



Host MicroRNA-217 Promotes White Spot Syndrome Virus Infection by Targeting *Tube* in the Chinese Mitten Crab (*Eriocheir sinensis*)

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MicroRNAs (miRNAs), a group of small molecule non-encoding RNAs, are key post-transcriptional regulators of gene expression that are implicated in many biological processes. In the current study, miR-217 from *Eriocheir sinensis* was selected for studying its roles during host–virus interaction. Overexpression or silencing of miR-217 led to considerable effects on white spot syndrome virus (WSSV) replication, implying that miR-217 played a positive role in WSSV infection. In insect High Five cells, miR-217 significantly inhibited *Tube* gene expression by binding to the 3′-untranslated region of the *Tube*. Overexpression of miR-217 in crab led to downregulation of *tube* expression. Knockdown of *Tube in vivo* led to significant enhancement of WSSV infection and inhibited the expression of five antimicrobial peptide (AMP) genes (*Anti-lipopolysaccharide factor ALF1, ALF2, ALF3; Crustin Crus1, Crus2*) in WSSV-challenged crabs. Overexpression of miR-217 also led to downregulation of these AMP genes in WSSV-challenged crabs. Our results showed that host miRNA played positive roles in virus infection by regulation of host *tube* gene, which is the key component of Toll signaling pathway.

Keywords: *Eriocheir sinensis*, WSSV, miR-217, toll signaling pathway, *Tube*, antimicrobial peptides

INTRODUCTION

MicroRNAs (miRNAs) are a class of small non-coding RNAs that are found in diverse eukaryotic organisms (Carrington and Ambros, 2003). MicroRNAs are endogenous, 18 to 26 nucleotides (nt) long, and are processed from the hairpin transcripts cleaved by two RNase III enzymes, namely, Drosha and Dicer (Bernstein et al., 2001; Bartel, 2004). The mature miRNA strand is incorporated into the RNA-induced silencing complex, wherein mRNA degradation or protein translation repression is controlled mainly through acting on the 3′-untranslated region (UTR) of target mRNAs (Schwarz et al., 2003; Meister and Tuschl, 2004; Bagga et al., 2005). Since *lin-4* and *let-7*, the first miRNAs, were discovered in *Caenorhabditis elegans* (Lee et al., 1993; Reinhart et al., 2000), numerous miRNAs have been found to be involved in the regulation of diverse biological processes, including development, differentiation, apoptosis, oncogenesis, and immune activation (Ambros, 2004; Lecellier et al., 2005; Pedersen et al., 2007). Host miRNAs are believed to be key regulators of virus–host interactions (Triboulet et al., 2007; Umbach and Cullen, 2009). For example, human

miR-125a has been shown to suppress the replication of hepatitis B virus by binding the viral transcript encoding the surface antigen and interfering with its expression (Mosca et al., 2014). MiR-155 inhibits Japanese encephalitis virus replication and negatively modulates innate immune responses in JEV-infected human microglial cells (Pareek et al., 2014). Two host cellular miRNAs, namely, miR-320a and miR-140, were found to inhibit mink enteritis virus infection into feline kidney cells by repression of its receptor, feline transferrin receptor (Sun et al., 2014). Host miR-26a suppresses the replication of porcine reproductive and respiratory syndrome virus by upregulating the expression of type I interferon, as well as the IFN-stimulated genes MX1 and ISG15 (Li L. et al., 2015). However, a small amount of information is available regarding the pathways mediated by host miRNAs in invertebrates.

Invertebrates lack antibodies and complements; however, they can initiate a rapid and effective innate immune response against pathogenic organisms (Uematsu and Akira, 2008). Recognizing invading microbial pathogen, which is conducted by a set of so-called pattern recognition receptors (PRRs), is an essential step in innate immune responses (Bischoff et al., 2004). Toll-like receptors or Tolls, which initiate innate immune responses by sensing pathogen-associated molecular patterns (Kawai and Akira, 2010) and then activating evolutionarily conserved Toll signal transduction (Imler and Hoffmann, 2001), are the most extensively studied PRRs. Toll signaling occurs through several adaptor proteins, such as myeloid differentiation factor 88 (MyD88), Tube, and Pelle (Valanne et al., 2011). Upon ligand binding, MyD88 recruits the activated Toll receptor and the cytosolic adaptor Tube to transfer Toll signaling. Then, Pelle is recruited to the vicinity of Tube, forming a trimeric complex (MyD88-Tube-Pelle) mediated by the death domain of Tube. Finally, the signal is transmitted to the Dorsal/Cactus complex that regulates the Toll-dependent gene expression, such as *antimicrobial peptides* (AMPs), as well as a substantial number of other innate immune responsive genes (Belvin and Anderson, 1996; Lemaitre and Hoffmann, 2007; Valanne et al., 2011). The adaptor Tube is an essential component of the Toll signaling pathway. The *Drosophila melanogaster* Tube not only participates in dorsoventral axis formation during development but is also involved in regulating diverse downstream signaling as well as the response against gram-positive bacteria and fungi (Lemaitre et al., 1996; Wang et al., 2006). The mud crab *Scylla paramamosain*, SpTube, together with SpPelle and SpMyD88, may form a trimeric complex in the Toll signaling cascade and are shown to be involved in the immunity against gram-positive and gram-negative bacteria (Li et al., 2013). *EsTube* might play important roles in the innate immune response to exogenous pathogenic stimulation and the development of the gonads in the Chinese mitten crab, *Eriocheir sinensis* (Yu et al., 2014). Therefore, Tube may have a vital role in initiating and activating the immune system. However, the host-virus interaction between *Tube* and miRNAs is not addressed at present.

White spot syndrome virus (WSSV) is one of the most virulent agents threatening the shrimp culture industry worldwide (Escobedo-Bonilla et al., 2008). Recently, one report showed that WSSV also caused serious disease, thereby threatening

the Chinese mitten crab aquaculture industry (Ding et al., 2015). Given the breadth of miRNA-mediated regulation of immunity, the role of host miRNAs in WSSV infection is of significant interest. Herein, the crab miR-217 targeting *Tube* was characterized in this study. Our findings indicated that miR-217 first directly targeted *Tube*. Then, it regulated the expression of AMP genes. Finally, miR-217 promoted the WSSV infection in *E. sinensis*.

MATERIALS AND METHODS

Crab Culture and WSSV Challenge

Approximately 40 g of *E. sinensis* was raised in a group of 30 individuals at room temperature. Three crabs from each group were randomly selected for the PCR detection of WSSV with WSSV-specific primers (5'-TATTGTCTCTCCTGACGTAC-3' and 5'-CACATTCTTCACGAGTCTAC-3') to ensure that the crabs were virus-free before experiments. Then, the virus-free crab was infected with 100 μ l WSSV solution for each crab at 10^5 copies/ml by intramuscular injection. At different times post-infection (0, 2, 4, 6, 12, 24, 36, 48, 60, and 72 h), the hemolymph was collected from treated crabs and subsequently centrifuged at 2000 rpm at 4°C for 10 min to harvest the hemocytes. Other tissues (heart, hepatopancreas, gills, muscles, intestines, nerves, and eyestalk) from the five untreated crabs were also collected for later use.

Detection of Crab miR-217 Using Real-Time PCR

Total RNAs were extracted from the preceding issues mentioned using the mirVana miRNA isolation kit following the instructions of the manufacturer (Ambion, USA). cDNA for miRNA qRT-PCR was then synthesized with All-in-one™ miRNA First-Strand cDNA Synthesis Kit (GeneCopoeia™, USA) according to the protocol of the manufacturer. Quantitative real-time PCR was carried out according to the protocol of All-in-one™ miRNA qRT-PCR Detection Kit (GeneCopoeia™, USA) using the miR-217 special primer (5'-CGCTACTGCATCAGGAAC TGA-3'). Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as a control. The first-strand cDNA synthesis was obtained by using the PrimeScript™ RT reagent Kit (Perfect Real Time) (Takara, Dalian, China), and qRT-PCR was carried out using 2 \times SYBR Premix Ex Taq kit (Takara, Japan) with *GAPDH*-specific primers (5'-CTGCCCAAACATCATCCCATC-3' and 5'-CTCTCATCCCCAGTGAAATCGC-3').

Silencing Or Overexpression of miR-217 in Crab

The anti-miRNA-217 oligonucleotide (AMO-miR-217: 5'-ATC CAATCAGTTCCTGATGCAGTA-3', 10 nM) and WSSV (10^5 copies/ml) were coinjected into crab to silence the expression of miR-217. As a control, the sequence of AMO-miR-217 was scrambled, yielding AMO-miR-217-scrambled (5'-CAC TAAGTGGATGCTAAACTTCTC-3'). The AMO-miR-217-scrambled (10 nM) and WSSV (10^5 copies/ml) were included in the injections. The oligonucleotides were sequentially injected into the same crab twice at a 16 h interval to avoid the

degradation of AMO-miR-217 or AMO-miR-217-scrambled in crab. WSSV alone (10^5 copies/ml) and phosphate buffer saline (PBS) were also injected into crab. At 24, 36, and 48 h after the last injection, the hemolymph specimens selected at random from three crabs were mixed and subjected to subsequent analysis.

The mimic of miR-217 (5'-UACUGCAUCAGGAACUGA UUGGAU-3') was synthesized with an *in vitro* transcription T7 kit for siRNA synthesis (Takara, Japan). The sequence of miR-217 was scrambled to generate the control miR-217-scrambled (5'-CUCAAGGAUGCGUUAAGGUUACUA-3'). miRNA (20 μ g) and WSSV (10^5 copies/ml) were coinjected into healthy crabs to overexpress miR-217. At 16 h after the coinjection, the miRNA (20 μ g) (100 μ l /crab) was injected into the same crab. As controls, miR-217-scrambled, WSSV (10^5 copies/ml), and PBS were included in the injections. At different time points after the last injection, the hemolymph specimens from three crabs were selected. All the preceding assays described were biologically repeated for three times.

Quantitative Analysis of WSSV Copies by Real-Time PCR

The genomic DNA was extracted to quantify the WSSV copies in crab. qRT-PCR was performed with WSSV-specific primers (5'-TTGGTTTCATGCCCGAGATT-3' and 5'-CCTTGGTCAGCC CCTTGA-3') and a TaqMan probe (5'-FAM-TGCTGCCGTCTC CAA-TAMRA-3'). The plasmid containing a DNA fragment of 1,400 bp from the WSSV genome was used as the internal standard of real-time PCR (Liu et al., 2009). The 25 μ l PCR reaction solutions contained 12.5 μ l of the Premix ExTaq (Takara, Japan), 0.5 μ l of 10 μ M primers each, 1 μ l of 10 μ M TaqMan fluorogenic probe, 1 μ l of DNA template, and 9.5 μ l of distilled water. The pre-denaturalization stage of the PCR program was 95°C for 1 min, followed by the amplification stage comprising 40 cycles of 95°C for 30 s, 52°C for 30 s, and 72°C for 30 s.

Prediction and Identification of Target Genes of miR-217 in Crab

Owing to the insufficient crab genome sequence, the assembled crab EST sequences (unpublished) were used to predict the target genes of miR-217 in crab. Three computational algorithms, including TargetScan 5.1 (<http://www.targetscan.org>), miRanda (<http://www.microrna.org/>), and Pictar (<http://pictar.mdc-berlin.de/>), were conducted. The data predicted by the three algorithms were overlapped and calculated.

Plasmid Constructions

To explore the interaction between miR-217 and its target gene *Tube*, the enhanced green fluorescent protein (*EGFP*) gene was amplified from the pEGFP vector (BD Biosciences, USA) using *EGFP* forward and reverse primers (5'-AAGAGCTCGGAT CCCC GG TAC-3' and 5'-AATCTAGAGTCGCGGCCGCTT TA-3') and cloned into the pIZ/V5-His vector (Invitrogen, USA) to produce a control EGFP construct. Subsequently, the *Tube* 3'UTR was cloned downstream of the *EGFP* gene using the XbaI and SacII restriction sites. The primers used for the amplification of *Tube* 3'UTR were 5'-GCTCTAGATGATGGCTGCATTCAC

TTGG-3' and 5'-TCCCCGCGGTAATTACATTGATGATAT CA-3'. The sequence of *Tube* 3'UTR complementary to the miR-217 seed sequence (ATGCAGT) was randomly mutated to CGTACTG, generating the EGFP- Δ Tube construct. The amplification of Δ Tube 3'UTR was conducted by PCR with sequence-specific primers 5'-GGATAGAAAATGTGTCCACGT ACTGACCTCATGG-3' and 5'-ATCTTCTTACCATGAGGT CAGTACGTGGACACATT-3'. Recombinant plasmids were confirmed by sequencing.

Cell Culture, Transfection, and Fluorescence Assays

Insect High Five cells (Invitrogen) were cultured at 28°C in Express Five serum-free medium (SFM, Invitrogen) containing L-glutamine (Invitrogen). The cells were cotransfected with the EGFP, EGFP-*Tube*, or EGFP- Δ Tube plasmid and the synthesized miR-217 (miR-217-mimic) using the Cellfectin transfection reagent (Invitrogen) according to the protocol of the manufacturer. The concentrations of miR-217-mimic and the plasmid were 200 nM/well and 400 ng/well, respectively. As a control, the control miRNA was included in the cotransfection. All the miRNAs were synthesized by Shanghai GenePharma Co., Ltd. (Shanghai, China). At 48 h after cotransfection, the EGFP fluorescence of the cells was measured by a Flex Station II microplate reader (Molecular Devices, USA) at 490/510 nm of excitation/emission (Ex/Em).

RNAi Assays in Crab *In vivo*

Based on the sequences of crab *Tube* gene, siRNA (*Tube*-siRNA: 5'-CCCAAACGGCAACCCUCAU-3') was synthesized according to the design rule for siRNA. The sequence of siRNA was randomly scrambled to generate the control siRNA, *Tube*-siRNA-scrambled (5'-UCACCUACGCACCACACAG-3'). To silence *Tube* expression, *Tube*-siRNA (20 μ g) and WSSV (10^5 copies/ml) were coinjected into each crab. Sixteen hours later, the siRNA was injected into the same crab at 20 μ g/crab. WSSV (10^5 virus copies/ml) alone was used as a positive control. At different times (24, 36, and 48 h) after the injection of siRNA, the crab hemocytes was collected and stored at -80°C for later use.

Quantification of mRNA with Real-Time PCR

The mRNA levels of *Tube* and *AMP* genes in crab were determined using qRT-PCR. The *Tube* primers were 5'-ATTGTG CTGCTGGAGTTGCTGAC-3' and 5'-CATCGTCCGTCGCTT CTTCTTGG-3'. *Anti-lipopolysaccharide factor 1 (ALF1)*, *ALF2*, *ALF3*, *Crustin 1 (Cru1)*, and *Crustin 2 (Cru2)* were detected with sequence-specific primers (ALF1-RT-F: 5'-GACGCAGGAGGA TGCTAAC-3', and ALF1-RT-R: 5'-TGATGGCAGATGAAG GACAC; ALF2-RT-F: 5'-GACCCTTTGCTGAATGCTTGA-3', and ALF2-RT-R: 5'-CTGCTCTACAATGTCGCCTGA-3'; ALF3-RT-F: 5'-ACGAGGAGCAAGGAAAGAAAG-3', and ALF3-RT-R: 5'-TTGTGCCATAGACCAGAGACTT-3'; Crus1-RT-F: 5'-GCTCTATGGCGGAGGATGTCA-3', and Crus1-RT-R: 5'-CGG GCTTCAGACCCACTTTAC-3'; Crus2-RT-F: 5'-GCCACCTC CCAAACCTAT-3', and Crus2-RT-R: 5'-GCAAGCGTCACA GCAGCACT-3'). Crab *GAPDH* was used as the internal

reference. The $2^{-\Delta\Delta CT}$ method was used to calculate the relative fold-change of the mRNA expression level (Livak and Schmittgen, 2001).

Statistical Analysis

Numerical data were analyzed using one-way analysis of variance to calculate the mean and standard deviation of triplicate assays. Unpaired sample *t*-test was conducted, and the differences were considered significant if $P < 0.05$ or $P < 0.01$.

RESULTS

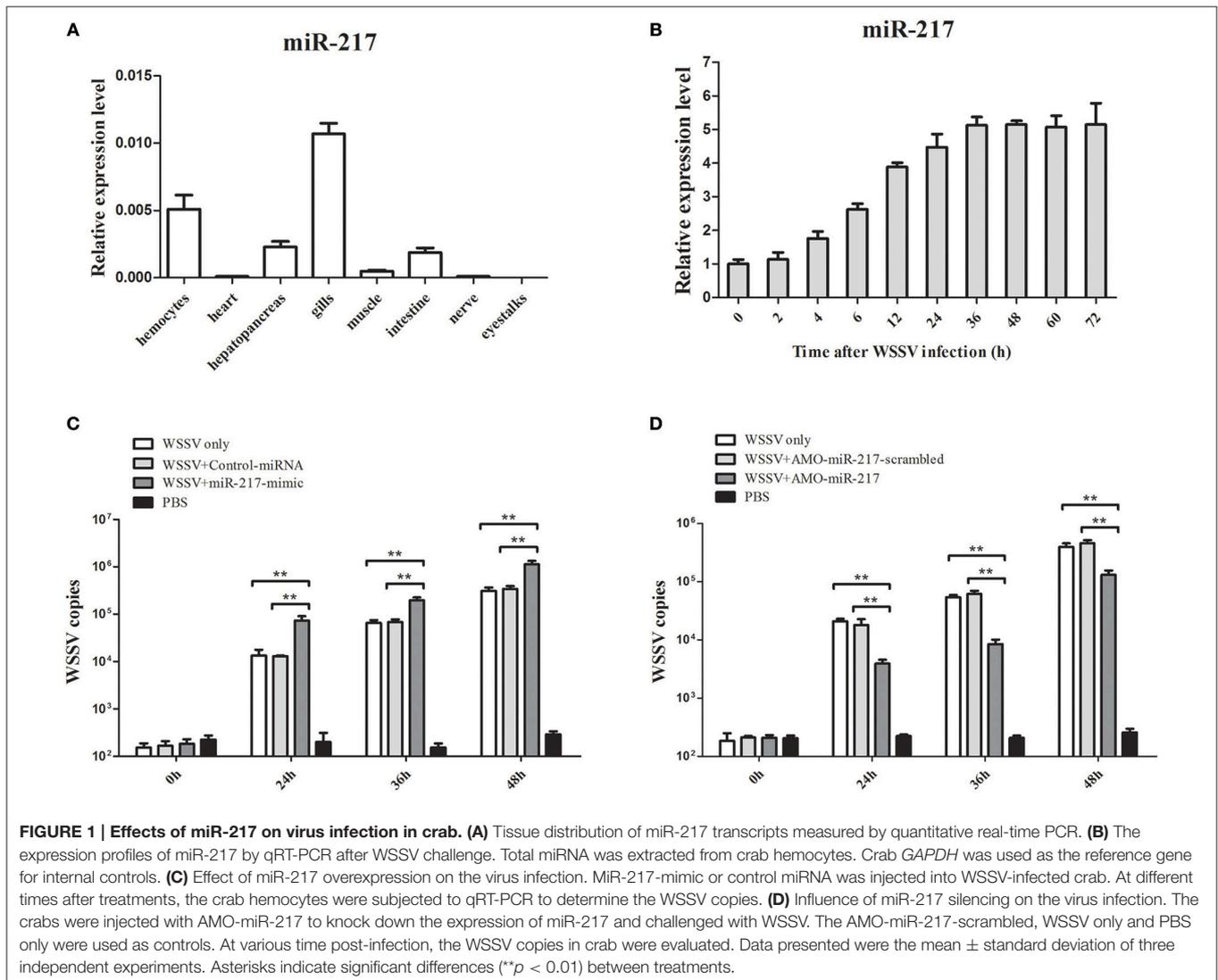
Effects of miR-217 on Virus Infection in Crab

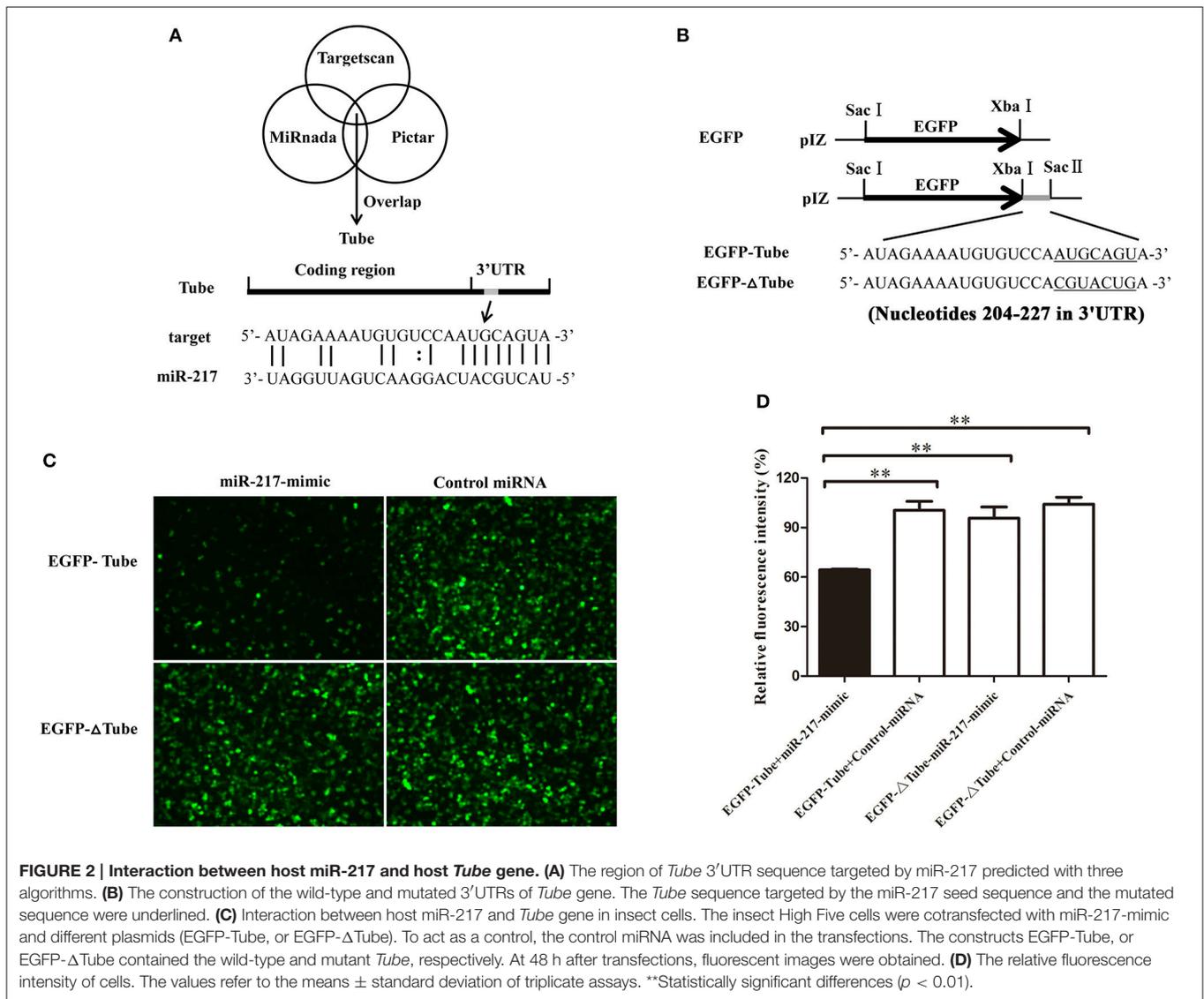
MiR-217 expression in several tissues was performed by qRT-PCR (Figure 1A). *GAPDH* was used as a reference. MiR-217 was mainly expressed in gills and hemocytes, with a relatively low expression level in the hepatopancreas, intestine, and muscle. The expression changes of miR-217 in WSSV-challenged crab

were detected to explore the relationship between miR-217 and WSSV infection. From the result of qRT-PCR, the WSSV infection led to a significant increase of miR-217 expression level in the hemocytes of crab (Figure 1B). MiR-217-silenced or overexpressed crabs were analyzed with the assays of the WSSV copies to further evaluate the role of miR-217 in the virus infection. The data presented that the WSSV copies were significantly increased by the miR-217 overexpression and significantly decreased by the miR-217 silencing compared with the control (Figures 1C,D), indicating that miR-217 played a positive role in regulation of the WSSV infection.

Interaction between Host miR-217 and Host *Tube* Gene *In vitro*

Growing evidence has indicated that host miRNAs play important roles in host–virus interactions in invertebrates. In this study, the prediction results indicated that the crab miR-217 could target the host *Tube* (GenBank Accession No. KC011815), an important adaptor protein of the Toll pathway (Figure 2A).





MiR-217-mimic and the plasmid EGFP-*Tube* were cotransfected into High Five cells because a crab cell line was unavailable (Figure 2B) to evaluate the direct interaction between miR-217 and *Tube*. The results revealed that the fluorescence intensity in the cotransfected cells was significantly weaker than that in the controls, showing that miR-217 targeted the *Tube* gene (Figures 2C,D). EGFP-Δ*Tube* was constructed to investigate the specificity of the interaction between miR-217 and *Tube* 3'UTR. The result showed that miR-217-mimic had no effect on the expression of EGFP in miR-217-mimic and EGFP-Δ*Tube* cotransfected cells, showing that miR-217 seed sequence was required to target the *Tube* 3'UTR.

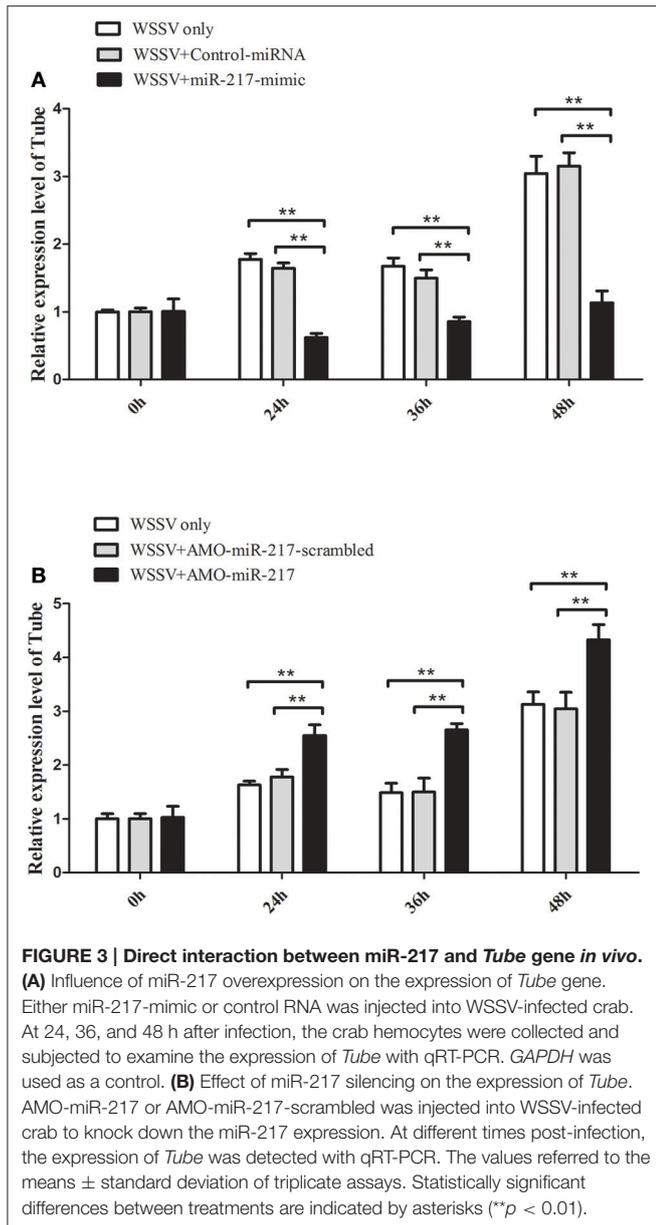
Interaction between miR-217 and *Tube* Gene *In vivo*

MiR-217-mimic was injected into WSSV-infected crab to reveal the *in vivo* interaction between miR-217 and *Tube* gene in crab.

The results indicated that overexpression of miR-217 led to a significant decrease in the transcript levels of the *Tube* gene at 24, 36, and 48 h post-infection (Figure 3A). AMO-miR-217 was injected into WSSV-infected crab to inhibit the expression of endogenous miR-217. The results showed that crab *Tube* expression was enhanced by AMO-miR-217 (Figure 3B). These results showed that changes in miR-217 levels could alter the expression of the host gene *Tube* in crab *in vivo*.

Roles of *Tube* in Crab-Virus Interaction

QRT-PCR was used to analyze the *Tube* mRNA level after WSSV infection. At 24, 36, 48, 60, and 72 h in response to WSSV infection, the crab *Tube* was significantly upregulated (Figure 4A). The expression of the crab *Tube* gene was silenced by sequence-specific siRNA, followed by the examination of WSSV copies to further examine the roles of *Tube* in virus infection. The qRT-PCR result showed that the expression of *Tube* was repressed by the gene-specific siRNA from 24 h to



48 h after siRNA injection (Figure 4B). The mutation-siRNA had no effect on the expression of *Tube*. The data indicated that the *Tube*-siRNA was highly sequence specific, which could silence the *Tube* gene in crab *in vivo*. When the *Tube* gene had been silenced by *Tube*-siRNA, the numbers of WSSV genome copies/mg in crab hemocytes significantly increased compared with the control (WSSV only and *Tube*-siRNA-scrambled) (Figure 4C). The data indicated that *Tube* gene played important roles in WSSV replication.

AMP Genes Expression Are Regulated by *Tube*

AMP genes are important effectors of the Toll pathway in invertebrates. The expression of *Tube* was knocked down in crab to reveal the relationship between *Tube* and AMP genes

ALF1 (Accession No. DQ793214), *ALF2* (GU014699), *ALF3* (HQ850572.1), *Crus1* (GQ200832), and *Crus2* (GQ200833). Under the conditions wherein the *Tube* gene expression was silenced, five AMP gene expression levels were detected by qRT-PCR. The results are shown in Figure 5, wherein the mRNA transcriptions of AMPs were increased by different degrees of magnitude after the WSSV challenge. However, the expression levels of these AMPs were significantly decreased in the *Tube*-siRNA experimental group compared with the controls (*Tube*-siRNA-scrambled, WSSV only). Therefore, *ALF1*, *ALF2*, *ALF3*, *Crus1*, and *Crus2* are possibly the downstream genes of *Tube*.

AMP Genes Expression Are Regulated by miR-217

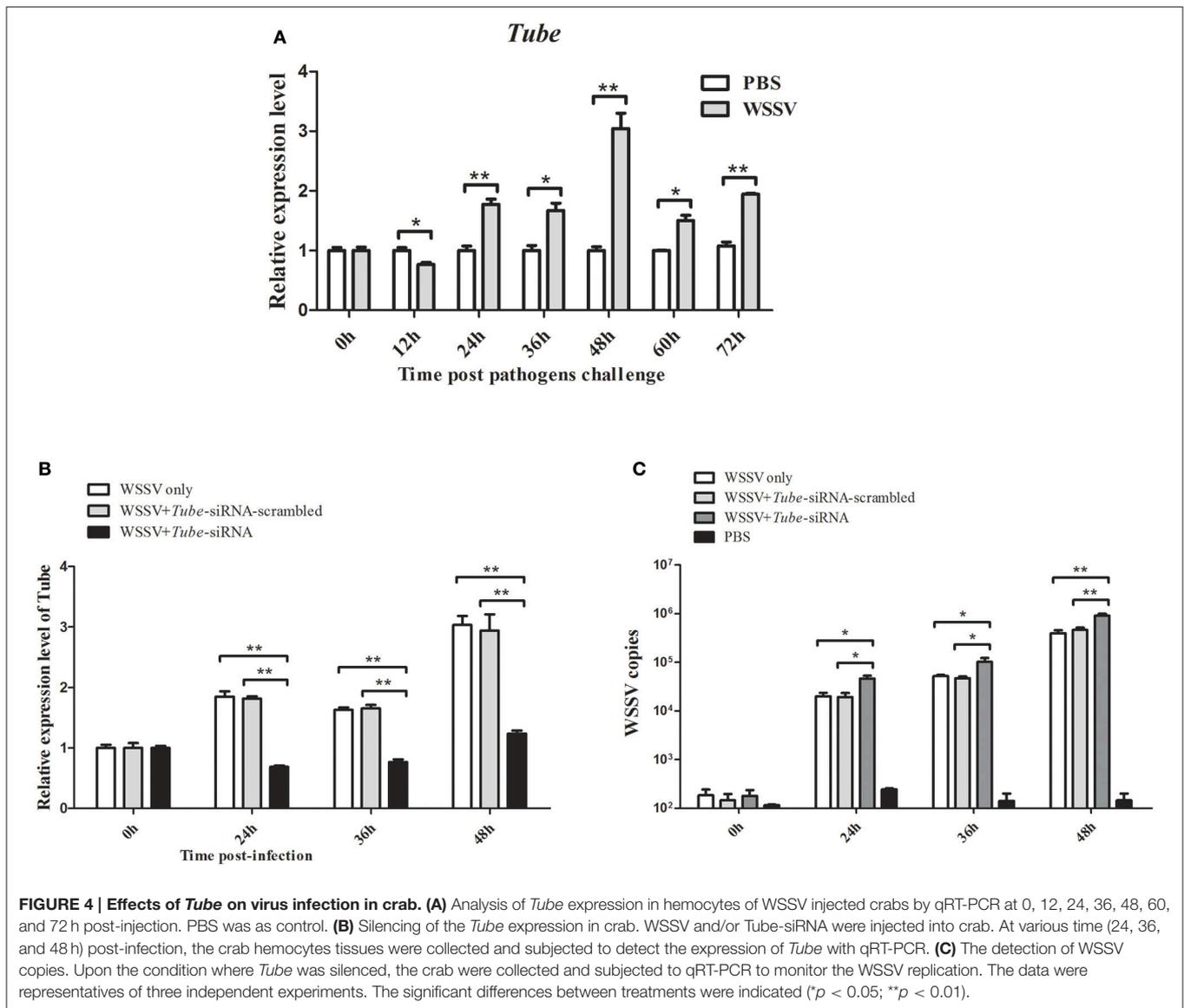
All the preceding results described implied that five AMP genes are the downstream effector genes regulated by *Tube*. Therefore, the pathway mediated by host miR-217 was further explored. The results indicated that the expression levels of *ALF1* (Figure 6A), *ALF2* (Figure 6B), *ALF3* (Figure 6C), *Crus1* (Figure 6D), and *Crus2* (Figure 6E) are significantly downregulated when miR-217 is overexpressed in crab. By contrast, the control miRNA injection had no effect on the transcription of these AMP genes. The knockdown of miR-217 by AMO-miR-217 led to a significant increase in the expression levels of *ALF1* (Figure 7A), *ALF2* (Figure 7B), *ALF3* (Figure 7C), *Crus1* (Figure 7D), and *Crus2* (Figure 7E) at 24 h to 48 h post-infection compared with those of WSSV only. By contrast, the control AMO-miR-217-scrambled had no effect on the transcriptions of AMPs, thereby suggesting that the host miR-217, *ALF1*, *ALF2*, *ALF3*, *Crus1*, and *Crus2* shared the same pathway.

Expression of *ALF1*, *ALF2*, *ALF3*, *Crus1*, and *Crus2* during Virus Infection in Crab

The genes of *ALF1*, *ALF2*, *ALF3*, *Crus1*, and *Crus2* were characterized and their expression patterns were performed in crabs to evaluate their roles in viral infection. As shown in Figures 8A–E, the mRNAs of *ALF1*, *ALF2*, *ALF3*, *Crus1*, and *Crus2* were upregulated post-WSSV infection at several time points. By contrast, the transcripts of the five AMP genes did not obviously change after PBS challenge. The data demonstrated that the five AMP genes might be involved in anti-viral innate immune defense. These findings together revealed that host miRNA (miR-217) inhibited the Toll signaling pathway by targeting the host *Tube* gene, resulting in the enhancement of virus replication in crabs (Figure 8F).

DISCUSSION

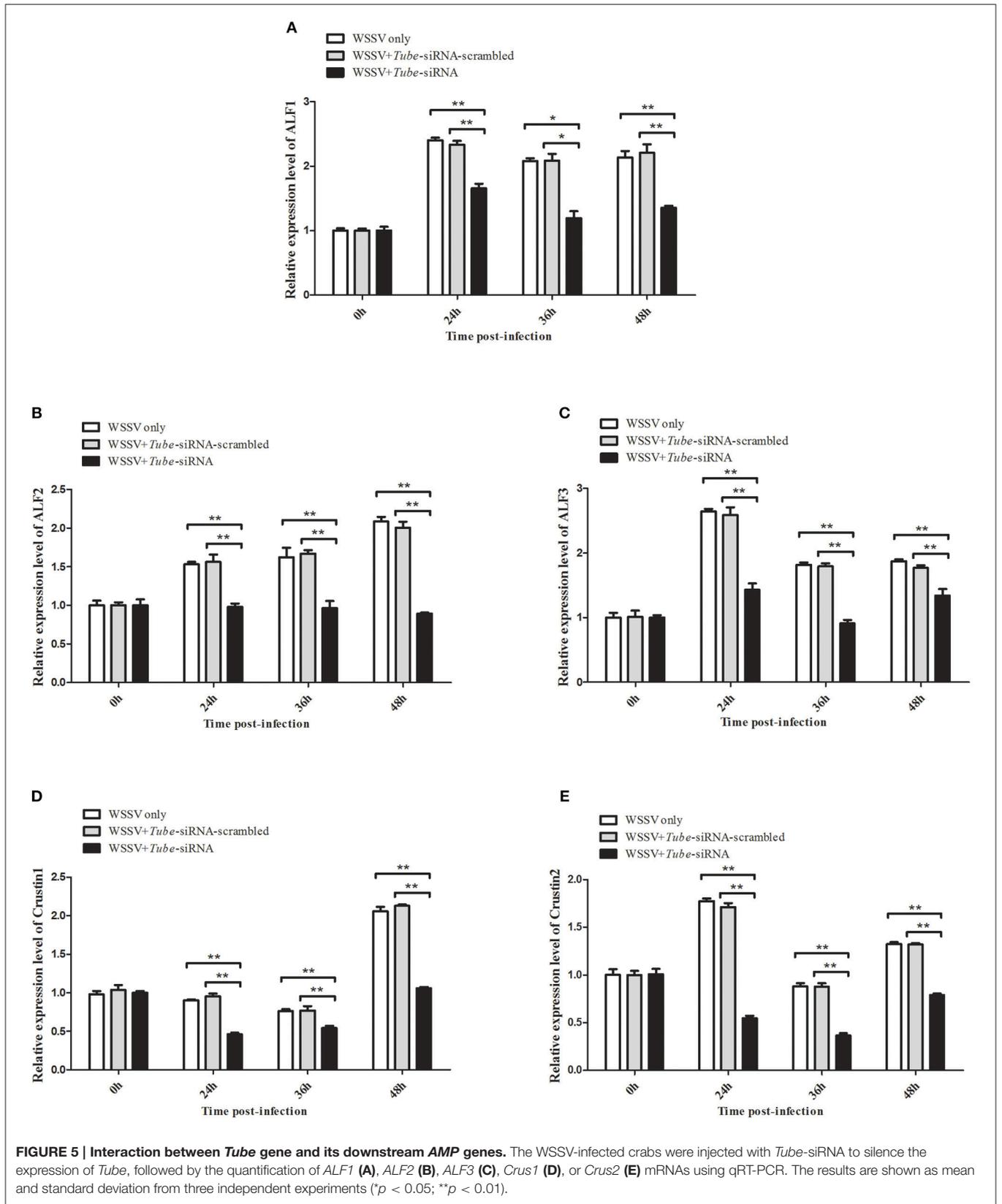
Although Toll signaling pathway has been implicated in the processes of virus infection, its function in host–virus interaction remains to be unfamiliar. MiRNAs are post-transcriptional regulatory molecules that could regulate gene expression and participate in numerous key biological processes by inhibiting mRNA translation or promoting mRNA decomposition (Meister and Tuschl, 2004; Bagga et al., 2005). Tumor-secreted miRNAs (miR-21 and miR-29a) can function as ligands, binding to the

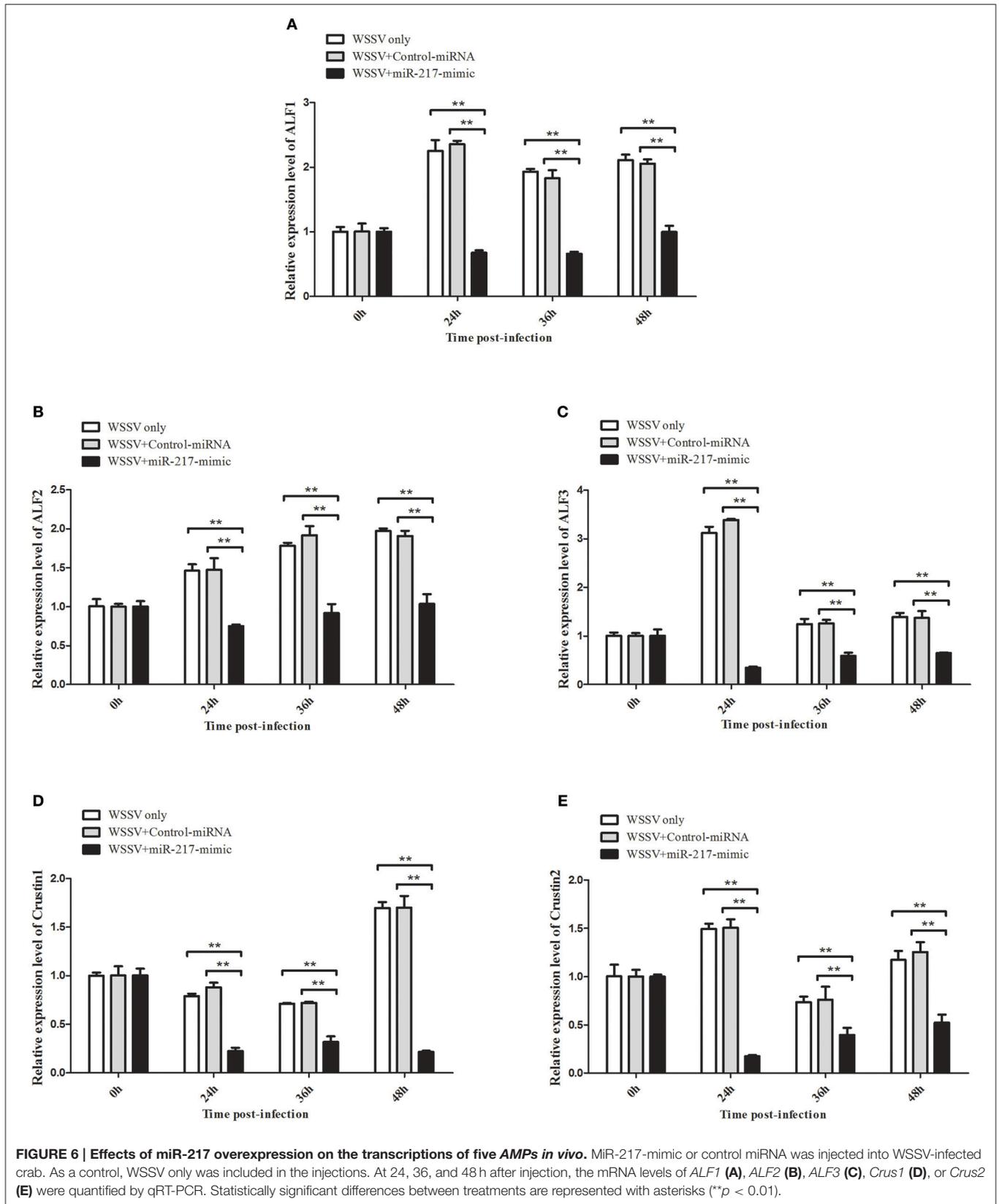


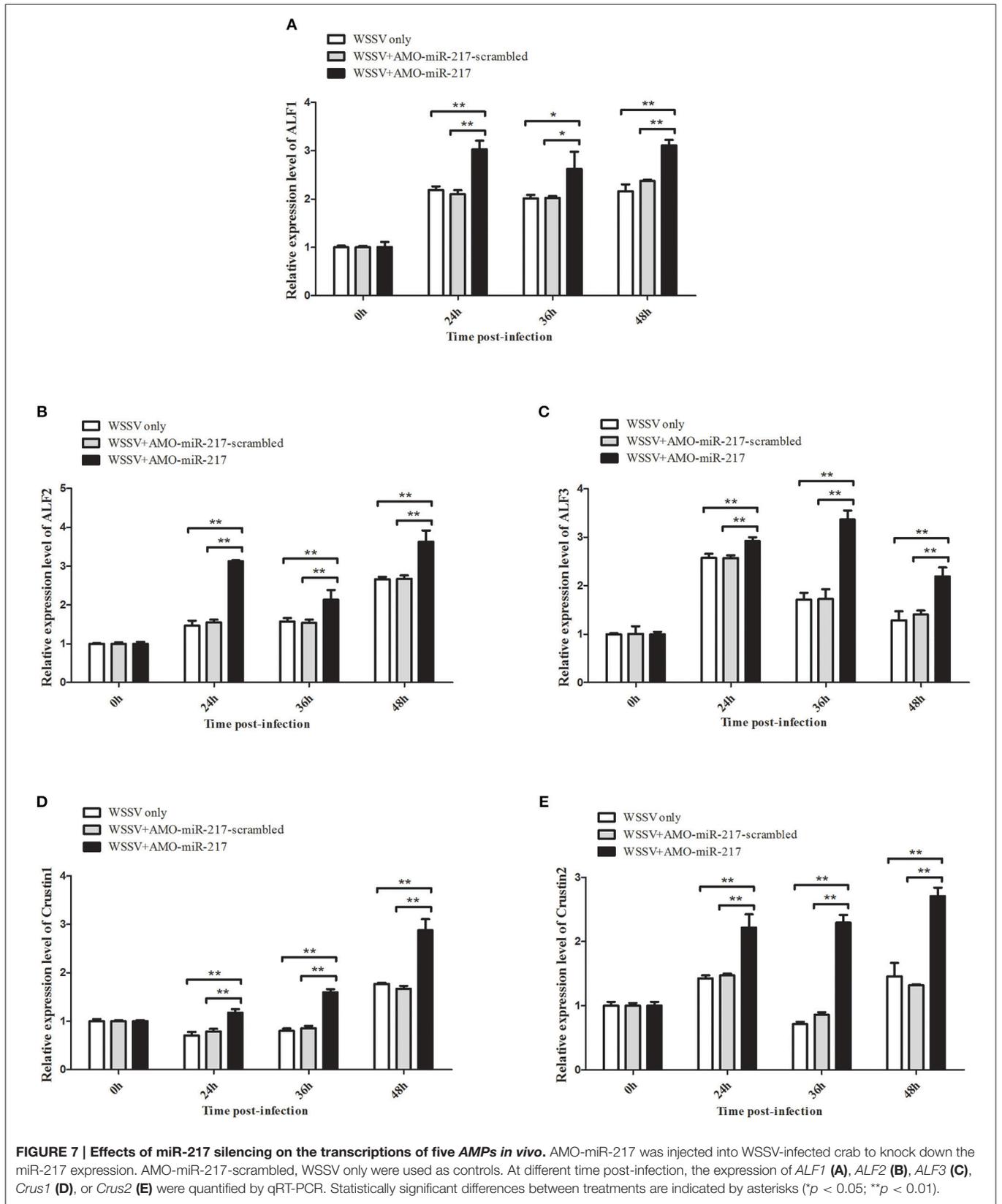
receptors of the Toll-like receptor (TLR) family, namely, murine *TLR7* and human *TLR8* (Fabbri et al., 2012). Human miR-9 was rapidly increased after *TLR4*-activation, which operated a feedback control of the NF- κ B-dependent responses by fine-tuning the expression of a key member of the NF- κ B family (Bazzoni et al., 2009). MiR-105 was reported as a modulator of *TLR2* translation in human gingival keratinocytes (Benakanakere et al., 2009). MiR-146 could control TLR and cytokine signaling through a negative feedback regulation loop involving the downregulation of IL-1 receptor-associated kinase 1 and TNF receptor-associated factor 6 protein levels (Taganov et al., 2006). However, miRNAs of crustaceans that mediate the regulation of key components of the host Toll pathway are still not well studied.

In the current study, we reported that crab-specific miR-217 played positive roles in WSSV infection by negatively regulating host Toll signaling pathway in *E. sinensis*. MiR-217 inhibited the

Toll signaling pathway by targeting the *Tube* gene. *Tube*, which is the key component of host Toll pathway, played pivotal roles in numerous biological activities, such as survival, development, and innate immunity (Yu et al., 2014). The *Tube* could activate or repress a broad range of downstream genes and be involved in forming a sophisticated network in response to various stress signals. In many cases, an individual miRNA can target a unique gene and lead to subsequent changes of the biological processes associated with this target gene. The overexpression of crab miR-217 through the injection of miR-217-mimic led to the enhancement of WSSV copies, implying that miR-217 plays a positive role in WSSV replication. In the multinuclear-activation of galactosidase indicator cells, human miR-217 could influence the transactivation of Tat-induced human immunodeficiency virus 1 through the downregulation of *SIRT1* expression (Zhang et al., 2012). In crustaceans, host miRNAs were also found to







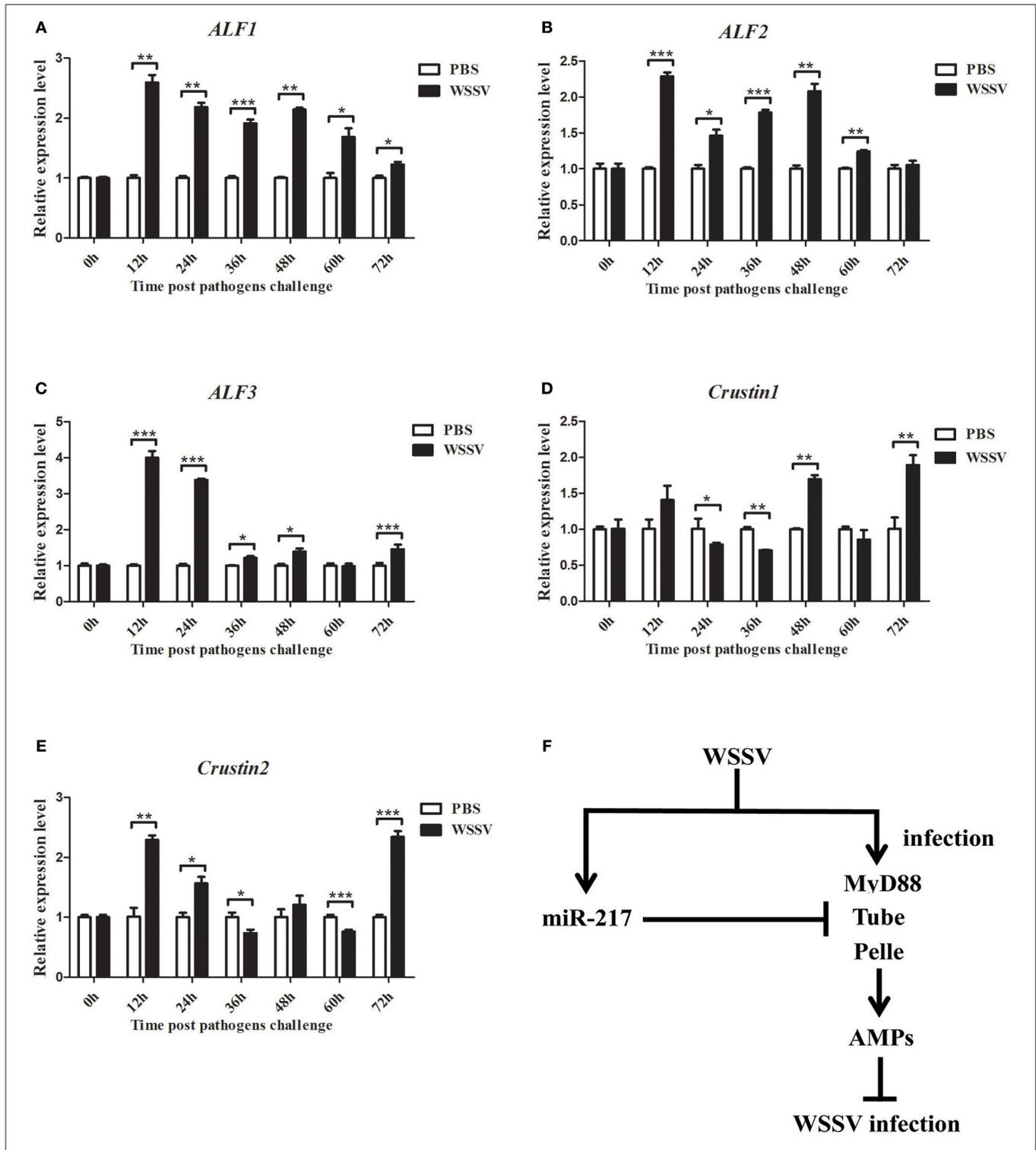


FIGURE 8 | Expression of *ALF1*, *ALF2*, *ALF3*, *Crus1*, and *Crus2* during virus infection in crab. qRT-PCR method was used to analysis the expression of *ALF1* (A), *ALF2* (B), *ALF3* (C), *Crus1* (D), and *Crus2* (E) in the hemocytes of *E. sinensis* at 0, 12, 24, 36, 48, 60, and 72 h after WSSV challenge. The mRNA levels of these five AMPs were analyzed and standardized according to *GAPDH* mRNA levels. Asterisks indicate significant differences ($*p < 0.05$; $**p < 0.01$; $***p < 0.001$) compared with values of the control. Error bars represent \pm S.D. of the three independent PCR amplifications and quantifications. (F) Model for the role of host miR-217 in virus-host interaction.

be involved in virus infection. In *Marsupenaeus japonicus*, host miR-1000 was reported to contribute considerable effects on apoptosis and WSSV replication through targeting *p53* (Gong et al., 2015). Shrimp miR-7 could serve as an antiviral factor by interacting with viral *wsv477* (Huang and Zhang, 2012). In *Macrobrachium rosenbergii*, host miR-9041 and miR-9850 played positive roles in WSSV replication by targeting the *STAT* gene (Huang Y. et al., 2016). Our investigations revealed that crab miR-217 led to a decrease in the transcript level of the *Tube* gene, resulting in the enhancement of WSSV copies.

AMP genes are effector molecules in the innate immunity of invertebrates (Hancock et al., 2006). The *AMPs* of major crustaceans are represented by three cationic peptide families: *ALFs*, *crustins*, and *penaeidins* (Tassanakajon et al., 2010). *ALFs* are cationic *AMPs* with the ability to bind and neutralize lipopolysaccharide and are crucial bioactive substances in the innate immunity of crustaceans (Aketagawa et al., 1986). *Crustins* are cationic cysteine-rich *AMPs* (Liu et al., 2015). In *E. sinensis*, three *ALF* genes (*ALF1*, *ALF2*, and *ALF3*) and two *Crustin* genes (*Crus1* and *Crus2*) were identified. The upregulated expression of the five *AMP* genes in WSSV-challenged crabs may indicate their possible roles in anti-virus immune defense. *ALF* from *Fenneropenaeus chinensis* played a key role in inhibiting WSSV replication (Li S. et al., 2015). In *Penaeus monodon*, the enhanced crustin-like *AMP* conferred significant protection from WSSV infection (Antony et al., 2011). The knockdown of *Tube* gene resulted in a reduction of *ALF1*, *ALF2*, *ALF3*, *Crus1*, and *Crus2* transcripts in crabs. These results suggested that crab *AMP* genes could be modulated by the host Toll pathway, which is also the case in *Drosophila* (Lemaitre and Hoffmann, 2007). Similarly, in *Procambarus clarkii*, *PcToll* was involved in the regulation of *Crus*

expression (Wang et al., 2015). The knockdown of *Dorsal* in *M. rosenbergii* resulted in the suppression of *Crus* transcripts (Huang X. et al., 2016). The silencing of the *PmMyD88* gene resulted in a decrease of expression of two *Crus* genes in *Litopenaeus vannamei* (Arayamethakorn et al., 2017). Therefore, we identified several *AMP* genes regulated by crab *tube* in our study. Further experiments indicated that overexpression of miR-217 inhibited the expression of these *AMP* genes. Hence, our findings together revealed that host miRNA (miR-217) targeting the host *tube* gene caused the inhibition of the expression of *AMP* genes, resulting in the enhancement of virus replication in crabs.

AUTHOR CONTRIBUTIONS

YH, KH, and QR carried out the experiments. YH, WW, and QR designed the experiments and analyzed the data. YH and QR contributed reagents/materials. YH and QR wrote the manuscript. All authors gave final approval for publication.

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Conflict of Interest Statement: 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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