SCHOOL OF AGRICULTURAL AND VETERINARY SCIENCES - UNESP

CAMPUS OF JABOTICABAL

MECHANISM OF ACTION OF Cry1Ac TOXIN FROM *Bacillus thuringiensis* IN *Helicoverpa armigera* (LEPIDOPTERA: NOCTUIDAE)

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Agricultural Engineer

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TÍTULO DA TESE: MECHANISM OF ACTION OF Cry1Ac TOXIN FROM *Bacillus thuringiensis* IN *Helicoverpa armigera* (LEPIDOPTERA: NOCTUIDAE)

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Igor Henrique Sena da Silva was born in Sete Lagoas-MG, on December 26th, 1989, son of Carlos José da Silva and Rosa Lucia Oliveira de Sena da Silva. He completed high school in 2007, where he joined on the Brazilian Army in 4º Anti-aircraft Artillery Group (4º GAAE) in your hometown, where he remained for 2 years. He started his academic career in 2009 in the Environmental Management course at Faculdades Promove in Sete Lagoas. In 2010, he was approved at Federal University of São João Del Rei, MG, in Agricultural Engineering course, where he became Agricultural Engineering bachelor's in 2015. During graduation, worked for 4 years at EMBRAPA Maize and Sorghum in different areas: Agroecology, soil microbiology and entomology with Dr. Fernando Valicente. In 2015, he joined the master's course in Agricultural Entomology at the São Paulo State University, School of Agricultural and Veterinary Sciences, *campus* of Jaboticabal-SP (FCAV/UNESP). In 2017, initiated your doctorate course in the same course and university, both under supervision of Professor Dr. Ricardo Antônio Polanczyk. He managed take part of his courses in Mexico in the masters (4 months) and doctorate (8 months) at the Instituto de Biotecnología from UNAM, Cuernavaca, MOR, Mexico, under supervision of Dra. Alejandra Bravo, with financial support of FAPESP. Furthermore, he participated in Mexico and the United States international meetings presenting his research results. In 2018, he was awarded at the 2nd Scientific Day promoted by Monsanto as the best doctoral project in the entomology sub-area, gaining a cash prize and a mentoring with the Entomology leader of Monsanto in Brazil. On the same year, he was awarded as the best oral presentation at the 27th Entomology Brazilian Congress promoted by Entomology Brazilian Society in Gramado, Rio Grande do Sul, Brazil. Currently, he works in market development of bioproducts at "Bionat Soluções Biológicas" (Essere Group). He acts in Entomology/Crop protection area with biological control of insect pests with the use of entomopathogens.

"O mapa do que chamamos de realidade é um mosaico de ideias
em constante mutação onde a incerteza é
a mola propulsora da criatividade".

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I TRIBUTE THIS THESIS…

To the **462,092 lives** lost by Covid-19 until May 31, 2021, in Brazil, and to all the frontline health professionals who are risking their lives for the lives of the others. Yes, you are the true heroes and heroines of this country, the true "mitos" of this nation.

May better days come for all of us!

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SUMMARY

MECHANISM OF ACTION OF Cry1Ac TOXIN FROM *Bacillus thuringiensis* IN *Helicoverpa armigera* (LEPIDOPTERA: NOCTUIDAE)

ABSTRACT - *Helicoverpa armigera* (Hübner, 1805) (Lepidoptera: Noctuidae), known as Cotton bollworm is an insect pest of global importance in cotton crop. Nonetheless, it also attacks several other important economic crops worldwide, such as soybeans, corn, sorghum, wheat, beans, tomatoes, and ornamental plants. This insect is susceptible to some insecticidal Cry toxins from *Bacillus thuringiensis* (Bt) expressed in transgenic plants (Bt plants) or used in biopesticides. However, the capacity to evolve resistance to Bt plants have been threaten the technology in the long term. The most common mechanisms of resistance of lepidopterans to Cry toxins are mutations linked to Cry toxins receptors resulting in reduced binding. Thus, the identification and characterization of the putative Cry receptors is fundamental to better understand the mode of action of Cry toxins, retarding resistance evolution and producing Cry toxins more effective against insect pests. Previously, we identified *H. armigera* prohibitin (PHB) as a Cry1Ac-binding protein. The aim of this work was to further analyzed the potential role of PHB as a Cry toxin receptor in comparison to cadherin (CAD), a midgut protein (MP) well-recognized as Cry1Ac-receptor. In addition, to characterize the interaction of those two MP with different Cry1A toxins. In this way, *Ha*PHB-2 midgut protein and *HaCAD* toxin binding region fragment (TBR) from *H. armigera* were expressed in *Escherichia coli* cells and qualitative and quantitative binding assays with different Cry1 toxins were performed, as well as competition assays. We demonstrated that Cry1Ab, Cry1Ac and Cry1Fa toxins bound to *Ha*PHB-2 similarly as to *Ha*CAD-TBR. *Ha*PHB-2 protein competed with Cry1Ac binding to *H. armigera* BBMVs. A reduction of toxin binding to *Ha*BBMV was observed in the presence of *Ha*PHB-2 in a concentration dependent way. Furthermore, different Cry1Ab mutant toxins located in domain II (Cry1Ab-F371A and Cry1Ab-G439D) or domain III (Cry1Ab-L511A and Cry1Ab-N514A), that were previously characterized to be affected in receptor binding, were analyzed regarding to their binding interaction with *Ha*PHB-2 and toxicity against *H. armigera*. One β-16 mutant (Cry1Ab-N514A) showed increased binding to *Ha*PHB-2 that correlated with six-fold higher toxicity against *H. armigera* while the other β-16 mutant (Cry1Ab-L511A) that was affected in binding to *Ha*PHB-2 lost toxicity against *H. armigera*. We have found that the β-16 region from domain III of Cry1Ab is involved in interaction with *Ha*PHB-2 and toxicity. This work identified a region of Cry1Ab involved in binding to *Ha*PHB-2 from a Lepidoptera insect suggesting that this midgut protein may participate as a novel receptor in the mechanism of action of the Cry1 toxins in *H. armigera.* This is the first characterization of *Ha*PHB-Cry1A interaction, supporting that *Ha*PHB-2 also participates in the mechanism of action of Cry1Ab toxin in *H. armigera*, presenting new insights of the mode of action of Cry1 toxins in this important global pest.

Keywords: Cotton bollworm, Cry toxins, mechanism of action, resistance management, cadherin, prohibitin.

MECANISMO DE AÇÃO DA TOXINA Cry1Ac DE *Bacillus thuringiensis* EM *Helicoverpa armigera* (LEPIDOPTERA: NOCTUIDAE)

RESUMO - Helicoverpa armigera (Hübner, 1805) (Lepidoptera: Noctuidae), conhecida como lagarta Helicoverpa é uma praga de importância global na cultura do algodão. No entanto, também ataca várias outras culturas de importância agrícola em todo o mundo, como soja, milho, sorgo, trigo, feijão, tomate e plantas ornamentais. Este inseto é suscetível a algumas toxinas inseticidas Cry de *Bacillus thuringiensis* (Bt) expressas em plantas transgênicas (plantas Bt) ou usadas em bioinseticidas. No entanto, a capacidade de desenvolver resistência às plantas Bt tem ameaçado a tecnologia a longo prazo. Os mecanismos mais comuns de resistência dos lepidópteros às toxinas Cry são mutações ligadas aos receptores das toxinas, resultando na redução da ligação. Assim, a identificação e caracterização dos receptores Cry é fundamental para melhor compreender o modo de ação das toxinas e retardar a evolução da resistência e assim produzir novas toxinas Cry mais eficazes contra os insetos-praga. Previamente, nós identificamos a proibitina (PHB) de *H. armígera* como uma proteína de ligação a Cry1Ac. O objetivo deste trabalho foi analisar a potencial função de PHB como receptor da toxina Cry em comparação com a caderina (CAD), uma proteína do intestino médio bem conhecida como receptor de Cry1Ac. Além disso, caracterizar a interação dessas duas proteínas de membrana à diferentes toxinas Cry1A. Desta forma, a proteína *Ha*PHB-2 e um fragmento da região de ligação da toxina a *HaCAD* de *H. armigera* foram expressos em células de *Escherichia coli* e ensaios de ligação qualitativos e quantitativos com diferentes toxinas Cry1 foram realizados, bem como ensaios de competição. Nós demonstramos que as toxinas Cry1Ab, Cry1Ac e Cry1Fa se ligaram ao *HaPHB*-2 de forma semelhante a *HaCAD*. A proteína *Ha*PHB-2 competiu com a ligação de Cry1Ac as BBMVs de *H. armigera*. Uma redução da ligação da toxina às *Ha*BBMVs foi observada na presença de *HaPHB*-2 em uma forma dependente da concentração. Além disso, diferentes toxinas mutantes de Cry1Ab localizadas no domínio II (Cry1Ab-F371A e Cry1Ab-G439D) ou domínio III (Cry1Ab-L511A e Cry1Ab-N514A), que haviam sido previamente caracterizadas como afetadas na ligação ao receptor, foram analisadas em relação à sua interação de ligação com *Ha*PHB-2 e toxicidade contra *H. armigera*. Uma toxina mutante em β-16 (Cry1Ab-N514A) mostrou ligação aumentada a *Ha*PHB-2, o que correlacionou com toxicidade seis vezes maior contra *H. armigera*, enquanto outro mutante de β-16 (Cry1Ab-L511A) que foi afetado na ligação a *HaPHB*-2 perdeu toxicidade contra *H. armigera*. Nós encontramos que a região β-16 do domínio III de Cry1Ab está envolvida na interação com *Ha*PHB-2 e toxicidade. Este trabalho identifica a região de Cry1Ab envolvida na ligação ao *Ha*PHB-2 de um inseto da ordem Lepidoptera, sugerindo que esta proteína de membrana pode participar como um novo receptor no mecanismo de ação das toxinas Cry1 em *H. armigera*. Esta é a primeira caracterização da interação *Ha*PHB-Cry1A, corroborando que *Ha*PHB-2 também participa do mecanismo de ação da toxina Cry1Ab em *H. armigera*, aumentando a compreensão sobre o modo de ação das toxinas Cry1 nesta importante praga global.

Palavras-chave: Lagarta Helicoverpa, toxinas Cry, mecanismo de ação, manejo de resistência, caderina, proibitina.

LIST OF THE TABLES

LIST OF THE FIGURES

LIST OF ABBREVIATIONS

3D-Cry toxin - Cry three-domain toxin family

ABC - ATP-binding cassette transporter

ALP - Alkaline phosphatase

ANOVA - Analysis of variance

APN – Aminopeptidase-N

BBMVs - Brush border membrane vesicles surface

BSA - Bovine serum albumin

Bt - *Bacillus thuringiensis*

CAD - Cadherin-type protein

cAMP - Adenosine 3'-5' monophosphate adenosine

CBP - Colorado Potato Beetle

dsRNA - Double-stranded RNA

EST - Expressed sequence tag

GPI – Glycosylphosphatidylinisotol

ICPs - Insecticidal Cry proteins

igG - Gamma immunoglobulin

LC – Lethal concentration

LC-MS - Liquid chromatography mass spectrometry

N-GalNac - N-acetylgalactosamine

P252 - 252 kDa protein

PBS - Phosphate buffer saline

PHB – Prohibitin

PKA - Protein kinase A

PMSF - Phenylmethylsulfonyl fluoride

PVDF - Polyvinylidene difluoride membrane

RNAi - RNA interference

Sip - Secreted insecticide protein

SPFH - Stomatin/prohibitin/flotillin/Hfl KC

TBR - Toxin binding region

Vip - Vegetative insecticide protein

1. INTRODUCTION

The Cotton bollworm, *Helicoverpa armigera* (Hübner, 1805) (Lepidoptera: Noctuidae), is a worldwide polyphagous insect pest. In Brazil, the first report of this insect was in 2013/2014, attacking soybean crops in Goias and Bahia states, and cotton in Mato Grosso state (Czepak et al., 2013; Leite et al., 2014; Gonçalves et al., 2019). Its control costs more than U$ 2 billion dollars, worldwide (Tay and Gordon, 2019). The most important challenge is related to its capacity to evolve resistance to chemical insecticides (Durigan et al., 2017; Sparks et al., 2020) and biopesticides such as the insecticidal proteins produced by *Bacillus thuringiensis* (Bt) bacterium (Xu et al., 2005; Xiao et al., 2014; Tay et al., 2015). Bt are gram-positive bacteria that produce different insecticidal toxins that show toxicity against several insect species of agricultural importance, or that are vectors of human diseases (Bravo et al., 2007). These proteins have been used worldwide showing several advantages compared to chemical control strategies since they are highly specific against target pests, are harmless to vertebrates and biodegradable (Glare et al., 2012; Lacey et al., 2015; James, 2018).

The mode of action of Cry proteins produced by Bt is a complex process, involving the interaction of these toxins with different receptors in the larval midgut epithelium, triggering toxin oligomerization and insertion of the oligomer into the membrane, resulting in pore formation in the apical membrane of the midgut cells and the insect death (Vachon et al., 2012; Gómez et al., 2014). Several studies have identified potential Cry toxin receptors in different lepidopteran insects, including, cadherin-like (CAD) proteins, glycosylphosphatidylinisotol-anchored (GPI) aminopeptidases (APN) and alkaline phosphatases (ALP) proteins; polycalin, a 270 kDa-glycoconjugate; a 250 kDa protein named P252; an α-amylase, and ATP-binding cassette (ABC) transporter proteins family, such as ABCC2, ABCC3 and ABCA2 transporters (Jurat-Fuentes and Adang, 2004; Pigott and Ellar, 2007; Fernandez-Luna et al., 2010; Heckel, 2012; Liu et al., 2018; Gómez et al., 2018a; Bing-Jie et al., 2019; Shabbir et al., 2020). However, it is still possible that additional proteins could be involved in the mode of action of Cry toxins.

The most common mechanisms of resistance to Cry toxins are mutations linked to Cry toxins receptors resulting in reduced toxin binding (Ferré and Van Rie, 2002; Tabashnik and Carrière, 2017). The Cry1Ac-binding proteins in the early and late instars of *H. armigera* by means of pull-down assay followed by protein identification by LC-MS analysis was carried out (Da Silva et al., 2018). Among the proteins identified, prohibitin-2 (*Ha*PHB-2) was identified as Cry1Ac-binding protein preferentially expressed in the early instars (Da Silva et al., 2018). Previously a PHB-1 that binds Cry4Ba was identified in *Aedes aegypti* (Diptera: Culicidae) (Bayyareddy et al., 2009) and it was shown that Cry3Aa bind both PHB-1 and PHB-2 in the coleopteran larvae *Leptinotarsa decemlineata* (Coleoptera: Chrysomelidae) (Ochoa-Campusano et al., 2013). In addition, PHB proteins may function as receptor for Vi capsular polysaccharide from *Salmonella typhi* in intestinal epithelial human cells and for dengue virus in *A. aegypti* cells (Sharma and Qadri, 2004; Kuadkitkan et al., 2010).

The aim of this work was to (1) further analyze the binding interaction of *Ha*PHB-2 with different Cry1 toxins and their toxicity against *H. armigera* neonate larvae, (2) to evaluate the binding affinity of different Cry1 toxins (Cry1Ab, Cry1Ac and Cry1Fa toxins) used in Bt plants with *Ha*PHB-2 protein, (3) to identify the Cry1Ab toxin domain regions that participates in the binding interaction to *Ha*PHB-2 and *Ha*CAD from *H. armigera*. In this way, the *Ha*PHB-2 midgut protein was cloned and expressed in *Escherichia coli* cells to perform semi-quantitative binding assays in comparison to a *HaCAD* toxin binding region (TBR) fragment previously described since *Ha*CAD has been shown to be a functional receptor of Cry1Ac in *H. armigera* (Liu et al., 2009; Wang et al., 2016). This work is the first characterization of *Ha*PHB-Cry1A interaction, supporting that *Ha*PHB-2 also participates in the mechanism of action of Cry1Ab toxin in *H. armigera*.

2. LITERATURE REVIEW

2.1. Cotton bollworm, *Helicoverpa armigera* (Hübner, 1805)

2.1.1 The damage of *Helicoverpa armigera*

Helicoverpa armigera (Hübner, 1805) (Lepidoptera: Noctuidae), known as Cotton bollworm is an insect pest of global importance in cotton crop. Nonetheless, there are more than 180 plant species reported as hosts of *H. armigera* (Tay et al., 2013). It attacks several other important crops worldwide, such as cotton, maize, soybean, beans, tomatoes, sorghum, wheat, sunflower, fruit, vegetables, ornamental plants, and some weeds (Lammers and McLeod, 2007; Avila et al., 2013; Fathipour and Naseri, 2011; Pratissoli et al., 2015) (Figure 1).

It is estimated that the annual worldwide loss caused by this species in different crops reached 5 billion dollars (Arnemann et al., 2019; Gonçalves et al., 2019). In Brazil, the damage caused by *H. armigera* to soybean during 2012/13 season was estimated 0.8 billion dollars (Bueno and Sosa-Gómez, 2014), demonstrating the importance and the knowledge needs about the damage caused by this insect pest in Brazilian soybean fields (Stacke et al., 2018).

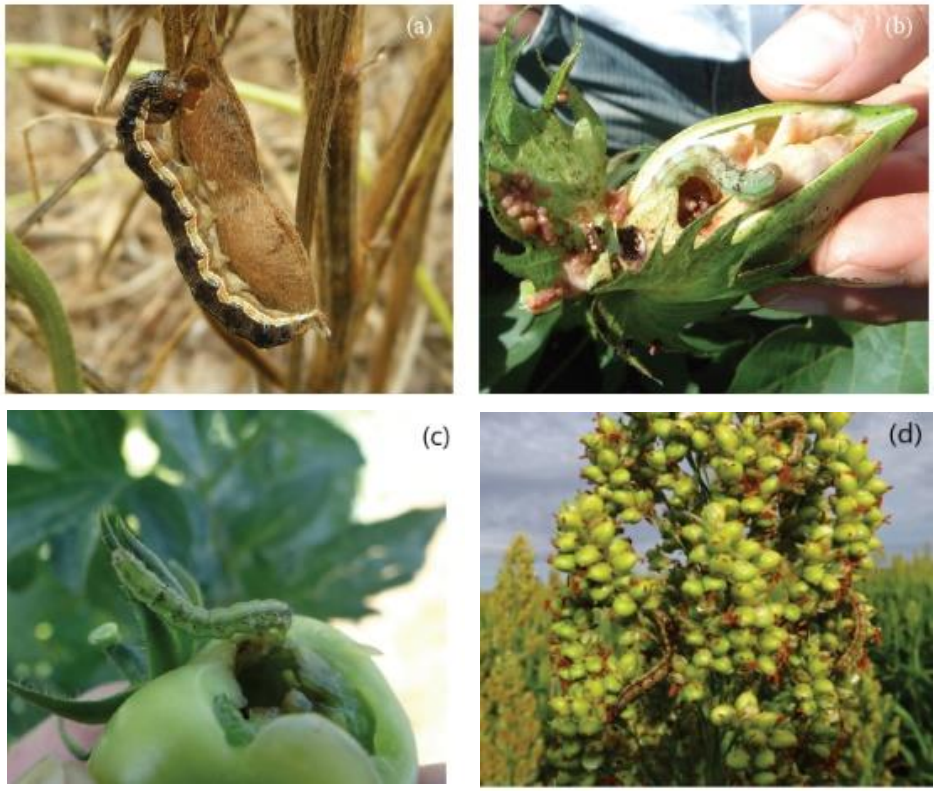

Figure 1. *H. armigera* larvae attacking soybean pods (a), cotton apple (b), tomatoes (c), and sorghum panicle. Photos: Cecilia Czepack (a), Lucia Madalelna Vivan (c), Felipe Zulbac (c), and Luis Henrique Kasuya (d).

Artificial defoliation in the vegetative stage and removal of pods in the reproductive stage have been used in attempts to understand the damage caused by *H. armigera* in soybean and to provide values for an economic threshold control (Timsina et al., 2007). Acordding to Rogers and Brier (2010) the loss of soybean yield by *H. armigera* larvae depends on the maturation stage and the potential yield of soybean plants, climatic conditions and, especially, the density of the larvae. In addition, the potential damage from natural feeding may result in a variety of injury events, including complete consumption of pods, consumption of developing pods and seeds, and destruction of the apical growing points, which reduce the plant's compensatory ability, reducing the yield production (Stacke et al., 2018).

Stacke et al. (2018) demonstrated the large potential of *H. armigera* to damage soybean plants during the reproductive stage in Brazil. The major damage from feeding by *H. armigera* occurs during pod-filling reproductive stage (> R5.1) of soybeans. There are significant reductions in pods/m^2 and seeds/pod with increased *H. armigera* density, showing that relatively few larvae/m^2 can cause significant reductions in seed yield, despite the demonstration of increased plant capacity compensation when the damaged occurred in R2 stage.

2.1.2. Origin and geographical distribution of *Helicoverpa armigera*

H. armigera is an endemic species from Africa, Europe, continental Asia, Japan, New Zealand, New Caledonia, Australia, New Guinea, eastern Indonesia, Kiribati, and Polynesia regions (Lammers and MacLeod, 2007). In January 2013, it was the first official report of its occurrence in Brazil, attacking soybean and cotton crops in Bahia, Mato Grosso and Goiás states (Czepak et al., 2013). *H. armigera* outbreaks occurred in the same year in a wide geographical area (EMBRAPA, 2013) and constantly associated with reports of control failures of pyrethroid pesticides (Durigan et al., 2017). However, a posterior report was made describing the occurrence of *H. armigera i*n citrus orchard in São Paulo state (SP). High infestation levels of *H. armigera* were observed in October 2012, in Botucatu, SP (Bueno et al., 2014).

In Brazil, the *H. armigera* incursion has resulted in over 800 million dollars in losses and control costs since 2012 (Bueno and Sosa-Gómez, 2014; Da Silva et al.,

2014; Mastrangelo et al., 2014; Pomari-Fernandes et al., 2015). Since them, the occurrence of *H. armigera* in South America has been reported in other countries, at the same year in Paraguay (SENAVE, 2013), in 2014 in Argentina (Murúa et al., 2014) and 2016 in Uruguay (Castiglioni et al., 2016). However, with the extent of the infested area, it is likely that *H. armigera* is present in whole South America, even before its first detection (Lepidoptera Noctuidae) (Sosa-Gómez et al., 2016; Pinto et al., 2017).

Although the first report of this species in Brazil was made in 2013, Sosa-Gómez et al. (2016) emphasize that this species has probably been present in Brazil since 2008 and has gone unnoticed by farmers due to its similarity to *Helicoverpa zea* (Bodie) (Lepidoptera: Noctuidae). Pinto et al. (2017) using molecular screening, confirmed the presence of *H. armigera* in Bt-crops of soybean and cotton, and non Bt-crops of soybean, cotton, and maize. Mixed infestations of *H. armigera* with *H. zea* were found in non Bt-maize in Viçosa, Southeastern of Minas Gerais state.

After the South America spreading the insect migrated to Central and North America. *H. armigera* has been reported in Puerto Rico attacking bean crops was detected in September 2014 (APHIS, 2014) and 2015, the United States Department of Agriculture's Sanitary Inspection service reported the first occurrence of *H. armigera* in United States, attacking tomato crops in Florida (APHIS, 2015). Since them, the researchers have been warning farmers for monitoring and developing tactics of control to stop the pest spreading in USA producing centers (CABI, 2020).

Kriticos et al. (2015) estimated that the arrival of *H. armigera* into North America would put at risk an extra 78 billion dollars of agricultural output. Naturally, biosecurity managers and others in the Americas who may be impacted by the spread of *H. armigera* are eager to understand the potential geographical range and abundance of this notorious pest species better. Strategic control tactics to contain or eliminate invasive pests depend on an accurate spatial characterization of the invasion and dispersion processes of the species in its new territory (Kriticos et al., 2015; Gonçalves et al., 2019).

2.1.3. External morphology of *Helicoverpa armigera*

H. armigera is morphologically very similar to the Brazilian native pest *H. zea.* In larval stages, they are morphologically indistinguishable, which made data collection concerning geographical distribution and dispersion of this pest in Brazil difficult (Pogue, 2004). Leite et al. (2017) detected a high intraspecific gene flow among populations collected in both countries (Brazil and United States). However, there has been a geographic limit to gene flow among *H. zea* individuals from South and North America. A comparative structure analysis suggested a natural hybridization between *H. armigera* and *H. zea* in Brazil (Leite et al., 2017).

Cordeiro et al. (2020) demonstrated that the landscape composition and bioclimatic variables influence the introgression rate between *H. armigera* and *H. zea* in agricultural areas. The frequency of hybridization varied from 15 to 30% depending on the statistical analyses. These methods showed more congruence in estimating that hybrids contained approximately 10% mixed ancestry (i.e., introgression) from either species. This question has been arisen after the invasion of *H. armigera* in the America continent and the simultaneous occurrence of these two species in the same geographical region, which could dramatically impact the evolution of host ranges and resistance management which cause great impact on pest management (Gonçalves et al., 2019; Cordeiro et al., 2020).

H. armigera is a holometabolic insect, which means, complete metamorphosis (egg-larva-pupa-adult). The eggs of this species are initially yellowish white in color with a shiny appearance early and turn brown near the larval hatching. The females oviposit during the night and the eggs are placed alone or in small groups, preferably on the abaxial (bottom) side of the leaves or on stems, flowers, fruits, and terminal shoots of the host plants (Mensah, 1996; Ávila et al., 2013).

The *H. armigera* larval phase has six instars in tropical conditions (Figure 2A-2H and 3A-3F). The initial instars have a yellowish white to reddish-brown color with a cephalic capsule between dark brown and black. The larvae feed initially on the tenderest parts of plants, which they can produce a type of web, characteristic of noctuid. As the caterpillars develop, they have brown stripes laterally on the chest, abdomen and on the head, the type of artificial diet used in laboratory can influence its color (Ávila et al., 2013). After the sixth instar, it releases a reddish secretion before burrowing into the soil to construct a pupal chamber with silk (Figure 3G-3I). The lateral

and dorsal parts of the body increase in size and remain motionless for the next six days through the end of pupation (Queiroz-Santos et al., 2018).

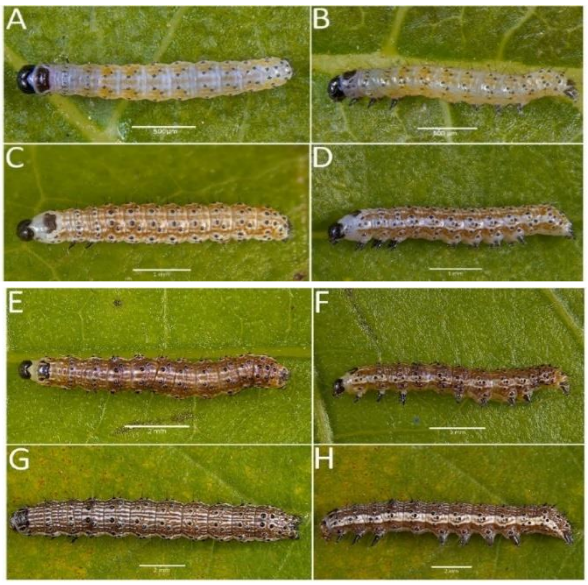

Figure 2. Larvae of *Helicoverpa armigera*. A, first instar in dorsal view; B, first instar in lateral view; C, second instar in dorsal view; D, second instar in lateral view; E, third instar in dorsal view; F, third instar in lateral view; G, fourth instar in dorsal view; H, fourth instar in lateral view. Adapted of Queiroz-Santos et al., 2018.

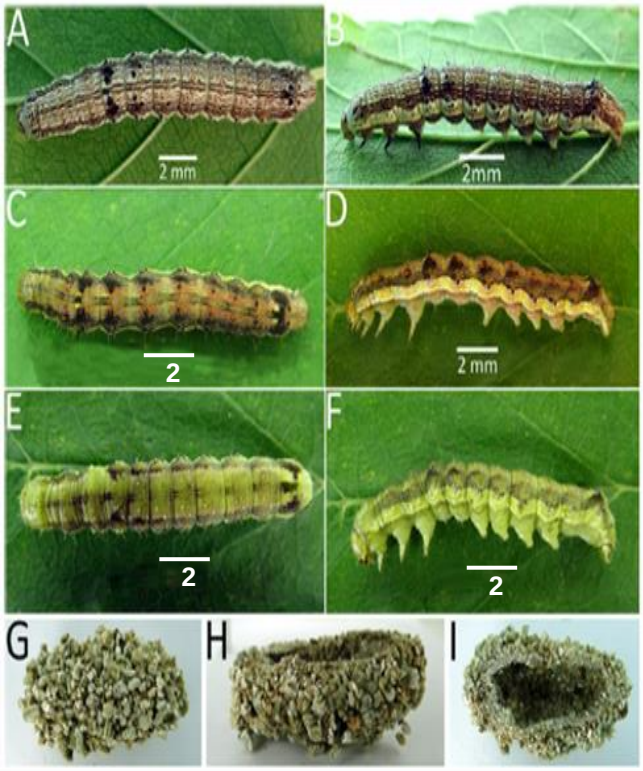

Figure 3. Larvae and pupal chamber of *Helicoverpa armigera*. A, fifth instar in dorsal view; B, fifth instar in lateral view; C, sixth instar in dorsal view; D, sixth instar in lateral view; E, sixth instar in dorsal view; F, sixth instar in dorsal view; G, pupal chamber in dorsal view; H, pupal chamber in lateral view; I, pupal chamber in ventral view. Adapted of Queiroz-Santos et al., 2018.

From the fourth instar (Figure 2E-2F), the larvae start to have dark and clearly visible tubers in the dorsal region of the first abdominal segment, which helps in their identification and differentiation from other species of *Heliothinae* genus (Czepak et al., 2013). Furthermore, a peculiarity of this species is the slightly leathery coat, which has often been related to the high resistance by the chemical insecticides, especially pyrethroids, organophosphates and carbamates, which acts by contact (Ahmad et al., 2001; McCaffery and Nauen, 2006; Durigan et al., 2017).

Figure 4. Pupa of *Helicoverpa armigera*. A, ventral view; B, dorsal view; C, lateral view. Adapted of Queiroz-Santos et al., 2018.

The adult's integument is dense, smooth, edges of segments well marked. The *H. armigera* adults presents on the forewings, a line with seven to eight spots, also with a broad brown cross section in the central part, and a comma shaped. The

posterior wings color are lighter, and they have a dark brown border, with a light spot in the center of the wings (Queiroz-Santos et al., 2018) (Figure 5).

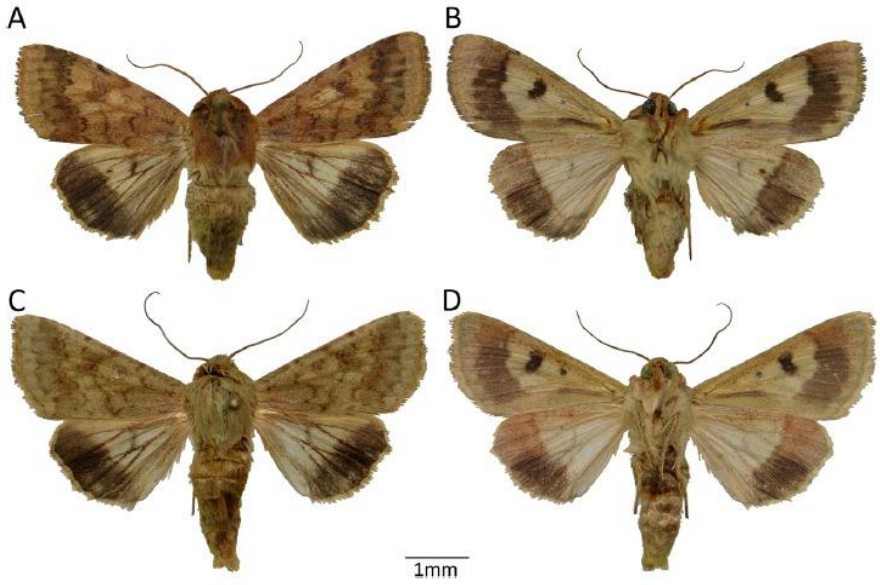

Figure 5. Adult of *Helicoverpa armigera*. A, female in dorsal view; B, female in ventral view; C, male in dorsal view; D, male in ventral view. Adapted from Queiroz-Santos et al., 2018.

2.1.4. Bioecological aspects and life cycle of *Helicoverpa armigera*

The main bioecological aspects of *H. armigera* are polyphagia, high migratory capacity, high reproductive capacity, facultative diapause, and fast life cycle. These physiological and ecological characteristics facilitate their survival, even in unstable habitats and favor their adaptation to seasonal changes (Naseri et al., 2010; Tay and Gordon, 2019). According to Jadhav et al. (2013) all these characteristics facilitated the rapid dispersion of this species around the globe.

The fertility of *H. armigera* females in field conditions is in the range of 500-1000 eggs, depending on the environmental conditions. However, in laboratory a unique *H. armigera* female can oviposit up to 3000 eggs (Mironidis and Savoupolou-Soultani, 2008). Often, the fertility is intimated related to the adult longevity (usually 7-20 days in laboratory conditions), climatic conditions and availability of hosts (Maelzer and Zalucki, 1999; Soleimannejad et al., 2010; Truzi et al., 2019).

For example, in subtropical and temperate regions, *H. armigera* diapauses at pupal stage during the winter months. In tropical regions, few populations of *H. armigera* enter diapause, this is due to the high rainfall regime and the high abundance of host plants that allow the pest to survive throughout the year (Maelzer and Zalucki, 1999; Wang et al., 2020).

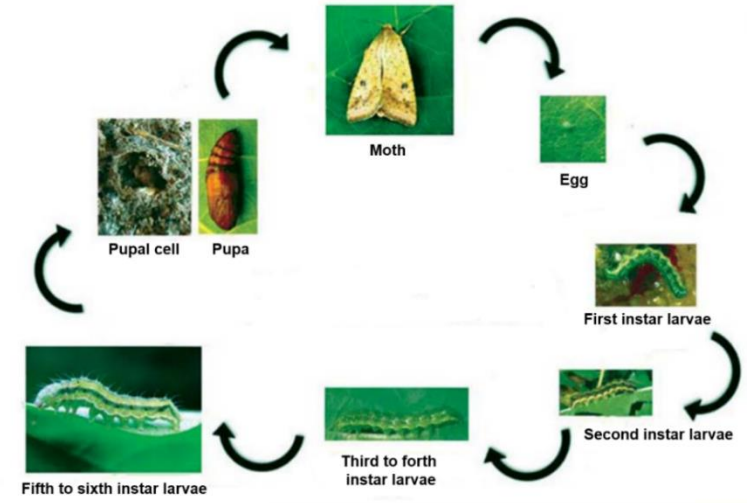

Figure 6. Life cycle of *Helicoverpa armigera*. (Source: Graniza, available in https://www.grainsa.co.za/control-of-bollworm-in-soybeans).

In general, the photoperiod decreased together with low temperatures during the larval and pre-pupal stages determine the proportion of the *H. armigera* population that enters in diapause, with spring/winter temperatures determining the time of emergence (Duffield and Dillon, 2005). The whole life cycle is usually completed in an average of 30 days, 20 to 50 days depending on the prevailing temperatures (Figure 6) (Pogue, 2004; Ávila et al., 2013). The moths then emerge from the pupae and fly to start another life cycle in the season.

Silva et al. (2018) evaluated the biotic potential and life table of *H. armigera* from different host plants (citrus, corn, and cotton) and Brazilian states (São Paulo, Distrito Federal, and Bahia) in artificial diet, under laboratory conditions. The parental progeny collected in cotton fields from Bahia had a higher biotic potential, a higher reproductive rate, and a better fecundity compared to the insects from remaining regions. The authors discussed that the greatest biotic potential of the Bahia progeny may be due to increased selection pressure from the insecticide used (organophosphate and pyrethroid) on cotton crops compared to that of other crops, as well due to the massive

adoption of Bt cotton-producing areas of that state from 2013 outbreaks. On this set of factors may have accelerated the biotic potential of the species in cotton crops in Bahia, which implies a population increased and control difficulty.

2.1.5. Management of *Helicoverpa armigera* in Brazil

The effective monitoring of eggs, caterpillars, pupae, and adults of *H. armigera* is the key factor for implementing effective pest management strategies. Through the knowledge of this information, the tactics control will be defined, such as, which tactic of control (chemical, biological, behavioral, etc.) product choice and dose (Avila et al., 2013).

The *H. armigera* management in Brazil has been done with the use of chemical insecticides and Bt plants expressing Cry1Ac toxin, specially Bt-soybean and Bt-cotton. In chemical control, are used insecticides in the seed treatment (ST) and foliar spraying (FS). The ST has been used for the insect pests that attack the initial phase of the crop and reducing FS during the crop development. In addition to chemical insecticides and Bt plants, biopesticides based in Bt bacterium and entomopathogenic virus (HzNPV) has been used successfully by farmers in Brazil since the pest entrance and it stays in use to the present day.

Perini et al. (2016) evaluated chemicals and biological insecticides to control *H. armigera* on soybean: Premio® 200 (chlorantraniliprole), Belt® 480 (flubendiamide), Avatar® 150 (Indoxacarb), Pirate® 240 (chlorfenapyr), Tracer® 480 (spinosad), Atabron® 50 + Lannate® (Chlorfluazuron + Methomyl), Intrepid® 240 (Methoxyfenozide), Ampligo® 50 (Lambda-cyhalothrin + Chlorantraniliprole), Orthene® 750 (acephate), Dipel and Bt Control (*B. thuringiensis* kurstaki), Gemstar® and HaNPV CCAB® (*Helicoverpa zea nucleopolyhedrovirus*) were evaluated. The biological treatment Bt Control® was efficient to control small larvae (100% mortality with 10 DAS) and the HzNPV CCAB® were efficient to control small and large larvae (100% mortality with 10 DAS). The chemical treatments: chlorantraniliprole, flubendiamide, chlorfenapyr, acephate and spinosad were efficient to control *H. armigera* with 90, 90, 90, 90 and 72%, respectively. Thus, the authors discuss the

integration of different control tactics (chemical and biological) for *H. armigera* management.

However, difficulties in controlling *H. armigera* have been reported with the use of some insecticides in Brazil. Resistance cases of *H. armigera* to carbamate insecticides (Group 1A), organophosphates (Group 1B), pyrethroids (Group 3A), spinosyns (Group 5), avermectins (Group 6), oxadiazines (Group 22A), Bt proteins (Group 11) among others have been documented in Brazil and other countries (Yang et al., 2013; Durigan et al., 2017; Silva et al., 2020; IRAC, 2021).

The rotation of insecticides with different mechanisms of action has been one of the most effective strategies for chemical resistance management. However, it must be integrated with other control tactics of pest management, such as monitoring and the use of Bt crops for example. In the case of Bt crops (Group 11), the refuge strategy must always be used to delay the resistance processes achievement and not compromise the control effectiveness (Tabashnik and Carrière, 2017; IRAC, 2021).

2.1.6 Microbial control of *Helicoverpa armigera*

In Brazil, 29 biopesticides are (bacteria, viruses and entomopathogenic fungi) registered for the *H. armigera* control (Table 1). From these, 17 bioproducts are based on bacterium *B. thuringiensis*, 10 products are based on baculovirus and 2 products are based in *Isaria fomosorea,* an entomopathogenic fungi.

As was discussed in the last section, the use of chemical insecticidal remains the most used for the *H. armigera* control, both in areas of soybean-Bt and cotton-Bt and conventional crops. However, since 2013, with the pest entrance to Brazil, the use of biopesticides based in *B. thuringiensis* bacterium and entomathogenic virures increased substantially and has been very efficient in the *H. armigera* management.

For better outcome, mostly, the biological control has been used in association with compatible chemical insecticides. There are several benefits with use of biological control with microorganisms when compared to chemical insecticides: They are very specific for the target pest, which means friendliness with the environment, and very specific for the target pest, as well as, selectiveness to natural enemies and are very safe for human (Bravo et al., 2007).

Table 1. Biological products registered for *Helicoverpa armigera* control in Brazil.

PRODUCTS	ACTIVE INGREDIENT	MICRORGANISM	FORMULATION	COMPANY*
Able	*Bacillus thuringiensis*	Bacterium	SC	Mitsui & Co (Brasil)
Agree	*Bacillus thuringiensis*	Bacterium	WP	Bio Controle
Armigen	VPN-HzSNPV	Virus	SC	Agbitech
Bac Control Max EC	*Bacillus thuringiensis*	Bacterium	EC	Vectorcontrol
Bac Control Max WP	*Bacillus thuringiensis*	Bacterium	WP	Vectorcontrol
Biolep Protection	*Bacillus thuringiensis*	Bacterium	SC	Simbiose
BTControl	*Bacillus thuringiensis*	Bacterium	SC	Simbiose
Challenger	*Isaria fomosorea*	Fungus	SC	Koppert do Brasil
Costar	*Bacillus thuringiensis*	Bacterium	WG	Mitsui & Co (Brasil)
Dipel	*Bacillus thuringiensis*	Bacterium	SC	Sumitomo Chemical
Dipel WG	*Bacillus thuringiensis*	Bacterium	WG	Sumitomo Chemical
Diplomata K	HearNPV	Virus	SC	Koppert do Brasil
Gemstar LC	VPN-HzSNPV	Virus	SC	Mitsui & Co (Brasil)
Gemstar-Max	VPN-HzSNPV	Virus	SC	Mitsui & Co (Brasil)
Helicovex	*Bacillus thuringiensis*	Bacterium	SC	FMC Química do Brasil
Helymax EC	*Bacillus thuringiensis*	Bacterium	EC	Ballagro Agro
Hz-NPV CCAB	VPN-HzSNPV	Virus	SC	CCAB Agro
Javelin WG	*Bacillus thuringiensis*	Bacterium	WG	Mitsui & Co (Brasil)
Lepigen	AcMNPV	Virus	SC	Agbitech
Octane	*Isaria fomosorea*	Fungus	SC	Koppert do Brasil
Owner	HearNPV	Virus	SC	Koppert do Brasil
Stregga EC	*Bacillus thuringiensis*	Bacterium	EC	Vectorcontrol
Surtivo Plus	AcMNPV+SfMNPV+ HearMNVP+ChinMNPV	Virus	SC	Agbitech
Surtivo Soja	HearMNVP+ChinMNPV	Virus	SC	Agbitech
Tarik WP	*Bacillus thuringiensis*	Bacterium	WP	Vectorcontrol
Thuricide	*Bacillus thuringiensis*	Bacterium	WP	Bio Controle
Thuricide SC	*Bacillus thuringiensis*	Bacterium	SC	Bio Controle
Verpavex	AgMNPV	Virus	SC	Andermatt do Brasil
Winner Max EC	*Bacillus thuringiensis*	Bacterium	EC	Vectorcontrol

EC – Emulsifiable concentrate; SC – Suspension concentrate; WG – Water dispersible granule; WP – Wettable powder.
* Company holding the registration number in Brazil.

2.2. *Bacillus thuringiensis* Berliner (Berliner, 1911)

B. thuringiensis is a gram-positive bacterium, belonging to the Bacillaceae family and the cereus Group, which includes *Bacillus anthracis*, *Bacillus mycoides*, *Bacillus pseudomicoydes* and *Bacillus weihenstephanensis* (Sauka and Benitende, 2008). This group of bacteria is characterized mainly by the formation of endospores and by growing in the presence of oxygen. *B. anthracis* and *B. cereus* are mammalian pathogens, *B. anthracis* is the causative agent of anthrax, an acute disease, and often lethal to humans and animals, whereas, *B. cereus* is a human opportunistic, pathogen, which can cause gastroenteritis, eye infections and periodontal disease, among other diseases (Ibrahim et al., 2010). Bt has a complex life cycle, which has been commonly found in soil, water, plants, stored cereals, and dead insects (Argolo-Filho and Loguercio, 2014).

Bt in its sporulation phase produces crystalline inclusions, known as δ-endotoxins (Cry and Cyt toxins). In addition, it also secrets Vip (vegetative insecticide protein) and Sip (secreted insecticide protein) proteins in its vegetative phase (Schnepf et al., 1998; Chakroun et al., 2016). This characteristic of producing crystal proteins during its sporulation phase is distinguished from other members of the cereus Group. Bt toxins have insecticidal properties against several species of agricultural importance (mainly lepidopterans and coleopterans) and mosquitoes that are vectors of human diseases, whether in the application of bioinsecticides and/or transgenic plants that express toxins of this bacterium, the well-known Bt plants (Heckel et al., 2007; Bravo et al., 2012). Bt toxins are highly specific for their hosts and have gained worldwide importance as an alternative to chemical insecticides. In addition, has no toxicity against humans or other vertebrates (Bravo et al., 2011).

2.2.1. History of *Bacillus thuringiensis* and its insecticidal activity spectral

The insecticidal activity of the bacterium *B. thuringiensis* (Bt) was recognized long before the bacterium was identified, some reports have suggested that Bt was previously used in ancient civilizations such as Egyptian and Chinese (reviewed by Sanahuja et al., 2011). However, this bacterium was first isolated in 1901 by the

Japanese biologist Shigetane Ishiwatari, when investigating the cause of disease that had been killing populations of silkworms, *Bombyx mori* (Lepidoptera: Bombycidae). Later, in 1911, the same bacterium was isolated by Ernst Berliner from the Mediterranean flour moth, *Ephestia kuehniella* (Lepidoptera: Pyralidae) in province of Thuringia, Germany, for this reason it was named *Bacillus thuringiensis* Berliner (Siegel, 2001).

Bt has been used successfully as a biopesticide for more than 60 years. More recently, genes encoding their toxins have been used to transform plants, knowing as Bt plants, transgenic plants that express Cry toxins with activity for some insect pests that cause damage to main crops. Currently, the Cry toxins constitute the largest group of insecticidal proteins produced by Bt. To date, the Bt Toxin Nomenclature Committee (Crickmore, 2020) has classified 78 different types (Cry1 to Cry78) of Cry proteins, varying of 369 (Cry34) to 1,344 amino acids (Cry43), including three-domain and ETX_MTX2 family proteins from Bt and *Lysinibacillus sphaericus* (Adang et al., 2014), with individual toxins showing well documented toxicity against lepidopterans, coleopterans, hemipterans, dipterans, nematodes (human and animal parasites, and free living; Rhabditida) some snails and/or human-cancer cells of various origins (Aronson et al., 1986; Xu et al., 2005; Marco and Manuel, 2012; Bravo et al., 2011; reviewed by Palma et al., 2014).

2.2.2. Cry toxins and their functional domains

The three-dimensional structure of eight Cry toxins with different insecticidal specificities has been resolved, among them, Cry1Aa (specific for Lepidoptera), Cry2Aa (specific for Diptera and Lepidoptera), Cry3Aa, Cry3Bb and Cry8Ea (specific for Coleoptera), Cry4Aa and Cry4Ba (specific for Diptera) and Cry5Ba toxins (specific for nematodes) (Grochulski et al., 1995; Morse et al., 2001; Li et al., 1991; Galitsky et al., 2001; Guo et al., 2009; Boonserm et al., 2005; Boonserm et al., 2006; Hui et al., 2012) (Figure 7). All these structures display a high degree of similarity with a three-domain organization, suggesting a similar mode of action of the Cry three-domain toxin family (3D-Cry toxin), despite the low amino acid sequence identity between these toxins (Bravo et al., 2007) (Figure 7).

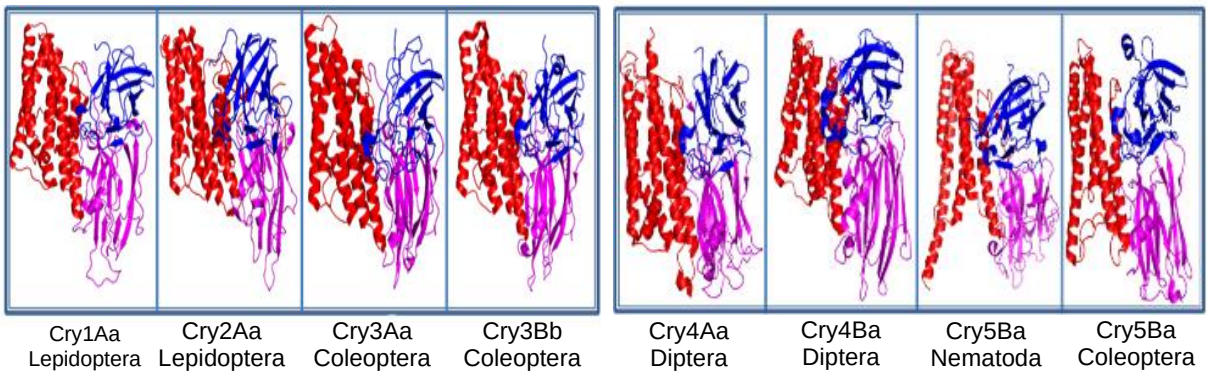

Figure 7. Three-dimensional structure of Cry toxins with different specificities. Domain I is colored in red, domain II and III are pink and blue, respectively. (PDB 1CIY, 1I5P, 1DLC, 1JI6, 2C9K, 3EB7, 4D8M). Adapted from Flores-Escobar (2014).

The figure 8 shows the Cry1Aa toxin: the domain I is a bundle of 7–8 α-helices with a centrally located hydrophobic α-helix 5 and has been involved in oligomerization and pore formation. Domain II, a three β-sheet structure, is involved in receptor binding, oligomerization, and membrane insertion. Domain III participates in receptor binding and possibly membrane insertion (Adang et al., 2014).

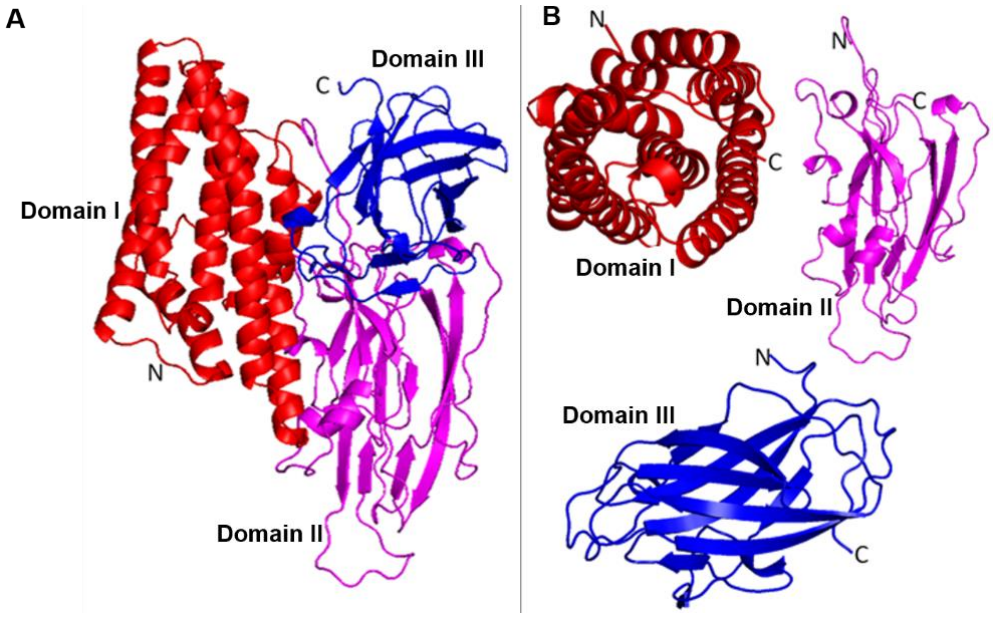

Figure 8. Structural topology of the Cry1Aa toxin (PDB: 1C1Y). A, domain I is colored in red, domain II and domain III are represented in pink and blue, respectively. B, rotation in z of domain I, domain III is rotated 90 °. Adapted from Flores-Escobar (2014).

2.2.3. Domain I

The domain I forms a cluster of seven antiparallel α-helices with the α-helix 5 on the middle, the outer helices, the α-3, α-4, α-6, and α-7 helices possess an amphipathic nature (Figure 9). The amino acid composition of these helices is arranged in the following way: charged or polar residues are exposed to the solvent while hydrophobic residues are oriented towards the central helix (Pigott and Ellar, 2007; Adang et al., 2014).

These characteristics, more with the structural identity that the domain possesses with the membrane insertion domain from hemolysin, colicin and the translocation membrane domain of diphtheria toxin allow us to infer that such domain is involved in pore formation (Flores-Escobar, 2014). The analysis by site-directed mutagenesis has made it possible to determine that the domain I region involved in pore formation in the cell membrane is the hydrophobic hairpin between α-4 and 5 helices, which undergoes a conformational rearrangement of the tertiary structure to form ion channels within the membrane (Figure 9) (Zavala et al., 2011).

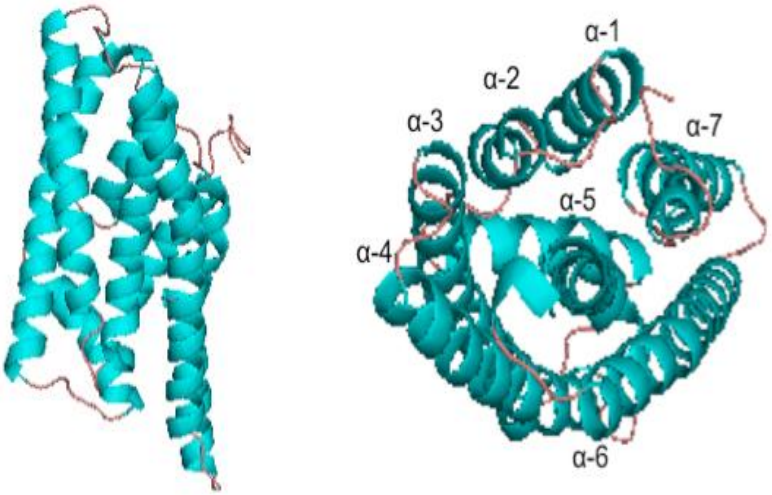

Figure 9. Structural arrangement of domain I. **A**, Seven β-sheets antiparallel. **B**, Angle showing the 6 α-amphipathic helices and the hydrophobic α-5 **central** helix. Adapted from Pacheco (2010).

2.2.4. Domain II

This domain is in the central region of the protein primary structure and is formed by three β-sheets forming a “prism” arrangement (Figure 10). Each sheet contains 3-4 β-sheets with a “Greek key” type topology and these sheets converge at the apex of the prism which has 3 loops, the loops correspond to a hairpin that joins two β-chains of each “Greek key” (Figure 10) (Pacheco, 2010).

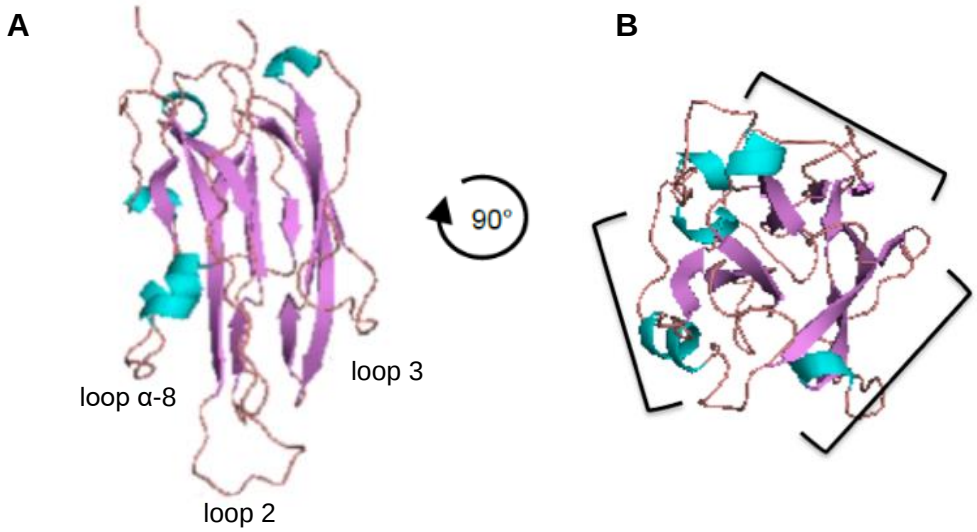

Figure 10. Structural arrangement of domain II. **A**, Domain II of Cry1Aa toxin, with the apex of the prism with the 3 important loops in receptor binding protein. **B**, 3-β sheets that form the prism faces (brackets). Adapted from Pacheco (2010).

Structurally, this domain is the most variable of the Cry toxins, it contains loops of different length, conformation and sequence and binding to their receptor, having an important function in determining specificity (Jurat-Fuentes and Crickmore, 2017). The length of the β-chains is also highly variable, the toxins Cry2Aa and Cry4Ba are extreme examples (Boonserm et al., 2006). This variability in domain II suggests that it can be a determining factor of the specificity of the toxins (Bravo et al., 2012; Pardo-López et al., 2013). Gómez et al. (2006) demonstrated that the monomeric toxin binds to Bt-R1 through loops 2 and 3 of domain II promoting the formation of the pre-pore inducing some structural changes, then the pre-pore interacts with APN through β-16 of domain III promoting membrane insertion and cell death.

The similarities between the domain II top and the complementary determining region of immunoglobulins suggest that this region is involved in receptor binding;

subsequently mutations in these loops demonstrated that these regions are important in determining specificity for receptor molecules (Flores-Escobar, 2014). The structure of domain II has been compared to other proteins with a β-prism structure, including vitellin and the plant lectin of jacalin or agglutinin. The structural similarity between domain II and the lectin domains has allowed the speculation that domain II can bind carbohydrates (Pigott and Ellar, 2007).

2.2.5. Domain III

The domain III is a β-sandwich of two antiparallel β-sheets. Both sheets are composed of five chains, with the outer sheet exposed to the solvent and the inner sheet packed towards domain II (Figure 11). Domain III shows less structural variability than domain II, and the main differences are found in the length, orientation, and sequence of the loops. The importance of these differences is particularly evident in Cry1Aa and Cry1Ac toxin, where a loop forms a binding cavity for N-acetylgalactosamine (N-GalNac), which is involved in the recognition of this sugar at the APN receptor (Burton et al., 1999; Lee et al., 1999). Domain III has been shown to have significant similarity to carbohydrate-binding protein modules found in glycoside hydrolases, lyases, and esterases, this fact suggests that some Cry toxins, such as Cry1Ac toxin, can bind carbohydrates in this region (Burton et al., 1999). In several studies, this domain has been implicated as a determinant of specificity, as well as receptor binding. The β-16 of domain III of the Cry1Ab toxin has been mapped as the region of interaction with APN1 and ALP receptors (Arenas et al., 2010; Flores-Escobar et al., 2013).

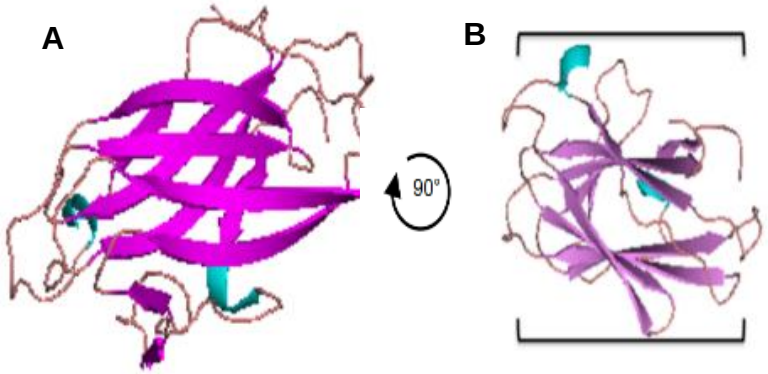

Figure 11. Structural arrangement of domain III. A, the folding type "sandwich" with the 2 β-sheets (brackets). Adapted from Pacheco (2010).

2.3. Mode of action of Cry toxins

The mode of action of Cry toxins is a complex process, involving their interaction with different receptors in the larval midgut epithelium, triggering toxin oligomerization and insertion of the oligomer into the membrane, resulting in pore formation in the apical membrane of the midgut cells and the insect death (Vachon et al., 2012; Gómez et al., 2014). One of the most important characteristics of the Cry proteins is their high specificity to target insects (Jurat-Fuentes and Crickmore, 2017). This specificity is largely determined by the Cry proteins specific binding to the receptors present on the brush border membrane vesicles (BBMVs) (Pigott and Ellar, 2007; Bravo et al., 2011).

Different receptors present on the BBMVs have been reported, such as a cadherin-type (CAD), aminopeptidase-N (APN), alkaline phosphatase (ALP) receptor and recently transporters from the ABC family (ABCC2 and ABCC3) (Pigott and Ellar, 2007; Jurat-Fuentes and Crickmore, 2017; Zhao et al., 2021). This mechanism can be even more complex, since other molecules may be involved, such as glycolipids (Griffitts et al., 2003), α-amylases (Fernandez-Luna et al, 2010) and an ABC-type carrier (ABCC2) (Zhou et al., 2016). Thus, site-directed mutagenesis has been extensively used to further understand the participation of specific protein regions of Cry toxins in their mode of action (Pardo-López et al., 2009). However, this technique can be effectively in an applied strategy to improving insecticidal activity against insect target pests (Soberón et al., 2007; Gómez et al., 2018b).

The main techniques for studies in Bt mode of action consist of the use of molecular tools, such as RNA interference (RNAi) and Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) (Gómez et al., 2018b; Guo et al., 2018). Both tools allow to “knock out” the possible receptors located in the midgut membrane and evaluate their participation in the toxicity of Cry toxins. Other techniques employed in the receptors identification that interact with Cry toxins include: ELISA binding assays, SPR resonance, ligand blotting, western blotting, pull-down (immunoprecipitation) assay and liquid chromatography mass spectrometry (LC-MS) sequencing (Pigott and Ellar, 2007; Arenas et al., 2010; Flores-Escobar et al., 2013; Zhou et al., 2016, Da Silva

et al., 2018; Peña-Cardeña et al., 2018). In addition, cell lines of different susceptible and resistant insects have been successfully used to use all these techniques (Soberón et al., 2017).

The mechanism of action of Cry proteins has been extensively studied in the order Lepidoptera, especially in *Manduca sexta* (Lepidoptera: Sphingidae). Currently, two main models to describe the Cry toxins molecular mechanism activity is the sequential binding model proposed by Bravo et al. (2004) and the signal transduction model proposed by Zhang et al. (2006). The Bravo's model was proposed based on data obtained of the Cry1Ab toxicity in *M. sexta*. The second model was proposed by heterologous expression of the *M. sexta* cadherin-like protein in High Five™ cell line from *Trichoplusia ni* (Lepidoptera: Noctuidae). Both have been proposed to describe the activity of the Cry1Ab toxin. In addition, a third model has been proposed by Jurat-Fuentes and Adang (2006) to explain the mode of action of the Cry1Ac protein in *Chloridea virescens* (Lepidoptera: Noctuidae). This model suggests that the cytotoxicity of the Cry toxin is due to the combined effects of osmotic lysis and intracellular signaling. Then, elements of both models, proposed by Bravo et al. (2004) and Zhang et al. (2006) are incorporated.

The signal transduction model suggests that a unique interaction of the toxin with the primary receptor induces the cell death in susceptible insects. For the other hand, the sequential binding model involves interaction with, at least, three molecules after the crystal ingestion, resulting in pore formation on the midgut membrane epithelium of the susceptible insect. Both models agree on the first steps of protein solubilization and activation (Vachon et al., 2012).

Currently, several researchers have dedicated to further understand the mode of action of Bt. The studies performed has as main goals: (1) studying structure of toxins and their different domains; (2) to understand the mechanisms of resistance of target insects to Bt toxins that have been used in Bt plants; (3) further understand how the specificity of the different Bt toxins occurs, with a focus on pyramiding genes against possible target insects; (4) producing novel mutant toxins with increased activity for target insects and toxins with combined domains to improve the action spectrum of the toxins (chimera toxins).

Understanding the mode of action of Bt toxins is essential for the development of more potent toxins, which have greater durability and are able of retarding the resistance evolution. However, in the last decade, the two models proposed to explain the mechanism of action of Bt toxins have attracted considerable attention from researchers and generated abundant literature. The main aspects of the two models are detailed and discussed on the next sub-chapters.

2.3.1. Sequential binding model, the Bravo's model

The sequential binding model was suggested by Bravo et al. (2004) and has been particularly well defined through the Cry1Ab toxin activity in *M. sexta* (Figure 12). The protein crystal is ingested by a susceptible insect and is dissolved due to the high pH in the insect midgut, the protoxin is then processed proteolytically by alkaline proteases present in the lumen, releasing an active toxic fragment of 65 kDa. The Cry protein undergoes a complex sequential binding event with the different receptors resent in BBMVs, resulting in its membrane insertion, pore formation, osmotic lysis, and subsequent insect death.

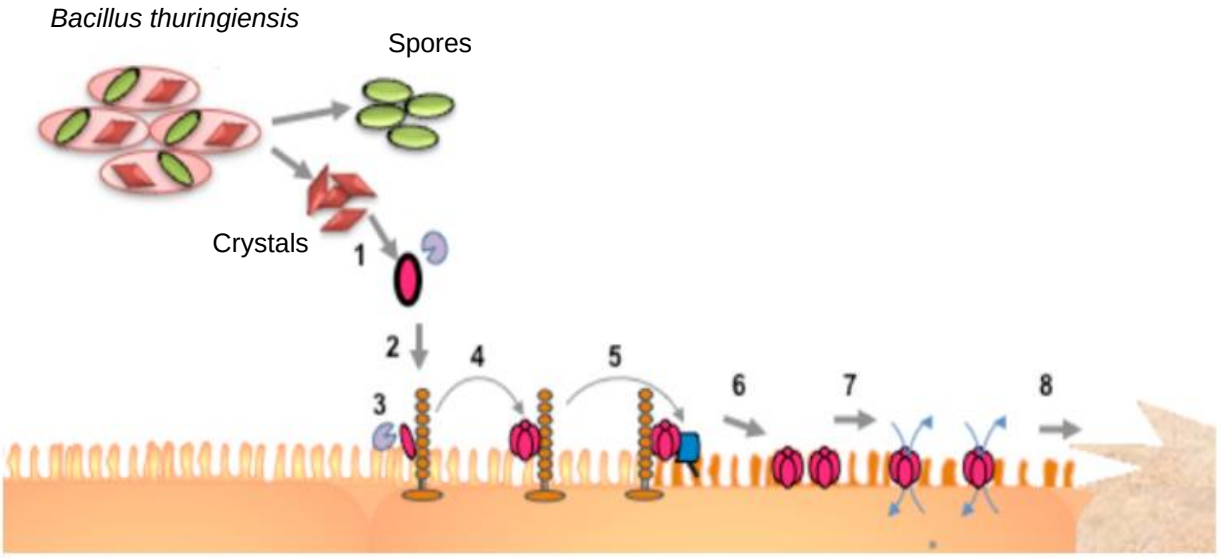

Figure. 12. Mechanism of action of Cry1A's toxins in *M. sexta*. 1 – The crystals are ingested are solubilized. 2 – The protoxins ate released and proteolytically activated. 3 – The activated fragment binds to CAD. 4 – The toxin oligomerizes and binds to APN (5). 6 – The oligomer inserts into the lipid rafts of the membrane, forming pores that

allow passage of ions and other molecules. 7 – The membrane destabilizes the osmotic balance, and the cell dies (8). Adapted from Pacheco (2010).

The first Cry toxin interaction occurs through of domain II and domain III exposed regions with APN and ALP of *M. sexta* (Gómez et al., 2006). This interaction occurs with low affinity. However, this binding concentrates the activated toxin on the BBMVs midgut surface, which binds with high affinity to the second receptor, a CAD-like protein, through of domain II exposed loops, including α-2 and α-8, and particularly loop 3 in *M. sexta*, *C. virescens* and *B. mori* (Atsumi et al., 2005; Xie et al., 2005; Gómez et al., 2006).

The interaction with CAD facilitates the proteolytic cleavage of the end N-terminal including the α-1 of domain I, which induces the oligomer formation, known as the pre-pore structure (Gómez et al., 2002). The oligomeric structure of the toxin gains a high affinity for receptors anchored in GPI, ALP and APN, involving loop 2 of domain II (Arenas et al., 2010). The interaction of the pre-pore structure with ALP and APN finally leads to membrane insertion into the membrane, causing the pore formation, osmotic shock, cell lysis and insect death by septicemia (Pardo-López et al., 2013). The main advantage of the sequential binding model resides in the fact that it provides a conceptual framework for the experimental study of the mechanism by which Bt Cry toxins form pores, with each of its steps being, at least in principle, amenable to experimental verification (Vachon et al., 2012).

2.3.2. Transduction signal model, the Zhang's model

Zhang et al. (2006) proposes that the correlation between pore formation and cytotoxicity has not been adequately demonstrated and suggests an alternative mode of action model in which the Cry1Ab protein kills insect cells exclusively by osmotic lysis (Figure 13).

As reviewed by Vachon et al. (2012), according to this model (Figure 13), cytotoxicity is mediated by the specific binding of Bt toxins to their cadherin receptors. This activates otherwise undescribed Mg^{2+}-dependent (Zhang et al., 2005) and adenylyl cyclase/protein kinase A (PKA) (Zhang et al., 2006) signaling pathways that lead to necrotic cell death. While the toxins can interact non-specifically with

membrane lipids, assembled into oligomers, and even insert into the membrane, this has no consequence for the target cells because, as the authors claim (Zhang et al., 2005), ‘‘membrane-incorporated oligomer complex does not form lytic pores in the membrane and has no toxic effect on cells’’.

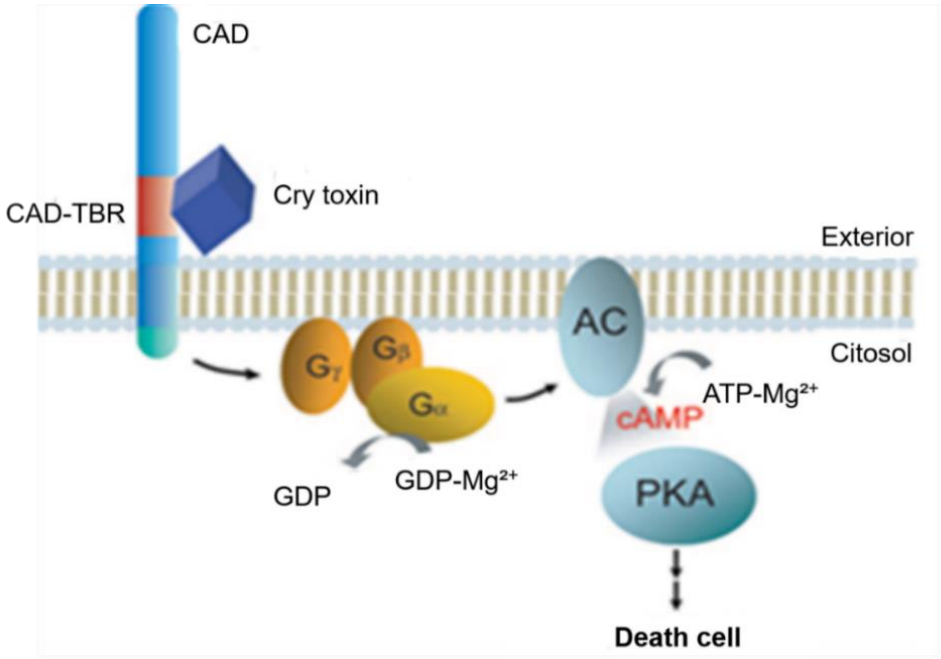

Figure. 13. Signal transduction model. Adapted from Zhang et al. (2006).

In this model, the monomeric protein Cry1Ab specifically binds to the cadherin-type receptor and initiates an Mg2 + dependent signaling cascade. This cascade stimulates the protein G synthesis and them, the protein adenylate cyclase. Finally, the accumulation of cyclic adenosine 3'-5' monophosphate adenosin (cAMP) and activation of the (PKA) protein occurs. The PKA protein, once activated, leads to destabilization of the cytoskeleton cells, also the membrane ion channels and after the cell death (Figure 13).

2.4. Interaction of receptors to Cry1A toxins

The identification of the binding-proteins of Cry1A toxins has been performed basically by *in vitro* techniques, such as pull-down assays, ligand blot assays, and recently, through proteomics studies. However, there are few results involving these

binding-proteins, as participants in the mechanism of action of Cry1A proteins *in vivo*. An essential part to further understand the mechanism of action of Cry toxins, is identifying the receptors involved in toxin interaction and their participation in toxicity.

In the last 10 years, these advances have been significant, especially in Lepidoptera. Thus, different proteins have been described as receptors for Cry toxins, such as CAD, APN, ALP, a 270 kDa glycoconjugate, P252 (250 kDa protein), an α-amylase and, recently, several ABC-type transport proteins, such as ABCC2, ABCC3, ABCA2 (Pigott and Ellar, 2007; Fernandez-Luna et al., 2010; Heckel, 2012; Pardo-López et al., 2013; Tay et al., 2015; Gómez et al., 2018a; Zhao et al., 2021). In addition, it has been suggested that other molecules may be involved in this interaction, such as glycolipids and other proteins present in “lipid rafts”, regions of micro membrane domains, such as flotillin (FLT), prohibitin (PHB), V-ATPase and actin (Griffitts et al., 2003; Bayyareddy et al., 2009; Ochoa-Campuzano et al., 2013; Da Silva et al., 2020).

2.4.1. Cadherin-like protein

The cadherin-like proteins (CAD) are a large family of adhesion proteins that are important for the mutual association in vertebrate cells. These molecules play an important role in the mechanisms of cell differentiation, conferring adhesion specificities to the cells. The cadherins are considered important regulators of morphogenesis because they control cell polarity and tissue morphology (Gumbiner, 1996; Angst et al., 2001). These proteins are defined by the presence of Calcium-binding or repeat domains of cadherin. CAD proteins can be approximately 110 amino acids, they are mostly glycosylated and are generally membrane anchored by a domain transmembrane (Pigott and Ellar, 2007; Flores-Escobar, 2014).

The CAD-like proteins of the lepidopterans, unlike those described in other Eukaryotes, they have been found in the brush border membrane vesicles of the midgut (BBMVs), the target site of the Cry toxins (Pigott and Ellar, 2007). CAD is one of the most important Bt toxin receptors because it has important roles in toxin oligomerization (reviewed by Xiao and Wu, 2019). The expression of the CAD proteins varies during the larvae development and progressively increases from the first to the fifth larval development stage of *M. sexta* (Midboe et al., 2003). Flores-Escobar et al.

(2013) analyzed the expression of ALP, APN1 and CAD in all larval development of *M. sexta*. The authors have found that the CAD and APN1 expression is increased during larval development, while ALP is produced through larval development, with a higher expression during the third instar and a slightly lower expression in the last larval instar.

Lepidopteran CAD proteins have been extensively studied as Cry toxin receptors. There is consistent evidence that suggests their participation in the mechanism of action of Cry toxins (Table 2). The first CAD protein reported interacting with Cry toxins in a lepidopterous insect was in *M. sexta*, the BT-R1, a 210 kDa glycoprotein. This protein bound to Cry1Ab and Cry1Ac in ligand blot assays (Francis and Bulla, 1997). In several *in vitro* tests, the CAD-like receptor has been shown to participate in the mechanism of action of Cry1A toxins. The heterologous expression of the CAD in lepidopteran insects, such as *M. sexta* (Meng et al., 2001; Dorsch et al., 2002; Hua et al., 2004; Zhang et al., 2005), *B. mori* (Nagamatsu et al., 1998; Nagamatsu et al., 1999), *C. virescens* (Jurat-Fuentes and Adang, 2006) and *Ostrinia nubilalis* (Lepidoptera: Crambidae) (Flannagan et al., 2005), confers susceptibility to Cry1A toxins.

Moreover, mutations in the cadherin gene (*cad*) are associated with resistance to Cry toxins in several lepidopteran insects, such as *C. virescens* (Gahan and Heckel, 2001) *H. zea* (Fritz et al., 2019), *Pectinophora gossypiella* (Lepidoptera: Gelechiidae) (Fabrick et al., 2014; Fabrick et al., 2020; Wang et al., 2018; Wang et al., 2019), *T. ni* (Badran et al., 2016), *Chilo suppressalis* (Lepidoptera: Crambidae) (Zhang et al., 2017a), and *H. armigera* (Wang et al., 2016; Liu et al., 2009; Peng et al., 2010; Xiao et al., 2017). The Cry1Ac toxin-binding region of *H. armigera* cadherin (*HaCAD*) and the membrane-proximal region of *HaCAD* are required for Cry1Ac toxicity (Wang et al., 2005a; Xiao et al., 2017; Ma et al., 2019).

Table 2. Interaction of CAD protein from lepidopteran insects with Cry toxins from *Bacillus thuringiensis*.

Insect	**Toxin**		**Reference**
	***In vitro* assays**	***In vivo* assays**	
Manduca sexta	Cry1Aa, Cry1Ab and Cry1Ac		Francis and Bulla, 1997

	Cry1Ab		Dorsch et al., 2002
	Cry1Ab		Gómez et al., 2003
	Cry1Aa, Cry1Ab and Cry1Ac		Hua et al., 2004
	Cry1Ab		Pacheco et al., 2009
		Cry1Ab	Flores-Escobar et al., 2013
Bombix mori	Cry1Aa, Cry1Ab and Cry1Ac		Nagamatsu et al., 1998
	Cry1Aa		Adegawa et al., 2017
Chloridea virescens		Cry1Ac	Gahan and Heckel., 2001
Helicoverpa armigera	Cry1Ac		Jurat-Fuentes and Adang, 2006
	Cry1Ac		Wang et al., 2005a; Liu et al., 2009; Peng et al., 2010; Ma et al., 2019
		Cry1Ac	Xu et al., 2005; Wang et al., 2016
	Cry1Ab, Cry1Ac and Cry1Fa		Da Silva et al., 2020
Ostrinia nubilalis	Cry1Ab		Flannagan et al., 2005
Ostrinia furnacalis	Cry1Ah		Shabbir et al., 2020
Pectinophora gossypiella		Cry1Ac	Morin et al., 2003
	Cry1Ac		Wang et al., 2018; Wang et al., 2019
Spodoptera frugiperda	Cry1Ab		Gómez et al., 2020
Chilo suppressalis		Cry1C and Cry2A	Zhang et al., 2017a

2.4.2. Aminopeptidase N

The aminopeptidase family is a class of enzymes that play an important role in protein breakdown during digestion. They catalyze the cleavage of amino acids located at the amino terminal-end of peptides and proteins. These enzymes are widely distributed in prokaryotic and eukaryotic organisms (Gonzales and Robert-Baudoy, 1996), additionally it has been suggested that they participate in the transport of peptides through the BBMVs (Antonov et al., 1984). Insect aminopeptidases belong to the aminopeptidase N (APN) family and are abundantly found in the midgut membrane (Wang et al., 2005b). Four classes of APN isoforms have been identified in Lepidoptera (Nakanishi et al., 2002; Angelucci et al., 2008; Gómez et al., 2018a). These different isoforms of APN have different specificities to the N-terminal residues of protein substrates and are anchored by GPI to the cell membrane (Knight et al., 1994; Hua et al., 1998; Jenkins et al., 2000; Pacheco et al., 2009).

Five isoforms of APNs have been reported: APN1, APN2, APN3, APN4 and APN 5 in *M. sexta* (Knight et al., 1994, Denolf et al., 1997, Angelucci et al., 2008; Martinez de Castro et al., 2017; Gómez et al., 2018a). However, *in vivo* participation of APNs in the mechanism of action of the Cry1Ab, Cry1Ac and Cry1Ca toxin has been studied in lepidopterous (Gill and Ellar, 2002; Rajagopal et al., 2002; Yang et al., 2010; Gómez et al., 2018a) (Table 3). After silencing the *apn* gene, the *Spodotera litura* (Lepidoptera: Noctuidae) larvae were less susceptible to the effect of the Cry1Ca toxin (Rajagopal et al., 2002).

In another lepidopteran*, Diatraea saccharalis* (Lepidoptera: Pyralidae), *in vivo* tests by Yang et al. (2010) demonstrated that silencing three APN isoforms resulted in decreased susceptibility to Cry1Ab toxin. Pull-down assays using *Spodoptera frugiperda* (Lepidoptera: Noctuidae) BBMVs identified APN, APN1, and APN2 isoforms as Cry1Ca-binding proteins (Gómez et al., 2018a). Those authors silenced the expression of APN1 transcript, by double-stranded RNA (dsRNA) feeding, and they demonstrated that silenced larvae are more tolerant of the Cry1Ca toxin, identifying APN1 as a functional receptor of Cry1Ca.

In vivo participation of APN in the mechanism of action of Cry toxins has been associated with the appearance of resistance to Cry1 toxins, and it has been correlated with decreased expression in transcripts or mutations in *apn* gene. When the HaAPN1 gene was silenced by RNAi, the susceptibility of *H. armigera* to Cry1Ac was strongly

reduced (Sivakumar et al., 2007). In the case of a strain resistant of *Spodoptera exigua* (Lepidoptera: Noctuidae) to Cry1C toxin, decreased expression of the APN1 transcript has been associated with resistance. In *H. armigera* (96-SBtR strain), a mutation in *apn* gene has been related to the appearance of resistance in this strain (Zhang et al., 2009). In a *T. ni* resistant strain to Cry1Ac toxin, both the expression, of *apn1* and the APN1 protein isoform were decreased, which led them to conclude that APN1 may be participating in the mechanism of action of Cry1Ac in that insect pest (Tiewsiri and Wang, 2011).

Table 3. Interaction of different APN isoform proteins from lepidopteran insects with Cry toxins from *Bacillus thuringiensis*.

Insect	Toxin		Class of APN	Reference
	***In vitro* assays**	***In vivo* assays**		
Manduca sexta	Cry1Aa, Cry1Ab and Cry1Ac		APN	Masson et al., 1995
		Cry1Ac	APN	Gill and Ellar, 2002
	Cry1Ab		APN1	Arenas et al., 2010
	Cry1Aa, Cry1Ab and Cry1Ac		APN1	Flores-Escobar et al., 2013
	Cry2Ab		APN2	Onofre et al., 2017
	Cry1Ab		APN1	Peña-Cardeña et al., 2018
Bombix Mori	Cry1Aa and Cry1Ab		APN3	Nakanishi et al., 2002
	Cry1Aa		APN	Yaoi et al., 2004
Diatraeae saccharalis		Cry1Ab	APN1	Yang et al., 2010
		Cry1Ac	APN1	Sivakumar et al., 2007

Helicoverpa armigera		Cry1Ac	APN1	Zhang et al., 2009
	Cry1Ac		APN, APN1, APN2, APN3, APN4 and APN5	Zhou et al., 2016
	Cry1Ah		APN1	Zhou et al., 2017
	Cry1Ac		APN1, APN2, APN3 and APN4	Da Silva et al., 2018
Lymantria dispar	Cry1Ac		APN1 and APN2	Valaitis et al., 1997
Plutella xylostella	Cry1Aa and Cry1Ab		APN3	Nakanishi et al., 2002
	Cry1Ac		APN1	Denolf et al., 1997
Spodoptera exigua		Cry1Ca	APN1, APN2, APN3 and APN4	Herrero et al., 2005
Spodoptera litura		Cry1Ca	APN	Rajagopal et al., 2002
Spodoptera frugiperda	Cry1AbMod		APN1, APN3, APN4 and APN5	Martínez de Castro et al., 2017
		Cry1Ca	APN1	Gómez et al., 2018a
	Cry1Ab		APN1	Gómez et al., 2020
Trichoplusia ni		Cry1Ac	APN1	Tiewsiri and Wang, 2011
Ostrinia furnacalis	Cry1Ah		APN	Shabbir et al., 2020

2.4.3. Alkaline phosphatase

The alkaline phosphatases (ALP) are hydrolases responsible for catalyzing the removal of groups phosphate from many types of molecules, including nucleotides, proteins, and alkaloids. Mostly, ALP is found in all animals and are primarily located in epithelium midgut cells of insects. It has been proposed that insect's ALP participate in the absorption of metabolites and transportation processes (Eguchi, 1995; Flores-Escobar., 2014).

ALPs have been identified as Cry toxin receptors (Pigott and Ellar, 2007; reviewed by Bravo el al., 2012). For example, ALP from several lepidopterous have binds to Cry1Ac toxin, such as *M. sexta* (McNall and Adang, 2003), *C. virescens* (Jurat-Fuentes and Adang, 2004) and *H. armigera* (Da Silva et al., 2018) (Table 4). It has also been suggested their functional role as Cry toxins-receptors in different insect orders, Cry1Ab in *M. sexta* (Arenas et al., 2010; Gómez et al., 2018a; Gómez et al., 2020), Cry1B in the *Anthonomous grandis* (Lepidoptera: Curculionidae) (Martins et al., 2010), Cry4Ba (Dechklar et al., 2011), Cry11Aa (Fernández-Luna et al., 2010) and Cry11Ba (Hua et al., 2009) in *A. aegypti*. ALP has been associated with different mechanisms of resistance to Cry1 toxins in several insect pests in laboratory and field conditions, such as *Plutella xylostella* (Lelidoptera: Plutellidae), *S. exigua*, *S. frugiperda*, *H. amigera*, *C. virescens* and *C. suppressalis* (Xiao and Wu, 2019). In *H. armigera* and *S. frugiperda*, the level of ALP that bound to the midgut membrane was significantly lower in resistant strains than in susceptible (Jurat-Fuentes et al., 2011).

Arenas et al. (2010) reported that Cry1Ab-L511A mutant, located in domain III of the Cry1Ab toxin, is affected in binding to ALP, and its toxicity is severely reduced in *M. sexta*. It was the first time that the ALP is a protein that interacts with the Cry1Ab toxin. Another important contribution from Arenas et al., 2010 was the observation that both proteins, APN and ALP interact with the toxin Cry1Ab, suggesting that both may have functional role in Cry1Ab toxicity. That hypothesis was confirmed by Flores-Escobar et al. (2013).

The authors demonstrated that the downregulation of APN1 and ALP expression by RNAi correlated with a reduction of transcript and protein levels. In addition, toxicity analysis of the three Cry1A proteins in ALP or APN1silenced larvae

showed that Cry1Aa relies similarly on both receptor molecules for toxicity. In contrast, RNAi experiments showed that ALP is more important than APN for Cry1Ab toxicity, while Cry1Ac relied principally on APN1. These results indicated that ALP and APN1 have a differential role in the mode of action of Cry1A toxins, suggesting that *Bt kurstaki* produces different Cry1A toxins that in conjunction target diverse midgut proteins to exert their insecticidal effect.

The exact function of ALPs as important receptors for Bt toxins remains unclear. According to Xiao and Wu (2019), the glycosyl on ALP binds the toxins, which may help the toxin accumulate, accelerate oligomerization of the Bt toxin by CAD and eventually cause cell perforation by binding to the ABC transporters.

Table 4. Interaction of ALP protein from lepidopteran insects with Cry toxins from *Bacillus thuringiensis*.

Insect	Toxin		Reference
	***In vitro* assays**	***In vivo* assays**	
Manduca sexta	Cry1Ac		McNall and Adang, 2003
	Cry1Ab		Arenas et al., 2010
		Cry1Ab	Flores-Escobar et al., 2013
	Cry1Aa		Adegawa et al., 2017
Chloridea virescens		Cry1Ac	Jurat-Fuentes et al., 2002; Jurat-Fuentes and Adang, 2004
Spodoptera exigua		Cry2Aa	Yuan et al., 2017
Helicoverpa armigera		Cry1Ac	Jurat-Fuentes et al., 2011
		Cry1Fa	Jurat-Fuentes et al., 2011
	Cry1Ac		Da Silva et al., 2018

2.4.4. Prohibitin

Prohibitin (PHB) is a conserved protein in divergent species from prokaryotes and eukaryotes. Two homologous and evolutionarily conserved members of the PHB family — prohibitin-1 (PHB1) and prohibitin-2 (PHB2) — are ubiquitously expressed in eukaryotic cells and assemble into large ring complexes with a diameter of ~20 nm, composed of multiple, alternating PHB1 and PHB2 subunits (Tatsuta and Langer, 2017). These two subunits share more than 50% identity and can form heteroligomers (Mishra et al., 2006). The loss of either PHB-1 or PHB-2 leads to the rapid turnover of its assembly partner, indicating that PHB are only active in the assembled, hetero-oligomeric complex. Both PHB-1 and PHB-2 belong to the SPFH (stomatin/prohibitin/flotillin/Hfl KC) family of proteins with representatives in all kingdoms. SPFH-family members function as scaffold proteins and membrane organizers in various cellular membranes (Langhorst et al., 2005; Wei et al., 2017).

PHB protein is typically associated with lipid rafts in insect cells. Lipid rafts are membrane microdomains rich in cholesterol, sphingolipids, GPI-anchored proteins are selectively located in lipid rafts and are implicated in different processes of the membrane, such as ion channel regulation, membrane protein chaperoning, vesicle and protein trafficking, membrane–cytoskeletal coupling, formation of specialized membrane structures and several cell-signaling responses (Morrow and Parton, 2005; Browman et al., 2007).

Recently, PHB has been studied as receptors for Cry toxins in different insects. PHB has been previously identified as Cry4Ba binding protein in *A. aegypti* (Bayyareddy et al., 2009) and as Cry3Aa binding protein in *L. decemlineata* (Ocho-Campuzano et al., 2013). Those authors demonstrated that PHB is an essential protein in their mode of action since its silencing affected the larval viability.

3. MATERIAL AND METHODS

3.1. Expression, purification, and activation of Cry1A toxins

Bt *kurstaki* HD-73 strain expressing Cry1Ac or crystalliferous Bt 407$^-$ strain expressing Cry1Ab (Meza et al., 1996) or Cry1Fa (Pacheco et al., 2009) proteins or Cry1Ab mutant proteins (G439D, F371A, N514A and L511A) (Arenas et al., 2010;

Torres-Quintero et al., 2018; Gómez et al., 2018b) were grown at 30 °C until complete sporulation for 3 days in nutrient broth sporulation medium (Schaeffer et al., 1965). For Bt 407$^-$ strain expressing Cry1Ab and Cry1Fa proteins, the growth medium was supplemented with erythromycin at 10 µg.ml^{-1}. Spores/crystals were washed three times in 0.3 M NaCl, 10 mM EDTA, pH 8.0. Crystal inclusions were solubilized in an alkaline buffer (50 mM Na_2CO_3 and $NaHCO_3$, 0.2% β-mercaptoethanol, pH 10.5) for 1 h at 37 °C.

Trypsin activated toxins were obtained by treatment of soluble protoxins with trypsin (TPCK treated trypsin from bovine pancreas, SIGMA Aldrich, St. Louis, MO, USA) in a mass ratio of 1: 20 (trypsin: toxin) for 1 h at 37 °C. Phenylmethylsulfonyl fluoride (PMSF) (1 mM final concentration) was added to stop proteolysis. Finally, the Cry toxins were purified with HP HiTrap ion exchange chromatographic column (GE Healthcare) according to manufacturer's protocol. The final concentrations of the purified proteins were determined by Bradford method using bovine serum albumin (BSA) as a standard and separated by SDS-PAGE electrophoresis.

3.2. Construction of domains II and III Cry1Ab mutants

Substitutions G439D, F371A, S587A, N514A and L511A were produced by site-directed mutagenesis (QuikChange; Stratagene) according to manufacturer's protocol using the pHT315 (Meza et al., 1996) plasmid harboring the *cry1Ab* gene as template. The corresponding mutagenic primers are presented in the Table 5. Candidate mutated plasmids were purified from *Escherichia coli* (Enterobacteriales: Enterobacteriaceae) cells and single-point mutations verified by DNA sequencing in the facilities of Instituto de Biotecnología, Universidad Nacional Autónoma de México, Cuernavaca, MOR, México. *E. coli* SCS110 (*dam dcm* mutant strain) cells, and purified plasmids from those cells were transformed in Bt 407 strain (Lereclus et al., 1989) by electroporation and selected in LB broth at 30 °C supplemented with 10 µg.ml^{-1} of erythromycin. The expression and purification of the Cry1Ab constructions are described in section 3.1.

Table 5. Oligonucleotides used for site-directed mutagenesis.

Mutants	Sequence (5' - 3')	Region
F371A	CCA CTT TAT ATA GAA GAC CTG CTA ATA TAG GCA TAA ATA ATC [a]	Loop 2 of domain II
G439D	TGT TTC AAT GTT TCG AAG TGG CTT TAG TAA TAG TAG TGT AAG	Loop 3 of domain II
L511A	GGC CAG ATT TCA ACC GCG AGA GTA AAT ATT ACT GCA	β-16 of domain III
N514A	TCA ACC TTA AGA GTA GCG ATT ACT GCA CCA TTA TCA	β-16 of domain III

[a] Sites of amino acids substitutions are underlined.

3.3. Midgut dissection and BBMV purification from *Helicoverpa armigera*

Five grams of *H. armigera* 3rd instar midgut tissue were dissected as described by Wolfersberger (1993). The larvae were previously refrigerated for 15 min and the midgut sectioned in the fourth pair of abdominal appendages and the first pair thoracic appendages. The peritrophic membrane and Malpighi tubules were removed. The midgut was flushed with MET buffer (0.3 M mannitol, 17 mM Tris-HCl, 5 mM EGTA, 1 mM EDTA, 10 mM Hepes, 2 mM DTT, 0.1 mM PMSF, 100 $\mu g.ml^{-1}$ leupeptatin, 100 $\mu g.ml^{-1}$ pepstatin, 50 $\mu g.ml^{-1}$ neomycin sulphate and pH 7.4). The midgut was centrifuged at 6,425 × g for 15 min at 4 ºC and suspended in MET buffer + distilled water (v/v) and immediately stored in -80 ºC freezer until the requirement.

H. armigera brush border membrane vesicles (*Ha*BBMV) were purified by differential precipitation method using $MgCl_2$ as described by Wolfersberger (1993). The midgut was suspended in MET buffer (1:20; w/v) and homogenized in blender-polytron homogenizer (Glass-Col® Terre Haunt, USA). After homogenization, 24 mM $MgCl_2$ was added and followed by 15 min ice incubation. After incubation, the samples were centrifuged at 6,000 × g for 15 min at 4 °C, the supernatant was recovered and centrifuged at 20,000 × g for 30 min at 4 °C. Supernatant was discarded and the pellet suspended in MET buffer + 24 mM $MgCl_2$ (v/v). The centrifugations were repeated, and the pellet was suspended in MET buffer + distilled water and stored at -80 °C.

BBMV concentration was determined by Lowry DC protein assay (BioRad, Hercules, CA, USA) using bovine serum albumin as a standard (Pierce). The enrichment of APN in BBMV was determined as previously reported (Da Silva et al., 2018), showing that APN activity was 13-fold higher in BBMV than the initial homogenate.

3.4. Heterologous expression of PHB and CAD-TBR from *Helicoverpa armigera* in *Escherichia coli* cells

The CAD from *H. armigera* (*Ha*CAD) used in this work (GenBank accession number JN836550) was previously characterized, and a 1097 bp gene fragment containing the *Ha*CAD toxin binding region (*Ha*CAD-TBR) was previously cloned in pET22b (Liu et al., 2009; Xiao et al., 2017). *Ha*PHB-2 gene (GenBank accession number XM_021345859) from *H. armigera* larvae was cloned in pET SUMO (Thermo scientific, Waltham, MA) and heterologous expressed in *E. coli* cells.

3.4.1. RNA extraction

Total RNA from *H. armigera* 3rd instar larvae midgut was extracted. Forty intestines were extracted as described in BBMV preparation section. Four pools, 10 intestines represented a biological quadruplicate were extracted. During dissection, midgut was stored in tubes dipped in a dry-ice bath and then stored at -80 °C. 30 mg of each midgut pool were used as template for total RNA extraction. The RNeasy Plus Mini Kit (Qiagen, Gathersburg, MD, USA) was used following the manufacturer instructions. Three sample of 1 µl each were separated for quantification of the RNA integrity extracted. RNA samples were quantified by colorimetry using Agilent 2100 Bioanalyzer apparatus (Agilent Technologies).

3.4.2. cDNA synthesis

Reverse transcriptase reaction (RT-PCR) was performed using RNA samples as template (section 3.4.1). The SuperScript ™ First-Strand Synthesis SuperMix (Invitrogen) Kit was used following the manufacturer instructions. The denaturation and annealing process was performed using 8 µL of RNA (1 µg total RNA), 2 µL RT enzyme

mix, 10 µL RT reaction mix, final volume of 20 µL. The reaction was gently mixed and incubated in thermocycler (Applied Biosystems). Cycles in the thermal cycler were: 25 °C for 10 min, 50 °C for 30 min, 85 °C for 5 min and 37 °C for 20 min. After third cycle, digestion mix containing 1 µL (2 $U.\mu L^{-1}$) of *E. coli* RNase was added. cDNA samples were quantified by spectrophotometry in NanoDrop 2000.

3.4.3. Amplification of *phb* gene by conventional PCR

Gene encoding prohibitin (*phb*) (900 bp) were amplified using the cDNA as template (section 3.4.2) by conventional PCR. The expressed sequence tag (EST) of the gene were obtained from GenBank (https://www.ncbi.nlm.nih.gov/genbank): *phb* (access XM_021345859). Oligonucleotides were designed from EST sequence (Forward: 5' – ATG GCA CAA AGT AAG CTT – 3'; Reverse: 5' – TTA CTT AGT CAG TTT C – 3'). Oligonucleotide was design and analyzed using the Gene Runner program version 3.05 (Hastings Software, Inc). Entire *phb* gene were amplified for subsequent cloning of PCR products.

The PCR reactions conditions were 100 ηg cDNA, 10 µM of each oligonucleotide, 0.5 mM dNTPs, 1U Taq DNA polymerase (Thermo Fisher Scientific), 1X High Fidelity PCR buffer, 2 mM $MgSO_4$ and ultrapure water to final volume of 25 µL. PCR cycles used were: initial denaturation at 94 °C for 5 min, followed by 30 denaturation cycles at 94 °C for 30 s, 30 s of annealing (55 °C for *cad* and 57 °C for *phb*) and extension of 72 °C for 45 s. Finally, a final extension of 68 °C for 10 min. In relation to *phb* gene, an additional step was performed to add adenylate residues to PCR product ends and facilitate the insert binding to pET SUMO vector thymidylate ends (5' overhang). Thus, 0.2 µL of Taq DNA polymerase (Fermentas) were added in reaction end, followed by incubation at 68 °C for 10 min. Expected PCR product sizes were analyzed on agarose gel (1%) and then purified with PCR Clean-Up System Kit (Promega).

3.4.4. Cloning into pET-SUMO vector

The PCR product containing *phb* gene were cloned in pET-SUMO (Champion ™ pET-SUMO TA Cloning®) vector (Invitrogen). For the insert-vector binding reaction were used: A volume of 1 µL binding buffer (10x, Invitrogen), 100 ng PCR products, 100 ng pET SUMO vector, 1 µL T4 DNA ligase enzyme (2 $U.\mu L^{-1}$, Invitrogen) and ultrapure water to final volume of 10 µL. The binding reaction were maintained at 15 °C for 16 h. The construction was sub cloned into *E. coli* DH5α cells. The plasmids were purified with Wizard SV Gel and PCR Clean-Up System Kit (Promega).

For the colony's selection with construction in correct orientation, conventional PCRs were performed. Forward primer and reverse primer vector (5' – TAGTTATTGCTCAGCGGTGG – 3') were used. The PCR reactions conditions were 4 µL total lysate, 0.2 µM each forward oligonucleotide of each gene, 0.2 µM oligo T7 reverse vector, 0.2 mM dNTPs, 1 U Taq DNA polymerase (Phusion High-Fidelity DNA Polymerase Thermo Fisher Scientific), 1x High Fidelity PCR buffer, 2 mM $MgSO_4$ and ultrapure water to final volume of 25 µL. PCR cycles used: initial denaturation at 94 °C for 5 min followed by 30 cycles (30 s of denaturation at 94 °C, 30 s 55 °C and 1 min and 30 s extension at 68 °C) and final extension of 68 °C for 10 min.

After correct insertion confirmation, the positive plasmids were verified by DNA sequencing at in Bioenergy Research Institute from Technology Department in São Paulo State University (Jaboticabal, SP, Brazil) using Sanger method. Finally, *phb* positive constructions were transformed into *E. coli* BL21 (DE3) cells for expression analysis.

3.4.5. *Escherichia coli* BL21 (DE3) cells preparation

The cells were plated in LB broth and incubated at 37 °C for 16 h in BOD. After growth, one isolated colony was transferred to 5 ml of LB liquid broth at 37 °C for 16 h under agitation. A volume of 1 ml of the culture overnight was transferred to 100 mL of LB broth until reach OD_{600} between 0.4 and 0.6. The culture was centrifuged at 6,000 × g for 10 min at 4 ºC. Supernatant was discarded and the pellet suspended in 10 ml $CaCl_2$ 0,1M and glycerol 20%. After centrifugation, 50 µL aliquots were immediately frozen in liquid nitrogen and stored at -80 °C.

For efficiency evaluation of the cells, the vector pUC19 DNA™ (Thermo Fisher Scientific) was used. A volume of 50 µg of vector were mixed with 50 µl of competent cells tube. The mix cells were gently shaken and incubated on ice for 30 min. A thermal shock at 42 °C for 45 s was given and then incubated on ice for 2 min. 900 µL of SOC (glucose 20 % and $MgCl_2$ 1 M in SOB broth (20 g tryptone, 5 g yeast extract, 0.58 g NaCl, 0.2 g KCl, pH 7.5 to 1L)) broth was added to cells and incubated at 37 °C for 1 h under agitation. Finally, 200 µL of transformed cells were plated in LB broth supplemented with 50 $\mu g.ml^{-1}$ ampicillin, IPTG 50 $\mu g.ml^{-1}$ and X-Gal 80 $\mu g.ml^{-1}$.

3.4.6. Transformation into *Escherichia coli* BL21 (DE3) cells

The PHB construction and CAD-TBR (Liu et al., 2009; Xiao et al., 2017) from *H. armigera* were used for competent *E. coli* BL21 (DE3) cells transformation. In Eppendorf tube containing 200 µL of *E. coli* BL21 (DE3) cells was added 3 µl of each construction and incubated 30 min on ice. After incubation on ice, a thermal shock at 42 °C for 2 min was given, followed by incubation for 5 min on ice. After the shock, 600 µL of LB broth was added and incubated at 37 °C for 1h with under agitation. 20 µL of cells were plated in LB broth (10 g tryptone, 5 g yeast extract, 10 g NaCl in 1L) supplemented with 100 $\mu g.mL^{-1}$ kanamycin for *Ha*PHB or ampicillin for *Ha*CAD and incubated at 37 °C for 16 h. In day next, colonies growth was verified and used to analysis expression.

3.4.7. Expression analysis of recombinant proteins from *Helicoverpa armigera*

A volume of 500 µL of overnight culture containing *HaCAD* fragment and PHB-2 from *H. armigera* was added into 50 mL of LB broth supplemented with 100 $\mu g.ml^{-1}$ kanamycin or ampicillin. The culture was incubated at 37 °C with shaking at 300 × g until OD_{600} nm reached 0.6. The proteins expression was induced with 1 mM IPTG. After induction, the culture was maintained for 4 h at 37 °C under agitation. Cells were harvested by centrifugation at 6,250 × g for 10 min. The cells were suspended into 5 mL STE buffer (10 mM Tris-HCl, 1 mM EDTA, 8 M urea [pH8]) and subjected to 10 sonication pulses of 10 s. The samples were centrifuged at 12,500 × g for 10 min,

generating soluble and insoluble fractions. Samples of these fractions were verified on SDS-PAGE (12%) to evaluate the expression and solubility of the induced proteins.

3.4.8. Purification of recombinant proteins from *Helicoverpa armigera*

For purification of the CAD-TBR and PHB recombinant proteins from *H. armigera*, the cells previously suspended in STE buffer and sonicated were centrifuged at 16,000 × g for 10 min. The supernatants were recovered and purified by Ni-NTA agarose chromatography affinity column (Quiagen). The column was equilibrated with phosphate buffer saline (PBS) 1x and washed with 3 ml 2 of 5 mM imidazole. The proteins were eluted with different concentrations: 10 mL of 35 mM; 5 fractions of 1 ml of 250 mM and 5 ml 500 mM. Finally, fractions containing the recombinant proteins were analyzed on SDS-PAGE (12%). The highest purity fractions were concentrated by centrifugation 30 kDa cutoff amicon filters (Millipore) and quantified by Bradford method using BSA as standard.

3.5. Binding of Cry1A toxins and competition assays

3.5.1. Binding of Cry1Ac toxin to *Helicoverpa armigera* BBMV

Binding of Cry1Ac activated toxin to BBMV from 3rd instar larvae from *H. armigera* (*Ha*BBMV) was performed. 2.5-20 nM of purified toxin with 10 µg BBMV protein for 1 h at room temperature in 100 µl of binding buffer (PBS, 0.1%, BSA, 0.1% Tween 20, pH 7.6) was incubated. A control of *Ha*BBMV without toxin was included in the assay. After incubation, the unbound toxin was removed by centrifugation for 10 min at 12,850 × g. The pellet containing *Ha*BBMV and bound toxin was washed twice with 100 µl binding buffer, suspended in 10 µl of PBS, and mixed with 10 µl sample loading Laemmli buffer 2X (0.125 mM Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, and 0.01% bromophenol blue). Samples were boiled 3 min, loaded in 10% SDS-PAGE gels and electro transferred to polyvinylidene difluoride membrane (PVDF) (Immobilion-P, Bio-Vin). The PVDF membrane was blocked with BSA 0.5% for 1 h under agitation, and bound Cry1Ac toxin was revealed by western blot using anti-

Cry1Ac antibody (1/10,000 dilution; 1 h) as primary antibody. As secondary antibody, a goat anti-rabbit antibody coupled to horseradish peroxidase (HRP) was used (Santa Cruz Biotechnology, Dallas, TX, USA) (1/10,000 dilution; 1 h), followed by luminol (Santa Cruz Biotechnology Inc.), according to the manufacturer's instructions.

3.5.2. Competition assays of Cry1Ac toxin to BBMV with *Ha*PHB-2

Heterologous competition binding assays of Cry1Ac toxin to *Ha*BBMV were done using different concentrations of *Ha*PHB-2 as competitor. For these assays, 10 µg of *Ha*BBMV were incubated with 5 nM of activated Cry1Ac toxin in the presence of different molar excesses of *Ha*PHB-2 (100, 200 and 500-fold) in 100 µl of binding buffer at room temperature for 1 h. Unbound proteins were removed by centrifugation at 12,850 × g for 10 min at 4 °C and washed twice. The *Ha*BBMV pellet containing the bound proteins were suspended in 10 µl H2O, mixed with 10 µl of Laemmli 2X sample buffer, and boiled for 3 min. Samples were loaded on 12% SDS-PAGE gel and electrotransfered to PVDF membrane. The membrane was blocked with 1X PBS + Tween 20 (2%), under agitation for 1 h and bound protein was recognized using anti-Cry1Ac antibody (1:20,000 dilution) and secondary goat anti-rabbit antibody coupled to HRP (1:20,000 dilution), followed by luminol as described above.

3.5.3. ELISA binding assays

Purified recombinant *Ha*PHB-2 or *HaCAD* protein fragment were used to coat 96-well plate ELISA (1 µg/well) (Rochester, NY, USA). Activated Cry1Ab, Cry1Ac, Cry1Fa or domain II and III Cry1Ab mutants at different concentrations (0-200 nM) were incubated with the receptor-coated ELISA plates. Comparative analysis was also performed with the different Cry1Ab mutants used at non-saturated conditions, 5 nM of each mutant protein was compared with the 5 nM of Cry1Ab toxin. Unbound toxin was removed with PBS and followed by three washes with PBS supplemented with 0.1% Tween 20.

Bound toxins were detected using their respective polyclonal antibody (anti-Cry1Ac, anti-Cry1Ab or anti-Cry1Fa) (1:20,000 dilution) and secondary goat anti-rabbit

antibody conjugated with HRP enzyme (1:20,000 dilution) (Bio-Rad, Hercules, CA, USA). Finally, o-phenylenediamine (Sigma) and H_2O_2 were used as substrates for peroxidase activity detection. Reaction was stopped by adding 50 µl of 5 M HCl and OD_{490} was measured using an ELISA microplate reader (PerkinElmer, Waltham, MA, USA). Negative controls were performed in parallel, where the HaPHB-2 or *Ha*CAD proteins were not used to coat the ELISA plate wells. The data shown here were obtained after subtracting, the data from negative controls to the samples containing the receptors.

3.5.4. Ligand blotting assay

Different amounts of *Ha*PHB-2 and *Ha*CAD proteins were separated by SDS-PAGE (12% acrylamide) and transferred to PVDF membrane. After renaturation and blocking, the blots were incubated for 1 h with 10 nM of Cry1Ac toxin in washing buffer (0.5% Tween 20 in PBS 1X) at room temperature. Unbound toxin was removed by washing three times for 10 min in washing buffer, and bound toxin was identified by western blots assays incubating the blots with anti-Cry1Ac antibody (1:10,000 dilution; 1 h). As secondary antibody, a goat anti-rabbit secondary antibody coupled to HRP was used (1:10,000 dilution; 1 h), followed by luminol as described above.

3.6. Toxicity bioassays

Toxicity bioassays of Cry1Ab Wt and Cry1Ab mutants were performed with *H. armigera* neonate larvae by the surface contamination method. Different concentrations of crystals/spores (25 to 5,000 ng of Cry toxin/cm^2 of artificial diet) were applied to the diet surface contained in 128-well polystyrene plates (Bio-BA-128 bioassay trays; C-D International, Inc.). A total of 48 larvae per toxin concentration were used (one larva per well). The mortality was recorded after 7 days, larvae were considered dead if no movement was apparent and the medium lethal concentration (LC_{50}) was estimated by Probit analysis (Polo-PC LeOra Software). The fiducial limits in each LC_{50} value were estimated.

For relative expression *cad*, *alp*, *apn* and *phb* genes, toxicity assays of dose-response of Cry1Ac exposure to *H. armigera* neonate larvae were performed. Six different concentrations of crystals/spores were assayed (from 5 to 100 ng of Cry1Ac toxin/cm^2 of artificial diet). A total of 96 larvae per toxin concentration were used (one larva per well). In both assays, the plates were incubated at 26 °C, with 65% ± 5% relative humidity and a 14 h light 10 h-1 dark cycle. All the toxicity bioassays were performed in triplicate. The mortality was recorded after 7 days and the LC_{50} and LC_{90} was estimated by Probit analysis (Polo-PC LeOra Software). The fiducial limits in each LC_{50} and LC_{90} value were estimated.

After the exposure of Cry1Ac toxin exposure to *H. armigera* neonate larvae, the LC_{90} was estimated to use in the new mortality bioassay. The surviving larvae of this experiment were used for quantitative real-time PCR (qRT-PCR), the relative expression of *cad*, *apn*, *alp* and *phb* genes from *H. armigera* was evaluated.

3.7 Quantitative real-time PCR (qRT-PCR)

To analyze the relative expression of midgut membrane proteins of *H. armigera*, a subset of differentially expressed genes (DEGs) was evaluated by quantitative real time PCR (qRT-PCR) with primers designed using Primer3Plus (Untergasser et al. 2007). Total RNA was extracted from ten 3rd instar larvae exposed and not exposed to Cry1Ac toxins and qRT-PCR assays were performed. The RNeasy Plus Mini Kit (Qiagen, Gathersburg, MD, USA) was used following the manufacturer instructions. All the experiments were performed in triplicate. A pool of RNA samples from two samples was used to optimize the qPCR reactions for each primer pair. The pooled RNA was treated with DNase I, and the first strand of cDNA was generated with the High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor kit following the manufacture's protocol (Thermo Fisher Scientific).

Table 6. Oligonucleotides used in qRT-PCR.

Oligonucleotides	Sequences
APN1 – F	5' – AGA CGA CGA ATG GGC TGA A – 3'

APN1 – R	5’ – TGA CAT TAG CTT GCG TGG C – 3’
ALP – F	5’ – GTC TGA ACC CAC TCT CGC T – 3’
ALP – R	5’ – TGT CCA TCT CCA GCG TCT C – 3’
CAD – F	5’ – CGA TGA GCT GCC GAT GTT C – 3’
CAD – R	5’ – GCC GGT TTC CTT GTC GAT C – 3’
PHB – R	5’ – AAG CCG CTG AAA TGT TGG G – 3’
PHB – F	5’ – ACA AGT CGT CGA AGG TGG G – 3’
GAPDH – F	5’ – TTG ATG GAC CCT CTG GAA AAC – 3’
GAPDH – R	5’ – TTA GCA ACA GGA ACA CGG AAA – 3’
β-actin – F	5’ – GTT GCT GCG TTG GTA GTA GAC A – 3’
β-actin – R	5’ – CGA TGG GGT ACT TGA GGG TAA – 3’

The cDNA was diluted and used for qPCR in a total reaction volume of 13 ul containing 6.5 ul SYBR Green (Thermo Fisher Scientific), 1 ul cDNA (100 ng) template and an optimized number of primers. For the qPCR assay, ABI 7300 (Applied Biosystems, Foster City, CA, USA) were used. Each optimized reaction amplified a single product with a single peak for the melting. The selected genes were verified with following cycling conditions: 94 °C for 120 s, followed by 35 cycles of 94 °C for 40 s, 56 °C for 60 s. The melting curve was used to analyze the specificity of the qPCR product. After the qPCR optimization, to ensure the reliability of the results, we carried out three biological replications for each individual RNA sample. β-actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) genes were selected as internal reference genes. The relative gene expression values were evaluated using the $2^{-\triangle\triangle Ct}$ method.

3.8. Phylogenetic analysis

A total of 15 PHB-2 amino acid sequences from different insects including lepidopteran, dipteran and coleopteran insects were aligned using Muscle 3.7 alignment (64) and a maximum likelihood Phylogenetic tree was constructed using PhyML version 3.0 (65) with a bootstrap of 500 replicates. Finally, a rooted tree was displayed by using TreeDyn (66). GenBank accession numbers of the selected PHB-2 sequences are as follows: XP_021201534.1 *Helicoverpa armigera*; XP_028169275.1 *Ostrinia furnacalis*; XP_011548688.1 *Plutella xylostella*; XP_026730391.1 *Trichoplusia ni*; XP_022826859.1 *Spodoptera litura*; ADQ90002.1 *Spodoptera frugiperda*; XP_030037509.1 *Manduca sexta*; XP_026752648.1 *Galleria mellonella*; NP_001040326.1 *Bombyx mori*; AAEL012282 *Aedes aegypti*; KXJ68175.1 *Aedes albopictus*; KFB49098.1 *Anopheles sinensis*; ETN60638.1 *Anopheles darlingi*; XP_023029964.1 *Leptinotarsa decemlineata*; and XP_974101.1 *Tribolium castaneum*.

3.9. Statistical analysis

The relative apparent binding affinities (*Kd*) values with Standard Error were determined from Scatchard plots analysis of ELISA assays and significance *P* values were determined for each analysis. Significant differences of the ELISA binding assays of Figures 21 and 24 were determined by using one-way analysis of variance (ANOVA). Significance *P* values < 0.05. Data were analyzed using GraphPad Prism 7 (version 5.0b). About the ELISA binding assays, all experiments were done in triplicate. Comparison of binding data were analyzed by t-test using GraphPad Prism 7 (version 5.0b), and Scatchard plots analysis were used to obtain the *Kd*. Data of the ELISA binding assays were analyzed by using one-way analysis of variance (ANOVA) showing significant differences ($P < 0.05$). For the toxicity bioassays, the LC_{50} was estimated by Probit analysis (Polo-PC LeOra Software).

4. RESULTS

4.1. Cry1A and Cry1Ab mutant production

The activated toxins were obtained by treatment of soluble protoxins with trypsin in a mass ratio of 1: 20 (trypsin: toxin). Then, 1 mM PMSF was added to stop proteolysis. The Cry toxins were purified with HP HiTrap ion exchange chromatographic column and the purified proteins concentrations were determined by Bradford method using BSA as standard.

The figure 14A to 14E shows the Cry1A Wt (Cry1Ab, Cry1Ac and Cry1Fa protoxins), the Cry1Ab mutants protoxins and activated toxins profiles. It was obtained equivalent bands of 130 and 65 KDa, compatible with the expected size for their non-activated and activated forms of the toxins, respectively.

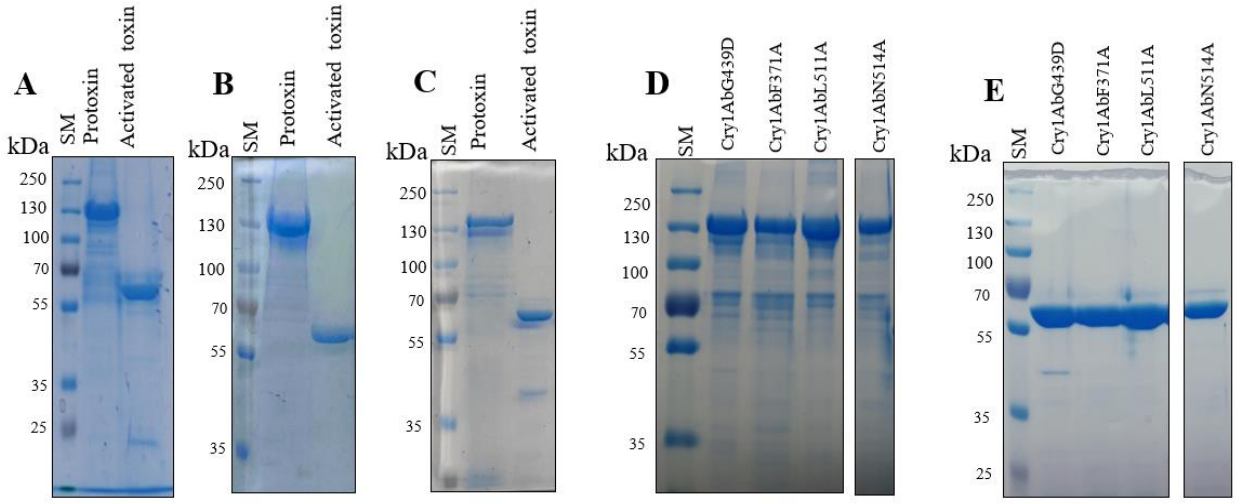

Figure 14. Expression of Cry1Ab, Cry1Ab and Cry1Fa Wt toxins, protoxins (130 kDa) and activated toxins (65 kDa) are shown on SDS-PAGE (10%). **A**, Cry1Ab; **B**, Cry1Ac; **C**, Cry1Fa; **D**, Protoxins of Cry1Ab mutants; **E**, Activated toxin of Cry1Ab mutants. Toxic fragments were activated with trypsin. **SM**, size markers of 250 kDa™.

4.2. Heterologous expression of CAD and PHB from *Helicoverpa armigera* in *Escherichia coli* cells

4.2.1. RNA extraction

The RNA virtual bands 18S and 28S are showed in the Figure 15. The total RNA quantification and integrity parameters of each sample are showed in the Table 7, RNA was analyzed in Agilent 2100 Bioanalyzer. The samples extracted from the pool containing 10 *H. armigera* midgut presented lower RIN number than recommended in some samples. Even though these samples showed characteristic bands of RNA in

the gel generated by Bioanalyzer. Probably, the excess of material for extraction resulted in bands that interfered in RIN calculation, making it difficult the correct identification of the bands corresponding to ribosomal subunits. Due to high amounts of protein, fat acid or polysaccharides, after an additional centrifugation step, the supernatant (which contains RNA, DNA, and proteins) was transferred to new tube. Thus, it was possible to obtain high quality total RNA, samples CH2 and CH3 (Table 7).

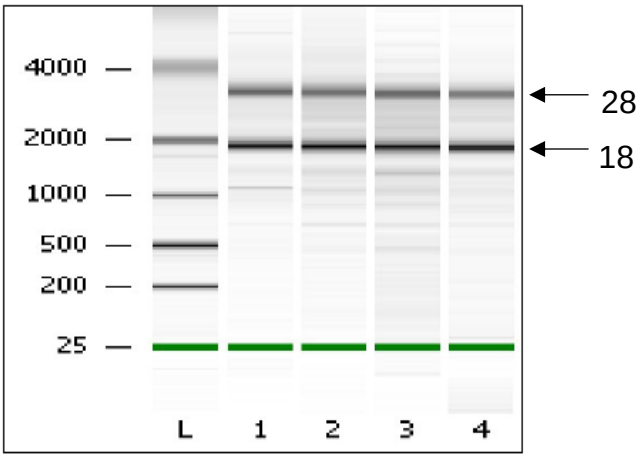

Figure 15. Electrophoresis analysis of 3rd instar total midgut RNA of *Helicoverpa armigera,* generated by Agilent 2100 Bioanalyzer. L, 1 Kb Ladder; lane 1 to 4: total midgut RNA of *H. armigera* (four pools of ten midgut each).

Table 7. Total RNA quantification extracted of 3rd instar midgut larvae of *Helicoverpa armigera*.

Sample	Concentration (ng of RNA/µl)	rRNA rate [28s/18s]	Total volume	Total amount (ng)	RIN [a]
CH1	60	0,6	30	1800	N/A
CH2	99	0,7	30	2970	8
CH3	48	0,6	30	1440	7,7
CH4	96	0,6	30	2880	N/A

[a] RIN: RNA integrity number

4.2.2. cDNA synthesis

The cDNA was synthetized using SuperScript™ First-Strand Synthesis System for RT-PCR (Invitrogen) kit according to the manufacture instructions. It was obtained 0,72 µg.µl^{-1} of cDNA.

4.2.3. Genes amplification by PCR

The Figure 16 shows the *phb* gene amplification by conventional PCR from *H. armigera* 3rd instar larvae. The complete sequence of *phb* (900 bp) gene was amplified.

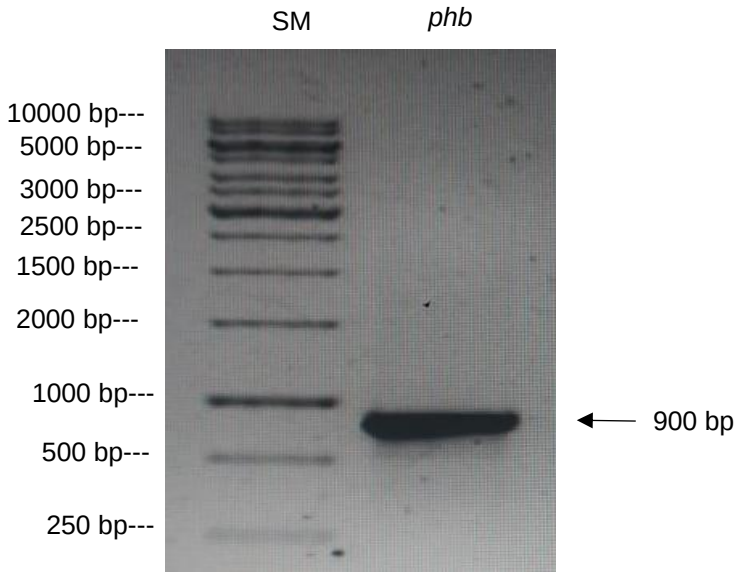

Figure 16. Gel electrophoresis in 1% agarose stained with ethidium bromide (10 mg/ml) containing amplification products of prohibitin (*phb*) (900 bp) from *Helicoverpa armigera* 3rd instar larvae. SM, size molecular marker GeneRuler 1kb DNA Lader.

4.2.4. Construction's confirmation and gene sequencing

The colony PCRs were performed to verify the insertion and correct orientation on the vector (Figure 17). For confirmation of the *phb* insertion, T7 of the pET-SUMO vector reverse primer was used according to instructions of the manufacturer. Among the five colonies tested, four of them were inserted in correct orientation (Figure 17). The positive colonies were sequenced for plasmids confirmation and to analyze possible mutations in the nucleotide sequences.

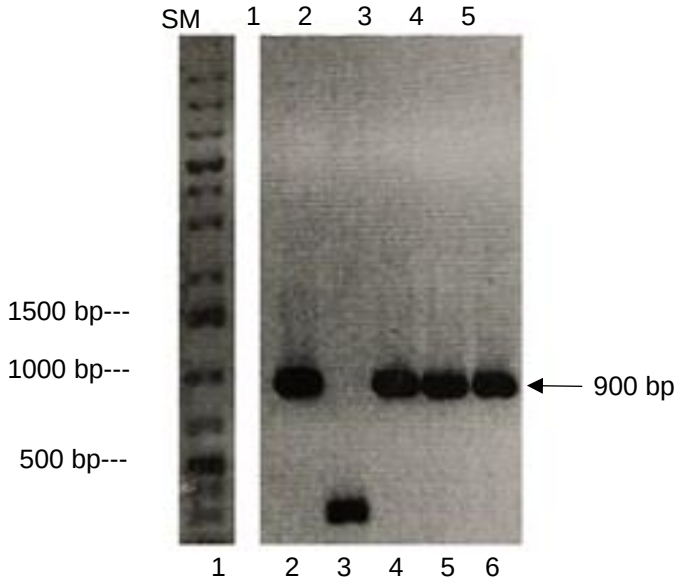

Figure 17. Electrophoresis on 1% agarose gel stained with ethidium bromide (10 mg.ml^{-1}) containing colonies on pET-SUMO vector. Lanes 2 to 6 correspond to five *phb* gene PCR products amplified by T7 primer reverse of the vector. Lane SM corresponds to molecular marker GeneRuler 1kb DNA Ladder.

The sequencing of *phb* was performed in Bioenergy Research Institute from Technology Department in São Paulo State University (Jaboticabal, SP, Brazil) using Sanger method. The sequencing of the *phb* gene is showed:

ATGGCACAAAGTAAGCTTAACGATATGGCGGGCAAATTCGCCAAGGGTGGACCCCCTGGACT
CAACGCCGGCCTCAAAGTGGTCGCCGTTGTAGGTGCGGCAGCCTATGGCATCTCGCAATCCT
TGTTTACGGTTGAGGGTGGTCATCGTGCCATCATGTTCAACAGAATAGGAGGAATTCAGCAA
CACGTCATGAGCGAGGGTATGCACTTCCGTATACCTTGGTTCCAATACCCTATCATTTATGA
CATTAGGTCCAGACCTCGCAAGATTTCATCACCCACCGGATCTAAGGATTTACAAATGGTCA
ACATTTCTTTGAGAGTACTCTCTCGTCCTGATGCGAGCTCATTGCCTACAATGTACAGACAG
CTTGGCACTGATTATGATGAGAAGGTGCTGCCATCAATTTGCAATGAAGTATTAAAATCTGT
TGTTGCTAAGTTCAATGCTTCACAGCTAATCACTCAGCGTCAGCAGGTGTCCCTTCTGATCA
GGAGAGAGTTGGTGGAACGAGCAGCCGATTTCAATATTATACTGGATGATGTCTCTCTGACT
GAACTGAGCTTTGGTAAAGAGTACACTGCTGCTGTTGAGGCTAAACAAGTTGCTCAGCAGGA
AGCTCAGCGAGCTGCTTTCGTTGTGGAAAGAGCCAAGCAAGAGCGTCAGCAGAAGATTGTTC
AAGCTGAGGGTGAAGCTGAAGCCGCTGAAATGTTGGGAAAAGCTATGGGTATGAACCCTGGT
TACTTGAAGCTGCGTAAGATCCGTGCCGCTCAGAGCATTTCCAGAATGATTGCTCAGTCACA

AAACCGTGTCTTCTTGCCTGGCAACAGCTTGATGATCAACCTCCAGGACCCCACCTTCGACG
ACTTGTCTGAGAAACTGACTAAGAAGAAGTAA

4.2.5. Expression of recombinant proteins from *Helicoverpa armigera*

The Figures 18A and 18B shows the protein profile of *Ha*PHB-2 and *HaCAD* -TBR fragment after purification. Both recombinant proteins were quantified by Bradford method and used for binding and competition assays with Cry1A purified toxins.

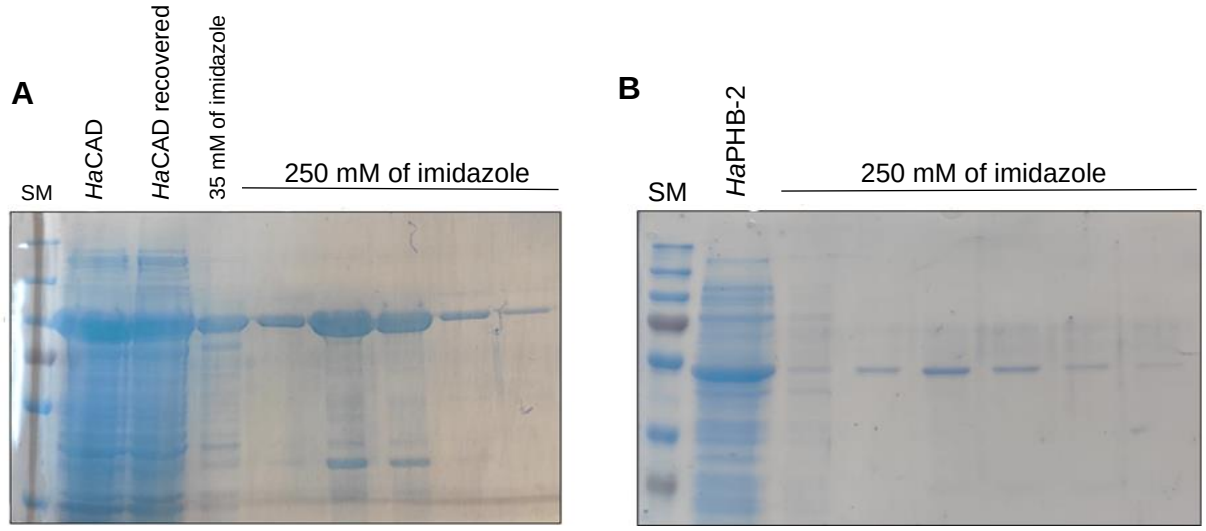

Figure 18. Expression of recombinant proteins of *Helicoverpa armigera* in polyacrylamide gel 12% stained with Comassie blue. The proteins were induced with 1mM of IPTG and recovered using 25 to 500 mM of imidazole. **A**, *Ha*CAD-TBR; **B**, *Ha*PHB-2. **SM**, size marker of 250 kDa™ (BioRad).

4.3. Binding and competition assays

4.3.1. Binding of Cry1Ac toxin to *Helicoverpa armigera* BBMVs

To analyze the binding of Cry1Ac to *Ha*BBMV, qualitative binding assays were performed. Cry1Ac bound to *Ha*BBMV in a toxin concentration dependent manner (Figure 19). The analysis of the densitometry of the bands shown in Figure 19 by ImageJ, revealed an apparent binding affinity (*Kd*) of 8.51 ± 1 nM.

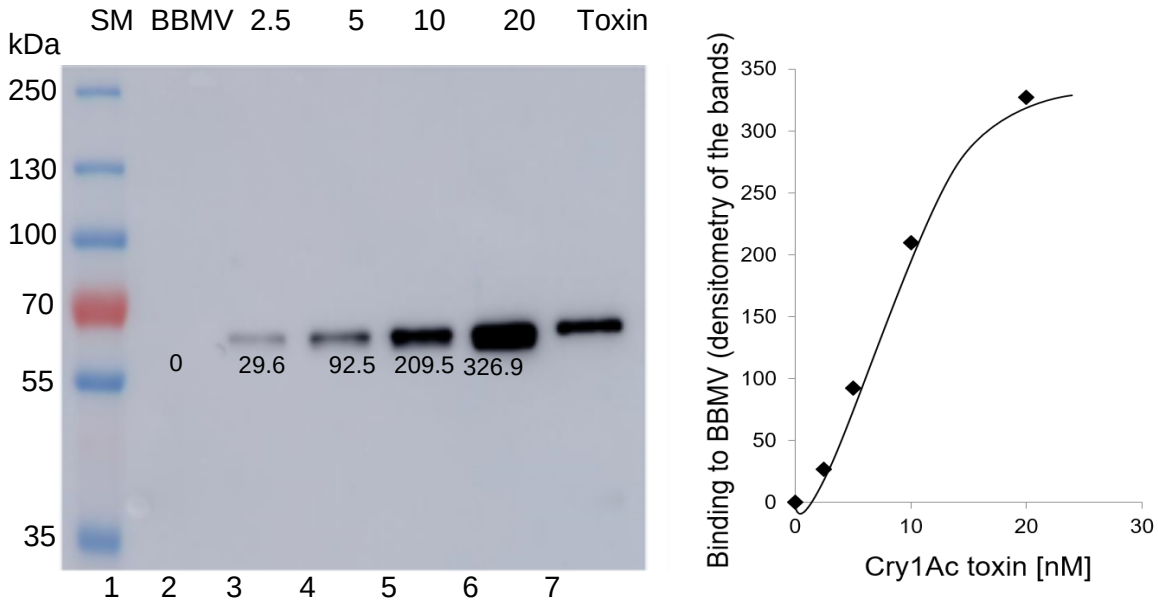

Figure 19. Binding interaction of Cry1Ac toxin to BBMV from *Helicoverpa armigera*. A total of 10 µg of *Ha*BBMV from 3rd larval instar were incubated with 2.5 to 20 nM of Cry1Ac toxin (lane 3 to 6). A negative control of *Ha*BBMV without toxin incubation (lane 2) and positive control loading 2.5 nM of Cry1Ac toxin directly into the SDS-PAGE (lane 7), were included in the figure. The optical density of the 65 kDa band was measured by using ImageJ program. **SM**, size marker of 250 kDa™ (BioRad). Right plot shows the densitometry analysis of the bands accordingly to protein concentration used in the binding assay.

4.3.2. Competition assays of Cry1Ac toxin to *Ha*BBMVs with *Ha*PHB-2

To determine if *Ha*PHB-2 is involved in the binding of Cry1Ac to *Ha*BBMV, the recombinant *Ha*PHB-2 protein was purified from *E. coli* cells and used as competitor in Cry1Ac binding competition assay to *Ha*BBMV. Five nM of Cry1Ac toxin was incubated with 10 µg of *Ha*BBMV in the absence or in the presence of different fold molar excesses of *Ha*PHB-2 (100 to 500 nM). A reduction of toxin binding to *Ha*BBMV was observed in the presence of *Ha*PHB-2 in a concentration dependent way (Figure 20). Densitometry analysis of the bands by ImageJ revealed a 2.8 and 13.2-fold reduction of Cry1Ac binding in the presence of 100 and 200 molar fold excess of *Ha*PHB-2, respectively, while a complete competition of binding was observed when 500-fold molar units of *Ha*PHB-2 was used as competitor.

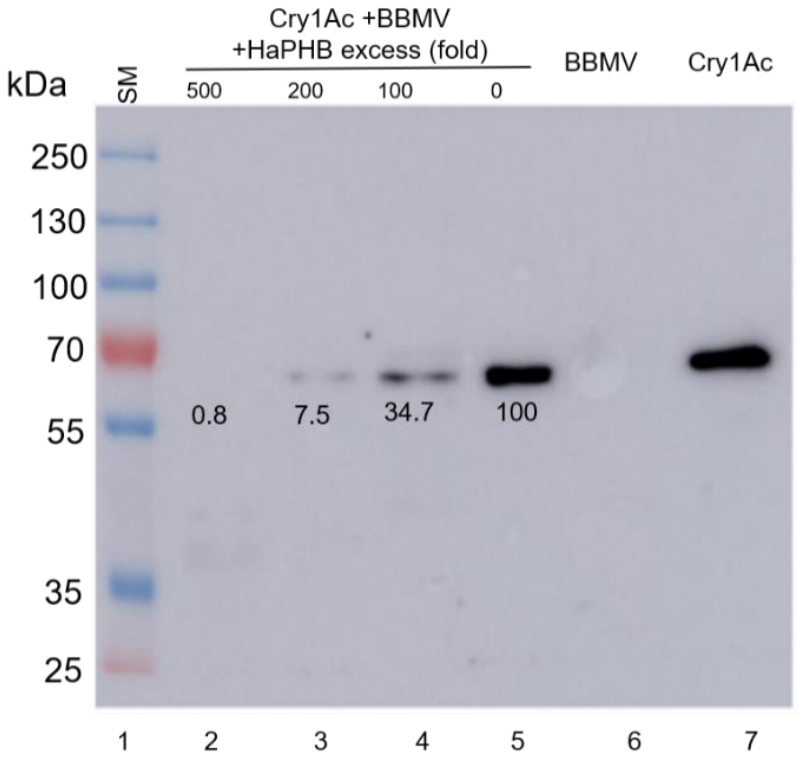

Figure 20. Competition of 5 nM of Cry1Ac toxin to 10 µg of 3rd larval instar *Helicoverpa armigera* BBMVs with 0 to 500-fold of *Ha*PHB (lane 2 to 5). Two controls were performed, a negative one using BBMVs without incubation with toxin (lane 6) and positive one using 2.5 nM of Cry1Ac toxin without BBMVs (lane 7). The optical density of the 65 kDa bands was measured by using ImageJ program (http://imagej.nih.gov/ij/). **SM**, size marker of 250 kDa™ (BioRad).

4.3.3. Binding of Cry1A toxins to CAD fragment and PHB proteins from *Helicoverpa armigera*

To further analyze the interaction of different Cry1 proteins to *Ha*PHB-2, the binding of Cry1Ab, Cry1Ac and Cry1Fa toxins to heterologous expressed *Ha*PHB-2 proteins by ELISA binding assays were determined. As control, we included a *Ha*CAD fragment that was reported to contain the toxin-binding region (TBR) (Xiao et al., 2017). Binding of Cry1 toxins to both proteins, *Ha*CAD-TBR and *Ha*PHB-2, were saturable and Cry1Ac and Cry1Fa showed slightly higher binding to both receptors compared to Cry1Ab (Figure 21).

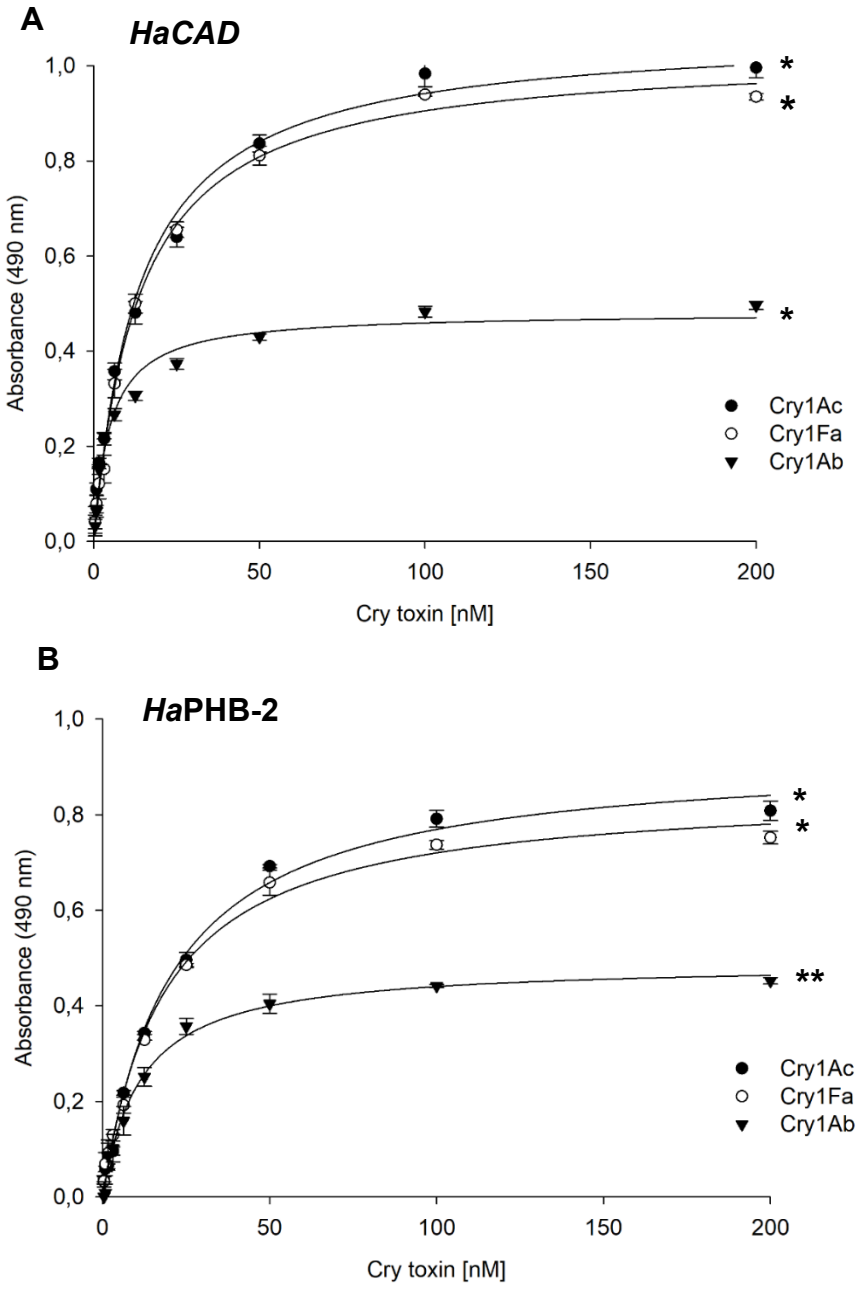

Figure 21. ELISA binding analysis of Cry1A toxins to the recombinant *Ha*CAD-TBR and *Ha*PHB-2 proteins from *Helicoverpa armigera* expressed in *Escherichia coli* cells. Different asterisks indicate statistically significant different data analyzed one-way ANOVA analysis (*P* value < 0.05).

The analysis of the apparent binding affinities obtained after total binding analysis showed that Cry1Ac and Cry1Fa toxins interact with *Ha*CAD-TBR with two-fold higher apparent binding (Cry1Ac *Kd* = 4.65 ± 1.6 nM and Cry1Fa *Kd* = 5.46 ± 0.9

nM) than Cry1Ab (*Kd* = 9.12 ± 0.72 nM) (*P* value < 0.04 which is statistically significant) (Figure 21A). A similar pattern was observed in the interaction of Cry toxins with *Ha*PHB-2, since Cry1Ac and Cry1Fa presented slightly higher apparent binding (Cry1Ac *Kd* = 6.65 ± 1.84 nM and Cry1Fa *Kd* = 7.75 ± 1.86 nM) than Cry1Ab (*Kd* = 9.68 ± 0.89 nM). However, the *P* value < 0.39 from these data indicated that these differences were not statistically significant (Figure 21B).

4.3.4. Ligand blot

In addition to binding assays, ligand blot analysis of Cry1Ac toxin to the recombinant *Ha*PHB-2 and *Ha*CAD-TBR proteins from *H. armigera* purified from *E. coli* cells were performed to evaluate the specific binding of the toxin to both receptors (Figure 22A and 22B).

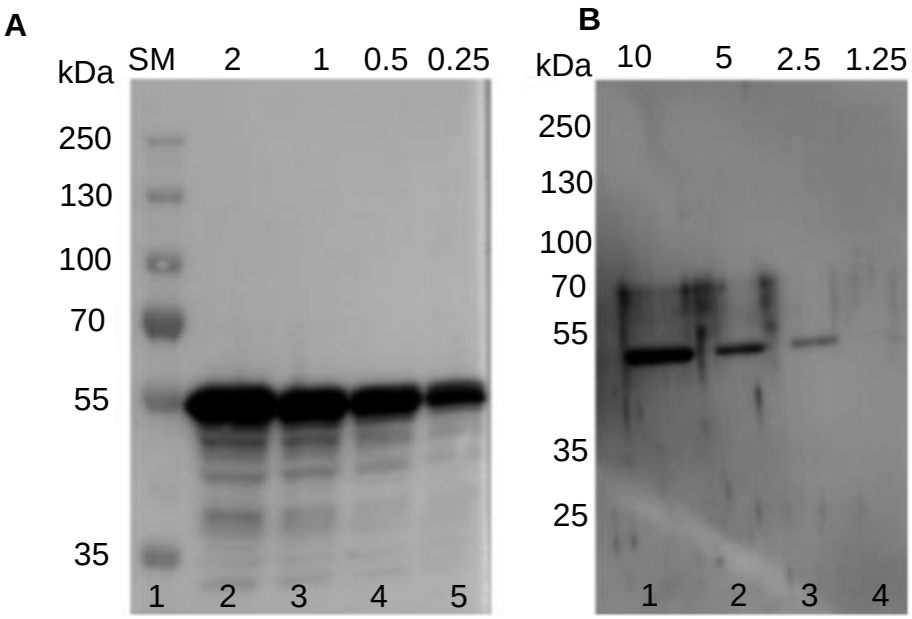

Figure 22. Ligand blot assays showing binding of Cry1Ac toxin to *HaCAD* -TBR and *Ha*PHB-2 recombinant proteins from *Helicoverpa armigera*. Cry1Ac (10 nM) was bound to different amounts of *Ha*CAD-TBR fragment (**A**) (0.25 to 2 µg) or to *Ha*PHB-2 (**B**) (1.25 to 10 µg). Bound Cry1Ac protein was revealed by using anti-Cry1Ac antibody and secondary HRP coupled anti-rabbit antibody. SM, size marker of 250 kDa™ (BioRad).

The binding of 10 nM Cry1Ac to different amounts of the purified *Ha*CAD-TBR protein of 47.5 kDa (Figure 22A) and to the purified *Ha*PHB-2 fused to SUMO protein

resulting in a 45 kDa protein (Figure 22B). Figure 22 shows that Cry1Ac binds to both receptor proteins of equal way. Although, *HaCAD* -TBR was able to bind to toxin in lower concentration, as 0.25 µg, while PHB was not. The binding was dependent of protein concentration.

4.3.5. Cry1Ab-binding regions involved in *Ha*PHB-2 interaction and their correlation in toxicity against *Helicoverpa armigera*

To identify the Cry1Ab toxin regions involved in the interaction with *Ha*PHB-2 and the role of *Ha*PHB-2 in toxicity, its binding to *Ha*PHB-2 and toxicity against *H. armigera* larvae was characterized. Different domain II and domain III Cry1Ab mutants previously characterized as affected in binding to different receptor molecules in *M. sexta* or *S. frugiperda* was used. Regarding Cry1Ab domain II mutations, two mutants affected in toxicity against *M. sexta* was used in those assays, Cry1Ab-F371A and Cry1Ab-G439D mutants. In the case of domain III mutations, two Cry1Ab β-16 mutants were analyzed.

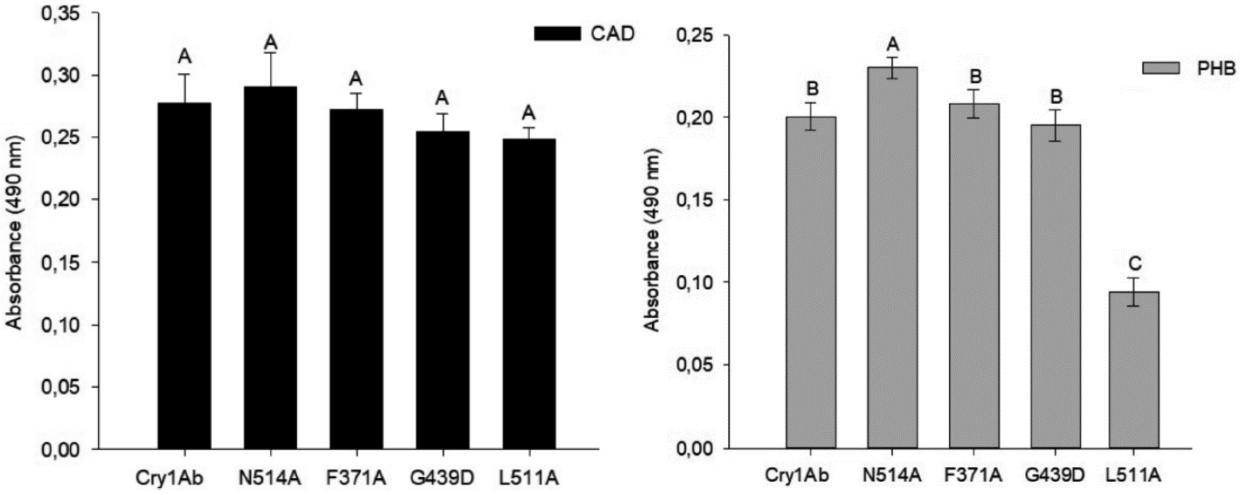

Figure 23. Comparative binding analyses of Cry1Ab mutants to *Ha*CAD -TBR and *Ha*PHB-2. Recombinant *Ha*PHB-2 or *Ha*CAD -TBR protein fragments (1 µg/well) were used to coat 96-well ELISA plate. The binding of 5 nM of each toxin mutant was analyzed to these two receptors and detected with anti-Cry1Ab, antibody as primary antibody and HRP conjugated anti-rabbit antibody as secondary antibody. The absorbance was determined at 490 nm and the data were analyzed by using GraphPad Prims 7 software. Different letters indicate statistical differences determined by one-way ANOVA analysis (*P* value < 0.05).

To further confirm these data, additional ELISA binding assays of these four Cry1Ab mutants to HaPBH-2 were performed. The data revealed that Cry1Ab-N514A gained higher apparent binding to *Ha*PHB-2 (*Kd* = 2.54 ± 1.16 nM) compared with the Cry1Ab toxin, that showed *Kd* value of 7.12 ± 0.89; while Cry1Ab-L511A showed less binding to *Ha*PHB-2 (*Kd* = 13.12 ± 0.72 nM), ANOVA analysis of these data indicated that these differences were statistically different $P < 0.05$ (Figure 24).

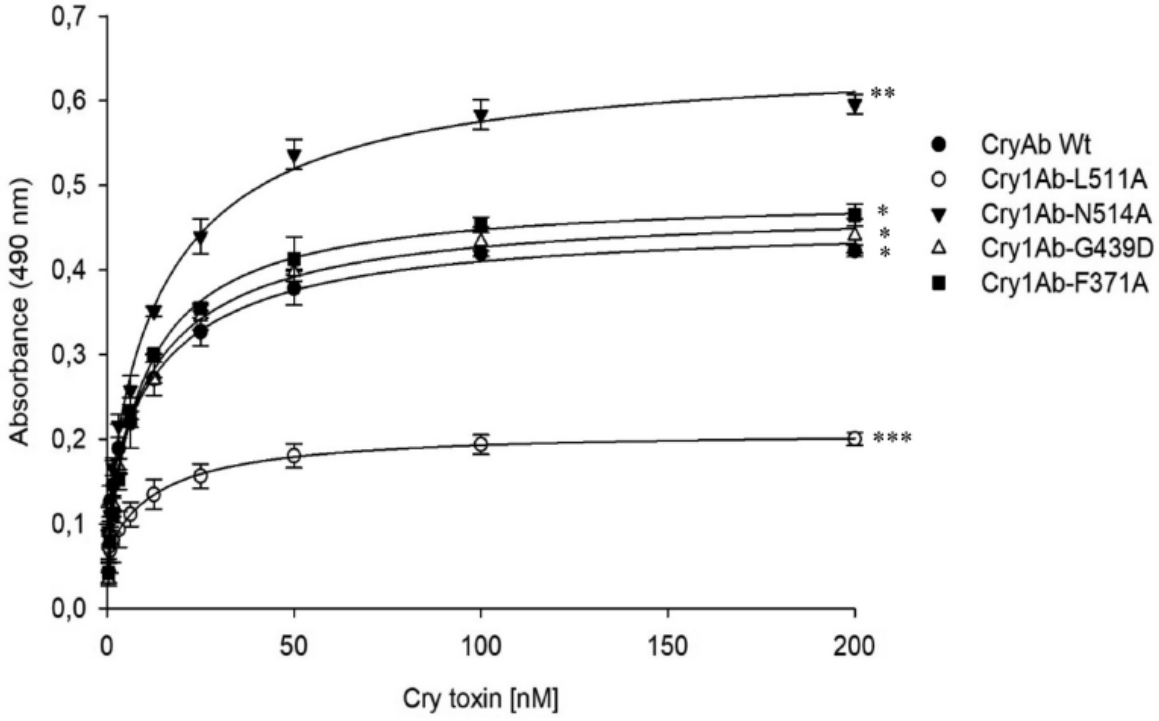

Figure 24. ELISA Binding analysis of different Cry1Ab mutants to recombinant *Ha*PHB-2 protein expressed in *Escherichia coli* cells. The absorbance was determined at 490 nm and the data were analyzed by using GraphPad Prism 7 software to obtain relative binding affinities (*Kd*) by Scatchard analysis. Different asterisks indicate statistically significant different data analyzed by one-way ANOVA analysis (*P* value < 0.05).

It is important to note that total binding of Cry1Ab-L511A mutant to *Ha*PHB-2 was reduced significantly. Interestingly, the binding of Cry1Ab-N514A or Cry1Ab-L511A mutants correlated with their toxicity against neonate *H. armigera* larvae, since Cry1Ab-N514A mutant was ~ 6-fold more toxic (LC_{50} = 43 ng/cm² (30-62 fiducial limits) than Cry1Ab (LC_{50} = 260 ng/cm² (178-398 fiducial limits), while Cry1Ab-L511A mutant lost toxicity, showing to be at least 20-fold less toxic (LC_{50} estimated value > 5000 ng/cm²) compared with Cry1Ab (Table 8). The two domain II Cry1AbF371A or Cry1Ab-

G439D mutants bound to *Ha*PHB-2 with similar apparent binding (*Kd* = 6.65 ± 0.61 nM or *Kd* = 6.98 ± 0.78 nM, respectively, *P* values < 0.05) (Figure 24). Table 7 shows that Cry1Ab-G439D mutant was affected in insecticidal activity, while Cry1AbF371A was not.

Table 8. Insecticidal activity of Cry1Ab wild type and domain II and III mutants against *Helicoverpa armigera* from Brazil.

Toxin	LC_{50} ng/cm² (fiducial limits) [a]	LC_{90} ng/cm²	Slope [b]	χ^2 [c]
Cry1Ab	260 (178 - 398)	> 3,000	1.16 ± 0.19	1.63
Cry1AbL511A	> 5,000	> 50,000	1.25 ± 0.20	0.93
Cry1AbN514A	43 (30 - 62)	415 (220 - 1283)	1.30 ± 0.20	1.04
Cry1AbG439D	> 2,000	> 30,000	1.06 ± 0.21	1.24
Cry1AbF371A	244 (97 - 445)	> 2,000	1.36 ± 0.21	6.36

[a] Concentration killing 50% and 90% of the population with 95% fiducial limits in parentheses. Units are ng of Cry toxin per cm² of artificial diet (25 to 5000 ng/cm² were used); [b] Slope ± standard error; [c] Chi-square.

4.4. qRT-PCR analysis

In order to evaluate the differentially expressed unigenes (DEGs) expressed in *H. armigera* 3rd instar larvae exposed and not exposed to Cry1Ac toxin, bioassay toxicity using LC_{90} was performed (Figure 25). As expected, it was observed 10% of survivor in treatment exposed to LC_{90} (400 ng.cm$^{2-1}$) of Cry1Ac toxin. Those survivors' insects in both treatments were used to qRT-PCR analyzes. Our results demonstrated that all the genes evaluated were shown to be downregulated (Figure 26). However, *cad, apn* and *phb* expression genes in *H. armigera* larvae exposed to LC_{90} of Cry1Ac toxin was significantly different to the larvae not exposed to toxin. On the other hand, the regulation of *apn1* in treatment fed with Cry1Ac toxin was not significantly different to exposed larvae (Figure 26).

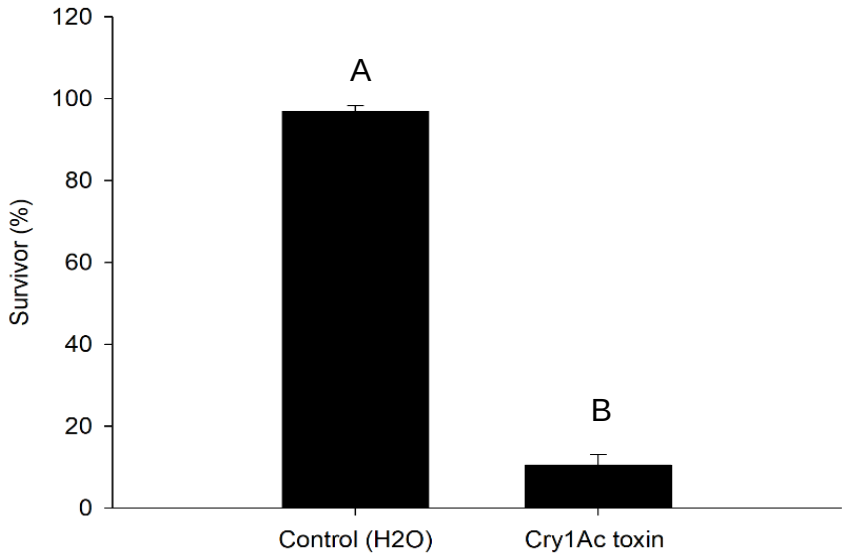

Figure 25. Survival of *H. armigera* neonate larvae exposed to Cry1Ac toxin (400 ng of toxin/cm² of artificial diet) and a control group not exposed. Asterisks above bars indicates significant differences (*P* > 0.05) between larvae exposed and not exposed to Cry1Ac toxin, n. s = not significant.

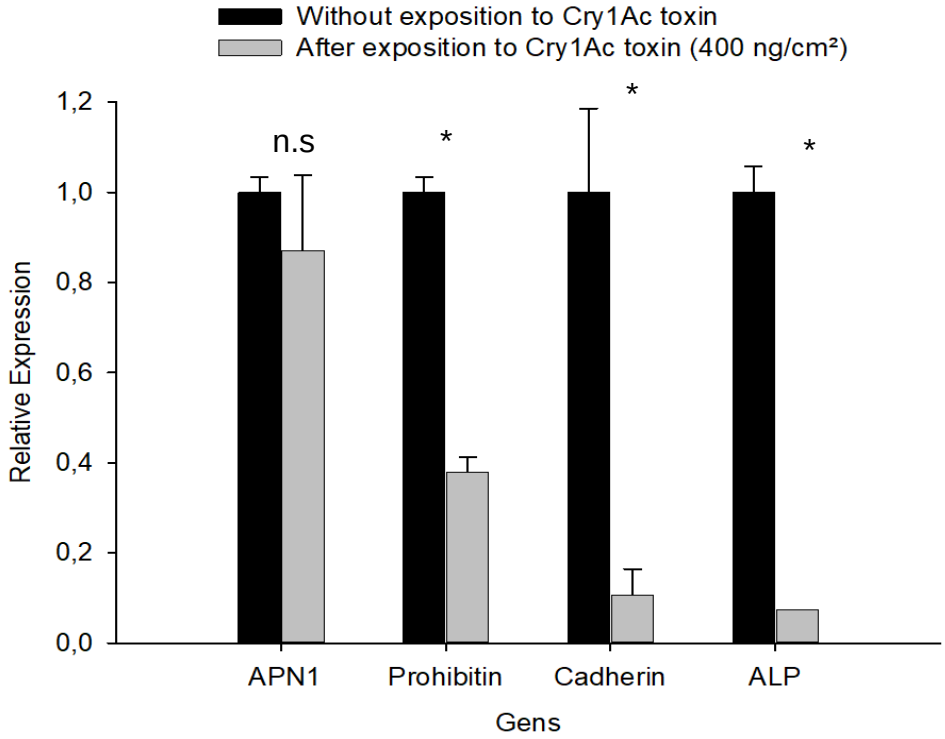

Figure 26. Relative expression of *cad, alp, apn1 and phb* midgut membrane proteins of *H. armigera* under exposure to Cry1Ac toxin (400 ng/cm²). Asterisks above bars indicates significant differences (*P* > 0.05) between larvae exposed and not exposed to Cry1Ac toxin, n. s = not significant.

4.5. PHB-2 is a highly conserved protein among different insect orders

Once cloned the *Ha*PHB-2 region, we took advantage of the previously annotated PHB-2 protein from *H. armigera* (GenBank accession number XM_021345859). *Ha*PHB-2 is a 299 amino acids protein with a predicted molecular weight of 33 kDa.

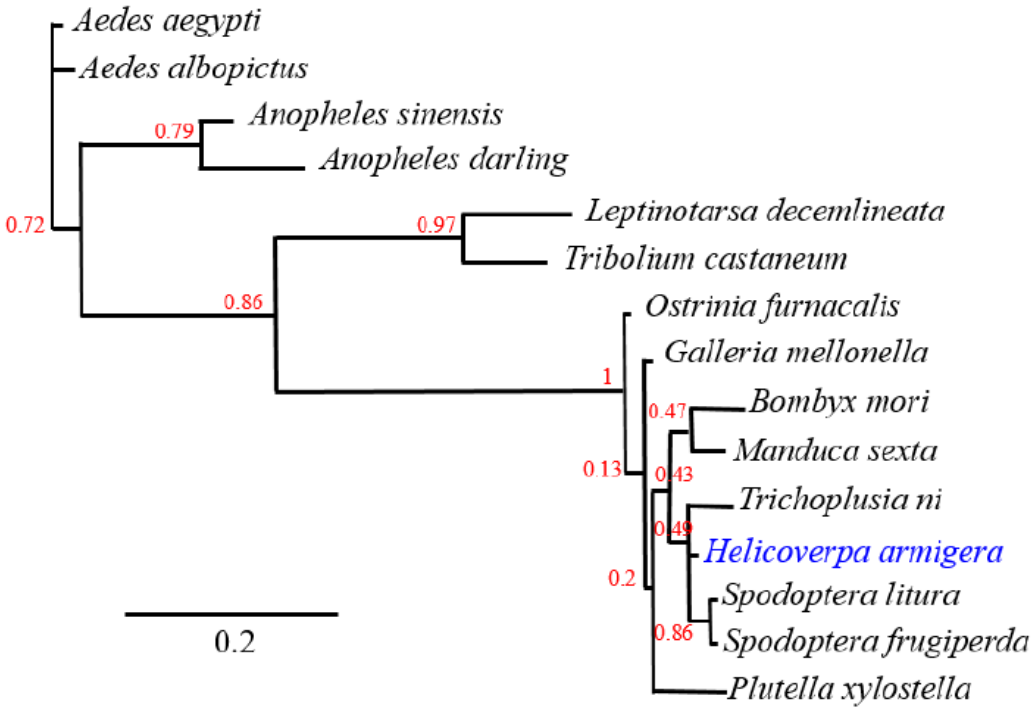

Figure 27. Phylogenetic rooted tree of the insect PHB-2 amino acid sequences. A phylogenetic rooted tree was constructed using 15 PHB-2 sequences. The bootstrap values of 500 replications are expressed as percentages and shown at each branch point. GenBank accession numbers are as follows: XP_021201534.1 *Helicoverpa armigera*; XP_028169275.1 *Ostrinia furnacalis*; XP_011548688.1 *Plutella xylostella*; XP_026730391.1*Trichoplusia ni*; XP_022826859.1 *Spodoptera litura*; ADQ90002.1 *Spodoptera frugiperda*; XP_030037509.1 *Manduca sexta*; XP_026752648.1 *Galleria mellonella;* NP_001040326.1 *Bombyx mori*; AAEL012282 *Aedes aegypti*; KXJ68175.1 *Aedes albopictus*; KFB49098.1 *Anopheles sinensis*; ETN60638.1 *Anopheles darlingi*; XP_023029964.1 *Leptinotarsa decemlineata*; and XP_974101.1 *Tribolium castaneum*.

Phylogenetic sequence analysis with other 15 PHB-2 sequences from different insect orders (Lepidoptera, Coleoptera and Diptera) revealed that PHB-2 proteins from lepidopteran species are clustered in the same compact branch, while the PHB-2 from

coleopteran insects and from dipteran insects are more distantly arranged in independent branches. The *Ha*PHB-2 is more closely related to PHB-2 protein from *Spodoptera spp.* and from *Trichoplusia ni* (Figure 27).

5. DISCUSSION

It has been demonstrated that *H. armigera* shows variable susceptibility to different Cry toxins. Frankenhuyzen (2009) analyzed 24 Cry toxins and demonstrated that seven toxins were active against *H. armigera*: Cry1Ac, Cry1Ah, Cry1Fa, Cry2Aa, Cry2Ab, Cry2Ac and Cry2Af, while other four Cry toxins (Cry1Aa, Cry1Ab, Cry1Ia and Cry9Aa) were classified as having potential toxicity. This differentially susceptibility has been explained by the specific mode of action of the Cry toxins and has been extensively discussed by Jurat-Fuentes and Crickmore (2017). The authors propose that the Bt specificity is related to seven different levels into the mode of action: (1) exposure to the insecticidal toxin, (2) crystal solubilization, (3) toxin processing and stability, (4) toxin circulation, (5) capacity to crossing the peritrophic matrix, (6) binding to receptors and (7) post-binding events, such as non-specific interactions, binding reversibility, toxin oligomerization and membrane insertion.

The identification of the midgut binding proteins that participates on Cry toxicity is crucial to further understand their function in the mechanism of action of these Cry proteins. The mode of action of Cry toxins is a complex process, involving their interaction with different receptors in the larval midgut epithelium, triggering toxin oligomerization and insertion of the oligomer into the membrane, resulting in pore formation in the apical membrane of the midgut cells and the insect death (Vachon et al., 2012; Gómez et al., 2014). The Cry toxins, including Cry1Ab toxin, is composed by a three-dimensional (3D) structure, which domain I, is composed of seven α-helices that is involved in the pore formation, and oligomerization of the toxin. Domain II is composed by two-β sheets that form a β-prism structure with exposed loops and is involved in the midgut receptors interaction. Domain III is composed by a β-sandwich with anti-parallel β-sheets, which also are involved in the midgut receptors interaction (Bravo et al., 2007; Pardo-López et al., 2013; Adang et al., 2014; Crickmore et al., 2020).

One critical step for toxicity to lepidopteran insects depends on their interaction with different receptors present on the midgut. In this way, several receptors have been reported, such as CAD-like protein, GPI-anchored proteins, such APN and ALP (Pigott and Ellar, 2007; Jurat-Fuentes and Crickmore, 2017; Da Silva et al., 2018). In addition, recently transporters from the ABC family (ABCC2 and ABCC3) have been reported in several lepidopteran (Xiao et al., 2014; Zhou et al., 2016; Ocelotl et al., 2017; Boaventura et al., 2020; Zhao et al., 2021).

In this work, the binding capacity of three Cry1 toxins to purified *Ha*PHB-2 and to *HaCAD* -TBR proteins from *H. armigera* that were previously identified as Cry1Ac binding protein (Xu et al., 2005; Liu et al., 2009; Peng et al., 2010; Zhang et al., 2017b; Da Silva et al., 2018; Wang et al., 2016) was evaluated. Cry1Ac bound to *H. armigera* BBMV with high affinity (Fig. 16; *Kd* = 8.51 nM), which confirms previous binding analyses of Cry1Ac to BBMV from this insect pest (Estela et al., 2004; Sebastião et al., 2015).

Estela et al. (2004) analyzed the binding of ^{125}I-labeled Cry1Ab protein (^{125}I-Cry1Ab) and ^{125}I-Cry1Ac to *Ha*BBMV in competition experiments with 11 non-labeled Cry proteins. The authors demonstrated that that Cry1Aa, Cry1Ab, and Cry1Ac competed for common binding sites. In addition, it was demonstrated that Cry1Ac and Cry1Ab use different epitopes for binding to *Ha*BBMV. Sebastião et al. (2015) analyzed the toxicity and binding capacity of Cry1Aa, Cry1Ab, Cry1Ac and Cry1Ca to BBMVs from *H. armigera* neonate larvae. They concluded that Cry1Ac toxin is the most toxic to *H. armigera*, followed by Cry1Ab and Cry1Aa toxins, while the Cry1Ca toxin did not present toxicity. In addition, the Cry1Aa, Cry1Ab and Cry1Ac toxins were able to bind to *H. armigera* BBMVs, but share the same receptor with each other, which indicates that these proteins should be avoided in pyramided Bt plants.

The Cry1Ac protein is one of the most active toxins against *H. armigera* and has been widely used in transgenic soybean (Bt-soybean) and cotton (Bt-cotton) with resistance to larvae that cause defoliation in crops (Tabashnik et al., 2013; Tay and Gordon, 2019). However, the resistance evolution of *H. armigera* populations have been threaten the technology sustainability (Liu et al., 2010; Dandan et al., 2019). The susceptibility of *H. armigera* field populations to Cry1Ac protein has been monitored since Bt cotton was commercialized in 1997 in China. Dandan et al. (2019) reported

that the IC_{50} values (concentration producing 50% inhibition of larval development to 3rd instar) among different strains ranged from 0.004 to 0.212 µg/mL, the percentage survival at a diagnostic concentration (IC_{99}, 1.0 µg/mL) ranged from 0 to 22.2%, and the percentage of field populations yielding survivors at diagnostic concentration (PSD) increased from 0 in 2006 and 2007 to 80% in 2015.

In Brazil, Bt-soybean and Bt-cotton have been largely used in Brazil to control *H. armigera* and other species of the subfamiliy Heliothinae. In the harvest 2018/2019 70% and 60% of the cotton and soybean cultivated in Brazil was Bt-cotton and Bt-soybean (ISAA, 2019). In the case of Bt-soybean, the MON 87701 × MON 89788 event (Intacta RR2 PRO® technology) expressing Cry1Ac toxin, launched in 2013 in Brazil, is recommended for control of some soybean pests, such as the Soybean looper (*Chrysodeixis includens*) (Lepidoptera: Noctuidae), Velvetbean caterpillar (*Anticarsia gemmatalis*) (Lepidoptera: Erebidae), Tobacco budworm (*Chloridea virescens*) (Lepidoptera: Noctuidae), and Bud borer (*Crocidosema aporema*) (Lepidoptera: Tortricidae). However, this technology provided high levels of control against *H. armigera*, since its first report into Brazil. Dourado et al. (2016) reported high levels of susceptibility of *H. armigera* to Intacta RR2 PRO® Bt soybean. The mean Cry1Ac LC_{50} ranged from 0.11 to 1.82 µg/mL of diet among *H. armigera* field populations collected from crop seasons 2013/14 to 2014/15 in Brazil.

Dourado et al. (2016) also assessed the risk of resistance to the Cry1Ac protein expressed by MON 87701 × MON 89788 soybean in Brazil and conducted studies to evaluate the baseline susceptibility of *H. armigera* to Cry1Ac. MON 87701 × MON 89788 soybean exhibited a high level of efficacy against *H. armigera* and most likely met the high dose criterion against this target species in leaf tissue dilution bioassays up to 50 times. Besides, high susceptibility to MON 87701 × MON 89788 soybean, and low frequency of resistance alleles across the main soybean-producing regions was reported, supporting the assumptions of a high-dose/refuge strategy. Nevertheless, a new technology will be commercialized by the Bayer company in 2021/2022, the Intacta 2 Xtend® technology. In addition to Cry1Ac toxin, it also possessed Cry1A.105 and Cry2Ab2 toxins which extends protection to other new two soybean insect pests, the Cotton bollworm (*H. armigera*) and Black armyworm (*Spodoptera cosmioides*) (Lepidoptera: Noctuidae) (https://plataformaintacta2xtend.com.br).

H. armigera is a polyphagous pest, with capacity to feed more than 100 species of plants around the world, including economically important crops such as cotton, corn, soybeans, tomatoes, tobacco, beans, fruits, and ornamental plants (Talekar et al., 2006; Ávila et al., 2013). *H. armigera* has several bioecological adaptations that makes it an important global insect pest. In addition to polyphagia, *H. armigera* has great mobility and variable diapause (Naseri et al., 2009; Fathipour and Naseri, 2011). In terms of fertility, the female may oviposit more than 2000 eggs during only one cycle, depending on the host. The pest has a great capacity for dispersal, and it has been observed that the adults can travel distances of up to 1000 km on nocturnal flights. All characteristics are dependent on the environmental conditions and features related to the pest population (Ávila et al., 2013).

In Brazil, the first report of its occurrence took place in 2013, attacking soybean and cotton crops in Bahia, Mato Grosso and Goiás states (Czepak et al., 2013). *H. armigera* population outbreaks occurred in the same year in a wide geographical are (EMBRAPA, 2013) and constantly associated with reports of control failures of pyrethroid pesticides (Durigan et al., 2017). Since that year, the use of biopesticides based in *B. thuringiensis* bacterium and entomathogenic virures (HzNPV) has been increased substantially by many efforts of Public research institutions, universities, and industry (Valicente, 2014). The biological control with use of Bt and Baculovirus are tools inside of the Integrated Pest Management (IPM), which should be used for several reasons: it does not pollute the environment, are very specific for the target pest, which means, does not kill other organisms, such as the natural enemies of the pests, does not contaminate the final product to be consumed. In this way, the use of the microbial insecticides is safe for both the environment, for humans and their benefits are numerous when compared to chemical insecticides.

*Ha*PHB-2 has been identified as a binding-protein for Cry1Ac in 2nd instar *H. armigera* midgut larvae by pull-down assay and LC-MS sequencing (Da Silva et al., 2018). Interestingly, Da Silva et al. (2018) evaluated early (2nd instar) and late (5th instar) stages of *H. armigera* and *Ha*PHB was identified only in the 2nd instar larvae, the larval stage more sensible to Cry1Ac toxin of the pest, indicating that this toxin may be participating for higher intoxication in early stages. In this work, we further analyzed

the potential role of *Ha*PHB-2 as Cry toxin receptor in comparison to CAD, well recognized as Cry1Ac-receptor using qualitative and quantitative binding assays.

We show that 500 molar fold concentration of *Ha*PHB-2 protein competed the binding of Cry1Ac to *Ha*BBMV, supporting that *Ha*PHB-2 is able to titrate Cry1Ac binding at a large excess. Those results indicate that it may participate as receptor protein of Cry1Ac toxin in *H. armigera*, although more studies are needed to uncover its specific role in the mechanism of action of Cry proteins. It is possible that *Ha*PHB-2 could also compete with the binding of the toxin to other receptors, since it has been shown that Cry1Ab domain III β-16 region is also involved in binding to *M. sexta* APN and ALP receptors (Arenas et al., 2010). Thus, it is possible that stearic hindrances may affect binding to these receptors resulting a significant reduction in Cry1Ac binding in the presence of this high excess of *Ha*PHB-2 protein. Also, it is known that CAD receptor is much less abundant than other receptors such as ALP and APN (Zhang et al., 2012). Thus, this competition could also reflect this differential expression levels of Cry-receptors in *Ha*BBMV. However, it remains to be analyzed.

Arenas et al. (2010) suggested that APN and ALP fulfill two roles in *M. sexta* Cry1Ab toxin intoxication. Firstly, APN and ALP are initial receptors promoting the localization of toxin monomers in the *M. sexta* BBMVs before interaction with CAD. Then, APN and ALP function as secondary receptors mediating oligomer insertion into the membrane. Furthermore, Da Silva et al. (2018) also identified ALP only in the early stages of the pest, indicating that it may have a predominant role in toxin action because Cry toxins are highly effective against the neonate larvae since that receptor also binds to BBMVs only in early stages of the insect. Our hypothesis is that *Ha*PHB-2 has similar function of ALP in Cry1Ac intoxication in *H. armigera.* However, its role functional remains to be further elucidated.

Our data shows that PHB-2 functions as Cry1-binding protein in Lepidopteran insect. Using ELISA binding assays, we demonstrated that Cry1Ab, Cry1Ac and Cry1Fa toxins binds to *Ha*CAD and *Ha*PHB-2 with similar apparent binding at the nM range (Figure 21). In addition, the binding of Cry1Ac to *Ha*CAD -TBR and *Ha*PHB-2 protein was further confirmed by ligand blotting assays (Figure 22). Previously, PHB-1 was identified as Cry binding protein in two other insect orders, in *A. aegypti* for Cry4Ba (Bayyareddy et al., 2009) and in *L. decemlineata* for Cry3Aa (Ochoa-Campuzano et

al., 2013) by pull-down assays. Besides, silencing studies of PHB-1 by RNAi *in L. decemlineata*, known as Colorado Potato Beetle (CPB), showed that PHB-1 is an essential protein, since its silencing strongly affected the larvae viability (Ochoa-Campuzano et al., 2013). Furthermore, the combination of PHB-1 silencing with Cry3Aa toxin treatment potentialized the CPB larval mortality evidencing the feasibility of utilizing RNAi strategies to complement existing Bt based crop protection methods. This work is the first report and characterization of PHB as Cry1-binding protein in an insect of Lepidoptera order, since that other reports has been done in other insect orders.

Bayyareddy et al. (2009) identified flotillin-1 (FLT-1) and PHB-1 as Cry4Ba binding proteins on 2D blots. Flotillins are structural proteins with detergent resistant lipid rafts. These proteins are highly conserved proteins that anchor lipid rafts with actin cytoskeleton via their stomatin/prohibitin/flotillin/HflK/C (SPFH) domain (Morrow and Parton, 2005; Langhorst et al., 2007). Also, PHB-1, like FLT-1 also has an SPFH domain and it typically is associated with lipid rafts (Browman et al., 2007). SPFH domain proteins are membrane-associated through N-terminal hydrophobic regions or, in the case of FLT, by palmitoylation (Browman et al., 2007). Those authors propose that likely the lipid raft proteins, FLT-1 and PHB-1 are co-localize with the GPI-anchored APNs and ALPs in insect BBMVs and might be participating in Cry1 intoxication in their respective target insects. It is very important to mention that FLT-1 was not analyzed in this work. However, this protein may be participating together with PHB-2 for Cy1Ac intoxication in *H. armigera*, since that was reported that this protein contains the same domains (SPFH) (Langhorst et al., 2007). Howsoever, it needs to be evaluated.

PHB-2 is a conserved protein in divergent species from prokaryotes and eukaryotes, and this protein is typically associated with lipid rafts in eukaryotic cells (Tatsuta and Langer, 2017). Lipid rafts are membrane microdomains rich in cholesterol, sphingolipids, GPI-anchored proteins, which are selectively located in lipid rafts and are implicated in different processes of the membrane, such as ion channel regulation, membrane protein chaperoning, vesicle and protein trafficking, membrane–cytoskeletal coupling, formation of specialized membrane structures and several cell-

signaling responses (Langhorst et al., 2005; Morrow and Parton, 2005; Browman et al., 2007).

When lipid rafts were isolated from *M. sexta* or *C. virescens* BBMVs that had been previously incubated with activated and biotinylated Cry1Ac, most of the toxin was found associated with this lipid fraction as a single band which presumably corresponds to its monomeric form since the authors make no mention of its apparent molecular mass or of the presence of oligomers (Zhuang et al., 2002; Vachon et al., 2012). In this case, most of the toxin was detected, associated with lipid rafts, as a widespread streak of protein ranging in apparent molecular mass from well below 160 kDa to well above 250 kDa, which was interpreted as corresponding to its oligomeric form. This PHB-2 co-localization with other proteins associated with lipid rafts, such as GPI-anchored proteins, ALP and APN, suggested a similar function of PHB-1 with those well documented Cry1-receptor proteins. However, its role functional in *H. armigera* lipid rafts needed to be further analyzed isolating their lipid rafts and evaluating their binding capacity to Cry1 toxins.

Here, the results revealed that differentially expressed unigenes (DEGs) were expressed significantly in *H. armigera* 3rd instar larvae exposed to Cry1Ac toxin compared to larvae group not exposed. The differences in DEG in the two treatments (larvae exposed and not exposed to Cry1Ac toxin) revealed that all the genes evaluated in this work were downregulated, highlighting *cad*, *alp* and *phb* genes. Cadherin fragment in *H. armigera* contains toxin-binding region TBR that increases Cry1Ac activity against *H. armigera* larvae since this type of interaction between *HaCAD* -TBR-Cry1Ac receptors induce the oligomerization of the toxin (Gómez et al., 2002; Pacheco et al., 2009). It can explain the expression decreased of these genes when neonate larvae were exposed to Cry1Ac toxin in the diet.

Corroborating with our results, downregulation of *alp*, *apn* or *cad* genes have been reported in other lepidopteran resistant insects to Cry toxins (Wang et al., 2005; Xu et al., 2005; Zhang et al., 2009; Yang et al., 2011; Wei et al, 2018). However, it is the first report of downregulation of *phb* gene expression in insect species exposed to a Cry toxin. Wei et al. (2018) employed RNAseq to investigate the midgut genes response to *H. armigera* strains with different levels of resistance (LF5, LF10, LF20, LF30, LF60, and LF120) to Cry1Ac. The results revealed that a series of DEGs were

expressed significantly in resistant strains compared with the LF-susceptible strain. Nine trypsin and ALP2, were downregulated significantly in all the six resistant strains and further verified by qRT-PC. For the *H. armigera* Cry1Ac-resistant Bt-R strain, it was identified that a deletion mutation of APN3 and the downregulation of *cad* lead to Cry1Ac resistance gene caused a more than 2,971-fold resistance to Cry1Ac in the BtR strain (Wang et al., 2005; Zhang et al., 2009). Also, lower expression of the *cad* gene resulted in a 564-fold Cry1Ac-resistance in *H. armigera* strain (Xu et al., 2005) and a 100-fold Cry1Ab-resistant in *Diatraea saccharalis* strain (Yang et al., 2011).

In this work, we showed that the Cry1Ab-L511A, a domain II Cry1Ab mutant, that was affected in binding to PHB-2 show to be affected in Cry1Ab toxicity. Disruption of toxin binding to larval midgut receptors is the most common mechanism of resistance of target insects to Cry toxins (reviewed by Kebede, 2020). According Heckel et al. (2020), mutations in either ABC transporters or CAD are the most potent resistance mechanisms of lepidopteran to Cry toxins discovered so far. Although most such mutations have the drastic effect of deleting the protein, a few can provide resistance with only minor structural changes and that mutations that have survived over evolutionary time to give rise to differences among insect species in the host ranges of Cry toxins.

Also, the insertion of transposons, which can confer resistance to chemical insecticides, can also cause resistance to Bt toxins by disrupting genes encoding Bt receptor proteins (Li et al., 2007; Fabrick et al., 2011). Wang et al. (2019) reported that a 3,370-bp insertion in *cad* gene associated with resistance to Bt toxin Cry1Ac in Pink bollworm (*Pectinophora gossypiella*) (Lepidoptera: Gelechidae), a global cotton pest. They have found the allele (r15) harboring this insertion in a field population from China. A strain homozygous for r15 had 290-fold resistance to Cry1Ac, little or no cross-resistance to Cry2Ab, and completed its life cycle on Bt cotton producing Cry1Ac.

It was proposed that *HaCAD* -TBR is involved in recruiting Cry1Ac to localize it in a good position for its interaction with the ABCC2, resulting in efficient toxin membrane insertion enhancing Cry1Ac toxicity (Ma et al., 2020). Furthermore, the *HaCAD* toxin-binding region (TBR), specifically the CAD repeat-11, the same CAD repetition used in this work, was necessary to enhance Cry1Ac toxicity with ABCC2. Those authors showed that the expression of *H. armigera* CAD (*HaCAD* -GFP) in Hi5

cells induces susceptibility to Cry1Ac and enhanced Cry1Ac toxicity when co-expressed with *H. armigera* ABCC2 (HaABCC2-GFP), since Cry1Ac toxicity increased 735-fold compared to Hi5 cells expressing *HaCAD* -GFP alone or 28-fold compared to HaABCC2-GFP alone.

Mutations in CAD were reported to be involved in Cry1Ac resistance in several other lepidopteran larvae, such *C. virescens* (Gahan and Heckel, 2001)*, H. zea* (Fritz et al., 2019), *T. ni* (Badran et al., 2016), *C. suppressalis* (Zhang et al., 2017a), and *H. armigera* (Wang et al., 2016; Liu et al., 2009; Zhang et al., 2017b).

To analyze the possible toxin regions involved in binding of Cry1 toxins to *Ha*PHB-2, we made use of a series of Cry1Ab domain II or domain III mutants that have been previously shown to affect binding to different receptors, such as CAD, ALP or APN in *M. sexta* (Pacheco et al., 2009; Arenas et al., 2010; Torres-Quintero et al., 2018) or *S. frugiperda* (Gómez et al., 2018a). Domain II loop 2 (Cry1Ab-F371A) or loop 3 (Cry1Ab-G439A) mutants were able to bind to *Ha*PHB-*2*, while domain III β-16 mutants (Cry1Ab-N514A and Cry1Ab-L511A) showed a correlative effect on binding to *Ha*PHB-2 and toxicity to *H. armigera*. Mutant Cry1Ab-N514A showed 6-fold higher toxicity than the Cry1Ab against *H. armigera*, which correlated with its higher apparent binding to *Ha*PHB-2 compared with Cry1Ab, while Cry1AbL511A showed to be severely affected in toxicity, which directly correlated with a lower binding interaction with *Ha*PHB-2 (Figure 24 and Table 8).

Those results indicate that domain III β-16 region of Cry1Ab toxin plays an important role in binding interaction with PHB-2 and suggest that *H. armigera Ha*PHB-2 could be a functional receptor of Cry1Ab and Cry1Ac toxins. However, we cannot discard that other regions of the toxin, such as domain II exposed loops, could also be involved in binding to *Ha*PHB-2, since only two domain II loop mutants were analyzed. Also, we cannot discard that Cry1Ab domain III is involved in binding to other midgut receptors also explaining its defects in toxicity. Nonetheless, this remains to be analyzed.

APN and ALP are both GPI-anchored proteins, and it was shown that these proteins are selectively included in lipid rafts from *M. sexta*, and *H. virescens* midgut cells (Zhuang et al., 2002; Munro, 2003; Bravo et al., 2004). The APN and ALP proteins have been identified as Cry toxins-receptors in several lepidopteran insects (Pigott and

Ellar, 2007; Gómez et al., 2007; Mitsuhashi and Miyamoto, 2019). Since domain III β-16 region from Cry1Ab has been shown to be involved in the binding interaction of this protein to ALP or APN, facilitating Cry1 toxin oligomer membrane insertion (Pacheco et al., 2009; Arenas et al., 2010; Flores-Escobar et al., 2013), we speculate that PHB-2 may have a similar role in Cry toxin mode of action. However, this hypothesis remains to be evaluated in the future.

Our results showed that a mutation in β-16 region from domain III in Cry1Ab as responsible to increase toxicity against *H. armigera* (Table 8). In equal way, the β-16 region from domain III of Cry1Ab has been shown to be involved in interactions with receptors such as ALP or APN in different lepidopteran insects. Alanine-scanning mutagenesis of amino acids of Cry1Ab β-16 (^{509}STLRVN^{514}) revealed that certain β-16 mutations, such as N514A, resulted in increased toxicity of Cry1Ab for *S. frugiperda* without affecting the toxicity for other lepidopteran larvae, such as *M. sexta* larvae (Gómez et al., 2018). Also, exhaustive mutagenesis of N514 was performed, showing that the Cry1Ab N514F, N514H, N514K, N514L, N514Q, and N514S mutations increased the toxicity toward *S. frugiperda*. Some of the Cry1Ab domain III mutants characterized here against *H. armigera*, specially Cry1Ab-N514A which also provided increased toxicity against *S. frugiperda* could be useful engineered insecticidal Cry toxins for control those important insect pests in the field.

Regarding domain II mutants analyzed, the toxicity data showed that Cry1AbG439D mutant located in loop 3 of domain II, loss toxicity against *H. armigera*. However, ELISA binding assays showed that Cry1Ab-G439D was not affected in *HaCAD* -TBR binding (Figure 24). It was previously reported that loop 3 region of Cry1Ab and Cry1Ac is an important region involved in binding interaction with multiple receptors from *M. sexta*, since it binds to CAD repeat 12 (CR12) and to *M. sexta* ALP and APN proteins (Xie et al., 2005; Torres-Quintero et al., 2018). Thus, it is possible, that this mutant is affected in toxicity against *H. armigera* due to its defects in binding interaction with these other receptors.

It was reported before that Cry1Ab-G439D mutant was specifically affected in its binding interaction with one region of CAD protein in *M. sexta* that corresponds to CR12 region (Torres-Quintero et al., 2018). Nevertheless, the *HaCAD* -TBR analyzed here contains two Cry1Ab binding sites and the other Cry1Ab binding site may

compensate the binding to *HaCAD* -TBR in these *in vitro* assays, explaining the observed interaction. It is also possible that the defect in toxicity of this mutant against *H. armigera* could be due to lower binding to other receptors, such as HaABCC2 that has been shown to be important for Cry1Ac toxicity in *H. armigera* (Xiao et al., 2014; Zhou et al., 2016), such as also important in other lepidopteran (Ocelotl et al., 2017; Boaventura et al., 2019; Min-Hui et al., 2019).

In view of the results obtained and presented in this work, we would like to propose novel insights in the mode of action of the Cry1Ab toxin in *H. armigera,* including the prohibitin participation as Cry1Ab-binding protein. In this model, besides to binding to ALP and APN GPI-anchored proteins, Cry1Ab could be also interacting with prohibitin with a binding affinity *Kd* value of 9.68 nM by the domain III β-16 (Figure 28A and 28B). To propose these novel insights of mode of action of Cry1Ab, we incorporate the results found in this work using *H. armigera* as model insect with data previously published using *M. sexta* as model insect (Gómez et al., 2002; Gómez et al., 2004; Bravo et al., 2004; Gómez et al., 2006; Pacheco et al., 2009; Arenas et al., 2010; Flores-Escobar et al., 2013).

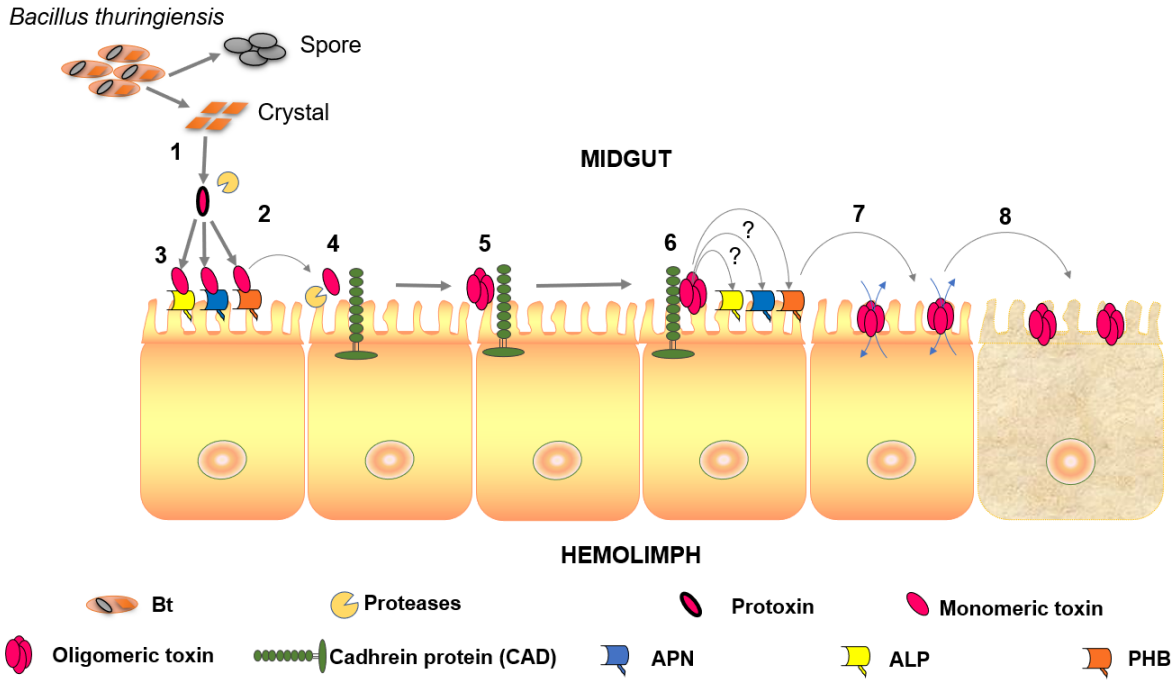

Figure 28. Mode of action of Cry1Ab toxin in *Helicoverpa armigera* incorporating novels insights of interaction with prohibitin (PHB). 1 – The crystals are ingested and solubilized due to the high alkalinity present in the midgut lumen of the insect. The released protoxins are proteolytically cleaved by proteases present in the midgut (2) generating an activated toxin fragment. The first binding interaction of Cry1Ab

activated toxin is a low-affinity interaction with ALP and APN or PHB receptors (3). This interaction concentrates the toxin in BBMVs where it binds to cadherin (4), that CAD interaction promotes the further proteolytic cleavage of the N-terminal end including helix α-1 of domain I leading to toxin oligomerization (5). With the oligomer pre-pore formation, this structure gains higher affinity to receptors such as APN, ALP or also according to our data PHB-2 (6) binding-proteins and inserts itself into the lipid rafts of the membrane (7), forming pores that allow passage of ions and molecules (8) destabilizing the osmotic balance, causing the cell death, and leading to the insect death. Adapted of Pardo López et al. (2013).

This new view of the Cry1Ab toxin mode of action in *H. armigera* is supported by the results obtained through the (1) ELISA binding assays of the Cry1Ab toxin and (2) Cry1Ab mutants to *Ha*PHB shown in figure 21, 23 and 24, (3) by the ligand blotting assays of the Cry1Ab toxin to *Ha*PHB shown in figure 22B and by the (4) toxicity data of Cry1Ab toxin in *Helicoverpa armigera* neonate larvae presented in table 8. Our data suggested an important role of prohibitin in Cry1Ab interaction before pore formation.

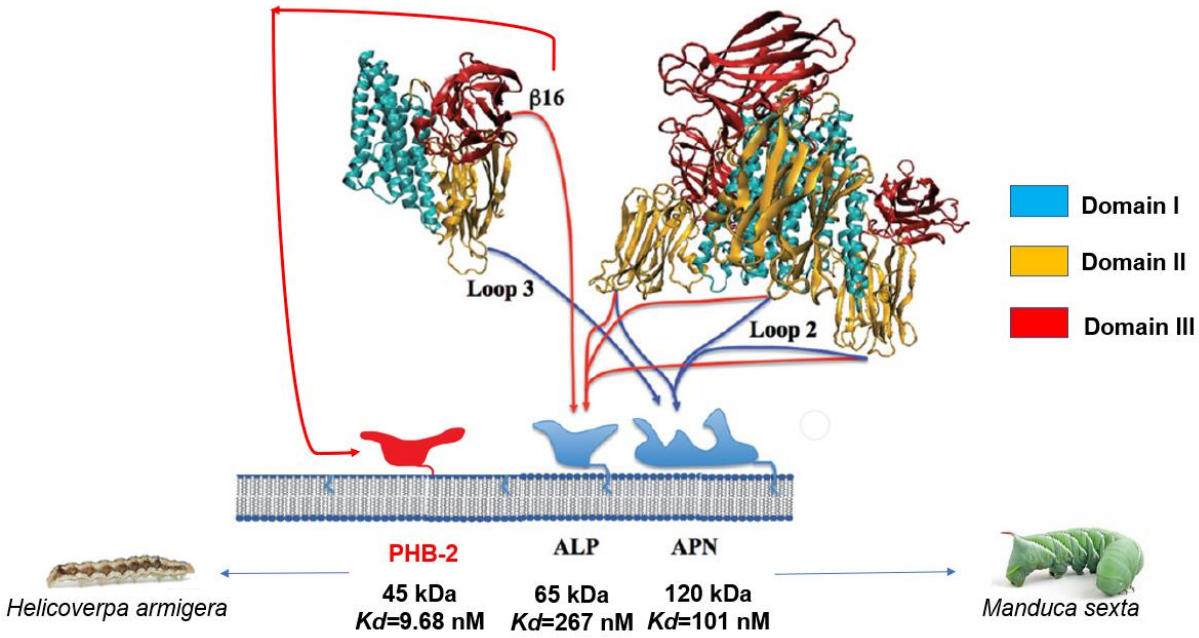

Figure 29. Schematic representation of the mechanism of action of 3d-Cry toxins in Lepidoptera at the molecular level using novel insights of interaction of Cry1Ab toxin with prohibitin (PHB-2) in *Helicoverpa armigera*. Adapted of Pardo López et al. (2013).

The Figure 29 shows a schematic representation of the updated mechanism of action of 3d-Cry toxins in Lepidoptera at the molecular level highlighting the first

interaction of low-affinity binding between Cry1Ab domain III β-16 to ALP in *Manduca sexta* (*Kd* = 267 nM) and with higher affinity with *Helicoverpa armigera* (*Kd* = 9.68 nM). It also shows a diagrammatic representation of the epitopes in 3d-Cry toxins that are involved in the binding interaction with ALP, APN, and PHB-2 receptors in *M. sexta* and *H. armigera*.

As review by Pardo-López et al. (2013), in *M. sexta* larvae the first binding interaction of activated Cry1Ab toxin is a low-affinity interaction with ALP and APN receptors (*Kd* = 101 nM for APN and 267 nM for ALP). The interaction with APN occurs through exposed loop 3 of domain II and with ALP through strand β-16 of domain III (Pacheco et al., 2009; Arenas et al., 2010). ALP and APN are highly abundant proteins anchored to the membrane by a GPI anchor and we proposed that PHB-2 could be also anchored by GPI and may play a similar role that APN and ALP in the toxicity of Cry toxins. However, this hypothesis needs to be experimentally tested by silencing PHB in combination with silencing assays of the other Cry1Ab receptors such as ALP and APN, to clearly demonstrate if their functions are redundant.

Following the intoxication with Cry1Ab, the interaction of these toxins with GPI-anchored proteins concentrates 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 (*Kd* = 1 nM) (Gómez et al., 2006). The CAD interaction involves, at least, three epitopes in the CAD corresponding to extracellular regions named CR7, CR11 and CR12, where CR12 is proximal to the cadherin membrane domain (Pacheco et al., 2009; Xiao et al., 2017). It is known that these CAD protein epitopes 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 a-1 of domain I (Gómez et al., 2002; Atsumi et al., 2008). Also, it is proposed that the cleavage of helix α-1 may result in the exposition of 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 pre-pore oligomer structure before insertion into the membrane (Gómez et al., 2002; Pacheco et al., 2009; Arenas et al., 2010; Pardo-López et al., 2013).

After the CAD interaction and formation of the Cry1Ab oligomeric structure, it was shown that Cry1Ab-oligomeric structure increases its affinity to ALP and APN receptors up to 200-fold and that this interacion occurs by the loop 2 of domain II region

of the toxin (*Kd* = 0.6 nM for APN and 0.5 nM for ALP) (Arenas et al., 2010). In this case, the possible role of ALP and APN proteins after pre-pore binding could be to induce its insertion into the membrane, leading to pore formation and to cell lysis (Pardo-López et al., 2006; Arenas et al., 2010). Similarly, according to our data, we also hypothesis that Cry1Ab oligomeric structure could increases its affinity to PHB-2 and induce its insertion into the membrane and increasing the toxicity of the Cry toxin (Fig. 28). However, this last hypothesis still requires to be evaluated *in vitro* using binding assays with PHB-2 interacting with Cry1Ab oligomers and their response in toxicity assays.

In summary, this work presents a binding interaction characterization of the Cry1 toxins to *Ha*PHB-2 from *H. armigera* and propose novel insights in mode of action of Cry1Ab against *H. armigera,* an important global pest. According to our results, we speculated that *Ha*PHB-2 is a novel binding site in *H. armigera*. Although its specific role in the mechanism of action of Cry toxins and any potential mechanism of resistance of *H. armigera* to these toxins remain to be further elucidated. Thus, further functional studies, such as RNA interference or CRISPR, may be carried out to determine the role functional in the mode of action of Cry1 in *H. armigera*.

6. CONCLUSIONS

H. armigera neonate larvae have *phb, alp* and *cad* genes are downregulated when exposed to Cry1Ac toxin.

HaCAD -TBR and *Ha*PHB-2 are Cry1-binding proteins (Cry1Ab, Cry1Ac and Cry1Fa) for *H. armigera* and *Ha*PHB-2 competes by the same Cry1Ac-binding site.

β-16 mutant (Cry1Ab-N514A) showed increased binding to *Ha*PHB-2 that correlated with six-fold higher toxicity against *H. armigera* while β-16 mutant (Cry1AbL511A) was affected in binding to *Ha*PHB-2 and lost toxicity against *H. armigera.*

β-16 mutant from domain III of Cry1Ab is involved in interaction with *Ha*PHB-2 and toxicity against *H. armigera.*

Novel insights in mode of action of Cry1Ab against *H. armigera* were proposed.

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