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The warm temperature acclimation protein *Wap65* as an immune response gene: Its duplicates are differentially regulated by temperature and bacterial infections

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Abstract

The warm temperature acclimation related 65 kDa protein (*Wap65*) in teleost fish shares high structural similarities with mammalian hemopexins. Recent studies using microarray analysis indicated that this temperature acclimation protein may also be involved in immune responses. To provide evidence of its potential involvement in immune responses after bacterial infections, we have identified and characterized two types of *Wap65* genes in channel catfish, referred to as *Wap65*-1 and *Wap65*-2, respectively. While *Wap65*-1 and *Wap65*-2 are both structurally similar to the mammalian hemopexins, they exhibit highly differential patterns of spatial expression. *Wap65*-1 was expressed in a wide range of tissues, whereas *Wap65*-2 was only expressed in the liver. Their regulation with warm temperature and bacterial infections was also highly different: *Wap65*-1 was constitutively expressed, whereas *Wap65*-2 was highly regulated by both warm temperature and bacterial infections, and warm temperature and bacterial infections appeared to synergistically induce the expression of *Wap65*-2. The great contrast of expression patterns and regulation of the two catfish *Wap65* genes suggested both neofunctionalization and partitioning of their functions. Phylogenetic analysis indicated that the duplicated catfish *Wap65* genes were evolved not only from whole genome duplication, but also from tandem, intrachromosomal gene duplications. Taken together, the results of this study suggest that *Wap65* genes are not only important for its classical role as a warm temperature acclimation protein, but more importantly, may also function as an immune response protein.

Keywords: Fish; Catfish; Hemopexin; *Wap65*; Immunity; Infection; Temperature; Genome

1. Introduction

Water temperature is one of the most important environmental factors that affect the physiology and behavior in poikilothermic animal including fish (Kikuchi et al., 1995; Hirayama et al., 2004). Physiological responses that compensate for temperature changes are often referred to as a acclamatory response during seasonal temperature fluctuations (Hazel and Prosser, 1974; Johnston and Temple, 2002). The markedly significant acclimation response were observed in eurythermal fish such as carp and catfish, which can survive in a wide range of temperature ranging from near 0 °C to over 30 °C (Kikuchi et al., 1997; Hirayama et al., 2003). Adaptation of such a range of temperature requires significant physiological and genome expression changes. Changes of a whole set of genes have been recently identified through the use of microarray technology (Ju et al., 2002; Gracey et al., 2004; Buckley et al., 2006). In particular, the expression level of certain genes such as myosin,

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lactate dehydrogenase, Δ^9-desaturase and the warm temperature acclimation-related 65 kDa protein gene (*Wap65*), have been reported to be regulated to adapt to the temperature changes (Segal and Crawford, 1994; Tiku et al., 1996; Kikuchi et al., 1997; Watabe, 2002; Hirayama et al., 2003).

Wap65 was initially identified in goldfish, and since then has been identified from a number of fish including carp, medaka and pufferfish (Kikuchi et al., 1995; Kinoshita et al., 2001; Hirayama et al., 2003, 2004). The teleost *Wap65* is most homologous to the mammalian hemopexin, but orthologies have not been established. Hemopexin is a mammalian plasma glycoprotein synthesized in the liver, functioning as a scavenger of free heme with high affinities (Altruda et al., 1985; Nikkila et al., 1991; Morgan et al., 1993; Tolosano and Altruda, 2002). Much studies of *Wap65* to date has been focused on its expression during temperature acclimation (Watabe et al., 1993; Kikuchi et al., 1995, 1997, 1998). Expression of *Wap65* in goldfish was drastically induced (40-fold) upon water temperature shift from 10 to 30 °C (Kikuchi et al., 1997). Similar results were obtained with studies in common carp (Kinoshita et al., 2001). In consideration of its function as a heme scavenger, several studies have explored the potential of *Wap65* involvement in immune response, as iron is one of the pivotal elements during bacterial infections. In goldfish, *Wap65* was reported to respond to bacterial lipopolysaccharides (LPS) and its expression was induced two-fold after exposure of goldfish to LPS (Kikuchi et al., 1997). However, exposure of medaka to LPS did not induce the expression of *Wap65* (Hirayama et al., 2004). Thus, the involvement of *Wap65* in immune response is uncertain, and the literature on *Wap65* during bacterial infection has been lacking. Recently, through the use of an oligo-based microarray, we have identified *Wap65* gene is among the upregulated genes after bacterial infection with *Edwardsiella ictaluri* (Peatman et al., 2007,2008). Such results demand further characterization of *Wap65* genes, their expression, and their potential roles in immune responses. In this study, we identified two *Wap65* genes, cloned and sequenced their cDNAs, cloned and sequenced their corresponding genes, characterized the gene structure and organization and conducted detailed analysis of *Wap65* genes under different temperature regime, and with bacterial infections. Here we report that the two channel catfish Wap65 genes are differentially expressed in their spatial distribution, and are differentially regulated with warm temperature and bacterial infections.

2. Materials and methods

2.1. Fish and challenge experiments

Channel Catfish (Kansas random strain) fingerlings were grown out for 1 year with approximately 14 cm of body length (average weight 45.4 g) were acclimated at 20 °C for 5 weeks before artificial bacterial challenges and warm water temperature treatment. To determine catfish *Wap65* genes expression in various tissues, 12 tissues including blood, brain, gill, heart, head kidney, intestine, liver, muscle, spleen, stomach, skin and trunk kidney were collected. For temperature treatment, fish were divided into two groups and gradually temperature was slowly raised in a period of 48 h from 20 to 24 or 28 °C, respectively. Before temperature treatment, a total of 12 fish were sampled from 20 °C aquarium as control. Challenge experiments were conducted on the fish in 24 and 28 °C aquariums following established protocols for ESC challenges with modification (Dunham et al., 1993; Baoprasertkul et al., 2005). The control groups were kept in 24 and 28 °C aquaria without bacterial challenge. The liver samples were collected on both challenged group and control group on three time points after the challenge: 1, 3 and 7 days post-challenge. The tissue samples were flash frozen in liquid nitrogen immediately after collection of samples, and stored at −80 °C until RNA extraction.

2.2. Tissue collection and RNA preparation

To determine *Wap65* genes expression in various healthy catfish tissues, samples of each tissue from 12 fish were pooled for RNA isolation. Similarly, the liver tissues of 12 fish at each time point in each group of the ESC challenge experiment were pooled for RNA isolation. TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was used for RNA extraction and the concentration of total RNA was quantified by Ultrospec 1100 pro (Amersham Biosciences, Piscataway, NJ, USA). Total RNAs were subsequently used for the analysis of gene expression.

2.3. Identification and sequencing of the catfish Wap65 cDNAs

Partial cDNA sequence of the channel catfish *Wap65* genes was initially identified from EST sequences (Li et al., 2007) using BLAST similarity comparisons. Complete cDNA sequences of *Wap65*-2 were obtained by sequencing of the existing EST clone. For *Wap65*-1, 3′-Rapid amplification of cDNA ends (3′-RACE) was conducted to obtain the complete cDNA sequences.

3′-RACE was conducted with Smart RACE cDNA amplification Kit (Takara, Santa Ana, CA, USA) following manufacturer's instructions, using gene-specific primers as listed in Table 1. The resulting cDNA fragment from 3′-RACE was cloned into a pGEM-T easy cloning kit vector (Promega, Milwaukee, WI, USA) and sequenced using an ABI PRISM 3130XL automated sequencer (Applied Biosystems, Foster City, CA, USA).

2.4. BAC library screening and BAC DNA isolation

Overgo probes were designed from the cDNA sequences for the screening of *Wap65* genes from the CHORI-212 BAC library (Wang et al., 2007). Overgo hybridization was conducted according to methods of a web protocol (http://www.tree.caltech.edu) with modifications (Baoprasertkul et al., 2005; Bao et al., 2006a,b). Briefly, overgo primers were purchased from Sigma Genosys (Woodlands, TX, USA), and labeled with ^{32}P-dATP and ^{32}P-dCTP (Amersham) in 10 mg/ml bovine serum albumin (BSA), overgo labeling reaction 1× buffer (Ross et al., 1999) and incubated for 1 h at room temperature with Klenow polymerase

Table 1
Primers and their sequences used in this study

Primers	Primer sequence (5′ to 3′)	Utilizations
SP6	ATTTAGGTGACACTATAG	Sequencing
T7	TAATACGACTCACTATAGGG	Sequencing
Wap65-1-Ova	AGGCCATGAAGTGGTTCTCTGTAG	Overgo probes
Wap65-1-Ovb	AGCATCCAAATGGTCCGGAATACC	Overgo probes
Wap65-1-f1	GGTAATTGGGGCTAGTGACC	For *Wap65*-1 primer walking sequencing
Wap65-1-r1	GGTCACTAGCCCCAATTACC	For *Wap65*-1 primer walking sequencing
Wap65-1-f2	TTTCTCCAAGTTCGACCCCA	For *Wap65*-1 primer walking sequencing
Wap65-1-r2	TGGGGTCGAACTTGGAGAAA	For *Wap65*-1 primer walking sequencing
Wap65-1-f3	ACATGTCGATGCTGCCTTCG	For *Wap65*-1 primer walking sequencing
Wap65-1-r3	CGAAGGCAGCATCGACATGT	For *Wap65*-1 primer walking sequencing
Wap65-1-f4	CTGTGGAGCAGATGGTGTGT	For *Wap65*-1 primer walking sequencing
Wap65-1-r4	ACACACCATCTGCTCCACAG	For *Wap65*-1 primer walking sequencing
Wap65-1-f5	TGTAGCCGGTGGTCTCACTT	For *Wap65*-1 primer walking sequencing
Wap65-1-r5	AAGTGAGACCACCGGCTACA	For *Wap65*-1 primer walking sequencing
Wap65-2-f1	GGAAAGGTATGCAAATGTAAGA	For *Wap65*-2 primer walking sequencing
Wap65-2-r1	GAGTCCCATTACAATGCCA	For *Wap65*-2 primer walking sequencing
Wap65-2-f2	GACAGGAATCAAACCCCTGA	For *Wap65*-2 primer walking sequencing
Wap65-2-r2	TCTGGGTTAAAATTCATTTGTT	For *Wap65*-2 primer walking sequencing
Wap65-2-f3	ACTTGCAACCAAAGGTTAAA	For *Wap65*-2 primer walking sequencing
Wap65-2-r3	GGAAATCAGGAAATTTATTCAA	For *Wap65*-2 primer walking sequencing
Wap65-2-f4	TCCTTCCATGATCTTACAATG	For *Wap65*-2 primer walking sequencing
Wap65-2-r4	GTGTGAGTTGACACAAAGCA	For *Wap65*-2 primer walking sequencing
Wap65-2-f5	GTGACAGAAGTGCTGGCTG	For *Wap65*-2 primer walking sequencing
Wap65-2-r5	TGGAGTTTAATGCGTTTATGA	For *Wap65*-2 primer walking sequencing
Wap65-1 probe f	GTGGAGCAGATGGTGTGTCAGT	Probe for Southern blot
Wap65-1 probe r	TACAGACAGCGCTGACAGAGAC	Probe for Southern blot
Wap65-2 probe f	AGAGCCCTGCTACATTCATTAC	Probe for Southern blot
Wap65-2 probe r	GCACAGGTGGTTCTCAATCTTT	Probe for Southern blot
Wap65-1RT f	CACTATGAGAGCCCTGCTACA	RT-PCR of *Wap65*-1
Wap65-1RT r	ACAAAGGACGAGAGGTAGGAA	RT-PCR of *Wap65*-1
Wap65-2RT f	AATCGGCAGCACATTACACA	RT-PCR of *Wap65*-2
Wap65-2RT r	ATTCCCCACCAACAAACACT	RT-PCR of *Wap65*-2
β-Actin f	AGAGAGAAATTGTCCGTGACATC	RT-PCR internal contol
β-Actin r	CTCCGATCCAGACAGAGTATTG	RT-PCR internal contol
Wap65-1qRT f	CAGATGGTGTGTCAGTAGTGATTG	Quantitive RT-PCR of *Wap65*-1
Wap65-1qRT r	CAGAAGATGGAAGGTGGGTG	Quantitive RT-PCR of *Wap65*-1
Wap65-2qRT f	CTTGGTATTCTTTTGTGCGTG	Quantitive RT-PCR of *Wap65*-2
Wap65-2qRT r	AGAGTGCGGTTCCTTTCTCG	Quantitive RT-PCR of *Wap65*-2
18s RNA f	TGCGCTTAATTTGACTCAACAC	Quantitive RT-PCR internal contol
18s RNA r	CGATCGAGACTCACTAACATCG	Quantitive RT-PCR internal contol

(Invitrogen). Sephadex G50 spin columns were used to remove unincorporated nucleotides. Probes were denatured at 95 °C for 10 min and added into hybridization tubes that had been under pre-hybridization for 2 h with the hybridization solution. High-density BAC filters containing the whole CHORI-212 BAC library were purchased from Children's Hospital of the Oakland Research Institute (CHORI, Oakland, CA, USA). The filters were hybridized at 54 °C for 18 h in 50 ml hybridization solution (1% BSA, 1 mM EDTA at pH 8.0, 7% sodium dodecyl sulfate, 0.5 M sodium phosphate, pH 7.2). The filters were washed at room temperature and exposed to X-ray film at −80 °C for 24 h. Initially, overgo probes were used for both *Wap65* genes. However, the overgo probes of *Wap65*-2 failed to allow identification of positive BAC clones. Therefore, cDNA probes were used for the identification of BAC clones containing *Wap65*-2 gene. The plasmid IpLvr00390 harboring *Wap65*-2 cDNA was used as template for the amplification of the cDNA insert using PCR with SP_6 and T_7 primers (Table 1). The PCR amplified segment of the *Wap65*-2 cDNA was gel-purified, and labeled using random primer labeling method using a kit from Roche Diagnostics (Indianapolis, IN, USA) following manufacturer's protocols.

Positive clones were identified according to the clone distribution pattern from CHORI. Positive clones were picked and cultured in 2× YT medium. After overnight culture, BAC DNA was isolated using R.E.A.L. Prep 96 BAC DNA isolation kit (Qiagen, Valencia, CA, USA) as we previously described (Xu et al., 2006, 2007).

2.5. Genomic DNA sequencing

Positive BAC clones were sequenced directly using the primer-walking method. The sequencing primers are listed in Table 1. Ten picomoles of each sequencing primer were used for sequencing reactions. BAC sequencing was performed in a 10 μl reaction using the BigDye Terminator v3.0 Ready Reac-

tion kit (Applied Biosystems, Foster City, CA, USA) following manufacturer's instructions. Sequencing reaction products were analyzed on an ABI PRISM 3130XL automated sequencer.

Sequences were analyzed by using DNASTAR software packages (Lasergene, Madison, WI, USA). BLAST searches were conducted to determine gene identities. The MegAlign program of the DNASTAR package was used for sequence alignments using ClustalW (Serapion et al., 2004). NCBI's Spidey program was used for alignment of genomic and cDNA sequences to identify splicing junctions.

2.6. Southern blot analysis

Southern blot analysis was conducted to determine genomic copy numbers of the *Wap65* genes. Genomic DNA was isolated from three individuals of channel catfish using method as previously described (Liu et al., 1998). A total of 10 μg genomic DNA was completely digested with restriction endonuclease *Eco*R I, *Hin*d III and *Pst* I separately (New England Biolabs, Beverly, MA, USA). The digested DNA samples were electrophoresed on 0.8% agarose gel at 20 V for 24 h. The DNA was transferred to a piece of Immobilon nylon membrane (Millipore, Bedford, MA, USA) by capillary transfer with 0.4 M NaOH overnight. DNA was fixed to the membrane by UV crosslinking using a UV Stratalinker 2400 (Stratagene, La Jolla, CA, USA) with the auto crosslink function. The membrane was washed in 0.5% SDS (w/v) at 65 °C for 15 min and then pre-hybridized in 50% formamide, 5× SSC, 0.1% SDS (w/v), 5× Denhardt's and 100 μg/ml sonicated and denatured Atlantic salmon sperm DNA (100 μg/ml) overnight. Hybridization was conducted overnight at 42 °C in the same solution with appropriate probes added. Hybridization probes were obtained by PCR amplification of a portion of the cDNAs using primers listed in Table 1. The probe was labeled using the random primer labeling method (Sambrook et al., 1989) with a labeling kit from Roche Diagnostics and ^{32}P-dCTP. The hybridized membrane was washed three times in 0.2× SSC for 15 min each. The membranes were then wrapped in Saran wrap and exposed to Kodak BioMax MS film for autoradiography.

2.7. Phylogenetic analysis

The relevant sequences of *Wap65* genes were retrieved from GenBank for multiple amino acid sequence alignments using ClustalW. Phylogenetic trees were drawn by the neighbor joining method (Saitou and Nei, 1987) within the Molecular Evolutionary Genetics Analysis (MEGA 3.1) package (Kumar et al., 2004). Data were analyzed using Poisson correction, and gaps were removed by complete deletion. The topological stability of the trees was evaluated by 10,000 bootstrapping replications.

2.8. RT-PCR analysis

RT-PCR reactions were conducted by using the SuperScript™ First-strand Synthesis System (Invitrogen). RT reactions were conducted in 20 μl reactions containing the following: 1 μg DNase-I-treated RNA, 1 μl (40 uM) oligo dT primers, 1 μl (10 mM) dNTP mixture, 1 μl RNase inhibitor, 1× RT reaction buffer and 50 U superscript™ II reverse transcriptase. Detailed procedures followed the manufacturer's protocols. After RT reaction, 1 μl of the RT products was used as a template for PCR using JumpStart *Taq* polymerase (Sigma, St. Louis, MO, USA). The reactions included the gene-specific primers and also the primers for β-actin serving as an internal control (Table 1). The reactions were completed in a thermocycler with the following thermoprofiles: denaturation at 95 °C for 3 min followed by 30 cycles of 94 °C for 30 s, 53 °C for 30 s and 72 °C for 1 min. Upon completion of PCR, the reaction was incubated at 72 °C for an additional 10 min. The RT-PCR products were analyzed by electrophoresis on a 1.0% agarose gel and documented with a gel documentation system (Nucleotech Corp., San Mateo, CA, USA).

2.9. Quantitative real time PCR analysis

Quantitative real time RT-PCR (qRT-PCR) analysis was conducted using a LightCycler (Roche Applied Science). Concentration and quality of total RNA was determined by spectrophotometry. One-step real time RT-PCR was carried out in a LightCycler using a Fast Start RNA Master SYBR Green I Reagents Kit (Roche Applied Science) following manufacturer's instructions. The 18S ribosomal RNA gene was used for normalization of expression levels. The triplicate fluorescence intensities of the control and treatment products for each gene, as measured by crossing point (Ct) values, were compared and converted to fold differences by the relative quantification method using the Relative Expression Software Tool 384 v. 1 (REST). PCR specificity was assessed by melting curve analysis.

3. Results

3.1. Identification and sequence of Wap65 cDNAs

A total of 26 partial cDNA sequences similar to *Wap65* genes were initially identified from the channel catfish ESTs (Li et al., 2007). Cluster analysis indicated that these ESTs belong to two clusters. An EST clone containing the full length cDNAs of *Wap65*-2 of channel catfish was directly sequenced to obtain the full length cDNA sequences. For *Wap65*-1, the existing EST sequences were used to design RACE primers to obtain the 3′-portion of the cDNA. The RACE products were cloned and sequenced.

Wap65-1 cDNA encode a protein of 478 amino acids and included a 5′-UTR of 55 bp and a 3′-UTR of 336 bp. Similarly, *Wap65*-2 cDNA encode a protein of 443 amino acids and included a 5′-UTR of 21 bp and a 3′-UTR of 78 bp. A typical polyadenylation signal sequence AATAAA existed in 7 bp upstream of the poly A tail in *Wap65*-1 and 11 bp upstream of the poly A tail in *Wap65*-2. Both *Wap65*-1 and *Wap65*-2 cDNA sequences have been deposited to GenBank with the accession number of EU030383 and EU030384.

Analysis of the deduced amino acid sequences by multiple sequence alignments indicated that the *Wap65* genes are moderately conserved through evolution (Fig. 1). At the amino acid

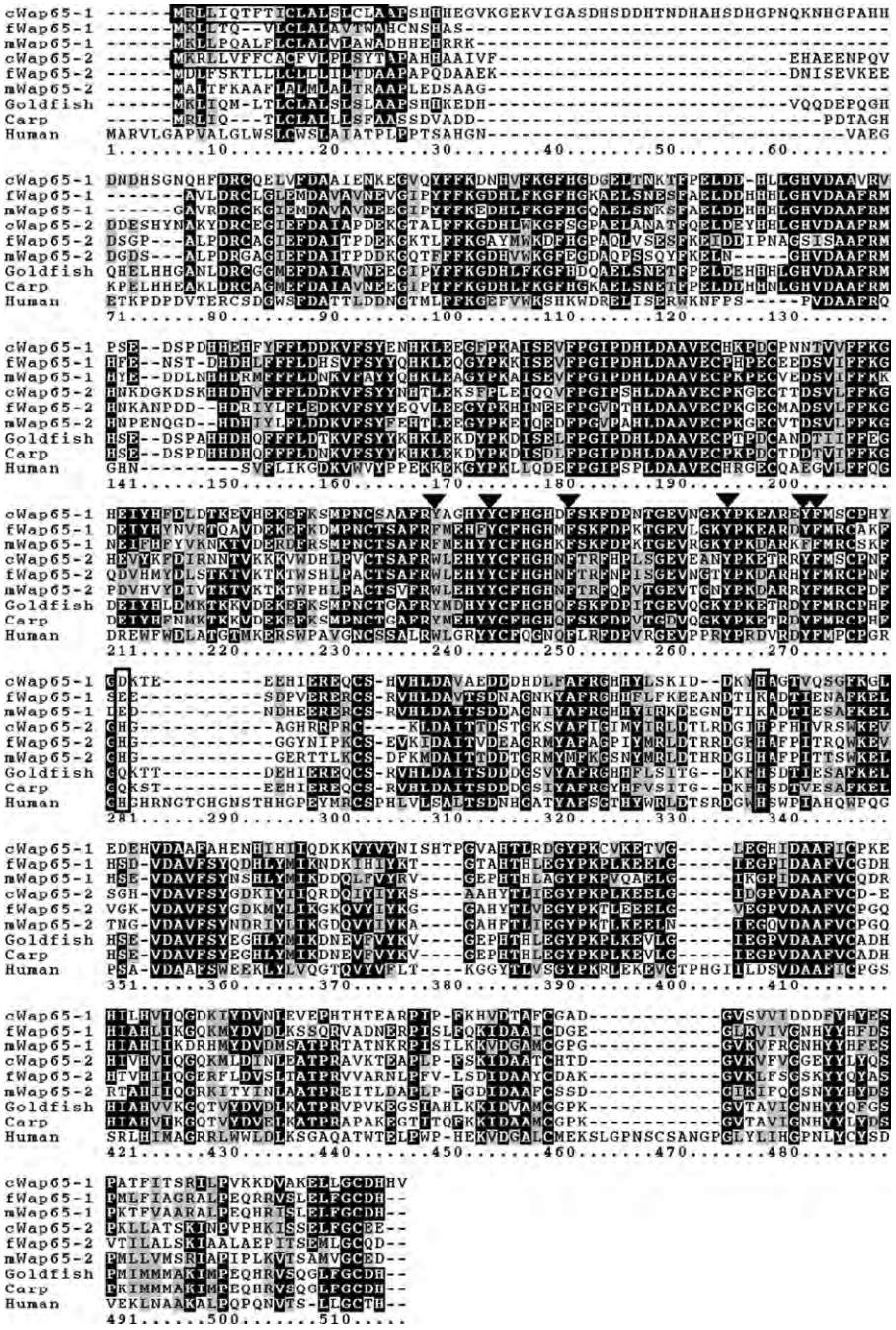

Fig. 1. Comparison of amino acid sequences of the channel catfish *Wap65* genes with *Wap65* genes from medaka, *Takifugu*, goldfish and carp and human hemopexin gene. The signal peptides of two *Wap65* genes are indicated by horizontal open boxes. The conserved histidine residues are indicated by vertical open boxes. Triangles indicate the conserved aromatic residues in the heme-binding pocket.

level, *Wap65*-2 genes are more evolutionarily conserved than *Wap65*-1 genes (Table 2). For instance, the catfish *Wap65*-2 is 66.3% similar to that of *Takifugu*, but *Wap65*-1 of catfish is only 56.7% similar to that of carp, although catfish is much more phylogenetically related to carp than to *Takifugu*. At present, only one *Wap65* gene has been identified from carp. The *Wap65*-1 showed 40.3–56.9% identity with those *Wap65* protein sequences of medaka, *Takifugu*, goldfish and carp and 30.1% identity with human hemopexin protein. The *Wap65*-2 showed 44.5–66.3% identity with that of medaka, *Takifugu*, goldfish and carp and 35.1% identity with human hemopexin protein (Table 2). The heme-binding pocket is well conserved among all *Wap65* genes (Fig. 1), suggesting conservation of their function as heme scavengers. Cysteine residues are essential to the structural integrity of hemopexin (Nakaniwa et al., 2005), which were conserved in catfish *Wap65*s, as well as other fish species. Histidine residues, which were previously reported as necessary for the high affinity of mature hemopexin for heme (Paoli et al., 1999), were conserved on the 250th and 291st amino acid in *Wap65*-2; however, the residue corresponding to

Table 2
Pairwise similarities of selected *Wap65* proteins

Catfish-1	Fugu-1	Medaka-1	Catfish-2	Fugu-2	Medaka-2	Carp	Goldfish	Human	
	52.7	52.8	43.1	40.3	44.5	56.7	56.9	30.1	Catfish-1
		71.3	47.3	44.5	50.4	67.5	66.4	32.2	Fugu-1
			45.9	45.6	49.5	65.8	66.3	32.1	Medaka-1
				66.3	59.0	46.6	43.1	37.0	Catfish-2
					57.0	46.7	44.8	38.0	Fugu-2
						51.7	49.7	35.1	Medaka-2
							86.8	30.8	Carp
								31.2	Goldfish
									Human

the 250th residue (histidine) in *Wap65*-2 was replaced by aspartic acid in *Wap65*-1. Seven conserved aromatic residues, which can help define the heme pocket, were identified through crystal structure analysis of rabbit hemopexin (Paoli et al., 1999). The six conserved aromatic residues were also identified in the hemo binding site of *Wap65*-2 gene. In *Wap65*-1, only the 231st residue was replace by Tyr, all the other five aromatic residues were retained. The predicted signal peptides, retaining 19 amino acids, were identified in both *Wap65* genes. The signal peptides have similar length to those of *Wap65* in other teleost fish.

3.2. Phylogenetic analysis of Wap65 genes

In order to analyze the catfish *Wap65* genes in the larger context of vertebrate *Wap65* genes, phylogentic analysis was conducted (Fig. 2). The phylogenetic tree clearly shows that *Wap65* genes are duplicated in the teleost lineage as *Wap65*-1 genes from *Takifugu*, *Tetraodon*, zebrafish, medaka formed a distinct clade, whereas *Wap65*-2 genes formed a separate clade. In contrast, there are only one hemopexin gene in mammalian species (Fig. 2).

3.3. Characterization of the Wap65 genes

BAC clones containing *Wap65*-1 and *Wap65*-2 genes were identified by hybridizations of a high-density BAC filter. A total of 2 BAC clones were identified to be positive to *Wap65*-1 and 4 clones were identified to be positive to *Wap65*-2 probes (Table 3). The BAC clone 153_B20 and 037_F12 was used for sequencing of *Wap65*-1 and *Wap65*-2, respectively. The entire coding region plus portions of upstream and downstream regions of *Wap65*-1 and *Wap65*-2 genes were sequenced, generating 5598 bp for *Wap65*-1 gene and 9413 bp for *Wap65*-2 gene. The gene sequences have been deposited to GenBank with the accession numbers of EU030385 and EU030386.

Table 3
Positive clone of *Wap65*-1 and *Wap65*-2 gene in BAC library CHOIR 212

Genes	Positive location in BAC library
Wap65-1	153_B20
Wap65-1	176_E15
Wap65-2	37_F12
Wap65-2	45_H24
Wap65-2	90_G10
Wap65-2	81_I21

Sequence analysis indicated the presence of 10 exons and 9 introns for both *Wap65* genes. The gene organization of the catfish *Wap65* genes are highly similar to that of known genes from several fish species, as well as to that of the hemopexin genes of mammalian species. The exon positions and sizes were well conserved through evolution (Fig. 3).

3.4. Determination of genomic copy numbers of Wap65 genes

Genomic Southern blot analysis was conducted to determine the copy numbers of the *Wap65* genes in the catfish genome. *Wap65*-2 is present as a single copy gene in the catfish genome as only a single band was generated using three different restriction endonucleases (Fig. 4A). Initial analysis of *Wap65*-1 using entire cDNA probes suggested the presence of many bands, making assessment of copy numbers difficult. In order to provide an assessment of its copy numbers, a short cDNA probe was then generated using PCR such that no restriction sites were allowed to reside within the probe. Thus, every single band lit up in the Southern blot would suggest a separate copy of the gene in the genome. As shown in Fig. 4B, four bands were largely produced using EcoR I, Hind III and Pst I, with some variations among individuals of fish. This suggested that the channel catfish genome included four *Wap65*-1 gene copies. Taken these results together with the BAC filter screening results, the presence of four copies of the gene and only two positive clones identified from the high-density BAC filter may suggest tandem duplications or highly under representation of the gene sequences in the BAC library. To differentiate these two possibilities, we also conducted Southern blot analysis using isolated BAC DNA. As shown in Fig. 5, three to four bands were produced from a single BAC DNA using the same set of restriction enzymes, suggesting the presence of all four copies of *Wap65*-1 gene within this BAC clone as there is no restriction cutting sites within the short cDNA probe.

Individual variations in restriction patterns were noted in the Southern blot analysis. For instance, restriction digestion using EcoR I and Pst I in fish 3 produced different restriction patterns as compared to fish 1 and fish 2. These individual variations most likely came from allelic polymorphism of the *Wap65*-1 gene.

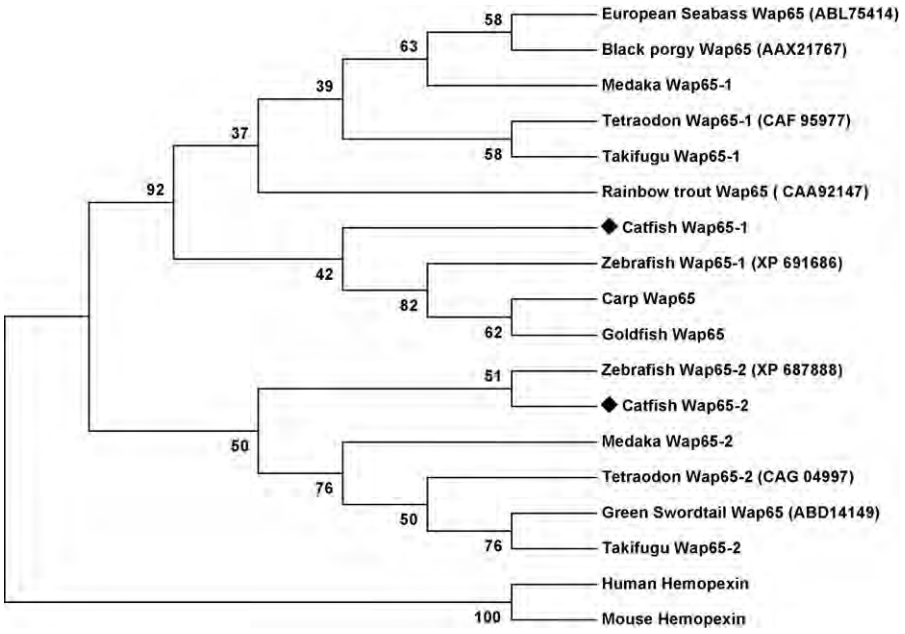

Fig. 2. Phylogenetic analysis of catfish *Wap65* genes with related vertebrate Wap65 genes and mammalian hemopexin sequences. The phylogenetic tree was drawn from ClustalW generated multiple sequence alignment of amino acid sequences using the neighbor-joining method within the MEGA 3.1 package. Data were analyzed using Poisson correction and gaps were removed by complete deletion. The topological stability of the neighbor joining trees was evaluated by 10,000 bootstrapping replications, and the bootstrapping percentage values are indicated by numbers at the nodes. Two types of catfish *Wap65* genes are marked with black diamonds.

3.5. Analysis of Wap65 expression in various tissues

Duplicated genes often exhibit spatially or temporally partitioned expression (Postlethwait et al., 2004). In order to gain insight into gene expression in relation to function, RT-PCR analysis was conducted using RNA isolated from various healthy tissues of channel catfish. As shown in Fig. 6, the two *Wap65* genes exhibit very different spatial expression patterns. *Wap65*-

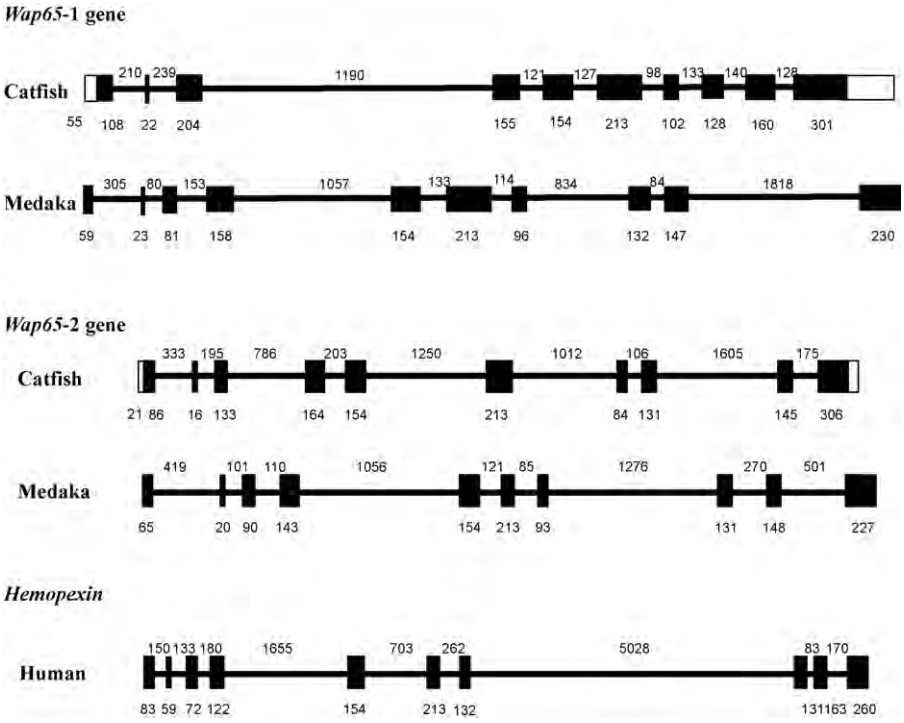

Fig. 3. Schematic representation of *Wap65* gene structure and organization from catfish and medaka as well as hemopexin gene structure and organization from human. Exons are represented by solid boxes and UTR regions are represented by open boxes. Exon sizes in base pairs are shown below of the boxes. Introns are represented by a line and the size in base pairs is shown on the top of the line in the parentheses.

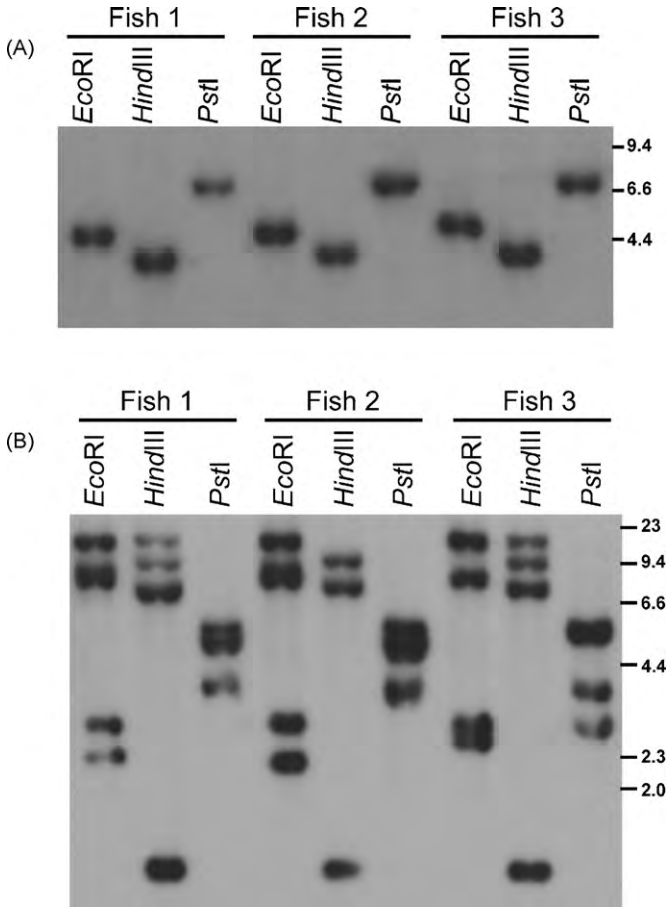

Fig. 4. Southern blot analysis of catfish *Wap65*-2 (A) and *Wap65-1* (B) using genomic DNA of three catfish individuals. Genomic DNA was digested with *Eco*R 1, *Hind* III or *Pst* I, electrophoresed through a 0.8% agarose gel, transferred to nylon membrane, hybridized to the cDNA probes of *Wap65* genes, and processed as detailed in the Material and methods. Molecular weight standards are indicated on the right margin in kb.

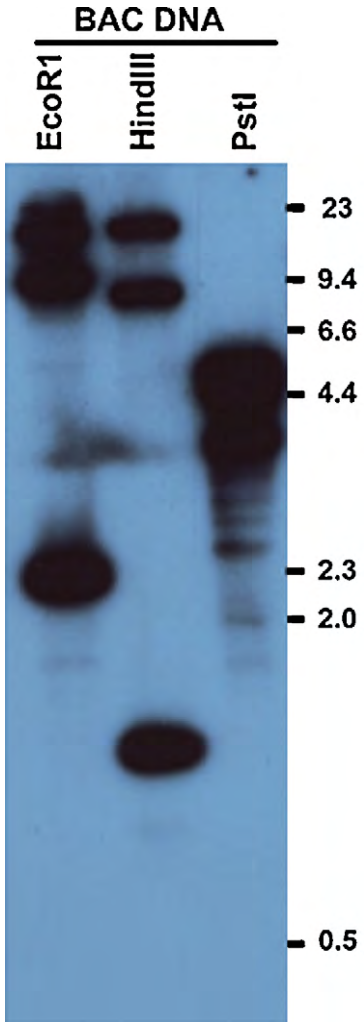

Fig. 5. Southern blot analysis of catfish *Wap65-1* gene using BAC DNA from clone 153_B20. The BAC DNA was digested with *Eco*R 1, *Hind* III or *Pst* I, electrophoresed through a 0.8% agarose gel, transferred to nylon membrane, hybridized to the cDNA probes of *Wap65* genes, and processed as detailed in the Materials and methods. Molecular weight standards are indicated on the right margin.

1 is expressed in essentially all the tissues analyzed including blood, brain, gill, intestine, head kidney, trunk kidney, liver, muscle, skin, spleen, stomach and heart. It is expressed most highly in the liver, and abundantly in most tissues except the head kidney in which *Wap65*-1 was expressed low. In contrast, *Wap65*-2 was only expressed in the liver, but not in any other analyzed tissues.

3.6. Differential expression profiles of Wap65 genes after bacterial infection and warm temperature treatment

Previous analysis using microarrays in our laboratory indicated that a *Wap65*-like gene was highly up-regulated (23-fold) after bacterial infection with *E. ictaluri* (Peatman et al., 2007). That suggested that *Wap65* genes could be involved in host immune responses during bacterial pathogenesis. However, previous infection experiments was conducted at 28 °C, and therefore, it is unknown if the elevated expression was regulated by bacterial infection, or by elevated temperature. In particular, *Wap65* genes have been believed to be one of the major players in warm water temperature acclimation in fishes (Kikuchi et al., 1997; Kinoshita et al., 2001). It is also to our greatest interest to determine if the *Wap65* genes are involved in immune response or not. In the first of this set of experiments, bacterial infection was carried out at 24 °C. *Wap65*-1 expression did not seem to change with or without bacterial infection, whereas *Wap65*-2 expression was elevated with infection (Fig. 7A and B). Not only bacterial infection regulated the expression of *Wap65*-2 gene, so did the temperature. When the experiment was conducted at 28 °C, expression of *Wap65*-2 was significantly up-regulated 3 days after water temperature treatment, but its expression almost returned normal 7 days after temperature treatment. Along with temperature treatment, bacterial infection drastically induced the expression of *Wap65*-2, at day 1, day 3 and day 7 after infection (Fig. 7C). In contrast, neither temperature treatment, nor bacterial infection or both had any effect on the expression patterns of weap65-1 (Fig. 7D). Taken together, these results indicated that *Wap65*-1 is constitutively

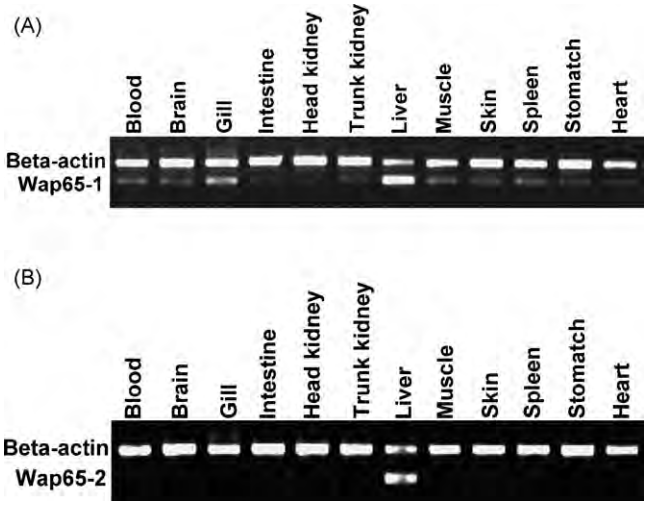

Fig. 6. RT-PCR analysis of catfish *Wap65*-1 (A) and *Wap65*-2 (B) expression in various healthy channel catfish tissues. RT-PCR products were analyzed on an agarose gel. The positions of the RT-PCR amplified bands of β-actin and *Wap65*s are indicated on the left margin. The names of tissue are labeled on the top of each lane.

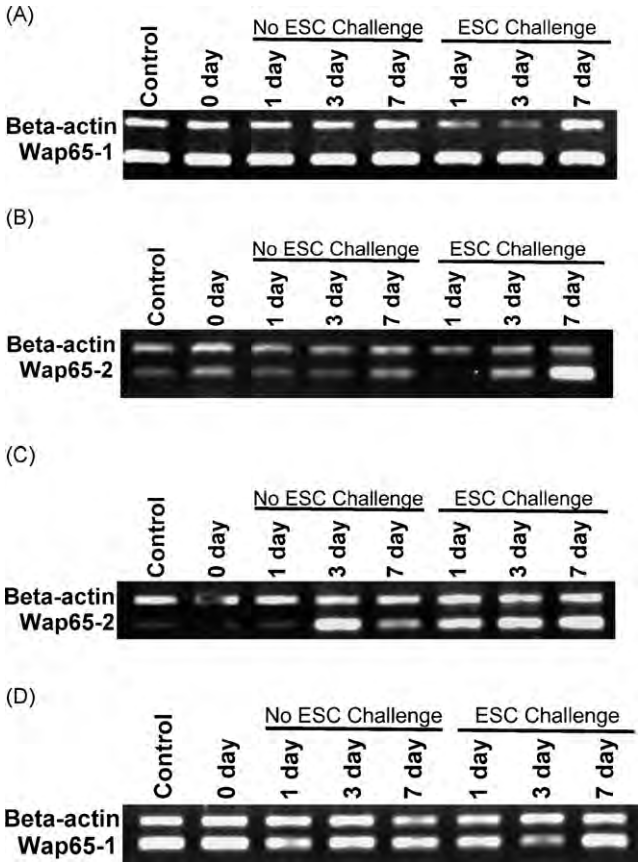

Fig. 7. RT-PCR analysis of *Wap65* gene expression after warm temperature treatment and bacterial challenge with *Edwardsiella ictaluri*. (A) *Wap65*-1 gene expression after the water temperature was increased to 24 °C. "No ESC challenge" lanes represent expression of *Wap65*-1 at 24 °C without infection while "ESC challenge" lanes represent expression of *Wap65*-1 at 24 °C and the fish were infected with *Edwardsiella ictaluri*. (B) *Wap65*-2 gene expression after the water temperature was increased to 24 °C. "No ESC challenge" lanes represent expression of *Wap65*-2 at 24 °C without infection while "ESC challenge" lanes represent expression of *Wap65*-2 at 24 °C and the fish were infected with *Edwardsiella ictaluri*. (C) *Wap65*-2 gene expression after the water temperature was increased to 28 °C. "No ESC challenge" lanes represent expression of *Wap65*-2 at 28 °C without infection while "ESC challenge" lanes represent expression of *Wap65*-2 at 28 °C and the fish were infected with *Edwardsiella ictaluri*. (D) *Wap65*-2 gene expression after the water temperature increasing to 28 °C. "No ESC challenge" lanes represent expression of *Wap65*-1 at 28 °C without infection while "ESC challenge" lanes represent expression of *Wap65*-1 at 28 °C and the fish were infected with *Edwardsiella ictaluri*. Control samples were collected from 20 °C aquarium before the water temperature was increased. The liver samples were collected at four time points after the water temperature increased to 24 or 28 °C. On each temperature, the fish was divided to two groups; one of these two groups was challenged using *E. ictaluri*. The time points are indicated on top of each lane.

expressed, while *Wap65*-2 is regulated by both temperature and bacterial infection, and the regulatory activities appeared to be synergetic.

In order to better quantify the expression changes of *Wap65*-2 after temperature treatment and bacterial infection, real time quantitative RT-PCR was conducted. As shown in Fig. 8A, *Wap65*-2 gene reached highest level on the third day after the water temperature increased from 20 to 24 or 28 °C. The response to temperature was more dramatic when the final temperature was higher. *Wap65*-2 expression was up approximately 18-fold at 28 °C as compared to the control, but it was up only 2-fold at 24 °C as compared to the control (Fig. 8A).

Wap65-2 expression responds to both temperature and bacterial infection in a synergistic fashion. While bacterial infection at 24 °C led to approximately five-fold elevated expression of *Wap65*-2, coupling of 28 °C along with bacterial infection led to 131-fold up in *Wap65*-2 expression at 7 days after infection. Even at earlier time points of 1 day and 3 days after infection, *Wap65*-2 expression was up 16- and 21-fold as compared to the control at 28 °C (Fig. 8B). These results confirmed RT-PCR experiments, and suggested that *Wap65*-2 is not only a warm temperature acclimation protein, but also an immune response protein.

4. Discussion

In this study, we identified and characterized two *Wap65* genes from channel catfish, and analyzed their expression and regulation by temperature and bacterial infections. For the first time, we demonstrated that *Wap65*-2 gene is not only regulated by temperature, but also by immune stimulations such as bacterial infections. The catfish *Wap65* genes are structurally similar to the mammalian hemopexin family of genes. The heme-binding domains are highly conserved, suggesting similar functions to the mammalian hemopexins (Satoh et al., 1994). In mammalian species, hemopexin serves as a heme transporter: scavenging heme from circulation and thereby preventing oxidative tissue damage caused by heme-catalyzed radical formation. Only a single copy hemopexin gene was identified from the human genome. A total of five copies of *Wap65* genes were found in the channel catfish genome, resulting from both whole genome duplications and from tandem intrachromosomal duplications. They have been mapped to BACs allowing comparative mapping analysis using the BAC library

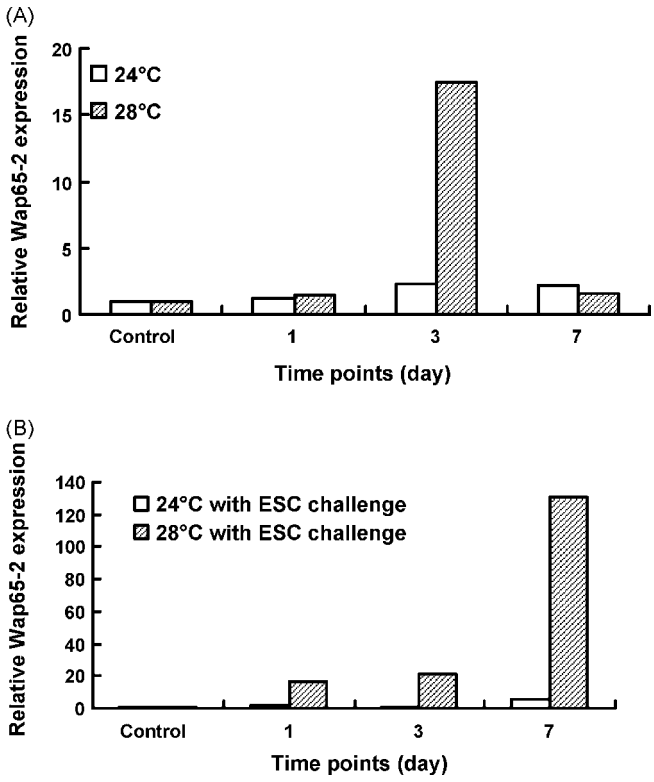

Fig. 8. Quantitative RT-PCR analysis of *Wap65*-2 gene expression after warm temperature treatment at 24 or 28 °C and bacterial challenge with *Edwardsiella ictaluri*. (A) The *Wap65*-2 gene expression after water temperature was increased to 24 and 28 °C without *E. ictaluri* challenge. (B) The *Wap65*-2 gene expression on water temperature was increased to 24 and 28 °C with *E. ictaluri* challenge. Open bar, 24 °C; sketched bar, 28 °C.

and physical map resources (Wang et al., 2007; Xu et al., 2007). The expression patterns of the two *Wap65* genes were determined. The channel catfish *Wap65*-1 was expressed in a wide range of tissues while *Wap65*-2 was liver specific. The two types of *Wap65* genes were differentially regulated with warm temperature and bacterial infections, suggesting their differential functions.

Phylogenetic analysis revealed that the *Wap65* genes are duplicated in teleost genomes. There are two types of the *Wap65* genes in *Tetraodon nigroviridis*, *Takifugu rubripes*, *Danio rerio* and Japanese medaka (Hirayama et al., 2003; Nakaniwa et al., 2005). In the catfish genome, two types of *Wap65* genes were identified. *Wap65*-2 was a single copy gene while *Wap65*-1 has four copies in the catfish genome. Only one copy of *Wap65* gene was reported from carp and goldfish, but the likely course for this is that the second copy has not been characterized. In contrast to the teleost situation, there is only one hemopexin gene in human and mouse. Phylogenetic analysis indicated that the two copies of the *Wap65* genes in teleost genomes were derived from the whole genome duplication in the teleost radiation, consistent with the 3R hypothesis (Ohno, 1970; Steinke et al., 2006).

The channel catfish *Wap65* genes were evolved also through the mechanism of tandem duplication in addition to the whole genome duplication. Southern blot analysis suggested that four copies of the *Wap65*-1 gene were present in the catfish genome. Further analysis using BAC DNA demonstrated that all four copies could have resided within a single BAC clone, evidence for tandem gene duplications. While whole genome duplication has caught the major attention of the scientific community, extensive tandem gene duplications may exist in catfish and other teleosts such as zebrafish (Bao et al., 2006a,b; Peatman et al., 2006; Peatman and Liu, 2007). *Wap65*-1 adds to the existing list of tandemly duplicated genes in channel catfish (Peatman and Liu, 2007).

Of the two *Wap65* genes in teleosts, *Wap65*-2 was better conserved in sequence identities through evolution as greater sequence identities existed among many *Wap65* genes with the mammalian hemopexins. In relation to this greater level of sequence conservation, the expression pattern of *Wap65*-2 was more similar to that of hemopexins. Hemopexin is a major heme-binding plasma glycoprotein synthesized in the liver. Such a pattern of liver-specific expression has been well conserved through evolution to include the teleost *Wap65*-2. However, the duplicated copy of the *Wap65* gene, *Wap65*-1, exhibited a drastically different spatial expression pattern. It was expressed essentially in all tissues, although most abundantly in the liver (Fig. 6). In relation to the expression patterns, the duplicated genes could have evolved to gain highly differential functions. With the whole duplicated teleost genomes, the duplicated genes tend to have a high rate of gene loss of the duplicated copies (Woods et al., 2005). However, in many instances, as in the cases of the classical cases of the *Hox* genes (Amores et al., 1998; Duboule, 2000), the retaining copies of duplicated genes tend to have neofunctions or to have spatial and/or temporal partitioning of functions (Postlethwait et al., 2004).

The duplicated catfish *Wap65* genes exhibit highly different expression patterns and regulation after warm temperature treatment and bacterial infections. Such differences could reflect their neofunctionalization as well as partitioning of their functions. First, as discussed above, the two genes are expressed with highly spatial differences and patterns. While *Wap65*-1 is universally and constitutively expressed, *Wap65*-2 was limited to the liver and was highly regulated by warm temperature treatment and bacterial infections. Such expression patterns could indicate that *Wap65*-2 is more important in warm temperature acclimation and in immune responses than *Wap65*-1. On the other hand, *Wap65*-1 could have evolved to harbor new functions that warrant additional studies. While previous research has focused on the roles of warm temperature acclimation, the up-regulation of *Wap65*-2 with LPS treatment in goldfish (Kikuchi et al., 1997) and with bacterial infection observed here in catfish strongly suggest that *Wap65*-2 may also be a player in the defense responses of the host upon infection. Our previous studies found that many immune related genes were induced after the infection of ESC (Bao et al., 2005, 2006a,b; Baoprasertkul et al., 2005; Chen et al., 2005; Q. Wang et al., 2006; Y. Wang et al., 2006; Xu et al., 2005). Another linkage to the immune response is the potential role of *Wap65* genes in heme-binding as iron is important in the regulation of bacterial pathogenesis. Many genes involved in iron homeostasis were found to be highly upregulated after bacterial infection in catfish (Peatman et al., 2007,2008).

The transcriptional response of *Wap65*-2 gene appeared to be quite slow, days after warm temperature treatment or bacterial

infection. This delayed response may suggest that it is likely a consequence of many other upstream regulatory gene actions. *Wap65*-2 was the highest 3 days after temperature shift and 7 days after infection. Similar results were found with warm temperature shift in goldfish where *Wap65*-2 gene expression peaked 3 days after temperature shift (Kikuchi et al., 1997).

It is most interesting that warm temperature and bacterial infection jointly regulate the expression of *Wap65*-2 gene in a synergistic way. Temperature treatment at 28 °C alone caused an elevation of expression by 17-fold; along with this temperature treatment, bacterial infection caused an elevation of expression by 131-fold (Fig. 8). Clearly warm temperature and bacterial infection each contribute to the induced expression of *Wap65*-2, and together, the extent of gene expression induction was the largest. The mechanism of how such very different stimuli caused gene induction is unknown at present, but certainly is of great interest for further study.

In conclusion, the two types of *Wap65* genes from catfish exhibit highly differential expression patterns in spatial distribution and regulation after warm water treatment. This study also demonstrated that expression of *Wap65*-2 is regulated by bacterial infections, suggesting the roles of *Wap65*-2 in immune responses. In spite of the lack of details at present as to how *Wap65*-2 is involved in host defense responses, it is time to pay great attention to its role in immune responses, as well as its role as a warm temperature acclimation protein.

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