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Effect of chronic ammonia nitrogen stress on the SOD activity and interferon-induced transmembrane protein 1 expression in the clam *Cyclina sinensis*

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Ammonia nitrogen plays a crucial part in oxidative stress in aquatic animals. To elucidate the effect of ammonia nitrogen stress on the superoxide dismutase (SOD) activity and interferon-induced transmembrane protein 1 (*IFITM1*) expression in the clam *Cyclina sinensis*, clams were exposed to ammonia nitrogen (8.07 mg/L) for 768 h (32 days) and then challenged with *Vibrio parahaemolyticus*. The results showed that the SOD activity in the hepatopancreas of *C. sinensis* exposed to ammonia nitrogen first increased and then decreased with time, returning to the control group's normal level at 768 h. Following infection with *V. parahaemolyticus*, the SOD activity in the hepatopancreas fluctuated over time. The SOD activity in clams infected with *V. parahaemolyticus* at 144 h did not return to the control group's normal level. The full-length cDNA of *CsIFITM1* was 2,434 bases in length, including a 2,301-bp open reading frame (ORF) encoding 714 amino acids, with a putative molecular weight of 83.86 kDa. *CsIFITM1* contains an RNA helicase domain (DEXHc_RLR, DR) and a Helicase_C (HC) domain. The transcriptional levels of *CsIFITM1* were upregulated by exposure to ammonia nitrogen and were significantly higher from 6 to 768 h compared to the control (0 h) ($p < 0.05$). Following infection with *V. parahaemolyticus*, the transcript levels of *CsIFITM1* in the hepatopancreas were upregulated and were significantly higher from 6 to 144 h, in contrast to those of the control (0 h) ($p < 0.05$). The present data provide the first evidence of the SOD activity and *CsIFITM1* transcript levels being able to reflect the effect of ammonia on the clam *C. sinensis*.

KEYWORDS

cyclina sinensis, ammonia nitrogen exposure, vibrio challenge, SOD activity, IFITM1 gene

Introduction

As an unavoidable factor, ammonia nitrogen easily accumulates in the aquaculture system (Barbieri and Bondioli, 2013; Egnew et al., 2019). The accumulation of total ammonia nitrogen (TAN) in the water environment can ultimately lead to severe problems such as oxidative stress (Ge et al., 2022a), gill hyperplasia (Zuffo et al., 2021), inefficient feed utilization (Silva et al., 2018), and poor immune response of aquatic animals (Mangang and Pandey, 2021). As a result, some opportunistic bacteria such as *Vibrio parahaemolyticus* may cause serious vibriosis (Ni et al., 2020). In some cases, it could even lead to death in aquatic animals (Barbieri et al., 2019). Aquatic animals suffer from oxidative stress, which causes the accumulation of reactive oxygen species (ROS) (Ge et al., 2022a), and the scavenging capacity induces lipid peroxidation (Debbarma et al., 2021). To protect organisms from such oxidative stress, antioxidant enzymes are likely to be activated. Therefore, a change in the antioxidant enzyme activities is one of the reliable and sensitive tools for the evaluation of oxidation resistance in aquatic animals (Ni et al., 2020; Debbarma et al., 2021). Superoxide dismutase (SOD) is one of the key antioxidant enzymes (Ge et al., 2021). It can reduce oxidative damage by eliminating ROS and enhancing the antioxidant capacity (Sinha et al., 2015). Ammonia nitrogen exposure may activate the antioxidant system and alter the activities of antioxidant enzymes (Ghelichpour et al., 2019).

Interferon (IFN) is one of the major multifunctional cytokines that play vital roles in the innate immune response in aquatic animals (Wan and Chen, 2008; Zhang et al., 2020). Among the IFN-responsive genes that collectively regulate the multifunctional effects of IFNs is the interferon-induced transmembrane (IFITM) protein family (Johnson et al., 2006; Wan and Chen, 2008). Members of the IFITM family are likely to be expressed basally in various tissues and cells. They may play a crucial part in the promotion and maintenance of the pluripotent state of an organism's cells (Johnson et al., 2006). All of the IFITM proteins share a conserved short topology, two transmembrane (TM) domains, and highly variable amino and carboxy termini (Zhang et al., 2020). Thus far, in aquatic animals, *IFITM1*, *IFITM2*, *IFITM3*, and *IFITM5* have been annotated in the fish genome (Johnson et al., 2006). The *IFITM* family comprises the known innate immune effectors involved in the regulation of immunoreaction, such as endocytosis, immune cell signaling, cell physiology, and antioxidative damage (Baird et al., 2001; Zhu et al., 2013). When organisms are under oxidative stress, they may synthesize some proteins, such as IFNs, interleukins, heat shock proteins, and the IFITM proteins (Ghelichpour et al., 2019). In humoral immunity, the transcriptional levels of members of the IFITM family can reflect the current immune status of aquatic animals (Johnson et al., 2006).

As one of the most important economic bivalves, the clam *Cyclina sinensis* is widely distributed in the coastal areas of East Asia, and the clam industry is growing rapidly (Ge et al., 2022b). The clam grows fast, tastes delicious, and has great market demand (Ge et al., 2021; Liao et al., 2022). The clam is highly adaptable to ammonia nitrogen (Ni et al., 2022). However, when the accumulation of ammonia nitrogen reaches the threshold level, this can have serious effects on the clam, including oxidative stress and poor immune response (Ni et al., 2021; Ge et al., 2022a). Therefore, it is essential to evaluate the effects of ammonia nitrogen on antioxidant enzymes and immunoreaction (Chai et al., 2022). Because a change in the SOD activity and *IFITM1* expression level can reflect the current immune status of aquatic animals, we assessed the SOD activity and the *IFITM1* transcription response in clams exposed to chronic ammonia nitrogen and following infection with *V. parahaemolyticus*. The present study provides a theoretical basis for the research on the detoxification mechanisms in marine animals.

Materials and methods

Experimental animal

C. sinensis (2.99 ± 0.45 g each) from Lianyungang Zhongchuang Aquaculture Company were transported to the experimental base of Jiangsu Ocean University. To acclimate the clams to laboratory conditions, they were stored in concrete tanks (0.8 m × 0.8 m × 0.5 m) with 200 L aerated seawater for 10 days. During the acclimation and the experiment, the seawater temperature was maintained at 24 ± 0.5°C, with pH at 8.0 ± 0.4, dissolved oxygen (DO) ≥ 4.9 mg/L, and TAN < 0.09 mg/L (Chen et al., 2021). Clams were fed twice daily with a mixture of alive microalgae (*Isochrysis zhangjiangensis* and *Nannochloropsis oceanica*) at a density of 2×10^4 cells/ml.

Six clams were dissected and various tissues collected, which were then frozen in liquid nitrogen for RNA extraction (Ni et al., 2022).

Long-term chronic ammonia nitrogen stress

Seven hundred and twenty selected clams were randomly stored in six concrete tanks (200 L water) at a density of 120 clams per tank, with three replicates for each treatment. According to the 96-h median lethal concentration (LC_{50}-96 h) TAN for *C. sinensis* (Ni et al., 2022), the TAN level in the experimental group was set at 8.07 mg/L. To achieve the designed level of TAN, a stock solution of NH_4Cl (1.0 g/L) was used. The control group was natural seawater. The other management conditions were the same as those used during the temporary rearing period, and the stress experiment was carried

out for 768 h. During the test, the seawater was changed twice daily to maintain the concentration of TAN.

Three individuals in each treatment were randomly selected at different time points after the clams were exposed to ammonia nitrogen (0, 3, 6, 12, 24, 48, 96, 192, 384, and 768 h). The hepatopancreas was collected and then frozen in liquid nitrogen for the SOD activity analysis and total RNA isolation. The SOD activity was determined using kits purchased from Nanjing Jiancheng Bioengineering Institute (Ge et al., 2022a).

Vibrio challenge

To determine the effect of ammonia nitrogen stress on the disease resistance of the clam, *Vibrio* challenge tests were further performed. After the ammonia nitrogen stress experiment, clams in the experimental group were transferred into the normal seawater tank with three replicates and challenged with *V. parahaemolyticus*. The clams were immersed and infected with *V. parahaemolyticus* at a level of 1×10^7 CFU/ml for 1 week (Ni et al., 2020). During the infection experiment, to achieve the level of *V. parahaemolyticus* (1×10^7 CFU/ml), all of the seawater was replaced twice daily. The control group was natural seawater without the addition of *V. parahaemolyticus*.

Three individuals in each treatment were randomly selected at different time points during the infection experiment (0, 3, 6, 12, 24, 48, 96, 120, and 144 h). The hepatopancreas was collected and then frozen in liquid nitrogen for the SOD activity analysis and total RNA isolation.

IFITM1 gene cloning and sequence analysis

The CDS sequence of the *IFITM1* gene was derived from the clam whole-genome sequencing complementary DNA (cDNA) library (Wei et al., 2020). To clone the full-length cDNAs, rapid amplification of cDNA ends PCR (RACE-PCR) was conducted (Ni et al., 2022). The primers required for *IFITM1* gene cloning are shown in Table 1. The *IFITM1* sequence was verified by DNA sequencing and analyzed using the BLAST program (Zhang et al., 2020). The NCBI database was used to predict the ORF of the *IFITM1* gene. Sequence homology retrieval and alignment were also performed. The ExPASy ProtParam program was utilized to predict the molecular weight and isoelectric points of the IFITM1 protein. According to a previous report, multiple sequence alignments were generated and a phylogenetic tree was constructed (Ni et al., 2022).

TABLE 1 Primers used for the cloning of *IFITM1* in *Cyclina sinensis*.

Primer	Sequence (5′–3′)	Sequence information
IFITM1-5GSP	CTTCCGTCTTACGCATACATTCTTC	5′RACE outer amplification primer
IFITM1-5GSP	ACAAGTCTTCCGTCTTACGCATACA	5′RACE outer amplification primer
IFITM1-5NGSP	TCGGCATCATGTCGCTTAATAGTGT	5′RACE inner amplification primer
IFITM1-3GSP	CAACAGGAAGGCAAACGGATAACG	3′RACE outer amplification primer
IFITM1-3GSP	GGCTTCAAATGTTCCTCTTTCACTG	3′RACE outer amplification primer
IFITM1-3NGSP	TTCAAAGACAACAAGGGACAAAGGT	3′RACE inner amplification primer
M13F	GTTGTAAAACGACGGCCAG	Positive clone verification
M13R	CAGGAAACAGCTATGAC	Positive clone verification
UPM-long	CTAATACGACTCACTATAGGGCAAGCAGT GGTATCAACGCAGAGT	RACE-PCR outer layer amplification
NUP	AAGCAGTGGTAACAACGCAGAGT	RACE-PCR inner layer amplification

RACE, rapid amplification of cDNA ends.

Quantitative real-time PCR analysis

To analyze the transcriptional level of *IFITM1* messenger RNA (mRNA), quantitative real-time PCR (qRT-PCR) was carried out with the SYBR method (Ge et al., 2022b). The primers required for qRT-PCR are shown in Table 2. Each sample was in triplicate. The $2^{-\Delta\Delta Ct}$ method was applied to calculate the mRNA transcriptional level with β-actin as the internal control (Ge et al., 2022b).

TABLE 2 Specific primers for the quantitative real-time PCR (qRT-PCR) of *IFITM1* from *Cyclina sinensis*.

Primer	Sequence (5′–3′)	Sequence information
IFITM1—upstream	AAACGCTCATCTTGTCCTTGG	qRT-PCR
IFITM1—downstream	GTCTTCTTCCAGTGGCGGTAT	qRT-PCR
β-actin—upstream	CACCACAACTGCCGAGAG	Reference gene
β-actin—downstream	CCGATAGTGATGACCTGACC	Reference gene

Statistical analysis

Data were analyzed with one-way ANOVA using SPSS. 18 (Ge et al., 2019). Duncan's multiple comparison tests were performed when significant differences were detected in the ANOVA. Difierences were considered statistically significant when $p < 0.05$ (Zhang et al., 2020).

Results

Activity of SOD in the clam exposed to long-term ammonia nitrogen

The SOD activity in the hepatopancreas of *C. sinensis* exposed to ammonia nitrogen first increased and then

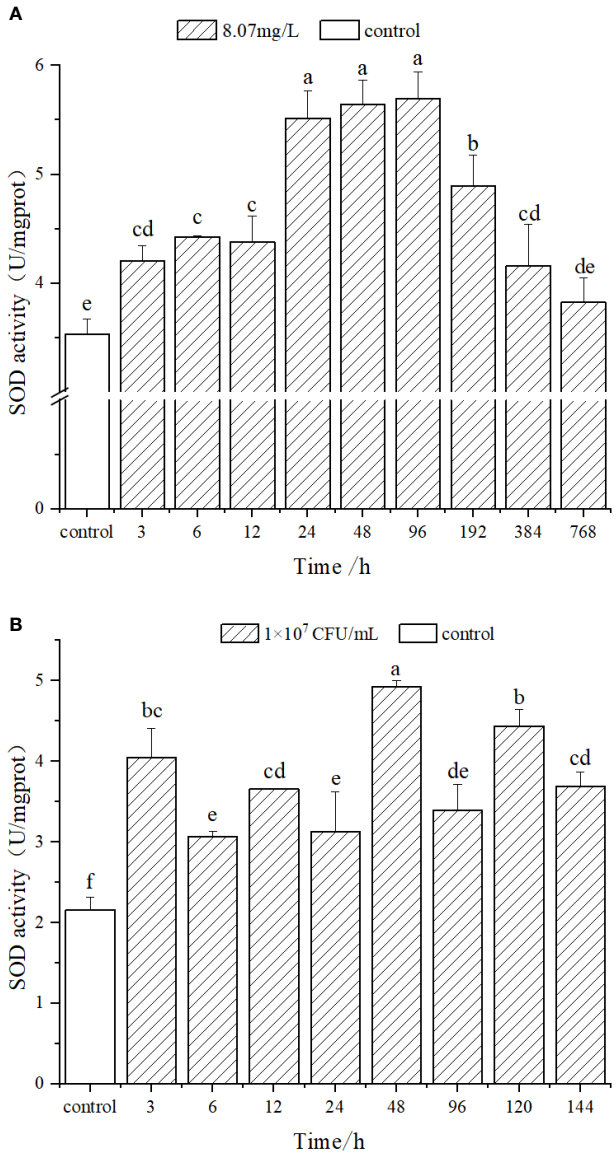

FIGURE 1
Superoxide dismutase (SOD) activity in the hepatopancreas tissue of *Cyclina sinensis* under long-term ammonia. **(A)** SOD activity in *C sinensis* exposed to long-term ammonia. **(B)** SOD activity in *C sinensis* infected with *Vibrio parahaemolyticus*. The control group in **(A)** comprised clams raised in natural seawater. The control group in **(B)** included clams raised in natural seawater without the addition of *V. parahaemolyticus*. The *same lowercase letters* indicate non-significant differences between the different stress time points ($p > 0.05$); otherwise, the difference is significant ($p < 0.05$).

decreased with time (Figure 1A). The SOD activity in the experimental group increased to the maximum value at 96 h. No significant difference was found at 24 and 48 h ($p > 0.05$), whereas the SOD activity was significantly higher than that at other time points ($p < 0.05$). At 768 h, the SOD activity returned to the control group's normal level. Following infection with *V. parahaemolyticus*, the SOD activity fluctuated with time (Figure 1B). The SOD activity from 3 to 144 h in the clams infected with *V. parahaemolyticus* was significantly higher than that in the control group ($p < 0.05$).

Identification of *CsIFITM1*

As shown in Figure 2, the full-length cDNA of *CsIFITM1* was 2,434 bases in length, including a 5′ untranslated region (UTR) of 34 bp and a 3′-UTR of 99 bp with a poly(A) sequence. It contained a 2,301-bp ORF encoding 714 amino acids, with a putative molecular weight of 83.86 kDa and a theoretical isoelectric point of 11.18. The *CsIFITM1* protein contained putative transmembrane domains, but did not contain a signal peptide (Figure 3). Multiple sequence alignment showed that it contained an RNA helicase domain (DEXHc_RLR, DR) located at amino acid residues 70–265 and a Helicase_C (HC) domain located at amino acid residues 468–564 (Figure 4). Phylogenetic analysis showed that *CsIFITM1* formed a cluster with the *IFITM1* of *Mercenaria mercenaria*, *Crassostrea gigas*, *Crassostrea virginica*, *Dreissena polymorpha*, and *Mytilus edulis*, but not with the *IFITM1* of *Haliotis rufescens*, *Haliotis rubra*, and *Pomacea canaliculata* (Figure 5). Expression analysis revealed that the *CsIFITM1* mRNA was constitutively expressed in the adductor muscle, mantle, gill, axon foot, hepatopancreas, and gonad, with higher levels of mRNA detected in the hepatopancreas and the adductor muscle (Figure 6).

CsIFITM1 transcript levels in the hepatopancreas of clams exposed to ammonia nitrogen and *Vibrio* challenge

The transcript levels of *CsIFITM1* in the hepatopancreas of the clams exposed to ammonia nitrogen were determined at different time points after exposure using qRT-PCR (Figure 7A). Exposure to ammonia nitrogen upregulated the transcriptional levels of *CsIFITM1*, which reached the peak at 192 h post-exposure. The expression levels of *CsIFITM1* were significantly higher from 6 to 768 h in clams exposed to ammonia nitrogen than those of the control (0 h) ($p < 0.05$).

Following infection with *V. parahaemolyticus*, the transcript levels of *CsIFITM1* in the hepatopancreas were upregulated (Figure 7B). The transcriptional levels of *CsIFITM1* were also upregulated by *V. parahaemolyticus* infection, which reached the peak at 48 h post-infection. The transcript levels of *CsIFITM1* in clams infected with *V. parahaemolyticus* from 6

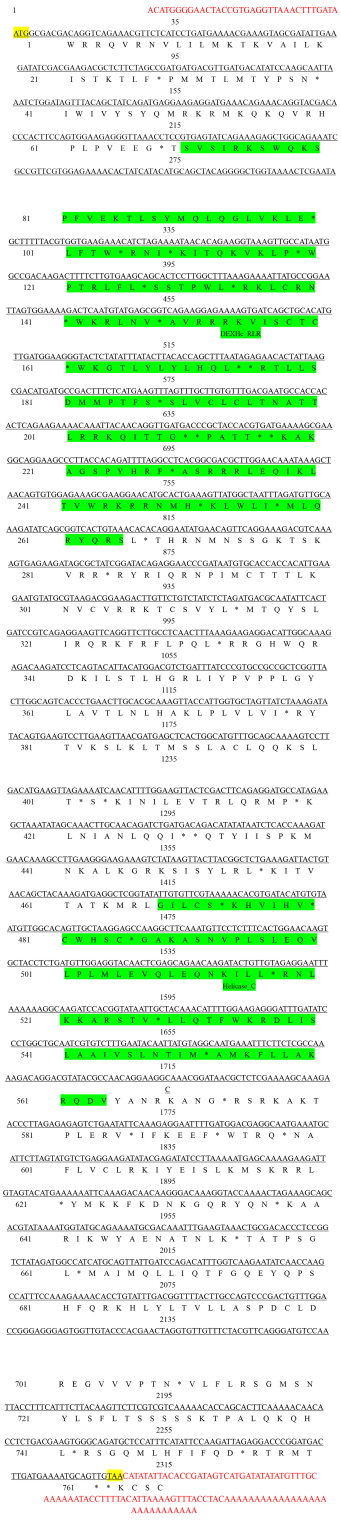

FIGURE 2
The cDNA of *IFITM1* and the deduced amino acid sequence of *Cyclina sinensis*. The *yellow part* indicates the start codon ATG and the stop codon TAA, the *underlined area* indicates the open reading frame (ORF), and the *green part* indicates the conserved domain of *IFITM1*.

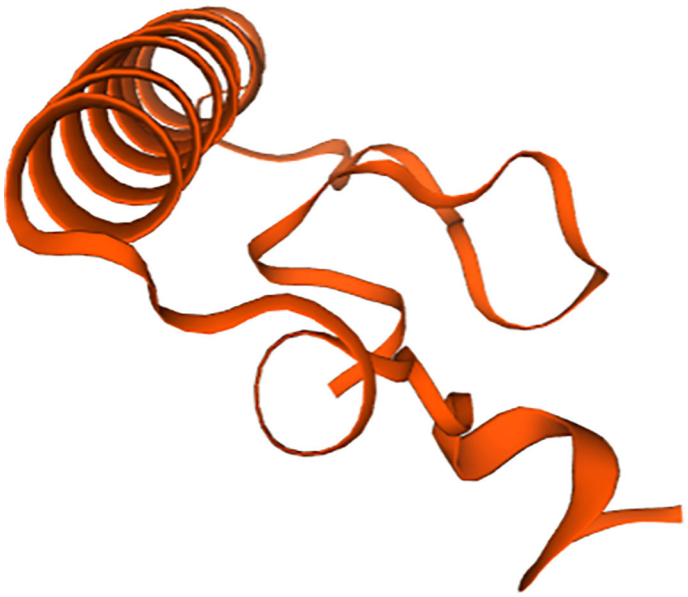

FIGURE 3
Prediction of the protein model of *IFITM1*.

to 144 h were significantly higher, in contrast to those of the control (0 h) ($p < 0.05$).

Discussion

The accumulation of ammonia nitrogen in water can ultimately lead to serious oxidative stress (Barbieri and Bondioli, 2013). As one of the key antioxidant enzymes, SOD can reduce oxidative damage (Ge et al., 2021). In the current study, the SOD activity in the hepatopancreas of *C. sinensis* exposed to ammonia nitrogen first increased and then decreased with time. This may be because aquatic animals have to activate antioxidant enzymes in order to deal with oxidative stress (Ni et al., 2022). This result showed that exposure to ammonia nitrogen could activate the SOD in the clam, and activating SOD can reduce the oxidative damage induced by ammonia nitrogen stress. At 768 h, the SOD activity returned to the control group's normal level, which is probably due to the toxicity of the low level of ammonia nitrogen (8.07 mg/L) being low. On the other hand, the 768-h recovery period may have been adequate to compensate for the SOD activity in clams exposed to ammonia nitrogen (Ni et al., 2022). This indicated that the SOD activity in clams exposed to ammonia nitrogen (8.07 mg/L) for 768h could return to normal levels. Bacterial infection can cause serious oxidative stress (Kumar, 2021). In the current study, the SOD activity from 3 to 144 h in the clams infected with *V. parahaemolyticus* was significantly higher than that in the control. This showed that SOD can be activated in clams infected with *V. parahaemolyticus*. The SOD activity in clams infected with *V. parahaemolyticus* for 144 h did not return to the control group's normal level. The reason may be that the bacterial infection may have caused irreparable damage to the antioxidant system, or it could be due to the 144-h recovery time being too short for the recovery of the SOD activity (Ge et al., 2022a).

In the current study, we cloned the *IFITM1* gene from the clam *C. sinensis*. The full-length cDNA of *CsIFITM1* was 2,434 bp in length, including a 2,301-bp ORF encoding 714 amino acids, with a putative molecular weight of 83.86 kDa. The *CsIFITM1* protein possessed putative transmembrane domains, which showed that the protein contained the typical structural features of IFITMs (Wan and Chen, 2008). This indicated that *CsIFITM1* may be a cell surface molecule (Tanaka et al., 2005). As is well known, members of the IFITM family are expressed in various cells in mammals (Bailey et al., 2014). In the present study, the expression analysis showed that, although at a different level, the *CsIFITM1* mRNA was constitutively broadly expressed in all the selected tissues, indicating that the *IFITM1* gene may be widely distributed among tissues and is expressed in various cells in *C. sinensis*. This is probably because immune cells (including leukocytes and lymphocytes) are widely distributed in various tissues (Desai et al., 2017).

The *IFITM1* gene in mammals is induced by IFNs, and the mRNA transcript level of this gene can increase as much as 100-fold upon induction by IFNs (Wan and Chen, 2008). In the present study, the transcript levels of *CsIFITM1* were upregulated by exposure to ammonia nitrogen and were

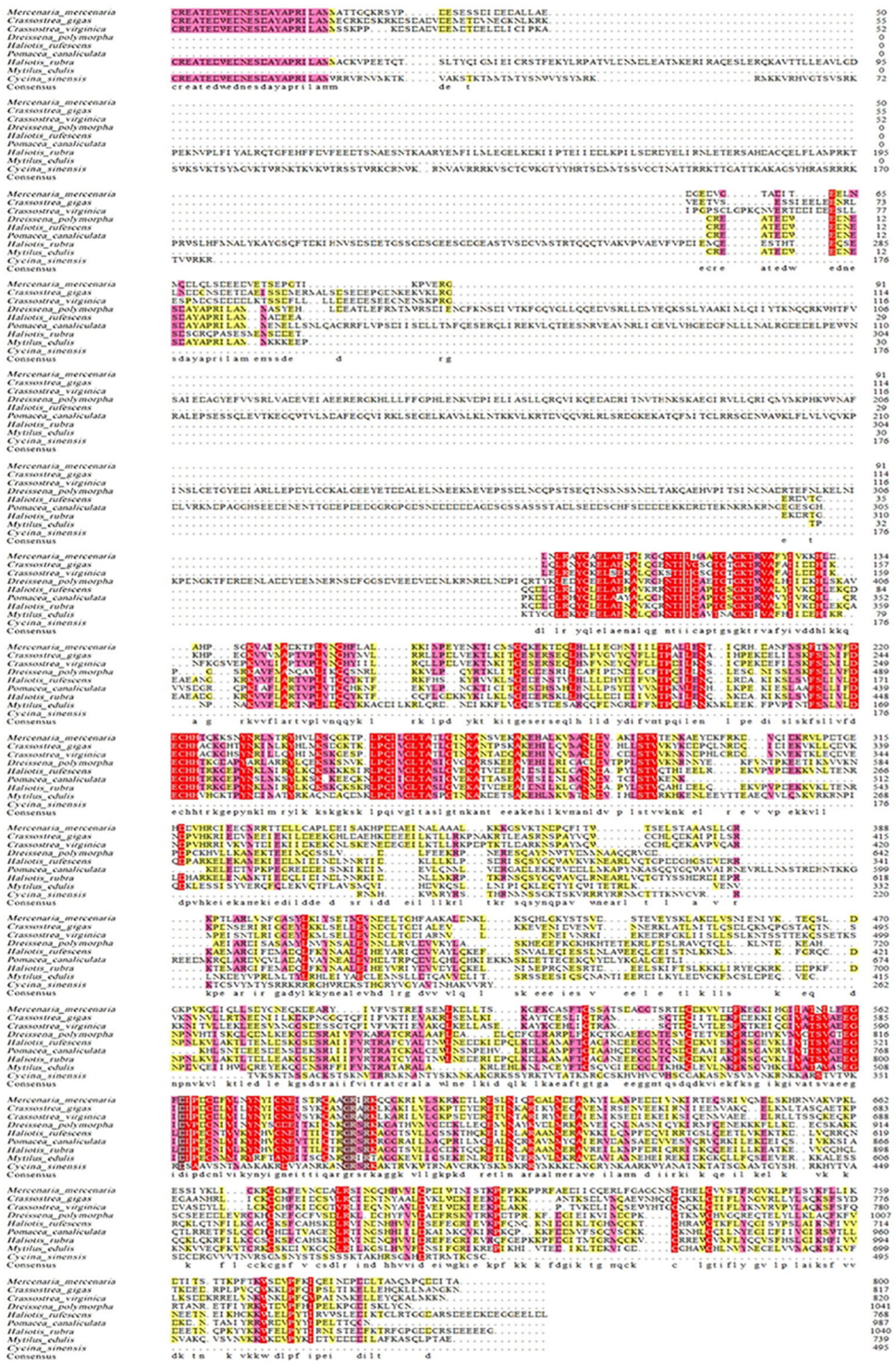

FIGURE 4
Alignment of the *IFITM1* amino acids from *Cyclina sinensis* and other species. All *shaded regions* represent residues sharing homology. The *light red regions* represent homology above 75%, while the *dark red regions* represent 100% homology. *Dots* denote amino acid deletion.

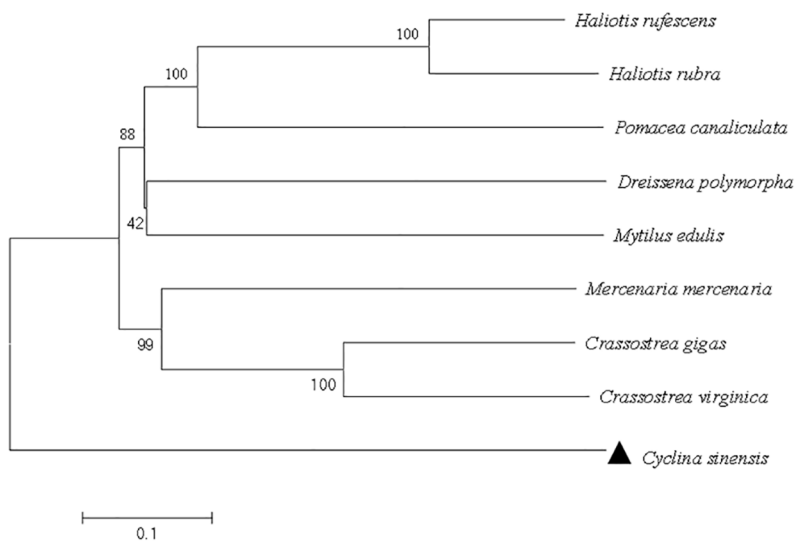

FIGURE 5
Phylogenetic tree derived from multiple alignment of the *IFITM1* amino acids from *Cyclina sinensis* and other species.

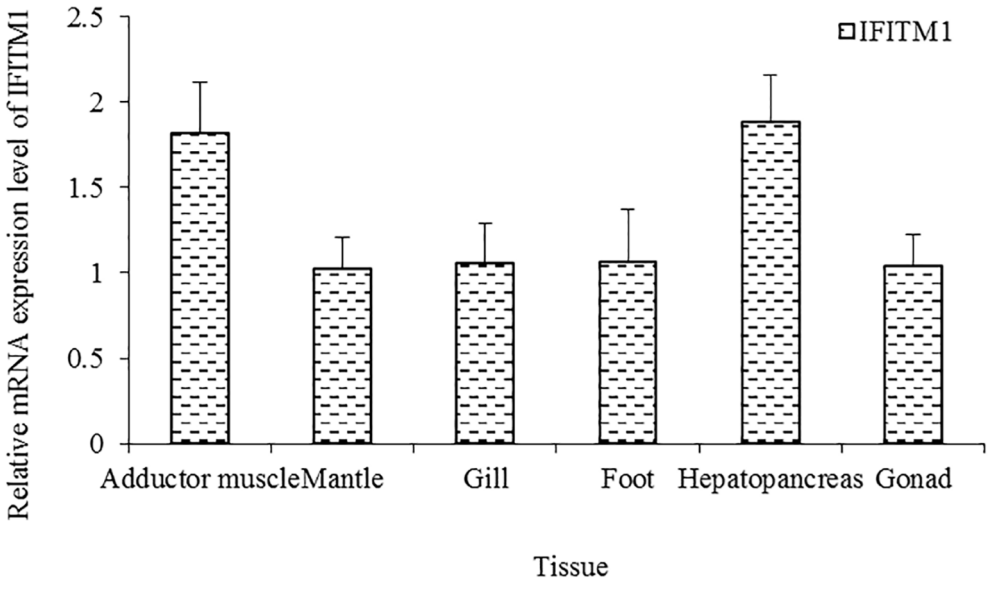

FIGURE 6
Expression of the *IFITM1* gene in various tissues of *Cyclina sinensis*.

significantly higher from 6 to 768 h in the clams exposed to ammonia nitrogen compared to the control. This result indicated that the gene expression of *CsIFITM1* could be induced by ammonia nitrogen stress. This is probably because ammonia nitrogen exposure can stimulate the *CsIFITM1* gene (Tanaka et al., 2005) or induce IFNs (Zou et al., 2005). Following infection with *V. parahaemolyticus*, the transcript levels of *CsIFITM1* in hepatopancreas were upregulated, indicating that the gene expression of *CsIFITM1* can be induced by infection with *V. parahaemolyticus*, suggesting that the *CsIFITM1* gene may be involved in the immune response of *C. sinensis*. This observation is consistent with the reported role of *IFITM* in the large yellow croaker (*Larimichthys crocea*), which can be stimulated by *Vibrio alginolyticus* (Zhang et al., 2021).

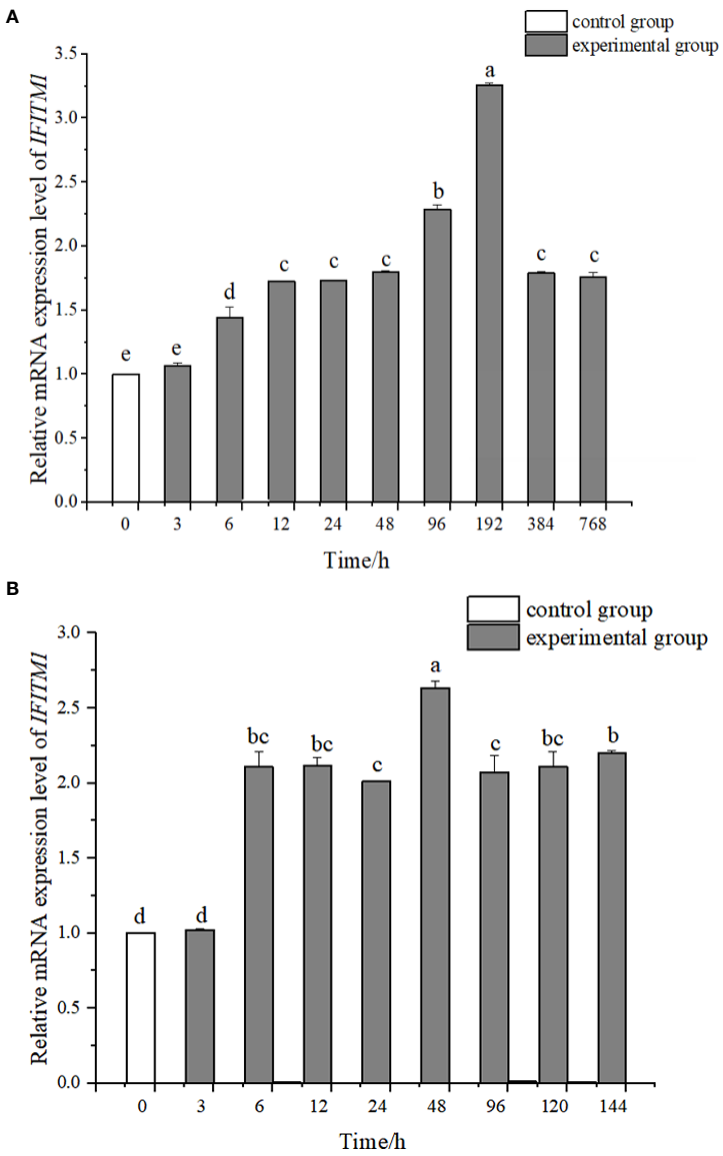

FIGURE 7
Expression of the *IFITM1* gene in the hepatopancreas tissue of *Cyclina sinensis*. **(A)** Gene expression in *C sinensis* exposed to long-term ammonia. **(B)** Gene expression in *C sinensis* infected with *Vibrio parahaemolyticus*. *Different letters in the column* indicate significant difference ($p < 0.05$).

In summary, exposure to ammonia nitrogen can activate the SOD in the clam, and activating SOD can reduce the oxidative damage induced by ammonia nitrogen stress. Furthermore, infection with *V. parahaemolyticus* may cause irreparable damage to the antioxidant system. The *IFITM1* gene from *C. sinensis* was cloned, and the *CsIFITM1* mRNA was broadly expressed in all the tissues selected. The *CsIFITM1* protein possessed the typical structural features of members of the IFITM family. The gene expression of *CsIFITM1* can be induced by ammonia nitrogen stress and *V. parahaemolyticus* infection.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding authors.

Author contributions

HG: Conceptualization, methodology, and writing—review and editing. QN: Formal analysis and writing—original draft. JL: Software, validation, and investigation. ZD: Project administration, conceptualization, and funding acquisition. SC: Investigation. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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