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Characterization of field evolved resistance to transgenic Cry1Fa maize in Spodoptera frugiperda (J. E. Smith)

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To the Graduate Council:

I am submitting herewith a dissertation written by Siva Rama Krishna Jakka entitled "Characterization of field evolved resistance to transgenic Cry1Fa maize in Spodoptera frugiperda (J. E. Smith)." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Plants, Soils, and Insects.

Juan Luis Jurat-Fuentes, Major Professor

We have read this dissertation and recommend its acceptance:

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Characterization of field evolved resistance to transgenic Cry1Fa maize in *Spodoptera frugiperda* (J. E. Smith)

A Dissertation Presented for the Doctor of Philosophy Degree

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Siva Rama Krishna Jakka

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DEDICATION

This work is dedicated to

My parents Panakalu Jakka, Nagamma Jakka and

my wife Radhika.

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ABSTRACT

Transgenic Bt crops expressing Cry and Vip toxins from *Bacillus thuringiensis* (*Bt*) have been increasingly planted to manage insect pest damage on agricultural crops. The high adoption of Bt-based insecticidal technologies suggests an increase selection pressure for the evolution of resistance in insect populations. So far, nine insect species have developed field evolved resistance to Bt crops, yet the mechanisms involved in field evolved resistance are unknown. In the present study, the resistance mechanism in field evolved resistance to maize producing Cry1Fa in *Spodoptera frugiperda* collected in fields from Puerto Rico was characterized. High levels of resistance to Cry1Fa have been observed in *S. frugiperda* with recessive and autosomal mode inheritance. Binding experiments showed the reduced binding of Cry1Fa toxin to brush border membranes of resistant (456) larvae compared to susceptible (Benzon) larvae. The same binding reduction was observed for Cry1A toxins, but not for Cry1Ca toxin. This reduced binding signifies the modification of a common Cry1Fa-Cry1A toxin binding site. Comparison of receptor protein levels revealed reduced alkaline phosphatase (ALP) levels in resistant compared to susceptible larvae. This reduced expression of ALP phenotype was linked to Cry1Fa resistance in *S. frugiperda*.

In cross-resistance studies using bioassays, reduced susceptibility to Cry1Ab and Cry1Ac toxins was detected and no differences in susceptibility to purified Cry1Bb, Cry1Ca, and Cry1Da toxins or Xentari WG and Dipel ES pesticidal formulations compared to susceptible larvae was detected. The cross-resistance patterns observed in these bioassays are in agreement with data from competition experiments indicating an

altered binding site for Cry1A and Cry1Fa toxins in 456 larvae. The only difference detected in fitness cost studies was a significant increase in the larval developmental time in resistant insects, which could result in emergence asynchrony between susceptible and resistant moths. The lack of fitness costs was also supported by stable resistance after 12 generations of rearing in the absence of a selective (transgenic maize) agent. This work is the first study on field level resistance to a Bt crop. Results from this study will help to understand resistance mechanisms responsible for field-level resistance and formulate improved resistance management practices.

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CHAPTER I

Interactions between *Bacillus thuringiensis* toxins and binding sites on midgut membranes from target insect pests.

1.1. Abstract

Bacillus thuringiensis (Bt) is a valuable source of insecticidal proteins used to control many economically important crop pests. Currently pesticides based on Bt are the most widely used biopesticide worldwide, and genes encoding the Cry and Vip Bt toxins have been successfully bioengineered into crop plants (Bt crops) for insect pest control. The high levels of use and adoption of Bt-based insecticidal technologies suggest an increase selection pressure for the evolution of resistance in insect populations. So far, nine insect species have developed field-evolved resistance to Bt crops. While the mechanisms involved in this resistance are yet unknown, studies on laboratory selected insects suggest alteration or modification of toxin binding to midgut receptors as the most common mechanism of resistance to Bt toxins. Furthermore, alteration of a common binding site has also been found to result in high levels of cross-resistance. In consequence, current resistance management strategies rely on sequential or simultaneous use of Bt toxins is not sharing common binding sites in target pests. The most widely reported method of predicting cross-resistance among Bt toxins the development of binding site models from toxin binding competition data. Hence the present chapter aims to review the literature on binding site models for Bt toxins in economically-important lepidopteran larvae.

1.2. Introduction

Bacillus thuringiensis (Bt) is an aerobic, gram-positive, facultative, ubiquitous soil bacterium. This bacterium produces insecticidal toxins during the vegetative and the sporulation phase of growth that are classified into different families based on amino acid sequence similarity. The most studied Bt toxins are the crystalline (Cry) proteins, a family currently containing 229 described halotypes (Crickmore, 2012). Diverse Cry toxins are active against a number of species in the orders Lepidoptera, Coleoptera, Diptera, Hymenoptera, Hemiptera, and Blattaria (van Frankenhuyzen, 2009). Pesticides based on Bt toxins and spores, or transgenic crops expressing Bt toxins are highly toxic to certain groups of insects and are considered environmentally safe due to their high specificity and unique mode of action (Lambert and Peferoen, 1992).

1.3. Bt toxin mode of action

Insecticidal Cry toxins from Bt target the insect midgut cells to compromise the gut epithelium barrier and facilitate the onset of septicemia (Raymond et al., 2010). Advancements in the description of this multistage process have been recently reviewed (Aronson and Shai, 2001; Faust et al., 1974; Gill et al., 1992; Ibrahim et al., 2010; Jurat-Fuentes and Adang, 2006; Louloudes and Heimpel, 1969; Pardo-Lopez et al., 2013; Rajamohan et al., 1998; Soberon et al., 2010; Tojo, 1986; Vachon et al., 2012; Whalon and Wingerd, 2003). Briefly, after ingestion of the crystalline inclusions by insect larvae they solubilized to release Cry protoxins in the insect gut fluids. These Cry protoxins are then cleaved by insect midgut proteinases to form protease-stable active Cry toxins (Bravo et al., 2002; Choma and Kaplan, 1990). The activated Cry protein then binds to

specific receptors at the midgut epithelium (Pigott and Ellar, 2007). It is proposed that the cadherin, aminopeptidase N (APN), and alkaline phosphatase (ALP) proteins are involved in Bt toxin binding by interacting sequentially with different toxin structures. The monomeric toxin form first binds to a primary receptor, cadherin-like protein, to induce further proteolytic processing and the formation of Cry oligomers (Gomez et al., 2002). Oligomeric structures then bind to secondary receptors APN or ALP concentrated in membrane microdomains, resulting in insertion of the oligomers and pore formation (Arenas et al., 2010; Bravo et al., 2004). These pores induce osmotic imbalance leading to cell death, disruption of the insetinal epithelium, septicemia and ultimately the death of the insect (Knowles and Dow, 1993). An alternative model suggests that monomer binding to cadherin is sufficient to trigger an intracellular signal transduction pathway that leads to cell death without the involvement of the oligomeric form (Zhang et al., 2006). However, the construction of modified Cry toxins able to oligomerize independently of interacting with cadherin has demonstrated that monomer binding to cadherin is not sufficient for toxicity (Soberon et al., 2007).

1.4. Insect resistance to Bt

The extensive exposure to Bt pesticidal formulations and widespread planting of transgenic Bt crops place a strong selection pressure on pest populations. Therefore, observed evolution of resistance by target pests has jeopardized the continued success of Bt crops (Bravo and Soberon, 2008; Ferre and Van Rie, 2002; Gould, 1998; Tabashnik, 1994). Laboratory-selected resistance to Bt toxins has been detected in several insects, such as the Indianmeal moth, *Plodia interpunctella* (McGaughey, 1985), tobacco

budworm, *Heliothis virescens*, (Gould et al., 1992), diamondback moth, *Plutella xylostella* (Tabashnik et al., 1994a), beet armyworm, *Spodoptera exigua* (Moar et al., 1995), European corn borer, *Ostrinia nubilalis* (Huang et al., 1999; Siqueira et al., 2004), pink bollworm, *Pectinophora gossypiella* (Tabashnik et al., 2004), corn earworm, *Helicoverpa zea* (Anilkumar et al., 2008) and cotton bollworm, *Helicoverpa armigera* (Akhurst et al., 2003; Gunning et al., 2005).

Three lepidopteran insect pests have developed resistance to formulated Bt microbial insecticide sprays in greenhouse conditions, *P. interpunctella* (McGaughey, 1985), *P. xylostella* (Tabashnik, 1994), and the cabbage looper, *Trichoplusia ni* (Janmaat and Myers, 2003). More importantly, field-evolved resistance to commercial transgenic Bt crops resulting in field control failures or reduced efficacy has been documented for the fall armyworm, *Spodoptera frugiperda* resistant to Cry1F corn in Puerto Rico (Storer et al., 2010), the African stem borer, *Busseola fusca* resistant to Cry1Ab corn in South Africa (van Rensburg, 2007), and *P. gossypiella* resistance to Cry1Ac cotton in India (Dhurua and Gujar, 2011).

Theoretically, any changes in insect gut physiology and/or biochemistry that affect one or more steps in the mode of action of Bt toxins could interfere with toxicity and confer resistance. Alteration of midgut proteases, which are critically involved in solubilization and proteolytic processing of Cry proteins in the insect midgut, has been found in several Bt-resistant insect strains generated by laboratory selections (Li et al., 2004; Oppert et al., 1997). Modification of midgut binding sites for Cry toxins resulting in reduced toxin binding has been reported as the mechanism responsible for the highest

levels of resistance to Cry toxins (Estada and Ferre, 1994; Ferre et al., 1991a; Ferre and Van Rie, 2002; Tabashnik et al., 1994a). Alternative resistance mechanisms have been proposed, including retention of the Cry toxin by the midgut peritrophic matrix (Hayakawa et al., 2004), aggregation of Cry toxin proteins by midgut esterases (Gunning et al., 2005b), elevated melanization activity of the hemolymph and midgut cells (Ma et al., 2005; Rahman et al., 2004), increased rate of repair or replacement of affected epithelial cells (Martinez-Ramirez et al., 1999), and possibly increased antioxidation activities in Bt resistant insects (Candas et al., 2003). It is possible that mechanisms for Bt resistance in insects may be multifaceted.

Data from resistance studies suggest that binding of Cry toxins to the receptors present in the midgut brush border membrane is a critical step in the Cry toxin mode of action (Ferre and Van Rie, 2002). Alterations in the proposed Cry receptors has been shown to be linked or associated with resistance, including cadherin-like proteins (Gahan et al., 2001; Morin et al., 2003), APN (Tiewsiri and Wang, 2011), and ALP (Jurat-Fuentes et al., 2011). Furthermore, alteration of a common toxin binding site has been found in cases where insects evolved cross-resistance to toxins not present in the selection environment. Resistance management strategies rely on sequential or simultaneous use of toxins that recognize different binding sites. The most used method of predicting cross-resistance among Cry toxins involves determining if they share a common binding site in a given insect.

1.5. Cry toxin binding experiments

Analyses of Cry toxin binding to brush border membrane vesicles (BBMV) from target insects have been performed with different methods, including binding assays with radiolabeled toxin, surface plasmon resonance (SPR), binding assays with antisera or biotinylated Cry toxins in blots (Bravo et al., 1992; Gouffon et al., 2011; Hernandez and Ferre, 2005; Herrero and Ferre, 2001a; Jurat-Fuentes and Adang, 2001; Jurat-Fuentes et al., 2002; Karim et al., 2000; Sayyed et al., 2000). The non-radioactive methods are safe to use but limited to only qualitative detection of Cry toxin binding, whereas, methods with radiolabeled toxins are sensitive and can be used to measure qualitative and quantitative binding of Cry toxins. Radiolabeling of Cry toxins is achieved by incorporation of iodine-125 isotope into tyrosine residues of the toxin through oxidation (Hofmann et al., 1988). The most commonly used labeling method involves chloramine-T as oxidizing agent, which in some cases has been proposed to affect biological function of Cry toxins (Luo et al., 1999). The use of an increased ratio of toxin to iodine has been proposed to overcome potential effects of chloramine-T labeling (Hernandez-Rodriguez et al., 2012). Alternatively, Cry toxins can also be radiolabeled using weaker oxidizing conditions, such as in the case of iodo-beads (Pierce).

Non-radiolabeling methods that have been used to detect the binding of Cry toxins include the use of antisera or biotinylated Cry toxins in blots, and SPR. Surface plasmon resonance (SPR) detects changes in polarized light as reflected from the surface of a sensor chip on which the toxin or BBMV are attached. Since the amine coupling used to attach the toxin to the chip can affect toxin function, attachment of BBMV

proteins has been suggested as a preferred strategy (Luo et al., 1997). Attachment of the BBMV to the sensor also allows for the performance of binding competition experiments (Hua et al., 2001a; Li et al., 2004; Masson et al., 1996). Biotinylated Cry toxins have been used to perform binding assays by detection with streptavidin. This method cannot be used for quantitative measurement of Cry toxin binding to BBMV. Blotting methods with antisera or biotinylated toxins have also been used for qualitative estimation of Cry toxin binding to BBMV proteins and to identify the BBMV proteins interacting with Cry toxins (Garczynski, 1991; Karim et al., 2000; Knowles et al., 1991; Lee et al., 2006).

1.6. Procedures for review

The Agriculture Databases through Web of Knowledge and PubMed were searched for literature presenting data relevant to Bt toxins binding to BBMV from selected lepidopteran insect pests based on their economic importance. In the preparation of the present review, published papers on Bt toxins and BBMV binding experiments conducted with radiolabeled or non-radiolabeled toxins to study binding patterns, including homologous and heterologous binding competition assays to develop binding site models in BBMV of the target insect were considered. Nearly 50 papers fulfilled these requirements, of which 75% presented data from experiments using radiolabeled toxins. The other 25% of the publications presented data using biotinylated toxins, immunoblotting, or SPR to conduct binding studies. The models proposed in this review are derived from synthesizing the information presented in these publications. All the published competition assays was examined and elaborate on potential binding site models, although binding sites detected when using high concentrations of competitor

need to be taken cautiously due to the possibility of artifacts related to high toxin concentrations.

1.7. Binding site model in *Heliothinae* group

The Heliothinae subfamily of Lepidoptera contains some of the most crop-damaging insect pests worldwide, including *H. virescens, H. zea*, *H. armigera*, and *Helicoverpa punctigera*. Many reports identified Cry1A, Cry1F, Cry1J, Cry2A, and vegetative insecticidal proteins (Vip) as effective insecticidal toxins to control this group of insects (Chakrabarti et al., 1998; Lambert et al., 1996; Liao et al., 2002). Cry1A toxins display high affinity saturable binding, while Cry1Fa, Cry1Ja and Cry2A toxins bound saturably with a lower affinity, resulting in differences in bio efficacy these toxins against Heliothinae insects (Caccia et al., 2010; Karim et al., 2000).

Homologous competition assays using Cry1Aa, Cry1Ab, Cry1Ac, Cry1Fa, Cry2Aa, Cry2Ab, Cry2Ae and Vip3A toxins revealed that these toxins bind specifically to BBMV from larvae of *H. virescens, H. zea*, and *H. armigera* (Caccia et al., 2010; Hernandez and Ferre, 2005; Jurat-Fuentes and Adang, 2001). In these assays, Cry2Aa toxins displayed less specific binding compared to Cry1A or Cry1Fa toxin, (Hernandez-Rodriguez et al., 2008). Within the Cry1A toxins, the affinity constant (*Kd*) value for Cry1Ac was the lowest, evidence of tighter binding of this toxin, which is the most active against Heliothine larvae.

Heterologous competition experiments using Cry1A toxins revealed that while Cry1Aa binds to a unique site shared with Cry1Ab and Cry1Ac, Cry1Ab and Cry1Ac share an additional site and Cry1Ac also has a unique site (Estela et al., 2004; Vanrie et

al., 1989). Competition assays between Cry1A and Cry1Fa toxins suggest the existence of shared Cry1A-Cry1Fa sites and also the presence of sites not recognized by Cry1Fa (Gouffon et al., 2011; Hernandez and Ferre, 2005; Jurat-Fuentes and Adang, 2001). The Cry1Ja toxin also shares a binding site with Cry1Ab and Cry1Ac (Hernandez and Ferre, 2005; Karim et al., 2000), while Cry2A (Gouffon et al., 2011; Jurat-Fuentes et al., 2003) and Vip3A toxins (Lee et al., 2006) bind to unique sites. Toxins in the Cry2A family, including Cry2Aa, Cry2Ab and Cry2Ae, have been shown to share binding site in heliothine BBMV (Hernandez-Rodriguez et al., 2008). According to the proposed model from these data (Fig. 1) there are six of binding sites (receptors A, B, C, D, E, and F, respectively) for Cry1, Cry2A and Vip toxins. Receptor A binds the Cry1Aa, Cry1Ab, Cry1Ac, Cry1Fa, and Cry1Ja toxins; receptor B binds Cry1Ab and Cry1Ac; receptor C binds only Cry1Ac; receptor D binds only Cry1Fa; receptor E binds only Cry2A (Cry2Aa, Cry2Ab and Cry2Ae) toxins, and receptor-F binds only the Vip3A toxin.

Ligand blot analyses using Cry1A toxins and *H. virescens* BBMV revealed that receptor A could be composed of the cadherin (210-kDa protein) and N-aminopeptidases (APNs) (170, 130, and 110 kDa) proteins (Banks, 2001; Luo et al., 1997; Oltean et al., 1999). Cry1A toxins bind to *H. virescens* cadherin repeats 7, 11, (Fabrick and Tabashnik, 2007; Gomez et al., 2001; Hua et al., 2004; Xie et al., 2005) and 12, and to repeats 10 and 11 of *H. armigera* cadherin (Wang et al., 2005). A 130-kDa protein binding both Cry1Ab and Cry1Ac was proposed to constitute receptor B, while several proteins of less than 100-kDa in size specific for Cry1Ac have been suggested to constitute receptor C (Jurat-Fuentes and Adang, 2001). In this regard, alkaline

phosphatase (63-kDa glycoprotein) would be a candidate for receptor C based on its affinity for Cry1Ac (Jurat-Fuentes et al., 2002). An unidentified 49-kDa protein binding to Cry2Ab has been proposed to constitute receptor D (Caccia et al., 2010; Gouffon et al., 2011) and 80 and 100-kDa proteins binding to Vip3A could be proposed to represent receptor E (Lee et al., 2006).

Reports are available on Cry1Ac binding to BBMV being inhibited by N-Acetyl-galactosamine (GalNAc) (Knowles et al., 1991; Luo et al., 1997); in contrast, Cry1Ab binding has been found not to be inhibited by this sugar (Luo et al., 1997). This same pattern of inhibition was also found with sialic acid, indicating that Cry1Ac, but not Cry1Ab, requires sugar residues to bind to BBMV in *H. virescens* (Luo et al., 1997). The fact that, Cry1Ab and Cry1Ac share binding sites in competition experiments seems to be in contradiction to the binding inhibition results with sugars. Furthermore, results from ligand blots have generally shown that, under denaturing conditions, Cry1Ac and Cry1Ab bind to different BBMV proteins (Jurat-Fuentes and Adang, 2001; Oddou, 1993). This paradox could be explained by proposing that binding sites in BBMV may be oligomeric complexes of glycosylated membrane proteins (Knowles et al., 1991; Luo et al., 1997). Binding of Cry1Ac and Cry1Ab could take place through different epitopes of multimeric receptor and at the same time hinder binding of the heterologous toxin by impeding access to a nearby site. This hindering does not exclude the possibility that both toxins could also share identical epitopes, but in this case full binding might require anchorage of the Cry protein to both the shared and the non-shared epitopes (Sayyed et al., 2000).

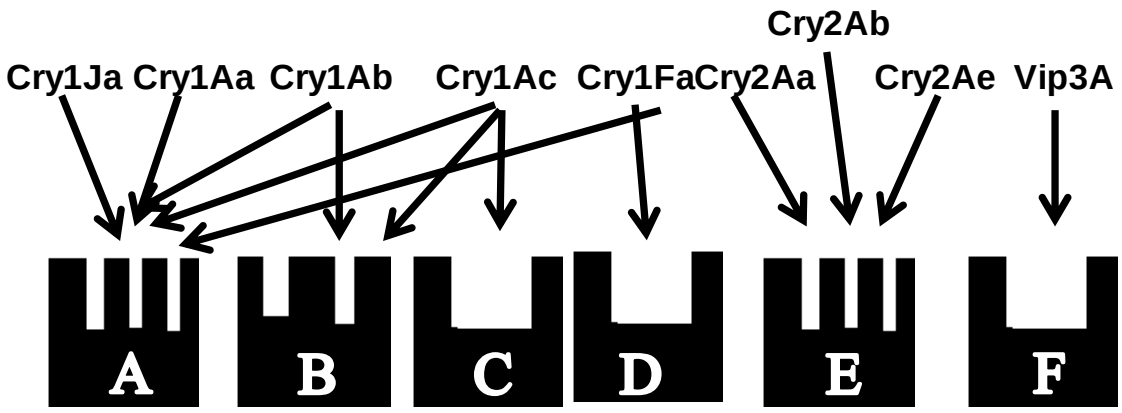


Fig.1. Model proposed for binding of *B. thuringiensis* toxins to sites on the larval midgut membrane in considered members of the *Heliothinae* group.

1.8. Binding site model in *Pectinophora gossypiella*

The pink bollworm, *P. gossypiella,* is the most destructive pest, after the Heliothinae group, of cotton worldwide. Previous studies demonstrated that Cry1Aa, Cry1Ab, Cry1Ac, Cry1Da, Cry1Ea, Cry1Ja, Cry2Aa, and Cry9Ca were highly toxic, while Cry1Bb and Cry1Ca were somewhat less toxic to larvae of *P. gossypiella* (Tabashnik et al., 2000). The same pattern of specificity is also confirmed using binding of these toxins in BBMV of *P. gossypiella* (Gonzalez-Cabrera et al., 2003;Herrero et al., 2001;Karim et al., 2000). Saturation binding assays with *P. gossypiella* BBMV suggested saturable and high affinity binding of Cry1Ab and Cry1Ac, while Cry1Aa and Cry2A toxins bound to BBMV with much lower affinity (Karim et al., 2000). Competitive heterologous binding assays with Cry1Aa toxin demonstrated that Cry1Ab and Cry1Ac recognize all the binding sites for Cry1Aa in *P. gossypiella* BBMV (Karim et al., 2000). When using labeled Cry1Ab, only Cry1Ac was able to compete for all the

Cry1Ab binding sites, suggesting the existence of a shared binding site for all Cry1A toxins and a second site only recognized by Cry1Ab and Cry1Ac. Furthermore, lack of full competition of labeled Cry1Ac binding by Cry1Ab, supported a third binding site unique for Cry1Ac (Karim et al., 2000). Qualitative competition tests with biotinylated Cry1Ac and unlabeled Cry1Ja competitor suggested that Cry1Ja shares binding sites with Cry1Ac (Herrero et al., 2001b). Heterologous competition assays in an alternative *P. gossypiella* strain supported the existence of shared Cry1A-Cry1Ja binding sites, although the low specific binding in these experiments prevented detection of binding to the Cry1Ac unique binding site (Gonzalez-Cabrera et al., 2003). Competition experiments with labeled Cry1Ab or Cry1Ac toxins showed they did not share binding sites with Cry1Ba, Cry1Ca, Cry2Aa, or Cry9Ca (Gonzalez-Cabrera et al., 2003). Ligand blot data showed that Cry1Ab binds to a cadherin (210-kDa) protein and Cry1Ac binds to an APN (120-kDa) protein in *P. gossypiella* BBMV (Morin et al., 2003). Based on available data a binding site model can be proposed with five binding sites (receptors A, B, C, D, and E, respectively) in BBMV from *P. gossypiella* interacting with Cry toxins (Fig. 2.) Receptor A binds the Cry1Aa, Cry1Ab, Cry1Ac, Cry1Fa and Cry1Ja toxins; receptor B binds Cry1Ab and Cry1Ac; receptor C binds only Cry1Ac; and receptor D binds only Cry2A (Cry2Aa, Cry2Ab andCry2Ae) toxins.

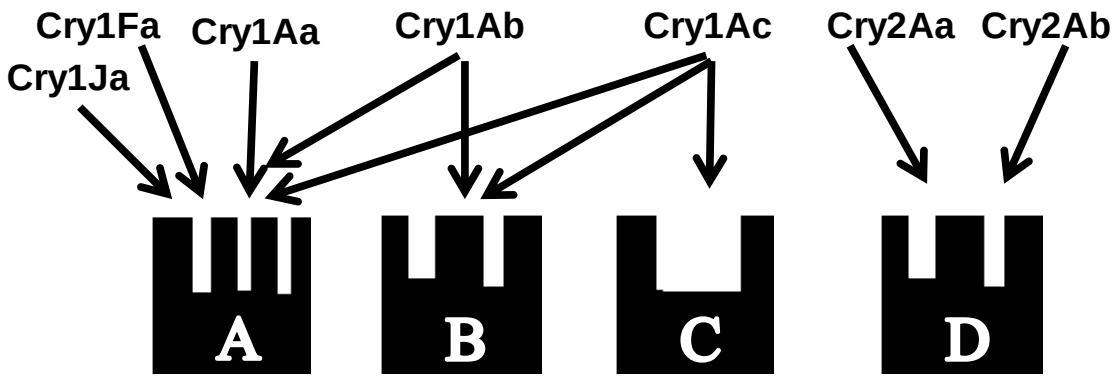


Fig. 2. Model proposed for binding of *B. thuringiensis* toxins to sites in the larvae midgut membranes of *P. gossypiella*.

1.9. Binding site model in *Spodoptera* species

The *Spodoptera* genus contains species that are worldwide pests of many economical important crops, and their highly polyphagous feeding behavior also makes them damaging secondary pests on alternative plant hosts. Relevant species include *Spodoptera frugiperda, S. exigua, S. littoralis,* and *S. litura*. Data from bioassays demonstrated that Cry1Bb, Cry1Ca, Cry1Da, Cry1Fa, and Vip3A toxins are highly active, while Cry1Ja and Cry2A are marginally active and Cry1A and Cry1E toxins are reported to be not effective against larvae of *Spodoptera* spp. (Bai et al., 1993; Luo et al., 1999; van Frankenhuyzen, 2009). Based on this information, transgenic crops producing Cry1Fa toxin were commercialized to control *Spodoptera* species. Effects of radiolabeling on Cry1Fa toxin prevented the use of this method to examine Cry1Fa binding to BBMV (Luo et al., 1999) until recent modifications of the iodination protocol were demonstrated to allow detection of specific Cry1F binding to BBMV (Hernandez-Rodriguez et al., 2012).

Homologous competition assays demonstrated that Cry1Fa binds with high affinity to *S. frugiperda* BBMV (Hernandez-Rodriguez et al., 2012). Heterologous competition assays using radiolabeled Cry1Ac toxin demonstrated that Cry1Fa recognizes all the Cry1Ac binding sites in *S. frugiperda* and *S. exigua* BBMV (Luo et al., 1999). Sharing of Cry1Fa and Cry1Ac binding sites was also confirmed using biotinylated Cry1Fa toxin and *S. exigua* BBMV (Hernandez and Ferre, 2005). This observation contradicts the toxicity of these proteins towards these insects, as Cry1Ac displays marginal activity against *Spodoptera* larvae. A plausible explanation for this observation may be that binding of Cry1Ac to a non-functional receptor creates steric hindrance and prevents binding of Cry1Fa to its receptor (Luo et al., 1999).

Both Cry1Ca and Cry1Bb toxin bound with high affinity to *S. frugiperda* and *S. exigua* BBMV (Luo et al., 1999; Rang et al., 2004). In heterologous competition assays with BBMV from the same insects both Cry1Ca and Cry1Fa competed radiolabeled Cry1Bb binding, although a unique population of binding sites for Cry1Bb was also detected. This population of binding sites is only detected when using high concentrations of competitor, suggesting that it is a low affinity site for Cry1Bb binding. In case of Cry1Ca, all its binding sites were recognized by Cry1Fa, while Cry1Bb was unable to compete Cry1Ca binding to a site observed when using high competitor concentrations, indicative of low affinity (Luo et al., 1999; Rang et al., 2004) . Reports using iodinated or biotinylated toxins have demonstrated lack of competition between Cry1Ca and Cry1A toxins in *Spodoptera* BBMV (Rang et al., 2004) or midgut sections (Aranda et al., 1996).

Binding experiments using radiolabeled Cry1Ab and BBMV from *S. exigua* revealed the existence of specific high affinity binding sites (Sena et al., 2009). In contrast, binding experiments using biotinylated Cry1Ab and midgut sections of *S. frugiperda* larvae suggested that binding of this toxin is non-specific (Aranda et al., 1996). Binding of Cry1Ac to *Spodoptera* BBMV has also been reported to be of high affinity (Garczynski, 1991;Hernandez and Ferre, 2005; Luo et al., 1999), although this toxin displays marginal toxicity towards these insects. Heterologous competition studies with ^{125}I-Cry1Ab and unlabeled Cry1Aa and Cry1Ac showed that all three toxins share a common binding site, and that Cry1Ac recognized all the Cry1Ab binding sites in *S. exigua* BBMV (Escriche et al., 1997). Heterologous competition between labeled Cry1Ac and unlabeled Cry1Ab suggests the potential existence of a small population of low affinity binding sites unique for Cry1Ac toxin (Rang et al., 2004). In contrast, lack of full competition of Cry1Ab binding by Cry1Aa suggests the existence of a population of Cry1Ab-Cry1Ac binding sites that is not recognized by Cry1Aa, although additional competition assays with labeled Cry1Aa toxin are needed to confirm this hypothesis. Data from heterologous competition with unlabeled Cry1Fa or Cry1Ja support that, these toxins bind to all the Cry1Ab binding sites in *Spodoptera* spp. (Hernandez and Ferre, 2005; Luo et al., 1999), although activities of these toxins against *Spodoptera* larvae are drastically different.

Binding interactions between biotinylated Cry1D toxin and histological midgut sections from *S. frugiperda* larvae revealed the specific binding of Cry1Da (Aranda et al., 1996). Although this toxin is very active against *Spodoptera* larvae, no information from

heterologous competition binding studies is available to place this toxin in the current binding site model. Competition binding experiments conducted with BBMV from *S. frugiperda* using radiolabeled Cry1Ea and unlabeled Cry1Ac, Cry1Ba, Cry1Ca, and Cry1Ea revealed that Cry1Ea toxin binds specifically to a unique binding site which is not shared with any of the other tested toxins. (Rang et al., 2004).

Data for binding of Vip3A toxin to BBMV from *Spodoptera* spp. is available from indirect binding competition tests. Competition binding assays using biotinylated Cry1Fa or radiolabeled Cry1Ab revealed that Vip3Aa and Vip3Af toxins do not share binding sites with Cry1Fa or Cry1Ab on *S. frugiperda* BBMV (Sena et al., 2009). In the same work, Vip3Aa and Vip3Af toxins were shown to bind specifically to the *S. frugiperda* BBMV and share binding sites.

Based on the available data, a binding site model can be proposed consisting of six different binding sites in BBMV from *Spodoptera* spp. larvae (Fig. 3). The first site (site A) would bind Cry1A (Cry1Aa, Cry1Ab, Cry1Ac), Cry1Fa and Cry1Ja toxins, while Cry1Aa has a unique binding site B not shared with other Cry1 toxins. Cry1Bb and Cry1Ca share binding site C, and Cry1Bb has an additional binding site D not shared with any of the tested toxins. In contrast to this model, a report from Luo et al., (1999) suggested that Cry1Fa shared binding sites with Cry1Ca, although this sharing has not been observed by alternative multiple authors (Aranda et al., 1996; Rang et al., 2004), suggesting that the results from Luo et al., (1999) may be artifactual. In our model Cry1Ea binds to site E and Vip3Aa and Vip3Af toxins share population of binding sites F, which is exclusive for Vip3A toxins.

Ligand blot analyses of Cry toxin binding to BBMV proteins from *Spodoptera* spp revealed proteins of 200-kDa and 180-kDa that bound Cry1Aa and Cry1Ab in *S. exigua* BBMV, while the same toxins recognized a 150-kDa protein in BBMV from *S. litura* (Oddou, 1993). In contrast, Cry1Ac bound to 130-kDa and 115-kDa proteins in BBMV of *S. frugiperda* and to a 125-kDa protein in BBMV from *S. littura* larvae. Using radiolabeled Cry1Ac, Garczynski et al. (1991) reported binding of Cry1Ac to a 148-kDa protein in BBMV from *S. frugiperda*. According to our model these unidentified proteins would represent binding site A, although no common protein bands were observed for all three Cry1A toxins. This inconsistency between results from BBMV binding assays and ligand blots have been reported previously (Daniel et al., 2002) and are probably due to the denaturing conditions of ligand blots.

A fragment encompassing the membrane proximal region of a cadherin from midgut membranes of *S. frugiperda* was reported to bind Cry1Fa toxin (Abdullah et al., 2009). A similar fragment from a homologous cadherin from *S. exigua* was shown to enhance toxicity of Cry1C and Cry1B toxins (Lu et al., 2012), although no binding data was provided. The Cry1Ca toxin has also been reported to bind to a protein of low molecular mass (40-44 kDa) in BBMV from *S. littoralis* (Sanchis and Ellar, 1993). The only Cry1Ca receptor with published functional data is a 108-kDa aminopeptidase (APN) from *S. litura* (Agrawal et al., 2002), since silencing expression of this APN resulted in reduced susceptibility to Cry1Ca (Rajagopal et al., 2002). This protein is a candidate for binding site C, although binding of Cry1B or Cry1Fa has not been tested to date.

Although no binding competition assays have been reported using Cry1Da toxin, this toxin was reported to bind to an unidentified 65-kDa protein in *S. littoralis* midgut (BenFarhat-Touzri et al., 2013). Two BBMV proteins of 55 and 100-kDa from *S. littoralis* were shown to bind biotinylated Vip3Aa16 toxin (Abdelkefi-Mesrati et al., 2011), suggesting that they may represent binding site F.

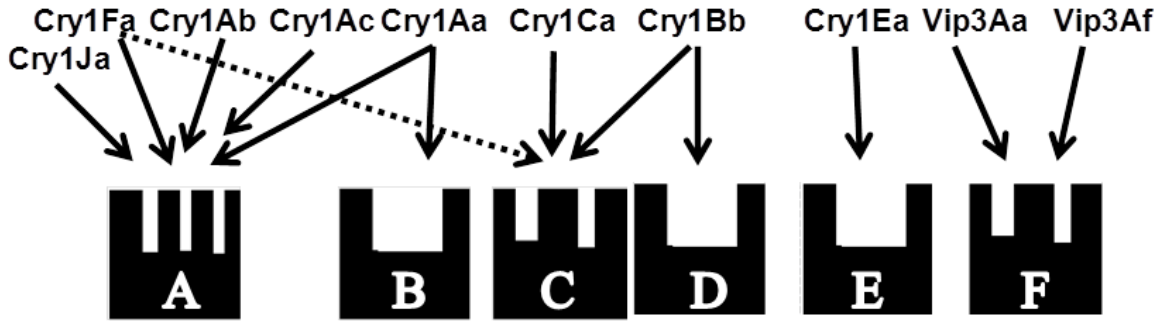


Fig. 3. Model proposed for binding of *B. thuringiensis* toxins to sites in the larvae midgut membranes of *Spodoptera* spp. larvae midgut membrane. Binding proteins correlated with specific sites are listed. Dashed arrows indicate contradiction among authors.

1.10. Binding site model in *Plutella xylostella*

Diamondback moth is a major pest of crucifer crops worldwide and is reported to develop resistance to many groups of insecticides, including Bt pesticides (Ferre et al., 1991;Sayyed et al., 2000; Tabashnik et al., 1997b). Several Cry toxins (Cry1Aa, Cry1Ab, Cry1Ac, Cry1B, Cry1Ca, Cry1Fa and Cry1Ja) from Bt have been reported to kill larvae of *P. xylostella* with high efficacy (Ballester et al., 1994;Mohan and Gujar, 2002; Sayyed et al., 2008).

Binding assays of Cry toxins with BBMV from *P. xylostella* confirmed the existence of specific binding for Cry1A, Cry1Fa, Cry1Ja, Cry1B, and Cry1Ca toxins (Ballester et al., 1999;Ferre et al.,1991;Higuchi et al., 2007;Sayyed et al., 2008). Binding patterns of Cry1Aa toxins revealed that this toxin binds to a high-affinity and a low affinity binding site in BBMV of *P. xylostella.* Heterologous competition observed among Cry1A toxins revealed that Cry1Ab and Cry1Ac compete binding of labeled Cry1Aa, although binding to Cry1Aa-specific binding sites is also detected. The results obtained in the reciprocal binding of ^{125}I-Cry1Ab and ^{125}I- Cry1Ac indicated that these toxins bind to one of two Cry1Aa binding sites (Ballester et al., 1999). Heterologous competition of ^{125}I-Cry1Ab and ^{125}I-Cry1Ca binding revealed that Cry1Fa shared binding sites with Cry1Ab but not with Cry1Ca (Granero et al., 1996). Further binding competition assays with radiolabeled Cry1Aa, Cry1Ab, and Cry1Ac confirmed that Cry1F shares Cry1A binding site and that Cry1B and Cry1Ca recognized alternative binding sites (Ballester et al., 1999). Lack of shared binding sites between Cry1Ba and Cry1Ca was reported from heterologous competition assays with radiolabeled Cry1Ca (Ferre et al., 1991).

An integrative model for the Cry toxin binding sites in *P. xylostella* developed from the published data is shown in Fig. 4. According to this model, *P. xylostella* contains at least four binding sites involved in binding of Cry1 toxins. Cry1Aa, Cry1Ab, Cry1Ac toxins, Cry1Fa and Cry1Ja compete for binding to a common binding site A, while Cry1Aa binds with low affinity to this shared site and with high affinity to a Cry1Aa-specific binding site B. Both Cry1B and Cry1C each bind to specific binding

sites C and D, respectively, which are not shared with other toxins. This model is supported by the fitting of the homologous competition data for Cry1Aa to a two-site model, whereas the homologous competition data for Cry1Ab, Cry1Ac, and Cry1C, Cry1B fits a one-site model (Ballester et al., 1999). Ligand blot experiments showed that Cry1Aa, Cry1Ab, and Cry1Ac bound to 105-kDa and 52-kDa proteins in *P. xylostella* BBMV, while Cry1Aa recognized a 117-kDa protein and both Cry1Ab and Cry1Ac bound to a 97-kDa protein (Higuchi et al., 2007). Western blotting with specific antisera identified the 105-kDa protein as an APN, suggesting that this protein may represent binding site A in the model. Antisera against cadherin and a midgut membrane protein (P252) were shown to reduce Cry1Ac, but not Cry1Aa or Cry1Ab, binding to *P. xylostella* BBMV, which would suggest the potential existence of Cry1Ac-specific binding sites not supported by competition binding assays.

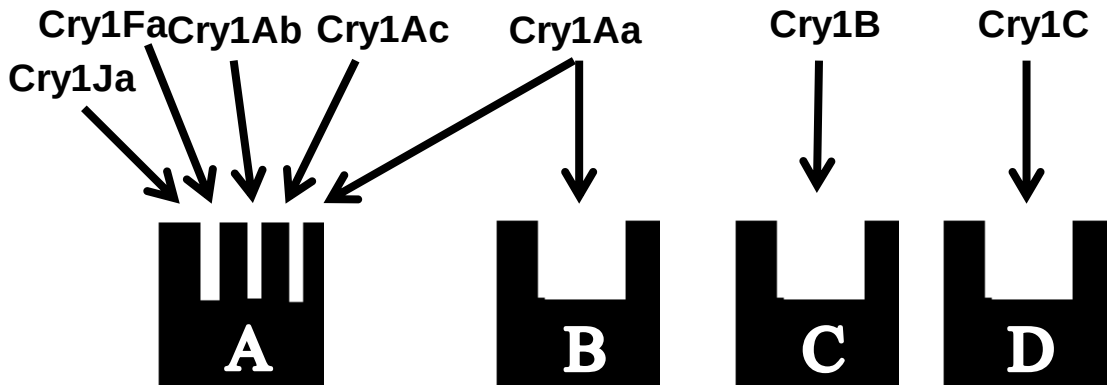


Fig. 4. Model proposed for binding of *B. thuringiensis* toxins to sites in the larval midgut membrane at *P. xylostella*.

1.11. Binding site model in *Ostrinia nubilalis*

The European corn borer is one of the most important damaging pests in corn. A number of Cry toxins from Bt, namely Cry1Ab, Cry1Ac, Cry1B, Cry1Fa, Cry9C, and Cry9E, were reported to kill larvae of *O. nubilalis* (van Frankenhuyzen, 2009). Binding experiments using radiolabeled Cry1Ab and Cry1Ac confirmed that these toxins bind specifically to *O. nubilalis* BBMV (Denolf et al., 1993). Results from heterologous binding suggested the existence of a shared Cry1Ab-Cry1Ac binding site for which higher binding affinity for Cry1Ab correlates with higher activity of this toxin to larvae of *O. nubilalis* (Denolf et al., 1993; Li et al., 2004). Competition experiments performed using histological midgut sections and biotinylated Cry1Ab demonstrated that this toxin does not share binding sites with Cry1B (Denolf et al., 1993). Homologous binding competition assays with radiolabeled Cry1Ab revealed specific and saturable binding of

this toxin to *O. nubilalis* BBMV (Hua et al., 2001). Heterologous competition experiments confirmed that Cry1Ab and Cry1Ac bind to common sites, and that part of these sites are recognized by Cry1Aa (Crespo et al., 2011). In contrast, Cry1B could not compete with Cry1Ab toxin binding, supporting the observations from the histological sections (Denolf et al., 1993). In binding assays using Cry1Fa and Cry1Ab against BBMV from *O. nubilalis,* competition was only observed when using high concentrations of Cry1Fa, suggesting that the Cry1Ab sites are low affinity binding sites for Cry1Fa. In support of this hypothesis, binding competition analysis using SPR revealed the existence of a high and a low affinity binding site for Cry1Fa in *O. nubilalis* BBMV (Hua et al., 2001). In a field-derived strain of *O. nubilalis* resistant to Cry1Ab only Cry1Aa binding was reduced, while binding of Cry1Ab and Cry1Ac, or Cry1Fa toxins were unchanged (Crespo et al., 2011). These results suggest the existence of a population of binding sites shared by Cry1A toxins that are not relevant to Cry1Fa toxicity. Competition assays between Cry1Aa and Cry1Fa would be needed to test whether these toxins share binding sites. Lack of significant competition between radiolabeled Cry1Ab and Cry9 toxins suggests that these toxins have independent binding sites in *O. nubilalis* BBMV (Hua et al., 2001).

Based on the available Cry toxin binding data a five binding site model can be proposed for BBMV of *O. nubilalis* (Fig. 5). A population of binding sites is shared between all Cry1A (Cry1Aa, Cry1Ab and Cry1Ac) toxins (site A), while Cry1Ab and Cry1Ac also share a second population of binding sites (site B) that is probably recognized as a low affinity binding site for Cry1Fa. A high affinity binding site for

Cry1Fa would represent site C. A fourth group of binding sites (site D) binds to Cry1Ba, and the fifth group of binding sites (site E) is recognized by Cry9C and Cry9E toxins.

Ligand blotting analyses to identify Cry binding proteins in BBMV of *O. nubilalis* revealed that Cry1Ab, Cry1Ac and Cry1Fa bind to proteins of approximately 154-kDa and 220-kDa identified through immunoblots as aminopeptidase N and cadherin, respectively (Hua et al., 2001; Pereira et al., 2010). In addition, Cry1Ab also recognized 145-kDa and 167-kDa proteins identified as APNs by immunoblotting. In an alternative report, cadherin protein bands of 220-, 170-, and 160-kDa were identified to bind Cry1Ab toxin in ligand blots with *O. nubilalis* BBMV (Flannagan et al., 2005). The cadherin and APN receptors recognized by Cry1Fa and Cry1Ab/Cry1Ac could be considered as part of binding site B in proposed model.

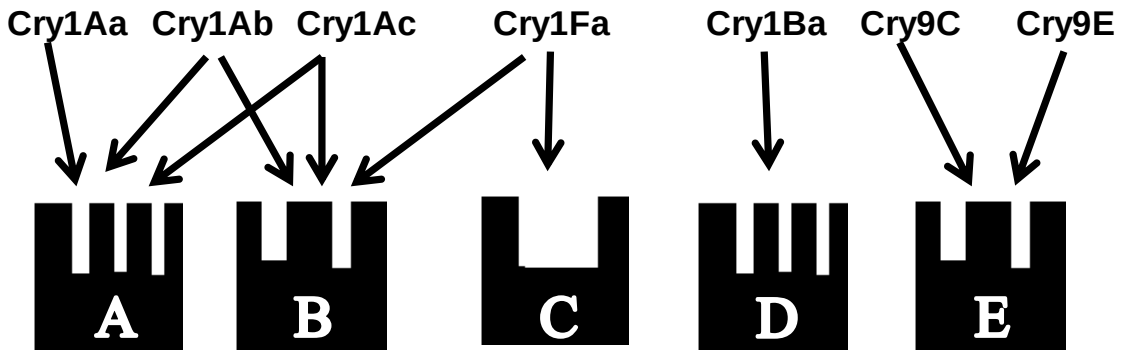


Fig. 5. Model proposed for binding of *B. thuringiensis* toxins to sites in thelarval midgut membranes of *O. nubilalis*.

1.12. Conclusions

B. thuringiensis (Bt) is the most widely used biological insecticide and produces diverse insecticidal toxins during its development. These toxins are highly toxic to some insects, and harmless to most other organisms. Binding of Bt toxins to specific receptor sites on the epithelial membrane is a key step in toxin specificity to kill target insects. A common feature in binding specificity in Lepidoptera is sharing of a binding site between Cry1Aa, Cry1Ab, and Cry1Ac toxins. In most cases, at least some of these binding sites are also shared with Cry1Fa and Cry1Ja toxins. These observations suggest that these toxins should not be combined in transgenic crops due to increased risk of cross-resistance. Although there is a report on the contrary sharing binding of Cry1Ca and Cry1A in *Spodoptera* spp., in all insects examined Cry1A and Cry1Ca toxins do not share binding sites. At least in *Spodoptera* spp. Cry1Ba and Cry1Ca share some binding sites, although this phenomenon has not been observed in other species. Unique Cry2A and Vip3A toxins are commonly described. These conclusions have important implications for resistance management. Since alteration of binding sites is the most common mechanism resulting in high levels of resistance, data on Cry toxin binding site models are critical to determine toxins that are safe for use in combination in transgenic crops (pyramiding). These pyramiding crops can control insect pests very effectively and delay resistance for an extended period. Based on the binding site models developed in this review combinations of toxins may be suggested for effective control and to delay resistance in target pests (Table 1). These combinations of toxins expressed in crops

could also be used to control the insects that had already developed resistance to Cry toxins.

Table 1. Combinations of two Cry toxins proposed for gene pyramiding in novel transgenic crops, based on binding site models proposed in this review.

Target insect species	**Combination of Cry toxins**
Heliothinae	Cry1Ac+Cry2A
	Cry1Ac+ Vip3A
	Cry2Ab+Vip3Aa
P. gossypiella	Cry1Ac+Cry2A
Spodoptera spp.	Cry1Fa+Cry1C/Cry1B
	Cry1C/Cry1B+Cry1Ab
	Cry1Fa +Vip3A
P. xylostella	Cry1Ac+Cry1B
	Cry1Ac+Cry1Ca
O. nubilalis	Cry1Fa+Cry1B
	Cry1Ab+Cry1B
	Cry1Fa+Cry9

CHAPTER II

Reduced Cry1Fa toxin binding to midgut alkaline phosphatase is the mechanism for field-evolved resistance to Bt maize in the fall armyworm, *Spodoptera frugiperda* (J. E. Smith).

2.1. Abstract

Transgenic Bt crops expressing Cry and Vip toxins from *B. thuringiensis* have been increasingly planted to manage insect pest damage on agricultural crops. However, augmented adoption of Bt crops has resulted in field-evolved resistance in at least nine insect species to Bt maize and Bt cotton, indicating the long-term use of Bt technology is in jeopardy. Diversified resistance mechanisms have been reported in laboratory selected insects with varied methods of selection, but resistance mechanisms in field-evolved insects remains unclear due to unavailability of field-evolved resistant insects. In the present study the resistance mechanism in *Spodoptera frugiperda* (J. E. Smith) with field-evolved resistance to maize producing Cry1Fa toxin was characterized. Resistance in strain 456 *S. frugiperda* was continued to be recessive and autosomal trait. Binding experiments showed the reduced binding of Cry1Fa toxin to brush border membranes from resistant compared to susceptible larvae. The same binding reduction was observed for Cry1A toxins, but not for Cry1Ca toxin, suggesting modification of a common Cry1Fa-Cry1A toxin binding site. Comparison of protein levels among putative aminopeptidase (APN) and alkaline phosphatase (ALP) receptors revealed highly reduced ALP levels but not changes in APN levels comparing resistant and susceptible larvae. The present work represents the first characterization of a field-evolved resistance mechanism to Bt toxins in target insects, and it illustrates a direct association between resistance and reduced Cry1Fa toxin binding due to reduction of ALP protein levels controlled at the transcriptional level.

2.2. Introduction

Bacillus thuringiensis (Bt) is a gram positive bacterium that produces crystalline inclusions composed of insecticidal Cry and Vip proteins. These Cry proteins are selectively insecticidal to larvae from various insect orders (Pigott and Ellar, 2007). Currently, more than 220 Cry proteins have been described based on their amino acid sequence similarities from the various Bt strains collected across the world (Crickmore, 2012). Several Bt strains have been used as biological insecticides to manage some economic pests on agricultural crops and public health (Gould, 1998). The mode of action of Cry toxins in target insects starts by the ingestion and solubilization of the crystalline inclusions to release as protoxins in larval gut. Then protoxins are cleaved by the action of insect enzymes to produce active toxin in alkaline midgut conditions. Activated toxin travels through the peritrophic matrix and binds to receptors on the brush border membrane of the insect midgut epithelium (Bravo et al., 2007). It was proposed that the cadherin-like, (APN) and (ALP) proteins are involved in toxin binding by interacting sequentially with different toxin structures (Pigott and Ellar, 2007). The monomeric toxin form binds to the cadherin-like proteins (primary receptors) to induce further proteolytic processing and formation of oligomeric toxin (Gomez et al., 2002). The oligomeric toxins then bind to APN or ALP (Arenas et al., 2010; Pardo-Lopez et al., 2006) that drive the proteins into the membrane microdomains to cause pore formation (Bravo et al., 2004). The formation of the insertion pores results in osmotic imbalance of the membrane epithelium leading to osmotic cell lysis, swelling of the intestine cells and

death of the gut cells (Knowles and Dow, 1993). After enterocyte death, bacteria in the gut lumen are able to invade the insect hemocoel, causing septicemia and ultimately insect death (Broderick et al., 2006).

Transgenic Bt crops expressing one or multiple Bt toxins to manage a wide range of insect pests have been commercialized since 1996. These transgenic Bt crops provide efficient management of target insects without harming non-target organisms and the environment (Betz et al., 2000). The increased Bt crops usage represents an elevated selection pressure on target insects, which has recently resulted in field evolved resistance in *P. gossypiella* to Cry1Ac cotton from India (Dhurua and Gujar, 2011), *S. frugiperda* to Cry1Fa maize in Puerto Rico (Storer et al., 2010b), *B. fusca* to Cry1Ab maize in South Africa (van Rensburg, 2007) and *D. v. virgifera* to Cry3Bb1 maize in the U.S. (Gassmann et al., 2011).

Any alterations in insect midgut physiology and/or biochemistry that affect one or more steps in the mode of action of Bt toxins may confer resistance. While some clues on the different possible resistance mechanisms have been obtained using laboratory selected insect strains (Ferre et al., 1991b; Gahan et al., 2010; Gunning et al., 2005; Hayakawa et al., 2004; Li et al., 2004; Rahman et al., 2004), the resistance mechanism resulting under field conditions is not known. The most reported resistance mechanism in laboratory-generated strains is the alteration of Cry toxin binding to the receptors present in brush border membrane (Ferre and Van Rie, 2002). Modification or loss of the proposed membrane receptors, including cadherin-like proteins (Gahan et al., 2001; Morin et al., 2003), APN (Tiewsiri and Wang, 2011), and ALP (Jurat-Fuentes et al.,

2011) has been associated with resistance to Bt toxins. However, numerous factors including continuous selection with high Cry toxin levels, production of activated Cry toxins in plants, and plant secondary metabolites and Cry toxin interactions may contribute to differences in resistance mechanisms selected under laboratory and field conditions.

The present study was aimed to identify the field-evolved resistance mechanism in a strain of *S. frugiperda* (strain 456) associated with transgenic maize expressing Cry1Fa toxin (event TC1507) in Puerto Rico (Blanco et al., 2010). Resistance to TC1507 in this *S. frugiperda* strain was characterized using Cry1Fa toxin efficacy assays, Cry toxin binding, receptor expression assays and linkage analysis. High levels of resistance to Cry1Fa and Cry1Ab toxins were found but not to Dipel or Cry1Ca in larvae from the 456 strain. Resistance was transmitted as a recessive autosomal trait. Results from binding experiments showed reduced binding of Cry1Fa and Cry1A toxins correlated with resistance. Furthermore, reduced Cry1Fa toxin binding levels were associated with reduced levels of ALP, and phenotypic linkage between reduced ALP levels and resistance to transgenic Bt maize was confirm. These data represent the first characterization of a mechanism responsible for field-evolved resistance to a transgenic Bt crop.

2.3. Materials and Methods

2.3.1. Plant culture

Transgenic Bt event TC 1507 expressing Cry1Fa and isogenic non Bt event maize seed were supplied by Dow AgroSciences (Indianapolis, IN). Transgenic Bt maize Mon 810 expressing Cry1Ab and its isogenic line were provided by Dr. Blanco (Southern

Insect Management Research Unit, USDA-ARS-MSA, Stoneville, MS 38776, USA). These Bt and non Bt plants were grown in the greenhouse at 16:8 (L:D) photoperiod, 24± 2 °C and 50% RH conditions. Three to four seeds from each event were sowed in a 20 cm diameter pot containing garden soil and maintained in a greenhouse under 26 ± 2°C, 65 ± 5% RH and 16:8 (L:D) photoperiod.

2.3.2. Insect strains

For the present study a resistant (456), susceptible (Benzon) and two F_1 heterozygote strains of *S. frugiperda* were used to characterize the resistance mechanism. The susceptible strain (Benzon) was generated using egg masses purchased from Benzon Research (Carlisle, PA), and it was maintained by rearing the neonates on non Bt isogenic TC1507 maize (provided by Dow AgroSciences, Indianapolis, IN) V6-V7 leaf tissue until third instar and then transferred to meridic diet (BioServ) until larval stage completion. The resistant *S. frugiperda* strain was received from Dr. Blanco laboratory (Southern Insect Management Research Unit, USDA-ARS-MSA, Stoneville, MS 38776, USA). This strain was developed from egg masses collected in maize fields from the Isabela region in Puerto Rico. This resistant *S. frugiperda* strain displayed more than 7,500 fold resistance to Cry1Fa toxin compared to susceptible strains (Blanco et al., 2010). Neonates from strain 456 eggs were reared on V6-V7 stage TC1507 maize leaf tissue until third instar, and then transferred to meridic diet until completion of larval stage. Two F_1 heterozygote strains were created by crossing reciprocally between resistant and susceptible *S. frugiperda* adults. These hybrids strains were named as 456M (456♂XBenzon♀) and 456F (456♀X Benzon♂). All the four strains were maintained in a incubator with 26±2 ºC temperature, 65%± 5% RH and 16:8 (L:D) photoperiod.

2.3.3. Toxin production and purification

Two strains Bt strain HD-73 producing Cry1Ac obtained from *Bacillus* Genetic Stock Center (Colmbus, OH, USA), and a Bt strain producing Cry1Ab toxin (kindly provided by Dr. Luke Masson), were used to produce toxins used in the present study. Purified Cry1Fa and Cry1Ca toxins were provided by Dow AgroSciences (Indianapolis, IN). Production and purification of all four toxins were done according to protocols described elsewhere (Herrero et al., 2004; Jurat-Fuentes and Adang, 2001b). Briefly, crystals were solubilized in 50 mM carbonate buffer pH 10 containing 0.1 % 2-βmercaptoethanol. Solubilized protoxin was activated by incubation with midgut fluids (1% v/v) obtained from dissected fourth instar *S. frugiperda* larvae. Activated toxins were loaded on a HiTrap HP Q anion exchange column (GE Healthcare) pre-equilibrated with Buffer A (50 mM Na_2CO_3 pH 9.8). Purified toxins were eluted from the column using a linear gradient of 1M NaCl. Toxin containing fractions were verified by SDS-10% PAGE electrophoresis, and toxin concentration was determined using the method of Bradford (Bradford, 1976) with BSA as standard. Toxin samples were stored at –80°C until used.

2.3.4. Labeling of Cry toxins

Purified Cry1Ab, Cry1Ac, Cry1Ca, and Cry1Fa toxins were radio-labeled with iodine-125 using the chloramine-T method as described previously (Van Rie et al., 1989). For Cry1Ab, Cry1Ac, and Cry1Ca radio-labeling 1µg of pure toxins were incubated with 0.5 mCi of iodine-125 (PerkinElmer), whereas pure toxin of Cry1Fa 20 µg was used to avoid reduced Cry1Fa biological activity due to iodination (Hernandez-Rodriguez et al., 2012). In a typical labeling reaction, 0.5 mCi of iodine-125 was added to the above

mentioned toxin concentrations in presence of 18 mM chloramine T (1/3 v) in PBS pH 7.5 and incubated for 60 seconds. The reaction was stopped by adding 23 mM potassium metabisulfite (1/4 v) in water and added 1M NaI (1/4 v) to quench the reaction. The mixture was loaded onto a PD-10 column (GE Life Sciences) equilibrated with buffer column (20 mM Tris-HCl pH 8.65, 150 mM NaCl, 0.1% BSA) and eluted in twenty 0.5 ml fractions. Fractions containing radiolabeled toxin were detected by measuring radioactivity in aliquots (5 µl) from each eluted fraction in a Wizard-2 automatic gamma meter (Perkin Elmer). The presence of radiolabeled toxin was confirmed by separating proteins in elution fractions by SDS-10%PAGE gel and exposing the dried electrophoresis gels to photographic film at -80°C for diverse times until bands were detected in the developed photographic film (Gouffon et al., 2011). The specific activities of Cry toxins were calculated based on the input toxin and the total sample radioactivity. Specific activities were 3.49 mCi/pmol for Cry1Ab, 2.02 mCi/pmol for Cry1Ac, 0.36 mCi/pmol for Cry1Ca and 0.036 mCi/pmol for Cry1Fa.

2.3.5. Biotinylation of Cry toxins

Biotinylation of Cry1Fa and Cry1Ca toxins was performed using EZ-Link NHS-LC-Biotin (Pierce) as described elsewhere (Jurat-Fuentes and Adang, 2001a). After labeling, free biotin was eliminated from the toxin sample by extensive dialysis and samples quantified as for purified toxins. Biotinylation was then evaluated (data not shown) using Western blots probed with streptavidin conjugated to horseradish peroxidase (HRP).

2.3.6. Brush border membrane vesicle (BBMV) preparation

Midguts were dissected from fourth instar larvae to prepare BBMV by the differential magnesium precipitation method (Wolfersberger et al., 1987), but substituting mannose for sucrose in all solutions (Jurat-Fuentes et al., 2002). Briefly, midguts homogenized in SET buffer (250 mM sucrose, 17 mM Tris [pH 7.5], 5 mM EGTA), containing a protease inhibitor cocktail (Complete, Roche). An equal volume of $MgCl_2$ buffer (24 mM $MgCl_2$ +250 mM sucrose) was added to homogenates and incubated on ice for 15 min. The mixture was centrifuged at 2,500 x *g* for 15 min and the supernatant was collected and centrifuged at 27,000 x *g* for 30 min. The resulting pellet was suspended in one and half the original volume of SET buffer and $MgCl_2$ buffer and then the centrifugation cycle repeated. The final BBMV pellet was re-suspended in ice-cold PBS buffer (135 mM NaCl, 2 mM KCl, 10 mM Na2HPO4, 1.7 mM KH2PO4, pH 7.5) and the protein concentration determined using the Bradford assay (Bradford, 1976). Purity of BBMV preparations was determined by estimating the specific activities of APN or/and ALP in initial midgut tissue homogenates and final BBMV (Jurat-Fuentes and Adang, 2004). Specific activities of APN and ALP were measured using leucine p-nitroanilide, and *p*-nitrophenyl phosphate disodium (*p*NPP) as substrates, respectively (Terra and Ferreira, 1994). Specific activities for APN and ALP were enriched 6-8 and 4-6 fold, respectively, in the BBMV preparations compared to those in initial midguts homogenates.

2.3.7. Plant based bioassays

Survival of larvae from strains 456, Benzon and F1 hybrids was compared in bioassays with maize leaf tissues. Bioassays were performed using V6-V7 leaves from

transgenic maize plants producing Cry1Fa or Cry1Ab toxins and non-transgenic isolines. A leaf section of 2.5L x 1.5W cm was placed in a 29.57 mL plastic cup containing 1% agar to prevent desiccation of leaf tissues. A single larva from each strain was released into the bioassay cup and kept in an incubator with 24±2°C, 50% RH, and 18:6 (L: D), and new leaf tissue was added as needed. Larval mortalities were recorded seven days after larval release. Thirty-two larvae per treatment were used from each strain and each treatment was replicated twice. Data from bioassays calculated as percent mortalities and mean larval and percent pupal growth inhibition were analyzed using analysis of variance (ANOVA). Means were separated at α = 0.05 by using Fisher's protected least square difference and Tukey's multiple comparison tests were used to determine significance at $p<0.05$ (SPSS 21).

2.3.8. Western blotting and qualitative binding assays

The different levels of ALP protein in BBMV from resistant, susceptible and larvae from F_1 hybrids were analyzed using immunoblotting. BBMV proteins (25 µg) were solubilized in SDS-PAGE sample buffer (Laemmli, 1970) and heat denatured before separation through SDS-10%PAGE electrophoresis. Proteins were then transferred overnight at 4°C to a polyvinylidene difluoride (PVDF) filter in transfer buffer (25 mM Tris, 192 mM glycine, 0.1% SDS and 20% methanol) using constant current (20 mVolts). After transfer, filters were blocked in PBS containing 01% Tween-20 and 3% BSA for 1 h at room temperature, and then they were probed for 1 h with primary antibody (1:10,000 dilution) prepared against the membrane-bound form of ALP from *Anopheles gambiae* (from Dr. Gang Hua and Dr. Mike Adang, University of Georgia, USA). Filters were washed six times for 10 min each with washing buffer

(PBS, 0.1% Tween-20 and 0.1% BSA) and then probed with secondary antibody (goat anti-rabbit conjugated to HRP, 1:30,000 dilution) for 1 h at room temperature. After washing as before, blots were developed using enhanced chemiluminescence (SuperSignal West Pico, Pierce) following manufacturer's instructions.

For qualitative evaluation of toxin binding, binding assay using BBMV proteins (25 µg) and Cry1Fa or biotinylated Cry1Ca (1 µg) was performed. Binding reactions (100 µl final volume) were performed as for radiolabeled toxins. After washing, resulting BBMV pellets were used for SDS-10%PAGE electrophoresis as described above. Proteins were transferred to PVDF filters and blocked as above, and then probed for 1 h with anti-Cry1Fa antisera (1:10,000 dilution) or streptavidin-HRP conjugate (1:25,000 dilution). After washing Cry1Fa blots were probed for 1 h with goat anti-rabbit-HRP conjugate (1:30,000 dilution). Blots were developed as described above.

2.3.9. Saturation binding of ^{125}I-Cry toxins to BBMV

Specific binding of Cry toxins to BBMV was tested using saturation binding assays. A constant amount (0.22 nM for Cry1Ab and Cry1Ac, 0.57 nM for Cry1Ca, and 4.5 nM for Cry1Fa) of radiolabeled toxins was incubated with increasing amounts of receptor (BBMV) in a final reaction volume of 0.1 ml of binding buffer (PBS pH 7.5 plus 0.1% BSA) for 1h at room temperature. An excess (300 fold) of unlabeled Cry1 toxin was used to calculate non-specific binding. After incubation for 1h at room temperature, binding reactions were stopped by centrifugation (16,000 x *g* for 10 min) and pellets containing BBMV and bound toxins were washed twice with 0.5 ml of ice-cold binding buffer. The radioactivity in final pellets was measured in a Wizard-2 gamma detector. Specific toxin binding was determined by subtracting non-specific from total binding.

Data shown are the mean values calculated from at least two independent experiments performed in duplicate with their corresponding standard errors.

2.3.10. Quantification of alkaline phosphatase (ALP) and aminopeptidase-N (APN) activities

Specific ALP and APN enzymatic activities in BBMV (1 µg) were measured as described elsewhere (Jurat-Fuentes and Adang, 2004) using ρ-nitrophenyl phosphate disodium (ρNPP) in ALP buffer (100 mM Tris/HCl, pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$) or leucine-ρ-nitroanilide (Sigma, St. Louis, MO, USA) in sodium phosphate buffer (10 mM Na_2HPO_4, pH 7.5, 135 mM NaCl, 2 mM KCl) as substrates for ALP and APN, respectively. Enzymatic activities were monitored for 5 minutes as changes in OD at 405 nm wavelength at room temperature in a microplate reader (BioTek), and the maximum initial velocity (Vmax) was calculated using the associated KC4 Data Analysis Software. One enzymatic unit was defined as the amount of enzyme that would hydrolyze 1.0 µmole of substrate to chromogenic product per minute at the specific reaction pH and temperature. Data shown are the mean specific activities obtained from at least three independent BBMV batches for each strain measured at least in triplicate (n=9). Statistical significance for differences in activity was determined through analysis of variance (ANOVA) using the Holm-Sidak multiple pairwise comparison test (overall significance level = 0.01), using the SigmaPlot v.11.0 software (Systat Software Inc., San Jose, CA, USA). Since APN activity data failed an equal variance test, in this case used ANOVA on ranks (Kruskal-Wallis test, overall significance level = 0.01) to determine statistical significance.

2.3.11. Linkage analysis of 456 resistance and ALP expression

We tested linkage between the reduced ALP phenotype and resistance by detecting levels of ALP protein in BBMV from larvae of a hybrid strain after exposure to non-transgenic maize or Cry1Fa-producing maize leaf tissue. The hybrid strains was originated by crossing mtohs of the 456 and Benzon strains and then sib-mating the resulting F1 generation to obtain an F2 generation containing all three potential resistance genotypes (ss, sr, and rr). Neonate larvae from eggs of the F2 generation were reared until third instar on either fresh maize leaf tissue from plants expressing Cry1Fa toxin (n=96) or the corresponding non-transgenic isoline (n=32). Larvae surviving these treatments were then moved to artificial diet until fourth instar, when their midgut was dissected and individually used to prepare BBMV as previously described (Jurat-Fuentes and Adang, 2004). Levels of ALP in BBMV were detected using immunoblots as described above.

2.4. Results

2.4.1. Resistance of strain 456 to Bt maize

Larvae from the 456 strain was previously reported to display > 7,500-fold resistance to Cry1Fa toxin compared to susceptible larvae from laboratory and field populations (Blanco et al., 2010). This resistant strain has been under continuous selection for 15 generations on TC1507 maize. Leaf tissue bioassays showed 94% survival of larvae from the 456 strain on TC1507 maize leaf tissues, while no survivors were detected in the Benzon strain (Fig. 6A). In hybrid (Benzonx456) strains marginal survival (between 5-7%) independently of the sex of the resistance parent in the cross,

supporting autosomal recessive inheritance of resistance was detected. Less than 10% mortality was observed for all strains in bioassays with leaf tissue from isogenic line (data were not shown).

Although transgenic maize expressing Cry1Ab toxin (event MON810) does not effectively control *S. frugiperda* (Abel and Adamczyk, 2004; Lynch et al., 1999), I was interested in testing for cross-resistance to this maize variety in larvae from the 456 strain. A significant differences in mortality between larvae from the Ben (15.6% mortality) compared to larvae from the 456 (9.4% mortality) strain detected in bioassays with fresh maize leaf tissue of event MON810 (Fig. 6A). These differences were also observed when comparing the weight of the surviving larvae from each strain (Fig. 6B). While larvae of the Ben strain (fourth instar) had significantly reduced weight when fed MON810 compared to non-transgenic maize leaf tissue, no significant differences were detected for larvae of the 456 strain.

Considering that Cry1Fa and Cry1A toxins share binding sites in BBMV from *S. frugiperda* larvae, and that Cry1Ca and Cry1Fa share a second binding site not recognized by Cry1A toxins, were interested in testing susceptibility to Cry1Ca in larvae of the 456 strain. Bioassays with purified Cry1Ca toxin revealed no significant differences in susceptibility to Cry1Ca when comparing neonate larvae from the Ben and 456 strains (Fig. 6C). The LC50s calculated from corrected mortality data were 20 ng/cm^2 and 22.5 ng/cm^2 for larvae from the Ben and 456 strains, respectively.

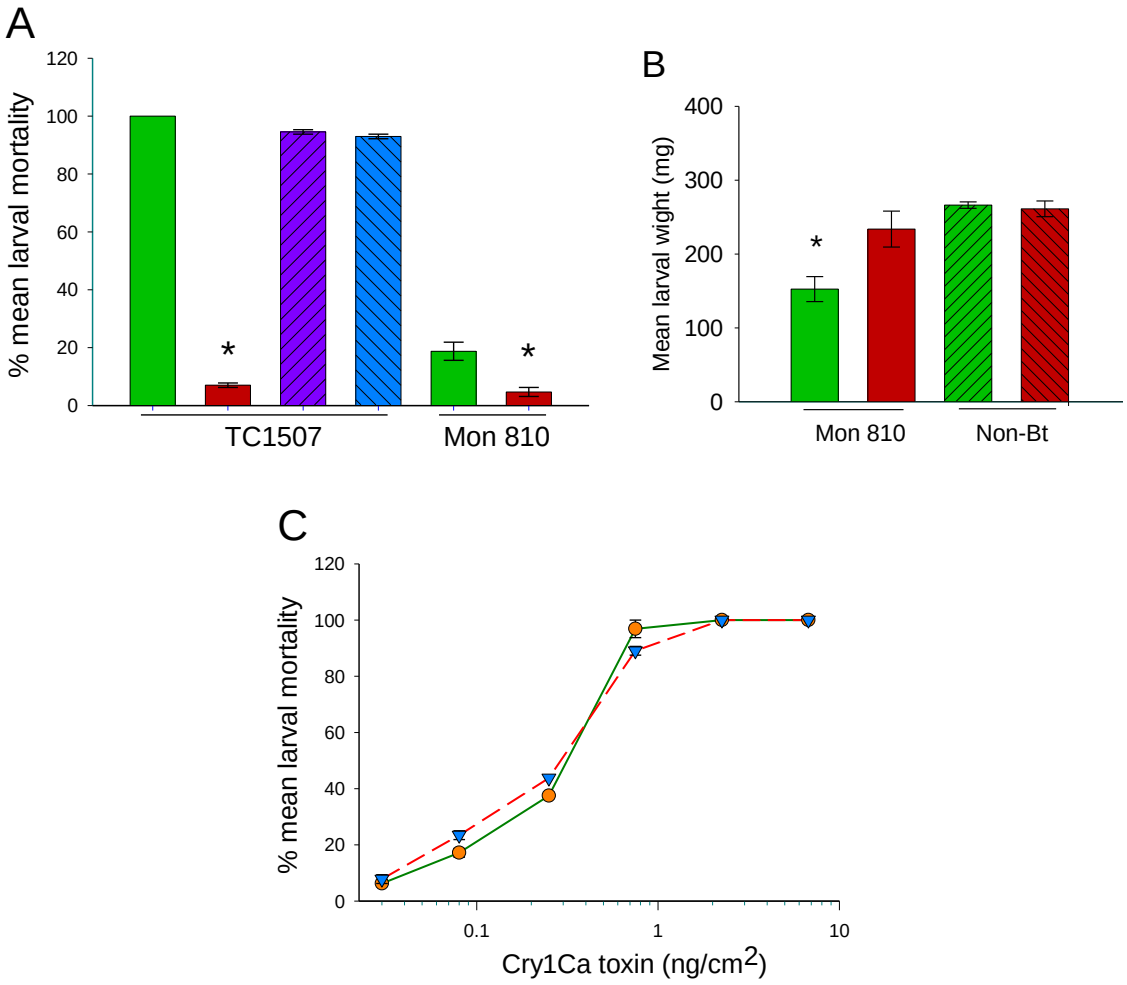
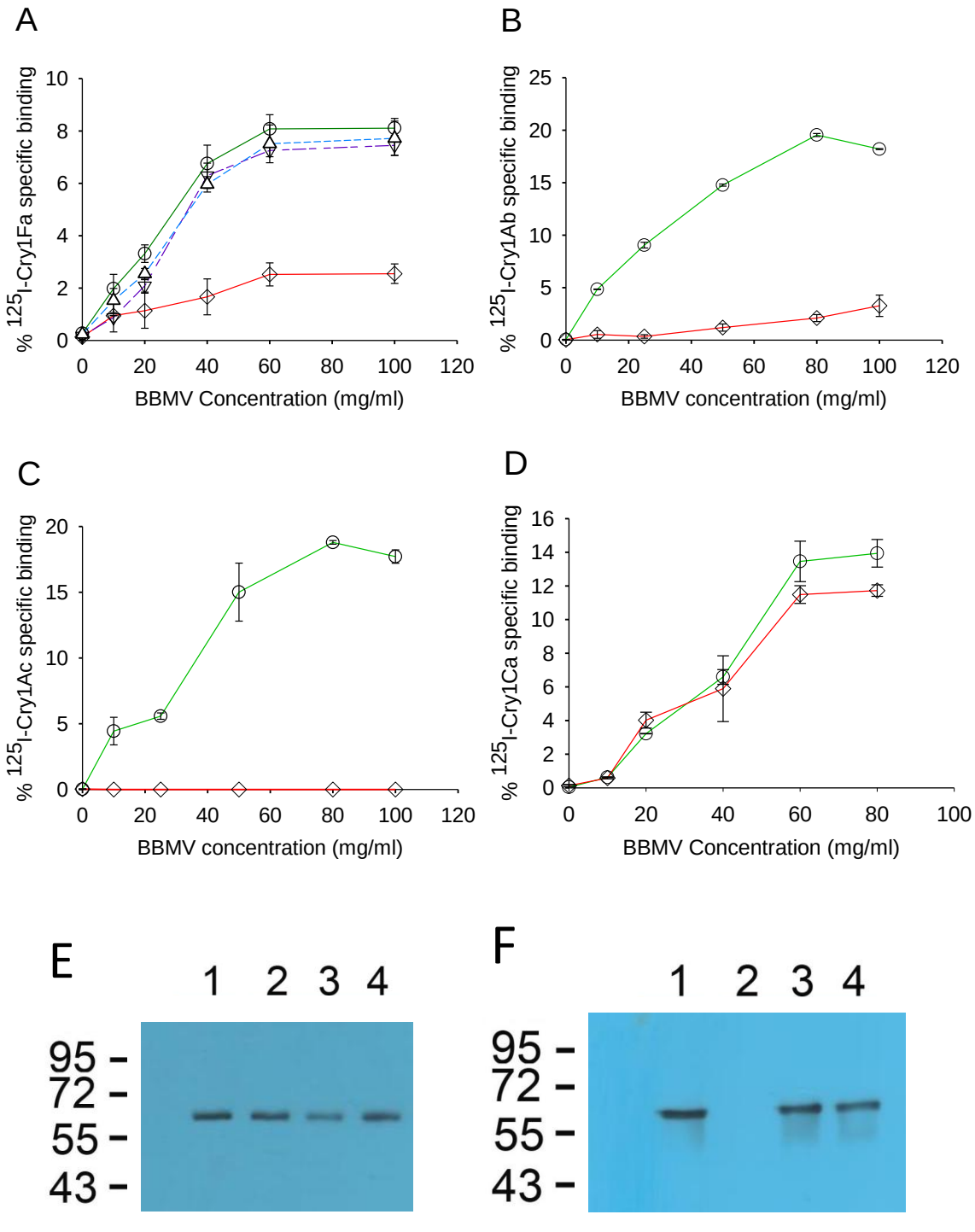


Fig. 6. Bioassays with fresh maize leaf tissue or artificial diet against neonate *S. frugiperda* larvae from Benzon (bar 1 in green), 456 (bar 2 in red), 456♀ x Ben♂ (bar 3 in violet) and Ben♀ x 456♂ (bar 4 in blue). A) Larval mortalities on leaf tissue of Cry1Fa Bt maize (TC1507) or Cry1Ab maize (MON810). B) Weight of larvae from the Benzon and 456 strains after feeding on leaf tissue Bt maize event MON810 or non-Bt isoline. C) Susceptibility of Benzon and 456 larvae to purified and activated Cry1Ca toxin.

2.4.2. Specific binding of Cry1A, Cry1Fa, and Cry1Ca toxins to BBMV

Results from binding assays with BBMV and radiolabeled Cry1Fa toxin revealed reduced toxin binding in strain 456 compared to Benzon, while no significant reduction was observed when comparing Benzon to F1 hybrids (Fig7A). Similar results were obtained for radiolabeled Cry1Ac and Cry1Ab toxins (Fig 7B and 7C). In contrast, no significant binding reduction was observed when using radiolabeled Cry1Ca and BBMV of strain 456 compared to Benzon (Fig 7D). The same reductions in Cry1Fa but not Cry1Ca binding in BBMV from 456 compared to Benzon or F1 hybrid larvae were also detected using immunoblotting (Fig. 7E and 7F).

Fig. 7. Binding of radiolabeled Cry1Fa (A), Cry1Ab (B), Cry1Ac (C) or Cry1Ca (D) purified activated toxins to BBMV from midguts of larvae from the Benzon (green) or 456 (red) strains was tested in binding assays. Specific Cry1Fa toxin binding to BBMV from larvae of crosses 456M (violet) and 456F (blue) are also shown in (A). Binding of biotinylated Cry1Ca (E) or Cry1Fa (F) to BBMV from Benzon (lane 1), 456 (lane 2), 456M (lane 3) and 456F (lane 4) detected using Western blotting (E) or immunoblotting (F).


A
% 125I-Cry1Fa specific binding
BBMV Concentration (mg/ml)
B
% 125I-Cry1Ab specific binding
BBMV concentration (mg/ml)
C
% 125I-Cry1Ac specific binding
BBMV concentration (mg/ml)
D
% 125I-Cry1Ca specific binding
BBMV Concentration (mg/ml)
E
1 2 3 4
95
72
55
43
F
1 2 3 4
95
72
55
43

2.4.3. Reduced Cry1Fa toxin binding associated to reduced expression of ALP

Since Cry toxins bind to APN and ALP in target insect midguts, and reduced Cry1Fa toxin binding in larvae from the 456 strain was detected, APN and ALP protein levels using specific enzyme activity of APN and ALP proteins in BBMV of strain 456, Benzon and F1 hybrids were compared to test the effect of these proteins levels on Cry1Fa binding. The specific activity of ALP (Fig. 8A) in midguts of larvae from strain 456 was reduced 75% compared to activity in Benzon and F_1 hybrids (df=3, F=0.066, P=<0.0001), whereas the APN specific activity (Fig. 8B) was not significantly different between the strains (df=3, F=4804.667, P=<0.975).

Western blots used to further test the variation in ALP protein levels in BBMV from 456 compared to Benzon strain. To detect the APN and ALP proteins antisera against the *M. sexta* APN and *A. gambiae* ALP, respectively were used. For blots with antisera to APN a ~110 kDa protein was detected, while a ~ 68-kDa protein was detected with antisera against ALP. In agreement with the specific activity assays, highly reduced levels of ALP protein in BBMV from 456 compared to Benzon were observed (Fig. 9A). Moreover, ALP levels in larvae from crosses between Benzon and 456 moths displayed levels of ALP intermediate between the susceptible and resistant parents. In contrast, we did not observed differences in the intensity of APN protein bands in Western blots (Fig.9B) in BBMV from all strains.

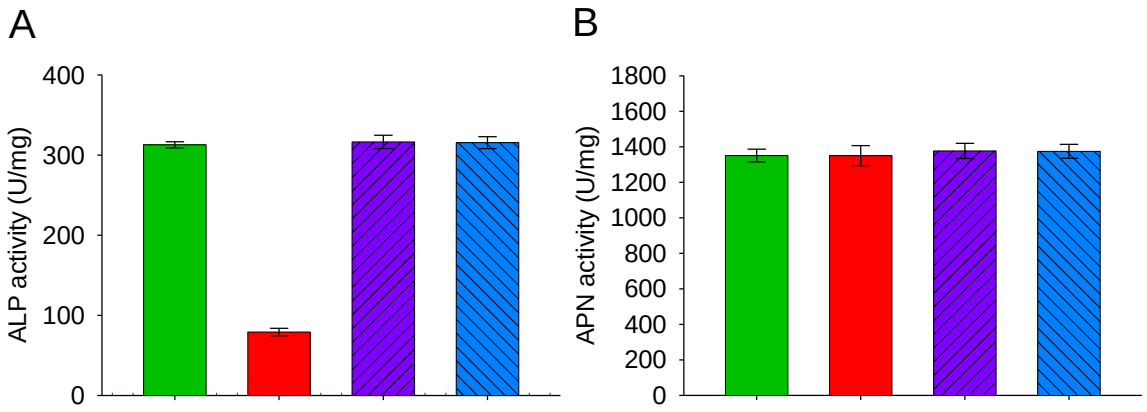


Fig. 8. Reduced ALP but not APN activity levels in BBMV from resistant *S. frugiperda* larvae. BBMV proteins from susceptible (Ben), F1 reciprocal crosses and resistant (456) strains of *S. frugiperda* were used in specific activity experiments. ALP (A) or APN (B) BBMV proteins with Benzon (bar 1in green), 456 strain (bar 2 in red color), 456F (bar 3 in blue) and 456M (bar 4 in violet).

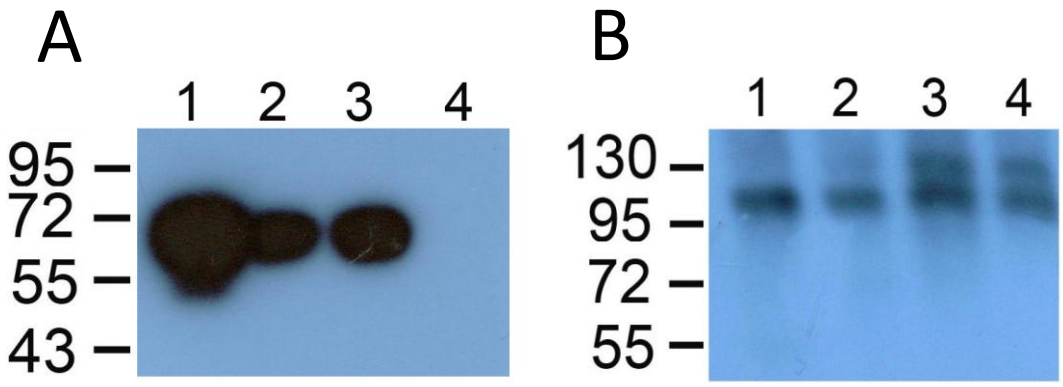


Figure 9.- Reduced ALP but not APN protein levels in BBMV from resistant *S. frugiperda* larvae. ALP and APN protein levels in BBMV from susceptible (Ben), F_1 hybrids and resistant (456) strains of *S. frugiperda* were detected using antisera against the *M. sexta* APN and *A. gambiae* ALP, respectively. ALP (A) or APN (B) BBMV proteins with strain Benzon (line 1), 456F (line 2), 456M (line 3) and 456 strain (line 4).

2.4.4. Reduced ALP protein expression phenotype is genetically linkage to Cry1Fa resistance

Linkage experiment was conducted to test for linkage between the reduced ALP protein level phenotype and resistance to Cry1Fa-producing maize. For this experiment an F2 strains containing all potential resistance genotypes were used, which were exposed to non-transgenic maize or maize expressing the Cry1Fa toxin. Then levels of ALP in larvae that survived exposure to the Cry1Fa maize compared to larvae from the control treatment were tested. When neonate larvae of this strain were exposed to TC1507 leaf tissue we detected 77 % mortality, while only 5% mortality was detected in larvae exposed to non-transgenic maize. These mortality results suggest a potential 1:2:1 proportion of (ss:sr:rr) genotypes in this strain. There were no significant differences in the weight of the surviving larvae from both treatments (data not shown). Using Western blots and BBMV prepared from individual midguts, three ALP phenotypes (high, intermediate, and low levels) according to the strength of the chemiluminescence signal in the Western blots were detected (Fig. 10A). Within the subgroup analyzed (31 larvae) 8 larvae with high, 14 with intermediate, and nine with low ALP levels were detected, which approximate the putative 1:2:1 genotype ratio observed in the mortality assays. In contrast, all the larvae surviving exposure to TC1507 maize displayed low levels of ALP

protein (Fig.10B).

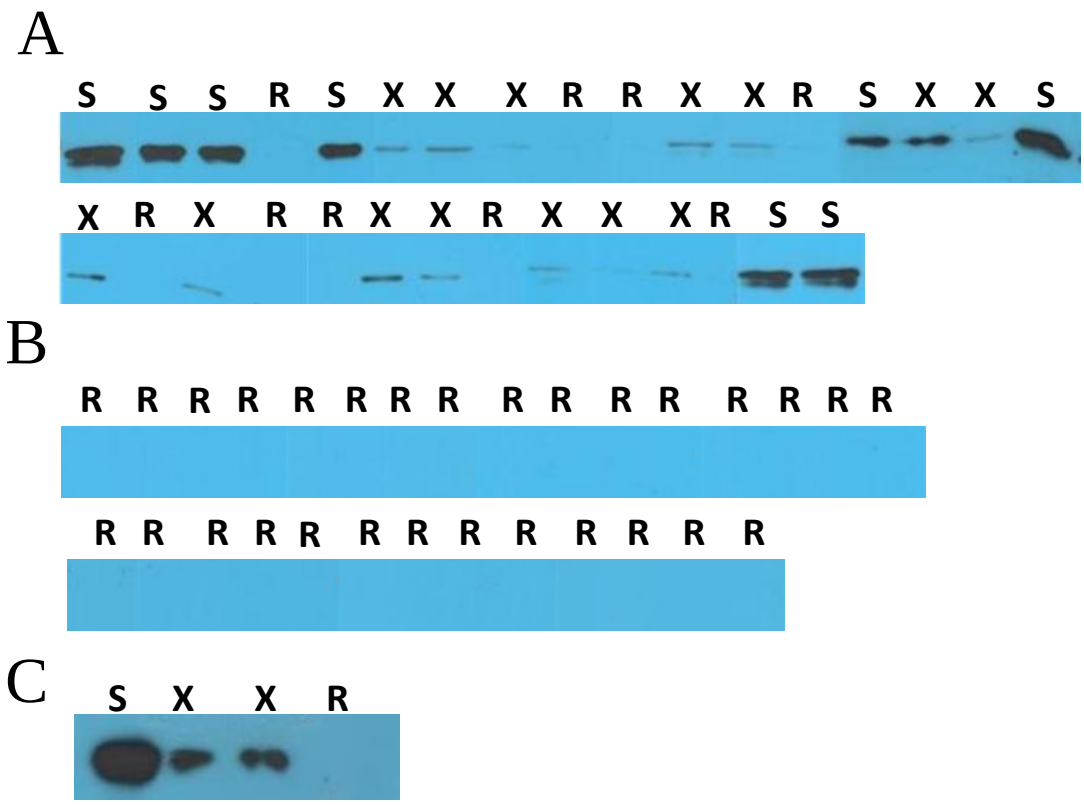


Fig. 10. Reduced ALP protein levels are linked to resistance against TC1507 maize. Neonates from this strain were exposed to fresh leaf tissue from non-transgenic (A) or TC1507 corn (B) until thirds instar. Individual larvae were classified as S (high), X (intermediate) and R (low) ALP levels based on comparison with ALP levels in strain Benzon (S), F1 hybrids (X) and 456 (R) as shown in (C) using antisera against *A. gambiae* mALP.

2.5. Discussion

The use of transgenic crops expressing insecticidal proteins from Bt has revolutionized insect pest management. While evolution of resistance in field pest populations is one of the main threats to the future utility of this technology, there is no available data on field-selected resistance mechanisms to these Bt crops. Field evolved resistance to Bt crops has been reported *in S. frugiperda* (Storer et al., 2010), *P. gossypiella* (Dhurua and Gujar, 2011), *B. fusca* (van Rensburg, 2007) and *D. v. virgifera* (Gassmann et al., 2011). Field evolved resistance in *S. frugiperda* from Puerto Rico to TC1507 maize producing Cry 1Fa toxin was characterized in the present study. In agreement with previous reports of resistance in *S. frugiperda* from Puerto Rico (Storer et al., 2010), the strain 456 displayed high levels of resistance to TC1507 maize with an autosomal and recessive mode of inheritance. In contrast, field-evolved resistance in *D. v. virgifera* was reported to be transmitted as a non-recessive trait (Gassmann et al., 2011). The genetics of resistance transmission in other cases of field-evolved resistance has not been addressed to date. In agreement with previous reports (Storer et a1., 2010), larvae from the 456 strain were also found to be cross-resistant to maize producing Cry1Ab toxin, a toxin sharing binding sites with Cry1Fa in *S. frugiperda* (Escriche et al., 1997), while no cross-resistance was observed to purified Cry1Ca toxin. While a common binding site for Cry1Ca and Cry1Fa was reported by Luo et al (1999), present binding data are in agreement with the rest of published reports (Van Rie et al., 1990b; Herrero et al., 2001; Lee et al., 1995; MacIntosh et al., 1991; Tabashnik et al., 1994; Ballester et al., 1999; Ferre et al., 1991; Masson etal., 1995) supporting the existence of a shared Cry1A-Cry1Fa binding site that is not recognized by Cry1Ca. This lack of cross-

resistance to toxins not sharing binding sites with the selective toxin was also observed in field resistant *P. gossypiella* (not cross-resistant to Cry2Ab) and *D. v. virgifera* (not cross-resistant to Cry34/35Ab). Although speculative, this observation may suggest that altered binding sites may be involved in resistance in those cases. Reports of laboratory-selected resistant strains suggest that high levels of resistance to Cry toxins are associated with altered toxin binding (Ferre and Van Rie, 2002).

As expected from the high levels of resistance to Cry1Fa and the cross-resistance pattern observed in strain 456, highly reduced Cry1Fa and Cry1A toxin binding to BBMV when compared to BBMV from susceptible larvae was detected. This pattern of resistance and cross-resistance indicates that a common binding site shared between Cry1Fa, Cry1Ab and Cry1Ac is altered in midguts of larvae from strain 456. Studies on Cry toxin interactions with cadherin, APN and ALP in target insect midguts identified these proteins as functional receptors (Piggott and Ellar, 2007). Reduced binding of Cry1A toxins in resistant insects was previously genetically linked to mutations in cadherin proteins in *H. virescens* (Gahan et al., 2001), *P. gossypiella* (Morin et al., 2003), *S. frugiperda* (Rahman et al., 2012) and *P. xylostell* (Baxter et al., 2008). Alterations in expression of APN genes have been reported to be linked with resistance to Cry1Ac in *Trichoplusia ni* (Wang et al., 2010). In *S. exigua* resistance to Cry1Ca was reported to be due to reduced expression of an APN Cry1Ca (Agrawal, et al., 2002). In agreement with this observation, knockdown of APN expression in *S. littoralis* larvae resulted in reduced susceptibility (Rajagopal et al., 2002). While protein bands representing APN and cadherin proteins from *Spodoptera* spp. have been reported to bind Cry1A or Cry1Fa

toxins (Rahman et al., 2012), there are no available data on functional receptors for Cry1A or Cry1Fa toxins in *Spodoptera* spp. Previous reports demonstrated an association between reduced ALP expression and resistance to Cry1Ac and Cry1Fa toxins in *H. virescens*, *H. armigera* and *S. frugiperda* larvae (Juan Luis et al., 2004). Increased release of ALP from the midgut epithelium into the gut fluids has been proposed as a mechanism of resistance against Cry1Ac in a laboratory-selected strain of *H. zea* (Caccia et al., 2012) The potential role of ALP as functional receptor has been reported for Cry1Ab in *M. sexta* (Arenas et al., 2010), Cry1Ac in *H. armigera* (Ning et al., 2010), mosquitocidal Cry toxins in mosquito larvae (Martins et al., 2010; Dechklar et al., 2011; Fernandez et al., 2006; Hua et al., 2009) and Cry3Aa in *Tenebrio molitor* (Zuniga-Navarrete et al., 2012). This array of reports supports a role for ALP as binding site for Cry toxins in diverse taxonomic insect orders. In present study a significant reduction of ALP protein levels in midguts from larvae of strain 456 compared to the Benzon strain was observed. When including hybrid larvae in specific activities analyses the reduced ALP levels were directly associated with reduced Cry1Fa-Cry1A toxin binding and resistance to TC1507 and MON810. Furthermore, present study able to demonstrate phenotypic linkage between reduced ALP expression and resistance to maize event TC1507. Further research is needed to understand the molecular level receptor role of ALP to Cry1Fa toxin in *S. frugiperda*.

The present study is the first report focused on characterization of mechanisms responsible for field-evolved resistance to a Bt crop. The results from this study provide an opportunity to update the current resistance management practices to delay and control

episodes of resistance for sustainability of Bt crop technology. The use of multiple combinations of Cry toxins with no common binding sites in pyramided crops could delay the resistance development and provide effective control of resistant insects. Pyramided maize expressing Cry1Fa, Cry1A.105, and Cry2Ab2 (SmartStax) was shown to effectively control resistant *S. frugiperda* from Puerto Rico. Based on present results, it would be expected that the mortality induced by SmartStax maize is due to Cry2Ab2 toxin, since both Cry1Fa and Cry1A.105 would be expected to share binding sites in *S. frugiperda*. However, Cry2Ab2 is not considered highly effective against *S. frugiperda* (Hernández-Martínez et al., 2008). Although further work would be needed to understand this seemingly contradictory observation, potential explanations include increased susceptibility to Cry2Ab2 in Cry1Fa-resistant larvae, and formation of heteroligomers (Cry1-Cry2) resulting in increased susceptibility. According to present data Cry1Ca is a toxin amenable for use in pyramided crops to delay resistance evolution in *S. frugiperda*. Further research is needed to identify molecular mechanism responsible for ALP down-regulation resulting in resistance. This will advance the use of genomic methods to develop DNA-based biomarkers for detecting Bt resistance in the field.

Present study conclude that the high survival of *S. frugiperda* resistant larvae on TC1507 leaf tissue is a result of reductions in Cry1Fa binding associated with decreased expression of ALP Disruption of Cry toxin binding to membrane receptors is the best characterized and most frequently reported mechanism responsible for high levels of resistance in laboratory selected insects (Ferre and Van Rie, 2002). Since this mechanism was also described in field and greenhouse populations selected with

commercial Bt pesticides (Wang et al., 2007), my findings support the importance of reduced toxin binding for high levels of resistance regardless of the Bt technology used (Bt sprays or transgenic crops).

CHAPTER III

Testing for cross-resistance in *Spodoptera frugiperda* (J. E. Smith) with field-evolved resistance to Bt maize

3.1. Abstract

The fall armyworm, *Spodoptera frugiperda* (J. E. Smith) (Lepidoptera: Noctuidae), is an important sporadic insect pest on various agricultural crops in U.S.A. and across the world. Transgenic corn producing Cry1Fa or Cry1Ab toxins from *Bacillus thuringiensis* (Bt) have been used to control larvae of *S. frugiperda*. Evolution of resistance by insect pests is the greatest threat to the continued success of transgenic crops producing Bt toxins. Previous studies showed that *S. frugiperda* developed resistance to Cry1Fa expressing corn event TC1507 in Puerto Rico. However, the existence of cross-resistance to alternative Cry toxins in these insects has not been reported to date. A resistant strain of *S. frugiperda* originally established from field-collected insects from Puerto Rico to study potential cross-resistance patterns was used. As expected, larvae of the resistant *S. frugiperda* strain (456) exhibited dramatically reduced susceptibility to purified Cry1Fa toxin compared to a susceptible strain. Similarly, reduced susceptibility to Cry1Ab, and Cry1Ac toxins in larvae from the 456 compared to control strains was detected. In contrast, no differences in susceptibility were detected in bioassays with purified Cry1Bb or Cry1Da purified toxins, or with Xentari WG or Dipel ES pesticidal formulations. The cross-resistance patterns observed in present bioassays are in agreement with data from competition experiments indicating an altered binding site for Cry1A and Cry1Fa toxins in 456 larvae. These data support the use of Cry1Bb, Cry1Ca, or Cry1Da to control *S. frugiperda* resistant to Cry1Fa, and the use of these toxins in pyramiding efforts for effective management of *S. frugiperda*.

3.2. Introduction

The fall armyworm, *Spodoptera frugiperda* (J. E. Smith) has been one of the most common insect pests on many plant species and recently evolved as a sporadic pest on corn, cotton and soybean crops (Buntin et al., 2004). This pest has developed resistance to most conventional insecticides and has caused economic damage on corn crops during migration across the southern U. S. A. The first generation Bt maize and cotton hybrids expressing Cry toxins from *Bacillus thuringiensis* (Bt) were used to control lepidopteran insect pests, including secondary targets like S. *frugiperda*. The mode of action of these Cry toxins involves binding to specific receptors on the midgut brush border membrane followed by insertion into the membrane, leading to cell lysis by osmotic shock and death of the insect by septicemia (Bravo et al., 2011). The efficacy of Bt crops to control target insect pest depends on the specificity of the Cry toxins expressed in that crop (van Frankenhuyzen, 2009). The first commercialized Bt maize expressing Cry1Ab toxin was highly efficacious to control corn stalk-boring insect pests such as *O. nubilalis*, but was less effective in suppressing *Helicoverpa zea* and *S. frugiperda* (Buntin, 2008). The TC1507 corn event expressing the Cry1Fa toxin is highly active against both stalk borers and *S. frugiperda*, but is still not effective in controlling *H. zea* (Siebert et al., 2008). High levels of adoption representing high selection pressure on *S. frugiperda* coupled with unique climatic and geographic isolation conditions and year-round cultivation, resulted in development of field-evolved resistance to event TC1507 in *S. frugiperda* populations from Puerto Rico (Storer et al., 2010).

Previous reports demonstrate in many instances development of Bt resistance in target insects results from altered or reduced binding of Cry toxins to their binding sites (Ferre and Van Rie, 2002). Furthermore, alteration of a common binding site has been responsible for development of cross-resistance to Cry toxins not present in the selection environment (Lee et al., 1995;Sayyed et al., 2000; Tabashnik et al., 1997b). Based on this information, current insect resistance management strategies support the sequential or simultaneous use of different Bt toxins binding to alternative sites to reduce the probability of resistance evolution. In this strategy, insects would need to evolve simultaneous alterations in diverse binding sites to become cross-resistant to both toxins. The best method of predicting the risk of cross-resistance among Bt toxins is by determining their binding patterns and identifying common binding sites in a given target insect. Sharing of common binding sites has been reported for diverse Cry toxins in several insect species, including *Plodia interpunctella* (Ferre et al., 1991; Vanrie et al., 1990)), *P. xylostella* (Ferre et al., 1991; Granero et al., 1996; Tabashnik et al., 1994a; Tabashnik et al., 1997a), *H. virescens* (Lee et al., 1995), *Leptinotarsa decemlineata* (Loseva et al., 2002), *P. gossypiella* (Gonzalez-Cabrera et al., 2003a; Tabashnik et al., 2000), *H. armigera* (Estela et al., 2004; Luo et al., 2007), *O. nubilalis* (Crespo et al., 2011), and *S. exigua* (Hernandez-Martinez et al., 2009).

An alternative approach to estimate cross-resistance patterns is by testing efficacy of Bt toxins against resistant strains of the target insect. Diverse patterns of cross-resistance have been reported for Cry toxins in different insects, but commonalities are clear in some cases. For instance, "Mode 1" resistance involves resistance to a Cry1A

toxin associated with reduced toxin binding and cross-resistance to other Cry1A toxins, Cry1Fa, and Cry1Ja, but not to Cry1Ca or Cry2 toxins. This pattern of cross-resistance is explained by the lack of shared binding sites among Cry1A, Cry1Ca, and Cry2 toxins, while Cry1A, Cry1Fa, and Cry1Ja share binding sites in multiple species (Ballester et al., 1999; Escriche et al., 1997; Escriche et al., 1994; Estela et al., 2004; Karim et al., 2000). Examples of this pattern of cross-resistance has been described for strains of *P. xylostella* (Tabashnik et al., 1994c), *H. virescens* (Lee et al., 1995) , *Diatraea saccharalis* (Wu et al., 2009), and *P. gossypiella* (Zhao et al., 2001). In contrast, there are also reports of Cry1Ca-resistant strains with cross-resistance to Cry1A, Cry1Fa, and Cry1J toxins in *P. xylostella* (Tabashnik et al., 2000), cross-resistance between Cry2Ab and Cry1Ac in *H. armigera* (Mahon et al., 2007), lack of cross-resistance to Cry1Fa in Cry1Ab-resistant *O. nubilalis* (Siqueira et al., 2004), and cross-resistance to Cry1Ca and Cry1Da in Cry1Ab-resistant *S. exigua* (Hernandez-Martinez et al., 2009). These cases of cross-resistance suggest that in Bt resistance may not be related to alterations in toxin binding (Anilkumar et al., 2008;Hernandez-Martinez et al., 2009). In above cases, cross-resistance patterns can only be identified using bioassays.

In previous study, I characterized the mechanism in *S. frugiperda* resistant to TC1507 maize expressing Cry1Fa toxin as a modification in Cry1Fa toxin binding sites on the brush border membranes of midgut cells. The study of cross-resistance patterns in S. frugiperda to Bt toxins is essential for development of effective resistance management strategies. Despite the high levels of field evolved resistance to Cry1Fa documented in *S. frugiperda* from Puerto Rico, there are no reports of cross-resistance patterns in these

insects. To address this knowledge gap, the objective of this study was to identify cross-resistance patterns in a *S. frugiperda* strain originated from Puerto Rico displaying high levels of resistance to TC1507 corn. First used toxin binding assays to determine the pattern of shared binding sites among Cry1Ab, Cry1Fa and Cry1Ca toxins, and then performed bioassays to confirm that cross-resistance was associated with shared binding sites. To examine potential cross-resistance in Bt maize-resistant *S. frugiperda* to Bt pesticides, compared susceptibility of Benzon and 456 strains against Xentari WG and Dipel ES formulations. In an attempt to identify effective Cry toxins active against Cry1Fa-resistant *S. frugiperda* also performed bioassays with purified Cry1Bb, Cry1Da, and Cry1Ea toxins. Present study goal was to assess the risk of cross-resistance to Bt pesticides and individual toxins in *S. frugiperda* larvae resistant to Bt maize. The data obtained helped identify effective Cry toxins for gene pyramiding and control of Cry1Fa-resistant *S. frugiperda*.

3.3. Materials and Methods

3.3.1. Insect strains

Two *S. frugiperda* strains were used in this study. Eggs of the susceptible Benzon strain were purchased from Benzon Research (Benzon Research Inc., Carlisle, PA). The resistant strain (456) was originated from egg masses collected in maize fields in Puerto Rico in 2010, and displayed >7,500-fold resistance to Cry1Fa toxin in laboratory bioassays compared to the Benzon strain (Blanco et al., 2010). Neonates of the 456 strain have been kept under continuous selection until 3rd instar on leaf tissue (the V5-V7 stage) from TC1507 corn plants, while larvae of the Benzon strain have been maintained on

similar material from the isogenic line. Larval development from the 3rd instar stage until pupation was continued on artificial diet (fall armyworm diet, BioServ). These two strains were separately maintained in two incubators with 25 ± 2°C and 65 ± 5% RH under L:D 16:8 h.

3.3.2. Bt toxins and formulations

For the present study purified toxins (Cry1Ab, Cry1Ac, Cry1Da, Cry1Ea and Cry1Fa) as well as commercial-grade formulations Di-Pel ES (32,000 IU mg−1, Abbott Laboratories, Chicago, IL) and Xentari WG (35,000 diamondback moth U mg−1, Valent Biosciences, Libertyville, IL) was used to test the cross-resistance in strain 456. Cry1Ab and Cry1Ac produced from Bt strains and Cry1Bb (EG5847), Cry1Da,(ECE129) and Cry1Ea (ECE127) expressing recombinant *Escharichia coli* strains were obtained from *Bacillus* Genetic Stock Collection, Columbus, OH). A recombinant Bt strain producing Cry1Ca toxin (Rang et al., 2004)) was kindly provided by Dr. Jean Louis Schwartz (University of Montreal, Canada), while a Bt strain producing Cry1Fa was kindly supplied by Dr. Chenxi Liu (Chinese Academy of Agricultural Sciences, Beijing, China).

3.3.3. Toxin preparation

Production and purification of Cry1Da and Cry1Ea were as described by Sayyed et al., (2005). Cry1Ca and Cry1Fa toxins were produced and purified as described in Perera et al., (2009). Protoxins were activated by treatment with 0.1% (v/v) of midgut fluids obtained from actively feeding 4th instar *S. frugiperda* larvae for 1 h at room temperature. After clearing samples by centrifugation at 10,000 g x min, the activated toxins in the supernatant were loaded on a HiTrap HP Q 5 ml anion exchange column

(GE Life Sciences) equilibrated in 50 mM sodium carbonate buffer (pH 9.8), and eluted with a linear gradient of 1M NaCl. The purity of the activated toxins was monitored by SDS-10% PAGE. Fractions containing purified toxin were pooled and samples maintained at –80°C until used.

3.3.4. Competition experiments

Brush border membrane vesicles (BBMV) were dissected from fourth instar *S. frugiperda* larvae and used to prepare BBMV by the differential magnesium precipitation method (Wolfersberger, 1987), as modified byJurat-Fuentes et al., (2002). The final BBMV preparations were kept at −80°C until used. Purified Cry1Ab, Cry1Ac, and Cry1Ca toxins (1 µg) were radio-labeled with 0.5 mCi of iodine-125 (Perkin Elmer) using the chloramine T method as described elsewhere (Van Rie et al., 1989). In the case of Cry1Fa toxin, 20 µg were labeled to detect specific binding as reported elsewhere (Hernandez-Rodriguez et al., 2012). Purity of labeled toxin preparations was monitored by SDS-10%PAGE electrophoresis and autoradiography.

Competition experiments were performed by incubating 40 µg/ml of *S. frugiperda* BBMV with 0.22 nM (^{125}ICry1Fa and ^{125}I-Cry1Ab) or 0.57 nM (^{125}I-Cry1Ca) toxins for 1 hour at room temperature in the presence of increasing concentrations of unlabeled competitor. After incubation, samples were washed twice with 0.5 ml of ice-cold binding buffer (20 mM Bis-Tris, pH 6.0, 100 mM KCl, supplemented with 0.1% BSA), and radioactivity in the final pellets measured in a Wizard-2 detector (Perkin Elmer). The percentage of labeled toxin bound with increasing competitor concentrations was determined relative to amount of toxin bound in the absence of competitor (considered

100% binding). Data presented are the mean percentages from at least two independent experiments performed in duplicate.

3.3.5. Assessing cross-resistance

Seven concentrations for each toxin in PBS buffer (135 mM NaCl, 2 mM KCl, 10 mM Na_2HPO_4, 1. 7 mM KH_2PO_4, pH 7.5) and formulation were tested in the bioassays. Controls included PBS buffer. Bioassays were conducted by the surface contamination method using 128-well bioassay trays (C-D international, Pitman, NJ) A volume of 50 µL test solution was added to the top of solid meridic diet (fall armyworm diet, BioServ) previously poured in each well, spread evenly on the surface and allowed to dry in a laminar flow hood. Bioassays with XenTari WG and Di-Pel ES were conducted by the diet incorporation method, dispensing 2 ml of diet containing the pesticide per well. Six concentrations of XenTari WG and Di-Pel ES were tested. After drying, one neonate was transferred to each well using a fine camel's-hair paintbrush, then the well-sealed with an adhesive bioassay tray lid. Bioassays trays were maintained in an incubator at 25 ± 2°C and 65% RH under 16:8 L: D. Neonates (n=32) from each *S. frugiperda* strain were tested on each dose, and each bioassay replicated twice. Mortalities were recorded seven days after neonate inoculation. Lethal concentrations killing 50% of the insects (LC_{50}) values were estimated from mortality data by probit analysis using the Polo-Plus v.2.1 program (LeOra software, Petaluma, CA.).

3.4. Results

3.4.1. Binding competition assays

As previously demonstrated in chapter-II, specific binding of ^{125}I-Cry1Fa, ^{125}I-Cry1Ab, and ^{125}I-Cry1Ca toxins to BBMV proteins from both resistant and susceptible *S. frugiperda* larvae. Results from homologous competition experiments with labeled Cry1Fa, Cry1Ab, or Cry1Ca confirmed specific high affinity binding of the three toxins to BBMV from larvae of the Benzon strain (Fig. 11). As expected from our previous work, only Cry1Ca was able to bind specifically to BBMV from larvae of the 456 strain (Fig. 11C). Heterologous competition assays showed that for BBMV from the Benzon strain, Cry1Fa shared all its binding sites with Cry1Ab, but not with Cry1Ca (Fig. 11A). This common Cry1Ab-Cry1Fa site was confirmed when using radiolabeled Cry1Ab and unlabeled Cry1Fa as competitor (Fig.11B). In contrast, Cry1Ca bound uniquely and not shared its binding sites with Cry1Ab or Cry1Fa on BBMV from the Benzon strain. In case of BBMV from strain 456, did not observe competition between Cry1Fa and Cry1Ab, but homologous competition was observed for Cry1Ca (Fig. 11C). These differences between the binding patterns of the strains support alteration of a shared Cry1A-Cry1Fa binding site, while Cry1Ca binding was unaltered.

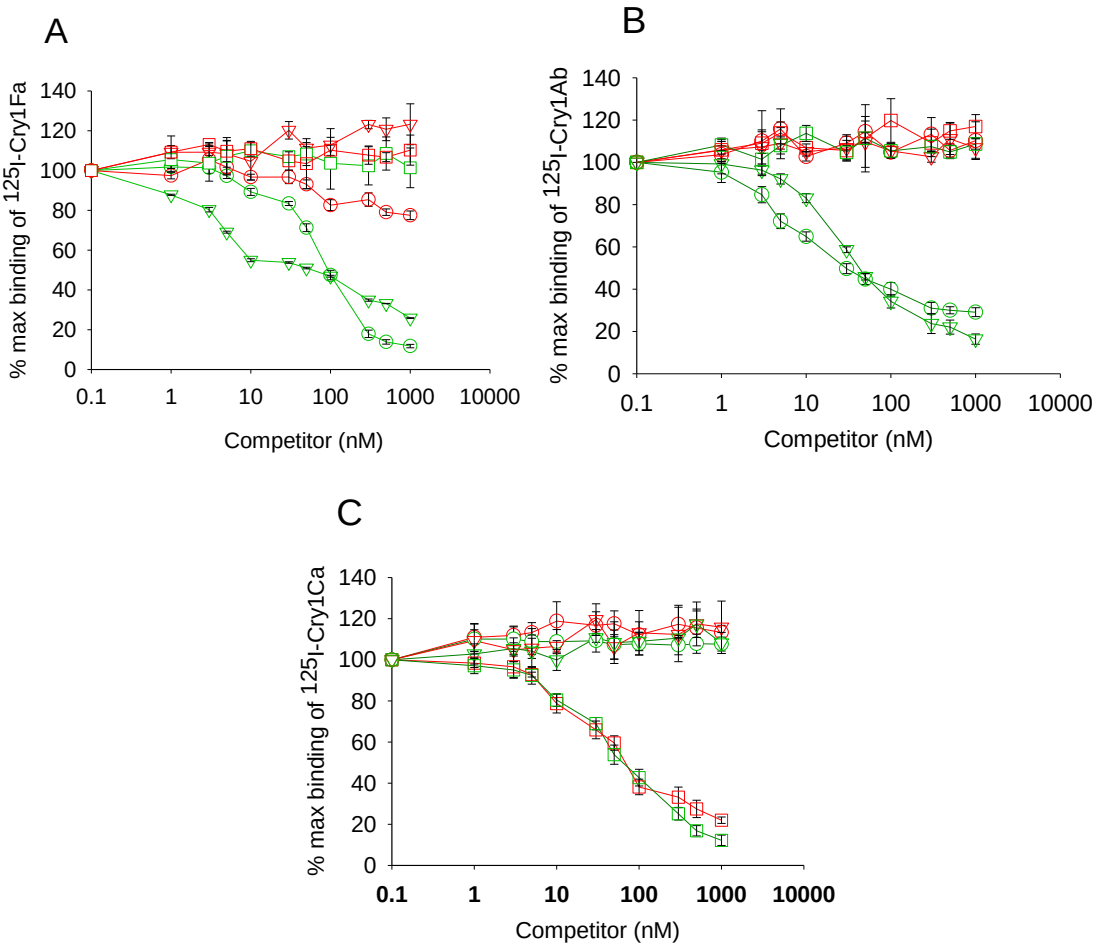


Fig. 11. Binding competition between ^{125}I-Cry1Fa or ^{125}I-Cry1Ab or ^{125}I-Cry1Ca with increasing concentrations of unlabeled Cry1Ab, Cry1Fa, or Cry1Ca to BBMV from Benzon and 456 strains of *S. frugiperda* associated with TC1507 maize producing Cry1Fa. In figures showed the competitions among Cry1Fa with Benzon (green circles), Cry1Fa with 456 (red circles); Cry1Ab with Benzon (green triangles), Cry1Ab with 456 (red triangles); and Cry1Ca with Benzon (green circles), Cry1Ca with 456 (red circles).

3.4.2. Bioassays with Cry1Fa, Cry1Ab, and Cry1Ac toxins

To test susceptibility to Cry1Fa in strain 456 larvae, a diet contaminated bioassays were conducted with purified Cry1Fa toxin against larvae from the Benzon and 456 strains. In these bioassays the calculated LC_{50} value for the Benzon strain was 194.75 ng/cm^2 whereas no LC_{50} value could be calculated for strain 456 due to marginal (6.25 %) mortality detected at the highest concentration tested (10,000 ng/cm^2) (Table 2.).

To test the cross-resistance pattern in strain 456, bioassays with purified Cry1Ab and Cry1Ac toxins was also performed. The LC_{50} value obtained for Benzon larvae was 865 ng/cm^2 diet for Cry1Ab and >2,000 ng/cm^2 diet for Cry1Ac toxin (Table 2). In contrast, only observed <5% mortality of larvae from the 456 strain after treatment with the highest tested Cry1Ab and Cry1Ac toxin concentrations, respectively. No larval mortality was detected in any of the controls for both strains. Given the low activity of Cry1Ac against *S. frugiperda*, and to better examine potential cross-resistance to Cry1Ac, we studied growth of larvae from each strain during treatment with 2,000 ng/cm^2 of Cry1Ac. Comparisons of growth between the 456 and Benzon strains showed a mass increase of 16.5% for larvae of the 456 compared to Benzon strain (Fig. 12). This difference was not detected when the insects were grown on meridic diet with PBS buffer.

3.4.3. Susceptibility of Bt maize-resistant *S. frugiperda* to Bt pesticides

Since larvae from the 456 strain were cross-resistant to Cry1A toxins, susceptibility of these larvae to commercial Bt pesticides containing combinations of multiple Cry toxins was tested. Bioassays with Xentari WG and DiPel ES formulations

were performed as they are based on the Bt vars. *aizawai* and *kurstaki*, respectively. The LC_{50} values for both formulations are presented in Table 2, and they support lack of cross-resistance to both pesticides in 456 larvae. Based on percentage of the pesticide volume used, both strains were slightly more susceptible to Xentari WG (LC_{50} 0.38-0.4%v/v) than to DiPel ES (1-1.15%v/v).

3.4.4. Bioassays with Cry1Da, Cry1Bb, and Cry1Ea

To identify effective Cry toxins to control larvae of the 456 strain, bio-efficacy of toxins with previously reported toxicity against *S. frugiperda*: Cry1Bb and Cry1Da were tested. In addition, Cry1Ea was included in present tests as a toxin with activity similar to Cry1A toxins. The LC_{50} values for Cry1Bb and Cry1Da for both strains 456 and Benzon are presented in Table 1. Both toxins were able to control larvae from the 456 and Benzon strains at similar concentrations. In case of the Cry1Ea toxin, it was not able show any activity against larvae from the 456 or Benzon strains, even at the highest tested concentration. Similarly to Cry1Ac, in present study I performed growth inhibition comparisons between Benzon and 456 larvae after treatment with 2,000 ng/cm^2 of Cry1Ea toxin. In contrast to results with Cry1Ac, Cry1Ea did not detect significant differences between larvae from each strain after treatment with Cry1Ea for 7 days (Fig. 12).

Table 2. Median lethal concentrations (LC50) and 95% fiducial limits based on larval mortality of susceptible (Benzon) and resistant (456) strains of *S. frugiperda* associated with TC1507 maize expressing Cry1Fa toxin to six purified Cry toxins and two Bt formulations.

Toxin	Strain	Slope±SE	LC_{50} (ng/cm^2)	95% Fiducial limits (ng/cm^2)	$\chi 2$
Cry1Ab	Benzon	0.652±0.075	813.99	490.45 -1581.1	2.564
	456	0.807±0.12	9842.20	4361.5-40891	3.091
Cry1Ac	Benzon	1.018±0.01	>2000	1745.92-4115.32	3.654
	456	2.205±0.83	NA	NA	1.749
Cry1Bb	Benzon	0.695±0.08	344.63	223.2-583.06	3.663
	456	0.773±0.07	369.545	247.61-596.59	4.131
Cry1Da	Benzon	1.089±0.80	81.95	63.96-104.92	1.463
	456	1.09±0.087	89.82	66.87-120.75	1.686
Cry1Ea	Benzon	0.859±0.02	>2000	2456.74 - 6523.44	3.248
	456	1.023±0.43	NA	NA	4.612
Cry1Fa	Benzon	0.984±0.35	194.75	89.52-389.46	1.86
	456	2.154±0.41	NA	NA	4.56
Xentari WG	Benzon	1.583±0.21	0.383	0.23-1.24	2.657
	456	2.521±.008	0.425	0.324-1.869	3.424
DiPel ES	Benzon	1.86±0.14	1.199*	0.63-2.49	5.522
	456	1.84±0.14	1.201	0.608-2.651	4.086

*Concentration is %v/v

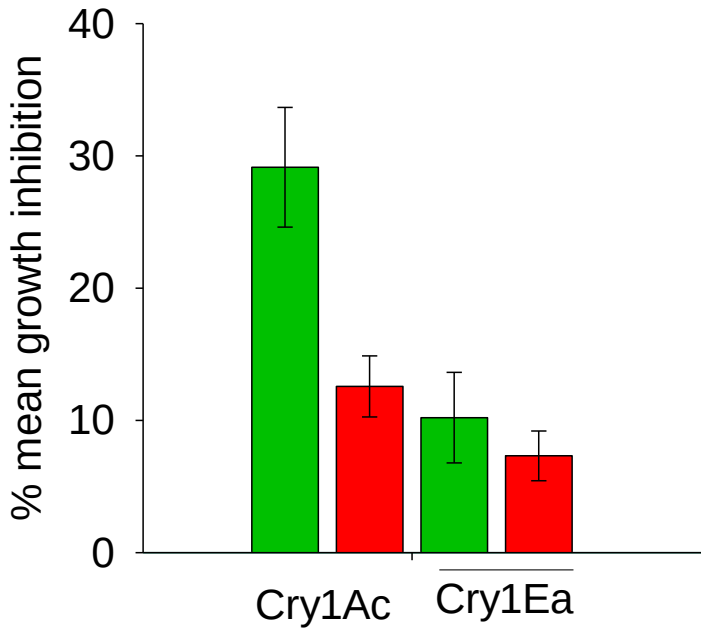


Fig. 12. Larval growth inhibition (% means ± SEM) of Benzon (bar 1 in green) and 456 (bar 2 in red) strains of *S. frugiperda* associated with TC1507 maize expressing Cry1Fa toxin when exposed to a diet treated with purified Cry1Ac, or Cry1Ea toxins at 7th day after inoculation of neonates.

3.5. Discussion

Populations of *S. frugiperda* in Puerto Rico developed high levels of resistance to corn event TC1507 producing Cry1Fa toxin, and this resistance has remained stable even long after removal of TC1507 maize from the local markets (Storer et al., 2012). This was the first documented example of field-evolved resistance to a Bt crops in U.S. territory. Availability of a strain generated from *S. frugiperda* egg masses from Puerto Rico, allows for characterization of field-evolved resistance to Bt maize. In this study provide data on cross-resistance patterns in *S. frugiperda* with field-evolved resistance to Bt maize. This information is important to identify efficacious toxins to manage resistant *S. frugiperda* through *cry* gene staking or pyramiding efforts. Data clearly support that resistance to Cry1Fa in 456 larvae is through alteration of toxin binding and that these larvae display cross-resistance to toxins that share this binding site with Cry1Fa.

As previously reported (Hernandez and Ferre, 2005; Luo et al., 1999), and present binding competition experiments support the existence of a shared binding site between Cry1Fa and Cry1A toxins, which is lacking or modified in larvae from the 456 strain. This common binding site is not shared by Cry1Ca toxin, which has alternative binding sites on *S. frugiperda* BBMV. Previous reports demonstrate the development of Bt resistance and cross-resistance by alteration or modification of a shared binding site on brush border membranes (Ferre and Van Rie, 2002). Based on our binding competition data, a population of binding sites A is recognized by Cry1Fa, Cry1Ab, and Cry1Ac, while a second sites B is recognized by Cry1Ca. Alteration of site A in 456 larvae is responsible for resistance to Cry1Fa and Bt maize event TC1507 (chapter-II) (Fig. 13).

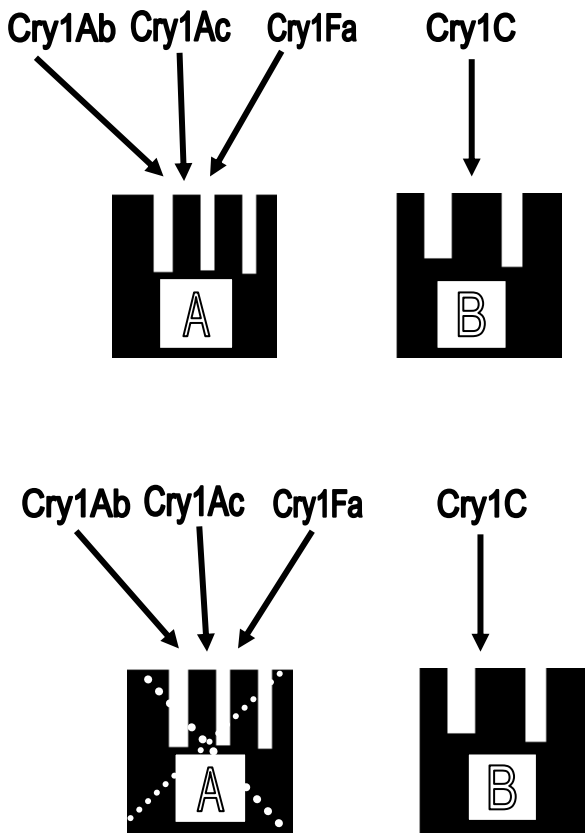


Fig. 13. Model proposed for binding of Cry1A, Cry1Fa and Cry1Ca to binding sites in *S. frugiperda* mid-gut membrane from susceptible (A) and resistant; (B) strains. Dashed arrow indicates that the modified or altered binding site A, which resulted in reduced or loss of binding of Cry1Fa, Cry1Ac and Cry1Ab to site A.

The proposed binding model is further supported by our bioassay data in which we detected cross-resistance with Cry1Ab but not with Cry1Ca. While present study was unable to detect significant mortality using Cry1Ac, growth inhibition assays demonstrated reduced toxin effects on larvae from the 456 compared to Benzon strain. However, binding site model proposed based on present study is in contrast to previous reports suggesting that Cry1Fa and Cry1Ca share a common binding site in *S. frugiperda*

BBMV (Luo et al., 1999). All other published binding studies in lepidopteran species support independent binding sites for Cry1Fa/Cry1A and Cry1Ca toxins (Ballester et al., 1999; Banks et al., 2001; Gonzalez-Cabrera et al., 2003b; Granero et al., 1996b; Luo et al., 1999). In addition, strains of *S. frugiperda*, and *S. littoralis* resistant to Cry1A/Cry1Fa not shown cross-resistance to Cry1Ca toxin (MullerCohn et al., 1996) While there are examples of cross-resistance between Cry1A and Cry1C toxins in *S. exigua* (Hernandez-Martinez et al., 2009; Moar et al., 1995), it has been attributed to protease-mediated mechanisms (Anilkumar et al., 2008; Candas et al., 2003; Li et al., 2004; Oppert et al., 1997). Based on this information, and while I do not know the reason for our discrepancy in binding results with the data of Luo et al (1999) present study conclude that Cry1Fa and Cry1Ca do not share binding sites in *S. frugiperda*.

Larvae from strain 456 were not cross-resistant to Xentari WG or DiPel ES pesticidal formulations. In the case of DiPel ES, this product is composed of spores and toxins produced by Bt var. *kurstaki*, which include Cry1Aa, Cry1Ab, Cry1Ac, and Cry2A and Cry2B (Mohan, 2001). Based on this composition, activity of DiPel ES towards larvae of the 456 strain of *S. frugiperda* would be due to Cry2A and Cry2B toxins. However, these toxins are not considered highly active towards *S. frugiperda* (van Frankenhuyzen, 2009). One possibility explaining activity of DiPel ES against 456 larvae is insecticidal activity of the Bt spores in the product. In support of this hypothesis, *S. exigua* larvae resistant to Cry1C suggest that use of formulations containing multiple Cry proteins and spores could delay the development of resistance in field populations than only Cry toxins used. Furthermore, development of resistance to a

spore-crystal mixture could not be achieved in target insects. Mechanisms other than binding will be required to develop resistance in target insects. Susceptibility of larvae from strain 456 to Xentari WG may be easily explained by the Cry1Ca and Cry1Da toxins produced by *B. thuringiensis* var. *aizawai* (Moar et al., 1995).

Present study also tested susceptibility of 456 larvae to Cry1Bb and Cry1Da, which are reportedly very active against *S. frugiperda*, and Cry1Ea, which displays levels of activity similar to Cry1Ac. The rationale for testing Cry1Ea was to test for the possibility that resistance to Cry1Fa could result in increased susceptibility to another toxin. In these bioassays, larvae from strain 456 were highly susceptible to Cry1Bb and Cry1Da, while Cry1Ea was not effective. The same results were observed with larvae from the susceptible strain. In contrast to results with Cry1Ac, present study did not detect differences in growth inhibition between susceptible and 456 larvae after exposure to Cry1Ea. These data support that alteration of the Cry1A-Cry1Fa binding site in 456 larvae does not affect binding of Cry1Bb or Cry1Da. In support of altered binding, the existing of two separate high affinity Cry1 binding sites in *S. exigua* and *S. frugiperda* was reported. One of these two binding sites bind to Cry1Ac, and the other site is for Cry1Bb and Cry1Ca toxins independently (Luo et al., 1999). Although I do not know the reason for the low activity of Cry1Ea against *S. frugiperda*, present study data may suggest that alterations in the Cry1A-Cry1Fa binding site do not affect binding of this toxin.

The results of this study provide evidence that field-evolved resistance to Cry1Fa in *S. frugiperda* is related to alteration of binding sites shared with Cry1A toxins. Based

on this information, new Bt maize hybrids producing multiple toxins not sharing binding sites with Cry1Fa should effectively control resistant *S. frugiperda*. Thus, Cry1Fa-resistant *S. frugiperda* from Puerto Rico are highly susceptible to SmartStax® and PowerCore® maize variety combined with Cry1F, Cry1A.105 and Cry2Ab2 toxins (Storer et al., 2012). New maize hybrids expressing Cry1Bb or Cry1Ca or Cry1Da toxins would also be able to overcome Cry1Fa-resistant *S. frugiperda* larvae. The absence of cross-resistance to Xentari WG and Di-Pel in strain 456 has important practical implications for rotation of maize hybrids with these pesticides as a potential approach to manage Bt resistance.

CHAPTER IV

Fitness costs in *Spodoptera frugiperda* with field-evolved resistance to Bt maize

4.1. Abstract

Increasing adoption of transgenic crops expressing *cry* and *vip* toxin genes from *Bacillus thuringiensis* (Bt) crops represents an augmented risk for development of insect resistance. This risk would be greatly influenced by the existence of fitness costs associated with resistance alleles. Most available data on fitness costs in resistance to Bt toxins available to date have been derived from laboratory-selected insect strains. In this work, fitness costs associated with high levels of field-evolved resistance to Bt maize event TC1507 was determined in a strain of the fall armyworm, *S. frugiperda* (J. E. Smith) originated from Puerto Rico. The present study compared fitness parameters in susceptible (Benzon), resistant (456) and hybrid individuals when reared on meridic diet, maize or soybean leaf tissue, and cotton reproductive tissues. Measured fitness parameters included larval survival, larval and pupal weights, developmental time of larval, pupal and adult stages, reproductive traits, and sex ratio. The only difference detected in resistant compared to susceptible insects was a significant increase in their larval developmental time, which could result in emergence asynchrony between susceptible and resistant adults. The present study detected increased fitness of hybrids (heterosis), probably due to the diverse genetic backgrounds of the susceptible and resistant strains used. To further test the importance of the detected fitness costs in resistance, present study monitored stability of resistance through several generations of rearing in the absence of selective pressure. Data from present study demonstrate the lack of fitness costs relevant to the stability of field-evolved resistance in *S. frugiperda*.

4.2. Introduction

The fall armyworm, *Spodoptera frugiperda* (J. E. Smith), is an economically important pest of maize, cotton, soybean, and many grasses in the Southern U.S.A. (Sparks, 1986). Larvae of this insect cause economic damage by feeding on the maize whorl and reproductive parts of cotton and soybean plants (Barros et al., 2010). Due to seasonal migration from Caribbean islands and Central America through Florida and Texas, respectively (Nagoshi et al., 2008), *S. frugiperda* can cause sporadic damage even in maize-growing regions where the insect does not overwinter. The biological characteristics of *S. frugiperda*, coupled with suboptimal control and widespread resistance to many insecticidal groups, make control difficult.

Transgenic crops that express insecticidal *cry* and/or *vip* protein genes from the bacterium *Bacillus thuringiensis* (Bt) provide the opportunity to control insect pests that are difficult to manage with synthetic insecticides, maximize crop yields, and conserve beneficial arthropods (Shelton et al., 2002). Hence, transgenic Bt maize event TC1507 expressing the *cry1Fa* toxin gene was developed to control stalk borers and secondary insect pests, including *S. frugiperda* (Siebert et al., 2008). Widespread and continuous planting of Bt maize coupled to geographic isolation and unique climatic conditions were hypothesized to favor development of field-evolved *S. frugiperda* resistance to event TC1507 in Puerto Rico. This resistance has remained stable in maize-growing areas of Puerto Rico, even six years after removal of event TC1507 from the regional market (Storer et al., 2012).

The development of resistance to insecticides is usually associated with relevant fitness costs, defined as adverse effects on the ability to survive and/or reproduce in the absence of selection pressure. These fitness costs can greatly influence evolution of insect resistance to Bt crops in field populations by affecting viability of homozygous resistant and/or heterozygous insects (Carriere and Tabashnik, 2001). Current resistance management for Bt crops is based on recessive resistance and the high dose/refuge strategy (Gould, 1998), which involves planting of suitable non-Bt host crops to maintain large numbers of susceptible individuals and minimize matings between homozygous resistant adults emerging from Bt fields. The heterozygous individuals generated by this approach are expected to be controlled by the high dose toxin levels produced by Bt crops.

In this scenario, recessive fitness costs affecting the duration of developmental periods may contribute to asynchronous (non-random) matings of resistant and susceptible adults in the refuge (Gould, 1998; Tabashnik et al., 1994b), thus decreasing the efficiency of the strategy. In contrast, non-recessive fitness costs affecting heterozygous individuals would decrease their viability and contribute to decrease the frequency of resistance alleles in the population. In other cases, fitness costs associated with Bt resistance can interact with host plant (Bird and Akhurst, 2007; Carriere et al., 2005; Janmaat and Myers, 2006; Raymond et al., 2007b), secondary chemicals (Carriere et al., 2004), insect pathogens, and natural enemies (Gassmann et al., 2006; Raymond et al., 2007a). If fitness costs are high on alternative host plants and also non-recessive, then increasing the abundance of these plants would help delay the evolution of

resistance (Tabashnik et al., 2003). These observations demonstrate that characterization of fitness costs associated with field-evolved resistance to Bt crops is highly important to the development of effective resistance management practices.

Diverse results on the existence of fitness costs have been reported from studies using laboratory-selected insect strains. In these reports, the existence of fitness costs was evaluated by comparing life cycle traits (survival, mass parameters, developmental periods and fertility parameters) between susceptible and resistant insects, or by monitoring the stability of Bt resistance in hybrid strains containing susceptible and resistant alleles (reviewed by Gassmann et al., 2009). Fitness costs, such as reduced survival (Groeters et al., 1994), lower larval growth rate (Liu et al., 1999), and reduced fecundity and mating success (Groeters et al., 1993), usually render resistance to Bt unstable. However, in other cases, such as Cry1Ac-resistant *H. virescens*, no fitness costs were detected (Gould and Anderson, 1991). Stability of resistance to Bt pesticides was also reported in field-selected *P. xylostella* (Sayyed and Wright, 2001;Tabashnik et al., 1995; Tang et al., 1997).

The present study was aimed to estimate fitness costs in a field-evolved Bt maize-resistant *S. frugiperda* strain. Here I report results of life-cycle trait comparisons (weights, developmental times and reproductive parameters) among resistant, susceptible and heterozygous insects, and the stability of Bt resistance in a hybrid strain containing a mixture of all susceptible and resistant genotypes.

4.3. Material and Methods

4.3.1. Insect strains

A susceptible *S. frugiperda* strain (Benzon) was started from purchased egg masses (Benzon Research, Carlisle, PA). This strain was maintained on leaf tissue from non-transgenic isolines to TC1507 maize. The *S. frugiperda* strain 456 resistant to Bt maize event TC1507 was developed from egg masses collected in maize fields around the Juana Diaz area (Puerto Rico), and initially displayed high levels of resistance to Cry1Fa toxin (Blanco et, al., 2010). Resistance in the 456 strain is transmitted as an autosomal recessive trait (Chapter-II). Neonates of strain 456 were selected to third instar with fresh TC1507 maize leaf tissue of the V6-V8 leaf stage for 20 generations in the laboratory. Heterozygous strains were generated by sexing pupae from the Benzon and 456 strains and crossing 100 emerging adults of the opposite sex from each strain. The strains were named according to the sex of the resistant adults in the cross as 456M (males were from strain 456) or 456F (females from strain 456). All strains were reared in an incubator (Percival) at 25 ± 2°C and 65% RH under L:D 16:8.

4.3.2. Host plants

Non-Bt maize seed (2T780 isogenic to event TC1507) was supplied by Dow AgroSciences (Indianapolis, IN), while conventional (AG 430) soybean and cotton (phytogen 315) were supplied by Dr. Scott D. Stewart (West Tennessee Research and Education Center, Jackson, TN). Plants were grown in 20 cm pots containing a commercial garden soil and maintained in a greenhouse under 26 ± 2°C, 65 ± 5% RH and

L:D 16:8. Staggered sowings of all three types of host plants were used to get synchronized development so that plant parts would be available for experiments.

4.3.3. Life trait comparisons

Various fitness cost parameters of individuals from the Benzon, 456, and hybrid strains were estimated during feeding on meridic diet (BioServ, Frenchtown, NJ), non-Bt maize, soybean, and cotton plant tissues. Based on *S. frugiperda* larval feeding preference (Ali et al., 1990), different plant tissues from maize (leaves of V6-V8 stage), soybean (leaves), and cotton (squares, flowers and bolls) were used in the experiments. Individual neonate larvae (32 per treatment, replicated twice) from each of the four *S. frugiperda* strains were placed in 29.57 ml plastic cups containing the specific food source, and reared in an incubator at 25 ± 2°C, 65%± 5 RH, and LD 16:8 photoperiod. For experiments with plant tissues, 1% agar plugs were included in the plastic cups to preserve humidity, and plant material was replaced when necessary. Larvae in the cups were checked daily until they reached the pupal stage. Percent survival was estimated by counting the number of larvae of each strain that developed into pupae in comparison to the initial larval population. Fourth-instar larvae and 4-day old pupae were weighed using a balance.

Pupae were sexed and equal numbers of male and female pupae (16 each) from each strain and treatment were combined in 1 L plastic jars. After emergence, adults were fed a 10% sucrose solution and ovipositional jars were covered with paper towels to prevent escape and as an egg-laying substrate. The daily number of egg masses laid in each jar was recorded until the end of the oviposition period, and the egg masses were

transferred daily to individual plastic bags (10x8 inches) for hatching. The number of egg masses with hatching neonates was counted daily to assess reproductively (hereafter referred as fertility). Total duration (in days) of the larval, pupal, and adult stages as well as number of egg masses and hatched egg masses were recorded. To determine the sex ratios after feeding on the different food sources, neonates from the eggs collected in the experimental procedures above were reared to pupation in the specific food source and then the number of male and female pupae recorded.

4.3.4. Stability of resistance

A colony containing a mix of all susceptible, heterozygous, and resistant genotypes was generated from sib mating of heterozygous colonies to assess stability of resistance. Heterozygous colonies (456M and 456F) were generated as described above (these represented generation 1). Moths (100 males and 100 females) from each heterozygous colony were placed in a 60 x 60 cm^2 styrofoam container for mating and egg laying. Neonates (n=350) hatched from the eggs laid were reared on meridic diet (these represented generation 2). This procedure was continued for a total of 12 generations.

To test the level of resistance in 456 strain larvae in the absence of TC1507 selection reared up to 12 generations, the present study used bioassays with a discriminatory Cry1Fa dose were used. Based on bioassays with Benzon, 456, and heterozygous neonates and purified Cry1Fa toxin, the LC_{95} dose (3.8µg/cm^2) as the discriminatory dose that would kill 95% of the homozygous susceptible and heterozygous larvae was selected, while only inducing marginal mortality among 456 larvae (~5%).

The stability of resistance in the generated strain was tested by comparing percentage mortality observed in bioassays of neonates from parental (Benzon), F1, F_2, F_5, F_{10}, and F_{12} generations using the discriminatory Cry1Fa dose.

4.3.5. Statistical analysis

Differences in percent larval survival, weight gained by larval and pupal stages, developmental time, egg mass laid, and egg mass hatch parameter means for each strain and crosses were analyzed by analysis of variance (ANOVA) using SAS release 9.2 (SAS Institute, Cary, NC). PROC MIXED (PROC UNIVARATE and PROC GPLOT) was used to ensure the assumptions of homogeneity of variance and normality. Developmental times, fecundity and fertility data were log transformed to normalize variances and for equal distribution. The means were separated at α = 0.05 by Fisher's protected least square difference (LSD; PROC MIXED) using SPSS Base 21.0 for Windows (SPSS Inc., Chicago, IL). Sex ratios were analyzed by Chi-square-of-fit to a 1:1 (female/male) ratio. Host and fitness parameter interactions for each strain were analyzed by using two-way analysis of variance (SPSS-12). The discriminatory (LC_{95}) dose of Cry1Fa was calculated using the Polo-Plus v.2.1 program (LeOra software, Petaluma, CA.). The two-way ANOVA details of fitness costs parameters were presented in the appendix.

4.4. Results

4.4.1. Larval survival

Survivorship of four strains of neonate larvae was tested in bioassays with four food sources (Fig. 14). No significant difference was detected (df=3, F=0.359, P=0.784)

among the *S. frugiperda* strains (Benzon, 456, 456M, and 456F). However, a significant difference (df=3, F=4.009, P=0.046) in larval survivorship was observed among food sources. The interaction of *S. frugiperda* strain and food source showed no significantly different (df=9, 32, F=0.129, P=0997). In the strains, the percentage of larval survival on cotton reproductive tissues (flowers>squares>bolls) was significantly greater than when larvae were fed cotton leaf tissue (data not shown). Based on this observation cotton reproductive tissues were chosen for further studies.

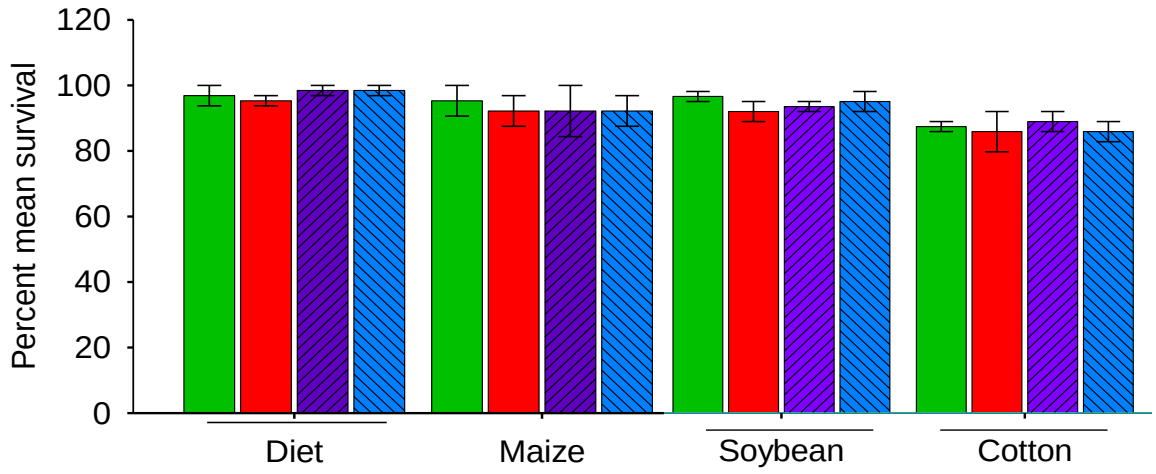


Figure 14. Mean (± SEM) percent larval survival of Benzon (bar 1 in green), 456 (bar 2 in red), 456F (bar 3 in violet) and 456M (bar 4 in blue) strains of *S. frugiperda* on four tested four food sources in absence of Cry1Fa toxin, (n=32/replication; two replications of each strain on four food sources). Data analyzed α = 0.05 by using Fisher's protected least square difference (LSD; PROC MIXED) (SAS Institute 2002).

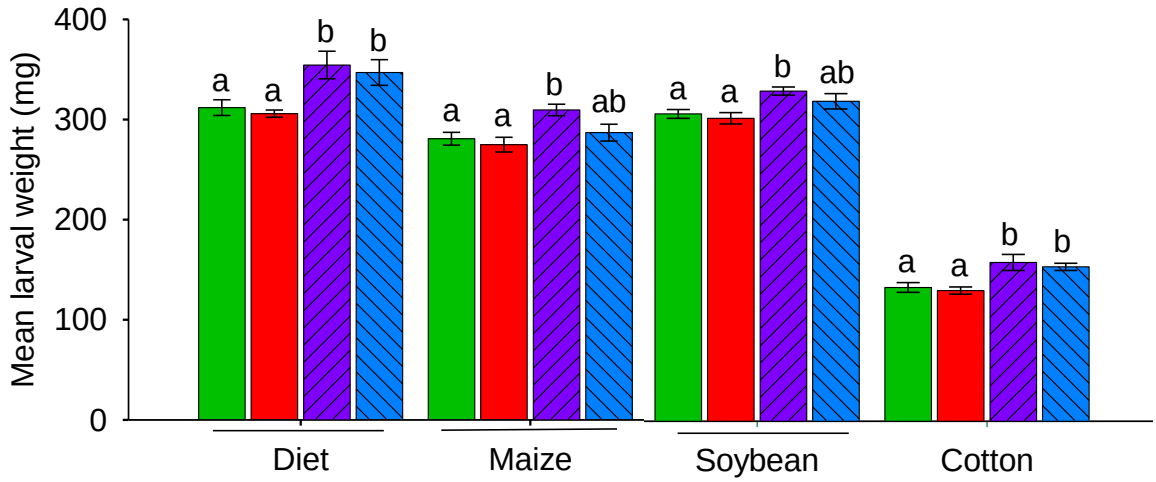
4.4.2. Larval and pupal weights

The weight gained during development by larvae and pupae of strains Benzon, 456, 456M, and 456F after feeding on meridic diet, maize, soybean or cotton is shown in

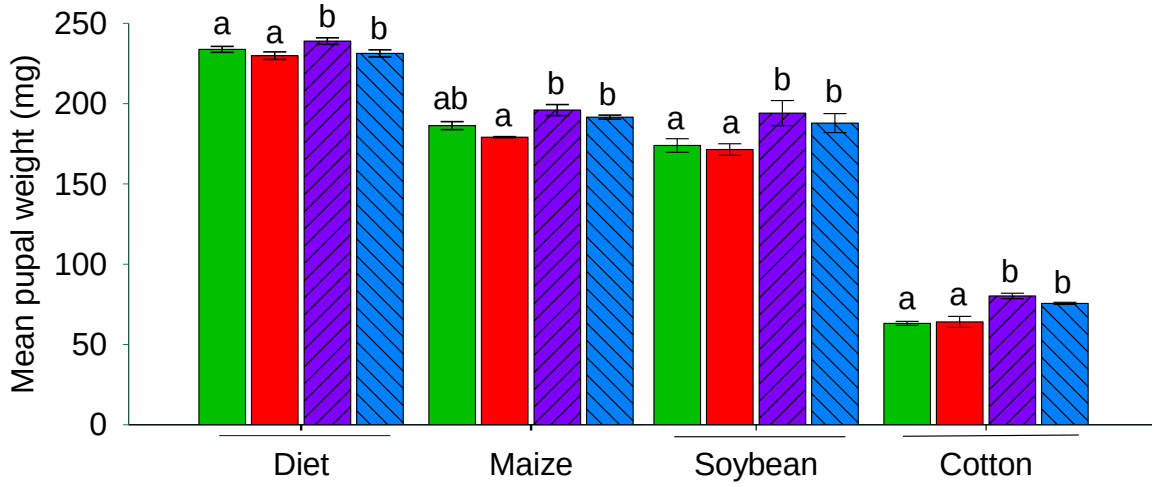
Figures 2A and 2B, respectively. No significant differences in larval or pupal weight when comparing individuals were detected from the Benzon and 456 strains (Fig. 15A and 15B). Statistical analysis confirmed significantly increased weight gain in 456M and 456F larvae (df=3, F=39.23, P=0.0001) and pupae (df=3, F=35.618, P=0.0001) compared to larvae from the Benzon and 456 strains, suggesting the existence of heterosis. This heterosis was observed among all four tested food sources in larvae (df=3, F=1087.29, P=0.0001) and pupae (df=3, F=3213.45, P=0.0001). The weight in 4^{th} instar larvae was 8-9% higher in 456M and 456F compared to Benzon and 456 larvae (Fig. 15A). Statistical analyses of strain and food interactions indicated that the larval (df=9, 1024, F=1.746, P=0.294) and pupal weights (df=9, 1024, F=2.646, P=0.071) followed a similar hybrid vigor trend in all food sources.

Figure 15. Mean (+ SEM) larval (A) and pupal (B) weights of Benzon (bar 1in green), 456 (bar 2 in red), 456F (bar 3 in violet) and 456M (bar 4 in blue) strains of *S. frugiperda* on four food sources in absence of Cry1Fa toxin.

A


Mean larval weight (mg)
400
300
200
100
0
a a b b
a a b ab
a a b ab
a a b b
Diet
Maize
Soybean
Cotton

B


Mean pupal weight (mg)
250
200
150
100
50
0
a a b b
ab a b b
a a b b
a a b b
Diet
Maize
Soybean
Cotton

4.4.3. Developmental times

Comparison of the duration of larval, pupal and adult life stages among all strains on the four food sources only showed a significant difference in length of the larval period. Larval developmental times were significantly different among strains (df=3, F=529.38, P=0.0001) and food sources (df=3, F=10309.58, P=0.0001). The shortest larval developmental time (13-15 days) was monitored for all tested strains when meridic diet was used as food source. When compared to meridic diet, the maximum delay in larval stage development was observed when larvae fed on cotton plant reproductive tissues, followed by maize and soybean leaf tissues. Larvae from the 456 strain required significantly longer developmental periods compared to the other three strains in all food sources. The interaction between larval stage duration and food source confirmed a significant delay in development of larvae from the 456 strain (df=9, 1002, F=7.866, P=0.511) (Table 3).

When comparing the number of days required to complete development for each larva using meridic diet as a food source, the majority/higher percentage of larvae from strains 456 (59.38%) and 456M (71.88%) required 15 days to complete development, while the majority of larvae in Benzon (59.38%)and 456F (46.88%) required 14 days. The remaining percentage of larvae in each strain displayed similar developmental times. In the case of maize leaf tissue the majority of larvae from strain 456 (57.81%) required 17 days to complete development, one day longer than the majority of larvae from strains 456F (42.19%) and 456M (54.69%), and two days longer than the majority of larvae in strain Benzon (46.88%). When using soybean leaf tissue, a higher percentage of larvae

from strains 456 (54.69%) and 456M (48.44%) required 21 days to complete development, compared to 19 days in the majority of larvae (64.06%) from Benzon and 20 days in the majority of larvae (48.43%) from strain 456F. Lastly, when reproductive cotton tissues were used as food source, a 2-day delay in larval development was observed when comparing the majority of larvae in strain 456 (65.63%) and 456M (68.75%) to larvae in the Benzon (46.88%) and 456F (53.13%) strains (Table 3).

No significant differences among the four tested strains in pupal (df=3, F=32.028, P=0.687) or adult development (df=3,1002, F=4.784, P=0.063) was not detected. However, a significant difference among the tested four food sources for the length of pupal (df=3, F=565.070, P=0.001) and adult development (df=3, F=222.33, P=0.001) time was detected. The longest pupal developmental time was observed when larvae were fed on cotton tissues, followed by soybean, maize, and meridic diet. Longer pupal development time generally corresponded with shorter adult lifespan. The interaction between strain and food was not significantly different between the four strains on all tested food sources for pupal (df=9, 1002, F=2.152, P=0.897) and adult (df=9, 1002, F=1.513, P=0.138) development (Table 4).

Table 3. Mean (+ SEM) durations of larval, pupal and adult development periods (in days) for the Benzon, 456, 456M, and 456F strains of *S. frugiperda* depending on the food source.

Life cycle developmental time* (days)

Strain	Larval				Pupal				Adult			
	Diet[+]	Maize[+]	Soybean[+]	Cotton[+]	Diet[+]	Maize[+]	Soybean[+]	Cotton[+]	Diet[+]	Maize[+]	Soybean[+]	Cotton[+]
Benzon	13.80± 0.08[a]	14.92± 0.08[a]	19.36± 0.06[a]	20.94± 0.09[a]	8.14± 0.07[a]	8.42± 0.21[a]	9.20± 0.10[a]	9.63± 0.31[a]	15.3± 0.30[a]	13.28± 0.36[a]	13.03± 0.48[a]	12.38± 0.19[a]
456	15.03± 0.08[c]	17.08± 0.08[d]	21.45± 0.06[d]	22.97± 0.07[c]	8.31± 0.16[a]	8.61± 0.20[a]	9.70± 0.35[a]	9.89± 0.45[a]	15.6± 0.18[a]	13.38± 0.31[a]	13.02± 0.49[a]	12.38± 0.19[a]
456F	14.16± 0.09[b]	15.77± 0.09[b]	19.83± 0.09[b]	21.47± 0.06[b]	8.72± 0.36[a]	8.48± 0.24[a]	9.36± 0.18[a]	9.77± 0.38[a]	15.1± 0.41[a]	13.22± 0.39[a]	12.63± 0.69[a]	12.28± 0.14[a]
456M	14.23± 0.12[b]	16.50± 0.07[c]	21.05± 0.09[c]	22.75± 0.06[c]	8.44 ± 0.22[a]	8.53± 0.23[a]	9.50± 0.25[a]	9.83± 0.41[a]	15.6± 0.16[a]	13.27± 0.37[a]	12.81± 0.59[a]	12.38± 0.19[a]

* Developmental time data was analyzed (P = 0.05) by using Fisher's protected least square difference (LSD; PROC MIXED)

[+]Food data within a column followed by the same latter are not significantly different and different letters are significantly different (developmental times were log transformed to normalize variances).

Table 4. Mean percent larval development periods distribution (in days) recorded for the Benzon, 456, 456M, and 456F strains of *S. frugiperda* depending on the food source*.

Food source	Day	Benzon	456	456F	456M
Meridic diet	13	29.69±0.23	0.000±0.00	18.75±0.44	32.81±0.58
	14	59.38±0.31	18.75±0.56	46.88±0.20	28.13±0.72
	15	10.94±0.24	59.38±0.82	34.38±0.55	71.88±0.64
	16	0.000±0.00	21.88±0.72	0.000±0.00	0.000±0.00
Maize	14	34.38±0.38	0.000±0.00	0.000±0.00	0.000±0.00
	15	46.88±0.41	0.000±0.00	40.63±0.46	0.000±0.00
	16	18.75±0.54	17.19±0.75	42.19±0.15	54.69±0.55
	17	0.000±0.00	57.81±0.33	17.19±0.82	40.63±0.12
	18	0.000±0.00	25.00±0.40	0.000±0.00	0.000±0.00
Soybean	19	64.06±0.11	0.000±0.00	34.38±0.76	0.000±0.00
	20	35.94±0.53	0.000±0.00	48.44±0.40	23.44±0.41
	21	0.000±0.00	54.69±0.00	17.19±0.85	48.44±0.10
	22	0.000±0.00	45.31±0.00	0.000±0.00	28.13±0.34
Cotton	20	29.69±0.45	0.000±0.00	0.000±0.00	0.000±0.00
	21	46.88±0.56	0.000±0.00	53.13±0.32	0.000±0.00
	22	23.44±0.83	18.75±0.92	46.88±0.65	28.13±1.02
	23	0.000±0.00	65.63±0.45	0.000±0.00	68.75±0.84
	24	0.000±0.00	15.63±0.84	0.000±0.00	3.13±0.55

* Frequency distribution of percent larval developmental periods data were analyzed (P = 0.05) by using Fisher's protected least square difference (LSD; PROC MIXED).

4.4.4. Adult emergence

The observed delayed larval development greatly influenced the time of adult emergence. Thus, adults from the 456 strain were 2-3 days delayed in emergence compared to adult moths from the Benzon strain. A higher percentage of the adult emergence in strain 456 coincided with adult emergence in the heterozygous strains compared to Benzon (Fig. 16).

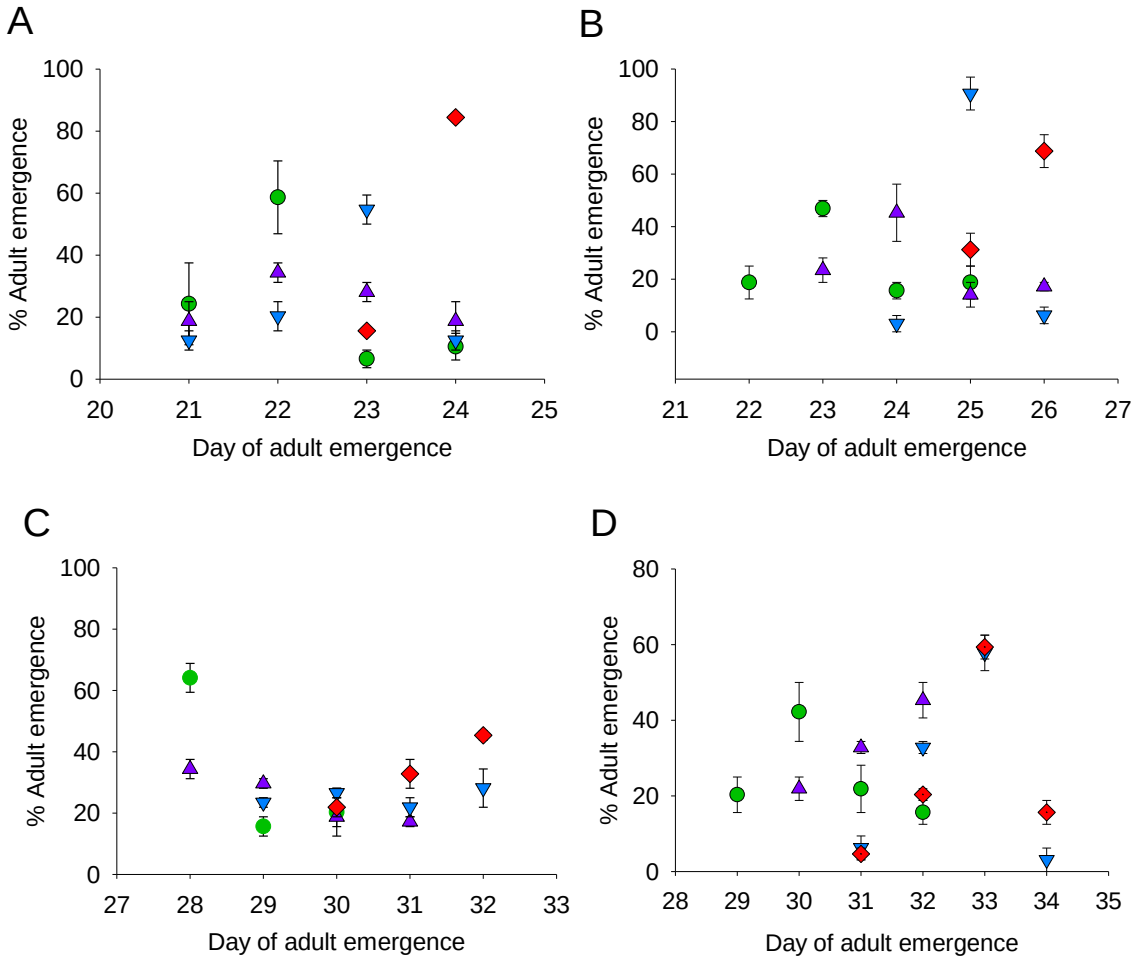


Figure 16. Percent adult's emergence recorded for each strain (Benzon (green circle), 456M (blue downward triangle), 456F (violet upward triangle) and 456 (red diamond)

fed with meridic diet (A), maize leaf tissue (B), soybean leaf and pod tissues (C) and cotton reproductive tissues (D).

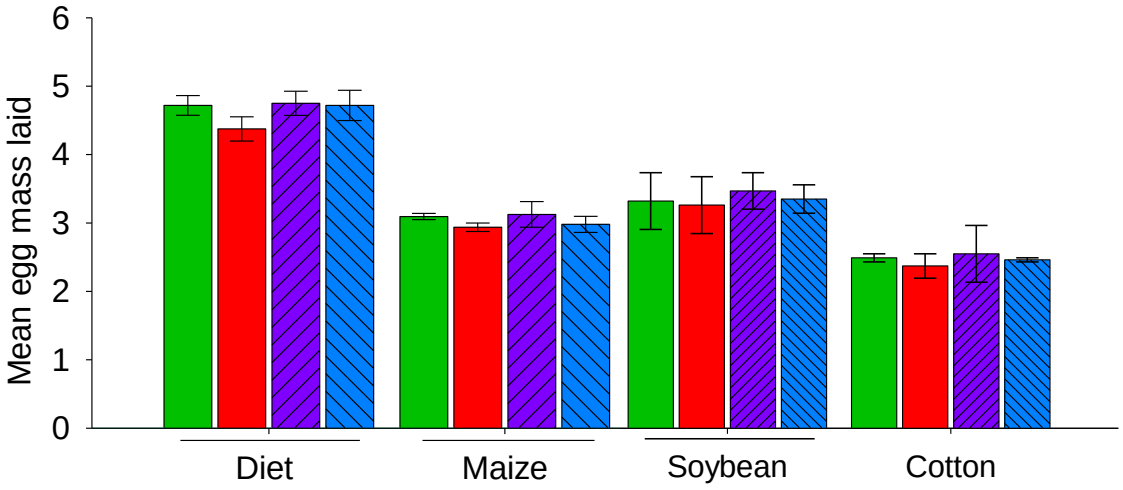
4.4.5. Average number of egg masses laid and egg mass hatchability

The mating period for all tested strains lasted for 7 days after adult emergence. Active participation in mating behavior was observed during the first three days of emergence, with much lower mating frequency observed in the remaining four days. The number of egg masses laid were not significantly different among the strains (df=3, 2537 F=0.464, P=0.708), although the average number of egg masses was significantly different among food sources (df=3, 2537, F=27.964, P=0.001). The non significant interaction (df=9, 2537, F=0.037, P=1.0) between the strains and food sources supported similar fecundity trends in all strains with every tested food source. A higher percent of egg mass hatch (fecundity) was recorded for all strains when fed on meridic diet, followed by soybean, maize, and with cotton, which had the lowest fecundity (Fig. 17A).

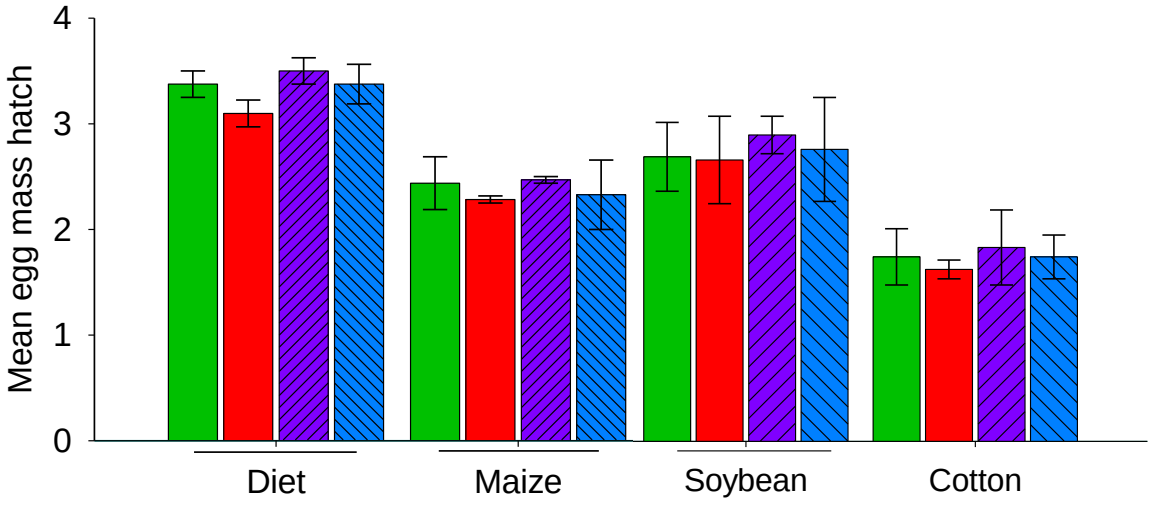
Statistical analysis of the number of egg masses hatched per number of egg masses laid demonstrated no significant differences among the four strains (df=3, 2535, F=0.282, P=0.839). However, hatchability differed significantly among the four food sources (df=3, 2535, F=24.869, P=0.001). Higher hatchability was observed when larvae were fed on soybean, followed by maize and diet, and cotton resulting in the lowest percent of hatchability (df=3,9, F=8.099, P=0.006). Statistical analysis of interactions between strain and food source indicated no significant differences in percent egg mass hatchability (df=9, 32, F=1.682, P=0.225) (Fig. 17B and 17C).

Figure 17. Mean (+ SEM) number of egg masses laid (A), hatched (B) and percent hatch (C) by Benzon (bar 1 in green), 456 (bar 2 in red), 456F (bar 3 in violet) and 456M (bar 4 in blue) strains of *S. frugiperda* when fed tested four food sources in absence of Cry1Fa toxin.

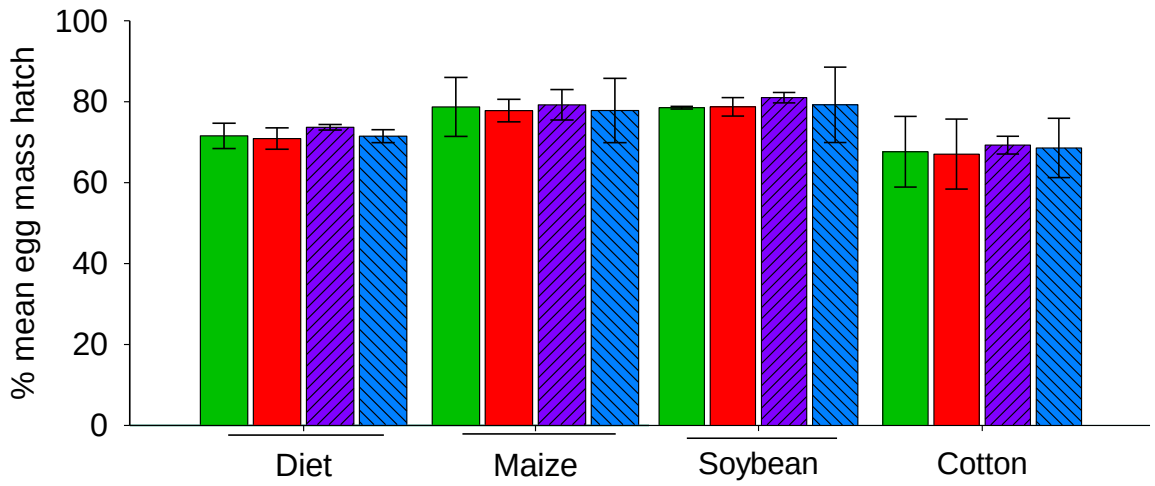
A


Mean egg mass laid
0
1
2
3
4
5
6
Diet
Maize
Soybean
Cotton

B


Mean egg mass hatch
0
1
2
3
4
Diet
Maize
Soybean
Cotton

C


% mean egg mass hatch
100
80
60
40
20
0
Diet
Maize
Soybean
Cotton

4.4.6. Sex ratio

The male to female sex ratio was estimated by sexing pupae from larvae of a second generation reared on a specific food source. Chi-square analyses showed that the sex ratio was not significantly different from the expected 1:1 ratio in any of the treatments (Table 5). A two-way ANOVA showed no significant difference in sex ratio among the four stains (df=3, 9, F=0.326, P=0.807) or among the different food sources (df=3, 9, F=1.495, P=0.281). There is no significant interaction (df=9, 32, F=1.838, P=0.189) between strain and food and sex ratio patterns were similar among the four strains on all tested food sources.

Table 5. Sex ratio estimated among adult strains of Benzon, 456, 456M and 456F emerged from after fed with meridic diet, maize and, soybean leaf tissue and cotton reproductive tissues in absence of Cry1Fa toxin.

	Strain	Observed frequency		Expected frequency		χ^2		Male/Female
		Female	Male	Female	Male	(*df*=1)	P_{val}	
Artificial diet	Benzon	94	92	93	93	0.022	0.883	0.9787
	456	80	85	82.5	82.5	0.520	0.697	1.0625
	456F	82	74	78	78	0.412	0.528	0.9024
	456M	76	75	75.5	75.5	0.007	0.935	0.9868
Maize	Benzon	88	84	86	86	0.093	0.760	0.9545
	456	76	79	77.5	77.5	0.058	0.809	1.0395
	456F	92	99	95.5	95.5	0.257	0.612	1.0761
	456M	95	93	94	94	0.021	0.884	0.9789
Soybean	Benzon	82	86	84	84	0.095	0.758	1.0488
	456	81	83	82	82	0.024	0.876	1.0247
	456F	79	87	83	83	0.386	0.535	1.1013
	456M	95	95	95	95	0.000	1.000	1.0000
Cotton	Benzon	80	85	82.5	82.5	0.152	0.687	1.0625
	456	81	79	80	80	0.025	0.874	0.9753
	456F	77	80	78.5	78.5	0.057	0.811	1.0390
	456M	80	84	82	82	0.098	0.757	1.0500

4.4.7. Stability of resistance

To test the existence of fitness costs in individuals from the 456 (resistant) compared to Benzon (susceptible) and hybrid strains, an experiment was performed to test stability of resistance in a strain (Benx456) containing all possible genotypes for resistance to Cry1Fa according to Mendelian inheritance. The Cry1Fa LC_{95} concentration (3.88 µg/cm^2) using generation 1 (heterozygous) larvae in diet surface contamination assays was determined (Fig. 18A). This toxin concentration was chosen as a discriminatory dose to detect the presence of resistant individuals in a generation, as it resulted in >95% mortality of homozygous (Benzon) and heterozygous (from Benzon x 456 crosses) larvae but induced marginal (<5%) mortality among 456 larvae. Using this discriminatory dose, susceptibility in the Ben456 strain during sequential sib mating and rearing in meridic diet was tested for 12 generations. Bioassays with the discriminatory Cry1Fa dose against neonate larvae did not detect significant differences (df=3,7, F=2.121, P=0.24) in susceptibility among larvae from the 2, 5, 10, and 12 generations. The 70-74% larval mortality detected in these generations supported the stability in the absence of a selective regime of the predicted proportion of homozygous resistant individuals in the populations considering Mendelian inheritance of resistance (Fig. 18B).

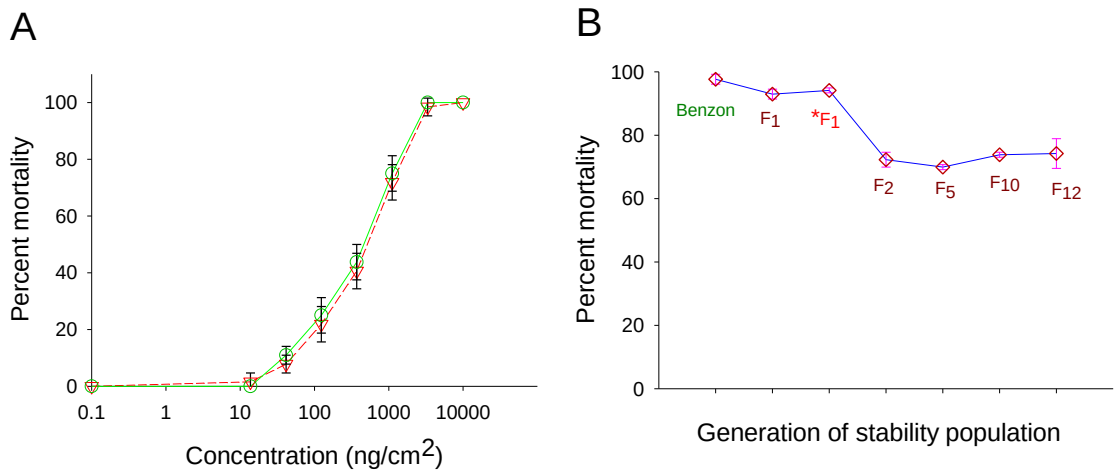


Figure 18. Stability of resistance to the Cry1Fa toxin in the *S. frugiperda* population associated with Tc-1507 Bt maize was tested by rearing stability population on meridic diet in the absence of Cry1Fa selection pressure. Cry1Fa LC_{95} dose discrimination value (3.85µg/cm^2) was calculated for heterozygous population in generation 1 using surface diet contamination bioassays (Fig. A). The larval populations from generation 2, 5, 10 and 12 were tested for their mortalities using the Cry1Fa LC_{95} concentrations (Fig. B).

4.5. Discussion

Results of life cycle trait comparisons and resistance stability studies support the absence of relevant fitness costs in *S. frugiperda* larvae with field- evolved resistance to Bt maize producing Cry1Fa toxin. All the considered fitness parameters (survival, weight, developmental times, fecundity, and fertility) tested on diverse food sources (meridic diet, and leaf tissue of maize and soybean, and reproductive tissues of cotton) were similar among the susceptible and Bt maize-resistant strains, with the exception of a prolonged larval developmental period in resistant insects. The fitness data obtained support that the suitability of the food source to *S. frugiperda* strains varied in the order of meridic diet>maize=soybean>cotton. Similar data obtained with two alternative

hybrid strains support the lack of sex-linked fitness costs in resistance to Bt maize. Data from resistance stability experiments revealed that resistance was stable within the tested 12 generations on meridic diet, further supporting the lack of significant fitness costs associated with resistance.

Previous studies have shown that reproductive capacity of many lepidopteran species is proportional to the nutrient reserves acquired during larval stages, and is correlated with larval and pupal mass gain (Leahy and Andow, 1994). In the present study, no significant difference in larval and pupal weight between resistant and susceptible strains was observed, but a comparatively increased weight in hybrid larvae and pupae was detected. A number of reports suggest that this increased weight gain in heterozygotes is due to crosses between strains of different genetic background (Carriere et al., 2006; Gassmann et al., 2008). This increased weight gain in heterozygotes was concomitant with increased number of egg masses laid in the first day compared to 456 and Benzon strains, although these egg masses were sterile. Previous reports suggested that increased fecundity in heterozygotes is due to heterosis (Carriere et al., 2001a;Gahan et al., 2005; Sayyed and Wright, 2001). However, in subsequent egg-laying periods no significant fecundity differences among all four strains were detected. No differences in fertility were detected.

The success of current high dose/refuge strategies for Bt crops to hinder the development of Bt resistance in target insects relies on synchronized adult emergence among resistant and susceptible populations (Liu et al., 1999). The lengthening of the larval period detected for the 456 strain would directly affect the random mating

assumption of current insecticide resistance management (IRM) models. According to present data, susceptible adults would emerge 2-3 days before resistant adults, so that older susceptible moths would mate with the emerging naive resistant adults, increasing the probability of unsuccessful matings. In experiments with Bt-resistant *P. gossypiella*, 80% random mating occurred within 3 days of adult emergence. An average delay of 5.7 days in emergence of resistant compared to susceptible moths resulted in their asynchronous mating, although synchronous mating was observed between heterozygotes and susceptible adults. In *S. frugiperda* the average mating period is three days after adult emergence (Simmons and Marti, 1992), and after three days there is a reduction in the male capacity to inseminate females, resulting in reduced laid egg numbers (fecundity) and reduced egg viability (Murua et al., 2008; Rogers and Marti, 1994). Considering this information, it is possible that unsuccessful matings may occur between resistant and susceptible *S. frugiperda* in the non-Bt maize refuge, as suggested by present developmental data with strains 456 and Benzon. Furthermore, a higher percentage of adult emergence was synchronized between the 456 and heterozygote populations, which would favor matings between moths carrying at least one copy of the resistance allelle. These scenarios would facilitate an increase in the frequency of the resistance allele in the population.

Stable resistance to Bt var. *kurstaki* in a strain of *P. xylostella* was reported by Tang et al. (1997). This strain remained ~ 300 fold resistant for 10 generations in the absence of selection in the laboratory. In contrast, unstable resistance was documented for strains of *P. xylostella* resistant to Cry1C (Liu et al., 2001) and *Trichoplusia ni*

resistant to Bt var. *kurstaki* (Di-pel) (Janmaat and Myers, 2003). The existence of fitness costs, especially when transmitted as a dominant trait, would favor resistance unstability. On the other hand, resistance stability could be a result of inherently fixed resistance alleles (Tabashnik et al., 1994b). A *S. frugiperda* strain containing a mixture of susceptible, heterozygous and resistant genotypes was developed to monitor stability of resistance and asses the existence of significant fitness costs, when the resistance allele is not fixed in the population. Stability of resistance in this strain supports that under tested conditions field-evolved resistance to Bt maize is not associated with significant fitness costs.

Various physiological mechanisms associated with the steps in the mode of action of Bt toxin could be altered in resistant insects (Ferre and Van Rie, 2002; Taylor and Feyereisen, 1996). In the 456 strain a reduction of Cry1Fa toxin binding to the midgut brush border membrane was identified as mechanism responsible for resistance. This binding reduction is due to reduced expression of at least two alkaline phosphatase (ALP) genes in the midgut of 456 larvae (Chapter II). Altered target sites could induce deleterious effects due to the disruption of physiological processes (Uyenoyama, 1990). Mutations in cadherin, APN, or ALP can cause fitness costs by alteration of cell recognition/signaling/communication, maintenance of cell structure, morphogenesis, angiogenesis, and phosphorylation function in proteolysis, active absorption of metabolites and transport processes in which these proteins are involved (Macintosh et al., 1991). Alternatively, Roush and McKenzie (1987), described as pleiotropic effects or modifier genes could ameliorate fitness costs and stabilize resistance in the absence of

selection pressure in insect strains developed resistance to synthetic pesticides. Thus, reduced expression of selected ALP genes may be compensated by the alternative ALP genes, resulting in normal ALP enzymatic function in 456 larvae.

In field conditions, the magnitude and dominance of fitness costs in Bt-resistant insects can be affected by the suitability of crop hosts used in the refuge. Suitability of host crops to susceptible populations is also relevant, as a less suitable host crop can result in fitness costs and can promote increased frequency of resistance (Carriere and Tabashnik, 2001b). Thus, knowledge of the interaction between host plants and fitness costs associated with resistance to Bt crops could be helpful in guiding the choice of refuge cultivars. Janmaat and Myers (2005) reported that in *T. ni* the magnitude of fitness costs associated with Bt resistance increased with declining host plant suitability. Bird and Akhurst (2007) reported that fitness of *H. armigera* heterozygotes from crosses between susceptible and Cry1Ac-resistant moths was reduced when reared on sorghum compared to cotton or pigeon pea. In present assays a detrimental effect of any of the tested food sources in fitness of larvae from susceptible, resistant, or hybrid strains was not detected. However, better overall performance of all the tested strains in soybean and maize compared to cotton was detected. Based on these observations, the choice of refuge host would not be relevant in prevention of *S. frugiperda* resistance to Bt maize.

Resistance to Bt maize (TC1507) in *S. frugiperda* populations from Puerto Rico was transmitted as a recessive autosomal trait (Storer et al., 2010). Based on this observation, one would predict that removal of the selective agent (event TC1507) would result in reduced resistance frequency if resistance-linked fitness costs exist. However,

resistance to Cry1Fa in 2012 was still detected at similar levels compared to 2006, when the Cry1Fa maize was removed from the regional market (Storer et al., 2012). Factors that may affect resistance stability, apart from fitness costs, include the presence of similar mode of action proteins (Cry1Ab and Cry1Ac) in transgenic maize and cotton, use of Bt-based formulations, geographic isolation of populations, and non-random mating between susceptible and resistant adults. Data from present research suggest stability of Cry1Fa resistance in *S. frugiperda* after rearing for 12 generations in the absence of selection. This stability may be due to observed lack of fitness costs in the 456 strain. In this regard, the slightly delayed adult emergence in the 456 strain could contribute to asynchronous mating and stabilize resistance. While speculative at this point, this aspect may also be contributing to stability of resistance in field populations from Puerto Rico.

The study of fitness costs and resistance stability in *S. frugiperda* resistant to Cry1Fa-producing maize provides a better understanding of effectiveness of resistance management practices at the field level. Knowledge acquired from these studies provides an important opportunity to assess current resistance management practices. These data support the potential importance of diverse factors that are not considered in the current high dose/refuge IRM strategy for Bt crops in determining stability of field-evolved resistance, including potential non-random mating, lack of relevant fitness costs, and low influx of susceptible alleles in resistant populations. These factors may help explain the reported stability of resistance to event TC1507 in Puerto Rico. Alternative transgenic maize crops expressing multiple genes with diverse mode of action (pyramiding) are

expected to effectively control *S. frugiperda* resistant populations (Storer et al., 2012). Present data emphasize that lack of fitness costs and stability of resistance in field-evolved Cry1Fa resistant *S. frugiperda* need to be considered in assessment of effective insect resistance management regulatory framework.

6. SUMMARY AND CONCLUSIONS

The use of Bt cotton and Bt maize expressing insecticidal protein genes from *Baciilus thuringiensis* has revolutionized insect pest management by killing only target pests without affecting other non-target insect species. Field-evolved resistance development in target pest populations has become the major threat to the future utility of Bt crops. So far few species have been identified for their field-evolved resistance to Bt maize or Bt cotton, but there is no available data on resistance mechanisms associated with field level resistance. The present study provides the first characterization of a mechanism responsible for field-evolved resistance to Bt maize expressing the Cry1Fa toxin in *Spodoptera frugiperda*.

Leaf tissues and diet contamination bioassays indicated that larvae from *S. frugiperda* strain 456 are highly resistant to maize producing the Cry1Fa toxin with recessive and autosomal mode of inheritance. Binding and blotting experiments showed the reduced binding of Cry1Fa toxin to brush border membranes of resistant larvae compared to susceptible larvae. The binding reduction was also observed for Cry1A toxins but not for Cry1Ca toxin. This binding pattern is explained by shared binding sites for the Cry1A-Cry1Fa toxins that are not recognized by Cry1Ca toxin. Immunoblot detection of Cry1Fa binding receptors and specific activity assays revealed reduced levels of alkaline phosphatase (ALP) and no significant reduction in protein levels of aminopeptidase (APN) in resistant compared to susceptible larvae. The positive correlation between survival in maize producing Cry1Fa toxin and reduced ALP protein levels in resistant larvae was confirmed using a phenotypic linkage analysis. Further

research is needed to understand the role of ALP as a functional Cry1Fa-Cry1A receptor in *S. frugiperda*. Identification of interacting regions in Cry1Fa with receptors in BBMV from *S. frugiperda* will allow recognition of potential cross-resistance patterns with alternative available active toxins. Once the genomic differences responsible for reduced ALP expression in resistant *S. frugiperda* are identified they may be used to develop DNA-based biomarkers for detecting resistance in field *S. frugiperda* populations.

Larvae from the 456 strain exhibited cross-resistance to Cry1Ab-expressing maize and purified Cry1Ac toxin, but not to purified Cry1Bb, Cry1Ca, and Cry1Da toxins, or Xentari WG or Dipel ES pesticidal formulations. These bioassay results agree with the existence of a shared Cry1Fa-Cry1A binding site in *S. frugiperda*. The reduced expression of ALP in the resistant strain could cause potential fitness costs in the absence of Cry1Fa selection. Present study only able to detect delayed larval development in the resistant compared to susceptible strain. This delay resulted in asynchronous adult emergence between susceptible and resistant moths. Stable resistance allele frequency was observed after rearing in the absence of Cry1Fa toxin for 12 generations.

The long-term success of Bt crops expressing Cry insecticidal proteins will depend on the development of resistance in target insects. Considering binding patterns and bio-efficacy detected in our work, the development of cross-resistance in *S. frugiperda* will be rare among Cry1F and Cry1Ca, and Cry1Fa and Cry1Da toxins, and thus the strategy of combining these Cry toxins in pyramided crops is advisable.

The lack of fitness costs and cross-resistance patterns in *S. frugiperda* need to be considered in formulating effective resistance management methods. A combination of

other insect pest control methods along with pyramided crops expressing multiple Bt toxins have to be included in regulatory framework for delaying or avoiding development of resistance in target insects to transgenic Bt technology.

7. References

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APPENDIX

Two-way ANOVA for larval survival.

Source	Type III Sum of Squares	Df	Mean Square	F	Sig.
Strain	31.738	3	10.579	0.359	0.784
Food	354.004	3	118.001	4.009	0.046
Replication	30.518	1	30.518	1.037	0.335
Food * strain	34.180	9	3.798	0.129	0.997
Strain * Rep	79.346	3	26.449	0.899	0.479
Food * Rep	84.229	3	28.076	0.954	0.455
Error	264.893	9	29.433		
Total	282128.906	32			

R Squared = 0. 70 (Adjusted R Squared = -0.04)

Two-way ANOVA for larval weight.

Source	Type III Sum of Squares	Df	Mean Square	F	Sig.
Strain	200450.293	3	66816.764	39.227	0.000
Food	5555983.887	3	1851994.629	1087.287	0.000
Replication	10829.004	1	10829.004	6.358	0.057
Strain * Food	26771.973	9	2974.664	1.746	0.294
Strain * Rep	10568.262	3	3522.754	2.068	0.233
Food * Rep	15536.230	3	5178.743	3.040	0.123
Error	1705021.191	1002	1703.318		
Total	81705700.000	1024			

R Squared = 0. 99 (Adjusted R Squared = 0.99)

Two-way ANOVA for pupal weight.

Source	Type III Sum of Squares	Df	Mean Square	F	Sig.
Strain	40694.168	3	13564.723	35.618	0.0001
Food	3671433.543	3	1223811.181	3213.447	0.0001
Rep	1392.223	1	1392.223	3.656	0.056
Strain * Food	9068.129	9	1007.570	2.646	0.065
Strain * Rep	350.730	3	116.910	.307	0.820
Food * Rep	7456.043	3	2485.348	6.526	0.071
Error	381221.441	1002	380.841		
Total	33199436.000	1024			

R Squared = 0.91 (Adjusted R Squared = 0.91)

Two-way ANOVA for larval developmental time.

Source	Type III Sum of Squares	Df	Mean Square	F	Sig.
Strain	0.305	3	0.102	529.377	0.0001
Food	5.935	3	1.978	10309.579	0.0001
Replication	0.002	1	0.002	7.866	0.511
Food * Strain	0.025	9	0.003	14.690	0.605
Strain * Rep	0.007	3	0.002	12.538	0.581
Food * Rep	0.058	3	0.019	100.382	0.018
Error	0.190	1002	0.000		
Total	1614.092	1024			

R Squared = 0. 995(Adjusted R Squared = 0.984)
Data were log10 transformed.

Two-way ANOVA for pupal developmental time.

Source	Type III Sum of Squares	Df	Mean Square	F	Sig.
Strain	0.047	3	0.016	32.028	0.687
Food	0.829	3	0.276	565.070	0.001
replication	0.006	1	0.006	12.399	0.224
Strain * Food	0.009	9	0.001	2.152	0.897
Strain * rep	0.001	3	0.000	0.434	0.920
Food * rep	0.000	3	0.000	0.266	0.869
Error	0.490	1002	0.000		
Total	934.920	1024			

R Squared = 0.72 (Adjusted R Squared = 0.72)
Data were log10 transformed.

Two-way ANOVA for adult longevity.

Source	Type III Sum of Squares	Df	Mean Square	F	Sig.
Strain	0.027	3	0.009	4.784	0.063
Food	1.271	3	0.424	222.326	0.000
Rep	0.003	1	0.003	1.318	0.251
Food * Strain	0.026	9	0.003	1.513	0.138
Strain * Rep	0.002	3	0.001	0.291	0.832
Food * Rep	0.004	3	0.001	0.737	0.530
Error	1.909	1002	0.002		
Total	1304.732	1024			

R Squared = 0.65 (Adjusted R Squared = 0.64)
Data were log10 transformed.

Two-way ANOVA for adult emergence (total larval and pupal developmental time).

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Strain	0.196	3	0.065	391.293	0.0001
Food	3.667	3	1.222	7330.923	0.0001
Rep	8.820E-6	1	8.820E-6	0.053	0.818
Food * Strain	0.009	9	0.001	6.217	0.0001
Strain * Rep	0.004	3	0.001	7.602	0.0001
Food * Rep	0.023	3	0.008	45.119	0.0001
Error	0.167	1001	0.000		
Total	2099.953	1024			

R Squared = 0.96(Adjusted R Squared = 0.96)
Data were log10 transformed.

Two-way ANOVA for average number of egg masses laid.

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Strain	0.983	3	0.328	0.464	0.708
Food	59.258	3	19.753	27.964	0.0001
Rep	0.625	1	0.625	0.885	0.347
Food * Strain	0.233	9	0.026	0.037	1.000
Strain * Rep	0.097	3	0.032	0.046	0.987
Food * Rep	3.416	3	1.139	1.612	0.185
Error	1792.062	2537	0.706		
Total	3086.000	2560			

R Squared = 0.35(Adjusted R Squared = 0.26)
Data were log10 transformed.

Two-way ANOVA for average number of egg masses hatched.

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Strain	0.505	3	0.168	0.282	0.839
Food	44.569	3	14.856	24.869	0.000
Rep	0.075	1	0.075	0.125	0.724
Food * Strain	0.628	9	0.070	0.117	0.999
Strain * Rep	0.632	3	0.211	0.353	0.787
Food * Rep	2.951	3	0.984	1.647	0.177
Error	1514.380	2535	0.597		
Total	2305.000	2558			

R Squared = 0.32(Adjusted R Squared = 0.23)
Data were log10 transformed.

Two-way ANOVA for percent average egg masses hatch.

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Strain	176.309	3	58.770	2.047	0.178
Food	697.602	3	232.534	8.099	0.006
Rep	0.043	1	0.043	0.002	0.970
Food * Strain	434.652	9	48.295	1.682	0.225
Strain * Rep	26.574	3	8.858	0.309	0.819
Food * Rep	0.126	3	0.042	0.001	1.000
Error	258.415	9	28.713		
Total	184750.594	32			

R Squared = 0.838(Adjusted R Squared = 0.441)

Two-way ANOVA for sex ratio.

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Strain	0.004	3	0.001	0.326	0.807
Food	0.016	3	0.005	1.495	0.281
Rep	0.000	1	0.000	0.037	0.852
Food * Strain	0.059	9	0.007	1.838	0.189
Strain * Rep	0.014	3	0.005	1.328	0.325
Food * Rep	0.022	3	0.007	2.066	0.175
Error	0.032	9	0.004		
Total	33.340	32			

R Squared = 0.782 (Adjusted R Squared = 0.248)

VITA

Siva Rama Krishna Jakka was born on June 15th, 1978 in Sankarapuram, in the State of Andhra Pradesh, India. Through his work in the family's farm he became interested in research on transgenic Bt technology and insect pest interactions. For Siva, the inspiration to excel in agricultural research resulted from his family farming exposure and a genuine interest to change farmer's lives for the better. Siva received his B.Sc. (Agriculture) degree from the Agricultural College at ANGR Agricultural University in (Bapatla, India), and his M. Sc. (Agricultural Entomology) from Tamil Nadu Agricultural University (Coimbatore, India). His M. Sc. Research project involved the study of temporal and spatial variations in insecticidal activity and Cry1Ac expression in transgenic Bt cotton. He also worked on various projects related to transgenic Bt crops and insect interactions at the Indian Agricultural Research Institute (IARI, New Delhi), the Directorate of Sorghum research (DSR, Hyderabad) and the College of Agriculture (ANGRAU, Hyderabad). During 2004-2007, he worked for MAHYCO seeds as Bt-cotton (BG-I & BG-II) coordinator for the Andhra Pradesh State to investigate the impact of new transgenic Bt-cotton hybrids on insect pests. During 2007-2008 he worked in maize breeding and hybrid development programs as a senior research associate in Pioneer overseas corporation (Bangalore, India). Siva received a graduate student assistantship from the University of Tennessee in August, 2009 to pursue his PhD degree in Plants, Soils and Insects at the Department of Entomology and Plant Pathology. He joined Dr. Jurat-Fuentes' laboratory where he studied the molecular characterization of resistance mechanism in field-evolved resistant *Spodoptera frugiperda* (fall armyworm)

to transgenic Bt maize. Siva presented his research work at various scientific meetings in the USA and abroad, including the meeting of the Society for Invertebrate Pathology (2011 and 2012), the Entomological Society of America (2010, 2011 and 2012) and the Tennessee Entomological Society (2010 and 2011). He was awarded a student travel award by the Society for Invertebrate Pathology to attend its annual meeting in 2012. He served as the Vice-president of the graduate student association in the Department of Entomology and Plant Pathology during 2010-2011 and President in 2011-2012. Siva successfully completed his doctoral degree in the spring of 2013 and looks forward to a challenging and promising future associated with entomology and transgenic Bt technology research.