UC Riverside

UC Riverside Electronic Theses and Dissertations

Title

Toxin Binding Receptors and the Mode of Action of Bacillus thuringiensis subsp. israelensis Cry Toxins

Permalink

https://escholarship.org/uc/item/6cr35910

Author

Lee, Su Bum

Publication Date

2013

Peer reviewed|Thesis/dissertation

eScholarship.org Powered by the California Digital Library
University of California

UNIVERSITY OF CALIFORNIA
RIVERSIDE

Toxin Binding Receptors and the Mode of Action of *Bacillus thuringiensis* subsp. *israelensis* Cry Toxins

A Dissertation submitted in partial satisfaction
of the requirements for the degree of

Doctor of Philosophy

in

Environmental Toxicology

by

Su Bum Lee

December 2013

Dissertation Committee:
Dr. Sarjeet S. Gill, Chairperson
Dr. Michael E. Adams
Dr. Howard S. Judelson

Copyright by
Su Bum Lee
2013

The Dissertation of Su Bum Lee is approved:

Committee Chairperson

University of California, Riverside

ACKNOWLEDGEMENTS

There are a number of people without whose supports I could not have made it to the completion of PhD program. I would like to greatly appreciate the people who helped me during PhD program.

First of all, I would like to thank Dr. Sarjeet S. Gill for giving me invaluable guidance and support during the course of my research. It has been my honor to have Dr. Sarjeet S. Gill as my mentor and dissertation adviser. I had a lot of opportunities in research field and have learned a number of things under his advice. I express my deep gratitude to him.

I am also very grateful to Dr. Howard S. Judelson and Dr. Michael E. Adams for serving as members of my dissertation committee and for their constructive criticism and evaluation of my research. I thank Dr. David A. Eastmond and Dr. Donald A. Cooksey who has served on my qualifying committee.

I express my thanks to my present and past lab members who helped my research and shared all of their knowledge and experience with me. Dr. Jainwu Chen and Dr. Supaporn Likitvivatanavong shared their valuable information on *Bacillus thuringiensis* and experience on molecular techniques. I sincerely appreciate Dr. Karly Aimanova for her help on immunohistochemical analysis and teaching on mosquito work. I would like thank Dr. Nadia Qureshi, Swati Chawla, and Francesca Della Ripa for their help and understanding. My thanks go to Amy Evans and Dr. Wanyoike Kang'ethe for their friendship. I also thank undergraduate students, Valeree, Jacqueline, Amanda, and Nancy,

who helped my mosquito work.

I want to thank to my old friends, Do-Hyoung and Sung-Hoon. Without them, I cannot imagine my life in America. They encouraged and helped me get through difficulties in my personal life. I also thank visiting professor, Dr. Yongman Yu and Dr. Yeonho Je for great discussion.

Finally, I would like to express my deep appreciation and share this special moment with my wonderful wife, Jihyon, who has patiently supported me with love and my son, Junseo. I love all of you. I express my special thanks to my parent and parent-in-law for giving me endless supports in Korea.

ABSTRACT OF THE DISSERTATION

Toxin Binding Receptors and the Mode of Action of *Bacillus thuringiensis* subsp. *israelensis* Cry Toxins

by

Su Bum Lee

Doctor of Philosophy Graduate Program in Environmental Toxicology
University of California, Riverside, December 2013
Dr. Sarjeet S. Gill, Chairperson

Cadherins play pivotal roles in the toxicity of *Bacillus thuringiensis* Cry proteins. Here I tested and showed that an *Aedes* cadherin (AAEL007478) and an N-cadherin (AAEL000597) are involved in the *in vivo* toxicity of Cry11A toxin to *Aedes aegypti*. *Aedes* cadherin was stably expressed in a mosquito cell line and these cells showed increased sensitivity (37% death) to Cry11Aa toxin. These results show *Aedes* cadherin mediates Cry11Aa toxicity, but since high toxicity was not obtained, an additional secondary receptor may be needed for manifestation of full toxicity. Using a whole genome screen to identify genes that are altered during Cry11Aa intoxication, I identified an N-cadherin gene (AAEL000597) that was significantly down-regulated. An EGF-LamG fragment from this N-cadherin bound Cry11Aa with high affinity and competed with Cry11Aa binding to mosquito midgut membranes. Moreover, N-cadherin-silenced mosquitoes showed tolerance to Cry11Aa, implying that this cadherin is involved in mediating Cry11Aa toxicity. I also showed that this N-cadherin interacts with an amino acid on loop α-8 of Cry11Aa, which is different from that which interacts

with the *Aedes* cadherin binding region. These data suggest that Cry11Aa probably has two different pathogenic pathways that act through two different cadherins in *Ae. aegypti*.

I also established a Cry11A-resistant strain to determine which mode of action is involved in *Aedes* Cry11Aa resistance. Brush border membranes from this strain (G30) bound Cry11Aa less compared to the binding in the wild type (WT), implying Cry11Aa resistance resulted from altered receptor binding affinity, but not proteolytic activity since no change in the latter was observed. Using RNA-seq analyses, immunoblot assays and mass spectrometry, we found the N-cadherin (AAEL000597) and an alkaline phosphatase (ALP, AAEL003298) were down-regulated in Cry11A-resistant larvae midgut. These results strongly suggest that N-cadherin and ALP are associated with Cry11Aa resistance in *Ae. aegypti*.

In summary, based on data in the literature and my work, I demonstrate that two different pathways of Cry11Aa toxicity are possible; one involving an N-cadherin and the other a combination of *Aedes* cadherin and an ALP in *Ae. aegypti*. Moreover, N-cadherin and ALP not only mediate Cry11Aa toxicity, but they were appear to be associated with Cry11Aa resistance. ALP has been proposed as a secondary receptor mediating Cry11Aa toxicity with *Aedes* cadherin, and hence attenuation of its expression can lead to Cry11A resistance.

Table of Contents

List of Figures

Page

List of Tables

Page

Chapter 1

General Background

1. Introduction to *Aedes aegypti*

Mosquitoes are major pests in human health because they transmit pathogens, such as viruses and parasites through blood feeding causing serious human disease including malaria, filariasis, West Nile fever, and dengue fever. These disease outbreaks are very frequent in tropical and subtropical regions, where environmental conditions are ideal for mosquito breeding, and result in billions of disease cases and millions of deaths worldwide annually.

Among mosquitoes, *Aedes aegypti* is a primary disease vector in urban areas transmitting viruses that cause chikungunya, yellow fever, and dengue fever (WHO, 2002; Tomori, 2004; Ligon, 2006). Yellow fever, for example, is a serious disease in Africa and South America: 200,000 infections annually resulting in 30,000 deaths in spite of vaccine usage (WHO, 1998). Dengue fever is a serious arboviral disease of the Americas, Asia, and Africa (Figure 1) and causes 1,000 million infections and 25,000 deaths worldwide annually (WHO, 1997). Moreover, there is no effective vaccine for dengue fever and the incidence of dengue fever is on the increase (Figure 2). Therefore, control of their vector, *Ae. aegypti*, is the only reasonable preventive option.

For a long time, attempts to manage *Aedes* mosquitoes have used chemical, biological, and physical methods. Chemical insecticides such as DDT, malathion, or

pyrethroids have been used worldwide since the 1940s. Physical methods were also attempted in many sites, where breeding sites were eliminated or predators were added to remove larvae. This strategy, a combination of insecticide treatment and breeding site elimination, seemed to contribute to the successful control of *Ae. aegypti* (Gomez-Dantes et al., 2009). Unfortunately, the widespread use of insecticide has resulted in outbreaks of resistant *Aedes* mosquitoes to insecticides in the Americas (Harris et al., 2010; Rodríguez et al., 2007). Insecticides are often toxic to non-target organisms and contribute to environmental contamination, while physical methods have limitations in their application. Therefore, biological methods are considered an alternative, including the introduction of parasites and predators, or use of pathogens to target mosquitoes. Among the pathogens used to control mosquito larvae are various bacterial strains, including *Bacillus thuringiensis* and *Lysinibacillus sphaericus*. *B. thuringiensis* subspecies have high insecticidal activity, low toxicity to other organisms, and a lack of resistance development to this bacterium in the field. Thus this bacteria strain has been used worldwide for the control of *Ae. aegypti* as well as other mosquito species.

2. *Bacillus thuringiensis*

Bacillus thuringiensis (Bt) is a Gram-positive bacterium, which was discovered and isolated by Ishiwata Shigetane in 1901 as the cause of the disease that was killing the silkworm populations (Luthy et al., 1981). This bacterium was named *B. thuringiensis* by Berliner in 1911 and classified as a member of the *Bacillus cereus* group in the *Bacillaceae* family, which contains *B. anthracis*, *B. cereus*, *Bacillus mycoides*, *Bacillus*

pseudomycoides and *Bacillus weihenstephanensis* (Rasko et al., 2005). Bt has a notable property – it is pathogenic to insects. In 1956, the crystal protein inclusions that are produced during sporulation were associated with the insecticidal activity (Angus, 1956) and their expression was shown to be encoded by transmissible plasmids (Gonzalez et al., 1982). These inclusions contain one or more proteins called Cry and Cyt toxins (Hofte et al., 1989). These proteins are highly selective to the target insect, but are harmless to humans and vertebrates such that they have been used to control lepidopteran, dipteran and coleopteran insect pests in agriculture and public health (Crickmore et al., 1998).

2.1. *Bacillus thuringiensis* strains and crystal proteins

A large number of Bt strains were isolated from diverse environments including soil, insects, stored-product dust, or coniferous leaves (Bernharda et al., 1997; Martin et al., 1989). Identified Bt strains from many countries were classified primarily based on serotype according to their H flagella antigenic determinants (de Barjac et al., 1990; Lecadet et al., 1999). A total 69 serotypes and 82 serovars were defined and named as subspecies in Bt. However, serotyping with Bt flagella did not reflect their insecticidal activity. For example, the *Bacillus thuringiensis* subsp. *morrisoni* is toxic against insects from the orders Lepidoptera, Coleoptera, and Diptera. Therefore, the prediction of insecticidal activity was based on individual Cry and Cyt toxins encoded by Bt plasmids. Individual Bt toxins have insecticidal activity that is limited to a few species in one particular order of Lepidoptera, Diptera, Coleoptera, and Hymenoptera, although a few

toxins have been shown to be toxic to two or three orders (de Maagd et al., 2001; Guerchicoff et al., 2001).

Numerous crystal proteins were isolated and first classified according to their insecticidal activities (Hofte et al., 1989). CryI toxins were toxic to lepidopteran insects; CryII toxic to lepidopteran and dipteran insects; CryIII toxic to coleopteran insects; CryIV toxic to dipteran insects. However, the Cry and Cyt toxin nomenclature was subsequently changed and is now based on their amino acid sequence (Crickmore et al., 1998). Thus, the first number means that toxins have less than 45% sequence identity (Cry1, Cry2, etc), the subsequent capital letter means there is less than 78% sequence identity (Cry1A, Cry1B, etc), and finally a lowercase letter means less than 95% sequence identity (Cry1Aa, Cry1Ab, etc). Even though this classification based on amino acid sequence did not correspond exactly to all insecticidal activity of toxins, some Cry toxin groups showed toxicity to similar insect orders. For instance, Cry1 toxins are toxic to Lepidoptera, while Cry3 toxins are toxic to Coleoptera.

2.2. Mosquitocidal Bt strains and Bti

To find more potent bacterial strains that affect mosquitoes, many Bt strains as well as other bacterial species, *L. sphaericus* or *Clostridium bifermentans* subsp. *malaysia*, were discovered and assayed for bioactivity. Some Bt strains, including Bt serovars *israelensis*, *jegathesan*, *medellin* and *morrisoni*, were found to be highly toxic to mosquito larvae and contained mosquitocidal crystal proteins (Table 1). Among them, *Bacillus thuringiensis* subsp. *israelensis* (Bti) was one of the most highly toxic strains to

mosquitoes, particularly to *Ae. aegypti* (Ragni et al., 1996). Bti was isolated by Goldberg and Margalit in 1977 (Goldberg et al., 1977) and has high toxicity to Dipteran insects, primarily mosquito and black fly larvae (Mittal, 2003; Lacey, 2007). Accordingly, Bti was applied for control of mosquitoes, particularly human disease vectors such as *Ae. aegypti* (the vector of dengue fever), *Simulium damnosum* (the vector of onchocerciasis) and *Culex* species (vectors of filariasis and West Nile fever). Mutant screening of this bacterium showed that Bti toxicity depended on the presence of a megaplasmid (Faust et al., 1983), later called pBtoxis. Complete sequencing of this pBtoxis plasmid was performed and four Cry toxins and three Cyt toxins are encoded by this plasmid: Cry4Aa, Cry4Ba, Cry10Aa, Cry11Aa, Cyt1Aa, Cyt1Ca and Cyt2Ba (Ben-Dov et al., 1999; Berry et al., 2002; Guerchicoff et al., 1997).

2.3. Bti crystal proteins

Individual Cry and Cyt toxins or their combinations were previously tested to estimate their mosquitocidal activity against seven species of mosquitoes that belong to three genera including *Aedes*, *Anopheles*, and *Culex* mosquitoes (Angsuthanasombat et al., 1992; Delecluse et al., 1993; Abdullah et al., 2003; Boonserm et al., 2003; Poncet et al., 1995). LC_{50} values of each toxin were summarized and showed their toxicity to mosquito species (Otieno-Ayayo et al., 2008). For instance, Cry4Aa has high toxicity to *Culex pipiens*, but low toxicity to *Ae. aegypti*, while Cry4Ba has high toxicity to *Ae. aegypti* and *Anopheles* mosquitoes but low toxicity to *Culex* mosquitoes. Cry11Aa is highly toxic to *Ae. aegypti* and *Cu. pipiens.* However, Cyt1Aa has low toxicity to three

mosquito genera. Evaluating only the toxicity towards *Ae. aeypti*, Cry11Aa was found to be the most active toxin in the Bti crystal inclusions (Crickmore et al., 1995; Delecluse et al., 1993).

The structures of the Cry4Aa and Cry4Ba toxins have been determined by X-ray crystallography or computational modeling (Boonserm et al., 2005; Fernandez et al., 2005; Boonserm et al., 2006). These structures are similar and composed of three structural domains like previously analyzed other Cry toxins (Li et al., 1991; Grochulski et al., 1995). Based on these, the Cry11Aa structure has been modeled (Fernandez et al., 2005). The N-terminal Domain I is composed of seven α-helices implicating its role in membrane insertion and pore formation (Parker et al., 1989). Domain II is formed by three antiparallel β-sheets with exposed loop regions and is the most variable of part of the toxin structure (de Maagd et al., 2001). This diversity in Domain II is suggested to be involved in receptor binding (Li et al., 1991). Domain III in the C-terminal region is composed of a β-sandwich with two antiparallel β-sheets indicating involvement in insect selectivity and receptor binding (Schwartz et al., 1997). The structures of the Cyt toxins was also determined by X-ray crystallography. The Cyt2Aa toxin is composed of a single α-β domain composed of two outer layers of α-helix hairpins wrapped around a β-sheet (Li et al., 1996). The Cyt1Aa structure has a typical cytolysin fold that allows the α-helical layers to swing away, exposing the β-sheet to insert into the membrane (Cohen et al., 2011).

3. Mechanism of Bt toxicity in Lepidoptera

3.1. Mode of action

The toxins are ingested by susceptible larvae and translocated to the midgut. The crystal inclusions are dissolved in the alkaline environment of insect midgut, and the protoxins are cleaved by midgut proteases at the N- and C-terminal ends to yield activated monomeric toxin (Choma et al., 1990). Cleaved toxins then bind to primary receptors such as a cadherin protein located in the brush border membrane of target insect midguts (Schnepf et al., 1998; de Maagd et al., 2001; Gill et al., 1992).

Two models of the mode of action of solubilized and activated Cry toxin have been proposed in Lepidoptera (Figure 3). The first proposed mechanism is the pore-forming model (Soberon et al., 2009). This model is based on the cell-swelling and bursting symptom in Cry toxin-treated Lepidoptera, in which the Cry toxin undergoes oligomerization before insertion of the oligomeric Cry toxin into the membrane (Bravo et al., 2004; Schnepf et al., 1998). Toxin binding triggers additional protease cleavages by eliminating helix α-1 and facilitating formation of oligomers (Gomez et al., 2002; Jimenez-Juarez et al., 2007). The toxin oligomers bind to the secondary receptors, glycosylphosphatidyl-inositol (GPI)-anchored proteins like aminopeptidase and alkaline phosphatases (Bravo et al., 2004; Jurat-Fuentes et al., 2004). Finally, this binding triggers membrane insertion of oligomers and formation of lytic pores which kill cells and ultimately the insect (Schnepf et al., 1998; Aronson et al., 2001).

The signal-cascade model, on the other hand, has been proposed as another mechanism (Zhang et al., 2006; Zhang et al., 2005). Cry toxins bind to a cadherin protein from Lepidoptera, which is expressed in the apical cell membrane and triggers a signal cascade pathway. This interaction activates a G protein and then adenylyl cyclase, thereby increasing the amount of cAMP. The increased cAMP turns on protein kinase A, which finally causes cell death. In addition, this model suggests that toxin binding actuates exocytosis of the cadherin receptor from intracellular vesicles to the cytoplasmic membrane.

The models mentioned above show that toxin binding to receptor is a key point in toxicity. Also interaction between receptor proteins and toxins determines Cry toxin selectivity (Jenkins et al., 2000). Therefore, the identification of receptors that interact with Cry toxin is important for understanding the molecular basis of insect selectivity. Many putative Cry toxin receptors have been reported and their function has been studied in lepidopteran insects - cadherin proteins, aminopeptidases N (APN), alkaline phosphatases (ALP) and glycolipids (Gill et al., 1995; Griffitts et al., 2005; McNall et al., 2003; Vadlamudi et al., 1993; Gahan et al., 2001; Nagamatsu et al., 1998; Knight et al., 1995; Jurat-Fuentes et al., 2004; Sangadala et al., 1994).

3.2. Cadherin

The cadherin protein is important since it is the first event in the interaction of Cry toxins with the brush border membrane of the target insect midgut (Bravo et al., 2005). Several cadherins have been identified as Cry toxin binding proteins and shown to

be involved in Cry toxin toxicity in Lepidoptera like *Manduca sexta, Bombyx mori, Heliothis virescens, Helicoverpa armigera, Pectinophora gossypiella,* and *Ostrinia nubilalis* (Vadlamudi et al., 1995; Nagamatsu et al., 1998; Gahan et al., 2001; Xu et al., 2005; Morin et al., 2003; Flannagan et al., 2005). Binding regions were mapped with Cry1Ab toxin and Bt-R_1, cadherin receptor from *M. sexta*. Two loop regions, loop α-8 and loop 2 on domain II, in Cry1Ab were involved in the interaction between Cry toxin and Bt-R_1 (Gomez et al., 2003). Correspondingly, cadherin repeat 7 (^{869}HITDTNNK^{876}) and cadherin repeat 11 (^{1331} IPLPASILTVTV^{1342}) in Bt-R_1 were mapped as Cry1Ab toxin binding region (Gomez et al., 2001; Dorsch et al., 2002). Toxin binding cadherin mutations have resulted in the development of resistant lepidopteran insects. A *H. virescens* population selected in the laboratory contained a retrotransposon insertion that disrupted expression of the full-length cadherin gene (Gahan et al., 2001). In *P. gossypiella*, a resistant population was obtained from cotton fields in Arizona that revealed cadherin deletion mutations (Morin et al., 2003). *H. armigera* populations were obtained from a selected population in the laboratory and a field population crossed and selected with offspring. Their resistance was associated with retrotransposon insertions and a stop codon mutation in the extracellular domain (Xu et al., 2005; Yang et al., 2007). Recently, a field-selected resistant *H. armigera* population was reported that had a deletion in the intracellular domain of cadherin (Zhang et al., 2012).

3.3. GPI-anchored receptors

GPI-anchored proteins such as aminopeptidase N (APN) and alkaline phosphatase (ALP) were identified as Cry toxin-binding receptors in Lepidoptera. While many GPI-anchored receptors were identified in Lepidoptera, how they bind the toxin is still not clear. However, resistant Lepidopteran species support the fact that GPI-anchored receptors are involved in Cry toxin toxicity. As mentioned above, a *H. virescens* population selected in the laboratory showed a lack of ALP (Jurat-Fuentes et al., 2003). A *Spodoptera exigua* resistant strain to Cry1Ca and a *Trichoplusia ni* to Cry1Ac also showed reduced APN transcripts (Herrero et al., 2005; Tiewsiri et al., 2011). A laboratory-selected *H. armigera* with Cry1Ac contained a deletion mutation in APN (Zhang et al., 2009). GPI-anchored receptors played an important role in membrane insertion and pore formation (Zhuang et al., 2002; Lorence et al., 1997). Cry toxin binding to GPI-anchored receptors in *M. sexta* actuated the localization of APN and ALP in lipid rafts microdomains where Cry toxin inserts and forms pores (Chen et al., 2005; Zhuang et al., 2002).

3.4. ABC transporter

The most recently identified protein involved in resistance is an ATP-binding cassette transporter (ABC transporter). In a *H. virescens* resistant strain that was separated from the cadherin mutation, an ABC transporter mutation in an ABCC2 was linked to Cry1Ac resistance and caused the loss of Cry1Ac binding on brush border membrane vesicles (Gahan et al., 2010). In addition, a strain of *B. mori* that was resistant

to Cry1Ab toxin also revealed a mutation in a homologous ABC transporter. In this case, a single amino acid insertion in an outer loop of the transmembrane region caused resistance and introduction of a susceptible gene into resistant strain rescued toxin susceptibility (Atsumi et al., 2012).

4. Mechanism of Bti toxin in *Aedes aegypti*

As mentioned in section 2.2 above, Bti has been used for decades for the control of mosquitoes including *Ae. aegypti*, an important disease vector. Despite long-term usage of Bti, resistant development to Bti in *Ae. aegypti* has not been reported yet from mosquitoes in the field (Mittal, 2003). Bti produces mosquitocidal crystalline inclusions during sporulation. Ingested crystalline inclusions from Bti are solubilized in the alkaline midgut environment of the mosquito and releases protoxin (Knowles et al., 1989). The protoxins are proteolytically activated by midgut protease and result in the formation of active toxins. Activated toxins then bind receptor proteins on midgut epithelial cells (Feldmann et al., 1995; Yamagiwa et al., 2002). In Bti-treated *Ae. aegypti*, epithelial cells in midgut swell, are damaged, and finally burst (Singh et al., 1986). This symptom is similar to that previously reported in Lepidoptera. Based on these symptoms, it was suggested that Cry toxin may be involved in making a pore in the microvilli membrane resulting in cell swelling and lysis (Knowles et al., 1987). On the other hand, Bti-treated epithelia cells also showed another symptom: cells were detached from each other and cell arrangement was disrupted (Singh et al., 1986). This result suggests that it is possible

that Cry toxin or other virulence factor may interact with proteins related to cell-cell junction.

Many putative Cry toxin receptors have been identified in mosquitoes. APNs from *Anopheles quadrimaculatus* and *Ae. aegypti* were identified and bound Cry11Ba from *Bacillus thuringiensis* subsp. *jegathesan* (Btj), and a cadherin receptor from *Anopheles gambiae* was identified and bound Cry4Ba (Abdullah et al., 2006; Hua et al., 2008; Zhang et al., 2008). In *Ae. aegypti*, Cry11Aa bound four proteins (200, 100, 65 and 62 kDa) in brush border membrane vesicles (BBMV) from *Ae. aegypti* midgut epithelia (Fernandez et al., 2006). Among them, the 65 kDa protein was identified as a GPI-anchored ALP and a functional receptor of Cry11Aa in *Ae. aegypti* midgut cells (Fernandez et al., 2006). Biotin-labeled Cry11Aa also bound three different proteins which had molecular masses of 140, 95, and 44 kDa in a pull-down assay with *Ae. aegypti* larval midgut (Chen et al., 2009). MS-MS mass spectrometry identified the 140 and 95 kDa proteins as APNs. Cry4Ba toxicity was mediated by GPI-anchored proteins. ALP (AAEL015070) bound Cry4BA with high affinity (Thammasittirong et al., 2011) and Sf9 cells expressing ALP were more sensitive to Cry4Ba and undergo cell lysis (Dechklar et al., 2011). Moreover, APNs-silenced larvae showed an increase in resistance to Cry4Ba toxicity (Saengwiman et al., 2011).

4.1. Cadherin in mosquitoes

The cadherin proteins have been known to be the primary receptors of Cry toxins in Lepidoptera as well as in Coleoptera (Vadlamudi et al., 1993; Nagamatsu et al., 1998;

Fabrick et al., 2009). Mosquito cadherins homologous with Bt-R_1 mediating Cry1Ab toxicity in Lepidoptera were also identified as functional receptors in *Ae. aegypti* and *An. gambiae* (Chen et al., 2009; Hua et al., 2013; Hua et al., 2008).

Aedes cadherin was immunolocalized on the apical membrane of distal and proximal caeca and posterior midgut epithelial cells, but not on the apical membrane of anterior midgut, in an identical fashion to Cry11Aa binding. Toxin binding regions were mapped with partial fragments of *Aedes* cadherin containing cadherin repeats. The cadherin repeats (CR7-CR11) nearest the membrane proximal extracellular domain bound Cry11Aa with high affinity (*Kd* = 16.7 nM). Further, this fragment interacted with loop α-8 and loop 2 in Cry11Aa domain II. Binding to *Aedes* cadherin is associated with the toxicity of Cry toxin. Cadherin-silenced larvae showed increased tolerance for Cry11A, but not for Cry 4Ba, which does not bound to the cadherin repeats (CR7-CR11) (Rodríguez-Almazán et al., 2012; Chen et al., 2009). A cytotoxicity test, determined with mosquito cells expressing the full-length cadherin stably, showed Cry11Aa killed 37% of cells expressing the cadherin compared to control cells expressing only GFP (Chapter 2).

In *An. gambiae*, two *Anopheles* cadherins (AgCad1 and AgCad2) were identified as putative receptors for Cry4Ba and Cry11Ba (Hua et al., 2013; Hua et al., 2008). AgCad1 was localized on the microvilli in posterior midgut and its cadherin repeat (CR9-CR11) and membrane proximal extracellular domain bound Cry toxin. However, this AgCad1 had high affinity for Cry4Ba (*Kd* = 13-23 nM) while *Aedes* cadherin did not bind Cry4Ba. Moreover, AgCad1 fragments synergized Cry4Ba toxicity in *An. gambiae* as well as *Ae. aegypti* (Park et al., 2009). AgCad2 which shares 14% identity to AgCad1

showed high affinity (*Kd* = 11.8 nM) with Cry11Ba from Btj (Hua et al., 2013). This AgCad2 fragment also inhibited Cry11Ba binding to brush border vesicles and decreased Cry11Ba toxicity in larvae.

4.2. GPI-anchored receptor in mosquitoes

As previously mentioned, Bti toxicity in mosquitoes was mediated by GPI-anchored proteins including APN, ALP and α-amlyase (Chen et al., 2009; Fernandez et al., 2006; Fernandez-Luna et al., 2010). In *Anopheles* mosquitoes, APN and ALP have not yet been identified as receptors for Bti-toxins but have been shown to be receptors for a Btj toxin. APN and ALP from *An. gambiae* were identified as functional receptors of mosquitocidal Cry11Ba toxin from Btj (Hua et al., 2009; Zhang et al., 2008). A 106-kDa *Anopheles* APN was localized on the microvilli of the posterior and a truncated fragment bound Cry11Ba with high affinity (*Kd* = 6.4 nM) and inhibited Cry11Ba toxicity to *An. gambiae* larvae. Mapping regions mediating toxicity identified two regions which oppositely affected toxicity: one region prevented Cry11Ba from binding to brush border membrane vesicle and another region increased Cry11Ba binding and toxicity to *Anopheles* larvae (Zhang et al., 2010). Another *Anopheles* ALP was also isolated that bound Cry 11Ba with high affinity (*Kd* = 23.9 nM), and inhibited Cry11Ba toxicity (Hua et al., 2009). A new type of GPI-anchored receptor was identified as a receptor of Cry4Ba and Cry11Aa in *Anopheles albimanus*, the disease vector for malaria (Fernandez-Luna et al., 2010). Ligand blot assays found a 70 kDa GPI-anchored protein which was determined to be an α-amylase by mass spectrometry. Recently, α-amylase (AgAmy1)

and α-glucosidase (Agm3) were identified as a functional receptor of Cry11Ba in *An. gambiae*. AgAmy1 localized in salivary gland and posterior midgut microvilli and Agm3 was observed in posterior midgut microvilli. Both AgAmy1 and Agm3 bound Cry11Ba with high affinity (*Kd* = 37.6, 21.1 nM) and feeding with a mixture of their inclusion bodies and Cry11Ba reduced Cry11Ba toxicity (Zhang et al., 2013).

In *Ae. aegypti*, APN and ALPs were identified as functional receptors of Bti toxins, Cry4Ba and Cry11Aa (Chen et al., 2009; Fernandez et al., 2006; Jiménez et al., 2012). A 140-kDa *Aedes* APN (AAEL012778) was identified as a putative receptor of Cry11Aa by pull-down assay and mass spectrometry (Chen et al., 2009). This APN was immunolocalized on the apical membrane of posterior midgut epithelial cells and bound Cry11Aa with high affinity (*Kd* = 8.5 nM). Interestingly, both *Aedes* cadherin and *Aedes* APN showed high affinity for the same Cry11Aa toxin, while Lepidoptera APN had low affinity for Cry1A toxin. These results suggest that Bti toxicity in mosquitoes probably has a different mechanistic pathway from that in Lepidoptera. Other *Aedes* APNs (AAEL005808, AAEL012778, and AAEL012783) have been shown to be receptors for Cry4Ba. Individually these APN-silenced larvae showed tolerance to Cry4Ba toxicity (Saengwiman et al., 2011).

Aedes ALP was further investigated as a functional receptor of Cry4Ba and Cry11Aa toxins. *Aedes* ALP (AAEL009077) was first observed in ligand blot assays with Cry11Aa and localized in the same regions as the Cry11Aa toxin binding sites, the microvilli membranes of caeca and the posterior midgut. Furthermore, mapping of binding regions in *Aedes* ALP determined that two regions (R59-F102 and N257-I296)

interacted with Cry11Aa domain II loop α-8 and domain II loop β18-β19, respectively (Fernandez et al., 2009). Silencing of *Aedes* ALP in the midgut resulted in reduced Cry11Aa toxicity (Jiménez et al., 2012). Other *Aedes* ALPs also interacted with Cry4Ba toxin. Proteomic analysis showed Cry4Ba was localized in lipid rafts from brush border membrane vesicle of larvae and bound three ALPs (AAEL003298, AAEL003313, and AAEL015070) (Bayyareddy et al., 2012; Bayyareddy et al., 2009). Moreover, *Aedes* ALP (AAEL015070) bound Cry4Ba with high affinity (*Kd* = 14 nM) and mediated Cry4Ba toxicity in Sf9 cells expressing *Aedes* ALP (AAEL015070) (Dechklar et al., 2011; Thammasittirong et al., 2011). *Aedes* ALP (AAEL009077) binding Cry11Aa also interacted with Cry4Ba domain II loop 2 and was involved in Cry4Ba toxicity to *Aedes* larvae (Jiménez et al., 2012).

4.3. Cytolytic endotoxin

Cyt toxins are found in dipteran-active Bt strains and synergized the toxic effect of Cry toxins (Wu et al., 1994). Furthermore, Cyt1Aa reduced resistance in Cry11Aa-resistant *Cu. quinquefasciatus* from greater than 1,000 fold to less than 8 fold (Wirth et al., 1997). Later, Cyt1Aa was revealed to function as a membrane-bound receptor of Cry11Aa in *Ae. aegypti* (Perez et al., 2005). Cyt1Aa bound BBMV, enhanced Cry11Aa binding to BBMV, interacted with Cry11Aa and facilitate the formation of oligomeric structure (Perez et al., 2007). Cyt2Aa from *Bacillus thuringiensis* subsp. *darmstadiensis* (Btd) also synergized Cry4Ba toxicity against *Ae. aegypti* and *Cu. quinquefasciatus* (Promdonkoy et al., 2005). Further analysis of the interaction between Cry4Ba and

Cyt1Aa showed loop β2-β3 and β4-β5 of Cry4Ba, particularly Tyr^{332} and Phe^{364}, are involved in synergistic interactions with Cyt2Aa (Lailak et al., 2013).

5. Mosquito resistance against Bti

Insect resistance is a major issue for pest control with chemical or biological pesticide. However, while Bti has been used for more than three decades for mosquito control, no resistance has been detected in the field. In contrast, resistance has been observed from the use of lepidopteran-active Cry toxins in a variety of insects (Tabashnik et al., 2008). Therefore, some mosquito strains were developed in laboratories to elucidate the mechanism of Bti toxins.

5.1. Bt resistance in *Culex* mosquitoes

Early research tried to develop resistant mosquitoes with Bti producing diverse Cry toxins. These laboratory selections with *Ae. aegypti* and *Cu. pipiens* evolved resistance slowly and to lower levels (Goldman et al., 1986; Saleh et al., 2003). However, resistance development was more rapid to a subset of toxins found in Bti. For example, *Culex quinquefasciatus* selected with the different number of Cry toxins from Bti: one (Cry11Aa), two (Cry4Aa and Cry4Ba), three (Cry4Aa, Cry4Ba, and Cry11Aa), and four (Cry4Aa, Cry4Ba, Cry11Aa, and Cyt1Aa) toxins (Georghiou et al., 1997) leading to different rates of insect resistance. A 28-time selection with a single Cry11Aa showed the highest resistance ratio (900 fold), but selection with four toxins resulted in the lowest resistance level (3.2 fold). Two toxins and three toxins reached high resistance ratio (120

fold and 91 fold). The cross-resistance patterns of these strains were observed with combinations of mosquitocidal Cry toxins and all four strains showed the cross resistance to Bti toxins as well as Cry11Ba from Btj (Cheong et al., 1997; Wirth et al., 1998). For example, Cry11Aa-resistant strains revealed high resistance ratio to Cry4Aa and Cry4Ba (41.6 fold), to Cry4Aa, Cry4Ba, and Cry11Aa (13.5 fold), and Cry11Ba (53.1 fold). However, all strains showed very low resistance level to Bti (1.1 fold) and Btj (2.8 fold) strains. Since mosquitoes selected with Cyt1Aa showed low resistance, mosquitoes were selected with Cry11Aa, Cyt1Aa, or a mixture of Cry11Aa and Cyt1Aa (Wirth et al., 2005; Wirth et al., 1997). One strain selected with only Cry11Aa achieved high resistance in 18 generations, but the strains selected by Cyt1Aa (Cyt1Aa or a mixture of Cry11Aa and Cyt1Aa) did not develop resistance.

5.2. Bt resistance in *Aedes* mosquitoes

As a laboratory-selected mosquito, a *Ae. aegypti* resistant strain (LiTOX strain) was developed with toxic leaf litter containing Bti toxins. After 18-time selection, this strain obtained 3.4-fold resistance ratio against toxic leaf litter and showed different resistance ratios for Cry4Aa, Cry4Ba, Cry11Aa, and Cyt1Aa toxin (Paris et al., 2011). Cry4Aa- or Cry4Ba-treated *Ae. aegypti* had 30-fold and 13-fold resistance compared to susceptible *Ae. aegypti*. However, LiTOX strain showed 6-fold and 3-fold resistance for Cry11Aa and Cyt1Aa toxins. To find the loci showing the highest inter-strain genetic differentiation, the genome of LiTOX and susceptible strains were mapped and compared (Bonin et al., 2009). Two genes, a cadherin (AAEL001196) and a leucine aminopeptidase

(AAEL007892), contained high nucleotide polymorphisms and were significantly down-regulated. Another genome scan with LiTOX strain was performed using amplified fragment length polymorphism (AFLP) and 454 pyrosequencing. This scan identified 11 outliers located on different supercontigs containing aminopeptidase (AAEL012918) and trypsin (AAEL004543) (Paris et al., 2012). This LiTOX strain was further selected until 30 generations finally obtained 67-fold, 9-fold, and 9-fold resistance ratio for Cry4Aa, Cry4Ba, and Cry11Aa (Tetreau et al., 2012). Enzyme activity test with midgut showed metalloproteinase activity was significantly reduced in LiTOX strain. Furthermore, transcription changes and differentially expressed proteins were investigated using DNA microarray and differential in gel electrophoresis (DIGE). Moreover, the expression of previously identified receptors (cadherin, APN, and ALP) for Bti toxins was analyzed in RT-qPCR. This combined analysis showed ALP (AAEL003298, receptor for Cry4Ba) was consistently down-regulated in all assays and APN (AAEL012774, receptor for Cry11Aa) was up-regulated in all assays.

6. Specific objectives of this study

Bti has high activity against disease vector mosquitoes such as *Ae. aegypti* and has been used for mosquito control. However, its mechanism is still poorly understood unlike the mechanism of Cry toxins in Lepidoptera. To elucidate a toxic mechanism of Bti in mosquito, many putative receptors for Bti toxins were tested and laboratory-selected *Ae. aegypti* were analyzed. However, these results imply that Bti action may involve more than one mechanism or one receptor, target proteins of Cry toxins may be

different or shared, and some toxins may bind to more than one receptor. For instance, the LiTOX strain shows different resistance ratios for individual toxins (Paris et al., 2012; Tetreau et al., 2012) and a cadherin and APNs in *Ae. aegypti* binds Cry11Aa with very high affinity (Chen et al., 2009; Chen et al., 2009). Moreover, two cadherins in *An. gambiae* are involved in Bti toxicity (Park et al., 2009; Hua et al., 2013). Therefore, to understand Bti toxicity in mosquitoes, further studies are needed to identify the receptors involved in toxicity and additional pathways for Bti toxicity should be examined in *Ae. aegypti*. This dissertation focuses on finding a receptor protein for Cry11Aa in Cry11Aa-resistant *Ae. aegypti*. Moreover, I attempt to show that a novel cadherin acts as a receptor for Cry11Aa and investigate whether this novel cadherin is associated with Cry11Aa resistance in mosquitoes.

Hypothesis: Receptor proteins may be involved in Cry11Aa toxicity in *Aedes aegypti*.

Hypothesis 1: The *Aedes* cadherin may mediate the *in vivo* toxicity of Cry11Aa.

Our lab previously cloned a full-length *Aedes* cadherin from *Ae. aegypti* larvae and reported this protein binds Cry11Aa with high affinity (*Kd* = 16.7 nM) (Chen et al., 2009). Binding to *Aedes* cadherin is associated with the toxicity of Cry toxin. Cadherin-silenced larvae shows increased tolerance for Cry11A, but not for Cry 4Ba, which does not bind to cadherin repeats (CR7-CR11) (Rodríguez-Almazán et al., 2012; Chen et al., 2009). Based on these results, I investigated shether *Aedes* cadherin mediates the cytotoxicity of Cry11Aa with cell line expressing *Aedes* cadherin. *Manduca* cadherin was

expressed in High Five cells and showed the high toxicity of Cry1Ab without a secondary receptor (Zhang et al., 2005). In contrast, APN was suggested as a secondary receptor triggering membrane insertion and pore formation (Bravo et al., 2004; Jurat-Fuentes et al., 2004). Therefore, I tested whether *Aedes* cadherin could mediated Cry11Aa toxicity without a secondary receptor.

Aim 1.1: Determine if *Aedes* cadherin mediates the cytotoxicity of Cry11Aa with cell line expressing *Aedes* cadherin.

Hypothesis 2: N-cadherin protein may be involved in Cry11Aa toxicity.

As mentioned above, Bti action may involve more than one mechanism or one receptor. To find more functional receptors of Bti toxins, I further investigated all cadherin proteins that are in the *Aedes* genome. Based on previous research and our microarray data, I found the N-cadherins (AAEL000597 and AAEL001196) were significantly altered in Cry11Aa-treated larvae midgut. Therefore, I investigated whether N-cadherin is involved in Cry11Aa toxicity. I first tested whether N-cadherins bind Cry11Aa and N-cadherin-silenced larvae obtain tolerance to Cry11Aa toxicity. Moreover, I investigated which regions are interacted between Cry11Aa and N-cadherin.

Aim 2.1: Test whether Cry11Aa binds to N-cadherin since Cry11Aa binds to normal cadherin proteins.

Aim 2.2: Examine the hypothesis that N-cadherins are involved in Cry11Aa toxicity *in vivo*.

Aim 2.3: Investigate regions interacting between Cry11Aa and N-cadherin.

Hypothesis 3: Receptor proteins may play an important role in Cry11Aa-resistant mosquitoes.

To investigate the mechanism of Bti action in *Ae. aegypti*, we selected mosquitoes with only Cry11Aa, the most active toxin in Bti and obtained a Cry11Aa-resistant *Aedes* mosquito strain. Since resistancd mechanisms in lepidopteran insects are linked to a change in Cry toxin receptors, I expected a functional receptor of Cry11Aa would be significantly altered, for example in a mutation or a change in transcript levels in Cry11Aa-resistant mosquitoes. Therefore, transcript changes of all genes in Cry11Aa-resistant larvae midgut were analyzed using Illumina sequencing. Previously identified receptors were further investigated to determine if any of the known receptors contain any mutations or the receptor expression levels are significantly altered using Sanger sequencing and RT-qPCR.

Aim 3.1: Develop a Cry11A resistant *Ae. aegypti* strain.

Aim 3.2: Investigate an important receptor protein mediating a resistance.

Aim 3.3: Identify genes which are altered in expression or contain sequence variants.

7. References

Abdullah, M. A., Alzate, O., Mohammad, M., McNall, R. J., Adang, M. J. and Dean, D. H. (2003). Introduction of *Culex* toxicity into *Bacillus thuringiensis* Cry4Ba by protein engineering. *Appl Environ Microbiol* **69**: 5343-5353.

Abdullah, M. A., Valaitis, A. P. and Dean, D. H. (2006). Identification of a *Bacillus thuringiensis* Cry11Ba toxin-binding aminopeptidase from the mosquito, *Anopheles quadrimaculatus*. *BMC Biochem* **7**: 16.

Angsuthanasombat, C., Crickmore, N. and Ellar, D. J. (1992). Comparison of *Bacillus thuringiensis* subsp. *israelensis* CryIVA and CryIVB cloned toxins reveals synergism in vivo. *FEMS Microbiol Lett* **73**: 63-68.

Angus, T. A. (1956). Association of toxicity with protein-crystalline inclusions of Bacillus sotto Ishiwata. *Can J Microbiol* **2**: 111-121.

Aronson, A. I. and Shai, Y. (2001). Why *Bacillus thuringiensis* insecticidal toxins are so effective: unique features of their mode of action. *FEMS Microbiol Lett* **195**: 1-8.

Atsumi, S., Miyamoto, K., Yamamoto, K., Narukawa, J., Kawai, S., Sezutsu, H., Kobayashi, I., Uchino, K., Tamura, T., Mita, K., Kadono-Okuda, K., Wada, S., Kanda, K., Goldsmith, M. R. and Noda, H. (2012). Single amino acid mutation in an ATP-binding cassette transporter gene causes resistance to Bt toxin Cry1Ab in the silkworm, *Bombyx mori*. *Proc Natl Acad Sci* **109**: E1591-E1598.

Bayyareddy, K., Andacht, T. M., Abdullah, M. A. and Adang, M. J. (2009). Proteomic identification of *Bacillus thuringiensis* subsp. *israelensis* toxin Cry4Ba binding proteins in midgut membranes from *Aedes* (Stegomyia) *aegypti* Linnaeus (Diptera, Culicidae) larvae. *Insect Biochem Mol Biol* **39**: 279-286.

Bayyareddy, K., Zhu, X., Orlando, R. and Adang, M. J. (2012). Proteome analysis of Cry4Ba toxin-interacting *Aedes aegypti* lipid rafts using geLC-MS/MS. *J Proteome Res.* **11**: 5843-5855.

Ben-Dov, E., Nissan, G., Pelleg, N., Manasherob, R., Boussiba, S. and Zaritsky, A. (1999). Refined, circular restriction map of the *Bacillus thuringiensis* subsp. *israelensis* plasmid carrying the mosquito larvicidal genes. *Plasmid* **42**: 186-191.

Bernharda, K., Jarrettb, P., Meadowsb, M., Buttb, J., Ellisb, D. J., Robertsb, G. M., Paulia, S., Rodgersc, P. and Burgesb, H. D. (1997). Natural Isolates of *Bacillus thuringiensis*:Worldwide Distribution, Characterization, and Activity against Insect Pests. *J Invertebr Pathol* **70**: 59–68.

Berry, C., O'Neil, S., Ben-Dov, E., Jones, A. F., Murphy, L., Quail, M. A., Holden, M. T., Harris, D., Zaritsky, A. and Parkhill, J. (2002). Complete sequence and organization of pBtoxis, the toxin-coding plasmid of *Bacillus thuringiensis* subsp. *israelensis*. *Appl Environ Microbiol* **68**: 5082-5095.

Bonin, A., Paris, M., Tetreau, G., David, J. P. and Despres, L. (2009). Candidate genes revealed by a genome scan for mosquito resistance to a bacterial insecticide: sequence and gene expression variations. *BMC Genomics* **10**: 551.

Boonserm, P., Davis, P., Ellar, D. J. and Li, J. (2005). Crystal structure of the mosquito-larvicidal toxin Cry4Ba and its biological implications. *J Mol Biol* **348**: 363-382.

Boonserm, P., Ellar, D. J. and Li, J. (2003). Crystallization and preliminary X-ray diffraction studies of a mosquito-larvicidal toxin from *Bacillus thuringiensis* subsp. *israelensis*. *Acta Crystallogr D Biol Crystallogr* **59**: 591-594.

Boonserm, P., Mo, M., Angsuthanasombat, C. and Lescar, J. (2006). Structure of the functional form of the mosquito larvicidal Cry4Aa toxin from *Bacillus thuringiensis* at a 2.8-angstrom resolution. *J Bacteriol* **188**: 3391-3401.

Bravo, A., Gill, S. S. and Soberon, M. (2005). *Bacillus thuringiensis*: Mechanisms and Use. In Comprehensive Molecular Insect Science. Gilbert, L.I., Kostas, I., and Gill, S.S.(Ed). pp175-205. Elsevier.

Bravo, A., Gomez, I., Conde, J., Munoz-Garay, C., Sanchez, J., Miranda, R., Zhuang, M., Gill, S. S. and Soberon, M. (2004). Oligomerization triggers binding of a *Bacillus thuringiensis* Cry1Ab pore-forming toxin to aminopeptidase N receptor leading to insertion into membrane microdomains. *Biochim Biophys Acta* **1667**: 38-46.

Chen, J., Aimanova, K. G., Fernandez, L. E., Bravo, A., Soberon, M. and Gill, S. S. (2009). *Aedes aegypti* cadherin serves as a putative receptor of the Cry11Aa toxin from *Bacillus thuringiensis* subsp. *israelensis*. *Biochem J* **424**: 191-200.

Chen, J., Aimanova, K. G., Pan, S. and Gill, S. S. (2009). Identification and characterization of *Aedes aegypti* aminopeptidase N as a putative receptor of *Bacillus thuringiensis* Cry11A toxin. *Insect Biochem Mol Biol* **39**: 688-696.

Chen, J., Brown, M. R., Hua, G. and Adang, M. J. (2005). Comparison of the localization of *Bacillus thuringiensis* Cry1A delta-endotoxins and their binding proteins in larval midgut of tobacco hornworm, *Manduca sexta*. *Cell Tissue Res* **321**: 123-129.

Cheong, H., Dhesi, R. K. and Gill, S. S. (1997). Marginal cross-resistance to mosquitocidal *Bacillus thuringiensis* strains in Cry11A-resistant larvae: presence of Cry11A-like toxins in these strains. *FEMS Microbiol Lett* **153**: 419-424.

Choma, C. T., Surewicz, W. K., Carey, P. R., Pozsgay, M., Raynor, T. and Kaplan, H. (1990). Unusual proteolysis of the protoxin and toxin from *Bacillus thuringiensis:* structural implications. *Eur J Biochem* **189**: 523-527.

Cohen, S., Albeck, S., Ben-Dov, E., Cahan, R., Firer, M., Zaritsky, A. and Dym, O. (2011). Cyt1Aa toxin: crystal structure reveals implications for its membrane-perforating function. *J Mol Biol.* **413**: 804-814.

Crickmore, N., Bone, E. J., Williams, J. A. and Ellar, D. J. (1995). Contribution of the individual components of the δ-endotoxin crystal to the mosquitocidal activity of *Bacillus thuringiensis* subsp. *israelensis*. *FEMS Microbiol Lett* **131**: 249-254.

Crickmore, N., Zeigler, D. R., Feitelson, J., Schnepf, E., Van Rie, J., Lereclus, D., Baum, J. and Dean, D. H. (1998). Revision of the nomenclature for the *Bacillus thuringiensis* pesticidal crystal proteins. *Microbiol Mol Biol Rev* **62**: 807-813.

de Barjac, H. and Frachon, H. (1990). Classifiction of *Bacillus thuringiensis* strains. *Entomophaga* **35**: 233-240.

de Maagd, R. A., Bravo, A. and Crickmore, N. (2001). How *Bacillus thuringiensis* has evolved specific toxins to colonize the insect world. *Trends Genet* **17**: 193-199.

Dechklar, M., Tiewsiri, K., Angsuthanasombat, C. and Pootanakit, K. (2011). Functional expression in insect cells of glycosylphosphatidylinositol-linked alkaline phosphatase from *Aedes aegypti* larval midgut: a *Bacillus thuringiensis* Cry4Ba toxin receptor. *Insect Biochem Mol Biol.* **41**: 159-166.

Delecluse, A., Poncet, S., Klier, A. and Rapoport, G. (1993). Expression of cryIVA and cryIVB Genes, Independently or in Combination, in a Crystal-Negative Strain of *Bacillus thuringiensis* subsp. *israelensis*. *Appl Environ Microbiol* **59**: 3922-3927.

Dorsch, J. A., Candas, M., Griko, N. B., Maaty, W. S., Midboe, E. G., Vadlamudi, R. K. and Bulla, L. A. (2002). Cry1A toxins of *Bacillus thuringiensis* bind specifically to a region adjacent to the membrane-proximal extracellular domain of BT-R_1 in *Manduca sexta*: involvement of a cadherin in the entomopathogenicity of *Bacillus thuringiensis*. *Insect Biochem Mol Biol* **32**: 1025-1036.

Fabrick, J., Oppert, C., Lorenzen, M. D., Morris, K., Oppert, B. and Jurat-Fuentes, J. L. (2009). A novel tenebrio molitor cadherin is a functional receptor for *Bacillus thuringiensis* Cry3Aa toxin. *J Biol Chem* **284**: 18401-18410.

Faust, R. M., Abe, K., Held, G. A., Iizuka, T., Bulla, L. A. and Meyers, C. L. (1983). Evidence for plasmid-associated crystal toxin production in *Bacillus thuringiensis* subsp. *israelensis*. *Plasmid* **9**: 98-103.

Feldmann, F., Dullemans, A. and Waalwijk, C. (1995). Binding of the Cry1VD toxin of *Bacillus thuringiensis* subsp *israelensis* to larval dipteran midgut proteins. *Appl Environ Microbiol* **61**: 2601-2605.

Fernandez-Luna, M. T., Lanz-Mendoza, H., Gill, S. S., Bravo, A., Soberon, M. and Miranda-Rios, J. (2010). An alpha-amylase is a novel receptor for *Bacillus thuringiensis* subsp. *israelensis* Cry4Ba and Cry11Aa Toxins in the malaria vector mosquito *Anopheles albimanus* (Diptera: Culicidae). *Environ Microbiol* **12**: 746-757.

Fernandez, L. E., Aimanova, K. G., Gill, S. S., Bravo, A. and Soberon, M. (2006). A GPI-anchored alkaline phosphatase is a functional midgut receptor of Cry11Aa toxin in *Aedes aegypti* larvae. *Biochemical J* **394**: 77-84.

Fernandez, L. E., Martinez-Anaya, C., Lira, E., Chen, J., Evans, A., Hernandez-Martinez, S., Lanz-Mendoza, H., Bravo, A., Gill, S. S. and Soberon, M. (2009). Cloning and epitope mapping of Cry11Aa-binding sites in the Cry11Aa-receptor alkaline phosphatase from *Aedes aegypti*. *Biochemistry* **48**: 8899-8907.

Fernandez, L. E., Perez, C., Segovia, L., Todriguez, M. H., Gill, S. S., Bravo, A. and Soberon, M. (2005). Cry11Aa toxin from *Bacillus thuringiensis* binds its receptor in *Aedes aegypti* mosquito larvae through loop α-8 of domain II. *FEBS Lett* **579**: 3508-3514.

Flannagan, R. D., Yu, C. G., Mathis, J. P., Meyer, T. E., Shi, X., Siqueira, H. A. and Siegfried, B. D. (2005). Identification, cloning and expression of a Cry1Ab cadherin receptor from European corn borer, *Ostrinia nubilalis* (Hubner) (Lepidoptera: Crambidae). *Insect Biochem Mol Biol* **35**: 33-40.

Gahan, L. J., Gould, F. and Heckle, D. G. (2001). Identification of a gene associated with Bt resistance in *Heliothis virescens*. *Science* **293**: 857-860.

Gahan, L. J., Pauchet, Y., Vogel, H. and Heckel, D. G. (2010). An ABC transporter mutation is correlated with insect resistance to *Bacillus thuringiensis* Cry1Ac toxin. *PLoS Genet* **6**: e1001248.

Georghiou, G. P. and Wirth, M. C. (1997). Influence of exposure to single versus multiple toxins of *Bacillus thuringiensis* subsp. *israelensis* on development of resistance in the mosquito *Culex quinquefasciatus* (Diptera: Culicidae). *Appl Environ Microbiol* **63**: 1095-1101.

Gill, S. S., Cowles, E. A. and Francis, V. (1995). Identification, Isolation, and Cloning of a *Bacillus thuringiensis* CryIAc Toxin-binding Protein from the Midgut of the Lepidopteran Insect *Heliothis virescens*. *J Biol Chem* **270**: 27277-27282.

Gill, S. S., Cowles, E. A. and Pietrantonio, P. V. (1992). The mode of action of *Bacillus thuringiensis* endotoxins. *Annu Rev Entomol* **37**: 615-636.

Goldberg, L. J. and Margalit, J. (1977). A bacterial spore demonstrating rapid larvicidal activity against *Anopheles sergentii*, *Uranotaenia unguiculata*, *Culex univitattus*, *Aedes aegypti* and *Culex pipiens*. *Mosq News* **37**: 355-358.

Goldman, I. F., Arnold, J. and Carlton, B. C. (1986). Selection for resistance to *Bacillus thuringiensis* subspecies israelensis in field and laboratory populations of the mosquito *Aedes aegypti*. *J Invertebr Pathol* **47**: 317-324.

Gomez-Dantes, H. and Willoquet, J. R. (2009). Dengue in the Americas: challenges for prevention and control. *Cad Saude Publica* **25**: 19-31.

Gomez, I., Dean, D. H., Bravo, A. and Soberon, M. (2003). Molecular basis for *Bacillus thuringiensis* Cry1Ab toxin specificity: two structural determinants in the *Manduca sexta* Bt-R_1 receptor interact with loops alpha-8 and 2 in domain II of Cy1Ab toxin. *Biochemistry* **42**: 10482-10489.

Gomez, I., Oltean, D. I., Gill, S. S., Bravo, A. and Soberon, M. (2001). Mapping the epitope in cadherin-like receptors involved in *Bacillus thuringiensis* Cry1A toxins interaction using phage display. *J Biol Chem* **276**: 28906-28912.

Gomez, I., Sanchez, J., Miranda, R., Bravo, A. and Soberon, M. (2002). Cadherin-like receptor binding facilitates proteolytic cleavage of helix alpha-1 in domain I and oligomer pre-pore formation of Bacillus thuringiensis Cry1Ab toxin. *FEBS Lett* **513**: 242-246.

Gonzalez, J. M., Jr., Brown, B. J. and Carlton, B. C. (1982). Transfer of *Bacillus thuringiensis* plasmids coding for delta-endotoxin among strains of *B. thuringiensis* and *B. cereus*. *Proc Natl Acad Sci U S A* **79**: 6951-6955.

Griffitts, J. S., Haslam, S. M., Yang, T., Garczynski, S. F., Mulloy, B., Morris, H., Cremer, P. S., Dell, A., Adang, M. J. and Aroian, R. V. (2005). Glycolipids as Receptors for *Bacillus thuringiensis* Crystal Toxin. *Science* **307**: 922 - 925.

Grochulski, P., Masson, L., Borisova, S., Pusztai-Carey, M., Schwartz, J. L., Brousseau, R. and Cygler, M. (1995). *Bacillus thuringiensis* CryIA(a) insecticidal toxin: crystal structure and channel formation. *J Mol Biol* **254**: 447-464.

Guerchicoff, A., Delecluse, A. and Rubinstein, C. P. (2001). The *Bacillus thuringiensis* cyt genes for hemolytic endotoxins constitute a gene family. *Appl Environ Microbiol* **67**: 1090-1096.

Guerchicoff, A., Ugalde, R. A. and Rubinstein, C. P. (1997). Identification and characterization of a previously undescribed cyt gene in *Bacillus thuringiensis* subsp. *israelensis*. *Appl Environ Microbiol* **63**: 2716-2721.

Harris, A. F., Rajatileka, S. and Ranson, H. (2010). Pyrethroid resistance in *Aedes aegypti* from Grand Cayman. *Am J Trop Med Hyg* **83**: 277-284.

Herrero, S., Gechev, T., Bakker, P. L., Moar, W. J. and de Maagd, R. A. (2005). *Bacillus thuringiensis* Cry1Ca-resistant *Spodoptera exigua* lacks expression of one of four Aminopeptidase N genes. *BMC Genomics* **6**: 96.

Hofte, H. and Whiteley, H. R. (1989). Insecticidal crystal proteins of *Bacillus thuringiensis*. *Microbiol Rev* **53**: 242-255.

Hua, G., Zhang, Q., Zhang, R., Abdullah, A. M., Linser, P. J. and Adang, M. J. (2013). AgCad2 cadherin in *Anopheles gambiae* larvae is a putative receptor of Cry11Ba toxin of *Bacillus thuringiensis* subsp. *jegathesan*. *Insect Biochem Mol Biol.* **43**: 153-161.

Hua, G., Zhang, R., Abdullah, M. A. and Adang, M. J. (2008). Anopheles gambiae Cadherin AgCad1 Binds the Cry4Ba Toxin of *Bacillus thuringiensis israelensis* and a Fragment of AgCad1 Synergizes Toxicity. *Biochemistry* **47**: 5101-5110.

Hua, G., Zhang, R., Bayyareddy, K. and Adang, M. J. (2009). *Anopheles gambiae* alkaline phosphatase is a functional receptor of *Bacillus thuringiensis jegathesan* Cry11Ba toxin. *Biochemistry* **48**: 9785-9793.

Jenkins, J. L. and Dean, D. H. (2000). Exploring the mechanism of action of insecticidal proteins by genetic engineering methods. *Genet Eng* **22**: 33-54.

Jimenez-Juarez, N., Munoz-Garay, C., Gomez, I., Saab-Rincon, G., Damian-Almazo, J. Y., Gill, S. S., Soberon, M. and Bravo, A. (2007). *Bacillus thuringiensis* Cry1Ab mutants affecting oligomer formation are non-toxic to *Manduca sexta* larvae. *J Biol Chem* **282**: 21222-21229.

Jiménez, A. I., Reyes, E. Z., Cancino-Rodezno, A., Bedoya-Pérez, L. P., Caballero-Flores, G. G., Muriel-Millan, L. F., Likitvivatanavong, S., Gill, S. S., Bravo, A. and Soberón, M. (2012). *Aedes aegypti* alkaline phosphatase ALP1 is a functional receptor of *Bacillus thuringiensis* Cry4Ba and Cry11Aa toxins. *Insect Biochem Mol Biol* **42**: 683-689.

Jurat-Fuentes, J. L. and Adang, M. J. (2004). Characterization of a Cry1Ac-receptor alkaline phosphatase in susceptible and resistant *Heliothis virescens* larvae. *Eur J Biochem* **271**: 3127-3135.

Jurat-Fuentes, J. L., Gould, F. L. and Adang, M. J. (2003). Dual resistance to *Bacillus thuringiensis* Cry1Ac and Cry2Aa toxins in *Heliothis virescens* suggests multiple mechanisms of resistance. *Appl Environ Microbiol* **69**: 5898-5906.

Knight, P. J., Knowles, B. H. and Ellar, D. J. (1995). Molecular cloning of an insect aminopeptidase N that serves as a receptor for *Bacillus thuringiensis* CryIA(c) toxin. *J Biol Chem* **270**: 17765-17770.

Knowles, B. H., Blatt, M. R., Tester, M., Horsnell, J. M., Carroll, J., Menestrina, G. and Ellar, D. J. (1989). A cytolytic delta-endotoxin from *Bacillus thuringiensis* var. *israelensis* forms cation-selective channels in planar lipid bilayers. *FEBS Lett* **244**: 259-262.

Knowles, B. H. and Ellar, D. J. (1987). Colloid-osmotic lysis is a general feature of the mechanism of action of *Bacillus thuringiensis* δ-endotoxins with different insect specificity. *Biochimica et Biophysica Acta* **924**: 509-518.

Lacey, L. A. (2007). *Bacillus thuringiensis* serovariety *israelensis* and *Bacillus sphaericus* for mosquito control. *J Am Mosq Control Assoc* **23**: 133-163.

Lailak, C., Khaokhiew, T., Promptmas, C., Promdonkoy, B., Pootanakit, K. and C., A. (2013). *Bacillus thuringiensis* Cry4Ba toxin employs two receptor-binding loops for synergistic interactions with Cyt2Aa2. *Biochem Biophys Res Commun* **435**: 216-221.

Lecadet, M. M., Frachon, E., Dumanoir, V. C., Ripouteau, H., Hamon, S., Laurent, P. and Thiéry, I. (1999). Updating the H-antigen classification of *Bacillus thuringiensis*. *J Appl Microbiol* **86**: 660-672.

Li, J., Koni, P. A. and Ellar, D. J. (1996). Structure of the mosquitocidal delta-endotoxin CytB from *Bacillus thuringiensis* sp. *kyushuensis* and implications for membrane pore formation. *J Mol Biol* **257**: 129-152.

Li, J. D., Carroll, J. and Ellar, D. J. (1991). Crystal structure of insecticidal delta-endotoxin from *Bacillus thuringiensis* at 2.5 A resolution. *Nature* **353**: 815-821.

Ligon, B. L. (2006). Reemergence of an unusual disease: the chikungunya epidemic. *Semin Pediatr Infect Dis* **17**: 99-104.

Lorence, A., Darszon, A. and Bravo, A. (1997). The pore formation activity of *Bacillus thuringiensis* Cry1Ac toxin on *Trichoplusia ni* membranes depends on the presence of aminopeptidase N. *FEBS Lett* **414**: 303-307.

Luthy, P. and Ebersold, H. R. (1981). *Bacillus thuringiensis* delta-endotoxin: Histopathology and molecular mode of action. In: Pathegenesis of Invertebrate Microbiol Disease. Davison, E. W. (Ed). pp235-267. Allenheld, Osmun & Co. Publishers, Inc., Totowa. N.J.

Martin, P. A. and Travers, R. S. (1989). Worldwide Abundance and Distribution of *Bacillus thuringiensis* Isolates. *Appl Environ Microbiol* **55**: 2437-2442.

McNall, R. J. and Adang, M. J. (2003). Identification of novel *Bacillus thuringiensis* Cry1Ac binding proteins in *Manduca sexta* midgut through proteomic analysis. *Insect Biochem Mol Biol* **33**: 999-1010.

Mittal, P. K. (2003). Biolarvicides in vector control: challenges and prospects. *J Vector Borne Dis* **40**: 20-32.

Morin, S., Biggs, R. W., Sisterson, M. S., Shriver, L., Ellers-Kirk, C., Higginson, D., Holley, D., Gahan, L. J., Heckel, D. G., Carriere, Y., Dennehy, T. J., Brown, J. K. and Tabashnik, B. E. (2003). Three cadherin alleles associated with resistance to *Bacillus thuringiensis* in pink bollworm. *Proc Natl Acad Sci U S A* **100**: 5004-5009.

Nagamatsu, Y., Toda, S., Yamaguchi, F., Ogo, M., Kogure, M., Nakamura, M., Shibata, Y. and Katsumoto, T. (1998). Identification of *Bombyx mori* midgut receptor for *Bacillus thuringiensis* insecticidal CryIA(a) toxin. *Biosci Biotechnol Biochem* **62**: 718-726.

Otieno-Ayayo, Z. N., Zaritsky, A., Wirth, M. C., Manasherob, R., Khasdan, V., Cahan, R. and Ben-Dov, E. (2008). Variations in the mosquito larvicidal activities of toxins from *Bacillus thuringiensis* ssp. *israelensis*. *Environ Microbiol* **10**: 2191-2199.

Paris, M. and Despres, L. (2012). Identifying insecticide resistance genes in mosquito by combining AFLP genome scans and 454 pyrosequencing. *Mol Ecol* **21**: 1672-1686.

Paris, M., Melodelima, C., Coissac, E., Tetreau, G., Reynaud, S., David, J. P. and Despres, L. (2012). Transcription profiling of resistance to Bti toxins in the mosquito *Aedes aegypti* using next-generation sequencing. *J Invertebr Pathol* **109**: 201-208.

Paris, M., Tetreau, G., Laurent, F., Lelu, M., Despres, L. and David, J. P. (2011). Persistence of *Bacillus thuringiensis israelensis* (Bti) in the environment induces resistance to multiple Bti toxins in mosquito. *Pest Manag Sci* **67**: 122-128.

Park, Y., Hua, G., Abdullah, M. A., Rahman, K. and Adang, M. J. (2009). Cadherin fragments from *Anopheles gambiae* synergize *Bacillus thuringiensis* Cry4Ba's toxicity against *Aedes aegypti* larvae. *Appl Environ Microbiol* **75**: 7280-7282.

Parker, M. W., Pattus, F., Tucker, A. D. and Tsernoglou, D. (1989). Structure of the membrane-pore-forming fragment of colicin A. *Nature* **337**: 93-96.

Perez, C., Fernandez, L. E., Sun, J., Folch, J. L., Gill, S. S., Soberon, M. and Bravo, A. (2005). *Bacillus thuringiensis* subsp. *israelensis* Cyt1Aa synergizes Cry11Aa toxin by functioning as a membrane-bound receptor. *Proc Natl Acad Sci U S A* **102**: 18303-18308.

Perez, C., Munoz-Garay, C., Portugal, L. C., Sanchez, J., Gill, S. S., Soberon, M. and Bravo, A. (2007). *Bacillus thuringiensis* ssp. *israelensis* Cyt1Aa enhances activity of Cry11Aa toxin by facilitating the formation of a pre-pore oligomeric structure. *Cell Microbiol* **9**: 2931-2937.

Poncet, S., Delécluse, A., Klier, A. and Rapoport, G. (1995). Evaluation of Synergistic Interactions among the CryIVA, CryIVB, and CryIVD Toxic Components of *B. thuringiensis* subsp. *israelensis* Crystals. *J Invertebr Pathol* **66**: 131-135.

Promdonkoy, B., Promdonkoy, P. and Panyim, S. (2005). Co-expression of *Bacillus thuringiensis* Cry4Ba and Cyt2Aa2 in *Escherichia coli* revealed high synergism against *Aedes aegypti* and *Culex quinquefasciatus* larvae. *FEMS Microbiol Lett* **252**: 121-126.

Ragni, A., Thiery, I. and Delecluse, A. (1996). Characterization of six highly mosquitocidal *Bacillus thuringiensis* strains that do not belong to H-14 serotype. *Curr Microbiol* **32**: 48-54.

Rasko, D. A., Altherr, M. R., Han, C. S. and Ravel, J. (2005). Genomics of the *Bacillus cereus* group of organisms. *FEMS Microbiol Rev* **29**: 303-329.

Rodríguez-Almazán, C., Reyes, E. Z., Zúñiga-Navarrete, F., Muñoz-Garay, C., Gómez, I., Evans, A. M., Likitvivatanavong, S., Bravo, A., Gill, S. S. and Soberón, M. (2012). Cadherin binding is not a limiting step for *Bacillus thuringiensis* subsp. *israelensis* Cry4Ba toxicity to *Aedes aegypti* larvae. *Biochem J* **443**: 711-717.

Rodríguez, M. M., Bisset, J. A. and Fernández, D. (2007). Levels of insecticide resistance and resistance mechanisms in *Aedes aegypti* from some Latin American countries. *J Am Mosq Control Assoc* **23**: 420-429.

Saengwiman, S., Aroonkesorn, A., Dedvisitsakul, P., Sakdee, S., Leetachewa, S., Angsuthanasombat, C. and Pootanakit, K. (2011). *In vivo* identification of *Bacillus thuringiensis* Cry4Ba toxin receptors by RNA interference knockdown of glycosylphosphatidylinositol-linked aminopeptidase N transcripts in *Aedes aegypti* larvae. *Biochem Biophys Res Commun* **407**: 708-713.

Saleh, M. S., El-Meniawi, F. A., Kelada, N. L. and Zahran, H. M. (2003). Resistance development in mosquito larvae *Culex pipiens* to the bacterial agent *Bacillus thuringiensis* var. *israelensis*. *J Appl Microbiol* **127**: 29-32.

Sangadala, S., Walters, F. S., English, L. H. and Adang, M. J. (1994). A mixture of *Manduca sexta* aminopeptidase and phosphatase enhances *Bacillus thuringiensis* insecticidal CryIA(c) toxin binding and 86Rb(+)- K+ efflux in vitro. *J Biol Chem* **269**: 10088-10092.

Schnepf, E., Crickmore, N., Van Rie, J., Lereclus, D., Baum, J., Feitelson, J., Zeigler, D. R. and Dean, D. H. (1998). *Bacillus thuringiensis* and its pesticidal crystal proteins. *Microbiol Mol Biol Rev* **62**: 775-806.

Schwartz, J. L., Potvin, L., Chen, X. J., Brousseau, R., Laprade, R. and Dean, D. H. (1997). Single-site mutations in the conserved alternating-arginine region affect ionic channels formed by CryIAa, a *Bacillus thuringiensis* toxin. *Appl Environ Microbiol* **63**: 3978-3984.

Singh, G. J. P., Schouest, L. P. and Gill, S. S. (1986). The toxic action of *Bacillus thuringiensis* var. *israelensis* in *Aedes aegypti in vivo*: I. The relevance of midgut lesions to its poisoning syndrome. *Pestic Biochem Physiol* **26**: 36-46.

Soberon, M., Gill, S. S. and Bravo, A. (2009). Signaling versus punching hole: How do *Bacillus thuringiensis* toxins kill insect midgut cells? *Cell Mol Life Sci* **66**: 1337-1349.

Tabashnik, B. E., Gassmann, A. J., Crowder, D. W. and Carriere, Y. (2008). Insect resistance to Bt crops: evidence versus theory. *Nat Biotechnol* **26**: 199-202.

Tetreau, G., Bayyareddy, K., Jones, C. M., Stalinski, R., Riaz, M. A., Paris, M., David, J. P., Adang, M. J. and Després, L. (2012). Larval midgut modifications associated with Bti resistance in the yellow fever mosquito using proteomic and transcriptomic approaches. *BMC Genomics* **13**: 248.

Thammasittirong, A., Dechklar, M., Leetachewa, S., Pootanakit, K. and Angsuthanasombat, C. (2011). *Aedes aegypti* membrane-bound alkaline phosphatase expressed in *Escherichia coli* retains high-affinity binding for *Bacillus thuringiensis* Cry4Ba toxin. *Appl Environ Microbiol* **77**: 6836-6840.

Tiewsiri, K. and Wang, P. (2011). Differential alteration of two aminopeptidases N associated with resistance to *Bacillus thuringiensis* toxin Cry1Ac in cabbage looper. *Proc Natl Acad Sci U S A* **108**: 14037-14042.

Tomori, O. (2004). Yellow fever: the recurring plague. *Crit Rev Clin Lab Sci* **41**: 391-427.

Vadlamudi, R. K., Ji, T. H. and Bulla, L. A. J. (1993). A Specific Binding Protein from *Manduca sexta* for the Insecticidal Toxin of *Bacillus thuringiensis* subsp. *bediner*. *J Biol Chem* **268**: 12334-12340.

Vadlamudi, R. K., Weber, E., Ji, I., Ji, T. H. and Bulla, L. A., Jr. (1995). Cloning and expression of a receptor for an insecticidal toxin of *Bacillus thuringiensis*. *J Biol Chem* **270**: 5490-5494.

WHO (1997). Dengue haemorrhagic fever: diagnosis, tratment, prevention and control, Geneva: World Health Organization.

WHO (1998). Yellow fever-Technical Consensus Meeting, In: Emerging and other Communcable Diseases, Surveillance and Control, Geneva: World Health Organization.

WHO (2002). Dengue and Dengue Haemorrhagic Fever, Geneva: World Health Organization.

Wirth, M. C., Delecluse, A., Federici, B. A. and Walton, W. E. (1998). Variable cross-resistance to Cry11B from *Bacillus thuringiensis* subsp. *jegathesan* in *Culex quinquefasciatus* (Diptera: Culicidae) resistant to single or multiple toxins of *Bacillus thuringiensis* subsp. *israelensis*. *Appl Environ Microbiol* **64**: 4174-4179.

Wirth, M. C., Georghiou, G. P. and Federici, B. A. (1997). CytA enables CryIV endotoxins of *Bacillus thuringiensis* to overcome high levels of CryIV resistance in the mosquito, *Culex quinquefasciatus*. *Proc Natl Acad Sci U S A* **94**: 10536-10540.

Wirth, M. C., Park, H. W., Walton, W. E. and Federici, B. A. (2005). Cyt1A of *Bacillus thuringiensis* delays evolution of resistance to Cry11A in the mosquito *Culex quinquefasciatus*. *Appl Environ Microbiol* **71**: 185-189.

Wu, D., Johnson, J. J. and Federici, B. A. (1994). Synergism of mosquitocidal toxicity between CytA and CryIVD proteins using inclusions produced from cloned genes of *Bacillus thuringiensis*. *Mol Microbiol* **13**: 965-972.

Xu, X., Yu, L. and Wu, Y. (2005). Disruption of a cadherin gene associated with resistance to Cry1Ac {delta}-endotoxin of *Bacillus thuringiensis* in *Helicoverpa armigera*. *Appl Environ Microbiol* **71**: 948-954.

Yamagiwa, M., Ogawa, R., Yasuda, K., Natsuyama, H., Sen, K. and Sakai, H. (2002). Active form of dipteran-specific insecticidal protein cryllA produced by *Bacillus thuringiensis* subsp. *israelensis*. *Biosci Biotechnol Biochem* **66**: 516-522.

Yang, Y., Chen, H., Wu, Y., Yang, Y. and Wu, S. (2007). Mutated cadherin alleles from a field population of *Helicoverpa armigera* confer resistance to *Bacillus thuringiensis* toxin Cry1Ac. *Appl Environ Microbiol* **73**: 6939-6944.

Zhang, H., Wu, S., Yang, Y., Tabashnik, B. E. and Wu, Y. (2012). Non-recessive Bt toxin resistance conferred by an intracellular cadherin mutation in field-selected populations of cotton bollworm. *PLoS One* **7**: e53418.

Zhang, Q., Hua, G., Bayyareddy, K. and Adang, M. J. (2013). Analyses of α-amylase and α-glucosidase in the malaria vector mosquito, Anopheles gambiae, as receptors of Cry11Ba toxin of Bacillus thuringiensis subsp. jegathesan. *Insect Biochem Mol Biol.*: In press.

Zhang, R., Hua, G., Andacht, T. M. and Adang, M. J. (2008). A 106-kDa aminopeptidase is a putative receptor for *Bacillus thuringiensis* Cry11Ba toxin in the mosquito *Anopheles gambiae*. *Biochemistry* **47**: 11263-11272.

Zhang, R., Hua, G., Urbauer, J. L. and Adang, M. J. (2010). Synergistic and inhibitory effects of aminopeptidase peptides on *Bacillus thuringiensis* Cry11Ba toxicity in the mosquito *Anopheles gambiae*. *Biochemistry* **49**: 8512-8519.

Zhang, S., Cheng, H., Gao, Y., Wang, G., Liang, G. and Wu, K. (2009). Mutation of an aminopeptidase N gene is associated with *Helicoverpa armigera* resistance to *Bacillus thuringiensis* Cry1Ac toxin. *Insect Biochem Mol Biol* **39**: 421-429.

Zhang, X., Candas, M., Griko, N. B., Rose-Young, L. and Bulla, L. A., Jr. (2005). Cytotoxicity of *Bacillus thuringiensis* Cry1Ab toxin depends on specific binding of the toxin to the cadherin receptor BT-R_1 expressed in insect cells. *Cell Death Differ* **12**: 1407-1416.

Zhang, X., Candas, M., Griko, N. B., Taussig, R. and Bulla, L. A. J. (2006). A mechanism of cell death involving an adenylyl cyclase/PKA signaling pathway is induced by the Cry1Ab toxin of *Bacillus thuringiensis*. *Proc Natl Acad Sci* **103**: 9897-9902.

Zhuang, M., Oltean, D. I., Gomez, I., Pullikuth, A. K., Soberon, M., Bravo, A. and Gill, S. S. (2002). *Heliothis virescens* and *Manduca sexta* lipid rafts are involved in Cry1A toxin binding to the midgut epithelium and subsequent pore formation. *J Biol Chem* **277**: 13863-13872.

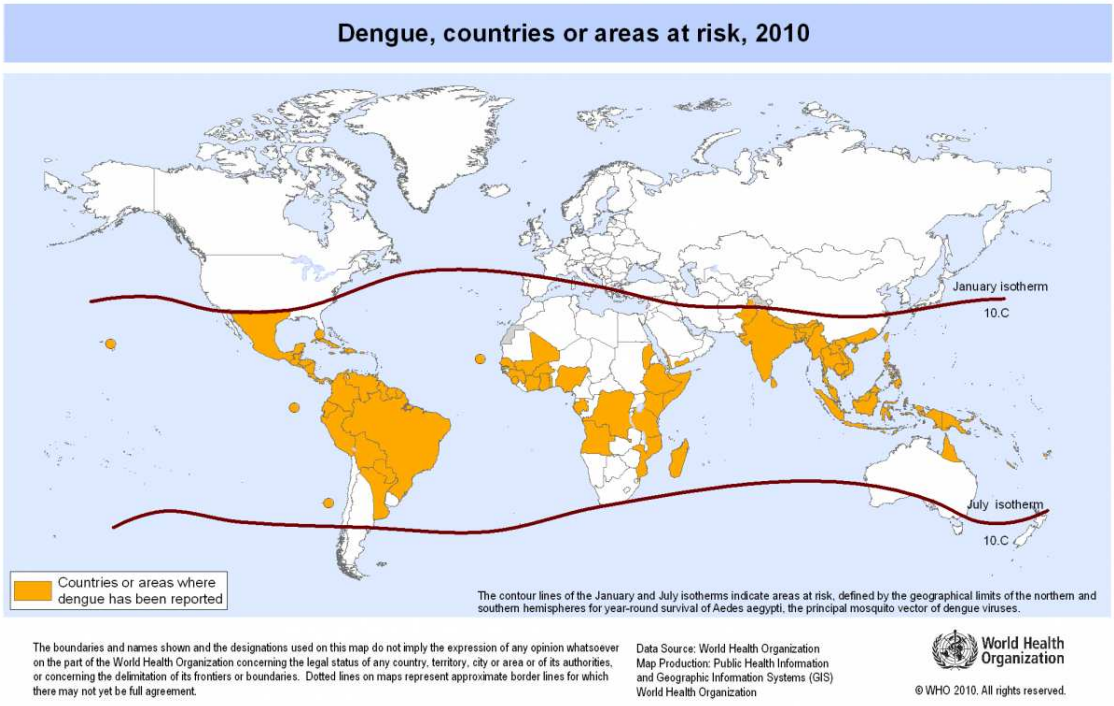


Figure 1.1. Dengue fever occurs in subtropical and tropical regions in the world. Data from WHO.

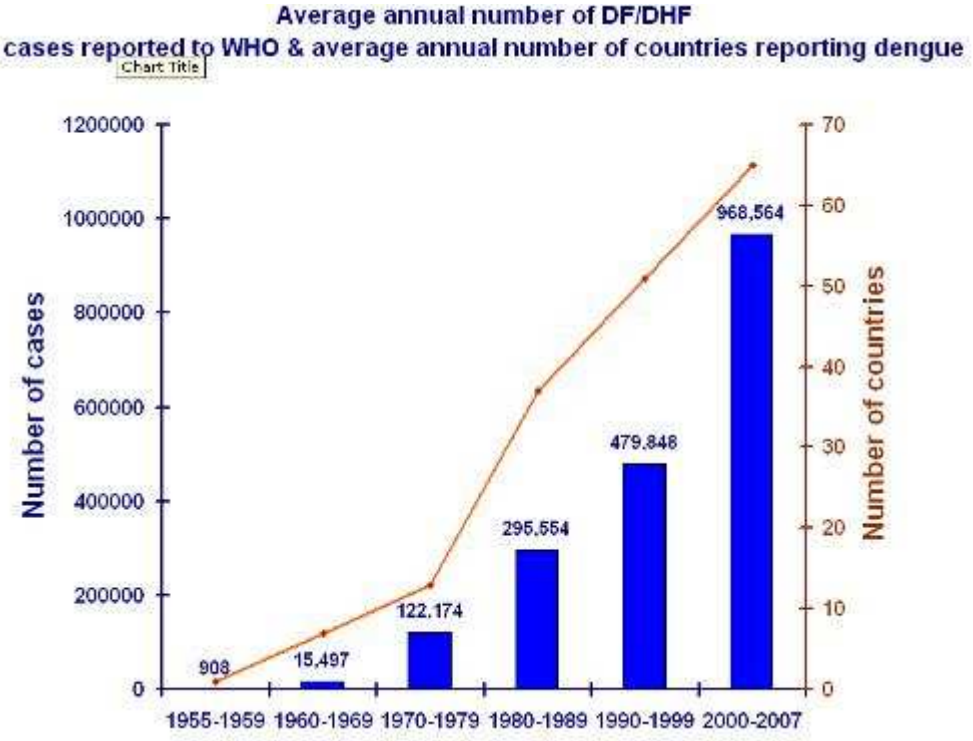


Figure 1.2. Dengue fever and dengue hemorrhagic fever incidents are increasing. Data from WHO.

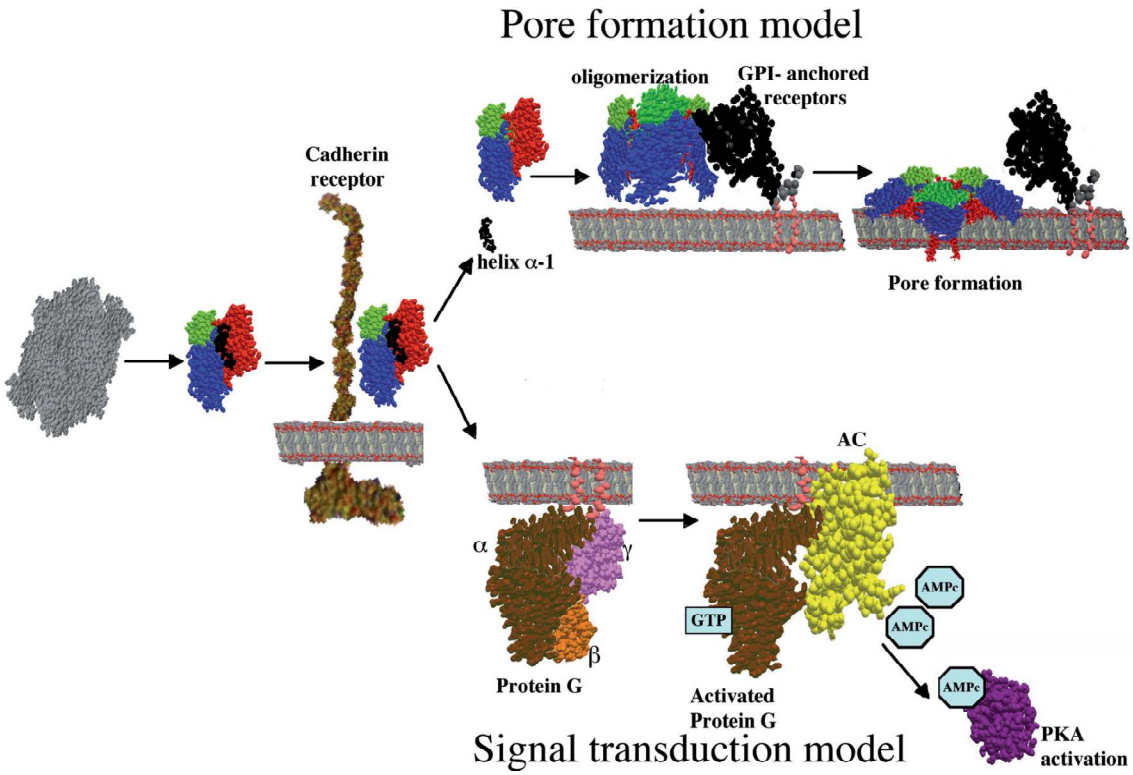


Figure 1.3. Two models are suggested for the mode of action of Cry toxin in Lepidoptera. On binding to cadherin, the Cry toxin oligomerizes, inserts into membranes, and forms pores (pore formation model) or alternatively after binding to cadherin, it activates a G protein, adenylyl cyclase, and protein kinase A resulting in cell death (signal transduction model). Figure from Soberon et al, 2009.

Table 1.1. Summary of Cry and Cyt toxin specificity for Diptera, Figure from Natural Resources Canada (www.nrcan-rncan.gc.ca). Toxins are indicated as active (●), not active (○) or possibly active (?).

Genus	Species	1Ab	1Ac	1Ba	1Bc	1Bd	1Ca	1Gb	1Ia	2Aa	2Ab	2Ac	3Aa	4Aa	4Ba	9Bb	9Ec	10Aa	11Aa	11Ba	11Bb	15Aa	16Aa	17Aa	19Aa	19Ba	20Aa	24Ba	24Ca
Aedes	*aegypti*	●		○			●			?	?	○	○	●	●	○		?	●	●	●	○	●	○	○		●	○	●
Aedes	*triseriatus*									●																			
Anopheles	*stephensi*									●				●	●			○	●	●			●	○	●	○		○	
Anopheles	*gambiae*						●				●			●	●														
Culex	*pipiens*									○				●	○		○	○	●	●			●	○	●	●		○	
Culex	*fatigans*									●																			
Culex	*pervigalans*								○																				
Culex	*quinquefasciatus*						●			?				●	●				●	●	●								
Culex	*tritaeniorhynchus*																												
Anopheles	*quadrimaculatus*									●																			
Anopheles	*albimanus*																		●	●	●								
Chironomus	*tepperi*													○	●			○	●										
Chironomus	*riparius*																												
Tipula	*paludosa*													○	○				○										
Tipula	*oleracae*								○						●				●										
Lucilia	*cuprina*			●																									
Lucilia	*sericata*																												
Calliphora	*stygia*																												
Glossina	*mortisans*		●																										
Musca	*domestica*			●	●	○		○		○			○			○													
Drosophila	*melanogaster*									○																			
Clogmia	*albipunctata*																												
Liriomyza	*trifoli*																												

Table 1.1. Continued.

Genus	Species	27Aa	29Aa	30Aa	30Ba	30Ca	30Fa	31Aa	32Aa	32Ba	32Ca	32Da	33Aa	39Aa	40Aa	40Ba	44Aa	47Aa	48Aa	49Aa	48/49A	54Aa	cyt1Aa	cyt1Ab	cyt1Ba	cyt2Aa	cyt2Ba	cyt2Bc
Aedes	*aegypti*	○	○	○	○	○	●		○	●	●	●					●		○	○	○	●	●	●		●	●	●
Aedes	*triseriatus*																											
Anopheles	*stephensi*	●	○	○	○	○			○					●	○	○	●						●	●			●	●
Anopheles	*gambiae*																		○	○	○		●			●		
Culex	*pipiens*	○	○	○	○	○		○					○	●	○	○	●						●	●		●	●	●
Culex	*fatigans*																											
Culex	*pervigalans*																											
Culex	*quinquefasciatus*								○										○	○	●		●			●	●	●
Culex	*tritaeniorhynchus*								○																			
Anopheles	*quadrimaculatus*																											
Anopheles	*albimanus*																											
Chironomus	*tepperi*																						●					
Chironomus	*riparius*																		○	○								
Tipula	*paludosa*																						●					
Tipula	*oleracae*																											
Lucilia	*cuprina*																	●					●			○		
Lucilia	*sericata*																						●			○		
Calliphora	*stygia*																						●			○		
Glossina	*mortisans*																											
Musca	*domestica*																											
Drosophila	*melanogaster*																											
Clogmia	*albipunctata*												○															
Liriomyza	*trifoli*																								●			

Chapter 2

Aedes cadherin mediates *in vivo* toxicity of *Bacillus thuringiensis* Cry11A toxin to *Aedes aegypti*

Abstract

Cadherin plays an important role in the toxicity of *Bacillus thuringiensis* Cry proteins. We previously cloned a full-length *Aedes* cadherin from *Aedes aegypti* larvae and reported this protein binds Cry11Aa toxin from *Bacillus thuringiensis* subsp. *israelensis* with high affinity, ≈ 16.7 nM. We also have data showing that a cadherin fragment synergizes Cry11A toxicity to mosquito larvae by 2-3 fold. Based on these results, we investigated if *Aedes* cadherin is involved in *in vivo* toxicity of Cry11A toxin to *Ae aegypti*. We established a mosquito cell line stably expressing the full-length *Aedes* cadherin. We investigated receptor expression by western blotting with a cadherin-specific antibody and by immunofluorescence staining using confocal microscopy. The toxicity of Cry11A to these cells was analyzed in a cytotoxicity assay using activated Cry11A toxin at final concentrations up to 400 nM. Cells expressing the *Aedes* cadherin showed sensitivity to the toxin. Cry11A toxin at 400 nM killed approximately 37% of the cells in 3 h. These results show the *Aedes* cadherin plays a pivotal role in Cry11A-mediated toxicity to *Ae. aegypti* larvae, but since high toxicity was not obtained, an additional receptor may be needed for manifestation of full toxicity.

Introduction

Bacillus thuringiensis, is a member of the *Bacillus cereus* group in the *Bacillaceae* family, which also contains *B. anthracis*, *B. cereus*, *B. mycoides*, *B. pseudomycoides* and *B. weihenstephanensis* (Rasko et al., 2005). Unlike the other bacillus species *B. thuringiensis* is pathogenic to insects by producing insecticidal proteins, which consists of one or more proteins, called Cry or Cyt toxins (Gill et al., 1992). Because these proteins are highly selective to the target insect and harmless to humans and vertebrates, they have been used for the control of lepidopteran, dipteran and coleopteran insect pests in agriculture and public health (Crickmore et al., 1998).

B. thuringiensis subsp. *israelensis* (Bti) has been used for the control of mosquitoes, like *Aedes aegypti*, an important vector of human diseases such as dengue fever, chikungunya and yellow fever. Bti also has high toxicity to other human disease vectors like *Simulium damnosum* (the vector of onchocerciases), *Culex* species (vectors of filariasis and West Nile fever) and to a less extent some *Anopheles* species (vectors of malaria) (Margalith et al., 2000). The control of *Ae. aegypti* and *Culex* species has been attempted by eliminating breeding sites, using predators and by using chemical insecticides. However, this control is still difficult because of limitations in application or the development of insect resistance. Because Bti has high insecticidal activity and the low toxicity to other organisms, it is used as an alternative and environmental friendly method for control of mosquito and black fly populations.

The mode of action of Cry toxins is fairly well understood in Lepidoptera. The toxins are ingested by susceptible larvae and transported to midgut. The crystal inclusions dissolve in the alkaline environment of midgut and the protoxins are cleaved by midgut proteases (Choma et al., 1990). Cleaved toxins then bind specific protein receptors located in the brush border membrane of target insect midguts (Schnepf et al., 1998; de Maagd et al., 2001). Toxin binding triggers formation of lytic pores, which kill midgut cells and ultimately the insect (Schnepf et al., 1998; Aronson et al., 2001). Interaction between receptor proteins and toxins determines Cry toxin selectivity (Jenkins et al., 2000). Therefore, the identification of receptors that interact with Cry toxin is important to understand the molecular basis of insect selectivity.

Many putative Cry toxin receptors have been reported and their function has been studied in lepidopteran insects – cadherins, ABCC transporters, aminopeptidases (APN), alkaline phosphatases (ALP) and glycolipids (Gill et al., 1995; Griffitts et al., 2005; McNall et al., 2003; Vadlamudi et al., 1993; Atsumi et al., 2012; Sangadala et al., 1994). The cadherins represent a large family of glycoproteins that are classically responsible for intercellular contact. An insect cadherin is important since it is the first event in the interaction of Cry toxins with the brush border membrane of target insect midgut (Bravo et al., 2005). In Lepidoptera, binding of Cry toxins to cadherin causes a mild denaturation of Cry toxin and proteolytic cleavage of helix α-1. Cleavage of this helix results in a conformational change and the formation of a molten globule state of the monomer exposing hydrophobic regions (Gomez et al., 2002). This conformational change is thought to result in the formation of oligomers that bind to a second protein, which is

thought to be APN or ALP anchored to the membrane by a GPI anchor (Bravo et al., 2004; Fernandez et al., 2006). APN facilitates oligomer insertion into membranes of midgut epithelial cells, through lipid rafts (Bravo et al., 2004). Inserted oligomers form a lytic pore, which leads to cell death and ultimately that of the insect.

As previously mentioned, Bti has high activity against disease vector mosquitoes like *Ae. aegypti* and has been used for mosquito control. However, its mechanism in mosquitoes is still largely unknown unlike the mechanism of Cry toxins in Lepidoptera. This bacterium has a megaplasmid, pBtoxis, which encodes a number of toxins (Cry4Aa, Cry4Ba, Cry10Aa, Cry11Aa, Cyt1Aa, Cyt1Ca and Cyt2Ba) (Berry et al., 2002). Among them, Cry11Aa is probably the most active toxin to *Ae. aegypti* (Chilcott et al., 1988). Fernandez et al. (2005) reported domain II of Cry11Aa is important in receptor recognition and binding. Domain II contains four putative loop regions, α-8, 1, 2 and 3. It was revealed that loop α-8 in Cry11Aa was involved in toxicity and receptor binding by competitive binding assay, peptide-displaying phages and mutagenesis (Fernandez et al., 2005).

Many putative Cry toxin receptors were identified in mosquitoes (Likirvivatanavong et al., 2011). An APN from *Anopheles quadrimaculatus* bound Cry11Ba, and a cadherin receptor from *An. gambiae* was identified and bound Cry4Ba (Abdullah et al., 2006; Hua et al., 2008; Zhang et al., 2008). In *Ae. aegypti*, Cry11Aa bound four proteins (200, 100, 65 and 62 kDa) in brush border membrane vesicles (BBMV) isolated from *Ae. aegypti* midgut epithelia (Fernandez et al., 2006). Among them, a 65kDa protein was identified as a GPI-anchored ALP and a functional receptor of

Cry11Aa toxin in *Ae. aegypti* midgut cells. In addition, no APN activity was observed among GPI-anchored proteins that bound Cry11Aa toxin. In a previous study, we showed that the *Aedes* cadherin bound Cry11Aa with high affinity (Chen et al., 2009). We cloned an *Aedes* cadherin cDNA (AAEL007478 and AAEL007488), which is homologous to the lepidopteran Bt-R_1 that mediates Cry1A toxicity in Lepidoptera. A partial fragment of the *Aedes* cadherin bound Cry11Aa with high affinity. This finding suggests that the cadherin is associated with the insecticidal activity of this Cry toxin. Some ALPs were suggested as a receptor of Bti toxins. ALP (AAEL009077) bound Cry11Aa and mediated Cry11Aa as well as Cry4Ba toxicity (Fernandez et al., 2009; Rodríguez-Almazán et al., 2012). AAEL003298, AALE003313, and AAEL015070 were located in lipid rafts and bound Cry4Ba (Bayyareddy et al., 2012; Bayyareddy et al., 2009). Moreover, *Aedes* ALP (AAEL015070) bound Cry4Ba with high affinity and mediated Cry4Ba toxicity in Sf9 cells expressing *Aedes* ALP (AAEL015070) (Dechklar et al., 2011; Thammasittirong et al., 2011).

Based on these results, we investigated further whether *Aedes* cadherin mediates Cry11A toxicity *in vivo*. *Aedes* cadherin cDNA was cloned into a pACTIN.SV expression vector and expressed in the C6/36 mosquito cell line for stable expression cell line. We determined the ability of the Cry11A protein to cause cytotoxicity with cells expressing *Aedes* cadherin.

Materials and Methods

Cell culture

C6/36 (*Aedes albopictus*) cells were grown and maintained in L15 medium (Gibco, Grand Island, NY) supplemented with 10% FBS (Gibco), 1% L-Glutamine (Gibco) and 1% Penicillin-Streptomycin (Gibco) at 27°C. The cells were grown as a monolayer in T-25 culture flasks (BD Falcon, Franklin Lakes, NJ) or 6-well tissue culture plate (Corning, Tewksbury, MA).

Construction of cadherin in pACTIN.SV

A full-length *Aedes* cadherin cDNA (AaeCad) cloned into pCR2.1 vector was obtained from Jianwu Chen, Department of Cell Biology and Neuroscience, University of California, Riverside, CA. To remove the 5' and 3' UTRs, partial AaeCad fragments (5EM and 3EM) were prepared with a set of primers (Table 2.1). The 5' end modified fragment (5EM) was amplified using a sense primer (5EM-S), which has restriction enzyme sites (NotI and StuI), a Kozak sequence (CCACC) and a start codon, and an antisense primer (5EM-A), which has a restriction enzyme site (Bstz17I). The 3' end modified fragment (3EM) was constructed from two fragments (3EM1 and 3EM2). The 3EM1 fragment was amplified using a sense primer (3EM1-S), which has a BlpI restriction enzyme site and an antisense primer (3EM1-A), which has a HA-tag (TACCCATACGACGTCCCAGACTACGCT). To insert the enhanced GFP gene, the 3EM2 was amplified from the pHyperD9a vector using a sense primer (3EM2-S), which

has a partial HA-tag and an antisense primer (3EM2-A), which has a stop codon and restriction sites (NheI, PmeI and SacI). To construct 3EM, the 3EM1 fragment was cloned into pCR2.1 vector (Invitrogen, Grand Island, NY) and subsequently digested in a solution (20 µl) containing 1 µg sample, 1 x bovine serum albumin (BSA), 1 x NEB buffer, and 1 µl each restriction enzymes (ZraI and SacI) for 6 h at 37°C. After the reaction mixture was separated in a DNA gel, the target band was cut and purified from the gel with Gel DNA Recovery kit (Zymo research, Irvine, CA). The amplified 3EM2 was also digested with ZraI and SacI and similarly purified. The digested 3EM1 and 3EM2 were ligated in 20 µl ligation solution containing 5 µl samples, 1X T4 DNA Ligase Reaction Buffer, and T4 DNA ligase (New England Biolabs, Ipswich, MA) overnight at 16°C. All PCR products were cloned into the pCR2.1 vector (Invitrogen, Grand Island, NY) and fully sequenced (Institute for Integrative Genome Biology, University of California, Riverside, CA). To construct the modified AaeCad, the 5EM and AaeCad were separately digested with NotI and Bstz17I, run in DNA gel, purified, ligated like above. Next, this construct and the 3EM were digested with Blp I and Sac I, run in DNA gel, purified, and ligated like above. To construct an expression vector, the modified AaeCad in pCR2.1 vector and pACTIN.SV vector were separately digested with Xba I and Sac I overnight at 37°C, run in DNA gel, purified with Gel DNA Recovery kit, and ligated with T4 DNA ligase for 1 day at 4°C (Figure 2.1A).

The blasticidin-resistance sequence from pCoBlast vector (Invitrogen) was amplified using a sense primer (BLA-S), which has a restriction enzyme site (BglII), a Kozak sequence, and a start codon, and an antisense primer (BLA-A), which has a

restriction enzyme site (PmlI). To construct a vector to be used for co-expression, the *Bla* gene and pIE1.SV were separately digested with BglII and PmlI, run in DNA gel, purified, and ligated like above (Figure 2.1C).

The pACTIN.SV, pIE1.SV, and pACTIN.SV vectors including enhanced GFP gene ORF (*EGFP*) (Figure 2.1B) were obtained from Huynh and Zieler (National Institutes of Health, Bethesda, MD) (Chen et al., submitted).

Cell transfection

To construct a stable cell line expressing *Aedes* cadherin, pACTIN.SV containing the cloned AaeCad (5 µg) together with pIE1.SV containing a blasticidin-resistance gene (1 µg) used for selection, were co-transfected into C6/36 cells using the FuGENE6 transfection reagent (Roche Applied Science, Madison, WI) following the manufacturer's protocol. Another stable C6/36 cell line expressing GFP was made using pACTIN.SV containing an enhanced GFP gene ORF (5 µg) together with pIE1.SV containing a blasticidin gene (1 µg), using identical conditions as described above. In brief, 1.5×10^5 C6/36 cells were plated in 6-well plate and incubated overnight at 27°C. Plasmid transfection mixtures were prepared by mixing 6 µg of plasmids and 9 µl FuGENE 6 transfection reagent with 91 µl L15 medium. The transfection mixture was incubated for 15 min at room temperature before use and added to the cells in a drop-wise manner. The cells were further incubated at 27°C for 3 days. The media was removed and replaced with the fresh medium containing blasticidin (15 µg). One week later, the media was replaced with the media containing 7.5 µg blasticidin. The selecting media was replaced

every 3-4 days until cell colonies were observed. Colonies expressing green fluorescent protein were picked under an inverted fluorescence microscope (Nikon ECLIPSE TE2000-S) and transferred to 24-well plate. Homogeneous cells were grown and maintained in the fresh medium containing blasticidin (7.5 µg) at 27°C.

Western blotting with cells

Cells expressing AaeCad or EGFP were harvested, washed with phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO4, 2 mM KH_2PO_4, pH7.4) 2 times and resuspended in SDS-PAGE sample buffer. The collected cells were boiled for 10 min and centrifuged at 10,000×g for 10 min to remove insoluble material. The supernatants were loaded in SDS polyacrylamide gel (8%), and the electrotransferred to nitrocellulose membrane. The membrane was blocked with blocking solution (PBS, 5% Skim milk and 0.1% Tween-20) for 1 h at room temperature washed with PBST (PBS and 0.1% Tween-20). The blocked membrane was incubated with an anti-HA antibody (a rabbit polyclonal IgG, Santa Cruz Biotechnology, Dallas, TX) or an anti-cadherin fragment antibody detecting AaeCad CR7-11 or an anti-AaeCad peptide antibody (CISYSIDESTLETHGENLPTT) (Chen et al., 2009) from Jianwu Chen, Department of Cell Biology and Neuroscience, University of California, Riverside, CA (1:3,000) overnight at 4°C. The membrane was washed with PBST, and then subsequently incubated with anti-rabbit horseradish peroxidase (HRP, 1:5,000) secondary antibody (Sigma, St. Louis, MO) for 1 h at room temperature. After washing with PBST, the HRP

activity was revealed with a luminal substrate (Thermo Scientific, Lafayette, CO) and exposed to an X-ray film in a darkroom.

Immunolocalization of AaeCad

C6/36 cells (1 x 10^5 cells) were plated in a slide chamber (Fisher, Hampton, NH) and incubated overnight at 27°C. The cells were washed three times with PBS and then fixed in 4% paraformaldehyde in PBS for 1 h at room temperature. The fixed cells were blocked with blocking solution (PBS, 2% BSA) for 1 h at room temperature. The cells were then incubated with anti-cadherin fragment antibody (1:100 dilution) in PBS including 1% BSA for 1 h at room temperature, washed three times with washing buffer (PBS, 0.1% BSA, and 2% Goat Serum) and incubated with Cy3-conjugated goat anti-rabbit IgG (1:1000, Jackson Immuno Research, West Grove, PA) in PBS including 0.1% BSA and 2% Goat Serum for 1 h at room temperature in dark condition. The fluorescence was observed using Zeiss 510 confocal microscope (Institute for Integrative Genome Biology).

Purification of Cry11A toxin

B. thuringiensis strains expressing Cry11Aa (Chang et al., 1993) were grown in nutrient broth sporulation medium containing 25 µg/ml erythromycin at 30°C for 4-5 days (Lereclus et al., 1995). The inclusion bodies for Cry11Aa were isolated as previous reported (Cowles et al., 1995). Briefly, after cell autolysis, the spores and crystal inclusions were harvested by centrifugation at 10,000xg for 10 min at 4 ˚C, and washed

three times with 1 M NaCl, 10 mM EDTA, pH 8.0. The spore and crystal mixture was resuspended in the same buffer and then centrifuged at 15,000xg for 2 h on a discontinuous NaBr gradient (42%, 45%, 49%, 52%, and 56%) in SW28 swing rotor. The purified Cry11A inclusions were washed, solubilized in 50 mM Na_2CO_3 pH 10.5 buffer, and then activated by trypsin (1:10 w/w) at 37°C, and stored at -80°C until needed.

Cytotoxicity test

The toxicity of Cry11A to C6/36 cells expressing AaeCad or EGFP was analyzed by using a MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) cytotoxicity assay (Chow et al., 1989). In brief, 6 x 10^4 cells were plated in each well of a 96-well cell culture dish and incubated overnight at 27°C. The plate was centrifuged at 1,000xg for 5 min. The medium was removed and replaced with the fresh medium containing the activated Cry11A toxin (finally 50 – 400 nM) and the cells were incubated for 3 h at 27°C. After centrifugation at 1,000xg for 5 min, the medium was removed and replaced with a mixture of 200 µl fresh medium and 20 µl MTT solution (5 mg/ml MTT in PBS) and the cells were incubated 2 h at 27°C. The plate was centrifuged at 1,000xg for 5 min and the medium was removed. Isopropanol-HCl-SDS solution (120 µl) was added to each well and the absorbance was read at 570 nm with a microplate reader (Molecular Devices, Sunnyvale, CA).

Results

Construction of expression vectors

To construct a clone that would express AaeCad in a mosquito cell line, the AaeCad cDNA was slightly modified (Figure 2.1). The 5'UTR was removed and a Kozak sequence was added at the 5'end of AaeCad. Also the 3'UTR was removed and an HA-Tag and EGFP was added at the 3'end of AaeCad to facilitate detection of expression. The modified AaeCad of approximately 6021 bp in length was cloned into pACTIN.SV vector. To facilitate the selection of cell lines, blasticidin-resistance sequence was cloned into the pIE1.SV vector (Figure 2.1C). This vector was co-transfected with pACTIN.SV-AaeCad to develop stable cell lines. After selections for two weeks, cells formed colonies and colonies expressing green fluorescent protein were picked up and subsequently grown to analyze the AaeCad expression.

AaeCad are stably expressed in cells

To determine the expression of AaeCad cloned into pACTIN.SV, non-soluble proteins were extracted and detected with an anti-HA antibody, an anti-cadherin fragment antibody or an anti-cadherin peptide antibody. We detected a band around 250 kDa in AaeCad-transfected cells that was not detected in control cells expressing only EGFP (Figure 2.3). Hence the full length cadherin is expressed in these cells as a 250kDa protein.

To determine the sub-cellular location of AaeCad, immunolocalization of AaeCad in cells was analyzed using an anti-cadherin fragment antibody and a secondary antibody conjugated red fluorescence. Cells expressing AaeCad and control cells expressing only EGFP were stained without permeabilization to detect AaeCad localized in the plasma membrane and analyzed under confocal microscopy. Receptor expression was also determined by detecting the fluorescence from EGFP. Control cells expressed EGFP (Figure 2.4A) and did not have specific immunofluorescence staining (Figure 2.4B). AaeCad was expressed and localized mostly in the cytoplasm, in addition to the plasma membrane (Figure 2.3C). Red immunofluorescence staining without cell permeabilization showed clearly that AaeCad was also localized in the plasma membrane (Figure 2.4D).

Cells expressing AaeCad are more sensitive to Cry11Aa toxin

The toxicity of Cry11Aa to these cells was analyzed by a MTT cytotoxicity assay using activated Cry11A toxin at final concentrations of 50 - 400 nM. After cells were incubated with activated Cry11Aa for 3 h, live cells were analyzed by measuring reduced MTT. Control cells expressing the EGFP protein were insensitive to Cry11Aa toxin even at concentrations up to 400 nM. However, cells expressing AaeCad showed significant sensitivity to the toxin from 200 nM (29% death). Cry11A toxin at 400 nM killed approximately 37% of the cells in 3 h (Figure 2.5).

Discussion

The cadherin receptor plays an important role in Cry toxin toxicity in lepidopteran insects (Bravo et al., 2005). Therefore, an AaeCad that was the most homologous to cadherin of Lepidoptera was identified and investigated to determine if it is a functional receptor of Cry11Aa toxin in *Ae. aegypti*. Previous research reported that a partial fragment of AaeCad bound Cry11Aa with high affinity (Chen et al., 2009). Further, silencing of AaeCad expression *in vivo* lead to increased tolerance against Cry11Aa toxicity (Rodríguez-Almazán et al., 2012).

Based on these results, we tested whether AaeCad mediates Cry11Aa toxicity in a mosquito cell line, C6/36 expressing AaeCad. To test the cytotoxicity of Cry11Aa, we established a stable cell line expressing AaeCad. In a similar approach *Heliothis* and *Manduca* cadherins were transiently expressed in *Drosophila* S2 cells. These cell lines showed maximum 20% and 5% mortality for Cry1Ac and Cry1Ab, respectively at 330 nM (Jurat-Fuentes et al., 2006). In contrast, *Manduca* cadherin was stably expressed in *Trichoplusia* High Five cells and showed 80% mortality for Cry1Ab at 180 nM (Zhang et al., 2005). Previous higher cytotoxicity test resulted from cell lines, which stably expressed co-ordinal cadherins. Therefore, we stably expressed the *Aedes* cadherin in the mosquito cell line, C6/36 from *Aedes albopictus* by co-transfecting blasticidin-resistance gene and selecting with blasticidin.

AaeCad expression in the C6/36 cell line was determined with three different antibodies. The immunoblotting showed only the 250 kDa band was observed in cell

extracts and AaeCad degradation products were not detected showing the full length cadherin was stably expressed. Furthermore, localization of AaeCad in cells was determined with EGFP fluorescence and an anti-cadherin fragment antibody detecting CR7-11. EGFP fluorescence showed the AaeCad was expressed and localized in the cytoplasm as well as the plasma membrane. Expression in the cytoplasm is likely due to overexpression of AaeCad. In previous binding assays, Cry11Aa bound to only CR7-11 of AaeCad with high affinity (Chen et al., 2009). Since the antibody was directed to this fragment, the red immunofluorescence staining obtained without cell permeabilization shows that the CR7-11of AaeCad is localized on cell membrane, and is accessible to extracellular toxins.

The cell line expressing AaeCad showed increased sensitivity to Cry11Aa. The cytotoxicity test showed 29 and 37% cell death with 200 and 400 nM of activated Cry11Aa toxin, respectively. In contrast, Cry11Aa toxin did not kill control cells expressing EGFP. The level of mortality obtained is lower than the cytotoxicity obtained with High Five cell line stably expressing *Manduca* cadherin although the toxicity is higher than that obtained from transient expressing cells (Zhang et al., 2005; Jurat-Fuentes et al., 2006). These results suggest that a secondary receptor may be required for full toxicity of the Cry11Aa to be manifest. The toxicity of Cry toxin in Lepidoptera requires secondary receptors like APN or ALP (Bravo et al., 2004; Jurat-Fuentes et al., 2004). In *Ae. aegypti*, two APNs were identified as a Cry11Aa receptor (Chen et al., 2009). However, these APNs bind monomer Cry11Aa with high affinity, while lepidopteran APN showed low affinity for monomer and high affinity for oligomer

(Bravo et al., 2004; Fernandez et al., 2006). *Aedes* ALPs were localized in caeca and posterior mdigut of *Aedes* larvae. These are the same regions which Cry toxins from Bti bound (Ravoahangimalala et al., 1995; Chen et al., 2009). This result suggests that an ALP possibly functions as a secondary receptor for Cry11A toxicity. Previously three ALPs were tested and the ALP (AAEL009077) bound Cry11Aa and Cry4Ba (Fernandez et al., 2009). However, this ALP was involved in both Cry11Aa and Cry4Ba toxicity (Jiménez et al., 2012). Therefore, future research is required whether the other ALP actually mediates Cry11Aa toxicity as a secondary receptor.

Consequently, Cry11Aa killed cells stably expressing AaeCad while Cry11Aa did not kill control cells expressing EGFP. However, an additional receptor may be required for full toxicity and *Aedes* ALP is proposed as an additional receptor. Therefore, future research aimed to investigate the role of ALP in Cry11Aa toxicity is needed.

References

Abdullah, M. A., Valaitis, A. P. and Dean, D. H. (2006). Identification of a *Bacillus thuringiensis* Cry11Ba toxin-binding aminopeptidase from the mosquito, *Anopheles quadrimaculatus*. *BMC Biochem* **7**: 16.

Aronson, A. I. and Shai, Y. (2001). Why *Bacillus thuringiensis* insecticidal toxins are so effective: unique features of their mode of action. *FEMS Microbiol Lett* **195**: 1-8.

Atsumi, S., Miyamoto, K., Yamamoto, K., Narukawa, J., Kawai, S., Sezutsu, H., Kobayashi, I., Uchino, K., Tamura, T., Mita, K., Kadono-Okuda, K., Wada, S., Kanda, K., Goldsmith, M. R. and Noda, H. (2012). Single amino acid mutation in an ATP-binding cassette transporter gene causes resistance to Bt toxin Cry1Ab in the silkworm, *Bombyx mori*. *Proc Natl Acad Sci* **109**: E1591-E1598.

Bayyareddy, K., Andacht, T. M., Abdullah, M. A. and Adang, M. J. (2009). Proteomic identification of *Bacillus thuringiensis* subsp. *israelensis* toxin Cry4Ba binding proteins in midgut membranes from *Aedes* (Stegomyia) *aegypti* Linnaeus (Diptera, Culicidae) larvae. *Insect Biochem Mol Biol* **39**: 279-286.

Bayyareddy, K., Zhu, X., Orlando, R. and Adang, M. J. (2012). Proteome analysis of Cry4Ba toxin-interacting *Aedes aegypti* lipid rafts using geLC-MS/MS. *J Proteome Res.* **11**: 5843-5855.

Berry, C., O'Neil, S., Ben-Dov, E., Jones, A. F., Murphy, L., Quail, M. A., Holden, M. T., Harris, D., Zaritsky, A. and Parkhill, J. (2002). Complete sequence and organization of pBtoxis, the toxin-coding plasmid of *Bacillus thuringiensis* subsp. *israelensis*. *Appl Environ Microbiol* **68**: 5082-5095.

Bravo, A., Gill, S. S. and Soberon, M. (2005). *Bacillus thuringiensis*: Mechanisms and Use. In Comprehensive Molecular Insect Science. Gilbert, L.I., Kostas, I., and Gill, S.S.(Ed). pp175-205. Elsevier.

Bravo, A., Gomez, I., Conde, J., Munoz-Garay, C., Sanchez, J., Miranda, R., Zhuang, M., Gill, S. S. and Soberon, M. (2004). Oligomerization triggers binding of a *Bacillus thuringiensis* Cry1Ab pore-forming toxin to aminopeptidase N receptor leading to insertion into membrane microdomains. *Biochim Biophys Acta* **1667**: 38-46.

Chang, C., Yu, Y. M., Dai, S. M., Law, S. K. and Gill, S. S. (1993). High-level cryIVD and cytA gene expression in *Bacillus thuringiensis* does not require the 20-kilodalton protein, and the coexpressed gene products are synergistic in their toxicity to mosquitoes. *Appl Environ Microbiol* **59**: 815-821.

Chen, J., Aimanova, K. G., Fernandez, L. E., Bravo, A., Soberon, M. and Gill, S. S. (2009). *Aedes aegypti* cadherin serves as a putative receptor of the Cry11Aa toxin from *Bacillus thuringiensis* subsp. *israelensis*. *Biochem J* **424**: 191-200.

Chen, J., Aimanova, K. G., Pan, S. and Gill, S. S. (2009). Identification and characterization of *Aedes aegypti* aminopeptidase N as a putative receptor of *Bacillus thuringiensis* Cry11A toxin. *Insect Biochem Mol Biol* **39**: 688-696.

Chilcott, C. and Ellar, D. J. (1988). Comparative Toxicity of *Bacillus thuringiensis* var. *israelensis* Crystal Proteins *in vivo* and *in vitro*. *Journal of General Microbiology* **134**: 2551-2558.

Choma, C. T., Surewicz, W. K., Carey, P. R., Pozsgay, M., Raynor, T. and Kaplan, H. (1990). Unusual proteolysis of the protoxin and toxin from *Bacillus thuringiensis:* structural implications. *Eur J Biochem* **189**: 523-527.

Chow, E. and Gill, S. (1989). A rapid colorimetric assay to evaluate the effects of *Bacillus thuringiensis* toxins on cultured insect cells. *Journal of Tissue Culture Methods* **12**: 39-42.

Cowles, E. A., Yunovitz, H., Charles, J. F. and Gill, S. S. (1995). Comparison of toxin overlay and solid-phase binding assays to identify diverse CryIA(c) toxin-binding proteins in *Heliothis virescens* midgut. *Appl Environ Microbiol* **61**: 2738-2744.

Crickmore, N., Zeigler, D. R., Feitelson, J., Schnepf, E., Van Rie, J., Lereclus, D., Baum, J. and Dean, D. H. (1998). Revision of the nomenclature for the *Bacillus thuringiensis* pesticidal crystal proteins. *Microbiol Mol Biol Rev* **62**: 807-813.

de Maagd, R. A., Bravo, A. and Crickmore, N. (2001). How *Bacillus thuringiensis* has evolved specific toxins to colonize the insect world. *Trends Genet* **17**: 193-199.

Dechklar, M., Tiewsiri, K., Angsuthanasombat, C. and Pootanakit, K. (2011). Functional expression in insect cells of glycosylphosphatidylinositol-linked alkaline phosphatase from *Aedes aegypti* larval midgut: a *Bacillus thuringiensis* Cry4Ba toxin receptor. *Insect Biochem Mol Biol.* **41**: 159-166.

Fernandez, L. E., Aimanova, K. G., Gill, S. S., Bravo, A. and Soberon, M. (2006). A GPI-anchored alkaline phosphatase is a functional midgut receptor of Cry11Aa toxin in *Aedes aegypti* larvae. *Biochemical J* **394**: 77-84.

Fernandez, L. E., Martinez-Anaya, C., Lira, E., Chen, J., Evans, A., Hernandez-Martinez, S., Lanz-Mendoza, H., Bravo, A., Gill, S. S. and Soberon, M. (2009). Cloning and epitope mapping of Cry11Aa-binding sites in the Cry11Aa-receptor alkaline phosphatase from *Aedes aegypti*. *Biochemistry* **48**: 8899-8907.

Fernandez, L. E., Perez, C., Segovia, L., Todriguez, M. H., Gill, S. S., Bravo, A. and Soberon, M. (2005). Cry11Aa toxin from *Bacillus thuringiensis* binds its receptor in *Aedes aegypti* mosquito larvae through loop α-8 of domain II. *FEBS Lett* **579**: 3508-3514.

Gill, S. S., Cowles, E. A. and Francis, V. (1995). Identification, Isolation, and Cloning of a *Bacillus thuringiensis* CryIAc Toxin-binding Protein from the Midgut of the Lepidopteran Insect *Heliothis virescens*. *J Biol Chem* **270**: 27277-27282.

Gill, S. S., Cowles, E. A. and Pietrantonio, P. V. (1992). The mode of action of *Bacillus thuringiensis* endotoxins. *Annu Rev Entomol* **37**: 615-636.

Gomez, I., Sanchez, J., Miranda, R., Bravo, A. and Soberon, M. (2002). Cadherin-like receptor binding facilitates proteolytic cleavage of helix α-1 in domain I and oligomer pre-pore formation of *Bacillus thuringiensis* Cry1Ab toxin. *FEBS Lett* **513**: 242-246.

Griffitts, J. S., Haslam, S. M., Yang, T., Garczynski, S. F., Mulloy, B., Morris, H., Cremer, P. S., Dell, A., Adang, M. J. and Aroian, R. V. (2005). Glycolipids as Receptors for *Bacillus thuringiensis* Crystal Toxin. *Science* **307**: 922 - 925.

Hua, G., Zhang, R., Abdullah, M. A. and Adang, M. J. (2008). Anopheles gambiae Cadherin AgCad1 Binds the Cry4Ba Toxin of *Bacillus thuringiensis israelensis* and a Fragment of AgCad1 Synergizes Toxicity. *Biochemistry* **47**: 5101-5110.

Huynh, C. Q. and Zieler, H. (1999). Construction of modular and versatile plasmid vectors for the high-level expression of single or multiple genes in insects and insect cell lines. *J Mol Biol.* **288**: 13-20.

Jenkins, J. L., Lee, M. K., Valaitis, A. P., Curtiss, A. and Dean, D. H. (2000). Bivalent sequential binding model of a *Bacillus thuringiensis* toxin to gypsy moth aminopeptidase N receptor. *J Biol Chem* **275**: 14423-14431.

Jiménez, A. I., Reyes, E. Z., Cancino-Rodezno, A., Bedoya-Pérez, L. P., Caballero-Flores, G. G., Muriel-Millan, L. F., Likitvivatanavong, S., Gill, S. S., Bravo, A. and Soberón, M. (2012). *Aedes aegypti* alkaline phosphatase ALP1 is a functional receptor of *Bacillus thuringiensis* Cry4Ba and Cry11Aa toxins. *Insect Biochem Mol Biol* **42**: 683-689.

Jurat-Fuentes, J. L. and Adang, M. J. (2004). Characterization of a Cry1Ac-receptor alkaline phosphatase in susceptible and resistant *Heliothis virescens* larvae. *Eur J Biochem* **271**: 3127-3135.

Jurat-Fuentes, J. L. and Adang, M. J. (2006). The *Heliothis virescens* cadherin protein expressed in *Drosophila* S2 cells functions as a receptor for *Bacillus thuringiensis* Cry1A but not Cry1Fa toxins. *Biochemistry* **45**: 9688-9695.

Lereclus, D., Agaisse, H., Gominet, M. and Chaufaux, J. (1995). Overproduction of encapsulated insecticidal crystal proteins in a *Bacillus thuringiensis* spo0A mutant. *Biotechnology (N Y)* **13**: 67-71.

Likirvivatanavong, S., Chen, J., Evans, A. M., Bravo, A., Soberon, M. and Gill, S. S. (2011). Multiple receptors as targets of Cry toxins in mosquitoes. *J Agric Food Chem* **59**: 2829-2838.

Margalith, Y. and Ben-Dov, E. (2000). Biological Control by *Bacillus thuringiensis* subsp. *israelensis*. In: Insect Pest Management: Techniques for Environmental Protection. Rechicgl, J. E. and Rechcigl, N. A. (Ed). pp243-301. CRC Press.

McNall, R. J. and Adang, M. J. (2003). Identification of novel *Bacillus thuringiensis* Cry1Ac binding proteins in *Manduca sexta* midgut through proteomic analysis. *Insect Biochem Mol Biol* **33**: 999-1010.

Rasko, D. A., Altherr, M. R., Han, C. S. and Ravel, J. (2005). Genomics of the *Bacillus cereus* group of organisms. *FEMS Microbiol Rev* **29**: 303-329.

Ravoahangimalala, O. and Charles, J. F. (1995). *In vitro* binding of *Bacillus thuringiensis* var. *israelensis* individual toxins to midgut cells of *Anopheles gambiae* larvae (Diptera: Culicidae). *FEBS Lett* **362**: 111-115.

Rodríguez-Almazán, C., Reyes, E. Z., Zúñiga-Navarrete, F., Muñoz-Garay, C., Gómez, I., Evans, A. M., Likitvivatanavong, S., Bravo, A., Gill, S. S. and Soberón, M. (2012). Cadherin binding is not a limiting step for *Bacillus thuringiensis* subsp. *israelensis* Cry4Ba toxicity to *Aedes aegypti* larvae. *Biochem J* **443**: 711-717.

Sangadala, S., Walters, F. S., English, L. H. and Adang, M. J. (1994). A mixture of *Manduca sexta* aminopeptidase and phosphatase enhances *Bacillus thuringiensis* insecticidal CryIA(c) toxin binding and 86Rb(+)- K+ efflux in vitro. *J Biol Chem* **269**: 10088-10092.

Schnepf, E., Crickmore, N., Van Rie, J., Lereclus, D., Baum, J., Feitelson, J., Zeigler, D. R. and Dean, D. H. (1998). *Bacillus thuringiensis* and its pesticidal crystal proteins. *Microbiol Mol Biol Rev* **62**: 775-806.

Thammasittirong, A., Dechklar, M., Leetachewa, S., Pootanakit, K. and Angsuthanasombat, C. (2011). *Aedes aegypti* membrane-bound alkaline phosphatase expressed in *Escherichia coli* retains high-affinity binding for *Bacillus thuringiensis* Cry4Ba toxin. *Appl Environ Microbiol* **77**: 6836-6840.

Vadlamudi, R. K., Ji, T. H. and Bulla, L. A. J. (1993). A specific binding protein from *Manduca sexta* for the insecticidal toxin of *Bacillus thuringiensis* subsp. *bediner*. *J Biol Chem* **268**: 12334-12340.

Zhang, R., Hua, G., Andacht, T. M. and Adang, M. J. (2008). A 106-kDa aminopeptidase is a putative receptor for *Bacillus thuringiensis* Cry11Ba toxin in the mosquito *Anopheles gambiae*. *Biochemistry* **47**: 11263-11272.

Zhang, X., Candas, M., Griko, N. B., Rose-Young, L. and Bulla, L. A., Jr. (2005). Cytotoxicity of *Bacillus thuringiensis* Cry1Ab toxin depends on specific binding of the toxin to the cadherin receptor BT-R_1 expressed in insect cells. *Cell Death Differ* **12**: 1407-1416.

Table 2.1. Primers used for cloning of AaeCad.

Primers	Sequences (5'-3')
5EM-S	GCGGCCGC AGGCCT CCACC ATG GATATGATAATGTG
5EM-A	GTATAC TTTCCTTCTATTTCATTGG
3EM1-S	GCTTAGC CCCGCTATGATGGAGCACATC
3EM1-A	AGCGTAGTCTGGGACGTCGTATGGGTA GAATCGATGTGTCAGTTCATCG
3EM2-S	GACGTC CCAGACTACGCT ATGGTGAGCAAGGGCGAGGA
3EM2-A	GAGCTC TTA CTTGTACAGCTCGTCCATGC
BLA-S	AGATCT CCACCATGGCCAAGCCTTTG
BLA-A	CACGTG TTAGCCCTCCCACAC

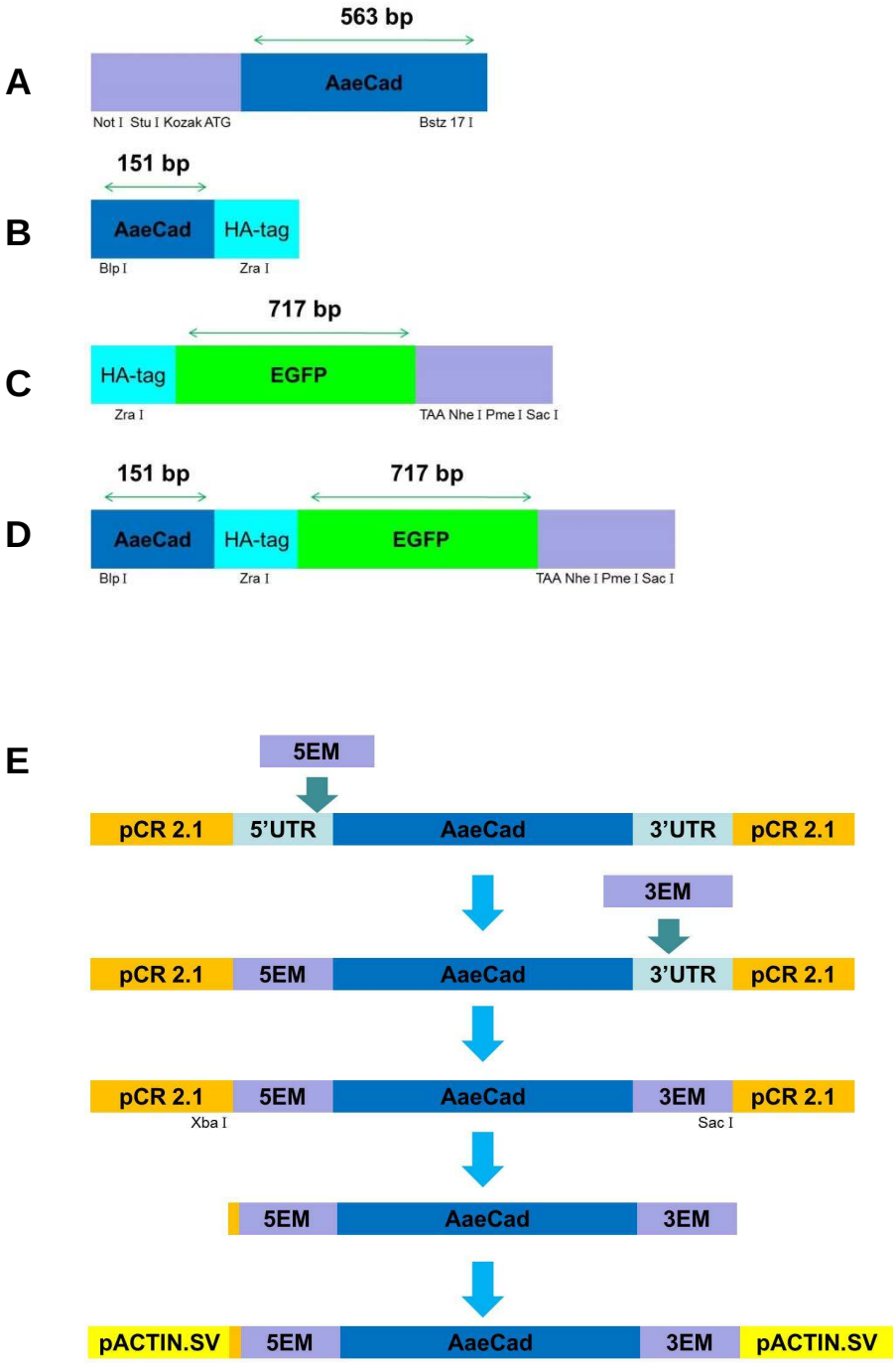

A
563 bp
AaeCad
Not I Stu I Kozak ATG
Bstz 17 I
B
151 bp
AaeCad
HA-tag
Blp I
Zra I
C
717 bp
HA-tag
EGFP
Zra I
TAA Nhe I Pme I Sac I
D
151 bp
717 bp
AaeCad
HA-tag
EGFP
Blp I
Zra I
TAA Nhe I Pme I Sac I
E
5EM
pCR 2.1
5'UTR
AaeCad
3'UTR
pCR 2.1
3EM
pCR 2.1
5EM
AaeCad
3'UTR
pCR 2.1
pCR 2.1
5EM
AaeCad
3EM
pCR 2.1
Xba I
Sac I
5EM
AaeCad
3EM
pACTIN.SV
5EM
AaeCad
3EM
pACTIN.SV

Figure 2.1. *Aedes* cadherin (AaeCad) is modified and cloned into pACTIN.SV expression vector. (A) 5EM was amplified with a sense primer (5EM-S) which has restriction enzyme sites (NotI and StuI), a Kozak sequence (CCACC), and a start codon and an antisense primer (5EM-A) which has a restriction enzyme site (Bstz17I). (B) 3EM1 was amplified with a sense primer (3EM1-S) which has a BlpI restriction enzyme site and an antisense primer (3EM1-A) which has a HA-tag (TACCCATACGACGTCCCAGACTACGCT). (C) 3EM2 was amplified from pHyperD9a vector with a sense primer (3EM2-S) which has a partial HA-tag and an antisense primer (3EM2-A) which has a stop codon and restriction sites (NheI, PmeI and SacI). (D) 3EM was constructed from 3EM1 and 3EM2 using restriction sites (ZraI and SacI). (E) 5EM was cloned into AaeCad in pCR2.1 with NotI and Bstz17I, subsequently 3EM was cloned into BlpI and SacI. The modified AaeCad was cloned into pACTIN.SV using restriction sites (XbaI and SacI).

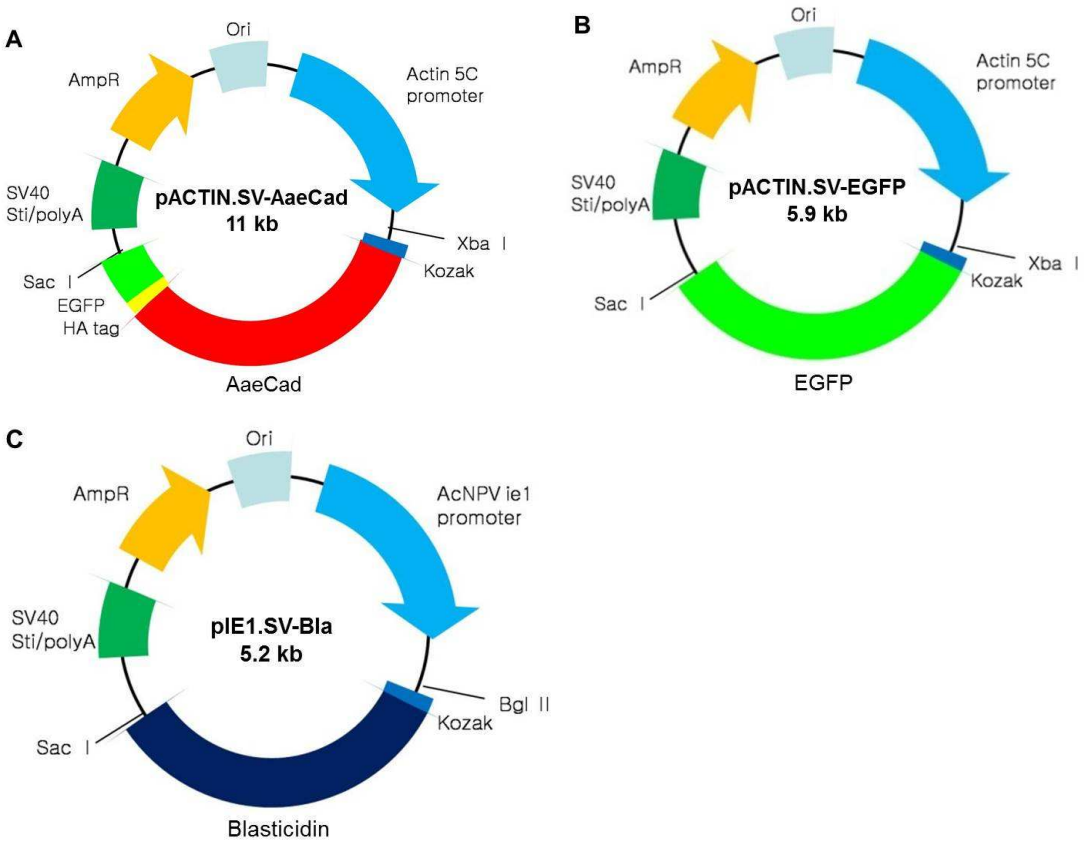


Figure 2.2. Construction of expression vectors. (A) pACTIN.SV-AaeCad. Modified *Aedes* cadherin (AaeCad) from *Ae. aegypti* larvae of approximately 6021 bp in length was cloned into pACTIN.SV vector. (B) pACTIN.SV-EGFP. The pACTIN.SV vector including the *EGFP* gene was used for a control cell line. (C) pIE1.SV-Bla. A blasticidin-resistance sequence (*bla* gene) from the pCoBlast vector was cloned into pIE1.SV vector. To make a stable cell line, pACTIN.SV-AaeCad and pIE1.SV-Bla / pACTIN.SV-EGFP and pIE1.SV-Bla were co-transfected into C6/36 (*Aedes albopictus*) cells and then cells were grown and maintained in fresh medium containing blasticidin.

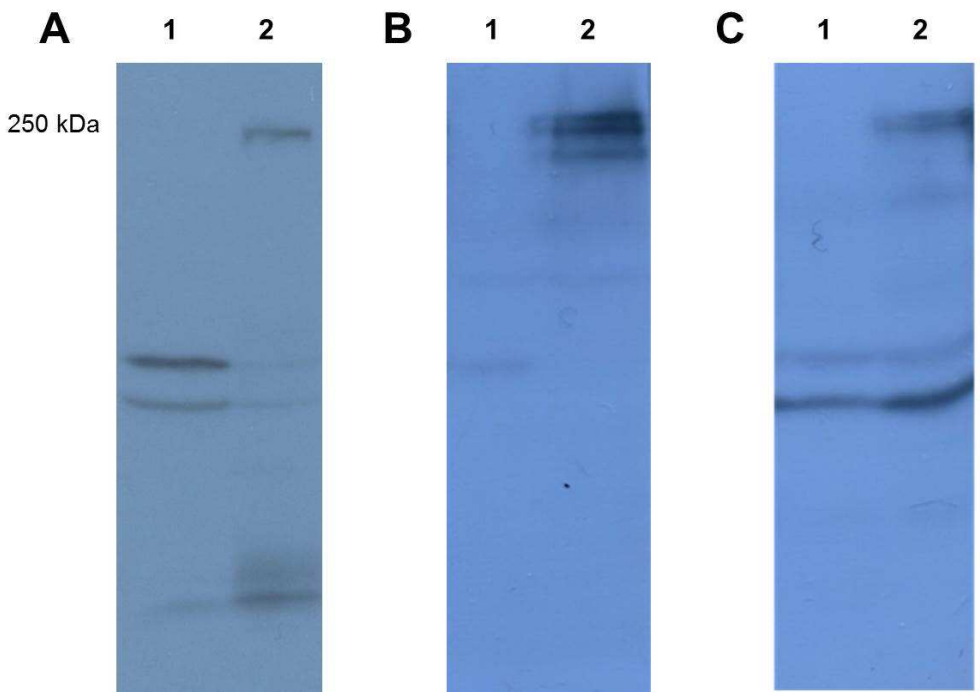


Figure 2.3. AaeCad is stably expressed in AaeCad-transfected cells. To determine the expression of *Aedes* cadherin cloned into pACTIN.SV, non-soluble proteins were extracted and detected with an anti-HA antibody (A), an anti-cadherin fragment antibody (B) or an anti-cadherin peptide antibody (C). In all cases, we detected a band around 250 kDa in AaeCad-transfected cells. The 250 kDa protein represents the full-length cadherin. Lane 1: EGFP-transfected cells, 2: AaeCad-transfected cells.

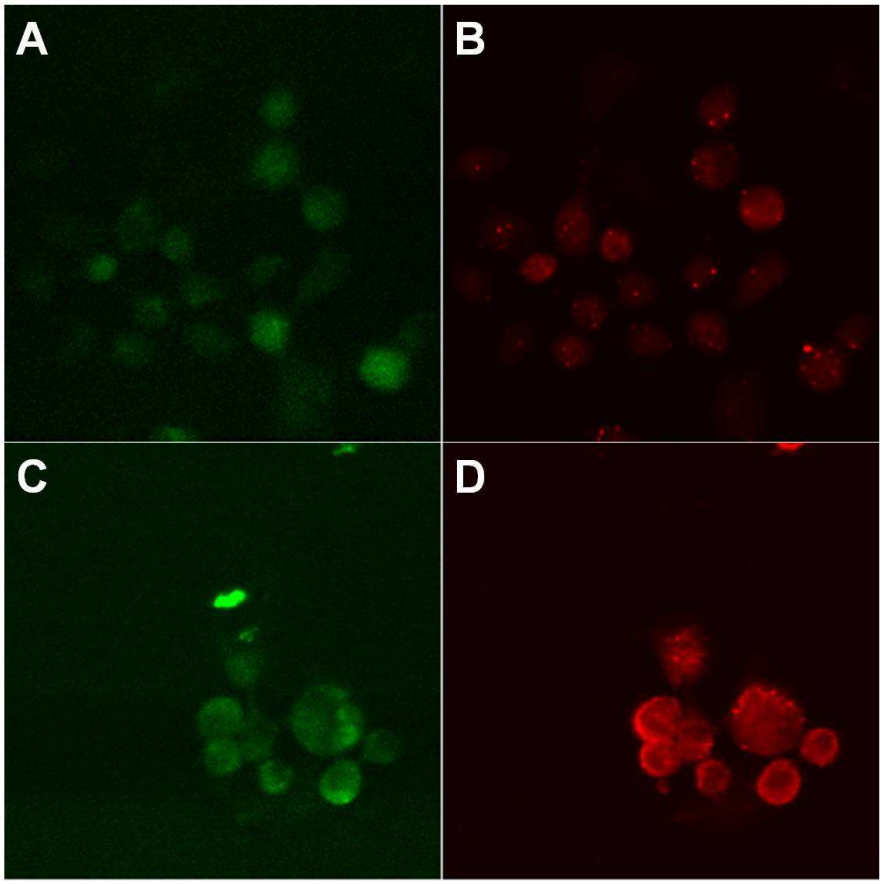


Figure 2.4. AaeCad is localized in the plasma membrane. To determine the sub-cellular location of AaeCad, receptor expression was determined by immunofluorescence staining under confocal microscopy or by detecting fluorescence from EGFP. EGFP fluorescence showed AaeCad was expressed and localized mostly in the cytoplasm, in addition to the plasma membrane (C). Red immunofluorescence staining without permeabilization showed clearly that AaeCad were localized in the plasma membrane (D). (A) Fluorescence from control cells expressing only EGFP. (B) Immunostaining of control cells using anti-cadherin fragment antibody. (C) Fluorescence from cells expressing AaeCad-EGFP. (D) Immunostaining of cells expressing AaeCad-EGFP using anti-cadherin fragment antibody.

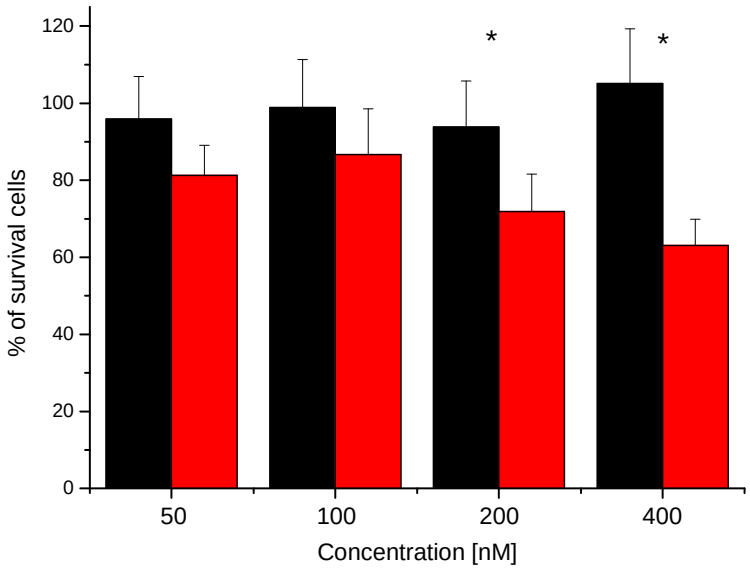


Figure 2.5. Cells expressing AaeCad are more sensitive to Cry11Aa toxin. The toxicity of Cry11A to these cells was analyzed in a MTT cytotoxicity assay using activated Cry11A toxin at final concentrations of 50 - 400 nM. After cells were incubated with activated Cry11A for 3 hours, live cells were analyzed by measuring reduced MTT. Control cells expressing the EGFP protein (black) were insensitive to Cry11A toxin up to 400 nM. However, cells expressing AaeCad (red) showed sensitivity to the toxin and Cry11A toxin at 400 nM killed approximately 37% of the cells in 3 hours (n=3, t-test, $p < 0.05$).

Chapter 3

A novel *Aedes aegypti* cadherin is a functional receptor of the Cry11Aa toxin from *Bacillus thuringiensis* subsp. *israelensis*

Abstract

Bacillus thuringiensis subsp. *israelensis* (Bti) has been used to control *Aedes aegypti* for more than three decades. However, its mechanism is still unclear because Bti produces at least three mosquitocidal toxins (Cry4Aa, Cry4Ba, and Cry11Aa), and these toxins bind multiple proteins (*Aedes* cadherin, alkaline phosphatase, and aminopeptidase N) that appear to be involved in toxicity. Using a whole genome screen to identify genes that are altered during Cry11Aa intoxication, we identified two cadherin genes that were significantly down-regulated: AAEL000597 and AAEL001196. To further characterize the role of the two cadherins in Cry11Aa toxicity, we cloned separately, expressed and purified the two functional domains (cadherin repeat domains and EGF-LamG domains) from AAEL000597 and AAEL001196. The binding affinity of Cry11Aa to these fragments was analyzed by ELISA, and we showed the EGF-LamG fragment from AAEL000597 bound Cry11Aa with high affinity (*Ki* = 12.0 nM). Furthermore, this domain competed with Cry11Aa binding to mosquito midgut membranes. To show if the cadherin is involved in modulating Cry11Aa toxicity *in vivo*, we used dsRNA to silence the expression of the AAEL000597 cadherin in larval midgut. At doses that killed 50% of

control mosquitoes only 30% of the AAEL000597-silenced *Aedes* mosquitoes died. These data suggest that AAEL000597-silenced mosquitoes has tolerance to Cry11Aa, implying that this cadherin is involved in mediating Cry11Aa toxicity. We further investigated the binding regions of EGF-LamG domain from AAEL000597 and loop regions from Cry11Aa toxin. We found two separated regions of EGF-LamG domain interacted with Cry11Aa toxin and, correspondingly, two loops of Cry11Aa toxin are involved in toxin binding.

Introduction

Bacillus thuringiensis, a spore forming bacterium, produces during sporulation insecticidal crystalline inclusions consisting of proteins known as Cry and Cyt toxins (Gill et al., 1992). One of the subspecies *B. thuringiensis* subsp. *israelensis* (Bti) produces toxins that are highly selective to dipteran insects, particularly mosquitoes and black flies (Margalith et al., 2000). Hence Bti has been used largely for control of these insects, including *Aedes aegypti*, an important disease vector for a long time (Lacey, 2007). However, despite long-term usage of Bti, its mechanism in mosquitoes is still poorly understood.

A proposed mechanism of Cry toxin in Lepidoptera is the pore-forming model (Soberon et al., 2009). This model is based on symptoms observed in Cry toxin-treated Lepidoptera, and on the structure of Cry toxin. The model proposes the toxin undergoes oligomerization and then inserts into the membrane leading to pore formation that causes

cell-swelling and bursting (Bravo et al., 2004; Schnepf et al., 1998). Binding to membrane proteins, including cadherin (Gahan et al., 2001; Nagamatsu et al., 1998; Vadlamudi et al., 1993), aminopeptidase N (APN) (Gill et al., 1995; Knight et al., 1995), and alkaline phosphatase (ALP) (Jurat-Fuentes et al., 2004; Sangadala et al., 1994) is necessary to trigger activation, oligomerization and membrane insertion of the Cry toxin. The most recently identified protein is an ATP-binding cassette transporter (ABC transporter). A mutation in a homologous ABC transporter in *Heliothis virescens* was linked to Cry1Ac resistance as well as *Bombyx mori* resistance to Cry1Ab (Gahan et al., 2010; Atsumi et al., 2012). Another proposed mechanism is the signal-cascade model that is based on cell cultures that express the lepidopteran cadherin protein (Zhang et al., 2005; Zhang et al., 2006). After the Cry toxin binds cadherin, the cell line initiates a cell death program that occurs via activation of adenylyl cyclase and protein kinase A. In addition, a number of lepidopteran insects have shown pretty high resistance to several Cry toxins that have been used for their control (Bravo et al., 2008). The resistance mechanisms were observed in these insects involving mutations or deletions on receptor proteins, defects in receptor binding, or lack of receptor proteins (Bravo et al., 2008). Based on these results, receptor proteins play a pivotal role in Cry toxin toxicity in Lepidoptera.

Based on the proposed mechanisms in Lepidoptera, cadherins are important receptors mediating Cry toxin toxicity. In *Ae. aegypti*, a 200 kDa *Aedes* cadherin (combined sequences of AAEL007478 and AAEL007488) was identified as a Cry11Aa receptor (Chen et al., 2009). *Aedes* cadherin has high affinity to Cry11Aa (*Kd* = 16.7 nM), substantially lower affinity to Cry4Aa, but did not bind Cry4Ba. *Aedes* cadherin also

bound Cry11Ba, a homologous toxin produced by *Bacillus thuringiensis* subsp. *jegathesan* (Chen et al., 2009; Likitvivatanavong S, 2011). In the case of *An. gambiae,* two *Anopheles* cadherins (AgCad1 and AgCad2) were identified as putative receptors for Cry4Ba and Cry11Ba (Hua et al., 2013; Hua et al., 2008). AgCad1 had high affinity with Cry4Ba and AgCad1 fragment synergized Cry4Ba toxicity in *An. gambiae* as well as *Ae. aegypti* (Park et al., 2009). AgCad2 which shares 14% identity to AgCad1 showed high affinity (*Kd* = 11.8 nM) with Cry11Ba (Hua et al., 2013). AgCad2 fragment inhibited Cry11Ba binding to brush border vesicles and decreased Cry11Ba toxicity in larvae.

The different affinities of Cry toxins of identified receptor proteins between Cry toxins suggest that the target proteins of Cry toxins may be different and at least two mechanisms are likely involved in Bti toxicity to *Ae. aegypti* (Likirvivatanavong et al., 2011; Rodríguez-Almazán et al., 2012). Based on these results, we screened all cadherin proteins in *Ae. aegypti* and analyzed them according to molecular weight and expression. Using a whole genome screen to identify genes that are altered during Cry11Aa intoxication, we identified two cadherin genes (AAEL000597 and AAEL001196), which encode proteins of around 200 kDa, were significantly down-regulated. Phylogenetic analysis showed they were homologous to N-cadherins. Therefore, we hypothesize that N-cadherins are involved in Cry11Aa toxicity in *Ae. aegypti*. To test this hypothesis, we examined whether Cry11Aa binds N-cadherins, and if N-cadherin silencing influences Cry11Aa toxicity. We also investigated binding regions of N-cadherin and Cry11Aa.

Materials and Methods

Cry 11Aa toxin preparation and loop peptides

B. thuringiensis strains expressing Cry11Aa (Chang et al., 1993) or Cry11Aa loop α-8 mutant proteins (Fernandez et al., 2005) were grown in nutrient broth sporulation medium containing 25 µg/ml erythromycin at 30°C for 4-5 days (Lereclus et al., 1995). The inclusion bodies for Cry11Aa were isolated as previous reported (Cowles et al., 1995). Briefly, after cell autolysis, the spores and crystal inclusions were harvested by centrifugation at 10,000xg for 10 min at 4 ˚C, and washed three times with 1 M NaCl, 10 mM EDTA, pH 8.0. The spore and crystal mixture was resuspended in the same buffer and then centrifuged at 15,000xg for 2 h on a discontinuous NaBr gradient (42%, 45%, 49%, 52%, and 56%) in SW28 swing rotor. The purified Cry11A inclusions were washed, solubilized in 50 mM Na_2CO_3 pH 10.5 buffer, and then activated by trypsin (1:10 w/w) at 37°C, and stored at -80°C until needed. Cry11Aa loop α-8 mutant proteins, E266A and V262A, were prepared in a similar manner. For biotin-labeled Cry11Aa, the purified and activated Cry11Aa was biotinylated and purified using a Sephadex G25 column following the manufacturer's protocol (GE Healthcare Life Science, Pittsburgh, PA). The loop α-8, 1, 2, and 3 sequences in Domain II were obtained from Cry11Aa model analysis (Fernandez et al., 2005). The loop peptides were synthesized, lyophilized, and stored at -20°C until needed (GenScript, Piscataway, NJ; Table 3.3).

Expression and sequence analysis of cadherin proteins

Early fourth-instar *Ae. aegypti* larvae were treated with Cry11Aa at the LC_{10} concentration level for 24 h. Total RNA were then extracted from dissected midguts of surviving larvae using TRIzol. RNA was also prepared from midguts of *Ae. aegypti* larvae that were untreated. These RNA samples were then sent to Roche NimbleGen (Madison, WI) for probing microarrays prepared by the company using data obtained from the *Ae. aegypti* genome (Nene et al., 2007). The expression data were normalized and the transcript expression levels in the midgut of untreated and Cry11Aa-treated *Ae. aegypti* larvae were analyzed using $\log_2$ ratio. Data of cadherin expression level in a population of *Ae. aegypti* larvae that were exposed to Bti in the field (Paris et al., 2012) were obtained from VectorBase (http://www.vectorbase.org).

A set of cadherin sequences from *Ae. aegypti*, *Drosophila melanogaster* and *Bombyx mori*, and Bt-R_1-homologous cadherin sequences from *Anopheles gambiae*, *Heliothis virescens* and *Manduca sexta* were obtained from VectorBase, FlyBase (http://flybase.org/), SilkDB (http://silkworm.genomics.org.cn), and NCBI (http://www.ncbi.nlm.nih.gov). The protein sequences were aligned using ClustalX and a phylogenetic tree was then produced by maximum likelihood in the Mega 5 program. Cadherin sequences were analyzed by Lasergene (DNAstar) for molecular weight, HMMTOP (http://www.enzim.hu/hmmtop) to identify the transmembrane domains, and MyHits (http://myhits.isb-sib.ch/cgi-bin/index) and Prosite (http://prosite.expasy.org/scanprosite/) to identify protein motifs and domains.

Cloning and expression of partial cadherin fragments

Two functional domain regions, a cadherin repeat and an EGF-LamG domain, were identified in each of the two cadherins of interest, namely AAEL001196 and AAEL000597. These domains, the cadherin repeat and EGF-LamG domain of AAEL001196 (here named 1196CR and 1196EGF) and of AAEL000597 (named 597CR, and 597EGF) were amplified from *Ae. aegypti* larvae midgut cDNA using primers based on the *Ae. aegypti* genome (Table 3.3) (Nene et al., 2007). The amplified PCR products were cloned into a pCR2.1 vector and fully sequenced (Institute for Integrative Genome Biology, University of California, Riverside, CA).

To express protein domains, each fragment was cloned into the pQE30 series expression vector (Qiagen) and transformed into the M15 (pREP4) strain. The bacterial strains were individually grown at 37°C and the N-terminal His-tagged recombinant proteins were induced by adding 1 mM isopropyl β-D-thiogalactoside (IPTG) for 5 h at 37°C. The bacteria were then lysed and the protein inclusion bodies were harvested using a B-PER Bacterial Protein extraction reagent (Pierce, Rockford, IL). The lysates were centrifuged at 15,000xg for 15 min and the pellets then dissolved in 0.1 M NaOH and dialyzed in PBS pH 7.4 overnight at 4°C. The crude protein concentrations were quantified using the BCA assay (Pierce). This mixture was separated by 10% SDS-PAGE gel and the percent of the recombinant protein in the crude mixture was assessed by quantification of scanned images of the gel using Image J software (NIH). The solubilized proteins were stored at -80°C until used.

Five overlapping fragments (Table 3.3) from the EGF-like and Laminin G domain of AAEL000597 (namely, 597EGFa, b, c, d, and e) were amplified from 597EGF, cloned into the pQE30 expression vector as described above. The protein fragments were expressed in M15 cells, purified by lysis, dissolved in 0.1 M NaOH, and dialyzed in PBS for use in competition binding assays.

Binding assays

ELISA binding assay was performed as previously described (Perez et al., 2005) using purified domain fragments (597CR, 597EGF, 1195CR, 1196EGF, 597EGFa-e) from the AAEL001196 and AAEL000597, and a cadherin repeat domain (CR7-11) from *Aedes* cadherin (AaeCad, AAEL007478) (Chen et al., 2009). In brief, 0.4 µg Cry11Aa in 50 mM $NaHCO_3$ pH 9.6 coating buffer was added to each well in a 96-well microtiter plate. After an overnight incubation at 4°C, the plates were washed three times with PBST buffer (PBS, 0.1% Tween 20), then treated with blocking buffer (PBS, 0.5% gelatin, 0.1% Tween 20) for 1 h at 37°C. After washing three times with PBST, 0.01 – 1,000 nM protein solutions in PBST and 0.1% gelatin were transferred to the coated wells and incubated for 2 h at 37°C. The protein solutions were discarded and the plates were washed three times with PBST. To detect proteins bound to Cry11Aa, anti-His antibody (1:5,000) was added to each well and incubated for 2 h at 37°C and then washed three times with PBST. Subsequently, goat-anti mouse-alkaline phosphatase antibody (1:1,500) was incubated in each well for 1 h at 37°C. After three-time washing with PBST, the phosphatase activity in each well was determined by adding freshly prepared substrate (3

mM nitrophenyl phosphate) and the absorbance read at 405 nm with a microplate reader (Molecular Devices, Sunnyvale, CA).

A modified competitive binding assay was performed with loop peptides of Cry11Aa or Cry11Aa mutant proteins. Briefly, 0.4 µg purified 597EGF in 50 mM $NaHCO_3$ pH 9.6 coating buffer was coated in 96-well plate overnight at 4°C, washed three times with PBST, and blocked with PBST for 1 h at 37°C. Biotin-labeled Cry11Aa (10 nM) was mixed with 0.01 – 1,000 nM of the loop peptides (loop α-8, 1, 2, and 3), activated Cry11Aa mutant proteins (E266A and V262A), or activated Cry11Aa as a control. The mixtures were pre-incubated in PBST and 0.1% bovine serum albumin (BSA) for 1 h at room temperature, transferred into plates, and then incubated for 2 h at 37°C. After three-time washing with PBST, a streptavidin-horseradish peroxidase (HRP) conjugate (1:1,500) was incubated into each well for 1 h at 37°C and washed three times with PBST. The HRP activity was revealed adding a luminol substrate (Thermo Scientific, Lafayette, CO). An X-ray film was placed over the microplate and the spot densities were measured and quantified by Image J software and Origin (Origin Lab, Northampton, MA).

Preparation of Brush Border Membrane Vesicles (BBMV).

BBMV were isolated from dissected midguts of early fourth-instar *Ae. aegypti* larvae as reported (Nielsen-Leroux et al., 1992). Briefly, midguts were resuspended and homogenized in ice-cold buffer A (0.3 M mannitol, 0.5 M EGTA, 20 mM Tris-Cl, pH 7.4) including protease inhibitor cocktail (Roche) and 1 mM phenylmethylsulfonyl

fluoride (PMSF). The homogenate was added with $MgCl_2$ (finally 12 mM) and kept on ice for 20 min. The mixture was centrifuged at 3,000xg for 15 min at 4°C and the supernatant was collected and kept on ice. The pellet was resuspended in ice-cold buffer A and retreated as above for the first homogenization. The collected supernatant was centrifuged at 14,000xg for 60 min at 4°C. The pellet was resuspended in buffer A and protein concentration was quantified using the BCA assay. The freshly prepared BBMV was immediately used for binding assay.

Competition of cadherin fragments to Cry11A binding to BBMV

Competitive binding assays were performed with BBMV prepared above in a 96 well format as previously described (Likitvivatanavong S, 2011). Briefly, 4 µg BBMVs in 50 mM $NaHCO_3$ pH 9.6 coating buffer were added to each well of a 96-well plate and incubated overnight at 4°C. The plate was washed three times with PBST and blocked with PBST for 1 h at 37°C. Biotin-labeled Cry11Aa (10 nM) was mixed with 0.01 – 1,000 nM protein solutions including 597CR, 597EGF, 1195CR, 1196EGF, AaeCad, or 597EGFa-e and pre-incubated in PBST and 0.1% BSA for 1 h at room temperature. The mixtures were transferred into each well and incubated for 2 h at 37°C. After three-time washing with PBST, a streptavidin-HRP conjugate (1:1,500) was incubated into each well for 1 h at 37°C and washed three times with PBST. The HRP activity was revealed adding a luminol substrate (Thermo Scientific) and then exposing an X-ray film over plate. The spot densities were measured and quantified by Image J software and Origin (Origin Lab).

Gene silencing of AAEL000597 and AAEL001196 cadherins

Specific ~500 bp regions from the EGF and LamG domains of AAEL000597 or AAEL001196 were amplified and cloned into LITMUS 28i including T7 promoters. dsRNAs were synthesized using the HiScribe T7 In Vitro Transciption kit (NEB, Ipswich, MA) following the manufacturer's protocol and stored in -20°C until used. LacZ dsRNA were similarly prepared for use as controls.

Cadherin silencing in *Ae. aegypti* larvae midgut was performed as described previously (Cancino-Rodezno et al., 2010). In brief, 200 µg dsRNAs were diluted in the DNA-condensation buffer to a final volume of 4 ml, mixed with 0.8 ml Enhancer buffer by vortexing for 1 sec, and then incubated for 5 min room temperature. The samples were mixed with Effectene (1.3 ml) by vortexing for 10 sec and then incubated for 10 min at room temperature to allow transfection-complex formation. These mixtures were added to dechlorinated water (final volume 10 ml) containing 200 first-instar *Ae. aegypti* larvae. After 16 h, the dsRNA-fed larvae were transferred to fresh water and the larvae fed dog food and yeast (3:1), and then reared under standard conditions (29°C, 8:12 h light:dark) until they were early fourth instar. These larvae were then used for bioassays or dissected to determine the expression of target genes. For bioassays, 25 early fourth-instar larvae were transferred into plastic cups containing 200 ml fresh tap water and incubated with Cry11Aa at a dose that gives an LC_{50} at 24 h. The LC_{50} values were determined with wild-type larvae using different Cry11Aa concentrations before bioassays with dsRNA-treated larvae were performed. Larval mortality of larvae treated with dsRNA was then determined.

Quantitative Real-time PCR (qPCR)

Total RNA was extracted from Cry11Aa- or dsRNA-treated larvae midgut using TRIzol reagent. The cDNA was synthesized from total RNA of each sample using SuperScript III (Invitrogen), diluted, and then 5-µl aliquots were used as the templates for qPCR. Primers were designed that were specific to AAEL000597, AAEL001196, and actin (AAEL011197) as a reference gene for quantification. Our microarray data showed the actin expression (AAEL011197) was not changed (-0.05, -0.06, or -0.07 fold) in Cry11Aa-treated larvae midgut at LC_{10}, LC_{50}, or LC_{90} compared to untreated larvae midgut. The primers were designed to give products that have similar properties in terms of nucleotide length and %GC content. PCR conditions, including the template cDNA, primer concentrations and annealing temperatures, were adjusted for amplification efficiencies (Efficiency 90 – 110%) for all genes. Optimized PCR master mix (20 µl) contained the following components: 10 µl iQ SYBR Green supermix (Bio-Rad), 5 µl cDNA and 10 µM each primer. The qPCR was performed using CFX Real-time PCR (Bio-Rad). The optimized thermal program consisted of: one cycle of 95°C/1 min and 40 cycles of 95°C/1 min, 62°C/1 min, and 72°C/1 min, followed by a final extension of one cycle 72°C/5 min. Following qPCR, the homogeneity of the PCR product was confirmed by melting curve analysis (Ririe et al., 1997). Quantification of the transcript level or relative copy number of the gene was conducted according to the Pfaffl method (Pfaffl, 2001). The qPCR was repeated with Cry11Aa- or dsRNA-treated larvae midguts to give three biological replicates.

Immunolocalization of AAEL000597 cadherin in *Aedes aegypti* larval midguts.

Peptide (Table 3.3, -CPQTEEVCSQSEQTS-) was commercially synthesized (Genscript) from AAEL000597 sequence and conjugated to a maleimide-activated KLH carrier protein (Pierce) via the cysteine introduced at the NH_2-terminal according to the manufacturer's protocol. The conjugated peptide was used to immunize rabbits five times for antibody development and serum from the third bleeding was used for immunohistochemistry.

AAEL000597 cadherin localization was detected in larval guts as previously reported (Chen et al., 2009). Briefly, whole fourth-instar larvae were fixed in 4% paraformaldehyde (PFA) overnight at 4°C, washed in PBSTx (PBS and 0.1% Triton X-100), and dehydrated in a 20, 40, 70, 96, and 100% ethanol series. The tissues were incubated in ethanol/xylene mixtures (70/30, 30/70, then 0/100) and placed in 100% paraffin for 24 h at 55°C. The tissues were embedded in paraffin blocks, 8-10 μm thick sections cut, placed on to poly-L-lysine slides with 1% gelatin, and the slides dried for 1-2 days at 40°C.

For immunolocalization, the tissue sections were washed with 100% xylene, rehydrated in 100, 70, 40, and 20% ethanol and rinsed in deionized water and PBSTx. After blocking with 2% BSA and PBSTx, the tissue sections were incubated overnight at 4°C with anti-N-cadherin antibody diluted 1:20 in 1% BSA and PBSTx. After washing with 0.1% BSA, 2% goat serum, and PBSTx, the tissue sections were incubated for 1 h in the dark with secondary antibodies, Cy3-conjugated goat-anti-rabbit (1:100) for the cadherin and Phalloidin-Alexa 488 (1:100) for actin F. The images were obtained with

laser-scanning confocal Zeiss Axioplan microscope (LSM Zeiss 510, Institute of Integrative Genome Biology, University of California, Riverside).

Results

Cadherins in *Aedes aegypti*

Because two cadherins have been implicated in mosquitocidal toxicity of Cry toxins, in particular Cry11 proteins (Hua et al., 2013; Hua et al., 2008), we screened all putative cadherin genes in the *Aedes* genome (Nene et al., 2007). We found a total of 18 cadherin genes in the genome (Table 3.1). All the identified cadherins were analyzed for changes in transcript expression in Cry11Aa-treated larvae midgut using whole genome microarrays or the LiTOX strain (Tetreau et al., 2012). In larvae surviving Cry11Aa-treatment at the LC_{10} for 24 h, seven of the cadherin transcripts were significantly altered ($P < 0.05$) (Table 3.1). Four of these showed increased expression (AAEL000246, 006955, 007299, and 011166), while three were down regulated (000597, 001074, and 001196). Of these the 001074, 001196, 007299 and 011166 were also significantly altered in the LiTOX strain (Tetreau et al., 2012).

Since we pulled down proteins of around 200kDa using Cry11Aa as a bait (Fernandez et al., 2006), we focused on cadherins that putatively are approximately 200 kDa in molecular weight. Eight cadherins were identified as proteins of this size (Table 3.1). Of the seven transcripts that had significant changes in expression, only four of the transcripts encode for proteins of about 200 kDa (000597, 001074, 001196, and 006955)

(Table 3.1). Of these four the most significant change, we observed with 001196 (P = 0.0001). Hence, further work was done with this gene and a closely related gene 000597. Quantitative real-time PCR confirmed that the expression of these two transcripts was suppressed in Cry11Aa-treated larvae midgut - AAEL000597 (-61.9%) and AAEL001196 (-70.1%) (Table 3.2). Interesting in the LiTOX strain (Tetreau et al., 2012), AAEL001196, was also significant suppressed (Table 3.1).

To further characterize these cadherins, amino acid sequences of all the cadherins were aligned with other cadherin sequences from *Anophele gambiae*, *Drosophila melanogaster*, *Bombyx mori, Heliothis virescens*, and *Manduca sexta* by clustalX and analyzed using the maximum likelihood method (Figure 3.1). The phylogenetic tree showed AAEL000597 and AAEL001196 were highly homologous with the classical cadherin, N-cadherin of *D. melanogaster*, which mediates cell-cell junctions between epithelial cells.

The putative functional and structural domains of the N-cadherins were analyzed by HMMTOP, Prosite, and MyHits. The putative *Aedes* N-cadherins have two large domains in the extracellular region. One domain contains six (AAEL001196) or eight (AAEL000597) cadherin repeats (CR) in the N-terminal region of both proteins. The other domain contains three or four EGF-like domains and two LaminiG domains. (Figure 3.2).

Cry11Aa binds N-cadherin, particularly the EGF-like and LamG domain

To investigate if Cry11A binds the two N-cadherins, the CR domain and the EGF-LamG domains from AAEL000597, namely 597CR (92 kDa), 597EGF (67 kDa), and those from AAEL001196, namely 1196CR (75 kDa), and 1196EGF (70 kDa), were individually cloned into the pQE30 series expression vector, expressed, and purified for binding assays (Figure 3.2, Table 3.3). Dose-dependent ELISA binding assays demonstrated Cry11Aa binds the 597EGF fragment best, but less so the 597CR, 1196CR, and 1196EGF fragments (Figure 3.3A). AaeCad CR7-11 from *Aedes* cadherin, which binds with high affinity to Cry11Aa, was used as a positive control.

Furthermore, each fragment was tested for competitive binding affinity with brush border membrane vesicles (BBMV) from *Ae. aegypti* midgut to determine which fragment has the highest affinity for the Cry11Aa toxin. Competitive binding assays showed the 597EGF domain had the highest apparent affinity to Cry11Aa toxin compared to other fragments (Figure 3.3B). The *Ki* for 597EGF was 12.0 nM compared to 30.2 nM for AaeCad CR7-11 (Table 3.5). The *Ki* values for 597CR, 1196CR and 1196EGF were substantially higher.

N-cadherin mediates mosquitocidal Cry11Aa toxicity

To test if either of the two N-cadherins are involved in Cry11Aa toxicity *in vivo*, the expression of N-cadherins (AAEL001196 and AAEL 000597) was silenced in larval midgut using dsRNA (Cancino-Rodezno et al., 2010). First instar larvae were fed with effectene-coated dsRNA (Table 3.3) obtained from from AAEL000597 (597dsRNA),

AAEL001196 (1196dsRNA), or LacZ (LacZdsRNA) as a control for 16 h and then reared until the fourth instar. Quantitative real-time PCR showed significantly reduced transcript changes of AAEL000597 (-66%) or AAEL001196 (-81%) in 597dsRNA- or 1196dsRNA-treated larvae midgut compared to lacZdsRNA-treated larvae midgut (Table 3.4). Cry11Aa treatment of dsRNA-treated larvae at the LC_{50} levels showed that this dose killed 50% of the lacZ-silenced larvae, but only 30% of AAEL000597-silenced *Aedes* larvae (Figure 3.4). AAEL001196 also showed low mortality (33%), but due to large variability in the bioassays the values are not statistically significant. These results imply that the AAEL000597 N-cadherin and likely AAEL001196 are indeed involved in mediating Cry11Aa toxicity.

Cry11Aa toxin binds two regions in the EGF-like and LamG domain

Since the AAEL000597 attenuates Cry11Aa toxicity and its EGF-like and LamG domain binds the Cry11A toxin, we investigated which region of this AAEL000597 domain interacts with Cry11Aa. Five overlapping His-tagged fragments from the EGF-LamG domain of AAEL000597 (named as 597EGFa, b, c, d, and e) were cloned, expressed, and the proteins purified for ELISA binding assay (Table 3.3, Figure 3.5A). The five fragments were incubated with immobilized Cry11Aa toxin and the bound fragments were detected by anti-His antibody. Three of the fragments, 597EGFa, 597EGFb, and 597EGFe, bound Cry11Aa, but not 597EGFc and 597EGFd (Figure 3.5B). To determine if any of these fragments competed with Cry11Aa binding to *Aedes* larval midgut BBMV, competitive binding assay were performed using the five overlapping

fragments with BBMV. Each fragment was incubated with biotin-labeled Cry11Aa in BBMV-coated well. Not surprisingly all fragments competed with Cry11Aa binding to BBMV, but the 597EGFb and 597EGFe fragments could compete the best with Cry11A binding, giving relatively low *Ki* values; 597EGFb (*Ki*, 5 nM) and 597EGFe (*Ki*, 12.6 nM). These values are very similar to that obtained with full length 597EGF fragment (*Ki*, 12 nM).

Two loops in Cry11Aa domain II binds to AAEL000597

Previous analyses of Cry11Aa identified four loop regions of domain II (loop α-8, loop 1, loop 2, loop 3) may be related to toxin binding to BBMV (Fernandez et al., 2005) (Table 3.3). To analyze if any of these loop regions are also involved in Cry11Aa binding the EGF-LamG domain, synthetic peptides corresponding to these four putative loop regions were synthesized and used for competitive binding assays. These binding assays showed that primarily loop α-8 and to some extent loop 2 bound 597EGF and competed with Cry11Aa toxin binding, but not loops 1 and 3 (Figure 3.6A). To identify specific amino acids involved in the loop α-8, which had bound with greatest affinity, loop α-8 toxin mutants were analyzed. The mutant E266A of Cry11Aa competed with biotin-labeled Cry11Aa binding to 597EGF, but mutant V262A of Cry11Aa did not compete (Figure 3.6B). Interestingly, V262 and not E266 is involved in binding to the *Aedes* cadherin (AAEL007478). Therefore, different amino acids in the same loop α-8 are involved in binding to two different cadherins in *Ae. aegypti*.

N-cadherins are localized on the apical membrane epithelial cells

To determine the localization of N-cadherins, anti-N-cadherin antibody was developed with AAEL000597 peptide (-CPQTEEVCSQSEQTS-). The anti-N-cadherin antibody detected proteins on the apical membrane of posterior midgut (Figure 3.7D, E, F). However, immunofluorescence was not observed on gastric caeca, anterior midgut, and Malpighian tubules (Figure 3.7A, B, C). The Cry11Aa toxin also binds the posterior midgut (Charles et al., 1983; Chen et al., 2009).

Discussion

Previous studies with lepidopteran insects demonstrated that cadherins are important receptor proteins mediating Cry toxin toxicity (Gahan et al., 2001; Vadlamudi et al., 1993; Vadlamudi et al., 1995; Yang et al., 2007; Zhang et al., 2006). Based on these results, we and others showed that cadherins are also involved in mediating Cry toxicity in mosquitoes. In the case of *An. gambiae*, two *Anopheles* cadherins (AgCad1 and AgCad2) were identified as putative receptors for Cry4Ba and Cry11Ba (Hua et al., 2013; Hua et al., 2008). AgCad1 is the most homologous cadherin to *Manduca* cadherin, and AgCad2 shares 14% identity to AgCad1. In *Ae. aegypti*, an *Aedes* cadherin that was the most homologous cadherin to *Manduca* cadherin was identified as a functional receptor for Cry11Aa. Because two cadherins have been implicated in mosquitocidal toxicity of Cry toxins, in particular Cry11 proteins (Chen et al., 2009; Hua et al., 2013;

Hua et al., 2008), it is possible cadherins other than that those previously reported may mediate the toxicity of Bti toxins.

Hence we screened all putative cadherin genes in the *Aedes* genome (Nene et al., 2007). Of the 18 cadherin genes identified in the genome, a number showed altered expression patterns upon exposure to LC_{10} levels of Cry11Aa (Table 3.1). One of these cadherins (AAEL001196) was very significantly altered, as was a related cadherin (AAEL000597). Both were approximately 200 kDa in molecular weight, a size we previously identified as Cry11Aa interacting proteins (Fernandez et al., 2006). Phylogenetic analysis showed these were N-cadherins, which are present in cell-cell junctions between epithelial cells. Based on these preliminary results, we hypothesized that N-cadherins are involved in the toxicity of Cry11Aa in *Ae. aegypti*.

To prove this hypothesis, the structure of N-cadherins was analyzed. Each of the cadherin had two functional domains; cadherin repeat domains (CR) and EGF-LamG domains (EGF). Based on previous data, which showed that in both lepidopteran and mosquito cadherins the Cry toxins all interact with the CR domains, we expected the Cry11Aa toxin would bind to the CR domains of both AAEL000597 and AAEL001196. However, surprisingly it is the EGF-LamG domain of AAEL000597 that binds Cry11A with the greatest affinity and not the CR domain, and this domain is important in Cry11Aa toxin binding to *Aedes* midgut BBMV. Further, while both the AAEL000597 and AAEL001196 transcripts are suppressed upon Cry11Aa exposure, it is the AAEL000597 cadherin that apparently plays a more critical role, since the EGF-like and

LamG domain of AAEL000597 has the highest affinity (*Ki* = 12.0 nM), which is lower than that for AaeCad (AAEL007478) (30.2 nM) (Chen et al., 2009).

These results further suggest that Cry toxins prefer to bind protein regions that are near the membrane. Similarly in *Aedes* cadherin Cry11Aa binds the proximal CR7-11 regions (Chen et al., 2009); the most proximal to the cell membrane (CR12-MPED) of *Manduca* cadherin mediates cytotoxicity with Cry1Ab (Hua et al., 2004); and CR11-MPED of *Anopheles* cadherin synergizes Cry4Ba toxicity (Hua et al., 2008). According to these results, it appears that Cry toxins prefer to bind to the close to transmembrane domain regardless of the type of binding domain. Potentially this binding to membrane proximal domains is critical for subsequent intoxication processes, such as membrane insertion and pore formation or cell signaling.

To determine if N-cadherins mediate Cry11Aa toxicity *in vivo*, the N-cadherins were silenced in larvae midgut. AAEL000597-silenced larvae had tolerance for Cry11Aa toxicity and finally showed only 30% death while 50% of control larvae were killed at same concentration. This mortality (30%) is the same as that obtained from *Aedes* cadherin (AAEL007478) silencing in larvae midgut. *Aedes* cadherin-silenced larvae showed 30% mortality at Cry11Aa LC_{50} concentration while 50% of control larvae were killed at the same concentration (Rodríguez-Almazán et al., 2012). Moreover, this result is consistent with the binding assay data obtained with the N-cadherin fragments. Silencing of N-cadherin (AAEL000597), which binds Cry11Aa with good affinity, resulted in larvae that are more tolerant against Cry11Aa, while silencing of N-cadherin

(AAEL001196), which has low-binding affinity for Cry11Aa, showed non-significant mortality changes. Therefore, receptor binding is a key step in the toxicity of Cry toxin.

Furthermore, we tested which region of EGF-LamG domain is involved in Cry11Aa binding. Among five-overlapping regions, two separated regions, 597EGFb and 597EGFe, bound Cry11Aa. In the *Manduca* cadherin three CR repeats, CR7, CR11, and CR12 were identified as Cry1Ab binding regions (Gomez et al., 2001; Dorsch et al., 2002; Hua et al., 2004). In contrast, *Aedes* cadherin showed CR8, 9, 10, 11 bound Cry11Aa in ELISA assay (Chen et al., 2009). However, this binding assay with *Aedes* cadherin was performed without a competitor, thus a competitive binding assay might provide more specific binding regions. Also in *Aedes* ALP (AAEL009077) two regions interacted with Cry11Aa (Fernandez et al., 2009). These results suggest that Cry11Aa needs to interact with two at least distinct receptor domains possibly facilitating a conformation change in the toxin that is likely need for membrane insertion.

Correspondingly, two Cry11Aa loops bind the EGF-like and LamG domain of N-cadherin (AAEL000597), and were identified as loop α-8 and 2 in Cry11Aa domain II. *Manduca* cadherin interacted with loop α-8, 2, and 3 in Cry1Ab toxin domain II and *Aedes* cadherin bound loop α-8 and 2 in Cry11Aa toxin domain II (Chen et al., 2009; Gomez et al., 2003). In addition, *Aedes* ALP (AAEL009077) interacted with loop α-8 of Cry11Aa domain II and loop β18-β19 of Cry11Aa domain II (Fernandez et al., 2009). Thus the same Cry11Aa loops bind *Aedes* cadherin as well as N-cadherin (AAEL000597). However, different amino acids on loop α-8 are involved; the Cry11Aa mutant E266A

lost binding with *Aedes* cadherin, while the mutant V262A lost binding with N-cadherin. Hence, the two cadherins in *Ae. aegypti* have distinct Cry11Aa binding epitopes.

In immunolocalization in larval gut, N-cadherin was detected on the apical membrane of posterior midgut. This result indicates that N-cadherin may mediate Cry11A toxicity following previously suggested pore forming or signal cascade models. However, previous reports also suggest possible mechanism with N-cadherin. In Bti-fed larvae guts, epithelia cells were detached from each other and cell arrangement was disrupted implying Cry toxins disrupted adhesion or tight junction (Singh et al., 1986). N-cadherin basically mediates cell-cell junction between epithelial cells. Therefore, it is still needed to determine if N-cadherin localizes between epithelial cells and Cry toxin causes unstable structure of N-cadherin.

In summary, we isolated a novel cadherin that mediates Cry11Aa toxicity in *Ae. aegypti*. This N-cadherin (AAEL000597) bound Cry11Aa with high affinity and was involved in Cry11Aa toxicity. Moreover, two binding regions in N-cadherin interacted with two loops of Cry11Aa. Currently, five different proteins have been identified as Cry11Aa receptors; cadherin (AAEL007478), APNs(AAEL012778 and AAEL008155), ALP (AAEL009077), and N-cadherin (AAEL000597) (Chen et al., 2009; Chen et al., 2009; Fernandez et al., 2009). Silencing receptors in larvae midgut revealed these receptors mediate Cry11Aa toxicity *in vivo* (Rodríguez-Almazán et al., 2012). However, receptor binding regions on Cry11Aa were slightly different for each receptor. These results implicate that Cry11Aa may involve more than one mechanism or one receptor.

Here we propose that Cry11Aa takes pathogenic pathway through *Aedes* cadherin as well as N-cadherin.

References

Atsumi, S., Miyamoto, K., Yamamoto, K., Narukawa, J., Kawai, S., Sezutsu, H., Kobayashi, I., Uchino, K., Tamura, T., Mita, K., Kadono-Okuda, K., Wada, S., Kanda, K., Goldsmith, M. R. and Noda, H. (2012). Single amino acid mutation in an ATP-binding cassette transporter gene causes resistance to Bt toxin Cry1Ab in the silkworm, *Bombyx mori*. *Proc Natl Acad Sci* **109**: E1591-E1598.

Bravo, A., Gomez, I., Conde, J., Munoz-Garay, C., Sanchez, J., Miranda, R., Zhuang, M., Gill, S. S. and Soberon, M. (2004). Oligomerization triggers binding of a *Bacillus thuringiensis* Cry1Ab pore-forming toxin to aminopeptidase N receptor leading to insertion into membrane microdomains. *Biochim Biophys Acta* **1667**: 38-46.

Bravo, A. and Soberon, M. (2008). How to cope with insect resistance to Bt toxins? *Trends Biotechnol* **26**: 573-579.

Cancino-Rodezno, A., Alexander, C., Villasenor, R., Pacheco, S., Porta, H., Pauchet, Y., Soberon, M., Gill, S. S. and Bravo, A. (2010). The mitogen-activated protein kinase p38 is involved in insect defense against Cry toxins from *Bacillus thuringiensis*. *Insect Biochem Mol Biol* **40**: 58-63.

Chang, C., Yu, Y. M., Dai, S. M., Law, S. K. and Gill, S. S. (1993). High-level cryIVD and cytA gene expression in *Bacillus thuringiensis* does not require the 20-kilodalton protein, and the coexpressed gene products are synergistic in their toxicity to mosquitoes. *Appl Environ Microbiol* **59**: 815-821.

Charles, J. F. and de Barjac, H. (1983). [Action of crystals of *Bacillus thuringiensis* var. *israelensis* on the midgut of *Aedes aegypti* L. larvae, studied by electron microscopy]. *Ann Microbiol (Paris)* **134A**: 197-218.

Chen, J., Aimanova, K. G., Fernandez, L. E., Bravo, A., Soberon, M. and Gill, S. S. (2009). *Aedes aegypti* cadherin serves as a putative receptor of the Cry11Aa toxin from *Bacillus thuringiensis* subsp. *israelensis*. *Biochem J* **424**: 191-200.

Chen, J., Aimanova, K. G., Pan, S. and Gill, S. S. (2009). Identification and characterization of *Aedes aegypti* aminopeptidase N as a putative receptor of *Bacillus thuringiensis* Cry11A toxin. *Insect Biochem Mol Biol* **39**: 688-696.

Cowles, E. A., Yunovitz, H., Charles, J. F. and Gill, S. S. (1995). Comparison of toxin overlay and solid-phase binding assays to identify diverse CryIA(c) toxin-binding proteins in *Heliothis virescens* midgut. *Appl Environ Microbiol* **61**: 2738-2744.

Dorsch, J. A., Candas, M., Griko, N. B., Maaty, W. S., Midboe, E. G., Vadlamudi, R. K. and Bulla, L. A. (2002). Cry1A toxins of *Bacillus thuringiensis* bind specifically to a region adjacent to the membrane-proximal extracellular domain of BT-R_1 in *Manduca sexta*: involvement of a cadherin in the entomopathogenicity of *Bacillus thuringiensis*. *Insect Biochem Mol Biol* **32**: 1025-1036.

Fernandez, L. E., Aimanova, K. G., Gill, S. S., Bravo, A. and Soberon, M. (2006). A GPI-anchored alkaline phosphatase is a functional midgut receptor of Cry11Aa toxin in *Aedes aegypti* larvae. *Biochemical J* **394**: 77-84.

Fernandez, L. E., Martinez-Anaya, C., Lira, E., Chen, J., Evans, A., Hernandez-Martinez, S., Lanz-Mendoza, H., Bravo, A., Gill, S. S. and Soberon, M. (2009). Cloning and epitope mapping of Cry11Aa-binding sites in the Cry11Aa-receptor alkaline phosphatase from *Aedes aegypti*. *Biochemistry* **48**: 8899-8907.

Fernandez, L. E., Perez, C., Segovia, L., Todriguez, M. H., Gill, S. S., Bravo, A. and Soberon, M. (2005). Cry11Aa toxin from *Bacillus thuringiensis* binds its receptor in *Aedes aegypti* mosquito larvae through loop α-8 of domain II. *FEBS Lett* **579**: 3508-3514.

Gahan, L. J., Gould, F. and Heckle, D. G. (2001). Identification of a gene associated with Bt resistance in *Heliothis virescens*. *Science* **293**: 857-860.

Gahan, L. J., Pauchet, Y., Vogel, H. and Heckel, D. G. (2010). An ABC transporter mutation is correlated with insect resistance to *Bacillus thuringiensis* Cry1Ac toxin. *PLoS Genet* **6**: e1001248.

Gill, S. S., Cowles, E. A. and Francis, V. (1995). Identification, Isolation, and Cloning of a *Bacillus thuringiensis* CryIAc Toxin-binding Protein from the Midgut of the Lepidopteran Insect *Heliothis virescens*. *J Biol Chem* **270**: 27277-27282.

Gill, S. S., Cowles, E. A. and Pietrantonio, P. V. (1992). The mode of action of *Bacillus thuringiensis* endotoxins. *Annu Rev Entomol* **37**: 615-636.

Gomez, I., Dean, D. H., Bravo, A. and Soberon, M. (2003). Molecular basis for *Bacillus thuringiensis* Cry1Ab toxin specificity: two structural determinants in the *Manduca sexta* Bt-R_1 receptor interact with loops alpha-8 and 2 in domain II of Cy1Ab toxin. *Biochemistry* **42**: 10482-10489.

Gomez, I., Oltean, D. I., Gill, S. S., Bravo, A. and Soberon, M. (2001). Mapping the epitope in cadherin-like receptors involved in *Bacillus thuringiensis* Cry1A toxins interaction using phage display. *J Biol Chem* **276**: 28906-28912.

Hua, G., Jurat-Fuentes, J. L. and Adang, M. J. (2004). Bt-R_1a extracellular cadherin repeat 12 mediates *Bacillus thuringiensis* Cry1Ab binding and cytotoxicity. *J Biol Chem* **279**: 28051-28056.

Hua, G., Zhang, Q., Zhang, R., Abdullah, A. M., Linser, P. J. and Adang, M. J. (2013). AgCad2 cadherin in *Anopheles gambiae* larvae is a putative receptor of Cry11Ba toxin of *Bacillus thuringiensis* subsp. *jegathesan*. *Insect Biochem Mol Biol.* **43**: 153-161.

Hua, G., Zhang, R., Abdullah, M. A. and Adang, M. J. (2008). *Anopheles gambiae* Cadherin AgCad1 Binds the Cry4Ba Toxin of *Bacillus thuringiensis israelensis* and a Fragment of AgCad1 Synergizes Toxicity. *Biochemistry* **47**: 5101-5110.

Jurat-Fuentes, J. L. and Adang, M. J. (2004). Characterization of a Cry1Ac-receptor alkaline phosphatase in susceptible and resistant *Heliothis virescens* larvae. *Eur J Biochem* **271**: 3127-3135.

Knight, P. J., Knowles, B. H. and Ellar, D. J. (1995). Molecular cloning of an insect aminopeptidase N that serves as a receptor for *Bacillus thuringiensis* CryIA(c) toxin. *J Biol Chem* **270**: 17765-17770.

Lacey, L. A. (2007). *Bacillus thuringiensis* serovariety *israelensis* and *Bacillus sphaericus* for mosquito control. *J Am Mosq Control Assoc* **23**: 133-163.

Lereclus, D., Agaisse, H., Gominet, M. and Chaufaux, J. (1995). Overproduction of encapsulated insecticidal crystal proteins in a *Bacillus thuringiensis* spo0A mutant. *Biotechnology (N Y)* **13**: 67-71.

Likirvivatanavong, S., Chen, J., Evans, A. M., Bravo, A., Soberon, M. and Gill, S. S. (2011). Multiple receptors as targets of Cry toxins in mosquitoes. *J Agric Food Chem* **59**: 2829-2838.

Likitvivatanavong S, C. J., Bravo A, Soberón M, Gill SS. (2011). Cadherin, alkaline phosphatase, and aminopeptidase N as receptors of Cry11Ba toxin from *Bacillus thuringiensis* subsp. *jegathesan* in *Aedes aegypti*. *Appl Environ Microbiol* **77**: 24-31.

Margalith, Y. and Ben-Dov, E. (2000). Biological Control by *Bacillus thuringiensis* subsp. *israelensis*. In: Insect Pest Management: Techniques for Environmental Protection. Rechicgl, J. E. and Rechcigl, N. A. (Ed). pp243-301. CRC Press.

Nagamatsu, Y., Toda, S., Yamaguchi, F., Ogo, M., Kogure, M., Nakamura, M., Shibata, Y. and Katsumoto, T. (1998). Identification of *Bombyx mori* midgut receptor for *Bacillus thuringiensis* insecticidal CryIA(a) toxin. *Biosci Biotechnol Biochem* **62**: 718-726.

Nene, V., Wortman, J. R., Lawson, D., Haas, B., Kodira, C., Tu, Z. J., Loftus, B., Xi, Z., Megy, K., Grabherr, M., Ren, Q., Zdobnov, E. M., Lobo, N. F., Campbell, K. S., Brown, S. E., Bonaldo, M. F., Zhu, J., Sinkins, S. P., Hogenkamp, D. G., Amedeo, P., Arensburger, P., Atkinson, P. W., Bidwell, S., Biedler, J., Birney, E., Bruggner, R. V., Costas, J., Coy, M. R., Crabtree, J., Crawford, M., Debruyn, B., Decaprio, D., Eiglmeier, K., Eisenstadt, E., El-Dorry, H., Gelbart, W. M., Gomes, S. L., Hammond, M., Hannick, L. I., Hogan, J. R., Holmes, M. H., Jaffe, D., Johnston, J. S., Kennedy, R. C., Koo, H., Kravitz, S., Kriventseva, E. V., Kulp, D., Labutti, K., Lee, E., Li, S., Lovin, D. D., Mao, C., Mauceli, E., Menck, C. F., Miller, J. R., Montgomery, P., Mori, A., Nascimento, A. L., Naveira, H. F., Nusbaum, C., O'Leary, S., Orvis, J., Pertea, M., Quesneville, H., Reidenbach, K. R., Rogers, Y. H., Roth, C. W., Schneider, J. R., Schatz, M., Shumway, M., Stanke, M., Stinson, E. O., Tubio, J. M., Vanzee, J. P., Verjovski-Almeida, S., Werner, D., White, O., Wyder, S., Zeng, Q., Zhao, Q., Zhao, Y., Hill, C. A., Raikhel, A. S., Soares, M. B., Knudson, D. L., Lee, N. H., Galagan, J., Salzberg, S. L., Paulsen, I. T., Dimopoulos, G., Collins, F. H., Birren, B., Fraser-Liggett, C. M. and Severson, D. W. (2007). Genome sequence of *Aedes aegypti*, a major arbovirus vector. *Science* **316**: 1718-1723.

Nielsen-Leroux, C. and Charles, J. F. (1992). Binding of *Bacillus sphaericus* binary toxin to a specific receptor on midgut brush-border membranes from mosquito larvae. *Eur J Biochem* **210**: 585-590.

Paris, M., Melodelima, C., Coissac, E., Tetreau, G., Reynaud, S., David, J. P. and Despres, L. (2012). Transcription profiling of resistance to Bti toxins in the mosquito *Aedes aegypti* using next-generation sequencing. *J Invertebr Pathol* **109**: 201-208.

Park, Y., Hua, G., Abdullah, M. A., Rahman, K. and Adang, M. J. (2009). Cadherin fragments from *Anopheles gambiae* synergize *Bacillus thuringiensis* Cry4Ba's toxicity against *Aedes aegypti* larvae. *Appl Environ Microbiol* **75**: 7280-7282.

Perez, C., Fernandez, L. E., Sun, J., Folch, J. L., Gill, S. S., Soberon, M. and Bravo, A. (2005). *Bacillus thuringiensis* subsp. *israelensis* Cyt1Aa synergizes Cry11Aa toxin by functioning as a membrane-bound receptor. *Proc Natl Acad Sci U S A* **102**: 18303-18308.

Pfaffl, M. (2001). A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* **29**: e45.

Ririe, K., Rasmussen, R. and Wittwer, C. (1997). Product differentiation by analysis of DNA melting curves during the polymerase chain reaction. *Anal Biochem* **245**: 154-160.

Rodríguez-Almazán, C., Reyes, E. Z., Zúñiga-Navarrete, F., Muñoz-Garay, C., Gómez, I., Evans, A. M., Likitvivatanavong, S., Bravo, A., Gill, S. S. and Soberón, M. (2012). Cadherin binding is not a limiting step for *Bacillus thuringiensis* subsp. *israelensis* Cry4Ba toxicity to *Aedes aegypti* larvae. *Biochem J* **443**: 711-717.

Sangadala, S., Walters, F. S., English, L. H. and Adang, M. J. (1994). A mixture of *Manduca sexta* aminopeptidase and phosphatase enhances *Bacillus thuringiensis* insecticidal CryIA(c) toxin binding and 86Rb(+)- K+ efflux in vitro. *J Biol Chem* **269**: 10088-10092.

Schnepf, E., Crickmore, N., Van Rie, J., Lereclus, D., Baum, J., Feitelson, J., Zeigler, D. R. and Dean, D. H. (1998). *Bacillus thuringiensis* and its pesticidal crystal proteins. *Microbiol Mol Biol Rev* **62**: 775-806.

Singh, G. J. P., Schouest, L. P. and Gill, S. S. (1986). The toxic action of *Bacillus thuringiensis* var. *israelensis* in *Aedes aegypti in vivo*: I. The relevance of midgut lesions to its poisoning syndrome. *Pestic Biochem Physiol* **26**: 36-46.

Soberon, M., Gill, S. S. and Bravo, A. (2009). Signaling versus punching hole: How do *Bacillus thuringiensis* toxins kill insect midgut cells? *Cell Mol Life Sci* **66**: 1337-1349.

Tetreau, G., Bayyareddy, K., Jones, C. M., Stalinski, R., Riaz, M. A., Paris, M., David, J. P., Adang, M. J. and Després, L. (2012). Larval midgut modifications associated with Bti resistance in the yellow fever mosquito using proteomic and transcriptomic approaches. *BMC Genomics* **13**: 248.

Vadlamudi, R. K., Ji, T. H. and Bulla, L. A. J. (1993). A specific binding protein from *Manduca sexta* for the insecticidal toxin of *Bacillus thuringiensis* subsp. *bediner*. *J Biol Chem* **268**: 12334-12340.

Vadlamudi, R. K., Weber, E., Ji, I., Ji, T. H. and Bulla, L. A., Jr. (1995). Cloning and expression of a receptor for an insecticidal toxin of *Bacillus thuringiensis*. *J Biol Chem* **270**: 5490-5494.

Yang, Y., Chen, H., Wu, Y., Yang, Y. and Wu, S. (2007). Mutated cadherin alleles from a field population of *Helicoverpa armigera* confer resistance to *Bacillus thuringiensis* toxin Cry1Ac. *Appl Environ Microbiol* **73**: 6939-6944.

Zhang, X., Candas, M., Griko, N. B., Rose-Young, L. and Bulla, L. A., Jr. (2005). Cytotoxicity of *Bacillus thuringiensis* Cry1Ab toxin depends on specific binding of the toxin to the cadherin receptor BT-R_1 expressed in insect cells. *Cell Death Differ* **12**: 1407-1416.

Zhang, X., Candas, M., Griko, N. B., Taussig, R. and Bulla, L. A. J. (2006). A mechanism of cell death involving an adenylyl cyclase/PKA signaling pathway is induced by the Cry1Ab toxin of *Bacillus thuringiensis*. *Proc Natl Acad Sci* **103**: 9897-9902.

Table 3.1. The N-cadherins (AAEL000597 and AAEL001196) have significantly reduced expression in Cry11Aa-treated larvae midgut. Cadherin proteins in *Aedes aegypti* were analyzed in molecular weight and gene expression in Cry11Aa-treated larvae midgut or LiTOX strain. Eight cadherin proteins were around 200 kDa and four of these cadherins showed significant transcript expression changes in Cry11Aa-treated larvae at LC_{10} (underlined).

		LC_{10}		LiTOX		
Gene ID	MW (kDa)	Fold	P-value	Fold	P-value	Description
AAEL000246	389	0.98	0.02	-	-	Stan
AAEL000597	<u>197</u>	<u>-0.77</u>	0.005	1.2	0.58	N-cadherin
AAEL000700	343	0.39	0.35	1.0	0.75	Dachsous (Ds)
AAEL000717	66.5	0.16	0.69	1.0	0.38	Dachsous (Ds)
AAEL001074	<u>197</u>	<u>-1.01</u>	0.001	1.8	0.02	Cad89D
AAEL001196	<u>187</u>	<u>-1.03</u>	0.0001	-1.0	0.03	N-cadherin
AAEL006534	<u>181</u>	-0.37	0.25	1.6	0.16	Fat like
AAEL006955	<u>193</u>	<u>0.69</u>	0.02	1.2	0.55	Cad88D
AAEL007299	74.9	1.39	0.0001	-1.3	0.04	Cad96Cb
AAEL007478	<u>198</u>	0.34	0.59	-1.2	0.31	Bt-R_1
AAEL008314	40.6	0.26	0.55	1.3	0.39	Calsyntenin
AAEL008318	151	0.20	0.70	1.1	0.35	Calsyntenin
AAEL008421	<u>191</u>	0.04	0.86	1.2	0.13	Cad99C
AAEL009616	<u>202</u>	0.57	0.06	1.2	0.58	Cad74A
AAEL011164	268	0.61	0.09	1.1	0.45	Fat2
AAEL011166	121	1.43	0.0001	1.2	0.04	Fat2
AAEL012421	159	0.0006	0.99	1.7	0.02	DE-cadherin
AAEL013873	99.1	0.42	0.48	-	-	DE-cadherin

Table 3.2. The N-cadherin (AAEL000597 and AAEL001196) transcripts are down-regulated in Cry11Aa-treated larvae midgut compared to that in untreated larvae midgut.

		LC_{10}		
Gene ID	MW (kDa)	Fold	P value	qPCR (%)
AAEL000597	197	-0.77	0.005	-62±10
AAEL001196	187	-1.03	0.0001	-70±21

Table 3.3. Sequences of partial N-cadherin fragments, loop peptide sequences of Cry11Aa toxin and N-cadherin dsRNA sequences.

	Protein Sequence	Molecular mass (kDa)	Amino acid No.
597CR	IRIGIAD - IAGITDE	89.3	48-845
597EGF	KAESCRS - QEGQTLK	65.5	991-1561
1196CR	IRYKITS - VVYNYQG	72.1	8-653
1196EGF	CMCNARE - ALKFSTS	66.4	890-1474
597a	KAESCRS - FYREQFD	22.7	991-1190
597b	RGYPRLL - TFKQQSY	22.7	1091-1290
597c	PTHYRWN - LDGGEGR	22.9	1191-1390
597d	VKYALSF - DPFECVD	23.1	1291-1490
597e	AVDDGQW - QEGQTLK	22.4	1362-1561
Loop α-8	GVSIPVNYNEWY	1.4	257-268
Loop 1	DIPARENRGVH	1.4	298-309
Loop 2	FTQWFQSTLYG	1.4	386-396
Loop 3	LTYNRIEYDSPTTEN	1.8	447-461
Peptide (for antibody)	CPQTEEVCSQSEQTS	1.7	1234-1248
	Nucleotide Sequence	Length	bp No.
597dsRNA	AAGGCGGAAT - GATGCGACTT	508 bp	2971-3478
1196dsRNA	AAGGGTATCC - ATTGGCAATT	507 bp	2999-3505

Table 3.4. N-cadherin (AAEL000597 and AAEL001196) transcripts are down-regulated in the midgut of dsRNA-treated larvae. AAEL000597 expression in 597dsRNA-treated larvae midgut was 64% down-regulated compared to one in LacZdsRNA-treated larvae midgut. AAEL001196 expression in 1196dsRNA-treated larvae midgut was 64% down-regulated compared to one in Lac dsRNA-treated larvae midgut

Gene ID	Transcript change, % of dsRNA-treated larvae midgut
AAEL000597	-64±15
AAEL001196	-81±16

Table 3.5. N-cadherin, particularly the EGF-like and LamG domain of AAEL000597, binds Cry11Aa with high affinity. Specifically the 597EGFb and e fragments interact with Cry11Aa.

Cadherin Fragments	*Ki* (nM, average)[a]
Aedes Cadherin CR7-11	30.2
1196CR	159
1196EGF	79.4
597CR	100
597EGF	12.0
597EGFa	21.9
597EGFb	5.0
597EGFc	27.5
597EGFd	27.5
597EGFe	12.6

[a]*Ki* values were obtained from the concentration corresponding to half the saturation response of specific binding.

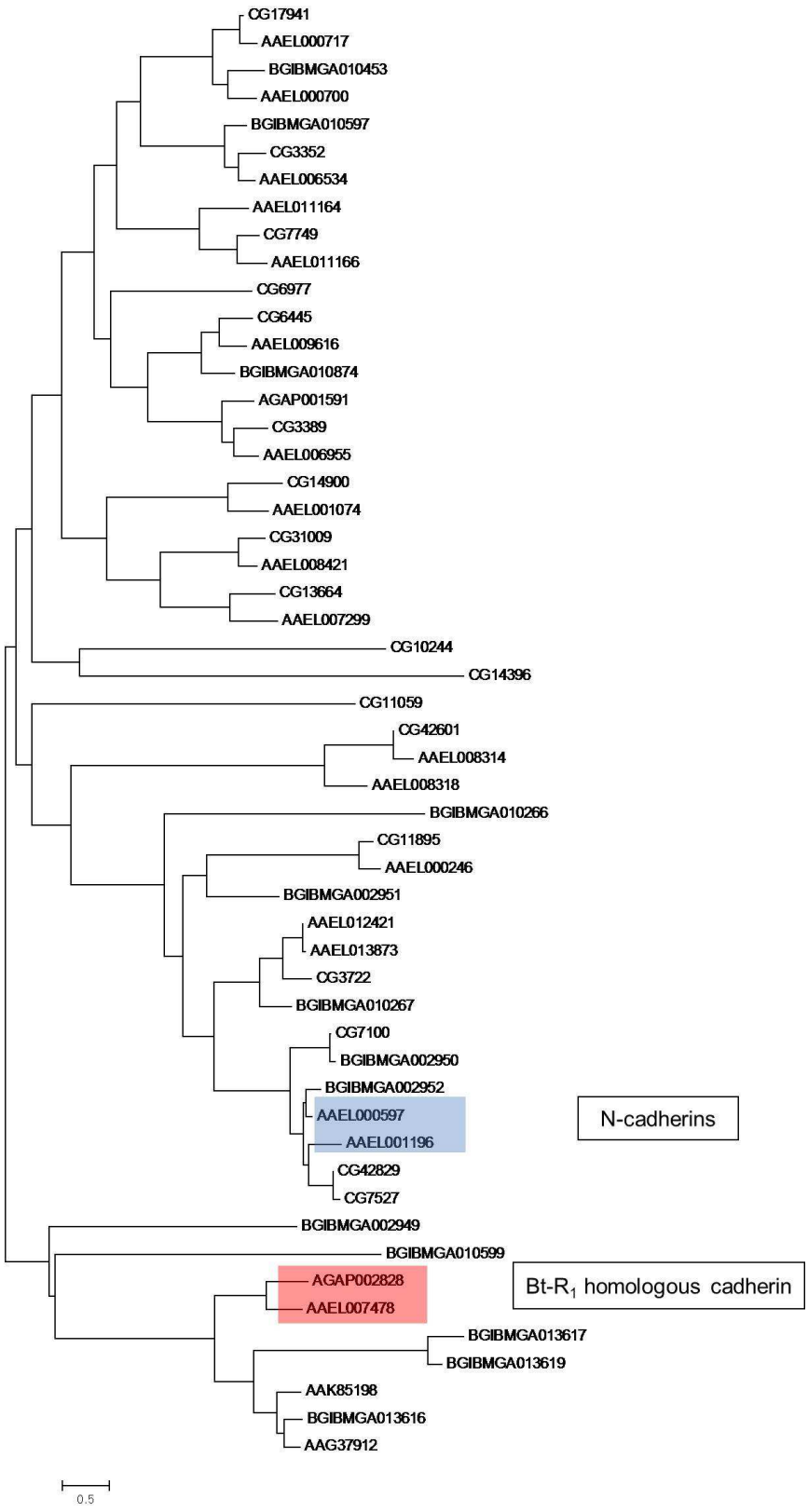

CG17941
AAEL000717
BGIBMGA010453
AAEL000700
BGIBMGA010597
CG3352
AAEL006534
AAEL011164
CG7749
AAEL011166
CG6977
CG6445
AAEL009616
BGIBMGA010874
AGAP001591
CG3389
AAEL006955
CG14900
AAEL001074
CG31009
AAEL008421
CG13664
AAEL007299
CG10244
CG14396
CG11059
CG42601
AAEL008314
AAEL008318
BGIBMGA010266
CG11895
AAEL000246
BGIBMGA002951
AAEL012421
AAEL013873
CG3722
BGIBMGA010267
CG7100
BGIBMGA002950
BGIBMGA002952
AAEL000597
AAEL001196
CG42829
CG7527
BGIBMGA002949
BGIBMGA010599
AGAP002828
AAEL007478
BGIBMGA013617
BGIBMGA013619
AAK85198
BGIBMGA013616
AAG37912
0.5
N-cadherins
Bt-R1 homologous cadherin

Figure 3.1. AAEL000597 and AAEL001196 are classified as N-cadherins in phylogenetic analysis with mosquito and lepidopteran cadherins. The phylogenetic tree was obtained from ClustalX alignment followed by using the maximum likelihood method with mosquito and lepidopteran cadherin sequences. Cadherin sequences of *Aedes aegypti* and *Anopheles gambiae* were obtained from Vectorbase (sequences have the prefix AAEL and AGAP). Cadherin sequences of Lepidoptera were obtained from NCBI and SilkDB (AAK: *Heliothis virescens*, AAG: *Manduca sexta* and BGIBMGA: *Bombyx mori*).

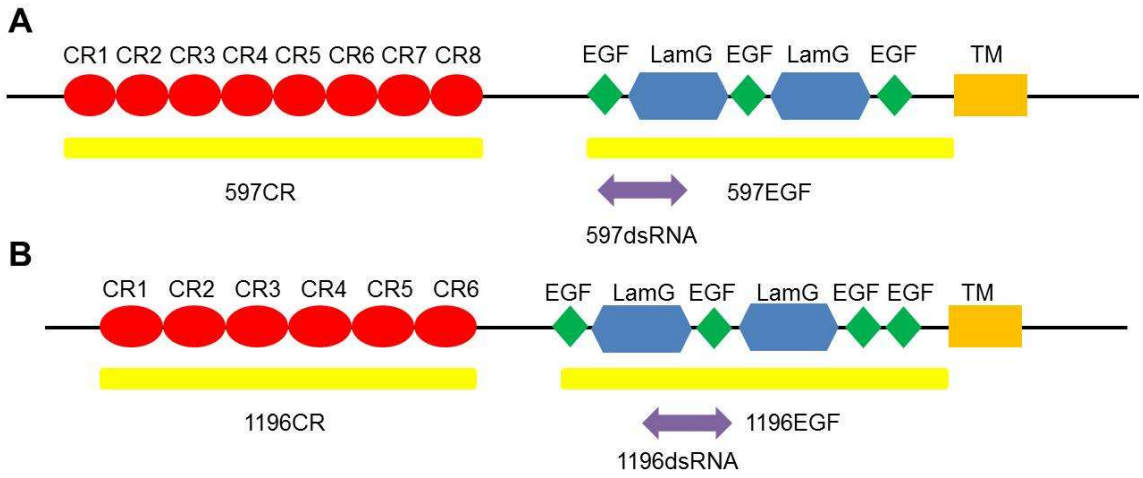


Figure 3.2. N-cadherins contain two functional domains; cadherin repeat domain and EGF-like and LamG domain. Full amino acid sequences were analyzed by MyHits to identify functional domains and motifs in the N-cadherins. Each N-cadherin contains six or eight cadherin repeats domains (CR), three or four EGF-like domains (EGF), two Laminin G domains (LamG) and a Transmembrane domain (TM). Two fragments from each N-cadherin (597CR, 597EGF, 1196CR, and 1196EGF) were amplified from *Aedes* midgut cDNA and used for binding assay (yellow rectangles). Approximately 500 bp dsRNAs (arrows) were synthesized from 597EGF or 1196EGF to silence N-cadherin expression. (A) AAEL000597. (B) AAEL001196.

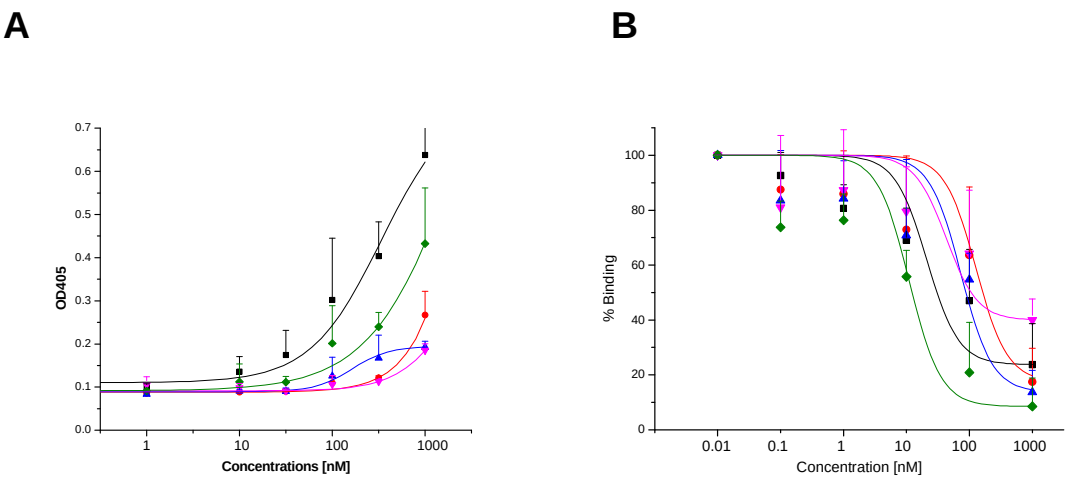


Figure 3.3. Cry11Aa binds to AAEL000597, particularly the EGF-LamG domain, but not AAEL001196. (A) Each fragment (0.01 – 1000 nM) was incubated with Cry11Aa and then the bound protein was detected by ELISA. The 597EGF fragment showed the best dose-dependent binding to immobilized Cry11Aa, and 1196CR, 1196EGF, 597CR bound less. (B) Each fragment (0.01 – 1000 nM) was competed with Cry11Aa binding to *Aedes* BBMV. In competitive binding assays, 597EGF showed higher affinity compared to other fragments (*Ki* = 12.0 nM). *Aedes* cadherin CR7-11 (■), 597CR(▼), 597EGF(◆), 1196CR(●), and 1196EGF(▲).

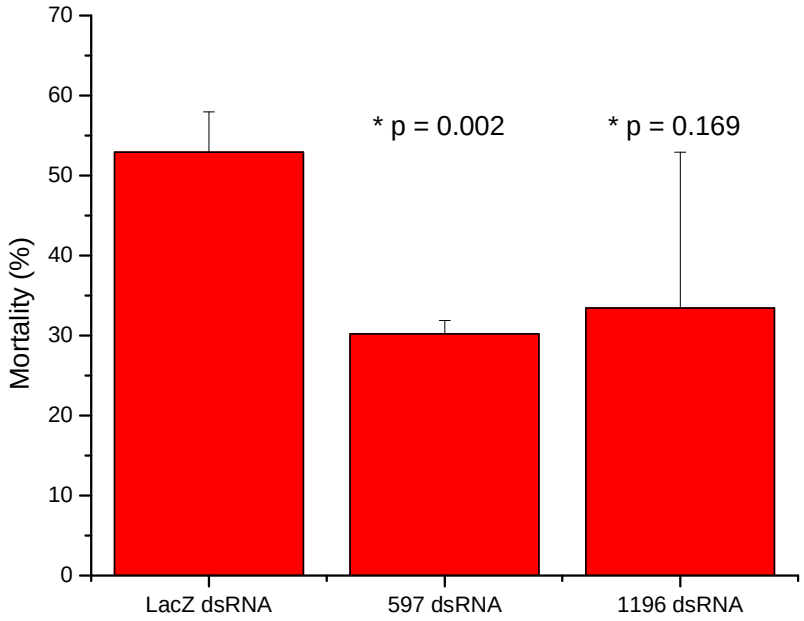


Figure 3.4. AAEL000597-silenced larvae have increased tolerance for Cry11Aa implying that N-cadherin (AAEL000597) mediates mosquitocidal Cry11Aa toxicity *in vivo*. First-instar larvae were fed effectene-coated dsRNA to silence N-cadherins. At doses that killed 50% of control mosquitoes (lacZ dsRNA) only 30% of AAEL000597-silenced *Aedes* mosquitoes (597dsRNA) died, suggesting N-cadherin (AAEL000597) is involved in toxicity. The 597 transcript of AAEL000597-silenced larvae was significantly reduced (-66%) when compared to control (lacZ-silenced larvae).

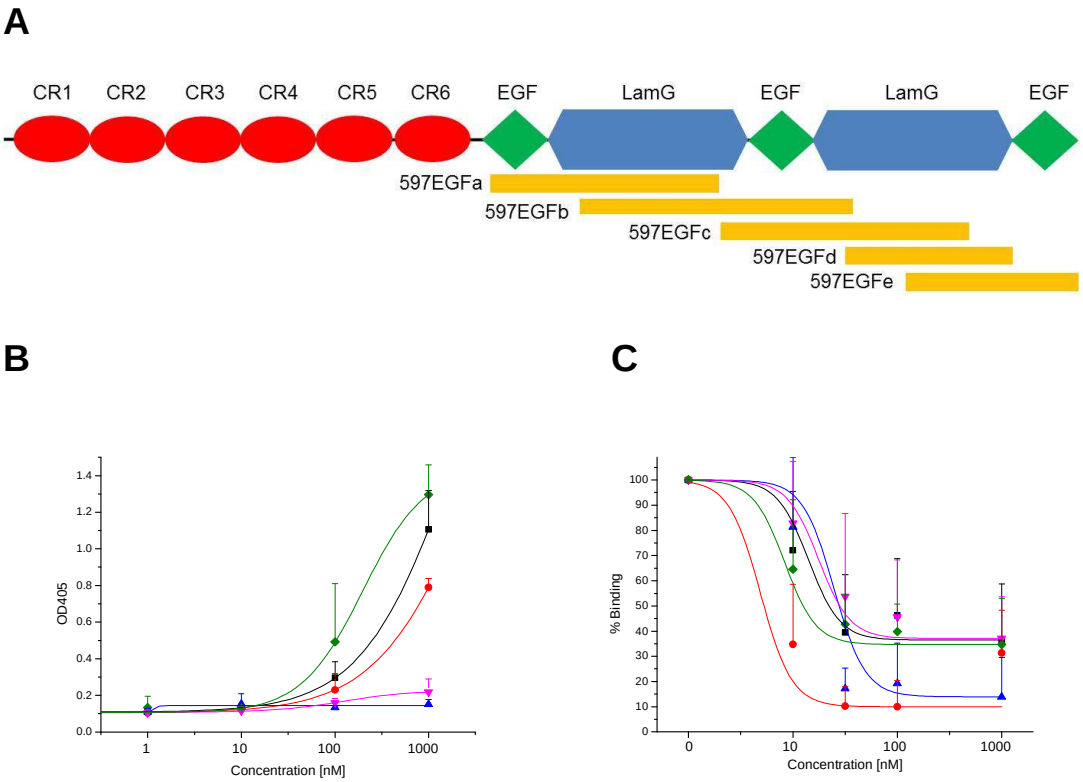


Figure 3.5. Cry11Aa binds two regions in the EGF-LamG domain of AAEL000597. (A) Five overlapping fragments were cloned from larval midgut cDNA and expressed to analyze binding with Cry11Aa. (B) Each fragment (0.01-1000 nM) was incubated with Cry11Aa and then the bound protein concentration was detected by ELISA. The 597EGFa (■), 597EGFb (●), and 597EGFe (◆) fragments showed dose-dependent binding to immobilized Cry11A, but not 597EGFc (▲) and 597EGFd (▼). (C) Each fragment was incubated with biotin-labeled Cry11Aa in BBMV-coated wells. All fragments competed with BBMV to bind Cry11Aa, but 597EGFb (●) and 597EGFe (◆) showed the highest affinity for Cry11Aa (Table 3.5, *Ki* = 5.0, 12.6 nM).

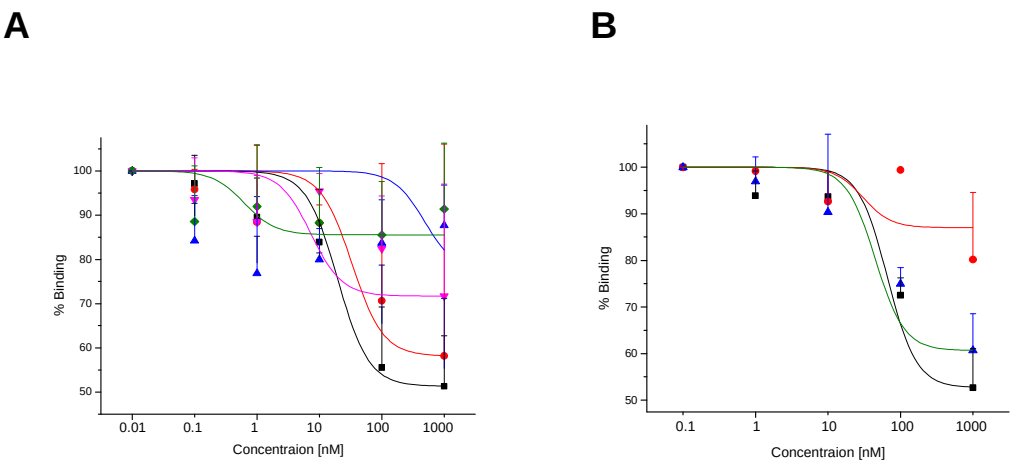


Figure 3.6. Two loops of Cry11Aa domain II bind the EGF-LamG domain of AAEL000597. (A) Loop peptides competed with Cry11Aa on immobilized 597EGF fragments. Loop α-8 and loop 2 peptides competed with biotin-labeled Cry11Aa, but not the loop 1 and loop 3 pepides. Cry11Aa (■), loop α-8 (●), loop 1 (▲), Loop 2 (▼), and loop 3 (◆). (B) Cry11Aa mutants were competed with Cry11Aa on immobilized 597EGF. The Cry11Aa mutant E266A (▲) competed with biotin-labeled Cry11Aa like Cry11Aa (■), but the mutant V262A of Cry11Aa (●) did not compete.

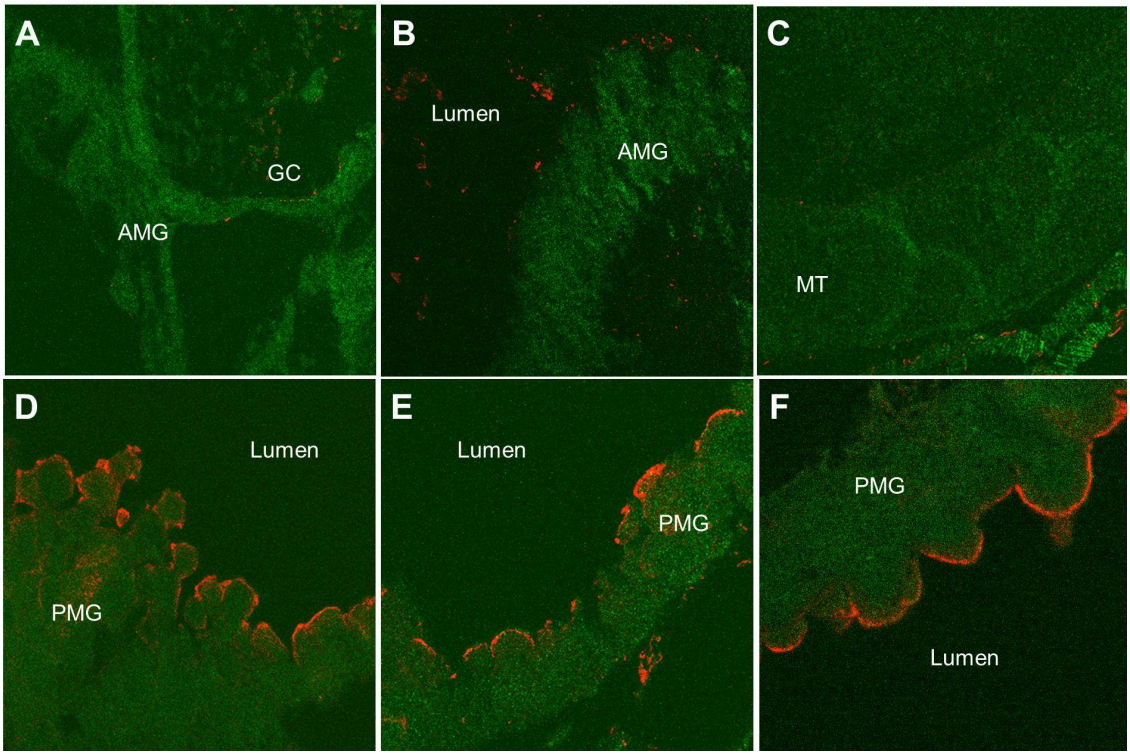


Figure 3.7. N-cadherins are localized on the apical membrane of posterior midgut. N-cadherin localization was determined with anti-N-cadherin antibody and subsequently Cy3-linked secondary antibody (red). The cell and tissue structures were visualized by phalloidin (green). Red immunofluorescence showed N-cadherins are localized on the apical side of the posterior midgut (PMG, D, E, and F). N-cadherin was not detected in anterior midgut (AMG, A), gastric caeca (GC, B), and malpighian tubules (MT, C).

Chapter 4

Investigation of a receptor associated with Cry11Aa resistance in *Aedes aegypti*

Abstract

Bacillus thuringiensis subsp. *israelensis* (Bti) has been widely used as an alternative method for the control of mosquito populations. However, the mechanism of Bti toxins is still not fully understood. To elucidate the mechanism of Bti toxins, we selected an *Aedes aegypti* population that shows high level resistance to Cry11Aa toxin. After 30 selections with Cry11Aa toxin, the larvae showed a 124-fold resistance ratio for Cry11Aa (Strain G30). G30 larvae showed strong cross resistance with Cry4Aa (66 fold resistance), but weaker cross resistance to Cry4Ba (13 fold) and Cry11Ba (2 fold). Moreover, removing Cry11Aa selection pressure made resistant larvae more susceptible quite quickly (G25-5, 20 fold resistance). G30 larvae midgut did not show any difference in protease activity compared to susceptible larvae (WT). However, G30 brush border membrane vesicles (BBMV) bound Cry11Aa less compared to WT and G25-5 BBMV implying receptor proteins mediate Cry11Aa resistance in *Ae. aegypti*. To identify receptor proteins associated with Cry11A resistance, transcript changes of all genes in the larval midgut were analyzed using Illumina sequencing. The transcripts of 23 genes were significantly increased and 115 genes were down regulated in G30 larvae midgut compared to WT and G25-5. However, no known receptor genes identified as receptors

of Cry11Aa (cadherin, alkaline phosphatase, and aminopeptidase N) were not altered found in this analysis. The genes for the identified functional receptors (*Aedes* cadherin, ALP1, APN1, and APN2) in G30 larvae midgut did not contain any mutation in their sequences nor was there any change in their expression levels compared to WT. N-cadherin (AAEL000597), a novel receptor of Cry11Aa, did not contain any mutation in G30 larvae gDNA. However, the N-cadherin transcript in G30 larvae midgut was expressed at lower levels compared to WT larvae midgut. These results were consistent with immunoblot assays using BBMV, where N-cadherin protein levels were reduced (-38%) in G30 larvae midgut, while the *Aedes* cadherin (AAEL007478) levels were unchanged. In addition, the ALP (AAEL003298) protein was expressed at reduced levels (-40%) in the G30 strain as analyzed by immunoblot and mass spectrometry. These results strongly suggest that N-cadherin and ALPs are associated with Cry11Aa resistance in *Ae. aegypti*.

Introduction

Aedes aegypti is an important vector of human diseases such as dengue fever, chikungunya, yellow fever that are transmitted through blood feeding by the mosquito (WHO, 2002; Tomori, 2004; Ligon, 2006). One approach to decrease the prevalence of these diseases has been to control *Aedes* mosquitoes. One of the agents used for this control is *Bacillus thuringiensis* subsp. *israelensis* (Bti). Bti also has high toxicity to other human disease vectors, including *Culex* (the vector of West Nile virus and

filariasis), Simulium (the vector for onchocerciasis), and to a less extend *Anopheles* spp (vectors for malaria) (Margalith et al., 2000). The high insecticidal activity and the low toxicity to other organisms has resulted in the rapid use of Bti for control of mosquito and black fly populations for more than three decades. However, its mechanism is still unclear because Bti produces a number of mosquitocidal toxins.

This bacterium contains a megaplasmid, pBtoxis, which encodes the proteins Cry4Aa, Cry4Ba, Cry10Aa, Cry11Aa, Cyt1Aa, Cyt1Ca and Cyt2Ba (Berry et al., 2002). Of the principal Bti proteins, Cry4Aa, Cry4Ba and Cry11Aa, have been identified as he major active toxins against mosquitoes (Chilcott et al., 1988), while Cyt1Aa has low mosquitocidal activity. However, Cyt1A is a synergist of the active Cry toxins, acting as a surrogate receptor for these toxins in the mosquito midgut (Perez et al., 2005; Perez et al., 2007). The biological activity of many of the other proteins is still unknown.

Among the toxins in Bti, Cry11Aa is important for the control of *Ae. aegypti* because it is the most active toxin (Chilcott et al., 1988) and shows high affinity (*Kd* ~ 28.9 nM) to the brush border membrane of *Ae. aegypti* (Chen et al., submitted). Cry11Aa bound to four proteins of 62, 65, 100 and 200 kDa in brush border membrane vesicles (BBMV) from *Ae. aegypti* midgut epithelia (Fernandez et al., 2006). Among them, the 65kDa protein was identified as a GPI-anchored ALP (AAEL009077) and a functional receptor of Cry11Aa in *Ae. aegypti* midgut cells, and localizes to the gastric caeca and posterior midgut (Fernandez et al., 2006). The 200 kDa protein was expected to be a cadherin protein. *Aedes* cadherin (AAEL007478 and AAEL007488), which is homologous with Bt-R_1 that mediates Cry1A toxicity in Lepidoptera, was cloned and a

partial cadherin fragment bound the Cry11Aa toxin with high affinity (Chen et al., 2009). Moreover, cadherin-silenced mosquitoes had increased tolerance for Cry11Aa toxicity (Rodríguez-Almazán et al., 2012). Additionally Cry11Aa pull-down assays coupled with mass spectrometry identified 95 and 140-kDa proteins (Chen et al., 2009). The 140-kDa protein was identified as an APN (AAEL012778) (Chen et al., 2009). This APN immunolocalizes to the apical membrane of posterior midgut epithelial cells and bound Cry11Aa with high affinity (*Kd* = 8.5 nM). Interestingly, both *Aedes* cadherin and APN showed high affinity for the same Cry11Aa. The 95-kDa protein is also an APN (AAEL008155) (Chen et al., submitted). This APN immunolocalizes to the apical membrane of gastric caeca.

Interestingly resistance to Bti has not been reported yet in the field, while some lepidopteran insects have developed field resistance to other Bt toxins (Mittal, 2003). Therefore, to elucidate toxin mechanisms of action, resistant lab strains have been developed. As a laboratory-selected mosquito, *Culex quinquefasciatus* was selected with single or multiple toxins from Bti (Georghiou et al., 1997). After 28 generations, the selected strain with a single Cry11Aa showed the highest resistance ratio (at least 1,000 fold), but selection with four toxins including Cyt1Aa resulted in the lowest resistance level (3.2 fold). The cross-resistance patterns of these strains were observed with combinations of mosquitocidal Cry toxins and all four strains showed the cross resistance to Bti toxins as well as Cry11Ba from *Bacillus thuringiensis* subsp. *jegathesan* (Btj) (Cheong et al., 1997; Wirth et al., 1998). For example, Cry11Aa-resistant strains revealed a high resistance ratio to Cry4Aa and Cry4Ba (41.6 fold), but very low resistance to Bti

(1.1 fold) and Btj (2.8 fold) strains. These results suggest that single Cry toxins trigger the development of more rapid resistance, but Cyt1Aa prevents resistant development because Cyt1Aa functions as a surrogate membrane-bound receptor for Bti Cry toxins in mosquitoes (Perez et al., 2005). With *Ae. aegypti,* a field collected strain that was selected with toxic leaf litter containing Bti toxins for 30 generations (LiTOX strain) showed low resistance ratio (3.5 fold) to a Bti mixture (Tetreau et al., 2012). But this strain showed 67-fold, 9-fold, and 9-fold resistance ratio for Cry4Aa, Cry4Ba, and Cry11Aa compared to susceptible *Ae. aegypti* . Transcript changes and differential protein expression analyses with LiTOX showed ALP (AAEL003298) and APN (AAEL012776) in LiTOX larvae midgut were down-regulated. These results imply that ALP and APN are possible receptors mediating Cry toxin resistance in mosquitoes.

Based on these results we developed resistant *Aedes aegypti* with the Cry11Aa toxin, the most active toxin in Bti to this species, to elucidate the molecular mechanisms of its action. The Cry11Aa-resistant *Ae. aegypti* was analyzed to determine if protease or other enzyme activities were involved in Cry11Aa resistance. To find a link to Cry11Aa resistance, midgut transcript changes were analyzed using RNA-seq. Furthermore, transcript and protein expression levels of previously identified receptor proteins for Cry11Aa were investigated using qPCR and immunoblot assay.

Materials and Methods

Rearing Cry11Aa-treated *Aedes aegypti*

Adult *Ae. aegypti* were reared at 29°C, 8:12 h light:dark, and 50% humidity and fed 10% sugar water or blood fed once a week, and eggs collected on moist filter paper. Larvae were reared in deoxygenated tap water at the same photoperiod and fed a mixture of dog food and yeast (3:1).

Selection of a Cry11A resistant mosquito colony and bioassays

EMS (ethyl methansulfonate) mutagenesis was used to obtain resistant *Aedes* mosquitoes. Adult males of a highly heterogeneous *Aedes* aegypti colony were fed sugar water for 24 h containing 10 mM EMS to cause random mutations. EMS-treated male adults were mated with untreated virgin females, which after mating were allowed to blood feed and lay eggs. To monitor the development of resistance to Cry11Aa and determine Cry11Aa concentrations needed for selection, bioassays were performed for each generation. In brief, 25 of early fourth-instar larvae were transferred to plastic cups containing 200 ml tap water and then fed Cry11Aa at different concentration for 24 h. Bioassay results were analyzed by Probit (EPA) or Origin program (Origin Lab, Northampton, MA). Then early fourth-instar larvae (approximately 2000 larvae) were treated with Cry11Aa at the LC_{80} or LC_{90} for 24 h. Surviving larvae were transferred to fresh water and reared until the next generation. The G25-5 strain was constructed as a

control to determine reversion of resistance. This G25 strain that had 77-fold resistance ratio for Cry11Aa was reared without toxin treatment for five generations.

Cry toxins preparation

For crude Cry toxin production, *B. thuringiensis* strains producing Cry4Aa, Cry4Ba, Cry11Aa, or Cry11Ba (Chang et al., 1993; Delecluse et al., 1995), were grown in nutrient broth sporulation medium containing 25 µg/ml erythromycin at 30°C for 4-5 days for cell autolysis to occur (Lereclus et al., 1995). Spores and crystal inclusions were harvested 10,000xg for 10 min at 4°C, washed twice with sterilized water, and stored in water at -80°C until used.

The inclusion bodies for Cry11Aa were isolated as previously reported using NaBr gradients (Cowles et al., 1995). In brief, the harvested spores and crystal inclusions were washed 3 times with 1 M NaCl, 10 nM EDTA pH 8.0. This mixture was resuspended in the same buffer and then centrifuged at 15,000xg for 2 h on a discontinuous NaBr gradient (42%, 45%, 49%, 52%, and 56%) in SW28 swing rotor. The purified Cry11A inclusions were washed, solubilized in 50 mM Na_2CO_3 pH 10.5 buffer, activated by trypsin (1:10 w/w) at 37°C, and stored at -80°C until needed. For biotin-labeled Cry11Aa, the purified and activated Cry11Aa was biotinylated and purified using a Sephadex G25 column following the manufacturer's protocol (GE Healthcare Life Science, Pittsburgh, PA).

Preparation of Brush Border Membrane Vesicles (BBMV)

BBMV were isolated from dissected midguts of early fourth-instar *Ae. aegypti* larvae as reported (Nielsen-Leroux et al., 1992). Briefly, frozen midguts were resuspended and homogenized in ice-cold buffer A (0.3 M mannitol, 0.5 M EGTA, 20 mM Tris-Cl, pH 7.4) including a protease inhibitor cocktail (Roche, Madison, WI) and 1 mM phenylmethylsulfonyl fluoride (PMSF). $MgCl_2$ (finally concentration 12 mM) was added to the homogenate and then kept on ice for 20 min. The mixture was centrifuged at 3,000xg for 15 min at 4°C and the supernatant was collected and kept on ice. The pellet was resuspended in ice-cold buffer A and treated as above for the first homogenization. The combined supernatants were then centrifuged at 14,000xg for 60 min at 4°C. The pellet was resuspended in buffer A and protein concentration was quantified using the BCA assay. BBMV were used fresh.

Alkaline phosphatase and Aminopeptidase assay

ALP and APN enzymatic activities were measured using *p*-nitrophenyl phosphate and leucine-*p*-nitroanilide (Sigma, St.Louis, MO) as a substrates following previous methods (Jurat-Fuentes et al., 2004; Chen et al., 2009; Fernandez et al., 2006). Freshly prepared BBMVs (5 µg) were mixed with ALP buffer (100 mM Tris/HCl, pH 9.5, 100 mM NaCl, 5 mM MgCl2, 1.25 mM *p*-nitrophenyl phosphate) or APN buffer (20 mM Tricine, pH 8.0, 0.4% methanol, 0.005% bovine serum albumin, 0.18 mM leucine-*p*-nitroanilide) in a final volume of 200 µl. The same concentration of bovine serum albumin (BSA) in ALP or APN buffer was used as a background control. Enzymatic

activities were monitored as changes in the absorbance at 405 nm for 5 min at room temperature in a microplate reader (Molecular Devices, Sunnyvale, CA).

Protease activity test

Total protease activity in wild-type and the G30 strains was analyzed as published (Forcada et al., 1996). Dissected midguts were thawed, resuspended, and homogenized in ice-cold extraction buffer (50 mM Tris-Cl, pH 7.4). After centrifugation at 12,000xg for 15 min at 4°C, the supernatant was transferred to fresh tube and quantified using the BCA assay (Pierce, Rockford, IL). The Cry11Aa protoxin (10 µg) was mixed with 0.1 µg midgut extraction in 50 mM Na_2CO_3, pH 10 including 10 mM dithiothreitol (DTT) and incubated for 5, 10, 15, 20 min at 37°C. A control was incubated with only buffer for 20 min at 37°C. Protease activity was stopped by adding Laemmli sample buffer and heating the samples for 5 min at 100°C. Digested samples were analyzed in SDS polyacrylamide gel (10%). SDS polyacrylamide gel was stained in Coomassie stain solution (0.1% Coomassie brilliant blue, 10% acetic acid, and 40% methanol) and subsequently destained in destain solution (10% acetic acid and 20% methanol). The stained band densities were measured and quantified with Image J software (NIH) and analyzed with Origin program (Origin Lab).

Binding assay

The kinetics of Cry11Aa binding were performed with BBMV prepared above in a 96-well format as previously described (Likitvivatanavong S, 2011). In brief, 4 µg

BBMVs in 50 mM $NaHCO_3$ pH 9.6 coating buffer were added into each well of a 96-well plate and incubated overnight at 4°C. The plate was then washed three times with PBST (PBS pH 7.4 and 0.1% Tween 20) and blocked with PBST for 1 h at 37°C. For total binding, biotin-labeled Cry11Aa (0.1 – 100 nM) in 100 µl binding buffer (PBST pH 7.4, 0.1% Tween 20, and 0.1% bovine serum albumin) were added into the BBMV-coated plates and incubated for 2 h at 37°C. For nonspecific binding, parallel wells were incubated under identical conditions, except in the presence of 10 µM cold Cry11Aa. The plate was washed with PBST three times and then incubated with streptavidin-horseradish peroxidase (HRP) conjugate (1:1,500) for 1 h at 37°C. After washing three times with PBST, HRP activity was detected with a luminol substrate (Thermo Scientific, Lafayette, CO) and the plated exposed to an X-ray film in a darkroom. The spot densities were measured and quantified with Image J software (NIH) and analyzed with Origin program (Origin Lab). Specific binding was obtained as total binding minus nonspecific binding and the dissociation constant (*Kd*) was obtained from the concentration corresponding to half the saturation response of specific binding.

Transcriptome sequencing and bioinformatics

Midgut transcript changes in the wild-type, G30, G25-5 strains were analyzed by RNA-seq (BGI Americas, Cambridge, MA). Total RNA was extracted from dissected midguts and mRNA was isolated with magnetic beads. The mRNA is fragmented into short fragments, then cDNA is synthesized using the mRNA fragments as templates. The cDNA was purified, resolved for end repair and single nucleotide A (adenine) addition,

and connected with adapters. Suitable cDNA were selected for the PCR amplification as templates. The samples were quantified and assessed using Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) and ABI StepOnePlus Real-Time PCR System (Invitrogen, Grand Island, NY). The library was sequenced using Illumina HiSeq™ 2000 for 30 million paired-end reads (~100 bp each end) per each sample. Three biological repeats were performed for each of the three strains. All sequence reads were assembled using *de novo* transcriptome assembly (Grabherr et al., 2011) and the identified genes were functionally annotated using blastX (Evalue < 0.0001) with a variety of databases including Gene Ontology, Nr, KeGG, SwissProt, COG as well as the *Ae. aegypti* genome, Aedes-aegypti-Liverpool_TRANSCRIPTS_AaegL1.3.fa.gz (https://www.vectorbase.org) (Nene et al., 2007). Gene expression levels were calculated using the FPKM method (Fragments Per kb per Million fragments) (Mortazavi et al., 2008) and differential gene expression were analyzed with significantly expressed genes (Qvalue > 0.8) between each groups using NOISeq method (Tarazona et al., 2011). In order to avoid library size bias, NOISeq method corrects the counts by a factor closely related to the sequencing depth (SD); the number of counts per million reads (the number of read counts for each gene x 10^6/SD). To screen gene mutations, the identified genes were clustered and aligned to other similar genes (more than 70% identity) and the number of gaps was quantified.

Mutation screening with direct sequencing

Four genes were selected to screen for mutations; *Aedes* cadherin (AAEL007478, AAEL007488), N-cadherin (AAEL000597), and alkaline phosphatase (ALP, AAEL009077). Primers were designed based on gene and transcript sequences of the *Ae. aegypti* genome (http://www.vectorbase.org). gDNA was extracted from G30 larvae with DNAzol (Molecular Research Center, Cincinnati, OH) following the manufacture's protocol. Exons regions of N-cadherin and ALP in G30 were amplified and sequenced for comparison of WT and G30 midgut genes. Because the *Aedes* cadherin gene sequence is not complete, mutation screening of the *Aedes* cadherin was performed with cDNA prepared from fourth-instar larvae midgut using TRIzol and SuperScript III (Invitrogen). Five fragments covering the whole *Aedes* cadherin sequence were amplified, sequenced and compared.

Quantitative Real-time PCR (qPCR)

Total RNA was extracted from WT and G30 larvae midgut using TRIzol. cDNA was synthesized from total RNA of each sample with SuperScript III (Invitrogen), diluted, and 5-µl aliquots were used as template for qPCR. Respective primers specific to the target genes (*Aedes* cadherin, N-cadherin, alkaline phosphatase, aminopeptidase Ns) and actin (AAEL011197) as a reference gene for quantification were designed to have similar properties in terms of nucleotide length and %GC content. Our microarray data showed the actin expression (AAEL011197) was not changed (-0.05, -0.06. or -0.07 fold) in Cry11Aa-treated larvae midgut at LC_{10}, LC_{50}, LC_{90} compared to untreated larvae midgut.

PCR conditions, including the template cDNA, primer concentrations and annealing temperatures, were adjusted for amplification efficiencies (Efficiency 90 – 110%) for all genes. Optimized PCR master mix (20 µl) contained the following components: 10 µl iQ SYBR Green supermix (Bio-Rad), 5 µl cDNA and 10 µM each primer. The qPCR was performed using CFX Real-time PCR (Bio-Rad). Optimized thermal program consisted of: one cycle of 95°C/1 min and 40 cycles of 95°C/1 min, 62°C/1 min, and 72°C/1 min, followed by a final extension of one cycle 72°C/5 min. Following qPCR, the homogeneity of the PCR product was confirmed by melting curve analysis. Quantification of the transcript levels or relative copy number of the genes was conducted according to the Pfaffl method (Pfaffl, 2001). Quantitative PCR was performed three times using independently prepared midgut cDNA.

Western blotting and mass spectrometry

Peptide (-CPQTEEVCSQSEQTS-) was commercially synthesized (GenScript, Piscataway, NJ) from the AAEL000597 sequence and conjugated to a maleimide-activated KLH carrier protein according to the manufacturer's protocol (Pierce) via the cysteine introduced at the NH_2-terminal. The conjugated peptide was used to immunize rabbits five times for antibody development. Serum was pre-adsorbed with the expressed fragment of AAEL001196 (EGF-like and LamG domain) to remove nonspecific binding to AAEL001196. The anti-cadherin peptide antibody (*Aedes* cadherin-specific antibody), anti-ALP polyclonal antibody, and anti-APN polyclonal antibody were obtained from Jianwu Chen, Department of Cell Biology and Neuroscience, University of California,

Riverside, CA. Freshly prepared BBMVs from WT and G30 midgut were quantified by BCA protein assay kit (Pierce). The same amount of BBMVs (10 µg) were heated at 70°C for 5 min, separated by SDS polyacrylamide gels and electrotransferred to nitrocellulose membranes. The membranes were blocked with blocking solution (PBS, 5% Skim milk and 0.1% Tween-20) for 1 h at room temperature and then washed with PBST (PBS and 0.1% Tween-20). The blocked membranes were incubated overnight at 4°C with primary antibody (1:3,000), washed with PBST, and then subsequently incubated with anti-rabbit horseradish peroxidase (HRP, 1:5,000) secondary antibody (Sigma) for 1 h at room temperature. After washing with PBST, the HRP activity was revealed with a luminol substrate (Thermo Scientific) and exposed to an X-ray film in a darkroom. The band densities of target proteins were measured and quantified using Image J software (NIH) and analyzed with Origin program (Origin Lab).

Freshly prepared BBMVs (10 µg) from WT and G30 midgut were separated by SDS polyacrylamide gels. SDS polyacrylamide gel was stained in coomassie stain solution (0.1% Coomassie brilliant blue, 10% acetic acid, and 40% methanol) and subsequently destained in destain solution (10% acetic acid and 20% methanol). Approximately 65 kDa protein bands from WT and G30 were excised from the gel , digested by trypsin, and analyzed by TOF mass spectrometry (Institute for Integrative Genome Biology, University of California, Riverside, CA)

Results

Ae. aegypti develops high level resistance to Cry11A

A heterogeneous *Ae. aegypti* lab colony that was occasionally supplemented with field collected mosquitoes were further treated with EMS to increase heterogeneity. Early fourth-instar *Ae. aegypti* larvae of this colony were then selected with Cry11Aa toxin at the LC_{50} levels with G0 and then the LC_{90} concentration level during 30 generations. For each generation, a bioassay was performed with susceptible and resistant larvae to determine the LC_{50} and LC_{90} values. After 20 generations, resistant larvae developed at least a 30-fold resistance ratio compared to susceptible larvae (WT) at the LC_{50} level (Table 4.1). A few of the generations (G17, G22 and G26) were not selected to maintain a robust population. After 30 generations, a 124-fold resistance ratio compared to WT at LC_{50} was obtained (Figure 4.1, 4.4A).

We also found non-selected larvae showed decreased Cry11A susceptibility. Therefore, a strain was established from G25 that showed 77-fold resistance ratio. The G25 larvae were reared without any further selection with Cry11Aa for five generations (G25-5). The G25-5 strain showed significantly lower Cry11Aa resistance, 20-fold at the LC_{50} level. (Figure 4.5).

The Cry11Aa resistance strain shows cross resistance to Cry4Aa

To investigate if the Cry11Aa-resistant larvae were cross resistant to the other Bti toxins, the Cry11A resistant larvae were in addition bioassayed with the Cry4Aa, Cry4Ba,

and Cry11Ba toxins (Figure 4.2, Table 4.2). With G30 larvae, the bioassay was performed three times and dose-response values were analyzed by probit to determine LC_{50} values (Figure 4.4, Table 4.2). G30 larvae showed resistance ratios of 124-fold, 66-fold, 13-fold, and 2-fold for Cry11Aa, Cry4Aa, Cry4Ba, and Cry11Ba toxins (Figure 4.3). Thus the Cry11Aa-resistant larvae showed substantial cross resistance at the LC_{50} level for Cry4Aa, a lesser amount to Cry4Ba but not to Cry11Ba. These results suggest that Cry11Aa and Cry4Aa may share some similar toxicity mechanisms, but these are different from that of Cry4Ba and Cry11Ba. Notably the LC_{50} values for Cry4Ba and Cry11Ba do not change much from generations 24 to 30, but these do change for Cry11Aa and CryAa (Tables 4.1 and 4.2)

Protease activity in Cry11A resistant larval mosquito midgut

In order to test if G30 resistance is associated with midgut proteolytic activity, digestion of the Cry11Aa protoxin with midgut proteases was determined. Total protease was extracted from WT and G30 larvae midgut and incubated with Cry11Aa protoxin for 5-20 min. The band densities of three proteins (76, 36, 32 kDa) were measured using Image J program. No difference in the digestion patterns of the Cry11Aa toxin were observed between WT and G30 larvae (Figure 4.6A). As shown in Figure 4.6, band densities of the 72 kDa protoxin incubated at same time were similar; Lane 1 (WT, 28) and 5 (G30, 30) of 5 min, 2 (WT, 23) and 6 (G30, 20) of 10 min, 3 (WT, 21) and 7 (G30, 17) of 15 min, 4 (WT, 20) and 8 (G30, 18) of 20 min. Moreover, the sum of three

proteins was similar at each lane implying Cry11Aa protoxin degradation was not significantly changed between WT and G30 (Figure 4.6B).

Cry11A binding affinity to BBMV is slightly altered in resistant *Aedes aegypti*

A competitive binding assay was performed to measure the Cry11Aa binding affinity to the larval midgut BBMV. To determine specific binding affinity, BBMVs from WT, G30, and G25-5 were incubated with biotin-labeled Cry11Aa in absence or presence of unlabeled Cry11Aa. Dissociation constant (*Kd*) values were calculated as a concentration corresponding to half the saturation value. While WT and G25-5 midgut showed similar binding affinities (*Kd* = 10.0 and 9.9 nM, respectively), the G30 midgut showed reduced binding affinity for Cry11Aa (*Kd* = 15.7 nM) (Figure 4.7). These data suggest that Cry11Aa has decreased binding to the G30 midgut membrane and implies that receptor alteration may contribute to Cry11Aa resistance in *Ae. aegypti*.

Checking for mutations in known receptors

To analyze if any of the previously identified Cry11Aa receptors were mutated, we analyzed these genes by direct sequencing and the transcripts by Illumina and Sanger sequencing. In RNA-seq analysis, one gap (three nucleotides insertion) in the G30 larval midgut from *Aedes* cadherin (AaeCad) transcript was observed but not that in APN1 and APN2 transcripts (Table 4.4). However, Sanger sequencing of G30 larval midgut cDNA showed there was no mutation in this transcript. Surprisingly, RNA-seq did not detect the Ncad and ALP1 sequences. But Sanger sequencing of exons of these genes did not show

any mutation that would lead to a change in protein sequence although the Ncad gene showed many polymorphisms.

Transcriptome analysis of Cry11A resistant *Aedes*

To determine if there were any specific changes in midgut transcript expression in the resistant *Aedes* larvae, RNA-seq was performed with the WT, G30 and G25-5 strains. Differentially expressed genes were identified by comparing G30 and WT, G30 and G25-5, WT and G25-5 midgut (Figure 4.8A). From this analysis, the significantly up-regulated or down-regulated genes in G30 larvae midgut were determined (Figure 4.8B, C). A total of 23 genes in G30 larvae midgut were significantly up-regulated compared to WT and G25-5, and 6 of these genes are functionally annotated (Table 4.5). Three different enzymes were highly expressed in the G30 strain: aldose-1-epimerase (AAEL010590), acylphosphatase (AAEL001490), and serine-type enodpeptidase (AAEL007938). A total of 115 genes in G30 larvae midgut were significantly down-regulated compared to WT and G25-5, and 26 of these genes are functionally annotated (Table 4.6). Tubulin alpha chain (AAEL006642) and cpg binding protein (AAEL011688) were the most reduced in G30 larvae midgut. Two kinases (AAEL00080 and AAEL000006) and 12 other enzymes were significantly down-regulated on G30 larvae midgut. However, none of the known receptor proteins for Cry11Aa were detected. Among the unannotated genes, 6 genes from up-regulated genes and 12 genes from down-regulated genes were determined as hypothetical proteins. The rest of genes were not identified from *Aedes* genome database,

and were analyzed using Blastp and Blastx methods. However, no functional domain or homologous gene was found.

Transcript changes of GPI-anchored proteins

RNA-seq analysis revealed 9 ALPs and 17 APNs were expressed in the midgut. Comparing the levels of these transcripts in the G30, WT, and G25-5 strains revealed that two ALPs (AAEL013330 and AAEL015070) in G30 were significantly down-regulated compared to WT, but not G25-5 (Table 4.8). One ALP (AAEL003286) was highly up-regulated in G30 larvae midgut, but ALP1 (AAEL009077), a receptor protein for Cry11Aa (Fernandez et al., 2009), was not significantly changed in qPCR analysis (Table 4.7). In case of APNs, two APNs (AAEL008158 and AAEL008162) were significantly reduced in G30 compared to WT, but not G25-5 (Table 4.8). However, previously identified APN receptors for Cry11Aa (APN1 and APN2) (Chen et al., 2009) were not significantly changed or only slightly up-regulated and this result was confirmed by qPCR (Table 4.8). Total ALP and APN activity was also tested with WT and G30 BBMVs and both enzyme activities were not significantly changed between WT and G30 BBMVs (Table 4.3).

Immunoblot assays with anti-ALP or anti-APN polyclonal antibody were performed with WT and G30 BBMVs. A 65 kDa band, the size for most ALPs, in G30 was significantly reduced (-40%) compared to WT (Figure 4.9). Therefore, the 65 kDa bands from WT and G30 were excised and analyzed in TOF Mass spectrometry. Two ALPs (AAEL003309 and AAEL003298) were identified and AAEL003298 ALP in G30

was down-regulated (-44%) compared to WT (Table 4.9). Additionally, 140 kDa bands with anti-APN polyclonal antibody in WT and G30 BBMVs were detected and not significantly changed (-11%).

Transcript changes in cadherin expression

Changes in cadherin transcripts were determined in G30 larvae midgut using RNA-seq analyses and qPCR (Table 4.6, 4.7). Four cadherins were analyzed, but their expressions were not changed in G30 larvae midgut. Furthermore, the expression of AaeCad and Ncad were analyzed in qPCR. The expression of Ncad (AAEL000597) in G30 was highly reduced compared to WT, while AaeCad (AAEL007478) was not changed (Table 4.7). These results were confirmed by immunoblot assay with antibodies for *Aedes* cadherin and N-cadherin (AAEL000597) (Figure 4.9). N-cadherin revealed 38% reduction in G30 larvae midgut compared to WT while *Aedes* cadherin was not changed between G30 and WT (Figure 4.9B).

Discussion

After 30 generations of selection with single Cry11Aa toxin, *Ae. aegypti* larvae (G30) showed a 124-fold resistance ratio compared to susceptible larvae (WT). However, this strain had lower resistance levels to the other Bti mosquitocidal Cry toxins, namely Cry4Aa, Cry4Ba, and Cry11Ba, a homologous Cry11 toxin from Btj. The resistance levels observed were 66 fold with Cry4Aa, while resistance levels to Cry4Ba and

Cry11Ba were lower or essentially absent, 13 fold and 2 fold, respectively. These results are consistent with previous research with *Culex quinquefasciatus* (Georghiou et al., 1997). Cry11Aa-resistant *Cu. quinquefasciatus* showed high resistance ratio to Cry11Aa (at least 1,000 fold), a mixture of Cry4Aa and Cry4Ba (41.6 fold), but low resistance to Cry11Ba (6.8-9.2 fold) (Cheong et al., 1997; Wirth et al., 1998; Wirth et al., 2010). These results suggest that *Ae. aegypti* and *Cu. quinquefasciatus* have similar pathogenic mechanism for Cry11Aa toxin.

To determine a resistance mechanism, proteolytic activity and binding affinity were determined with WT and G30 larvae midgut. *Heliothis virescens* showed different digestion patterns between susceptible and resistant strain (Forcada et al., 1996). However, WT and G30 strain showed similar digestion patterns and protoxin activation implying proteolytic activity was probably not involved in Cry11Aa resistance. In contrast, a competitive binding assay revealed that the G30 midgut showed reduced binding affinity for Cry11Aa while WT and G25-5 midgut showed similar binding affinities. These data suggests that Cry11Aa has decreased binding affinity to the G30 midgut membrane and implies that receptor alteration may contribute to Cry11Aa resistance in *Ae. aegypti*. The most common mechanism of resistance in Lepidoptera carried changes of binding affinity of toxin receptors (Bravo et al., 2008). Cry toxin resistance in Lepidoptera was associated with mutations in receptor proteins such as cadherin, ALP, APN or ABC transporter (Gahan et al., 2001; Jurat-Fuentes et al., 2004; Herrero et al., 2005; Atsumi et al., 2012). Based on these results, this research focused on finding a receptor protein linked to Cry11Aa resistance.

Previously identified receptor proteins of Cry11Aa are *Aedes* cadherin (AAEL007478), N-cadherin (AAEL000597), APNs (AAEL012778 and AAEL08155), and ALP (AAEL009077) (Chen et al., 2009; Chen et al., 2009; Fernandez et al., 2009). These receptors were further analyzed to determine if there were any mutations or the expression of receptors was altered. Moreover, other possible receptor proteins were investigated with the transcriptome analysis of whole genes using RNA seq. Based on these analyses, the expression of N-cadherin (AAEL000597) and ALP (AAEL003298) were changed but these proteins showed not to associated mutations. Potentially such expression changes could lead to Cry11Aa resistance.

We showed N-cadherin (AAEL000597) bound Cry11Aa with high affinity and AAAEL000597-silenced larvae in midgut showed increased tolerance against Cry11Aa (Chapter 3). In G30 larvae midgut, both transcript levels and protein expression of N-cadherin were consistently reduced (-56%, -38%) implying down-regulated N-cadherin may be associated with Cry11Aa resistance. These results are different from previous research in Lepidoptera. The most frequently observed mechanism in Lepidoptera results from a cadherin mutation or deletion that results in premature truncation of the protein (Yang et al., 2007; Gahan et al., 2001). However, research with the sugarcane borer reported that reduction of cadherin expression was associated with Cry1Ab-resistant *Diatraea saccharalis* (Yang et al., 2011). Accordingly, these data suggest that N-cadherin could be involved in Cry11Aa resistance in *Ae. aegypti*.

ALP (AAEL003298) was also significantly down-regulated in G30 larvae midgut. The 65 kDa band detected by an anti-ALP polyclonal antibody was analyzed in TOF

mass spectrometry and showed ALP (AAEL003298) expression was reduced (-44%) in G30 larvae midgut. In a *H. virescens* resistance strain, reduced expression of ALP was observed (Jurat-Fuentes et al., 2004). Decreased expression of ALP suggests that ALP (AAEL003298) may be linked to Cry11Aa resistance. Chapter 2 shows *Aedes* cadherin mediates Cry11Aa toxicity, but needs an additional receptor for full toxicity. We expect this ALP (AAEL003298) possibly functions as a secondary receptor with *Aedes* cadherin. However, more investigation is still needed. The transcript level of ALP was not changed in RNA seq analyses. Moreover, this ALP (AAEL003298) binds Cry4Ba and is localized to *Ae. aegypti* lipid rafts (Bayyareddy et al., 2009; Bayyareddy et al., 2012). These results suggest this ALP (AAEL003298) is likely involved in Cry11A and Cry4Ba toxicity. However, the G30 strain showed low resistant ratio to Cry4Ba. Therefore, future research aimed to determine if ALP (AAEL003298) actually mediates Cry4Ba toxicity is required.

In summary, Cry11Aa resistance in *Ae. aegypti* was associated with decreased toxin binding to larval midgut membranes. Therefore, receptor proteins binding Cry11Aa were tested to see if they contain mutations or their expression is altered in Cry11Aa-resistant larvae midgut. These investigations found two receptor proteins, N-cadherin (AAEL000597) and ALP (AAEL003298), which were down-regulated in G30 larvae midgut. In Chapter 3, we mentioned Cry11Aa toxicity takes two mechanisms in *Ae. aegypti*. Here we propose that N-cadherin (AAEL000597) not only is involved in Cry11Aa toxicity but also mediates Cry11Aa resistance. This would be an additional mechanism then those previously proposed. Moreover, we suggest that ALP

(AAEL003298) is involved in Cry11Aa toxicity with *Aedes* cadherin likely involving the pore-forming model.

References

Atsumi, S., Miyamoto, K., Yamamoto, K., Narukawa, J., Kawai, S., Sezutsu, H., Kobayashi, I., Uchino, K., Tamura, T., Mita, K., Kadono-Okuda, K., Wada, S., Kanda, K., Goldsmith, M. R. and Noda, H. (2012). Single amino acid mutation in an ATP-binding cassette transporter gene causes resistance to Bt toxin Cry1Ab in the silkworm, *Bombyx mori*. *Proc Natl Acad Sci* **109**: E1591-E1598.

Bayyareddy, K., Andacht, T. M., Abdullah, M. A. and Adang, M. J. (2009). Proteomic identification of *Bacillus thuringiensis* subsp. *israelensis* toxin Cry4Ba binding proteins in midgut membranes from *Aedes* (Stegomyia) *aegypti* Linnaeus (Diptera, Culicidae) larvae. *Insect Biochem Mol Biol* **39**: 279-286.

Bayyareddy, K., Zhu, X., Orlando, R. and Adang, M. J. (2012). Proteome analysis of Cry4Ba toxin-interacting *Aedes aegypti* lipid rafts using geLC-MS/MS. *J Proteome Res.* **11**: 5843-5855.

Berry, C., O'Neil, S., Ben-Dov, E., Jones, A. F., Murphy, L., Quail, M. A., Holden, M. T., Harris, D., Zaritsky, A. and Parkhill, J. (2002). Complete sequence and organization of pBtoxis, the toxin-coding plasmid of *Bacillus thuringiensis* subsp. *israelensis*. *Appl Environ Microbiol* **68**: 5082-5095.

Bravo, A. and Soberon, M. (2008). How to cope with insect resistance to Bt toxins? *Trends Biotechnol* **26**: 573-579.

Chang, C., Yu, Y. M., Dai, S. M., Law, S. K. and Gill, S. S. (1993). High-level cryIVD and cytA gene expression in *Bacillus thuringiensis* does not require the 20-kilodalton protein, and the coexpressed gene products are synergistic in their toxicity to mosquitoes. *Appl Environ Microbiol* **59**: 815-821.

Chen, J., Aimanova, K. G., Fernandez, L. E., Bravo, A., Soberon, M. and Gill, S. S. (2009). *Aedes aegypti* cadherin serves as a putative receptor of the Cry11Aa toxin from *Bacillus thuringiensis* subsp. *israelensis*. *Biochem J* **424**: 191-200.

Chen, J., Aimanova, K. G., Pan, S. and Gill, S. S. (2009). Identification and characterization of *Aedes aegypti* aminopeptidase N as a putative receptor of *Bacillus thuringiensis* Cry11A toxin. *Insect Biochem Mol Biol* **39**: 688-696.

Chen, J., Likitvivatanavong, S., Aimanova, K. G. and Gill, S. S. (submitted). A 104 kDa *Aedes aegypti* aminopeptidase N is a putative receptor for the Cry11Aa toxin from *Bacillus thuringiensis* subsp. *israelensis*. *Insect Biochem Mol Biol.*

Cheong, H., Dhesi, R. K. and Gill, S. S. (1997). Marginal cross-resistance to mosquitocidal *Bacillus thuringiensis* strains in Cry11A-resistant larvae: presence of Cry11A-like toxins in these strains. *FEMS Microbiol Lett* **153**: 419-424.

Chilcott, C. and Ellar, D. J. (1988). Comparative Toxicity of *Bacillus thuringiensis* var. *israelensis* Crystal Proteins *in vivo* and *in vitro*. *Journal of General Microbiology* **134**: 2551-2558.

Cowles, E. A., Yunovitz, H., Charles, J. F. and Gill, S. S. (1995). Comparison of toxin overlay and solid-phase binding assays to identify diverse CryIA(c) toxin-binding proteins in *Heliothis virescens* midgut. *Appl Environ Microbiol* **61**: 2738-2744.

Delecluse, A., Rosso, M. L. and Ragni, A. (1995). Cloning and expression of a novel toxin gene from *Bacillus thuringiensis* subsp. *jegathesan* encoding a highly mosquitocidal protein. *Appl Environ Microbiol* **61**: 4230-4235.

Fernandez, L. E., Aimanova, K. G., Gill, S. S., Bravo, A. and Soberon, M. (2006). A GPI-anchored alkaline phosphatase is a functional midgut receptor of Cry11Aa toxin in *Aedes aegypti* larvae. *Biochemical J* **394**: 77-84.

Fernandez, L. E., Martinez-Anaya, C., Lira, E., Chen, J., Evans, A., Hernandez-Martinez, S., Lanz-Mendoza, H., Bravo, A., Gill, S. S. and Soberon, M. (2009). Cloning and epitope mapping of Cry11Aa-binding sites in the Cry11Aa-receptor alkaline phosphatase from *Aedes aegypti*. *Biochemistry* **48**: 8899-8907.

Forcada, C., Alcicer, E., Garceri, M. D. and Martinez, R. (1996). Differences in the midgut proteolytic activity of two *Heliothis virescens* strains, one susceptible and one resistant to *Bacillus thuringiensis* toxins. *Arch Insect Biochem Physiol* **31**: 257-272.

Gahan, L. J., Gould, F. and Heckle, D. G. (2001). Identification of a gene associated with Bt resistance in *Heliothis virescens*. *Science* **293**: 857-860.

Georghiou, G. P. and Wirth, M. C. (1997). Influence of exposure to single versus multiple toxins of *Bacillus thuringiensis* subsp. *israelensis* on development of resistance in the mosquito *Culex quinquefasciatus* (Diptera: Culicidae). *Appl Environ Microbiol* **63**: 1095-1101.

Grabherr, M., Haas, B., Yassour, M., Levin, J., Thompson, D., Amit, I., Adiconis, X., Fan, L., Raychowdhury, R., Zeng, Q., Chen, Z., Mauceli, E., Hacohen, N., Gnirke, A., Rhind, N., di Palma, F., Birren, B., Nusbaum, C., Lindblad-Toh, Friedman, N. and Regev, A. (2011). Full-length transcriptome assembly from RNA-Seq data without a reference genome. *Nat Biotechnol* **29**: 644-652.

Herrero, S., Gechev, T., Bakker, P. L., Moar, W. J. and de Maagd, R. A. (2005). *Bacillus thuringiensis* Cry1Ca-resistant *Spodoptera exigua* lacks expression of one of four Aminopeptidase N genes. *BMC Genomics* **6**: 96.

Jurat-Fuentes, J. L. and Adang, M. J. (2004). Characterization of a Cry1Ac-receptor alkaline phosphatase in susceptible and resistant *Heliothis virescens* larvae. *Eur J Biochem* **271**: 3127-3135.

Lereclus, D., Agaisse, H., Gominet, M. and Chaufaux, J. (1995). Overproduction of encapsulated insecticidal crystal proteins in a *Bacillus thuringiensis* spo0A mutant. *Biotechnology (N Y)* **13**: 67-71.

Ligon, B. L. (2006). Reemergence of an unusual disease: the chikungunya epidemic. *Semin Pediatr Infect Dis* **17**: 99-104.

Likitvivatanavong S, C. J., Bravo A, Soberón M, Gill SS. (2011). Cadherin, alkaline phosphatase, and aminopeptidase N as receptors of Cry11Ba toxin from *Bacillus thuringiensis* subsp. *jegathesan* in *Aedes aegypti*. *Appl Environ Microbiol* **77**: 24-31.

Margalith, Y. and Ben-Dov, E. (2000). Biological Control by *Bacillus thuringiensis* subsp. *israelensis*. In: Insect Pest Management: Techniques for Environmental Protection. Rechicgl, J. E. and Rechcigl, N. A. (Ed). pp243-301. CRC Press.

Mittal, P. K. (2003). Biolarvicides in vector control: challenges and prospects. *J Vector Borne Dis* **40**: 20-32.

Mortazavi, A., Williams, B., McCue, K., L, S. and Wold, B. (2008). Mapping and quantifying mammalian transcriptomes by RNA-Seq. *Nat Methods* **5**: 621-628.

Nene, V., Wortman, J. R., Lawson, D., Haas, B., Kodira, C., Tu, Z. J., Loftus, B., Xi, Z., Megy, K., Grabherr, M., Ren, Q., Zdobnov, E. M., Lobo, N. F., Campbell, K. S., Brown, S. E., Bonaldo, M. F., Zhu, J., Sinkins, S. P., Hogenkamp, D. G., Amedeo, P., Arensburger, P., Atkinson, P. W., Bidwell, S., Biedler, J., Birney, E., Bruggner, R. V., Costas, J., Coy, M. R., Crabtree, J., Crawford, M., Debruyn, B., Decaprio, D., Eiglmeier, K., Eisenstadt, E., El-Dorry, H., Gelbart, W. M., Gomes, S. L., Hammond, M., Hannick, L. I., Hogan, J. R., Holmes, M. H., Jaffe, D., Johnston, J. S., Kennedy, R. C., Koo, H., Kravitz, S., Kriventseva, E. V., Kulp, D., Labutti, K., Lee, E., Li, S., Lovin, D. D., Mao, C., Mauceli, E., Menck, C. F., Miller, J. R., Montgomery, P., Mori, A., Nascimento, A. L., Naveira, H. F., Nusbaum, C., O'Leary, S., Orvis, J., Pertea, M., Quesneville, H., Reidenbach, K. R., Rogers, Y. H., Roth, C. W., Schneider, J. R., Schatz, M., Shumway, M., Stanke, M., Stinson, E. O., Tubio, J. M., Vanzee, J. P., Verjovski-Almeida, S., Werner, D., White, O., Wyder, S., Zeng, Q., Zhao, Q., Zhao, Y., Hill, C. A., Raikhel, A. S., Soares, M. B., Knudson, D. L., Lee, N. H., Galagan, J., Salzberg, S. L., Paulsen, I. T., Dimopoulos, G., Collins, F. H., Birren, B., Fraser-Liggett, C. M. and Severson, D. W. (2007). Genome sequence of *Aedes aegypti*, a major arbovirus vector. *Science* **316**: 1718-1723.

Nielsen-Leroux, C. and Charles, J. F. (1992). Binding of *Bacillus sphaericus* binary toxin to a specific receptor on midgut brush-border membranes from mosquito larvae. *Eur J Biochem* **210**: 585-590.

Perez, C., Fernandez, L. E., Sun, J., Folch, J. L., Gill, S. S., Soberon, M. and Bravo, A. (2005). *Bacillus thuringiensis* subsp. *israelensis* Cyt1Aa synergizes Cry11Aa toxin by functioning as a membrane-bound receptor. *Proc Natl Acad Sci U S A* **102**: 18303-18308.

Perez, C., Munoz-Garay, C., Portugal, L. C., Sanchez, J., Gill, S. S., Soberon, M. and Bravo, A. (2007). *Bacillus thuringiensis* ssp. *israelensis* Cyt1Aa enhances activity of Cry11Aa toxin by facilitating the formation of a pre-pore oligomeric structure. *Cell Microbiol* **9**: 2931-2937.

Pfaffl, M. (2001). A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* **29**: e45.

Rodríguez-Almazán, C., Reyes, E. Z., Zúñiga-Navarrete, F., Muñoz-Garay, C., Gómez, I., Evans, A. M., Likitvivatanavong, S., Bravo, A., Gill, S. S. and Soberón, M. (2012). Cadherin binding is not a limiting step for *Bacillus thuringiensis* subsp. *israelensis* Cry4Ba toxicity to *Aedes aegypti* larvae. *Biochem J* **443**: 711-717.

Tarazona, S., García-Alcalde, F., Dopazo, J., Ferrer, A. and Conesa, A. (2011). Differential expression in RNA-seq: a matter of depth. *Genome Res* **21**: 2213-2223.

Tetreau, G., Bayyareddy, K., Jones, C. M., Stalinski, R., Riaz, M. A., Paris, M., David, J. P., Adang, M. J. and Després, L. (2012). Larval midgut modifications associated with Bti resistance in the yellow fever mosquito using proteomic and transcriptomic approaches. *BMC Genomics* **13**: 248.

Tomori, O. (2004). Yellow fever: the recurring plague. *Crit Rev Clin Lab Sci* **41**: 391-427.

WHO (2002). Dengue and Dengue Haemorrhagic Fever, Geneva: World Health Organization.

Wirth, M. C., Delecluse, A., Federici, B. A. and Walton, W. E. (1998). Variable cross-resistance to Cry11B from *Bacillus thuringiensis* subsp. *jegathesan* in *Culex quinquefasciatus* (Diptera: Culicidae) resistant to single or multiple toxins of *Bacillus thuringiensis* subsp. *israelensis*. *Appl Environ Microbiol* **64**: 4174-4179.

Wirth, M. C., Walton, W. E. and Federici, B. A. (2010). Evolution of resistance to the *Bacillus sphaericus* Bin toxin is phenotypically masked by combination with the mosquitocidal proteins of *Bacillus thuringiensis* subspecies *israelensis*. *Environ Microbiol* **12**: 1154-1160.

Yang, Y., Chen, H., Wu, Y., Yang, Y. and Wu, S. (2007). Mutated cadherin alleles from a field population of *Helicoverpa armigera* confer resistance to *Bacillus thuringiensis* toxin Cry1Ac. *Appl Environ Microbiol* **73**: 6939-6944.

Yang, Y., Zhu, Y. C., Ottea, J., Husseneder, C., Leonard, B. R., Abel, C., Luttrell, R. and Huang, F. (2011). Down regulation of a gene for cadherin, but not alkaline phosphatase, associated with Cry1Ab resistance in the sugarcane borer *Diatraea saccharalis*. *PLoS One* **6**: e25783.

Table 4.1. Toxicity of Cry11A to resistant and susceptible *Aedes* at each generation during selection.

	LC_{50} (ul/200 ml)			LC_{90} (ul/200 ml)		
	Susceptible strain	Resistant strain	Fold	Susceptible strain	Resistant strain	Fold
G11	0.18	0.47	3	1.76	3.29	2
G12		0.09			0.67	
G13	0.14	0.53	4	0.85	3.48	4
G14		0.53			3.48	
G15	0.07	0.27	4	1.19	2.87	2
G16	1.51	4.46	3	12.2	46.2	4
G17						
G18		2.51			52.1	
G19	0.19	1.31	7	2.50	32.2	13
G20	17.3	483	28	122	11800	96
G21		305			9630	
G22		1880			25300	
G23		71.1			57600	
G24	16.2	843	52	704	27700	39
G25	13.9	1070	77	115	96100	834
G26		195			8400	
G27		173			3260	
G28	5.97	455	76	40.2	6800	169
G29	7.62	719	94	67.9	16000	236
G30	6.12	758	124	32.6	30800	943

Table 4.2. Resistance levels in *Aedes aegypti* larvae to Cry4Aa, Cry4Ba, and Cry11Ba and toxin concentrations at the four generations tested.

		LC_{50} (ul/200 ml)			LC_{90} (ul/200 ml)		
		Susceptible strain	Resistant strain	Fold	Susceptible strain	Resistant strain	Fold
Cry4Aa	G24	12.2	270	22	673	7660	11
	G25	44.2	632	14	814	8050	10
	G28	4.15	279	67	26.4	3130	119
	G30	2.62	174	66	11.5	1100	95
Cry4Ba	G24	14.9	106	7	175	688	4
	G25	10.2	160	16	54.9	1770	32
	G28	7.86	85.7	11	29.3	711	24
	G30	5.26	69.2	13	38.2	1540	40
Cry11Ba	G24	16.3	27.8	2	114	191	2
	G25	8.20	10.6	1	33.4	82.9	2
	G28	5.64	12.4	2	41.5	119	3
	G30	2.92	6.67	2	15.2	35.4	2

Table 4.3. Total ALP and APN activities are not changed between susceptible larvae midgut (WT) and G30 larvae midgut.

Strain	ALP activity[a] uM x ml^{-1} x min^{-1} / ug	APN activity[a] uM x ml^{-1} x min^{-1} / ug
WT	233	327
G30	224	255

[a]To determine ALP and APN activities, BBMVs from WT and G30 were determined using *p*-nitrophenyl phosphate as an ALP substrate and leucine-*p*-nitroanilide as an APN substrate. The specific activities of ALP and APN were enriched in both BBMVs, but did not show any difference in activities between WT and G30.

Table 4.4. Previously identified receptors do not contain any mutations in the G30 larvae. Mutations in target genes were screened using RNA seq and direct sequencing. NI: non-identified gene in Illumina sequencing. X: no mutation in screened gene. -: non-checked gene.

	Gene ID	Gap in illumina sequencing	Mutation screening
AaeCad	AAEL007478	1	X
Ncad	AAEL000597	NI	X
ALP1	AAEL009077	NI	X
APN1	AAEL012778	0	-
APN2	AAEL008155	0	-

Table 4.5. Transcript expression of a number of genes is significantly up-regulated in the midgut of the G30 strain compared to that in the WT and G25-5 strains (Qvalue > 0.8).

Gene ID	Aedes ID	Description	Log_2	Qvalue
CL1839.Contig1_All	--	--	13.1	0.96
CL3653.Contig2_All	AAEL002812-RA	conserved hypothetical protein	11.7	0.91
Unigene22204_All	--	--	10.8	0.84
CL1312.Contig1_All	AAEL002812-RA	conserved hypothetical protein	10.7	0.84
CL1032.Contig2_All	AAEL010590-RA	aldose-1-epimerase	10.7	0.83
CL1202.Contig4_All	AAEL002798-RA	conserved hypothetical protein	8.11	0.88
Unigene8501_All	--	--	8.03	0.87
CL3942.Contig3_All	AAEL009555-RA	Niemann-Pick Type C-2	5.31	0.82
CL2413.Contig1_All	--	--	4.77	0.83
Unigene20178_All	AAEL001490-RA	acylphosphatase	4.34	0.82
Unigene5390_All	AAEL007938-RA	serine-type endopeptidase	4.33	0.88
Unigene9801_All	AAEL004631-RA	actin	4.13	0.87
CL78.Contig3_All	AAEL007938-RA	serine-type endopeptidase	4.13	0.88
Unigene5370_All	AAEL001673-RA	actin	4.10	0.87
CL10.Contig2_All	AAEL001673-RA	actin	3.91	0.87
Unigene11783_All	AAEL001673-RA	actin	3.80	0.87
Unigene15664_All	--	--	3.76	0.84
Unigene11784_All	AAEL004631-RA	actin	3.75	0.87
CL690.Contig2_All	AAEL010163-RA	conserved hypothetical protein	3.74	0.84
Unigene5055_All	AAEL001767-RC	conserved hypothetical protein	3.56	0.80
Unigene11739_All	AAEL004631-RA	actin	3.39	0.86
Unigene10591_All	--	--	3.00	0.80
Unigene19449_All	AAEL004157-RA	hypothetical protein	2.21	0.80

Table 4.6. Transcript expression of a number of genes is significantly down-regulated in the midgut of the G30 strain compared to that in the WT and G25-5 strains (Qvalue > 0.8).

Gene ID	Aedes ID	Description	Log_2	Qvalue
Unigene2241_All	--	--	-13.8	0.97
Unigene2577_All	--	--	-12.9	0.95
Unigene400_All	--	--	-12.2	0.93
CL1091.Contig1_All	--	--	-12.2	0.93
Unigene2591_All	--	--	-12.2	0.93
Unigene2593_All	--	--	-12.0	0.93
Unigene1023_All	--	--	-11.8	0.92
Unigene713_All	--	--	-11.6	0.90
Unigene2317_All	--	--	-11.6	0.90
Unigene1911_All	--	--	-11.5	0.90
Unigene1160_All	--	--	-11.5	0.90
Unigene1177_All	--	--	-11.5	0.90
Unigene2558_All	--	--	-11.5	0.90
Unigene1588_All	--	--	-11.5	0.90
Unigene744_All	--	--	-11.4	0.89
Unigene2032_All	--	--	-11.3	0.89
Unigene2352_All	--	--	-11.3	0.89
Unigene1152_All	--	--	-11.3	0.89
Unigene1506_All	--	--	-11.3	0.88
CL793.Contig2_All	--	--	-11.2	0.88
Unigene1013_All	--	--	-11.2	0.88
Unigene2484_All	--	--	-11.2	0.88
Unigene1001_All	--	--	-11.2	0.88
Unigene1507_All	--	--	-11.2	0.88
Unigene1141_All	--	--	-11.1	0.87
Unigene2043_All	--	--	-11.1	0.87
Unigene2035_All	--	--	-10.9	0.86
Unigene352_All	--	--	-10.9	0.86
CL2806.Contig1_All	AAEL006642-RA	tubulin alpha chain	-10.9	0.85
Unigene2225_All	--	--	-10.9	0.85
Unigene2036_All	--	--	-10.9	0.85
Unigene1772_All	--	--	-10.9	0.85
Unigene732_All	--	--	-10.9	0.85
Unigene874_All	--	--	-10.8	0.85
Unigene1504_All	--	--	-10.8	0.84
Unigene752_All	--	--	-10.8	0.84

Table 4.6. Continued.

Gene ID	Aedes ID	Description	Log_2	Qvalue
Unigene2600_All	--	--	-10.7	0.84
Unigene1814_All	--	--	-10.7	0.84
Unigene24398_All	AAEL011688-RA	cpg binding protein	-10.7	0.84
CL1091.Contig2_All	--	--	-10.7	0.84
Unigene670_All	--	--	-10.7	0.84
Unigene1247_All	--	--	-10.7	0.84
CL3007.Contig1_All	--	--	-10.7	0.83
Unigene2178_All	--	--	-10.7	0.83
Unigene360_All	--	--	-10.7	0.83
Unigene90_All	--	--	-10.7	0.83
Unigene1424_All	--	--	-10.6	0.83
Unigene772_All	--	--	-10.6	0.83
Unigene24252_All	--	--	-10.6	0.82
CL4222.Contig2_All	--	--	-10.6	0.82
Unigene1246_All	--	--	-10.6	0.82
Unigene2650_All	--	--	-10.6	0.82
Unigene9771_All	--	--	-10.5	0.81
Unigene1908_All	--	--	-10.5	0.81
Unigene2050_All	--	--	-10.5	0.81
Unigene2057_All	--	--	-10.5	0.80
CL1091.Contig3_All	--	--	-10.4	0.80
Unigene688_All	--	--	-10.4	0.80
Unigene10724_All	AAEL008467-RA	cysteine synthase	-6.06	0.80
Unigene2109_All	--	--	-5.60	0.85
Unigene10723_All	AAEL008467-RA	cysteine synthase	-5.47	0.84
Unigene179_All	--	--	-5.36	0.82
CL2032.Contig1_All	AAEL011772-RA	DNA repair protein rad50	-5.36	0.81
Unigene18411_All	AAEL017225-RA	hypothetical protein	-5.35	0.87
Unigene9736_All	AAEL005159-RA	latent nuclear antigen	-5.07	0.89
Unigene8746_All	--	--	-5.01	0.82
Unigene20083_All	AAEL010656-RA	leucine-rich immune protein	-4.80	0.82
CL429.Contig1_All	--	--	-4.73	0.81
Unigene19900_All	AAEL007836-RA	hypothetical protein	-4.71	0.87
Unigene9777_All	AAEL003589-RA	transcription factor	-4.67	0.88
CL2518.Contig2_All	AAEL013584-RA	conserved hypothetical protein	-4.55	0.86
Unigene20282_All	--	--	-4.43	0.83
Unigene2326_All	--	--	-4.33	0.85
Unigene2629_All	--	--	-4.27	0.84
Unigene5412_All	AAEL003589-RA	transcription factor	-4.18	0.87
CL212.Contig1_All	AAEL009124-RA	cytochrome P450	-4.16	0.88

Table 4.6. Continued.

Gene ID	Aedes ID	Description	Log_2	Qvalue
CL4457.Contig10_All	AAEL001054-RA	glutathione transferase	-4.15	0.88
Unigene17648_All	AAEL009127-RA	cytochrome P450	-4.04	0.86
Unigene19656_All	--	--	-4.04	0.84
Unigene10506_All	AAEL011167-RA	cathepsin	-3.85	0.85
CL3560.Contig1_All	--	--	-3.80	0.85
Unigene11811_All	AAEL013845-RA	ER resident protein	-3.79	0.82
Unigene13977_All	AAEL012864-RA	conserved hypothetical protein	-3.75	0.84
CL2466.Contig1_All	AAEL005156-RA	hypothetical protein	-3.74	0.88
CL4918.Contig3_All	AAEL002670-RA	AMP dependent ligase	-3.67	0.86
Unigene12816_All	AAEL010386-RA	glucosyl transferases	-3.63	0.85
Unigene13553_All	--	--	-3.40	0.86
CL1690.Contig2_All	AAEL000525-RA	histone H2A	-3.37	0.81
Unigene2270_All	--	--	-3.30	0.85
Unigene13339_All	AAEL007758-RA	conserved hypothetical protein	-3.24	0.86
CL3604.Contig2_All	AAEL009556-RA	Niemann-Pick Type C-2	-3.19	0.85
CL2308.Contig1_All	AAEL001887-RB	glutamine synthetase 1, 2	-3.15	0.83
Unigene12860_All	AAEL009556-RA	Niemann-Pick Type C-2	-3.14	0.85
Unigene12338_All	AAEL008651-RA	conserved hypothetical protein	-3.12	0.82
Unigene12602_All	--	--	-3.09	0.86
CL2346.Contig2_All	AAEL002495-RA	conserved hypothetical protein	-3.09	0.85
Unigene19120_All	--	--	-3.03	0.82
Unigene10043_All	--	--	-3.03	0.86
Unigene18633_All	AAEL009566-RA	apolipoprotein D	-3.02	0.85
Unigene14655_All	AAEL010037-RA	phosphoglucomutase	-2.91	0.83
CL3691.Contig3_All	--	--	-2.90	0.84
CL2855.Contig1_All	AAEL014949-RA	conserved hypothetical protein	-2.87	0.81
Unigene13338_All	AAEL007758-RA	conserved hypothetical protein	-2.85	0.83
Unigene16880_All	AAEL001057-RA	lipase	-2.83	0.82
CL3569.Contig1_All	AAEL000080-RA	phosphoenolpyruvate carboxykinase	-2.83	0.84
Unigene1196_All	AAEL005617-RA	UTP-glucose-1-phosphate uridylyltransferase 2	-2.83	0.81
Unigene14437_All	AAEL010104-RA	predicted protein	-2.78	0.82
CL3569.Contig2_All	AAEL000006-RA	phosphoenolpyruvate carboxykinase	-2.60	0.82
CL3246.Contig1_All	AAEL007951-RA	glutathione transferase	-2.60	0.82
Unigene15427_All	AAEL004112-RA	Thioredoxin Peroxidase	-2.52	0.81
Unigene14226_All	AAEL009467-RA	conserved hypothetical protein	-2.51	0.82
Unigene7070_All	--	--	-2.48	0.81
Unigene1236_All	--	--	-2.46	0.82
CL4663.Contig1_All	AAEL001297-RA	conserved hypothetical protein	-2.37	0.81
CL3754.Contig2_All	AAEL012696-RA	sterol carrier protein-2	-2.21	0.80

Table 4.7. N-cadherin (AAEL000597) transcript levels are attenuated in G30 larvae midgut, but not that of the other known receptors.

		vs WT		vs G25-5		qPCR (%)
		Log_2	Qvalue	Log_2	Qvalue	
AaeCad	AAEL007478	-0.61	0.41	-0.50	0.39	45±22.5
Ncad	AAEL000597	-	-	-	-	-56±26.2
ALP1	AAEL009077	-	-	-	-	23±30.6
APN1	AAEL012778	0.37	0.30	0.34	0.32	46±45.8
APN2	AAEL008155	0.58	0.40	0.31	0.29	89±35.2

Transcript changes of identified receptors for Cry11Aa (AaeCad, Ncad, ALP1, APN1, and APN2) were determined in G30 larvae midgut using Illumina sequencing and qPCR. Ncad was significantly down-regulated in G30 larvae midgut compared to WT larvae midgut.

Table 4.8. RNA seq identified a number of cadherins, ALPs, and APNs. One ALP (AAEL003286) was significantly up-regulated, two ALPs (AAEL01330 and AAEL015070) were significantly down-regulated, and two APNs (AAEL008158 and AAEL008162) were significantly down-regulated compared to WT.

	vs WT		vs G25-5	
	Log_2	Qvalue	Log_2	Qvalue
Cadherin				
AAEL007299	-0.29	0.14	-0.01	0.07
AAEL007488	-0.61	0.41	-0.50	0.39
AAEL011164	-0.11	0.13	-0.04	0.09
AAEL012421	0.13	0.12	-1.03	0.41
ALP				
AAEL003286	5.16	0.84	1.56	0.42
AAEL003289	-1.63	0.69	-0.68	0.47
AAEL003297	-1.43	0.46	-0.39	0.23
AAEL003298	0.05	0.10	-0.13	0.18
AAEL003309	0.23	0.22	-0.54	0.43
AAEL003313	-0.53	0.38	-0.17	0.20
AAEL003905	0.73	0.32	-1.11	0.39
AAEL013330	-4.23	0.86	-2.01	0.79
AAEL015070	-3.06	0.84	-2.05	0.79
APN				
AAEL003227	0.71	0.40	1.36	0.66
AAEL008155	-0.07	0.11	0.27	0.27
AAEL008158	-2.33	0.81	-1.50	0.74
AAEL008162	-2.26	0.81	-1.40	0.72
AAEL008163	-0.11	0.15	0.53	0.42
AAEL009108	-0.41	0.31	-0.49	0.40
AAEL011292	0.08	0.10	-0.64	0.29
AAEL012099	0.05	0.09	0.02	0.09
AAEL012110	-2.81	0.76	-0.66	0.42
AAEL012217	2.27	0.74	0.77	0.41
AAEL012774	1.33	0.67	0.57	0.44
AAEL012776	0.24	0.22	0.21	0.23
AAEL012778	0.37	0.30	0.34	0.32
AAEL012779	0.27	0.23	0.61	0.44
AAEL012781	0.36	0.27	0.50	0.38
AAEL012783	-0.73	0.47	-0.23	0.24
AAEL013899	-0.76	0.48	-1.01	0.62

Table 4.9. ALP (AAEL003298) is down-regulated in G30 BBMV.

Gene ID	WT		G30		G30 vs WT
	Score	TOF MS ES+ (m/z)	Score	TOF MS ES+ (m/z)	
AAEL003309	260	1.54e3	251	1.57e3	102%
AAEL003298	175	1.51e3	134	848	56%

WT and G30 BBMVs were separated in SDS-PAGE gel and 65 kDa bands were analyzed by TOF Mass spectrometry. Two ALPs (AAEL003309 and AAEL003298) were identified, AAEL003309 ALP was not changed between WT and G30, and AAEL003298 ALP was lower expressed (-44%) in G30 compared to WT.

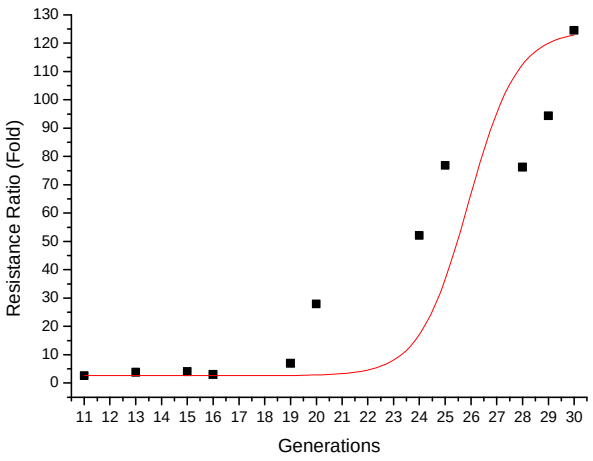


Figure 4.1. *Aedes aegypti* develops high level Cry11Aa resistance after twenty generations. Early fourth-instar larvae were selected at LC_{90} concentration. Resistance ratios were obtained by comparing the LC_{50} values to that obtained with susceptible larvae (WT). Bioassays showed the G30 larvae had a 124-fold resistant ratio.

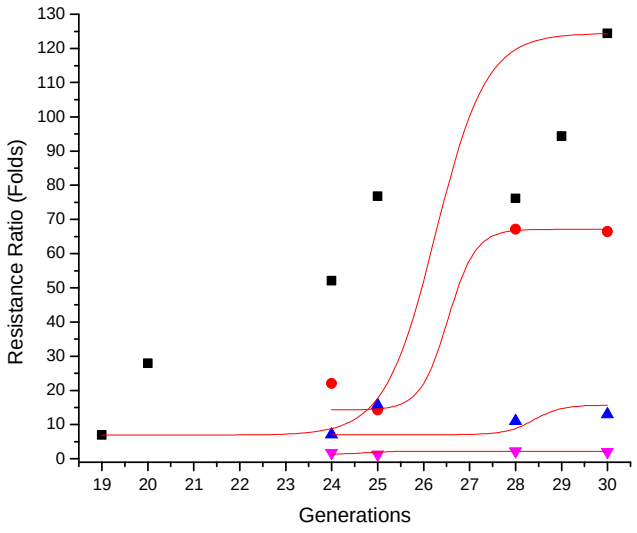


Figure 4.2. *Aedes* larvae developed resistance against Cry11Aa and Cry4Aa but not to CryBa and Cry11Ba. Resistance ratios for individual toxins from mosquitocidal *Bacillus thuringiensis israelensis* (Bti) and *Bacillus thruringiensis jegathesan* (Btj) strains that were tested at four generations during resistance development to Cry11Aa. Cry11Aa (■),Cry4Aa (●) and Cry4Ba (▲) from Bti, and Cry11Ba (▼) from Btj. Resistance ratios for individual toxins were obtained by comparing the LC_{50} to that of susceptible.

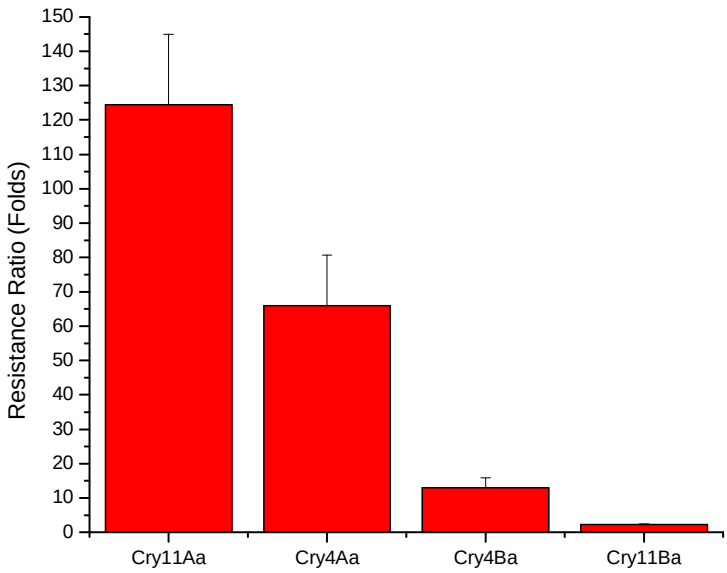


Figure 4.3. G30 show high level resistance to Cry11A and reveals cross resistance to Cry4Aa. Resistance levels of G30 to mosquitocidal Cry toxins were determined with individual Cry toxins; Cry11Aa, Cry4Aa, and Cry4Ba from *Bacillus thruringiensis israelensis* and Cry11Ba from *Bacillus thruringiensis jegathesan*. G30 larvae had high resistance levels to Cry11Aa (124 fold) and Cry4Aa (66 fold), but were relatively susceptible to Cry4Ba (13 fold) and Cry11Ba (2 fold).

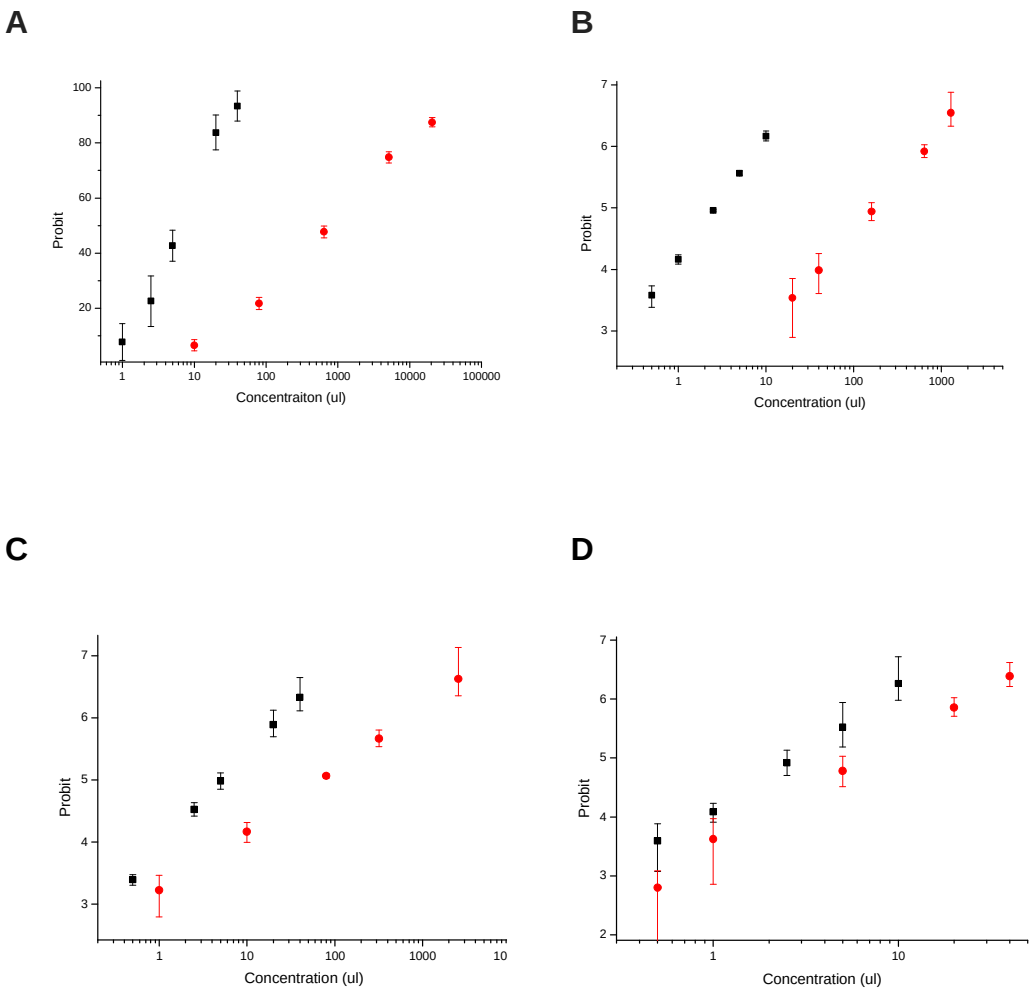


Figure 4.4. Probit analysis of Cry11Aa and Cry4Aa toxins show significant differences in the toxicity to WT (■) and G30 (●), that were not observed with Cry4Ba and Cry11Ba, (A) Cry11Aa, (B) Cry4Aa, (C) Cry4Ba, (D) Cry11Ba.

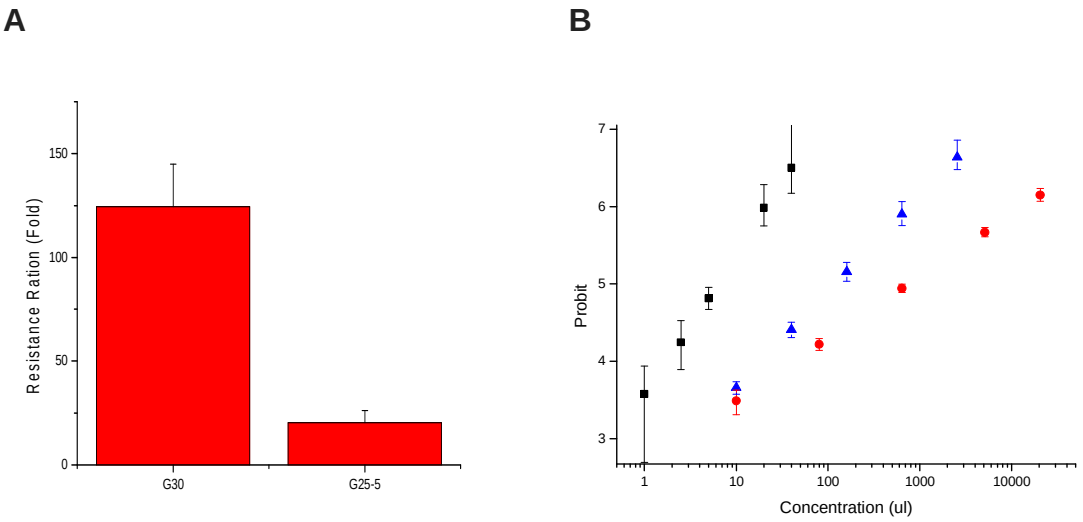


Figure 4.5. Cry11Aa resistant larvae show significant loss of resistance after four generations of non-selection. G25 larvae showing 77-fold resistance ratio were reared without Cry11Aa treatment for five generations, after which the G25-5 showed only a 20-fold resistance ratio compared to susceptible larvae. (A) From G25 larvae, G30 strain was selected by Cry11Aa and finally had 124-fold resistance ration. However, the G25-5 strain has only a 20-fold resistance ratio after removal of selection pressure for five generations. (B) Probit analysis of WT (■), G30 (▲), and G25-5 (●) strain with Cry11Aa toxin.

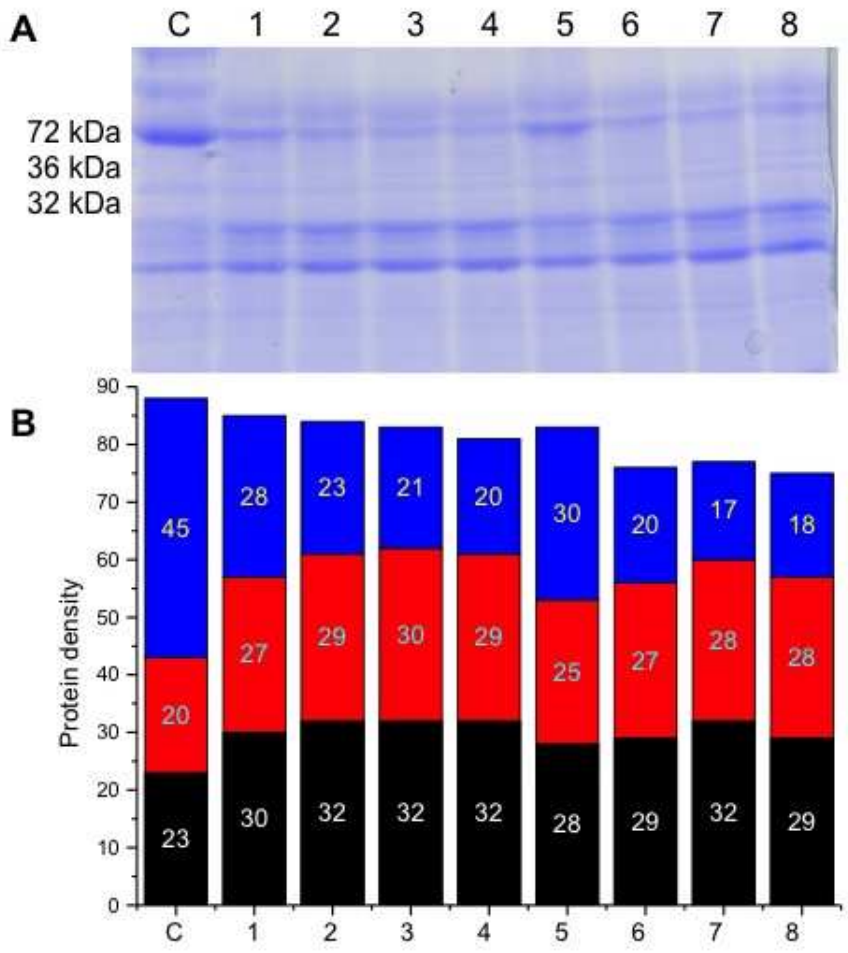


Figure 4.6. Proteolytic activities of WT and G30 larvae midgut are unchanged. Cry11Aa protoxin was incubated with midgut protein extraction including crude midgut protease and then analyzed in SDS-PAGE gel. (A) SDS-PAGE gel showing the results of Cry11Aa protoxin digested with midgut proteolytic extracts. (B) Band densities of three band were calculated and plotted; 72 kDa (blue), 36 kDa (red), and 32 kDa (black). C: Control, 1: 5 min with WT, 2: 10 min with WT, 3: 15 min with WT, 4: 20 min with WT, 5: 5 min with G30, 6: 10 min with G30, 7: 15 min with G30, 8: 20 min with G30.

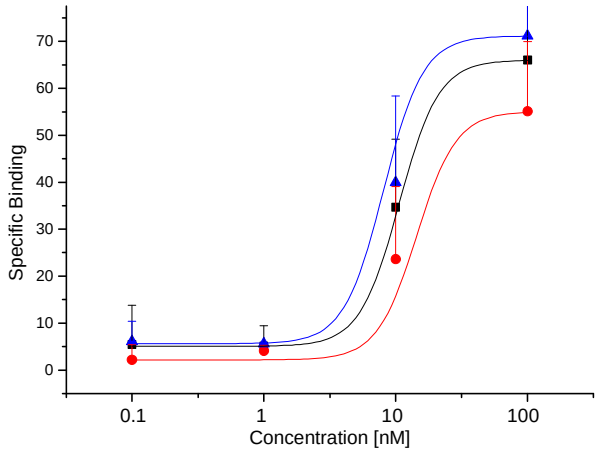
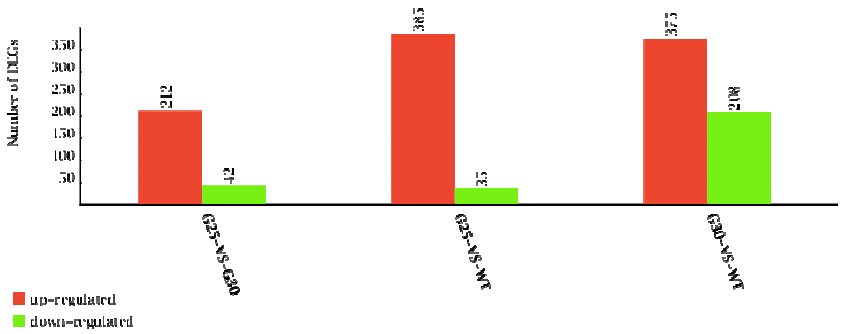
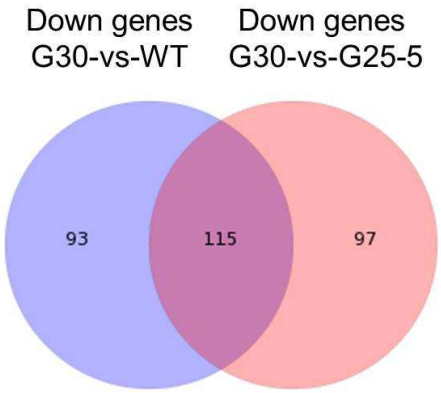


Figure 4.7. Cry11Aa has a lower affinity to midgut membrane prepared from the G30 larvae than the affinity to membranes prepared from WT and G25-5. Binding affinity of WT (■), G30 (●), and G25-5 (▲) was tested with early fourth-instar larvae midguts. Specific binding of biotin-labeled Cry11Aa to BBMV was obtained from total binding minus nonspecific binding. Cry11Aa binding affinity for G30 BBMV was slightly reduced (*Kd*= 10.0 nM for WT, 15.7 for G30, 9.9 nM for G25-5).

A



B



C

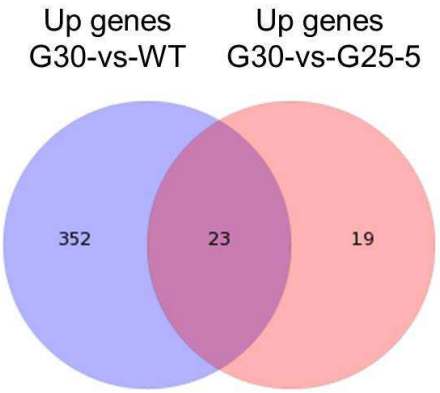


Figure 4.8. A total of 23 genes in G30 are significantly up-regulated and a total of 115 genes in G30 are significantly down-regulated compared to WT and G25-5. Transcript changes in larvae midgut were analyzed using Illumina sequencing. (A) Gene differential expression was analyzed by comparing to WT, G30, and G25-5 each other. (B) A total of 115 genes were significantly down-regulated in G30 midgut compared to WT and G25-5. (C) A total of 23 genes were significantly up-regulated in G30 midgut compared to WT and G25-5.

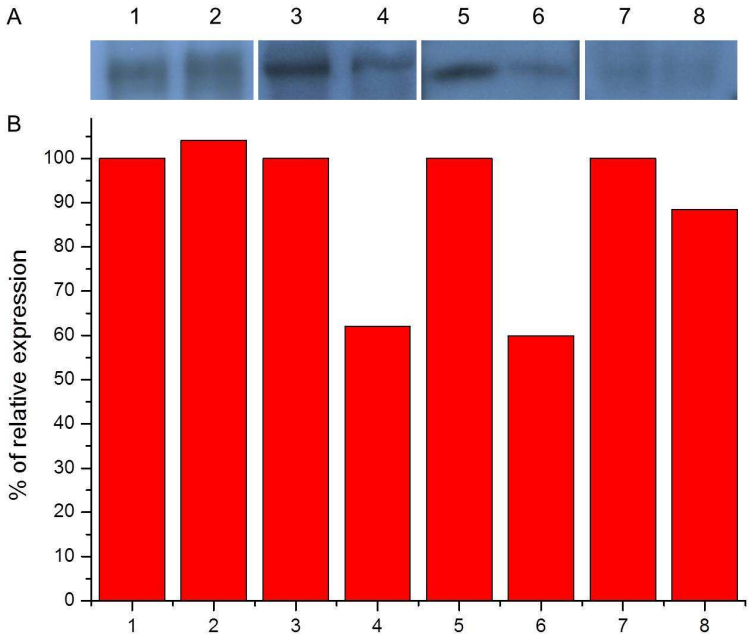


Figure 4.9. N-cadherin and ALPs are significantly reduced in G30 larvae midgut compared to WT while *Aedes* cadherin and APNs are not changed between G30 and WT. (A) To determine the expression of target proteins in WT and G30 larvae midgut, BBMVs were extracted, loaded (10 µg) in SDS-PAGE gel, transferred in membrane, and incubated with the anti-AaeCad peptide antibody, anti-Ncad peptide antibody, anti-ALP polyclonal antibody, and anti-APN polyclonal antibody. (B) The band densities of target proteins were measured and quantified using Image J software and compared using Origin program. The expression of N-cadherin (-38%) and ALP (-40%) in G30 were significantly reduced compared to ones in WT. 1: *Aedes* cadherin in WT, 2: *Aedes* cadherin in G30, 3: AAEL000597 in WT, 4: AAEL000597 in G30, 5: ALP in WT, 6: ALP in G30, 7: APN in WT, 8: APN in G30.

Chapter 5

General conclusions

The objective of this dissertation is to elucidate the molecular mechanisms of *Bacillus thuringiensis* subsp. *israelensis* Cry toxins. The research focused on identification and functional characterization of cadherins mediating Cry11Aa toxicity in *Aedes aegypti*.

Bacillus thuringiensis subsp. *israelensis* (Bti) is a subspecies of *Bacillus thuringiensis* producing crystalline inclusions known as Cry and Cyt toxins. Cry toxins from Bti have insecticidal properties and are highly selective to Dipteran insects, particularly mosquitoes and black flies (Margalith et al., 2000). Because of these advantages, Bti has been largely used for control of mosquitoes including *Aedes aegypti*, an important disease vector (Lacey, 2007). Despite its long-term usage in the field, the molecular mechanisms of Bti action are not well understood. Here I investigated receptor proteins that could mediate Bti toxicity based on previous studies and proposed models in Lepidoptera: the pore-forming model (Soberon et al., 2009) and the signal-cascade model (Zhang et al., 2006; Zhang et al., 2005). For Cry toxin activity, both models basically need the first important event, that is binding to cadherins in the brush border membrane of the target insect midgut (Bravo et al., 2005). Resistance mechanisms to Cry toxins in Lepidoptera have been reported because of a lack of Cry toxin binding to cadherin proteins caused by mutation or deletions in the cadherin gene. Based on these results, we

previously cloned a full-length *Aedes* cadherin, the most homologous cadherin to Lepidoptera cadherin from *Aedes aegypti* larvae and reported this protein binds Cry11Aa toxin from Bti with high affinity (Chen et al., 2009). Furthermore, this cadherin was silenced in larval midgut and cadherin-silenced mosquitoes had tolerance for Cry11Aa toxicity (Rodríguez-Almazán et al., 2012). On the other hand, I investigated if another cadherin is involved in Cry11Aa toxicity. Using a whole genome screen to identify genes that are altered during Cry11Aa intoxication, I identified two cadherin genes that were significantly down-regulated; AAEL000597 and AAEL001196. Based on these results, I investigated if these cadherins are involved in Cry11Aa toxicity *in vivo* and which cadherin is associated with Cry11Aa-resistant mosquitoes.

In the first objective of this study, I investigated if the *Aedes* cadherin (AaeCad) mediates Cry11Aa toxicity in a cell line expressing AaeCad. I established an *Aedes* C6/36 cell line stably expressing AaeCad. The full-length AaeCad was detected without AaeCad degradation in immunoblotting and immunofluorescence staining showed AaeCad was localized in the plasma membrane. Cells expressing AaeCad were significantly more sensitive to Cry11Aa (37% mortality at 400 nM), while control cells expressing the EGFP protein were insensitive to Cry11Aa. This result indicates that *Aedes* cadherin mediates the *in vivo* toxicity of Cry11Aa. Additionally, the level of mortality is lower compared to High Five cell line stably expressing *Manduca* cadherin and requiring only *Manduca* cadherin triggering cell death program for Cry1Ab toxicity (Zhang et al., 2005). These results suggest that a secondary receptor may be required for full toxicity of the Cry11A to be manifest. In *Ae. aegypti,* alkaline phosphatases (ALP) appear to be more

important than aminopeptidase Ns (APN) for Cry11Aa toxicity (Fernandez et al., 2006), even though APNs bind Cry11Aa with high affinity (Chen et al., 2009). Previously three ALPs were tested and the ALP (AAEL009077) bound Cry11Aa and Cry4Ba (Fernandez et al., 2009). However, this ALP was involved in both Cry11Aa and Cry4BA toxicity (Jiménez et al., 2012). Therefore, it is required to find an ALP as a secondary receptor for only Cry11Aa.

The second objective of this research focused on finding novel receptor proteins involved in Bti toxicity. Since cadherins are critical proteins, I focused on finding if there are additional novel cadherins in *Ae. aegypti* that are involved in the toxicity of Cry proteins. I found, using whole genome microarrays, two cadherin genes (AAEL000597 and AALE001196), which were significantly down-regulated during Cry11Aa intoxication. Cry11Aa bound the EGF-LamG domain of AAEL000597 with high affinity and AAEL000597-silenced larvae showed increased tolerance to Cry11Aa toxicity. Furthermore, two regions in EGF-LamG domain of AAEL000597 interacted with Cry11Aa. In addition, loop α-8 and 2 of Cry11Aa domain II bound to EGF-LamG of AAEL000597. Based on these results, I suggest some possible molecular mechanisms for Cry11Aa toxicity. First, Cry toxins prefer to bind protein regions near the transmembrane region. In *Manduca* cadherin, *Aedes* cadherin, and *Aedes* N-cadherin, the extracellular region near the transmembrane domain of receptors all bind Cry toxins with high affinity, regardless the function of receptor domain (Gomez et al., 2001; Hua et al., 2004). These results imply that Cry toxins require an interaction with a component of membrane for Cry toxicity. Second, Cry11Aa seems to require two binding regions of receptors.

Cry11Aa interacted with two regions of *Aedes* ALP (AAEL009077) (Fernandez et al., 2009) and N-cadherin (AAEL000597) just like that in *Manduca* cadherin, in which CR7, CR11, and CR12 were identified as binding regions of Cry1Ab (Gomez et al., 2001; Dorsch et al., 2002; Hua et al., 2004). *Aedes* cadherin showed CR8, 9, 10, 11 bound Cry11Aa in ELISA assay (Chen et al., 2009). However, this binding assay with *Aedes* cadherin was performed without a competitor, thus a competitive binding assay might provide more specific binding regions. Last, *Aedes* cadherin and N-cadherin bind to different Cry11Aa epitopes. Both cadherins showed high affinity to Cry11Aa and bind loop α-8 and loop 2 of Cry11Aa (Chen et al., 2009). However, using Cry11Aa mutant proteins (E266A and V262A) we showed the mutant E266A of Cry11Aa bound N-cadherin (AAEL000597) but not the *Aedes* cadherin, while the mutant V262E bound the *Aedes* cadherin but not N-cadherin (AAEL000597). These results suggest that Cry11Aa has two different binding epitope in larvae midgut implying two pathogenic pathways could be involved in Cry11Aa toxicity. However, additional studies are needed to determine how N-cadherin mediates Cry11Aa toxicity in larvae midgut. I expect Cry11Aa toxicity through N-cadherin may be involved in cadherin stability related with calcium ion binding (Candas et al., 2002). Collectively, I propose that Cry11Aa takes at least two toxic mechanisms by binding to *Aedes* cadherin and N-cadherin. *Aedes* cadherin-involved mechanism may lead to the pore-forming pathway as suggested in lepidopteran insects. However, Cry11Aa toxicity through N-cadherin-mediated mechanisms may result from an unstable structure of N-cadherin caused by Cry11Aa binding.

The final research investigated what mechanisms are involved in Cry11Aa resistance in *Ae. aegypti* that would help further elucidation of Bti toxicity mechanisms. We established a resistant *Aedes* population against Cry11Aa by selecting with Cry11Aa for 30 times. This strain (G30) showed a 124-fold resistance ratio for Cry11Aa compared to susceptible larvae (WT). I investigated the resistant mechanism based on previously reported resistance mechanisms in Lepidoptera (Bravo et al., 2008). Total proteolytic activity causing Cry11Aa degradation was not changed in WT and G30 larvae midgut. Total ALP and APN activity also showed similar activity between WT and G30 larvae midgut. However, Cry11Aa binding affinity was slightly reduced in G30 larvae midgut compared to WT larvae midgut. Therefore, I hypothesized that a Cry11Aa receptor may be associated with Cry11Aa resistance in *Ae. aegypti*. Based on previous research, *Aedes* cadherin (AAEL007478), N-cadherin (AAEL000597), APNs, and ALPs were examined to detremine if they are involved in Cry11Aa resistance. The transcript and protein expression levels of N-cadherin (AAEL000597) were down-regulated. ALP (AAEL003298) also revealed that its protein expression was reduced, although its transcript levels were unchanged. As mention in chapter 3, N-cadherin (AAEL000597) binds Cry11Aa with high affinity and mediates Cry11Aa toxicity. Based on these results, I propose that N-cadherin (AAEL000597) not only mediates Cry11Aa toxicity but also is associated with Cry11Aa resistance by reducing its expression in *Ae. aegypti*. Additionally, ALP (AAEL003298) was also linked to Cry11Aa resistance by down-regulating its expression. However, further research is still required known if ALP (AAEL003298) interacts with Cry11Aa even though this ALP bound Cry4Ba (Chen et al.,

2009; Bayyareddy et al., 2009; Bayyareddy et al., 2012). As mentioned in chapter 2, Cry11Aa requires *Aedes* cadherin as well as an additional receptor for full toxicity. I expect the identified ALP (AAEL003298) could function as a secondary receptor for mosquitocidal Cry toxins.

Consequently, this research demonstrated that the mode of action of Cry11Aa in *Ae. aegypti* is complex. According to the results obtained from this research, I showed that *Aedes* cadherin (AAEL007478) mediates Cry11Aa toxicity, but needs an additional receptor protein, as suggested an ALP, for full toxicity. In research with Cry11Aa resistant mosquitoes, ALP (AAEL003298) was down-regulated. Therefore, I propose that ALP (AAEL003298) may be a secondary receptor since ALP is associated with Cry11Aa resistance. I also found a new type of cadherin, N-cadherin. This N-cadherin binds Cry11Aa with high affinity and is involved in Cry11Aa toxicity. Binding epitopes suggested that N-cadherin follows a pathogenic mechanism that is different from *Aedes* cadherin. Furthermore, I found that this N-cadherin is associated with Cry11Aa resistance in *Ae. aegypti* by changing the expression of N-cadherin.

References

Bayyareddy, K., Andacht, T. M., Abdullah, M. A. and Adang, M. J. (2009). Proteomic identification of *Bacillus thuringiensis* subsp. *israelensis* toxin Cry4Ba binding proteins in midgut membranes from *Aedes* (Stegomyia) *aegypti* Linnaeus (Diptera, Culicidae) larvae. *Insect Biochem Mol Biol* **39**: 279-286.

Bayyareddy, K., Zhu, X., Orlando, R. and Adang, M. J. (2012). Proteome analysis of Cry4Ba toxin-interacting *Aedes aegypti* lipid rafts using geLC-MS/MS. *J Proteome Res.* **11**: 5843-5855.

Bravo, A., Gill, S. S. and Soberon, M. (2005). *Bacillus thuringiensis*: Mechanisms and Use. In Comprehensive Molecular Insect Science. Gilbert, L.I., Kostas, I., and Gill, S.S.(Ed). pp175-205. Elsevier.

Bravo, A. and Soberon, M. (2008). How to cope with insect resistance to Bt toxins? *Trends Biotechnol* **26**: 573-579.

Candas, M., Francis, B. R., Griko, N. B., Midboe, E. G. and Bulla, L. A., Jr. (2002). Proteolytic cleavage of the developmentally important cadherin BT-R_1 in the midgut epithelium of *Manduca sexta*. *Biochemistry* **41**: 13717-13724.

Chen, J., Aimanova, K. G., Fernandez, L. E., Bravo, A., Soberon, M. and Gill, S. S. (2009). *Aedes aegypti* cadherin serves as a putative receptor of the Cry11Aa toxin from *Bacillus thuringiensis* subsp. *israelensis*. *Biochem J* **424**: 191-200.

Chen, J., Aimanova, K. G., Pan, S. and Gill, S. S. (2009). Identification and characterization of *Aedes aegypti* aminopeptidase N as a putative receptor of *Bacillus thuringiensis* Cry11A toxin. *Insect Biochem Mol Biol* **39**: 688-696.

Dorsch, J. A., Candas, M., Griko, N. B., Maaty, W. S., Midboe, E. G., Vadlamudi, R. K. and Bulla, L. A. (2002). Cry1A toxins of *Bacillus thuringiensis* bind specifically to a region adjacent to the membrane-proximal extracellular domain of BT-R_1 in *Manduca sexta*: involvement of a cadherin in the entomopathogenicity of *Bacillus thuringiensis*. *Insect Biochem Mol Biol* **32**: 1025-1036.

Fernandez, L. E., Aimanova, K. G., Gill, S. S., Bravo, A. and Soberon, M. (2006). A GPI-anchored alkaline phosphatase is a functional midgut receptor of Cry11Aa toxin in *Aedes aegypti* larvae. *Biochemical J* **394**: 77-84.

Fernandez, L. E., Martinez-Anaya, C., Lira, E., Chen, J., Evans, A., Hernandez-Martinez, S., Lanz-Mendoza, H., Bravo, A., Gill, S. S. and Soberon, M. (2009). Cloning and epitope mapping of Cry11Aa-binding sites in the Cry11Aa-receptor alkaline phosphatase from *Aedes aegypti*. *Biochemistry* **48**: 8899-8907.

Gomez, I., Oltean, D. I., Gill, S. S., Bravo, A. and Soberon, M. (2001). Mapping the epitope in cadherin-like receptors involved in *Bacillus thuringiensis* Cry1A toxins interaction using phage display. *J Biol Chem* **276**: 28906-28912.

Hua, G., Jurat-Fuentes, J. L. and Adang, M. J. (2004). Bt-R_1a extracellular cadherin repeat 12 mediates *Bacillus thuringiensis* Cry1Ab binding and cytotoxicity. *J Biol Chem* **279**: 28051-28056.

Jiménez, A. I., Reyes, E. Z., Cancino-Rodezno, A., Bedoya-Pérez, L. P., Caballero-Flores, G. G., Muriel-Millan, L. F., Likitvivatanavong, S., Gill, S. S., Bravo, A. and Soberón, M. (2012). *Aedes aegypti* alkaline phosphatase ALP1 is a functional receptor of *Bacillus thuringiensis* Cry4Ba and Cry11Aa toxins. *Insect Biochem Mol Biol* **42**: 683-689.

Lacey, L. A. (2007). *Bacillus thuringiensis* serovariety *israelensis* and *Bacillus sphaericus* for mosquito control. *J Am Mosq Control Assoc* **23**: 133-163.

Margalith, Y. and Ben-Dov, E. (2000). Biological Control by *Bacillus thuringiensis* subsp. *israelensis*. In: Insect Pest Management: Techniques for Environmental Protection. Rechicgl, J. E. and Rechcigl, N. A. (Ed). pp243-301. CRC Press.

Rodríguez-Almazán, C., Reyes, E. Z., Zúñiga-Navarrete, F., Muñoz-Garay, C., Gómez, I., Evans, A. M., Likitvivatanavong, S., Bravo, A., Gill, S. S. and Soberón, M. (2012). Cadherin binding is not a limiting step for *Bacillus thuringiensis* subsp. *israelensis* Cry4Ba toxicity to *Aedes aegypti* larvae. *Biochem J* **443**: 711-717.

Soberon, M., Gill, S. S. and Bravo, A. (2009). Signaling versus punching hole: How do *Bacillus thuringiensis* toxins kill insect midgut cells? *Cell Mol Life Sci* **66**: 1337-1349.

Zhang, X., Candas, M., Griko, N. B., Rose-Young, L. and Bulla, L. A., Jr. (2005). Cytotoxicity of *Bacillus thuringiensis* Cry1Ab toxin depends on specific binding of the toxin to the cadherin receptor BT-R_1 expressed in insect cells. *Cell Death Differ* **12**: 1407-1416.

Zhang, X., Candas, M., Griko, N. B., Taussig, R. and Bulla, L. A. J. (2006). A mechanism of cell death involving an adenylyl cyclase/PKA signaling pathway is induced by the Cry1Ab toxin of *Bacillus thuringiensis*. *Proc Natl Acad Sci* **103**: 9897-9902.