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CHARACTERISATION OF INSECTICIDE RESISTANCE IN *Plutella xylostella* (THE DIAMONDBACK MOTH).

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I hereby declare that this thesis has not been submitted, either in the same or

different form, to this or any other University for a degree

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Abbreviations

APS: Ammonium persulphate

bp: base pairs

cAMP: Cyclic adenosine monophosphate

Cry: Crystal toxin

Cyt: Cytolytic toxin

DNA: Deoxyribonucleic acid

DTT: Dithiothreitol

E.coli: *Escherichia coli*

EDTA: Ethylene-diamine-tetraacetic acid

Kb: Kilobase

kDa: kilodalton

nAChR: Nicotinic acetylcholine receptor

PAGE: Polyacrylamide gel electrophoresis

PCR: Polymerase chain reaction

Px: *Plutella xylostella*

RGB: Resolving gel buffer

rRNA: Ribosomal ribonucleic acid

SDS: Sodium dodecyl sulphate

SGB: Stacking gel buffer

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

Tris: Tris(hydroxymethyl)aminomethane

APN: Aminopeptidase-N

GPI: Glycosylphosphatidyl-inositol

ALP: Alkaline phosphate

Abstract

Cry toxins are δ- endotoxins produced by *Bacillus thuringiensis*. They are toxic against different insect orders and are highly specific. However some of the insects have developed resistance to Cry toxins. Resistance to Cry1Ac in *Heliothis virescens, Pectinophora gossypiella* and *Helicoverpa armigera* has been linked to mutations in the cadherin gene. *Plutella xylostella* has also developed resistance to Cry1Ac but resistance to Cry1Ac in *Plutella xylostella* has not been linked to the cadherin gene. Previous studies have shown that a modified Cry1Ac toxin lacking helix-α1 of domain I is effective against insects which have developed resistance due to mutations in their cadherin gene. So it was decided to make modified Cry1Ac toxin lacking helix-α1 of domain I and check its effectiveness against the Cry1Ac resistant NOQA strain of *Plutella xylostella*. A modified *cry1Ac* toxin gene was created and expressed in *E.coli*. Bioassays conducted on the NOQA strain with modified and non-modified Cry1Ac showed that modified Cry1Ac was in fact the less effective toxin. This supports the hypothesis that the mechanism of resistance in NOQA population is not cadherin based.

A previous study has shown that field based resistance to spinosad in a *Plutella xylostella* strain collected from Pearl City, Hawaii is due to a point mutation in the ninth intron splice junction of nAChR Pxα6. Hence it was decided to check whether or not other spinosad resistant lepidopteran insects have similar mechanisms of resistance (i.e. splice-site mutation) as this population. PCR was performed to amplify nAChR intron 9 (including the splice junction) from a spinosad resistant *Spodoptera litura* population collected from the fields of Pakistan, but we were unable to amplify this region. Unfortunately the *Spodoptera litura* population was lost, so we could not carry on the experiments further. It was also decided to check whether *Plutella xylostella* NOQA population has the same splice site mutation as *Plutella xylostella* Pearl City population. Whether NOQA population is resistant to spinosad is not known. Sequencing showed that there was no splice site mutation present in NOQA.

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Chapter 1

Introduction

1.1. *Bacillus thuringiensis*

 Bacillus thuringiensis (Bt) is a gram positive spore forming bacteria. It forms a spore when it is in an adverse condition i.e. when nutrients become limiting. Bt produces protein crystals in the cytoplasm of the mother cell during sporulation (Schnepf et al., 1998). The protein crystals are insoluble protoxins when they are synthesized. Protoxins dissolve and become active in the insect gut. Protoxins require extremes of pH for dissolving, which is present in the insect gut. Protoxins are activated by insect gut protease (Knowles and Dow, 1993).

The δ- endotoxins consist of two multigenic families, Cry and Cyt. Cry proteins are toxic to different insect orders. They are toxic to Lepidoptera, Coleoptera, Hymenoptera, Diptera and also to nematodes. But Cyt proteins are toxic mostly against Diptera (Gomez et al., 2007). *Bacillus thuringiensis* is the most commonly used biopesticide to control insects which cause damage to the crops (Crickmore, 2005).

Bt based biopesticides are used as spray, granular or solid form to control insects. These biopesticides are commercially available under different names. *Bacillus thuringiensis kurstaki* strains are available in the name of Biobit, Dipel, Thuricide etc. and *Bacillus thuringiensis israelensis* strains are available in the name of Vectobac, Bactimos etc. (Cranshaw,2008).

Another method to control insects is to express the Cry genes in plants and these types of plants are known as transgenic Bt plants (Bravo and Soberon, 2008).

Prolonged and continuous use of Bt toxin has led to the development of resistance in three species of Lepidoptera in granary, open field and greenhouse. *Plutella xylostella* has developed Bt toxin resistance in the open field, *Trichoplusia ni* has developed Bt toxin resistance in the greenhouse and *Plodia interpunctella* has developed Bt toxin resistance in the granary (Heckel et al., 2007). Many other species of insects have developed resistance to Bt toxin by selection under laboratory conditions (Heckel et al., 2007; Griffiths and Aroian, 2005).

Bacillus thuringiensis strains have been isolated from different habitats such as soil, leaves, insects, freshwater, grain dust, mills, annelids and insectivorous mammals (Meadows et al., 1992; Espinasse et al., 2003; Martinez and Caballero, 2002; DeLucca

et al., 1984; Hendriksen and Hansen, 2002; Swiecicka et al., 2002).

Bacillus thuringiensis and *Bacillus cereus* are closely related. There is ample evidence which suggests that *Bacillus thuringiensis* and *Bacillus cereus* should be considered a single species. Biochemical and morphological methods of classifying bacteria could not differentiate *B.thuringiensis* from *B.cereus*. Molecular methods such as chromosomal DNA hybridization, 16S rRNA sequence comparison, amplified fragment length polymorphism analysis, genomic restriction digest analysis suggest that they are single species. The only notable phenotypic difference between *B.cereus* and *B.thuringiensis* is that most of the *B.thuringiensis* strains produce parasporal crystals (Carlson et al., 1994; Carlson et al., 1996; Keim et al., 1997; Schnepf et al., 1998; Prieto-Samsonov et al., 1997).

Flagellar (H) serotype method is the most preferred method for classifying the *B.thuringiensis* strains. Till now *B.thuringiensis* strains have been classified into more than 69 H serotype (Xu and Cote, 2008).

1. 2. *B.thuringiensis* Genome

Genome size of *B.thuringiensis* strains range from 2.4 to 5.7 million base pairs. Most of the *B.thuringiensis* strains have many extrachromosomal elements. Some of these extrachromosomal elements are circular and others are apparently linear (Carlson et al., 1994; Carlson et al., 1996).

Most of the Cry genes are present on large plasmids. *B.thuringiensis* possesses a large variety of transposable elements. These transposable elements include insertion sequences and transposons. The study of Cry1A genes showed that these genes were flanked by two sets of inverted repeated sequences. Nucleotide sequence analysis analysis of these repetitive elements showed that they were insertion sequence. They have been given the name IS231 and IS232. The IS231 and IS232 belong to IS4 and IS21 family of insertion sequence respectively. The first transposable element to be discovered in *B.thuringiensis* was Tn4430. It was isolated serendipitously from *B.thuringiensis* when it got inserted into a conjugative plasmid pAMβ1 transferred from *Enterococcus faecalis* (Mahillon et al., 1994; Kronstad and Whiteley, 1984; Lereclus et al., 1984; Lereclus et al., 1983).

1. 3. Expression of Cry Genes

Expression of Cry genes are either sporulation dependent or sporulation independent.

Most of the Cry genes are sporulation-specific genes i.e. they are expressed during sporulation. Mechanism of sporulation has been studied extensively in *Bacillus subtilis*. Sporulation is controlled by sigma factors. Each sigma factor recognizes a specific promoter and directs the transcription of that specific gene. These sigma factors are σ^A (primary sigma factor of vegetative cell) and five factors σ^H, σ^F, σ^E, σ^G and σ^K which become activated during sporulation. These five factors have been written in order of their appearance during sporulation. σ^A and σ^H factors are active before the septum formation i.e. in predivisional cell, σ^F and σ^G are active in forespore and σ^E and σ^K are active in mother cell (Losick and Stragier, 1992; Errington, 1993; Schnepf et al., 1998). Cry1Aa, Cry1Ab, Cry1Ac, Cry1Ba, Cry2Aa, Cry4Aa, Cry4Ba, Cry11Aa, Cry15Aa etc shows sporulation dependent expression and are expressed only in the mother cell compartment (Schnepf et al., 1998).

Cry1Aa gene possesses two overlapping promoters BtI and BtII. Brown and Whiteley isolated two sigma factors σ^{35} and σ^{28} from *B.thuringiensis.* σ^{35} directs transcription of Cry1Aa from BtI and σ^{28} directs transcription of Cry1Aa from BtII. BtI promoter is active from early to mid sporulation and BtII is active from mid sporulation to end of spore formation (Brown and Whiteley, 1988; Brown and Whiteley, 1990).

Amino acid sequence comparison of σ^{35} with σ^E and σ^{28} with σ^K shows that they are nearly identical. These comparison results suggest that σ^{35} of *B.thuringiensis* is a homolog of σ^E of *B.subtilis* and σ^{28} of *B.thuringiensis* is a homolog of σ^K of *B.subtilis* (Brown and Whiteley, 1988; Brown and Whiteley, 1990; Schnepf et al., 1998).

Adams and his colleagues showed that σ^{35} and σ^{28} can restore sporulation in σ^E and σ^K defective strains of *B.subtilis*. Their in vitro transcription assays showed that σ^{35} and σ^{28} can recognize the *B.subtilis* promoters recognized by σ^E and σ^K containing polymerase (Adams et al., 1991).

All the sporulation specific Cry genes such as Cry1Aa, Cry1Ab, Cry1Ac, Cry1Ba, Cry2Aa, Cry4Aa, Cry4Ba, Cry11Aa, Cry15Aa etc. are transcribed by either or both of the σ^E or σ^K forms of RNA polymerase (Baum and Malvar, 1995; Schnepf et al., 1998).

1. 3. 1. Sporulation Independent Cry Gene Expression

The Cry3Aa gene is an example of sporulation independent Cry gene expression. Several studies have shown that Cry3Aa gene is expressed during vegetative growth. The Cry3Aa promoter is similar to the promoter recognized by σ^A (the primary sigma factor of vegetative cells). Expression of Cry3Aa was increased and prolonged in

mutant strains of *B.subtilis* and *B.thuringiensis* which were unable to initiate sporulation. These results indicate that Cry3Aa gene expression is not dependent on sporulation. Cry3Aa gene is activated by genes regulating the transition from exponential growth to the stationary phase (Agaisse and Lereclus, 1995).

1. 3. 2. Cry mRNA Stability

The high level of toxin production in *B.thuringiensis* is due to the stable Cry mRNA. Production of stable mRNA maximizes gene expression. The half-life of Cry mRNA is about 10 minutes. The half-life of Cry mRNA is at least five times greater than the average half-life of bacterial mRNA (Agaisse and Lereclus, 1995).

Wong and Chang showed that fusion of Cry1Aa terminator fragment to the distal ends of either penicillinase (PenP) gene of *B.licheniformis* or the interleukin2 cDNA from human jurkat cell line increased the half lives of the mRNAs transcribed from these fusion genes in *E.coli* and *B.subtilis*. The half-lives of these fusion gene transcripts increased almost three times. The results suggest that Cry1Aa gene termination sequence acts as a positive retroregulator (Wong and Chang, 1986).

The Cry1Aa terminator sequence possesses an inverted repeat sequence. The inverted repeat sequence has the potential to form stable stem-loop structure. It has been proved that processive activities of $3^{'}$-$5^{'}$ exoribonucleases are disrupted by $3^{'}$ stem-loop structure. Therefore it is possible that Cry1Aa terminator may be involved in Cry mRNA stability by protecting it from exonucleolytic degradation from the $3^{'}$. Similar terminator sequence has been found downstream of various Cry genes (Agaisse and Lereclus, 1995).

Cry3Aa gene promoter region is composed of at least three domains: an upstream region from -635 nucleotide position to -553 nucleotide position, an internal region from -553 nucleotide position to -367 nucleotide position and a downstream region from -367 nucleotide position to +18 nucleotide position. Full expression of Cry3Aa gene requires the upstream region and the downstream region. The downstream region is involved in post-transcriptional event. Cry3Aa gene produces a stable mRNA whose $5^{'}$ end corresponds to nucleotide position -129. Deletion of nucleotides from positions -189 to -129 (60bp) showed no detectable effect on Cry3Aa expression level or on the position of $5^{'}$ end of transcript. Therefore Aggaise and Lereclus proposed that transcription of Cry 3Aa gene initiated at nucleotide position -558. This transcript is then processed from nucleotide -558 position to nucleotide -130 position generating a

stable mRNA whose 5' end corresponds to nucleotide position-129 (Agaisse and Lereclus, 1994; Agaisse and Lereclus, 1995).

Fusion of the Cry3Aa 5' untranslated region (from -129 nucleotide position to -12 nucleotide position) to the 5' region of lacZ reporter gene which is transcribed by the *B.subtilis* xylA promoter increased the stability of the lacZ fusion mRNA and also increased the production of β galactosidase by about 10 times. Deletion and mutation analysis suggest that a Shine-Dalgarno related sequence (GAAAGGAGG) from nucleotide positions -125 to -117 is the determinant of Cry3Aa stability. This Shine-Dalgarno sequence has been given the name STAB-SD. The interaction between the 3' end of 16S rRNA and STAB-SD could be the reason for the stability of Cry3Aa mRNA. Thus the binding of 30S ribosomal subunit to STAB-SD may protect Cry3Aa mRNA from 5'-3' ribonuclease activity. Therefore giving rise to a stable Cry3Aa mRNA with the 5' end at -129 position (i.e. the extent of 30S subunit protection) (Agaisse and Lereclus, 1996).

1. 4. Crystallization of δ- endotoxins

Cry proteins generally form crystal inclusions in the mother cell compartment of *B.thuringiensis*. Crystals have different shapes. The shapes of the crystals depend on their protoxin composition. Cry1 crystal has bi-pyramidal shape. Cry2, Cry3A and Cry3B crystals have cuboidal, flat rectangular and irregular shapes respectively. Cry11A crystal has rhomboidal shape and Cry4A and Cry4B crystals have spherical shapes (Schnepf et al., 1998).

Several Cry1 genes have been expressed in *E.coli* and *B.subtilis*. These Cry1 genes were able to direct the synthesis of biologically active inclusions in *E.coli* and *B.subtilis*. Thus suggesting that 130 to 140 kDa Cry1 proteins can spontaneously form crystals independent of the host bacteria. It is assumed that cysteine rich C-terminal halves of the Cry1 protoxins play an important role in maintenance of the parasporal inclusion structure. Cry3Aa (73kDa) protoxin does not possess cysteine rich C-terminal region. But the three dimensional structure of Cry3Aa protein shows the presence of four intramolecular salt bridges. These intramolecular salt bridges might be involved in providing stability to the crystal inclusion (Ge et al., 1990; Shivakumar et al., 1986; Bietlot et al., 1990; Li et al., 1991; Gill et al., 1992).

1. 5. Cry toxin Nomenclature and specificity of Cry toxins towards different insects

All the Cry genes whose sequences are available have been assigned to 218 holotypes by percent amino acid sequence identity. Each holotype is given a primary (Arabic number), secondary (uppercase letter), tertiary (a lowercase letter) and quaternary (another Arabic number) rank. Toxins with less than 45% amino acid sequence identity differ in primary rank. If the toxins have less than 78% and 95% amino acid identity they differ in secondary and tertiary rank respectively. Quaternary ranks have been given to those toxins which are more than 95% identical or identical but the sequences have been obtained independently. Quatneray rank is optional and is used only for the sake of clarity (Crickmore et al., 2011; Crickmore et al., 1998; de Maagd et al., 2001).

Fig 1: Cry toxins branches are colour coded according to insect order specificity of the toxin. Red: Coleoptera specific; Green: Lepidoptera specific; Blue: Diptera specific; Magenta: Nematode specific; Yellow: Hymenoptera specific (de Maagd et al., 2003).
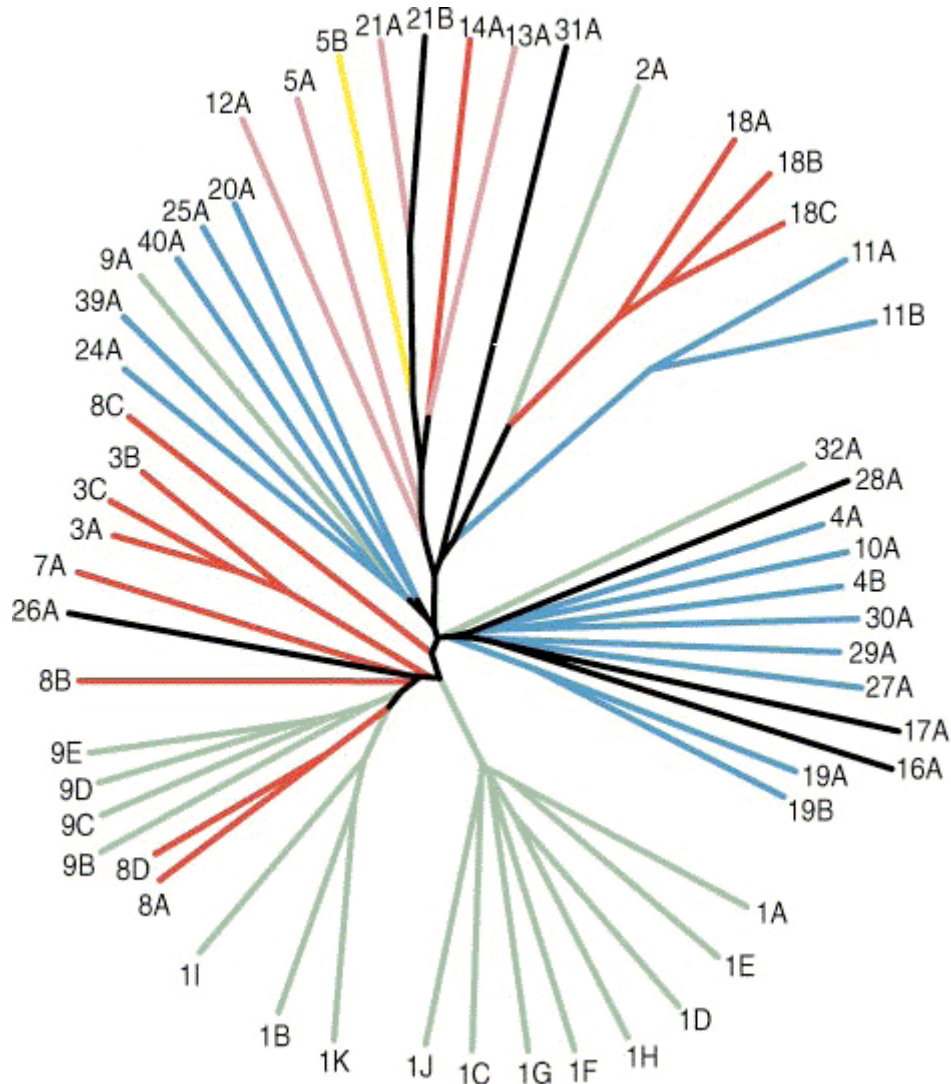
1. 6. Cry toxin diversity

The remarkable diversity of Cry proteins is assumed to be due to a high degree of genetic plasticity. Many Cry genes possess transposable elements. It is assumed that transposable elements are involved in gene amplification (or gene duplication) of Cry genes. Thus gene amplification (or gene duplication) of Cry genes may lead to the evolution of new toxins. Most of the Cry genes are present on plasmids and horizontal transfer of these plasmids may result in the creation of new toxins and strains (Piggott and Ellar, 2007; de Maagd et al., 2001).

Complete amino acid sequence alignment of the Cry proteins showed that most of the Cry proteins have five conserved blocks. The alignment result suggests that these conserved regions may be important for toxin function or stability. Block1 includes helix α-5 of domain I. Block2 covers helix α-7 of domain I and the first β strand of domain II. Block 3 includes the last β-strand of domain II. The last β-strand of domain II is involved in interaction between domain I and domain III. Blocks 4 and 5 are present in domain III (Hofte and Whiteley, 1989; Grochulski et al., 1995; Li et al., 1991).

1. 7. Three dimensional structure of Cry protein:

The majority of Cry toxins consist of three functional domains, domain I, II and III.

1. 7. 1. Domain I

Domain I which is present at the N-terminus consists of a bundle of seven alpha helices. It possesses a central helix surrounded by six helices. Outer helices are amphipathic in nature. Polar amino acid residues are generally projected towards the solvent and hydrophobic amino acid residues are projected towards the central helix. Polar groups are present in the interhelical space. All the polar groups in Cry3Aa are either hydrogen bonded or involved in the salt bridges. Domain I has striking similarities to the pore forming domain of Colicin A (a bacterial toxin). Helices α-4 and α-5 may form helical hairpin and initiate membrane insertion and thus pore formation. Helix α-5 is the most conserved region in the family of 3 domain Cry toxin. The role of domain I is in membrane insertion (Li et al., 1991).

1. 7. 2. Domain II

Domain II consists of three antiparallel β sheets. These sheets are packed together in

such a way that they form a β-prism with pseudo three fold symmetry. Two sheets are exposed to the solvent and the third sheet packs against domain I. Surface loop of the β sheets show similarities to immunoglobin antigen binding sites thus suggesting a role in receptor binding (Li et al., 1991; Boonserm et al., 2005; Pigott and Ellar, 2007).

Site directed mutagenesis studies of Cry1A toxins have shown that loop α-8 (present in the junction of domain I and domain II) and loop2 and loop3 regions of domain II are involved in receptor binding and toxicity (Rajamohan et al., 1996a; Rajamohan et al., 1995; Lee et al., 2000; Lee et al., 2001; Gomez et al., 2002b).

1. 7. 3. Domain III

Domain III forms a β sandwich structure. In the β-sandwich arrangement two antiparallel β-sheets pack together and resembles that of a "Jelly roll". The outer sheet is exposed to the solvent and the inner sheet pack against domain II (Li et al., 1991; Boonserm et al., 2005).

It is suggested that domain III possibly plays an important role in initial binding to the receptor and domain II may be responsible for the secondary, irreversible binding (Lee et al., 1999).

Domain swapping experiments have suggested that Cry1Ab domain III is involved in binding to midgut receptor in *Spodoptera exigua* (de Maagd et al., 1996).

1. 8. Mode of action of Cry toxin:

Until now two models have been proposed for the mode of action of the Cry1A toxins.

1. Pore formation model

2. Signal transduction model

In both the models initial steps are identical. These initial steps include ingestion, solubilization and proteolytic activation and primary receptor binding (Soberon et al., 2009; Knowles and Dow, 1993; Bravo et al., 2002).

1. 8. 1. Ingestion: Cry toxins are gut poisons so they must be first eaten by the susceptible larvae (Knowles and Dow, 1993).

1. 8. 2. Solubilization and Proteolytic activation: Cry1A toxins are synthesized as inactive protoxins of around 130-140 kDa. In most of the lepidopterans, protoxins are solubilized by alkaline conditions present in the midgut. Solubility of the crystals depends on the composition of the crystals (Knowles and Dow, 1993; Aronson et al.,

1991; Soberon et al., 2009).

After solubilization protoxins are activated by gut proteases. Trypsin-like or chymotrypsin-like enzymes are the major gut proteases. Gut protease typically removes some 500 amino acids from the C- terminus and 25-30 amino acids from the N-terminus of Cry1A. The active forms of toxins are of 65-55 kDa and are protease resistant (Knowles and Dow, 1993; Bravo et al., 2002; Schnepf et al., 1998).

Seven specific proteolytic cleavages occur at C-terminus end in a sequential manner. Each proteolytic cleavage produces a 10 kDa fragment which is rapidly proteolyzed further to small peptides (Gill et al., 1992).

Activated Cry1Ac comprises of amino acids from 29th position (N-terminus) to 623rd position (C-terminus). The 29th position and 623rd position residues are isoleucine and lysine respectively (Rukmini et al., 2000).

1. 8. 3. Receptor binding: The binding of Cry toxin to insect midgut epithelial receptors determines the specificity and toxicity of the Cry toxin. The relation between binding and toxicity was first shown by using brush border membrane vesicles (BBMV) from microvilli. The technique used to show the correlation between binding and toxicity was developed by Wolfersberger (Pigott and Ellar, 2007). Later Liang et al. showed that the rate constant of irreversible binding had a better correlation with toxicity than maximum extent of binding (Liang et al., 1995).

There are at least four different protein receptors that interact with Cry1A toxins. These are: a cadherin- like protein (CADR), a glycosylphosphatidyl-inositol (GPI)-anchored aminopeptidase-N (APN), a GPI-anchored alkaline phosphatase (ALP) and a 270 kDa glycoconjugate (Gomez et al., 2007).

1. 8. 3. 1. Cadherin

Cadherin proteins represent a diverse family of glycoprotein. Cadherin proteins have a variety of functions which includes cell adhesion, migration, cytoskeletal organization and morphogenesis. These proteins are transmembrane proteins. These proteins have two domains: cytoplasmic domain and extracellular ectodomain. Ectodomain contains several cadherin repeats. Classical cadherins are present primarily within adherens junctions which are involved in cell-cell adhesion. Cadherin like protein in lepidopteran species have been found on the apical membrane of midgut columnar epithelial cells which is the target site for Cry toxins. Much research has been done on lepidopteran

cadherin- like proteins as Cry1A receptors and there is good evidence which suggest that cadherin-like proteins play a very important role in toxin susceptibility. BT-R$_1$ a 210- kDa glycoprotein was the first cadherin-like protein shown to interact with Cry toxins. BT-R$_1$ was identified in *Manduca sexta* BBMV (Angst et al., 2001; Gumbiner, 1996; Vadlamudi et al., 1993; Gomez et al., 2007).

BT-R$_1$ contains a signal peptide, an extracellular ectodomain containing 11 cadherin repeats, a cadherin repeat 12- membrane proximal extracellular domain (CR12-MPED), a transmembrane domain and a small cytoplasmic domain (Vadlamudi et al., 1995; Chen et al., 2007). Francis and Bulla carried out ligand blot assays to show that Cry1Aa, Cry1Ab and Cry1Ac bind to BT-R$_1$ (Francis and Bulla, 1997).

Drosch et al. showed that Cry1Ab was cytotoxic to COS-7 and Human Embryonic Kidney (HEK) 293 cells expressing BT-R$_1$. Thus suggesting that BT-R$_1$ mediates cell death upon Cry toxin binding (Drosch et al., 2002).

Gomez et al. suggested that BT-R$_1$ promotes the conformational change in Cry1Ab when Cry1Ab binds to BT-R$_1$. The conformational change exposes helix α1 for proteolytic degradation and allows the formation of pre pore toxin oligomer (Gomez et al., 2002 a, Gomez et al., 2002 b).

A cadherin like protein BtR175 acts as a Cry1Aa receptor in *Bombyx mori* (Nagamatsu et al., 1998). Nagamatsu et al. showed that *Spodoptera frugiperda* 9 (Sf 9) cells expressing BtR175 swell when exposed to Cry1Aa.They suggested that swelling may be due to the formation of ion channels in cell membrane (Nagamatsu et al., 1999).

COS 7 cells expressing BtR175 showed susceptibility to Cry1Aa (Tsuda et al., 2003).

Genetic studies carried by Gahan et al. showed that a single major gene is responsible for resistance in *Heliothis virescens* YHD2 strain which has developed resistance in the lab. This gene was assigned to Linkage Group 9 (LG9). They tested whether gene encoding APNs and Cadherin like proteins in *Heliothis virescens* mapped to LG9. The gene encoding APNs were rejected because they did not map to LG9. Cadherin like proteins were not isolated from *Heliothis virescens* so they searched for and found a gene homologous to BtR175 and named it BtR4. The protein from BtR4 was 70% identical to BtR175 and was named HevCaLP. Subsequently BtR4 gene was mapped in YHD2 strain and it was found to be located on LG9. BtR4 gene disruption by a long terminal repeat-type retrotransposon is suggested as the reason for resistance in YHD2 strain (Gahan et al., 2001).

Jurat-Fuentes et al. showed that expression of HevCaLP was necessary for Cry1Aa

toxin binding to BBMV in *Heliothis virescens* (Jurat-Fuentes et al., 2004). Xie et al. showed that Cry1Ac and Cry1Ab could also bind to *Heliothis virescens* cadherin (Xie et al., 2005).

 1. 8. 3. 2. APN: APN is a GPI anchored exopeptidase. Lepidopteran APNs have been divided into five different classes. They have a number of functions in a wide range of species. In lepidopteran larval midgut they work together with endopeptidases and carboxypeptidases to digest proteins present in the insect diet (Herrero et al., 2005; Gomez et al., 2007; Wang et al., 2005).

Out of nine known class1 APNs five of them have been checked for their ability to bind to the Cry toxins. A 120 kDa Class1 APN from *M.sexta* was shown to bind to Cry1Ac, Cry1Ab and Cry1Aa. Cry1Ac binds to two different sites in Class1 APN. Out of these two sites one of the site is shared by all the three Cry1A toxins (Masson et al., 1995; Pigott and Ellar, 2007).

A 170 kDa class1 APN from *H.virescens* was shown to bind to Cry1Ac, Cry1Ab and Cry1Aa. All the three Cry1A toxins bind to two sites of APN from *H.virescens* (Luo et al., 1997; Pigott and Ellar, 2007).

Class 3 APN from *Lymantria dispar* binds to Cry1Ac but not to Cry1Aa or Cry1Ab (Valaitis et al., 1995; Pigott and Ellar, 2007).

Class 3 APN from *H.armigera* was expressed exogenously in *T.ni* cells. The expressed protein was highly glycosylated and enzymatically active and was of 120 kDa in size. The ligand blot analysis showed that Cry1Ac reacted with the expressed protein i.e. class 3 APN but Cry1Aa and Cry1Ab did not react with class 3 APN (Rajagopal et al., 2003; Pigott and Ellar, 2007).

So it can be summarized that Cry1Aa and Cry1Ab binds to class1 APN. Cry1Ac binds to class1 and class 3 APN. Cry1Ac has broader specificity than Cry1Aa and Cry1Ab. Cry1Ac binds to class1 APNs at two sites. It shares one site with Cry1Aa and Cry1Ab. Binding to other site is dependent on Gal NAC(N-acetylgalactosamine) and this region is threonine rich and predicted to be highly glycosylated.Cry1Ac also binds to class3 APN at this region (Pigott and Ellar, 2007; Gomez et al., 2007).

 1. 8. 3. 3. ALP: ALPs also act as Cry toxin receptors but the researches on ALPs are very limited as compared to APNs and Cadherin-like protein. Recent researches suggest that ALP may act as Cry1Ac receptor in *Maduca sexta* and *Heliothis virescens.* In

Heliothis virescens ALP is a 68 kDa GPI-anchored membrane glycoprotein and in *Manduca sexta* it is a 65-kDa BBMV protein (Jurat-Fuentes and Adang, 2004; McNall and Adang, 2003).

1. 8. 4. Pore formation model:

According to this model (fig 1) activated Cry1A toxins bind to the primary receptor (i.e. cadherin receptor). Binding of toxin to cadherin facilitates further proteolytic cleavage of toxin at its N terminal end, thereby eliminating helix alpha1 of domain I. This cleavage leads to the oligomerisation of monomeric toxin. Oligomerisation of toxin increases the binding affinity of toxin to the secondary receptor which is a glycosylphosphatidyl-inositol (GPI)-anchored aminopeptidase-N (APN) in *Manduca sexta* and a GPI-anchored alkaline phosphatase (ALP) in *Heliothis virescens*. After binding to the secondary receptor, oligomerised toxin inserts into lipid micro domain where these secondary receptors are localised and creates pore in apical membrane of midgut cells. This induces osmotic shock, bursting of midgut cells and finally leads to the insect death (Soberon et al., 2009).

1. 8. 5. Signal transduction model:

According to this model (fig 2) toxicity of Cry1Ab protein is due to the activation of a Mg2+ dependent signal cascade pathway. Cry1Ab toxin binds to the primary receptor (i.e. cadherin receptor) and triggers the pathway. Cry1Ab toxin and primary receptor interaction activates a guanine nucleotide-binding protein (G protein) which in turn leads to the activation of an adenylyl cyclase. Activated adenylyl cyclase promotes the production of intracellular cAMP. Increased levels of cAMP activate protein kinase A which in turn activates an intracellular pathway resulting in cell death (Zhang et al., 2006; Soberon et al., 2009).
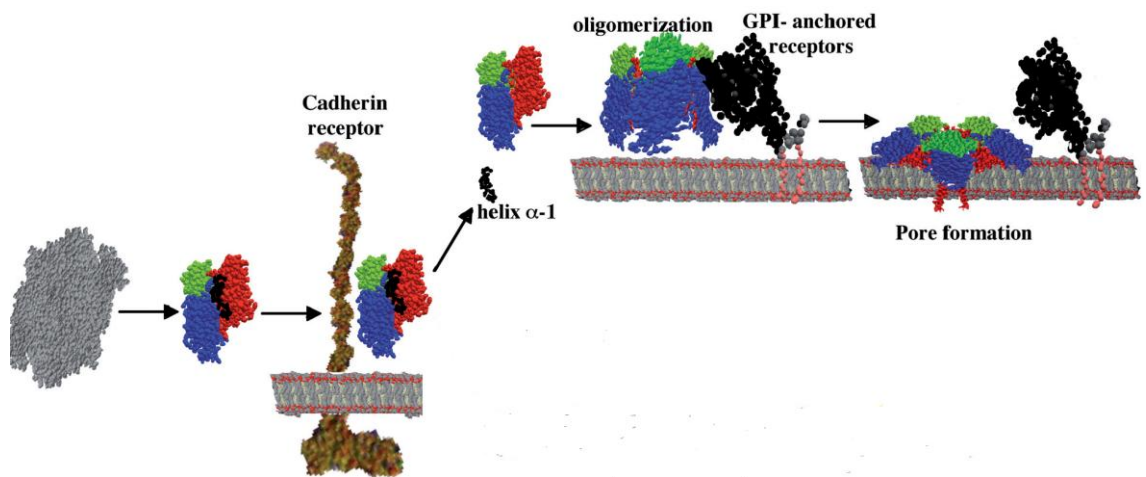
Fig 2 a: Diagrammatic representation of Cry1A toxins mode of action according to the pore formation model (Soberon et al., 2009).
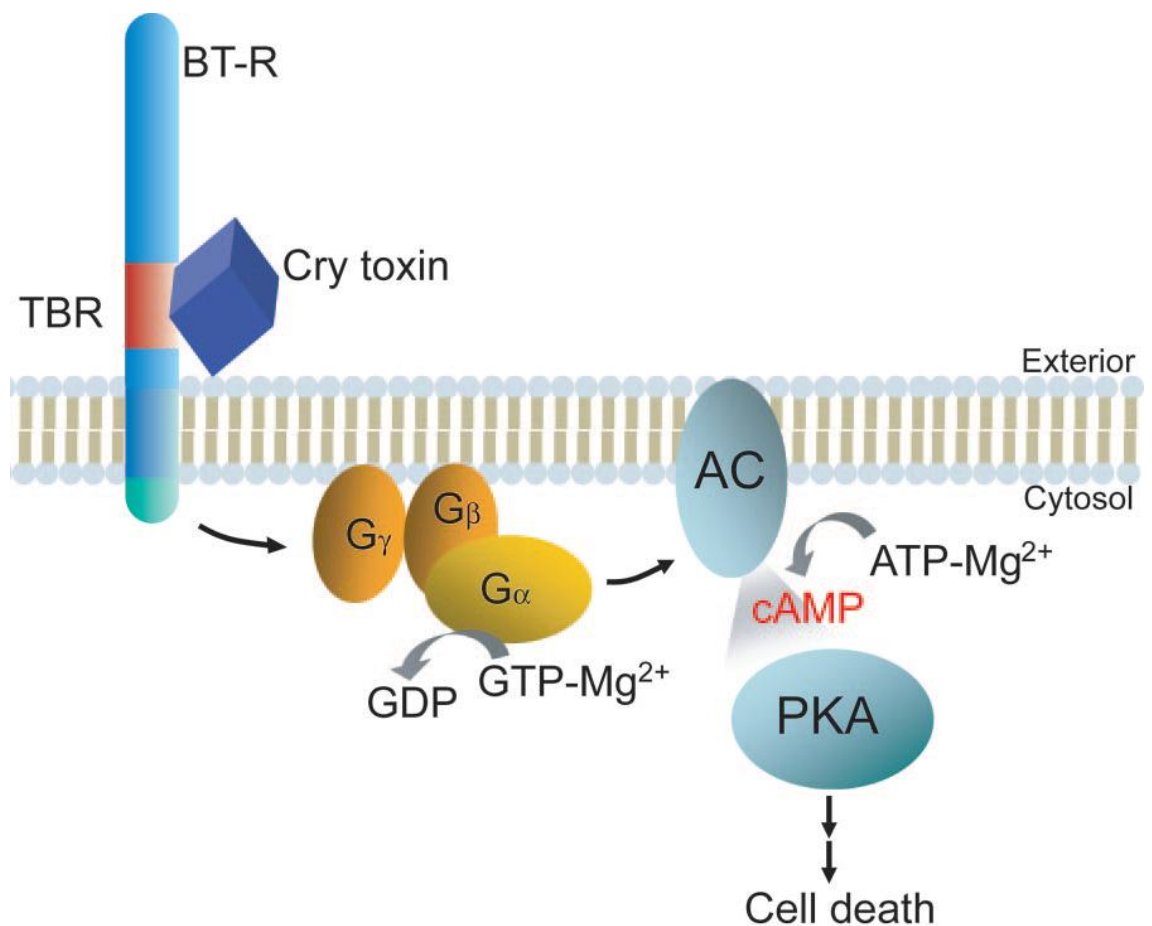
Fig 2 b: Diagrammatic representation of mode of action of Cry1Ab according to the signal transduction model (Zhang et al., 2006).

1. 9. Mechanism of resistance to Bt:

Changes in the biochemical and physiological processes of the insect gut and its content

can alter the mode of action of Bt and reduces the effectiveness of the Bt toxin considerably leading to insect resistance. Development of resistance involves various mechanisms. The mechanism of resistance depends on the type of insect, toxin and Bt strain. Defective solubilization, insufficient proteolytic activation, over proteolysis (i.e. toxin degradation), binding of toxin molecules to non-functional binding sites, defects in functional binding sites, defect in pore formation and increased cellular repair have been reported as mechanisms of resistance. But there is also a possibility of involvement of other mechanisms as well (Bruce et al., 2007; Griffitts and Aroian, 2005).

Many different resistance mechanisms have been proposed but the best characterized resistance mechanisms to date involve receptor inactivation at the midgut membrane or solubilization-activation of the crystal proteins. Receptor mediated mechanism may include loss of Cry toxin binding sites or binding of toxin molecules to non-functional binding sites. Resistance mechanisms based on solubilization and proteolysis may involve changes in the gut pH or changes in proteinases involved in protoxin activation (Bruce et al., 2007; Griffitts and Aroian, 2005; Ma et al., 2005).

1. 9. 1. Binding disruption:

In many resistant populations of *P. xylostella* reduced binding of Cry toxins was found (Sayyed et al., 2000; Sayyed et al., 2004; Sayyed et al., 2005). NOQA population of *P. xylostella* showed high levels of resistance to Cry1Aa, Cry1Ab, Cry1Ac, Cry1Fa and Cry1Ja but not to Cry1Ba, Cry1Bb, Cry1Ca. Experiments conducted with BBMV of NOQA population and Cry1Ac showed a dramatic reduction in the binding of Cry1Ac. Binding of Cry1Ab was virtually absent but binding of Cry1Aa to BBMV was unaltered. These results are in agreement with the proposed model for binding of Cry toxins to the brush border membrane of mid gut cells of susceptible *Plutella xylostella* larvae. According to this proposed model (fig 3) a binding site (site 1) is recognized only by Cry1Aa and another binding site (site 2) is shared by Cry1Aa, Cry1Ab and Cry1Ac. This suggests that modification in site 2 affects binding of all three toxins but Cry1Aa also binds to site1 and it is proposed that site 1 is probably not involved in toxicity. This may explain why NOQA population is resistant to these three toxins. The absence of cross-resistance to Cry1B and Cry1C was also observed in NOQA population. Cry1B binds to site 3 and Cry1C binds to site 4 and these two sites are not shared with the other toxins (Ferre and Van Rie, 2002; Griffitts and Aroian, 2005).
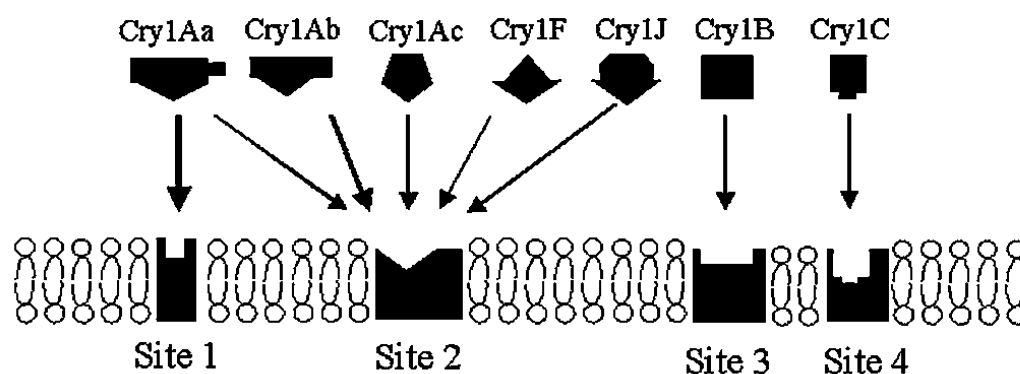
Fig 3: Suggested model for binding of Cry toxins to the brush border membrane of mid gut cells of susceptible *Plutella xylostella* larvae (Ferre and Van Rie, 2002).

Similar results were obtained with a *P.xylostella* colony from Pennsylvania (PEN). The population showed reduced binding to Cry1Ac and Cry1Ab but binding to Cry1Aa was unaltered. *P.xylostella* Loxa colony also showed similar results. These results showed that there is relationship between reduced binding and resistance. This was named as type I binding-site alteration by the researchers (Tabashnik et al., 1997; Ferre and Van Rie, 2002).

A *P.xylostella* colony (PHI) from the Philippines showed resistance to Cry1Aa, Cry1Ab and Cry1Ac but was susceptible to Cry1Ca, Cry1Fa and Cry1Ja. This population showed reduced binding of Cry1Ab but binding to Cry1Aa and Cry1Ac was unaltered. Similar results were obtained from *P.xylostella* SERD-3 population from Malaysia. This population showed reduced binding to Cry1Ab but not to Cry1Aa, Cry1Ac and Cry1Ca. These results were explained by type II binding site alteration. According to type II binding site alteration hypothesis alteration of site2 affects only one Cry protein which normally binds to this site. This explains that there is only a partial overlap of the binding epitopes of the different toxins (Tabashnik et al., 1997; Wright et al., 1997; Ferre and Van Rie, 2002).

In *P. xylostella* major Cry1A resistance gene has been mapped to AFLP (Amplified Fragment Length Polymorphism) linkage group22 (LG22) (Baxter et al., 2005; Heckel et al., 1999; Heckel et al., 2007).

Linkage analysis showed that none of the eight aminopeptidases, one alkaline phosphatase, one intestinal mucin, one glycosyltransferase and a homologue of a Cry1A binding protein from *B.mori* genes map to LG22. Till now genetic approach has failed to identify the resistance gene in NOQA and other *P.xylostella* population but it has conclusively removed 13 known genes including cadherin as candidates for resistance

genes (Baxter et al., 2005; Jurat-Fuentes and Adang, 2004; Sarauer et al., 2003; Griffitts et al., 2003; Hossain et al., 2005).

Xu and Wu showed that BBMV of Cry1Ac resistant *Helicoverpa armigera* strain GYBT had lower binding affinity to Cry1Ac as compared to the BBMV of Cry1Ac susceptible *Helicoverpa armigera* strain GY (Xu and Wu, 2008).

Wang et.al showed that BBMVs from Cry1Ac resistant strain of *T.ni* lack specific affinity for binding to Cry1Ab and Cry1Ac (Wang et al., 2007).

Reduced binding was also observed in resistant strains of *H.virescens* and *S.exigua* (Lee et al., 1995; Moar et al., 1995).

1. 9. 2. Altered Proteolytic Activation:

Altered proteolytic activation has been reported as mechanism of resistance in many insects including: *Spodoptera littoralis, Pieris brassicae, Heliothis virescens, Plodia interpunctella, Choristoneura occidentalis, Melolontha melolontha, Ostrinia nubialis and Leptinotarsa decemlineata* (Lecadet and Martouret, 1987; Jaquet et al., 1987; Oppert et al., 1997; Valaitis et al., 1999; Wagner et al., 2002; Li et al., 2004; Loseva et al., 2002).

Some insects resistant to Bt were found to have higher proteolytic activity or a relative higher concentration of proteases in the gut. Toxin sensitivity may be affected by types and or by the activity levels of gut proteases. Research was conducted on *P. brassicae, Mamestra brassicae* and *S. littoralis.* It was found that there was a direct correlation between the toxicity of Bt subsp.*thuringiensis,* gut protein concentration and protease activity (Oppert, 1999).

In *Helicoverpa armigera* toxin degradation was suggested as the mechanism of resistance. The excessive degradation was caused by chymotrypsin like proteases (Shao et al., 1998).

Excessive toxin degradation was also the suggested cause for resistance to Bt in *Choristoneura fumiferana* (Pang and Gringorten, 1998).

Another possible mechanism of resistance is sequestration of toxin by gut proteases and this mechanism has been reported in *C.fumiferana* (Milne et al., 1995).

The toxic effect of 14 different Bt strains were studied on *P.brassicae, H.virescens, S.littoralis.* There was a large variation in relative toxicities. These variation in relative toxicities depended on what the insects were fed i.e. they were fed crystals or solubilized crystals or invitro-activated toxins (Jacquet et al., 1987).

In Bt subsp. *entomocidus* resistant population of *Plodia interpunctella* significantly lower soluble gut proteinase activities were found. When phenotypic expression of gut proteinases was compared between susceptible and resistant population to Bt *subsp entomocidus* the absence of a major serine proteinase activity was observed in the resistant population. This proteinase is involved in Bt protoxin activation .Loss of proteinase could lead to toxin resistance. Bt resistance and loss of proteinase is genetically linked (Oppert et al., 1997).

In a resistant colony of *H.virescens* (CP73-3) slower activation of Cry1Ab protoxin and faster degradation of Cry1Ab toxin was observed when compared with susceptible population (Ferre and Van Rie, 2002).

Several resistant populations were reported to have more susceptibility towards activated toxin than protoxin. Resistant population of *P.xylostella* showed more susceptibility towards activated Cry1Ac and Cry1Ca toxin than Cry1Ac and Cry1Ca protoxin. Similarly resistant population of *P. interpunctella* and *O. nubialis* showed more susceptibility towards activated Cry1Ab toxin than Cry1Ab protoxin. The results from these populations suggest that only protoxin is affected by the resistance mechanism but not the activated toxin. The reason for this might be that resistant insects are able to reduce the rate of toxin activation or causes over-proteolysis of protoxin which leads to toxin degradation (Sayyed et al., 2001; Sayyed et al., 2005; Bruce et al. , 2007; Herrero et al., 2001; Li et al., 2005).

1. 10. Strategies to improve the efficacy of Cry toxin

1. 10. 1. Serine protease inhibitors:

Serine protease inhibitors have been shown to increase the efficacy of insecticidal activity of Cry toxin in three different insect orders (Coleoptera, Lepidoptera and Diptera). Serine protease inhibitors are present in legume seeds. At higher concentration serine protease inhibitors kill insects. Serine protease inhibitors when used at a concentration 10^5 times below their insecticidal level with Cry toxins enhance the insecticidal activity of the Cry toxins. Genetically modified tobacco plant expressing *Cucurbita maxima* trypsin protease inhibitor with Cry toxin showed six times increase in insecticidal activity of Cry toxin when compared with genetic modified tobacco plant expressing only Cry toxin against *H.virescens*. It is suggested that protease inhibitor enhances Cry toxin efficacy by inhibiting gut proteases or by preventing the degradation of membrane bound receptors (MacIntosh et al., 1990; Pardo-Lopez et al., 2008).

1. 10. 2. Chitinase:

It has been shown that chitinase when used with Cry toxin has a synergistic effect. Chitinase increases the potency of Cry toxin by ten times. It is suggested that chitinase increases the larvicidal effect of Cry toxin by forming holes in the peritrophic membrane there by making it easy for the Cry toxin to bind to the receptors (Ding et al., 2008; Regev et al., 1996; Pardo-Lopez et al., 2008).

1. 10. 3. 23.3 kDa CR12-MPED (membrane – proximal extracellular domain)

CR12-MPED is the functional receptor region of cadherin for Cry1Ab binding and cytotoxicity. This 23.3kDa peptide fragment was fed with Cry1Ab to *M.sexta* larvae. CR12-MPED peptide fragment increased the toxicity of Cry1Ab. CR12-MPED peptide was also shown to enhance the toxicity of Cry1Ac in other lepidopteran insects. It was suggested that when CR12-MPED is fed with Cry1A toxin, CR12-MPED increases the number of binding sites in the microvilli of the insects by binding to the microvilli thereby increasing the probability of Cry1A interaction with receptor thus increasing the Cry1A toxicity (Chen et al., 2007).

1. 10. 4. Mutations to increase the toxicity of Cry proteins:

Rajamohan et al. showed that asparagine which is present at 372nd position of Cry1Ab amino acid sequence, lies in loop 2 of domain II, when substituted with alanine or glycine increased the toxicity of Cry1Ab by 8-fold against *Lymantria dispar* (Rajamohan et al., 1996 b).

A triple mutation in Cry1Ab was shown to increase the toxicity by 36 times against *L.dispar*. Substitutions were made at 282nd (located in α-helix 8), 283rd (located in α-helix8) and 372nd (located in loop 2) positions. At 372nd position asparagine was substituted by alanine. At 282nd position alanine was substituted by glycine and at 283rd position leucine was replaced by serine (Rajamohan et al., 1996 b).

1. 10. 5. Domain swapping to increase the toxicity of Cry toxin:

Cry1Ab is moderately toxic to *Spodoptera exigua*. Domain III of Cry1Ab when swapped with domain III of Cry1Ca increased the toxicity of Cry1Ab hybrid (Cry1Ab having domain III of Cry1Ca) toxin against *S.exigua* (de Maagd et al., 1996).

1. 10. 6. Cry1A modified toxins:

Cry1A modified toxins have been created by removing the helixα1 of domain I. Cry modified toxins do not require interaction with cadherin to form oligomers. So they bypass the cadherin receptor and bind directly to the GPI-anchored receptor and insert into lipid micro domain where these GPI-anchored receptors are localised and pores in the apical membrane of midgut cells are created (fig 4). This induces osmotic shock, bursting of midgut cells and finally leads to the insect death. Cry1A modified toxins are effective against those insects which are resistant to native Cry1A toxins and whose mechanism of resistance is linked to mutation in cadherin gene (Bravo and Soberon, 2008; Soberon et al., 2009).
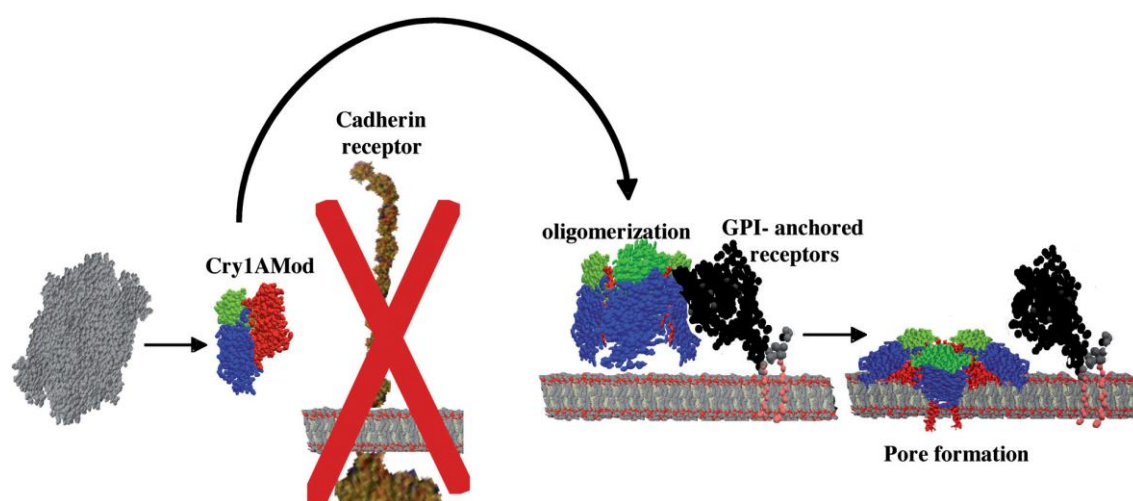
Fig 4: Diagrammatic representation of mode of action of modified Cry1A toxins (Soberon et al., 2009).

1. 11. Esterases and Resistance:

Esterase has recently been proposed to be involved in Cry1Ac resistance in silver selected strain of *H. armigera*. Cry1Ac-resistant *H.armigera* larvae showed higher esterase activity than Cry1Ac susceptible larvae. Overproduced nonspecific esterases which belong to a class of serine hydrolases and found in insect gut were proposed to bind to and sequester Cry1Ac toxin (Gunning et al., 2005).

Nonspecific esterases have been involved in insecticide resistance in many insects because these enzymes have the ability to hydrolyze insecticidal esters and have the ability to sequester xenobiotics (Gunning et al., 2005).

Schizaphis graminum has developed resistance to organophosphate insecticides. The resistance to organophosphate insecticide is associated with elevated esterase activity.

Two types of esterases TypeI and TypeII were involved in resistance mechanism in *S.graminum*. Resistance is due to the increased levels of esterase because presence of more esterase can bind to more insecticide molecules and sequester them (Ono et al., 1999).

Overproduction of esterase is a common mechanism of resistance to organophosphate insecticides in *Culex pipiens*. Esterase overproduction in *Culex pipiens* is either due to gene regulation or due to gene amplification (Qiao et al., 1998; Rooker et al., 1996; Guillemaud et al., 1996).

1. 12. Glutathione-s-transferase (GST) and resistance:

The glutathione-s-transferases are a large group of multifunctional enzyme involved in the metabolism of wide range of xenobiotics including insecticides (Enayati et al., 2005). Xenobiotic metabolism is the set of metabolic pathways which detoxify xenobiotics (xenobiotic metabolism, wikipedia, online accessed on 24th May2010).

GST catalyses the conjugation of reduced GSH and xenobiotics. It is a nucleophilic addition reaction. Conjugation makes the product more water soluble and therefore it can be readily excreted (Enayati et al., 2005).

Increased GST activity in insects has been associated with insecticide resistance. The role of GST has been proved in many cases of organophosphate insecticide resistance. GST detoxifies organophosphate via two distinct pathway: a) O-dealkylation b) O-dearylation e.g. *Plutella xylostella* resistance to parathion and methyl parathion (Enayati et al., 2005).

1. 13. General structure of GST:

GSTs are non allosteric enzymes. They are dimeric and have a molecular mass around 26 kDa.Each monomer has one active site and active sites function independently. Each monomer has two distinct domains (domain I and domain II) linked by short hexamer (Salinas and Wong, 1999).

Domain I consists of four stranded β pleated sheet flanked by α-helices. It has "βαβαββα" motif. N-terminal end is present in this domain. The glutathione binding site is also present in this domain. Domain II is larger than domain I. It contains five amphipathic α-helices. These helices are arranged in right handed spiral. C-terminus and hydrophobic site (H-site) is present in this domain (Salinas and Wong, 1999).

1. 14. Mixed Fuction Oxidases (MFOs) and resistance:

MFOs are also known as P450 enzymes or Cytochrome P450 monooxygenases. P450s are multifunctional enzymes. They play an important role in growth, development, feeding, resistance to pesticide and tolerance to plant toxins in insects (Scott and Wen, 2001; Feyereisen, 1999).

P450- mediated detoxification is one of the most important mechanisms of resistance to insecticides in many insects (Scott and Wen, 2001).

P450 enzymes modify the xenobiotics by incorporating an oxygen atom into a variety of functional groups of xenobiotics which helps them prepare for rapid excretion (Terriere, 1984).

1. 15. *Plutella xylostella*:

Plutella xylostella is the most destructive pest of crucifers throughout the world (excluding Europe).It is present from temperate to tropical region. It is generally thought to have originated in the Mediterranean region but more recently it has been suggested that *Plutella xylostella* originated in South Africa because of rich and diverse fauna of *Plutella xylostella* parasitoids (Sayyed et al., 2002). Its extent of damage is such that it sometimes causes more than 90% crop loss. It causes a loss of more than one billion US dollars per year. In tropical and sub-tropical climate *Plutella xylostella* can cause damage throughout the year except during the rainy season. Its larvae feed on the plant parts which are above the ground thereby reducing the yield and quality of the produce. Thus the marketability of the produce is significantly reduced (Sayyed et al., 2002; Talekar and Shelton, 1993).

An absence of natural enemies especially parasitoids in many non indigenous areas, its ability to migrate long distance and its ability to produce large numbers of offspring are considered to be the major causes of the high pest status of *Plutella xylostella* in most parts of the world(Sayyed et al., 2002).

Diamondback moths (*Plutella xylostella)* have become resistant to every insecticide used extensively against them. Factors which help in the development of resistance in *Plutella xylostella* are rapid turnover of insect generation, high fecundity and reproductive potential, a long and continuous growing season, large area under crucifer cultivation and frequent insecticide application. These are the reasons for widespread insecticide resistance in South East Asia (Talekar and Shelton, 1993; Sayyed et al.,

2002).

Plutella xylostella has developed high levels of resistance to *Bacillus thuingiensis* (Bt) in the open field. The first case of field resistance to Bt in *Plutella xylostella* was reported from Hawaii. Populations from areas where Dipel (a product of Bt) was used at higher levels showed less susceptibility to Bt than populations that had been treated at lower levels. The highest level of resistance obtained from a population from a heavily treated area was 30-fold. When laboratory selection of this population was done using Dipel resistance increased rapidly to over 1000-fold. A diamondback moth colony (BL) from Philippines which was exposed regularly to Dipel in field condition showed more than 200-fold resistance to Cry1Ab. A *Plutella xylostella* colony (Loxa A) from Florida showed more than 1500-fold resistance to Javelin (a commercial formulation of Btk NRD12) in the second generation after the colony was collected from the field. Another *Plutella xylostella* colony (SERD3) from Malaysia showed considerable levels of resistance to Btk and Bta. This SERD3 population was reared for seven generations without selection. It showed 330-fold resistance to Btk and 160-fold resistance to Bta. Another *Plutella xylostella* colony (UNSEL-MEL) from Malaysia's Melaka region showed field resistance to Btk products. It also showed resistance to Cry1Ac, Cry1Ab and Bta. When they were Cry1Ac selected (1Ac SEL-MEL) it showed more than 95-fold increase in resistance to this toxin during five generations but when they were selected with Cry1Ab(1Ab SEL-MEL), Btk (Btk SEL-MEL) or Bta(Bta SEL-MEL) they showed only tenfold or less increase in resistance to these toxins (Ferre and Van Rie, 2002).

1. 16. Spinosad resistance in insects:

Plutella xylostella has developed resistance to spinosad (a biopesticide) at a very rapid rate. Six of twelve field population of *Plutella xylostella* collected from Hawaii showed high level of resistance towards spinosad (Baxter et al., 2010). Spinosad resistance in *Plutella xylostella* has also been reported in the US, Thailand and Malaysia (Sayyed et al., 2004 b; Baxter et al., 2010).

High level of resistance to spinosad has also been reported in field population of *Spodoptera exigua*, *Heliothis virescens* and *Musca domestica* (Perry et al., 2007).

The active compounds of spinosad are macrocyclic lactones, spinosyn A and spinosyn D. These compounds are produced by the actinomycetes *Saccharopolyspora spinosa* during fermentation (Thompson et al., 2000; Baxter et al., 2010).

Spinosad affects the central nervous system of the insects. It primarily targets the nicotinic acetylcholine receptor (nAChR) causing neuromuscular fatigue. Insects experience tremors and paralysis and finally die (Salgado, 1998; Thompson et al., 2000; Baxter et al., 2010).

nAChR consists of five subunits. These five subunits are arranged around a central cation -permeable channel. Each subunit consists of four transmembrane regions (TM1-TM4) and one extracellular N-terminal domain. TM2 region is located in the ion channel and a large intracellular loop is present between TM3 and TM4 region.The extra cellular N-terminal domain contains the Cys-loop and acetylcholine (ACh) binding site. Cys-loop consists of two cysteine residues and 13 amino acid residues in between the two cysteines. The ACh binding site has several distinct regions (loops A-F) at subunit interfaces. Subunits that are essential for ACh binding are called alpha subunits. Alpha subunits have two adjacent cysteines in loop C which is required for ACh binding. The subunits which do not have two adjacent cysteine residues are known as non alpha or beta, delta, epsilon or gamma subunits. For receptor function at least two alpha subunits are required. Acetylcholine binds to the extracellular N-terminal domain (Rinkevic and Scott, 2009; Baxter et al., 2010; Sattelle et al., 2005).

Twelve subunits of nAChR have been identified in *Bombyx mori* (Shao et al., 2007) and *Tribolium castaneum* (Rinkevic and Scott, 2009). Eleven subunits have been identified in *Apis mellifera* (Rinkevic and Scott, 2009) and 10 in *Drosophila melanogaster* (Sattelle et al., 2005). Subunit diversity of nAChR in insects is due to alternate exon splicing, exon exclusion or A-to-I pre-mRNA editing (Baxter et al., 2010; Grauso et al., 2002).

Baxter et al. showed that field based resistance to spinopsad in a *Plutella xylostella* (Px) strain collected from Pearl city, Hawaii is due to a point mutation in the ninth intron splice junction of nAChR Px alpha6 gene (Baxter et al., 2010).

Perry and his colleagues showed that deletion of D alpha 6 subunit of nAChR causes high level of resistance to spinosad in *Drosophila melanogaster* without being lethal. D alpha 6 strain of *Drosophila melanogaster* showed 1181 fold resistance to spinosad (Perry et al., 2007).

1. 17. Aims

1. Mutations in the cadherin gene have been linked to Cry1Ac resistance in *Heliothis virescens, Pectinophora gossypiella* and *Helicoverpa armigera* (Morin et al., 2003; Gahan et al., 2001; Xu et al., 2005).

According to Soberon et al. deletion of helix α1 of domain I from Cry1A toxins leads to the formation of oligomers without binding to the cadherin receptors. Thus these modified toxins overcome resistance by bypassing the cadherin receptor binding. So the Cry1A modified toxins are effective against those insects which are resistant to native Cry1A toxins and whose mechanism of resistance is linked to mutation in the cadherin gene (Soberon et al., 2007; Bravo and Soberon, 2008).

According to Baxter et al. resistance to Cry1A toxins in two strains of *Plutella xylostella*, SC1 and NOQA, were not linked to mutations in the cadherin gene (Baxter et al., 2005; Baxter et al., 2008).

So it was decided to make Cry1Ac modified toxin and check whether it is effective or not against *Plutella xylostella* NOQA population whose resistance mechanism has not been linked to the Cadherin gene.

2. Baxter and his colleagues showed that field based resistance to spinosad in a *Plutella xylostella* strain collected from Pearl city, Hawaii is due to the point mutation in the ninth intron splice junction of nAChR Pxα6 (Baxter et al., 2010).

Hence it was decided to check whether or not other spinosad resistant lepidopteran insects have similar mechanism of resistance (i.e. splice-site mutation) as *Plutella xylostella* Pearl population.

For this reason it was decided to work on spinosad resistant *Spodoptera litura* population collected from the fields of Pakistan.

It was also decided to check whether *Plutella xylostella* NOQA population has the same mechanism of resistance as *Plutella xylostella* Pearl City population. NOQA population is resistant to Cry1Ac but whether this population is resistant to spinosad is not known.

Chapter 2

Materials and methods

2. 1. Bacterial strains

2. 1.1. *E.coli*

2. 1. 1. 1. JM 109

Genotype: endA1 glnV44 thi-1 relA1 gyrA96 recA1 mcrB$^+$ Δ(lac-proAB) e14- [F' traD36 proAB$^+$ lacI^q lacZΔM15] hsdR17(r$_K$$^-m_K$$^+$).

2. 1. 1. 2. DH5α

Genotype: F$^-$ endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG Φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169, hsdR17(r$_K$$^-$ m$_K$$^+$), λ– .

2. 1. 1. 3. BL21

Genotype: *E. coli* B F- *dcm ompT hsdS*(r$_B$- m$_B$-) gal [malB$^+$]$_{K-12}$(λ^S).

2. 1. 2. *B.thuringiensis*

2. 1. 2. 1. 78/11

Acrystalliferous strain of *Bacillus thuringiensis* subsp *israelensis*.

2. 2. Plasmids

pSV2: *E.coli*-Bt shuttle vector, pSVP27A: *E.coli*-Bt expression vector, pGEM-T easy vector by Promega.

2. 2. 1. Recombinant Plasmids

pGEM 1Ac, pGEM 1AcD(2-50): given to me by Dr.Neil Crickmore.

2. 3. *Plutella xylostella* strain

2. 3. 1. NOQA: Cry 1Ac resistant population.

2. 4. Culture media

2. 4. 1. LB (Luria-Bertani) media:

Tryptone: 10 g; Yeast Extract: 5 g; NaCl: 10 g; Water: to 1 L.

The pH was adjusted to 7.5 with 5M NaOH.

2. 4. 2. LB- agarose plates: 15 g/l agar added to Luria-Bertani media

2. 5. Polymerase chain reaction to create modified Cry1Ac toxin:

PCR was used to create modified Cry1Ac toxin using Pfu Ultra 6 Kb program (table 1).

Cycle	Number of Cycles	Temperature	Time
Initial Denaturation	1	95^0C	2 minutes
Denaturation+ Annealing+Extension	30	95^0C 55^0C 72^0C	30 seconds 30 seconds 6 minutes
Final extension	1	72^0C	10 minutes

Table 1: Pfu ultra 6kb program

The forward primer was designed to anneal from 57th amino acid residue position and reverse primer was designed to anneal from start codon.

Forward Primer: 5'GTGTTAGGACTAGTTGATATAATATGGG 3'

Reverse Primer: 5'CATAAGTTACCTCCATCTCTTTTATTAAG 3'

The reaction mixture was introduced into the PCR machine which included Primers (forward primer 0.5 µl of 100 pmol/µl, reverse primer 0.5 µl of 100 pmol/µl), pGEM 1Ac as template DNA 5 µl, water 19 µl and Agilent's Pfu ultra II hotstart 2X master mix 25µl.

After the PCR, PCR product was run on 1% agarose gel (0.3 g of agarose in 30 ml of TBE buffer). The mixture was heated until the agarose dissolved. The solution was allowed to cool but not allowed to set. 0.5 µl of gel-red (fluorescent nucleic acid gel stain) was added to the solution and allowed to solidify, with gel comb inserted to create wells.

10 X TBE contains Tris 108 g, Boric acid 55 g, EDTA 7.44 g and Water up to 1 L.

2. 6. Purification of PCR product from the gel

Purification of PCR product from the gel was performed using Qiagen kit. The PCR product was run on 1% agarose gel and the required band was excised from the gel and added to eppendorf tube. Then, 600 µl of solubilisation buffer, QG was added into the eppendorf to dissolve the excised agarose gel. The tube was incubated at 60^0C until the excised agarose gel was completely dissolved, occasionally mixing the tube by inverting it. 200 µl of isopropanol was added to the tube and mixed by inverting the tube several times. The solution was then transferred to the column and centrifuged at 14 k for 1 minute in eppendorf centrifuge 5418. The flow-through was discarded and 500 µl of solubilisation buffer was added to the column and was centrifuged for 1 minute. The flow-through was discarded and after that 750 µl of wash buffer PE was added to the column and centrifuged for 1 minute. The flow-through was discarded and another 1 min spin was given to the column. The column was placed into a clean eppendorf tube. 15 µl of elution buffer EB was added to the column and was left to stand for 1 min and then centrifuged for 1 min. 5 µl of sample was run on 1% agarose gel.

2. 7. Ligation of Purified PCR product

For ligation to occur purified PCR product- 4 µl, ligation buffer- 5µl, ligase enzyme (T_4 DNA ligase)- 1µl was added to a tube and kept at room temperature for 4 hours and then at 4^0C for overnight.

2. 8. Transformation in *E.coli*

The ligated product was to be introduced into *E.coli* JM 109, BL21 and DH5α strain. So JM 109, BL21 and DH5α were inoculated in 100 ml of LB- broth separately and grown till optical density of 0.4-0.8. The broth containing JM 109, BL21 and DH5α were poured into three different centrifuge tubes under sterile conditions and centrifuged using rotor SLA 1500 (10k for 10 min). Pellets obtained were washed in 100 ml of cold sterile water and again centrifuged using the same program. Supernatants were poured off and pellets obtained were washed in 1 ml of cold sterile water. The JM109, BL21 and DH5α cells were then transferred to three different eppendorf tubes and were centrifuged at 14k for 1 minute in eppendorf centrifuge 5418. Pellets were resuspended

in 200 µl of cold sterile water. 2 mm cuvettes were cooled on ice before use. 50 µl of cells were transferred to small eppendorf tubes and 1 µl of ligated PCR product were added to these tubes and were mixed. The following settings were applied to the genepulser (Biorad): 1.8 KV, 200 Ohms, 25 µF. The cuvette containing the cells and ligated PCR product (DNA) was placed in the genepulser and the two red buttons on genepulser were simultaneously pressed until a beep was heard. A sterile Pasture pipette was used to wash the cells out of the cuvette with 0.5-1 ml LB. The cells were incubated at 37^0C for 1 hour and plated on the agar plates containing ampicillin (100µg/ml). Plates were incubated overnight at 37^0C. Some of the colonies were picked up and streaked onto another agar plate containing ampicillin.

2. 9. Miniprep of transformed *E.coli* cells

Miniprep is performed to elute the plasmid from the bacteria.

Miniprep was performed using Qiagen's QIAprep spin miniprep kit. The colonies were scraped off from the plate and suspended in 250 µl of Buffer P1 in eppendorf tubes. Then they were vortexed. After that 250 µl of Buffer P2 was added to each eppendorf tube and was mixed throroughly by inverting the tube 8-10 times without vortexing. A volume of 350 µl of Buffer N3 was added to each tube and was mixed thoroughly without vortexing. The tubes were centrifuged at 14k rpm for 10 minutes; the supernatants obtained were taken into Q1Aprep spin columns and centrifuged for 1 minute. The flow-throughs were discarded. Spin columns were washed with 0.5 ml of Buffer PB and centrifuged for 1 min. The flow-throughs were discarded. After that spin columns were washed with 0.75 ml of Buffer PE and centrifuged for 1 minute. The flow-throughs were discarded and columns were centrifuged for additional 1 minute to remove any residual wash buffer. The spin columns were then placed in clean 1.5 ml eppendorf tubes and 50 µl of elution buffer EB was added to the each column. The tubes were left to stand for 1 minute and centrifuged for 1 minute at 14k rpm.

2. 10. Ligation of pSV2 and pSVP27A with Cry1AcD (1-56) and Cry1AcD (2-50)

For ligation to occur Cry1AcD (1-56)- 3 µl,SV2- 2 µl Ligation Buffer- 4µl, Ligase enzyme (T$_4$ DNA ligase)- 1µl ; Cry1AcD(2-50)- 3 µl,SV2- 2 µl Ligation Buffer- 4µl, Ligase enzyme (T$_4$ DNA ligase)- 1µl; Cry1AcD (1-56)- 3 µl, pSVP27A- 2 µl Ligation Buffer- 4µl, Ligase enzyme (T$_4$ DNA ligase)- 1µl; Cry1AcD (2-50)- 3 µl, pSVP27A- 2 µl Ligation Buffer- 4µl, Ligase enzyme (T$_4$ DNA ligase)- 1µl were added to the tubes

and kept at room temperature for 4 hours and then at 4^0C for overnight.

2. 11. Transformation in *Bacillus thuringiensis* (Bt)

The ligated product was to be introduced into Bt 78/11 and HD73 strain. So 78/11 and HD73 were inoculated in 100 ml of LB- broth separately and grown at 30^0C till optical density of 0.4-0.8. The rest of the steps followed were similar to the transformation in *E.coli*.

2. 12. Miniprep of transformed *Bacillus thuringiensis* cells

Colonies were scraped off from the chloramphenicol containing agar plates and resuspended in 250 µl of P1 buffer containing 10 mg/ml lysozyme and were incubated in water bath at 37^0C for 30 minutes to 1 hour and the rest of the procedures were similar to the *E.coli* miniprep.

2. 13. Rapid size screen

30 µl of the rapid size screen solution (pre-warm) was added to each of the eppendorf tube. Individual bacterial colonies were picked by sterile toothpicks and were resuspended in the solution. The tubes were incubated in water bath at 37^0C for 5 minutes followed by incubation on ice for 5 minutes. The samples were spun at 14k rpm for 5 minutes. 15 µl was loaded onto a 1% agarose gel.

Rapid size screen solution consists of:

Water   6.5 ml

EDTA   100 µl (500 mM)

Sucrose   1 g (30%)

SDS   150 µl (10%)

NaOH   2.5 ml (0.5 M)

KCl   600 µl (1.4 M)

Bromophenol Blue to colour the solution.

2. 14. Restriction digest

Restriction digest was done to digest the DNA obtained after miniprep. Restriction digests were performed by using different restriction enzymes according to the requirement of the experiments. The constituents of restriction digest included water, DNA to be digested, digest buffer and required restriction enzymes. The volume of

buffer and restriction enzymes used were 1 µl and 0.2 to 0.5 µl respectively. Water volume depended on volume of DNA used and number and volume of enzymes used. Buffer 1-4 to be used depended on the activity of the enzyme in that particular buffer (as described by NEB). The total digest volume was made up to 10 µl and incubated at described temperature for 1 hour. After incubation the total volume was run on 1% agarose gel.

2. 15. Harvesting of protein from *E.coli*

500 ml of LB media containing ampicillin was inoculated with transformed colonies of JM109, BL21 and DH5α i.e. all the transformed strains of *E.coli* were inoculated separately in 500 ml of LB media containing ampicillin were incubated in incubator shaker at desired temperatures for 2-3 days. After that they were transferred to centrifuge bottles of 1 L. The volumes were made up to 1 L by adding sterile water. The tubes were centrifuged at 6.5k for 10 minutes at 4^0C in a JLA 8.100 rotor. The supernatants were removed and 30 ml of sterile water was added to each centrifuge flask to resuspend the pellets. The samples were transferred to tubes of 100 ml volume for sonication. Sonication was done to cells for 4 minutes (with a gap of 1 minute after every 1 minute of sonication). After sonication the samples were transferred to 100 ml centrifuge tubes and centrifuged at 12k for 15 minutes in a SS34 rotor. The supernatants were removed and 5-10 ml of sterile water was added to each tube to resuspend the pellet. The protein harvested was stored in cold room.

2. 16. Harvesting of protein from *Bacillus thuringiensis*

Transformed colonies of *Bacillus thuringiensis* (78/11) were plated on chloramphenicol containing agar plates and incubated at 30^0C for 2-3 days. After that colonies were scraped off from the plates and resuspended in 30 ml sterile water. Sonication was done to the cells for 4 minutes (with a gap of 1 minute after every 1 minute of sonication) rest of the steps was similar to harvesting of protein from *E.coli*.

2. 17. Preparation of SDS gel

All the protein studies were done using SDS-PAGE. Two glass plates and comb were cleaned with ethanol and dried thoroughly before assembly. 1% agarose solution was used to seal the bottom of the plates to prevent any leakage. The gels were made up of a resolving gel (7.5% acrylamide) and a stacking gel. The resolving gel solution consists

of:

Water   2 ml

RGB   1 ml

Acrylamide (30%)   1 ml

400mg/ml APS   8 µl

TEMED   4 µl

TEMED and APS were added last because they are the polymerisation agent. The resolving gel solution was poured between the plates. 120 µl of water saturated butanol was added on top of the resolving to prevent oxygen from reaching the solution. The gel was left to set for 20-25 minutes. After the gel was set butanol was washed off and the stacking gel was poured on top of the resolving gel and the comb was inserted immediately.

Stacking gel solution consists of:

Water   1 ml

SGB   500 µl

Acrylamide (30%) 333 µl

400mg/ml APS   4 µl

TEMED   2 µl

The gel was left to set for 30 minutes.

RGB: Tris   18.18g

     SGB   0.4 g

   Water   up to 100 ml

     pH 8.8

SGB: Tris   6.06 g

     SDS   0.4 g

   Water   up to 100 ml

   pH 6.8

2. 17. 1. Sample preparation for SDS-PAGE

Sample   5 µl

Loading buffer

+ 2 mercaptoethanol   5 µl

The samples were boiled for 5 minutes and a quick spin was given to the samples and the samples were loaded on the gel.

Loading Buffer: SDS   2 g

                    EDTA   6 mg

                  BPB dye   20 mg

                     RGB   5 ml

                 Glycerol   50 ml

             Water   up to 100 ml

2. 17. 2. Running and developing of gel

The gel was run at 200 V for 30 minutes. After that gel was stained for 25 minutes and then destained for 25 minutes.

SDS running buffer (10 X): Tris   7.6 g

                         Glycine   36 g

                       SDS   2.5 g

                   Water   up to 250 ml

Stain: Methanol   250 ml

        Water   225 ml

 Acetic acid   25 ml

Coomassie blue   1.25 g

Destain: Methanol   250 ml

         Water   225 ml

 Acetic acid   25 ml

2. 18. Solubilisation and Trypsin activation

Solubilisation was done using Na_2CO_3 buffer of pH 10.9 and 10 mM DTT. A desired volume of Cry1AcD (1-56) and Cry1AcD (2-50) and Cry1Ac wild type were centrifuged for 5 minutes. The supernatant was removed and same amount of carbonate buffer with DTT was added on to the pellet. The tubes were incubated in water bath for 1 hour at 37^0C. After incubation 5 μl of samples were taken out (total solubilised sample) and the rest of the samples were centrifuged for 10 minutes at 14k. After that 5 μl of supernatants were taken out (supernatant solubilised sample). The total solubilised samples (5μl) and supernatant solubilised samples (5μl) along with loading buffer (5μl) were loaded on 7.5% SDS gel.

For trypsin activation Na_2CO_3 buffer pH 9.5 and 2 mg/ml trypsin was used. A desired volume of Cry1AcD (1-56) and Cry1AcD (2-50) and Cry1Ac wild type were

centrifuged for 5 minutes. The supernatant was removed and same amount of carbonate buffer with trypsin was added on to the pellet. The tubes were incubated in water bath for 1 hour at 37^0C. After incubation 5 µl of samples were taken out (total trypsinised sample) and the rest of the samples were centrifuged for 10 minutes at 14k. After that 5 µl of supernatants were taken out (supernatant trypsinised sample). The total trypsinised samples (5µl) and supernatant trypsinised samples (5µl) along with loading buffer (5µl) were loaded on 7.5% SDS gel.

2. 19. Gel comparison of Cry1AcD (1-56) and Cry1AcD (2-50) with Cry1Ac wild type.

4 mg/ml concentration of Cry1Ac wild type was diluted to the concentrations of 0.2 mg/ml and 0.1 mg/ml. These two concentrations of Cry1Ac wild type along with Cry1AcD (1-56) and Cry1AcD (2-50) whose concentrations were unknown, were run on 7.5% SDS gel. This was done to estimate the concentration of Cry1AcD (1-56) and Cry1AcD (2-50).

2. 20. Leaf dip Bioassay

Leaf dip bioassay was performed according to the Sayyed et al. paper (Sayyed et al., 2000). The leaf discs were cut out from the Chinese cabbage leaves and were dipped in 120 µg/ml of Cry1Ac wild type (positive control), 120 µg/ml of Cry1AcD (1-56), 120 µg/ml of Cry1AcD (2-50) and control (water+ triton).The leaf discs were allowed to dry at ambient temperature. Leaf discs were then placed in petri dishes containing filter paper moistened with water. Ten late second instar larvae were put in each petri dish and they were kept at 25^0C. In total five replications were used. Mortality was calculated after 5 days.

2. 21. Extraction of genomic DNA from *Plutella xylostella* NOQA population

Genomic DNA of NOQA was extracted using extraction buffer (50 mM Tris-HCl, pH 8, containing 2% SDS, 0.75M NaCl, 10 mM EDTA and 100 µg/ml proteinase K. Each larva was mashed up in the extraction buffer. The samples were incubated at 65^0C for 30 minutes. Larval homogenates were then deproteinised with phenol: chloroform: isoamyl alcohol (25:24:1). The deproteinisation step was repeated twice. After deproteinisation the samples were centrifuged at 10k for 5 minutes. Nucleic acid was precipitated by adding cold isopropanol and incubated at -20^0C for 2 hours. Extraction

buffer, phenol: chloroform: isoamyl alcohol (25:24:1) and isopropanol were used in same amount. After incubation at -20^0C for 2 hours the samples were centrifuged at 14k for 30 minutes. The supernatants were removed and the pellets were vaccum dried for for 15 minutes. The pellets were resuspended in 200 µl TE (10 mM Tris-HCl, pH 8, 1mM EDTA) and incubated with RNAseA (100µg/ml) for 30 min in water bath at 37^0C. The integrity and purity of DNA samples were checked on 1% agarose gel (Waldschmidt et al., 1997).

2. 22. Extraction of genomic DNA from *Spodoptera litura*

Extraction of genomic DNA from *Spodoptera litura* was done using Qiagen's DNeasy Blood and Tissue kit. Extraction of genomic DNA was performed as per the instruction of manufacturer's handbook (DNeasy Blood and Tissue handbook).

2. 23. PCR to amplify ninth intron splice junction of nAChR

PCR was performed using new high fidelity program (table 2) to amplify ninth intron splice junction of nAChR.

Cycle	Number of cycles	Temperature	Time
Initial denaturation	1	94^0C	2 min
Denaturation+Annealing + Extension	10	94^0C 50^0- 55^0 C 68^0 C	10 sec 1 min 10 sec 2 min 30 sec
	20	94^0 C 50^0- 55^0 C 68^0 C	15 sec 30 sec 2 min 30 sec
Final extension	1	72^0 C	7 min

Table 2: New High Fidelity Program.

Forward Primer Exon 9: 5' GCATCATGTTCATGGTGGCG 3'

Reverse Primer Intron 9: 5' CCCGATAATCGTCGGAATTTG 3'

The reaction mixture was introduced into the PCR machine which included Primers (forward primer 0.5 µl of 100 pmol/µl, reverse primer 0.5 µl of 100 pmol/µl), genomic DNA as template DNA 5 µl, water 19 µl and Roche's new high fidelity master mix 25

μl.

2. 24. Ligation of purified PCR product with pGEM-T easy vector

For ligation to occur Purified PCR product- 3 μl, pGEM-T easy vector- 1 μl, Ligation Buffer- 5μl and Ligase enzyme (T_4 DNA ligase)- 1μl was added to a tube and kept at room temperature for 4 hours and then at 4^0C for overnight.

Chapter 3

Creating modified Cry1Ac and checking its effectiveness against *Plutella xylostella* NOQA population.

3. 1. Introduction

Mutation in cadherin gene has been linked to Cry1Ac resistance in *Heliothis virescens, Pectinophora gossypiella* and *Helicoverpa armigera* (Morin et al., 2003; Gahan et al., 2001; Xu et al., 2005).

According to Soberon et.al, deletion of helix α1 of domain I from Cry1A toxins leads to the formation of oligomers without binding to the cadherin receptors. Thus these modified toxins overcome resistance by bypassing the cadherin receptor binding. So the Cry1A modified toxins are effective against those insects which are resistant to native Cry1A toxins and whose mechanism of resistance is linked to mutation in cadherin gene (Soberon et al., 2007; Bravo and Soberon, 2008).

According to Baxter et.al resistance to Cry1A toxins in two strains of *Plutella xylostella* SC1 and NOQA were not linked to mutation in the cadherin gene (Baxter et al., 2005; Baxter et al., 2008).

So it was decided to make Cry1Ac modified toxin and check whether it is effective or not against *Plutella xylostella* NOQA population whose resistance mechanism has not been linked to the cadherin gene.

Three dimensional structure of Cry1Ac has not yet been determined by X-ray crystallography. Cry1Ac amino acid sequence is very similar to the Cry1Aa amino acid sequence. Three dimensional structure of activated Cry1Aa toxin has been determined by X-ray crystallography (Grochulski et al., 1995). Domain I of Cry1Aa and Cry1Ac are identical except at 148th (Cry1Aa and Cry1Ac alignment result) and 248th (Masson et al., 1994; Cry1Aa and Cry1Ac alignment result) position.

So it was decided to align Cry1Aa and Cry1Ac (fig 5) to predict the α-helices of domain I and β strands of domain II and domain III of Cry1Ac.

```
                                               α1                    α2a
Cry1Aa    MDNNPNINECIPYNCLSNPEVEVLGGERIETGYTPIDISLSLTQFLLSEFVPGAGFVLGL 60
Cry1Ac    MDNNPNINECIPYNCLSNPEVEVLGGERIETGYTPIDISLSLTQFLLSEFVPGAGFVLGL 60
          ************************************************************


                      α2b                      α3
Cry1Aa    VDIIWGIFGPSQWDAFLVQIEQLINQRIEEFARNQAISRLEGLSNLYQIYAESFREWEAD 120
Cry1Ac    VDIIWGIFGPSQWDAFLVQIEQLINQRIEEFARNQAISRLEGLSNLYQIYAESFREWEAD 120
          ************************************************************


                   α4                           α5
Cry1Aa    PTNPALREEMRIQFNDMNSALTTAIPLLAVQNYQVPLLSVYVQAANLHLSVLRDVSVFGQ 180
Cry1Ac    PTNPALREEMRIQFNDMNSALTTAIPLFAVQNYQVPLLSVYVQAANLHLSVLRDVSVFGQ 180
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                  *********************************:****************************

                      α6                                    α7
Cry1Aa  RWGFDAATINSRYNDLTRLIGNYTDYAVRWYNTGLERVWGPDSRDWVRYNQFRRELTLTV 240
Cry1Ac  RWGFDAATINSRYNDLTRLIGNYTDYAVRWYNTGLERVWGPDSRDWVRYNQFRRELTLTV 240
                  **********************************************************

                                    β1a       β1b     α8a           α8        β2
Cry1Aa  LDIVALFSNYDSRRYPIRTVSQLTREIYTNPVLENFDGSFRGMAQRIEQNIRQPHLMDIL 300
Cry1Ac  LDIVALFPNYDSRRYPIRTVSQLTREIYTNPVLENFDGSFRGSAQGIERSIRSPHLMDIL 300
                  *******.**********************************  ** **:.**.*******

                      β3r    β3                β4                 β5        β6
Cry1Aa  NSITIYTDVHRGFNYWSGHQITASPVGFSGPEFAFPLFGNAGNAAPP-VLVSLTGLGIFR 359
Cry1Ac  NSITIYTDAHRGYYYWSGHQIMASPVGFSGPEFTFPLYGTMGNAAPQQRIVAQLGQGVYR 360
                  ********.***: ****** *************:***.*. *****   :*:   * *::*

                                β7r    β7            β8       β9
Cry1Aa  TLSSPLYRRIILGSGPNNQELFVLDGTEFSFASLTTNLPSTIYRQRGTVDSLDVIPPQDN 419
Cry1Ac  TLSSTLYRRPFN-IGINNQQLSVLDGTEFAYG-TSSNLPSAVYKSGTVDSLDEIPPQNN 418
                  ****.****  :    * ***.* *******::.   ::****::**: ******* ****:*

                            β10            β11            β12    β13
Cry1Aa  SVPPRAGFSHRLSHVTMLSQAAG--AVYTLRAPTFSWQHRSAEFNNIIPSSQVTQIPLTK 477
Cry1Ac  NVPPRQGFSHRLSHVSMFRSGFSNSSVSIIRAPMFSWIHRSAEFNNIIASDSITQIPAVK 478
                  .**** **********:*: .. .   :* :*** *** **********.*...:*** .*

              β13b     β14         β15       β16                   β17
Cry1Aa  STNLGSGTSVVKGPGFTGGDILRRTSPGQISTLRVNITAPL-----SQRYRVRIRYASTT 532
Cry1Ac  GNFLFNG-SVISGPGFTGGDLVRLNSSGNNIQNRGYIEVPIHFPSTSTRYRVRVRYASVT 537
                  .. * .* **:.********::* .*.*:    * .*:     * *****:****.*

              β18         β19                β20    β21      β22
Cry1Aa  NLQFHTSIDGRPINQGNFSATMCSGSNLQSGSFRTVGFTTPFNFPNGSSVFTLSAHVFNS 592
Cry1Ac  PIHLNVNWGNSSIFSNTVPATATSLDNLQSSDFGYFESANAFTSSLGN---IVGVRNFSG 594
                  :::::.. .. .* .....**  * *.****..*  .  :..*. . *.    :..: *..

              β23
Cry1Aa  GNEVYIDRIEFVPAEVTFEAEYDLERAQKAVNELFTSSNQIGLKTDVTDYHIDQVSNLVE 652
Cry1Ac  TAGVIIDRFEFIPVTATLEAEYNLERAQKAVNALFTSTNQLGLKTNVTDYHIDQVSNLVT 654
                   * ***:**:*. .*:****:********* ****.**:****:************

Cry1Aa  CLSDEFCLDEKQELSEKVKHAKRLSDERNLLQDPNFRGINRQLDRGWRGSTDITIQGGDD 712
Cry1Ac  YLSDEFCLDEKRELSEKVKHAKRLSDERNLLQDSNFKDINRQPERGWGGSTGITIQGGDD 714
                  **********:********************.**:.**** :*** ***.********

Cry1Aa  VFKENYVTLLGTFDECYPTYLYQKIDESKLKAYTRYQLRGYIEDSQDLEIYLIRYNAKHE 772
Cry1Ac  VFKENYVTLSGTFDECYPTYLYQKIDESKLKAFTRYQLRGYIEDSQDLEIYLIRYNAKHE 774
                  ********* ***********************:***************************

Cry1Aa  TVNVPGTGSLWPLSAQSPIGKCGEPNRCAPHLEWNPDLDCSCRDEGKCAHHSHHFSLDID 832
Cry1Ac  TVNVPGTGSLWPLSAQSPIGKCGEPNRCAPHLEWNPDLDCSCRDGEKCAHHSHHFSLDID 834
                  ********************************************    **************

Cry1Aa  VGCTDLNEDLGVWVIFKIKTQDGHARLGNLEFLEEKPLVGEALARVKRAEKKWRDKREKL 892
Cry1Ac  VGCTDLNEDLGVWVIFKIKTQDGHARLGNLEFLEEKPLVGEALARVKRAEKKWRDKREKL 894
                  ************************************************************

Cry1Aa  EWETNIVYKEAKESVDALFVNSQYDRLQADTNIAMIHAADKRVHSIREAYLPELSVIPGV 952
Cry1Ac  EWETNIVYKEAKESVDALFVNSQYDQLQADTNIAMIHAADKRVHSIREAYLPELSVIPGV 954
                  *************************:**********************************

Cry1Aa  NAAIFEELEGRIFTAFSLYDARNVIKNGDFNNGLSCWNVKGHVDVEEQNNHRSVLVVPEW 1012
Cry1Ac  NAAIFEELEGRIFTAFSLYDARNVIKNGDFNNGLSCWNVKGHVDVEEQNNQRSVLVVPEW 1014
                  *************************************************:*********

Cry1Aa  EAEVSQEVRVCPGRGYILRVTAYKEGYGEGCVTIHEIENNTDELKFSNCVEEEVYPNNTV 1072
Cry1Ac  EAEVSQEVRVCPGRGYILRVTAYKEGYGEGCVTIHEIENNTDELKFSNCVEEEIYPNNTV 1074
                  *****************************************************:******
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Cry1Aa      TCNDYTATQEEYEGTYTSRNRGYDGAYESNSSVPADYASAYEEKAYTDGRRDNPCESNRG 1132
Cry1Ac      TCNDYTVNQEEYGGAYTSRNRGYN----EAPSVPADYASVYEEKSYTDGRRENPCEFNRG 1130
            ******..**** *:********:    . .********.****:******:**** ***


Cry1Aa      YGDYTPLPAGYVTKELEYFPETDKVWIEIGETEGTFIVDSVELLLMEE 1180
Cry1Ac      YRDYTPLPVGYVTKELEYFPETDKVWIEIGETEGTFIVDSVELLLMEE 1178
            * ******.*************************************
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Fig 5: Amino acid sequence alignment of Cry1Aa and Cry1Ac. Amino acid residues highlighted in yellow represent α helices of domain I; amino acid residues highlighted in bright green represent α8a, α8 and β-strands of domain II; amino acid residues highlighted in turquoise represent β-strands of domain III.

A previous student had made deletion from 2^{nd} amino acid position to 50^{th} (phenylalanine) amino acid position from Cry1Ac gene removing the helix-α1.

But we later found out that modified Cry1Ac toxin made by Soberon et al. lacked 56 amino acid residues from N-terminus i.e. from 1^{st} to 56^{th} (phenylalanine) position (Franklin et al., 2009; Soberon et al., 2007).

So it was decided to delete the amino acid residues from 2^{nd} to 56^{th} position.

Around 60 amino acid residues, which include helix-α1, from the amino terminus of Cry1Ac are protease susceptible i.e. they are cleaved by protease (Aronson et al., 1999).

3. 2. Use of Polymerase chain reaction to create Cry1Ac modified toxin

The primers were designed by keeping the above criteria in mind. The forward primer was designed to anneal from 57^{th} amino acid residue position and reverse primer was designed to bind from start codon. This was done to obtain the desired deletion.

Forward Primer: 5$^{'}$GTGTTAGGACTAGTTGATATAATATGGG 3$^{'}$

Reverse Primer: 5$^{'}$ CATAAGTTACCTCCATCTCTTTTATTAAG 3$^{'}$

Polymerase chain reaction was performed to obtain the desired deletion. The reaction mixture which included designed primers, template DNA, master mix and water was introduced into the PCR machine and amplified using Pfu ultra 6kb program (as described in methods 2. 5). pGEM1Ac was used as template.

The size of pGEM1Ac is 7103 base pairs (bp). Following the deletion of amino acid residues from 2^{nd} to 56^{th} position, the estimated size of the PCR product should be 6938 bp. 1μl of PCR product was run on 1% agarose gel. A very prominent band was observed around 8Kb (fig 6) and two very faint bands were observed at 4Kb and 3Kb.

3 kb

L1   L2
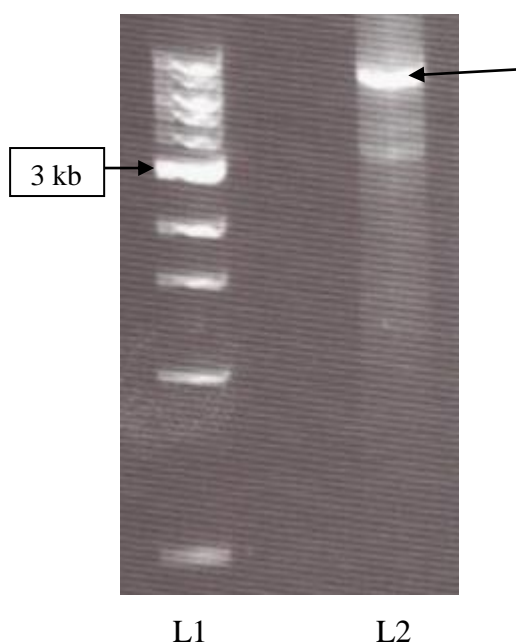
Fig 6: PCR products of Pfu ultra 6kb program. L1= 1kb Marker (sizes of 1 kb Marker bands are 0.5 kb, 1 kb, 1.5 kb, 2 kb, 3 kb (brightest band), 4 kb, 5 kb, 6 kb, 8 kb and 10 kb respectively from bottom to top on agarose gel); L2= PCR products. Arrow is showing PCR product of around 8 kb which was excised from the gel and purified.

3. 3. Purification of PCR product

As the PCR product obtained was not clean so it was decided to run the PCR product on 1% agarose gel. The band around 8Kb was excised from the gel and purified (as described in methods 2. 6). 5µl of purified product was run on 1% agarose gel along with 1Kb marker. A band of around 8Kb was observed (fig 7).

Fig 7: Purified PCR product. L1= 1 kb Marker(sizes of 1 kb Marker bands are 0.5 kb, 1 kb, 1.5 kb, 2 kb, 3 kb (brightest band), 4 kb, 5 kb, 6 kb, 8 kb and 10 kb respectively from bottom to top on agarose gel); L2= PCR product purified from the gel.

3. 4. Ligation of purified PCR product

Ligation (as described in methods 2. 7) was performed to ligate the ends of purified PCR product. This purified PCR product (pGEM 1Ac) should contain deletion so it was named pGEM 1AcD.

3. 5. Transformation of *E.coli* JM109 with pGEM 1AcD and mini-prep of pGEM 1AcD

E.coli JM109 strain was transformed with ligation mix which contained pGEM 1AcD (as described in methods 2. 8) and grown on agar plate containing ampicillin. Only 4 colonies grew on the plate. 3 colonies were picked up from the plate and miniprep was performed (as described in methods 2. 9) to elute pGEM 1AcD from *E.coli* JM109 strain.

3. 6. Restriction digest to verify whether deletion has taken place or not

To check whether deletion has taken place or not restriction digest was performed on minipreped colonies 1, 2, 3 and pGEM 1Ac using HaeIII enzyme. HaeIII restriction enzyme cuts pGEM1Ac and pGEM1AcD 19 times. There is only one difference. In Cry1Ac one of the fragments is 1193 bp while it is 1028 bp in Cry1AcD. Rest of the

fragments are of equal size. This information was obtained by using NEB Cutter.

But from the restriction digest result (Fig 8) it was not possible to confirm that deletion had been made. So it was decided to send the minipreped sample for sequencing. Minipreped Colony 1 sample was sent for sequencing because it had same number of bands and bands were of almost similar size as pGEM1Ac.

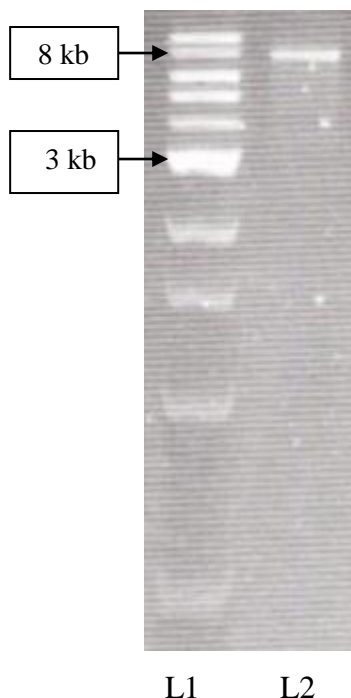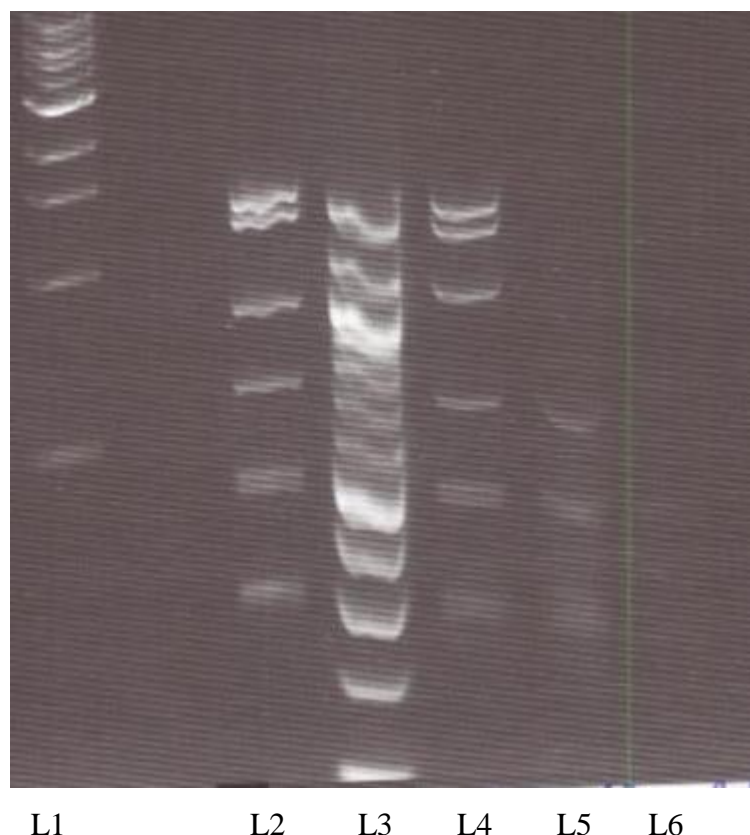Fig 8: HaeIII restriction digest of pGEM 1Ac and pGEM 1AcD. L1= 1 kb Marker (sizes of 1 kb marker bands are 0.5 kb, 1 kb, 1.5 kb, 2 kb, 3 kb (brightest band), 4 kb, 5 kb, 6 kb, 8 kb and 10 kb respectively from bottom to top on agarose gel); L2= pGEM 1Ac ; L3= 100 bp Marker (sizes of 100 bp marker bands are 100 bp, 200 bp, 300bp, 400 bp, 500/517 bp, 600 bp, 700 bp, 800 bp, 900 bp, 1000 bp, 1200 bp and 1517 bp respectively from bottom to top on agarose gel); L4= pGEM 1AcD colony 1; L5= pGEM 1AcD colony 2; L6= pGEM 1AcD colony 3.

The sequencing result showed that additional five bases were deleted including the desired deletion. The extra five bases which were deleted included the start codon (ATG).

```
                                          RBS
pGEM1Ac    CATAATGAATTGGTATCTTAATAAAAGAGATGGAGGTAACTTATGGATAACAATCCGAAC 3240
pGEM1AcD   CATAATGAATTGGTATCTTAATAAAAGAGATGGAGGTAAC------------------- 3220
           ****************************************

pGEM1Ac    ATCAATGAATGCATTCCTTATAATTGTTTAAGTAACCCTGAAGTAGAAGTATTAGGTGGA 3300
pGEM1AcD   ------------------------------------------------------------

pGEM1Ac    GAAAGAATAGAAACTGGTTACACCCCAATCGATATTTCCTTGTCGCTAACGCAATTTCTT 3360
```

```
pGEM1AcD          -----------------------------------------------------------

pGEM1Ac    TTGAGTGAATTTGTTCCCGGTGCTGGATTTGTGTTAGGACTAGTTGATATAATATGGGGA 3420
pGEM1AcD   --------------------------GTGTTAGGACTAGTTGATATAATATGGGGA 3250
                                     ****************************
```

A small part of the alignment result of pGEM 1Ac and pGEM 1AcD which shows the deleted nucleotides from the pGEM 1AcD. Nucleotides highlighted in yellow represent ribosomal binding site (RBS). Nucleotides highlighted in turquoise represent start codon of Cry1Ac.

GTG could act as start codon in bacteria.

In the Cry1Ac deleted sequence GTG is near to the ribosome binding site (only 4 bases apart). So this protein could be expressed in bacteria.

3. 7. Expression of Cry1AcD protein

The Cry1AcD (1-56) and Cry1AcD (2-50) proteins were expressed in three different *E.coli* strains JM 109, BL21 and DH5α. These three *E.coli* strains were incubated at 30^0C and 25^0C and proteins were harvested from them after two days (as described in methods 2.15) and were run in SDS-PAGE (figs 9, 10, 11, 12).

The expression of Cry1AcD (1-56) and Cry1AcD (2-50) proteins harvested from *E.coli* strains incubated at 25^0C were higher when compared with the expression of Cry1AcD (1-56) and Cry1AcD (2-50) proteins harvested from *E.coli* strains incubated at 30^0C.

Difference in expression of Cry1AcD (1-56) was observed when harvested from three different strains (JM109, BL21 and DH5α) at same temperature i.e. at 25^0C. Cry1AcD (1-56) harvested from DH5α showed slightly higher expression as compared to Cry1AcD (1-56) harvested from JM109 and BL21.

Difference in expression of Cry1AcD (2-50) was also observed when harvested from three different strains (JM109, BL21 and DH5α) at same temperature i.e. at 25^0C. Cry1AcD (2-50) harvested from DH5α showed slightly higher expression as compared to Cry1AcD (2-50) harvested from JM109 and BL21.

L1 L2 L3 L4
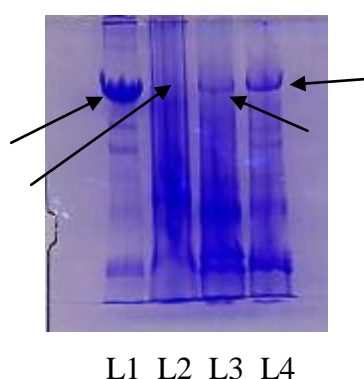
Fig 9: Expression of Cry1AcD (1-56) in JM109, BL21 and DH5α strains of *E.coli*. at 25^{0}C. L1= Cry1Ac wild type used as reference protein; L2= Cry1AcD (1-56) expressed in JM109; L3= Cry1AcD (1-56) expressed in BL21; L4= Cry1AcD (1-56) expressed in DH5α. Arrows are showing the expressed recombinant protein.

L1 L2 L3 L4
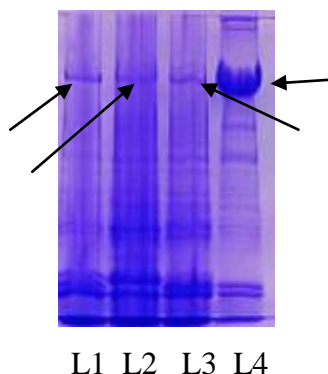
Fig 10: Expression of Cry1AcD (2-50) in JM109, BL21 and DH5α strains of *E.coli*. at 25^{0}C. L1= Cry1AcD (2-50) expressed in DH5α; L2= Cry1AcD (2-50) expressed in BL21; L3= Cry1AcD (2-50) expressed in JM109; L4= Cry1Ac wild type used as reference protein. Arrows are showing the expressed recombinant protein.

L1   L2   L3   L4
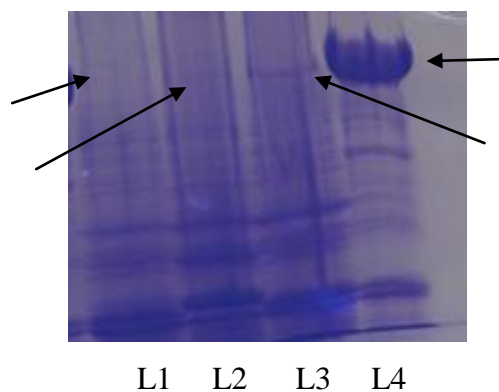
Fig 11: Expression of Cry1AcD (1-56) in JM109, BL21 and DH5α strains of *E.coli*. at 30^{0}C. L1= Cry1AcD (1-56) expressed in DH5α; L2= Cry1AcD (1-56) expressed in BL21; L3= Cry1AcD (1-56) expressed in JM109; L4= Cry1Ac wild type used as reference protein. Arrows are showing the expressed recombinant protein.

L1   L2   L3   L4
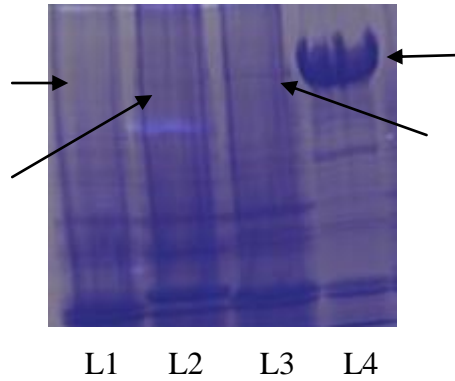
Fig 12: Expression of Cry1AcD (2-50) in JM109, BL21 and DH5α strains of *E.coli*. at 30^{0}C.
L1= Cry1AcD (2-50) expressed in DH5α; L2= Cry1AcD (2-50) expressed in BL21;
L3= Cry1AcD (2-50) expressed in JM109; L4= Cry1Ac wild type used as reference protein.
Arrows are showing the expressed recombinant protein.

3. 8. Solubilisation of Cry1AcD (1-56) and Cry1AcD (2-50)

Solubilisation experiments (as described in methods 2. 18) were performed on
Cry1AcD (1-56) and Cry1AcD (2-50) harvested from all the three strains (JM109,
BL21 and DH5α) of *E.coli* to know whether or not Cry1AcD (1-56) and Cry1AcD (2-
50) protoxins were released from their respective crystals.

Fig 13 shows that solubilisation of Cry1AcD (1-56) harvested from all the three strains
(DH5α, BL21 and JM109) had taken place because the solubilised bands of Cry1AcD
(1-56) were observed almost near to the solubilised band of Cry1Ac wild type (i.e.
around 130 kDa). Thus suggesting that Cry1AcD (1-56) protoxins had been released
from the Cry1AcD (1-56) crystals.

L1 L2 L3 L4 L5 L6 L7 L8

Fig 13: Comparison of solubilisation of Cry1Ac wild type with Cry1AcD (1-56) from JM109,
BL21& DH5α (grown at 25^{0}C). L1= supernatant of solubilised sample (Cry1AcD (1-56)) from
DH5α; L2= supernatant of solubilised sample (Cry1AcD (1-56)) from BL21; L3= supernatant
of solubilised sample (Cry1AcD (1-56)) from JM109; L4= supernatant of solubilised sample
Cry1Ac wild type (used as reference protein); L5= total solubilised sample (Cry1AcD (1-56))
from DH5α; L6= total solubilised sample (Cry1AcD (1-56)) from BL21; L7= total solubilised
sample (Cry1AcD (1-56)) from JM109; L8= total solubilised sample Cry1Ac wild type (used

as reference protein). Arrows are showing the solubilised recombinant protein.

Fig 14 shows that solubilisation of Cry1AcD (2-50) harvested from DH5α and JM109 has taken place because the solubilised bands of Cry1AcD (2-50) were observed almost near to the solubilised band of Cry1Ac wild type (i.e. around 130 kDa) but Cry1AcD (2-50) harvested from BL21 did not solubilise because no band was observed near to the solubilised band of Cry1Ac wild type. Thus suggesting that Cry1AcD (2-50) protoxins (from DH5α and JM109) have been released from the Cry1AcD (2-50) crystals but Cry1AcD (2-50) protoxin (from BL21) had not been released from the crystal.

L1 L2 L3 L4   L5 L6 L7 L8
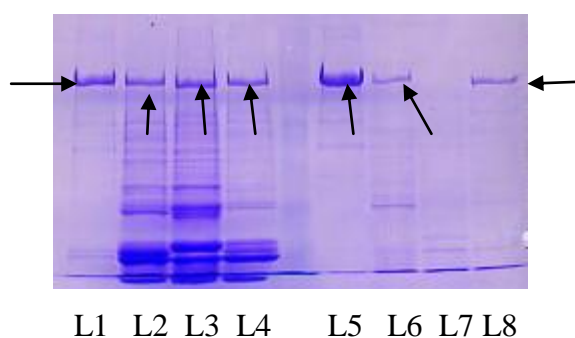
Fig 14: Comparison of solubilisation of Cry1Ac wild type with Cry1AcD (2-50) from JM109, BL21& DH5α (grown at 25^{0}C). L1= total solubilised sample Cry1Ac wild type (used as reference protein); L2= total solubilised sample (Cry1AcD (2-50)) from JM109; L3= total solubilised sample (Cry1AcD (2-50)) from BL21; L4= total solubilised sample (Cry1AcD (2-50)) from DH5α; L5= supernatant of solubilised sample Cry1Ac wild type (used as reference protein); L6= supernatant of solubilised sample (Cry1AcD (2-50)) from JM109; L7= supernatant of solubilised sample (Cry1AcD (2-50) from BL21; L8= supernatant of solubilised sample (Cry1AcD (2-50) from DH5α. Arrows are showing the solubilised recombinant protein.

3. 9. Trypsin digest of Cry1AcD (1-56) and Cry1AcD (2-50)

Trypsin digest experiment (as described in methods 2. 18) is performed to activate the protein in vitro. This experiment helps us to know whether the activated toxin is stable or not stable.

Fig 15 shows that trypsin activated Cry1AcD (1-56) (from DH5α and JM109) was stable because the bands were observed near the trypsin activated Cry1Ac (wild type) band (i.e. around 65 kDa) but trypsin activated Cry1AcD (1-56) (from BL21) was not stable because no band was observed in that lane near the trypsin activated Cry1Ac (wild type) band (i.e. around 65 kDa).
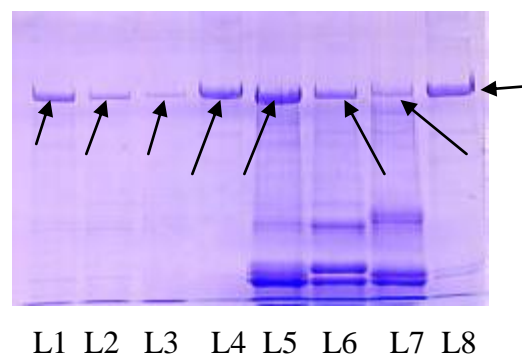

L1 L2 L3 L4 L5 L6 L7 L8
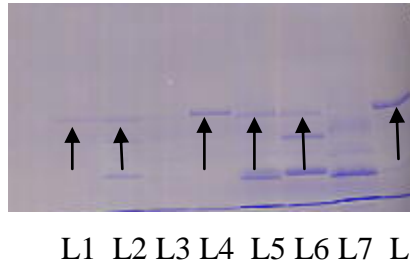
Fig 15: Comparison of trypsin digest of Cry1Ac wild type with Cry1AcD (1-56) from JM109, BL21& DH5α (grown at 25^0C). L1= supernatant of trypsinised sample (Cry1AcD (1-56)) from DH5α; L2= supernatant of trypsinised sample (Cry1AcD (1-56)) from JM109; L3= supernatant of trypsinised sample (Cry1AcD (1-56)) from BL21; L4= supernatant of trypsinised sample Cry1Ac wild type (used as reference protein); L5= total trypsinised sample (Cry1AcD (1-56)) from DH5α; L6= total trypsinised sample (Cry1AcD (1-56)) from JM109; L7= total trypsinised sample (Cry1AcD (1-56)) from BL21; L8= total trypsinised sample Cry1Ac wild type (used as reference protein). Arrows are showing the trypsin activated recombinant protein.

Fig 16 shows no bands near the total trypsinised band of Cry1Ac wild type (i.e. around 65 kDa) in total trypsinised lanes of Cry1AcD (2-50) (from DH5α, JM109) but the bands were observed in supernatant of typsinised Cry1AcD (2-50) (from DH5α and JM109) lanes and the bands were near to the supernatant of trypsinised Cry1Ac (wild type) band. The absence of band in total trypsinised lanes of Cry1AcD (2-50) (from DH5α and JM109) may be due to some technical error. Fig 16 suggest that trypsin activated Cry1AcD (2-50) (from DH5α and JM109) was stable but trypsin activated Cry1AcD (2-50) (from BL21) was not stable as no band was observed, in lanes L3 and L7, near the trypsinised band of Cry1Ac wild type.

L1 L2 L3 L4 L5 L6 L7 L8
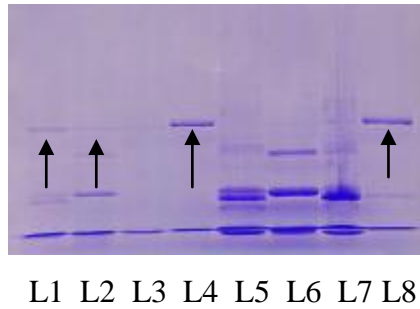
Fig 16: Comparison of trypsin digest of Cry1Ac wild type with Cry1AcD (2-50) from JM109, BL21& DH5α (grown at 25^0C). L1= supernatant of trypsinised sample (Cry1AcD (2-50)) from DH5α; L2= supernatant of trypsinised sample (Cry1AcD (2-50)) from JM109; L3= supernatant of trypsinised sample (Cry1AcD (2-50)) from BL21; L4= supernatant of trypsinised sample Cry1Ac wild type (used as reference protein); L5= total trypsinised sample (Cry1AcD (2-50)) from DH5α; L6= total trypsinised sample (Cry1AcD (2-50)) from JM109; L7= total trypsinised sample (Cry1AcD (2-50)) from BL21; L8= total trypsinised sample Cry1Ac wild type (used as reference protein). Arrows are showing the trypsin activated recombinant protein.

For leaf dip bioassay (as described in methods 2. 20) it was decided to use Cry1AcD (1-56) and Cry1AcD (2-50) which were harvested from DH5α (grown at 25^0C) because Cry1AcD (1-56) and Cry1AcD (2-50) from DH5α were slightly better expressed, solubilised and stable than Cry1AcD (1-56) and Cry1AcD (2-50) harvested from JM109. Cry1AcD (1-56) from BL21 was expressed and solubilised but not stable when activated with trypsin. Cry1AcD (2-50) from BL21 was expressed but did not solubilise.

3. 10. Protein concentration determination of Cry1AcD (1-56) and Cry1AcD (2-50) from DH5α grown at 25^0C.

The mutant proteins, Cry1AcD (1-56) and Cry1AcD (2-50) were run along with varying concentrations of Cry1Ac (as described in methods 2. 19). By observing the gel (fig 17), Cry1AcD (1-56) and Cry1AcD (2-50) concentrations were estimated as 0.1mg/ml because the band intensity of these two mutant proteins matched with band intensity of Cry1Ac (wild type) of 0.1 mg/ml concentration.
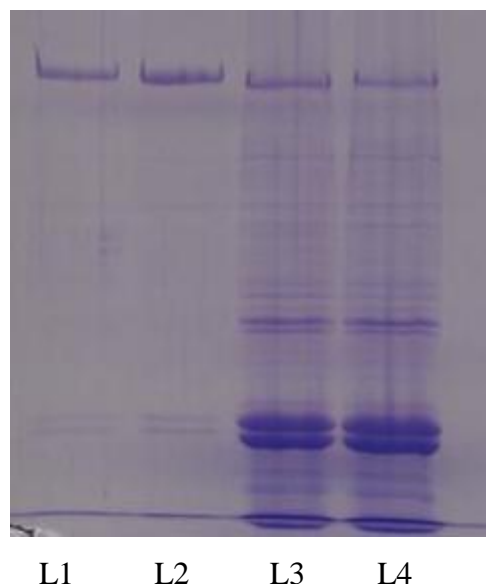
Fig 17: Protein concentration determination of Cry1AcD (1-56) and Cry1AcD (2-50). L1= Cry1Ac wild type 0.1 mg/ml; L2= Cry 1Ac wild type 0.2 mg/ml; L3= Cry1AcD (1-56); L4= Cry1AcD (2-50).

3. 11. Leaf dip bioassays using Cry1AcD (1-56) and Cry1AcD (2-50) from DH5α grown at 25^0C.

Toxicity assays with mutant recombinant proteins Cry1AcD (1-56) and Cry1AcD (2-50) and with Cry1Ac (wild type) were performed against the resistant (NOQA) population of *Plutella xylostella*. The toxicity results are summarised in table 3.

The toxicity results showed that mutant toxins Cry1AcD (1-56) and Cry1AcD (2-50) were less toxic when compared with Cry1Ac (wild type) at a similar toxin concentration (i.e. 120μg/ml) against NOQA (resistant population of *P.xylostella*).16% and 8% mortality were observed after feeding NOQA with 120μg/ml of Cry1AcD (1-56) and Cry1AcD (2-50) respectively. When fed with 120μg/ml of Cry1Ac (wild type) 56% mortality was observed.

Population	Toxin	Concentration of toxin (µg/ml)	Total number of insects used	Total number of insects alive	Total number of insects dead	% mortality
NOQA	Cry1Ac (wild type)	120	50	22	28	56%
NOQA	Cry1AcD (1-56)	120	50	42	8	16%
NOQA	Cry1AcD (2-50)	120	50	46	4	8%
NOQA	No toxin used	No toxin used	50	48	2	4%

Table 3: Toxicity assays of Cry1AcD (1-56), Cry1AcD (2-50) and Cry1Ac wild type against resistant, NOQA, population of *P.xylostella*.

Even though Cry1AcD (1-56) and Cry1AcD (2-50) from DH5α showed proper solubilisation and activated Cry1AcD (1-56) and Cry1AcD (2-50) were stable but still they showed very less toxicity towards NOQA. So it was decided to change the expression system for Cry1AcD (1-56) and Cry1AcD (2-50) and see if there were any changes in toxicity of these two mutant toxins. Therefore it was decided to express Cry1AcD (1-56) and Cry1AcD (2-50) in *Bacillus thuringiensis* (Bt) 78/11 strain.

For expressing Cry1AcD (1-56) and Cry1AcD (2-50) in Bt they have to be cloned in a shuttle vector. So it was decided to use pSV2 and pSVP27A vectors. Both of them are *E.coli*-Bt shuttle vectors. pSVP27A also possesses Cyt1A promoter.

For cloning Cry1AcD (1-56) and Cry1AcD (2-50) into pSV2 and pSVP27A they have to be first excised from pGEM vector. The scheme for the construction of these vectors are shown in diagrams 1 and 2.

3. 12. Restriction digest to separate Cry1AcD (1-56) and Cry1AcD (2-50) from pGEM vectors

For excising Cry1AcD (1-56) and Cry1AcD (2-50) from pGEM plasmid SalI and SphI enzymes were used. SalI and SphI enzymes were used because these enzymes do not cut in between the Cry 1AcD (1-56) and Cry1AcD (2-50) genes. SalI and SphI excises pGEM 1AcD (1-56) and pGEM 1AcD (2-50) at three positions. The sizes of pGEM 1AcD (2-50) fragments when digested with SalI and SphI should be 3961 bp, 2943 bp and 52 bp (information obtained using NEB cutter). Similarly the sizes of pGEM 1AcD (1-56) fragments when digested with SalI and SphI should be 3938 bp, 2943 bp and 52 bp (information obtained using NEB cutter).

It was decided to excise 3961 bp band from pGEM 1AcD (2-50) and 3938 bp from pGEM 1AcD (1-56) because these bands possess Cry1Ac promoter, RBS and Cry1AcD gene (information obtained using NEB cutter).

In fig 18 three bands were observed in lane 2 (L2) and lane 3 (L3). Bands near 4Kb and 3Kb, were double (SalI + SphI) digested products of pGEM 1AcD (1-56) and pGEM 1AcD (2-50). Bands near 4 Kb were excised from the agarose gel. Bands in between 6Kb and 8Kb were observed because pGEM 1AcD (1-56) and pGEM 1AcD (2-50) were partially digested.

L1   L2   L3
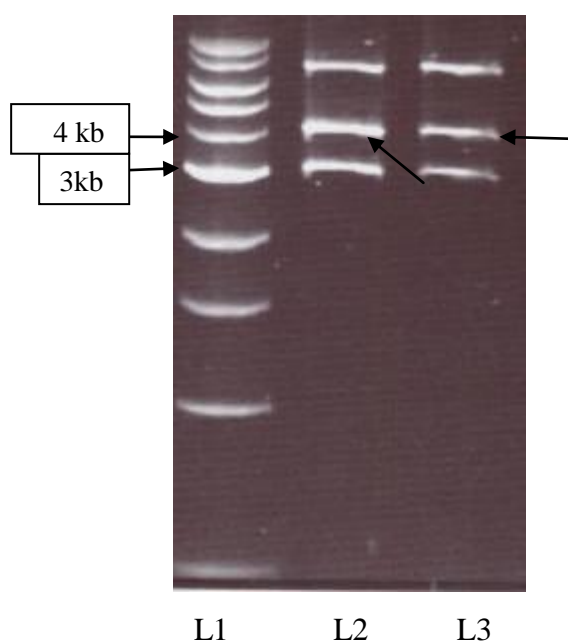
Fig 18: SalI + SphI digested pGEM 1AcD (1-56) and pGEM 1AcD (2-50). L1= 1Kb marker (sizes of 1 kb marker bands are 0.5 kb, 1 kb, 1.5 kb, 2 kb, 3 kb (brightest band), 4 kb, 5 kb, 6 kb, 8 kb and 10 kb respectively from bottom to top on agarose gel); L2= SalI+SphI digested pGEM 1AcD (1-56); L3= SalI+SphI digested pGEM 1AcD (2-50). Arrows showing the bands excised

from the gel.

3. 13. Extraction of 4 kb band

Double (SalI + SphI) digested products pGEM 1AcD (1-56) and pGEM 1AcD (2-50) were run on 0.8% agarose gel. The bands near 4 Kb were cut out and purified (as described in methods 2. 6). 5µl of purified products were run on 1% agarose gel (fig 19).

L1   L2   M
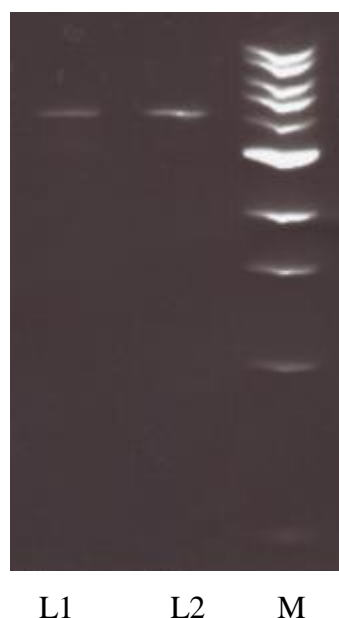
Fig 19: Gel purified 4 Kb products (Cry1AcD (1-56)) and (Cry1AcD (2-50)). L1= Gel purified 4Kb product (Cry1AcD (1-56)); L2= Gel purified 4Kb product (Cry1AcD (2-50)); M= 1Kb marker (sizes of 1 kb marker bands are 0.5 kb, 1 kb, 1.5 kb, 2 kb, 3 kb (brightest band), 4 kb, 5 kb, 6 kb, 8 kb and 10 kb respectively from bottom to top on agarose gel).

3. 14. Double digestion of pSV2 and pSVP27A plasmid with Sal I and Sph I enzyme

pSV2 when digested with Sal I and Sph I produces fragments of 4925 bp and 16 bp and Sal I and Sph I digested pSVP27A produces fragments of 5572 bp and 16 bp (information obtained using NEB cutter).

It was decided to excise pSV2 band near 5 Kb (fig 20) and pSVP27A band near 6Kb (fig 20). Sal I and Sph I digested pSV2 and pSVP27A were run on 1% agarose gel. The pSV2 band near 5 Kb and pSVP27A band near 6 Kb were cut out and purified. 5 µl of purified products were run on 1% agarose gel (fig 21).
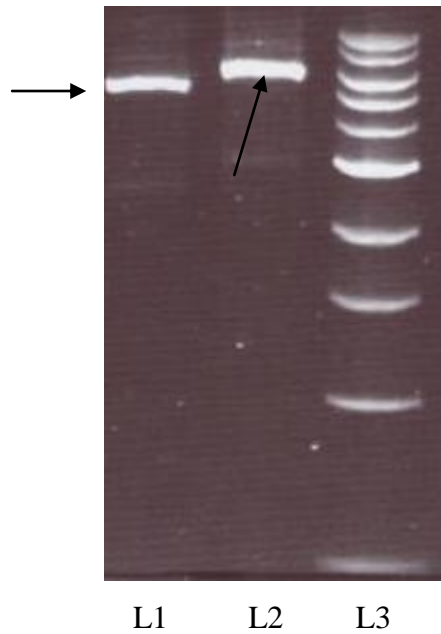
Fig 20: Sal I + Sph I digested pSV2 and pSVP27A plasmid. L1= Sal I + Sph I digested pSV2; L2= Sal I + Sph I digested pSVP27A; L3= 1Kb marker (sizes of 1 kb marker bands are 0.5 kb, 1 kb, 1.5 kb, 2 kb, 3 kb (brightest band), 4 kb, 5 kb, 6 kb, 8 kb and 10 kb respectively from bottom to top on agarose gel). Arrows showing the bands to be excised from the gel.
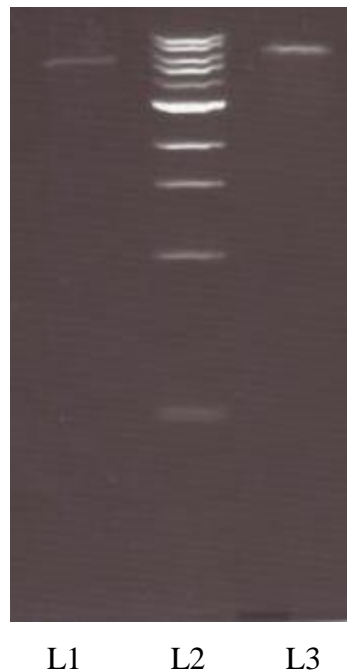
Fig 21: gel purified pSV2 and pSVP27A. L1= Gel purified pSV2; L2= 1 Kb marker (sizes of 1 kb marker bands are 0.5 kb, 1 kb, 1.5 kb, 2 kb, 3 kb (brightest band), 4 kb, 5 kb, 6 kb, 8 kb and 10 kb respectively from bottom to top on agarose gel); L3= Gel purified pSVP27A.

3. 15. Ligation of Cry1AcD (1-56) and Cry1AcD (2-50) with pSV2 and pSVP27A

Ligations (as described in methods 2. 10) were performed to ligate gel purified Cry1AcD (1-56) and Cry1AcD (2-50) with gel purified pSV2 and pSVP27A plasmids.

3. 16. Transformation of *E.coli* JM109 strain with ligation mixes

E.coli JM109 strain cells were transformed (as described in methods 2. 8) with ligation mixes containing pSV2 1AcD (1-56), pSV2 1AcD (2-50), pSVP27A 1AcD (1-56), pSVP27A 1AcD (2-50) and were grown on agar plates containing ampicillin.

The transformed JM109 possessing pSV2 1AcD (2-50), pSV2 1AcD (1-56), pSVP27A 1AcD (2-50), pSVP27A 1AcD (1-56) were plated on agar plates containing ampicillin, only 8, 1, 4 and 7 colonies grew on them respectively.

All of these colonies were picked up and subcultured on ampicillin containing agar plates.

3. 17. Rapid Size Screen of the subcultured colonies

Rapid Size Screen (as described in methods 2.13) was performed on all these colonies to know whether these colonies might possess the constructs i.e. recombinant plasmids (pSV2 1AcD (2-50), pSV2 1AcD (1-56), pSVP27A 1AcD (2-50), pSVP27A 1AcD (1-56) or not (fig 22 a, b, c).

Fig 22 a showed that colony 1(L2) might possess pSV2 1AcD (1-56) and colony 1 (L3), 2 (L4) and 5 (L7) might possess pSV2 1AcD (2-50) because the bands in the lanes of these colonies were above the control band (pSV2) (L1).

Fig 22 b showed that JM 109 colony 2 (L3) and JM 109 colony 3 (L4) might possess pSVP27A 1AcD (2-50) because the bands in the lanes of these two colonies were above the band in control lane (pSVP27A) (L1). Colony 7 (L7) and 8 (L8) might possess pSV2 1AcD (2-50) because the bands in the lanes of these two colonies were above the band in control lane (pSV2) (L6).

Fig 22 c showed that JM 109 colony 4 (L5) and JM 109 colony 7 (L8) might possess pSVP27A 1AcD (1-56) because the bands in the lanes of these two colonies were above the band in control lane (pSVP27A) (L1).
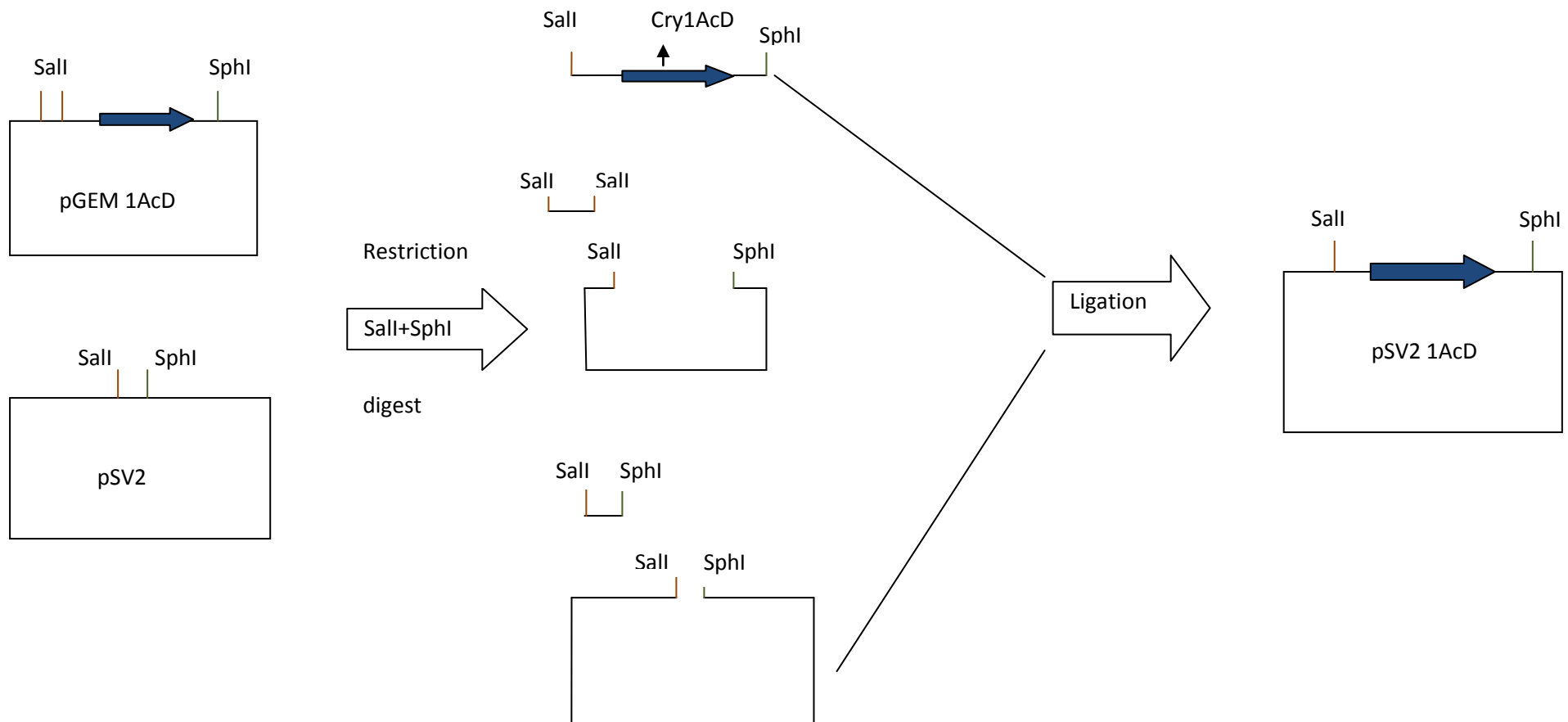
Diagram 1: Construction of recombinant plasmid pSV2 1AcD
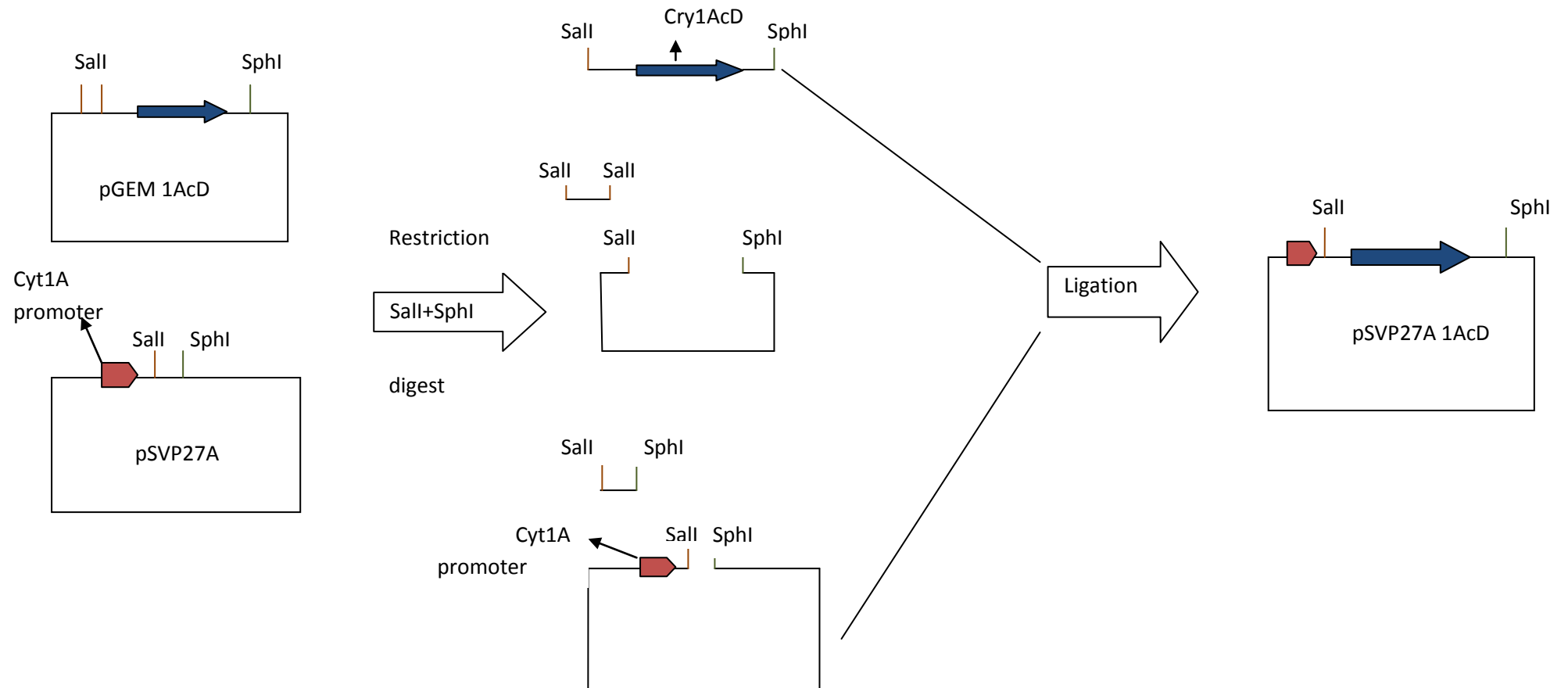
Diagram 2: Construction of recombinant plasmid pSVP27A 1AcD

22 a.

L1   L2   L3   L4   L5   L6   L7   L8

22 b.

L1   L2   L3   L4   L5   L6   L7   L8

22 c.

L1   L2   L3   L4   L5   L6   L7   L8
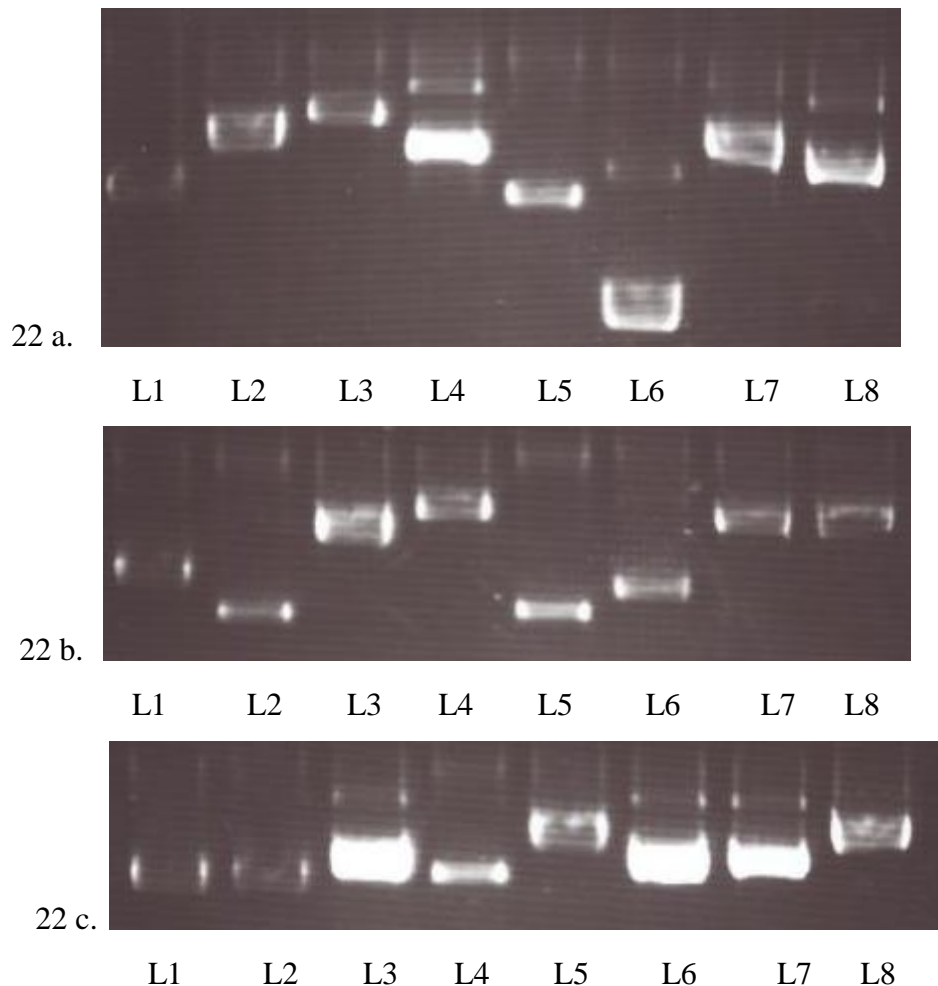
Fig 22 a: Rapid Size Screen of JM 109 colonies supposed to possess pSV2 1AcD (1-56) and pSV2 1AcD (2-50). L1= JM 109 possessing pSV2 (used as control); L2= JM 109 colony1 supposed to possess pSV2 1AcD (1-56); L3= JM 109 colony 1 supposed to possess pSV2 1AcD (2-50); L4= JM 109 colony 2 supposed to possess pSV2 1AcD (2-50); L 5= JM 109 colony 3 supposed to possess pSV2 1AcD (2-50); L6= JM 109 colony 4 supposed to possess pSV2 1AcD (2-50); L7= JM 109 colony 5 supposed to possess pSV2 1AcD (2-50); L8= JM 109 colony 6 supposed to possess pSV2 1AcD (2-50).

Fig 22 b: Rapid Size Screen of JM 109 colonies supposed to possess pSVP27A 1AcD (2-50) and pSV2 1AcD (2-50). L1= JM 109 possessing pSVP27A (as control); L2= JM 109 colony1 supposed to possess pSVP27A 1AcD (2-50); L3= JM 109 colony 2 supposed to possess pSVP27A 1AcD (2-50); L4= JM 109 colony 3 supposed to possess pSVP27A 1AcD (2-50); L5= JM 109 colony 4 supposed to possess pSVP27A 1AcD (2-50); L6= JM 109 possessing pSV2 (used as control); L7= JM 109 colony 7 supposed to possess pSV2 1AcD (2-50); L8= JM 109 colony 8 supposed to possess pSV2 1AcD (2-50).

Fig 22 c: Rapid Size Screen of JM 109 colonies supposed to possess pSVP27A 1AcD (1-56). L1= JM 109 possessing pSVP27A (used as control); L2= JM 109 colony 1 supposed to possess pSVP27A 1AcD (1-56); L3= JM 109 colony 2 supposed to possess pSVP27A 1AcD (1-56); L4= JM 109 colony 3 supposed to possess pSVP27A 1AcD (1-56); L5= JM 109   colony 4 supposed to possess pSVP27A 1AcD(1-56);   L6= JM 109 colony 5 supposed to possess

pSVP27A 1AcD (1-56); L7= JM 109 colony 6 supposed to possess pSVP27A 1AcD (1-56); L8= JM 109 colony 7 supposed to possess pSVP27A 1AcD (1-56).

3. 18. Miniprep of colonies whose bands were above the control band

So it was decided to miniprep those colonies whose bands were above the control bands, to extract the recombinant plasmids.

3. 19. Restriction digests of recombinant plasmids

All the eluted recombinant plasmids were restriction digested (as described in methods 2. 14) with SalI and SphI enzymes. The correctly formed constructs (i.e. recombinant plasmid) pSV2 1AcD (1-56) and pSV2 1AcD (2-50) should be 8863 bp and 8886 bp respectively and pSVP27A 1AcD (1-56) and pSVP27A 1AcD (2-50) should be 9510 bp and 9533 bp respectively. When pSV2 1AcD (1-56) and pSV2 1AcD (2-50) are double digested with SalI and SphI two fragments of sizes 4925bp and 3938 bp and 4925 bp and 3961 bp should be produced respectively and when pSVP27A 1AcD (1-56) and pSVP27A 1AcD (2-50) are double digested with SalI and SphI two fragments of sizes 5572 bp and 3938 bp and 5572 bp and 3961 bp should be produced respectively (information obtained using NEB cutter).

The result (fig 23) indicated that pSV2 1AcD (2-50) construct was formed because the fragments produced by double digestion with SalI and SphI in lanes L2, L5 and L6 were near 5 Kb and 4Kb bands of 1Kb marker. Thus colonies 1, 7 and 8 only possessed the correctly and fully formed construct pSV2 1AcD (2-50). The pSV2 1AcD (1-56) construct was not formed because the fragments produced by double digestion with SalI and SphI in lane L7 were not near 5 Kb and 4Kb bands of 1Kb marker.

L1   L2   L3   L4   L5   L6   L7
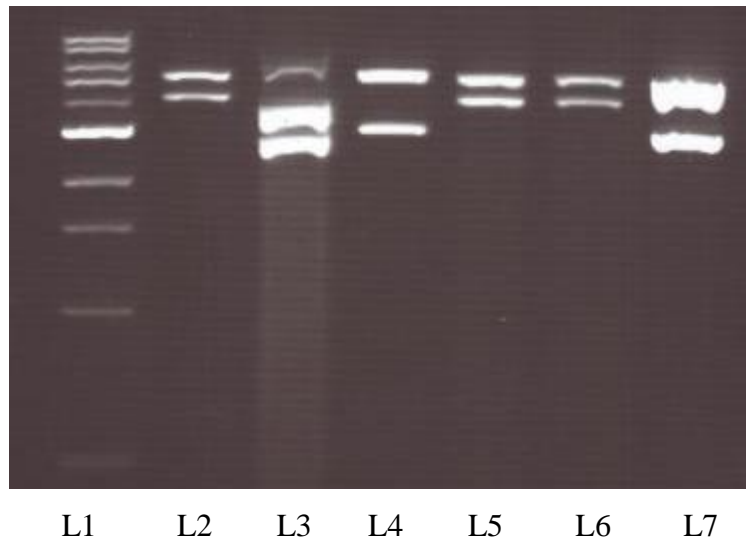
Fig 23: SalI + SphI restriction digest of recombinant plasmids supposed to be pSV2 1AcD (2-50) and pSV2 1AcD (1-56). L1= 1Kb marker (sizes of 1 kb marker bands are 0.5 kb, 1 kb, 1.5 kb, 2 kb, 3 kb (brightest band), 4 kb, 5 kb, 6 kb, 8 kb and 10 kb respectively from bottom to top on agarose gel); L2= SalI+SphI digested recombinant plasmid supposed to be pSV2 1AcD (2-50) eluted from JM 109 colony 1; L3= SalI+SphI digested recombinant plasmid supposed to be pSV2 1AcD (2-50) eluted from JM 109 colony 2; L4= SalI+SphI digested recombinant plasmid supposed to be pSV2 1AcD (2-50) eluted from JM 109 colony 5; L5= SalI+SphI digested recombinant plasmid supposed to be pSV2 1AcD (2-50) eluted from JM 109 colony 7; L6= SalI+SphI digested recombinant plasmid supposed to be pSV2 1AcD (2-50) eluted from JM 109 colony 8; L7= SalI+SphI digested recombinant plasmid supposed to be pSV2 1AcD (1-56) eluted from JM 109 colony1.

Fig 24 shows that only colony 3 possessed the fully and correctly formed construct pSVP27A 1AcD (2-50) because the fragments produced by double digestion with SalI and SphI were near 6 Kb and 4Kb. The pSVP27A 1AcD (1-56) construct was not formed.

L1   L2   L3   L4   L5
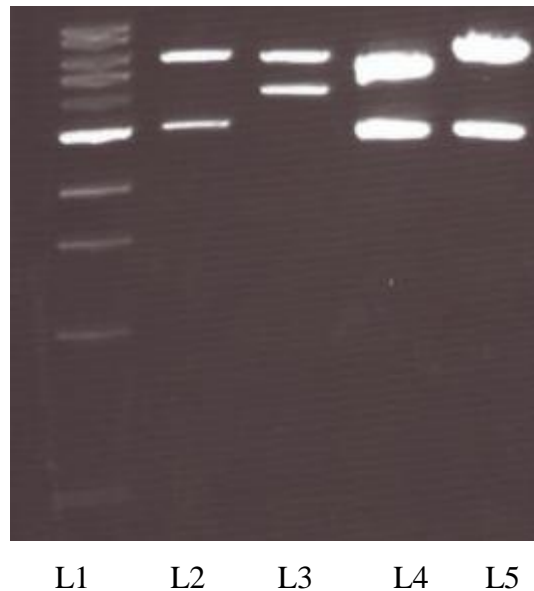
Fig 24: SalI + SphI restriction digest of recombinant plasmids supposed to be pSVP27A 1AcD (2-50) and pSVP27A 1AcD (1-56). L1= 1Kb marker (sizes of 1 kb marker bands are 0.5 kb, 1 kb, 1.5 kb, 2 kb, 3 kb (brightest band), 4 kb, 5 kb, 6 kb, 8 kb and 10 kb respectively from bottom to top on agarose gel); L2= SalI+SphI digested recombinant plasmid supposed to be pSVP27A 1AcD (2-50) eluted from JM 109 colony 2; L3= SalI+SphI digested recombinant plasmid supposed to be pSVP27A 1AcD (2-50) eluted from JM 109 colony 3; L4= SalI+SphI digested recombinant plasmid supposed to be pSVP27A 1AcD (1-56) eluted from JM 109 colony 4; L5= SalI+SphI digested recombinant plasmid supposed to be pSVP27A 1AcD (1-56) eluted from JM 109 colony 7.

So it was decided to transform *Bacillus thuringiensis* 78/11 strain with only the fully and correctly formed constructs i.e. pSV2 1AcD (2-50) and pSVP27A 1AcD (2-50).

3. 20. Transformation of Bt 78/11 with pSV2 1AcD (2-50) and pSVP27A 1AcD (2-50)

Bacillus thuringiensis 78/11 strain were transformed (as described in methods 2. 11) with pSV2 1AcD (2-50) and pSVP27A 1AcD (2-50) and the transformed cells were grown on agar plate containing chloramphenicol.

In 78/11(pSV2 1AcD (2-50)) plate 15 colonies grew and in 78/11 (pSVP27A 1AcD (2-50)) plate 20 colonies grew.

To confirm transformed *Bacillus thuringiensis* 78/11 strain possessed the correctly formed constructs (i.e. recombinant plasmid) pSV2 1AcD (2-50) and pSVP27A 1AcD (2-50), it was decided to elute these constructs from 78/11 and transform them back into *E.coli* JM109 strain.

For that one colony each from 78/11 (pSV2 1AcD (2-50)) and78/11 (pSVP27A 1AcD (2-50)) plates were picked up and were subcultured on another agar plate containing

chloramphenicol.

Minipreps (as described in methods 2. 12) were performed to elute these constructs from *Bacillus thuringiensis* 78/11 strain.

3. 21. Transformation of JM109 with constructs

Both the constructs were eluted from *Bacillus thuringiensis* 78/11 strain. After that *E.coli* JM109 were transformed (as described in methods 2. 8) with these eluted constructs and were grown on agar plates containing ampicillin. Around 100 colonies grew on both plates. One colony from each plate was picked up and was subcultured on another plate containing ampicillin. These two subcultured colonies were minipreped to elute the constructs.

3. 22. Restriction digests of the Constructs

Double digest of the constructs were performed using SalI and SphI enzymes.

The result (fig 25) showed that the construct (L2) when digested with SalI and SphI enzymes produced fragments of around 5 Kb and 4 Kb thus the result confirmed that *Bacillus thuringiensis* 78/11 possessed the fully and correctly formed construct pSV2 1AcD (2-50). Similarly the construct (L3) when digested with SalI and SphI enzymes produced fragments of around 6Kb and 4Kb thus the result confirmed that *Bacillus thuringiensis* 78/11 possessed the fully and correctly formed construct pSVP27A 1AcD (2-50).

L1   L2   L3
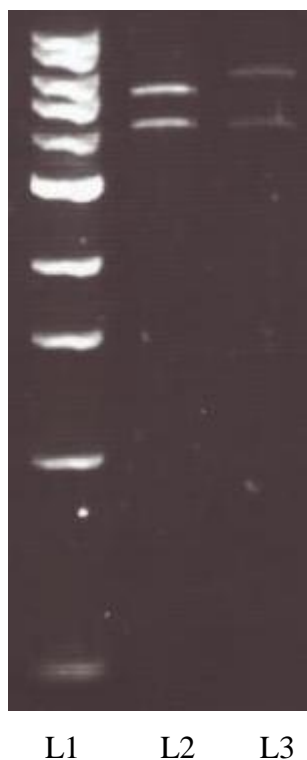
Fig 25: SalI and SphI digested constructs pSV2 1AcD (2-50) and pSVP27A 1AcD (2-50). L1= 1Kb Marker (sizes of 1 kb marker bands are 0.5 kb, 1 kb, 1.5 kb, 2 kb, 3 kb (brightest band), 4 kb, 5 kb, 6 kb, 8 kb and 10 kb respectively from bottom to top on agarose gel); L2= Construct (pSV2 1AcD (2-50)) digested with SalI and SphI; L3= Construct (pSVP27A 1AcD (2-50)) digested with SalI and SphI.

So it was decided to grow these two colonies, one colony possessing pSV2 1AcD (2-50) and another colony possessing pSVP27A 1AcD (2-50), on agar plates containing chloramphenicol at 30^0C for two days. After two days colonies were scrapped off from the plates to harvest (as described in methods 2. 16).

The result (fig 26) showed that Cry1AcD (2-50) was not expressed in *Bacillus thuringiensis* 78/11 strain because no bands were observed near Cry1Ac (wild type) band in lanes L2 and L3 and also no crystals were observed when viewed under a microscope.
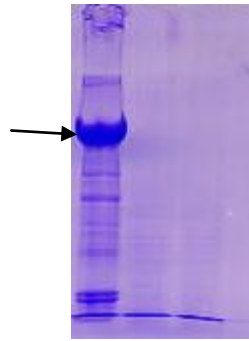
L1 L2 L3

Fig 26: Gel to show the expression of Cry1AcD (2-50) harvested from *Bacillus thuringiensis* 78/11. L1= Cry1Ac (wild type) used as reference protein; L2= Cry1AcD (2-50) harvested from *Bacillus thuringiensis* 78/11 possesing pSV2 1AcD (2-50) recombinant plasmid; L3= Cry1AcD (2-50) harvested from *Bacillus thuringiensis* 78/11 strain possesing pSVP27A 1AcD (2-50) recombinant plasmid. Arrow is showing Cry1Ac (wild type).

3. 23. Discussion

Mutations in the cadherin gene have been linked to Cry1Ac resistance in *Heliothis virescens, Pectinophora gossypiella* and *Helicoverpa armigera* (Morin et al., 2003; Gahan et al., 2001; Xu et al., 2005).

It has been suggested that binding of Cry1A toxins to cadherin receptors promote the conformational change in Cry1A toxins. The conformational change exposes helix α1 of domain I for proteolytic degradation and allows the formation of a pre pore toxin oligomer (Gomez et al., 2002 a; Gomez et al., 2002 b).

Mutations in the cadherin gene slow down the oligomer formation of Cry1A proteins. Mutation in cadherin gene does not stop oligomerisation of Cry1A proteins completely. This may be supported from the fact that higher concentrations of Cry1A toxins are still capable of killing the Cry1A resistant insects with cadherin gene mutation (Gahan et al., 2010).

Soberon et al. showed that deletion of helix α-1 of domain I from Cry1A toxins leads to the formation of oligomers without binding to the cadherin receptors. Thus these modified toxins overcome resistance by bypassing the cadherin receptor binding. So the Cry1A modified toxins are effective against those insects which are resistant to native Cry1A toxins and whose mechanism of resistance is linked to mutations in cadherin gene (Soberon et al., 2007; Bravo and Soberon, 2008).

Modified Cry1Ac and Cry1Ab lacking helix α-1 were much more effective against Cry1Ac and Cry1Ab resistant *P. Gossypiella* than native Cry1Ac and Cry1Ab. Modified Cry1Ab was also much more effective against cadherin silenced *M. sexta* than native Cry1Ab (Soberon et al., 2007).

Soberon et al. also showed that modified Cry1A toxins were less potent than native Cry1A toxins against Cry1A susceptible larvae of *P.gossypiella*. The reason suggested by them for this lesser potency was that relative to native Cry1A toxin modified toxin had lower stability in the mid gut or had reduced oligomer forming ability (Soberon et al., 2007).

Franklin et al. showed that modified Cry1Ab and Cry1Ac were more effective than native Cry1Ab and Cry1Ac against Cry1Ac and Cry1Ab resistant *T.ni* larvae. However the role of cadherin in *T.ni* resistance has not been determined yet (Franklin et al., 2009). In fact the mechanism of resistance to Cry1Ac and Cry1Ab is unknown in *T.ni* (Bravo and Soberon, 2008).

According to Baxter et.al resistance to Cry1A toxins in two strains of *P.xylostella*, SC1

and NOQA, is not linked to the cadherin gene (Baxter et al., 2005; Baxter et al., 2008).
So it was decided to make Cry1Ac modified toxin and check whether it is effective or not against *P.xylostella* NOQA population whose resistance mechanism has not been linked to the cadherin gene.

PCR was used to create the modified toxin. Primers were designed to delete amino acids from 2nd position to 56th position from the N-terminal region of Cry1Ac. Deletion of amino acids from 2nd position to 56th position was chosen because the modified toxin developed by Soberon et.al also lacked 56 amino acid residues from the N-terminal region (Franklin et al., 2009).

The sequencing result showed that an extra amino acid methionine (start codon ATG) was also deleted but in bacteria GTG could also act as start codon. In the Cry1Ac deleted (Cry1AcD) sequence GTG is near to the ribosome binding site (only 4 base apart). So this modified protein could be expressed in bacteria. Expression was undertaken in three different *E.coli* strains (JM 109, BL21 and DH5α). Since it has previously shown that expression of modified Cry1Ac can be strain dependent (personal communication between Dr. Neil Crickmore and Dr. M. Soberon). The modified Cry1Ac toxins (Cry1AcD (1-56) and Cry1AcD (2-50)) were expressed in all these three strains at 30^0C and 25^0C. But the expression of modified toxins (Cry1AcD (1-56) and Cry1AcD (2-50)) were better at 25^0C than at 30^0C. The reason for this could be that Cry1AcD (1-56) and Cry1AcD (2-50) proteins produced at 30^0C were degraded faster than Cry1AcD (1-56) and Cry1AcD (2-50) proteins produced at 25^0C.

Differences in the expression of the same protein (Cry1AcD (1-56) or Cry1AcD (2-50)) were observed when harvested from three different strains (JM109, BL21 and DH5α) of *E.coli* at same temperature i.e. at 25^0C.

Cry1AcD (1-56) and Cry1AcD (2-50) harvested from DH5α were slightly better expressed as compared to Cry1AcD (1-56) and Cry1AcD (2-50) harvested from JM109 and BL21.

The resulting inclusion bodies from all these three strains (JM109, BL21, DH5α) were checked for the characteristic pattern of alkali solubility and stability associated with the full length Cry1Ac (wild type) toxin.

Cry1AcD (1-56) from all the three strains solubilised. Cry1AcD (2-50) from JM109 and DH5α solubilised but Cry1AcD (2-50) from BL21 did not solubilise. Trypsin activated Cry1AcD (1-56) and Cry1AcD (2-50) from DH5α and JM109 were stable but trypsin activated Cry1AcD (1-56) and Cry1AcD (2-50) from BL21 were not stable. Thus

suggesting that Cry1AcD (1-56) and Cry1AcD (2-50) from DH5α and JM109 strains may be properly folded.

The results of Cry1AcD (1-56) and Cry1AcD (2-50) expression, solubilisation and trypsin activation from all the three strains have been summarised in the table 4 given below.

Strain	Cry1AcD(1-56) Expression	Cry1AcD(1-56) Solubility	Cry1AcD(1-56) Trypsin activation
DH5α	++ +	+++	+++
JM109	++	++	++
BL21	++	++	_ _
Strain	Cry1AcD(2-50) Expression	Cry1AcD(2-50) Solubility	Cry1AcD(2-50) Trypsin activation
DH5α	+++	+++	+++
JM109	++	++	++
BL21	++	_ _	_ _

Table 4: Expression, solubility and trypsin activation of Cry1AcD (1-56) and Cry1AcD (2-50) from three different strains of *E.coli* (DH5α, JM109 and BL21). + = shows that protein has been expressed, solubilised or stable upon trypsin activation. _ = shows that protein has not been expressed, solubilised or stable upon trypsin activation.

So by looking at the results it was decided to use Cry1AcD (1-56) and Cry1AcD (2-50) from DH5α for leaf dip bioassay.

Toxicity assays with Cry1AcD (1-56), Cry1AcD (2-50) and Cry1Ac (wild type) were performed against the resistant (NOQA) population of *P.xylostella*.

The toxicity results showed that modified toxins Cry1AcD (1-56) and Cry1AcD (2-50) were very less toxic when compared with Cry1Ac (wild type) at similar toxin concentration (i.e. 120μg/ml).

The reason for less effectiveness of Cry1AcD (1-56) and Cry1AcD (2-50) against resistant NOQA population, whose mechanism of resistance has not been linked to mutation in cadherin gene, may be due to their lower stability in the midgut or may be due to their decreased oligomer forming ability.

Even though Cry1AcD (1-56) and Cry1AcD (2-50) from DH5α showed proper solubilisation and activated Cry1AcD (1-56) and Cry1AcD (2-50) were stable they still showed less toxicity towards NOQA. So it was decided to change the expression system

for Cry1AcD (1-56) and Cry1AcD (2-50) and see if there were any changes in toxicity of these two mutant toxins. Therefore it was decided to express Cry1AcD (1-56) and Cry1AcD (2-50) in *Bacillus thuringiensis* (Bt) 78/11 strain.

For expressing Cry1AcD (1-56) and Cry1AcD (2-50) in Bt strains Cry1AcD (1-56) and Cry1AcD (2-50) genes were first excised from pGEM vector and then cloned into *E.coli*-Bt shuttle vectors pSV$_2$ and pSVP27A. pSVP27A possesses Cyt1A promoter. So it was also decided to be used to see whether or not it creates any difference in Cry1AcD (1-56) and Cry1AcD (2-50) expression.

The result showed that only two constructs pSV$_2$ 1AcD (2-50) and pSVP27A 1AcD (2-50) were fully and correctly created. These two constructs were then introduced into Bt 78/11.

To confirm 78/11 strains possessed the correctly and fully formed constructs, the constructs were eluted from 78/11 strain and introduced into *E.coli* JM109 strain.

The result confirmed that Bt 78/11 strain possessed the fully and correctly formed constructs pSV$_2$ 1AcD (2-50) and pSVP27A 1AcD (2-50).

But the modified proteins Cry1AcD (1-56) and Cry1AcD (2-50) were not expressed in Bt 78/11 strain.

The results showed that modified Cry1Ac i.e. Cry1AcD (1-56) and Cry1AcD (2-50) are not effective against NOQA population. According to Baxter et al. resistance to Cry1Ac in NOQA population is not linked to mutation in cadherin gene (Baxter et al., 2005; Baxter et al., 2008). So the result strengthens the hypothesis that the mechanism of resistance in NOQA population is not cadherin based.

Future work:

Franklin et.al showed that modified Cry1Ac was more effective than native Cry1Ac against Cry1Ac resistant *T.ni* larvae. However the role of cadherin in *T.ni* resistance has not been determined yet (Franklin et.al, 2009).

So the modified Cry1Ac should be used against other resistant lepidopteran insects whose resistance mechanism has been linked and has not been linked to mutation in cadherin gene. If it is effective then modified Cry1Ac toxin would counter or delay insect resistance to Cry1Ac.

Modification could also be made in other Cry toxins which have similar structure as Cry1A toxins, form oligomers and induce pores. It would be interesting to see whether these other modified Cry toxins lacking helix α-1 can kill resistant insects that have

altered receptor or not (Soberon et al., 2007).

As modified Cry1Ac toxins were not expressed in Bt 78/11 strain, alternative Bt hosts could be used. Perhaps expressing the modified toxin in a Bt strain that already expresses a Bt toxin, for example HD73 that expresses Cry1Ac, may facilitate the stable expression of the mutant.

Chapter 4

Mechanism of resistance to spinosad in lepidopteran insects

4. 1. Introduction

Baxter and his colleagues showed that field based resistance to spinosad in a *Plutella xylostella* strain collected from Pearl city, Hawaii is due to the point mutation in the ninth intron splice junction of nAChR Pxα6. A point mutation at the 5' donor site of intron 9 (GT changed to AT) causes mRNA mis-splicing which leads to the addition of 40 bases into the mRNA of the resistant population. This mutation causes a premature termination codon between transmembrane domain 3 and 4 and is the likely functional cause of resistance in *Plutella xylostella* strain collected from Pearl city, Hawaii (Baxter et al., 2010).

Hence it was decided to check whether or not other spinosad resistant lepidopteran insects have similar mechanism of resistance as *Plutella xylostella* Pearl population i.e. whether this splice site region was a hot spot for mutation.

For this reason it was decided to work on a spinosad resistant *Spodoptera litura* population collected from the fields of Pakistan.

So it was decided to amplify nAChR intron 9 (including the splice junction) of *Spodoptera litura.*

The primers were designed by keeping this criterion in mind. Forward primer was designed on exon 9 and reverse primer on intron 9.

As the nAChR sequence of *Spodoptera litura* was not available, BLAST (NCBI) was performed using the nAChR exon 9 nucleotide sequence of *Plutella xylostella* G88 population. This was done because *Plutella xylostella* and *Spodoptera litura* belongs to the order Lepidoptera. nAChR exon 9 sequence of those insects which showed maximum score, identity and query coverage were selected and these sequences were aligned with *Plutella xylostella* nAChR exon 9 sequence using Clustal W program. The forward primer was designed from the region where the nucleotide sequences were highly similar after alignment.

```
G88            CTGCATCATGTTCATGGTGGCGTCGTCGGTGGTGCTGACCGTGGTGGTGCTCAACTACCA 73
H.virescens    CTGCATCATGTTCATGGTGGCTTCCTCCGTCGTCTCCACCATACTGATCCTCAACTACCA 1320
B.mori         CTGTATTATGTTCATGGTGGCCTCGTCTGTGGTGCTCACCGTTGTGGTGTTGAACTACCA 964
```

Nucleotide sequences highlighted in yellow is the region from where nAChR exon 9 forward primer has been designed.

Forward Primer Exon 9: 5' GCATCATGTTCATGGTGGCG 3'

The reverse primer was designed from the intron 9 sequence of *Plutella xylostella* alone because the alignment scores of different insects were very low.

```
G88           AAAATTGCTAGCCTGGCTAAATCAATTATAATAGCACAAAGTTCTGTGCTCCGGAACAAA 596
T.castaneum   -AAAATTGCT----TGACCA------TTACAAC-------------GTACT--GGAACGG- 165
H.virescens   -GCGTTGCT-----GGCGAA-----CTACAAC--------------ACCCTGGAGCGA- 211


G88           TTCCGACGATTATCGGGATTATTATAGGGAACTATTTTTATATCATACAGTATACTGGCT 656
T.castaneum   ------------------------------------------------CCGGTT 171
H.virescens   -----------------------------------------------CCGGTG 217
```

Nucleotide sequences highlighted in yellow is the region from where nAChR intron 9 reverse primer has been designed.

Reverse Primer Intron 9: 5' CCCGATAATCGTCGGAATTTG 3'

4. 2. Genomic DNA extraction from *Spodoptera litura*

Genomic DNA of *Spodoptera litura* was extracted (as described in methods 2. 22) using Qiagen Dneasy Kit.

4. 3. PCR to amplify the region supposed to possess the mutation

The reaction mixture which included designed primers (forward and reverse) template DNA (genomic DNA *Spodoptera litura*) and master mix was introduced into PCR machine and amplified using new high fidelity program (as described in methods 2. 23). The best annealing temperature was obtained by repeating the experiment at six different temperatures (from 50^0 to 55^0 C). Annealing temperature at 54^0 C showed the best result (fig 27).

The PCR product was run on 1% agarose result. Fig 27 shows the presence of a prominent band near 2 Kb and the other band was present below 500 bp but it was very faint.

L1   L2

Fig 27: PCR product of New High Fidelity Program.L1 = 1 Kb Marker (sizes of 1 kb marker bands are 0.5 kb, 1 kb, 1.5 kb, 2 kb, 3 kb (brightest band), 4 kb, 5 kb, 6 kb, 8 kb and 10 kb respectively from bottom to top on agarose gel) ; L2 = PCR product.

As the intron 9 size of *Spodoptera litura* is not known so it was decided to purify the band near 2 Kb from the gel.

4. 4. Purification of PCR product

As the PCR product obtained was not clean so it was decided to run the PCR product on 1% agarose gel. The band near 2 Kb was excised from the gel and purified (as described in methods 2. 6). 2µl of purified product was run on 1% agarose gel along with 1Kb marker. A band near 2 Kb was observed (fig 28).

L1   L2

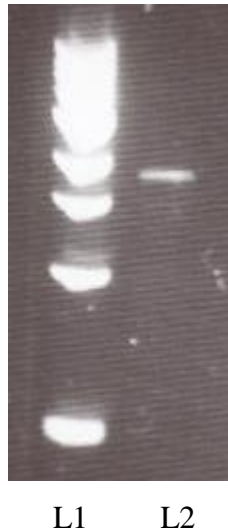Fig 28: Gel purified PCR product. L1 = 1 Kb Marker (sizes of 1 kb marker bands are 0.5 kb, 1 kb, 1.5 kb, 2 kb, 3 kb (brightest band), 4 kb, 5 kb, 6 kb, 8 kb and 10 kb respectively from bottom to top on agarose gel); L2 = PCR product purified from the gel.

4. 5. Ligation of purified PCR product with pGEM-T easy vector

The purified PCR product was ligated (as described in methods 2. 24) with pGEM-T easy vector.

4. 6. Transformation of *E.coli* JM 109 strain with ligation mix

E.coli JM 109 strain was transformed with the ligation mix and was grown on ampicillin containing agar plate. Around 30 colonies grew on the plate. Eight colonies were randomly picked up. These Eight colonies were subcultured on ampicillin containing agar plate.

4. 7. Rapid Size Screen of subcultured colonies

Rapid Size Screen (as described in methods 2. 13) was performed on randomly picked 8 colonies. Rapid Size Screen was performed on all these colonies to know whether these colonies might possess the construct i.e. recombinant plasmid or not.

Fig 29 shows that colonies 6, 20, 23 were slightly higher than the other colonies on the 1% agarose gel. So these three colonies 6, 20 and 23 were picked up and were then grown in 1.5 ml LB for 3-4 hours in the incubator.

Miniprep (as described in methods 2. 9) was performed to elute the plasmids from these three colonies.

L1   L2   L3   L4   L5   L6   L7   L8

Fig 29: Rapid Size Screen of subcultured colonies. L1 = *E.coli* JM109 colony 5; L2 = *E.coli* JM109 colony 6; L3 = *E.coli* JM 109 colony 12; L4 = *E.coli* JM109 colony 15; L5 = *E.coli* JM109 colony 20; L6= *E.coli* JM109 colony 23; L7= *E.coli* JM109 colony 27; L8 = *E.coli* JM109 colony 30.

4. 8. Restriction digest on the plasmids eluted from colonies 6, 20 and 23

Restriction digest was performed on the eluted plasmids to confirm whether they possess the insert (i.e. the purified PCR product) or not. From Promega technical manual (pGEM-T and pGEM-T easy vector systems) EcoRI enzyme was chosen for restriction digest. EcoRI cuts the pGEM-T easy vector two times. Restriction digest

samples were run on 1% agarose gel.

Fig 30 shows the presence of three bands in colony 6 and colony 20 lanes.

Thus the result confirmed that plasmids eluted from colonies 6 and 20 possessed an insert (i.e. the purified PCR product).

L1   L2   L3   L4
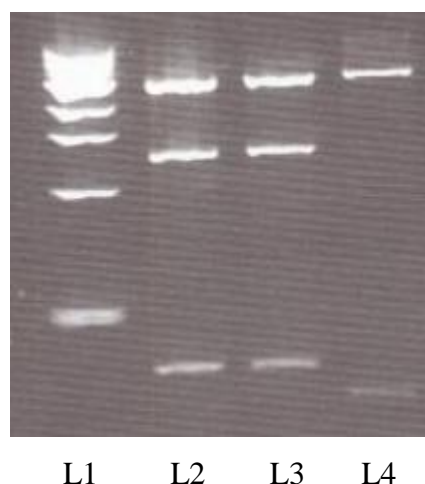
Fig 30: Restriction digest of plasmids eluted from *E.coli* JM109 colonies 6, 20, 23.
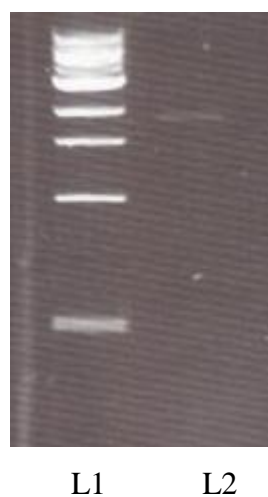L1= 1Kb marker (sizes of 1 kb marker bands are 0.5 kb, 1 kb, 1.5 kb, 2 kb, 3 kb (brightest band), 4 kb, 5 kb, 6 kb, 8 kb and 10 kb respectively from bottom to top on agarose gel); L2= Plasmid eluted from colony 6 and digested with EcoRI; L3= Plasmid eluted from colony 20 and digested with EcoRI; L4= Plasmid eluted from colony 23 and digested with EcoRI.

So the plasmid eluted from colony 20 confirmed to possess the insert was sent for sequencing.

NCBI, nucleotide blast was performed on the sequencing result but no match was found. So EMBOSS Transeq (EMBL-EBI) program was used to translate nucleotide sequence to a protein sequence. NCBI, protein blast was performed using all six frame result. No desired match was found.

So it was decided to change the reverse primer. Reverse primer was designed on nAChR exon 10. For that NCBI blast was performed using nAChR exon 10 nucleotide sequence of *Plutella xylostella* G88 population. nAChR sequence of those insects were selected which showed maximum score, identity and query coverage. nAChR exon 10 sequence of these insects were aligned with nAChR exon 10 sequence of *Plutella xylostella* G88 population using clustal W program. The reverse primer was designed from the region where the nucleotide sequences were highly similar after alignment.

```
B.mori        AACAGGATGCGAGAGTTGGAGCTAAAAGAGCGTTCGTCTAAGTCGCTGTTAGCCAACGTG 1095
H.virescens   ACGAGGATGAGGGAGCTGGAACTGAAGGAGAGGTCGTCGAAGTCCTTGCTGGCGAATGTT 1140
G88           AACCGCATGAGGGAGCTGGAGCTCAAGGAGAGGTCCTCAAAGTCTCTGCTGGCGAATGTG 150
```

Nucleotide sequences highlighted in yellow is the region from where nAChR exon10 reverse primer has been designed.

Reverse primer exon 10: 5' ACATTCGCCAGCAGAGACTT 3'

Unfortunately further experiments could not be conducted due to the loss of the *Spodoptera litura* population.

It was also decided to check whether *Plutella xylostella* NOQA population has same mechanism of resistance as *Plutella xylostella* Pearl City population. Whether NOQA population is resistant to spinosad is not known.

So it was decided to amplify the ninth intron splice junction of nAChR of NOQA. The primers were designed by keeping this criterion in mind.

As the nAChR sequence of *Plutella xylostella* is available so the forward primer was designed on exon 9 and reverse primer on intron 9.

Forward Primer Exon 9: 5' GCATCATGTTCATGGTGGCG 3'

Reverse Primer Intron 9: 5' CCCGATAATCGTCGGAATTTG 3'

Genomic DNA of NOQA was extracted (as described in methods 2. 21). The extracted genomic DNA of NOQA was run on 1% agarose gel (fig 31).

Fig 31 shows the presence of a single clear band.

Fig 31: Genomic DNA of NOQA.

PCR was performed using New High Fidelity Program. The best annealing temperature was obtained by repeating the experiment at five different temperatures (from 50^0 to 54^0C). Annealing temperature at 54^0C showed the best result (fig 32).

Fig 32 shows the presence of a very prominent band near 500bp and one other band that was very faint and well below 500bp. As the primers were designed to amplify around 500 bp, the band around 500 bp was purified from the gel.

L1 L2

Fig 32: PCR product of NOQA. L1= 1 Kb marker (sizes of 1 kb marker bands are 0.5 kb, 1 kb, 1.5 kb, 2 kb, 3 kb (brightest band), 4 kb, 5 kb, 6 kb, 8 kb and 10 kb respectively from bottom to top on agarose gel); L2= PCR product.

Purified PCR product of NOQA was sent for sequencing with forward and reverse primer.

Sequencing result obtained was aligned with Pearl (spinosad resistance) and G88 (spinosad susceptible) genomic DNA from exon 9 to exon 10 (which includes intron 9) using ClustalW program.

```
 G88         TGCTCAACTACCACCACCGCACCGCCGACATACACGAGATGCCGCAGTGGGTGAGTACCT 120
NOQAForward  TGCTCA-CTACCACCACCGCACCGCCGACATACACGAGATGCCGCAGTGGGTGAGTACCT  60
NOQAreverse  TGCTCAACTACCACCACCGCACCGCCGACATACACGAGATGCCGCAGTGGGTGAGTACCT 106
 Pearl       TGCTCAACTACCACCACCGCGCCGCCGACATACACGAGATGCCGCAGTGGATGAGTACCA 120
```

 Nucleotides highlighted in yellow are the splice site junction of exon 9 and intron 9 of nAChR in G88, NOQA and Pearl City populations of *Plutella xylostella*

The alignment result showed that there was no splice site mutation present in NOQA population of *Plutella xylostella* i.e. GT was present at the splice site junction of exon 9 and intron 9 of nAChR, as in G88 (spinosad susceptible) population of *Plutella xylostella*.

4. 9. Discussion

Baxter and his colleagues showed that field based resistance to spinosad in a *Plutella xylostella* strain collected from Pearl city, Hawaii is due to the point mutation in the ninth intron splice junction of nAChR Pxα6. A point mutation at the 5' donor site of intron 9 (GT changed to AT) causes mRNA mis-splicing which leads to the addition of 40 bases into the mRNA of the resistant population. This mutation causes a premature termination codon between transmembrane domain 3 and 4 and is the likely functional cause of resistance in *Plutella xylostella* strain collected from Pearl city, Hawaii (Baxter et al., 2010).

Perry and his collegues showed that deletion of D alpha 6 subunit of nAChR causes high level of resistance to spinosad in *Drosophila melanogaster* without being lethal. D alpha 6 strain of *Drosophila melanogaster* showed 1181 fold resistance to spinosad (Perry et al., 2007).

Hence it was decided to check whether or not spinosad resistant *Spodoptera litura* population collected from the fields of Pakistan has a similar mechanism of resistance (i.e. splice-site mutation) as *Plutella xylostella* Pearl population.

So it was decided to amplify the nAChR intron 9 (including the splice junction) of *Spodoptera litura*.

As the nAChR sequence of *Spodoptera litura* is not available so BLAST (NCBI) was performed using the nAChR exon 9 nucleotide sequence of *Plutella xylostella* G88 population. This was done because *Plutella xylostella* and *Spodoptera litura* belong to the order Lepidoptera. nAChR exon 9 sequence of those insects which showed maximum score, identity and query coverage were selected and these sequences were aligned with *Plutella xylostella* nAChR exon 9 sequence using Clustal W program. The forward primer was designed from the region where the nucleotide sequences were highly similar after alignment.

The reverse primer was designed from the intron 9 sequence of *Plutella xylostella* because the alignment scores of different insects were very low.

PCR was performed to amplify the nAChR intron 9 (including the splice junction) of *Spodoptera litura*.

The results showed the presence of a prominent band near 2 Kb and the other band present was below 500 bp but it was very faint.

As the intron 9 size of *Spodoptera litura* is not known so it was decided to purify the band near 2 Kb from the gel as it was the most prominent band.

The 2 Kb band was purified from the gel and cloned into pGEM-T easy vector and was then transformed into *E.coli* JM109 strain. Rapid Size Screen was performed on selected colonies. The result showed that colonies 6, 20 and 23 might possess the recombinant plasmid.

Plasmids eluted from the colonies 6, 20 and 23 were restriction digested with EcoRI. EcoRI cuts the pGEM-T easy vector two times.

The plasmids eluted from colonies 6, 20 when digested with EcoRI produced 3 bands. Thus confirming that plasmids eluted from these two colonies possessed the insert (i.e the purified PCR product).

So the plasmid eluted from colony 20 confirmed to possess the insert was sent for sequencing.

NCBI, nucleotide blast was performed on the sequencing result but no match was found. So EMBOSS Transeq (EMBL-EBI) program was used to translate nucleotide sequence to a protein sequence. NCBI, protein blast was performed using all six frame result. No desired match was found.

Thus PCR performed to amplify the nAChR intron 9 (including the splice junction) of *Spodoptera litura* was unsuccessful.

So it was decided to change the reverse primer. Reverse primer was decided to be designed on nAChR exon 10. For that NCBI, blast was performed using nAChR exon 10 nucleotide sequence of *Plutella xylostella* G88 population. nAChR sequence of those insects were selected which showed maximum score, identity and query coverage. nAChR exon 10 sequence of these insects were aligned with nAChR exon 10 sequence of *Plutella xylostella* G88 population using clustal W program. The reverse primer was designed from the region where the nucleotide sequences were highly similar after alignment.

Unfortunately further experiments could not be conducted due to the loss of *Spodoptera litura* population.

 Future Work on *Spodoptera litura*:

Forward primer exon 9 and reverse primer exon 10 would be used to carry on the PCR and rest of the experiments would be similar to the previous work and if it does not work then total RNA would be extracted from spinosad resistant *Spodoptera litura* and then cDNA would be generated from it. Then cDNA would be cloned and sequenced. From cDNA sequence it would be looked for whether intron splicing occurred after 40

bp or not because in resistant population (Pearl) of *Plutella xylostella* intron splicing occurred after 40 bp at a second GT splice site (Baxter et al., 2010) or splicing occured anywhere else in this gene or more generally whether there was evidence for any mis-spliced transcripts or other significant mutations anywhere.

It was also decided to check whether *Plutella xylostella* NOQA population has same splice site mutation as *Plutella xylostella* Pearl City population. Whether NOQA population is resistant to spinosad is not known.

So it was decided to amplify the ninth intron splice junction of nAChR of NOQA. The primers were designed by keeping this criterion in mind.

As the nAChR sequence of *Plutella xylostella* is available so the forward primer was designed on exon 9 and reverse primer on intron 9.

PCR was performed using New High Fidelity Program. The result showed the presence of a very prominent band near 500bp and the other band was very faint and well below 500bp. As the primers were designed to amplify around 500 bp so the band around 500 bp was decided to be purified from the gel.

Purified PCR product of NOQA was sent for sequencing with forward and reverse primer.

Sequencing result obtained was aligned with Pearl (spinosad resistance) and G88 (spinosad susceptible) gDNA from exon 9 to exon 10 (which includes intron 9) using ClustalW program.

The alignment result showed that there was no splice site mutation present in NOQA population of *Plutella xylostella* i.e. GT was present at the splice site junction of exon 9 and intron 9 of nAChR, as in G88 (spinosad susceptible) population of *Plutella xylostella*.

So it may be possible that NOQA population is not resistant to spinosad or if it is resistant then there might be a different mutation.

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