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Identification, annotation and expression analysis of 29 Rho GTPase genes from channel catfish (*Ictalurus punctatus*) after bacterial infections

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ABSTRACT

The Rho family GTPases are a group of small monomeric G proteins, which are molecular switches in signaling pathways. They have been known to regulate a diverse range of cellular processes including actin cytoskeleton rearrangement and microtubule dynamics. In particular, their participations in immune responses are also significant. However, little information of the Rho GTPases is available in teleost including channel catfish, an economically important species and one of the best teleost models for immunological research. In this study, Rho GTPase genes were identified from channel catfish and well annotated by phylogenetic and syntenic analyses. Their expression profiles were determined in channel catfish healthy tissues and infected tissues. Altogether seven Rho GTPase genes were significantly regulated after bacterial infection, with six genes in the gill after *Flavobacterium columnare* challenge and two genes in the intestine in response to *Edwardsiella ictaluri*. All the differentially expressed genes were up-regulated soon after bacterial infection. Different expression patterns between the two experiments were observed, which may be attributed to tissue-specific regulation or pathogen-specific regulation. These results suggested that Rho GTPases play important roles in immune responses to bacterial pathogens, setting a foundation for future investigation on Rho GTPases.

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1. Introduction

Rho GTPases, members of the Ras superfamily of small GTPases, are relatively small and evolutionarily conserved proteins. They are distinguished from other small GTPases with the presence of an insert region in the GTPase domain (Wennerberg and Der, 2004). Rho GTPases present in all studied eukaryotic organisms (Boureux et al., 2007), however, their number of genes varies among organisms. The yeast *Saccharomyces cerevisiae* has 6 Rho GTPase genes (Eitzen and Logan, 2012), while mammals contain 20 Rho GTPases (Heasman and Ridley, 2008). Mammalian Rho family can be divided into 8 subfamilies: Rho (RhoA, RhoB, and RhoC), Rac (Rac1, Rac2, Rac3, and RhoG), Cdc42 (Cdc42, RhoJ/TCL, and RhoQ/TC10), Rnd (Rnd1, Rnd2/RhoN, and Rnd3/RhoE), RhoUV (RhoU/Wrch and RhoV/Chp), RhoDF (RhoD and RhoF/Rif), RhoH, and RhoBTB (RhoBTB1 and RhoBTB2) (Burridge and Wennerberg, 2004; Heasman and Ridley, 2008). As important components in signal transduction pathways, Rho GTPases act as molecular switches cycling between a GTP-bound state (active) and a GDP-bound state (inactive). The cycling activity is tightly controlled by three factors: (a) guanine nucleotide exchange factors (GEFs) which activate the switch by promoting the exchange of GDP for GTP (Schmidt and Hall, 2002); (b) GTPase-activating proteins (GAPs) that inactivate the switch by stimulating the hydrolysis of GTP (Bos et al., 2007); (c) guanine nucleotide dissociation inhibitors (GDIs) which dissociate the inactive switch from membrane to prevent spontaneous activation (Jaffe and Hall, 2005; Sadok and Marshall, 2014). In the active GTP-bound form, Rho proteins interact with downstream targets (or effectors) to trigger diverse cellular processes: the regulation of the actin cytoskeleton (Supplementary Table S1) and microtubule dynamics, and thereby the regulation of a vast array of processes including morphogenesis, cell polarity, migration, cell

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division and adhesion, and vesicular trafficking, as well as gene transcription regulation (Aspenström et al., 2007; Aspenstrom et al., 2004; Bokoch, 2005; Burridge and Wennerberg, 2004; Etienne-Manneville and Hall, 2002; Heasman and Ridley, 2008). Furthermore, Rho small GTPases have been reported to participate in cancer metastasis (Li et al., 2014), embryonic development (Settleman, 1999), and cell wound repair (Verboon and Parkhurst, 2015). Recent studies have shown that Rho GTPases play vital roles in innate immunity, mostly depending on phagocytic leukocytes, but their roles in immunity are not definitely clear. On the one hand, leukocytes respond to invading pathogens through directed migration and phagocytosis regulated by Rho GTPases (Bokoch, 2005; Chimini and Chavrier, 2000). On the other hand, Rho GTPases are involved in NADPH oxidase complex assembly and implicated in regulating the oxidase activity of nicotinamide adenine dinucleotide phosphate (NADPH) to produce reactive oxygen species to promote bacteria killing (Bokoch, 2005; Bokoch and Diebold, 2002).

Channel catfish (*Ictalurus punctatus*) is the predominant aquaculture species in the United States. However, due to serious diseases, the catfish industry has been declining in recent years. Among these disease challenges, two bacterial diseases had the most severe impact on the catfish industry and caused the largest economic losses (Wagner et al., 2002). Columnaris disease, caused by the Gram-negative bacterium *Flavobacterium columnare*, is a devastating disease which affects most fresh water fish species worldwide, particularly in farm-raised aquaculture species such as channel catfish (Plumb and Hanson, 2011). Enteric septicemia of catfish (ESC), caused by the Gram-negative bacterium *Edwardsiella ictaluri*, is the most prevalent disease widely distributed throughout the catfish industry worldwide (Hawke et al., 1981).

In order to prevent and control these diseases, enormous efforts have been made to investigate the molecular mechanisms underlying host immune responses to bacteria. However, the roles of signal transduction factors during disease responses have not been studied thoroughly. As pivotal components in signaling pathways during host defense, Rho GTPases remain uncharacterized in channel catfish. In this study, we aim to identify and annotate the Rho family genes in channel catfish, and examine their expression profiles after bacterial infections to provide insight into their roles in host defense responses.

2. Materials and methods

2.1. Sequence identification and analysis

The Rho GTPase genes were identified from the transcriptome databases (Li et al., 2012; Liu et al., 2012) and the whole genome database of channel catfish (Liu et al., 2016). First, the Rho protein sequences of various vertebrates, including human (*Homo sapiens*), mouse (*Mus musculus*), chicken (*Gallus gallus*), frog (*Xenopus tropicalis*), zebrafish (*Danio rerio*), medaka (*Oryzias latipes*), tilapia (*Oreochromis niloticus*), fugu (*Takifugu rubripes*), and stickleback (*Gasterosteus aculeatus*), were retrieved from Ensembl (http://useast.ensembl.org) and NCBI (http://www.ncbi.nlm.nih.gov). Then, they were used as query sequences to search against the transcriptome databases by utilizing TBLASTN program with the cutoff E-value of e^{-5}. To verify the cDNA sequence, BLASTN program was performed by aligning with the whole genome sequence with the cutoff E-value of e^{-10}. Furthermore, ORF finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) and FGENESH (Solovyev et al., 2006) were used for gene prediction. The predicted amino acid sequences were further confirmed by searching against NCBI non-redundant (NR) protein sequence database using BLASTP program. Simple molecular Architecture Research Tool (SMART, http://smart.embl.de) was used to identify the protein domains and further supported by conserved domain search via BLASTP.

2.2. Phylogenetic and syntenic analysis

To further identify Rho GTPases in channel catfish, phylogenetic analyses were conducted using amino acid sequences from various organisms, including human, mouse, chicken, frog, and several teleost fish species. Multiple sequences were aligned by Clustal Omega (Sievers et al., 2011), MUSCLE (Edgar, 2004), and MAFFT v7 (Katoh and Standley, 2013) with the L-INS-i, E-INS-i and G-INS-i strategies. The best-scoring alignment was determined by MUMSA (Lassmann and Sonnhammer, 2006). The program ProtTest (Darriba et al., 2011) was used to determine the appropriate evolutionary model based on Bayesian Information Criterion score. Through the maximum likelihood method, phylogenetic trees were constructed by MEGA6 with bootstrap test of 1000 replicates (Tamura et al., 2013). When molecular evolutionary relationship could not support the precise gene annotation, syntenic analysis was performed to provide additional evidence for orthologous relationship. First, certain genome scaffolds and chromosomes were retrieved by searching against channel catfish genome database using deduced Rho amino acid sequences of channel catfish. Second, Rho GTPase and neighbor genes were identified from the retrieved genome scaffolds by FGENESH and BLASTP. Then, Genomicus (Louis et al., 2012) and Ensembl genome database were utilized to obtain the syntenic regions covering Rho GTPase of human and zebrafish for the comparison with those of channel catfish.

2.3. Protein structure analysis

I-TASSER Suite 5.0 (Yang et al., 2015) was utilized to conduct protein three-dimensional (3D) structure prediction with the amino acid sequences of channel catfish Rho GTPases, and graphical representations were prepared using PyMol (http://www.pymol.org). Certain representative Rho protein data of human were obtained from Protein Data Bank (http://www.rcsb.org) and visualized by PyMol in order to compare with those of channel catfish.

2.4. Expression of Rho GTPase genes in healthy tissues

Meta-analysis was conducted to examine the expression patterns among channel catfish healthy tissues (gill, intestine, liver, skin, barbel, testis, and ovary) using Illumina RNA-Seq datasets from previous studies (Li et al., 2012; Liu et al., 2016; Sun et al., 2012; Wang et al., 2013; Zeng et al., 2016). All of these datasets were downloaded from NCBI Sequence Read Archive (SRA). Sequencing reads were first quality-evaluated by the popular FastQC tool (Andrews, 2010). They were then subjected to quality control with FASTX toolkit (Gordon and Hannon, 2010) to trim biases in the 5′ sequences (Hansen et al., 2010), with Trimmomatic (Bolger et al., 2014) to trim adaptor sequences and low quality reads (quality score less than 20 and read length short than 35). Moreover, in order to normalize the sequencing reads abundance and gene expression among various tissues, we applied the normalization strategy of Sailfish (Patro et al., 2014) with RPKM (reads per kilobase per million mapped reads) given the normalization comparison results reported by Li et al. (2015). In brief, Sailfish implements an efficient expectation-maximization algorithm for mRNA abundance estimation normalization and corrects numerous types of systematic bias in RNA-Seq experiments. RPKM was further used to correct for difference in both library size and gene length (Mortazavi et al., 2008).

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2.5. Expression of Rho GTPase genes after bacterial infections

Expression analyses after bacterial infections were conducted using available RNA-Seq datasets from previous studies of channel catfish in response to *F. columnare* infection (Sun et al., 2012) and *E. ictaluri* infection (Li et al., 2012). Briefly, the trimmed high quality reads in RNA-Seq datasets were first mapped onto the channel catfish reference transcript sequences including Rho gene transcripts using CLC genomics workbench software package. Restrictive mapping parameters were set: at least 95% of bases in one read were mapped to the reference, and a maximum of two mismatches was allowed. The total number of mapped reads for each transcript was determined, and then it was normalized to obtain reads per kilobase of exon model per million mapped reads. The differentially expressed genes were determined by absolute fold change value $\geq$ 1.5, the proportion-based Kal's test with P-value < 0.05, and total mapped reads ≥ 5.

3. Results

3.1. Identification of Rho GTPase genes in channel catfish

A total of 29 Rho GTPase genes were identified from channel catfish transcriptome databases and confirmed with the channel catfish genome sequence. The characteristics of these genes, including cDNA length, 5′ and 3′ untranslated regions, amino acid length, predicted RHO domain positions, chromosomes where they are, and GenBank accession numbers, are summarized in Supplementary Table S2. RhoD was not identified in channel catfish. Multiple amino acid sequence alignment of human and catfish Rho GTPases demonstrated conserved regions and common characteristics, including the Rho insert region (Supplementary Fig. S1).

3.2. Phylogenetic and syntenic analysis

Phylogenetic analysis was first conducted to annotate the Rho GTPase genes in channel catfish. The overall phylogenetic tree demonstrated Rho GTPases of channel catfish were subdivided into 8 subfamilies (Supplementary Fig. S2), in consistence with previous studies of the Rho GTPase family (Aspenström et al., 2007; Burridge and Wennerberg, 2004; Heasman and Ridley, 2008; Ridley, 2006). To better annotate catfish Rho GTPases, the detailed phylogenetic analysis for each subfamily was performed subsequently (Supplementary Figs. S3 and S4). In the detailed phylogenetic analysis, eight catfish genes (Cdc42l, Cdc42l2, Rac1a, Rac1b, RhoB, RhoCa, RhoCb, and RhoF) failed to fall into proper clade as expected to be in the same clade with the genes from zebrafish, which is most closely related to catfish among the studied organisms. For instance, the catfish RhoF GTPase gene was placed at the edge of fish subclade, rather than clustered with its counterpart in zebrafish (Supplementary Fig. S4b). Therefore, syntenic analyses were further conducted for these insufficiently annotated catfish genes, with conserved syntenic blocks identified for all of them (Fig. 1). For example, the neighboring region of RhoF GTPase gene, setd1b-rhof-tmem120b, was well conserved among genomes of human, zebrafish and channel catfish. In summary, combing phylogenetic and syntenic analyses allowed concrete annotation for catfish Rho GTPase genes.

3.3. Copy numbers of Rho GTPase genes in channel catfish

Gene copy numbers of the Rho family genes of representative vertebrates were investigated and summarized in Supplementary Table S3. The copy numbers of Rho GTPase genes are generally conserved among mammals, birds, reptiles, and amphibians, while different gene copies are found among various fish species. In channel catfish, eight genes (RhoA, RhoC, RhoG, RhoU, Rac1, Rnd1, Rnd3, and RhoBTB2) have two copies, and one gene (Cdc42) has three copies. The duplication of Rho GTPase genes among fish species may be derived from the teleost-specific whole genome duplication (Meyer and Van de Peer, 2005). The teleost-specific genome duplication, however, does not account for all of these duplicated genes. For instance, the two copies of the gene for RhoA or Rnd1 are on the same chromosome and relatively far from each other (data not shown), suggesting that they are intrachromosomal duplication (Liu et al., 2016). For those Rho GTPase genes with only a single copy, the duplicated copy after the whole genome duplication was probably lost (Brunet et al., 2006). It was noted that RhoD was not present in channel catfish and the representative ray-finned fishes. Further investigation showed that RhoD was in tetrapods (mammals, birds, and reptiles) and one lobe-finned fish (*Latimeria chalumnae*) which was a close relative of tetrapods (Amemiya et al., 2013). This likely reflected that the RhoD gene was gained in the sarcopterygian (lobe-finned fishes and tetrapods) genome during evolution.

3.4. Tertiary structure of channel catfish Rho GTPases

Protein structure comparisons between channel catfish and human revealed highly conserved tertiary structure of Rho GTPases. The 3D structures of channel catfish Rho GTPases are very similar with those of human, especially for the insert region and essential GTP hydrolysis sites (Supplementary Fig. S5). Tertiary structures of other channel catfish Rho GTPases are provided in Supplementary Fig. S6.

3.5. Tissue expression of Rho GTPase genes in healthy channel catfish

The expression patterns of all the 29 Rho GTPase genes were determined using existing RNA-Seq datasets from healthy channel catfish tissues. As shown in Fig. 2A, most Rho GTPase genes were ubiquitously expressed with tissue- and gene-specific patterns. In mouse tissues, RhoA, Rac1, and Cdc42 appeared to be the most ubiquitously expressed and relatively highly expressed Rho family members (Boureux et al., 2007). In this study, RhoA (RhoAa and RhoAb), Rac1 (Rac1a and Rac1b), and Cdc42 (Cdc42, Cdc42l1, and Cdc42l2) were also ubiquitously expressed with a relatively high expression level, suggesting their basic and important roles in normal cells. Some genes, such as RhoGd, RhoJ, RhoV, Rnd1, and Rnd1l, displayed a narrow expression in studied tissues, suggesting these members evolved toward specific functions (Boureux et al., 2007).

3.6. Expression analysis of Rho GTPase genes after bacterial infections

The roles of Rho GTPases in disease responses are not well understood in fish, and less so with channel catfish. In this study, the expression profiles of Rho small GTPases were determined from gill tissues after *F. columnare* infection, and from intestine tissues after *E. ictaluri* infection. In *F. columnare* challenge, expression analysis indicated that six genes, including Rac1a, Rac2, RhoGb, Cdc42l, RhoB, RhoC, were significantly up-regulated at the early stage (4 h or 24 h) with a modest level of approximately 1.5–2.0 folds (Fig. 2B). After *E. ictaluri* infection, RhoC and RhoUb were significantly up-regulated with approximately 1.5–3.0 fold changes at 24 h and 3 h, respectively (Fig. 2C). The differential gene expression patterns suggested their involvements in acute immune responses against bacterial infections. Three Rho GTPases in the Rac

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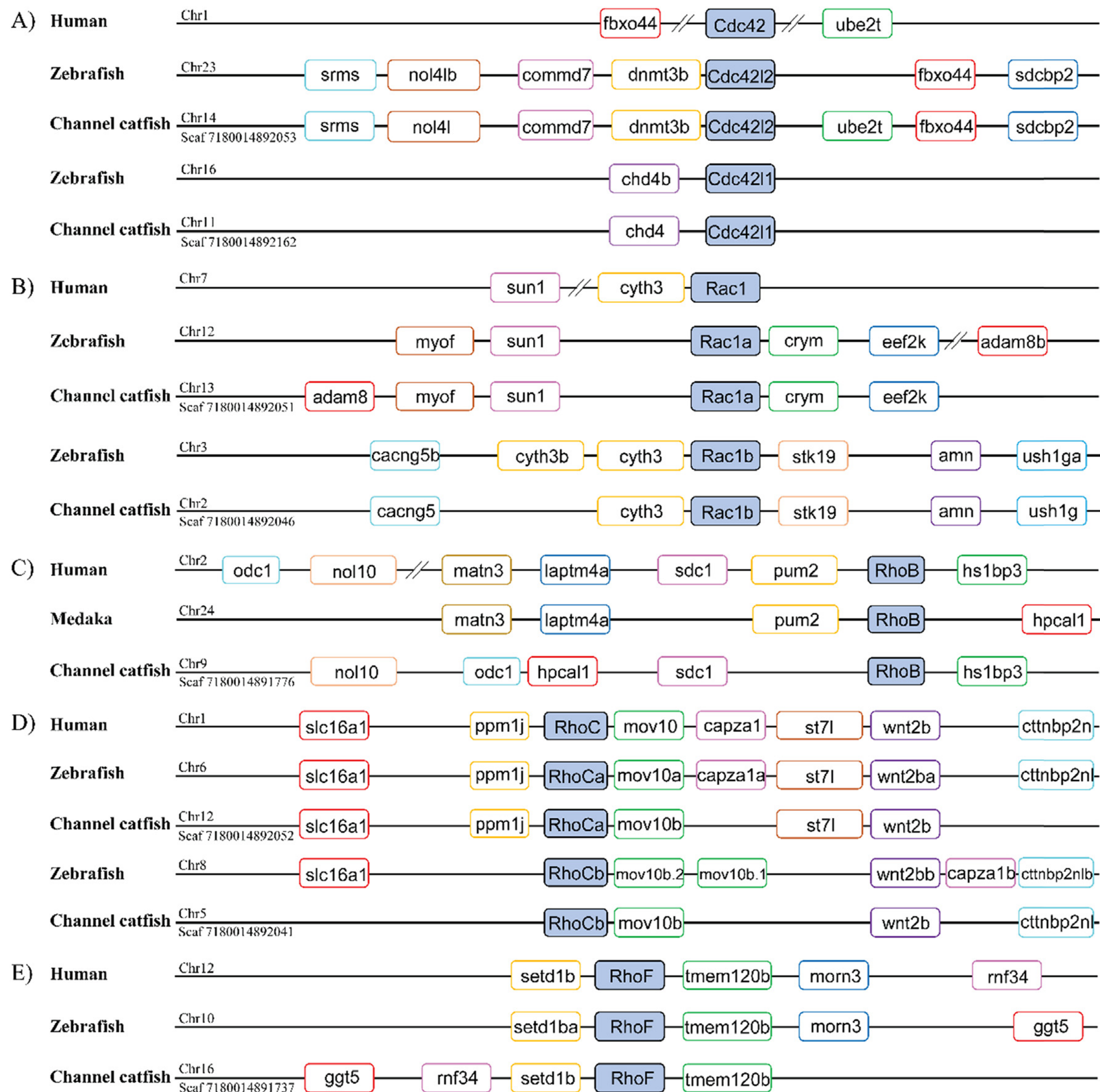

Fig. 1. Syntenic analyses of channel catfish (A) Cdc42, (B) Rac1, (C) RhoB, (D) RhoC, and (E) RhoF GTPase gene with corresponding genes from human and zebrafish (or Medaka). Genes with orthologous relationship are aligned vertically. Full gene names are provided in Supplementary Table S6.

subfamily, including Rac1a, Rac2, and RhoGb, were significantly regulated after *F. columnare* infection. Rac1 and Rac2 were reported to be involved in a variety of cellular processes including adhesion and migration by cytoskeletal rearrangement (Gu et al., 2003). In addition, they contributed to the NADPH oxidase complex assembly and activation to combat with the invading bacteria (Bokoch, 2005; Bokoch and Diebold, 2002; Dinauer, 2003). In the present study, Rac1a expression was up-regulated in the gill at 4 h after *F. columnare* infection, and continued to be up-regulated at 24 h when the expression was significantly altered. The up-regulated expression pattern was consistent with the observations in studies of other aquaculture species. In grass carp, Rac1 was shown to be inducible by *Aeromonas hydrophila* in vivo and in vitro (Hu et al., 2016); in turbot, the expression level of Rac1 increased significantly at 8 h and 24 h in liver after challenge with *Vibrio harveyi*, suggesting an immunologic function of this Rho family member (Jia and Zhang, 2009). In this study, Rac2 was found to be differentially expressed at 24 h after *F. columnare* infection. Various studies on zebrafish indicated that Rac2 was important in host defense. Using the embryonic zebrafish model, Deng et al. (2011) reported that Rac2 signaling played an essential part in the regulation of neutrophil mobilization and polarization in vivo. Furthermore, the role of zebrafish Rac2 was determined by using Rac2-specific small-molecule inhibition in vitro (Tell et al., 2012). Similarly, RhoGb was found to be significantly up-regulated in this study, consistent with the observation in zebrafish. One zebrafish study showed that RhoGb expression was significantly up-regulated during *Mycobacterium marinum* infection, suggesting its vital roles in immune responses (Salas-Vidal et al., 2005). Cdc42 was well known to have a conserved role in regulating cell shape, motility, and phagocytosis in many cell types such as neutrophils (Szczur et al., 2006), monocytes (Weber et al., 1998), and macrophages (Hoppe and Swanson, 2004). Chen et al. (1996) reported that Cdc42 was required for cytoskeletal rearrangement and bacterial internalization in monkey kidney cells after the infection of bacterial pathogen *Salmonella typhimurium*. Of three Cdc42 copies (Cdc42, Cdc42l, and Cdc42l2) in channel catfish, only Cdc42l was significantly up-regulated, suggesting its involvement in immune responses after bacterial infection. In the channel catfish Rho subfamily, RhoB and RhoC were significantly altered after *F. columnare*

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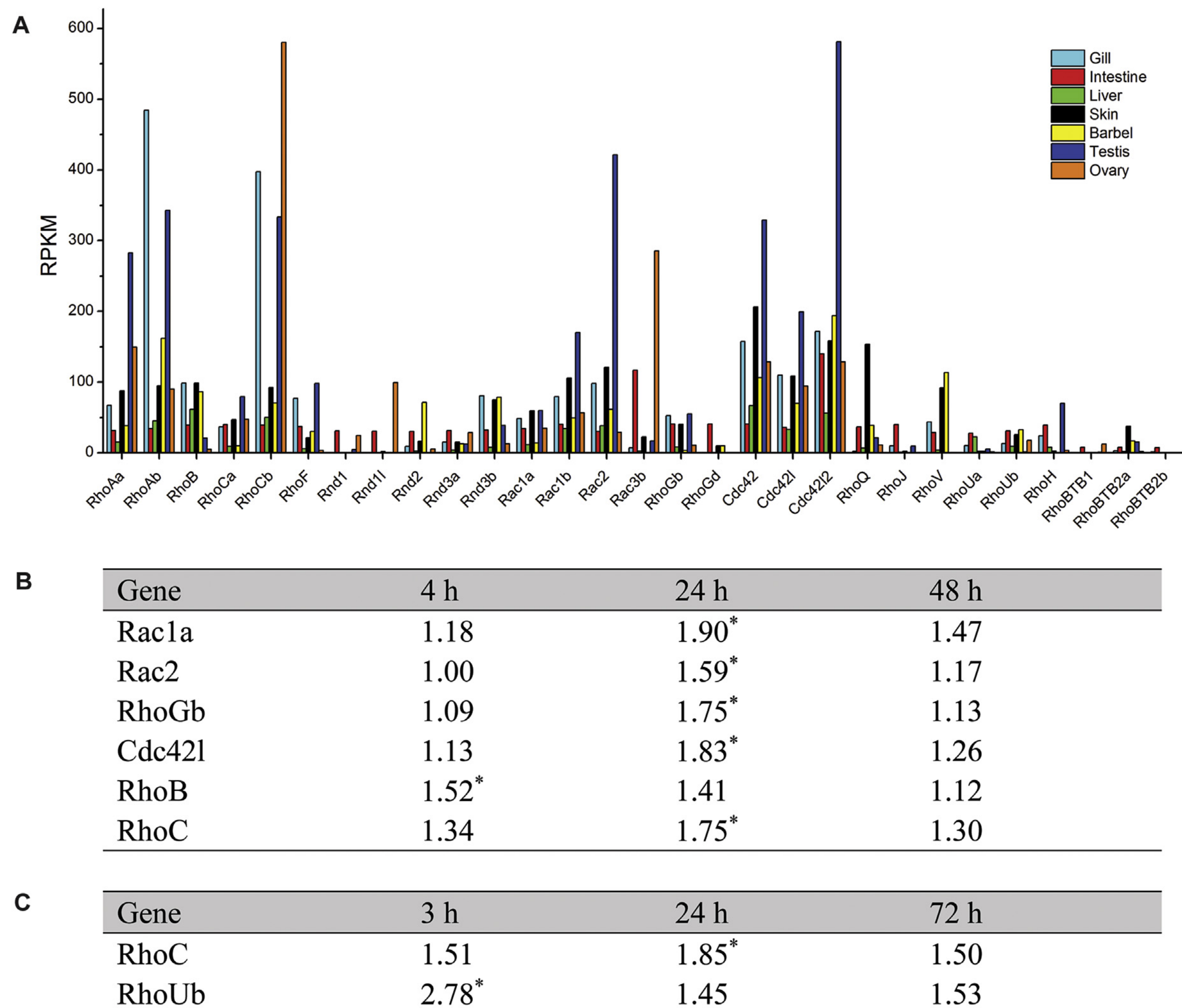

B

Gene	4 h	24 h	48 h
Rac1a	1.18	1.90*	1.47
Rac2	1.00	1.59*	1.17
RhoGb	1.09	1.75*	1.13
Cdc42l	1.13	1.83*	1.26
RhoB	1.52*	1.41	1.12
RhoC	1.34	1.75*	1.30

C

Gene	3 h	24 h	72 h
RhoC	1.51	1.85*	1.50
RhoUb	2.78*	1.45	1.53

Fig. 2. (A) Expression profiles of Rho GTPase genes in healthy channel catfish tissues. Gene expression levels are presented as RPKM after normalization by Sailfish, and the x-axis shows the names of the studied genes. (B) Differentially expressed Rho GTPase genes in gill tissues of channel catfish after *F. columnare* infection. Asterisks indicate significant differences compared to the control (P-value < 0.05). (C) Differentially expressed Rho GTPase genes in intestine tissues of channel catfish after *E. ictaluri* infection. Asterisks indicate significant differences compared to the control (P-value < 0.05).

infection. RhoB was predominantly up-regulated at 4 h post-infection. This observation was in consistence with the results of cDNA microarray analysis in human colonic CaCo-2 cells, in which RhoB was significantly up-regulated after 4 h of treatment with exotoxin of *Clostridium difficile* (Gerhard et al., 2005). For RhoC, extensive studies have been conducted concentrating on its contribution on promoting metastatic behavior. For instance, Stoletov et al. (2007) demonstrated that RhoC-induced morphological change and invasion played an important role during the early stage of cancer cell metastasis using the zebrafish model. It has been indicated that RhoC regulated metastasis by controlling cytoskeleton organization and cell motility (Clark et al., 2000). In channel catfish, RhoC was the only Rho GTPase gene that showed significantly up-regulated expressions in both columnaris and ESC challenges, implying its crucial roles in disease defense responses. In addition, mRNA expression of RhoUb was statistically significant after infection with *E. ictaluri* in this study. In a zebrafish study, RhoUb was suggested to be implicated in the neural development and function (Dickover et al., 2014). RhoU, which shared sequence similarities with Rac and Cdc42, was reported to regulate cell adhesion and motility (Ory et al., 2007).

All differentially expressed Rho GTPases in this study were observed to be up-regulated. The expressions of significantly up-regulated genes were relatively transient as each of them was only detected at one time point after infections. All of these significantly expressed genes were regulated during the first 24 h post-challenge and then returned to normal levels. This observation can be explained by the response pattern in innate immune systems, in which only immediate defense against infection rather than long-lasting response is provided (Alberts et al., 2002). The extent of up-regulation was fairly modest, with all genes having fold changes less than 2 times, and the only exception was RhoUb. Furthermore, different regulation patterns after bacterial infections of the two pathogens may be attributed to tissue-specific regulation or pathogen-specific regulation. It is worth noting that not all significant changes of Rho GTPases definitely result in immune outcomes. Some changes in the expression of these genes could be an indirect outcome of the immunological challenges due to the complex cellular roles of Rho GTPases. Besides, the Rho GTPases expression can be influenced by pathogenesis, during which bacteria intentionally change actin cytoskeleton organization and achieve entry into non-phagocytic host cells by secreting effector

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proteins (Popoff, 2014).

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.dci.2016.10.005.

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