รายงานวิจัยฉบับสมบูรณ์

โครงการ การโคลนและการแสดงออกของยีนของตัวตอบรับสำหรับโปรตีนสารพิษชนิด Cry4 จากเซลย์บุกระเพาะลูกน้ำยุง *Aedes aegypti*

(Cloning and expression of a receptor gene for *Bacillus thuringiensis* Cry4 toxins from *Aedes aegypti* larval gut epithelial cells)

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สัญญาเลขที่ RSA4680005

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ผู้วิจัย

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สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย

Abstract

Project Code: RSA4680005

Project Title: Cloning and expression of a receptor gene for *Bacillus thuringiensis* Cry4 toxins from *Aedes aegypti* larval gut epithelial cells

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Project Period: 1 June 2003 – 31 May 2006. (with extension to 30 May 2007)

Objectives: To determine if the putative Cry toxin receptor, aminopeptidase N (APN), is indeed function as the receptor in the *Aedes aegypti* larvae. **Methods:** We originally proposed to use the toxin-overlay method to answer the above question. However, we have developed the RNAi technique that can be easily performed in the *A. aegypti* larvae. **Results:** Ingestion (via soaking) of *A. aegypti* larvae with gene-specific dsRNA resulted in targeted suppression. The 2-day old larvae were soaked in aminopeptidase N (APN) dsRNA at various concentrations (0.05, 0.1 and 0.2 μg/μl). It was found that at all concentration there were reduced APN transcripts. The highest suppression (~100%) was found at high concentration used (0.2 and 0.1 μg/μl); at lower concentration (0.05 μg/μl) it showed ~ 57% reduction. This knockdown effect is sequence-specific since ingestion of dsGFP (green fluorescent protein) did not reduce APN transcripts. Moreover, the silencing effect was still effective after 36 hours after the initial ingestion. However, toxicity test suggested that the APN isoform tested may not be the functional receptor to Cry4B toxin. **Conclusion:** A simple and effective method of soaking the mosquito larvae in the solution of dsRNA can strongly and specifically silenced the target gene. In so doing, we have successfully knocked down the larval APN gene as soon as 12 hours. Even though, the receptor to Cry4B toxin was not found; we have developed the method that can be employed in high-throughput reverse genetic screening in the mosquito larvae. **Suggestions:** Since multiple proteins (receptors) are found to be able to bind to Cry toxins, at least, *in vitro*, it is possible that for the toxin to be effective it needs to employ that many receptors. Therefore, simply targeting a single receptor may not have any effect – multiple knockdown of such proteins may be needed to reduce the toxicity of Cry toxin.

Keywords: *Aedes aegypti*, *Bacillus thuringiensis*, Aminopeptidase N, Cry toxin, RNAi.

บทคัดย่อ

รหัสโครงการ: RSA4680005

ชื่อโครงการ: การโคลนและการแสดงออกของยีนของตัวตอบรับสำหรับโปรตีนสารพิษชนิด Cry4 จาก
เซลล์บุกระเพาะลูกน้ำยุง *Aedes aegypti*

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วัตถุประสงค์:เพื่อตรวจสอบว่าอะมิโนเปบทิเดสเอ็น (aminopeptidase N, APN) ทำหน้าที่เป็นตัวตอบรับต่อสารพิษ Cry ในลูกน้ำยุงลายหรือไม่ **วิธีการทดลอง:**วิธีการ toxin-overlay ถูกเลือกที่จะนำมาใช้ตอบคำถามข้างต้น อย่างไรก็ตามเทคนิค RNAi ซึ่งทางเราพัฒนาขึ้นสามารถตรวจสอบจุดประสงค์ดังกล่าวในลูกน้ำยุงลายได้ง่าย เทคนิคดังกล่าวจึงถูกนำมาใช้ **ผลการทดลอง:**ลูกน้ำยุงลายที่ได้รับ RNA สายคู่ (dsRNA) ซึ่งจำเพาะต่อยีนเป้าหมายเข้าสู่ร่างกายทางปากโดยการแช่กับสารละลาย dsRNA พบว่ามีการยับยั้งการแสดงออกของยีนเป้าหมาย โดยลูกน้ำยุงลายอายุ 2 วันถูกแช่ในสารละลาย dsRNA ของยีนอะมิโนเปบทิเดส เอ็น (APN) ที่ความเข้มข้นต่างๆ (0.05 - 0.2 μg/μl) ผลการทดลองพบว่า ที่ทุกความเข้มข้นของ dsRNA มีการลดลงของการแสดงออกของยีน APN การยังยั้งการแสดงออกของยีนสูงที่สุด (~100%) ที่การใช้ dsRNA ความเข้นข้นสูง (0.2 และ 0.1 μg/μl) โดยที่ dsRNA ความเข้นข้นต่ำที่สุดที่ใช้ (0.05 μg/μl)พบการยับยั้งประมาณ 57% การยับยั้งการแสดงออกของยีนเป็นแบบจำเพาะต่อลำดับเบส เนื่องจากการได้รับ dsRNA ของยีน โปรตีนเรืองแสง (green fluorescent protein) ไม่มีผลต่อการลดลงของยีน APN นอกจากนั้นการยับยั้งยังคงมีประสิทธิผลภายหลัง 36 ชั่วโมงนับจากการได้รับ dsRNA เริ่มต้น อย่างไรก็ตาม ผลการทดสอบความเป็นพิษพบว่า APN อาจไม่ใช่ตัวตอบรับต่อสารพิษ Cry4 **สรุปและวิจารณ์ผลการทดลอง:**การแช่ลูกน้ำยุงลายในสารละลาย dsRNA ซึ่งเป็นวิธีที่ง่ายและมีประสิทธิภาพสามารถยับยั้งการแสดงออกของยีนเป้าหมายได้อย่างชัดเจนและมีความจำ เพาะเราประสบความสำเร็จในการยับยั้งการแสดงออกของยีน APN ในลูกน้ำได้ตั้งแต่ชั่วโมงที่12 โดยถึงแม้ว่าจะไม่สามารถหาตัวตอบรับต่อสารพิษ Cry4B **ข้อเสนอแนะสำหรับงานวิจัยในอนาคต:** เนื่องจากมีการพบว่าโปรตีนตัวตอบรับหลายชนิดสามารถจับกับสารพิษ Cry ได้ อย่างน้อยในสภาพแวดล้อมที่ทำเทียมขึ้น (*in vitro*) เป็นไปได้ว่าสารพิษต้องการตัวตอบรับหลายชนิดสำหรับการทำงานอย่างมีประสิทธิภาพ ดังนั้นการค้นหาโปรตีนตัวตอบรับเพียงตัวเดียว จึงไม่มีผลใดๆ การยับยั้งการแสดงออกของยีนของโปรตีนหลายชนิดดังกล่าวอาจจำเป็นสำหรับการลดความเป็นพิษของสารพิษ Cry

คำหลัก: *Aedes aegypti*, *Bacillus thuringiensis*, Aminopeptidase N, Cry toxin, RNAi.

เนื้อหาสรุปของงานวิจัย

Introduction

Mosquito-borne diseases are still prevalent in the tropics and especially in Thailand. Some of the diseases that are carried by mosquitoes are malaria and dengue fever. Millions of lives, worldwide, are lost each year due to these diseases. Thus, effective methods for mosquito control are needed. Synthetic insecticides have long been used to control the spread of mosquitoes. The best known among them is dichloro-diphenyl-trichloroethane or DDT. It is the most widely used chemical insecticide to fight mosquitoes. However, DDT-resistant strains of mosquitoes have arises (Suarez et al., 1990). Furthermore, due to its well- documented environmental hazards, its use is of limited in the future. Thus, alternative forms of insecticides that are both effective and environmental friendly are of great interest. One of these is the *Bacillus thuringiensis* (*Bt*) β-endotoxins family. They are the most widely used bioinsecticide against mosquitoes and other insects (Lambert and Peferoen, 1992). This is because *Bt* toxins are highly effective; in addition, they have high target-specificity, that is, they do not killed insects indiscriminately unlike synthetic insecticide (reviewed in Siegel, 2001).

Bt β-endotoxins are formed as crystalline proteins or Cry toxins inclusions during the bacteria sporulation cycle. Upon ingestion of the toxins by the insect larvae, Cry proteins undergo solubilization and subsequent activation through proteolytic processing in the gut of the insect larvae. Activated Cry proteins then bind to their specific receptors present on the apical brush border of gut cells, causing destructions of these gut epithelial cells through cell-lysis; thus, killing the insect larvae (reviewed in Schnepf *et al*., 1998; de Maagd *et al*., 2001), terminating the transmission cycle.

Thus far, at least 143 *Bt* β-endotoxins have been identified from various subspecies of *Bt* (reviewed in Crickmore *et al*., 1998, 2002). Furthermore, some of these β-endotoxins have been found to be effective against nematodes, mites and protozoa (Feitelson, 1993; Feitelson *et al*., 1992), raising the stakes in understanding the mechanisms of *Bt* β-endotoxins and their receptors even more. However, so far, only two receptors have been positively identified only in lepidopteran insects, a cell-surface enzyme, aminopeptidase N (APN) (*Manduca sexta*, Knight *et al*., 1995; *Heliothis virescens*, Gill *et al*., 1995; *Bombyx mori*, Yaoi *et al*., 1999), and a cadherin-like membrane protein (Vadlamudi *et al*., 1993).

Of particular interest here is the fact that, to date, only scant information on the nature of the receptor for any mosquito species is available, considering that this dipteran insect is a major disease carrier world-wide. For instance, using toxin overlay assay, Cry11Ba toxin was found to be able to bind to APN from the *Anopheles quadrimaculatus* mosquito larvae (Abdullah *et al*., 2006), and Cry4Ba can bind to an *Anopheles gambiae* CadLP (Hua *et al*., 2008). However, none of these studies have clearly demonstrated *in vivo* that these putative receptors indeed serve as Cry toxin receptor in the mosquito larvae.

Since *Aedes agypti* mosquito belongs to the diptera order, limited information is available on its toxins-receptor interactions. Nonetheless, it was demonstrated that β-endotoxins from the *isralensis* subspecie are effective against dipteran insects (van Frankenhuuyzen, 1993). So far, five endotoxins from this *Bt* subspecie have been identified and cloned. They are: Cry 4A (Ward and Ellar, 1988), Cry 4B (Angsuthanasombat *et al*., 1987), Cry 10A (Thorne *et al*., 1986), Cry 11A (Donovan *et al*., 1988) and Cyt 1A (Waalwijk *et al*., 1985).

Besides the identification and characterization of Cry toxins and their receptors, other studies have investigated the molecular nature of Cry toxins and receptor interactions. For example, the mechanism of ion channel formation of the toxin receptors complex (Sangadala *et al*., 1994; Schwartz *et al*., 1997; Carroll *et al*., 1997), the mechanism of intrinsic insect specificity of different Cry toxins (de Maagd *et al*., 1999; Rajamohan *et al*., 1995) and the mechanism of insect resistance to these toxins (Ferre *et al*., 1991; MacIntosh *et al*., 1991).

Thus, a large gap in further understanding this basic mechanism of toxin-receptor interaction in the mosquito larva is missing. Therefore, it is of critical important to be able to positively identify the receptor for, at the least, Cry4Ba toxins; and it is imperative that this basic question needs to be answered as the information obtained can lead to further improvement in the eradication of mosquitoes. For instance, by understanding the nature of *Bt* toxins and their receptors, we can gain valuable insights; and potentially, may improve the lethality of the toxins themselves. Thus, the overall goal of this study is to determine if APN, a candidate membrane-bound protein present in *Aedes aegypti* mosquito larval gut cells, act as a fortuitous receptor for *Bti* Cry4Ba endotoxin. This is because in our previous study (Pootanakit *et al*., 2003), we have identified and cloned two APN isoforms from this mosquito, thus our focus in this study is on APN.

Our original proposal was to identify the putative receptor to Cry4B toxin via the standard toxin-overlay assay. However, since then we have developed the technique of RNA interference (RNAi) which can be easily performed in *A. aegypti* larvae. Therefore, tt seems a better strategy to use RNAi technique to determine if APN also served as the receptor to Cry4B toxin.

In the mosquitoes, RNAi has been used to knock down gene expression in the adults (Blandin *et al*., 2002; Attardo *et al*., 2003), cell lines (Levashina *et al*., 2001; Hoa *et al*., 2003), and more recently in the larvae (Blitzer *et al*., 2005). In the larval and adult mosquitoes, the dsRNAs, in so far studies, were introduced via microinjection which is both time consuming and tedious. But, in our laboratory, we have developed a quicker and less tedious method. Specifically, we have found that those larvae that were soaked in dsAPN RNA resulted in reduction of APN gene expression. However, when tested for Cry4B toxicity test, the mortality of the larvae was not decreased. This suggested that APN (at least the isoform we tested) is not important for Cry4Ba toxicity.

Materials and Methods

Plasmid construction for APN dsRNA synthesis

A schematic diagram showing the construction of pET17b-hairpin APN to generate the APN dsRNA is shown in figure 1. In order to do this, two complementary APN fragments of approximately 680 bp and 1,000 bp were subcloned into a pET17b vector (Invitrogen, USA) in an inverted head-to-head orientation (this will produce hairpin APN RNA when transcribed and will produce dsAPN RNA when digested with RNase A). Specifically, two PCR reactions were needed. The first PCR was performed per the manufacturer's recommendations in a 50 µl reaction mixtures consisting of 100 ng of the obtained APN template (Pootanakit *et al*., 2003), 1 µM of APN primers having *Nco*I and *Xba*I sites (apnF2: 5'-aaaccatggcggccgtttggtcggg-3' and apnR995: 5'-aaatctagacgtgggcaatgattgaagccg-3'), 200 µM dNTPs and 0.5 units of DyNAzyme II DNA polymerase (Finnzyme, Finland). PCR conditions were as followed: (1) 94°C, 2 min – hot start; (2) 94°C, 30 sec; (3) 50°C, 30 sec; (4) 72°C, 1 min. Steps 2 – 4 were repeated for 35 cycles with an additional final extension time of 10 min. The obtained APN PCR product of ~1,000 bp was gel-purified, digested with *Nco*I and *Xba*I and then ligated to *Nco*I- and *Xba*I-digested pLITMUS28i vector (Promega, USA) using T4 DNA ligase (Promega). Next, a second PCR was performed to obtain the 680 bp APN amplicon; the PCR condition was as above except that the primers employed contained *Pst*I and *Xho*I sites: stem680APN_f (5'-aaactgcaggtcacctac gagttcctgg-3') and stem680APN-b (5'-aaactcgagcgtgggcaatgattgaagccg-3'). Then, both the 680 bp APN amplicon and the pLITMUS28i-1000APN vector were double-digested with *Pst*I and *Xho*I, and then ligated. Finally, this recombinant plasmid was digested with *Xba*I and *Xho*I to give a construction cassette of ~1.7 kb which contained both the 680 and the 1,000 bp APN fragments; it was then subcloned into a pET17b vector.

hairpin APN and dsAPN RNAs production

For APN hairpin RNA production, the recombinant pET17b vector was first transformed into an RNase III-deficient E. coli HT115 (DE3) strain using the standard molecular biology protocol (Sambrook *et al*., 1989). After the IPTG induction, the bacteria cells were centrifuged at 8,000x *g*, at 4° C, for 5 min and was lysed by resuspending in 1 ml of 0.1% SDS and then incubated in boiling water for 2 min. Next, 50 µg of RNase A (10 mg/ml) was added to the lysed cells and was further incubated at 37º C, for 30 min to eliminate single-stranded RNAs and also to convert the hairpin APN RNA into dsAPN RNA. The dsAPN RNA was extracted by Tri-Reagent (Molecular Research Center, USA). Finally, the RNA pellet was dissolved in soaking buffer [10.9 mM Na_2HPO_4, 5.5 mM KH_2PO_4, 2.1 mM NaCl, 4.7 mM NH_4Cl, 3 mM spermidine and 0.05% gelatin] (Maeda *et al*., 2001).

Detection of dsRNA

RNase digestion assay was used to detect the amount of dsRNA expressed from the *E. coli*. After dsRNA induction to produce, the *E. coli* cells were collected by centrifugation at 8,000x *g* at 4°C. Total RNA extraction was performed by Tri-Reagent (Molecular Research Center, USA) as described by the manufacturer. After obtaining total RNA from the *E. coli* cells, 20 µg of total RNA was treated with 0.1 µg RNaseA in reaction buffer [0.3 M NaCl, 0.030 M sodium citrate] and incubated at 37 °C for 10 min to eliminate single-stranded RNA. Gel electrophoresis was performed to check the dsRNA. To confirm duplex structure of dsRNA, RNase III (New England BioLabs, USA) digestion was also performed. After obtaining the total RNA from *E. coli* cells, 20 µg of total RNA was treated with 1.3 unit of RNase III in reaction buffer [50 mM NaCl, 10 mM $MgCl_2$, 1mM dithiothreitol] and incubated at 37 °C for 30 min to eliminate double stranded RNA. Then gel electrophoresis was performed.

Rearing of A. aegypti mosquito larvae

A. aegypti eggs were obtained from the mosquito rearing facility, Institute of Molecular Biology and Genetics, Mahidol University. They were hatched by placing a paper sheet containing *A. aegypti* eggs in a chamber filled with tap water. The paper was removed after 2 hours. The mosquito larvae were reared and fed with fish diet pellet at 30 ºC for 2 days.

Ingestion of dsAPN RNA

Single colony of HT115(DE3) strain containing pET17b-hairpin APN RNA expressing a 700 bp hairpin APN was grown for 16-18 hr in 2xYT medium with 100 µg/ml amplicillin at 37°C and 12.5 µg/ml tetracycline. The culture was diluted 100-fold in 2xYT medium and allowed to grow until OD_{600} = 0.4-0.6. Then the expression of the hairpin APN RNA was induced by the addition of IPTG to a final concentration of 0.4 mM, and the culture was incubated with shaking for 4 hr at 37 °C. 50×10^8 cells of the *E.coli* expressing hairpin APN RNA were collected by centrifugation at 8,000x *g* 4°C for 5 min. The *E. coli* pellet was resuspended in 1 ml of 0.1% SDS and then incubated in boiling water for 2 min. The lysed *E.coli* cells were treated with 50 µg of RNaseA and incubated for 30 min at 37 °C to eliminate single stranded RNA. After digestion with RNaseA, the dsAPN RNA was extracted by Tri-Reagent (Molecular Research Center, USA) as described by the manufacturer. In the final step of the RNA extraction, the dried RNA pellet was dissolved in soaking buffer and stored at -80ºC or used immediately. The amount of the APN dsRNA was analyzed by 1.0% agarose gel electrophoresis. Twelve 2-day old *A. aegypti* larvae were incubated in 350 µl of the diluted dsAPN RNA at various concentrations (0.2, 0.1 and 0.05 µg/µl) and durations (12, 24 and 36 hr) at room temperature in microcentrifuge tubes. After the incubation, the dsAPN RNA was discarded and the larvae were washed twice with distilled water.

A. aegypti larval midgut total RNA isolation

To excise the midgut from the larvae, mosquito larvae were incubated in ice-cold distilled water. The gut was dissected from the larvae under dissecting microscope (Zeies, Germany) using fine-tip forceps by pulling the head and tail ends of the larvae apart. The food sac inside the gut and the foregut and hind gut were also removed. The larval midguts were kept in Tri-Reagent (Molecular Research Center, USA) before RNA extraction. After soaking with dsAPN RNA, the isolated midgets were soaked in Tri-Reagent (Molecular Research Center, USA) and homogenized by glass-rod homogenizer for 40 strokes. The RNA extraction method was as described by the manufacturer. Briefly, after homogenization, the homogenate was incubated at room temperature for 5 min, then 0.2 volumes of chloroform was added, followed by vigorously shaking for 15 sec and incubation at room temperature for 15 min. The mixture was centrifuged at 13,000x *g*, 4 ºC for 15 min. Then the aqueous phase was transferred to a new tube. To precipitate RNA, 0.5 volume of isopropanol was added, followed by incubation at room temperature for 10 min. The precipitated

RNA was collected by centrifugation at 13,000x *g*, 4 ºC for 15 min. The RNA pellet was washed with 1 volume of 75% ethanol. Then it was centrifuged at 13,000x *g*, 4 ºC for 10 min and air dried (10 min). The dried RNA pellet was dissolved in DEPC-treated water and stored at -80 ºC or used immediately.

Semi-quantitative multiplex RT-PCR

To monitor APN mRNA level in *A. aegypti* larvae, semi-quantitative RT-PCR was performed. The ribosomal protein S7 gene was used as the endogenous control. To eliminate the contaminated DNA in the total extracted RNA sample, the sample was treated with DNase I. The reaction contained total extracted RNA, 1 units of RNase-free DNase (Fermentus, Lithuania), 1X DNase reaction buffer [10 mM Tris-HCl, 2.5 mM $MgCl_2$, 0.1 mM $CaCl_2$] and DEPC-treated water. The mixture was incubated at 37 ºC for 40 min. To stop the reaction, 1 µl of 25 mM EDTA was added and the mixture was incubated at 65 ºC for 10 min. The cDNAs of total mRNAs were synthesized in the mixture containing 1 µg of total RNA with 2 µg of random hexamer and RNase-free sterile distilled water. The reaction was incubated at 70 ºC for 5 min and the solution was quickly cooled on ice. The following components: 1X RevertAid H-Minus MMLV reverse transcriptase reaction buffer, 1.0 mM dNTPs, and RNase-free sterile distilled water were added into the previous mixture and incubated at 25 ºC for 5 min. Then 200 units of RevertAid H-Minus MMLV reverse transcriptase was added into the reaction mixture. The solution was gently mixed and the cDNA synthesis was incubated at 25ºC for 10 min, then 42ºC for 60 min. The reaction was stopped by incubation at 70 ºC for 15 min.

Next, multiplex PCR was performed to monitor the APN mRNA level in *A. aegypti* larvae. The ribosomal protein S7 gene (RpS7) was used as the endogenous control. APN and RpS7 (internal control) gene were amplified by ApnF/apnR680 and RpS7f/RpS7r primers respectively. The reaction was performed by using 4 µl of the cDNA products. The components of PCR reaction were 0.4 µM of each primer, 200 µM of dNTPs mix, 1X PCR buffer (50 mM Tris-HCl pH 9.0, 1.5 mM $MgCl_2$, 15 mM $(NH_4)_2SO_4$ and 0.1% Triton X-100), 0.5 units of DyNAzyme II and sterile distilled water. The temperature profile for PCR amplification was performed by holding at 94ºC for 2 min, denaturation at 94ºC for 30 sec, annealing at 53ºC for 30 sec, and extension at 72ºC for 1 min. The target DNA was amplified for 25 cycles and subsequently held at 72ºC for 10 min. The PCR product was analyzed by agarose gel electrophoresis.

Mosquito larvicidal assay

After soaking the *A. aegypti* larvae in dsAPN RNA in a 1.5 ml microcentrifuge tube for 12, 24 or 36 hr, 1×10^8 *E. coli* JM 109 cells expressing Cry4Ba protoxin were fed to the larvae to a final volume of 1 ml/tube. The percent mortality was recorded after 24-hr incubation at room temperature. Three independent experiments were performed and a total of 100 dsAPN RNA-soaked larvae were used in each experiment.

Results

Production and analysis of dsAPN RNA

To produce dsAPN RNA, *E. coli* strain HT115 (DE3) was chosen because this strain is deficient in RNase III, an enzyme that normally degrades a majority of dsRNAs in the bacterial cell. The *E. coli* HT115 strain was transformed with pET17b-hairpin APN and was then induced by IPTG to express hairpin APN. After total RNA extraction from the *E. coli* cells and treatment with RNase A, a band of approximately 750 bp which showed resistant to the degradation by RNaseA was found (Figure 2, lane 1). In addition, other discrete bands of RNA were also observed. For undigested RNA, beside the 750 bp band, additional band of RNA which was approximately 1,300 bp was also found. This larger band might be the intact hairpin structure which contained 679 bp stem plus a 370 bp of loop structure. The structure of stem was also confirmed by the addition of RNase III into the total RNA. It was found that both bands of approximately 750 bp and 1,300 bp disappeared (Figure 2).

Silencing of APN gene by soaking the larvae in dsAPN RNA

Semi-quantitative multiplex PCR showed that soaking of *A. aegypti* larvae in dsAPN RNA resulted in an almost complete decrease (~100 % reduction as determined by the Scion Image version 4.0.2) of APN transcript at all time points (12, 24 and 36 hr) and concentrations (0.2, 0.1 and 0.05 µg/µl) (Figure 3a), except for those larvae incubated at the lowest concentration (0.05 µg/µl) for only 12 h (~57 % reduction). To test whether this knockdown was specific, the larvae were also soaked in the double-stranded green fluorescent protein (dsGFP) RNA of ~ 450 bp (from Dr. Witoon Tirasophon, Mahidol University). After soaking in dsGFP RNA, it was found that APN gene expression was not inhibited at all concentrations tested (1.0 and 0.1 µg/µl) (Figure 3b). This suggested that the silencing of APN gene in *A. aegypti* larvae is sequence-specific and is both dose- and time-dependent.

Mosquito-larvicidal assay

As almost 100% decrease in APN transcripts was achieved by soaking the *A. aegypti* larvae in dsAPN RNA, the role of APN as receptor to Cry4Ba toxin can then be tested. Specifically, the larvae were soaked in soaking buffer containing 0.1 mg/ml dsAPN RNA solution for 24 or 36 hours. Then, they were fed with *E. coli* expressing Cry4Ba protoxin. It was found that for the 24 hours-soaked larvae, the mortality of larvae in dsAPN RNA slightly increase when compared to the control (77% vs. 84%; figure 4). For the 36 hours-soaked larvae, there was no different between the larvae in dsAPN RNA and those in soaking buffer alone (around 75% mortality; figure 4).

Discussion

To evaluate the functional role of APN in insecticidal activity of Cry4Ba toxin RNAi technique was employed to specifically inhibit the APN expression *in vivo* by soaking the larvae in dsAPN RNA solution. It was found that the APN transcripts can be suppressed when the larvae were incubated in the dsAPN RNA solution. In *C. elegans*, dsRNA uptake is mediated by transmembrane protein, SID-1 which has been shown to function as channel for dsRNA (Winston *et al*., 2002; Feinberg and Hunter, 2003). The homologs of sid-1 with capacity to uptake dsRNA have also been reported from human (Duxbury *et al*., 2005) and grasshopper (Dong and Friedrich., 2005). Sequence analysis by BLAST search against the genome of *A. aegypti* by using *C. elegans* (accession number NM 071971), humans (accession number NM_017699) and grasshopper (accession number AY879097) sid-1 coding DNA sequences as queries suggested that *A. aegypti* lacks sid-1 homologue. This implied that the dsRNA might not be uptake by SID-1 protein in *A. aegypti* larvae or they might be taken up by a different mechanism.

A report in *S. litura* larvae showed that silencing of APN gene lead to reduction in toxicity to Cry1C, providing an evidence for the role of APN as a *Bt* toxin receptor in the midgut of insects (Rajagopal *et al*., 2002). Here, toxicity assay was performed in *A. aegypti* larvae to determine the functional role of APN as receptor to Cry4Ba toxin after the APN transcripts were suppressed by soaking in dsAPN RNA. Surprisingly, the mortality at 24 hours for dsAPN RNA soaked larvae was higher than the larvae in soaking buffer alone after feeding with Cry4Ba toxin. The increase mortality may be because the larvae were previously stressed the first time from high osmolarity generated from dsRNA.

After soaking the larvae with dsAPN RNA for 36 hours followed by toxicity test, the mortality of larvae was not much different. This may be because when incubated in dsRNA for a longer time, the osmotic pressure within the larvae and dsRNA solution is in equilibrium.

Since, the reduction of mortality was not observed by toxicity assay after soaking in dsAPN RNA. This suggested that this APN isoform is not important for Cry4Ba toxicity. It is possible that this APN isoform may not be the receptor to Cry4Ba toxin or there are more than one receptor to Cry4Ba toxin might be present in the larval midgut. Besides APN protein, several studies in other insects reported that cadherin-like protein (Vadlamudi *et al*., 1993) and, more recently, alkaline phosphatase (Fernandez *et al*., 2006) served as the receptor to Cry toxins. In *A. aegypti* larvae, the alkaline phosphatase was shown to present in midgut cells and was reported to serve as receptor to Cry11Aa toxin (Fernandez et al., 2006). In addition, the result in our laboratory suggested that cadherin-like protein transcript is also present in the *A*. *aegypti* larvae midgut. These two candidate proteins might also act as receptor to Cry4Ba toxin in the larvae. Another reason may be because the APN protein may not yet have been completely turnovered after 24 or 36 hr of soaking in dsAPN RNA.

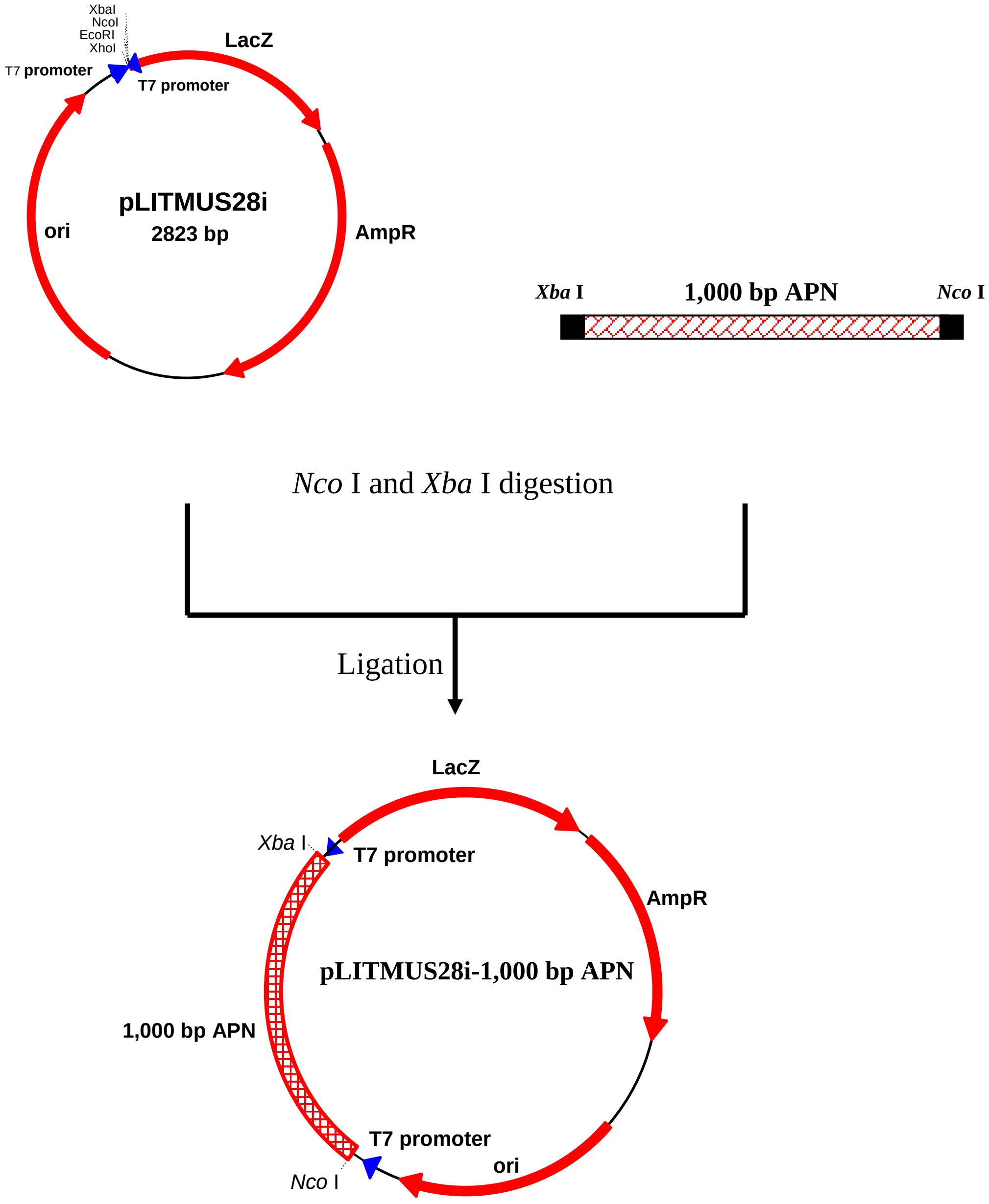
Because there are at least two APN isoforms in *A. aegypti* larvae (Pootanakit et al., 2003), other isoforms may also serve as receptor to Cry4Ba toxin. Although, to trigger RNAi effect, only 21-25 nucleotides precursor was required to activate the degradation of their homologous mRNA (Elbashir et al., 2001); however, from the alignment of these two APN isoforms, there was not a region that showed identical 21-25 nucleotides between these two APN isoforms. Therefore, the introduction of one APN isoform might not knockdown the other.

In conclusion, we have shown here that using a simple method of soaking the *A. aegypti* larvae in the solution of dsRNA can indeed strongly and specifically silenced the target gene. In so doing, we have successfully knocked down the larval APN gene transcript as soon as 12 hours. Although, we were unable to determine if APN is a functional Cry4Ba receptor in the *A*. *aegypti* larvae, this simple method will, nonetheless, enable experiments in high-throughput reverse genetic screening in the mosquito larvae.

Figure 1

Schematic diagram showing the construction of pET17b-hairpin APN.

Two complementary APN fragments of approximately 680 bp and 1,000 bp were subcloned into a pET17b vector in an inverted head-to-head orientation.


XbaI
NcoI
EcoRI
XhoI
T7 promoter
T7 promoter
LacZ
pLITMUS28i
2823 bp
ori
AmpR
Xba I
1,000 bp APN
Nco I
Nco I and Xba I digestion
Ligation
LacZ
Xba I
T7 promoter
AmpR
pLITMUS28i-1,000 bp APN
1,000 bp APN
T7 promoter
ori
Nco I

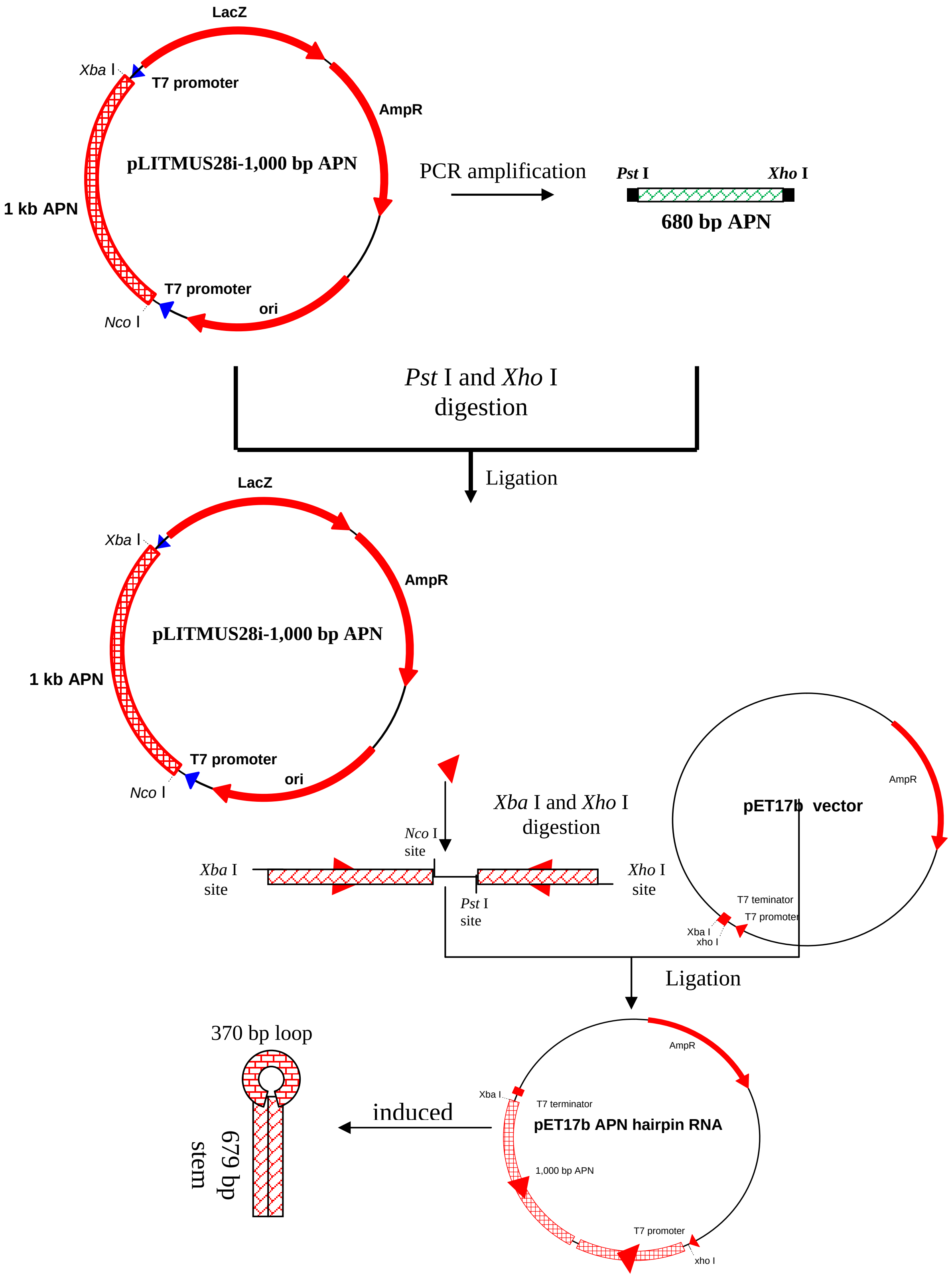

LacZ
Xba I
T7 promoter
AmpR
pLITMUS28i-1,000 bp APN
1 kb APN
T7 promoter
ori
Nco I
PCR amplification
Pst I
Xho I
680 bp APN
Pst I and Xho I
digestion
Ligation
LacZ
Xba I
AmpR
pLITMUS28i-1,000 bp APN
1 kb APN
T7 promoter
ori
Nco I
Xba I and Xho I
digestion
Nco I
site
Xba I
site
Pst I
site
Xho I
site
pET17b vector
AmpR
T7 teminator
T7 promoter
Xba I
xho I
Ligation
AmpR
Xba I
T7 terminator
pET17b APN hairpin RNA
1,000 bp APN
T7 promoter
xho I
induced
370 bp loop
679 bp
stem

Figure 2

Production and analysis of dsAPN RNA.

Total RNA extracted from *E. coli* expressing hairpin APN RNA showed two major bands of approximately 1,300 bp and 750 bp (lane 1). Upon RNase A treatment (lane 3), only the 750 bp band is still present, suggesting that this band is in dsRNA form. And, as expected, RNase III treatment completely degraded the two bands (lane 2). Lane M1 is *Hin*d III DNA marker. Lane M2 is 100 bp DNA ladder.

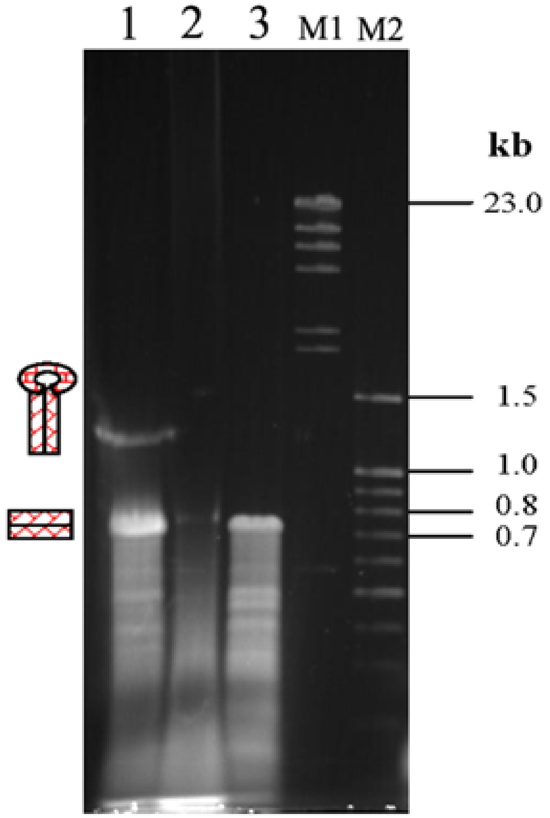
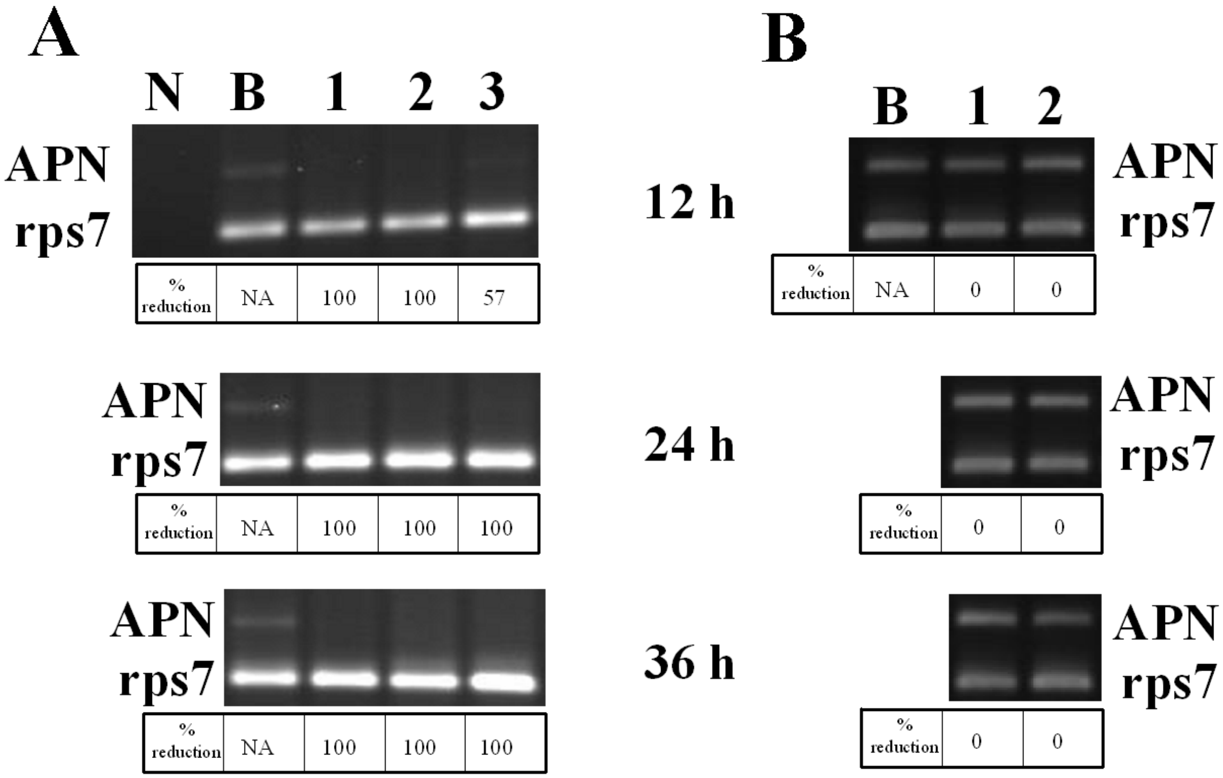

1
2
3
M1
M2
kb
23.0
1.5
1.0
0.8
0.7

Figure 3

Soaking of 2-day old *A. aegypti* larvae in dsAPN RNA resulted in specific suppression of APN expression. Before soaking, the amount and integrity of dsAPN and dsGFP RNAs obtained were analyzed by running it in a 1% agarose gel.

(A) Semi-quantitative multiplex PCR was performed on the mosquito larvae that were soaked in dsAPN RNA at 0.2 (lane 1), 0.1 (lane 2) and 0.05 µg/µl (lane 3) for 12 h (top panel), 24 h (middle panel) and 36 h (bottom panel). Lane N is negative PCR control; lane B is soaking buffer control. The reduction of APN expression is indicated in the tables below the gels.

(B) Semi-quantitative multiplex PCR was also performed on the mosquito larvae that were soaked in dsGFP RNA at 0.1 (lane 1) and 1.0 µg/µl (lane 2) for 12 (top panel), 24 (middle panel) and 36 h (bottom panel). Lane B is soaking buffer control. The reduction of APN expression is indicated in the tables below the gels.



A, 12 h

% reduction	NA	100	100	57

A, 24 h

% reduction	NA	100	100	100

A, 36 h

% reduction	NA	100	100	100

B, 12 h

% reduction	NA	0	0

B, 24 h

% reduction	0	0

B, 36 h

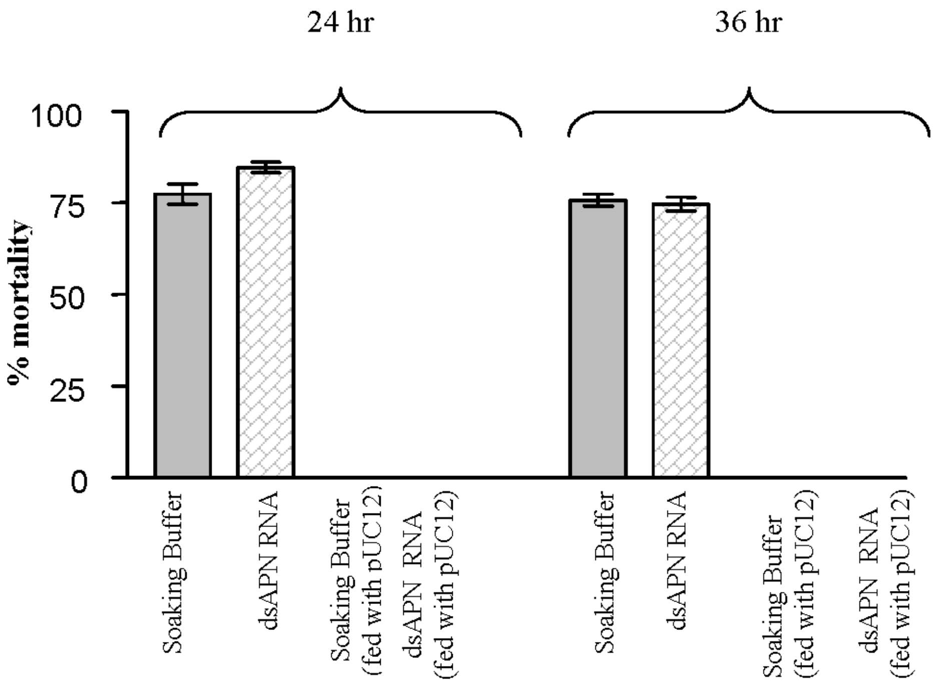
% reduction	0	0

Figure 4

Mosquito-larvicidal assay of *A. aegypti* larvae soaked in dsAPN RNA.

Comparison of larvicidal activity of *E. coli*-expressing Cry4Ba toxin between larvae in soaking buffer alone and to larvae soaked in 0.1 mg/ml dsAPN RNA for 24 or 36 hr. As negative controls, the larvae were fed instead with *E. coli* containing pUC12 after soaking in the appropriate buffers. The experiments were repeated at least 3 times.

	% mortality			
	Soaking for 24 hr		Soaking for 36 hr	
	Soaking buffer	0.1 mg/ml dsAPN RNA	Soaking buffer	0.1 mg/ml APN dsRNA
Mean	77.4	84.0	75.7	74.7
SEM	2.8	2.1	1.7	1.9



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Project outputs

Presentation

1. Dedvisitsakul, P., Saengwiman, S. and Pootanakit, K. (2006) Injection or ingestion of long double-stranded RNA causes specific interference in *Aedes aegypti* larvae. TRF Meeting, held at Cha-am, Petchaburi. October 12 -14. (Abstract # P-S2C-08).
2. Dedvisitsakul, P. and Pootanakit, K. (2004) Silencing of aminopeptidase N gene in *Aedes aegypti* larvae by RNA interference. 30th Congress on Science and Technology of Thailand, Muang Thong Thani. Oct 19-21.