MODE OF ACTION ANALYSES OF CRY1 TOXINS FROM *BACILLUS THURINGIENSIS* IN SUSCEPTIBLE AND RESISTANT *HELIOTHIS VIRESCENS*.

by

JUAN LUIS JURAT-FUENTES

(Under the Direction of MICHAEL J. ADANG)

ABSTRACT

Development of resistance is one of the concerns in the use of *Bacillus thuringiensis* Cry proteins as insecticides. After ingestion, Cry toxins are processed in the insect midgut to activated toxins that bind to receptors in the midgut cells and form pores that cause cell death by osmotic lysis. Alterations in any of these steps can lead to resistance.

In this dissertation, I expanded a toxin-binding model to study the mechanisms of resistance in three Cry1Ac laboratory-selected strains of *Heliothis virescens* (tobacco budworm). In toxin binding assays Cry1A, Cry1Fa and Cry1Ja toxins shared a population of binding sites (receptor A) in midgut brush border membrane vesicles (BBMV) from susceptible *H. virescens*. Two populations of receptors were only recognized by Cry1Ab and Cry1Ac (receptor B), or Cry1Ac (receptor C). According to ligand blotting, aminopeptidase-N proteins of 170- and 110-kDa constituted receptor A, a 130-kDa protein constituted receptor B, while different proteins of less than 100-kDa in size constituted receptor C. After continuous selection with Cry1Ac, the YHD2 strain showed increased resistance to Cry1Ac when compared to previous reports. Using toxin-

binding experiments and ligand blots, I demonstrated that increased resistance correlated with reduced toxin binding that was not due to absence of toxin binding molecules in YHD2 BBMV. Lectin blots with soybean agglutinin (SBA) demonstrated that resistance correlated with altered glycosylation of YHD2 BBMV proteins.

In contrast to the YHD2 strain, CXC and KCBhyb Cry1Ac-resistant strains developed cross-resistance to Cry2Aa. These strains were also cross-resistant to Cry1Aa and Cry1Ab toxins. Toxin binding competition experiments demonstrated that only binding of Cry1Aa was reduced in both strains. Altered Cry1Aa but not Cry1Ab or Cry1Ac toxin binding had been previously reported for the YHD2 strain, suggesting that a similar resistance mechanism is occurring. Since Cry1Ac and Cry2Aa did not share binding proteins, resistance to Cry2Aa in CXC and KCBhyb is due to a different mechanism.

I conclude that at least two mechanisms of resistance are present in each of the resistant strains studied. In the case of the CXC and KCBhyb strains different resistance mechanism have evolved to confer resistance to very different Cry toxins.

INDEX WORDS: *Bacillus thuringiensis*, Cry1Ac, Cry2Aa, resistance, *Heliothis virescens*, N-acetylgalactosamine, altered glycosylation, reduced binding, cross-resistance, N-aminopeptidase.

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DEDICATION

This work is dedicated to the persons that encouraged and supported me through the completion of this degree. There are no words enough to thank my parents Jose Luis Jurat Perez and Luisa Fuentes Anaya, who encouraged and supported me in all possible ways through my academic career. A special dedication to Sodeya, whose love, support and inspiration greatly contributed to finishing this work. I would also like to dedicate this work to all my relatives.

To all of you, this is also your work. We always regret the things we do not do.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Since the beginning of agriculture, human populations have strived to improve crop yields. This has led to an increased selection pressure on the cultivars and on the animals that naturally feed on these plants. Increased selection pressure, as well as the reproductive characteristics of insects has resulted in development of resistance to methods used to control these organisms. Insects have developed resistance against most chemical insecticides, prompting the search for effective and more environmentally safe control alternatives. The insecticides based on *Bacillus thuringiensis* represent one of the most important and safe alternatives to chemical pesticides.

1. ***Bacillus thuringiensis***

Bacillus thuringiensis is a Gram-positive, spore-forming bacterium that produces an array of virulence factors during sporulation, including phospholipases, exotoxins, vegetative insecticidal proteins (VIPs), cytolytic toxins (Cyt toxins) and parasporal crystal proteins (Cry proteins) (Tanada and Kaya, 1993). Most of these factors are active against insects, which is evidence for the hypothetical ecological niche for *B. thuringiensis* as an entomopathogen.

B. thuringiensis was first isolated by Ishiwata in 1901 from diseased *Bombyx mori* (silkworm) larvae (Ishiwata, 1901). Berliner (1915) coined the name *Bacillus thuringiensis* for a bacterium isolated from *Anagasta kuehniella* (Mediterranean flour moth) larvae from the German region of Thuringia. Although production of a parasporal body was observed, it was not until five decades later that Angus associated insecticidal activity with presence of the parasporal crystal inclusions (Angus, 1956). These

parasporal crystals are composed of a combination of one or more proteins called Cry proteins (for Crystal proteins) or δ-endotoxins.

Production of these parasporal inclusions is the main factor that differentiates *B. thuringiensis* from closely related species like *Bacillus cereus* (Schnepf *et al.*, 1998). In fact, *B. thuringiensis* mutant strains that do not produce crystals cannot be distinguished from *B. cereus* (Tanada and Kaya, 1993). Both *B. cereus* and *B. thuringiensis* are capable of inducing septicemia in the insect hemocoel, and they are so closely-related that both species are included as members of *B. cereus* sensu lato (Schnepf *et al*., 1998).

Production of the parasporal Cry proteins confers on *B. thuringiensis* an evolutionary advantage over other entomopathogens by promoting insect midgut colonization. Thus, Cry toxin action results in midgut disruption and establishment of a favorable environment for *B. thuringiensis* spore germination. Although other virulence factors produced by the bacteria contribute to this process, the parasporal proteins are considered to be the prominent insecticidal factors in this process (Schnepf *et al*., 1998). Cry toxins or δ-endotoxins are the most widely studied of all entomocidal toxins (Tanada and Kaya, 1993).

Because of their high potency and specificity, Cry toxins have been used in insecticide formulations for insect pest control. Insecticides based on *B. thuringiensis* toxins are the most widely used biologically produced pest control agent (Betz *et al*., 2000). To improve Cry toxin stability in the field, *cry* genes were introduced in plants to produce transgenic cultivars that synthesized their own insecticidal toxins (Adang *et al*., 1993; Perlak *et al*., 1991)

2. ***cry*** **genes and transgenic Bt crops.**

Most *cry* genes encoding for Cry toxins are located in large plasmids (Gonzalez *et al*. 1981), although they have also been detected in chromosomal DNA (Carlson and Kolstø, 1993). These genes are expressed at high levels during the sporulation phase and their products accumulate in crystal inclusions (Schnepf *et al*., 1998). A variety of transcriptional, posttranscriptional and posttranslational mechanisms regulate this expression (Agaisse and Lereclus, 1995) to produce large amounts of Cry proteins that are stored in a crystalline inclusion by the spore.

Selected *cry* genes have been cloned and expressed in other organisms, such as other bacteria and plants, to improve efficacy of Cry toxins in the field. For example, *cry* genes were introduced in the bacteria *Pseudomonas fluorescens* to produce concentrated biopesticide formulations of encapsulated Cry toxins with improved persistence in the field (Schnepf *et al*., 1998). When introduced in plants, the A-T rich *Bacillus* DNA generated potentially deleterious sequences in the host plant cells, limiting the levels of Cry toxin production in plants. Examples of such sequences include polyadenylation sites and AU-rich repeats that functions in RNA processing and turnover, respectively in eucaryotic cells. Expression of *cry* genes in plants was greatly improved when the original *Bacillus* DNA sequence was modified to increase the usage of preferred plant codons and eliminate A-T rich sequences (Adang *et al*., 1993). Tobacco expressing Cry toxin was the first transgenic Bt plant produced (Barton *et al*., 1987). Since then, other major crops like potato, maize, cotton and rice have been genetically engineered (Betz *et al*., 2000; De Maagd *et al*., 1999). All of these transgenic crops express different Cry toxins through nucleus-directed expression, although high levels of *cry1Ac* and *cry2Aa*

gene expression have also been obtained through chloroplast-directed expression in tobacco leaves (McBride *et al*., 1995; Kota *et al*., 1999).

In 1995-1996 transgenic Bt potato, cotton and corn were commercialized (Reviewed in De Maagd *et al*., 1999), and other Bt transgenic crops are now available (Betz *et al*., 2000). The main advantages of these transgenic crops over conventional cultivars include: reduction in chemical applications, the absence of harmful effects against beneficial fauna, continuous delivery of toxin in plant tissues, and absence of chemical pollutants in the ecosystem (Betz *et al*., 2000). In this regard, beneficial insects not only are unaffected but sometimes favored by the use of these transgenic cultivars (Poppy, 2000; Schuler *et al*., 2001).

Fears of gene flow to wild plant relatives and development of insect resistance due to increased selection pressure have limited the wide acceptance of Bt transgenic plants as efficient and safe biocontrol methods (De Maagd *et al*., 1999).

3. Cry toxin nomenclature

Höfte and Whiteley (1989) were the first authors to attempt a systematic nomenclature for Cry toxins. For the primary ranking in this system, *cry* genes were assigned a Roman numeral according to the insecticidal activity of the respective crystal protein. Thus, *cryI* genes encoded crystal proteins that were toxic against lepidopterans; *cryII* genes encoded toxins active against both lepidopterans and dipterans; proteins from *cryIII* genes were toxic against coleopterans, and *cryIV* genes encoded proteins toxic only toxic to dipterans. Inconsistencies with this scheme of classification appeared as the activity range of different toxins was elucidated. For example CryIB belonging to the

cryI group was found to be toxic to both coleopteran and lepidopteran insects (Bradley *et al*., 1995).

To resolve these inconsistencies, Crickmore *et al*. (1998) proposed a revision of the nomenclature by assignment of names in relation to evolutionary divergence as determined from phylogenetic analysis of *cry* genes and amino acid sequence analysis of Cry toxins. For the Cry toxins, Roman numerals were substituted by Arabic numerals in the first rank and further ranks were assigned according to the percent amino acid identity of the specific toxin with other toxins in that rank. The boundaries of the first, second and third ranks were 45, 78 and 95% sequence identity respectively (Crickmore *et al.*, 1998). The first rank includes toxins with up to 45% sequence identity. The second rank, which groups toxins with 46% to 78% sequence identity is denoted by the first capital letter in the toxin nomenclature. The third rank groups toxins with identities between 78-96% and is denoted by the second letter in lower case. For example, the toxins used in this dissertation research are Cry1 toxins, most of which are in the Cry1A family. Sequences with more than 96% identity were considered as alleles of the same gene. This system has assigned unique names to 153 crystal proteins (Crickmore *et al*., 1998).

4. Cry toxin structure

The structure of four *B. thuringiensis* toxins was resolved through X-ray crystallography: Cry3Aa (Li *et al*., 1991), Cry1Aa (Grochulski *et al*., 1995), Cyt2A (Li *et al*., 1996) and Cry2Aa (Morse *et al*., 2001). From these studies, it was concluded that Cry and Cyt toxins have very distinctive features that are probably related to their

different mode of action (Li *et al*., 1996). Although Cry1Aa, Cry2Aa and Cry3A toxins are active against different insect orders (Lepidoptera for Cry1Aa , Lepidoptera and Diptera for Cry2Aa and Coleoptera for Cry3A) and their amino acid sequences are only about 27-36% identical (Crickmore *et al*., 1998), they show a remarkable similarity in their three dimensional structure (Grochulski *et al*., 1995; Morse *et al*., 2001). These toxins have three clearly delimited structural domains (Fig. 1.1). Domain I consists of a bundle of seven amphipathic antiparallel α-helices with helix 5 in the center of a ring-like structure formed by the other helices (Fig. 1.2). Domain II and III both consist of β-sheets in different conformations. Domain II consists of three antiparallel β-sheets in a Greek key topology, while domain III consists of two antiparallel β-sheets arranged in a jellyroll topology (Grochulski *et al*., 1995; Li *et al*., 1991; Morse *et al*., 2001).

After comparing the amino acid sequence of different Cry toxins, Höfte and Whiteley (1989) described five highly conserved blocks of amino acids among different Cry toxins. Based on these conserved blocks, three toxin groups were distinguished (Schnepf *et al*., 1998). Toxins in group 1 (Cry1, Cry3, Cry4, Cry7-10, Cry16, Cry17, Cry19, and Cry20) contained all five conserved blocks, while group 2 (Cry5, Cry12-14, and Cry21) and group 3 (Cry2, Cry11 and Cry18) possessed blocks 1, 2, 4, and 5 or blocks 1 and 2, respectively. These blocks were located at limiting regions between structural domains (blocks 2 and 3), in domain I (block 1 is located in helix 5), and in domain III. Since these regions are predicted to be involved in toxin pore-formation, contact between domains, or in hydrophobic stretches that likely contribute to toxin folding, they directly influence toxin tertiary structure and ultimately toxin mode of action (Schnepf *et al*., 1998). Conservation of these five blocks between Cry1 and Cry3

toxins would thus explain the identical three-domain structure observed in these toxins even though they share low sequence identity (Schnepf *et al*., 1998).

5. Structure-function association

Cry toxins domains show structural similarity with colicin from *Escherichia coli* and α-toxin from *Staphylococcus aureus* and some plant lectins, e.g. jacalin, KM+ lectin, and *Maclura pomifera* agglutinin (Burton *et al*., 1999; English and Slatin, 1992; Li *et al*. 1991; Parker and Pattus, 1993). The role of each Cry toxin domain in the mode of action has been studied based on functional analogy with these structurally similar proteins.

Isolated domains II-III bound to insect midgut membranes in a reversible fashion, suggesting that domain I is involved in irreversible toxin binding (insertion) in the membrane and domains II and III in binding specificity (Flores *et al*., 1997). In fact, the amphipathic helices of domain I are similar to the pore-forming domains of other bacterial toxins like colicin A (Li *et al*. 1991). Based on this similarity, Hodgman and Ellar (1990) proposed a "penknife" model for Cry toxin insertion, as described for colicin A (Lakey *et al*. 1992). According to this model, helices α5 and α6 of Cry toxin domain I would open in a penknife structure that inserts into the membrane. Since these two helices are the furthest away from the membrane after toxin binding to the receptor, they would have to "flip out" of domain I, leaving the rest of the alpha helices bound to the toxin receptor (Knowles, 1994). The predicted area of the hydrophobic faces of the amphipathic helices, as well as the high degree of conservation of the α5 and α6 amino acid sequences among Cry toxins, supported this model (Knowles, 1994).

Alternatively, Li *et al*. (1991) proposed an "umbrella" model for Cry toxin insertion, similar to the model proposed for the membrane-translocating domain of diphtheria toxin, and *Pseudomonas* exotoxin A (Parker and Pattus, 1993). According to this "umbrella " model, a central hydrophobic α-helical hairpin formed by α–helices 4 and 5 in the center of the alpha-helical bundle of domain I inserts in the membrane of the host cell, leaving the rest of the helices flatten out on the exterior of the membrane (Li *et al.* (1991). Restriction of intramolecular movement in this central region of domain I from Cry1Aa by disulfide bonds resulted in toxins that lost their ability to form ion channels in lipid bilayers when compared with native Cry1Aa toxin (Schwartz *et al*., 1997a). Studies on the interactions between the different α-helices from domain I with lipid bilayers, indicated that helices 4 and 5 insert into the membrane while the other helices lie on the membrane surface in an "umbrella-like" fashion (Gazit *et al*., 1998). Using Cry1Aa domain I mutants, Masson *et al*. (1999) demonstrated that helix 4 lines the lumen of the toxin-produced ion channel. In agreement with this result, mutagenesis of Cry1Ac helix 4 led to decreased membrane permeation and toxicity with unchanged toxin binding and aggregation, evincing a direct role of helix 4 in the properties of the channel (Kumar and Aronson, 1999). Synthetic helices 4 and 5 were extremely active in membrane permeation when compared to the rest of the helices in domain I, and the loop between them was necessary for efficient insertion (Gerber and Shai, 2000). Taken together, all these results support the "umbrella" model and emphasize the important role of the helix 4–helix 5 hairpin of domain I in Cry toxin pore formation.

Oligomerization of toxins in the membrane leads to formation of a channel with two rings of negatively charged amino acids lining the lumen (Vié *et al*., 2001).

Experiments using Atomic Force Microscopy (AFM) showed that Cry1 toxin channels in lipid mono- and bilayers are formed by oligomerization of four toxin molecules in a tetramer (Vié *et al*. 2001).

At the apices of the β-sheets of Domain II there are three loops that extend outwards from the toxin (Fig. 1.1). Li *et al*. (1991) proposed that these loops were involved in specificity of binding, based on their similarity to antigen-binding sites. In agreement with this hypothesis, these loops determined toxin binding specificity and cross-resistance in *P. xylostella* (Granero *et al*., 1996; Tabashnik *et al*., 1996). This prediction was also extensively studied by mutagenetic substitution of loop residues in Cry1Aa (Rajamohan *et al*., 1996b), Cry1Ab (Rajamohan *et al*., 1996a, 1996b), Cry1Ac (Smedley and Ellar, 1996), and Cry3A toxins (Wu and Dean, 1996). In these studies, changes in toxin binding after loop mutagenesis were directly related to changes in toxicity, suggesting that binding specificity determined by domain II is involved in toxin activity (Schnepf *et al*., 1998). Effects of domain II loops mutagenesis were insect and toxin specific, in other words, specific loops were important for binding to midgut cells from a specific insect (Dean *et al*., 1996). Chimeric-scanning mutagenesis suggested that the determinants of specificity in Cry2Aa are located in a different region of domain II when compared with Cry1 toxins (Morse *et al*., 2001).

Interestingly, domain II has the same folding pattern as certain carbohydrate binding proteins (Burton *et al*., 1999). More specifically, domain II has β-sheets arranged in Greek key motifs. This folding pattern is present in the plant lectins jacalin (Sankaranarayanan *et al*., 1996), *Maclura pomifera* agglutinin (Lee *et al*., 1996), and the KM+ lectin from *Artocarpus integrifolia* (Rosa *et al*., 1999). The vitelline membrane

outer layer protein I from hen's eggs, a carbohydrate modifying enzyme, also has this specific arrangement of Greek key structures (Shimizu *et al*., 1994). Most of these lectins bind to N-acetylgalactosamine (GalNAc) residues, which are considered to be a recognition epitope for Cry1Ac binding (Knowles and Ellar, 1986; Knowles and Ellar, 1991). The predicted carbohydrate binding properties of domain II have not been studied.

Domain III was hypothesized to be involved in toxin stability and protection against proteolytic toxin degradation (Li *et al*., 1991). Mutagenesis of one of the conserved amino acid blocks located in domain III of Cry1Aa led to mutants that bound to insect midguts as wild type Cry1Aa, but had reduced *in vivo* toxicity due to altered channel formation activity (Chen *et al*., 1993), indicating a role for domain III in channel formation. Amino acid mutagenesis in a conserved region in one of the β-sheets of domain III resulted in mutant toxins with reduced toxicity and ion channel formation in *B. mori* midguts and brush border membrane vesicles (BBMV), implicating that at least this region of domain III may be involved in pore formation (Chen *et al*., 1993, Wolfersberger *et al*., 1996). Exchange of domains II and III between closely related toxins resulted in active hybrids with altered specificity (Ge *et al*., 1989; Schnepf *et al*., 1990). In the case of *Spodoptera exigua* (beet armyworm) and *Manduca Sexta* (tobacco hornworm), toxicity followed domain III in these hybrid toxins, evincing that this domain may be responsible for effective receptor binding (De Maagd *et al*., 1996, 2000). In contrast, toxin binding in *Plutella xylostella* (diamondback moth) followed domain II and not domain III in hybrid toxins (Ballester *et al*., 1999). Binding of Cry1Ac to 120-kDa midgut toxin binding proteins from *Lymantria dispar* (gypsy moth) and *M. sexta* was also mediated by domain III (Lee *et al*., 1995; De Maagd *et al*., 1999). These results

suggested that different insects have different binding specificity determinants in Cry toxins. Burton *et al*. (1999) identified the GalNAc binding site for Cry1Ac in a structural pocket of domain III. Mutant Cry1Ac toxins with altered GalNAc binding pocket showed toxin binding not inhibited by GalNAc, and slightly decreased toxicity *in vivo* against *M. sexta* (Burton *et al*., 1999), *H. virescens* and *L. dispar* (Lee *et al*., 1999). These results were evidence that domain III lectin-like binding was not directly related to toxicity.

In summary, it has been proposed that domain I is involved in pore formation, domain II in binding specificity, and domain III in ion channel function and receptor binding, depending on the toxin and insect considered. Interactions between the three domains of Cry1 toxins were also shown to be important for toxin activity (Rang *et al*., 1999).

6. Mode of action of Cry1 toxins.

6.1 The midgut of lepidopteran larvae.

Based on microscopic observation of insect tissues, the midgut was determined to be the site of action of Bt Cry toxins (Heimpel and Angus, 1959). However, Cry1 toxins were also active in intrahemocoelic injections in larvae of *L. dispar* and the fleshfly *Neobellieria bullata* (Cerstiaens *et al*., 2001).

Lepidopteran larvae spend most of their time feeding, exponentially increasing their weight until reaching the pupal stage. This intensive digestive activity is reflected in the midgut being nearly half the weight of the larva (Dow, 1986). The gut lumen of most insects is surrounded by a peritrophic matrix, composed of chitin and glycoproteins,

that separates the gut contents from the epithelium, protecting the gut epithelial cells from abrasion (Lehane, 1997). This matrix is produced continuously by the whole midgut epithelium (type I peritrophic matrix). In larvae of most Lepidoptera, the 4 to 10 nm pores of this peritrophic matrix allow for digestive enzymes and small substances to pass through it (Spence and Kawata, 1993).

The lepidopteran gut is a single-layer epithelium resting on a basal membrane. Four main types of cells can be distinguished in this epithelium: goblet, columnar, endocrine and regenerative cells. Goblet cells are directly involved in creating and maintaining the electrochemical gradient of pH and ions across the midgut epithelium (Knowles, 1994). The columnar cells are responsible for most absorptive processes, while regenerative cells reside in specific nidi differentiate into either columnar or goblet cells resulting in epithelium renovation. The midgut columnar cells make most digestive enzymes. Many of these enzymes are secreted to the midgut lumen, and some remain bound to the epithelial cells (Terra and Ferreira, 1995). Herbivorous lepidopteran larval midgut juices are characterized by a high trypsin or chymotrypsin-like serine protease activity, which is optimal at pH 10-11 (Purcell *et al*., 1992).

Lepidopteran digestive systems are characterized by a high concentration of K^+ in the lumen, maintained by potent electrogenic K^+ transport from the hemocoel to the lumen side. The activities of a V-ATPase membrane proton pump and a H^+/K^+ exchanger located on the apical membrane of goblet cells produce a net K^+ flux to the gut lumen (Harvey *et al.*, 1983). This process generates an electrical gradient that drives most of the nutrient absorptive processes (Giordana *et al*., 1982). The lepidopteran midgut lumen has a characteristically alkaline pH, exceeding pH 12 in some cases (Dow,

1986). The pH gradient between the midgut lumen and the hemocoel is maintained by the enormous electrical potential difference across the midgut generated by the electrogenic K^+ transport (Dow and O'Donnell, 1990).

6.2 Pathophysiology of Cry1 toxins in the insect midgut.

The first experimental observations of the pathology of Bt toxins suggested that they disrupted cell permeability in a way similar to valinomycin (Angus, 1968). Microscopic observations of the midgut pathology of Cry toxins *in vivo* included increased vesiculation inside the damaged cells, deformation of mitochondria and endoplasmic reticulum, swelling and disintegration of the microvilli, cells sloughing from epithelium, and development of new epithelial cells from regenerative nidi (Kinsinger and McGaughey, 1979; Nishiitsutsuji-Uwo and Endo, 1981; Oron *et al.*, 1984). Similar effects were also observed in Bt toxins active against coleopteran and phthirapteran insects (Lacey and Federici, 1979; Hill and Pinnock, 1998). These midgut symptoms are also characteristic of fasting (DeLello *et al*., 1984), intoxication with tannins (Steinly and Berenbaum, 1985), and chemical stress (Felton and Dahlman, 1984), suggesting that the midgut response to Cry toxins is in part related to a depressed metabolism. Although initial pathogenesis is localized to the midgut, pathological effects of *B. thuringiensis* have also been observed after four days post-infection in the integument, Malpighian tubules, nerve ganglia, tracheoles and fat body cells, probably due to bacterial systemic infection (Salama and Sharaby, 1985).

The main physiological result of Bt toxicity is disruption of the pH and K^+ gradients through the midgut epithelium, resulting in increased pH and K^+ concentrations

in the hemolymph (Nishiitsutsuji-Uwo and Endo, 1981). Increased glucose intake and disappearance of glycogen granules has also been observed, reflecting an increase in metabolism possibly to maintain gut regulatory mechanisms (Nishiitsutsuji-Uwo and Endo, 1981).

Bt Cry1 toxins were toxic against some cultured lepidopteran cell lines, showing responses comparable to midgut epithelial cells *in vivo* (Hofmann and Lüthy, 1986). Cytotoxicity of the Cry1 toxins was dependent on the insect cell line and could be inhibited by specific carbohydrates (McCarthy, 1994). Bt toxins from the strain *israelensis* induced hemolysis of mammalian erythrocytes and cytopathological changes in other mammalian cells (Thomas and Ellar, 1983). Since cell cultures are maintained at conditions different from those prevailing in the insect midgut and since they lack midgut receptors, high (non-physiological) toxin concentrations needed to be used (Himeno *et al.*, 1985; Knowles and Ellar, 1986) limiting the application of the conclusions obtained from these experiments to *in vivo* conditions (Wolfersberger, 1995). Primary cells isolated directly from midgut tissue are another *in vitro* model for Bt toxins. Exposure of *H. virescens* cultured midgut cells to doses of Cry1Ac that killed some columnar and goblet cells, resulted in a 40-100% increase in stem and differentiating cell numbers (Loeb *et al*., 2001). The interpretation was that cells challenged with toxin, stimulated differentiation of stem cells replacing dead cells. This response is analogous to a wound response in vertebrates.

6.3 Ingestion, solubilization and activation.

For Cry1 toxins in lepidopteran larvae, the high alkaline conditions of the midgut lumen (Dow, 1986) favor the initial steps of the toxin mode of action. After ingestion by a susceptible lepidopteran larvae, Bt crystals are solubilized in the alkaline insect midgut to 130-140-kDa protoxin forms (Hoffmann *et al*., 1988). Both pH and crystal composition influence the degree of crystal solubility and thus, the activity of the crystal suspension against specific insects (Aronson, 1995; Aronson *et al*., 1991; Gringorten *et al*., 1992). The reducing midgut environment also plays a role in crystal dissolution *in vivo* (Tanada and Kaya, 1993). Under *in vitro* conditions, Bt crystals can be solubilized in alkaline solutions with reducing agents, such as β-mercaptoethanol.

Solubilized protoxins are activated by endogenous trypsin or chymotrypsin-like midgut proteases, sequentially removing portions from the C-terminus portion of the protoxin to yield a 55-65- kDa activated toxin core. This toxin core is resistant to further proteolysis (Choma *et al.*, 1990). In some cases, extracellular proteases produced by *B. thuringiensis* also have a role in toxin activation (Kumar and Venkateswerlu, 1998). Midgut proteases remove around 500 amino acids from the C-terminus and 28 amino acids from the N-terminus of the protoxin, yielding the core toxin fragment that represents most of the N-terminus of the protoxin. Lack of N-terminus activation yielded toxins with reduced activity (Bravo *et al*., 2002). Generally, although activation is necessary for toxin activity it does not determine specificity (Knowles and Ellar, 1986). Thus, different larval midgut extracts or commercial proteases can be used *in vitro* to activate a Cry toxin without affecting its specificity.

6.4 Binding to receptors.

Activated Cry toxins traverse the insect peritrophic matrix and interact with the brush border membrane of the midgut cells (Ryerse *et al*., 1990; Bravo *et al.*, 1992). *B. thuringiensis* synthesizes exochitinases at the time of sporulation that degrade the peritrophic matrix, increasing the amount of toxin presented to the midgut epithelium (Ding *et al.*, 1998; Sampson and Gooday, 1998). Toxins bind specifically to receptors on the apical brush border membrane of susceptible midgut cells both *in vitro* (Hofmann *et al*., 1988) and *in vivo* (Aranda *et al*., 1996).

To study the toxin-binding characteristics and identify the toxin receptor, a larval midgut brush border epithelium model was needed. Wolfersberger *et al.* (1987) first isolated vesicles composed of the apical brush border membrane of midgut epithelial cells, where Bt toxin mode of action takes place. These brush border membrane vesicles (BBMV) from lepidopteran midguts have provided a midgut brush border epithelium model to study the interaction of Bt toxins with midgut receptors *in vitro*.

Hofmann *et al*. (1988a) and Van Rie *et al*. (1989) studied toxin binding using ^{125}I-radiolabeled Cry toxins and insect BBMVs. Competition of toxin binding by unlabeled competitors indicated that Cry toxins bound in a specific, saturable, and reversible manner, suggesting the presence of specific toxin receptors in the BBMV (Hofmann *et al*., 1988a). Binding of radiolabeled Cry toxins to BBMV from *Pieris brassicae* (Hofmann *et al*., 1988a), *Heliothis virescens*, *Manduca sexta* and *Spodoptera littoralis* (Van Rie *et al*., 1989, 1990), *Lymantria dispar* (Kwak *et al*., 1995; Wolfersberger, 1990), *Plutella xylostella* (Ferré *et al*., 1991) and other insects (Escriche *et al*., 1997; Herrero *et al*., 2001; Karim and Dean, 2000; Lee *et al*., 1997) has been reported. Based on results

from toxin binding competition experiments, models for toxin binding to BBMV from these insects have been developed. These models are important to study the potential interactions between different Cry toxins in the insect midgut.

Although binding results demonstrated a direct correlation between toxin binding and activity *in vivo*, some toxins did not follow this relationship (Garczynski *et al*., 1991; Luo *et al*., 1999a; Van Rie *et al*., 1990a; Wolfersberger, 1990). This observation suggested that specific toxin binding to BBMV was not necessarily related to toxicity and that subsequent steps were required for activity *in vivo*. Toxin binding to BBMV has been shown to be a two-stage process where initial reversible toxin binding is followed by irreversible binding (Ihara *et al*., 1993; Liang *et al*., 1995). Irreversible binding was shown to be due to toxin insertion in the BBMV membrane and was directly related to toxin activity *in vivo* (Ihara *et al*., 1993).

6.5 Toxin insertion and pore formation.

The observation that Cry toxins formed pores in the plasma membrane of CF1 cells led to the hypothesis that they kill the midgut epithelial cells by colloid osmotic lysis (Knowles and Ellar, 1987). According to this hypothesis, formation of toxin pores would lead to a net inflow of ions and water from the midgut lumen in the midgut epithelial cells, resulting in cell swelling and eventual lysis. Purified Cry1 toxins disrupted the short circuit current in the insect midgut (Liebig *et al*., 1995; Peyronnet *et al*., 1997) and permeated phospholipid vesicles and planar lipid bilayers (PLBs), although the toxin concentrations needed were 1,000-fold higher than their *in vivo* insecticidal concentrations (Schwartz *et al*., 1997c; Slatin *et al*., 1990; Smedley *et al*., 1997). When

fused with PLBs, midgut brush border membrane material reduced the amount of Cry toxin needed for pore formation 100 to 1000-fold (Lorence *et al*., 1995; Peyronnet *et al*., 2001; Schwartz *et al*., 1997b).

Aggregation of several Cry toxin molecules seems to be necessary for the formation of functional pores on midgut vesicles (Aronson, *et al*., 1999; Gómez *et al*., 2002; Maddrell *et al*., 1988). These aggregates consist of a minimum of four toxin molecules per pore (Vié *et al*., 2001), and they are physically confined to the target membrane (Aronson, 2000). Specific asparagine residues of helix 4 are involved in toxin oligomerization (Tigue *et al*., 2001).

Inconsistencies on the characteristics of the pores formed by Cry1 toxins have been found when different *in vitro* systems were used to study them. Non-selective pores have been described in BBMV (Hendrickx *et al*., 1990; Carroll and Ellar, 1997), while anionic and cation-specific channels have been described in BBMV (Kirouac *et al*., 2002; Lorence *et al*., 1995), insect midgut cells (Sacchi *et al*., 1986; Schwartz *et al*., 1991) and PLBs (Schwartz *et al*., 1993). These results suggested that despite the presence of receptors pore formation can also be modulated by other factors, such as interaction between the toxin domains (Rang *et al*., 1999), pH (Butko *et al*., 1994; Schwartz *et al*., 1993), or lipid membrane composition (Vié *et al*., 2001). Consequently, different values for the size of the Cry1 toxin pore have been determined under different systems and conditions. Cry1 toxins pores of between 1-2.5 nm in diameter were described in *M. sexta* BBMV (Carroll and Ellar, 1997) and Sf9 cells (Villalon *et al*., 1998). On the other hand, Schwartz *et al*. (1997a) reported that Cry1Aa formed small channels of 0.6 nm in diameter in lipid bilayers.

Although the main toxicity mechanism of Cry1 toxins is generally accepted to be a type of osmotic lysis, effects on intracellular calcium concentration have been described as early steps in toxicity (Monette *et al*., 1997; Potvin *et al*., 1998; Schwartz *et al*., 1991). Extracellular [Ca^{2+}] stimulated Cry1C toxicity to Sf9 and UCR-SE-1a cells in a dose-dependent manner, and this effect was related to increased concentration of intracellular [Ca^{2+}] (Monette *et al*., 1997). These results suggest that rise in cellular [Ca^{2+}] related to toxin exposure represent an early step in the activity of the toxin. The rise in [Ca^{2+}] concentration may be a general response of susceptible insect cells to *B. thuringiensis* toxins (Potvin *et al*., 1997).

7. Bt receptors.

Receptors are defined as binding molecules that transduce binding (molecular recognition) to a response (Burt, 1986). In the Bt toxin literature this term has been incorrectly used to refer to membrane surface proteins or other molecules that bind Cry toxins. Cry toxin binding molecules can only be considered as functional toxin receptors when their expression in cell lines that are insensitive to Cry toxins renders these cell lines susceptible to the toxins.

Putative Cry toxin toxin-binding proteins were identified in several insects by probing blots of separated BBMV proteins with labeled toxins (Garczynski *et al*., 1991; Knowles *et al*., 1991; Lee *et al*., 1996; Martínez-Ramírez *et al*., 1994; Oddou *et al*., 1991; Jurat-Fuentes and Adang, 2001). This technique for detecting Cry toxin binding proteins is called ligand blotting. Results obtained by probing blotted BBMV proteins with labeled toxin, sometimes conflict with toxin binding site models developed from

membrane vesicle binding assays (Lee and Dean, 1996). These inconsistencies are thought to be due to the denaturing conditions employed in ligand blotting in contrast to the native conditions used in vesicle binding assays (Daniel *et al*., 2002).

Cry toxin binding proteins have also been described from solubilized BBMV proteins using affinity chromatography purification (Bagchi, 2000; Banks *et al*., 2001; Denolf *et al*., 1997; Gill *et al*., 1995; Indrasith and Hori, 1992; Lee *et al*., 1996; Valaitis *et al*., 2001) and immunoprecipitation (Vadlamudi *et al*., 1995; Nagamatsu *et al*., 1998a). These binding proteins have been identified by N-terminal sequencing and homology searches. Although a 270-kDa glycoconjugate in *L. dispar* (Valaitis *et al*., 2001) has been demonstrated to bind Cry1A toxins with high affinity, most of the identified Cry1 toxin binding proteins belong to two major protein families: N-aminopeptidases (APN) and cadherin-like proteins.

7.1 N-Aminopeptidases.

Aminopeptidases are enzymes present in the cytosol, subcellular organelles and membranes of plant and animal cells that catalyze the hydrolysis of amino acid residues from the amino terminus of peptides (Taylor, 1996). They are involved in a variety of physiological processes, such as protein processing and turnover, regulation of non-hormonal and hormonal peptides, viral infection, tissue invasion and cell cycle control (Taylor, 1996).

Aminopeptidases that bind Cry1 toxins have been identified by amino acid homology and enzymatic activity in BBMV from different Lepidoptera: *Manduca sexta* (Knight *et al*., 1994; Sangadala *et al*., 1994; Denolf *et al*., 1997; Masson *et al*., 1995, Luo

et al., 1996), *Lymantria dispar* (Valaitis *et al*., 1995; Valaitis *et al*., 1997), *Plutella xylostella* (Denolf *et al*., 1997), *B. mori* (Yaoi *et al*., 1997), *Trichoplusia ni* (Lorence *et al*., 1997), *Epiphyas postvittana* (Simpson and Newcomb, 2000), *Helicoverpa armigera* (Ingle *et al*., 2001) and *Heliothis virescens* (Gill *et al*., 1995; Luo *et al*., 1997; Oltean *et al*., 1999; Banks *et al*., 2001). Although tight toxin binding (Masson *et al*., 1995; Valaitis *et al*., 1997) and toxin pore formation (Lorence *et al*., 1997; Luo *et al*., 1997) are dependent on APNs, some reports suggest that this binding is not related to toxicity *in vivo* (Burton *et al*., 1999; Jenkins *et al*., 1999; Simpson and Newcomb, 2000).

Lipids in the cell membrane associated with the 120-kDa APN from *M. sexta* enhanced toxin binding and pore formation dependent on this APN (Sangadala *et al*., 2001). The distribution of specific APNs in membrane lipid rafts of insect midgut cells may also be important for their potential as Cry toxin receptors (Zhuang *et al*., 2002). These lipid rafts have also been suggested to be important for oligomerization of other bacterial toxins like aerolysin from *Aeromonas hydrophyla* (Abrami and Van der Goot, 1999). Although APNs that bind Cry1 toxins have been expressed in cultured cells (Luo *et al*., 1999, Simpson and Newcomb, 2000) binding and toxicity of Cry1 toxins to these cells have not been observed. An explanation for the lack of binding is that APN expressed in insect cells is not correctly glycosylated. Experiments to express APNs in cultured cells that bind Cry1 toxins independently of sugars, such as the 120-kDa APN from B. mori (Yaoi *et al*. 1997) are not published.

7.2 Cadherin-like proteins.

Cadherins are transmembrane proteins that mediate Ca^{2+} dependent cell-cell adhesion, provide extracellular information to the cell, and participate in tissue morphogenesis and maintenance of the epithelium (Isacke and Horton, 2000). These proteins are usually localized in adhesion junctions in mature epithelial cells. Although they are exposed on the surface of the cell, they possess a cytoplasmic C-terminus domain that interacts with the actin cytoskeleton in intracellular signal transduction (Isacke and Horton, 2000).

A cadherin-like protein (HevCadLP) has been hypothesized to be directly related to high levels of resistance against Cry1Ac and cross-resistance to other Cry1 toxins in a laboratory-selected *H. virescens* strain (Gahan *et al*., 2001). Binding of Cry1 toxins to the HevCadLP protein has not been reported to date.Cadherin-like proteins mediating Cry1A toxin binding from *Manduca sexta* (Vadlamudi *et al*., 1993; Keeton *et al*., 1997; Keeton *et al*., 1998) and *B. mori* (Nagamatsu *et al*., 1998a, 1998b) have been identified and cloned. Purified BTR175 cadherin-like protein from *B. mori* bound Cry1Aa with high affinity (2.6 nM), but not Cry1Ab or Cry1Ac (not active against *B. mori*) (Jenkins and Dean, 2001). When expressed in *Spodoptera frugiperda* Sf9 cells, BTR175 rendered these cells susceptible to Cry1Aa (Nagamatsu *et al*., 1999). Antibodies produced against this protein blocked toxin binding to *B. mori* BBMV and reduced Cry1Aa toxicity *in vivo* (Nagamatsu *et al*., 1998a). The Cry1Ab-binding cadherin-like protein BTR1 from *Manduca sexta* was cloned in Sf21 cells (Meng *et al*., 2001). The expressed BTR1 protein was secreted in the medium. The purified protein bound Cry1Ab with high affinity (1.1 nM) (Meng *et al*., 2001). Antibody mimics of a specific region of the BTR1

protein facilitated the formation of a Cry1Ab oligomer necessary for toxin pore-formation *in vitro* (Gómez *et al*., 2002).

Although the intercellular location of cadherin proteins at cell-cell interaction points would suggest that they are unavailable to active Cry toxins, results obtained by Nagamatsu *et al*. (1998a, 1999) and Meng *et al*. (2001) strongly indicate that BtR175 and BtR1 may function as Cry1A toxin receptors. Identification of proteins that function as bacterial toxin receptors but are not directly exposed to toxins has been previously reported (Tafazoli *et al*., 2000; Lecuit *et al*., 2001). For example, the bacteria *Listeria monocytogenes* interacts with E-cadherin from host human epithelial cells to initiate successful host cell invasion (Lecuit *et* al., 2001). Since E-cadherin is located in the cell-cell interaction regions, it was hypothesized that the sloughing and regeneration of gut cells would favor temporary exposure of regions containing E-cadherin to *L. monocytogenes* (Lecuit *et al*., 2001). Translocation of the bacteria *Yersinia enterocolitica* across the midgut epithelium is dependent on *Yersinia* invasin binding to β1 integrins from the host cell (Isberg and Leong, 1990). Integrins are localized to the tight junction areas of the basolateral surface in enterocytes and are involved in actin distribution (Beaulieu, 1992). Invasin interacts with β1 integrins and perturbs actin structure and distribution, leading to paracellular translocation of the bacteria (Tafazoli *et al*., 2000).

Localization of BTR1, BTR175 and HevCadLP proteins on insect midgut cells and their accessibility to Cry1 toxins has not been reported to date. A recent report suggests that BTR1 is not preferentially distributed on the midgut epithelium microvilli but on other regions of midgut cells (Bravo *et al*., 2002). This would suggest that

cadherin-like proteins may not be exposed to Cry toxins in the insect midgut epithelium, questioning the role of these proteins as functional toxin receptors.

7.3 Carbohydrates as binding epitopes on Cry1 toxin receptors.

Various carbohydrates inhibit binding (Knowles *et al*., 1991; Hofmann *et al*., 1988a; Denolf *et al*., 1997) and toxicity (Ellar *et al*., 1985; Knowles *et al*., 1984) of Cry1 toxins suggesting that these toxins recognize carbohydrate moieties on their receptors. The specific interaction of Cry1Ac with GalNAc is well characterized. This sugar inhibits Cry1Ac toxin binding to the *M. sexta* 120-kDa APN (Garczynski *et al*., 1991) and *H. virescens* 170-kDa APN (Luo *et al*., 1997). Since these binding proteins contain GalNAc (Knowles *et al*., 1991; Lee *et al*., 1996), Cry1Ac recognizes this sugar in these APNs. To test this hypothesis, the putative Cry1Ac GalNAc-binding pocket localized to domain III was altered with site directed mutagenesis (Burton *et al*., 1999; Lee *et al*., 1999). As expected, the mutant toxin (^{509}QNR^{511}-AAA) did not bind to *M. sexta* 120-kDa APN (Burton *et al*., 1999; Jenkins *et al*., 1999), although it bound to 110-kDa and 170-kDa APNs in *H. virescens* (Banks *et al*., 2001). Surprisingly, the mutant toxin had only 2-4 fold reduction of *in vivo* toxicity against both *M. sexta* and *H. virescens* when compared with wild type Cry1Ac, suggesting that toxicity was not caused by GalNAc-dependent binding alone (Burton *et al*., 1999; Carroll *et al*., 1997; Jenkins *et al*., 1999; Lee *et al*., 1999). GalNAc-dependent binding of Cry1Ac in *M. sexta* was localized to the posterior midgut, while a GalNAc-independent toxin binding mechanism was distributed along the entire midgut (Carroll *et al*., 1997). Binding of Cry1Ac to purified APN proteins incorporated in lipid monolayers had an initial reversible GalNAc-sensitive

phase followed by a second irreversible GalNAc-insensitive binding phase (Cooper *et al*., 1998; Jenkins *et al*., 2000). It has also been suggested that Cry1Ac binding to GalNAc moieties could facilitate conformational changes in domain I that may result in toxin insertion (Li *et al*., 2001; Derbyshire *et al*., 2001).

8. Resistance to Bt toxins.

8.1 Resistance to Cry toxins

Development of insecticide resistance requires long term/high level exposure and genetic variation in large insect populations. Resistance to chemical insecticides is one of the many reasons why *B. thuringiensis* is commonly used today. Sprayable Bt-based biopesticides were used with success in the late 1930s to control *Ostrinia nubilalis* (European corn borer). After the commercialization of Bt in the USA in 1957 (Van Frankenhuyzen, 1993), Bt sprays were used to control *Choristoneura fumiferana* (spruce budworm) and *L dispar* (gypsy moth) (Mott *et al.*, 1961). To increase efficiency of insect control, transgenic crops expressing Bt toxins were engineered and commercialized in the late 1990s (reviewed in De Maagd *et al*., 1999). The major concern of this transgenic technology is the increase in selection pressure resulting in development of resistance. To date, only *P. xylostella* has developed resistance to Bt toxins in the field (Tabashnik *et al*. 1990; Tang *et al*., 1997). Other insects have become highly resistant to purified Bt toxins after selection in the laboratory (Ferré and Van Rie, 2002). Bt sprays may lead to pest resistance more quickly than use of Bt plants due to degradation of the Cry toxins in the environment, resulting in insects being exposed to low concentrations of toxins (Roush, 1994). The expression of high levels of toxin in transgenic crops (high dose

strategy) together with stability of the toxins in these plants would reduce the possibility of resistance (Gould, 1998). Nevertheless, insects have the potential to develop resistance to both. Based on the frequency of resistance alleles in wild populations of *H. virescens*, Gould *et al.* (1997) predicted safe use of Bt crops for ten years before resistance would appear. The fact that resistance to Bt plants in the field has not been reported to date may be due to the short time (7 years) of Bt plant use. The main challenge for the future use of this technology is to develop strategies to delay resistance to Bt plants.

One of these strategies called gene stacking, uses combinations of Cry toxins with different mode of action simultaneously or in alternation (Gould, 1998). For example, transgenic Bt cotton expressing two Cry toxins was shown to confer a wider range of activity and increased efficacy against *H. virescens* (Stewart *et al*., 2001). The success of this strategy relies on the difficulty for the insect of becoming cross-resistant to different Cry toxins simultaneously. This consideration highlights the importance of knowing in greater detail the potential interactions between different Cry toxins as well as the differences in their modes of action.

8.2 Mechanisms of resistance.

Alterations in any of the steps in the mode of action of Bt toxins could potentially lead to resistance. Specific precipitation of Cry1 toxins by a gut juice elastase (Milne *et al*., 1995, 1998) as well as degradation of toxin (Pang and Gringorten, 1998) has been shown to prevent toxicity of Cry1Aa against *C. fumiferana* larvae. Toxin degradation and reduced activation have been suggested as sources of resistance in laboratory-

selected strains of *Plodia interpunctella* (Oppert *et al*., 1994), *H. virescens* (Forcada *et al*., 1996) and *P. xylostella* (Sayyed *et al*., 2001). Surprisingly, no quantitative difference in protease activity was found between susceptible and resistant *H. virescens* (Forcada *et al*., 1996, 1999). In the case of *P. interpunctella*, protease activity was reduced or increased in different resistant strains with respect to susceptible insects (Oppert *et al*., 1994). A genetic linkage between resistance and absence of a gut protease in *P. interpunctella* was also established (Oppert *et al*., 1997). This type of resistance mechanisms has been hypothesized to occur in insect strains with low levels of resistance (between 10-128-fold) to Cry1 toxins (Ferré and Van Rie, 2002).

Differences in the regenerative properties of *H. virescens* midgut epithelium as observed by scanning electron microscopy have also been reported as a potential mechanism of resistance to Cry1 toxins (Forcada *et al*., 1999; Martínez-Ramírez *et al*., 1999). A major concern with these experiments was that the sublethal concentrations of toxins were almost inactive against resistant larvae, thus, the recovery of the midgut epithelium in the resistant insects was probably overestimated. However, there is evidence for a midgut defense mechanism against Cry toxins in rice moth (*Corcyra cephalonica*) (Chiang *et al*., 1986) and baculovirus infection in *H. virescens* larvae (Hoover *et al*., 2000) that is related to stem cell maturation and sloughing of affected cells. Furthermore, sublethal concentrations of Cry1 toxins induce an increase in the rate of stem cell maturation in midgut cultured cells when compared to control cultures (Loeb *et al*., 2001).

Alteration of toxin binding to midgut cells is the best-characterized mechanism of resistance against Cry1 toxins, and was demonstrated for field-resistant *P. xylostella*

(Ferré *et al*., 1991). Reduced ^{125}I-Cry1 toxin binding to BBMV from resistant insects when compared to susceptible controls has been reported for strains of *P. interpunctella* (Van Rie *et al*., 1990; Herrero *et al*., 2001), *P. xylostella* (Ballester *et al*., 1994; Ballester *et al*., 1999; Ferré *et al*., 1991; Tabashnik *et al*., 1994; Tang *et al*., 1997), *H. virescens* (McIntosh *et al*., 1991; Lee *et al*., 1995), and *Leptinotarsa decemlineata* (Loseva *et al*., 2002). Reduced toxin binding has also been demonstrated by immunohistochemical analysis of midgut sections from *P. xylostella* larvae treated with Cry1 toxins (Bravo *et al*., 1992; Escriche *et al*., 1995). However, surface plasmon resonance measurements of Cry1 toxin binding to BBMV from the same *P. xylostella* resistant strains did not record differences in binding affinity when compared to BBMV from susceptible insects (Masson *et al*., 1995). This discrepancy between radiolabeled toxin binding and surface plasmon resonance was proposed to be due to the BBMV processing recquired for plasmon resonance. For example, a BBMV sonication step necessary in plasmon resonance measurements could have disrupted the masking of the toxin receptors in the BBMV from the resistant insects (Masson *et al*., 1995).

Recently, two groups have identified and cloned genes involved in resistance of *H. virescens* (Gahan *et al*., 2001) and the nematode *Caenorhabditis elegans* (Griffitts *et al*., 2001) to Cry toxins. In the YHD2 strain of *H. virescens*, disruption of a cadherin-superfamily gene by retrotransposon-mediated insertion was linked with resistance to Cry1Ac (Gahan *et al*., 2001). There is indirect (Wilson, 1993) and direct (Berrada and Fournier, 1997) evidence for transposable element-associated insecticide resistance in *Drosophila melanogaster*. Retrotransposon insertion in YHD2 insects resulted in the absence of a full-length transcript of the cadherin-like protein (HevCadLP). Gahan *et al*.

(2001) hypothesized that the absence of HevCadLP from midguts was the mechanism of resistance in this strain.

C. elegans strains resistant to Cry toxins were obtained after mutagenesis with ethyl methanesulfonate (Marroquin *et al*., 2000). These resistant nematodes did not undergo midgut degeneration when treated with Cry toxins, as susceptible worms did. Analysis of the mechanism of resistance in one of these mutant strains (bre-5), led to the identification and cloning of a gene that encodes a β-1,3-galactosyltransferase protein (Griffitts *et al*., 2001). Loss or reduction in the activity of this enzyme resulted in resistant nematodes that were defective in Bt toxin insertion in gut cell membranes. Since these worms were resistant to Cry5B and Cry14A, and both toxins are active against nematodes and insects, the authors hypothesized that resistance mechanisms similar to the one in bre-5 worms could occur in insects (Griffitts *et al*., 2001).

In one report APN proteins have been hypothesized to be involved in resistance to Bt toxins (Zhu *et al*., 2000). In this case, a conserved single amino acid change in the APN sequences of three Cry1Aa-resistant *P. interpunctella* strains in a region corresponding to a putative binding site for Cry1Aa toxin was related to resistance (Zhu *et al*., 2000).

8.3 Genetics of resistance.

In all cases of resistance due to reduced toxin binding, the resistance trait is recessive or partially recessive in a major autosomal gene, and cross-resistance extends to Cry proteins that share binding sites in the BBMVs (Ferré and Van Rie, 2002). This suggests that Cry toxins with different binding sites in the BBMV should be considered

for gene stacking strategies for resistance management. Gould *et al*. (1997) estimated the frequency of resistance alleles in wild populations of *H. virescens* to be 3 out of 2,050 (or 1.5×10^{-3}). Assuming this frequency, it was estimated that under the current regulations for Bt crops, it would take at least 10 years before *H. virescens* Bt resistance arises in field populations (Gould *et al*., 1997). In all reported cases, resistance to Bt is autosomal, and in most cases only one locus is involved, although coexistence of resistance mechanisms due to alteration of more than one gene has been postulated for some Bt-resistant insect strains (Heckel *et al*., 1997; Wright *et al*., 1997; Herrero *et al*., 2001). In most cases, resistance was unstable, and declined when selection pressure was relieved (Tabashnik *et al*., 1994). This was possibly related to the fitness costs associated with resistance genes in the absence of an environment that would favor resistant insects.

9. *Heliothis virescens*: the tobacco budworm.

9.1 Biology of *H. virescens*.

In the larval stage, the noctuid *Heliothis virescens* (tobacco budworm) causes important economic damage in cotton, tobacco, tomato and other crops. In 1997, nearly 30 million dollars were spent to control *H. virescens* in cotton fields in Georgia alone (Roberts *et al*., 1997). Even after this disbursement, the estimated damage to the cultivars was more than 17 million dollars.

Found in Central and South America, this species breeds only in the southern part of the USA, although adults may be found further north. The adults are light olive to brownish olive in color, with three dark bands on their forewings and with dark margins on white hind wings (Roberts and Guillebeau, 1999). Larvae are yellowish white to

greenish, reddish brown, or black with pale stripes running lengthwise on the body. The larvae feed on foliage, vegetative and flower buds of tobacco, cotton, soybeans, geranium and other crops. The life cycle of *H. virescens* lasts about a month, and there are several generations per year in the field. Pupae overwinter in the upper layer of soil until adults emerge in the spring.

Different methods have been used to control *H. virescens*. This insect is well known for its ability to develop resistance and cross-resistance to insecticides in the field (Heckel *et al*., 1997; Zhao *et al*., 1996). A number of diseases, parasites, and predators attack budworms, but additional methods are necessary for effective control. A number of Cry1 toxins are highly effective against *H. virescens* (Van Frankenhuyzen and Nystrom, 2002). Transgenic Bt-cotton expressing Cry1Ac (the most active toxin against *H. virescens*) was commercialized in 1996 to control this insect in the field (De Maagd *et al*., 1999). Just one year after its introduction in the market, Bt-cotton reduced insecticide application by 300,000 gallons and gave a 14% increase in yield (De Maagd *et al*., 1999). To keep this technology useful and effective in the future, studies aimed at characterizing the resistance mechanisms against Bt toxins that *H. virescens* may develop in the field are essential.

9.2 Receptors for Cry1 toxins in *H. virescens*.

The first Cry toxin receptor model for *H. virescens* was proposed by Van Rie *et al*. (1989) using radiolabeled Cry1A in toxin binding competition experiments to BBMV

from this insect. According to this model, one population of receptors (receptor A) bound all Cry1A toxins (Cry1Aa, Cry1Ab and Cry1Ac). A second population of receptors (receptor B) bound Cry1Ab and Cry1Ac, while a third population of receptors (receptor C) bound only Cry1Ac. Although there were some quantitative discrepancies, this model explained the results from binding competition (Van Rie *et al*., 1989).

Multiple proteins in BBMV from *H. virescens* have been found to bind Cry1 toxins (Aronson, 1995; Cowles *et al*., 1995; Garczynski *et al*., 1991; Knowles *et al*., 1991; Oddou *et al*., 1991, 1993). Cry1C bound to a 40-kDa protein; Cry1Aa, Cry1Ab and Cry1Ac bound to a 170-kDa protein, while Cry1Ac additionally bound a more complex pattern of proteins of 140-, 120-, 105-, 90-, 81-, 68-, 64-, and 50-kDa. The 170-kDa (Oltean *et al*., 1999) and 120-kDa (Gill *et al*., 1995) Cry1Ac-binding proteins have been cloned and identified as APNs. The 120-kDa APN or $BTBP_1$ (for *B. thuringiensis* toxin binding protein) bound Cry1Ac on ligand blots. This binding was inhibited by GalNAc or when $BTBP_1$ was expressed in *E. coli*, suggesting that Cry1Ac recognized carbohydrate moieties on this glycoprotein (Gill *et al*., 1995). Purified 170-kDa APN bound Cry1Aa, Cry1Ab, and Cry1Ac in surface plasmon resonance assays (Luo *et al*., 1997). Binding of Cry1Ac to 170-kDa APN was GalNAc-dependent (Luo *et al*., 1997). The same purified APN when reconstituted into phospholipid vesicles catalyzed Cry1Aa, Cry1Ab and Cry1Ac but not Cry1C or Cry1Ea pore formation (Luo *et al*., 1997). Together, these results indicated that the 170-kDa APN functioned as receptor A in the toxin-binding model. Recently, the 120 and 170-kDa APNs have been shown to be partitioned into lipid rafts on the membranes of BBMV from *H. virescens* (Zhuang *et al*., 2002). Furthermore, integrity of the lipid rafts was shown to be essential for formation of

pores by Cry1A toxin, suggesting that these membrane microdomains might be involved in toxin aggregation and pore formation (Zhuang *et al*., 2002). Although the identity of receptor B has not been addressed, Lee *et al*. (1995) proposed two BBMV proteins of 120- and 80-kDa in size that were only recognized by Cry1Ac in ligand blots to constitute receptor C.

A 130-kDa APN that bound Cry1Aa and Cry1Ac copurified with the 170-kDA APN (Oltean *et al*., 1999). The 130-kDa and 170-kDa seemed to be derived from the same gene. Both proteins showed GalNAc-dependent Cry1Aa and Cry1Ac binding on ligand blots, although the affinity of binding was different for each toxin (Oltean *et al*., 1999). Differential glycosylation was hypothesized to be responsible for different Cry1Ac binding affinity to these proteins (Oltean *et al*., 1999).

Cry1 toxin binding proteins in *H. virescens* are extensively glycosylated (Gill *et al*., 1995; Knowles *et al.*, 1991; Oltean *et al*., 1999). Binding of Cry1Ac to BBMV or purified toxin-binding proteins is inhibited by GalNAc, suggesting an important role for this sugar in toxin binding in this insect (Gill *et al*., 1995; Lee *et al*., 1999; Luo *et al*., 1997; Oltean *et al*., 1999). Nevertheless, Cry1Ac binding to a novel 110-kDa APN from *H. virescens* was shown to be GalNAc-independent (Banks *et al*., 2001).

9.3 Resistance to Bt in *H. virescens*.

Although various resistant strains of *H. virescens* have been developed after laboratory selection with Cry toxins, the mechanism of resistance has only been characterized for three strains (Ferré and Van Rie, 2002). The SEL *H. virescens* resistant strain originated by selection with Cry1Ab for 13 generations, with Dipel (a commercial

product containing Cry1Aa, Cry1Ab, Cry1Ac, and Cry2Aa) for 4 generations, and then with Cry1Ab again for another 4 generations (MacIntosh *et al*., 1991). This strain became resistant to Dipel (57-fold), Cry1Ab (71-fold), and Cry1Ac (16-fold). When toxin binding to BBMV from this strain in binding competition assays was studied, the dissociation constants for Cry1Ab and Cry1Ac were 2-fold to 4-fold higher in the resistant strain, although a 6-fold and 4-fold increase respectively, in the binding site concentration was also found in the BBMV from resistant insects. Results from binding competition experiments confirmed that the receptor specificity in resistant insects was modified for Cry1Ab and Cry1Ac (MacIntosh *et al*., 1991). Resistance in this strain was transmitted as a recessive character (Sims and Stone, 1991).

Gould *et al*. (1992) generated the CP73-3 resistant strain after selection of individuals from a field-collected *H. virescens* population with Cry1Ac for 17 generations. This strain developed 50-fold resistance to Cry1Ac, but also 53-fold and 13-fold cross-resistance to Cry2A and Cry1Ab respectively. These were unexpected results, since Cry1A and Cry2A toxins have very different amino acid sequences and were thought to bind to different receptors and display different modes of action (English *et al*., 1994; Morse *et al*., 2001). No significant differences in toxin binding were found between susceptible and resistant insects with competition binding experiments using BBMV (Gould *et al*., 1992). These findings demonstrated that the resistance mechanism was different from the one hypothesized by MacIntosh *et al*. (1991). Results from genetic crosses evinced that the resistance trait in the CP73-3 strain was recessive at low toxin concentrations but additive at high toxin concentrations (Gould *et al*., 1992). Subsequent analysis of the proteolytic activity of midguts in larvae from the CP73-3

strain revealed that these insects degraded protoxin more slowly and processed active toxin faster than a susceptible strain (Forcada *et al*., 1996). Surprisingly, total proteolytic activity using azocasein as substrate was the same in susceptible and resistant larvae. A subset of adults form the CP73-3 strain was mated with adults from a susceptible *H. virescens* strain to obtain a new strain (CXC) that was only 20-fold resistant to Cry1Ac. This resistant strain had a gut protease pattern different from that of either parental strain, although no quantitative differences in total proteolytic activity were found (Forcada *et al*., 1999). No correlation was found between resistance and the presence of any specific protease. The CXC strain has been selected with Cry2Aa, and resistance to this toxin has increased about 5-fold (Dr. F. Gould, personal communication).

Martínez-Ramírez *et al*. (1999) compared the histopathological effects and growth reduction in the CP73-3 and a susceptible strain (CPN) after treatment with doses of Cry1Ac that were sublethal to CPN individuals. They found that midgut cells from the two strains were similarly affected, but the CP73-3 insects recovered faster after treatment, suggesting that enhanced epithelium recovery could be a mechanism of resistance. The Cry1Ac-selected *H. virescens* KCB strain also developed cross-resistance to Cry2Aa (Forcada *et al*., 1999). Enhanced midgut epithelium recovery after intoxication with sublethal doses of Cry1Ac and a distinct midgut protease pattern were also observed for these insects (Forcada *et al*., 1999), although direct correlations between protease presence and resistance could not be established (Forcada *et al*., 1999). Exposure to sublethal toxin concentrations has been demonstrated to increase numbers of differentiating and stem cells in *H. virescens* midgut cell cultures (Loeb *et al*., 2001). Furthermore, resistance to baculovirus infection due to rapid sloughing of midgut cells

and renewal of midgut epithelium has also been demonstrated in *H. virescens* (Hoover *et al*., 2000).

The YHD2 resistant strain was generated after selection with Cry1Ac of a subset of individuals from a field-collected susceptible strain (YDK). After 29 generations of selection, YHD2 insects were 10,000-fold resistant to Cry1Ac (highest level of resistance to Cry toxins described for *H. virescens*) (Gould *et al*., 1995). YHD2 larvae were cross-resistant to toxins with high amino acid sequence homology to Cry1Ac, like Cry1Aa (32-fold) or Cry1Ab (2,000-fold), but also to toxins very different from Cry1Ac, like Cry1Fa (3,600-fold) (Gould *et al*., 1995). Cross-resistance to Cry2Aa was only 16-fold, suggesting a mechanism of resistance different from the one present in CP73-3 or CXC strains. Cross-resistance to other toxins (Cry1C, Cry1B) was also low. The resistance trait was incompletely recessive (Gould *et al*., 1995). Only binding of Cry1Aa was reduced in the YHD2 strain, while Cry1Ab and Cry1Ac binding was similar to binding in the susceptible YDK strain (Lee *et al*., 1995). There were no major differences in the Cry1A binding proteins detected on ligand blots of BBMV from susceptible and resistant larvae (Lee *et al*., 1995). However, toxin binding proteins of 170- and 140-kDa from YHD2 insects migrated more closely to each other than the respective proteins in YDK insects. This observation suggests that potential differences in protein glycosylation or sequence modified the apparent molecular size of the proteins. Heterologous binding competition experiments confirmed the model proposed by Van Rie *et al*. (1989) in BBMV from both YDK and YHD2 insects (Lee *et al*., 1995). The authors concluded that resistance in YHD2 was due to alteration of the Cry1A receptor (receptor A), while

receptors B and C were still able to bind Cry1Ab and Cry1Ac but did not confer toxicity (Lee *et al*., 1995).

Using Quantitative Trait Loci (QTL) analysis, Heckel *et al*. (1997) identified a major locus (*BtR*-4) on linkage group 9 of *H. virescens* that was responsible for 40 to 80% of Cry1Ac resistance in YHD2, although other linkage groups also contributed to resistance. This locus contained a cadherin-superfamily gene that was disrupted by retrotransposon-mediated insertion in insects from the YHD2 strain (Gahan *et al*., 2001). Disruption of the gene led to absence of a cadherin-like protein (HevCadLP for *H. virescens* cadherin-like protein) from midguts of YHD2 insects. The YHD2 strain has been under continuous Cry1Ac selection since 1995, and Cry1Ac resistance has increased (Kota *et al*., 1999). This suggests that additional resistance mechanisms have emerged in YHD2 insects.

10. Dissertation rationale.

There were two main objectives for the research presented in this dissertation: first, expand the current model of Cry toxin binding in *H. virescens* BBMV by including Cry1Fa and Cry1Ja toxins, and second, study the mechanisms that lead to increased resistance and cross-resistance against Cry toxins in three independent strains of *H. virescens*.

For the first objective, I wanted to study the potential interactions between Cry1Fa and Cry1Ja with Cry1A toxins in *H. virescens* BBMV, for a further evolution of the toxin-binding model proposed by Van Rie *et al*. (1989). Cry1Fa was of special interest because it is a candidate for gene stacking with Cry1Ac in future transgenic Bt

crops. Determinants of Cry1Ac binding-specificity have been localized to domain II regions (Ge *et al*., 1991; Schnepf *et al*., 1990). Although Cry1A toxins do not share high similarity in amino acid sequence with Cry1Fa or Cry1Ja, these toxins share high similarity in the exposed loops of domain II (Tabashnik *et al*., 1996). The loops of domain II have been demonstrated to determine binding specificity (Rajamohan *et al*., 1996a, 1996b, 1996c; Smedley and Ellar, 1996). Accordingly, Cry1A, Cry1Fa and Cry1Ja have been shown to share binding sites in *P. xylostella* (Granero *et* al., 1996; Herrero *et al*., 2001). Cross-resistance between Cry1A, Cry1Fa and Cry1Ja toxins has also been reported in *P. xylostella* (Tabashnik *et al.,* 1994, 1996, 2000) and *H. virescens* (Gould *et al*., 1995). These findings imply that insects can become resistant to toxins to which they have never been exposed if they share a population of receptors that catalyzes toxicity.

From these results, I hypothesized that Cry1Fa and Cry1Ja would share binding sites with Cry1A toxins in *H. virescens* BBMV. These binding sites would be recognized by the exposed loops of domain II, which share high similarity in Cry1A, Cry1Fa and Cry1Ja toxins (Tabashnik *et al*., 1996). To test my hypothesis, I used competition-binding experiments with *H. virescens* BBMV, ^{125}I-labeled Cry1A toxins and unlabeled heterologous competitors to determine wether the Cry1 toxins would compete with high affinity for a shared binding site. This shared binding site would be analogous to receptor A from the model proposed by Van Rie *et al*., (1989). Using ligand blotting, I was also able to propose a more complete toxin-binding model for *H. virescens* and Cry1 toxins. These results are detailed in Chapter Two of this dissertation.

In Chapter Three of this dissertation I report the results of studies of the mechanism for increased resistance against Cry1Ac in the YHD2 strain of *H. virescens*. In this strain, Lee *et al*. (1995) had found that only Cry1Aa toxin binding to BBMV was greatly reduced, while Cry1Ab and Cry1Ac binding was not affected when compared to a susceptible strain. Interestingly, they found no differences in the pattern of toxin binding proteins between BBMV from susceptible and resistant insects on ligand blots. To explain these results, the authors hypothesized that resistance in the YHD2 strain was due to alteration of a shared Cry1A binding protein (receptor A). Binding of Cry1A toxins to other receptor populations (receptor B and C) was not involved in toxicity. Lack of expression of the HevCadLP protein in YHD2 insects was proposed as the mechanism to explain resistance in YHD2 (Gahan *et al*., 2001). This implies that HevCadLP is part of receptor A and that toxin binding to this protein is important for toxicity.

Based on these results, I hypothesized that if Cry1Ac binding to receptors B and C in the BBMV from YHD2 was not related to toxicity, it would not lead to toxin pore formation. To test this hypothesis, I performed analyses of pore formation by Cry1Ac in BBMV from the YHD2 strain and a control susceptible strain (YDK). The results from these experiments demonstrated that Cry1Ac did not make pores in BBMV from YHD2, as predicted. However, when I tried to reproduce the toxin binding experiments of Lee *et al*. (1995), I observed that Cry1Ac did not bind to BBMV from YHD2. Furthermore, neither Cry1Aa, Cry1Ab nor Cry1Fa bound to BBMV from this strain. This was evidence that the YHD2 strain had changed since the analyses of Lee *et al*. (1995). YHD2 insects had been under continuous selection with Cry1Ac, resulting in increased resistance to Cry1Ac (Kota *et al*., 1999). Since this increased resistance could not be

explained only by absence of HevCadLP from YHD2 midguts, I considered that other mechanism/s of resistance had evolved in these insects. To test this hypothesis I used Cry1Aa, Cry1Ab, Cry1Ac and Cry1Fa toxins to compare BBMV from YHD2 and YDK strains at the toxin binding and pore formation levels to investigate if any of these steps of the toxin mode of action was altered in the YHD2 insects.

Results from experiments on binding of Cry1A and Cry1Fa toxins, their pore formation and ligand blotting demonstrated that the YHD2 larvae had changed since the analyses of Lee *et al*. (1995). As detailed in Chapter Three, Cry1Aa, Cry1Ab, Cry1Ac and Cry1Fa binding and pore formation abilities were altered in BBMV from YHD2 insects. Since Cry1Ac recognizes GalNAc as a binding epitope (Derbyshire *et al*., 2001), I proposed that changes in midgut protein glycosylation could be responsible for decreased Cry1Ac toxin binding to BBMV from YHD2. To test this hypothesis, I used lectin blots with soybean agglutinin (SBA), which specifically recognizes GalNAc moieties (Pereira *et al*., 1974). The results presented in Chapter Three confirm that altered glycosylation of at least two specific BBMV glycoproteins of 68- and 63-kDa correlates with reduced toxin binding and increased resistance to Cry1Ac in the YHD2 strain. Proteins of these sizes were classified as constituting receptor C in the binding model from Chapter Two. Resistance to Cry toxins due to altered glycosylation has been demonstrated in the nematode *C. elegans* (Griffitts *et al*., 2001).

The resistance mechanisms in the CXC and KCBhyb *H. virescens* strains were then studied in Chapter Four of this dissertation. These strains were derived from laboratory Cry1Ac-selected strains that were backcrossed to susceptible adults and then further selected with Cry2Aa. Both strains were highly resistant to Cry1Ac and Cry2Aa,

and also cross-resistant to Cry1Aa, Cry1Ab and Cry1Fa. Since I had shown that cross-resistance to Cry1Aa, Cry1Ab and Cry1Fa in the YHD2 strain was due altered toxin binding (Chapter Three), I postulated that the same mechanism was present in the CXC and KCBhyb strains. To test this hypothesis, I performed toxin binding and homologous binding competition. The results (Chapter Four) suggest the presence of a mechanism similar to the one observed by Lee *et al*. (1995) in the YHD2 strain. Since this mechanism is related to binding site alteration and Cry1Ac and Cry2Aa do not share binding sites in *H. virescens* BBMV (Jurat-Fuentes and Adang, 2001), it does not explain resistance to Cry2Aa in the CXC and KCBhyb strains. The existence of additional mechanisms in the CXC and KCBhyb strains may explain resistance to Cry2Aa.

Taken together, the results from Chapters Three and Four of this dissertation demonstrate that *H. virescens* can develop a broad array of potential resistance mechanisms against Cry toxins. This information is extremely important in designing strategies aimed to delay or overcome resistance to Bt crops in the field.

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LIST OF ABBREVIATIONS USED

APN- Aminopeptidase –N

BBMV- Brush Border Membrane Vesicles

Bt- *Bacillus thuringiensis*

GalNAc- N-Acetylgalactosamine

PLBs- Planar Lipid Bilayers

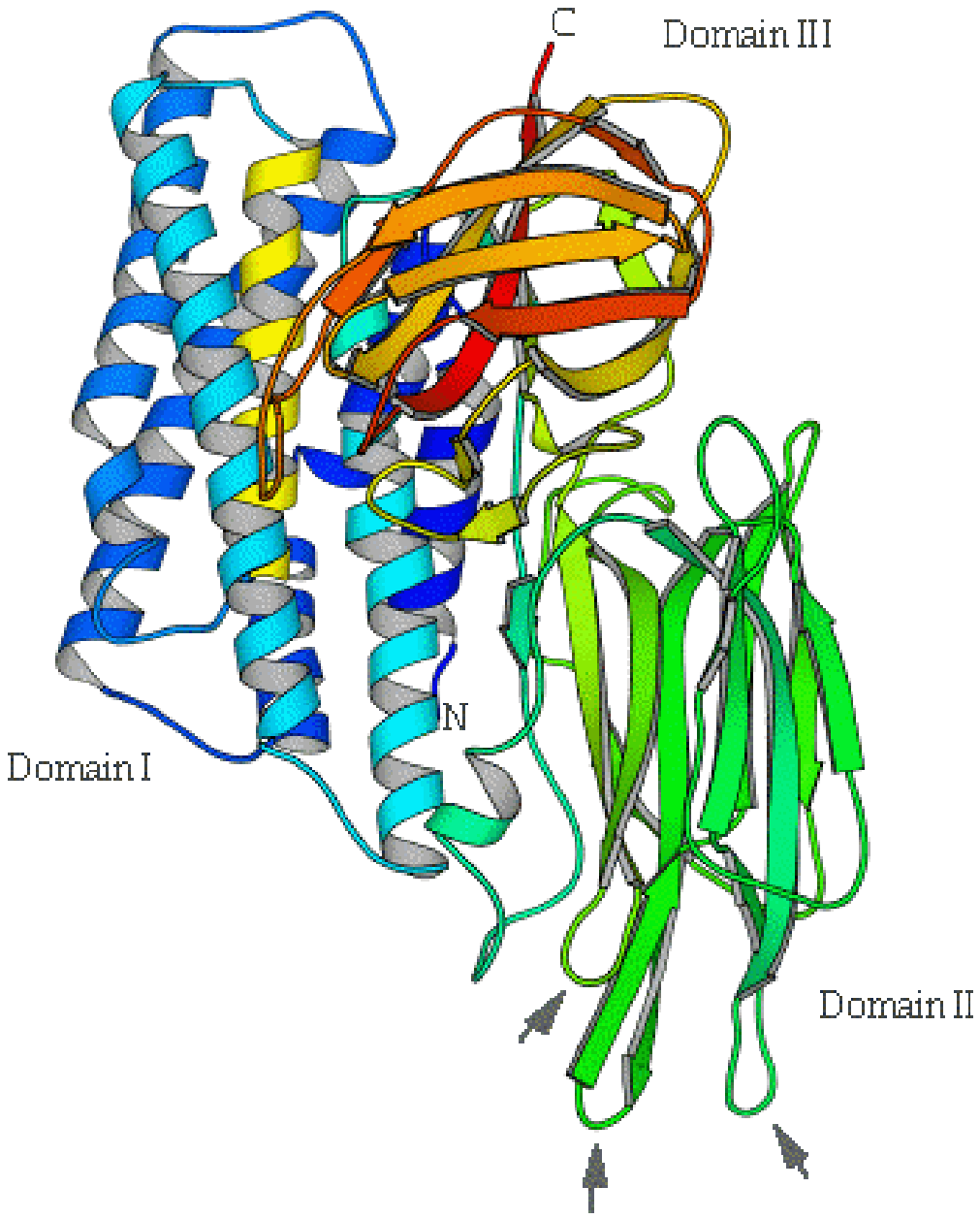

Figure 1.1- Schematic view of the three-dimensional domain structure of Cry1Aa (Li *et al*., 1991). Arrows point to the three surface exposed loops of domain II.

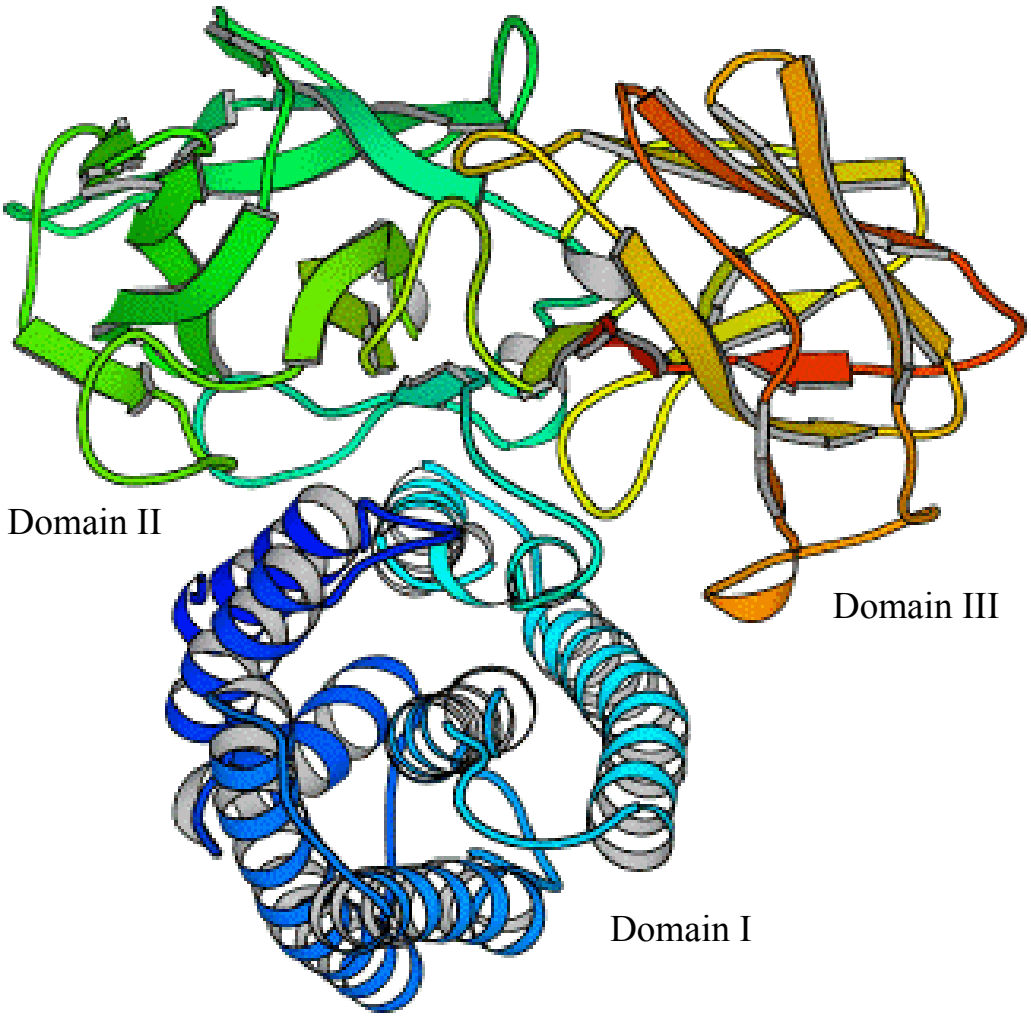

Figure 1.2- Top view of the structural domains of Cry3A (Li *et al.*, 1991). Note the two central alpha-helices (α4 and α5) in the helical bundle of domain I.

CHAPTER 2

IMPORTANCE OF CRY δ-ENDOTOXIN DOMAIN II LOOPS FOR BINDING SPECIFICITY IN *HELIOTHIS VIRESCENS* (L.).[1]

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Applied and Environmental Microbiology
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ABSTRACT

We constructed a model for *Bacillus thuringiensis* Cry1 toxin binding to midgut membrane vesicles from *Heliothis virescens*. Brush border membrane vesicle binding assays were performed with five Cry1 toxins that share homologies in domain II loops. Cry1Ab, Cry1Ac, Cry1Ja and Cry1Fa competed with ^{125}I-Cry1Aa, evidence that each toxin binds to the Cry1Aa binding site in *H. virescens*. Cry1Ac competed with high affinity (K_{com}= 1.1 nM) for ^{125}I-Cry1Ab binding sites. Cry1Aa, Cry1Fa and Cry1Ja also competed for ^{125}I-Cry1Ab binding sites though the K_{com} values ranged from 212-318 nM. Cry1Ab competed for ^{125}I-Cry1Ac binding sites (K_{com}= 79.7 nM) with higher affinity than Cry1Aa, Cry1Fa or Cry1Ja. Neither Cry1Ea nor Cry2Aa competed with any of the ^{125}I-Cry1A toxins. Ligand blots prepared from membrane vesicles were probed with Cry1 toxins to expand the model of Cry1 receptors in *H. virescens*. Three Cry1A toxins, Cry1Fa and Cry1Ja recognized 170-kDa and 110-kDa proteins that are probably aminopeptidases. Cry1Ab, Cry1Ac, and to some extent Cry1Fa, also recognized a 130-kDa molecule. Our vesicle binding and ligand blot results support a determinant role for domain II loops in Cry toxin specificity for *H. virescens*. The shared binding properties for these Cry1 toxins correlate with observed cross-resistance in *H. virescens*.

INTRODUCTION.

Bacillus thuringiensis (Bt) produces parasporal crystals composed of Cry proteins during the sporulation phase of growth (Adang, 1991; Schnepf *et al*., 1998). Most Cry proteins are toxic to insects and Cry1 proteins are specifically toxic to larvae of Lepidoptera. Cry1 crystals are solubilized in the midgut of larvae releasing 130-140 kDa protoxins that are subsequently processed to 55- to 60-kDa active toxins by midgut proteinases. Passing through the peritrophic membrane, Cry toxins bind to specific receptors on the brush border membrane of the midgut cells (Van Rie *et al*., 1989, 1990) in a reversible manner. An irreversible binding phase, attributed to toxin insertion into the midgut membrane takes place (Liang *et al*., 1995), followed by toxin oligomerization (Aronson *et al*., 1999) and pore formation. The osmotic shock resulting from toxin-induced pores leads to cell lysis, gut paralysis and insect death (Knowles, 1994).

Cry toxins consist of three structural domains (Grochulski *et al*., 1995; Li *et al*., 1991) that are associated with different steps of toxin mode-of-action. Domain I, composed of α–helices, is involved in pore formation (Li *et al*., 1991; Schwartz *et al*., 1997). Domain II, composed mainly of β-sheets, contains the primary determinants that specify binding to receptors on the midgut brush border (Ballester *et al*., 1999; Dean *et al*., 1996; Rajamohan *et al*., 1996a, 1996b). Domain III (also composed of β-sheets) has been implicated in toxin stability and binding specificity in some insects (Burton *et al*., 1999; De Maagd *et al*., 1996).

Since the commercial introduction of Bt corn, cotton and potatoes in 1996, Bt toxins have become one of the most important tools for pest insect control. However, the development of resistance by insects challenges their future efficacy. Insects are capable

of developing high levels of resistance to Bt toxins after laboratory (Gould *et al*., 1992, 1995; Oppert *et al*., 1994; Whalon *et al*., 1993) or field (Shelton *et al*., 1993; Tabashnik *et al*., 1990) selection. Resistant insects are often cross resistant to Bt toxins that were not in the environment of selection (Gould *et al*., 1992, 1995; Tabashnik *et al*., 1994, 1996), suggesting that the mechanism/s of acquired resistance can be effective against toxins that have never been used against that insect. Although several mechanisms of resistance have been proposed (Forcada *et al*., 1996; Oppert *et al*., 1997), the best-documented mechanism is the alteration of binding to the specific receptors in the midgut (Ferré *et al*., 1991; Lee *et al*., 1995; Van Rie *et al*., 1990).

Toxins that share high homology in the loops of domain II (Tabashnik *et al*., 1996) often share midgut binding sites (Ballester *et al*., 1999; Escriche *et al*., 1997), and display cross-resistance. For example, Cry1F and Cry1A toxins have a common high-affinity binding site on brush border membrane vesicles (BBMV) prepared from *P. xylostella* (Granero *et al*., 1996). Cry1Ac-resistant *P. xlylostella* show greatly reduced Cry1Ac binding and cross-resistance to Cry1Fa. Though direct binding studies of Cry1F are not reported, it appears that when *P. xylostella* adapted to Cry1Ac toxin the modification that reduced Cry1Ac binding also reduced Cry1Fa toxicity. Cry1Ja, another toxin that shares high homology in sequence in the loops of Domain II with Cry1A toxins, presents a similar pattern of cross-resistance in *P. xylostella* (Tabashnik *et al*., 1996).

H. virescens, the subject of this study, is an important pest of cotton in the U.S. and the major target of transgenic cotton expressing the Bt *cry1Ac* gene. *H. virescens* selected for Cry1Ac resistance in the laboratory is cross resistant to Cry1Aa, Cry1Ab and

Cry1Fa toxins (Gould *et al*., 1995). In *H. virescens*, a model of three populations of receptor molecules for Cry1A toxins is generally accepted (Garczynski *et al*., 1991; Luo *et al*., 1997; Oddou *et al*., 1991; Van Rie *et al*., 1989). According to this model, receptor A (previously identified as a 170-kDa aminopeptidase N [APN]), binds Cry1Aa, Cry1Ab and Cry1Ac. Receptor B binds Cry1Ab and Cry1Ac, and receptor C only binds Cry1Ac. Alteration of Receptor A is implicated as a mechanism for *H. virescens* resistance to Cry1A toxins (Lee *et al*., 1995). The 170-kDa APN elicits binding and pore formation by all the three Cry1A toxins (Luo *et al*., 1997). Other authors (Lee *et al*., 1995; Oltean *et al*., 1999) have suggested that a 130-kDa aminopeptidase may also function as receptor A. The 170-kDa APN in *H. virescens* was found to be a low affinity-binding site for Cry1Ac, while the 130-kDa aminopeptidase showed high affinity (32.1 nM) for this toxin. The 170-kDa and 130-kDa APN may be the product of differential post-translational glycosylation of the same protein precursor (Oltean *et al*., 1999).

The objective of this study was to determine if toxins that share homology in the loops of domain II share binding sites on BBMV from *H. virescens*. Four of the toxins (Cry1Aa, Cry1Ab, Cry1Ac, and Cry1Fa) are highly active against *H. virescens* while Cry1Ja has low toxicity. We then used ligand blotting to determine the molecular sizes of toxin binding proteins for each Cry1 toxin. By integrating data from vesicle binding experiments, ligand blotting and published results we further developed the model of Cry1A toxin binding in *H. virescens* to include Cry1Fa and Cry1Ja toxins.

MATERIALS AND METHODS.

2.1 Insect bioassays.

The *H. virescens* strain used in this work was started from insects collected in Alabama by Dr. J. Graves (Louisiana State University). The colony has been maintained on artificial diet (Southland products, Lake Village, Arkansas) in the laboratory for 23 generations.

For insect bioassays, at least five dilutions of each toxin were prepared in 20 mM Na_2CO_3 (pH 9.6) and 50 µl aliquots were applied uniformly over the surface of artificial diet in 2 cm^2 wells (EC-International). Each toxin dilution was assayed with at least 12 neonate larvae, and the bioassay repeated three times. Mortality was scored after 7 days. LC_{50} values and the slopes of concentration-mortality regression lines were obtained using the Polo-PC program (Russell *et al*., 1977).

2.2 Bacterial strains and toxin purification.

Bt strain HD-37 and HD-73 producing Cry1Aa and Cry1Ac respectively, were obtained from the *Bacillus* Genetic Stock Collection (Columbus, Ohio). Bt strains producing Cry1Fa and Cry1Ja were obtained from Ecogen Inc. (Langhorne, PA). Bt strain MR522 producing Cry1Ea was obtained from Dow Agrosciences (San Diego, CA). An *Escherichia coli* carrying the Bt NRD-12 *cry1Ab* toxin gene was kindly provided by Luke Masson (National Research Council of Canada, Montreal).

Toxins were prepared and purified from Bacilli as described elsewhere (Luo *et al*., 1999). Cry1Ab inclusions were prepared from *E. coli* NRD-12 and toxin purified as previously described (Masson *et al*., 1989). Fractions containing pure toxin (as

determined by gel electrophoresis) were pooled, quantified by the Bradford (1976) protein assay using BSA as standard and stored at –80^{o}C until used.

2.3 Gel electrophoresis.

Purified Cry1 toxin samples were analyzed by sodium dodecyl sulfate-10% polyacrylamide gel electrophoresis (SDS-PAGE). Briefly, 10 μg toxin (for gels stained with Coomassie brilliant blue R-250) or 10^5 cpm (for ^{125}I-Cry1A toxins) per lane were used in SDS-PAGE. Gels were stained with Coomassie brilliant blue R-250 or exposed to Kodak XAR-5 film with an intensifying screen at –80^{o}C for 1 hour.

2.4 Iodination and biotinylation of Cry1A toxins.

Labeling of purified Cry1A toxins with Na^{125}I was done using the Chloramine-T method (Garczynski *et al*., 1991). Toxin (1 μg) was labeled with 0.5 mCi of Na^{125}I (Amersham-Pharmacia). The specific activities were 6.2 μCi/μg for Cry1Aa, 10.5 μCi/μg for Cry1Ab, and 28.4 μCi/μg for Cry1Ac (based on input toxin).

For toxin biotinylation, 0.5 mg of purified toxin was incubated (1:30 molar ratio) with EZ^{TM}-Link sulfo-NHS-LC-Biotin (Pierce) for 30 minutes at room temperature. To remove excess biotin, samples were dialyzed overnight in 20 mM Na_2CO_3, 200 mM NaCl, pH 9.6. Biotinylated toxins were quantified as described above and stored at –80^{o}C until used.

2.5 Midgut isolation and BBMV preparation.

Midguts were dissected from fifth instar *H. virescens* larvae, washed in ice-cold SET buffer (250 mM sucrose, 17 mM Tris [pH 7.5], 5 mM EGTA) and kept at –80^{o}C until used. BBMV were prepared by the differential magnesium precipitation method (Wolfersberger *et al*., 1987), as modified by Carroll and Ellar (1993). Briefly, midguts homogenized in SET buffer containing protease inhibitors (complete® tablets, Pierce) were mixed with one volume of 24 mM $MgCl_2$, 250 mM sucrose and kept on ice for 15 minutes. Solutions were centrifuged at 2,500 x *g* for 15 minutes, the pellets discarded, and the supernatant further centrifuged at 27,000 x *g* for 30 minutes. The final pellet consisting of purified BBMV was suspended in ice-cold TBS (25 mM Tris [pH 7.5] 2 mM KCl, 135 mM NaCl) and protein concentration quantified by the method of Bradford (1976) as before. BBMV were frozen in dry ice and kept at -80°C until used. After thawing, BBMV were centrifuged for 10 min at 13,500 x *g* and resuspended in binding buffer for binding assays (see below).

Aminopeptidase specific activity was used as an enzymatic marker of enrichment for brush border membranes (Terra *et al*., 1994), using leucine-ρ-nitroanilide as substrate. Typical activity enrichment in the BBMV preparations was 5 to 7 times the activity measured in the initial midgut homogenates.

2.6 Binding of ^{125}I-Cry1A to BBMV.

For qualitative binding experiments, increasing amounts of BBMV were incubated with either 0.5 nM (^{125}I-Cry1Aa) or 0.1 nM (^{125}I-Cry1Ab and ^{125}I-Cry1Ac) labeled toxin in 0.1 ml (final volume) of binding buffer (25 mM Tris [pH 7.5], 3 mM

KCl, 135 mM NaCl, 0.1% BSA) for 1 hour at room temperature. After incubation, samples were centrifuged at 13,500 x *g* for 10 min, the pellets washed twice with 0.5 ml cold binding buffer. Non-specific binding was determined by adding 1000 nM of the respective unlabeled toxin to the reaction mixtures. Radioactivity was measured in a Beckman model 4000 Gamma detector.

Homologous and heterologous competition experiments were done by incubating 40 μg (for Cry1Aa), 10 μg (for Cry1Ab), or 5 μg (for Cry1Ac) of BBMV with 0.5 nM (^{125}I-Cry1Aa) or 0.1 nM (^{125}I-Cry1Ab and ^{125}I-Cry1Ac) labeled toxin for an hour at room temperature. Increasing amounts of unlabeled homologous or heterologous competitor were used to compete binding. Competition reactions were stopped by centrifugation at 13,500 x *g* for 10 min. and the pellets washed as before. Data were analyzed using the LIGAND program (Munson and Rodbard, 1980) to obtain a representative value of the binding affinity constant and the concentration of receptors for all toxins. K_{com} is used as the binding constant instead of K_d, due to the two-step binding process (reversible plus irreversible) taking place (Burton *et al*., 1999; Wu and Dean, 1996).

2.7 Ligand blotting.

BBMV proteins (15 μg) were separated by SDS-8% PAGE and transferred to a polyvinylidiene difluoride Q membrane filter (PVDF) (Millipore). The filter was blocked for one hour in 3% BSA in TBST (25 mM Tris [pH 7.5], 3 mM KCl, 135 mM NaCl, 0.1% Tween-20) and then cut into strips for the different treatments after washing. Subsequent incubations and washes were done in 0.1% BSA TBST.

Ligand blots with radiolabeled toxins were done by incubating the filters with 1 x 10^6 cpm of ^{125}I-labeled toxin in 10 ml of 0.1% BSA TBST for three hours. After this, membranes were washed before exposure to film overnight at -80°C.

For biotinylated toxin ligand blots, the blocked filters were incubated with biotin-toxins (1.6 nM) for one hour. After washing, filters were incubated with anti-biotin (Sigma) antibody (1:50,000 dilution) for an hour. Binding proteins were visualized using the ECL kit (Amersham-Pharmacia) following the manufacturer's instructions.

For the ligand blots with antibodies against the toxins, blocked filters were incubated with toxin (5 nM) for one hour. After washing, filters were incubated with rabbit polyclonal antibodies against Cry1Ac or Cry1Fa (1:5,000 dilution) for an hour. After washing, filters were incubated with donkey anti-rabbit peroxidase conjugated antibody (1:30,000 dilution) (Amersham-pharmacia) for another hour. Binding proteins were visualized with an ECL kit as for biotinylated toxins.

RESULTS.

3.1 Toxicity of Cry1 toxins to *H. virescens*.

As shown in Figure 2.1, each purified toxin appeared as a single band on SDS-PAGE after staining or autoradiography for ^{125}I-labeled Cry1A toxins. Table 2.1 shows the results of bioassays conducted with *H. virescens*. As previously reported, Cry1Aa, Cry1Ab, Cry1Ac and Cry1Fa are highly toxic to *H. virescens*, but not Cry1Ea or Cry1Ja (Ge *et al*., 1991; Lee *et al*., 1995; Oddou *et al*., 1991; Van Rie *et al*., 1989, 1990). Cry1Aa was more toxic than in some previous reports (Van Rie *et al*., 1989, 1990). Bioassays with an independent Cry1Aa preparation (kindly provided by L. Potvin,

National Research Council of Canada, Montreal) gave the same toxicity results. Peptide mapping analyses confirmed the identity and purity of our Cry1Aa preparation (data not shown).

As activity of biotinylated Cry1Fa and Cry1Ja toxins are unknown, we performed bioassays with these modified toxins. Activity of these biotinylated toxins against *H. virescens* was the same as unmodified Cry1Fa and Cry1Ja.

3.2 Binding of ^{125}I-Cry1A toxins to BBMV.

Total binding experiments done with ^{125}I-labeled toxins and various concentrations of BBMV provided the basis for selecting BBMV concentrations appropriate for subsequent competition experiments. The levels of ^{125}I-Cry1Aa, ^{125}I-Cry1Ab and ^{125}I-Cry1Ac binding shown in Figure 2.2 were similar to previous studies (Lee *et al*., 1995; Van Rie *et al*., 1989). The amounts of BBMV needed to reach maximum binding were different for the three toxins, and they correlate directly with their *in vivo* activity against *H. virescens*. Thus, the most toxic toxin, Cry1Ac reached maximum specific binding at 50 μg/ml of BBMV, while Cry1Aa (the least toxic of the Cry1A toxins tested) maximum binding was increasing up to 1 mg/ml. These binding data identified the lowest BBMV concentration that gave maximal specific binding. According to the binding observed, 40 μg (for Cry1Aa), 10 μg (for Cry1Ab) and 5 μg (for Cry1Ac) were selected as the BBMV concentrations for binding competition experiments.

3.3 Competitive binding with ^{125}I-Cry1A toxins.

Using unlabeled Cry1Aa, Cry1Ab, Cry1Ac, Cry1Fa, and Cry1Ja as competitors, we performed heterologous binding competition experiments. As negative controls we also tested Cry2Aa and Cry1Ea toxins and neither toxin competed with labeled Cry1A toxins (Cry2Aa data not shown).

When using ^{125}I-Cry1Aa, high affinity competitive binding was observed with Cry1Aa, Cry1Ab and Cry1Ac (Figure 2.3). These toxins competed up to 90% of ^{125}I-Cry1Aa binding at low nM concentrations. Cry1Fa and Cry1Ja both competed with ^{125}I-Cry1Aa, although the levels of affinity were lower, as reflected in the K_{com} (Table 2.2). Cry1Ja and Cry1Fa competed 90% of the ^{125}I-Cry1Aa binding, although ten times more toxin was required to reach the 90% level of competition in the case of Cry1Fa. The fact that these unlabeled toxins competed up to 90% of the ^{125}I-Cry1Aa binding is evidence that these toxins all recognize the single population of Cry1Aa binding sites present in the BBMV.

In competition experiments, only the heterologous Cry1Ac and the homologous Cry1Ab competed with high-affinity for ^{125}I-Cry1Ab binding sites. While Cry1Aa, Cry1Fa and Cry1Ja reduced the amount of ^{125}I-Cry1Ab bound, relatively higher concentrations of competitor toxins were needed and lower total competition was observed. These results support a two binding site model, whereby Cry1Aa, Cry1Fa and Cry1Ja have low-affinity and Cry1Ac high affinity for one Cry1Ab site. Only the homologous toxin and Cry1Ac recognize this Cry1Ab binding site.

In heterologous competition assays Cry1Aa competed with a low affinity (K_{com} = 300.0 nM) for 40% of the ^{125}I-Cry1Ac binding sites. Cry1Ab showed higher affinity

(K_{com} = 79.7 nM) than Cry1Aa for ^{125}I-Cry1Ac binding sites, competing for more than 60% of the ^{125}I-Cry1Ac binding. Cry1Ac K_{com} was 2.6 nM and maximal competition was 90%. Cry1Fa and Cry1Ja competed with low affinity for a Cry1Ac binding site. These binding results are evidence for a population of receptors recognized exclusively by Cry1Ac, a second population recognized by Cry1Ac and Cry1Ab and a third population recognized by Cry1Ac, Cry1Aa, Cry1Ab, Cry1Fa and Cry1Ja.

3.4 Ligand blot analyses.

Ligand blotting with BBMV was performed to relate the *H. virescens* Cry1 receptor model constructed from BBMV binding studies, with Cry1 toxin binding proteins. Ligand blotting with ^{125}I-labeled Cry toxins has inherent challenges due to differences in toxin labeling efficiency and, as with Cry1F (Luo *et al*., 1999), loss of toxicity attributed to modification of critical amino acids. We employed ^{125}I-labeled toxins, biotinylated toxins and anti-Cry protein antibodies to ensure that toxin binding proteins visualized on blots were a consequence of toxin recognition and not the labeling technique.

Binding molecules detected with ^{125}I-Cry1A toxins are shown in Fig. 2.4 (lanes 2-4). Each ^{125}I-Cry1A toxin bound to 170-kDa and 110-kDa proteins. Apart from these common molecules, ^{125}I-Cry1Ab and ^{125}I-Cry1Ac also bound to a 130-kDa protein. ^{125}I-Cry1Ac also bound to 100-kDa and smaller-sized molecules.

We used biotinylated Cry1Fa and Cry1Ja to detect binding molecules on blots (Fig 2.4B). As a comparison, ligand blots with biotinylated Cry1A toxins are also shown (lanes 5, 6 and 7). Biotinylated Cry1A toxins recognized the same molecules as

radiolabeled Cry1A toxins. Although both Cry1Fa and Cry1Ja bound to the 170-kDa and 110-kDa molecules, unlike Cry1Ab and Cry1Ac, binding to the 130-kDa protein was almost absent.

Direct detection of bound toxin on blots with anti-toxin antibodies avoids the covalent modification of the toxin, but requires antibodies against each toxin studied. Immunoblots with Cry1Ac and Cry1Fa revealed the same patterns of binding molecules as seen with biotinylated toxins (Fig 2.4, lanes 10-12). In these experiments, Cry1Fa bound to the 130-kDa protein when using higher amounts of toxin (lane 12).

DISCUSSION.

The objective of this study was to construct a model for the binding sites in *H. virescens* recognized by five Cry1 toxins sharing high homology in domain II loops.

We determined the *in vivo* potencies of the selected toxins. Cry1Aa, Cry1Ab, Cry1Ac and Cry1Fa were highly toxic to *H. virescens* while Cry1Ja only kill larvae at high concentrations. Cry1Aa was about 10-fold more toxic than reported by Van Rie *et al*. (1989), but in agreement with Ge *et al*. (1991). Since the LC_{50} values for Cry1Ab and Cry1Ac are similar to reported values, there may be differences in Cry1Aa susceptibility between populations of *H. virescens*. We also tested the activity of biotinylated Cry1Fa and Cry1Ja toxins, since the toxicities of these modified toxins were unknown. Bioassay results are evidence that biotinylated Cry1Fa and Cry1Ja toxins retain their activity against *H. virescens*.

Cry1A binding to *H. virescens* fits a three-site model (Van Rie *et al*., 1989) (Fig 2.5). As expected from this model (Lee *et al*., 1995; Van Rie *et al*., 1989), Cry1Aa,

Cry1Ab and Cry1Ac competed with high affinity (1 nM range) for ^{125}I-Cry1Aa binding sites. Based on Cry1Fa and Cry1Ja competition with ^{125}I-Cry1Aa and ^{125}I-Cry1Ab we conclude that those toxins recognize receptor A. Cry1Fa and Cry1Ja had high affinity (12.6 nM and 2.8 nM respectively) for receptor A in ^{125}I-Cry1Aa binding assays. The Cry1Fa and Cry1Ja K_{com} values for ^{125}I-Cry1Ab and ^{125}I-Cry1Ac were of considerably lower affinity (212 nM-340 nM) indicating these toxins have low affinity for the receptor shared with these toxins (receptor A). The ability of Cry1Ja toxin to bind with high affinity, but not kill is analogous to Cry1Ac binding to BBMV from *Spodoptera frugiperda* (Garczynski *et al*., 1991).

Receptor B is a high-affinity binding site for Cry1Ab and Cry1Ac (Table 2.2) (Van Rie *et al*., 1989). Cry1Aa, Cry1Fa and Cry1Ja do not recognize receptor B. The competition of Cry1Ab for ^{125}I-Cry1Ac binding (K_{com}= 79.7 nM) is possibly the composite of competition for receptors A and B.

Cry1Ac was the only toxin tested to recognize Receptor C (Fig 2.5). Van Rie *et al*. (1989) proposed the existence of receptor C. An argument for receptor C follows. Since Cry1Ac competes all ^{125}I-Cry1Aa (Fig 2.3A) and ^{125}ICry1Ab (Fig 2.3B), but the reciprocal heterologous competition was not detected (Fig 2.3C), there must exist a population of receptors unique to Cry1Ac and this population is receptor C.

Our ligand blotting results were internally consistent for detection of similar-sized binding proteins by three techniques (^{125}I-labeled Cry1A toxins, antibodies against biotin-labeled Cry toxins, or antibodies against Cry1Ac and Cry1Fa). Similar patterns have been previously reported for ^{125}I-Cry1A toxins (Cowles *et al*., 1995; Garczynski *et al*., 1991; Luo *et al*., 1997). ^{125}I-labeled Cry1A toxins bound to 170-kDa and 110-kDa

proteins on ligand blots. Cry1Fa and Cry1Ja bound the 170-kDa and 110-kDa proteins. The 170-kDa protein is an isoform of APN. Both 170-kDa and 110-kDa proteins reacted with anti-aminopeptidase N serum (data not shown).

Although ligand blot data has to be interpreted with caution (Lee *et al*., 1996), in our model of Cry1 binding proteins (Figure 2.5), binding competition experiments and ligand blots observations agree. Based on the definition of receptor A being recognized by Cry1Aa, Cry1Ab and Cry1Ac, receptor A would be comprised of 170-kDa and 110-kDa proteins. How do we reconcile this model with the previous designation of the 170-kDa APN as a low affinity binding site for Cry1Ac and receptor A (Oltean et al., 1999)?. It is not surprising that multiple molecules bind related Cry1A toxins, since this has been shown previously in *Manduca sexta* (Schwartz *et al*., 1997). Schwartz *et al.* (1997) reported that multiple binding proteins were associated in a complex containing glycosylphosphatidyl inositol-anchored proteins. Also, it is possible that the high affinity Cry1Ac binding site is a consequence of combined affinity for the 170-kDa and 110-kDa molecules.

Modification of receptor A in a Cry1Ac-selected strain (YHD2) of *H. virescens* is implicated in high levels of resistance against Cry1Ac and cross-resistance to Cry1Aa and Cry1Ab (Lee *et al*., 1995). Apparently, the observed loss of Cry1Aa binding in strain YHD2 was due to a modification of Receptor A (Lee *et al*., 1995). Our vesicle binding and ligand blot results may explain the *H. virescens* cross-resistance to Cry1Fa (Gould et al., 1995) and Cry1Ja (F. Gould, unpublished personal communication) observed in strain YHD2: the modification of the shared receptor A also affects Cry1Fa

and Cry1Ja binding and toxicity. This highlights the important role of the shared receptor A for toxicity in *H. virescens*, as suggested previously (Lee *et al*., 1995).

Cry1Ab and Cry1Ac also recognized a 130-kDa molecule. The 130-kDA molecule was recognized slightly by Cry1Aa, but not by biotinylated Cry1Fa and Cry1Ja. Cry1Fa binding to the 130-kDa molecule was visualized only when high amounts of toxin (10 nM) were used, suggesting that the 130-kDa protein is a low affinity-binding molecule for Cry1Fa. The 130-kDa protein recognized by Cry1Ac andCry1Ab on ligand blots is a candidate for receptor B.

Molecules of 100-kDa and smaller size were recognized only by ^{125}I-Cry1Ac. These molecules may constitute receptor C, although some of these proteins seem to also bind Cry1Ja and Cry1Fa. We occasionally detected a 205-kDa molecule that bound Cry1 toxins, although this observation seemed to depend on the efficacy of the transfer due to the high molecular size of this molecule. Cry1A binding molecules of this size in ligand blots have been previously described for *M. sexta* (Martínez-Ramírez *et al*., 1994).

The 170-kDa and 110-kDa proteins bind Cry1A toxins and toxins with the highest homology in domain II loops: Cry1Fa and Cry1Ja. This domain II homology suggests that binding to receptor A is specified with the loops of domain II. In agreement with this notion, greatly decreased toxicity and reduced binding to the 170-kDa protein has been reported for a Cry1Ab with mutated domain II loop 2 amino acids (Rajamohan *et al*., 1996a). Specificity of binding to receptor B and C seems not to be related to this homology, since high homology does not relate to competition for these sites.

Our data also implies that resistance in *H. virescens* strain YHD2 is directed against the homologous domain II loops in these toxins. This conclusion is especially

relevant when considering strategies to decrease the development of *H. virescens* resistance to Bt toxins. Thus, toxins with low homology to Cry1A toxins in domain II loops are reasonable alternative toxins to Cry1A toxins in Bt plant and biopesticide formulations. In support of this strategy, high levels of toxicity against the resistant YHD2 strain are reported for transgenic tobacco plants producing Cry2Aa2 (Kota *et al*., 1999). Cry2Aa2 clusters in a group distant from Cry1A toxins in a domain II loops on a sequence similarity dendrogram (Tabashnik *et al*., 1996).

The availability of receptor models for Cry toxins provides a framework for exploring mechanisms of resistance and reduces the chance of selecting toxin combinations that promote cross-resistance. Our results encourage further investigations into the relationship between domain II loops and toxin binding in *H. virescens*.

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FIGURES AND TABLES.

Table 2.1 Toxicity of purified Cry1 toxins to neonate larvae of *Heliothis virescens* .

Toxin	LC_{50}(95% fiducial limits)[a]	Slope ± SE[b]
Cry1Aa	5.0 (2.9-7.4)	1.8 ± 0.3
Cry1Ab	1.5 (0.8-2.3)	1.7 ± 0.2
Cry1Ac	0.5 (0.1-1.1)	1.6 ± 0.2
Cry1Fa	1.9 (1.2-2.5)	3.2 ± 0.7
Cry1Ja	660 (464.3-921.4)	3.2 ± 0.9
Cry1Ea	>9000	2.8 ± 0.6
Cry1Fa-Biotin	1.9 (0.5-5.1)	1.4 ± 0.2
Cry1Ja-Biotin	793 (246-5989)	3.2 ± 1.2

[a] LC_{50} is expressed ng of protein per square centimeter of artificial diet.
[b] SE, standard error of the mean.

Figure 2.1- SDS-PAGE (A) and autoradiography (B) analyses of purified and ^{125}I-labeled Cry1 toxins. Lane 1, Cry1Aa; lane 2, Cry1Ab; lane 3, Cry1Ac; lane 4, Cry1Fa; lane 5, Cry1Ea; lane 6, Cry1Ja; lane 7, ^{125}I-Cry1Aa; lane 8, ^{125}I-Cry1Ab; and lane 9, ^{125}I-Cry1Ac. Positions of molecular mass markers (in kilodaltons) are indicated on the left.

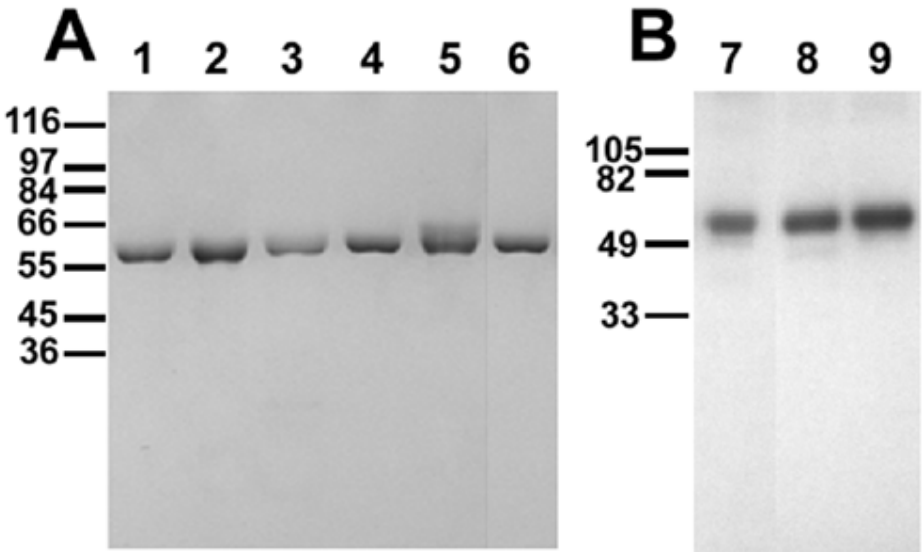
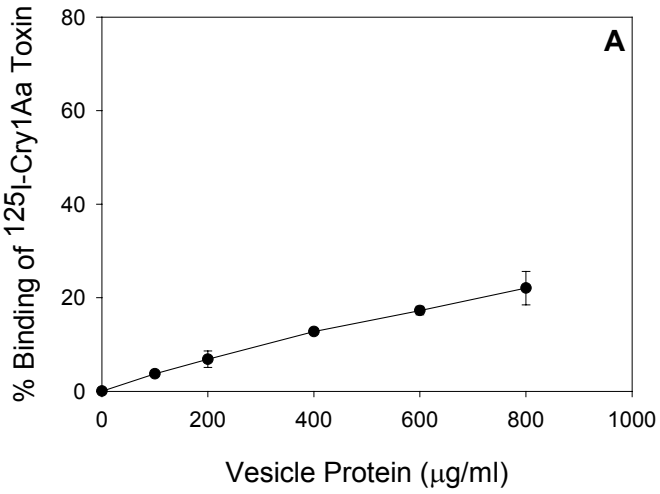
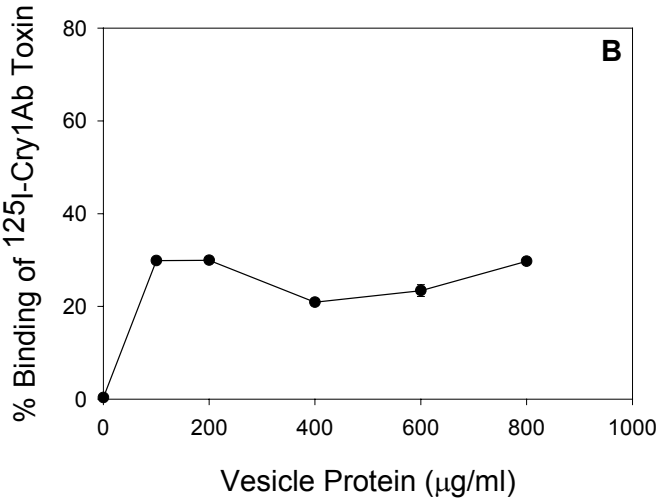

A
1
2
3
4
5
6
116
97
84
66
55
45
36
B
7
8
9
105
82
49
33

Figure 2.2- Specific binding of ^{125}I-Cry1Aa (A), ^{125}I-Cry1Ab (B) and ^{125}I-Cry1Ac (C) toxins to *H. virescens* BBMV. Vesicles at the concentrations indicated were incubated with ^{125}I-Cry1A toxins. Binding is expressed as a percentage of input ^{125}I-Cry1A. Binding in the presence of 1000 nM unlabeled homologous toxin was subtracted from total binding. Maximum non-specific binding was 20% (for ^{125}I-Cry1Aa) or 40% (for ^{125}I-Cry1Ab and ^{125}I-Cry1Ac) of total binding. Each data point is the average of the means based on independent trials with duplicate samples. Standard deviation of the mean values are depicted by error bars.


A
% Binding of 125I-Cry1Aa Toxin
0
20
40
60
80
0
200
400
600
800
1000
Vesicle Protein (μg/ml)


B
% Binding of 125I-Cry1Ab Toxin
0
20
40
60
80
0
200
400
600
800
1000
Vesicle Protein (μg/ml)

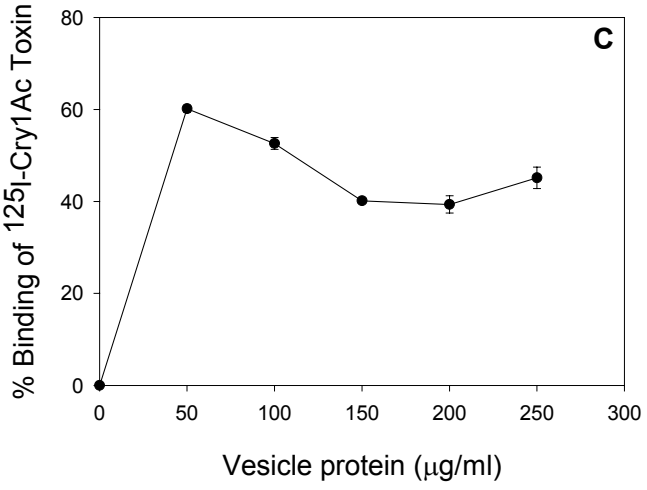
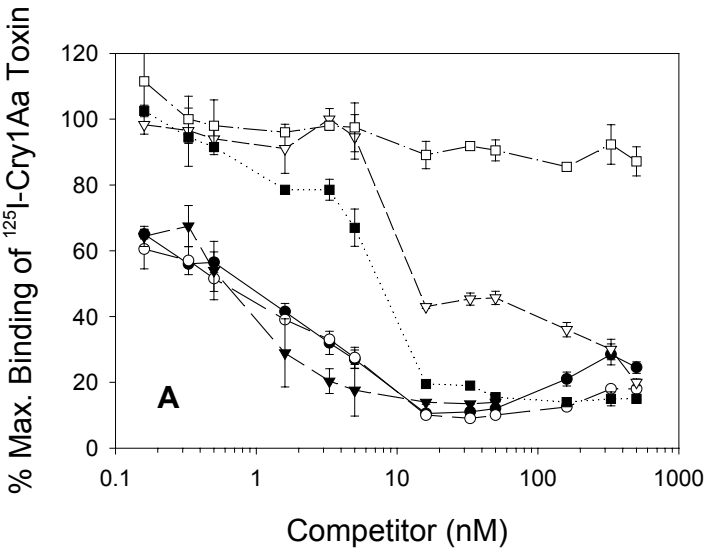
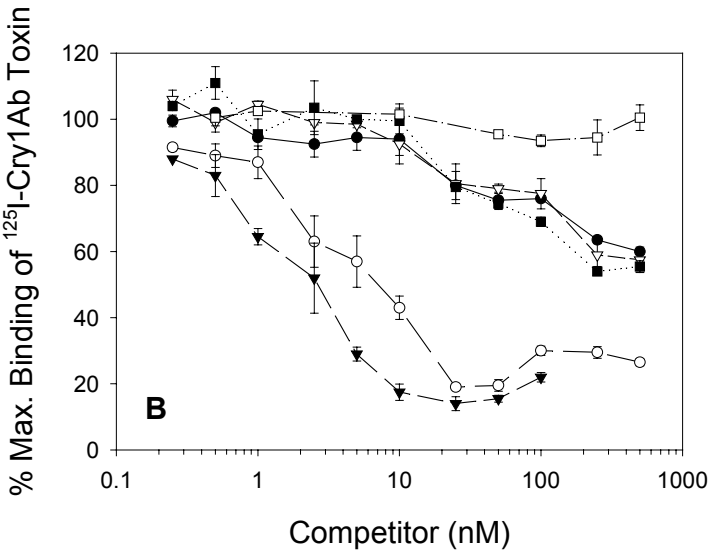

C
% Binding of 125I-Cry1Ac Toxin
0
20
40
60
80
0
50
100
150
200
250
300
Vesicle protein (μg/ml)

Figure 2.3- Binding competition between ^{125}I-Cry1Aa (A), ^{125}I-Cry1Ab (B), and ^{125}I-Cry1Ac (C) and unlabeled Cry1Aa (●), Cry1Ab (○), Cry1Ac (▼), Cry1Fa (▽), Cry1Ja (■), or Cry1Ea (□). *H. virescens* BBMV were incubated with ^{125}I-Cry1A toxins at a concentration of 0.5 nM (^{125}I-Cry1Aa) or 0.1 nM (^{125}I-Cry1Ab and ^{125}I-Cry1Ac) plus increasing concentrations of unlabeled toxins. Binding was expressed as a percentage of the maximum amount of toxin bound during incubation with labeled toxin. Each data point is a mean based on data from independent trials. Standard deviation of the mean values are depicted by error bars.


% Max. Binding of ^{125}I-Cry1Aa Toxin
120
100
80
60
40
20
0
A
0.1
1
10
100
1000
Competitor (nM)


% Max. Binding of ^{125}I-Cry1Ab Toxin
120
100
80
60
40
20
0
B
0.1
1
10
100
1000
Competitor (nM)

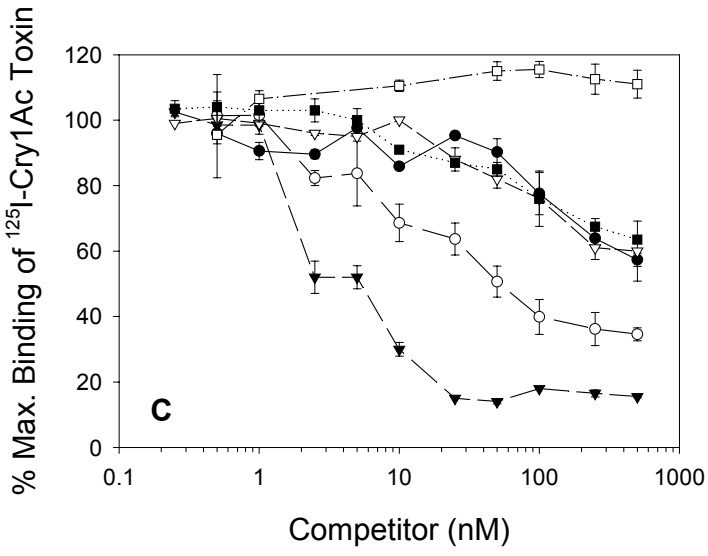

% Max. Binding of ^{125}I-Cry1Ac Toxin
120
100
80
60
40
20
0
C
0.1
1
10
100
1000
Competitor (nM)

Table 2.2- K_{com}s and concentrations of binding sites (R_ts) of Cry1 toxins on BBMV from *H. virescens*.

	^{125}I-Cry1Aa		^{125}I-Cry1Ab		^{125}I-Cry1Ac	
Toxin	K_{com} (nM) ± SE	R_t (nM) ± SE	K_{com} (nM) ± SE	R_t (nM) ± SE	K_{com} (nM) ± SE	R_t (nM) ± SE
Cry1Aa	1.2 ± 0.4	0.2 ± 0.0	318.0 ± 66.0	69.5 ± 13.0	300.0 ± 46.0	142.00 ± 15.6
Cry1Ab	2.0 ± 0.6	0.3 ± 0.2	3.5 ± 0.5	1.1 ± 0.1	79.7 ± 22.0	16.6 ± 3.7
Cry1Ac	2.0 ± 0.9	0.4 ± 0.1	1.1 ± 0.2	0.2 ± 0.0	2.6 ± 0.9	0.9 ± 0.1
Cry1Fa	12.6 ± 0.4	5.0 ± 0.6	297.5 ± 62.5	64.2 ± 11.4	236.5 ± 29.5	97.2 ± 10.2
Cry1Ja	2.8 ± 1.0	0.5 ± 0.1	212.0 ± 83.0	6.5 ± 2.1	340.0 ± 69.0	34.9 ± 23.7

Figure 2.4- Ligand blot analyses of SDS-PAGE separated *H. virescens* BBMV proteins. Panel A: lane 1, BBMV proteins transferred to a PVDF filter detected by Coomassie blue staining; lanes 2-4, autoradiography of blots incubated with: lane 2, ^{125}I-Cry1Aa; lane 3, ^{125}I-Cry1Ab; lane 4, ^{125}I-Cry1Ac. Panel B: blots were incubated with biotinylated toxins, then anti-biotin antibody-peroxidase conjugate and detection was enhanced by chemiluminescence. Lane 5, Cry1Aa; lane 6, CryAb; lane 7, Cry1Ac; lane 8, Cry1Fa; and lane 9, Cry1Ja. Panel C: blots were incubated with 5 nM Cry1Ac (lane 10), 5 nM Cry1Fa (lane 11), or 10 nM Cry1Fa (lane 12). Primary antibody was anti-Cry1Ac or Cry1Fa sera, secondary antibody was anti-rabbit peroxidase and detection was enhanced by chemiluminescence. Positions of molecular mass markers (in kilodaltons) are indicated on the left of each panel.

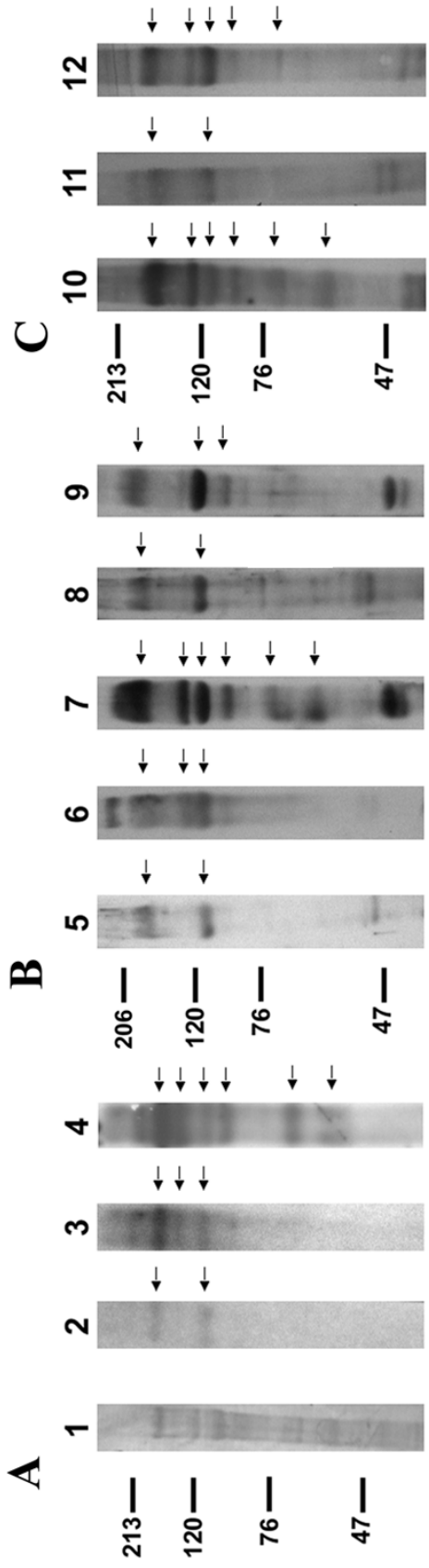
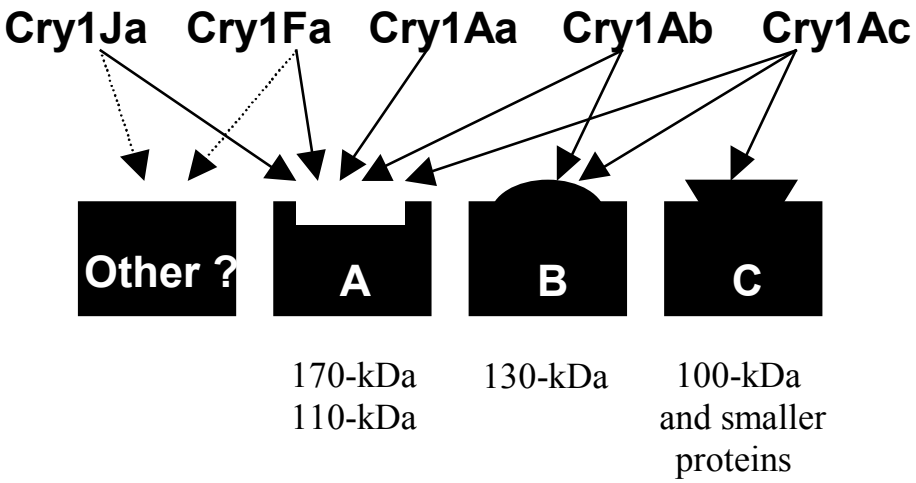

A
1
213
120
76
47
2
3
4
B
206
120
76
47
5
6
7
8
9
C
213
120
76
47
10
11
12

Figure 2.5- Model proposed for binding of *B. thuringiensis* Cry1 toxins to sites in the *H. virescens* midgut membrane. Binding proteins correlated with specific sites are listed. Dashed arrows indicate predicted, but not determined sites.


Cry1Ja
Cry1Fa
Cry1Aa
Cry1Ab
Cry1Ac
Other ?
A
B
C
170-kDa
110-kDa
130-kDa
100-kDa
and smaller
proteins

CHAPTER 3

INCREASED LEVELS OF *HELIOTHIS VIRESCENS* RESISTANCE AND CROSS-RESISTANCE TO *BACILLUS THURINGIENSIS* CRY1 TOXINS CORRELATE WITH REDUCED TOXIN BINDING, PORE FORMATION AND ALTERED GLYCOSYLATION.[1]

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[1] Jurat-Fuentes, Juan Luis, Fred L. Gould, and Michael J. Adang
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ABSTRACT

Binding and pore formation abilities of Cry1A and Cry1Fa *Bacillus thuringiensis* toxins were analyzed using brush border membrane vesicles (BBMV) prepared from susceptible (YDK) and resistant (YHD2) strains of *Heliothis virescens*. ^{125}I-labeled Cry1Aa, Cry1Ab and Cry1Ac toxins did not bind to BBMV from the resistant YHD2 strain, while specific binding to susceptible YDK vesicles was observed. Binding assays showed a reduction in Cry1Fa binding to BBMV from resistant larvae when compared to susceptible BBMV. In agreement with this binding reduction, neither Cry1A nor Cry1Fa toxins altered the permeability of membrane vesicles from resistant larvae, as measured by a light scattering assay. Ligand blotting experiments using BBMV and ^{125}I-Cry1Ac did not differentiate susceptible from resistant larvae. Iodination of BBMV surface proteins suggested that putative toxin-binding proteins were exposed on the surface of the BBMV from resistant insects.

BBMV protein blots probed with the N-acetylgalactosamine-specific lectin soybean agglutinin (SBA), revealed altered glycosylation patterns for BBMV proteins from resistant insects of 63- and 68-kDa in size. The F1 progeny of crosses between susceptible and resistant insects were similar to the susceptible strain when tested by toxin binding assays, light scattering assays and lectin blots with SBA. These results suggest that a dramatic reduction in toxin binding is responsible for the increased resistance and cross-resistance to Cry1 toxins observed in the YHD2 strain of *H. virescens*, and that this trait correlates with altered glycosylation of specific BBMV glycoproteins.

INTRODUCTION.

Development of resistance by target insect pests is one of the major concerns associated with long-term use of insecticides based on *Bacillus thuringiensis* (Bt) δ-endotoxins. These so-called Cry toxins are synthesized during sporulation as proteinaceous crystals (Schnepf *et al*., 1998). Upon ingestion by a susceptible insect, the Cry1 protoxin form (130 to 140-kDa) is solubilized and activated by midgut proteinases to a toxic form (55 to 65-kDa) that binds to specific receptors on the brush border membrane of midgut cells. After binding, toxins oligomerize and insert in the membrane forming pores that lead to cell lysis and insect death (Aronson *et al*., 1999).

To date, only *Plutella xylostella* (diamondback moth) has attained high levels of resistance and cross-resistance to Bt insecticides in the field (Ferré and Van Rie, 2002), while other insect species have developed resistance after laboratory selection. Although different mechanisms of resistance have been proposed, the best characterized is the alteration of binding to specific receptors in the midgut (Ferré and Van Rie, 2002).

Heliothis virescens (tobacco budworm) has shown the ability to develop resistance and cross-resistance to Bt toxins after selection in the laboratory (MacIntosh *et al*., 1991; Gould *et al*., 1992, 1995). In this insect, three populations of receptors (A, B and C) for Cry1 toxins have been proposed (Van Rie *et al*., 1989; Jurat-Fuentes and Adang, 2001). According to this model, receptor A binds Cry1A, Cry1Fa and Cry1Ja toxins; receptor B binds Cry1Ab and Cry1Ac; and receptor C binds only Cry1Ac.

Notably, the Cry1Ac-selected YHD2 strain developed the highest level of resistance reported to this toxin, and cross-resistance to Cry1Aa, Cry1Ab and Cry1Fa (Gould *et al*., 1995). When the binding properties of Cry1A toxins to brush border

membrane vesicles (BBMV) prepared from midguts of resistant YHD2 larvae were analyzed, only Cry1Aa showed reduced binding. Both Cry1Ab and Cry1Ac bound specifically to BBMV from YHD2 with the same parameters as BBMV from susceptible YDK larvae (Lee *et al*., 1995). No differences in toxin stability or irreversible binding of Cry1Ac were detected between susceptible and resistant larvae in the same study. These results suggested that alteration of binding to the shared Cry1A receptor was the mechanism of resistance against these toxins.

Recently, a retrotransposon-mediated disruption of a cadherin-superfamily gene has been proposed as a mechanism to explain resistance to Cry1A toxins in YHD2 (Gahan *et al*., 2001). This disruption would prevent full-length expression of a putative toxin binding protein named HevCadLP (for *Heliothis* cadherin-like protein). The potential role of the HevCadLP protein in Cry1 toxin mode of action has not been demonstrated yet. Although hypothetical, absence of this protein in YHD2 insects would explain the absence of Cry1Aa binding observed by Lee *et al.* (1995). This hypothesis identifies the HevCadLP as the shared Cry1A receptor (receptor A) in *H. virescens*.

The YHD2 strain has been under continuous Cry1Ac selection since the experiments conducted by Lee *et al.* (1995), and the resistance ratio is believed to have increased (F. Gould, unpublished data). This increased resistance suggests that YHD2 has changed, and that additional mechanism(s) of resistance to Cry1Ac may have developed in this strain, besides lack of full-length HevCadLP expression. In fact, the mechanism reported by Gahan *et al.* (2001) would only explain 40-80% of Cry1Ac resistance in YHD2, and other linkage groups contribute to Cry1Ac resistance in this strain (Heckel *et al*., 1997).

In the present study, our bioassays with YDK and YHD2 neonates indicated that resistance to Cry1Ac has increased when compared to Lee *et al.* (1995). Furthermore, both Cry1A and Cry1Fa toxins showed reduced binding to BBMV from YHD2 insects, and this reduction correlated with reduced toxin pore formation in this strain. Ligand blots with radiolabeled Cry1Ac toxin confirmed that toxin-binding molecules were still present in BBMV from YHD2 larvae. Labeling of BBMV surface proteins attested the availability of potential Cry1Ac binding proteins on the surface of BBMV from YHD2. Lectin blots with soybean agglutinin (SBA) detected differences in glycosylation between some BBMV proteins from YDK and YHD2 insects. These differences correlated with transmission of susceptibility in the F1 progeny from crosses between susceptible and resistant adults.

These results suggest that an additional resistance mechanism is currently working in the YHD2 strain. This additional mechanism leads to reduced Cry1Ab and Cry1Ac toxin binding and pore formation relative to the results from Lee *et al.* (1995). Reduced toxin-binding correlates with altered glycosylation of specific BBMV proteins.

MATERIALS AND METHODS.

2.1 Insect strains and bioassays.

Heliothis virescens strains YDK and YHD2, as well as bioassay protocols have been previously described (Gould *et al*., 1995). F1 larvae were obtained after mating YDK to YHD2 adults, as described in Gould *et al.* (1995). For bioassays, five concentrations of each toxin were tested in diet incorporation bioassays with neonate

larvae for 7 days. Mortality data were analyzed using the probit procedure in SAS (SAS institute, 1982) with correction for control mortality.

Due to the high levels of resistance of YHD2 larvae to Cry1Ac, 10-day growth tests of neonates on toxin-incorporated diet were also carried out to obtain more accurate resistance ratio values for this toxin. Larval weight was log-transformed and a regression of log weight on Cry1Ac toxin concentration was run. The toxin concentration needed to decrease 10-day larval weight to 1/10 of the expected weight (when no toxin was present on diet) was estimated. Resistance ratios were calculated as the concentration of toxin that decreased growth rate by 10-fold for YHD2 or larvae from the F1 generation of YDKxYHD2 crosses divided by the concentration estimated for YDK.

2.2 Bacterial strains and toxin purification.

Bacterial strains producing individual toxins, toxin activation and purification were as described in Jurat-Fuentes and Adang (2001). Fractions containing pure toxin (as determined by SDS-PAGE) were pooled and stored at –80^{o}C until used. Protein concentrations were determined using the method of Bradford (1976).

2.3 Midgut isolation and BBMV preparation.

Midguts were isolated from fifth instar *H. virescens* larvae, washed in ice-cold MET buffer (250mM mannitol, 17mM Tris/HCl, 5mM EGTA, pH 7.5), frozen on dry ice and stored at –80°C.

BBMV were prepared as described elsewhere (Wolfersberger *et al*., 1987). The final BBMV pellet was suspended in ice-cold TBS buffer (25 mM Tris/HCl [pH 7.5], 3

mM KCl, 100 mM NaCl), and protein concentration determined using the method of Bradford (1976).

Aminopeptidase activity, a marker enzyme for lepidopteran brush border membrane preparations (Terra and Ferreira, 1994), was assayed using leucine *p*-nitroanilide as substrate (Garczynski and Adang, 1995). Aminopeptidase activity was 5 to 8 times higher in BBMV preparations relative to the initial homogenate (data not shown).

2.4 Labeling of Cry1 toxins.

Cry1A toxins were labeled with $Na^{125}I$ using the chloramine-T method (Garczynski *et al*., 1991). Toxins (1 μg) were labeled with 0.5 mCi of $Na^{125}I$. Specific activities of ^{125}I-labeled toxins were 20-40 mCi/mg (based on input toxin). The purity of ^{125}I-labeled toxins was verified by SDS-PAGE and radiography (not shown).

Cry1Ac and Cry1Fa were biotinylated with EZ-Link Sulfo-NHS-LC-Biotin (Pierce) as previously described (Jurat-Fuentes and Adang, 2001).

Radiolabeling (Cry1Aa, Cry1Ab, Cry1Ac) or biotinylation (Cry1Ac and Cry1Fa) do not alter Cry1 toxin activity (Jurat-Fuentes and Adang, 2001; Van Rie *et al*., 1989).

2.5 Binding experiments with intact BBMVs.

Specific binding assays were performed three times in duplicate as described in Jurat-Fuentes and Adang (2001). Radioactivity was measured with a Beckman Gamma 4000 detector. The amount of non-specific binding was determined by adding 1 μM of

the respective unlabeled toxin to the initial reaction mixtures. Specific binding was expressed as the difference between total and non-specific binding.

Qualitative binding assays were performed with 200 μg/ml BBMV proteins and 0.1 nM ^{125}I-Cry1Ac in 0.1 ml of binding buffer (TBS plus 0.1% BSA) at room temperature for 1 hour. For biotinylated toxins, BBMV were incubated with 12 nM biotinylated Cry1Ac or Cry1Fa. After centrifugation, pellets were washed twice with 0.5 ml of binding buffer, and the final BBMV pellets were solubilized and electrophoresed in 10% SDS-PAGE. For ^{125}I-toxins, gels were dried and exposed to film (Kodak XAR-5) for one hour to detect the presence of bound toxin.

Bound biotinylated toxins were detected by Western blotting. BBMV and bound toxin were electrophoresed and transferred to polyvinylidiene difluoride Q membrane filters (PVDF) (Millipore) in transfer buffer (48 mM Tris, 390 mM glycine, 0.1% [w/v] SDS, 20% methanol, pH 8.3). After blocking in TBST buffer (25 mM Tris/HCl [pH 7.5], 3 mM KCl, 100 mM NaCl, 0.1% Tween-20) containing 3% BSA, membranes were incubated with streptavidin-peroxidase conjugate in TBST plus 0.1% BSA for one hour. Biotinylated toxins were visualized using ECL (Amersham Pharmacia) reagents following the manufacturer's instructions.

2.6 BBMV permeability assay.

BBMV solute permeability was studied using the recordings of 450 nm light scattered at a 90^{o} angle from incidence by a BBMV suspension, in a stop flow spectrofluorimeter (model RSM 1000, On-line Instrument Systems, Bogart, GA).

Freshly prepared BBMV were suspended in 10 mM Tris/HCl (pH 7.5), diluted to 0.4 mg/ml in the same buffer and incubated for 1 hour on ice followed by 10 min incubation at room temperature before starting the light scattering experiments. Baseline measurements were obtained by injecting the same volume of BBMV and isosmotic buffer (10 mM Tris/HCl, pH 7.5) into the cuvette in the spectrofluorimeter sample compartment. Incident 450-nm light scattered at 90^{o} from incidence was monitored for 60 seconds by obtaining five measurements per second. To determine the amount of light scattered after osmotically induced shrinkage, measurements were obtained after simultaneous injection of same volumes of BBMV and hyperosmotic solution (500 mM KCl, 10 mM Tris/HCl, pH 7.5). In preliminary trials, the response of BBMV from YDK insects to Cry1Ac was dose dependent, while the non-toxic Cry1Ea had no effect on overall BBMV permeability (data not shown). Based on these results, a 100 nM dosage of toxin (final concentration in cuvette) in hyperosmotic buffer was selected.

Because YHD2 insects are resistant to all toxins used in this study, nystatin, a pore forming macrolide, served as a positive control. 835 units of nystatin (Sigma) in DMSO were added to the hyperosmotic buffer. DMSO in hyperosmotic buffer had no effect on BBMV permeability (data not shown).

Toxin-induced pore formation on the BBMV was monitored as a decrease in the amount of light scattered due to re-swelling of the vesicles. To quantify these data, rates of swelling of BBMV during a 1-minute period after toxin addition were calculated using the RSM-1000 data fitting software. Kinetic swelling traces were fitted to a first order process ($Y=A_0.e[-k_1.t]$) as best fit suggested by the robust global fitting program (I. B. C. Matheson©, On-line Instrument Systems, Bogart, GA). Results are expressed as the

calculated rate for each toxin treatment minus the rate observed for hyperosmotic solution alone. Presented rates are the mean of at least three measurements from independent BBMV preparations and light scattering experiments.

2.7 Ligand blotting.

For ligand blots, BBMV proteins (15 μg) were separated by SDS-PAGE and electrotransferred onto PVDF filters. Protein-blotted membranes were blocked with TBST buffer containing 3% BSA, then incubated with 0.05 nM ^{125}I-Cry1Ac at room temperature for 90 minutes. After washing, filters were allowed to dry and then exposed to Kodak XAR-5 film with an intensifying screen at –80^{o}C for 24-48 hours.

Immunoblots to detect aminopeptidase-N (APN) were performed using a 1:3,000 dilution of serum developed against the 120-kDa aminopeptidase-N (APN) protein from *Manduca sexta.* Bound antibodies were detected using anti-rabbit-horseradish peroxidase conjugate (SIGMA) and visualized by enhanced chemiluminescence (Santa-Cruz Biotechnology, Santa Cruz, CA).

Lectin blots were conducted by probing BBMV proteins on PVDF filters with 1 μg/ml of peroxidase-labeled soybean agglutinin (SBA) (Sigma) for one hour. After washing, SBA blots were developed using enhanced chemiluminescence substrate (Santa-Cruz Biotechnology, Santa Cruz, CA).

The intensity of SBA recognition of specific BBMV proteins was quantified by spot densitometry using AlphaImager^{TM} software (Alpha Innotech Corporation, San Leandro, CA). This program assigns a percentage of Integrated Density Value (IDV) to each protein, taking the sum of signal intensity of all the proteins as 100% IDV. The

density values obtained from at least four independent experiments were used on One-way ANOVA statistical tests with $\alpha<0.050$ (SigmaStat statistical software, SPSS Science, Chicago, IL) to test for statistically significant differences in SBA recognition between the strains.

2.8 Radio-iodination of BBMV surface proteins.

BBMV surface proteins were radio-iodinated with $Na^{125}I$ using a lactoperoxidase-catalyzed reaction (Huber and Morrison, 1973). All reactions were carried out on ice with ice-cold materials. Briefly, 100 μg of BBMV proteins were suspended in 100 μl of PBS buffer (135 mM NaCl, 2 mM KCl, 10 mM Na_2HPO_4, 1.7 mM KH_2PO_4, pH 7.5) including protease inhibitors (complete®, Pierce). Lactoperoxidase (0.4 mg/ml from bovine milk, Sigma) was included in each reaction. $Na^{125}I$ was treated with 1 mM sodium sulfite to reduce any I_2 (permeable through cell membrane) to I^- (does not permeate through membrane) and then 0.15 mCi was added to each reaction. Reactions were carried out by adding six 10 μl aliquots of a 0.03 % hydrogen peroxide solution at 2-min intervals, and were stopped by centrifugation. ^{125}I-BBMV pellets were washed four times with ice-cold PBS and resuspended in 150 μl of the same buffer. Ten microliters of each sample were loaded on 8%-SDS-PAGE. Gels were dried and exposed to Kodak XAR-5 photographic film with an intensifying screen at –80^{o}C for less than one hour to detect radiolabeled surface proteins.

RESULTS.

3.1 Bioassays.

Cry1A and Cry1Fa toxins were highly active against YDK, but not YHD2 larvae (Table 3.1). Cry1Ea was inactive against YDK and YHD2 larvae. YHD2 larvae were resistant to Cry1Ac, and cross-resistant to Cry1Aa, Cry1Ab, and Cry1Fa as previously reported (Gould *et al*., 1995; Lee *et al*., 1995).

No mortality of YHD2 larvae was observed using 2 mg/ml of Cry1Ac toxin. Since Lee *et al.* (1995) reported 0.5 mg/ml as the LC_{50} for this toxin in YHD2, this is evidence that resistance to Cry1Ac has increased after further laboratory selection. In our bioassays, we did not observe any mortality at the highest Cry1Aa, Cry1Ab or Cry1Fa concentrations used, and when compared with the results for YDK larvae, cross-resistance to these toxins was evident.

Although mortality for YHD2 was not observed at any toxin concentration tested, the 1,000 μg/ml concentration of Cry1Ac caused decreased growth of the YHD2 larvae. Therefore, to obtain a resistance ratio for this toxin we performed 10-day growth tests of neonates on diet with incorporated Cry1Ac (Table 3.1). From these growth bioassays, a 73,700-fold resistance ratio against Cry1Ac was obtained for YHD2 larvae. In these experiments, larvae from the F1 generation of the crossing between YDK and YHD2 adults were 6-fold resistant to Cry1Ac. Since susceptibility of the F1 larvae was independent of the sex of the resistant progenitor, maternal-inheritance or sex linkage of Cry1Ac resistance in YHD2 were considered unimportant.

Results from our bioassays show that resistance to Cry1Ac has increased in the YHD2 strain, when compared to previous reports (Lee *et al*., 1995), and that resistance is

inherited as an incompletely recessive autosomal trait, as previously demonstrated (Gould *et al*., 1995).

3.2 Binding assays performed with the susceptible (YDK) and resistant (YHD2) strains.

We tested BBMV prepared from YDK and YHD2 larvae for their ability to bind three ^{125}I-labeled Cry1A toxins. Figure 3.1 shows that all ^{125}I-Cry1A toxins bound specifically to BBMV from susceptible YDK larvae. Maximum specific binding of each ^{125}I-Cry1A toxin to YDK BBMV was in agreement with Lee *et al.* (1995). For BBMV from the resistant YHD2 strain, the lack of ^{125}I-Cry1Aa binding was anticipated based on Lee *et al.* (1995). Unexpectedly, ^{125}I-Cry1Ab and ^{125}I-Cry1Ac did not bind to YHD2 BBMV (Fig. 3.1). Even at the highest BBMV concentration tested no specific binding to BBMV from YHD2 was measured for any of the ^{125}I-Cry1A toxins tested. This indicated that the YHD2 strain had undergone genetic changes in toxin binding since tested by Lee *et al.* (1995).

We also measured ^{125}I-Cry1Ac binding to BBMV prepared from larvae of the F1 progeny from matings of susceptible and resistant moths. ^{125}I-Cry1Ac bound specifically to F1 BBMV, though the extent of specific binding was slightly reduced when compared to YDK BBMV (Fig. 3.1C). ^{125}I-Cry1Ac binding was independent of the sex of the resistant parent, evidencing that the binding trait is not sex-linked (data not shown). Binding data for F1 BBMV suggested a recessive inheritance of the reduced binding.

Because Cry1Fa was highly toxic to YDK larvae and it shares binding sites with Cry1A toxins in *H. virescens* BBMV (Jurat-Fuentes and Adang, 2001), we tested the

hypothesis that YHD2 larvae cross-resistant to Cry1Fa had reduced Cry1Fa binding. Since iodination inactivates Cry1Fa (Luo *et al*., 1999), we biotinylated Cry1Fa and its binding to BBMV was detected by enhanced chemiluminescence. The intensity of toxin signal seen in Fig. 3.2 provided a qualitative measure of binding. As a control, we compared Cry1Ac binding detected by two methods: radiography of bound ^{125}I-Cry1Ac (Fig. 3.2A) and Western blotting with biotinylated Cry1Ac (Fig. 3.2B). Decreased toxin binding to YHD2 BBMV was observed by both techniques. Figure 3.2C shows the results of the biotinylated Cry1Fa binding experiment performed with susceptible and resistant BBMV. Cry1Fa binding to YDK was observed as for Cry1Ac, and Cry1Fa binding to YHD2 BBMV was undetectable.

Results from toxin-binding experiments demonstrate that increased resistance and cross-resistance in the YHD2 strain correlated with reduction of toxin binding.

3.3 Permeability Assays.

The rationale for permeability assays follows. If YHD2 resistance to Cry1 toxins was due to reduced toxin binding, toxin-induced pore formation should be reduced in YHD2 BBMV. The light scattering technique indirectly measures a Cry toxin's capacity to permeate membranes, i.e. form pores (Carroll and Ellar, 1993). In a hyperosmotic environment, Cry toxin pores allow entry of KCl and water into BBMV, resulting in BBMV swelling and decreased scattered light. The rate of BBMV reswelling provides a measure of the rate of membrane permeation by the toxins.

The results of light scattering experiments performed with Cry1Ab, Cry1Ac, and Cry1Fa toxins are shown in Fig. 3.3. Each toxin increased membrane permeability of

YDK, but not YHD2 BBMV. Nystatin, a pore forming antibiotic, permeated both YDK and YHD2 vesicles, confirming that both BBMVs could respond in the light scattering assay. The light scattering experiments supported the conclusion that Cry1Ab, Cry1Ac and Cry1Fa toxin binding was reduced in brush border membrane vesicles of YHD2 larvae when compared to YDK BBMV.

Using the light scattering technique, we measured Cry1-induced membrane permeation in BBMV from the F1 larvae used in binding experiments (Fig. 3.3). The rationale was that since toxin binding was observed in F1 BBMV, toxin-induced pore formation would also be restored. Cry1Ab and Cry1Ac induced rates of swelling in F1 BBMV were intermediate between YHD2 and YDK BBMV. These results agreed with the binding data and provided further evidence of the recessive nature of the resistance trait**.**

3.4 Toxin binding molecules in susceptible and resistant BBMV.

We performed ligand blotting to ascertain if reduced toxin binding was correlated to absence of toxin binding molecules in the YHD2 vesicles. The patterns of ^{125}I-Cry1Ac toxin-binding molecules in BBMV from YDK and YHD2 appeared identical (Fig. 3.4B). Identical results were obtained when using ^{125}I-Cry1Aa, ^{125}I-Cry1Ab or biotinylated Cry1Fa as probes (data not shown).

A specific 170-kDa APN protein from *H. virescens* BBMV suggested to represent a shared Cry1A binding protein has been shown to catalyze both toxin binding and pore formation (Luo *et al*., 1997). Because of this, we studied the possibility that absence of this toxin binding molecule would lead to the reduced toxin binding observed in the

BBMV binding assays. Immunoblots performed with BBMV proteins from YDK and YHD2 were probed with serum developed against the 120-kDa APN from *M. sexta*, which cross-reacts with APNs from *H. virescens* (Banks *et al*., 2001). As shown in Fig. 3.4C, no detectable changes in either quantity or size of the 170-kDa APN were observed when comparing BBMV from YDK and YHD2 insects.

To test the possibility that content of BBMV surface proteins was changed in resistant larvae, BBMV surface proteins were radiolabeled using a lactoperoxidase-catalyzed reaction. As shown in Fig. 3.4D, no obvious differences in surface exposed proteins were observed between BBMV from YDK or YHD2 insects.

Therefore, absence of toxin binding molecules did not appear to be the reason for toxin binding reduction to BBMV from YHD2.

3.5 Protein blots of YHD2 and YDK BBMV probed with SBA.

The first step of Cry1Ac recognition of its midgut receptors is dependent on N-acetylgalactosamine (GalNAc) moieties (Jenkins *et al*., 2000). Therefore, we tested for GalNAc residues on BBMV proteins by probing blots with the GalNAc-specific lectin from soybean (SBA). SBA bound to BBMV proteins of 170- and 120-kDa, as expected from previous analyses of *H. virescens* BBMV (Knowles *et al*., 1991; Lee *et al*., 1996). Several proteins of less than 100-kDa in size were also detected by our SBA blots (Fig. 3.5). Interestingly, some of these proteins exhibited reduced SBA recognition in BBMV from YHD2, especially proteins of 68- and 63-kDa. These proteins regained SBA recognition levels observed for YDK vesicles in BBMV from F1 crosses (Fig. 3.5, lane 3). To quantify these differences, binding of SBA to the 68- and 63-kDa BBMV proteins

was quantified by spot densitometry (Fig. 3.6). One-way ANOVA statistical tests revealed that SBA recognition of the 68- and 63-kDa proteins was decreased in BBMV from the YHD2 insects when compared to YDK or F1 vesicles. Densitometry measurements of SBA binding to the 120-kDa APN in the different strains were used as a control. These results suggest a correlation between decreased toxin binding, resistance and altered glycosylation of these proteins.

DISCUSSION.

Reduced binding of Cry1 toxins is a widely documented mechanism of insect resistance to *B. thuringiensis* toxins (Ferré and Van Rie, 2002). However, for *H. virescens* the role of reduced toxin binding in insect resistance has been less clear (MacIntosh *et al*., 1991; Lee *et al*., 1995). Specifically, Lee *et al.* (1995) reported that while Cry1Aa binding to BBMV from YHD2 larvae was reduced, Cry1Ab and Cry1Ac binding was unchanged. The binding data presented here for Cry1Aa, Cry1Ab, Cry1Ac and Cry1Fa are evidence that increased levels of resistance and cross-resistance to these toxins in the *H. virescens* strain YHD2 are due to reduced toxin binding.

Using the light scattering technique we correlate for the first time decreased binding with absence of toxin-induced pore formation in BBMV from resistant larvae. This absence of pore formation on BBMV from the YHD2 strain was observed for all Cry1 toxins tested and is evidence for the important effect of toxin binding reduction in toxicity.

Western blotting and light scattering experiments also allowed us to study Cry1Fa binding and pore formation in BBMV from susceptible and resistant insects. This toxin

is highly toxic to *H. virescens* larvae and has been considered as an alternative to Cry1Ac in transgenic crops aimed to control this insect. Our results provide evidence that cross-resistance to Cry1Fa in YHD2 is also due to a dramatic decrease in toxin binding that prevents pore formation. This suggests that the same mechanism of resistance may confer cross-resistance to distantly related toxins.

Inheritance of resistance in the YHD2 strain has been reported to be autosomal and incompletely recessive, although closer to codominance than to complete recessivity (Ferré and Van Rie, 2002; Gould *et al*., 1995) as in other cases of resistance to Cry1 toxins due to toxin binding reduction (Ferré and Van Rie, 2002). Our binding and pore formation data are in agreement with this genetic characterization of YHD2 resistance. BBMV from the F1 crosses showed levels of binding and pore formation between YDK and YHD2 parents. Toxin binding and pore formation were not dependent on the sex of the resistant parent, suggesting that the resistance trait is not sex-linked (data not shown).

Strain YHD2 has changed during the past five years: resistance to Cry1Ac in this strain has increased, and Cry1A toxin binding is virtually eliminated. In 1995, toxin binding to BBMV from YHD2 larvae was only reduced for Cry1Aa (Lee *et al*., 1995). Recently, Gahan *et al.* (2001), have proposed a mechanism of resistance for YHD2 based on retrotransposon-mediated disruption of a cadherin-superfamily gene that results in the absence of a wild-type form of a specific cadherin-like protein (HevCadLP) in the midgut of YHD2 insects. Cadherin-like proteins have previously been reported to bind Cry1A toxins in *Bombyx mori* (Nagamatsu *et al*., 1998) and *Manduca sexta* (Keeton *et al*., 1997), although binding of Cry1A toxins to a cadherin-like protein in *H. virescens* has only been hypothesized (Banks *et al*., 2001). Absence of HevCadLP would account for

40% to 80 % of Cry1Ac resistance in YHD2 insects (Heckel *et al*., 1997). Insects for the work of Gahan *et al.* (2001) were obtained when Lee *et al.* (1995) performed their toxin binding experiments (F. Gould, personal communication). Thus, absence of HevCadLP may explain the Cry1Aa binding reduction observed by Lee *et al.* (1995). The fact that resistance in YHD2 has increased and that Cry1Ab and Cry1Ac binding is also reduced at the time of the current experiment, suggests that an additional mechanism of resistance has appeared in this strain. Alternatively, this additional mechanism could have also already existed in the YHD2 strain in 1995 and it has become more effective or widespread in the YHD2 colony after laboratory selection.

H. virescens has multiple binding proteins for Cry1Ac (Cowles *et al*., 1995; Jurat-Fuentes and Adang, 2001). Mutations in Cry1Ac domain III revealed the existence of multiple functional receptors in *M. sexta* (Burton *et al*., 1999) and *H. virescens* (Lee *et al*., 1999). One class of receptor depends on Cry1Ac recognition of GalNAc, while a second class is GalNAc independent (Lee *et al*., 1999). Though speculative, the fact that Cry1Ac binding is absent in YHD2 and that reduction in toxin binding correlates with increased resistance, suggests that while a single site may be most important, other binding sites contribute to toxicity. Lee *et al*. (1995) proposed the existence of 'null receptors' for Cry1 toxins in *H. virescens* BBMV. These 'null receptors' accounted for Cry1Ab and Cry1Ac binding, but not killing in YHD2 insects. Our results suggest that these "null" receptors actually have a role in toxicity, since reduction of Cry1Ab and Cry1Ac toxin binding to these receptors appears to correlate with increased resistance in the YHD2 strain.

Reduced toxin binding could be due to absence of specific binding proteins in BBMV from YHD2 insects. To study this possibility, we performed ligand blotting and immunoblots with BBMV proteins. No differences in ^{125}I-Cry1Ac toxin-binding molecules were observed between BBMV from susceptible and resistant larvae. The same results were obtained using ^{125}I-Cry1Aa, ^{125}I-Cry1Ab or biotinylated Cry1Fa toxin as probes (data not shown). ^{125}I-Cry1Ac bound to 170-, 120-, and 110-kDa aminopeptidases plus other smaller-sized proteins in BBMV from both YDK and YHD2 insects. This pattern of Cry1 toxin binding molecules has been previously described in *H. virescens* (8, 14, 22, 25). Our results from ligand blotting are evidence that toxin-binding molecules are still present in the BBMV from YHD2 insects. Lack of differences in toxin binding molecules from ligand blots between Cry1A susceptible and resistant insects has been previously reported (Lee *et al*., 1995; Luo *et al*., 1997; Mohammed *et al*., 1996) and could be due to denaturing conditions involved in SDS-PAGE electrophoresis and ligand blotting. Furthermore, radio iodination of BBMV surface proteins did not reveal any difference between YDK and YHD2 vesicles.

Strain YHD2 has a transposon disrupting the open reading frame in the cadherin-like HevCadLP gene (Gahan *et al*., 2001). We did not detect a Cry1Ac binding protein of the expected ~200-kDa size on ligand blots of susceptible larvae. A phenomenon is reported for BtR175, the cadherin-like protein from *B. mori*, that may explain this observation. Native BtR175 binds Cry1Aa with high affinity, but Cry1Aa binding on ligand blots is greatly reduced (Nagamatsu *et al*., 1998). Alternatively, the BBMV purification method may not be selective for HevCadLP.

The importance of carbohydrate moieties in Cry1Ac (Knowles *et al*., 1991), Cry1Ab5 (Denolf *et al*., 1997) and Cry1B (Hoffmann *et al*., 1988) toxin binding to insect BBMV has been reported previously. More specifically, Cry1Ac recognizes GalNAc moieties in APN as a first binding step (Jenkins *et al*., 2000). Recently, Griffitts *et al.* (2001) presented evidence for a glycosyltransferase enzyme being the source of altered glycosylation leading to resistance and cross-resistance to Bt toxins in *Caenorhabditis elegans.* To study if altered glycosylation was related to Cry1Ac resistance in YHD2, lectin blotting using SBA was performed. Although it also recognizes galactose with low affinity, SBA is the lectin with the highest affinity for binding to GalNAc moieties (Pereira *et al*., 1974). SBA lectin blots showed decreased SBA recognition of several brush border membrane proteins in resistant BBMV. These proteins were readily recognized by SBA in both susceptible and F1 BBMV. Decreased SBA recognition is probably due to altered glycosylation of these proteins. Spot densitometry was used to quantify SBA recognition of two proteins of 68- and 63-kDa in BBMV from YDK, YHD2 and F1 insects. Statistical tests confirmed that SBA recognition of these proteins was significantly reduced in YHD2 BBMV when compared to either YDK or F1 BBMV proteins. There were no statistically significant differences between SBA recognition of the 68- or 63-kDa proteins from YDK and F1. Thus, changes in glycosylation of the 68- and 63-kDa proteins seem to correlate with reduced toxin binding and resistance. Preliminary experiments in our laboratory confirm that Cry1Ac binds to the carbohydrate moieties recognized by SBA (GalNAc) in these proteins (Jurat-Fuentes *et al.*, in preparation). Proteins of 68- and 63-kDa containing GalNAc have been described in mosquito midgut microvillae (Wilkins and Billingsley, 2001).

Cry1A and Cry1Fa toxins share high sequence homology in a series of protruding loops in domain II of the three-domain tertiary structure of these proteins (Tabashnik *et al*., 1996). These loops are involved in toxin binding specificity to *H. virescens* BBMV proteins (Jurat-Fuentes and Adang, 2001). The pattern of cross-resistance in YHD2 follows selection against this domain II sequence homology. Although speculative, this may suggest that binding of domain II to a shared binding site is altered in YHD2 larvae. Interestingly, domain II of Cry1Aa shares structural similarity to the plant lectins jacalin (Sankaranarayanan *et al*., 1996) and *Maclura pomifera* agglutinin (Lee *et al*., 1998), among other carbohydrate binding proteins (Burton *et al*., 1999). Both jacalin and *M. pomifera* lectins bind with high affinity to Galβ(1,3)GalNAc residues, although they also recognize other galactose and galactosamine derivatives, which complicates glycoprotein analyses with these lectins. From our results, we hypothesize a resistance mechanism in YHD2 insects that confers reduced binding of various Cry1 toxins by altering a shared carbohydrate binding epitope recognized by domain II of these toxins.

Altered glycosylation is known to have important consequences in pathogenesis (Bond *et al*., 1997; Chabot *et al*., 2000) as well as insect resistance (Heckel *et al*., 1997; Small and Hemingway, 2000). In some cases, modifications of specific carbohydrates mask recognition epitopes used by pathogens (Varki, 1993). In our case, reduced toxin binding and pore formation due to altered glycosylation of toxin-binding moieties might be one of the potential molecular mechanisms involved in Cry1 resistance in YHD2. Further work aimed to identify these toxin-binding glycoproteins and the specific alterations that lead to reduced toxin binding is currently being addressed in our laboratory.

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FIGURES AND TABLES.

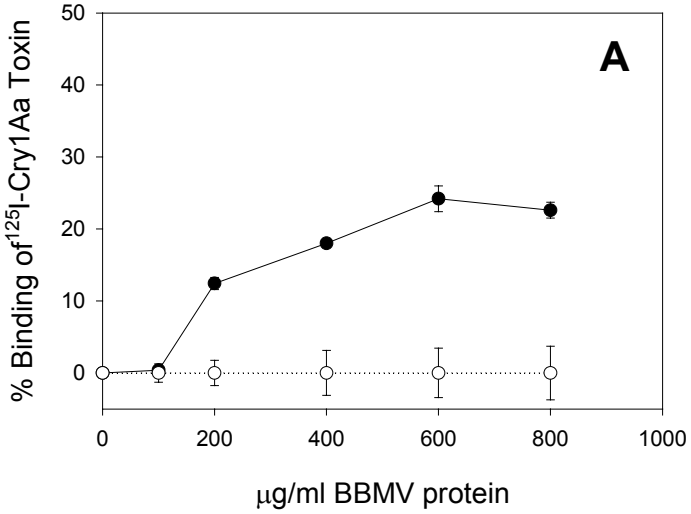
Table 3.1- Toxicity and resistance ratios of Cry1 toxins to susceptible (YDK) and resistant (YHD2) *H. virescens* neonates.

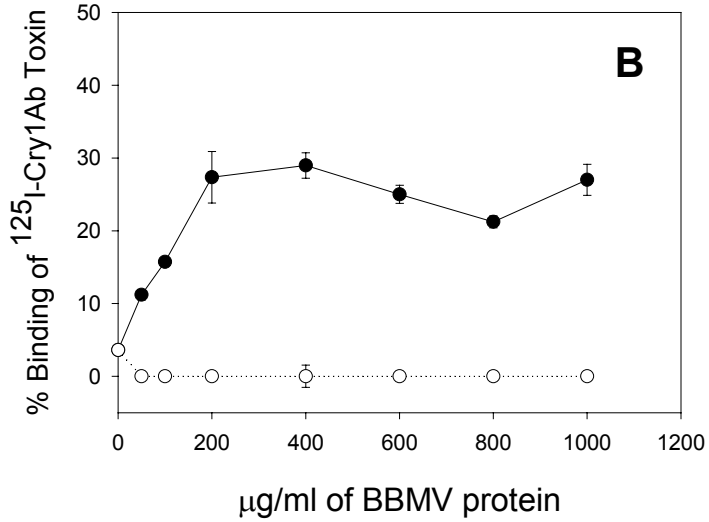
Toxin	YDK LC_{50} (μg/ml)	YHD2 LC_{50}(μg/ml)	RR[a]
Cry1Aa[b]	2.07 (1.06-4.87)	>55[c]	>25
Cry1Ab	7.56 (4.49-19.55)	>900[c]	>119
Cry1Ac	0.93 (0.33-1.39)	>2,000[c]	>2,000
Cry1Fa	3.86 (2.67-5.54)	>130[c]	>33
Cry1Ea	>560	>560	N/A

Strain	intercept	slope	Cry1Ac concentration for 1/10 growth[d]	RR
YDK	2.570	-23.55	0.042	1.0
YDK x YHD2	2.797	-3.436	0.291	6.8
YHD2 x YDK	2.776	-4.707	0.212	5.0
YHD2	2.887	-0.0003	3125.0	73,703

[a] Resistance ratio (LC_{50} YHD2/LC_{50} YDK).
[b] Cry1Aa LC_{50} values are expressed in micrograms per square centimeter of diet.
[c] No mortality observed at this concentration of toxin.
[d] Cry1Ac concentration values are expressed in micrograms of toxin per milliliter of diet.
N/A not applicable.

Figure 3.1- Specific binding of ^{125}I-labeled Cry1Aa (A), Cry1Ab (B), and Cry1Ac (C) toxins to BBMV from YDK (●), YHD2 (O), or F1 (▼) insects. Vesicles at the indicated concentrations were incubated with ^{125}I-Cry1A toxins at 0.3 nM (Cry1Aa) or 0.1 nM (Cry1Ab and Cry1Ac) for one hour. Binding reactions were stopped by centrifugation. Nonspecific binding in the presence of 1,000 nM unlabeled toxin was subtracted from total binding. Bars denote standard error of the mean.


A
% Binding of 125I-Cry1Aa Toxin
0
10
20
30
40
50
0
200
400
600
800
1000
μg/ml BBMV protein


B
% Binding of 125I-Cry1Ab Toxin
0
10
20
30
40
50
0
200
400
600
800
1000
1200
μg/ml of BBMV protein

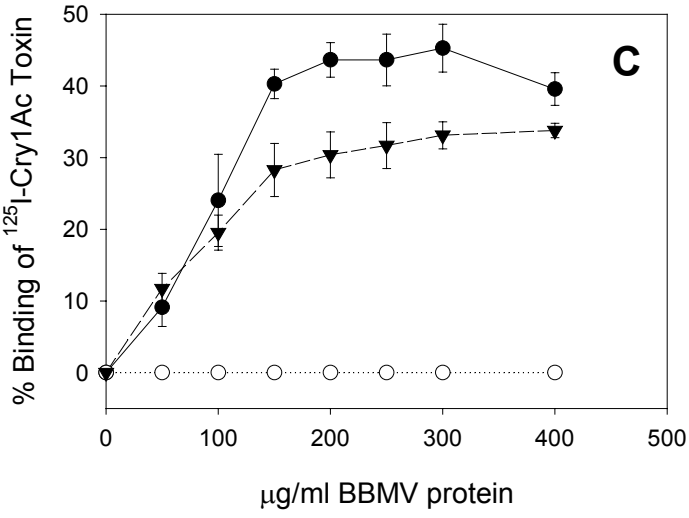

C
% Binding of 125I-Cry1Ac Toxin
0
10
20
30
40
50
0
100
200
300
400
500
μg/ml BBMV protein

Figure 3.2- Binding of ^{125}I-Cry1Ac (A), biotinylated Cry1Ac (B) and biotinylated Cry1Fa (C) toxins to BBMV from YDK (lanes 1) or YHD2 (lanes 2) insects. Toxins were incubated with BBMV proteins (20 μg) for one hour. Binding reactions were stopped by centrifugation and washed pellets were separated by SDS-PAGE. Gels were dried and autoradiographed (A) or transferred to PVDF filters. Biotinylated Cry1Ac (B) and Cry1Fa (C) were detected with streptavidin-peroxidase conjugate and enhanced chemiluminescence.

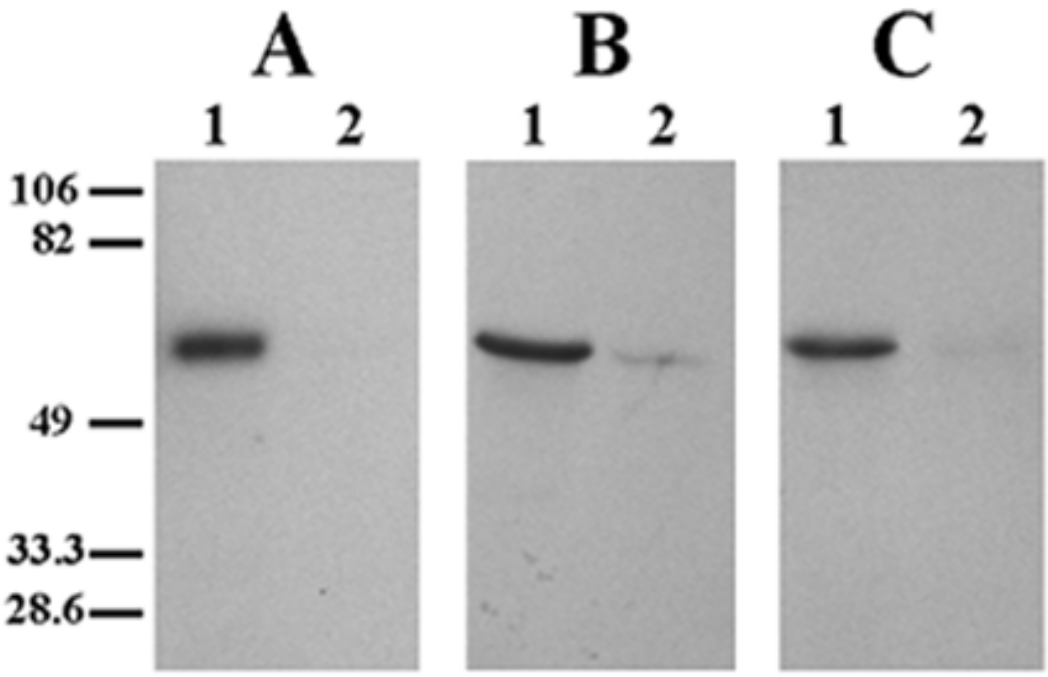

A
B
C
1
2
1
2
1
2
106
82
49
33.3
28.6

Figure 3.3- Rates (decrease in scattered light per second) of BBMV swelling after challenge with a KCl hyperosmotic solution containing a specific Cry1 toxin. YDK (black bars), F1 (gray bars), and YHD2 (white bars) vesicles were mixed with a hyperosmotic solution containing toxin or nystatin. The rates of swelling after shrinkage as calculated from 1-min. measurements are shown with standard errors of the mean.

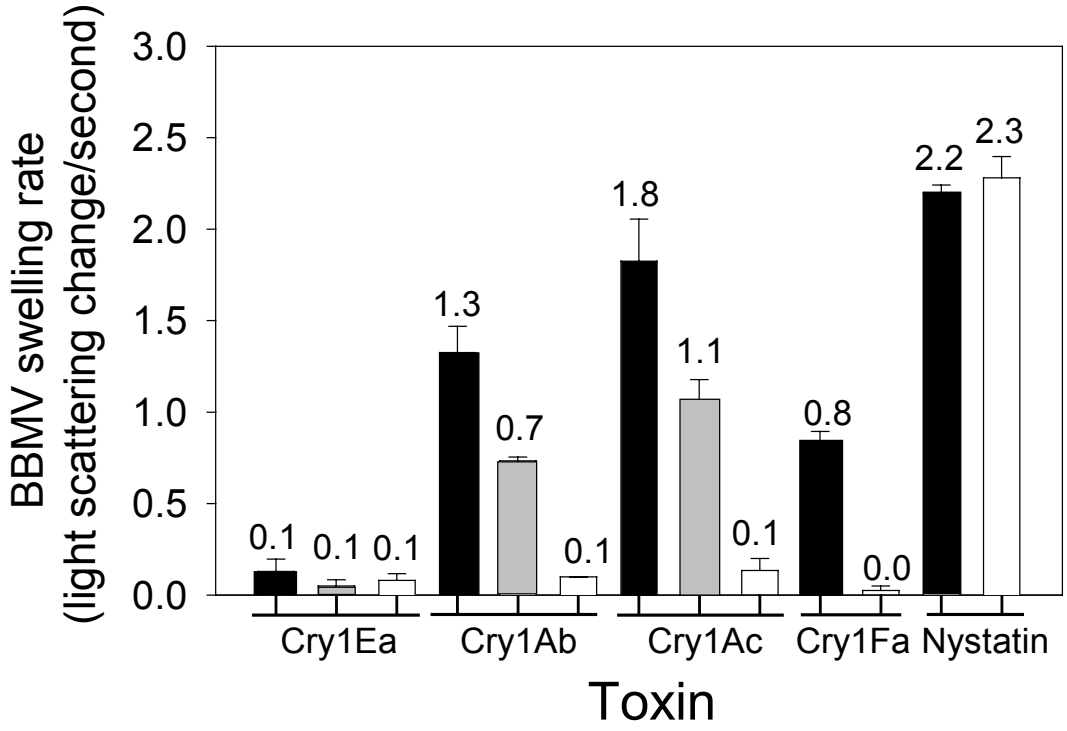

BBMV swelling rate
(light scattering change/second)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0.1
0.1
0.1
1.3
0.7
0.1
1.8
1.1
0.1
0.8
0.0
2.2
2.3
Cry1Ea
Cry1Ab
Cry1Ac
Cry1Fa
Nystatin
Toxin

Figure 3.4- Ligand blotting, immunoblotting and BBMV surface protein radio-iodination. For ligand blotting, 20 μg of BBMV proteins from YDK (lane 1) or YHD2 (lane 2) insects were separated by SDS-PAGE and either Coomassie blue stained (A) or electrotransferred to PVDF filters (B and C). Filters were probed with 0.1 nM ^{125}I-Cry1Ac toxin for one hour (B), then washed, dried and exposed to photographic film for 12 hours. Immunoblotting was completed by probing BBMV proteins with serum against the 120-kDa APN from *M. sexta* (C). For BBMV surface protein iodination (D), 100 μg of BBMV proteins from YDK (lane 1) or YHD2 (lane 2) insects were radiolabeled as described in the methods section.

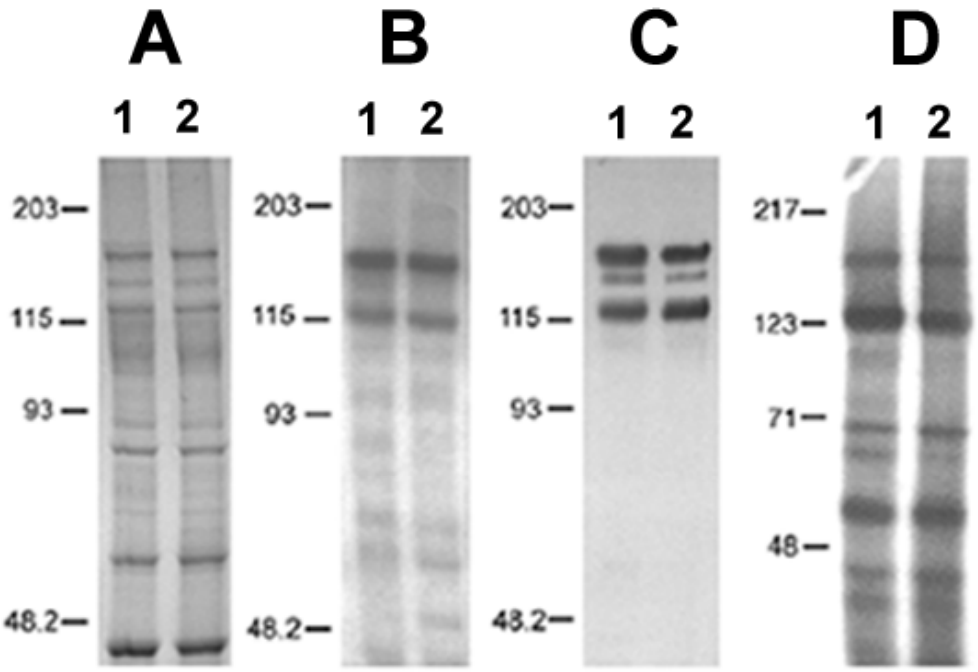

A
1 2
203
115
93
48.2
B
1 2
203
115
93
48.2
C
1 2
203
115
93
48.2
D
1 2
217
123
71
48

Figure 3.5- SBA lectin blots of BBMV from YDK, YHD2 and F1 larvae. BBMV proteins (15 μg) from YDK (lane 1), YHD2 (lane 2), or F1 (lane 3) insects were separated by SDS-PAGE, electrotransferred to PVDF filters and probed with peroxidase-conjugated SBA to detect GalNAc moieties. Detection was by enhanced chemiluminescence. Arrows indicate proteins selected for spot densitometry analysis.

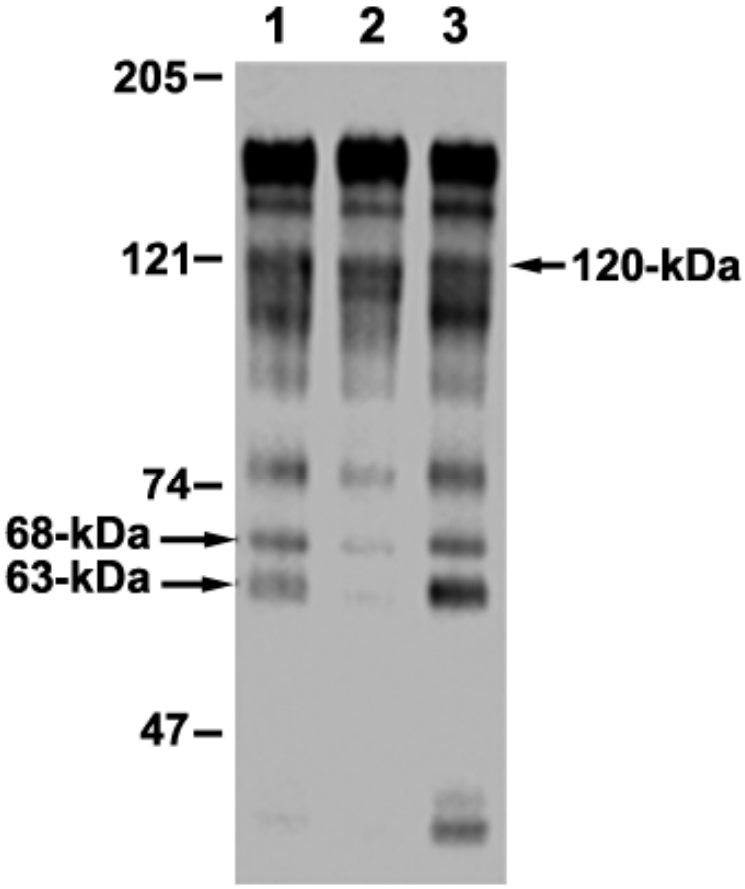
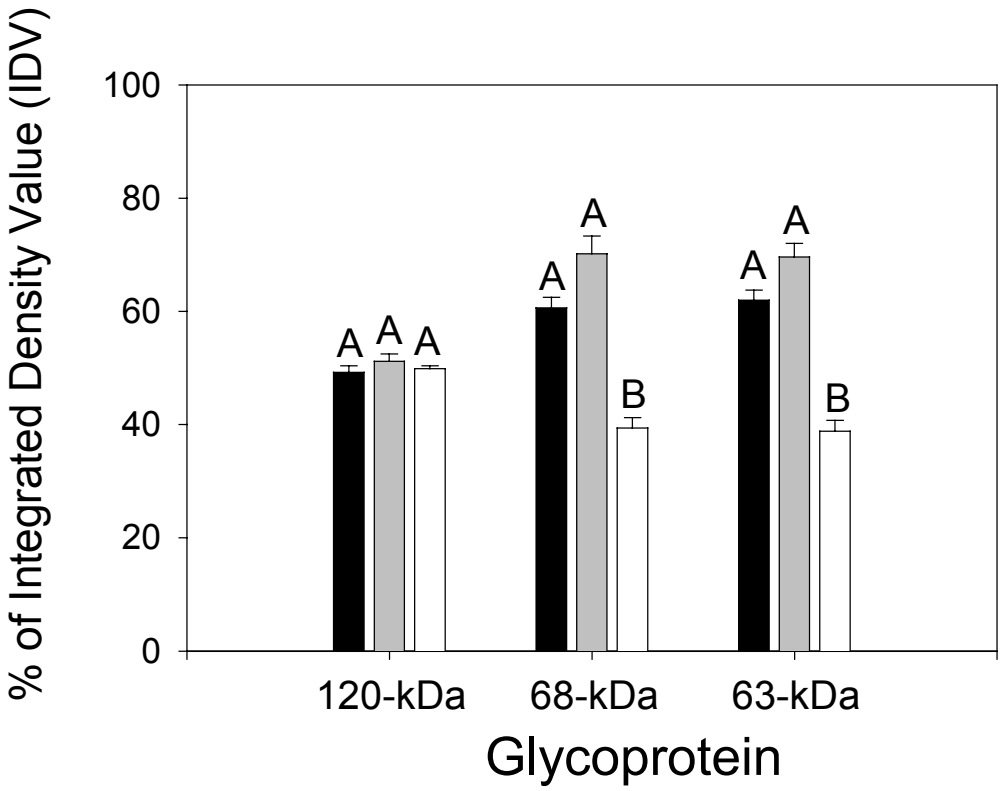

1
2
3
205
121
74
47
120-kDa
68-kDa
63-kDa

Figure 3.6- Spot densitometry of SBA binding to 120-, 68- and 63-kDa BBMV proteins from YDK (black bars), F1 (gray bars), and YHD2 (white bars) larvae. Binding of SBA to protein bands was analyzed using the AlphaImager™ software as detailed in the methods section. Different letters denote significative differences at the 95% confidence level for intensity of SBA binding within a specific protein.


% of Integrated Density Value (IDV)
100
80
60
40
20
0
A
A
A
A
A
B
A
A
B
120-kDa
68-kDa
63-kDa
Glycoprotein

CHAPTER 4

DUAL RESISTANCE TO *BACILLUS THURINGIENSIS* CRY1AC AND CRY2A IN *HELIOTHIS VIRESCENS* SUGGESTS MULTIPLE MECHANISMS OF RESISTANCE.[1]

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ABSTRACT

Development of resistance against Cry toxins from *Bacillus thuringiensis* (Bt) is one of the main concerns in the use of transgenic Bt plants. The use of combinations of Cry toxins in Bt transgenic crops also known as gene stacking, has been proposed as a strategy to delay resistance. This strategy relays on the inability of insects to develop resistance to both toxins simultaneously. For this, very different toxins with distinct modes of action should be selected. In the case of Bt cotton simultaneous expression of Cry1Ac and Cry2Aa has been proposed to delay resistance in *Heliothis virescens* (tobacco budworm). However, the CXC and KCBhyb Cry1Ac–selected laboratory strains of *H. virescens* developed high levels of cross-resistance to Cry1Aa, Cry1Ab and Cry2Aa, suggesting that this insect can develop cross-resistance to similar but also very different toxins. In the present study, we analyzed the potential role of reduced toxin binding in resistance and cross-resistance in the CXC and KCBhyb resistant strains. In specific toxin binding and binding competition experiments only Cry1Aa binding was affected in both resistant strains, while binding and insertion of Cry1Ac was unaffected when compared to susceptible insects. Since Cry1Aa and Cry2Aa do not share binding proteins in *H. virescens*, an additional mechanism for resistance against Cry1Ac and Cry2Aa should be present in these insects. Our results suggest simultaneous occurrence of at least two mechanisms of resistance in CXC and KCBhyb insects, one of them being related to altered toxin binding. The result of both mechanisms is cross-resistance to Cry1Ac and Cry2Aa. This highlights the genetic potential of *H. virescens* to become cross-resistant to very different Cry toxins. However, if two independent mechanisms of

resistance are required to attain resistance to both toxins, gene stacking in transgenic plants will be useful to delay the onset of resistance.

INTRODUCTION.

The insecticidal toxins produced by *Bacillus thuringiensis* are considered the most successful commercial biological insecticides (Betz *et al*., 2000). These so-called δ-endotoxins or Cry toxins have a unique mode of action (Knowles, 1994). After ingestion by a susceptible insect, the crystal proteins are solubilized and activated to an active toxin core. Activated toxins bind irreversibly (Liang *et al*., 1995) to specific receptors on the brush border membrane of the insect midgut and oligomerize (Aronson *et al*., 1999) to form pores that result in cell death by osmotic shock (Knowles, 1994).

Genes encoding these toxins have been cloned and introduced in plants to develop transgenic Bt plants. These plants produce their own Cry toxins, increasing insect control efficiency without being toxic against vertebrates or non-target insects (Betz *et al*., 2000). One of the main issues on the use of these plants for insect control in the field is the potential for development of resistance by target insects due to increased selection pressure (De Maagd *et al*., 1999). Alteration of any steps in the toxin mode of action can potentially result in decreased susceptibility.

Based on the frequency of resistance genes in wild populations of *H. virescens*, Gould *et al*. (1997) predicted episodes of resistance to Bt transgenic plants after 10 years of use. No episodes of resistance to Bt plants in the field have been reported after more than 6 years of use. However, from resistance development in laboratory-selected insects, it is clear that the genetic potential for resistance is present (De Maagd *et al*.,

1999). To delay development of resistance against transgenic Bt crops, different strategies have been proposed (Gould, 1998). One of these strategies is to express combinations of distinct Cry toxins in transgenic plants. These Cry toxins would be selected because they have different mechnaims of action. Bt cotton containing Cry1Ac and Cry2Ab will be the first commercial plant of this type. The success of this approach depends on the potential for development of resistance against both toxins in the insect (Gould, 1998).

Several Cry1-resistant *H. virescens* strains have been developed after laboratory selection with Cry1Ac (Ferré and Van Rie, 2002). This is the most active toxin against *H. virescens*, and it is produced by transgenic Bt cotton aimed to control this insect in the field (De Maagd *et al*., 1999). Cry1Ac-selected *H. virescens* developed cross-resistance to Cry1A and Cry1Fa toxins (Gould *et al*., 1995; MacIntosh *et al*., 1991), which share high homology in the binding specificity determining region with Cry1Ac (Tabashnik *et al*., 1996). Expression of high levels of Cry2Aa in cotton chloroplasts led to more effective control of *H. virescens* larvae (Stewart *et al*., 2001) and susceptibility of a Cry1Ac-resistant *H. virescens* strain (Kota *et al*., 1999).

The CP73-3 and KCB Cry1Ac laboratory-selected strains of *H. virescens* developed cross-resistance against Cry2A toxins (Forcada *et al*., 1999; Gould *et al*., 1992). This was not expected, since Cry1Ac and Cry2Aa share low sequence identity and show differences in their modes of action (English *et al*., 1994; Morse *et al*., 2001). These strains were crossed to susceptible adults to increase colony fitness and the resultant strains (CXC from CP73-3 and KCBhyb from KCB) were further selected with Cry2Aa.

The mechanisms of resistance in the CP73-3, CXC, KCB and KCBhyb strains have been studied (Forcada *et al*., 1996, 1999; Gould *et al*., 1992; Martínez-Ramírez *et al*., 1999). Toxin binding was not affected in the CP73-3 strain when compared to susceptible insects (Gould *et al*., 1992). Different patterns of midgut proteases may occur in larvae from susceptible, CP73-3, CXC and KCB strains, although no direct correlations between midgut protease activity and resistance could be established (Forcada *et al*., 1996, 1999). Additionally, insects from the CP73-3 and KCB strains displayed enhanced epithelium recovery after challenge with sublethal doses of Cry1Ac (Forcada *et al*., 1999; Martínez-Ramírez *et al*., 1999).

In the present study we report the toxin binding characteristics of Cry1A and Cry1Fa toxins to brush border membrane vesicles (BBMV) from the CXC and KCBhyb *H. virescens* strains. Our goal was to investigate any potential resistance mechanism related to altered toxin binding in the CXC and KCBhyb strains. The results from this study are evidence that *H. virescens* has the genetic potential to simultaneously develop different mechanisms of resistance against ditinct Cry toxins.

MATERIALS AND METHODS.

2.1 Insect strains and bioassays.

The CXC resistant *H. virescens* strain was founded by mating an already Cry1Ac resistant strain to susceptible insects to increase colony fitness (Forcada *et al*., 1999). The parental resistant strain was resistant to Cry1Ac (50-fold), Cry1Ab (12-fold) and Cry2Aa (52-fold) (Gould *et al*., 1992). The CXC strain was further selected with

Cry2Aa2 in the laboratory for more than 24 generations to increase resistance against this toxin (Kota *et al*., 1999).

The KCBhyb strain was developed by crossing adults of the resistant KCB strain (Forcada *et al*., 1999) with susceptible individuals to increase colony fitness. The parental KCB strain was resistant to Cry1Ac and cross-resistant to Cry2A (Forcada *et al*., 1999). To increase resistance to Cry2A, KCBhyb insects have been further selected with Cry2A.

Derivation of the YDK (susceptible) and YHD2 (resistant) strains and bioassay protocols with Cry1Ac and Cry2Aa toxins are described in Gould *et al*. (1995). Briefly, the YDK strain is a susceptible population of insects that serves as a control for resistant YHD2 larvae. The YHD2 strain was selected for Cry1Ac resistance.

2.2 Brush Border Membrane Vesicles (BBMV) preparation.

Frozen dissected midguts from CXC, KCBhyb, YDK and YHD2 fifth instar larvae were kindly supplied by Dr. Fred Gould (North Carolina State University, Raleigh, NC). BBMV from all the strains were prepared as described in Jurat-Fuentes and Adang, 2001). BBMV protein concentration was determined by the method of Bradford (1976) using BSA as standard and BBMV kept at –80°C until used.

2.3 Bacterial toxin production, purification and labeling.

Bacterial strains and methods for Cry1 toxin production and purification are described in Jurat-Fuentes and Adang (2001). Purity of Cry1 toxins was assessed by the presence of a single protein band in SDS-PAGE (data not shown). Toxin concentration

was determined by the method of Bradford (1976) with BSA as standard. Purified toxins were kept at –80°C until used.

Cry1A toxins (1 μg) were radiolabeled with 0.5 μCi of ^{125}I using the chloramine T method (Garczynski *et al*., 1991). Specific activities of the radio-iodinated toxins were 13-27 μCi/μg based on input toxin.

Cry1Ac and Cry1Fa toxins were biotinylated as described in Jurat-Fuentes and Adang (2001) and stored at –80°C until used.

2.4 Toxin binding to BBMV.

Methods for ^{125}I-Cry1A toxin specific binding and binding competition experiments were as previously described (Jurat-Fuentes and Adang, 2001). Specific binding of ^{125}I-Cry1A toxins to BBMV from CXC, KCBhyb, YDK and YHD2 insects was calculated as the difference between the total ^{125}I-Cry1A toxin bound minus non-specific binding determined by including 1000 nM unlabeled homologous competitor in the reaction. From the results of binding competition experiments, a representative value of the dissociation constant (K_{com}) and concentration of receptors (R_t) for each toxin in BBMV from each of the strains was calculated using the LIGAND program (Munson and Rodbard, 1980).

Irreversible binding of ^{125}I-Cry1Ac to BBMV from CXC, KCBhyb, and YDK insects was measured as the amount of toxin dissociated from BBMV through time after addition of excess unlabeled competitor as described in Luo *et al*. (1999). Toxin binding reactions were conducted for one hour prior to the addition of competitor.

2.5 Western blotting.

Quantitative binding of biotinylated Cry1Fa and Cry1Ac toxins to BBMV proteins from KCBhyb, YDK and YHD2 larvae was analyzed using Western blot analysis.

BBMV proteins (20 μg) were incubated with 12 nM biotinylated Cry1Ac or Cry1Fa in 0.1 ml of binding buffer (PBS plus 0.1% BSA) at room temperature for 1 hour. Binding was stopped by centrifugation and pellets were washed twice with 0.5 ml of binding buffer. Final BBMV pellets were solubilized and electrophoresed in 10% SDS-PAGE and transferred to polyvinylidiene difluoride Q membrane filters (PVDF) (Millipore) in transfer buffer (48 mM Tris, 390 mM glycine, 0.1% [w/v] SDS, 20% methanol, pH 8.3). After blocking in PBST buffer (PBS plus 0.1% Tween-20) containing 3% BSA, membranes were incubated with streptavidin-peroxidase conjugate (SIGMA) in PBST plus 0.1% BSA for one hour. Biotinylated toxins were visualized using ECL (Amersham Pharmacia) reagents following the manufacturer's instructions.

RESULTS.

3.1 Cry1Ac and Cry2Aa activity against different *H. virescens* strains.

Both Cry1Ac and Cry2Aa toxins were highly active against YDK insects (Table 4.1). The LC_{50} values obtained for both toxins were similar to previous reports (Gould *et al*., 1995; Kota *et al*., 1999).

Different levels of resistance to Cry1Ac were observed in the CXC, KCBhyb and YHD2 strains relative to the YDK strain. Resistance to Cry1Ac in the CXC strain (200-fold) was greater than previously reported (Kota *et al*., 1999), demonstrating an increase

in resistance to this toxin after selection with Cry2Aa. As shown in Table 4.1, the LC_{50} of KCBhyb insects was about 180-fold higher than the LC_{50} for YDK larvae. Insects from the YHD2 strain were notoriously resistant to Cry1Ac as previously reported (Gould *et al*., 1995, Lee *et al*., 1995). No mortality of larvae from this strain was observed even at the highest toxin concentration tested.

In agreement with previous studies (Gould *et al*., 1995; Kota *et al*., 1999), insects from YHD2 were only about 4-fold resistant to Cry2Aa, while CXC and also KCBhyb insects were highly resistant to this toxin (more than 250-fold).

Although other Cry1 toxins were not tested in our bioassays, the CXC, KCBhyb and YHD2 strains are also cross-resistant to Cry1Aa, Cry1Ab and Cry1Fa (F. Gould, unpublished data).

3.2 Specific toxin binding to BBMV.

To investigate specificity of ^{125}I-Cry1A toxin binding to BBMV from the CXC, KCBhyb, YHD2, and YDK strains, we measured the extent of specific toxin binding. The amounts of ^{125}I-Cry1A toxins specifically bound were dependent on the strain from which BBMV were prepared (Fig. 4.1). Since BBMV from the YHD2 strain do not bind specifically any ^{125}I-Cry1A toxin (Chapter Three), they were used as a negative control for toxin binding.

In agreement with data presented in Chapter Three, each ^{125}I-Cry1A toxin bound specifically to BBMV from YDK insects. Saturation of ^{125}I-Cry1Aa specific binding was not reached for the BBMV protein concentrations tested. Maximum specific binding of ^{125}I-Cry1Ab (35%), and ^{125}I-Cry1Ac (50%) was observed at 400 and 200 μg/ml of

BBMV proteins, respectively. These percentages represent the maximum amount of ^{125}I-Cry1A toxins that can bind specifically to BBMV and also helped determine the BBMV concentrations to be used in subsequent binding competition experiments.

Maximum specific binding of ^{125}I-Cry1A toxins to BBMV from CXC and KCBhyb was slightly decreased relative to BBMV from YDK. Thus, in both cases only 20% of ^{125}I-Cry1Ab and 40% of ^{125}I-Cry1Ac was specifically bound to BBMV from these strains. Furthermore, no saturation was observed for the binding of ^{125}I-Cry1Aa to BBMV from CXC (as observed for YDK) and binding of this toxin to BBMV from KCBhyb was negligible (Fig. 4.1A). This decrease in the percentage of input ^{125}I-Cry1A toxins that can bind to BBMV suggests a reduction of available toxin binding sites in the BBMV from the CXC and KCBhyb strains in respect to YDK.

3.3 Binding of biotinylated Cry1Fa to BBMV.

Larvae from the KCBhyb strain are cross-resistant to Cry1Fa (F. Gould, unpublished data). Cry1A and Cry1Fa toxins share a common binding site in *H. virescens* BBMV (Jurat-Fuentes and Adang, 2001). This binding site is the only binding site recognized by Cry1Aa. Since binding of Cry1Aa was decreased in the BBMV from the KCBhyb strain, we studied the potential role of altered toxin binding in cross-resistance to Cry1Fa in this strain.

Binding of biotinylated Cry1Fa was quantitatively studied by Western blotting of BBMV proteins from the YDK and KCBhyb strains after incubation with the biotinylated-toxin. BBMV from the YHD2 resistant strain were included in the analysis as a control, since these vesicles have greatly decreased Cry1Fa binding (Chapter Three

of this dissertation). As an internal control, we also studied binding of biotinylated Cry1Ac to BBMV from YDK, KCBhyb and YHD2 insects.

As expected from the results of specific toxin binding experiments and from previous reports, biotinylated Cry1Ac bound BBMV from YDK and KCBhyb strains, but binding to BBMV from the YHD2 was highly reduced (Fig. 4.2A). On the other hand, biotinylated Cry1Fa bound to YDK and KCBhyb but not to BBMV from YHD2 (Fig. 4.2B). These results suggest that cross-resistance to Cry1Fa in the KCBhyb strain is not due to altered toxin binding.

3.4 Competition of ^{125I}-Cry1A binding to BBMV.

To quantify potential differences in the amounts or affinities of toxin binding sites, we performed homologous competition binding experiments. ^{125}I-Cry1A toxins were incubated with BBMV from CXC, KCBhyb, and YDK larvae in the presence of increasing concentrations of homologous unlabeled toxin (Fig. 4.3). The binding dissociation constant (K_{com}) and the concentration of receptors (R_t) for each of the ^{125}I-Cry1A toxins in BBMV from the CXC, KCBhyb and YDK strains were calculated (Table 4.2).

As expected from the specific toxin binding experiments, binding of ^{125}I-Cry1Aa was highly reduced in BBMV from KCBhyb (Fig. 4.3A). In fact, we were not able to obtain a binding dissociation constant for this toxin in BBMV from KCBhyb. The binding affinity of ^{125}I-Cry1Aa to BBMV from the CXC strain was also reduced (about 6-fold increase in the dissociation constant) when compared to BBMV from YDK insects. This increase in the dissociation constant was accompanied with a 5-fold

increase in the concentration of binding sites in BBMV from CXC when compared to YDK. These results show that at the time these assays were conducted the CXC and KCBhyb insects could be differentiated by ^{125}I-Cry1Aa toxin-binding assays. In KCBhyb larvae resistance was correlated with highly reduced Cry1Aa binding.

No significant differences in the binding dissociation constants or concentration of binding sites for ^{125}I-Cry1Ab between BBMV from the CXC, KCBhyb and YDK strains were found. The ^{125}I-Cry1Ab dissociation constant for the YDK strain was about 5 fold higher than previously reported (Gould *et al*., 1992; Jurat-Fuentes and Adang, 2001; Lee *et al*., 1995).

The calculated binding parameters for ^{125}I-Cry1Ac binding to BBMV from YDK were in agreement with previous reports (Lee *et al.*, 1995). Similar binding dissociation constants and binding site concentrations were also calculated for Cry1Ac binding to BBMV from CXC and KCBhyb.

In summary, binding competition experiments showed that Cry1Aa binding sites in KCBhyb were greatly reduced in number, absent or modified to reduce toxin binding affinity. Cry1Aa binding to BBMV from CXC was also altered. Quantitative binding parameters for Cry1Ab and Cry1Ac were the same for BBMV from resistant (CXC and KCBhyb) and susceptible (YDK) larvae, suggesting that resistance to these toxins is not due to reduced binding. Alternatively, since CryAb and Cry1Ac bind to the Cry1Aa binding site (Jurat-Fuentes and Adang, 2001), the binding assays may not be sensitive enough to detect reduced binding of Cry1Ab and Cry1Ac to this shared binding site.

3.5 ^{125}I-Cry1Ac irreversible binding to BBMV.

Cry1 toxins undergo a reversible binding phase before toxin insertion in the membrane (Liang *et al*., 1995). Binding of Cry1Ac was not altered in BBMV from the Cry1Ac-resistant CXC and KCBhyb strains when compared to BBMV from YDK insects. One possible explanation for this observation was that the measured Cry1Ac binding was not followed by toxin insertion on the BBMV membrane. To test this possibility, we measured the percentage of ^{125}I-Cry1Ac binding to BBMV that was irreversible. In these experiments, bound ^{125}I-Cry1Ac was challenged with an excess of unlabeled Cry1Ac competitor after a one-hour incubation period. If binding were irreversible (presumably due to toxin insertion into the membrane), then bound ^{125}I-Cry1Ac would not be displaced by the competitor.

As shown in Fig. 4.4, ^{125}I-Cry1Ac binding to BBMV from the YDK, CXC, and KCBhyb strains was not competed by an excess of unlabeled Cry1Ac, suggesting irreversible binding of ^{125}I-Cry1Ac to these vesicles.

These results suggest that resistance to Cry1Ac in these strains is not related to altered binding or toxin insertion on the brush border membrane of midgut cells.

4. DISCUSSION.

Wild populations of *H. virescens* carry the genetic potential for developing resistance to Cry1 toxins (Gould *et al*., 1997). This prospect threatens the control of this insect by transgenic Bt crops. Expressing combinations of Cry toxins that have distinct modes of action is a tactic proposed to delay the onset of resistance (Gould, 1998).

In the U.S. Bt cotton that controls *H. virescens* expresses Cry1Ac (De Maagd *et al*., 1999), the most active toxin against this insect (Van Frankenhuyzen and Nystrom, 2002). All Cry1A, Cry1Fa and Cry1Ja toxins share high homology in the binding specificity determining region with Cry1Ac (Tabashnik *et al*., 1996). Consequently, these toxins share a common binding site in BBMV from susceptible *H. virescens* larvae (Jurat-Fuentes and Adang, 2001). Cross-resistance to Cry1A, Cry1Fa and Cry1Ja toxins in Cry1Ac-selected insects is common (Ferré and Van Rie, 2002).

Cry2A toxins show low sequence homology with Cry1 toxins (Tabashnik *et al*., 1996) and have distinct receptor binding epitopes (Morse *et al*., 2001). Accordingly, Cry2A toxins have a distinct mode of action (English *et al*., 1994) and do not share binding sites with Cry1A toxins (Jurat-Fuentes and Adang, 2001; Karim and Dean, 2000). As would be expected from these differences, Cry1-selected strains of *H. virescens, Plutella xylostella* and *Pectinophora gossypiella* did not develop cross-resistance to Cry2A toxins (Gould *et al*., 1995; Tabashnik *et al*., 1996, 2000; Zhao *et al*., 2001).

Unexpectedly, two independent laboratory strains of *H. virescens* selected with Cry1Ac (CP73-3 and KCB), developed cross-resistance to Cry2Aa (Gould *et al*., 1992; Forcada *et al*., 1999). Binding of Cry1Ab and Cry1Ac to BBMV from the CP73-3 strain was not altered, suggesting that resistance to these toxins in this strain was not due to reduced binding (Gould *et al*., 1992).

The Bt-resistant strains that were the subjects of this study were derived from CP73-3 and KCB. Adults from those strains were backcrossed to susceptible adults to increase colony fitness and the progeny colonies designated CXC and KCBhyb. CXC

and KCBhyb strains were further selected with Cry2Aa, and our bioassays (Table 4.1) demonstrate that resistance against both Cry1Ac and Cry2Aa has increased to similar levels in both strains when compared to previous reports (Kota et al., 1999). This observation suggests that resistance to Cry2Aa conferred increased cross-resistance to Cry1Ac.

Resistance to Cry1A toxins in several strains of *H. virescens* has been associated with altered binding (Lee *et al*., 1995; MacIntosh *et al*., 1991). In this study toxin binding was analyzed in the CXC and KCBhyb strains. BBMV from the CXC and KCBhyb strains had less of each ^{125}I-Cry1A toxin bound at saturation as compared to vesicles from the susceptible YDK strain. The most dramatic change was the lack of ^{125}I-Cry1Aa binding to KCBhyb BBMV. The simplest explanation for this observation is that the number of available binding sites, or the binding affinity of the toxins for the BBMV from the resistant strains was reduced.

Toxin binding competition experiments demonstrated a slightly decreased ^{125}I-Cry1Aa binding affinity and increased concentration of binding sites in BBMV from the CXC strain when compared to YDK vesicles. This phenomenon of decreased affinity accompanied by increased number of binding sites has been observed in other resistant *H. virescens* strains (MacIntosh *et al*., 1991). Because BBMV from the KCBhyb strain had greatly reduced ^{125}I-Cry1Aa binding, we were not able to calculate any binding parameters for this toxin.

We did not detect any significant difference in the binding of ^{125}I-Cry1Ab or ^{125}I-Cry1Ac toxins to BBMV from CXC or KCBhyb when compared to BBMV from YDK insects. In fact, irreversible binding experiments demonstrated that more than 90% of the

Cry1Ac toxin was irreversibly bound (inserted) on the BBMV membrane from CXC, KCBhyb and YDK insects. This suggests that a small proportion of Cry1Ac binding mediates toxicity. Lee *et al*. (1995) made a similar observation after binding analyses of YHD2 *H. virescens* larvae. In each resistant strain the critical population of binding sites seems to be the Cry1Aa site.

In *H. virescens* BBMV, Cry1Aa shares its only binding site with Cry1Ab, Cry1Ac, Cry1Fa and Cry1Ja toxins (Jurat-Fuentes and Adang, 2001). Since the KCBhyb strain showed greatly reduced Cry1Aa toxin binding and cross-resistance to Cry1Fa (F. Gould, unpublished results), we studied the possibility that Cry1Fa binding was also affected in this strain. Western blot analyses revealed that biotinylated Cry1Fa bound to both BBMV from KCBhyb and YDK insects.

The mechanisms of resistance functioning in the CXC and KCBhyb strains affected Cry1Aa but not Cry1Ab, Cry1Ac or Cry1Fa binding. This pattern of altered Cry1 binding was also described for the Cry1Ac-resistant YHD2 strain of *H. virescens* (Lee *et al*., 1995). However, cross-resistance to Cry2Aa in the YHD2 strain was low (Gould *et al*., 1995). This would be expected, since Cry1A and Cry2Aa toxins do not share binding sites in *H. virescens* (Jurat-Fuentes and Adang, 2001). The fact that at least binding of Cry1Aa is altered in CXC and KCBhyb insects, yet the strains have Cry2Aa resistance, suggests the existence of two resistance mechanisms in these strains.

One mechanism may be responsible for part of the resistance to Cry1Ac and cross-resistance to Cry1Aa, CryAb and Cry1Fa. This mechanism may operate in the CXC, KCBhyb and YHD2 resistant strains, since these strains have reduced Cry1Aa but not Cry1Ab, Cry1Ac or Cry1Fa binding. The differences in Cry1Aa binding between

CXC and KCBhyb may be evidence for differences in the effectiveness of this mechanism of resistance. Even in the well-characterized YHD2 strain multiple genes contribute to resistance. From 40 to 80% of resistance to Cry1Ac in the YHD2 strain was due to lack of expression of a cadherin-like protein (HevCadLP) in the resistant larval midgut (Gahan *et al*., 2001). Since the YHD2 larvae had little cross-resistance to Cry2Aa, HevCadLP is probably not recognized by this toxin. Thus, absence of HevCadLP would not explain resistance to Cry2Aa in the CXC and KCBhyb strains. To explain toxin binding not leading to toxicity, Lee *et al* (1995) proposed the existence of "null receptors" in the YHD2 strain that would allow for irreversible binding of the toxins but not for toxicity. In this respect, Cry1Ac has been shown to bind to BBMV specifically without conferring susceptibility in *Spodoptera frugiperda* and *Lymantria dispar* (Garczynski *et al*., 1991; Wolfersberger, 1990).

A second mechanism of resistance present in CXC and KCBhyb insects would confer part of the Cry1Ac resistance and all the resistance to Cry2Aa. This resistance mechanism must be related to an alteration of a step in toxin action shared by both Cry1Ac and Cry2Aa toxins. Although these toxins have different modes of action at the membrane level, they both undergo the same initial steps of solubilization and activation in the insect midgut juice. Differential midgut protease activity was proposed as a mechanism of resistance for the CXC strain and the parental strains of CXC (CP73-3) and KCBhyb (KCB) (Forcada *et al*., 1996; 1999). However, a correlation between expression of specific proteases and resistance could not be established for any of the strains (Forcada *et al*., 1999). Alternatively, enhanced midgut epithelium regeneration after Cry toxin challenge was proposed as a potential mechanism of resistance in the

CXC and KCB strains (Forcada *et al*., 1999). Faster rates of midgut epithelium renewal have been associated with resistance against *B. thuringiensis* (Chiang *et al*., 1986) and baculovirus infection (Hoover *et al*., 2000) in *H. virescens*. Furthermore, challenge of *H. virescens* midgut cell cultures with sublethal doses of Cry1Ac induced an increase in the number of stem and differentiating cells when compared to controls (Loeb *et al*., 2001).

The second resistance mechanism was presumably enhanced by further selection with Cry2Aa. This would result in increased resistance to Cry2Aa and Cry1Ac, but not cross-resistance to Cry1Aa, Cry1Ab or Cry1Fa. Bioassays of CXC and KCBhyb larvae to study if resistance to Cry1Aa, Cry1Ab and Cry1Fa toxins segregates independently to resistance against Cry1Ac and Cry2Aa will help support or reject the two-mechanism model for resistance in these strains.

Resistance to Cry toxins due to a combination of resistance genes in the same insect strain has been previously reported for *H. virescens* (Heckel *et al*., 1997), *P. xylostella* (Wright *et al*., 1997), and *Plodia interpunctella* (Herrero *et al*., 2001). More specifically, in a Cry1Ab resistant strain of *P. interpunctella* reduced toxin binding and a protease-mediated mechanism were observed (Herrero *et al*., 2001).

Our results are evidence for the existence of multiple resistance mechanisms in both the CXC and KCBhyb strains of *H. virescens*. This highlights the broad variety of potential resistance mechanisms that *H. virescens* may develop to cope with very different Cry toxins. Dual resistance to Cry1Ac and Cry2Aa in these laboratory strains raises questions as to how *H. virescens* in the field will respond to transgenic cotton producing Cry1Ac and Cry2A proteins. This information is extremely important when

designing and implementing strategies aimed at delaying resistance and cross-resistance to insecticides based on this toxins and Bt transgenic crops.

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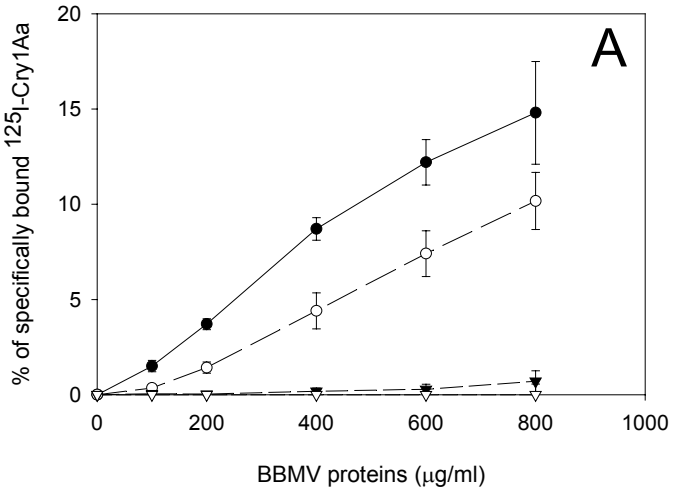
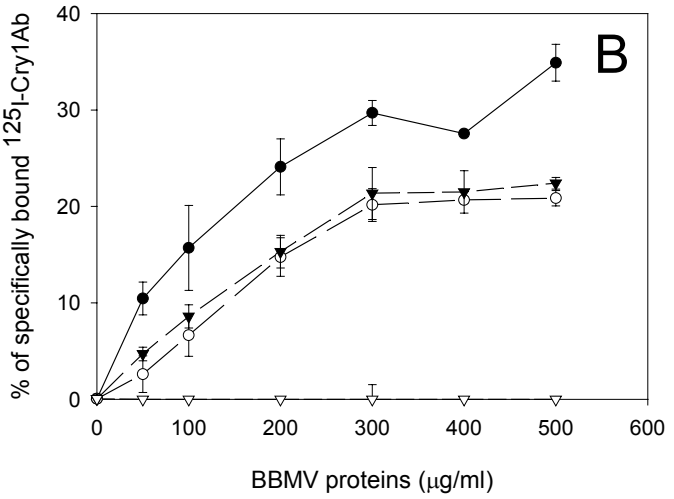
FIGURES AND TABLES.

Table 4.1.- Susceptibility of control and selected *H. virescens* strains to Cry1Ac and Cry2Aa toxins from *B. thuringiensis*.

Toxin	Strain	LC_{50} (μg/ml)(lower -upper 95% C. I)	Slope	RR
Cry1Ac	YDK	0.73 (0.33-1.39)	0.93	NA
	YHD2	no mortality at 2000 μg/ml	NA	>2,800
	CXC	211.20 (104.40-343.60)	1.46	289.31
	KCBhyb	137.00 (84.89-200.00)	2.00	187.67
Cry2Aa	YDK	4.30 (2.33-6.36)	3.31	NA
	YHD2	14.22 (8.40-21.33)	1.97	3.30
	CXC	no mortality at 1,000 μg/ml	NA	>250
	KCBhyb	no mortality at 1,000 μg/ml	NA	>250

Figure 4.1- Specific binding of ^{125}I-Cry1Aa (A), ^{125}I-Cry1Ab (B), ^{125}I-Cry1Ac (C), toxins to increasing concentrations of BBMV from YDK (●), CXC (○), KCBhyb (▼), and YHD2 (▽) *H. virescens* strains. Vesicles at the concentrations indicated were incubated with ^{125}I-Cry1A toxins. Binding is expressed as a percentage of input ^{125}I-Cry1A. Binding in the presence of 1000 nM unlabeled homologous toxin was subtracted from total binding. Maximum non-specific binding was 20% (for ^{125}I-Cry1Aa) or 40% (for ^{125}I-Cry1Ab and ^{125}I-Cry1Ac) of total binding. Each data point is the average of the means based on independent trials with duplicate samples. Error bars depict standard deviation of the mean values.


A
% of specifically bound 125I-Cry1Aa
BBMV proteins (μg/ml)
0
5
10
15
20
0
200
400
600
800
1000


B
% of specifically bound 125I-Cry1Ab
BBMV proteins (μg/ml)
0
10
20
30
40
0
100
200
300
400
500
600

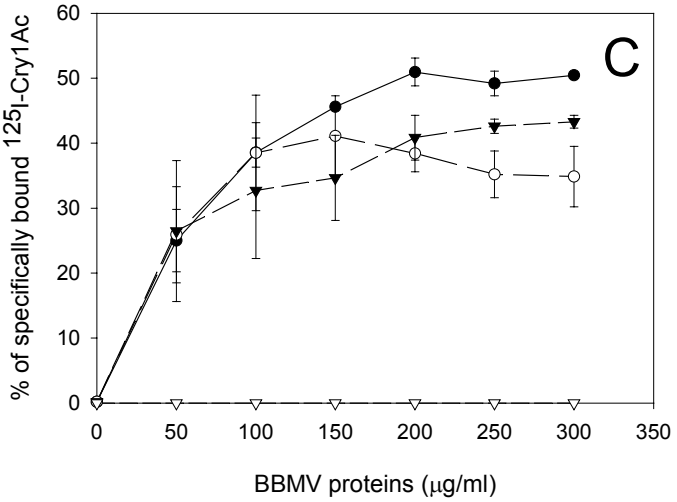

C
% of specifically bound 125I-Cry1Ac
BBMV proteins (μg/ml)
0
10
20
30
40
50
60
0
50
100
150
200
250
300
350

Figure 4.2- Binding of Cry1Ac (A) and Cry1Fa (B) biotinylated toxins to BBMV from YDK, YHD2, and KCBhyb insects. Toxins were incubated with BBMV proteins (20 μg) for one hour. Binding reactions were stopped by centrifugation and washed pellets were separated by SDS-PAGE. Gels were dried and transferred to PVDF filters. Biotinylated toxins were detected with streptavidin-peroxidase conjugate and enhanced chemiluminescence.

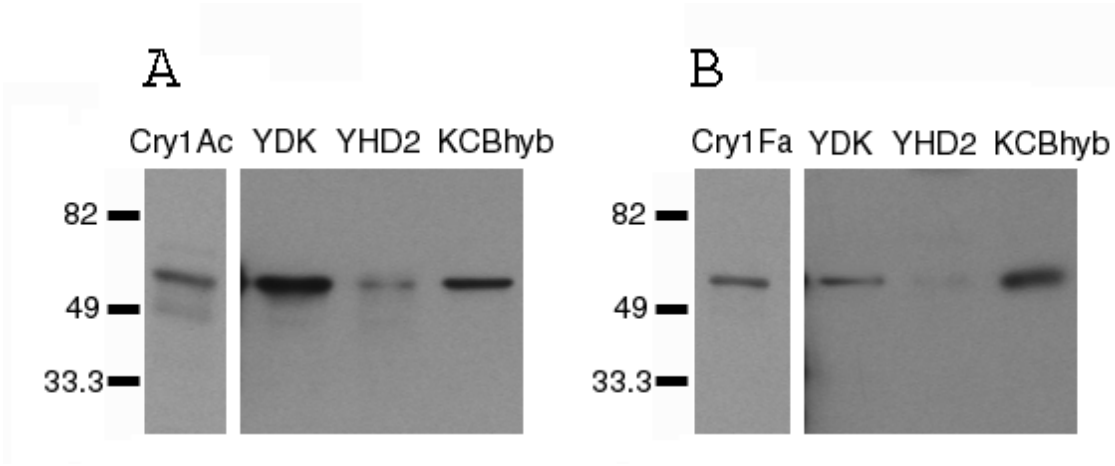
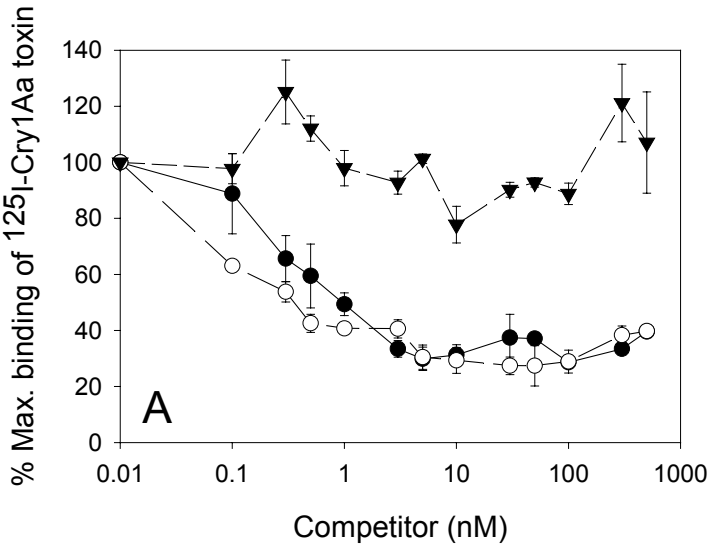
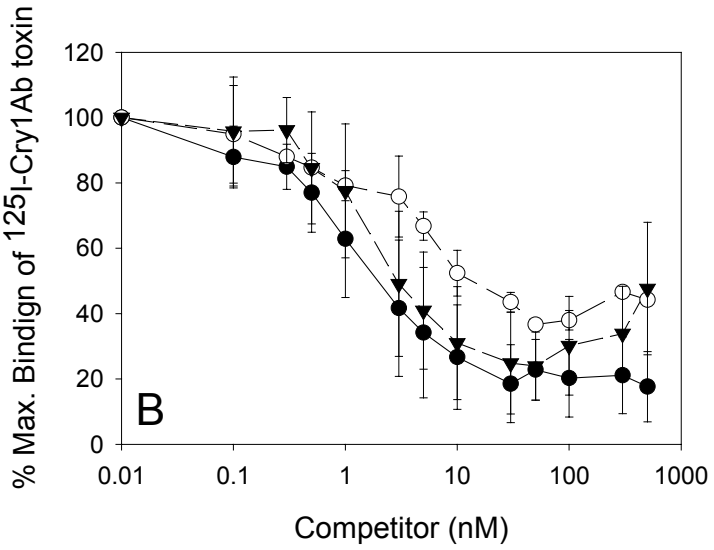

A
Cry1Ac
YDK
YHD2
KCBhyb
82
49
33.3
B
Cry1Fa
YDK
YHD2
KCBhyb
82
49
33.3

Figure 4.3- Binding competition between ^{125}I-Cry1Aa (A), ^{125}I-Cry1Ab (B), and ^{125}I-Cry1Ac (C) and unlabeled homologous competitors. BBMV from the YDK (●), CXC (○), KCBhyb (▼), and YHD2 (▽) *H. virescens* strains were incubated with ^{125}I-Cry1A toxins (0.1 nM) and increasing concentrations of unlabeled homologous toxins for one hour. Binding was expressed as a percentage of the maximum amount of toxin bound during incubation with labeled toxin. Each data point is a mean based on data from independent trials. Error bars depict standard deviation of the mean values.


% Max. binding of 125I-Cry1Aa toxin
140
120
100
80
60
40
20
0
A
0.01
0.1
1
10
100
1000
Competitor (nM)


% Max. Bindign of 125I-Cry1Ab toxin
120
100
80
60
40
20
0
B
0.01
0.1
1
10
100
1000
Competitor (nM)

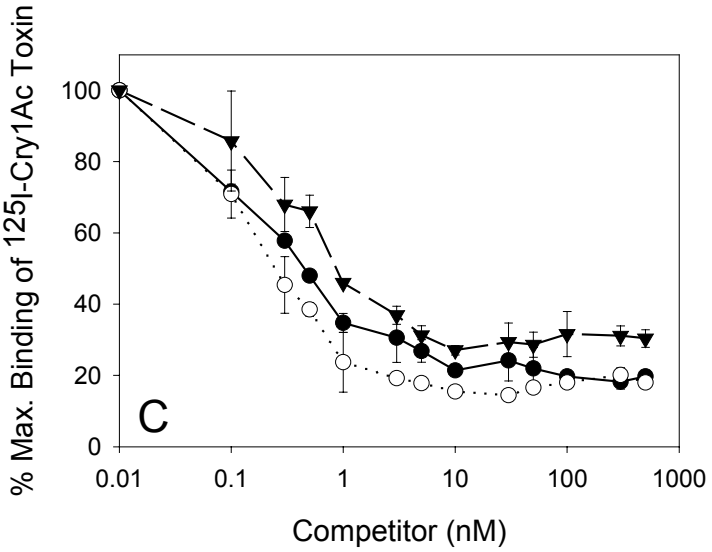
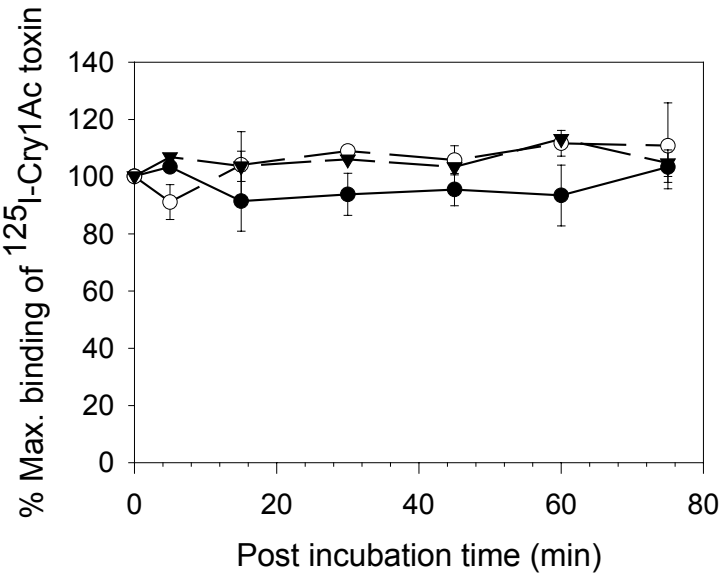

% Max. Binding of 125I-Cry1Ac Toxin
100
80
60
40
20
0
C
0.01
0.1
1
10
100
1000
Competitor (nM)

Table 4.2- Binding parameters of ^{125}I-Cry1A toxins to BBMV from CXC, KCBhyb and YDK *H. virescens* strains.

	CXC		KCBhyb		YDK	
Toxin	K_{com} (nM) ± SE	R_t (nM) ± SE	K_{com} (nM) ± SE	R_t (nM) ± SE	K_{com} (nM) ± SE	R_t (nM) ± SE
Cry1Aa	3.58 ± 2.50	0.26 ± 0.14	-----	------	0.44 ± 0.07	0.05 ± 0.01
Cry1Ab	18.65 ± 4.45	4.33 ± 0.84	13.70 ± 4.24	3.50 ± 0.84	10.37 ± 1.52	3.44 ± 0.80
Cry1Ac	3.39 ± 1.47	0.40 ± 0.13	0.02 ± 0.01	0.04 ± 0.01	1.14 ± 1.06	0.16 ± 0.12

Figure 4.4- Irreversible binding of ^{125}I-Cry1Ac toxin to BBMV from YDK (●), CXC (○), and KCBhyb (▼) insects. One hour after initiation of the binding reaction, 1000 nM of unlabeled Cry1Ac was added to the mixture. The time on the *x* axis represents postincubation time after addition of the unlabeled competitor. Each data point is a mean based on data from independent trials. Error bars depict standard deviation of the mean values.


% Max. binding of 125I-Cry1Ac toxin
140
120
100
80
60
40
20
0
0
20
40
60
80
Post incubation time (min)

CHAPTER 5

GENERAL DISCUSSION AND CONCLUSION

The insecticidal toxins produced by *Bacillus thuringiensis* (Bt) during sporulation are considered the best alternatives to synthetic pesticides for insect pest control. One of the main concerns in the use of Bt sprays and transgenic crops is the development of resistance. Insects have evolved resistance against most classes of synthetic insecticides and biocontrol methods (Mullin and Scott, 1992). Although the mechanisms by which insects attain resistance vary, Bt resistance does occur. Only *Plutella xylostella* has developed resistance to Bt toxins in the field (Tabashnik *et al*., 1990). However, other insects have shown the potential for development of resistance under laboratory conditions (Ferré and Van Rie, 2002). Laboratory-selected strains of *Heliothis virescens* have evolved high levels of resistance to Cry1 toxins (Forcada *et al*., 1996; Forcada *et al*., 1999; Gould *et al*., 1992; Gould *et al*., 1995).

Importance of domain II for binding specificity

Several approaches may delay the onset of resistance to Bt transgenic crops in the field (Gould, 1998). One approach is to alternate or combine Cry toxins that recognize different receptors in the insect midgut or that have different modes of action (Gould, 1998). This approach is based on the principle that resistance to toxins that have different modes of action requires multiple resistance genes. Therefore, resistance would be more difficult to develop. A detailed understanding of toxin binding and the receptors recognized by Cry toxins is necessary for the success of this approach.

The main objective of Chapter Two of this dissertation was to expand a previous toxin-binding model for Cry1 toxins in *H. virescens* BBMV (Van Rie *et al*., 1989) by including Cry1Fa and Cry1Ja toxins. I chose to use Cry1Fa for three reasons. First, its

high activity against *H. virescens* (Van Frankenhuyzen and Nystrom, 2002); second, its high homology in the loops of domain II with Cry1A toxins (Tabashnik *et al*., 1996), and third, its ability to share binding sites with Cry1A toxins in other insects (Granero *et al*., 1996). Furthermore, cross-resistance to Cry1Fa in Cry1A-resistant insects is common (Tabashnik *et al*., 1996; Gould *et al*., 1995). Cry1Ja was used because it shares all the features stated above for Cry1Fa, except that its activity against *H. virescens* is negligible. Thus, I expected Cry1Ja to recognize a receptor different from toxins highly active against this insect. Based on these lines of evidence, I hypothesized that Cry1A, Cry1Fa and Cry1Ja toxins would share binding sites in BBMV from susceptible *H. virescens* larvae.

The results from binding competition experiments in Chapter Two demonstrate that Cry1A, Cry1Fa and Cry1Ja toxins do share a high affinity-binding site in *H. virescens* BBMV. According to my results, receptor A from the model proposed by Van Rie *et al*. (1989) represents this binding site shared by Cry1A, Cry1Fa and Cry1Ja toxins. Neither Cry1Fa nor Cry1Ja bound to receptors B or C, although binding of these toxins to other receptors cannot be excluded by my results, since I did not perform binding competition experiments with labeled Cry1Fa or Cry1Ja toxins. Binding competition studies using labeled Cry1Fa and Cry1Ja toxins would help to distinguish binding sites specific for these toxins. Loss of activity of Cry1Fa after iodination (Luo *et al*., 1999) complicates the feasibility of binding analysis with this toxin.

Using ligand blotting, I classified Cry1 binding proteins as belonging to one of the three populations of receptors predicted by the toxin-binding model. Although ligand-blotting data should be interpreted with caution because of the denaturing conditions used

(Daniel *et al*., 2002; Lee *et al*. 1996; Mohammed *et al*., 1996), the pattern of binding proteins detected in ligand blots agreed with the results from binding competition analyses. Cry1A, Cry1Fa and Cry1Ja toxins bound to 170- and 110-kDa proteins. These proteins constitute receptor A in the binding model. The high homology in the loops of domain II of Cry1A, Cry1Fa, and Cry1Ja toxins (Tabashnik *et al*., 1996) suggests that these loops are important determinants for binding to receptor A. Cry1Ab and Cry1Ac also recognized a 130-kDa protein, and Cry1Ac additionally bound to several proteins smaller than 100-kDa in size. Thus, according to the binding model, the 130-kDa protein would constitute receptor B, while the proteins recognized by Cry1Ac only would constitute receptor C.

The 170-kDa and 110-kDa proteins have been identified as N-aminopeptidases (APN) (Luo *et al*., 1997; Banks *et al*., 2001). Purified 170-kDa catalyzed Cry1A toxin pore formation (Luo *et al*., 1997), and both Cry1Ac and Cry1Fa toxins bind to 170- and 110-kDa APNs (Banks *et al*., 2001). Cry1Ac toxin recognizes different binding epitopes on these binding proteins. Recognition of the 170-kDa APN is mostly GalNAc-dependent (Luo *et al*., 1997; Oltean *et al*., 1999), while binding to the 110-kDa APN is GalNAc-independent (Banks *et al*., 2001).

Cry1Ja bound with high affinity to receptor A but showed little toxicity to *H. virescens* larvae. Although hypothetical, this observation can be explained by one of the proteins constituting receptor A not being directly involved in toxicity. The existence of "null receptors" that would bind toxins but not mediate pore formation has been proposed (Lee *et al*., 1995). In support of this hypothesis, Banks *et al.* (2002) found that expressed 110-kDa APN in *Drosophila melanogaster* DS-2 cells did not promote cell killing by

Cry1Fa or Cry1Ac. Thus, Cry1Ja may have a higher binding affinity for the 110-kDa APN protein than for the 170-kDa APN. If so, such a result would be in accord with the suggestion that only high affinity toxin binding to the 170-kDa protein in receptor A is involved in toxicity and that the 110-kDa APN may act as a "null receptor" in this population of receptors. Alternatively, there could be other variables in *H. virescens* that affect Cry1Ja activity.

Banks *et al*. (2001) detected a 210-kDa protein that bound Cry1Ac and Cry1Fa toxins. A protein of this size was seldom detected in the ligand blots that I reported in Chapter Two. Although I do not know the reason for this infrequent detection, it could be due to variable electrotransfer or protein degradation. Cry1 toxin-binding proteins of this size have been identified as cadherin-like proteins and are considered to be functional Cry1 toxin receptors in *Manduca sexta* (Meng *et al*., 2001) and *Bombyx mori* (Nagamatsu *et al*., 1999). This 210-kDa protein may be part of receptor A population. Further experimentation is needed to confirm this possibility.

A 130-kDa APN protein from *H. virescens* BBMV, a product of the same gene that encodes the 170-kDa APN, binds Cry1Aa and Cry1Ac (Oltean *et al*., 1999). Interestingly, this protein was detected in fractions from chromatographic separation of BBMV proteins, but was not observed in ligand blots of total BBMV, suggesting that it may be a degradation product of a BBMV protein (Oltean *et al*., 1999). This observation may explain why this protein was not detected in the BBMV protein ligand blots with Cry1Aa described in Chapter Two.

Based on their similar sizes, the 130-kDa protein that bound Cry1Ab and Cry1Ac toxins in ligand blots is probably the 120-kDa APN cloned by Gill *et al*. (1995). Binding

of Cry1Ab and Cry1Ac to this protein might increase activity of these toxins against *H. virescens* when compared with Cry1Aa, which only binds to receptor A. Additionally, the Cry1Ac-binding proteins smaller than 100-kDa in size (receptor C) could confer on this toxin a higher level of toxicity (Cry1Ac is the most active toxin against *H. virescens*) than Cry1Ab or Cry1Fa.

The results described in Chapter Two indicate that high homology in the protruding loops of domain II in Cry1 toxins are directly involved in binding to receptor A, but not receptor B or C. Since transgenic Bt cotton expresses Cry1Ac, my results predict that toxins that are active against *H. virescens* but that do not show high homology with Cry1Ac in domain II would be good candidates for gene stacking strategies to delay resistance against these crops. Cry2Aa does not share sequence homology with Cry1A, Cry1Fa or Cry1Ja in the loops of domain II (Tabashnik *et al*., 1996) and has different binding specificity determinants (Morse *et al*., 2001). In support of this prediction, Cry2Aa was highly toxic against a highly Cry1Ac resistant *H. virescens* strain (Kota *et al*., 1999). Transgenic cotton plants expressing Cry2Aa and Cry1Ac toxins displayed higher efficacy for *H. virescens* control (Stewart *et al*., 2001).

Mechanism for increased Cry1Ac resistance in YHD2

The *H. virescens* YHD2 strain has been used in different studies as a model to study insect resistance against Cry1 toxins (Gahan *et al*., 2001; Gould *et al*., 1997; Heckel *et al*., 1997a, 1997b; Lee *et al*., 1995). This strain originated after laboratory selection with Cry1Ac and developed high levels of resistance to Cry1Ac (10,000-fold), and cross-resistance to Cry1Aa (about 30-fold), Cry1Ab (more than 1,000-fold), and

Cry1Fa (more than 3,000-fold) (Gould *et al*., 1995; Lee *et al*., 1995). Cross-resistance to other toxins (Cry1B, Cry1C, Cry2Aa) was low (Gould *et al*., 1995). These levels of resistance and cross-resistance were related to alteration in a shared receptor that led to reduced Cry1Aa but not Cry1Ab nor Cry1Ac toxin binding to BBMV from YHD2 insects (Lee *et al*., 1995). According to these observations and the toxin binding model from Chapter Two, alteration of either the 170- or 110- and possibly the 210-kDa proteins would lead to resistance in the YHD2 insects, although Cry1Ab and Cry1Ac toxins could still bind to any other receptors of receptor A that do not participate in toxicity ("null receptors") and to receptors B and C. In fact, Lee *et al*., (1995) proposed that receptors B and C were enhancers of toxicity only if toxins were binding to receptor A. Retrotransposon-mediated disruption of a cadherin-superfamily gene correlated with 40% to 80% of Cry1Ac resistance in YHD2 (Gahan *et al*., 2001), although other genes are involved in resistance (Heckel *et al*., 1997a).

The YHD2 strain was continuously selected with Cry1Ac, and resistance to Cry1Ac increased (Kota *et al*., 1999; Chapter Three). Increased resistance in YHD2 can only be explained by development of new mechanisms or increased effect of previous mechanisms of resistance. The main objective of Chapter Three was to study the mechanism involved in increased levels of resistance to Cry1Ac in YHD2.

Our toxin binding experiments demonstrated that none of the Cry1A or Cry1Fa toxins was able to bind to BBMV from the YHD2 strain, in contrast to the results of Lee *et al.* (1995). This discrepancy was probably due to the existence of new resistance mechanisms in the YHD2 insects. Binding of Cry1Ac to BBMV from insects of the F1 generation of crosses between susceptible (YDK) and resistant (YHD2) adults confirmed

that resistance to Cry1Ac was an incompletely recessive trait, since BBMV from the F1 larvae did not bind the same amount of toxin that the susceptible parents, but more than the resistant BBMV. Resistance in YHD2 has been previously reported to be incompletely recessive (Gould *et al*., 1995). Ligand blotting and radiolabeling of BBMV surface proteins demonstrated that the Cry1Ac binding proteins detected in BBMV from YDK insects are present in BBMV from the YHD2 strain. This suggested that reduced toxin binding was probably due to a modification rather than absence of these proteins.

Cross-resistance in YHD2 to toxins that share high homology in the sequence of domain II loops with Cry1Ac suggests the existence of a resistance mechanism that affects Cry1 toxin binding specified by these loops. Although the GalNAc to Cry1Ac interaction site has been localized to domain III of the toxin (Burton *et al*., 1999), the potential for carbohydrate binding of domain II has not been studied. Moreover, domain II of Cry1Aa has a similar structural folding pattern observed in different carbohydrate binding proteins (Burton *et al*., 1999). Cry1 toxin receptors are glycoproteins (Knowles *et al.*, 1991; Nagamatsu *et* al., 1998; Oltean *et al*., 1999; Vadlamudi *et al*., 1995; Valaitis *et al*., 2001), and carbohydrates on these proteins are important for toxin binding (Denolf *et al*., 1997; Ellar *et al*., 1985; Hofmann *et al*., 1988; Knowles *et al*., 1984). Several bacterial toxins recognize glycan moieties in their receptors as a first step in binding to target cells (Hilaire *et al*., 1994; Wang *et al*., 1999). Alteration of the glycosylation of these receptors can lead to reduction of toxin binding (for a review see Varki, 1993). Alteration of glycosyltransferase function is directly related to resistance against Cry5B toxins in *Caenorhabditis elegans* (Griffitts *et al*., 2001).

Lectin blotting using the GalNAc-specific soybean agglutinin lectin (SBA) provided evidence for a correlation between altered glycosylation in YHD2, toxin binding and resistance. At least two proteins (68- and 63-kDa in size) were differentially glycosylated in BBMV from YHD2 when compared to YDK and F1 BBMV. Considering this information, I propose the existence of a resistance mechanism in YHD2 that results in reduced binding of Cry1 toxins by alteration of a shared carbohydrate binding epitope in the receptor recognized by domain II of Cry1 toxins. How might glycosylation be related to Bt resistance? One possibility is that the 68 and 63-kDa proteins are directly involved in the Cry1Ac mode of action and that their altered glycosylation directly affects toxin binding. Alternatively, altered glycosylation may affect recognition of other toxin binding proteins in the midguts from YHD2 larvae.

Proteins of 65- and 62-kDa in size have been identified in brush border membrane proteins from the mosquito *Aedes aegypti* as binding proteins for Cry4B and Cry11A *B. thuringiensis* toxins (Buzdin *et al*., 2002). These mosquito proteins did not bind Cry1Ab, and binding of Cry4B and Cry11A toxins was not GalNAc-dependent. Glycoproteins of 68- and 63-kDa that contain GalNAc residues have been noted in mosquito midgut (Wilkins *et al*., 2001). A 66-kDa glycoprotein is a receptor in human intestine cells for the hemolysin toxin from the bacterium *Aeromonas sobria* (Wang *et al*., 1999).

This study presents future directions for research. The 68- and 63-kDa glycoproteins from *H. virescens* BBMV need to be identified. Their potential involvement in toxin action should be determined. Knowledge of the specific glycosylation steps altered in YHD2 larvae is necessary to understand how altered glycosylation contributes to Bt resistance.

Mechanism for resistance to Cry1Ac in strains cross-resistant to Cry2Aa

As proposed in Chapter Two, if resistance to Cry1Ac in *H. virescens* is due to altered toxin binding, then we would expect cross-resistance to toxins that share high homology in the loops of domain II with Cry1Ac. Cry2Aa toxin has low homology with Cry1Ac in the loops of domain II (Tabashnik *et al*., 1996), resulting in different binding specificity-determining regions (Morse *et al*., 2001) and mode of action (English *et al*., 1994) than those of Cry1Ac. Cry1Ac and Cry2Aa do not share binding sites in BBMV from *H. virescens* (Jurat-Fuentes and Adang, 2001). Therefore, resistance against Cry1Ac due to altered toxin binding should not affect binding or activity of Cry2Aa. Surprisingly, two Cry1Ac-selected *H. virescens* strains developed high levels of cross-resistance to Cry2Aa (Gould *et al*., 1992; Forcada *et al*., 1999). Resistance in these strains was not related to reduced Cry1Ab and Cry1Ac binding (Gould *et al*., 1992). These Cry1Ac and Cry2Aa-resistant strains were backcrossed with susceptible adults, and the resulting hybrid strains (CXC and KCBhyb) were further selected with Cry2Aa to increase resistance against this toxin.

My bioassays indicated that resistance to Cry1Ac and Cry2Aa in the CXC and KCBhyb insects increased after further selection with Cry2Aa when compared with parental strains (Kota *et al*., 1999, Forcada *et al*., 1999). Possibly, the same mechanism is involved in resistance to Cry1Ac and Cry2Aa in both strains. Development of concurrent resistance to both toxins would be achieved if a common step in the mode of action of both toxins was altered. Additionally, the observation that both CXC and KCBhyb strains developed cross-resistance to toxins that share binding sites with Cry1Ac

prompted the question of the potential role of altered toxin binding in resistance in these strains. The main goal of Chapter Four was to investigate if altered toxin binding was related to resistance to Cry1Ac and cross-resistance to other Cry1 toxins in the CXC and KCBhyb strains.

Toxin binding competition experiments demonstrated that binding of Cry1Aa to both CXC and KCBhyb insects is reduced when compared to susceptible (YDK) insects. In the case of CXC insects, a 6-fold decrease in Cry1A binding affinity was measured. Binding of Cry1Aa to KCBhyb BBMV was negligible. Binding of Cry1Ab, Cry1Ac and Cry1Fa to BBMV from both resistant strains was not affected when compared with BBMV from the YDK strain. Furthermore, Cry1Ac binding was irreversible, as observed in the susceptible BBMV. Interestingly, this pattern of reduced Cry1Aa but not Cry1Ab, Cry1Ac or Cry1Fa toxin binding was described for the YHD2 strain before it was further selected for increased Cry1Ac resistance (Lee *et al*., 1995). This result implies that reduced binding of Cry1Aa may always be observed (independently of the levels of resistance) in Cry1Ac-resistant strains. According to this model, binding of Cry1Aa would be a good marker to test for resistance to Cry1Ac in *H. virescens* BBMV. This observation could be especially useful considering that binding of Cry1Ac to many different sites may preclude detection of changes in Cry1Ac binding.

Reduced Cry1Aa binding to BBMV from both CXC and KCBhyb larvae suggests that at least one mechanism of resistance related to alteration of toxin binding exists in these strains. The fact that the level of Cry1Aa binding is higher in CXC than in KCBhyb may be a result of slight differences in the same mechanism.

Resistance in the YHD2 strain was explained by the absence of a cadherin-like protein (HevCadLP) in the midguts of resistant insects (Gahan *et al*., 2001). If the same mechanism existed in the CXC and KCBhyb strains, it would explain the similar pattern of reduced Cry1Aa binding and cross-resistance to Cry1Fa observed in CXC, KCBhyb and YHD2 larvae. The different levels of resistance to these toxins in CXC, KCBhyb and YHD2 may reflect slight differences between the resistance mechanism in each strain. For example, HevCadLP may be modified but not absent from the midguts of CXC and KCBhyb, and thus confer lower levels of resistance to Cry1Ac.

Since cross-resistance to Cry2Aa cannot be explained by alteration of a Cry1A binding site, additional resistance mechanisms should be present in CXC and KCBhyb insects. This additional mechanism of resistance in CXC and KCBhyb must be related to alteration of a step in the mode of action shared by both Cry1Ac and Cry2Aa toxins. Although these toxins have different modes of action, they both undergo the same initial steps of solubilization and activation in the insect midgut juice. Resistance due to differential midgut protease activity has been previously proposed for the CXC strain (Forcada *et al*., 1999). This strain possesses a specific protease activity that correlates with resistance (Dr. B. Oppert, personal communication). Studies on pre- and post-binding events in Cry1Ac and Cry2Aa action will generate more information on this second mechanism.

Conclusions.

Selection for insect resistant strains under laboratory conditions cannot predict whether resistance will develop in the field or which resistance mechanism will evolve.

However, these strains provide important information about possible resistance mechanisms. In natural populations, the gene pool would be much higher, and this would certainly affect development of resistance. The results obtained after studying laboratory-selected strains of *H. virescens* in this dissertation demonstrate that this insect can develop distinct resistance mechanisms against very different Cry toxins.

My comparison between the CXC, KCBhyb and YHD2 resistant strains presents evidence that at least one of these mechanisms seems to evolve frequently after Cry1Ac selection. This mechanism results in cross-resistance to toxins that share high homology in the loops of domain II with Cry1Ac, and is characterized by reduced Cry1Aa binding. My results also demonstrate that either simultaneous or alternative use of different Cry toxins does not guarantee delay of resistance or prevent cross-resistance in *H. virescens*.

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