

CHARACTERIZATION AND FUNCTIONAL ANALYSES OF MIDGUT PROTEINS
INVOLVED IN *BACILLUS THURINGIENSIS* SUBSP. *JEGATHESAN* CRY11BA TOXICITY
TO THE MALARIA MOSQUITO *ANOPHELES GAMBIAE*

by

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(Under the Direction of MICHAEL J. ADANG)

ABSTRACT

Cry11Ba, a crystal component produced by *Bacillus thuringiensis* subsp. *jegathesan*, is highly toxic to a broad spectrum of mosquito larvae. The toxicity of Cry11Ba correlates with its functional binding to receptors on mosquito larval midgut epithelial cells. Receptors identified as mediating Cry11Ba toxicity include cadherin, aminopeptidase N and alkaline phosphatase in *An. gambiae* larvae. The objectives of this dissertation research are to investigate the functional roles of receptors in mediating Cry11Ba toxin that lead to its high mosquitocidal potency and to further understand the mechanism of Cry toxin action in mosquito larvae. In the first part of my dissertation, I describe two glycosylphosphatidylinositol (GPI)-anchored proteins, α -amylase (AgAmy1) and α -glucosidase (Agm3) acting as putative binding receptors of Cry11Ba. Immunohistochemistry revealed their different locations in *An. gambiae* larvae. Enzyme-linked immunosorbent assay showed that AgAmy1 and Agm3 proteins bound to Cry11Ba with high affinities (37.6 nM and 21.1 nM) through shared binding sites on Cry11Ba. AgAmy1 and Agm3

neutralized Cry11Ba toxicity against *An. gambiae* larvae. The results provide evidence that AgAmy1 and Agm3 are putative receptors of Cry11Ba in *An. gambiae* larvae.

Cadherins are essential receptors of Cry toxins in several Orders of insects, and loss of a midgut cadherin by mutation or gene silencing can cause resistance. In the second part of my dissertation, I establish the *in vivo* role of cadherin AgCad1 as a binding receptor of Cry11Ba using chitosan/DsiRNA nanoparticle-mediated RNAi in *An. gambiae* larvae. Larvae became more tolerant when cadherin AgCad1 was silenced. Because AgCad1 was co-suppressed by AgCad2 DsiRNA, the role of AgCad2 in Cry11Ba toxicity could not be determined. We conclude that AgCad1 and possibly AgCad2 are involved in Cry11Ba toxin *in vivo* in *An. gambiae* larvae.

INDEX WORDS: *Bacillus thuringiensis jegathesan* (Btjg); Cry11Ba; *Anopheles gambiae* α -amylase (AgAmy1); *Anopheles gambiae* α -glucosidase (Agm3), BBMV; *Anopheles gambiae* cadherin 1 (AgCad1); *Anopheles gambiae* cadherin 2 (AgCad2); Dicer substrate short interfering RNA (DsiRNA); RNA interference (RNAi)

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DEDICATION

I dedicate this dissertation to my beloved parents and my younger brother for their love, support and encouragement.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Mosquitoes in the genus *Anopheles* have proven to be the major sources of malaria transmission to humans. Malaria is a severe public health problem in most tropical areas. According to the latest report from the World Health Organization (WHO), 207 million people were infected with malaria worldwide and approximately 627,000 people died of malaria in 2012 (<http://www.who.int/malaria>). The pathogen causing malaria is the parasite *Plasmodium*, which is transmitted when an infected mosquito bites a human.

Vector control gives economical and effective strategy for reducing disease transmission. There are a variety of commercial pesticides that are used to control mosquitoes, including adulticides, such as synthetic pyrethroids and organophosphates, and larvicides, such as methoprene and temephos. The appearance of insect resistance to chemical pesticides has fostered scientists' interest in the utilization of biopesticides in pest management programs. *Bacillus thuringiensis* (Bt) is a naturally occurring and environmentally friendly bacterium used in control that has highly toxic effect on target insects with no known toxicity on non-target organisms. The strain of Bt subsp *israelensis* (Bti) has been widely used as a tool for mosquito control. Bti is highly toxic for *Aedes* and *Anopheles* larvae, but is less effective against *Culex* larvae (Poncet et al., 1995). To offset this limitation, *Lysinibacillus sphaericus* with highly toxic Binary toxins was developed as a *Culex* larvicide. However, field resistance to *L. sphaericus* by *Culex* populations has been observed in locations as diverse as France, China, and Brazil (Chevillon et al., 2001; Rao et al., 1995; Silva-Filha and Regis, 1997; Su and Mulla, 2004; Yuan

et al., 2000). Alternative Cry toxins and Bt strains with higher potency are needed to control *Culex* and other genera of mosquitoes.

The search for additional mosquitocidal Bt strains led to the discovery of *Bacillus thuringiensis* subsp. *jegathesan* (Btjeg) in Malaysia (Seleena et al., 1995). Btjeg crystals have the highly toxic Cry11Ba as a component, a toxin with the highest potency and host range of all known Cry toxins against *Aedes*, *Anopheles* and *Culex* (Delecluse et al., 1995). A combination of Cry11Ba with Bin or Cry11Aa was reported to have enhanced toxicity to susceptible mosquito larvae, and overcame *Culex* resistance. Their different mechanisms may benefit pest management by delaying the emergence of resistance and increasing toxin potency against a wide range of host specificities (Servant et al., 1999). Elucidating the Cry11Ba mode of action will contribute to the design of novel approaches for more efficient mosquito control and pest management.

1.1 Bacillus thuringiensis

Bacillus thuringiensis (Bt) is a gram-positive aerobic bacterium that produces insecticidal crystalline inclusions during sporulation of the mother cell. A member of the Bacillaceae family, Bt produces three classes of insecticidal proteins during its vegetative and sporulation phases, which are Cry (Crystal proteins) and Cyt (Cytolytic toxin) proteins during the sporulation phase, and Vip (vegetative insecticidal proteins) during the vegetative phase (Adang et al., 2014). Cry and Cyt toxins are produced as parasporal crystals (Hofte and Whiteley, 1989). But the third group, Vips, as well as two mosquitocidal toxins (MTX) proteins from *L. sphaericus*, are produced as soluble proteins and, thus, are not classified as part of the Cry family (de Maagd et al., 2003). Cry toxins were previously classified into four groups according to their target insects: CryI (Lepidoptera-specific), CryII (Lepidoptera- & Diptera-specific), CryIII (Coleoptera-

specific), and CryIV (Diptera-specific) (Hofte and Whiteley, 1989). Currently, Cry toxins are classified into 300 families in about 75 primary subgroups based on amino acid sequence identities (Adang et al., 2014). The Cyt toxins present in some Bt parasporal crystals were first discovered in mosquitocidal Bti (Goldberg, 1977). The Cyt toxins themselves have less toxicity to mosquito larvae as compared to Cry toxins (Perez et al., 2005). The mosquitocidal Cyt1A toxin is also toxic to coleopteran but not to lepidopteran species (Soberon et al., 2013).

The destruction of crops and forests by pests can have severe economic consequences, and infectious diseases can be devastating to human populations. For the past five decades, Bt has been successfully used as a biopesticide for controlling pests that are harmful to crops, forests and humans. Bt toxins were introduced in the pesticide market through insecticidal formulations and transgenic plants. The first Bt-based formulation, known as ‘Sporeine’, was developed to control caterpillars in France in 1938 (Lambert and Peferoen, 1992). The first documented commercial use of Bt was to control lepidopteran pests in the United States in 1958 (Lambert and Peferoen, 1992). The range of insect species susceptible to Bt was expanded to Diptera with the discovery of Bti that was found to have highly toxic effects on mosquito and black fly larvae (Goldberg, 1977). In 1983, *Bt* variety *tenebrionis* was reported to be toxic against beetles in the Tenebrionidae family (Krieg et al., 1983). The spectrum of Bt has broadened to Lepidoptera, Diptera, Coleoptera, Hymenoptera, and Hemiptera (de Maagd et al., 2003; Schnepf et al., 1998). Several Bt Cry toxins in the parasporin group are considered non-toxic to insects but have activity against cancer cells (Ohba et al., 2009). Since the successful cloning of the first Bt *cry* gene (Schnepf and Whiteley, 1981), recombinant Bt genes have been modified for increased efficacy (Deist et al., 2014) and engineered to construct transgenic plants for agricultural pest control. To date, more than 175 million hectares of transgenic plants have

been grown in 30 countries, yielding an extensive increase in crop products compared to 1.7 million hectares in 1996 (<http://www.isaaa.org>). The main commercialized Bt transgenic plants on the market include corn, cotton and the recently introduced rice and soybean (Romeis et al., 2006).

1.2 Importance of mosquito control and mosquitocidal bacteria

Mosquitoes transmit deadly diseases that threaten human health at individual and societal levels. Approximately 30 of the 400 known mosquito species are capable of transmitting pathogens to humans. The three most important groups of mosquito vectors are *Anopheles* species (spp.) from the Anophelinae subfamily and *Culex* spp. and *Aedes* spp. from the Culicinae subfamily. Diseases vectored by these mosquitoes include malaria, yellow fever, dengue fever, Chikungunya, and West Nile Virus. By far malaria presents the highest health risk to humans. As previously mentioned, 207 million people worldwide were infected with malaria in 2012. Of that number, 627,000 died (<http://www.who.int/malaria>). From 1999-2013, the West Nile Virus was reported to infect 39,557 humans in the U.S with 1,668 fatalities reported and many others becoming severely ill [Centers for Disease Control and Prevention (CDC; 2014)].

With the health care risks that mosquitoes pose for humans, various approaches to controlling mosquito populations have been implemented. One of these approaches uses Bti and *L. sphaericus* as active ingredients in larvicidal products, which are available from suppliers in many countries world-wide. Bti is more effective against *Aedes* and *Anopheles* larvae (Poncet et al., 1995), while *L. sphaericus* is considered more effective against *Culex* larvae (Singer, 1990). With homology to toxins produced by *Bacillus*, toxins in strains of *Clostridium bifermentans* (de Barjac et al., 1990), *Bacillus circulans* (Darriet and Hougard, 2002), and *Brevibacillus laterosporus* (Favret and Yousten, 1985) were each shown to have equal to or less toxicity than

that of Bti against dipterans. Another strain that shows high toxicity is *Bacillus morrisoni*, which is as toxic as Bti against mosquito larval species (Padua et al., 1980). *Bt* subsp. *jegathesan* (Btjeg), which contains the most toxic Cry11Ba crystal protein, has shown a high potency and broad spectrum against different mosquito species (Delecluse et al., 1995).

1.2.1 Cry proteins of Bti

The use of bacteria as a tool for mosquito control has been implemented increasingly since the discovery of Bti (Goldberg, 1977). The crystal inclusions produced by Bti, including Cry (4Aa, 4Ba, 11Aa, 10Aa) and Cyt (1Aa, 2Ba), are encoded by a 128 kb plasmid known as pBtoxis (Ben-Dov, 2014; Porter et al., 1993). Bti proteins differ from each other, resulting in a range of toxicity among different mosquito genera. For example, Cry4Aa is toxic to *Aedes* and *Culex* larvae but not *Anopheles*, while Cry4Ba, which shares 35% sequence identity to Cry4Aa, is highly toxic to *Aedes* and *Anopheles* larvae but not *Culex* (Poncet et al., 1995). Cry11Aa is highly toxic to mosquito species belonging to the *Culex*, *Aedes* and *Anopheles* genera (Delecluse et al., 1995). A Cyt toxin has a single α - β domain in its structure and is interpreted as being distantly related to members of the Cry family (Butko, 2003; Manasherob et al., 2006). Cyt toxin has been reported to have cytolytic toxicity to mammalian and insect cell lines, and fatal effects on mice via injection (de Maagd et al., 2003; Thomas and Ellar, 1983). In addition, synergism of Cyt toxin to Cry toxin toxicity has been observed against dipteran insects (Chilcott and Ellar, 1988; Perez et al., 2005; Sayyed et al., 2001; Thiery and Hamon, 1998).

1.2.2 Cry proteins of Btjeg

Found after Bti, Btjeg is another Bt strain that is highly toxic against mosquito larvae and has parasporal crystal components that are distinct from those of Bti. Parasporal crystals produced by Btjeg contain eight protoxins that include Cry11Ba, Cry19Aa, Cry24Aa, Cry25Aa, Cyt2Bb, as well as the three newly described Cry30Ca, Cry60Aa, and Cry60Ba protoxins (Sun et al., 2013). The 81 kDa Cry11Ba of Btjeg has the highest toxicity of known Cry proteins to *Culex*, *Anopheles*, and *Aedes* (Delecluse et al., 1995). It is 58% identical to Cry11Aa, but exhibits 7-37 times greater toxicity against different mosquito species (Delecluse et al., 1995). Structurally, the principle proteinase-digestive sites on Cry11Aa were found to be different from those in Cry11Ba, which may contribute to their different toxicities (Delecluse et al., 1995). A stretch of five cysteine residues at the carboxyl terminal of Cry11Ba was also considered crucial for crystal formation, which might be involved in the higher toxicity of Cry11Ba compared to Cry11Aa (Delecluse et al., 1995). Mutants of loops α 8, 1 and 3 in domain II of Cry11Ba showed different levels of reduced toxicity against *Ae. aegypti*, *An. stephensi*, and *Cu. quinquefasciatus* mosquito larvae, and these mutants were considered as important residues for Cry11Ba intoxication (Likitvivanavong et al., 2009). The combination of Cry11Ba with a Bti or Bin toxin increases toxin potency up to 10-fold against susceptible *Ae. aegypti* and *Cu. pipiens* larvae (Servant et al., 1999). In addition, Cry11Ba toxin combined with *L. sphaericus* Binary toxin can partially overcome *Culex* resistance to Bin toxin (Servant et al., 1999).

1.3 Cry toxin structure and function

Cry toxins possess a similar tertiary structure (shown in Figure 1.1), but distinctive toxin specificities against a variety of insect groups. To date, the structures of Cry toxins have been

elucidated using X-ray crystallographic methods (Figure 1), including Cry1Aa (Grochulski et al., 1995), Cry1Ac (Derbyshire et al., 2001), Cry2Aa (Morse et al., 2001), Cry3Aa (Li et al., 1991), Cry3Bb (Galitsky et al., 2001), Cry4Aa (Boonserm et al., 2006), Cry4Ba (Boonserm et al., 2005), Cry8Ea (Guo et al., 2009) and Cry5B (Hui et al., 2012). The common 3-D topology suggests a similar mode of action among Cry toxins. Specifically, the N terminal domain I consists of seven α -helices in a bundle, notably with helix 5 encircled in the center (Li et al., 1991). Domain II consists of antiparallel β -sheets, forming a ‘Greek key’ topology with a short alpha-helix that connects to domain I. Loops in domain II are exposed to the apex, acting as links between each β -sheet. The structure of domain II is the most variable of the three domains, which enables it to work as a determinant for toxin specificity (Li et al., 1991). The C terminal domain III consists of a lectin-like sandwich with two antiparallel β -sheets forming a ‘jelly roll’ topology (Li et al., 1991). Five strands of the outer sheet are exposed to the solvent, and five strands of the inner sheet face domains I and II (Grochulski et al., 1995).

Toxin domain I confers its intramolecular activity by inserting itself into a membrane via a hydrophobic helical hairpin. Domain I amphipathic helices attach on the surface of the membrane utilizing hydrophobic α 4 and α 5 helices to insert into the phospholipid bilayer (Li et al., 1991). Site-directed mutagenesis on the pore-forming region α 4 and α 5 helices has altered Cry1Ac toxicity to lepidopteran insects (Kumar and Aronson, 1999; Masson et al., 1999; Wu and Aronson, 1992). Cry4Ba toxicity against *Ae. aegypti* larvae was greatly reduced after a glutamine was substituted in the middle of helix α 4 (Uawithya et al., 1998). Evidence also supported the role of helix α 3, which is adjacent to helix α 4, as a principle region on the toxin molecule involved in pore formation (Vachon et al., 2002).

The representative domain II has a hyper-variable region with loops exposed at the apex. Domain II is involved in receptor binding, particularly with exposed loops acting as receptor binding sites (Lu et al., 1994; Rajamohan et al., 1996c). Toxin binding to receptors takes at least two steps. The first step is the initial reversible binding that reflects recognition of receptors like cadherin, APN and ALP. In the second step, the irreversible binding is associated with toxin insertion into the membrane of susceptible insects (Liang et al., 1995). Truncated domain II and III of Cry1Ab without domain I retains the ability to bind to receptors on the microvilli of epithelial cells in a manner similar as wild type toxin (Flores et al., 1997). Site-directed mutagenesis studies on domain II of Cry1A were employed by exchanging residues on loops formed by the β -sheet, demonstrating the critical role of loops for receptor binding and insect toxicity (Gomez et al., 2006; Lee et al., 1996; Lu et al., 1994; Rajamohan et al., 1996a; Rajamohan et al., 1996b; Rajamohan et al., 1996c). As for mosquitocidal toxins, the loop $\alpha 8$ mutant of Cry11Aa attenuated its toxicity against *Ae. aegypti* and affected the toxin-receptor interactions. Synthetic loop $\alpha 8$ competed with wild-type Cry11Aa binding to membrane vesicles, suggesting that loop $\alpha 8$ is the binding site of Cry11Aa to membrane proteins (Fernandez et al., 2005). Non-conservative residue substitutions on loops 1 and 2 of domain II abolished Cry4Ba toxicity against *Ae. aegypti* and *An. quadrimaculatus*, whereas loop 3 mutants enhanced Cry4Ba toxicity against *Culex* species (Abdullah et al., 2003). Substitutions on Cry1C domain loops caused a distinctive reduction of Cry1C toxicity against *Spodoptera littoralis* and *Ae. aegypti* larvae (Abdul-Rauf and Ellar, 1999). Loops $\alpha 8$, 1 and 3 were shown to be involved in Cry11Ba toxicity against *Anopheles*, *Culex* and *Aedes* larvae (Likitvivatanavong et al., 2009). Collectively, all of these reports corroborated the critical role of epitopes on loop regions of domain II for receptor binding and toxin specificity.

Domain III consists of two antiparallel β -sheets with less variability in structure than domain II. There are two long loops that extend between β -sheets, and which differ in sequence and orientation. Domain III has been shown to be essential for toxin stability, of activated toxin and its loops are involved in receptor binding in a reversible manner (Gomez et al., 2006; Rajamohan et al., 1995). Domain III of Cry1Ac with a lectin-like structure was bound specifically to N-acetylgalactosamine (GalNAc) on the receptor of aminopeptidase N (de Maagd et al., 1999; Derbyshire et al., 2001). A mutant in domain III of Cry1Ac was shown to be involved in the disruption of the sugar-binding pocket, and can cause loss of toxin binding to the N-acetylgalactosamine moiety on the 120-kDa aminopeptidase N in *Maduca sexta* (Burton et al., 1999; Jenkins et al., 1999).

1.4 Cry toxin mode of action

Although widely investigated, the detailed mechanism of Cry toxin action in insect larvae midgut is still controversial (Adang et al., 2014; Vachon et al., 2012). It is generally accepted that Cry toxin recognizes proteins on brush border membranes in a multiple-step binding process to exert toxicity. The crystalline inclusions are cleaved at inter-chain disulfide bonds to yield Cry protoxins in suitable gut physicochemical conditions (Du et al., 1994). The soluble protoxin is then proteolytically cleaved by intestinal proteases into an activated form (Choma et al., 1990; Terra and Ferreira, 1994). These activated toxins traverse through the peritrophic matrix (Hayakawa et al., 2004; Rees et al., 2009) and then bind to specific proteins localized on apical brush border membrane microvilli of the insect midgut (Bravo et al., 2011; Pigott and Ellar, 2007). The ‘sequential binding’ model describes the binding of Cry toxin to receptors such as cadherin and glycosylphosphatidylinositol (GPI) anchored proteins in a step-by-step fashion (Pardo-Lopez et al., 2013). Moreover, toxin binding to the extracellular cadherin domain induces

further proteolytic cleavage of helix $\alpha 1$ from domain I, resulting in the formation of a pre-pore oligomer (Bravo et al., 2007; Gomez et al., 2002). Toxin oligomer formation promotes a high affinity binding to GPI-anchored aminopeptidase N and alkaline phosphatase (Pigott and Ellar, 2007). In addition to these proteins, intracellular molecules such as actin, flotillin, prohibitin and V-ATPase are recognized as Cry-binding molecules (Bayyareddy et al., 2009; McNall and Adang, 2003). Toxin insertion into the cell membrane forms ion permeable channels, which allows small molecules like inorganic ions and amino acids to pass through the cell membrane; this eventually results in colloid-osmotic lysis of epithelial cells, followed by septicemia and death of the insect (Adang et al., 2014; Soberon et al., 2009).

Two alternative models, known as the ‘penknife’ and ‘umbrella’ models, propose that the helical $\alpha 5$ and $\alpha 6$ structures in domain I govern the process of membrane insertion (Knowles, 1994). Both of these models are derived from colicin, a well-characterized bacterial toxin, with a structure that possesses pore-forming properties similar to Cry toxins (Lakey et al., 1992). The ‘penknife’ model postulates that only helices $\alpha 5$ and $\alpha 6$ in domain I are inserted into the plasma membrane through a targeting helical ‘hairpin’, segregating the remaining helices lying on the surface of the membrane (Hodgman and Ellar, 1990). In contrast, the widely accepted ‘umbrella’ model proposes that helices $\alpha 4$ and $\alpha 5$ are like the shaft of an ‘umbrella’ with the central helices insert into the phospholipid bilayer with the remaining helices splatted across the membrane surface like ribs of an umbrella (Li et al., 1991). Domain I is considered to be the major determinant for pore formation, allowing the central helices $\alpha 4$ and $\alpha 5$ penetrate into the membrane in an antiparallel manner (Gazit et al., 1998). In support of this model, helix $\alpha 4$ -loop- $\alpha 5$ was synthesized and shown to be active by itself in inserting and forming ion channels into the cell membrane (Gerber and Shai, 2000). Furthermore, helix $\alpha 5$ of Cry3A and Cry1Ac were

also suggested to be important for toxin assembly and membrane permeation (Gazit and Shai, 1993). A significant loss of toxicity was observed from mutagenesis of residues on helix α 4 of Cry1Ac, which correlates with its role in formation of an ion channel (Kumar and Aronson, 1999). A mutant of serine 176 at the C-terminal end of α - helix 5 of Cry1Ab was identified as playing a crucial role for ion transport action through the brush border membrane (Alzate et al., 2009). In addition to the ‘penknife’ and ‘umbrella’ models, a relatively recent ‘buried dragon’ model portrays nearly the whole activated toxin as penetrating into the membrane (Tomimoto et al., 2006). According to this model, α -helix 1 is cleaved prior to membrane insertion, and is the only region of toxin that does not insert into the epithelial membrane (Nair and Dean, 2008).

In contrast to the pore-formation model, the cell signaling transduction model (Zhang et al., 2005; Zhang et al., 2006) postulates that Cry toxin action in Lepidoptera causes cell death by inducing a cell death pathway. Data supporting the cell signaling model comes from studies of Cry toxin action in cultured cells. The model describes an interaction between toxin monomer and cadherin, which induces activation of the G protein ($G\alpha$) coupled receptor and adenylyl cyclase, which triggers a magnesium-dependent intracellular signaling pathway (Zhang et al., 2006). The increased cAMP activates the protein kinase A, which triggers a cascade of cell physiologies and causes the programmed death of the cell by lysis (Zhang et al., 2006).

In addition to the proposed models described above, another mechanistic view claimed that Bt toxin exerts its lethal effect upon collaboration with insect gut bacteria (Broderick et al., 2006; Broderick et al., 2009). Their studies reported that the toxicity of Bt toxin was attenuated when larvae were pretreated with a mixture of four dietary antibiotics to eliminate gut bacteria in *Ly. dispar* larvae (Broderick et al., 2006) and later again, across a range of Lepidopteran larvae (Broderick et al., 2009). In these studies, Bt toxicity could be restored when larvae was orally

administered with *Enterobacter* bacterium isolated from susceptible *Ly. dispar*, demonstrating that gut microbiota play a role in supporting Bt toxin for its pathogenic effects (Broderick et al., 2006; Broderick et al., 2009).

This hypothesis challenges the widely accepted receptor-based Bt toxin action as described above. Recently, Crickmore et al., (2010) debated their study in considering the effects that prior-exposed antibiotics pose for Bt toxin component and the consistency of cooperation between gut bacteria and Bt toxin in Lepidopteran hosts. Much of the claim proposed by Broderick et al., (2006) was initiated based on the observation from a previous report that Bt toxicity could be synergized by a gut microbial zwittermycin A (Broderick et al., 2000), however, the consistency of gut bacteria in assisting Bt virulence needs to interpret with attention. Previously, gut bacterium was reported to produce antibiotic-like substances to suppress the activity of Bt toxin (Jarosz et al., 1979), or to suppress the growth of Bt in *Ho. magnanima* larvae (Takatsuka et al., 2000). Additionally, the prior treatment of antibiotics was proved to play a role on the antibiotic-sensitive Bt toxin to suppress its toxicity against *Ma. sexta* larvae (Johnston et al., 2009) and *Pl. xylostella* larvae (Raymond et al., 2009), evident that the reduced Bt toxicity may not correlate with the elimination of gut microbiota.

1.5 Cry toxin binding receptors in midgut of mosquito larvae

Receptor binding is the major determinant for Cry toxin action and host specificity. The mechanism of mosquitocidal Cry toxin was recently reported to occur according to a multi-step binding process (Likitvivatanavong et al., 2011). A number of proteins on the brush border membrane of mosquito larvae have been identified as putative binding receptors of mosquitocidal Cry toxins. Two cadherins were postulated to work as receptors of Cry4Ba and Cry11Ba respectively in *An. gambiae* larvae (Hua et al., 2013; Hua et al., 2008). Binding

between Cry11Aa with *Ae. aegypti* BBMV was inhibited by anti-cadherin antibody, and the cadherin binding site to Cry11Aa could be competed by Cry11Ba and Cry4Aa, suggesting shared binding sites of toxins on an *Ae. aegypti* cadherin (Chen et al., 2009a). A number of GPI-anchored proteins were identified as potential receptors of Cry toxins in mosquito larvae, including aminopeptidase N (Abdullah et al., 2006; Chen et al., 2009b; Zhang et al., 2008), alkaline phosphatase (Fernandez et al., 2006; Hua et al., 2009), α -amylase (Fernandez-Luna et al., 2010; Zhang et al., 2013) and α -glucosidase (Zhang et al., 2013). Identified binding proteins considered putative receptors for mosquitocidal Cry toxins are listed in Table 1.1.

1.5.1. Cadherin

Cadherin, which is a member of a glycoprotein superfamily, functions as a Ca^{+} -dependent transmembrane protein to maintain cell-cell homophilic adhesion and intercellular connections (Gumbiner, 1996; Takeichi, 1991). The prototypical cadherin consists of a cytoplasmic domain (CYTO), transmembrane domain (TM), membrane proximal extracellular domain (MPED), cadherin repeats (CR), and signal leading peptide (SP). A 210-kDa cadherin protein known as Bt-R1 in *Ma. sexta* midgut was first identified as a binding receptor of Cry1Ab (Vadlamudi et al., 1995). Cry1Ab toxin was shown to bind Bt-R1 with high affinity, and the binding epitopes on Bt-R1 were narrowed down to a series of amino acids (Gomez et al., 2003; Hua et al., 2004a). Expression of a form of cadherin with a CR12 deletion in cultured insect cells led to the loss of Cry1Ab-mediated toxin cytotoxicity, demonstrating the importance of CR12 in cadherin for Cry1Ab toxicity (Hua et al., 2004b). Functional evidence focusing on cytolytic analyses of Cry toxins effect on cadherin-expressing cells further supports the role of cadherin as a Cry1A receptor in different insect species (Flannagan et al., 2005; Jurat-Fuentes and Adang, 2006; Nagamatsu et al., 1999). Sequence analyses of cadherins in Cry1A-resistant insect species

established a correlation between mutated cadherins and larval resistance to Cry1A toxin in *Helicoverpa virescens* (Gahan et al., 2001), *Pe. gossypiella* (Morin et al., 2003), and *He. armigera* (Xie et al., 2005). Mechanistically, cadherin was found to play a principle role in Cry1Ac oligomerization, in which toxin binding to cadherin facilitated proteolytic digestion and removal of helix $\alpha 1$, inducing formation of a 250-kDa toxin oligomer (Gomez et al., 2002; Rausell et al., 2004). An association between cadherin and Cry1Ac toxin oligomer formation was also reported to occur in *He. armigera* (Peng et al., 2010). In Coleoptera, cadherin TmCad1 bound to Cry3Aa with high affinity and their interaction triggered Cry3Aa toxin oligomerization (Fabrick et al., 2009). As for mosquitoes, the *Ae. aegypti* cadherin fragment CR8-11 binds to Cry11Aa on loop $\alpha 8$ and loop 2 in domain II with high affinity, and Cry11Ba competed for a binding site, but Cry4Ba did not compete with Cry11Aa (Chen et al., 2009a). Two *Anopheles* cadherins, AgCad1 and AgCad2 were identified to be working as putative binding receptors for Cry4Ba and Cry11Ba, respectively, in *An. gambiae* (Hua et al., 2013; Hua et al., 2008).

Cadherin synergism of Cry toxicity was first discovered when a peptide containing a last cadherin repeat, CR12 of Bt-R1, enhanced Cry1Ac toxicity to *Ma. sexta* (Chen et al., 2007). It was later found that the longer CR10-12 region of Bt-R1 was even more effective than CR12 as a synergist of Cry1A and Cry1C toxicity against lepidopteran insects (Abdullah et al., 2009). In Coleoptera, the Cry-binding CR repeat of corn rootworm cadherin is capable of synergizing Cry3Aa and Cry3Bb toxicity against several coleopteran species (Park et al., 2009a). A peptide containing CR9 and MPED of lesser mealworm, *Alphitobius diaperinus*, cadherin bound Cry3Bb toxin with high affinity and synergized Cry3Bb toxicity significantly against *Al. diaperinus* larvae (Hua et al., 2014). Synergism conferred by cadherin repeats to Cry4Ba toxicity was also reported

in *An. gambiae* and *Ae. aegypti* larvae (Hua et al., 2013; Hua et al., 2008; Park et al., 2009b). The crucial role of *Ae. aegypti* cadherin for Cry11Aa toxicity was further demonstrated when cadherin-silenced larvae became resistant to Cry11Aa toxin and cadherin expressed insect cells showed sensitivity to Cry11Aa and other toxins (Lee et al., 2014).

1.5.2 GPI-anchored proteins

The α -amylase and α -glucosidase proteins belong to the GH13 family of glycoside hydrolases (GH), known as the α -amylase family (MacGregor et al., 2001). According to the Carbohydrate-Active Enzymes (CAZy) classification system, family GH13 is classified into 37 subfamilies, including α -amylase and α -glucosidase subfamilies (Cantarel et al., 2009; MacGregor et al., 2001; Stam et al., 2006). Proteins in the α -amylase family are able to enzymatically cleave α -1, 4-glycosidic bonds, allowing α -amylase-type proteins to cleave glycosidic linkages in starch, glycogen and related polysaccharides into glucose. The α -amylase proteins have three structural domains designated as A, B, and C (Brzozowski and Davies, 1997). The catalytic domain is arranged in the form of a $(\beta/\alpha)_8$ barrel fold in domain A, while domain B is a small region which protrudes between strand β_3 and helix α_3 (Janecek et al., 1997; Rodenburg et al., 1994). Members of the α -amylase family share seven conserved sequence regions that work as the active sites for starch and oligosaccharide degradation (Janecek, 1997). In *An. albimanus*, a 70 kDa GPI-anchored protein in BBMV was identified as an α -amylase, and ligand blot analysis showed the ability of *E. coli* expressed α -amylase to bind to Cry4Ba and Cry11Aa, but not Cry3Aa, which is not toxic to *An. albimanus* larvae (Fernandez-Luna et al., 2010). Homologous competition binding assays revealed the role of α -amylase as a putative binding receptor of both Cry4Ba and Cry11Aa in *An. albimanus* (Fernandez-Luna et al., 2010).

α -glucosidase cleaves individual glucosyl residues from various glycoconjugates, including α - or β -linked polymers of glucose (Zhang et al., 2012). α -glucosidases are very similar to maltase and α -amylase proteins with about 39-43% amino acid identities (Darboux et al., 2001). In *Cu. pipiens*, α -glucosidase has a predicted signal leading peptide at the N-terminus and GPI-anchorage at the C-terminus, which tethers the protein to the brush border membrane in the midgut (Darboux et al., 2001). α -glucosidase is the specific binding receptor of Bin toxin from *L. sphaericus* in *Culex* larvae (Darboux et al., 2001; Darboux et al., 2002). Bin toxin contains two subunits called BinA and BinB (Arapinis et al., 1988). The toxicity of Bin toxin depends upon interaction between the BinB subunit and α -glucosidase, followed by cytopathological events conferred by the BinA subunit (Darboux et al., 2001). Bin toxin is highly toxic to susceptible *Culex* larvae, less toxic to *Anopheles* larvae and has little activity against *Aedes* species (Darboux et al., 2001; Opota et al., 2008). Recently, fourteen α -glucosidases from various mosquito species were identified through a bioinformatics approach (Gabrisko, 2013), including the binding receptors of Bin toxin in mosquito larvae: Agm3 (Opota et al., 2008), Cpm1 (Darboux et al., 2001), and Cqm1 (Romao et al., 2006).

Aminopeptidase N (APN) is a zinc-binding metalloprotease and was identified as a GPI-anchored protein in the cell membrane of the mosquito midgut. APN is a digestive glycoprotein that cleaves N-terminal amino acids of polypeptides. APNs have been identified as binding receptors of Cry1A toxins in *Ma. sexta* (Denolf et al., 1997; Knight et al., 1994; Sangadala et al., 1994), in *He. virescens* (Banks et al., 2001; Gill et al., 1995; Luo et al., 1997), in *Ly. dispar* (Valaitis et al., 1995), and in *Bo. mori* (Yaoi et al., 1997). The role of APN in Cry toxin action was further demonstrated in different mosquito species including *Ae. aegypti* (Chen et al., 2013; Likitvivatanavong et al., 2011), *An. gambiae* (Zhang et al., 2008) and *An. quadrimaculatus*

(Abdullah et al., 2006). Investigation of regions of APN binding to Cry11Ba revealed a synergistic effect of specific truncated APN fragments for Cry11Ba toxicity to *An. gambiae* larvae (Zhang et al., 2010), and the same characteristic was also observed for *Aedes* APN fragments which enhanced Cry11Aa toxicity against *Ae. aegypti* larvae (Chen et al., 2013).

Alkaline phosphatase is a membrane-bound protein belonging to the metalloenzyme family (Cabrero et al., 2004). Brush border ALP was considered a possible receptor of Cry1Ac in *Ma. sexta* based on enzyme inhibition data and on the ability of an ALP and APN mixture to catalyze Cry1Ac-induced pore formation in liposomes (Sangadala et al., 1994). Subsequently Cry1Ac was reported to bind specific forms of *Ma. sexta* ALP on blots (McNall and Adang, 2003). ALP was first identified as a receptor of a Cry toxin in *He. virescens* (Jurat-Fuentes and Adang, 2004). The involvement of ALP in Cry toxicity to susceptible insects is now reported for other lepidopteran, dipteran (Fernandez et al., 2006; Hua et al., 2009), and coleopteran larvae (Martins et al., 2010; Zuniga-Navarrete et al., 2013). Reduced levels of ALP transcripts and ALP protein are common in Cry-resistant strains of lepidopteran larvae, demonstrating an important role for ALP in Cry toxin action and resistance (Jurat-Fuentes et al., 2011). Additionally, a low transcript level of ALP was found in an *Ae. aegypti* Bti-resistant strain, which revealed the potential role of ALP in directing Cry toxin resistance in mosquito larvae (Tetreau et al., 2012). Mutagenesis studies on *Aedes* ALP mapped two regions, R59-G102 and N257-I296, as containing essential binding sites of Cry11Aa on domain II and III respectively (Fernandez et al., 2009). Insect cells expressing *Aedes* ALP were rendered susceptible to Cry4Ba toxicity (Dechklar et al., 2011). The *in vivo* functional role of ALP associated with Cry toxin toxicity was confirmed when ALP-silenced *Ae. aegypti* larvae showed a tolerant response against Cry11Aa and Cry4Ba (Jimenez et al., 2012).

1.5.3 ATP binding cassette transporter (ABC transporters)

ABC transporters are trans-membrane proteins responsible for transporting toxic molecules out of the cell and are major factors in multidrug resistance (Heckel, 2012). ABC transporter was thought to be important for toxin action by facilitating toxin binding and toxin insertion in pore formation (Gahan et al., 2010). An inactivating mutation in ABCC2 gene was reported to be genetically linked with *He. virescens* resistance to Cry1A toxin and was involved in the loss of Cry1A binding to membrane vesicles (Gahan et al., 2010). The principal role of ABC transporter as a mechanism of Cry toxin resistance was further verified when a single tyrosine insertion in an outer loop between two trans-membrane regions conferred *Bo. mori* resistance to Cry1Ab (Atsumi et al., 2012; Tanaka et al., 2013). Although evidence has established the critical role of ABC proteins for the Cry toxin mechanism, how the interaction between ABCC2 and Cry toxin results in mortality of insect larvae has not been elucidated.

1.6 Mosquito larval alimentary tract

The biology of the alimentary tract of mosquito larvae differs from adults in structure and function due to their different feeding habits. Once adults have emerged, females are highly mobile in order to seek blood meals for reproduction. When pathogen-infected females take blood meals from hosts like humans or livestock, infectious diseases can be transmitted to the hosts through saliva secreted by female adults. Mosquito larvae, however, feed on plants and algae in water and thus live as non-vectors. The alimentary tract of mosquito larvae, particularly the midgut, is the target region where gut-active Bt Cry proteins exert toxicity. An understanding of larval gut morphology may provide a basis of knowledge for developing effective vector-control strategies for pest management.

The alimentary tract of mosquito larvae is a long tubular structure that takes up the major part of the larval body. It is divided into the foregut, midgut, and hindgut. The foregut includes the esophagus, where a food bolus is passed towards the terminus of the foregut by muscular activity. The peritrophic matrix is a tube-like structure consisting of chitins and proteoglycans, which separates the food bolus from midgut cells (Terra, 2001). The food materials must be small enough to penetrate the peritrophic matrix and enter the ectoperitrophic space for further digestion and absorption (Terra, 1996). Mosquito adults have a type I peritrophic matrix, a bag-like structure encasing the blood meals, whereas mosquito larvae have a type II peritrophic matrix with an open tube-like structure extending from midgut to hindgut (Shao et al., 2001). In mosquito larvae, the midgut is divided into four parts: caecum, gastric caecum, anterior and posterior midgut (Zhuang et al., 1999). At least four types of midgut epithelial cells are present in the epithelial layer, including columnar cells, goblet cells, endocrine cells and regenerative cells (Hakim et al., 2010; Zhuang et al., 1999). The most numerous columnar cells are elongated with microvilli facing the gut lumen. Microvilli allow a broad surface of digestive enzymes to be exposed and accessible for food digestion in the gut lumen (Zieler and Dvorak, 2000). The posterior midgut is involved in the absorption of amino acids and lipids. Cry toxin receptors are localized on the apical side of epithelial cells in posterior midgut, the region where Cry toxins exert their toxicity (Zhang et al., 2013; Hua et al., 2013; Hua et al., 2009; Zhang et al., 2008). The Malpighian tubules are localized at the anterior region of the hindgut. The hindgut begins at the proventriculus, followed by the ileum and rectum and is responsible for the reabsorption of ions and water before food waste passes out of the body.

1.7 RNA interference

RNA interference was first discovered as double-stranded RNA (dsRNA) induces an effective suppression of the complementary mRNA in a target-specific manner in the nematode *Caenorhabditis elegans* (Fire et al., 1998). A long dsRNA is cleaved by an RNase III enzyme, Dicer, into a double-stranded small interfering RNAs (siRNA) about 21-23 nucleotides in length (Meister et al., 2004). The siRNA duplex incorporates onto a protein complex known as RNA-induced silencing complex (RISC) and then unwound to expose the antisense guide strand. The guide strand directs the RISC complex to bind to the target mRNA via complementary base pairing. This leads to an endonuclease cleavage processed by Argonaute, known as 'slicer', to degrade the mRNA and inhibit protein translation (Schwarz et al., 2002).

RNAi is a potent intracellular response and provides an ideal platform for the study of gene function *in vivo* via reverse genetics. RNAi can be initiated by either exogenous dsRNA or endogenous stem-loop microRNAs. dsRNA is widely used as a genetic tool due to the involvement of RNAi in the natural antiviral response to degrade the transposable element of dsRNA viruses (Keene et al., 2004; Zamboni et al., 2006; Anderson et al., 2005). Alternatively, microRNA was found to act as a naturally occurring short RNA, which regulates gene expression to mediate host biological development (Lee et al., 1993). Mimicking the natural microRNAs, short hairpin RNA (shRNA) has been employed to suppress gene expression through RNAi in mammalian cells lines (Brummelkamp et al., 2002).

Since the discovery by Caplen et al., (2001) that chemically synthesized siRNA can efficiently trigger RNAi in *C. elegans* and cell lines from human and mice, siRNA has been utilized as a functional genetic tool for RNAi-based gene silencing. In comparison to the dsRNAs approach, small DsiRNAs of less than 30 bp were demonstrated to be able to bypass the

interferon (IFN) response generated by the host immune system (Pardo-López et al., 2013). In addition, dsRNA uptake requires the involvement of a RNA channel transporter, SID protein, to initiate RNAi processing (Feinberg et al., 2003). As the SID gene is thought to be absent or weakly expressed in dipterans (Gordon and Waterhouse, 2007), siRNAs with short length have the advantage of being permeable to membrane entry. Alternative functional delivery systems like transfection reagents, liposomes, and chitosan have been used to increase the efficiency of RNAi in mosquito larvae (Cancino-Rodezno et al., 2010; Jimenez et al., 2012; Zhang et al., 2010).

Recently, Dicer substrate siRNA (DsiRNA) has been introduced into the commercial scientific market as a potent effector of RNAi. In cells, Dicer-processed siRNA has a 2-nt overhang at the 3' end, which binds to RISC for the subsequent degradation event. DsiRNA is synthesized as a 25-27 nt duplex because siRNA of 25-30 nt was shown to have 100-fold more potency for gene silencing compared to 21-nt siRNAs at the same region (Kim et al., 2005). DsiRNA was synthesized containing additional bases added to the 3' end of the sense strand and 5' end of the antisense strand for preferential binding as a dicer substrate. Maximum efficiency is obtained as a 2 base 3'-overhang on the antisense strand and 2 DNA bases on the 3' sense strand forming a blunt end (IDT; Coralville, IA, USA). This modification provides Dicer a single preferential PAZ-domain binding site to initiate the cleavage efficiently. The appropriate cleavage by Dicer on the synthetic DsiRNA is thought to link with an increased potency of siRNA incorporation onto RISC for the mRNA degradation process (Rose et al., 2005).

Mysore et al., (2013) obtained a complete absence of axon guidance gene semaphorin-1a expression in *Ae. aegypti* larvae using chitosan/DsiRNA nanoparticle delivery system. Later again, the same research group reported 77% silencing of the gene encoding “single-minded” in

the brain, which led to a reduced expression of odorant receptor in the olfactory system (Mysore et al., 2014). The chemically synthesized 27 nt duplex RNAs with dicer substrate described in Chapter 3 were designed to increase the potency and efficiency of the gene silencing pathway, with the expectation that the ‘Dicer’ substrate would be interpreted by *An. gambiae* larvae as being involved in the transfer of siRNA to incorporate with RISC.

1.7.1 RNAi research for the study of Bt toxin mode of action

RNAi has been used as an effective tool to investigate the roles of receptors for Bt toxin activity *in vivo*. In Lepidoptera, silencing cadherin expression through injection and feeding dsRNAs suppressed Cry1Ca toxicity against *Sa. exigua*, evidence of its role as a functional receptor of Cry1Ca toxin (Park and Kim, 2013; Ren et al., 2013). Silencing of APN through dsRNA injection in fifth-instar *S. litura* resulted in a resistant response to Cry1C toxin (Rajagopal et al., 2002). Furthermore, dsRNA-silenced APN in *He. armigera* caused decreased larval susceptibility to Cry1Ac, evidence for an *in vivo* role for APN in Cry1Ac toxin (Sivakumar et al., 2007). Moreover, knockdown of an antimicrobial peptide, gloverin through injection of dsRNA in *Sa. exigua* resulted in increased toxicity of a Cry toxin, which retarded the process of larvae molting to pupae (Hwang and Kim, 2011). In Coleoptera, gene-silencing of cadherin through injection of dsRNA led to a dramatic tolerance in *An. diaperinus* (lesser mealworm) and *Tenebrio molitor* (yellow mealworm) larvae to Cry3Bb and Cry3Aa toxins, respectively (Fabrick et al., 2009; Hua et al., 2014). As for dipterans, three APN isoforms (APN2783, APN5808, and APN2778) were silenced through dsRNA soaking in *Ae. aegypti* larvae, with one isoform, APN2783, identified as a predominant APN for Cry4Ba toxicity (Saengwiman et al., 2011). The knockdown of GPI-anchored ALP through dsRNA in liposomes conferred *Ae. aegypti* larvae tolerance to Cry11Aa, but not Cry4Ba toxin (Jimenez et al., 2012).

Silencing of cadherin in transgenic *Ae. aegypti* larvae led to a decreased larval susceptibility to Cry11Aa but not to other toxins, indicating a principle role of this cadherin in Cry11Aa toxicity (Lee et al., 2014). Moreover, gene silencing of the heat shock protein through dsRNA in liposomes resulted in a four-fold increased tolerance of *Ae. aegypti* larvae to Cry11Aa (Cancino-Rodezno et al., 2012).

1.8 Rationale and goals of the research

The overall goal of my research is to investigate the mechanism of Cry11Ba action in the midgut of *An. gambiae* larvae through *in vitro* and *in vivo* approaches. I focus on the mechanism of the high toxicity of Cry11Ba to mosquito larvae (Delecluse et al., 1995) to answer unresolved questions posed by previous investigators (Hua et al., 2013; Hua et al., 2009; Zhang et al., 2008; Zhang et al., 2010). My research will help us understand the basis for Cry11Ba's excellent potency and may suggest strategies for effectively incorporating Cry11Ba in mosquito larvicides.

Receptor binding is a crucial step for toxin activity and my research has examined the nature and function of two types of Cry11Ba receptors in *An. gambiae* larvae. Chapter 2 presents the results of my identification and characterization of an α -amylase and α -glucosidase as receptors of Cry11Ba. In a previous study, Zhang et al. (2008) extracted GPI-anchored brush border proteins that bound Cry11Ba coupled in bead complex. Those authors identified a Cry11Ba-binding APN in the mixture, but left a 70-kDa protein unidentified. This protein was subsequently identified by mass spectrometry as α -amylase and is a subject of my research. A related protein, α -glucosidase, is considered a receptor of Bin toxin in *An. gambiae* (Opota et al., 2008). My hypothesis was that α -amylase (AgAmy1) but not α -glucosidase (Agm3) works as the receptor that mediate Cry11Ba toxicity. The cloning and characterization of α -amylase and α -glucosidase are described in Chapter 2. The locations of both proteins in the larval midgut were

established. Results show that both α -amylase and α -glucosidase bind Cry11Ba and are involved in Cry11Ba action.

Cadherins AgCad1 and AgCad2 are located on the midgut brush border membrane of *An. gambiae* larvae (Hua et al., 2013; Hua et al., 2008). AgCad2 binds and is a putative receptor of Cry11Ba (Hua et al., 2013). In contrast AgCad1 binds Cry11Ba with low affinity, but peptides of AgCad1 bind and synergize Cry4Ba toxicity to *An. gambiae* larvae (Hua et al., 2008). In Chapter 3, I describe results of my research investigating the *in vivo* roles of AgCad1 and AgCad2 in Cry11Ba toxicity by RNAi in *An. gambiae* larvae. Chapter 3 reports that AgCad1 is a functional receptor that mediates Cry11Ba toxicity.

1.9 References

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Figure 1.1. The 3-D crystal structure of Cry1Aa structure modified by PyMOL Version 1.7 (<http://www.pymol.org/>) using file (id:1CIY) downloaded from the Resource for Structure Bioinformatics Protein Data Bank at (<http://www.rcsb.org.pdb>). Cry1Aa amino acid sequence was obtained from NCBI database (GenBank: AAP40639.1) and the protein structure was analyzed according to Grochulski et al., 1995. Panel A, overview of three domains of Cry1Aa, in which domain I is shown in red, domain II in yellow, and domain III in blue. Domain I with α -helices in different colors is shown in Panel B. Panel C shows domain II with named α -helices and loops. Domain III with two antiparallel β -sheets in yellow is shown in Panel D.

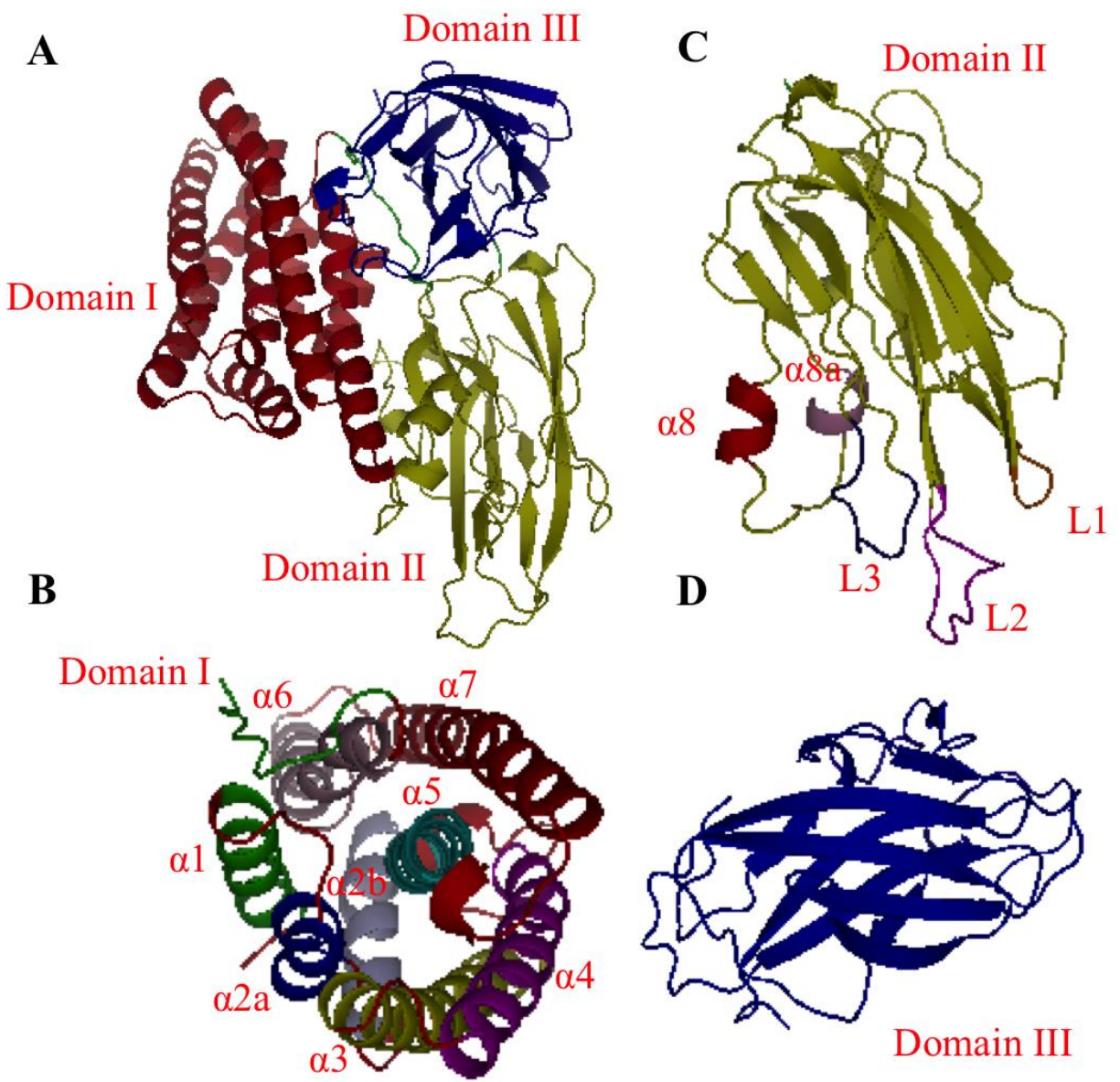


Table 1.1. Receptors of Cry toxins studied in mosquitoes

	GPI-anchored proteins			
	Cadherin	APN	ALP	Amy
<i>Ae. aegypti</i>	<p>Cry11Aa</p> <p>(Chen et al., 2009a)</p> <p>(Rodriguez-Almazan et al., 2012)</p> <p>Cry11Ba</p> <p>(Likitvitanavong et al., 2011)</p>	<p>Cry11Aa</p> <p>(Chen et al., 2009b)</p> <p>Cry11Ba</p> <p>(Likitvitanavong et al., 2011)</p> <p>Cry4Ba</p> <p>(Bayyareddy et al., 2009)</p>	<p>Cry11Aa</p> <p>(Fernandez et al., 2006)</p> <p>Cry4Ba, Cry11Aa</p> <p>(Jimenez et al., 2012)</p> <p>(Thammasittirong et al., 2011)</p> <p>(Bayyareddy et al., 2009)</p> <p>Cry11Ba</p> <p>(Likitvitanavong et al., 2011)</p>	
<i>An. gambiae</i>	<p>Cry4Ba</p> <p>(Hua et al., 2008)</p> <p>Cry11Ba</p> <p>(Hua et al., 2013)</p>	<p>Cry11Ba</p> <p>(Zhang et al., 2008)</p>	<p>Cry11Ba</p> <p>(Hua et al., 2009)</p>	<p>Cry11Ba</p> <p>(Zhang et al., 2013)</p>
<i>An. quadrimaculatus</i>		<p>Cry11Ba</p> <p>(Abdullah et al., 2006)</p>		
<i>An. albimanus</i>				<p>Cry4Ba, Cry11Aa</p> <p>(Fernandez-Luna et al., 2010)</p>

CHAPTER 2

ANALYSES OF α -AMYLASE AND α -GLUCOSIDASE IN THE MALARIA VECTOR MOSQUITO, *ANOPHELES GAMBIAE*, AS RECEPTORS OF CRY11BA TOXIN OF *BACILLUS THURINGIENSIS* SUBSP. *JEGATHESAN*

Zhang Q., Hua G., Bayyareddy K., and Adang M. J., 2013. *Insect Biochemistry and Molecular Biology*. (43) 907-915. Reprinted here with permission of the publisher.

Abstract

Bacillus thuringiensis subsp. *jegathesan* produces Cry11Ba crystal protein with high toxicity to mosquito larvae. The Cry11Ba toxicity is dependent on its receptors on mosquito larval midgut epithelial cells. Previously, a cadherin-like protein (AgCad2), aminopeptidase (AgAPN2) and alkaline phosphatase (AgALP1) were reported to be involved in regulation of Cry11Ba toxicity on *Anopheles gambiae* larvae. Here, the cDNAs encoding α -amylase (AgAmy1) and α -glucosidase (Agm3) were cloned from *An. gambiae* larva midgut. Both are glycosylphosphatidylinositol (GPI) anchored proteins on brush border membranes (BBMV). Immunohistochemistry revealed their localization on different regions of the larval midgut. AgAmy1 and Agm3 bound Cry11Ba with high affinity, 37.6 nM and 21.1 nM respectively. Cry11Ba toxicity against *An. gambiae* larvae was neutralized by both AgAmy1 and Agm3. The results provide evidence that both AgAmy1 and Agm3 function as receptors of Cry11Ba in *An. gambiae*.

Keywords: *Bacillus thuringiensis*; Bt; Cry toxin; mosquitocidal; amylase, glucosidase

Abbreviations: *Bacillus thuringiensis* (Bt); bovine serum albumin (BSA); brush border membrane vesicles (BBMV); amylase (Amy1); *Anopheles gambiae* glucosidase (Agm3); cross-reacting determinant (CRD); peptide mass fingerprinting (PMF); polymerase chain reaction (PCR)

2.1. Introduction

Species of the bacterium *Bacillus thuringiensis* (Bt) produce insecticidal proteins during vegetative and sporulation growth phases. The high potency of the various proteins has enabled the successful use of Bt proteins for pest control in agriculture, forestry and disease vector control applications. Bt subsp. *israelensis* is an important larvicide for mosquito and black fly control and Bti crystals are comprised of Cry4Aa, Cry4Ba, Cry10Aa, Cry11Aa, Cyt1Aa and Cyt2Ba toxins (Berry et al., 2002).

Limitations in the target range of Bti have encouraged the search for Bt strains with more potency and broader toxicity. The bacterium *Bacillus thuringiensis* subsp. *jegethesan* (Delecluse et al., 1995) was recognized as having high potency and a complement of Cry proteins distinct from Bti. The protein Cry11Ba, one of the seven major polypeptides produced by Bt subsp. *jegethesan* (Btjeg) (Kawalek et al., 1995; Ragni et al., 1996), is the most toxic of known Cry proteins against *Aedes*, *Anopheles* and *Culex larvae* (Breman et al., 2004). It is a crystal protein which shares 58% identity with Bt Cry11Aa toxin but exhibits 7-37 times higher powerful mosquitocidal activity than Cry11Aa (Delecluse et al., 1995). The combination of Cry11Ba with Bti or with Bin toxin could greatly increase its toxicity to mosquitoes (Servant et al., 1999).

The general concept for the mode of action in either Lepidoptera or mosquito is that Bt crystal proteins are activated by the alkaline environmental gut of the susceptible larvae. The activated toxins bind to a primary receptor on the gut brush border membrane (BBMV), known as cadherin to form oligomers. Afterwards, the toxin undergoes conformation changes to continue binding to the second receptors to subsequently insert into the epithelium cell membrane to form pores (Aronson and Shai, 2001; Bravo et al., 2004; Jurat-Fuentes and Adang, 2004). These secondary receptors are glycosylphosphatidylinositol (GPI)-anchored proteins,

such as aminopeptidases (APNs), alkaline phosphatases (ALPs) (Abdullah et al., 2006; Fernandez et al., 2006; Hua et al., 2009; Jurat-Fuentes and Adang, 2004) amylase (Fernandez-Luna et al., 2010) and glucosidase (Opota et al., 2008) on the epithelial cell membranes.

Glycoside hydrolases in family GH 13, the α -amylase family, catalyze the breakdown of starch and related polysaccharides into sugars via cleavage of α -1, 4-glycosidic bonds. The α -amylases (EC 3.2.1.1) are calcium metalloenzymes that can be classified into 37 subfamilies including α -amylase and α -glucosidase subfamilies (Stam et al., 2006). A bioinformatic approach was recently used to identify fourteen α -glucosidases in *Ae. aegypti*, *An. gambiae* and *C. quinquefasciatus* (Gabrisko, 2013). An α -amylase in *A. albimanus* has been identified as a binding receptor of Cry4Ba and Cry11Aa (Fernandez-Luna et al., 2010). The α -glucosidases (EC 3.2.1.20) cleave individual glucosyl residues from various glycoconjugates including alpha- or beta-linked polymers of glucose. In addition, α -glucosidase has been demonstrated to be the specific binding receptor of mosquitocidal Bin toxin from *L. sphaericus* (Darboux et al., 2001; Darboux et al., 2002). The action of Bin toxin, which is composed of A and B subunits, depends on the binding property of BinB subunit with a glycosylphosphatidylinositol (GPI)- α -glucosidase. Subsequent cytopathological events induced by BinA subunit leads to the final mortality (Darboux et al., 2001). To date, α -glucosidases Cpm1 and Cqm1 have been cloned and proven to be Bin toxin binding receptors in *Cu. pipiens* and *Cu. quinquefasciatus*, respectively; there is evidence based on high affinity binding that Agm3 in *An. gambiae* is also a Bin receptor (Darboux et al., 2001; Darboux et al., 2002; Fernandez-Luna et al., 2010; Opota et al., 2008; Pauchet et al., 2005; Romao et al., 2006). An orthologue of Cqm1 glucosidase, Aam1 from *Ae. aegypti*, does not bind Bin toxin, a result in agreement with the natural refractoriness of *Ae. aegypti* larvae to Bin toxin (Ferreira LM et al., 2010).

An α -amylase is involved in Cry4Ba and Cry11Aa toxicities to *A. albimanus* and, in this study, the 70-kDa GPI-anchored protein extracted by Cry11Ba affinity chromatography from *An. gambiae* BBMV (Zhang et al., 2008) is identified as α -amylase. We cloned cDNAs encoding α -amylase (AgAmy1) and the related α -glucosidase (Agm3), which is considered the Bin receptor, from midgut of *An. gambiae* larvae. While we expected AgAmy1 to bind Cry11Ba with high affinity, Agm3 also bound Cry11Ba with high affinity and both maltase-like proteins functionally interact with toxin *in vivo* as evidenced by Cry11Ba *in vivo* bioassays.

2.2. Materials and Methods

2.2.1 Mosquitoes

An. gambiae mosquitoes (CDC G3 stain) were reared at 27°C with a 16h: 8h light-dark photoperiod. Larvae (ca. 200/pan) were kept in dechlorinated water and fed with ground TetraMin daily until pupation. Adults were given *ad libitum* access to 10% sucrose water normally and fed after fertilization with restrained mice as described previously (Zhang et al., 2008). Early fourth instar larvae were collected for insecticidal bioassays, immunohistochemistry and extraction of BBMV.

2.2.2 Preparation of activated Cry11Ba toxin

The Cry11Ba-producing Bt strain 407 was grown in sporulation medium with erythromycin (20 μ g/ml) as described (Zhang et al., 2008). Spores and crystals were harvested by centrifugation, washed, and crystals purified by NaBr step gradient centrifugation according to described methods (Zhang et al., 2008). Crystals were solubilized to protoxin in 100 mM 3-(cyclohexylamino) propanesulfonic acid (CAPS), pH10.6, supplemented with 0.05% β -mercaptoethanol overnight at 5°C. Insoluble material was removed by 10,000 g centrifugation for 10 min, and the supernatant was passed through a 0.45 μ M BA85 membrane filter

(Whatman). For insect bioassays, protoxin was dialyzed overnight at 5°C against 20 mM sodium carbonate pH 9.6. Otherwise, protoxin was activated into toxin using TPCK-treated bovine pancreatic trypsin (Sigma) at a mass ratio of 1:10 (trypsin:protoxin) for 2 hr at 37°C. Toxin was purified by fast protein liquid chromatography (Akta Explorer) with a Bio-Scale mini High Q cartridge column (Bio-Rad, Richmond CA) with a linear gradient of 0-0.5M NaCl in 100mM CAPS (10.6). Toxin was dialyzed against 20 mM sodium carbonate pH 9.6 and stored in aliquots at -80°C for later use in binding assays.

2.2.3 Midgut dissection, RNA methods and cloning α -amylase and α -glucosidase cDNAs.

Midguts were dissected from fourth instar *An. gambiae* larvae as previously described (Zhang et al., 2008). Total RNA was prepared using a total RNA mini kit according to the manufacturer's instructions (Bio-Rad, Richmond CA) and cDNA was prepared from total RNA using reverse transcriptase (Gibco-BRL Superscript II) and oligo(dT)17. The cDNA encoding α -amylase and α -glucosidase were amplified from midgut cDNA as follows. For AgAmy1, the 70-kDa BBMV protein that bound Cry11Ba in (Zhang et al., 2008) was identified by peptide mass fingerprinting (PMF) (Supplementary Figure 1) as being the expression product of SNAP transcript (SNAP00000012401); primers AgAmy1-F1 and AgAmy-R1 (Table 1) were designed to amplify AgAmy1. For Agm3, primers Agm3-F1 and -R1 (Table 1) were designed based on Agm3 sequence (GenBank: EU165335.1). The PCR products were cloned into pGEM-Teasy vector (Promega). The DNA inserts were sequenced in both forward and reverse directions at the Molecular Genetics Instrumentation Facility at University of Georgia. The sequencing data indicated these two products were identical to *An. gambiae* α -amylase (SNAP00000012401) and α -glucosidase Agm3 (GenBank: EU165335.1) respectively.

2.2.4 Cloning and production of AgAmy1 and Agm3 in *E. coli*.

Plasmids were constructed for expressing AgAmy1 and Agm3 without their signal peptides in *E. coli*. The primer sets AgAmy1T-F3/AgAmy1-R1 and Agm3T-F2/Agm3R1 (Table 1) were used to amplify the respective coding regions in pGEM-AgAmy1 and pGEM-Agm3. The PCR reaction conditions were 1 U Taq polymerase (Qiagen) with 25 cycles of 94°C for 30 sec, 55°C for 30 sec and 72°C for 2 min. The PCR products were purified with a Qiaex II gel extraction kit (Qiagen), digested with *NdeI/NotI* for AgAmy1 and *NcoI/NotI* for Agm3 and cloned into protein expression vector pET-30a (+) (Novagen, Madison, WI) yielding pET-AgAmy1 and pET-Agm3 each with a C-terminal 6-His-tag. The coding region of each clone was sequenced in both directions to assure accuracy of PCR and cloning (Georgia Genomics Facility). Plasmids were transformed into *E. coli* strain BL21-CodonPlus (DE3)/pRIL, and over expressed in *E. coli* by culturing in one liter LB broth with induction of 1 mM isopropyl β -D-thiogalactopyranoside when the OD600nm reached 0.5-0.6. The culture was harvested by centrifugation and pellet was suspended in buffer (50 mM Tris-HCl pH 8.0, 10 mM EDTA, 15% w/v sucrose) with 20 – 40 mg lysozyme (Sigma) for 1hr at 37°C. The *E. coli* inclusions were collected by centrifugation, washed with 0.1 M NaCl, 2% Triton X-100, 20 mM Bis-Tris (pH 6.5), and then sonicated on ice in the same buffer supplemented with 2% sodium deoxycholate. The inclusions were washed in deionized H₂O, suspended in 10 ml deionized H₂O, and stored on ice at 5°C.

2.2.5 Polyclonal antiserum preparation against AgAmy1 and Agm3 and reduction of cross-reactivity between antisera.

A region of AgAmy1 and Agm3 was selected as an antigen for raising antibodies by aligning the primary amino sequence of each protein using ClustalW and then selecting the

region of least similarity between AgAmy1 and Agm3. Figure 1C shows the aligned sequences of each protein with the C-terminal regions being the most dissimilar (26% identity). The C-terminal region of AgAmy1 encoding a 20-kDa peptide was amplified with AgAmy1T-F2 and AgAmy1-R1 primers (Table 1), cloned into pET30A and the resulting plasmid called pET-AgAmy1t. A region from the C-terminus of Agm3 encoding a 30-kDa peptide was obtained by cleaving pET-Agm3 with NdeI, releasing a DNA fragment encoding the N-terminal half of the protein, and ligating the plasmid yielding pET-Agm3t. Plasmids pET-AgAmy1t and pET-Agm3t were transformed into *E. coli* strain BL21-CodonPlus (DE3)/pRIL and heterologous proteins were over-expressed, inclusion bodies prepared as described above, and then purified over an immobilized Nickel column (GE Healthcare) as described in (Zhang et al., 2008). Peptide purity was confirmed by SDS-PAGE and polyclonal antisera produced in New Zealand White rabbits (HsdOkd:NZW) at Harlan Laboratories (Indianapolis, IN).

Anti-AgAmy1 and Agm3 sera each detected both AgAmy1 and Agm3 on western blots (data not shown). To remove cross-reactive antibodies we separately coupled soluble Agm3 or AgAmy1 (25 mg each) to 0.5 g cyanogen-activated sepharose 6MB (Sigma) according to the manufacturer's instructions. After saturating coupling sites on beads with 2M ethanolamine (pH 8.0) and washing twice with the same buffer, beads were collected by centrifugation (1,000 rpm \times 1min) at 5°C. Each antiserum (100 μ l) was diluted to 1 ml with Na₂CO₃/NaHCO₃ (pH 9.6) and diluted anti-AgAmy1 serum added to the Agm3-beads and anti-Agm3 serum added to the AgAmy1-beads. The bead suspensions were rotated overnight at 5°C and then the beads were allowed to settle and the supernatant collected. The supernatant was then dialyzed against 20mM Na₂CO₃/NaHCO₃ pH 9.6 overnight and stored at -80°C for later used in western blotting and immunohistochemistry.

To verify the specificity of the cleared antisera, 0.5 µg of AgAmy1 or Agm3 inclusions and 20 µg of BBMV were subjected to 10% SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) filter. The PVDF filter was blocked by 3% bovine serum albumin (BSA) in PBST (PBS+0.1% Tween 20). The filters were incubated with the cleared anti-AgAmy1 or anti-Agm3 serum in 1:100,000 dilutions at room temperature for 2 hr. After washing, the filters were probed with anti-rabbit IgG horseradish peroxidase conjugate (Pierce) (1:25,000 dilution) by 0.1% BSA in PBST for 30 min at room temperature. The signals were detected with an ECL kit (GE Healthcare) and developed on X-ray film.

2.2.6 Phosphatidylinositol-specific phospholipase C (PI-PLC) treatment and GPI-anchor detection

BBMV from whole early fourth instar larvae of *An. gambiae* G3 were extracted by the MgCl₂ precipitation method (Silva-Filha et al., 1997) and stored at -80°C until use. Bt PI-PLC was produced in *E. coli* harboring plasmid pHN1403-PLC (provided by F. J. Sharom, University of Guelph, Guelph) as previously described (Hua et al., 2009). Two aliquots of BBMV were treated either with PI-PLC at mass ratio of 1:10 (PI-PLC:BBMV) or with 1 × PBS buffer as control for 2 hr at 37°C. BBMV were pelleted from PI-PLC released proteins by centrifugation at 13,000 g at 5°C, the supernatant was collected by centrifugation at 5 °C for analysis. Aliquots were separated on 10% SDS-PAGE and transferred to PVDF filter. The GPI-anchored proteins released by PI-PLC on the PVDF filter were probed with polyclonal antibody against the cross-reacting determinant (CRD) (kindly provided by Dr. K. Mensa-Wilmot, University of Georgia), or antisera of AgAmy1 or Agm3 of 1:5,000, 1:100,000, 1:100,000 dilutions respectively.

2.2.7 Immunohistochemistry

Fourth instar *An. gambiae* larvae were fixed in 5% paraformaldehyde for 5hr, then 10% overnight at 5°C. Fixed larvae were dehydrated in a series of ethanol (50%, 70%, 95%, 95%, and 100%) for 1 hr each at room temperature and kept in 100% ethanol overnight at 5°C. The larvae were defatted in 100% ethanol, xylene 1:1 (v/v) twice, then in 100% xylene twice for 1 h per step. Paraffin was melted at 58°C in heat-proof vessel. The samples were infiltrated by 1:1 (v/v) xylene: melted paraffin (v/v) for 1 h at 58°C and transferred to melted paraffin for 1 h three times. After being embedded, the whole larvae in paraffin block were sectioned into 8 µm thickness and placed on slides. Slides were heated at 70°C for 15 min to melt paraffin, soaked in xylene for 10 min three times, subsequently dried in air until tissue turns white. The slides were microwaved in the antigen-retrieval unmasking solution (Vector lab) for exposure of antigenic determinants. The slides were blocked in blocking buffer (5% w/v BSA, 0.2% v/v Tween 20 in PBS) for 2 hr and then incubated with anti-AgAmy1 or anti-Agm3 sera at dilution of 1:5,000 in blocking buffer overnight at 5°C overnight. After washing with same blocking buffer to remove the unbound antibodies, the slides were further incubated with goat-anti-rabbit IgG-conjugated with Alexa-488 (Molecular Probes, Eugene, Ore.) for 2 h in darkness. The slides were mounted with a 1:1 (v:v) glycerol to PBST medium and covered with a coverslip. The localization of AgAmy1 or Agm3 was observed under epifluorescence illumination microscopy.

2.2.8 Microtiter plate binding assays

An enzyme-linked immunosorbent assay for measuring Agm3, AgAmy1, and AgALP1 peptide binding to Cry11Ba in microplates was performed as described (Zhang et al., 2008). Peptides were biotinylated using N-hydroxy peptides and dialyzed as described previously. A microtiter plate assay for Cry11Ba (1.0 µg) in 100 µl coating buffer (100 mM Na₂CO₃, pH9.6)

was added to wells of microtiter plate (high-binding 96-well, Immulon 2HB; Thermo Fisher Scientific Inc., Waltham, MA) overnight at 4 °C. In the saturation binding assay, biotinylated Agm3 or AgAmy1 peptides were diluted to a series of concentrations (0.5nM to 20 nM in 100 µl of coating buffer) with or without a 500-fold molar excess of the unlabeled Agm3 or AgAmy1 peptides after the plates were blocked as previously reported (Zhang et al., 2008). In the competition binding assay, Cry11Ba coated at 1.0 µg of /well was incubated with 20 nM biotinylated Agm3, AgAmy1 or AgALP1 peptide with an increasing concentration of unlabeled homologous and heterologous peptides of AgAmy1, Agm3 or AgALP1. Results of saturation and competition reactions were measured under absorbance at 450 nm using microplate reader after following the reaction condition presented in (Zhang et al., 2008). Data were analyzed in best fit curves by one site saturation binding equation for saturation binding assay or one site competition binding equation for competition binding assay using SigmaPlot software (Version 11; Systat Software, Inc., San Jose, CA).

2.2.9 Bioassay

An. gambiae susceptibility to Cry11Ba protoxin or protoxin with a mixture of AgAmy1 or Agm3 proteins was assessed through bioassay on the early 4th instar larvae. Larvae were fed with soluble Cry11Ba only or mixed with AgAmy1 or Agm3 inclusion bodies at 1:100 molar ratio (toxin: peptide). A total of ten larvae in 2 ml deionized water suspension with two technical replicates of three biological replicates were tested in Costar culture plate at 27 °C. Bioassay was tested with a positive control of AgALP (Hua et al., 2009) and a negative control of AgAPN2tb (Zhang et al., 2010). The mortality was recorded after 24hr and analyzed by Sigma Plot Software (Version 11; Systat Software Inc., San Jose, CA).

2.3. Results

2.3.1 Identification and cloning of putative orthologous α -amylase and α -glucosidase in *An. gambiae*

We reported a protein of 70-kDa molecular size that was extracted by a Cry11Ba-bead complex from a mixture of proteins released by PI-PLC treated *An. gambiae* BBMV. In this study we separated the same Cry11Ba-extracted proteins by SDS-PAGE, excised the 70-kDa band from a stained SDS-gel and identified the protein by PMF analysis as α -amylase (Fig. S1). The best match was the protein encoded by SNAP00000012401 in SNAP transcript database of *An. gambiae* genome, but the corresponding gene was not in the official gene set. We manually annotated the sequence to a gene of which open reading frame (ORF) extends from nt 22585473 to nt 22587355 at chromosome 3R, which is 270 base pairs upstream of 5' end of AGAP008963 (Vectorbase *Anopheles* genome database). This gene, named *AgAmy1* in this study, encodes a protein of 604 amino acids (Fig. 1C) with a molecular mass of 68.754 kDa and a predicted isoelectric point of 4.9 (http://web.expasy.org/compute_pi/). The predicted GPI-anchored cleavage site at Glu⁵⁷⁹ of *AgAmy1* is in agreement with its release from BBMV by PI-PLC treatment (Zhang et al., 2008). Three asparagines predicted to be putative N-glycosylation sites are indicated in Fig1C. The putative calcium binding sites are predicted at positions 135, 201 and 235 of *AgAmy1*. The residues that are part of the active site for α -amylase family starch and oligosaccharide-degrading enzymes are structurally conserved (Janecek, 1997).

The *L. sphaericus* Bin toxin receptor α -glucosidase *Agm3* (Genbank ABW98683.1) (Opota et al., 2008) was cloned from cDNA prepared from *An. gambiae* gut tissue encodes a 67.092 kDa protein with 588 amino acids (Fig. 1C) and a predicted isoelectric point of 5.4. The *Agm3* protein shares 42% amino acid identity with *AgAmy1*.

2.3.2 Specificity of anti-AgAmy1 and anti-Agm3 antibodies and GPI-anchorage of brush border AgAmy1 and Agm3

Rabbit anti-sera prepared against *E. coli*-produced 20-kDa AgAmy1t and 30-kDa Agm3t peptides detected the expected 70-kDa protein in BBMV from *An. gambiae* larvae, however each anti-serum detected both *E. coli* produced peptides (data not shown). Apparently, even though the peptide immunogens have only 26% amino acid identity the shared antigenic sites that caused cross-reactivity between the sera. To solve this problem AgAmy1 and Agm3 were separately coupled to beads and anti-AgAmy1 serum was passed through the Agm3 column and anti-Agm3 serum was passed through the AgAmy1 column. Results from western blot analysis demonstrated that after this treatment each antiserum only recognized its own antigens. The anti-AgAmy1 serum detected only amylase AgAmy1 produced in *E. coli*, but not the same amount of glucosidase Agm3 (Fig. 2A: lanes 1 and 2) and cleared anti-Agm3 serum detected Agm3, but not AgAmy1 (Fig. 2B: lanes 1 and 2). Importantly, both cleared sera still detected a single protein band of about 70-kDa in BBMV of *An. gambiae* gut (Fig. 2A and B, lane 3).

2.3.3 AgAmy1 and Agm3 on brush border membrane have GPI anchorage.

AgAmy1 and Agm3 protein sequences were analyzed on-line with 'PredGPI' software (Pierleoni et al., 2008) to predict GPI-anchorage. Glu⁵⁷⁹ of AgAmy1 and Arg⁵⁶³ of Agm3 were predicted to be the ω -site for GPI modification with typical GPI-anchor motifs (Fig. 1C). PI-PLC was used to further verify whether these two proteins were GPI-anchored on the brush border membrane of gut epithelial cells. After aliquots of BBMV were treated with PI-PLC or buffer only, the released proteins were analyzed by Western blotting using anti-CRD serum. The anti-CRD serum detected several bands (Fig. 3A, lane 1), that agreed with the results observed before (Zhang et al., 2008), and no proteins were detected in control buffer treatment (Fig 3A,

lane 2). Anti-AgAmy1 (Fig 3B, lane 1) and anti-Agm3 (Fig, 3C, lane 1) sera each detected a band of about 70-kDa within the PI-PLC released proteins. Because these two anti-sera specifically recognized their own antigens, the 70-kDa band detected by anti-CRD should contain both Agm3 and AgAmy1 proteins due to their close molecular sizes. Anti-Agm3 also strongly detected a small protein or peptide (Fig. 3C, lane 1) that was not detected by anti-CRD antibodies suggesting that this peptide may be a degradation product of Agm3 that does not have a cleaved, but attached GPI anchor.

2.3.4 Localization of AgAmy1 and Agm3 in An. gambiae

Mosquito midguts provide suitable alkaline environment for digestive enzymes to digest and absorb nutrition from the ingested foods. To further confirm that we had cloned two food-digestion related enzymes, their localizations were observed with immunohistochemistry on sectioned 4th instar larvae *An. gambiae*. The results revealed that these two proteins are located in different regions of the larval midgut; AgAmy1 was found in salivary gland and posterior midgut microvilli (Fig. 4A), whereas Agm3 was only observed in posterior midgut microvilli (Fig. 4C). Meanwhile, pre-immune sera did not give any signal in the gut tissue (Fig. 4B). The results of immunolocalization further indicated that these proteins were present on *An. gambiae* digestive tract, especially on the brush border epithelial cells.

2.3.5 Binding affinities of AgAmy1 and Agm3 to Cry11Ba

The binding affinities of AgAmy1 and Agm3 to Cry11Ba were determined with a microplate binding assay as described before (Hua et al., 2009; Zhang et al., 2008). The biotin-labeled AgAmy1 and Agm3 specifically and saturably bound to Cry11Ba toxin (Fig. 5A & B). Using a one-site saturation binding model, a K_d of 37.8 ± 11.2 nM was calculated for AgAmy1 binding to Cry11Ba (Figure 4A); a K_d of 21.1 ± 4.9 nM was calculated for Agm3 binding. Both

proteins bound to Cry11Ba in high affinities though K_d values were a little lower than those of AgAPN2 (6.4 nM) (Zhang et al., 2008), but close to AgALP1 (23.9 nM) (Hua et al., 2009). Alkaline phosphatase AgALP1, one of the receptors of Cry11Ba in *An. gambiae* larval midgut, shares the same binding site with AgAPN2 on Cry11Ba (Hua et al., 2009). To determine whether the pattern of shared binding sites on Cry11Ba extends to AgAmy1 and Agm3, we probed Cry11Ba bound to wells of a microtiter plate with biotin-labeled AgAmy1, Agm3 or AgALP1 in the presence of increased amounts of unlabeled homologous or heterologous peptide. Competition binding assays revealed that the binding of biotin-AgAmy1 was competed by unlabeled AgAmy1, Agm3 and AgALP1 (Fig 6A). Both biotin-Agm3 and biotin-AgALP1 were competed by these three peptides in the same pattern as shown in Figure 6B & 6C. Our competition binding results demonstrated that AgAmy1, Agm3 and AgALP share the same binding sites on Cry11Ba, and according to previous results (Hua et al., 2009), with AgAPN2.

2.3.6 Effects of AgAmy1 and Agm3 peptides on Cry11Ba larvicidal activity.

We tested the abilities of AgAmy1 and Agm3 which bind Cry11Ba with high affinity to neutralize Cry11Ba toxicity to larvae. We fed 4th instar larvae Cry11Ba alone or with inclusion bodies of *E. coli*-produced AgAmy1 and Agm3 and recorded larval mortality. AgAmy1 and Agm3 inclusion bodes reduced Cry11Ba toxicity from 90% larval mortality to 30% ($P < 0.005$) and 10% ($P < 0.005$), respectively for AgAmy1 and Agm3 (Fig 7). As controls we included AgALP1 inclusions with known inhibitory effect on Cry11Ba toxicity (Hua et al., 2009) and AgAPN2tb inclusions that synergize Cry11Ba toxicity (Zhang et al., 2010) and the effects on larval mortality were as expected (Fig 7).

2.4. Discussion

Glycoside hydrolases (i.e. glucosidases) in the α -amylase family act as receptors of mosquitocidal Bt Cry and *L. sphaericus* Bin toxins (Fernandez-Luna et al., 2010; Opota et al., 2008). Specifically, an α -amylase is a receptor of Cry4Ba and Cry11Aa in *An. albimanus* and α -glucosidases Cpm1, Cqm3 and Agm3 are receptors of Bin toxin in *Cu. pipiens*, *Cu. quinquefasciatus* and *An. gambiae*, respectively (Darboux et al., 2001; Opota et al., 2008; Romao et al., 2006). In this study we showed that AgAmy1 and Agm3 are high affinity binding proteins and putative receptors of Cry11Ba in *An. gambiae*. AgAmy1 and Agm3 proteins produced in *E. coli* from cloned midgut cDNA bound Cry11Ba with high affinity ($K_d = 38$ and 21 nM, respectively), binding association values comparable to Cry11Ba receptor protein AgALP1 ($K_d = 24$ nM) (Hua et al., 2009), but lower than the binding constants calculated for AgAPN2 (6 nM) and AgCad2 (12 nM) (Hua et al., 2013; Zhang et al., 2008). In competition binding assays AgAmy1 and Agm3 bound the same site(s) on Cry11Ba, a site shared with AgALP1 (Fig. 5). Since AgALP1 and AgAPN2 share binding sites on Cry11Ba (Hua et al., 2009), competition binding results indicate that the four GPI-anchored BBM proteins are bound by the same site(s) on Cry11Ba. The ability of peptides of AgAmy1, Agm3, AgALP1 and AgAPN2 to each reduce Cry11Ba toxicity when fed with toxin to larvae underscores the relevance of the shared binding site(s) to Cry11Ba larval toxicity,

The uniquely high potency of Cry11Ba against *Aedes*, *Anopheles* and *Culex* species (Delecluse et al., 1995) implies that aspects of Cry11Ba action differ from other mosquitocidal Cry toxins. It was suggested that high toxicity might be due to either differential activation by midgut proteases or a differential stability involving the carboxy-terminal part of Cry11Ba (Delecluse et al., 1995). Another not exclusive possibility is that Cry11Ba has a particularly

effective mechanism of action at the midgut brush border level. Cadherin, alkaline phosphatase and aminopeptidase have been identified as receptors of Cry11Ba in *Ae. aegypti* (Likitvivanavong et al., 2011) and *An. gambiae* (Hua et al., 2013); (Hua et al., 2009) (Zhang et al., 2008). With the present identification of AgAmy1 and Agm3 we can extend the model of Cry11Ba action in *An. gambiae* larvae (Hua et al., 2013), which integrates Cry11Ba interactions with midgut proteins. AgCad2 is a high affinity binder and receptor of Cry11Ba ($K_d = 12$ nM) (Hua et al., 2013), a role similar to AeCad in *Aedes* larvae (Likitvivanavong et al., 2011). In *Aedes*, Cry11Aa contact with AeCad induces toxin oligomerization (Likitvivanavong et al., 2011), which would be followed by membrane insertion mediated by GPI-anchored APNs and ALPs. Unlike *Ae. aegypti*, larvae of *An. gambiae* have a second cadherin (AgCad1) that bound Cry11Ba with low affinity ($K_d = 766$ nM), which we interpret as AgCad1 having minimal involvement in Cry11Ba action. In *An. gambiae* larvae AgAmy1, Agm3 and AgALP1 each bind Cry11Ba with similar association constants ($K_d = 20-40$ nM) at the same site on toxin. The ability of Cry11Ba to bind AgAPN2 with a greater overall binding affinity ($K_d = 6$ nM) and at multiple sites on AgAPN2, suggests a more important role for AgAPN2 in Cry11Ba toxicity. The differential inhibitory or enhancement effects when specific AgAPN2 peptides were fed with Cry11Ba to larvae, is also evidence that Cry11Ba interacts differently with AgAPN2 *in vivo* than it does to AgALP1, AgAmy1 and Agm3. Overall, the number of GPI-anchored proteins recognized by Cry11Ba is unique among Cry toxins, a factor which correlates positively with high mosquitocidal potency.

Our experimental results in this study only supported part of hypothesis that amylase was another receptor of Bt Cry11Ba in *An. gambiae*. But for glucosidase, Agm3 actually acted as the receptor for both Cry11Ba from Bt subsp. *jegathesan* and binary toxin from *L. sphaericus*. The

common binding motifs within these receptors appears very interesting for further study that might be reveal the real mechanism of Bt Cry11Ba toxic action to mosquitoes.

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Figure legends

Fig 2.1. (A) Diagram of *An. gambiae* AgAmy1 and Agm3 molecules and PCR primer locations.

(B) Protein sequences of AgAmy1 and Agm3 analyzed using ClustalW

(<http://www.genome.jp/tools/clustalw/>). Symbols below the sequences denote: an * (asterisk) indicates positions which have a single, fully conserved residue, a : (colon) indicates conservation between groups with strongly similar properties, and a . (period) indicates conservation between groups of weakly similar properties. The amino acid predicted to be the ω -site for GPI-anchor attachment is underlined and in italic. Asparagines predicted to be N-glycosylation sites are double underlined and in italic. Putative calcium binding sites are highlighted in grey.

Fig 2.2. Western blots to test the specificity of anti-AgAmy1 and anti-Agm3 sera and the presence of AgAmy1 and Agm3 in *An. gambiae* BBMV. Inclusion bodies of recombinant *E.coli* expressed AgAmy1 (0.5ug) (lane 1) and Agm3 (0.5ug) (lane 2), and *An. gambiae* BBMV (20 ug) (lane 3) were separated on 10% SDS-page and transferred to PVDF membrane. Anti-AgAmy1 or anti-Agm3 incubated with the designated membrane exhibited specific detection for the target inclusions. The specific antiserum of AgAmy1 detects the presence of AgAmy1 in BBMV, whereas anti-Agm3 detects Agm3 in BBMV.

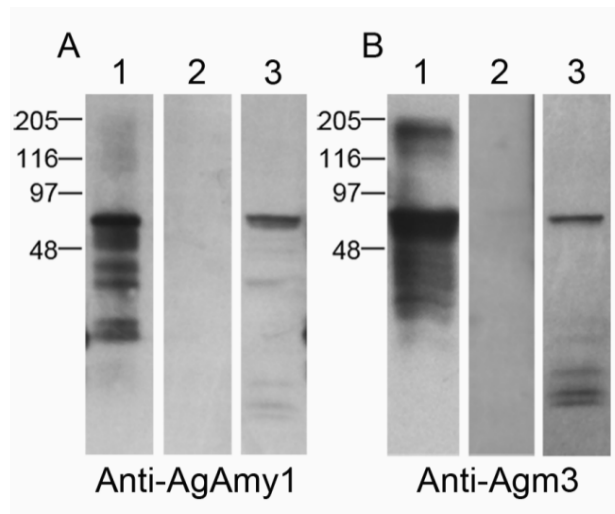


Fig 2.3. PI-PLC release of AgAmy1 and Agm3 from *An. gambiae* BBMV. BBMV were treated with PI-PLC (lane 1) or PBS buffer only (lane 2), pelleted by centrifugation, washed and soluble proteins separated by SDS-PAGE and blotted to PVDF membrane filters. The membranes were probed with anti-CRD (Panel A), anti-AgAmy1 (Panel B) and anti-Agm3 (Panel C).

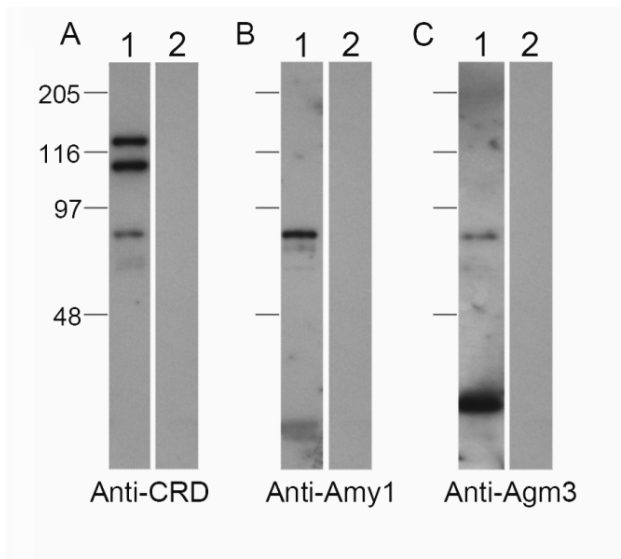
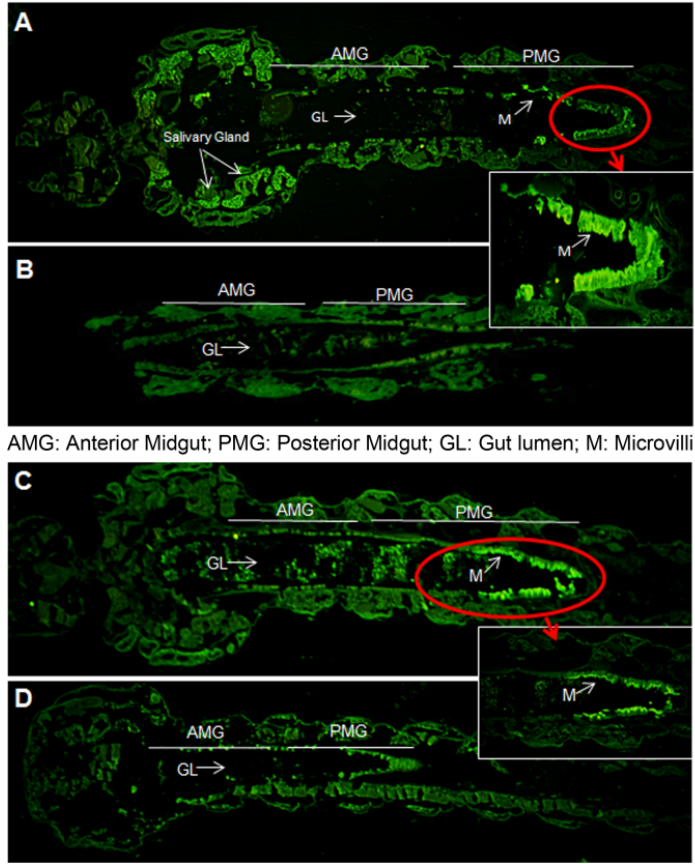


Fig 2.4. Immunolocalization of AgAmy1 and Agm3 in *An. gambiae* 4th instar larvae. (A) Sectioned larvae probed with anti-AgAmy1. Inserts are enlargements of the microvilli in posterior midgut. (B) Control section of Figure 3A probed with preimmune AgAmy1. (C) Sectioned larvae probed with anti-Agm3. (D) Control section of Figure 3C probed with preimmune Agm3. AMG: Anterior Midgut; PMG: Posterior Midgut; GL: Gut Lumen; M: Microvilli.



AMG: Anterior Midgut; PMG: Posterior Midgut; GL: Gut lumen; M: Microvilli

Fig 2.5. Saturation binding assays of Cry11Ba to AgAmy1 and Agm3. Microtiter plates coated with 1 μg of Cry11Ba toxin were incubated with an increasing nM concentration of biotinylated AgAmy1 or Agm3 peptide alone or with 500-fold molar excess of unlabeled AgAmy1 or Agm3 peptide to determine specific binding.

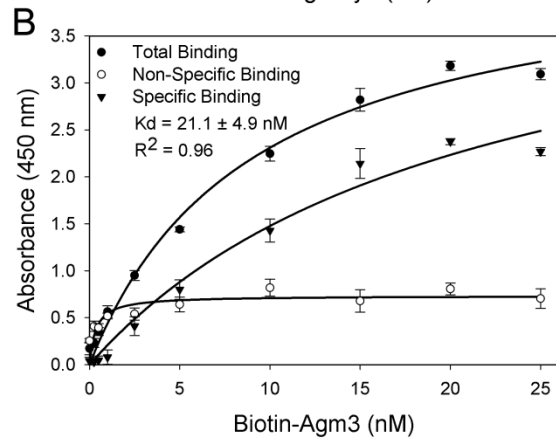
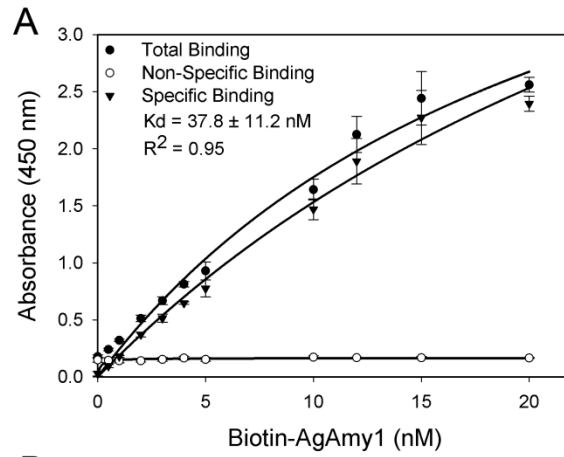


Fig 2.6. Homologous and heterologous competition binding assay of among AgAmy1, Agm3 and AgALP to Cry11Ba toxin. After 1.0 ug of Cry11Ba toxin was coated in microtiter 96-well plate, 20 nM biotinylated Agm3, AgAmy1 or AgALP peptides were incubated with an increasing concentration of unlabeled AgAmy1, Agm3 or ALP peptides, and applied to the toxin-coated plate to determine the competition activity among three of the proteins.

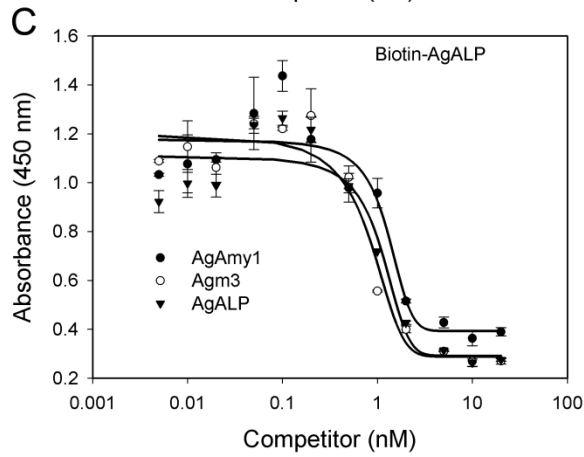
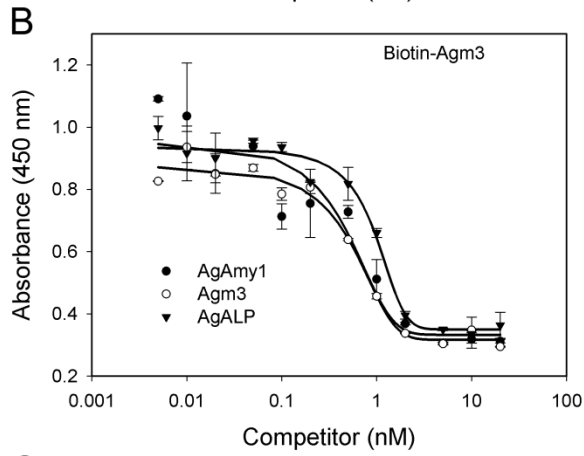
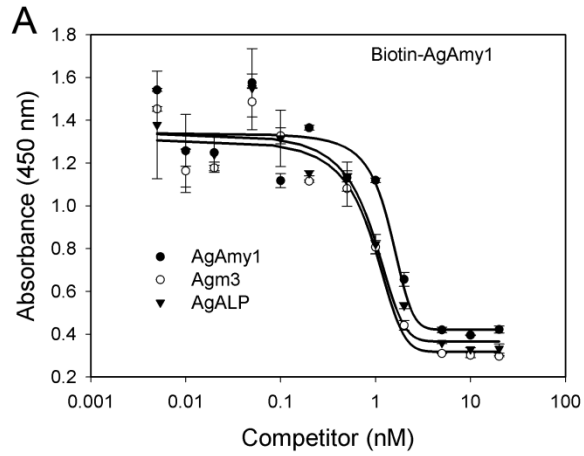


Fig 2.7. *In vivo* bioassay of soluble Cry11Ba against *An. gambiae* 4th instar larvae with or without the AgAmy1 and Agm3 inclusions. Larvae were fed with soluble Cry11Ba only or mixed with AgAmy1 or Agm3 inclusion bodies at 1:100 molar ratio (toxin: peptide). A total of ten larvae in 2ml deionized water suspension repeats were tested in Costar culture plate at 27 °C. Bioassay was tested with a positive control of AgALP, and a negative control of AgAPN2 (a peptide with synergistic effect on soluble Cry11Ba toxicity).

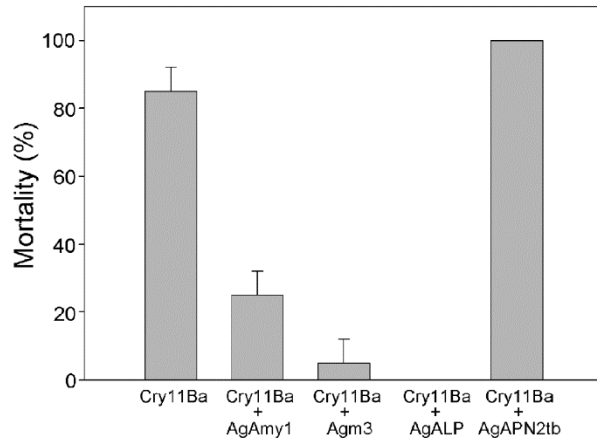


Table 2.1. Primers used in this study

Primer name	Sequence (5'-3')
AgAmy1-F1:	5'-ATG CGT ATT GCA GTG AGT GCA CTA GTG CTG GCA GC-3'
Ag-Amy-R1:	5'-TTA GAA GAG CGA TCG CAG CAC CGC TGC CAC CAG CA-3'
Agm3-F1:	5'-AG TTT TAC CGA CCA TTG GTC ACC G-3'
Agm3-R1:	5'-AAA CAA ATG CTC AAC AGC TGC CAA CAA G-3'
AgAmy1T-F3:	5'-CGA CCA TAT GGC CGA GCT GGA CTG G-3'
Agm3T-F2:	5'-AGA CCC ATG GAG CAT GCC ACG TTC TA-3'
AgAmy1t-F2:	5'-GAT CCA TAT GGA GGA CAA CCG GGA CAT TAC G-5'

CHAPTER 3

CHITOSAN/DSIRNA NANOPARTICLE TARGETING IDENTIFIES AGCAD1 CADHERIN
IN *ANOPHELES GAMBIAE* LARVAE AS AN *IN VIVO* RECEPTOR OF CRY11BA TOXIN
OF *BACILLUS THURINGIENSIS* SUBSP. *JEGATHESAN*

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Abstract

The Cry11Ba protein of *Bacillus thuringiensis* subsp. *jegathesan* crystals has uniquely high toxicity against a spectrum of mosquito species. The high potency of Cry11Ba against *Anopheles gambiae* is caused by recognition of multiple midgut proteins including glycosyl phosphatidylinositol-anchored alkaline phosphatase AgALP1, aminopeptidase AgAPN2, α -amylase AgAmy1 and α -glucosidase Agm3 that bind Cry11Ba with high affinity and function as putative receptors. The cadherin AgCad2 in *An. gambiae* larvae also binds Cry11Ba with high affinity ($K_d = 12$ nM) and is considered a putative receptor, while cadherin AgCad1 bound Cry11Ba with low affinity ($K_d = 766$ nM), a property not supportive for a Cry11Ba receptor role. Here, we show the *in vivo* involvement of AgCad1 in Cry11Ba toxicity in *An. gambiae* larvae using chitosan/DsiRNA nanoparticles to inhibit AgCad expression in larvae. Cry11Ba was significantly less toxic to AgCad1-silenced larvae than to control larvae. Because AgCad1 was co-suppressed by AgCad2 DsRNAi, the involvement of AgCad2 in Cry11Ba toxicity could not be ascertained. The ratio of AgCad1:AgCad2 transcript level is 36:1 for gut tissue in 4th instar larvae. Silencing AgCad expression had no effect on transcript levels of other binding receptors of Cry11Ba. We conclude that AgCad1 and possibly AgCad2 in *An. gambiae* larvae are functional receptors of Cry11Ba toxin *in vivo*.

Keywords: *Bacillus thuringiensis*; Bt; Cry toxin; mosquitocidal; cadherin; RNAi; DsiRNA

Abbreviations: *Bacillus thuringiensis* (Bt); bovine serum albumin (BSA); brush border membrane vesicles (BBMV); *Anopheles gambiae* alkaline phosphatase (AgALP1); *Anopheles gambiae* aminopeptidase N (AgAPN2); *Anopheles gambiae* cadherin 1 (AgCad1); *Anopheles gambiae* cadherin 2 (AgCad2); *Anopheles gambiae* glucosidase (Agm3); cadherin repeat (CR);

quantitative polymerase chain reaction (RT-qPCR); polymerase chain reaction (PCR); signal peptide (SP); transmembrane (TM).

3.1. Introduction

Protozoa and viruses vectored by mosquitoes cause severe human diseases including malaria, Dengue, West Nile Virus and chikungunya. Disease transmission can be disrupted by strategies that target the adult stage with chemical pesticides, or by targeting the larval stage by treating breeding sites with either insect growth regulators or the entomopathogenic bacterium *Bacillus thuringiensis* (Bt) subsp. *israelensis* (Bti). When applied to mosquito breeding sites, formulations of Bti spores and parasporal crystals are highly effective against larval stages of *Aedes* and *Anopheles* but less effective against *Culex* species (Poncet et al., 1995).

The mosquitocidal activity of Bti involves its proteinaceous crystals that are composed of Cry4Aa, Cry4Ba, Cry10Aa, Cry11Aa, Cyt1Aa and Cyt2Ba proteins (Ben-Dov, 2014; Berry et al., 2002). Concerns regarding the potential for mosquito resistance and the desire to have greater activity against certain species led to the discovery of *Bt* subsp. *jegathesan* (Btjeg) (Selena et al., 1995). The crystals of Btjeg have eight insecticidal proteins (Sun et al., 2013), of which Cry11Ba has been studied in the most detail (Delecluse et al., 1995) as Cry11Ba is the single most active Cry protein across the three major genera of disease-vectoring mosquitoes.

Midgut proteins in the midgut brush border of larvae bind Cry toxins and mediate events culminating in larval death [for recent reviews refer to (Adang et al., 2014; Pardo-López et al., 2013; Vachon et al., 2012)]. Alkaline phosphatases and aminopeptidases are midgut receptors of Bti Cry4Ba and Cry11Aa, and Btjeg Cry11Ba toxins in *Aedes aegypti* (Fernandez et al., 2006; Jimenez et al., 2012; Likitvivatanavong et al., 2011). An aminopeptidase binds Cry11Ba in *Anopheles albimanus* (Abdullah et al., 2006) and receptor function was established for a related aminopeptidase in *An. gambiae* larvae (Zhang et al., 2008). An α -amylase is a receptor of Cry4Ba and Cry11Aa in *An. albimanus* and for Cry11Ba in *An. gambiae* (Fernandez-Luna et al.,

2010; Zhang et al., 2013). Cadherin was originally identified as a Cry1Ab receptor on the brush border membrane of *Manduca sexta* (Vadlamudi et al., 1993). Cadherin was found not only to be involved in toxin binding, but the cadherin repeats (CR) showed a synergistic effect to Cry1A toxin in lepidopterans (Abdullah et al., 2009; Chen et al., 2007; Peng et al., 2010) and to Cry4Ba toxin in dipterans (Zhang et al., 2008; Park et al., 2009). In *Ae. aegypti*, cadherin alone was identified as a binding receptor of Cry11Aa and Cry11Ba (Bravo et al., 2011; Lee et al., 2014). As for *An. gambiae*, cadherin AgCad2, which shares only 14% identity at the amino acid level with the Cry4Ba-binding AgCad1, bound Cry11Ba with high affinity and cadherin repeats (CR) peptides showed an inhibitory effect to Cry11Ba toxicity against *An. gambiae* larvae (Hua et al., 2013). *Aedes* cadherin mediates *in vivo* toxicity of Cry11Aa to *Ae. aegypti* larvae, as evidenced when cadherin-silenced *Ae. aegypti* larvae became less susceptible to Cry11Aa (Lee et al., 2014; Rodriguez-Almazan et al., 2012).

RNAi has been employed as an effective tool to investigate the *in vivo* roles that receptors have for Cry toxin toxicity. Silencing of GPI-anchored APN isoforms (APN2783, APN5808, and APN2778) led to different levels of tolerance to Cry4Ba toxicity in *Ae. aegypti* larvae (Saengwiman et al., 2011). One of the APN isoforms (APN2778) was previously reported as a binding receptor of Cry11Aa (Chen et al., 2009). Another GPI-anchored protein, ALP1, was silenced in *Ae. aegypti* larvae, resulting in a tolerant response against Cry11Aa but not Cry4Ba (Jimenez et al., 2012). Moreover, silencing of heat shock protein, ATP synthase beta subunit or actin caused 2 to 4-fold increased susceptibility to Cry11Aa in *Ae. aegypti* larvae (Cancino-Rodezno et al., 2012).

While the success of RNA inhibition as a functional approach in *Ae. aegypti* larvae is well-established, it is less so in *An. gambiae* larvae. Zhang et al (2010) reported successful gene

silencing using chitosan-entrapped dsRNA nanoparticles in *An. gambiae*. The recent success of Mysore et al., using chitosan-DsiRNA nanoparticles to achieve successful gene silencing in *Ae. aegypti* (Mysore et al., 2014; Mysore et al., 2013), prompted our use of chitosan/DsiRNA nanoparticle-mediated RNAi to investigate the *in vivo* roles of two cadherins, AgCad1 and AgCad2, for Cry11Ba toxicity in *An. gambiae* larvae. We expected AgCad2, but not AgCad1, to have an *in vivo* role in Cry11Ba toxicity to larvae, but our observed results did not support this hypothesis. Rather, our results support the role of AgCad1, and possibly AgCad2, as mediating Cry11Ba toxicity in *An. gambiae* larvae.

3.2. Materials and Methods

3.2.1 Insects

An. gambiae (CDC G3 strain) were maintained at 27°C with a light-dark photoperiod of 16h : 8h as described (Zhang et al., 2008). Larvae of the appropriate age were collected and used for RNAi and other experiments.

3.2.2. Purification of Cry11Ba protoxin

The Bt strain 407, harboring plasmid pJEG80.1 encoding Cry11Ba (Delécluse et al., 1995) was grown in sporulation medium with erythromycin as described previously (Zhang et al., 2008). Spores and crystals were harvested by centrifugation and washed according to (Zhang et al., 2008). Crystals were purified from spores by centrifugation through a 30-60% (w/v) NaBr step gradient at 47,000 g for 2 h. Purified crystals were washed twice with deionized H₂O and then dissolved in 25 ml 100 mM 3-(cyclohexylamino) propanesulfonic acid (CAPS), pH10.6, supplemented with 0.05% β-mercaptoethanol overnight at 5°C to release protoxin. The solution was clarified by centrifugation at 10,000 g for 10 min and filtration through a 0.45 μM BA85 membrane filter (Whatman). The solution was dialyzed overnight at 5°C against 20 mM sodium

carbonate pH 9.6. Protoxin was bioassayed against early 4th instar larvae to calculate an LC₆₀ dosage for use in RNAi experiments (described below).

3.2.3 Preparation of chitosan/DsiRNA mediated nanoparticles for mosquito RNAi

Cadherin knockdown was performed by orally feeding chitosan/DsiRNA nanoparticles to *An. gambiae* larvae. Two DsiRNAs, DsiRNA-a and DsiRNA-b corresponding to different regions of each cadherin gene AgCad1 or AgCad2 were designed with the Integrated DNA Technologies (IDT; Coralville, IA, USA) website at <http://www.idtdna.com/site>. Scrambled sequences to AgCad2 (DsiRNAs) lacking a target to any known *An. gambiae* gene were determined by blast search against the NCBI database and used as a negative control. Two of the DsiRNAs of scrambled sequence to AgCad2 were selected using a software tool available on Genscript: <https://www.genscript.com/ssl-bin/app/scramble>. Food pellets mixed with chitosan without DsiRNAs was used as another control. All DsiRNAs were chemically synthesized by IDT Company (Coralville, IA, USA), with sense and antisense sequences shown in Table 3.1. The method of preparation of chitosan/DsiRNA nanoparticles was adapted from Zhang et al., and Mysore et al. (Mysore et al., 2013; Zhang et al., 2010b). Two DsiRNA constructs targeting the same cadherin were combined to make one chitosan/DsiRNAs nanoparticle pellet. Each pellet containing 32 µg of each of the two DsiRNAs was dissolved in 50 µl 50mM Na₂SO₄. The combined DsiRNA were incorporated with 100 µl 0.04% chitosan. After centrifuging at 13,000 g, the pellet was mixed with 6 mg of food (Tetra Min, tropical flakes chopped with a bladed coffee grinder) with dry yeast (2:1 ratio of food: dry yeast) and coated in 15 µl 2% agarose (Fisher Scientific) gel. The embedded pellet was divided into four pieces, and a piece was fed to 65 larvae every 4 h continuously for 3 days for 18 feedings in total. Larvae were maintained in 50

small container (24 oz.), and collected four h after the final feeding for total RNA extraction and bioassay.

3.2.4 RNA extraction and cDNA synthesis

Total RNA extracted from larvae was used to synthesize cDNA for testing the transcript levels of AgCad1 and AgCad2. Eight larvae in three biological replicates were collected from each of the two separated feeding biological replicates and soaked in 200 µl of TRIzol reagent (Ambion) in a microfuge tube. Samples were homogenized using a cordless motor-driven pellet pestle (Grainger) and centrifuged at 12,000 g 30 min at 4°C. The supernatant was collected and added to 40 µl chloroform and mixed vigorously at room temperature for 5 min, and centrifuged as above for 15 min at 4°C. The upper aqueous phase containing the RNA was collected and mixed with an equal volume of 100% isopropanol at room temperature for 10 min, and centrifuged for 10 min at 4°C to pellet total RNA. The RNA pellet was washed with 75% ethanol, air dried and dissolved in 25 µl of diethyl pyrocarbonate-treated water. Total RNA amount was determined by NanoDrop Spectrophotometer (N-1000). cDNA was synthesized from 1.5 µg of total RNA using SuperScript III First-Strand Synthesis System (Invitrogen) with an Oligo dT primer following the manufacturer's directions.

3.2.5 Quantitative real-time PCR (RT-qPCR)

RT-qPCR was carried out to evaluate the transcript levels of AgCad1 and AgCad2 as previously described (Hua et al., 2014). Primers for amplifying AgCad1 (VectorBase: AGAP002828) and AgCad2 (VectorBase: AGAP001591) were designed using Primer 3 software (v 0.4.0) (<http://frodo.wi.mit.edu/>); the reference gene RPS3 (Vectorbase: AGAP001910) primers were the same as used previously by Zhang et al. (Zhang et al., 2010b). Primers RPS7 (Vectorbase: AGAP010592) were provided by Dr. Judith Willis (University of Georgia) that

were used in their previous study (Togawa et al., 2008). Primers were verified to amplify a single gene and tested for efficiency (see Table 3.4). Data were normalized to the geometric mean of RPS3 and RPS7 using the $\Delta\Delta C_t$ calculation method (Livak and Schmittgen, 2001). Details for primers and RT-qPCR will be found in Table 3.4.

To evaluate the stability of AgCad1 and AgCad2 transcript levels across the feeding period, larvae were collected on the 3rd, 4th, 5th and 6th day after hatching for total RNA extraction and cDNA synthesis as described above. RT-qPCR was performed to detect the transcript level of AgCad1 and AgCad2. The resulting values in 4-, 5-, and 6-day-old larvae were compared to that in 3-day-old larvae as a standard.

The relative percentage of transcript levels of non-target genes, Agm3 (VectorBase: AGAP008961), AgAPN2 (Vectorbase: AGAP013188), AgALP1 (Vectorbase: AGAP004578) were measured by RT-qPCR for larvae fed with cadherin DsiRNAs or scrambled control as described above. Data are presented as means \pm SEM of three replications (n = 3). The primers used in RT-qPCR are listed in Table 3.4.

To examine AgCad1 and AgCad2 transcript levels in head, gut and whole body, larval head and gut were dissected from fourth instar larvae and collected separately with whole body in duplicates for RNA extraction and cDNA synthesis as described above. All cDNAs were diluted 100 times as template to examine the transcript levels of AgCad1 and AgCad2 by RT-qPCR.

The relative quantity (ΔC_q) of AgCad1 to AgCad2 is shown in Table 3.4. Results were calculated with the formula: Relative Quantity sample (GOI) = $(1+E)^{(C_{q(\text{Min})} - C_{q(\text{sample})})}$ according to the instruction Manual of CFX Connect Real-Time PCR Detection Systems (<http://www.biorad.com/webroot/web/pdf/lsr/literature/10021337.pdf>), where E represents the

efficiency of primer calculated as $E=1$. $Cq_{(MIN)} = \text{Average } Cq \text{ for AgCad2}$; $Cq(\text{sample}) = \text{Average } Cq \text{ for AgCad1}$; GOI = Gene of interest.

3.2.6 Bioassay

Soluble Cry11Ba, prepared as described above, was added to a twelve-well Costar culture plate (Corning) to test toxicity to *An. gambiae* larvae. To determine the lethal concentration (LC_{60}) of Cry11Ba, *An. gambiae* larvae were treated with soluble Cry11Ba in 2 ml deionized H_2O in a twelve-well culture plate. Mortality of larvae was recorded after 24 h. The mean lethal concentration (LC_{60}) was calculated by probit analysis using SAS program SAS 9.3. For RNAi bioassays, *An. gambiae* larvae fed with AgCad1-DsiRNAs, AgCad2-DsiRNAs, food without DsiRNAs and scrambled DsiRNAs were exposed to soluble Cry11Ba at the LC_{60} dosage. Bioassays were performed using ten larvae in four replicates at 27 °C. Mortality was recorded after 24 h. Results were analyzed using t test from Sigma Plot Software (Version 11; Systat Software Inc., San Jose, CA).

3.3. Results

3.3.1 Relative percentage of AgCad1 and AgCad2 transcript levels in *An. gambiae* larvae after RNAi treatment

To investigate the *in vivo* roles of AgCad1 and AgCad2 cadherins in Cry11Ba toxicity, two DsiRNAs were designed and synthesized (Table 3.1) for each AgCad gene (Fig. 3.1A). The DsiRNAs were incorporated into chitosan/DsiRNA nanoparticles, and then embedded with food in agarose pellets and fed to larvae (Mysore et al., 2013; Zhang et al., 2010b). Three-day-old *An. gambiae* larvae were fed AgCads-DsiRNA for three days and then transcript levels of AgCad1 and AgCad2 were measured by RT-qPCR. DsiRNAs of AgCad1 significantly ($P < 0.05$) knocked down its target gene, AgCad1 to 49% of the food control, but did not decrease the transcript level

of AgCad2 gene significantly ($P > 0.05$) (Fig. 3.1B). AgCad2 transcript level was repressed 80% after feeding with AgCad2-DsiRNAs, significantly different from the control ($P < 0.05$) (Fig. 3.1C). It was unexpected to find that AgCad2-DsiRNAs also significantly repressed transcript level of AgCad1 by 35% ($P < 0.05$).

3.3.2 Relative percentage of Agm3, AgAPN2, and AgALP1 transcript levels after feeding with AgCad-DsiRNAs

As an off-target effect was observed on AgCad1 by AgCad2-DsiRNAs, RT-qPCR was performed to investigate whether AgCads-DsiRNAs could interfere with the transcript levels of other non-target genes in *An. gambiae* larvae. The relative transcript levels of Agm3, AgAPN2 and AgALP1 were evaluated by RT-qPCR after feeding with AgCads-DsiRNAs. These genes were chosen because they encode binding proteins and putative receptors of Cry11Ba in *An. gambiae* larvae (Hua et al., 2009; Zhang et al., 2013; Zhang et al., 2008). Agm3, AgAPN2 and AgALP1 transcript levels showed no significant change ($P > 0.05$) after feeding with AgCad1-DsiRNAs, AgCad2-DsiRNAs, or scrambled DsiRNAs (Fig. 3.2). Therefore, AgCads-DsiRNAs did specifically interfere with cadherin gene expression and there was no off-target effect detected for transcripts encoding Agm3, AgAPN2, or AgALP1 proteins.

3.3.3 Comparison of AgCad1 and AgCad2 transcript levels between 4-, 5-, and 6-day-old larvae relative to 3-day-old larvae, and relative abundance of AgCads in 6-day-old larvae

The transcript levels of AgCad1 and AgCad2 in untreated *An. gambiae* larvae were examined for the three developmental days that correspond to the toxin-treatment days. Using the same amount of total RNA for cDNA synthesis, the transcript levels of AgCad1 and AgCad2 were analyzed in 3-, 4-, 5- and 6-day-old larvae. As shown in Fig. 3.3, transcript levels of AgCad1 and AgCad2 showed no significant changes for 3-day to 6-day-old larvae ($P > 0.05$).

These results support the conclusion that reduced AgCad expression in 6-day old larvae after DsiRNA treatments can be attributed to RNAi effects and not to possible effects on rate of development in AgCad transcript levels from day 3 to day 6.

The relative amounts of AgCad1 and AgCad2 transcripts in head, guts and whole body preparations from 4th instar *An. gambiae* larvae were determined by RT-qPCR. The levels of AgCad2 and AgCad1 transcripts were equal in cDNA samples prepared from head, but AgCad1: AgCad2 ratio in gut was about 36:1 and the overall AgCad1: AgCad2 transcript ratio in whole body samples was 5:1 (Table 3.2). The relative transcript levels in 4th instar *An. gambiae* larvae for the five genes analyzed in this study by RT-qPCR are AgAPN2>Agm3>AgALP≥AgCad1>AgCad2.

3.3.4 Toxicity of Cry11Ba on cadherin-silenced An. gambiae larvae

The effects of reduced cadherin expression on Cry11Ba toxicity to larvae was examined as follows. Larvae were fed with DsiRNAs as administered in section 3.1 and then exposed to 3.33 µg/ml soluble Cry11Ba (an LC₆₀ dosage). The recorded mortality after 24 h showed that larvae fed with AgCad-DsiRNAs became more tolerant to Cry11Ba as compared to those fed with scrambled DsiRNAs or food only (Fig. 3.4). Toxicity of Cry11Ba was reduced by 50% after *An. gambiae* larvae were fed with AgCad1-DsiRNAs (P<0.05) and by 42% on those fed with AgCad2-DsiRNAs (P<0.05) compared to the food control group. There was no significant difference in the reduced toxicity between the AgCad1 and AgCad2 DsiRNA groups at (P>0.05), evidence that AgCad1 and possibly AgCad2 are functional binding receptors of Cry11Ba. AgCad2 involvement in Cry11Ba toxicity cannot be unambiguously concluded because the AgCad1 transcript level was decreased in larvae fed with AgCad2-DsiRNAs.

3.4. Discussion

RNAi gene silencing in insect larvae established specific midgut cadherins in coleopteran, dipteran and lepidopteran larvae as necessary for Cry-mediated toxicity. For example, in *Ae. aegypti* larvae knockdown of AeCad by feeding with dsRNA encapsulated in Effectene liposomes resulted in reduced Cry11Aa-mediated mortality from 70% for the control group to 30% for AeCad dsRNA-fed larvae, leading to the conclusion that cadherin binding is a limiting step in Cry11Aa toxicity to *Aedes* (Rodriguez-Almazan et al., 2012). In the current study feeding both of AgCad1 and AgCad2 DsiRNAs on chitosan-nanoparticles reduced AgCad transcript levels (Fig. 3.1) without reducing other known Cry toxin receptors (Fig. 3.2), and reduced Cry11Ba-mediated mortality from 60% for larvae fed scrambled DsiRNA to 30% and 35% mortality for AgCad1- and AgCad2-DsiRNA, respectively (Fig. 3.4). Since specific AgCad1 transcript reduction increased tolerance to Cry11Ba, we conclude that limited availability of AgCad1 reduced Cry11Ba binding and toxicity.

The presence of AgCad1 and AgCad2 cadherins in *An. gambiae* larval midgut, and the *in vitro* binding of Cry11Ba to AgCad1 CR11-MPED ($K_d=760$ nM) and AgCad2 CR14-MPED ($K_d=12$ nM) (Hua et al., 2013), made it important that DsiRNAs used in experiments be cadherin-specific; yet the 48% nucleotide identity between the cadherins, presented challenges in designing gene-specific DsiRNAs. Our intent was to design two 25-27 nt DsiRNAs for each AgCad that minimized matches to the other cadherin and to additional off-target *An. gambiae* sequences. Our design strategy was successful for AgCad1 DsiRNAs as evidenced by suppression of only AgCad1 and not AgCad2 transcript levels (Fig. 3.1), yet the off-target 35% reduction of AgCad1 by AgCad2 DsiRNAs (Fig. 3.1) suggests that the strategy was not optimal. It is possible that the 65% identity match between AgCad2-DsiRNA-b and a region in AgCad1

(shown in Table 3.3) triggered an interference response to AgCad1 transcripts. Alternatively, there might have been an indirect effect where silencing AgCad2 by 80% negatively affected AgCad1 transcript levels. Recently, silencing of cadherin, ALP, or APN in *M. sexta* was shown to affect transcript levels of each other by an as-yet unknown mechanism (Gomez et al., 2014). However in our study, when larvae were treated with AgCad1- and AgCad2-DsiRNA-nanoparticles, the off-target effects did not extend to the other putative Cry11Ba receptors, AgALP1, AgAPN2 and Agm3 (Fig. 3.2).

Although knockdown of AgCads in larvae increased tolerance to Cry11Ba, it is notable that decreasing AgCad2 transcripts by 80% and AgCad1 transcripts by 35% in the same groups of larvae did not confer more tolerance to Cry11Ba than with larvae fed the AgCad1-DsiRNAs alone (Fig. 3.4). This seeming conflict contrasts with studies where knock-down of a single cadherin caused a major decrease in Cry toxicity. In the coleopteran mealworm *Alphitobius diaperinus*, when cadherin AdCad1 was silenced by 80%-90%, larval susceptibility to Cry3Bb was reduced by 90% (Hua et al., 2014). In *Ae. aegypti*, cadherin silenced *Ae. aegypti* larvae showed 40-fold more tolerance to Cry11Aa (Lee et al., 2014). Why didn't an 80% decrease in AgCad2 transcript levels confer a significant level of Cry11Ba tolerance to larvae? One explanation is that the amount of AgCad2 localized to 4th instar posterior midgut of larvae, is naturally limiting conferring a minimal role in Cry11Ba toxicity. This 'limiting amount' hypothesis is suggested by the 36-fold ratio of AgCad1; AgCad2 (Table 3.2).

Cry11Ba is remarkable among the Cry toxins for its high affinity *in vitro* binding to AgAPN2, AgALP1, AgAmy1 and Agm3. So how can AgCad1 be involved in Cry11Ba toxicity *in vivo*, when its binding peptide (AgCad CR14-MPED) binds with a low dissociation constant ($K_d = 760$ nM)? This issue was raised previously when we observed that AgCad1-CR11-MPED

peptide competed Cry11Ba from binding to BBMV prepared from *An. gambiae* larvae, but could not significantly neutralize Cry11Ba toxicity (Hua et al., 2013). A variation of the ‘sequential binding model’ (Pardo-López et al., 2013) of Cry toxin action may apply to Cry11Ba action in *An. gambiae*. The ‘sequential binding’ model involves initial reversible Cry binding to APN and ALP followed by high affinity binding to cadherin. Cry binding at the cadherin CR site closest to the brush border membrane is proposed to induce cleavage of the N-terminal α -helix of Cry toxin resulting in oligomer formation and a pre-pore structure that has high affinity for APN and ALP (Pacheco et al., 2009). Similar to CR peptides that induce oligomer formation and synergy of Cry toxins (Lee et al., 2014), we reported a synergistic effect of an AgAPN2 peptide on Cry11Ba toxicity to larvae (Zhang et al., 2010a). In *An. gambiae*, Cry11Ba binding to AgAPN2 may alter Cry11Ba conformation resulting in an increase in binding affinity to AgCad1; this hypothetical action is suggested by the ‘ping-pong’ action of Cry1A toxins in *M. sexta* (Pacheco et al., 2009).

In sum, our results support the conclusion that AgCad1 and possibly AgCad2 are involved in Cry11Ba toxicity to *An. gambiae* larvae. Further experimentation is needed to deduce if AgCad2 is involved in Cry11Ba action. However, the results presented in this study do not support a significant role for AgCad2 in Cry11Ba’s action.

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Figure Legends

Fig. 3.1. Diagram of DsiRNA locations for AgCad1 and AgCad2 genes and their transcript levels after RNAi treatments in *An. gambiae* larvae. Panel A: Diagrams of AgCad1 and AgCad2 showing the regions of each cadherin synthesized for DsiRNAi experiments. Arrows indicate primer locations used to detect the transcript levels of the target genes by RT-qPCR. Relative transcript levels of AgCad1 and AgCad2 after larvae were fed with AgCad1-DsiRNAs or AgCad2-DsiRNAs to that in food control are shown in Panel B and C respectively. Relative transcript levels of the target genes were obtained by normalization to two endogenous reference genes. Data were presented as means \pm SEM of four replications (n = 4).

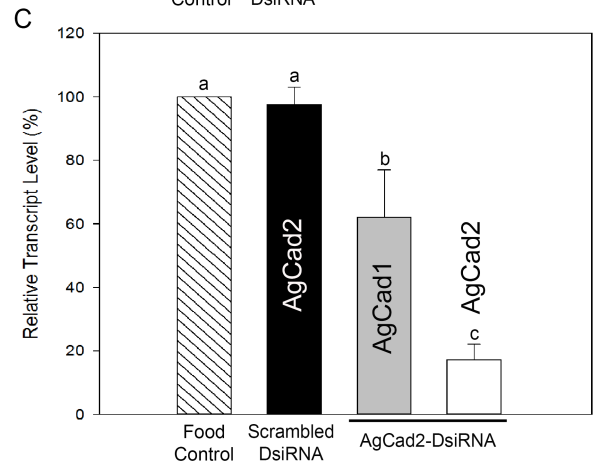
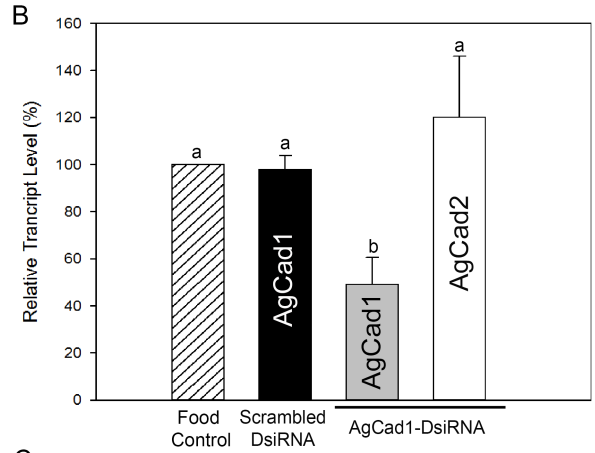
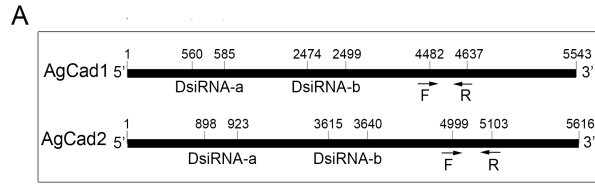


Fig. 3.2. Relative transcript levels of the non-target genes, Agm3, AgAPN2, and AgALP1 transcript levels in larvae that were fed with cadherin DsiRNAs and scrambled control. Transcript levels of these genes showed no significant change ($P \geq 0.05$) compared to food control. Data were presented as means \pm SEM of three replications ($n = 3$).

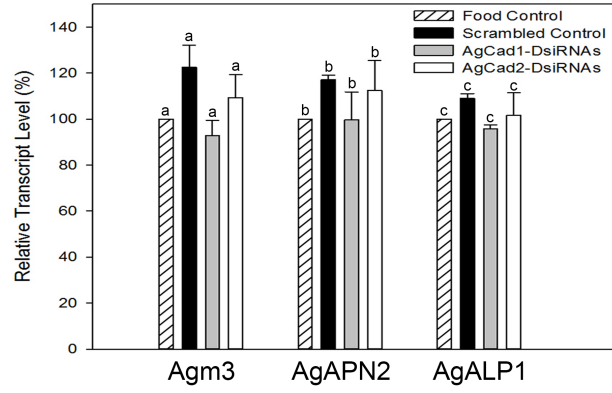


Fig. 3.3. Transcript levels of AgCad1 and AgCad2 across the DsiRNA feeding period from 3rd day to 6th day larvae. *An. gambiae* larvae were collected on 3rd, 4th, 5th and 6th days for total RNA extraction. Relative transcript levels of AgCad1 and AgCad2 from 4th, 5th and 6th days were compared to that of 3rd day larvae. Results showed that AgCad1 and AgCad2 transcripts have no significant difference ($P \geq 0.05$) from Day 3 to Day 6. Data were presented as means \pm SEM of three replications (n = 3).

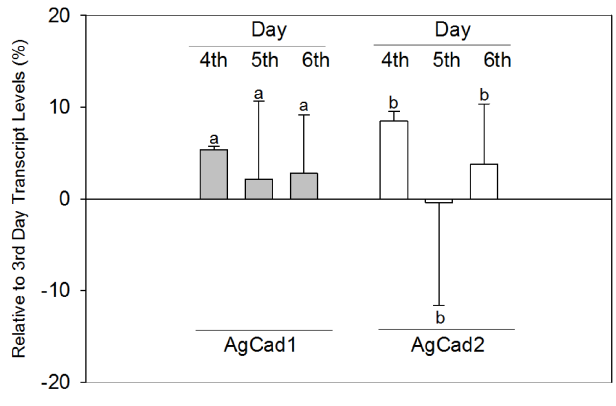


Fig. 3.4. Cry11Ba toxicity to *An. gambiae* larvae after feeding with cadherin DsiRNAs. AgCad1 and AgCad2 silenced larvae were more tolerant to Cry11Ba. *An. gambiae* larvae fed with AgCad1-DsiRNAs, AgCad2-DsiRNAs, food control without DsiRNA or scrambled sequences to AgCad2 (DsiRNAs) were exposed to Cry11Ba protoxin at LC₆₀ dosage. Mortality was recorded after 24 hr. Histograms show the average of three independent replicates with standard errors.

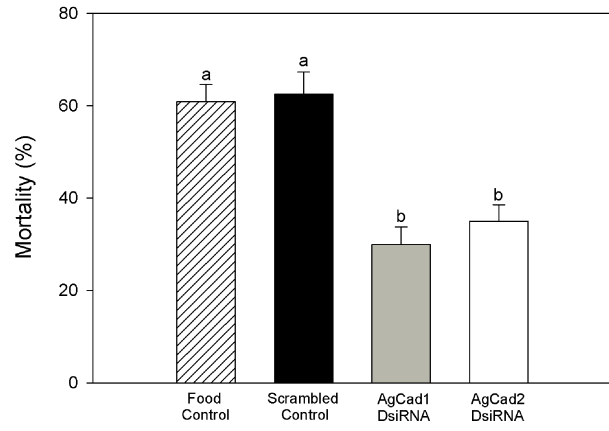


Table 3.1. DsiRNAs synthesized from IDT Company used for RNAi

AgCad1-DsiRNA-a sense:	CUACGAGGUGCAUCAGCGUAGCAGT
AgCad1-DsiRNA-a antisense:	CUGAUGCUCACGUAGUCGCAUCGUCA
AgCad1-DsiRNA-b sense:	CGAUAAUGCUCUUACUUUGACAAC
AgCad1-DsiRNA-b antisense:	UUGCUAUUACGAGGAAUGAAACUCUUG

AgCad2-DsiRNA-a sense:	CGAGGAUGAUCUAUUCUCUUUCAAC
AgCad2-DsiRNA-a antisense:	ACGCUCCUACUAGAUAAAGAGAAAGUUG
AgCad2-DsiRNA-b sense:	GGUCAACUGAGCUUCGACAUUGUGG
AgCad2-DsiRNA-b antisense:	UGCCAGUUGACUCGAAGCUGUAACACC

Scrambled Control DsiRNA sense:	GCUCAUAUUCUAAGAUUGACCUCUG
Scrambled Control DsiRNA antisense:	CGAGUAUAAGAUUCUAACUGGAGAC

Table 3.2. Relative transcript levels of AgCad1 to AgCad2 in Head, Gut and Whole Body of 4th instar *An. gambiae* larvae.

Body parts	EXP I			EXP II		
	Cq±SE		Relative Transcript level of AgCad1/AgCad2	Cq±SE		Relative Transcript level of AgCad1/AgCad2
	AgCad1	AgCad2		AgCad1	AgCad2	
Head	26.63±0.35	26.50±0.33	0.91	27.07±0.19	27.18±0.11	1.07
Gut	23.36±0.14	28.56±0.19	36.75	23.73±0.12	28.85±0.18	34.65
Whole body	26.74±0.15	29.09±0.31	5.11	26.29±0.25	28.62±0.39	5.02

The quantification cycle (Cq) values for AgCad1 and AgCad2 with standard errors obtained by RT-qPCR were shown in the table. The transcript level (ΔCq) for AgCad1 to AgCad2 was calculated with the formula described in section 2.5.

Name	AgCads-dsRNAs and non-target cadherins	Identity (%)
gAgCad1 AgCad2-DsiRNA-a	CGAGAACGCTATATTTAACTTCATC 949 CGAGGATGATCTATTCTCTTTCAAC 923 * * * * * * * * * * * * * * * *	64%
gAgCad1 AgCad2-DsiRNA-b	CATCGATGATGATGGTGGCATATA 3677 GGTCAACTGAGCT - - TCGACATTG 3637 * * * * * * * * * *	36%
gAgCad2 AgCad1-DsiRNA-a	AGCGGAGGACAGTCCGCCGGGGAT 560 CT ACGAGGTGCATCAGCGTAGCAG 584 * * * * * * * * * *	43%
gAgCad2 AgCad1-DsiRNA-b	CGATGCG--CTGGGCTCGGGGTCA 2460 CGATAATGCTCCT TACTTT GACA 2497 * * * * * * * * * *	43%

Table 3.3. Alignments and identities of the regions designed for DsiRNAs of AgCad1 and AgCad2 to DNA sequences of the non-target cadherins. Sequences are labeled with names as follows: gAgCad1 (genome sequence for AgCad1), gAgCad2 (genome sequence for AgCad2), AgCad1-DsRNA-a, AgCad1-DsRNA-b, AgCad2-DsRNA-a, and AgCad2-DsRNA-b. An ‘*’ (asterisk) represents an identical DNA base was highlighted in gray. The dissimilar DNA bases or gaps in the sequences are shown without highlights. The multiple sequence alignment was performed using ClustalW at <http://www.genome.jp/tools/clustalw/>

Table 3.4. RT-qPCR information

Experimental design	
Definition of experimental and control groups	Experimental Group: <i>A. gambiae</i> larvae fed with AgCads-DsiRNAs. Food Control Group: <i>A. gambiae</i> larvae fed with food only. Scramble Control Group: <i>A. gambiae</i> larvae fed with scrambled DsiRNA sequences to AgCad2. Larvae of different ages. Head, gut, and whole bodies of 4 th instar larvae.
Number per sample	n=8 (n=20 for head)
Sample	
Description	For each experiment 3 RNA extractions were performed with 2 independent experimental groups. Real time PCR was performed using three replicates for each cDNA.
Nucleic acid extraction	
Procedure	RNA was extracted with TRIzol reagent (Ambion).
Quantification	NanoDrop 1000 Spectrophotometer
Purity	260/280 analysis (value 2-2.04)
Reverse transcription	
Procedure/kit	SuperScript III First-Strand Synthesis System (Invitrogen), Oligo-dT primer.
Amount of RNA	1.5µg of total RNA
Reaction volume	20µl
Temperature and time	65°C for 5min; 55°C for 50min; 85°C for 5min
RT-qPCR target information	
Sequence accession number	AgCad1 (AGAP002828); AgCad2 (AGAP001591); RPS3 (AGAP001910); RPS7 (AGAP010592); Agm3 (AGAP008961); AgAPN2 (AGAP013188); AgALP1 (AGAP004578)

RT-qPCR oligonucleotides			
	Primers (5'-3')	Amplicon length	E (%)
Primer sequences	AgCad1/F: CCGAACCAGAACGGGTACTA AgCad1/R: TCCGGTTGATCAATCTCCTC	155bp	100
	AgCad2/F: GGACCAAGAGCGACATGTTT AgCad2/R: AAGCTTTTCCACGTGCGTAT	104bp	100.1
Amplicon length	AgRPS3/F: GCTGGGCATCAAGGTCAAG AgRPS3/R: ATCTCATCCTTCGGCTCAAC	108bp	100
Primer efficiency	AgRPS7/F: GTGAGGTCGAGTTCAACAACAAGAA AgRPS7/R: GGCACCGGCACGTAGATGA	52bp	100.6
	Agm3/F: GCAGTGGGACAACCTCCATTT Agm3/R: CGGAAGTGCTTGAGCGTACT	132bp	100.1
	AgAPN2/F: GGAATCGTGACGAGATCCAT AgAPN2/R: CCAACATCAACAGTCCAACG	102bp	100
	AgALP1/F: GAGACGATGTGGCCGTATTT AgALP1/R: CCGATTGGTGGGAATGTATCC	163bp	99.1
RT-qPCR protocol			
Complete reaction conditions	Bio-Rad's MyiQ Real-Time PCR Detection System		
Reaction volume/amount of cDNA/primers/polymerase	Reaction volume: 20µl Primer: 2.5µM cDNA: 5 µl of 1/100 diluted cDNAs Polymerase: iQ SYBR® Green Supermix (Bio-rad)		
Complete thermo cycling parameters	95°C for 2min followed by 39 cycles of 95°C for 10s and 57°C for 30s		
RT-qPCR instrument	CFX Connect™ Real Time PCR Detection System		
Data analysis			
Statistical methods for results significance	t-test from Sigma Plot Software (Version 11; systat Software Inc., San Jose, CA)		
Analysis of expression stability of endogenous reference genes	RPS3 (Zhang et al., 2010) and RPS7 (Togawa et al., 2008) were validated as stable reference genes within a larval stage. While both are from the same family and have similar functions, we were unable to identify other suitable reference genes to normalize our data due to potential effects in response to Bt treatment.		

CHAPTER 4

GENERAL DISCUSSION AND CONCLUSION

Proteins on brush border membranes of the midgut in a susceptible insect larva are selectively bound by Cry toxin allowing toxin to insert into the membrane and initiate events that kill the insect. A midgut protein is considered a Cry receptor when binding has functional significance, which is typically defined as leading to death of the insect. Cry toxins bind to receptor proteins with high affinity ($K_d = 2 \text{ nM}$ to 20 nM) or lower affinity ($K_d = 100 \text{ nM}$ or less). The lepidopteran-specific Cry1Ab toxin binds aminopeptidase with low affinity as a monomer, but with high affinity when oligomerized into a pre-pore structure (Pacheco et al., 2009). Aminopeptidases, alkaline phosphatases, and cadherins are established as Cry receptors in Lepidoptera, Coleoptera and Diptera (Adang et al., 2014).

Alkaline phosphatases and aminopeptidases are receptors of mosquitocidal Cry toxins in *Aedes aegypti* and *Anopheles gambiae*. These abundant brush border enzymes are receptors of Cry4Ba and Cry11Aa in *A. aegypti* larvae (Fernandez et al., 2006; Jimenez et al., 2012; Likitvivatanavong et al., 2011). Aminopeptidase was first reported to bind Cry11Ba tightly in *A. quadrimaculatus* (Abdullah et al., 2006), and then a related *An. gambiae* aminopeptidase was identified as a receptor of Cry11Ba (Zhang et al., 2008). The identification of alkaline phosphatase as a receptor of Cry11Ba in *An. gambiae* larvae was consistent with the enzyme's accepted role in Cry toxin action (Hua et al., 2009).

Chapter 2 reports my identification of glycosylphosphatidylinositol-anchored α -amylase,

AgAmy1, and α -glucosidase, Agm3, as putative receptors of Cry11Ba in *An. gambiae*. Previously, an α -amylase was identified as a possible receptor of Cry11Aa and Cry4Ba in midgut of *A. albimanus* (Fernandez-Luna et al., 2010). The α -glucosidase Agm3 I describe in Chapter 2 is the receptor of Bin toxin from *L. sphaericus* in *An. gambiae* larvae (Opota et al., 2008). cDNAs encoding AgAmy1 and Agm3 were cloned from larval midgut and expressed as full-length 68.8-kDa and 67.1-kDa proteins, respectively, and as truncated peptides in *E. coli*. The truncated peptides were used to raise antisera in rabbits, which, after being cleared of cross-reactive antibodies, were used to detect α -amylase and α -glucosidase in *An. gambiae* BBMVs (Figure 2.2) and in larval gut. The AgAmy1 protein was localized on salivary gland and posterior midgut, and Agm3 on posterior midgut (Figure 2.4). Cry11Ba bound *E. coli*-produced Agm3 or AgAmy1 with high affinity ($K_d = 20\text{-}40$ nM). AgAmy1, Agm3 and alkaline phosphatase, AgALP1, share a binding site on Cry11Ba. With AgALP1 and AgAPN2 sharing the same binding site(s) on Cry11Ba (Hua et al., 2009), it appears that Cry11Ba can bind any one of four GPI-anchored proteins (AgALP1, AgAPN2, AgAmy1 and Agm3) to its receptor binding epitopes. The receptor binding epitopes of Cry11Ba are localized on loop $\alpha 8$ and loops 1 and 3 in domain II of Cry11Ba (Likitvivatanavong et al., 2009). It is not known which Cry11Ba loop epitope actually binds to a GPI-anchored protein.

The possible roles of AgAmy1 and Agm3 in Cry11Ba toxicity to larvae were assessed by feeding larvae with Cry11Ba protoxin mixed with AgAmy1, Agm3, AgALP1, or AgAPN2b peptide and then recording larval mortality. The ability of AgAmy1, Agm3 and AgALP1 to reduce Cry11Ba toxicity is evidence that the shared binding site is critical to Cry11Ba larval toxicity (Figure 2.7). The bioassay results also support α -amylase Amy1 and α -glucosidase Agm3 as binding receptors for Cry11Ba in *An. gambiae* larvae. My results suggest a model

where the uniquely high potency of Cry11Ba against *Aedes*, *Anopheles* and *Culex* species is due to the number, and possibly diversity, of GPI-anchored proteins that function as receptors in the midgut of larvae.

Cadherin proteins are critical receptors of Cry toxins in susceptible insect larvae [see (Adang et al., 2014) for a recent review and Chapter 1 of this dissertation]. Cadherin can trigger formation of a pre-pore Cry toxin oligomer (Bravo et al., 2007); the oligomer is then thought to bind a GPI-anchored protein with high affinity and insert into midgut membrane (Pigott and Ellar, 2007). In *An. gambiae*, two cadherin proteins were identified in 4th instar larvae, which share 14% amino acid identity. Cadherin AgCad2 bound Cry11Ba with high affinity ($K_d=12$ nM) in *An. gambiae* larvae, while AgCad1 showed a substantially lower binding affinity ($K_d=766$ nM) with Cry11Ba (Hua et al., 2013). Accordingly, AgCad2 but not AgCad1 was considered a likely receptor of Cry11Ba in *An. gambiae* larvae (Hua et al., 2013).

In Chapter 3 of my dissertation, I describe my use of chitosan/DsiRNA nanoparticles to investigate the *in vivo* involvement of AgCad1 and AgCad2 cadherins in Cry11Ba toxicity to *An. gambiae* larvae. This is the first report of chitosan/DsiRNA-mediated RNAi as a tool for studying Cry toxin mode of action.

Due to the high affinity interaction between AgCad2 and Cry11Ba, I hypothesized that AgCad2, but not AgCad1, is an *in vivo* receptor of Cry11Ba. After larvae were fed with AgCad1-DsiRNAs, the AgCad1 level was reduced by 49% relative to control larvae (Fig 3.1B) and larvae were more tolerant to Cry11Ba (Figure 3.3). While the transcript level of AgCad2 was reduced by 80% in larvae fed chitosan/AgCad2 DsiRNAs there was co-suppression given to AgCad1. Although larvae fed with chitosan/AgCad2 DsiRNAs were more tolerant to Cry11Ba,

presumably due to the co-suppression of AgCad1 by AgCad2 DsiRNAs, any involvement of AgCad2 in Cry11Ba toxicity might be obscured by a lower level of AgCad1 in larvae.

Results showing reduction of AgCad2 transcript without an apparent change in larval susceptibility to Cry11Ba were unexpected as AgCad2 binds Cry11Ba with high affinity. To address this seeming conflict, I measured the relative transcript levels of AgCad1 and AgCad2 in the 4th instar larval gut, and calculated an AgCad2 to AgCad1 ratio of 1:36 ratio (Table 3.2). One explanation is that although AgCad2 binds Cry11Ba with 12 nM affinity (Hua et al., 2013), the small amount of AgCad2 in membrane relative to other Cry11Ba-binding proteins is not sufficient to mediate Cry11Ba toxicity *in vivo*. Interestingly, *Aedes* larvae also have a cadherin called AeCad that binds Cry11Ba with high affinity and was considered a receptor of Cry11Ba (Likitvivanavong et al., 2011). However, while silencing AeCad in transgenic mosquitoes greatly reduced Cry11Aa toxicity to larvae, those larvae exhibited no increased tolerance to Cry11Ba toxicity (Lee et al., 2014). Considering the parallels between Cry11Ba action in *Aedes* and *Anopheles* species with respect to identified receptors and larval toxicity, it is quite possible that AgCad2 is not a receptor of Cry11Ba toxicity in *Anopheles*.

The identification of AgCad1 as a receptor of Cry11Ba in larvae was not predicted due to the low affinity, $K_d = 760$ nM, for Cry11Ba to AgCad1 peptides (Hua et al., 2013). One possibility is that AgCad1 has a high-affinity binding site, but that site is not in the CR11-MPED region tested for Cry11Ba binding (Hua et al., 2013). Another possibility is suggested by the ‘sequential binding’ model (Pardo-Lopez et al., 2013). This model involves toxin reversible binding to cadherin receptor that triggers an additional protease cleavage of α -helix1 in domain I, leading to an assembly of a pre-pore oligomer that binds with high affinity to aminopeptidase or alkaline phosphatase. Although speculative, it is possible that the roles for Cry11Ba receptor

binding are reversed from those of Cry1A toxins (Pardo-Lopez et al., 2013). If Cry11Ba binding to a receptor protein such as AgAPN2 induces cleavage and oligomer formation then the oligomer might have increased binding affinity to AgCad1. The synergistic properties of peptide AgAPN2tb that increase Cry11Ba toxicity to larvae (Zhang et al., 2010) are similar to the synergistic properties of toxin-binding cadherin fragments which induce toxin oligomerization (Lee et al., 2014). In *An. gambiae*, Cry11Ba may bind to APN first to cause toxin conformational changes followed by binding to AgCad1, in support of the hypothetical ‘ping-pong’ model proposed for Cry1A action in *M. sexta* (Pacheco et al., 2009).

In conclusion, results from my dissertation research firstly demonstrated the role of two GPI-anchored proteins, α -amylase and α -glucosidase, as binding receptors of Cry11Ba in *An. gambiae* larvae. From the second part of my project on AgCads, I concluded the functional role of cadherin AgCad1 as a receptor of Cry11Ba toxin *in vivo*. The role of AgCad2 for Cry11Ba toxicity in *An. gambiae* larvae could not be ascertained based on our results. This research provides greater knowledge about key receptors involved in Cry11Ba action in mosquito larvae. It also illustrates the complicated role that cadherins have in Cry toxin action.

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