

Chapter 2

Bacillus thuringiensis Applications in Agriculture

Zenas George and Neil Crickmore

Abstract *Bacillus thuringiensis* (*Bt*) and its insecticidal toxins have been used in agronomical pest control for decades. The mechanism of action of *Bt* toxins on insect pest involves specific molecular interactions which makes *Bt* a popular choice for pest control. The specificity of action of *Bt* toxins reduces the concern of adverse effects on non-target species, a concern which remains with chemical insecticides. Different strains of *Bt* are known to express different classes of toxins which in turn target different insects. *Bt* and its toxins can be formulated into powder or liquid sprays or expressed in transgenic plants. To maximize the effect of *Bt* toxins, multiple toxins are often combined when making *Bt* formulations or expressed in transgenic plants. Though *Bt* is a very effective biological control agent, there are concerns over the development of resistance by insect species and also the narrow spectrum of activity of individual toxins. To address these concerns, new strains of *Bt* expressing novel toxins are actively sought and existing toxins are genetically modified for improved activity.

Keywords Parasporal crystal proteins · Mode of action · Resistance · Use of Bt products · Synergism

2.1 Introduction

Bacillus thuringiensis is a Gram positive spore forming bacteria grouped into the *Bacillus cereus* group of *Bacilli* which produces proteinaceous insecticidal crystals during sporulation which is the distinctive feature between it and other members of the *Bacillus cereus* group (Read et al. 2003; Rasko et al. 2005). *Bacillus thuringiensis* was originally discovered in 1902 by a Japanese biologist Shigetane Ishiwatari who isolated it from diseased silkworm, *Bombyx mori* but it was formally characterised in 1915 by Ernst Berliner of Germany who isolated it from diseased larva of *Ephestia kuehniella* (flour moth caterpillars) in Thuringia province and linked it to the cause of a disease called Schlaffsucht (Milner 1994).

Z. George (✉) · N. Crickmore
Department of Biochemistry, School of Life Sciences, University of Sussex, Falmer,
BN1 9QG Brighton, UK
e-mail: n.crickmore@sussex.ac.uk

Bacillus thuringiensis readily proliferates when environmental conditions such as temperature and nutrient availability are favourable whilst the formation of spores have been shown to be triggered by internal and external factors including signals for nutrient starvation, cell density and cell cycle progression (Hilbert and Piggot 2004). The life cycle of *Bt* can be divided for convenience into phases and these are Phase I: vegetative growth; Phase II: transition to sporulation; Phase III: sporulation; and Phase IV: spore maturation and cell lysis (Hilbert and Piggot 2004; Berbert-Molina et al. 2008). The production of the characteristic insecticidal (Cry) proteins deposited in crystals in the mother cell have been shown to mainly start from the onset of sporulation (Sedlak et al. 2000; Xia et al. 2005; Guidelli-Thuler et al. 2009; Pérez-García et al. 2010). A number of *cry*-genes have been shown to be transcribed from two overlapping promoters BtI and BtII by RNA polymerases that contain sporulation dependent sigma factors σ^E and σ^K (Sedlak et al. 2000; Hilbert and Piggot 2004) and a mutation in the consensus region of σ^E has been shown to inhibit transcription from BtI and BtII promoters (Sedlak et al. 2000). It has also been shown that some *Bt* insecticidal proteins are produced and secreted into the culture medium during vegetative growth (Estruch et al. 1996; Donovan et al. 2001; Shi et al. 2004; Bhalla et al. 2005; Leuber et al. 2006; Milne et al. 2008; Singh et al. 2010; Abdelkefi-Mesrati et al. 2011).

As well as the Cry toxins *Bt* produces additional virulence factors including phospholipase C (Palvannan and Boopathy 2005; Martin et al. 2010), proteases (Hajaij-Ellouze et al. 2006; Brar et al. 2009; Infante et al. 2010) and hemolysins (Gominet et al. 2001; Nisnevitch et al. 2010). The virulence factors are controlled by the pleiotropic regulator PlcR and it has been demonstrated that cytotoxicity of *Bt* is PlcR dependent (Ramaraio and Lereclus 2006). Deletion of the *plcR* gene has been shown to result in a drastic reduction in the virulence of *Bt* in orally infected insects (Salamitou et al. 2000). The production of virulence factors by *Bt* is necessary but not enough for *Bt* to be called a pathogen (Fedhila et al. 2003) but its production of proteins that have been proved beyond doubt to be independently insecticidal justifies its name as an insect pathogen (Frankenhuyzen 2009).

The insecticidal proteins in the crystalline bodies produced during sporulation have been shown to contain two types of insecticidal proteins namely Cry toxins and Cyt-toxins and there are one or more toxins produced and packaged into a single crystal or multiple crystals by a *Bt* strain (de Maagd et al. 2001). The Cry toxins acquired the mnemonic Cry from the fact that they are found in the crystal while the Cyt-toxins acquired the mnemonic Cyt because of their in vitro cytolytic activity (Crickmore et al. 1998).

Schnepf and Whiteley (1981) confirmed that the insecticidal ability of *Bt* is as a result of the proteins that it produces by first cloning and heterologously expressing a toxin gene in *E. coli* which showed insecticidal activity to *Manduca sexta* just as the wild type *Bacillus thuringiensis* var. kurstali HD-1 from which it was cloned did. Since this discovery, a great number of other genes have cloned and expressed and the process of *Bt* toxin gene discovery is still ongoing. In order to differentiate between one *Bt* insecticidal gene and the other, the discoverers of the genes gave them arbitrary names like 4.5, 5.3 and 6.6-kb-class genes (Kronstad and Whiteley 1986), *bta* gene (Sanchis et al. 1989), *cry* gene (Donovan et al. 1988) and Type A and

Type B (Hofte et al. 1988) among others. With a steady growth in the number of cloned and characterised novel insecticidal genes coming through, an attempt was made to organise the ever growing data. The first attempt to produce an organised systematic nomenclature of *Bt* insecticidal genes was dependent on the insecticidal activity of the protein they code for to assign a primary rank to the gene and with this system, genes that encode proteins toxic to lepidopteran insects were called *cryI* genes, while lepidopteran and dipteran protein genes were called *cryII* genes, *cryIII* genes were those ones that encoded proteins toxic to coleopterans and *cryIV* genes encoded proteins toxic to dipterans alone (Hofte and Whiteley 1989). Though this system provided a framework for naming newly cloned novel toxins, it was short of a robust system of nomenclature that is able to accommodate new genes without ambiguity. The discovery of wild type gene like *cryIB* that codes for a toxin toxic to both lepidoptera and coleoptera (Bradley et al. 1995) threw the system off balance as it did not have room to accommodate a toxin with such spectrum of activity. Also, toxins like CryIC that had toxicity to both diptera and lepidoptera (Smith and Ellar 1994) did not have a place in the (Hofte and Whiteley 1989) system of nomenclature.

With the difficulty of accommodating newly discovered genes in the (Hofte and Whiteley 1989) nomenclature system arising, there was a need to come up with a robust system of nomenclature and (Crickmore et al. 1998) came up with a system that is based on sequence similarity rather than function based. In the Crickmore et al. system, the mnemonic root was combined with a series of numerals and letters assigned in a hierarchical fashion to indicate degrees of phylogenetic divergence which was estimated by phylogenetic tree algorithms. The mnemonic Cyt was used for parasporal crystal proteins from *Bacillus thuringiensis* that exhibits hemolytic activity or any protein that has obvious sequence similarity to a known Cyt protein and mnemonic Cry was assigned to a parasporal crystal proteins from *Bacillus thuringiensis* that exhibits some experimentally verifiable toxic effect to a target organism, or any protein that has obvious sequence similarity to a known Cry protein. With this system for naming Cry and Cyt proteins widely accepted, it has also been adopted for the naming of the vegetatively produced *Bacillus thuringiensis* toxins and this family of proteins has been given the mnemonic Vip. A website which hosts all the *cry*, *cyt* and *vip* cloned genes has been established and is frequently updated as new genes are discovered.

The 3-D crystal structure of Cry-proteins including coleopteran specific Cry8Ea1 (Guo et al. 2009), Cry3Aa (Li et al. 1991) and Cry3Bb (Galitsky et al. 2001), dipteran specific Cry4Aa (Boonserm et al. 2006) and Cry4Ba (Boonserm et al. 2005), lepidopteran specific Cry1Aa (Grochulski et al. 1995), lepidopteran/dipteran specific Cry2Aa (Morse et al. 2001) have been resolved through X-ray crystallographic methods of their activated forms. Also, the 3-D structure of Cyt-proteins have been resolved including activated Cyt2Ba (Cohen et al. 2008) and unprocessed Cyt2Aa (Li et al. 1996). Figure 2.1 is the crystal structure of Cry8Ea1 (Guo et al. 2009) showing the three domain organisation typical of all resolved 3-D structures of Cry toxins while Fig. 2.2 is the 3-D structure of Cyt2Ba which shows overall similarity to 3-D structure of Cyt2Aa that had previously been resolved.

Fig. 2.1 3-D crystal structure of Cry8Ea determined at 2.20 Å (PDB code: 3EB7). The three domains of the protein are represented with different colours with domain I coloured *blue*, domain II coloured *green* while domain III is coloured *red*. (Guo et al. 2009)

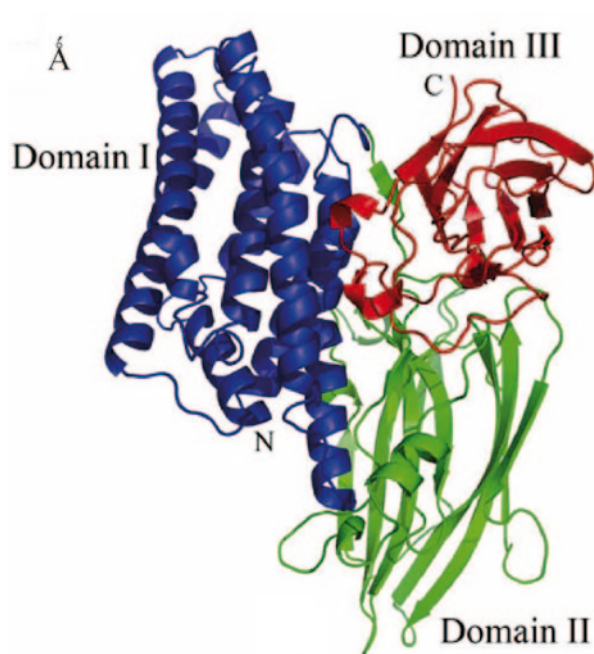
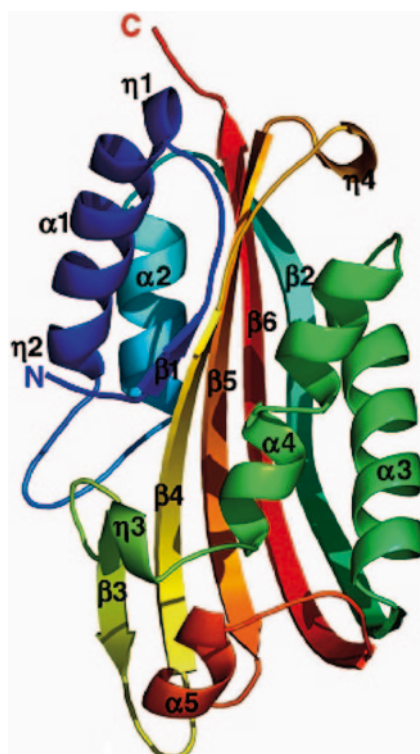


Fig. 2.2 Crystal structure of a monomer of Cyt2Ba determined at 1.80 Å (PDB code: 2RCI). The ‘rainbow’ colouring scheme is used in colouring the molecule starting with *blue* at the N-terminal and ending with *red* at the C-terminal. (Cohen et al. 2008)



Though different Cry toxins have been shown to have specific targets in their insecticidal activity, the overall 3-D fold of many of them has been shown to be the same (see Fig. 2.1), comprising of three domains (de Maagd et al. 2003). Domain I has been shown to compose of seven α -helices in which the central helix- $\alpha 5$ is hydrophobic and is encircled by six other amphipathic helices. The helical domain I has been shown to share structural similarities with other pore forming bacterial toxins like cytolysin A (Mueller et al. 2009), diphtheria toxin and colicin A (Parker and Pattus 1993). Each of the outer helices of domain I is known to be amphipathic in nature and most of the helices are longer than 30 Å in length (Pigott and Ellar 2007). Domain II is made up of three antiparallel β -sheets packed together to form a β -prism with pseudo threefold symmetry (Li et al. 1991). Two of the sheets are composed of four strands in a Greek key motif and are solvent exposed (Boonserm et al. 2006). The third sheet packs against domain I and is arranged in a Greek-key-like motif with three strands and a short alpha-helix (Pigott and Ellar 2007). The structure of domain II has been compared to those of other β -prism proteins with carbohydrate-binding properties (de Maagd et al. 2003), including vitelline (Shimizu et al. 1994) and *Maclura pomifera* agglutinin (Lee et al. 1998) and it reveals a great topological similarity. Domain III has been shown to contain two antiparallel β -sheets that adopt a β -sandwich fold with the jelly roll topology (Boonserm et al. 2006). Both sheets are composed of five strands, with the outer sheet facing the solvent and the inner sheet packing against domain II. Two long loops extend from one end of the domain and interact with domain I (Grochulski et al. 1995). Domain III shows less structural variability than domain II, and the main differences are found in the lengths, orientations, and sequences of the loops (Boonserm et al. 2005). Domain III has been compared to other carbohydrate-binding protein domains and it shows great degree of similarity (de Maagd et al. 2003) and similarity of domain III was also found with those of domain 4 of the pore-forming toxin aerolysin which is involved in maintenance and stability of the heptameric toxin complex (Lesieur et al. 1999).

Proposed mechanisms of action of Cry and Cyt toxins from *Bt* include pore formation in which *Bt* toxins induce cell death by forming ionic pores following insertion into the membrane, causing osmotic lysis of midgut epithelial cells in their target insect (Knowles and Ellar 1987; Haider and Ellar 1989; Grochulski et al. 1995; Schnepf et al. 1998; Bravo et al. 2004; Rausell et al. 2004). Also, a relatively new mechanism of action of Cry toxins have been proposed which involves the activation of Mg^{2+} -dependent signal cascade pathway that is triggered by the interaction of the monomeric 3-domain Cry toxin with the primary receptor, the cadherin protein BT-R₁ (Zhang et al. 2005, 2006; Soberón et al. 2009). The triggering of the Mg^{2+} -dependent pathway has a knock-on effect and initiates a series of cytological events that include membrane blebbing, appearance of nuclear ghosts, and cell swelling followed by cell lysis (Zhang et al. 2006). The Mg^{2+} -dependent signal cascade pathway activation by Cry toxins have been shown to be analogous to similar effect imposed by other pore forming toxins on their host cells when they are applied at subnanomolar concentration (Parker and Pattus 1993; Nelson et al. 1999; Menzies and Kourteva 2000; Soberón et al. 2009; Porta et al. 2011).

Though the two mechanisms of action seem to differ, with series of downstream events following on from toxin binding to receptors on target cell membranes, there

is a degree of commonality in that initially the crystals have to be solubilised in vivo (Aronson et al. 1991; de Maagd et al. 2001; Soberón et al. 2009) or in vitro (Lambert et al. 1992; Bradley et al. 1995; Zhang et al. 2005, 2006) and activated by proteases before (Zhang et al. 2005, 2006) and/or after binding (Gómez et al. 2002; Bravo et al. 2004; Jiménez-Juárez et al. 2007; Soberón et al. 2009) to receptors such as cadherin. The midgut of lepidopteran and dipteran insects have been shown to be alkaline (Berebaum 1980; Gringorten et al. 1993) and this enhances the solubility of Cry toxins (Bravo et al. 2004; Soberón et al. 2009). Those of coleoptera are neutral or slightly acidic and in vitro solubilisation of Cry1Ba (Bradley et al. 1995) and Cry7Aa (Lambert et al. 1992) has been shown to enhance the activity of these toxins towards *Leptinotarsa decemlineata*.

With the pore forming model (Knowles and Ellar 1987; Haider and Ellar 1989; Grochulski et al. 1995; Schnepf et al. 1998; Bravo et al. 2004; Rausell et al. 2004), an ingested crystal toxin is solubilised in the alkaline environment of the insects midgut releasing protoxins which are initially processed by midgut proteases. The initial cleavage of a Cry1A protoxin by the gut proteases results in the removal of the C-terminal half and about 30 amino acid residues from the N-terminal thus releasing active toxin monomers which bind to receptors such as cadherin (Atsumi et al. 2008; Bel et al. 2009; Chen et al. 2009; Fabrick et al. 2009a; Muñoz-Garay et al. 2009; Obata et al. 2009; Pacheco et al. 2009a; Arenas et al. 2010) or proteins anchored to the membrane by GPI-anchored proteins such as aminopeptidase N (Arenas et al. 2010). The initial binding of the activated toxins to receptors is proposed to result in a conformational change which facilitates a second cleavage that removes the N-terminal helix α -1, by a membrane-bound protease. The removal of helix α -1 results in the formation of oligomers that are membrane insertion competent (Bravo et al. 2004). The binding of Cry toxins to the cadherin-like receptors have been shown to involve specific interactions of the variable loop regions in domain II and III with cadherin epitopes (Nair et al. 2008; Chen et al. 2009; Pacheco et al. 2009a; Soberón et al. 2009).

The oligomerised activated toxin that is bound to membrane receptors then inserts the central hydrophobic helix α -4 and 5 (Nair et al. 2008) into the apical membrane of midgut cells causing osmotic shock, bursting of the midgut cells and finally ending in the insect death (Knowles and Ellar 1987; Haider and Ellar 1989; Grochulski et al. 1995; Schnepf et al. 1998; Bravo et al. 2004; Rausell et al. 2004). The pore formation model as proposed by Bravo et al. (2004) for Cry1A toxins is presented in Fig. 2.3.

Cyt-toxins have also been shown to effect killing of its insect targets through un-specific binding to midgut membrane lipids followed by membrane insertion which leads to pore formation and insect death (Li et al. 1996; Cohen et al. 2008; Zhao et al. 2009; Rodriguez-Almazan et al. 2011).

The activation of Mg^{2+} -dependent signal cascade pathway that is triggered by the interaction of the monomeric 3-domain Cry toxin with the primary receptor, the cadherin protein (Zhang et al. 2005, 2006; Soberón et al. 2009) has been shown to trigger a pathway involving stimulation of the stimulatory G protein α -subunit and adenylyl cyclase (AC), increased cyclic adenosine monophosphate (cAMP) levels, and activation of protein kinase A (PKA). Activation of the AC/PKA signalling pathway initiates a series of cytological events that include membrane blebbing, appearance of nuclear ghosts, and cell swelling followed by cell lysis (Zhang et al. 2005, 2006).

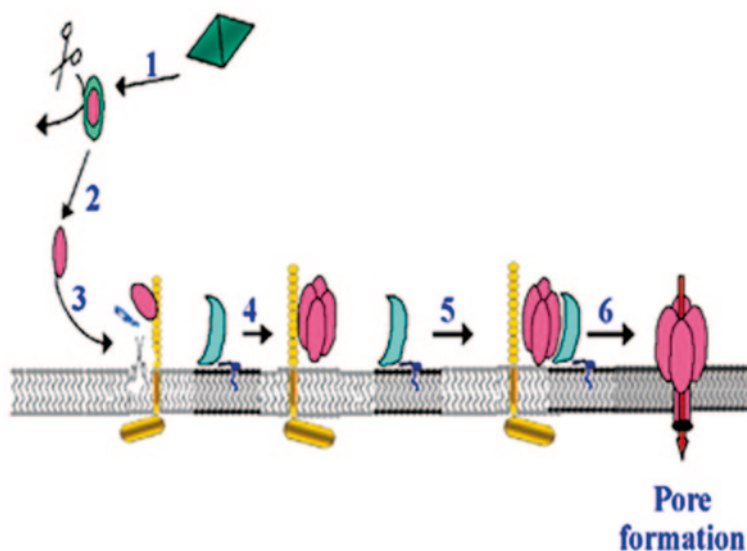


Fig. 2.3 Model of the mode of action of Cry1A toxins. 1 Crystal toxin solubilisation, 2 Initial cleavage by gut proteases, 3 Toxin monomer binding to receptors and second cleavage by membrane bound protease, 4 Membrane insertion-competent oligomer formation, 5 Binding of oligomeric toxin to receptors, 6 Lytic pore formation. (Bravo et al. 2004)

2.2 Methods of Application of *Bt* and Its Products in Agriculture

Bacillus thuringiensis and its products have been formulated into various forms for application as biological control agents. Such formulations could be solid (powdery or granulated) or liquid. Presently there are over 400 of *Bt* based formulations that have been registered in the market and most of them contain insecticidal proteins and viable spores though the spores are inactivated in some products (Ahmedani et al. 2008). Formulated *Bt* products are applied directly in the form of sprays (Ali et al. 2010). An alternative, and highly successful, method for delivering the toxins to the target insect has been to express the toxin-encoding genes in transgenic plants (Barton et al. 1987; Vaeck et al. 1987; Qaim and Zilberman 2003; Walter et al. 2010; Chen et al. 2011).

2.3 Advantages of Using *Bt* Products Over Chemical Agents in Agricultural Practices

With their specific insecticidal effect on insect pests in the orders coleoptera (beetles and weevils) (López-Pazos et al. 2010; Sharma et al. 2010), diptera (flies and mosquitoes) (Pérez et al. 2007; Roh et al. 2010), hymenoptera (bees and wasps)

(Garcia-Robles et al. 2001; Sharma et al. 2008) and lepidoptera (butterflies and moths) (Baig et al. 2010; Darsi et al. 2010) and to non-insect species such as nematodes (Cappello et al. 2006; Hu et al. 2010), *Bt* toxins have taken centre stage as the major biological control agent and widely preferred to chemical insecticides.

Various assessments have been carried out to check for the safety of *Bt* toxins from sprays or transgenic plants to non-target species in the environment and it has been shown to be mostly environmentally friendly without significant adverse effects (Kapur et al. 2010; Walter et al. 2010; Chen et al. 2011; Randhawa et al. 2011) though there has recently been a laboratory observation that seemed to implicate a commercial *Bt aizawai* strain in the reduction of reproduction in bumblebee (*Bombus terrestris*) workers when applied at a concentration of 0.1% through sugar water and pollen (Mommaerts et al. 2010).

The increased popularity of biological control agents over synthetic chemicals is because of the non-selective lethal effect of the latter agents (Moser and Obrycki 2009; Kristoff et al. 2010; Shah and Iqbal 2010; Eriksson and Wikteliuss 2011; Stevens et al. 2011) and the rapid development of resistance by insect pests to synthetic insecticides (Ahmad et al. 2008).

2.4 Threat to Continuous Use of *Bt* as Biological Control Agent

2.4.1 Development of Resistance and Cross Resistance

The continued relevance of *Bt* toxins in the control of insect and non-insect pests is threatened by the development of resistance by the pests in the field (Sayyed et al. 2004) and laboratory reared populations (Pereira et al. 2008; Fabrick et al. 2009b). There have been reports of insect populations resistant to a particular toxin showing resistance to other toxins to which they have not previously been exposed, a term known as ‘cross-resistance’ (Pereira et al. 2008; Sayyed et al. 2008; Gong et al. 2010; Xu et al. 2010).

There have been a number of proposed modes of resistance of insect pests to *Bt* toxins including reduction of binding of toxins to receptors in the midgut of insects, reduced solubilisation of protoxin, alteration of proteolytic processing of protoxins and toxin degradation and or precipitation by proteases (Bruce et al. 2007). The understanding of the mechanism of action of *Bt* toxins (Knowles and Ellar 1987; Haider and Ellar 1989; Grochulski et al. 1995; Schnepf et al. 1998; Bravo et al. 2004; Rausell et al. 2004) have enhanced the experimental verification of some of the modes.

The most studied and experimentally verified mode of resistance is ‘mode 1’ which is characterized by recessive inheritance, reduced binding by at least one Cry1A toxin, and negligible cross-resistance to Cry1C (Tabashnik et al. 1998; Heckel et al. 2007).

Alteration of protease profile in the midgut of Cry1Ac resistant *Helicoverpa armigera* affected the proteolytic processing of Cry1Ac resulting in the production of 95 and 68 kDa toxin as opposed to the active 65 kDa toxin produced by midgut protease from susceptible population (Rajagopal et al. 2009) suggesting a linkage between improper processing of *Bt* toxin and development of resistance. Sayyed et al. (2005) also demonstrated that a field collected resistant population of *Plutella xylostella* (SERD4) which was subsequently selected in the laboratory using Cry1Ab and named Cry1Ab-SEL was more sensitive to trypsin-activated Cry1Ab compared to Cry1Ab protoxins again suggesting a defect in processing although no such defect could be identified.

Brush border membrane vesicles from a laboratory selected population of *Ostrinia nubilalis* resistant to Cry1F were found binding the toxin as well as those from a susceptible population and furthermore no differences in activity of luminal gut proteases or proteolytic processing of the toxin were observed (Pereira et al. 2010). This failure to implicate defects in binding or toxin processing in the resistant strain indicates either alternative resistance mechanisms or limitations in the assays used.

2.4.2 *Narrow Spectrum of Activity*

Apart from resistance by pests being a major threat to the future of *Bt* products, the problem of efficacy and spectrum of activity (Regev et al. 1996; de Maagd et al. 2001) remain. In contrast to many synthetic insecticides most *Bt* toxins cloned have a narrow spectrum of activity (Kao et al. 2003; Shu et al. 2009). Only a small minority of toxins (such as Cry1Ba) show activity that spans two to three insect orders (Zhong et al. 2000).

2.5 **Strategies to Ensure Continuing Use of *Bt* and Its Products in Agriculture**

2.5.1 *Continuous Search for Bt Strains Expressing Toxins with Improved Activity*

The inability of many existing *Bacillus thuringiensis* toxins to overcome the resistance developed by insect species in field (Sayyed et al. 2004) and in laboratory reared populations (Pereira et al. 2008; Fabrick et al. 2009b) is problematic and efforts are continuing to search for *Bt* strains expressing novel toxins with improved activity.

Currently, about 600 insecticidal genes have been cloned from various *Bt* strains and their sequences deposited at the *Bt* toxin nomenclature website (http://www.lifesci.sussex.ac.uk/home/Neil_Crickmore/Bt/). Of these a large number have been

heterologously expressed and found to be either independently (Song et al. 2003; Gonzalez-Cabrera et al. 2006; Ibargutxi et al. 2008; Xue et al. 2008; Hu et al. 2010) or in combination (Sharma et al. 2010) toxic to specific insect species in one or more orders.

2.5.2 Use of Synergism Between *Bt* Products or Between *Bt* Products and Other Substances

Most of the toxins cloned have narrow spectrum of activity (Kao et al. 2003; Shu et al. 2009) while some expressed toxins like Cyt1Aa show a weak toxicity to mosquitoes on their own but show synergistic activity when combined with other toxins like Cry4Ba and Cry11Aa (Fernandez-Luna et al. 2010).

To boost the efficacy of *Bt* insecticidal toxins and overcome resistance posed by insect pests, the use of other proteins like cadherin fragments have been shown to be a successful strategy (Chen et al. 2007; Abdullah et al. 2009; Pacheco et al. 2009b; Park et al. 2009; Peng et al. 2010). The use of a toxic compound, gossypol derived from the cotton plant has also been used in combination with Cry1Ac to boost its efficacy against a resistant population of *Helicoverpa zea* (Anilkumar et al. 2009). Co-expression of chitinase, an enzyme that is known to disrupt chitin present in the peritrophic membrane in the midgut of insects, has been shown to have an enhanced effect on the efficacy of Cry1Ac against *Helicoverpa armigera* (Ding et al. 2008) and Cry1C against *Spodoptera littoralis* (Regev et al. 1996). Also combinations of Cry toxins have proven to be a very useful strategy employed in boosting efficacy and fighting resistance (Jurat-Fuentes et al. 2003; Kaur 2006; Avisar et al. 2009). The combination of Cry1Ac and Cry2Ab showed a synergistic effect to *Helicoverpa armigera* (Ibargutxi et al. 2008).

It has also been shown that a mixture of crystal protein and spores from the same strain can result in a synergistic insecticidal activity (Johnson and McGaughey 1996; Tang et al. 1996; Johnson et al. 1998).

2.5.3 Genetic Manipulation of Existing *Bt* Strains and Its Toxin Genes for Improved Activity

With insights gained into the structure of Cry toxins (Li et al. 1991; Grochulski et al. 1995; Galitsky et al. 2001; Morse et al. 2001; Boonserm et al. 2005, 2006) and their mechanism of action (Zhang et al. 2005, 2006; Bravo et al. 2007; Gómez et al. 2007; Pacheco et al. 2009a) molecular genetics has been employed in an attempt to alter or broaden the activity of a given toxin. Herrero et al. (2004) demonstrated that replacing single residues in loops 2 and 3 of domain II with and residues 541–544 in

domain III of Cry1Ca with alanine resulted in lower toxicity to *Spodoptera exigua* while its toxicity to *Manduca sexta* was not affected. Swapping domain III of three toxins with little or no activity against *Spodoptera exigua* (Cry1Ac, Cry1Ba and Cry1Ea) with domain III of Cry1Ca resulted in an improved toxicity towards this pest (de Maagd et al. 2000).

Abdullah et al. (2003) remodeled domain II loops of Cry4Ba to resemble that of Cry4Aa and generated mutants that showed improved toxicity to *Culex quinquefasciatus* and *Culex pipiens* of >700-fold and >285-fold respectively. In a similar study, Cry19Aa, a mosquitocidal toxin with specificity toward *Anopheles stephensi* and *Culex pipiens* but with no measurable activity against *Aedes aegypti*, was made more than 42,000-fold more toxic to *Aedes aegypti* by engineering domain II loops 1 and 2 to resemble that of Cry4Ba (Abdullah and Dean 2004). Also, Liu and Dean (2006) introduced mosquitocidal activity to Cry1Aa through rational design to the sequence of loops 1 and 2 based on a sequence alignment with Cry4Ba, a naturally occurring mosquitocidal toxin.

Replacement of domain III of Cry1Ba with Cry1Ac resulted in an improved activity to *Heliothis virescens* (Karlova et al. 2005). Naimov et al. (2001) created a hybrid protein by replacing domain II of Cry1Ba with that of Cry1I which resulted in activity against *Leptinotarsa decemlineata* that was comparable to that of Cry3Aa.

Site directed mutagenesis has great potential to alter toxin-encoding genes particularly when sufficient structural information is available to inform the choice of mutations (Cammack et al. 2006; Fleming et al. 2010; Moustafa et al. 2010; de Maagd et al. 2001, 2003). The understanding of the domain structure and function of Cry1Ac enhanced the use of site directed mutagenesis by Kim et al. (2008) to effect changes to domain I and II that resulted in mutants that showed improve activity to *Ostrinia furnacalis* and *Plutella xylostella*. In another example a triple mutation (N372A, A282G and L283S) in domain II loop of Cry1Ab resulted in a 36-fold increase in toxicity to *Lymantria dispar* and this correlated with an increased binding affinity of greater than 18-fold to brush border membrane vesicles which also resulted in higher toxin concentration at the binding site (Rajamohan et al. 1996).

Natural evolutionary trends have been used in analysing the divergence and host specificity in Cry toxins (de Maagd et al. 2001) and biotechnological techniques like gene shuffling has been used in artificially directing the evolution of new genes with novel characteristics (Stemmer 1994a, b; Zhao and Arnold 1997; Lassner and Bedbrook 2001; Craveiro et al. 2010). Craveiro et al. used DNA shuffling technique to produce four variants of Cry11A12synth and Cry11A12 that, unlike the parent toxins, had toxicity against *Telchin licus licus*. Shan et al. (2011) used error-prone PCR and staggered extension process (StEP) shuffling combined with Red/ET homologous recombination to investigate the insecticidal activity of Cry1Ac and isolated a toxin variant designated as T524N which has increased insecticidal activity against *Spodoptera exigua* larvae while its original insecticidal activity against *Helicoverpa armigera* larvae was retained.

The understanding of the regions that are bound to receptors and how protoxins are processed to a functional form has led to creation of manipulated toxins that mimic the in vivo processing of toxins (Pardo-López et al. 2009). Bravo et al. (2004) demonstrated that the processing of a protoxin to an active toxin in the midgut of a susceptible insect involves the initial cleavage of the protoxin by soluble proteases followed by a second cleavage by membrane bound proteases that removes helix α -1 while the toxin is bound to its receptor. Deletion of the amino-terminal region including helix α -1 of Cry1A toxins resulted in variants that formed oligomers in the absence of cadherin receptor and which killed insects that had developed resistance to Cry1A toxins through mutations in the cadherin gene. The modified toxins were also effective against insects which had acquired reduced susceptibility to native *Bt* toxins due to diminished expression of cadherin protein by cadherin gene silencing through RNA interference (Soberón et al. 2007). Mandal et al. (2007) demonstrated that in vitro truncation of Cry2Aa at the N-terminal of 42-amino acid residues resulted in an improved toxicity against *Spodoptera littoralis* and *Agrotis ipsilon* which was consistent with an observation made by Morse et al. (2001) that the structure of the Cry2Aa protoxin revealed a 49-amino acid N-terminal section preceding helix α -1 that was cleaved in vivo to generate an active toxin and which masked other parts of the toxin believed to interact with the surface of the target cell.

Apart from truncation of the N-terminal residues of domain I to generate toxins with improved activity, Wu et al. (2000) also created mutants (R345A, Δ Y350, Δ Y351) that involved deletions and specific mutations in loop I of Cry3A domain II which resulted in improved activity against *Tenebrio molitor*.

2.5.4 Use of Gene Stacking

This approach involves the expression of two or more *Bt* toxins with differing spectrum of activities and/or mechanism of action in transgenic plants to control insect pests which has the advantage of controlling pests from many orders as opposed to the narrow spectrum that a single toxin can control. It also has the advantage of reducing development of resistance because if the toxins used are such that there is little potential for cross-resistance between them, therefore there has to be resistance alleles at independent loci before an insect can develop resistance to the stacked toxins, which is a rare event (Gould 1998).

The effectiveness of this method relies on the fact that development of resistance by insect to the stacked toxins would be through similar mechanism e.g. reduced toxin binding and therefore if stacked toxins have different binding sites, it would be difficult for an insect to develop resistance to all the stacked toxins. This assumption has been challenged though as two toxins, Cry1Ac and Cry2Aa that are believed to have different binding sites in *Heliothis virescens* (tobacco budworm) have been shown to have cross-resistance (Jurat-Fuentes et al. 2003) which provokes a rethink on advantages of gene stacking.

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