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Molecular evidence for the existence of an aryl hydrocarbon receptor pathway in scallops *Chlamys farreri*

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ABSTRACT

The aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor that controls the expression of a diverse set of genes. In this study we cloned full-length cDNAs encoding an AhR homologue (designated *Cf*AhR, Accession number: *FJ588640*) from scallop *Chlamys farreri*. The *Cf*AhR sequence was constituted by an open reading frame (ORF) of 2466 bp encoding 821 amino acids. The predicted molecular weight was 93.0 kDa. The *Cf*AhR showed a high conservation of the residues and domains essential to the function of AhR, including basic helix–loop–helix (bHLH) and Per-ARNT-Sim (PAS) domains. Phylogenetic analysis demonstrated that it was clustered within the invertebrate AhR branch. *Cf*AhR expression was detected in gill, digestive gland, ovary, spermary, mantle and adductor, and the highest transcription level was observed in gill. Recombinant plasmid *Cf*AhR-pET32a (designated r*Cf*AhR) was successfully expressed in *Escherichia coli* BL21. To investigate the molecular detoxification mechanism of benzo(*a*)pyrene (BaP) detoxification-related genes (AhR; aryl hydrocarbon receptor nuclear translocator, ARNT; heat shock protein 90, HSP90; cytochrome P450 1A1, CYP1A1; glutathione S-transferase pi, GST-pi and P glycoprotein, Pgp) in *C. farreri* gill, real-time quantitative PCR analysis revealed that the mRNA expression level of *Cf*AhR, xenobiotic-metabolizing enzymes and efflux transporters was induced by BaP and was sensitive to BaP exposure time and concentration, suggesting that BaP influenced the expression of a putative AhR/ARNT signaling pathway in scallops. Our results support the possibility that *Cf*AhR genes are early molecular indicators of BaP through a putative CYP signaling pathway in marine bivalves.
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1. Introduction

The aryl hydrocarbon receptor (AhR) is a ligand-activated transcriptional factor that dimerizes with aryl hydrocarbon receptor nuclear translocator (ARNT) belonging to the Per-ARNT-Sim-basic helix–loop–helix protein family. AhR resides in the cytoplasmic compartment of cells as a multiprotein chaperone complex that includes the heat shock protein 90 (HSP90) (Hoffman et al., 1991; Whitlock, 1999; Kazlauskas et al., 2001). In mammals, AhR translocates into the nucleus upon the binding of various small molecules into the pocket of its single-ligand binding domain. The AhR-ARNT complex binds to the xenobiotic response element (XRE) motif and activates a battery of genes to mediate toxicity of persistent chemicals such as benzo(*a*)pyrene (BaP) (Ou and Ramos, 1995; Nagata et al., 2007; Joseph et al., 2008) whose toxicity occurs through activation of the AhR as well as induction of cytochrome P450 enzymes and a number of phase II enzymes including several glutathione S-transferase (GST) isoforms (Hayes and McMahon, 2001; Rushmore and Tony, 2002.; Sogawa et al., 2004; Kawajiri and Fujii-Kuriyama, 2007) as well as ATP-binding cassette (ABC) efflux transporters (Leslie et al., 2005; Xu et al., 2005).

The cloning and sequencing of the AhR and its dimerization partner ARNT at the end of the last century have provided new opportunities and tools to further our understanding of the AhR-dependent signal transduction pathway and the mechanism of toxicity action (Hahn et al., 1998). So far, genes of AhR and ARNT homologues have been identified from several species in vertebrate and invertebrate (Karchner et al., 1999; Tanguay et al., 1999; Butler et al., 2001). Various studies have proved the presence of AhR in bivalves, including *Crassostrea gigas* (EKC23432), *Dreissena polymorpha* (AAZ83700), *Ruditapes philippinarum* (ACM16807), *Mya arenaria* (AAF70378), *Pinctada martensii* (AHV83477), *Haliotis diversicolor* (AGG55386) and *Aplysia californica* (XP_005096897). Following the cloning of the AhR and ARNT, an extensive effort to map their functional and interactional domains was initiated (Dolwick et al., 1993; Jain et al., 1994; Ma et al., 1995; Poland et al., 1994; Whitelaw et al., 1993a,b). As others reported, the molecular mechanism through AhR that regulates genes transcription had been well studied in animals exposed to contaminants including 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin (TCDD) (Butler et al., 2001; Yamauchi et al., 2005; Hanssona et al., 2003). However there is no previous report on recombinant expression and functional characterization of the AhR in bivalves. In the current studies,

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Table 1
Nucleotide sequences of primers in this article.

Label	Forward and reverse primers (5′–3′)	Expect products	Efficiency
Clone of DNA sequences			
AhR-F	AAYCCNAGYAARCGNCAYC	974 bp	
AhR-R	AARTCNGGYTTRCTRTTYTTRTA		
AhR-5'GSP	AGCCAACGGTAGGCAATTAGACCTGAAC		
AhR-3'GSP	CAGAATGGGAGGACGGATGAGCCACAAT		
Quantitative real-time PCR			
AhR-qF	AAAAGATCCTGGCCCTCATC	154 bp	1.97
AhR-qR	ACTAAAGACTTCCGCTGGCT		
HSP90-qF	ATTACGTCCCTGCCCTTTGTAC	238 bp	2.21
HSP90-qR	ATGATCCTTCCGCAGGTTCA		
ARNT-qF	ACGGGAACAACTGTCCACTA	272 bp	2.21
ARNT-qR	AGTTTTTGATGTAGCCCGTG		
CYP1A1-qF	GCAAGGGAGGACAAGCAACTA	199 bp	2.10
CYP1A1-qR	GGCGTAAGGAATGCCATGAAG		
GST-qF	AGTTTGGTTTGGCGGGAG	187 bp	2.00
GST-qR	AGCAAATGTTGTTAGAACTGAATC		
Pgp-qF	GGTCAGCCAGGAACCAATACT	199 bp	2.15
Pgp-qR	CTGTTTCTGTCCACCTGAGAGTT		
β-actin-qF	TTCTTGGGAATGGAATCTGC	137 bp	1.97
β-actin-qR	TCTGCGATACCTGGGAACAT		
Clone of cDNA sequences and prokaryotic expression			
*Cf*AhR-Sac I	CGGAGCTCATGGTGGAAGACTGG		
*Cf*AhR-Xho I	CGCTCGAGTCTGGATCGTCACT		
Vector primer			
M13-47	CGCCAGGGTTTTCCCAGTCACGAC		
RV-M	GAGCGGATAACAATTTCACACAGG		
S. Tag	GGTTCTGGTTCTGGCCAT		
T7 terminator	GTTATTGCTCAGCGG		

Degenerate bases: Y, C/T; R, A/G; N, A/T/G/C.

the understanding of AhR in bivalves and the effects of BaP on the AhR/ARNT signaling pathway are limited.

Studies in mammals have shown that the AhR/ARNT complex binds to XREs, and subsequently, this complex binds to XREs upstream of the AhR gene battery including CYP1A, CYP1A2, CYP1B1, glutathione transferase, and ABC efflux transporters (Nebert and Dalton, 2006; Hahn, 2002; Rowlands and Gustafsson, 1997). In particular, the AhR-mediated CYP1A enzyme signaling pathway, which is involved in metabolism of xenobiotic such as β-naphthoflavone (β-NF), BaP and other Polycyclic Aromatic Hydrocarbons (PAHs), has been well studied in vertebrates (Billiard et al., 2006). Although the mechanism of activation for the AhR/ARNT signaling pathway is not yet known in invertebrates, in the female scallop *Chlamys farreri*, 0.025 μg/L BaP activates transcription of AhR, ARNT, CYP1A1 homologues (Tian et al., 2013). The expression patterns of CYP genes were measured in the rotifer *Brachionus koreanus* in response to BaP (Kim et al., 2013). Likewise, GSTs, the Phase II enzymes, provided cellular protection against the toxic effects of a variety of endogenous and environmental chemicals (Doyen et al., 2008), which are shown to be effective catalysts of PAH detoxification in bivalves (Banni et al., 2010). Several studies have demonstrated that the expression of the GST in clams seems to be regulated by BaP (Hoarau et al., 2002). Further, PAHs can be directly pumped out of the cells by ABC efflux transporters such as P glycoprotein (Pgp). The induction of Pgp-like genes and/or proteins has also been found in different tissues of bivalves living in polluted environments (Medeiros et al., 2008). But the study also showed that, in the cnidarian *Nematostella vectensis*, AhR does not bind to ARNT for dimerization (Reitzel et al., 2014), and *N. vectensis* AhR fails to bind TCDD and β-NF. Although there is no evidence to support a typical AhR/ARNT signaling pathway to respond to xenobiotic substances in invertebrates, many studies about the function of this pathway indicated that it is not to be neglected that there is a way related to AhR for xenobiotic detoxification in bivalves.

The scallop *C. farreri* is a commercial species native from southern Siberia to China. It distributes at shore area and sessile nature. The scallop filters large amounts of water by the siphon extended out to the water for their nutritional and respiratory needs, so they accumulate environmental pollutants and are often considered as an indicator organism in coastal seawater and sediment pollution assessments. The objective of this study is to characterize *Cf*AhR and investigate the response of mRNA expression of the AhR/ARNT signal pathway and the induction of multiple phase I and II biotransformation enzymes and efflux transporters in the scallops exposed to BaP.

2. Materials and methods

2.1. Scallops and exposure

Healthy scallops (*C. farreri*) were collected from the Bay of Pacific Corner (Yellow Sea, Qingdao, China) and acclimated in tanks containing aerated sand-filtered seawater (salinity 31‰, pH 8.1) at 12 ± 0.5 °C for one week before the exposure test. During the acclimatization period, the water in each tank was renewed completely once daily and the scallops were fed with dried powder of *Spirulina platensis* (30 mg for each individual) per day.

The scallops were randomly divided into five experimental groups with three replicates. BaP concentrations of 0, 0.025, 0.5, 1 and 2 μg/L were chosen for exposure based on contents of BaP in coastal seawater of Qingdao (Liu et al., 2012). Scallops were not fed during the treatment period. All other conditions were kept the same as those used for acclimatization. Six scallops for each replicate were sampled at 0, 0.5, 1, 3, 6 and 10d. Gills from six scallops were excised and mix grinded, total RNA was extracted immediately and stored at −80 °C until expression analysis. The gill of untreated scallops was collected from six scallops to clone *Cf*AhR and the gill, digestive gland, ovary, spermary, mantle and adductor of untreated scallops were sampled from another six scallops to determine the tissue distribution of *Cf*AhR.

2.2. Cloning the internal fragment of CfAhR

Total RNA was extracted from 100 mg gill tissues using TRIzol (Takara, Dalian, China), according to the manufacturer's instructions. RNA quantity, purity and integrity were examined by both native RNA electrophoresis on 1.0% agarose gel and the UV absorbance ratio at 260 nm and 280 nm (Multiskan Go 1510, Thermo scientific, Finland). The contaminating genomic DNA of 1 μg of total RNA was eliminated using gDNA Eraser (Takara, Dalian, China) at 42 °C for 2 min, and then cDNA was synthesized by PrimeScript RT Enzyme Mix (Takara, Dalian, China) at 37 °C for 15 min with adaptor primer oligo(dT) (Takara, Dalian, China) following the protocol of the manufacturer.

An internal fragment of *Cf*AhR was amplified using degenerate primers designed by the sequences of *D. polymorpha* (GenBank accession no. AAZ83700), *Drosophila melanogaster* (GenBank accession no. AF039570), *Danio rerio* (GenBank accession no. AAY42958) and *Rattus norvegicus* (GenBank accession no. NP_037281). The PCR reaction was performed in a total volume of 25 μL using Gradient Mastercycler (Eppendorf, Germany), PCR mixture containing reaction buffer with 15 mM $MgCl_2$, 0.8 mM dNTP mix, 1 mM each primer, template cDNA and 0.05 U of rTaq DNA Polymerase (Takara, Dalian, China). For *Cf*AhR amplification, RT-PCR cycles were conducted at 95 °C for 3 min followed by 35 cycles of 94 °C for 30 s, 54 °C for 1 min, 72 °C for 1 min, and a final cycle of 72 °C for 7 min. PCR products were analyzed by electrophoresis on 1.0% agarose gel stained with ethidium bromides in 1 × TAE buffer. The resulting PCR products were purified using the TIANgel Midi Purification Kit (TIANGEN, China) and ligated into pMD 19-T vector (Takara, Dalian, China). Vectors containing cloned inserts were transformed into *Escherichia coli* DH5α and incubated overnight at 37 °C. Positive clones were identified by blue/white screening and PCR screening with M13-47 and RV-M primers, and then sequenced on both strands.

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