REVIEW ARTICLE

Bacillus thuringiensis insecticidal three-domain Cry toxins: mode of action, insect resistance and consequences for crop protection

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Abstract

Bacillus thuringiensis bacteria are insect pathogens that produce different Cry and Cyt toxins to kill their hosts. Here we review the group of three-domain Cry (3d-Cry) toxins. Expression of these 3d-Cry toxins in transgenic crops has contributed to efficient control of insect pests and a reduction in the use of chemical insecticides. The mode of action of 3d-Cry toxins involves sequential interactions with several insect midgut proteins that facilitate the formation of an oligomeric structure and induce its insertion into the membrane, forming a pore that kills midgut cells. We review recent progress in our understanding of the mechanism of action of these Cry toxins and focus our attention on the different mechanisms of resistance that insects have evolved to counter their action, such as mutations in cadherin, APN and ABC transporter genes. Activity of Cry1AMod toxins, which are able to form toxin oligomers in the absence of receptors, against different resistant populations, including those affected in the ABC transporter and the role of dominant negative mutants as antitoxins, supports the hypothesis that toxin oligomerization is a limiting step in the Cry insecticidal activity. Knowledge of the action of 3d-Cry toxin and the resistance mechanisms to these toxins will set the basis for a rational design of novel toxins to overcome insect resistance, extending the useful lifespan of Cry toxins in insect control programs.

Introduction

In future years the world will be facing a food shortage crisis, posing a challenge for agriculture in increasing food production. Crop damage due to insects, fungi, bacteria and viruses could account for up to 35% of total losses. Improvements to existing pest control programs are therefore urgently sought. Some of the chemical insecticides that are currently used to control insect pests are extremely toxic to nontarget organisms and in many cases are deleterious to the health of humans and animals, inducing important human diseases, such as cancer and immune system disorders. In addition, chemical insecticides are recalcitrant, breaking down only slowly, leading to soil and water pollution. Finally, many insects have developed resistance to different chemical pesticides, resulting in inefficient insect control programs (Devine & Furlong, 2007).

The use of microbial insecticides as substitutes for chemical products is an alternative for insect control in main crops. Biological insecticides based on entomatopathogenic bacteria are based mainly on *Bacillus thuringiensis* (*Bt*). *Bt* relies on insecticidal toxins, such as Cry and Cyt toxins, during its pathogenic process. Other biological insecticide products that are commercially available are based on *Serratia entomophila* and *Bacillus sphaericus*, which produce Sep and Bin insecticidal toxins, respectively (Hurst *et al.*, 2007; Charles and Nielsen-LeRoux 2000). In addition, the bacteria *Xenorhabdus* and *Photorhabdus* spp. belonging to the family *Enterobacteriaceae* associated with entomopathogenic nematodes also produce potent insecticidal toxins that could represent additional alternatives for insect control (Ffrench-Constant & Bowen, 2000).

In this review we focus on the description of the group of three-domain Cry (3d-Cry) toxins produced by *Bt*.

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These proteins are produced as crystal inclusions during the sporulation phase of growth of the bacteria (Bravo *et al.*, 2007). 3d-Cry toxins represent a viable alternative for the control of insect pests in agriculture and of disease vectors of importance in public health (Crickmore *et al.*, 2011). They are highly specific to their target insects, killing a limited number of species. 3d-Cry toxins are innocuous to humans, vertebrates and plants, and are completely biodegradable. However, only a few *Bt* strains have been used so far to produce insecticidal spray products, representing around 2% of the total insecticidal market. Nevertheless, some *cry* toxin genes have been introduced into transgenic crops, providing an effective way to control insect pests in agriculture and lowering the worldwide use of field-applied chemical pesticides (James, 2010). For example, important benefits were documented in *Bt*-cotton, such as a 70% reduction in insecticide applications in fields in India, resulting in savings of up to US$30 ha^{-1} and an 80–87% increase in cotton yields (Qaim & Zilberman, 2003). In transgenic plants, the Cry protein is produced continuously inside the cells, the toxin is protected from UV inactivation and is highly effective against chewing insects that eat plants but also against boring insects that make holes inside the plant tissue, which are more difficult to control with classical chemical pesticides than insects that remain on the plant surface. In 2010, transgenic corn and cotton producing *Bt* toxins were planted on more than 58 million hectares worldwide (James, 2010). The Cry1Ab and Cry1Ac proteins that are expressed in corn and cotton *Bt*-plants are active against the main lepidopteran insect pests that affect these crops (James, 2010).

The primary threat to the long-term efficacy of *Bt* toxins is the evolution of resistance by pests. It has been shown that insects can develop resistance to *Bt* toxins in the laboratory and to *Bt* sprays in the field (Ferré & Van Rie, 2002; Janmaat & Myers, 2003; Tabashnik *et al.*, 2008; Kruger *et al.*, 2009; Storer *et al.*, 2010; Dhurua & Gujar, 2011; Gassmann *et al.*, 2011; Zhang *et al.*, 2011). Here we review the different mechanisms of Cry toxin resistance that have been described in lepidopteran pests. Recently, a novel mechanism of resistance was reported in three different insect populations, *Heliothis virescens*, *Plutella xylostella* and *Trichoplusia ni*, where resistance was shown to be genetically linked to mutant alleles of an ATP-binding cassette transporter (ABC transporter) subfamily C, member 2 (ABCC2; Gahan *et al.*, 2010; Baxter *et al.*, 2011). This protein has not been described either as a binding protein of Cry toxins or as part of the mechanism of action of Cry toxins. In addition, it was recently reported that mutant toxins named Cry1AMod, which are able to oligomerize in the absence of toxin receptors, are also able to overcome the high levels of resistance induced by mutations in the ABC transporter (Tabashnik *et al.*, 2011). We will integrate these data in the analysis of the action of 3d-Cry toxins and discuss the role of toxin oligomerization in the insecticidal activity of these toxins.

The group of 3d-Cry toxins

The mayor determinants of the insecticidal properties of *Bt* bacteria are the δ-endotoxins produced during bacterial sporulation, which form two multigenic groups, *cry* and *cyt* (de Maagd *et al.*, 2001). Cry proteins are specifically toxic to different insects orders such as *Lepidoptera*, *Coleoptera*, *Hymenoptera* and *Diptera*. In contrast, Cyt toxins are mostly found in *Bt* strains active against *Diptera*, although few exceptions of Cyt proteins active against Coleopteran larvae have been documented (Federici & Bauer, 1998; Guerchicoff *et al.*, 2001).

A Cry protein is a parasporal inclusion protein that exhibits a toxic effect to a target organism, or any protein that has obvious sequence similarity to a known Cry protein (Crickmore *et al.*, 2011). The nomenclature of Cry proteins is based on their primary sequence identity, and they have been classified in 70 subgroups. Different proteins not related phylogenetically form part of this classification, such as the group of 3d-Cry toxins, the Mtx-like Cry toxins and Bin-like Cry toxins. Among these toxins, the lineage of 3d-Cry toxins represents the largest group with more than 53 different subgroups of Cry toxins (Crickmore *et al.*, 2011). One particular feature of the members of the 3d-Cry group is the presence of protoxins with two different lengths, 65 and 130 kDa. The main difference between the 65- and 130-kDa 3d-Cry toxin is the C-terminal extension that is found in the 130-kDa protoxins and is dispensable for toxicity, as it is cleaved by proteases present in the larval midgut (de Maagd *et al.*, 2001). The N-terminal region of all 3d-Cry genes codifies for the N-terminal fragment of protoxin (composed of 20–60 residues depending in the toxin) and active toxin, which is composed of approximately 600 amino acid residues. The 3d-Cry toxins are globular molecules containing three distinct domains connected by single linkers. The crystal structure of different trypsin-activated Cry toxins, such as Cry1Aa (lepidopteran specific), Cry3Aa, Cry3Bb and Cry8Ea (coleopteran specific), Cry4Aa and Cry4Ba (dipteran specific) and the Cry2Aa protoxin (dipteran-lepidopteran specific), has been determined (Li *et al.*, 1991; Grochulski *et al.*, 1995; Galitsky *et al.*, 2001; Morse *et al.*, 2001; Boonserm *et al.*, 2005, 2006; Guo *et al.*, 2009). Although the sequence identity among these toxins is low, the overall structural topology of the three structural domains is quite similar. Figure 1 shows the structure alignments between Cry1Aa and other 3d-Cry toxin structures as determined using the FATCAT algorithm and dynamic

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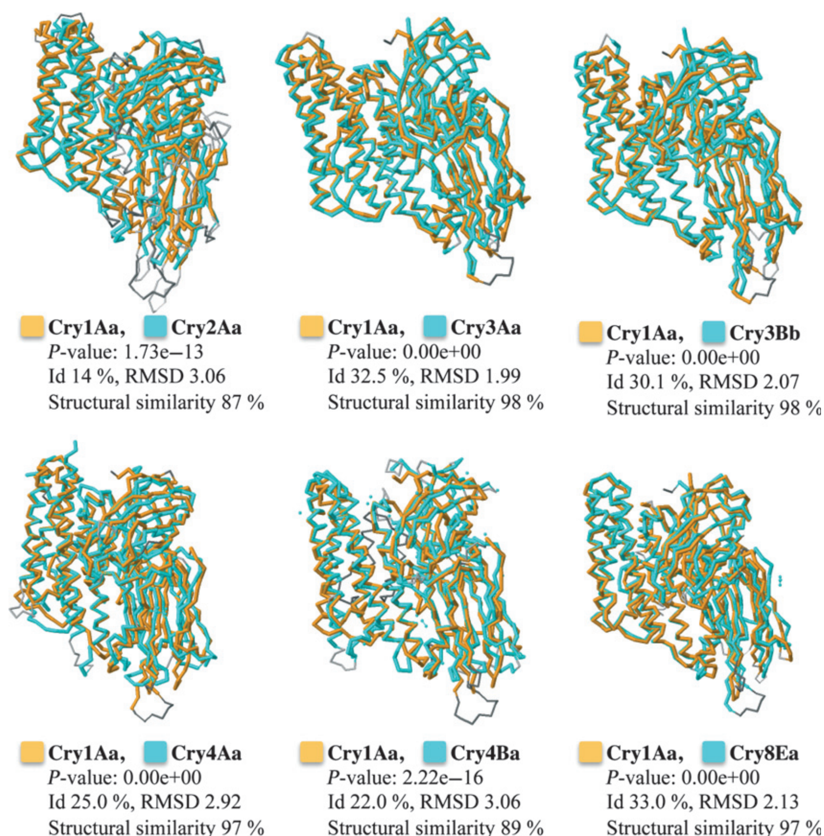

Fig. 1. Structural alignments between Cry1Aa and other 3d-Cry toxin structures as determined using FATCAT algorithm and dynamic programming. Cry1Aa, pdb 1CIY; Cry2Aa, pdb 1I5P; Cry3Aa, pdb 1DLC; Cry3Bb, pdb 1JI6; Cry4Aa, pdb 2C9K; Cry4Ba, pdb 1W99; Cry8Ea, pdb 3eb7. Id%, percentage sequence identity in the alignments; RMSD, measure of the average distance between the atoms of superimposed proteins. Significant results ($P < 0.001$) are shown.

programming. According to FATCAT, a pair with a *P*-value probability lower than 0.05 indicates that the two proteins are significant similar. The data presented in Fig. 1 indicates that all 3d-Cry structures are significantly similar.

Phylogenetic analysis of Cry toxins shows that the great variability in the biocidal activity of the 3d-Cry group has resulted from the independent evolution of the three structural domains and the domain III swapping among different toxins. These two processes have generated proteins with similar modes of action but with different specificities (de Maagd *et al.*, 2001).

Mechanism of action of 3d-Cry toxins in *Lepidoptera*

When susceptible larvae ingest the 3d-Cry protoxin, it is solubilized and activated by gut proteases, generating a toxic fragment of approximately 60 kDa, composed of the three-domain structure described above. The activated toxin goes through a complex sequence of binding events with the different insect gut Cry-binding proteins, leading to membrane insertion and pore formation (Bravo *et al.*, 2004; Pigott & Ellar, 2007; Pacheco *et al.*, 2009b). Cry toxins form pores in the apical membrane of larvae midgut cells, destroying the cells and killing the larvae (Soberón *et al.*, 2009). It was proposed that the ion channel activity of 3d-Cry toxins leads to a colloidal osmotic lysis of the midgut cells given that the 3d-Cry toxins induce the formation of nonselective channels permeable to cations, anions and neutral solutes, and that the influx of water results in cell swelling and eventual lysis (Knowles and Ellar 1987).

Figure 2 shows the morphological changes in midgut cells of *Manduca sexta* larvae after intoxication with the toxin. In these pictures the toxin was immunolocalized in the apical microvilli of the midgut cells, as reported by Bravo *et al.* (1992).

Receptor identification and role of these receptors in 3d-Cry toxin action

The interaction of Cry1 toxins with different proteins present in *Lepidoptera* midgut cells is a complex process

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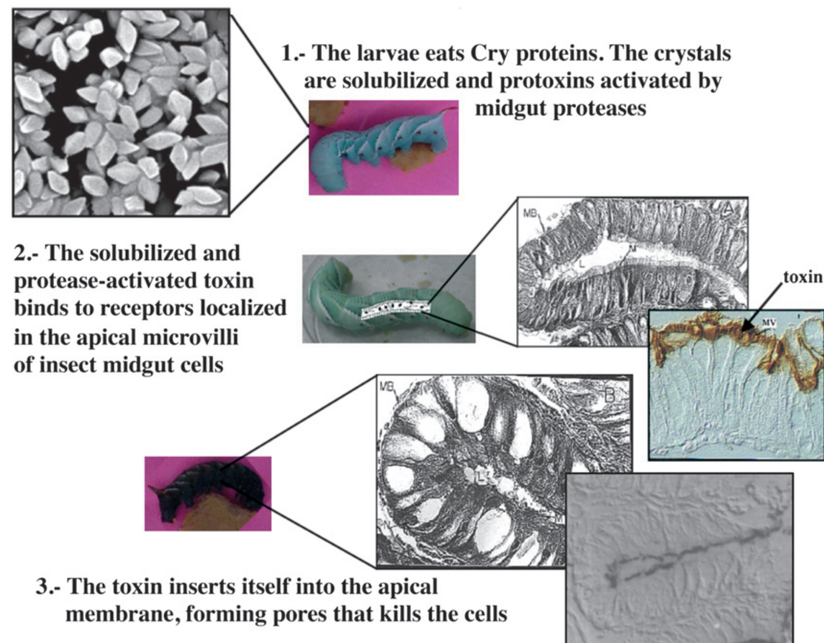

Fig. 2. Schematic representation of the mechanism of action of 3d-Cry toxins in *Lepidoptera* at the cellular level, showing the immunolocalization of Cry toxin during intoxication (Bravo *et al.*, 1992).

involving multiple membrane proteins such as cadherin-like proteins (CAD), aminopeptidase N (APN) and alkaline phosphatase (ALP; Soberón *et al.*, 2009; Pigott & Ellar, 2007). Besides these membrane proteins, other components have been identified due to their capacity to interact with 3d-Cry toxins such as glycolipids or intracellular proteins, such as V-ATPase subunit A and actin (McNall & Adang, 2003; Griffits *et al.*, 2005; Krishnamoorthy *et al.*, 2007; Bayyareddy *et al.*, 2009) . The role of the intracellular proteins that bind 3d-Cry toxins in the insecticidal activity of these toxins remains to be analyzed.

Figure 3 shows a schematic representation of the updated mechanism of action of 3d-Cry toxins in *Lepidoptera* at the molecular level. It also shows a diagrammatic representation of the epitopes in 3d-Cry toxins that are involved in the binding interaction with CAD, ALP and APN receptors. In lepidopteran larvae the first binding interaction of activated Cry1A toxins is a low-affinity interaction with ALP and APN receptors (K_d = 101 nM for APN and 267 nM for ALP). The interaction with APN occurs through exposed loop 3 of domain II and interaction with ALP through strand β-16 of domain III (Masson *et al.*, 1995a; Pacheco *et al.*, 2009b; Arenas *et al.*, 2010). ALP and APN are highly abundant proteins anchored to the membrane by a glycosyl phosphatidylinositol anchor (Upadhyay & Singh, 2011). The interaction with ALP and APN concentrate the activated toxin in the microvilli membrane of the midgut cells where the toxin is then able to bind in a high-affinity interaction to the CAD receptor (K_d = 1 nM; Vadlamudi *et al.*, 1995; Gómez *et al.*, 2006; Pacheco *et al.*, 2009b; Arenas *et al.*, 2010). The interaction with CAD is a complex interaction that involves three epitopes in the CAD corresponding to extracellular regions named CR7, CR11 and CR12, where CR12 is proximal to the membrane domain of the cadherin. These epitopes of CAD protein interact with exposed loops 2, 3 and α-8 from domain II of the toxin, promoting further proteolytic cleavage of the N-terminal end including helix α-1 of domain I (Fig. 3; Gómez *et al.*, 2002; Atsumi *et al.*, 2008). It is evident that cleavage of helix α-1 exposes buried hydrophobic regions of domain I, and it was hypothesized that cleavage of helix α-1 is necessary to trigger the formation of a toxin prepore oligomer structure before insertion into the membrane (Gómez *et al.*, 2002; Pacheco *et al.*, 2009b; Arenas *et al.*, 2010). Later, it was shown that Cry1AMod toxins deleted of the N-terminal region including helix α-1 were able to form oligomers in the absence of the CAD receptor (Soberón *et al.*, 2007). It was also shown that a *M. sexta* CAD fragment containing Cry1Ab binding sites CR11 and CR12 enhanced Cry1Ab toxicity when fed to the larvae along with the Cry1Ab protein (Chen *et al.*, 2007). Similar CAD fragments isolated from different insect orders enhanced the toxicity of other Cry toxins such as

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the CAD fragment from *Anopheles gambiae*, which synergizes activity of Cry4Ba in this mosquito species (Hua *et al.*, 2008), or the CAD fragment from the coleopteran *Diabrotica virgifera*, which enhances Cry3Aa toxicity to susceptible coleopteran larvae (Park *et al.*, 2009). The enhancement in toxicity of the CAD fragment isolated from *M. sexta* larvae was later shown to correlate with higher Cry1Ab oligomer formation (Pacheco *et al.*, 2009a). The oligomeric prepore structure can be produced in solution after activation of protoxin in the presence of a fragment of cadherin containing the toxin binding sites (Pacheco *et al.*, 2009a); this oligomeric structure has been purified by size exclusion chromatography, revealing that is able to make pores in black lipid bilayers (Rausell *et al.*, 2004b). Overall, these data support the proposition that interaction of the toxin with the CAD receptor is important to promote oligomerization of Cry toxins.

The oligomeric Cry structure increases its affinity to ALP and APN receptors up to 200-fold (K_d = 0.6 nM for APN and 0.5 nM for ALP; Fig. 3). In this high-affinity interaction the domain II loop-2 region of Cry1Ab toxin is involved (Arenas *et al.*, 2010). The role of ALP and APN proteins after prepore binding is to induce its insertion into the membrane, leading to pore formation and finally to cell lysis (Fig. 3; Pardo-López *et al.*, 2006; Arenas *et al.*, 2010).

An alternative model of the mode of action of Cry toxin was given by Zhang *et al.* (2006). In this model it was proposed that binding to cadherin triggers an intracellular pathway that results in activation of a G protein with subsequent activation of adenylyl cyclase, raising cAMP levels and activating a protein kinase A that in turn leads to cell death without involvement of oligomer formation, toxin pore formation or participation of other receptors such as GPI-anchored proteins. Nevertheless, as discussed below, the construction of nontoxic Cry toxin mutants affected in toxin oligomerization or in pore formation activity without affecting binding to cadherin receptor showed that binding to cadherin is not sufficient for toxicity (Vachon *et al.*, 2002, 2004; Jiménez-Juárez *et al.*, 2007; Girard *et al.*, 2008; Rodríguez-Almazán *et al.*, 2009). Furthermore, Cry1AMod toxins that lacked the amino-terminal end including helix α-1 were able to skip cadherin interaction and kill Cry1A-resistant insects affected in the cadherin gene, supporting the hypothesis that binding to cadherin is not sufficient to induce toxicity in the midgut cells of target insects (Soberón *et al.*, 2007).

Oligomerization and pore formation activity of 3d-Cry toxins

Oligomerization of 3d-Cry toxins and pore formation activity of Cry1A toxins are essential steps in the mode of

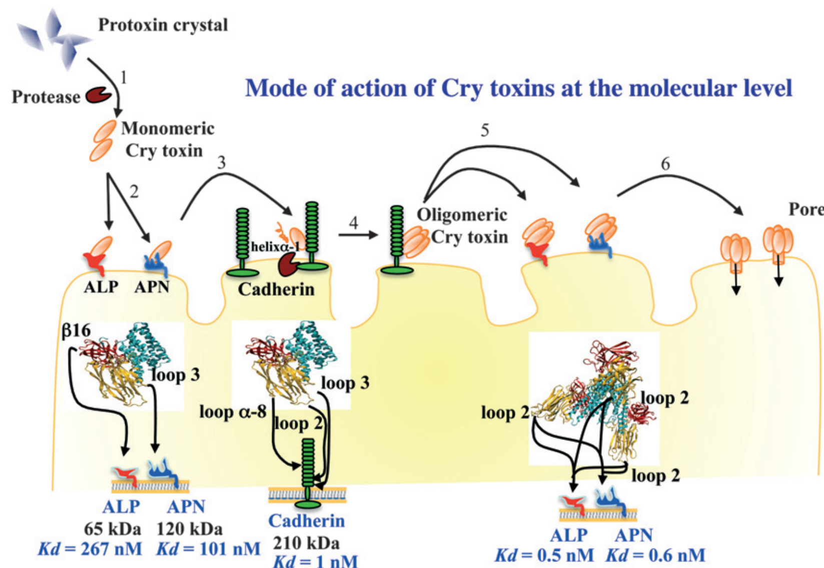

Fig. 3. Schematic representation of the mechanism of action of 3d-Cry toxins in *Lepidoptera* at the molecular level. 1, the larvae ingest the 3d-Cry protoxin, which is solubilized in the midgut lumen of the larvae due to high pH and reducing conditions and activated by gut proteases, generating the toxin fragment. 2, the monomeric 3d-Cry toxin binds ALP and APN receptors; in a low-affinity interaction, the toxin is then located in close proximity to the membrane. 3, the monomeric 3d-Cry toxin binds the CAD receptor in a high-affinity interaction and this interaction induces proteolytic cleavage of the N-terminal end of the toxin, including helix α-1 of domain I. 4, the cleaved 3d-Cry toxin is then able to oligomerize in a toxin prepore oligomer. 5, the oligomeric 3d-Cry structure binds to ALP and APN receptors with high affinity. 6, the prepore inserts into the membrane causing pore formation.

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action of these toxins. Different procedures have been used to measure pore formation activity of different 3d-Cry toxins, such as light scattering of liposomes or brush border membrane vesicles (BBMVs; Haider & Ellar, 1989; Carroll & Ellar, 1993); leakage of $^{86}Rb^{+}$–K^{+} and leakage of calcein from BBMVs (English *et al.*, 1991; Rausell *et al.*, 2004a) ; analysis of single-channel currents in black lipid bilayers (Schwartz *et al.*, 1993; Lorence *et al.*, 1995); analysis of ion-amino acid cotransport (Hendrickx *et al.*, 1990); analysis of changes in membrane potential (Lorence *et al.*, 1995; Muñoz-Garay *et al.*, 2006); and studies of short-circuit currents in midgut tissues (Liebig *et al.*, 1995). The size of the pore induced by 3d-Cry toxins estimated by osmotic protection assays suggested a diameter of approximately 2.4 nm under high pH (Carroll & Ellar, 1997).

Mutations in some residues of domain I, specifically in helices α-3 or α-4, resulted in complete loss of toxicity to *M. sexta* larvae (Vachon *et al.*, 2002, 2004; Jiménez-Juárez *et al.*, 2007; Girard *et al.*, 2008; Rodríguez-Almazán *et al.*, 2009). The nontoxic mutants located in these α-helices showed altered oligomerization or membrane insertion, respectively, severely affecting pore formation. However, these mutants did not affect binding interactions with membrane proteins, retaining similar binding affinity with the CAD receptor to the wild-type toxin, indicating that binding to the CAD receptor is not enough to kill the larvae and that oligomerization of Cry toxin and pore formation are essential steps for toxicity (Jiménez-Juárez *et al.*, 2007; Girard *et al.*, 2008; Rodríguez-Almazán *et al.*, 2009).

In addition, it was shown that genetically engineered Cry1A modified toxins (Cry1AMod) that were deleted at the N-terminal region, including helix α-1 of domain I, were able to form oligomeric structures without interacting with the CAD receptor and showed similar binding to *M. sexta* APN receptor (Soberón *et al.*, 2007; Muñóz-Garay *et al.*, 2009). The Cry1AMod toxins were able to kill the *Pectinophora gossypiella* population resistant to Cry1A toxins due to mutations in the cadherin gene and *M. sexta* larvae that were tolerant to Cry1Ab toxin due to CAD silencing by RNA interference (RNAi; Soberón *et al.*, 2007), showing that even in the absence of CAD in the resistant *P. gossypiella* larvae or the tolerant *M. sexta* larvae, the oligomers of Cry1AMod toxins could still bind APN and ALP receptors and kill the larvae. These data confirmed that the principal role of CAD after toxin binding is to induce the removal of helix α-1, promoting the formation of toxin oligomer (Soberón *et al.*, 2007; Muñóz-Garay *et al.*, 2009).

Oligomerization of 3d-Cry proteins has been described for toxins active against different insect orders, such as Cry1, Cry3, Cry4B and Cry11A, which are active against lepidopteran, coleopteran and dipteran larvae (Aronson *et al.*, 1999; Tigue *et al.*, 2001; Rausell *et al.*, 2004a; Likitvivatanavong *et al.*, 2006; Muñoz-Garay *et al.*, 2006; Pérez *et al.*, 2007; Herrero *et al.*, 2004) . The oligomeric structure of 3d-Cry toxin allows it to interact with the membrane lipid bilayer, forming stable pores that show high open probability, in contrast to the toxin monomer, which has marginal interaction with the liposomes inducing multiple subconducting states, and showing unstable traces with current jumps of intermediate levels that are difficult to resolve (Schwartz *et al.*, 1993; Rausell *et al.*, 2004b).

The number of monomers that are associated in the oligomeric structure remains unclear, as two-dimensional crystallographic analysis showed a trimeric organization of the membrane-associated structure of Cry1AbMod and Cry4Ba (Ounjai *et al.*, 2007; Muñóz-Garay *et al.*, 2009) while an oligomer structure composed of four subunits was shown by atomic force microscopy studies performed with two different 3d-Cry toxins, the Cry1Aa and Cry4Ba toxins (Vié *et al.*, 2001; Puntheeranurak *et al.*, 2005) or by photobleaching of fluorescently labeled Cry1Aa toxin (Groulx *et al.*, 2011). Cry4Ba represents a special toxin as it is able to oligomerize when proteolytically activated *in vitro* in the absence of cadherin receptor and these oligomeric structures have pore formation activity in planar lipid bilayers (Rodríguez-Almazán *et al.*, 2012). It is interesting to note that Cry4Ba toxin preferentially inserts into liposomes in an aggregated form with a fourfold symmetry rather than as a single monomeric molecule, supporting the suggestion that oligomeric structures are able to interact with the lipid bilayer (Puntheeranurak *et al.*, 2005). These data correlate with measurements of pore formation where the monomeric toxins showed a marginal effect, and the oligomeric structure works like an ionic pore, confirming that the oligomeric structure of 3d-Cry toxins is the intermediate that is responsible for insertion into the membrane (Rausell *et al.*, 2004b; Muñoz-Garay *et al.*, 2006).

Role of dominant negative mutants of Cry1Ab as antitoxins and demonstration that toxin oligomerization is a necessary step in the action of 3d-Cry toxin

Dominant negative (DN) inhibitors of different pore-forming toxins produced by bacteria have been developed. The DN proteins are mutant toxins that are able to form oligomeric structures but show reduced membrane insertion and pore formation (Vinion-Dubiel *et al.*, 1999; Sellman *et al.*, 2001; Wai *et al.*, 2003). These powerful inhibitors act at substoichiometric levels, in contrast to other toxin mutants that compete for receptor binding

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which require 10-fold or higher concentrations to inhibit toxicity. Therefore, the DN mutant monomers assemble into oligomers together with the wild-type toxin inactivating the wild-type toxin *in vivo*, providing an effective strategy to counter action of the toxin. Toxin mutants showing a DN phenotype also provide unequivocal evidence that oligomerization is a key step in the mode of action of pore-forming toxins (Vinion-Dubiel *et al.*, 1999; Sellman *et al.*, 2001; Wai *et al.*, 2003).

The nontoxic α-helix 4 mutants of Cry1Ab, Cry11Aa and Cry4B toxins showed a DN phenotype, being able to inhibit the toxicity of their corresponding wild-type toxins (Rodríguez-Almazán *et al.*, 2009; Carmona *et al.*, 2011). Figure 4 shows a schematic representation of the DN phenotype of Cry toxins. The DN mutants of Cry1Ab inhibited the insertion of native Cry1Ab into the membrane, blocking wild-type toxicity and functioning as antitoxins (Rodríguez-Almazán *et al.*, 2009; Carmona *et al.*, 2011; Fig. 4). In addition, the Cry1Ab DN mutant functions as an antitoxin of other 3d-Cry toxins such as Cry1Aa, Cry1Ac and Cry1Fa, while Cry11Aa and Cry4Ba DN mutants inhibit the toxicity of both Cry11Aa and Cry4Ba toxins, suggesting that in some cases 3d-Cry toxins have the potential to form hetero-oligomers with different 3d-Cry toxins (Rodríguez-Almazán *et al.*, 2009; Carmona *et al.*, 2011). *In vivo* hetero-oligomerization may represent an important advantage for some Cry toxin combinations to improve their toxicity against specific targets. It was reported that Cry11Aa and Cry4Ba have synergistic activity, in some cases showing up to 10-fold higher activity against mosquitoes in the mixture than the expected mortality from the individual toxins (Poncet *et al.*, 1995; Fernandez-Luna *et al.*, 2010). The synergism between Cry1A toxins was also documented previously; Cry1Ab and Cry1Ac synergized against *Chilo partellus* larvae showing up to fivefold higher activity when both toxins are present in the bioassay (Sharma *et al.*, 2010). Similarly, Cry1Aa and Cry1Ac have a synergistic effect against *Lymantria dispar* larvae, increasing their toxicity 4.9-fold when the larvae were fed with a mixture of toxins and in this case it was demonstrated that the combination of the two toxins resulted in greater pore formation activity than the individual toxins (Lee *et al.*, 1996). Most *Bt* strains produce more than one 3d-Cry toxin, suggesting that hetero-oligomerization of different 3d-Cry toxins could be selected during evolution of Cry toxins as a mechanism to modulate the insect specificity and toxicity of these toxins.

Conformational changes of 3d-Cry toxins during membrane insertion

Insertion of the toxin into the membrane is one of the less well-characterized steps in the mechanism of action of 3d-Cry toxins, and elucidation of the toxin structure when inserted into the membrane is important to a full understanding of the mechanism of action of the 3d-Cry toxin. The interaction of 3d-Cry toxin oligomer with the APN and ALP receptor proteins is a high-affinity interaction and the hypothesis is that binding to these receptors is important to trigger a conformational change that is necessary to insert the toxin into lipid rafts to form a pore that eventually kills the midgut cells (Zhuang *et al.*, 2002; Bravo *et al.*, 2004; Pardo-López *et al.*, 2006).

Previous studies performed with mutant toxins in which disulfide bridges were introduced between domains I and II demonstrated that unfolding of the protein in the region linking domain I and II is a necessary step for pore formation, as bridged mutants could not form functional ion channels in lipid bilayers in the oxidized state,

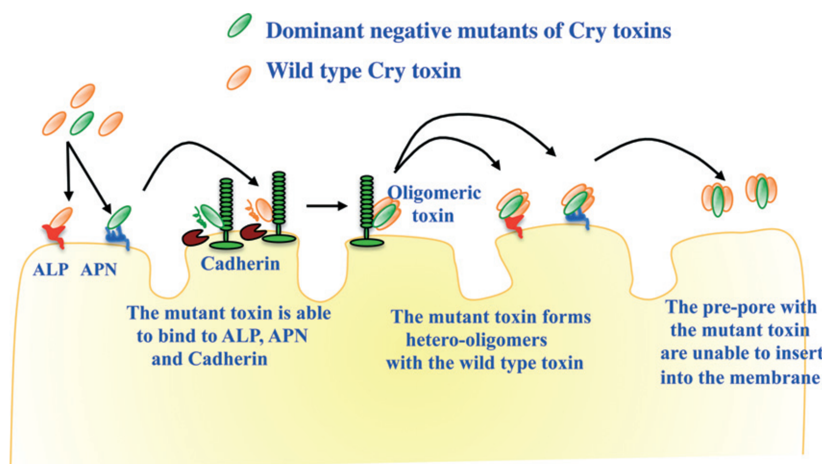

Fig. 4. Schematic representation of the DN phenotype of 3d-Cry toxins affected in helix α-4.

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but upon reduction regained parental toxin channel activity (Schwartz *et al.*, 1997). Additional supporting evidence was the finding that mutations in residue D242 of Cry1Aa, which is involved in a salt-bridge linking domains I and II of this toxin, resulted in a faster rate of pore formation, suggesting that increases in molecular flexibility, due to the removal of this salt bridge, facilitated toxin insertion into the membrane (Coux *et al.*, 2001). Analysis of urea and thermal denaturation showed that oligomerization of Cry1Ab toxin increased the flexibility of the toxin structure, especially at high high pH, similar to the pH that is found in the midgut lumen of lepidopteran insects (Rausell *et al.*, 2004c).

The most accepted model of Cry toxin insertion into the membrane proposed that the hydrophobic hairpin formed by helices α-4 and α-5 inserts into the phospholipid bilayer whereas the rest of amphipathic helices of domain I are spread on the membrane surface in an umbrella-like conformation (Fig. 5; Schwartz *et al.*, 1997; Kumar & Aronson, 1999; Girard *et al.*, 2008, 2009). Numerous mutagenesis studies performed in helices α-4 and α-5 of Cry1A toxins or in other regions of domain I support this model. One of the most interesting reports suggested that the hydrophilic face of helix α-4 faces the lumen of the pore. The authors introduced a Cys residue in substitution of an Asp acid residue in mutant D136C of Cry1Aa. The negative charge of aspartic acid was restored *in situ* by MTSES, a reagent that is capable of passive diffusion into the channel lumen and is able to introduce a negative charge by a covalent interaction with a Cys residue. Restoration of the negative charge in this position resulted in restitution of ionic conductance to the levels of the wild-type Cry1Aa toxin, indicating that D136 faces the lumen pore (Masson *et al.*, 1999).

Studies of pronase digestion assays of Cry1Aa toxin after interaction with BBMV isolated from *Bombyx mori*

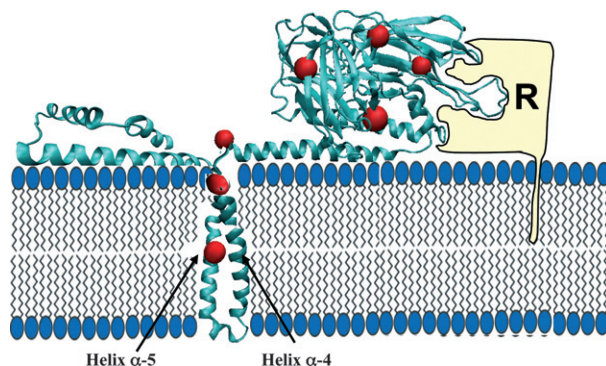

Fig. 5. Umbrella model of toxin insertion into the membrane. The residues that were substituted by Cys in Zavala *et al.* (2011) are shown as red balls, showing that only two residues (T122 and V171) were buried into the membrane upon membrane insertion.

showed that BBMV-bound Cry1Aa was highly resistant to pronase digestion. The protected fragments were identified by Western blot assays using antibodies raised against different regions of the toxin, showing that only helix α-1 from domain I and a region comprising β-strands 1–5 of domain II were digested by the protease, and suggesting that the rest of the toxin was protected due to membrane insertion (Tomimoto *et al.*, 2006). The authors call this model the dragon buried insertion model. However, a different interpretation could be that during toxin oligomerization strong contacts among monomeric toxins could bury the specific proteolytic sites, resulting in protease-protection.

Finally, an alternative model of toxin insertion into the membrane proposed that the complete Cry3Aa toxin inserts into the bilayer, except for the first three α-helices, without mayor conformational changes in the toxin structure upon insertion into the membrane. This model was based on analysis of the activation energy of denaturation of Cry3Aa toxin when it is bound to synthetic lipid vesicles (Loseva *et al.*, 2001). Nair & Dean (2008) also proposed that all domains of Cry1Ab toxin insert into the membrane based on a study of single-Cys mutants labeled with fluorescent probes such as 1,5 IAEDANS. However, their conclusions were shown to be incorrect, as they attributed false characteristics to this fluorophore (Zavala *et al.*, 2011). Single Cys mutants were also introduced in the different domains of Cry1Ab toxin and were labeled with the same dye, 1,5 IAEDANS, and also with Alexa-Flour 350; their exposure to the solvent or to the lipid bilayer was analyzed using differing hydrophilic collisional quenchers such as KI or acrylamide, as well as membrane-associated quenchers, showing that most of the toxin remains exposed to the solvent upon pore formation and only a small region of domain I, comprising helices α-4 and α-5, is involved in membrane insertion (Zavala *et al.*, 2011). Overall these data support the umbrella model of toxin insertion (Fig. 5).

Note that in many other pore-forming toxins produced by different bacteria, such as anthrax toxin, aerolysin, alpha-hemolysin and CDC-toxins, membrane insertion involved only a small part of these proteins while the rest of the protein remains outside of the membrane (Parker & Feil, 2005). Cry toxins from *Bt* are not the exception; they also insert a small part of the protein into the membrane bilayer to induce pore formation.

Resistance mechanisms to 3d-Cry toxins in different lepidopteran insects

In theory, resistance to 3d-Cry toxins could occur by blocking any step in the mechanism of action described above (Fig. 3). In fact, resistant insect populations

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selected in laboratory conditions have shown that resistance can be developed by different mechanisms, such as altered activation of Cry toxins by midgut proteases (Keller *et al.*, 1996; Oppert *et al.*, 1997; Li *et al.*, 2004), sequestering the toxin by glycolipid moieties (Ma *et al.*, 2011) or esterases (Gunning *et al.*, 2005), by inducing an elevated immune response (Rahman *et al.*, 2004; Hernandez-Martinez *et al.*, 2010) and by alteration resulting in reduced binding to insect gut membranes. Toxin binding to BBMVs has been reported to be linked to mutation in toxin receptors (Ferré & Van Rie, 2002; Heckel *et al.*, 2007) or mutations in other molecules such as the recently described mutations in the ABCC2 transporter in lepidopteran larvae or mutations affecting glycolipid biosynthesis in the nematode *Caenorhabditis elegans*, which also resulted in animals that showed reduced binding of the toxin to their midgut membranes and became resistant to Cry1A toxins or Cry5 toxin, respectively (Griffits *et al.*, 2005; Gahan *et al.*, 2010; Baxter *et al.*, 2011). It was proposed that Cry1Ab and Cry1Ac could bind to the ABCC2 protein in its open state where some hydrophobic surfaces of the channel are exposed to the outside and it was hypothesized that this binding interaction could facilitate membrane insertion of the toxin oligomer (Gahan *et al.*, 2010). Note that among all these mechanisms of resistance, the most common mechanism of toxin resistance is the reduction in toxin binding to midgut cells, which in different resistant insect species include mutations in Cry toxin receptors such as CAD, ALP or APN or mutations in the ABCC2 transporter (Gahan *et al.*, 2001, 2010; Jurat-Fuentes *et al.*, 2004, 2011; Herrero *et al.*, 2005; Baxter *et al.*, 2011). Figure 6 shows the different mechanisms of Cry toxin resistance that have been described in different lepidopteran insects.

Alterations in protease activity may result in tolerance to Cry toxin action. This is the case of *Plodia interpunctella* larvae, where the lack of major trypsin-like gut proteases was shown to be genetically linked to larval survival after Cry1Ac intoxication in the laboratory-selected 198r strain (Oppert *et al.*, 1997). The *P. interpunctella* 198r resistant line showed reduced activation of the protoxin, resulting in a 10-fold decrease in the insecticidal activity of Cry1Ab (Johnson *et al.*, 1998). Similarly, the *Ostrinia nubilalis* HD-1 *Bt kurstaki* resistant strain, named KS-SC, showed 47-fold greater resistance to Dipel (containing Cry1Aa, Cry1Ab, Cry1Ac and Cry2Aa) due to reduced protoxin activation (Li *et al.*, 2004, 2005). However, it was shown that this strain has similar susceptibility to *Bt*-corn as the susceptible line, given that the plant enzymes are able to hydrolyze transgenic toxin, suggesting that resistant insects due to reduced proteinase activity do not represent a threat to the efficacy of *Bt* crops (Li *et al.*, 2007). By contrast, in *Spodoptera littoralis* the increased activity of gut proteases resulted in reduced

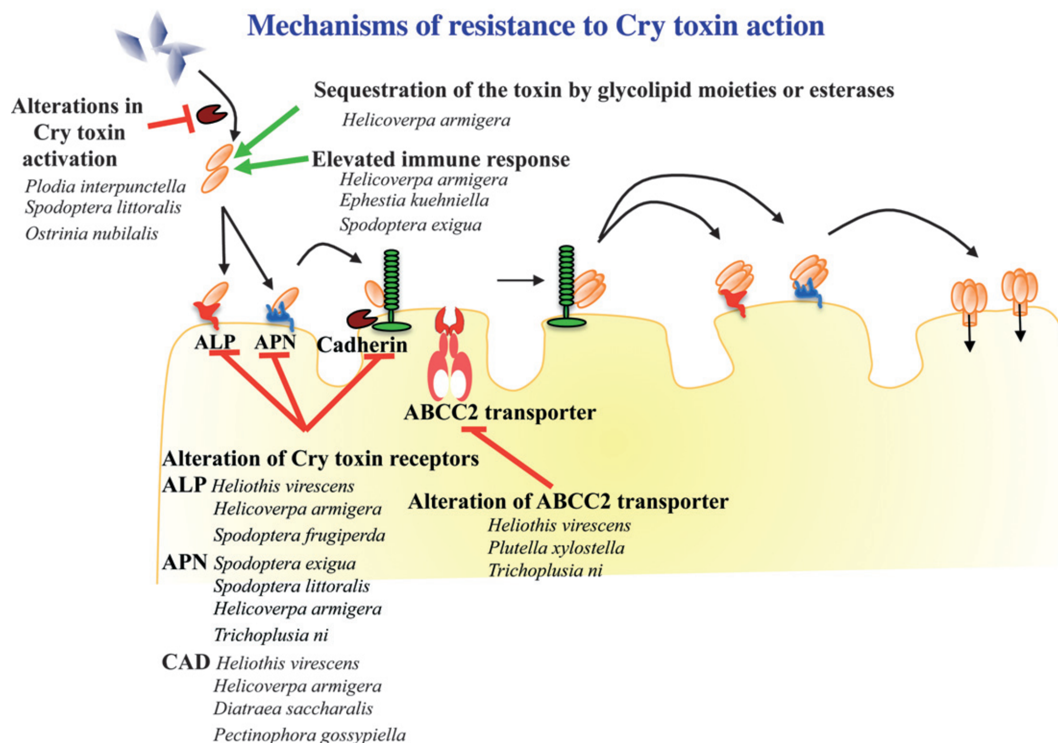

Fig. 6. Schematic representation of the different mechanisms of resistance to 3d-Cry toxin described in lepidopteran insects.

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susceptibility to Cry1C, possibly due to over-degradation and inactivation of the toxin (Keller *et al.*, 1996).

It has been shown that some 3d-Cry-tolerant insects such as *Helicoverpa armigera* released lipid particles into the gut lumen. The glycolipids that are present in these lipid particles bind Cry1Aa and Cry2Ab toxins resulting in toxin sequestration in the gut lumen, thereby affecting the interaction of the toxins with specific receptors on midgut cells, acting as a trap for mature toxins (Ma *et al.*, 2011). In addition, it was reported that sequestration of Cry1Ac in the 275-fold tolerant *H. armigera* strain is also due to binding of the toxin to esterases (Gunning *et al.*, 2005).

The role of elevated immune responses that result in a 16-fold greater tolerance to 3d-Cry toxins in *Ephestia kuehniella* or 12-fold resistance in *H. armigera* larvae has been documented. In these insects the rate of melanization reactions was increased, resulting in tolerance to 3d-Cry toxin intoxication (Rahman *et al.*, 2004; Ma *et al.*, 2005). In *Spodoptera exigua* an elevated immune response was described by analyzing differential gene expression in a 100-fold more resistant population to the *Bt*-formulated product Xentari (Hernandez-Martinez *et al.*, 2010). These data indicated that alterations in insect gut physiology could result in resistance to *Bt* toxins.

There are multiple examples in the literature reporting that 3d-Cry toxin phenotypes of resistance are associated with mutations affecting 3d-Cry receptor genes or mutations that affect transcription of 3d-Cry toxin receptor genes by a trans-regulatory mechanism. The first examples that were reported described mutations linked to the cadherin gene, but later mutations in other receptors such as APN and ALP were also documented. Among the CAD mutations linked to resistance in *Lepidoptera*, the first report was in *H. virescens*; genetic analysis revealed that the cadherin gene in the YHD2 strain was interrupted by a retrotransposon, preventing the translation of a full-length protein. The CAD protein in this resistant line is not detectable by Western blot analysis, resulting in more than 10 000-fold greater resistance to Cry1Ac (Gahan *et al.*, 2001; Jurat-Fuentes *et al.*, 2004). The second example was in an AZP-R-resistant strain of *P. gossypiella* that showed 3100-fold greater resistance to Cry1Ac, where three mutated cadherin gene alleles were genetically linked to resistance to Cry1Ac. The *r*1 allele has a deletion mutation of 24 bp that results in two amino acid substitutions and the omission of eight amino acids. The *r*2 allele has a 202-bp deletion creating a frame shift that introduces a stop codon and the *r*3 allele has a 126-bp deletion that eliminates 42 amino acids. All three deletions were located upstream of the previously identified toxin-binding region in the cadherin protein (Morin *et al.*, 2003). In addition, an insertion of a retrotransposon was described in the mutation of the *r*3 allele of *P. gossypiella* (Fabrick *et al.*, 2011). In *H. armigera* a deletion between exons 8 and 25 was found in the cadherin gene and demonstrated to be responsible for production of a truncated protein in the GYBT resistant strain, which shows 564-fold greater resistance to Cry1Ac-activated toxin (Xu *et al.*, 2005). Finally, in the 100-fold greater Cry1Ab-resistant *Diatraea saccharalis* Cry1Ab-RR strain, it was reported that lower expression of the cadherin gene was associated with resistance to Cry1Ab toxin. The role of CAD protein in this insect was further demonstrated by RNAi analysis, where the downregulation of CAD expression by gene silencing was functionally correlated with a decrease in Cry1Ab susceptibility (Yang *et al.*, 2011).

Regarding mutations affecting the GPI-anchored receptors, the first report that showed that APN1 is associated with resistance was performed in *S. exigua*, where a lack of expression of APN1 correlated with high levels of resistance to Cry1C (Herrero *et al.*, 2005). These data are in agreement with the report that experimental depression of RNAm levels of APN1 by RNAi in *S. litura* resulted in higher tolerance to intoxication with Cry1C toxin (Rajagopal *et al.*, 2002), suggesting that the reduced expression of APN in *Spodoptera* species might indicate resistance to Cry1C toxin. Later, a deletion mutant in the APN1 gene of the *H. armigera* BtR strain, which shows 2971-fold greater resistance to Cry1Ac, was associated with resistance in this insect (Zhang *et al.*, 2009). In another report, two mutations in the aminopeptidase-P gene of the *O. nubilalis* Cry1Ab-resistant ECB strain (3100-fold greater resistance) were identified by expressed sequence tag analysis and these mutations were associated with resistance in different resistant *O. nubilalis* strains (Khajuria *et al.*, 2011). The participation of this protein in 3d-Cry toxin action was further demonstrated by RNAi assays, where silenced larvae in the *Onapp* gene were highly tolerant to Cry1Ab toxin, confirming the participation of this aminopeptidase protein in action of the toxin (Khajuria *et al.*, 2011). Finally, it was reported that in the *Trichoplusia ni* resistant strain GLEN-Cry1Ac-BCS (1000-fold greater resistance to Cry1Ac), the APN1 was significantly downregulated at the protein level (0.11 relative to the susceptible strain) and transcript level (0.026). The 110-kDa APN protein was not detected by Western blot in the resistant strain. The downregulation to APN1 expression was genetically linked to the resistant phenotype but was not linked to mutations in the APN1 gene, suggesting that an additional mutation is responsible for the low expression of APN1 by a post-translational modification event (Tiewsiri & Wang, 2011). Later, a mutation in the ABCC2 transporter was identified in this resistant strain, as described below (Baxter *et al.*, 2011).

Regarding the ALP receptor, its role as a putative binding protein was inferred from analysis of the YHD2 resistant

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strain from *H. virescens*, where it was shown that resistance to Cry1Ac was linked to lower expression levels of a specific binding protein that was later identified as ALP (Jurat-Fuentes *et al.*, 2002). Recently, reduced levels of midgut membrane-bound alkaline phosphatase (mALP) were found to be a common feature in strains of Cry-resistant *H. virescens*, *H. armigera* and *Spodoptera frugiperda* when compared with susceptible larvae (Jurat-Fuentes *et al.*, 2011).

Recently, mutations in a different protein were reported in three different lepidopteran insects included laboratory-selected Cry1Ac-resistant *H. virescens* YEE and YHD3 strains and two field-evolved Cry1Ac-resistant larvae of the *P. xylostella* NO-QA strain, originally isolated from Hawaii and *T. ni* GlenBtR and GipBtR strains isolated from greenhouses in Canada. In these resistant insects, the resistance mechanism was genetically linked to mutant alleles of ABCC2 (Gahan *et al.*, 2010; Baxter *et al.*, 2011). In *H. virescens* three different resistant strains were analyzed: YFO affected in the cadherin gene only; YHD3 affected in the cadherin and ABCC2; and YEE affected only in the ABCC2 transporter. The YDH3 and YEE mutants showed a deletion of 22 bp in exon 2 of ABCC2 (Gahan *et al.*, 2010). YHD3 was 10 000-fold more resistant, and was able to grow in 200 μg Cry1Ac per mL of diet, while the YFO population showed lower resistance levels, as it was able to grow on a diet containing at most 5 μg Cry1Ac toxin per mL (a concentration of toxin 40 times lower than YHD3). The YEE strain, affected only in the ABCC2 transporter, was at least 10 times more resistant than the cadherin-affected YFO strain; strain YEE was able to grow in a diet containing 50 μg mL^{-1} Cry1Ac toxin. These data indicated that mutations in ABCC2 transporter generate higher levels of resistance to 3d-Cry toxins than cadherin mutations and differently affected the binding of 3d-Cry toxins to BBMV from *H. virescens* (Gahan *et al.*, 2010). In the case of resistant *P. xylostella* strain NO-QA, a 30-bp deletion in exon 20 of the ABCC2 transporter was identified, which was predicted to remove the final transmembrane and C-terminal regions of the ABCC2 transporter, suggesting that the ATP-binding loop should be nonfunctional, leaving the channel in the closed state (Baxter *et al.*, 2011). The Cry1A-resistant population of *P. xylostella* from Pennsylvania, the PEN strain, shares the genetic locus associated with resistance with the NO-QA strain, suggesting that the PEN strain is also affected in the ABCC2 transporter (Tabashnik *et al.*, 1997). In *T. ni* the mutation that is responsible for resistance of GlenBtR and GipBtR strains is also linked to the ABCC2 transporter (Baxter *et al.*, 2011).

The ABC transporters are found ubiquitously in all species studied (Dean *et al.*, 2001; Labbé *et al.*, 2011). These are integral membrane proteins that bind ATP and use the energy of ATP hydrolysis to transport substrates across the membrane. Eight subfamilies (A–H) have been described in mammals and insects (Dean *et al.*, 2001; Labbé *et al.*, 2011). A subset of the ABC subfamily C possesses an additional N-terminal domain composed of five transmembrane helices and members of this subset are known as long multidrug-resistant proteins (MRPs); ABCC2 belongs to this subset and is also called MPR2. In mammals, ABCC2 is predominantly expressed in excretory organs and physiological barriers, such as epithelial cells, and was localized in liver, kidney and intestine (Payen *et al.*, 2002). ABCC2 transporters are involved in ion transport and toxin secretion, being able to transport different substrates such as xenobiotics and heavy metals, conferring resistance to antibiotics, chemotherapeutic drugs and herbicides (Dean *et al.*, 2001). In zebra fish, highest expression of the ABCC2 gene was found in intestinal cells (Long *et al.*, 2011). In insects, the ABC transporters have been implicated in uric acid metabolism, development and insecticide resistance (Labbé *et al.*, 2011). In *Bombyx mori*, 51 putative ABC transporter genes and 15 ABCC transporter genes have been identified (Liu *et al.*, 2011).

The ABC transporters have not been described as binding sites for Cry toxins and the direct binding of Cry toxins to ABCC2 transporters from *H. virescens*, *T. ni* and *P. xylostella* has not been investigated. However, mutation in the ABCC2 transporter in the *H. virescens* YEE strain correlated with a loss of Cry1Ab and Cry1Ac binding to BBMVs from this strain. In fact, strains YEE and YFO showed a complementary pattern; strain YFO, affected in the ABCC2 transporter, still bound Cry1Aa, whereas strain YFO, which is affected only in the cadherin gene, lost the ability to bind Cry1Aa and was able to bind Cry1Ab and Cry1Ac (Gahan *et al.*, 2010). In *T. ni* strain GlenBtR, the binding of Cry1Ab and Cry1Ac is also affected (Wang *et al.* 2007). However, this strain has an additional mutation in a trans-acting factor that reduced the transcript levels of APN1 (Janmaat *et al.*, 2004; Tiewsiri & Wang, 2011), and therefore it is difficult to judge if reduced binding of Cry1Ab and Cry1Ac to BBMVs from this strain is due to the mutation in the ABCC2 transporter or to the low expression of APN1 protein. Finally, in *P. xylostella* strain NO-QA, binding data relating to Cry1Ab and Cry1Ac are not as clear, with two studies reporting that this strain is affected (Ferré *et al.*, 1991; Tabashnik *et al.*, 1994b) whereas two other studies showed that Cry1Ac and Cry1Ab were able to bind to immobilized BBMVs from NO-QA in SPR assays or to the microvilli of midgut tissue sections of NO-QA, respectively (Escriche *et al.*, 1995; Masson *et al.*, 1995b). It is clear that mutations of the ABCC2 transporter are

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responsible of for the high resistance levels to Cry toxins in these three lepidopteran species but the exact role of the ABCC2 transporters in the mechanism of action of Cry toxins remains to be elucidated.

Strategies to overcome resistance to 3d-Cry toxins

High levels and constitutive expression of 3d-Cry proteins in *Bt* plants represent a strong pressure for selection of insect populations with increasing resistance to 3d-Cry toxins. As resistance to 3d-Cry proteins in most cases has been shown to be a recessive trait, in many countries a refuge strategy has been used to avoid selection of *Bt*-resistant populations. This strategy proposes the use of refuge zones, where non-*Bt* crops are cultivated adjacent to *Bt* plants. This procedure aims to maintain a population of susceptible insects that will be able to mate with resistant insects, resulting in insect heterozygous progeny that are susceptible to the toxin, thus delaying the appearance of resistance in the field. This practice is in part responsible for forestalling *Bt* resistance in insects, even after 15 years of extensive use of *Bt* crops. Modeling studies have shown that the refuge strategy has been successful in delaying appearance of resistance of *P. gossypiella* to *Bt*-cotton in the United States and explains the appearance of resistance of the same insect species to *Bt*-cotton in India (Tabashnik *et al.*, 2010). However, despite the use of refuge zones, insect-resistant populations have been found in *Bt*-fields.

The first documented case of resistance to *Bt*-crops was *H. zea* to *Bt*-cotton expressing Cry1Ac in the US (Tabashnik *et al.*, 2008). However, resistance of *H. zea* to *Bt*-cotton is controversial as it was suggested that the definition of resistance as an increase in the frequencies of resistance alleles based on bioassays performed under laboratory conditions without apparent field failures was not precise (Moar *et al.*, 2008). Nevertheless, other cases of resistance to *Bt*-crops followed, such as *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), *P. gossypiella* to *Bt*-cotton expressing Cry1Ac in India (Bagla, 2010) and in China (Wan *et al.*, 2012), and *H. armigera* to *Bt*-cotton expressing Cry1Ac in China (Liu *et al.*, 2010) or Cry2A in Australia (Downes *et al.*, 2010). In Puerto Rico and South Africa, extensive field failures were also observed. These data suggest that resistance to *Bt*-crops is an emerging problem that is likely to endanger this technology if counteractions to solve it are not undertaken.

In addition, resistance to sprayed *Bt*-formulated products have also evolved in the field (McGaughey, 1985; Tabashnik *et al.*, 1994a; Janmaat & Myers, 2003) and in laboratory conditions (reviewed by Ferré & Van Rie, 2002), supporting the suggestion that insects have multiple ways to become resistant to the action of 3d-Cry toxins.

A different strategy to overcome resistance to 3d-Cry toxins is expression of more than one 3d-Cry toxin with a different mechanism of action in the same plant, for instance two Cry toxins that bind to different receptor molecules. The possibility of evolving resistance to two different 3d-Cry toxins is greatly diminished because multiple mutations would be necessary to evolve resistance to both toxins (Bravo & Soberón, 2008). Novel Cry proteins active against important pests or novel engineered Cry proteins with improved insecticidal activities are expected to be introduced into transgenic crops, diminishing the possibility of resistant insects appearing (Bravo & Soberón, 2008). In addition, novel insecticidal proteins produced by other bacteria such as *Serratia*, *Xenorhabdus* and *Photorhabdus* spp. could potentially be used in insect control programs in combination with 3d-Cry toxins from *Bt* (Bravo *et al.*, 2011). In fact, the next-generation *Bt* crops produce more than one Cry toxin, reducing the possibility of the development of resistant insects and controlling species of different insect orders such as *Coleoptera* and *Lepidoptera*. Gene pyramiding of different 3d-Cry toxins such as the *Bt*-cotton Bollgard II (Monsanto, MI), expressing Cry1Ac and Cry2Ab proteins that bind to different receptor molecules, is already available. Another example of *Bt*-plants expressing a different *cry* gene combination is SmartStax (Monsanto, MI and Dow AgroSciences, IN), a *Bt*-corn expressing Cry1F along with Cry2Ab and Cry1Ab.105 (a hybrid toxin containing domain I and domain II of Cry1Ab protein; domain III from Cry1F and the C-terminal region from Cry1Ac protein) as well as two Cry toxins active against coleopteran insects (Cry34Ab/Cry35Ab and Cry3Bb). SmartStax promised to control multiple insect pests and slow the evolution of resistance due to use of a toxin combination with different mechanisms of action, such as 3d-Cry (Cry1Ab and Cry3Bb) and Bin-Cry toxins (Cry34Ab/Cry35Ab). However, the development of resistance to multiple toxins is also possible, as shown in *H. virescens* CXC and KCBhyb strains or *P. gossypiella* BX-R, which are resistant to Cry1Ac and Cry2Aa toxins (Jurat-Fuentes *et al.*, 2003; Tabashnik *et al.*, 2009). In addition, a significant increase in the frequency of Cry2Ab-resistant alleles in Australian field populations of *H. puntigera* due to adoption of *Bt*-cotton expressing Cry1Ac and Cry2Ab (Downes *et al.*, 2010) provides an early warning of the increase in resistant insects in *Bt*-crop fields.

The construction of Cry1AMod toxins that are able to form oligomeric structures in the absence of the CAD receptor have been shown to be able to kill a *P. gossypiella*-resistant population that as mentioned above was shown

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to be linked to mutations in the cadherin gene. Cry1A-Mod toxins also killed *M. sexta* larvae silenced in cadherin gene expression by RNAi that showed high tolerance to Cry1Ab intoxication (Soberón *et al.*, 2007). A follow-up study showed that Cry1AMod toxins were effective to reduced resistance levels in resistant populations of six other species of major crop pests, *P. xylostella* strain NO-QA, *O. nubilalis* strain ECB, *D. saccharalis* strain Cry1Ab-RR, *H. armigera*, *T. ni* strains GlenBtR and Gip-BtR, and *H. virescens* strains YEE and YHD3 (Franklin *et al.*, 2009; Tabashnik *et al.*, 2011). As mentioned above, these resistant populations have different genetic bases of resistance, suggesting that Cry1AMod toxins have the potential to counter insect resistance when resistance is linked to mutations affecting cadherin expression, but also counters resistance linked to other genes such as mutations in the ABCC2 transporter gene and OnAPP. Interestingly, Cry1AMod toxins were not particularly effective against the *H. virescens* YFO population containing a mutation only in the cadherin gene (Tabashnik *et al.*, 2011), probably due to a loss of potency of Cry1A-Mod toxins in the different lepidopteran insects analyzed compared with the wild-type toxin. In *H. virescens*, the loss of potency of Cry1AcMod toxin is so important that the Cry1AcMod toxin is not able to reduce the resistant levels of strain YFO, which shows moderate resistance levels, while Cry1AcMod is effective against the double ABCC2-cadherin mutant, YHD3 resistant strain, which shows much higher resistance levels than the single cadherin or ABCC2 mutants. The decrease in potency of Cry1AMod against susceptible lines when compared with native Cry1A toxins was not related to toxin stability against proteases and the reason for the loss of toxicity remains to be determined (Tabashnik *et al.*, 2011). Note that the loss of potency of Cry1AMod toxins varies among different insect species and in *M. sexta* larvae no loss of potency was reported against Cry1AbMod toxin, which showed similar toxicity as the native Cry1Ab toxin (Muñóz-Garay *et al.*, 2009; Tabashnik *et al.*, 2011). A possible explanation is that deletion of the amino-terminal region of Cry1A toxins in Cry1AMod might result in subtle conformational changes in epitopes involved in toxin interaction with additional receptors such as APN and ALP, reducing the *in vivo* insertion of oligomers into the membrane. Another possible explanation could be that, *in vivo* the Cry1AMod toxins might have a reduced rate of oligomer-forming ability than native toxins which may suggest that cadherin interaction is more effective in inducing oligomerization in close contact with GPI-anchored receptors, APN and ALP, and to the membrane. Finally, it is possible that the cadherin-induced cell death by intracellular signaling could not be triggered by Cry1AMod toxin and in some insects these intracellular effects could be more important for toxicity than in other insects such as *M. sexta*. Regardless, the fact that Cry1A-Mod toxins are highly effective in overcoming resistance of insect populations affected in the ABCC2 transporter may indicated that the role of this transporter in the mechanism of action of Cry toxins is involved in promoting oligomerization of the toxin. Thus, a toxin that is able to form oligomers in the absence of receptors is able to overcome resistance to resistant colonies affected in the ABCC2 transporter. Oligomerization of 3d-Cry toxins is a crucial and limiting step in the mechanism of action of 3d-Cry toxins and if Cry1AMod toxins enhance oligomer formation close to the brush border of epithelial cells the probability of membrane insertion may increase, thus explaining the ability of Cry1AMod to overcome different mechanisms of resistance. Overall, these results suggest that the mode of action of Cry1A toxins involves additional molecules that may have a role as toxin receptors and that Cry1AMod toxins have the potential to counter resistance based on different mechanisms (Tabashnik *et al.*, 2011).

Finally, it is important to mention that the Cyt1Aa toxin, a different toxin produced by some *Bt* strains, proved to be effective in overcoming resistance to different 3d-Cry toxins such as Cry4Aa, Cry4Ba and Cry11Aa in resistant populations of the mosquito larvae *Culex quinquefasciatus* (Wirth *et al.*, 1997). Cyt1Aa has low toxicity to the mosquito larvae but it synergizes the toxicity of 3d-Cry toxins, improving the insecticidal effect of the mixture (Crickmore *et al.*, 1995). It was proposed that Cyt1Aa might be functioning as a Cry-receptor as these proteins interact with high affinity (K_d = 0.4 nM) and binding of Cyt1A to Cry11Aa induced the oligomerization and pore formation activity of Cry11Aa toxin (Pérez *et al.*, 2005, 2007). Furthermore, it was shown that mutations in the binding regions of these proteins that affected their binding interaction severely reduced their synergistic activity (Pérez *et al.*, 2005, 2007).

Cyt toxins are mostly found in *Bt* bacteria that are active against mosquitoes in combination with different dipteran-specific 3d-Cry toxins. Cyt toxins are not able to synergize the toxicity of other 3d-Cry toxins that are active against different insect orders such as *Coleoptera* or *Lepidoptera*. It was reported that Cyt1Aa was toxic to the colepteran larvae *Chrysomela scripta* (Federici & Bauer, 1998) and Cyt1C to the coleopteran pests *Leptinotarsa decemlineata*, *Tribolium castaneum* and *Diabrotica* spp. (Rupar *et al.*, 2000). It was shown that Cyt1Aa was able to suppress resistance to Cry3Aa in *C. scripta* (Federeci & Bauer, 1998). It would be interesting to determine if these Cyt toxins interact with the coleopteran-specific 3d-Cry toxins and are able to synergize their toxicity. Understanding the mechanism of action and synergism of Cyt

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toxins is extremely important because they may represent a great potential to deal with insect resistance.

Concluding remarks

Bt 3d-Cry toxins are valuable tools for insect control, as sprays or expressed in transgenic plants. The appearance of resistant insects could compromise this technology. However, only a limited number of Cry proteins are now produced in transgenic crops or sprays. Thus, screening of novel 3d-Cry proteins that show toxicity against insect pests is likely to provide new *cry* genes useful for insect control either as sprays or in transgenic plants.

Understanding the mode of action of 3d-Cry toxins and how insects respond to the attack of Cry proteins will allow the development of new, more efficient *Bt* crops and spray products. In this review we have discussed data showing that oligomerization of 3d-Cry toxins is a key limiting step in the toxicity of these toxins. Thus, Cry1A-Mod toxins, which readily form oligomers, have been shown to counter different mechanisms of resistance in lepidopteran insects. Cry1AMod toxins are just one example of the toxin modifications that were based on the basic understanding of the toxin's mechanism of action. To use the Cry1AMod toxins in the fields, either as transgenic crops or sprays, it is necessary to demonstrate that these proteins are nontoxic to other organisms. We have already demonstrated that Cry1AMod toxins are selective, as they still require contact with the APN and ALP receptors to be lethal. They are not toxic to other lepidopteran insects such as *Agrotis ipsilon* (another corn pest that is not susceptible to Cry1A toxins), and to species of other insect orders such as the dipterans *Aedes aegypti* and *Anopheles albimanus*, or against coleopteran pests, such as *Leptinotarsa texana* and *Tribolium castaneum* (A. Bravo, unpublished data). We have also shown that Cry1AMod toxins are stable and efficiently produced when expressed in transgenic tobacco plants and that these plants are able to kill wild-type and cadherin-silenced *M. sexta* larvae (Porta *et al.*, 2011).

Bt crops are considered a friendly environmental technology, greatly reducing the dependence on chemical insecticides and having a positive impact on the environment. It is anticipated that transgenic plants may be used for a longer time than expected, as novel technologies such as provided by Cry1AMod toxins have great potential to counter resistant insects in the field.

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