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Pongsopee Attasart

บทคัดย่อ

รหัสโครงการ: RSA5880008

ชื่อโครงการ: การศึกษาการเข้าสู่เซลล์ของอาร์เอ็นเอสายคู่และการแพร่กระจายแบบ systemic ของ RNAi ในกุ้งขาว

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ระยะเวลาโครงการ: 3 ปี

เทคนิค RNAi ได้มีการนำมาใช้ในงานวิจัยด้านกุ้งอย่างแพร่หลายเพื่อการศึกษาการทำงานของยีนและการพัฒนาให้ได้วิธีการป้องกันกุ้งจากไวรัส แต่ยังขาดความรู้ความเข้าใจในกระบวนการนำอาร์เอ็นเอสายคู่เข้าสู่เซลล์กุ้ง ซึ่งจากการศึกษาก่อนหน้านี้พบว่าในสัตว์ไม่มีกระดูกสันหลังจะมีกระบวนการนำอาร์เอ็นเอสายคู่เข้าสู่เซลล์อยู่ 2 วิธีคือ กระบวนการที่อาศัยการส่งผ่าน SID1 ซึ่งเป็นโปรตีนบนผิวเซลล์ และกระบวนการที่อาศัย endocytosis ในโครงการนี้ต้องการศึกษาว่าการนำอาร์เอ็นเอสายคู่เข้าสู่เซลล์กุ้งและการแพร่กระจาย RNAi ในกุ้งอาศัยกระบวนการใดบ้าง ในการศึกษาความเกี่ยวข้องของ SID1 ในการนำอาร์เอ็นเอสายคู่เข้าสู่เซลล์กุ้ง เริ่มจากการให้อาร์เอ็นเอสายคู่แบบเป็นลำดับ โดยกุ้งจะได้รับอาร์เอ็นเอสายคู่เพื่อกระตุ้นการแสดงออกของยีน SID1 ก่อนแล้วจึงทำการให้อาร์เอ็นเอสายคู่ตัวที่สอง (ที่มีความจำเพาะกับยีน STAT หรือยีน CHC ของกุ้ง) เพื่อไปลดการแสดงออกของยีนเป้าหมาย จากการส่องภายใต้กล้องคอนโฟคอลพบว่าเซลล์เม็ดเลือดของกุ้งที่มีการกระตุ้นการแสดงออกของ SID1 ก่อนจะมีจุดแสดงฟลูออเรสเซนท์ (Cy3) ของอาร์เอ็นเอสายคู่ตัวที่สองมากกว่าเซลล์ของกุ้งที่ไม่ได้มีการกระตุ้น SID1 และยังพบว่ากุ้งที่มีการกระตุ้นให้มี SID1 มากขึ้นมีการลดระดับของยีนเป้าหมายที่จำเพาะกับอาร์เอ็นเอสายคู่ตัวที่สอง (ในเนื้อเยื่อเหงือก) มากกว่ากุ้งควบคุม ซึ่งถ้าทำการให้อาร์เอ็นเอสายคู่ตัวที่สองโดยการกินก็ให้ผลทำนองเดียวกัน แสดงให้เห็นว่า SID1 มีส่วนเกี่ยวข้องกับการนำอาร์เอ็นเอสายคู่เข้าสู่เซลล์เหงือกกุ้งและการแพร่กระจาย RNAi ในตัวกุ้ง นอกจากนี้ยังทำการทดสอบความเกี่ยวข้องของกระบวนการ endocytosis ในการนำอาร์เอ็นเอสายคู่เข้าสู่เซลล์ตับและเหงือกกุ้งโดยการฉีดด้วย โดยจะทำการยับยั้งกระบวนการนี้ก่อนด้วยตัวยับยั้ง (chlorpromazine และ bafilomycin-A1) แล้วจึงตามด้วยการฉีดอาร์เอ็นเอสายคู่ตัวที่สอง พบว่าการลดระดับของยีนเป้าหมายในเนื้อเยื่อตับจะลดลงเมื่อมีการยับยั้ง endocytosis ก่อน แต่จะไม่พบการเปลี่ยนแปลงนี้ในเหงือก แสดงว่ากระบวนการ endocytosis มีส่วนเกี่ยวข้องในการนำอาร์เอ็นเอสายคู่เข้าสู่เซลล์ตับ แต่ไม่เกี่ยวข้องในเซลล์เหงือก นอกจากนี้ยังได้ทำการทดสอบว่ากระบวนการ endocytosis มีส่วนเกี่ยวข้องในการนำอาร์เอ็นเอสายคู่เข้าสู่กุ้งโดยการกินด้วยหรือไม่ โดยการใช้ตัวยับยั้งก่อนการให้กุ้งกินอาหารที่มีอาร์เอ็นเอสายคู่ ผลการทดลองพบว่ามีการลดระดับการแสดงออกของยีนเป้าหมายเฉพาะในกุ้ง (ทั้งในเนื้อเยื่อตับและเหงือก) ที่ไม่ได้รับการยับยั้งกระบวนการ endocytosis มาก่อน แต่กุ้งที่ได้รับตัวยับยั้งไม่มีการเปลี่ยนแปลงยีนเป้าหมาย แสดงให้เห็นว่ากุ้งใช้กระบวนการ endocytosis ในการนำอาร์เอ็นเอสายคู่เข้าสู่เซลล์ตับที่ได้รับอาร์เอ็นเอสายคู่โดยการกิน

Abstract

Project Code: RSA5880008

Project Title: Characterization of dsRNA cellular uptake and systemic RNAi in shrimp (*Litopenaeus vannamei)*

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Project Period: 3 years

RNA interference (RNAi) technology has been widely applied to shrimp research for functional genomics, as well as for investigation of its potential anti-viral applications. However, the mechanism of extracellular dsRNA uptake into shrimp cells has not been determined. In invertebrates, uptake of an extracellular dsRNA can occur through two different mechanisms; the transmembrane channel-mediated mechanism, requires a multispan transmembrane protein called systemic RNA interference defective 1 (SID1), and endocytosis-mediated mechanism. This project, we aim to investigate the molecular mechanisms underlying the uptake of dsRNA and systemic RNAi in shrimp (*Litopeneaus vannamei*). To elucidate the role of *Lv*SID-1 in dsRNA uptake, a strategy of sequential introduction of dsRNAs was employed. Shrimp were initially injected with a long dsRNA to induce *Lv*SID-1 mRNA expression before administration of the second dsRNA (dsRNA-STAT or dsRNA-CHC). Under a confocal microscope, the Cy3 signal of second dsRNA in the *Lv*SID-1 induced hemocytes was significantly higher than the signal in naïve hemocytes. Significantly, improved suppression of STAT and CHC was found in gills of the *Lv*SID-1 induced shrimp. Similar result was observed when shrimp was pre-injected with dsRNA-GIH to induce expression of the *Lv*SID1 and fed with diet containing dsRNA-STAT. These results indicate that the *Lv*SID1 participates in the uptake of the injected dsRNA and involves in systemic RNAi in shrimp. The possible involvement of endocytosis in the delivery of injected dsRNA into shrimp hepatopancreatic and gill cells was also evaluated. Clathrin-mediated endocytosis was inhibited through the injection of shrimp with two pharmacological endocytosis inhibitors (chlorpromazine and bafilomycin-A1) before injection of dsRNA directed against STAT (dsSTAT). Inhibition of clathrin-mediated endocytosis showed a reduction of STAT suppression in the shrimp hepatopancreas. In contrast, neither chlorpromazine nor bafilomycin-A1 effectively blocked dsSTAT inhibition of STAT in gill tissue, suggesting that clathrin-mediated endocytosis participates in dsRNA uptake into hepatopancreas but not gills. Moreover, mechanism for uptake of dsRNA from environment (food) was also evaluated. Chlorpromazine was used to block the clathrin-mediated endocytosis before continuous feeding the formulated food containing dsRNA specific to shrimp Rab7 gene for 6 days. Level of Rab7 mRNA suppression in gill and hepatopancreas tissues were monitored every day (12 hours after feeding of each day) by RT-PCR. Suppression of Rab7 mRNA was detected in gill and hepatopancreas tissues at day6 only in dsRNA-Rab7 fed shrimp without chlorpromazine treatment. In contrast, the Rab7 mRNA level was not changed in dsRNA-Rab7 fed shrimp treated with chlorpromazine. The results indicate that the clathrin-mediated endocytosis participates in dsRNA uptake in shrimp by feeding.

Keywords: RNAi, dsRNA, uptake, systemic and shrimp

Executive Summary

RNA interference approach is probably the most powerful technique and has been widely applied to inhibit both DNA and RNA viruses in shrimp. The exogenous dsRNA can be introduced into shrimp either by injection or by oral feeding bacteria expressing dsRNA however the latter has much lower efficacy. Even though the oral feeding method is more practical in shrimp farm, its efficiency in triggering dsRNA-mediated gene silencing needs to be improved. Since the effectiveness of fed RNAi technique relies on the systemic RNAi, hence, the parameters required to obtain a robust systemic RNAi response have to be analyzed. This project, we aim to investigate the molecular mechanisms underlying the uptake of dsRNA and systemic RNAi in shrimp (*Litopeneaus vannamei*).

In invertebrates, uptake of an extracellular dsRNA can occur through two different mechanisms; the transmembrane channel-mediated mechanism, requires a multispan transmembrane protein called systemic RNA interference defective 1 (SID1), and endocytosis-mediated mechanism. The involvement of the *Lv*SID1 on dsRNA uptake in shrimp was evaluated by over expression of the *Lv*SID1. The *Lv*SID1 induced shrimp showed level of STAT suppression more than that of the *Lv*SID1 non-induced shrimp. Similar result was observed when shrimp was pre-injected with dsRNA-GIH to induce expression of the *Lv*SID1 and fed with diet containing dsRNA-STAT. These results indicate that the *Lv*SID1 participates in the uptake of the injected dsRNA and involves in systemic RNAi in shrimp.

Inhibition of clathrin-mediated endocytosis pathway by chlorpromazine affected the dsRNA uptake by injection into hepatopancreas but not into gills. Moreover, suppression of Rab7 mRNA was significantly reduced in the dsRNA-Rab7 fed shrimp pre-treated with chlorpromazine. The results indicate that the clathrin-mediated endocytosis participates in dsRNA uptake in shrimp hepatopancreas but not gills by both feeding and injection. This information may help in improving the potency of dsRNA mediated anti-virus in shrimp by fed RNAi in the future.

1. Introduction

RNA interference (RNAi) is a mechanism which exists in many eukaryotic organisms that can be activated by exogenous double-stranded RNA (dsRNA) for the destruction of invading pathogens, such as viruses, in a sequence-dependent manner (Fire, 1999; Hannon, 2002). Because of its specificity, thus, the RNAi approach is probably the most powerful technique and has been widely applied to inhibit both DNA and RNA viruses in shrimp (Robalino et al., 2005; Yodmuang et al., 2006; Tirasophon W et al., 2007; Attasart et al., 2009; Ongvarrasopone et al., 2010; Attasart et al., 2011). The exogenous dsRNA can be introduced into shrimp either by injection or by oral feeding bacteria expressing dsRNA however the latter has much lower efficacy (Attasart et al., 2013; Sanitt et al., 2014). Therefore, the details or parameters affecting its low efficiency in triggering dsRNA-mediated gene silencing need to be investigated. Moreover, understanding the molecular mechanisms underlying the systemic RNAi may help in improving the potency of anti-virus in shrimp by fed RNAi.

To achieve efficient RNAi, in general, two main steps; 1) uptake of extracellular dsRNA into cells and 2) systemic spread of RNAi throughout the whole body, has to be concerned. In invertebrate, the cellular uptake of dsRNA can be classified into two mechanisms. First, the systemic RNA interference-deficient 1 (sid1), a protein with nine transmembrane domains characterized as an RNA channel transporter (Feinberg and Hunter, 2003), was required for systemic RNAi in nematode Caenorhabditis elegans (Winston et al., 2002). Ectopic expression of Cesid1 protein facilitated uptake of dsRNA into Drosophila melanogaster Schneider S2 cells (Feinberg and Hunter, 2003). Second, the receptor mediated endocytosis has been shown to be involved in dsRNA uptake in Drosophila S2 cells (Saleh et al., 2006) and some insects such as beetle (Tomoyasu et al., 2011), fly (Ulvila et al., 2006) and ticks (Aung et al., 2011).

The core RNAi machineries are not involved in systemic RNAi spreading in C.elegans but they affect the cellular RNAi efficacy (Parrish and Fire, 2001). It has been shown that siRNA production are not necessary for spreading of the RNAi effect, at least in the mutant conditions, suggesting that dsRNA itself may be the transmitting factor for RNAi spreading. This conclusion was supported by the preference for uptake of dsRNA longer than 50 bp into S2 cells overexpressing the C.elegans sid1 gene (Feinberg and Hunter, 2003). However, at present, no experimental evidence clearly confirmed this speculation.

In shrimp, the suppression of shrimp endogenous genes (Rab7 and STAT) via feeding was detected not only in the hepatopancreas but also in the gills indicating the successful systemic induction of RNAi via oral delivery of dsRNA (Attasart et al., 2013). The sid1-like has been identified in pacific white leg shrimp recently (Labreuche et al., 2010). It encoded a putative protein that was predicted to contain 11 transmembrane domains similar to sid1 protein in C.elegans suggests it may function as a tramsmembrane channel for dsRNA. Moreover, its expression was strongly up-regulated after shrimp receiving long dsRNAs with arbitrary sequences indicating that it may account for the systemic RNAi effect in shrimp. Nevertheless, the sid1 homologs have been identified in most insects such as silkworm (Kobayashi et al. 2012) and migratory locust (Luo et al. 2012) however they are not required for cellular

dsRNA uptake. Hence, this study will be conducted to determine whether the presence of LvSID1 is involved in uptake of dsRNA into cells and correlated with the systemic RNAi in shrimp. In addition, characterizing and understanding the mechanisms involved in the systemic RNAi process in shrimp fed with bacteria expressing dsRNA will allow us for determining the rate limiting steps in anti-virus approach by feeding.

2. Objectives

2.1 To determine *LvSID1* expression in normal and dsRNA-treated shrimp (*L. vannamei*) (by both injection and feeding)

2.2 To evaluate the role of *LvSID1* on dsRNA uptake and systemic RNAi in shrimp.

2.3 To characterize the systemic RNAi process in shrimp fed with bacteria expressing dsRNA

3. Methods

3.1 Determination of *LvSID1* expression in different tissues

3.1.1 In normal shrimp

To investigate tissue distribution of *LvSID1*, the level of *LvSID1* mRNA expression in shrimp tissues (hepatopancreas, gill and muscle) will be determined by real-time RT-PCR. Total RNA will be extracted from each 10 mg tissue of 500 mg shrimp and used to synthesize cDNA by random hexamer primer and reverse transcriptase enzyme. PCR amplification will be performed using primers corresponding to the shrimp *LvSID1* in a real-time PCR machine (realplex, Eppendrof). Elongation factor-1-alpha (EF-1α) will be amplified as an internal control with their specific primers. The relative level of *LvSID1* mRNA normalized with EF-1α will be compared among different tissues.

3.1.2 In dsRNA-treated shrimp

In this study, we plan to investigate the response of *LvSID1* expression if shrimp are treated with dsRNA by injection and by feeding. Both specific dsRNA (dsRNA-STAT and dsRNA-Rab7) and non-specific dsRNAs such as dsRNA-gfp, dsRNA-ns1 and dsRNA-rr2 that corresponding to shrimp STAT, Rab7, jelly fish green fluorescence protein, *Peneous monodon* densovirus ns1 and white spot syndrome virus rr2 genes, respectively, will be used for shrimp treatment. For injection, 1.5 μg of each dsRNA and saline will be injected into individual shrimp (about 300mg). Then, total RNAs from different tissues (hepatopancreas, gill and muscle) will be extracted at 48 hours post injection. In case of feeding, the diet will be formulated using bacteria expressing dsRNA-gfp and dsRNA-Rab7 and used to feed shrimp at 10% body weight/meal, 2 meals /day. The control shrimp will be fed with commercial diet or formulated diet with bacteria without dsRNA. Total RNA will then be isolated at 2, 4 and 6 days after feeding, and used to synthesize cDNA. This cDNA will act as a template for amplification with *LvSID1* and actin specific primers. PCR product will then be analyzed by agarose gel electrophoresis. The relative amount of *LvSID1* (normalized by actin) will be monitored and compared between treated and non-treated shrimp.

3.2 Investigation of the role of *LvSID1* on dsRNA uptake and systemic RNAi

To determine whether the *LvSID1* is involved in dsRNA cellular uptake, level of shrimp endogenous gene (STAT) suppression will be monitored under the *LvSID1* expression being knocked down and induced. In this experiment, the STAT will be suppressed by two methods; by injection (for determining the role of *LvSID1* on cellular dsRNA uptake) and by feeding bacteria expressing dsRNA-STAT (for determining the role of *LvSID1* on systemic RNAi). By injection, the dsRNA-STAT will directly contact with the cells in every tissues after introduction. While, feeding, only RNAi signals (? intact molecule of long dsRNA-STAT or processed dsRNA-STAT) from epithelial cells of hepatopancreas will spread through haemolymph circulation to reach cells in the distant tissues (gill and muscle).

3.2.1 In *LvSID1* knocked down shrimp

3.2.1.1 Construction of dsRNA-*SID1* expression cassette in pET-17b plasmid

In order to knockdown *LvSID1* using dsRNA, the region of *LvSID1* like cDNA from *L. vannamei* (GenBank: HM234688) will be selected for cloning of inverted-repeat cassette. To amplify 600bp and 400bp fragments for cloning, we will design primers specific to this region and then amplify using Vent® DNA polymerase. These two fragments were then cloned into the pET17b vector (under the T7 promoter) in an inverted direction. The correct recombinant plasmid (pET-17b-st*SID1*) will be confirmed by restriction analysis and sequencing.

3.2.1.2 Expression of dsRNA and dsRNA extraction

Each dsRNA will be produced in *E.coli* HT115. The overnight culture was diluted and grown at 37 °C until OD600 reached 0.4 before induction with isopropyl-β-D-thiogalactopyranoside (IPTG) for 4 h. The bacterial cells were then centrifuged at 6000 × g for 5 min at 4 °C. DsRNA will be extracted according to the modified protocol from Posiri *et al*. (2013), bacterial cell pellet will be resuspended in 75% ethanol in PBS at 100 μl /1 OD cell. After incubation at room temperature for 5 min, the treated cells will be collected by centrifugation at 8000 × g for 5 min at 4 °C. The cell pellet will be resuspended in 150 mM NaCl and incubated at room temperature for 1 hour. The cell suspension will then centrifuged at 12000 × g at 4 °C for 5 min to generate a cell-free supernatant that contains dsRNA. The extracted dsRNA will be diluted and the concentration will be estimated by agarose gel electrophoresis. Then 2 μg of dsRNA will be digested with RNase A or RNase III to assess relative double-stranded structure.

3.2.1.3 Evaluation of STAT suppression by injection or feeding after *LvSID1* knocked down.

We hypothesize that the level of STAT suppression will be decreased in *LvSID1* knocked down shrimp if systemic RNAi requires *LvSID1*. To test this, 300 mg shrimp will be injected with 1.50 μg of dsRNA- *LvSID1* into haemolymph, NaCl and dsRNA-gfp will be used as a control. Five shrimps /group will be collected at 24 and 48 hours post injection, total RNAs from different tissues (hepatopancreas, gill and muscle) will be isolated. The relative expression of *LvSID1* will be determined by RT-PCR using *LvSID1* specific primers and normalized with actin. The optimal condition demonstrating

LvSID1 knocked down will be used for further STAT suppression evaluation (by injection and by feeding). For injection: The *LvSID1* knocked down shrimp will be injected with dsRNA-STAT into haemolymph. For feeding: the *LvSID1* knocked down shrimp will be fed with formulated diet containing bacteria expressing dsRNA-STAT at 10% body weight/meal, 2 meals/day. Total RNAs will be isolated from different tissues (hepatopancreas, gill and muscle) at 12 and 24 hrs after injection or at 2, 4 and 6 days after feeding. Total RNA will then be used to synthesize cDNA and STAT product will be amplified with STAT specific primer and actin as an internal control. PCR product will then be analyzed by agarose gel electrophoresis. The level of amplified STAT of different shrimp tissues will be relatively quantified and compared between normal and *LvSID1* knocked down shrimp.

3.2.2 In *LvSID1* induced shrimp

3.2.2.1 Testing of STAT suppression after *LvSID1* induction.

We hypothesize that the level of STAT suppression will be increased in *LvSID1* induced shrimp if *LvSID1* is essential for systemic RNAi. According to the previous information that *LvSID1* expression was up-regulated when shrimp was treated with dsRNA, therefore we will use dsRNA-gfp injection to induce *LvSID1* expression in this study. The 300 mg shrimp will be injected with 1.5 μg dsRNA-gfp into haemolymph then leave for 48 hours. After that we will test STAT suppression by injection or feeding. Total RNA will be isolated and the STAT suppression will be analyzed by RT-PCR. PCR product will then be analyzed by agarose gel electrophoresis. The level of amplified STAT will be relatively quantified among different tissues and compared between normal and *LvSID1* induced shrimp.

3.3 Characterization of the systemic RNAi process after feeding with bacteria expressing dsRNA

In this study, we will determine the RNAi signals that spread throughout the shrimp haemolymph at various time points after feeding. The 5g shrimp will be fed with the formulated diet containing bacteria expressing dsRNA-STAT at 10% body weight/meal, 2 meals/day. Total RNAs will be extracted from haemolymph of treated shrimp that will be collected at 6 hrs (after complete digestion process), 2, 4, and 6 days after feeding. Northern blot analysis will be used to detect dsRNA molecules in the extracted RNA from haemolymph. The DIG-labeled probe specific to STAT sequence will be performed according to the manufacturer's protocol.

4. Results

4.1. Expression of the *Lv*SID1 mRNA in normal shrimp

The relative level of *LvSID1* mRNA expression in three different tissues (hepatopancreas, gill and muscle) of normal shrimp were determined by quantitative real-time RT-PCR using primers corresponding to *Lv*SID1 cDNA (GenBank: HM234688) and shrimp elongation factor alpha 1 gene (Ef1α) for normalization. The highest expression level of *Lv*SID1 mRNA was detected in hepatopancreas, which was approximately 3 and 45 times higher than that in gill and muscle, respectively (figure 1).

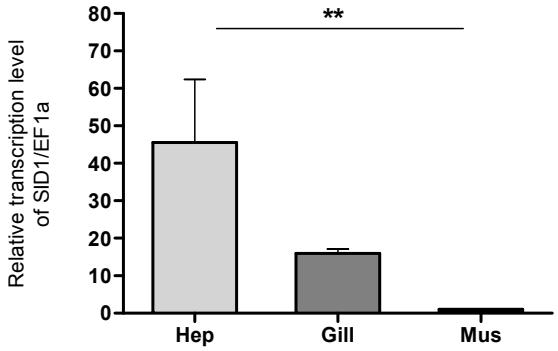


Figure 1 The relative expression of *Lv*SID1 mRNA in normal shrimp. Ten milligrams of hepatopancreas (Hep), gill (Gill) and muscle (Mus) were separately collected from *L. vannamei* shrimp (n=4). The extracted RNA from each tissue was used to determine the relative expression of *Lv*SID1 mRNA by quantitative real-time RT-PCR. The relative level of *Lv*SID1 mRNA normalized by Ef1α mRNA level were plotted using GraphPad Prism 5 program, The statistical analysis was performed by Krukal-Wallis test at *p*-value less than 0.01 (**).

4.2 Response of the *Lv*SID1 mRNA expression to long dsRNA

Expression of the *Lv*SID1 mRNA in shrimp injected with dsRNA

300 mg shrimps were injected with approximately 1.5 μg of long dsRNA (300-400 bp). To determine the response of *Lv*SID1 expression to long dsRNA with arbitrary sequences, four different dsRNAs such as dsRNA-STAT (dsSTAT, 406 bp), dsRNA-GFP (dsGFP, 380 bp), dsRNA-NS1 (dsNS1, 425 bp) and dsRNA-RR2 (dsRR2, 398 bp) that correspond to shrimp signal transduction and transcription protein, jelly fish green fluorescence protein, *Penaeus monodon* densovirus ns1 and white spot syndrome virus rr2 genes, respectively were used in this experiment. At 48 hours post dsRNA injection, the *Lv*SID1 mRNA expression in gill tissue was up-regulated when shrimp was injected with any of those dsRNAs. Notable, the level of *Lv*SID1 mRNA induction among the dsRNA treated shrimp were different, in which the dsGFP gave the strongest induction while the others (dsNS1, dsRR2 and dsSTAT) gave comparable effect (figure 2).

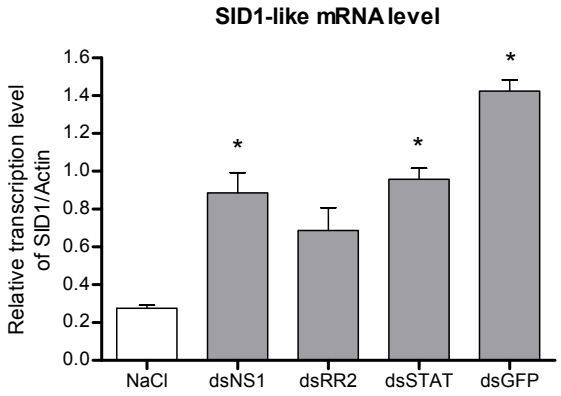


Figure 2 The response of *Lv*SID1 mRNA expression to long dsRNAs. Shrimp (*L. vannamei*) were injected with 400 bp long dsRNAs (dsNS1, dsRR2, dsSTAT and dsGFP, N= 8, 8, 4, 3 and 5, respectively). At 48 hours post injection, the level of *Lv*SID1 mRNA in gill tissue of the treated shrimp was determined by semi-quantitative RT-PCR. The relative level of *Lv*SID1 mRNA normalized by actin mRNA was plotted. Data is presented as mean ± SEM, the statistical analysis was performed by Krukal-Wallis test, * indicates significance at *p*-value <0.05.

To evaluate the induction effect in different tissues (hepatopancreas, gill and muscle) and the longevity of this effect, shrimp were injected with dsGFP at 5.0 μg/g shrimp and the *Lv*SID1 mRNA expression was followed at 2, 3, 4 and 8 days post dsRNA injection (dpi). The induction of *Lv*SID1 mRNA was observed in gill and muscle of the treated shrimp. The increasing level in gill was found up to 5 folds from the control at 2 dpi and gradually decreased thereafter. In muscle, the strength of this effect was high (4 folds from the control) at day 2 and then reduced sharply. The induction effect can be prolonged up to a week especially in gill tissue. The response of *Lv*SID1 to the dsRNA at lower doses was determined, dsGFP were injected into shrimp haemolymph at 5.0, 2.0 and 0.6 μg/g shrimp followed by determination of *Lv*SID1 mRNA expression at 12, 24 and 48 hours post injection (hpi) in both hepatopancreas and gill tissue. Similar level of *Lv*SID1 induction with different dose of dsGFP in gill tissue was observed in all tested conditions. In contrast, there was no difference of *Lv*SID1 mRNA expression in hepatopancreas of the dsRNA-injected shrimp when compared with the control.

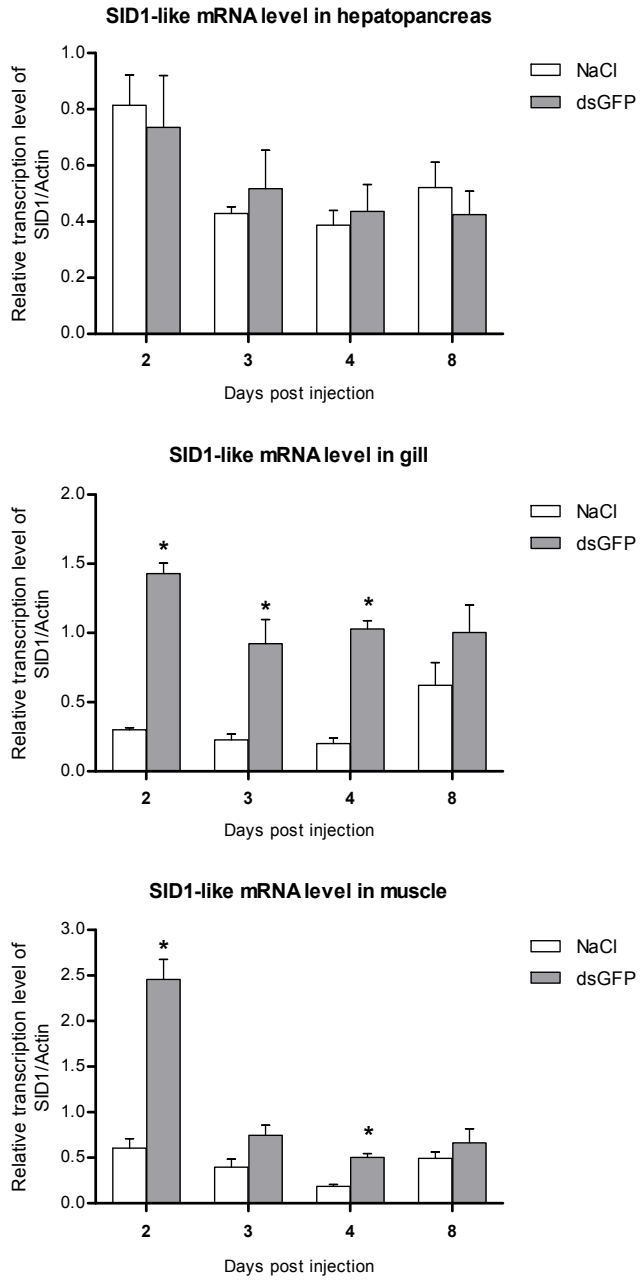


Figure 3 Longevity of the induction effect of dsGFP on *Lv*SID1 mRNA expression. 1.5 µg of dsGFP was injected into 300 mg shrimp. Hepatopancreas, gill and muscle of an individual shrimp (N=4) were isolated at different time points (2, 3, 4 and 8 dpi). Relative level of *Lv*SID1 transcript normalized by actin was quantified by semi-quantitative RT-PCR. Data is presented as mean ± SEM, the statistical analysis was performed by Mann Whitney test (t-test), * indicates significant difference of SID1-like level at *p*-value <0.05 between dsRNA injected group and NaCl control.

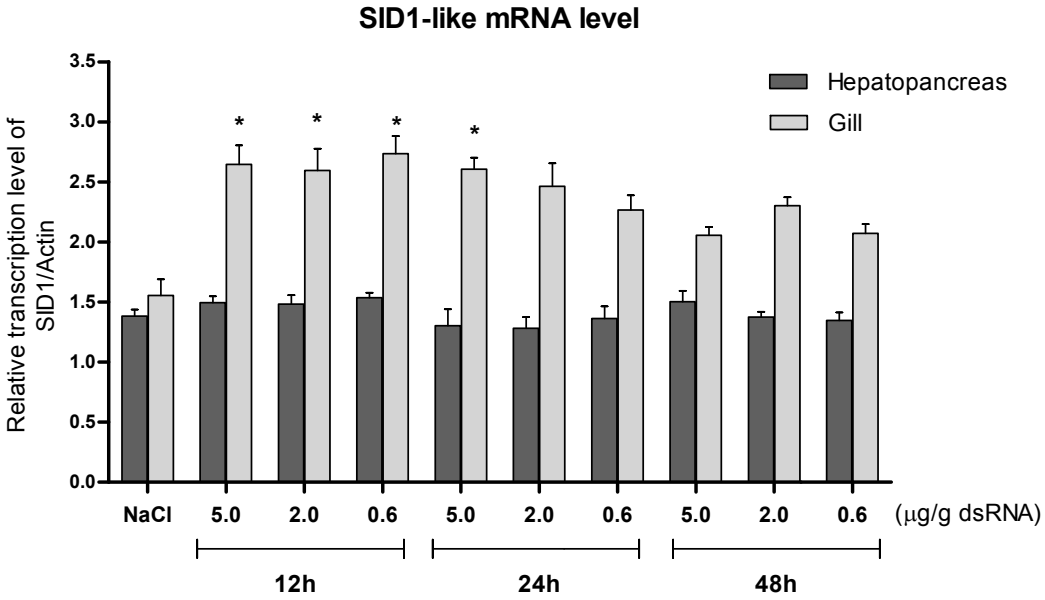


Figure 4 The response of *Lv*SID1 to various amount of dsGFP. The graph presents the relative expression of *Lv*SID1 mRNA normalized by actin in hepatopancreas and gill tissues of shrimp injected with 5.0, 2.0 and 0.6 μg of dsGFP/g shrimp collected at different time points (12h; N=4, 24h; N=4, 48h; N=5). Data is presented as mean ± SEM, the statistical analysis was performed by Krukal-Wallis test. * indicates significant difference of SID1-like level at *p*-value <0.05 between dsRNA injected group and NaCl control.

Expression of the *Lv*SID1 mRNA in shrimp fed with bacteria expressing dsRNA

In case of feeding, shrimp were fed with formulated diet containing bacteria expressing dsRNA-Rab7 (dsRab7) at 10% body weight/meal, 2 meals /day for 6 days. Expression of the *Lv*SID1 mRNA was induced in gill tissues after feeding for 4 and 6 days, however no suppression of Rab7 expression was observed in both gill and hepatopancreas tissues at those time points. Like the injection experiment, induction of *Lv*SID1 mRNA expression in hepatopancreas was not detected. These results suggest that *Lv*SID1 mRNA expression was up-regulated in gill but not in hepatopancreas when shrimp receiving the dsRNA introduced by injection or feeding.

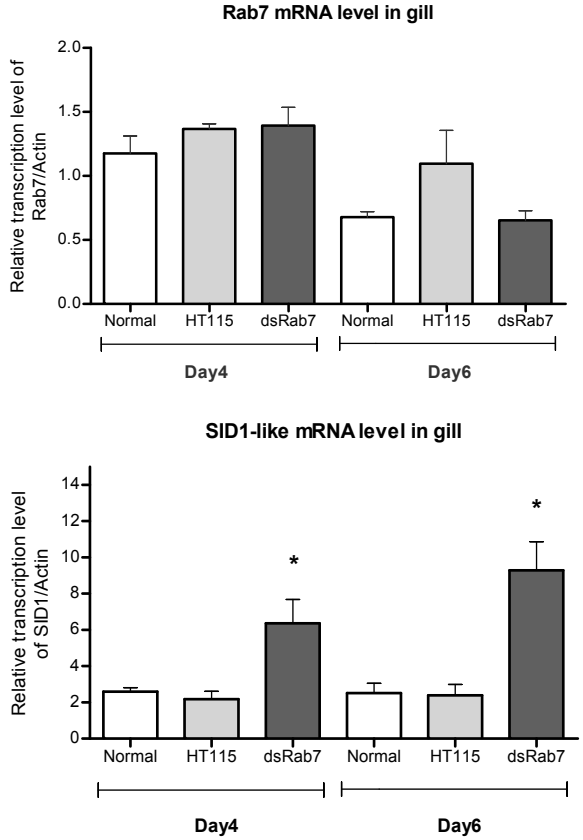


Figure 5 The response of *Lv*SID1 to fed dsRab7 in gill tissue of shrimp Gills were collected from each individual shrimp (n=5) fed with either commercial diet (normal), diet containing bacterial cells without dsRNA expression plasmid (HT115) or diet containing bacteria expressing dsRab7. Relative level of mRNA transcript was determined by semi-quantitative RT-PCR. A): The relative level of *Lv*SID1 mRNA at 4 (Day4) and 6 days (Day6) of feeding. B): The relative level of Rab7 mRNA at 4 (Day4) and 6 days (Day6) of feeding. Data is expressed as mean ± SEM, * indicates significant difference of SID1-like level of dsRab7 fed shrimp from the normal control at *p*-value <0.05.

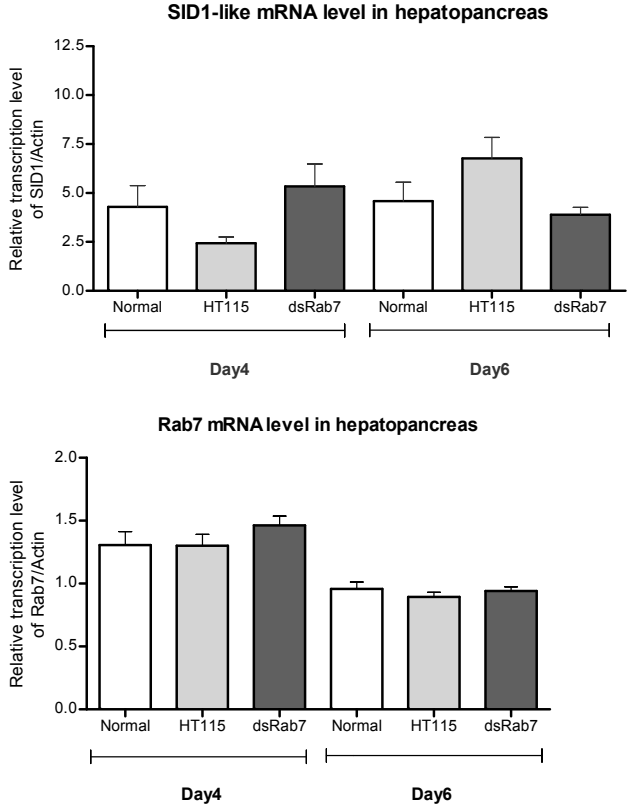


Figure 6 The response of *Lv*SID1 to fed dsRNA-Rab7 in hepatopancreas of shrimp. Hepatopancreas was collected from each individual shrimp (n=5) fed with either commercial diet (normal), diet containing bacterial cells without dsRNA expression plasmid (HT115) or diet containing bacteria expressing dsRab7. Relative level of the *Lv*SID1 mRNA was determined by semi-quantitative RT-PCR. A): The relative level of SID1-like mRNA at 4 (Day4) and 6 days (Day6) of feeding. B): The relative level of Rab7 mRNA at 4 (Day4) and 6 days (Day6) of feeding. Data is presented as mean ± SEM.

4.3 Investigation of the role of *Lv*SID1 on dsRNA uptake and systemic RNAi In *LvSID1* knocked down shrimp

The pET17b-stSID1 plasmid containing the cassette for expression of dsRNA specific to *Lv*SID1 gene (dsSID1) was constructed. To amplify 400 bp (sense) and 600 bp (anti-sense with loop region), two primers were designed based on the *Lv*SID1 cDNA sequence of *L. vannamie* (GenBank accession no. HM234688) (figure 7). These two fragments were then cloned into the pET17b vector under the T7 promoter in the format of inverted repeat. The dsSID1 was produced in *E.coli* HT115 (RNase III defective strain) and extracted by ethanol method. Shrimp were injected with 1.5 μg of the dsSID1 into haemolymph, then the *Lv*SID1 mRNA expression in hepatopancreas and gill were observed at 48 and 72 hours post dsRNA injection. The level of *Lv*SID1 mRNA expression in hepatopancreas was decreased at 48 hours but not different from the control level of shrimp injected with NaCl (figure 9). Thereafter, approximately 40% reduction of the *Lv*SID1 mRNA level was detected at 72 hours post injection. Moreover, the dsGFP did not show suppression effect on the *Lv*SID1 mRNA expression. These results

indicate dsSID1 could suppress the target gene with sequence specific manner. However, in gill tissue, the *Lv*SID1 mRNA expression was up-regulated by injection of the dsGFP. The level of *Lv*SID1 mRNA of the dsSID1 treated shrimp was reduced when compared with that of the shrimp injected with dsGFP but was not different from the saline group (figure 8). It indicated that the dsSID1 has two effects on the *Lv*SID1 mRNA expression; it can induce the expression of *Lv*SID-1 mRNA and trigger the degradation of *Lv*SID1 mRNA at the same time. From these results, the suppression of *Lv*SID1 mRNA expression was still ineffective to investigate *Lv*SID1 function in shrimp

1621 AGCAGATGCA CAAGTTGCAG AGATAGGCTT CCAGAATAAT GTTTTGATTA TGGCAGTATT

1681 TACAGCACTT CCAACCACAG AACTTGTGAG GTCATATCTG AAGTTGCTGC TGTACCATGG

*Xba*I **stSID1-F2**

1741 CCAAGAGGAC CAGTGCTTTT TCAATTCCCG CTGTCTGACA GCATTTGGTA CCCTGCCTGA

stSID1-F1 ***Xho*I**

1801 TTTTGCTCGA GTCTTCACCA ATATTGGATA CCTCTTGTGT GGTGCAGCTT TTATTATCAT

1861 AGTGAAAGAA CACAAGAAAT TTACTGAGAA CATTCTCCGA CAGTATGGTG CAAATAATAG

1921 TGTTGGTGTA AGCAGACATT ATGGATTGTT TATGTCTGTT GGTTATGGAC TCTTCATCCA

1981 GGGTGTTATG TCATCCTTAT ATCACACTTG CCCAAACAGC GTTACTATCA GATTCGACAT

2041 GATGTTCGTG TACGTAGTGG CAGTCGCTGC TGTTGTGAGC ATGTGGGGAT TCCGCCACGG

2101 TGATGTCACA CACCATGTTT ACCCCACAAT GGTTATGGTC GGTATGATAC TTCTGATGGC

2161 AGAGGCGCGT GAATGGGTCA GTCAGGCAGC CTTCTGGACT GTACTGTCTC TGTGTTATGT

stSID1-R2 ***Eco*RI**

2221 CTTCCTTATG GTGACAAACA CCATACTGCT CACAAAGTAT GGAGTTTGGT CTTTCTCTCC

2281 ATACAAGATG CTAATGGTCT GGAAGGGATG GAGACCCGTT GCAGAAAAAT TGCGTAATGA

2341 ATTGTGGGGT TCAGCAACGA CTGCCAAGCC ACTGCAGATT GTGAGGATTG TCATAGGACT

***Eco*RI** **stSID1-R1**

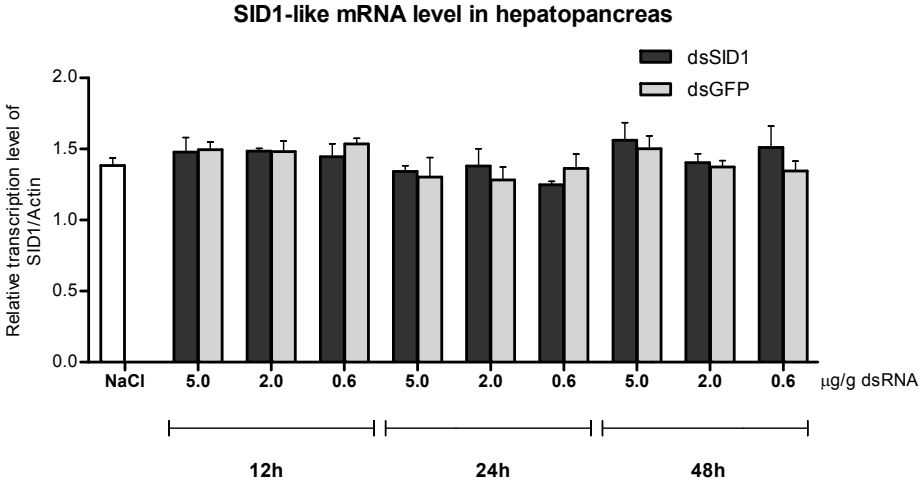
2401 GGTGGTGAAT TCTGCAATTA TCCTGTTTGG TTGCTTAGCT GATCCAAATA TCTACAGTTA

2461 TATCCTCATG GTGTGCCTCA TAAACATGGG ACTTTACTTC TTAAATTATG TAATTGCAAA

2521 GATATGCGAG AGGGAAAGTG TGAGAGCTCT TCCCTCGATA GCTCTGGGAA TATCCTTGAT

Figure 7 Primer regions for *Lv*SID1 sense and anti-sense fragments amplification. Four primers (stSID1-F1, stSID1-R1, stSID1-F2 and stSID1-R2) were designed based on the *L. vannamei* cDNA sequences (GenBank: HM234688)**.** Primers stSID1-F1 + stSID1-R1 and stSID1-F2 + stSID1-R2 were used for the synthesis of anti-sense fragment (652 bp) and sense fragment (418 bp), respectively. The restriction enzyme sites providing the directional cloning are indicated at 5' end of stSID1-F2 (*Xba*I) and stSID1-R2 (*Eco*RI) primers. The gray colour shade represents the region of each primer while the underline represents an internal restriction enzyme site used for cloning.

A)



B)

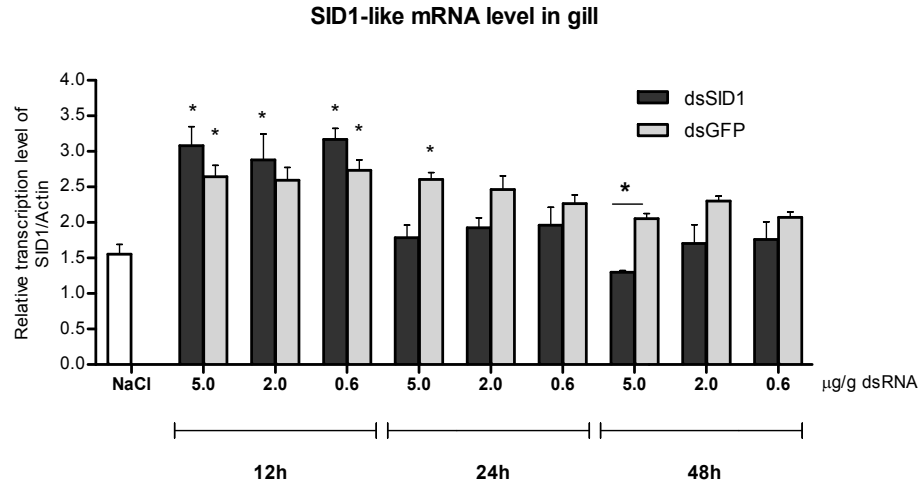


Figure 8 Suppression of *Lv*SID1 mRNA by dsSID1 injection. Shrimp were injected with dsSID1 or dsGFP at 5.0, 2.0 or 0.6 μg/g shrimp. The level of *Lv*SID1 mRNA in hepatopancreas (A) and gill (B) tissues of the treated shrimp were determined at 12, 24 and 48 hpi. The relative expression of *Lv*SID1 mRNA normalized by actin was quantified from the semi-quantitative RT-PCR results and plotted by using GraphPad Prism 5 program. Data is demonstrated as mean ± SEM (12h; N=4, 24h; N=4, 48h; N=5). The statistical analysis was performed using non-parametric test. * indicates significant difference of *Lv*SID1 level at *p*-value <0.05 between dsRNA injected group and NaCl control.

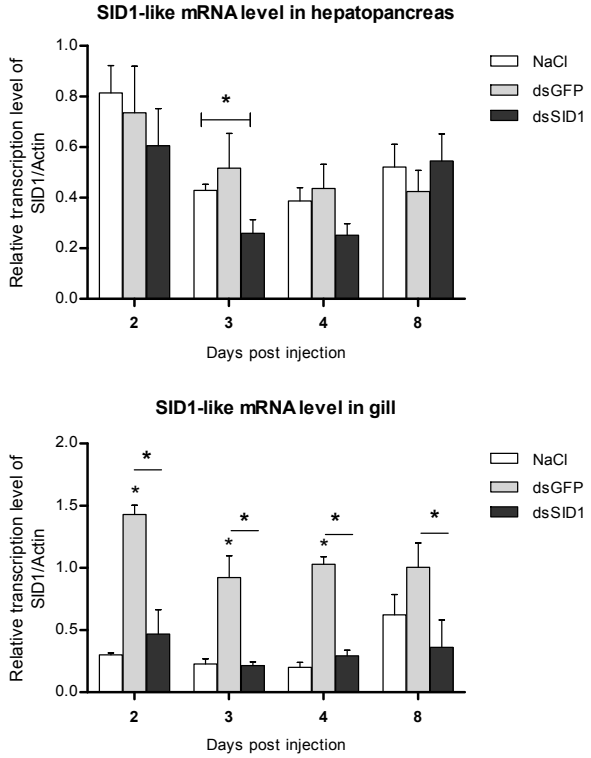


Figure 9 Suppression of *Lv*SID1 mRNA by dsSID1 injection at longer time. Shrimp were injected with dsSID1 or dsGFP at 5.0 µg/g shrimp or NaCl (N=4). The levels of *Lv*SID1 mRNA in hepatopancreas (A) and gill (B) tissues of the treated shrimp were determined at 2, 3, 4 and 8 days post injection. The relative expression of *Lv*SID1 mRNA normalized by actin was quantified from the semi-quantitative RT-PCR results and plotted as mean ± SEM by using GraphPad Prism 5 program. The statistical analysis was performed by Krukal-Wallis test. * indicates significant difference of *Lv*SID1 level at *p*-value <0.05 between dsRNA injected group and NaCl control.

In *LvSID1* induced shrimp

To determine whether the *LvSID1* is involved in dsRNA cellular uptake, level of shrimp endogenous gene (STAT) suppression was monitored under the *LvSID1* expression being induced. In this experiment, the STAT was suppressed by two methods; by injection (for determining the role of *LvSID1* on cellular dsRNA uptake) and by feeding bacteria expressing dsRNA-STAT (for determining the role of *LvSID1* on systemic RNAi). By injection, the dsRNA-STAT was directly contact with the cells in every tissues after introduction. While, feeding, only RNAi signals (? intact molecule of long dsRNA-STAT or processed dsRNA-STAT) from epithelial cells of hepatopancreas was spread through haemolymph circulation to reach cells in the distant tissue (gill).

We hypothesize that the level of STAT suppression will be increased in *LvSID1* induced shrimp if *LvSID1* is essential for dsRNA uptake and systemic RNAi. According to the previous information that *LvSID1* expression was up-regulated when shrimp was treated with dsRNA, therefore we injected dsRNA-ns1 to induce *LvSID1* expression in this study. The 300 mg shrimp will be injected with 1.5 µg dsRNA-ns1 into

haemolymph then leave for 6 days. After that, STAT suppression was established by injection or feeding with dsRNA-STAT. Total RNA will be isolated from gill tissues and the STAT suppression was analyzed by RT-PCR. PCR product was analyzed by agarose gel electrophoresis. Relative level of STAT mRNA expression was compared between normal, non-induced and *LvSID1* induced shrimp.

Six days after injection of dsRNA-ns1, the dsRNA-ns1 up-regulated expression of the *Lv*SID1 mRNA but did not affect expression level of STAT mRNA (figure 10). Relative transcript of STAT was clearly reduced in all shrimp receiving dsRNA-STAT at 12 hours post injection (hpi) compared to the control. Interestingly at 24 hpi, the *Lv*SID1 induced shrimp showed level of STAT suppression (71%) more than that of the *Lv*SID1 non-induced shrimp with approximately 44% reduction significantly (*p*-value <0.05) (figure 11). Similar result was also observed when shrimp was fed with diet containing dsRNA-STAT. Shrimp with induced-*Lv*SID1 had higher level of STAT suppression when compared with the control shrimp with normal *Lv*SID1 (figure 12). These results indicate that the *Lv*SID1 participates in the uptake of the dsRNA and involves in systemic RNAi in shrimp.

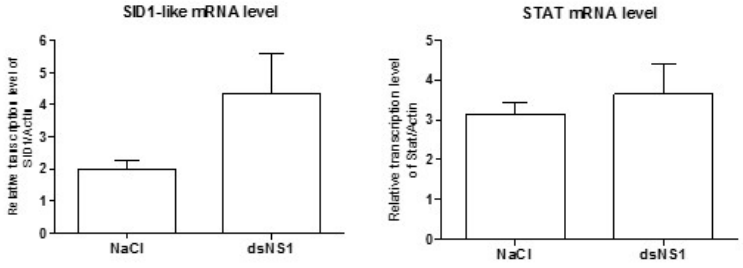


Figure 10 Relative transcriptional level of *Lv*SID1-like and STAT in gill tissue. Shrimp were pre-injected with NaCl and dsNS1. At 6 days post injection, gills were collected from each shrimp for determination of the relative *Lv*SID1 (left) and STAT (right) mRNA level before injection of dsSTAT.

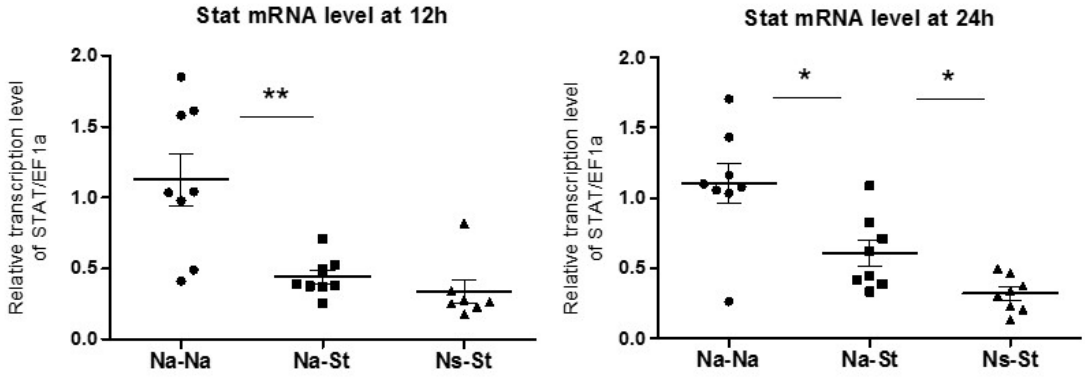


Figure 11 Effect of the *Lv*SID1 on uptake of injected dsSTAT. After the dsNS1 injection for 6 days: The dsSTAT was injected into the pre-treated shrimp. At 12 and 24 hours post dsSTAT injection, relative STAT mRNA expression of each shrimp was measured and plotted in the graph**.** Data is presented as mean ± SEM. The statistical analysis was performed by Mann Whitney t test (A), Krukal-Wallis test (B) at *p*-value less than 0.05 (*) and 0.01 (**). Na-Na = NaCl followed by NaCl, Na-St, Ns-St indicate NaCl, dsNS1 followed by dsSTAT, respectively.

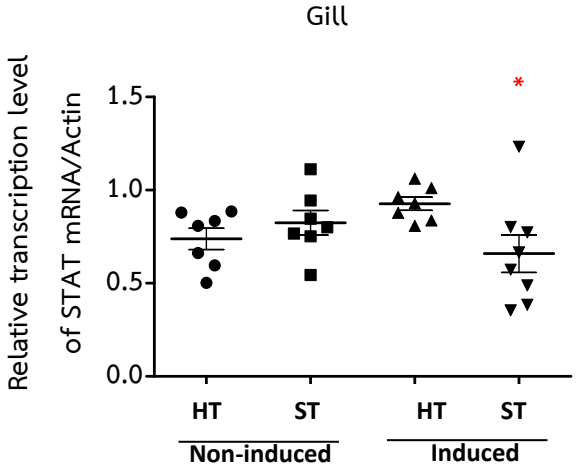


Figure 12 Effect of the *Lv*SID1 on uptake of fed dsSTAT in gills. After the dsNS1 injection for 6 days: shrimp were fed with the diet containing dsSTAT for 6 days. At 48 hours post last feeding, relative STAT mRNA expression in gills of each shrimp was measured and plotted in the graph**.** Data is presented as mean ± SEM. The statistical analysis was performed by Mann Whitney t test (A), Krukal-Wallis test (B) at *p*-value less than 0.05 (*). HT = diet containing HT115 without dsRNA, ST = diet containing dsSTAT, non-induced = shrimp injected with NaCl, Induced = shrimp injected with dsNS1 to induce the *Lv*SID1.

4.4 Investigation of the requirement of endocytosis for dsRNA uptake in shrimp

The level of STAT mRNA suppression induced by injection of dsSTAT was used as a marker to determine whether endocytosis is involved in the cellular uptake of dsRNA into shrimp gill and hepatopancreatic cells. Shrimp were pre-injected with drug inhibitors (Cpz and BafA) to block clathrin coated pit endocytosis before injection of dsSTAT. Thereafter, expression of STAT mRNA in the hepatopancreas and gill tissues of the treated shrimp was monitored and compared with the expression levels of control shrimp (without drug treatment). The relative transcript of STAT in the hepatopancreas of the untreated shrimp was significantly suppressed by approximately 40% from the control levels (STAT mRNA level of shrimp injected with drug-dissolving solvents (NaCl for Cpz or EtOH for BafA, respectively) (Figure 13). However, suppression level of shrimp pre-treated with two doses of Cpz or BafA were significantly reduced to approximately 10% from the control levels (STAT mRNA level of shrimp injected

with Cpz or BafA without dsSTAT). The results indicated that inhibition of clathrin-mediated endocytosis affected the dsSTAT uptake in shrimp hepatopancreas resulting in reduction of RNAi-mediated STAT suppression. The effect of the two endocytosis inhibitors on STAT suppression was also determined in gill tissue of the same shrimp. In contrast to hepatopancreas, the results showed levels of STAT suppression after blocking with two drugs (Cpz and BafA) were not significantly reduced (Figure 14), indicating that inhibition of clathrin-mediated endocytosis did not affect the uptake of dsSTAT in shrimp gill tissue.

Moreover, mechanism for uptake of dsRNA from environment (food) was also evaluated. Chlorpromazine, drug inhibitor, was used to block the clathrin-mediated endocytosis before continuous feeding the formulated food containing dsRNA specific to shrimp Rab7 gene for 6 days. Level of Rab7 mRNA suppression in gill and hepatopancreas tissues were monitored every day (12 hours after feeding of each day) by RT-PCR. Suppression of Rab7 mRNA was detected in gill and hepatopancreas tissues at day6 only in dsRNA-Rab7 fed shrimp without chlorpromazine treatment. In contrast, the Rab7 mRNA level was not changed in dsRNA-Rab7 fed shrimp treated with chlorpromazine (figure 15). The results indicate that the clathrin-mediated endocytosis participates in dsRNA uptake in shrimp by feeding.

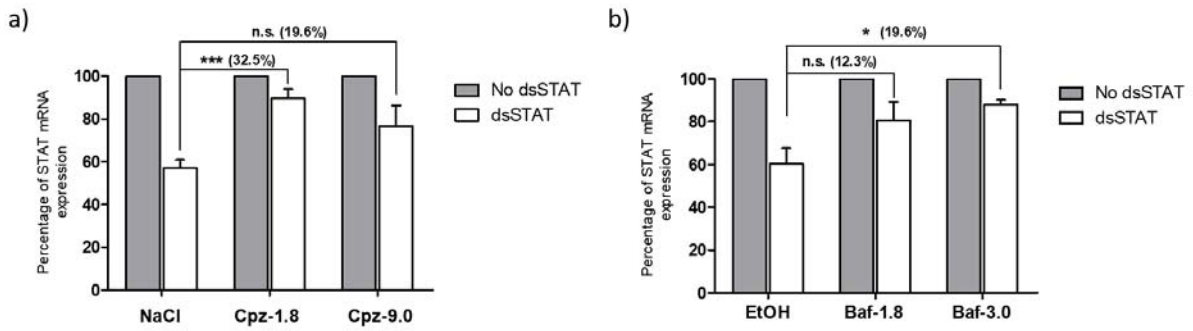


Figure 13. Effect of inhibitors (chlorpromazine (Cpz) and bafilomycin A1 (Baf)) on endocytic uptake of injected dsRNA into shrimp hepatopancreatic cells.

Shrimp (n=3-9/group) were pre-injected with the solvents [NaCl (Na) or EtOH (Et)] or drug inhibitors [(a) Cpz (at 1.8 or 9 µg/g of shrimp) or (b) Baf (at 1.8 or 3 µg/g of shrimp)] before injection of NaCl (no dsSTAT) or dsSTAT (St). Twelve hours later, relative expression of STAT mRNA in the hepatopancreas of shrimp in each group was monitored by RT-PCR and plotted as mean +/- SEM. The percentage of STAT mRNA expression of the drug treated and untreated shrimp are presented. The statistical analysis was performed using the Mann-Whitney test at *p*-value less than 0.05 (*) and less than 0.001 (***).

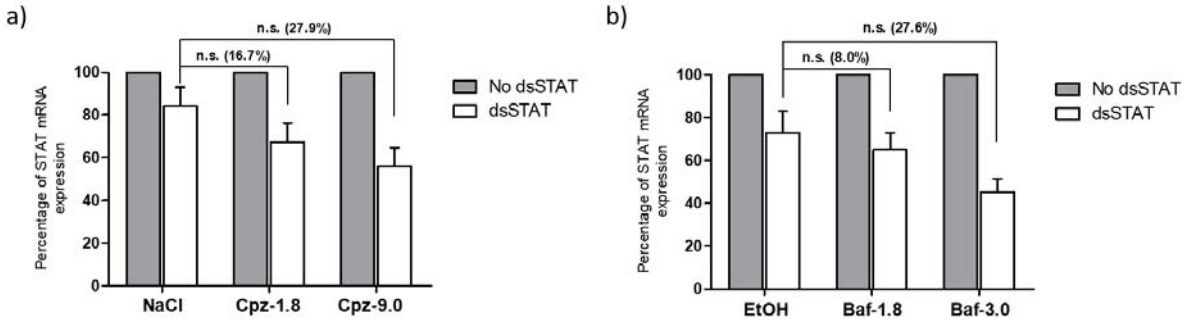


Figure 14. Effect of inhibitors (chlorpromazine (Cpz) and bafilomycin A1 (Baf)) on endocytic uptake of injected dsRNA into shrimp gill cells.

Shrimp (n=3-9/group) were pre-injected with the solvents [NaCl (Na) or EtOH (Et)] or drug inhibitors [(a) Cpz (at 1.8 or 9 µg/g of shrimp) or (b) Baf (at 1.8 or 3 µg/g of shrimp)] before injection of NaCl (no dsSTAT) or dsSTAT (St). Twelve hours later, relative expression of STAT mRNA in hepatopancreas of shrimp in each group was monitored by RT-PCR and plotted as mean +/- SEM. The percentage of STAT mRNA expression of the drug treated and untreated shrimp are presented. The statistical analysis was performed using the Mann-Whitney test.

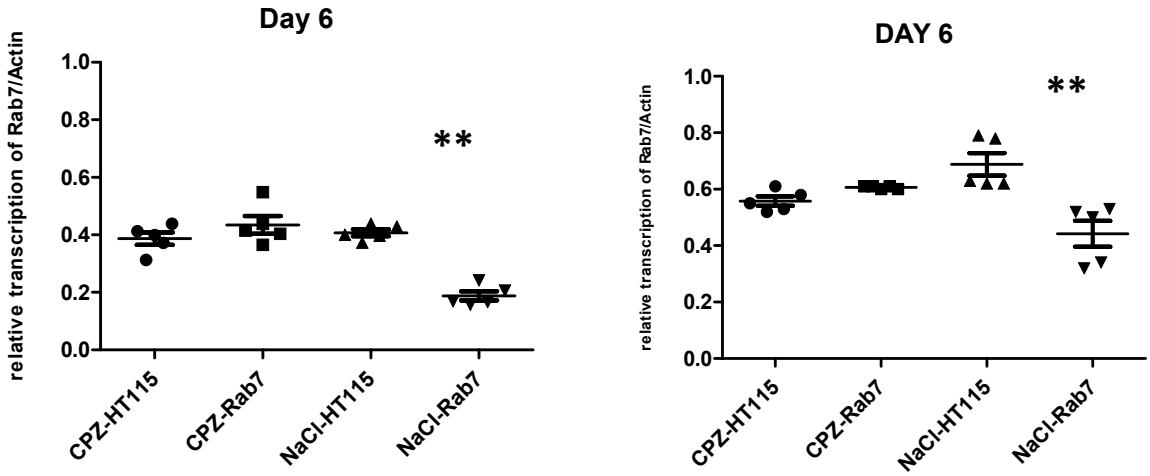


Figure 15 Determination of endocytic pathway in dsRNA uptake using chlorpromazine (CPZ). Shrimp were pre-treated with chlorpromazine for 12 hrs before continuous feeding with diet containing dsSTAT for 6 days. At 12 hours post feeding, relative Rab7 mRNA expression of each shrimp was measured and plotted in the graph (left: hepatopancreas, right: gills)**.** Data is presented as mean ± SEM. The statistical analysis was performed by Mann Whitney t test (A), Krukal-Wallis test (B) at *p*-value less than 0.05 (*). HT115 = diet containing HT115 without dsRNA, Rab7 = diet containing dsRab7, NaCl = shrimp injected with NaCl, CPZ = shrimp injected with chlorpromazine.

4. Conclusions

Expression of *Lv*SID1 mRNA was detected in three examined tissues (hepatopancreas, gill and muscle) of normal shrimp. The highest expression level of *Lv*SID1 mRNA was observed in hepatopancreas, which was approximately 3 and 45 times higher than that in gill and muscle, respectively.

The *Lv*SID1 mRNA expression was up-regulated in gill and muscle tissues but not in hepatopancreas when shrimp was injected with long dsRNAs. This induction response was also detected when shrimp was fed with diet containing dsRNA. Like the injection, induction of the *Lv*SID1 mRNA expression was not detected in hepatopancreas. These results suggest that the *Lv*SID1 mRNA expression was up-regulated in gill but not in hepatopancreas when shrimp receiving the long dsRNA introduced by injection or feeding.

The recombinant plasmid for producing dsRNA specific to the *Lv*SID1 mRNA was constructed in this study. However, we couldn't knock down the *Lv*SID1 mRNA expression effectively (only 40% suppression) by the injected dsRNA-SID1. This was the result of the induction effect of dsRNA on the *Lv*SID1 mRNA expression. Therefore, the role of *Lv*SID1 could not be evaluated by suppression of *Lv*SID1 approach. The involvement of the *Lv*SID1 on dsRNA uptake in shrimp was evaluated by over expression of the *Lv*SID1 instead.

The *Lv*SID1 induced shrimp showed level of STAT suppression more than that of the *Lv*SID1 non-induced shrimp. Similar result was observed when shrimp was pre-injected with dsRNA-GIH to induce expression of the *Lv*SID1 and fed with diet containing dsRNA-STAT. These results indicate that the *Lv*SID1 participates in the uptake of the injected dsRNA and involves in systemic RNAi in shrimp.

Inhibition of clathrin-mediated endocytosis pathway by chlorpromazine affected the dsRNA uptake by injection into hepatopancreas but not into gills. Moreover, suppression of Rab7 mRNA was significantly reduced in the dsRNA-Rab7 fed shrimp pre-treated with chlorpromazine. The results indicate that the clathrin-mediated endocytosis participates in dsRNA uptake in shrimp hepatopancreas but not gills by both feeding and injection.

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5. Outputs

5.1 International publication

1. Maruekawong, K., Tirasophon, W., Panyim, S., **Attasart, P**. 2018. Involvement of LvSID-1 in dsRNA uptake in Litopenaeus vannamei. Aquaculture 482: 65-72. (Impact factor = 2.710)

2. Maruekawong, K., Panyim, S., **Attasart, P**. 2018. Endocytosis participates in cellular uptake of injected dsRNA into hepatopancreas but not gill of *Litopenaeus vannamei*. Aquaculture (under review)

3. Maruekawong, K., Panyim, S., **Attasart, P**. 2018. Environmental RNAi requires both clathrin-mediated endocytosis and SID-1 for dsRNA uptake in *Litopenaeus vannamei* (manuscript in preparation)

5.2 Poster

1. Maruekawong, K., **Attasart, P**., Tirasophon, W. 2014. Silencing of sid1-like gene by specific double-stranded RNA in *Litopenaeus vannamei*. TSB International Forum (Green Bioprocess Engineering), Bangkok International Trade and Exhibition Centre (BITEC), Thailand (September 17-18).

5.3 Student

1. Miss Kamonwan Maruekawong (M.Sc. 2015)