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The molecular mechanism of Nrf2-Keap1 signaling pathway in the antioxidant defense response induced by BaP in the scallop *Chlamys farreri*

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ABSTRACT

In this study, we cloned the full-length cDNA of the Kelch-like ECH-associated protein 1 (Keap1) from the scallops *Chlamys farreri (C. farreri)*. Sequences alignment and phylogenetic analysis showed that CfKeap1 was highly specific in the scallops, and the amino acid sequence identity value is closer to that in zebrafish Keap1b and *Nothobranchius furzeri* Keap1b than Keap1a. The highest transcription level of CfKeap1 expression was detected in the digestive glands. The gene expressions of CfKeap1, NF-E2-related nuclear factor 2 (Nrf2), Superoxide Dismutase (SOD), Catalase (CAT) and Glutathione Peroxidase (GPx) in digestive glands were evaluated by quantitative real-time PCR (qRT-PCR) after being exposed to benzo(a)pyrene (BaP) (0.25, 1and 4 μg/L) for 15 days, which indicated that the activation of Nrf2 and Keap1 expression can be significantly induced under BaP exposure. RNA interference (RNAi) experiments were conducted to examine the expression profiles of CfKeap1, Nrf2, antioxidant genes (Cu/Zn-SOD, CAT and GPx), mitogen-activated protein kinase (MAPKs) and protein kinase C (PKC) signaling pathways key genes in digestive glands and gills when exposed to BaP. Results showed that the mRNA level of CfKeap1 was significantly decreased by 60.69% and59.485%. The changes of CfKeap1 and Nrf2 suggested that the enhancement of Keap1 expression stimulating Nrf2 degradation. Furthermore, the expression of antioxidant genes were consistent with the Nrf2 gene, which suggesting that Nrf2-Keap1 signaling pathway is required for the induction of antioxidant genes. Besides, the changes of PKC, c-Jun N-terminal kinase (JNK) and p38 genes expression suggested that PKC and MAPKs signaling pathways played a synergistic role with Nrf2-Keap1 signaling pathway in the anti-oxidative defense system of bivalve molluscs. In conclusion, these data demonstrated that Keap1 can sense nucleophilic or oxidative stress factors to regulate the Nrf2 signaling pathway together with Cul3-based E3 Ubiquitin Ligase (E3), and the Nrf2-Keap1 signaling pathway played an important role in modulating gene expression of antioxidant enzymes in bivalve mollusks.

1. Introduction

In vertebrates, many studies have shown that organisms could develop an antioxidant defense system including enzymatic and non-enzymatic mechanisms to cope with oxidative stress [1,2] and Nrf2-Keap1 signaling pathway plays an important role in the transcriptional activation of an array of antioxidant and detoxification genes [1,3]. In molluscs, Wang (2018) found Nrf2-Keap1 signaling pathway could be induced by PAHs and the mRNA expression levels of antioxidant defense gene showed a similar pattern to Nrf2 under the PAHs exposure which confired the essential roles of Nrf2-Keap1 signaling pathway in the antioxidant defense system [4]. In the previous study, Keap1 has been identified as an important factor interacting with the Neh2 (Nrf2-ECH homology domain 2) degron domain of Nrf2, which played an important negative regulatory role for Nrf2 [5]. Keap1 is composed of five major domains: BTB/POZ domain, adouble glycine repeat (DGR) domain, intervening region (IVR) domain, N-terminal structure (NTR) and C-terminal structure (CTR). The BTB/POZ domain as a dimer structure for a Cul3-dependent ubiquitin-protein isopeptide ligase complex to maintain the steady-state levels of Nrf2. Besides, the IVR domain contains cysteine residues with the strongest Keap1 activity. It is a functional regulatory region of the whole protein, which not only participates in the reaction of electrophiles and oxidant, but also is Cys273, Cys288 and other cysteine sites which can relate to the ubiquitination of Nrf2. Mutation of Cys-273 or Cys-288 in the Keap1 IVR domain to alanine or serine reduces Keap1-dependent ubiquitination and increases Nrf2 stability in cultured cells, suggests that these residues are crucial for the Nrf2-repressing activity of Keap1 [5,6].

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Cysteine residues corresponding to Cys-273 and Cys-288 in the intervening region of mouse Keap1 are conserved among Keap1 orthologs in vertebrates and invertebrates. In the study of fish species, many scholars have discovered that fish have two types of Keap1, Keap1a and Keap1b [7–9]. Both Keap1a and Keap1b contain the cysteine residue corresponding to Cys-288 and Cys-273 and both Keap1a and Keap1b are able to facilitate the degradation of Nrf2 protein and repress Nrf2-mediated target gene activation. However, the analysis about the structure and subtypes of the Keap1 gene in mollusks has not been reported.

Numerous mammalian studies have shown that the activity of Nrf2 is primarily controlled by Keap1 [10]. What's more, the research of *Crassostrea gigas* [11] *and Ruditapes philippinarum* [4] also showed the Keap1 can sense oxidative stress and regulate the mRNA expression levels of Nrf2 to start and switch off of Nrf2 signaling pathway. Under quiescent conditions, Keap1 recruits Nrf2 into the Cul3-containing E3 ubiquitin ligase complex for ubiquitin conjugation and subsequent proteasomal degradation [6]. Under oxidative and electrophilic stresses, Keap1 functions as a sensor, causing the stress signals lose its activity to interact with Nrf2, thereby reducing the degradation of Nrf2 and translocating it to the nucleus. Then Nrf2 binds to antioxidant/electrophile-response elements located in the regulatory regions of many defense enzyme genes to enhance cell survival [9,12]. Furthermore, some scholars believe that the activation and regulation of Nrf2 is based on the mechanism of “hinge and latch model” [13]. Therefore, this model has been proposed that based on the discovery that Keap1 homodimer binds to a single Nrf2 molecule through DLG and ETGE regions two distinct binding sites within the Neh2 domain of Nrf2. Once the electrophilic reagents or oxidants are stimulated, the relatively low affinity DLG region will be dissociated from the DGR region. However, a high affinity for ETGE is still firmly bound to DGR. Therefore, Nrf2 cannot be identified and degradated by the ubiquitin ligase. At the same time, the entire process of Keap1 site is still occupied by Nrf2, a new generation of Nrf2. Due to the site saturated cannot be combined, so it translocates to the nucleus [13,14]. Besides, Sun (2007) identified Keap1 as a key postinduction repressor of Nrf2 and demonstrated that a nuclear export sequence (NES) in Keap1 was required for termination of Nrf2-antioxidant response element (ARE) signaling by escorting nuclear export of Nrf2 [15].

At present, studies on many numerous mammalian suggested that several signaling pathways such as mitogen-activated protein kinase (MAPKs), protein kinase C (PKC) and phosphatidylinositol-3-kinase (PI3K) are involved in regulation of Nrf2-Keap1 signaling pathway activation [16–19]. Hayes (1999) found that, the transactivation domain of Nrf2 contains the MAPK protein phosphate site by analyzing the amino acid sequence [20]; Levy (2009) found that the Nrf2 which was phosphorylated by JNK can increase the expression of Nrf2 signaling pathway target genes in the research of human bronchial epithelial cells [21]. Niture(2009) demonstrated that the PKC signaling pathway induced the phosphorylation of Nrf2-S40 and resulted in the dissociation of Nrf2 from Keap1. Besides, the interfering of PKC RNA reduced the basal level of antioxidant defense gene expression. In the research of Park (2013), RNA interference (RNAi) technology was used to investigate the effect of p38 on the nuclear translocation of Nrf2, and the expression of HO-1 in this study was inhibited [22]. In addition, Kay (2011) used RNAi technology and pharmacological inhibitors of PKC, p38 genes to reduce the nuclear translocation of Nrf2 and the activity of anti-oxidation element (ARE) [23]. There are just a few studies about the regulation of Nrf2-Keap1 signaling pathway activation in mammalian [24–33], and the molecular studies about it on mollusk have not been reported. By means of introducing dsRNA molecules into organisms or cells to inhibit the expression of a gene, RNAi technology has become a very powerful tool for reverse genetics to characterize the function of a novel gene, and it was widely used in many aquatic organisms such as zebrafish [34], *Litopenaeus vannamei* [35–37], *Crassostrea gigas* [38] and so on. However, such investigations about the regulation of Nrf2-Keap1 signaling pathway activation induced by PAHs in bivalves have not been reported.

C. farreri is a commercially important bivalve that is widely distributed along the coast of China, therefore the protection of anthropogenic contaminants is essential for survival and food safety. In this study, we first cloned and characterized the Keap1 gene from the *C. farreri (*CfKeap1), and then measured the expression levels of the Keap1 gene in the digestive glands, gills, adductor muscle, mantle and gonad by qRT-PCR. In order to contribute to a better understanding of the detoxification mechanisms of Nrf2-Keap1 signaling pathway in bivalves, gene expression of Keap1, Nrf2, Cu/Zn SOD, CAT and GPx in the digestive glands were evaluated by qRT-PCR after being exposed to benzo(a)pyrene (BaP). What's more, we used RNAi technology to explore the regulation of Nrf2-Keap1 signaling pathway activation to verify the role of Keap1 in the antioxidant defense signaling pathway in molluscs. The objective of this study is to investigate the molecular mechanism of the regulation of Nrf2-Keap1 signaling pathway activation in the antioxidant defense response induced by PAHs in the scallops *C. farreri*.

2. Materials and methods

2.1. Experimental animals and sample preparation

Healthy scallops (*C. farreri*) (shell length: 6.28 ± 0.60 cm; shell height: 2.41 ± 0.25 cm; mass: 30 ± 2.5 g) were collected from the rope-growing cultures at Red Island (Qingdao, China) and acclimated to laboratory condition in aquarium. The seawater was continuously aerated, and salinity, temperature and pH were maintained at 30‰, 12 ± 0.5 °C and 8.1. Water was fully renewed and the scallops were fed with dried powder of *Spirulina platensis* (30 mg for each individually) daily. Tissues from the digestive glands were collected from 6 healthy scallops, snap-frozen in liquid nitrogen and stored at −80 °C. Total RNA was extracted from the digestive glands using RNAiso Plus reagent according to the manufacture's protocol (TaKaRa, Dalian, China). The integrity of the total RNA was verified by 1.2% agarose gel electrophoresis, and the UV absorbance ratio at 260 nm and280 nm (Ultrospec 2100 pro, Amersham, USA). First-strand cDNA was synthesized from 1 μg of the total RNA using a PrimeScriptTM reverse transcriptase kit (TaKaRa, Dalian, China).

2.2. Cloning the full-length cDNA of CfKeap1

The partial sequences of CfKeap1 were obtained from the transcriptome of the scallops *C. farreri* established by our laboratory (accession number: SRR5192169). The sequences of primers were listed in Table 1 and all of the primers were obtained from Sangon Biotech (Shanghai) Co.,Ltd. The PCR reaction was performed using a Gradient Mastercycler (Eppendorf, Germany) in a total volume of 25 μL PCR mixture containing 2.5 μL 10 × reaction buffer with 15 mmol L^{-1} $MgCl_2$, 2 μL of 10 mmol L^{-1} dNTP mix, 1 μL of 25 μmol L^{-1} of each primer, 2 μL template cDNA, 16 μL MilliQ water, and 0.5 μL of Bio-Ready rTaq DNA Polymerase (5 U μL^{-1}) (TaKaRa, China). The PCR reaction was 95 °C for 3 min followed by 31 cycles of 94 °C for 30 s, 56.5 °C for 1 min, 72 °C for 3 min, and a final cycle of 72 °C for 7 min. The products were analyzed by electrophoresis in 1.0% agarose gel. The result of PCR products were purified using the TIANgel Midi Purification Kit (TIANGEN BIOTECH, Beijing, China) and ligated into pMD 18-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.

The full-length of CfKeap1 was obtained by the procedures of rapid amplification of cDNA ends (RACE) method using the SMART™ RACE cDNA amplification kit (Clontech, USA). 5′ and 3′ RACE-PCRs were

Table 1
Primer sequences of all genes used in the present study listed.

Primer name	Sequences (5′ → 3′)
Keap1-F	TTGTAGGCGAGGAAAAAT
Keap1-R	GCATACAACAGTCCTTCTAA
M13-47	CGCCAGGGTTTTCCCAGTCACGAC
RV-M	GAGCGGATAACAATTTCACACAGG
5′RACE-Keap1	GGAACAGCCATCGGACACTTGGAAAAC
3′RACE-Keap1	TGTCGTCAACCGCCTCATGTATGCA
RT-Nrf2-F	ACAACATGACCGTGGTGAG
RT-Nrf2-R	TGGTGTAGTGTAGTTCGCC
RT-Keap1-F	TTACAGGTGGTATGCGTGA
RT-Keap1-R	ACACACATTCATTTCGTTCA
RT-SOD-F	CTAAAACAGCCCAATACGAC
RT-SOD-R	TGGAAGCTCATTCTTTCACT
RT-CAT-F	GTACTGAACAGAAATCCCAAGAAC
RT-CAT-R	GAGAACAGACGACCCTGTAACAT
RT-GPx-F	GGAAAAACGCGTCAGAGATATT
RT- GPx-R	GAAGTTCCATGCCACATCTGTC
RT-PKC-F	GAGGATGAGGAAGTGAGTGA
RT-PKC-R	GGAGGAGGTTTGTGATGAG
RT-JNK-F	GGTAGACCCAGAAAAAAGG
RT-JNK-R	TGTGCTCCTGCTCATCTACT
RT-p38-F	AGCAGTAGAGAGGCAAGGAA
RT-p38-R	ACTGGGCAAGGTAAGGATG
β-actin-qF	TTCTTGGGAATGGAATCTGC
β-actin-qR	TCTGCGATACCTGGGAACAT
DsKeap1-F	TAATACGACTCACTATAGGGGTGGTATGCGTGAGGAAG
Keap1-F	GTGGTATGCGTGAGGAAG
DsKeap1-R	TAATACGACTCACTATAGGGGCATACAACAGTCCTTCTAA
Keap1-R	GCATACAACAGTCCTTCTAA

performed using specific primers (Table 1). Amplification methods were conducted by following the protocol of manufacturers. Sequence analysis and database comparison were performed using BLASTx search of the GenBank. The amino acid sequences, protein molecular mass and isoelectric point (pI) were predicted using lasergene 5.1 (DNA STAR inc., Madison, USA). Multiple protein sequence alignment was performed with ClustalW by the Meglign program (DNASTAR, Inc). The unrooted trees were built by the Molecular Evolutionary Genetic Analysis 5 programme (http://www.megasoftware.net) using Neighbour-joining method, consisting of 10 000 trials with bootstrap.

2.3. BaP exposure experiments

The scallops were put in aquarium (1 L water per mussel) after collection and were accilimated for 7 days in filtered seawater pumped from the Red Island (Qingdao, China) at laboratory condition. According to previous investigations PAHs concentrations in seawater from different sea areas were quite different, ranging from 0.0015 to 4.48 μg/L [1]. But several investigations suggested that as one of the most carcinogenic PAHs, the BaP pollution was also serious in parts of China sea. For instance, the water solubility of BaP is 16 nM (i.e. 4.037 μg/L). Accordingly, the present study selected 0, 0.25, 1 and 4 μg/L BaP to mimic the natural contaminant concentrations of PAHs. BaP was first dissolved in acetone, and then added to seawater to a final acetone concentration of 0.0025%. The scallops were randomly divided into four experimental groups (0, 0.25, 1 and 4 μg/L BaP exposure) with three replicates. All the conditions were maintained the same as those used for acclimation. Six scallops for each replicate were sampled at days 0, 1, 3, 6, 10 and 15. digestive glands from the six scallops were excised, mixed and grinded, and total RNAs were extracted immediately and stored at −80 °C until expression analysis.

2.4. Quantification of tissue distribution and expression of Nrf2-Keap1 signaling pathway key genes in experiment of BaP exposed scallops using qRT-PCR

The mRNA expressions of Keap1 genes in various tissues, including digestive glands, gills, adductor muscle, mantle and gonad of unexposed scallops, and the expression of Keap1 in the digestive glands of scallops from the BaP exposure experiment were determined by SYBR Green quantitative real-time RT-PCR.

Firststrand cDNA synthesis for the qRT-PCR was performed using 1 μg of total RNA as a template for cDNA synthesis by PrimeScript™ RT reagent Kit with gDNA Eraser (Perfect Real Time) and following the instructions of the manufacturer (Takara, Dalian, China). SYBR Green qRT-PCR amplification was performed in the Real-Time Thermal Cycler 5100 (Thermo scientific, Finland) with the total volume of 10 μL containing 5 μL of a 2 × SYBR Premix Ex Taq (Takara, Dalian, China), 1 μL of cDNA (1 μg/μL), 0.2 μL of forward and reverse primers and 3.6 μL of dH_2O (Takara, Dalian, China). The qRT-PCR program was used as: denaturation program (95 °C for 3 min), amplification and quantification program repeated 40 times (95 °C for 10 s, 53 °C for 20 s, and 72 °C for 30 s with a single fluorescence measurement), melting curve program (60–95 °C with a heating rate of 0.3 °C per second) and finally a cooling step to 40 °C. Dissociation curve analysis of amplification products was performed at the end of each qRT-PCR reaction to confirm that only one qRT-PCR product was amplified and detected. Gene specific primers used for the qRT-PCR are presented in Table 1 and in which β-actin was the housekeeping gene for the rest of the analyses. The amplification efficiency of each primer pair was calculated using a dilution series of cDNA. All amplification efficiencies were between 94 and 98%. The relative expression levels of the target genes were calculated with the $2^{-\Delta\Delta Ct}$ method [1] and normalized with β-actin.

2.5. Synthesis of dsRNAs

Based on the CfKeap1 cDNA sequence, a fragment of nucleotide sequences from position 269–740 of ORF was used to design (Table 1) and amplify the target site of the dsRNA for RNAi. The 472 bp fragment was subcloned to pGM-T vector (Tiangen) for obtaining the recombinant plasmid. As a negative control, the GFP gene fragment was synthesized by the plasmid P EGFP-C1 vector (Clontech).

Recombinant plasmids (pD7-CfKeap1 and pD7-GFP) were established. The sense and antisense DNA templates in *vitro* transcription were generated using pD7-CfKeap1 and pD7 GFP recombinant plasmids as templates via the PCR approach. Therefore, dsRNA-CfKeap1 and dsRNA-GFP were synthesized in vitro with Transcription T7 Kit (TaKaRa) following the manufacturer's protocol. The dsRNA was stored at −80 °C.

A total of 90 healthy scallops were divided into three groups: CfKeap1-dsRNA group, GFP-dsRNA group, and phosphate buffer saline (PBS) group, and the three experimental groups were cultured under 4 μg/L BaP exposur. The first group, as the experimental group, was injected CfKeap1-dsRNA with a concentration of 50 μg in 100 μL of PBS buffer (pH 7.6) per scallops [1]. Meanwhile, the GFP-dsRNA and PBS groups were taken as two types of negative controls and separately injected with volumes equivalent to those applied to the experimental group. At 1, 2 and 3 days post-injection, scallops testes from each group were sampled for RNA extraction. The mRNA expressions of PKC, JNK, p38, Nrf2, Keap1, Cu/Zn SOD, CAT and GPx in each group were determined by SYBR Green quantitative real-time as described in Section 2.4.

2.6. Data analysis

All data presented were the mean values of three independent sets of experiments. Each value was expressed as means ± standard deviation (S.D.). All data were tested for normality (Kolmogorov–Smirnov) and homogeneity of variance (Levene). Statistical analysis was carried out by one-way ANOVA using the Duncan's test to evaluate whether the means were significantly different ($P < 0.05$). Moreover, spearman correlation analysis was used to evaluate the correlations between the level of biomarker genes

Fig. 1. Multiple alignment of deduced amino acid sequence of CfKeap1. BTB domain and DGR domain are share in blue box and pink box, respectively. Intervening region (IVR) is located between BTB domain and DGR domain. The six Kelch repeats (in pink) are main structural features of the DGR domains. GenBank accession numbers for the protein sequences are as follows: *Danio rerio* keap1b (AB271119.1); *Helicoverpa armigera* Keap1 (KU355788.1); *Drosophila melanogaster* Keap1 (DQ372684.1).

expression and PAHs exposure concentrations. The statistical software SPSS 19.0 was used for all statistical calculations.

3. Results

3.1. Cloning, multiple sequences alignment and phylogenetic analysis

The full-length cDNA of the CfKeap1 encompassed 2,257bp (GenBank accession no. KX227455.1), containing a 5′ untranslated region (UTR) of 230 bp, a 3′ UTR of 251 bp, and an ORF of 1776 bp. The ORF encoded a polypeptide of 591 amino acids with the predicted molecular mass of 66.3 kDa and theoretical pI of 6.58. Alignment of the CfKeap1 with Keap1 sequences from several invertebrates demonstrates that CfKeap1 shared a identity of 50.9% to *Drosophila melanogaster* (DQ372684.1), and 51.5% to *Helicoverpa armigera* (KU355788.1) (Fig. 1). The phylogenetic analysis including 24 representative Keap1 proteins sequences from vertebrates and invertebrates revealed that the Keap1 genes of each species were clustered into three different branchs and CfKeap1 was clustered with *D. melanogaster* and *H. armigera* (Fig. 2).

3.2. Tissue distribution expression of the CfKeap1 mRNA

The tissue-specific expression of the CfKeap1 in five different scallops tissues was assessed by qRT-PCR (Fig. 3), and the CfKeap1 transcripts were expressed in all tissues examined except the adductor muscle. Despite the variability among tissues, the digestive glands consistently showed the highest levels in each individual compared to other tissue.

3.3. Quantitative analysis of the Nrf2-Keap1 signaling pathway key genes expression after BaP exposure

The mRNA levels of Nrf2, Keap1, Cu/Zn-SOD, CAT and GPx were evaluated by qRT-PCR in digestive glands of *C. farreri* exposed to BaP with the nominal concentrations of 0, 0.25, 1 and 4 μg/L (Figs. 4 and 5). No scallops died during the exposure experiment. The melting curve profile was clear with single peaks.

The mRNA expression of Keap1 in digestive glands had shown in Fig. 4. At the 1 d of exposure, it was significantly higher in each exposure group compared with the control group ($p < 0.05$). The expression of Keap1 significantly decreased at day 6 after exposure ($p < 0.05$) in the BaP low-dose group. By the end of exposure, the mRNA levle of Keap1 in the BaP middle-dose group was still higher than the control group.

The mRNA expression of Nrf2, Cu/Zn-SOD, CAT and GPx in the digestive glands had shown in Fig. 5. At the earlier stage of exposure, the mRNA expression of Nrf2 was significant differences in each exposure group compared with the control group ($p < 0.05$) especially in high-dose group. At day 6 after exposure, the expression levels began to decline. By the end of exposure, the mRNA levle of Nrf2 in each exposure group were still higher than the control group. At day 1, the mRNA level of Cu/Zn-SOD was no significant difference between each exposure group and the control group ($p > 0.05$). Significant increase in Cu/Zn-SOD mRNA expression was detected in BaP middle-treated group at day 6 and day 10 ($p < 0.05$). The expression of CAT significantly increased at day 6 and day 10 after exposure ($p < 0.05$). As time progressed, the expression levels began to decline. By the end of exposure, the mRNA levels of CAT showed no significant difference compared to the control group ($p > 0.05$). At the earlier stage of exposure, the expression of GPx was no significant differences in each exposure group compared with the control group ($p < 0.05$), then it began to increase gradually and showed maximum value in BaP low-dose group at day 10 after exposure ($p > 0.05$).

3.4. CfKeap1 mRNA expression profile stimulated by CfKeap1-dsRNA

We determined the CfKeap1, Nrf2, Cu/Zn-SOD, CAT, GPx, PKC, JNK and p38 expression levels after injection of CfKeap1-dsRNA in the digestive glands and gills of *C. farreri*. The relative expression level of

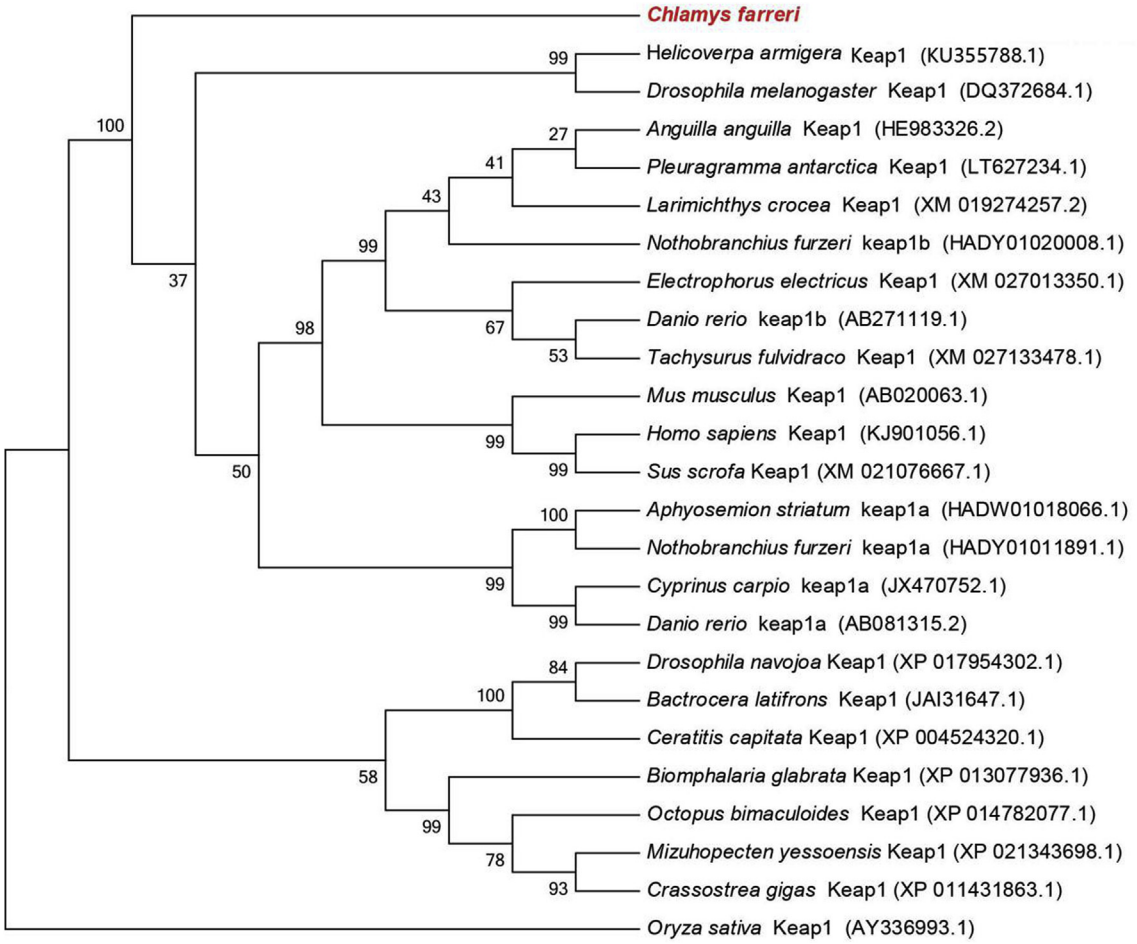

Fig. 2. Phylogenetic tree based on multiple alignment (ClustalX) of various Keap1 protein sequences from various vertebrates and invertebrates. Tree wasgenerated using neighbour-joining method, and the percent concordance based on 10 000 bootstrap iterations is shown at the nodes. GeneBank sequences in the tree include: *Pleuragramma* Keap1 (LT627234.1); *Anguilla anguilla* Keap1 (HE983326.2); *Larimichthys crocea* Keap1 (XM_019274257.2); *Tachysurus fulvidraco* Keap1 (XM_027133478.1); *Electrophorus electricus* Keap1 (XM_027013350.1); Ctenopharyngodon idella Keap1 (KJ729125.1); *Danio rerio* keap1b (AB271119.1); *Nothobranchius furzeri* keap1b (HADY01020008.1); *Danio rerio* keap1a (AB081315.2); *Nothobranchius furzeri* keap1a (HADY01011891.1); *Aphyosemion striatum* keap1a (HADW01018066.1); *Cyprinus carpio* keap1a (JX470752.1); *Mus musculus* Keap1(AB020063.1); *Drosophila melanogaster* Keap1(DQ372684.1); *Oryza sativa* Keap1 (AY336993.1); *Helicoverpa armigera* Keap1 (KU355788.1); *Homo sapiens* Keap1 (KJ901056.1); *Rattus norvegicus* Keap1 (NM_057152.2); *Sus scrofa* Keap1 (XM_021076667.1); *Mizuhopecten yessoensis* Keap1 (XP_021343698.1); *Crassostrea gigas* Keap1 (XP_011431863.1); *Biomphalaria glabrata* Keap1(XP_013077936.1); *Octopus bimaculoides* Keap1 (XP_014782077.1); *Drosophila navojoa* Keap1(XP_017954302.1); *Bactrocera latifrons* Keap1(JAI31647.1); *Ceratitis capitata* Keap1 (XP_004524320.1).

CfKeap1 mRNA in the digestive glands and gills were examined by qRT-PCR for determining the RNAi effect. At 48 h post injection, the expression amount of CfKeap1 mRNA showed significant difference between the GFP-dsRNA group and the PBS group, the relative expression level of CfKeap1 mRNA in the CfKeap1-dsRNA group was significantly decreased by 60.69% in the digestive glands and 59.485% in the gills compared to the negative control PBS group or GFP-dsRNA group (Fig. 6).

The mRNA expression of Nrf2, Cu/Zn-SOD, CAT and GPx in the digestive glands and gills had shown in Fig. 7. As the expression of Keap1 gene was inhibited in CfKeap1-dsRNA group, the expression of Nrf2, Cu/Zn-SOD, CAT and GPx gene were also significantly different from the control group. At day 1 after injection, the mRNA expression of Nrf2 in the digestive glands showed maximum value in CfKeap1-dsRNA group, then it began to fall gradually and showed no significant difference compared to the control group ($p > 0.05$). The change of Cu/Zn-SOD and CAT gene expression in the digestive glands were consistent with that of Nrf2 gene and the mRNA expression of Cu/Zn-SOD and CAT in the gills at 3days after injection was lower than the control group. What' more, the change of GPx in the digestive glands and gills showed no significant difference in the CfKeap1-dsRNA group compared to the control group ($p > 0.05$) at day 1 after injection, and the mRNA expression of GPx was higher than the control group level at day 2 after injection.

The mRNA expression of PKC, JNK and p38 in the digestive glands and gills had shown in Fig. 8. At the earlier stage of injection, the expression of PKC, JNK and p38 in the digestive glands were significant differences in CfKeap1-dsRNA group compared with the control group ($p < 0.05$), then the expression of JNK began to decrease gradually and it showed no significant difference compared to the control group ($p > 0.05$) at day 2 after injection. The expression of PKC in the gills was significant higher in CfKeap1-dsRNA group compared with the control group ($p < 0.05$) at day 1 and 2 after injection, then it began to fall gradually and showed significant lower than the control group ($p > 0.05$). What' more, the change of p38 in the gills showed no significant difference in the CfKeap1-dsRNA group compared to the control group ($p > 0.05$) at day 1 after injection, and showed maximum value in CfKeap1-dsRNA group at day 2 after injection ($p < 0.05$).

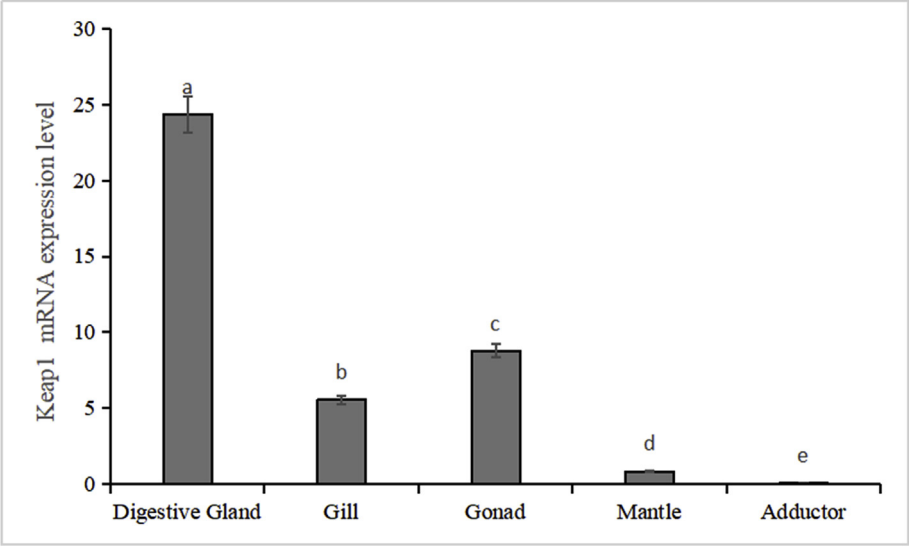

Fig. 3. Tissue distributions of the CfKeap1 transcripts were measured by SYBR Green qRT-PCR. Relative expression showing qRT-PCR products from total RNA isolated from digestive gland, gill, adductor muscle, mantle and gonad of unexposed *C. farreri*. Values are shown as mean ± S.D. (n = 6).

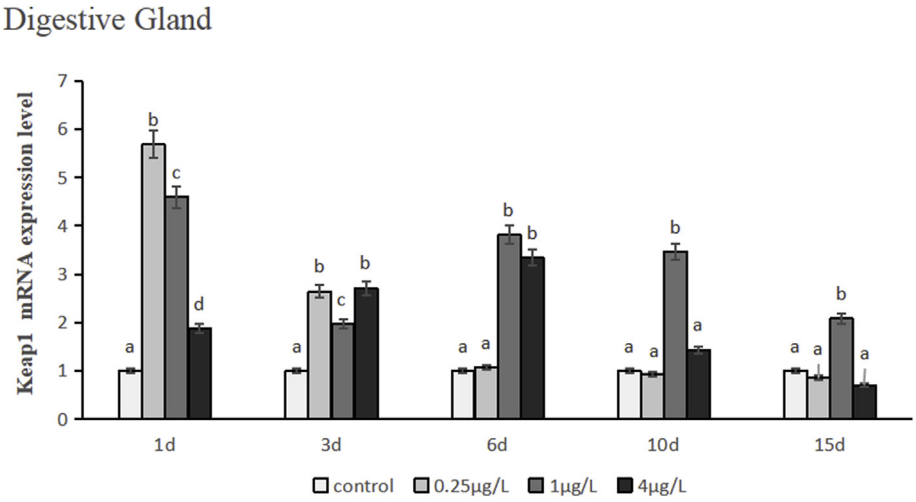

Fig. 4. Temporal expressions of CfKeap1 transcripts of *C. farreri* in the digestive glands exposed to BaP. One-way ANOVA Duncan's test was applied. Values are shown as mean ± S.D. (n = 6). Significant difference compare to the control expression level at the same sampling time was marked with different letter at $p < 0.05$.

4. Discussion

The detoxification response of PAHs in shellfish is a complex process that involves aryl hydrocarbon receptor (AhR) pathway, phase I, phase II xenobiotic-metabolizing enzymes and phase III transporters [39–44], which could produce a large number of active intermediates and reactive oxygen species (ROS) substances to disturbed the normal physiological function of aquatic organisms [41,42]. To prevent damage caused by ROS, organisms have developed an antioxidant defense system including enzymatic and non-enzymatic to cope with oxidative stress [2]. Nrf2-Keap1 signaling pathway plays an important role in the transcriptional activation of an array of antioxidant and detoxification genes.

In the current study, a full-length cDNA sequence of the Keap1 was successfully cloned from the *C. farreri* for the first time. The *C. farreri* Keap1 contains five major domains: a BTB/POZ domain, DGR domain, IVR domain, NTR and CTR, total 591 amino acid residues. Stogios and Prive'predicted that more than 53 members of the BTB-Kelch protein family exist in human [45]. Some of them, such as Mayven, KLHL20, and KLHL5, show relatively high similarity to Keap1. The phylogenetic analysis showed that the Keap1 genes of each species were clustered into three different branchs and CfKeap1 was clustered with *Drosophila melanogaster* and *Helicoverpa armigera* and belong to different categories with other vertebrates. Researchers believe that the main cause of this phenomenon is that the three species belong to the invertebrates and there are certain differences in homology suggesting that the Keap1 gene of the *C. farreri* is highly specific. In this research work, *D. melanogaster* and *H. armigera* have a BTB/POZ domain with the highest amino acid sequence identity (50.9% and 51.5%) to that of CfKeap1. This value is close to that in *zebrafish* Keap1b and *Nothobranchius furzeri* Keap1b. Li (2008) found that keap1b is highly expressed in the gut and liver of zebrafish [46] What's more, the similar results can be confirmed in the experiment of Giuliani (2014), which suggesting that the *Cf*Keap1 is similar to the Keap1b in bone fish [1]. So we believe that CfKeap1 and Keap1b genes have similar biological functions.

As a master regulator of cellular responses, Keap1 is widely found in insects, fish, mammals and abundantly expressed in the liver, kidneys, intestine and other organs continuously exposed to environment [47]. In particular, Nrf2-Keap1 signaling pathway is important in protecting the liver, since the absence of Nrf2-Keap1 signaling pathway increases hepatic lipid peroxidation [48]. Consistent result was observed in this study, the CfKeap1 transcripts were expressed in all examined tissues

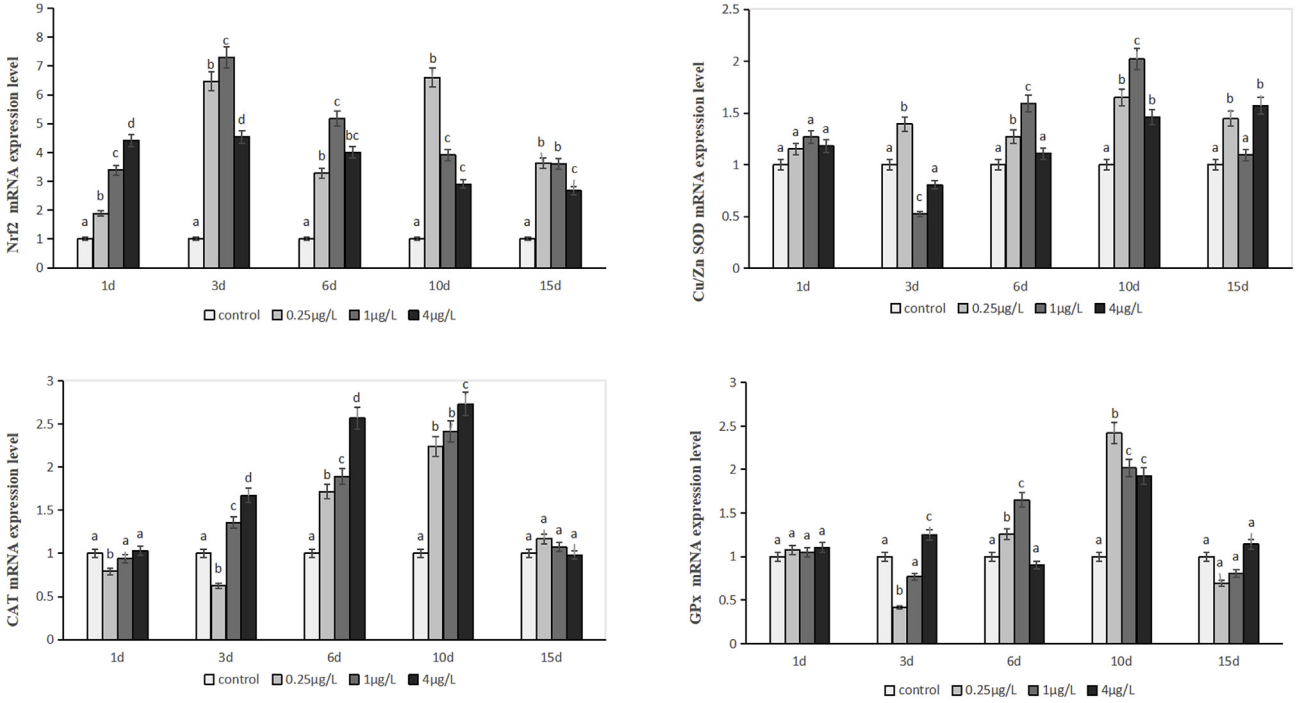

Fig. 5. Temporal expressions of Nrf2, Cu/Zn-SOD, CAT and GPx transcripts of *C. farreri* in the digestive glands exposed to BaP. Values are expressed as means ± SD (n = 6). One-way ANOVA Duncan's test was applied. Significant difference compare to the control expression level at the same sampling time was marked with different letter at $p < 0.05$.

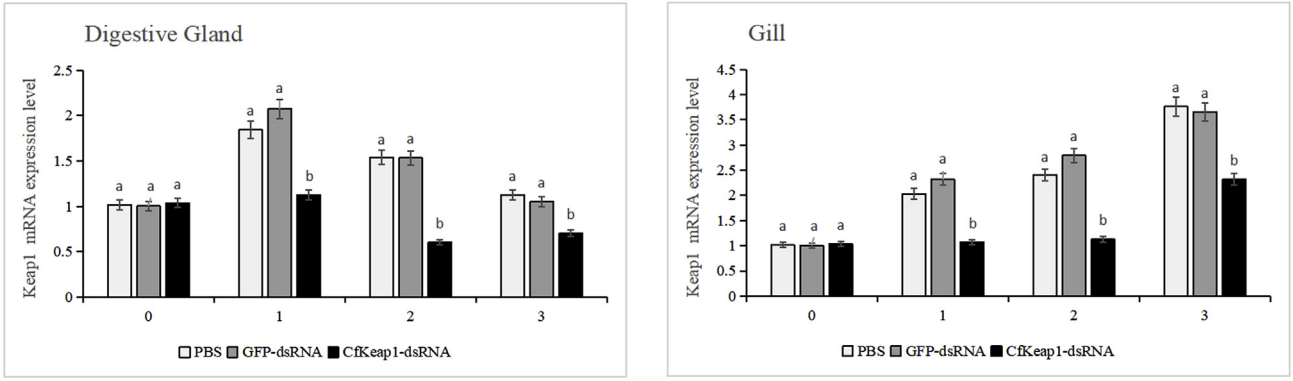

Fig. 6. Temporal expressions of Keap1 transcripts of *C. farreri* in the Scallop digestive glands and gills exposed to BaP at 1–3 days post dsRNA-Keap1 injection. One-way ANOVA Duncan's test was applied. Values are shown as mean ± S.D. (n = 6). Significant difference compare to the control expression level at the same sampling time was marked with different letter at $p < 0.05$.

except adductor muscle, indicating that CfKeap1 was synthesized under non-stimulated conditions. Besides, the digestive glands showed the highest levels consistently consistent with the previous findings in the liver of human, carp and bone fish [49], suggesting that CfKeap1 was important in the process of detoxification and the digestive glands was the major site of xenobiotic metabolism in molluscs [50,51]. In summary, the studies of the CfKeap1 expressions in the scallops tissues will be necessary for full understanding of the tissue-specific regulation and these information may be essential to explore the physiological function of CfKeap1.

The Nrf2-Keap1 signaling pathway target genes encode enzymes including the synthesis and conjugation of glutathione, antioxidant enzymes, drug-metabolizing enzymes, transporters, and pentose phosphate pathway enzymes [1], which could participate in the detoxification response of PAHs [1]. It can be speculated that an increase in Nrf2-Keap1 signaling pathway activity is able to elevate the resistance of PAHs stress. Zheng(2016) demonstrated that the 8 mg/L Zn and 32,64 μ g/L Hg exposure could increase the mRNA levels of Keap1 and Nrf2 gene in large yellow croaker [31,32,52]. In addition, in the research of *R. philippinarum* the expression of Nrf2 significantly increased at day 1 and day 6 after BaP exposure and by the end of BaP exposure (15 days), the mRNA levels of Nrf2 in the BaP exposure groups were still higher than the control group [4]. Consistent result was observed in our research, the expression of Keap1 and Nrf2 significantly increased at day 1 after BaP exposure ($p < 0.05$), what's more, the mRNA levels of Nrf2 and Keap1 in 1 μg/L BaP exposure group were still higher than the control group at the end of exposure. In this research, the activation of Nrf2 and Keap1 expression can be significantly induced by BaP exposure, suggesting the Nrf2-Keap1 signaling pathway may play an important role in regulating antioxidant genes. Similar results have been reported in *Danio rerio* [25,52,53], *Anguilla anguilla* [1], *Cyprinus carpio* [1] and *Antarctic silverfish* [1].

Numerous mammalian studies have shown that the most several important antioxidant enzymes are SOD, which detoxifies superoxide anions, CAT can reduces H_2O_2, GPx which reduces both H_2O_2 and organic peroxides by a glutathione-dependent reaction. It has also been

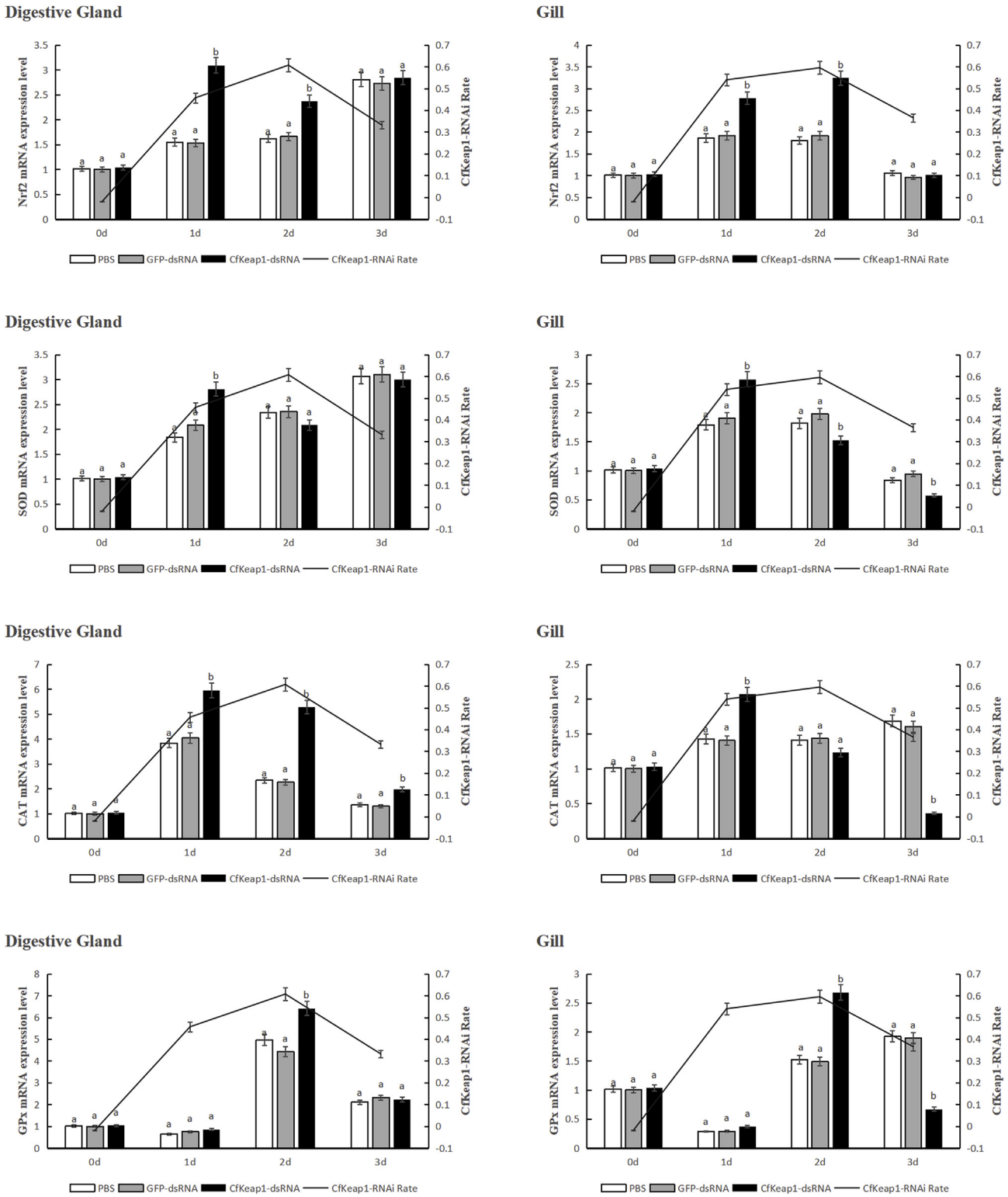

Fig. 7. Temporal expressions of Nrf2, Cu/Zn-SOD, CAT and GPx transcripts of *C. farreri* in the digestive glands and gills exposed to BaP at 1–3 days post dsRNA-Keap1 injection. One-way ANOVA Duncan's test was applied. Values are shown as mean ± S.D. (n = 6). Significant difference compare to the control expression level at the same sampling time was marked with different letter at $p < 0.05$.

widely reported that the intracellular levels of some nonenzymatic antioxidants, such as glutathione, influenced the activity of the enzymatic antioxidants [54]. As the most powerful antioxidant enzyme, SOD has been widely studied and used as a marker of antioxidant defense [55,56]. The results of this study showed that, the Cu/Zn-SOD mRNA expression was significantly increased on the first day, indicating that more antioxidants were produced in the tissue to relieve the oxidative stress of the body. This is in accordance with the study of the effect of BaP on the activity of SOD enzyme in scallop *Chlamys farreri* and *R. philippinarum* [1]. Beyond that, the mRNA expression levels of Cu/Zn-SOD, GPx and CAT showed a similar pattern to *that of* Nrf2. The changes of antioxidant genes expression were consistent with

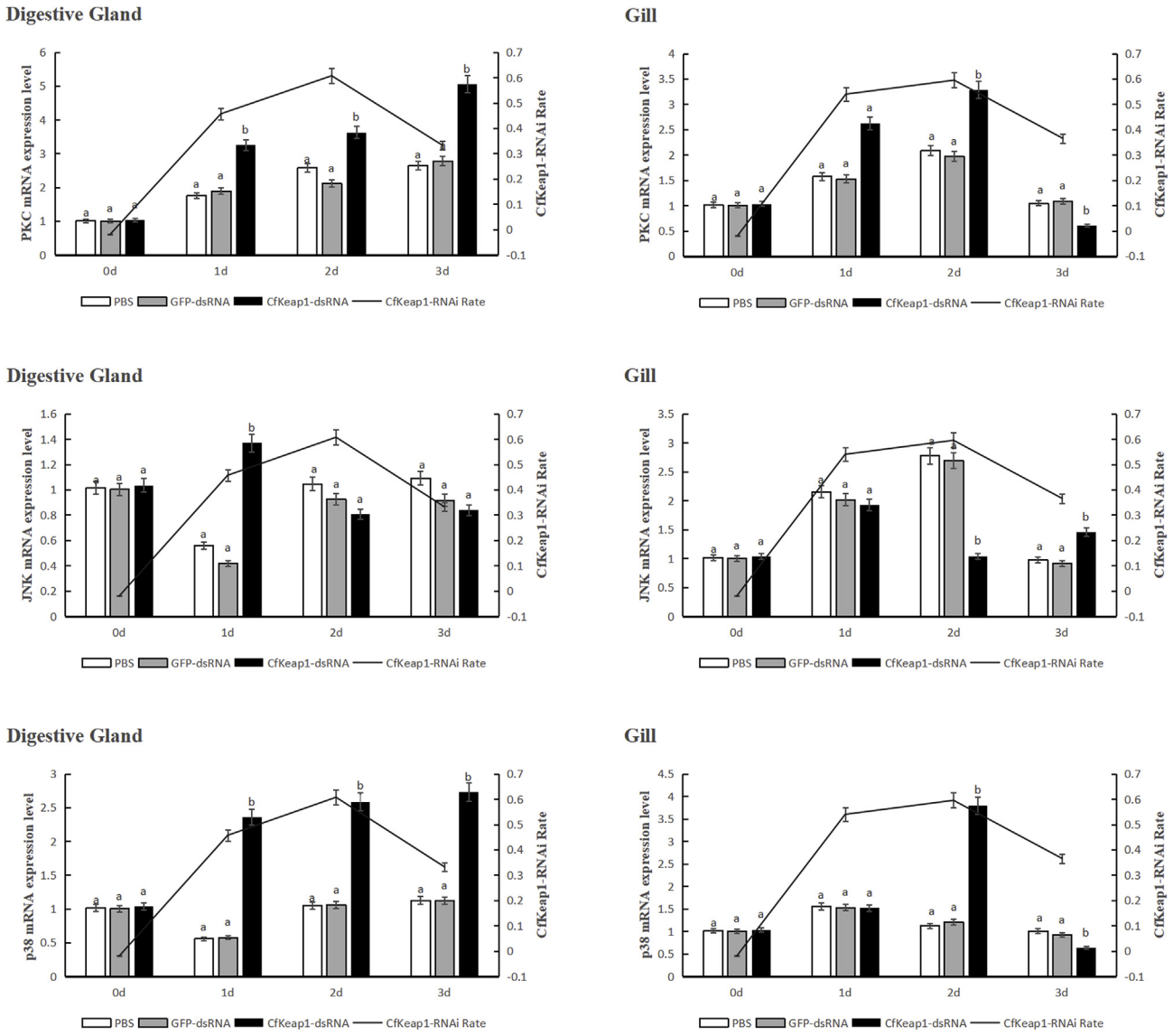

Fig. 8. Temporal expressions of PKC, JNK, p38 transcripts of *C. farreri* in the clam digestive glands and gills exposed to BaP at 1–3 days post dsRNA-Keap1 injection. One-way ANOVA Duncan's test was applied. Values are shown as mean ± S.D. (n = 6). Significant difference compare to the control expression level at the same sampling time was marked with different letter at $p < 0.05$.

the Nrf2 gene, suggesting that Nrf2 is required for the induction of antioxidant genes. Similar results have been reported in *R. philippinarum*'s research [1,4].

In order to contribute to a better understanding of the regulation of Nrf2-Keap1 signaling pathway activation and the detoxification mechanisms of Nrf2-Keap1 signaling pathway in bivalves, we used RNAi technology to verify the role of Keap1 in the antioxidant defense signaling pathway of the molluscs. It is well known that, RNAi technology has become a very powerful tool for reverse genetics to characterize the function of a novel gene in many aquatic organisms [34–38]. In this study, the relative expression level of CfKeap1 mRNA in the CfKeap1-dsRNA group was significantly decreased by 60.69% in the digestive glands and 59.485% in the gills compared to the negative control group or GFP-dsRNA group, and RNAi effect was not detected using a dsRNA of the GFP. These results indicating this interference effect of the target sequence was specificity, which was similar to previous researches [37,38].

Sun (2007) identified Keap1 as a key postinduction repressor of Nrf2 and demonstrated that nuclear export sequence (NES) in Keap1 was required for the termination of Nrf2-antioxidant response element (ARE) signaling by escorting nuclear export of Nrf2 [15]. In order to verify the role of Keap1 in the antioxidant defense signaling pathway in molluscs, we studied the relationships between the Keap1 and Nrf2 mRNA levels. In this research, there was a negative relationship between the mRNA levels of Nrf2 and Keap1 that indicating the nuclear export mechanism and degradation mechanism of Nrf2 were hindered after the expression of Keap1 gene was inhibited, resulting the expression of Nrf2 gene in the CfKeap1-dsRNA groups was significantly higher than the control groups. Besides, the mRNA expression levels of Cu/Zn-SOD, GPx and CAT showed a similar pattern to that of Nrf2. The changes of antioxidant genes expression were consistent with the Nrf2 gene, suggesting that Nrf2 is required for the induction of antioxidant and detoxification genes.

Niture (2009) used RNAi technology to demonstrate that the PKC effected the dissociation of Nrf2 from Keap1 and reduced the basal level of antioxidant defense gene expression [19]. In the research of Chang (2013), RNAi technology was used to investigate the effect of p38 on the nuclear translocation of Nrf2, and the expression of HO-1 in this study was inhibited [22]. In addition, Kim (2011) used RNAi technology and pharmacological inhibitors of PKC, p38 genes to reduce the

nuclear translocation of Nrf2 and the activity of anti-oxidation element (ARE) [23]. Similar results can be obtained in our study, the expression of PKC, JNK and p38 in the CfKeap1-dsRNA groups were significantly differences compared with the control group at the earlier stage of injection, and the expression of PKC in the gills was significant higher in CfKeap1-dsRNA group compared than the control group at day 1 and 2 after injection. The changes of PKC, JNK and p38 genes expression were consistent with the Nrf2 gene suggesting that there was significant effect on PKC and MAPKs signaling pathways after the inhibition of Keap1 gene expression. PKC and MAPKs signaling pathways play a synergistic role in the regulation of Nrf2-Keap1 signaling pathway activation in the anti-oxidative defense signaling system of bivalve molluscs.

In conclusion, we identified and characterized full-length cDNAs of Keap1 genes from the *C. farreri* for the first time. We found that Keap1 and Nrf2 was induced by BaP and the mRNA expression levels of SOD, GPx and CAT showed a similar pattern to Nrf2 under the BaP exposure, suggesting their essential roles in this process. Our study used RNAi technology to explore the regulation of Nrf2-Keap1 signaling pathway activation to verify the role of Keap1 in the antioxidant defense signaling pathway in molluscs for the first time. This study provides not only an expanded perspective on the collective roles of Keap1, but also a foundation for further researches on the mechanism of detoxification and antioxidation mechanisms of BaP in the scallops in a molecular level.

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