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## ***Bacillus thuringiensis*: A story of a successful bioinsecticide**

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### **Abstract**

*Bacillus thuringiensis* (Bt) bacteria are insect pathogens that rely on insecticidal pore forming proteins known as Cry and Cyt toxins to kill their insect larval hosts. At least four different non-structurally related families of proteins form the Cry toxin group of toxins. The expression of certain Cry toxins in transgenic crops has contributed to an efficient control of insect pests resulting in a significant reduction in chemical insecticide use. The mode of action of the three domain Cry toxin family involves sequential interaction of these toxins with several insect midgut proteins facilitating the formation of a pre-pore oligomer structure and subsequent membrane insertion that leads to the killing of midgut insect cells by osmotic shock. In this manuscript we review recent progress in understanding the mode of action of this family of proteins in lepidopteran, dipteran and coleopteran insects. Interestingly, similar Cry-binding proteins have been identified in the three insect orders, as cadherin, aminopeptidase-N and alkaline phosphatase suggesting a conserved mode of action. Also, recent data on insect responses to Cry toxin attack is discussed. Finally, we review the different Bt based products, including transgenic crops, that are currently used in agriculture.

### **Keywords**

*Bacillus thuringiensis*; Cry toxins; Mode of action; Pore formation; Bt crops

## **1. Introduction**

Control of insect pests in agriculture and of insect vectors of important human diseases is mainly achieved using chemical insecticides. However, the use of these chemical pesticides has led to several problems, including environmental pollution and increase in human health effects, such as cancer and several immune system disorders. The selection of insect resistant populations has also caused significant and major outbreaks of secondary pests (Devine and Furlong, 2007). Although microbial insecticide have been proposed as substitutes for chemicals their use is limited since most microbes show a narrow spectrum of activity that enables them to kill only certain insect species. Moreover, they have low environmental persistence and they require precise application practices, since many of these pathogens are specific to young insect larval stages or are sensitive to irradiation.

The most successful insect pathogen used for insect control is the bacterium *Bacillus thuringiensis* (Bt), which presently is ~2% of the total insecticidal market. Bt is almost

exclusively active against larval stages of different insect orders and kills the insect by disruption of the midgut tissue followed by septicemia caused probably not only by Bt but probably also by other bacterial species (Raymond et al., 2010). Bt action relies on insecticidal toxins that are active during the pathogenic process but these bacteria also produce an array of virulence factors that contribute to insect killing (reviewed in Bravo et al., 2005). Upon sporulation, Bt produces insecticidal crystal inclusions that are formed by a variety of insecticidal proteins called Cry or Cyt toxins. These toxins show a highly selective spectrum of activity killing a narrow range of insect species. The Cry and Cyt toxins belong to a class of bacterial toxins known as pore forming toxins (PFT) that are secreted as water-soluble proteins that undergo conformational changes in order to insert into the membrane of their hosts. Despite the limited use of Bt products as sprayable insecticides, Cry toxins have been introduced into transgenic crops providing a more targeted and effective way to control insect pests in agriculture. Concomitantly, this approach has resulted in significant reduction in the use of chemical insecticides in places where this technology has been embraced (James, 2009).

The mode of action of Cry toxins has mostly been studied in lepidopteran insects and has been reviewed recently (Bravo et al., 2005, 2007). Also, the identification of insect midgut proteins that bind Cry toxins and mediate toxicity and insect specificity has also been previously reviewed (Pigott and Ellar, 2007). In this review we will summarize recent work regarding the mode of action of Cry toxins in different insect orders, the identification of new Cry toxin insect binding proteins and the binding of Cry toxins to insect midgut proteins depending on the oligomeric state of the toxin. Finally we discuss recent genetic studies of mechanisms of resistance to Cry toxins and their most important applications.

## 2. Cry toxins: a diverse and large family of insecticidal proteins

Cry toxins are classified by their primary amino acid sequence and more than 500 different *cry* gene sequences have been classified into 67 groups (Cry1–Cry67) (Crickmore et al., 2010). These *cry* gene sequences have been divided into at least four phylogenetically non-related protein families that may have different modes of action: the family of three domain Cry toxins (3D), the family of mosquitocidal Cry toxins (Mtx), the family of the binary-like (Bin) and the Cyt family of toxins (reviewed in Bravo et al., 2005). Also, some Bt strains produce additional insecticidal toxins named VIP. VIP toxins, in contrast to Cry, are produced during the vegetative growth phase. At least three VIP toxins have been characterized, VIP1/VIP2, a binary toxin, and VIP3 (Estruch et al., 1996; Warren, 1997).

The largest Cry family is the 3D-Cry group that is formed by at least 40 different groups with more than 200 different gene sequences. The three dimensional structure of seven different 3D-Cry toxins have been solved, Cry1Aa, Cry2Aa, Cry3Aa, Cry3Ba, Cry4Aa, Cry4Ba and Cry8Ea (Li et al., 1991; Grochulski et al., 1995; Morse et al., 2001; Galitsky et al., 2001; Boonserm et al., 2005, 2006; Guo et al., 2009). Fig. 1 shows the three-dimensional structure of Cry8Ea that was the most recent structure released in the PDB database and has high similarity at the structural level with the other 3D-Cry that have been crystallized before (Guo et al., 2009). Domain I, a seven  $\alpha$ -helix bundle, is implicated in membrane insertion, toxin oligomerization and pore formation. Domain II is a beta-prism of three anti-parallel  $\beta$ -sheets packed around a hydrophobic core with exposed loop regions that are involved in receptor recognition, and domain III, is a  $\beta$ -sandwich of two anti-parallel  $\beta$ -sheets. Both domain II and III are implicated in insect specificity by mediating specific interactions with different insect gut proteins (reviewed in Bravo et al., 2007). The three dimensional structure is conserved among members of the 3D-Cry family suggesting proteins from this family may share a similar mechanism of action even though they show very low amino acid sequence similarity. In Fig. 1 the three dimensional structure of Cyt2Ba

toxin shows a single domain of two outer layers of  $\alpha$ -helix hairpins wrapped around a  $\beta$ -sheet that is also highly similar to Cyt2Aa toxin that was previously crystallized (Cohen et al., 2008).

Phylogenetic analysis of the 3D-Cry toxin family revealed that Cry toxin variability evolved by two main processes; the independent evolution of the three functional domains and by domain III swapping among different toxins (Bravo, 1997; de Maagd et al., 2001). Fig. 2 shows several natural examples of domain III swapping, revealed by different toxins that share a domain III with highly similar amino acid sequence as Cry8Ca and Cry1Jc; Cry1Bd and Cry1Ac; Cry1Cb, Cry1Eb and Cry1Be; Cry8Aa, Cry1Jb, Cry1Ba and Cry9Da etc. Domains II and III are involved in binding of Cry toxins to insect midgut proteins, thus domain III swapping could have led to the selection of proteins with different insect specificities. In addition, *in vitro* construction of hybrid Cry proteins by interchanging domain III among different Cry toxins has been reported. An example of such *in vitro* novel-Cry constructions is the Cry1Ab hybrid toxin that contains the domain III of Cry1C toxin (1Ab-1Ab-1C). This hybrid toxin showed ten times increased insecticidal activity against *Spodoptera exigua* larvae than either of the parental proteins (de Maagd et al., 2000). More recently, a hybrid toxin containing domains I and II from Cry3Aa and domain III from Cry1Ab was shown to be toxic to *Diabrotica virgifera virgifera* in contrast to Cry3Aa and Cry1Ab that showed no toxicity to this insect (Walters et al., 2010).

### 3. How Cry toxins kill their hosts

#### 3.1. The case of lepidopteran insects

One of the most interesting features of Cry toxins is their insect specificity. Insect specificity is largely determined by the specific binding of Cry toxins to surface proteins located in the microvilli of larvae midgut cells. In the case of lepidopteran insects, Cry1 binding proteins have been identified as cadherin-like proteins, glycosylphosphatidylinositol (GPI)-anchored aminopeptidase-N (APN), GPI-anchored alkaline phosphatase (ALP) a 270 kDa glycoconjugate and a 250 kDa protein named P252 (reviewed in Pigott and Ellar, 2007). In addition, glycolipids were proposed to act as Cry toxin receptors in lepidopteran insects as was demonstrated for the nematode *Caenorhabditis elegans* (Garczynski and Adang, 2000; Griffiths et al., 2005). Cry1A toxins binds to cadherin proteins of at least six lepidopteran species, *Manduca sexta*, *Bombyx mori*, *Heliothis virescens*, *Helicoverpa armigera*, *Pectinophora gossypiella* and *Ostrinia nubilalis* (reviewed in Pigott and Ellar, 2007). Insect cadherins that bind Cry toxins belong to a subset of the family of cadherin proteins and are composed of an ectodomain formed by 11 to 12 cadherin repeats (CR), a transmembrane domain and an intracellular domain (Bel and Escriche, 2006). In the case of APN, proteins belonging to at least five different subfamilies from *B. mori*, *H. armigera*, *H. virescens*, *Lymantria dispar*, *M. sexta* and *Plutella xylostella* have been found to bind Cry1 toxins (reviewed in Pigott and Ellar, 2007). ALP has been characterized as Cry1A binding protein in *H. virescens* and *M. sexta* (reviewed in Pigott and Ellar, 2007). The 270 kDa glycoconjugate was identified as Cry1Ac binding protein in *L. dispar* (reviewed in Pigott and Ellar, 2007). Recently *B. mori* P252 that binds Cry1Ac was identified as a chorophyllide-binding protein (Pandian et al., 2008). Table 1 shows the receptor molecules of Cry toxins identified in lepidopteran insects. Identification of Cry1Ac binding proteins by a proteomic approach after separation in 2D SDS-PAGE gels of brush border proteins from *M. sexta* and *H. virescens*, revealed that Cry1Ac also binds to V-ATPase subunit A and actin indicating that the mode of action of Cry toxins may involve binding of the toxin with other components of the midgut cells but their role in the mechanism of action remains to be analyzed (McNall and Adang, 2003; Krishnamoorthy et al., 2007).

Although the binding kinetic parameters of the interaction of Cry1 proteins with the cadherin, APN and ALP binding proteins have not been fully analyzed, it is generally accepted that Cry1 toxins bind cadherin proteins with high affinity at the nM range; for instance Cry1Ab binds *M. sexta* cadherin with a 1 nM apparent binding affinity. In contrast, APN and ALP show lower binding affinities that are in the range of more than 100 nM binding affinity (reviewed in Pigott and Ellar, 2007).

The mode of action of Cry1Ab toxin has been particularly well defined in *M. sexta*. The 3D-Cry toxins such as Cry1A are produced as protoxins that are dissolved and processed proteolytically by insect proteases releasing the active toxic fragment composed of the three domain structure as described in Fig. 1. Two groups of protoxins have been reported, large protoxins such as Cry1Aa of 130 kDa and short protoxins of 70 kDa such as Cry2Aa. Large protoxins lose half of the C-terminal end and 20 to 50 amino acids of the N-terminal end by proteolytical cleavages while short protoxins are processed primarily at the N-terminal end. The activated toxin goes through complex sequential binding events with the different insect gut Cry-binding proteins leading to membrane insertion and pore formation. The first binding interaction of the activated Cry1Ab toxin occurs through exposed amino acid regions of domain II (specifically through loop 3) and domain III (through strand  $\beta$ -16) of the toxin with *M. sexta* ALP and APN that are highly abundant low affinity binding sites for the toxin (Gómez et al., 2006; Pacheco et al., 2009b). These binding interactions concentrate the activated toxin in the microvilli membrane of the midgut cells, where the toxin then binds with high affinity through exposed domain II loops to the cadherin receptor including loops  $\alpha$ -8, 2 and particularly loop 3 in *M. sexta*, *H. virescens* and *B. mori* (Xie et al., 2005; Gómez et al., 2006; Atsumi et al., 2008). This interaction with cadherin receptor facilitates further proteolytic cleavage of the N-terminal end including helix  $\alpha$ -1 of domain I that induces the formation of a toxin pre-pore oligomer (Gómez et al., 2002; Atsumi et al., 2008). The oligomeric structure of the toxin showed an important increase of 200 fold in its affinity to GPI anchored receptors, ALP and APN, involving domain II loop 2 region (Arenas et al., 2010). The binding of the pre-pore to the GPI-anchored proteins leads finally to the insertion into the membrane causing pore-formation and cell lysis (Pardo et al., 2006). Recent data showed that formation of oligomers by Cry1Ab toxin is an essential step in the mode of action of this toxin. First,  $\alpha$ -helix 3 of domain I was identified as a potential oligomerization region and point mutations in some residues of  $\alpha$ -helix 3 resulted in complete loss of toxicity to *M. sexta*. It was shown that these non-toxic mutants were affected in oligomer formation indicating that oligomer formation is essential for toxicity (Jimenez-Juarez et al., 2007). In addition, characterization of domain I  $\alpha$ -helix 4 mutants revealed that these mutants were also severely affected in toxicity against *M. sexta* but, in contrast to  $\alpha$ -helix 3 mutants described above, the point mutations in  $\alpha$ -helix 4 were able to form oligomeric structures that were affected in membrane insertion (Rodríguez-Almazan et al., 2009). Interestingly, the non toxic  $\alpha$ -helix 4 mutants showed a dominant negative phenotype since they were capable of inhibiting Cry1Ab toxicity, membrane insertion and pore formation when mixed in low protein:protein ratios. The isolation of dominant negative phenotype indicates that the non-toxic  $\alpha$ -helix 4 mutants were capable of forming hetero-oligomers with the wild type Cry1Ab toxin, inhibiting its insertion into the membrane (Rodríguez-Almazan et al., 2009). Also, it was shown that a *M. sexta* cadherin fragment containing a Cry1Ab binding site enhanced Cry1Ab toxicity when fed to the larvae along with the Cry1Ab protein (Chen et al., 2007). The enhancement in toxicity was later shown to correlate with Cry1Ab oligomer formation (Pacheco et al., 2009a). In the pore formation model of Cry toxin action, binding to cadherin facilitates the proteolytic removal of domain I  $\alpha$ -helix 1 promoting oligomer formation. It was shown that genetically engineered Cry1Ab and Cry1Ac modified toxins (Cry1AbMod and Cry1AcMod) that were deleted at N-terminal region including domain I  $\alpha$ -helix 1, were able to kill the *P. gossypiella* populations resistant to Cry1Ac toxin due to mutations in the cadherin gene (Soberón et al., 2007). These data

show that even in the absence of cadherin protein the Cry1AMod toxins are active against resistant larvae and that the primary role of cadherin binding is the removal of  $\alpha$ -helix 1 promoting oligomer formation (Soberón et al., 2007). Fig. 3 shows the molecular events that lead to Cry toxin membrane insertion and pore formation. As seen in Fig. 3, two GPI anchored receptors are involved in Cry1Ab toxicity to *M. sexta*. However, ALP seems to play a more important role in toxicity than APN since ALP is produced at higher levels at the first larval instars with low levels of expression in the fourth and fifth larval instars, while APN shows the opposite, low expression levels in the first larval instars and increased expression at the third until the fifth larval instars (Arenas et al., 2010). The expression profile of ALP correlates with the sensitivity of *M. sexta* larvae to Cry1Ab toxin since younger larvae are more sensitive to the toxin.

The Cry1Ab pre-pore oligomer was proposed to be a tetrameric structure based on the apparent molecular size of the oligomeric structure after SDS-PAGE electrophoresis (Gómez et al., 2002). Nevertheless, two dimensional array images of membrane inserted Cry4Ba and later of Cry1AbMod toxins revealed a trimeric organization (Ounjai et al., 2007; Muñoz-Garay et al., 2009). Interestingly, the three dimensional structure of Cry4Ba, obtained from a Cry4Ba truncated form that lacked domain I  $\alpha$ -helices 1 and 2A, revealed a trimeric structure with a contact interface involving domain I  $\alpha$ -helices 3, 4 and 6 (Boonserm et al., 2005). This structure agrees with the phenotype of  $\alpha$ -helix 3 mutants that were affected in oligomer formation (Jiménez-Juárez et al., 2007). Recently, a structural model of Cry4Aa pre-pore trimer was reported and the authors proposed that the mechanism of membrane insertion may involve the insertion of three domain I  $\alpha$ -helix 4-helix 5 hairpins to form a stable transmembrane pore (Taveecharoenkool et al., 2010).

As mentioned previously there is a significant consensus that Bt Cry toxins are pore forming proteins that cause cell lysis by producing an osmotic shock. Nevertheless, an alternative model of the mode of action of Cry toxins was proposed based on data obtained with transfected H5 *Tricoplusia ni* cultured cells expressing the *M. sexta* cadherin gene (Zhang et al., 2006). This model proposes that insect cell death is triggered by the binding of monomeric Cry1Ab toxin to cadherin receptor resulting in increased cAMP cellular levels by activation of adenylyl cyclase. Then, cAMP activates protein kinase-A resulting in cell death related to oncosis (Zhang et al., 2006). In this signal transduction model, neither GPI-anchored receptors nor oligomer formation are involved in Cry toxicity (Zhang et al., 2006).

### 3.2. The case of dipteran insects

There is an increasing interest in determining the mode of action of Cry toxins in mosquitoes since these insects are important vectors of human diseases such as dengue, yellow fever and malaria among others. Interestingly there are multiple Cry toxins with low primary sequence similarities that show toxicity against mosquitoes like, Cry1, Cry2, Cry4, Cry11, Cry29 etc. However, one particular Bt strain has been used worldwide for the control of mosquitoes, *Bt* var *israelensis* (Bti). Bti produces crystal inclusions formed principally by Cry4Aa, Cry4Ba, Cry11Aa and Cyt1Aa (Margalith and Ben-Dov, 2000). Bti shows high toxicity to *Aedes aegypti* vector of dengue and yellow fevers and *Culex* sp. species but moderate toxicity to *Anopheles gambiae*, a vector of malaria (Margalith and Ben-Dov, 2000). One interesting feature of these four Bti toxins is that they show a synergistic effect. The toxicity of the whole Bti crystal inclusion is much higher than the sum of the individual toxicities of each one of the Cry and Cyt proteins in this crystal (reviewed in Bravo et al., 2005). Cyt1Aa was shown to synergize the activity of the three Cry toxins and to overcome resistance of the *Culex* sp. populations to the Cry toxins in *Culex* sp. (Khasdan et al., 2001; Wirth et al., 1997).

Receptor identification of mosquitocidal proteins has been performed primarily in *Ae. aegypti*, *An. gambiae*, *An. quadrimaculatus* and *An. albimanus*. As in lepidopteran insects, cadherin proteins have been identified in *Ae. aegypti* and *An. gambiae* showing binding to Cry11Aa and Cry4Ba respectively (Hua et al., 2008; Chen et al., 2009b). In *Ae. aegypti*, cadherin also serves a receptor of Cry11Ba toxin that was isolated from the Bt var *jegathesan* strain but showed lower affinity to Cry4Ba protein (Chen et al., 2009b; Likitvivatanavong et al., 2010). An *An. gambiae* cadherin fragment containing the Cry4Ba binding site enhanced the toxicity of Cry4Ba in both *An. gambiae* and *Ae. aegypti* larvae suggesting its active role as a receptor of Cry4Ba in these mosquitoes species (Hua et al., 2008; Park et al., 2009a). In the case of *Ae. aegypti* cadherin, it was shown that an anti-cadherin antibody competed binding of Cry11Aa to *Ae. aegypti* BBMV (Chen et al., 2009b). In both *Ae. aegypti* and *An. gambiae*, cadherin is located in the microvilli of the caeca and in the microvilli of the posterior gut cells, that are the same sites where Cry11Aa and Cry4Ba bind (Hua et al., 2008; Chen et al., 2009b). Regarding GPI anchored proteins, both APN and ALP have been identified in *An. gambiae*, *An. quadrimaculatus* and *Ae. aegypti* as Cry4Ba, Cry11Aa and Cry11Ba binding proteins (Fernández et al., 2006; Abdullah et al., 2006; Zhang et al., 2008; Hua et al., 2009; Chen et al., 2009a; Likitvivatanavong et al., 2010). Interestingly, a GPI-anchored  $\alpha$ -glucosidase was identified as a Cry4Ba binding molecule in *A. albimanus* larvae (Fernández-Luna et al., 2010). The *Ae. aegypti* ALP is involved in Cry11Aa toxicity since a peptide-phage (P1-BBMV) that bound ALP, competed the binding and toxicity of Cry11Aa (Fernández et al., 2006). The ALP1 isoform was identified as the Cry11Aa receptor and two ALP Cry11Aa binding sites in this receptor were shown to bind domain III<sup>561</sup>RVQSQNSGNN<sup>570</sup> and domain II loop  $\alpha$ -8 regions of Cry11Aa toxin (Fernández et al., 2009). Previous work identified Cry11Aa loop  $\alpha$ -8 as an important toxin region involved in Cry11Aa binding to *Ae. aegypti* BBMV and toxicity and more recently with the cadherin receptor (Fernández et al., 2005; Chen et al., 2009b). ALP1 was later shown to bind Cry4Ba and Cry11Ba (Fernández et al., 2009; Likitvivatanavong et al., 2010). In *A. gambiae* an ALP was also identified as Cry11Ba binding protein (Hua et al., 2009).

Two APN isoforms (AaeAPN1 and AaeAPN2) were identified in *Ae. aegypti* by Cry11Aa pull down experiments (Chen et al., 2009a). Protein fragments from both APN isoforms were produced in *E. coli* and shown to inhibit binding of Cry11Aa to BBMV, suggesting their active role in Cry11Aa binding to insect membranes (Chen et al., 2009a). In the case of *An. gambiae* and *An. quadrimaculatus* larvae, two APN's were also identified as Cry11Ba binding proteins (Abdullah et al., 2006; Zhang et al., 2008). Interestingly, Cry11Ba binds both *An. quadrimaculatus* and *An. gambiae* APN molecules with a very high binding affinity of 0.56 nM and 6.4 nM respectively (Abdullah et al., 2006; Zhang et al., 2008). These results suggest that APN may have a more important role in the toxicity of Cry11Ba in these two Anopheline species. In fact, it was recently shown that certain *A. gambiae* APN protein fragments enhanced Cry11Ba toxicity as has been shown for cadherin protein fragments (Zhang et al., 2010).

Table 1 shows the Cry toxin receptors identified in different mosquito species. The fact that similar Cry binding proteins are involved in the mechanism of action of Cry toxins in both lepidopteran and dipteran insects suggests the Cry toxins have a conserved mode of action. However, the precise role of the Cry toxins receptors identified in mosquitoes in the mode of action of Cry toxins still remains to be determined. As in lepidopteran insects cadherin binding might facilitate oligomer formation while binding of Cry oligomer to GPI-anchored ALP or APN receptors might be necessary to facilitate membrane insertion. Nevertheless, the high binding affinity of Anopheline APN's to Cry11Ba is substantially different from what has been reported in lepidopteran insects and further studies on the differential role of

APN and cadherin in monomer/oligomer binding in mosquitoes are necessary to determine the precise role of toxin binding to these receptor molecules.

The analysis of Cry4Ba binding proteins by mass spectrometry in *Ae. aegypti* BBMV, revealed two lipid rafts associated proteins, flotillin and prohibitin, as well as cytoplasmic actin, besides ALP and APN, thus suggesting that additional proteins as well as intracellular proteins may have an active role in the mode of action of Cry toxins in mosquitoes (Bayyareddy et al., 2009).

One of the most interesting features of Bti toxins is the synergistic effect of Cyt1Aa on Cry4Aa, Cry4Ba and Cry11Aa toxins activity. Cry4Ba and Cry11Aa bind Cyt1Aa through domain II loop regions that are involved in receptor interaction (Pérez et al., 2005; Cantón et al., 2011). Moreover single point mutations in the Cyt1Aa binding epitopes involved in the binding interaction with Cry4Ba and Cry11Aa toxins, showed a correlation between Cyt1Aa binding to these toxins and synergism (Pérez et al., 2005; Cantón et al., 2011). These results suggest that Cyt1Aa functions as a surrogate receptor for Cry4Ba and Cry11Aa. In the case of Cry11Aa, it was shown that its binding to Cyt1Aa facilitates the formation of a 250 kDa oligomeric structure of Cry11Aa, which is competent in pore formation suggesting that Cyt1Aa fulfills at least the role of a cadherin receptor regarding oligomer formation (Pérez et al., 2007).

### 3.3. The case of coleopteran insects

In the case of Cry toxins active against coleopteran insects, Cry binding proteins have been identified in *Tenebrio molitor*, *Diabrotica virgifera virgifera*, *Leptinotarsa decemlineata* and *Anthonomus grandis* (Fabrick et al., 2009; Park et al., 2009b; Ochoa-Campuzano et al., 2007; Martins et al., 2010). A cadherin protein from *T. molitor* was identified as a Cry3Aa binding protein, and it was shown to facilitate Cry3Aa oligomer formation. Moreover, silencing of the cadherin gene by feeding dsRNA showed that the silenced beetles were resistant to Cry3Aa indicating an active role of cadherin on Cry3Aa toxicity (Fabrick et al., 2009). A cadherin protein was also identified as a Cry3Aa receptor in *Diabrotica virgifera virgifera*. A fragment of this cadherin protein containing the membrane proximal cadherin repeats 8–10 bound Cry3Aa and Cry3Bb toxins with high affinity (Kd of 12 and 1.4 nM, respectively) and enhanced Cry3Aa and Cry3Bb toxicity to different coleopteran insects (Park et al., 2009b). In the case of *L. decemlineata*, an ADAM 3 metalloprotease was identified as Cry3Aa receptor. Binding of Cry3Aa to ADAM-3 through domain II loop 1 enhanced Cry3Aa pore-formation activity suggesting that this binding interaction is important for Cry3Aa toxicity (Ochoa-Campuzano et al., 2007). The only GPI-anchored protein identified in coleopteran insects as a putative Cry receptor was an ALP from *A. grandis* that bound Cry1B toxin (Martins et al., 2010). Table 1 shows the Cry toxin receptors identified in different coleopteran pests.

Overall, the identification of similar Cry binding proteins in the three different insect orders and the fact that Cry toxins share a similar three domain fold, suggests that the mode of action of Cry toxins is conserved in different insect orders.

## 4. Opposing the attack of Cry toxins

The identification of cellular components involved in a defense response to Cry toxins could provide tools for enhancing Cry toxicity against certain insects. The most important contributions on the identification of these cellular responses were achieved in *C. elegans* that is sensitive to Cry5B and Cry21 toxins. Nevertheless, it should be pointed out that although Cry5B and Cry21 are members of the 3D Cry toxin family, it has been shown that Cry5Ba is internalized into the host cell cytoplasm (Griffitts et al., 2003) and no pore

formation activity of these toxins has been documented until today indicating that probably the mode of action of Cry toxins in this organism may be different from that of Cry toxins in insects. A microarray analysis of *C. elegans* gene expression in the presence of Cry5B toxin, revealed the mRNA up-regulation of MAPK p38 (PMK-1), SEK-1 (a MAPKK immediately upstream of p38) and JNK kinases (Huffman et al., 2004). JNK and p38 MAPK pathways are mainly associated to stress-associated stimuli, and they are collectively termed stress-activated protein kinases.

The relevance of SEK-1 and PMK-1 in the defense pathway was demonstrated by feeding *C. elegans* *sek-1* or *pmk-1* mutants animals with Cry5B showing a hypersensitive response, indicating that SEK-1 and PMK-1 kinases participates in the protection of nematodes against Cry5B toxin action (Huffman et al., 2004).

To identify downstream targets of the p38 pathway that are specifically activated in response to Cry5B, differences in transcript levels *C. elegans* wild type or p38 silenced animals in the presence of Cry5B revealed two p38 dependent transcripts named *ttm-1* and *ttm-2* (Huffman et al., 2004). The role of these proteins in Cry5B defense was determined by analyzing silenced animals using dsRNA and again they were hypersensitivity to Cry5B intoxication (Huffman et al., 2004). The *ttm-1* gene shares homology with the human zinc transporter ZnT-3, suggesting a possible role in removing cytotoxic cations (Huffman et al., 2004). Additional genes identified as targets of p38 pathway after Cry5B action in *C. elegans* showed that the stress response to the unfolded proteins of the endoplasmic reticulum (UPR) resulted in the hypersensitive phenotype to Cry5B exposure (Bischof et al., 2008). Recently, a whole genome approach in the nematode led to the identification of hypoxia response and the signal transduction ERK pathway as additional responses to Cry5B intoxication (Bellier et al., 2009; Chen Cha et al., 2010).

In the case of insects, the role of p38 pathway on Cry toxin defense was reported in the lepidopteran *M. sexta* and the dipteran *Ae. aegypti* (Cancino-Rodezno et al., 2010). Treatment of *M. sexta* or *Ae. aegypti* larvae with a medium lethal concentration dose of Cry1Ab or Cry11Aa, respectively, resulted in a fast activation of p38 by phosphorylation. The activation of p38 was not observed when the insect larvae were treated with non-toxic Cry1Ab- or Cry11Aa-mutants affected in pore formation, indicating that p38 phosphorylation is triggered after pore formation by Cry toxin and not by the interaction with receptor proteins (Cancino-Rodezno et al., 2010). Finally, silencing of p38 protein by feeding dsRNA in *M. sexta* and *Ae. aegypti*, resulted in hypersensitivity of both insect larvae to Cry toxin intoxication, supporting again that p38 pathway has a protective function in insects (Cancino-Rodezno et al., 2010).

## 5. Cry toxins as bioinsecticide products

Different Bt products have been developed for insect control in agriculture and also against mosquitoes species. Most of these products are based on spore-crystal preparations derived from a few wild-type strains such as *B. thuringiensis* var. *kurstaki* (Btk) HD1 that express Cry1Aa, Cry1Ab, Cry1Ac and Cry2Aa proteins or HD73 that produces Cry1Ac; *B. thuringiensis* var. *aizawai* HD137 which produces slightly different Cry toxins such as Cry1Aa, Cry1B,a Cry1Ca and Cry1Da; *B. thuringiensis* var. *san diego* and *B. thuringiensis* var. *tenebrionis*, which produce Cry3Aa toxin and Bti containing Cry4A, Cry4B, Cry11A and Cyt1Aa toxins. Btk products are effective in controlling many leaf-feeding lepidopterans that are important crop pests or forest pest defoliators (reviewed in Soberón et al., 2009). Bt *aizawai* based products are especially active against lepidopteran larvae that feed on stored grains. Bt *san diego* and Bt *tenebrionis* based products, are suited for beetle



pests in agriculture. Finally, Bti based products are used for the control of mosquitoes that are vector of human diseases as dengue fever and malaria (reviewed in Soberón et al., 2009).

Bt based sprayable products have limited use in agriculture since Cry toxins are specific to young larval stages, are sensitive to sun radiation and have a limited activity against borer insects. Nevertheless, an important breakthrough in the reduction of chemical insecticides in agriculture came with the development of transgenic crops that are able to express Cry toxins (James, 2009). In transgenic plants the Cry protein is produced continuously, protecting the insecticidal toxin from UV degradation and specifically targets chewing and boring insects. In 2009 more than 40 million hectares of Bt-crops were grown world-wide resulting in a significant reduction on the use of chemical insecticides and contributing in some cases to the suppression of certain insect pests like *P. gossypiella*, a pest of cotton (James, 2009; Tabashnik et al., 2010). Presently the most important Bt-crops are soya, corn, cotton and canola (James, 2009). Commercial Bt cotton expresses the Cry1Ac protein for the control of lepidopteran pests as *H. zea* and *P. gossypiella* among others. A second generation Bt-cotton produces Cry2Ab besides Cry1Ac as a resistance managing mechanism (see below). Bt-corn expressing Cry1Ac effectively controls lepidopteran pests as *H. virescens* and *O. nubilalis* (Christou et al., 2006). The next generation of commercial Bt-corn express a series of toxin including Cry34Ab/Cry35Ab binary toxin and Cry3Bb to control coleopteran pests such as *Diabrotica virgifera* and also Cry1A, Cry2Ab and Cry1F for the control of lepidopteran pests including also *Spodoptera frugiperda* (Christou et al., 2006). Although not commercially available yet, VIP3 has been also successfully produced in transgenic corn (Christou et al., 2006).

## 6. A threat to the technology: resistance to Cry toxins

The major threat to the use of Bt crops is the appearance of insect resistance. Resistance to Cry toxins can be developed by mutations in the insect pests that affect any of the steps of the mode of action of Cry toxins. Laboratory selected resistant insect populations have shown that resistance can be developed by different mechanisms including alteration of Cry toxins activation (Oppert et al., 1997), sequestering the toxin by lipophorin (Ma et al., 2005) or esterases (Gunning et al., 2005), by elevated immune response (Hernández-Martínez et al., 2010) and by alteration of toxin receptors resulting in reduced binding to insect gut membranes (reviewed in Griffiths and Aroian, 2005). In the case of *C. elegans* mutations affecting glycolipid biosynthesis resulted in resistance to Cry5 toxin (Griffiths et al., 2005). The most common mechanism of toxin resistance in insect pests until now is the reduction in toxin binding to midgut cells, that in different insect species include mutations in Cry toxin receptors as cadherin, ALP or APN (Gahan et al., 2001; Morin et al., 2003; Herrero et al., 2005; Jurat-Fuentes et al., 2004; Zhang et al., 2009). Recently, a resistant allele of a *H. virescens* resistant population was identified as a mutation in a gene coding for an ABC transporter molecule. This mutation affected binding of Cry1A toxin to brush border membrane vesicles indicating that this ABC transporter molecule is a novel Cry1A toxin receptor probably involved in the later stages of oligomer membrane insertion (Gahan et al., 2010). In fact, the most frequently phenotype of insect resistance, denoted as “Mode 1 of Resistance”, is characterized by the reduction of one Cry1A toxin binding, cross resistance of Cry1Aa, Cry1Ab and Cry1Ac and lack of resistance to Cry1C. In several lepidopteran insects, the mode 1 of resistance is linked to mutations in the cadherin gene (Gahan et al., 2001; Morin et al., 2003; Xu et al., 2005). In field conditions three lepidopteran insect pests have evolved resistance to formulated Bt products, *Plodia interpunctella*, *Plutella xylostella* and *T. ni* (McGaughey, 1985; Tabashnik, 1994; Janmaat and Myers, 2003). In recent years, at least four cases of resistance to Bt crops have been documented, *H. zea* to Bt-cotton expressing Cry1Ac in United States (Tabashnik et al., 2008), *S. frugiperda* to Bt-corn expressing Cry1F in Puerto Rico (Storer et al., 2010), *Busseola fusca* to Bt-corn expressing

Cry1Ab in South Africa (van Rensburg, 2007), and *P. gossypiella* to Bt-cotton expressing Cry1Ac in India (Bagla, 2010).

Appearance of insect resistance to Cry toxins in transgenic crops has been delayed by the so called “High Dose Refuge Strategy”. This strategy entails planting a significant percentage of non-Bt plants in the proximity of Bt-crops that express a high dose of Cry toxin. Non-Bt plants refuges are intended to maintain a population of susceptible insects. Susceptible insects to Cry toxins mate with resistant individuals that are potentially selected on Bt-plants producing susceptible offspring due to the recessive characteristic of resistant alleles (Tabashnik et al., 2008). Modeling studies have shown that refuge strategy has been successful in delaying appearance of resistance of *P. gossypiella* to Bt-cotton in the United States and explains the reason for the appearance of resistance of the same insect species to Bt-cotton in India (Tabashnik et al., 2010). Recently it has been shown that the release of sterile *P. gossypiella* females in an eradication program along with the use of Bt-cotton can efficiently slow down the frequency of resistant alleles in the field (Tabashnik et al., 2010). This strategy can be used instead of the refuge strategy to avoid significant crop losses since non-Bt plants will suffer damage from insect attack. This strategy may be particularly relevant in countries where the refuge strategy is difficult to implement.

Other strategies to cope with the appearance of insect resistance is the use of gene stacking of different Cry toxins with different mode of action in the same plant (reviewed in Bravo and Soberón, 2008). This includes for instance the Bt-cotton Bollgard II expressing Cry1Ac and Cry2Ab proteins that bind to different receptor molecules. Also Bt-corn plant expressing Vip3 along with Cry1Ab and Bt-corn with three Cry toxins against lepidopteran insects (Cry1A1.05, Cry2Ab and Cry1F) and two Cry toxins against coleopteran insects (Cry34Ab/Cry35Ab and Cry3Bb). As mentioned previously Cry1AMod toxins skip cadherin receptor and have been shown to be able to kill *P. gossypiella* resistant population that is linked to mutations in the cadherin gene. Cry1AMod toxins also killed *M. sexta* larvae that were silenced of the cadherin gene expression by dsRNA and that showed high tolerance to Cry1Ab intoxication (Soberón et al., 2007). More recently, Cry1AcMod was shown to be effective against the *T. ni* field-resistant population indicating that this resistant population may be linked to mutations in the cadherin gene (Franklin et al., 2009).

## 7. The future

Bt Cry toxins have been shown to be a valuable tool for insect control, especially with the development of transgenic plants expressing Cry toxins. This technology has been shown successful in diminishing the use of chemical insecticides (James, 2009). As pointed out earlier, the appearance of resistant insects could threaten this technology. However, only a limited number of Cry proteins are now produced in transgenic crops. New Cry proteins that are active against important pests will be introduced to transgenic crops diminishing the possibility for the appearance of resistant insects. In fact the next generation Bt crops produce more multiple Cry toxins reducing the possibility of the development of insect resistance while controlling different insect order species as coleopteran and lepidopteran insects. Gene stacking in crops will continue with the introduction of novel Cry genes identified by screening novel Bt isolates or by introducing novel Cry genes engineered to have improved insecticidal activities against important insect pests. Understanding the mode of action of these toxins and how insects respond to the attack of Cry proteins will allow the development of new, more efficient Bt crops and spray products. Therefore, we foresee a brilliant future in the use Bt Cry proteins to control important insect pests in agriculture reducing to a greater extent the dependence in chemical insecticides and having a positive impact helping to conserve a healthier environment.

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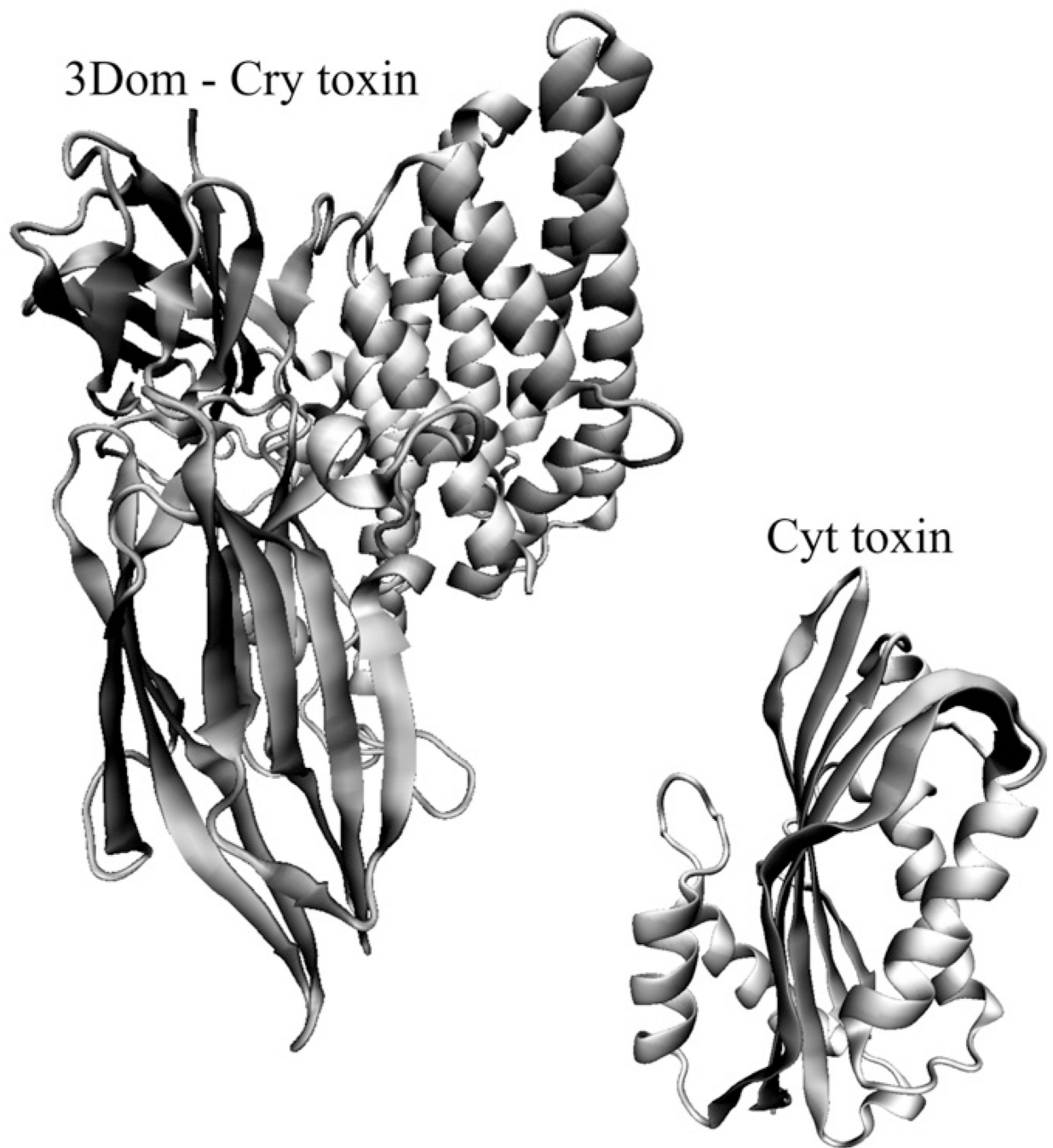
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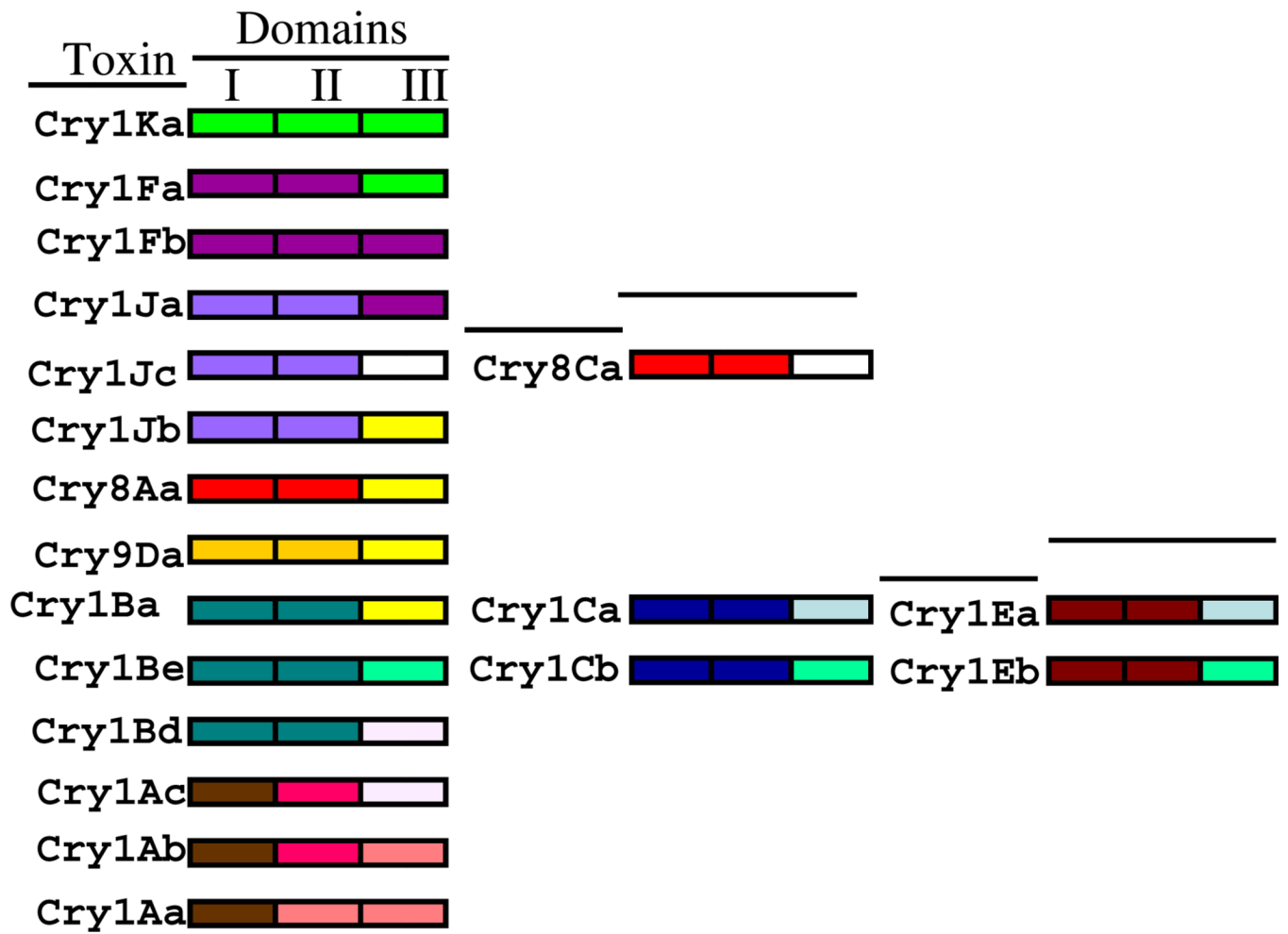
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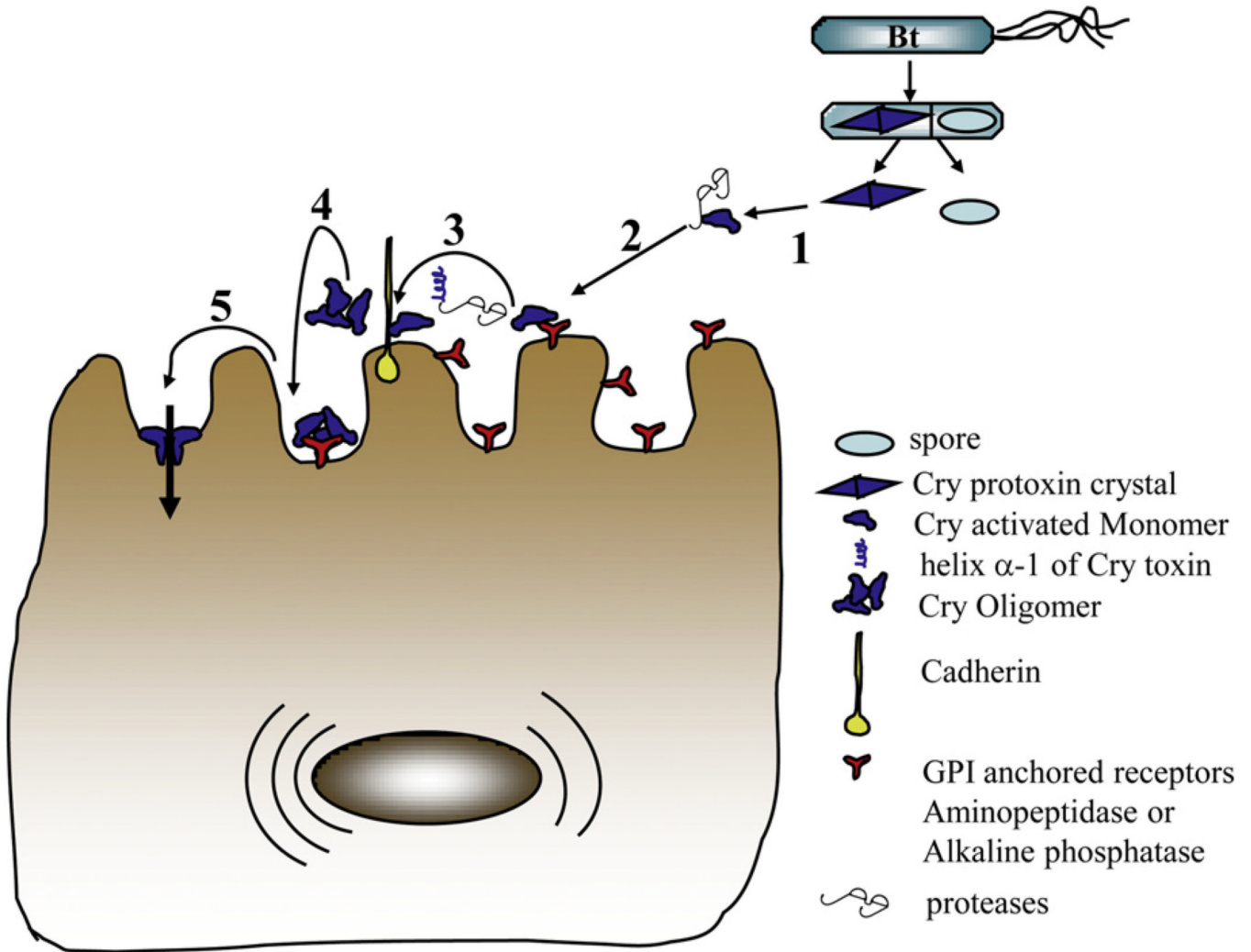




**Fig. 1.** Three dimensional structures of insecticidal toxins produced by *Bacillus thuringiensis*. The structure of the 3D-Cry toxin is that of Cry8Ea and the Cyt corresponds to Cyt2Ba.



**Fig. 2.** Natural examples of domain III swapping among *Bacillus thuringiensis* Cry toxins. Colors represent similar amino acid sequences in the three domains of different 3D Cry toxins (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).



**Fig. 3.** Mode of action of Cry1A toxins in the lepidopteran *M. sexta*. 1. Solubilization and proteolytic processing of Cry1A protoxin by midgut proteases. 2. Binding of monomeric 3D Cry1A to highly abundant GPI-anchored aminopeptidase-N and alkaline phosphatase. 3. Binding of monomeric Cry1A toxin to cadherin receptor and further proteolysis of domain I  $\alpha$ -helix 1. 4. Oligomer formation and binding of the oligomeric structure to GPI-anchored aminopeptidase-N and alkaline phosphatase receptors. 5. Insertion of the oligomeric Cry1A structure into the membrane.

**Table 1**

Midgut Cry toxin binding proteins in three different insect orders.

Insect order	Insect species <sup>a</sup>	Cry-binding protein
Lepidoptera	Ms, Hv, On, Ha, Bm, Pg	Cadherin
	Ld	270 Glycoconjugate
	Bm	P252
	Ms, Bm, Hv, Ld, Px	APN
	Ms, Hv	ALP
	Ag, Ae	Cadherin
Diptera	Ag, Ae, Aq	APN
	Ag, Ae, Aq	ALP
	Aa	alpha-glucosidase
	Tm, Dv	Cadherin
Coleoptera	Lde	ADAM 3 metalloprotease
	Ag	ALP

<sup>a</sup> *Manduca sexta* (Ms), *Heliothis virescens* (Hv), *Ostrinia nubilalis* (On), *Helicoverpa armigera* (Ha), *Bombyx mori* (Bm), *Pectinophora gossypiella* (Pg), *Limantria dispar* (Ld); Diptera, *Anopheles gambiae* (Ag), *Anopheles quadrimaculatus* (Aq), *Anopheles albimanus* (Aa), *Aedes aegypti* (Ae). Coleoptera, *Tenebrio molitor* (Tm), *Diabrotica virgifera* (Dv), *Anthonomus grandis* (Ag), *Leptinotarsa decemlineata* (Lde).