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Short communication

Identification and expression profile of a new cytochrome P450 isoform (CYP414A1) in the hepatopancreas of *Venerupis* (*Ruditapes*) *philippinarum* exposed to benzo[*a*]pyrene, cadmium and copper

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ABSTRACT

With the objective to identify promising molecular biomarkers for marine pollution monitoring, a new cytochrome P450 gene was identified from *Venerupis* (*Ruditapes*) *philippinarum* and classified as a member of a new subfamily, CYP414A1. Phylogenetic analysis showed that CYP414A1 was closely related to members of the CYP2 family. CYP414A1 mRNA expression was significantly induced by 50 μg/L B[*a*]P at 96 h, while no significant change was found in 5 μg/L B[*a*]P-exposed samples. For heavy metals exposure, the expression of CYP414A1 was significantly up-regulated by Cd but sharply depressed by Cu exposure. These results suggested that CYP414A1 responded to various xenobiotics stresses, and could be used as a candidate biomarker of heavy metals and B[*a*]P.

1. Introduction

Cytochrome P450s (CYPs) comprise one of the largest and most versatile heme-thiolate protein families, which can catalyze the oxidation of a wide variety of exogenous compounds or xenobiotics including drugs, toxicants and chemical carcinogens (Gonzalez, 2005). The link between these exogenous compounds acting as induction agents and the induced CYPs metabolizing them has been established in both mammalian and insect detoxification systems (Correia, 1995; Zhou et al., 2010). Currently, increasing CYPs sequences

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Table 1 – Primers used in the this study.

Primer	Sequence (5′-3′)	Sequence information
P1 (reverse)	TTGAGTTCAAGTAGCGTTCT	5′ RACE primer for CYP414A1
P2 (reverse)	TCTTCTTGGTCGAAACTTGG	5′ RACE primer for CYP414A1
P3 (forward)	AGGGATTCATTCGCAACAGC	3′ RACE primer for CYP414A1
P4 (forward)	CTGCTAACGAAAGTAGAGGG	3′ RACE primer for CYP414A1
P5 (forward)	AGGACCGAGGTCATGTTTAG	Real time primer for CYP414A1
P6 (reverse)	GGATTTAGAGTTGTCGCCAG	Real time primer for CYP414A1

have been described and can be found in regular updates (http://drnelson.utmem.edu:homepage.html) (Nelson, 2009). Researches upon CYPs have been mainly focused on the biochemical and toxicological aspects in higher vertebrates and insects (Zhou et al., 2010). CYPs and associated activities have also been demonstrated in several marine invertebrates belonging to the phyla Cnidaria, Annelida, Mollusk, Crustacea and Echinodermata (de Toledo-Silva et al., 2008; Rewitz et al., 2006). However, only 12–58 CYPs sequences or expressed sequence tags (ESTs) from bivalve mollusks have been deposited in GenBank so far (Zanette et al., 2010). The molecular features and expression profiles of CYPs in bivalve mollusks are still deficient compared with those of insects and vertebrates. In this study, we cloned the full-length cDNA of CYP414A1 from *Venerupis* (*Ruditapes*) *philippinarum* and investigated the transcriptional response of CYP414A1 to B[*a*]P, Cd, and Cu exposure.

2. Materials and methods

2.1. Samples

All the adult clams *V.* (*R.*) *philippinarum* (shell length: 3.0–4.0 cm, Zebra pedigrees) were purchased from local unpolluted culturing farm (Bohai Sea, Yantai, China) and acclimatized for 10 days before the commencement of the experiment. The sea water was aerated continuously, and salinity and temperature maintained at 32 part per thousand (ppt) and 25 °C throughout the experiment. Clams were fed with *Chlorella vulgaris Beij* daily and the seawater was renewed daily. After the acclimatization period, the clams were randomly divided into eight flat-bottomed rectangular tanks and each tanks contained 30 individuals.

For the exposure experiment, clams were treated with different toxicants at following final concentrations: B[*a*]P (5 and 50 μg/L dissolved in DMSO), Cd (as $CdCl_2$, 10 and 40 μg/L) and Cu (as $CuCl_2$, 10 and 40 μg/L). The concentration of heavy metals and B[*a*]P have been previously reported in some heavily polluted sites of Bohai Sea and the coastal waters of southeast China (Zhang, 2001; Zhang et al., 2004a, 2004b). The clams cultured in the normal filtered sea water (FSW) and FSW containing 0.002% DMSO (v/v) were used as the blank and control groups, respectively. The toxicant-laden seawater was renewed daily, and clams were fed daily during exposure time. The hepatopancreas of four individuals from each treatment were randomly sampled after exposure for 24, 48 and 96 h respectively. Gills, hepatopancreas, adductor muscles, mantles and haemocytes from untreated clams were also collected to determine the tissue distribution of CYP414A1. After collection, the samples were flash-frozen in liquid nitrogen immediately and stored at −80 °C until total RNA was extracted for analysis.

2.2. cDNA cloning and sequence analysis of CYP414A1

Based on the EST sequence of *V.* (*R.*) *philippinarum* cDNA library, four specific primers (P1–P4) (Table 1) were designed to clone the full-length cDNA of CYP414A1 by nested PCR strategy. The RNA extraction, cDNA synthesis and PCR amplification were performed according to previously described (Li et al., 2010). CYP414A1 cDNA sequence was analyzed using the Blast algorithm at NCBI web site (http://www.ncbi.nlm.nih.gov/blast), and the deduced amino acid sequence was analyzed with the Expert Protein Analysis System (http://www.expasy.org/). Multiple alignments of CYP414A1 were performed with the ClustalW Multiple Alignment program (http://www.ebi.ac.uk/clustalw/) and Multiple Alignment Show program (http://www.bioSoft.net/sms/index.html). A neighbor-joining (NJ) phylogenetic tree was constructed by MEGA 4.0 with 1000 bootstrap replicates.

2.3. CYP414A mRNA expression in different tissues and the response to B[a]P, Cd, and Cu exposure

Tissue distribution and the response of hepatopancreas CYP414A1 transcript to B[*a*]P, Cd, and Cu exposure was measured by quantitative real-time RT-PCR in Applied Biosystem 7500 fast Real-time PCR System. Gene-specific primers P5 and P6 (Table 1) were designed to amplify a PCR product of 147 bp, which was sequenced to verify the PCR specificity. A pair of clam β-actin primers was used to amplify a 121 bp fragment as the internal control. The PCR was carried out in a total volume of 50 μL, containing 25 μL of SYBR Green PCR Master Mix (Applied Biosystems), 20 μL of the diluted cDNA, 0.5 μL of each of primers (10 μmol/L), and 4 μL of DEPC-treated water. The thermal profile for RT-PCR was 50 °C for 2 min and 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 31 s. After amplification, data acquisition and analysis were performed using the Sequence Detection Software (SDS vers. 2.3, Applied Biosystems). The $2^{-\Delta\Delta Ct}$ method was chosen as the calculation method. All datasets were analyzed in terms of relative mRNA expression as means ± S.D. One-way analysis of variance (one-way ANOVA) was performed on all data using SPSS 13.0 statistical software, and *P* values less than 0.05 were considered statistically significant.

3. Results and discussion

3.1. *cDNA cloning and sequence analysis of CYP414A1*

The obtained sequence of *V.* (*R.*) *philippinarum* CYP414A1 cDNA (HQ234335) was shown with the deduced amino acid sequence in Fig. 1, which contained an open reading frame of 1485 bp encoding a polypeptide of 495 amino acids with the predicted molecular weight of 56.77 kDa. This sequence was named by the CYP Nomenclature Committee (Professor David Nelson, personal communication) as a member of a new subfamily, CYP414A1. The putative signal peptide was identified by SignalP program analysis with the cleavage site located after position 24 (AYS-NT). Blast and phynogenetic analysis indicated a close relationship between the CYP414A1 and CYP2 family (Fig. 2). Alignments of the CYP414A1 with CYP2 sequence from other species (Fig. S1) demonstrated the highly similarities of the conserved regions among different species, suggesting that these regions in the CYP proteins are involved in functions essential for the enzymes, despite the relatively low overall sequence similarity (Jørgensen et al., 2005). The signature motif FXXGXRXCXG (434-447 aa) in CYP414A1 was essential for heme binding in P450 cytochromes (Yang et al., 2008). In addition, it is noteworthy that there was a motif (138-153 aa) near the N-terminal of CYP414A1, which was distinct from that in CYP2 family.

3.2. *Tissue distribution of CYP414A1 gene*

CYPs are found in virtually all tissues and organs of marine invertebrates, especially hepatic-like organs and steroidogenic tissues (Rewitz et al., 2006). Similar phenomenon was also observed in our study, the CYP414A1 transcript was found to be most abundantly expressed in hepatopancreas (Fig. 3), which is in agreement with previous studies establishing hepatopancreas as the major organ for metabolism of organic compounds and the main site of biotransformation activities (Livingstone, 1998).

3.3. *Modulation of CYP414A1 gene expression in hepatopancreas of clam exposed to B[a]P, Cd and Cu*

Both organic and inorganic contaminants pose great threats to marine ecosystem and even human beings through food chains (Rainbow, 1995). Thus it is necessary to assess the toxicological effects of contaminants with biomarker discovery for the environmental monitoring. Recently, xenobiotic metabolizing enzymes (XMEs) reflecting the effects of contaminations on resident biota have been employed to monitor environmental pollutants (Sarkar et al., 2006). To our knowledge, researches on the expression and regulation of XMEs in mollusks, especially for CYPs, are still deficient compared with those in vertebrates and insects. As a typical and widely distributed mollusks, *V.* (*R.*) *philippinarum* occurs at high densities around the heavily polluted Bohai Sea (Liang et al., 2004), and has been listed as a sentinel organism in "Mussel Watch Program" launched in China. This specie exhibits distinct biochemical and genetic responses

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1    M  D  E  T  D  Y  S  T  I  T  T  I  L  I  F  L  I  T  F  L
1    ATGGATGAAACGGATTACAGCACCATAACAACAATACTGATTTTCTTAATAACATTTTTG
21   L  A  Y  S  N  T  R  R  Q  K  R  V  P  P  G  P  A  L  F  P
61   TTAGCTTATTCTAACACAAGACGACAAAAAAGGGTGCCGCCTGGACCTGCCTTGTTTCCT
41   I  I  G  N  L  P  S  L  A  T  E  D  T  L  G  R  L  K  E  L
121  ATTATCGGAAATCTCCCATCGTTGGCTACGGAAGATACGTTGGGCAGATTAAAGGAACTA
61   R  K  K  Y  G  D  V  F  G  L  Y  T  G  S  K  L  L  V  F  L
181  CGAAAAAAGTATGGTGACGTTTTTGGACTATATACCGGAAGTAAACTTCTCGTGTTTCTA
81   N  G  Y  D  V  I  H  D  A  L  I  K  K  G  S  K  F  M  F  R
241  AATGGTTATGATGTAATTCATGACGCGCTGATAAAGAAAGGATCCAAATTTATGTTCAGA
101  A  L  P  S  F  D  Q  E  E  D  I  H  T  K  G  L  I  F  T  K
301  GCTCTGCCAAGTTTCGACCAAGAAGAAGATATACATACAAAAGGGTTAATATTTACGAAA
121  G  T  K  W  K  E  G  R  S  F  T  L  A  V  L  Q  E  I  C  Y
361  GGGACAAAGTGGAAGGAAGGTAGAAGTTTTACATTAGCAGTATTACAAGAAATCTGTTAT
141  N  D  K  G  F  V  E  H  L  V  D  Y  E  V  N  D  L  T  E  T
421  AATGATAAAGGTTTTGTAGAACATTTAGTAGATTACGAGGTGAATGACTTGACAGAGACA
161  I  L  K  F  D  E  P  F  D  I  E  R  Y  L  N  S  S  V  Q  N
481  ATTCTTAAATTTGATGAGCCATTTGATATAGAACGCTACTTGAACTCAAGCGTTCAAAAT
181  V  I  F  Q  V  V  Y  G  H  R  F  D  L  N  D  E  G  L  H  W
541  GTGATATTTCAAGTCGTGTACGGACATCGATTTGATCTAAATGATGAAGGCTTGCATTGG
201  F  Q  K  F  V  R  T  S  S  E  E  F  L  K  R  E  V  I  L  N
601  TTTCAGAAATTTGTCCGGACCAGTTCAGAAGAATTTTTGAAAAGGGAAGTAATTCTAAAT
221  C  L  P  F  L  Q  N  L  P  G  D  L  L  R  I  Q  K  T  R  D
661  TGTCTCCCATTCTTACAAAATCTACCGGGAGATCTTTTAAGAATTCAAAAAACTAGGGAT
241  S  F  A  T  A  K  E  F  L  T  N  F  I  E  D  L  K  K  Q  S
721  TCATTCGCAACAGCTAAGGAATTTTTGACAAATTTTATCGAAGATTTGAAGAAACAGAGT
261  R  N  T  Q  R  T  T  Y  V  E  C  Y  L  D  S  I  A  A  N  E
781  AGAAACACGCAAAGAACAACTTATGTTGAATGCTACTTAGATAGCATTGCTGCTAACGAA
281  S  R  G  I  E  S  T  F  D  E  E  D  L  K  I  A  A  Y  H  L
841  AGTAGAGGGATAGAAAGTACATTCGATGAAGAAGATTTAAAGATAGCGGCATATCATCTC
301  G  V  A  G  F  E  T  T  A  V  T  I  R  W  I  L  L  H  L  I
901  GGTGTGGCAGGGTTTGAAACCACCGCTGTCACAATTAGATGGATACTCCTACACCTAATA
321  R  N  P  H  I  Q  D  K  L  N  A  E  V  E  T  V  L  G  K  E
961  AGGAATCCACACATACAGGATAAATTGAACGCGGAGGTAGAAACTGTGCTTGGAAAGGAG
341  P  P  S  I  E  D  R  K  R  M  P  Y  M  Q  A  V  M  L  E  G
1021 CCTCCGTCAATTGAAGATAGGAAACGAATGCCGTACATGCAAGCGGTAATGTTGGAAGGA
361  L  R  I  S  H  V  V  P  L  S  M  P  H  T  V  E  Q  D  T  L
1081 CTCCGAATTTCTCACGTGGTACCGCTATCAATGCCGCACACTGTTGAACAAGATACCTTA
381  F  R  G  Y  L  I  P  E  N  C  T  V  L  P  V  L  S  T  A  L
1141 TTCCGGGGATACCTTATTCCAGAGAATTGCACAGTTTTGCCAGTTCTCAGCACCGCTTTA
401  K  D  P  D  V  W  K  D  P  E  E  F  I  P  E  R  F  L  N  A
1201 AAGGATCCGGATGTTTGGAAGGACCCTGAAGAATTTATACCGGAAAGATTTCTGAACGCG
421  E  G  N  D  V  I  V  P  K  E  F  I  P  F  S  L  G  P  R  S
1261 GAAGGAAATGACGTCATAGTTCCAAAAGAGTTCATCCCATTTTCATTAGGACCGAGGTCA
441  C  L  G  E  T  L  A  T  I  E  I  F  L  F  L  T  G  L  V  Q
1321 TGTTTAGGGGAGACGTTAGCAACAATTGAAATATTTCTGTTTTTGACAGGACTTGTTCAA
461  K  L  R  F  L  P  E  K  E  G  V  V  P  D  Q  K  G  K  L  A
1381 AAGTTGAGATTTTTACCGGAAAAGGAAGGCGTTGTTCCGGATCAAAAAGGAAAACTGGCG
481  T  T  L  N  P  S  S  F  K  M  K  V  E  K  R  *
1441 ACAACTCTAAATCCGAGTAGTTTCAAAATGAAAGTTGAGAAACGCTAA
```

Fig. 1 – The complete nucleotide and deduced amino acid sequence of CYP414A1. The predicted signal peptide is underlined. The highly conserved regions were highlighted in gray and the distinct motif was marked in red. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

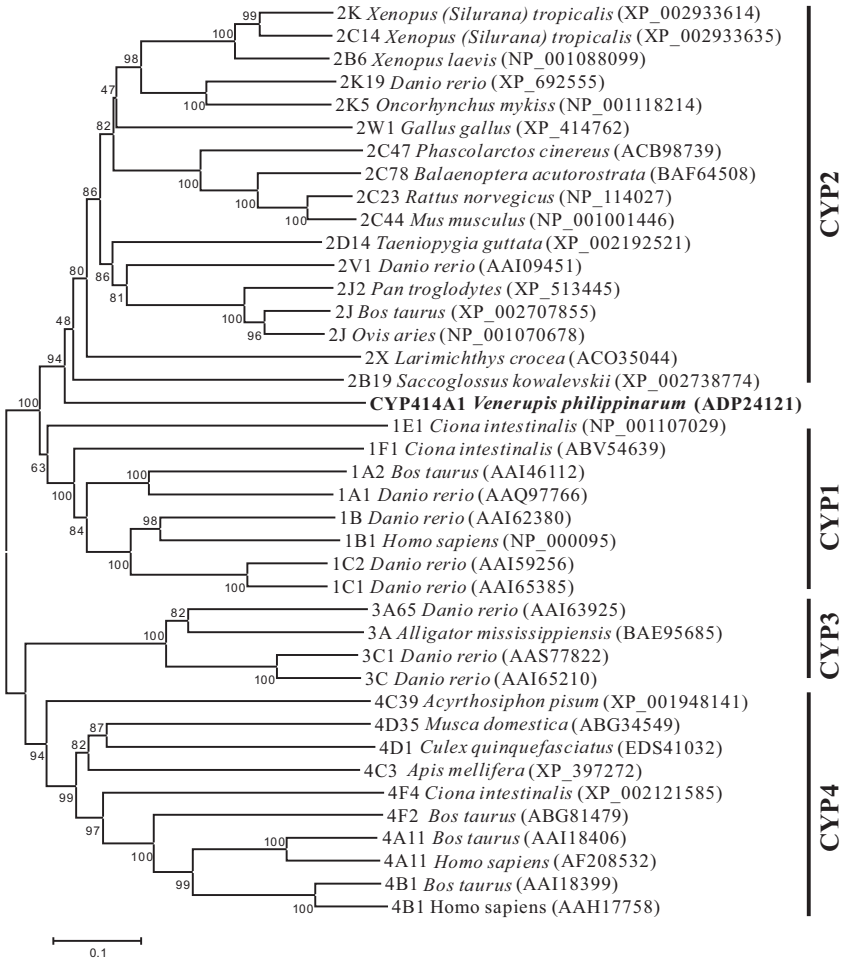

Fig. 2 – Phylogenetic tree constructed by the neighbor-joining method in MEGA software based on the P450 sequences. CYP414A1 obtained in this study was shown in bold. Numbers next to the branches indicated bootstrap value of each internal branch in the phylogenetic tree nodes from 1000 replicates.

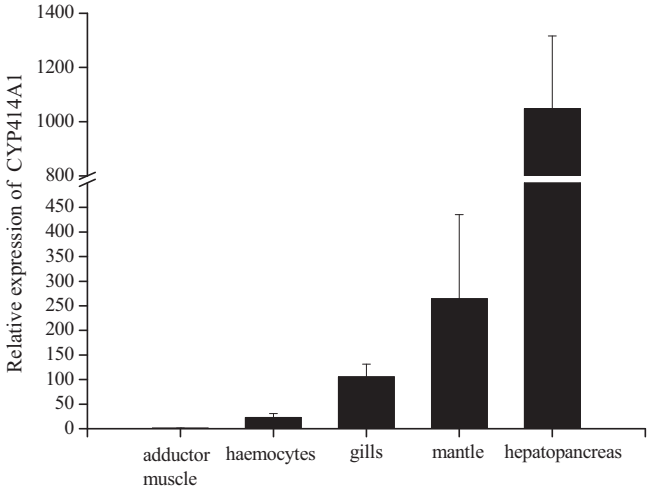

Fig. 3 – Tissue distribution of CYP414A1 mRNA in adult clams detected by Real-time PCR. CYP414A1 transcript level in haemocytes, gills, mantle, and hepatopancreas is normalized to that of adductor muscle in blank clams (untreated). Each bar represented mean ± S.D ($n = 4$).

when exposed to heavy metals and PAHs (Ji et al., 2006; Moraga et al., 2002), possessing the requisite features of a promising indicator species. As a matter of fact, *V.* (*R.*) *philippinarum* has been substantiated a good bioindicator for marine environmental pollution, especially heavy metal pollution, as well as aquatic toxicology (Ji et al., 2006; Park et al., 2008).

A number of studies have correlated CYPs induction with B[*a*]P challenge (Rewitz et al., 2006). In this work, the exposed group with 5 μg/L B[*a*]P showed no significant changes in CYP414A1 expression during the whole experimental period, while 50 μg/L B[*a*]P-treated sample showed significant increase (5.6-fold; $P < 0.05$) in gene expression of CYP414A1 after exposure for 96 h. The similar expression inductions of CYP1A and CYP1C by 5 mg/L B[*a*]P were also observed in *Fundulus heteroclitus* (Wang et al., 2010). Additionally, mRNA expression of CYP1A in *Boreogadus saida* and *Danio rerio* also showed significant inductions following B[*a*]P exposure (Nahrgang et al., 2009; Thompson et al., 2010). This well-characterized induction is perhaps mediated by promoting the binding of the liganded aryl hydrocarbon receptor (AHR) and AHR nuclear translocator (ARNT) to aromatic hydrocarbon-responsive element (AHREs) located in the

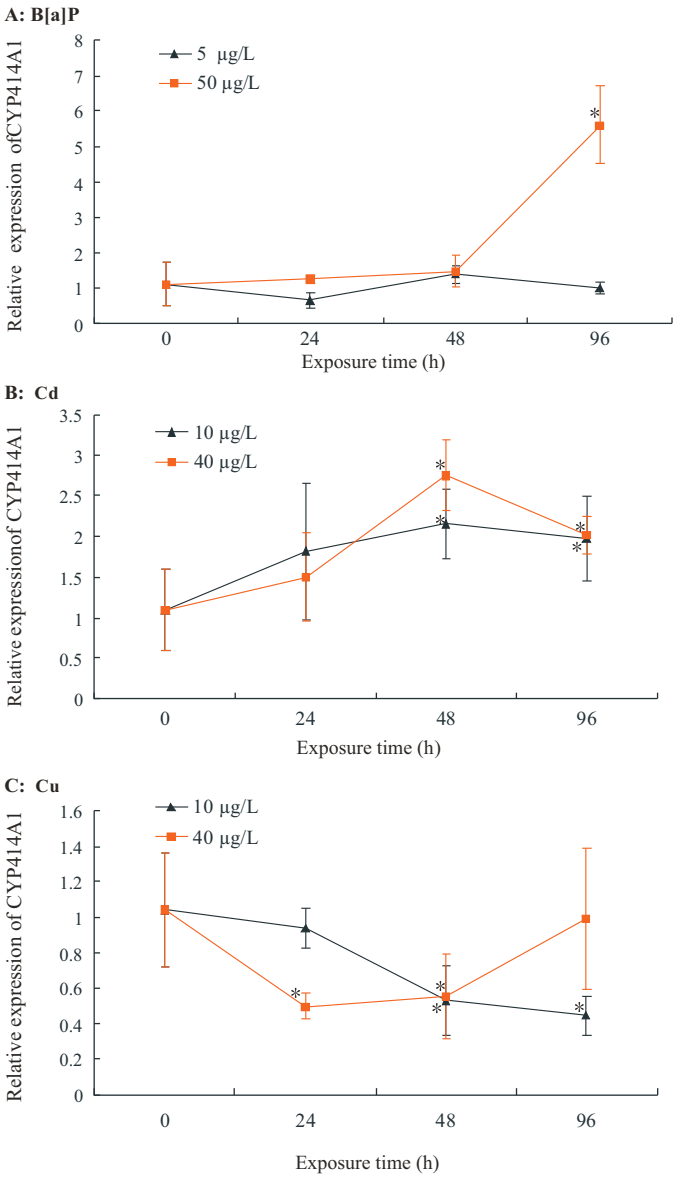

Fig. 4 – Temporal expression of CYP414A1 transcript in hepatopancreas of clams after exposed to different concentrations of B[*a*]P (A), Cd (B) and Cu (C). Each bar represented mean ± S.D. (*n* = 4). Significance across control was indicated with an asterisk at *P* < 0.05.

5′-flanking regions of the CYP genes (Neuhold et al., 1989; Whitlock, 1999).

Cd has been demonstrated to stimulate or inhibit the liver and kidney cytochrome P450 isoforms in rat (Plewka et al., 1999, 2004). Cu and other metals have also been reported to increase CYP1A mRNA in cultured cells (Korashy and El-Kadi, 2004). In the present study, the expression level of CYP414A1 was significantly up-regulated by 10 and 40 μg/L Cd at 48 and 96 h ($P < 0.05$), with a maximal response (2.8-fold) appeared at 48 h by 40 μg/L Cd (Fig. 4B). With respect to Cu exposure (Fig. 4C), the expression level of CYP414A1 decreased significantly ($P < 0.05$) after exposure for both 48 (0.53-fold) and 96 h (0.44-fold) by 10 μg/L Cu. When the concentration increased to 40 μg/L, the expression level of CYP414A1 dropped obviously to 0.5-fold ($P < 0.05$) of control group from 24 to 48 h. It was hypothesized that the regulation in mRNA expression was perhaps due to alterations in mRNA stability and/or processing or a decrease in the transcription rate of the CYP414A1 gene. Previous study described that multiple transcription start and polyadenylation sites may contribute to the regulation of grape cytochrome P450 gene expression in response to *Xylella fastidiosa* infection (Cheng et al., 2010). In addition, cytochrome P450 isoforms in humans are responsible for the oxidative metabolism of the majority of drugs and xenobiotics (Bozcaarmutlu and Arinç, 2007). Henczová et al. (2008) had reported that heavy metals can interact with hepatic microsomal cytochrome P450, and cause complex changes in the cytochrome P450-dependent metabolism in fish. Hereby, the regulation in the CYP414A1 expression could be induced by the production of reactive oxygen species generated by Cd and Cu

exposure (Company et al., 2004). Further studies of CYP414A1 transcriptional initiation sites and its antioxidant defense may be a key step towards understanding its transcriptional regulatory mechanism.

In conclusion, this is an initial report of cDNA cloning, tissue distribution and expression regulation of a new CYP414A1 gene in V. (R.) *philippinarum* exposed to typical marine environmental pollutants. The present study provides clues for further elucidating the function and regulation mechanism of CYP414A1 in V. (R.) *philippinarum*.

Conflicts of interest

Nothing declared.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.etap.2011.08.015.

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