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Molecular Cloning, Characterization and Expression Analysis of Wap65 Gene from the Crucian Carp

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Authors' Contributions
XW and JC conceived the study, wrote the manuscript. JC and XZ performed the bioinformatics analysis and conducted the experiments. ZW and DK helped in data collection. XQ and HW helped in manuscript preparation.

Key words
Warm temperature acclimation-related 65 kDa protein, Gene cloning, qRT-PCR, Heat stress, Crucian carp

ABSTRACT

The Wap65 is a warm temperature acclimation-related protein, and it plays an important role in adapting temperature shock. But the research about Wap65 in crucian carp is very limited. In this study, the CDS of *Wap65* was firstly cloned and characterized from crucian carp (*Carassius carassius*). This sequence is 1338bp that encodes a polypeptide of 445 amino acids. The calculated molecular weight of crucian carp Wap65 protein (CcWap65) is 50.8kDa, containing a signal peptide cleavage site between amino acids 18 and 19. The CcWap65 is a hydrophilic protein and no trans-membrane topological proteins. The SMART analysis revealed that CcWap65 contains three hemopexin-like repeats (E-value < 0.05). The crucian carp *Wap65* was mainly expressed in liver, with limited expression observed in intestine, skeletal muscle and kidney. *CcWap65* was significantly up-regulated in the liver of crucian carp after heat stress, suggesting that increase in Wap65 gene may be related to high water temperature stress and play important roles in high water temperature environment of crucian carp.

INTRODUCTION

Fish are poikilothermic animals and their physiology is fundamentally affected by environmental temperatures (Cho *et al*., 2012). Temperature is able to affect the survival, growth, and reproduction of fish. For this reason, the acclimation response to temperature change is a pivotal reaction in maintaining their homeostasis under new environmental conditions. It is necessary to study temperature-related genes, which would be beneficial to investigate fish modulation by thermal treatment and establish a scientific base for breeding of thermotolerant fish.

Among the physiological response to warm temperatures, the synthesis of warm-temperature acclimation-associated protein 65 kDa protein (Wap65) has been clearly demonstrated (Kikuchi *et al*., 1995; Kim *et al*., 2013). Teleost Wap65 is most closely homologous to mammalian hemopexin. Moreover, the Wap65s contain hemopexin-like domains. The Wap65s have the same function with mammalian hemopexin, including iron homeostasis, anti-oxidant protection, bacteriostatic defense, nerve regeneration and gene expression promoting cell survival (Kim *et al*., 2013; Muller-Eberhard, 1993; Stred and Messina, 2003). Wap65 was first identified as an abundant cytosolic protein in eurythermal fish such as common carp *Cyrinus carpio* and goldfish *Carassius auratus* acclimated to 30°C (Kikuchi, 1993; Watabe *et al*., 1993), and since has been identified in several fish species, including flounder, mud loach, sea bass, sea bream, plunderfish, swordtail fish, channel catfish, black progy, medaka and pufferfish (Cho *et al*., 2012; Kim *et al*., 2013; Stred and Messina, 2003; Nakaniwa *et al*., 2005; Hirayama*et al*., 2003; Clark and Burns, 2008; Aliza *et al*., 2008; Kikuchi *et al*., 1995).

In this study, we report the cloning and characterization of the coding sequense (CDS) encoding full sequence of a Wap65 protein in the crucian carp (*Carassius carassius*). It has been submitted to DDBJ and the accession ID is LC010912. We also studied the differential expression of crucian carp Wap65 in different tissues and its expression level in liver after heat stress. These results suggest that increase in Wap65 gene is related to high water temperature stress and play important roles in high water temperature environment of crucian carp.

MATERIALS AND METHODS

Ethics and methods

This study was approved by the Animal Care and Use

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Committee of the Key Laboratory of Mariculture in North China (Dalian, Liaoning). All surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.

Experimental animals and tissue collection

The crucian carps (10-12cm) were collected for the heat stress experiment. The heat stress experiment was conducted at the Genetic Engineering Laboratory of Dalian Ocean University. In this experiment, a total of 23 individuals were transferred to the experimental tanks. These individuals were kept in water at 23°C for 48 h to acclimate before the studies. Meanwhile, of the 23 individuals, 3 continued to be kept at 23°C as control group and other 20 as treatment group. The treatment groups were conducted the heat stress experiment. Water temperature was increased for the experimental fish through heat exchangers by approximate 1°C / 30min until the first individual showed loss of equilibrium (LOE). And then stop heating and maintain this temperature. The last 3 individuals showing LOE were quickly removed from the tank and sampled.

Isolation of total RNA, synthesis of first strand cDNA and cloning of the crucian carp Wap65-1

Total RNAs were isolated from gill, heart, intestine, skeletal muscle, kidney, liver and brain in treatment group and control group using TRIzol reagent (Takara) according to the manufacture's instructions. The first strand cDNA was synthesized with PrimeScript TM RT-PCR kit (Takara, Dalian China), according to the manufacturer's instructions (Qiu et al., 2014).

To clone the CDS of crucian carp *Wap65* (*CcWap65*), a pair of primers WF and WR (Table I) were designed according to the conserved regions of goldfish (GenBank: D50437.1) and common carp (GenBank: AB052623.1) in 5'-UTR and 3'-UTR, respectively, using the Primer 5 Software. The PCR amplification was performed with one cycle at 94°C for 4 min; 30 cycles at 94°C for 30s, 53°C for 90s, 72°C for 30s, one cycle at 72°C for 7min. PCR product were isolated and cloned into pMD-19-T Vector (Takara, Dalian China) to sequence.

Sequence analysis

Sequence similarity analyses were performed using the Blast program at the National Center for Biotechnology Information (NCBI). The CDS of crucian carp *Wap65* was determined using BioEdit, and then translated into the corresponding amino acid sequence. Multiple protein sequence alignments were performed with ClustalX1.83. The phylogenetic analysis was performed using the neighbor-joining method (Bootstraping =1,000) of MEGA6 software. The composition and physicochemical character, signal peptide, subcellular localization, rans-membrane topological structure and hydrohobicity or hydrophilicity were analyzed by ProtParam at http://web.expasy.org/protparam, SignalP at http://www.cbs.dtu.dk/services/SignalP, TMHMM at http://www.cbs.dtu.dk/services/TMHMM, Wolfpsort at http://wolfpsort.org and ProtScale at http://web.expasy.org/protscale.

Table I.- Primers used in the present study.

Primer	Sequence (5'- 3')
WF	TGTCTCACCAGAGGACCCTG
WR	GCACATGCTGTAATGGCAGC
WqF	CCCTGAGTTGGATGAACATC
WqR	CCACTGCAGCATCCAAATGG
β-actinF	TGCAAAGCCGGATTCGCTGG
β-actinR	AGTTGGTGACAATACCGTGC

Tissue expression and quantification of CcWap65

We applied the $2^{-\Delta\Delta CT}$ method to study the differential expression of *CcWap65* in 7 crucian carp tissues (gill, heart, intestine, skeletal muscle, kidney, liver and brain) and the relative mRNA levels of *Wap65* in the liver of crucian carp between the treatment group and control group. β-actin was used as reference gene. All the primers (WqF, WqR, β-actinF and β-actinR) are listed in Table I. qRT-PCR was carried out using SYBR® Premix Ex Taq™ kit (Takara, Dalian China) and the PCR amplification was quantified according to the manufacturer's instruction. PCR reactions consisted of 1.5 µl first strand cDNA, 7.5 µl SYBR Green (Roche Applied Science), 0.3µl ROX, 0.6 µl each of 10 µM forward and reverse primers, and 4.5 µl nuclease-free water. qRT-PCR conditions were as following: 1 cycle at 95ºC for 30 sec, 40 cycles at 95ºC for 5 sec and 34 sec at 60ºC. At the end, a dissociation stage was added: 5 sec at 95ºC, 30 sec at 60ºC and 30 sec at 95ºC.

RESULTS AND DISCUSSION

Cloning and characterization of CcWap65 CDS

We used ORF finder to find the open reading frame of *CcWap65* and did multiple sequence alignment with the CDS of goldfish and common carp *Wap65*s to determine the CDS of *CcWap65*. This CDS includes one 1338bp fragment which was submitted to DDBJ (accession ID: LC010912), encoding polypeptides of 445 amino acids. The calculated molecular weights is 50.8 kDa. The SignalP program predicts that this CDS contains a signal peptide cleavage site between amino acids 18 and 19 (Fig. 1). The hydrophilicity and hydrophobicity of CcWap65 was displayed by ProtScale, the scale Kyce and Doolittle. It indicates that CcWap65 is a hydrophilic protein because its hydrophilic regions are significantly higher than the

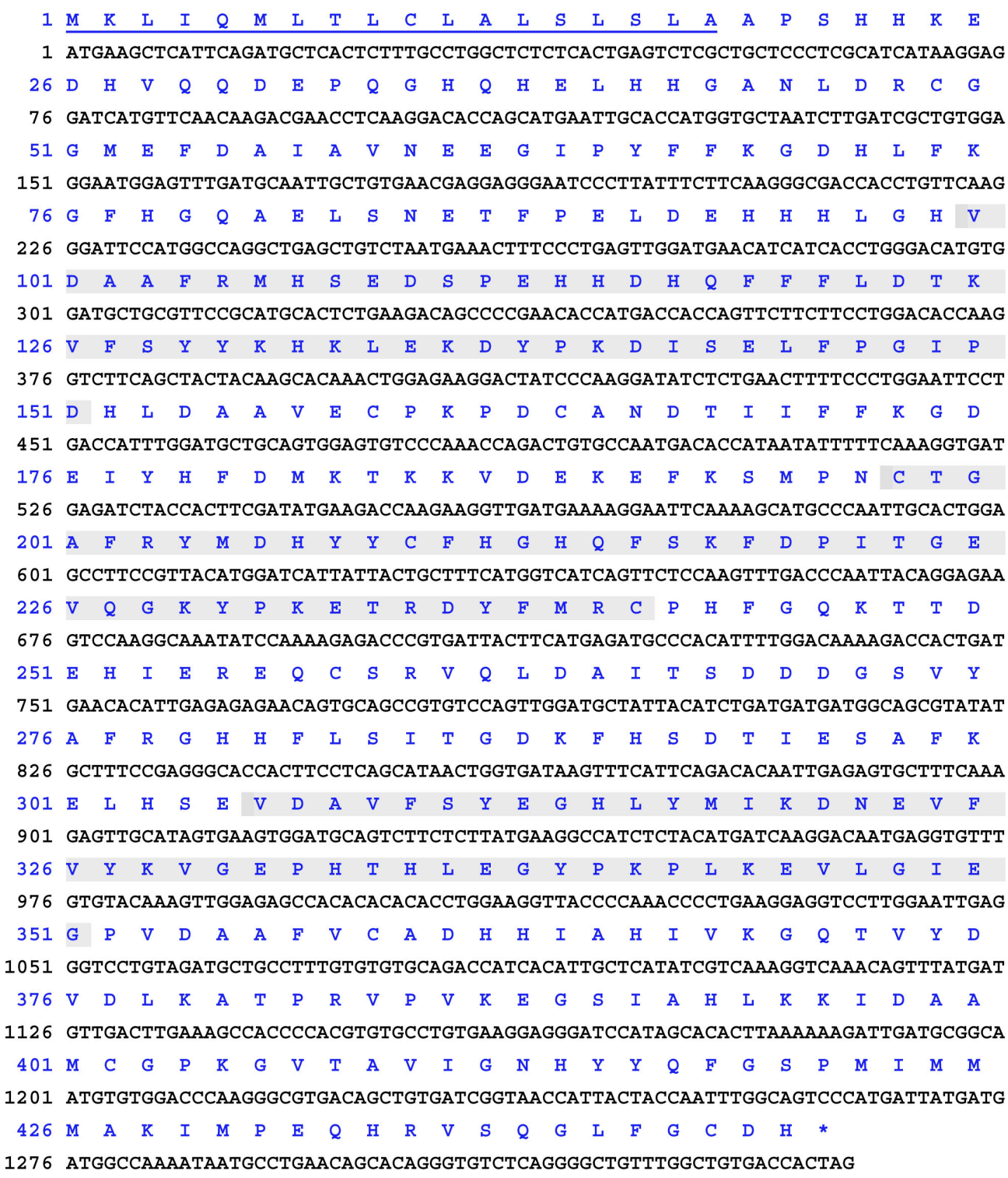

Fig. 1. Nucleotide sequence and deduced amino acid sequence of crucian carpWap65. Nucleotide sequences were determined as described in experimental procedures. The nucleotide sequences are shown below the deduced amino acid sequences. The nucleotide and amino acid numbers are indicated to the left of the sequence. The signal peptide is indicated in the line. The hemopexin-like repeats are indicated by shadows.

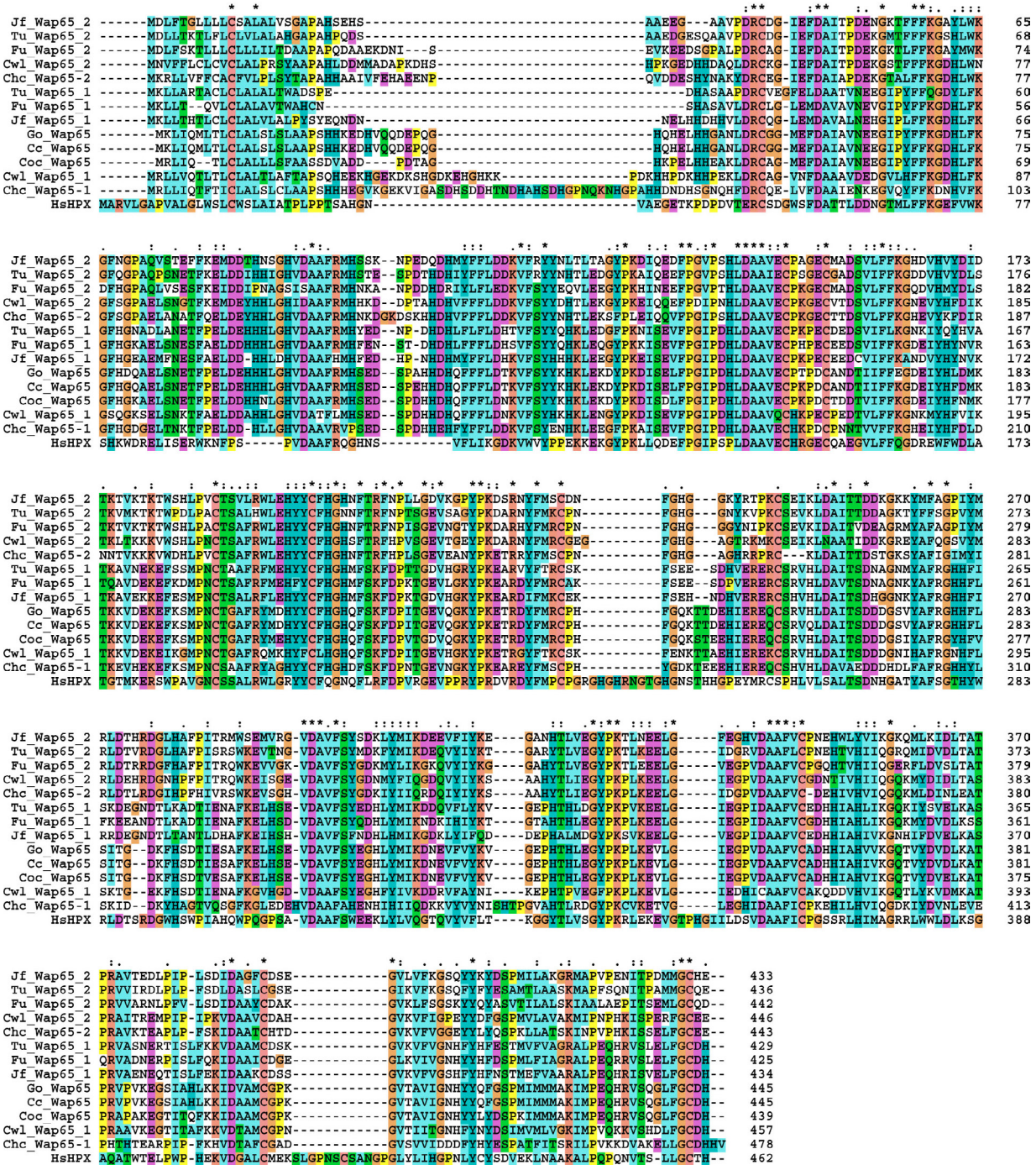

Fig. 2. Multiple alignment of deduced amino acid sequences for Wap65-like proteins. Abbreviation: Jf, Japanese flounder; Tu, Turbot; Fu, Fugu; Cwl, Chinese weather loach; Chc, Channel catfish; Go, Goldfish; Cc, Crucian carp; Coc, Common carp; HsHPX, Human hemopexin. The other 13Wap65 protein sequences aligned were: Japanese flounder Wap65_1 (*Paralichthys olivaceus*, KC521544.1), Japanese flounder Wap65_2 (*P. olivaceus*, KC521545.1), Chinese weather loach Wap65_1 (*Misgurnus mizolepis*, JN230714.1), Chinese weather loach Wap65_2 (*M. mizolepis*, JN230715.1), Channel catfish Wap65_1 (*Ictalurus punctatus*, EU030383.1), Channel catfish Wap65_2 (*I. punctatus*, EU030384.1), Turbot Wap65_1 (*Scophthalmus maximus*, KJ160506.1), Turbot Wap65_2 (*S. maximus*, KJ160507.1), Fugu Wap65_1 (*Takifugu rubripes*, AB125932.1), Fugu Wap65_2 (*T. rubripes*, AB125933.1), Common carp Wap65 (*Cyprinus carpio*, AB052623.1), Goldfish Wap65 (*Carassius auratus*, D50437.1), Human hemopexin (Genbank ID: NM_000613.2).

hydrophobic regions. The trans-membrane topological structure of CcWap65 were predicted by TMHMM, and the result shows the peptide chains of CcWap65 is inside membrane, which indicates that it is no trans-membrane topological proteins. A simple k-nearest neighbor (Knn) classifier was used to predicted subcellular localization in Wolfpost tool. CcWap65 may position in extracellular because of the max value of Knn. The SMART analysis reveals that CcWap65 encoded polypeptide contains three Hemopexin-like repeats (E-value < 0.05) (Fig. 1).

Based on a multipe sequence alignment with representative orthologues from other fish species and human hemopexin, the CcWap65 polypeptide shares varying degrees of homology with their corresponding orthologues (Fig. 2). The CcWap65 shows the highest amino acid sequence identities to the goldfish Wap65 (98%) and common carp (87%). The identity to human hemopexin is 33%. In addition, the identities of CcWap65 to other Wap65-1s are higher than that to Wap65-2. In addition, the phylogenetic tree was constructed by neighbor-joining algorithms of MEGA6, and bootstrapping was performed 1000 times to obtain support values for each branch in Figure 3. It can be seen that the Wap65s were classified into major branches, Wap65-1, Wap65-2 and Human hemopexin. This shows that the division appears between Wap65-1 and Wap65-2 in the evolutionary process, which may cause different function. The CcWap65 located in the first branch, containing Wap65-1 subfamily.

The primary role of the hemopexin is to bind free heme with very high affinity and thus protects against heme toxicity, sequesters heme from pathogens, and helps conserve valuable iron in mammals (Dooley *et al*., 2010). CcWap65 has the same function with human hemopexin because it contains hemopexin-like repeats, and hemopexin-like domains and the cysteine (C) were very conserved (Fig. 2). In addition, CcWap65 is stability protein, have trans-membrane topological structure and signal peptide. Moreover, its subcellular localization is extracellular. This show CcWap65 is a secreted protein and its function is also consistent with the binding and transport of heme.

Tissue expression and expression after heat stress

Quantitative RT-PCR (qRT-PCR) was used to determine relative tissue distribution of Wap65 gene expression in 7 crucian carp tissues including gill, heart, intestine, skeletal muscle, kidney, liver and brain. The CcWap65 gene was mainly expressed in liver, with limited expression observed in intestine, skeletal muscle and kidney (Fig. 4A).

Among the experiment of heat stress, when water temperature was 39°C, the first individual showed LOE. The qRT-PCR results showed that *CcWap65* gene expression level in the liver of crucian carp after heat stress. The *CcWap65* was significantly up-regulated in treatment group, suggesting that *CcWap65* may be involved in the response process of high temperature induction (Fig. 4B).

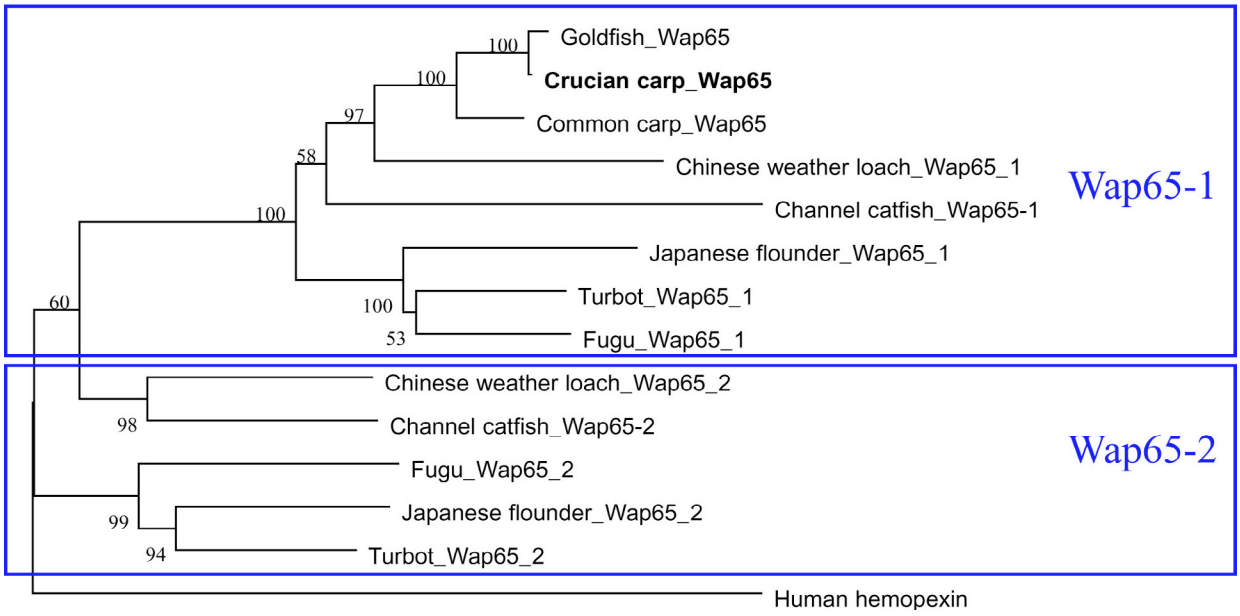

Fig. 3. Phylogenetic analysis of Wap65 proteins, including crucian carp Wap65 and another 13 Wap65 protein sequences (Bootstraping=1,000). Neighbor-joining phylogenetic tree was constructed based on the sequence as below utilizing the sequence analysis tool MEGA6, and bootstrapping was performed 1000 times to obtain support values for each branch. The Wap65s were classified into major branches, Wap65-1, Wap65-2 and Human hemopexin.

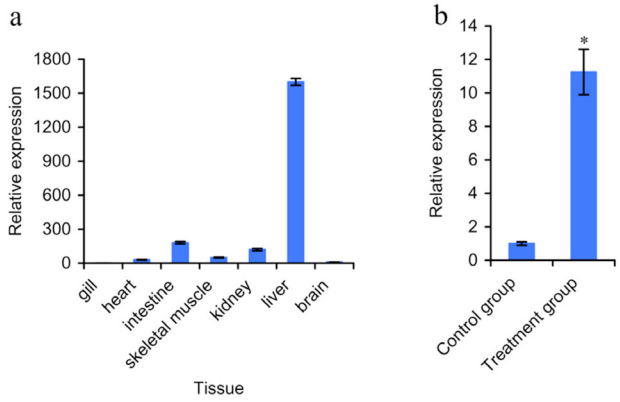

Fig. 4. Expression pattern of crucian carp *Wap65* mRNA in different experiment. **a,** Relative expression of Wap65 in crucian carp determined by qRTPCR. The Y-axis represents normalized relative expression values of Wap65. Tissue RNA samples are labeled along the X-axis; **b,** Fold induction of crucian carp *Wap65* gene after heat treatment in the liver. Relative *Wap65* expression was expressed as fold change over control samples taken at the same time interval as normalized to change in expression in the *β-action* control (n=3, **P*<0.05).

Teleost Wap65 is most closely homologous to mammalian hemopexin, which contains hemopexin-like domains. The Wap65s have the same function with mammalian hemopexin, including iron homeostasis, anti-oxidant protection, bacteriostatic defense, nerve regeneration and gene expression promoting cell survival. Hemopexin is a mammalian plasma glycoprotein that is mainly synthesized in liver (Dooley *et al*., 2010; Kikuchi *et al*., 1995). For instance, rat hemopexin was first detected in liver on day 24 of gestation and rapidly increase during the postnatal period (Dooley *et al*., 2010; Nikkila *et al*., 1991). *Wap65* as many important functional genes, has a few isomers, *Wap65-1* and *Wap65-2*. In the previous studies, the *Wap65-1* of fugu, madaka, and channel catfish are mainly expressed in liver, with limited expressed in other tissues, while the *Wap65-2* is only expressed in liver (Hirayama *et al*., 2003; Nakaniwa *et al*., 2005; Sha *et al*., 2008). The difference of expressed characteristic of *Wap65-1* and *Wap65-2* shows that function differentiation has been appeared, which is consistent with the view of Sarropoulou *et al*. (2010). In the present study, the Cc*Wap65* was mainly expressed in liver, with limited expression observed in the intestine, skeletal muscle and kidney. This expression pattern is consistent with Wap65-1 in fish species.

Wap65 is a protein related to temperature, and plays an important role in adapting to the water temperature changes. In the the previous studies, both Kikuchi *et al*. (1993) and Watabe *et al*. (1993) found the expression of *Wap65* is significantly up-regulated in common carp and goldfish acclimated to 30°C by using RT-PCR. Moreover, the expression of channel catfish *Wap65s* are up-regulated after heat stress (Sha *et al*., 2008; Delanghe and Langlois, 2001). Many studies showed that the expression of Wap65 is significantly up-regulated after the heat stress in other fish, such as mud loach (Cho *et al*., 2012), flounder (Kim *et al*., 2013), antarctic plunder fish (Clark and Burns, 2008), sea bass and sea bream (Pierre *et al*., 2010). Wap65 can regulate stress response to temperature change and protect the body from harm because it has the same function with human hemopexin. Therefore, in this study we reported for the first time that the CDS encoding the full sequence of a Wap65 protein is cloned and characterized in the crucian carp. We also studied the differential expression of crucian carp Wap65 in different tissues and its expression level in liver after heat stress. These results suggest that increase in Wap65 gene is related to high water temperature stress and play important roles in high water temperature environment of crucian carp.

CONCLUSION

In the present study, we first clone and characterize the CDS of *Wap65* from crucian carp (*Carassius carassius*). This sequence is 1338bp that encodes a polypeptide of 445 amino acids with a calculated 50.8kDa. With the sequence analysis, the CcWap65 is a secreted hydrophilic protein with no trans-membrane topological proteins, and its function is also consistent with the binding and transport of heme. qRT-PCR results indicated that the *CcWap65* was mainly expressed in liver after heat stress, with limited expression observed in the intestine, skeletal muscle and kidney. The *CcWap65* was also significantly up-regulated in treatment group. These results suggest that increase in Wap65 gene may play important roles in high water temperature environment of crucian carp, which would be beneficial to investigate fish modulation by thermal treatment and establish a scientific base for breeding of thermotolerant fish.

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Conflict of interest statement

We declare that we have no conflict of interest.

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