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E-mail:xbywb@ouc.edu.cn

Expression Analysis of the Insulin-Like Growth Factors I and II During Embryonic and Early Larval Development of Turbot (*Scophthalmus maximus*)

WEN Haishen*, QI Qian, HU Jian, SI Yufeng, HE Feng, and LI Jifang

Fisheries College, *Ocean University of China*, *Qingdao* 266003, *P. R. China*

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Abstract The insulin-like growth factors I and II (IGF-I and IGF-II) are important proteins involved in fish growth and development. Here, we report the isolation of IGF-II and expression analysis of IGFs in turbot *Scophthalmus maximus*, aiming to clarify their function in embryonic and larval development of fish. The deduced IGF-II gene is 808 bp in full length, which encodes a protein of 219 amino acids and is 93% similar with that of *Paralichthys olicaceus* in amino acid sequence. The tissue abundance and the expression pattern of IGFs in a turbot at early development stages were investigated *via* reverse transcription-polymer chain reaction. Result showed that the IGF-I and IGF-II genes were widely expressed in tissues of *S. maximus*. IGF-I was detected in all tissues except intestines with the highest level in liver, while IGF-II transcript presented in all tissues except muscle. At the stages of embryonic and larval development, the mRNA levels of IGFs sharply increased from the stage of unfertilized egg to post larva, followed by a decrease with larval development. However, there was an increase in IGF-I at the embryonic stage and IGF-II at the gastrula stage, respectively. These results suggested that IGFs play important roles in cell growth and division of the turbot. Our study provides reference data for further investigation of growth regulation in turbot, which can guarantee better understanding of the physiological role that IGFs play in fish.

Key words *Scophthalmus maximus*; insulin-like growth factor; cloning; expression analysis

1 Introduction

The insulin-like growth factors I and II (IGF-I and IGF-II) are two highly homologous mitogenic polypeptides which function ubiquitously and show diverse effects on the development, growth, and metabolism of animals (reviewed by Werner and LeRoith, 1996). The IGF system, including IGF receptor and its binding protein (IGFBP), plays important roles in regulating vertebrate growth (Reinecke and Collet, 1998). IGFs are single-chain polypeptides initially translated as prepropeptides with a leader signal peptide at N-terminal, which is followed by domains B, C, A, and D and domain E trailer at C-terminal (Tse *et al*., 2002).

In early researches, the IGF genes have been cloned in many vertebrates, and their expression modes have been studied. The evidenced is that teleost IGFs are initially produced in liver, and hepatic tissues synthesize IGFs as well. The IGFs act through local paracrine and autocrine pathways as sensitive regulators of testicular function (Reinecke and Collet, 1998; Gao *et al*., 2012). IGF-I has been shown to exhibit a wide range of biological actions including stimulation of cell division and differentiation as well as protection from protein degradation and apoptosis. In addition, IGF-I acts as a regulator of endocrine factors such as growth (Jones and Clemmons, 1995; Upton *et al*., 1998). IGF-II is a potent mitogenic and survival factor involved in the regulation of growth, development, and reproduction in animals (Yuan *et al*., 2011).

The IGF-I cDNA sequence has been cloned in a number of teleosts including chinook salmon (Wallis and Devlin, 1993), coho salmon (Duguay *et al*., 1992), catfish (McRory and Sherwood, 1994), gilthead seabream (Duguay *et al*., 1996), goldfish (Kermoun *et al*., 1998), and turbot (Duval *et al*., 2002). IGF-II amino acid sequence was first reported in human in 1978 (Rinderknecht *et al*., 1978), and then found in many fish species. Though Duval *et al*. (2002) have determined the partial sequence of IGF-II in turbot, it is meaningful to further study the full-length IGF-II in this species for better understanding its physiological function. Despite the knowledge that IGFs play crucial roles in the growth of mammals (De Pablo *et al*., 1993; Rosen *et al*., 1993; Scavo *et al*., 1991), information is lacking on the function of the IGF system in the early development of fish. Duguay *et al*. (1996) found that IGFs are expressed in gilthead seabream dur-

* Corresponding author. Tel: 0086-532-82031825
E-mail: wenhaishen@ouc.edu.cn

ing larval development, whereas Greene and Chen (1997) suggested that IGFs are expressed in embryos of rainbow trout. Despite a study on the regulation between IGFs and growth hormone (GH) release in turbot (Duval *et al*., 2002), the expression pattern of IGFs genes during the embryonic and larval developmental stages of this species is unknown.

The turbot *Scophthalmus maximus* is an important commercial flatfish species inhabiting European waters. It was first introduced into China for farming in 1992, and as a result of successful artificial breeding, commercial culture of the species has spread rapidly along the coast of North China since 1999 (Lei and Zhan, 2001). Previously, we have cloned the IGFBP-1 and -2 genes and analyzed their expression at the stages of adult and early development (Hu *et al*., 2012). In the present study, we cloned IGF-II gene from liver of *S. maximus* and analyzed the expression of IGF-I and IGF-2 genes during the stages of embryogenesis and early larval development in order to understand how the growth of turbot is regulated. The results will contribute to understanding the physiological characteristics of *S. maximus* and providing a reference data for rational breeding of this fish species.

2 Materials and Methods

2.1 Experimental Fish

Adult turbot (*S. maximus*) were obtained from coastal area of Shandong Province during a period from September 2009 to next May. The fish were anesthetized with MS-222 (Sigma, St. Louis, MO, USA) and sacrificed after decapitation. Tissues including ovary, intestine, muscle, gills, kidney, liver, stomach, heart, brain, spleen, and skin were collected from adult turbot and immediately frozen in liquid nitrogen, then stored at −80℃ till use. Additionally, unfertilized eggs (0-hour-post-fertilization, 0HPF); fertilized eggs in the gastrula (18HPF), embryonic (80HPF) and hatching (120HPF) stages; and the prophase of hatching (13-day-post-hatching, 13DPH) and post larva (25DPH) were obtained from a commercial hatchery and immediately frozen in liquid nitrogen, then stored at −80℃ till use.

2.2 Total RNA Extraction and Reverse Transcription

Total RNA was extracted using Trizol reagent (Invitrogen, USA). The intactness and purity of RNA extracts were determined with a UV spectroscopy at 260 and 280 nm. Total RNA (1 μg) was subjected to reverse transcription in a total volume of 10 μL with random primers and M-MLV reverse transcriptase (Takara, Japan).

2.3 Molecular Cloning and Sequence Characterization of IGF-II cDNA

The strategy for isolating IGF-II full length cDNA involved the generation of a partial cDNA from liver by reverse transcription-polymerase chain reaction (RT-PCR) with degenerate primers firstly, then the amplification of 5’ and 3’-cDNA ends by rapid amplification of cDNA ends (RACE), and finally the generation of a cDNA that encodes the complete coding region in a single set of RT-PCR. For IGF-II gene fragment amplification, the primers IGF2f and IGF2r (Table 1) were designed using the IGF-II amino acid sequences of tilapia (*Oreochromis niloticus*), gilthead seabream (*Sparus aurata*), rainbow trout (*Oncorhynchus mykiss*), and European seabass (*Dicentrarchus labrax*). The 50-μL PCR reaction contained 2 μL of cDNA from liver tissue following the manufacturer’s instructions (Takara, Japan). PCR amplification was performed using the following touchdown PCR cycling conditions: pre-denaturation at 94℃ for 5 min; 10 cycles of 94℃ for 30 s, 65℃ (decreasing by 1℃ each cycle) for 30 s, and 72℃ for 30 s, followed by additional 25 cycles of 94℃ for 30 s, 55℃ for 30 s and 72℃ for 30 s; and final extension at 72℃ for 10 min. According to the IGF-II fragment, PCR was performed with the 5’ and 3’ RACE primers and nested-PCR primers (listed in Table 1) designed using Primmer5.0 and DNAMAN software; the 5’ and 3’ RACE reactions were obtained

Table 1 Primers used for *Scophthalmus maximus* IGF-II gene cloning and IGFs (I and II) gene expression analysis

Primer name	Primer sequence (5’–3’)	Length of amplified fragment
For partial cloning		
IGF2f	GACGCCCTGCAGTTCgtntgygarga	
IGF2r	GGCCTGCTCCTGGgcyttdatytt	381 bp
For cloning 5’-end		
IGF2 (5-1)	CGCTCGTTCAAGTGCTGCTTCCTG	
IGF2 (5-2)	GGGGTCCGTGGGTCCTCCAGTTG	350 bp
For cloning 3’-end		
IGF2 (3-1)	AACAGGAAGTCCCGAGGAAGCAGCA	
IGF2 (3-2)	AAAAGTTTCGGAGGCAGGCGGAGA	250 bp
For expression analysis		
IGF1F	AGCCACACCCTCTCACTACTG	
IGF1R	AGCCTCTCTCTCCACACACAA	127 bp
IGF2-F	CTCCAACCAAATAACCCC	
IGF2-R	TTCCTCTACGATCCCACG	374 bp
18SF	CCTGAGAAACGGCT ACCACATC	
18SR	CCAAT TACAGGGCCTCGAAAG	119 bp

with a SMART™ RACE cDNA Amplification Kit (Clontech, USA). Products of the degenerate PCR and RACE reactions were electrophoresed on 1.5% agarose gel. The DNA band of expected size was purified with a TIAN Gel Extraction Kit (TIANGEN, China). The purified DNA fragments were then cloned into PGM-T vector (TIANGEN, China), propagated in *E. coli* DH5, and sequenced using an ABI3730XL sequencer.

2.4 Phylogenetic Analysis and Sequence Analysis

The presence and location of putative signal peptide cleavage sites and potential N-glycosylation sites in the amino acid sequences were predicted using the prediction servers of the Center for Biological Sequence Analysis (http://www.cbs.dtu.dk/services/). Amino acid sequences were retrieved from GenBank (Altschul *et al*., 1990), and the sequences of IGF-II were aligned with the homologs of other fish. Multiple protein sequence alignments were performed in Clustal X 1.81 (Thompson *et al*., 1997). Phylogenetic analyses of full-length amino acid sequences were conducted using MEGA 2.0 (Tamura *et al*., 2007). A rooted phylogenetic tree was constructed using the neighbor-joining algorithm (Saitou and Nei, 1987), and the data were re-sampled *via* 1000 bootstrapping replicates.

2.5 RT-PCR Analysis

The mRNA abundances of IGFs genes were detected by semi-quantitative RT-PCR assays. Tissue expression of IGFs genes was detected with a mature-stage sample. Total RNA extracts from the tissues, including ovary, intestine, muscle, gills, kidney, liver, stomach, heart, brain, spleen, and skin, were treated as described above. Temporal expression of IGFs genes was investigated in unfertilized and fertilized eggs at the gastrula (18HPF), embryonic (80HPF), and hatching (120HPF) stages, prophase of hatching (13DPH) and post larva (25DPH).

RT-PCR was carried out using a Takara Taq™ kit (Takara, Japan) according to the manufacturer's instructions. The PCR program was set as follows: degeneration at 94℃ for 5 min, followed by 34 cycles of 94℃ for 30 s, 62℃ for 30 s (both IGFs), 72℃ for 30 s, and final extension at 72℃ for 10 min (primers listed in Table 1), using 18S rRNA as an internal standard. The PCR program of 18S rRNA was 94℃ for 5 min, followed by 25 cycles of 94℃ for 30 s, 61℃ for 30 s, and 72℃ for 30 s, and final extension at 72℃ for 10 min. Each reaction product was resolved on a 1.5% agarose gel containing ethidium bromide and visualized on a gel system (Tanon, China).

3 Results

3.1 Characteristics of IGF-II cDNA

The cloned IGF-II cDNA (GenBank Accession No.: JN032705) is 880 bp in full length, which contains a 165-bp 5'-untranslated terminal region (UTR), a 55-bp 3'-UTR, and a 657-bp open reading frame. It encodes a protein of 219 amino acids, and the amino acid contains a signal peptide and domains B, C, A, D, and E with 6 conserved cysteine residues in domain B and domain A (Fig.1).

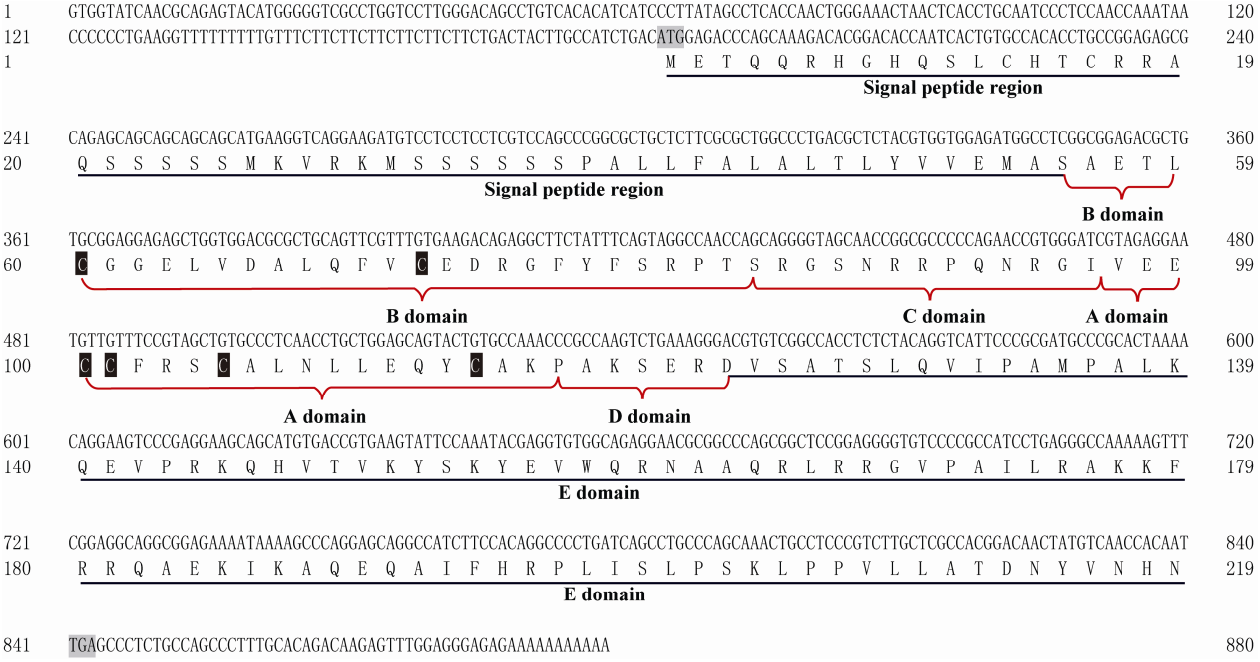

Fig.1 Full sequence of *Scophthalmus maximus* IGF-II. The domains B, C, A, D, and E are shown after the signal peptide region; 6 cysteine residues are indicated by black background and white letters.

3.2 Phylogenetic Relationship

Results of the sequence comparison analysis showed that the IGF-II gene of turbot is highly similar with those of redbanded seabream (*Pagrus auriga*), gilthead seabream (*Sparus aurata*), white seabream (*Diplodus sargus*), and

orange-spotted grouper (*Epinephelus coioides*) (Table 2). Results of the phylogenetic analysis on deduced amino acids showed that IGF-II belongs to the super IGF family, with four receptor binding sites in domains A and B. In the comparison of IGF-II amino acid sequence of turbot with those of *Paralichthy solivaceus* (AF091454.1), *Cynoglossus semilaevis* (FJ608668.1), and *Epinephelus coioides* (AY552787.1), the four domains B, C, A, and D are more conservative while domain E and signal peptide region are less conservative (Fig.2). In the phylogenetic tree (Fig.3), bony fish clustered as a branch; wild boar, chicken, and claw toad form a branch; three sole fish (turbot, Japanese flounder, and half slippery tongue sole) form a small branch. Results of sequence comparison showed that the closest relative of *S. maximus* is Japanese flounder (*P. olivaceus*), followed by half slippery tongue sole (*C. semilaevis*).

Table 2 Homologs of *Scophthalmus maximus* IGF-II

GenBank accession number	Species	Similarity
AB362310.1	*Pagrusauriga* 1062	93%
AY996778.1	*Sparusaurata* 1050	89%
AB362312.2	*Diplodussargus* 1042	93%
AY552787.1	*Pinepheluscoioides* 1029	92%
HM164111.1	*Sinipercachuatsi* 1014	92%
AY198185.1	*Siganusguttatus* 985	91%
AF091454.1	*Paralichthysolivaceus* 965	93%

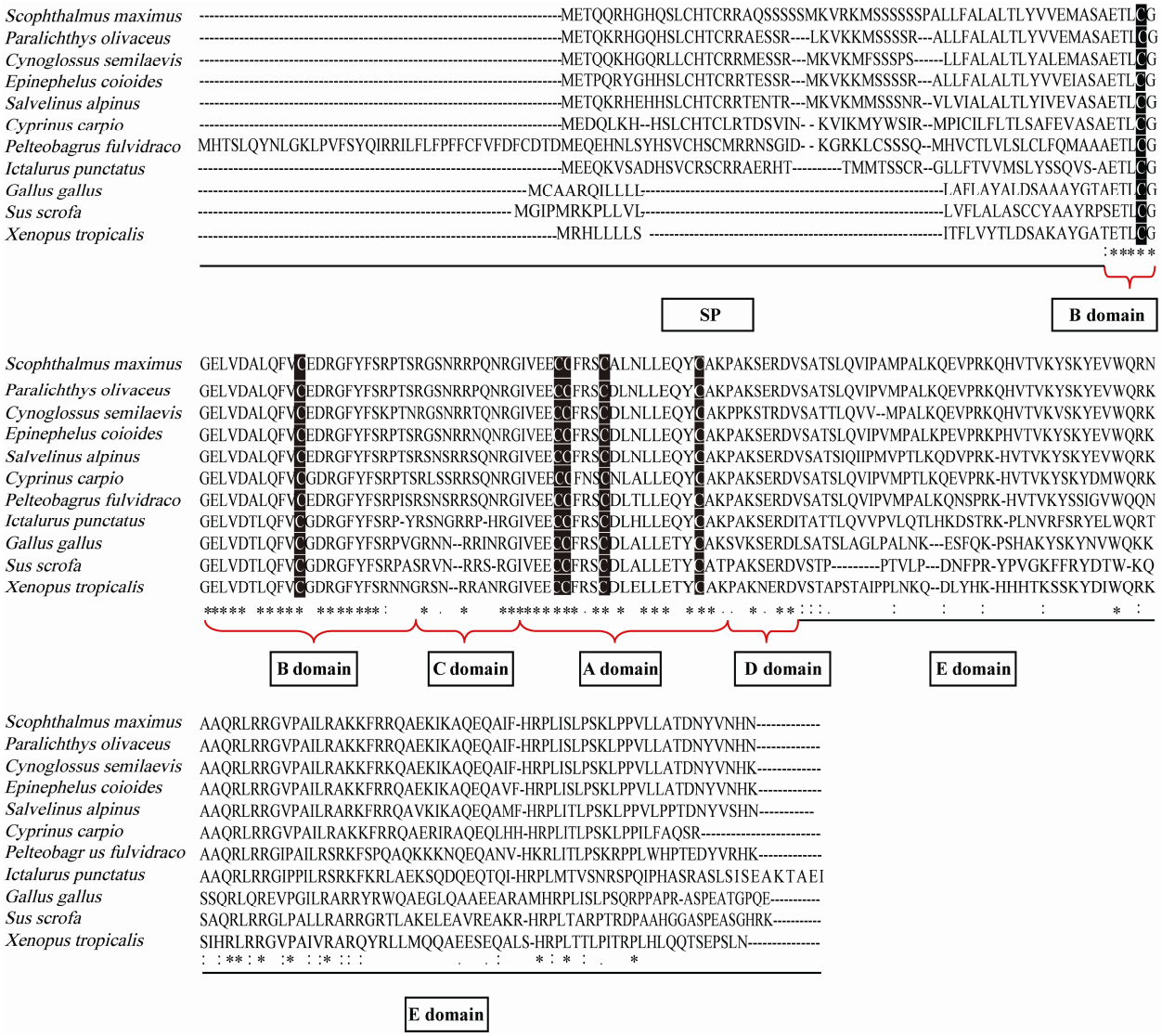

Fig.2 Alignment of amino acid sequences of *Scophthalmus maximus* IGF-II and those of other species. The identical, highly and less conserved amino acid residues are indicated by (*), (:), and (.), respectively; black backgrounds and white words indicate the 6 cysteine residues.

3.3 Tissue Distribution

The IGF-I gene was expressed in nine tissues of turbot except intestine (Fig.4). The IGF-I gene expression level was the highest in liver, followed by brain, spleen, kidney, heart, stomach, gills and skin, but hardly detected in muscle and ovary. On the other hand, the IGF-II gene was detected in all tissues (Fig.5). Its expression levels in spleen, brain, heart, stomach, liver, kidney, gills and intestine is higher than that in skin and muscle.

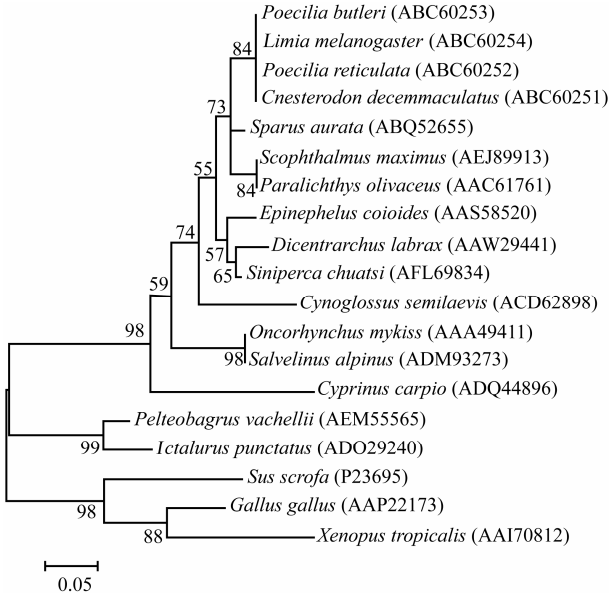

Fig.3 Phylogenetic tree constructed with amino acid sequences of IGF-II obtained in this study and those retrieved from GenBank. Phylogenetic analysis of IGF-II amino acid sequences inferred from the neighbor-joining method. GenBank accession numbers are in brackets after species names. Bootstrap values are indicated (1000 replicates).

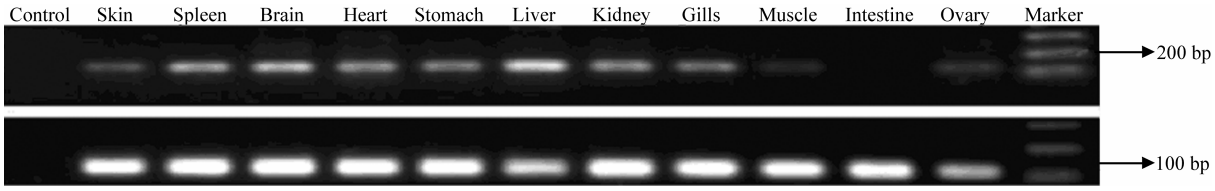

Fig.4 The tissue distribution of IGF-I gene transcript abundance in *Scophthalmus maximus* assayed by RT-PCR. The integrity of RNA from each turbot tissue was detected by uniform amplification of 18S rRNA transcripts (lower line).

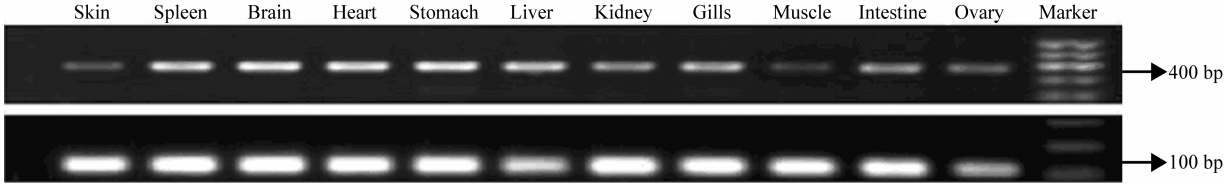

Fig.5 The tissue distribution of IGF-II gene transcript abundance in *Scophthalmus maximus* assayed by RT-PCR. The integrity of RNA from each turbot tissue was detected by uniform amplification of 18S rRNA transcripts (lower line).

3.4 Gene Expression Profiling

Results of the RT-PCR assay (Fig.6) showed that during embryonic and larval development, the IGF-I mRNA abundance decreased from the unfertilized egg stage (0HPF) to the lowest level in the gastrula stage (18HPF), followed by a sharp increase at embryonic stage (80HPF) and a subsequent decrease at hatching stage (120HPF). The IGF-I gene mRNA abundance finally increased to the highest at post larva stage (25DPH). IGF-II gene showed a different expression pattern from IGF-I gene did, sharply increasing from unfertilized egg stage (0HPF) to gastrula stage (18HPF), then greatly decreased at embryonic stage (80HPF) and subsequently increased to the highest at post larva stage (25DPH).

4 Discussion

In this study, IGF-II gene was cloned from turbot liver. Additionally, the tissue distribution of IGFs and their expression patterns during embryonic and larval development of turbot *S. maximus* were examined. Compared with other teleosts by amino acid sequence alignment, IGF-II of *S. maximus* shared the greatest homology with that of *Paralichthys olivaceus* (91%). The high similarity between different IGF sequences suggested that these polypeptides are of importance to the growth and development of vertebrates. The results of amino acid sequence analysis revealed that IGF-II was divided into highly fragmented N-terminal signal peptide, conservative do-

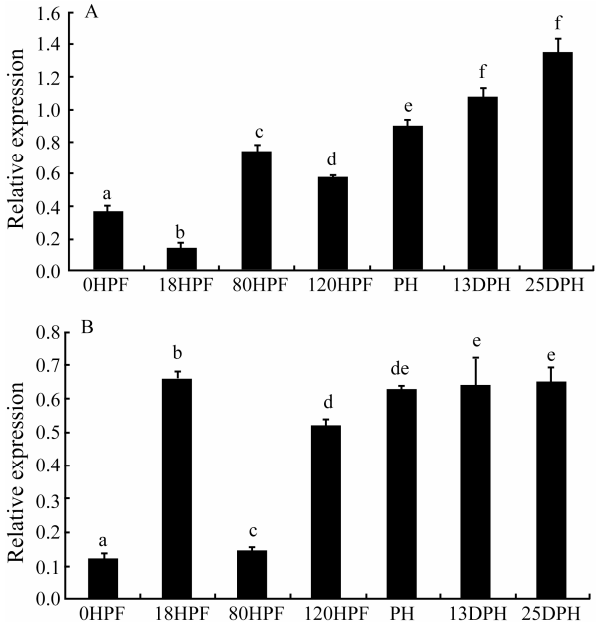

Fig.6 Expression of IGF-I (A) and IGF-II (B) genes during embryonic and larval developmental stages of *Scophthalmus maximus*. UE, unfertilized egg; PL, post larva; 13DPH, 13-day-post-hatching; PH, prophase of hatching; HS, hatching stage; ES, embryonic stage; GS, gastrula stage. The different letters indicate significant differences ($P<0.05$).

mains B, C, A, and D, and a less conservative domain E at C-terminus, with 6 conserved cysteine residues in the domains A and B. There are cysteine residues and IGF receptors binding protein amino acid residues in mammals' IGF, which can explain why turbot has similar physiological function with mammals. This result is similar with that reported in IGF-II of *Psetta maxima* (Duval *et al*., 2002). Taking into account our previous finding of IGFBP in turbot (Hu *et al*., 2012), we speculated that IGF-II could combine with 18 cysteine residues of IGFBP through the formation of disulfide bond.

It is well known that IGF-I stimulates the growth, differentiation, proliferation and metabolism of cells (Langdahl *et al*., 1998). In the present study, the IGF-I gene expression level of turbot liver was significantly higher than those of other tissues. Similar results had been reported in *Sparus aurata* (Duguay *et al*., 1996; Perrot *et al*., 1999), *Cyprinus carpio* (Tse *et al*., 2002; Vong *et al*., 2003), *Oreochromis niloticus* (Caelers *et al*., 2004), *Soleasene galensis* (Funes *et al*., 2006), *Dicentrarchus labrax* (Patruno *et al*., 2008), *Anguilla japonica* (Moriyama *et al*., 2006), and *Oreochromis hornorum* (Gao *et al*., 2012). The high expression of IGF-I in turbot liver indicated that IGF-I played a key role in the physiological function of liver. In addition, the nutritional conditions, ambient temperature, salinity and development state could affect IGF-I gene expression in liver (Moriyama *et al*., 2006; Gao *et al*., 2012). Although IGF-I is produced mainly in liver, it is also synthesized in a variety of tissues, including ovary, muscle, gills, kidney, stomach, heart, brain, spleen, and skin. This tissue distribution pattern of IGF-I was similar to that found in other fish species (Reinecke and Collet, 1998; Reinecke *et al*., 2006), which indicated that paracrine and autocrine actions of IGF-I were involved in organ-specific functions. The expression level of IGF-I gene in intestine was not detected in turbot, although it had been found low in other teleost species such as common carp (Vong *et al*., 2003) and rainbow trout (Greene *et al*., 1997).

As compared to IGF-I, IGF-II showed a different expression pattern in turbot tissues. IGF-II gene was highly expressed in turbot spleen, brain, heart, stomach, kidney, gill and intestine except for liver. In Zanzibai tilapia, IGF-II gene was highly expressed in intestine, spleen, stomach, kidney and pituitary (Gao *et al*., 2012), whereas in *Sparus aurata*, *Cyprinus carpio*, *Solea senegalensis*, and *Dicentrarchus labrax*, the IGF-II gene expression level was the highest in liver (Duguay *et al*., 1996; Tse *et al*., 2002; Funes *et al*., 2006; Terova *et al*., 2007). The different expression patterns of IGF-II gene in various teleosts suggested that IGF gene expression was species-dependent. In addition, the expression of IGFs genes in turbot was weaker in muscle than in other tissues, despite IGFBPs were not detected in muscle in our previous study (Hu *et al*., 2012). We speculated that the function of IGFs in muscle was controlled by other IGFBP family members. While the expression level of IGF-II gene in brain was higher than that of IGF-1, IGFBP-2 was highly expressed in brain of turbot (Hu *et al*., 2012). It was possible that IGFBP-2 had higher affinity to IGF-II than to IGF-I (Binkert *et al*., 1989).

Studies showed that IGFs were synthesized at the early embryonic development stage. In Japanese flounder, IGF-I gene was expressed from the early embryonic stage and its expression was regulated at different embryonic stages. IGF-I mRNAs are hardly detected in early embryos, and the zygotic IGF-I gene transcripts gradually increased from the eye lens formation to pre-hatching stage (Zhang *et al*., 2012). IGF-I gene was detected in the embryos and larvae in *Sparus aurata*, *Oncorhynchus mykiss*, *Cyprinus carpio*, *Ictalurus punctatus* and hybrid channel catfish (*Ictalurus punctatus* × *I. furcatus*) (Perrot *et al*., 1999; Greene *et al*., 1999; Tse *et al*., 2002; Peterson *et al*., 2005). In addition, the IGF-1 gene transcription had been found in unfertilized eggs in gilthead seabream and rainbow trout (Perrot *et al*., 1999; Greene *et al*., 1999). However, IGF-I mRNA was not detected in *Siganus guttatus* unfertilized eggs but was first expressed in larvae soon after hatching (Ayson *et al*., 2002). In the present study, the expression levels of IGF-I gene in unfertilized eggs, different embryonic development, and larvae stages were significantly different, implicating that IGF-I gene played an important regulatory role in embryo and larvae development.

In mammals, the circulating level of IGF-II gene was high before birth and declined afterwards (Moses *et al*., 1979). In the present study, results indicated that IGF-II gene was highly expressed after birth. Many studies demonstrated that IGF-II played a role not only in the embryonic period, but also at stages thereafter (Duguay *et al*., 1996; Perrot *et al*., 1999; Tse *et al*., 2002; Vong *et al*.,

2003; Caelers *et al*., 2004; Funes *et al*., 2006; Patruno *et al*., 2006; Hevroy *et al*., 2007; Radaelli *et al*., 2008). Furthermore, our study showed that the expression level of IGF-II gene in the gastrula stage was higher than those in other embryo and larvae development stages, consistent with our pervious findings of IGFBPs (Hu *et al*., 2012). Together these results proved that there existed a relationship between IGF-II and IGFBPs in early embryo development. As a whole, the relative expression of IGF-II gene was less abundant than that of IGF-I gene in turbot, different from result in common carp. The IGF-II mRNA level was in fact higher than that of IGF-I in all tissues examined with an exception of liver (Tse *et al*., 2002). It is presumed that IGF-I played more important role than IGF-II for cell growth and tissue differentiation in the process of embryonic development in physiology during the early development of turbot (Chen *et al*., 2010). Additionally, the GH-dependent IGFs mRNA accumulation has been reported in teleosts (Sakamoto and Hirano, 1993), and our next work is to explore the function of the IGF system in GH release.

In conclusion, the full-length cDNA of IGF-II gene was isolated from turbot (*S. maximus*) liver in the present study. The result demonstrated that liver was the major tissue for IGF-I gene expression in turbot. In addition, we found that IGFs genes widely expressed in a variety of other tissues. To our knowledge, this study presented the first evidence of IGFs genes expression at the stages of embryonic and larval development, which showed that both IGFs genes played an important role at late embryonic stages. These findings contributed to understanding the functional mechanism of IGF system in teleosts.

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