Screening for Cry proteins with unique receptors in fall armyworm (*Spodoptera frugiperda*) (J.E. Smith) and corn earworm (*Helicoverpa zea*) (Boddie)

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DEDICATION

To my mother for her endless love and support.

Thank you, Mommy.

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ABSTRACT

Transgenic crops producing insecticidal Cry proteins from *Bacillus thuringiensis* (*Bt*) have been efficiently used for insect control since 1996. These transgenic *Bt* crops are responsible for benefits such as reduced rates of chemical insecticide use. Intensive use of *Bt* crops has led to an increase in the development of resistance to the insecticidal proteins that they express. New Cry proteins with unique modes of action are needed so that they may be used in gene pyramiding, a method commonly used to delay resistance to Cry proteins in insects. My thesis research involved identifying Cry proteins suitable for gene pyramiding by testing the toxicity of proteins whose activity and mode of action were unknown, as well as developing truncated cry proteins that lack the specific domain responsible for pore formation in targeted insects. These truncated proteins missing the pore formation domain but containing the binding and accessory domains, could eventually be used in bioassays to determine if new proteins have a distinct mode of action. When the truncated protein and a native Cry toxin are both fed to the insect, the binding ability of the native protein would be reduced, as the two proteins would compete for binding sites. Cloning and expression of truncated Cry1Fa domains produced successful cloning results, but revealed that issues with low expression levels and a high rate of inclusion body formation must be resolved in order for competition bioassays to be conducted with the truncated proteins. Results from bioassays indicated that Cry9Aa is very active against *S. frugiperda* and may share binding sites with Cry1F. Additionally, it was found that Cry1Ba, Cry1Da, and Cry9Aa were active against *H. zea*. These results identify candidate proteins for testing of shared binding sites with Cry proteins in *Bt* corn

and cotton. Lack of shared binding sites would allow for the use of these proteins in gene pyramiding efforts to delay resistance evolution. Additional work is needed to develop a protocol that will allow expression of biologically active truncated *Bt* proteins for use in bioassays.

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CHAPTER I: INTRODUCTION

Abstract

Cry insecticidal proteins, produced by the bacterium, *Bacillus thuringiensis*, are produced during the sporulation phase and are known to target a wide variety of insect pests. For many decades they have been sprayed topically on plants to provide protection from insect pests, but since 1996, have been expressed by transgenic Bt crops, which has provided an estimated yearly benefit of over $100 billion. The majority of Cry proteins follow a three domain structure that allows for the protein to bind to receptors in the insect's gut, then form a pore that will lead to the death of the insect. A major appeal of these proteins is their ability to be highly specific in the insect species to which they are toxic. Since their initial use, many instances of resistance have been observed across the globe. Many strategies have been devised to combat the development of resistance which has slowed, but not prevented the development of resistant populations. The discovery of new Cry proteins with unique modes of action has also allowed for this method of biological pest control to continue as a highly effective method of protecting crops.

Mode of action and resistance mechanisms to Cry toxins

1.1 Introduction and brief history

Bacillus thuringiensis (*Bt*) is a gram-positive bacterium that synthesizes parasporal crystalline proteins, known as δ-endotoxins or Cry insecticidal proteins. When resources are limited, *Bt* vegetative cells undergo sporulation and produce proteinaceous crystals that may contain cytolytic (Cyt) and/or Cry proteins. The known Cry proteins usually have insecticidal activity against a specific group of insects, yet as a protein family target a broad range of insects (Yu et al., 1997). The *Bt* bacterium is also capable of producing insecticidal proteins during the vegetative stage, known as vegetative insecticidal proteins (Vip). Among the *Bt* insecticidal proteins, Cry toxins are the most thoroughly studied and have been shown to be highly effective at managing targeted insect crop pests that threaten food security (Koch et al., 2015). Additionally, Cry toxins demonstrate toxicity towards insects that vector diseases of humans, proving that their benefit spans both agriculture and public health (Mulla, 1990).

The *Bt* bacterium was first discovered in 1902 by Japanese biologist Shigetane Ishiwatari when investigating the cause of sotto disease, which caused silkworm larvae to suddenly collapse, giving the disease its name. He determined the disease was caused by a rod shaped, pore forming bacterium that he isolated from the guts of silkworms and named as *Bacillus sotto* (Aoki and Chigasaki, 1916). In 1911, the bacterium was rediscovered by German scientist Ernst Berliner, who isolated it from flour moths (Berliner, 1915). He named the bacterium as *Bacillus thuringiensis*, and was first to publish his results, explaining why this stands as the accepted name today. In 1951, the

effectiveness of *Bt* was measured on the alfalfa caterpillar (*Colias eurytheme*) (Boisduval) in test plots of alfalfa, and it was found that *Bt* reduced the population of the caterpillar below the economic threshold within just a few days (Steinhaus, 1951). After this, formulations of sprays containing *Bt* would be produced and commercialized. As of last year, 88% of cotton fields in the U.S are planted with *Bt* cotton. This is an increase from the 37% that were planted with *Bt* cotton in 2001 and this value is expected to continue growing (USDA, 2020a).

Another use of *Bt* insecticidal proteins for pest control is their production by transgenic plants. Tobacco modified to express insecticidal proteins from *Bt* were first produced in 1985 in a Belgian laboratory, but were not commercially successful (Milner 1994). European corn borer (*Ostrinia nubialis*) (Hübner*)* was the primary targeted pest upon introduction of *Bt* corn. With larval damage hidden in the stalks and bolls, this insect resulted in unpredictable infestations that were not properly managed by topically applied pesticides. A three-year long, multi-state survey that followed the introduction of *Bt* crops reported an increase in yield and a decrease in the number of farmers scouting for European corn borer damage (Pilcher et al., 2002). Within 20 years (1996-2016), just under 100 million hectares of land were planted with *Bt* crops (Tabashnik and Carriere, 2017). As of 2020, 88% of all cotton planted in the U.S. is genetically engineered and insect-resistant (USDA, 2020b). In this introduction, I will discuss what is currently known about the intoxication process of *Bt* proteins, the development of resistance, and current methods devised to combat the development of resistance.

1.2 Cry toxin nomenclature/classification

Since the discovery of *Bt*, many of its insecticidal proteins have been identified, requiring a simple system for classifying known and new proteins whose insecticidal activity may not be well known. The nomenclature of Cry insecticidal proteins has traditionally been based on pair-wise amino acid sequence identity to previously named toxins. A unique name incorporating four ranks may be assigned to a newly discovered protein. This includes the use of Arabic numbers for the first and fourth rank, as well as uppercase and lowercase letter for the second and third rank, respectively. Proteins share at least 45% sequence identity if they share the same first rank, 78% sequence identity if they also share the same secondary rank, and 95% sequence identity if they also share the same tertiary rank (Crickmore et al., 1998). Next generation sequencing methods have allowed for over 800 different Cry genes belonging to 75 classes (Cry1 to Cry73) to have been discovered so far (Crickmore, 2016). This naming protocol paved the way for new proteins whose toxicity and mode of action may be hypothesized based on sequence similarity but are ultimately unknown.

Advances in prediction of three-dimensional structures of Cry proteins has led to a newly proposed nomenclature for pesticidal proteins (including Cry toxins) that includes structural information (Crickmore, 2020). In this system, different groups of Cry insecticidal proteins exist based on their three-dimensional structure, including Mtx-like, Bin-like, and three domain proteins (Crickmore et al., 2020). Between these three distinct groups, three-domain proteins make up the majority of known Cry proteins, which is more than 53 different subgroups of Cry toxins (Crickmore et al., 2020).

Activity of Cry proteins has been documented against a variety of different insect orders, as well as nematodes, mites, and protozoa (Ye et al., 2012). Some Cry proteins have even been found to be active against insects across multiple insect orders (Van Frankenhuyzen, 2013) (Fig 1.2.a). The impressive diversity of Cry toxins that enables them to have insecticidal activity against so many different species of insects is believed to be due to a high degree of genetic plasticity. Many Cry genes are associated with transmissible plasmids and flanking transposable elements, which are assumed to facilitate gene amplification, allowing for new toxins to evolve from established ones (de Maadg et al., 2001).

1.3 Three-domain Cry toxin structure and mode of action

Most Cry proteins display a number of up to five highly conserved blocks in their sequence (Fig. 1.2). The conservation of these blocks suggests they are important for toxin function/stability. The majority of Cry proteins displaying these conserved blocks follow a three-domain 3D structure (Crickmore et al., 2020a).

In this three-domain structural model (Fig. 1.3), Domain I is composed of an α-helix bundle, Domain II by a β-prism of three antiparallel β-sheets, and Domain III is formed by two anti-parallel β-sheets that form a β-sandwich. While the primary sequences of three-domain Cry proteins may vary significantly, their structural alignment can be very similar. For example, although sequence identity between Cry1Aa and Cry3Aa, Cry3Bb, Cry4Aa, or Cry8Ea are <45% identical, they are all at least 97% structurally similar.

These structural similarities suggest similarities in the mode of action (de Maagd et al., 2001). Intoxication begins with the ingestion of the crystalline protein body, which is then solubilized under the alkaline conditions of the insect's midgut. Proteases within the gut process the protoxin to an activated toxin form by removing the C-terminus end of the protein as well as an N-terminal peptide. Four models have been proposed for the mechanism in which the insect is killed by the toxins (Fig. 1.4). In one of the proposed mechanisms (a), the activated insecticidal protein binds sequentially to alkaline phosphatase (ALP) or aminopeptidase N (APN) on the surface of the midgut epithelial cells, and then to cadherin. Binding to cadherin facilitates oligomerization and pore formation. Outward extensions of Domain II form loops directly involved in receptor recognition and binding (de Maagd et al., 2001). These loops are the most diverse region of Cry proteins as expected from recognition of different receptors by distinct toxins (Schnepf et al., 1990; Gill et al., 1992; Knowles, 1994; Lu et al., 1994). In some cases, Domain III also has a role in receptor recognition (Bosch et al., 1994; Masson et al., 1994, Schnepf et al. 1990). The sequential binding /pore formation model (b) also includes the possibility that Cry protoxin binds to cadherin allowing for oligomer pre-pore formation, and then binding to the APN or ALP receptor to result in membrane insertion. Alternatively, insertion and oligomerization in the membrane plane can happen after binding to APL, ALP, or cadherin (c), or an intracellular cell death mechanism may be activated after interaction with cadherin (d) (de Maagd et al, 2001).

The specific receptor that the Cry protein binds to, as well as its affinity for the insecticidal protein, is determined by the pairing of the toxin and insect (Piggot and Ellar,

2007). For instance, Domain III of Cry1Ac is necessary in receptor binding and toxicity against soybean looper (*Chrysodeixis includens*) (Walker) but the specificity of the same toxin against velvetbean caterpillar (*Anticarsia gemmatalis*) (Boisduval) is mainly determined by Domain II, particularly the less conserved regions in that domain (Mushtaq et al., 2018). After binding, a process that involves Domain I will result in Cry protein insertion in the cell membrane to form a pore that is suggested to be approximately only 2.4 nm in diameter under high pH conditions (Carroll and Ellar, 1997). This membrane pore then leads to osmotic cell death. Disruption of the midgut epithelium from enterocyte death allows for bacteria present in the gut to invade the hemocoel, leading to the death of the insect.

1.4 Resistance to three domain Cry toxins

Evolution of resistance to Cry toxins in targeted insects threatens the long-term use of *Bt* crops and pesticides for pest control. The first lepidopteran pest reported to have developed resistance to a *Bt* insecticidal formulation was the Indian meal moth (*Plodia interpunctella*) (Hübner). Within a laboratory setting, this insect was able to develop resistance to a *Bt* spray within only a few generations. Previous to this study, it was believed that resistance to *Bt* Cry proteins was unlikely to occur (McGaughey, 1985). No cases of resistance were reported within the first few years of *Bt* crop use, but based on cases of resistance to *Bt* sprays, there was concern over this eventually happening (Roush, 1997). The first case of resistance to a *Bt* crop in a lepidopteran insect was

reported in 2002, only six years after their introduction in 1996. This case of resistance was in *H. zea* to Cry1Ac produced by *Bt* corn (Tabashnik et al., 2013).

Numerous instances of resistance to three domain Cry toxins in pesticides and transgenic crops have been reported in both the laboratory as well as in the field (Ferré & Van Rie, 2002; Janmaat & Myers, 2003; Tabashnik *et al*., 2008; Kruger *et al*., 2009; Storer *et al*., 2010; Dhurua & Gujar, 2011; Gassmann *et al*.,2011; Zhang *et al*., 2011). Understanding the mechanism and genetic basis of these cases of resistance is a useful tool in combatting and delaying the development of resistance (Fabrick et al., 2020). It has been found that the most common mechanism of resistance is alteration to binding sites (Jurat-Fuentes et al., 2021). Other mechanisms of resistance include reduced solubilization, incomplete toxin processing, toxin immobilization, and other alterations within the insect that lead to reduced susceptibility (Jurat-Fuentes et al., 2021).

1.5 Insect Resistance Management (IRM) strategies

Methods intended to delay the development of insect resistance are known as Insect Resistance Management (IRM). Several IRM strategies have been proposed based on criteria identifying and producing pesticides that may have a long-term efficacy in delaying the onset of resistance. In the case of *Bt* proteins, these include demonstrating a high mortality effect towards homozygous-susceptible insects by individual *Bt* proteins expressed or present in the pesticide. This ensures maximum susceptibility and reduces the possibility of "redundant killing", which is where two or more cry proteins utilize the same mode of action, not offering any increased benefit from the presence of the additional Cry protein. The second criterion is that the probability of high levels of cross-

resistance between the *Bt* components is low. The third criterion is to ensure that some of the individuals in a population remain untreated with the use of refuge cropping. The final criterion is that the individual toxins must demonstrate relatively equal persistence, as not to encourage the development of resistance to a subset of the toxins produced by the plant.

For *Bt* proteins produced by transgenic (GM) crops, the high dose and refuge strategy has been proposed as an efficient IRM tool to delay resistance (Ferre et al., 2008). This strategy assumes single locus recessive resistance and requires that GM crops expressing *Bt* toxins express a sufficiently high concentration of the toxins to ensure that ≥95% of the heterozygous individuals are killed, as well as 99.99% of homozygous non-resistant individuals (Andow & Hutchison, 1998; US EPA, 2018). In addition, the refuge crops involve growing non-*Bt* crops in alternating rows or plots near *Bt* crops, allowing susceptible insects to survive and mate with the resistant moths that may have developed in the *Bt* crop. Because the mutations that allow for the individuals to be resistant are recessive, these mating pairs will produce susceptible heterozygote offspring that are controlled by the *Bt* plants. The downside of this method is that it requires additional land and other resources to maintain the refuge, which does not provide yield to the farmer (Hellmich and Hellmich, 2012).

Another strategy to delay resistance involves the use of transgenic plants expressing the genes of two or more *Bt* proteins that utilize different modes of action. This strategy is known as gene pyramiding and has the potential to delay resistance more effectively than single-toxin plants and may require fewer or smaller refuges to

accompany these *Bt* crops (Roush, 1998). This method is a useful strategy by broadening the range of insect pests controlled by each transgenic variety and at the same time delaying the development of resistance in the insect, particularly when the mutation is recessive and associated with reduced fitness of the resistant individual in the presence of both *Bt* and non-*Bt* plants (Gould et al., 2016). Other strategies for delaying resistance, such as temporal and spatial crop rotation should still be deployed in tandem to optimize the use of *Bt* crops (Manyangarirwa, 2006).

Since 2005, the majority of newly registered *Bt* crop varieties express proteins representing at least two modes of action (Fig. 1.5). Examples for Lepidopteran pests include Bollgard® II (Cry1Ac + Cry2Ab) and WideStrike® (Cry1Ac + Cry1F) cotton, Genuity® VT Double PRO® (Cry1A.105 + Cry2Ab), Optimum® IntrasectTM (Cry1Fa + Cry1Ab), Agrisure® VipteraTM 3220 (Cry1Ab + Cry1Fa + VIP3A), PowerCore® (Cry1A.105 + Cry2Ab2 + Cry1F), SmartStax® (same proteins as in PowerCore® with Cry3Bb + Cry34/35Ab1 to provide protection against coleopteran pests), and Pioneer Hi-Bred/Dupont (Cry34Ab1/Cry35Ab1 and Cry1F) corn (US EPA, 2015).

The traditional method of identifying *Bt* proteins amenable for gene pyramiding includes first identifying proteins that have a high likelihood of being insecticidally active against a particular species. Competition binding assays are then necessary, which includes the use of a radiolabeled (iodine-125 isotope), biotinylated or fluorescently-labeled toxin alongside a non-radiolabeled toxin in a solution containing midgut brush border membrane vesicles (BBMV). The amount of binding measured when only the labeled toxin is applied to the BBMV is compared to the amount of binding measured

when the labeled and unlabeled proteins are co-administered. By applying two toxins to the receptors and measuring the amount of the labeled toxin that binds in the presence of the unlabeled one, this method quantifies the amount that the two proteins are competing over the same receptors. If binding of the labeled toxin is not reduced when co-administered, it can be concluded that the unlabeled toxin does not recognize the same binding sites as the labeled toxin. When labeled toxin binding is reduced by the competitor, it indicates the toxins bind to the same receptors. Toxins found to bind to different receptors may be suitable in gene pyramiding (Jakka et al., 2015).

1.6 Biological model

The fall armyworm (*Spodoptera frugiperda*) (J.E. Smith) (Lepidoptera: Noctuidae), and the corn earworm (*Helicoverpa zea*) (Boddie) (Lepidoptera: Noctuidae), will serve as the biological models in this study. Both species are among the most significant crop pests across North and South America. They are polyphagous and feed on major crops including maize, soybean and cotton, as well as a variety of other plant species (Sparks, 1979; Fitt, 1989). Severe infestations of these pests can be devastating, resulting in significant crop loss (Cruz et al., 1983; Gore et al., 2003). These species are also highly mobile. For example, populations of fall armyworm in North America will overwinter in the southern United States and migrate to Canada in the summer months (Sparks, 1979). Moreover, *S. frugiperda* has invaded most of Africa, Eastern Asia and Australia to become a global super-pest (CABI, 2017).

Field-evolved resistance to *Bt* crops, defined as at least 50% of the individuals in a population demonstrating reduced susceptibility, was reported as early as 2002 for corn

earworm (Ali et al., 2006) and 2006 for fall armyworm (Storer et al., 2010). The primary Cry toxins in *Bt* crops used for control have been Cry1F for fall armyworm and Cry1Ac for corn earworm, but resistance has been observed against both proteins, as well as when *Bt* crops expressed Cry1Ac and Cry2Ab (Matten, 2007, Matten et al.,2008, Luttrell et al., 2004, Tabashnik et al., 2008, Tabashnik et al.,2009). For fall armyworm, resistance to Cry1F was due to an indel in the *SfABCC2* gene that renders the protein a non-functional receptor for Cry1Fa as well as Cry1A.105 , which share mode of action (Banerjee, 2017; Flagel, 2018). It was hypothesized that the occurrence of resistance to Cry1F in fall armyworm populations in the contiguous U.S. is due to migration of insects from Puerto Rico (Huang et al., 2014). However, resistance alleles from Puerto Rico have not been detected in the mainland U.S. (Banerjee, 2017; Flagel, 2018). The ability for these mutations to develop and the ease of migration by lepidopteran pests, highlights the need to identify additional *Bt* proteins to which these species are susceptible.

CHAPTER II: CLONING AND EXPRESSION OF TRUNCATED CRY1F PROTEINS

Abstract

Bacillus thuringiensis (*Bt*) insecticidal proteins have been used in controlling Lepidopteran pests of corn and cotton throughout the past 20 years, due to their effectiveness and high specificity. The fall armyworm (*Spodoptera frugiperda*) is targeted by the Cry1F protein produced by both corn and cotton. The evolution of resistance in pest insects poses the biggest threat to sustainability of transgenic crops producing *Bt* proteins. Populations of *S. frugiperda* developed resistance to Cry1F in Puerto Rico by 2007, within three years of registration. Resistance was linked to reduced binding to a mutant receptor. Pyramiding of *Bt* toxin genes resulting in plants producing multiple toxins is highly beneficial to delay resistance evolution as long as the toxins recognize different receptors, i.e. have different mode of action. The overarching objective of this project was to use truncated Cry1Fa toxin fragments as non-toxic competitors of binding that could be used in high-throughput protocols identifying novel insecticidal proteins that may be suitable for gene pyramiding with Cry1Fa. In this study, the three structural domains described for Cry1Fa were cloned as candidate competitors and produced in a heterologous system. The truncated Cry1Fa domain peptides allow for *in vivo* testing of the ability for these proteins to prevent binding and toxicity of full length Cry1Fa toxin against larvae of *S. frugiperda*.

Introduction

The evolution of resistance in pest insects poses the biggest threat to sustainability of transgenic crops producing *Bt* proteins. One of the key steps in the Cry1 mode of action is the recognition of receptors in target midgut cells. Pyramiding of Bt toxin genes resulting in plants producing multiple toxins that recognize distinct receptors is highly beneficial to delay resistance evolution. An improved method for identifying proteins amenable to gene pyramiding was proposed by Jerga et al. (2019). In this method, an amino acid substitution in Domain I of Cry1Ab was introduced to eliminate the pore-forming activity of the proteins, rendering them inactive in the intoxication of the insect. Because this mutation doesn't affect the protein's receptor binding function, the disabled Cry1Ab protein applied in combination with and in excess of unaltered Cry1Ab was capable of reducing insect mortality in bioassays. To explain further, because the proteins competed for the same receptor, binding of the unaltered protein was reduced. This result was quantifiable as a decrease in mortality compared to when only the unaltered protein is fed to the insects. If the proteins did compete for the same receptor, the disabled protein would not cause reduced binding of the unaltered protein to result in mortality at an equivalent rate of when only the unaltered protein is applied. This method proved to be valuable in identifying whether the modes of action between two proteins overlap or are unique.

As an alternative to the disabled insecticidal protein method proposed by Jerga at al. (2019), we propose a method allowing performance of competition bioassays with truncated or isolated proteins missing domains responsible for pore formation in the

novel insecticidal protein. Flores et al. (1997) determined that Domains II and III of Cry1Ab are still capable of receptor recognition and binding to brush border membrane vesicles from tobacco hornworm (*Manduca sexta*) (Linnaeus) and cabbage looper (*Trichoplusia ni*) (Hübner), confirming the role that Domain II and III play in receptor binding as well as their ability to occupy receptors after the removal of Domain I. Our approach would involve using an excess of truncated protein to compete for the receptor with the wild type toxin.

In order to provide proof of concept, this project focuses on Cry1Fa as a model protein to demonstrate that a truncated Cry1Fa protein can be produced as a biologically active. The truncated Cry1Fa proteins could later be used as competitor to identify Cry insecticidal proteins with different mode of action so that they may be expressed alongside Cry1Fa in transgenic plants.

Research Design and Methodology

Bacterial stocks

A clone of *Bacillus thuringiensis* (HD73) mutant strain harboring the *cry1Fa* toxin core gene in the pHT315 vector under the control of the *cry1Ac* promoter was generously provided by Dr. Jie Zhang (institute of Plant Protection, Chinese Academy of Agricultural Sciences, Beijing, China) and described elsewhere (Zhao et al., 2015)

Objective one: Cloning of Cry1Fa domains in Escherichia coli

Amplification of Cry1Fa protein domains

The nucleotide sequence for the Cry1Fa insecticidal protein from the NCBI database (accession number JE980828.1) was used to design primers (Table 2.1) amplifying each of the three structural domains, which were predicted based on a 3D structure generated in Swiss Model software hosted at Expasy (Switzerland). Additionally, primers were designed to contain restriction sites that would allow ligation into the pTrcHis expression vector (Fig. 2.1). Considering previous reports of issues dealing with the solubility of the isolated Domain II of Cry1A toxins (Wacobi and Yasura, 1995), fragments containing Domains II and III of Cry1Fa were also cloned, which in the case of the Cry1Ab toxin was shown to be soluble (Flores, 1997).

Genomic DNA was purified from the *Bt* HD73 containing the *cry1Fa* toxin core, using the Qiagen miniprep kit (Germany), following the purification protocol provided by the manufacturer. The amplification reaction for the domain II+III protein contained 12.5 µL PCR Master Mix (Thermo Fisher Scientific, MA, USA), 5 µl of 2.5 µmol forward domain II primer, 5 µl of 2.5 µmol reverse domain III primer, 2 µl of roughly 1 µm/µL sample of Cry1Fa template DNA, and 0.5 µL of sterile H_2O. Domain II and Domain III samples were prepared with the same ingredients and concentration, but with the II or III forward and reverse primers, respectively. Amplification was performed using a program including a denaturation step at 98°C for 30 seconds, followed by 30 cycles of denaturation at 98°C for 10 seconds, primer annealing at 43°C for 10 seconds, and extension at 72°C for 75 seconds. Upon completion of the cycling steps, a final extension

at 72°C for 5 minutes was done and then the reaction was stored at 4°C. The molecular size of amplicons was analyzed using agarose gel electrophoresis.

Ligation to expression vector

The amplified *cry1F* domains and purified plasmid pTrcHis A were digested with Hind III and Xho I in NEBuffer 2.1 for one hour at 37ºC in separate reactions. Restriction enzyme digest products were electrophoresed in a 1% agarose gel and purified using Qiaquick gel purification kit (Qiagen, Germany). Ligation reactions with insert and vector (10:1 molar ratio) were performed with T4 DNAse ligase (Invitrogen, Carlsbad, CA, USA) overnight at 4ºC. The reaction was inactivated by incubating at 65ºC for 20 minutes.

Transformation of recombinant plasmid into E. coli

Ligation reaction products were transformed by heat shock into chemically competent *E. coli* Lemo21(DE3) cells (New England Biolabs, USA), and transformation reactions were seeded on LB agar plates containing 50 µg/µl ampicillin for selection of successful transformants. To confirm transformants, individual colonies were grown in 5 ml LB media and 50 ug/ml ampicillin cultures overnight, and then plasmid DNA was purified using the Qiaprep Miniprep kit (Qiagen, Germany). Purified plasmids were sequenced at the University of Tennessee Genomics Core using the universal primers designed for the expression vector. Samples were also analyzed using agarose gel electrophoresis for the expected amplicon size.

Bacterial stocks in 25% glycerol were prepared from individual colonies picked from LB plates with 100 μg/ml ampicillin. After colonies were grown in cultures as described below, stocks were made mixing 500 μl of culture media and 500 μl of sterile 50% glycerol. Glycerol stocks were store at -80^{o}C.

Objective two: Heterologous expression, solubilization, and purification of Cry1Fa domains

Heterologous expression of Cry1Fa domains

Preliminary expression of Cry1Fa domains followed a protocol provided by Thermo Scientific (Massachusetts, USA) and began with inoculation of LB media containing ampicillin (50 μg/ml) with a single recombinant *E. coli* colony. This was performed for each of the recombinant *E. coli* samples containing domain II, domain III, and combined domains II+III. Bacteria were grown overnight at 37^{o}C with shaking, and then 0.2 ml of the overnight culture was used to inoculate flask containing 500 ml of LB containing ampicillin (50 μg/ml). The cultures were grown at 37^{o}C with shaking to an OD_{600} = 0.55-0.6, when the cells should be in mid-log growth phase. A 1 ml sample of the culture at this point in the expression process was collected and after centrifugation at 10,000 rpm for 10 minutes the pellet was collected and stored at 4^{o}C. This sample served as the time zero for comparison with the final expression product. Isopropyl β- d-1-thiogalactopyranoside (IPTG), which serves to silence the lac operon and increase transcription in the cells, was added from a 1 M stock to the flask for a final concentration of 1 mM. Cultures were grown for a minimum of five additional hours at 37^{o}C with shaking and pellets from 1 ml culture samples were extracted at one-hour

intervals and stored at 4°C. Depending on the time that individual cultures reached the OD_{600} value at which they were induced, some cultures were incubated up to an hour longer. Once all time points were collected, culture pellets were resuspended in 100 µl of 20 mM phosphate buffer (0.0049 M NaH_2PO_4, 0.0151 M Na_2HPO_4, pH 6.8), and then flash frozen in liquid nitrogen. Samples were thawed at 42°C and the freeze/thaw cycle was repeated 2–3 additional times to lyse cells, and the final pellet containing insoluble protein was refrigerated. Aliquots (15 µl) of the pellet and supernatant samples were mixed with an equal volume of 2X SDS-PAGE Sample Loading Buffer (4% SDS, 10% beta-mercaptoethanol, 20% glycerol, 0.1% Tris pH 6.8, and 0.005% of bromophenol blue), heat denatured (100°C for 10 minutes) and resolved in a 10% SDS polyacrylamide gel. After electrophoresis, gels were stained with Coomassie blue (Thermo Fisher Scientific, MA, USA). A negative control for expression, consisting of pellets from cultures of TOP10 *E. coli* containing the empty pTrcHis/CAT vector was included. These bacteria containing the pTrcHis/CAT vector harbor the chloramphenicol acetyl transferase (CAT) gene encoding a CAT protein of approximately 30 kDa that is expected to appear at approximately 0.5 hours after IPTG induction and continue for more than 4 hours afterwards. The optimal time post IPTG-induction to harvest the cells, based on the amount of protein expression detected after SDS gel electrophoresis of the samples collected hourly and was determined to be between three and seven hours. Small modifications to this protocol, including increasing ampicillin selection concentration to 100 µg/ml, were made in attempts to increase production of soluble recombinant protein.

Production of Cry1Fa domains for further tests was done in 2L flasks with 1 L of LB plus ampicillin (100 ug/ml). selection plates prepared from the glycerol stocks and grown to an optical density$_{600}$ of 0.5 to 0.6. Protein expression was induced by adding IPTG to achieve a total 0.4 mM concentration. Cultures were then kept overnight (roughly 16 hours) at 16^{o}C before collection.

Solubilization of Cry1Fa truncated proteins

Cultures were centrifuged at 10,000 g and 4^{o}C to collect bacterial pellets. Pellets were then resuspended in 50 ml of solubilization buffer (50 mM Na_2CO_3, 0.1 M NaCl, pH 9.5), and then 0.1% 2-mercaptoethanol and 10 mg/ml of DNase I (RNase-free, New England Biolabs, USA) were added to the samples, reducing overall viscosity. The samples were then sonicated at 10 kHz for 95 seconds with 10 second on/off intervals (Q700 sonicator, Sonica LLC., Newtown, USA). After incubation overnight at 4^{o}C with mild agitation, the samples were centrifuged at 10,000 rpm and 4^{o}C. The supernatant was transferred to a new container and stored at 4^{o}C and the pellet was resuspended in 50 ml of solubilization buffer.

Western blotting

A Western blot was first performed to verify that the proteins were solubilized. Samples (15 µl) were mixed with 3 µl of 6x SDS-PAGE sample loading buffer (5X solution of 250 mM Tris·HCl, pH 6.8, 10% SDS, 30% (v/v) Glycerol, 10 mM DTT, 0.05% (w/v) Bromophenol Blue) and incubated for 10 minutes at 100^{o}C to denature the proteins. After cooling, tubes containing samples were centrifuged and 15 µl loaded into

lanes of a 12% SDS-PAGE gel along with 10 μL of Precision Plus Protein™ Dual Color size reference ladder (Bio-Rad, Hercules, CA, USA). Replicated gels were prepared and samples were electrophoresed for roughly 1.5 hours at 200V. One of the gels was stained in Coomassie Blue (Fisher BioReagents, NH, USA), overnight to visualize the total protein content of samples in the gel.

The other gel was transferred to a polyvinylidene difluoride (PVDF) filter by electroblotting in Towbin's transfer buffer (192 mM glycine, 25 mM Tris, 0.1% SDS, 20% methanol) at 60 V for 45 minutes, until the size reference ladder was no longer visible in the gel. After transfer, filters were blocked for 1 hour with constant shaking in blocking buffer (1X PBS, 0.1% Tween-20, 3% w/v BSA). Filters were then probed with a 1:25,000 dilution of 6X-Histag HRP primary antibody, or a 1:20,000 dilution of Cry1Fa rabbit polyclonal antisera added to the blocking buffer and incubated for an additional hour with constant shaking. The blocking buffer was then discarded and the membrane washed five times in washing buffer (1X TBS, 0.1% Tween-20 with 0.5% w/v BSA) with 10 min incubations between washes. For detection of anti-Cry1F, filters were probed with a 1:2,000 dilution of goat anti-rabbit HRP secondary antibody in washing buffer for 1 hour, and then washed as before. The filters were developed using SuperSignal West Pico PLUS Chemiluminescent Substrate (Thermo Scientific, MA, USA) as instructed by the manufacturer.

Purification of proteins via liquid affinity chromatography

The supernatant of solubilized samples (using method described above) were used for protein purification via affinity chromatography in an ÄKTA Pure FPLC (Cytiva Life Sciences, USA). A HisTrap™ HP column was affixed to the system to purify recombinant proteins containing a 6x histidine tag. The column was rinsed with buffer A (20 mL sodium phosphate, 0.5 M NaCl, 5 mM imidazole, pH 7.4). After running samples through the machine, those that bound to the column were eluted off of it. This was done using a linear gradient from 0% to 100% of buffer B (20 mL sodium phosphate, 0.5 M NaCl, 1 M imidazole, pH 7.4) over 5 ml. All fractions were collected after completion and stored at 4^{o}C. Samples where the UV increased (indicating the presence of protein) or where a UV peak was expected were electrophoresed in a 12% SDS-PAGE gel and a western blot was performed using with method described above with 1:20,000 dilution of Cry1Fa antisera.

Due to the samples having additional proteins present, the Cry1Fa domain concentration was estimated via Bradford assay alongside three concentrations of bovine serum albumin (BSA) (Thermo Fisher Scientific, MA, USA), rather than the overall concentration of protein in the sample. A Western blot was also performed after subsequent purification steps to check for the presence of the protein of interest in different eluted fractions.

Results

Cloning of Cry1Fa domains in E. coli

Throughout the cloning process, samples were electrophoresed in a 1% agarose gel after each step to ensure that the products were present and had the expected molecular size. After performing a miniprep from cultures of two *Bt* clones producing Cry1Fa, purified plasmid DNA was electrophoresed in a 1% agarose gel (A and B in Fig. 2.2). It was expected that the plasmid size would be over 10,000 bp as this contained the toxin gene and pHT315 vector. The cultures were grown in media containing ampicillin to ensure that the cultures would not be contaminated. Based on the cultures been resistant to the selection antibiotic and the size of the plasmid band in the gel, the purified plasmid from the Cry1Fa gene was detected in both lanes.

After PCR amplification using primers designed to amplify regions of Cry1Fa that would contain domains II (DII), III (DIII), and II plus III (DII+III), the resulting amplicons were examined by electrophoresis in an agarose gel (Fig. 2.3). Amplicons of the expected size were detected for DII (482 bp), DIII (443 bp) and DII+DIII (925bp). These amplicons were purified for ligation into the pTrcHis vector and transformation into *E. coli* LEMO21(DE3) cells.

Successful transformants from selection plates were used for production and purification of plasmids in minipreps, which were examined by electrophoresis (Fig. 2.4). Plasmid DNA was present in the miniprep samples, but due to the plasmids being circular supercoiling resulted in two bands being visible in lanes two through five. Despite supercoiling, the main bands on the gel for lanes two through five show bands that begin

at roughly 4.5 kbp, 5 kbp, 5.5 kbp, and 5 kbp for the empty vector, DIII, DII+III, and DII, respectively. The sizes for each of these were 4414 bp, 4857 bp, 5339 bp, and 4896 bp, respectively. Purified plasmids were submitted to DNA sequencing to ensure that the expected sequence was present without any errors that may cause a frame shift or incorrect reading orientation, confirming proper alignment between the insert region of the plasmid and the corresponding sequence of Cry1Fa. Furthermore, DNA sequencing confirmed that the inserts were in the correct reading frame and direction, although an additional codon was detected at the 5' end of the inserted Cry1Fa region for both DII and DII+DIII inserts directly after the restriction enzyme cutting site used for ligation, probably the result of primer design. Considering that this was a complete codon and its location at the beginning of the sequence, it would have no effect on the protein that is produced through translation.

Heterologous expression, solubilization, and purification of Cry1Fa domains

Transformants were grown and induced to express their respective recombinant protein with IPTG induction. After collection of cultures, confirmation that the proteins of interest were expressed and solubilized was achieved in two ways: total protein staining and Western blots. Proteins extracted from bacterial cultures were electrophoresed and then gels stained to analyze total protein content in each sample (Fig. 2.5). The presence of the recombinant protein band was examined comparing samples prior and after induction. If the recombinant protein was produced, a dark band would be present at the expected molecular size of 36 kDa after induction but not in the pre-induction culture. Contrary to the expected result, a band of 36 kDa was observed in both

induced and not-induced sample. This result suggests that the amount of recombinant protein produced by these cultures may be low. Samples containing isolated DII and DIII proteins were included in all of the following steps. Due to reduced solubility and activity that was reported by Flores et al. (1997) for the single domain truncated proteins, emphasis will be put on achieving expression and solubilization of DII+III protein from this point on.

Automated Western blots of proteins from expression cultures prior to and five hours post induction with IPTG induction were probed with Cry1Fa antisera and goat anti-rabbit IgG secondary antibody to detect the expressed Cry1Fa fragments. Bands of the expected molecular sizes for DII (21.5 kDa), DIII (20.8 kDa), and DII+III (36 kDa) were observed in induced samples (Fig. 2.6). The bands detected were determined to be 31kDa, 29kDa, and 45kDa in size for DII, DIII, and DII+III, respectively. Our group has previously observed this deviation of size determinations from expected values when using the automated Western blotting unit. The intensity of the bands detected at the same size in the stained gel that the amount of protein that is expressed is minimal.

When checking the samples after solubilization by SDS-PAGE using total protein staining, we expected to observe the recombinant protein band in the post-solubilization supernatant rather than the pellet. This confirms that the protein was no longer in inclusion body form. While protein was visible in all samples at the expected molecular size of the DII+III protein, the intensity of the band in the pre-solubilization sample indicates the proteins of interest were exclusively in the supernatant (Fig. 2.7a).

Western blotting of the samples before and after solubilization showed multiple bands in each line being recognized by the Cry1Fa antisera (Fig. 2.7b). Bands of the expected molecular size for recombinant proteins were present in the supernatant after solubilization, indicating that those proteins were no longer in inclusion bodies. However, much of the protein was still present in the pellet indicating that not all of the protein was solubilized.

Samples of solubilized DII+III Cry1Fa domains were purified in an FPLC by affinity purification using the 6xHis-tag at the N-terminus of the recombinant protein. This was also used as an attempt to concentrate the proteins. The absorption of UV light indicates that a large amount of protein passes through the column rather than binding (Fig. 2.8). It was expected that the recombinant protein would bind to the HisTrap HP column and be eluted during the linear increase to 100% of Buffer B containing imidazole. However, this was not the case, as no UV peak was detected in fractions. Only a small peak occurred after transitioning to 100% of buffer B.

Based on the chromatogram, samples from fractions where the UV increased were tested by Western blotting using Cry1Fa antisera to detect the presence of the DII+III protein (Fig. 2.9). That blot did not detect bands for fractions during the 0-100% increase of imidazole, which was when the 36 kDa DII+III protein expected to be eluted from the column. In contrast, fractions 11 and 12 showed a faint band slightly lower than the expected size of DII+DIII that could potentially be the protein of interest, yet the number of other bands recognized by the Cry1Fa antisera at a greater intensity make it difficult to

confirm. Nevertheless, based on the UV peak height and the amount of other proteins present, this would represent an extremely small of amount of purified DII+DIII protein.

Discussion

Successful identifications of DNA and protein at their expected molecular sizes in agarose gels, SDS-PAGE gels, and Western blots indicates that the DII, DIII, DII+III proteins were successfully cloned and expressed. This is also supported by DNA sequencing performed on plasmids purified from transformed cells. To be used in competition bioassays, the truncated proteins need to be expressed and solubilized in high quantity.

Observed problems with identifying the proteins after solubilization and after FPLC purification likely stem from two issues. The first potential issue was the low expression levels detected. Protein expression in this system is controlled by the lac operon and influenced by a number of factors. The promoter used in this study, the T7 *lac*O promoter, was suitable with expression in the cell line used, LEMO21(DE3). Commercially available products were used to ensure that compatibility, strength of the promotor, metabolic state of the cells, and copy number would not be concerns. Based on the results from the Western blots, I concluded that the proteins of interest were present. However, low levels of expression were observed despite testing different conditions under which the cell cultures were grown and the proteins later expressed. Proteins were also detected in the samples prior to IPTG induction that matched the expected molecular

size. In the absence of IPTG it is not uncommon for cells to produce a basal level of expression from the lac promoter. This is referred to as “leaky expression”, which could explain the detection of recombinant protein in the uninduced samples (Nielsen et al., 2007). Optimizing the expression protocol could include checking the amount of protein expressed after induction at different OD600 values or testing additional IPTG concentrations for induction. These variables may influence the amount of protein the cells are able to produce before nutrients in the media are depleted.

The second potential issue is the formation of insoluble proteins, which was evident by the proteins present in the pellet after centrifugation. Flores at al. experienced similar issues with solubility when cloning and expressing truncated domains of Cry1Ab (Flores et al., 1997). Insoluble proteins typically are the result of misfolding, and the need to form disulfide bonds results in aggregation. These proteins often do not demonstrate biological activity when aggregated (Singh et al., 2015). As reported by Francis and Page (2010), *E. coli* cells have an average success rate of producing soluble proteins that is around 40-60%. While some inclusion bodies have been shown to have considerable biological activity, these are characterized by a loose arrangement of protein molecules, allowing them to be solubilized easily (Jevsevar et al., 2005; Peternel and Komel, 2010).

The amount of the protein of interest present in the pelleted sample after expression and even after sodium carbonate solubilization indicates that these proteins were aggregating in very densely packed clusters. While other methods may be capable of solubilizing a higher percentage of the expressed protein, they often include the use of a denaturant. These methods would require an additional procedure of refolding the

proteins, which could result in misfolding, producing truncated Cry proteins whose Domain II is incapable of binding.

The addition of more steps (solubilization using a denaturant, refolding) allows for an increased likelihood of the proteins misfolding, reducing their chance of being bioactive. Ideally, the proteins of interest would be expressed at a high enough rate and in sufficient amounts for performing competition bioassays. Additionally, a high percentage of those would be need to soluble and properly folded to allow for bioactivity. Modifications to the expression protocol recommended by Invitrogen were made in hopes of producing proteins that were more easily solubilized. These included decreasing the incubation temperature, decreasing the IPTG concentration, and increasing the length of time after induction, as these have previously been found to contribute to a decreased rate of inclusion body formation (Carrio, 2005). Changing these variables can help to slow down cell processes, leading to reduced protein aggregation. Another study reported production of soluble and active recombinant cyclomaltodextrinase when induced at a concentration of 0.05 mM IPTG, but not when a concentration of 0.1 mM was used (Turner et al., 2005). To produce an increased amount of soluble DII+III proteins using the cells already prepared, it would be beneficial to try out different modifications made to the expression protocol and to compare the proportionate amount of DII+III protein in the supernatant that is detected in a Western blot.

To prove that the proposed method for more efficiently identifying Cry insecticidal proteins is practical and effective, the proteins must be properly folded and biologically active, which would be determined after initial bioassays. Results of binding

experiments with truncated Cry1Ab domains II and III protein confirmed that the protein maintained binding functionality in the absence of domain I (Aranda et al., 1997). It is likely that the truncated protein containing Domains II and III of Cry1Fa would also retain binding functionality based on what is known about the structure and sequence homology of Cry proteins sharing the same primary ranking. Performing competition binding assays with the recombinant Cry1Fa domains would help confirm a number of other factors which were not confirmed in this study, such as the folding of the proteins and their ability to bind within the insect's gut. Additional protocol optimization steps could be taken after initial competition binding assays to produce proteins that are folded in the native configuration of Cry1Fa, but without the pore forming domain. While several issues might have arose that have required additional experiments to achieve truncated Cry proteins that would demonstrate an ability to block binding of native Cry1Fa proteins, it is expected that, based on previous research, this method would be possible (Flores, 1997; Jerga et al., 2019).

CHAPTER III: DETERMINATION OF TOXICITY AND RECEPTOR BINDING OF CRY1BA, CRY1DA, AND CRY9AA IN *SPODOPTERA FRUGIPERDA* AND *HELICOVERPA ZEA*

Abstract

In North America alone, the potential economic losses due to agricultural insect pests are estimated to be in the billions when proper control methods are not available. Within the United States, nearly 85% of the commercial corn crops in 2016 contained *Bt* insecticidal traits. The development of resistance to Cry insecticidal proteins produced in transgenic crops presents an urgent need to identify additional insecticidal proteins with activity against the most damaging pests that also utilize novel modes of action. Identifying Cry proteins suitable in gene pyramiding due to their toxicity and selective receptor binding would be useful in delaying the inevitable development of resistance. In this study, plasmids containing the gene to express Cry1Ba, Cry1Da, and Cry9Aa proteins were used to produce and purify these toxins. After cloning and protein expression, bioassays were conducted to determine their activity in fall armyworm (*Spodoptera frugiperda*) and corn earworm (*Helicoverpa zea*). It was found that Cry9Aa was active against fall armyworm, with an LC50 value of 0.95 µg/cm^2. Bioassays with earworm found that Cry1Ba, Cry1Da, and Cry9Aa were active and had LC50 values of 1.27µg/cm^2, 1.66 µg/cm^2, 2.2 µg/cm^2, respectively.

Introduction

Environmental benefits of the use of insecticidal proteins from *Bacillus* thuringiensis (*Bt*) over traditional chemical insecticides include a high level of effectiveness, high target specificity, and a reduced environmental cost. For instance, *Bt* insecticidal proteins have contributed to a decrease in the amount of chemical pesticides used worldwide (Benbrook, 2012). The *Bt* cells rely on proteins, such as Cry and Vip toxins, that are produced during its pathogenic process to intoxicate the insect. Sprays and granules have been formulated to include *Bt* strains that produce certain toxins. Modern *Bt* technology has allowed for insecticidal proteins to be incorporated into the genome of plants, allowing for them to produce the proteins (Abbas, 2018). The effectiveness of *Bt* crops has resulted in an increase in their use. Worldwide, the planting area of *Bt* crops has grown from an initial 1.1 million hectares in 1999, to over 100 million hectares by 2017 (Tabashnik and Carriere, 2017; Briefs, 2017).

Feeding on at least 100 species of plants in 29 families, including corn and soybean, *H. zea* is a damaging pest that threatens food security in North America. Larvae of this insect destroy the economically important parts of the plants, by boring into the fruits and flowers (Pearce, 2017). Serving as the main pest of corn in South and North America, *S. frugiperda* is a highly polyphagous pest that in more recent years, has invaded Yemen, western Australia, and numerous countries in Africa (FAO, 2019; IPPC, 2020). *Bt* crops producing Cry and Vip3A proteins have been used to control these and other damaging lepidopteran pests since 1996 (US EPA, 1998).

The evolution of resistance to *Bt* toxins and other insecticides occurs at different rates, even in closely related pests, such as *H. zea* and *Helicoverpa virescens*. This highlights the importance of using *Bt* toxins in which the target insects are highly susceptible as well as meeting high-dose standards (Tabashnik et al., 2013). For instance, while Cry1Ac is used in many transgenic crops and *Bt* sprays as a highly effective toxin towards *H. zea*, it is only moderately toxic (Karim, 2000). In 1998, the EPA received reports of crop failure from *H. zea*, but laboratory studies found no reduction in sensitivity to Cry1Ac in this species or a reduction in *Bt* expression in plants damaged by the pest (US EPA, 1998). The low relative activity of Cry1Ac probably contributed to evolution of resistance (Reisig et al., 2018). Long-term studies investigating cases of resistance to cotton expressing pyramided Cry toxins in this species have found varying levels of susceptibility across the southern United States (Reisig et al., 2018).

In *S. frugiperda*, resistance to corn producing Cry1F in Puerto Rico was first reported in 2006 (Storer et al., 2010). Banerjee et al. (2018) reported that this resistance is due to a mutation in the ABCC2 subfamily C2 gene that causes this Cry1F receptor to be non-functional. This mutation was found to be both autosomally inherited and highly recessive, indicating that not meeting the high-dose recommendation was likely a contributing factor (Tabashnik et al., 2013).

As mentioned in section 1.8, the use of gene pyramiding as a method of delaying resistance can be highly effective, especially when other tools such as the high dose strategy and refuge cropping are also utilized. This chapter was aimed to identify one or more Cry toxins that were both highly active against *S. frugiperda* or *H. zea* and utilized

a dissimilar mode of action to Cry1Ac, so that they may be suitable for gene pyramiding alongside this toxin. The three toxins tested in this project include Cry1Ba, Cry1Da, and Cry9Aa, which have been the subject of varying amounts of research. Publications detailing work on these toxins on lepidopteran species, particularly the two species involved in this study, are detailed below.

Pinto et al. (2011) reported Cry1Ba as toxic towards *S. frugiperda*, exhibiting a lethal concentration killing 50% of individuals (LC50) of 10.88 μg/cm^2 when applied topically to leaf disks and fed to second instar larvae. Higher rates of effectiveness towards *S. frugiperda* were reported for Cry1Bb (Luo et al., 1999). While these toxins have 95% similar sequence identity, their few dissimilarities allow for a change in the activity against this species. Available data on Cry1Ba activity against *H. zea* suggests low toxicity with LC50 >1 μg/cm^2 (Karim et al., 2000).

Cry1D bioassays indicated this protein is highly toxic to *S. frugiperda* with an LC50 of 77 ng/cm^2 (Aranda et al., 1996). A Cry1Da mutant, Cry1Da_7, also demonstrated toxicity towards a Vip3A-resistant strain of *S. frugiperda* (Wang et al., 2019). The variation in susceptibility to Cry1D and Cry1B among different populations of *S. frugiperda* was studied and determined to vary over a 280-fold in LC50 (ng/cm^2) for insects found in Brazil and Colombia (Monnerat, 2006).

While no data exist demonstrating that Cry9Aa is toxic towards *S. frugiperda* or *H. zea*, insecticidal activity was shown towards other economically important insects belonging to the family Noctuidae, such as *Plutella xylostella* and *Spodoptera exigua* (Kuvshinov et al., 2001, Naimov, 2014). In *Helicoverpa armigera*, feeding assays

showed that Cry9Aa had an LC50 of 727.84 ng/cm^2 when applied to artificial diet. When tested alongside Cry1Ac, the LC50 for Cry9Aa was found to be 18,200 times greater than for Cry1Ac. This indicates a comparatively low level of activity in this species, although Cry9Aa and Cry1Ca showed a synergistic effect when applied in combination (Li and Bouwer, 2014).

Overall, the amount of data available for the two noctuid pest species involved in this study, particularly *S. frugiperda*, indicates that Cry1Ba, Cry1Da, and Cry9Aa may be suitable as traits expressed by transgenic crops. Based on the information available, we tested Cry9Aa in *S. frugiperda* and Cry1Ba, Cry1Da, and Cry9Aa in *H. zea*. Additionally, the PR1 strain of *S. frugiperda* was used in bioassays with Cry9Aa as an additional method of checking if receptors are shared in that insect.

Research Design and Methodology

Objective one: Transformation, expression, solubilization, and purification of Cry1Ba, Cry1Ca, and Cry9Aa

Clones

E. coli clones containing the *cry1Ba*, *cry1Ca*, and *cry9Aa* genes were provided by the *Bacillus* Genetic Stock Center (BGSC) at The Ohio State University in Columbus, USA. All clones contain the *cry* gene and pTZ19R vector in DH5-alpha cells. Accession numbers for the clones are: ECE 128 (Cry1Ba), ECE 129 (Cry1Da), ECE 130 (Cry9Aa). Plasmid-cured *Bt* were also provided by the BGSC with accession numbers 4D11, 487, and 4Q8. Clones of *Bacillus thuringiensis* (HD73) producing Cry1Ac or Cry1Fa toxins

were received from the BGSC and Dr. Jie Zhang (Institute of Plant Protection, Beijing, China), respectively.

Production of crystalline toxins

Plasmids from bacterial clones were purified from a 5 ml LB media culture containing 100 ug/ml ampicillin using a Qiagen Miniprep Kit. The plasmids were then transformed into One Shot™ INV110 Chemically Competent cells (Thermo Fisher Scientific, MA, USA) using the manufacturer's instructions. Chemical transformations were prepared from 5 mL cultures of these cells and a miniprep was performed again to prepare non-methylated plasmids suitable for expression in *Bacillus thuringiensis* (*Bt*) cells.

Initially, *Bt* strains 4D11, 3Q7, 3Q8, and 4D12 were considered as hosts for Cry1Ba, Cry1Da, and Cry9Aa toxin plasmids. However, based on results from preliminary experiments, we selected strains 4Q7 and 4Q8 for transformation. Strains were grown on three LB plates containing no antibiotic, 100 μg/ml ampicillin, and 50 μg/ml kanamycin. Electrocompetent cells were prepared by growing 1ml of overnight starter culture (LB media, no antibiotic, grown at 28^{o}C and 160 RPM) in 100 ml LB media cultures containing no antibiotic at 28^{o}C and 160 RPM to an OD_{600} of 0.6, and then collecting cells by centrifugation (5,000 RPM, 4^{o}C). Pellets were washed with ice-cold sterile H_2O, and centrifuged again (5,000 RPM and 4^{o}C). This washing step was repeated two additional times. Pellets were then resuspended in a cold 10% glycerol solution, aliquoted (200 μl) and stored at -80^{o}C until used. Roughly 5 ug of plasmid DNA for each Cry toxin was added to 200 μl of electrocompetent cells and electroporated at

1.5 kV, 400 Ohm, and 25 uF in a 0.1 cm cuvette. Immediately after electroporation, 3 ml of prewarmed Brain Heart Infusion (BHI) medium was added to the mixtures and incubated at 37ºC for 1 hour with gentle shaking. The cultures were then used to seed BHI plates containing 100 µg/µl ampicillin, which were incubated at 30ºC overnight. Two colonies for each of the Cry9Aa, Cry1Ba, and Cry1Da toxin plasmids were selected and 5 ml cultures were prepared for each in 1/3 Tryptic Soy Broth (TSB) media containing 100 µg/ml ampicillin. All cultures grew successfully and plasmids were purified using a Qiagen Miniprep kit. Purified plasmid DNA samples were then electrophoresed in a 1% agarose gel to determine successful transformants.

Expression of Cry proteins

After transformant verification based on the presence of plasmids, one liter cultures of 1/3 TSB containing 100 µg/ml of ampicillin were inoculated with the 4Q7 and 4Q8 *Bt* clones containing plasmids with the Cry1Ba (4Q7), Cry1Da (4Q7), and Cry9Aa (4Q8) toxin genes. A small streak of the clones grown on selection plates was collected and deposited into 1 ml of sterile water and incubated at 70°C for 40 minutes to kill all vegetative cells and synchronize cultures. These inoculums were added to the one liter cultures and incubated for three days at 30ºC and 160 rpm. To verify that the samples had grown crystals and that >90% of the cells had achieved autolysis, 5 µl of the cultures was observed under a microscope under 100x magnification to detect refractive *Bt* spores and crystals.

Cultures were collected by centrifugation at 10,000 rpm and 4ºC for 10 minutes. The supernatants were decanted and pellets were resuspended in 50 ml of washing buffer

(1 M sodium chloride solution containing 0.1% Triton X-100). After fully resuspending the pellet in the buffer, the centrifuge bottles were sonicated in a water bath sonicator for 5 minutes. The bottles were centrifuged again under the same conditions as before and the washing process was repeated two more times followed by three washes in deionized water. The final pellets were stored overnight at -20ºC.

Solubilization and purification

To solubilize the protein crystals containing the Cry proteins, the pellets were resuspended in solubilization buffer (50 mM sodium carbonate and 0.1 M sodium chloride with freshly added 1% 2-mercaptoethanol) and sonicated in a water bath for five minutes before incubation overnight at 30ºC and 160 rpm. The following day, the solutions were centrifuged at 15,000 rpm and 4ºC in a JA-20 rotor for 30 minutes. The supernatant was transferred to 50 ml Falcon tubes and 0.2 mg of bovine trypsin (Sigma Aldrich, MO, USA) was added per ml of protoxin solution. The tubes were incubated at 37ºC for 1 hour to process Cry protoxins to toxins, and then centrifuged again at 15,000 rpm to pellet impurities and contaminants prior to purification of Cry toxins.

Activated toxins were purified by anion exchange liquid chromatography using a HiTrap Q HP (Cytiva Life Sciences, MA, USA) column connected to an AKTA Pure chromatography system (GE LifeSciences) equilibrated in buffer A (50mM Na_2CO_3, pH 9.8). A linear gradient of approximately 0-30% over 5ml, 30% to 55% over 60ml, 55-100% over 5ml of elution buffer B (buffer A containing 1 M NaCl) was used to elute the toxin into 1 ml fractions. Adjustments to the buffer B gradient were made based on the percent of buffer B at which the peak was noticed. Additionally, the

elution gradient was optimized to prevent large amounts of precipitation of the toxin during elution due to high protein concentration. Fractions believed to contain activated toxin were identified based on the UV peak detected by the AKTA Pure. These fractions were examined by 12% SDS PAGE electrophoresis and Coomassie staining. The concentration of the protein of interest present in each sample was determined by densitometry of bands in an SDS PAGE gels using BSA as a standard as follows. Fractions containing the same toxin (Cry1Ba, Cry1Da, etc.) were pooled together and an aliquot loaded alongside BSA at concentrations of 0.5-2-10 µg/µl, in 12% SDS PAGE gels. Gels were then electrophoresed at 200 V and stained in Coomassie blue (Thermo Fisher Scientific, MA, USA). Toxin concentration was determined via Bradford assay. Each toxin was loaded into a 12% SDS-PAGE gel at amounts 20 µl and concentrations of bovine serum albumin (BSA) loaded beside them at an equal amount included 2 µg/ml, 1 µg/ml, and 0.5 µg/ml. Samples were stored at -80ºC until further testing.

Objective two: Bioassays of Cry9Aa in S. frugiperda *and Cry1Ba, Cry1Da, and Cry9Aa in* H. zea

Bioassays

Artificial diet feeding assays were conducted with *H. zea* and *S. frugiperda* neonates from eggs purchased from Benzon Research (Carlisle, PA). Eggs of the PR1 population of *S. frugiperda* were obtained from colonies maintained in our laboratory. Larvae of this strain are resistant to Cry1F, Cry1Ab and Cry1Ac, as described elsewhere (Banerjee, 2017). Preliminary bioassays with four ten-fold different concentrations ranging from 0.05 µg/cm^2 to 50 µg/cm^2 as well as a negative buffer control were used to

determine a range of concentrations to use for dose bioassays. Replicates included 16 larvae per treatment and two technical replicates. For *S. frugiperda*, replicated dose bioassays were only performed for Cry9Aa, as data already have been published on the toxicity of Cry1Ba and Cry1Da towards this species (Aranda et al., 1996; Pinto et al., 2011). Because these earlier studies indicated the toxicity of Cry1Ba is lower than Cry9Aa, bioassays with Cry1Ba using the values tested for Cry9Aa were used to compare the response to the two toxins in the insects used in this study. For *H. zea*, Cry1Ba, Cry1Da, and Cry9Aa toxins were all tested in three biological replicates containing two technical replicates (16 larvae per technical replicate) to determine LC50 values.

Toxins were diluted using elution buffer and overlaid on solid beet armyworm diet (Frontier Scientific, DE, USA) previously dispensed into wells of a 128-well bioassay tray (Frontier Scientific, DE, USA). One neonate (<24h post-hatch) was placed into each well and trays sealed with 16-well tray lids (Frontier Scientific, DE, USA). Trays were incubated for seven days in an environmental chamber at 27°C, relative humidity of 60%, and a photoperiod of 14 hours of light and 10 hours of darkness. Toxin efficacy was evaluated based on percent insect mortality at day seven. Insects not moving when lightly prodded with the tip of a mechanical pencil were considered as dead. Additional data collected from the bioassays included number of insects stunted (less than 0.5 cm in length) and the averaged weight of surviving individuals for each treatment. An estimation of the lethal concentration needed to kill 50% of the insects present (LC50) or severely stunt 50% of them was calculated using probit analysis (Polo Plus, LeOra, California, USA). This was done by considering mortality values from all

replicates where the natural mortality rate was below 17%. Larvae were considered moribund or severely stunted if they were estimated to be in their third instar or below after the seven-day period. Insects between the second and fourth instar were measure with a ruler and those larger than 0.5 cm were considered fourth instar. All larvae smaller than 0.5 cm in length were counted as moribund.

Objective three: Competition binding assays

Biotinylation of purified Cry toxins

Calculations were made to prepare 1mg/ml of biotinylated toxin. The toxin was added to an Eppendorf tube and PBS was added to result in a total volume of 1 ml. 1.39 mg of biotin was added to 250 µl of DMF in a tube and vortexed thoroughly. The DMF solution containing biotin was added to the tube containing toxin and PBS based on the molar ration of a 65 kDa toxin so that a 3 M excess of biotin was added to each tube. This equaled 50 µl of the DMF solution to each tube. Tubes containing toxin, biotin, DMF, and PBS were incubated on ice for 30 minutes. The contents of the tube were transferred to a 3 ml Slide-A-Lyzer Dialysis Cassette (Thermo Fisher Scientific, MA, USA). Cassettes were immersed in PBS for at least 20 seconds and the corner of the cassette was dabbed on a paper towel. The contents of the tubes were loaded following the instructions provided by the manufacturer. The cassettes were immersed in PBS for two hours at room temperature with a continuous light vortexing. The cassettes then were transferred to four liters of PBS and dialyzed over night at 4^{o}C with continuous light vortexing. The biotinylated toxin was extracted from the cassettes the next day following the manufacturer's instructions. The toxin was aliquoted into multiple tubes at quantities of

20 µl per tube and stored at -80ºC until defrosted for use. Biotinylated toxins (20 µl + 4µl of 6X loading buffer) were electrophoresed in a 12% agarose gel then transferred to a PVDF membrane. A western blot was performed using a 1:20,000 concentration of streptavidin-HRP conjugate to check that biotinylation was successful.

Cry1Ac and Cry1Ba/Cry9Aa/Cry1Da in BBMV from H. zea

Brush border membrane vesicles (BBMV) were prepared from fourth instar larvae of *H. zea*. Midguts were excised from the insect by cutting between the meso and metathoracic legs and between the first and second prolegs. The gut was pulled out of the insect and soaked in ice-cold PBS buffer. Guts were blotted dry and cleaned of extra material, then deposited into liquid nitrogen and stored at -80ºC. A slightly modified magnesium chloride precipitation method (Wolfersburger, 1987) was used for BBMV preparation. Frozen guts were weighed after thawing and then resuspended in 13 volumes of ice-cold SET buffer (250 uM sucrose, 5 mM EGTA, 17 mM Tris-HCl, pH 7.5) containing 1 Complete Proteinase Inhibitor tablet (Roche) per 50 ml of buffer. The guts were homogenized on ice in an electric homogenizer (Poly Tron 2100, Kinematica, USA) for two bursts of 30 seconds with 30 seconds rest in between at 30,000 rpm. The guts were further homogenized on ice with seven strokes of the pestle of an ice-cold Dounce homogenizer. An aliquot of this crude homogenate (100 µl) was saved for measuring enzymatic enrichment. One volume of cold magnesium chloride buffer (24 mM MgCl2, 250 mM sucrose) was added to the homogenate and incubated on ice for 15 minutes. The homogenate was then centrifuged at 1,600 x *g* for 15 minutes at 4ºC. The supernatant was transferred to a new tube and centrifuged at 20,000 x *g* for 30 minutes at 4ºC.

Enzymatic enrichment of the BBMV was then measured. This was done by measuring the protein concentrations of both the prepared BBMV sample and the raw homogenate. Samples were diluted to 0.1ug/µl in room temperature PBS, then 10µl of the diluted raw homogenate was added to the wells (three per sample) of a 96 well Microplate (Thermo Fisher Scientific, MA, USA). 100µl of 50mg/ml of L-Leucine was added to 20ml of PBS then vortexed and allowed to settle for at least 15 minutes. In Gen5 (BioTek, VT, USA), settings were selected to read wells A1-B6, measure the kinetic energy including a kinetic step for five minutes at one-minute intervals, and produce a 405 curve. 200µl of prepared L-leucine buffer was added to wells B1-B6. 190µl of the prepared L-leucine buffer was added to the wells containing the diluted BBMV and raw homogenate, then the assay was started immediately after. Enrichment values were calculated by dividing the V-max values of the BBMV by the V-max value of the homogenate.

Binding assays with *H. zea* BBMV and biotinylated Cry1Ac contained two control groups. One contained only 0.3 ug of biotinylated Cry1Ac and 30 ug of BBMV. This served as the negative control (no competition for binding). The other contained 0.3 ug of biotinylated Cry1Ac, 150 ug of non-biotinylated Cry1Ac, and 30 ug of BBMV. This served as the positive control (competition for binding). Three other samples were set up to include 0.3 ug of biotinylated Cry1Ac, 30 ug of BBMV, and 150ug of the competitor toxin being tested (Cry1Ba, Cry1Da, and Cry9Aa). These five samples constitute one replicate and three replicates were performed. A 500-fold excess of the unlabeled toxin compared to the biotin-labeled toxin was used for all tubes containing

both he labelled and unlabeled toxins. This ration was used because a 500-fold increase was expected to produce a reduction in the intensity of the band that would be easily visible if competition were present. The amount of BBMV, labelled toxin, and unlabeled toxin that would be needed to achieve each concentration was calculated. The amount of PBS + 0.1% BSA to achieve a total volume of 150 µl was added to an Eppendorf tube first. The other ingredients were added to the Eppendorf tubes in the order of non-biotinylated toxin, biotinylated toxin, then BBMV. Tubes were vortexed before BBMV was added, then vortexed again and centrifuged very briefly after BBMV was added. Tubes were incubated at room temperature for one hour. After, tubes were centrifuged at 15,000 RPM for 10 minutes at 4^{o}C. The supernatant was decanted and 500ml of PBS +0.1% BSA was added to all tubes to wash the pellets. The tubes were vortexed until the pellet disappeared and centrifuged again at 15,000 RPM and 4^{o}C for 10 minutes. This washing step was repeated two more times. The pellets were resuspended in 15 µl of 6x SDS-PAGE loading buffer. An additional sample containing 4.8 ug of 0.0125 ug/µl of biotinylated toxin for a total of 0.06 ug was prepared. To this sample, 10.2 ug of 6x SDS-PAGE loading buffer was added. All tubes were heat denatured at 100^{o}C for 10 minutes. Tubes were cooled on ice for ca. three minutes then centrifuged briefly to collect all liquid at the bottom of the tube. Samples were loaded into a 10% SDS-PAGE gel and electrophoresed at 200 V for 1.25 hours. A Western blot was performed on the membrane using a 1:40,000 dilution of streptavidin HRP and the protocol described in chapter II.

Cry1Ac and Cry1Ba/Cry9Aa/Cry1Da in BBMV from *S. frugiperda*

Preparation of BBMV from *S. frugiperda* was done using the same method described above. Binding assays also were done using the same method described above, but only two replicates were prepared and loaded onto separate gels. All the samples described were tested, as well as Vip3A to serve as an additional negative control.

Results

Transformation, heterologous expression, solubilization, and purification of Cry1Ba, Cry1Ca, and Cry9Aa

Glycerol stocks of *E. coli* were grown and plasmids purified and analyzed by restriction site cleavage and electrophoresis to confirm they contained the pTZ19R vector harboring the Cry1Ba, Cry1Da, or Cry9Aa genes (Fig. 3.2). Plasmids were digested with restriction enzymes that were identified to cut the pTZ19R vector. The expected size of all three toxin plasmids when cut to produce a single band was around 4,600 bp (2,862 bp vector + 1,750 kb toxin gene insert). This size was observed for products from linearization of ECE 129 (Cry1Ba), the band for ECE 129 (Cry1Da) was closer to 6 kb, while the band for ECE 130 (Cry9Aa) was close to 10 kb.

Prior to transforming the plasmids into the plasmid cured *Bt*, they needed to be transformed into a strain of *E. coli* intended to produce non-methylated clones of the plasmids. Plasmids transformed into One Shot INV110 chemically competent (non-methylating) cells (Invitrogen, CA, USA) were grown in cultures then purified using the same methods. This was performed to achieve cells suitable for the following steps.

Transformation was done following the manufacturer's standard transformation protocol. Plasmids were purified using Qiaprep miniprep kit (Qiagen, Germany).

Electrophoretic analysis of plasmid minipreps of transformants cultures confirmed the presence of plasmid DNA (Fig. 3.3). An unexpected number of bands were present in the lanes where no restriction enzymes were used, compared to a previous analysis (Fig. 3.3). Additionally, the bands present in the lanes digested with Nco I appeared to be the same size as those present in the lanes with uncut plasmid.

To ensure that the *Bt* bacteria growing in the cultures was producing both spores and crystal inclusions, 5 µl of the media after two days of incubation was examined under the phase contrast objective of a microscope (Fig. 3.4). Both spore and newly forming crystals were visible within the cells. After two additional days, cultures were checked again and many of the cells had autolysed and crystals were free-floating in the media by this time. Cultures were collected, crystals solubilized, and activated before purification by anion exchange.

Chromatograms from the purification process showed a clear UV peak eluting from the column at around 45-50% buffer B in all four toxin types (Fig. 3.5a). This elution is commonly observed for other Cry proteins in our laboratory. Fractions electrophoresed in 12% agarose gels with BSA then calculated in Bradford assay revealed concentration of toxin band related to BSA (Fig. 3.5b).

Bioassays of Cry9Aa in S. frugiperda and Cry1Ba, Cry1Da, and Cry9Aa in H. zea

Bioassays revealed that species is sensitive to Cry9Aa (Fig. 3.6). Cry1Fa, a toxin that was known to be toxic to the *S. frugiperda* strain from Benzon, was tested alongside Cry9Aa for comparison. The LC50 estimated for Cry1Fa was 0.42 μg/cm^2 when considering moribund larvae as dead. In comparison, the LC50 for Cry9Aa when including moribund larvae was 0.95 μg/cm^2. When only including dead larvae, the LC50 values for Cry1Fa and Cry9Aa were 0.44 and 0.39 μg/cm^2, respectively. To obtain the LC50 value for dead larvae for Cry9Aa the natural response had to be ignored to fit the probit model. Bioassays with Cry1Ba in *S. frugiperda* revealed that this population is more susceptible to Cry1Ba than Cry9Aa (Table. 3.1).

Bioassays with the Cry1F-resistant strain of *S. frugiperda* PR1 revealed that the Cry9Aa toxin had little effect on the mortality of these insects (Fig. 3.7). Response values at the highest dosage tested were comparable to the natural response. Bioassays with Cry1Fa confirmed that the population maintains a high level of resistance to that toxin (Table 3.2). When determining potential stunting by measuring mean weight of surviving larvae at each dosage, we detected a decrease in weight as concentration of toxin increased, suggesting a stunting effect (Fig. 3.8). While a decrease in weight with increasing Cry9Aa concentrations was detected, the mean weight of the larvae in the negative control was lower than for the toxin doses tested. The natural mortality rate of the PR1 insects (negative control) and the highest dosage of Cry9Aa that was tested in PR1 were compared using a t-test in SigmaPlot 11.0 (IBM SPSS Inc., Chicago, IL, USA). This produced a p-value of 0.333, showing that they are not significantly different.

Neonate *H. zea* were sensitive to all toxins tested in this study. Results from bioassays with the Benzon strain of *H. zea* performed with purified Cry1Ba, Cry1Da, and Cry9Aa toxins are shown in Figure 3.9. The LC50 values for Cry1Ac, Cry1Ba, Cry1Da, and Cry9Aa when including dead and moribund larvae in the affected group were 0.66 μg/cm^2, 1.27 μg/cm^2, 1.66 μg/cm^2, and 2.20 μg/cm^2, respectively. When only considering dead larvae, the LC50 values were 1.14 μg/cm^2, 1.94 μg/cm^2, 1.86 μg/cm^2, and 3.35 μg/cm^2 for Cry1Ac, Cry1Ba, Cry1Da, and Cry9Aa, respectively. Of the toxins that were fed to the insects and not previously known to be toxic to this species, Cry1Ba was the most active, but overlapping confidence intervals indicate that the LC50 values are possibly closer (Table 3.2). Measurement of larval weights revealed a decrease in weight compared to the negative control group in all toxin types (Fig. 3.10).

Competition binding assays

Purified toxins were labeled with biotin and biotinylation was tested by detection of labeled proteins by streptavidin-HRP in Western blots (Fig. 3.11). The main signal for all lanes corresponded to the expected size of roughly 65 kDa for all toxins. BMMV enrichment assays resulted in an average enrichment value of 3.48 for *H. zea* and 4.93 for *S. frugiperda*. Binding assays with biotinylated-Cry1Ac, H. zea BBMV, and the competitor toxins produced bands at the expected size of 65 kDa in all lanes (Fig. 3.12). An increased intensity is present in the lanes containing the positive control for competition (lanes 5, 10 and 15 starting from the left) shows an increase in the intensity

of the band when both the biotinylated toxin and competitor is present. Other lanes containing toxin and competitor are present at an intensity equal to or less than the lane containing only BBMV and the biotinylated toxin for each replicate. The lane containing 0.06 µg of Cry1Ac only confirms the Western blot is effective.

Discussion

I successfully cloned Cry proteins into *Bacillus thuringiensis* (*Bt*), expressed in crystalline form, and purified via Fast Liquid Chromatography. Results confirm that Cry1Ba, Cry1Da, and Cry9Aa demonstrate toxicity towards *H. zea* and Cry9Aa demonstrates toxicity towards *S. frugiperda*. Inability to complete binding assays prevents us from confirming if these toxins may be suitable for use in gene pyramiding.

Despite the plasmid DNA in early steps not being found at the expected molecular size, we continued to transformation. The presence of additional bands or bands at the incorrect size when restriction enzymes were used may be due to low efficiency of the enzyme or its respective buffer, causing only partial cutting. For the plasmids present after transformation into *Bt* cells, it is possible that the extra bands present may be due to additional plasmids present, despite the cells being labeled as "plasmid-cured."

Results from bioassays with *H. zea* revealed the toxicity of Cry9Aa. While Cry9Aa toxicity in related species is not a determinant of their activity in this species, toxicity values were similar (Jurat-Fuentes and Crickmore, 2017). The activity of Cry9Aa in *Helicoverpa armigera* was much higher than Cry1Ac, which held true for *H. zea*. Li and Bouwer (2014) discovered a synergistic effect between Cry9Aa and Cry1Ca in *H.*

armigera. It would be interesting to test this ability for Cry9Aa to synergize other toxins in *H. zea*.

High variability in the toxicity of Cry1Da in *H. zea* may be attributed to a few factors. After purification, the fractions were saved individually, then fractions to be used for bioassays were defrosted the day of use. This may have resulted in increased variability among biological replicates. Variation in the natural response (mortality rate) among biological replicates may be responsible for the larger confidence intervals. Performing additional replicates and excluding those with a natural response of higher than 10% mortality would most accurately determine the LC50 value of this and other toxins being tested.

Bioassays performed with Cry1Ba by Aranda et al. (1996) indicated a low level of activity against *H. zea*. Testing of Cry1Ba was repeated in this study for two main reasons. The highest dosage tested in that study (1 μg/cm^2) was much lower than the highest initial values tested here (50 μg/cm^2). Additionally, the strain of *H. zea* used in that study is unknown and may differ from the ones used in this work. Testing the activity of Cry1Ba against *H. zea* revealed an LC50 value of 1.27 μg/cm^2 when including moribund insects. Based on Monnerat's 2006 publication comparing the different LC50 values between insects of the same species found in different regions (Monnerat, 2006), none of the LC50 values found are any bit surprising in comparison to those found in previous studies with strains not sourced from Benzon.

Bioassays conducted with *S. frugiperda* revealed the level of Cry9Aa in this strain, as well as Cry1Ba's toxicity in relation to it. Previous studies testing the activity of

Cry9Aa in other lepidopteran species were considered as a reference when conducting bioassays with this species. Most interestingly, larvae from the Cry1Fa-resistant *S. frugiperda* strain PR1 were found to be resistant to Cry9Aa. Cross-resistance to Cry1Ab and Cry1Ac also has been reported in a strain of Cry1Fa-resistant *S. frugiperda* (Storer, 2010). Due to the mutation that enables resistance in PR1 insects (Banerjee, 2017), it would be expected that these toxins, along with others from the Cry1 family of proteins have a lower level of activity. Mutations to the ATP binding cassette transporter proteins have been reported as the cause for many instances of resistance to Cry1 family proteins, even in other species (Atsumi et al., 2012; Xiao et al., 2014; Coates and Siegfried, 2015; Park et. Al., 2014; Gahan et al., 2014). Based on the binding assay results in this study with Benzon and the PR1 strains of *S. frugiperda*, it can be concluded that ABCC2 is a receptor for Cry9Aa in *S frugiperda*. It was hoped that a Cry toxin so different from Cry1Fa would recognize a different receptor, making it suitable for gene pyramiding, but that seems to not be the case. It can also be inferred insects of other species that are resistant to Cry1Fa and other toxins in the Cry1 family likely would be resistant to Cry9Aa. It would be of interest to test other Cry9 toxins to see if reduced susceptibility is conferred to these as well.

To identify toxins that may be used alongside Cry1Ac or Cry1Fa in gene pyramiding, successful completion of binding assays with *H. zea* and *S. frugiperda* would be necessary. Multiple methods exist for labeling the toxin. The method used in this study involves biotin labeling. Another method involves the radiolabeling, which includes the use of the radioactive isotope of iodine. To demonstrate that the labeled toxin is

functional, the homologous unlabeled toxin should reduce the amount of biotinylated toxin that is able to bind. The increased intensity of the band containing both the labeled and unlabeled toxin could be due to several factors. The methods described by Gonzalez-Cabrera (2006) for the biotin binding experiment were followed, so the issue likely lies in the preparation of the BBMV or toxins. To determine if the Cry1Ba, Cry1Da, and Cry9Aa compete for binding sites with Cry1Ac in *H. zea* and Cry1Fa in *S. frugiperda*, these binding assays should be repeated by isolating variables in the experiment to determine the cause of the issue in the positive control.

CHAPTER IV: CONCLUSIONS

Truncation of Cry genes to produce proteins containing isolated domains for use in competition bioassays may prove to be a time and resource efficient method of identifying Cry toxins suitable for gene pyramiding. Progress towards that goal has shown the ease of identifying the fragments to be cloned and all steps prior to expression of the recombinant proteins. The methods used are standard in cloning protein expression. Early steps in this method can be applied to many other Cry proteins without the need to identify a mutation that disables the protein's binding ability. As discussed previously, most Cry proteins possess a conserved structure. This would allow for relatively similar cut regions to be applied in many different Cry proteins. This method could also be applied to non-Cry insecticidal proteins, as long as the regions responsible for binding and pore formation are separate.

Testing of additional expression and solubilization methods is needed to produce a higher quantity of biologically active proteins for use in bioassays. Determining the conditions under which the cultures produce an optimal amount of soluble and active truncated proteins could be a key step in making this method patentable and widely applicable. Flores el al. (1997) reported issues with producing soluble and active proteins in the truncated Cry1Ab protein only containing domain II. Additionally, they also reported low expression levels. We assume similar issues with expression levels and solubility will be experienced with other three domain Cry proteins, regardless of the expression system or host cells used.

To confirm competition for binding sites exists between the truncated Cry1Fa protein and the native proteins, binding assays should be performed with these proteins.

This would confirm that any reduced mortality seen in the *in vitro* method is due to reduced binding of the competitor truncated protein. This proposed method could prove to be very useful in combatting the development of resistance to *Bt* insecticidal proteins, as this reduces the amount of work involved in identifying candidate toxins. Continuing the use of this method, it would be best to use other Cry proteins already produced by commercially available *Bt* crops, particularly those in which insects have already developed resistance. These include Cry1Ac, Cry1Ab, and Cry2Ab, among many others (Tabashnik & Carriere, 2020).

Completion of the binding assays will be necessary for determining if the Cry1Ba, Cry1Da, and Cry9Aa proteins would be candidates for use in gene pyramiding alongside Cry1Fa or Cry1Ac. This will reveal whether the proteins recognize receptors different from ABCC2 in *S. frugiperda* and alkaline phosphatase (ALP) and aminopeptidase N (APN) in *H. zea* (Banerjee, 2017; Caccia, 2012). If binding assays reveal no competition between the toxin being tested and the biotin labeled Cry1Ac, further research should be conducted to identify the receptors they bind to if the toxins are eventually to be used in transgenic crops.

Through the work, we provide insight into the structure of three domain Cry proteins. The predominance of insoluble proteins after expression and the low level of solubilization when using non-denaturant solvents may indicate the need for domain I in maintaining protein structure and function. Continuing this research by conducting bioassays and binding assays with the truncated proteins could further elucidate the role of domain I in maintaining the structure of the toxin. While Flores et al. (1997) found

success in producing biological active proteins missing domain I, it could be possible that this doesn't hold true for a truncated Cry1Fa protein.

The results from binding assays with Cry1Fa-resistant *S. frugiperda* offered the most unexpected results of this thesis work. As mentioned in the discussion section of chapter three, it was expected that due to their structural and sequence dissimilarities, that Cry1Fa and Cry9Aa would recognize different binding sites. While it is possible that Cry9Aa recognizes more than one receptor, such as the case with Cry1Ac in *H. zea*, this seems unlikely due to fact that the mortality of the PR1 insects is similar when fed cry9Aa and Cry1Fa at the concentrations tested (Cassia et al., 2012). This result may present an opportunity to study the similarities between Cry1Fa, Cry9Aa and other families of Cry toxins believed to recognize ABCC2 as a receptor, as this may offer important information about the development of resistance in *S. frugiperda* and other species which are susceptible to Cry toxins that recognize ABCC2.

Additional experiments will need to be conducted so that the results of chapter two or three will be publishable. Proposed solutions to the issues encountered were detailed in the discussion sections of each chapter. To most efficiently determine the issue that is occurring in the binding assays, an individual variable, such as the preparation of the BBMV or non-biotinylated toxin should be isolated in experiments to determine which may be contributing. A similar approach should be used to achieve expression of soluble domain II+III proteins. Because expression was achieved but needs to be optimized, experiments that test the result of small changes to the expression protocol should be conducted.

After production of soluble and active truncated domains as well as identification of Cry proteins that are active and utilize a new mode of action, the effectiveness of the proposed truncated toxin method could be demonstrated using these proteins. This would serve as a proof of concept for the truncated domains method, as well as an extra method for determining the level of activity and compatibility of the toxins tested in this study whose mode of action is still unknown. The third chapter demonstrates the additional steps that could be bypassed with the *in vitro* method proposed for chapter one. The issues encountered with performing binding assays serve as a testament to why the proposed method would be an improvement over the traditional multistep method.

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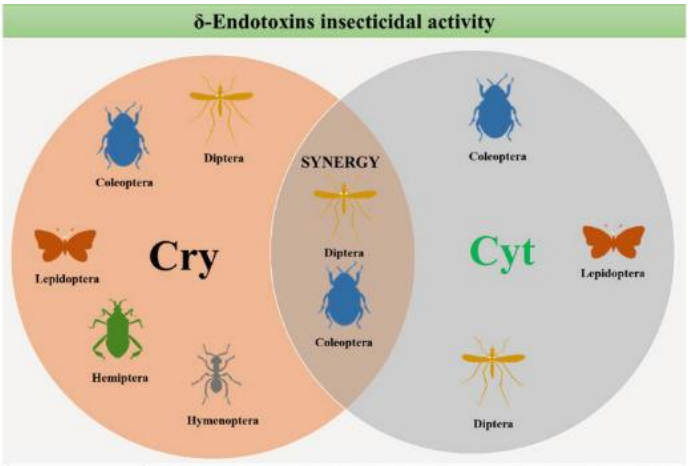
APPENDIX



Diptera	Cry1(A-C), Cry2(A-B), Cry4(A-C), Cry10(A), Cry11(A-B), Cry16(A), Cry17(A), Cry19(A-C), Cry20(A), Cry21(A-H), Cry24(A-C), Cry25(A), Cry27(A), Cry29(A-B), Cry30(A-G), Cry32(B-D), Cry39(A), Cry40(A-D), Cry44(A), Cry47(A), Cry48(A),Cry49(A), Cry52(A-B), Cyt1(A-D), Cyt2(A-D), Cyt3(A)
Coleoptera	Cry1(A-I), Cry2(A), Cry3(A-C), Cry7(A), Cry8(A-G), Cry9(D), Cry14(A), Cry18(A-C), Cry22(A-B), Cry23(A), Cry26(A), Cry28(A), Cry34(A-B), Cry35(A-B), Cry36(A), Cry37(A), Cry38(A), Cry43(A-C), Cry55(A), Cyt1(A), Cyt2(C)
Lepidoptera	Cry1(A-K), Cry2(A-B), Cry7(B), Cry8(D) Cry9(A-C,E), Cry15(A), Cry19(A-C), Cry20(A-B), Cry22(A), Cry32(A), Cry51(A), Cry54(A-B), Cry59(A-B), Cyt2(B)
Hemiptera	Cry3(A), Cry5(A), Cry22(A)
Hymenoptera	Cry2(A), Cry3(A), Cry11(A)

Fig. 1.1 - Venn Diagram showing overlap of insecticidal activity of Cry and Cyt toxins and list of Cry toxins (black) that are active against insects of each Order (Fernández-Chapa et al., 2019)

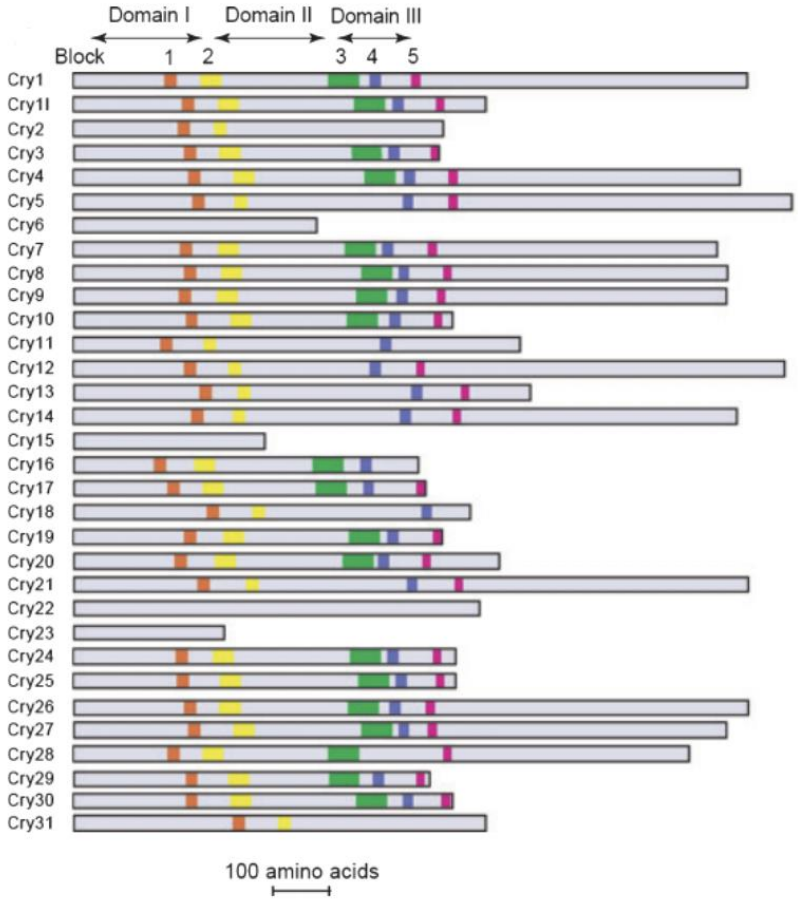


Fig. 1.2 - Alignment of 32 families of Cry toxins. Orange, yellow, green, purple, and pink regions show five conserved blocks of the protoxin sequence that are present in the majority of the proteins (de Maagd et al., 2001).

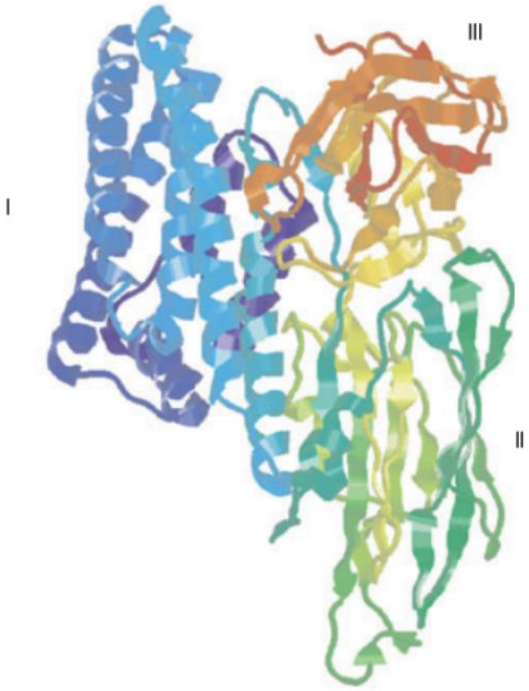


Fig. 1.3 - Conserved three-dimensional structure of Cry toxins showing domains I through III (de Maagd et al., 2001)

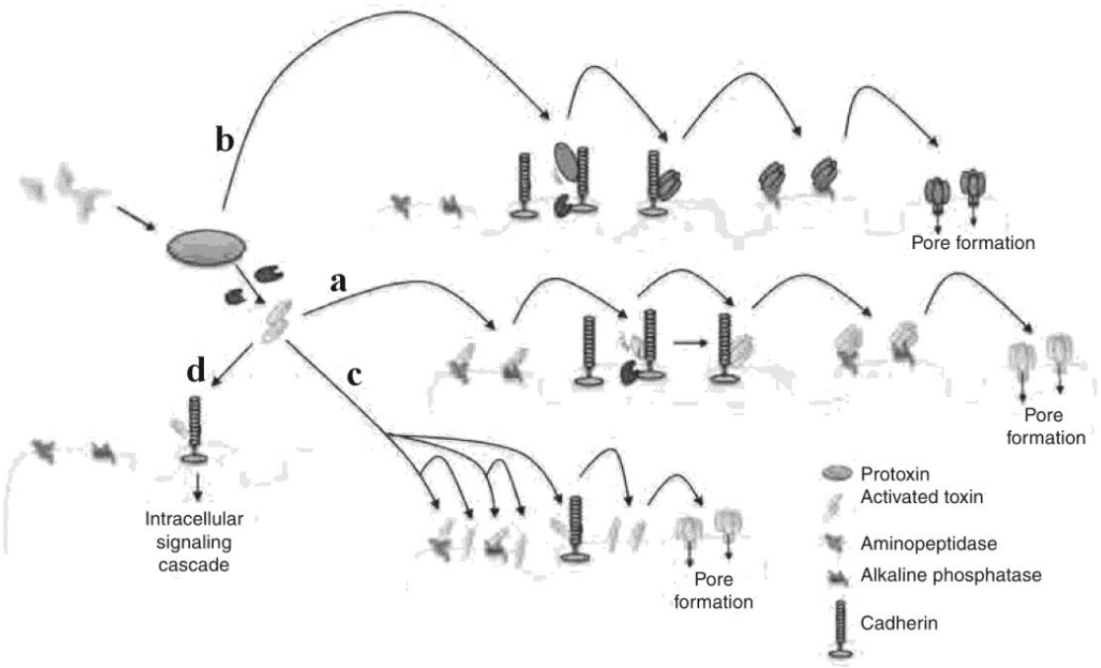


Fig. 1.4 - Model of sequential interaction of three domain Cry proteins with midgut receptors for pore formation. Mechanisms of binding leading to pore formation include the sequential binding pore formation models of activated toxins (a) and protoxins (b), the pore formation model of monomeric activated toxins (c), and the signal transduction model (d). (Pardo-Lopez et al., 2013).

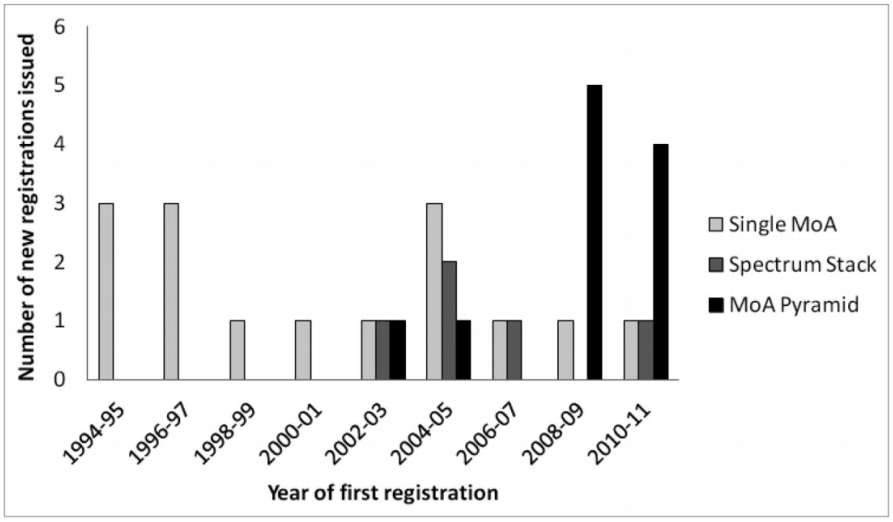


Fig. 1.5 - New product registrations (1994-2010) showing the number of new varieties of *Bt*-protected crops registered for commercial use with the US Environmental Protection Agency each year, categorized by IRM strategy. Projects are categorized as "single mode of action (MoA)", if equipped with a single protein, "Spectrum Stack" if multiple proteins are present with a single MoAs against each target pest, or "MoA Pyramid" if it employs two or more MoA against one or more target pests (Storer et al., 2012).

Table 2.1 - Primers used for amplification and sequencing of *cry1Fa*.

Primer Name	Target Sequence Name	Sequence 5' to 3'	Restriction Site 5'	Restriction Site 3'	For Cloning Into/Use
DomI Fwd	Domain I of Cry1F	CCGCTCGAGATGGAGAATAATATTCAAATC	XhoI	none	Cloning into pTRHis
DomI Rev	Domain I of Cry1F	CCCAAGCTTGTCCATAAGATGGGG	none	HindIII	Cloning into pTRHis
DomII Fwd	Domain II of Cry1F	CCGCTCGAGCCCCATCTTATGGAC	XhoI	none	Cloning into pTRHis
DomII Rev	Domain II of Cry1F	CCCAAGCTTAATTGTATTTGT	none	HindIII	Cloning into pTRHis
DomIII Fwd	Domain III of Cry1F	CCGCTCGAGACAAATACAATT	XhoI	none	Cloning into pTRHis
DomIII Rev	Domain III of Cry1F	CCCAAGCTTATCATATTCTGC	none	HindIII	Cloning into pTRHis
pTrcHis Forward	5' in pTrcHis vector	GAGGTATATATTAATGTATCG	none	none	PCR/Sequencing
pTrcHis Reverse	3' in pTrcHis vector	GATTTAATCTGTATCAGG	none	none	PCR/Sequencing

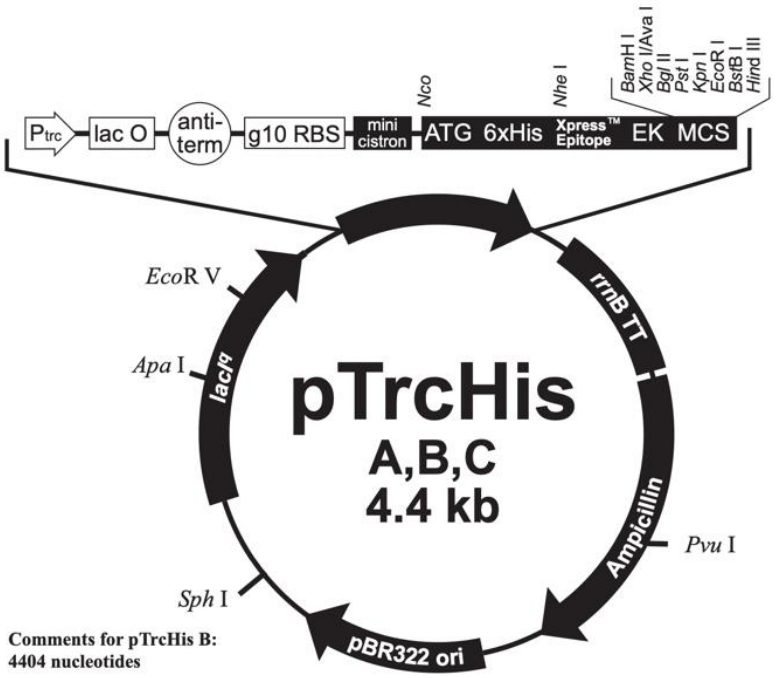


Fig. 2.1 - Map of pTrcHis A vector.

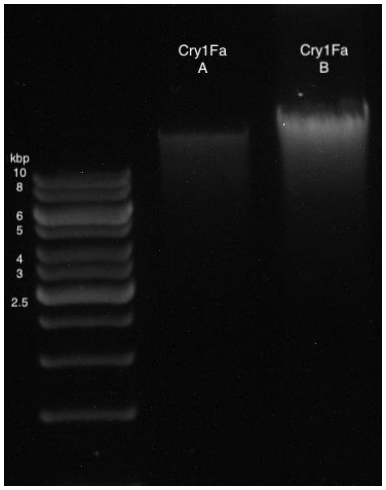


Fig. 2.2 - 1% agarose gel examining purified plasmids from *Bt* bacterial cultures containing Cry1Fa gene. Lanes: 1 – 1kb DNA size reference marker ladder (sizes in kilobases), 2 – Plasmid from clone A, 3 – Plasmid from clone B.

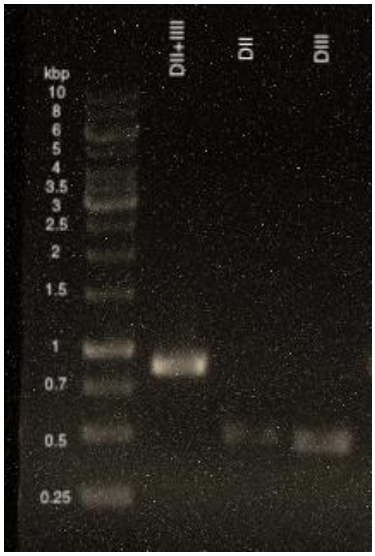


Fig. 2.3 - 1% agarose gel containing amplicons of DII, DIII, and DII+III after PCR amplification. Lanes: 1 – 1kB ladder, 2 – amplicon for DII+DIII, 3 – amplicon for DII, 4 – amplicon for DIII.

Fig. 2.4 - Plasmids purified from LEMO21(DE3) cell transformants of Cry1Fa DII, DIII and DII+III in pTrcHis vector. Lanes: 1- 1kB ladder, 2 – pTrcHis vector not containing an insert, 3 – pTrcHis vector containing DIII insert, 4 – pTrcHis vector containing DII+II insert, 5 – pTrcHis vector containing DII insert.

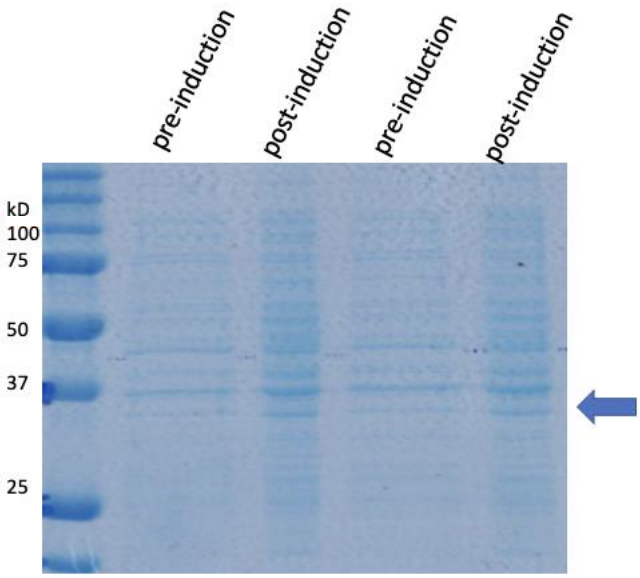


Fig. 2.5 - Coomassie stained 12% SDS PAGE gel containing samples from two cultures (a and b) of DII+III before and after IPTG induction. All samples were diluted ¼ in PBS buffer prior to loading in the gel to achieve clearer bands. Lanes: 1 – Precision Plus All Blue Ladder, 2 – Pre-IPTG induction sample of DII+III from culture a), 3 – sample from culture a) after 18 hours after inducing protein expression with 0.4 mM IPTG, 2 – Pre-IPTG induction sample of DII+III from culture b), 3 – sample b) 18 hours after inducing protein expression with 0.4 mM IPTG. The arrow next to the gel indicates the expected size for the recombinant DII+DIII protein band.

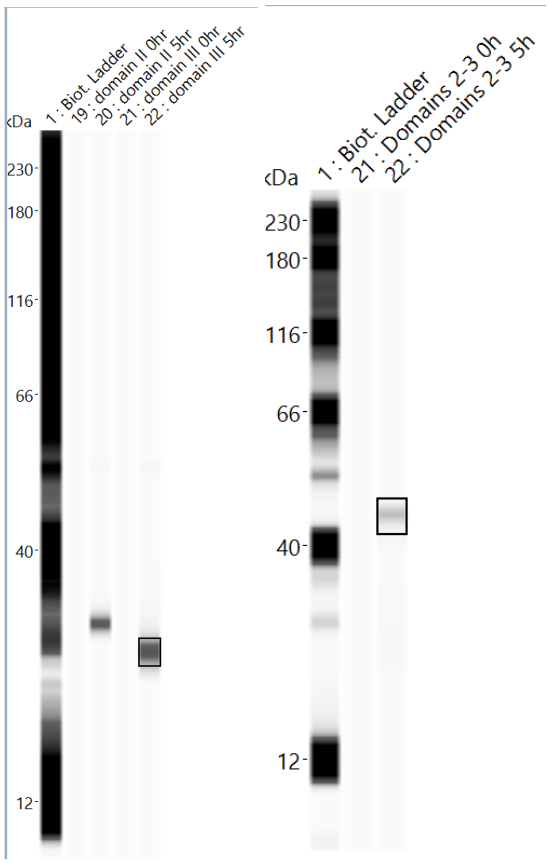


Fig. 2.6 - Western blot using Wes automated unit to detect for DII, DIII, and DII+III recombinant protein before and five hours after induction. Proteins were detected using 1:20,000 Cry1Fa antisera followed by 1:25,000 goat anti-rabbit HRP. Lanes: 1 – Wes 12-230 kDa ladder, 2 - Pre-IPTG induced sample of DII, 3 – sample of DII, six hours after inducing protein expression with 1M IPTG, 4 - Pre-IPTG induced sample of DIII, 5 – sample of DIII, six hours after inducing protein expression with 1M IPTG, 6 - Wes 12-230 kDa ladder, 7 - Pre-IPTG induced sample of DII+III, 8 – sample of DII+III, six hours after inducing protein expression with 1M IPTG

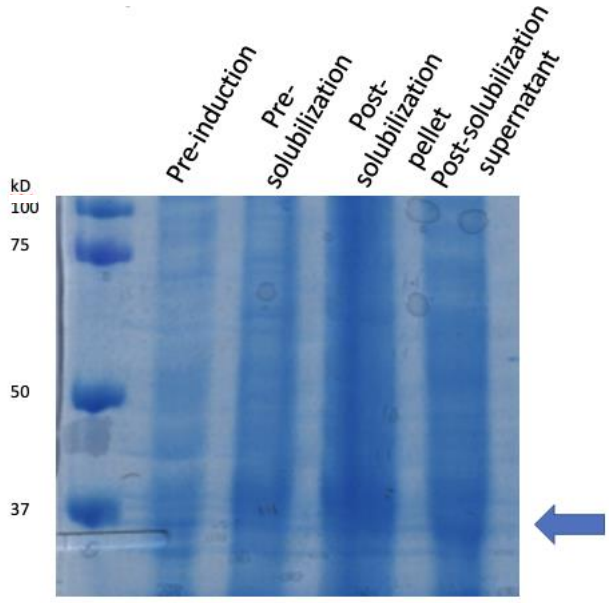


Fig. 2.7a - 12% SDS PAGE gel of DII+III samples Lanes: 1- Precision Plus Dual Color ladder, 2 - cell culture prior to induction, 3 - cell culture after IPTG induction but before solubilization, 4 – resuspended pellet of culture after solubilization with carbonate buffer, 5 - supernatant of culture after solubilization.

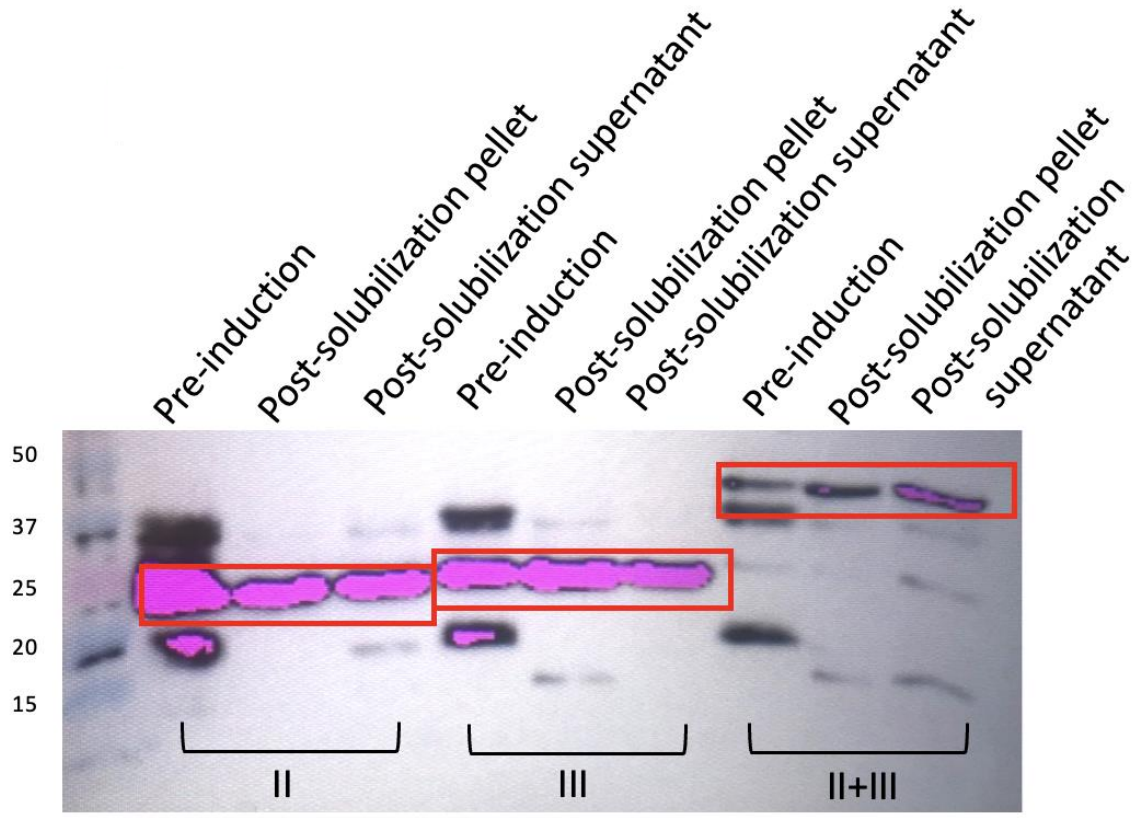


Fig. 2.7b - Western blot of pre-induction, post solubilization pellet, and post solubilization supernatant samples detected with Cry1Fa antisera. Lanes: 1 – Precision Plus Dual Color ladder (Bio-Rad, USA), 2 – DII cell culture after IPTG induction but before solubilization, 3 – resuspended pellet of DII culture after solubilization with carbonate buffer, 4 - supernatant of DII culture after solubilization with carbonate buffer. 5 – DIII cell culture after IPTG induction but before solubilization, 6 – resuspended pellet of DIII culture after solubilization with carbonate buffer, 7 - supernatant of DIII culture after solubilization with carbonate buffer. 8 – DII+III cell culture after IPTG induction but before solubilization, 9 – resuspended pellet of DII+III culture after solubilization with carbonate buffer, 10 - supernatant of DII+III culture after solubilization with carbonate buffer.

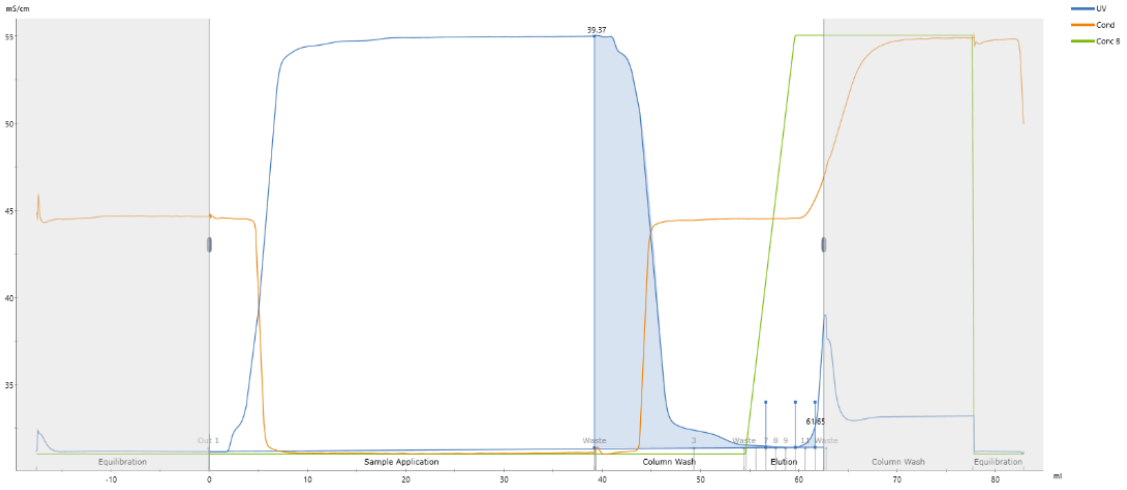


Fig. 2.8 - Chromatogram of recombinant DII+DIII protein purification. Blue line shows measurement of UV absorbance at a fixed wavelength of 280 nm. Percent of buffer B flowing through FPLC is represented by the green line. Conductivity (orange line) measures salt concentration.

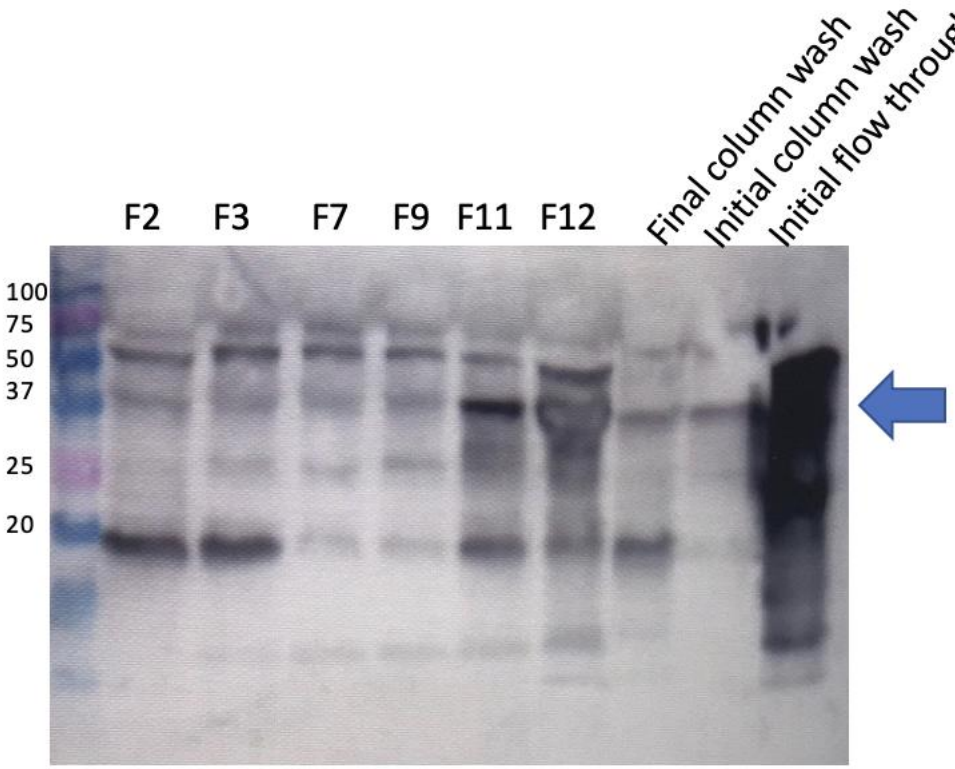


Fig. 2.9 - Western blot of FPLC fractions containing peaks probed with Cry1Fa antisera to detect DII+DIII protein. Lanes: 1 – Precision Plus Dual Color ladder, 2 – fraction two, 3 – fraction three, 4 – fraction seven, 5 – fraction nine, 6 – fraction 11, 7 – fraction 12, 8 – fraction from final column wash, 9 – fraction from initial column wash, 10 – fraction from initial flow through.

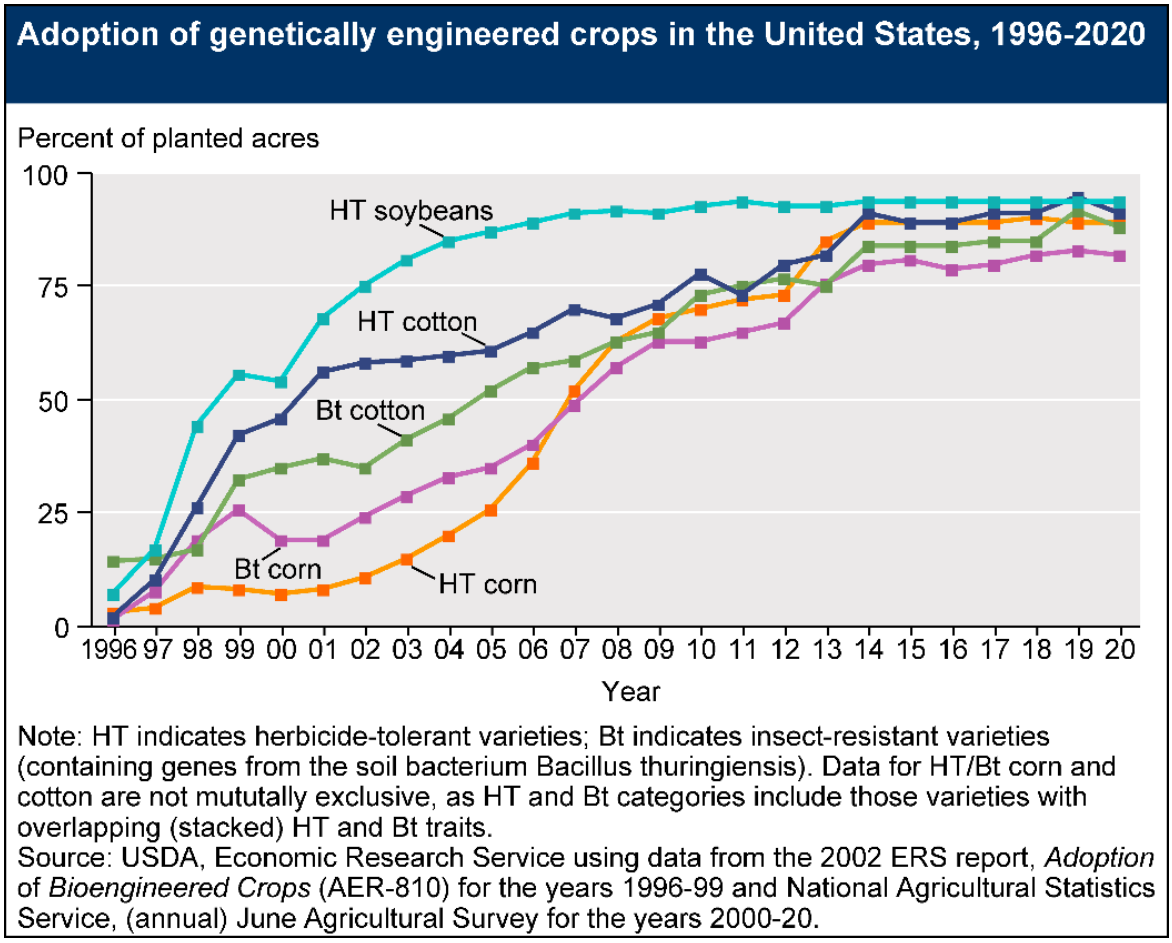


Fig. 3.1 - Increase in use of *Bt* and herbicide tolerant genetically engineered crops from 1996 to current (USDA, 2021a).

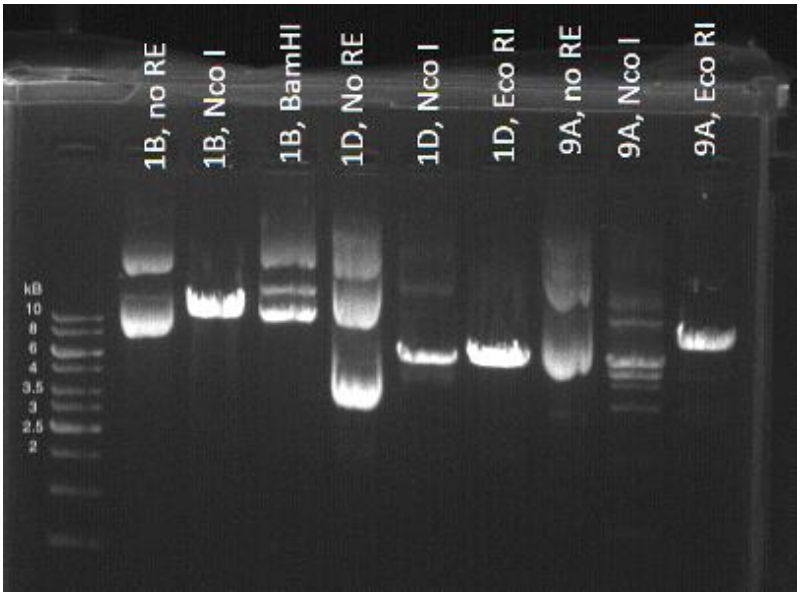


Fig. 3.2 - Restriction cleavage analysis of plasmids carrying the Cry1Ba/Cry1Da/Cry9Aa genes. Restriction enzymes used to linearize to single band are shown for each lane. Lanes: 1 – 1kB DNA ladder, 2 – uncut plasmid from ECE 128 (Cry1Ba in pTZ19R), 3 – plasmid from ECE 128 digested with Nco I, 4 – plasmid from ECE 128 digested with Bam HI, 5 – uncut plasmid from ECE 129 (Cry1Da in pTZ19R), 6 – plasmid from ECE 129 digested with Nco I, 7 – plasmid from ECE 129 digested with Eco RI, 8 – uncut plasmid from ECE 130 (Cry9Aa in pTZ19R), 9 – plasmid from ECE 130 digested with Nco I, 10 – plasmid from ECE 130 digested with Eco RI.

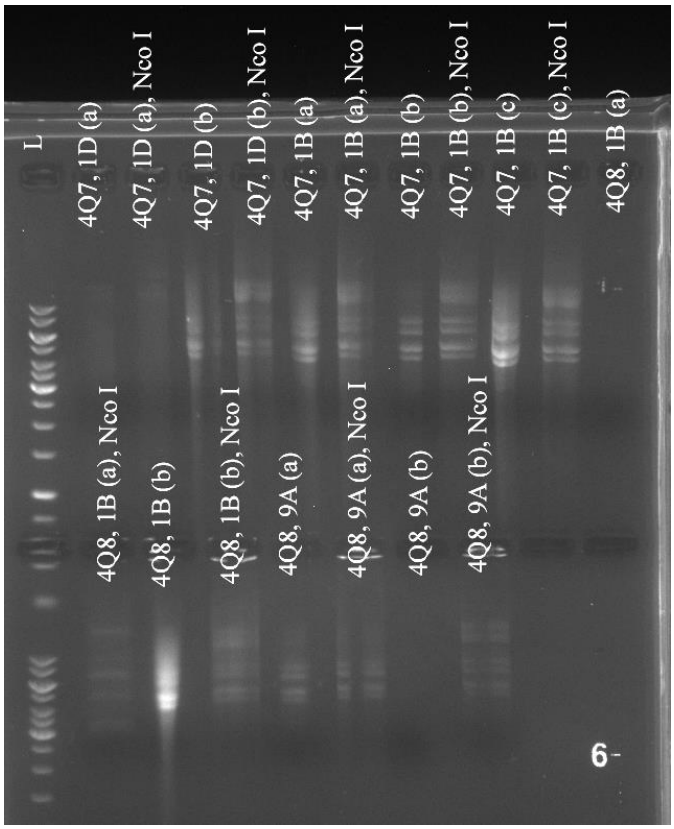


Fig. 3.3 - Agarose gel of plasmids and restriction enzyme digests of plasmids from 4Q7 and 4Q8 transformants. Lanes: 1 – 1kB DNA ladder, 2/4 – plasmid from 4Q7 cells containing Cry1Da in pTZ19R, 3/5 – plasmid from 4Q7 cells containing Cry1Da in pTZ19R digested with Nco I, 6/8 plasmid from 4Q7 cells containing Cry1Ba in pTZ19R, 7/9 – plasmid from 4Q7 cells containing Cry1Ba in pTZ19R digested with Nco I, 10/12 - plasmid from 4Q7/4Q8 cells containing Cry1Ba in pTZ19R, 11/14 – plasmid from 4Q7/4Q8 cells containing Cry1Ba in pTZ19R digested with Nco I, 13 – 1kB DNA ladder, 15 – plasmid from 4Q8 cells containing Cry1Ba in pTZ19R, 16 – plasmid from 4Q8 cells containing Cry1Ba in pTZ19R digested with Nco I, 17/19 - plasmid from 4Q8 cells containing Cry9Aa in pTZ19R, 18/20 – plasmid from 4Q8 cells containing Cry9Aa in pTZ19R digested with Nco I.

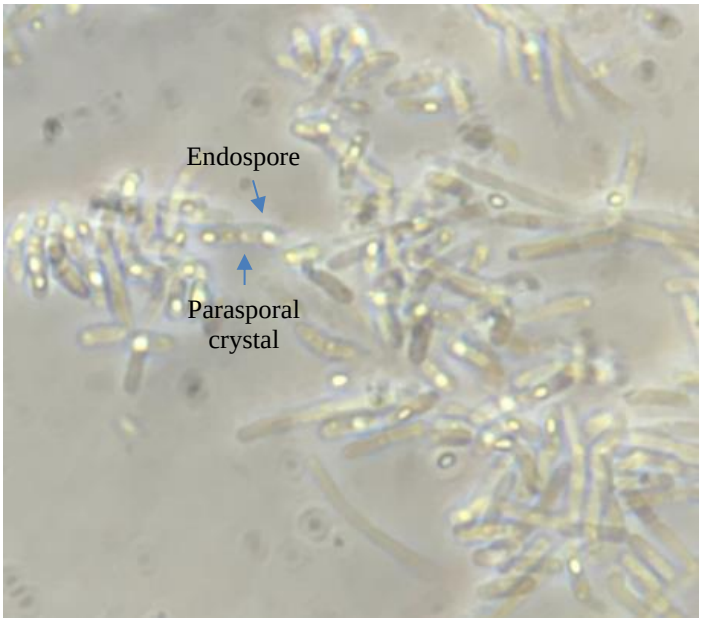


Fig. 3.4 - Phase contrast photograph of *Bt* 4Q7 transformed with Cry1Da after two days of incubation in 1/3 TSB media containing 100 ug/ml ampicillin at 28ºC at 160 RPM. Endospore and early growth parasporal crystals were present in some cells.

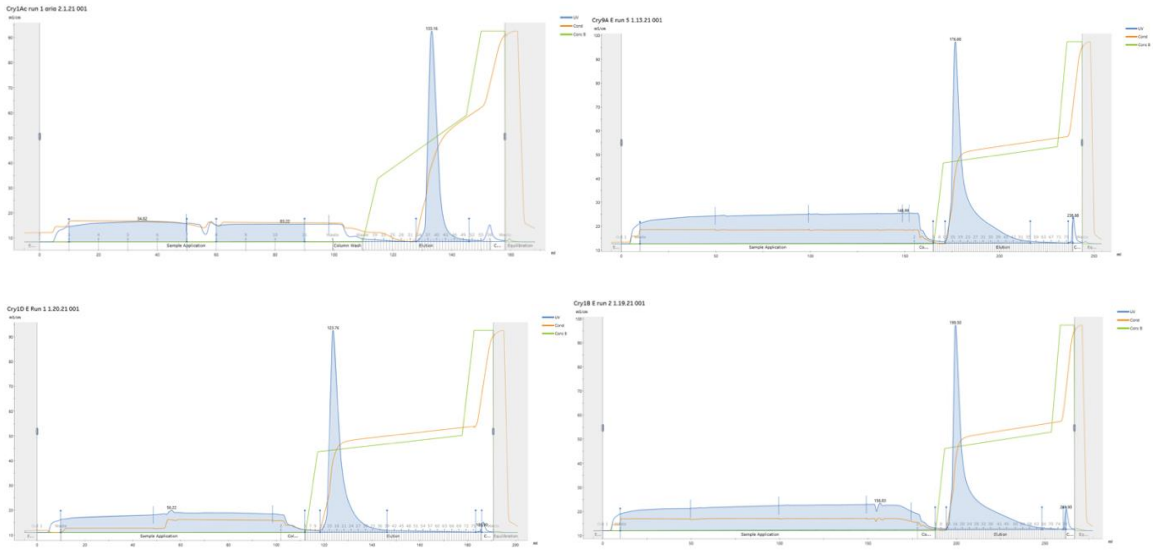


Fig. 3.5a - Chromatograms for anion exchange purification of Cry1Ac, Cry9Aa, Cry1Da and Cry1Ba proteins. Blue line shows measurement of UV absorbance at a fixed wavelength of 280 nm. Percent of buffer B flowing through FPLC is represented by the green line. Conductivity (range line) measures salt concentration.

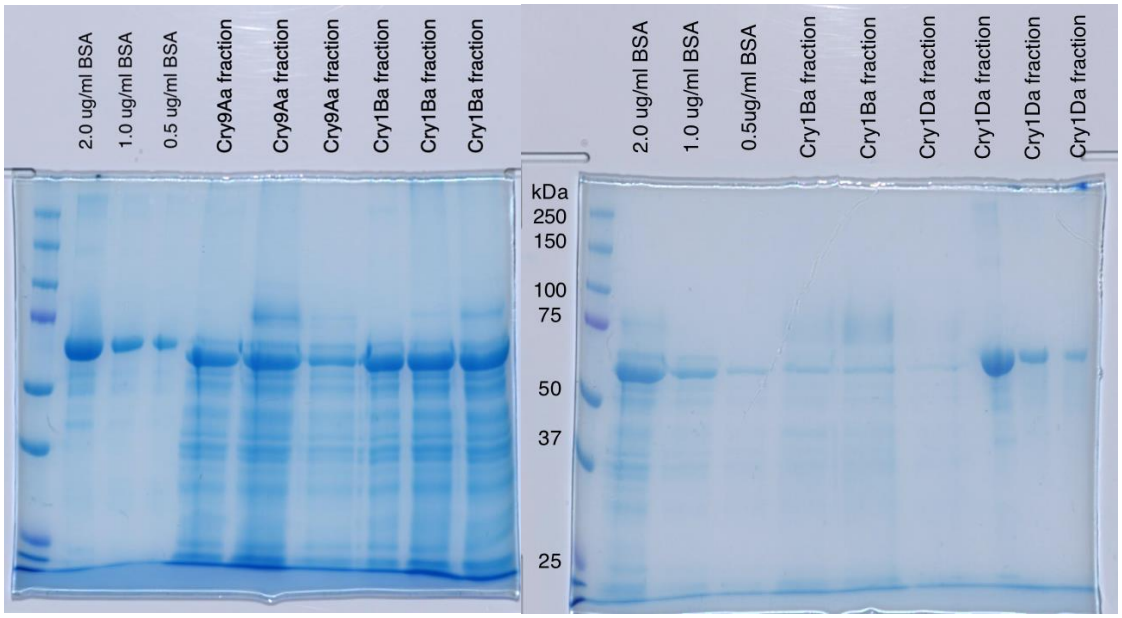


Fig. 3.5b - Coomassie stained gel of samples of Cry1Ba and Cry9Aa (left gel) and Cry1Da and Cry1Ba (right gel). Included in the gels were three concentrations of BSA to estimate the concentration of toxin in fractions.

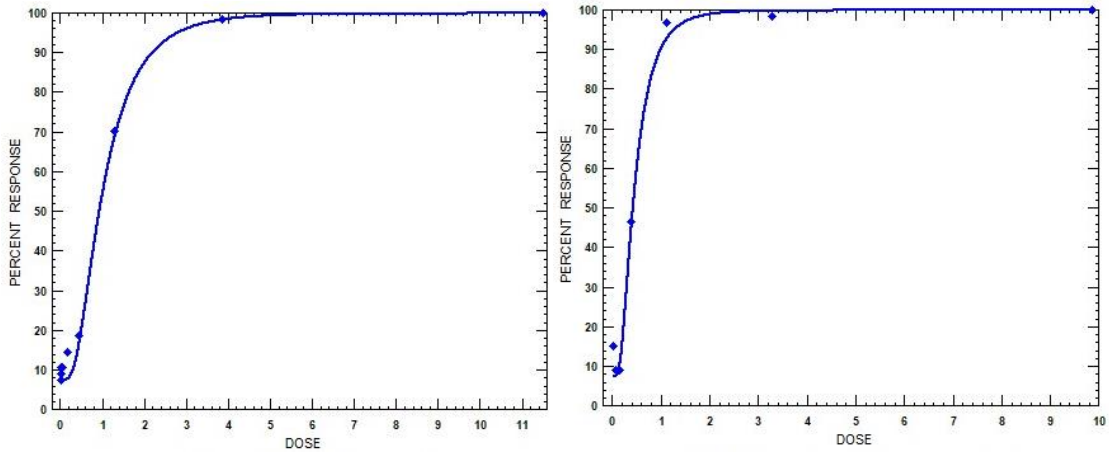


Fig. 3.6 - Probit curves generated by Polo Plus based on all bioassay biological and technical replicates of Cry1Fa (left panel) and Cry9Aa (right panel) for the Benzon strain of *S. frugiperda*. Response values for dosages are represented by blue dots. Probit curve illustrated by blue line.

Table 3.1 - Bioassay results of *S. frugiperda* when Cry1Ba and Cry9Aa. Tested as same concentrations to determine relative toxicity of Cry1Ba in Benzon strain.

Cry1Ba conc. (ug/cm^2)	0.005	0.016	0.142	1.275	11.48	Buffer
Alive	30	30	27	0	0	30
Moribund + dead	2	2	4	32	32	2
Dead	2	2	4	32	32	2
Total	32	32	31	32	32	32
Percent dead	6.25	6.25	12.9	1	1	6.25
Percent moribund	6.25	6.25	12.9	1	1	6.25
Total weight (mg)	4438.4	2676.1	518.4	0	0	5069.2
Individual weight	147.9	89.2	19.2	0	0	169.0

Cry9Aa conc. (ug/cm^2)	0.005	0.016	0.142	1.275	11.48
Alive	31	31	32	2	0
Moribund + dead	0	1	1	32	31
Dead	0	1	0	30	31
Total	31	32	32	32	31
Percent dead	0	3.1	0	93.3	1.0
Percent moribund	0	3.1	3.1	1.0	1.0
Total weight (mg)	4888.9	3289.7	1297.0	0.4	0
Mean weight	157.7	106.1	40.5	0.2	0

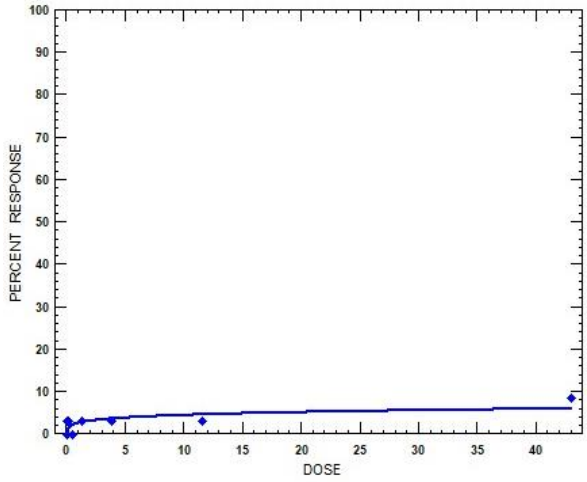


Fig 3.7 - Probit curve generated by Polo Plus based on all bioassay biological and technical replicates of Cry9Aa for the PR1 strain of *S. frugiperda*. Response values for dosages represented by blue dots. Probit curve illustrated by blue line.

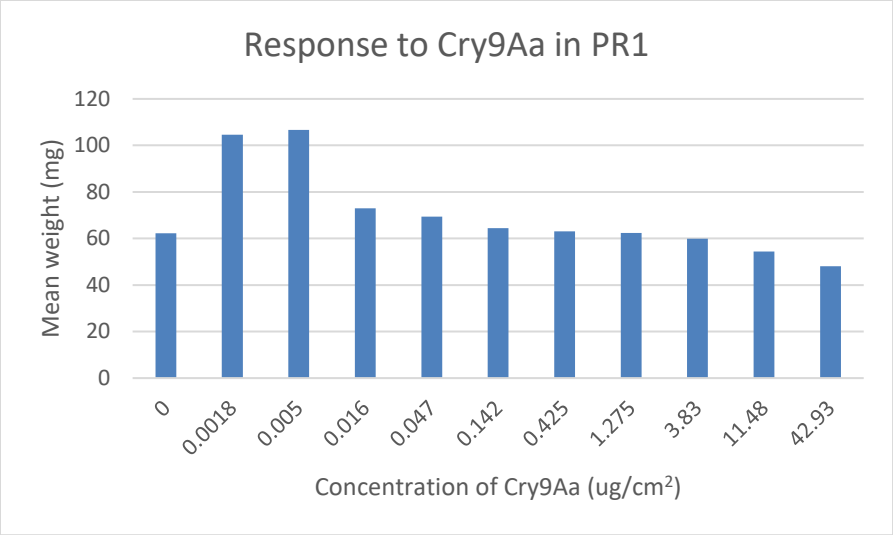


Fig. 3.8- Mean weight in PR1 larvae surviving increasing concentrations of Cry9Aa in bioassays. Bars represent the average individual weight of larvae subjected to each concentration of Cry9Aa. A treatment of buffer alone (0) served as negative control.

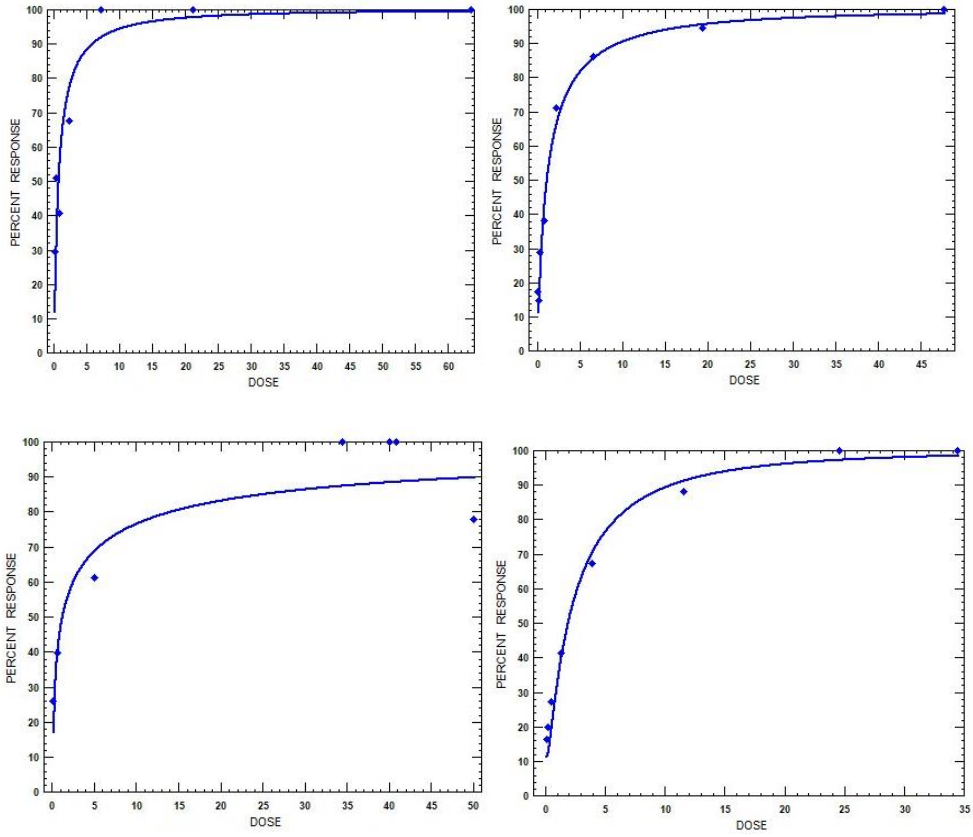


Fig. 3.9 - Probit curves generated by Polo Plus based on all bioassay biological and technical replicates for Cry1Ac, Cry1Ba, Cry1Da, and Cry9Aa for *H. zea*. Response values for dosages represented by blue dots. Probit curve illustrated by blue line. Natural response was considered in estimates, which is why the percent of response begins after 10 for all curves.

Table 3.2 - Comparison of LC50 for Cry1Ba, Cry1Da, Cry9Aa, and Cry1Ac in H. zea and Cry9Aa and Cry1Fa *S. frugiperda*. Results from all bioassays (biological and technical) combined. Mortality classified as all insects dead or less than 3rd instar after seven days.

Insect	Toxin	LC50 (ug/cm^2)	95% CI	Slope
H. zea	Cry1Ba	1.265	0.908-1.605	1.91E-02
H. zea	Cry1Da	1.661	0.122-6.654	1.07E-02
H. zea	Cry9Aa	2.196	1.177-3.275	3.87E-02
H. zea	Cry1Ac	0.422	0.079-1.158	7.33E-03
S. frugiperda	Cry9Aa	0.954	0.781-1.106	0.299
S. frugiperda	Cry1Fa	0.415	0.180-0.706	0.201

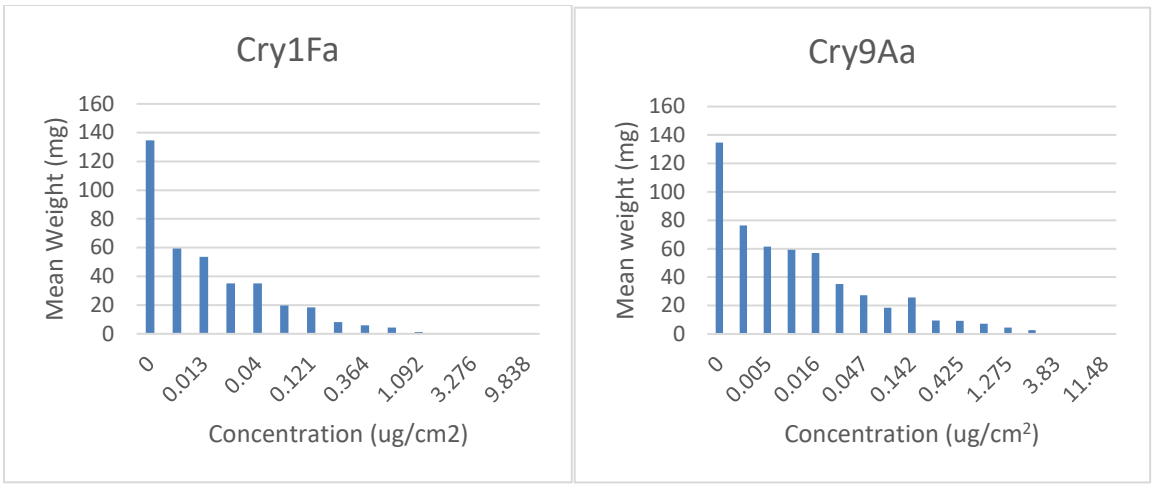


Fig. 3.10 - Mean weight of *S. frugiperda* larvae surviving increasing dosages of Cry1Fa (left) and Cry9Aa (right) in bioassays. Bars represent the average individual weight of larvae subjected to each concentration of Cry9Aa. A treatment of buffer alone (0) served as negative control.

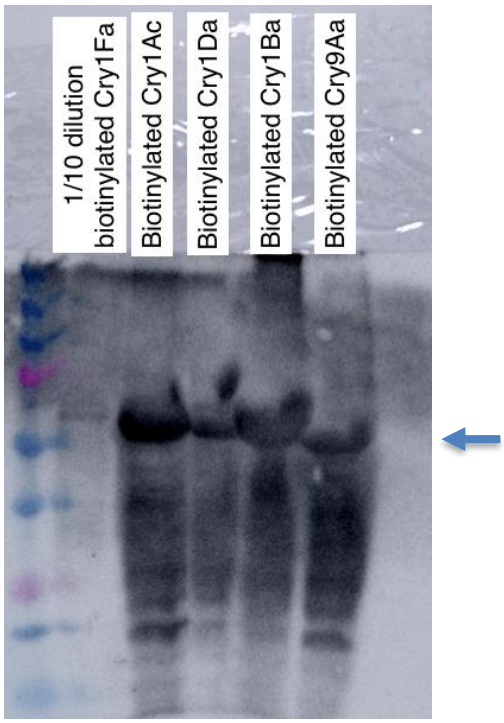


Fig. 3.11 - Western blot of biotinylated Cry toxins detected with streptavidin-HRP. Lanes: 1 – Precision Plus Dual Color protein ladder (Bio-rad, USA), 2 – 1/10th dilution of Cry1Fa (previously biotinylated), 3 – Biotinylated Cry1Ac, 4 – Biotinylated Cry1Da, 5 – Biotinylated Cry1Ba, 6 – Biotinylated Cry9Aa.

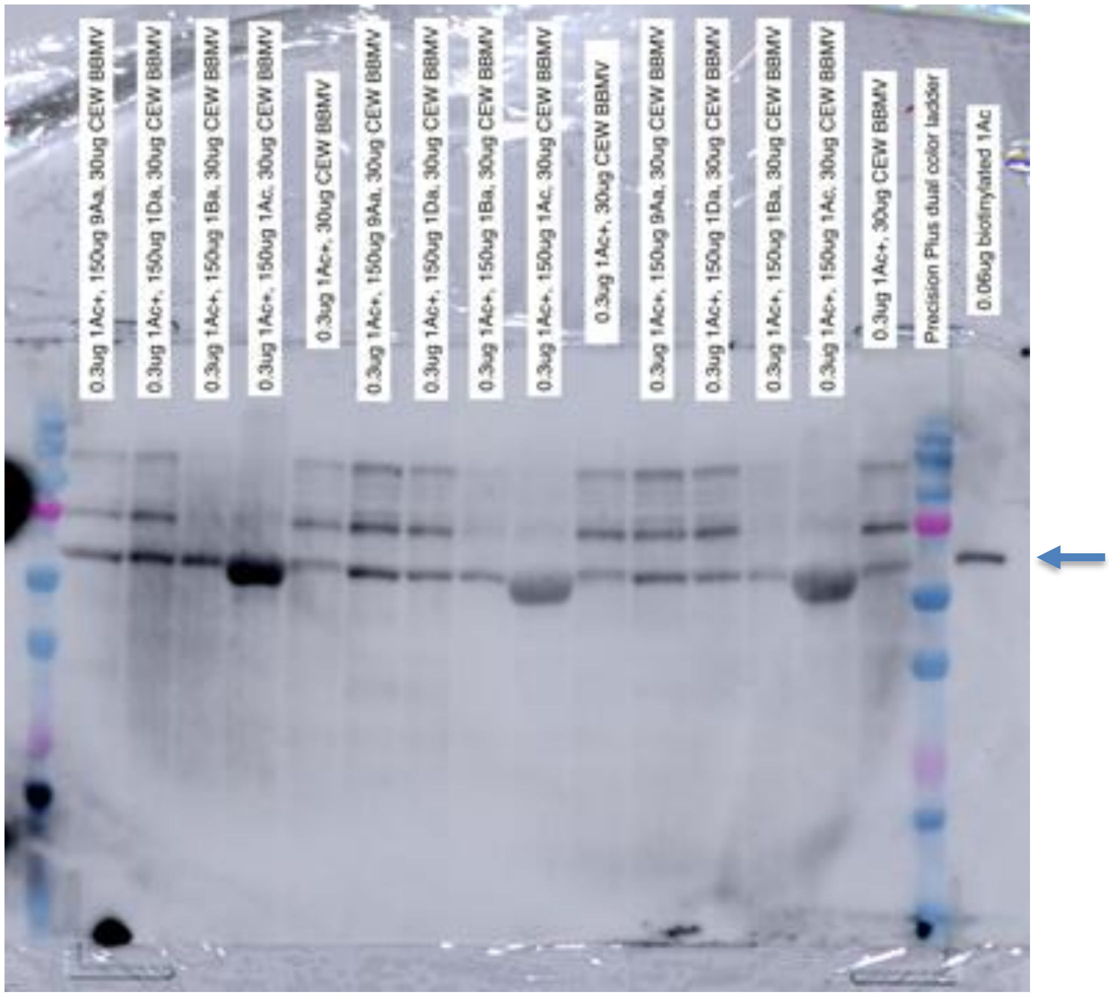


Fig. 3.12 - Western blot of Binding assays. Competition between biotinylated Cry1Ac and non-biotinylated toxin at a 500x excess in BBMV prepared from *H. zea* larvae.

VITA

Aria C. Deluna is a native of san Antonio, Texas and graduated with a Bachelor of Science in Entomology from Texas A&M University. As an undergraduate, she completed research on various topics ranging from antimicrobial natural product of marine bacteria to density-dependent phenotypic plasticity in locusts. Her masters research addressed the need to identify additional Cry proteins to which *Spodoptera frugiperda* and *Helicoverpa zea* are susceptible, as well as develop a Cry protein that is capable of receptor binding but not pore formation for use as a novel tool of identifying Cry proteins amenable for gene pyramiding. She is continuing her education at the University of Florida as a McKnight Doctoral Fellow to study the role of chironomids in the transmission and persistence of *Vibrio cholerae.*