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Cloning and characterization of Δ6/Δ5 fatty acyl desaturase (Fad) gene promoter in the marine teleost *Siganus canaliculatus*

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ABSTRACT

The rabbitfish *Siganus canaliculatus* was the first marine teleost demonstrated to have the ability of biosynthesizing long-chain polyunsaturated fatty acids (LC-PUFA) from C_{18} PUFA precursors, and all genes encoding the key enzymes for LC-PUFA biosynthesis have been cloned and functionally characterized, which provides us a potential model to study the regulatory mechanisms of LC-PUFA biosynthesis in teleosts. As the primary step to clarify such mechanisms, present research focused on promoter analysis of gene encoding Δ6/Δ5 fatty acyl desaturase (Fad), a rate-limiting enzyme catalyzing the first step in the conversion of C_{18} PUFA to LC-PUFA. First, 2044 bp promoter sequence was cloned by genome walking, and the sequence from −456 bp to +51 bp was determined as core promoter by progressive deletion mutation. Moreover, binding sites of transcription factors (TF) such as CCAAT enhancer binding protein (C/EBP), nuclear factor 1 (NF-1), stimulatory protein 1 (Sp1), nuclear factor Y (NF-Y), activated protein 1 (AP1), sterol regulatory element (SRE), hepatocyte nuclear factor 4α (HNF4α) and peroxisome proliferator activated receptor γ (PPARγ) were identified in the core promoter by site-directed mutation and functional assays. Moreover, NF-1 and HNF4α were confirmed to interact with the core promoter region by gel shift assay and mass spectrometry. This is the first report of the promoter structure of a Δ6/Δ5 Fad in a marine teleost, and a novel discovery of NF-1 and HNF4α binding to the Δ6/Δ5 *Fad* promoter.

1. Introduction

It is known that long-chain polyunsaturated fatty acids (LC-PUFA) such as eicosapentaenoic (EPA; 20:5n-3), docosahexaenoic (DHA; 22:6n-3) and arachidonic (ARA; 20:4n-6) acids are essential fatty acids (EFA) for normal growth and development of vertebrates including fish. Freshwater fish generally have the ability to convert linoleic (LNA; 18:2n-6) and α-linolenic (ALA; 18:3n-3) acids into LC-PUFA, and thus these two C_{18} precursors can satisfy the EFA requirements of these species. On the contrary, most marine teleosts require LC-PUFA as EFA due to the low or absent capability of LC-PUFA biosynthesis (Yone, 1978; Watanabe, 1982; Kanazawa, 1985). Accordingly, fish oil (FO) rich in LC-PUFA must be added to compound feed for the aquaculture of marine fish. The limited resource and high price of FO seriously restricted the development of marine aquaculture. Consequently, many studies have been conducted to develop feasible and sustainable alternatives of FO, and vegetable oils (VO) which is rich in C_{18} precursors but devoid of LC-PUFA, have been considered as the prime candidates (Turchini and Francis, 2009). However, replacement of dietary FO by VO results in reduced flesh n-3 LC-PUFA contents of farmed fish, which could significantly compromise their nutritional quality for humans, and possibly have other negative effects on fish growth and health (Turchini and Francis, 2009; Geay et al., 2015). Over the last decade, therefore, considerable efforts have been made to understand the

Abbreviations: ALA, α-linolenic acid (18:3n-3); AP1, activated protein 1; ARA, arachidonic acid (20:4n-6); C/EBP, CCAAT enhancer binding protein; DHA, docosahexaenoic acid (22:6n-3); DR1, direct repeat 1 element; EFA, essential fatty acid; EPA, eicosapentaenoic acid (20:5n-3); Fad, fatty acyl desaturase; FAS, fatty acid synthase; GATA-2, GATA binding protein 2; HEK 293T cell, human embryonic kidney 293T cell; HNF4α, hepatocyte nuclear factor 4α; LC–MS, liquid chromatography coupled with tandem mass spectrometry; LC-PUFA, long-chain polyunsaturated fatty acids; LNA, linoleic acid (18:2n-6); NF-1, nuclear factor 1; NF-Y, nuclear factor Y; PPARγ, peroxisome proliferator activated receptor γ; PUFA, polyunsaturated fatty acids; Sp1, stimulatory protein 1; SRE, sterol regulatory element; TF, transcription factor; TSS, transcription start site; UTR, untranslated region

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molecular basis of endogenous LC-PUFA biosynthesis, so as to facilitate the efficient and effective utilization of sustainable plant lipid sources while maintaining the nutritional quality of farmed fish, especially marine teleosts.

The biosynthesis of LC-PUFA from C_{18} precursors requires a series of desaturations and elongations catalyzed by fatty acyl desaturases (Fad) and elongases of very long-chain fatty acids (Elovl), respectively (Sprecher, 1981). At present, most studies have focused on the cloning and characterization of genes encoding key enzymes in LC-PUFA biosynthesis, including Fad and Elovl. Irrespective of specificity, Fad in teleosts all belong to the FADS2 gene cluster, which includes Δ6 Fad, Δ6/Δ5 Fad, Δ5 Fad and Δ4 Fad. So far, Δ6 Fad has been found in more than fifteen fish species (Castro et al., 2016), while Δ6/Δ5 Fad has been reported in one marine teleost, rabbitfish (*Siganus canaliculatus*) (Li et al., 2008), and four freshwater fish including zebrafish (*Danio rerio*) (Hastings et al., 2001), Mexican whitefish (*Chirostoma estor*) (Fonseca-Madrigal et al., 2014), Nile tilapia (*Oreochromis niloticus*) (Tanomman et al., 2013) and striped snakehead (*Channa striata*) (Kuah et al., 2016). Δ5 Fad is reported in Atlantic salmon (*Salmo salar*) (Hastings et al., 2004) and rainbow trout (*Oncorhynchus mykiss*) (Abdul Hamid et al., 2016). Δ4 Fad is identified in vertebrates first by our group in rabbitfish (Li et al., 2010), followed by Senegalese sole (*Solea senegalensis*) (Morais et al., 2012), Mexican silverside (*Chirostoma estor*) (Fonseca-Madrigal et al., 2014), striped snakehead (*Channa striata*) (Kuah et al., 2015), and primates, the baboon (Park et al., 2015).

The accumulating studies demonstrated that the activity of Fad was regulated mainly at the transcription level (Nakamura and Nara, 2004). For examples, the expression of Atlantic salmon Δ6 *Fad* was up-regulated by replacement of dietary FO with VO (Zheng et al., 2004). Moreover, the transcription activity of Atlantic salmon Δ6 *Fad* promoter was much higher than that of marine fish Atlantic cod (Zheng et al., 2009). In contrast, the mRNA expression of Atlantic cod Δ6 *Fad* did not respond to dietary lipid and its activity was also weak (Tocher et al., 2006). The expression of European sea bass Δ6 *Fad* was increased by VO treatment, however, its activity and protein content were not changed, suggesting a potential regulatory mechanism of Δ6 Fad activity at post-transcriptional level (Geay et al., 2010). However, the detailed regulatory mechanisms of LC-PUFA biosynthesis in teleosts are largely unknown at present, which are important for increasing the efficient and effective utilization of sustainable VO in feed while maintaining the nutritional quality of farmed fish, especially marine teleosts.

Rabbitfish *S. canaliculatus* is an economically important marine teleost, which is widespread along the Indo-West Pacific coast and farmed in southeastern Asia including China. What is worthy specially mentioned is that *S. canaliculatus* was the first marine teleost demonstrated to have the ability of biosynthesizing LC-PUFA from C_{18} PUFA precursors (Li et al., 2008; Xie et al., 2015). Moreover, all genes encoding the key enzymes for LC-PUFA biosynthesis have been cloned and functionally characterized including a Δ4 Fad (the first report in vertebrates) and a Δ6/Δ5 Fad (the first report in marine teleosts) (Li et al., 2010), and two elongases (Elovl4 and Elovl5) (Monroig et al., 2012). These provide us a good model for investigating the regulatory mechanisms of LC-PUFA biosynthesis in teleosts. Recently, studies at transcriptional levels have demonstrated the roles of HNF4α and Lxr-Srebp in *Fad* expression (Dong et al., 2016; Zhang et al., 2016a). At post-transcriptional level, miR17 targeted at the 3′ untranslated region (3′ UTR) of Δ4 *Fad* and downregulated its gene expression, and miR-33 was involved in the regulation of LC-PUFA biosynthesis probably through targeting *insig1* in rabbitfish (Zhang et al., 2016b). Moreover, the structure of rabbitfish Δ4 *Fad* promoter was characterized and HNF4α was identified as a TF of *Fad* genes, for the first time in vertebrates (Dong et al., 2016).

Δ6/Δ5 Fad is a rate-limiting enzyme catalyzing the first step in the conversion of C_{18} PUFA to LC-PUFA, and thus understanding the regulatory mechanisms of this gene is important for comprehensively exploring the regulation mechanisms of LC-PUFA biosynthesis in teleost. As the first step to the goal, the present study focused on the promoter structure analysis of rabbitfish Δ6/Δ5 *Fad* gene, including the cloning of promoter sequence, determination of core promoter region by progressive deletion mutation, identification of possible binding sites for TFs by a combination of bioinformatics analysis and site-directed mutagenesis. Moreover, TFs interacting with core promoter were confirmed by gel shift assay and liquid chromatography coupled with tandem mass spectrometry (LC–MS). The results will increase our understanding on the regulatory mechanisms of LC-PUFA biosynthesis in vertebrates, which at last contribute to the optimization and/or enhancement of the LC-PUFA pathway in teleosts.

2. Materials and methods

2.1. Ethics statement

Nan Ao Marine Biology Station (NAMBS) of Shantou University provided wild rabbitfish for the present study. In order to minimize the suffering of rabbitfish, 0.01% 2-phenoxyethanol (Sigma-Aldrich, St. Louis, MO, USA) was used for anesthesia. Based on the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978), all the experiments with fish in the present research were assessed as correct and reasonable by the Institutional Animal Care and Use Committee of Shantou University (Guangdong, China).

2.2. Cloning of Δ6/Δ5 Fad gene promoter

Genomic DNA was extracted from muscle tissue of rabbitfish *S. canaliculatus* (Sambrook and Russell, 2001) and used for thermal asymmetric interlaced PCR (TAIL PCR) to clone the Δ6/Δ5 Fad gene promoter with forward primer AP4 in the Genome Walking Kit (TaKaRa, Dalian, China) and three reverse primers SP1, SP2, SP3 (Table 1), which was designed from the cloned Δ6/Δ5 Fad mRNA of *S.*

Table 1
Primers used in present study.

Subject	Primers	Nucleotide sequence
TAIL PCR for 5′ flanking sequence cloning	SP1	5′-GTCTGCTTTCCTATGGTCACGTTG-3′
	SP2	5′-GTGCTCCGTCCACATTCGAGTT-3′
	SP3	5′-TCATCCTCCTCAAATAGG-3′
Pfu PCR for deletion mutant construction	DF3	5′-CCCGATATCTTGCTGACGTAAAGTGTGGA-3′
	DF2	5′-CCCGATATCGGCCATTTGATTAACTCTGCT-3′
	DF1	5′-CCCGATATCGGAGCACGGTCAACGTGAC-3′
	DR	5′-CCCAAGCTTCATCTTCACTGCTGTCTCTGCTT-3′
EMSA for gel shift	BF (5′ biotinlabeled)	5′-GTATTTCATCAGACTGTTTCCGT-3′
	BR (5′ biotinlabeled)	5′-CAAACGTTGTCTGCTTTCCTAT-3′
	UF (5′ unlabeled)	5′-GTATTTCATCAGACTGTTTCCGT-3′
	UR (5′ unlabeled)	5′-CAAACGTTGTCTGCTTTCCTAT-3′

Restriction sites underlined are *Eco*RV (5′-GATATC-3′) and *Hin*dIII (5′-AAGCTT-3′) in expression vector pGL4.10.

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