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Disabled insecticidal proteins: A novel tool to understand differences in insect receptor utilization

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ABSTRACT

The development of insect resistance to pesticides via natural selection is an acknowledged agricultural issue. Likewise, resistance development in target insect populations is a significant challenge to the durability of crop traits conferring insect protection and has driven the need for novel insecticidal proteins (IPs) with alternative mechanism of action (MOA) mediated by different insect receptors. The combination or "stacking" of transgenes encoding different insecticidal proteins in a single crop plant can greatly delay the development of insect resistance, but requires sufficient knowledge of MOA to identify proteins with different receptor preferences. Accordingly, a rapid technique for differentiating the receptor binding preferences of insecticidal proteins is a critical need. This article introduces the Disabled Insecticidal Protein (DIP) method as applied to the well-known family of three-domain insecticidal proteins from *Bacillus thuringiensis* and related bacteria. These DIP's contain amino acid substitutions in domain 1 that render the proteins non-toxic but still capable of competing with active proteins in insect feeding assays, resulting in a suppression of the expected insecticidal activity. A set of insecticidal proteins with known differences in receptor binding (Cry1Ab3, Cry1Ac.107, Cry2Ab2, Cry1Ca, Cry1A.105, and Cry1A.1088) has been studied using the DIP method, yielding results that are consistent with previous MOA studies. When a native IP and an excess of DIP are co-administered to insects in a feeding assay, the outcome depends on the overlap between their MOAs: if receptors are shared, then the DIP saturates the receptors to which the native protein would ordinarily bind, and acts as an antidote whereas, if there is no shared receptor, the toxicity of the native insecticidal protein is not inhibited. These results suggest that the DIP methodology, employing standard insect feeding assays, is a robust and effective method for rapid MOA differentiation among insecticidal proteins.

1. Introduction

Insect-protected row crops expressing insecticidal proteins (IPs) derived from the entomopathogenic bacterium *Bacillus thuringiensis* (Bt) have transformed farming practices in many countries (Abrol and Shankar, 2012). The insecticidal traits resulting from transgene expression of IPs provide these crops with robust and effective protection from insect herbivory, a benefit that even extends to non-transgenic crops grown in proximity to the transgenic crops, due to area-wide insect pest suppression (Tabashnik, 2010). Bt proteins expressed in plants may be used in their native form or after considerable engineering and improvement (Siebert et al., 2012; Koch et al., 2015; Gowda et al., 2016; Badran et al., 2016). When ingested by a susceptible insect these proteins become activated by gut proteases, bind to cognate receptors in the insect gut, and form transmembrane pores that eventually kill the insect (Vachon et al., 2012; Pardo-Lopez et al., 2013). As is the case for synthetic insecticides, insect pest populations can evolve resistance to insecticidal proteins (Tabashnik et al., 2013; Melo et al., 2016). There are many mechanisms through which resistance could emerge, but the dominant phenomenon seems to be receptor-mediated wherein the resistant insects exhibit alterations in key receptors or lower their expression such that the toxin is no longer recognized (Tabashnik et al., 2013; Melo et al., 2016; Tabashnik et al., 1997). Accepted strategies for curtailing insect resistance development

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include the planting of a non-transgenic refuge and the stacking of insect resistance traits that operate via apparently different mechanisms of action (MOA), which are typically characterized as differences in receptor binding (Devos et al., 2013; Deitloff et al., 2016; Carrière et al., 2015). The discovery of new efficacious IPs that target insect receptors distinct from those that are recognized by currently deployed IPs in commercial insect-protected crops is of paramount importance for the sustainability of this pest management strategy (Zhao et al., 2003; Bates et al., 2005).

Clearly, knowledge of IP MOA is the key component in identifying new IPs for deployment in next-generation insect-protected crops (Granero et al., 1996; González-Cabrera et al., 2003; Estela et al., 2004; Jurat-Fuentes and Crickmore, 2017). There are several published methods used to study IP MOA including ligand blots (Keeton et al., 1998; Banks et al., 2001), *in vitro* binding experiments with labeled IPs (Jakka et al., 2015) and isolated insect gut brush-border membrane vesicle (BBMV) preparations (Martin and Wolfersberger, 1995), pull-down experiments using immobilized or immuno-precipitated IPs (Luo et al., 1997), insect cell-based assays using cloned insect receptor genes (Tanaka et al., 2013; Onofre et al., 2017), and the use of resistant insect colonies (Tabashnik et al., 2000a,b; Herrero et al., 2001; Siqueira et al., 2004). Apart from resistant colonies, the aforementioned methods provide only a partial representation of an IP's receptor preferences due to the highly challenging and complex nature of the systems under study. Given the rapidly increasing numbers of known IPs (Bravo et al., 2012), there is a need for a simple, inexpensive, and robust method for MOA differentiation across multiple insect species. The method presented in this paper, applied to 3-domain Cry endotoxins (3dCry) with similar insecticidal spectrum of activity (Table 1) (Koch et al., 2015; Pardo-Lopez et al., 2013; Bravo et al., 2012; Xu et al., 2014; Adang et al., 2014), fulfills this previously unmet need and enables practical decisions regarding the usefulness of any particular IP in the field without exact knowledge of the actual receptors that mediate toxicity towards the target pests.

2. Materials and methods

2.1. Cloning and expression

The insecticidal proteins used in this study include Cry1Ab3 (Crickmore et al., 2018), Cry1Ac.107, Cry1A.105, Cry1A.1088, Cry1Ca (alias Cry1Ca.pEG1649), and Cry2Ab2 (Crickmore et al., 2018). Cry1Ac.107 differs from the holotype Cry1Ac1 protein (Crickmore et al., 2018) at 6 positions with the following substitutions: Y206H, V227I, A245S, R254T, R289G, and Y313E. Cry1Ca differs from the holotype Cry1Ca1 protein (Crickmore et al., 2018) at four positions with the following substitutions: E124A, R148A, R294A, and D453H. Cry1A.105 and Cry1A.1088 are chimeric Cry1A proteins with domain 3 sequences different from those of either Cry1Ab3 or Cry1Ac.107. All native proteins were expressed in the acrystalliferous (Cry-) Bt host strain EG10650 transformed with the applicable expression plasmids as follows: Cry1Ab3, pMON78408; Cry1Ac.107, pMON74111; Cry1A.105, pMON74124; Cry1A.1088, pMON112761; Cry2Ab2, pMON72815. To make the DIP variants, site-directed mutagenesis was done using the QuikChange kit with 5 ng of the above plasmids and 125 ng mutagenic primers that were complementary at the 5' end and ranged in length between 60 and 100 bp (Liu and Naismith, 2008). Following an initial denaturation at 95 °C for 30 s, the reaction mixtures were submitted to 18 cycles of successive denaturation (95 °C), annealing (55 °C), and extension (68 °C) for 30 s, 1 min, and 8 min, respectively, using a thermal cycler (Applied Biosciences) equipped with a hot-top. The DpnI-treated DNA was transformed into TOP10 *Escherichia coli* cells and the identity of the constructs were confirmed by DNA sequencing. The DIP variants were expressed in the same acrystalliferous (Cry-) Bt host strain EG10650. Single colonies from glycerol stock of each of the *Bt* strains were isolated on Luria Broth (LB) agar plates supplemented with 5 μg/mL chloramphenicol at 30 °C following overnight growth and used to inoculate 2.5 mL LB starter culture containing 3 μg/mL chloramphenicol. Cells were grown at 25 °C on a rotating roller drum overnight, then diluted into 500 mL Bt media (Donovan et al., 1988) containing 3 μg/mL chloramphenicol in a 2 L baffled flask and continued to grow at 20 °C and 250 rpm for 65 h. Sporulation and crystal formation in the culture was verified by phase-contrast optical microscopy of a 2 μL aliquot of the *Bt* culture. Upon confirmation of the presence of crystals, the partially lysed sporulated cells were harvested by centrifugation at 4 °C and 10,000 × *g* for 10 min. The pellet was then re-suspended in 125 mL TX wash buffer containing 10 mM TRIS at pH 7.5 and 0.005% Triton X-100 supplemented with 0.1 mM PMSF, incubated at 250 rpm and 4 °C for 15 min and centrifuged again as above. The resulting pellet was resuspended in 50 mL 1x PBS at pH 7.4 containing 0.1% Triton X-100, 2 mM $MgCl_2$ and 10 units/mL benzonase, incubated at 250 rpm and 4 °C for 2 h and centrifuged at 4 °C and 10,000 × *g* for 10 min. Subsequently, the pellet, containing the spore/crystal mixture, was subjected to resuspension and centrifugation in the above TX buffer twice more.

2.2. Protein purification, activation, and crosslinking

Insecticidal proteins and their variants were solubilized from their respective spore/crystal mixture as described above with the exception that the *Bt* pellet was subjected to extra wash steps in 150 mL 50 mM Na-carbonate at pH 9 and 15 °C for 30min, and following centrifugation again, in 150 mL 50 mM Na-carbonate at pH 10 that was supplemented with 1 mM TCEP, 1 mM PMSF, 1 mM EDTA and 1 mM benzamidine. After shaking incubation at 250 rpm in a buffer composed of 100 mL 50 mM Na-carbonate at pH 11, 5 mM TCEP, 1 mM PMSF, 1 mM EDTA, and 1 mM benzamidine and incubated for 60 min while shaking at 250 rpm and 22 °C. The insoluble debris was pelleted by centrifugation and the full-length toxin was purified on a Q-Sepharose anion exchange column and optionally subjected to trypsinization, follow-up purification to remove the protease. Intact molecular weight determination using Q-Tof LC/MS provided the weight difference between full-length and truncated form of the protein and was used to assess the N- and C-terminus of activated protein core (Jerga et al., 2016). Spot densitometry using BSA standard on SDS-PAGE was used to quantitate the protein samples.

Table 1
Insecticidal proteins used in this study.

NIP'S	Domain.1*	Domain.2*	Domain.3*	Domain.4-6**	Commercial
CrylAb3	**1Ab**	**1Ab**	**1Ab**	**1Ab**	corn
CrylAc.107	**1Ab**	**1Ab**	**1Ac** (40)[a](41)[b]	**1Ac** (89)[a]	cotton/soy
CrylA.105	**1Ab**	**1Ab** (70)[c]	**1Fa** (54)[a](42)[b](39)[c]	**1Ac** (89)[a]	corn
CrylA.1088	**1Ac** (98)[a]	**1Aa** (71)[a,c]	**1Ax** (35)[a](39)[c]	**1Ac** (89)[a]	
Cry2Ab	**2Ab** (25)[a]	**2Ab** (14)[a]	**2Ab** (26)[a]	N/A	cotton/corn

*Domain composition of NIP's based on Bt toxin holotype nomenclature; number in paranthesis indicates the % sequence identity in reference to the corresponding domains: a, NIP vs. CrylAb; b, CrylAc.107 vs. CrylA.105; c, CrylA.105 vs. CrylA.1088

**Domains 4-6 of Cryl NIP's are protoxin domains that get digested in vivo and thus not part of the active ingredient

2.3. Labeling of Cry proteins with electrochemiluminescent tag

Cry1A.1088[Q180C], Cry1A.105[Q180C], Cry1Ab3[Q180C], and the Cry1Ab3-DIP3 cysteine variants were labeled according to manufacturer's specification using MSD Sulfo-TAG™ linked to an iodoacetyl group that can modify solvent exposed cysteine residues in the protein. The cysteine variants were buffer exchanged using a 40 kDa MWCO desalting ZEBA column pre-equilibrated with 50 mM Na-carbonate at pH 10.5 and 250 mM NaCl following manufacturer's specifications. Tributylphosphine to 200 μM final concentration was mixed with the eluted protein sample and the reaction was started with the addition of MSD Sulfo-TAG™ iodoacetamide at a ratio of 2.5 labels to protein. The labeling reaction was monitored by intact molecular weight MS analysis and upon completion, the unincorporated MSD tags were removed by purifying the labeled toxin on a HiPrep 16/60 Sephacryl S200HR gel filtration column (GE) pre-equilibrated with 50 mM Na-carbonate at pH 10.5 and 250 mM NaCl. The chromatography was performed at 4 °C and 1.5 ml/min flow-rate. The labeled toxin fractions consistent with retention times of the monomeric protein were pooled, concentrated to 1–2 mg/mL and subjected to intact molecular weight MS analysis. Samples were aliquoted, flash frozen in liquid nitrogen and stored at −80 °C. Protein concentration was determined by Bradford assay (Bio-Rad).

2.4. Brush border membrane preparation and characterization

Brush border or microvillar membrane (BBM) was prepared using 3rd instar whole larvae via the cation differential precipitation method using 10 mM calcium chloride, which was a modified version of the procedure implemented for *P. brassicae* by Wolfersberger et al. (1987). The total protein concentration in both the BBM sample and the initial insect homogenate was determined by Bradford assay (Bio-Rad) according to manufacturer's protocol and the quality of BBM preparation was evaluated based on a partial biochemical characterization measuring specific alkaline phosphatase (ALP) and leucine aminopeptidase (APN) (Sangadala et al., 1994) enzyme activities of both the BBM fraction and the initial insect homogenate.

2.5. Competitive binding assay

Competitive binding assay was performed according to a standard protocol. Toxin aliquots were removed from the −80 °C freezer, incubated on a 37 °C heat block until thawed and transferred to wet ice. The thawed aliquots of the same toxin lot were pooled and dialyzed in a Slide-A-Lyzer (Thermo Sci., P/N 66380) cassette against 1000x (V/V) buffer containing 50 mM Na-carbonate pH 9, 150 mM NaCl at 4 °C for approximately 2 h, followed by protein concentration determination by the Bradford (Bio-Rad) method. BBM aliquot(s) were removed from the −80 °C freezer and incubated on a 37 °C heat block until thawed and then diluted to 0.01 mg/mL (total protein concentration) in 1x PBS at pH 7.4 on wet ice. In order to solution coat MSD electrodes with insect gut proteins, a 10 μL aliquot of this BBM mixture (100 ng gut proteins) was pipetted into the wells of a 384-well bare carbon electrode MSD plate (Meso Scale Discovery Multi-Array™[1] eledctrochemiluminescence plate-based technology), which was then covered with Biomek aluminum foil, centrifuged at 900 × *g* for 10 s and incubated on a titer plate shaker at 500 rpm and 4 °C for ~ 3 h. The remaining mixture was vacuum-aspirated and the immobilized gut proteins were washed once with 75 μL PBS at pH 7.4. The unoccupied sites in the wells of the MSD plate was blocked by 75 μL 1000 ppm fatty acid free BSA in PBS at pH 7.4 incubated in the wells at 500 rpm for 120 min. The excess BSA was removed by vacuum aspiration and the wells were washed once with 75 μL PBS at pH 7.4. Mixtures containing fixed amount of labeled toxin and increasing concentration of non-labeled toxin were prepared in a 96-well dilution plate via serial dilution in a buffer containing 50 mM Na-carbonate at pH 9.0, 150 mM NaCl and 1000 ppm BSA; 12.5 μL aliquots were transferred to the 384-well MSD plate, centrifuged at 900 × *g* for 10 s and incubated on a Jitterbug-2 plate shaker at 25 °C and 500 rpm for 60 min. The unbound toxins were removed from the wells by vacuum aspiration and washed three times with 75 μL PBS at pH 7.4 in succession. An aliquot of 20 μL Buffer P (proprietary buffer from Meso Scale Diagnostics) at 1x was added per well of the MSD plate, centrifuged at 900 × *g* for 10 s and incubated on a Jitterbug-2 plate shaker at 25 °C and 500 rpm for 20 min. Then the MSD plate was loaded in a Sector Imager 6000 plate reader (Meso Scale Discovery), and the counts per well were collected.

[1] ™ Multi-array is a trademark of Meso Scale Diagnostics, LLC.

2.6. Insect bioassay

Artificial diet feeding assays were conducted with the following lepidopteran species: tobacco hornworm (THW, *Manduca sexta* Linnaeus), corn earworm (CEW, *Helicoverpa zea* Boddie), European corn borer (ECB, *Ostrinia nubilalis* Hübner), tobacco budworm (TBW, *Chloridea virescens* Fabricius, formerly *Heliothis virescens* Fabricius), southwestern corn borer (SWC, *Diatraea grandiosella* Dyar), and soybean looper (SBL, *Chrysodeixis includens* Walker). Insect eggs were obtained from laboratory colonies: CEW and TBW from Benzon Research (Carlisle, PA), ECB, SWC, and SBL from Monsanto Company (Union City, TN), and THW from Carolina Biological Supply Company (Burlington, NC). To conduct the feeding assays, 200 μL of an artificial, agar-based diet was dispensed into each well of a 96-well bioassay plate and allowed to cool and solidify. Protein samples (20 μL) were overlaid onto the diet surface and the diet allowed to dry to remove excess moisture. Individual wells containing the treated diet were infested with neonate larvae (< 24 h post-hatch), targeting one larva per well. Plates were sealed with Mylar® film, ventilated with an insect pin, and incubated for 5 days in an environmental chamber at a target temperature of 27 °C, a target relative humidity of 60%, and a photoperiod of 14 h of light and 10 h of dark. Toxin efficacy was evaluated based on either insect mass, mortality or stunting at day 5. Statistical analyses were performed using multiple comparisons after ordinary one-way ANOVA and post-hoc Tukey test ($\alpha = 0.05$) using GraphPad Prism (GraphPad Software Inc.).

2.7. Structure determination

The purified sample of the Cry1Ab-DIP tryptic core protein containing the I109C and E129C substitutions was treated with iodoacetamide (IA) using a 10X molar amount of IA. Diffraction-quality crystals were obtained from a protein sample containing 0.3 mM protein, 3 mM IA, 30 mM carbonate-pH 10, and 100m NaCl. Plate-like crystals that grew in rosette clusters were obtained using a precipitant that was 0.1 M MES-pH 6.0, 14% (w/V) PEG6K. A 2.2 Å data set was collected remotely at the SER-CAT 22-ID beam line in the APS Synchrotron at Argonne National Labs. These data were reduced using the HKL package (Otwinowski et al., 1997). The crystal was space group C2, with a = 122.9 Å, b = 50.0 Å, c = 97.4 Å, and a β = 111.1°. The structure was solved by the molecular replacement method using the Phaser package (McCoy et al., 2007) in CCP4i (Collaborative, 1994) with a prior 2.6 Å Cry1Ab-based structure. There was one molecule in the asymmetric unit. Refinement was performed using Refmac5 (Murshudov et al., 1997), and map-fitting was done using Coot (Emsley and Cowtan, 2004). The current structure has an R-work/R-free = 20.7%/26.7% and extends from Gly32 to Arg619.

The purified sample of the Cry1Ca-DIP tryptic core protein containing the I87C and F152C substitutions yielded X-ray diffraction quality crystals from a precipitant solution that was 20% (w/v) PEG 8000, 0.1 M Tris-HCl (pH 8.5) buffer, 0.1 M MgCl2, 20% (w/v) PEG 400. A 3.0 Å data set was collected remotely at the SER-CAT 22-ID

beamline. The crystals are space group P212121, with a = 166.8 Å, b = 240.2 Å, c = 242.7 Å, and all angles 90°. The structure was solved by the molecular replacement method, using CCP4i (Collaborative, 1994) protocols noted previously, with a Cry1Aa-based phasing model (Grochulski et al., 1995). There are six molecules in the crystallographic asymmetric unit, and they are arranged as a dimer of two trimers. Crystallographic refinement and map-fitting were done using procedures cited above for the Cry1Ab-DIP tryptic core work. The current structure has R-factors of R-work = 21.9% and R-free = 25.7% extends from Gly32 to Asp616. A particularly noteworthy feature in the molecule is a disulfide between Cys87 to Cys152, and there was good-density in all six molecules of the asymmetric unit to justify this.

Crystals were obtained from trypsin-treated Cry1A.105 protein at 6 mg/mL using the hanging drop vapor diffusion crystallization method, which was conducted in Limbro plates with a reservoir solution that was 5% (w/v) PEG4000, 0.1 M sodium citrate-pH 6.0, and 10% (V/V) isopropanol. Micro-seeding was required to obtain crystals sizable for X-ray data collection. Droplets containing 2 μL protein, 4 μL reservoir solution, and 0.5 μL of 0.01x strength seed stock worked best. A 96% complete, 3.0 Å resolution data set was collected on-site at the SER-CAT 22-ID beamline. These data were reduced using the HKL package (Otwinowski et al., 1997). The trypsinized Cry1A.105 crystals are space group C2, with a = 124.4 Å, b = 49.4 Å, c = 103.7 Å, and a β = 108.7°. Molecular replacement phasing was conducted using the Phaser program (McCoy et al., 2007) in the CCP4 package (Collaborative, 1994), using the PDB available Cry1Aa structure (Grochulski et al., 1995) as a phasing model. There was one protein molecule in the crystallographic asymmetric unit. Iterative map-fitting and refinement were initially done using programs O (Jones et al., 1991) and X-PLOR (Brunger, 1992), respectively, and were later done using programs Coot (Emsley and Cowtan, 2004) and Refmac5 (Murshudov et al., 1997). The structure extends from Tyr33 to Ala609, and there are two gaps between N372 and N376, and between N516 and P520. The structure was refined using 98–3.0 Å data, and it has R-factors of R-work = 20.0% (95% of data), and R-free = 30.0% (5% of data).

2.8. Sequence comparison

The primary sequence of the insecticidal proteins in each domain was determined based on domain boundaries in the crystal structure, and sequence comparisons were made using available software (Edgar, 2004).

3. Results

3.1. Homologous competition between Cry1Ab3 and disabled versions of Cry1Ab3

Mutations in domain 1 of Cry1A insecticidal proteins have been shown to disable ion channel/pore forming activity as well as insecticidal activity, consistent with the recognized mode of action of these insecticidal toxins (Fig. 1) (Vachon et al., 2004; Girard et al., 2008; Girard et al., 2009; Schwartz et al., 1997; Alzate et al., 2006; Rodríguez-Almazán et al., 2009). We hypothesized that a sub-class of these variants should retain the proteolytic stability and receptor binding properties of the native Cry protein, and that these variants should compete with their native counterpart in insect feeding assays resulting in suppression of insecticidal activity in a dose-dependent manner. Based on protein structure analysis as well as on the previously reported inactivating substitutions in Cry1, Cry2 and Cry4 proteins, we selected three Cry1Ab3 variants to study: R99E (Cry1Ab3-DIP1), E129K/D136N (Cry1Ab3-DIP2), and I109C/E129C (Cry1Ab3-DIP3). The DIP1 and DIP2 variants were previously described by Rodríguez (Rodríguez-Almazán et al., 2009), and DIP2 was reported to act as "dominant negative" variant that inhibits Cry1Ab activity towards *Manduca sexta* (tobacco hornworm, THW) via oligomerization with native Cry1Ab monomers, resulting in a loss of ion channel or pore-forming activity. The dominant-negative (Herskowitz, 1987) effect on Cry1Ab activity was observed at a 1:1 molar ratio, suggesting that the variant protein can poison the native oligomer (Carmona et al., 2011). Based on the reported crystal structure of Cry1Ac [4W8J, PDB] (Evdokimov et al., 2014), the DIP3 variant was designed such that the surface-exposed cysteine residues are optimally positioned for intramolecular N,N′-ethylene-bis(iodoacetamide) chemical crosslinking of two domain 1 helices with the intent to inhibit structural rearrangement reported to be critical for pore-formation (Fig. 1) (Ludueña and Roach, 1981; Gazit et al., 1998). Insect bioassays were run with the native Cry1Ab3 protein and the disabled Cry1Ab3 proteins to 1) establish concentrations of Cry1Ab3 required to cause mortality or significant stunting of each insect species tested, 2) confirm that the disabled proteins retain little or no insecticidal activity at high concentrations, and 3) assess whether the disabled proteins can suppress the insecticidal activity of Cry1Ab3. Interestingly, the disabled Cry1Ab3-DIP3 protein was observed to be completely inactive towards multiple lepidopteran species in the absence of any cross-linking. Accordingly, subsequent studies employed the non-crosslinked version of Cry1Ab3-DIP3. The wild-type Cry1Ab3 protein was mixed with each disabled protein at molar ratios of 1:1 and 1:10 and tested in insect bioassay to determine if the disabled protein could suppress the activity of the Cry1Ab3 protein. Fig. 2 shows the results of a bioassay with *Manduca sexta*. In this example, the 0.04 ppm sample of Cry1Ab3 caused severe stunting (mass reduction) of the larvae while the 4 ppm samples of the disabled Cry1Ab3 proteins showed no significant activity. At a 1:1 ratio, the disabled proteins had little or no effect on Cry1Ab3 activity, although the Cry1Ab3:Cry1Ab3-DIP2 mixture was less active than Cry1Ab3 alone. At a 1:10 ratio, both Cry1Ab3-DIP2 and Cry1Ab3-DIP3 effectively suppressed the activity of Cry1Ab3, while Cry1Ab3-DIP1 was less effective in suppressing the activity of its native counterpart. In another example, using *O. nubilalis* (European corn borer, ECB) as the insect test species, the concentration of Cry1Ab3 was sufficient to cause 100% mortality of ECB larvae, allowing us to record suppression of mortality (Fig. 3). The data shown in Fig. 3 demonstrate that 1) the three disabled proteins exhibit no significant activity towards ECB when tested at 50 ppm, 2) the three disabled proteins have no significant impact on the activity of Cry1Ab3 when presented in a 1:1 molar ratio, and 3) all three disabled proteins suppress the activity of Cry1Ab3 when presented in a 1:10 molar ratio. Additional studies with *Heliothis virescens* (tobacco budworm, TBW) as the test species demonstrated that 1) Cry1Ab3-DIP1 retained significant activity on TBW at the concentration tested and thus could not be used to suppress the activity of Cry1Ab3, 2) Cry1Ab3-DIP2 and Cry1Ab3-DIP3 exhibit little or no activity against TBW, 3) neither Cry1Ab3-DIP2 nor Cry1Ab3-DIP3 impacts the activity of Cry1Ab3 when presented in a 1:1 molar ratio, and 4) both Cry1Ab3-DIP2 nor Cry1Ab3-DIP3 suppress the activity of Cry1Ab3 when presented in a 1:10 molar ratio (Suppl. Fig. 1). Feeding assays with TBW and other insect species showed that both Cry1Ab3-DIP1 and Cry1Ab3-DIP2 exhibit some insecticidal activity at high concentrations. In addition, Cry1Ab3-DIP2 partially inhibited the insecticidal effect of Cry1Ab3 at a 1:1 ratio by Rodríguez-Almazán et al. (Rodríguez-Almazán et al., 2009). Based on these observations, we decided to use Cry1Ab3-DIP3 for all subsequent studies because it was inactive on all tested insect species and was consistently non-inhibitory to its native counterpart at 1:1 molar ratio, but consistently exhibited mass-action based competition at high molar ratios (1:10–1:25), suggesting that inhibition is caused by competition between the inactive monomeric variant and the active toxin for limited receptor sites. *In vitro* binding assessments demonstrated that Cry1Ab3-DIP3 binds to brush border membranes similarly to its native counterpart (Suppl. Fig. 2). To demonstrate that the Cry1Ab3-DIP3 protein is not capable of forming ion channels, we conducted planar lipid bilayer experiments to compare the tryptic core of Cry1Ab3 with the tryptic

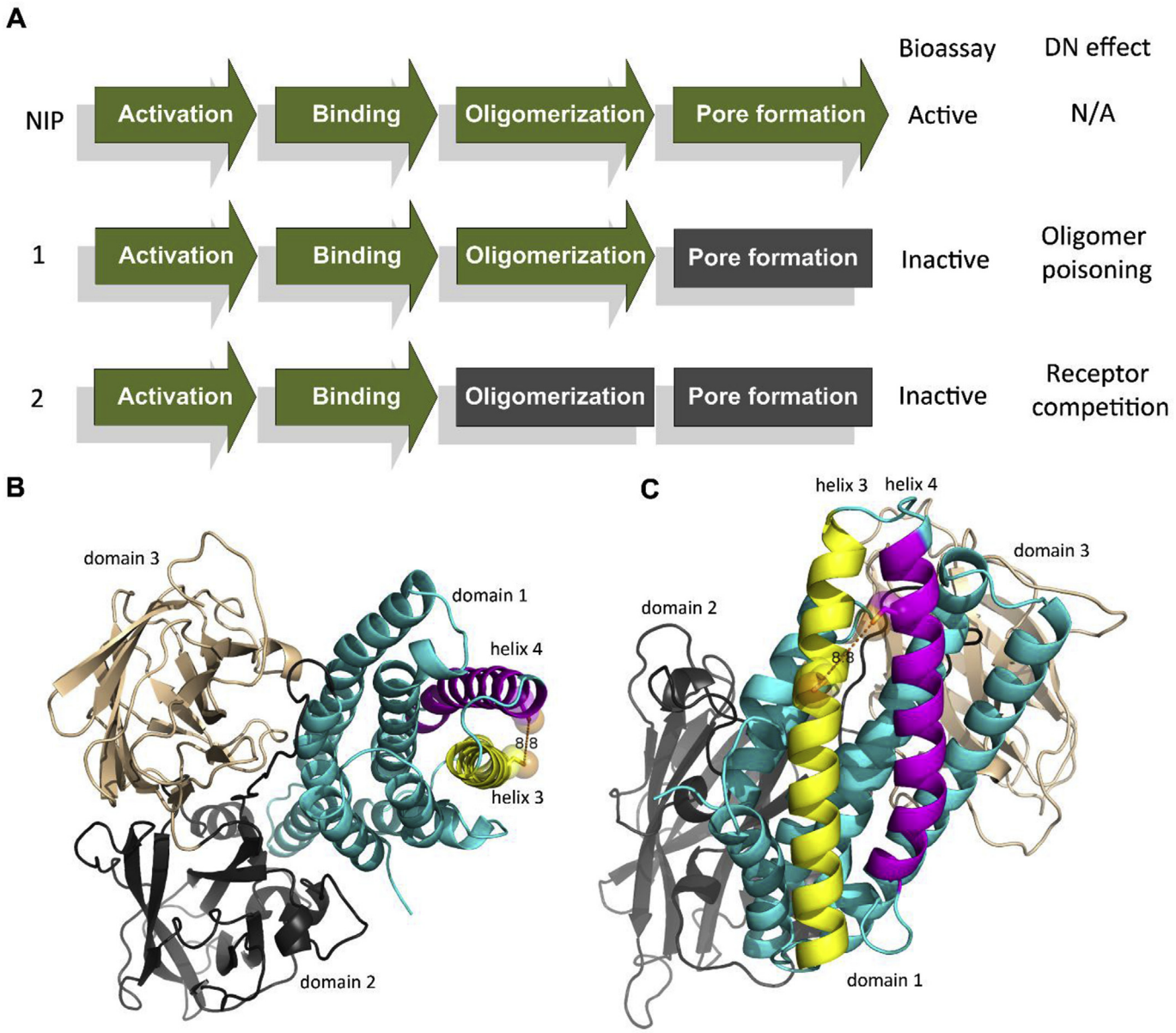

Fig. 1. Model and structure. The mechanistic steps (green arrows) of the three-domain Cry protein MOA model, and the steps that are deficient (grey boxes) in the two archetypes of inhibitory variants **(A)**. Crystal structure of the Cry1Ab3 disabled insecticidal protein (DIP) variant with its two cysteines in domain 1 **(B & C)**.

core of the non-crosslinked Cry1Ab3-DIP3. Ion channel activity was readily detected in the assay with the wild-type protein after 1 h of incubation. In contrast, no ion channel activity was evident after 3 h of incubation with Cry1Ab3-DIP3 tryptic core (Suppl. Fig. 3) consistent with the hypothesis that this protein is disabled with respect to membrane permeabilizing activity.

3.2. Heterologous competition between diverse Cry proteins and Cry1Ab3-DIP3

Further studies assessed the ability of Cry1Ab3-DIP3 to suppress the activity of heterologous Cry proteins including Cry1A.105, Cry1A.1088, Cry1Ca, and Cry2Ab2. In the first study, solubilized preparations of Cry1Ab3-DIP3 and trypsinized + crosslinked Cry1Ab3-DIP3 were tested in a larval feeding assay with the southwestern corn borer (SWC), *Diatraea grandiosella,* a test species that is highly sensitive to all the native proteins included in this study. Both forms of Cry1Ab3-DIP3 were insecticidally inactive but suppressed the activity of the Cry1A proteins Cry1A.105 and Cry1A.1088 towards SWC when presented in diet at a molar ratio of 1:25 native protein: Cry1Ab3-DIP3. The more distantly related Cry1Ca and Cry2Ab2 proteins retained full insecticidal activity when tested against applicable target pests despite the 25-fold molar excess of Cry1Ab3-DIP3 (Fig. 4), consistent with numerous studies showing that these proteins interact with insect midgut receptors distinct from those recognized by Cry1Ab3 (see Discussion). Similar feeding assays with *Manduca sexta* indicate that the results of heterologous competition may vary with the insect species tested. Cry1Ab3-DIP3 suppressed the activity of Cry1Ab3 towards *Manduca sexta* but had no impact on the activity of the Cry1A.105, Cry1A.1088, Cry1Ca and Cry2Ab2 proteins (Suppl. Figure 4).

3.3. Other disabled Cry1A proteins based on the domain 1 mutations in Cry1Ab3-DIP

The Cry1Ac.107, Cry1A.105, and Cry1A.1088 proteins share nearly identical domain 1 sequences with Cry1Ab3 yet contain distinct domain 2 or domain 3 sequences (Suppl. Fig. 5). Variants of these proteins containing the Cry1Ab3-DIP3 I109C-E129C substitutions were

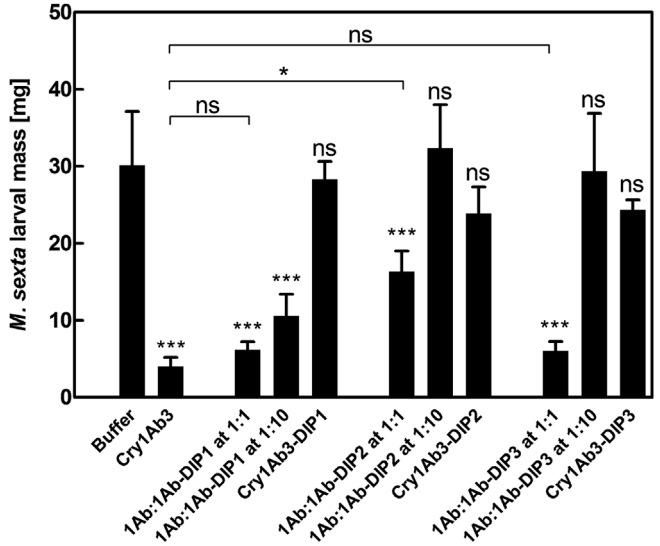

Fig. 2. Disabled Cry1Ab3 proteins suppress the activity of Cry1Ab3 in the *Manduca sexta* feeding assay. Cry1Ab3 protein was tested at a concentration of 0.04 μg/mL and the Cry1Ab3 disabled proteins tested at a concentration of 4 μg/mL. Mixtures contained Cry1Ab3 at 0.04 μg/mL and disabled proteins at 0.04 μg/mL and 0.4 μg/mL, a 1:1 or 1:10 molar ratio of Cry1Ab3 protein to disabled protein, respectively. Mean insect mass was used as a measure of Cry1Ab3 efficacy, and the data were plotted as mean ± SD. Statistical analysis was done using multiple comparisons after ordinary one-way ANOVA and post-hoc Tukey test ($\alpha = 0.05$); the symbols above the bars indicate these results in reference to the buffer treatment (negative control), whereas symbols above the connector lines inform about differences between the connected treatment groups (ns, $P > 0.05$; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

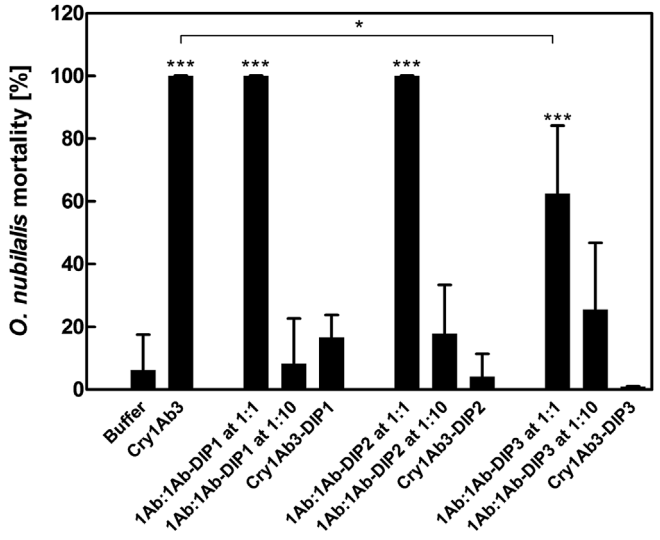

Fig. 3. Disabled Cry1Ab3 proteins suppress the activity of Cry1Ab3 in the *Ostrinia nubilalis* feeding assay. Cry1Ab3 protein was tested at a concentration of 5 μg/mL and the Cry1Ab3 disabled proteins tested at a concentration of 50 μg/mL. Mixtures contained Cry1Ab3 at 5 μg/mL and disabled proteins at 5 μg/mL and 50 μg/mL, a 1:1 or 1:10 molar ratio of Cry1Ab3 protein to disabled protein, respectively. Mean insect mortality was used as a measure of Cry1Ab3 efficacy, and the data were plotted as mean ± SD. Statistical analysis was done using multiple comparisons after ordinary one-way ANOVA and post-hoc Tukey test ($\alpha = 0.05$); the symbols above the bars indicate these results in reference to the buffer treatment (negative control), whereas symbols above the connector lines inform about differences between the connected treatment groups (ns, $P > 0.05$; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

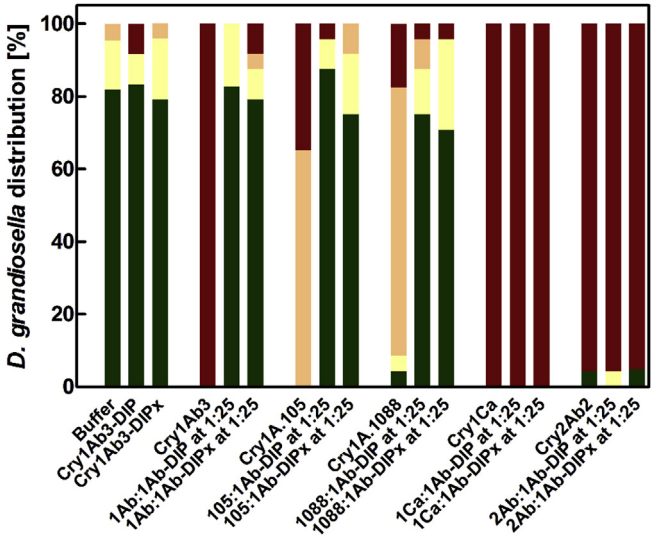

Fig. 4. The protoxin form of Cry1Ab3-DIP3 and the cross-linked (X) trypsin-activated form of Cry1Ab3-DIP3 suppress the activity of Cry1A-related proteins in the *Diatraea grandiosella* feeding assay. Cry proteins were evaluated at a concentration of 5 μg/mL while the disabled proteins Cry1Ab3-DIP3 and Cry1Ab3-DIP3X were evaluated at concentrations of 125 μg/mL and 62.5 μg/mL, respectively, representing a 25-fold molar excess over the native toxin concentration. Insecticidal activity of bioassay treatments was reported based on phenotypic distribution of each insect (technical replicates) across the following four phenotypic categories, dead or stunted more than 75%, red; stunted between 50 and 75%, orange; stunted between 25 and 50%, yellow; stunted less than 25%, green. The bar height is proportional to the percent of *D. grandiosella* insects in each of these phenotypic categories.

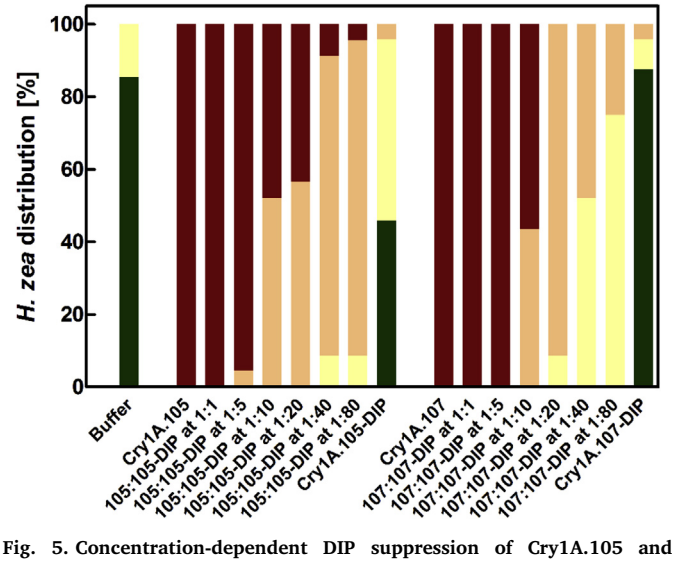

Fig. 5. Concentration-dependent DIP suppression of Cry1A.105 and Cry1Ac.107 activity in feeding assays with *Helicoverpa zea*. The Cry1A.105 and Cry1Ac.107 proteins were evaluated at a concentration of 10 μg/mL with increasing amounts of the disabled Cry1A.105-DIP3 and Cry1Ac.107-DIP3 proteins, respectively. Homologous suppression of activity was observed at a 10:1 molar challenge ratio between competitor (DIP) and native protein. Insecticidal activity of bioassay treatments was reported based on phenotypic distribution of each insect (technical replicates) across the following four phenotypic categories, dead or stunted more than 75%, red; stunted between 50 and 75%, orange; stunted between 25 and 50%, yellow; stunted less than 25%, green. The bar height is proportional to the percent of *H. zea* insects in each of these phenotypic categories.

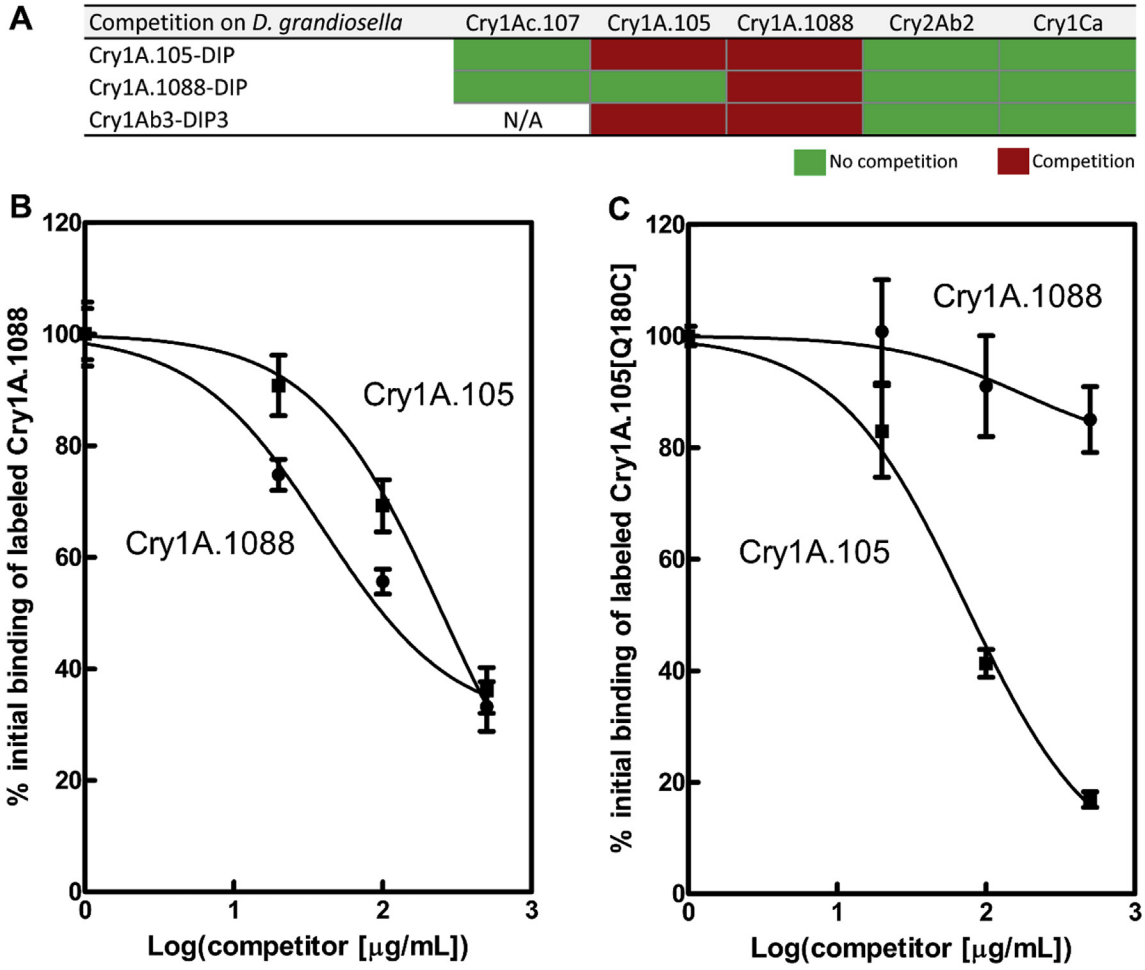

Fig. 6. Concentration-dependent competition DIP assays *in vivo* and brush border membrane toxin binding *in vitro* reveal new MOA on *Diatraea grandiosella*. Heat map indicating assay outcome when insecticidal activity of Cry1Ac.107, Cry1A.105, Cry1A.1088, Cry2Ab2 and Cry1Ca was challenged with three different DIP probes; changes in insecticidal activity in the absence vs. in the presence of each DIP probe, used at 10- to 25-fold molar excess of the native toxin, were recorded as competition when the change is statistically significant (red), or no competition when the change is not significant (green) **(A)**. *In vitro* competition toxin binding to BBM isolated from *D. grandiosella*: **(B)** A fixed dose of labeled Cry1A.1088 was mixed with Cry1A.1088 (●) and Cry1A.105 (■) non-labeled competitors at increasing concentrations and incubated with BBM. Binding is expressed as a percentage of maximum binding of labeled toxin without the non-labeled toxin competitor and is plotted against the logarithm (base 10) of the concentration of competitor. The data points are the mean of eight replicates and the error bars represent the standard error of the means. **(C)** Binding of labeled Cry1A.105 in the presence of increasing concentrations of Cry1A.1088 (●) and Cry1A.105 (■) competitors.

evaluated in insect feeding assays as soluble full-length protoxins and shown to be inactive in the non-crosslinked forms, as was observed with Cry1Ab3-DIP3. In each case, the DIP proteins were shown to exhibit homologous competition with their native IPs. We also tested whether full-length crystalline forms of DIPs could substitute for the soluble full-length forms of IPs in insect bioassay. Sucrose gradient-purified crystal preparations of Cry1Ac.107-DIP3 and Cry1A.105-DIP3 were tested along with crystal preparations of the native IPs in feeding assay with *Helicoverpa zea* (corn earworm, CEW). Both Cry1A.105-DIP3 and Cry1Ac.107-DIP3 exhibited strong competition with their respective native proteins in the CEW assay, with complete suppression of mortality observed at 20-40X molar excess of the DIP (Fig. 5).

3.4. Heterologous competition between Cry1A native and disabled proteins

The studies with Cry1Ab3-DIP3 demonstrated that the activities of Cry1A.1088 and Cry1A.105 can be selectively abrogated depending on the insect species tested. The expanded set of Cry1A DIP probes enabled a study of heterologous competition among different IPs, again using SWC as the test species due to its sensitivity to all of the IPs included in this work. As expected, the Cry1Ab3-DIP3, Cry1A.105-DIP3, and Cry1A.1088-DIP3 proteins each exhibited homologous competition with their respective native IPs and no competition with the more distantly related Cry1Ca and Cry2Ab2 proteins (Fig. 6A). With respect to Cry1A heterologous competition, neither the Cry1A.105-DIP3 nor Cry1A.1088-DIP3 protein exhibited competition with the Cry1Ac.107 protein, suggesting that the unique domain 3 of Cry1Ac.107 (Suppl. Fig. 5) is critical for its SWC activity. Interestingly, we observed that while Cry1Ab3-DIP3 and Cry1A.105-DIP3 suppressed the activity of Cry1A.1088 toward SWC, Cry1A.1088-DIP3 failed to suppress the activity of Cry1A.105 in the SWC feeding assay. This lack of reciprocity was recapitulated in binding assays with SWC brush border membranes (Fig. 6B and C), supporting the conclusion from the feeding assay that these proteins exhibit a partial overlap in receptors.

3.5. Disabled protein variants for Cry2Ab2 and Cry1Ca

Domain 1 sequences of Cry1Ca and Cry2Ab2 are very different from that of the Cry1Ab3 Domain 1 (57.6% and 25.3% identity, respectively), therefore we decided to approach the discovery of disabling mutations for these two proteins in a more generalized manner. Assuming that chemical crosslinking of two proximal helices in Domain

1 may be enough to disable these proteins (and that mutations that fit this criterion may be fortuitously disabled even without crosslinking) we used structural information to select a set of double cysteine mutations for each toxin such that the two thiols are positioned and oriented favorably for EBI crosslinking. Taking into account structural flexibility, we selected residue pairs with β-carbon atoms (Cβ) 7.3–12 Å apart and pointing in the same direction. In cases where reactivity of the proposed amino acid substitutions might be sterically hindered by surrounding bulkier residues, the potentially conflicting residues were mutated to smaller ones (e.g. alanine or serine) depending on the expected hydrophobicity at those positions. With these criteria in mind, 61 Cry1Ca and 44 Cry2Ab2 designs were created and expressed in Bt, and the variants that expressed were tested in insect diet bioassay: the Cry1Ca variants were tested on SWC and the Cry2Ab2 variants were tested on CEW at an applied concentration of 1000 ppm. Given that Cry1A-like disabled toxin candidates were inactive without crosslinking, we tested non-crosslinked Cry1Ca and Cry2Ab2 variants first and identified several promising (i.e. inactive) protein variants for the follow-up homologous competition assay. Cry1Ca-DIP (N98C_D143C) and Cry2Ab2-DIP (G119C_N123A_L156C_R160A) satisfied the criteria for use as disabled proteins for competition studies, exhibiting homologous competition in insect assays with Cry1Ca and Cry2Ab2, respectively, at a high molar excess of the DIP but not at a 1:1 molar ratio. Comparative analysis between Cry1Ca and Cry2Ab2 using their respective DIP probes in the *C. includens* feeding assay showed that the insecticidal activity of these two distantly related three domain toxins is affected only by their respective DIPs (Suppl. Figure 6).

3.6. Disabling mutations and the three-dimensional protein structure of disabled toxins

Members of the three-domain toxin class include proteins with significant structural similarity and differences in receptor utilization is primarily associated with sequence diversity in domain 2/3 receptor epitopes. Beyond functional equivalence tests conducted between native and disabled proteins in insect feeding assays, we also set out to assess whether introduction of domain 1 mutations could alter the structure of these disabled toxin probes. We solved the crystal structure of several Cry1 proteins including two disabled toxins, Cry1Ab3-DIP3 and Cry1Ca-DIP. A structural alignment of Cry1A.105, Cry1Ab3-DIP3 and Cry1Ca-DIP, with the Cry1Aa tryptic core sequence from the 1CIY PDB entry (Grochulski et al., 1995) is shown in Supplementary Figs. 7–10, and the root-mean-square deviation (r.m.s.d.) between matched atoms is as follows: Cry1Ab3-DIP3 (0.69 Å for 3884 atoms), Cry1A.105 (0.74 Å for 3677 atoms), and Cry1Ca-DIP (0.93 Å) suggesting that all of the Cry1 structures align well and display the characteristic three domain features present in Cry1Aa (Grochulski et al., 1995) including the seven-helix bundle of Domain 1 (res.33-253 in Cry1Aa), a prism-shaped Domain 2 (res. 254-461 in Cry1Aa) made up of three antiparallel β-sheets, and a Domain 3 (res.462-609 in Cry1Aa) which is an antiparallel β-sandwich adopting a jelly roll fold.

4. Discussion

The development of resistance to insect-protected crops is an ongoing challenge that requires effective stewardship of the technology as well as the discovery of insecticidal proteins with new modes of action (MOA) for future crop protection needs. Since the development of high-level resistance to insect-protected crops in field populations tends to be receptor-mediated, new insecticidal proteins will be most useful if they utilize receptors distinct from those utilized by insecticidal proteins presently expressed in commercialized crops. Robust and practical methods for differentiating the receptor binding of novel insecticidal proteins are needed because conventional methods (*in vitro* binding assays, isolation of field-relevant resistant colonies, RNAi suppression of receptors) are technically challenging, limited in scope, and are not suitable for high-throughput application.

Herskowitz published a seminal reference (Herskowitz, 1987) that introduced the dominant negative concept and defined that “a dominant negative mutant protein will retain an intact, functional subset of the domains of the parent, wild-type protein, but have the complement of this subset either missing or altered so as to be non-functional”. Given that three-domain Cry proteins form oligomers, an inactive variant capable of interacting with the parent protein will be inhibitory as it causes the formation of non-functional oligomers (Fig. 1A, option 1). Rodríguez-Almazán et al. (Rodríguez-Almazán et al., 2009) reported that the Cry1Ab[E129K/D136N] variant acted as a “dominant negative” variant inhibiting Cry1Ab activity towards *Manduca sexta* (tobacco hornworm) via oligomerization with native Cry1Ab monomers, resulting in a loss of ion channel or pore-forming activity of the mixed oligomer. Herskowitz also indicated that a monomeric protein deficient in oligomerization can also be inhibitory if there is limiting amount of substrate. Bt receptors, which are key in conferring the spectrum of insecticidal activity to three-domain Cry proteins, are displayed on the midgut epithelium and are generally less abundant than the insecticidal proteins used. Thus, a monomeric DIP variant that is deficient in self-oligomerization, but otherwise have unaltered receptor binding domain (s) would compete against its native counterpart on a target insect if it is mixed with the NIP in large excess (Fig. 1A, option 2). Furthermore, such a DIP variant when mixed with a heterologous insecticidal protein (NIP) that shares receptor(s) with the DIP, would reduce the insecticidal activity in a dose dependent manner due to the ensuing receptor competition between NIP and DIP proteins. The method described here relies on the use of three-domain Cry proteins with amino acid substitutions in domain 1 that render the insecticidal protein inactive, presumably due to impairment of ion channel activity. The disabled insecticidal proteins (DIPs) communicated in this study suppressed *in vivo* the insecticidal activity of their homologous native insecticidal protein in a concentration-dependent manner, presumably because they still retain the independent receptor binding specificity associated with domains 2 and/or 3.

It was interesting to observe that the disabled Cry1Ab3-DIP3 protein was completely inactive towards multiple lepidopteran species in the absence of any cross-linking. The crystal structure provided evidence that the two cysteine residues in helices 3 & 4 of domain 1 (Fig. 1) are not oxidized to a disulfide bridge, but rather they comprise free sulfhydryl groups in the I109C/D129C variant. In addition, we also found that both the soluble, pre-proteolyzed form of this toxin and its precursor crystal/spore preparation were disabled. The fact that the crystal/spore preparation of the native Cry1Ab has insecticidal activity on these target lepidopteran pests indicates that the insect lumen in these pests has a reducing environment sufficient to reduce multiple disulfide bridges interconnecting the protoxins in the crystalline form. Thus, it is unlikely that this protein would re-oxidize between helices 3 & 4 in the lumen following ingestion. A more plausible disabling mechanism is the disruption of self-oligomerization (Fig. 1A, option 2). Numerous contributions are already published on elucidating the function of helix 3 and 4 of Cry1A proteins by characterizing single point mutants, and these studies suggest that some of the helix 3 and 4 positions are critical for insecticidal activity and pore-formation. Vachon and Girard et al. (Vachon et al., 2004; Girard et al., 2008, 2009) showed that cysteine mutagenesis of the helix 4 E129 position resulted in loss of bioassay activity as well as loss of pore-formation (osmotic shock) in brush border membrane; later, the same group communicated that E129C has unaltered BBMV binding inferred from the pore-formation assay set up as competition between wild-type toxin and variant. Because their competitive binding assay is set up with a pore-formation assay read-out, the results cannot readily distinguish between competition binding and oligomer poisoning. Additional insights to the same position were published by Rodríguez-Almazán et al. (Rodríguez-Almazán et al., 2009) showing that the E129K variant is inhibiting its native counterpart via oligomer poisoning (Fig. 1A, option

1). Regarding the helix 3 positions, Jimenez-Juarez et al. (2007) reported on a helix 3 mutagenesis study that identified two variants, R99E (used as control in our Cry1Ab-DIP1 study) and Y107E, that lost its insecticidal activity, and were characterized as non-functional oligomers with reduced stability. Taken together, our hypothesis was that helix 3 & 4 comprise an extensive surface for self-oligomerization that is fully disrupted upon stacking key mutations in helix 3 & 4. Single point mutants showed partial reduction of self-oligomerization and therefore a concomitant oligomer poisoning dominant negative effect in competition assays; thus, these probes are not ideal if one wants to observe receptor utilization in isolation. Our studies suggest that the 109C/D129C mutations in Cry1Ab completely disrupt the oligomerization step, given that (i) a large excess of DIP competitor was required to inhibit the homologous native protein, and (ii) Cry1A.105-NIP was not competed by Cry1A.1088-DIP even though these are chimeric proteins that share the same domain 1 whose function is associated with oligomerization and pore-formation step.

Our initial studies with Cry1Ab3 variants demonstrated that Cry1Ab3-DIP3, containing the I109C/E129C substitutions, satisfied our criteria for use as a DIP in MOA studies. The protein exhibited 1) no significant insecticidal activity towards any of the lepidopteran species tested, 2) no detectable ion channel activity in planar lipid bilayer experiments, 3) no apparent differences in susceptibility to processing with trypsin, 4) no significant competition in bioassays with the native Cry1Ab3 protein at molar concentrations of 1:1, and 5) significant competition with Cry1Ab3 in feeding assays with multiple lepidopteran species when presented in a molar excess of ≥ 10. Crosslinking of the engineered cysteine residues with EBI was not required to inactivate the protein, a feature that provides additional uses for the protein as discussed below. This method thus enables researchers to classify Cry proteins or other insecticidal proteins into groups that are likely to share receptor binding sites and to prioritize insecticidal proteins that appear to operate via an independent MOA, as evidenced by the absence of any competition in insect bioassays. Finally, because the method only requires a validated insect bioassay, it is possible to assess competition quickly across a wide range of insect target species.

A comparison of the Cry1A.105, Cry1Ac.107, Cry1A.1088, and Cry1Ab3 tryptic core structures shows near-identical arrangement of residues in Domain 1, which led us to believe that the same amino acid substitutions introduced into Cry1Ab3-DIP3 should work equally well in these proteins. Indeed, these variants (designated Cry1A.105-DIP3, Cry1Ac.107-DIP3, and Cry1A.1088-DIP3) were completely inactive in insect bioassays and showed competitive inhibition of their native protein's activity at high molar ratios. This set of Cry1A proteins containing highly related domain 1 sequences, but distinct domain 2 and/or domain 3 sequences, afforded us the opportunity to evaluate heterologous competition across multiple lepidopteran species. These studies provided insight into the potential utility of these chimeric proteins for use as insect control traits. For example, the strong competition observed between Cry1A.1088 and Cry1A.105-DIP3 in bioassays with SBL, SWC, and CEW suggests that the Cry1A.1088 protein would not provide an additional MOA to transgenic corn or soy events expressing the Cry1A.105 protein. The absence of reciprocity evidenced by the failure of Cry1A.1088-DIP3 to compete with Cry1A.105 (Fig. 6) in the SWC assay suggests that the Cry1A.105 protein recognizes at least one additional receptor that is sufficient for efficacy in this species. Conceivably, more extensive testing of DIPs could enable a complete reconstruction of receptor spectra across commercially relevant insect pests and insecticidal proteins.

Heterologous competition experiments conducted thus far with the Cry1A, Cry1Ca, and Cry2Ab2 proteins and their respective DIPs have demonstrated a lack of competition between the Cry1A proteins and Cry1Ca, between the Cry1A proteins and Cry2Ab2, and between the Cry1Ca and Cry2Ab2 proteins. These results are very much in agreement with the consensus view that Cry1C-, Cry2-, and Cry1A-like proteins represent three distinct MOAs without significant overlap in receptor preferences, based on studies conducted with field-relevant resistant insect colonies, and binding experiments with radioactively labeled toxins to brush-border membrane preparations.

Since chemical cross-linking was not required for inactivation of the DIP variants described in this paper, their use is not dependent on purification or chemical modification. Accordingly, the proteins can be used as solubilized full-length protoxins (Fig. 2), as trypsin-processed core toxins (Fig. 4), or even as crude or purified parasporal crystals (Fig. 5), depending on the specific experimental need. For example, the ease with which full-length DIPs can be produced in *Bt* makes them particularly suitable for high-throughput screens in which novel insecticidal protein compositions are queried for competition with multiple DIPs.

The development of disabled insecticidal proteins provides a simple method to quickly differentiate the receptor binding preferences of novel insecticidal proteins from existing proteins of commercial value, using the results of conventional insect feeding assays as the read-out for receptor competition. This assessment can be made without prior knowledge of specific receptors or even detailed aspects of the protein's interaction with the target insect pest. Application of this method greatly facilitates the identification of insecticidal proteins with potential value for insect resistance management. Ongoing research is focused on further establishing the concordance between heterologous competition observed in traditional binding assays employing 125I-labeled proteins with isolated brush border membranes and heterologous competition observed in disabled toxin bioassays across multiple lepidopteran species (Juan Ferre, personal communication). Additional studies are needed to develop and validate disabled insecticidal proteins from other structural classes, especially those that are disabled in the pore-forming function without chemical modification (Jerga et al., 2016).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ibmb.2018.12.006.

References

Abrol, D.P., Shankar, U., 2012. Integrated Pest Management: Principles and Practice. CABI, Cambridge, MA.

Adang, M.J., Crickmore, N., Jurat-Fuentes, J.L., 2014. Diversity of Bacillus thuringiensis crystal toxins and mechanism of action. Adv. Insect Physiol. 47, 39–87.

Alzate, O., You, T., Claybon, M., Osorio, C., Curtiss, A., Dean, D.H., 2006. Effects of disulfide bridges in domain I of Bacillus thuringiensis Cry1Aa delta-endotoxin on ion-channel formation in biological membranes. Biochemistry 45, 13597–13605.

Brunger, A.T., 1992. The free R value: a novel statistical quantity for assessing the accuracy of crystal structures. Nature 355, 472–475.

Badran, A.H., Guzov, V.M., Huai, Q., Kemp, M.M., Vishwanath, P., Kain, W., Nance, A.M., Evdokimov, A., Moshiri, F., Turner, K.H., Wang, P., Malvar, T., Liu, D.R., 2016. Continuous evolution of Bacillus thuringiensis toxins overcomes insect resistance. Nature 533, 58–63.

Banks, D.J., Jurat-Fuentes, J.L., Dean, D.H., Adang, M.J., 2001. Bacillus thuringiensis Cry1Ac and Cry1Fa delta-endotoxin binding to a novel 110 kDa aminopeptidase in Heliothis virescens is not N-acetylgalactosamine mediated. Insect Biochem. Mol. Biol. 31, 909–918.

Bates, S.L., Zhao, J.Z., Roush, R.T., Shelton, A.M., 2005. Insect resistance management in GM crops: past, present and future. Nat. Biotechnol. 23, 57–62.

Bravo, A., Gomez, I., Porta, H., Garcia-Gomez, B.I., Rodriguez-Almazan, C., Pardo, L., Soberon, M., 2012. Evolution of Bacillus thuringiensis Cry toxins insecticidal activity. Microb. Biotechnol. 6, 17–26.

Carmona, Daniela, R-A, C., Muñoz-Garay, Carlos, Portugal, Leivi, Pérez, Claudia, de Maagd, Ruud A., Bakker, Petra, Soberón, Mario, Bravo, Alejandra, 2011. Dominant negative phenotype of Bacillus thuringiensis Cry1Ab, Cry11Aa and Cry4Ba mutants suggest hetero-oligomer formation among different cry toxins. PLoS One 6, 19952.

Carrière, Y., Crickmore, N., Tabashnik, B.E., 2015. Optimizing pyramided transgenic Bt crops for sustainable pest management. Nat. Biotechnol. 33, 161–168.

Collaborative, 1994. The CCP4 suite: programs for protein crystallography. Acta Crystallogr. D 50, 760–763.

Crickmore, N., Baum, J., Bravo, A., Lereclus, D., Narva, K., Sampson, K., Schnepf, E., Sun, M., Zeigler, D.R., 2018. Bacillus Thuringiensis Toxin Nomenclature. http://www.btnomenclature.info/.

Deitloff, J., Dunbar, M.W., Ingber, D.A., Hibbard, B.E., Gassmann, A.J., 2016. Effects of refuges on the evolution of resistance to transgenic corn by the western corn

rootworm, Diabrotica virgifera virgifera LeConte. Pest Manag. Sci. 72 (1), 190.
Devos, Y., Meihls, L.N., Kiss, J., Hibbard, B.E., 2013. Resistance evolution to the first generation of genetically modified Diabrotica-active Bt-maize events by western corn rootworm: management and monitoring considerations. Transgenic Res. 22, 269–299.
Donovan, W.P., Dankocsik, C.C., Gilbert, M.P., Gawron-Burke, M.C., Groat, R.G., Carlton, B.C., 1988. Amino acid sequence and entomocidal activity of the P2 crystal protein. An insect toxin from Bacillus thuringiensis var. kurstaki. J. Biol. Chem. 263, 561–567.
Emsley, P., Cowtan, K., 2004. Coot: model-building tools for molecular graphics. Acta Crystallogr. D 60, 2126–2132.
Estela, A., Escriche, B., Ferre, J., 2004. Interaction of Bacillus thuringiensis toxins with larval midgut binding sites of Helicoverpa armigera (Lepidoptera: Noctuidae). Appl. Environ. Microbiol. 70, 1378–1384.
Evdokimov, A.G., Moshiri, F., Sturman, E.J., Rydel, T.J., Zheng, M., Seale, J.W., Franklin, S., 2014. Structure of the full-length insecticidal protein Cry1Ac reveals intriguing details of toxin packaging into in vivo formed crystals. Protein Sci. 23, 1491–1497.
Gazit, E., La Rocca, P., Sansom, M.S., Shai, Y., 1998. The structure and organization within the membrane of the helices composing the pore-forming domain of Bacillus thuringiensis δ-endotoxin are consistent with an “umbrella-like” structure of the pore. Proc. Natl. Acad. Sci. 95, 12289–12294.
Girard, F., Vachon, V., Prefontaine, G., Marceau, L., Su, Y., Larouche, G., Vincent, C., Schwartz, J.L., Masson, L., Laprade, R., 2008. Cysteine scanning mutagenesis of alpha4, a putative pore-lining helix of the Bacillus thuringiensis insecticidal toxin Cry1Aa. Appl. Environ. Microbiol. 74, 2565–2572.
Girard, F., Vachon, V., Prefontaine, G., Marceau, L., Schwartz, J.L., Masson, L., Laprade, R., 2009. Helix alpha 4 of the Bacillus thuringiensis Cry1Aa toxin plays a critical role in the postbinding steps of pore formation. Appl. Environ. Microbiol. 75, 359–365.
González-Cabrera, J., Escriche, B., Tabashnik, B.E., Ferré, J., 2003. Binding of Bacillus thuringiensis toxins in resistant and susceptible strains of pink bollworm (Pectinophora gossypiella). Insect Biochem. Mol. Biol. 33, 929–935.
Gowda, A., Rydel, T.J., Wollacott, A.M., Brown, R.S., Akbar, W., Clark, T.L., Flasinski, S., Nageotte, J.R., Read, A.C., Shi, X., Werner, B.J., Pleau, M.J., Baum, J.A., 2016. A transgenic approach for controlling Lygus in cotton. Nat. Commun. 7, 12213.
Granero, F., Ballester, V., Ferré, J., 1996. Bacillus thuringiensis crystal proteins CRY1Ab and CRY1Fa share a high affinity binding site in Plutella xylostella (L.). Biochem. Biophys. Res. Commun. 224, 779–783.
Grochulski, P., Masson, L., Borisova, S., Pusztai-Carey, M., Schwartz, J.-L., Brousseau, R., Cygler, M., 1995. Bacillus thuringiensisCrylA(a) insecticidal toxin: crystal structure and channel formation. J. Mol. Biol. 254, 447–464.
Liu, H., Naismith, J.H., 2008. An efficient one-step site-directed deletion, insertion, single and multiple-site plasmid mutagenesis protocol. BMC Biotechnol. 8, 1.
Herrero, S., Oppert, B., Ferre, J., 2001. Different mechanisms of resistance to Bacillus thuringiensis toxins in the indianmeal moth. Appl. Environ. Microbiol. 67, 1085–1089.
Herskowitz, I., 1987. Functional inactivation of genes by dominant negative mutations. Nature 329, 219–222.
Jakka, S., Ferré, J., Jurat-Fuentes, J.L., 2015. In: In: Soberón, M., Gao, Y., Bravo, A. (Eds.), Cry Toxin Binding Sites and Their Use in Strategies to Delay Resistance Evolution. Bt resistance: Characterization and Strategies for Gm Crops Producing bacillus Thuringiensis Toxins Chapter 13. CAB International, Boston (MA), USA, pp. 138.
Jerga, A., C. D, Zhang, C., Fu, J., Kouadio, J.K., Wang, Y., Duff, S.M.G., Howard, J.E., Rydel, T.J., Evdokimov, A.G., Ramaseshadri, P., Evans, A., Bolognesi, R., Park, Y., Haas, J.A., 2016. Mechanistic insights into the first Lygus-active β-pore forming protein. Arch. Biochem. Biophys. 600, 1–11.
Jimenez-Juarez, N., Munoz-Garay, C., Gomez, I., Saab-Rincon, G., Damian-Almazo, J.Y., Gill, S.S., Soberon, M., Bravo, A., 2007. Bacillus thuringiensis Cry1Ab mutants affecting oligomer formation are non-toxic to Manduca sexta larvae. J. Biol. Chem. 282, 21222–21229.
Jones, T.A., Zou, J.Y., Cowan, S.W., Kjeldgaard, M., 1991. Improved methods for building protein models in electron density maps and the location of errors in these models. Acta Crystallogr. A 47, 110–119.
Jurat-Fuentes, J.L., Crickmore, N., 2017. Specificity determinants for Cry insecticidal proteins: insights from their mode of action. J. Invertebr. Pathol. 142, 5–10.
Keeton, T.P., Francis, B.R., Maaty, W.S., Bulla Jr., L.A., 1998. Effects of midgut-protein-preparative and ligand binding procedures on the toxin binding characteristics of BT-R1, a common high-affinity receptor in Manduca sexta for Cry1A Bacillus thuringiensis toxins. Appl. Environ. Microbiol. 64, 2158–2165.
Koch, M.S., Ward, J.M., Levine, S.L., Baum, J.A., Vicini, J.L., Hammond, B.G., 2015. The food and environmental safety of Bt crops. Front. Plant Sci. 6, 283.
Ludueña, R.F., Roach, M.C., 1981. Interaction of tubulin with drugs and alkylating agents. 1. Alkylation of tubulin by iodo[14C]acetamide and N,N'-ethylenebis(iodoacetamide). Biochemistry 20, 4437–4444.
Luo, K., Sangadala, S., Masson, L., Mazza, A., Brousseau, R., Adang, M.J., 1997. The heliothis virescens 170 kDa aminopeptidase functions as "receptor A" by mediating specific Bacillus thuringiensis Cry1A delta-endotoxin binding and pore formation. Insect Biochem. Mol. Biol. 27, 735–743.
Martin, F.G., Wolfersberger, M.G., 1995. Bacillus thuringiensis delta-endotoxin and larval Manduca sexta midgut brush-border membrane vesicles act synergistically to cause very large increases in the conductance of planar lipid bilayers. J. Exp. Biol. 198, 91–96.
McCoy, A.J., Grosse-Kunstleve, R.W., Adams, P.D., Winn, M.D., Storoni, L.C., Read, R.J., 2007. Phaser crystallographic software. J. Appl. Crystallogr. 40, 658–674.
Melo, A.L., Soccol, V.T., Soccol, C.R., 2016. Bacillus thuringiensis: mechanism of action, resistance, and new applications: a review. Crit. Rev. Biotechnol. 36, 317–326.
Murshudov, G.N., Vagin, A.A., Dodson, E.J., 1997. Refinement of macromolecular structures by the maximum-likelihood method. Acta Crystallogr. D 53, 240–255.
Onofre, J., G. M. O, Peña-Cardeña, A., García-Gomez, B.I., Pacheco, S., Gómez, I., Bravo, A., Soberón, M., 2017. Identification of aminopeptidase-N2 as a Cry2Ab binding protein in Manduca sexta. Peptides 98, 93.
Otwinowski, Z., Minor, W., 1997. Methods in enzymology. In: In: Carter Jr.C.W., Sweet, R.M. (Eds.), Macromolecular Crystallography, Part A, vol 276 Academic Press, New York.
Pardo-Lopez, L., Soberon, M., Bravo, A., 2013. Bacillus thuringiensis insecticidal three-domain Cry toxins: mode of action, insect resistance and consequences for crop protection. FEMS Microbiol. Rev. 37, 3–22.
Edgar, R.C., 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res. 32, 1792.
Rodríguez-Almazán, C., Zavala, L.E., Muñoz-Garay, C., Jiménez-Juárez, N., Pacheco, S., Masson, L., Soberón, M., Bravo, A., 2009. Dominant negative mutants of Bacillus thuringiensis Cry1Ab toxin function as anti-toxins: demonstration of the role of oligomerization in toxicity. PLoS One 4, 5545.
Sangadala, S., Walters, F.S., English, L.H., Adang, M.J., 1994. A mixture of Manduca sexta aminopeptidase and phosphatase enhances Bacillus thuringiensis insecticidal CryIA(c) toxin binding and 86Rb(+)-K(+) efflux in vitro. J. Biol. Chem. 269, 10088–10092.
Schwartz, J.L., Juteau, M., Grochulski, P., Cygler, M., Préfontaine, G., Brousseau, R., Masson, L., 1997. Restriction of intramolecular movements within the Cry1Aa toxin molecule of Bacillus thuringiensis through disulfide bond engineering. FEBS (Fed. Eur. Biochem. Soc.) Lett. 410, 397–402.
Siebert, M.W., Nolting, S.P., Hendrix, W., Dhavala, S., Craig, C., Leonard, B.R., Stewart, S.D., All, J., Musser, F.R., Buntin, G.D., Samuel, L., 2012. Evaluation of corn hybrids expressing Cry1F, cry1A.105, Cry2Ab2, Cry34Ab1/Cry35Ab1, and Cry3Bb1 against southern United States insect pests. J. Econ. Entomol. 105, 1825–1834.
Siqueira, H.A.A., Moellenbeck, D., Spencer, T., Siegfried, B.D., 2004. Cross-resistance of Cry1Ab-selected *Ostrinia nubilalis* (Lepidoptera: Crambidae) to *Bacillus thuringiensis* δ-endotoxins. J. Econ. Entomol. 97, 1049–1057.
Tabashnik, B.E., 2010. Communal benefits of transgenic corn. Science 330, 189–190.
Tabashnik, B.E., Brevault, T., Carriere, Y., 2013. Insect resistance to Bt crops: lessons from the first billion acres. Nat. Biotechnol. 31, 510–521.
Tabashnik, B.E., Liu, Y.B., Malvar, T., Heckel, D.G., Masson, L., Ballester, V., Granero, F., Ménsua, J.L., Ferré, J., 1997. Global variation in the genetic and biochemical basis of diamondback moth resistance to Bacillus thuringiensis. Proc. Natl. Acad. Sci. 94, 12780–12785.
Tabashnik, B.E., Johnson, K.W., Engleman, J.T., Baum, J.A., 2000a. Cross-resistance to Bacillus thuringiensis toxin Cry1Ja in a strain of diamondback moth adapted to artificial diet. J. Invertebr. Pathol. 76, 81–83.
Tabashnik, B.E., Liu, Y.B., de Maagd, R.A., Dennehy, T.J., 2000b. Cross-resistance of pink bollworm (Pectinophora gossypiella) to Bacillus thuringiensis toxins. Appl. Environ. Microbiol. 66, 4582–4584.
Tanaka, S., Miyamoto, K., Noda, H., Jurat-Fuentes, J.L., Yoshizawa, Y., Endo, H., Sato, R., 2013. The ATP-binding cassette transporter subfamily C member 2 in Bombyx mori larvae is a functional receptor for Cry toxins from Bacillus thuringiensis. FEBS J. 280, 1782–1794.
Vachon, V., Laprade, R., Schwartz, J.L., 2012. Current models of the mode of action of Bacillus thuringiensis insecticidal crystal proteins: a critical review. J. Invertebr. Pathol. 111, 1–12.
Vachon, V., Prefontaine, G., Rang, C., Coux, F., Juteau, M., Schwartz, J.L., Brousseau, R., Frutos, R., Laprade, R., Masson, L., 2004. Helix 4 mutants of the Bacillus thuringiensis insecticidal toxin Cry1Aa display altered pore-forming abilities. Appl. Environ. Microbiol. 70, 6123–6130.
Wolfersberger, M.L., P Maurer, A., Parenti, P., Sacchi, F.V., Giordana, B., Hanozet, G.M., 1987. Preparation and partial characterization of amino acid transporting brush border membrane vesicles from the larval midgut of the cabbage butterfly (Pieris brassicae). Comp. Biochem. Physiol. A 86, 301–308.
Xu, C., Wang, B.C., Yu, Z., Sun, M., 2014. Structural insights into Bacillus thuringiensis Cry, Cyt and parasporin toxins. Toxins 6, 2732–2770.
Zhao, J.Z., Cao, J., Li, Y., Collins, H.L., Roush, R.T., Earle, E.D., Shelton, A.M., 2003. Transgenic plants expressing two Bacillus thuringiensis toxins delay insect resistance evolution. Nat. Biotechnol. 21, 1493–1497.