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**Understanding the nature of specificity of
Bacillus thuringiensis Cry2A toxins against
*Plutella xylostella***

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Submitted for the award of Degree of Doctor of Philosophy

Department of Biochemistry

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Declaration

Work not submitted elsewhere for examination

I hereby declare that this thesis has not been submitted in whole or in part to this or any other university for the award of a degree.

Aminah Barqawi

Dedication

This work is dedicated to my great and wonderful father, who encouraged me and wanted me to pursue this PhD, but unfortunately, he passed away during my study. I pray this makes him proud.

Acknowledgement

First of all, I thank God for giving me strength to be able to complete my PhD journey.

I would like to express my special appreciation and thanks to my supervisor Dr. Neil Crickmore for his expertise, patience, guidance and support. I am very grateful to be a part of his lab team. You have been a great supervisor to me.

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Abstracts

Bacillus thuringiensis (Bt) is a gram positive spore forming bacterium that has entomopathogenic properties. It has the ability to synthesize insecticidal proteins in the sporulation phase of its life cycle and package them as parasporal crystals. These so-called Cry proteins are known to have insecticidal activity against many insect orders including Hymenoptera, nematodes, Lepidoptera, Diptera and Coleoptera. This study aimed to understand the basis of the specificity of the Cry2A family of toxins against the diamondback moth *Plutella xylostella*. The Cry2A toxins were characterized and bioassayed against *P. xylostella*, the results of which were used to inform the design of hybrid and mutant proteins. These were used to establish the relationship between toxin sequence and function with respect to their activity against this insect. The results showed that both domain I and II of the Cry2A toxins influenced specificity towards *P. xylostella* and that different populations of this insect show dramatically different susceptibilities to individual Cry2A toxins. Two different expression systems were compared and whilst differences in yield and purity were observed, there were no differences in the specific activity of the expressed toxins. Finally, an attempt was made to interchange the activity of Cry2Ac against *P. xylostella* and the activity of Cry41Aa against human cancer cells through domain swaps.

List of abbreviations

AC: Adenylate cyclase
APS: Ammonium persulfate
BBMV: brush border membrane vesicle
Bp: base pairs
BSA: Bovine Serum Albumin
Bt: Bacillus thuringiensis
°C: Degrees Celsius
Chymo: chymotrypsin
Cry: Crystal
Cry2AcNT: Cry2Ac N-terminus
Cry2AbNT: Cry2Ab N-terminus
Cry2ADI: Domain I of Cry2A toxin
Cry2AW/O DI: Cry2A toxin without domain I
Cry2AW/O DII: Cry2A toxin without domain II
Cry2AW/O DIII: Cry2A toxin without domain III
Cyt: Cytolytic
KDa: Kilo Dalton
DNA: Deoxyribonucleic Acid
G: gram
E.coli: Escherichia coli
IPTG: Isopropyl β -D-1-thiogalactopyranoside
L: Litre
LB: Luria Broth
LC50: Half maximal lethal concentration
MW: molecular weight
M: Molar concentration

ml: Millilitre

Mg/ml: Milligram per millilitre

MJ: midgut juice

OD: optical density

ORF: open reading frame

PAGE: Polyacrylamid Gel Electrophoresis

PBS: Phosphate buffer saline

PCR: Polymerase Chain Reaction

pGEM - pGEM-T Easy vector systems

PS: Parasporin

RGB: Resolving gel buffer

SDS: Sodum Dodecyl sulphate

SGB: stacking gel buffer

SoL: solubilised

TBE: tris-borate EDTA

TEMED: *N,N,N',N'*-tetramethylethylenediamine

TX-100: triton X-100

V: volt

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1. Introduction

1.1 *Bacillus thuringiensis*

Bacillus thuringiensis (Bt) is a gram-positive and spore-forming bacterium. It is a member of the *Bacillus cereus* family which also include *B. cereus*, *B. anthracis* and *B. mycooides* (Rasko *et al.*, 2005). It was first isolated by Japanese biologist Ishiwata in 1902 from diseased larvae of silkworm, *Bombyx mori* and formally described by Berliner in 1915 (Milner *et al.*, 1994). Also, Bt has been isolated from different ecological habitats such as soil, plant surfaces, stored crop products and insects, and also occurs naturally in the gut of caterpillars and several types of moths and butterflies (Madigan & Martinko, 2005). The Cry proteins are known to have insecticidal activity toward different insect orders including Lepidoptera, Diptera and Coleoptera and Hymenoptera (Hofte and Whiteley, 1989, de Maagd *et al.*, 2001). In addition some of Cry toxins have shown activity against non-insect species particularly nematodes, mites and protozoa (de Maagd *et al.*, 2001, Wei *et al.*, 2003).

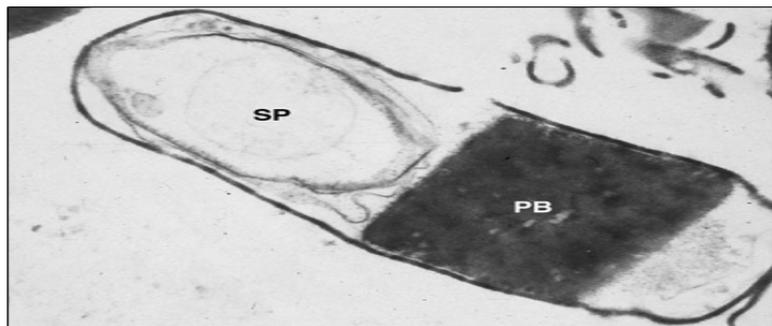


Figure 1.1. Transmission electron micrograph of a sporulating Bt cell. δ -endotoxins Adapted from (deMaagd *et al.*, 2001)

However, Bt is distinguishable from other member of *B. cereus* group due to its production of large crystalline inclusion bodies that contain entomocidal protein protoxins figure (1.1) (Asano *et al.*,1997). Such crystals consist of two kinds of δ -endotoxins called the crystal (Cry) protein family, named so because of their existence within the crystal, whereas the cytolysin (Cyt) protein family are named so because they have specified in vitro cytolytic activity (de Maagd *et al.*, 2001; Crickmore *et al.*, 1998). The third family of Bt toxin known as vegetative insecticidal proteins (Vips) which is produced from Bt during vegetative growth phase (Palma *et al.*, 2014).

Three-domain		ETX-MTX		Bin		Parasporin	
Cry1Aa	Cry2Aa	Cry8Aa	Cry15Aa	Cry31Aa	Cry40Aa	Cry58Aa	Cyt1Aa
Cry1Ab	Cry2Ab	Cry8Ab		Cry31Ab	Cry40Ba		Cyt1Ab
Cry1Ac	Cry2Ac	Cry8Ac	Cry16Aa	Cry31Ac	Cry40Ca	Cry59Aa	Cyt1Ba
Cry1Ad	Cry2Ad	Cry8Ad		Cry31Ad	Cry40Da	Cry59Ba	Cyt1Ca
Cry1Ae	Cry2Ae	Cry8Ba	Cry17Aa				Cyt1Da
Cry1Af	Cry2Af	Cry8Bb		Cry32Aa	Cry41Aa	Cry60Aa	Cyt2Aa
Cry1Ag	Cry2Ag	Cry8Bc	Cry18Aa	Cry32Ab	Cry41Ab	Cry60Ba	Cyt2Ba
Cry1Ah	Cry2Ah	Cry8Ca	Cry18Ba	Cry32Ba	Cry41Ba		Cyt2Bb
Cry1Ai	Cry2Ai	Cry8Da	Cry18Ca	Cry32Ca	Cry41Ca	Cry61Aa	Cyt2Bc
Cry1Ba	Cry2Aj	Cry8Db		Cry32Cb			Cyt2Ca
Cry1Bb	Cry2Ak	Cry8Ea	Cry19Aa	Cry32Da	Cry42Aa	Cry62Aa	Cyt3Aa
Cry1Bc	Cry2Ba	Cry8Fa	Cry19Ba	Cry32Ea			
Cry1Bd		Cry8Ga	Cry19Ca	Cry32Eb	Cry43Aa	Cry63Aa	Vip1Aa
Cry1Be	Cry3Aa	Cry8Ha		Cry32Fa	Cry43Ba		Vip1Ab
Cry1Bf	Cry3Ba	Cry8Ia	Cry20Aa	Cry32Ga	Cry43Ca	Cry64Aa	Vip1Ac
Cry1Bg	Cry3Bb	Cry8Ib	Cry20Ba	Cry32Ha	Cry43Cb		Vip1Ad
Cry1Bh	Cry3Ca	Cry8Ja		Cry32Hb	Cry43Cc	Cry65Aa	Vip1Ba
Cry1Bi		Cry8Ka	Cry21Aa	Cry32Ia			Vip1Bb
Cry1Ca	Cry4Aa	Cry8Kb	Cry21Ba	Cry32Ja	Cry44Aa	Cry66Aa	Vip1Bc
Cry1Cb	Cry4Ba	Cry8La	Cry21Ca	Cry32Ka			Vip1Ca
Cry1Da	Cry4Ca	Cry8Ma	Cry21Da	Cry32La	Cry45Aa	Cry67Aa	Vip1Da
Cry1Db	Cry4Cb	Cry8Na	Cry21Ea	Cry32Ma	Cry45Ba		
Cry1Dc	Cry4Cc	Cry8Pa	Cry21Fa	Cry32Mb		Cry68Aa	Vip2Aa
Cry1Ea		Cry8Qa	Cry21Ga	Cry32Na	Cry46Aa		Vip2Ab
Cry1Eb	Cry5Aa	Cry8Ra	Cry21Ha	Cry32Oa	Cry46Ab	Cry69Aa	Vip2Ac
Cry1Fa	Cry5Ab	Cry8Sa		Cry32Pa		Cry69Ab	Vip2Ad
Cry1Fb	Cry5Ac	Cry8Ta	Cry22Aa	Cry32Qa	Cry47Aa		Vip2Ae
Cry1Ga	Cry5Ad		Cry22Ab	Cry32Ra		Cry70Aa	Vip2Af
Cry1Gb	Cry5Ba	Cry9Aa	Cry22Ba	Cry32Sa	Cry48Aa	Cry70Ba	Vip2Ag
Cry1Gc	Cry5Ca	Cry9Ba	Cry22Bb	Cry32Ta	Cry48Ab	Cry70Bb	Vip2Ba
Cry1Ha	Cry5Da	Cry9Bb		Cry32Ua			Vip2Bb
Cry1Hb	Cry5Ea	Cry9Ca	Cry23Aa	Cry32Va	Cry49Aa	Cry71Aa	
Cry1Ia		Cry9Da		Cry32Wa	Cry49Ab		Vip3Aa
Cry1Ib	Cry6Aa	Cry9Db	Cry24Aa			Cry72Aa	Vip3Ab
Cry1Ic	Cry6Ba	Cry9Dc	Cry24Ba	Cry33Aa	Cry50Aa		Vip3Ac
Cry1Id		Cry9Ea	Cry24Ca		Cry50Ba	Cry73Aa	Vip3Ad
Cry1Ie	Cry7Aa	Cry9Eb		Cry34Aa			Vip3Ae
Cry1If	Cry7Ab	Cry9Ec	Cry25Aa	Cry34Ab	Cry51Aa	Cry74Aa	Vip3Af
Cry1Ig	Cry7Ba	Cry9Ed		Cry34Ac			Vip3Ag
Cry1Ja	Cry7Bb	Cry9Ee	Cry26Aa	Cry34Ba	Cry52Aa		Vip3Ah
Cry1Jb	Cry7Ca	Cry9Fa			Cry52Ba		Vip3Ai
Cry1Jc	Cry7Cb	Cry9Ga	Cry27Aa	Cry35Aa			Vip3Ba
Cry1Jd	Cry7Da			Cry35Ab	Cry53Aa		Vip3Bb
Cry1Ka	Cry7Ea	Cry10Aa	Cry28Aa	Cry35Ac	Cry53Ab		Vip3Ca
Cry1La	Cry7Fa			Cry35Ba			
Cry1Ma	Cry7Fb	Cry11Aa	Cry29Aa		Cry54Aa		Vip4Aa
Cry1Na	Cry7Ga	Cry11Ba	Cry29Ba	Cry36Aa	Cry54Ab		
Cry1Nb	Cry7Gb	Cry11Bb			Cry54Ba		Slp1Aa

Figure 1.2. List of Bt toxins. Toxins listed according to their primary rank, the majority of Cry toxins belong to three domain Cry toxins family coloured with blue, two other toxins Bin and ETX/MTX2 like are coloured with pink and orange respectively. Those toxins colour in red belong to the parasporin class. Cry toxin highlighted with other colour indicated toxins are that are not currently classified into existing Cry toxin family (taken from Adang *et al.*, 2014).

1.2 Bt toxins nomenclature

Hofte and Whiteley (1989) introduced the first systematic nomenclature for toxins which was based on the spectrum of activity of proteins as well as their size and apparent relatedness to nucleotide and amino acid sequences (Hofte and Whiteley 1989). Currently, the crystal toxins are classified based on amino acid sequence homology, where each protoxin acquired a name consisting of the mnemonic Cry (or Cyt) and four hierarchical ranks, consisting of number, capital letters, lower case letter and number depending on its place in phylogenetic tree. Proteins with less than 45% identity differ in primary rank and 78%, 95%, and 100% identity differ in secondary, tertiary and quaternary rank respectively (Crickmore *et al.*, 1998).

1.2.1 Cry toxin

Cry toxins are officially defined as proteins that have significant sequence similarity to existing toxins within the nomenclature or be a *B. thuringiensis* parasporal inclusion protein that exhibits pesticide activity, or some experimentally verifiable toxic effect to a target organism (Crickmore *et al.*, 1998). However, the name of Cry toxin derives from the fact that it forms a parasporal crystal. Cry toxins family contain about 300 different crystal proteins, which have been classified to 74 different types of Cry proteins figure 1.2 (Crickmore *et al.*, 2016). Cry proteins are known to have insecticidal activity against different insect orders including Lepidoptera, Diptera and Coleoptera and Hymenoptera, snail and human cancer cell figure 1.3 (Palma *et al.*, 2014).

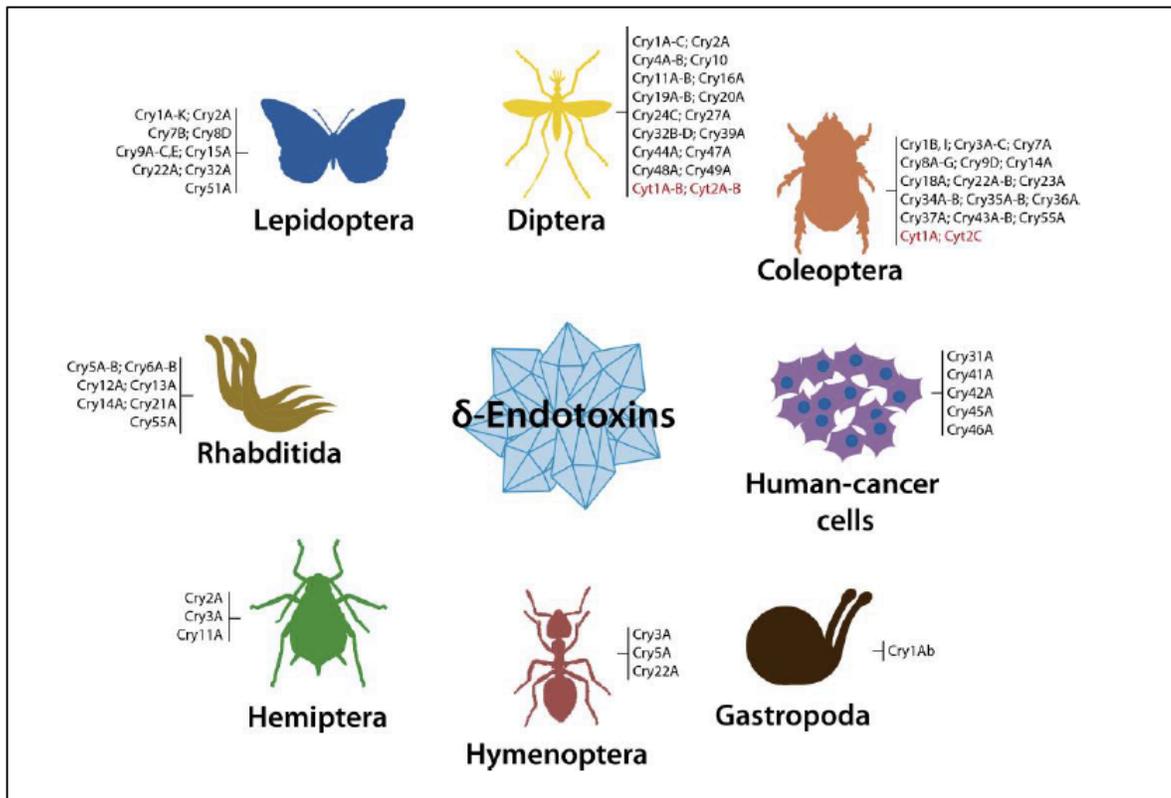


Figure 1.3. Represents the host spectrum of Bt toxins (Cry and Cyt) based on its target specificity (taken from Palma *et al.*, 2014).

Comparing the Cry proteins has led to the location of shared sites that are critical in insect specificity and toxic function (Pigott and Ellar, 2007). Höfte and Whiteley (1989) generated alignments between 13 Cry protein sequences, showed high degree of diversity among the Cry proteins, and revealed the existence of five conserved blocks of amino acids that are prevalent in most Cry proteins. This was made up along with additional work done by (Adang *et al.*, 2014) (Figure 1.4). Block 1 exists in domain I, blocks 4 and 5 are contained in domain III while blocks 2 and 3 span the junction between domains I and II and domains II and III respectively (Höfte and Whiteley, 1989). Cry toxins exist in long and short forms (130-140kDa and 70kDa) and many three domains Cry proteins have the five conserved blocks.

The blocks indicate that the sequence of amino acid could play a critical role in toxin function or stability. The conserved blocks exist within the N-terminal part of the longer toxins, which is approximately of equal length with the shorter toxins. The C-terminal part has a protoxin domain that is absent within the toxins having smaller length and lacks any of the five conserved blocks (Pigott and Ellar, 2007).

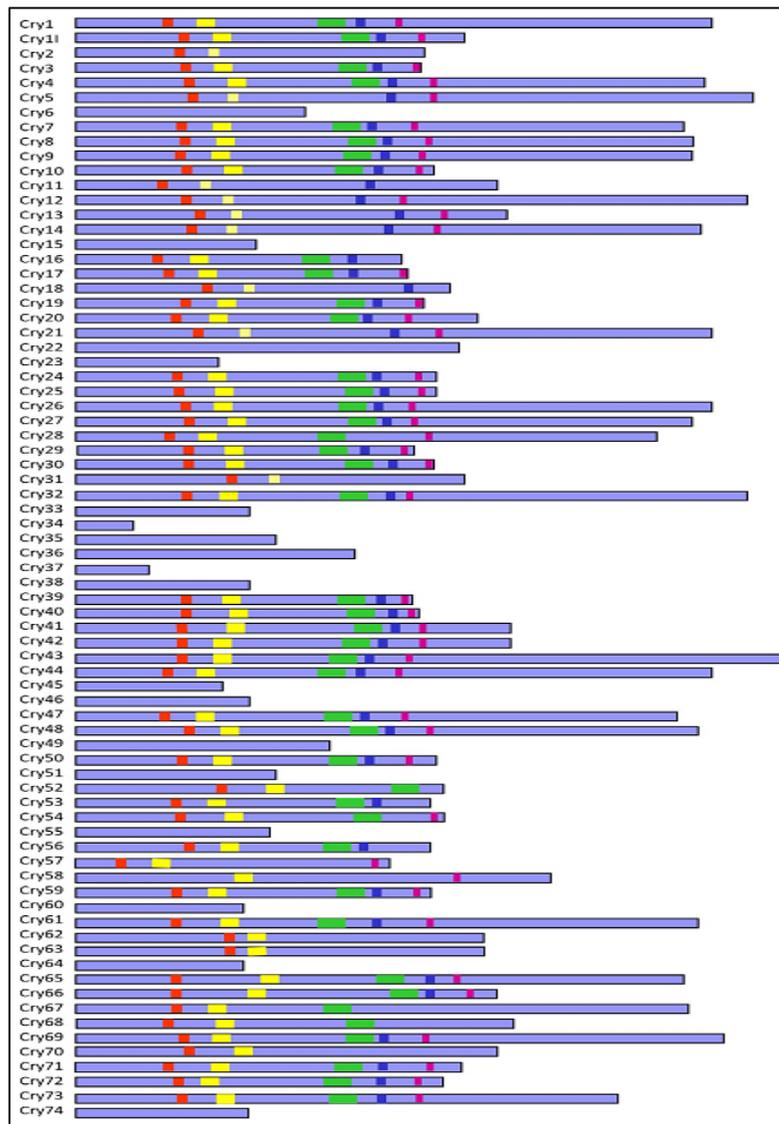


Figure 1.4. Represent different Bt Cry toxins. The lengths of Cry protoxins and position of the five conserved blocks are shown as colored inserts (taken from Adang *et al.*, 2014).

The crystal structure of various three domain proteins have been solved using X-ray crystallographic techniques: these include Cry1Aa (Grochulski *et al.*, 1995), Cry2Aa (Morse *et al.*, 2001), Cry3Aa (Li *et al.*, 1991), Cry3Bb (Galitsky *et al.*, 2001), Cry4Aa (Boonserm *et al.* 2006), and Cry4Ba (Boonserm *et al.* 2005), and Cry8Ea1 (Guo *et al.* 2009). Figure (1.5) shows the structure of several three domain Cry toxins.

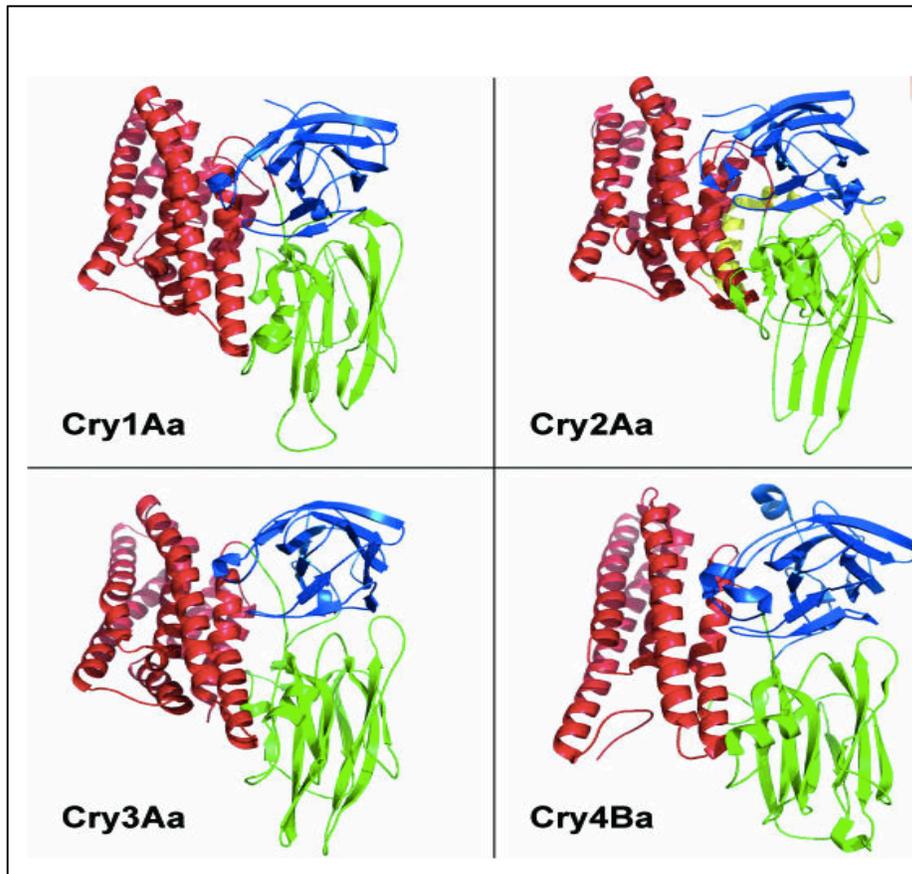


Figure 1.5. Represents the structure of three domain Cry toxins (Cry1Aa, Cry2Aa, Cry3Aa and Cry4Ba). Each domain indicated by colour, domain I shown in red, domain II in green and domain III in blue (taken from Pigott and Ellar, 2007).

The three domain Cry toxins consist of three structural domains and share a high degree of topological similarity. The domain I N-terminal is composed of seven α -helix bundle that

exceeds 30Å in length and can span a hydrophobic cellular membrane (Pigott and Ellar 2007). The central hydrophobic α -helix 5 is enclosed by 6 amphipathic helices (de Maagd *et al.*, 2001). This domain I has a similar function to another pore-forming bacterial toxin, later it was proven to be true that domain I is responsible for toxin membrane insertion and pore formation (Li *et al.*, 1991). Domain II is composed of three antiparallel β -sheets, which are responsible for receptor binding and specificity (Schnepf *et al.*, 1998). In the case of domain II there are three loops suggested to be involved in receptor binding (Adang *et al.*, 2014). Domain III constitutes two anti-parallel β -sandwich sheets in a jellyroll topology. The two β -sheets have 5 β -strands, the internal sheet is attached to the second domain whereas the external sheet is exposed to solvent (George and Crickmore, 2012, Li *et al.*, 1991). The third domain is involved in receptor binding and possibly pore formation, also it seems to have other function, which is maintaining the structural integrity of the toxin by protecting it from proteolysis (Deist *et al.*, 2014). The ETX_MTX2 family includes 11 members that were classified under distinct primary rankings in the nomenclature: Cry15, Cry23, Cry33, Cry38, (Coleoptera), Cry51 (Coleoptera and Hemiptera), Cry60 (Diptera), Cry45 (Parasporin-4) and Cry64 (Parasporin-5) (human cancer cells). They all show features of the ETX/MTX2 family that include the MTX2 protein from *Lysinibacillus sphaericus* and the *Clostridium perfringens* epsilon toxin (Adang *et al.*, 2014). However, they have a different structure from the three domain toxins, but are still believed to act via forming pores in the membranes of the target cells (Bokori-Brown *et al.*, 2011). An example of the ETX/MTX structure is depicted in (figure 1.6).

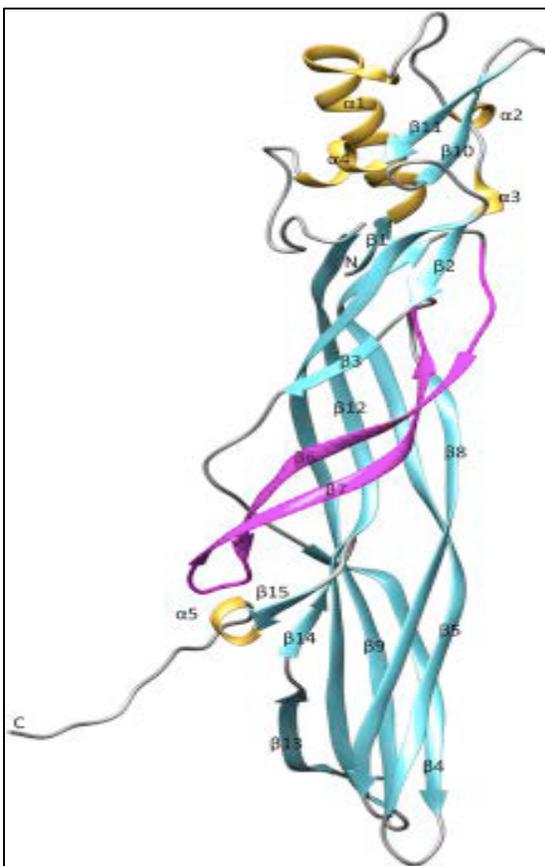


Figure 1.6. Three dimensional structure of Cry51Aa1. The structure of Cry51Aa1 was solved at 1.65 Å resolution; PDB 4PKM. The secondary structure components include 5 helices and 15 β -strands presented in yellow and cyan respectively. The amphipathic β -hairpin is shown in magenta (Xu *et al.*, 2015a).

The Bin-like toxins are a class that is constituted of 13 toxins. Their name is derived from the fact that they resemble the two homologous components (Bin A and Bin B) of the mosquitocidal binary toxin from *L. sphaericus*. Although their molecular structure as well as their mechanism of action is unclear, there is some evidence that they form pores and that one of the components acts intracellular (Berry, 2012). (Figure 1.7) represents the 3-D structure of Bin A and Bin B of *L. sphaericus*. More also, during the vegetative growth phase of Bt they synthesis an insecticidal protein which is further secreted into the growth medium. They constitute what is referred to as vegetative insecticidal proteins (Vips) and it has been to have activity against lepidopteron, coleopteran and some homopteran insects. They are

classified into Vip1, Vip2, Vip3 and Vip4 based on their amino sequence similarities. Vip1 and Vip2 are known as the binary toxin and they show insecticidal activity against some coleopteran, whereas Vip3 toxins are active against lepidopteron. The host spectrum of the Vip4Aa1 toxin is still unknown to date (Palme *et al.*, 2014).

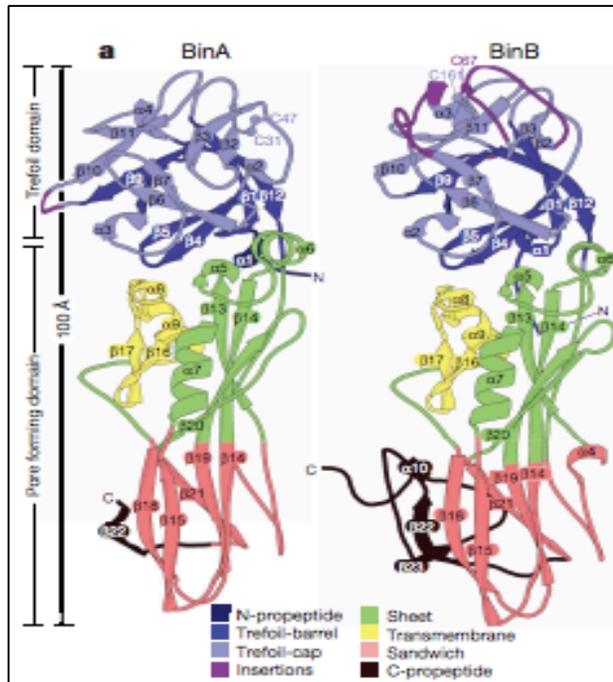


Figure 1.7. Three dimensional structure of Bin A and Bin B of *L. sphaericus*. Structural similarity between Bin A and Bin B composed of trefoil and pore-forming domains. Each molecule is approximately 100 Å long and 25-30 Å in diameter. α and β represent carbohydrate-binding molecules where the structural differences are suggested to be located (Colletier *et al.*, 2016).

1.2.2 Cyt Toxins

Cyt toxins from *B. thuringiensis* are cytolytic to different insect and mammalian cells. They have been classified into three major classes Cyt1, Cyt2 and Cyt3. However, Cyt toxins produced by various strains of Bt and these toxins shown specific toxicity to Dipteran insects but are cytolytic to a broad range of cells including red blood cells in vitro (Promdonkoy and

Ellar, 2003). The structure of two members of this group, Cyt1Aa and Cyt2Ba have been elucidated and reveal a single domain three-layer alpha-beta protein with a unique fold (figure 1.8) (Cohen *et al.*, 2008 and 2011). The mechanism of action is unclear and may be either pore forming (Promdonkoy and Ellar, 2000) or aggregation on the target cell surface leading to the destruction of the lipid bilayer in a detergent-like manner (Butko, 2003, Rodriguez-Almazan *et al.*, 2011).

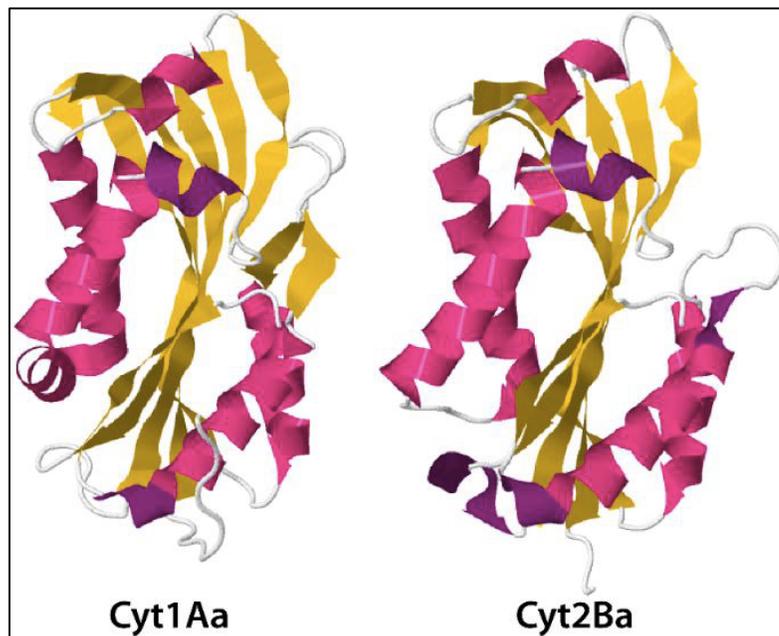


Figure 1.8. Three-dimensional structure for toxin Cyt1Aa and activated Cyt2Ba monomer from PDB accession numbers 3RON [20] and 2RCI [66], respectively. Adapted from (Cohen *et al.*, 2008 and 2011).

1.2.3 Cry2A family of toxins

The Cry2A proteins comprise a group of 11 small toxins with molecular weight between 61-72kDa, and occur in cuboidal crystals produced by Bt (Donovan *et al.*, 1988, Nicholls *et al.*, 1989, Dankocsik *et al.*, 1990). The Cry2A proteins are a subset of the Cry toxins that possess

the dual activity spectra to Dipteran and Lepidopteran insects (Donovan *et al.*, 1988 and Yamamoto *et al.*, 1981). However, the Cry2Aa toxin is highly toxic to both moth and mosquito.

Of the Cry2A proteins the only structure of Cry2Aa has been elucidated, it is composed of 633-amino acids, the N-terminal region consists of 49- amino acids peptide which gets cleaved upon activation to be able to form a mature toxin (Morse *et al.*, 2001). Moreover, the three domains structure of Cry2Aa protein has the same topology to those of the activated toxins of Cry3Aa (Li *et al.*, 1991) and Cry1Aa (Grochulski *et al.*, 1995), despite having little sequence identity to Cry2Aa (20% of Cry3Aa and 17% of Cry1Aa).

On the basis of modeling studies using Cry2Aa, Morse *et al.* (2001) suggested that proteolytic activation of the toxin may involve the cleavage of 49 N-terminal amino acids resulting in the exposure of residues consisting of a potentially binding toxin-receptor surface.

1.3 Parasporins: Anti-cancer toxins

Mizuki *et al* (1999) reported a non-insecticidal parasporal inclusion isolated from Bt strains that were cytotoxic to human cancer cells (Mizuki *et al.*, 1999 and Ohba *et al.*, 2000). There are a great number of Bt strains with no known insecticidal activity. However, much work done by Mizuki *et al.* (1999), screen a large number of Bt strains to investigate their cytotoxic activity against human leukemia T-cell and their hemolytic activity against sheep erythrocytes (Mizuki *et al.*, 1999). Out of 1684 strains with no hemolytic activity, 42 strains were shown to have cytotoxic activity against MOTL-4, and they did not show significant

insecticidal activity when tested against 11 insect species of five orders: Lepidoptera, Diptera, Orthoptera, Dictyoptera and Isoptera. The three selected strains namely 84-HS-1, 89-T-26-17 and 90-F45-14 were non-insecticidal, non-hemolytic and were found to be cytotoxic to Leukemia T cell and other human cancer cells and the first two strains were able to discriminate between Leukemia and normal T-cell with a preferential activity against the cancer line (Mizuki *et al.*, 1999 and Ohba *et al.*, 2009). Researches have provided evidence that Bt strains with parasporin activities are common in Bt natural populations occurring in Japan, Vietnam, Canada, Caribbean, Malaysia and India (Ohba *et al.*, 2009 and Gonzalez *et al.*, 2011). To date 19 parasporins have been discovered and placed in the list of parasporins by the committee of parasporin and they have been classified this toxin into six main classes based on the primary amino acid sequence of protoxins, namely parasporin 1(PS1), parasporin 2 (PS2), parasporin 3(PS3), parasporin 4 (PS4), parasporin 5 (PS5) and parasporin 6 (PS6) (<http://parasporin.fitc.pref.fukuoka.jp/intro.html>). Moreover, like other Cry toxin a novel parasporin is assigned a four rank name based on the degree of sequence identity to previously identified toxins (Crickmore *et al.*, 1998). Out of all six parasporins, parasporin 3 is the topic of this research.

1.3.1 Parasporin-3 (Cry41Aa)

Parasporin-3, also known as Cry41Aa is an 88kDa protein produced by Bt strain A1462 discovered within Japanese soil. Once solubilised and activated through proteolytic cleavage in a comparable way to insecticidal Cry proteins, it generated a 64kDa product that was cytotoxic to human tumour cell lines HL60 (myeloid leukaemia cells) and HepG2 (Hepatocellular carcinoma) (figure 1.9) although non-toxic to Jurkat, HeLa, and mammalian

non-cancerous cell lines (Yamashita *et al.*, 2005). Moreover, it was viewed that the cytotoxic consequences of treatment by means of activated Cry41Aa on HepG2 cells - principally cell inflammation and lysis - resemble the outcome of insecticidal Cry proteins on pest mid gut epithelial cells (Yamashita *et al.*, 2005).

Cry41Aa is encoding by an operon of three open reading frames orf1, 2 and 3, they are located and oriented in the same direction. In addition, all orfs had putative ribosome binding sites; ORF1 consists of a protein with a predicted molecular weight of 19 KD and has no recognized role. ORF2 contains a protein with an approximate molecular weight of 93 KD, which has a cytotoxic activity against human cancer cells. ORF 3 is contain a protein with molecular weight of 82 KD, it involves in crystallization and expression (Yamashita *et al.*, 2005). Cry41Aa is the most closely related parasporin to the insecticidal Cry toxins, although it does contain a C-terminal beta-trefoil ricin domain not normally found in insecticidal Cry proteins. The five preserved blocks are found in ORF 2 that demonstrate tough homology with the insecticidal Cry proteins proposing that Cry41Aa contains the three-domain arrangement feature of insecticidal Cry proteins (Ohba *et al.*, 2009). Cry41Aa has the narrowest activity spectrum among the PS proteins. It is cytotoxic to HepG2 and HL60 cells but harmless to numerous other tumour-cell lines which suggests that Cry41Aa contains an inherent specificity to these lines. The configuration resemblance between Cry41Aa and connected insecticidal Cry proteins together with the comparable cytotoxic consequences on objective cells cause, to assume that Cry41Aa “destroys particular tumour cells through a receptor-mediated system comparable to that of Cry proteins of insecticidal nature” (Yamashita *et al.*, 2005). Exploring the specificity of Cry41Aa to tumour-cell lines might have possible applications in cancer treatment (Ohba *et al.*, 2009).

Cell	Origin	EC ₅₀ (µg/ml)	
		P2	P3
Human			
MOLT-4	Leukemic T cell	>10	>10
Jurkat	Leukemic T cell	>10	>10
HL60	Myeloid leukemia	1.32	1.25
HeLa	Uterus cervix cancer	>10	>10
TCS	Uterus cervix cancer	>10	>10
Sawano	Uterus cancer	>10	>10
HepG2	Hepatocyte cancer	2.80	1.86
A549	Lung cancer	>10	>10
CACO-2	Colon cancer	>10	>10
T cell	Normal T cell	>10	>10
UtSMC	Normal uterus	>10	>10
HC	Normal hepatocyte	>10	>10
MRC-5	Normal lung	>10	>10
Simian			
Vero	African green monkey kidney	>10	>10
COS-7*	African green monkey kidney	>10	>10
Murine			
NIH3T3-3	Mouse embryo	>10	>10

Figure 1.9. Cytotoxicity spectra and levels of PS-3. The cytotoxic effect of proteinase K activated PS-3 was tested on several mammalian cell lines using MTT cell viability assay and EC₅₀s were determined. P2 and P3 represent Cry41Aa1 and Cry41Ab1 respectively (Yamashita *et al.*, 2005).

1.4 Mode of action of *Bacillus thuringiensis* Cry toxins:

There are a number of models to explain the ability of Cry toxins to kill particular insects. The general accepted model was proposed by Knowles and Ellar, and is known as the colloid-osmotic lysis model (Knowles and Ellar, 1987).

Their study was based on the experiment carried out on three domain Cry toxins on lepidopteran larvae. First, the Bt crystals are ingested by insect larvae, then solubilized in the alkaline environment of the insect's gut. Research done by Angus, (1954) showed the importance of alkaline environment for solubilisation (Angus, 1954). The findings were

confirmed by Du *et al.*, (1994) where only the insects with digestive juice able to solubilize the crystal were susceptible to Cry toxin (Du *et al.*, 1994, Jurat-Fuentes and Crickmore, 2017). The protoxin is cleaved by mid gut proteases to the toxic core, activated toxins bind to a specific receptor in the gut brush border membrane. Following toxin-receptor interaction, toxin oligomerization occurs. In vitro studies demonstrated that oligomerization of monomeric Cry toxins requires interaction with cell membrane (Güereca and Bravo, 1999). Following oligomerization, the toxin inserts itself into membrane, causes nonspecific pores in the membrane of the target cells. The presence of pores promotes changes in membrane potential, ion imbalance, influx of water, cell swelling, colloid-osmotic lysis of gut cells, and larval death (Knowles and Dow, 1993). There are many studies that aim to clarify the mechanism of toxicity, including receptor toxin complexes as well as revealed crystal structure of Cry toxins. Some of the main models are explained below.

1.4.1 The Bravo model

The Bravo model based on experiments done with Cry1Ab and *Manduca sexta*, and suggested that the binding of Cry1Ab requires two different receptors, aminopeptidase N (APN) and cadherin-like protein (Bt-R1) (Bravo *et al.*, 2004). The model proposes sequentially binding of activated monomers to these receptors. This study was based on Immunoprecipitation experiments which showed that initial Cry1Ab toxin binding to Bt-R1 followed by binding to APN. Binding to (BT-R1) promotes the cleavage of helix α -1 of domain I by membrane-associated proteases, which leads to the formation of oligomer toxin which then bind to APN, which then drives the toxin into detergent-resistant membrane (DRM) where toxin insertion occurs resulting in formation of pores and subsequent cell lysis

and death (figure 1.10). APN was observed in samples immunoprecipitated purified oligomeric toxin structure, whereas cadherin (Bt-R1) was detected in samples immunoprecipitated with the monomeric Cry1Ab, which proposed that the interaction of two receptors happened sequentially with different structural states of the toxin to promote its efficient membrane insertion (Bravo *et al.*, 2004). Supportive data comes from Gomes *et al.*, 2002 who showed that Cry1Ab oligomer was toxic against *M. sexta* (Gomes *et al.*, 2002). However, its insertion into membrane vesicles was more efficient compared to the monomeric Cry1Ab, indicating that the oligomerization process is necessary for the insertion of the toxin into the target cell membranes (Rausell *et al.*, 2004b).

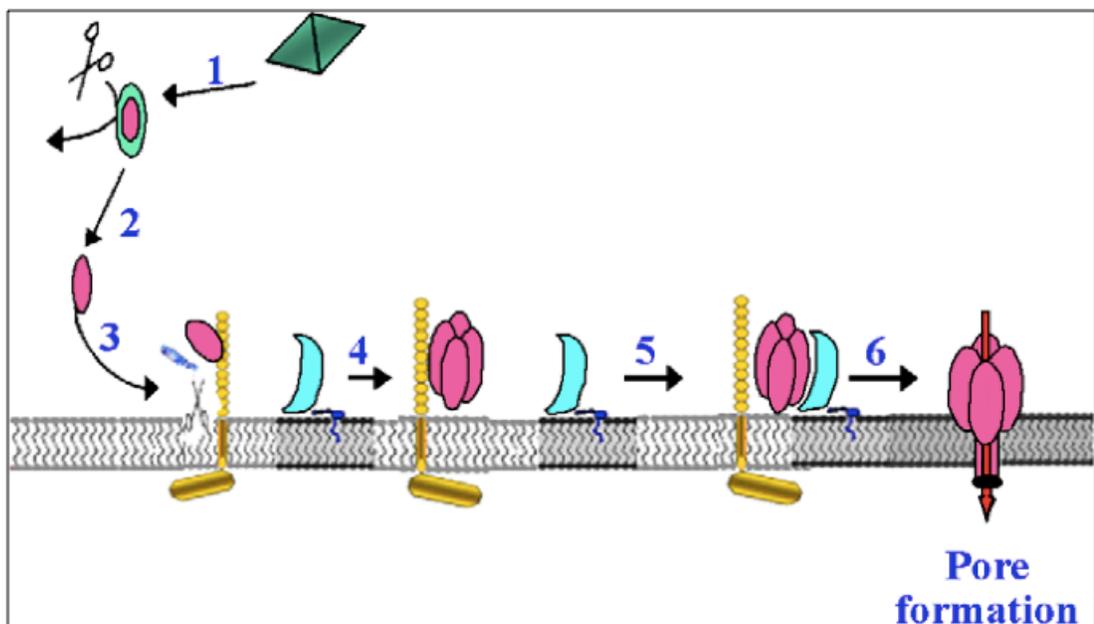


Figure 1.10. The mode of action of Cry1A toxin according to Bravo model. The mode of action of Cry toxins involved several steps (1) crystal solubilization; (2) protoxin proteolytic activation; (3) monomer binding to Bt-R₁ and cleavage of helix a-1; (4) pre-pore oligomeric structure formation; (5) oligomer binding to APN and mobilization to DRM; (6) pore formation in DRM (Bravo *et al.*, 2004).

1.4.2 The Zhang model

The second mode of action of Cry toxins proposed by Zhang *et al.* (2005) suggested that the death of the cell is associated with the binding of the monomeric form of Cry1Ab toxin to a respective receptor BT-R1 which provokes toxicity. However, the interaction of toxin with the cell membrane does not cause lytic pores and does not lead to cell death, but was linked to Mg^{2+} -dependent signal cascade pathway (Zhang *et al.*, 2005).

The study of the model was carried out using Cry1Ab and undifferentiated ovarian cell of cabbage looper *Trichoplusia ni*. This cell line named (S5) was engineered to stably express cadherin BT-R1 receptor on cell surface. Western blot experiment demonstrated that, it was the only monomer form of the toxin that is incorporated into the membrane expressing BT-R1 receptors, but the oligomeric form of Cry1Ab was detected in membrane of both susceptible and non-susceptible cells. Furthermore, blocking the interaction of the cadherin binding site prevented toxicity and cell death but not incorporation of oligomeric toxin to cell membrane. In addition, the toxin oligomers remain incorporated for several generations without any toxic effect (Zhang *et al.*, 2005).

Zhang *et al.*, (2005) showed that the toxin binding Cry1Ab to cadherin receptor BT-R1 receptor still occurred in the presence of EDTA and EGTA chelators, but EDTA prevented subsequent cell death. Adding Mg^{2+} to susceptible cells pre-exposed to EDTA restored Cry1Ab and microscopic findings showed that removal of this cation prevented swelling of susceptible cells. Apparently binding of Cry1Ab to cadherin receptor

BT-R1, required for cytotoxicity, is associated with Mg^{2+} dependent signal cascade pathway (Zhang *et al.*, 2005).

Zhang *et al.*, (2006) proposed that the mode of action, which includes adenylyl cyclase (AC), protein Kinase A (PKA) signaling pathway, as demonstrated in (figure 1.11), in this proposed model he suggested that, the binding of Cry1Ab toxin to cadherin receptor (BT-R1) stimulates a G protein –coupled receptor (GPCR) and adenylyl cyclase, resulting in an increase in the level of cAMP and activation of protein kinase A (PKA), all these have been shown to disturb the host cell's structural and functional integrity resulting to its death (Zhang *et al.*, 2006). Induction of the adenylyl cyclase protein kinase A pathway is manifested by sequential cytological changes that include membrane blebbing, appearance of ghost nuclei, cell swelling, and lysis (Zhang *et al.*, 2006).

Pre-treatment of S5 cells with PKA inhibitors prevented these phenotypic changes of cells and protected them from Cry toxin action. In addition, inhibition of G α s (NF449) and AC (ddADP) resulted in lack of cAMP production and reduced the Cry1Ab cytotoxicity while the activator (FSK) and potentiator (pCPT-cAMP) of cAMP sensitized the cells and enhanced cytotoxicity (Zhang *et al.*, 2006).

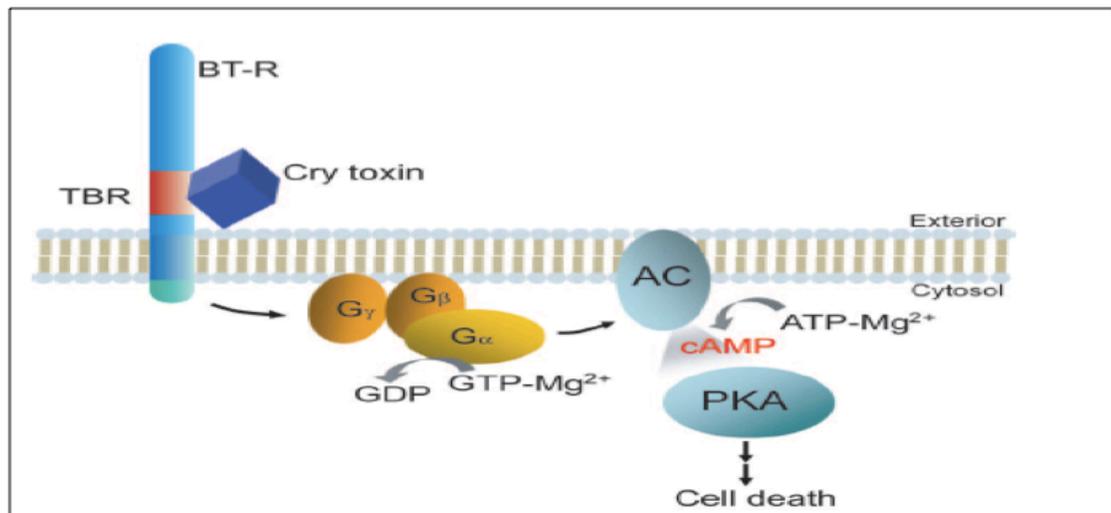


Figure 1.11. The Zhang model for mode of action of Cry toxin – Mg^{2+} dependent signal cascade pathway: It implicates AC/PKA signalling pathway. It states that monomeric form of the toxin is required in binding to cadherin receptor. The binding induces coupled G protein which in turn activates adenylyl cyclase and a rise in cytoplasmic cAMP. cAMP levels activate protein kinase A which phosphorylates target proteins resulting the disruption of channels and eventual cell death (Zhang *et al.*, 2006).

1.5 *Plutella xylostella*

The diamondback moth (DBM) *Plutella xylostella*, is the most destructive insect pest of crucifers crops. In 1953, the diamondback moth was the first insect to develop resistance to DDT (Ankersmit, 1953, Johnson, 1953). In addition, several countries reported that diamondback moth developed resistance to every synthetic insecticide used towards it in the field (Talekar and Shelton 1993). However, *P. xylostella* is one of the most cosmopolitan species of Lepidoptera and is found wherever crucifer crops are grown (Talekar and Shelton, 1993), and it is reported as the first insect to show resistance to insecticidal toxin from *Bacillus thuringiensis* (Tabashnik *et al.*, 1990). Control failure and insecticidal resistance are widespread especially in countries that have tropical climates for instance Southeast Asia, Central America, the Caribbean, and the southeastern United States. The economical

production of crucifers crops seems to be impossible in some of these areas (Metcalf *et al.*, 1980). However, the use of natural enemies of *P. xylostella*, such as larval parasitoids, as biological control agents is often hampered by the indiscriminate use of insecticides in futile attempts to overcome resistance (Lim, 1986). Insecticidal *B. thuringiensis* toxins were therefore highly useful as a substitute for broad-spectrum synthetic insecticides as they provided efficient control of *P. xylostella* with minimal non-target effects on parasitoids and predators (Sastrosiswojo and Sastrodihardjo, 1986). *P. xylostella* resistance to *B. thuringiensis* toxin is now widespread in the field (Ferre and Van Rie, 2002, Sayyed *et al.*, 2005, Tabashnik *et al.*, 1990) and thus there is a great risk that unwise use of insecticides will lead to the selection of multiply resistant pest populations.

Furthermore, an estimated cost of managing this pest was around U.S. \$1 billion (Talekar and Shelton, 1993), but recently in 2012 the cost of managing the diamondback moth with the loss of crops as updated was approximately between U.S. \$4 - U.S. \$5 billion per year (Zalucki *et al.*, 2012).

1.4.3 Life cycle of *Plutella xylostella*

Diamondback moth (*Plutella xylostella*) has four stages in its life cycle adult, egg, larva and pupa (Talekar and Shelton, 1993). The development time of each stage is dependent on temperature (Sarnthoy *et al.*, 1989, Golizadeh *et al.* 2007). Also, the host crop can affect development rate (Hoy *et al.* 1988). Adults moths are active at dusk and at night, and mating occurs at dusk and they start mating in the same day that adults emerge, females lay their

eggs a short time after mating, and the perfect time for laying eggs before midnight around 7:00 and 8:00 PM (Talekar and Shelton, 1993).

Single female can lay up to 356 eggs in their life cycle, and they are laid on both the upper and lower leaf surfaces but preferably on the lower surface away from direct sunlight and where there is protection from the wind and the rain, the normal life length for the adults around two weeks but they can live for seven or eight weeks (Shrivastava and Dhaliwal, 2014). According to Capinera (2015), the life expectancy for an adult males and females of diamondback moth is about 12 and 16 days. The number of generations varies from 13 to 16 per year (Talekar and Shelton, 1993, Gautam *et al.*, 2018). Eggs are small, oval, flattened and yellow to pale green, eggs start hatching around five to six days under normal field conditions (Talekar and Shelton, 1993).

Larvae of diamondback moth have four instars and they need a maximum time to complete development from 9 to 30 days. The early stage larvae are small, colourless to yellow, and have a dark head capsule, mid to later stage of larva is green (Shrivastava and Dhaliwal, 2014). The duration of completing the development of the four larvae instars depends on temperature (Liu *et al.*, 2002; Golizadeh *et al.*, 2007).

The pupae are yellow to green and it can be seen in a loose silk cocoon, usually found on the lower or outer leaves. The duration of complete development of the pupae is around 4 to 15 days. Once the pupal period is over, the DBM has gone through complete metamorphosis, adult will emerge between 1:00 and 4:00 PM with a peak at 2:00 PM and they feed on dew and water drops and soon starts mating and begins the cycle all over again (figure 1.12) (Talekar and Shelton, 1993). The general colour of the moth is brownish grey; the female is

usually paler than the male. The inner borders of the forewings are whitish; when united on the dorsal area, the wings form the typical diamond shaped markings. The sexes are identified by the anal abdominal segment; in males it is divided longitudinally in the ventral area, whereas in females it is not divided (figure 1.13) (Carlos R and Carlos C, 1986).

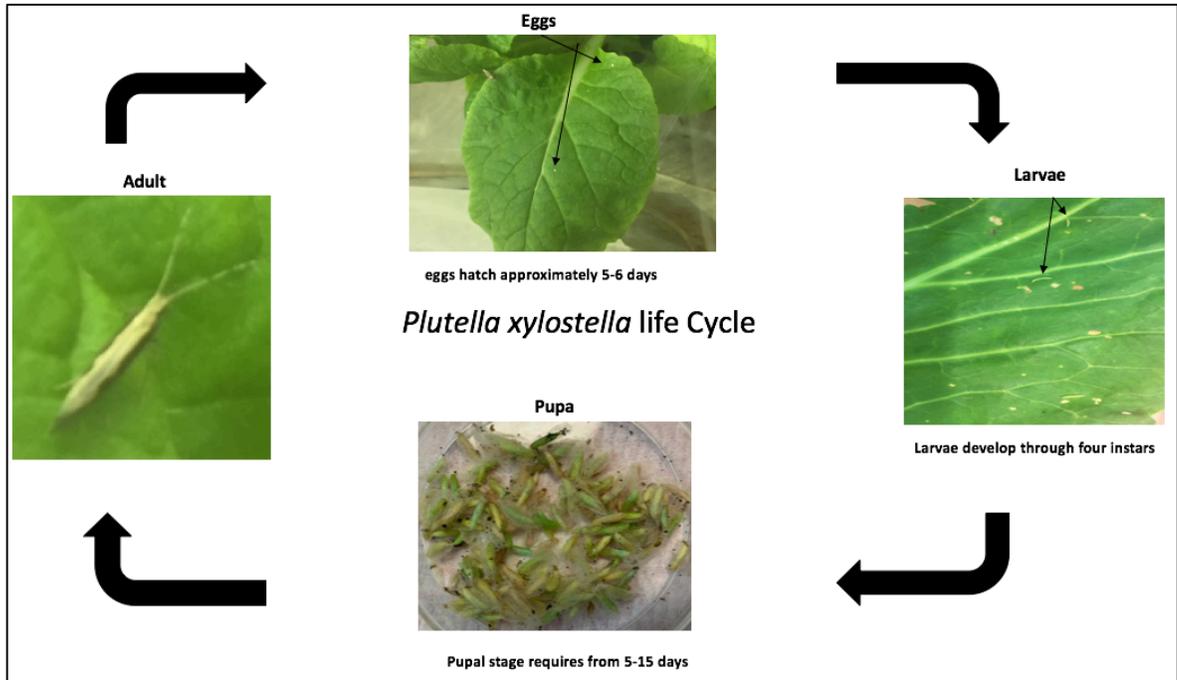


Figure 1.12. Represent the life cycle of diamondback moth showing the four stages for *Plutella xylostella*, adult, eggs, larvae and pupa. Each picture was taken from the lab.

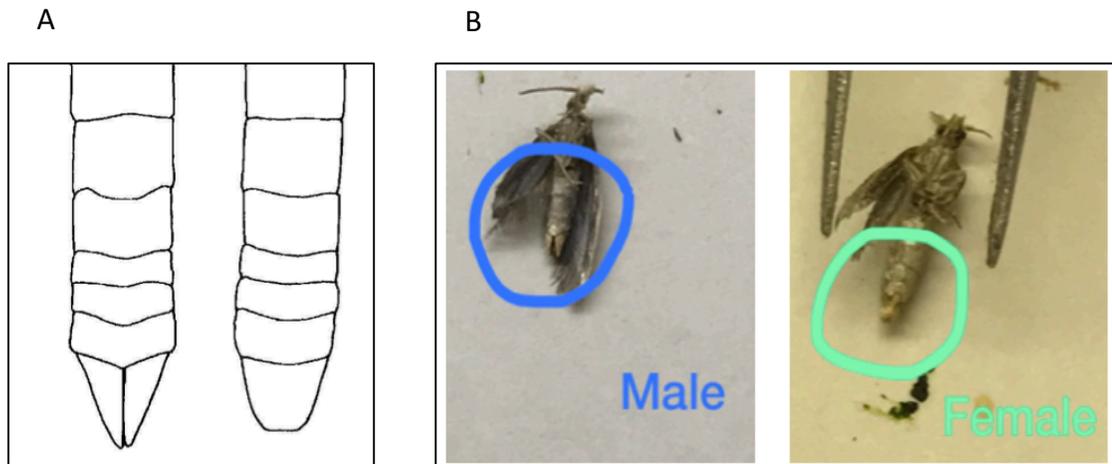


Figure 1.13. (A) Adult ventral abdominal segment for Male and Female of diamondback moth (Carlos R and Carlos C et al.1986). (B) Picture taken from the lab showing the difference between male and female.

1.5.2 Control of *Plutella xylostella*

The control of *Plutella xylostella* is more necessary now than ever before considering the fact that this insect pest has affected crops, such as cabbage, cauliflower, broccoli, brussel sprouts etc. In more than 100 countries across the global (Gautam *et al.*, 2018). This has therefore resulted in high economic loss in many countries that depend on agriculture as a major source of income generation. Farmers have, for the past 30 years, relied on the use of synthetic (chemical) insecticides for the control of the *P. xylostella*. but the ease of resistance to these chemicals, which introduction led to the stoppage of the control methods that were in use previously, has stimulated interest for the search for a newer, more effective, and reliable methods for the control of *P. xylostella* (Talekar and Shelton, 1993). Therefore, here we would review some of the old methods that have been used in the past for the control of *P. xylostella* populations and the current practices that are in use at the moment. These, for the

purpose of clarity, we classified into two namely; cultural and the scientific methods for *P. xylostella* population control.

1.5.2.1 Cultural control methods

Some of the cultural practices that have been used in the past and some of which are being reintroduced today for the control of *P. xylostella* populations are as follows:

1. Pre-season clean-up

This method involves removal of all plant debris and weeds from the greenhouse. This is because many pests, including *P. xylostella* are found on other crops or broadleaf weeds. Therefore, it is very important to avoid growing other crops next to the greenhouse and also prevent the growth of broadleaf weeds near them (Gautam *et al.*, 2018).

2. Balanced use of fertilizers

The balanced use of fertilizers has been found to play important role in the control of insect pest growth for instance, fertilizer applications method that favor the use of nitrogen surplus has been shown to cause excessive growth of aphids and other pests whereas, the application of potassium at desired levels has been shown to reduce the growth of insect pests (Gautam *et al.*, 2018).

3. Pinching and pruning

This practice involves the pinching-off, of damage plant parts, flower and spotted leaves probably containing insect larvae or egg deposits in the greenhouse. These plant debris are placed immediately in a closed container before being disposed-off.

This practice is effective in reducing the pest populations of all targeted insects including *P. xylostella* (Gautam *et al.*, 2018).

4. The use of trap crops

This is a method that has found common applications before the advantage of modern organic insecticides (Talekar and Shelton, 1993). The practice involves the use of economically less important plant that are highly desired by *P. xylostella* within a commercial cabbage field. The desired crop, e.g. mustard (*Brassica hirta*) or rape (*B. juncea*) attract the *P. xylostella* thus sparing the commercially important crops such as cabbage, brussel sprouts etc. from being attacked by the pest with the advent of insecticidal resistance, this has found, application in developing countries (Gautam *et al.*, 2018, Talekar and Shelton, 1993).

1.5.2.2 Scientific control methods

The scientific control methods mainly consist of the use of both chemical and biological insecticides in the field, and the use of biological control in reducing the populations of *P. xylostella* in the field.

1. Chemical insecticides

This method has been the most prevalent in use for the control of *P. xylostella* in the past 50 years (Gautam *et al.*, 2018). Therefore, the over reliance on this method for insect control led to a lot of abuse in its usage, whereas application rates, decreased effectiveness and eventual breakdown of control efficiency. Farmers increased the dosage to over 25 times its required dose, which led to decrease effectiveness and cases of insect resistance (Kalra *et al.*, 1997). Some of

the chemical insecticides that have been used in the past for the control of *P. xylostella* are organochlorines, organophosphate, carbamates, synthetic pyrethroids (Gautam *et al.*, 2018).

2. Biological insecticides

The biological insecticide, which is derived from Bt, has found application in the control of *P. xylostella*, mostly, because of their environmentally friendlier nature, high level of specificity and lack of toxicity to other useful organisms in the environment. This method still finds application in the control of *P. xylostella* as many new combinations and genetic engineering approaches are being used to develop insecticides from Bt, despite many cases of resistance of *P. xylostella* to Bti products being reported (Sarfraz and Keddie, 2004).

3. Biological control

The biological control method employed the fact that at all stages of development of the *P. xylostella* from egg to adult. It is being attacked by numerous parasitoids and predators with parasitoids being the most widely studied. Therefore, the biological control strategy introduced the growth of a population of predators that do not feed on cabbage but rather *P. xylostella* such as birds and spiders to control the population of *P. xylostella* in the field. In addition, parasitoids which feed in the eggs such as *Trichogrammatodea*, larva belong to *Diadegma* and *Cotesia* genera are used to halt the growth of *P. xylostella* hence reducing their populations in the field (Talekar and Shelton, 1993). In addition, mating disruption methods which may be classified under biological control is utilized in the control of *P. xylostella* populations. The female diamondback moth secretes

sex pheromone which consist of three components, (Z-11-16: OAC), (Z-11-16: OH) and (Z-11-16: Ald), that attracts male diamondback moth. Therefore, this pheromone is synthetically produced and sprayed in the field to disrupt the males from mating with the female as a population control strategy (Ando *et al.*, 1979, Chisholm *et al.*, 1979, Chow *et al.*, 1977 and Talekar and Shelton, 1993).

1.6 Mechanism of resistance of Cry toxin in *Plutella xylostella*

Resistance to Cry toxins and other insecticides by *Plutella xylostella* mainly occur due to their intensive use over time (Sarfraz and Keddie, 2004). Intensive use of synthetic insecticides in the control of *Plutella xylostella* led to the elimination of natural enemies and have more reliance on the insecticides and subsequent development of resistance. Also, the fact that *Plutella xylostella* are migratory in nature and hence they can move into a new region where the control agent is not available, hence farmers then have to rely on insecticides leading to more resistance. Farmers may also attempt to grow crops with the use of conventional insecticides due to market pressure resulting from the need of pest free crops hence leading to more reliance on insecticides and hence resistance (Sayed *et al.*,2002, Chapman *et al.*,2002). More also, the fact there are several steps involved in processing the crystal toxin into an activated toxin may increase the possibility of insect populations to develop various means of resistance to these toxins (Schnepf *et al.*,1998).

Genetic diversity plays significant role in the development of resistance to Bt, this is because insect population maintained in the laboratory have a considerably low level of genetic

diversity compare to the field population, probably due to exclusion of many factors such as dilution of resistance due to mating with susceptible populations, absence of fitness costs as a result of environmental factors and absence of natural enemies such as prey and predators found in the field (Schnepf *et al.*, 1998). *Plutella xylostella* was the first insect known to develop resistance to Bt in the field (Schnepf *et al.*, 1998). Resistance, which is directly proportional to the amount of formulated Bt product (Dipel) used in this treatment can develop in theory as a result of blockage of any of the various steps involved in processing the toxin (figure 1.14) as previously mentioned by different mechanisms highlighted below.

Altered activation of Cry toxins by midgut proteases has been reported to result in a lack of activation of the toxin and hence no toxicity against the insect (Li *et al.*, 2004, Oppert *et al.*, 1997). Sequestration of the toxin, which could be either glycolipid moieties (Ma *et al.*, 2011) or esterases (Gunning *et al.*, 2005), by the induction of elevated immune response, which all result in reduced binding of the toxin to insects' gut membranes resulting in lack of toxicity (Schnepf *et al.*, 1998). The binding of Cry toxins to BBMV has been associated with mutation in toxin receptors (Ferre and Van Rie, 2002, Heckel *et al.*, 2007) such as ABCC2. It was proposed that Cry1Ab and Cry1Ac could bind to the ABCC2 protein in its open state where some hydrophobic surfaces of the channel are exposed to the outside and at the same hypothesised that this binding interaction could facilitate membrane insertion of the toxin oligomer (Gahan *et al.*, 2010). Therefore, it is of note to state that among all mechanism of toxin resistance outlined above, the most common resistance mechanism in lepidoptera is the reduction in toxin binding to midgut cells, which may result from mutation in different toxin receptors such as CAD (cadherins), ALP (alkaline phosphatases) or APN (aminopeptidases)

and ABCC2 transporter depending on the specific insect species (Gahan *et al.*, 2001, Jurat-Fuentes *et al.*, 2004).

other factors that could result in lack of activity of the toxin apart from the resistance mechanisms outlined above, which have to do with the bioassay procedures and hence affect the rate of toxins interaction or uptake by insect resulting in low activity are intraspecies variation in toxin susceptibility which seemed to occur between test colonies obtained from different parts of the world, a variation of 1-2 orders of magnitude, even among insects from the same geographical region or colony may vary by 1 order of magnitude between cohorts or successive generations (Frankenhuyzen, 2009). The larval age may also influence the susceptibility of Cry toxins to the insect population in question as it is generally discovered that susceptibility decreases with an increase in the larval age (McNeil and Dean, 2011). Reduced amount of toxin on the leaf during leaf- dip bioassay as a result of evaporation of the toxin or its drying out due to atmospheric oxygen leading to reduce or no toxin to be ingested by the insect, which may be mistaken for insect resistance.

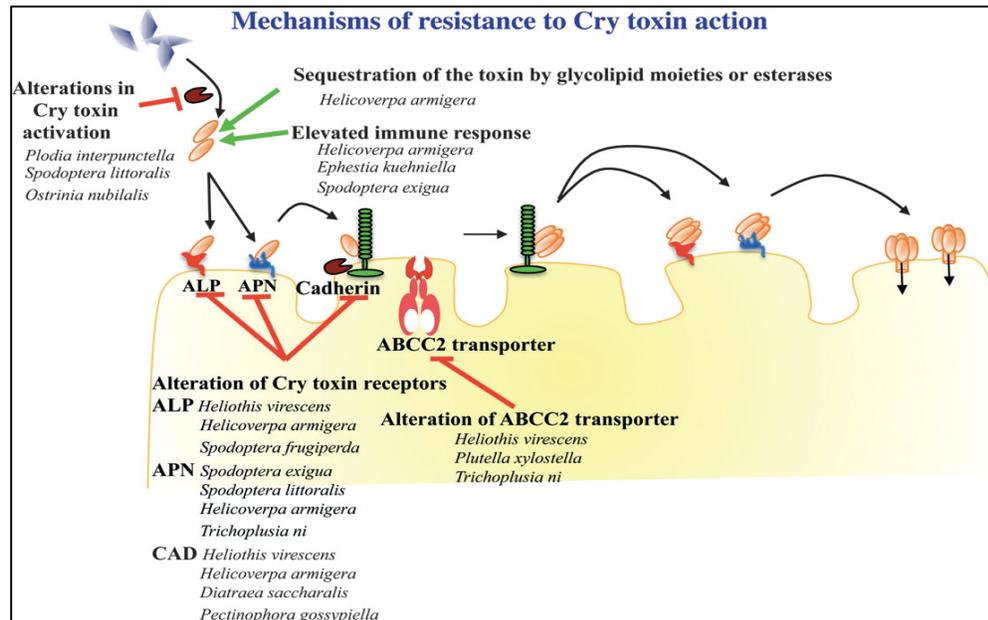


Figure 1.14. Schematic representation of different mechanisms of resistance to Cry toxin described in lepidopteran insects (Pardo-Lopez *et al.*, 2013).

1.7 Determining the specificity of Cry toxins:

Amongst the methods used for the qualitative assessment of Cry toxins is specificity determination. This is because the knowledge of the specificity of a toxin against particular insects can help us understand which regions of a toxin are important for individual target insects. Therefore, the specificity of crystal proteins is defined as the range of species or taxa that it affects whereas the range of toxins that affects the species is known as corollary. This information was compiled by van Frankenhuyzen (2009) to review the specificity of various toxins against different orders of insects utilizing published results from the literature and considering the following criteria: (i) the method of toxin preparation was not considered to have altered the results from the published data. (ii) Crystals toxins were considered active when they evoked a response at any concentration and inactive when they failed to response

at the highest concentration. (iii) Toxins are classified as active in case of contrasting results. Lastly, the review is based on the current knowledge of the crystals proteins at the time and calcified across orders and within as follows.

1.7.1 Determination the specificity of Cry toxin across orders

Order specificity was examined across toxin families at the secondary rank and summarized in (figure 1.15). It appears that, the number of crystal proteins showing cross-order activity has increased considerably since the publication of the first classification by Höfte and Whiteley (1989). However, they distinguished four main pathotypes based on order specificity: Lepidoptera specific (CryI, now Cry1), Coleoptera-specific (CryIII, now Cry3), Diptera-specific (CryIV, now Cry4, Cry10, Cry11; and CytA, now Cyt1A), and CryII (now Cry2), the only family known at that time to have dual (Lepidoptera and Diptera) specificity. Cross-order activity is now apparent in 15 of the 87 pesticidal crystal protein families (not counting the 5 cancer-cell active parasporin families) as depicted in (figure 1.15).

		Coleoptera	Collembola	Diptera	Blattaria	Hemiptera	Hymenoptera	Isoptera	Lepidoptera	Neuroptera	Orthoptera	Siphonoptera	Thysanoptera	Echinostomida	Rhabditida	Cancer cells			Coleoptera	Collembola	Diptera	Blattaria	Hemiptera	Hymenoptera	Isoptera	Lepidoptera	Neuroptera	Orthoptera	Siphonoptera	Thysanoptera	Echinostomida	Rhabditida	Cancer cells		
Cry 1A																		Cry 19B																	
Cry 1B																		Cry 20A																	
Cry 1C																		Cry 21A																	
Cry 1D																		Cry 22A																	
Cry 1E																		Cry 22B																	
Cry 1F																		Cry 23A																	
Cry 1G																		Cry 24B																	
Cry 1H																		Cry 24C																	
Cry 1I																		Cry 27A																	
Cry 1J																		Cry 29A																	
Cry 1K																		Cry 30A																	
Cry 2A																		Cry 30B																	
Cry 3A																		Cry 30C																	
Cry 3B																		Cry 31A																	
Cry 3C																		Cry 32A																	
Cry 4A																		Cry 32B																	
Cry 4B																		Cry 32C																	
Cry 5A																		Cry 32D																	
Cry 5B																		Cry 33A																	
Cry 6A																		Cry 34A																	
Cry 6B																		Cry 34B																	
Cry 7A																		Cry 35A																	
Cry 7B																		Cry 35B																	
Cry 8A																		Cry 36A																	
Cry 8B																		Cry 37A																	
Cry 8C																		Cry 38A																	
Cry 8D																		Cry 39A																	
Cry 8E																		Cry 40A																	
Cry 8F																		Cry 40B																	
Cry 8G																		Cry 41A																	
Cry 9A																		Cry 42A																	
Cry 9B																		Cry 43A																	
Cry 9C																		Cry 43B																	
Cry 9D																		Cry 44A																	
Cry 9E																		Cry 45A																	
Cry 10A																		Cry 46A																	
Cry 11A																		Cry 47A																	
Cry 11B																		Cry 48A																	
Cry 12A																		Cry 49A																	
Cry 13A																		Cry 51A																	
Cry 14A																		Cry 55A																	
Cry 15A																		Cyt 1A																	
Cry 16A																		Cyt 1B																	
Cry 17A																		Cyt 2A																	
Cry 18A																		Cyt 2B																	
Cry 19A																		Cyt 2C																	

Figure 1.15. Specificity of Cry and Cyt toxin families (secondary rank) across orders. Toxin families are indicated as being active with black fill, not active with white fill, possibly active grey fill, or not tested. Toxin families for which no bioassay data are available are not shown (Cry1L, 7C, 8H, 18B, 18C, 21B, 24A, 25A, 26A, 28A, 30D, 42A, 50A, 52A, 53A, 54A, Cyt1C), (van Frankenhuyzen, 2009).

1.7. 2 Determination the specificity of Cry toxin within orders:

Van Frankenhuyzen (2009) assigned the specificity within orders for three main species of insects, lepidoptera, diptera, and coleoptera, also other insect orders were grouped together

in this project we focused on the Cry2A toxins so for that reason we show orders that Cry2A toxins appeared to be active to.

1. Lepidoptera

In 1182 bioassays, about 59 holotype toxins have been tested against 71 species. These species susceptibility spectra are depicted in (figure 1.16) by rows. However, the toxins, which show specificity toward this order of insects from group of Cry2A, were (Cry2Aa, Cry2Ab, Cry2Ac, Cry2Ae and Cry2Af). *P. xylostella* was the most commonly tested species (9.8% of complete bioassays), followed by *Spodoptera exigua* (8.7%), *Heliothis virescens* (6.4%), *Manduca sexta* (5.9%), *Trichoplusia ni* (5.3%), *Ostrinia nubilalis* (5.1%), *Helicoverpa armigera* (4.8%), *Heliothis zea* (4.0%) and *Bombyx mori* (3.9%) as shown in (Figure 1.16).

P. xylostella, and in the case of Cry2Ac and Cry2Ae were stated in this review to be non-active against *P. xylostella*, Cry2Af was tested against one insect species *Helicoverpa armigera*. Activity spectra of the 59 tested holotype toxins are represented by columns in (figure 1.17). Toxins in the Cry1, Cry2 and Cry9 families accounted for 96.2% of all bioassays, with Cry1 and in particular Cry1A toxins accounting for, respectively, 80% and 36.6%. The broadest range of toxins was tested against *P. xylostella* (43 toxin types), which was one of only 12 species that were tested against 15 toxins or more as indicated in (figure 1.17) below.

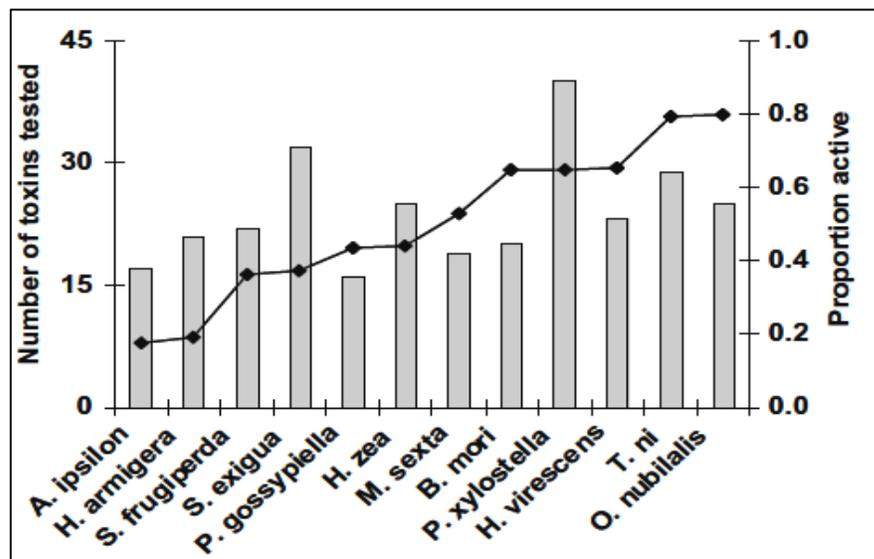


Figure 1.17. Permissiveness of the most frequently tested species as indicated by the proportion of toxin types that displayed toxicity (Van Frankenhuyzen, 2009).

The Cry1Ac protein was tested against the biggest amount of species (56) and tested against 15 or more species only 14 toxins figure 1.18 below. However, among the Cry2A group it appears that Cry2Aa is more active than Cry2Ab, as shown in the (figure 1.18) below.

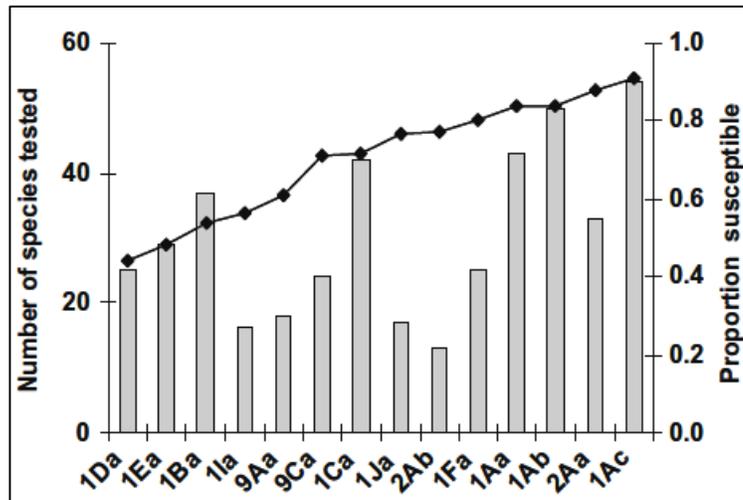


Figure 1.18. Activity range of holotype toxins that were tested against at least 15 species as indicated by the proportion of species that were susceptible (Van Frankenhuyzen, 2009).

1.7.3 Determination of domain/domains responsible for the specificity of Cry2A toxins:

The current focus of Cry2A toxin research is towards the identification of the region that confers specificity to Dipteran and Lepidopteran insects. Cry2Aa is largely considered to have dual specificity for the orders Lepidoptera and Diptera. The direct comparison between the amino acid alignments of Cry2Aa and Cry2Ab can be used in understanding why the toxicity spectrum of Cry2Ab and Cry2Aa differ. Experiments involving homologue-scanning mutagenesis helped in ascertaining the responsible amino acids for conferring the specificity differences between Lepidopteran and Dipteran orders (Hofte and Whiteley, 1989, Liang and Dean, 1994).

Hofte and Whiteley (1989) created hybrids through homologue scanning mutagenesis and domain swaps, and substitution of Cry2Ab residues into Cry2Aa as depicted in (figure 1.19).

Hybrids with increasing amounts of Cry2Ab at the N terminus were created and the chimeric proteins tested against *Manduca sexta* and *Aedes aegypti* to measure the established toxicity change compared to the usual results of Cry2Aa. Additionally, hybrids with increasing amounts of Cry2Aa at the N-terminus were created. Markedly, there was the generation of 72 hybrid Cry2Aa/Cry2Ab proteins in total. Based on the bioassay data for the hybrids 15, 14,7, and 6, it was postulated that hybrids 13 and 5 boundaries bordered minimum required Cry2Aa amino acids number that is necessary to confer toxicity to *Aedes aegypti*. However, it was considerably lower than the usual levels of toxicity thereby suggesting that other amino acids may contribute significantly to the Dipteran specificity.

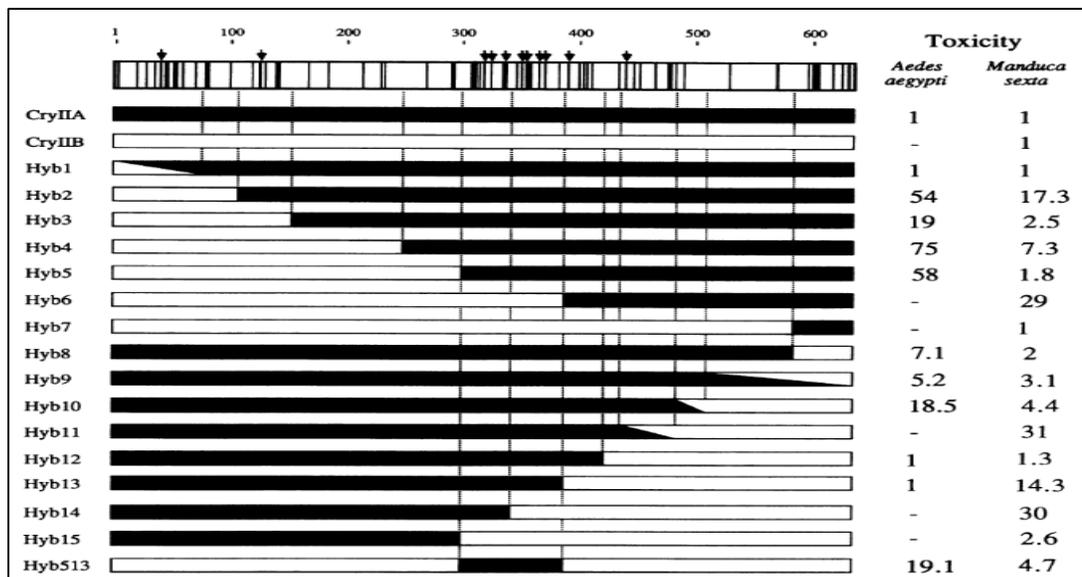


Figure 1.19. Cry2Aa (shaded bar), Cry2Ab (non-shaded bar), and hybrid gene products (combination of the two patterns) and their toxicities to *A. aegypti* and *M. sexta*. All of the toxicities are relative to that of Cry2Aa (value of 1); a fivefold difference in toxicity is considered significant. The bar at the top of the figure is a diagram depicting a FASTP alignment of the Cry2Aa and Cry2Ab polypeptides; vertical lines represent differences between the two, and arrows above the bar denote the locations of non-conservative changes. Vertical broken lines show locations of the hybrid junctions determined by restriction mapping (hybrids 1, 9, 10, and 11) and DNA sequence analysis (hybrids 2 to 4, 6, 8, 12 to 14, and 513). The dotted lines extend upwards to the alignment diagram to show where the junctions are located with regard to the amino acid differences that exist between the two polypeptides (Widner and Whiteley, 1990).

Another study done by Liang and Dean (1994), who investigated the region responsible for specificity for Cry2A against mosquito larvae and gypsy moth larvae by the creation of hybrids between Cry2Aa and Cry2Ab. Cry2Aa possesses insecticidal activity against lepidopteran and dipteran insects while Cry2Ab is active against lepidopteran and non-active against dipteran insect.

In an attempt to find the specificity region of Cry2A against lepidopteran and dipteran insects, the putative domain II of Cry2Aa was substituted by domain II of Cry2Ab. They used the sequence alignment for the toxic domains of Cry proteins done by Hodgman and Ellar (1990). Domain II of Cry2Aa was aligned between amino acid 278 and amino acid 487, encoded by the naturally existing *NheI*-*NarI* fragment of the Cry2Aa gene. The DNA fragment-encoding domain II of Cry2Ab (also aligned between amino acids 278 and 487) was amplified and they introduced the *NheI* and *NarI* sites into the fragment ends by PCR. Using this technique, they generated a recombinant gene DL105 as depicted in (figure 1.20).

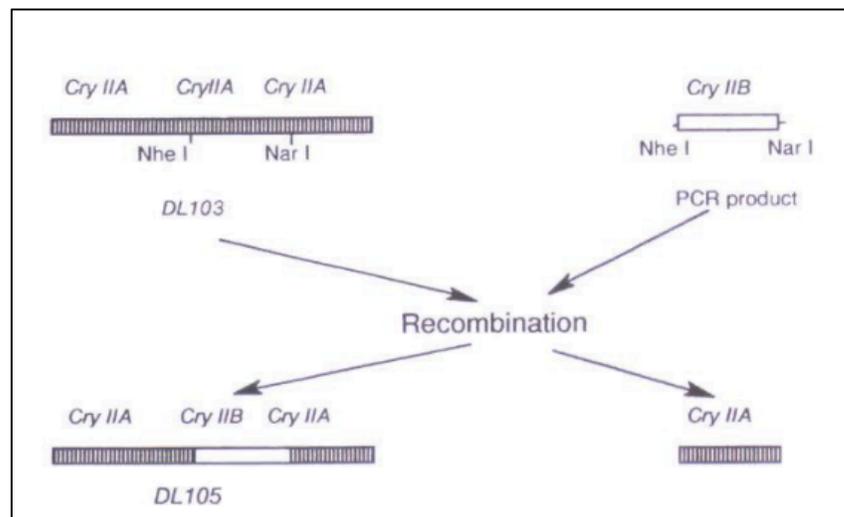


Figure 1.20. Replacement of domain II of Cry2A with domain II of Cry2Ab. The filled bar indicates DNA from Cry2Aa, the open bar from Cry2Ab. Gene DL103 is a wild-type Cry2Aa gene. Gene DL105 is a recombinant gene which had domain I and domain III of Cry2Aa origin and domain II of Cry2Ab origin (Taken from Liang and Dean, 1994).

Two restriction sites were introduced, which is *MluI* and *XhoI* into both Cry2Aa wild type (DL103) and the hybrid created (DL105) which divide the domain II of both genes into three regions as shown in (figure 1.22).

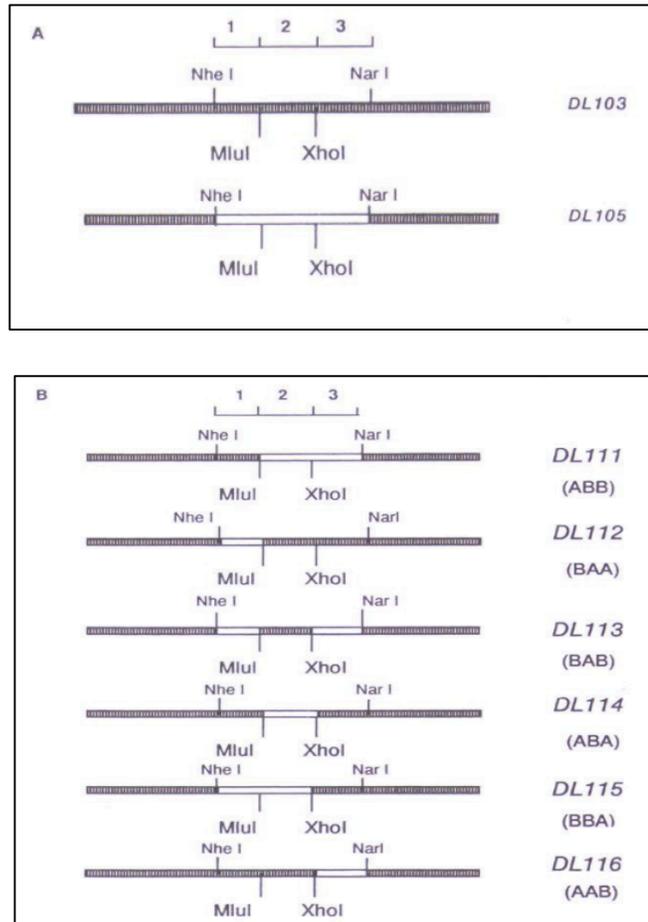


Figure 1.22A. *MluI* and *XhoI* sites introduced into DL103 and DL105. *MluI* and *XhoI* almost equally divide domain M into three regions, which have been named (from N-terminal to C-terminal) regions 1, 2, and 3, *NheI* and *NarI* are naturally occurring sites bordering domain II of Cry2Aa. **B.** Homologue-scanning mutagenesis of domain II of Cry2Aa. Letters in parentheses indicate the origin of each region in domain II of each mutant. A. Cry2Aa origin; B, Cry2Aaborigin (Taken from Liang and Dean, 1994).

These are named region 1, region 2 and region 3 and comprised of amino acids 278-340, 341-412, and 413-487, respectively. They made six chimeric proteins in total (table 1.23) by changing one or two regions of domain II of Cry2Aa with that of Cry2Ab. The following shows the six multiple hybrids generated from chimeric-scanning mutagenesis

involving substitution between the three fragments created between the domain II of Cry2Aa and that of Cry2Ab, and their toxicities against both *Aedes aegypti* and *Lymantria dispar*.

Protein	Domain II Origin	LC₅₀ of <i>Aedes aegypti</i> (95% confidence interval) (ng/ml)	ID50 of <i>L. dispar</i> (95% confidence interval) (ng)
DL103	AAA	65.5 (41.1-100)	102 (77-100)
DL105	BBB	>10 ⁵	305 (226-418)
DL111	ABB	ND	ND
DL112	BAA	1.23x10 ⁵ (2.82x10 ⁴ -8.33x10 ⁵)	126 (85.7-187)
DL113	BAB	1.50x10 ⁵ (1.05x10 ⁵ -1.02x10 ⁶)	88.7 (58-129)
DL114	ABA	ND	ND
DL115	BBA	>10 ⁵	3200 (1340-51900)
DL116	AAB	52.2 (25.7-107)	90.6 (57.7-136)

Table 1.1. Showing mutagenesis done to Cry2 toxins, measuring the lethal concentration and infection dose to *A. aegypti* and *L. dispar* respectively. Letters indicate the origin of each of the three regions- A= Amino acid section taken from Cry2Aa, B= Amino acid section taken from Cry2Ab. Protein marked Cry2Ab is made wholly of Cry2Ab residues, protein marked DL105 is a Cry2Aa with the three regions of domain II substituted by Cry2Ab residues (Taken from Liang and Dean, 1994).

The bioassay was conducted and the data in table 1.23 indicates that the wild type Cry2Aa was toxic against *Aedes aegypti* larvae, and show some activity toward *Lymantria dispar*, but Cry2Ab was less active about three times than Cry2Aa against *Lymantria dispar* larvae. The research done by Dankocsik *et al.* (1990) who used the mixes of spores and crystal, they gain a similar ratio of toxicities of these two wild-type toxins. When domain II of Cry2Aa was substituted by domain II of Cry2Ab, the hybrid protein (Hybrid105) was three times less toxic to *Lymantria dispar* than wild-type Cry2Aa and showed a similar toxicity to wild-type Cry2Ab. As with wild-type Cry2Ab, it did not show any mosquitocidal activity even at higher concentration of 100 100µg/ml. These results indicated that the specificity regions of Cry2Aa against *L. dispar* larvae and *A. aegypti* larvae are both located in domain II. All mutants they created by switching regions inside domain II displayed different behavior. Though DL116 was as active as wild-type Cry2Aa against the larva of both insects, DL115 has lost its toxicity to both insects. DL112 and DL113 showed similar toxicity to both insects. While they remained as toxic to *L. dispar* larvae as wild-type Cry2Aa, their toxicities against *A. aegypti* larvae were both reduced by about 2000-fold. Therefore, the result from this experiment showed that the specificity region of Cry2A for *A. aegypti* larvae was located within both region 1 and 2 within these amino acids (307-382), this result supported by Widner and Whiteley (1990) who discovered the specificity region of Cry2A against *A. aegypti* within these amino acids (304- 382). However, their data shows switching region 1 from Cry2Aa to Cry2Ab did not affect protein stability, crystal-forming ability, or *L. dispar* toxicity (DL112 and DL113) but did affect the *A. aegypti* toxicity indicates that the difference in region 1 (amino acids 278-340) between Cry2Aa and Cry2Ab is related only to mosquitocidal activity. However, region 2 of Cry2Aa was also needed in order to produce an

active toxin against the mosquito. Also, they found the specificity region against gypsy moth larvae located in region 2 of domain II of Cry2 (Liang and Dean, 1994).

Recent work carried out by Shu *et al.*, 2016 to study the relationship between sequence and function involved the creation of chimeric genes between Cry2Aa and Cry2Ad through the creation of single crossover recombinants by the technique of template change PCR reaction (TC-PCR). The hybrid created involve changing domain I of Cry2Ad with Cry2Aa, changing domain III of Cry2Ad with Cry2Aa and making some few amino acid changes within domain II in each case and testing them against four insects *O. furnacalis*, *C. suppressalis*, *H. armigera* and *P. xylostella*. they found that hybrids involving change in domain I and III of Cry2Ad and Cry2Aa respectively do not result in any change in activity relative to the wild type whereas, specific amino acid change in domain II give rise to differing activity each specific to some particular insects. For instance, changing amino residues 410 and 411 is specific to *O. furnacalis*, and changing three amino acids 430,433 and 439 is specific for *C. suppressalis* and *H. armigera*, finally changing at position 443,446 and 447 is specific for *P. xylostella*. This confirmed that involvement of domain II in various insects' specificities as speculated by previous researches (Shu *et al.*, 2016).

Another research was done by Shu *et al.*, 2017 to identify and characterize *cry2* gene in a collection of 300 Bt strains led to the discovery of a novel cry toxin known as Cry2Aa17, whose domain I resembled that of Cry2Ab, domain II and III resembled Cry2Aa (figure 1.22). The toxicity profile of this novel toxin was determined through bioassay against *Aedes aegypti* and *Spodeptera exigua* respectively. It was found that the toxicity profile against these two insects resembled those of Cry2Ab thus implicating domain I in specificity determination. This led to hybrid creation between Cry2Aa and Cry2Ab and testing them

against *Aedes aegypti* mosquito, which then confirmed the involvement of domain I in toxicity determination (Shu *et al.*, 2017).

More, recent work carried out by (Joseph unpublished) on *Aedes aegypti* found that the toxicity of Cry2A toxins was associated with the N-terminal 49 amino acids. He went further to screen the N-terminal sequence in order to locate the specific residues involved in this activity through bioinformatics and mutagenesis. It was then discovered that only four amino acids (E, R, T, D) located at positions 27, 43, 44, and 45 respectively were responsible for the activity of Cry2A toxins against *Aedes aegypti*, as he showed that changing the amino acids QKNN of Cry2Ab, which is known to possess no activity against *Aedes aegypti*, found in these locations to ERTD converts Cry2Ab in to an active toxin against this insect (Joseph unpublished).

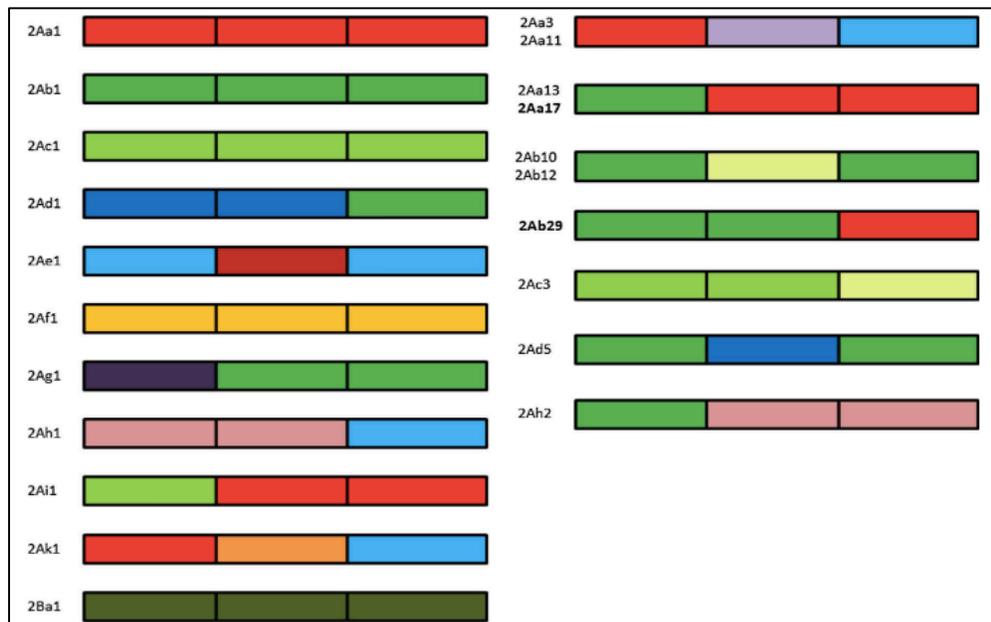


Figure 1.23: Domain configurations of the Cry2A toxins. Each toxin is split into the three domains identified from crystallographic studies of Cry2Aa (Morse *et al.*, 2001). Figure taken from (Shu *et al.*, 2017).

1.8 Aims and Objectives

The objective of this research is to understand the nature of the specificity in *Bacillus thuringiensis* toxins against *Plutella xylostella* which belongs to the order Lepidoptera and also to attempt to convert an insecticidal toxin into an anticancer toxin and vice versa. To do this, literature reviews, data collection, growing of *Plutella* culture, growing of bacterial culture, DNA and Protein sequences analysis using bioinformatics tools such as Neb Cutter, Gen Bank, Expasy, Clustal Omega etc. and theory applications will be carried out.

- To investigate the toxicity of Cry2A toxins against *Plutella xylostella* by carrying out a comprehensive set of bioassays to obtain reliable toxicity data on the larvicidal activities of Cry2A family against *Plutella xylostella*.
- Use the bioassay results to create hybrids to allow us to better understanding the basis of specificity against *Plutella xylostella* and identify associations between toxin sequence and activity.
- To create an improved expression system (using Bt) in order to produce larger amounts of purer toxin.
- Attempt to understand any population-dependent differences in susceptibility through toxin activation and binding assays.
- Attempt to convert an insecticidal toxin into a cancer toxin and vice versa.

2. Materials and methods

2.1 The insect population

- **Gen-88 population**

Gen-88 is a population susceptible to Cry1Ac, and adapted to artificial diet. Gen-88 was obtained from Dr. Ben Raymond, centre for ecology and hydrology (Oxford, UK) and maintained in the lab on a sterile artificial Hoffman's diet at 25°C under 16h photophase.

- **SBT population**

The SBT population is a susceptible population, named after the place that it was collected. The name of the farm is "Sitio Boa Terra" Jaboticabal, Sao Paulo, Brazil. The collection date was 5th of July 2010. It was collected from cabbage plants in an area with no history of insecticide application. For insect rearing, the methodology used was as described by Barros and Vendramim (1999).

- **NO-QA population**

This insect population is originally from Hawaii collected from a watercress farm in Pearl City on the Island of Oahu, in the year 1989. It is a highly resistant population having been selected with Cry1Ac many times.

2.2 Bacterial strains and plasmids

Bacteria species and reagents for this study were provided by the Crickmore Laboratory of the School of Life Sciences, at the University of Sussex.

2.2.1 Bacterial strains

1) *A-Bacillus thuringiensis*

NO	Strain	Toxin
1	pGEM	Cry2Aa
2	pEB	Cry2Aa9
3	pEB	Cry2Aa17
4	pEB	Cry2mAa17
5	pEB	Cry2Ab4
6	pEB	Cry2Aab29
7	pGEM	Cry2Ab-916
8	pGEM	Cry2Ab
9	pGEM	Cry2Ac
11	pGEM	Cry2Ad
12	pEB	Cry2Ah
13	pEB	Cry2Ax

1) *-Bacillus thuringiensis*

NO	Strain	Application
1	IPS78/11	A crystal minus strain of <i>Bacillus thuringiensis</i> subsp <i>israelensis</i> used as a host for expression

2)-*Escherichia coli*

NO	Strain	Application
1	DH5 α	host for transformation
2	BL21	host for expression

2.2.2. Plasmids

- pGEM T-easy plasmid

This plasmid was used for cloning and expression and it was obtained from (Promega.USA).

- pSV27B40B plasmid

This plasmid is a *Bt*- shuttle vector was modified from vector pSV2, which was derived from pBR322 vector and used for expression in *Bt* (Crickmore and Ellar 1992, Crickmore *et al.*, 1994).

- pEP plasmid

The pEB cloning/expression vector using *Bam*HI and *Sal*I (Shu *et al.*, 2016).

- pBL plasmid

It is the phagemid pBLUESCRIPT (Strata gene) it consists of an f1 phage origin of DNA replication (Sikorski and Hieter, 1989).

2.3 Buffers/Solution

- 10 x TBE buffer: 108g of Tris, 55g of Boric Acid, 40 ml of EDTA and 2 l of distilled water.
- 10 x SDS Running buffer: 7.6g Tris, 36g Glycine, 2.5g SDS and distilled water to 250ml.
- Resolving gel buffer (RGB): 18.18g Tris, 0.4g SDS and distilled water to 100ml, pH 8.8.

- Stacking gel buffer (SGB): 6.06g Tris, 0.04g SDS, distil water to 100ml, pH 6.8
- 2 x protein gel sample loading buffer: 2g SDS, 6mg EDTA, 20mg BPB, 5ml RGB, 50 ml Glycerol and distilled water to 100ml.
- DNA gel sample loading buffer: 0.05% bromophenol blue, 40% sucrose, 0.1M EDTA pH 8.0 and 0.5% SDS.
- Coomassie Brilliant Blue stain: methanol, distilled water, acetic acid (10:9:1 V/V/V) and Coomassie Brilliant Blue G 250 (0.25% W/V).
- De- staining: methanol, distilled water and acetic acid (10:9:1 V/V/V).
- PBS: 80 g of NaCl, 2 g of KCl, 14.4 g of Na₂HPO₄, 2.4 g of KH₂PO₄, 1 l of dH₂O, pH 7.4
- M.E.T buffer (250mM mannitol, 5mM EGTA, 17mM Tris-HCL) pH 7.5
- Dry blotting buffer: 39mM Glycine, 48mM Tris, 0.037% SDS, 20% Methanol.
- PBS/Tween: 50ml 10XPBS, 450ml water, 100 µl Tween20.
- ECL: 10 ml of 100 mM Tris pH 8.5, 3 µl of H₂O₂, 25 µl of 14.7 mg/ml p-coumaric acid, 50 µl of 88.6 mg/ml luminol.
- Binding buffer: PBS with 0.1% Tween 20 and 0.1% BSA, pH 7.4.

2.4 Reagent and Enzymes

The following reagents were purchased from Thermo Fisher Scientific methanol, 1-butanol, acetic acid, sucrose, magnesium chloride and glycine. SDS, acrylamide/bis-acrylamid 30%, TEMED, Tris base, Tris-HCL, Brilliant Blue G, Bromophenol Blue, B-mercaptoethanol, BSA fraction V, chloramphenicol, mannitol, EGTA, p-coumaric acid,

luminol, hydrogen peroxide, chymotrypsin and lysozyme were obtained from Sigma. Enzymes were purchased from New England Biolabs *DpnI*, *HindIII*, *SacI*, *BsaI*, *XbaI*, also Blue Protein standard, DNA ladder 1Kb and T4 ligase. Hae III was purchased from Promega. HCl, NaOH, ethanol, Ponceaus S and Coomassie Blue stain were obtained from AnalaR BDH. Agarose and LB broth buffered capsules were from Melford Biolaboratories. Triton X-100 was from BDH chemical Ltd (Poole England). Gel Red nucleic acid was from Biotium.

2.5 Antibodies

- Anti-rabbit IgG (whole molecule)-peroxidase antibody produced in goat. Affinity isolated antibody, SIGMA (A0545).
- Primary antibody which is anti Cry2A antibody, was provided by Dr. Crickmore lab.

2.6 Experimental protocols

Design of PCR primers for amplification of DNA fragments:

The primers used to create hybrids were obtained from Eurofins Genomics.

Cry2- domain I

Forward primer 5'P-ATGAATAATGTATTGAATAAYGGAAG- 3'

Reverse premier 5'P-TTTAAATAACGACCAGATRGAKACATA- 3'

PGEM Cry2 without domain I

Forward primer 5' - TATCAAAGCCTTCTAGTATCTTCYG- 3'

Reverse primer 5' -ATAAAATTCCTCCTTAATCGAATTC- 3'

Cry2Ac-domain III

Forward primer 5'P- AATAATATCTATGACACTCATGAAAATG- 3'

Reverse primer 5'P- TTAATAAAGTGGTGGGAAGATTAGTTG- 3'

PGEM Cry2Ac without domain III

Forward primer 5'-GGTTTGAGTGAATCACTAGTGAATTC- 3'

Reverse primer 5'-TTTTCTGTTATGCACTGTTACAAG- 3'

Cry2Ab-domain III

Forward primer 5'P- AATAATATCCATGCTGTTTCATGAAAATG- 3'

Reverse primer 5'P- TTAATAAAGTGGTGAAATATTAGTTG- 3'

pSVP27B40B

*Xba*I Forward primer 5'P-TTCTAGATGAATAATGTATTGAATARYGG-3'

B40B Reverse primer 5'-TCAAAGCTTAATAAAGTGGTGRAAG-3'

*Hind*III Reverse primer 5'-TCAAAGCTTAATAAAGTGGTGRAAG-3'

Cry2ADII

Forward primer 5'P-TATCAAAGCCTTCTAGTATCTTCYG- 3'

Reverse primer 5'P-TTTTCTGTTATGCACTGTTACAAG-3'

PGEM Cry2Ab without domain II

Forward primer 5'-AATAATATCCATGCTGTTTCATGAAAATG- 3'

Reverse primer 5'- TTAAATAACGACCAGATRGAKACATA-3'

Cry41AaDI

Forward primer 5'P- TACAGCGCAGATGTAAGGGATG -3'

Reverse primer 5'P- AAGTCCCGGATCATAACGTTGG -3'

Cry41AaDIII

Forward primer 5'P- GATTTGAACAATATAATATCACAGTATAG- 3'

Reverse primer 5'P-AGTGGTTAAGCCAATACCCATAC -3'

2.6.1 The reaction conditions for PCR amplification were set as follows

The initial warming of lid at 98C° for 4 minutes, initial denaturation at 95C° for 2 minutes, denaturation at 95C° for 20 seconds, annealing of the primers at 50C° or 51C° for 20 seconds (depending on the primer's optimum annealing temperature), elongation at 72C° for 2 minutes or 45 seconds (depending on length of amplicon) and final extension at 72C° for 3 minutes. The cycle from initial denaturation to primer extinction was set to repeat for 30 cycles after which the sample was cooled to 4 C°.

In table below the PCR reaction mix is shown

Component	Amount
PfuUltra Hotstart 2x Master Mix	25.0 µl
Forward Primer (10ng/µl)	1.0 µl
Reverse Primer (10ng/µl)	1.0 µl
Template DNA (10ng/µl)	1.0 µl
dH ₂ O	22.0 µl
Total Volume:	50 µl

2.6.2 Agarose Gel Electrophoresis

In this study 1% agarose concentration was used: 0.3 g of Agarose Low EEO was added to 30 ml of 1x TBE (Tris-Borate-EDTA) and heated until completely dissolved. The mixture was allowed to cool then 1.5 μ l of a 1 in 3 dilution of Gel Red was added before pouring the solution onto a gel casting tray. The gel was allowed to cast, then 5 μ l of purified product was added to 1 μ l of loading buffer and a total of 6 μ l of each sample was loaded onto gel.

1 Kb ladder (New England Biolabs) was used as a marker depending on the desired fragment size. The gels were run at 120 Volt in 1x TBE buffer in electrophoresis chamber then the bands of amplified products were visualized under ultraviolet light and in some instances excised from the gel for further use and analysis.

2.6.3 Column Purification of PCR products

QIAquick PCR Purification Kit (QIAGEN) was used in order to separate the PCR products from the other components present in the mixture.

A digestion with *DpnI* enzyme was performed on the PCR products by adding 1 μ l *DpnI* to each PCR product then incubating at 37C° for 30 minutes. 1x volume of PCR products was mixed with 5x volumes of PB buffer (chaotropic buffer). A QIAprep column was placed in 2ml collection tube. The sample was applied to the QIAprep column and centrifuge for 1 min at 13, 000 rpm. the flow-through was discard and the QIAprep column was placed back to the same tube, 750 μ l of Buffer PE was added to the bound DNA in the QIAprep column and centrifuged at 13,000 rpm for 1 min. flow-through was discard and the QIAprep column re-centrifuge for 1 min to remove any traces of buffer. The QIAprep column was placed in a

clean 1.5ml Eppendorf tube. To elute the DNA 30 μ l of EB buffer (10Mm Tris-Cl, PH 8.5) was added to the centre of membrane, the column was left for 1 min after than centrifuged for 1min. The purified PCR product was analysed on 1% agarose gel.

2.6.4 Gel purification using QIAprep kit

PCR reaction mixes were run on 1% Agarose gel and required bands excised and purified. The excised bands were placed in a clean microcentrifuge tube and purified according to the QIAprep gel extraction kit protocol. According to this protocol three volumes of buffer QG was added to one volume of excised gel in microcentrifuge tube, the tubes were incubated at 50⁰C water bath for 10 minutes with inverting the tube 4-6 times to enhance dissolution. After the excised gel completely dissolved then the tubes were centrifuged for 30 seconds at 13.000rpm. Supernatant was applied from microcentrifuge tube to the QIAprep2.0 spin column by pipetting, then it was centrifuged for 30 seconds at 13.000 rpm, the QIAprep2.0 spin column was washed by adding 500 μ l of QG (solubilisation buffer) and centrifuged for 30 seconds at 14.000 rpm and the flow-through was discarded, and the Qiaprep2.0 spin column was washed by adding 750 μ l of PE, centrifuged for 30 seconds and the flow-through was discarded, centrifuged for 1 minute at 14.000xg to remove residual wash buffer. The QIAprep 2.0 column was placed in a clean 1.5ml Eppendorf tube. To elute the DNA, 30 μ l of EB buffer (10Mm Tris-Cl, pH 8.5) was added to the centre of membrane, the column was allowed to stand for 1 min after then centrifuged for 1min at 13.000 rpm.

2.6.5 PCR ligation

The ligation reaction was set up following the recommended ligation ratio of 1:3 to 1:5 of vector to insert in the right estimated proportion based on the intensity of their respective bands on a gel, plus 1µl of ligase buffer and 0.5µl of DNA Ligase then adding deionised water to make a total of 10µl. This left overnight at room temperature for ligation reaction to take place.

The other ligation reaction that we later adopted in this work was the one using the Blunt/TA master mix reagent from New England BioLabs which proved to be much more efficient and time saving compare to the above ligation procedure. The protocols are as outlined below:

1. The master mix was transferred to ice prior to reaction set up. The tube was mixed by finger flicking before use
2. 20-100ng of the vector was combined with a 3- fold molar excess of insert and the volume was adjusted to 5µl with deionised water.
3. 5µl of the Blunt/TA master mix was added to the above mixture by pipetting up and down 7-10 times or by finger flicking
4. The above mixture was incubated at room temperature (25⁰C) for 15 minutes and placed on ice

The above ligation mixture was then used for transformation or stored at -20⁰C for future use.

2.6.6 *E.coli* strain transformation

We employed two methods (NEB-5 α and DH5- α transformation) in this research for *E.coli* transformation.

i. Microelectroporation method

E. coli strains were grown on 25ml L-agar at 37°C overnight. The strains were then prepared by transformation by scraping a culture from the agar plate using a sterile toothpick into 100ml L-broth and grown in a shaking incubator till it reached an OD of over 0.4. The cells were then harvested by centrifugation- the 100ml of broth containing the *E. coli* was spun at 10,000rpm for ten minutes, the supernatant discarded and the pellet washed and re-suspended in 100ml of deionised water at 4°C. The re-suspended cells were centrifuged again at 10,000rpm for a further ten minutes and the pellet re-suspended in approx 1ml of 4°C water, which was then transferred to a 1.5ml Eppendorf. The Eppendorf was centrifuged for 1min and the pellet re-suspended in 200 μ l of 4°C sterile water.

50 μ l of the *E. coli* cell suspension was transferred to a small Eppendorf tube and stored on ice- 1 μ l of the ligated DNA was added and mixed before being transferred into a sterile electroporation cuvette and tapped to ensure the liquid cells settled on the bottom. The gene pulser was set to 1.8kV, 200Ohms and 25 μ F, the electroporation cuvette placed in it and a short electrical pulse was applied to the cells. The cells and 0.5ml of L-broth were mixed with a sterile Pasteur pipette and transferred into small glass bottles, before being left to rest for one hour at room temperature.

ii. Heat shock method

The protocol outlined below is the one specified by the New England BioLab for transformation using this method. The protocol is as follows:

- i. A tube containing 50µl of NEB 5-α competent *E. coli* cells was thawed on ice for 10 minutes
- ii. 2µl of the ligation mixture was added to the 50µl NEB 5-α competent *E. coli* cells above and carefully flick the tube 4-5 times to mix the cells and DNA. It was not vortexed
- iii. The mixture was placed on ice for 30 minutes without mixing
- iv. The above mixture was then heat shocked at exactly 42⁰C for exactly 30 seconds without mixing.
- v. It was then placed on ice for 5 minutes
- vi. 950µl of recovery media (SOC) maintained at room temperature was added to the mixture
- vii. It was then placed on a shaker maintained at 37⁰C, 250 rpm and was allowed to rotate for 1 hour
- viii. 500µl of the above was poured on an ampicillin plate that was warmed at 37⁰C and the plate was placed in an incubator maintained at 37⁰C overnight.

2.6.7 Picking transformation colonies

Successful transformants were identified as colonies. White colonies were picked using a toothpick and streaked onto ampicillin (100µg/ml) L-agar plates. Plates were incubated overnight at 37°C.

2.6.8 Harvesting of the transformed colonies

Bacteria cells were scraped from the plate, then QIAprep kit was used by re-suspending it in 250µl of buffer P1 in Eppendorf tube and thoroughly mixed. 250µl of buffer P2 was added to the mixture and inverted 5-6 times until the solution became clear. Followed by, 350µl of N3 (neutralising buffer) and mixed immediately and thoroughly by inverting the tube 4–6 times. The mixture was centrifuged for 10 minutes at 13,000 rpm and supernatant transferred to a column in an Eppendorf tube. This was spun for 30 seconds, and the flow-through was discarded. 500µl of PB buffer was added to bound DNA and spun for 30 seconds. The column was then washed again by adding 750µl of PE buffer and centrifuging for 30–60 seconds, discarding the flow-through and then spun again for a further 1 minute. The QIAprep column was transferred to a clean 1.5 Eppendorf tube and 50µl of EB buffer (10 mM Tris Cl, pH 8.5) was added to elute DNA and could rest for 1 minute. The column was spun for a minute and purified plasmids DNA were collected. The above procedure was done for *E.coli* transformed colonies, but in case of Bt colonies the cells was re-suspended in P1 buffer mentioned in step one is add together with lysozyme (5mg/ml) and the mixture was incubated for 30 minutes at 37 °C followed by the other steps.

2.6.9 Digestion of extracted plasmid DNA with restriction enzymes

Restriction digestion using *Hae*III enzyme was performed on the plasmids thus extracted to verify the integration of the correct insert. 2µl of purified plasmid DNA was digested by adding 0.5µl *Hae*III restriction enzyme, 1 µl of corresponding buffer and 6.5 sterile distilled was added to make a final volume of 10 µl. The reaction mixes were then incubated in water bath at 37⁰C for 30 minutes. Then run on a 1% agarose gel for comparison with the predicted banding profile produced by NEB cutter software.

2.6.10 Verification of mutation

To confirm presence of the correct mutation purified DNA from colonies after transformation into DH5α *E.coli* and IPS78/11 Bt were sent for sequencing in Eurofins Genomic. This was done using the primers below.

Cry2A-DI-II 5' CTTAATGCAGATGAATGGGG 3'

Cry2A-DII-III 5' GATTGTA ACTATTTCCATTCCCTC 3'

2.6.11 Expression and Harvesting of the Cry2 Toxins from *E. coli* (BL21)

Cells were plated out on agar plates with ampicillin (100µg/ml) and incubated at 37⁰C and left overnight. Then scraped from 100µg/ml ampicillin impregnated agar plates using a sterile loop into 500ml L-broth with 500µl of 100 mg/ml ampicillin. This was carried out over a flame to prevent contamination from the air and the bacteria left to incubate for three hours in a 37⁰C shaking incubator till an OD (optical density) 600nm of 0.4-0.6 was obtained by

using a spectrophotometer with pure L-broth as a base ($A_{600}=0$). 250 μ l 1M IPTG (Isopropyl β -D-1-thiogalactopyranoside) was added to stimulate expression of the toxin from a T7 promoter.

The L-broth left overnight at 25⁰C in the shaking incubator. The solution was then centrifuged (JA10) for 10 minutes at 10000 rpm and the pellet re-suspended in 30ml of de-ionised water and transfer to 50 ml plastic beaker. The sample was sonicated for 4 X 1 minute intervals with 1-minute rest then transfer cells to 50 ml centrifuge tube and centrifuged (JA25.50) for 30 minutes at 15,000 rpm. The pellet was re-suspended in 1ml of de-ionised water then re-sonicated using a smaller tip for 4 minutes at 20 second intervals. This was then centrifuged and the pellet re-suspended in 1ml of de-ionised water. In this stage, the crystals should be released from the cell and cell debris removed by using light microscopy to determine the purity of the sample also, use it to observe the size and level of expression of toxin crystals.

2.6.12 Expression and Harvesting of the Cry2 Toxins from *Bt* (IPS78/11)

Cells were plated out on agar plates with chloramphenicol (6 μ g/ml) and incubated at 30⁰C for three days. Then sporulated cells were scraped from the chloramphenicol plates using a sterile wire loop into 30 ml of sterile distilled water. The cells were lysed by sonicating it at full power for 4 minutes (1minute on, 1 minute off). This was then centrifuged (JA25.50) at 10000rpm for 10 minutes at 4⁰C and the supernatant was discarded, and the obtained pellet were re-suspended in 1ml of de-ionised water

2.6.13 Protein analysis by SDS-PAGE

7.5% SDS gels (Sodium Dodecyl Sulphate-Polyacrylamide Gel) were used throughout. The glass plates cleaned with ethanol and the bottom of the plates sealed with 200 μ l of 1% agarose gel (composed of 0.3g of Agarose in 30ml SDS running buffer) The resolving gel, consists of 18.18g Tris, 0.4g SDS in 100ml of distilled water at pH 8.8. It was made in a sterile glass bottle and pipetted into glass plates and a butanol water mix was added to prevent oxygen inhibition of resolving gel and allowed to set before finally adding Stacking gel solution.

SDS Resolving Gel Components	Volume added (μ l)
Water	2ml
RGB	1ml
Acrylamide stock	1ml
400mg/ml APS (Ammonium Persulphate)	8 μ l
TEMED	4 μ l

Once set, the butanol solution was removed and the gel washed with water, before adding the stacking gel. A ten-well comb was washed with ethanol and added immediately afterwards and the gel left to set for another 30 minutes. Once set, the comb was removed and the gel transferred to the electrophoresis apparatus, where it was washed in SDS running buffer, made by diluting 100ml of 10xSDS running buffer (7.6g Tris; 36g Glycine; 2.5g SDS and water up to 500ml) in 400 ml of de-ionised water. Then sample were mixed 1:1 with sample loading buffer in the presence of B-mercaptoetanol, boiled for 4 minutes then spun

for 30 second. The supernatant of each sample was loaded on to a gel which composed of two parts (stacking and resolving). Electrophoresis was carried out at 200V for 35 minutes in MiniPROTEAN® II Electrophoresis cell (Bio-Rad) using tris-glycine running buffer. Gels were later stained with Coomassie Blue for 20 min then de-stained until clear visualisation of the bands of interest. The fragment sizes were estimated using a protein ladder (New England BioLabs).

SDS Stacking Gel Components	Volume Added (µl)
Water	2x585 µl
SGB	500 µl
Acrylamide stock	333 µl
400mg/ml APS (Ammonium Persulphate)	4 µl
TEMED	2 µl

2.6.14 Protein concentration

Two methods were used to measure the protein concentration in this study. The first method was the Bradford method, which was used to measure the concentration of the *Plutalle xylostella* midgut juice protein and the densitometry method using Image J software to measure the Cry toxins concentration. The concentration of protein toxin was determined by running equal volumes of all proteins on SDS PAGE gel along with some known concentrations of BSA as a standard. Using Image J software, the concentration of the protein bands was measured as a peak shown as area under the curve, Excel program was used to

plot a graph of area under the curve versus the concentration of the known BSA standard. Then the concentration of the Cry toxin protein was determined using the equation of the line or by extrapolation. However, for determination of the concentration of the proteins present in BBMV extracts used in western blot and ligand blot experiments, Bradford method was applied using a Bio-Rad Protein Assay Kit (Bio-Rad) with BSA used as the standard. The concentration of each sample was determined by comparing its absorbance against a BSA standard curve.

2.6.15 Solubilisation and activation of crystal protein

Crystal protein were solubilised in 50mM sodium hydroxide (NaOH), the mixture was incubated in water bath at 37⁰C for 1 hour, before being spun for 1 minute at 13000 rpm. The supernatant was then collected, treated with chymotrypsin (1mg/mL) of PBS buffer and incubated for 10 minute at 37⁰C. Solubilised and activated samples were then spun for 1 minute at 13000 rpm and protease inhibitor was added.

2.6.16 Preparation of *P. xylostella* Midgut Protease

The *P. xylostella* midgut proteases were prepared by dissecting 20 midguts from third instar larvae of *P. xylostella* and midgut tissues were homogenized with 200 μ L of phosphate-buffered saline (PBS) buffer (50 mM, pH 7.4) and centrifuged at 25000g for 10 min at 4 °C. The supernatants, which contained midgut proteases, were labeled as *P. xylostella* midgut juice (PxMJ). PxMJ was stored at -80 °C until used. Protocol from (Xu *et al.*, 2016).

2.6.17 Preparation of BBMV from dissected midguts of lepidopteran larvae

Protocol from Dr. Juan Luis Jurat Fuentes as modified from (Wolfersberger *et al.*, 1987).

Dissected guts were thawed and weighed. Typically, to about 1 gram, 13 ml of ice cold MET buffer (250mM mannitol, 5mM EGTA, 17mM Tris-HCL) pH 7.5 with 1 tablet Roche Complete proteinase inhibitors to 100ml was added. It was homogenized at 30 sec + 30 sec rest +30 sec using electric homogenizer, speed 30, this was followed by 7 strokes in a 15 ml glass Dounce homogenizer. Then 13 ml of ice cold 24mM MgCl₂ +250mM sucrose was added to the homogenate and placed on ice for 30 min. It was centrifuged at 1600 x g x15 min at 4°C (rotor JA20 at approx. 4.500 rpm), and the supernatant was collected and centrifuged at 20,000 x g x30 min at 4°C (rotor JA20 at approx. 15.000 rpm). The pellet was re-suspended in the half volume of MET buffer. 1 volume of ice cold 24mM MgCl₂ +250mM sucrose was added, and allowed to stand on ice for 30 min, then centrifuged at 1600 x g x15 min at 4 °C (rotor JA20 at approx. 4.500 rpm), the pellet was discarded and the supernatant was centrifuged at 20,000 x g x30 min at 4°C (rotor JA20 at approx. 15.000 rpm). The pellet obtained constitutes the BBMV preparation. This was then re-suspended in at least 2ml of PBS pH7.5 (depending on size of pellet). It was aliquoted in Eppendorf tube 200 µl, stored at -20°C for short term or -80°C for long term.

2.6.18 Western Blotting

BBMV extracts were suspended in 1x SDS loading buffer and heated for 10 minutes in boiling water. 10-20 μ g of the extract was loaded in each well of a 7.5 % SDS-PAGE along with 5-10 μ g of activated chymotrypsin Cry2A toxin. Electrophoresis was carried out at 180mA for 45 minutes. Transfer was carried out in semi-dry transfer apparatus using semi-dry buffer (Tris-Base 48mM, Glycine 39 mM, Methanol 20%, SDS 0.04%) and nitro-cellulose membrane cut to gel measurements. Transfer sandwich was created using filter paper (pre-soaked in transfer buffer), nitro-cellulose membrane, and SDS-PAGE gel covered with another filter paper. Transfer was ran at 100mA for 1 hour. Nitro-cellulose membrane was stained using reversible Ponceau S stain (0.1g Ponceau S, 5mL acetic acid in 100mL ddH₂O) to confirm transfer onto to the membrane. Membrane was then washed with 1x PBS, then was blocked for 40 min at room temperature in 10ml of 1x PBS containing 2% tween and 5 μ l of anti-Cry2A antibody with constant shaking. Membrane was then washed with 1x PBS-T (1 litre 1x PBS, 200 μ L Tween-20) for 5 minutes 3 times, then membrane was incubated with 0.5 μ g/mL of secondary antibody in 5% blocking solution (0.5g skimmed milk, 10mL PBST) for 1 hour. Again, the membrane was washed with 1x PBS-T for 5 minutes 3 times before addition of 10mL ECL solution (5mL solution 1 250mM Luminol, 90mM P-coumaric acid, 1M Tris-HCL pH 8.5 and 5mL solution 2, 32 μ L of H₂O₂ (30%), 1M Tris-HCL pH 8.5). Membrane was developed using the signal (UVP ChemStudio imaging system).

2.6.19 Binding assays of Cry2Ac and Cry2Ab toxins to BBMV

Protocol from (Wang *et al.*, 2018) was and further modified according to the experiment. 20 or 10 ug of BBMVs from *P. xylostella* were incubated with increasing concentrations of Cry2Ab and Cry2Ac respectively, in a final volume of 100ul of binding buffer (PBS with 0.1% Tween 20 and 0.1%BSA, pH 7.4) for 1 h at room temperature. Binding reactions were stopped by centrifugation for 10 min at 18,000xg after which the BBMV pellet containing the bound toxin was washed with 0.1 ml of ice-cold binding buffer twice. A control of the highest concentration of toxins without BBMVs was also included to monitor toxin precipitation. Pellets were solubilized in 10ul of sample buffer, heat-denatured for 10 min at 100 °C, and loaded for SDS-PAGE.

2.6.20 Maintenance of *Plutella xylostella* population on cabbage plant (*Brassica pekinensis*)

Pupa and Chinese cabbage leaves (*Brassica pekinensis*) were placed in plastic cage with net fitted around it until adults emerge. The emerging adults were fed with honey diluted in water by soaking cotton wall in the honey then placing it in a small petri dish inside the cage and adding more of the honey when required. The adult moth remains in the cage and mate, after which they began laying eggs on the cabbage leaves, the eggs are maintained at 27°C and 70% relative humidity, with 16 hours light and 8 hours dark. When the eggs hatched and the larvae had reached to the second instar larvae, bioassays were performed and the remaining larvae were then be placed in plastic boxes, which contained non-organic cabbage (spring green) washed as out line in section 2.6.22. This was added or changed as required. When

the larvae started pupating, pupae were picked and placed into a small petri dish (labelled and dated) these were stored in the fridge sealed with cling film, pupae were stored in the fridge for up to 15 days after which they were used.

2.6.21 Leaf Dip Bioassay

Leaf disc dip bioassay as described by Sayyed *et al* (2000) was used, in which cabbage leaf discs (5cm in diameter) were cut with a metal punch and then dipped in to a toxin solution for about 10 seconds and then removed to dry for 1 hour. The toxin solution consists of 30ml deionised water and 15µl Triton x100, which is a detergent that increases the binding of the toxin to the leaf. Ten larvae of *Plutella xylostella* at the second instar stage were placed in 5cm petri dish, which is, has three 42.5mm No1 Whatman filter papers soaked in 600µl deionised water. The larva was allowed to feed on the leaves treated with 200ul in 1.8ml of the triton X 100, and mortality was assessed after 3 days.

In case there is no sufficient amount of toxin, 125µl of toxin was applied to each leaf by using paintbrush. But in this case, the toxin is prepared by centrifuging the toxin using table microcentrifuge at 14,000 rpm for 1 minute, then remove the supernatant and re-suspend the pellet in a mixture of water and triton x 100 same volume as that of the supernatant removed. Any further dilution required after the above, is done using a mixture of triton x100 and water. The leaf was then left to dry, and when it was completely dried, ten second instar larva placed in petri dish and kept at 23°C.

2.6.22 Preparation of cabbage for larval feeding

Non –organic cabbage bought from store (Spring Green), and was washed by soaking in a container full of water with approximately 10 ml of bleach added. This was then left to stand for 10 minutes. After which, it was washed with tap water and then dried by wiping with tissue paper. Once dried the cabbage is ready for use, it was stored in the refrigerator for later use.

2.6.23 Growing cabbage plant for bioassay

The cabbage plant was grown pots full of organic soil, by placing two Chinese cabbage seeds in each pot. This was then stored in an incubator maintained at 23°C and 60% humidity, regular watering to avoid dryness.

3. Identification of domain/domains that determine Cry2A specificity against *Plutella xylostella*

3.1 Introduction

Many researchers who aim to understand the region or amino acid residues responsible for the specificity of Cry toxins start by creating hybrids through domain swaps. This helps to identify the domain that could be responsible for specificity, and later they narrow down to specific amino acid residue(s) that may be responsible for the specificity of such Cry toxin (Höfte and Whiteley, 1989, Widner and Whiteley, 1990, Liang and Dean, 1994).

The results of previous homologue-scanning mutagenesis experiments identified the functional regions of the putative specificity region of Cry2A. As substitutions of residues 278-340 resulted in a loss of dipteran-specificity in Cry2Aa and substitution of residues 307-382 conferred Dipteran-specificity in Cry2Ab, the boundary of this putative Dipteran-specificity region falls between amino acids 307-340 (Widner and Whiteley, 1990; Morse *et al.*, 2001). Furthermore, Liang and Dean, (1994) identified region 2 in domain II in Cry2Aa responsible for high activity against mosquito (*Aedes aegypti*) and Gypsy Moth (*Lymantria dispar*), however Cry2Ab had no activity against *Aedes aegypti* but possessed a relatively low activity against *Lymantria dispar*. They divided the domain II of both toxins using two restriction enzyme namely *MluI* and *XhoI* by taking advantage of the naturally occurring *NheI* and *NarI* sites that borders the putative domain II. The result showed that amino acids 278-412 are required for Cry2Aa activity against mosquito and Gypsy Moth, which defined

domain II as the specificity determining region. However, another work done by student in our lab (Joseph, 2019) shows that the N-terminal of Cry2A is required for activity against mosquito (*Aedes aegypti*).

In this chapter we wanted to determine which domain/domains of Cry2A toxins is/are responsible for specificity against diamondback moth, *Plutella xylostella*. There are a lot of contradictory results from the literature available, with some reports claiming that a given Cry2A toxin kills an insect and others stating that it does not. We found this to be the case with Cry2Aa and Cry2Ab in *P. xylostella*. According to Pan *et al.*, (2014) and Lin *et al.*, (2007), who carried out bioassays using Cry2Ab30 and Cry2Ab10 against a susceptible population of *P. xylostella*, each was found to be toxic with LC50 values of 0.0103µg/ml and 90 µg/ml, respectively. Whereas, another researcher reported that Cry2Ab had no any activity against *P. xylostella* (Frankenhuyzen *et al.*, 2009), whilst Zhao (2001) stated that Cry2Ab has low activity against the same insect. Moreover, in the case of Cry2Aa toxin several works have been done to assess the toxicity of the toxin against different populations of *P. xylostella*, the result of the bioassays showed that Cry2Aa was toxic (Frankenhuyzen *et al.*, 2009; Tabashnik *et al.*, 1996; Ibargutxi *et al.*, 2005). While, some studies carried out by different researchers using the same toxin and insect reported that Cry2Aa has low level of toxicity against *P. xylostella* (Monnerat *et al.*, 2015 and Zago *et al.*, 2013). Therefore, it was important to test all of the wild type Cry2A toxins that we had in the lab to be able to establish our own bioassay data. We carried out bioassays after which we were able to find which among them were or were not active against *P. xylostella*. Based on these results, we carried out informed hybrid creation through domain swaps to be able to identify which part of Cry2A toxins is responsible for activity against *P. xylostella*.

3.2 Results

3.2.1 Confirmation Cry2A toxin identity and expression

The following, Cry2A toxins used in this research (Cry2Aa9, Cry2Aa17, mCry2Aa17, Cry2Ab4, Cry2Ab29, Cry2Ah1, and Cry2Ax) were provided and confirmed through DNA sequencing by the Institute of Plant Protection, Chinese Academy of Agricultural Sciences and were introduced into *E. coli* BL 21 strain by a previous student (Nicholas Stevens, 2015). The rest of Cry2 toxins (Cry2Aa2, Cry2Ab-916, Cry2Ab Cry2Ac, Cry2Ad and Cry2Ba) were confirmed by sequencing the N-terminus using a pGEM T7 primer. In addition, SDS-PAGE was running for the Cry2A toxins that we used in this research along with a known protein marker to be able to confirm the position of the proteins. A gel showing a selection of these toxins is depicted in (figure 3.1).

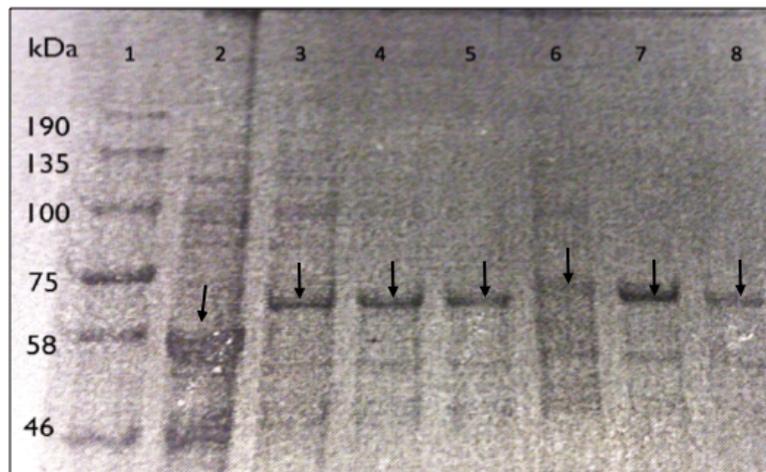


Figure 3.1. SDS-PAGE of the expression of Cry2A toxins. The position of the Cry proteins is indicated with arrows (1) protein Marker, (2) Cry2Ac, (3) Cry2Ab29, (4) Cry2Aa9, (5) Cry2Aa17, (6) Cry2Ab4, (7) Cry2Ba, and (8) mCry2Aa17.

Figure 3.1 indicated that, all Cry2A toxins that were cloned in *E. coli* were expressed but Cry2Ab4 which showed a lower level of expression. Furthermore, it is clear from the gel that all Cry2A toxins have a molecular weight between 58 and 72 kDa, and all the Cry2A toxins appeared at the expected molecular weight except Cry2Ac which had a lower than expected molecular weight. The plasmid DNA containing the *cry2Ac* gene was extracted and sequenced with the aim of confirming the identity of this Cry2Ac since it gives us a lower molecular weight, we therefore sent it for sequencing to confirm if it is the exact molecular weight or there is any error. Three primers were used to enable us to sequence the entire gene of *cry2Ac*.

T7 forward primer: 5'-TAATACGACTCACTATTAGGG-3'

Cry2F forward primer: 5'-TATTACCTTTATTTGCACAGGCA-3'

SP6 reverse primer: 3'-ATTTAGGTGACACTATAG-5'

We used the sequence of Cry2Ac1 from database as a template to help in filling the gaps when overlaps among the sequence received were identified. So, after we identify these regions where there are differences between the Cry2Ac we obtained from our sequencing result and the Cry2Ac1 sequence from database we carried out multiple sequence alignment between Cry2Ac1 from the database and Cry2Ac from our lab as shown in figure 3.3A.

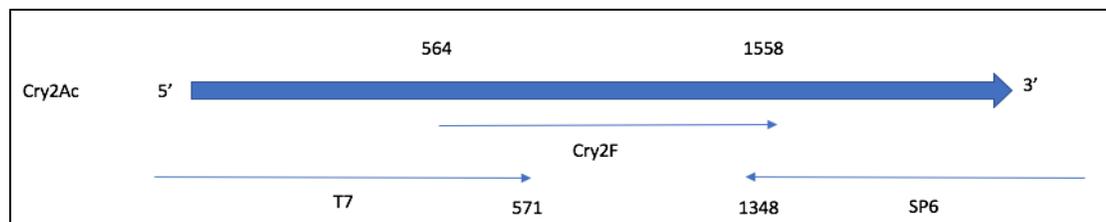


Figure 3.2. Schematic representation the region at which three primers sequenced and the nucleotide positions at which they started and stopped along the *cry2A* gene.

Cry2Ac1	ATGAATACTGTATTGAATAACGGAAGAAATACTACTTGTTCATGCACATAATGTAGTTGCT	60
Cry2Ac	ATGAATAATGTATTGAATAACGGAAGAAATACTACTTGTTCATGCACATAATGTAGTTGCT	60

Cry2Ac1	CATGATCCATTTAGTTTTGAACATAAATCATTAAATACCATAGAAAAAGAATGAAAAGAA	120
Cry2Ac	CATGATCCATTTAGTTTTGAACATAAATCATTAAATACCATAGAAAAAGAATGAAAAGAA	120

Cry2Ac1	TGAAAAAGAACTGATCATAGTTTATATGTAGCCCTATTGTGGAACTGTGGGTAGTTTT	180
Cry2Ac	TGAAAAAGAACTGATCATAGTTTATATGTAGCCCTATTGTGGAACTGTGGGTAGTTTT	180

Cry2Ac1	CTATTAAGAAAGTAGGGAGTCTTGTGGAAAAAGGATACTGAGTGAGTTACAGAATTTA	240
Cry2Ac	CTATTAAGAAAGTAGGGAGTCTTGTGGAAAAAGGATACTGAGTGAGTTACAGAATTTA	240

Cry2Ac1	ATTTTCCTAGTGGTAGTATAGATTTAATGCAAGAGATTTAAGAGCGACAGAACAATTC	300
Cry2Ac	ATTTTCCTAGTGGTAGTATAGATTTAATGCAAGAGATTTAAGAGCGACAGAACAATTC	300

Cry2Ac1	ATAAATCAAAGGCTTAATGCAGACACCCTTGGTCGTGTAATGCAGAATTGGCAGGTCTT	360
Cry2Ac	ATAAATCAAAGGCTTAATGCAGACACCCTTGGTCGTGTAATGCAGAATTGGCAGGTCTT	360

Cry2Ac1	CAAGCGAATGTGGCAGAGTTTAAATCGACAAGTAGATAATTTTTAAACCCTAATCAAAC	420
Cry2Ac	CAAGCGAATGTGGCAGAGTTTAAATCGACAAGTAGATAATTTTTAAACCCTAATCAAAC	420

Cry2Ac1	CCTGTCCTTTAGCAATAATTGATTGATTAGTAATACATTGCAGCAATTATTTCTAAGTAGA	480
Cry2Ac	CCTGTCCTTTAGCAATAATTGATTGATTAGTAATACATTGCAGCAATTATTTCTAAGTAGA	480

Cry2Ac1	TTACCACAGTCCAGATACAAGGCTATCAACTGTTATTATTACCTTTATTGCACAGGCA	540
Cry2Ac	TTACCACAGTCCAGATACAAGGCTATCAACTGTTATTATTACCTTTATTGCACAGGCA	540

Cry2Ac1	GCCAATTTCAATCTTTCTTTTATTAGAGGTGTCATCCTTAATGCAGATGAATGGGGCATT	600
Cry2Ac	GCCAATTTACATCTTTCTTTTATTAGAGATGTCATCCTTAATGCAGATGAATGGGGCATT	600

Cry2Ac1	TCAGCAGCAACAGTACGCACATATAGAGATCACCTGAGAAAATTCACAGAGATTACTCT	660
Cry2Ac	TCAGCAGCAACAGTACGCACATATAGAGATCACCTGAGAAAATTCACAAGAGATTACTCT	660
	***** ** *****	
Cry2Ac1	AATTATTGTATAAATCCGTATCAAATGCATTTAGAGGTTTAAACCACCGTTTACCCGAT	720
Cry2Ac	AATTATTGTATAAATACGTATCAAATGCATTTAGAGGTTTAAACCACCGTTTACCCGAT	720
	***** *****	
Cry2Ac1	ATGTTAGAAATTTAGAACATATATGTTTTTAAATGTATTGAATATGTCTTATCTGGTGC	780
Cry2Ac	ATGTTAGAAATTTAGAACATATATGTTTTTAAATGTATTGAATATGTATcATCTGGTGC	780
	***** ** *****	
Cry2Ac1	TTATTTAAATATCAAAGCCTTCTAGTATCTTCCGGCGCTAATTTATATGCGAGTGGTAGT	840
Cry2Ac	TTATTTAAATATCAAAGCCTTCTAGTATCTTCCGGCGCTAATTTATATGCGAGTGGTAGT	840

Cry2Ac1	GGTCCAACACAATCATTACAGCACAAAACCTGGCCATTTTTATATCTCTTTTCCAAGTT	900
Cry2Ac	GGTCCAACACAATCATTACAGCACAAAACCTGGCCATTTTTATATCTCTTTTCCAAGTT	900

Cry2Ac1	AATTCATAATTATGTATTAATGGTTTGAGTGGTGCTAGGACCACCATTACTTCCCTAAT	960
Cry2Ac	AATTCATAATTATGTATTAATGGTTTGAGTGGTGCTAGGACCACCATTACTTCCCTAAT	960

Cry2Ac1	ATGAATACTGTATTGAATAACGGAAGAAATACTACTTGTTCATGCACATAATGTAGTTGCT	60
Cry2Ac	ATGAATAATGTATTGAATAACGGAAGAAATACTACTTGTTCATGCACATAATGTAGTTGCT	60

Cry2Ac1	CATGATCCATTAGTTTTGAACATAAATCATTAAATACCATAGAAAAAGAATGGAAAGAA	120
Cry2Ac	CATGATCCATTAGTTTTGAACATAAATCATTAAATACCATAGAAAAAGAATGGAAAGAA	120

Cry2Ac1	TGGAAAAGAACTGATCATAGTTTATATGTAGCCCTATTGTGGAACTGTGGGTAGTTTT	180
Cry2Ac	TGGAAAAGAACTGATCATAGTTTATATGTAGCCCTATTGTGGAACTGTGGGTAGTTTT	180

Cry2Ac1	CTATTAAGAAAGTAGGGAGTCTTGTGGAAAAGGATACTGAGTGAGTTACAGAATTTA	240
Cry2Ac	CTATTAAGAAAGTAGGGAGTCTTGTGGAAAAGGATACTGAGTGAGTTACAGAATTTA	240

Cry2Ac1	ATTTTTCTAGTGGTAGTATAGATTTAATGCAAGAGATTTTAAGAGCGACAGAACAATTC	300
Cry2Ac	ATTTTTCTAGTGGTAGTATAGATTTAATGCAAGAGATTTTAAGAGCGACAGAACAATTC	300

Cry2Ac1	ATAAATCAAAGGCTTAATGCAGACACCCCTTGGTCGTGTAATGCAGAATTGGCAGGTCTT	360
Cry2Ac	ATAAATCAAAGGCTTAATGCAGACACCCCTTGGTCGTGTAATGCAGAATTGGCAGGTCTT	360

Cry2Ac1	CAAGCGAATGTGGCAGAGTTAATCGACAAGTAGATAATTTTTAAACCCTAATCAAAAC	420
Cry2Ac	CAAGCGAATGTGGCAGAGTTAATCGACAAGTAGATAATTTTTAAACCCTAATCAAAAC	420

Cry2Ac1	CCTGTTCCCTTAGCAATAATTGATTGAGTTAATACATTGCAGCAATATTTCTAAGTAGA	480
Cry2Ac	CCTGTTCCCTTAGCAATAATTGATTGAGTTAATACATTGCAGCAATATTTCTAAGTAGA	480

Cry2Ac1	TTACCACAGTTCAGATACAAGGCTATCAACTGTTATTATTACCTTTATTGACAGGCA	540
Cry2Ac	TTACCACAGTTCAGATACAAGGCTATCAACTGTTATTATTACCTTTATTGACAGGCA	540

Cry2Ac1	GCCAAATTCATCTTTCTTTTATTAGAGGTGCATCCTTAATGCAGATGAATGGGGCATT	600
Cry2Ac	GCCAAATTCATCTTTCTTTTATTAGAGGTGCATCCTTAATGCAGATGAATGGGGCATT	600

Cry2Ac1	TCAGCAGCAACAGTACGCACATATAGAGATCACCTGAGAAAATTCACAGAGATTACTCT	660
Cry2Ac	TCAGCAGCAACAGTACGCACATATAGAGATCACCTGAGAAAATTCACAGAGATTACTCT	660

Cry2Ac1	AATTATTGTATAAATCCGTATCAAACCTGCATTTAGAGGTTTAAACCACCGTTACCCGAT	720
Cry2Ac	AATTATTGTATAAATCCGTATCAAACCTGCATTTAGAGGTTTAAACCACCGTTACCCGAT	720

Cry2Ac1	ATGTTAGAATTTAGAACATATATGTTTTTAAATGTATTGAATATGTCTCTATCTGGTCG	780
Cry2Ac	ATGTTAGAATTTAGAACATATATGTTTTTAAATGTATTGAATATGTCTCTATCTGGTCG	780

Cry2Ac1	TTATTTAAATATCAAAGCCTTCTAGTATCTTCCGGCGCTAATTTATATGCGAGTGGTAGT	840
Cry2Ac	TTATTTAAATATCAAAGCCTTCTAGTATCTTCCGGCGCTAATTTATATGCGAGTGGTAGT	840

Cry2Ac1	GGTCCAACACAATCATTACAGCACAAAACCTGGCCATTTTTATATTCTCTTTCCAAGTT	900
Cry2Ac	GGTCCAACACAATCATTACAGCACAAAACCTGGCCATTTTTATATTCTCTTTCCAAGTT	900

Cry2Ac1	AATTCATTTATGTATTAATGGTTTGGAGTGGTGCTAGGACCACCATTACTTTCCCTAAT	960
Cry2Ac	AATTCATTTATGTATTAATGGTTTGGAGTGGTGCTAGGACCACCATTACTTTCCCTAAT	960

Figure 3.3A. Multiple sequence alignment of pGEM Cry2Ac from our lab and Cry2Ac1 from database. The DNA sequence from database display as Cry2Ac1.

The differences found based on the sequence alignment presented diagrammatically in figure 3.3A above are detailed in table 1, with these differences being verified and resolved accordingly.

Position	2Ac1	Our 2Ac	Verified
8	C	A	Yes
549-550	CA	AC	Yes
569	G	A	Yes
642	A	T	Yes
676	C	A	Yes
706-708	CAC	ACT	Yes
716	C	A	Yes
977	T	G	Yes
1146	A	G	Yes
1464	A	C	Yes
1560	C	A	Yes

Table 1. Showing nucleotide differences between our *cry2ac* and the database *cry2Ac1* and their relative positions. These differences are classified as either verified or not verified.

The table above, which shows areas of differences between our Cry2Ac and that of the data base was carefully studied to see if these are real differences or a case of error in the sequencing process, and fortunately, they were all verified by checking the raw sequencing data (chromatogram), as the entire sequence of the Cry2Ac1 from the data base was

efficiently covered by the sequence received from the sequencing results, which made the verification easier. Thus, we conclude that our Cry2Ac is essentially the same as Cry2Ac1 from the database. In addition, we carried out multiple amino acids sequence alignment between Cry2Ac from our lab and other Cry2A (Cry2Aa and Cry2Ab) to be able to know why Cry2Ac is shorter than that the rest of Cry2A toxins on the gel, shown in figure 3.3B below.

Cry2Ac1	MNTVLNNGRNTTCHAHNVVAHDPFSFEHKSLENTIEKEWKEWKRTHDSLYVAPIVGTVGSF	60
Cry2Ac	MNNVLNNGRNTTCHAHNVVAHDPFSFEHKSLENTIEKEWKEWKRTHDSLYVAPIVGTVGSF	60
Cry2Aa	MNNVLNSGRRTTICDAYNVVAHDPFSFEHKSLENTIEKEWKEWKRTHDSLYVAPVGTVGSF	60
Cry2Ab	MNNVLNSGRRTTICDAYNVVAHDPFSFEHKSLENTIEKEWKEWKRTHDSLYVAPVGTVGSF	60
	**.*	
Cry2Ac1	LLKKVGSLVGKRILSELQNLIFPPSGSIDLMQEIILRATEQFINQRLNADTLGRVNAELAGL	120
Cry2Ac	LLKKVGSLVGKRILSELQNLIFPPSGSIDLMQEIILRATEQFINQRLNADTLGRVNAELAGL	120
Cry2Aa	LLKKVGSLVGKRILSELQNLIFPPSGSIDLMQEIILRATEQFINQRLNADTLGRVNAELAGL	120
Cry2Ab	LLKKVGSLVGKRILSELQNLIFPPSGSIDLMQEIILRATEQFINQRLNADTLGRVNAELAGL	120
	*****.*	
Cry2Ac1	QANVAEFNRQVDNFLNPNQNPVPLAIIIDSVNTLQQLFSLRPLPQFQIQGYQLLLPLFAQA	180
Cry2Ac	QANVAEFNRQVDNFLNPNQNPVPLAIIIDSVNTLQQLFSLRPLPQFQIQGYQLLLPLFAQA	180
Cry2Aa	QANIREFNQVDNFLNPNQNPVPLAIIIDSVNTLQQLFSLRPLPQFQIQGYQLLLPLFAQA	180
Cry2Ab	QANVVEFNQVDNFLNPNQNPVPLAIIIDSVNTLQQLFSLRPLPQFQIQGYQLLLPLFAQA	180
	*****.*	
Cry2Ac1	ANFNLSFIRGVILNADEWGISAAATVRYRDHLRKFHRDYSNYCINPYQTAFRGLNHRRLPD	240
Cry2Ac	ANLHLSFIRDVILNADEWGISAAATVRYRDHLRKFHRDYSNYCINPYQTAFRGLNHRRLPD	240
Cry2Aa	ANMHLFSIRDVILNADEWGISAAATLRYRDYLRNYTRDYSNYCINPYQTAFRGLNHRRLPD	240
Cry2Ab	ANLHLSFIRDVILNADEWGISAAALRYRDYLRNYTRDYSNYCINPYQTAFRGLNHRRLPD	240
	*****.*	
Cry2Ac1	MLEFRTYMFNLFVFEYVSIWLSFKYQSLVSSGANLYASGSGPT--QSFTAQNWPFYLSLF	298
Cry2Ac	MLEFRTYMFNLFVFEYVSIWLSFKYQSLVSSGANLYASGSGPT--QSFTAQNWPFYLSLF	298
Cry2Aa	MLEFRTYMFNLFVFEYVSIWLSFKYQSLVSSGANLYASGSGPQQTQSFTAQNWPFYLSLF	300
Cry2Ab	MLEFRTYMFNLFVFEYVSIWLSFKYQSLVSSGANLYASGSGPQQTQSFTAQNWPFYLSLF	300
	*****.*	
Cry2Ac1	QVNSNYVNLGSGARTTITFPNIGGLPVYH-NSTLHFARINRYGGVSSSRIGQANLNQNF	357
Cry2Ac	QVNSNYVNLGSGARTTITFPNIGGLPGLTTQTTLHFARINRYGGVSSSRIGQANLNQNF	358
Cry2Aa	QVNSNYVNLGSGARTTITFPNIGGLPGSTTTHLSNARVNYSGVSSGLIGANLNHNF	360
Cry2Ab	QVNSNYVNLGSGARTTITFPNIGGLPGSTTTHLSNARVNYSGVSSGLIGANLNHNF	360
	*****.*	
Cry2Ac1	NISTLFNPLQTPFIRSWLDSGTDREGVATSTNWQSGAFETTL-LRFSIFSARGNSNFFPD	416
Cry2Ac	NISTLFNPLQTPFIRSWLDSGTDREGVATSTNWQSGAFETTL-LRFSIFSARGNSNFFPD	417
Cry2Aa	NCSTVLPPLSTPFVRSWLDSDGTDREGVATSTNWQTESFQTTLSLRCGAFSARGNSNFFPD	420
Cry2Ab	NCSTVLPPLSTPFVRSWLDSDGTDREGVATSTNWQTESFQTTLSLRCGAFSARGNSNFFPD	420
	*****.*	
Cry2Ac1	YFIRNISGVVGTISNADLARPLHFNEIRDIGTT-----AVASLVTVHNRKNNIYDTHEN	470
Cry2Ac	YFIRNISGVVGTISNADLARPLHFNEIRDIGTT-----AVASLVTVHNRKNNIYDTHEN	471
Cry2Aa	YFIRNISGVVPLVIRNEDLRLPHNEIRNIESPSGTPGGARAYLVSVHNRKNNIYAANEN	480
Cry2Ab	YFIRNISGVVPLVIRNEDLRLPHNEIRNIESPSGTPGGARAYLVSVHNRKNNIYAVHEN	480
	*****.*	
Cry2Ac1	GTMIHLAPNDYTGFTVSPIHATQVNNQIRTFISEKYGNGGDSLRFELSNPTARYTLRNGG	530
Cry2Ac	GTMIHLAPNDYTGFTVSPIHATQVNNQIRTFISEKYGNGGDSLRFELSNPTARYTLRNGG	531
Cry2Aa	GTMIHLAPNDYTGFTVSPIHATQVNNQIRTFISEKYGNGGDSLRFELSNPTARYTLRNGG	540
Cry2Ab	GTMIHLAPNDYTGFTVSPIHATQVNNQIRTFISEKYGNGGDSLRFELSNPTARYTLRNGG	540
	*****.*	
Cry2Ac1	NSYNLYLRVSSIGSSTIRVTINGRVYTA-NVNTTNDGVLNDGARFSDINIGNVVASAN	589
Cry2Ac	NSYNLYLRVSSIGSSTIRVTINGRVYTA-NVNTTNDGVLNDGARFSDINIGNVVASAN	590
Cry2Aa	NSYNLYLRVSSIGSSTIRVTINGRVYTVSNVNTTNDGVLNDGARFSDINIGNVVASAN	600
Cry2Ab	NSYNLYLRVSSIGSSTIRVTINGRVYTVSNVNTTNDGVLNDGARFSDINIGNVVASAN	600
	*****.*	
Cry2Ac1	TNVPLDIQVTFNGNPFQFELMNMIFVPTNLPPLY	622
Cry2Ac	TNVPLDIQVTFNGNPFQFELMNMIFVPTNLPPLY	623
Cry2Aa	TNVPLDINVTLSNGTQFELMNMIFVPTNLPPLY	633
Cry2Ab	SDVPLDINVTLSNGTQFELMNMIFVPTNLPPLY	633
	*****.*	

Figure 3.3B. Multiple amino acids sequence alignment of Cry2Ac from our lab, Cry2Aa, Cry2Ab and Cry2Ac1, from database.

From the amino acids alignment result between Cry2Ac and other Cry2A toxins it could be seen that Cry2Ac has only 623 amino acids residues, whereas Cry2Aa and Cry2Ab have 633 amino acids residues, this accounts for 10 amino acids (1kDa) difference, which cannot be visualised in SDS-PAGE gel system. Therefore, it is more likely that the physical properties of Cry2Ac cause it to run differently.

3.2.2 Control experiment for *E.coli* contaminants

After running the different Cry2A toxins on the gel we saw other proteins appear on the gel as shown in (figure 3.1). To ensure that, these other proteins do or not interfere in the toxicity of our toxin, we decided set up a control consisting of BL21contining pGEM plasmids in which Cry2Ab genes were cloned in the wrong orientation for expression. We electrophoresed these recombinants on SDS-PAGE gel to observe expression and then tested them toward *P. xylostella* (figure 3.4). It is obvious that from the gel there is no expression of the crystal protein as can be seen by the absence of any band in that position compared to Cry2Ac toxin used as positive control. To get the same concentration as Cry2Ac we compared equal volumes of samples prepared in the same way. For example, we worked out volume of samples that contains 400µg/ml Cry 2Ac and then use the same volume of control-1 and control-2.

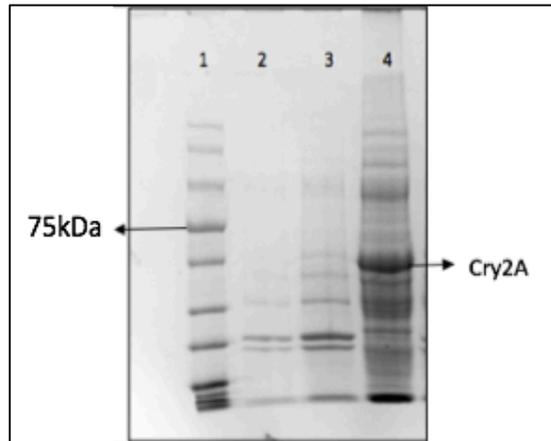


Figure 3.4. SDS-PAGE analysis of controls Cry2A along with the expressed Cry2Ac as a positive control. (1) Marker, (2) control-1, (3) control-2, (4) Cry2Ac and the arrow in the right indicate the position of the Cry2A toxin band.

Therefore, we carried out a qualitative bioassay using Cry2Ac toxin and *E.coli* proteins (control 1 and control 2) against *P. xylostella* to prove that these have no interference with bioassay result against this insect (figure 3.5).

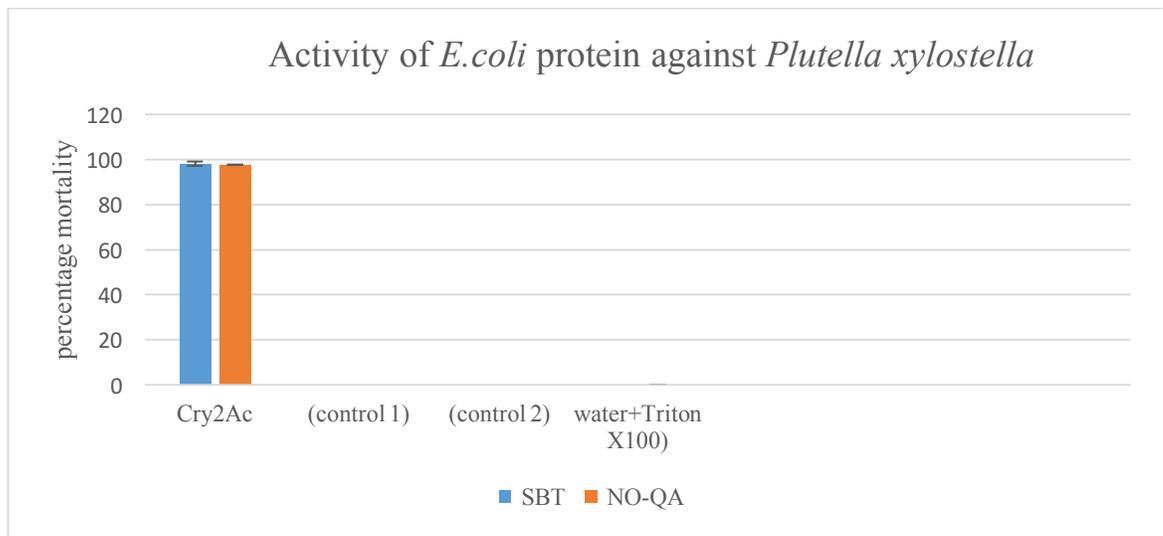


Figure 3.5. Evaluation of the level of activity of *E.coli* proteins against *Plutella xylostella*. The result for two *E.coli* proteins (control 1 and control 2) respectively, Cry2Ac as a positive control and water +triton x100 as a negative control. Percentage mortality was recorded in three different experiments after a period of three days.

It appears from the bioassay data that the two Control samples (control 1 and control 2) had no activity against *P. xylostella*, as the percentage mortality was 0%, in contrast the Cry2Ac the one that expressed and show high activity against *P. xylostella* as shown in bioassay data. This confirmed that the *E. coli* contaminants had no activity against both SBT and NO-QA populations of *P. xylostella*.

3.2.3 Result of bioassay for Cry2A toxins against Geneva-88, SBT and NO-QA populations of *Plutella xylostella*

The leaf dip bioassay experiment was carried out for all the available Cry2A toxins against a population of *P. xylostella* (Geneva-88) susceptible to Cry1Ac, following the method described in the materials and methods section. This showed that some of the toxins were toxic while others appeared to show little or no toxicity, the result is illustrated in (figure 3.6) below.

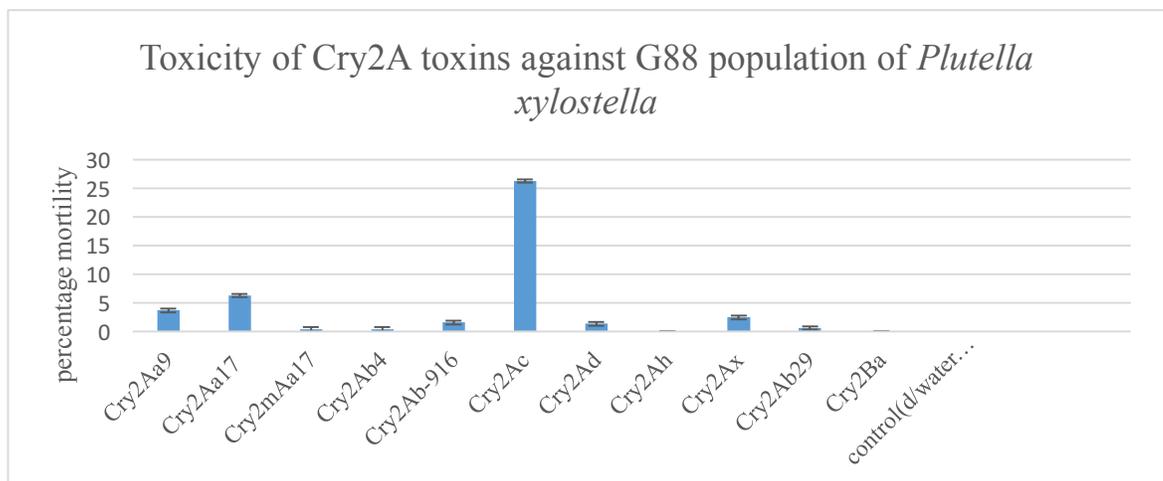


Figure 3.6. Evaluation of the level of toxicity of different Cry2A against *Plutella xylostella* Geneva-88 population. Percentage mortality recorded in three different experiments after a period of three days, the concentration of all Cry2A samples 400 µg/ml, water +tritonx100 as a negative control.

Figure 3.6 bioassay data showed that among those Cry2A toxins that were toxic against *P. xylostella* G88, Cry2Ac showed the highest level of toxicity, followed by Cry2Aa17, Cry2Aa9 Cry2Ax, Cry2Ab-916 and Cry2Ad whereas Cry2mAa17, which is a mutant of Cry2Aa17, and Cry2Ab29 showed the least level of toxicity, Cry2Ah and Cry2Ba did not have any activity. Thereafter, we used two different populations of *P. xylostella* since we lost the previous population (G-88). The SBT population which is a Cry1Ac susceptible population from Brazil, and the NO-QA population that is highly resistant to Cry1Ac, and test different Cry2A against them to see if we could gain the same result as G-88 population, but we focused on three Cry2A toxins namely: Cry2Aa, Cry2Ac and Cry2Ab, due to insect limitations and based on the fact that it may give a significant difference between Cry2Aa, Cry2Ac and Cry2Ab, and the previous bioassay results are contradictory in the literature for this reason we concentrate in these ones. We carried out bioassay against the two populations of *P. xylostella* SBT and NO-QA using Cry2Aa, Cry2Ab and Cry2Ac. The results from the bioassay conducted against these two different populations is shown in (figure 3.7).

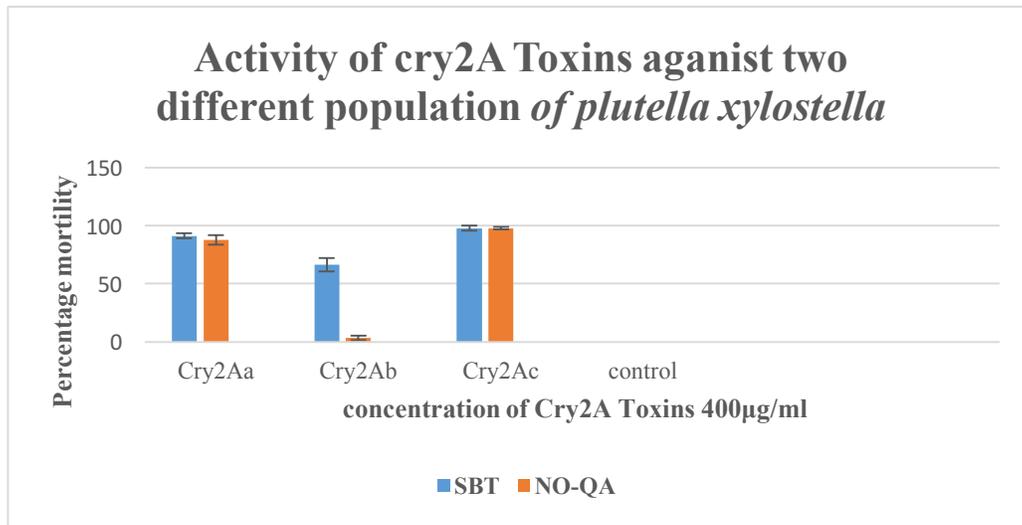


Figure 3.7. Evaluation of the level of toxicity of different Cry2A proteins against two different populations of *Plutella xylostella* (SBT and NO-QA). Percentage mortality was recorded in three different experiments after a period of three days, the concentration of all Cry2A samples was 400 µg/ml, water +tritonx100 as a negative control.

Figure 3.7 indicates that Cry2Aa, Cry2Ab, and Cry2Ac were all toxic against the SBT population of *P. xylostella*. In the case of the NO-QA population both Cry2Aa and Cry2Ac were toxic but Cry2Ab showed virtually no activity against this population. From all bioassay results it is obvious that some Cry2A toxins have different levels of activity against different population of *P. xylostella*. However, more bioassays were done to be able to establish LC₅₀ values as shown in Table2.

Toxin	Population	LC ₅₀ (µg/ml)	95% Confidence limits (µg/ml)
Cry2Aa	SBT	171.318	130.621-233.769
	NO-QA	191.737	167.265-223.364
Cry2Ab	SBT	204.910	197.962-237.010
Cry2Ac	SBT	156.558	106.636-241.649
	NO-QA	88.929	45.771-167.508

Table 2. Shows the LC50 for Cry2A toxins against the SBT and NO-QA populations of *Plutella xylostella*.

Tables 2 summarises the value of lethal concentration that can kill 50% of insects (LC₅₀) of Cry2A toxins against SBT and NO-QA populations of *P. xylostella* larvae. The level of toxicity of Cry2A were determined using SPSS software (statistical package for social science). Cry2Ac had the highest activity followed by Cry2Aa and Cry2Ab in the case of the SBT population, for NO-QA the Cry2Ac toxin also has a significant activity, followed by Cry2Aa.

3.3. Hybrid creation through domain swapping

Based on the result of bioassay data depicted in (figure3.7), we were able to discover that Cry2Ac and Cry2Aa were the most toxic toxins to the two populations of *P. xylostella* tested, whereas Cry2Ab was the one with no toxicity against NO-QA population but toxic toward SBT population. Therefore, since Cry2Ac and Cry2Aa toxins were toxic to both populations we decided to create hybrids between Cry2Ac and Cry2Ab by towards understanding which

of the domain(s) were responsible for their specificity against NQ-QA population of *P. xylostella* (figure 3.8).

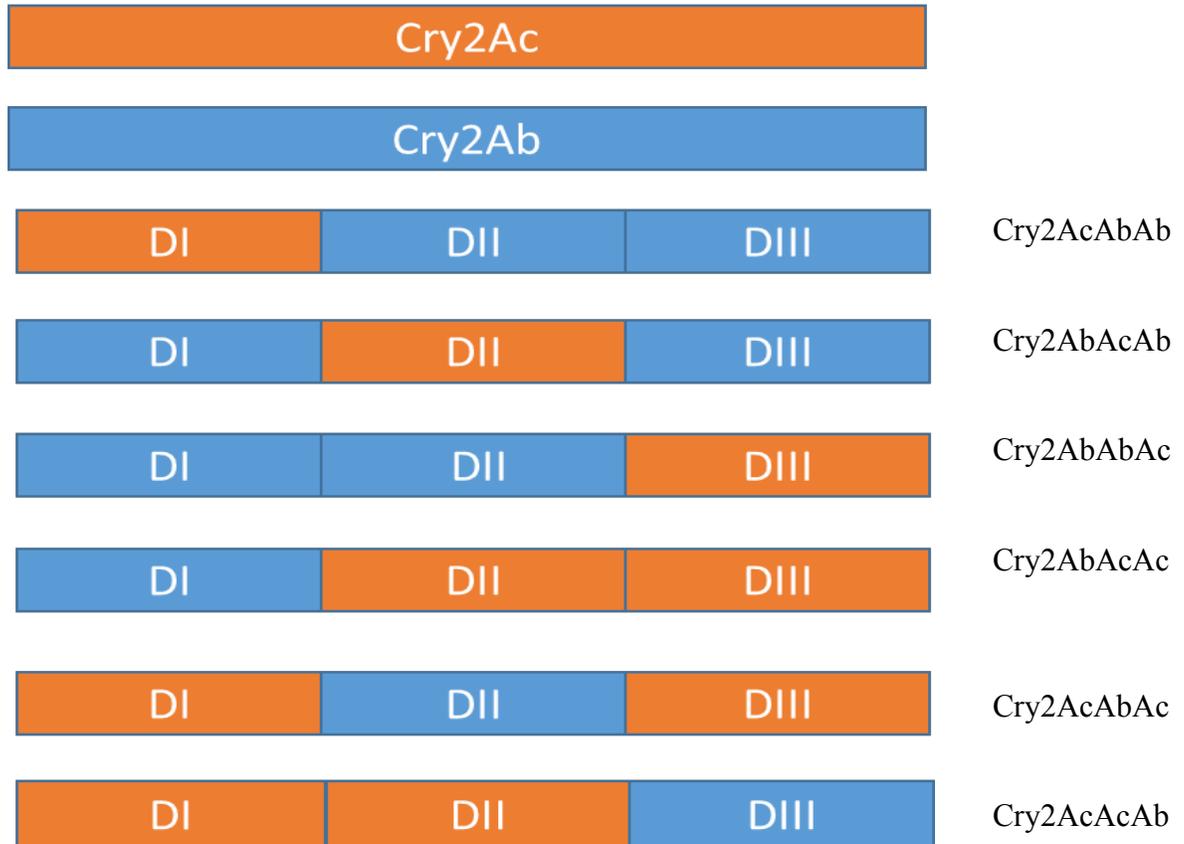


Figure 3.8. Schematic representation of the various hybrids created through domain swap. Cry2Ac represents the toxic protein, whereas Cry2Ab represents non-toxic protein.

Some previous study has implicated domain II to be responsible for specificity (Morse *et al.*, 2001). In addition, recent work carried out to identify Cry2A genes in a collection of 300 strains of *Bacillus thuringiensis*, led to the identification of a novel toxin (Cry2Aa17) which is a natural hybrid comprising of the Cry2Ab sequence in domain I and Cry2Aa sequence in domain II and III. When the toxicity profile of this toxin was assessed against *Aedes aegypti*, it was discovered that the toxicity profile matched those of Cry2Ab, thus implicating domain

I in specificity determination (Shu *et al.*, 2017). Therefore, we created hybrids through domain swaps involving all three domains. Our first task in this part of the research was to create hybrids between Cry2Ac and Cry2Ab through various domain swaps with the aim of identifying the domain that could be implicated in the specificity and/ or activity of Cry2A group of toxins against *P. xylostella*. Therefore, we started by creating hybrids involving domain I, swapping between these two important Cry2A toxins based on the data from Shu *et al.*, (2017) which showed that domain I could influence the specificity of Cry2A. After this domain III and domain II were created.

3.3.1. Creation of Domain I and III hybrids between Cry2Ac and Cry2Ab

To create hybrids through domain swapping, primers were designed (Table 3) using the primer select programs, and alignment between the all three domains sequences of Cry2Ac and Cry2Ab was conducted using Clustal Omega. The alignment results were used to design the forward and reverse primers for domain I amplification (figures 3.9 and 3.10).

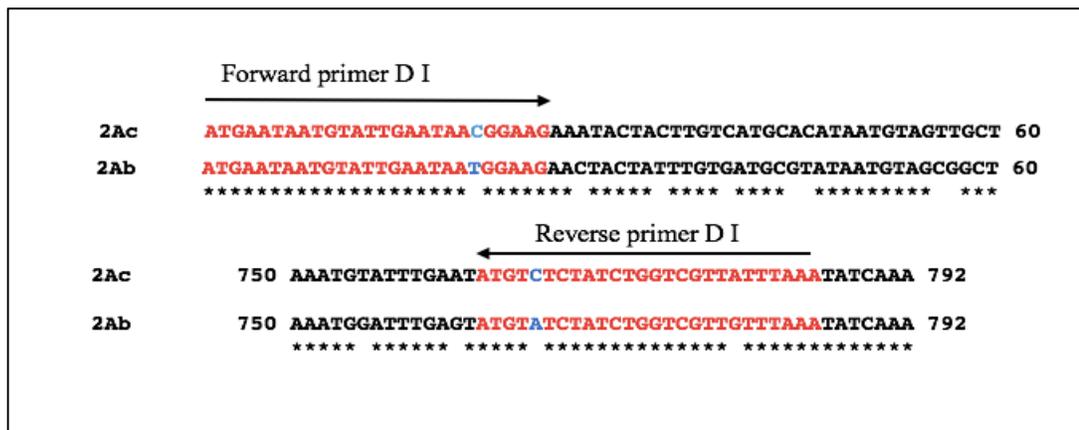


Figure 3.9. Alignment results between Cry2Ac and Cry2Ab showing the forward and reverse primer for domain I amplification indicated by arrow and degenerate base indicated by blue colour.

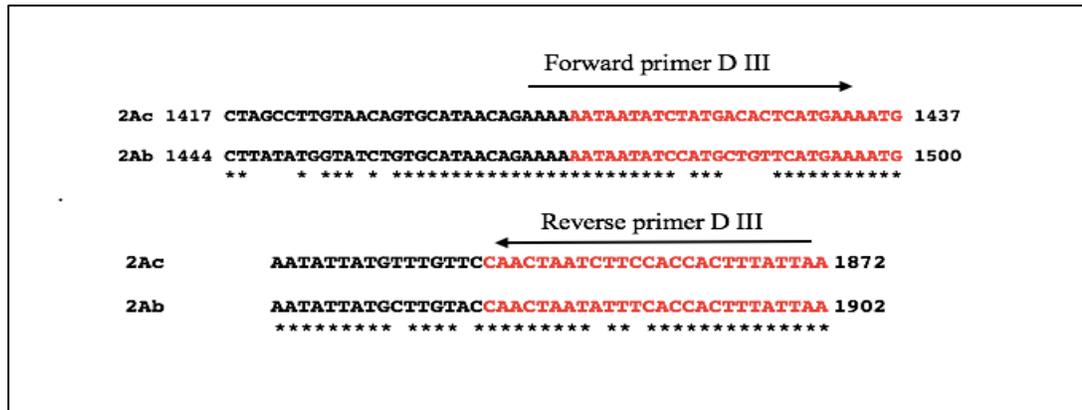


Figure 3.10. Alignment results between Cry2Ac and Cry2Ab showing the forward and reverse primer for domain III amplification indicated by arrow.

The strategy used for the creation of the hybrids involved the design of primers, which could amplify only domain I of one of the Cry2A toxin known to be active against the insect and the Domains II and III along with the pGEM plasmid of the other Cry2A which is non active. The two DNAs are then ligated together and introduced into *E.coli* after which the transformants were screened and protein grown from them as described in the materials and methods section. The plasmid diagram shows the strategy for the design of the creation of the hybrids through domain swaps as showing in (figure 3.11).

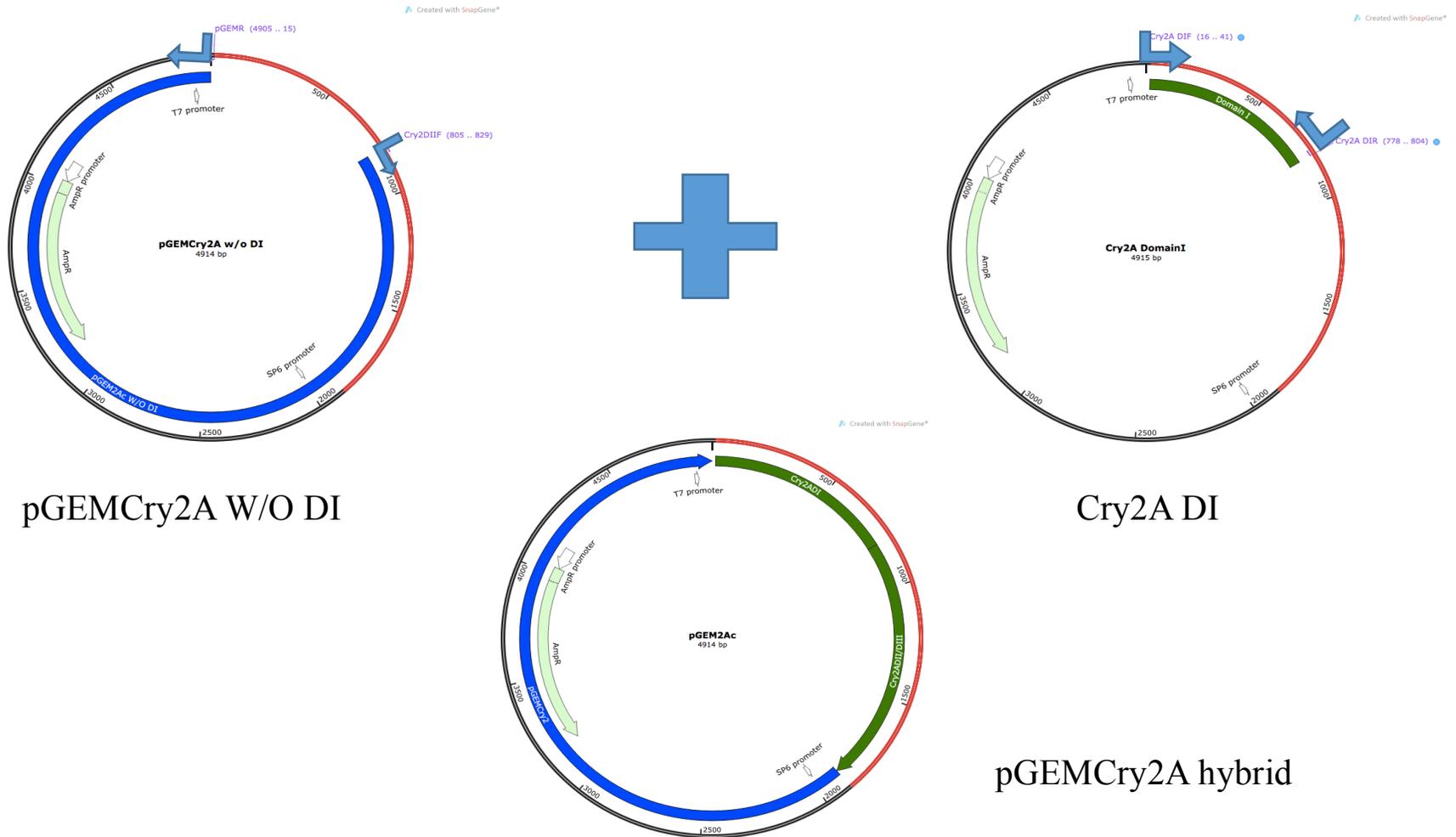


Figure 3.11. Schematic representation explaining the process of creating hybrids through domain I swap.

Domain I was amplified using Cry2A domain I forward primer and Cry2A domain I reverse primer, then we amplified pGEMCry2A without domain I which has domain II and domain III with the plasmid but without domain I, and this portion was also amplified using, Cry2ADIIF forward primer and pGEM reverse primer as depicted in (figure3.11).

The primers used for the amplification of the various domains, also used for the creation of the hybrids are depicted in table 3. In the primer that used to amplify domain I forward (Cry2ADIF) there is one area replaced with Y and for domain I reverse there are two areas replaced with R and K, we made these primers degenerate to allow these primers to bind DNA nucleotide in different Cry2A toxin.

Table 3. Show list of primers used to create hybrids through domains swap.

NO	Oligo Name	Sequence (5'-3')	Modification
1	Cry2DIF	ATGAATAATGTATTGAATAAYGGAAG	5'-PHO
2	Cry2DIR	TTTAAATAACGACCAGATRGAKACATA	'PHO
3	Cry2DIIF	TATCAAAGCCTTCTAGTATCTTCYG	
4	pGEMR	ATAAAATTCCTCCTTAATCGAATTC	
5	Cry2AcDIIF	AATAATATCTATGACACTCATGAAAATG	5'-PHO
6	Cry2ADIIR	TTAATAAAGTGGTGAAGATTAGTTG	5'-PHO
7	Cry2AbDIIF	AATAATATCCATGCTGTTTCATGAAAATG	5'-PHO
8	Cry2AbDIIR	TTAATAAAGTGGTGAATATTAGTTG	5'-PHO
9	pGEMDIIF	GGTTTGAGTGAATCACTAGTGAATTC	
10	2ACDIIR	TTTTCTGTTATGCACTGTTACAAG	

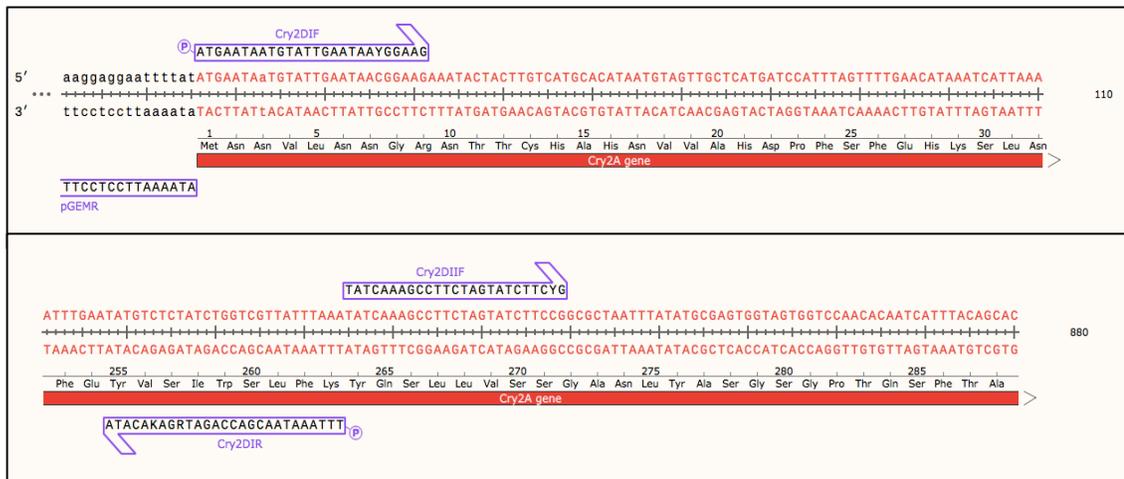


Figure 3.12. Schematic representation for forward and reverse primers used in PCR to create hybrids with domain I swap. Both primers were phosphorylated (Cry2ADIF and Cry2ADIR) to facilitate ligation of 5' phosphate group with the free 3' OH group on the amplified plasmid.



Figure 3.13. Schematic representation for forward and reverse primers used in PCR to create hybrids with domain III swap.

The PCR (PFU ultra 6kb) reaction was performed on pGEM Cry2A plasmid, and the amplified products were digested with *DpnI* to remove methylated template DNA, purified using QIAquick kit and electrophoresed on 1% agarose gels to prepare them for ligation. This is indicated in (figure 3.14) for domain I amplification.

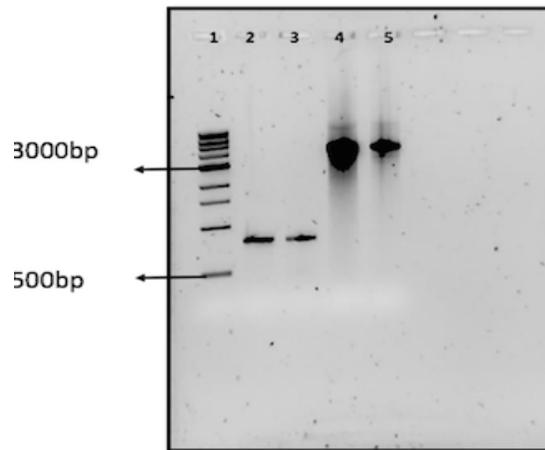


Figure 3.14. Confirmation of presence of amplified PCR products: Lane (1) Marker, lane (2) Cry2Ac domain I, lane (3) Cry2Ab domain I, lane (4) pGEM2Ac w/o domain I and lane (5) pGEM2Ab w/o domain I.

According to (figure 3.14) the size of the amplified products presents in the gel for Cry2A domain I was (789bp) and pGEM Cry2Ac without domain I, position on the gel was consistent with expected sizes (4125bp). The PCR products were then purified, the amplicons were ligated, and the circularized plasmid used to transform into NEB-5 competent *E. coli* cells. The transformed colonies were streaked on an ampicillin plate and extracted plasmid DNA later digested with *HaeIII* restriction enzyme to be able to know which of the colonies contain the right transformants. These colonies were electrophoresed on an agarose gel and the fragments obtained were compared to those generated using NEB cutter. Those colonies that appeared to have the right fragments were further digested with *BsaAI* to confirm if the inserted fragments were in the right orientation, this is because the domain I of Cry2A genes has no *HaeIII* restriction digest site, hence *HaeIII* cannot be used to confirm the orientation of the gene in domain I fragment.

For those hybrids involving domain II and III swapping, the second digestion was done by using the *SacI* restriction enzyme (figure 3.23 and 3.29).

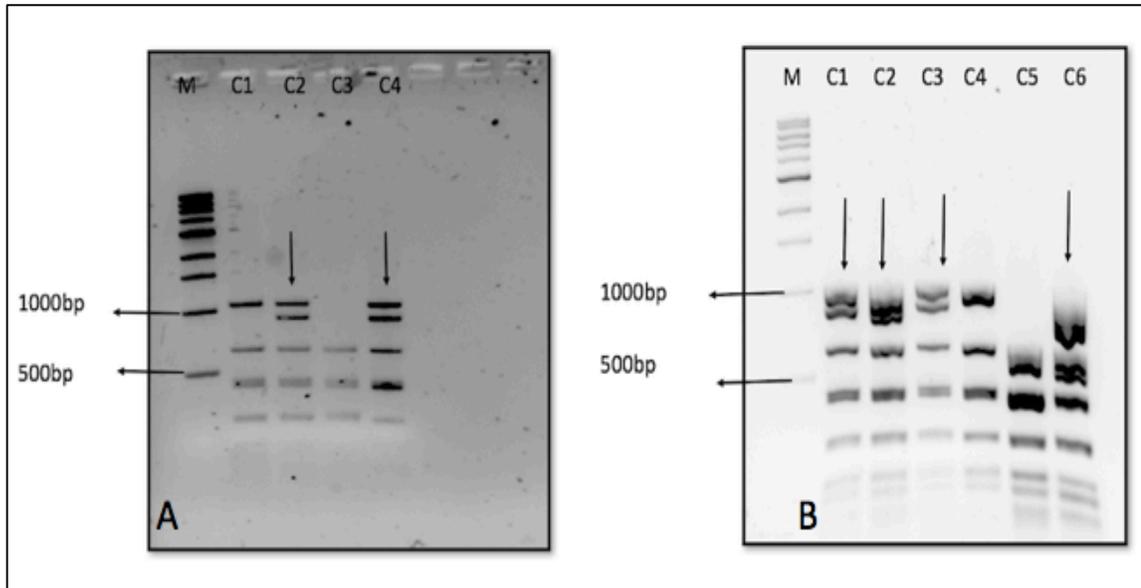


Figure 3.15. DNA banding pattern after *HaeIII* digest. Gel A shows Cry2AcAbAb clones and Gel B shows Cry2AbAcAc DNA clones digested with *HaeIII* restriction enzyme. The correct colonies are indicated by arrows.

Figure 3.15 shows that the colonies 2 and 4 from gel A and colonies 1, 2, 3, and 6 from gel B have the correct plasmids were compared with the fragments generated from NEB cutter prediction as indicated in (figure 3.16). Hence, they were further digested with *BsaAI* to determine which have the insert in the right orientation as shown in (figure 3.17).

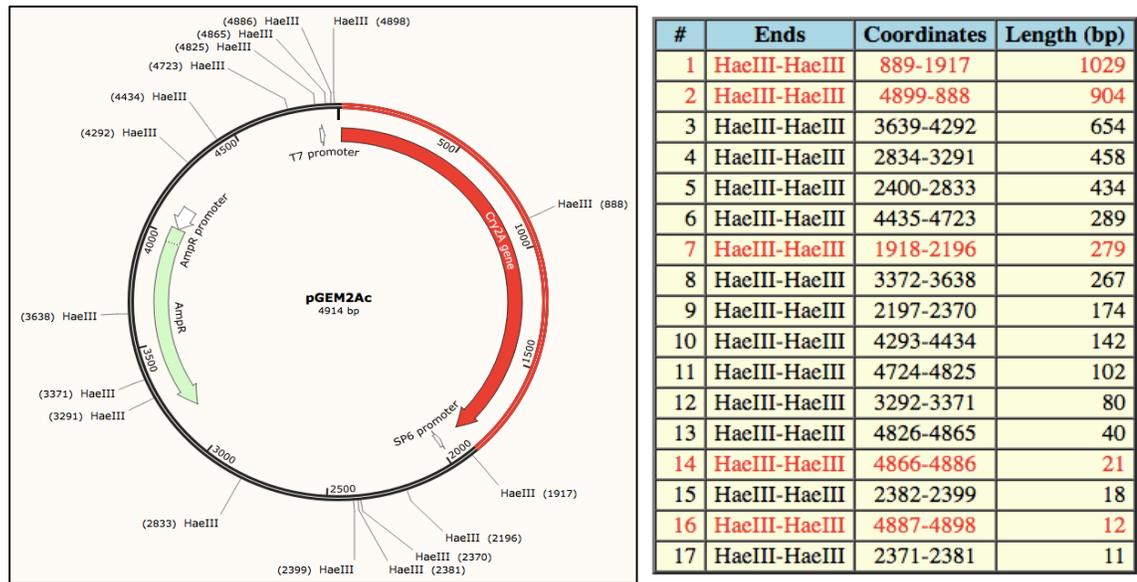


Figure 3.16. This illustrates the plasmid of pGEM2Ac and table lists the expected fragments digested with *HaeIII* restriction enzyme. Taken from NEB cutter website.

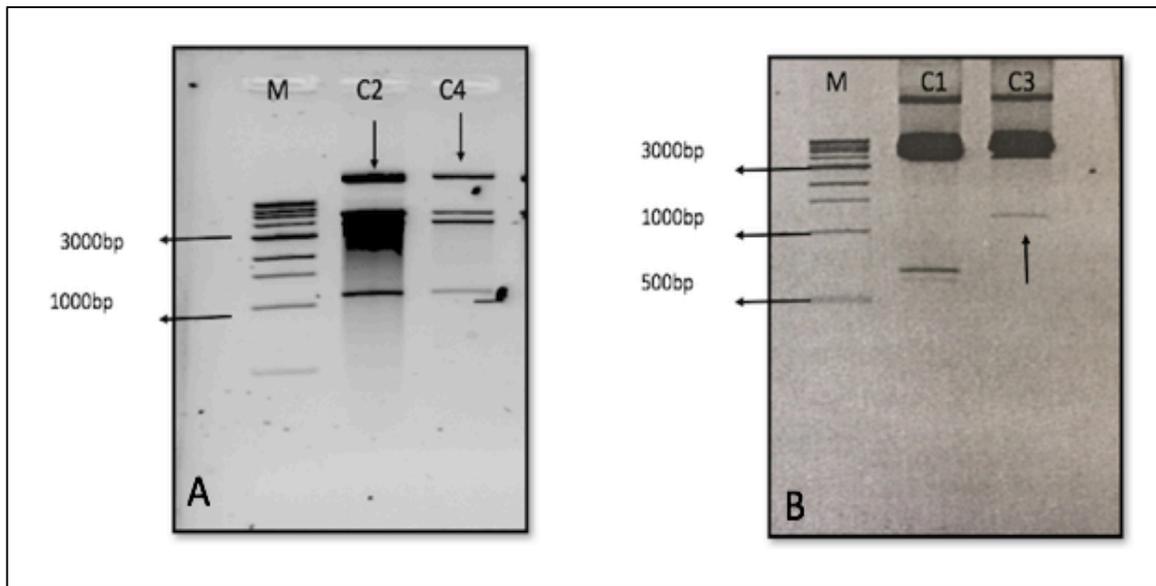


Figure 3.17. DNA banding pattern after digested with *BsaAI* restriction enzyme to confirm which of these inserts have the right orientation. Gel A is showing Cry2AcAbA clones digested with *BsaAI* restriction enzyme and gel B is showing Cry2AbAcAc clones digested with the same restriction enzyme the right colonies indicated by arrow.

Figure 3.17 shows that from gel A both colonies 2 and 4 seem to have the inserts in the right orientation after the *Bsa*AI restriction digest. Hence, they were both sent for sequencing, from which it was confirmed through sequence alignment with the construct that (Cry2AcAbAb) colony 2 from gel A and colony 3 that represent (Cry2AbAcAc) from gel B have the correct plasmids. The right hybrids through domain I swap were created, based on the alignment result we can confirm that domain I from the sequencing result (Cry2AcAbAb) is a match with Cry2Ac before the junction and after the junction between domain I and domain II, indicated by a red line as represented in (figure 3.19), the sequence matches Cry2Ab. Also, for the hybrid (Cry2AbAcAc) is matching with Cry2Ab before the junction but after the junction between domain I and II the sequence matches Cry2Ac (figure 3.20). Therefore, these hybrids were subsequently introduced to BL21 for the expression of their proteins.

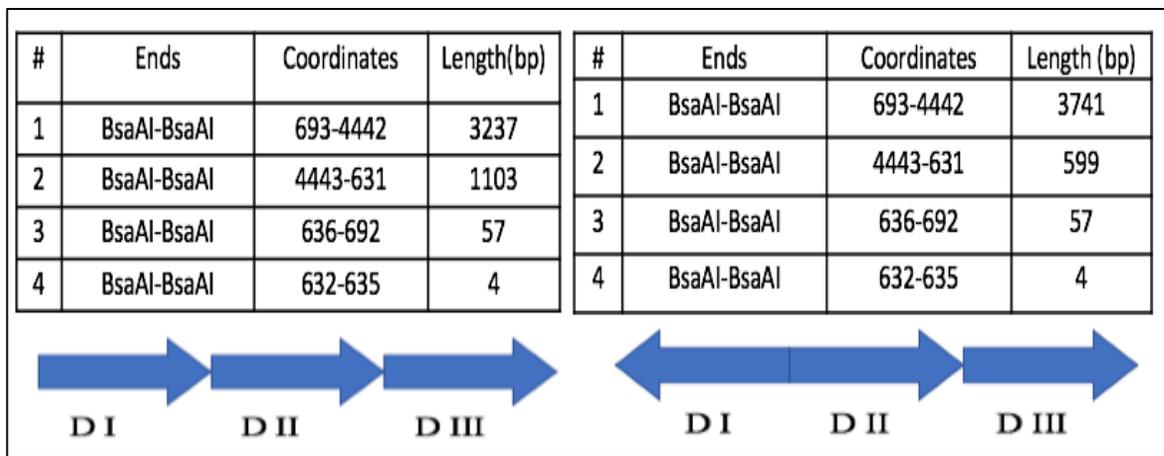


Figure 3.18. Restriction fragments generated from NEB cutter digested with *Bsa*AI restriction enzyme. Shows A orientation represented by arrow pointing towards the right direction, and table (2) shows B orientation represented by arrow pointing towards the left direction.

3.3.2. Creation of hybrids through domain III swapping

The hybrids used (pGEMCry2AcAcAb) and (pGEMCry2AbAbAc) were utilized to create another hybrid to ensure the swapping of domain III. PCR (PFU ultra 6kb) was performed on pGEM Cry2A plasmid, and the amplified products were digested with *DpnI*, purified and electrophoresed on 1% agarose gel.

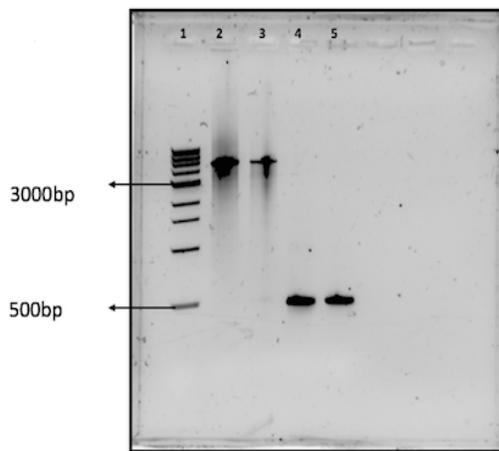


Figure 3.21. Confirmation of presence of amplified PCR products. Lane (1) Marker, lane (2) pGEM2Ac plasmid w/o domain III, lane (3) pGEM2Ab plasmid w/o domain III and lane, lane (4) Cry2Ac domain III (5) Cry2Ab domain III.

The amplified PCR products present in the gel (figure 3.21) show the position on gel consistent with the expected size of the products, which is (495bp) for Cry2Ac and Cry2Ab domain III and pGEM without domain III about (4505bp). The amplicons were ligated, and the circularized plasmid used to transform into NEB-5 competent *E. coli* cells. Extracted plasmid DNA was digested with *HaeIII* restriction enzyme (figure 3.22) as well as a second digestion by using *SacI* restriction enzyme to be able to know which colonies have the insert in the right orientation as shown in (figure 3.23) and sequencing was carried out to confirm the presence of correct constructs as shown in (figure 3.25 and 3.26).

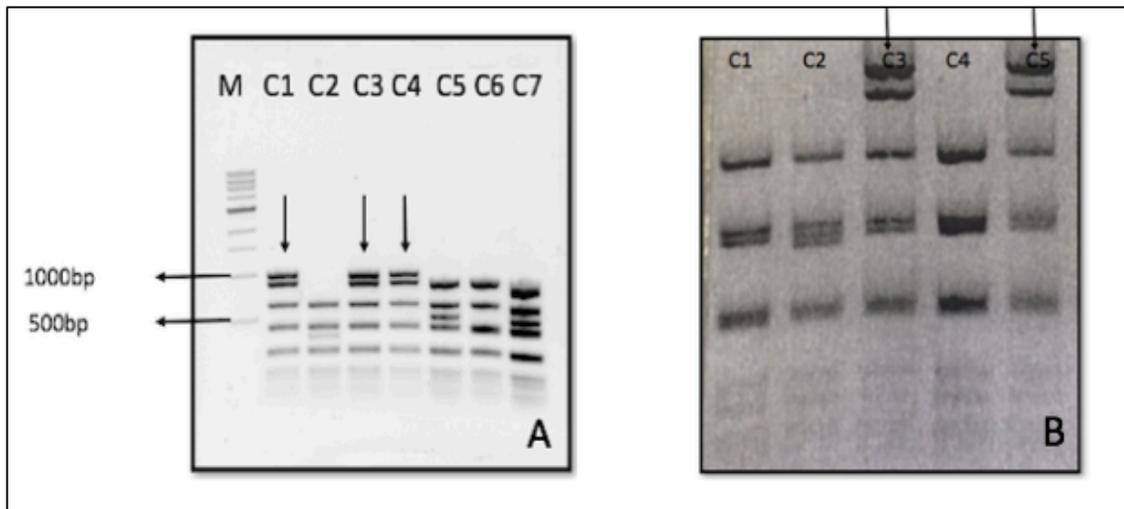


Figure 3.22. DNA banding pattern after *HaeIII* digest. Gel A shows Cry2AbAbAc clones and Gel B shows Cry2AcAcAb clones digested with *HaeIII* restriction enzyme. The correct colonies are indicated by arrows.

Figure 3.22 shows that colony 1, 3, and 4 for 2AcAcAb hybrid (Gel A) and for 2AbAbAc DNA (Gel B) hybrid colony 3 and 5 have the correct insert with the right transformants when compared with the fragments generated from NEB cutter prediction as indicated in (figure 3.31). Hence, they were further digested with *SacI* as shown in (figure 3.24).

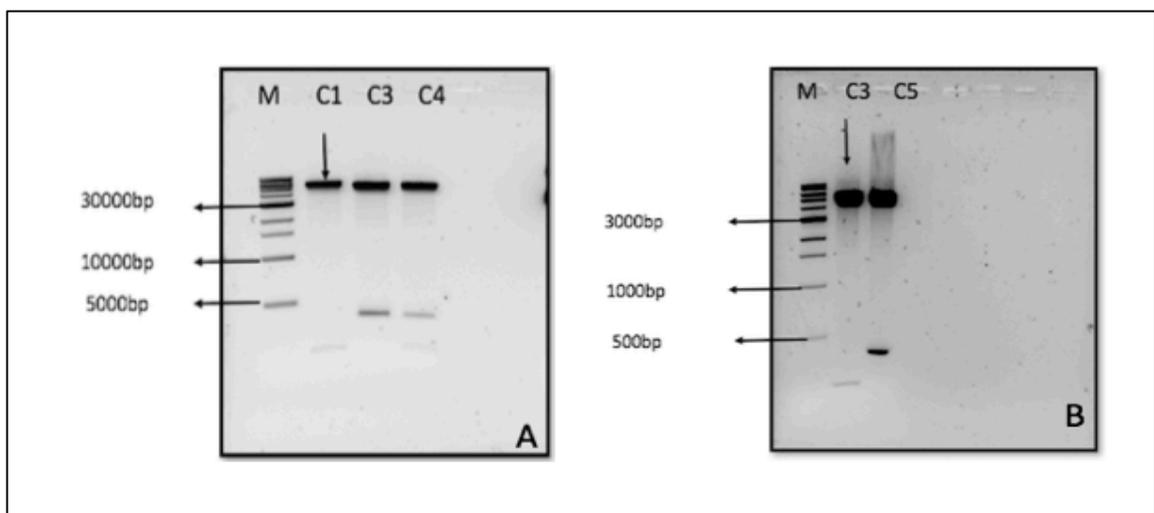


Figure 3.23. DNA banding pattern after digested with *SacI* restriction enzyme to confirm which of these inserts have the right orientation. Gel A is showing Cry2AbAbAc DNA colonies digested with *SacI* restriction enzyme and gel B is showing Cry2AcAcAb clones digested with the same restriction enzyme the right colonies indicated by arrow.

2Ab	ATAATGAAATAAGAAATATAGCAAGTCCTTCAGGAACACCTGGTGGAGCACGAGCTTATA	1440
2Ac	TAAATGAAATAAGAGATATAGGAACGACAG-----CAGTCGCTAGCC	1363
AcAcAb	TAAATGAAATAAGAGATATAGGAACGACAG-----CAGTCGCTAGCC ***** ** *	381
2Ab	TGGTATCTGTGCATAACAGAAAAATAATATCCATGCTGTTTCATGAAAATGGTTCATGA	1500
2Ac	TTGTAAACAGTGCATAACAGAAAAATAATATCTATGACACTCATGAAAATGGTACTATGA	1423
AcAcAb	TTGTAAACAGTGCATAACAGAAAAATAATATCCATGCTGTTTCATGAAAATGGTTCATGA * ** * ***** ** *****	441
2Ab	TTCATTTAGCGCCAAATGACTATACAGGATTTACTATTTGCGCCGATACATGCAACTCAAG	1560
2Ac	TTCATTTAGCGCCAAATGACTATACAGGATTTACCGTATCTCCAATACATGCCACTCAAG	1483
AcAcAb	TTCATTTAGCGCCAAATGACTATACAGGATTTACTATTTGCGCCGATACATGCAACTCAAG ***** * ** * *****	501
2Ab	TGAATAATCAAAACACGAACATTTATTTCTGAAAAATTTGGAAATCAAGGTGATTCCTTAA	1620
2Ac	TAAATAATCAAAATTCGAACGTTTATTTCCGAAAAATATGGTAATCAGGGTGATTCCTTGA	1543
AcAcAb	TGAATAATCAAAACACGAACATTTATTTCTGAAAAATTTGGAAATCAAGGTGATTCCTTAA * ***** ** ***** ** ***** ** *	561

Figure 3.26. Alignment of the sequence received from sequencing result of pGEM(Cry2AcAcAb) and the sequence of Cry2Ac and Cy2Ab. The sequence of the DNA obtained from DH5 and the sequence of Cry2Ac and Cry2Ab were aligned using Clustal Omega software. The red line indicates the junction between domain II and III.

3.3.3 Creation of hybrids through domain II swapping

To create hybrids with domain II swap the same primers listed in Table 3 were used and we used the PCR amplification products which when ligated could produce the required hybrids. For instance, in creating the hybrid Cry2AbAcAb we amplified 2AbAcAc without domain III and ligated it to 2Ab domain III to give rise to the hybrid 2AbAcAb. PCR (PFU ultra 6kb) was performed, and the amplified products were digested with *DpnI*, purified and electrophoresed on 1% agarose gel to prepare them for ligation. (Figure 3.27) indicates the purified PCR products for domain II swaps.

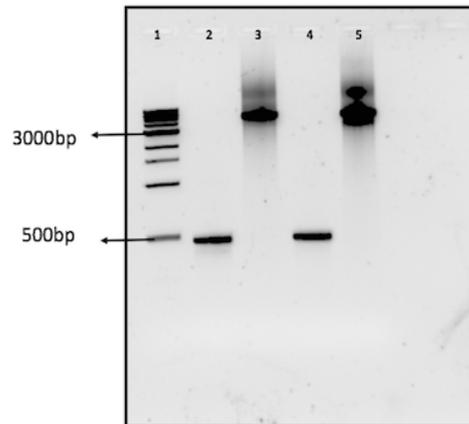


Figure 3.27. Confirmation of presence of amplified PCR products. Lane (1) Marker, lane (2) Cry2Ac domain III, lane (3) pGEMCryAcAb2Ab w/o domain III plasmid, lane (4) Cry2Ab domain III and lane (5) pGEM Cry2AbAcAc w/o domain III plasmid.

The amplified PCR products present in the gel in (figure 3.27) were consistent with the expected size for Cry2Ac and Cry2Ab domain III, also for pGEM Cry2A without domain III is around 4505bp. The amplicons were ligated and plasmid from resulting clones digested with *Hae*III restriction enzyme (figure 3.28). A second digestion using *Sac*I as shown in (figure 3.29) and sequencing were carried out to confirm the presence of right constructs as shown in (figure 3.30 and 3.31).

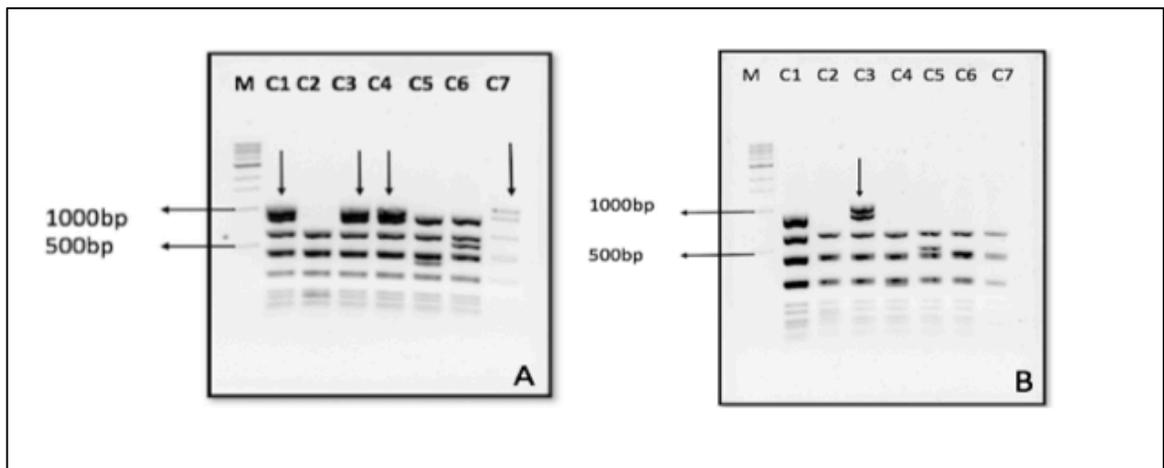


Figure 3.28. DNA banding pattern after *Hae*III digest. Gel A shows Cry2AcAbAc clones and Gel B shows Cry2AbAcAb clones digested with *Hae*III restriction enzyme. The correct colonies are indicated by arrows.

The DNA colonies with the right plasmids were compared with the fragments generated from NEB cutter prediction as indicated previously in (figure 3.16). Hence, they were further digested with *SacI* to be able to know which ones had the insert in the right orientation (figure 3.29).

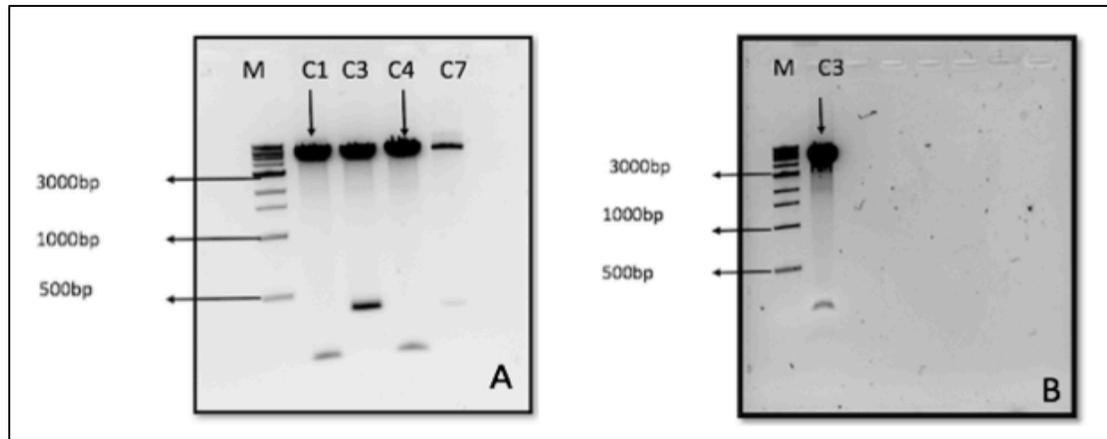


Figure 3.29. DNA banding pattern after digested with *SacI* restriction enzyme to confirm which of these inserts have the right orientation. Gel A is showing Cry2AcAbAc clones digested with *SacI* restriction enzyme and gel B is showing Cry2AbAcAb clones digested with the same restriction enzyme the right colonies indicated by arrow.

From the above (figure 3.29) the DNA gel A and B shows colony 1 and 4 from gel A and colony 3 from gel B seems to have the inserts in the right orientation after the *SacI* restriction digest. Hence, they all three were sent for sequencing, and were all confirmed through sequence alignment with their respective constructs to have the right sequences.

```

AbAcAb      TATTTTATCCGTAATATTTCTGGTGTGTTGGGACTATTAGCAACGCAGATTTAGCAAGA 188
2Ab         TATTTTATTCGTAATATTTCTGGAGTTCCTTTAGTTGTTAGAAATGAAGATTTAAGAAGA 1320
2Ac         TATTTTATCCGTAATATTTCTGGTGTGTTGGGACTATTAGCAACGCAGATTTAGCAAGA 1311
*****
abacab      CCTCTACACTTTAATGAAATAAGAGATATAGGAACGACAGC-----A 230
cry2ab     CCGTTACACTATAATGAAATAAGAATATAGCAAGTCCCTTCAGGAACACCTGGTGGAGCA 1380
Cry2Ac:    CCTCTACACTTTAATGAAATAAGAGATATAGGAACGACAGC-----A 1353
** *****
AbAcAb      GTCGCTAGCCTTGTAACAGTGCATAACAGAAAAATAAATATCCATGCTGTTCATGAAAAAT 290
2Ab        CGAGCTTATATGGTATCTGTGCATAACAGAAAAATAAATATCCATGCTGTTCATGAAAAAT 1440
2Ac        GTCGCTAGCCTTGTAACAGTGCATAACAGAAAAATAAATATCTATGACACTCATGAAAAAT 1413
*** * ** * *****
AbAcAb      GGTTCATGATTCATTTAGCGCCAAATGACTATACAGGATTTACTATTTTCGCCGATACAT 350
2Ab        GGTTCATGATTCATTTAGCGCCAAATGACTATACAGGATTTACTATTTTCGCCGATACAT 1500
2Ac        GGTACTATGATTCATTTAGCGCCAAATGACTATACAGGATTTACCGTATCTCCAATACAT 1473
** *****
AbAcAb      GCAACTCAAGTGAATAATCAAACACGAACATTTATTTCTGAAAAATTTGGAAATCAAGGT 410
2Ab        GCAACTCAAGTGAATAATCAAACACGAACATTTATTTCTGAAAAATTTGGAAATCAAGGT 1560
2Ac        GCCACTCAAGTGAATAATCAAATTCGAACGTTTATTTCCGAAAAATATGGTAATCAGGGT 1533
** *****

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Figure 3.30. Alignment of the sequence received from sequencing result of pGEM(Cry2AbAcAb) and the original sequence of Cry2Ac and Cy2Ab: the sequence of the hybrid DNA and the sequence of the Cry2Ac and Cry2Ab were aligned using Clustal Omega software, the red line indicated to the junction between domain II and III.

```

2Ac         GTAATATTTCTGGTGTGTTGGGACTATTAGCAACGCAGATTTAGCAAGACCTCTACACT 1321
2Ab         GTAATATTTCTGGAGTTCCTTTAGTTGTTAGAAATGAAGATTTAAGAAGACCGTTACACT 1380
AcAbAc     GTAATATTTCTGGAGTTCCTTTAGTTGTTAGAAATGAAGATTTAAGAAGACCGTTACACT 356
*****
2Ac         TTAATGAAATAAGAGATATAGGAACGACAGC-----AGTCGCTAGCC 1363
2Ab        ATAATGAAATAAGAATATAGCAAGTCCCTTCAGGAACACCTGGTGGAGCAGCAGCTTATA 1440
AcAbAc     ATAATGAAATAAGAATATAGCAAGTCCCTTCAGGAACACCTGGTGGAGCAGCAGCTTATA 416
*****
2Ac         TTGTAACAGTGCATAACAGAAAAATAAATATCTATGACACTCATGAAAAATGGTACTATGA 1423
2Ab        TGGTATCTGTGCATAACAGAAAAATAAATATCCATGCTGTTCATGAAAAATGGTTCATGA 1500
AcAbAc     TGGTATCTGTGCATAACAGAAAAATAAATATCTATGACACTCATGAAAAATGGTACTATGA 476
* ** * *****
2Ac         TTCATTTAGCGCCAAATGACTATACAGGATTTACCGTATCTCCAATACATGCCACTCAAG 1483
2Ab        TTCATTTAGCGCCAAATGACTATACAGGATTTACTATTTTCGCCGATACATGCAACTCAAG 1560
AcAbAc     TTCATTTAGCGCCAAATGACTATACAGGATTTACCGTATCTCCAATACATGCCACTCAAG 536
*****
2Ac         TAAATAATCAAATTCGAACGTTTATTTCCGAAAAATATGGTAATCAGGGTGATTCCCTTGA 1543
2Ab        TGAATAATCAAACACGAACATTTATTTCTGAAAAATTTGGAAATCAAGGTGATTCTTTAA 1620
AcAbAc     TAAATAATCAAATTCGAACGTTTATTTCCGAAAAATATGGTAATCAGGGTGATTCCCTTGA 596
* *****

```

Figure 3.31. Alignment of the sequence received from sequencing result of pGEM(Cry2AcAbAc) and the original sequence of Cry2Ac and Cy2Ab: the sequence of the hybrid DNA and the sequence of the Cry2Ac and Cry2Ab were aligned using Clustal Omega software, the red line indicated to the junction between domain II and III.

Next, all the confirmed constructs were introduced into BL21 *E. coli* for the expression of their respective proteins. These were then grown, the hybrid proteins were harvested, and a 7.5 % protein SDS-PAGE was performed to confirm the presence of the hybrid toxins along with the wild type as can be seen in (Figure 3.32) the gel below shows the six hybrids (from 4-6 in the gel) created through various domain swaps between the two Cry toxins.

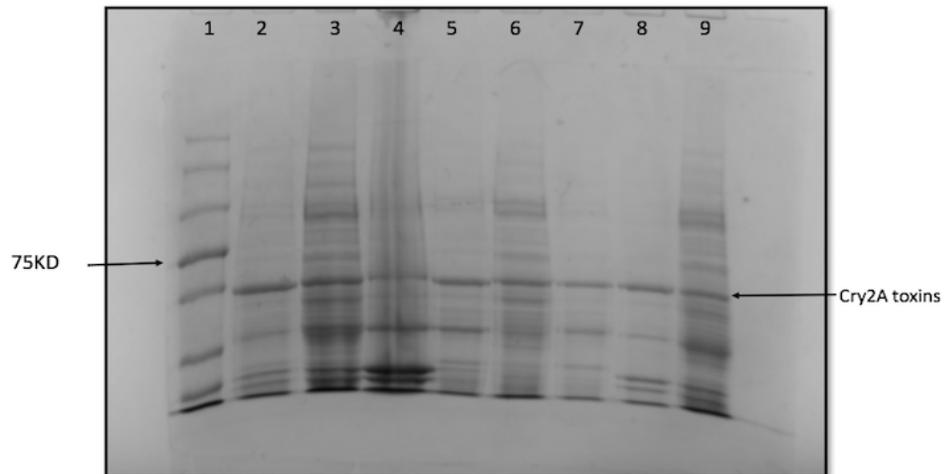


Figure 3.32. SDS-PAGE analysis of the various hybrid toxins created indicated by number compared their size with the wild type Cry2Ab and Cry2Ac. (1) Marker, (2) Cry2Ac, (3) Cry2Ab, (4) Cry2AcAbAb (5) Cry2AbAcAc, (6) Cry2AcAbAc, (7) Cry2AbAcAb, (8) Cry2AcAcAb and (9) Cry2AbAbAc.

Figure 3.32 clearly shows that all the hybrid proteins created through the various domain swaps were expressed as can be seen by the presence of the various bands indicated by the arrow. The toxicity level of all Cry2A hybrids were assessed by bioassay towards two different populations of *P. xylostella* SBT and NO-QA. The qualitative bioassay results to evaluate the level of toxicity of the various hybrids created on SBT and NO-QA populations of *P. xylostella* is shown in (figure 3.33 and 3.34).

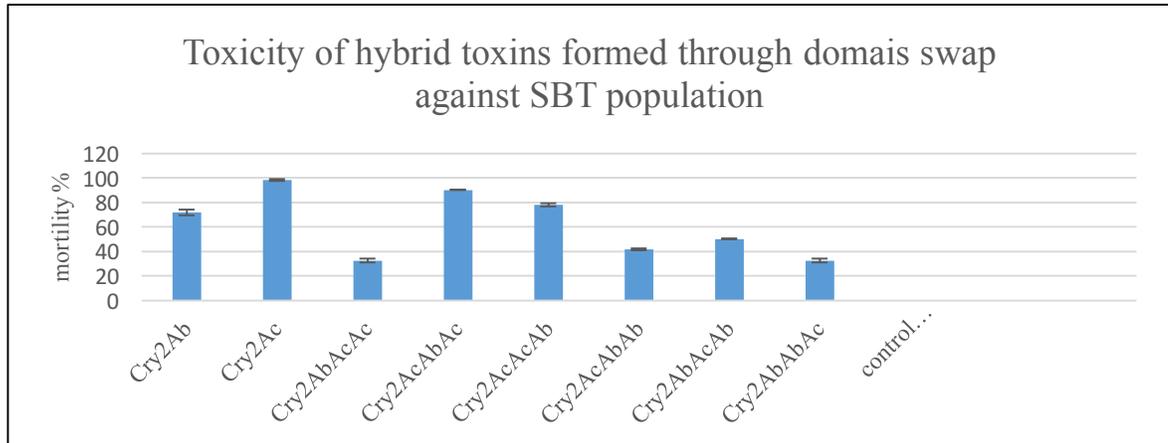


Figure 3.33. Evaluation of the level of toxicity of various hybrids created through domain swap between Cry2Ab and Cry2Ac against SBT population of *Plutella xylostella*. Cry2Ac as a positive control and water +tritonx100 as a negative control Percentage mortality was recorded in three different experiments after a period of three days. The concentration of each toxin was 400 µg/ml.

The results of the bioassays show that all the hybrids demonstrated activities against the SBT population of *P. xylostella*, which confirms that the hybrids were functional.

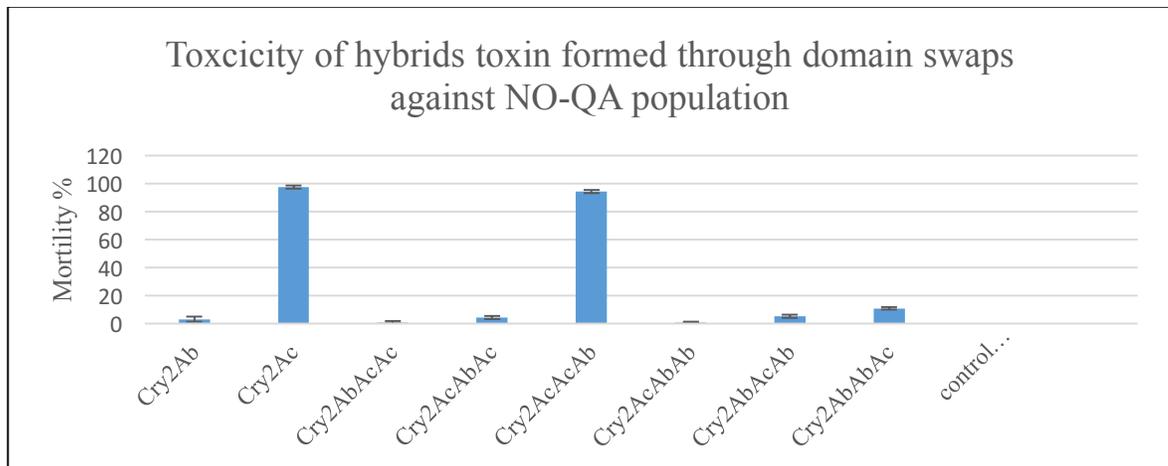


Figure 3.34. Evaluation of the level of toxicity of various hybrids created through domain swap between Cry2Ab and Cry2Ac against NO-QA population of *Plutella xylostella*. Cry2Ac as a positive control and water +tritonx100 as a negative control Percentage mortality recorded in three different experiments after a period of three days. The concentration of Cry2Awas 400 µg/ml. Hybrids involve Cry2Ab in domain I, II and III

Figures 3.34 illustrate that the level of toxicity of various hybrids against the NO-QA population of *P. xylostella*. Some of the hybrids show slight activity against the NO-QA population, but less than the bench mark of 10% set out for significance hence they are still considered as non-active except the hybrid which involve Cry2Ac in domain I and II, Cry2Ab in domain III (Cry2AcAcAb). Cry2Ab is non-toxic to this population but Cry2Ac is toxic when the hybrid has Cry2Ac in domain I and II, that is convert the toxicity to non-toxic one which Cry2Ab. From this result it is clear that domain I and domain II, but not domain III influences the toxicity toward the NO-QA population of *P. xylostella*.

3.3.4 Hybrids creation through N-terminal swapping

Previous work (Joseph unpublished) on *Aedes aegypti*, found that toxicity was associated with the N-terminal 49 amino acids in Cry2A toxins. He created hybrids between Cry2Ac which was toxic to *A. aegypti* and Cry2Ab, which was non-toxic, through swapping only the N-terminal 49 amino acids part from domain I of the toxic Cry2Ac to non-toxic Cry2Ab (figure 3.35). The result was that Cry2Ac lost its toxicity when it had Cry2Ab at the N-terminus, and Cry2Ab gained toxicity when it had Cry2Ac at the N-terminal. Therefore, the level of toxicity of these hybrids were tested against two different populations of *P. xylostella* to find out if the result would be the same or different from *A. aegypti*.

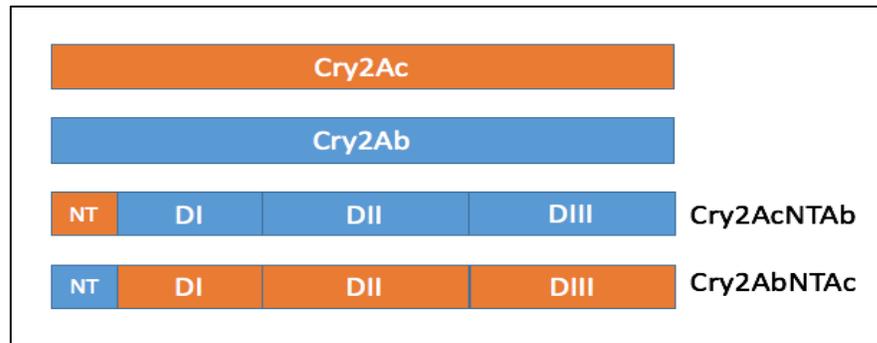


Figure 3.35. Schematic representation of hybrids created through domain swap. Cry2Ac represents toxin, whereas Cry2Ab represent non-toxic protein.

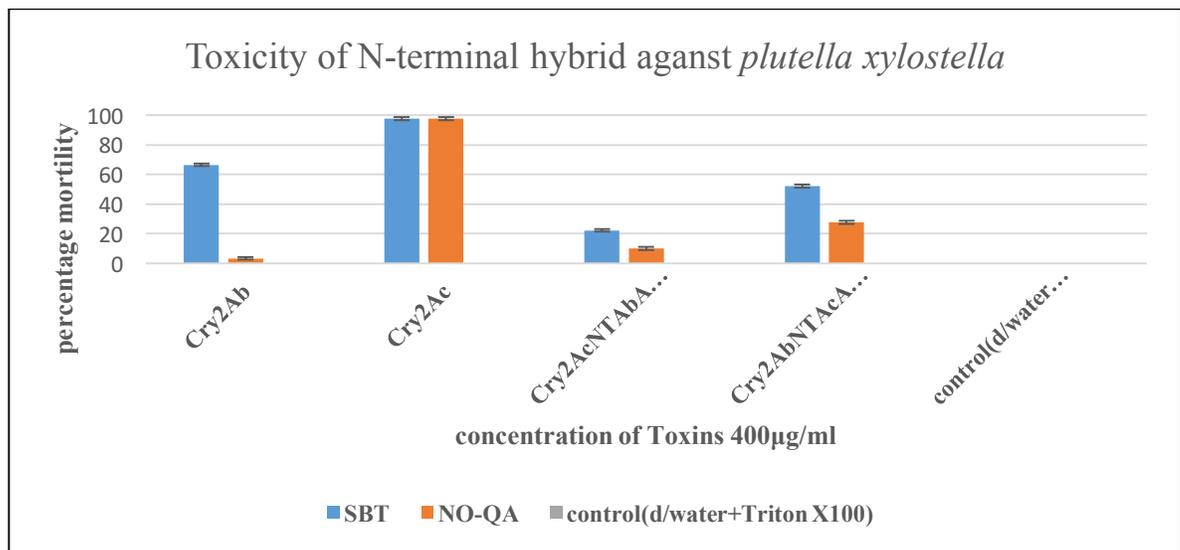


Figure 3.36 Evaluation of the level of toxicity of hybrids created through N-terminal swap between Cry2Ab and Cry2Ac against the NO-QA and SBT populations of *Plutella xylostella*. Cry2Ac as a positive control and water +tritonx100 as a negative control, Percentage mortality recorded in three different experiments after a period of three days. The concentration of all toxins was 400 µg/ml.

According to (figure 3.36), it appears that adding Cry2AcNT to Cry2Ab may have had slight effect in increasing activity, and adding Cry2AbNT to Cry2Ac have had slight effect in decreasing activity. we decided to create another hybrid consisting of Cry2Ac at the N-terminal portion of domain I and domain II, and Cry2Ab in the remaining parts to see whether

this N- terminal 49 amino acids association with domain II was enough to determine specificity for this reason Cry2AcNTAbAcAb was made (figure 3.37).

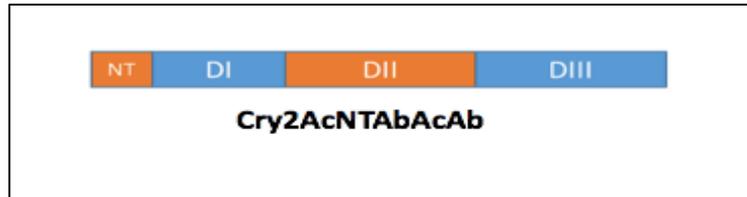


Figure 3.37 Schematic representation of hybrid formed between N-terminal and domains II of Cry2Ac, and Cry2Ab.

To create this hybrid (Cry2AcNTAbAcAb) the following primers were used. Some were phosphorylated to allow ligation of the 5' phosphate group with the free 3' OH group on the amplified plasmid.

Table 4. Show list of primers used to create hybrids between N-terminal and domain II.

NO	Oligo Name	Sequence	Modification
1	Cry2A DII F	5'-TATCAAAGCCTTCTAGTATCTTCYG-3'	5'-PHO
2	Cry2A DII R	5'-TTTTCTGTTATGCACTGTTACAAG-3'	5'-PHO
3	Cry2Ab w/o DII F	5'-AATAATATCCATGCTTGTTTCATGAAAATG- 3'	
4	Cry2Ab w/o DII R	5'-TTTAAATAACGACCAGATRGAKACATA-3'	

PCR (PFU ultra 6kb) was performed on pGEM Cry2A plasmids, the amplified products digested with *DpnI*, purified using gel purification methods, then run on 1% agarose gel to prepare them for ligation.

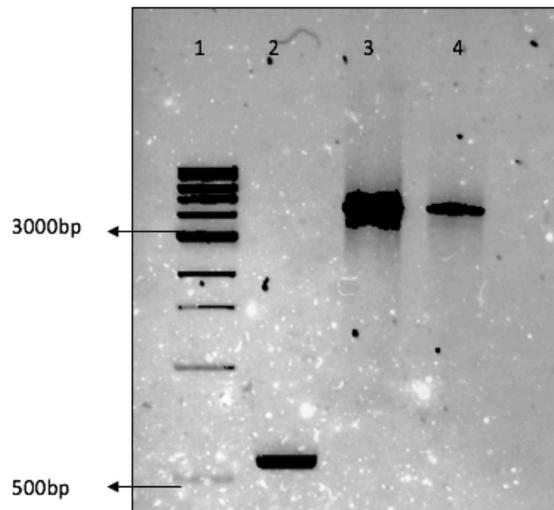


Figure 3.38. Confirmation of presence of amplified PCR products: PCR products was digested with *DpnI*, purified using QIAquick kit and run on 1% agarose gel along with the template and 1kb ladder (Marker) for size confirmation. Lane (1) Marker, lane (2) cry2Ac domain II, lane (3) Cry2AcNTAb w/o domain II, lane (4) the same sample in lane 3 but diluted 1:5.

The amplified PCR products which presented in (figure 3.38) were consistent with the expected size of Cry2Ac domain II and for pGEM Cry2AcNTAb without domain II. The amplicons were ligated, transformed into *E.coli*, digested with *HaeIII* restriction enzyme, and sequencing carried out to confirm the presence of the right constructs as shown in (figure 3.39).

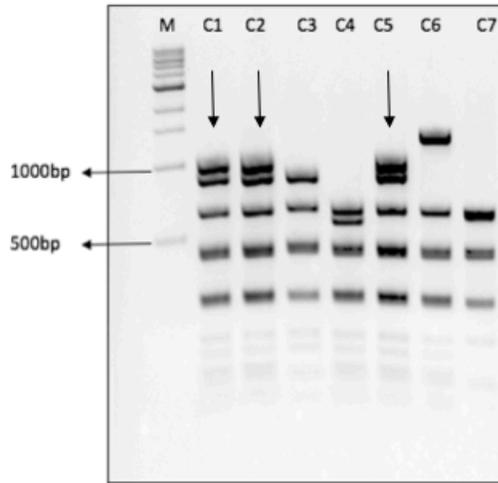


Figure 3.39 DNA banding pattern after *Hae*III digest: DNA minprep was conducted after each transformation step using QIAquick kit, digested with *Hae*III then run on 1% agarose gel along with the marker. Gel is showing Cry2AcNTAbAcAb DNA colonies digested with *Hae*III restriction enzyme, the right colonies number (1,3 and 5) indicated by arrow.

The DNA was then sequenced for final confirmation, and according to (figures 3.40 and 3.41) the right hybrids were created. We can also clearly see this from the alignment of the sequencing result and the sequence of Cry2Ab and Cry2Ac.

2Ab	ATGTTAGAATTTAGAACATATATGTTTTTAAATGTTTTGAGTATGTATCTATCTGGTCCG	780
2Ac	ATGTTAGAATTTAGAACATATATGTTTTTAAATGTTTTGAAATATGTaTcC-ATCTGGTCCG	780
AcNTAbAcAb	ATGTTAGAATTTAGAACATATATGTTTTTAAATGTTTTGAGTATGTATCTATCTGGTCCG	166

2Ab	TTGTTTAAATATCAAAGTCTTCTAGTATCTTCCGGTGCTAATTTATATGCAAGTGGTAGT	840
2Ac	TTATTTAAATATCAAAGCCTTCTAGTATCTTCCGGCGCTAATTTATATGCGAGTGGTAGT	840
AcNTAbAcAb	TTATTTAAATATCAAAGCCTTCTAGTATCTTCCGGCGCTAATTTATATGCGAGTGGTAGT	226
	** *****	
2Ab	GGACCACAGCAGACCCAATCATTTACTTCCACAAGACTGGCCATTTTTATATTCTCTTTTC	900
2Ac	GGTCCA-----ACACAATCATTTACAGCACAAAACCTGGCCATTTTTATATTCTCTTTTC	894
AcNTAbAcAb	GGTCCA-----ACACAATCATTTACAGCACAAAACCTGGCCATTTTTATATTCTCTTTTC	280
	** *** ** *****	
2Ab	ACCGTTACACTATAATGAAATAAGAAATATAGCAAGTCCCTTCAGGAACACCTGGTGGAGC	1379
2Ac	ACCTCTACACTTTAATGAAATAAGAGATATAGGAACGACAGC-----	1353
AcNTAbAcAb	ACCTCTACACTTTAATGAAATAAGAGATATAGGAACGACAGC-----	738
	*** ***** ** *	
2Ab	ACGAGCTTATATGCTATCTGTGCATAACAGAAAAATAAATATCCATGCTGTTTCATGAAAA	1439
2Ac	AGTCGCTAGCCTTGTAAACAGTGCATAACAGAAAAATAAATATCTATGACACTCATGAAAA	1413
AcNTAbAcAb	AGTCGCTAGCCTTGTAAACAGTGCATAACAGAAAAATAAATATCCATGCTGTTTCATGAAAA	798
	* *** * *****	
2Ab	TGGTTCATGATTCATTTAGCGCCAAATGACTATACAGGATTTACTATTTTCGCCGATACA	1499
2Ac	TGGTACTATGATTCATTTAGCGCCAAATGACTATACAGGATTTACCGTATCTCCAATACA	1473
AcNTAbAcAb	TGGTTCATGATTCATTTAGCGCCAAATGACTATACAGGATTTACTATTTTCGCCGATACA	858
	*** ***** * * * * *	

Figure 3.40. Alignment of the sequence received from sequencing result of pGEM(Cry2AcNTAbAcAb) and the original sequence of Cry2Ac and Cy2Ab: the sequence of the hybrid DNA and the sequence of the Cry2Ac and Cry2Ab were aligned using Clustal Omega software, the two red lines indicate the junction between domain I and II, then between domain II and III respectively in the desired hybrid.

```

2Ac      ATTTAGTTTTGAACATAAATCATTAATACCATAGAAAAAGAATGGAAAGAATGGAAAAG 128
2ab      ATTTAGTTTTCAACACAAATCATTAGATACCGTACAAAAGGAATGGACGGAGTGGAAAAA 128
2AcNTAb  ATTTAGTTTTGAACATAAATCATTAATACCATAGAAAAAGAATGGAAAGAATGGAAAAG 180
          ***** ** * ***** ** * ** * ***** ** * *****
2Ac      AACTGATCATAGTTTATATGTAGCCCCATTGTGGAACTGTGGGTAGTTTTCTATTAAA 188
2ab      AAATAATCATAGTTTATACCTAGATCCTATTGTGGAACTGTGGCTAGTTTTCTGTAAA 188
2AcNTAb  AACTGATCATAGTTTATACCTAGATCCTATTGTGGAACTGTGGCTAGTTTTCTGTAAA 240
          ** * ***** ** * ***** ***** ***** *****
2Ac      GAAAGTAGGGAGTCTTGTTGGAAAAGGATACTAGTGAGTTACAGAATTTAATTTTCC 248
2ab      GAAAGTGGGGAGTCTTGTTGGAAAAGGATACTAAGTGAGTTACGGAAATTTAATTTCC 248|
2AcNTAb  GAAAGTGGGGAGTCTTGTTGGAAAAGGATACTAAGTGAGTTACGGAAATTTAATTTCC 300
          ***** ***** ***** ***** ***** *****
2Ac      TAGTGGTAGTATAGATTTAATGCAAGAGATTTTAAGAGCGACAGAACAAATTCATAAATCA 308
2ab      TAGTGGTAGTACAAATCTAATGCAAGATATTTTAAGAGAGACAGAAAATTCCTGAATCA 308
2AcNTAb  TAGTGGTAGTACAAATCTAATGCAAGATATTTTAAGAGAGACAGAAAATTCCTGAATCA 360
          ***** * * ***** ***** ***** ***** * *****

```

Figure 3.41. Alignment of the sequence received from sequencing result of pGEM (Cry2AcNTAbAb Ab) and the sequence of Cry2Ac and Cy2Ab: the sequence of the hybrid DNA and the sequence of the Cry2Ac and Cry2Ab were aligned using Clustal Omega software, the red line indicated to the junction between N-terminus and domain I.

The hybrid (Cry2AcNTAbAcAb) was introduced into BL21, grown after, then the protein was harvested using the method described in material and methods chapter, then run on SDS-PAGE along with Cry2Ac and Cry2Ab wild type for size comparison as shown in (figure 3.42).

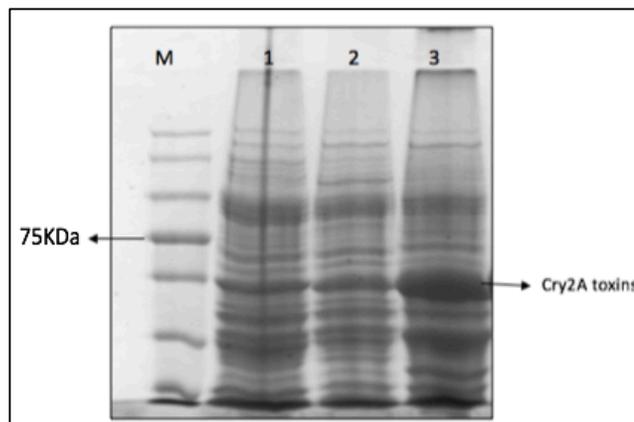


Figure 3.42. SDS-PAGE analysis of the hybrid toxin created comparing its size with the wild type Cry2Ab and Cry2Ac. (M) Marker, (1) Cry2Ab, (2) Cry2Ac, (3) Cry2AcNTAbAcAb.

Based on (figure 3.42), it is obvious that the hybrid protein created through domain swaps was well expressed as can be seen by the presence of the bands indicated by the arrow on the right side.

The toxicity level of the hybrid was assessed by carrying out bioassay on the NO-QA population of *P. xylostella*. The qualitative bioassay results to evaluate the level of toxicity of the hybrids created on NO-QA population of *P. xylostella* is shown in (figure 3.43).

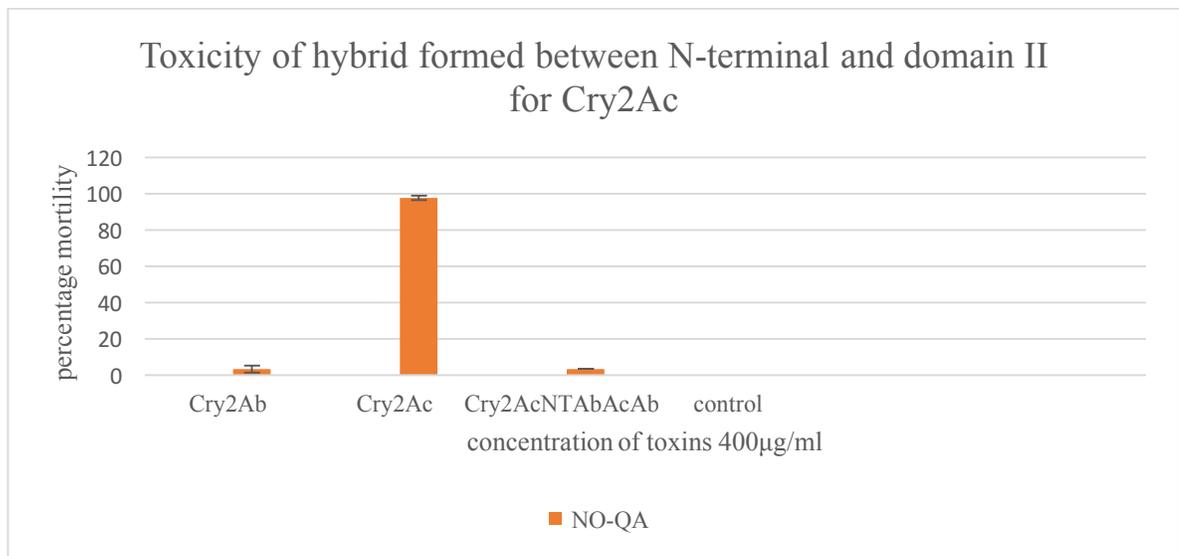


Figure 3.43. Evaluation of the level of toxicity of hybrid created through N-terminal and domain II of Cry2Ac, and Cry2Ab against NO-QA population of *Plutella xylostella*. Cry2Ac as a positive control and water +tritonx100 as a negative control Percentage mortality was recorded in three different experiments after a period of three days, the concentration of all toxins of Cry2Awas 400 µg/ml.

Figure 3.43 shows that the hybrid has little toxicity against NO-QA population of *P. xylostella*. Figure 3.34B had indicated that Cry2AcAcAb retained toxicity to NO-QA but this result indicated that more than just the N-terminal 49 amino acids of domain I is required.

3.4 Discussion

This research, which focused on understanding the basis of specificity of Cry2A group of toxins against *P. xylostella*, started by carrying out bioassay studies of all the wild type Cry2A toxins available in our lab. This is because there are contradictory results in the literature and, we wanted to establish our own bioassay data. After these bioassays, we then created some hybrids through different domain swaps to be able to understand the domain/domains that is/are responsible for activity in this group of toxins.

Based on the results we obtained from the bioassay we established that using the wild type toxins, Cry2Ac, Cry2Aa and Cry2Ab, were active against the SBT population of *P. xylostella*. Whereas, Cry2Ac and Cry2Aa toxins were active against the NO-QA population, Cry2Ab was non-active against the same population. In the case of Geneva- 88 the most toxic protein was Cry2Ac but unfortunately, we could not carry out further work with this population due to the fact that it was lost. Additionally, a review (Frankenhuyzen, 2009) indicated that Cry2Aa is toxic to *P. xylostella*. Moreover, a study conducted by Tabashnik *et al.*, (1996) indicated that the NO-QA population has no-cross resistance to Cry2Aa toxins. All these results support our findings that Cry2Aa is toxic against to diamondback moth.

In contrast, the findings of Monnerat *et al.* (1999), who tested various toxins of Cry1A, along with Cry2Aa, showed that Cry2Aa has lower activity with an LC₅₀ of (54.515 µg/ml). This is contrary to our findings because we discovered that Cry2Aa is active against both populations of *P. xylostella*. However, (Liu *et al.*, 2001) used different population of *P. xylostella*, LAB-PS (susceptible) and NO-QA and NO-95C (resistant). They discovered that Cry2Aa is less toxic to the susceptible population, suggesting there is no cross-resistance to

the resistance population. This result is similar to our findings which showed that the Cry2Aa toxin is active against the NO-QA population. Whilst research done by Zago *et al.* (2014) showed lower levels of Cry2Aa toxin resistance among several *P. xylostella* that had a history of low prior exposure to Cry2A containing products. This result supports our finding which shows that Cry2Aa has low level of resistance in case of SBT population of *P. xylostella*.

Furthermore, previous work done by (Pan *et al.*, 2014) claimed that Cry2Ab was significantly toxic against *P. xylostella* susceptible populations with LC₅₀ (0.0103 µg/ml). Additionally, Lin *et al.*, (2007) examined toxicity of the Cry2Ab toxin, and reported that it has high insecticidal activity against susceptible diamondback moths, with the value of LC₅₀ was (90 µg/ml), these results agreed with our findings, which showed that Cry2Ab was toxic towards SBT population. Some researchers who tested the toxicity of Cry2Ab against three different populations of *P. xylostella*, stated that Cry2Ab showed low level of toxicity (Zhao *et al.*, 2001). In contrast, a review by Frankenhuyzen (2009) reported that Cry2Ab and Cry2Ac had no insecticidal activity against *P. xylostella*. Our results showed that Cry2Ac is toxic against the SBT population of *P. xylostella*, which is contrary to what they reported. Hence, this justified why we carried out a fresh bioassay to be able to establish our baseline data.

Since we found out which Cry2A toxins were active against our populations and those that were non-active, the next hurdle was try to find out which among the three domains of Cry2A toxins influences activity against these two populations of *P. xylostella*. We carried out domain swaps as shown in (figure 3.8), involving Cry2Ac toxin known to be active against NO-QA population, and another one known to be non-active Cry2Ab.

The results confirmed that domain I and domain II of Cry2Ac are required for activity to the NO-QA population as shown in (figure 3.34), but not the N-terminus from domain I alone or N-terminus along with domain II that conferred activity based on our findings, in (figure 3.36 and 3.43).

These results appeared similar to the findings of Liang and Dean (1994) who discovered that some sequences in domain II are responsible for activity against mosquitos (*Aedes aegypti*) and gypsy moth (*Lymantria dispar*), similar to our findings that sequences comprising domain I and II influenced activity of Cry2A toxins against NO-QA population of *P. xylostella*. These, strengthened the fact that some specific sequences could determine activity of the toxins against different insects. In addition, other research suggests that some residues in domain II not domain I or III responsible for activity against dipteran insects (Widner and Whiteley, 1990; Morse *et al.*, 2001).

This difference in toxicity witnessed from the three different populations of *P. xylostella* obtained from two different countries is not surprising because previous research has shown that even among Cry toxins that are toxic against an order of insect, there exists significant differences in their level of specificities and or toxicity. This is due to intraspecies variation in toxin susceptibility which seemed to occur between test colonies obtained from different parts of the world: a variation of 1-2 orders of magnitude, even among insects from the same geographical region or colony may vary by 1 order of magnitude between cohorts or successive generations (Frankenhuyzen, 2009).

This might be the reason why our results appeared contradictory to others, because the bioassay was performed in different geographical locations, and research was performed in

different geographical locations that can affect the activity of Cry toxins to some insects. Moreover, the age of larvae could affect the result of bioassays. (McNeil and Dean, 2011) discovered that susceptibility decreased with decreasing larvae age. They might have used different population of *P. xylostella* from our population and that could lead to some variation in our bioassay results, hence, why they reported that this toxin is active but, in our case, not active. Therefore, differences in susceptibility of Cry toxins are population dependent as could be seen in research conducted by Ingber *et al.*, (2017) who worked with two different populations of Lepidopteran insects which were *Spodoptera frugiperda* corn-strain and rice-strain. These were tested on some Cry toxins, namely: Cry1Ab, Cry1Ac and Cry1F. In addition to these two strains, they created another hybrid between these two strains referred to as the rice-corn hybrid. Therefore, they carried out bioassays to compare the susceptibilities of these Cry toxins to the different strains of *S. frugiperda*, after which they discovered that all Cry toxins were active against only two strains; the corn-strain and the hybrid population. Most specifically Cry1F appeared to be much more active to these two strains. They discovered that these Cry toxins were non-active towards the rice-strain. These results are further confirmation of the fact that activity of Cry toxins are population dependent, in that these Cry proteins were toxic to the two populations of *Spodoptera frugiperda* but not the rice-strain population.

4. Comparative studies between *E. coli* and *Bt* expression systems for Cry2A toxins

4.1 Introduction

The idea behind this chapter was to see which expression system gave the best and clearest toxin, but also to check whether the expression system had any effect on activity. Based on the research done by Crickmore and Ellar, (1992) explaining the effects of the presence and/or absence of ORF2 using different vectors of *Bacillus thuringiensis* could have on the expression level of Cry2A toxin. This was achieved by inserting the *Cry2Aa* gene in different plasmid constructs using IPS78/11 *Bacillus thuringiensis* for expressing the *Cry2Aa* gene as shown in (figure 4.1), and they discovered that they got a high yield when they used a particular *Bacillus thuringiensis* expression system; clearly depicted in (figure 4.2) lane 6 and 7 which showed the high level of expression of Cry2Aa, and the large amount of protein obtained compared to other lanes. These two lanes 6 and 7 correspond to vector constructs pSVP27B40B and pSVP27B40P shown in (figure 4.2). Based on these results (figure 4.2), it was concluded that these two vectors gave an efficient expression of the Cry2Aa toxin compared to the other vector constructs, this showed the need for ORF2 for efficient expression. Therefore, based on these findings from this study we decided to use the same plasmid pSVP27B40B to express different wild type *cry2A* genes and several hybrid *cry2A* genes to be able to find out if we could get a higher yield of cleaner toxin and to see whether there would be any difference in activity of Cry2A toxins against the two populations of *Plutella xylostella*.

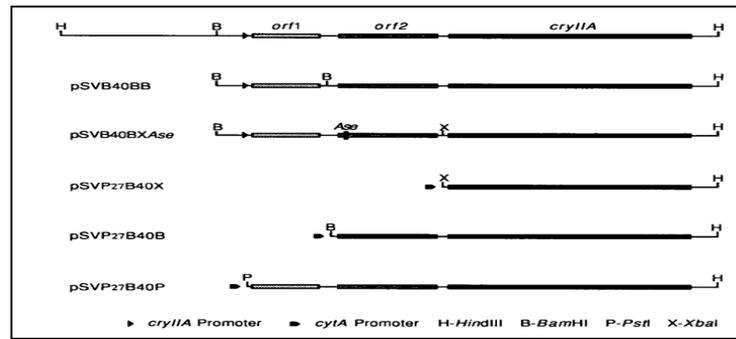


Figure 4.1. Represents construction of different vectors and restriction sites introduced by site-directed mutagenesis. The fragments were cloned into shuttle vector pSV2 (Crickmore and Ellar, 1992).

Below is the SDS gel as depicted by Crickmore and Ellar, 1992.

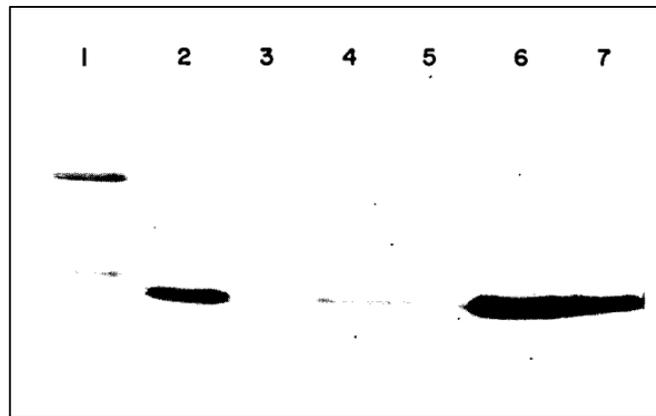


Figure 4.2. Represents the SDS-PAGE analysis of different expression of Cry2Aa in IPS78/11 in different vectors. Lane (1) Marker, Lane (2) pSVB40BB plasmid, Lane (3) pSV2, Lane (4) pSVB40BXAse, Lane (5) pSVP₂₇B40X, Lane (6) pSVP₂₇B40B and Lane (7) pSVP₂₇B40P (Crickmore and Ellar., 1992).

Furthermore, we would aim to carry out a binding assay towards finding if the activities of Cry2A toxins are related with their receptor binding ability. This is because we are using two different populations of *P. xylostella* to test for the activities of these toxins, and there may be high tendency of getting a toxin with differing toxicities to these populations and as such it will be very interesting to know if activity of toxins is related to their receptor binding

ability at all times. Various models that describe the different mechanisms of actions of Cry toxins to insects linked it to the ability of these toxins to bind to the receptors within the gut epithelium of these insects (Bravo et al., 2004). Therefore, the results would further shed more light if there may be other possible mechanism of action of the toxin in addition to the ones already proposed.

4.2 Results

4.2.1 Preparation of pSVP27B40B plasmid for introducing *cry2A* genes

In order to insert the various genes into pSVP27B40B, we create the restriction sites in pGEM plasmid to be able to excise *Cry2A* toxins genes, which has *XbaI* site upstream of the gene and *HindIII* sites downstream of the gene. Therefore, we need to have same two sites in pSVP27B40B plasmid to use it for the cloning, and interestingly it already has *Hind III* site, hence we need to introduce only the *XbaI* site. The same process was used to create these two restriction sites in pSVP27B40B that was used to insert the *Cry2A* toxin genes that have been removed from pGEM plasmid into pSVP27B40B to able be successfully insert the *cry2A* gene in to the pSVP27B40B plasmid.

We removed the *Cry2Aa* toxin gene that had been inserted into pSVP27B40B. We followed the technique used by Crickmore and Ellar, (1992) which involved the use of restriction sites to insert the gene. To remove the gene from the plasmid in order to create a site for inserting the new gene, the presence of restriction sites flanking the toxin genes are required as depicted in (figure 4.3).

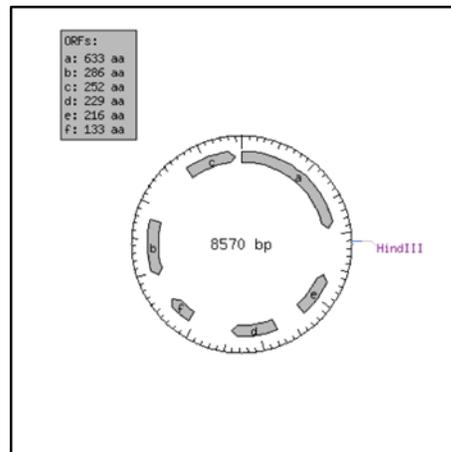


Figure 4.3. Map of pSVP27B40B plasmid with the *HindIII* site, *ORF a* represent *cry2Aa* gene. it taken from NEB-cutter web site

From figure 4.3 above, it is obvious that pSVP27B40B plasmid had only one restriction site for *HindIII* downstream the *cry2Aa* gene, which appears as *ORFa*, and but it has no any restriction site detected upstream the gene. Therefore, artificial insertion of a restriction site upstream of the gene needs to be done in order to exchange *cry2Aa* genes in the plasmid. As shown in (figure4.1), the additional restriction site of *XbaI* was successfully introduced upstream the *cry2Aa* gene to this plasmid pSVP27B40BX. However, the location of *XbaI* restriction site should be in the right position to ensure that *orf2* gene remains within the plasmid when the toxin gene is removed, because of the importance of *orf2* gene in crystallization and expression of the Cry2A toxins (Crickmore *et al.*, 1992). In order to incorporate an *XbaI* site into pSVP27B40B plasmid two primer were designed using PCR PRIMER SELECT program to be able to introduce the site in the plasmid using PCR.

No	Oligo Name	Sequence	Modification
1	<i>XbaI</i> Forward primer	5'-TTCTAGATGAATAATGTATTGAATARYGG-3'	
2	pSVP ₇₂ B40B Reverse primer	5'-TTCCTCCTTAAATATCTAGTTTTATAT-3'	

Table 4.1. Forward and reverse primers that were used to introduce the desired mutation.

As appeared in table (1), we made the primer degenerate by replacing two bases which do not appear similar in all Cry2Aa toxins by RY to be able to introduce *XbaI* site in pSVP27B40B plasmid, also to be able to excised *cry2A* genes from pGEM plasmid and use it to amplify the hybrid toxins as the different *cry2A* genes are degenerate at these two positions. In order to amplify the whole plasmid from where the primers bind to one point on the plasmid and replicate the DNA in opposite directions PCR (PFU ultra 6KB) reaction was performed on pSVP27B40BCry2Aa by using *XbaI* forward primer and pSVP27B40B reverse primer, the result was purified using gel purification method as described in chapter two (materials and methods), then run on 1% agarose gel (figure 4.4).

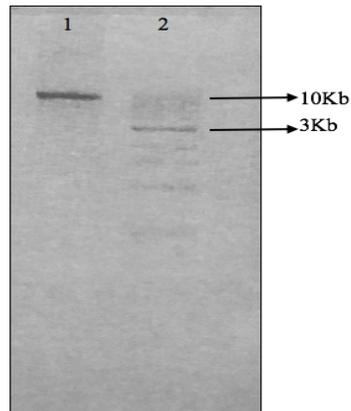


Figure 4.4. Confirmation of the presence of amplified PCR product. PCR product was purified and run on 1% agarose gel, lane (1) is pSVP27B40B plasmid containing *Cry2Aa* toxin gene and lane (2) is 1 Kb DNA marker for size comparison.

Based on the figure 4.4 the size of amplified product presence in lane 1, size was consistent with the expected size (8570 kb). The amplified product was self-ligated prior to introduction to DH5 α *E.coli* competent cells, once colonies of the electrotransformed DH5 α cells was grown, then they were harvested, and the DNA was miniprepmed using QIAprep kit. This DNA was digested with *Hind*III and *Xba*I to be able to ensure that the PCR had successfully introduced the *Xba*I site, and to remove the *cry2Aa* gene from the plasmid. The verification of successful presence of restriction site was carried out by digestion with *Hind*III, after than *Xba*I and *Hind*III+ *Xba*I, at the same time, by comparing the size of the fragment (expected band) generated by NEB cutter program (figure 4.5) with the size of resultant bands, after digestion with the enzyme which were then run it on 1% agarose gel (figure 4.6).

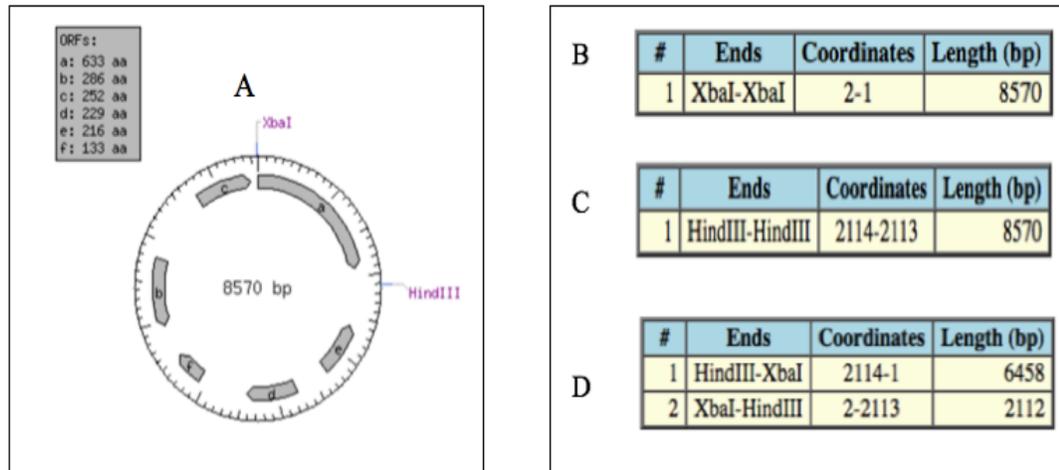


Figure 4.5. Schematic representation of pSVP27B40BXba plasmid and localization of different restriction sites of *XbaI* and *HindIII*. B, C, and D sizes of different fragments generated by digestion of pSVP27B40Bplasmid with *XbaI*, *HindIII* and with both enzymes respectively. It taken from NEB cutter program.

According to figure 4.5 (B and C), it is clear that when the plasmid is digested with *HindIII* the size of the band obtained from the gel was consistent to the predicted size (8570 kb), which is the size of the whole plasmid as shown in (figure 4.6). Similar result was obtained when the plasmid was digested with *XbaI*. However, to be able to confirm if the insertion of *XbaI* site was successful, the plasmid was digested with both enzymes giving two fragments with predicted sizes of 6458 bp and 2112 bp as shown in (figure 4.6). But due to overloading of the marker we cannot confirm the sizes of single digests but only one band from double digestion could be confirmed.

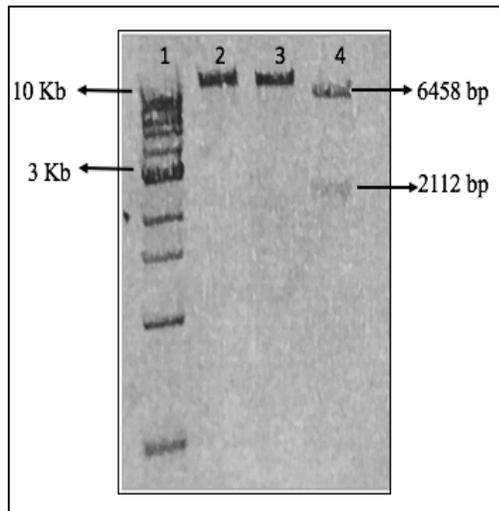


Figure 4.6. DNA banding pattern after digested with *XbaI*, *HindIII* and both enzymes. DNA was miniprepred after transformation using QIAprep kit digested with *XbaI* and *HindIII*. Lane (1) 1kb DNA marker, lane (2) plasmid DNA digested with *HindIII*, lane (3) plasmid DNA digested with *XbaI* and lane (4) plasmid DNA digested with both enzymes.

In order to clone *cry2A* genes, the new plasmid was cut with *HindIII* and *XbaI*, top band purified and run on 1% agarose gel to make sure we got the desired product. In this stage, the plasmid was ready for introducing various *cry2A* genes as shown in (figure 4.7).

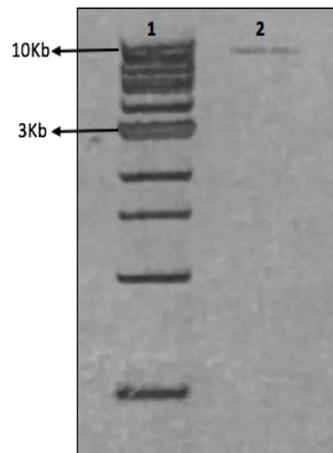


Figure 4.7. Purified DNA fragment of pSVP27B40B plasmid after digestion with both *XbaI* and *HindIII*. Lane (1) 1Kb DNA marker and lane (2) purified fragment of pSVP27B40BXba plasmid.

4.2.2 Isolation and amplification of *cry2A* genes from pGEM plasmid

We decided to extract all *cry2A* hybrid genes that were in pGEM plasmid and introduce them into the pSVP27B40BXba plasmid, in this case we needed to design primers to be able to introduce *Xba*I and *Hind*III either side of the amplified *cry2A* genes, table 2.

NO	Oligo name	Sequence	Modification
1	<i>Xba</i> I Forward primer	5'-TTCTAGATGAATAATGTATTGAATARYGG-3'	5'-PHO
2	<i>Hind</i> III Reverse primer	5'-TCAAAGCTTAATAAAGTGGTGRAAG-3'	

Table 4.2. Forward and reverse primers that were used to introduce the two restriction sites. The bases indicated by red are the start and stop codons respectively.

For the forward primer we used the same one that was used to introduce *Xba*I site to the pSVP27B40B plasmid, hence the sequence for this primer was made degenerate due to some few dissimilarities between the sequences of *cry2A* genes. The forward primer enabled us to insert *Xba*I site upstream of start codon of *cry2A* gene, but for the reverse primer we designed a new primer which has a mutation introduced, AAGCTT, giving rise to *Hind*III restriction site into the PCR product downstream of the *cry2A* gene (figure 4.8).

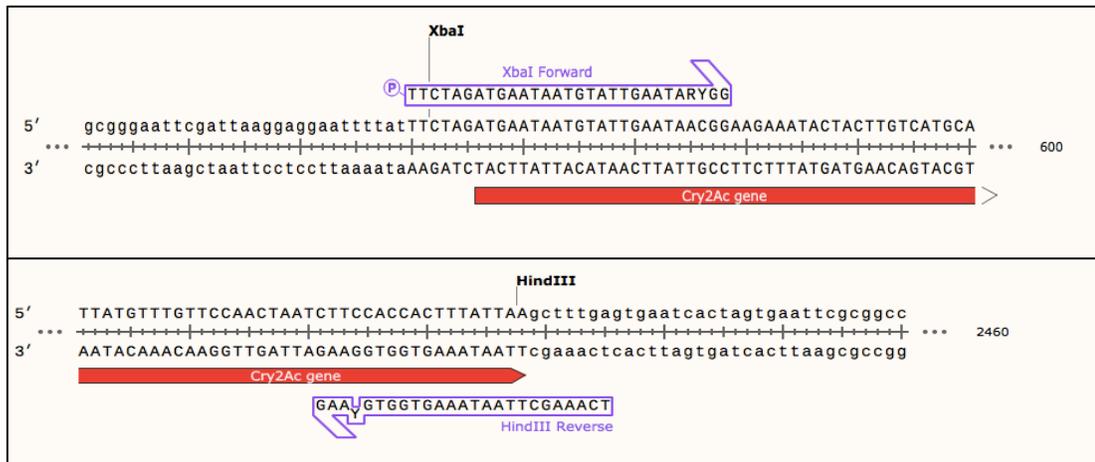


Figure 4.8. Schematic representation of forward and reverse primers used in the PCR reaction to amplify Cry2A toxin genes from pSVB40B plasmid.

According to (figure 4.8) the Cry2A and the hybrid genes were amplified from the pGEM plasmid using PCR (PFU ultra 6 KB program). The PCR amplified products were purified using QIAprep Spin Miniprep kit, then run on 1% agarose gel as shown in (figure 4.9).

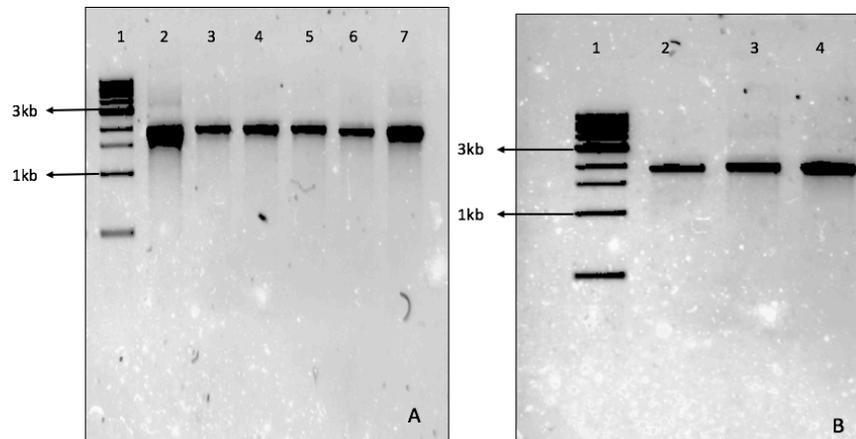


Figure 4.9. Confirmation of purified PCR products using QIAprep kit. Gel A lane (1) 1kb DNA marker, lane (2) Cry2Ac, lane (3) Cry2Ab, lane (4) Cry2AcNTAbAbAb, lane (5) Cry2AcAbAb, lane (6) Cry2AbAcAb, and lane (7) Cry2AbAbA. Gel B lane (1) 1Kb DNA Marker, lane (2) Cry2AbAcAc, lane (3) Cry2AcAbAc, and lane (4) Cry2AcAcAb.

It is clear that, from (figure 4.9) that the size of the amplified products present in the gel were consistent with expected size of size of the products which was (1876kb). After purification of the PCR, an enzyme digestion was performed, using both *Xba*I and *Hind*III restriction enzymes to expose the sticky ends of the PCR products to allow ligation of the gene into the digested pSVP27B40BXba plasmid. Afterwards, the digested PCR products were purified using column purification procedure and run again on 1% agarose gel as depicted in (figure 4.10).

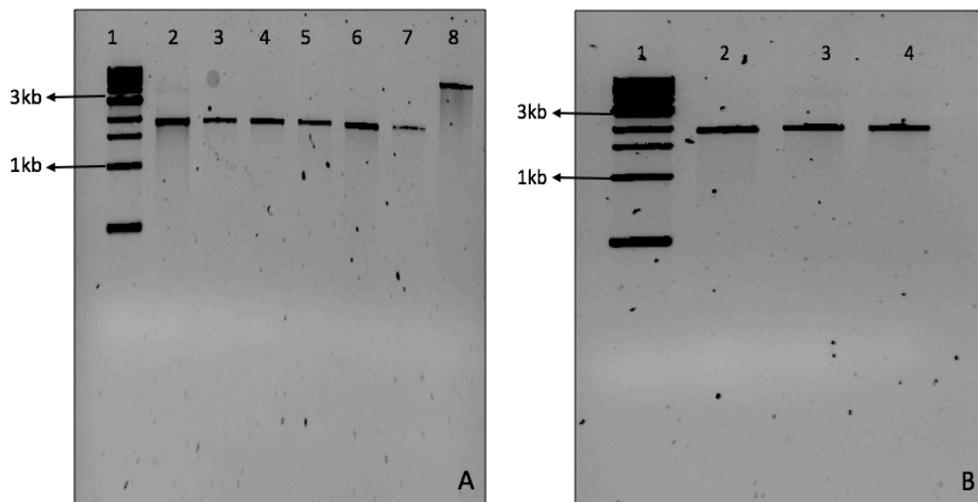


Figure 4.10. Confirmation of purified PCR product digested with *Xba*I and *Hind*III restriction enzymes. Gel A lane (1) 1kb DNA marker, lane (2) Cry2Ac, lane (3) lane Cry2Ab, (4) lane Cry2AcNTAbAbAb, (5) lane Cry2AcAbAb, (6) lane Cry2AbAcAb, (7) Cry2AbAbAc and lane (8) pSVP27B40BX plasmid. Gel B lane (1) 1kb DNA marker, lane (2) Cry2AbAcAc, lane (3) Cry2AcAbAc and lane (4) Cry2AcAcAb.

The amplicons were ligated to pSVP27B40BXba plasmid, and the circularized plasmid was used to transform NEB-5 competent *E.coli* cells. Extracted plasmid DNA was digested with *Hae*III restriction enzyme (figure4.12) to know which colonies have the right insert by comparing the size of fragments (expected band) taken from NEB cutter program in (figure

4.11). The right colonies were sent for sequencing, then the extracted DNA from these cells were introduced to *Bt* cells (IPS78/11) for expression.

1				2			
#	Ends	Coordinates	Length (bp)	#	Ends	Coordinates	Length (bp)
1	HaeIII-HaeIII	2664-5132	2469	1	HaeIII-HaeIII	2664-5132	2469
2	HaeIII-HaeIII	6988-884	2467	2	HaeIII-HaeIII	6988-884	2467
3	HaeIII-HaeIII	1390-2663	→1274	3	HaeIII-HaeIII	885-2663	→1779
4	HaeIII-HaeIII	6401-6987	587	4	HaeIII-HaeIII	6401-6987	587
5	HaeIII-HaeIII	885-1389	→505	5	HaeIII-HaeIII	5596-6053	458
6	HaeIII-HaeIII	5596-6053	458	6	HaeIII-HaeIII	5162-5595	434
7	HaeIII-HaeIII	5162-5595	434	7	HaeIII-HaeIII	6134-6400	267
8	HaeIII-HaeIII	6134-6400	267	8	HaeIII-HaeIII	6054-6133	80
9	HaeIII-HaeIII	6054-6133	80	9	HaeIII-HaeIII	5144-5161	18
10	HaeIII-HaeIII	5144-5161	18	10	HaeIII-HaeIII	5133-5143	11
11	HaeIII-HaeIII	5133-5143	11				

Figure 4.11. Illustrate table (1) and (2) with the differences in the size of the fragments between pSVP27Cry2Aa and pSVP27Cry2Ac respectively depicted by arrows. Taken from NEB cutter website.

Based on (figure 4.12), which showed the difference between pSVP27B40BCry2Aa and pSVP27B40Bcry2Ac after digestion with *HaeIII* enzyme, for Cry2Aa the difference in two fragments (1274bp) and (505bp), but for Cry2Ac the size is around (1779bp) with no 505bp band and the same result as Cry2Ac was obtained for Cry2Ab which holds for all the other hybrids. To confirm that, the digested DNAs were run on 1% agarose gel and the difference in the sizes of the digestion fragments were obvious in (figure 4.12). Showing the band around (1274 bp) and (505bp) for Cry2Aa and the band around (1779bp) for Cry2Ac and Cry2Ab.

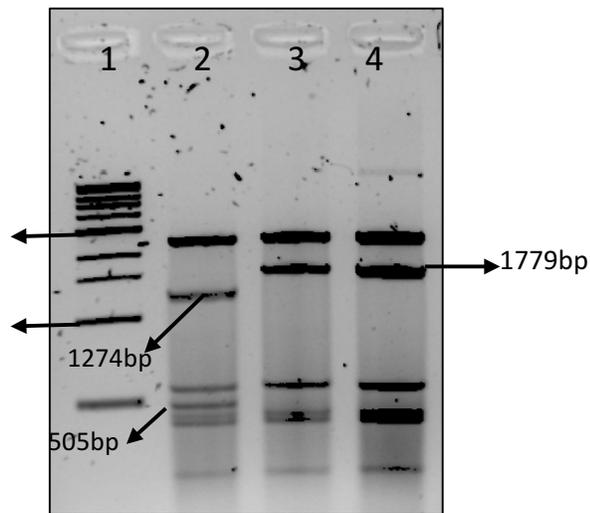


Figure 4.12. Predicted *Hae*III digestion profile of pSVP27B40BCry2Aa and pSVP27B40B, and the gel shows: DNA bands, when minpreped using QIAprep kit after transformation, then digested with *Hae*III, and run on agarose gel.

All the hybrids that were amplified from pGEM plasmid and introduced into pSVP27B40BXba plasmid were confirmed by sequencing, and using Clustal Omega software to align the DNA sequence, with the original sequence for the wild types that we used to create all the hybrids. All hybrids and wild type Cry2Ab and Cry2Ac that were successfully introduced into pSVP27B40B plasmid and further introduced into the accrystalliferous Bt strain IPS78/11 for expression, and IPS78/11 transformants were grown, the proteins were harvested, and crystals were observed under a phase contrast microscope, after which they were run on 7.5% SDS-PAGE gel along with a protein marker (figure 4.13).

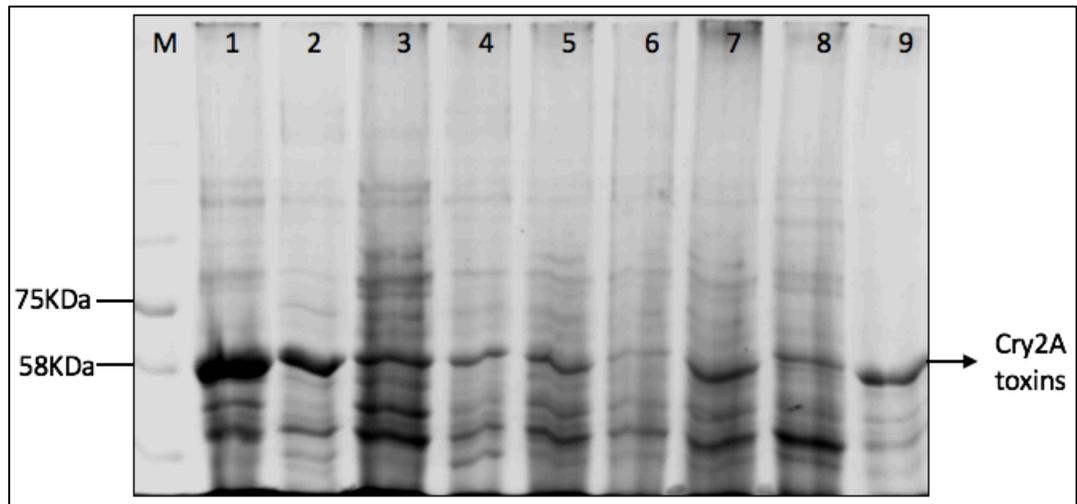


Figure 4.13. SDS-PAGE showing both the wild type and hybrid toxins expressed in Bt. The position of Cry2A proteins are indicated with arrow and these were compared with the standard protein marker. M (protein marker), lane (1) Cry2Ac, lane (2) Cry2Ab, lane (3) Cry2AcNTAbAbAb, lane (4) Cry2AcAbAb, lane (5) Cry2AbAcAb, lane (6) Cry2AbAbAc, lane (7) Cry2AbAcAc, lane (8) Cry2AcAbAc and lane (9) Cry2AcAcAb.

According to (figure, 4.13) all Cry2A hybrids and wild type toxins, which were introduced into pSV27B40B plasmid were expressed. From the gel above, it is obvious that Cry2Ac, Cry2Ab, Cry2AcNT and Cry2AcAcAb were well expressed, whereas, Cry2AbAbAc was not well expressed but also Cry2AcAbAb and Cry2AbAcAb were expressed, but the level of expression was not good enough for bioassay. However, from the gel we could clearly realize that Cry2Ac, Cry2Ab and Cry2AcAcAb were well expressed, therefore we decided to use those Cry toxins that were well expressed in Bt to make comparison with those expressed in *E.coli*. Hence, both *E.coli* and Bt expressed samples were run on an SDS-PAGE gel shown below in (figure 4.14).

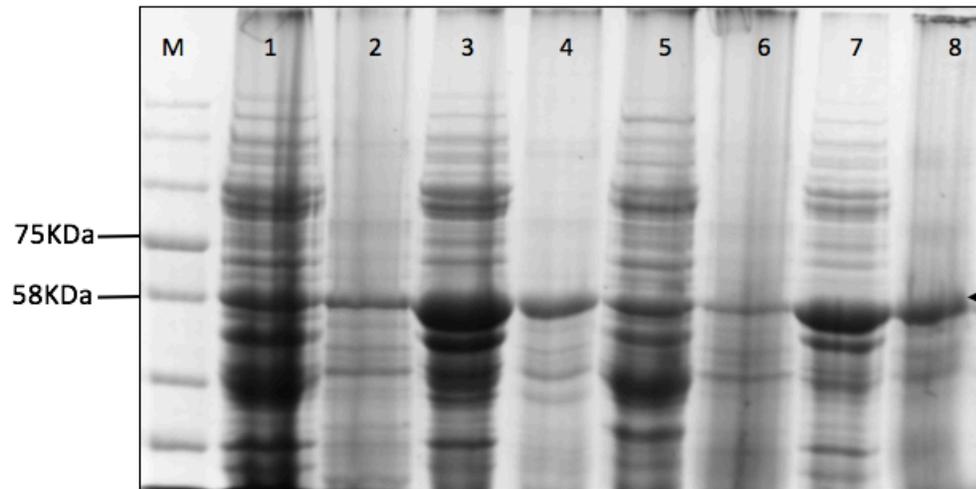


Figure 4.14. SDS-PAGE comparative analysis of Cry2A toxin expressed in *E.coli* BL21 and *Bt* IPS78/11. The position of Cry2A proteins are indicated with arrow and these were compared with the standard protein marker. M (protein marker), lane (1) Cry2Ab (*E.coli*), lane (2) Cry2Ab (*Bt*), lane (3) Cry2AcAcAb (*E.coli*), lane (4) Cry2AcAcAb (*Bt*), lane (5) Cry2AbAcAc (*E.coli*), lane (6) Cry2AbAcAc (*Bt*), lane (7) Cry2Ac (*E.coli*), lane (8) Cry2Ac (*Bt*).

From the result above in (figure 4.14), it appears that the level of purity of the toxin was higher in *Bt* than *E.coli* expression system, based on the intensity of the band from the SDS-PAGE gel. This is because those toxins expressed in *Bt* gave more clear bands with less contamination compared to those expressed in *E.coli*, based on the clarity of the various lanes observed from the gel. The next hurdle was to be able to assess the level of activity of these toxins expressed in these systems to see if there is any difference between them. We measured the concentration of some of these toxins as shown in (figure 4.15) and then carried out bioassay against the two different populations of *P. xylostella*, SBT and NO-QA.

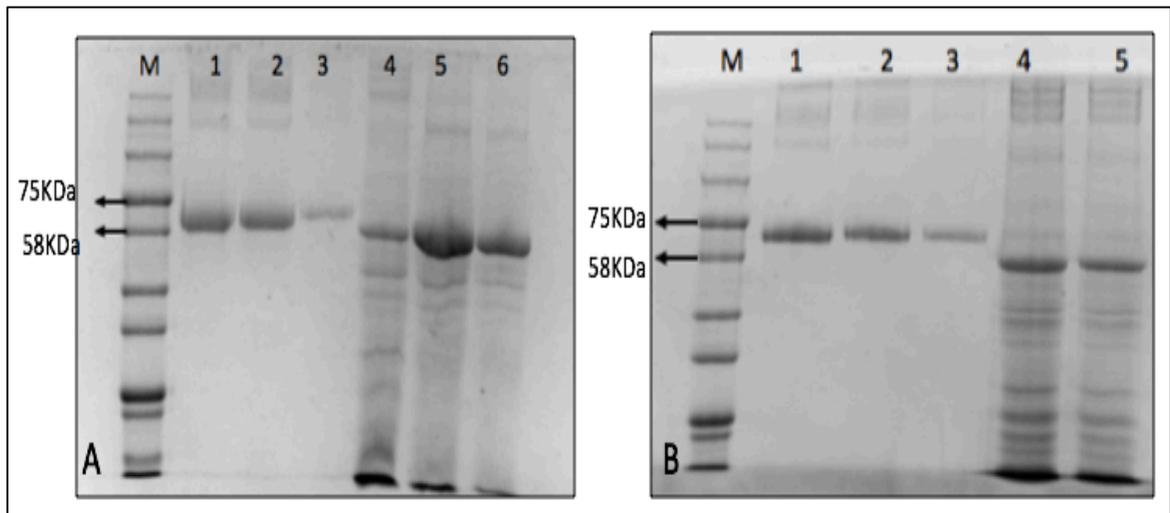


Figure 4.15A. SDS-PAGE used in measuring the concentration of the proteins expressed in Bt using densitometry method: M is a protein marker, lane (1to3) BSA standards (500, 250 and 125) $\mu\text{g/ml}$ respectively in gel A and B, lane (4) Cry2Ab, lane (5) Cry2Ac and lane (6) Cry2AcAcAb. Gel B lane (4) Cry2AbAcAb, lane (5) Cry2AbAcAc.

The concentration of proteins was found as follows for Cry2Ab (272.34 $\mu\text{g/ml}$), Cry2Ac (932.22 $\mu\text{g/ml}$), Cry2AcAcAb (575.83 $\mu\text{g/ml}$), Cry2AbAcAb (375.25 $\mu\text{g/ml}$) and Cry2AbAcAc (209.54 $\mu\text{g/ml}$).

The qualitative bioassay result to evaluate the level of toxicity of the toxins expressed in Bt compared to the toxins expressed in *E.coli* on SBT and NO-QA populations of *P. xylostella* is shown in (figures 4.16 and 4.17).

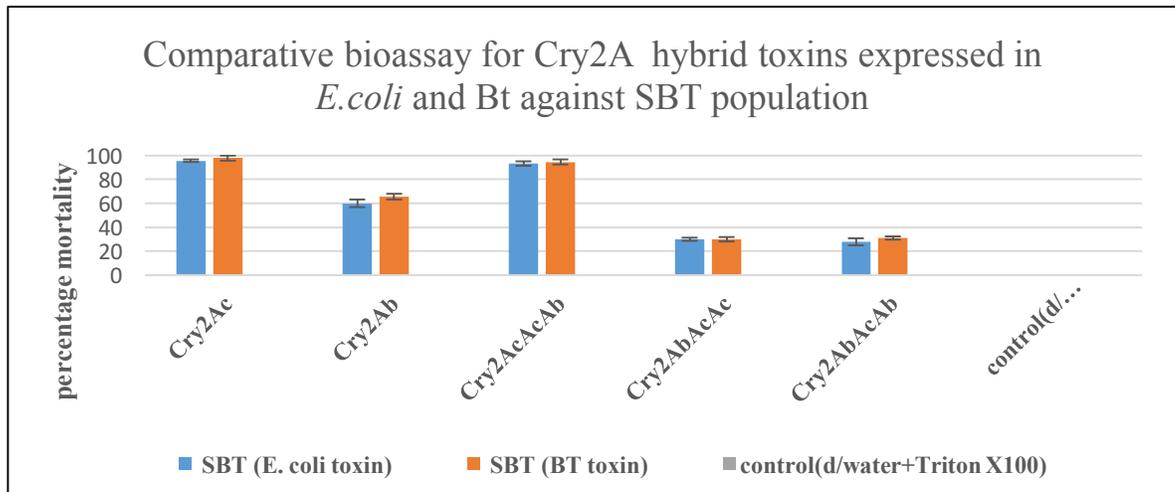


Figure 4.16. Evaluation of the level of toxicity of Cry2A wild type toxins and hybrids toxin expressed in Bt and the one expressed in *E.coli* against SBT populations of *Plutella xylostella*. Percentage mortality recorded in three different experiments after a period of three days, the concentration of all Cry2A toxins were 400ug/ml and Triton X100 +distilled water used as a control.

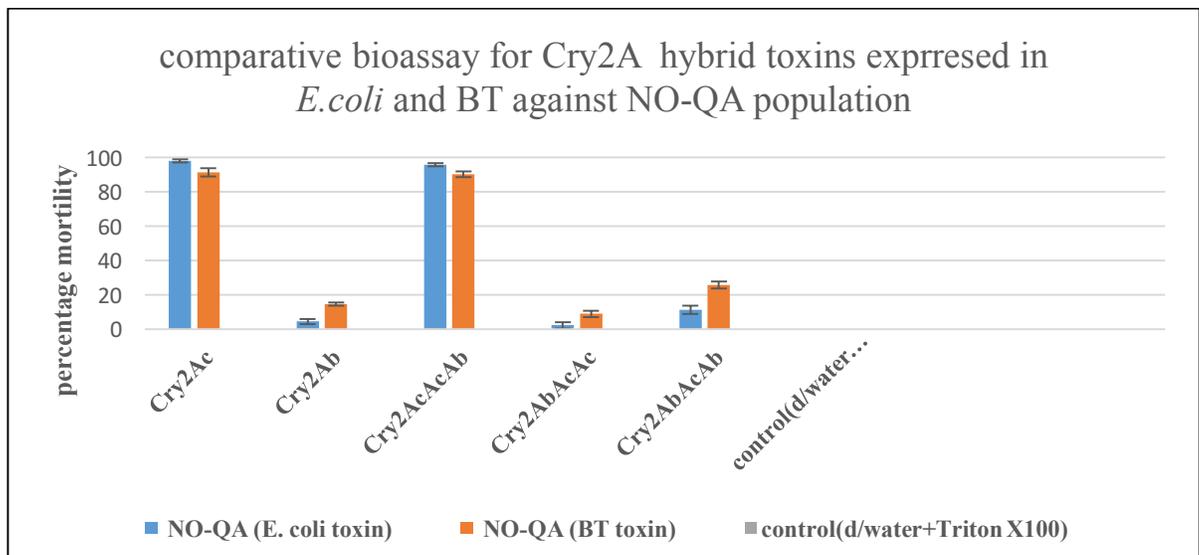


Figure 4.17. Evaluation of the level of toxicity of Cry2A wild type toxins and hybrids toxin expressed in Bt and the one expressed in *E.coli* against NO-QA populations of *Plutella xylostella*. Percentage mortality recorded in three different experiments after a period of three days, the concentration of all Cry2A toxins were 400ug/ml and Triton X100 +distilled water used as a control.

From the two graphs (figure 4.16 and 4.17), it could be observed that there was no significant difference in terms of activity for the toxins expressed in either *E.coli* or Bt expression system, implying that the system of expression used does not affect the activity of the toxin against the populations of insect in question. This could be seen as a further confirmation of the result that was obtained from the control experiment in the previous chapter, showing that the presence of some contaminants in *E.coli* expressed toxin does not affect the activity of the toxins.

4.3 Activation of Cry2A toxins expressed in Bt using chymotrypsin and mid gut juice of *Plutella xylostella*

This section seeks to understand the possible difference in binding as well as to establish if there is possible difference in activation among Cry2A toxins. Chymotrypsin was used as reference protease for activation of these toxins because it has been established that activation using chymotrypsin does mimic what actually happened in the insect midgut (Xu *et al.*, 2016). From our results we showed that a given Cry2A toxin, for instance Cry2Ab, could show different activity towards different populations of *Plutella xylostella*. This led us to investigate whether this difference in activity to different populations of *Plutella xylostella* by a given Cry2A toxin was related to the ability of the toxin to bind to the midgut of the population of *Plutella xylostella* under study. Therefore, we decided to carry out binding assay of Cry2A toxins against the two populations of the insect to be able to understand if the activity of the toxin towards a particular population of the insect has to do with its binding ability and to find out if differences in activation could associated with differences in activity.

As part of the procedure for the binding assay, the toxin had to be activated using a protease. Therefore, we decided to carry out an activation of Cry2A toxins using chymotrypsin and mid gut juice of both populations *Plutella xylostella*.

The toxins that were used as representatives of this group of toxins were Cry2Aa, Cry2Ab and Cry2Ac. Since we know that Cry2Aa and Cry2Ac toxins are toxic against both SBT and NO-QA population of *Plutella xylostella*, whereas Cry2Ab is toxic towards SBT population but non-toxic against NO-QA population. Therefore, we will study the activation pattern of these toxins with chymotrypsin and midgut juice to see whether this pattern associates with specificity, and when this is established then the activated forms of the toxins would be used to investigate their binding with BBMW of the two populations of *Plutella xylostella*. All Cry2A toxins used in this study were expressed in Bt, because we were able to get a clean toxin. In order to activate Cry2A toxins, the crystals were solubilized using NaOH buffer, then we activated Cry2Aa and Cry2Ac by adding 9µl of chymotrypsin to 1µl of the toxin for final concentration of 1mg/ml of chymotrypsin, but for Cry2Ab whose yield was low compared to Cry2Aa and Cry2Ac, 5µl of the toxin was add to 5µl of chymotrypsin for a final concentration 1mg/ml. These were all incubated for 5 minutes at 37⁰C.

The solubilized and activated samples were run in 7.5 SDS-PAGE gel as shown in (figure 4.18).

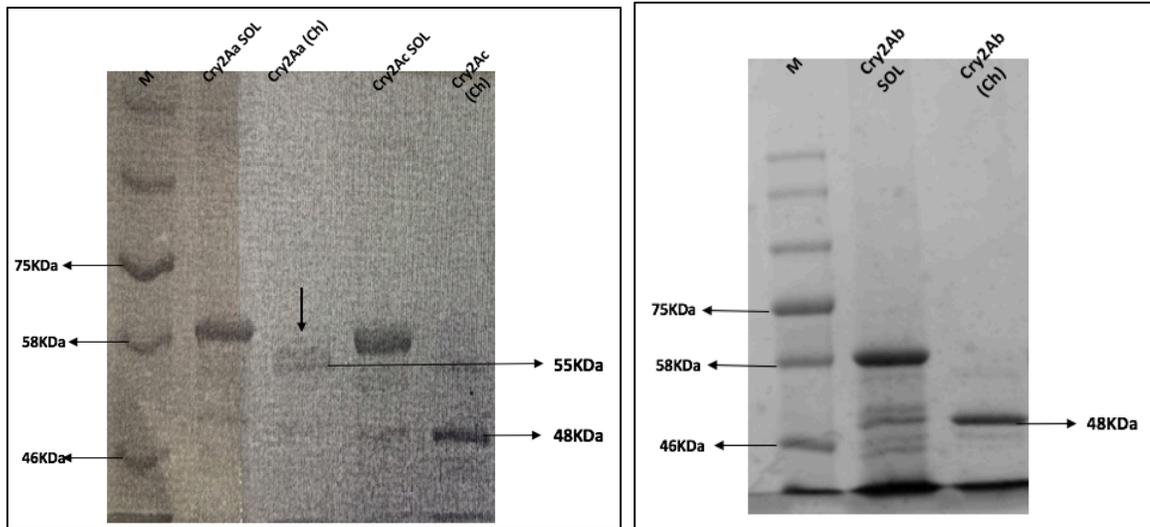


Figure 4.18. SDS- PAGE gel showing an activation of Cry2Aa, Cry2Ac and Cry2Ab by chymotrypsin: the final concentration of chymotrypsin +Cry2A toxins in all the lanes is 1mg/ml.

From the gel above (figure 4.18), it appears that the molecular weight of a complete digestion of Cry2Aa toxin by chymotrypsin is around 55 KDa as indicated by arrow in the gel, whereas Cry2Ac and Cry2Ab have their complete digestion bands around 48 KDa each (figure 4.18) A or B respectively.

We then carried out the activation of these three toxins, Cry2Ac, Cry2Aa and Cry2Ab using the midgut juice of prepared from both populations of *Plutella xylostella* using the procedure described in the material and methods chapter. The midgut juice of *Plutella xylostella* obtained from both populations were diluted 1 in 5 and 1 in 10 with PBS buffer before adding each to the Cry2A toxins as incubating toxin with undiluted midgut juice lead to complete

degradation of the toxin. The SDS-PAGE gel obtained from this activation is shown in (figure 4.19) for SBT population, and (figure 4.20) for the NO-QA population respectively.

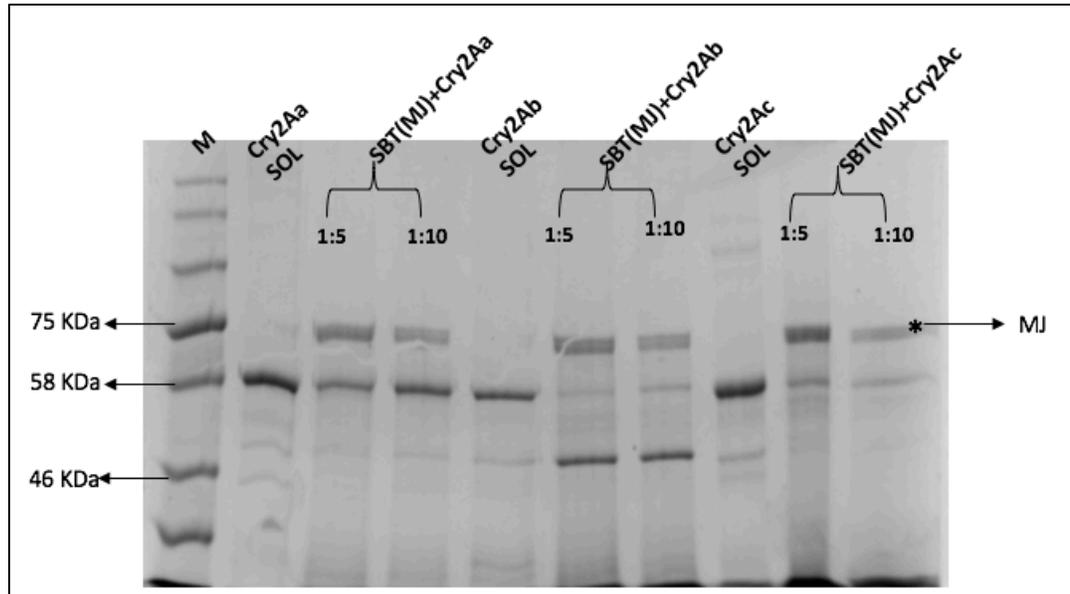


Figure 4.19. SDS-PAGE gel showing an activation of Cry2Aa, Cry2Ac and Cry2Ab by midgut juice of SBT population of *P. xylostella*. Cry2A toxins were solubilized for 1hour at 37°C in 50 mM sodium hydroxide and activated with (MJ) mid gut juice of SBT population using different dilutions for 5 minutes at 37 °C, the samples were run on 7.5 % SDS gel. (SOL) indicates solubilized protein.

From the above gel (Figure 4.19) for the activation of Cry2Ac, 2Aa and 2Ab using the midgut juice of SBT population of *Plutella xylostella*, it could be observed that there was no obvious activation of the toxin by the midgut juice in the case of Cry2Aa (lanes 3, and 4), and Cry2Ac (lanes 9 and 10), whereas it appears to be digestion of the toxin by the midgut juice in the case of Cry2Ab (lanes 6 and 7), where the toxin was digested from around 58 KDa to 48 KDa by midgut of SBT population of *Plutella xylostella*. The bands indicated by asterisk in the gel are from the midgut juice of the insect.

The gel showing the activation of the toxins by the mid gut juice of NO-QA population of *Plutella xylostella* is shown in (figure 4.20) below.

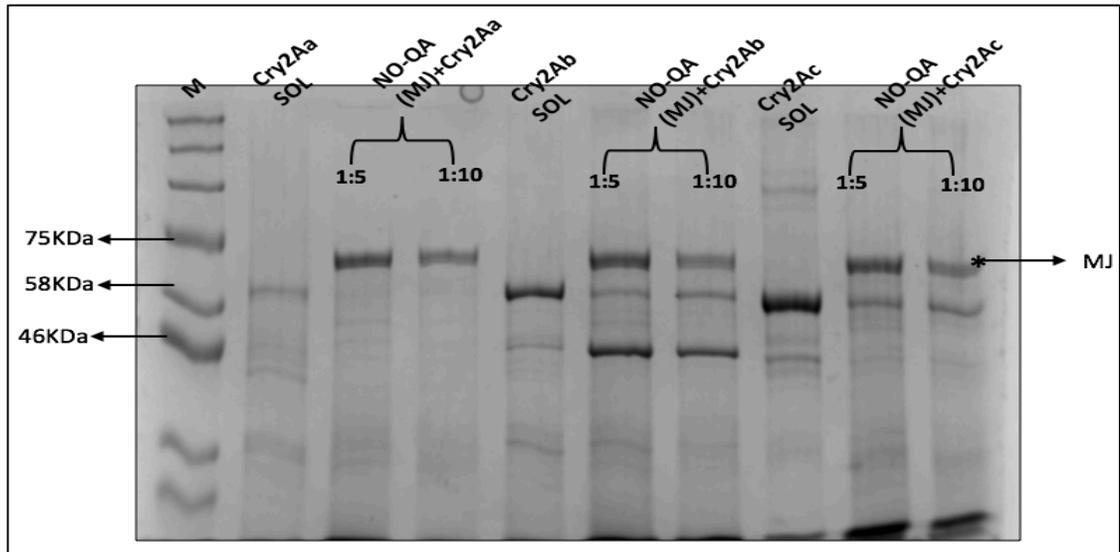


Figure 4.20A. SDS-PAGE gel showing an activation of Cry2Aa, Cry2Ac and Cry2Ab by mid gut juice of NO-QA population of *P. xylostella*. Cry2Atoxins were solubilized for 1hour at 37°C in 50 mM sodium hydroxide and activated with (MJ) mid gut juice of NO-QA population using different dilution for 5 minutes at 37 °C, the samples were run on 7.5 % SDS gel. (SOL) indicate solubilized protein.

From above gel (figure 4.20A) it appears that there was no digestion of the toxins by the midgut juice of NO-QA population of *Plutella xylostella* in the case Cry2Ac (lanes 9 and 10), whereas there was digestion of Cry2Ab by the mid gut juice (lanes 6 and 7), but for Cry2Aa due to the low concentration of the solubilized toxin we could not conclude the result from the gel, hence we repeated the SDS gel for only Cry2Aa toxin as shown in (figure 4.20B). The bands indicated by asterisk appeared to come from the midgut juice as they are absent in solubilized toxins, to confirm that we ran the solubilized toxin along with the midgut juice of both populations of *P. xylostella* as shown in (figure 4.21).

It seems that from (figure 4.20B) there was no digestion of the Cry2Aa toxin by the midgut juice of NO-QA population of *Plutella xylostella* (lanes 4 and 5) the band indicated by arrow

(MJ) come from the midgut juice.

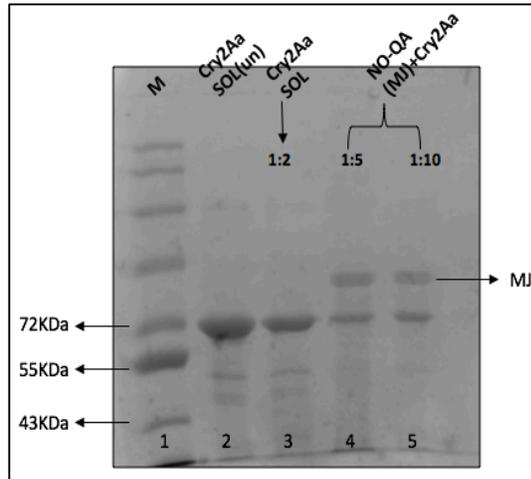


Figure 4.20B. SDS-PAGE gel showing an activation of Cry2Aa by mid gut juice of NO-QA population of *P. xylostella*. Cry2A toxins were solubilized for 1 hour at 37°C in 50 mM sodium hydroxid and activated with (MJ) mid gut juice of NO-QA population using different dilution for 5 minutes at 37 °C, the samples were run on 7.5 % SDS gel. (SOL) indicate solubilized protein and (SOL-un) indicate undiluted solubilized protein.

Therefore, it appears that the midgut juice obtained from the two populations of *Plutella xylostella* gave the same pattern of activation as regards the three Cy2A toxins, Cry2Aa, Cry2Ac and Cry2Ab used in this study, this could confirm that there is no correlation between how the toxin is cleaved and its specificity.

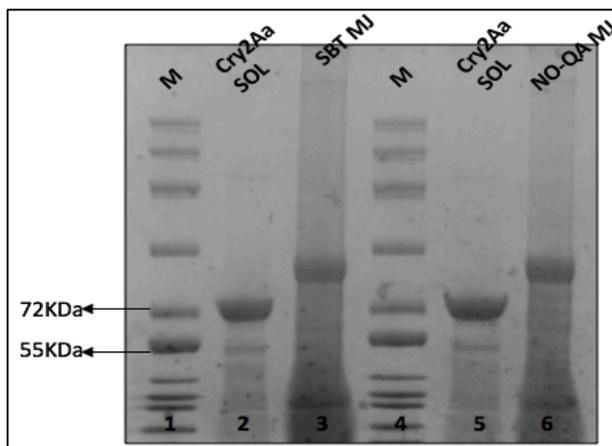


Figure 4. 21. SDS-PAGE gel showing solubilized Cry2Aa toxin run along with mid gut juice for both populations of *p.xylostella*. Lane (1 and 4) protein marker, lane (2 and 5) solubilized Cry2Aa, lane (2) mid gut juice for SBT population and lane (6) mid gut juice for NO-QA population.

4.4 Binding assay for Cry2A toxins against BBMV of SBT and NO-QA populations of *Plutella xylostella*

The binding assay was carried out for two representatives of Cry2A toxins, Cry2Ac and Cry2Ab as these were the two toxins that were used to create the hybrids and they showed different activity to NO-QA population of *Plutella xylostella* in this study. The binding assay will help us to find out if the difference in activity of a given toxin to two different populations of the same insect witnessed in this study in the case of Cry2Ab, which appeared to be toxic against SBT population of *Plutella xylostella* and non-toxic to NO-QA population, has to do with binding and/or non-binding of the toxin to the receptors of the insect. The binding assay was carried out following a method described by Wang *et al.*, (2018), as described in the materials and methods chapter of this project. The gels obtained from the western blot analysis are depicted in (figure 4.22 and 4.23).

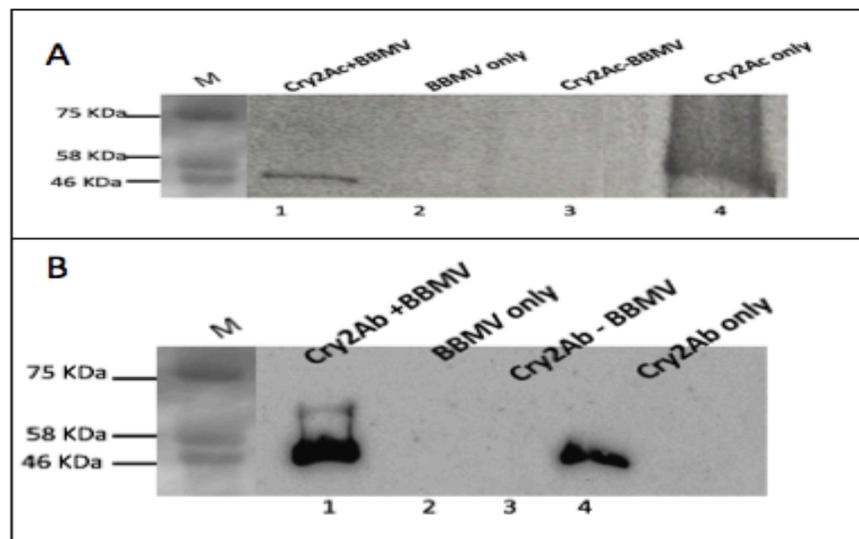


Figure 4.22. Western blot analysis showing binding assay of Cry2Ac and Cry2Ab toxins to BBMVs of NO-QA population of *Plutella xylostella*.

Figure 4.22 A, showed that there is binding interaction between Cry2Ac and the BBMV of NO-QA lane 1 showing a band at around 48 KDa corresponding to the molecular weight of the activated form of the toxin, which was used in the binding assay. Lane 2, corresponds to the BBMV preparation only, which shows no any band thus indicating the purity of the BBMV preparations and further showing that any binding interaction observed is solely between the activated toxin and the BBMV. Lane 3, showed the activated form of Cry2Ac given the same treatment as lane 1 but this time with no BBMV, to confirm there is no precipitation of the toxin and to show that the band in Lane 1 is as a result of binding interaction between Cry2Ac and the BBMV of the NO-QA population of *Plutella xylostella*. Lane 4 showed only the activated form of Cry2Ac and a validation of the results seen from the other lanes. Therefore, the results showed that there is binding between Cry2Ac and the BBMV of the NO-QA population of *P. xylostella* indicating that this toxin binds to the receptors within the midgut epithelium of NO-QA population of *Plutella xylostella*.

The binding assay between Cry2Ab and the BBMV of NO-QA population of *Plutella xylostella* in (figure 4.22 B) gave similar results as with the binding of Cry2Ac with the NO-QA population.

Therefore, the binding assay of these toxins, Cry2Ac and Cry2Ab to the SBT population of this insect is shown in (figure 4.23).

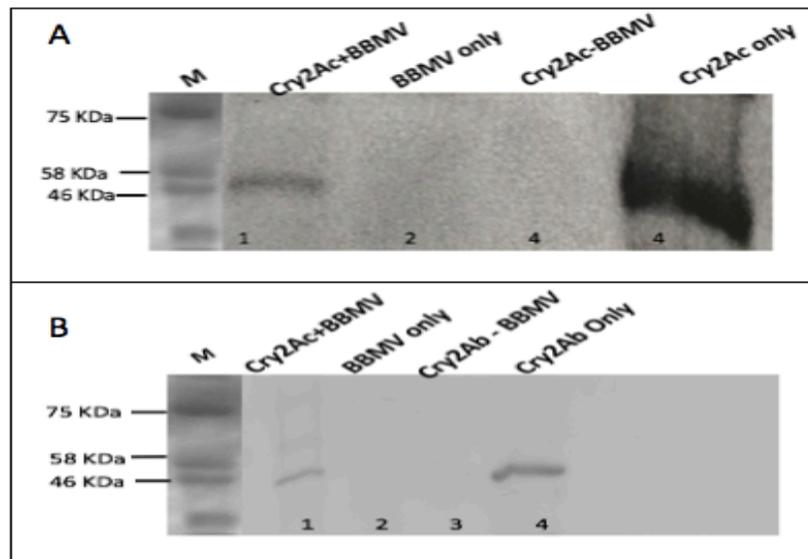


Figure 4.23 Western blot analysis showing binding assay of Cry2Ac and Cry2Ab toxins to BBMVs of SBT population of *Plutella xylostella*. Samples were run on 7.5% SDS-PAGE.

Figure 4.23 A, shows the binding assay of Cry2Ac to the BBMV of SBT population of *P. xylostella* and indicates that there is binding interaction between Cry2Ac and the BBMV of SBT (lane 1) giving a band at around 48 KDa corresponding to the molecular weight of the activated form of the toxin, which was used in the binding assay. Lane 2, corresponds to the BBMV preparation only, which gave no band thus, indicating the purity of the BBMV preparations and further showing that any binding interaction observed could solely be between the activated toxin and the BBMV of SBT population of the *Plutella xylostella*. Lane 3, showed the activated form of Cry2Ac given the same treatment as lane 1 but this time with no BBMV, this is to confirm that there is no precipitation of the toxin and to show that the band in Lane 1 is as a result of binding interaction between Cry2Ac and the BBMV of the SBT population of *Plutella xylostella*. Lane 4 showed only the activated form of Cry2Ac and

a validation of the results seen from the other lanes (1, 2 and 3). Therefore, the results showed that there is binding between Cry2Ac and the BBMV of the SBT population of *Plutella xylostella* indicating that Cry2Ac binds to the receptors within the midgut epithelium of SBT population of *Plutella xylostella*.

Figure 4.23 B, shows the binding assay between Cry2Ab and the BBMV of SBT population of *Plutella xylostella* and it appeared to give similar results as with the binding of Cry2Ac with the same population of the insect.

4.5 Discussion

This chapter involved comparative studies between *E.coli* and Bt expression system in terms of toxin yield and activity against the two different populations of *P.xylostella*.

Different strategies have been used in the past to improve the yield and activity of Cry proteins. For instance, Park *et al.*, (1999) showed that the combination of Cyt1 promoter and Cry3A STAB mRNA referred to as (Cyt1AP/STAB) led to the enhancement of protein synthesis in different Cry proteins, the level of which depends on the Cry protein in question. In addition, it has also been shown that co-expression of Cry2Aa toxin with a P20 helper protein resulted in a high yield of toxin and an increased activity against *E. kuehniella* and *A. aegypti* larvae respectively, also prevent the early degradation of the toxin within the insect mid gut (Elleuch *et al.*, 2016). The aim in this chapter was to compare two expression systems.

To achieve this, we adopted the work of Crickmore and Ellar, (1992) who showed the importance of *orf2* gene for the efficient expression and formation of crystal in Cry2Aa by constructing a vector called pSV27B40B where the *orf2* gene is situated upstream of the *cry2A* gene. Therefore, we used two restriction enzymes, *Xba*I and *Hind*III, to excise the gene from the vector and replaced it with our wild type and hybrid toxin genes, these were then expressed in Bt.

The level of expression of the toxins was higher in the *E.coli* expression system compared to the Bt expression system because of using the equal volume of product shown in (figure 4.14). Though, the *Bt* expressed toxin appeared to be clearer on the gel. This is seen most

clearly in the hybrid toxin Cry2AbAcAc expressed in *E.coli* lane 5 and Bt lane 6 in (figure 4.14).

The result obtained by Kumar and Udayasuriyan (2004) who cloned two insecticidal crystal genes of *Bt* namely: Cry2Aa and Cry2Ab, from new isolates of Bt 22-4 and 22-11 respectively by fusing the genes of both toxins down stream of Cry2Aa promoter and orf1 + orf2 sequences. They discovered low level of expression of both toxins in recombinant *Bt* strain whereas high level of expression of these genes, Cry2Aa and Cry2Ab, was achieved in the recombinant *E.coli* strain. This result is similar to ours in the sense that in (figure 4.14), the toxins expressed in *E.coli* expression system appeared to give a higher yield and hence expression levels compared to when same toxins were expressed in Bt expression system. Clearly, in our case it does not correspond to an activity. They explained the reason why they had a low level of expression of Cry2Aa /Cry2Ab in Bt, to be as a result of gene fusion brought about by the introduction of *Xho* restriction enzyme site just before the start codon, which displaced the Shine Dalgarno (SD) sequences (GGAGG) to 14 bp upstream of the start codon (ATG). Whereas, they discovered that in the wild type Cry2Aa/ Cry2Ab the Shine Dalgarno sequence was found to be highly conserved and is 8 bp upstream from the start codon. It was reported that the relative distance of the Shine Dalgarno sequence from the start codon determines the effectiveness of translation, the closer to the start codon (Kumar and Udayasuriyan, 2004).

The activities of the two differently expressed toxins were tested on two different populations of *P. xylostella* through bioassay as shown in (figure 4.16) for SBT population and (figure

4.17) for NO-QA populations. The bioassay results, which were presented on a graph, showed that there was no difference in activity between the toxin expressed in *E.coli* and Bt in both the two populations of *P. xylostella*. This showed that the expression system does not play a role in activities of these toxins against the two populations of *P. xylostella* in this instance.

Though the *E.coli* expressed toxins seem to look clearer on the gel compared to the Bt expressed toxin (figure 4.14), this does not influence their activities against the insect. Hence, a further insight into the fact that the presence of some other contaminants on the *E.coli* expressed toxins do not interfere with their activities as demonstrated in the control experiment depicted in (figure 3.3) in chapter three.

This clearly showed that different systems could be used to improve the yield and purity of different toxins expressed on a particular system but the system of expression used might not affect the activity of the toxin as shown in (figure 4.16 and 4.17) respectively.

We showed from our results that a given Cry2A toxin, for instance Cry2Ab, showed different activity towards the two populations of *Plutella xylostella*, whereas Cry2Ac showed the same activity. This led us to investigate if this difference in activity towards the two populations of *Plutella xylostella* by Cry2Ab toxin has to do with the ability of the toxin to bind to midgut of the population of *Plutella xylostella* or not. Therefore, we carried out binding assay of Cry2A toxins against the two populations to understand this, starting with their activation by chymotrypsin and midgut protease. The results which is displayed in (Figure 4.19), and it showed that both Cry2Ac and Cry2Ab were activated by chymotrypsin to a 48 KDa protein similar to what was obtained by previous researchers, who activated Cry2Ab with the same

protease (Xu *et al.*, 2016), who reported obtaining a 50 KDa band protein on complete digestion. The difference, which could be as a result of the different gel used and the experimental conditions. More also, the results indicated that midgut preparations from both SBT (figure 4.19, lanes 6 and 7) and NO-QA (figure 4.20, lanes 6 and 7) populations of *P. xylostella* activated Cry2Ab to protein of around 48 KDa similar to what was obtained in (Figure 4.19) when activated by chymotrypsin. However, it appears from our results that Cry2Ac is not activated by midgut proteases from both population of *P. xylostella* (figure 4.19, lanes 9 and 10) and the NO-QA population of *P. xylostella* (figure 4.20, lanes 9 and 10). Therefore, this may suggest that Cry2Ab and Cry2Ac have different mechanisms of activation by the two populations of *P. xylostella* though each of them is being activated the same way by chymotrypsin as could be seen in the amino acids sequence comparison between Cry2Ac and Cry2Ab depicted in figure (4.24)

Cry2Ac	ILSELQNLIFPSGSDILMQEILRATEQFINQRLNADTLGRVNAELAGLQANVAEFNRQVD	132
Cry2Ab	ILSELRNLIFFSGSTNLMQDILRETEKFLNQLNTDTLARVNAELTGLQANVEEFNRQVD	132
Cry2Aa	ILSELWGIIFFSGSTNLMQDILRETEQFLNQLNTDTLARVNAELIGLQANIREFNQVD	132
	*****.:*****.:***:*** **:*:*****:***.*****.*****.:*****.***	
Cry2Ac	NFLNPNQNPVPV A IIDSVNTLQQLFLSRLPQFQIQGYQLLLPLFAQAANLHLSFIRDVI	192
Cry2Ab	NFLNPNRNAV P SITSSVNTMQQLFLNRLPQFQMQGYQLLLPLFAQAANLHLSFIRDVI	192
Cry2Aa	NFLNPTQNPVP S ITSSVNTMQQLFLNRLPQFQIQGYQLLLPLFAQAANMHLSPIRDVI	192
	*****.:* **:*.* **:*:*****.*****.*****:*****.*****.:*****.**	

Figure 4.24. Multiple amino acids sequence alignment for Cry2Ac, Cry2Ab and Cry2Aa showing the cleavage site of chymotrypsin indicated by arrow.

The (figure 4.24) above showed that both Cry2Aa and Cry2Ab have Leu amino acid at position 144, a position which was shown by early study to be the cleavage site for chymotrypsin and *P. xylostella* midgut juice in Cry2Ab (Xu *et al.*, 2016). Hence, this explain why both Cry2Ac and Cry2Ab yield similar results on activation by chymotrypsin. However, this does not explain the differences in activation by the midgut juice of the two populations

of *P. xylostella*, which could mean that Cry2Ab and Cry2Ac have different activation sites with regard to two population of *P. xylostella*.

The activation results for Cry2Ab as regards to SBT population agrees with Morse hypothesis, who stated that the activation of Cry2Aa toxin led to the removal of the N-terminus 49-amino acids fragment resulting in the exposure of the residues comprising the putative receptor binding surface (Morse *et al*, 2001). But, this does not hold in case of the NO-QA population as Cry2Ab appeared to be non-toxic to this population.

More also, Cry2Aa and Cry2Ac were all activated by chymotrypsin to 48 KDa fragment which agreed with Morse's hypothesis, but when each of these toxins was activated by midgut juice from both insect populations there was no any activation. This suggest that with regards to SBT and NO-QA population of *P. xylostella*, Cry2Aa and Cry2Ac do not agree with the hypothesis put forward by Morse *et al.*, (2001). Therefore, this clearly showed that differences in activation of Cry2A toxins is not associated with activity because Cry2Ab which is activated to a 48 KDa protein is active to SBT population of the insect. Nevertheless, Cry2Ac and Cry2Aa, which are not activated at all also were active to both insect populations.

The results from the binding assay of Cry2Ab and Cry2Ac to the two populations of *P. xylostella* depicted in (figure 4.21 and 4.22) showed that both Cry2Ab and Cry2Ac bind to the BBMV preparations from both SBT and NO-QA populations of the insect. Therefore, this results might suggest the fact that, the activity of the toxins in the case of Cry2Ab, which appeared to be active against SBT population of the insect but not NO-QA population, is not related to its ability to bind to the receptors in the midgut epithelium of the insect since

Cry2Ab is able to bind to BBMV preparations from both populations of the insect. Our results are similar to findings by Li *et al.*, (2004), who showed that both Cry 1Ab and Cry IAc bind to BBMV preparations from resistant and susceptible populations of *Ostrinia nubilalis* with the same binding affinity, despite the fact that they are two populations of the same insect with different activities to these toxins. Therefore, both our results and those of Li *et al.*, (2004) indicate that the activity of a Cry toxin to an insect population may not necessarily be connected with its ability to bind to the receptors of the insect as there may be other mechanisms for susceptibility not necessarily involving receptor binding.

There appeared to be agreement also between our results and those of Ballester, *et al.*, (1999) who analysed the competitive binding assay of four Cry toxins, Cry1Aa, Cry1Ab, Cry1Ac and Cry1Af of *Bt* in a susceptible and resistance strains of *P. xylostella* from Philippines, Hawaii, and Pennsylvania, part of which findings showed that the binding of Cry1Aa to its binding site in the NO-QA and PEN populations of *P. xylostella* does not relate to its toxicity to these insect populations. More also, findings by Wolfersberger, (1990) who studied the binding affinity of Cry1Ab and Cry1Ac on the BBMV preparations from *P. xylostella* and discovered that there is an inverse relationship between the binding of these two toxins to the insect receptors and their toxicities, further buttressed this point. In addition, Luo *et al.*, (1999) found that there is no association between binding and toxicity, different insecticidal Cry toxins Cry1Ac, Cry1Ca and Cry1Bb have ability to bind similarly to the BBMV from *S. exigua* and *S. frugiperda*, but they have different toxicity toward both insects, their results showed that Cry1 toxin binding is necessary but not sufficient for toxicity. Also Ocelotel *et al.*, (2015) was found that the specific binding of Cry1Ac to the BBMV from resistant and

susceptible larvae of *Pectinophora gossypiella* was similar, this suggest that this binding is not sufficient to confer susceptibility to Cry1Ac.

Therefore, both our results and those of Li *et al.* (2004), Ballester, (1999) and Wolfersberger, (1990) indicate that, the activity of a Cry toxin to an insect population may not necessarily be connected with its ability to bind to the receptors of the insect or that the binding of the Cry toxin to the receptor in these instances is not sufficient enough to elicit toxicity. This is because some toxins may require additional binding to one or more receptors for activity which may be absent in some insects or population of insects as the case maybe. In addition, there may be other mechanisms for toxin activity not necessarily involving receptor binding or that the assay cannot actually measure binding to actual receptors but may just be measuring non-productive binding to BBMV.

5. Comparative studies between an insecticidal toxin Cry2Ac and an anticancer toxin Cry41Aa.

5.1 Introduction

The main plan for the work described in this chapter was to try to interconvert an anticancer toxin known as parasporin-3 (Cry41Aa) and an insecticidal toxin (Cry2Ac). Generally, it is believed that, *Bacillus thuringiensis* has acquired insecticidal activity in the course of co-evolution with insects through a host-parasite relationship. Many believe, however, that Bt as a species is a more versatile environmental bacterium, but not an obligate pathogen of insects (Argolo-Filho and Loguercio, 2013). This is supported by evidence in natural environments, because many Bt isolates possess no insecticidal activity, and it was observed that the number of those isolates that have no known insecticidal activity outnumber those ones that possess an insecticidal activity (Ohba *et al.*, 1996, Mizuki *et al.*, 1999). Among such Cry proteins which have no known insecticidal activity is Cry41Aa also known as parasporin-3, this is one among six different parasporins which are PS-1, PS-2, PS-3, PS-4, PS-5, and PS-6. The first one is parasporin-1 (Cry31Aa1) which has been found to be very toxic to different cancer cell lines particularly HeLa and HL-60 cell lines (Katayama *et al.*, 2007). Parasporin-2 (Cry46Aa1) has been linked to cytotoxic activity against HepG2, HL-60, Sawano, Jurkat and MOLT-4 cell lines (Ito *et al.*, 2004, Ohba *et al.*, 2009). Parasporin-3 (Cry41Aa1) shows cytotoxic effect against only two cancer cell lines, HL-60 (myeloid leukaemia cancer) and HepG2 (liver cancer) (Ohba *et al.*, 2009). Parasporin-4 (Cry45Aa1) when treated with pepsin produces a 27 KDa fragment which was highly cytotoxic towards CACO-2, Sawano, MOLT-4, TCS, and HL-60 cells (Okumura *et al.*, 2011). Parasporin-5

(Cry64Aa) has been shown to exhibit cytotoxic activity against different mammalian cell lines, however is showing that the toxin has a cytotoxic effect against 7 out of 18 mammalian cell lines such as (MOLT-4, HepG2, TCS, HeLa, COS7, Vero, and Sawano cells) and low to no cytotoxicity to the others (Ekino *et al.*, 2014). Parasporin-6 (Cry 63Aa) has been shown to exhibit toxicity against HepG2 and HeLa cells. But our main interest here is parasporin-3, Parasporin-3 (PS3) also known as Cry41Aa, is a three-domain Cry protein, activated by N- and C- terminal digestion (Ohba *et al.*, 2009). It has a typical three-domain structure with five block sequences commonly conserved in the non-insecticidal Cry proteins. It consists of 825 amino acid residues with a deduced molecular weight of 93,689, having low homology with insecticidal Cry proteins. Proteolytic digestion is required for activation of Cry41Aa: the 81 KDa precursor is converted to the 64 KDa toxic moiety by proteolytic processing of both N- and C-terminal regions (Okassov *et al.*, 2015, Ohba *et al.*, 2009). Krishnan *et al.*, (2017) reported that the ricin domain which is an additional amino acid sequence found in C-terminal of Cry41Aa was not involved in Cry41Aa toxicity. They also proposed that Cry41Aa is a pore-forming toxin and does not induce apoptosis. However, they detected morphological changes as well as membrane damage when the HepG2 cells were treated with activated Cry41Aa. Sequence comparison of Cry41Aa and insecticidal toxins led to the identification of loop 3 in domain II of Cry41Aa, which in insecticidal Cry toxins was previously shown to play a major role in receptor binding (Pacheco *et al.*, 2009). This exposed loop region, was shown to be important for the activity of Cry41Aa against HepG2 cells (Krishnan *et al.*, 2017). More work on Cry41Aa by Domanska, (2016) revealed that exposure to Cry41Aa gave rise to permeability of the membrane, which made the detection of both large and small size cytotoxic markers possible. Formation of channels in planar lipid

bilayers and biological membranes was induced by Cry41Aa, which activity on HepG2 cells was also inhibited by pre-treatment with EGTA. In addition, treatment of susceptible cells with Cry41Aa led to the activation of p38 MAPK, whose activity inhibition could not rescue cell viability (Domanska, 2016). The idea that parasporins may be fundamentally different to insecticidal Cry toxin, perhaps interacting with their target in different way. This forms the main aim of this chapter, which is trying to interconvert an anticancer toxin and an insecticidal one to test whether or not they are fundamentally different. We attempted to achieve the aim of this study by creating hybrids between Cry41Aa and an insecticidal toxin Cry2Ac, we used Cry2Ac as a representative of Cry2A toxins because based on our bioassay result it has a high activity to *P.xylostella* .

5.2 Result

5.2.1 Determination of Cry41Aa and Cry2Ac structures through structural prediction

The structures of three domain toxins were determined from previous literature by (Morse *et al.*, 2001). For the Cry2 toxins, it is only the structure of Cry2Aa that has been elucidated, therefore, we used Cry2Aa as a template to model the structure of Cry2Ac. The regions of Cry41Aa from domain I to domain III were all previously modelled out by PhD student (Elhigazi *et al.*, 2019).

The sequence showing the location of all the three different domains is depicted in (figure 5.1 and 5.2) below.



Figure 5.1. Represent the location of domain I, domain II and III in Cry41Aa toxin: domain I indicated by orange color, domain II indicated by black color and domain III indicated by green color. N terminal indicated by black color and ricin domain by red (Elhigazi *et al.* 2019).



Figure 5.2. Represent the location of domain I, domain II and III in Cry2Ac toxin: Domain I indicated by orange color, domain II indicated by black color and domain III indicated by green color.

5.2.2. Design of hybrids

In order to create different hybrids between Cry41Aa and Cry2Ac, we used two plasmids pGEMCry2Ac plasmid for Cry2Ac and pBS41Aa plasmid for Cry41Aa as shown in (figure 5.3).

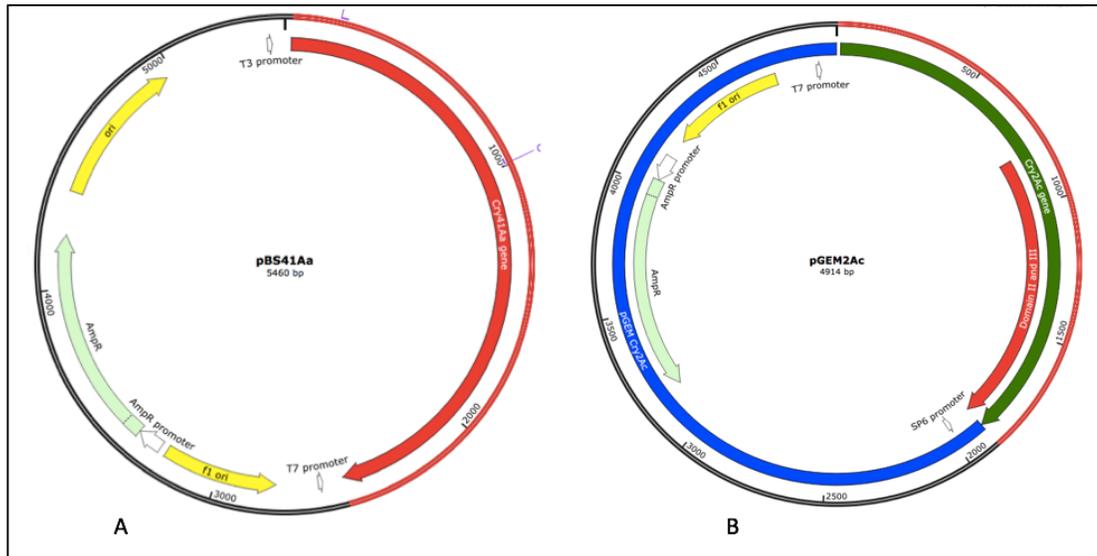


Figure 5.3. Plasmids used to create hybrid through domain swap (constructed using snap gene program). A Plasmid (pBS41Aa) encoding Cry41Aa. B plasmid (pGEM 2Ac) encoding Cry2Ac gene, both plasmids have ampicillin resistance gene.

We decided to exchange domains of Cry2Ac with that of Cry41Aa as shown in (figure 5.4), first we started by creating a hybrid which contains Cry41Aa domain I and Cry2Ac domain II and III in order to form hybrid (pGEMCry41Aa2Ac2Ac), based on research done by Shu *et al.*, (2017) who found that domain I of Cry2Aa responsible for the specificity of the toxin against *A.aegypti* larvae. As well as this domain I we want to move domain III from Cry41Aa into Cry2Ac to form (pGEMCry2Ac2Ac41Aa), as our previous result in chapter three showed that domains I and II, were responsible for *P.xylostella* specificity, final hybrid was to swap an insecticidal toxin domain II Cry2Ac into an anticancer toxin Cry41Aa domain I and III (Cry41Aa2Ac41Aa), due to domain II having a known role in specificity, Also, the result from previous homology-scanning mutagenesis experiment showed that the specificity region of Cry2A for *A.aegypti* larvae was located in domain II with both region 1 and 2 within these amino acids (307-382) and the specificity region against gypsy moth larvae located in region 2 of domain II of Cry2 (Liang and Dean, 1994). This result supported by Widner and Whiteley (1990) who found the specificity region of Cry2A against *A.aegypti* within these amino acids (304- 382).

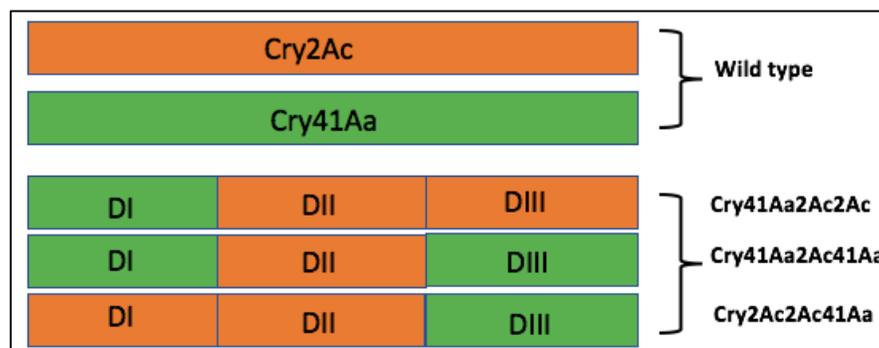


Figure 5.4. Schematic representation various created hybrids through domains swap between insecticidal toxin Cry2Ac and anticancer toxin Cry41Aa.

5.2.3 Creation of the hybrids through domain swap between Cry2Ac and Cry41Aa

In order to create hybrids through domains swapping as shown in (figure 5.5), primers were designed using primer select program, and these are listed in table (5.1) and were used for the PCR amplification products which were ligated to give the required hybrids. The PCR program (PFU ultra 6kb) was used, and the amplified products were mixed with 1 μ L of *DpnI* enzyme and incubated for 1 hour to digest parental/template strand. This was run on 1% agarose gel in order to prepare them for ligation, the gel below (figure 5.6) indicates the purified PCR products. The plasmid diagram showing the strategy for the design of the creation of the hybrids through domain swaps is shown in (figure 5.5).

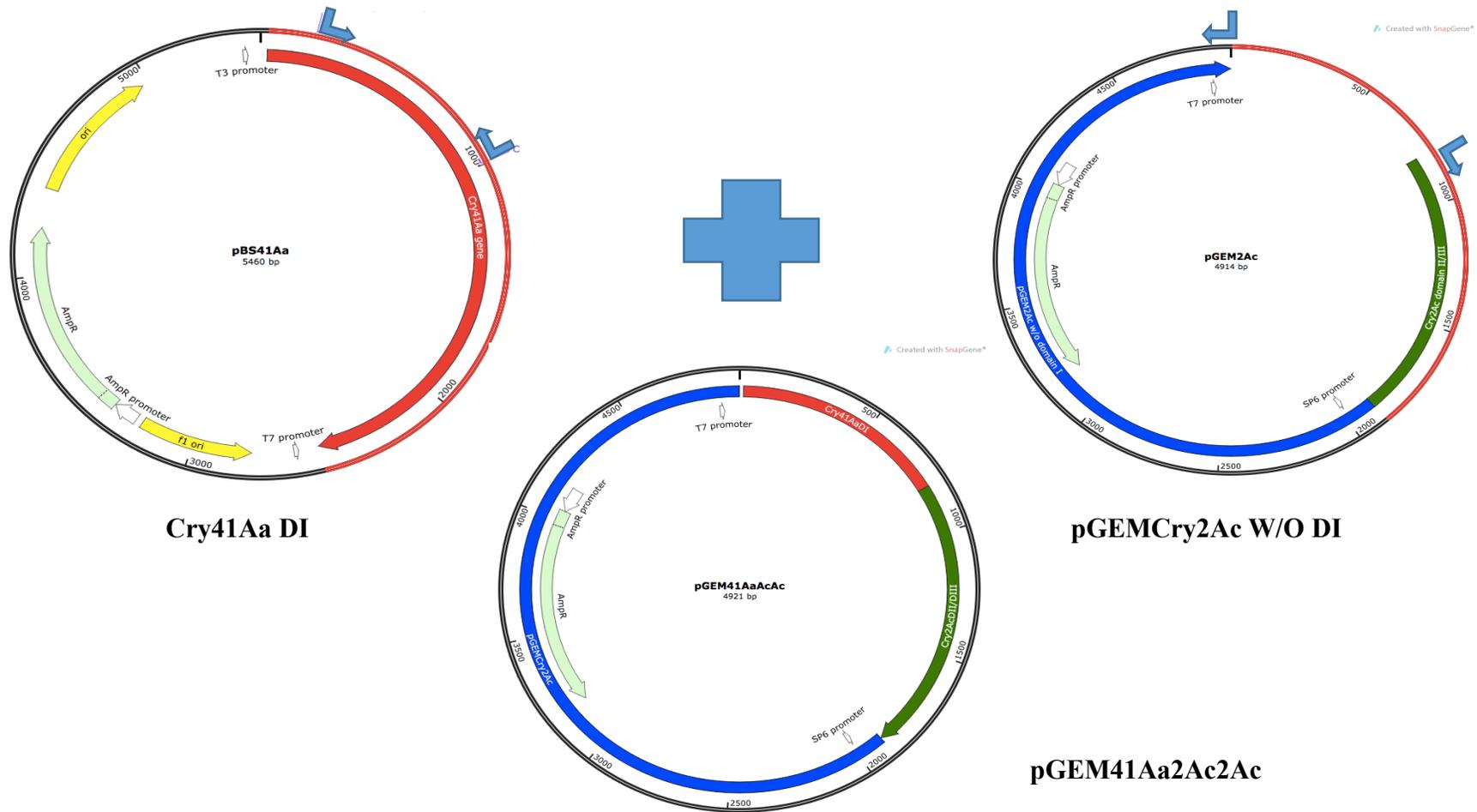


Figure 5.5. Schematic representation a design for creation hybrids through domain swap by using two plasmids pGEM Cry2Ac and pBSCry1Aa.

Domain I was amplified using Cry41Aa domain I forward and Cry41Aa domain I reverse, then we amplified pGEMCry2Ac without domain I which has domain II and domain III with the plasmid but without domain I, and this portion was also amplified using, Cry2ADIIF forward primer and pGEM reverse primer as depicted in (figure 5.5).

The primers used for amplification of various domains and creation of different hybrids, which design strategy is shown in (table 5.5).

N O	Oligo Name	Sequence (5'-3')	Modificatio n
1	Cry41Aa DIF	5' - TACAGCGCAGATGTAAGGGATG -3'	5'-PHO
2	Cry41Aa DIR	5' - AAGTCCCGGATCATAACGTTGG -3'	5'-PHO
3	Cry2ADIIF	5'-TATCAAAGCCTTCTAGTATCTTCCG-3	
4	pGEMR	5'-ATAAAATTCCTCCTTAATCGAATTC-3	
5	Cry41Aa DIIIIF	5'- GATTTGAACAATATAATATCACAGTATAG - 3'	5'-PHO
6	Cry41Aa DIIR	5'-AGTGGTTAAGCCAATACCCATAC -3'	5'-PHO
7	pGEM2ADIII F	5' - GGTTTGAGTGAATCACTAGTGAATTC - 3'	
8	Cry2Ac DIIR	5' - TTTTCTGTTATGCACTGTTACAAG -3'	

The table 5.1. Showing forward primers and reverse primers used to create hybrids through domain swap.

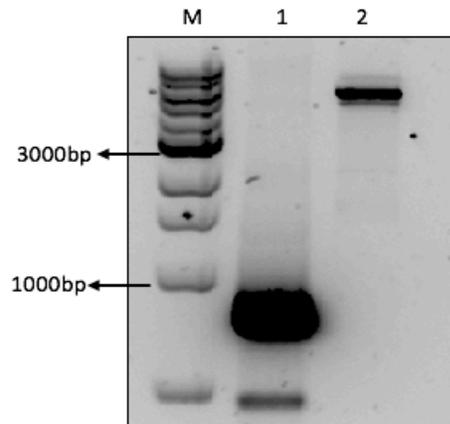


Figure 5.6. Confirmation of presence of amplified PCR products: PCR products was digested with *DpnI*, purified using Qiaquick kit and run on 1% agarose gel along with 1kb ladder (Marker) for size confirmation. Lane (M) Marker, lane (1) Cry41Aa domain I and (3) pGEMCry2Ac w/o domain I.

The amplified PCR products present in (figure 5.6) and the size of amplified products present in the gel was consistent with an expected size (795 bp) for Cry41Aa domain I and pGM2Ac without domain I (4262 bp). The amplicons were ligated Cry41Aa domain I and pGEMCry2Ac domain II and III, used to transform *E.coli*, colonies minpreped and plasmid was digested with *HaeIII* restriction enzyme as well as the sequencing were carried out to confirm the presence of right constructs as shown in (figure 5.7).

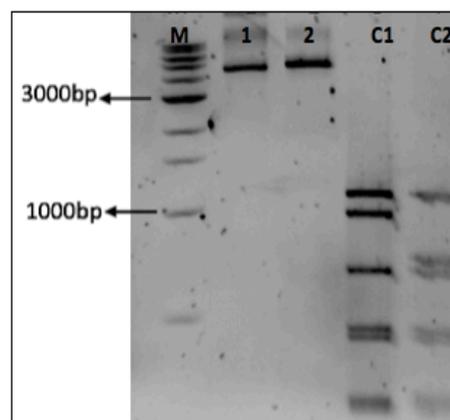


Figure 5.7. DNA minprep after each transformation step using QIAquick kit, digest with *HaeIII* then run on 1% agarose gel along with the marker. Gel is showing M (DNA marker), lane (1) DNA templateCry2Ac, lane (2) DNA template Cry41Aa, lane (C1) digested Cry2Ac with *HaeIII* and lane (C2) Cry41Aa2Ac2Ac DNA colony digested with *HaeIII* restriction enzyme.

Figure 5.7 shows that from the gel colony C2 seems to have the right plasmid when compared with the fragment generated from NEB cutter prediction as depicted in (figure 5.8). Hence, was sent for sequencing from which it was confirmed through sequence alignment with the expected construct.

A				B			
#	Ends	Coordinates	Length (bp)	#	Ends	Coordinates	Length (bp)
1	HaeIII-HaeIII	889-1917	1029	1	HaeIII-HaeIII	1039-206	1029
2	HaeIII-HaeIII	4899-888	904	2	HaeIII-HaeIII	445-1038	704
3	HaeIII-HaeIII	3639-4292	654	3	HaeIII-HaeIII	3789-4442	654
4	HaeIII-HaeIII	2834-3291	458	4	HaeIII-HaeIII	2984-3441	458
5	HaeIII-HaeIII	2400-2833	434	5	HaeIII-HaeIII	2550-2983	434
6	HaeIII-HaeIII	4435-4723	289	6	HaeIII-HaeIII	5049-285	301
	HaeIII-HaeIII	1918-2196	279	7	HaeIII-HaeIII	4585-4873	289

Figure 5.8. This illustrate the pGEMCry2Ac plasmid and the location of different restriction sites of *HaeIII*, table lists of the expected fragments digested with *HaeIII* restriction enzyme. Table (A) and (B) showed the differences in the size of the fragments between pGEMCry2Ac and pGEMCry41AaAcAc respectively depicted by yellow color. It taken from NEB cutter website.

5.2.4 Creating hybrids (Cry2Ac2Ac41Aa) and (Cry41Aa2Ac41Aa)

The amplified PCR products present in (figure 5.9) and the size of amplified products present in the gel was consistent with an expected size (4431bp) for pGEM2Ac without domain III and (594 bp) for Cry41Aa domain III. Domain III was amplified using primer Cry41Aa domain III forward and Cry41aA reverse, then we amplified pGEMCry2Ac without domain III using pGEMCry2A forward and Cry2A domain II reverse, the amplicons were ligated Cry41Aa domain III with pGEM2Ac domain I and domain II. In order to create hybrid involving domain II Cry2Ac, and domain I and III Cry 41Aa we amplified Cry41Aa2Ac2Ac without domain III and ligated with Cry41Aa domain III (figure 5.9). The primers used for

the amplification of the hybrid Cry41Aa2Ac2Ac are Cry41Aa domain III forward and Cry41Aa domain III reverse, whereas the ones used for the amplification of Cry41Aa2Ac2Ac without domain III, pGEM2A domain III forward and Cry2Ac reverse.

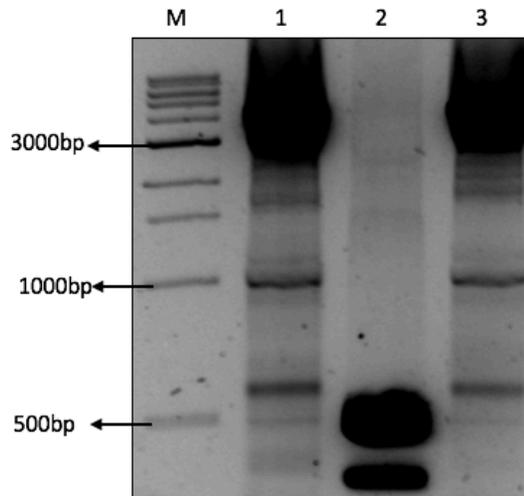


Figure 5.9. Confirmation of presence of amplified PCR products: PCR products was digested with *DpnI*, purified using QIAquick kit and run on 1% agarose gel along with 1kb ladder (Marker) for size confirmation. Lane (M) Marker, lane (1) pGEM Cry2Ac w/o domain III, lane (2) Cry41Aa domain III and lane (3) pGEM 41Aa 2Ac2Ac w/o DIII.

Circularized plasmid used to transform to *E.coli*, colonies minpreped and plasmids DNA was digested with *HaeIII* restriction enzyme to be able to know which of the colonies contain the right plasmid (figure 5.10). These colonies were electrophoresed on 1% agarose gel and the fragments obtained were compared to those generated using NEB cutters.

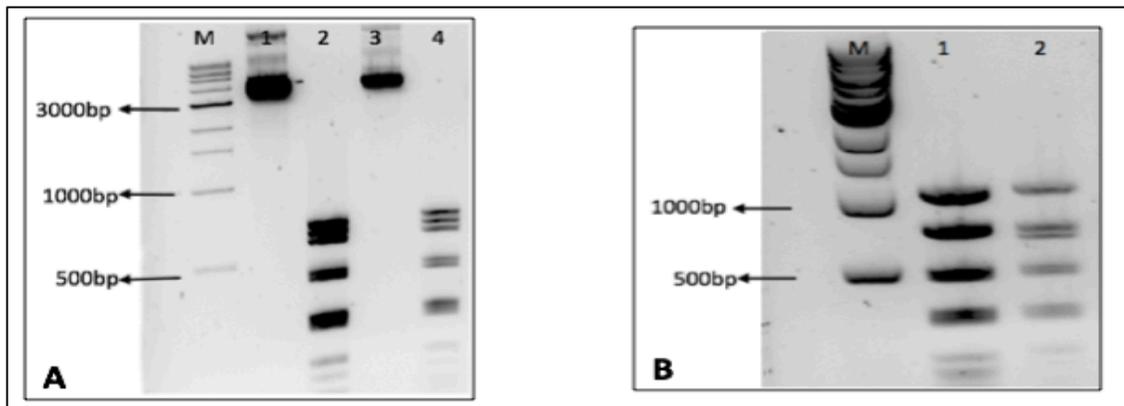


Figure 5.10. DNA miniprep after each transformation step using QIAquick kit, digest with *HaeIII* then run on 1% agarose gel along with the marker. Gel (A) is showing M (marker), lane (1) DNA template, lane (2) pSBCry41Aa2Ac41Aa colony digested with *HaeIII* restriction enzyme, lane (3) and lane (4) same samples with less concentration. Gel (B) lane (1) Marker, (2) pGEM2Ac2Ac41Aa digested with *HaeIII* and lane (3) the same sample with less concentration.

Figure 5.10 shows that, colony 2 from gel A and colony 1 from gel B appears to have the correct plasmids when compared with the fragment generated from NEB cutter prediction as depicted in (figure 5.11). hence, they were sent for sequencing, and were all confirmed through sequence alignment with their respective constructs to have the right sequence for the hybrid pBSCry41Aa2Ac41Aa, but for pGEMCry2AcAc41Aa hybrid was not successfully formed.

A				B			
#	Ends	Coordinates	Length (bp)	#	Ends	Coordinates	Length (bp)
1	HaeIII-HaeIII	335-1038	704	1	HaeIII-HaeIII	4965-888	904
2	HaeIII-HaeIII	3855-4508	654	2	HaeIII-HaeIII	3705-4358	654
3	HaeIII-HaeIII	1039-1644	606	3	HaeIII-HaeIII	889-1494	606
4	HaeIII-HaeIII	3050-3507	458	4	HaeIII-HaeIII	2900-3357	458
5	HaeIII-HaeIII	2616-3049	434	5	HaeIII-HaeIII	2466-2899	434
6	HaeIII-HaeIII	1645-1950	306	6	HaeIII-HaeIII	1495-1800	306
7	HaeIII-HaeIII	5115-285	301	7	HaeIII-HaeIII	4501-4789	289

Figure 5.11. This illustrate the pBSCry41Aa2Ac41Aa and pGEM2Ac2Ac41Aa plasmid and the location of different restriction sites of *HaeIII*, table lists of the expected fragments digested with *HaeIII* restriction enzyme. Table (A) and (B) showed the differences in the size of the fragments between pSBCry41Aa2Ac41Aa and pGEM2Ac2Ac41Aa respectively. Taken from NEB cutter website.

Next, the confirmed construct (Cry41Aa2Ac2Ac) was introduced into BL21 *E.coli* for expression of the encoded protein. This was grown, the hybrid protein was harvested, and a 7.5 % SDS-PAGE was performed to confirm the presence of the hybrid toxin along with the wild type (figure 5.12).

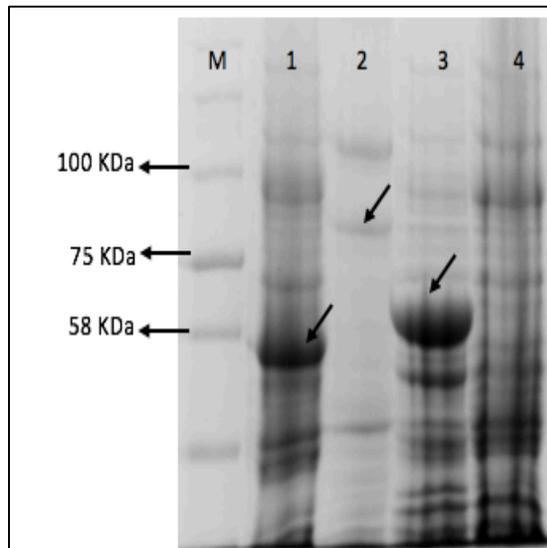


Figure 5.12. SDS-PAGE analysis of the wild type and hybrid toxins created indicated by number compared their size with the wild type Cry2Ac and Cry41Aa (M) protein marker, (1) Cry2Ac, (2) Cry41Aa, (3) Cry2Ac different sample and (4) Cry41Aa2Ac2Ac.

As can be seen in the (figure 5.12) the hybrid (pGEMCry41Aa2Ac2Ac) was not expressed, this could be as a result of misfolding of the protein. When we sent the DNA for sequencing for confirmation after *HaeIII* digest, we received the sequencing result and we discovered that the hybrid which involve domain I and III Cry41Aa and domain II Cry2Ac (Cr41Aa2Ac41Aa) also the hybrid involves domain III Cry41Aa and domain I and II Cry2Ac (Cry2Ac Ac 41Aa) had not been successfully created and so no expression studies were undertaken.

5.3 Discussion

This work which involved the interconversion of an anti-cancer toxin and an insecticidal toxin because we want to understand the functional relationship between the two toxins and how that translates into their activities against either insects or cancer cells. We attempted this through creating hybrids involving domains swaps between an anti-cancer toxin (Cry41Aa) and an insecticidal toxin (Cry2Ac), this was attempted to create three hybrids which was depicted in (figure 5.5), but unfortunately when we sent the DNA sample for sequencing, the sequencing result showed that there was no formation of two of the hybrids. We tried changing various reaction conditions for both the PCR and the primer but it still failed to work, and this fail could be as a result of the reaction condition or primers design, but due to the time constraints we could not continue with this work. Hence, we stopped at this point, but previous research carried out by (Elhigazi *et al.*, 2019) showed that swap between an anticancer toxin and insecticidal toxin is possible. The author exchanged loop3 between Cry41Aa and Cry1Ac which is an insecticidal toxin, and they discovered that Cry1Ac containing loop3 of Cry41Aa did not show anti-cancer activity. Furthermore, Cry41Aa hybrid containing loop3 of Cry1Ac failed to form stable protein, hence the activity could not be determined, and this failure to form a stable protein could be as a result of miss folding the protein. Considering that both the two Cry toxins in question have three domains structure, it was hoped that hybrid formation between the two toxins should not be too problematic

6. General discussion

This study was carried out basically to understanding the basis of specificity of the of Cry2A group of toxins against *P. xylostella*. Firstly, we carried out bioassay studies of all the wild type Cry2A toxins available in our lab because there are contradictory results in the literature. Therefore, we wanted to establish a more comprehensive and reliable bioassay data. Based on the results we obtained from the bioassay we established that the wild type toxins, Cry2Ac, Cry2Aa and Cry2Ab, were active against the SBT population of *P. xylostella*. Whereas, Cry2Ac and Cry2Aa toxins were active against the NO-QA population, while Cry2Ab was non-active against the same population.

After these bioassays, we then created some hybrids through different domain swaps to be able to understand the domain/domains that is/are responsible for activity in this group of toxins toward *P. xylostella*. Cry2Ab was active against SBT but not NO-QA population of *P. xylostella*, which could mean that differences in susceptibility of Cry toxins are population dependent as was observed by Ingber *et al.*, (2017) who worked with two different populations of lepidopteran insects which were *Spodoptera frugiperda* corn-strain and rice-strain.

In the case of Geneva- 88 population, Cry2Ac appeared to be the most active protein but unfortunately, we could not carry out further work with this population due to the fact that it was lost.

Previous studies have shown that Cry2Aa is active against *P. xylostella* similar to the result we obtained here as regards the two populations of *P. xylostella* used in this study (Tabashnik *et al.*, 1996, Monnerat *et al.*, 1999, Zago *et al.*, 2014)

Furthermore, previous studies also showed that Cry2Ab was active against *P. xylostella* (Pan *et al.*, 2014, Lin *et al.*, 2007, Zhao *et al.*, 2001). These results partially agreed with our findings, which showed that Cry2Ab was active towards SBT population.

In contrast, a review by Frankenhuyzen (2009) reported that, Cry2Ab and Cry2Ac had no insecticidal activity against *P. xylostella*. But our results showed that Cry2Ac is toxic against the all three populations of *P. xylostella*, which is contrary to what they reported. Hence, this justified why we carried out a fresh bioassay to be able to establish our baseline data.

Since we have established which Cry2A toxins were active against our populations and which were non-active, the next aspect of the study is to examine which of the three domains of Cry2A toxins influences activity against these two populations of *P. xylostella*. We carried out domain swaps as shown in (figure 3.9), involving a Cry2Ac toxin known to be active against NO-QA population, and another one known to be non-active, Cry2Ab. The results confirmed that both domain I and domain II of Cry2Ac are required in the hybrid for activity to the NO-QA population as shown in (figure 3.34), but not the N-terminus alone or N-terminus along with domain II that conferred activity based on our findings, in (figure 3.36 and 3.43).

These results partially agreed with the findings of Liang and Dean (1994) who discovered that some sequences in domain II are responsible for the specificity of Cry2Aa against mosquitos (*Aedes aegypti*) and gypsy moth (*Lymantria dispar*). These, therefore, affirm the

fact that some specific sequences could determine activity of the toxins against different insects. In addition, other research suggests that some residues in domain II not domain I or III responsible for the specificity of Cry2Aa against dipteran insects (Widner and Whiteley, 1990; Morse *et al.*, 2001).

We carried out comparative studies between *E.coli* and Bt expression system in terms of toxin yield and activity against the two different populations of *P. xylostella*. Though, we intend to compare two expression systems in terms of better yield and/or activity.

The results showed that the level of purity of the toxins was higher in Bt expression system compared to *E.coli* expression system as shown in (figure 4.14). Though, the Bt expressed toxin appeared to be clearer on the gel. This is seen most clearly in the hybrid toxin Cry2AbAcAc expressed in *E.coli* lane 5 and Bt lane 6 (figure 4.14).

The activities of the differently expressed toxins were tested on two different populations of *P. xylostella* through bioassay as shown in (figure 4.16) for SBT population and (figure 4.17) for NO-QA populations. The bioassay results showed that there was no difference in activity between the toxin expressed in *E.coli* and Bt in both the two populations of *P. xylostella*. This showed that the expression system does not play a role in activities of these toxins against the two populations of *P. xylostella* in this instance.

Though the *E.coli* expressed toxins seem to look clearer on the gel compared to the Bt expressed toxin (figure 4.14), this does not influence their activities against the insect. Hence, a further insight into the fact that the presence of some other contaminants on the *E.coli* expressed toxins do not interfere with their activities as demonstrated in the control experiment depicted in (figure 3.3) in chapter three.

This clearly showed that different systems could be used to improve the purity of different toxins expressed on a particular system but the system of expression used might not affect the activity of the toxin as shown in (figure 4.16 and 4.17) respectively.

Furthermore, we considered the relationship between toxin-receptor binding and activity because our results showed that Cry2Ab was active towards SBT but not NO-QA population of *Plutella xylostella*, whereas Cry2Ac showed activity against both populations. Hence the need to investigate if this difference in activity towards the two populations of *Plutella xylostella* by Cry2Ab toxin has to do with the ability of the toxin to bind to midgut of the population of *Plutella xylostella* or not. Therefore, we carried out binding assay of Cry2A toxins against the two populations to understand this, starting with their activation by chymotrypsin and the midgut protease. The results which is displayed in (Figure 4.19), and it showed that both Cry2Ac and Cry2Ab were activated by chymotrypsin to a 48 KDa protein respectively as observed by a previous study (Xu *et al.*, 2016), though they reported obtaining a 50 KDa band protein on complete digestion. More also, the results showed same pattern of activation for Cry2Ab by both the two populations of *P. xylostella* and chymotrypsin whereas Cry2Ac and Cry2Aa were activated by chymotrypsin but not obviously activated by midgut extract of either of the two populations of *P. xylostella*.

The activation results for Cry2Ab as regards to SBT population agrees with Morse's hypothesis, who stated that the activation of Cry2Aa toxin leads to the removal of the N-terminus 49-amino acids fragment resulting in the exposure of the residues comprising the putative receptor binding surface (Morse *et al.*, 2001). But, this does not hold in case of the NO-QA population as Cry2Ab has no activity against this population.

Cry2Aa and Cry2Ac, which were both activated by chymotrypsin to a 48 KDa protein agreed with Morse's hypothesis but not when activated by midgut juice from both insect populations as there was not any obvious activation.

Therefore, this clearly showed that differences in activation of Cry2A toxins is not associated with activity because Cry2Ab which is activated to a 48 KDa protein is active to SBT, but not NO-QA population of the insect. Nevertheless, Cry2Ac and Cry2Aa, which were not activated at all appeared to be active to both insect populations.

The results from the binding assay of Cry2Ab and Cry2Ac to the two populations of *P. xylostella* depicted in (figure 4.22 and 4.23) showed that both Cry2Ab and Cry2Ac bind to the BBMV preparations from both SBT and NO-QA populations of the insect. Therefore, this result might suggest the fact that the activity of the toxins, in the case of Cry2Ab, which appeared to be active against SBT population of the insect but not NO-QA population, is not related to its ability to bind to the insect receptors.

These findings agree with that of Li *et al.*, (2004), who demonstrated that both Cry1Ab and Cry1Ac bind to BBMV preparations from resistant and susceptible populations of *Ostrinia nubilalis* with the same binding affinity despite the fact that, they are only active to the susceptible population.

Previous binding studies also demonstrated similar scenario with the results we obtained from our binding studies (Ballester, *et al.*, 1999, Wolfersberger, 1990, Luo *et al.*, 1999, Ocelotel *et al.*, 2015).

Therefore, both our results and those of Li *et al.* (2004), Ballester, (1999) and Wolfersberger, (1990), Luo *et al.*, (1999), Ocelotel *et al.*, (2015) indicate that the activity of a Cry toxin to an insect population may not necessarily be connected with its ability to bind to the receptors of the insect or that the binding of the Cry toxin to the receptor in these instances is not sufficient enough to elicit toxicity. This is because some toxins may require additional binding to one or more receptors for activity which may be absent in some insects or population of insects as the case maybe. In addition, there may be other mechanisms for toxin activity not necessarily involving receptor binding or the assay may not be detecting productive binding.

We also attempted to convert an anti-cancer toxin into an insecticidal toxin to understand the functional relationship between the two toxins and how that translates into their activities against either insects or cancer cells. We created hybrids through domain swaps between an anti-cancer toxin (Cry41Aa) and an insecticidal toxin (Cry2Ac). Unfortunately, the sequencing results showed that there was no formation of hybrids in two of our constructs. Several attempts at recreating these hybrids failed and due the time constraints we could not continue with this work. Previous study carried out by (Elhigazi *et al.*, 2019) demonstrated that a swap between an anti-cancer toxin and insecticidal toxin is possible. The authors exchanged loop3 between Cry41Aa which is an anti-cancer toxin and Cry1Ac which is an insecticidal toxin, and they discovered that Cry1Ac containing loop3 of Cry41Aa does not show anti-cancer activity. Furthermore, Cry41Aa hybrid containing loop3 of Cry1Ac failed to form stable protein and as such the activity could not be determined. The inability to form a stable protein could be as a result of protein misfolding or instability of the various structure of the two combining protein.

Therefore, it is recommended that this work be continued by designing new primers and consider changing the PCR reaction conditions and the amount of both template DNAs, the primers, and buffers involved in the PCR reaction.

More also, it is recommended that the hybrids between Cry2Ac and Cry2Ab be tested on more populations of *P. xylostella* to see if the specificity determining residues could be further reduced to a particular domain or some amino acid motifs.

It is recommended that bioassay for the solubilized toxin, chymotrypsin activated toxins, and toxins activated by the midgut of the populations of *P. xylostella* be carried out to work out the mechanism of action of Cry2A toxins against *P. xylostella*.

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