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Effect of the Cry3 toxins on beetles

Ph.D. Thesis

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2013 České Budějovice

This thesis should be cited as:

Mostafa M. (2013). Effect of the Cry3 toxins on beetles. Ph.D. Thesis, in English, Faculty of Science, University of South Bohemia in České Budějovice, Czech Republic, 99 pp.

Annotation

The thesis consists of two articles. First publication presents data of the sensitivity of *Tribolium castaneum* to Cry3Aa and its modifications. Second article describes the identification of Cadherin-like protein in different stages and tissues of *Tenebrio molitor*.

Declaration [in Czech]

Prohlašuji, že svoji disertační práci jsem vypracoval samostatně, pouze s použitím pramenů uvedených v seznamu citované literatury.

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České Budějovice, 22.03.2013 Moataz Mostafa, M.Sc.

Financial Support

This work was supported by grant QI91A229 from the Ministry of Agriculture of the Czech Republic.

Acknowledgements

This dissertation would not have been possible without blessing and God's will.

First and foremost, I would like to thank the Ministry of Education, Youth and Sport for giving me chance to come and learn in Czech Republic through a full PhD scholarship. In addition, I would like to show my gratitude to my supervisor Prof. František Sehnal, for giving me the opportunity to become a PhD student in the Czech Republic and for providing me with the opportunity to work in his laboratory.

I would like to thank my colleague Suresh Sajwan, and Heba Yassin for their critical comments and helpful advice during my thesis work.

Special thanks are due to Klara Mičulková for her advice and help provided in course of my laboratory work.

I also would like to thank Prof. Dalibor Kodrik and Dr. Oxana Habuštová for giving me chance to work in their laboratories. My thanks also belong to Dr Saad Mousa, who received me in his lab for four months.

I wish to thank to my wife and my daughter Sara, who accompanied me in this journey and supported me during these four years.

Finally, I would like to dedicate this thesis to the spirit of my mother and my son Yassin

List of papers and auther's contribution

The thesis is based on the following papers:

Paper 1.

Mostafa M., Vlasák J., Sehnal F. (2013) Activities of modified Cry3A-type toxins on the Red Flour Beetle, *Tribolium castaneum* (Herbst). *J. Appl.Entomol*., accepted for publication.

Paper 2.

Mostafa M., Sehnal F. (2013) C*adherin* gene expression in distant populations and different developmental stages and tissues of *Tenebrio molitor*. *Biol. Chem.*, submitted.

***Moataz Mostafa**, author of this Ph.D. thesis, is the first author of papers (manuscripts). He was responsible for bioassay, laboratory processing, RNA extraction, PCR, sequence assembling, literature survey, and writing the manuscript (partly). Most of the raw data processing, as well as most of the statistical analyses were performed by him.*

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CONTENTS

CHAPTER 1

Background information

1. INTRODUCTION

Pesticide resistance is an increasingly urgent worldwide problem; it has been documented in more than 504 species of insects and mites. Over 270 weed species, over 150 plant pathogens, and about a half dozen species of rats are resistant to pesticides that once controlled them. Multiple resistance to more than one pesticide and to pesticides in more than one chemical class is increasing rapidly - there are over 1,000 insect/insecticide resistance combinations, and at least 17 species of insects are resistant to all major classes of insecticides (Georghiou and Lagunes-Tejada, 1991). In response to the resistance problem, the concentration or frequency of pesticide applications is often increased but this is often accelerates resistance development. To delay or prevent resistance development, we must use a variety of insecticides with different modes of action and design strategies for resistance management.

To avoid undesirable side effects of synthetic pesticides and prevent resistance development, the schemes of integrated pest management include other methods of pest control. They include applications of bioagents, such as the entomopathogenic, Gram positive bacterium *Bacillus thuringiensis* (Bt), which represents about 90% of all marketed bioagents (http://www.bt.ucsd.edu/organic_farming.html). Bt was first isolated by the Japanese scientist Ishiwata S., in 1901, from the silkworm larvae exhibiting the sotto disease. Ten years later, Berliner E., formally described the species from an isolate originating from *Anagasta kuehniella* collected in the German region of Thuringia, which gave the name to the species (Porcar and Juarez-Perez, 2003). There are more than a hundred Bt strains, each producing during spoluration specific type(s) of crystalline protein called δ-endotoxins. *B. thuringensis* var. *tenebrionis* contains a major polypeptide of 67 kDa and minor polypeptides of 73, 72, 55 and 46 kDa. During the stage I of the sporulation process, only the 73 kDa polypeptide could be detected, while the 67 kDa polypeptide was detected at stage II (Carroll et al., 1989). These major components of the inclusions are the δ-endotoxins. These proteins are toxic to certain insects and nematodes and are characterized by high specificity towards the target organisms. There are two

types of δ-endotoxins: the highly specific Cry (from crystal) toxins which act via specific receptors and the non-specific Cyt (cytolytic) toxins, with no known receptors (Porcar and Juarez-Perez, 2003). There are more than 150 Cry toxins that have been described from *B. thuringensis* and *Bacillus cereus* and classified based on their insecticidal action and amino acid sequences. Many Cry protein genes have been cloned and sequenced. Four major groups include Cry1 (active on Lepidoptera), Cry2 (active on Lepidoptera and Diptera), Cry3 (active on Coleoptera) and Cry4 (active on Diptera) (Ferre et al., 1995 and Schnepf et al., 1998). Proteins which are toxic for lepidopteran insects belong to the Cry1, Cry9, and Cry2 groups; toxins active against coleopteran insects are the Cry3, Cry7, and Cry8 proteins as well as the Cry1B and Cry2, which have dual activity. The Cry5, Cry12, Cry13, and Cry14 proteins are nematocidal, and the Cry2, Cry4, Cry10, Cry11, Cry16, Cry17, Cry19, and Cyt proteins are toxic for dipteran insects (Bravo et al., 1998).

The importance of Cry toxins has greatly increased in the last twenty years when the genes encoding them were transferred to plants. Genetically modified crops expressing Cry proteins are resistant to specific pest(s) without affecting other components of the agroecosystems.

1.1. *Bacillus thuringensis* (Bt) as a bio control agent

B. thuringiensis is one of the few microbes that have been successfully used in agricultural insect pest control. Liquid and powder formulations of spores and crystals from this bacterium have been used for more than 60 years on many crops (Gould, 1998; Bel et al., 2009). The use of Cry toxins from *B. thuringiensis* (Bt) to control insect pests is nearly obligatory in the protection of certain crops, such as the sweet corn. Unlike chemical insecticides, this biopesticide is highly specific for a narrow range of species and is non-toxic to vertebrates. Majority of beneficial invertebrates also tolerates Bt and its toxins. These features of Cry toxins promoted their wide use in form or spores applications as well as in transgenic crops (Betz et al., 2000; Whalon and Wingerd, 2003).

Commercial preparations of Bt spores and crystals are used to control harmful species of Lepidoptera, Coleoptera, Hymenoptera and Diptera, and also nematodes. Recently, the use of Cry toxins has increased dramatically following the introduction of Cry genes into plants (Van Rie, 2000; Shelton et al., 2002). These “Bt crops” have thus far proved to be an effective control strategy. In 2007, Bt maize and Bt cotton were grown on 28 million

hectares worldwide (James, 2008) and the acreage of GM crops continues to increase by about 10% annually. Evolution of resistance in the target pests is the major threat of using this new technology. Plantation schemes have been designed to reduce selection pressure posed by the widespread use of the Bt crops (Gould, 1998; Ferre and Van Rie, 2002; Tabashnik and Carrie`re, 2008). Bt also contains Cyt toxins (they are mostly found in Bt strains active against Diptera) that have been unsufficiently exploited so far (Bravo et al., 2007; Pigott and Ellar, 2007).

The Cry3 class of Bt Cry proteins is known for toxicity to coleopteran larvae including yellow mealworm, *T. molitor* ((Fabrick et al., 2009), the Colorado potato beetle, *Leptinotarsa decemlineata* (Rausell et al., 2004 and Park et al., 2009), Andean potato weevil, *Premnotrypes vorax* (Gomez et al., 2000), and Coffee berry borer (CBB), *Hypothenemus hampei* (Lopez-Pazos et al., 2009), but these biopesticide have had limited usage and sales (Gelernter W., 2004) due to the limited efficacy of Cry3-based biopesticides/plants and the success of competing chemical pesticides. Some of the proteins are toxic for Coleoptera only after in vitro solubilization because protoxin cannot be solubilized from the crystals at the neutral or weakly acidic pH of coleopteran midgut (de Maagd et al., 2001). Recently, in assessing the activities of 1% concentrations of Cry 34/35 and Cry1F on *T. castaneum*, Oppert et al. (2010) found no effect on the larval weights and mortality. The CryAa formulation Novodor that contains 3% of *B. thuringiensis ssp. tenebrionis* was tested against *Tribolium confusum* (Coleoptera: Tenebrionidae) larvae. Mortality levels ranging between 60-80% at 3000 ppm and 30-70% at 1500 ppm were established. Moreover, the addition of spinosad (1 ppm), which is based on metabolites actinomycete *Saccharopolyspora spinosa*, to treated diet was increased the mortality of the exposed *T. confusm* larvae (Athanassiou and Kavallieratos, 2009).

Cry1 toxins are a family of 130 to 140 kDa proteins that exhibit specific toxicities to numerous lepidopteran insects including tobacco budworm, *Heliothis virescens* (Gahan et al., 2001; Jurat-Fuentes and Adang, 2004, 2006), tobacco hornworm, *Manduca sexta* (Dorsch et al., 2002) cotton bollworm, *Helicoverpa armigera* (Xu et al., 2005), pink bollworm, *Pectinophora gossypiella* (Morin et al., 2003), European corn borer, *Ostrinia nubilalis* (Flannagan et al., 2005). Several Cry1 toxins including Cry1Bb, Cry1Ca and Cry1Fa are toxic to important agricultural pests belonging to the genus *Spodoptera* (Luo et al., 1999). Among the beneficial insects, increased mortality was reported for the green lacewing, *Chrysoperla carnea*, larvae that had been fed the larvae of *O. nubilalis*

(Lepidoptera: Crambidae) or *Spodoptera littoralis* (Lepidoptera: Noctuidae) that were reared on a transgenic corn expressing cry1Ab (Hilbeck et al., 1998).

1.2. Cry toxin structure and function

Bt Cry and Cyt toxins belong to a class of bacterial toxins known as pore-forming toxins (PFT) that are secreted as water-soluble proteins undergoing conformational changes in order to insert into, or to translocate across, cell membranes of the midgut epithelium of their host. There are two main groups of PFT: (i) the a-helical toxins, in which a-helix regions form the trans-membrane pore, and (ii) the β-barrel toxins, that insert into the membrane by forming a β-barrel composed of β-sheet hairpins from each monomer (Bravo et al., 2007).

The Cry proteins are globular molecules which contain three structural domains connected by single linkers. The molecular conformation of Cry toxins determines toxin specificity. There are seven structural conformation that have been resolved by X-ray crystallography: Cry1Aa, Cry1Ac, Cry2Aa, Cry3Aa, Cry3Ba, Cry4Aa, and Cry4Ba (Li et al., 1991; Grochulski et al., 1995; Morse et al., 2001; Galitsky et al., 2001; Boonserm et al., 2005, 2006).. These toxins considerably differ in their amino acid sequences and insect specificity but all retainsimilar conformation of the three domains.

The N-terminal domain (domain I) is a cluster of seven α helices, which are responsible for membrane insertion and pore-formation. Domain II consists of three antiparallel β sheets with exposed loop regions, and domain III is a β-sandwich (Li *et al.*, 1991; Bosch *et al*., 1994; Bravo et al., 2007). Three surface loops of domain II of Cry3A are involved in the receptor binding (Li et al., 1991; Wu and Dean, 1996). Domain I shares structural similarities with other PFT like colicin Ia and N and diphtheria toxin, while domain II and III share structural similarities with several carbohydrate-binding proteins - these similarities suggest that carbohydrate moieties could have important role in the mode of action of the Cry toxins (Bravo et al., 2007). This similarity may explain the increase of Cry1Ab toxicity against *Spodoptera exigua* after exchanging domain III of Cry1Ab with that of more active Cry1Ac protein; however, both toxins showed differences in the specific binding sites in intact BBMVs of *S. exigua*, which suggested that the resistance of S. exigua to Cry1Ac could be due to a loss of its specific binding site (de Maagd et al., 1996).

Moreover, Ge et al. (1991) showed that replacement of amino acid residues 450 to 612 (domain III) of Cry1Aa by those of Cry1Ac enhanced toxicity to *H. virescens* 300 fold. Other results suggested that domain III of Cry proteins is involved in receptor recognition. As well as, (Aronson et al., 1995) demonstrated that mutatios in domain III of Cry1Ac affected affinity to the membrane proteins in *M. sexta* and *H. virescens* BBMVs. Hence, both domains (II and III) play important roles in toxin binding.

1.3. Cry toxin-binding receptors

The first observation indicating that the midgut is disrupted during insect intoxication with *B. thuringiensis* was made by Heimpel and Angus (1959). Cry specific binding to the brush border membrane vesicles (BBMV) prepared from the epithelial midgut cells was described in 1988 (Hofmann et al., 1988). de Maagd et al. (1999) reviewed the three types of binding assays used to study Cry binding to BBMV: ligand binding to separated BBMV proteins on the Western blots, surface Plasmon resonance (SPR) technique, and use of intact BBMVs. Binding assays with BBMV freshly isolated from insect midgut is most commonly used in studies on the interactions between Bt Cry toxins and their receptors (Van Rie et al., 1989; Braun and Keddiet, 1997 and Hernandez et al., 2004). Toxin binding to midgut brush border sites, which is a key determinant of toxicity to insects, involves two steps (i) recognition by the receptor and (ii) irreversible association with the membrane (Van Rie et al., 1990). The loss of binding sites, as measured by reduced toxin binding, is correlated with resistance to Bt in *P. xylostella* larvae (Ferre et al., 1991; Tabashnik et al., 1994; Ballester et al., 1999). On the other hand, irreversible toxin binding to BBMV was reported to correlate with its insecticidal potential (Ihara et al., 1993; Liang et al., 1995). Similar, Wu and Dean (1996) found that there was a direct correlation between insecticidal potency and the intensity of binding. The potency of a Cry1 toxin can significantly decrease as the larvae age (Ali and Young, 1996; Rausell et al., 2000), and the variations in the potencies of Cry1 toxins for different lepidopteran species and different larval stages may reflect differences in any one of the prebinding, binding, and subsequent pore-forming that is required for full toxicity. By discovering why Cry1 toxins exhibit diverse potencies on different developmental stages or different species, it would be possible to engineer more potent toxins (Gilliland et al., 2002). The correlation between toxicity and binding was shown to be responsible for increased resistance to Cry1Ac and Cry1Ba during larval development in *M. sexta*, in which the third-instar BBMV had fewer bindig sites than in the neonate BBMV. In other species,

such as *Pieris brassicae*, *Mamestra brassicae*, *Spodoptera exigua*, and *Agrotis ipsilon*, it was not possible to detect positive correlations between insecticidal potency and changes in binding characteristics during development (Gilliland et al., 2002).

The extent of toxin binding does not always correlate with insecticidal activity. An example of binding that did not result in insect mortality was demonstrated in Cry1Ab toxin domain II mutants tested on *M. sexta*. The mutant exhibited similar binding to the BBMV as the wild-type Cry1Ab, but was 400-fold less toxic. In other trials, recombinant toxin binding was reversible, in contrast to the irreversible binding of the wild-type Cry1Ab toxin (Rajamohan et al., 1996). Luo et al. (1999) found no relationship between Cry1Ac, Cry1Ca, and Cry1Bb irreversibly bound to BBMV from *S. exigua* and *S. frugiperda* and toxicity. Also, there were no significant differences showed in Cry1C binding to resistant and susceptible strains of *P. xylostella* (Liu et al., 2000). For beneficial insect, the immunological detection of the toxin after direct feeding (in vivo binding experiments), and in vitro binding experiments with BBMV preparations showed that none of these approaches revealed presence of the Cry1Ab or Cry1Ac proteins in tested lacewing larvae, *Chrysoperla carnea*, This was consistentn with the bioassay results that showed no effect of these toxins on the larvae (Rodrigo-Simon et al., 2006).

On the other hand, multiple binding sites for the *B. thuringiensis* Cry1 toxins are present in many insects, for example, Aranda et al. (1996) reported that Cry1Ab and Cry1Ca recognized different binding sites in the brush border membrane of *S. frugiperda*, in *P. xylostella*, Cry1Ab and Cry1Fa recognized the same binding site, while Cry1Ca bound to a distinct site (Ferre et al., 1991; Wright et al., 1997). Luo et al. (1999) found that there are at least two high-affinity Cry1 binding sites in *S. exigua* and *S. frugiperda*; one site is for Cry1Ac, and the other site is for Cry1Bb and Cry1Ca, and Cry1Fa competed with Cry1Ac, Cry1Bb and Cry1Ca in both *Spodoptera* species.

Beside the Cry toxins, in recent years, a number of secreted insecticidal proteins called vegetative insecticidal proteins (Vip) have been identified during the vegetative growth phase of bacteria (Estruch et al., 1996). Vip3A toxin from *B. Thuringiensis* shows a high level of activity against a range of lepidopteran pests including *Agrotis ipsilon, S. fugiperda, S. exigua, H. virescens,* and *Helicoverpa zea*. Activated Vip3A toxin binds in *M. sexta* midgut to distinct 80 and 100 kDa BBMV proreins, unlike the well-known Cry1A toxin receptors, 120 kDa aminopeptidase N (APN) and 250 kDa cadherin-like molecules (Lee et al., 2006). Similar, Sena et al. (2009) found that Cry1Ab and Cry1Fa competed for the same binding sites, whereas Vip3Aa competed for those of Vip3Af.

The binding to midgut cell membrane receptors is the key step in Cry action. A variety of receptors have been identifed, including cadherin-like proteins (Gahan et al., 2001; Nagamatsu et al., 1998a,b; Vadlamudi et al., 1993, 1995), GPI-anchored aminopeptidases (Gill et al., 1995; Knight et al., 1995; Rajagopal et al., 2002; Sangadala et al., 1994) and alkaline phosphatases (Jurat-Fuentes and Adang, 2004). McNall and Adang (2003) identified a membrane-bound form of alkaline phosphatase (mALP) and actin as novel Cry1Ac toxin-binding proteins in the brush border midgut membrane proteome of *M. sexta* larvae. Krishnamoorthya et al. (2007) identified V-ATP synthase subunit A and actin as novel Cry1Ac binding proteins in *H. virescens* by using 2D gel electrophoresis and peptide mass fingerprinting (PMF). The V-ATPase enzyme is localized in the apical membrane of the goblet cells and represents the primary energy source for secretion and absorption by serving as an H+/K+ electrogenic transporter across the midgut epithelium (Wieczorek et al., 1999). *H. virescens* larvae have Three groups (A, B and C) of Cry1 binding sites were found in BBMV from *H. virescens*, larvae: Cry1Aa, Cry1Ab, Cry1Ac, Cry1Fa and Cry1Ja toxins share a common binding site (receptor A), Cry1Ab and Cry1Ac have an additional binding site (receptor B) and Cry1Ac is the only toxin that can recognize a third binding site (receptor C) (Van Rie et al., 1989; Jurat-Fuentes and Adang, 2001). Higher levels of resistance were correlated with reducing binding not only to site A, but also to sites B and C (Jurat-Fuentes et al., 2002; Jurat-Fuentes and Adang, 2004). The A group is especially critical for the Cadherin protein, HevCaLP, the B group of binding sites was associated with a 130- kDa APN, while the C binding sites include alkaline phosphatase, HvALP, (Jurat-Fuentes and Adang, 2001; Jurat-Fuentes and Adang, 2004). Moreover, Valaitis et al. (2001) discovered in *Lymantria dispar* a highly anionic glycoprotein (BTR-270) associated with the brush border. This protein bound Cry1Aa and Cry1Ab with higher affinity than Cry1Ac, mirroring the relative toxicity of these three toxins toward this species. On the other hand, Hossain et al. (2004) isolated a protein (P252) from the brush border of *B. mori* that showed specific binding to Cry1Aa, Cry1Ab, and Cry1Ac. Probing this protein with various lectins revealed extensive glycosylation.

The interaction between Cry toxins and their binding receptors have been studied extensively in lepidopteran insect; however, in 1994 was the first report of the identification of a single binding protein from a coleopteran insect, *T. molitor*-The protein binding specifically Cry3 appered as a single band of 144 kDa on radioligand and immunoblots of total protein extracted from the BBMV from the midgut of *T. molitor*

(Belifore et al., 1994). The binding assays and heterologous competition experiments showed that Cry3A, Cry3B and Cry3C toxins share a common binding site in *L. decemlineata*. Cry3Aa and Cry3Ca were able to compete for the binding of labelled chymotrypsinized Cry3Ba but Cry3Ba did not compete for the binding of the other two toxins (Rausell et al., 2004).

Generally, the receptor binding is essential for further toxin activation, toxin aggregation, and pore formation (Gomez et al., 2002). The specificity of Cry toxins is often determined by their interaction with the high affinity receptors, which are present in insect gut (Hernandez et al., 2004), and the major mechanism of resistance to the Cry toxin application is due to the reduction of toxin binding to this sites in the midgut membrane (Baxtera et al., 2008).

1.3.1 Cadherin-like protein

Cadherin proteins represent a large family of calcium-dependent, transmembrane glycoproteins that are responsible for maintaining the integrity of cell–cell contacts in multicellular organisms and arre thereby involved in cell–cell adhesion, migration, cytoskeletal organization, and morphogenesis (Nollet et al., 2000; Pigott and Ellar, 2007). The classical epithelial (E)-cadherin proteins are present in cell–cell junctions (Nelson et al., 1990).This type of Cadherin is also present in the midgut columnar cell membranes In *M. sexta* itnwas demonstrated that it did not occur in the foregut and hindgut (Aimanova et al., 2006). The expression of cadherin was shown to vary with developmental stage, with aprogressive increase from the first to the fifth instar in *M. sexta* larvae, while no cadherin expression was found in the eggs and in the midgut of adults (Midboe et al., 2003). Cadherin mRNA was found in the larvae as well as in adults and embryos, and for tissues, it was found in the gut, and testes. The qRT-PCR revealed that cadherin transcripts increased to high levels in the first three larval instars, dropped dramatically in the fourth instar, reached low levels in pupae, and increased again in adult males but less in females (Carriere et al., 2009).

Lepidopteran cadherin-like proteins have been extensively studied as Cry1A receptors. They seem to play critical role in mediating toxin susceptibility. A 210-kDa cadherin-like glycoprotein has been identified as a Cry1Ab binding protein in BBMV prepared from the midguts of *M. sexta* larvae (Vadlamudi et al., 1993, 1995), later, there were three Cry1Ab-binding sites which have been mapped in the *M. sexta* cadherin, BtR1, the first site ^{865}NITIHITDTNN^{875}, mapped using phage display, and was involved in binding loop 2 of Cry1Aa and Cry1Ab toxins (Gomez et al., 2001). A second region important for

toxin binding (Dorsch et al., 2002), was mapped to amino acids 1291–1360 and subsequently narrowed to ^{1331}IPLPASILTVTV^{1342} – it was shown to bind loop α-8 (Gomez et al., 2003). A third region, aa 1363–1464, was recently shown to be involved in toxin binding and cytotoxicity (Hua et al., 2004). A 220-kDa cadherin-like protein present in the BBMVs of *O. nubilalis* was identified as a receptor for the Cry1Ab toxin. It consists of 12 CRs and 14 N-glycosylation sites which are distributed along the extracellular domains of the *O. nubilalis* cadherin. Studies based on its expression in the Sf9 cells provided strong evidence that this molecule not only binds Cry1Ab but also mediates its insertion into the cell membrane and thereby kills the insect (Flannagan et al., 2005).

In *Bombyx mori*, a 219-amino-acid residue region (a1245–1464) of the 175 kDa cadherin-like protein was identified as a Cry1Aa binding protein (Nagamatsu et al., 1998a, b). More recently, Xie et al. (2005) reported that the Cry1Ac toxin binding region in *H. virescens* cadherin was mapped to a 40-amino-acid fragment, from amino acids 1422 to 1440, and this site overlaps with a Cry1Ab toxin-binding site, amino acids 1363–1464 reported in *M. sexta* (Hua et al., 2004).

Similar, Fabric and Tabashnik (2007) found a multiple binding sites for Cry1Ac in the cadherin protein BtR in the pink bollworm, *P. gossypiella* Binding occurred in CR8-CR9, CR10, and CR11, which which are immediately adjacent to the membrane proximal region, and, unlike the cadherins of *M. sexta* and *B. mori*, toxin binding was not seen in regions more distal from the membrane proximal region. The discovery of multiple binding sites for Cry1Ac is consistent with findings on the cadherin and Cry1A toxins in other Lepidoptera. BtR1 from *M. sexta* and BtR175 from *B. mori* contain at least three toxin binding sites including CR7, CR11, and CR12-MPED (Nagamatsu et al., 1998a, b; Gomez et al., 2003; Chen et al., 2007). Generally, a critical Cry1 toxin binding site is localized within the final cadherin repeat, CR12, of cadherins from tobacco hornworm *M. sexta* (Lepidoptera: Sphingidae) and tobacco budworm *H. virescens* (Lepidoptera: Noctuidae) (Hua et al., 2004; Xie, et al., 2005).

The mutations in cadherin gene lead to resistance to Cry1A toxins in the tobacco budworm, *H. virescens* (Gahan et al., 2001) - Cry1Ac resistance was tightly linked to a cadherin-encoding gene but not to the genes encoding aminopeptidases. Later, Morin et al. (2003) found that the pink bollworm, *P. gossypiella*, had three different cadherin alleles (r1, r2, and r3). This is linked to the resistance to Cry1Ac and the survival on transgenic Bt cotton following loss of at least eight amino acids from the extracellular

region of BtR. In these species, mutations produce mRNA transcripts that differ from the wild type due to partial deletions or to the presence of stop codons preventing production of functional proteins. Similar, Xie et al. (2005) reported that mutations in the region, to which the Cry1Ac binds through its loop 3, resulted in the loss of toxin binding. Mutations affecting the cadherin amino acids Leu1425 and Phe1429, which are critical for Cry1Ac toxin interaction, result in the loss of toxin binding, with a *KD* of <10_5 M. However, mutation of Gln1430 to an alanine increased the Cry1Ac affinity 10-fold. Most of the resistance to Cry1Ac has been shown to be conferred by mutations in the 12-cadherin-domain protein (Bel and Escriche, 2006; Pigott and Ellar, 2007). Recently, Bel et al., 2009 found a high variability in the cadherin gene in the Europe-R strain of *O. nubilalis*, with many deleterious mutations that would not contribute to the 220 kDa cadherin bands due to their variable sizes and a 4 bp insertion that produced a premature stop codon preventing the production of functional protein. Only 1 bp deletion with the 4 bp insertion produced truncated cadherins with no binding regions and no transmembrane and cytoplasmic domains. Other deletions produced mutated proteins with the signal peptide, about 8 CR, and the complete binding region, transmembrane and cytoplasmic domains. Existence of these mutations explained why low amount of cadherin protein in BBMV and low Cry1A binding were detected by comparison with the susceptible strain (Siqueira et al., 2006). The mutations in the cadherin gene that have been shown in *O. nubilalis* to be linked to the resistance to Cry1A are not due to single nucleotide substitutions but to major changes in the gene. This is also true for H. armigera, in which mutations occurred because of the deletion of about 8.8 kb (from exon 8 to intron 24) (Yang et al., 2006) or retrotransposon insertions (Yang et al., 2007) producing, in both cases premature stop codons.

On the other hand, production of the midgut cadherin protein (HevCaLP) in *H. virescens* was necessary for Cry1Aa binding in susceptible strains, while binding was not observed in a resistant strain (YHD2-B) (Jurat-Fuentes et al., 2004).

Cry3Aa and Cry3Bb insecticidal proteins of *B. thuringiensis* are used as biopesticides or in transgenic crops to control larvae of the leaf-feeding beetles and rootworms. Park et al. (2009) demonstrated that the cadherin fragment (CR8 to CR10) of western corn rootworm *Diabrotica virgifera virgifera* binds α-chymotrypsin-treated Cry3Aa and Cry3Bb toxins at high affinity (11.8 nM and 1.4 nM, respectively). This fragment has similarity to the Cry1A-binding region 2 (TBR 2) of *M. sexta* cadherin (Gomez et al., 2003). Recently, Fabric et al. (2009) identified the first functional Cry toxin receptor

cadherin from the coleopteran insect *T. molitor* (Coleoptera: Tenebrionidae), suggesting similarities in the mode of action of Cry toxins across insect orders.

1.3.2. Glycosylphosphatidylinositol (GPI)-anchored proteins bind toxin

1.3.2.1. Aminopeptidase-N (APN)

The APN family is a class of enzymes that cleaves neutral amino acids from the N terminus of polypeptides. They serve a variety of functions in a wide range of species, but in the lepidopteran larval midgut, they work in cooperation with endopeptidases and carboxypeptidases to digest proteins derived from the insect's diet (Pigott and Ellar, 2007).

APN is found abundantly in insect midgut where it is anchored to the membrane of epithelial cells by glycosyl phosphatidylinositol (GPI) moiety (kaur et al., 2007).

APN was identified as a receptor to Cry toxins in different insects (Banks et al., 2001; Budatha et al., 2007). Cry toxin interaction with APNs generally involves glycosylatedmoieties. For example, N-acetyl galactosamine (GalNAc) is an important determinant of Cry1Ac interaction with 120 kDa APN from *M. sexta* (Masson et al., 1995; Burton et al., 1999) and *H. virescens* (Gill et al., 1995). But some APNs are believed to bind toxins in a glycan-independent manner. It was first reported that putative Cry1Aa toxin-binding sites in *B. mori* APN were localized between 135-Ile and 198-Pro (Yaoi et al., 1997, 1999). The first aminopeptidase N (APN) identified as a Cry binding protein of Cry1Ac was found in *Manduca sexta* (Knight *et al.,* 1994). In addidition, *B. thuringiensis* Cry1Ac δ-endotoxins recognize 120 and 170 kDa aminopeptidase N (APN) molecules in BBMV from *H. virescens* (Gill et al., 1995; Luo et al., 1997). Moreover, *B. thuringiensis* Cry1Ac and Cry1Fa δ-endotoxins recognize the same 110, 120 and 170 kDa aminopeptidase N (APN) molecules in the BBMV from *H. virescens*, which are likely the sources of cross-resistance between both toxins (Banks et al., 2001) However, the binding assays have shown that Cry1Fa competes with low affinity for Cry1Ac sites in BBMV from *H. virescens* (Jurat-Fuentes and Adang, 2001).

Most of reports showed APNs as Cry toxin-binding proteins (Gill et al., 1995; Knight et al., 1995; Sangadala et al., 1994) in lepidopteran insects.APNs as toxin receptors have been identified from dipteran insects only recently. For example, APNs from Anopheles quadrimaculatus and An. gambiae bind Cry11Ba (Abdullah et al., 2006; Zhang et al., 2008). Moreover, Chen et al. (2009) performed the pull-down assays using biotinylated Cry11Aa toxin and solubilized brush border membrane vesicles from the midguts of Aedes aegypti larvae. An aminopeptidease N (APN), 140 kDa protein, was identified as a

putative receptor of the Cry11A toxin. APN appears to be a functional receptor, to which cadherin-induced toxin oligomers can bind according to the sequential toxin-binding model (Bravo et al., 2004).

1.3.2.2. Alkaline Phosphatase

Alkaline phosphatases, ALP, are GPI-anchored to the cell membrane. In insect larvae, alkaline phosphatases have been localized along the midgut, in Malpighian tubules, and in embryos. Alkaline phosphatases have been proposed to function in active absorption of metabolites and in transport processes (Eguchi, 1995). First observation of a correlation between reduced soybean agglutinin binding to 63- and 68-kDa midgut glycoproteins and the resistance to Cry1Ac toxin was done in the tobacco budworm, *H. virescens*, (Jurat-Fuentes and Adang, 2001). Jurat-Fuentes and Adang (2004) later identified the 68-kDa glycoprotein as a membrane-bound form of alkaline phosphatase (HvALP). Lectin blot analysis revealed existence of N-linked oligosaccharides containing terminal N-acetylgalactosamine required for [125I] Cry1Ac binding in the ligand blots. In another lepidopteran insect, *M. sexta*, a 65-kDa BBMV protein was identified as a Cry1Ac-binding protein by two-dimensional gel electrophoresis followed by ligand blot analysis. By database searches of peptide mass fingerprints and by detection with an ALP-specific antibody, it was identified as ALP (McNall and Adang, 2003). Similar to the lepidopteran ALPs, the ALP from *A. aegypti* has been identified as a putative receptor of Cry11Aa (Fernandez et al., 2006). This protein of 65 kDa is anchored to the membrane by GPI. Immunofluorescence studies showed that ALP localizes predominantly to the coeca and posterior midgut and has a distribution pattern similar to that of bound Cry11Aa (Fernandez et al., 2006).

1.3.3. A Disintegrin and Metalloprotease (ADAM)

Ochoa-Campuzano *et al.* (2007) found a new type of Cry3A toxin receptor by ligand blot and N-terminal analysis. This receptor was called ADAM metalloprotease (A Disintegrin and Metalloprotease). ADAM belongs to the metzincin subgroup of the zinc protease superfamily that includes modular transmembrane proteins implicated in the control of membrane adhesion and fusion and in growth factor shedding (Amour et al., 2002; Ochoa-Campuzano et al., 2007). ADAM as Cry3Aa receptors shares certain features with APN - both are metalloproteases, both are localized in membrane lipid rafts, and both interact with the toxin domain III.

1.4. Cry toxin mode of action

The understanding of Cry toxins mode of action is necessary for improving these products and ensuring their continued use. Long history of intensive research has established that their toxic effect is due primarily to their ability to form pores in the plasma membrane of the midgut epithelial cells of susceptible insects (Schnepf et al., 1998; Aronson and Shai, 2001; de Maagd et al., 2001; Promdonkoy and Ellar**,** 2003; Bravo et al., 2005; Vachon et al., 2012). Many details of Cry toxins mode of action have been characterized in lepidopteran insects, which may be considered as the ‘‘classical’’ model of Bt mode of action. Before reaching their functional pore-forming state, the Cry protoxins must go through several structural changes:

(1) Crystal proteins are ingested as protoxins, solubilized and activated by midgut proteases, resulting in the production of monomeric toxin. In the case of the Cry1A toxins, the 130-140 kDa protoxin is cleaved to a ~60-70 kDa active toxin (Li et al., 1991; Bravo et al., 2005). The activation includes removal of an N terminal peptide (25–30 amino acids for Cry1 toxins, 58 residues for Cry3A and 49 for Cry2Aa) and approximately half of the remaining protein from the C-terminus in the case of the long Cry protoxins (Bravo et al., 2007). The active molecule is made up from three domains which are, from N to C terminus, a membrane inserting (pore forming) seven α-helices bundle (Domain I), receptor-binding three antiparallel β-sheets (Domain II), and a tightly packed β-sandwich (Domain III) (Li et al., 1991).

(2) Binding of the toxin to specific membrane receptors on the apical membrane of midgut cells (Hofmann et al., 1988; Bravo et al., 1992; de Maagd et al., 2001). For Cry1A toxins, at least four different binding proteins have been described in different lepidopteran insects; a 120 kDa glycosylphosphatidyl-inositol (GPI)-anchored aminopeptidase-N (APN) (Knight *et al.*, 1994), cadherin-like protein of 210 kDa (Bt-R1) in *M. sexta* (Vadlamudi et al., 1995), 220 kDa in *O. nubilalis* (Flannagan et al., 2005), a 175 kDa protein (Bt-R175) in *B. mori* (Nagamatsu et al., 1998a), GPI-anchored alkaline phosphatase (ALP) (Jurat-Fuentes and Adang, 2004) and a 270 kDa glycoconjugate (Valaitis et al., 2001).

(3) Formation of a pre-pore oligomeric structure allowing insertion into the membrane and formation of pores which are permeable to small molecules such as inorganic ions, amino acids and sugars (Carroll and Ellar,1993).

In recent years, a rather elaborate model involving the sequential binding of the toxins to different membrane receptors has been developed to describe the events leading to membrane insertion and pore formation (Vachon et al., 2012). Research on the mode of action of Cry toxins has been dominated in recent years by three models, which proposes a somewhat complex sequence of events involving multiple receptors in an attempt to explain the mechanism of pore formation (Gómez et al., 2002; Bravo et al., 2004; Pacheco et al., 2009a), the signaling pathway model, which radically questions the sequence of events presented above by suggesting that pore formation does not play an essential role (Zhang et al., 2005, 2006), and a combination of the two above models has also been proposed (Jurat-Fuentes and Adang, 2006).

1.4.1. The sequential binding model

This model has been extensively reviewed by its authors in the last few years. In the first step, the toxin binds to the cadherin and the resulting conformational change favors proteolytic cleavage at the level of residue F50, located within the loop linking helices α1 and α2, i.e. the first helices on the N-terminal end of the pore-forming domain of the toxin molecule (Gómez et al., 2002). This cleavage had been observed for the first time by Aronson et al. (1999). Gomez et al. (2001 and 2002) demonstrated the proteolytic removal of helix a1, which allows the rest of the toxin to oligomerize and form a so-called pre-pore structure, by three different ways; incubation of Cry1Ab protoxin with a single chain antibody scFv73 that mimics the cadherin-like receptor, treatment with *M. sexta* midgut juice or trypsin, and incubated with brush border membrane vesicles from *M. sexta*, resulting toxin preparation was the 250 kDa oligomer that lacked helix α-1 of domain I. This oligomer, in contrast with the 60 kDa monomer, was capable of membrane insertion as judged by measuring toxin membrane insertion using the intrinsic fluorescence of tryptophan residues and also by the analysis of membrane permeability using black lipid bilayers (Rausell et al., 2004b). In 2007 Soberon et al. confirmed the importance of the helix α1 removal step in the sequential binding model by experiments conducted with engineered Cry1A modifications Cry1AbMod and Cry1AcMod and two genetically modified Cry1Ab and Cry1Ac proteins that lacked helix α-1. These compounds formed oligomers in vitro in the absence of cadherin receptor and killed resistant lepidopteran insects that had mutated cadherin gene. But before this evidence, Soberón et al. (2000) reported that the intermolecular interaction between Cry1Ab monomers is a necessary step for pore formation and toxicity: two Cry1Ab mutant proteins affected in different steps of their mode of action (F371A in receptor binding and

H168F in pore formation) bound efficiently to *M. sexta* BBMV from *M. sexta* larvae when mixed in a one to one ratio.

By immunoprecipitation of toxin-binding proteins (APN and BT-R1) by pure monomeric or oligomeric structures of Cry1Ab toxin showed that APN was preferentially observed after immunoprecipitation with the pure oligomeric toxin structure. In contrast, Bt-R1 was preferentially detected when the experiment was done with the monomeric Cry1Ab structure (Bravo et al., 2004). The binding to Bt-R1 is the first event in the interaction with the microvilli membrane. This initial binding promotes a conformation that facilitatates proteolytic cleavage of helix a-1 by a membrane-bound protease (Gomez et al., 2002). This cleavage results in the formation of a tetrameric pre-pore structure that is insertion competent (Gomez et al., 2002). The oligomeric toxin then binds to APN, which drives the pre-pore complex to detergent-resistant membranes (DRM) lipid rafts where it inserts into the membrane. Generally, all Cry1A-APN receptors are anchored to the membrane by a glycosylphosphatidyl- inositol (GPI) anchor (Knight et al., 1994; Lee et al., 1996). These GPI-anchored proteins are selectively included in lipid rafts that are conceived as spatially differentiated liquid-ordered microdomains in cell membranes (Schroeder et al., 1998). Lipid rafts are detergent-resistant membranes (DRM) enriched in glycosphingolipids, cholesterol and GPI-anchored proteins and are involved in signal transduction, sorting and trafficking of plasma membrane proteins in mammalian cells (Schroeder et al., 1998). Also, they function as pathogen portals for different viruses, bacteria and toxins (Cabiaux et al., 1997). Zhuang et al. (2002) demonstrated that the APNs in *M. sexta* and *H. virescens*, in contrast to the cadherin receptors, are located in DRM, and that the integrity of these microdomains is essential for Cry1Ab pore activity.

In a recent modification of this model (Pacheco et al., 2009a), an additional step was proposed in which the monomeric toxin first binds to the aminopeptidase, with low affinity but high capacity, before interacting with the cadherin, which is present in smaller amounts in the membrane, as was pointed out earlier by Pigott and Ellar (2007), but binds the toxin with higher affinity.

1.4.2. The signaling pathway model (Zhang model)

The previous model showed that Cry1A kills cells exclusively by osmotic lysis, while Zhang et al. (2005) reported that there are two kinds of interaction between toxin and cell. The first is a nonspecific toxin–lipid interaction, mediating assembly of Cry toxin molecules as oligomers and their insertion into membrane. The complex of membrane-incorporated oligomer does not form lytic pores in the membrane and has no toxic effect

on cells, while the second kind is specific interaction between Cry toxin and the cadherin receptor BT-R in *M. Sexta*, which initiates an $Mg2^{+}$-dependent signaling that promotes cell death, and the evidence that suggested that Cry1Ab induces an $Mg2^{+}$ signaling pathway was based on experiments examining the effect of the divalent cation chelators EDTA (chelator of $Ca2^{+}$ and $Mg2^{+}$), and EGTA($Ca2^{+}$-specific chelator) on toxin-susceptible cells heterologously expressing BT-R1 (S5). The result showed that the addition of $Mg2^{+}$to S_5 cells pretreated with EDTA restored Cry1Ab-mediated cytotoxicity, and by immunoblotting, it was proposed that Cry1Ab binding to BT-R1 was linked to an $Mg2^{+}$-dependent signaling pathway associated with cell death (Zhang et al., 2005).

Additional work suggested that receptor binding activates a signaling pathway involving stimulation of G protein, adenylyl cyclase (AC), increased cyclic AMP (cAMP) levels by 8 to 10 fold, and activation of protein kinase A (PKA), leading to destabilization of the cytoskeleton and ion channels and subsequent cell death (Zhang et al., 2006), and the evidence to suggest that Cry1Ab induces an AC/PKA signaling pathway was based on experiments examining the effect of two potent cell-permeable PKA inhibitors, H-89 (competitive inhibitor that interferes with the utilization of ATP by PKA) and myristoylated amide 14–22 (PKAI 14–22-amide) on cAMP production and cytotoxicity in S5 cells. The result showed that both inhibitors protected the cells from Cry toxin action. Thus the preventing of cAMP production by inhibitors of Gαs (NF449) and AC (ddADP) substantially reduced the Cry1Ab cytotoxicity, whereas the activator (FSK) and potentiator (pCPT-cAMP) of cAMP sensitized the cells and enhanced cytotoxicity (Zhang et al., 2006).

1.4.3. The Jurat-Fuentes Model

The Studies of Bt susceptible and resistant strains of the three strains of the tobacco budworm *Heliothis virescens,* YHD2, CXC and KCBhyb strains, have provided that the lack of *H. virescens* Cadherin-like protein (HevCaLP) in midgut epithelium correlated with high levels of resistance to Cry1Ac, and loss of HevCaLP also caused reduced Cry1Aa binding to BBMV from larvae of the YHD2 and KCBhyb strains, while Cry1Ab and Cry1Ac binding remained unchanged (Gould et al., 1992 and 1995; Lee et al., 1995; Gahan et al., 2001; Jurat-Fuentes et al., 2004). Continuous selection of larvae from the YHD2 strain with Cry1Ac resulted in a strain called YHD2-B that has 73-fold higher resistance than YHD2 and reduced Cry1Ab and Cry1Ac binding (Jurat-Fuentes et al., 2002). These changes imply that Cry1Ac binding sites other than HevCaLP are involved

in toxicity to *H. virescens* larvae, which was identified as a membrane-bound form of alkaline phosphatase (HvALP) that was GPI-anchored to the cell membrane (Jurat-Fuentes and Adang, 2004).

This information led Jurat-Fuentes and Adang (2006) to explain a new model for the Cry toxin mode of action. According to this model, binding to cadherin (HevCaLP) and GPI-anchored proteins (APN and HvALP) has a significant role for toxicity. Firstly, the solubilized Cry1Ac crystals are activated to crystal monomers that bind to HevCaLP. This binding results in activation of intracellular signaling pathways regulated by phosphatases (P), while, part of the Cry1Ac toxin may interact with actin and intracellular phosphatases. After binding to HevCaLP, toxin monomers are processed and form oligomers that bind to GPI anchored proteins (HvALP, APN). These proteins are concentrated in lipid rafts, and toxin binding may induce lipid raft aggregation, and inducing toxin insertion forming pores and activating intracellular signaling pathways, which regulated by the same type of phosphatases that regulate signaling through cadherins. Both the intracellular signals, which may activate apoptotic responses, and osmotic shock induced by toxin pore formation contribute to cell killing.

1.5. Enhancement of Cry toxins by toxin-binding fragments

Insect cadherin receptors are modular proteins composed of three domains, the ectodomain formed by 9–12 cadherin repeats (CR) depending on the insect species, the transmembrane domain and the intracellular domain (Pacheco et al., 2009b).

Toxin binding sites have been studied extensively in Lepidoptera special in the *M. sexta* cadherin receptor which the cadherin Bt-R1 serves as the primary binding protein for Cry1A toxins in *M. sexta,* and has multiple toxin binding region (TBRs) including the one located in CR12-MPED. It is interesting when some of TBR-induced toxicity inhibition, while the CR12-MPED-synergized Cry1A toxicity in *M. sexta*. Gomez et al. (2003) reported that cadherin fragments corresponding to CR7 and CR11 inhibited Cry1Ab toxin activity in *M. sexta*. In contrast, truncation analysis of cadherin receptor (BT-R1) in *M. sexta* revealed that the only fragment (EC11 or CR11) capable of binding the Cry1A toxins of *B. thuringiensis* was a contiguous 169-amino acid sequence adjacent to the membrane-proximal extracellular domain, and this toxin binding fragment acted as an antagonist to Cry1Ab toxin by blocking the binding of toxin to the tobacco hornworm, *M. sexta*, midgut and inhibiting insecticidal action (Dorsch et al., 2002). Nevertheless, it was reported that peptide CR12-MPED extracted from *E. coli* inclusion bodies enhanced

the activity of Cry1Ab in the same insect species (Chen et al., 2007) as well as the cadherin fragments corresponding to CR7 and CR11 regions which also enhanced the activity of Cry1Ac and Cry1Ab toxin to *M. sexta* larvae, but not as efficient as the CR12 fragment (Pacheco et al., 2009b). The CR12-MPED peptide bound brush border membrane vesicles with high affinity (*K*d =32 nM) and insect midgut microvilli but did not alter Cry1Ab or Cry1Ac binding localization in the midgut (Chen et al., 2007). This repeat (CR12) has been recognized for first time as an important region of cadherin mediating Cry1A toxicity since S2 cultured insect cells expressing cadherin receptor proteins containing CR12 in their surface become sensitive to Cry1Ab toxin (Hua et al., 2004). The enhanced activity of Cry1A toxins in the presence of toxin-binding cadherin fragments (CR12) correlates with oligomer formation through improving the probability of toxin binding to a second receptor, leading to enhanced insecticidal activity of Cry toxins (Pacheco et al., 2009b). If the binding residues within CR12 were removed, the resulting peptide lost the ability to bind toxin and lost its function as a toxin synergist.

On another lepidopteran insect, *H. armigera*, Peng et al. (2010a) reported that the toxin binding regions of *H. armigera* (HaCad1) can also enhance the toxicity of Cry1Ac and the synergism of HaCad1 to Cry1Ac toxins against *H. armigera*. In another study, the toxin binding region fragment of the *H. armigera* cadherin gene (Hacad1) was cloned and introduced into *B. thuringiensis* BMB171 and this co-express of HaCad1 and Cry1Ac in *B. thuringiensis* and enhance the insecticidal activity of Cry1Ac against the Lepidoptera insects including *H. armigera* and S. exigua by 5.1-fold and 6.5-fold, respectively (Peng et al., 2010b).

In Diptera, Hua et al. (2008) reported that the CR11-MPED region of *Anopheles gambiae* cadherin (AgCad1) enhanced Cry4Ba toxicity to A. gambiae larvae.

Coleopteran larvae ingesting the cadherin fragment (CR8-CR10) of western corn rootworm *Diabrotica virgifera virgifera* inclusions had increased susceptibility to Cry3Aa or Cry3Bb toxin for Colorado potato beetle *L. decemlineata*, southern corn rootworm *Diabrotica undecimpunctata howardi*, and western corn rootworm, and this enhancement was ranged from 3- to 13-fold (Park et al., 2009). On another way, a protein fragment containing the predicted toxin-binding region from *T. molitor* cadherin, rTmCad1p, was demonstrated to bind Cry3Aa toxin specifically and promote Cry3Aa toxin oligomerization (Fabric et al., 2009). Recently, Gao et al. (2011) found that rTmCad1p can potentiate Cry3Aa toxicity in field coleopteran pests of vegetables in China, including the spotted asparagus beetle (SAB), *Crioceris quatuordecimpunctata*,

the cabbage leaf beetle (CLB), *Colaphellus bowringi*, and the daikon leaf beetle (DLB), *Phaedon brassicae* 15.3, 7.9 and 4.3 fold respectively.

Another approach for enhance the toxicity of Cry toxins is to changing in the domain I structure which involved in membrane interaction. For example, two mutant toxins (A1 and A2) of Cry3A through the changing of site-specific in loop I of the receptor binding domain of Cry3A N-endotoxin altered the toxicity of this protein in coleopteran larvae including *T. molitor* (yellow mealworm), *L. decemlineata* (Colorado potato beetle) and *Chrysomela scripta* (cottonwood leaf beetle), and competitive binding assays of *L. decemlineata* brush border membrane vesicles (BBMV) revealed that binding affinities for the A1 and A2 mutant toxins were ca. 2.5-fold higher than for the wild-type Cry3 toxin. Similar binding assays with *C. scripta* BBMV revealed a ca. 5-fold lower dissociation rate for the A1 mutant as compared to that of Cry3A (Wu et al., 2000).

This strategy is a good candidate for development to control target pests, and the enhancers of *Bt* toxicity will represent an opportunity to improve currently available commercial products against diverse pests. Moreover, this discovery provides a novel strategy to enhance insecticidal activity and delay insect resistance in coleopteran pests to *Bt*-toxin-based biopesticides or transgenic crops (Gao et al., 2011).

2. OBJECTIVES

Bacillus thuringiensis is the most commonly used bioagent in insect pest control. The toxic activity is largely due to the crystalline (Cry) toxins that are highly specific for certain taxa of insects or nematodes. Cry toxins bind to specific receptors in the midgut and either derrange the uptake of nutrients or cause perforation of the gut wall leading to general sepsis and death. The receptors have been studied extensively in Lepidoptera but much less in the pest beetles. The sequences of one type of the receptors called cadherin 1 were elucidated in *Tribolium castaneum* and *Tenebrio molitor* in a US laboratory. This information opens door to the search of cadherins in other economically important beetles, and in course of that, the specific goals of my work were defined:

1) Assessing the sensitivity of *T. castaneum* and *T. molitor* larvae to the Cry3Aa toxin and its modifications

2) Identification of cadherin 1 ortholog in a Czech and a Romanian population of *T. molitor* - comparison with the previously analyzed US population

3) Check the presence of cadherin in different tissues and in different developmental times in *T. molitor*

4) Enhance the Cry3Aa activity in coleopteran larvae by a *T. molitor* cadherin fragment

Important goal of the development and improvement of *B. thuringiensis*-based pesticides is to compete with hazardous chemical pesticides and replace them as much as possible. Reaching this goal is impossible without detail structural and biochemical knowledge of the Cry toxins and their action. On the basis of such knowledge one could redesign toxin structure to customize them to desired host range, alter activity, improve stability, and delay or overcome resistance. Elucidation of the toxin-receptor interactions may stimulate development of synergists – application of cadherin fragments along with the toxin has already been shown to increase efficiency, and can possibly overcome or delay insect resistance to this biopesticide.

3. METHODOLOGY

Methods are briefly described in the manuscripts (chapters 2-4). Here I present detail description that can facilitate research of students working on similar subjects.

3.1. The sensitivity of *Tribolium castaneum* penultimate larval instar to Cry3 toxin

3.1.1. Insect rearing

T. castaneum, Red Flour Beetle (Coleoptera: Tenebrionidae) was reared in darkness at 25 ± 1°C and cca 60% humidity. The insects were fed wheat flour containing 5% yeast. The penultimate instar larvae were weighed and those with body mass 2 ± 0.4 mg were taken for the assay. Their mortality and body growth were checked after 10 days of toxin feeding.

3.1.2. Preparations of recombinant cry toxins

Recombinant Cry3 toxins were prepared from synthetic genes expressed in *Escherichia coli* BL21; modified genes contained spruce-consensus optimized codons and various combinations of N- terminal deletions and C-terminal sequence duplications.

3.1.3. Preparation of Cry3Aa toxin

The purification of Cry3Aa crystal from commercial insecticide Novodor™ 3% was done according to the method of Murray and Spencer (1966).

3.1.4. Insect toxicity assays

The activity of Cry3Aa derivatives was tested on the penultimate instar larvae of *T. castaneum*. The Stock solutions (2 mg protein per ml) of tested Cry3A derivatives were diluted with water to obtain protein concentrations 800, 400, 200 and 100 µg per ml. These solutions were thoroughly mixed with the diet in ratio 245 µl per 1750 mg diet and the mixtures were crushed to fine powders in ceramic mortars. Assuming that the solutions contained no other proteins, the toxin concentrations in the diet were assessed to be 112, 56, 28 and 14 µg/g (= ppm). The fresh preparations of the diet/toxin mixtures were kept in a humidity chamber for 3 h and then distributed by 50 mg per well (1 ml volume) in a titer-plate. Five groups of 5 pre-weighed larvae of similar size were used in a typical test. The results were analyzed with the aid of the regression analysis and One-Way Anova (the df, F, and P-value were established) included in the GraphPad Prism software (the homogeneity of variance was confirmed with the Bartlett's test.

3.1.5. Determination of the Cry3Aa and its modification in the diet

ELISA with a commercial kit from the GDIA Company (catalog number PSP 05900) was used to quantify Cry3Aa content in the diet.

3.2. Identification of cadherin 1 ortholog in a Czech and a Romania population of *T. molitor*

3.2.1. RNA isolation

3.2.1.1. Isolation of the *T. molitor* larval midgut

Freshly dissected midgut of *T. molitor* larvae were rinsed in a cold saline and kept in -80C. Total RNA was isolated with the Trizol reagent (Invitrogen).

Procedure

1. Trizol reagent was adding to the midgut sample (1 ml / 50-100 mg tissue) in a 1.5 eppendorf tube and homogenize well by using plastic Teflon.
2. The homogenized sample was incubated for 5 min at room temp.
3. Chloroform was added (1:5 of Trizol) and the mixture was shaken vigorously by hand for 15 sec and then incubated for 3 min at room temp.
4. The sample was centrifuged at 14,000 for 15 min at 4°C and the aqueous phase (middle white part) was transfered to a new eppendorf tube.
5. Isopropanol was added (1:2 of Trizol) and mixed well.
6. The sample was incubated for 15 min at room temp.
7. Centrifugation at 14,000 rpm for 10 min at 4°C, discard of the supernatant, and washing the pellet once with 70% ethanol (1:1 of Trizol).
8. Centrifugation at 7,500 for 5 min at 4°C.
9. The ethanol was removed and the RNA pellet was air dried for 5 to 10 min and then dissolved in DEPC treated autoclaved water.).
10. An aliquot of RNA was checked on denaturing agarose gel while the rest of RNA sample was kept at -80°C until use.

3.2.1.2. RNA concentration and purity determination

The RNA quality and quantity were determined by using a spectrophotometer at the absorbance of 260 and 280 nm. The RNA concentration was calculated from the absorbance value measured at 260 nm by the formula:

$$\text{RNA conc. } (\mu g/\mu l) = \frac{\text{A260 X dilution factor X 40}}{1000}$$

The RNA purity was determined by the absorbance ratio of A260/A280. The ratio should be between 1.8 and 2 which represents a high purity of RNA.

3.2.2. Isolation of *T. molitor* cadherin (TmCad1) cDNA and its fragments

3.2.2.1. Synthesis of the 1st strand cDNA

The first strand cDNA was synthesized using, 1 µg total RNA as template, oligo (dT) primer and the enzyme reverse transcriptase PrimeScript™ (Takara). The reaction conditions and mixtures were prepared in a micro tube as the following:

Oligo dT primer (50µM)	1 µl
dNTP Mixture (10 mM)	1 µl
Template RNA	1 µl
RNase free dH_2O	up to 10 µl

- Keep for 5 min. at 65 ^oC, and cool immediately on ice.
- The reaction mixture was prepared by combining the following reagents to a total volume
of 20 µl.

Template RNA Primer mixture	10 µl
5 X primeScript™ buffer	4 µl
RNase inhibitor (40 U/µl)	0.5 µl
primeScript™ RTase (200 U/µl)	1 µl
RNase free dH_2O	up to 20 µl

- Mix gently and incubate the reaction mixture immediately under the following conditions

50^oC	60 min
70^oC	15 min

- Stored the samples at -20^oC.

3.2.2.2. Amplification of the cDNA

The 1st strand cDNA, was used to obtain full-length cDNAs of TmCad1 with the primers described in the table (1), that were designed from the TmCad1 sequence deposited in the data base (GenBank: DQ988044.2)

Table (1): Nucleotide primers used to obtain the full length TmCad1 cDNA

Primer	Orientation	Position	Primer DNA Sequence
Tm4	Antisense	4131-4156	5’-TGTCCAGGTCGAGGTTAGATGGAGT-3´
Tm8	Antisense	3847-3866	5’-GAGCGGTTGTTTAAGGGTGA-3´
Tm9	Antisense	3019-3042	5’-TGTCACCTTCATCGTCATCTTTCC-3´
Tm12	Antisense	2380-2403	5’-CAACCCAGTCGGGAGTGTTCTCAT-3´
Tm15	Antisense	1136-1157	5’-AATGTCTTCAAGGATCAGCAGT-3´
Tm14	Antisense	996-1022	5’-GGCATCCACCGTAGCGAAGTTGTTCTC-3´
Fw1	Sense	20-42	5’-AGTACAGCAGTTCCGTAAGTGCG-3´
Tm13	Sense	490-517	5’-TCAAGAACTTGGACGACGAACATCCGAC-3´
Fw2	Sense	982-1006	5´-ATACCGTCGAAGACGAGAACAACTT-3´
Tm11	Sense	1943-1966	5’-CGACGCAGATTTGGAGTTCTCGAT-3´
Fw3	Sense	2370-2392	5´-AAGAGTGCTGATGAGAACACTCC-3´
Fw4	Sense	3840-3864	5´-GGAACAATCACCCTTAAACAACCGC-3´

Trikant	Universal		TGAGCAAGTTCAGCCTGGTTATTTTTTTTTTTTTTTTT

The reaction conditions were optimized and mixtures (25-µl total volume) consisted of the following:

10x buffer	2.5 µl
dNTPs (2.5 mM)	2.5 µl
$MgCl_2$ (50 mM)	2.5 µl
Forward primer (10 µM)	1.0 µl
Reverse primer (10 µM)	1.0 µl
Template DNA (50 ng/µl)	1.0 µl
Taq (5 U/µl)	0.2 µl
ddH_2O	up to 25 µl

Amplification was carried out in a Biometra T3000 Thermal Cycler (Göttingen, Germany). Programmed for 37 cycles as follows: 94°C/1 min (1 cycle); 94°C/30 sec, 58°C/30 sec, 72°C/45 sec (35 cycles); 72°C/7 min (1 cycle); 4°C (hold). Agarose (1.0%) electrophoresis was used for resolving the PCR products. The electrophoresis run was performed at 100 V. Bands were detected on UV-transilluminator and photographed.

3.2.2.3. Agarose gel electrophoresis

Reagents and solutions

50x TAE running buffer

Tris-base	52.42 g
EDTA-Na_2	37.2 g
ddH_2O	900 ml
Glacial acetic acid	57.1 ml

Then, pH was adjusted using 10 N NaOH solution and volume was completed to 1 L.

Running buffer

From the stock solution (50x), 20 ml was taken and completed to 1 L using ddH_2O.

Gel loading dye (6x)

Bromophenol blue	25 mg
Glycerol	50 ml
ddH_2O	up to 100 ml

Then, pH (8.0) was adjusted using 10 N NaOH solution (stored at -20°C).

Ethidium bromide stain (EtBr)

Ethidium bromide	10 mg

ddH_2O 1 ml

Then, stored in a dark bottle.

Procedure

1. Agarose gel (1%) was prepared by dissolving 0.5 g agarose powder in 50 ml running buffer (1x TAE buffer) and placed on heater till complete dissolution. Then, volume was adjusted to 50 ml ddH_2O. The solution was allowed to cool to 45°C and then EtBr was added (0.5 µl). The gel was poured into its tray with well-forming comb and allowed to complete solidification (about 30 min). Then, the comb was removed carefully and the gel was placed on submarine electrophoresis unit covered by 1x TAE running buffer.

2. Sample loading: 0.5 µl of loading dye (6x) was mixed with 2 µl of the cDNA and then loaded into the wells. Electrophoresis was done under constant voltage of 100 V.

3. UV detection and photography: samples were visualized using UV transilluminator and photographed.

3.2.2.4. Purification of the PCR products by MinElute Gel Extraction Kit (Qiagen)

1. Excising the DNA fragment from the agarose gel with a clean, sharp scalpel in a clean eppendorf tube.

2. 3 volumes of Buffer QG was adding to 1 volume of gel (300 µl/100 mg) and incubate at 50°C for 10 min (or until the gel slice has completely dissolved) and mix by vortexing the tube every 2–3 min during the incubation.

3. After the gel slice has dissolved completely, isopropanol was adding (100 µl/100 mg of gel slice).

4. Transfer the aliquot to the MinElute column and spin for 1 min at 14.000 rpm.

5. Discard the flow-through and 500 µl of buffer QG was adding to the spin column and centrifuge for 1 min at 14.000 rpm.

6. Discard the flow-through and 750 µl of buffer PE (for washing) was adding to the MinElute column and centrifuge for 1 min at 14.000 rpm.

7. Discard the flow-through and centrifuge the MinElute column for an additional 1 min.

8. Placed the MinElute column into a clean 1.5 ml microcentrifuge tube and 10 µl of Buffer EB (10 mM Tris·Cl, pH 8.5) or water was adding to the center of the membrane, and incubated the column for 1 min, before centrifuge for 1 min.

9. Agarose (1.0%) electrophoresis was used for resolving the PCR products. The electrophoresis run was performed at 100 V. Bands were detected on UV-transilluminator and photographed.

3.2.2.5. Cloning the PCR products (TmCad cDNAs) in pGEM-T Easy Vector (Promega)

Reagents and solutions

LB medium

Trypton	10.0 g
Yeast extract	5.0 g
NaCl	10.0 g
ddH_2O	up to 1 liter

Adjust pH to 7.0 with NaOH.

LB plates with ampicillin

Agar	15.0 g
LB medium	1 liter

Autoclave for 20 min, and after cooling to 50°C, the ampicillin 100μg/ml was adding.

Pour 30–35ml of medium into 85mm petri dishes, and store it at 4°C after hardiness.

100 mM IPTG (isopropyl β-Dthiogalactopyranoside)

IPTG	23.83 mg
ddH_2O	1 ml

X-Gal (50 mg/ml)

X-Gal	50 mg
N,N´-dimethylformamide	1 ml

Cover with aluminum foil and store at –20°C.

Ampicillin (sodium salt)

Ampicilline	100 mg
ddH_2O	1 ml

LB plates with ampicillin/IPTG/X-Gal

100mM IPTG	20 μl
50mg/ml X-Gal	40 μl

Spread over the surface of an LB ampicillin plate and allowed to absorb for 30 min. at 37°C prior to use.

$CaCl_2$ solution

$CaCl_2$	100 mM
Glycerol	10%

Solution was filter-sterilized or autoclaved.

Preparation of competent cells

1. A single colony of *E. coli* (DH5α) was inoculated into 5 ml LB medium and grown overnight at 37°C with shaking (180 rpm).

2. Two ml of the culture were inoculated into 200 ml LB medium in a sterile 1-liter flask and grown at 37°C with shaking at 180 rpm, to an OD_{590} of 0.37. Overgrowth of culture (beyond OD_{590} of 0.4) decreases the efficiency of transformation.

3. Culture was aliquoted into four 50-ml pre-chilled, sterile polypropylene tubes and left on ice for 5-10 min (cells should be kept cold for all subsequent steps).

4. Cells were centrifuged for 7 min at 3,000 rpm/4°C. Centrifuge was allowed to decelerate without brake.

5. Supernatant was poured off and each pellet was resuspended in 10 ml of ice-cold $CaCl_2$ solution (resuspension should be performed very gently and all cells kept on ice).

6. Cells were centrifuged for 5 min at 2,500 rpm/4°C. Supernatant was discarded and each pellet was resuspended in 10 ml of cold $CaCl_2$ solution, which was kept on ice for 30 min.

7. Cells were pelleted for 5 min at 2,500 rpm/4°C. Each pellet was resuspended in 2 ml of ice-cold $CaCl_2$ solution.

8. Cells were dispensed into pre-chilled, sterile polypropylene tubes (100 µl aliquots), which were frozen immediately at -80°C.

Ligation of vector and insert:

The ligation reaction was set up using the desired vector: insert molar ratio 1: 3, the reaction conditions and mixtures consisted of the following:

2X Rapid Ligation Buffer	5 µl
pGEM®-T Easy Vector (50ng)	1 µl
PCR product	3 µl
T4 DNA Ligase (3 Weiss units/µl)	1 µl

The reaction was incubated at 4°C for 16 h. The ligation reaction can be frozen and stored at –20°C or transformed into the host strain immediately. The reaction should not exceed 0.5% of the transformation reaction volume.

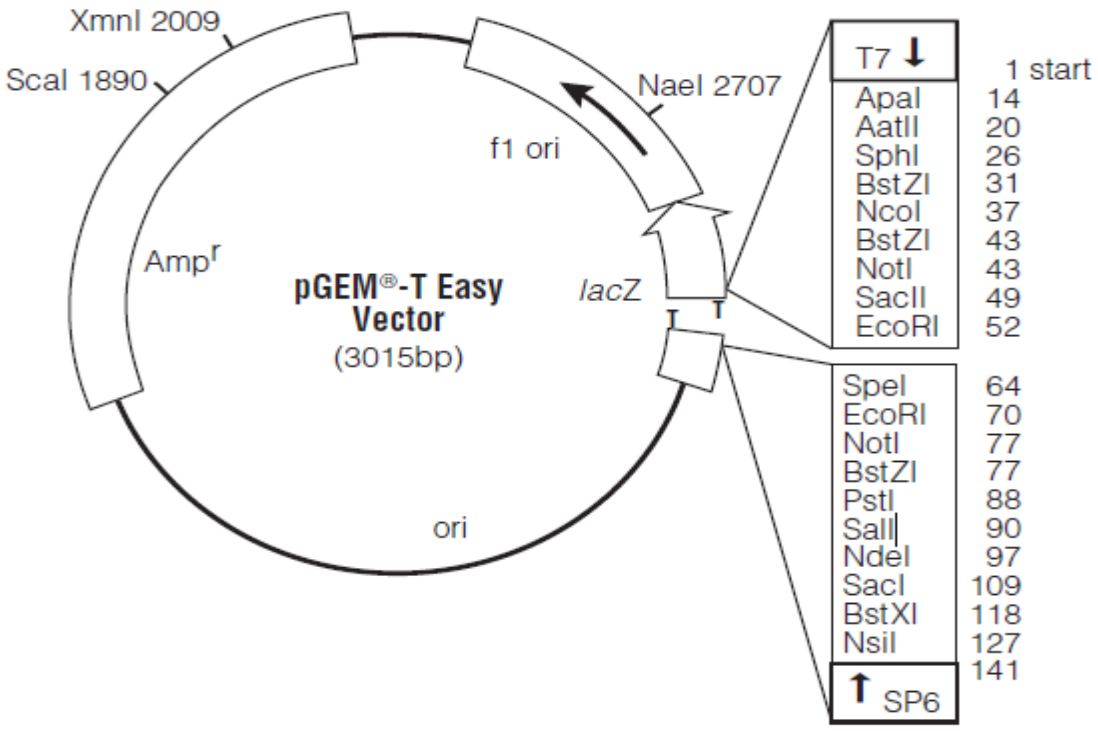

Figure 1: pGEM®-T Easy Vector circle map and sequence reference points. The map is available from Promega website (www.promega.com).

Transformation of competent cells with plasmid DNA

1- About 100 µl of bacterial suspension were transferred into a sterile eppendorf tube. Approx 1 ng of plasmid DNA was added to the competent bacterial cells in 1.5 ml microcentrifuge tubes and mixed gently without pipetting up and down or vortexing the tube.

2- The transformation mix was placed on ice for 30 min, and then exposed to heat shock at 42°C for 1 min.

3. The mixture was cooled on ice for 1 min, and then 900 µl of LB liquid media was added. The cells were incubated at 37°C for 1 h to allow the bacteria to express the antibiotic resistance marker encoded by the plasmid.

4. Cells were centrifuged for 1 min at 5,000 rpm and the pellet was resuspended in 100 µl LB media, followed by spreading on LB plates with ampicilline.

5. The plates were incubated overnight at 37°C. Only the transformed cells grew and gave separate colonies.

Master plate preparation and screening of recombinant clones by direct colony PCR

Approximately 25- 40 white clones and one blue clone were picked and spotted on LB agar plate containing 100µg/ml Ampicillin to make master plate. The master plate was incubated at 37°C for 12-16 hr. After bacterial growth, a small touch was picked for colony PCR, which was performed as following:

10x buffer	2.5 µl
dNTPs (2.5 mM)	2.5 µl
$MgCl_2$ (50 mM)	2.5 µl
Upstream Primer (specific primer of the insert)	1.0 µl
Downstream primer (specific primer of the insert)	1.0 µl
Taq (5 U/µl)	0.2 µl
ddH_2O	up to 25 µl

Amplification was carried out in a Biometra T3000 Thermal Cycler (Göttingen, Germany) programmed for 37 cycles as follows: 94°C/1 min (1 cycle); 94°C/30 sec, 58°C/30 sec, 72°C/45 sec (35 cycles); 72°C/7 min (1 cycle); 4°C (hold). Agarose (1.0%) electrophoresis was used for resolving the PCR products. The electrophoresis run was performed at 100 V. Bands were detected on UV-transilluminator and photographed.

Plasmid isolation

The plasmid was isolated from successful cloning of an insert into the pGEM®-T Easy Vector by using QIAprep Spin Miniprep Kit (Qiagen) as follows:

1. Inoculate a touch from the successful colony in 5 ml LB-media and grow overnight at 37 ^oC with shaking (180 rpm).
2. Transfer 1.5 ml of the culture to eppendorf tube and pellet cells at 8,000 rpm for 2 min. Pour off the supernatant and blot the inverted tube on a paper towel to remove excess media.
3. Add 250 µl buffer P1 (cell resuspension solution) and completely resuspend the cell pellet by vortexing or pipetting.
4. Add 250 µl buffer P2 (cell lysis buffer) and mix by inverting the tube 4 times (do not vortex).
5. Add 350 µl of buffer N3 (Neutralization) and immediately mix by inverting the tube 4 times (do not vortex). Centrifuge for 10 min at 13.000 rpm.
6. Transfer the supernatant to the QIAprep spin column, and centrifuge for 1 min at 13,000 rpm.

7. Discard the flow-through and wash the QIAprep spin column by adding 500 µl buffer PB and centrifuge for 1 min at 13,000 rpm.

8. Discard the flow-through and wash the QIAprep spin column by adding 750 µl buffer PE and centrifuge for 1 min at 13,000 rpm.

9. Discard the flow-through, and centrifuge for 1 min to remove residual wash buffer.

10. Place the QIAprep spin column in a clean eppendorf tube, and add 20-50 µl buffer EB or water to the center of the column, incubate for 1 min at room temperature, and centrifuge for 1 min.

Spectrophotometric determination of plasmid concentration

To quantitate the plasmid concentration, the plasmid DNA was diluted in dist. water 1: 100 or 1: 50 (depending on the plasmid copy number) and optical density was measured at 260 nm (A260) and 280 nm (A280). This measurement permitted direct calculation of the nucleic acid concentration using the formula

DNAconc. (µg/mL) = A260 × dilution factor × 50

Where 50 is the extinction coefficient of DNA. The ratio A260/A280 provides a reasonable estimate of the purity of the preparation. A pure sample of DNA has an A260/A280 ratio of 1.8 ± 0.05.

Sequence analysis

The sequence analysis and homology between TmCad1 Czech, Romania population and US population were identified using the NCBI BLAST, and the multiple sequence alignment with the BioEdit 5.09 program. The alignments of nucleotides and amino-acid residues were carried out using MEGA 4.0 software (Tamura et al., 2007).

3.3. Cadherin gene transcription in the midgut of different developmental times and different tissues in *T. molitor midgut*

3.3.1. Insect strain

The basic stock of *T. molitor* was of Czech origin and had been inbred for about 20 years. The insects were reared on wheat bran mixed with 5% yeast at 60% R.H., 25°C, darkness.

3.3.2. Preparation of different *T. molitor* tissues

Freshly ecdysed of last instar larvae, pupae and adults taken from standard cultures, larvae subjected to different feeding regimes (no food, water only, and standard diet), for one week after ecdysis, and adult females 4, 10, 12 and 14 days after emergence were anaesthetized in water and dissected. The foregut, which was recognized by the cuticle

lining, midgut, and hindgut (gut region posterior to the attachment of Malpighian tubuli) were taken from the larvae, while the midgut was taken from the pupae and adults stages and ovaries only from the adults – 14 days after emergence they contained chorionated eggs that were analyzed separately from the samples of whole ovaries. These females also provided the samples of fat body as well as from larval and pupal stages. All dissected organs were rinsed in cold saline and then kept at -80^{o}C. Cadherin expression during embryonic development was assessed from the analysis of eggs 1 - 6 days after oviposition.

3.3.3. RNA isolation and cDNA synthesis

After the isolation of the total RNAs from all tissues with the Trizol reagent (Invitrogen), the first strand cDNA was synthesized from 5 μg total RNA with the aid of the SuperScript III Reverse Transcriptase (Invitrogen) and the Trikant primer [5′-TGAGCAAGTTCAGCCTGGTTA$(T)_{19}$] described by (Fedič et al., 2003). One microliter of the reaction product was used as template for the amplification of specific cDNAs. The specific PCR forward and reverse primers were designed based on the published sequence (Fabric et al., 2009) to match cadherin region CR12 and the membrane-proximal extracellular cadherin domain (MPED), respectively:
FWD (5′-GGCAGCCATATGGACCAAACGGAGTATTTCACCAC-3′), and REV (5′-CGAGGATCCTTATCCGAGAACTACCGAAACAC-3′). PCR product included the toxin-binding region (nucleotides 3,963 to 4,548). RT-PCR was also used to determine relative abundance of cadherin across developmental stages and in different tissues**.** All PCRs were performed on the Biometra T3000 Thermal Cycler (Göttingen, Germany).

3.3.4. cDNA cloning and sequence analysis

PCR products from the larval, pupal and adult midgut and fat body and from the ovaries were purified by agarose electrophoresis, excised from the gel, isolated by MinElute Gel Extraction Kit (Qiagen), and ligated into a pGEM-T Easy vector used for transformation of the competent DH5-α cell. DNA from several positive clones was sequenced in both directions to eliminate possible errors caused by PCRs amplification and cloning. BLAST program was used to screen databases accessible through the National Center for Biotechnology Information internet server in search for homologous proteins. Pairwise sequence comparison was performed with ClustalW and the multiple sequence alignment with the BioEdit 5.09 program. The alignments of nucleotides and amino-acid residues were carried out using MEGA 4.0 software (Tamura et al., 2007)

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CHAPTER 2

Activities of modified Cry3A-type toxins on the red flour beetle, *Tribolium castaneum* (Herbst).

Moataz A.M. Mostafa, Josef Vlasák, František Sehnal

Journal of Applied Entomology (accepted)

CHAPTER 3

C*adherin* gene expression in distant populations and different developmental stages and tissues of *Tenebrio molitor*

Moataz A.M. Mostafa and František Sehnal

Biological Chemistry (submitted)

CHAPTER 4

Enhancement of *Bacillus thuringiensis* Cry3Aa toxicity to coleopteran larvae by a *Tenebrio molitor* cadherin fragment, CR12-MPED

Moataz A.M. Mostafa, Saad Mousa, Zdeňka Svobodová, František Sehnal

Prepared for submission

CHAPTER 5

Conclusions

The insecticidal Cry proteins are the main toxic component of the spores of *B. thuringiensis*, which are effective in killing a wide range of insect pests. The understanding the mode of action of the Cry toxins is essential for designing strategies to improve their activity and to counter pest resistance. My PhD work contributed to this aim in three areas.

Identification of Cadherin 1 protein as a putative Cry3 receptor gene

Cadherins are calcium-dependent cell adhesion proteins composed of an ectodomain formed in *Tenebrio molitor* TmCad1 by 12 cadherin repeats , a transmembrane domain and an intracellular domain. Our study confirms expression of the *TmCad1* gene, which was described in a US population of *T. molitor* (Fabric et al. 2009), in two European populations of this species. Most differences between the three populations occurred in "cadherin" domains 4, 8 and 12 and in the cytoplasm domain. Only 15 amino acid replacements were detected among putative translation products of the cDNAs based on the midgut RNA of the three populations. Six of these positions contained identical residues in both European populations, other mutations were unique: 5 times for the Czech and 4 times for the Romanian populations. This result indicates that the European populations are more related to each other than any of them to the US population. In addition, from the cadherin distribution in the gut, ovaries and fat body we assume that it functions as a cell adhesion molecule in different epithelia. Low cadherin expression in the midgut of newly ecdysed insects apparently indicates that the reconstruction of midgut wall, which occurs during molting, has not been fully completed. The absence of cadherin transcript during embryonic development and the stimulation of cadherin expression by feeding after the larval ecdysis indicate that this cadherin occurs only in functional epithelia

Activities of modified Cry3A-type toxins on the red flour beetle, *Tribolium castaneum* (Herbst)

One of the goals of our research was to overcome the problem of low toxicity of the natural Cry toxins by the design and production of Cry3Aa modifications. Using molecular biology techniques we prepared a nearly 30 recombinant modifications in order to understand the structure-activity relationships and possibly discover a derivative with improved insecticidal activity against *Tribolium castaneum*. However, none of the tested Cry3A derivatives matched in activity the natural or the recombinant Cry3Aa. We assume that our assay primarily revealed affinities of tested proteins to *Tribolium* Cry receptors that seem to be tuned to the natural Cry3Aa.

Enhancement of *Bacillus thuringiensis* Cry3Aa toxicity to coleopteran larvae by a *Tenebrio molitor* cadherin fragment, CR12-MPED.

In this paper we investigated possible synergistic effect of recombinant protein CR12-MPEDp covering cadherin region CR12 and the membrane-proximal extracellular cadherin domain, MPED () of the TmCad1 receptor on the insecticidal activity of Cry3Aa. Statistically significant synergistic effect was found in the tests with *T. molitor* larvae and apparent, but statistically not significant effect with the *T. castaneum* larvae. Thesedata are the first report on synergistic action of the CR12-MPED cadherin fragment. We conclude that the use of TmCR12-MPEDp in combination with Cry3Aa toxin should be considered for practical application in the control of coleopteran stored product pests.

Plan of future work

In response to resistance problem, the concentration or frequency of pesticide applications is often increased. So there is a need for a number of different insecticides with different modes of action, for evaluation of the potential for insect resistance to insecticides and for resistance management. The modern molecular methods are become a greatest task in that branch. Based on my knowledge of using the molecular biology techniques, I will apply a project on the biotechnology management of *Tuta absoluta* (Lepidoptera, Gelechiidae) to improve Tomato Plant Growth.

For Egypt, Tomato is one of the most important vegetable crops; it's grown all year round. However, production faces some problems. One of this problems is invasion with insect especially *Tuta absoluta* (tomato leafminer). It is a very harmful leaf mining moth with a strong preference for tomatoes. That pest also occurs on eggplants, sweet peppers as well as potatoes and various other cultivated plants. *Tuta absoluta* can cause 50-100% yield reduction on tomato crops and its presence may also limit the export of the product to several destinations.

So my aim in this project is to screen the response of some biological control agents as well as chemical agents that induced resistance under open field conditions in order to find an effective alternative method for controlling that pest to reduce using pesticide and its side effect in the consumer.