Identification of Myomaker in Yellowfin Seabream (Acanthopagrus latus) (Hottuyn, 1782) and its Transcriptional Regulation by Two MyoDs

Kecheng Zhu1,2,3, Peiying He1, Baosuo Liu1,2,3, Huayang Guo1,2,3, Nan Zhang1,2,3, Liang Guo1,2,3, Shigui Jiang1,2,3 and Dianchang Zhang1,2,3,*

1Key Laboratory of South China Sea Fishery Resources Exploitation and Utilization, Ministry of Agriculture and Rural Affairs; South China Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences, 510300, Guangzhou, Guangdong Province, PR China
2Guangdong Provincial Engineer Technology Research Center of Marine Biological Seed Industry, Guangzhou, Guangdong Province, PR China
3Guangdong Provincial Key Laboratory of Fishery Ecology and Environment, Guangzhou, Guangdong Province, PR China

ABSTRACT

Myomaker is a muscle-specific membrane protein that is essential for myoblast fusion. Myomaker is regulated by myoblast determination protein (MyoD), a muscle-specific basic helix-loop-helix (bHLH) transcription factor in higher vertebrates. However, the transcriptional regulatory mechanism of the myomaker gene has not been explored in marine fishes. In the present study, molecular cloning, bioinformatic analysis and transcriptional analysis of Acanthopagrus latus myomaker (Almyomaker) were performed. The open reading frame (ORF) sequence of Almyomaker is 858 bp, which encodes a polypeptide of 285 amino acids. Moreover, phylogenetic and gene structure analysis indicates that Almyomaker is highly conserved among vertebrates. The tissue distribution pattern shows that Almyomaker is highly expressed in white muscle than in other tissues. Furthermore, to explore whether two MyoDs are modulators of Almyomaker, a promoter analysis was performed using progressive deletion mutations of Almyomaker. The results of promoter activity assays show that Almyomaker expression is notably activated by two MyoD transregulatory activity of the Almyomaker promoter was observed to dramatically decrease after targeted mutation of the MyoD1 M1 and MyoD2 M2 binding sites. In summary, MyoD1 and MyoD2 play an important role in the regulation of Almyomaker expression and may promote myoblast fusion during muscle development and growth by modulating Almyomaker expression.

INTRODUCTION

The fusion of myoblasts is an important process to generate multinucleated myofibers during skeletal muscle regeneration and development (Kim et al., 2015). To date, several proteins, such as myoferlin (Doherty et al., 2005), myogenin and myoD (Tapscott, 2005), Myocyte enhancer factor 2s (mef2s) (Hinits and Hughes, 2007), Ras-related C3 botulinum toxin substrate 1 (Rac1) (Vasyutina et al., 2009), nephrin (Sohn et al., 2009), Junctional adhesion molecule b and c (Jamb and Jamm) (Powell and Wright, 2011; Shi et al., 2019), CD9 (Charrin et al., 2013), and CD81 (Charrin et al., 2013), involved in the process of muscle development, have been already identified. However, myomaker, also called transmembrane protein 8c (TMEM8c), is a muscle-specific protein that is absolutely indispensable for myoblast fusion and sufficient to promote fibroblast fusion with muscle cells in zebrafish (Danio rerio), mice (Mus musculus), and chickens (Gallus gallus) (Millay et al., 2013, 2014; Landemaine et al., 2014, 2016, 2018; Luo et al., 2015; Zhang et al., 2017). Knockdown of myomaker in zebrafish, mice, and chickens indicates that myomaker is necessary for myoblast fusion and that loss of myomaker function causes abnormal muscle development (Millay et al., 2013, 2014; Landemaine et al., 2014, 2015; Luo et al., 2015; Shi et al., 2018). The amino acid (aa) sequence of myomaker is highly conserved throughout vertebrate species (Millay et al., 2013), and its function in myogenesis is also conserved between mammals and fish (Landemaine et al., 2014). Myomaker is a hydrophobic protein...
consisting of 221 aa that localizes to intracellular vesicles and the plasma membrane in skeletal myocytes (Millay et al., 2013). The structural features of myomaker span the bilayer seven times with an extracellular N-terminal region and cytosolic C-terminal tail with unknown conserved functional domains (Millay et al., 2016).

Myogenin (MyoG) and MyoD, members of myogenic regulatory factors (MRFs), are pivotal transcription factors (TFs) in myogenesis and can control the transcription of most muscle-related genes (Braun et al., 1989; Edmondson and Olson, 1990; Berkes and Tapscott, 2005; Braun and Gautel, 2011). The two MRFs play a critical role in the regulation of myoblast differentiation. The transcription of myomaker is mediated through the activity of muscle-specific TFs MyoG and MyoD (Luo et al., 2015; Quinn et al., 2017; Zhang et al., 2017; Ganassi et al., 2018). Additionally, during the acute and chronic muscle regeneration process, MyoD can induce myomaker expression in muscle satellite cells and accelerate myoblast fusion. Then, the expression of myomaker decreased rapidly after fusion (Millay et al., 2014; Landemaine et al., 2014; Demonbreun et al., 2015; Zhang and Roy, 2017). In the MyoD signalling pathway, a downstream gene, myomixer, is activated by MyoD. Nonmyoblasts can fuse into multicellular cells when myomaker and myomixer are overexpressed (Bi et al., 2017; Quinn et al., 2017; Zhang et al., 2017). In avians, the essential role of myomaker is in myoblast fusion and shows that MyoD can regulate myomaker expression (Luo et al., 2015). Two highly conserved e-box mutations in the 5'-regulatory region sequence of the myomaker gene in mice and chickens have been verified, suggesting that the importance of the e-box is in regulating the transcription and expression of myomaker (Millay et al., 2014; Luo et al., 2015). Moreover, when two MyoD binding sites of the myomaker promoter are deleted in chickens, its promoter activity is significantly reduced, indicating that MyoDs have a positive regulatory effect on myomaker (Luo et al., 2015). The C-terminal region of myomaker is essential for the function of three cysteine residues, which are speculated to be palmitoylated (Millay et al., 2016). Myomaker displays N-terminal glycine predicted to sustain myristoylation (Bologna et al., 2004). However, the cellular mechanism of myomaker and the regulatory mechanism of its expression during myogenesis have not been determined in marine fish.

Yellowfin seabream (Acanthopagrus latus) (Hottuyun, 1782), Sparidae, and Perciformes are considered significant aquaculture fish in southern China due to their economic value. Nevertheless, the muscle growth rate is too low in A. latus. Consequently, this fish is known as a specific model for exploring regulatory mechanisms in muscle development in marine fish. In the present study, to investigate the underlying function of Almyomaker and transcriptional regulation of two AlMyoDs, this study focused on illuminating the consequence of MyoD in the activation of Almyomaker expression. A muscle-specific membrane protein gene from A. latus, myomaker (Almyomaker), was confirmed. Promoter activity assays via the mutation of potential MyoD binding sites are executed to determine key elements in the Almyomaker candidate sequence. The present study may contribute to further exploration of myomaker function in marine fish and help to elucidate the regulatory mechanism for myoblast fusion.

MATERIALS AND METHODS

Animals and tissues collection

Fish (body weight: 289 ± 18.5 g) were collected from Yangjiang Marine Fish Farm in Guangdong Province, China. For the present study, three healthy fish were used, and 13 tissues (heart, male gonad, eye, skin, brain, fin, spleen, small intestine, gill, white muscle, kidney, liver, and stomach) were isolated to analyze the expression of Almyomaker and help to elucidate the regulatory mechanism for myoblast fusion.

Gene cloning and bioinformatics of myomaker

Total RNA (1 μg) was extracted from A. latus white muscle by TRIzol Reagent (Takara, Japan). To synthesize cDNA, the Prime ScriptTM RT reagent Kit (Takara) was used according to the manufacturer’s instructions. Putative myomaker, MyoD1 and MyoD2 sequences were acquired according to genomic data of A. latus (Sequence Read Archive under BioProject PRJNA566024). To verify the accuracy of the derived sequence, gene-specific primers were designed (Table 1). The PCR system (volume) and procedure employed were described previously (Zhu et al., 2014). The amplified products, linked into the pEASY-T1 vector (TransGen Biotech, China), were purified by a DNA Purification Kit (Tiangen, China) and then sequenced (Invitrogen, China). Confirmed recombinants were transformed into competent Trans1-T1 cells (TransGen Biotech, China). A BLAST search on the supposed myomaker open reading frame (ORF) sequence further confirmed the accuracy and validity of the analysis. The derived aa sequence from the cloned Almyomaker ORF was aligned with other myomaker orthologue proteins from the NCBI and Ensembl databases (Fig. 2). Multiple sequence alignment was performed by ClustalX version 2.0 (Larkin et al., 2007) with default parameters. Phylogenetic analyses for all myomaker aa sequences were achieved using MEGA 6.0 (Tamura et al., 2013) with maximum likelihood (ML) methods (LG + G...
model, bootstrap 1000). All available myomaker genome sequences were obtained from Ensembl (http://asia.ensembl.org/) and Genome Browser (http://genome.ucsc.edu/cgi-bin/hgBlat). To predict the signal peptides and transmembrane domain, the Signal P3.0 server (http://www.cbs.dtu.dk/services/SignalP/), TMHMM (http://www.cbs.dtu.dk/services/TMHMM/), and SMART (http://smart.embl-heidelberg.de/) were used. Moreover, the genome structure and phylogenetic tree were embellished using Adobe PhotoShop CS6 (Adobe, San Jose, CA) and FigTree v1.4.2 (http://tree.bio.ed.ac.uk/software/figtree/), respectively.

Table 1.- Primers used for sequence cloning, deletion mutant construction, and qRT-PCR.

| Subject and Primers | Nucleotide sequence |
|---------------------|---------------------|
| Primers for sequence cloning | |
| Myomaker-ORF | CTGGCGATCCATGGCTGTCGGATATCT |
| Myomaker-ORF | CGGGGTACCCACACAAAATCCAGATCAGC |
| MyoD1-ORF-F | CGCGGATCCATGGATCTGTCCGACCTTCC |
| MyoD1-ORF-R | CCAGCTCGAGCTATAGGACTTGATAGATCA |
| MyoD2-ORF-F | CGGGGTACCAAACTTTAAAGGGCCAAGCTG |
| MyoD2-ORF-R | CGGGGTACCCTGAAGACATTCATACAGA |
| Deletion mutant construction | |
| Myomaker-pR | CGTGGTGCATCTCCACAGACT |
| Myomaker-pF1 | AAGCCAGGTATGGTTGTCAACTTT |
| Myomaker-pF2 | CGGCTCGAGCTGTGAGGACTTGATAGATCA |
| Myomaker-pF3 | CGGGGTACCCACACAAAATCCAGATCAGC |
| Myomaker-pF4 | CGGGGTACCCACACAAAATCCAGATCAGC |
| Myomaker-pF5 | CGGGGTACCCACACAAAATCCAGATCAGC |
| Primers for qRT-PCR | |
| MyoD1-ORF-F | CTGGCGATCCATGGCTGTCGGATATCT |
| MyoD1-ORF-R | CGGGGTACCCACACAAAATCCAGATCAGC |
| EF1α-F | AGTGGCGATCCATGGCTGTCGGATATCT |
| EF1α-R | AAGCCAGGTATGGTTGTCAACTTT |
| "ΔΔCt" method (Livak et al., 2001).

Plasmid construction, cell culture and dual-luciferase reporter assays

Briefly, to clone the target fragment of Almyomaker, genomic DNA was isolated from the white muscle of A. latus, as previously described in other marine fish (Sun et al., 2013). The ORF sequence upstream of the myomaker gene was obtained from genomic data of A. latus. Moreover, to obtain the recombinants of MyoD1 and MyoD2, the integrated sequences of MyoD1 and MyoD2 ORFs were inserted into the pCDNA3.1-Flag vector (Invitrogen, USA) using specific primers (Table I) (Zhu et al., 2020). To confirm the effect of AlMyoD1 and AlMyoD2 on Almyomaker expression, five different truncated regions from Almyomaker were amplified by peculiar primers with KpnI and XhoI restriction sites (Table I). The forward primers (Myomaker-pF1, Myomaker-pF2, Myomaker-pF3, Myomaker-pF4, and Myomaker-pF5) were designed with a 5′ KpnI site, and the common reverse primer (Myomaker-pR) was designed with a 3′ XhoI site (Table I). These primers were used to acquire the target region (Myomaker-pF1, 2,090 bp) and four truncated fragments (i) Myomaker-pF2, 1,483 bp; (ii) Myomaker-pF3, 1,211 bp; (iii) Myomaker-pF4, 436 bp and (iv) Myomaker-pF5, 302 bp (Fig. 5). PrimeSTAR Master Mix (Takara, Japan) was used to amplify the five truncated mutants. The PCR program consisted of 95°C for 4 min, followed by 30 cycles of 95°C for 40 s, 56°C for 40 s, and 72°C for 1 min. Subsequently, the PCR products were purified by the general DNA Purification Kit (Tiangen, China). A pGL3-basic (Promega, USA) vector and purified PCR products were digested with KpnI and XhoI and linked by T4 DNA ligase (Takara, Japan) overnight at 16°C. Recombined plasmids were extracted using the EndoFree Plasmid Giga Kit (Tiangen, China), and recombinants were validated by sequencing, as described above.

Furthermore, to investigate the possible function of MyoD1 or MyoD2 binding sites on the core myomaker promoter and 5’UTR sequences, three truncated mutations of recombinant plasmids were established. The TF binding site prediction (TFBS)-JASPAR database (http://jaspar.genereg.net/), TRANSFAC®, and MatInspector® were used to search for potential binding sites in the myomaker sequence with MyoD1 and MyoD2, respectively. Then, according to the manufacturer’s protocol, site-directed mutagenesis was conducted with a QuickChange II Site-Directed Mutagenesis Kit (Vazyme, USA) from the deletion mutant pGL3-basic-myomaker-p5, which was regarded as the wild-type plasmid. The prediction of three binding sites (M1, M2, and M3) was directly deleted, and the schematic diagram and sequences of homologous
TF binding sites are shown in Figure 6A and Table II, respectively. The role of TF binding site mutations on the promoter activity of Almyomaker was explained by a dual luciferase assay, as described below (Genecreate, China).

Table II.- Sequences of putative binding sites on AlMyomaker-P5 sequence.

| Putative binding sites | Nucleotide sequence | Mutated pattern |
|------------------------|---------------------|----------------|
| M1                     | GGACAGCTGGGT        | Deletion       |
| M2                     | GGACATCTACTT        | Deletion       |
| M3                     | GACTGAGGAACT        | Deletion       |

Human embryonic kidney (HEK293T) cells were cultured in DMEM (Gibco, USA) with 10% fetal bovine serum (FBS) (Invitrogen, USA) accompanied by 100 μg/mL streptomycin and 100 U/mL penicillin (Thermo Fisher Scientific, USA) at 37°C in a humidified incubator under 5% CO₂. The procedure of transfection and dual luciferase reporter assays were described by Li et al. (2017). Relative luciferase activities (the ratio of firefly and renilla luciferase activities) were measured and calculated using the VICTORTM X2 Multi-label Plate Reader (PerkinElmer, Inc., Waltham, MA, USA).

RESULTS

Sequence characterization of Almyomaker

The genomic sequence of Almyomaker is 3,383 bp, including 6 exons and 5 introns (Supplementary Fig. S1). The full-length ORF of Almyomaker is 855 bp (Fig. 1A), encoding peptides of 285 amino acids with a predicted molecular weight of 32.14 kDa and a theoretical isoelectric point of 9.44 (Accession No. MN266854). A conserved domain (DUF3522 superfamily starting from the 3rd amino acid and ending at the 185th amino acid) was detected using BLAST (Huang et al., 2019). Hydrophobicity profiles show that the Almyomaker protein includes more hydrophobic amino acids than hydrophilic amino acids (Fig. 1B). The results of TMHMM indicate that there are 6 obvious transmembrane domains in the Almyomaker protein (Fig. 1C), which is consistent with the analysis of goose Anser cygnoides (He et al., 2017).

Fig. 1. Sequence characterization of the Myomaker gene in Acanthopagrus latus. A, the nucleotide sequence of the Myomaker gene and the deduced amino acid sequence of T. ovatus. Initiation and termination codons are marked by green. Six transmembrane domains are shown in red; B, hydrophobicity profile of Myomaker; C, TMHMM posterior probabilities of Myomaker.

Statistical analysis

All trials were executed in triplicate in the present study. All values are shown with three replicates ± SE. Significant differences were calculated by one way ANOVA tests. P values <0.05 were considered to be significant.
Identification of Myomaker in Yellowfin Seabream

Fig. 2. Amino acid sequences of Myomaker homologues in vertebrates. A, amino acid alignment of Myomaker proteins from *A. latus* (Al), *Oreochromis niloticus* (On), *Xiphophorus maculatus* (Xm), *Oryzias latipes* (Ol), *Poecilia formosa* (Pf), *Danio rerio* (Dr), *Tetraodon nigroviridis* (Tn), *Gallus gallus* (Gg), *Latimeria chalumnae* (Lc), *Pelodiscus sinensis* (Ps), *Homo sapiens* (Hs), and *Mus musculus* (Mm). Conserved sequences are marked with asterisks. B, Percent identities of Myomaker amino acids compared to the above 12 Myomaker amino acids, and the accession numbers of the Myomaker sequences used are also listed. The myomaker amino acids in *A. latus* reveal sequence homology with those in the other metazoan species (Fig. 2A) and are most highly homologous with *Xiphophorus maculatus*, *Oryzias latipes*, *Poecilia formosa*, and *Tetraodon nigroviridis*. Moreover, a BLAST analysis indicated that the Almyomaker protein sequence shares high sequence identity with myomaker sequences from other teleosts containing tilapia (*O. niloticus*, 92%), platyfish (*X. maculatus*, 87.1%), medaka (*O. latipes*, 86.7%), and molly (*P. formosa*, 86.3%) and low sequence identity with humans (*Homo sapiens*, 56.5%) and mice (*Mus musculus*, 54.7%) (Fig. 2B).

Almyomaker structural and phylogenetic analyses

The genomic structural features and phylogenetic relationship of myomaker were determined and constructed in metazoans (Fig. 3). The lengths and distributions of the exons and introns of metazoan myomaker genes are displayed in Supplementary Table I. Six exons and five introns were identified in myomaker gene sequences,
except for zebrafish (*Danio rerio*), Tetraodon (*Tetraodon nigroviridis*), Chinese softshell turtle (*Pelodiscus sinensis*), Coelacanth (*Latimeria chalumnae*), chicken (*Gallus gallus*), *H. sapiens* and *M. musculus* myomaker, which only possess five exons and four introns. Furthermore, the sizes of the exon sequences reveal that there is nearly no diversity among species. Additionally, the phylogenetic tree was constructed with the full-length myomaker aa of various fish, mammalia, aves, and amphibia species. Almyomaker is grouped together with other Perciformes, such as *O. niloticus*. The phylogenetic tree clearly shows that fish evolve into one branch followed by Amphibians, Aves and then Mammals (Huang et al., 2019).

### Tissue expression of Almyomaker

The mRNA levels of *Almyomaker* are determined by qRT-PCR in various kinds of tissues. qRT-PCR shows that *Almyomaker* is broadly distributed in various tissues, and the abundance of mRNA varies among tissues. The highest *Almyomaker* transcriptions were detected in the white muscle followed by the brain and spleen, whereas the lowest *Almyomaker* expression levels were found in the liver, skin and stomach (Fig. 4).

Fig. 3. Phylogenetic relationship and structure of the *Almyomaker* gene with other vertebrates. Genome structure analysis of *Almyomaker* genes according to their phylogenetic relationship. Lengths of exons and introns of each *Myomaker* gene are displayed proportionally. Different colour boxes and lines represent exons and introns, respectively. The identical colour boxes represent homologous sequences.

Fig. 4. Relative expression levels of *Almyomaker* in different tissues. The twelve tissues are heart, gonad, eye, skin, brain, fin, spleen, small intestine, gill, white muscle, kidney, liver, and stomach. Different letters indicate significant differences (*p* < 0.05).
Identification of Myomaker in Yellowfin Seabream

Fig. 5. Promoter activity analysis of the *Almyomaker* gene. A, the structure of the *Almyomaker* promoter and 5'UTR region. Five recombinant plasmids were denoted myomaker-p1 (-1822 to +267), myomaker-p2 (-1215 to +267), myomaker-p3 (-943 to +267), myomaker-p4 (-168 to +267) and myomaker-p5 (-34 to +267). B and C, Transcriptional activity of the *Almyomaker* promoter. These plasmids were transfected along with the transcription factors MyoD1 (B) and MyoD2 (C) into HEK 293T cells. Dual-luciferase activity was driven by the *Almyomaker* promoter upon the transfection of pcDNA3.1-MyoD1, pcDNA3.1-MyoD2 or pcDNA3.1 into HEK 293T cells. Data are presented as the means of three replicates ± SE. Different letters indicate significant differences (*p* < 0.05).

![Promoter activity analysis](image)

Fig. 6. Construction of truncated mutants for the identification of predicted transcription factor (TF) binding sites in the *Almyomaker* candidate sequence. A, the nucleotide sequence and predicted binding sites in the core region of the *Almyomaker*-p5. TSS indicates the transcription start site. Effects of three mutants on *Almyomaker*-p5 promoter activity transfected with pcDNA3.1-MyoD1 (B) or pcDNA3.1-MyoD2 (C) or pcDNA3.1. Binding sites are shown with boxes. Data are presented as the means of three replicates ± SE. Different letters indicate significant differences (*p* < 0.05).

![Construction of truncated mutants](image)

Two *AlMyoD*s activate *Almyomaker* expression

The amplified candidate *Almyomaker* promoter and 5'UTR region (2,090 bp) are upstream nontranslational sequences. To comprehend the binding region of two MyoDs...
in the Almyomaker sequence, a full-length target fragment and 4 truncated mutants were inserted with a promoterless luciferase reporter vector, pGL3-basic (Promega, USA). The result of promoter activity analysis shows that the construct of myomaker-p5 (-34 bp to +267 bp) is higher than that of other constructs with cotransfection of MyoD1 or MyoD2, suggesting that the core binding region is located at -34 bp to +267 bp, which may include several MyoD1 or MyoD2 binding sites (Fig. 5). Therefore, the sequence of construct myomaker-p5 (-34 bp to +267 bp) was used for further functional analysis.

To understand the MyoD1 and MyoD2 binding sites in Almyomaker, the presumptive binding sites are mutated (Fig. 6A; Table II). The effects on promoter activity were investigated in 293T cells, together transfected with each mutant and MyoD1 or MyoD2. The results reveal that mutation of the M1 binding site (GGACAGCTGGGT, -14 bp to -3 bp) generates a marked reduction in promoter activity (Fig. 6B), showing that M1 is the MyoD1 binding site on the Almyomaker promoter. Moreover, mutation of the M2 binding site (GGACATCTACTT, +92 bp to +103 bp) resulted in a significant reduction in promoter activity (Fig. 6C), suggesting that M2 is the potential MyoD2 binding site in the Almyomaker gene 5’-UTR region. Notably, one predicted binding site (M3) does not activate luciferase activity with MyoD1 or MyoD2, suggesting that this site is not required for triggering Almyomaker expression with two MyoDs.

**DISCUSSION**

The present study investigated the potential mechanisms of the transcriptional regulation of myomaker by MyoD1 and MyoD2 in *A. latus*. The sequence and functional characterization, tissue expression and regulation relationship between two AlMyoDs and Almyomaker were determined. The Almyomaker aa sequence was 54.7%–92.0% identical to myomaker proteins from other teleosts.

In general, the expression of several genes depends on RNA polymerases and TFs binding to specific sequences on the promoters of target genes in eukaryotic organisms (O’Malley, 1977; Xie et al., 2018; Zhu et al., 2019). Furthermore, the integrity and activity of a promoter could regulate gene transcription. The TF MyoDs monitored skeletal muscle growth in mammals and altered the transcription of muscle-related genes in teleosts and avians (Millay et al., 2014; Luo et al., 2015). Notably, evidence has demonstrated that overexpression of MyoD could increase downstream gene myomaker expression in zebrafish (Bi et al., 2017; Quinn et al., 2017; Zhang et al., 2017). It was unclear whether MyoD could upregulate Almyomaker in marine fishes. In the present study, dual-luciferase reporter assays were conducted to realize regulatory mechanisms whereby MyoD1 and MyoD2 were believed to accommodate Almyomaker expression. The results of truncated mutants indicated that Almyomaker reporter activity was activated by the overexpression of two AlMyoDs. The core binding region in the Almyomaker promoter was -34 to +267 bp (Fig. 6). This evidence was the first to show that the expression of Almyomaker could be upregulated by both AlMyoD1 and AlMyoD2 in marine fishes.

MyoDs possess a highly conserved bHLH domain, which is necessary for heterodimerization with characteristic DNA binding to the e-box motif (consensus CANNTG) detected in the regulatory regions of their target genes (Murre et al., 1989; Sassar et al., 1989, 1991; Davis and Weintraub, 1992). Consequently, to investigate the two MyoD binding sites on the Almyomaker promoter, three mutant vectors were constructed. Mutation of the MyoD1 M1 binding site (GGACAGCTGGGT) and MyoD2 M2 binding site (GGACATCTACTT) resulted in observably decreased promoter activity (Fig. 6B, C), suggesting that the MyoD1 binding M1 site and MyoD2 binding M2 site were essential for Almyomaker promoter activity, respectively. The sequences of the above two binding sites were representative e-box motifs (Davis and Weintraub, 1992). In brief, MyoD1 and MyoD2 could mediate myomaker promoter expression by combining e-box motifs (M1 and M2 binding sites) in fish, respectively, which was consistent with the findings in *M. musculus* and *G. gallus* (Millay et al., 2014; Luo et al., 2015). Moreover, the MyoD2 M2 binding site was located in the 5’UTR, which was analogous to previous studies (Wu et al., 2018; Khan et al., 2019).

Phylogenetic analysis of myomakers in tetrapods and teleosts showed that a protein of uniform length (221 aa) was observed in ancestral nonteleost fish, and teleost myomaker sequences were classified into three groups according to myomaker protein length (Landemaine et al., 2019). In the present study, phylogenetic analysis showed that a typical phylogeny revealing the amino acid sequence of Almyomaker was closely matched to myomakers of *O. niloticus, X. maculatus, O. latipes, P. formosa*, and *T. nigroviridis*, with analogous lengths. This result was similar to that reported in Landemaine et al. (2019). A genome structure analysis showed that all myomakers contained 6 exons and 5 introns in metazoans, except for *D. rerio, T. nigroviridis, P. sinensis, L. chalumnae, G. gatlus, H. sapiens and M. musculus* myomaker, which only possessed 5 exons and 4 introns, suggesting that the last exon might be lost during evolution.

Previous studies have focused on the role of myomaker
during embryonic development and have determined that myomaker was indispensable for the facilitation of myoblast fusion in such species as D. rerio, G. gallus, and M. musculus (Millay et al., 2013; Landemaine et al., 2014; Luo et al., 2015; He et al., 2017; Zhang and Roy, 2017). The mRNA expression patterns of the myomaker gene during 8 different postnatal developmental stages in the Japanese flounder (Paralichthys olivaceus) showed that the expression of myomaker at 180 dph was higher than that at other periods (Huang et al., 2019). Moreover, myomaker is expressed only in skeletal muscle in M. musculus, G. gallus and Oncorhyncus mykiss (Millay et al., 2013; Landemaine et al., 2019). In the present study, the tissue-specific expression pattern revealed that the highest Almyomaker mRNA expression was detected in white muscle, showing that Almyomaker played an important role in muscle development. Almyomaker is also broadly expressed in other tissues; however, in mammals, chicks and fish, Myomaker is nearly undetectable in other organs (Millay et al., 2013; Landemaine et al., 2019). This discrepancy may be attributable to species diversity.

CONCLUSION

In conclusion, the full-length Almyomaker genome sequence was cloned. The tissue expression profile indicated that the mRNA level of Almyomaker was highest in the heart and gonad among the detected tissues. The luciferase activity analysis showed that the region from -34 bp to +267 bp includes the core binding region. Mutation analyses indicated that the activity of the Almyomaker promoter significantly decreased after the targeted mutation of the M1 and M2 binding sites with MyoD1 and MyoD2, respectively. This study mainly focused on the transcription of Almyomaker by MyoDs in heterologous cells. In the future, more attention should be paid to the mRNA or protein levels of Almyomaker after overexpression of MyoDs in endogenous cells.

ACKNOWLEDGMENTS

The study was supported by the Natural Science Foundation of Guangdong Province (2017A030310594), the National Infrastructure of Fishery Germplasm Resources Project (2019DKA30470) and the Science and Technology Infrastructure Construction Project of Guangdong Province (2019B030316030).

Supplementary material

There is supplementary material associated with this article. Access the material online at: https://dx.doi.org/10.17582/journal.pjz/20200116020138

Statement of conflict of interests

The authors declare no competing interests.

REFERENCES

Berkes, C.A. and Tapscott, S.J., 2005. MyoD and the transcriptional control of myogenesis. Semin. Cell Dev. Biol., 16: 585-595. https://doi.org/10.1016/j.semcdb.2005.07.006

Bi, P.P., Ramirez-Martinez, A., Li, H., Cannavino, J., McAnally, J.R., Shelton, J.M., Sanchez-Ortiz, E., Bassel-Duby, R. and Olson, E.N., 2017. Control of muscle formation by the fusogenic micropeptide myomixer. Science, 356: 323-327. https://doi.org/10.1126/science.aam9361

Bologna, G., Yvon, C., Duvaud, S. and Veuthey, A.L., 2004. N-Terminal myristoylation predictions by ensembles of neural networks. Proteomics, 4: 1626-1632. https://doi.org/10.1002/pmic.200300783

Braun, T., Büsschhausen-Denker, G., Bober, E., Tannich, E. and Arnold, H.H., 1989. A novel human muscle factor related to but distinct from MyoD1 induces myogenic conversion in 10T1/2 fibroblasts. EMBO J., 8: 701-709. https://doi.org/10.1002/j.1460-2075.1989.tb03429.x

Braun, T. and Gautel, M., 2011. Transcriptional mechanisms regulating skeletal muscle differentiation, growth and homeostasis. Nat. Rev. Mol. Cell Biol., 12: 349-361. https://doi.org/10.1038/nrm3118

Charrin S., Latil M., Soave S., Polesskaya A., Christien F., Boucheix C. and Rubinstein E., 2013. Normal muscle regeneration requires tight control of muscle cell fusion by tetraspanins CD9 and CD81. Nat. Commun., 4: 1674. https://doi.org/10.1038/ncomms2675

Davis, R.L. and Weintraub, H., 1992. Acquisition of myogenic specificity by replacement of three amino acid residues from MyoD into E12. Science, 256: 1027-1030. https://doi.org/10.1126/science.1317057

Demonbreun, A.R., Biersmith, B.H. and McNally, E.M., 2015. Membrane fusion in muscle development and repair. Semin. Cell Dev. Biol., 45: 48-56. https://doi.org/10.1016/j.semcdb.2015.10.026

Doherty, K.R., Cave, A., Davis, D.B., Delmonte, A.J., Posey, A. and Earley, J.U., 2005. Normal myoblast fusion requires myoferlin. Development, 132: 5565-5575. https://doi.org/10.1242/dev.02155

Edmondson, D.G. and Olson, E.N., 1990. A gene with homology to the myc similarity region of MyoD1 is expressed during myogenesis and is sufficient to...
activate the muscle differentiation program. Genes Dev., 4: 1450. https://doi.org/10.1101/gad.4.8.1450

Ganassi, M., Badodi, S., Quiroga, H.P.O., Zammit, F.S., Hnits, Y. and Hughes, S.M., 2018. Myogenin promotes myocyte fusion to balance fibre number and size. Nat. Commun., 9: 4232. https://doi.org/10.1038/s41467-018-06583-6

He, K., Ren, T., Zhu, S., Liang, S. and Zhao, A., 2017. Transiently expressed pattern during myogenesis and candidate miRNAs of Tmem8C in goose. J. Genet., 96: 39-46. https://doi.org/10.1007/s12041-016-0737-8

Hnits, Y. and Hughes, S.M., 2007. Mel2s are required for thick filament formation in nascent muscle fibres. Development, 134: 2511-2519. https://doi.org/10.1242/dev.007088

Huang, Y., Wu, S., Zhang, J., Wen, H., Zhang, M. and He, F., 2019. Methylation status and expression patterns of myomaker gene play important roles in postnatal development in the Japanese flounder (Paralichthys olivaceus). Gen. Comp. Endocrinol., 280: 104-114. https://doi.org/10.1016/j.ygcen.2019.04.017

Khan, K., Zhu, S., Liang, S. and Zhao, A., 2017. Activator of myoblast fusion and muscle formation. Cell, 58: 823-831. https://doi.org/10.1016/0092-8674(89)90935-5

Lassar, A.B., Davis, R.L., Wright, W.E., Kadesch, T., Murre, C., Vorona, A., Baltimore, D. and Weintraub, H., 1991. Functional activity of myogenic HLH proteins requires hetero-oligomerization with E12/E47-like proteins in vivo. Cell, 66: 305-315. https://doi.org/10.1016/0092-8674(91)90620-E

Li, S.L., Monroig, O., Wang, T.J., Yuan, Y.H., Navarro, J.C., Hontoria, F., Liao, K., Tocher, D.R., Mai, K.S. and Xu, W., 2017. Functional characterization and differential nutritional regulation of putative Elovl5 and Elovl4 elongases in large yellow croaker (Larimichthys crocea). Sci. Rep., 7: 2303. https://doi.org/10.1038/s41598-017-02646-8

Livak, K.J. and Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2−ΔΔCT method. Methods, 25: 402-408. https://doi.org/10.1016/meth.2001.1262

Luo, W., Li, E., Nie, Q.H. and Zhang, X.Q., 2015. Myomaker, Regulated by MyoD, Myog and miR-140-3p, Promotes chicken myoblast fusion. Int. J. Mol. Sci., 16: 26186-26201. https://doi.org/10.3390/ijms161125946

Millay, D.P., Gamage, D.G., Quinn, M.E., Min, Y.L., Mitani, Y., Bassel-Duby, R. and Olson, E.N., 2016. Structure-function analysis of myomaker domains required for myoblast fusion. Proc. natl. Acad. Sci. U.S.A., 113: 2116-2121. https://doi.org/10.1073/pnas.160101113

Millay, D.P., O’Rourke, J.R., Sutherland, L.B., Bezprozvannaya, S., Shelton, J.M., Bassel-Duby, R. and Olson, E.N., 2013. Myomaker is a membrane activator of myoblast fusion and muscle formation. Nature, 499: 301-305. https://doi.org/10.1038/nature12343

Millay, D.P., Sutherland, L.B., Bassel-Duby, R. and Olson, E.N., 2014. Myomaker is essential for muscle regeneration. Genes Dev., 28: 1641-1646. https://doi.org/10.1101/gad.247205.114

Murre, C., McCaw, P.S., Vaessen, H., Caudy, H., Jan, L.Y., Jan, Y.N., Cabrera, C.V., Buskin, J.N., Hauschka, S.D., Lassar, A.B., Weintraub, H. and Baltimore, D., 1989. Interactions between heterologous helix-loop-helix proteins generate complexes that bind specifically to a common DNA sequence. Cell, 58: 537-544. https://doi.org/10.1016/0092-8674(89)90434-0

O’Malley, B.W., Towle, H.C. and Schwartz, R.J., 1977. Regulation of gene expression in eucaryotes. Annu. Rev. Genet., 11: 239-275. https://doi.org/10.1146/annurev.ge.11.020177.001323
Powell, G.T. and Wright, G.J., 2011. Jamb and Jamc are essential for vertebrate myocyte fusion. *PLoS Biol.*, 9: e1001216. https://doi.org/10.1371/journal.pbio.1001216

Quinn, M.E., Goh, Q.N., Kurosaka, M., Gamage, D.G., Petraný, M.J., Prasad, V. and Millay, D.P., 2017. Myomemer induce fusion of non-fusogenic cells and is required for skeletal muscle development. *Nat. Commun.*, 8: 15665. https://doi.org/10.1038/ncomms15665

Shi, J., Cai, M.X., Si, Y.F., Zhang, J.S. and Du, S.J., 2018. Knockout of myomaker results in defective myoblast fusion, reduced muscle growth and increased adipocyte infiltration in zebrafish skeletal muscle. *Hum. Mol. Genet.*, 27: 3542-3554. https://doi.org/10.1093/hmg/ddy268

Si, Y.F., Wen, H.S. and Du, S.J., 2019. Genetic mutations in jamb, jamc, and myomaker revealed different roles on myoblast fusion and muscle growth. *Mar. Biotechnol.*, 21: 111-123. https://doi.org/10.1007/s10126-018-9865-x

Sohn, R.L., Huang, P., Kawahara, G., Mitchell, M., Guyon, J., Kalluri, R., Kunkel, L.M. and Gussoni, E., 2009. A role for nephrin, a renal protein, in vertebrate skeletal muscle cell fusion. *Proc. natl. Acad. Sci.*, 106: 9274-9279. https://doi.org/10.1073/pnas.0904398106

Sun, L.Y., Zhang, D.C., Guo, H.Y. and Zhu, C.Y., 2013. Isolation and characterization of 21 polymorphic microsatellites in golden pompano *Trachinotus ovatus*. *Conserv. Genet. Resour.*, 5: 1107-1109. https://doi.org/10.1007/s12686-013-9942-4

Tamura, K., Stecher, G., Peterson, D., Filipski, A. and Kumar, S., 2013. MEGA6: Molecular evolutionary genetics analysis, Version 6.0. *Mol. Biol.*, 3: 2725-2729. https://doi.org/10.1093/molbev/msr197

Vasyutina, E., Martarelli, B., Brakebusch, C., Wende, H. and Birchmeier, C., 2009. The small G-proteins Rac1 and Cdc42 are essential for myoblast fusion in the mouse. *Proc. natl. Acad. Sci.*, 106: 8935-8940. https://doi.org/10.1073/pnas.0902501106

Wu, X., Xu, F.L., Ding M., Zhang, J.J., Yao, J. and Wang, B.J., 2018. Characterization and functional analyses of the human HTR1A gene: 5’ regulatory region modulates gene expression in vitro. *BMC Genet.*, 19: 115. https://doi.org/10.1186/s12866-018-0708-6

Xie, D., Fu, Z., Wang, S., You, C., Monroig, Ó., Tocher, D.R. and Li, Y., 2018. Characteristics of the fads2 gene promoter in marine teleost *Epinephelus coioides* and role of Sp1-binding site in determining promoter activity. *Sci. Rep.*, 8: 5305. https://doi.org/10.1038/s41598-018-23668-w

Tapscott, S.J., 2005. The circuitry of a master switch: Myod and the regulation of skeletal muscle gene transcription. *Development*, 132: 2685-2695. https://doi.org/10.1242/dev.01874

Zhang, Q., Vashisht, A.A., O’Rourke, J., Corbel, S.Y., Moran, R., Romero, A., Miraglia, L., Zhang, J., Durrant, E. and Schmidt, C., 2017. The microprotein Minion controls cell fusion and muscle formation. *Nat. Commun.*, 8: 15664. https://doi.org/10.1038/ncomms15664

Zhang, W.B. and Roy, S., 2017. Myomaker is required for the fusion of fast-twitch myocytes in the zebrafish embryo. *Dev. Biol.*, 423: 24-33. https://doi.org/10.1016/j.ydbio.2017.01.019

Zhu, K.C., Chen, L.P., Zhao, J.K., Wang, H.J., Wang, W.M., Li, Z. and Wang, H.L., 2014. Molecular characterization and expression patterns of myogenin in compensatory growth of *Megalobrama amblycephala*. *Comp. Biochem. Physiol. B*, 170: 10-17. https://doi.org/10.1016/j.cbpb.2014.01.001

Zhu, K.C., Song, L., Guo, H.Y., Guo, L., Zhang, N., Liu, B.S., Jiang, S.G. and Zhang, D.C., 2019. Elolv4a participates in LC-PUFA biosynthesis and is regulated by PPARαβ in golden pompano *Trachinotus ovatus* (Linnaeus 1758). *Sci. Rep.*, 9: 4684. https://doi.org/10.1038/s41598-019-41288-w

Zhu, K.C., Liu, B.S., Guo, H.Y., Zhang, N., Guo, L., Jiang, S.G. and Zhang, D.C., 2020. Functional analysis of two MyoDs revealed their role in the activation of myomixer expression in yellowfin seabream (*Acanthopagrus latus*) (Hottuyn, 1782). *Int. J. Biol. Macromol.*, https://doi.org/10.1016/j.ijbiomac.2019.11.139
Supplementary Material

Identification of Myomaker in Yellowfin Seabream (Acanthopagrus latus) (Hottuyn, 1782) and its Transcriptional Regulation by Two MyoDs

Kecheng Zhu1,2,3, Peiying He1, Baosuo Liu1,2,3, Huayang Guo1,2,3, Nan Zhang1,2,3, Liang Guo1,2,3, Shigui Jiang1,2,3 and Dianchang Zhang1,2,3,*

1Key Laboratory of South China Sea Fishery Resources Exploitation and Utilization, Ministry of Agriculture and Rural Affairs; South China Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences, 510300, Guangzhou, Guangdong Province, PR China
2Guangdong Provincial Engineer Technology Research Center of Marine Biological Seed Industry, Guangzhou, Guangdong Province, PR China
3Guangdong Provincial Key Laboratory of Fishery Ecology and Environment, Guangzhou, Guangdong Province, PR China

Supplementary Table I.- Lengths of exons and introns of each Myomaker gene.

| Species                  | E1  | I1  | E2  | I2  | E3  | I3  | E4  | I4  | E5  | I5  | E6  |
|-------------------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Acanthopagrus latus     | 135 | 704 | 115 | 967 | 149 | 338 | 117 | 249 | 141 | 260 | 201 |
| Oreochromis niloticus   | 135 | 119 | 115 | 1187| 149 | 311 | 117 | 160 | 141 | 1467| 210 |
| Oryzias latipes         | 135 | 248 | 115 | 731 | 149 | 142 | 117 | 104 | 141 | 1715| 201 |
| Poecilia formosa        | 135 | 936 | 115 | 1144| 149 | 502 | 117 | 114 | 141 | 1209| 204 |
| Xiphophorus maculatus   | 135 | 919 | 115 | 848 | 149 | 1008| 117 | 114 | 141 | 4917| 204 |
| Danio rerio             | 135 | 1764| 115 | 694 | 149 | 1933| 117 | 99  | 147 | -   | -   |
| Tetraodon nigroviridis  | 135 | 75  | 115 | 658 | 149 | 79  | 117 | 159 | 144 | -   | -   |
| Pelodiscus sinensis     | 135 | 4396| 115 | 6029| 149 | 2988| 117 | 4519| 144 | -   | -   |
| Latimeria chalumnae     | 135 | 2095| 115 | 3509| 149 | 2558| 117 | 2984| 144 | -   | -   |
| Gallus gallus           | 135 | 2203| 115 | 1893| 149 | 589 | 117 | 904 | 144 | -   | -   |
| Homo sapiens            | 135 | 4421| 115 | 1151| 149 | 3266| 117 | 705 | 147 | -   | -   |
| Mus musculus            | 135 | 4413| 115 | 913 | 149 | 3472| 117 | 364 | 147 | -   | -   |

* Corresponding author: zhangdch@scsfri.ac.cn

0030-9923/2021/0001-0001 $ 9.00/0
Copyright 2021 Zoological Society of Pakistan
Supplementary Fig. S1. The full length of *Myomaker* genomic DNA in *Acanthopagrus latus*. Initiation and termination codons are marked by green. Exons are shown with yellow boxes.