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Expression and localization of grass carp *pkc-θ* (*protein kinase C theta*) gene after its activation

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ABSTRACT

Haemorrhagic disease caused by grass carp reovirus (GCRV) can result in large-scale death of young grass carp, leading to irreparable economic losses that seriously affect large-scale breeding. Protein kinase C (PKC, also known as PRKC) represents a family of serine/threonine protein kinases that includes multiple isozymes in many species. Among these, PKC-θ (PKC theta, also written as PRKCQ) is a novel isoform, mainly expressed in T cells, that is known to be involved in immune system function in mammals. To date, no research on immunological functions of fish Pkc-θ has been reported. To address this issue, we cloned the grass carp *pkc-θ* gene. Phylogenetic and syntenic analysis showed that this gene is the most evolutionarily conserved relative to zebrafish. Real-time quantitative PCR (RT-qPCR) indicated that *pkc-θ* was expressed at high levels in the gills and spleen of healthy grass carp. Infection with GCRV down regulated *pkc-θ* expression in the gills and spleen. Gene products that function upstream and downstream of *pkc-θ* were up regulated in the gill, but were down-regulated in the spleen. These results suggest that direct or indirect targeting of *pkc-θ* by GCRV may help the virus evade host immune defences in the spleen. Phorbol ester (PMA) treatment of Jurkat T cells induced translocation of grass carp Pkc-θ from the cytoplasm to the plasma membrane. This response to PMA suggests evolutionary conservation of an immune response function in fish Pkc-θ, as well as conservation of its sequence and structural domains. This study expanded our knowledge of the fish PKC gene family, and explored the role of *pkc-θ* in function of the grass carp immune system, providing new insights which may facilitate further studies of its biological functions.

1. Introduction

Protein kinase C (PKC, also known as PRKC) represents a family of serine/threonine protein kinases that includes multiple isozymes in many species [1]. Mammalian PKCs can be classified into four categories: 1) conventional/classical, 2) novel, 3) atypical, and 4) PKC-related, based on their ligand sensitivity and structural properties [2]. The conventional/classical PKCs (cPKCs) require diacylglycerol (DAG), Ca^{2+}, and phospholipids for activation. This group includes α (PRKCA), β (PRKCB), and γ (PRKCG) members. Novel PKCs (nPKCs) require DAG, but not Ca^{2+} for activation. This group includes δ (PRKCD), ε (PRKCE), η (PRKCH), and θ (PRKCQ) members. Atypical PKCs (aPKCs) need neither Ca^{2+} nor DAG for activation, but require phosphatidylserine. Atypical PKCs include ζ (PRKCI) and ι (PRKCZ) members [1,2]. Finally, the newly discovered PKC-related kinases (PRKs) define a fourth group consisting of at least three members, named PRK1, PRK2, and PRK3 [2].

Members of the *PKC* gene family are involved in a wide range of biological processes, including cell proliferation, apoptosis, and cell differentiation [3]. The specific biological functions of *PKC* gene family members depend on their unique domains [4]. Knowledge of the structures of PKCs is crucial to understanding their function [5]. Each family member has a conserved C1, C2, and serine/threonine protein kinase catalytic domain [3]. The C1 domain of PKC-θ is located between the C2 domain and the serine/threonine protein kinase catalytic domain. The C1 domain is the DAG and phorbol ester (PMA, also known as TPA) binding domain [6]. The C2 domain is a phosphotyrosine-binding module that also binds Ca^{2+} and phospholipids, and has been reported to play an important role in PKC-θ activation [6]. The

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serine/threonine protein kinase catalytic domains do not discriminate between target enzymes, and are highly conserved across the family of PKCs [3].

PKC-θ is the only PKC isoform that translocated to the immune synapses after antigenic stimulation of T cells [7–9]. After recruitment to the synapse, it interacts with various signalling molecules, transmitting T-cell receptor (TCR) signals and activating transcription factors, including nuclear factor-kappa B (NF-κB), activator protein-1 (AP-1), and nuclear factor of activated T cells C1 (NFATC1), ultimately inducing production of *Interleukin-2* (*IL-2*) [7–9]. PIP2-dependent kinase-1 (PDK-1) also plays an important role in NF-κB activation after T cell stimulation, by phosphorylating PKC-θ and recruiting it to lipid rafts [10]. In addition, PKC-θ physically interacts with the IkB kinase (IKK) complex in lipid rafts, so that PDK-1 can recruit IKK complexes to TCR signalling complexes via PKC-θ [10]. IL-2 can drive proliferation of T cells, B cells, and natural killer cells [11]. Therefore, PKC-θ is involved in important immune functions. In addition, NOTCH1 promotes T cell leukaemia-initiating activity by regulating the effects of RUNXs on PKC-θ and reactive oxygen species in T cell acute lymphoblastic leukaemia (T-ALL) [12]. Studies have shown that selective localization of PKC-θ is essential for PKC-θ-mediated downstream signalling. PKC-θ regulates intracellular signal transduction and mature T cell activation and proliferation through its unique localization [13]. In addition to its primary biological function in T cells, PKC-θ is also expressed in other cell types, where it is involved in multiple physiological and pathological processes. For example, high levels of PKC-θ expression in platelets can modulate signal transduction from different surface receptors, which are required for platelet activation, aggregation, and haemostasis [14,15].

Eleven *PKC* isoforms have been discovered in mammals thus far [4]. Many fish *pkc* sequences have been submitted to NCBI (https://www.ncbi.nlm.nih.gov/), including 50 fish *pkc*-θ sequences. Research on fish *pkc-θ* has mainly focused on analyses of expression during embryonic development, in apoptotic processes, and in the zebrafish central nervous system [1,16,17]. Although a wide range of mammalian *PKC* functions have been elucidated, the biological functions of fish *pkcs*, including the immunological functions of fish *pkc-θ*, have not been reported.

Grass carp occupy an important position in the fish breeding industry in China and around the world. In 2016, grass carp accounted for 13% of global freshwater fish farm production [18]. However, the grass carp industry is often affected by diseases, especially GCRV infection in juvenile fish, which causes haemorrhagic disease and underlies significant losses within the breeding industry. Since PKC-θ has important immune functions in mammals [7–10], we here cloned the grass carp *pkc-θ* gene and investigated its potential immunological function(s). This study provides important insights into the function(s) of *pkc-θ* in host immune defence mechanisms of grass carp and the roles of *pkc-θ* in the fish immune system.

2. Materials and methods

2.1. Grass carp, GCRV, and Jurkat T cells

Juvenile grass carp (15 ± 3 cm, 40 ± 10 g, 6 months old) used in these experiments were from the Guanqiao Experimental Base of the Institute of Hydrobiology, Chinese Academy of Sciences. The GCRV-GD108 virus solution used for GCRV infection was prepared by our laboratory, as previously described [19]. Human leukaemia Jurkat T cells, from the American Type Culture Collection (ATCC, cell clone number TIB-152), were maintained in DMEM medium (BioWhittaker; Walkersville, MD, USA) supplemented with 10% foetal bovine serum (Sigma; St Louis, MO, USA), 100 U/mL penicillin, 100 g/mL streptomycin, and 2 mM L-glutamine. Cells were cultured at 37 °C in an incubator with 5% CO_2, and were harvested in logarithmic growth phase for use in subsequent experiments.

2.2. Cloning of full-length grass carp pkc-θ cDNA

Using the zebrafish *pkc-θ* sequence (*D. rerio pkc-θ*, accession no. XM_009300385.3) from the NCBI database (http://www.ncbi.nlm.nih.gov/sites/gquery) as a reference, the DNA coding sequence of grass carp *pkc-θ* was obtained by performing a BLAST search of the grass carp genome [20]. Based on this sequence, gene-specific primers were designed for 5′ RACE (reverse primers) and 3′ RACE PCR (forward primers). The RACE method was performed in accordance with SMARTer® RACE 5′/3′ Kit instructions (Clontech; Kyoto, Japan). The resulting fragments were ligated into the pMD-18T vector and sequenced. The full-length cDNA sequence of grass carp *pkc-θ* was obtained, and overlapping sequences and vector sequences were discarded. All primers designed for gene cloning are listed in Supplementary Table 1.

2.3. Bioinformatics analysis of grass carp pkc-θ

Based on full-length cDNA and genomic sequences, the structure of the grass carp *pkc-θ* gene was manually drawn using a sequence alignment assembled using DNAMAN software. The predicted amino acid sequence was analysed using the Sequence Manipulation Suite (SMS, http://www.bioinformatics.org/sms) and Expert Protein Analysis System (ExPASy, https://www.expasy.org/) tools. The domains of the grass carp Pkc-θ protein sequence were predicted using the Simple Modular Architecture Research Tool (SMART, http://smart.embl-heidelberg.de/). Sequence similarity analysis between Pkc-θ proteins of grass carp and other species was performed by Multiple Alignment using Fast Fourier Transform (MAFFT, https://www.ebi.ac.uk/Tools/msa/mafft/). Based on the neighbour-joining (NJ) method, a phylogenetic tree of Pkc-θ proteins of grass carp and other species was constructed using MEGA (7.0) [21]. Synteny analysis of *pkc-θ* genes among multiple species was performed using Genomicus V92.01 (http://www.genomicus.biologie.ens.fr/) [22].

2.4. GCRV infection and sampling

Experimental fish were placed in a circulating water system (containing 100 U/mL penicillin, 100 g/mL streptomycin) at 28 °C with an aeration system to ensure sufficient dissolved oxygen, and observed for one week. Grass carp with normal skin colour, feeding behaviour, physical activity, and no abnormalities such as rotting gills or tail, or bleeding, were used for virus infection experiments.

Prior to GCRV infection, three fish were randomly selected as controls. From each control, 11 tissues (gill, liver, spleen, intestine, kidney, head kidney, muscle, skin, blood, brain, and heart) were analysed to determine normal tissue distribution and expression levels of grass carp *pkc-θ* mRNA. The remaining fish were intraperitoneally injected with a GCRV suspension (1.20×10^3 copies/μL) at a 2% volume-to-weight ratio (mL/g). Gills and spleens from three fish were analysed on days 1–6, 8, 11, and 14 post-infection. Samples on days 1–6 and 8 were taken from three live grass carp randomly selected during the incubation period (the first 8 days). Samples on days 11 and 14 were taken from the three live fish with the most obvious symptoms at the start of the onset period (8 days). All tissue samples were ground in TRIzol (Invitrogen; San Diego, CA, USA) and stored at −80 °C for use.

Experimental fish were anesthetized with an appropriate concentration of aqueous eugenol solution prior to sampling. With the utmost effort to minimize damage to these experimental fish, all operations were performed in accordance with the relevant policies and regulations of the U.K. Animals (Scientific Procedures) act 1986, and the Guidelines for the Care and Use of Laboratory Animals (Ministry of Science and Technology of China, 2006). Experimental procedures were endorsed by the Academic Committee of the Institute of Hydrobiology, Chinese Academy of Sciences (CAS).

2.5. Tissue distribution of grass carp pkc-θ

The mRNA expression levels of grass carp *pkc-θ* in the healthy control group were analysed using real-time quantitative PCR (RT-qPCR). Expression levels were calculated relative to *β-actin* (reference gene) using the $2^{-\Delta\Delta Ct}$ method. The mRNA expression level of grass carp *pkc-θ* in skin tissue was set as the benchmark for comparative tissue distribution [23,24]. Every sample was assayed in triplicate using a CFX96TM Real-Time PCR Detection System (Bio-Rad; Hercules, CA, USA). The primers used for RT-qPCR experiments are listed in Supplementary Table 2.

2.6. Expression of grass carp pkc-θ and its pathway-related genes after GCRV infection

RT-qPCR was used to analyse transcription levels of grass carp *pkc-θ* in gills and spleens at different time points (day 0, day 1, day 2, day 3, day 4, day 5, day 6, day 8, day 11, and day 14) after GCRV infection. In addition to *pkc-θ*, expression levels in gills and spleen of its upstream gene, *pdk-1*, and downstream target molecules, *ap-1, nfatc-1 and il-2 were* analysed at the same time points [20]. *β-actin* was used as the internal reference gene for expression analysis [24]. The relative expression levels of each gene in the corresponding tissue in the control group (day 0) was used as a benchmark (1.0) for comparative analysis. The $2^{-\Delta\Delta Ct}$ method was used to analyse relative gene expression level [23]. Primers used in these experiments are listed in Supplementary Table 2.

2.7. Transient transfection of Jurkat T cells and confocal microscopy

To construct a Pkc-θ-GFP expression plasmid, the open reading frame of grass carp *pkc-θ* was amplified using primers listed in Supplementary Table 1. The resulting PCR products were cloned into pGFP-N3 (Clontech), which expresses proteins of interest as N terminal fusions to green fluorescent protein. Cells (1.2×10^7) were transfected with 2.5 μg of Pkc-θ-GFP plasmid DNA by electroporation using a Gene Pulser instrument (BioRad; 270 V, 975 μF). At 48 h post-transfection, cells were stimulated with 200 ng/mL PMA or mock treated with phosphate-buffered saline (PBS) for 30 min. Cells were then fixed in 4% paraformaldehyde and permeabilized in PBS containing 0.5% Triton X-100 and 20 mM glycine. After five washes, cell nuclei were stained with Hoechst 33258 (Beyotime; Shanghai, China). Finally, mounted slides were analysed by confocal microscopy (LSM-510 META; Zeiss; Oberkochen, Germany) and images were processed with ImageJ (National Institutes of Health; Bethesda, MA, USA) and Adobe Photoshop (Adobe; San Jose, CA, USA) software.

2.8. Data analysis

RT-qPCR data were represented as mean ± standard deviation of three independent experiments. Data were analysed by two-tailed independent *t*-test. $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Characterization and sequence analysis of grass carp pkc-θ

The cDNA sequence (GenBank accession number: MH625916) of grass carp *pkc-θ* identified in this study includes a 2154-bp open reading frame (ORF) (including the TGA stop codon), a 164-bp 5′-untranslated region (UTR) and a 189-bp 3′-UTR region. Typical AATAAA signature sequences and poly-A tails (18 bp) were found in the 3′-UTR region (Fig. 1A). In addition, genomic structural analysis revealed that the ORF region of grass carp *pkc-θ* contains 17 exons and 16 introns (Fig. 1B). Grass carp Pkc-θ encodes a protein of 718 amino acids (aa), with an average molecular weight of 82.21 kDa, and a theoretical isoelectric point of 8.38. Domain predictions showed that grass carp Pkc-θ contains a conserved C2 domain (aa 4–101) near the N-terminus (Fig. 1A), and two conserved C1 domains (aa 166–215, aa 238–287). A conserved inhibitory pseudosubstrate site (RRGAIKQA, aa 151–158) was found near the N-terminus of the first C1 domain [25]. Near the C-terminus, an S_TKc domain (serine/threonine protein kinase catalytic domain, aa 390–644) and an S_TK_X domain (extension to Ser/Thr-type protein kinase, aa 645–708) were predicted (Fig. 1B). MAFFT sequence alignment analysis of homologous genes showed that the aa sequence of grass carp Pkc-θ was most similar to that of zebrafish (93.81%), followed by cave fish (90.78%). Overall, the aa sequence of grass carp Pkc-θ was more than 73% similar to its ortholog in other species (Fig. S1).

3.2. Phylogenetic and syntenic analysis of Pkc-θ

To perform phylogenetic analysis of Pkc-θ proteins among multiple species, an evolutionary tree (bootstrap = 1000) was constructed (Fig. S2A). All fish Pkc-θs were found to aggregate into one large branch that then clustered with other branches composed of African clawed frog, chicken, mouse, human, and cow. In the large branch containing fish species, the closest relationship occurred between grass carp and zebrafish or cavefish. The Pkc-θs of these three species can be clustered into one small branch that then aggregates with Pkc-θs of other fish species. These molecular evolution data are consistent with evolutionary law and confirm the high reliability of the grass carp Pkc-θ sequence.

To study the evolutionary conservation of *pkc-θ* gene structure in different species, synteny analysis was performed using Genomicus V92.01, the genomic map of grass carp, and NCBI BLAST programs. The results showed that, with the exception of cave fish, the genes upstream of *pkc-θ* were identical among these fish species, while the downstream genes adjacent to *pkc-θ* differed among fish species (Fig. S2B). Overall, the genes downstream of *pkc-θ* are relatively conserved in the genomic structure of grass carp, zebrafish, humans, mice, and chickens. In particular, the downstream gene immediately adjacent to *pkc-θ* was consistently *pfkfb3*, but subsequent adjacent genes varied. In addition, among these fish species, *pkc-θ* genes of grass carp and zebrafish had the highest conservation in genomic structure, that is, the order of upstream and downstream genes adjacent to the PKC-θ gene was most similar.

3.3. Tissue distribution of grass carp pkc-θ

RT-qPCR was used to analyse grass carp *pkc-θ* mRNA expression in 11 different tissues (skin, blood, brain, kidney, head kidney, spleen, intestine, heart, muscle, gills, and liver). Grass carp *pkc-θ* expression was lowest in skin tissue (1.0-fold, Fig. 2). Relative to expression levels in skin, grass carp *pkc-θ* was highly expressed in the spleen and gills (17.53-fold and 16.35-fold, respectively; $p < 0.05$). Moderate expression levels were detected in muscle, intestine, brain, blood, kidney, heart, head kidney, and liver (8.90-fold, 8.72-fold, 6.48-fold, 6.46-fold, 4.75-fold, 3.96-fold, 2.92-fold, and 2.08-fold, respectively; $p < 0.05$, compared to skin).

3.4. Expression of pkc-θ and its pathway-related genes in grass carp after GCRV infection

RT-qPCR was further used to analyse changes in expression of *pkc-θ*, its upstream gene, *pdk-1*, and response genes, *ap-1*, *nfatc-1*, and *il-2* at different time points (0, 1, 2, 3, 4, 5, 6, 8, 11, and 14 d) in the gills and spleen. Significant changes in *pkc-θ*, *pdk-1*, *ap-1*, *nfatc-1*, and *il*-2 mRNA expression were observed in the gill. On day 1 after GCRV infection, *pkc-θ* expression was significantly down-regulated (0.13-fold, $p < 0.05$, compared to day 0). Although *pkc-θ* increased by day 4 relative to day 1, it was still lower than basal expression level. In addition, the expression profiles of *ap-1*, *nfatc-1*, and *il-2* were quite consistent.

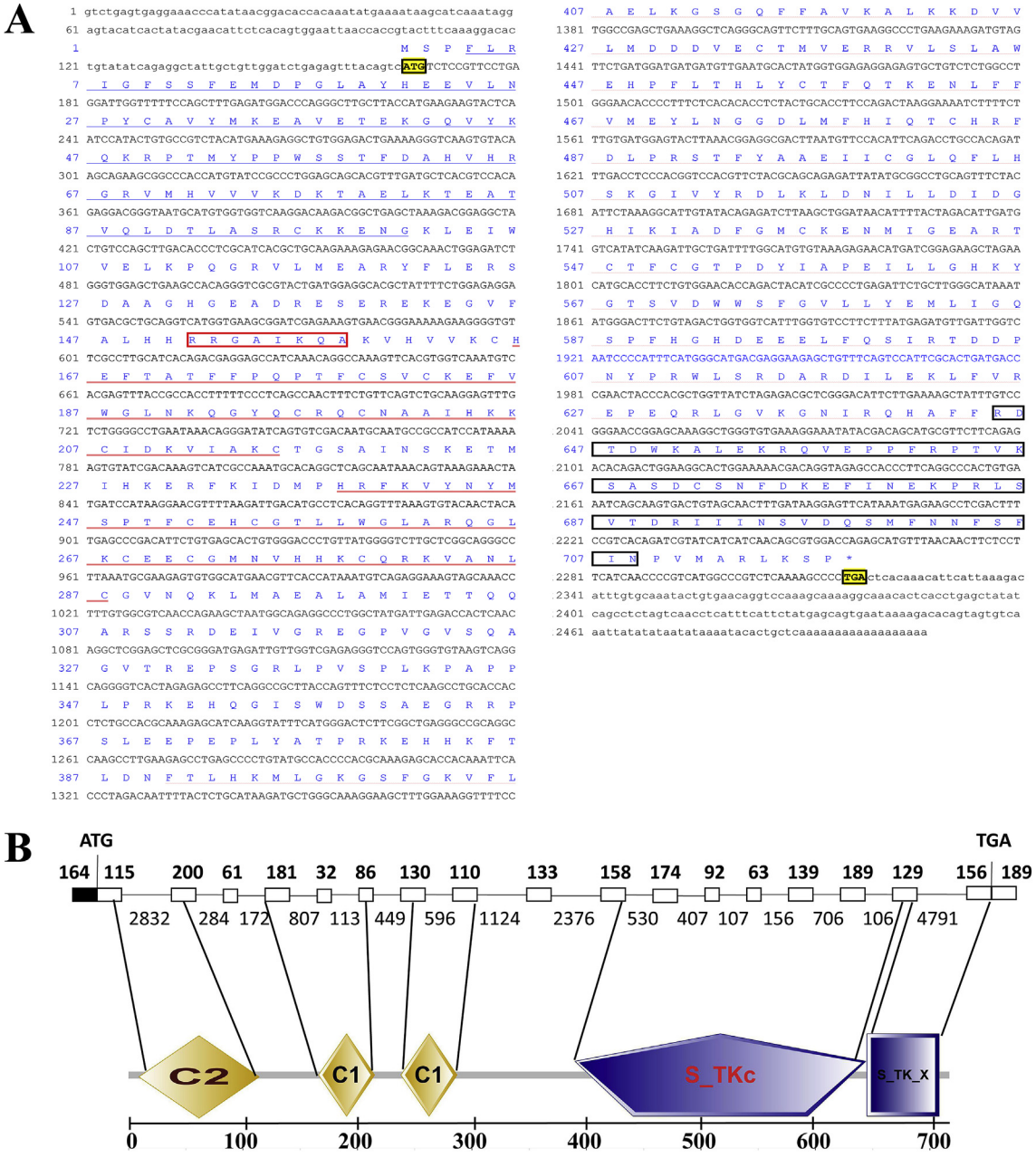

Fig. 1. cDNA sequence, genomic structure, and protein domain prediction of grass carp *pkc-θ* gene. (A) Full-length sequence of grass carp *pkc-θ* cDNA. The C2 domain (aa 4–101) and C1 domain (aa 166–215, aa 238–287) are indicated by a single underline in blue, and a double underline in red, respectively. The conserved inhibitory pseudosubstrate motif (RRGAIKQA; aa 151–158) is outlined in red. The S_TKc domain (serine/threonine protein kinases, catalytic domains; aa 390–644) and the S_TK_X domain (extension to Ser/Thr-type protein kinases; aa 645–708) are indicated with a red wavy line and a black square, respectively. The start and stop codons are both shown on a yellow background outlined in black. (B) Genomic structure and protein domain prediction of grass carp *pkc-θ*. White and black boxes represent exons and flanking non-coding regions, respectively. Horizontal lines indicate introns. Numbers indicate the number of bases in an intron or exon. Start and stop codons are indicated in the genome structure pattern diagram. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Peak *ap-1* and *nfatc-1* expression occurred on day 6 (10.69 and 11.36-fold, respectively; $p < 0.05$), and *il -2* reached its highest expression level on day 8 (4.43-fold, $p < 0.05$; Fig. 3). In the spleen, *pkc-θ*, *pdk-1*, *ap-1*, *nfatc-1*, and *il*-2 mRNA levels were all reduced throughout the GCRV infection, but transcriptional expression patterns of these genes still displayed similarity (Fig. 4). The above results suggest that *pkc-θ* and its related genes underwent significant changes in gill and spleen tissues in response to GCRV infection.

3.5. Localization of grass carp Pkc-θ in Jurkat T cells before and after PMA treatment

As shown in Fig. 5, grass carp Pkc-θ localization in Jurkat T cells showed significant changes before and after PMA treatment. In the PBS-treated control group, grass carp Pkc-θ was evenly dispersed in the cytoplasm of Jurkat T cells. After treatment of Jurkat T cells with PMA, grass carp Pkc-θ translocated from the cytoplasm to the cell membrane.

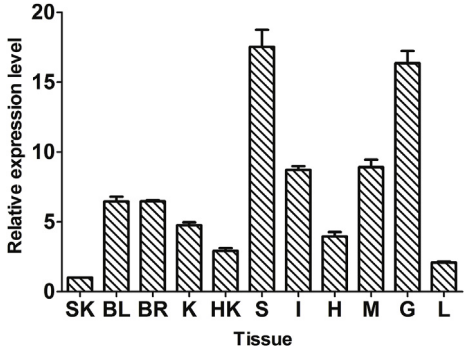

Fig. 2. Tissue distribution of *pkc-θ* in healthy grass carp. Tissues are abbreviated as follows: skin (SK), blood (BL), brain (BR), kidney (K), head kidney (HK), spleen (S), intestine (I), heart (H), muscle (M), gill (G), and liver (L). *β-actin* expression was used as an internal control for RT-qPCR experiments. Relative expression was calculated based on the ratio of gene expression in different tissues to that in skin. Results are based on three independent experiments and are expressed as mean ± standard deviation.

4. Discussion

Many studies of the structural domains of PKC proteins have been conducted that now contribute to in-depth understanding of their functions. In 1987, House et al. were the first to report that the N-terminus of the C1 domain contained an inhibitory pseudosubstrate region (RRGAIKQA) in PKCs of all species that had been discovered up to that time [30]. Other studies have shown that the pseudosubstrate region binds to the substrate binding site of the C-terminal kinase domain, allowing PKCs to be present in the cytoplasm in an inactive form in cells in a resting state [2,9,25,31]. In 1991, Hubbard et al. discovered a conserved motif of cysteine and histidine residues (H-X_{12}-C-X_2-C-$X_{13/14}$-C-X_2-C-X_4-H-X_2-C-X_7-C, C6—H2) in the C1 domains of mammalian PKCs, and those of *Saccharomyces cerevisiae*, *Drosophila melanogaster*, and *Caenorhabditis elegans* [32]. C6—H2 coordinates two zinc ions, accepts the activation signal of the upstream second messenger DAG, and binds PMA [2,9]. The C2 domain recognizes and binds Ca^{2+} and membrane phospholipids [4]. Domain prediction analysis showed that an S_TKc domain (the C-terminal kinase domain; aa 390–644) and an S_TK_X domain (the extension to Ser/Thr-type protein kinases; aa 645–708) were also present at the C-terminus of grass carp Pkc-θ (Fig. 1B). The N-terminus of grass carp Pkc-θ also contains a C2 domain (aa 4–101) and two C1 domains (aa 166–215, aa 238–287; Fig. 1). The most prominent discovery is that there are also conserved C6—H2 motifs in both C1 domains, and a conserved inhibitory pseudosubstrate region (RRGAIKQA, aa 151–158) near the N-terminus of the first C1 domain (Fig. 1A). The above structural analyses suggest that the domain characteristics of other species' PKC-θ proteins are also present in grass carp Pkc-θ. In short, grass carp Pkc-θ shares evolutionary

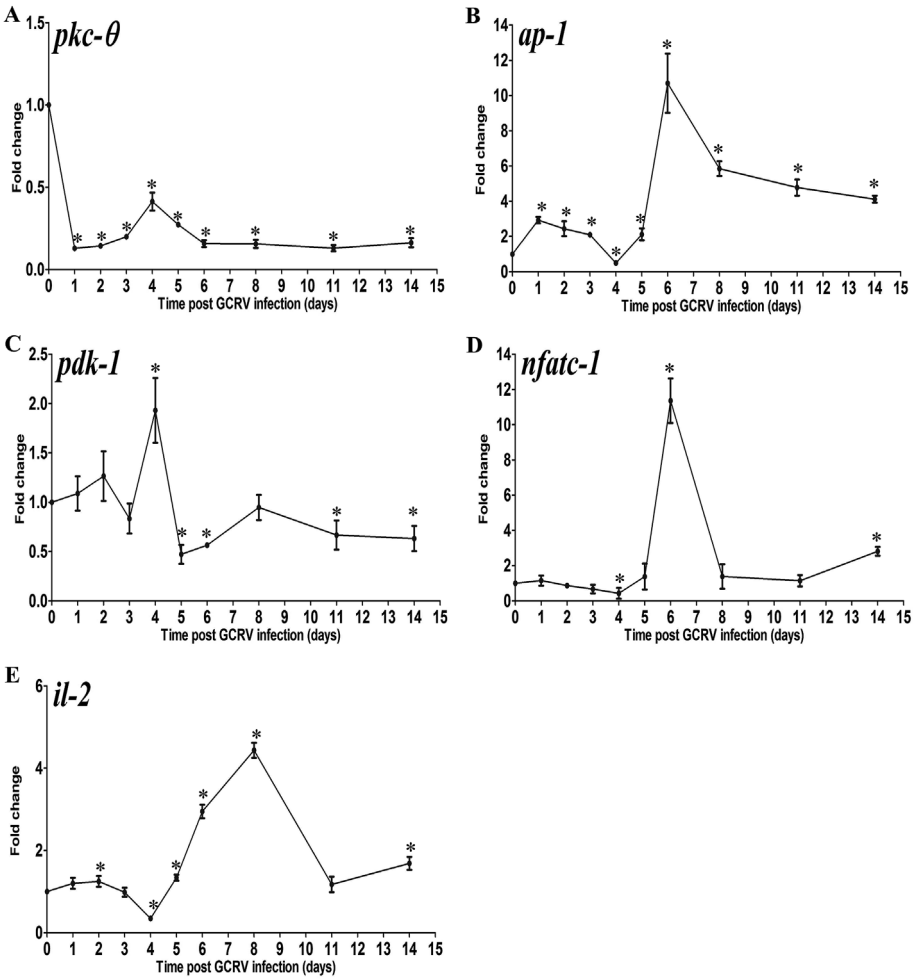

Fig. 3. Expression profile of grass carp *pkc-θ* and functionally interrelated genes in gills before and after GCRV infection. (A) *pkc-θ*, (B) *ap-1*, (C) *pdk-1*, (D) *nfatc-1*, (E) *il-2*. *β-actin* expression was used as an internal control for RT-qPCR experiments. Relative expression was calculated based on the ratio of gene expression in different tissues of the GCRV-infected group to that of the control group. Results are based on three independent experiments and are expressed as mean ± standard deviation.

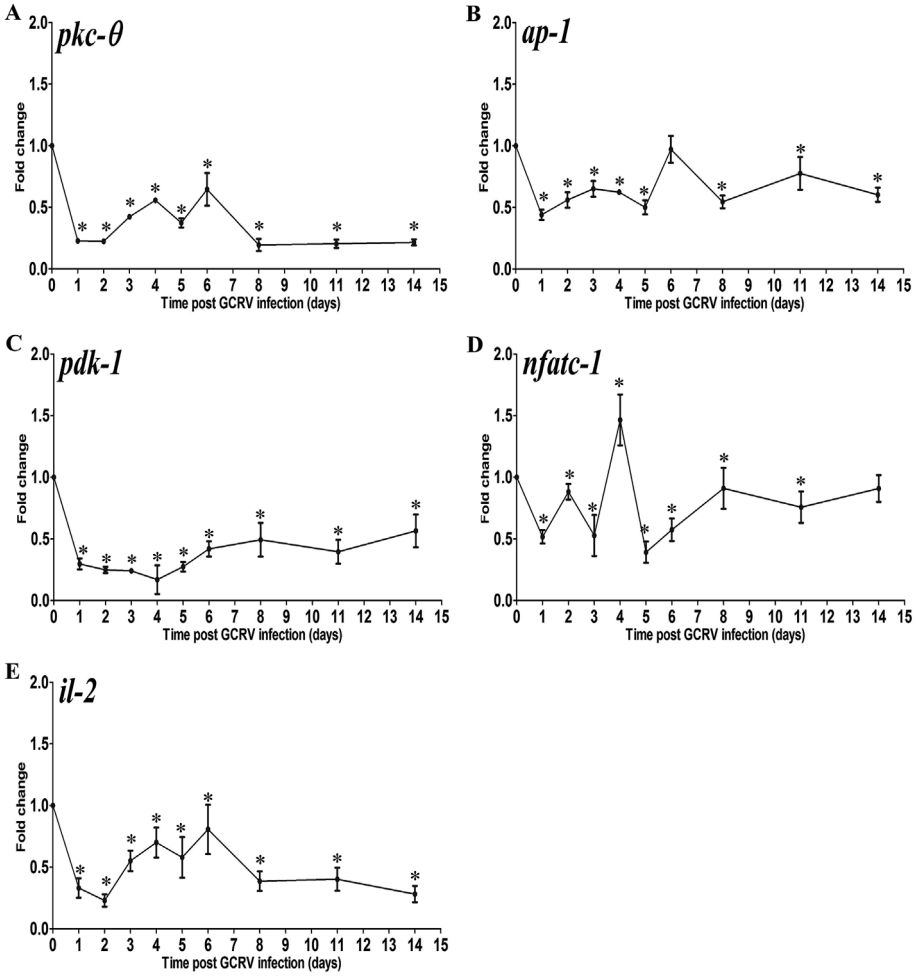

Fig. 4. Expression profile of grass carp *pkc-θ* and functionally interrelated genes in the spleen before and after GCRV infection. (A) *pkc-θ*, (B) *ap-1*, (C) *pdk-1*, (D) *nfatc-1*, (E) *il-2*. *β-actin* expression was used as an internal control for RT-qPCR. The relative expression was calculated based on the ratio of gene expression in different tissues of the GCRV-infected group to that of the control group. Results are based on three independent experiments and are expressed as mean ± standard deviation.

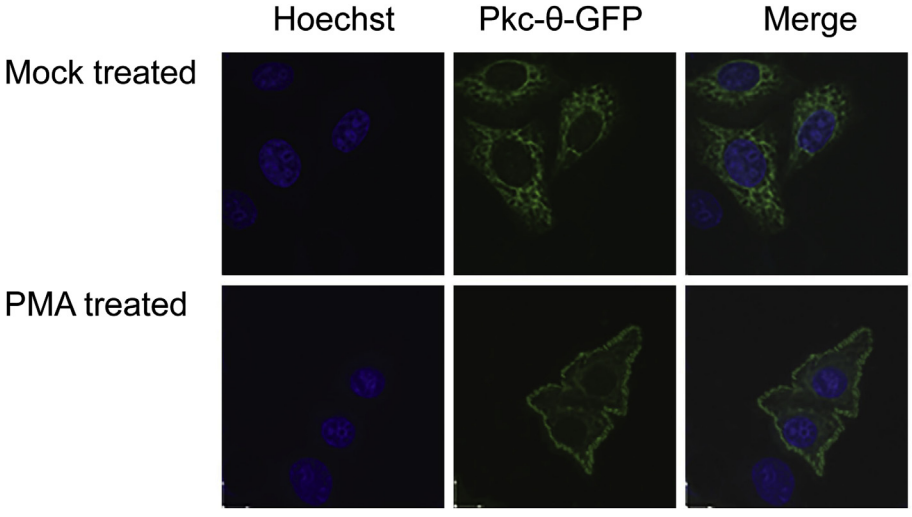

Fig. 5. Localization of grass carp Pkc-θ in Jurkat T cells before and after PMA treatment. Jurkat T cells were transfected with our Pkc-θ-GFP expression plasmid for 48 h and treated with PBS/PMA for 30 min. The mock-treated group was treated with PBS and the experimental group was treated with PMA. Green fluorescence indicates the subcellular localization of grass carp Pkc-θ expressed in fusion with GFP. Blue fluorescence indicates nuclei stained with Hoechst 33258. Membrane vs cytoplasmic localization was determined by the localization of green fluorescence relative to blue fluorescence. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

conservation of domains with those of other species.

Specific PKC functions depend on each PKC's specific biological structure [4]. Both stimulation of T cells and binding of DAG to the C1 region of PKCs can promote translocation of PKCs from the cytoplasm to the membrane, so that they are specifically located in contact regions (the locations of the immune synapses) between antigen-specific T cells and antigen presenting cells [2,30,33,34]. PKCs also translocate from the cytosol to a membrane-bound fraction in response to PMA (an activator of PKCs) treatment in a C1 domain dependent manner [6,33]. Based on the above-mentioned sequence conservation of grass carp Pkc-

θ protein domains, we further analysed its evolutionary conservation from the perspective of functional domain conservation. Treatment of Jurkat T cells with PMA induced translocation of overexpressed grass carp Pkc-θ from the cytoplasm to the membrane, whereas in cells treated with PBS, grass carp Pkc-θ remained dispersed throughout the cytoplasm (Fig. 5). The subcellular localization of grass carp Pkc-θ after T cell activation suggests that grass carp Pkc-θ is present in the cytoplasm in an inactive form in resting cells and can be translocated to the plasma membrane in response to PMA. These results suggest that grass carp Pkc-θ can respond to PMA stimulation similarly to studied Pkc-θs from other species.

We also analysed the potential immune function of grass carp *pkc-θ* via mRNA level expression studies. Studies in human tissues and related cell lines have shown that PKC-θ is highly expressed in hematopoietic stem cells, thymus, skeletal muscle, kidney, lung, cell lines derived from six human T cell malignancies (Molt-3, Hut-78, Jurkat, SupT1, H9 and CEM), and the histiocytic lymphoma cell line U-937, and expressed at low levels in the brain and testicles [25]. Results reported here show that the grass carp *pkc-θ* gene is mainly expressed in the spleen and gills (Fig. 2). The gills, as mucosal lymphoid tissue, can process and present antigens when the fish body is challenged, and the spleen is the largest peripheral lymphoid organ, where lymphocytes migrate and receive immune responses after antigen stimulation [26,27]. Since the gills and spleen are rich in T cells and are important immune organs of teleost fish [26,27], the high expression of *pkc-θ* mRNA in these tissues suggests its potential importance in the grass carp's immune response.

PKC-θ is required for efficient activation of transcription factors NF-κB, NFATC-1, and AP-1, and transcription of *IL-2*, serving as a bridge that propagates phosphorylation mediated signalling from the upstream kinase PDK-1 to downstream PKC-θ targets NF-κB, NFATC-1 and AP-1 [7–10,28]. To further explore the immunological function of grass carp *pkc-θ* in the gills and spleen, we analysed the effects of GCRV infection on expression profiles of *pkc-θ* and its functionally interrelated genes (*pdk-1*, *ap-1*, *nfatc-1*, and *il-2*). The results showed that levels of *pkc-θ* mRNA in the gills and spleen were lower than those in healthy grass carp ($p < 0.05$, Figs. 3A and 4A). Expression levels of *pdk-1*, *ap-1*, *nfatc-1*, and *il-2* in gills were upregulated in response to GCRV infection while their expression levels in spleen were decreased (Figs. 3 and 4). We speculate that slight upregulation of *pdk-1* may increase phosphorylation of Pkc-θ in the gills at early stages of GCRV infection, thus enhancing signal-mediated phosphorylation of Ap-1 and Nfatc-1. Upregulated transcription of *ap-1* and *nfatc-1* may be mediated by other regulatory factors related to the pathway. Finally, transcription of *il-2* is up regulated in the gills. In the spleen, however, *pdk*-1 mRNA levels were consistently low, thus Pdk-1 may not phosphorylate enough Pkc-θ to in turn activate Ap-1 and Nfatc-1. Also, transcription levels of *ap-1* and *nfatc-1* may be down regulated by other regulatory factors associated with the pathway, eventually contributing to low *il*-2 mRNA levels in the spleen.

In 2005, Marsland et al. found that PKC-θ is required for the recall phase of lymphocytic choroiditis virus (LCMV) antigen in $CD8^+$ T cells, and delayed PKC-θ signalling severely impairs memory T cell development [29]. Our studies suggest that reduced expression of *pkc-θ* and related cytokines may be one mechanism by which GCRV escapes host immune defences in spleen tissue. In short, *pkc-θ* expression may be a direct or indirect target of GCRV, allowing it to evade host immune defences in the spleen. GCRV appears to also have a more mature mechanism for evading host immune defences in the spleen that requires further study.

5. Conclusions

Grass carp *pkc-θ* was cloned and analysed in this study. The expression profiles of *pkc-θ* after GCRV infection, and PMA-induced translocation of Pkc-θ from the cytosol to the plasma membrane suggest the involvement of grass carp *pkc-θ* in responses to GCRV infection and PMA, and broaden our understanding of the grass carp immune system. This preliminary evidence that *pkc-θ* participates in the grass carp immune response may help to further elucidate functions of *pkc-θ* in the fish immune system.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.01.057.

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