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Identification of Differentially Regulated Genes in the Brain of *Limanda yokohamae* from Masan Bay, Korea

Jeong Hwan Oh, Hyo-Bang Moon[1] and Eun Sang Choe*

Department of Biological Sciences, Pusan National University, Busan 609-735, Korea
[1]Marine Environment Research Team, National Fisheries Research and Development Institute, Busan 619-705, Korea

Abstract - Transcriptomic changes in the brain of *Limanda yokohamae* were investigated to understand the environmental condition of Masan Bay, Korea. Differentially expressed genes (DEGs) in the brain of the flat fish from Masan Bay were identified by comparing those from the reference site Gangneung using annealing control primers-based polymerase chain reaction. The results demonstrated that two different kinds of the cytoplasmic ribosomal proteins, 40 s ribosomal protein S27a and ribosomal protein L6, were identified by the BLAST searching followed by sequence analysis. These findings suggest that environmental status of Masan Bay could hinder protein synthesis that is required for maintaining brain functions and thus cause the dysfunction of fish physiology.

Key words : ACP-based PCR, marine environmental contamination, Masan Bay, *Limanda yokohamae*

INTRODUCTION

Masan Bay located on the south cost of Korea is a semi-enclosed bay with a slower water exchange. Many studies demonstrate that Masan Bay is heavily contaminated with organic pollutants including endocrine disrupting chemicals due to the industrial development and increasing population over the past few decades (Khim *et al.* 1999; Im *et al.* 2002; Hong *et al.* 2003; Yim *et al.* 2005; Hyun *et al.* 2007; Kannan *et al.* 2007; Moon *et al.* 2007, 2008a, b). Due to the liphophilic and hydrophobic characteristics of organic pollutants, they prefer to adhere to particles in the sediments and are accumulated in the lipid rich tissues of marine organisms, which may adversely affect the physiology of marine organisms via food web.

Marine environmental contamination caused by the complex mixtures of various organic chemicals, but not by a single compound, disrupts vital physiological processes or interferes with regulatory steps in the fundamental metabolisms of marine organisms. All these events leading to the dysfunctions of physiology are known to be regulated by altered gene expression. Many studies have been conducted using cytochrome P450 (CYP450) families as biomarkers to determine the toxicological effects of organic compounds on fish in the field and laboratory studies (Peters *et al.* 1997; Carlson *et al.* 2002, 2004; Gravato and Santos 2002, 2003; Nacci *et al.* 2002; Greytak *et al.* 2005; Ortiz-Delgado *et al.* 2005; Hoffmann and Oris 2006; Jonsson *et al.* 2006; Patel *et al.* 2006).

Identification of differentially expressed genes (DEGs) in response to organic pollutants has advantage to access toxicological risk in contaminated marine environments like Masan Bay. The annealing control primer (ACP) system, a new differential display-polymerase chain reaction (PCR) technique, has been developed to determine DEGs under

* Corresponding author: Eun Sang Choe, Tel. 051-510-2272, Fax. 051-581-2962, E-mail. eschoe@pusan.ac.kr

various physiological conditions. Since the system has 3′-, 5′- end regions that are bridged with polydeoxyinosine linker, the structural characteristics improve the specificity of PCR amplification and the elimination of false-positive results (Hwang *et al.* 2003; Kim *et al.* 2004). Although the number of primers is limited, ACP-based PCR can be employed as a powerful tool in identifying DEGs in marine organisms in response to environmental stimuli. In this study, therefore, DEGs were identified to assume the condition of Masan Bay using ACP-based PCR in the brain of flat fish living in the sediments of Masan Bay, Korea. Marbled sole, *Limanda yokohamae* (*L. yokohamae*), was used as a model fish in this study because the fish is a resident species of the bay.

MATERIALS AND METHODS

1. Fish sample collection and preparation

The female *L. yokohamae* were captured from Gapo [weight (g), 83.38±2.76; length (cm), 18.54±0.06] in Masan Bay and Gangneug [weight (g), 116.4±5.2; length (cm), 22.46±2.14], a reference site during the period from April to June, 2005, as depicted in Fig. 1 (n=7 per site). The flat fish was sacrificed and the brain was immediately frozen in liquid nitrogen and stored at −80°C until RNAs were isolated.

2. Total RNA isolation and the first-strand cDNA synthesis

Total RNAs were isolated from the brain of *L. yokohamae* using easy-spin™ [DNA free] Total RNA Extraction Kit (iNtRON Biotechnology, Korea). Briefly, brain sample (100 mg) was homogenized in 1 mL of lysis buffer and then vigorously vortexed in room temperature for 10 sec. After adding 200 μL of chloroform, the solution was centrifuged at 13,000 rpm for 10 min at 4°C. Then the supernatant (400 μL) was transferred to a fresh 1.5 mL tube and 400 μL of binding buffer was added and mixed it well by 2~3 times gentle inverting. The supernatant was loaded to the column and centrifuged at 13,000 rpm for 30 sec. Following discarding the flow-through, the column was washed by adding washing buffer and centrifuged at 13,000 rpm for 1~2 min to dry the column membrane. The 50 μL of Elution buffer was added directly onto the membrane after the column was placed in a new 1.5 mL tube. Finally, the column was incubated for 1 min at room temperature and centrifuged 13,000 rpm for 1 min to elute total RNAs. The isolated total RNAs were used for the synthesis of the first-strand cDNAs by reverse transcriptase. Reverse transcription was performed for 1.5 h at 42°C in a final reaction volume of 20 μL containing 3 μL of the purified total RNAs, 4 μL of 5 X reaction buffer (Promega, WI, USA), 5 μL of dNTPs (each 2 mM), 2 μL of 10 μM dT-ACP1 [5′-CTGTGAATGCTGC GACTACGATIIIIIT(18)-3′], 0.5 μL of RNasin® RNase Inhibitor (40 U μL^{-1}; Promega), and 1 μL of moloney murine leukemia virus reverse transcriptase (200 U μL^{-1}; Promega). The first-strand cDNAs were diluted by the addition of 80 μL of ultra-purified water for the GeneFishing™PCR, and stored at −20°C until used.

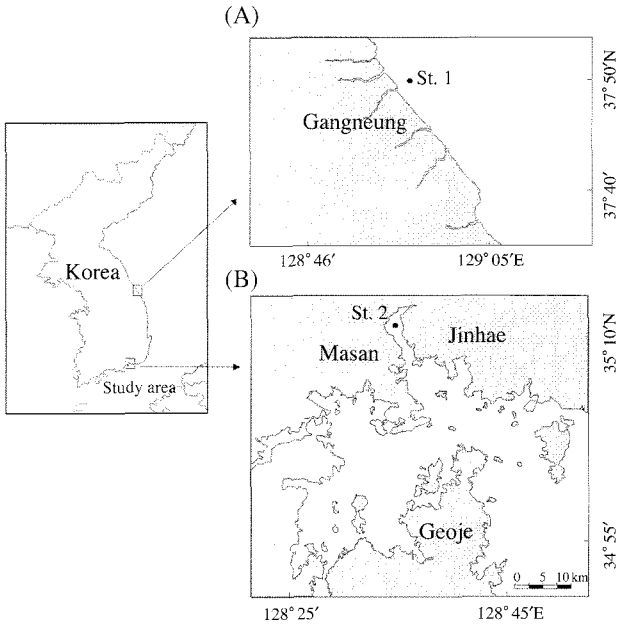

Fig. 1. Sampling stations showing the reference site Gangneung (A) and Gapo in the Masan Bay (B), Korea.

3. ACP-based PCR

DEGs were screened by ACP-based PCR method (Kim *et al.* 2004) using the GeneFishing™DEG kits (Seegene, Korea). Briefly, the second-strand cDNA synthesis was conducted at 50°C during one cycle of the first-stage PCR in a final reaction volume of 20 μL containing 3~5 μL (about 50 ng) of diluted first-strand cDNA, 1 μL of dT-ACP2 (10 μM), 1 μL of 10 μM arbitrary ACP, and 10 μL of 2 × Master Mix (Seegene). The PCR protocol for the second-strand

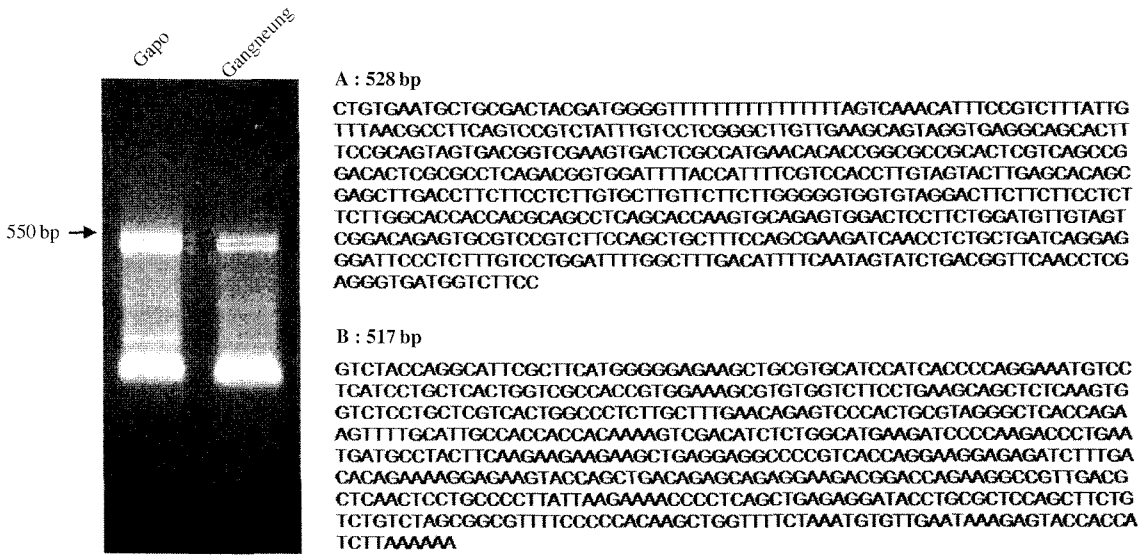

Fig. 2. Identified 2 up-regulated DEGs and corresponding DNA sequences in the brain of *L. yokohamae* from Gapo as compared with Gangneung, Korea.

synthesis was one cycle at 94°C for 1 min, followed by 50°C for 3 min, and 72°C for 1 min. After the second-strand DNA synthesis was completed, the second-stage PCR amplification protocol was 40 cycles of 94°C for 40 s, followed by 65°C for 40 s, 72°C for 40 s, followed by a 5 min final extension at 72°C. The amplified PCR products were separated in 2% agarose gel stained with ethidiumbromide. The differentially expressed band was selected as DEGs based on the size of PCR products (300 ~ 1,200 base pairs), intensity (at least more than two folds compared with control) and shape (sharp but not smear).

4. Direct sequencing

The differentially expressed bands were re-amplified and extracted from the gel by using the GENCLEAN®II Kit (Q-BIO gene, CA, USA), and directly sequenced with ABI PRISM®3100-*Avant*Genetic Analyzer (Applied Biosystems, CA, USA) using universal primer (5′-GTCTACCA GGCATTCGCTTCAT-3′).

5. Statistical analysis

The difference of an expression level between each group was determined by their density on agarose gel (mean ± SD). The statistical significance was analyzed with the student's *t*-test using GraphPad Prism 4 (GraphPad Software Incorporation, CA, USA) and set at $p < 0.05$.

RESULTS AND DISCUSSION

The status of environmental contamination of Masan Bay was determined by identifying DEGs in the brain of *L. yokohamae* as compared with those of Gangneung, Korea, using ACP-based PCR. Recently, the "omics" approach such as transcriptomics has been used to aid the risk assessment of pollutants and to assess the biological effect of toxicants on living organisms, which can offer an opportunity to identify a potential biomarker and gain a more comprehensive assessment (Waring *et al.* 2001; Williams *et al.* 2006, 2007). Thus, the identification of DEGs in marine organisms using genomic analysis may help to develop our understanding of the toxicological risk in environments that are contaminated, like Masan Bay.

The ACP technology has been applied in the identification of DEGs in fish (Yum *et al.* 2005) because of the high annealing specificity of primers (Hwang *et al.* 2003; Kim *et al.* 2004). In this study, mRNA expression profiles from Masan Bay were compared with the reference site Gangneung using the ACP-based PCR with 9 arbitrary primers. As shown in Fig. 2, two DEGs nearby 550 base pairs on the gel were found to be statistically different. Following sequ-

Table 1. Identification of DEGs by BLAST searching in the brain of *L. yokohamae* from Gapo in the Masan Bay, Korea

Identified DEGs	Description	Identities
A	40s ribosomal protein S27a mRNA [*Epinephelus coioides*]	464/513 (90%)
B	RPL6 mRNA for ribosomal protein L6 [*Solea senegalensis*]	428/483 (88%)

ence analysis and BLAST searching, two different kinds of the cytoplasmic ribosomal proteins, 40 s ribosomal protein S27a and ribosomal protein L6, were identified and the results were summarized in Table 1.

Ribosomes in the cellular process of translation in eukaryotes have both a small 40S subunit and a large 60S subunit which are composed of different RNA types and approximately 80 structurally distinct ribosomal proteins (Lodish *et al.* 2003). Although genome stores protein information, translation is a necessary step for producing functional proteins which play important roles in a variety of biological activities (Kapp and Lorsch 2004; Acker and Lorsch 2008). For this reason, the transcriptomic changes of ribosomal proteins critically affect on the assembly of each ribosome and protein biosynthesis. Thus, the present data indicate that the environmental condition of Masan Bay may alter fundamental biochemical reactions like protein biosynthesis in fish. These findings are supported by the fact that Masan Bay contaminated with the various pollutants alters the expression of endoplasmic reticulum stress proteins, cytochrome P450 1A and the activity of acetyl cholinesterase in *L. yokohamae* (Ahn *et al.* 2008; Jung *et al.* 2008). In addition, the concentration of toxic organic chemicals in sediments of Masan Bay is high enough to induce the physiological changes of flat fish inhabiting the Bay (Khim *et al.* 1999; Im *et al.* 2002; Hong *et al.* 2003; Yim *et al.* 2005; Yoo *et al.* 2006; Moon *et al.* 2007, 2008a). Taken together, these findings suggest that environmental status of Masan Bay could hinder protein synthesis that is required for maintaining brain functions and thus cause the dysfunctions of fish physiology.

In conclusion, we have performed experiments to determine transcriptomic changes in the brain of *L. yokohamae* from Masan Bay, Korea, as compared with the reference site Gangneung using ACP-based PCR. Although the limited primers were used in this study, identified genes may provide an informative insight into assess marine environmental risk.

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