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Short communication

pik3r3b, a novel immune-related gene in Nile tilapia (*Oreochromis niloticus*): Identification, expression and analysis of antibacterial activity

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ABSTRACT

A full-length cDNA encoding phosphatidylinositol 3-kinase regulatory subunit gamma b gene in Nile tilapia (*Oreochromis niloticus*), termed as *On-pik3r3b*, was identified and characterized in this study. The sequence analysis demonstrated that the full-length cDNA of *On-pik3r3b* was 2018 bp, containing a 5′ untranslated region (UTR) of 171 bp, an open reading frame (ORF) of 1422 bp and a 3' UTR of 425 bp. Its protein sequence displayed a high degree of identity with other fish. Using qPCR, the expression patterns of *On-pik3r3b* were investigated. In healthy Nile tilapia, the transcripts of *On-pik3r3b* were detected in all examined tissues, except the skin. Upon the stimulation with *Streptococcus agalactiae*, the *On-pik3r3b* expression level in liver, spleen, kidney and gill were significantly increased at 12 h after infection. The recombinant On-pik3r3b showed *in vitro* antibacterial activity, against *S. agalactiae* and *E. coli*. Our observation strongly indicates that *On-pik3r3b* is involved in the innate immune response in Nile tilapia.

1. Introduction

Nile tilapia (*Oreochromis niloticus*) is one of the most important economical freshwater fish widely cultured in many countries around the world. Especially in China, the production of tilapia was 1.7 million tons in 2015, accounting for nearly half of the global annual production. However, as the largest tilapia producer and exporter in the world, diseases of the cultured fish have occurred seriously and frequently in recent years due to the high stocking densities, poor water quality and degeneration of genetic characterization. Among the various pathogens, *Streptococcus agalactiae*, a Gram-positive bacteria that could affect many species of fish with significant morbidity and mass mortality in summer [1], has been one of the most serious pathogenic bacteria of tilapia. *S. agalactiae* has resulted in great economic loss and severely threatened the sustained development of tilapia culture [2,3]. Although application of antibiotics can effectively solve these problems, it has raised many other concerns, such as antibiotic resistance of pathogens, residue of antibiotics, environmental pollution and thus threatening human health. Therefore, it is necessary and urgent to improve disease resistance of tilapia using molecular technique.

Phosphatidylinositol 3-kinase (PI3K) has been known to play ubiquitous roles in cellular processes. It can regulate reorganization of the actin cytoskeleton [4], induction of cellular survival [5], protection of fibroblast from apoptosis [6], antigen and IL-2 growth factor-mediated activation in T cells [7,8] and membrane ruffling in T cells [9]. In the last decades, many studies have proved that the PI3K is involved in both innate and adaptive immunity [10–13], which is important for the activation of NF-κB pathway in different mechanisms [14,15]. In addition, PI3K is required for modifying the cytoskeleton dynamics, regulating membrane traffic and pseudopod extension, which is utilized by pathogenic bacteria for entry into host cell [16–18].

PI3K is a heterodimeric molecule, which composed of a regulatory and a catalytic subunit [19]. Phosphatidylinositol 3-kinase regulatory subunit gamma, which encoded by the *pik3r3* gene in human [20], has been shown to interact with insulin-like growth factor 1 receptor, insulin receptor substrate 1 and retinoblastoma protein [21–23]. Recent study in catfish demonstrated that several genes involved in PI3K pathway were associated with the resistance against columnaris.

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Moreover, *pik3r3b* is the closest flanking gene to the most significantly associated SNP [24]. These findings strongly implied the close relation between *pik3r3b* and the resistance against columnaris in catfish. However, few studies about the functional analysis of *pik3r3b* in immune response and disease resistance were carried out, especially in teleost.

In the present study, the full-length cDNA of *On-pik3r3b* in Nile tilapia was isolated and characterized. Using qPCR, expression analysis of *On-pik3r3b* were performed in various tissues of healthy fish, and four immune-related tissues of fish stimulated with *S. agalactiae* were selected for further analysis. Using On-Pik3r3b recombinant protein, the *in vitro* antibacterial activity was presented. This research may help us to expand more knowledge about the interaction between *S. agalactiae* and tilapia, and contribute to provide novel molecular resource for disease resistant breeding.

2. Materials and methods

2.1. Preparation of experimental fish

The Nile tilapia used in this study were purchased from Qingdao National Tilapia Seed Farm (Qingdao, China). Prior to experiment, the fish (average body weight of 100 g ± 10 g) were acclimatized in a 100 L fiber-reinforced plastic tank under 30 °C for one week.

2.2. Bacterial challenge and sampling

A virulent *S. agalactiae* strain HN016 [25,26] was used in the present study. The bacteria were inoculated in trypticase soy broth (TSB) medium at 28 °C for 12 h. After cultivation, the bacterial suspension were diluted to a concentration of 1×10^7 colony forming units (cfu) mL^{-1} with sterilized phosphate buffered saline (PBS). The fish of treatment group were injected intraperitoneally with 0.2 mL bacteria suspension and then cultured at 32 °C. As controls, the other fish were injected with sterilized PBS and maintained in a separate tank under the same culture condition.

Heart, liver, kidney, intestine, spleen, blood, brain, gill and skin (total nine tissues) were removed from healthy Nile tilapia, and immediately frozen by liquid nitrogen and stored at −80 °C. At time points of 0 h, 6 h, 12 h, 24 h, 48 h, 72 h and 96 h post-injection, four kinds of tissues (including liver, kidney, spleen and gill) were removed from treatment and control group, and frozen in liquid nitrogen immediately, followed by storage at −80 °C until usage. All samples were collected from at least three independent individuals for biological replicates.

The protocols were approved by the Yellow Sea Fisheries Research Institute's animal care and use committee, and conducted in accordance with the Guidelines of the committee. To minimize fish suffering, all tissues were removed under MS222 anesthesia.

2.3. Isolation of On-pik3r3b full-length cDNA

According to the Nile tilapia genome sequence data in NCBI (XM_019347047), we obtained a partial mRNA sequence with a pair of primers (Table 1) using PCR. It was identified as Nile tilapia *pik3r3b* by BLAST tools. Gene specific primers (GSP, Table 1) were designed according to this sequence. Total RNA from liver, spleen and kidney of healthy Nile tilapia was extracted using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). 1 μg mixed RNA (mixture composed with equal amount of liver, spleen and kidney total RNA) was used as template to synthesize the 5′- and 3′- ready-cDNA using the SMART RACE cDNA Amplification Kit (Clontech Inc., Moutain View, CA, USA) according the manufacturer's instructions. The two ready-cDNAs were then served as template to conduct rapid amplification of cDNA ends (RACE). Both 5′-RACE and 3′-RACE were conducted using the SMART RACE cDNA Amplification Kit. The outer PCR was performed using a touchdown PCR procedure: denaturation at 94 °C for 5 min; five cycles of 94 °C for 30 s and 72 °C for 3 min; five cycles of 94 °C for 30 s, 70 °C for 30 s, and 72 °C for 3 min; 25 cycles of 94 °C for 30 s, 68 °C for 30 s, and 72 °C for 3 min; then 72 °C for 10 min as an elongation. To obtain desired products, nested-PCR was performed using the diluted products (100 times) of last step as templates. PCR products were separated on a 1.0% agarose gel and the amplified fragments of expected size were purified with a Gel Extraction Kit (CWBIO, Beijing, China). Purified products were cloned into a pMD18-T vector (TaKaRa, Dalian, China), propagated in *Escherichia Coli* TOP10 (Tiangen, Beijing, China), and sequenced.

2.4. Sequence and phylogenetic analysis

The obtained sequences were assembled using the Lasergene DNASTAR 5.0 software package [27]. The cDNA sequence data were edited and translated into amino acid sequences using DNASTAR 5.0. The NCBI open reading frame (ORF) finder (http://www.ncbi.nlm.nih.gov/gorf/orfig.cgi) and the NCBI conserved domain search (http://www.ncbi.nlm.nih.gov/structure/cdd) were employed to predict the coding sequence and analyze the conserved domain [28], respectively. The putative signal peptide was predicted using Signal 4.0 [29]. Other vertebrate Pik3r3b sequences were retrieved from NCBI GenBank. Multiple alignments of amino acid sequences were performed using Clustal W 2.0 [30]. A phylogenetic tree based on deduced amino acid difference was constructed by Neighbor-joining (NJ) method using Mega 7.0 software [31].

2.5. Quantitation analysis of On-pik3r3b expression in Nile tilapia

Quantitative (q) PCR was employed to analyze the expression patterns of *On-pik3r3b* in Nile tilapia. Using PrimeScript RT reagent Kit with gDNA Eraser (TaKaRa), total RNA from tissues collected in section 2.1 was used as templates to synthesize the first strand cDNA. A pair of gene specific primers (Table 1) was designed based on the *On-pik3r3b* ORF sequence. qPCR was performed on a 7500 ABI real-time PCR system (Applied Biosystems, Foster City, CA, USA) using the first strand cDNA as template. The PCR amplification procedure was as follows: 95 °C for 30 s, then 40 cycles of 95 °C for 5 s, and 60 °C for 34 s. A disassociation curve analysis was followed to verify the specificity of the primers. Nile tilapia *ef1-α* was used as the internal control. Triplicate reactions were performed for each sample. The Cq values reflected the concentration of cDNA in each sample, and the $2^{-\Delta\Delta Ct}$ method [32] was performed to calculate the relative mRNA expression. All data were log-transformed and differences between groups were tested using one-way ANOVA followed by Duncan multiple comparison tests using SPSS 18.0 (IBM, New York, USA). Significance was set at $p < 0.05$.

2.6. Expression and purification of recombinant On-pik3r3b

The ORF region of *On-pik3r3b* was amplified by PCR with a pair of specific primers (Table 1). The PCR product was ligated into the plasmid expression vector pEAZY-E1 (TransGen, Beijing, China) and transformed into *E. Coli* TOP10 (Tiangen). The recombinant plasmids were extracted from the positive colonies, and sequenced to confirm the correctness and direction of the insert sequence. After sequencing, the correct recombinant plasmids were transformed into *E. Coli* BL21 (DE3) (Tiangen). The transformants were inoculated in LB medium at 37 °C with shaking at 200 rpm. When the culture reached an OD_{600} of 0.5–0.6, isopropyl-b-D-thiogalactopyranoside (IPTG) was then added to the medium to a final concentration of 0.5 mM, and then incubated for additional 6 h at 20 °C. The culture without IPTG was used as a negative control. The recombinant protein was analyzed by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

The On-pik3r3b recombinant protein was purified following the improved methods described by Kuhelij et al. [33]. Briefly, after

Table 1
PCR primer sequences used in the present study.

Primer name	Primer sequences (5′ to 3′)	Primer utilizations
On-Pik3-S	CTCATCAGCCATTACAGACA	Identification of partial mRNA sequence
On-Pik3-A	TACAGAGCAGGCATAGCAC	Identification of partial mRNA sequence
Pik-GSP3-1	GAACAGCCTAAAGCCTGATCTCATA	GSP for 3′ RACE
Pik-GSP3-2	GGGTGCTATGCCTGCTCTG	GSP for 3′ RACE nest PCR
Pik-GSP5-1	GTTTCTGGCGAACTCCTTTATGA	GSP for 5′ RACE
Pik-GSP5-2	GACTCATGTCTGTAATGGCTGA	GSP for 5′ RACE nest PCR
UPM long	CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT	RACE
UMP short	CTAATACGACTCACTATAGGGC	RACE
NUP	AAGCAGTGGTATCAACGCAGAGT	RACE
Pik -RT-S	GGGGAAGAAGTTGCAGGAATAC	qPCR
Pik -RT-A	TGAAGGCTTCAATAGCGGTTC	qPCR
On-EF-S	AAGATTGGCGGTATCGGAAC	qPCR internal reference
On-EF-A	GGATGATGACCTGAGCGTTG	qPCR internal reference
Pik -Ex-S	ATGTATAACACAGTCTGGACGACCA	Recombinant expression
Pik -Ex-A	TCATCTTCGTCCAGAGGGCA	Recombinant expression

collection by centrifuge, inclusion bodies were washed twice with 20 mL of buffer A (Supplementary Table 1), and then washed twice with 20 mL of buffer B (Supplementary Table 1). Finally, inclusion bodies were dissolved in 20 mL of cold dissolution buffer (Supplementary Table 1). The purified protein was obtained using His Trap FF crude columns (GE Healthcare, Uppsala, Sweden) according to the manufacturer's protocol. Using the gradient dialysate (Supplementary Table 1), refolding of the protein was achieved by dialyzing at 4 °C for 12 h at each step. After dialysis, the supernatant was collected by centrifugation, and then stored in −80 °C. The purified protein concentration was measured using a BCA Protein Assay Kit (Beyotime, Jiangsu, China).

2.7. Western blotting

Western blotting was performed to verify the On-pik3r3b recombinant protein. After 12% SDS-PAGE, the proteins on the gel were electro-transferred onto a PVDF membrane at 300 mA for 60 min. After blocking with 5% (w/v) skim milk at room temperature for 2 h, the membrane was incubated in the mouse anti-His-tag antibody (Beyotime) diluted 1:1000 with dilution buffer at 4 °C overnight. Subsequently, the membrane was washed three times with TBST buffer (Tris-buffered saline containing 0.05% Tween-20) and incubated in horseradish peroxidase (HRP) labeled goat anti-mouse IgG antibody (Beyotime) diluted 1:1000 with dilution buffer at room temperature for 1 h. The incubation was washed three times using TBST buffer, and then the bands were stained using the DAB substrate Kit (Solarbio, Beijing, China).

2.8. Antibacterial activity analysis of On-pik3r3b recombinant protein in vitro

The Antibacterial activity of On-pik3r3b recombinant protein was tested using both Gram-positive bacteria (*S. agalactiae*) and Gram-negative bacteria (*E. Coli*). The culture condition of experimental bacteria and Gram-stainability are listed in Supplementary Table 2. In the present study, the inhibition zone method was employed to analyze the antibacterial activity of On-pik3r3b recombinant protein. The method was carried out as followed: after cultivation overnight, the bacteria suspension were diluted to 10^2-10^3 cfu/mL with PBS buffer, and were then transferred to coated plates. Oxford cup with 100 μL 2.76, 0.55 mg/mL On-pik3r3b recombinant protein, boiled On-pik3r3b recombinant protein (2.76 mg/mL) and kanamycin (5 μg/mL) were added into each hole, respectively. Boiled protein and kanamycin were used as negative and positive control, respectively. The plates were cultured at optimal bacterial growth temperature for 12 h, and the inhibition zone was observed and photographed. All the assays were conducted in triplicate. Minimum inhibitory concentration (MIC) of On-pik3r3b recombinant protein was determine by a series of concentrations (2.76, 0.55, 0.11. 0.011 mg/mL). Bacterial binding assay of *On-pik3r3b* recombinant protein was performed using *S. agalactiae* and *E. coli*. In brief, both bacteria were inoculated overnight and 1 mL bacteria were precipitate by centrifuge at 6000 g for 5 min. The bacteria was washed three times with PBS and resuspended in 500 μL PBS (OD_{600} = 1.0). Subsequently, the resuspended bacteria were mixed with 100 μL recombinant protein (experimental) or 100 μL PBS (control group) and incubated at room temperature for 30 min. After washed three times with PBS, the mixture was added with 100 SDS loading buffer and boiled for 5min. The samples were subjected to SDS-PAGE and analyzed with Western blot using anti-His-tag antibody.

3. Results

3.1. On-pik3r3b cDNA cloning and sequence analysis

A PCR product amplified by the primer On-pik3-S and On-pik3-A was 780 bp, and showed a high similarity to other known *pik3r3b* sequences. Using 5′ and 3′ RACE, two amplified products of 667 bp and 647 bp were obtained, respectively. These three sequences were assembled into the full-length *On-pik3r3b* cDNA (GenBank accession no. QAV53947.1.) with 2018 bp in length. This complete cDNA contained a 171 bp 5′ UTR, a 1422 bp ORF, and a 425 bp 3' UTR with a single typical polyadenylation signal (AATAAA) between nucleotides 1975–1980 (Fig. 1).

On-pik3r3b ORF encoded a 473 amino acid putative protein with a predicted molecular weight of 55.28 kDa and a theoretical isoelectric point of 6.25. No signal peptide was found in the deduced protein. Three Src homology 2 (SH2) domains were found in the predicted On-Pik3r3b protein. N-terminal SH2 (nSH2) domain containing four phosphotyrosine binding pockets was located on amino acid 71–179, while C-terminal SH2 (cSH2) domain containing six phosphotyrosine binding pockets was located on amino acid 365–466. The inter SH2 (iSH2) helical domain resided on amino acid 184–344. Besides, a proline-rich motif was found in N-terminal (Fig. 2).

3.2. Multiple alignment and phylogenetic analysis

Using NCBI BLASTp search in other species, we found that On-Pik3r3b displays an extremely high identity of protein sequence with other teleost. For example, it shares 98%, 92%, 89%, 87% identity with *Paralichthys olivaceus*, *Larimichthys crocea*, *Danio rerio* and *Scleropages formosus*, respectively. While the identities to amphibians and mammals are lower (81% with *Rattus norvegicus* and *Mus musculus*, 82% with *Xenopus tropicalis* and 83% *Homo sapiens* and *Callithrix jacchus*).

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        ggaattcagatagaagacttagaagtaaacagatagattaacatcgaggcatatgacaggagcacacgatgattgtttctt      81
ttttctttttttaaatacgccggtcatttttactttgtttaatagcgtttttctaatacaaagactgcctggttaagctacaagaaagca      171
ATGTATAACACAGTCTGGACGACCAAAAAAGCCGGCGAGGAAGGAGACTGGAGGGACGTGATGATGCCCTACTCCACAGAGCTGATATTT      261
 M  Y  N  T  V  W  T  T  K  K  A  G  E  E  G  D  W  R  D  V  M  M  P  Y  S  T  E  L  I  F    30
TATCTGGAAATGGACCAACCGCCAGCCCTTCCTCCAAAGCCAACCAAGCCTCAGCAGCCCTCCTCGGTCGCAATGGCAGGGGGCAGCAGC      351
 Y  L  E  M  D  Q  P  P  A  L  P  P  K  P  T  K  P  Q  Q  P  S  S  V  A  M  A  G  G  S  S    60
AATGTCGCCAAGGATGGAGGAGCAGGAGGCTCCTTACAAGAGGCCGAATGGTACTGGGGTGACATATCCAGGGAGGAGGTGAATGATAAG      441
 N  V  A  K  D  G  G  A  G  G  S  L  Q  E  A  E  W  Y  W  G  D  I  S  R  E  E  V  N  D  K    90
CTCAGAGACATGCCTGATGGGACTTTCCTGGTTCGGGACGCGTCTACTAAGATGCAAGGCGACTACACGCTAACGCTAAGGAAAGGGGGC      531
 L  R  D  M  P  D  G  T  F  L  V  R  D  A  S  T  K  M  Q  G  D  Y  T  L  T  L  R  K  G  G   120
AATAACAAGCTGATAAAGATCTACCACAGAGATGGGAAGTACGGCTTTTCTGACCCCCTGACTTTCAGCTCAGTGGTGGAGCTCATCAGC      621
 N  N  K  L  I  K  I  Y  H  R  D  G  K  Y  G  F  S  D  P  L  T  F  S  S  V  V  E  L  I  S   150
CATTACAGACATGAGTCACTGGCTCAATACAACACCAAGCTGGATGTCAAGCTCATGTACCCCATCTCCCGCTTCCAGCAGGACCAGCTG      711
 H  Y  R  H  E  S  L  A  Q  Y  N  T  K  L  D  V  K  L  M  Y  P  I  S  R  F  Q  Q  D  Q  L   180
GTGAAGGAGGACAACATCGATGCAGTGGGGAAGAAGTTGCAGGAATACCATAATCAGTACCAGGAGAAAAGCAAAGAATATGACAGACTG      801
 V  K  E  D  N  I  D  A  V  G  K  K  L  Q  E  Y  H  N  Q  Y  Q  E  K  S  K  E  Y  D  R  L   210
TATGAAGAGTACACCAAGACATCACAGGAGATCCAGATGAAGCGAACCGCTATTGAAGCCTTCAATGAAACCATCAAGATTTTCGAGGAG      891
 Y  E  E  Y  T  K  T  S  Q  E  I  Q  M  K  R  T  A  I  E  A  F  N  E  T  I  K  I  F  E  E   240
CAATGCCACACGCAGGAGCGCTACAGCAAGGACTACATTGAGCGTTTTAGGCGTGAGAGCAATGATAAAGAGATTGAGCGCATCATGATG      981
 Q  C  H  T  Q  E  R  Y  S  K  D  Y  I  E  R  F  R  R  E  S  N  D  K  E  I  E  R  I  M  M   270
AACTATGAAAAGCTTAAATCTCGCCTCGGAGAGATCCACGACAGCAAGATGCGGCTGGAGCAGGACCTAAAGACGCAAGCCATGGACAAC      1071
 N  Y  E  K  L  K  S  R  L  G  E  I  H  D  S  K  M  R  L  E  Q  D  L  K  T  Q  A  M  D  N   300
CGGGAGACGGACAAAAAGATGAACAGCCTAAAGCCTGATCTCATACAGCTCCGCAAAATCAGGGATCAGTATCTAGTCTGGCTCAATCAT      1161
 R  E  T  D  K  K  M  N  S  L  K  P  D  L  I  Q  L  R  K  I  R  D  Q  Y  L  V  W  L  N  H   330
AAAGGAGTTCGCCAGAAACGAATTAATGATTGGCTGGGAATCAAGAATGAGAACACAGACGAAGGTTACTTTGTGAGTGAGGAAGATGAG      1251
 K  G  V  R  Q  K  R  I  N  D  W  L  G  I  K  N  E  N  T  D  E  G  Y  F  V  S  E  E  D  E   360
AACTTGCCTCACTATGATGAGAAGAACTGGTTTGTGGGGGATCTGAACAGGACACAGGCAGAGGAGCTGCTCCTGGGGAAGCCAGATGGA      1341
 N  L  P  H  Y  D  E  K  N  W  F  V  G  D  L  N  R  T  Q  A  E  E  L  L  L  G  K  P  D  G   390
GCTTTTCTTATCCGAGAAAGCAGCAAAAAAGGGTGCTATGCCTGCTCTGTAGTTGTGGAAGGGGAGGTGAAGCACTGTGTCATCTACAGT      1431
 A  F  L  I  R  E  S  S  K  K  G  C  Y  A  C  S  V  V  V  E  G  E  V  K  H  C  V  I  Y  S   420
ACACCACGTGGCTTTGGCTTTGCTGAGCCATACAACCTCTACAGCAGCCTGAAGGACCTGGTGCTCCACTACCACCAGACCTCCCTGGTG      1521
 T  P  R  G  F  G  F  A  E  P  Y  N  L  Y  S  S  L  K  D  L  V  L  H  Y  H  Q  T  S  L  V   450
CAGCACAATGACTCACTCAATGTGCGCCTAGCCTACCCTGTCTACGCACAGATGCCCTCTGGACGAAGATGAaccccaaccaacgatgct      1611
 Q  H  N  D  S  L  N  V  R  L  A  Y  P  V  Y  A  Q  M  P  S  G  R  R  *                    473
aaacaactacccccagaaggacgacacattttaccgtcttcaggataaaggagaaaagggagtgttatgaagcttggtggtggtggtatt      1701
ggttgaattacaaaacaaccctgtatccagacctgatgaacatcgatacaattttaacatttgctgcaacacacacatcagccaccttgg      1791
agttatatatttttacaagaacaattttggagggcattctttctaaagactgcttgatttgcacaaggaagttttaaatgtttgtgaatg      1881
tgaaaacaagagtgaccgaaggaaggaaatgaagtaggagacgtcagagtttcaggttgatcccgctgctacctaaactccagctcagac      1971
tttaataaaccagaaacccaaaaaaaaaaaaaaaaaaaaaaaaaaa                                              2018
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Fig. 1. The full-length cDNA and deduced amino acid sequences of *On-pik3r3b*. The coding region and untranslated regions are indicated in uppercase and lowercase letters, respectively. The amino acids are shown as single letter codes below the coding region. The initiation codon is bold, and the stop codon is indicated by an asterisk. The polyadenylation signal are underlined ( ), and the Poly-A tail are marked by wave line ( ).

Multiple sequence alignment indicated that the important structure, which included the iSH2 helical domain, nSH2 domain, cSH2 domain and proline-rich motif, were highly conserved from teleost to mammal (Fig. 2).

To clarify the phylogenetic relationship between Nile tilapia Pik3r3b and that of other species, a Neighbor-joining phylogenetic tree with a bootstrap of 1000 replications was constructed. AS shown in Fig. 3, the tree showed that all of the fish had a high homology and close relationship. *O. niloticus* and the other teleost were grouped together, forming a separate clade. The mammals was grouped into a separate cluster, and then grouped into another clade with amphibians.

3.3. Tissue distribution of On-pik3r3b in healthy Nile tilapia

In order to study the tissue distribution, we measured *On-pik3r3b* mRNA expression levels in different tissues of healthy Nile tilapia using qPCR. The results revealed that *On-pik3r3b* ubiquitously expressed in all examined tissues except the skin. The highest expression level was detected in kidney. Among the other tissues, the transcription was highly abundant in the liver, spleen and gill, weakly in the heart, blood and intestine, and negligibly in the brain (Fig. 4).

3.4. Expression analysis of On-pik3r3b in response to S. agalactiae

After *S. agalactiae* stimulation, the expression of *On-pik3r3b* in immune-related tissues of tilapia was measured by qPCR. As shown in Fig. 5, significant up-regulation of *On-pik3r3b* expression was observed in the kidney at 12 h after infection. At this time point, the expression level reached the peak with 9.86-fold compared to 0 h, and then dropped gradually. In the liver and spleen, the *On-pik3r3b* expression

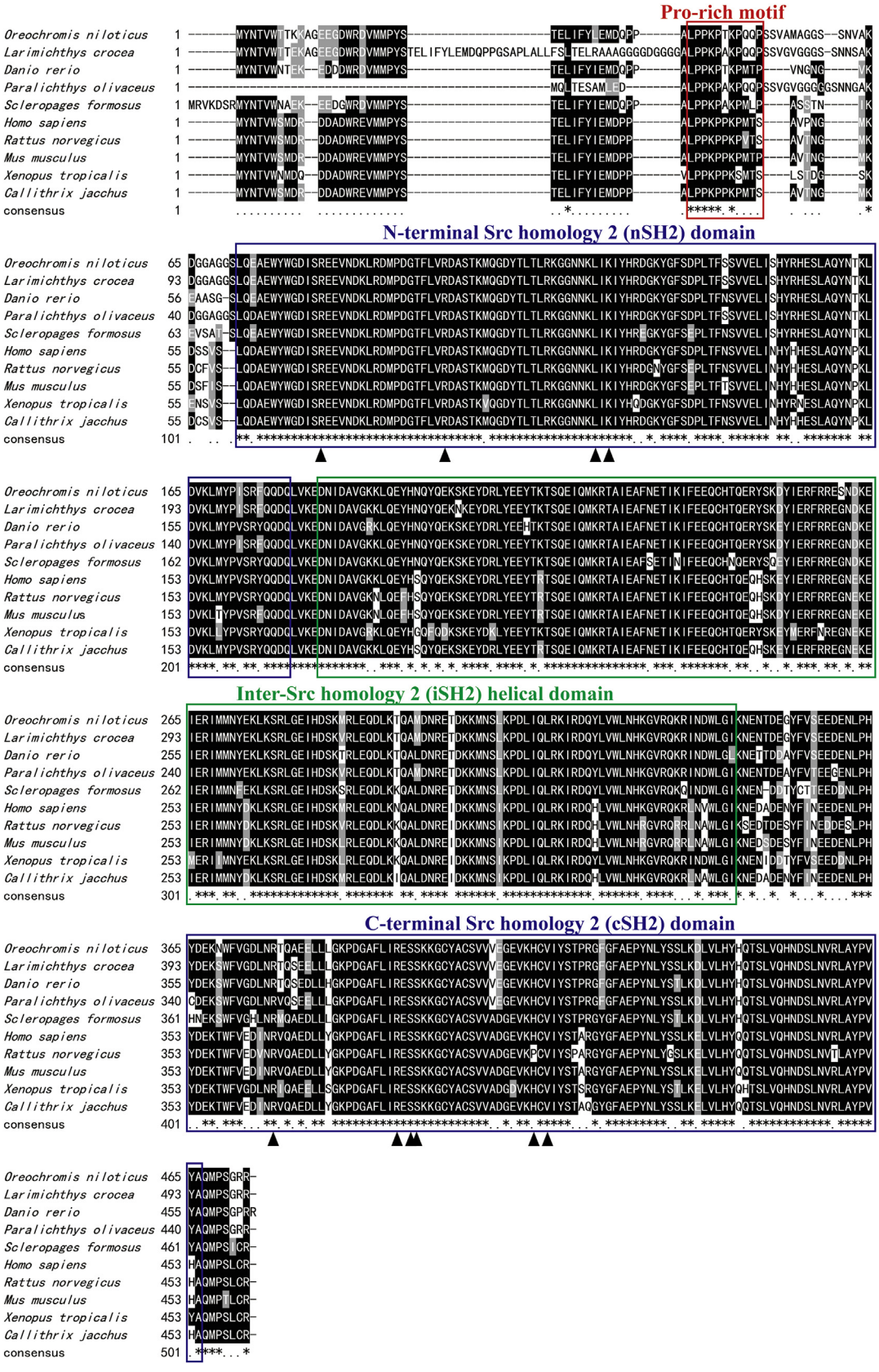

Fig. 2. Multiple alignment of the predicted On-Pik3r3b with amino acid sequences in other species. Sequences are aligned using MEGA 7.0. The identical or similar amino acids are shaded by BOXSHADE, and gaps are indicated by dashes. The N- and C-terminal SH2 domain are indicated by blue boxes. The phosphotyrosine binding pockets in SH2 domain are marked by triangles below the sequence. The inter SH2 helical domain and a proline-rich motif were indicated by green and red box, respectively. The GenBank accession numbers of protein sequences used in this section are as follows: *Danio rerio* (AAH45386.1), *Larimichthys crocea* (XP_027146471.1), *Paralichthys olivaceus* (AAS87047.1), *Scleropages formosus* (KPP72989.1), *Xenopus tropicalis* (NP_001017229), *Rattus norvegicus* (NP_071549), *Mus musculus* (NP_853616), *Homo sapiens* (Q92569) and *Callithrix jacchus* (JAB43925). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

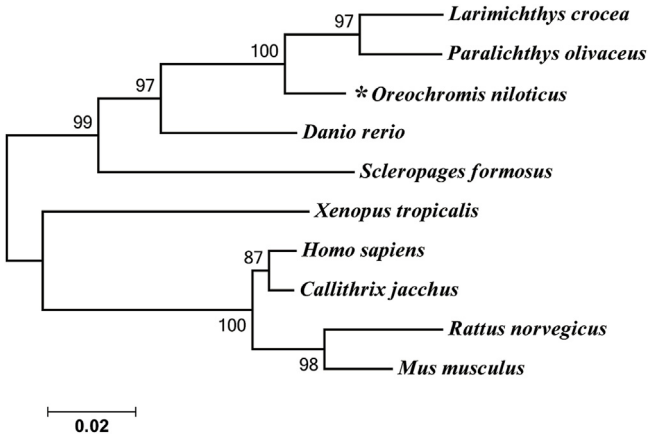

Fig. 3. Phylogenetic analysis of On-Pik3r3b protein sequences. A phylogenetic tree is constructed by neighbor-joining method using MEGA 7.0. The bootstrap values of 1000 replicates indicated next to the branches. Based on the protein sequence, the relations of different organisms are shown by dendrogram graphically. The scale bar and the branch lengths in terms of genetic distance is denoted above the tree. Protein sequences used in this analysis are as follows: *Danio rerio* (AAH45386.1), *Larimichthys crocea* (XP_027146471.1), *Paralichthys olivaceus* (AAS87047.1), *Scleropages formosus* (KPP72989.1), *Xenopus tropicalis* (NP_001017229), *Rattus norvegicus* (NP_071549), *Mus musculus* (NP_853616), *Homo sapiens* (Q92569) and *Callithrix jacchus* (JAB43925).

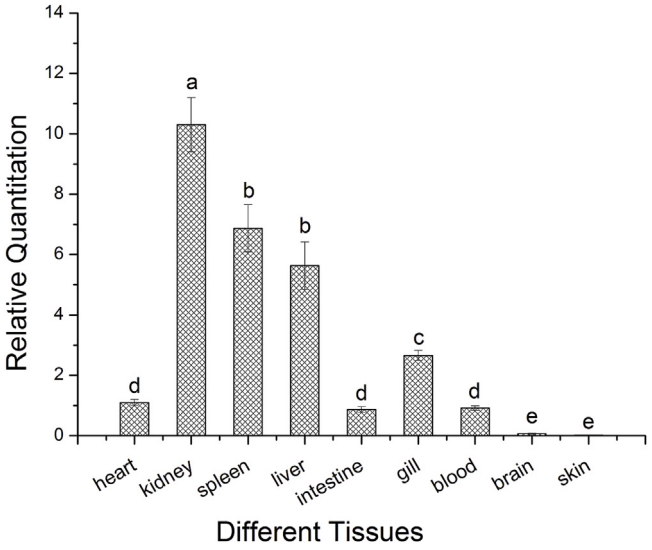

Fig. 4. The expression of *On-pik3r3b* in various tissues of healthy tilapia. Each vertical bar represents the Mean ± S.E. (n = 3). Letters above the bars indicate the Duncan grouping in SPSS. The statistical significance was set as $p < 0.05$.

was up-regulated and peaked (2.01- and 2.79-fold, respectively) at 12 h post infection, maintained a high level until 48 h and followed a decreasing at 72 h. In the gill, the expression level slightly increased from 6 h to 24 h, and peaked at 48 h. In controls, the expression of *On-pik3r3b* was not significantly changed (Fig. 5). These results showed *S. agalactiae* stimulation influenced the specific expression patterns of *On-pik3r3b* in various tissues of Nile tilapia.

3.5. Expression and purification of recombinant On-Pik3r3b

The recombinant plasmid was constructed successfully and named as pEAZY-OnPik3r3b. The transformed *E. coli* was induced and further analyzed using SDS-PAGE. After induced by IPTG, an intense band of approximately 58 kDa was observed, in accordance with the expected molecular mass (Fig. 6A). The target product was mainly detected in the pellet after sonication, suggesting that the On-Pik3r3b recombinant protein was not secreted into the supernatant but mainly existed as inclusion body (Fig. 6A). The purified recombinant protein was obtained by denaturing, renaturing and dialyzing, and then detected using SDS-PAGE (Fig. 6A).

To confirm the presence of recombinant protein, western blot analysis was performed. The result showed that a specific band reacted with antibody, indicating that On-Pik3r3b recombinant protein was exactly expressed (Fig. 6B).

3.6. Antibacterial assay and bacterial binding assay

The antibacterial activity of On-Pik3r3b recombinant protein against *S. agalactiae* and *E. coli* was examined. As shown in Fig. 7, the recombinant protein showed significant antibacterial activities against *S. agalactiae* and *E. coli*. In addition, the minimum inhibitory concentration (MIC) of On-Pik3r3b recombinant protein was determined and the MIC for both bacteria was ~0.11 mg/mL (Supplementary Fig. 1). Bacterial binding assay revealed that On-Pik3r3b recombinant protein could bind to *E. coli* and *S. agalactiae* (Supplementary Fig. 2).

4. Discussion

As the most important economical fishes in China, the development of Nile tilapia industry has been more and more rapidly. However, Nile tilapia was frequently subjected to disease in the past decades, which became an obstacle to its aquaculture. Therefore, understanding the immune system and immune response against infection better is an effective method for enhancing the immunity of cultured fish [34–36].

In the present study, we isolated and characterized the full-length cDNA of *pik3r3b* from Nile tilapia. Three SH2 domains were discovered in the deduced On-Pik3r3b protein sequence. These conserved domains displayed a very high sequence identity with other species. The alignment of amino acids sequences demonstrated that Pik3r3b was highly conserved in teleost, amphibians and mammals, which indicate similar functions of Pik3r3b among these species.

qPCR analysis revealed that *On-pik3r3b* mRNA was widely expressed in various tissues of healthy Nile tilapia. It is well accepted that PI3K plays an important role in immunity [10,11]. Notably, in the present study, the transcripts were mainly detected in several immune-related tissues, which included liver, kidney, spleen and gill. Given these findings, we speculated that *On-pik3r3b* play some roles in Nile tilapia immune response.

S. agalactiae was first reported as an opportunistic agent with broad host range, including mammals and fish [37]. Among fish, it could infect various saltwater and fresh water fish with significant morbidity and mass mortality. Particularly, Nile tilapia is very sensitive to *S. agalactiae*. After challenging with this kind of bacteria, the highest mortality coefficients occurred on the first day, and the accumulated mortality was 83.2% [38]. Therefore, to confirm our speculation, we measured the expression levels of *On-pik3r3b* in immune-related tissues after *S. agalactiae* infection by qPCR. In teleost, kidney, liver, spleen and gill are important immune-related organs, and play key roles in both innate and adaptive immunity. In our work, obvious up-regulating of the *On-pik3r3b* transcript levels was observed in these four organs after challenge. In the kidney, liver and spleen, the expression levels of *On-pik3r3b* were significantly up-regulated (9.86-fold, 2.01- and 2.79-fold compared to the controls, respectively) at 12 h after infection, while in the gill, the expression level began to increase at 6 h after infection. The fast and sensitive response to the pathogen supported evidence for *On-pik3r3b* involved in immune response of Nile tilapia.

In order to verify the immune function of *On-pik3r3b*, antibacterial assay was carried out. The recombinant On-Pik3r3b protein exhibited significant antibacterial activity against Gram-positive bacteria *S. agalactiae*, and medium antibacterial activity against Gram-negative

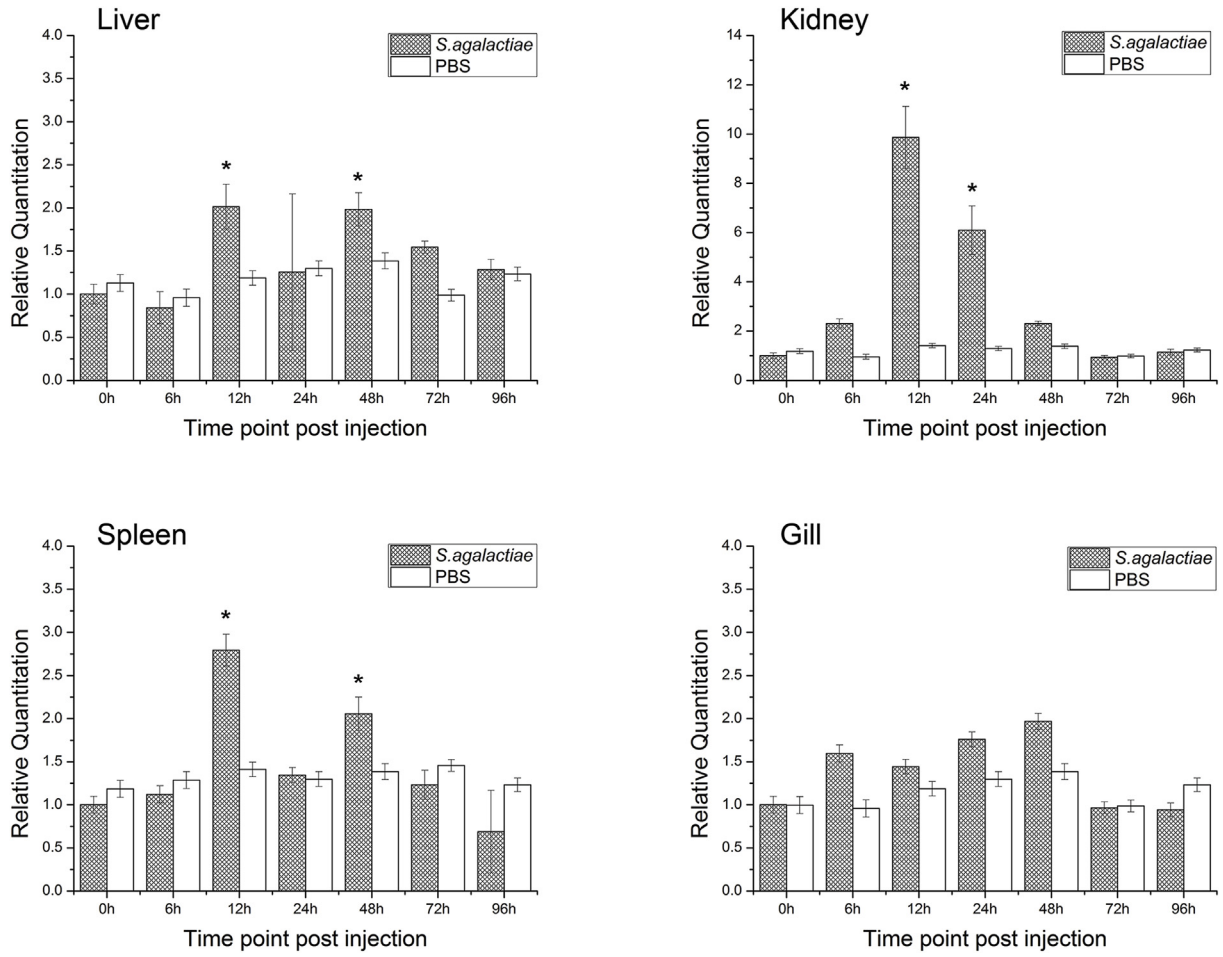

Fig. 5. Expression levels of *On-pik3r3b* in the liver, kidney, spleen and gill at different time points after *S. agalactiae* infection. Each vertical bar represents the Mean ± S.E. (n = 3). The asterisks above the bars indicate significant differences ($p < 0.05$) compared with values of the control (0 h).

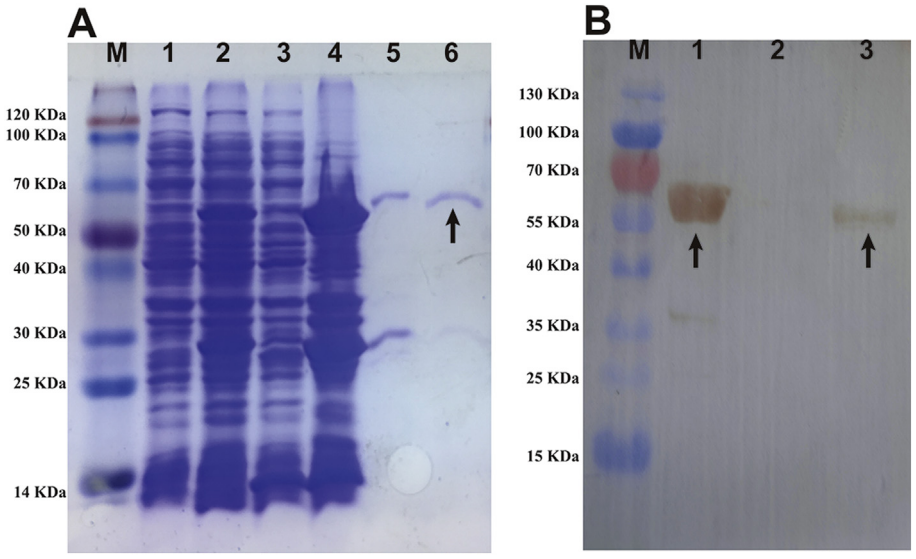

Fig. 6. SDS-PAGE (A) and Western blotting (B) analysis of On-Pik3r3b recombinant protein. (A) Lane M: protein marker; lane 1: *E. coli* without IPTG induction as negative control; lane 2: IPTG-induced *E. coli*; lane 3: the supernatant of IPTG-induced *E. coli* after the sonication; lane 4: the pellet of IPTG-induced *E. coli* after the sonication; lane 5: dissolved inclusion bodies; lane 6: purified On-Pik3r3b recombinant protein. **(B)** Lane M: protein marker; lane 1: the pellet of IPTG-induced *E. coli* after the sonication; lane 2: the supernatant of IPTG-induced *E. coli* after the sonication; lane 3: purified On-Pik3r3b recombinant protein.

bacteria *E. coli*, which required high protein concentration. Furthermore, we found that the antibacterial activity *in vitro* was not stable (data not shown). There were several reports about antibacterial activity of regulatory proteins in fish, including MDA5, LGP2 and MAVS in tilapia, complement regulatory protein in channel catfish, and Dctn5 in tongue sole [39–41]. Moreover, by performing the bacterial binding assay, we suggested that one explanation for the antibacterial activity of On-Pik3r3b was probably realized by its binding to bacteria cells and in turn inhibiting their growth, although the detailed mechanism was yet unknown. However, more evidences are needed to be proved, and further investigation is required to understand the molecular mechanism.

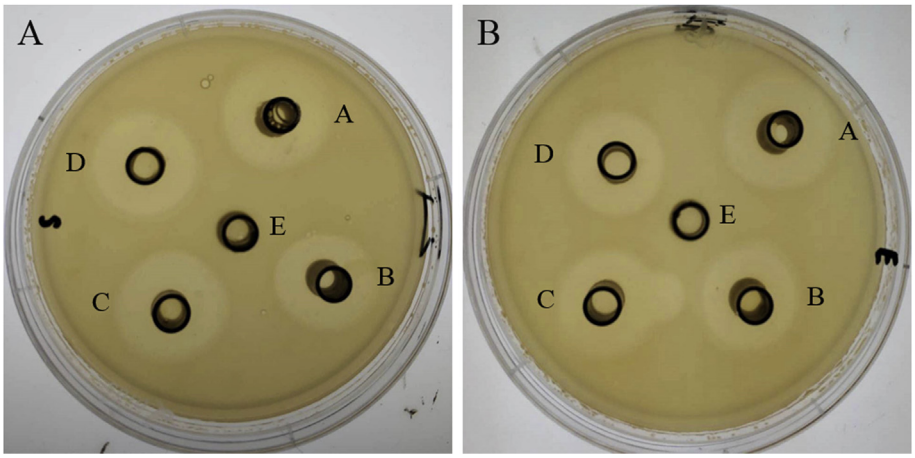

Fig. 7. Antibacterial activity assay of On-Pik3r3b recombinant protein against *S. agalactiae* (A) and *E. coli* (B) *in vitro*. A: 5 μg/mL kanamycin as positive control; B and C: 2.76 mg/mL On-pik3r3b recombinant protein; D: 0.55 mg/mL On-pik3r3b recombinant protein; E: Boiled On-pik3r3b recombinant protein (2.76 mg/mL) as negative control.

In summary, the full-length cDNA of *pik3r3b* was isolated and identified from Nile tilapia. *On-pik3r3b* transcripts were mainly expressed in some immune-related tissues and up-regulated in these tissues after pathogenic infection, which indicated its potential role in immune response of Nile tilapia. Furthermore, the purified recombinant On-Pik3r3b protein showed antibacterial activity *in vitro*. Our findings provide a new insight into the immune function of *pik3r3b* in teleost.

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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.2019.01.006.

Conflicts of interest

The authors have declared that no competing financial interests exist.

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