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RESEARCH PAPER

Ferritin Gene from the Swimming Crab (*Portunus trituberculatus*) Involved in Salinity Stress Adaptation

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Abstract

Ferritin is a highly conserved iron storage protein playing an important role in the iron metabolism and cell protection. Swimming crab (*Portunus trituberculatus*) is an important fishery and aquaculture species in China and water salinity has a significant effect on its physiological processes. In order to verify whether the ferritin gene contributed to swimming crab salinity adaptation, ferritin (*Pt*Fer) cDNA open reading frame (ORF) was cloned. Homologous amino acid sequence alignment of *Pt*Fer showed a1 higher similarity to the ferritin heavy chain than the light chain. The gene expression profiles of *Pt*Fer under different salinity treatments were investigated by semi-quantitative RT-PCR. To further validate the salinity tolerance functions of *Pt*Fer, we investigated the eukaryotic expression of *Pt*Fer recombinant plasmid in 239T cells under a series of salinity stress. The results showed that the survival rate of the cells transfected with *Pt*Fer gene recombinant plasmid was significantly higher than that of the cells transfected with plasmid without insert fragment during the low salinity challenges, which indicated that *Pt*Fer might possess a protective effect against low salinity stress. Therefore, our results together indicated that the *Pt*Fer gene plays an important role in swimming crab salinity adaptation physiological process.

Keywords: *Portunus trituberculatus*, ferritin, eukaryotic expression, salinity adaptation.

Introduction

Ferritins are the highly conserved iron storage and iron detoxification proteins that can sequestrate the excess free iron at the state (Fe^{2+}) to prevent the harmful reactive oxygen species (ROS) generation and reduce the oxidative damages (Harrison and Arosio, 1996; McCord, 1996; Bou-Abdallah, 2010). It has been shown to be ubiquitous in many organisms including Archea, eubacteria, plants and mammals but not in yeasts (Arosio *et al.*, 2009). Ferritins are mainly found in the cytoplasm as tiny dense particles in animals, and it also can be found in the serum, nuclei, mitochondria and the biological fluids in plants. There are multi-gene ferritins laid in the plastids and chloroplasts responding to different environmental stress. And the ferritins found in the mitochondria indicated the ferritins have a crucial role in the life of cells like respiration and apoptosis (Iancu, 1992; Thompson *et al.*, 2002; Theil, 2007; Recalcati *et al.*, 2008; Crichton and Declercq, 2010). The Ferritin was found be correlated with some diseases. For example, serum ferritin levels had been reported to be altered in the condition of thyroid disease and the low serum ferritin levels can reflect the hypothyroidism disease (Sachdeva *et al.* 2015). Also , the serum ferritin concentration was significantly related with metabolic syndrome and red meat consumption. In other ways , the oxidative stress markers (carbonyl groups, AOPP, and glycated hemoglobin) or hepatic damage markers (GGT, SGOT), the parameters of insulin resistance (HOMA, blood insulin, and blood glucose) also were showed had significant correlations with the serum ferritin concentrations (Felipe *et al.* 2015). The higher serum ferritin level was correlated with lower hospital admission GCS (Glasgow Coma Scale) which can be a indicator of severe traumatic brain injury (Simon *et al.* 2015). The pharmacological induction of ferritin leads to the osteoblastic transformation diminishment of the smooth muscle cells which would result in the calcification and the enhancement of ferritin synthesis (Becs *et al.* 2015).

In general, there are two basic ways affecting the ferritin synthesis. One is the iron production and release at translational level. At translational level, the translation of ferritin was regulated by the Iron Responsive Elements (IREs) and the repressors Iron Responsive Proteins IRP1 and IRP2 (Testa *et al.*, 1989; Hubert *et al.*, 1993; Arosio *et al.*, 2009). The IRPs untranslated regions were bind to IREs to

prevent ferritin mRNA translation at the condition of iron depletion. And the IRPs can also prevent IREs binding by an iron-sulfur cluster switch mechanism to let the ferritin translated under the condition of iron repletion (Pantopoulos, 2004; Rouault, 2006). The other way affecting the ferritin synthesis is at the transcriptional level when responding to oxidative stress. The ferritin synthesis is mediated by the antioxidant/electrophilic responsive element (ARE/EpRE) on the manner of activating the transcriptional factors (Hintze and Theil, 2005; Iwasaki *et al.*, 2006; Theil, 2007; Orino and Watanabe, 2008).

Till so far, there are lots of literatures demonstrate that the ferritin protein can offer valid protection mechanisms against the harmful effects of dioxygen damages (Caban-Hernandez *et al.*, 2012), bacterial infections (Rebl *et al.*, 2012) and DNA damages by the UV (Shimmura *et al.*, 1996). It also offers immunity protections for the vertebrates and invertebrates (Ong *et al.*, 2006), and protects the cells from injury under cold stress (0℃ or 4℃) (Zieger and Gupta, 2009). The salinity stress would induce Reactive oxygen species (ROS), and the ferritin can suppress ROS accumulation via maintaining iron metabolism and act as an anti-apoptotic protein (Calabretta *et al.*, 1986). The ferritin was also considered as an unknown osmotic regulation protein (Bundzikova *et al.*, 2008). For example, the ferritin (*PpFer4*) expression was significantly increased after being exposed to salinity challenges (Xi *et al.*, 2011), and the accumulation of ferritin can be lasted longer time after being treated in 50 mM NaCl + 50μM sodium nitroprusside than just being treated in NaCl solution, which can protect the barley (*Hordeum vulgare*) effectively (Li *et al.*, 2008). The up-regulations of the ferritin protein under 100 mmol/L NaCl challenges indicated that the ferritin protein might contribute to the soybean seeds germination responding to the salinity stress (Xu *et al*., 2011).

The swimming crab (*Portunus trituberculatus*), is a commercially important euryhaline crab species, and its distributions and migration route were influenced by the salinity change (Dai, 1977; Sun *et al*., 1984; Dai *et al.*, 1986; Xue *et al*., 1997; Xu and Liu, 2011). The water salinity condition is also an important factor for artificial propagation of the swimming crab, especially in larval development and molt stage (Ji, 2005). Therefore, the research on the genetic mechanisms to environmental salinity changes would possibly help the crab artificial propagation. Investigating the swimming crab genetic mechanisms responding to the environmental salinity changes will also be an interest for marine biology and ecology. A transcriptome analysis of the gills of *Portunus trituberculatus* under the salinity stress was conducted by the Illumina Deep Sequencing technology aimed to explain mechanism of osmoregulation in *P. trituberculatus* when faced salinity challenges (Lv *et al.* 2013). In our previous study, gill cDNA library of expressed sequence tags (ESTs) was constructed from the swimming crab exposed to two different salinity challenges (10 and 35ppt) (Xu *et al.*, 2010). A total of 2426 transcripts were selected for microarray construction based on criteria described in Xu *et al.* (2010). The results showed that the ferritin (*Pt*Fer) gene was found to have different expression levels in the swimming crab exposed to 10 or 35 ppt salinity challenges compared with the 25 ppt normal seawater, which indicated that the *Pt*Fer gene might be involve in the salinity acclimation (Xu and Liu, 2011). Although ferritin was considered as an unknown osmotic regulation protein (Bundzikova *et al.*, 2008), the functional study of ferritin in lower invertebrates were very limited, and none study has been conducted in the crustacean species to date. Therefore, our study could facilitate further understanding about the molecular mechanism of ferritin gene in lower invertebrates especially the crab during the salinity stress.

In order to verify the role of *Pt*Fer gene in the protection against salinity stress, the *Pt*Fer gene open reading frame (ORF) was cloned from the swimming crab. The gene expression profiles of *Pt*Fer under different salinity treatments were investigated by semi-quantitative RT-PCR. In addition, the metabolic activity of cells transfected with *Pt*Fer recombinant plasmid exposed to different salinity stresses were assessed by AlamarBlue. The results of our study would provide useful evidences for the further genetic improvement of *P. trituberculatus* propagated during the fluctuated salinity stress.

Materials and Methods

Samples Preparation and Rearing Conditions

All the healthy male *P. trituberculatus* crabs, averaging 150±25g in weight, were collected from Zhoushan Archipelago of the East China Sea. After being exposed to the 25ppt, 10ppt or 40ppt salinity challenges for 6h, tissues including stomach, abdominal muscle, dermis, heart, appendicular muscle, sex gland, liver, antennal gland, intestines and the 6th pair of gills were removed from the exposed crabs. Other crabs were acclimatized in tanks at 18°C, containing the water of 25ppt, 10ppt or 40ppt salinity, under continuous aeration for 3 days, then the 6th and 7th gills together at the time point 12h, 24h, 48h, 72h, 120h salinity challenges were extracted, respectively (Xu and Liu, 2011). The samples were kept frozen at -80°C for further study.

Total RNA Extraction and cDNA Synthesis

Total RNA from the swimming crabs was extracted using TRIZOL reagent (Invitrogen) following the manufacturer's protocol, and the concentration was determined at the absorbance 260/280nm ratios using a spectrophotometer

(Nanodrop 2000C, Thermo). The RNA was run on 1% agarose electrophoresis to check the integrity. The cDNA was reverse transcribed from the 2μg of RNA, using 1μl oligo(dT) primer, 2μl dNTP, 1μl M-MLV Reverse Transcriptase and 4μl buffer (Promega, USA), and ddH_20 in total volume of 25μl.

Cloning of *Pt*Fer Open Reading Frame (ORF)

In order to clone the *Pt*Fer open reading frame (ORF), we designed two specific primers based on the EST (PT0013D12) obtained from the ESTs library (Xu *et al.*, 2010) as following: *Pt*Fer-P1 (5′CCGAATTCATGTGTAGCCAAGTCCGCCAGA 3′, position: 1-22bp), *Pt*Fer-P2 (5′CGCTCGAGTTAAGCAAGCTCCTTGTCAAAC 3′, position: 492-513bp) with restriction enzyme *EcoR I* and *Xho I* sites respectively (underlined, see Figure 1). Then the PCR reaction mixture contained 10pmol of each primer 0.6μl, 10×PCR buffer 2.5μl, 10mM of each dNTP (with 1.5 mM $MgCl_2$) 2μl , H_20 18.1μl, 5U/μl TaqDNA polymerase 0.2μl in a total volume of 25μl (Aidlab Biotechnologies, China). The amplification was performed on a thermocycler (Bio-rad, USA), initially denatured 95°C for 5 min, 34 cycles of following: 30s at 95°C , 45s at 64°C , 1min extension at 72°C. The 34 cycles were followed by a final extension of 10 min, and cooling to 4°C before the PCR products were removed from the thermocycler.

The amplified products were visualized on 1% agarose gel electrophoresis with a DL2000 ladder (TAKARA, Dalian, China). The PCR productions were purified by using TIANgel Mini Purification Kit (TIANGEN, China), then inserted into pMD18-T vector (TAKARA, China) and transformed into the competent *Escherichia coli* DH5α cells (TIANGEN). Subsequently, cells were spread on to agar plates containing LB-ampicillin/IPTG/Xgal and incubated overnight at 37°C to promote selective growth of transformed colonies. Positive colonies were identified by white/blue selection and then subject to ABI 3730 DNA sequencing with T3 and T7 universal primers (Sangon, China).

cDNA Sequences Analysis, Multiple Sequence Alignment and Phylogenetic Analysis

The full-length cDNA sequence of *Pt*Fer was analyzed for similarity with the BLAST programs (Altschul *et al.*, 1997) at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/). The deduced amino acid sequences, molecular mass and theoretical isoelectric point (pI) were predicted by using the DNASTAR7.0 software. Alignment of the amino acid sequence of *Pt*Fer with those of other ferritins was analyzed by Clustalx software. Molecular phylogenetic trees were constructed using the neighbor-joining method from the phylogenetic component of the MEGA6.05 software, and the tree topology was evaluated by 1,000 replications bootstraps.

***Pt*Fer mRNA Tissues Expression Analysis**

Semi-quantitative RT-PCR was used to analyze the abundance of *Pt*fer mRNA in various tissues of *P. trituberculatus.* Total RNA (2μg) was isolated from the abdominal muscle, dermis, heart, sex gland, liver,

Figure 1. Nucleotide and deduced amino acid sequences of *Pt*Fer (GenBank accession AEK81609.1)
The nucleotide sequence is numbered from the 5′ end, and the single letter aa code is shown below the corresponding codon. Nucleotide and deduced amino acid residues are numbered on the left. The start code ATG and the termination code are shown underlined, and the * indicated the termination code. The sequence contains 513 nucleotides, translated to 170 amino acids with a predicted molecular mass of 19.4 kDa and a theoretical isoelectric point (pI) of 5.196. The designed primers (*Pt*Fer-P1/P2) are indicated by light grey.

antennal gland, stomach, appendicular muscle, intestines and the 6th pair gills of the crabs which were acclimated to salinity challenge of 10ppt, 25ppt or 40ppt for 6h. Another 2μg of total RNA of 6th and 7th gills of the crabs adapted to salinity challenge at the time points of 12h, 24h, 48h, 72h, 120h were also isolated. The *Pt*Fer mRNA was amplified using a pairs of specific primers: *Pt*Fer-P1 (5' CCGAATTCATGTGTAGCCAAGTCCGCCAGA 3') and *Pt*Fer-P2 (5' CGCTCGAGTTAAGCAAGCTCCTTGTCAAAC 3'). The amplification of ribosomal protein L8 (GenBank accession number GW399316), which was used as an internal PCR control, was performed using a pairs of specific primers: L8F (5′ GCGTACCACAAGTATCGCGT 3′) and L8R (5′ AGACCGACCTTCCTACCAGC 3′).

The PCR reaction mixture contained 10 μmol of each primer 1μl, cDNA template 1μl, 10×PCR buffer 2.5μl, dNTP 0.5μl, $MgCl_2$ 1.5μl, ddH_20 17μl and 5U/μl TaqDNA polymerase 0.2μl (Sangon, Shanghai, China) with a PCR protocol consisting of 95°C for 5 min, then 27 cycles of 30 s at 95°C, 45 s at 60°C, 1min at 72°C, after cycles was a final extension 72°C for 10 min. The RT-PCR reaction conditions of ribosomal protein L8 was denaturation at 95°C (5 min), 27 cycles at 95°C (30 s), 56°C (45 s), 72°C (1min), a final extension at 72°C (10 min). The relative expressions of ferritin were analyzed on 1.0% agarose gel electrophoresis with a DL2000 ladder (TAKARA, Dalian, China) by AlphaView (version 1.2.0.1) software. The relative expression of *Pt*Fer mRNA was determined on the ratio of band intensity *Pt*Fer to ribosomal protein L8. Statistical analysis was performed by Statistics17.0 with a one-way ANOVA, using a Duncan test and Independent Samples *t* Test examined significant differences. Differences were considered to be significant at $P<0.05$.

Construction of Eukaryotic Expression Vector EGFP-C2-*Pt*Fer

To construct the EGFP-C2-*Pt*Fer eukaryotic expression plasmid, the *Pt*fer ORF fragment was amplified by PCR with the primers shown as following: *Pt*fer-F1 (5' CCCTCGAGGATGTGTAGCCAAGTCCGCCAGA 3') and *Pt*Fer-F2 (5' CGGAATTCTTAAGCAAGCTCCTTGTCAAAC 3') with restriction enzyme sites of *XhoI* and *EcoRI*. The PCR productions were electrophoresed on 1% agarose gel and purified using a DNA purification kit (Aidlab, China), then were subcloned into a pMD18-T vector and transformed into *E. coli* DH5α cells. The inserted *Pt*Fer, being excised from pMD18-T vector by being digested with restriction endonuclease enzymes *XhoI* and *EcoRI* (Fermentas), was ligated into the *XhoI*, *EcoRI*-digested vector EGFP-C2 (TAKARA) at 16℃ overnight, and then was transformed into *E. coli* DH5α cells (TIANGEN). The target recombinant transformants were selected by using the kanamycin on the Luria Bertani (LB) agar plates. The recombinant plasmid was extracted by using a QIAGEN EndoFree Plasmid Maxi Kit according the manufacturer's protocol. Then, the target fragment was identified by digesting with the restriction enzyme *XhoI* and *EcoRI* and run on 1.0% agarose gel electrophoresis. The *Pt*Fer cDNA was finally sequenced by Sangon Biotech Company (Shanghai, China).

Cell Culture

Cell culture experiments were performed by using the human embryonic kidney 293T cells, cultured in Dulbecco's modified Eagle's medium/HIGH GLUCOSE (DMEM) (HyClone, America) with 10% fetal bovine serum FBS and penicillin- streptomycin (100U/ml-100ug/ml, HyClone, America), supplemented with 5% CO_2 at a humidified environment 37℃.

Expression of EGFP-C2-*Pt*Fer Recombinant Plasmid in 293T Cells against Salinity Challenges

The 293T cells were transiently transfected using the Entranster™-D transfection reagents (Engreen Biosystem Co, Ltd, China) with the expression vector EGFP-C2 either with or without *Pt*Fer cDNA fragment. The method was described as following: the cells were plated onto 96-well culture plates (Corning) at a density of $1.0x10^5$/ml along with 100μl culture medium per well without penicillin and streptomycin, and the cells were incubated at 37℃ until the cells grew to 50% confluence. The 2μg vector with or without *Pt*Fer insertion was pipetted into 25μl DMEM medium, and 9μl Entranster™-D transfection reagents were then pipetted into another 25μl DMEM medium. After incubated for 5 minutes, we mixed the two together and incubated for 30 minutes to get the transfection mixed reagents. 5μl transfection mixed reagents were added into the transfected cells in the 96-well plates per well and cultured for 6 hours. After 6 hours transient transfection, the culture medium was changed by the complete medium consisting of 10% fetal bovine serum, and 1% 100 U/ml penicillin/100 mg/ml streptomycin (Hyclone) to continue culturing for 12 hours.

To examine whether the *Pt*Fer mRNA was successfully expressed in the *Pt*Fer transfected cells, total RNA of transfected cells were extracted and checked by RT-PCR analysis by using the primer *Pt*Fer-F1 and *Pt*Fer-F2. Subsequently, the transfected cells were exposed to two different salinity challenges: high salinity challenge (100μl DMEM medium with 10μl 31% NaCl solution added per well) and low salinity challenge (50μl DMEM medium with 200μl ddH_20 added per well). Three groups were set for each salinity challenge: control group (normal

cells without transfection reagents), cells transfected by plasmid EGFP-C2 without an insertion and cells transfected by recombinant plasmid EGFP-C2-*Pt*Fer. And each group has at least three duplicates.

AlamarBlue were added to the culture medium (1/10 dilution in media) as an indicator of cell proliferation, then continued to culture for 24h, 36h, 48h, 60h and 72h. At each time point, the absorbance was measured at 490nm or 595nm on a FlexStation3 Benchtop Multi-Mode Microplate Reader (Molecular Devices, USA), The percentage reduction of AlamarBlue (%) was calculated as following equation: $[A_{LW}-(A_{HW}\times R_0)\ ]\times 100\%$ (A_{LW}=absorbance at lower wavelength minus the media blank, A_{HW}= absorbance at higher wavelength minus the media blank; $R_0=AO_{LW}/AO_{HW}$, AO_{LW} = Absorbance of AlamarBlue oxidized form in media at the lower wavelength – Absorbance of media only, AO_{HW} = Absorbance of AlamarBlue oxidized form in media at the higher wavelength – Absorbance of media only (www.abdserotec.com/alamarBlue).

Statistical Analysis

All the measurements (percentage reduction of alamarBlue (%)) were performed in duplicate for at least three replicates. The statistical analysis was performed using Statistics17.0. (SPSS Inc.) . The data were analyzed with one-way ANOVA by a Duncan test to compare the growth differences between the normal 293T cells, the cells transfected with plasmid EGFP-C2 without an insertion and the cells transfected with EGFP-C2-*Pt*Fer recombinant vector against different salinity challenges. The value are reported as mean ± standard deviation (SD), and different letters indicated the significance of differences at $P<0.05$.

Results

Identification of *Pt*Fer Open Reading Frame cDNA

The partial *Pt*Fer gene (PT0013D12) from the swimming crab was obtained from our previous constructed gill cDNA library (Xu *et al.*, 2010). By using primers (*Pt*Fer-P1 and *Pt*Fer-P2), the coding sequence of the full-length cDNA of *Pt*Fer was obtained in our study. The open reading frame of the *Pt*Fer gene is 513bp in length from the start codon (ATG) to the stop codon (TAA) coding for a protein of 170 amino acids (Figure 1) with a predicted molecular mass of 19.4 kDa and a theoretical isoelectric point (pI) of 5.196.

The deduced amino acid sequence protein of the *Pt*Fer performed by BLAST (BLASTP) of the NCBI database showed the amino acid sequence has 100% identities with the known ferritin protein (AEK81609.1) of *P. trituberculatus*, and the structural domain of *Pt*Fer contains the ferrihydrite nucleation center and iron ion channel, which indicates the *Pt*Fer we cloned was conformed to the basic characteristics of ferritin protein (Figure 2).

Phylogenetic Analysis of the *Pt*Fer Protein

The deduced amino acid sequence compared with the other known ferritin proteins were performed by the BLAST search against NCBI database. The results showed that the *Pt*Fer revealed high conserved amino acids with ferritin protein from *Arthropoda*, especially from crustacean species. The detailed comparisons were shown in Table 1.

Homologous amino acid sequence alignment of the various ferritin proteins from the *Arthropoda, Chordata, Mollusca and Tracheophyta* was performed by ClustalX. The results were shown in Figure 2. As shown in Table 1 and Figure 2, the deduced amino acid sequence of *Pt*Fer had the highest identities (95%) with *Scylla paramamosain* ferritin (ADM26622.1), while it shared comparatively higher identities with other crustacean species including *Fenneropenaeus indicus* (AEQ53930.1) (83%), *Eriocheir sinensis* (ADD17345.1) (82%), *Litopenaeus vannamei* (AAX55641.1) (82%) and *Fenneropenaeus chinensis* (ABB05537.1) (82%). It should be noticed that the *Pt*Fer showed higher conservative with heavy chains of ferritin than the light chains. For example, it shared higher identities with the *Homo sapiens*-H (AAA35832.1) (61%) and *Mus musculus*-H (NP_034369.1) (60%), but lower identities with the *Homo sapiens*-L (NP_000137.2) and *Mus musculus*-L (NP_034370.2) with the identities 50% and 45% (Table 1 and Figure 2).

A phylogenetic tree was generated by analyzing the homologous amino acid sequence of the various ferritin proteins. As shown in Figure 3, *Pt*Fer is clustered with the *Scylla paramamosain* (ADM26622.1) while it is not clustered into the clade of Vertebrata (Figure 3).

The *Pt*Fer mRNA Expression Levels in the Tissues

We then analyzed the *Pt*Fer mRNA expression and distribution in the examined tissues by semi-quantitative RT-PCR. The examined tissues included stomach, abdominal muscle, dermis, heart, appendicular muscle, sex gland, liver, antennal gland, intestines and the 6th pair of gills that were exposed to normal 25ppt salinity, 10ppt or 40ppt salinity challenges for 6h. As shown in Figure 4, at the first glance, under the low (10ppt) salinity challenges, the abdominal muscle and gill tissues had a very abundant expression level, while the antennal gland and dermis had a lower expression level. The expression of *Pt*Fer in appendicular muscle was higher than that in the 25ppt or 40ppt salinities ($P<0.05$). And the *Pt*Fer could be found in all the detected tissues under the normal (25ppt) salinity (the 1% agarose electrophoresis picture was not

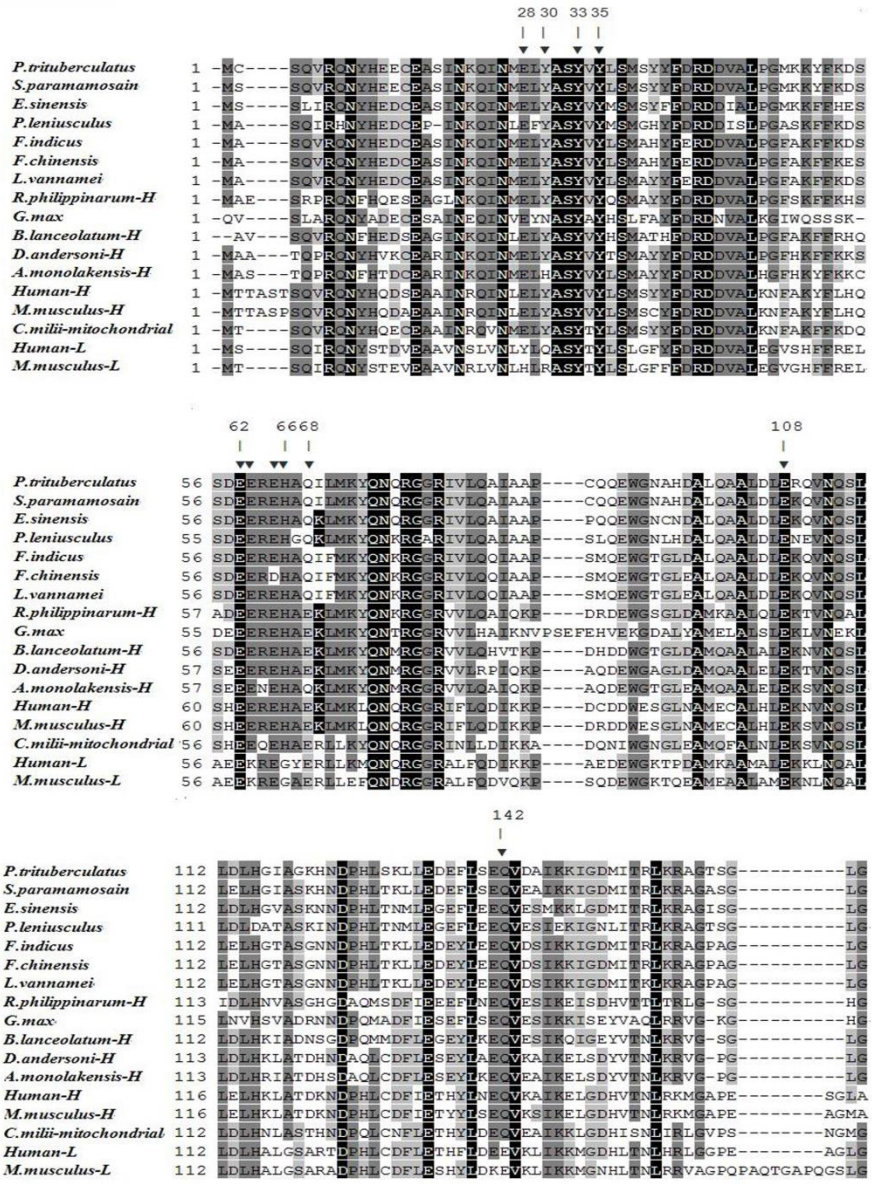

Figure 2. Alignment of the *Pt*Fer amino acid sequence of known ferritin proteins

Clustalx was used to compare the ferritin sequences. The amino acids are numbered along the left margin. Residues in black background indicate 100% of amino acid identities. Residues identical with the threshold of 80% in all sequences are shaded. And residues identical with the threshold of 50% in all sequences are light shaded. Arrowheads (▼) indicate the conserved residues important in vertebrate H-ferritin and they are involved in ferroxidase center. And the numbers were corresponding to numbering of human H ferritin sequence. The species names and GenBank accession numbers are given in Table 1.

published). The expression of *Pt*Fer in stomach and dermis in 25ppt was higher than that in the 10ppt or 40ppt ($P<0.05$). At the high salinity challenges (40ppt), the expression of *Pt*Fer in antennal gland had a higher level than that in the 25ppt or 40ppt ($P<0.05$).

To identify whether *Pt*Fer mRNA expression was time dependent under different salinity challenges, the *Pt*Fer mRNA expression of the *P. turberculartus* gills exposed to 10ppt or 40ppt salinity challenges in various times including 12h, 24h, 48h, 72h and 120h were detected by using semi-quantitative RT-PCR analysis. As shown in Figure 5, the *Pt*Fer mRNA expression had different expression levels under different salinity challenges. It was clear that the expression levels of *Pt*Fer seemed had no significant changes during the low salinity (10ppt) challenges. As for the high (40ppt) salinity stress, a significant up-regulated expression of *Pt*Fer mRNA was observed in the first 12-24h, then returned to the lower levels with the prolonged exposure time. It should be noticed that, compared with the condition of 10ppt, significant differences in the expression levels of *Pt*Fer mRNA were observed at 12h, 48h and

Table1. The related information of ferritin genes and the identities to *Pt*Fer of *P. trituberculatus*

Species	Common name	Class	Accession number	Number of amino acids	Identities
Scylla paramamosain	Green mud crab	Arthropoda	ADM26622.1	170Aa	95%
Fenneropenaeus indicus	Indian Prawn	Arthropoda	AEQ53930.1	170Aa	83%
Eriocheir sinensis	Chinese mitten crab	Arthropoda	ADD17345.1	170Aa	82%
Litopenaeus vannamei	Pacific white shrimp	Arthropoda	AAX55641.1	170Aa	82%
Fenneropenaeus chinensis	Chinese shrimp	Arthropoda	ABB05537.1	170Aa	82%
Pacifastacus leniusculus	Freshwater crayfish	Arthropoda	Q26061.1	181Aa	75%
Dermacentor andersoni-H like	Rocky Mountain Wood Tick	Arthropoda	AAR21568.1	172Aa	69%
Argas monolakensis-H	Mono lake bird tick	Arthropoda	ABI52633.1	174Aa	69%
Branchiostoma lanceolatum-H	Amphioxus	Chordata	AAN63032.1	175Aa	65%
Callorhinchus milii-mitochondrial	Elephant shark	Chordata	AFM90243.1	176Aa	65%
Ruditapes philippinarum-H like	Manila clam	Mollusca	AFU61136.1	171Aa	63%
Homo sapiens-H	Human	Chordata	AAA35832.1	183Aa	61%
Homo sapiens-L	Human	Chordata	NP_000137.2	175Aa	50%
Mus musculus-H	House mouse	Chordata	NP_034369.1	182Aa	60%
Mus musculus-L	House mouse	Chordata	NP_034370.2	183Aa	45%

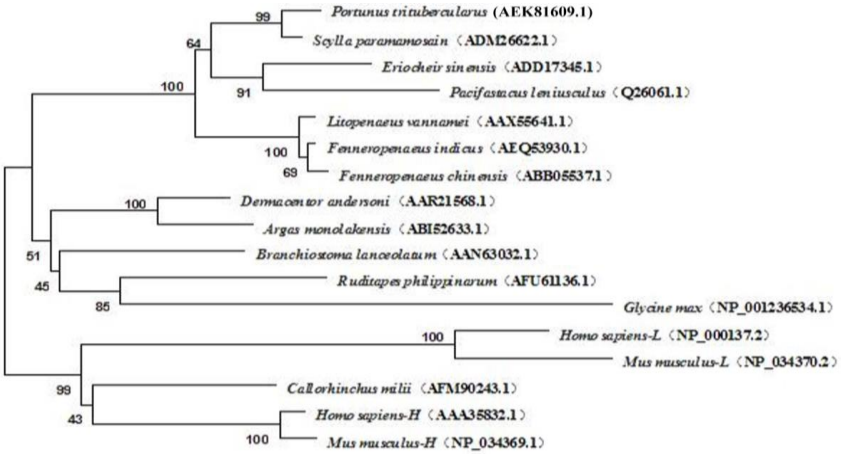

Figure 3. Phylogenetic analysis of *Pt*Fer. Alignment of amino acid sequences are produced by ClustalX, and the bootstrap neighbor-joining phylogeny tree was constructed by MEGA 6.05 (bootstrap=1,000). The species and accession numbers are the same as shown in Table 1. Branch lengths are proportional to estimates of evolutionary change. The number associated with each internal branch is the local bootstrap probability, which is an indicator of confidence.

48h during the high salinity challenges ($P<0.05$, Figure 5).

Eukaryotic Expression of *Pt*Fer in 293T Cells under Salinity Challenge

To validate the effect of recombinant plasmid EGFP-C2-*Pt*Fer on 293T cells' salinity tolerance, we compared the cell growth differences between the normal cells (control), cells transfected with the plasmid EGFP-C2 without an insertion and the cells transfected with the EGFP-C2-*Pt*Fer recombinant vector against salinity challenges. Before we conducted our salinity challenging experiments, the transfection success were verified by RT-PCR analysis. *Pt*Fer mRNA expressions were only detected in the cells transfected with EGFP-C2-*Pt*Fer (data not shown). The cell metabolic activity analysis was performed during the low (200µl H_20 with 50µl DMEM medium) and high (10µl 31% NaCl solution with 100µl DMEM medium) salinity challenges by measuring the percentage reduction of AlamarBlue (%).

The results showed that during the low salinity challenges, the cells transfected with EGFP-C2-*Pt*Fer recombinant vector exhibited less salinity tolerance than the normal cells, but higher resistance than the cells transfected with the plasmid EGFP-C2 without an insertion (Figure 6), which indicated that *Pt*Fer might have strong functions against low salinity challenges. As for the high salinity challenge, all the cells including the normal cells, the cells transfected with EGFP-C2-*Pt*Fer recombinant vector and the cells transfected with EGFP-C2 without an insertion showed no significant differences (the data not shown).

Discussion

In this study, we cloned the *Pt*Fer gene open reading frame (ORF) from *P. trituberculatus*, which containing 513bp in length coding for a protein of 170

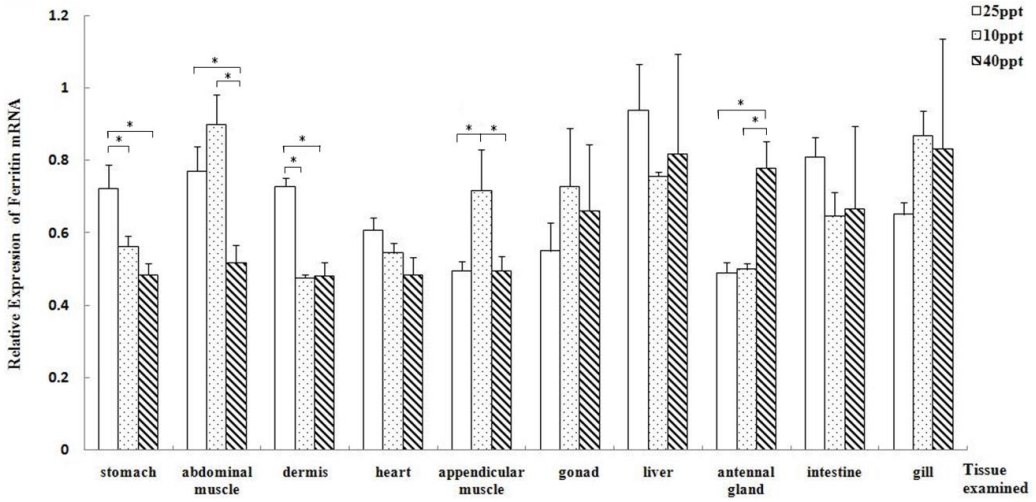

Figure 4. Relative expression of *Pt*Fer mRNA of the swimming crab exposed to normal salinity (25 ppt) and salinity challenges (10ppt or 40ppt) at the time of 6h, as determined by semi-quantitative RT-PCR. The examined tissues of three crabs in each group were collected. The tissues were including stomach, abdominal muscle, dermis, heart, appendicular muscle, gonad, liver, antennal gland, intestine, gills. The ribosomal protein L8 (RL8) was used as an internal and the relative expression levels of *Pt*Fer were obtained relative to RL8 expression. Values are expressed as means±SD of the relative variations (fold induction) between each treatment (10ppt or 40ppt) and the control sample (25ppt); asterisks beside the bars indicate statistically significant differences among the same tissues in three different salinity stress (*P<0.05).

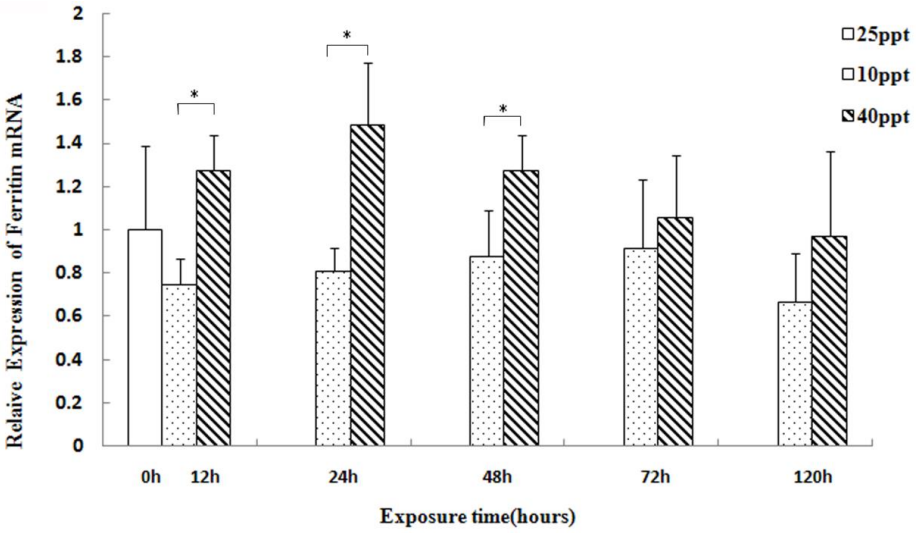

Figure 5. The relative *Pt*Fer mRNA expression levels in gill tissue at different time points in response to salinity challenges (10ppt or 40ppt) during the time of 12h, 24h, 48h, 72h and 120h. The 6th and 7th gills tissues of three crabs in each group were collected. Transcript levels for all samples were assessed by semi-quantitative RT-PCR, and the relative expression levels of *Pt*Fer were obtained relative to RL8 expression. Values are expressed as means±SD of the relative variations (fold induction) between each treatment (10ppt or 40ppt) and the control sample (25ppt); asterisks above the bars indicate statistically significant differences (*P<0.05) between the 10ppt and 40ppt; 0–120 h, sampling point after salinity challenge.

aa with a predicted molecular mass of 19.4 kDa and a theoretical isoelectric point (pI) of 5.196 (Figure 1). Using the BLAST analysis, we found the *Pt*Fer deduced amino acid sequence contained the eukaryotic ferritin domain and possessed the ferroxidase diiron center (ion binding site) (Figure 2). And the *Pt*Fer deduced amino acid sequence showed higher identities to the invertebrate species: 95% to *Scylla paramamosain*, 83% to *Fenneropenaeus indicus* , 82% to *Eriocheir sinensis*, 82% to *Litopenaeus vannamei*, 82% to *Fenneropenaeus chinensis*, 75% to *Pacifastacus leniusculus*, 69% to *Dermacentor andersoni*-H like and *Argas monolakensis*-H (Table 1). When compared with the vertebrate species, the deduced amino acid sequence of *Pt*Fer showed higher identities to the heavy chain ferritin than to the light chain: 61% to the *Homo sapiens*-H, 60% to the *Mus musculus*-H, 50% to the

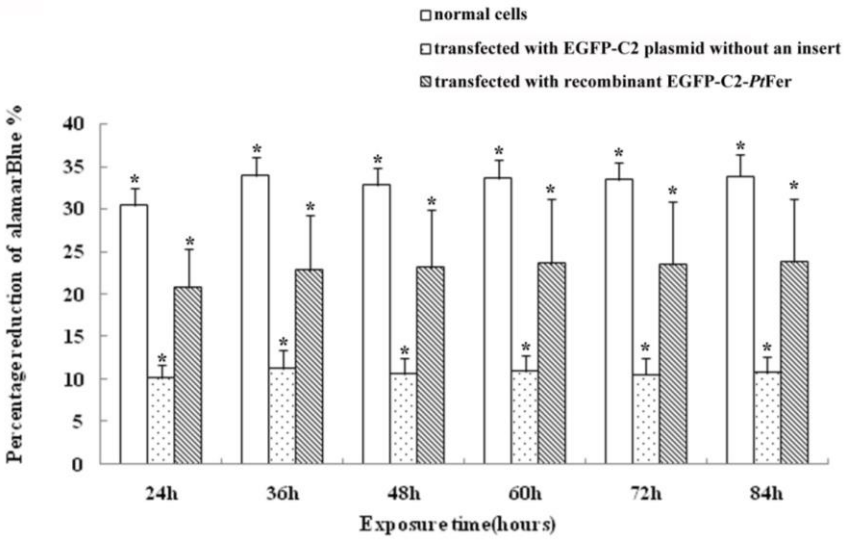

Figure 6. The cells metabolic activity exposure to the low (200μL H_20 with 50μL DMEM medium) salinity stress detected by Alamar Blue assay. The values are represented as means±SD, statistically significant differences (**p*<0.05) indicated as asterisks symbols and the compared was performed in the three cells in the same times.

Homo sapiens-L, 45% to *Mus musculus*-L (Table 1). The conserved residues Glu28, Tyr35, Glu62, Glu63, Glu65, His66, Glu68, Glu108, Gln142 (numbering by human H ferritin sequence) are the important ferroxidase centers in vertebrate H ferritin (Dhar *et al.*, 1993). And the multiple sequence alignment of ferritin protein sequence analysis showed they are obviously conserved in *Pt*Fer except for Glu68 (Figure 2). The residues Tyr30, Tyr33, Tyr35, Glu63 and His66 are consider as the sites of formation of Fe (III)-tyrosinate complex (Waldo *et al.*, 1993; Prochazkova *et al.*, 2011). Therefore, we could conclude that the *Pt*Fer is a homologue of H-type ferritin.

The phylogenetic analysis showed the *Pt*Fer and *Scylla paramamosain* were paraphyletic to *Eriocheir sinensis* and *Pacifastacus leniusculus*, and they clustered with other crustacean species including the *Litopenaeus vannamei*, *Fenneropenaeus indicus* and *Fenneropenaeus chinensis*, but separated from the insets and vertebrate species (Figure 3). The *Pt*Fer also had a similar subunit size with the ferritins of the vertebrate species ranging from 19kDa to 21kDa. In our previous study, prokaryotic expression plasmid pET28a(+)-*Pt*Fer was constructed and expressed in *E coli* DE3 (BL21). A band protein consistent with the predicted molecular weight of 19.4 kDa was observed by SDS-PAGE in the *E. coli* transformed by the recombinant plasmid pET28(+)-*Pt*Fer (Huang and Xu, 2013). The crustacea and vertebrates ferritins were suggested as the cytosolic ferritin located in cytoplasm of cells, whereas the insect ferritins were the different type secreted in yolk fluid, hemolymph and vacuoles (Locke and Nichol, 1992; Huang *et al.*, 1996; Arosio *et al.*, 2009). Therefore, we conclude that the *Pt*Fer is more similar to the vertebrates' ferritins, and it might have similar iron-storage functions on cell protections against oxidative stress since they all possess the conserved ferroxidase center residues to reduce the damage (Andrews *et al.*, 1992; Lin and Girotti, 1998).

The ferritin was a self-protection protein through sequestering the excess iron and dioxygen reaction products against the oxidant damage (Balla *et al.*, 1992; Theil, 2007). The iron responsive proteins (IRPs) triggered the labile iron pool (LIP) up and down in the cells to modulate the synthesis of ferritin subunits (Kakhlon *et al.*, 2001). For instance, down-regulation of the LIP would raise the expression of ferritin to protect the cells from oxidative damage (Kakhlon *et al.*, 2001). The mRNA expression analysis of *Pt*Fer gene showed that the *Pt*Fer gene was ubiquitously expressed in all the examined tissues under the normal salinity of 25ppt (data not shown). In fact, the ferritin gene was also widely expressed in various tissues of many Arthropoda species including stomach, heart, mantle, hematocytes, digestive gland, adductor muscle and gill (Durand *et al.*, 2004) and midgut gland, brain ganglion, hepatopancreas, eyestalk and muscle (Hsieh *et al.*, 2006). The extensive expressions of *Pt*Fer in various tissues of the swimming crab indicated *Pt*Fer played an important role in the physiology of *P. trituberculatus* and was synthesized continually under normal conditions.

In normal salinity (25ppt), the expression of *Pt*Fer gene in the hepatopancreas was more abundant compared with other tissues (P<0.05, the asterisk didn't marked), but there exist no significant difference among the three salinity challenges (25ppt, 10ppt and 40ppt) (Figure 4). The hepatopancreas was an important metabolic tissue under the condition of the reactive oxygen species (Soderhall and Cerenius, 1998). The high expression levels but no significant

variations during the salinities challenges indicated the role of *Pt*Fer in the hepatopancreas tissue might be related to metabolic process. The posterior gills and antennal gland were reported to play an important role in osmoregulation (Henry and Wheatly, 1992). It was clear that *Pt*Fer in the antennal gland showed a higher expression level when facing the high salinity stress (40ppt) (Figure 4). As for the gills, although there is no significant difference during the salinity challenges, the expression of *Pt*Fer was abundant in the gill tissues (Figure 4). The appendicular muscle in 10ppt had a higher expression compared with the expressions in 25ppt or 40ppt salinities, which indicated that the *Pt*Fer was more sensitive during the low salinity challenge (10ppt). As we know, stomach is known to play key roles in contacting with external environment (Clavero-Salas et al., 2007). Our results showed that the expression of *Pt*Fer in stomach had a comparable higher expression levels under normal salinity condition (25ppt), and was significantly dropped under low and high salinity stress (10ppt or 40ppt, $P<0.05$) (Figure 4), which indicated that low or high salinity stress (10ppt or 40ppt) might inhibit the normal metabolic activity of crab, and the PtFer could be regarded as a reflection of the inhibition brought by salinity variabilities. However, the correlations between osmotic regulations and energy savings were still unknown. As shown in Figure 4, high expression level of *Pt*Fer was also observed in the gill under the different salinity challenges, and our previous microarray assay showed that the *Pt*Fer gene was highly up-regulated in low salinity challenges (Xu and Liu, 2011), which together suggested that *Pt*Fer gene might play an important role in osmoregulation in the gills of *P. trituberculatus* especially under the low salinity (10ppt). Therefore, we selected the gill as the salinity sensitive tissue to conduct the time-dependent study (Figure 5).

From the tissue expression profiling results of the *Pt*Fer mRNA (Figure 4), we could suggest that salinity stress might disturb the iron balance and some specific tissues might generate more excess iron, which required more ferritin to keep the free iron at a possible lower level. Similar results were also found in the patients with deficiency of ceruloplasmin, a protein containing blue copper with ability of ferroxidase, had high iron level in the liver, pancreas and kindey and brain (Yoshida *et al.*, 1995). Furthermore, hemodialysis patients with lower iron level which was considered as "functional iron deficiency" might require the erythropoietin therapy; however, the normal or increased iron level was resist to the erythropoietin therapy because of the limited mobilization of ferritin iron (Adamson, 1994; Ponka, 1999). The two studies provide one possible explanation of the higher expression level *Pt*Fer in some specific tissues which might disturb physiology leading to facilitate the ferroxidase activity of *Pt*Fer to store the released iron. Similar to our result, the ferritin mRNA of euryhaline teleost, *Dicentrarchus labrax* showed up-regulation in the intestine during the freshwater-acclimated conditions (Boutet *et al.*, 2006), and the expression of ferritin in trunk kidney of juvenile ayu (*Plecoglossus altivelis*) was observed down-regulated when it was transferred from freshwater to brackish water (Chen *et al.*, 2009).

Since the gill was an important tissue for osmoregulation and detoxification (Clavero-Salas *et al.*, 2007), we therefore detected the *Pt*Fer expression levels in gill after different salinity challenges (10ppt or 40ppt). The *Pt*Fer expression showed a clear time-dependent response when exposed to high salinity stress (40ppt). The accumulation of *Pt*Fer reached the peak at 24h when facing the high salinity challenge (40ppt) and then began to decline to a lower level (Figure 5). The salinity inducible expression patten of *Pt*Fer was similar to the mangrove plant and pear (*Pyrus pyrifolia*) (Jithesh *et al*., 2006; Xu *et al.*, 2011). The ferritin expression level of those plants was increased high and then reduced in the later time of salinity stress, and the ferritin was deduced to contribute to stress defense or stress tolerance (Jithesh *et al.*, 2006; Xu *et al.*, 2011). Our result showed the high salinity treatment (40ppt) had a significant effect on the expression of *Pt*Fer, and we therefore deduced that the *Pt*Fer might contribute to *P. trituberculatus* salinity adaption (Figure 5).

In our previous study, prokaryotic expression plasmid pET28a(+)-*Pt*Fer was constructed and expressed in *E coli* DE3 (BL21). And it was found that *E. coli* cells transformed with pET28a(+)-*Pt*Fer were more resistant than the cells transformed with vector pET28a (+) without an insert when facing high salinity challenges (Huang and Xu, 2013). Subsequently, eukaryotic expression recombinant plasmid EGFP-C2-*Pt*CacyBP was constructed and the eukaryotic expression of *Pt*Fer in 293T cells under salinity challenges were also validated in this study. By using AlamarBlue to assess the transfected 293T cells metabolic activity exposed to the low (add 200μl H_20 to 50μl DMEM medium) or high (add 10μl 31% NaCl solution to 100μl DMEM medium) salinity stress, we found that the cells transfected by EGFP-C2-*Pt*Fer recombinant plasmid exhibited higher resistance than the cells transfected by EGFP-C2 without an insert during the low salinity challenge (Figure 6), which indicated that *Pt*Fer might have quite strong functions against low salinity stress.

It should be noticed that the normal cells without any transfection treatment revealed higher resistance than the transfected cells (Figure 6), which might due to the toxicity brought by the transfection reagents itself to some extent, or might because the ferritin create a certain burden to the transfected cells itself, and it might bring the illegitimate iron sequestration of the over accumulation H-ferritin. In fact, the overexpression of ferritin will affect the cellular phenotype and life cycle (Arosio and Levi, 2002). In another word, the ferritin might have a protective role in the cells exposed to oxidative stress, but it also

bring other problems such as iron starvation or being more sensitivity to oxidative damage. It was also found that overexpression of H-ferritin in HeLa cells increased the resistance to H_2O_2 toxicity but reduced cell growth (Cozzi *et al.*, 2000), and the over accumulation of ferritin in transgenic tobacco also lead to iron deficient but more resistant to methylviologen toxicity (Van Wuytswinkel *et al.*, 1999).

In conclusion, we identified the effective activity of *Pt*Fer and described its differential expression patterns in response to environmental salinity stress. Moreover, the eukaryotic recombinant expression plasmid of *Pt*Fer was constructed and their salinity tolerance abilities were also validated in our study. Our results indicated that *Pt*Fer protein possessed protective effect against salinity stress and *Pt*Fer protein might be involved in salinity adaptation physiological process in *P. trituberculatus*.

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