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# Identification of interacting proteins with aryl hydrocarbon receptor in scallop *Chlamys farreri* by yeast two hybrid screening

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ABSTRACT

The aryl hydrocarbon receptor (AhR) belongs to the basic-helix-loop helix (bHLH) Per-Arnt-Sim (PAS) family of transcription factors. AhR has been known primarily for its role in the regulation of several drug and xenobiotic metabolizing enzymes, as well as the mediation of the toxicity of certain xenobiotics, including 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). Although the AhR is well-studied as a mediator of the toxicity of certain xenobiotics in marine bivalves, the normal physiological function remains unknown. In order to explore the function of the AhR, the bait protein expression plasmid pGBKT7-*Cf*AhR and the cDNA library of gill from *Chlamys farreri* were constructed. By yeast two hybrid system, after multiple screening with the high screening rate medium, rotary verification, sequencing and bioinformatics analysis, the interactions of the *Cf*AhR with receptor for activated protein kinase C 1 (RACK1), thyroid peroxidase-like protein (TPO), Toll-like receptor 4(TLR 4), androglobin-like, store-operated $Ca^{2+}$ entry (SocE), ADP/ATP carrier protein, cytochrome b, thioesterase, actin, ferritin subunit 1, poly-ubiquitin, short-chain collagen C4-like and one hypothetical protein in gill cells were identified. This study suggests that the *Cf*AhR played fundamental roles in immune system homeostasis, oxidative stress response, and in grow and development of *C. farreri*. The elucidation of these protein interactions is of much importance both in understanding the normal physiological function of AhR, and as potential targets for further research on protein function in AhR interactions.



## 1. Introduction

Initial studies of the aryl hydrocarbon receptor (AhR) focused on its roles in regulating the induction of cytochrome P450, family 1 (CYP1) enzymes (Nebert et al., 2004) and mediating toxicity of dioxin like chemicals (Okey et al., 2005). This receptor belongs to the basic-helix-loop-helix (bHLH)/PAS (Period [Per]-Aryl hydrocarbon receptor nuclear translocator [ARNT]-Single minded [Sim]) family of heterodimeric transcriptional regulators (Barouki et al., 2007). bHLH/PAS proteins are involved in the control of diverse physiological processes such as circadian rhythms, organ development, neurogenesis, metabolism and in the stress response to hypoxia (Crews, 1998; Gonzalez and Fernandez-Salguero, 1998; Whitlock Jr., 1999). Nevertheless, increasing experimental evidence suggests physiological roles for the AhR in cell proliferation and differentiation, in liver and immune system homeostasis and in tumor development (Fernandez-Salguero et al., 1995; Lahvis and Bradfield, 1998; Bunger et al., 2003; Walisser et al., 2004a,b; Li et al., 2011). Ma and Whitlock examined differences in the growth rates of wild-type (Hepa1) and AhR-defective mouse cell lines and determined that the AhR influences G1 cell cycle progression (Ma and Whitlock, 1996). Animal studies have yielded increasing evidence that AhR possesses strong immunomodulation potential by controlling the differentiation of T lymphocytes and dendritic cells (Quintana et al., 2008; Esser et al., 2009; Nguyen et al., 2013). In fish, Carlson et al. have demonstrated that immune cells in fish possess all the machinery responsible for PAHs metabolism (Carlson et al., 2004). Moreover, AhR has been identified in spleen of fish suggesting a possible implication of this receptor in xenobiotic induced immunotoxicity (Merson et al., 2006). Besides, the characterization of AhR heterologous in invertebrates has provided evidence for the involvement of such proteins in development, regardless the fact that they are unable to bind xenobiotics (Crews and Brenman, 2006; Puga et al., 2005). In our earlier researches, the characterization of *Cf*AhR in scallop *Chlamys farreri* has been identified and reported (Cai et al., 2016). The complete sequence of *Cf*AhR homologue cDNA is 2890 bp (Accession number: FJ588640), encodes a polypeptide of 821 amino acids with the predicted molecular mass of 93.0 kDa. *Cf*AhR homologue transcripts are expressed in all tissues of scallop and gill consistently showed the highest levels in each individual compared to other tissues.

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Spineless is a bHLH-PAS *Drosophila melanogaster* protein considered to be an AhR ortholog. It plays a central role in defining the distal regions of both antennae and leg. Loss-of function alleles of Spineless cause several developmental defects: transformation of distal antenna into leg, deletion of distal leg (tarsal) structures, and reduction in size of most bristles (Duncan et al., 1998). Although AhR signal plays an important role, effect of physiology and homeostasis mechanism also is ambiguous however. On the other hand, to date, no evidence supported a typical AhR/ARNT signaling pathway in invertebrates that responds to xenobiotic substances and no results support such signaling, but many studies about function of this pathway on CYP450-mediated detoxification indicated that it is not to be neglected that there is a way related to AhR for xenobiotics detoxification in bivalves.

Bivalve is one of the most important marine commercial species in China. Due to their sessile nature, filter-feeding habits, and pollutant bioconcentration, bivalves are widely used as marine pollution sentinels (Goldberg et al., 1978). In this study, the bait protein expression plasmid of *Cf*AhR and yeast two hybrid cDNA library of gill cells from *C. farreri* were constructed and the yeast two-hybrid system was used to screen the interaction of *Cf*AhR in *C. farreri*. The goal of the present investigation is to identify any interaction protein from the gill of scallop by using the yeast two-hybrid screen. This study is aimed at discovering the interaction of *Cf*AhR in *C. farreri* and laying the foundation to explore the function of *Cf*AhR.

## 2. Materials and methods

### 2.1. Scallops and total RNA extraction

Adult *C. farreri* with shell length of $7.25 \pm 0.5$ cm, obtained from Pacific Corner (Yellow Sea, Qingdao, China). All the scallops acclimated to laboratory condition in aquarium (1 l water per scallop) for one week before formal experiments. They were randomly distributed and cultured under filtered seawater at a salinity of 30‰ with a temperature of $17 \pm 1$ °C and a pH of 8.1 in $30 \times 40 \times 50$ $cm^3$ tanks. Each replicate contained 30–40 scallops (three replicates were performed). Water was renewed completely and the scallops were fed with dried powder of *Spirulina platensis* (30 mg for each individually) daily. Six scallops for each replicates were sampled after fostered. Gill tissue was collected immediately frozen in liquid nitrogen and then stored at −80 °C until use. Trizol Reagent was used to isolate total RNA from tissues according to the manufacturer's instructions (Invitrogen, USA). RNA degradation and contamination was assessed on 1% agarose gels. RNA concentration was measured using Qubit RNA Assay Kit in a Qubit 2.0 Fluorometer (Life Technologies, CA, USA).

### 2.2. Construction of the bait protein expression plasmid pGBKT7-CfAhR

We have acquired the sequence of the *Cf*AhR gene in *C. farreri* and submitted it to GenBank (GenBank accession number: FJ588640). According to the sequence, the primers with restriction enzyme cutting sites were designed. The designed primers used to amplify the *Cf*AhR ORF were *Cf*AhR-F (5′GAGGACCTGCATATGATGGTGGAAGACTGGGTGAT′3, the underlined is restriction sites Nde I), *Cf*AhR-R (5′GATGGGCGGGAATTCTCACTGGATCGTCACTTTGG′3, the underlined is restriction sites EcoR I). Those primers were synthesized in Sangon Biotech (Shanghai, China).

The composition of the PCR system (25 μl) includes 16 μl $ddH_2O$, 2 μl dNTP (each is 0.02 μM) (Takara, Dalian, China), 10 pmol/l primer for 1 μl each (0.4 pM), 10 × Ex Taq buffer for 2.5 μl, pMD19-T-*CfAhR* DNA for 2 μl, and Ex Taq polymerase for 0.5 μl (5 U/μl) (Takara, Dalian, China). The PCR cycling conditions were an initial denaturation at 95 °C for 3 min followed by 30 cycles consisting of 94 °C for 30 s, 57 °C for 30 s, and 72 °C for 120 s, and a final extension step of 10 min at 72 °C.

The aimed genes were purified using Gel Extraction Kit (Takara, Dalian, China) from agarose gel. The purified products and pGBKT7 vector were digested by the endonuclease Nde I and EcoR I at 37 °C for 4 h and connection systems (20 μl) includes 11.4 μl or 11.8 μl nuclease free $H_2O$, 3.3 μl linearized vector pGBKT7 (about 150 ng), 1.3 μl *Cf*AhR (150 ng) insert DNA, and 5 × MasterMix for 4 μl (Takara, Dalian, China). Mixed completely and incubated for 30 min in ice bath. Then transformed to DH5α *E. coli*. The recombined plasmid was verified by sequencing.

### 2.3. Auto-activation and toxicity detection of the bait proteins expression plasmid pGBKT7-CfAhR

According to the instructions of Yeastmaker™ Yeast Transformation System 2 (Clontech), auto-activation and toxicity detection of the bait plasmid pGBKT7-*Cf*AhR was done. The correctly constructed plasmid pGBKT7-*Cf*AhR was transformed into the yeast strain Y2HGold, then coated 100 μl of a 1/10 dilution and a 1/100 dilution per plate on the three selective mediums SD/-Trp, SD/-Trp/X-α-Gal, and SD/-Trp/AbA /X-α-Gal to detect the auto-activation. The vector pGBKT7 and the bait plasmid pGBKT7-*Cf*AhR were, respectively, transformed into the yeast strain Y2HGold, and then respectively coated on the selective medium SD/-Trp. The toxicity of the bait protein expression plasmid pGBKT7-*Cf*AhR was detected based on growth situation of the colonies on plates.

### 2.4. Construction of the cDNA library

The total RNA populations were first used to synthesize the first-strand as directed as the manufacturer using the kit of Make Your Own 'Mate & Plate™' Library System (Clontech, USA) with a thermal cycler (Bio-Rad, USA), and then the resultant ss cDNAs serving as template were exponentially amplified by LD-PCR kit (Clontech, Advantage 2 PCR Kit, Cat. No. 639206) with the nested primers (5′ PCR primer and 3′ PCR primer) of SMART III™ (all primer sequences were provided in Table 1). The PCR products ($>200$ bp) were excised from 1% low melting Agarose gel and purified using CHROMA SPIN-400 columns (Clontech, USA). The purified ds DNAs, together with linearized pGADT7-Rec AD cloning vector (Clontech, Cat. No. 630304), were co-transformed into yeast competent cell Y187, where yeast repair enzymes restore the linearized plasmid to its circular form by recombining homologous sequences at the end of the ds cDNA and pGADT7-Rec. This reaction was performed using Yeastmaker™ Yeast Transformation System 2 (Clontech, Cat. No. 630439). In addition to culturing on SD/-Leu plates to select the transformants, a series of dilution of the transformed mixture also were spread on SD/Leu media to calculate the transformation efficiency and independent colonies. After culturing at 30 °C for 5 days, the positive transformants were harvested to form an Y2H library. Hemacytometer was used to measure the cell density of the Y2H library to make sure the cell density $\geq 2 \times 10^7$ cells/ml, which is essential for a high efficiency of the Y2H library screens. The constructed cDNA libraries were evaluated for number of independent clones and transformation efficiency from $10^{-2}$ dilution SD/-Leu plates. The harvested and pooled libraries were also plated on SD/-Leu plates at $10^{-2}$, $10^{-3}$, $10^{-4}$, and $10^{-5}$ dilutions to calculate cell density, library titer and library quantity. To exhibit the insert sizes of the ready Y2H library, 48 colonies were randomly picked out and as templates they were used to amplify cloned inserts.

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