Pakistan J. Zool., vol. 49(3), pp 915-921, 2017. DOI: http://dx.doi.org/10.17582/journal.pjz/2017.49.3.915.921

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

# Jun Cui, Xiaoxu Zhou, Zhicheng Wang, Derong Kong, Xuemei Qiu, Hongdi Wang and Xiuli Wang\*

College of Fisheries and Life Science, Dalian Ocean University, 52 Heishijiao Street, Shahekou District, Dalian 116023, China

### 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 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**

**F** ish 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



Article Information Received 06 August 2016 Revised 24 September 2016 Accepted 18 October 2016 Available online 02 May 2017

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 acclimationrelated 65 kDa protein, Gene cloning, qRT-PCR, Heat stress, Crucian carp

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

<sup>\*</sup> Corresponding author: xiuliwang417@sina.com 0030-9923/2017/0003-0915 \$ 9.00/0 Copyright 2017 Zoological Society of Pakistan

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 1) 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, ransmembrane 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.  $\beta$ -actin was used as reference gene. All the primers (WqF, WqR,  $\beta$ -actinF and  $\beta$ -actinR) are listed in Table I. gRT-PCR was carried out using SYBR® Premix Ex Taq<sup>™</sup> 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 95°C for 30 sec, 40 cycles at 95°C for 5 sec and 34 sec at 60°C. At the end, a dissociation stage was added: 5 sec at 95°C, 30 sec at 60°C and 30 sec at 95°C.

## **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 Expression Analysis of Crucian Carp Wap65 Gene

1	М	K	L	I	Q	М	L	т	L	С	L	A	L	S	L	S	L	A	A	Р	S	н	н	ĸ	Е
1	ATGAAGCTCATTCAGATGCTCACTCTTTGCCTGGCTCTCTCACTGAGTCTCGCTGCTCCCTCGCATCATAAGGAG																								
26	D	н	v	Q	Q	D	Е	Р	Q	G	н	Q	н	Е	L	н	н	G	Α	N	ь	D	R	С	G
76	GA	TCA	TGT	TCA	ACA	AGA	CGA	ACC	TCA	AGG	ACA	CCA	GCA	TGA	ATT	GCA	CCA	TGG	TGC	TAA	тст	TGA	TCG	CTG	TGGA
51	G	M	Е	F	D	A	I	A	v	N	Е	Е	G	I	Р	Y	F	F	K	G	D	н	$\mathbf{L}$	F	ĸ
151	GG.	AAT	GGA	GTT	TGA	TGC	AAT	TGC	TGT	GAA	CGA	GGA	GGG	AAT	CCC	TTA	TTT	CTT	CAA	GGG	CGA	CCA	ССТ	GTT	CAAG
76	G	F	н	G	Q	A	Е	L	S	N	Е	т	F	Р	Е	L	D	Е	н	н	н	L	G	н	V
226	GG.	ATT	CCA	TGG	CCA	GGC	TGA	GCT	GTC	TAA'	TGA	AAC	TTT	CCC	TGA	GTT	GGA	TGA	ACA	TCA'	TCA	ССТ	GGG	ACA	TGTG
101	D	Α	Α	F	R	М	н	S	Е	D	S	Р	Е	н	н	D	н	Q	F	F	F	$\mathbf{L}$	D	Т	K
301	GA	TGC	TGC	GTT	CCG	CAT	GCA	СТС	TGA	AGA	CAG	CCC	CGA	ACA	CCA	TGA	CCA	CCA	GTT	CTT	CTT	ССТ	GGA	CAC	CAAG
126	v	F	S	Y	Y	K	н	K	$\mathbf{L}$	Е	K	D	Y	Р	K	D	I	S	Е	$\mathbf{L}_{-}$	F	Р	G	I	Р
376	GT	CTT	CAG	CTA	CTA	CAA	GCA	CAA	ACT	GGA	GAA	GGA	CTA	TCC	CAA	GGA	TAT	CTC	TGA	ACT	TTT	CCC	TGG	AAT	TCCT
151	D	н	$\mathbf{L}$	D	A	Α	v	Е	С	Р	ĸ	Р	D	С	Α	N	D	т	I	I	F	F	к	G	D
451	GA	CCA	TTT	GGA	TGC	TGC	AGT	GGA	GTG	TCC	CAA	ACC	AGA	CTG	TGC	CAA	TGA	CAC	CAT	AAT	ATT	TTT	CAA	AGG	TGAT
176	Е	I	Y	н	F	D	м	ĸ	т	K	к	v	D	Е	K	Е	F	к	S	м	Р	N	C	т	G
526	GA	GAT	CTA	CCA	CTT	CGA	TAT	GAA	GAC	CAA	GAA	GGT	TGA	TGA	AAA	GGA	ATT	CAA	AAG	CAT	GCC	CAA	TTG	CAC	TGGA
201	A	F	R	Y	М	D	н	Y	Y	C	F	н	G	н	Q	F	S	К	F	D	Р	I	т	G	Е
601	GC	CTT	CCG	TTA	CAT	GGA	TCA	TTA	TTA	CTG	CTT	TCA	TGG	TCA	TCA	GTT	CTC	CAA	GTT	TGA	CCC	AAT	TAC	AGG.	AGAA
226	v	Q	G	K	Y	Р	K	Е	т	R	D	Y	F	M	R	C	Р	н	F	G	Q	к	т	т	D
676	GT	CCA	AGG	CAA	ATA	TCC	AAA	AGA	GAC	CCG	TGA	TTA	CTT	CAT	GAG	ATG	CCC	ACA	TTT	TGG	ACA	AAA	GAC	CAC	TGAT
251	Е	н	I	Е	R	Е	Q	C	S	R	V	Q	L	D	Α	I	т	S	D	D	D	G	S	V	Y
751	GA.	ACA	CAT	TGA	GAG	AGA	ACA	GTG	CAG	CCG	TGT	CCA	GTT	GGA	TGC	TAT	TAC	ATC	TGA	TGA	TGA	TGG	CAG	CGT.	ATAT
276	Α	F	R	G	н	н	F	L	S	I	т	G	D	к	F	н	S	D	т	I	Е	S	A	F	ĸ
826	GC	TTT	CCG	AGG	GCA	CCA	CTT	ССТ	CAG	CAT	AAC	TGG	TGA	TAA	GTT	TCA	TTC	AGA	CAC.	AAT	TGA	GAG	TGC	TTT	CAAA
301	Е	L	н	S	Е	V	D	Α	V	F	S	Y	Е	G	H	ь	Y	Μ	I	K	D	N	Е	V	F
901	GA	GTT	GCA	TAG	TGA	AGT	GGA	TGC	AGT	CTT	CTC	TTA	TGA	AGG	CCA	TCT	CTA	CAT	GAT	CAA	GGA	CAA	TGA	GGT	GTTT
326	v	Y	K	V	G	Е	Ρ	H	Т	н	$\mathbf{L}_{-}$	Е	G	Y	Ρ	K	Ρ	Ъ	K	Е	V	ь	G	I	Е
976	GT	GTA	CAA	AGT	TGG	AGA	GCC	ACA	CAC	ACA	ССТ	GGA	AGG	TTA	CCC	CAA	ACC	ССТ	GAA	GGA	GGT	ССТ	TGG	AAT	TGAG
351	G	Ρ	V	D	Α	Α	F	V	С	Α	D	н	H	I	Α	н	I	V	K	G	Q	т	v	Y	D
1051	GG	TCC	TGT	AGA	TGC	TGC	CTT	TGT	GTG	TGC	AGA	CCA	TCA	CAT	TGC	TCA	TAT	CGT	CAA	AGG	TCA	AAC	AGT	TTA	TGAT
376	v	D	L	к	Α	т	Ρ	R	v	Ρ	v	ĸ	Е	G	S	I	Α	н	ь	ĸ	к	I	D	Α	Α
1126	$\mathbf{GT}$	TGA	CTT	GAA	AGC	CAC	CCC	ACG	TGT	GCC	TGT	GAA	GGA	.GGG	ATC	CAT	AGC	ACA	CTT.	AAA	AAA	GAT	TGA	TGC	GGCA
401	M	C	G	Р	K	G	v	т	Α	v	I	G	N	н	Y	Y	Q	F	G	S	Ρ	M	I	М	Μ
1201	AT	GTG	TGG	ACC	CAA	GGG	CGT	GAC	AGC	TGT	GAT	CGG	TAA	CCA	TTA	CTA	CCA	ATT	TGG	CAG	TCC	CAT	GAT	TAT	GATG
426	M	A	ĸ	I	M	Р	Е	Q	н	R	v	S	Q	G	L	F	G	С	D	н	*				
1276	AT	GGC	CAA	AAT	AAT	GCC	TGA	ACA	GCA	CAG	GGT	GTC	TCA	GGG	GCT	GTT	TGG	CTG	TGA	CCA	CTA	G			

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.

917

J. Cui et al.

76		
Jf_Wap65_2	MDLFTGLLLLCSALALVSGAPAHSEHS	AAEEGAAVPDRCDG-IEFDAITPDENGKTFFFKGAYLWK
Tu_Wap65_2	MDLLTKTLFLCLVLALAHGAPAHPQDS	AAEDGESQAAVPDRCDG-IEFDAITPDEKGMTFFFKGSHLWK
Fu Wap65 2	MDLFSKTLLLCLLLILTDAAPAPQDAAEKDNIS	EVKEEDSGPALPDRCAG-IEFDAITPDEKGKTLFFKGAYMWK 7
Cwl_Wap65_2	MNVFFLCLCVCLALPRSYAAPAHLDDMMADAPKDHS	
Chc_Wap65-2	MKRLLVFFCACFVLPLSYTAPAHHAAIVFEHAEENP	QVDDESHYNAKYDRCEG-IEFDAIAPDEKGTALFFKGDHLWK 7
Tu_Wap65_1	MKLLARTACLCLALALTWADSPE	DHASAAPDRCVEGFELDAATVNEEGIPYFFQGDYLFK 6
Fu Wap65 1	<mark>MKLLT</mark> QVL <mark>CLALAVTWAHC</mark> N	SHASAVLDRCLG-LEMDAVAVNEVGIPYFFKGDHLFK 5
Jf Wap65 1	MKLLTHTLCLCLALVLALPYSYEQNDN	
Go Wap65	MKLIQMLTLCLALSLSLAAPSHHKEDHVQQDEPQG	<mark>HQHELHHGANLDRCGG-MEFDAIAVNEEGIPYFFKGDHLFK</mark> 7
Cc Wap65	MKLIQMLTLCLALSLSLAAPSHHKEDHVQQDEPQG	
Coc Wap65	PDTAG	<b>HKPELHHEAKLDRCAG-MEFDAIAVNEEGIPYFFKGDHLFK</b> 6
Cwl Wap65 1	MRLLVQTLTLCLALTLAFTAPSQHEEKHGEKDKSHGDKEHGHKK	PDKHHPDKHHPEKLDRCAG-VNFDAAAVDEDGVLHFFKGDHLFK 8
Chc Wap65-1	MRLLIQTFTICLALSLCLAAPSHHHEGVKGEKVIGASDHSDDHTNDHA	HSDHGPNQKNHGPAHHDNDHSGNQHFDRCQE-LVFDAAIENKEGVQYFFKDNHVFK 10
HSHPX	MARVLGAPVALGLWSLCWSLAIATPLPPTSAHGN	<mark>VAEGE</mark> TKPDPDVTERCSDGWSFDATTLDDNGTMLFFKGEFVWK 7
		* .:* :.: **.:* ****::* :* : ::*:: :
Jf Wap65 2	GFNGPAQVSTEFFKEMDDTHNSGHVDAAFRMHSSKNPEDQDHMYFFLDDKVF	RYYNLTLTAGYPKDIQEDFPGVPSHLDAAVECPAGECMADSVLFFKGHDVHVYDID 17
Tu Wap65 2	GFQGPAQPSNETFKELDDIHHIGHVDAAFRMHSTE SPDTHDHIYFFLDDKVF	r <mark>YYNHTLEDGYPKAIQEEFPGVP</mark> SHLDAAIECPSGECGADSVIFFKGDDVHVYDLS 17
Fu Wap65 2	DFHGPAQLVSESFKEIDDIPNAGSISAAFRMHNKANPDDHDRIYLFLEDKVF	<b>SYY</b> EQVLEE <mark>GYPKHINEEFPGVPTHLDAAVECPKGECMADS</mark> VLFFKGQDVHMYDLS 18
Cwl Wap65 2	GFSGPAELSNGTFKEMDEYHHLGHIDAAFRMHHKD DPTAHDHVFFFLDDKVF	<mark>SYYDHTLEKGYPKEIQQ</mark> EF <mark>PDIPNHLDAAVECPKGEC</mark> VT <mark>DS</mark> VLFF <mark>KGNEVYHFD</mark> IK 18
Chc Wap65-2	GFSGPAELANATFQELDEYHHLGHVDAAFRMHNKDGKDSKHHDHVFFFLDDKVF	SYYNHTLEKSFPLEIQOVFPGIPSHLDAAVECPKGECTTDSVLFFKGHEVYKFDIR 18
Tu Wap65 1	GFHGNADLANETFPELDEHHHLGHVDAAFRMHYED NP - DHDHLFLFLDHTVF	SYYOHKLEDGFPKNISEVFPGIPDHLDAAVECPKPECDEDSVIFLKGNKIYOYHVA 16
Fu Wap65 1	GFHGKAELSNESFAELDDHHHLGHVDAAFRMHFEN ST - DHDHLFFFLDHSVF	SYYOHKLEOGYPKKISEVFPGIPDHLDAAVECPHPECEEDSVIFFKGDEIYHYNVR 16
Jf Wap65 1	GFHGEAEMFNESFAELDD-HHLDHVDAAFHMHFEDHP-NHDHMYFFLDHKVF	SYHHHKLEEGYPKEISEVFPGIPDHLDAAVECPKPECEEDCVIFFKANDVYHYNVK 17
Go Wap65	GFHDQAELSNETFPELDEHHHLGHVDAAFRMHSED SPAHHDHOFFFLDTKVF	SYYKHKLEKDYPKDISELFPGIPDHLDAAVECPTPDCANDTIIFFEGDEIYHLDMK 18
Cc Wap65	GFHGOAELSNETFPELDEHHHLGHVDAAFRMHSED SPEHHDHOFFFLDTKVF	SYYKHKLEKDYPKDISELFPGIPDHLDAAVECPKPDCANDTIIFFKGDEIYHFDMK 18
Coc Wap65	GFHGKAELSNETFPELDDHHNLGHVDAAFRMHSED SPDHHDHOFFFLDNKVF	SYYKHKLEKDYPKDISDLFPGIPDHLDAAVECPKPDCTDDTVIFFKGDEIYHFNMK 17
Cwl Wap65 1	GSOGKSELSNKTFAELDDAHHLGHVDATFLMHSED SPDHHDHOFFFLDNKVE	SYHKHKLENGYPKDISEVFPGIPDHLDAAVOCHKPECPEDTVLFFKGNKMYHFVIK 19
Chc Wap65-1	GEHGDGELTNKTEPELDD - HLLGHVDAAVRVPSED SPDHHEHFYFFLDDKVF	SYENHKLEEGFPKAISEVFPGIPDHLDAAVECHKPDCPNNTVVFFKGHEIYHFDLD 21
HSHPX	SHKWDRELTSERWKNEPSPVDAAFROGHNSVELTKGDKVW	VYPPEKKEKGYPKILODEFPGIPSPLDAAVECHEGECOAEGVLFFOGDREWFWDLA 17
	*: :::*::*: * :*.* *:* **:*	* *
Jf Wap65 2	TKTVKTKTWSHLPVCTSVLRWLEHYYCFHGHNFTRENPLLGDVKGPYPKDSRNY	FMSCDNFGHGGKYRTPKCSETKLDATTTDDKGKKYMFAGPTYM 27
Tu Wap65 2	TKVMKTKTWPDLPACTSALHWLEHYYCFHGNNFTRFNPTSGEVSAGYPKDARNY	FMKCPNFGHGGNYKVPKCSEVKLDATTTDDAGKTYFFSGPVYM 27
Fu Wap65 2	TKTWKTKTWCHLDACTCAFDWLFHVYCFHCHNFTPFNDTCCFWNCTVDKDAPHY	FMPCDNFCHCCCVNTDKCSFVKTDATTVDFACDMVAFACDTVM 27
Cwl Wap65 2	TKI.TKKKVWSHI.PNCTSAFRWI.FHYYCFHCHSFTRFHPVSCEUTCEVPKDARNY	FMRCGEGFGHGAGTRKMKCSETKLNAATTDDKGREVAFOGSVVM 28
Chc Wap65-2	NNTVKKKVWDHI. PUCTSAFRWI. EHVYCFHCHNFTRFHPI. SCEVEANVPKETRP	FMSCPNFGHGAGHRPPRCKLDATTTDSTGKSVAFTGTMVT 28
Tu Wap65 1	TKAVNEKEESSMONCTAAFPFMEHYYCEHCHMESKEDDTTCDUHCBYDKEAPUV	FTDCSKRSFFSDHVFDFDCSPVHLDATTSDNACNMVAFDCHHFT 26
Fu Wap65 1	TOAVDEKEEKDMPNCTSAFREMEHEYCEHCHMESKEDPKTGEVLGKVPKEARDY	FMPCAKFSEESDPVEPEPCSPUHLDAVTSDNACNKVAFPGHHFL 26
Tf Wap65 1	TYAVEPREPERMINETERI PELEVYCENCIMETRICHUCKVDVERADD	
Go Wap65	TEXUDEVEEVEMDNCTCAEPVMDHYYCEHCHOESVEDDTTCEUOCVAPVETDDY	
Go_Wap65		
Cc_wap65		
Coc_wapos	TKKVDEKEFKSMPNCIGAFRIMEHIICFHGHQFSKFDFVIGDVQGKIPKEIRDI	FMRCPHFGQXSTEBHIEREQCSRVHLDATISDDDGSTIAFRGIHFV 2/
CWI_Wap65_1	TKKYDEKEIKGMPNCIGAF KUMANIFCLHGHQF SAFDFIIGEVHGAIFAEIKGI	
uaupv		
ASHFA	IGIMERSWFAVGNCSSRIKWIGRIICFQGNQFIRFDFVRGEVFFRIFRDVRDI	recedence of the stand of the s
	***.*•	** .*** *** ** * ***** *** *
.Tf Wap65 2	RIDTHRDGI HAFPTTRMWSEMURG-UDAVESUSDKI, VMTKDEEVETUKEG	ANHTIVEGYDETINEELG FEGHUDAAFVCPNEHWLVVIKGKOMLKIDITAT
Tu Wap65 2	RUDTVRDGLHAFPTSRSWKEVTNG-VDAVESYMDKEVMTKDEOVVTVKTG	ARYTLYEGYPKTLKEELGTDGRVDAAFLCPNEHTYHTTOGROMTDVDLTAT 37
Fu Wap65 2	RUDTRRDGFHAFPTTROWKEVVGK-VDAVESYGDKMYLTKGKOVYTYKGG	AHYTLVEGYPKTLEEELGVEGPVDAAFVCPGOHTVHTLOGEEFLDVSLTAT 37
Cwl Wap65_2	RUDEHRDGNHPEPTTROWKETSGE-VDAVESYGDNMYETOGDOVYTYKSA	AHYTLTEGYPKPLKEELGTEGPVDAAFVCGDNTTVHTTOGOKMYDTDLTAS 38
Chc Wap65-2	RLDTLRDGIHPFHIVRSWKEVSGH-VDAVFSYGDKIVIJORDOIVIVKSA	AHYTLIEGYPKPLKEELGIDGPVDAAFVC-DEHIVHVIOGOKMLDINLEAT 38
Tu Wap65 1	SKDEGNDTLKADTIENAFKELHSE-VDAVFSYEDHLYMIKDDOVFLYKVG	EPHTHLDGYPKPVKEELGIEGPIDAAFVCEDHHIAHLIKGOKIYSVELKAS 36
Fu Wap65 1	FKEEANDTLKADTIENAFKELHSD-VDAVFSYODHLYMIKNDKIHIYKTG	TANTHLEGYPKPLKEELGIEGPIDAAFVCGDHHIAHLIKGOKMYDVDLKSS 36
Jf Wap65 1	RRDEGNDTLTANTLDHAFKEIHSH-VDAVFSFNDHLHMIKGDKLYIFOD	DEPHALMDGYPKSVKEELGVEGPIDAAFVCEDHHIAHIVKGNHIFDVELKAS 37
Go Wap65	SITGDKFHSDTIESAFKELHSE-VDAVFSYEGHLYMIKDNEVFVYKVG	EPHTHLEGYPKPLKEVLGIEGPVDAAFVCADHHIAHVVKGOTVYDVDLKAT 38
Cc Wap65	SITGDKFHSDTIESAFKELHSE-VDAVFSYEGHLYMIKDNEVFVYKVG	EPHTHLEGYPKPLKEVLGIEGPVDAAFVCADHHIAHIVKGÕTVYDVDLKAT 38
Coc Wap65	SITGDKFHSDTVESAFKELHSE-VDAVFSYEGHLYMIKDNEVFVYKVG	EPHTHLEGYPKPLKEVLGIEGPVDAAFVCADHHIAHVIKGOTVYDVELKAT 37
Cwl Wap65 1	SKTGEKFHSDTIENAFKGVHGD-VDAAFSYEGHFYIVKDDRVFAYNIK	EPHTPVEGFPKPLKEVLGIEDHICAAFVCAKODDVHVIKGOTLYKVDMKAT 39
Chc Wap65-1	SKID DKYHAGTVOSGFKGLEDEHVDAAFAHENHIHIIODKKVYVYNISHTPO	VAHTLRDGYPKCVKETVGLEGHIDAAFICPKEHILHVIOGDKIYDVNLEVE 41
HSHPX	RLDTSRDGWHSWPIAHOWPOGPSA-VDAAFSWEEKLYLVOGTOVYVFLTK	GGYTLVSGYPKRLEKEVGTPHGIILDSVDAAFICPGSSRLHIMAGRRLWWLDLKSG 38
	** *:*:.	
Jf_Wap65_2	PRAVTEDLPIP-LSDIDAGFCDSEGVLVFKGSQYYKYDSPMI	LAKGRMAPVPENITPDMMGCHE 433
Tu_Wap65_2	PRVVIRDLPLP-FSDLDASLCGSEGIKVFKGSQFYFYESAMT	LAASKMAPFSQNITPAMMGCQE 436
Fu_Wap65_2	PRVVARNLPFV-LSDIDAAYCDAKGVKLFSGSKYYQYASVTI	ILALSKIAALAEPITSEMLGCQD 442
Cwl_Wap65_2	PRAITREMPIP-IPKVDAAVCDAHGVKVFIGPEYYDFGSPMV	IAVAKMIPNPHKISPERFGCEE 446
Chc_Wap65-2	PRAVKTEAPLP-FSKIDAATCHTDGVKVFVGGEYYLYQSPKI	JLATSKINPVPHKISSELFGCE 443
Tu_Wap65_1	PRVASNERTISLFKKVDAAMCDSKGVKVFVGNHFYHFESTMV	/FVAGRALPEQHRVSLELFGCDH 429
Fu_Wap65_1	QRVADNERPISLFQKIDAAICDGEGLKVIVGNHYYHFDSPMI	FIAGRALPEQRRVSLELFGCDH 425
Jf_Wap65_1	PRVAENEQTISLFEKIDAAKCDSSGVKVFVGSHFYHFNSTME	FVAARALPEQHRISVELFGCDH 434
Go_Wap65	PRVPVKEGSIAHLKKIDVAMCGPKGVTAVIGNHYYQFGSPMI	IMMMAKIMPEQHRVSQGLFGCDH 445
Cc_Wap65	PRVPVKEGSIAHLKKIDAAMCGPKGVTAVIGNHYYQFGSPMI	MMMAKIMPEQHRVSQGLFGCDH 445
Coc Wap65	PRAPAKEGTITQFKKIDAAMCGPKGVTAVIGNHYYLYDSPKI	IMMMAKIMPEQHRVSQGLFGCDH 439
Cwl_Wap65_1	PRAAVKEGTITAFKKVDTAMCGPNGVTIITGNHFYNYDSIMV	MLVGKIMPVQKKVSHDLFGCDH 457
Chc_Wap65-1	PHTHTEARPIP-FKHVDTAFCGADGVSVVIDDDFYHYESPAT	FITSRILPVKKDVAKELLGCDHHV 478
HSHPX	AGATWTELPWP-HEKVDGALCMEKSLGPNSCSANGPGLYLIHGPNLYCYSDVEK	LNAAKALPOPONVTS-LLGCTH 462

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).

918

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 hemopexinlike 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.

J. Cui et al.



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  $\beta$ -action control (n=3, \*P<0.05).

Teleost Wap65 is most closely homologous to mammalian hemopexin, which contains hemopexinlike 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 CcWap65 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.

### ACKNOWLEDGMENT

This project was supported by the grant of Dalian Ocean University (Grant No. 2012HYDX07).

### Conflict of interest statement

We declare that we have no conflict of interest.

# REFERENCES

Aliza, D., Ismail, I.S., Kuah, M.K., Shu-Chien, A.C. and Tengku Muhammad, T.S., 2008. Identification of Wap65, a human homologue of hemopexin

920

as a copper-inducible gene in swordtail fish, Xiphophorus helleri. *Fish Physiol. Biochem.*, **34**: 129-138. https://doi.org/10.1007/s10695-007-9153-6

- Cho, Y.S., Kim, B.S., Kim, D.S. and Nam, Y.K., 2012. Modulation of warm-temperature-acclimationassociated 65-kDa protein genes (Wap65-1 and Wap65-2) in mud loach (Misgurnus mizolepis, Cypriniformes) liver in response to different stimulatory treatments. *Fish Shellf. Immunol.*, **32**: 662-669.
- Choi, C.Y., An, K.W., Choi, Y.K., Jo, P.G. and Min, B.H.J., 2008. Expression of warm temperature acclimation-related protein 65-kDa (Wap65) mRNA, and physiological changes with increasing water temperature in black porgy, Acanthopagrus schlegeli. *Exp. Zool. A Ecol. Genet. Physiol.*, **309**: 206-214.
- Clark, M.S. and Burns, G., 2008. Characterisation of the warm acclimated protein gene (wap65) in the Antarctic plunderfish (*Harpagifer antarcticus*). *DNA Sequence: J. DNA Sequenc. Mapp.*, **19**: 50-55.
- Delanghe, J.R. and Langlois, M.R., 2001. Hemopexin: a review of biological aspects and the role in laboratory medicine. *Clin. Chim. Acta; Int. J. clin. Chem.*, **312**: 13-23.
- Dooley, H., Buckingham, E.B., Criscitiello, M.F. and Flajnik, M.F., 2010. Emergence of the acute-phase protein hemopexin in jawed vertebrates. *Mol. Immunol.*, 48: 147-152. https://doi.org/10.1016/j. molimm.2010.08.015
- Hirayama, M., Nakaniwa, M., Ikeda, D., Hirazawa, N., Otaka, T., Mitsuboshi, T., Shirasu, K. and Watabe, S., 2003. Primary structures and gene organizations of two types of Wap65 from the pufferfish Takifugu rubripes. *Fish Physiol. Biochem.*, **29**: 211-224. https://doi.org/10.1023/ B:FISH.0000045723.52428.5e
- Kikuchi, K., Watabe, S., Suzuki, Y., Aida, K. and Nakajima, H., 1993. The 65-kDa cytosolic protein associated with warm temperature acclimation in goldfish, *Carassius auratus. J. comp. Physiol. B.*, 163: 349-354. https://doi.org/10.1007/BF00265637
- Kikuchi, K., Yamashita, M., Watabe, S. and Aida, K., 1995. The warm temperature acclimation-related 65-kDa protein, Wap65, in goldfish and its gene expression. J. biol. Chem., 270: 17087-17092. https://doi.org/10.1074/jbc.270.29.17087
- Kim, Y.O., Park, E.M., Moon, J.Y., Nam, B.H., Kim, D.G., Kong, H.J., Kim, W.J., Jee, Y.J. and Lee, S.J., 2013. Genetic organization of two types of flounder warm-temperature acclimation-associated 65-kDa

protein and their gene expression profiles. *Biosci. Biotechnol. Biochem.*, **77**: 2065-2072. https://doi. org/10.1271/bbb.130263

- Muller-Eberhard, U. and Fraig, M., 1993. Bioactivity of heme and its containment. Am. J. Hematol., 42: 59-62. https://doi.org/10.1002/ajh.2830420112
- Nakaniwa, M., Hirayama, M., Shimizu, A., Sasaki, T., Asakawa, S., Shimizu, N. and Watabe, S., 2005. Genomic sequences encoding two types of medaka hemopexin-like protein Wap65, and their gene expression profiles in embryos. *J. exp. Biol.*, 208: 1915-1925. https://doi.org/10.1242/jeb.01570
- Nikkilä, H., Gitlin, J.D. and Muller-Eberhard, U., 1991. Rat hemopexin. Molecular cloning, primary structural characterization, and analysis of gene expression. *Biochemistry*, **30**: 823-829. https://doi. org/10.1021/bi00217a036
- Pierre, S., Coupe, S., Prevot-d'Alvise, N., Gaillard, S., Richard, S., Gouze, E., Aubert, J. and Grillasca, J.P., 2010. Cloning of Wap65 in sea bass (*Dicentrarchus labrax*) and sea bream (*Sparus aurata*) and expression in sea bass tissues. *Comp. Biochem. Physiol., Part B, Biochem. Mol. Biol.*, 155: 396-402.
- Qiu, X., Li, D., Cui, J., Liu, Y. and Wang, X., 2014. Molecular cloning, characterization and expression analysis of melanotransferrin from the sea cucumber *Apostichopus japonicus*. *Mol. Biol. Rep.*, 41: 3781-3791. https://doi.org/10.1007/s11033-014-3243-1
- Sarropoulou, E., Fernandes, J.M., Mitter, K., Magoulas, A., Mulero, V., Sepulcre, M.P., Figueras, A., Novoa, B. and Kotoulas, G., 2010. Evolution of a multifunctional gene: The warm temperature acclimation protein Wap65 in the European seabass Dicentrarchus labrax. *Mol. Phylogen. Evolut.*, **55**: 640-649. https://doi.org/10.1016/j. ympev.2009.10.001
- Sha, Z., Xu, P., Takano, T., Liu, H., Terhune, J. and Liu, Z., 2008. The warm temperature acclimation protein Wap65 as an immune response gene: its duplicates are differentially regulated by temperature and bacterial infections. *Mol. Immunol.*, 45: 1458-1469. https://doi.org/10.1016/j.molimm.2007.08.012
- Stred, S.E. and Messina, J.L., 2003. Identification of hemopexin as a GH-regulated gene. *Mol. cell. Endocrinol.*, 204: 101-110. https://doi.org/10.1016/ S0303-7207(03)00149-7
- Watabe, S., Kikurchi, K. and Aida, K., 1993. Cold- and warm-temperature acclimation induces specific cytosolic proteins in goldfish and carp. *Nippon Suisan Gakkaishi*, **59**: 151-156. https://doi. org/10.2331/suisan.59.151