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Identification of two ferritin genes and their expression profiles in response to bacterial challenge in noble scallop *Chlamys nobilis* with different carotenoids content

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ABSTRACT

As a major intracellular iron storage protein, ferritin plays important roles in iron homeostasis and innate immunity. In this study, two novel *ferritin* subunits from noble scallop *Chlamys nobilis* (*CnFer1* and *CnFer2*) were identified and analyzed. The open reading frame of *CnFer1* and *CnFer2* was 522 and 519bp long, encoding 173 and 172 amino acids, respectively. Both ferritins contained a putative iron-binding region signature (IBRS). Analysis of putative conserved domains showed the two *CnFer* genes contained three key domains of ferritin subunits, a ferroxidase diiron center (E25, Y32, E59, E60, H63, E105, and Q139), an iron ion channel (H116, D129, E132) and a ferrihydrite nucleation center (D58, E59, and E62) that present in M type subunits. A putative iron response element (IRE) was observed at both *CnFer* genes in the 5' UTR. Phylogenetic analysis result suggested that the two genes are cytoplasmic *ferritins* and have the closest evolution relationship with *ferritins* from *Mizuhopecten yessoensis*. The two *ferritin* genes were wildly expressed in examined tissues and the highest level was found in gill. After *V. parahaemolyticus* challenged, both *CnFer* genes were significantly up-regulated suggesting that they are important proteins involved in host immune defense. Moreover, under bacterial challenge, the expression levels of both two genes in Golden scallops (rich in carotenoids) were significantly higher than that in Brown scallops (less in carotenoids) which suggesting that carotenoids enhance the immunity in scallops to defense against the bacterial stress.

1. Introduction

The noble scallop *Chlamys nobilis*, one of the important edible marine mollusks, has been cultured extensively in the southern coastal area in China since 1980s [1]. To improve its productivity and stress resistance, a genetic breeding project has been carried out by our laboratory since 2008. A new variety named Nan'ao Golden Scallop (ID: GS-01-009-2014) that rich in carotenoids with golden shells, golden adductor muscle and mantle was bred by genetic selection [2]. The traditional noble scallops (Brown scallops) are brown shells, white adductor muscle and mantle with relatively lower total carotenoids content (TCC). Carotenoids are very effective antioxidants and serve many biological functions to human, such as providing antioxidant protection, enhancing defense capability, immune-competence and regulating gene expression [3,4]. More importantly, our previous study demonstrated a positive link between TCC and total antioxidant capacity (TAC) in the noble scallop [5]. The Golden scallop exhibit a stronger toleration to environmental stress than the brown scallops may be contributed to genes related to immunity can be up-regulated by carotenoids [6–9].

In marine environment, mollusks are exposed to a complex microbiota from the surrounding and subjected to various potential pathogens infection. Therefore, their survival is highly dependent on their antimicrobial defense systems. Due to lack of adaptive immunity, mollusk must rely on innate immunity to combat against invading pathogens [10]. As essential trace element, iron is important to all living organisms through its role in regulating metabolism, electron transport, oxidative phosphorylation and DNA biosynthesis [11]. Therefore, iron homeostasis is very important in living cells. Iron withholding is also an important innate defense mechanism that has received much attention

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Table 1
Primers used in this study of CnFers.

Primer	Sequence (5′-3′)	Information
long	CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT	RACE primer
short	CTAATACGACTCACTATAGGGC	RACE primer
NUP	AAGCAGTGGTATCAACGCAGAGT	RACE primer
CnFer1-31	CAGAAGCAAAAATGGCACAGACA	3′RACE primer
CnFer1-32	CTTTGACCGTGATGATGTTGCTC	3′RACE primer
CnFer1-51	TACTCCCCAAGTCCAGGTCCAAC	5′RACE primer
CnFer1-52	TCCTTGATGGCGTTCACCTGTTC	5′RACE primer
CnFer2-31	CATCACAGTGCTGAAGAAGGTCG	3′RACE primer
CnFer2-32	ACCCTACGGGACTAATCAGCCAA	3′RACE primer
CnFer2-51	CAGTGATTGGCTGATTAGTCCCG	5′RACE primer
CnFer2-52	TGGATTCAACTTGTTCATTCAGG	5′RACE primer
Fer1-RTF	GGACGAGGATGAATGGGGCTGTG	RT primer
Fer1-RTR	CTTGATGGCGTTCACCTGTTCCT	RT primer
Fer2-RTF	CCTGAATGAACAAGTTGAATCCA	RT primer
Fer2-RTR	ATTGGCTGATTAGTCCCGTAGG	RT primer
β-actinF	CAAACAGCAGCCTCCTCGTCAT	RT primer
β-actinR	CTGGGCACCTGAACCTTTCGTT	RT primer

from immunologists in recent years [12]. *Ferritins*, as important iron-chelating proteins, play crucial roles in the iron-withholding defense system [13]. This protein is ubiquitous in a wide range of organisms, such as bacteria, fungi, plants, invertebrates, and vertebrates, and shows several conserved features [14]. Typical *ferritins* are composed of 24 subunits that create a spherical structure with very high iron-binding capacity (approximately 4500 iron atoms) [15]. Iron, one of the essential trace elements required for the growth and survival of most organisms, plays a critical role in various biological processes, such as growth and differentiation, oxygen transport and storage, energy production, cell cycle, and DNA synthesis [13]. In marine mollusks, many researches have demonstrated that enhanced expression of cytosolic and secreted ferritin of invertebrate maybe involve in innate immune defenses after stimulation [16].

However, *ferritin* genes from noble scallop *C. nobilis* have yet to be identified or characterized. Therefore, present study aims to clone the *ferritin* genes from noble scallop *C. nobilis*, two novel *ferritin* genes (named as *CnFer1* and *CnFer2*) were cloned and the transcripts under *V. parahaemolyticus* stress were determined to identify their expression patterns and verify if carotenoids play a role in regulating ferritin genes. PBS (control) and an acute *V. parahaemolyticus* challenges on Golden and Brown scallops for 48 h were conducted.

2. Materials and methods

2.1. Experimental animals and microbes

In the present study, 150 Golden and 150 Brown scallops *Chlamys nobilis* with 12-month old were separately chosen from sea field of Nan'ao Marine Biology Experimental Station of Shantou University (Shantou, China), cleaned and maintained indoor by a 500 L tank with aerating seawater at 20 °C for a week before the experiment. During maintaining, scallops were fed with diatom (*Nitzschia closterium f. minutissima*) and tetraselmis (*Platymonas subcordiformis*). Water was completely changed daily and feces were removed from the bottom of the tank. The bacterium *V. parahaemolyticus* used in current study was preserved in our laboratory [6].

2.2. Bacterial challenge and tissue collection

To investigate the distribution of ferritin genes mRNA expression, eight tissues including the hemolymph, intestine, adductor muscle, mantle, gonad, hepatopancreas, gill and kidney were separately sampled from three Golden and Brown scallops. All these tissue samples were stored at −80 °C after addition 1 mL of Trizol reagent (Invitrogen)

A

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ATATTGATAACATTTGTCTTGCTGCGTCAGTGAACGTACAGGCAAATTTATCCTCCGCTT 60
ACCAAATTTCATCAATTTGACAATCGACAATTTAACATCCTTGTTGTACCTGACATCAGA 120
AGCAAAAATGGCACAGACACAACCTCGCCAGAACTTCCATGTGGAGACAGAAGCTGGAAT 180
        M  A  Q  T  Q  P  R  Q  N  F  H  V  E  T  E  A  G  I 18
CAACCGCCAGATCAACATGGAGTTGTACGCTTGCTACTGTTACCAACCCATGTCCTTTTA 240
  N  R  Q  I  N  M  E  L  Y  A  C  Y  C  Y  Q  P  M  S  F  Y 38
CTTTGACCGTGATGATGTTGCTCTGCCTGGCTTTGCAAAATACTTCAAGAAGGCCTCAGA 300
  F  D  R  D  D  V  A  L  P  G  F  A  K  Y  F  K  K  A  S  D 58
TGAGGAGCGTGAACACGCAGAAAAGTTCATGAAGTACCAGAACAAGAGGGGAGGCAGGAT 360
  E  E  R  E  H  A  E  K  F  M  K  Y  Q  N  K  R  G  G  R  I 78
TGTCCTTCAAGACATCAAGAAGCCGGACGAGGATGAATGGGGCTGTGCCCTGGATGCCAT 420
  V  L  Q  D  I  K  K  P  D  E  D  E  W  G  C  A  L  D  A  M 98
GCAGGTTGCCTTGGCTCTGGAGAAGAGTGTTAACCAATTTCTTCTGGATCTCCACGGTAT 480
  Q  V  A  L  A  L  E  K  S  V  N  Q  F  L  L  D  L  H  G  I 118
CGGGGATAAGCATGGTGATTCTCAGTTCATGGACTTCCTGGAGGGCGAATACCTGGAGGA 540
  G  D  K  H  G  D  S  Q  F  M  D  F  L  E  G  E  Y  L  E  E 138
ACAGGTGAACGCCATCAAGGAAATCTCCGACCACATCACCAACCTGAAGCGTGTTGGACC 600
  Q  V  N  A  I  K  E  I  S  D  H  I  T  N  L  K  R  V  G  P 158
TGGACTTGGGGAGTACTTGTACGACAAAGAGTCCATCAATGGATCCTAAACCTGAGGCAC 660
  G  L  G  E  Y  L  Y  D  K  E  S  I  N  G  S  *              174
TCTATGTGGAAGCTGCTTGCAGTGTTGAACATTTAGCCCAGAATGTGTTGCCTACTGAAA 720
TACCTTATAAACACACAGTAATCTGTAAATTTTATTCATAGAACTTTTTAAAAACAAGAT 780
TTAACACATTGAAGAAAATTTGTAAACAAAATCATGGGCACAAGAGAAAAAAAAGTTAAA 840
ATAATAAAAAAAAAATGTAAAATTTTAAAAAAAAAAAAAAAAAAAAAAAAA          892
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B

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CTCTCCACTTTGTTGTCGAATTGTCTCGCTGCGTCAGTGAACGTACAGACAAAGACGACA 60
AATTATTCGACATTTTCAGATTGTAAGCAAAATTTGCTGTTCTCCTCGAGATGGCCCAGA 120
                                                M  A  Q   4
CTCAACCGAGACAAAATTTCCACTCAGAAAGTGAAGCTAGCATCAACAAACAGATAAATT 180
T  Q  P  R  Q  N  F  H  S  E  S  E  A  S  I  N  K  Q  I  N   24
TGGAGCTGTATGCAAGCTACATTTATCAGTCAATGTCTTTTTACTTTGACCGAGATGACG 240
L  E  L  Y  A  S  Y  I  Y  Q  S  M  S  F  Y  F  D  R  D  D   44
TGGCACTTCCGGGATTCAGCAAATTTTTCAAAAAGGCATCTGACGAGGAACGAGAACATG 300
V  A  L  P  G  F  S  K  F  F  K  K  A  S  D  E  E  R  E  H   64
CAGAAAAGCTGATGAAGTACCAGAACAAGAGAGGAGGACGTATTGTACTACAGAACATCA 360
A  E  K  L  M  K  Y  Q  N  K  R  G  G  R  I  V  L  Q  N  I   84
CTAAGCCTGACCGTGACGAGTGGGGAAGTGGACTTGAAGCCATGCAGACAGCACTTTCAC 420
T  K  P  D  R  D  E  W  G  S  G  L  E  A  M  Q  T  A  L  S  104
TGGAGAAGAACGTCAACCAGTCACTTTAGACCTTCATGGTGTAGCTAACTCTCATGGTG 480
L  E  K  N  V  N  Q  S  L  L  D  L  H  G  V  A  N  S  H  G  124
ATCCACAGTTTTCCGATTTTATTGAGGAAACATTCCTGAATGAACAAGTTGAATCCATCA 540
D  P  Q  F  S  D  F  I  E  E  T  F  L  N  E  Q  V  E  S  I  144
AACAGCTGAGTGATTACATCACAGTGCTGAAGAAGGTCGGACCAGGGCTGGGAGAGTACC 600
K  Q  L  S  D  Y  I  T  V  L  K  K  V  G  P  G  L  G  E  Y  164
AGTTTGACAAAGAAACCCTACGGGACTAATCAGCCAATCACTGTAATGCTTATAATAGCA 660
Q  F  D  K  E  T  L  R  D  *                                 173
TGGACCAATGAGAGCAAATATTACATACCAATCATCTCTGATCAGTGCACAAACAGTTTC 720
AGTTAAAAAATGTCTTTGTTGAACTTACAGAAAATTATGAGATTGTAATCTTTACTTTTT 780
GGTTGTTTTATATTTCAATAATAACAAATGTATATATTAGGAACCAGACCAAGGGAATTT 840
AAGCATAAAAAGGACATATTTGAATTGTTTTGCTGCAAGAAATAAGGAATCTCTTTTCAT 900
AAAAAAAAAATTATTCAGATGTGTAGGTGTTGTTTGTGCTCAGTAGATATATTCCGACAA 960
AAATGGTCAACGTGTATTTATTTTCTTCATAGTGGAAATCCGTCTATCTCATTACCTAGA 1020
CAGCATTCCTATATGTTACATGTATACTAAAAAGTAATCGTGATACTTTATCAAATTTTT 1080
ATCTTTGGATATATTATACCTGTTAGACCAAACTATTCCATCATTTTCATTATTTTGATA 1140
TACAATGTTTACAAAATGTTACCAAATATTTCATATAAGTTTTAAAAAGCTGTTTTTTCT 1200
TTACTAAATGATATGTGTCATTTTTTTTTGCTTTTTCTTATGCTCTGGCCAGTGTTTATT 1260
GTTTTGTCTCAGGAAGTATGTATGTTTGTATATCATGTATTTGAGAATAAATATTGTCTT 1320
CAAATCTGAAAAAAAAAAAAAAAAAAAAAAAAAAA                          1355
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Fig. 1. Full-length nucleotide and deduced amino acid sequences of *CnFer1* (A) and *CnFer2* (B). The iron response element (IRE) sequences in the 5′-UTR of two cDNAs are in bold with the signature sequence 5′-CAGTGN-3′. The start (ATG) and stop (TAA) codons are marked in box. The ferritin iron-binding regions signature (IBRS) are italic and bold. The polyadenylation signal a in the 3′-UTR are represented underlined.

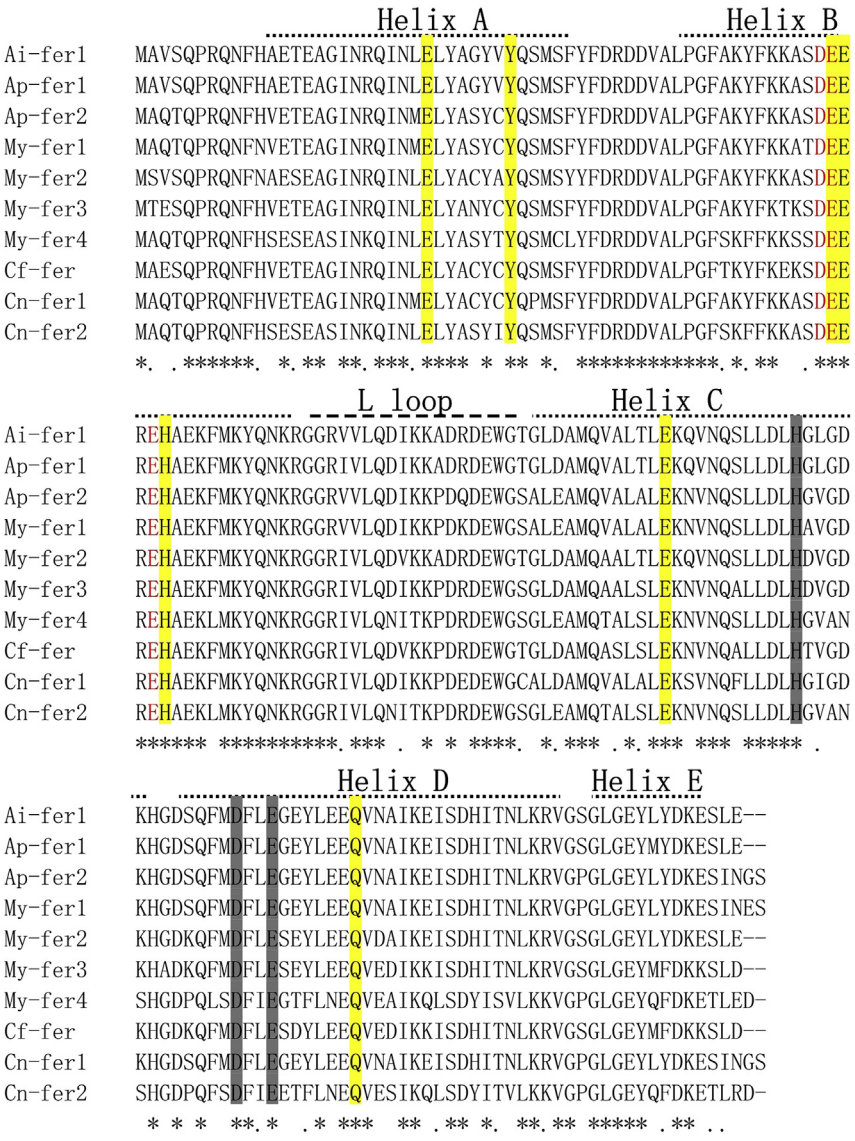

Fig. 2. Multiple sequence alignments of *CnFer1* and *CnFer2* with other ferritin subunits. *Ai-fer1* (*Argopecten irradians*, AEN71558.1), *Ap-fer1* and *2* (*Argopecten purpuratus*, ALV83426.1, ALV83427.1), *My-fer1*, *2*, *3* and *4* (*Mizuhopecten yessoensis*, AGK92812.1, AGK92813.1, AGK92814.1, AGK92815.1). The five a-helices (A-E) and an L-loop are marked above the sequences. Ferroxidase di-iron center sites are highlighted in yellow; ferrihydrite nucleation center sites are shown in red text; and iron ion channel sites are highlighted in gray. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

for subsequent RNA extraction.

100 Golden scallops and 100 Brown scallops were separately divided into two groups (50 scallops each group): control (scallops were injected with 100 μL PBS) and treatment (scallops were injected with100 μL of *Vibrio parahaemolyticus* (5×10^7 cfu mL^{-1} in PBS)). Then, they were maintained in four aerated 500 L tanks for 48 h. Six individuals were randomly sampled from each group at 3, 6, 12, 24, 36 and 48 h after injection, to investigate if difference in immune response does exist between two color scallops, and scallops without any treatment were randomly sampled at 0 h for control. Finally, hemolymph was drawn from each scallop with a disposable syringe (1 mL) on ice and centrifuged at 800 × *g* and 4 °C for 10 min. The hemocytes and gill were harvested for RNA isolation. All these samples were stored at −80 °C for the subsequent experiment.

2.3. Gene cloning and sequencing

The partial cDNA sequences of *ferritin* genes were obtained from our transcriptome assembly data [17], and based on the sequence, 5′ and 3′-RACE-Ready cDNA were conducted using SMARTer® RACE 5′/3′ Kit (TaKaRa, Japan). All primers were listed in Table 1. The PCR was performed in a 50 μL reaction mixture contained 2 μL 3′-RACE-Ready cDNA and 5′-RACE-Ready cDNA, 1 μL gene specific primer (GSP, 10 mM), 1 μL 10 UPM, 21 μL ddH_2O and 25 μL 2 × TransStart® FastPfu PCR Supermix (Transgen Biotech, China), respectively. The PCR parameters were as follow: initial denaturizing at 94 °C for 3 min; 35 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s and extension at 72 °C for 1min; and final extension at 72 °C for 10 min. The PCR product was detected on 1.0% agarose gel and purified using EasyPure Quick Gel Extraction Kit (Transgen Biotech, China). Then the purified product was cloned into pEASY®-T1 Cloning Vector (Transgen Biotech, China) and immediately transformed into Trans1-T1 Phage Resistant Chemically Competent Cells (Transgen Biotech, China). The positive recombinants were identified by colony PCR using M13 Primers (Transgen Biotech, China), and then sequenced by a commercial company (Sangon Biotech, Shanghai, China). The full length cDNA of *CnFer*s were aligned from the overlapping cDNA clones.

2.4. Bioinformatics sequence analysis

The full length cDNA sequences of *CnFer* genes were analyzed using Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nlm.nih.gov/blast) program, and the open reading frames (ORFs) were identified by ORF Finder (http://www.ncbi.nlm.nih.gov/projects/gorf/orfig.cgi). The molecular weights and pI of *CnFer* genes were calculated with the Expasy compute pI/MW tool (http://www.expasy.org/), and the

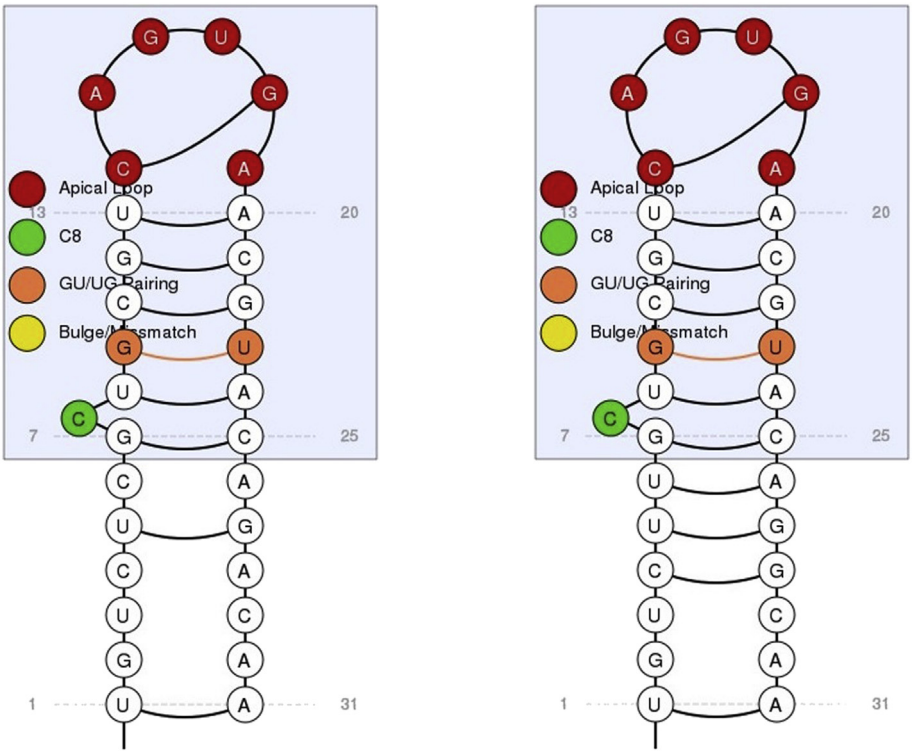

Fig. 3. IREs and stem-loop structures of *CnFer1* and *CnFer2* (by http://ccbg.imppc.org/sires/index.html). (A) *CnFer1* IREs. (B) *CnFer2* IREs.

signal peptides of *CnFer* genes deduced amino acid were predicted by SignaIP 4.1 Server (http://www.cbs.dtu.dk/services/SignalP/). The potential N-linked glycosylation sites were predicted by the Asn-X-Ser/Thr rule (http://cbs.dtu.dk/services/NetNGlyc). Phylogenetic tree was conducted by the MEGA 7.0 program using maximum-likelihood method. The ferritin domains were analyzed using the Simple Modular Architecture Research Tool (SMART) program (http://smart.embl-heidelberg.de/). The iron responsive element (IRE) and stem-loop structures of *CnFer* genes were predicted by the SIREs web server (http://ccbg.imppc.org/sires/).

2.5. Tissue-specific expressions of the ferritin genes

The spatial expression levels of all *ferritin* subunit genes were analyzed by LightCycler® 480 (Roche). Real-time PCR (RT-PCR) was conducted in a 20 μL reaction mixture contained 2 μL of 1:3 diluted original cDNA, 10 μL of 2 × SYBR Green Master mix (SYBR PrimeScript™ RT-PCR kit II, TaKaRa, Japan), 0.8 μL (10 mM) of each primer (Table 1) and 6.4 μL ddH_2O. The quantitative RT-PCR (qRT-PCR) parameters were as follow: an initial step at 95 °C for 30 s; 40 cycles of 95 °C for 5 s, and 60 °C for 30 s, a melting curve analysis from 65 °C to 95 °C and a cooling step of 40 °C for 10 min. The specific amplification of PCR products was confirmed by melting curve. The relative mRNA levels of *CnFer* genes were analyzed using the $2^{-\triangle\triangle Ct}$ methods [18]. The *β*-actin gene was used as the housekeeping gene [8].

2.6. Statistical analysis

The data were presented as the relative expression levels (mean ± S.D, n = 6). Differences in relative expression levels of ferritin genes among tissues and different time points were analyzed by one-way Analysis of Variance (ANOVA).

A general linear model (GLM) was undertaken to evaluate the fixed effects of "Scallop color, S" and "Time, T" and their interaction on mRNA transcripts in tissues. The model was:

$$Y_{ijk} = \mu + S_i + E_j + (S \times T)_{ij} + e_{ijk}$$

Where Y_{ijk} = the *CnFer* genes expression level of the k replicate in the i scallops' color from the j time; μ = overall constant; S_i = the fixed effect of scallops' color (i = 1, 2); T_j = the fixed effect of stress time (j = 1, 2, 3, 4, 5, 6, 7); $(S \times E)_{ij}$ = interaction effect between scallops' color and stress time; and e_{ijk} = random observation error.

All statistical analyses were done on a SAS system for windows (SAS 8.0, SAS Institute Inc., Cary, NC, USA) and significance for all analyses was set to $P < 0.05$ unless noted otherwise.

3. Results

3.1. cDNA cloning and sequence analysis of CnFers

The full-length cDNA sequences of *CnFer1* and *CnFer2* were 892 and 1355bp, which have been deposited in GenBank under accession no. MH982187 and MH982188. The open reading frame (ORF) of *CnFer1* and *CnFer2* was 522 and 519bp, encoding 173 and 172 amino acids, respectively (Fig. 1). The predicted molecular weight of *CnFer1* and *CnFer2* was 19.96 and 19.81 kDa, and the isoelectric point (pI) was predicted to be 4.89 and 5.04, respectively. Analysis of putative conserved domains showed that the two *CnFer* genes contained three key domains of the ferritin subunits, including a ferroxidase diiron center (E25, Y32, E59, E60, H63, E105, and Q139), an iron ion channel (H116, D129, E132) and a ferrihydrite nucleation center (D58, E59, and E62) that are present in M type subunits (Fig. 2). Signal peptide was no found at the N-terminus of both *ferritins*, glycosylation sites were absent in *CnFer1*. However, *CnFer2* has two predicted glycosylation sites (^{82}NITK and ^{109}NQSL). Both *ferritins* contained a putative iron-binding region signature (IBRS) (^{59}EEREHAEKFMKYQNKRGGR^{77}). This sequence is predicted to fold into a typical stem-loop secondary structure, containing a conserved CAGUGN loop and a C bulge located six nucleotides upstream of this loop (Fig. 3). The 5′ untranslated region (UTR) of *CnFer1* and *CnFer2* was 61 and 104 bp, respectively, and a putative iron response element (IRE) was observed in the 5′ UTR of both CnFers

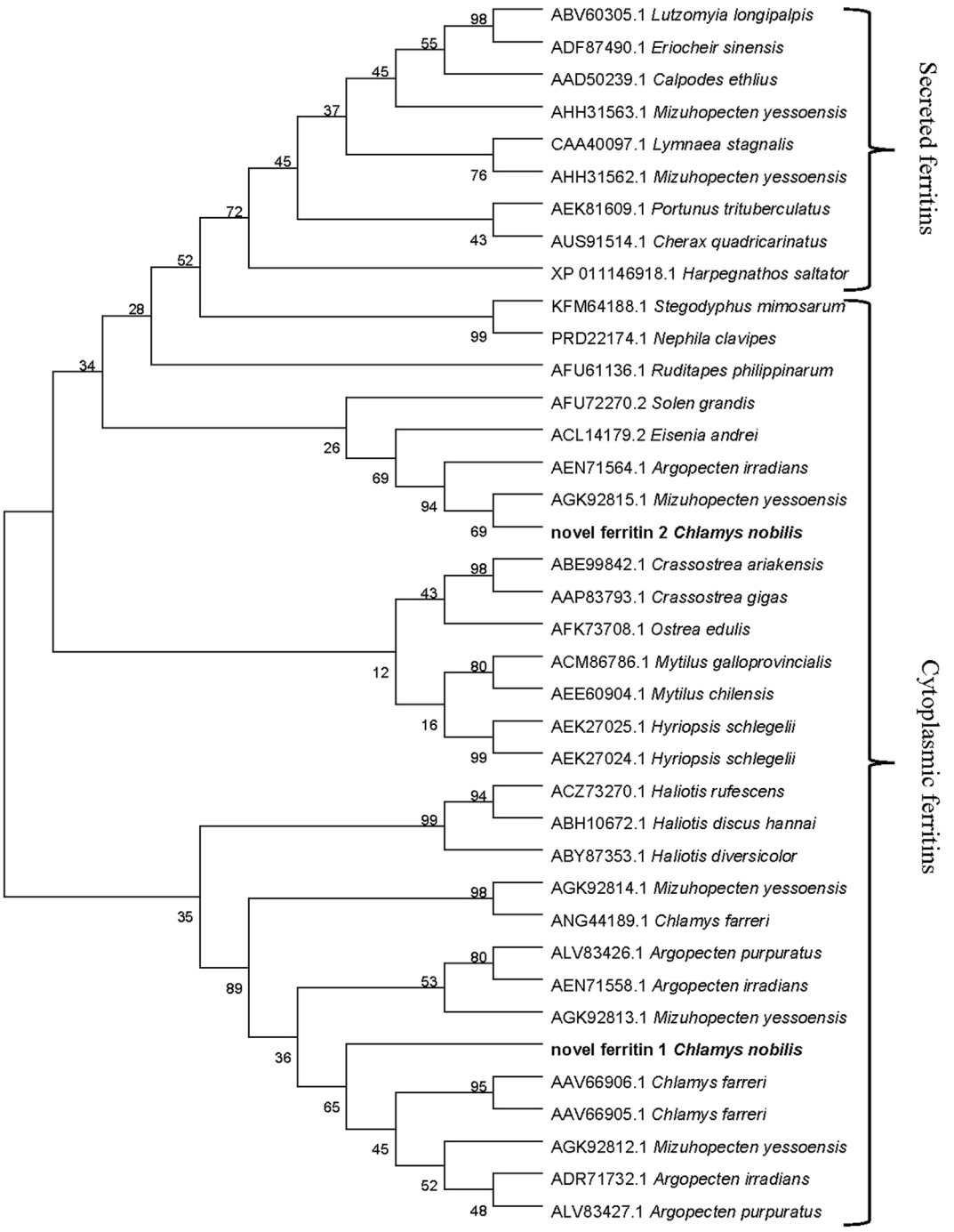

Fig. 4. Phylogenetic analysis of *CnFer1* and *CnFer2* with selected *ferritin* homologues from other species, the tree is constructed by the maximum likelihood method using the MEGA 7.0 program based on multiple sequence alignment generated with ClustalW. Bootstrap trials were replicated 1000 times to derive the confidence value.

(Fig. 1). The 3' UTR of *CnFer1* and *CnFer2* was 244bp and 727bp, respectively, and both contained a canonical polyadenylation signal sequence of AATAAA and a poly-A tail (Fig. 1).

The result of phylogenetic relationship between scallops *CnFer* genes and other mollusks *ferritins* showed t two well-defined clades. First group clustered together and then grouped with secreted *ferritins* (Fig. 4). The two *CnFer* genes of noble scallop were clustered with the cytoplasmic *ferritins* and showed the highest identity to *ferritins* from other scallop species, including *Mizuhopecten yessoensis*, *Chlamys farreri* and *Argopecten irradians*.

3.2. Tissue-specific expression profile of CnFer1 and CnFer2

The amplification efficiency of *CnFer1*, *CnFer2* and β-actin was 95.1%, 96.23% and 97.23%, respectively. Both *CnFer1* and *CnFer2* were expressed in all examined tissues including the adductor muscle, kidney, gonad, mantle, intestine, hepatopancreas, hemocytes and gill in Golden and Brown scallops, and significantly differences existed among different tissues. The highest level of *CnFer1* and *CnFer2* expression were in the gill (Fig. 5). Both *CnFer1* and *CnFer2* expression levels in hemocytes of Golden scallops were significantly higher ($P < 0.05$) than that in Brown scallops. Moreover, *CnFer2* expression level in hepatopancreas of Golden scallops was also significantly higher

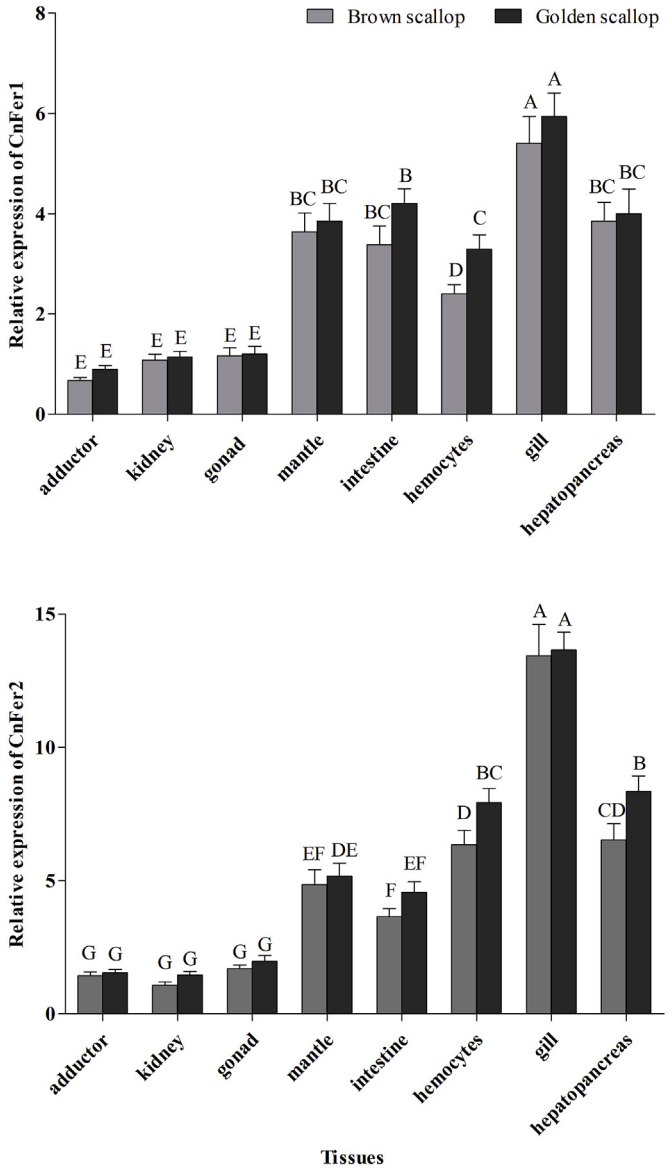

Fig. 5. *CnFer1* and *CnFer2* mRNA expression levels in different tissues of noble scallop detected by qRT-PCR. Vertical bars represent the mean ± SD (n = 6).

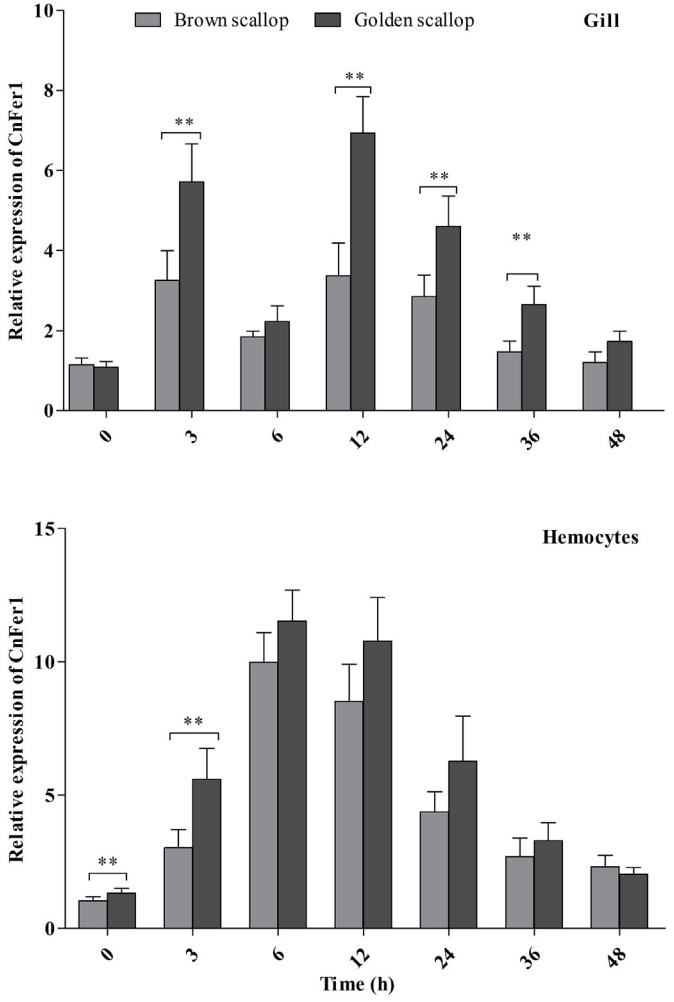

Fig. 6. Time-course expression levels of *CnFer1* mRNA in gill and hemocytes after *V. parahaemolyticus* challenge measured by qRT-PCR. Vertical bars represent the mean ± SD (n = 6). Significant differences between Brown and Golden scallops in challenged individuals were indicated with asterisks. **, $P < 0.01$.

($P < 0.05$) than that in Brown scallops.

3.3. Comparison of CnFer1 and CnFer2 transcript profiles between Golden and Brown scallops in response to bacterial challenge

As shown in Figs. 6 and 7, both *CnFer1* and *CnFer2* in gill and hemocytes were induced and increased after *V. parahaemolyticus* infection in Golden and Brown scallops. *CnFer1* of Golden scallops was significantly higher ($P < 0.01$) at 3, 12, 24 and 36 h than Brown scallops in gill, *CnFer2* of Golden scallops was significantly higher ($P < 0.01$) at 3, 6 and 12 h than Brown scallops in gill. In hemocytes, *CnFer1* of Golden scallops was significantly higher ($P < 0.01$) than Brown scallops only at 3 h, *CnFer2* of Golden scallops was significantly higher ($P < 0.01$) than Brown scallops at 6 and 24 h.

Analysis of variance in Tables 2 and 3 showed that scallops' color and time independently made significant impacts on *CnFer1* and *CnFer2* transcripts ($P < 0.05$), and their interactions were all significant ($P < 0.05$).

4. Discussion

According to ferroxidase and ferrihydrite nucleation sites, *ferritin* subunits can be classified into three types: H-, L- and M − type. H and L subunits possess ferroxidase center and ferrihydrite nucleation center, respectively, whereas M subunit has both [19,20]. *CnFer1* and *CnFer2* reported in this study possess both ferroxidase center and ferrihydrite nucleation center, indicating they belong to M subunit.

Ferritins are also categorized into cytosolic, secreted and mitochondrial *ferritins* based on their subcellular localization [21]. Cytosolic *ferritins* are involved in iron storage and regulation. Secreted *ferritins* serve as iron transfer or donor molecules and mitochondrial *ferritins* function as antioxidants by protecting mitochondria from iron-dependent oxidative damage [21]. In recent years, cytoplasmic *ferritins* have been widely reported in scallops, such as in bay scallop *Argopecten irradians* [22,23], Yesso scallop *Patinopecten yessoensis* [24] and Zhikong scallop *Chlamys farreri* [16]. Previous studies have shown that the ORFs of secreted *ferritins* are generally longer than cytoplasmic *ferritins* because of the presence of signal peptide at the N-terminus [16]. In the present study, the absence of signal peptide detected by bioinformatics analysis and encoding protein of 172 and 173 amino acids indicated that two *CnFer* genes are cytosolic *ferritins*. This was further confirmed by the result of phylogenetic analysis that showed the two genes were

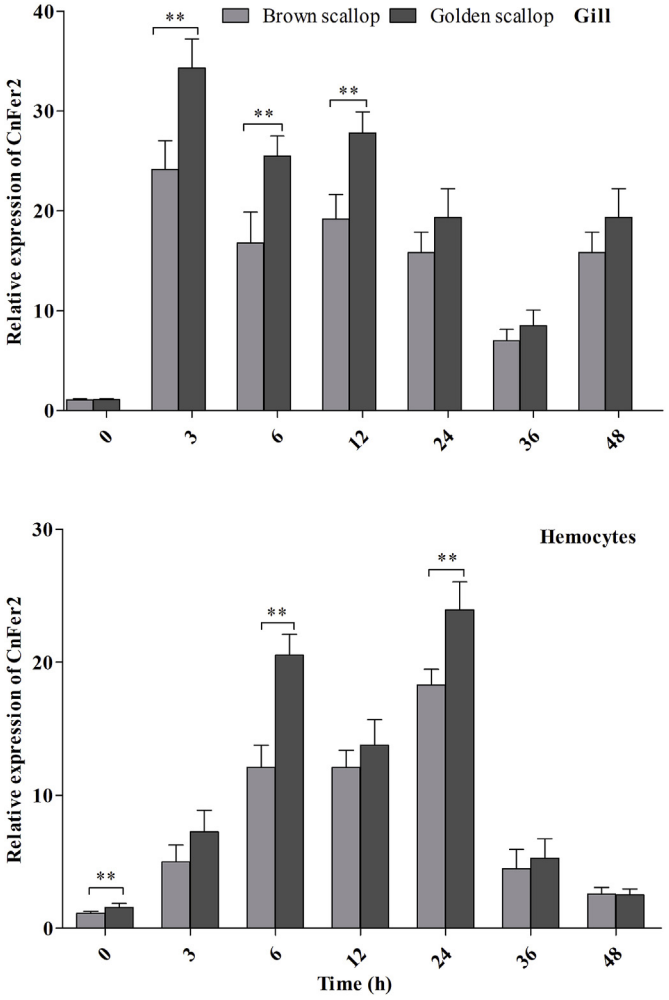

Fig. 7. Time-course expression levels of *CnFer2* mRNA in gill and hemocytes after *V. parahaemolyticus* challenge measured by qRT-PCR. Vertical bars represent the mean ± SD (n = 6). Brown and Golden scallops in challenged individuals were indicated with asterisks. **, $P < 0.01$.

clustered with known cytoplasmic *ferritins* rather than with secreted *ferritins*.

The IREs which bind with the iron regulatory proteins (IRPs) to regulate the synthesis of ferritin proteins are present in most of known cytosolic *ferritins* genes. IRPs bind to the IREs and block the initiation of ferritin protein synthesis when the iron level is low. Otherwise, the IRE/IRP interaction declines and increases the synthesis of ferritin proteins to enable iron storage [25]. The typical stem-loop motif of the IREs in the 5' UTRs were present in both *CnFer* genes, indicating that these mRNAs might be regulated by iron at the translational level.

Like other mollusks cytoplasmic *ferritins*, both *CnFer1* and *CnFer2* genes were expressed in all examined tissues, suggesting that the two genes were widely distributed in the tissues of the noble scallop. The highest level was both recorded in gill, indicating that the two genes preferentially contributes to the maintenance of iron homeostasis in this tissue. Moreover, *CnFer1* and *CnFer2* in hemocytes of Golden scallops exhibited a significantly higher expression levels than that of Brown scallops, this might be partially explained by higher total carotenoids content in Golden scallops. As we all know, hepatopancreas plays a role in iron storage and detoxication, whereas hemocytes are the main line of host defense in invertebrates. High expression of *ferritin* mRNA in hepatopancreas and hemocytes contributed to the high iron metabolism and host immune defense have been reported in *Patinopecten yessoensis* [24], *Crassostrea gigas* [26] and *Tegillarca granosa* [27].

Other than iron storage and detoxification, *ferritins* are also known to against microbial infection [22–24,26,27]. In recent years, many studies have found ferritin proteins are widely involved in the innate immunity response of marine invertebrates. In mollusks, *ferritin* genes expression levels can be up-regulated by bacterial infection suggesting that *ferritin* genes play important roles in immunity [22–24,26,27]. For example, in Pacific abalone *Haliotis discus hannai*, *ferritin* subunit was up-regulated in hemocytes when challenged with *Vibrio anguillarum* [28]. In this study, both *CnFer* genes were significantly up-regulated in hemocytes and gill after *Vibrio parahaemolyticus* challenged suggesting that they play important roles in bacterial stress. Similar results have been also reported in other molluscs. In Zhikong scallop *Chlamys farreri*, *ferritin* gene was also found significantly up-regulated after challenged with bacteria and virus [16]; in Yesso scallop *Patinopecten yessoensis*, six *ferritin* genes were significantly up-regulated by *Vibrio anguillarum* challenge [24,29].

Under *V. parahaemolyticus* challenge, Golden scallops enriched in carotenoids showed significantly higher *CnFer1* and *CnFer2* expressions compared to Brown scallops. The significantly different between Golden and Brown scallops is the carotenoids content [5]. Therefore, it is reasonable to conjecture that carotenoid content was the main factor resulting in the higher expression level of *CnFer1* and *CnFer2* in Golden scallops. Carotenoids has been reported to enhance the immunity and regulate gene expression [30–32]. Carotenoids supplementation has been reported to increase the *Betta splendens* immune response [30]. Dietary carotenoids have broad immunostimulating effects including enhance phenoloxidase activity and increase resistance to bacterial infection in the crustacean *Gammarus pulex* [31]. Carotenoids also can regulate retinoic acid signaling pathway during embryonic development in vertebrates [32] and regulate connexin 43 in human and animal cells [33]. Golden scallops also exhibited a stronger resistant to environmental and bacterial stresses than the brown scallops, where vitellogenin, *CnTRX* and *CnTLR* genes were up-regulated in Golden scallops as a respond to the invasion resistance of *V. anguillarum* [6,8,9], and *CuZnSOD* gene was up-regulated in Golden scallops under low temperature stress [7].

In conclusion, two *ferritin* subunits were cloned from noble scallop *C. nobilis*. Bioinformatic analyses suggested that these proteins were M-type cytoplasmic *ferritins*. Both *CnFer* genes were expressed in all tested tissues and significantly up-regulated by bacterial (*V. parahaemolyticus*) invasion suggesting that they play important roles involved in host immune defense. Moreover, expression levels of both *CnFer* genes in Golden scallops are significantly higher than those in Brown scallops under bacterial challenge indicating that carotenoids in the scallop can enhance the immunity to defense against the bacterial stress.

Table 2
Analyses of variance for *CnFer1* mRNA expression in hemocytes and gill of *C. nobilis*.

Source	df	Hemocytes			Gill		
		MS	F	*P*	MS	F	*P*
Scallops color (S)	1	32.1162333	32.42	< 0.001	41.0998220	130.05	< 0.001
Time (T)	6	163.8242825	165.38	< 0.001	29.926938	94.70	< 0.001
S × T	6	3.6823278	3.72	< 0.01	4.9739928	15.74	< 0.001
Error	70	0.990564			0.3160327		

Table 3
Analyses of variance for *CnFer2* mRNA expression in hemocytes and gill of *C. nobilis*.

Source	df	Hemocytes			Gill		
		MS	F	*P*	MS	F	*P*
Scallops color (S)	1	150.731256	83.61	< 0.001	558.518571	111.45	< 0.001
Time (T)	6	686.718822	380.92	< 0.001	1097.313104	218.97	< 0.001
S × T	6	30.551147	16.95	< 0.001	47.292271	9.44	< 0.001
Error	70	1.802796			5.011306		

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