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Molecular cloning and function of two tumor necrosis factor receptor-associated factors genes (*TRAF2* and *TRAF4*) from *Pinctada fucata martensii*

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Tumor necrosis factor receptor-associated factors (TRAFs) have been studied in a few mollusks and participate in various biological processes, like apoptosis, immune response, stress, and inflammatory response. However, TRAFs' function and mechanism of pearl oysters (*Pinctada fucata martensii*) are still unclear. In this study, the novel *PmTRAF2* and *PmTRAF4* from *P. f. martensii* were cloned by rapid amplification of complementary DNA ends and their mRNA expression were analyzed by quantitative real-time PCR (qPCR). The interacting protein of PmTRAF2 was verified by the yeast two-hybrid assay. The result shows that full-length of *PmTRAF2* and *PmTRAF4* cDNA were 2055 bp and 2365 bp, respectively. The deduced PmTRAF2 and PmTRAF4 proteins contain TRAF-type zinc finger domain and MATH domain, while PmTRAF4 lacks a RING finger domain. Multiple sequence alignment revealed that PmTRAF2 and PmTRAF4 had high homology with the ortholog of other species. Phylogenic analysis indicated that PmTRAF4 clustered with the homolog protein of *Mytilus edulis* and *Mytilus galloprovincialis*, and PmTRAF2 has the closest genetic relationship to *Crassostrea gigas* TRAF2. The qPCR analysis revealed that *PmTRAF2* and *PmTRAF4* were expressed in all six tissues, and both of them were significantly expressed in hepatopancreas and gill ($p < 0.01$). Under lipopolysaccharide (LPS) stimulation, polyinosinic acid (Poly I:C) stimulation, and nucleus insertion surgery, the transcripts of *PmTRAF2*, *PmTRAF3*, *PmTRAF4* and *PmTRAF6* in hepatopancreas were markedly changed at corresponding time points. These results have indicated that these genes may play a role in *P. f. martensii* innate immunity. Yeast two-hybrid assays show that PmTRAF2 interacts with PmTRAF6 but not PmTRAF3, potentially affecting downstream immune signaling pathways. Our findings provide new perspectives for further investigation of TRAFs' immune mechanisms in bivalves.

KEYWORDS

***Pinctada fucata martensii*, *PmTRAF2*, *PmTRAF4*, innate immunity, yeast two-hybrid assay**

1 Introduction

Tumor necrosis factor receptor-associated factors (TRAFs) are a family of cytoplasmic adapters that play important roles in innate and acquired immunity, promoting intracellular signal transduction *via* receptors, such as tumor necrosis factor receptor (TNFR), toll-like receptors-interleukin-1binding receptors (TLR/IL-1R), the Epstein–Barr virus protein LMP1, and the RIG-I–like receptor family, potentiating the recruitment of signal transduction proteins (Arch et al., 1998; Evans et al., 2018; Guo et al., 2020). Seven members of the TRAF family, TRAF1-7, have been identified and participated in a host of signaling pathways relative to immunity. TRAF2, a prototypical TRAF, combines with TNFR1, TNFR2, CD40, and LMP-1 to induce downstream signaling factors, participating in cell survival and apoptosis, immunity, and infection process (Han et al., 2003). TRAF1, TRAF2, TRAF3, TRAF5, and TRAF6 can be recruited by CD40, whose cytoplasmic tail has two TRAF-interacting motifs; TRAF2 and TRAF3 bind to PxQxT motif to positively regulate the downstream factor nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), while TRAF6 binds to another motif to activate NF-κB (Bishop, 2004; Hauer et al., 2005). In addition, TRAF4 modulated mitogen-activated protein kinase, c-Jun N-terminal kinase, Wnt/β-catenin, and NF-κB signaling pathways *via* interacting with other molecules in mammals subsequently exerting functions in immunity (Xu et al., 2002; Wang et al., 2014; Lalani et al., 2018).

Interacting with receptors to modulate pathways, TRAFs can function as a regulatory factor in physiological processes. TRAF1 can regulate pathways that can be mediated by TRAF2, and TRAF3 can increase TRAF6-mediated NF-κB transcriptional activity while inhibiting the TRAF2/5-mediated NF-κB signal pathway (Hauer et al., 2005; Xie et al., 2006; Foight and Keating, 2016). These findings have revealed that different members of the TRAFs' family interactions with others can result in different effects. TRAF members can interact to form homo- and hetero-dimers with distinct regulatory functions involved in signal transduction (Arron et al., 2002). In the CD40 signal pathway, TRAF1, TRAF2, TRAF3, and TRAF6 can form homo-dimers and bind CD40 receptors. TRAF3 and TRAF5 can form hetero-oligomers (Arron et al., 2002; Xie et al., 2006). These findings indicate that TRAFs may regulate immune-related pathways through their interactions. However, at present, the specific mechanism of the mutual regulation and binding of TRAFs is still unclear.

In recent years, most TRAFs from bivalves in marine mollusks have been identified and studied. Four *Chlamys nobilis* TRAF genes were identified, and their messenger RNA (mRNA) expression levels in hemolymph were significantly increased by exposure to *Vibrio parahaemolyticus* compared to the control group, which indicates that *TRAFs* are involved in host immunity (Zhang et al., 2021). *HcTRAF6* expression level was significantly up-regulated in gills and hemocytes in response to immune stimulation (*Aeromonas hydrophila* and lipopolysaccharide (LPS) stimulation) in *Hyriopsis cumingii* (Huang et al., 2018). In the oyster, *TRAF2* and *TRAF6* from *Crassostrea gigas* and *TRAF6* from *Mizuhopecten yessoensis* were induced to express upon bacterial infection (He et al., 2013; Huang et al., 2016; Mao et al., 2017). Although TRAFs can have an impact in response to immune-related stimulation, their functions and molecular mechanisms in different bivalves still need to be explored.

The oyster *Pinctada fucata martensii*, a type of seawater pearl oyster, relies primarily on innate immunity to defend against invading pathogens (Zhang et al., 2022). In previous studies, the *TRAF3*, *TRAF6*, and *TRAF7* genes from the pearl oyster were identified (Huang et al., 2012; Jiao et al., 2014; Lei et al., 2016); Huang et al. and Jiao et al. have observed that TRAF3 and TRAF6 are involved in the innate immune response to *V. alginolyticus* and LPS stimulation, respectively. However, less is well understood about two *P. f. martensii TRAFs*, *TRAF2* (*PmTRAF2*) and *TRAF4* (*PmTRAF4*), and the role of these genes in the immune response remains obscure. In this study, *PmTRAF2* and *PmTRAF4* were identified in *P. f. martensii* hemocyte transcriptome, and their expression levels were analyzed in different tissues. The expression levels of *PmTRAF2*, *PmTRAF3*, *PmTRAF4*, and *PmTRAF6* were analyzed in the hepatopancreas in response to LPS and polyinosinic acid (PolyI:C) stimulation, and nucleus insertion surgery. In addition, we used yeast two-hybrid to verify the interacting proteins. Our study offers new insights to further study the function of TRAF and the mechanism of shellfish immunity.

2 Materials and methods

2.1 Experimental sample

P. f. martensii (about 6-8 cm in shell length) were procured from Xuwen Breeding Base, Zhanjiang, Guangdong Province, China, and were temporarily cultured in seawater at 20-25°C for about 3 d to adapt to the indoor environment and conduct follow-up experiments.

2.2 Stimulation experiments and tissue sample collection

A total of 250 oysters *P. f. martensii* were randomly divided into four groups: 10 oysters in the tissue quantification group, 60 oysters in the LPS stimulation group, 60 oysters in the PolyI:C stimulation group, 60 oysters in the phosphate-buffered saline (PBS) group (control group), and 60 oysters in the nuclear insertion surgery.

In the tissue quantification group, mantle, gills, adductor muscle, hepatopancreas, hemolymph, and gonad were collected from each pearl oyster. The hemolymph was centrifuged at 3500 rpm for 5 min at 4°C, and the pellet (hemocytes) was collected and resuspended with 1 mL of Trizol reagent (Thermo-Fisher Scientific, Waltham, MA, USA).

The adductor muscle injection method was used in LPS and PolyI:C stimulation experiments. Each pearl oyster in the LPS stimulation group was injected with 100 μL LPS (10 μg/mL, diluted with sterile PBS), while the control group was injected with 100 μL PBS and the PolyI:C stimulation group was injected with 100 μL PolyI:C (10 μg/mL, diluted with sterile PBS) (Liang et al., 2022). At 0, 12, 24, 48, 72, and 96 h exposed to LPS and PBS, hepatopancreas were collected with 10 pearl oysters at each time point. All samples were stored at -80°C.

Sixty oysters were used to perform nucleus insertion surgery by an experienced technician at Xuwen Breeding Base, China. In nucleus insertion surgery, a piece of mantle graft from a donor oyster with a spherical shell bead or nucleus is transplanted into the gonad of the host oyster (Wu et al., 2020). At 0, 12, 24, 48, and 72 h after insertion, hepatopancreas samples were collected with 10 pearl oysters.

2.3 Cloning and sequence analysis

The partial sequences of *PmTRAF2* and *PmTRAF4* were from the hemocyte transcriptome of *P. f. martensii* in a previous study (Wang et al., 2017). To obtain the full-length sequences, we used Rapid amplification of complementary DNA (cDNA) ends to amplify the 5′ and 3′ ends of *PmTRAF2* and *PmTRAF4* (Liang et al., 2022). The primers are presented in Table 1. DNAMAN8.0 software was used to align and splice full-length nucleotide sequences, and open reading frames (ORF) finder was employed to predict ORF. The theoretical isoelectric point and molecular weight of amino acid sequences were predicted by ExPASy-ProtParam (https://web.expasy.org/protparam/). Protein domains were carried out on the SMART online sites. After performing multiple sequence alignments with ClustalW, the phylogenetic tree was constructed by MEGAX software using the neighbor-joining method.

2.4 Quantitative real-time PCR (qPCR) assay

Total RNA was extracted from the isolated *P.f. martensii* tissues (mantle, gills, adductor muscle, hepatopancreas, hemolymph, and gonad) and purified using Trizol reagent (Thermo-Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. The purified RNA was treated with RNase-free Dnase (Promega, Promega, Madison, WI, USA) to eliminate DNA contamination. RNA integrity was assessed *via* electrophoresis on a 1% agarose gel stained with ethidium bromide. The RNA concentration was determined based on the OD_{260}/OD_{280} ratio using a NanoDrop 2000 spectrophotometer (Thermo-Fisher Scientific, Waltham, MA, USA). RNA aliquots (2 μg) were used to synthesize cDNA using M-MLV reverse transcriptase (Promega, Promega, Madison, WI, USA).

To explore the potential role of *PmTRAF2* and *PmTRAF4*, we performed qPCR to detect their tissue-specific expression in all tissues and their temporal expression in the hepatopancreas after LPS stimulation. Reagents were mixed according to the instructions of the Dy NAmo Flash SYBR Green qPCR kit (ThermoFisher Scientific, Waltham, MA, USA), and fluorescence detection was performed on an Applied Biosystems 7500/7500 Fast Real-Time system. The PCR reaction steps were as follows: 95°C for 5 min; 95°C for 10 s, 60°C for 15 s, 72°C for 15 s, 40 cycles; 95°C for 10 s, 60°C for 60 s, 95°C for 1 s; 37°C for 30 s. The target gene's relative expression was calculated *via* the $2^{-\Delta\Delta CT}$ method. The qPCR primers are shown in Table 1. Actin was an internal reference primer. After completing all runs, data from three replicates of each sample were analyzed using 7500 System SDS software 2.0.1 (ABI, Applied Biosystems, Inc., Waltham, MA, USA). The baseline was automatically set by the software.

2.5 The yeast two-hybrid assay

To verify whether PmTRAF2 interacts with PmTRAF3 or PmTRAF6, we performed a yeast two-hybrid experiment using the GAL4 system. The coding sequence of the PmTRAF2 gene was inserted into the pGBKT7 vector, while the coding sequences of the PmTRAF3 and PmTRAF6 genes were respectively inserted into the pGADT7 and sequenced to screen for the correct plasmid. Two plasmids (0.3 μg each) and denatured salmon sperm DNA (0.1 mg) were added to yeasts. Then, 600 μL PEG/LiAc solution was added to yeasts and put in the water bath at 30°C for 45 min. Then, 70 μL DMSO was added and put in the water bath at 42°C for 20 min and placed in ice for 2 min. Transformed yeasts were obtained after centrifuging at 700 g for 5 min at room temperature. The pGBKT7-PmTRAF2/pGADT7-PmTRAF3 and pGBKT7-PmTRAF2/pGADT7-PmTRAF6 plasmids were co-transformed into yeast AH109 as experimental groups, respectively, while PGBKT7-53/PGADT7-T was used as a positive control group, pGBKT7-lam/pGADT7-T as a negative control group, and pGBKT7-PmTRAF2/pGADT7, pGADT7-PmTRAF3/pGBKT7, and pGADT7-PmTRAF6/pGBKT7 as self-activation assay groups. The co-transformed yeast cells were dispersed on selective SD/-Leu/-Trp and SD/-Ade/-His/-Leu/-Trp plates and cultured at 30°C for 5 d. Yeasts grown on SD/-Ade/-His/-Leu/-Trp plates were transferred to new SD/-Ade/-His/-Leu/-Trp plates and cultured for 2-4 d. If the yeasts turn blue, it indicates successful protein interactions.

TABLE 1 Primer sequences were used in this study.

Application	Primer	Sequence (5′-3′)
Cloning	PmTRAF2-ORF-F	GCATCGGGAAATTGAGAGGT
	PmTRAF2-ORF-R	TCCTGTTCGTCGTTATTCTGG
	PmTRAF4-ORF-F	TCGTCTTTGGGGAGGGATAG
	PmTRAF4-ORF-R	GAAAGTAATGGGAAGAGCGAAA
	PmTRAF2-5'-outer	GCAGCTTTAAGAAGCCCACGTCAG
	PmTRAF2-5'-inner	GAACTTGCATTCAGTGGCGTGTTT
	PmTRAF2-3'-outer	GTGGTGATGATGGGGGATTACGAC
	PmTRAF2-3'-inner	GCAGCTTTAAGAAGCCCACGTCAG
	PmTRAF4-5'-outer	GGGACATAACGCCTGTATATGGACCTC
	PmTRAF4-5'-inner	CAGTACGTTTATGGCACTCATTCCTCAT
	PmTRAF4-3'-outer	CCTTTCGCTCTTCCCATTACTTTCA
	PmTRAF4-3'-inner	GACACAGATAGAGATGCTCTCGGCTTT
qPCR	PmTRAF2-qPCR-F	GGGAGTGAGGACAACGCAGT
	PmTRAF2-qPCR-R	GTGCCACTGGTCCCGAGT
	PmTRAF4-qPCR-F	TACAGGCGTTATGTCCCTCAGC
	PmTRAF4-qPCR-R	CCATGTTAAACCTGGGGCACTT
	Actin-qPCR-F	CGGTACCACCATGTTCTCAG
	PmTRAF3-qPCR-F	GCCCGTTCCGTGTCCTAATTAC
	PmTRAF3-qPCR-R	GTAAAGGGTTTATACCACACTCATACC
	PmTRAF6-qPCR-F	GATGGAAACGCTTGTAGCGA
	PmTRAF6-qPCR-R	AGCACAGTCAAAGGGAGGAA
	Actin-qPCR-R	GACCGGATTCATCGTATTCC
Yeast two-hybrid	PmTRAF2-BD-F	CGGGATCCGTATGAACGACGCAGAAG
	PmTRAF2-BD-R	GGTTCTGCAGTCACAACGTCACTGTTGTTTTC
	PmTRAF3-AD-F	ATATGGCCATGGAGGCCAGTGAATTCATGTGTAAAATTTGTAATG
	PmTRAF3-AD-R	CGCTCGAGTCAAATGCTGTCTTCATAC
	PmTRAF6-AD-F	CTACAATTGTATGGCATCGAATGATAAC
	PmTRAF6-AD-R	CCGCTCGAGtcaAGAATTGTCTAGCACAG

2.6 Data analysis

All experimental results on qPCR were expressed as a mean ± standard deviation (S.D.) and were analyzed using SPSS 19.0 (IBM, Chicago, IL, USA). Differences in gene expression levels in the tissue quantification group and nucleus insertion surgery group were determined by a one-way analysis of variance with Duncan's Multiple Range test. Differences in gene expression levels between different groups at the same time point were analyzed by T-test. Statistical significance was defined at $p < 0.05$.

3 Results

3.1 Sequence analysis and characterization of *PmTRAF2* and *PmTRAF4*

The full-length sequences of *PmTRAF2* and *PmTRAF4* are obtained using RACE (Figure 1). The nucleotide sequence of *PmTRAF2* was 2055 bp, containing a 5'-untranslated region (UTR; 183 bp), a 3' UTR (288 bp) with a polyA tail (27 bp), and an ORF (1584 bp), encoding 527 amino acids (Figure 1A). The

A

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1     GGGTTGGTTAAGCAGTGGTATCAACGCAGAGTACATGGGGATTAATAAGGATTATCATCACCGAGTACATGAGGGAGTACAAGTCCGACG
91    ACCGTCTTGAAGGGAGTACTGAGAAGATTCACATGTACAAAACTGTTCGGAAATACAAGAAGCATTTGAAAGAAAAAAATTCTACAAAAC
181   AAAATGAACGACGCAGAAGCGCGTGGAGAGACTCCAATGGAAGTCGGGTATCCGACAGAGATATTTCCCCAAGGATTTGACGAGAAGCTG
          M  N  D  A  E  A  R  G  E  T  P  M  E  V  G  Y  P  T  E  I  F  P  Q  G  F  D  E  K  L
271   ATTTGTAACAGCTGTAACAAAGTGCTGAAGGATCCCGTACAAAGTTATTGTGGTCATAGATTCTGTAAACCTTGCATCAATAGGGAAATG
       I  C  N  S  C  N  K  V  L  K  D  P  V  Q  S  Y  C  G  H  R  F  C  K  P  C  I  N  R  E  M
361   AGTAAAGGCCAACATGTACGCTGTCAGACATGTATCCGAGAAGGAGCTGAAGAAGAAGACTACAGCATGATCAGCAAAGATCACATATTT
       S  K  G  Q  H  V  R  C  Q  T  C  I  R  E  G  A  E  E  E  D  Y  S  M  I  S  K  D  H  I  F
451   CCTGACAATGCACTCAGACGTGAGATGTTCAGCATGGAAGCAACTTGTCTGAATCAAGGATGTAATTGGTCTGGCAAATTCAGACATTTC
       P  D  N  A  L  R  R  E  M  F  S  M  E  A  T  C  L  N  Q  G  C  N  W  S  G  K  F  R  H  F
541   GAGAAACACGCCACTGAATGCAAGTTCAAACCAGTGACATGCCAACAATGTCATGGATTGGTTGAAGCCTCCAGATATAAACATCACCTT
       E  K  H  A  T  E  C  K  F  K  P  V  T  C  Q  Q  C  H  G  L  V  E  A  S  R  Y  K  H  H  L
631   GGAAATGACTGTCCAAAACGACCAATCAAATGCCAGTACTGCAAAGAGGATTTCTTCGCAGAGTCATACGAGGCCCACAAGTTAGAGTGT
       G  N  D  C  P  K  R  P  I  K  C  Q  Y  C  K  E  D  F  F  A  E  S  Y  E  A  H  K  L  E  C
721   CCTAAGTGTCCAGTGAAATGTGACGACTGTGGGAAAAAGAAGCTGACCAGAGATATGTTGCAACATCACTATAACAACGAATGTTCACAG
       P  K  C  P  V  K  C  D  D  C  G  K  K  K  L  T  R  D  M  L  Q  H  H  Y  N  N  E  C  S  Q
811   AGGAAGATTACGTGTCCTGCGGGATGTGATCCTGTAGAGCGTAGCAGGTTCTTGACACACCTTGATCAGAAATCCGGGTTCCATCTAAAC
       R  K  I  T  C  P  A  G  C  D  P  V  E  R  S  R  F  L  T  H  L  D  Q  K  S  G  F  H  L  N
901   TTTATCATGGATCAACTCAAAATTCTGTCGCAAGAAACCAATACAAAAGTCGGTCAATTAGCTCCAATGTCGGAAATCTTACAACTCAAA
       F  I  M  D  Q  L  K  I  L  S  Q  E  T  N  T  K  V  G  Q  L  A  P  M  S  E  I  L  Q  L  K
991   CAGAACGTGGAGCAACTCCAAGTAAGATTACAACAAGTAGGAGGACCAATACCTCCCGGGAGTGAGGACAACGCAGTGATGCAGCAACGA
       Q  N  V  E  Q  L  Q  V  R  L  Q  Q  V  G  G  P  I  P  P  G  S  E  D  N  A  V  M  Q  Q  R
1081  TGCCGAGCTCTTGAATTGAAAGTGAGCACATTTGAGGGAATCGTCACCACGCTGCATCGGGAAATTGAGAGGTGTCTCACCCATCTTGAT
       C  R  A  L  E  L  K  V  S  T  F  E  G  I  V  T  T  L  H  R  E  I  E  R  C  L  T  H  L  D
1171  GGCATAGAAAGGAGGAACTCTGGTAACTCGGGACCAGTGGCACAAAGAGTAGAGCAACTGGAACGTGCTCTTGCACAGAAAGACAAAGAA
       G  I  E  R  R  N  S  G  N  S  G  P  V  A  Q  R  V  E  Q  L  E  R  A  L  A  Q  K  D  K  E
1261  AATATGGAATTGAAACAGTACATTGAAGACAAATTGTCCGCCACTTACGATGGAGAACACGTATGGAGAATAGAAAACTGGTCAAAGTTA
       N  M  E  L  K  Q  Y  I  E  D  K  L  S  A  T  Y  D  G  E  H  V  W  R  I  E  N  W  S  K  L
1351  CGTAAGGATGCTATGTCAGGAATGAGGACCAGCATATTCTCTGTACCCTTTTATACAAGCAGACATGGATATAAAATGTGTGTTAGATTA
       R  K  D  A  M  S  G  M  R  T  S  I  F  S  V  P  F  Y  T  S  R  H  G  Y  K  M  C  V  R  L
1441  TATCCAAACGGCGATGGTATGGGTCGGGGGACGCATATATCGGTGTTCTTTGTGGTGATGATGGGGGATTACGACCCCGTACTCAAGTGG
       Y  P  N  G  D  G  M  G  R  G  T  H  I  S  V  F  F  V  V  M  M  G  D  Y  D  P  V  L  K  W
1531  CCATTCAAACATCGGGTCACATTCAGATTGGTCAACCAGAATAACGACGAACAGGATCAAACAGACTCTTTCAGACCTGACCCTAACAGC
       P  F  K  H  R  V  T  F  R  L  V  N  Q  N  N  D  E  Q  D  Q  T  D  S  F  R  P  D  P  N  S
1621  AGCAGCTTTAAGAAGCCCACGTCAGAAATGAACATAGCTAGCGGGTGTCCGCTCTTCATACCACTTACAAAAATTAATGACGCTAGCAGT
       S  S  F  K  K  P  T  S  E  M  N  I  A  S  G  C  P  L  F  I  P  L  T  K  I  N  D  A  S  S
1711  GGTTTTGTAAAAAATGACGTTCTTTTTCTGAAAACAACAGTGACGTTGAACTCTTGATAGTGGTATTTTTAAAAAAATTAATTCAGATTT
       G  F  V  K  N  D  V  L  F  L  K  T  T  V  T  L  N  S  *
1801  TTTTGTGATTTTTTTCTCATTTCAGGAAAGACAGTTTCTTGTTTTGTTTTTTTTTTTCGAAGAACCGCTTTCTGTAGAGGTCATGATTTT
1891  TTTCATCTTTGTCGCAGAAGAGATATTTAGCCATGTATATGACGTTCTGTACATTTGTACATACTGTATGTCAAATCATTTACTGAAAAT
1981  GTCTATGTTAAACACGATATATTTCAATATATGGTATCATAGACCTATAAAAAAAAAAAAAAAAAAAAAAAAAAA
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FIGURE 1 (Continued)

B

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1     GAATCAAAAAGAAGCAAAGGCAGGGACAGAGGGCAGACAACTCCTCAGCAAAAATATTCAGTGGATTACCTTGTACAGCAGGTGGTTATG
91    TTATCCATGGTTTTATGATTTAAAAACTTTCTAATAAATGATGGAATTACTGCATGATTTTTCGCAGTTGGAAAATTTTGTGAAATGTCA
                                                  M  M  E  L  L  H  D  F  S  Q  L  E  N  F  V  K  C  Q
181   ATATTGCAACTGTATGGGTGTGGAGAAAGAGTCCCCTACCTCGTCTTTGGGGAGGGATAGTATTTACAGAGCAAGTCTGAAATTACTCAG
        Y  C  N  C  M  G  V  E  K  E  S  P  T  S  S  L  G  R  D  S  I  Y  R  A  S  L  K  L  L  R
271   ACACATCAAAAATGCAGGAGTGTTCAGGTGTCCGGAAGATGACAGACCACTCGACTATGCACAGATTTACCCGGATGATGAGCTGACGAC
        H  I  K  N  A  G  V  F  R  C  P  E  D  D  R  P  L  D  Y  A  Q  I  Y  P  D  D  E  L  T  T
361   AGAAGTCATGAACAGTTTGATCCGGTGTAGATATCATAAGGAGGGATGCCGCTGGGTCGATAAACTCCAAAACCTACAGGCTCATTTAGA
        E  V  M  N  S  L  I  R  C  R  Y  H  K  E  G  C  R  W  V  D  K  L  Q  N  L  Q  A  H  L  D
451   CCAGTGTCCGTATGATTCGATCCAGTGCCCTAACAGCTGTACCAGGATGTTATCTCGTCTCTCATTGGATGATCATCTTGAGTTTTCTTG
        Q  C  P  Y  D  S  I  Q  C  P  N  S  C  T  R  M  L  S  R  L  S  L  D  D  H  L  E  F  S  C
541   TCCAAGAAGAATGGTCGTCTGTGAATTTTGCAATCAACAATTCCCTGGAGACTTATTTGAAAAGCAACATGCCGGTAATTGTCCGTACGA
        P  R  R  M  V  V  C  E  F  C  N  Q  Q  F  P  G  D  L  F  E  K  Q  H  A  G  N  C  P  Y  E
631   AGTGACATGGTGTGAAAATAAATGTGGAGCTAAGCTTGAGAGACGTTTCCTTGCCAATCACATGAGGAATGAGTGCCATAAACGTACTGT
        V  T  W  C  E  N  K  C  G  A  K  L  E  R  R  F  L  A  N  H  M  R  N  E  C  H  K  R  T  V
721   ACAATGTCCATACTGTAACAGGGACTTCGTCCAGGAGACTTTACAGACCCATCAGTACCAGTGCCCTAGATTCCCTGTGGCGTGCCCTAA
        Q  C  P  Y  C  N  R  D  F  V  Q  E  T  L  Q  T  H  Q  Y  Q  C  P  R  F  P  V  A  C  P  N
811   CAGGTGTGACCCGACAAAGATCCCCAGGGAGGAGGTGGAGGTCCATATACAGGCGTTATGTCCCTCAGCTACTGTATCTTGTACATTTAA
        R  C  D  P  T  K  I  P  R  E  E  V  E  V  H  I  Q  A  L  C  P  S  A  T  V  S  C  T  F  K
901   AGAAGCCGGATGCAAACATAAGTGCCCCAGGTTTAACATGGACAAGCATTTAGACAACAACATGAAGGGCCACCTGCAGCTCATGTGTGA
        E  A  G  C  K  H  K  C  P  R  F  N  M  D  K  H  L  D  N  N  M  K  G  H  L  Q  L  M  C  D
991   CCTGGTTGCTAAGCAACAAACTCAAATCACCCAATTGTGCAATGCTTTATACTCAATCACCCATGTGACAGACGGTACATTTGTGTGGAA
        L  V  A  K  Q  Q  T  Q  I  T  Q  L  C  N  A  L  Y  S  I  T  H  V  T  D  G  T  F  V  W  K
1081  AATCTCAAACTACAAGCAAAAATTCTTAGAGTCTGTTTACAAGAATGCAGAACTTGTCAGTGAACCGTTTTACACGTCTCGCTACGGATA
        I  S  N  Y  K  Q  K  F  L  E  S  V  Y  K  N  A  E  L  V  S  E  P  F  Y  T  S  R  Y  G  Y
1171  TAAAATGGCAGCTTCTCTATTTCTGAACGGAAACGGAGCCGGTGAGGGGAAATACATCTCAGTCTATATCAAAATCTTGCCAGGAGAGTA
        K  M  A  A  S  L  F  L  N  G  N  G  A  G  E  G  K  Y  I  S  V  Y  I  K  I  L  P  G  E  Y
1261  TGACAATATCTTGGAATGGCCTTTCGCTCTTCCCATTACTTTCAGTCTTTTTGATCAGTGTGTTGAGTTTGATTTCCGAGCAAACATTTC
        D  N  I  L  E  W  P  F  A  L  P  I  T  F  S  L  F  D  Q  C  V  E  F  D  F  R  A  N  I  S
1351  AGAAAGCTTTGTTCCTGACCCGACATGGAAACATTTCCAGAAGCCAAGAAAGGACACAGATAGAGATGCTCTCGGCTTTGGATATCCAAA
        E  S  F  V  P  D  P  T  W  K  H  F  Q  K  P  R  K  D  T  D  R  D  A  L  G  F  G  Y  P  K
1441  ATTTGTGTCGCATGAAATTCTCAAAACCAGGGACTACATCAAGGATGACAGCATAATCATTAAGGTCAAGGTCGATAACAATAAATTCAT
        F  V  S  H  E  I  L  K  T  R  D  Y  I  K  D  D  S  I  I  I  K  V  K  V  D  N  N  K  F  I
1531  TTCACCTTGAAGAGGTCAAATGTCATTTCAAAGTAACGTTCATTTCATTTTGGCTACATTAAATTAAAGATTGCAATCAGACTGTTACTC
        S  P  *
1621  ATGTATAATGTTTGTGACTTATATAATTGCAAAAGTGCTTTGTGATGTATGATGTATGTATGGGGTTATTTGGTTTGAATGATTGAGAAA
1711  TGATTATTTATTTTGATAGTTATTGTGTATTGATTGCTTGTGATTTTATGTTACCCAGTTATACTATACATTAATATAATAATTACTTTA
1801  AATGATTACAAATATCTAGCTAGGATAAGTTTACCTTAAGATATTATTAATTTTGTTATAATTCTCCAAGAACCAAAGTCAGGGAAATTC
1891  ATGTTGTCTGTTATATCGAGTATAATTAATAATGATCTTGTTGTCTTCTATTTCTGTGAATAACAATCAATAATGATGTAATATTATGGG
1981  GGATTCTTTACTTGTGATGTCGACGATATGATGGTGGTACCATATCACAAATAGCATTATTATTGACAACATTATGCCCTCCATCATTTA
2071  GAAATGATTATGCATGATGTTTTAATATGTTCTTGTTGTTCGGAGGACATCCTCATCGATCATCATGACTGCTGAATATAGCATCATTGA
2161  GTCTTTCTTCCTGCGGAGTACATAAGATGACCAGGTAGGATAGATGAGTACTTCTCGTCAACAATCCTTCTTCTCTACTACTTTTGTACT
2251  CCTCCACTATTTGTACATAATGGTATACACCTACTTTATGTTGGCAGCTAATATCAACAAGTATTTTGATGTTCATGTAATTAATTATTC
2341  TACACAAAAAAAAAAAAAAAAAAAA
```

FIGURE 1 (Continued)
The full-length sequences and deduced amino acids of *PmTRAF2* **(A)** and *PmTRAF4* **(B)**. Amino acids with double-underscore stand for the RING finger domain, light shadows for the TRAF-type zinc finger domain, light shadows with boxes for the TRAF_BIRC3_bd domain, and deep shades of gray for the MATH domain. * indicates the translation stop codon.

predicted molecular mass and theoretical isoelectric point (pI) of PmTRAF2 were 60.46 kD and 8.06, respectively. A RING finger domain, two TRAF-type zinc finger domains, a TRAF_BIRC3_bd domain, and a MATH domain were identified in the deduced PmTRAF2 protein.

The nucleotide sequence of *PmTRAF4* was 2365 bp, containing a 5' UTR (127 bp), a 3' UTR (825 bp) with a polyA tail (20 bp), and ORF (1413 bp), encoding 470 amino acids with a predicted molecular mass of 54.70 kD and theoretical isoelectric point (pI) of 7.15 (Figure 1B). The deduced amino acid sequence was composed of three TRAF-type zinc finger and a MATH domain.

3.2 Multiple sequence alignment and phylogenic relationships

PmTRAF2 was compared with TRAF2 sequences from *C. gigas*, *Homo sapiens*, *Mus musculus*, *Oncorhynchus mykiss*, and *Columba livia*, showing that PmTRAF2 shared the highest homology with TRAF2 from *C. gigas* (47.1%; Figure 2A). The comparison of PmTRAF4 with TRAF4 amino acid sequences from *H. sapiens*, *Branchiostoma belcheri*, *Mytilus coruscus*, *Aplysia californica*, and *Cryptotermes secundus* indicated that PmTRAF4 was highly similar to *M. coruscus* TRAF4 (70.4%) and *A. californica* TRAF4 (65.1%), and TRAF4 sequences were highly conserved (Figure 2B).

Our phylogenetic analysis revealed that proteins TRAF2 and TRAF4 from vertebrates and invertebrates clustered into two major branches. All TRAF4 and TRAF2 had a highly conserved structural domain composition (Figure 3B). PmTRAF4 clustered with *Mytilus edulis* TRAF4 and *Mytilus galloprovincialis* TRAF4 (Figure 3A). Furthermore, PmTRAF2 clustered with *C. gigas* TRAF2, and a distinct division between vertebrate and invertebrate TRAF2 sequences was observed in the TRAF2 branch (Figure 3A).

3.3 Expression levels of *PmTRAF2* and *PmTRAF4*

The qPCR analysis showed that *PmTRAF2* and *PmTRAF4* were expressed in adductor muscle, hemocytes, gill, mantle, hepatopancreas, and gonads, and both genes were significantly expressed in hepatopancreas and gill tissues ($p < 0.01$, Figure 4).

3.4 Expression of *PmTRAFs* response to LPS stimulation, PolyI:C stimulation, and nuclear insertion surgery

After LPS stimulation, the expression of *PmTRAF2* in the hepatopancreas of the LPS group was significantly up-regulated compared with the PBS group at 24 h, 48 h, and 96 h ($p < 0.01$); *PmTRAF4* expression was significantly up-regulated at 96 h ($p < 0.05$); *PmTRAF3* and *PmTRAF6* were significantly expressed at 12, 24 and 96 h compared with the control group. Additionally, *PmTRAF3* was significantly expressed at 48 h after the LPS challenge (Figure 5A).

Compared with the control group, the mRNA expression levels of *PmTRAF3* and *PmTRAF6* in the hepatopancreas began to increase at 6 h after PolyI:C stimulation and reached maximum expression level at 48 h and 24 h, respectively (Figure 5B). At 24, 48, and 72 h exposure to PolyI:C stimulation, *PmTRAF2* mRNA expression was rapidly increased. However, *PmTRAF4* mRNA was significantly decreased (Figure 5B).

In nuclear insertion surgery, the mRNA expression trends of *PmTRAF2* and *PmTRAF6* genes in the hepatopancreas are the same, showing decreased, then increased, and then decreased (Figure 6). *PmTRAF4* mRNA reached maximum expression level at 6 h. *PmTRAF3* mRNA rapidly increased at 6 h, decreased at 24 h and 48 h, and rapidly increased at 72 h and 96 h (Figure 6).

3.5 PmTRAF2 interacts with PmTRAF6

In this experiment, a yeast two-hybrid assay was performed to study the interaction between PmTRAF2 and PmTRAF3 or PmTRAF6. AH109 of pGBKT7-PmTRAF2/pGADT7 co-transformed growth, pGADT7-PmTRAF3/pGBKT7 co-transformed growth, and pGADT7-PmTRAF6/pGBKT7 co-transformed growth grew on the SD/-Leu/-Trp medium but not on the SD/-Leu/-Trp/-His/-Ade/x-a-gal medium, indicating that PmTRAF2, PmTRAF3, and PmTRAF6 did not activate themselves (Figure 7). The yeasts of the experimental, positive control, and negative control groups grew on SD/-Leu/-Trp medium, except that yeasts of the positive control group and experimental group pGBKT7-PmTRAF2/pGADT7-PmTRAF6 grew on the SD/-Leu/-Trp/-His/-Ade/x-a-gal medium and turned blue, while the negative control group and experimental group pGBKT7-PmTRAF2/pGADT7-PmTRAF3 did not grow, indicating an interaction between PmTRAF2 and PmTRAF6 (Figure 7).

4 Discussion

Worsening coastal water quality has brought various diseases to *P. f. martensii*, which lacks a specific immune system. Thus, studying the role of immune-related genes in pathogen response may improve the survival of *P. f. martensii* (He et al., 2019b). TRAFs are major signal transducers of the TNF receptor superfamily and other receptors, which can regulate various cellular activities and innate immune

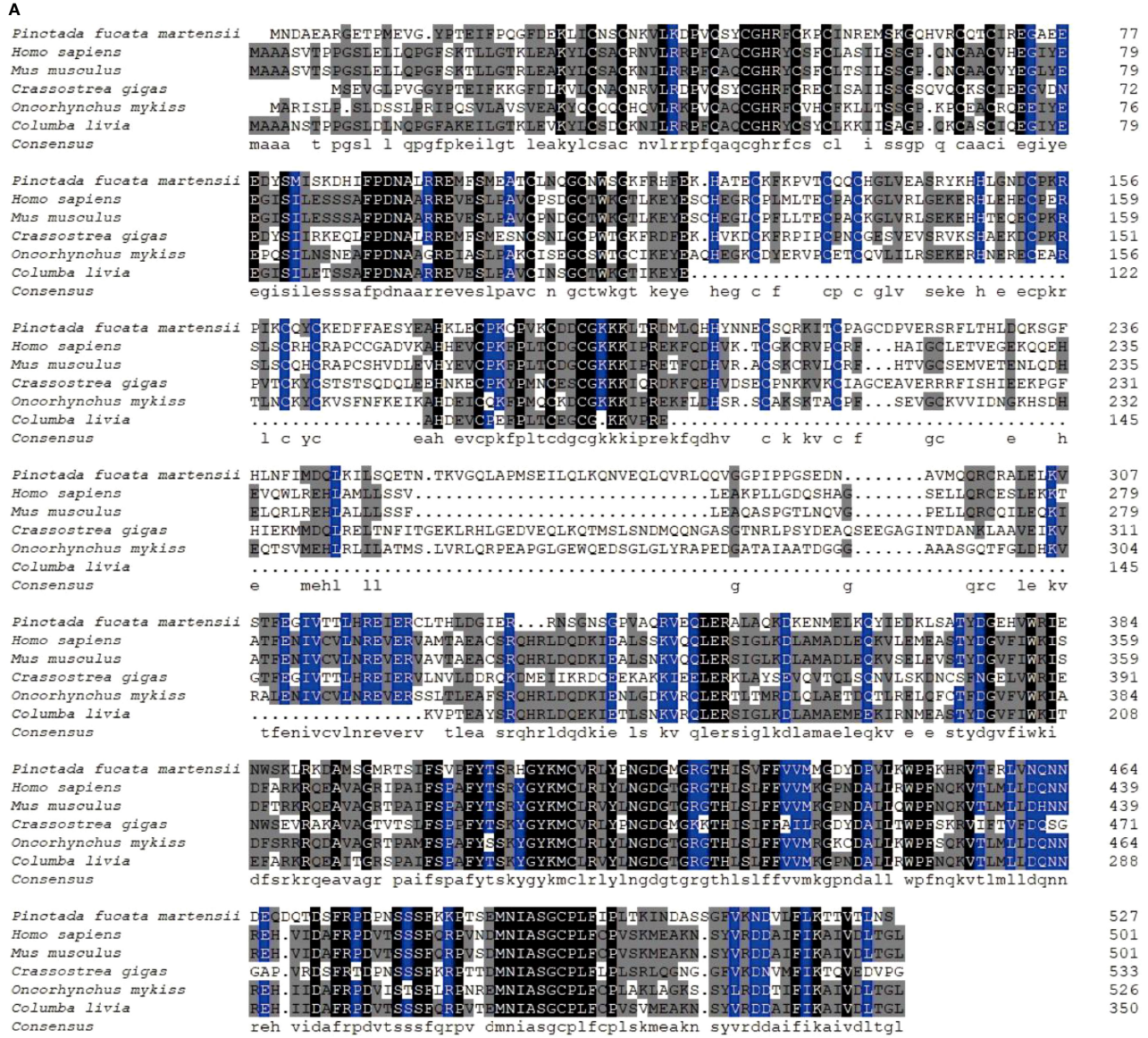

FIGURE 2 (Continued)

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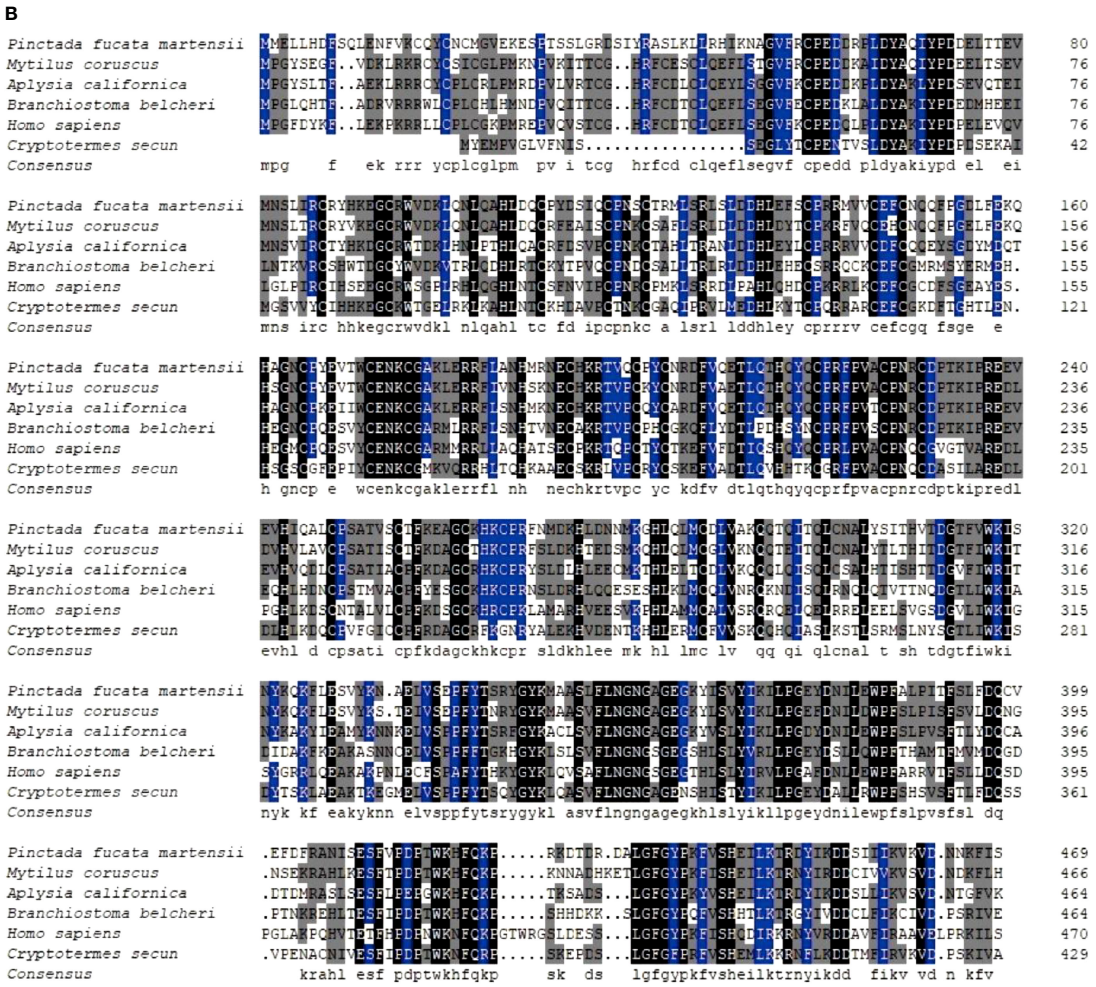

FIGURE 2 (Continued)
Alignment of PmTRAF2 **(A)** and PmTRAF4 **(B)** with their corresponding homologous amino acid sequences from other species, respectively. Identical residues are shown in black, while conserved residues in all sequences with more than 75% and 50% similarity are highlighted in blue and gray. The GenBank accession numbers of TRAF4 ortholog sequences are as follows: *Mytilus coruscus* (Accession no.: CAC5367166.1), *Aplysia californica* (Accession no.: XP_005107744.2), *Branchiostoma belcheri* (Accession no.: ABN04153.1), *Homo sapiens* (Accession no.: NP_004286.2), and *Cryptotermes secundus* (Accession no.: PNF16644.1). The GenBank accession numbers of TRAF2 ortholog sequences are as follows: *Homo sapiens* (Accession no.: NP_066961.2), *Mus musculus* (Accession no.: AAH03801.1), *Crassostrea gigas* (Accession no.: XP_034302657.1), *Oncorhynchus mykiss* (Accession no.: NP_001117865.1), and *Columba livia* (Accession no.: PKK17224.1).

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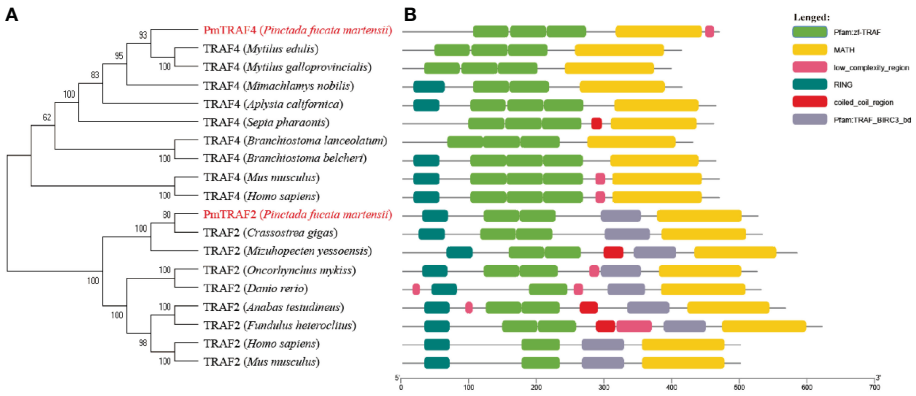

FIGURE 3
Phylogenic analysis **(A)** and protein domain information **(B)** of PmTRAF2 and PmTRAF4. The GenBank accession numbers of TRAF4 ortholog sequences are as follows: *Mytilus edulis* (Accession no.: CAG2230844.1), *Mytilus galloprovincialis* (Accession no.: VDI05192.1), *Mimachlamys nobilis* (Accession no.: QOS44542.1), *Aplysia californica* (Accession no.: XP_005107744.2), *Sepia pharaonis* (Accession no.: CAE1235462.1), *Branchiostoma lanceolatum* (Accession no.: CAH1232131.1), *Branchiostoma belcheri* (Accession no.: ABN04153.1), *Homo sapiens* (Accession no.: NP_004286.2), and *Mus musculus* (Accession no.: NP_033449.2). The GenBank accession numbers of TRAF2 ortholog sequences are as follows: *Crassostrea gigas* (Accession no.: XP_034302657.1), *Mizuhopecten yessoensis* (Accession no.: OWF39470.1), *Oncorhynchus mykiss* (Accession no.: NP_001117865.1), *Danio rerio* (Accession no.: XP_005165557.1), *Anabas testudineus* (Accession no.: XP_026198148.1), *Fundulus heteroclitus* (Accession no.: JAR83986.1), *Homo sapiens* (Accession no.: NP_066961.2), and *Mus musculus* (Accession no.: AAH03801.1).

responses, including cell survival, proliferation, and death (Jiao et al., 2014). In the present study, we cloned two novel TRAFs, *PmTRAF2*, *and PmTRAF4*, in *P. f. martensii*. The protein domain of PmTRAF2 and orthologs of other species contain the typical RING finger domain, TRAF-type zinc finger domain, TRAF_BIRC3_bd domain, and MATH domain (TRAF-C) (Figure 3B). The MATH domain is crucial for homo- and hetero-oligomerization of TRAFs and the interaction of upstream and downstream factors, while the RING finger domain is involved in the activation of downstream signaling pathways (Zapata et al., 2007; Ha et al., 2009), which helps to investigate the immune-related signaling pathways involved in PmTRAF2. The PmTRAF4 TRAF-type zinc finger domains and MATH domain and their numbers are similar to those of other mollusk and vertebrate TRAF4. However, PmTRAF4 lacks the RING finger domain, and its deletion may affect the transmission of downstream signaling proteins. The results of the phylogenetic analysis revealed that TRAF2 and TRAF4 were highly conserved across all species, and PmTRAF2 and PmTRAF4 were closer to bivalves' homologs, which suggests that PmTRAF2 and PmTRAF4 may have similar functions to other homologs.

TRAFs genes were detected and expressed at high levels in various immune-related tissues (gill, hemocytes, and hepatopancreas) in mollusks, suggesting their involvement in the host immune response. For instance, the mRNA of

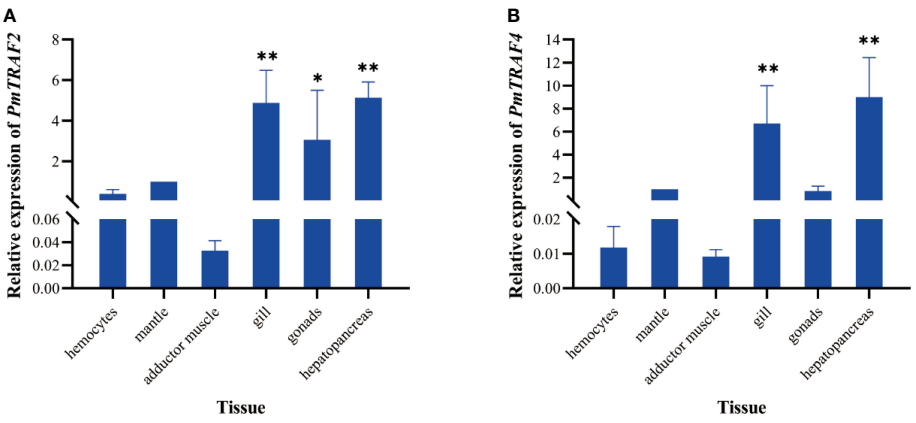

FIGURE 4
Expression distribution of *PmTRAF2* **(A)** and *PmTRAF4* **(B)** in different tissues. Error bars are expressed as the mean ± S.D. (n = 5). Asterisks denote significant differences: $^{**}p < 0.01$; $^{*}p < 0.05$.

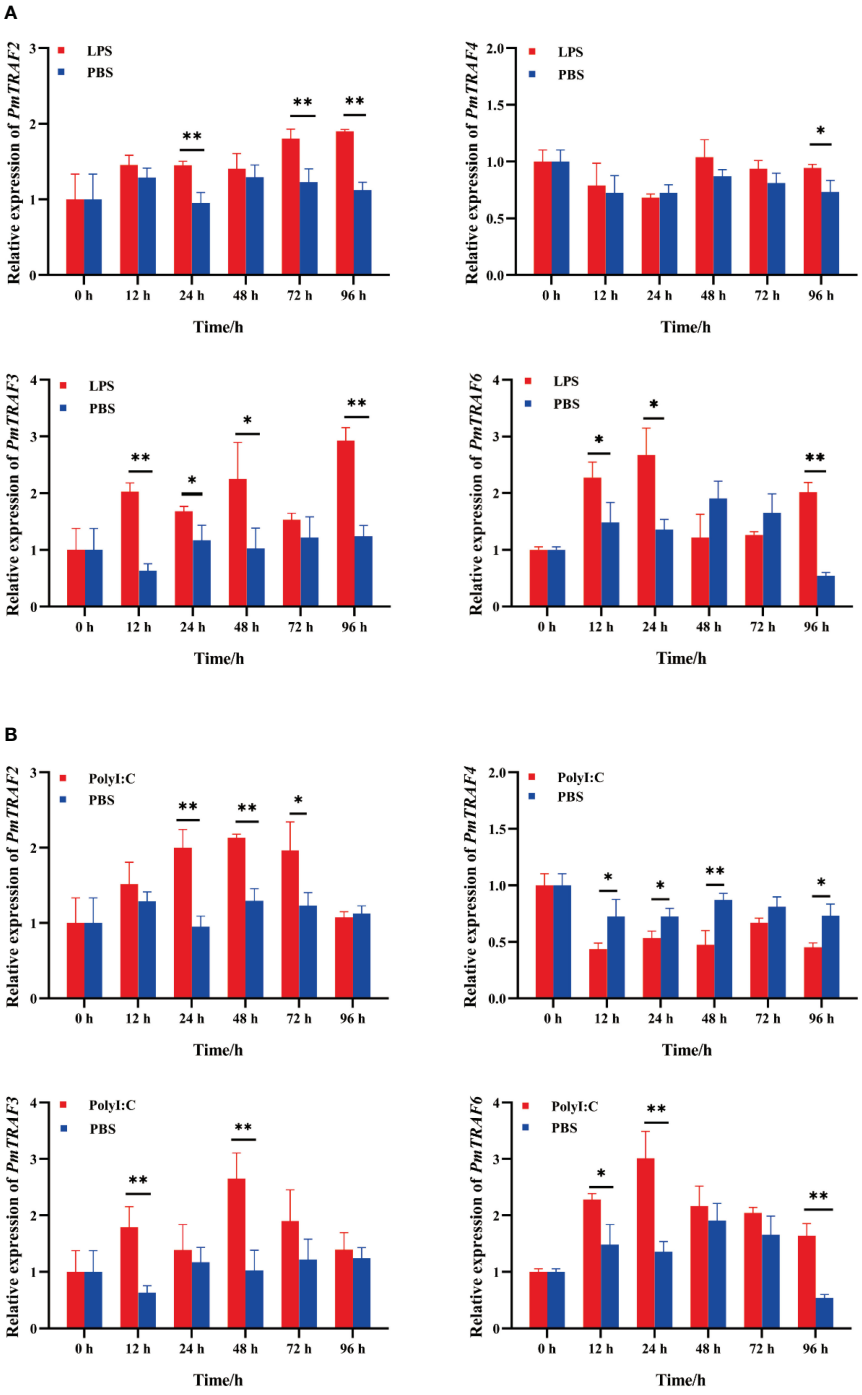

FIGURE 5
The mRNA expression of *PmTRAFs* in hepatopancreas after LPS challenge **(A)** and PolyI:C challenge **(B)**. Relative expression was expressed as the mean ± S.D. (n = 5). Significant differences in relative expression levels between two groups at the same time point are denoted by asterisks: $^{**}p < 0.01$; $^{*}p < 0.05$.

Patinopecten yessoensis's intact *TRAFs* (*TRAF2, TRAF3, TRAF4, TRAF6*, and *TRAF7*) were mainly abundant in gills and hemocytes (Wang et al., 2015). In *C. nobilis*, high expression levels of *CnTRAF2*, *CnTRAF3*, and *CnTRAF4* were observed in hemocytes, whereas *CnTRAF6* was highly expressed in gills (Zhang et al., 2021). *C. gigas TRAF3-S* and *TRAF3-L* transcripts were up-regulated in hemolymph and muscle relative to the gill of the internal reference sample (Huang

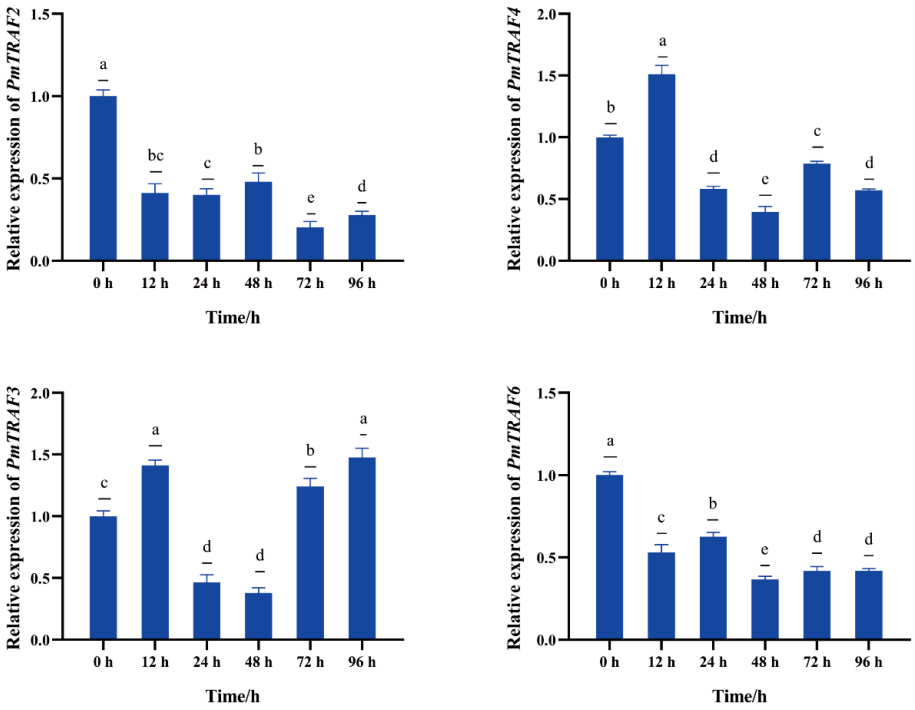

FIGURE 6
The mRNA expression of *PmTRAFs* in nuclear insertion surgery. Relative expression was expressed as the mean ± S.D. (n = 5). Different superscript letters indicate a significant difference in the groups ($p < 0.05$).

et al., 2014). Consistent with the above results, the mRNA transcripts of *PmTRAF2* and *PmTRAF4* were highly expressed in hepatopancreas and gills, indicating that they operated in immune defense. TRAF2 is one of the major recruitment targets for TRADD and TNFR5 proteins, and all three genes from *P. f. martensii* share high expression in gills and hepatopancreas (Hsu et al., 1996; He et al., 2019a; Wu et al., 2020). It can be speculated that PmTRADD and PmTNFR5 can recruit PmTRAF2 and play a role in apoptosis and other immune responses.

To investigate the function of TRAF family (*PmTRAF2*, *PmTRAF3*, *PmTRAF4* and *PmTRAF6*) in *P.f. martensii*'s immune response, their expression levels were examined in the hepatopancreas after three immune stimulation, including nuclear insertion surgery, LPS challenge, and PolyI:C challenge. Several reports have indicated that the TNF–TNFR system (TNFR, TRAF, and NF-κB family members) responded to the nuclear insertion surgery. These genes may be involved in allograft immunity in pearl oysters (Wu et al., 2020). Here, in hepatopancreas tissues, *PmTRAF3* and *PmTRAF4* transcripts with significant up-regulation at different times after nuclear insertion surgery, and *PmTRAF2* and *PmTRAF6* with down-regulation at all times, indicating that four TRAFs might participate in the immune defense at different time. The expression pattern of *PmTRAF2* was similar to *PmTRAF6*, which suggests that they may have the same regulation in response to nuclear insertion surgery. *PmTRAF3* and *PmTRAF4* transcripts were rapidly increased at 6 h, which illustrates that *PmTRAF3* and *PmTRAF4* may quickly involve in immune response after nuclear insertion surgery. In addition, four *TRAFs* mRNA in hepatopancreas were decreased at a short time (24 h and 48 h). However, in a longer period of time (5 d to 30 d), *PmTRAF2* transcripts in hemocytes were significantly upregulated on 5 d, 15 d, and 30 d after nuclear insertion surgery, while *PmTRAF3* was significantly upregulated on 15 d (Wu et al., 2020). The difference between the two results indicates that the downregulation of *PmTRAFs* mRNA in the hepatopancreas may be caused by time or that different tissues have different immune responses, which requires more experiments to verify.

Pathogen-associated molecular patterns (PAMPs) are highly conserved molecular structures that are common in pathogenic microorganisms and serve as ligand receptors for pattern recognition receptor (PRR) binding, and LPS and PolyI:C were typical PRR moleculars (Shen et al., 2022). *PmTRAF4* transcript was downregulated within 96 h exposure to PolyI:C. Contrary to this result, the transcript level of *Larimichthys crocea TRAF4* in gill was markedly upregulated at 6 h post PolyI:C (Chen et al., 2022). In *Patinopecten yessoensis*, *TRAF4* was upregulated in the hemocytes post-bacterial infection (*Micrococcus luteus* and

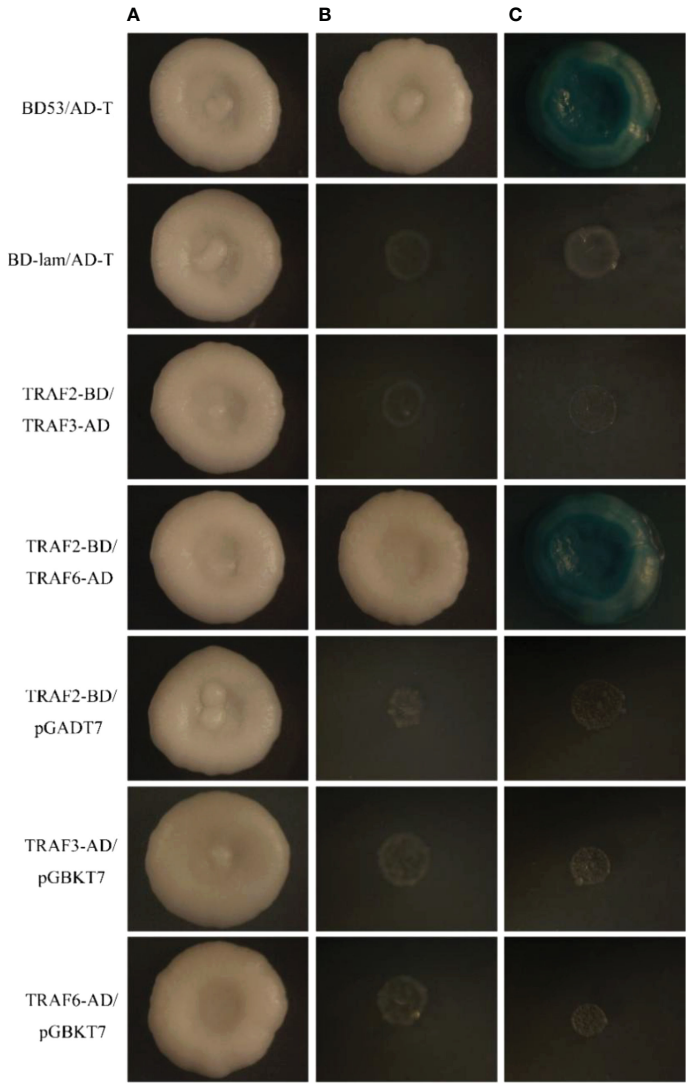

FIGURE 7
The yeast two-hybrid assay of PmTRAF2, PmTRAF3, and PmTRAF6. Co-transformed yeasts were cultured on SD/-Leu/-Trp medium **(A)** and SD/-Leu/-Trp/-His/-Ade medium **(B)**. Protein interactions were verified with SD/-Leu/-Trp/-His/-Ade medium containing x-a-gal **(C)**. AD represents pGADT7, and BD denotes pGBKT7. PGBKT7-53/PGADT7-T is the positive control group. pGBKT7-lam/pGADT7-T is used as the negative control group; pGBKT7-PmTRAF2/pGADT7- PmTRAF3 and pGBKT7-PmTRAF2/pGADT7-PmTRAF6 are expressed as experimental groups.

Vibrio anguillarum) (Wang et al., 2015). The above different phenomena suggest that *PmTRAF4* may have different expression patterns in innate defenses in response to pathogens. Furthermore, TRAF4 can inhibit innate immune signaling pathways, such as TRAF4 inhibits nucleotide-binding oligomerization domain 2-induced NF-κB activation (Marinis et al., 2011); TRAF4 inhibits the toll-like receptors signaling pathway by associating with p47phox and interacting with TRAF6 and toll-IL-1 receptor domain-containing adaptor-inducing IFN-β (Takeshita et al., 2005). Whether TRAF4 can inhibit the immune response to pathogens is worth exploring. *PmTRAF2*, *PmTRAF3*, and *PmTRAF6* were significantly induced to express at multiple time points within 96 h under LPS and PolyI:C, which suggests that their involvement in the innate immune system may be highly sensitive to the invasion of pathogenic microorganisms. Post LPS and PolyI:C infection, the expression levels of *PmTRAF3* and *PmTRAF6* increased sharply at 12 h, whereas *PmTRAF2* at 24 h, which may indicate that *PmTRAF3* and *PmTRAF6* were more sensitive to LPS and PolyI:C model pathogen invasion than *PmTRAF2*. However, *PmTRAF2* was responsive to LPS challenge in the gill tissue of *P. f. martensii*, with expression significantly up-regulated at 6 h and 12 h relative to the control group, which may be caused by the ability of gills to recognize and defend against LPS model pathogens rapidly (Wu et al., 2020; Zhang et al., 2022). Our results speculate that *PmTRAFs* (*PmTRAF2*, *PmTRAF3*,

PmTRAF4 and *PmTRAF6*) may be the important molecule in the host's immune defense.

Based on our studies, *PmTRAF2*, *PmTRAF3*, and *PmTRAF6* may exert a similar action at the transcript level. Therefore, these may have a coordinative effect on signaling pathways. TRAF2, TRAF3, or TRAF6 have been found to mediate CD40-induced activation of NF-κB (Lu et al., 2003). Thus studying the relationship between them is important. Furthermore, in a previous report, TRAFs members can participate in signal transduction by interacting to form homo- and hetero-dimers with distinct regulatory functions (Arron et al., 2002). The TRAF-C structural domain of TRAF3 interacts with the TRAF-N structural domain and zinc fingers 4 and 5 of TRAF2 to form multimers, which inhibits the capability of TRAF2 to induce the NF-κB activation (He et al., 2004). Using RNA interference and immunoblotting, the researchers found for the first time in nonhemopoietic cells that TRAF6 can regulate CD40 signaling by associating with TRAF2 (Davies et al., 2005). Therefore, yeast two-hybrid assays were performed to understand the relationship between TRAF2 and TRAF3, and TRAF6 in the present study, which is crucial for subsequent exploration of the downstream signaling. The yeast two-hybrid assays have revealed that PmTRAF2 interacts with PmTRAF6 while not interacting with PmTRAF3. The TRAF domain is crucial for homo- and hetero-oligomerization of TRAFs. However, TRAF6 forms a heterodimer by binding to the RING domain of TRAF2 *via* its RING domain (Das et al., 2021). How PmTRAF2 and PmTRAF6 form a heterodimer and their molecular mechanism for downstream signaling still need to be explored.

5 Conclusion

The novel *PmTRAF2* and *PmTRAF4* were identified from *P. f. martensii* and had highly conserved regions of amino acid sequences. *PmTRAF2* and *PmTRAF4* with abundant expression levels in hepatopancreas and gill. Transcripts of *PmTRAF2*, *PmTRAF3*, *PmTRAF6*, and *PmTRAF4* were markedly changed post- LPS injection, PolyI:C injection, and nuclear insertion surgery, indicating that these genes may play a role in *P. f. martensii* innate immunity. PmTRAF2 interacts with PmTRAF6 instead of PmTRAF3, potentially affecting downstream immune signaling pathways, such as NF-κB signaling pathways. The above results provide new perspectives for further investigation of TRAF's immune mechanisms in bivalves.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/supplementary material.

Author contributions

MZ: Investigation and designed the study, Analyzed all data, Writing - Original draft preparation; CS: Analyzed all sequencing data; HL: Conceptualization, Methodology, Writing- Reviewing and Editing; YW: Investigation, Analyzed all sequencing data; BL: Prepared the samples, Conclusion. 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.

Publisher's note

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