rad, USA), in accordance with the manufacturer's protocol.

For Western blot, proteins (100 μg/well) were separated by 10% SDS PAGE, and transferred to a PVDF membrane (400 mA, 1 h). Membranes were subjected to the following treatment: (i) exposure to SuperSignal Western Blot Enhancer (Thermo, USA); (ii) washing with ultrapure water for 2 min with shaking; (iii) two rinses with fresh ultrapure water; (iv) immersion in 5 ml Antigen Pretreatment Solution; (v) incubation at room temperature for 10 min with shaking; (vi) five rinses with ultrapure water; (vii)

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