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

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Key words
***Acanthopagrus latus*, Myomaker, MyoD, Promoter activity, Mutation analyses.**

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 more 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 MyoDs. Transcriptional 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 Jamc) (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, 2016; Landemaine *et al*., 2014; 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; 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

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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*) (Hottuyn, 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 *myomaker* expression level in different tissues. Then, the flash was frozen in liquid nitrogen and stored at -80°C until use.

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 I). 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 I.- Primers used for sequence cloning, deletion mutant construction, and qRT-PCR.

Subject and Primers	Nucleotide sequence
Primers for sequence cloning	
Myomaker-ORF	F: ATGGGTGCGTTTATTGCCAAGAT
Myomaker-ORF	R: TCACTTCCAGCCATTCATCT
MyoD1-ORF-F	CGCGGATCCATGGAGCTGTCGGATATCT
MyoD1-ORF-R	CCGCTCGAGCTATAGGACTTGATAGATCA
MyoD2-ORF-F	CGCGGATCCATGGATCTGTCCGACCTTCC
MyoD2-ORF-R	CCGCTCGAGTCAGAGCGGCTCCTGGATGCT
Deletion mutant construction	
Myomaker-pF1	CGGGGTACCCACACAAAATCCAGATCAGC
Myomaker-pF2	CGGGGTACCCGCCAAAAGGGTTAAATC
Myomaker-pF3	CGGGGTACCCTGAAGACATTCATACAGA
Myomaker-pF4	CGGGGTACCATGGCTACAATATGGCGTG
Myomaker-pF5	CGGGGTACCAAACTTTAAAGGGCCAAGCTG
Myomaker-pR	CCGCTCGAGCATGGTGGAGGGCCAGTAGACA
Primers for qRT-PCR	
Myomaker-F	CTGGGCGACTTTGATGAGC
Myomaker-R	AGTGATGATGAAGACAGCGGA
EF1α-F	AAGCCAGGTATGGTTGTCAACTTT
EF1α-R	CGTGGTGCATCTCCACAGACT

Real-time quantitative PCR analysis

Specific primers for real-time quantitative PCR (qRT-PCR) were designed by Primer Premier 5.0 (Premier Biosoft, USA) based on cloned nucleotide sequences (Table I) (Zhu *et al*., 2020). The *myomaker*, *MyoDs* and elongation factor 1-alpha (*EF-1α*) were tested and used as a target and reference gene, respectively. qRT-PCR was implemented in a quantitative thermal cycle (Mastercycler® eprealplex; Eppendorf, Germany). The PCR system (volume) was described above. The qRT-PCR program was conducted as follows: 95°C for 2 min followed by 40 cycles of 95°C for 10 s, 56°C for 10 s, and 72°C for 20 s. Gene expression was determined using the $2^{-\Delta\Delta Ct}$ 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 *Kpn*I and *Xho*I restriction sites (Table I). The forward primers (Myomaker-pF1, Myomaker-pF2, Myomaker-pF3, Myomaker-pF4, and Myomaker-pF5) were designed with a 5′ *Kpn*I site, and the common reverse primer (Myomaker-pR) was designed with a 3′ *Xho*I 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 *Kpn*I and *Xho*I 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% CO_2. The procedure of transfection and dual luciferase reporter assays were described by Li *et al.* (2017). Relative luciferase activities (the ratio of firefly and ranilla luciferase activities) were measured and calculated using the VICTOR™ X2 Multi-label Plate Reader (PerkinElmer, Inc., Waltham, MA, USA).

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.

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).

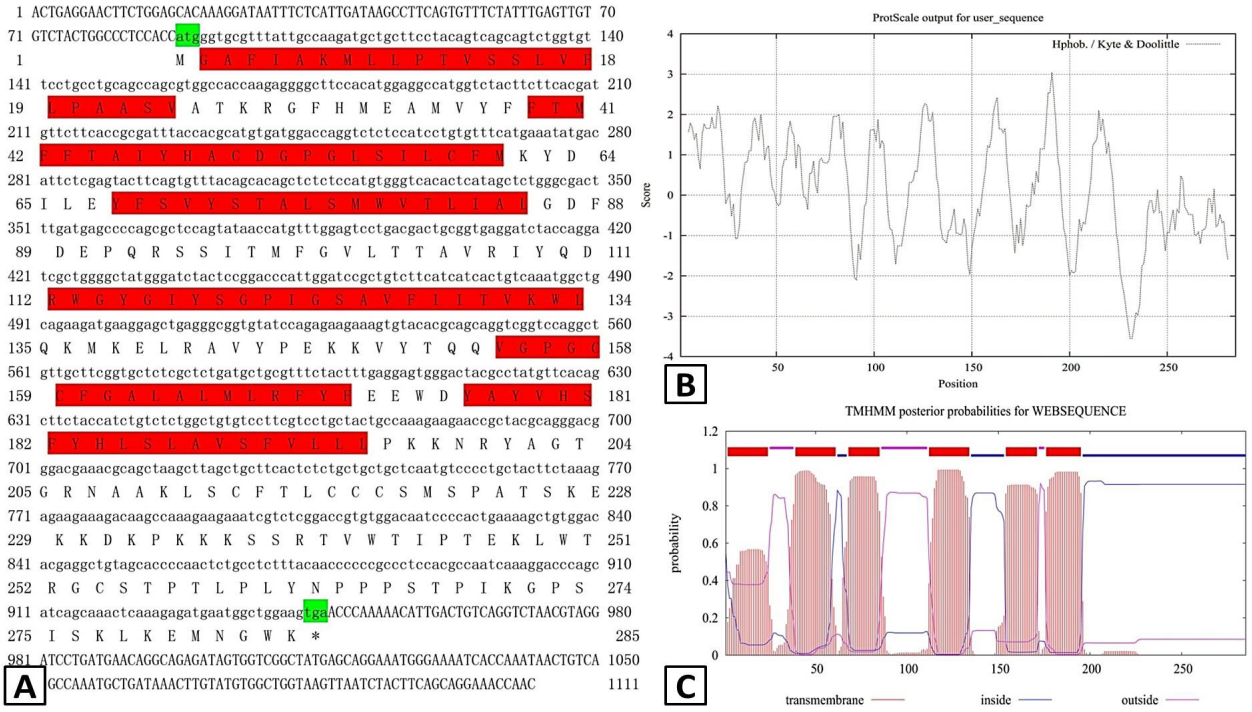

Fig. 1. Sequence characterization of the *Myomaker* gene in *Acanthopagrus latus*. A, the nucleotide sequence of the *Myomaker* gene and the deduced amino acid sequence of *T. ovatus*. Initiation and termination codons are marked by green. Six transmembrane domains are shown in red; B, hydrophobicity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

A

Total AA identity %													Species	No.
1	2	3	4	5	6	7	8	9	10	11	12			
100	92.0	87.1	86.7	86.3	79.7	71.5	61.0	59.7	58.7	56.5	54.7	1	*Acanthopagrus latus*	MN266854
-	100	89.2	90.3	88.5	78.1	73.2	58.0	60.5	60.1	63.6	55.0	2	*Oreochromis niloticus*	ENSONIT00000017480.1
-	-	100	83.9	97.6	65.3	69.8	57.0	59.4	59.1	56.1	54.7	3	*Xiphophorus maculatus*	XP_005812520.1
-	-	-	100	83.9	65.2	72.2	56.9	65.5	59.0	57.6	54.7	4	*Oryzias latipes*	XP_004072981.1
-	-	-	-	100	65.3	69.4	56.7	59.4	58.7	56.1	54.4	5	*Poecilia formosa*	ENSPFOT00000029545.1
-	-	-	-	-	100	88.6	77.7	80.9	79.6	75.1	72.9	6	*Danio rerio*	ENSDART00000161578.2
-	-	-	-	-	-	100	75.5	78.6	78.2	73.3	72.4	7	*Tetraodon nigroviridis*	ENSTNIT00000020078.1
-	-	-	-	-	-	-	100	83.2	95.0	86.9	85.5	8	*Gallus gallus*	ENSGALT00000034784.5
-	-	-	-	-	-	-	-	100	85.9	80.5	78.3	9	*Latimeria chalumnae*	XP_005990668.1
-	-	-	-	-	-	-	-	-	100	89.6	86.9	10	*Pelodiscus sinensis*	ENSPSIT00000008830.1
-	-	-	-	-	-	-	-	-	-	100	88.7	11	*Homo sapiens*	ENST00000339996.3
-	-	-	-	-	-	-	-	-	-	-	100	12	*Mus musculus*	ENSMUST00000009358.8

B

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 zerbrafish (*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).

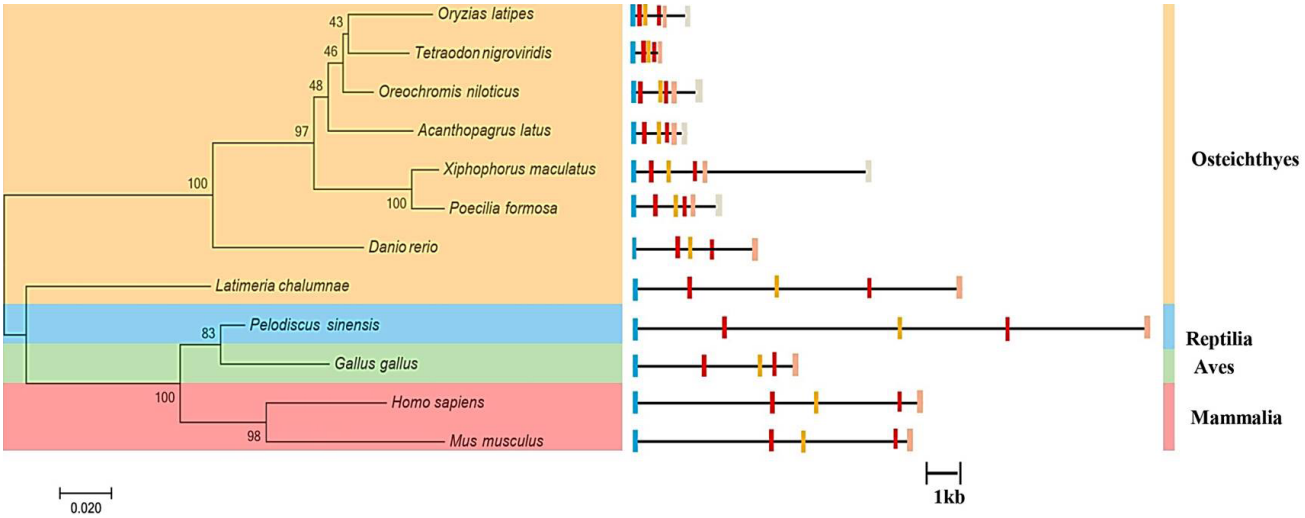

Fig. 3. Phylogenetic relationship and structure of the *Almyomaker* gene with other vertebrates. Genome structure analysis of *Almyomaker* genes according to their phylogenetic relationship. Lengths of exons and introns of each *Myomaker* gene are displayed proportionally. Different colour boxes and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

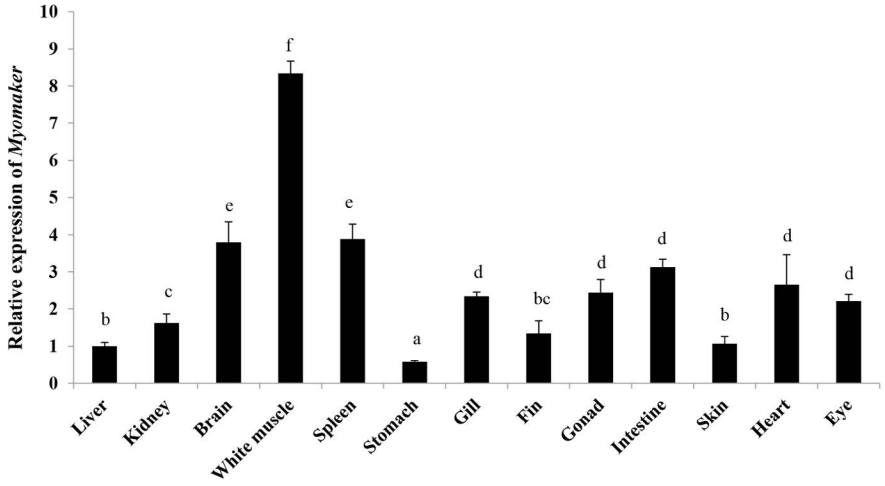

Fig. 4. Relative expression levels of *Almyomaker* in different tissues. The twelve tissues are heart, gonad, eye, skin, brain, fin, spleen, small intestine, gill, white muscle, kidney, liver, and stomach. Different letters indicate significant differences ($p < 0.05$).

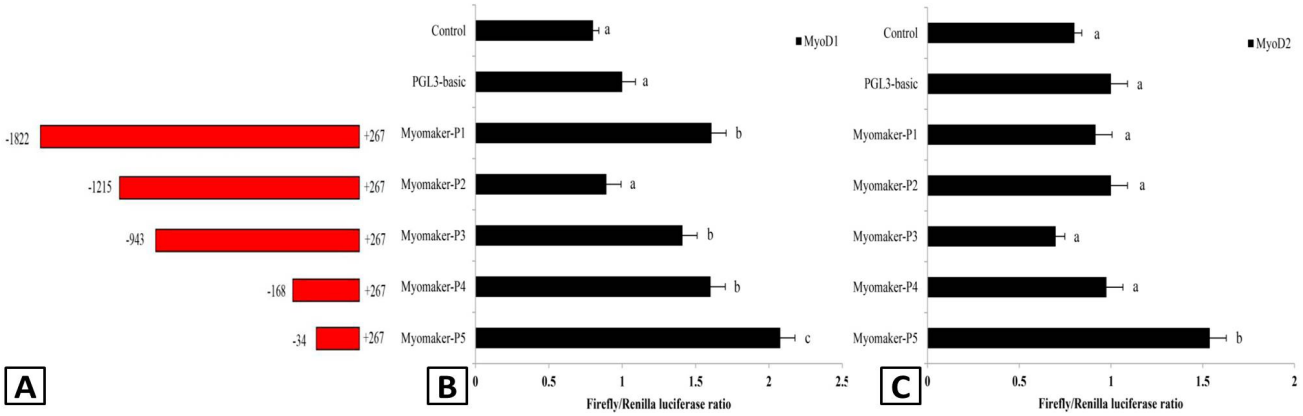

Fig. 5. Promoter activity analysis of the *Almyomaker* gene. A, the structure of the *Almyomaker* promoter and 5'UTR region. Five recombinant plasmids were denoted myomaker-p1 (-1822 to +267), myomaker-p2 (-1215 to +267), myomaker-p3 (-943 to +267), myomaker-p4 (-168 to +267) and myomaker-p5 (-34 to +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 ($p < 0.05$).

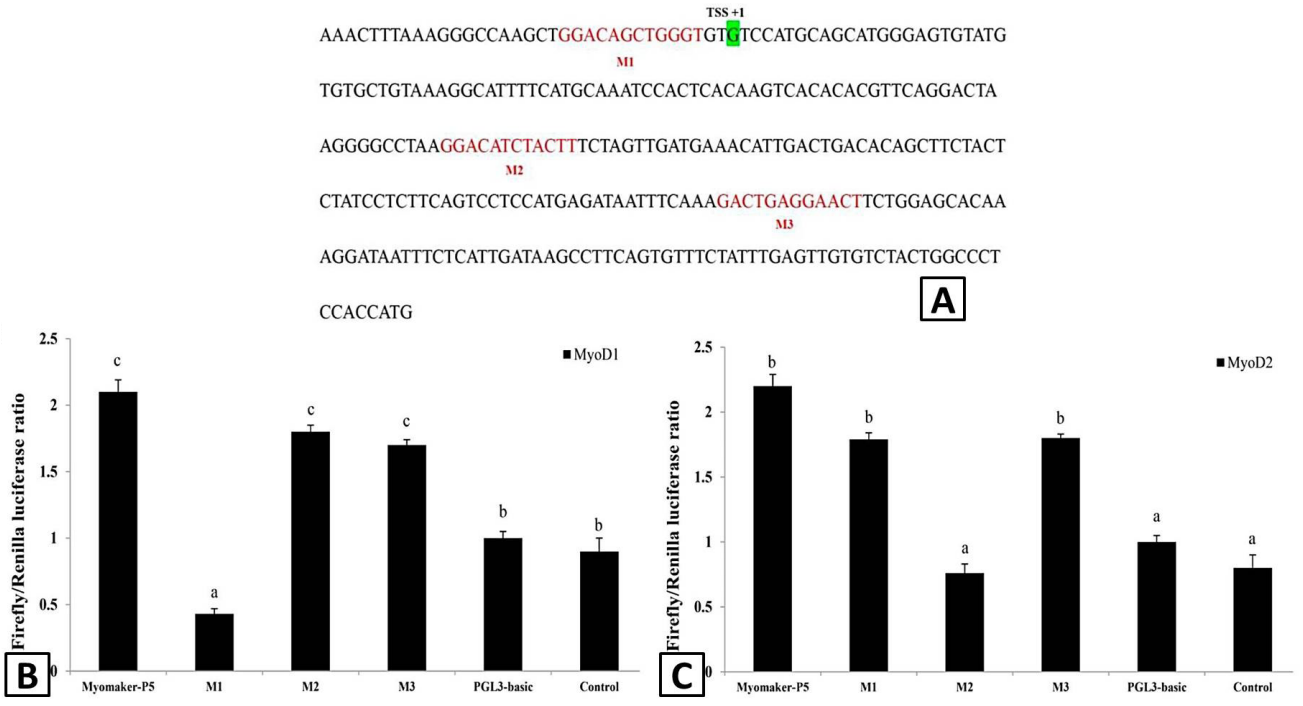

Fig. 6. Construction of truncated mutants for the identification of predicted transcription factor (TF) binding sites in the *Almyomaker* candidate sequence. A, the nucleotide sequence and predicted binding sites in the core region of the *Almyomaker-p5*. TSS indicates the transcription start site. Effects 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 ($p < 0.05$).

Two AlMyoDs 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 promoter-less 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; Lassar *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. gallus*, *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 *Oncorhynchus 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.

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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.

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