Molecular Characterization of the Ocular EST Clones from Olive Flounder, *Paralichthys olivaceus*

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ABSTRACT : The olive flounder (*Paralichthys olivaceus*) is one of the most widely cultured flatfish in Korea and Japan. During development, in a process known as metamorphosis, this fish reorients itself to lie on one side, the body flattens, and the eye migrates to the other side of the body. However, few studies have focused on molecule regulation mechanism of eye development in olive flounder. To reveal the molecular mechanism of eye development, we performed the studies on identification of genes expressed in the eye of olive flounder using EST and RT-PCR strategy. A total of 270 ESTs were sequenced, and 178 (65.9%) clones were identified as known genes and 92 (34.1%) as unknown genes. Among the 178 EST clones, 29 (16.3%) clones were representing 9 unique genes identified as homologous to the previously reported olive flounder ESTs, 131 (73.6%) clones representing 107 unique genes were identified as orthologs of known genes from other organisms. We also identified a kind of eye development associated proteins, indicating EST as a powerful method for identifying eye development-related genes of fish as well as identifying novel genes. Further functional studies on these genes will provide more information on molecule regulation mechanism of eye development in olive flounder.

Key words : Olive flounder, Eye, Expressed sequence tags (ESTs), Expression profile, Developmental biology.

INTRODUCTION

The olive flounder (*Paralichthys olivaceus*) is one of the most valuable commercial flatfish species that has well-established markets in Korea and Japan. Due to its high value as an important source of protein, investigations into the molecular mechanisms are required for the establishment of modern functional genomic approaches for the study of growth, development, reproduction, stress biology and molecular breeding. Previous studies on olive flounder were mainly focused on studying expression profiles from several kinds of tissues (Lee et al., 2003; 2006; 2007) and identification of genes involved in innate and acquired immunity (Aoki et al., 1999; Nam et al., 2000; 2003; Kono et al., 2001), but little molecular information was known with regard to the molecular mechanism of the eye development.

Flatfish (Pleuronectiformes), including olive flounder, comprise a biologically interesting group of fish. The asymmetry of flatfishes is one of the most striking body forms among vertebrates with both eyes being located on only one side (the ocular side) of the body. Migration of eye occurs at the beginning of the asymmetrical development. It was proposed that thyroid hormone may regulate eye migration in the same way as tail regression in tadpole metamorphosis (Inui & Miwa, 1985; Miwa & Inui, 1987; Yamano & Miwa, 1998). Bao et al. (2005) isolated differentially expressed gene, the splicing factor arginine/serine rich-3 (SFRS3) during metamorphosis

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involving eye migration in olive flounder and remained understanding the functions of upregulated expression of SFRS3 gene in relation to metamorphosis of flounder. Therefore, large-scale EST analysis is essential to adopt the cDNA microarray technology for comparative functional genomics, particularly to address the complex nature of gene expression involved in molecule regulation mechanism of eye development in olive flounder. As part of studies on the molecular mechanism of eye development, we report analysis of 270 clones from the olive flounder eye. This is the first report on large- scale eye gene discovery and the first large-scale examination of molecular compositions and expression profiles of the olive flounder eye.

MATERIALS AND METHODS

1. Experimental Fish and RNA Preparation

All experimental fish were raised at Genetics and Breeding Research Center, National Fisheries Research and Development Institute (NFRDI) and maintained in 10 tons flow-through tank at 15±1℃ under a natural photoperiod. Eye samples of 10 randomly selected fish were collected and frozen in liquid nitrogen until isolation of RNA. Pooled eye tissues were ground with a mortar/pestle and then homogenized with a hand-held tissue tearor using Trizol reagent (Invitrogen) according to the manufacturer's instructions. Poly (A)+ RNA was purified from total cellular RNA using the Poly A Tract mRNA isolation kit (Promega) according to the manufacturer's instructions.

2. cDNA Library Construction

A directional cDNA library of the eye was constructed using the Uni-ZAP XR cDNA synthesis/Gigapack cloning kit (Stratagene). cDNA synthesis was carried out using an oligo-$(dT)_{18}$ primer for the reverse transcription of approximately 5 ㎍ of mRNA and the libraries were constructed by directional cloning based on the manufacturers instruction manual. The primary library was amplified and aliquots of the amplified library were stored at both 4 and −70℃. Mass excision was performed and the cDNA inserts from the amplified Uni-ZAP XR library were rescued as pBluescript phagemids in SOLR *Escherichia coli*.

3. Sequencing and Bioinformatic Analysis

The plasmid cDNA library was plated to a density appropriate for picking individual colonies. Random clones were grown in 1.5-㎖ LB medium overnight in 12×75 ㎜ culture tubes. Plasmid DNA was prepared by alkaline lysis method (Sambrook et al., 1989) using the Qiagen Spin Column Mini-plasmid kits. Three microlitres of plasmid DNA (about 0.5-1.0 ㎍) were used in sequencing reactions. Single-pass sequencing of the 5'-termini of selected cDNA clones in phagemid form was performed using the ABI 3100 automatic DNA sequencer (PE Applied Biosystems) and the ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction kit (PE Applied Biosystems).

Bioinformatic analysis was conducted to determine gene identities using GeneMaster software (Ensoltek). ESTs were assembled in clusters of contigous sequences (contig) using ICAtools program (Parsons, 1995). Gene annotation procedures and homology searches of the sequenced ESTs have been locally done by BLASTX for amino acid similarity comparisons. Matches with the Expect value (E) less than $1.0\times e^{-4}$ were considered to be significant. All ESTs that were not identified as orthologs of known genes were designated as unknown EST clones.

4. Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

RT-PCR experiments were performed to study the expression of pupurin mRNA in olive flounder larvae from 7, 14 (premetamorphosis, stage 0), 21 (late preme-

tamorphosis), 27 (early metamorphic climax) and 34 (late metamorphic climax) days post-fertilization (dpf). Stages of development have been previously defined using descriptions of development in olive flounder (Minami, 1982; Miwa et al., 1988; Tanangonan et al., 1989; Keefe & Able, 1993). Total RNA was extracted from flounder larvae of different developmental stages using Trizol (Invitrogen). The quantity and purity of the RNAs were checked by electrophoresis on a 1% agarose gel with ethidium bromide staining. Next, 1 ㎍ total RNA was reverse-transcribed using an oligodT primer with SuperScript II Rnase H Reverse Transcriptase (Invitrogen) in a total reaction volume of 20 ㎕. The primer pair fpur-2F (5'-CAGCTGCGAGGATGGCTATG-3') and fpur-2R (5'-TGACGTGCACTCTCTCATTC-3') was used for amplifying purpurin fragment of 242 bp. The amplification conditions were: One cycle of 5 min at 94℃; 35 cycles of 30 s at 94℃, 30 s at 58℃, 30 s at 72℃; one cycle of 10 min at 72℃. As a positive control for RT-PCR, β-actin was amplified to determine the concentration of each template. The PCR products were subjected to 1.2% agarose gel electrophoresis and stained with ethidium bromide.

RESULTS AND DISCUSSION

1. EST Sequencing, Clustering and Similarity Search of ESTs

In the process of EST analysis from the olive flounder eye, 270 cDNA clones were single-pass sequenced from the 5' end, resulting in the characterization of cDNA clones that were longer than 100 bp after elimination of vector sequence. The number of clones sequenced from the cDNA library, the average size of inserts, and the redundancy of the obtained sequences, are given in Table 1. The average insert size was estimated to be 1.7±0.4 kb by PCR amplification of inserts from 20 randomly selected clones. The assembly program ICA tools software

Table 1. General characteristics of olive flounder eye ESTs

Total cDNA sequenced [a]	270
Average insert size [b]	1.7±0.4 kb
Average EST length	591 bp
EST clusters [c]	24
Singletons [d]	198
Redundancy [e]	17.8 %
ESTs with E value $< 1 \times e^{-4}$ (matched)	178 (65.9%)
ESTs with E value $\geq 1 \times e^{-4}$ (unknown)	92 (34.1%)

[a] Length of sequence used for comparison after editing (inserts <100 base pairs were excluded).
[b] The average insert size was calculated for 20 randomly selected cDNA clones.
[c] ESTs with 90% or greater identity over a 100 bp region were clustered together forming 24 EST clusters.
[d] 198 sequences did not sufficiently match any sequence in the data set to allow assembly.
[e] Redundancy=The number of genes / Total ESTs.

(Parsons, 1995) was used to organize the redundant ESTs into overlapping contigs. The results showed that the 270 brain ESTs were composed of 24 clusters and 198 singletons, suggesting that the overall redundancy of the library was 17.8%.

Gene annotation procedures and homology searches of the sequenced ESTs have been locally done by BLASTX for amino acid similarity comparisons. The ESTs with significant similarities ($E < 1\times e^{-4}$) to known proteins were evaluated to determine if the significant similarities were caused by simple amino acid matches. Of the 270 clones, 178 (65.9%) were identified as orthologues of known genes from olive flounder and other organisms. The remaining 92 (34.1%) clones could not be identified by similarity comparisons ($E \geq 1\times e^{-4}$); most of them are expected to derive from hitherto uncharacterized or novel genes, whereas some of them may be due to the fact that the homology is too low to detect in the region that is sequenced.

Table 2. Functional classification of identified ESTs in the eye library

Classification	Number of clones	Clones(%)	Number of gene	Redundancy factor
Cell structure/Cell communication	16	5.9	15	1.1
Cell structure/Motility	35	13.0	21	1.7
Cell/Organism defence	5	1.9	4	1.3
Gene/Protein expression	65	24.1	56	1.2
Metabolism	39	14.4	20	2.0
Unknown function	18	6.7	18	1.0

2. Functional Categorization of the Identified ESTs

The 178 identified genes were categorized into 6 functional groups (Table 2). Fifteen (5.9%) of the total ESTs were found to be homologous to genes involved in cell signaling/cell communication. The most abundant functional group consisted of genes involved in gene/protein expression for which there were 84 ESTs (31.1% of the total ESTs). Other ESTs were assigned to the following categories: cell structure/motility with 16 ESTs (5.9% of the total ESTs); cell/organism defence with 5 ESTs (1.9% of the total ESTs); metabolism with 84 ESTs (31.1% of the total ESTs).

Within the category gene/protein expression, several ESTs with homology to genes involved in the eye development were found. These include ESTs with significant sequence similarity to purpurin, plastin, peripherin, arrestin, lengsin and crystallins. Especially, the clones homologous to crystallins were most abundant (19 clones) accounting for 54.3% of the clones in this category. The presence of relatively high number of transcripts of these genes is not surprising as these genes are responsible for the optical properties of the eye lens (Bloemendal et al., 2004). Earlier studies have demonstrated that many of these globular proteins have been recruited from stress proteins and metabolic enzymes which are also expressed at lower levels for non-visual functions in many tissues (Wistow and Piatigorsky, 1988; Piatigorsky and Wistow, 1989). To our knowledge this is the first description of crystallins in olive flounder.

3. Expression Profile and Gene Identification

Expression profiles of the EST clones identified from the olive flounder eye are shown in Fig. 1. Of the 270 EST clones identified by BLASTX, 198 (73.3%) were singletons. Although redundancy will increase as the number of sequenced clones increases, the high percentage of singletons indicated that the complexity and coverage of this cDNA library from olive flounder ocular tissue was good. Of the 270 eye ESTs, 198 genes (73.3%) were sequenced only once; 51 genes (18.9%) were sequenced 2-5 times; 21 genes (7.8%) were sequenced over five times. These results suggest that the eye library has a

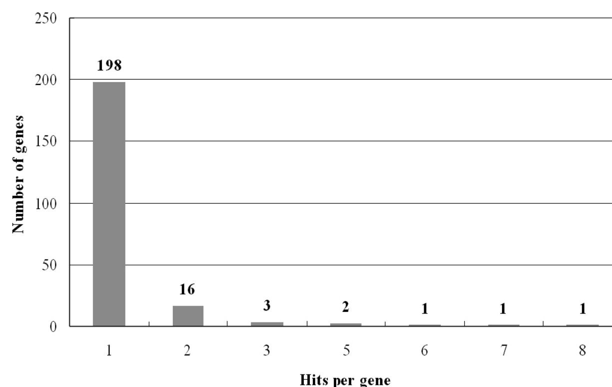

Fig. 1. Expression profiles and sequencing redundancy in the analysis of ESTs from the olive flounder eye. Of the 270 eye ESTs, 198 genes (73.3%) were sequenced only once; 51 genes (18.9%) were sequenced 2-5 times; 21 genes (7.8%) were sequenced over five times.

more even distribution of cDNA clones with relatively fewer abundant clones that tend to contribute redundant clones in EST projects.

One EST had significant sequence similarity to purpurin, which is a retina-specific adhesion molecule. The alignment showed that the putative sequence is 93.8, 93.8, 90.8 and 46.2% identical to those of salmon, goldfish, zebrafish, chicken and human purpurin, respectively (Fig. 2). Purpurin was originally isolated from cultured neural cells of developing chicken retina by Schubert et al. (Schubert & LaCorbiere, 1985; Schubert et al., 1986). Earlier studies have demonstrated that fish retinal purpurin plays a key molecule for cell differentiation and neurogenesis during early development of zebrafish retina. In the development of larval zebrafish retina, all retinal neurons may require purpurin for cell adhesion and survival (Tanaka et al., 2007; Nagashima et al., 2009). Although purpurin has been significantly detected in chicken and zebrafish retina, little molecular information was known with regard to its molecular mechanism in olive flounder. Therefore, to determine the expression pattern of purpurin in flounder larvae of different developmental stages, the transcriptional levels of purpurin gene in flounder larvae were detected at different time points after hatching using RT-PCR. The expression of purpurin in olive flounder during developmental stages is presented in Fig. 3. Purpurin mRNA was detected in all larval stages studied 7-34 days post-hatching with the expected fragment size of 683 bp. However, intensity of the mRNA signal decreased on following days, which is consistent with the temporal expression patterns previously reported in chicken and zebrafish (Berman et al., 1987; Nagashima et al., 2009). The results showed that purpurin mRNA is expressed at all stages examined during development and the mRNA levels of purpurin was found to remain roughly constant throughout embryo development.

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eyes1-C07   73  IDDDGSMTASSKGRVTLFGFWVVCADMAAQYSVPDPTTPGKMFMNYQGLASYLSSGGDNYWVIDTDYDNY
salmon      67  ..............................N.......................................
goldfish    67  ......................................................................
zedrafish   67  ......................................A...............................
chicken     67  VEE..................I................A....T...........................
human       63  V.ET.Q.S.TA....R.LNN.D.....VGTFT--.TED.A.F..K.W.V..F.QK.N.DH.IV.....T.

                *  * **    ** * * *   ***   ****  *  **  *   *   *       * *  Identity
eyes1-C07  143  AITYACRTVKEDGSCEDGYALIFSRNQRGLPPAIQRTVRQKQEDICMSGQFQPVLQSGAC  (100%)
salmon     137  ...................S......P.........I..G...E...A...E........  (93.8%)
goldfish   137  .........D..T.......V....P.........L.....DE...A............  (93.8%)
zedrafish  137  .........D....D...S.V....P.........L.....DE...A............  (92.3%)
chicken    137  .......S.......D...S......P.........I......E...............  (90.8%)
human      133  .VQ.S..LLNL..T.A.S.SFV...DPN....EA.KI...R..EL.LAR.YRLIVHN.Y.  (46.2%)
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Fig. 2. Alignment of deduced amino acid sequence of clone eyes1-C07 with salmon, goldfish, zebrafish, chicken and human purpurin. Identical amino acid residues with clone eyes1-C07 are indicated by dots (.). Asterisk (*) indicates the invariant and conserved residues in purpurin. The percentages in parentheses indicate the overall amino acid identities.

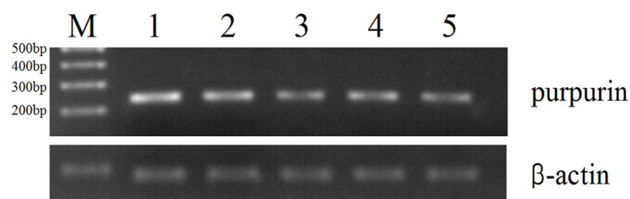

Fig. 3. RT-PCR analysis of purpurin expression during developmental stages. M, DNA marker; 1, 7 dpf; 2, 14 dpf; 3, 21 dpf; 4, 27 dpf; 5, 34 dpf. RT-PCR was used to detect the expression levels of purpurin mRNA from flounder larvae of different developmental stages; as positive control for RT-PCR, β-actin was amplified to determine the concentration of templates.

In conclusion, we describe the construction and assessment of the high quality olive flounder cDNA library which is a valuable resource for more detailed EST surveys and the identification of full-length genes of interest. This work identified 270 ESTs from a non-normalized cDNA library of the olive flounder eye. Expression profiles of these genes were revealed by their frequency in a cDNA library. We also identified a kind of eye development associated proteins, indicating EST as a powerful method for identifying eye development-related genes of fish as well as identifying novel genes. These results provide a useful starting point to explore the functional genomics of genes involved in the eye development, making it an important resource for studying the developmental and comparative biology of Pleuronectiformes.

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