**University of São Paulo**
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# Molecular characterization of fall armyworm (*Spodoptera frugiperda*) resistant to Vip3Aa20 protein expressed in corn

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Thesis presented to obtain the degree of Doctor in Science. Program: International Plant Cell and Molecular Biology

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# Molecular characterization of fall armyworm (*Spodoptera frugiperda*) resistant to Vip3Aa20 protein expressed in corn

versão revisada de acordo com a resolução CoPGr 6018 de 2011

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*To my heroes, mother and father (in memorian), and my lovely wife.*

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# Fields of Study

Major Field: Translational Plant Science

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## RESUMO

### Caracterização molecular da lagarta do cartucho (*Spodoptera frugiperda*) resistente a proteína Vip3Aa20 expressa em milho

Plantas Transgênicas expressando genes de *Bacillus thuringiensis* (Bt) tem sido usadas como alternativa ao controle químico para controle de insetos praga. A proteina Vip (Vegetative Insecticide Protein) cuja secreção é realizada durante fase de crescimento da bacteria é considerada como segunda geração de proteinas inseticidas em função desta não apresentar similaridade de sequencias com todas as outras proteinas cristal (Cry), apresentando ainda maior espectro de controle de pragas. Uma das pragas alvo desta proteina é a lagarta-do-cartucho do milho (*Spodoptera frugiperda*), considerada a mais importante na cultura do milho na América do Sul. Larvas desta espécie foram sempre controladas com inseticidas e mais recentemente, milho expressando proteínas Cry. No entanto, esta praga tem desenvolvido resistência para várias ferramentas de controle, trazendo preocupação para a sustentabilidade das taticas de controle geradas através da biotecnologia. Dessa forma, estudos de caracterização da resistencia envolvendo modo de ação e characteristicas genéticas envolvidas com resistência pode contribuir para melhorar estratégias de Manejo de Resistencia de Insetos (IRM) e aumentar a durabilidade destas tecnologias para o controle. Nesta dissertação, foi gerado dados proteômicos e de transcriptoma comparando uma população de *S. frugiperda* resistente a Vip3Aa20 com a susceptivel. No capítulo 2, abordamos as características de bio-ecologia da praga associado ao sistema de cultivo suportando o alto potencial adaptativo desta espécie para hibridos de milho expressando proteinas Bt no Brazil. No capitulo 3, estudos de proteômica mostrou que Vip-R1 e Vip-R2 quando comparado com SUS, não demostraram diferenças para ativação da proteina nem ausencia de ligação da proteína com receptor de membrana no intestino do inseto. Dados de transcriptoma descritos no capitulo 5 mostrou forte evidências de que a baixa expressão de genes relacionados ao sistema transportador ABC pode estar associado com resistência bem como genes da via de sinalização das proteínas G. Estes resultados serão discutidos em um contexto para suportar boas praticas de manejo de resistência para lagarta-do-cartucho e assim estender a durabilidade da tecnologia Viptera® no campo.


Palavras-chave: *Spodoptera frugiperda*; Vip3A; Manejo de resistência de insetos; ligação proteina-receptor; Transcriptoma; RNA-seq; ABC transportador

## ABSTRACT

### Molecular characterization of fall armyworm (*Spodoptera frugiperda*) resistant to Vip3Aa20 protein expressed in corn

Transgenic plants containing genes from *Bacillus thuringiensis* have been used as an alternative to chemical insecticides for insect pest control. The vegetative insecticidal proteins (Vip) secreted during the vegetative growth phase of bacteria are considered a second generation of insecticidal proteins since they do not share any structural or sequence homology with previously used crystal proteins (Cry) as well as having a wide insecticidal spectrum. One of the target pests for this protein is the fall armyworm (FAW) (*Spodoptera frugiperda*), the most important corn pest in South America. Previously it has been controlled by insecticides and corn expressing Cry proteins, but has rapidly evolved resistance to many control practices and remains a top concern for sustainable biotechnology control efforts. Thus, resistance characterization involving mode of action and genetics of resistance can help with Insect Resistance Management strategies, and improve the durability of control. In this dissertation, using two selected FAW population resistant to Vip3Aa20 Bt protein (Vip-R1and Vip-R2) we generated comparative proteomic and transcriptomic data among resistant and susceptible colonies. In the chapter 2, we bring FAW biology/ecology and Brazilian agriculture landscape data to support the high adaptive potential of this pest to genetically modified corn expressing Bt Cry proteins in Brazil. Proteomics studies in the chapter 3 revealed that neither Vip-R1 nor Vip-R2 showed difference between resistant and susceptible colonies either for Vip3Aa20 activation through proteolysis assay nor protein binding to the receptor. Transcriptomic sequencing and RNA-seq analysis in the chapter 4 showed strong evidence of ABC transporter genes associated with resistance as well as genes related to G-protein signaling pathway as downregulated. These results will be discussed in context of providing best management practices for managing FAW resistance to Vip, and extending the durability of Vip technology.

Keywords: Fall armyworm; Vip3A; Insect resistance management; Protein-receptor binding; Transcriptome; RNA-seq; ABC transporter

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# 1 INTRODUCTION

Brazil is one of the major food suppliers for the globe and has immense agricultural potential. Agricultural production is a strong foundation for Brazil's economy, and, in 2013, accounted for 23% of all its wealth. One of the most important crops is corn, whose area as well as yield has increased significantly in the last 15 years, driven by the adoption of a second planting season (corn planted immediately after soybean). The second corn planting season has surpassed the first crop in area: 10.5 million hectares are grown in the second compared to 5.3 million hectares in the first season. Indeed, the production of the second season has already surpassed the first, with 41.1 million tons compared to 25.8 million tons, respectively (CONAB 2016). This increase was facilitated by optimization of fertilizers and pesticides as well as the significant adoption of transgenic corn hybrids resistant to Lepidoptera species (13 million hectares of genetically modified corn tolerant to insects was planted), which represents 83% of the total market (USDA 2016).

The rapid adoption of genetically modified crops has been driven by various benefits, primarily related to the positive economic impact for the farmer. A worldwide study conducted by Brookes and Barfoot (2013), showed that the adoption of GMOs crops during the period of 1996 to 2011 brought an economic benefit to farmers of $98.2 billion in the period of 16 years; in 2011 alone this benefit was estimated at $19.8 billion.

The additional benefit showed in this study was the significant increase of corn productivity, which had increased in 195 million tons followed by the reduction of 45.2% (50 million of Kg) of insecticide active ingredient applied in corn fields worldwide in the same period (Brookes and Barfoot 2013).

Bt corn was developed by inserting specific genes from *Bacillus thuringiensis* (Bt) bacteria in its genome, which encode specific proteins to control some insect pests. In South America the highest benefit is from the control of *Spodoptera frugiperda* (fall armyworm (FAW) which is the most important pest in corn in this region due to its aggressive feeding habit which can drastically impact the potential yield losses.

Not different from other insect control tactics, one of the major risks associated with Bt crops is the potential for resistance evolution in target pests. Insects evolve in response to natural selection imposed by control methods, limiting their efficiency and viability in the long term (Hawthorne 1999). More than 500 species of insects have become resistant to conventional insecticides and there is concrete evidence that they can also adapt to Bt toxin (Gut et al. 2002).

The evolution of resistance of an insect population to a Bt toxins, is a process governed by a great number of factors that interact each other. These are related to the characteristics of the genetic background of the transgenic plant, to the bio ecology and genetics of the target pest, to the crop management and to the environment of the region (Maia 2004).

Several mechanisms of insect resistance have been described for Cry proteins (Ferre and Van Rie 2002, Frutos et al. 1999), however the most frequently reported is the the modification of the binding sites of Cry protein to receptors of membrane (Ferre and Van Rie 2002, Van Rie et al. 1990), which might be influenced by several genetic factors related to different gene expression profiles. However, the intoxication process as well as the mechanisms of resistance for some Bt protein as Vips remains unknown.

Studies involving the identification of modes of action of Bt proteins as well as the molecular mechanisms of insect resistance, are of great importance in order to better set resistance management strategies and contribute to slow down the evolution of insect resistance to Bt proteins. Such studies will also contribute to the development of new Bt products with different modes of action for managing FAW.

Thus, this research emphasized on the understanding of biochemical and molecular mechanisms of resistance. We used biochemical analysis approach (protein activation and protein-receptor binding studies) as well as transcriptome analysis to try to understand the molecular mechanisms of resistance as well as molecular pathways involved with Vip3Aa20 mode of action in FAW.

*Spodoptera frugiperda*

*S. frugiperda* (JE Smith, 1797) (Lepidoptera: Noctuidae) larvae, are the major pest of corn in all regions of South America (Blanco et al. 2016). In Brazil, this lepidopteran is popularly known as "lagarta-do-cartucho". In Argentina, Paraguay and Uruguay is popularly known as "gusano cogollero". In Central America as "barredora" and in North America as "fall armyworm" (FAW), "grass worm," "overflow worm" and "grass warm worm". This species infests and damages the young leaves of corn (Zea mays), but also can feed on kernels in North America. FAW is also frequently observed in rice (*Oryza sativa*) growing regions, for which it is a pest of great economic importance (Carvalho 1970). This insect is one of the most harmful species of the tropics, and responsible for losses that reach about $ 1 billion annually in Brazil (Waquil et al. 2008).

The biology and ecology of this pest has made FAW one of the highest pest pressures in the agroecosystem which itself also contributes to a high risk pest of resistance evolution

against control tactics. It has pronounced habit of polyphagy, with preference for gramineaes, but can attack leguminous plants. Grützmacher et al. 2000 described 23 families of plants as hosts of this species.

Metcalf et al. 1965, show that this pest can produce up to 10 generations a year in areas that do not have frost and have abundant food resources. The FAW has sexual reproduction which allows new allelic combinations through recombination. Genetic variation is quickly spread across different populations due to the high flight capacity of adults. They are able to migrate hundreds of kilometers after mating but before laying eggs (Metcalf et al. 1965). Long dispersal movement has been documented through meteorological synoptic maps, which have detected adults migrating from Mississippi, USA to Canada within 30 hours (Johnson 1995). Thus genetic variation, include that responsible for any resistance, can be spread not only far away, but also very quickly and infest new Bt areas expressing proteins with similar mode of action.

The reproductive output of FAW is also high and directly contributes to rapid population growth. Females can lay up to 1,800 eggs, usually on the upper layer of the corn leaves (Barros et al. 2010). After hatching, the neonate larvae tend to migrate to the new leaves of the plant. However, the notable cannibalism in this species can potentially offset rapid reproductive output, depending on initial larval movement and other factors. Thus due to its high reproductive capacity and adaptation, FAW infestations are usually quite large, resulting in expressive economic losses for corn growers not only in Brazil, but across the Americas (Waquil et al. 2008, García-Gutiérrez et al. 2012, Silva-Aguayo et al. 2010, Blanco et al. 2016).

*Bt proteins and its mode of action*

*Bacillus thuringiensis* is an entomo-pathogenic gram-positive bacterium which is characterized by the presence of crystalline inclusions formed during its sporulation. This bacterium is widely distributed throughout the world, mainly due to its sporulation capacity, which gives them a high resistance to heat and drought (Martin and Travers 1989). Although described as a soil bacterium, it has also been found in vegetables, water and insects.

The bacteria, at the time of sporulation, produces inclusion bodies containing a number of proteins (δ-endotoxins) with insecticidal activity: Cry proteins and Cyt proteins. Delta-endotoxins include all proteins produced by *B. thuringiensis* that accumulate in the cell of the body of parasporal inclusion (crystal) and toxic activity against the target organism.

In addition to Cry proteins, *B. thuringiensis* also produce a different class of proteins known as Vip (vegetative insecticidal protein). These proteins are produced by the bacteria during the vegetative stage of growth. Unlike delta-endotoxins, which are produced in the form of a protein crystal within the cell during sporulation, Vips are secreted into the nutrient growth medium (Estruch et al. 1996). Regarding sequence homology, Vips has no similarity with known δ-endotoxins (Estruch et al. 1996).

Despite these differences, the action of Vip proteins seem to be similar to the Cry proteins. The toxic activity appears to occur in the insect midgut epithelium, where binding to receptors in the intestinal cells is followed by progressive degeneration of the double epithelial layer (Yu et al. 1997).

The similarities in the gut reactions between delta-endotoxins and Vips may suggest similar mode of actions. Although Vips are understudied, the mode of action of δ-endotoxins of *B. thuringiensis* are described by two models (pore forming and G-protein signaling pathway models) (Soberon and Bravo 2009).

In general, for these models, ingestion is the first step of a series of events inside the insect that will lead to its death by starvation, sepsis or osmotic collapse. After ingestion, the crystals of *B. thuringiensis* pass mostly intact through the first portion of the digestive tube. Later, mainly due to the insect intestinal pH characteristics and crystal composition, these crystals are solubilized, releasing peptides without insecticidal activity which are called pro-toxins. There is evidence that the solubilizing rate depends on pH of the midgut. Studies with *Anagasta kuehniella*, an insect with a slightly alkaline intestinal pH, showed a slow dissolution of the crystal (Du et al. 1994). Alternatively, in *Bombyx mori*, whose intestinal pH is around 10, the symptoms begin within few minutes of ingestion. Each toxin will have specific and optimum conditions for solubilization, and toxins active against lepidopterans typically solubilize in alkaline pH, whereas coleopteran toxins are active at neutral pH (Koller et al. 1992).

Once the protein is solubilized, it is activated by the action of intestinal proteases, particularly serine proteases. The condition under which this activation takes place is important, because intestinal fluid can produce different variants of the same toxin with different activities (Haider et al. 1986). Once Bt protein is activated by proteases, it will bind to the primary receptor which is presented in the membrane of insect midgut. The Bravo model (Bravo et al. 2004) poses that once protein binds to the primary receptor, a α-helix 1 from domain III will be cleaved by serine protease and a hydrophobic region will be exposed, allowing the protein to start oligomerization and form a tetrameric pre-pore that preferentially

binds to a secondary receptor which is anchored to the membrane by glycosylphosphatidylinositol (GPI). The secondary receptor will help the protein to be introduced into the cell through the membrane and start the process of pore forming (Bravo et al. 2004).

The second model proposed by Zhang et al. 2006, shares initial steps with Bravo's model, however does not include protein oligomerization. Instead, once protein binds to the primary receptor, it will initiate an $Mg^2$ dependent signaling pathway that results in cell death. Protein-receptor binding stimulates the G protein pathway, which starts by induction of subunit-α that will join adenylyl cyclase present in the membrane. This reaction will stimulate the production of cAMP which will work as signal amplifier into the cell. Afterwards, cAMP will stimulate the kinase A protein cascade acting in the disturbing of cytoskeleton and consequently forming ion channels at the membrane.

*Insect resistance evolution to Bt crops*

Resistance is defined as the acquired ability through evolutionary processes of an organism to survive in response to selection pressure imposed by exposure to a toxic agent (ILSI / HESI 1998). Target pests evolve by natural selection in response to selection imposed by control methods, limiting their efficiency and long-term viability (Hawthorne 1999). Resistance cases are not exclusively to chemical products, but occur across a wide range of different control tactics including plant growth regulators (Ehrlich and Raven 1964), crop rotation (Levine et al. 2002), and biocontrol agents (Price et al. 1980) including toxins produced by *B. thuringiensis* or by transgenic plants expressing Bt toxins (Farias et al. 2014).

Insecticide that included Bt toxins have been used for over forty years before evidence of resistance occurred in the field. The first report of resistance to Bt insecticides occurred in the Philippines with *Plutella xylostella*. Other reports showed control failures of *P. xylostella* in the United States, Japan, Central America and China mainly with the chemicals Dipel® and Xentari® (Van Rie and Ferré 2000). Populations of several species of Lepidoptera, Coleoptera and Diptera, have developed resistance to Bt toxins in laboratory conditions (Neppl 2000). These results emphasize the potential of resistance to Bt crops in field conditions.

The development of resistance to Bt transgenic plants would nullify the benefits of this new technology used in millions of hectares worldwide. Most target pests continue to be susceptible to Bt crops; however field-evolved resistance has been published for some Lepidoptera: *Busseola fusca* in South Africa to Cry1Ab (Kruger et al. 2009), FAW to Cry1F in Puerto Rico (Matten et al. 2008), *Pectinophora gossypiella* to Cry1Ac in India (Bagla

2010), *Helicoverpa zea* to Cry1Ac+Cry2Ab in United States (Luttrell and Luttrell 2004), *Helicoverpa punctigera* to Cry1Ac+Cry2Ab in Australia (Downes et al. 2010), and FAW in Brazil to Cry1F and Cry1Ab (Farias et al. 2014, Omoto et al. 2016). To date, there are no reports of field resistance to Vip3Aa20 Bt protein in South America countries (Syngenta 2017, personal communication).

Insect resistance evolution to Bt toxins expressed in transgenic plants is affected by a number of interacting factors interact including the genetic background of transgenic plant, the bio-ecology and genetics of the target pest, the crop management and the environment (Maia 2003). Thus increased resistant allele frequency in a population is governed by: 1- Survivorship differences among individuals feeding on Bt crop; 2- Capacity of survivors from Bt crop generate viable offspring; 3- If survivors are present in a Bt field, the fitness differences between susceptible and resistant (Endler 1986).

The risk of FAW evolving resistance is very high, due to its biological and ecological characteristics and a strong adaptive capacity. In the chapter 2, I present FAW as a case study with strong evidence to support rapid resistance evolution influenced by ecological and evolutionary characteristics of FAW to Bt corn.

From a genetic variation perspective, resistance likely mostly originates from polymorphisms or mutations in the insect DNA. Bt proteins are expressed in plant tissues in high concentrations, the selection for increased survival on Bt plants mostly favor polymorphisms or mutations at single genes. On the other hand, proteins that are not highly active against a target pest can result small or moderate decreases in susceptibility, potentially involving multiple loci. Those mutations can also influence potential survivorships on Bt crops, however the resistance tends to be given by minor genes, and not one or two genes (Storer et al. 2003, Showalter et al. 2009).

From the biochemical basis of resistance, any change in the mode of action of a Bt toxin may result in selection for resistance. Examples include 1) a change in the binding site of the toxin to the membrane; 2) modification of the proteolysis activity in the insect gut; 3) increased speed of repair damaged epithelial tissue. Changes in the toxin binding site is the most likely to occur and it can generate the highest resistance levels. It has been described in both field and laboratory cases of resistance. This change has been detected in strains of *P. interpunctella* resistant to one or more Cry1A toxins family (Van Rie et al. 1990), *P. xylostella* (Ferré et al. 1991) and *H. virescens* (Lee et al. 1995). In the chapter 3 of our study, we tried to identify potential reduction of protein-receptor interaction which could be associated with resistance of FAW to Vip3Aa20.

Cross-resistance is defined as the resistance of a Bt toxin not present in the selection process which was influenced by exposing previously to other protein with similar mode of action (Tabashnik et al. 1997). Binding studies of Cry proteins have revealed a close correlation between cross-resistance development and common binding sites for these toxins. Insect species that share the same binding sites for certain toxins can also develop resistance other toxins that share the same binding sites as in *P. xylostella* (Granero et al. 1996), *H. virescens* (Jurat-Fuentes and Adang 2001), *D. virgefera virgifera* (Gassmann et al. 2014). Using heterologous competition assay, in chapter 3 we investigated the potential for cross resistance with Vip3Aa20 to other known Lepidopteran Cry protein.

From the molecular genetic basis of resistance, some research has been carried out to elucidate this evolution process. Regarding the mechanism that involves the changes of the proteolysis activity of the insect gut, Oppert et al. (1997) showed that there is a genetic correlation between Cry1Ac resistance and the absence of an intestinal protease in a strain of *P. interpuntella*. For *H. virescens*, resistant larvae show faster recovery of the epithelial tissue after its intoxication with sub lethal doses of Cry1Ac toxin (Martinez-Ramirez et al. 1999). The potential cause of resistance was investigated in our study through proteolysis assay using gastric fluids from the resistant colony to assess the protolithic activity of serine proteases to Vip3Aa20. This study is described in the chapter 3.

Insect resistance to Bt crops has been studied using various techniques, but only a few studies have demonstrated the potential of genes that are strongly associated with field resistance. In chapter 4, my work focused on trying to identify potential candidate genes that could be associated to field resistance of FAW to Vip3Aa20. I used a transcriptomic sequencing approach to select candidate genes that were differentially expressed among susceptible and resistant insects which could be playing key roles in resistance. With this study, described in the chapter 4, we expect to have indications on the molecular mechanisms of field resistance of FAW to Vip3Aa20.

This research will bring information about the potential pathways involved with mode of action of FAW to Vip3Aa20 and allow researchers to investigate new proteins with different mode of action.

In addition, the resistance characterization involving biochemical as well as transcriptome approach will drive a better understanding of risks for resistance evolution and orient us towards the adoption of best practices for resistance management as well as develop molecular markers associated to resistance allele which will allow Industry to monitor

resistance proactively and react to evolution of resistance alleles before unexpected damage appear in the field.

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# 2 ADAPTIVE POTENTIAL OF FALL ARMYWORM LIMITS Bt TRAIT DURABILITY IN BRAZIL

**Abstract**

The fall armyworm, *Spodoptera frugiperda* (J.E. Smith 1797) (Lepidoptera: Noctuidae) is the most important corn pest in South America. Larvae feed mostly on leaves, but also ears when population densities are high. This pest has been historically controlled with insecticide applications until many cases of resistance limited their efficacy. Transgenic corn varieties expressing *B. thuringiensis* proteins (Bt corn) have been a widely-adopted alternative to insecticides and, in the last eight years, have been the primary technology for fall armyworm control in Brazil. Since transgenic varieties require 10 to 15 years to be developed, strategies for Bt trait durability are critical. However, some Bt corn varieties lost the ability to control fall armyworm in just three years after their release in Brazil. Here we summarize the known Bt resistance in fall armyworm, a phenomenon perhaps never seen before in any part of the world. Furthermore, we suggest that the interaction between management practices adopted (or not adopted, e.g. refuge compliance) to delay the evolution of resistance and the ecological and evolutionary characteristics of fall armyworm are driving the rapid evolution of resistance to Bt corn in Brazil. As newer products emerge on the market, careful consideration will be needed to maximize trait durability.

Keywords: Resistance evolution; Bt corn; Fall armyworm; Insect ecology; Insect resistance management; Vip3A protein

## 2.1 Introduction

The fall armyworm (FAW), *Spodoptera frugiperda* (J.E. Smith 1797) (Lepidoptera: Noctuidae), is the major corn pest in Brazil and throughout South America (Blanco et al. 2016). In the last 4 years, Brazilian cotton and corn fields have experienced high FAW infestations causing large economic losses. Corn yield reductions caused by this pest can reach 34-38% (Carvalho 1970). When late instar larvae act as seedling cutworm, corn losses can reach up to 100% (Avila et al. 1997). Such population outbreaks are, in part, due to increased cultivation during the second corn season (corn planted immediately after soybean – Sept. to Jan., soybean; Feb. to June, corn) (Valicente et al. 2008). In 2015, the area of second corn season in Brazil was 65.46 % of the total, whereas the traditional summer season was 34.54% (CONAB 2016). As a consequence, FAW populations that used to peak during the summer (first corn season) are now found throughout the extended growing period, facilitated by so-called "green bridges"—plant hosts where FAW migrate from one crop to another in the same region, maintaining high populations.

Chemical control has widely been used in the last three decades to manage FAW infestations, although this tactic is not without concerns. Growers lack or do not adopt economic thresholds for chemical applications, and multiple immigrations are common in early stage corn fields. In addition, females usually lay eggs on the upper layer of the corn leaves and, after hatching, the neonates tend to quickly move to the whorl of the plant. Once hidden in the whorl, the exposure, and therefore efficacy, of chemical controls are limited (Young 1979). Thus, most insecticides are applied on a weekly basis to prevent neonates moving into the plant whorl (Cruz 1995). After neonates move to the whorl, one of the strategies used by growers is to increase the liquid volume and application doses, to ensure FAW mortality. However, aerial applications are frequently used across large farms, which use very low liquid volume and leads to sub-lethal dosage exposure. Association of larval behavior and chemical application may have promoted rapid resistance evolution of this species to many insecticides in Brazil (Diez-Rodriguez and Omoto 2001). The Arthropod Pesticide Resistance Database lists FAW resistance to 24 different active ingredients. Field resistance has been documented in 45 different locations among 8 countries. Brazil includes 25 cases, representing 55.5% of the total worldwide cases.

To help to overcome control challenges imposed by FAW, transgenic corn hybrids expressing insecticidal proteins derived from *Bacillus thuringiensis* (Bt) have been the most widely adopted technology in South America and across the world (NAS 2016). The rapid adoption of genetically modified crops has been driven by various benefits provided by this technology including effective insect control, reduced agricultural inputs (i.e. chemical pesticides) and positive economic impact for growers (NAS 2016). For example, between 1996 and 2011, genetically modified crops provided an economic benefit of $98.2 billion; in 2011 alone, this benefit was estimated at $19.8 billion. An additional benefit was the significant increase of corn productivity, which increased by 195 million tons globally (Brookes and Barfoot 2013). Brazil represents 8.8% (15.43 million hectares) of the total corn production in the world (USDA 2013), of which 12.1 million hectares were planted with genetically modified corn tolerant to insects (Bt corn) (ISAAA 2013). From 2008 to 2015, 5 different Bt proteins and 5 different pyramid products (those containing >1 Bt protein) were launched in Brazil for Lepidoptera control (Table 2.1) (CTNBio 2016).

Similar to insecticides, FAW can also develop resistance to Bt crops in response to the strong selection pressure that this technology imposes over field populations due to constitutive Bt protein expression (Storer et al. 2012). To manage resistance and ensure trait durability, Insect Resistance Management (IRM) practices for Bt crops must be a priority.

IRM strategies include refuge (i.e. non-Bt crops planted with Bt corn), high-dose, and pyramid products. The primary IRM strategy for insect resistance management is the adoption of refuge. Without Bt, the refuge should then produce a preponderance of susceptible insects (genotype ss) which will mate with potential homozygous resistant (*rr*) selected for in the Bt area. Assuming resistance is recessive, the offspring generated would be heterozygous (*rs*), and controlled by a Bt crop (Gould and Tabashnik 1998, Matten et al. 2008, Tabashnik et al. 2009).

Refuges are most effective when used in concert with a high-dose protein. Under high-dose, homozygous recessive (*ss*) and heterozygous (*rs*) insects cannot survive when exposed to the product; only homozygous resistant (*rr*) insects might survive (Tabashnik et al. 2004, Crespo et al. 2009). The high dose strategy has been somewhat successful in other Bt crops such as cotton expressing Cry1Ac to control *H. virescens* and *P. gossypiella*; these pests became resistant only 11 and 13 years, respectively, after introduction of Bt cotton (Tabashnik et al. 2013). Perhaps the best case of refuge/high-dose success has been with *Ostrinia nubilalis*, or the European corn borer. Once the most damaging corn pest in North America, this Lepidopteran has been substantially controlled by several Bt proteins (Hutchison et al. 2010).

Bt traits that are not high dose events might require multiple control tactics to maintain low resistance frequency. This strategy assumes that insect resistance to all tactics is unlikely, and at least one toxin will provide mortality. Pyramiding of Bt proteins is one such example adopted for manage insect resistance to Bt crops. Successful cases of pyramiding were reported in cotton expressing Cry1Ac+Cry2Ab to control *H. armigera* and *H. punctigera* in Australia; since its introduction in 2005, there has been no report for resistance for both species (Tabashnik et al. 2013). However, success of IRM will depend on other conditions such as initial low frequency of resistance for each protein, recessive resistance for each protein, fitness cost and incomplete resistance, lack of cross resistance, refuge strategy (block compared to integrated) and refuge compliance (Tabashnik et al. 2013, Carriére et al. 2010, Gassmann et al. 2009).

Unfortunately, IRM for FAW has been challenging. In Brazil, multiple Bt proteins are labelled for FAW control, either in single or pyramid combinations. Cry1Ab was first introduced in 2008, but unexpected damage by FAW occurred 3 years later. Similarly, Cry1F was introduced in 2009 and growers reported unexpected damage in several different regions of the country as early as 2012. A pyramid Bt product containing Cry1A.105+Cry2Ab2 was introduced in 2010, but yet unexpected damage in the field was first noticed in 2013.

Nowadays, this product can have as many as 3 insecticide applications in some regions of Brazil to achieve adequate FAW control while, in other regions, continue offering a good suppression for FAW as well as controlling secondary Lepidoptera pests. In addition, FAW resistance emerged in other South American countries where the same products expressing the same Cry proteins are grown. Of all the Bt proteins, FAW remains susceptible to only one, Vip3Aa20: a toxin produced in the vegetative stage of Bt (as opposed to a Cry endotoxin). Introduced in 2010, Vip3Aa20 has no reports of unexpected damage or product failures by FAW in the field. However, rapid adaptation of FAW to insecticides and almost all Bt Cry proteins makes Brazil a most challenging environment for the durability of any Bt technology.

In this chapter, we discuss FAW resistance evolution to Bt toxins in Brazil within a framework of three interacting factors: *i*) Genetics; *ii*) Biology and ecology; *iii*) Implementation of resistance management tactics. We suggest that these 3 factors enabled FAW to overcome Bt crops in an unexpected and unprecedented period of time in Brazil.

## 2.2 Genetics Characteristics of FAW and its Impact on Resistance Evolution to Bt Crops

*Resistance allele frequencies: assumptions and empirical estimations*

Resistance evolution tends to be faster when the initial frequency of resistance alleles is high in insect populations (Tabashnik 1994, Georghiou and Taylor 1977, Tabashnik and Croft 1982, Roush 1997). Due to its contribution for resistance evolution, the estimation and predictions of resistance through mathematical modeling could be substantially improved if the frequency of resistance was empirically estimated (Gould et al. 1997). In most cases, the estimation of resistance frequencies is usually absent and only assumed. Current methods of estimating frequencies are extremely laborious and usually performed only after the technology is launched and may not reflect true frequencies before the product introduction (Génissel et al. 2003). With F2 screen (for methodology see Andow and Alstad (1998)), frequencies have been estimated in a limited number of studies, mostly in Lepidoptera: *H. armigera* (Wu et al. 2002), *H. virescens* (Gould et al. 1997), *Helicoverpa zea* (Burd et al. 2003), *O. nubilalis* (Andow et al. 1998, Bourguet et al. 2003), *Scirpophaga incertulas* (Bentur et al. 2000) and *Pectinophora gossypiella* (Tabashnik et al. 2000). The frequency of FAW resistance to Vip3Aa20 in Brazil was estimated in 2013 and 2014 (Bernardi et al. 2015a), 3-4 years after its initial release. Data for Cry proteins is unknown, potentially jeopardizing models predicting resistance evolution.

The initial frequency of a new allele is calculated by the mutation rate and the population size (1/2N, for a diploid organism, where N is the population size). The

reproductive output of FAW is quite high, with 1 female able to produce, on average, 1,688 offspring (Barros et al. 2010). Hence, in one generation, FAW is expected to have very large populations. However, large population sizes also influence genetic and allelic diversity and reduce the potential of rare alleles (i.e. resistance) to be lost through random genetic drift. Species with higher population sizes are generally more adaptable and have a greater evolutionary response towards shifting selection pressures (Wright 1932). Integrated pest management (IPM) strategies, when deployed at a regional level, can enforce a population reduction, decreasing diversity and adaptation potential (Hutchison et al. 2010). However, in terms of IPM strategies, population reduction is often accompanied with a strong selection pressure such as insecticides or Bt traits. Before product failures, therefore, we may expect a genetic and population bottleneck, not due to random processes (e.g. environmental), but a selective bottleneck retaining only resistant individuals. The question remains whether or not Bt resistance is a novel mutation or a pre-existing polymorphism. Nonetheless, given the reproductive output and strong selection pressure of FAW, resistance can spread quite quickly.

High initial frequency of resistance alleles, provided mainly by a high frequency of homozygous resistant (*rr*) insects, can neutralize the benefit from any resistance management practices adopted. For example, the most useful strategies are a high dose/refuge; however if high in frequency, *rr* insects will also reside in refuge, and even potentially outnumber susceptible (*ss*). Additional control tactics in the refuge could control *rr* insects, but ss insects will likely suffer the same fate. It is possible that the rapid evolution of FAW resistance to Cry proteins in Brazil may be associated with higher initial frequency of resistance alleles in the field populations than originally assumed.

*The FAW resistance as a dominant trait facilitates rapid evolution*

Assuming that resistance is based on a single gene or allele, extended Bt trait durability depends on the inheritance of resistance, e.g. recessive or dominant (Storer et al. 2003). Trait inheritance of insect resistance to Bt crops can be measured by the heritability (*h*), which varies from 0 (completely recessive) to 1 (completely dominant) (Gould and Tabashnik 1998). This parameter can also be indirectly estimated through crossing experiments of resistant and susceptible individuals (Tabashnik et al. 2004, Gould and Tabashnik 1998). Generally speaking, if resistance is recessive, then an insect will need 2 copies of the resistant allele. Resistance is functionally recessive when the dose of protein expressed in the Bt plant is sufficient to kill all heterozygous insects (*rs*); however, some

survivorship of resistant homozygous insects (*rr*) is expected (Tabashnik et al. 2004, Crespo et al. 2009). Alternatively, functional dominance is seen when heterozygous (*rs*) are more likely to survive on Bt crops (i.e. needing 1 copy of the resistance allele).

As the dominance of resistance is classified as "functionally" completely dominant or completely recessive, it may also depend on protein expression of plant tissues (e.g. dosage). Cry protein expression in cotton and corn can be reduced as the plant matures (Dutton et al. 2004). Bt cotton expresses less Cry1Ac during the reproductive stages than in vegetative stages; thus, resistance can appear as recessive if insects feed on plants in vegetative stage but partially dominant during reproductive stages (Showalter et al. 2009). Alternatively, cotton plants expressing Vip3Aa19 tends to have stable expression throughout the plant's life cycle (Llewellyn et al. 2007).

The FAW resistance to Cry proteins appears to be dominant, with substantial heterozygote survival (Cry1F – Farias et al. 2015; Cry1Ab – Jakka et al. 2016). The FAW genetics combined with insufficient protein concentration to kill all heterozygous (*rs*) individuals (e.g. high-dose) likely facilitated the persistence of resistant alleles in heterozygotes and enabled FAW resistance evolution in Brazil.

*Absence or reduced fitness costs promotes survival of resistant FAW*

A fitness cost is a biological or ecological penalty that organisms encounter for carrying the resistance allele. The intensity of this penalty is influenced by the environment and genetics of the target pest (Carrière et al. 2010). In regards to the high dose/refuge strategy, fitness costs have most impact when resistant (*rr*) insects are less fit compared to susceptible individuals (*ss*) in the refuge (e.g. *ss* individuals are more likely to survive and reproduce on non-Bt plants than *rr* individuals). As the rationale of the refuge is to support the production of *ss* individuals, any survival of *rr* can potentially increase resistance evolution (Carrière and Tabashnik 2001). Bt durability can be very successful when resistance comes with a high fitness cost, is recessive, and refuges are abundant, even if the initial frequency of resistance is high (Carrière and Tabashnik 2001).

Assuming the initial frequency of resistance alleles is low and likely carried by heterozygous insects (*rs*), resistance evolution in this stage is largely governed by the difference of fitness between susceptible individuals (*ss*) and heterozygous (*rs*) feeding on refuge areas (assuming before widespread Bt adoption). Thus, the strength of the fitness cost is critical for individuals that carry the *r* allele to transmit resistance to the next generation. Data on the strength of fitness costs will help to better understand the resistance risks and

develop improved IRM strategies. Unfortunately for FAW resistance to Cry proteins, fitness costs are reduced or are absent when resistant larvae are fed non-Bt corn (Jakka et al. 2014, Santos-Amaya et al. 2016, Vélez 2013, Souza et al. 2016, Bernardi et al. 2015b) or when resistant larvae are fed on different host plants, limiting the effectiveness of the refuge. The absence of FAW fitness cost likely maintained resistant alleles in the population, hastening Bt resistance.

### 2.3. Biology of FAW and its Influence on Resistance Evolution

A species' life history traits have a strong influence on resistance evolution; these include developmental rate, sex ratio, generations per year and the timing and rate of reproduction. Evolution of resistance, for example, is directly correlated to the number of generations per year (Tabashnik and Croft 1982). The FAW is highly reproductively efficient in tropical areas, where the warmer temperature allows the more generations per year compared to temperate areas (no more than 2 generations in a year). In some tropical and sub-tropical regions (areas without frost) FAW can produce up to 10 generations during a year (Metcalf et al. 1965). In Brazil, this species can have, on average, 11.3 and 8.3 generations under laboratory and field conditions, respectively (Busato et al. 2005). Rapid generation turnover is facilitated by the presence multi-crop systems where different crops are grown at the same time and in succession year-round, which maintains high FAW density.

As previously mentioned, this pest has a high reproductive output which is also related to different host plants. A study on the reproductive capacity on different host plants showed that the number of eggs/females ranged from 1,341.5 up to 1,844.3 when, as larvae, FAW were fed on millet and corn leaves, respectively, and 1,844.3 and 1,838.7 when fed on soybean and cotton, respectively (Barros et al. 2010). Furthermore, this study confirmed a high oviposition capacity, especially on corn and cotton which are 2 hosts that provide selection pressure for Bt resistance. The net reproductive rate female/female per generation (R0) is statistically equal when larvae are grown on soybean (421.8 ± 107.0), millet (330.5 ± 42.4), cotton (372.2 ± 80.82), but higher in corn (501.7 ± 42.04).

Despite these differences, FAW females do not appear to exhibit a preference among host plants to lay eggs (Barros et al. 2010). Assuming 8 generations in the field, one reproductive female could be responsible for 14,752 offspring in a year (1,844 eggs x 8 generations). Resistant females, therefore, can rapidly produce resistant offspring, increasing resistance alleles.

*High migration capacity of FAW increases resistance allele dispersal*

Migratory capacity and gene flow influences the dispersal of Bt resistance alleles when species are not genetically structured and share similar environments across its range, such as common agronomic crop production practices and management tactics (Fuentes-Contreras et al. 2004). The speed of dispersal will depend on the initial frequency of the resistance alleles in the population as well as the dispersion characteristics of adults. In addition to a large reproductive capacity describe above, FAW adults are able to migrate hundreds of kilometers after mating but before laying eggs (Metcalf et al. 1965). Meteorological synoptic maps have recorded long distance dispersal of adults, detecting individuals migrating from Mississippi, USA to Canada within 30 hours (Johnson 1995). Adult insects have the potential to rapidly spread resistance alleles among regional and continental populations. However, some geographic barriers to migration of this species do exist, such as the Appalachian Mountains in eastern North America (Nagoshi et al. 2015). The FAW experiences Bt crops across much of its range (see below), expanding not only the distribution range, but the area that exclusively favors rr individuals.

*FAW polyphagy increases exposure to similar Bt proteins in multiple crop hosts*

The FAW is a polyphagous species that feeds on more than 80 species of plants, including the most important commercial crops of corn, cotton and soybean (Pogue 2002, Capinera 2008). The latter three crops are also used in succession or concomitant in some regions. The FAW life cycle is very similar among those host crops (24.2 days – corn; 27.4 – cotton; 26.4 – soybean; 24.5 – millet) (Barros et al. 2010). Individuals resistant to Cry1F do not appear to have a fitness cost when fed corn, soybean or cotton (Jakka et al. 2014). These results support the potential for those crops to keep multiple, and perhaps overlapping, generations of FAW, including those resistant to Bt, maintaining large population sizes in all Brazilian regions. In addition to commercial and cash crops, the larvae can feed on several host plants used for cover crops, occupying more than 90% of crop area. While none of these crops include Bt varieties, this practice likely maintains large FAW populations year-round (De Sá et al. 2009, Prasifka et al. 2009, Barros et al. 2010). Despite a wide option of larvae host plants in tropical regions, which could be considered as a natural refuge, it might not be sufficiently effective to manage resistance evolution of FAW in Brazil. There may be mating asynchrony between individuals selected in Bt fields and susceptible individuals generated in alternative hosts due to a distinct life-span influenced by different food sources.

*Complex detoxification system from adaptation to multiple host plants*

FAW feeding on different host plants may have facilitated adaptation to overcome many plant allelochemicals including expressing plant protease inhibitors (Jongsma et al. 1995, Brioschi et al. 2007, Dunse et al. 2010, Chikate et al. 2013), developing cell detoxification systems including cytochrome P450s and ABC transporters (Xie et al. 2012, Dermauw et al. 2013), and increasing metabolism of toxic compounds (Wadleigh and Yu, 1988, Sasabe et al. 2004, Li et al. 2007).

Detoxification is a major physiological trait related to host-plant evolution (Ehrlich and Raven 1964). Cytochrome P450s are a class of a detoxification enzymes metabolizing xenobiotic compounds in insects (Li et al. 2004, Sasabe et al. 2004, Rupasinghe et al. 2007). In general, insects with a wide host range have a diverse set of P450's, whereas specialists are more limited in number (Giraudo et al. 2015). For example, FAW has 100-120 P450 genes (Giraudo et al. 2015) while *Drosophila sechellia*, a specialist insect, has around 70 P450s (Good et al. 2014). Interestingly, P450s are also known as one of the main enzymes responsible for metabolizing many insecticide compounds (Rupasinghe et al. 2007), which could explain the capacity of FAW to rapidly adapt to several different active ingredients under selection pressure. Their role in Bt resistance has yet to be determined.

ABC transporters are also used in the detoxification process of host plant allelochemicals, and have frequently been reported to be associated with resistance to insecticides through the detoxification process (increased expression of ABC transporter genes). More recently, resistance to Bt crops was also associated to the ABC transporter system but due to mutations that decreased their expression (*H. virescens* to Cry1Ac, Gahan et al. 2010; *P. xylostella*, Baxter et al. 2011, Guo et al. 2015; *Spodoptera exigua*, Park et al. 2014; *Bombyx mory* to Cry1Ab, Atsumi et al. 2012; *H. armigera* to Cry1Ac and Cry1Ab, Xiao et al. 2014). However, mechanisms of FAW insecticide or Bt resistance have yet to be determined.

*FAW larvae movement is density dependent and influenced by strong cannibalism*

Pronounced FAW cannibalism is an additional ecological factor that plays a crucial role in population regulation. Despite the large reproductive output, it is common to find only one late instar larvae per corn plant. Cannibalism is affected by several factors with insect density associated with host plant architecture being the most important (Fischer 1961, Istock 1966, Sikand and Ranade 1975, Tschinkel 1978). On corn, females lay eggs in the upper surface of new leaves emerging from the whorl. After hatching, neonates drop into the whorl where they

remain agglomerated until they are induced to disperse, avoiding being easy prey from intraspecifics.

Larval movement plays a considerable role in insect resistance evolution to Bt crops, especially in integrated refuges (e.g. refuge in a bag) when feeding starts on Bt plants, but then moves to refuge to avoid toxin which can lead to sub-lethal doses. However, in Brazil, integrated refuges have not been implemented. Nonetheless, late instar larvae carrying resistance alleles can survive on Bt plants even if there is a "high dose" event (i.e. no movement, Miraldo et al. 2016). Most of the Cry proteins are not "high dose" for FAW (Santos-Amaya et al. 2015, Farias et al. 2015) and the potential of late instar survivorship is very high, accelerating resistance evolution. Assuming 50% of cporn plants are infested at with 1 larvae, and planted at 65,000 plants per hectare, around 32,500 larvae per hectare in the first generation can be produced.

### 2.4 Agroecosystem Landscape in Tropical Brazil Favors Resistance

Brazil's tropical climate associated with abundant rain in important agronomic crop growing areas promotes very efficient land useproduction. Two crop seasons per year is common for almost all regions, with some able to produce a third crop under pivot irrigation. Crop succession forms a year-round mosaic that includes many FAW hosts such as soybean, corn, wheat, cotton, or millet, and most of these produce Cry proteins (corn, cotton, and soybean, see above). For example, in Bahia State, some farms grow corn in the summer (from Sept. to Jan.), then cotton (Jan. to June) and, after cotton harvest, then plant soybean; all 3 hosts can express similar Cry proteins and place intense and consistent selection pressure in FAW for ten months of the year (Figure 2.1). The ecological interactions between FAW, Cry protein availability in multiple crops year-round, and the large expanse of agronomic crop production in the Brazilian landscape poses one of the biggest challenges to prevent FAW resistance.

The fast resistance evolution of this species to Bt corn expressing Cry proteins in Brazil might also be influencing the evolution of resistance in other South American countries that grow the same products. Interestingly, unexpected damage for Cry proteins has been observed in other countries such as Argentina, Paraguay, Uruguay and Colombia soon after the failures in Brazil (Blanco et al. 2016). We raise here two different, but not mutually exclusive, hypotheses for such "regional" resistance in several different countries in a short period of time: *i*) Resistance evolution developed locally due intense selection pressures; *ii*) Long-distance migration of FAW spread resistant alleles across South America.

A landscape genetic approach could help to test these two hypotheses as well as to understand the metapopulation dynamics of FAW. The question remains whether Brazil, with 16 million ha of corn (not to mention other Bt crops), is a potential source of FAW Bt resistance for other South American countries where production is significantly smaller (Argentina: 4 million ha, Paraguay: 700,000 ha, Uruguay: 83,200 ha, Colombia: 68,000 ha) and less crop per season (Figure 2.2). If this hypothesis is supported, FAW may adhere to the "Mainland-island Model" of metapopulation dynamics (Hanski and Gyllenberg l997), and allow industry to implement strategies toward reducing emigration from sources and help manage Bt resistance evolution across South America.

**2.5 Lack of Proper Adoption of IRM Strategies to Prevent FAW Resistance**

Among the three factors influencing resistance evolution (pest genetics, biology/ecology and resistance management), only resistance management is amenable to human intervention. Accordingly, industry, academic and government researchers (e.g. EMBRAPA) have developed and advertised the best practices for IRM for Bt crops in Brazil (Agrobio 2016).

The primary IRM strategy recommended is the refuge which will reduce the selection pressure for a proportion of the population. However, refuge compliance in Brazil has been one of the biggest challenges agricultural industry has faced since Bt crops launched. While informal surveys have been done, compliance data has not been published. However, expectations suggest that compliance is at or lower than 20%. In addition, resistance for Cry proteins launched since 2008 are not consistently recessive (Cry1F - Farias et al. 2015, Cry1Ab – Jakka et al. 2016), thus not adhering to the high dose/refuge strategy. Vip3Aa20 expressed in Agrisure Viptera® hybrids is a unique Bt corn event that adheres to high dose concept for FAW (Bernardi et al. 2016). This product has been on the market for over five years and in six different countries of South America (Argentina, Paraguay, Uruguay, Brazil and Colombia) and, to date, unexpected damage has not been reported in the field as well as in laboratory through susceptibility monitoring.

The refuge effectiveness also depends on the biology/ecological factors of each target pest. For example, when FAW larvae feed on corn leaves, volatiles are released that deter additional oviposition (De Moraes et al. 2001, Harmon et al. 2003). The refuge, under high FAW pressure, can be extensively damaged by larvae from the first migration into the field. Additional females that migrate will then likely prefer to lay eggs on Bt corn instead of the refuge (Téllez-Rodríguez et al. 2014). Furthermore, the refuge would likely generate susceptible adults only in the first migration of FAW, which may result in asynchronous mating times between adults emerging from Bt and refuge fields. A potential recommendation

would be to increase refuge size. Also, refuge could be planted with hybrids that support higher plant density (e.g. plant architecture), thereby increasing the number of susceptible insects being generated, as currently only one late instar larvae per whorl is common due to cannibalism.

A different strategy would include a partial insecticide spray program to protect some of the new corn leaves in the refuge. In Brazil, IRAC (Insecticide Resistance Action Committee) recommends to plant refuge and perform no more than two foliar applications until V6 corn stage when 20% of the plants reach a FAW damage score of 3 (Davis Scale) (Figure 2.3). This recommendation can generate a reasonable number of adults in early corn stages and could give some protection of at least two waves of new leaves which would be suitable for new females to lay eggs in later growth stages of the refuge (IRAC-BR 2016). This could make the refuge more effective for longer time periods. Unfortunately, this recommendation was released in 2015, after which wide-scale resistance to some Cry proteins had already occurred.

Pyramid strategy is also strongly recommended to manage resistance in Bt fields and has been used worldwide. The pyramid strategy discussed earlier uses two or more genes expressing different Bt proteins in the same hybrid. Extended durability is achieved when each single protein from that combination is able to control all susceptible individuals (Roush 1998, Brévault et al. 2013). When Bt corn was first launched in Brazil, only a single toxin expressed in hybrids were available, and the technology adoption was close to 75% in the first two years (Celeres 2013). Thus, the selection pressure imposed by a single product was very high. When the first pyramids were launched, the selection pressure had already occurred, increasing the frequency of resistance alleles to the pyramid. For example, Cry1F protein was commercialized in Brazil for at least five years as single toxin. In 2011, a pyramid containing Cry1A.102+Cry2Ab2 was released. One year later, the combination Cry1F+Cry1A.105+Cry2Ab2 was also introduced. Both pyramids had unexpected damage in the field within 2-3 years after launch (Table 1).

The rapid resistance evolution for pyramids might be also explained by the potential for cross resistance between proteins. Some of the proteins compete for the same binding sites in the receptor of the FAW midgut, causing unstable binding when one protein is in presence of the other, reducing the efficacy of both (Hernandez-Rodriguez et al. 2013, Sena et al. 2009). Such potential for cross resistance might be given by high sequence similarity between proteins. For example, Cry1A.105 has 99% of its amino acid sequence similar to Cry1F for domain III and is identical to Cry1Ab for domain I (Tabashnik et al. 2013). The multi-Bt crop

system combined with cross resistance among Cry proteins is further aggravated by the reduced refuge compliance. Thus, pyramid strategy for manage resistance of FAW to Bt crops in Brazil expressing Cry proteins has not reached expected benefits.

Another option for IRM is the use of chemical treatment in Bt corn. The use of insecticide to manage resistance in Bt crops can have positive (if only Bt field is treated), negative (if only refuge is treated) or neutral impact (if both refuge and Bt field are treated) (IRAC International 2016). The rationale of spraying the Bt field would be to kill any potential survivors selected on Bt areas (i.e. *rr* insects). However, similar challenges in application efficacy, such as protection in the ear, remain, and *rr* could still emerge, with the added determinant of insecticide exposure.

## 2.6 Conclusion

Bt crops are the fastest technology ever adopted for agronomic crop production which has brought benefits not only to growers but also to the environment, consumers and food security (NAS 2016). The high adoption of such technology has strongly contributed to a reduction of important crop pests in several regions and crops. Indeed, after Bt corn was first introduced in Brazil to control FAW, larvae practically disappeared from corn fields. However, Bt trait efficacy was broken very quickly not only in Brazil, but also in other South American countries due to fast resistance evolution of this pest to all Cry proteins.

Rapid resistance evolution seems to not have been influenced by any individual factor, but through the contribution of several different factors including genetics and biology/ecology characteristics of the species, the agricultural landscape and, especially, resistance management practices adopted. Immense and rapid reproduction, large scale dispersal, lack of fitness costs and high-doses, and poor refuge compliance have created a perfect storm that facilitated Bt resistance in FAW. Further research and implementation on IRM strategies would help understand the potential risk for resistance evolution before product launching and allow industry, academic, government and researchers to propose and improve proactive resistance management strategies.

Development of a perfect IRM system is challenging due to the interaction of biology, ecology and even society (e.g. refuge amounts, and compliance). Decision of any IRM strategy implementation starts on the farm and will largely influence trait durability. The refuge, which is a basic recommendation and has consensus among the scientific community and agriculture industry as one of the best practices, is poorly adopted in in Brazil. Academic, industry and government researchers and regulators should continue their collaborations to

develop solid, science-based recommendations and transfer these through the product chain in order to extend the durability of Bt technologies in the field for further generation of products. These groups should also invest in education and training of farmers and crop consultants for proper refuge implementation. As only 1 Bt product remains effective (Vip3Aa20), all agronomic groups have a vested interest in extending its durability.

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## Tables and Figures

Table 2.1. Commercial Bt corn for Lepidoptera control in Brazil, regulatory approval timelines and initial failure seen in the field.

| Company | Traits | Event Name | Trade Name | Approval Year | Field Failure Started |
|---|---|---|---|---|---|
| **Syngenta** | Cry1Ab | SYN-BT011-1 | Agrisure TL | 2008 | 2011 |
| | Vip3Aa20 | SYN-IR162-4 | Agrisure Viptera | 2009 | NF |
| | Cry1Ab+Vip3Aa20+ Gli | SYN-BT011-1 x SYN-IR162-4 x MON-00021-9 | Agrisure Viptera3 | 2010 | NF |
| **Monsanto** | Cry1Ab | MON-00810-6 | YieldGard | 2008 | 2011 |
| | Cry1A.105+Cry2Ab2 | MON-89034-3 | YieldGard VTPro | 2009 | 2013 |
| **Dow Agroscience** | Cry1F | DAS-01507-1 | Herculex I | 2008 | 2011 |
| | Cry1A.105+Cry2Ab2 +Cry1F | MON-89034-3 x DAS-01507-1 x MON-00603-6 | PowerCore | 2013 | 2014 |
| **Pioneer** | Cry1F | DAS-01507-1 x MON-00603-6 | Herculex I | 2009 | 2011 |
| | Cry1F+Cry1Ab | DAS-01507-1 x MON-00810-6 x MON-00603-6 | Optimum Intrasect | 2011 | 2012 |
| | Cry1F+Cry1Ab+ Vip3Aa20+Gli+Glu | DAS-01507-1 x MON-00810-6 x SYN-IR162-4xMON-00603-6 | Leptra | 2015 | NF |

NF – No failure reported
Field Failure Started for FAW– Based on growers reports and observations (data unpublished)

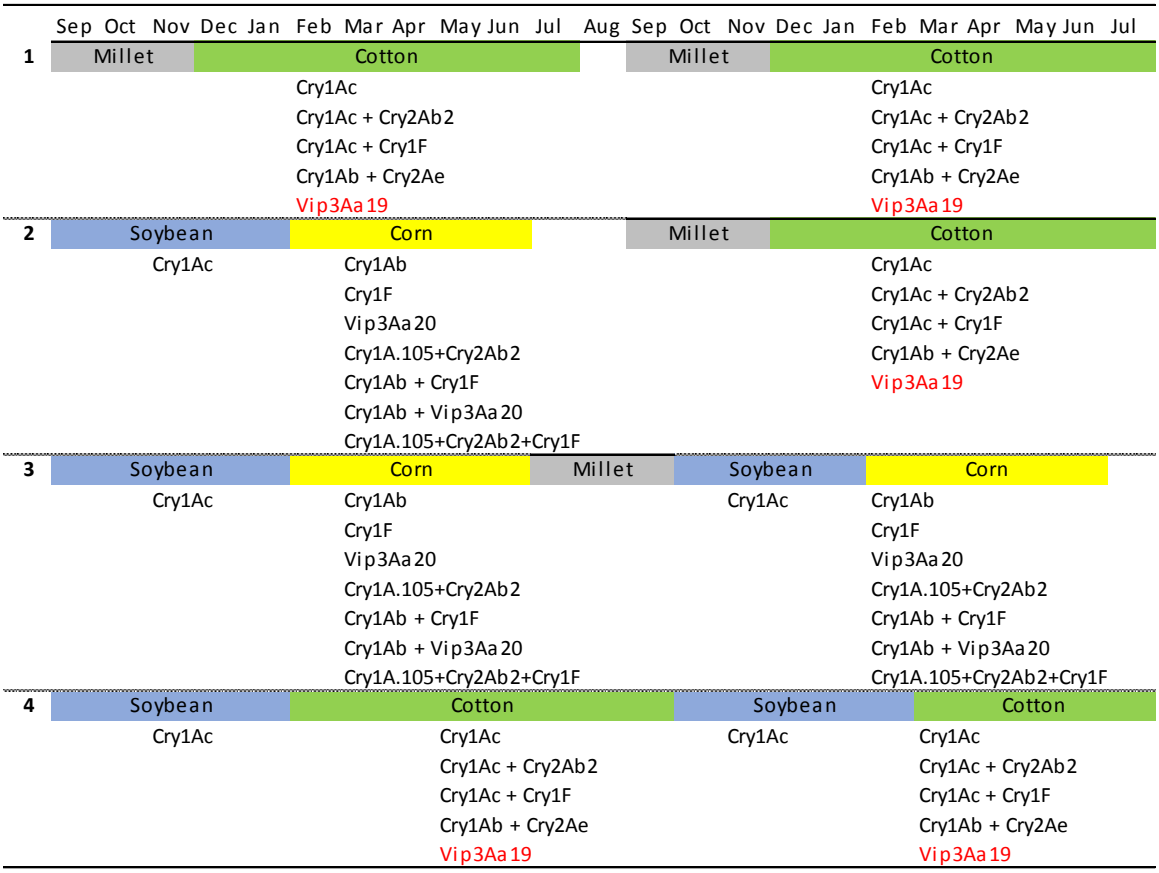


Figure 2.1. Four most common scenarios of agriculture production for Bt crops in Bahia and Mato Grosso States, Brazil. Intensive use of Cry proteins occurs concomitantly or in succession. Vip3Aa19 (red) in combination with other Cry protein is expected to be available by 2019 depending on regulatory approval. Figure from Prof. Celso Omoto from University of São Paulo University, ESALQ, with his permission.

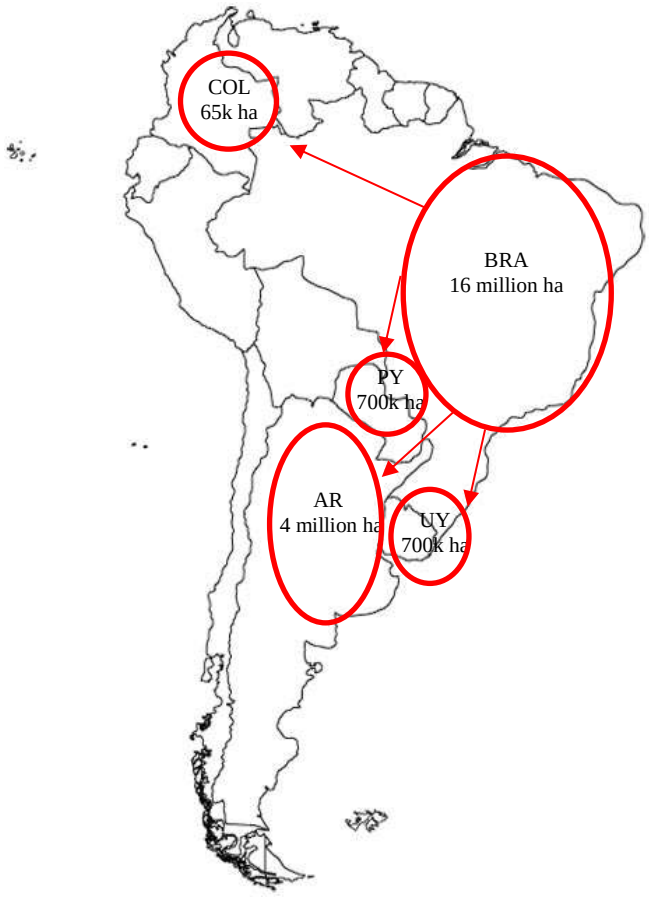


Figure 2.2. Countries in South America that cultivate Bt corn and area of hybrids market (not included white corn for human consumption). COL – Colombia; BRA-Brazil; PY-Paraguay; UY-Uruguay; AR-Argentina. Red circles represent the estimated size of potential Bt corn area (USDA 2016) and red narrows represent potential migration of FAW carrying resistant alleles.

Figure 2.3. Threshold for insecticide application in refuge area for FAW control. Limitation to two insecticide applications until V6 corn stage. Insecticide Resistance Action Committee - Brazil, 2016. Photo: IRAC-BR.

# 3 BT PROTEIN ACTIVATION AND RECEPTOR INTERACTION IN FALL ARMYWORM (*Spodoptera frugiperda*) RESISTANT TO VIP3Aa20 PROTEIN

**Abstract**

Transgenic plants containing genes from *Bacillus thuringiensis* have been used as an alternative to chemical insecticides for controlling important agricultural pests. Evolution of resistance in insect pests is the main threat to the sustainability of Bt crops. Extended sustainability requires understanding the biochemical as well as the genetics of modes of action and mechanisms of resistance to improve Insect Resistance Management (IRM). The vegetative insecticidal proteins (Vip), secreted during the vegetative growth phase of the bacteria (*Bacillus thuringiensis*), are considered a second generation of insecticidal proteins, since they do not share any structural or sequence homology with previously used crystal proteins (Cry), in addition to having a wide insecticidal spectrum. In this study, Vip3Aa20 proteolysis activity was assessed through protein activation using larval gastric extract from susceptible and resistant colonies of fall armyworm (FAW). The results suggested no difference between resistant and susceptible colonies in neither the total Vip3Aa20 activation nor time of activation. Additionally, we analyzed brush border membrane vesicles (BBMV) to determine if lack of protein-receptor binding was responsible for resistance. Using homologous and heterologous competition assays, there was no difference among resistant and susceptible colonies, and no competition with Cry1Ab. Our data suggest that resistance of FAW to Vip3Aa20 is neither associated to protein activation nor a lack of protein-receptor binding. Other downstream mechanisms in the Vip3Aa20 mode of action are likely responsible for resistance, which necessitates additional molecular analyses.

Keywords: Protein-receptor binding; Fall armyworm; Vip3A; Brush border membrane vesicles; *Bacillus thuringiensis*; Insect resistance

## 3.1 Introduction

The *Spodoptera* genus includes many insects that are important primary and secondary pests of various economically important crops. Among these, *Spodoptera frugiperda* (JE Smith), or the fall armyworm (FAW), is arguably the most important pest of agronomic crops in South America, especially in Brazil (Blanco et al. 2016, Valicente et al. 2008). This species has experienced higher infestations in recent years, causing great economic losses in several crops, especially corn and cotton. Many factors have led to this increase including insecticide resistance and the amount of second season corn (corn planted immediately after harvest soybean in the same year) (Santos et al. 2011). Currently, second season corn represents a larger acreage than summer season (CONAB 2016), and helps maintains FAW populations year-round.

In corn, cotton and soybean, crops expressing proteins from *Bacillus thuringiensis* (Bt) toxic to insect pests have been the most used tactic to control some Coleopteran and Lepidopteran insects, including FAW. The area cultivated with transgenic crops has

increased from 1.7 million acres in 1996 (the first planting) to 179.7 million hectares in 2015 worldwide. Thus, biotech crops are considered the most rapidly adopted agricultural technology in the history of modern agriculture (ISAAA 2015). Bt crops have been seen as a revolution to agricultural production in the last twenty years by reducing insecticide use and input costs associated with application (Brookes and Barfoot 2013). Additional benefits from Bt crops include effective control of target pests and reduced negative impact to non-target organisms (Marvier et al. 2007, Cattaneo et al. 2006, NAS 2016).

Commercial Bt crops constitutively express Cry proteins in most plant tissues. Once ingested by the insect, the protein is activated by serine-proteases in the midgut, in order to bind to specific insect receptors present in the cellular membrane. Binding causes Cry protein oligomerization and, after binding to secondary receptors, leads to protein introduction through the bi-layer membrane. After introduction, pores open in the cellular membrane of the insect midgut, causing septicemia and death (Luo et al. 1999, Hofmann et al. 1988, Jurat-Fuentes et al. 2003).

Regardless of the Cry protein (e.g. Cry1A, Cry1F, Cry2Ab etc) being expressed in plant tissue, the effectiveness of Bt crops depends on the specificity of insecticidal proteins themselves. Any changes of the mode of action of these proteins could potentially result in resistance by the target pest (Ferre and Van Rie 2002). The protein activation by serine-proteases and interactions between the activated protein and the receptor on the insect midgut through an irreversible binding are necessary steps for toxicity. Changes in Cry toxin binding have been associated with high levels of resistance in insect populations (Ferre and Van Rie 2002). Thus, Cry proteins that share high sequence similarity in the domains involved with protein binding might have a higher potential for cross-resistance (Tabashnik et al. 2013). Cross resistance must be considered with the introduction of new products or the adoption of pyramid strategy, which might influence the durability of the Bt crop (Carrière et al. 2015). In Brazil, five different Bt proteins and five different pyramid products were launched from 2008 to 2015 for Lepidoptera control in corn (CTNBio 2016), and most including Cry toxins. Despite multiple Bt Cry proteins, resistance evolution now impedes the efficacy of these crops for FAW control (Farias et al. 2014, Omoto et al. 2016); even pyramid products (containing more than 1 Bt trait) are failing, potentially due to cross resistance (Hernandez-Rodriguez et al. 2013, Sena et al. 2009).

Alternatively, the Vip3Aa20 Bt protein differs from Cry proteins in several characteristics. It represents a vegetative insecticidal protein (e.g. Vip) and an exotoxin (whereas Cry proteins are endotoxins). It is soluble in a pH ranging from 5 to 10, while most

Cry proteins have pH 7 to 10 as optimum. Vip3Aa20 works immediately following the ingestion (Lee et al. 2003). The mode of action slightly differs from Cry proteins as well. The activation process generates a 62 kDa toxin from its original 89 kDa form, followed by binding to a receptor resulting in 80 and 110 kDa complexes, substantially different from Cry1 proteins (Lee et al. 2003). It forms pores in the insect midgut, similar to Cry proteins; however the characteristics of ions channels formed in the membrane is different from channels formed by the action of Cry1 proteins (Lee et al. 2003). In addition, Vip3A does not share sequence homology with known Cry toxins, which reduces potential for cross resistance since there is likely not competition for the same receptors, a favorable condition for pyramid strategy (Estruch et al. 1996). Midgut receptors for Cry1 are very well characterized, but not for Vip. Proteomic studies involving Vip3Aa20 showed that ribosomal protein S2 might be a potential receptor, whose interaction is required for the proper mode of action and cell death in FAW and insect death of *Spodoptera litura* (Singh et al. 2010).

Bt corn expressing Vip3Aa20 was first introduced in Brazil in 2010 and, to date, provides effective FAW control. The resistance allele frequency was estimated to be extremely low (0.0009), in 11 different Brazilian regions (Bernardi et al. 2015). In addition, susceptibility monitoring data generated for this pest in laboratory assays over the last four years has not shown any unexpected survivorship in 10 different States of Brazil. However, field collections and subsequent laboratory mating (e.g. F2 screens) (Andow and Alstad 1998) generated two resistant FAW colonies to Vip3Aa20, Vip-R1 with lower resistance ratio (RR=7.6) and Vip-R2 with higher resistance ratio (RR>3,200) (Bernardi et al. 2015, Bernardi et al. 2016).

Using these Vip3Aa20 resistant and susceptible colonies, we tested two hypotheses which could be associated with resistance of FAW to Vip3Aa20: 1) reduced protein activation by the midgut extracts through serine-proteases proteolysis activity; 2) failure of protein binding to a receptor, which is the immediate step after protein activation and a pre-requisite for downstream events and insect cell death. We also generated supplementary information for homologous and heterologous competition with Cry1Ab comparing Vip-S2 against Vip-R2. Understanding of mechanisms of resistance of FAW to Vip3A20 before resistance occurs will allow industry, government and academic researcher to proactively develop and implement the best IRM strategies for this product and extend the durability for control.

## 3.2 Material and Methods

*Insect colonies and proteins*

Resistant colonies of FAW (Vip-R1 and Vip-R2) were established through the F2 screen methodology (Andow and Alstad 1998) and maintained under 25ºC ± 1ºC, humidity 70 ± 10% and light: dark 14:10 h) (Bernardi et al. 2015, Bernardi et al. 2016). Resistance of Vip-R2 colony was confirmed through dose-response study which showed a very high resistance ratio (RR>3,208) (Bernardi et al. 2016). This colony also demostrated to complete the cycle and generated viable offspring when feeding on Viptera® corn plants (Bernardi et al. 2016). The resistance ratio of Vip-R1 was also estimated through dose-response assay, which demonstrated to be low (RR = 7.6), but larvae could survive and generate offspring when fed Viptera® corn plants (Bernardi et al. 2015). Aspects related to phenotypic and genetic resistance characterization of Vip-R2 colony was published by Bernardi et al. (2016). A susceptible (Vip-S2) colony used for this study was established in the laboratory and kept over 12 years out of selection pressure on insecticide and Bt crops.

Vip3Aa20 protein (86.5% purity) was produced and purified by Syngenta Crop Protection and shipped on dry ice and stored in a -20 °C freezer. Cry 1Ab protein used in the heterologous competition study was produced and purified by the laboratory of Genetic of Bacteria at Sao Paulo State University – Jaboticabal Campus, through Bt strains maintained in the facility (Herrero et al. 2004).

*Proteolysis assay using Vip-R1, VipR2 and Vip-S2 FAW colonies*

Fourth instar larvae of FAW (Vip-R1, Vip-R2 and Vip-S2) were used for midgut extraction to obtain gastric extracts. Ten midguts from each colony (Vip-R1, Vip-R2 and Vip-S2) were removed from the larvae, suavely washed in MET buffer and placed in 1.5 mL tube and centrifuged for 10 minutes at 6,000 Xg. The supernatant was subsequently transferred to a new 1.5 mL tube, immediately frozen in liquid nitrogen and stored at -80 °C to maintain activity of serine proteases in the gastric extracts.

The proteolysis assay was performed three times for each colony. A solution containing 50 µg of 86.5% purity protoxin obtained from Syngenta was incubated with midgut extracts at a ratio of 1:100 (w:w, weight of midgut extract:weight of Vip3Aa20) in a final volume of 70 µL of water. This solution was then incubated over different times of protein activation (5, 10, 15, 20, 25, 30, 60, 120 min) under 30 °C in mixer table at 100 rpm. The reaction was stopped by adding 35 µL of buffer solution (62 mM Tris-HCl pH 6.8, 4% SDS, 20% glycerol, 5% β-

mercaptanol and 0.02% bromophenol blue) and heated for 5 minutes at 99 °C. The reaction was initially optimized using commercial trypsin (porcine pancreas – Sigma-Aldrich®) to set the best w:w proportion. To visualize proteolysis, the samples were electrophoresed in a 12% polyacrylamide gel (SDS-PAGE) using a vertical Mini Protean II (Bio-Rad© Laboratories, Richmond, EUA), filled with buffer (0.119 M glycine, 0.025 M Tris and 0.1% SDS) and subjected to a constant current of 60 mA and 200 V for 90 min. A marker for molecular weight was added to the gel. The stacking (in the top of vertical Mini Protean II) and separation (botton of vertical Mini Protean II) gels were prepared at 5% and 12% of acrylamide respectively. The reagents used in the preparation of the stacking gel (5%) were 0.5 mL of Tris-HCL (0.5M, pH 6.8); 0.33mL of acrylamide – Bis (30:0.08%); 0.125 mL of SDS (4%); 0.006 mL of TEMED (tetramethylenediamine); 0.02 mL of ammonium persuphate (10%); 1.105 mL of distilled water. For the separation gel (12%), we used 0.8 mL of Tris-HCL (2.25M, pH 8.8); 1.66 mL of acrylamide – Bis (30:0.08%); 0.115 mL of SDS (4%); 0.015 mL of TEMED (tetramethylenediamine); 0.065 mL of ammonium persuphate (10%) and 2.43 mL of distilled water. After electrophoresis, the polypeptide bands were stained with Coomassie-Brilliant Blue 0.02% colored gel (30% methanol, 10% acetic acid, 0.1% cupric sulfate and water) for 15 min and washed successively with a bleach solution (10% methanol, 10% acetic acid and water). The gel was analyzed and photo documented in a scanner (HP Scanjet 3570c).

Quantitative comparisons for the activation rate across the different incubation times was performed to determine the amount of inactivated protein (89 kDa) as well as the portion of activated protein (62 kDa) through density parameters using ImageQuant TL 8.1 software (GE Healthcare Bio Sciences AB, Uppsala, Sweden). Afterwards, the data were analyzed by analysis of variance through F test. Mean comparison was performed through Tukey test. Analyses were carried out using AgroEstat software (Barbosa and Maldonado 2014), using $P < 0.05$ for statistical significance.

*Vip3Aa20 protein activation and labeling*

Vip3Aa20 protein was activated by commercial trypsin (bovine trypsin - Sigma®) at 37 °C for 1 h 30 min while shaking at 200 rpm. The enzyme was then inactivated by centrifugation at 17,000 Xg, 4 °C for 10 min. Activated Vip3Aa20 was labeled with biotin, using the "biotinylation kit ECL Module" (GE Healthcare®). The labeling reaction consisted of incubation of 1,000 µg of Vip3Aa20 protein plus 40 µL of the biotinylation compound under moderate mixing at room temperature (22-25oC) for 2 hr. Afterwards the protein was

transferred to a G-25 column (P10 Desalting) (GE Healthcare®), and eluted using 20 mL of PBS. Samples were collected in a 1.5mL eppendorf tube, quantified through the Bradford method (Bradford 1976) and stored at 4 °C.

*Mid gut extraction and BBMV preparation*

Midgut of fourth-instar larvae of FAW from the Vip-R1, Vip-R2 and Vip-S2 colonies were extracted, washed and stored in cold MET buffer [250 mM mannitol, 17 mM Tris-HCl and 5 mM ethylene glycol-tetra-acetic acid (EGTA), pH 7.5], frozen in liquid nitrogen and stored at -80 °C (Hernandez et al. 2004) to be used for BBMV preparation (BBMVs). Vesicle preparation followed the differential magnesium precipitation method described by Wolfersberger et al. (1987), and quantified by the Bradford method (1976). Midgut samples (1g) were transferred to tubes containing 10 mL of homogenization buffer (300 mM mannitol, 17 mM Tris-HCl, 10 mM hepes, 5 mM EGTA, 2 mM DTT, 1 mM EDTA and 1 mM PMSF, pH 7.4) and subjected to cycles of 30 seconds paused by 30 seconds (9 times) at 3,000 rpm for homogenization. After homogenization, 10 mL of 24mM MgCl2 was added to the samples and incubated on ice for 15 min followed by centrifugation at 4,500 rpm for 15 min at 4 °C. The supernatant was collected and centrifuged at 10,000 rpm for 1 hr and 30 min at 4 °C. The pellet was resuspended in 2.5 mL of homogenization buffer plus 5 mL of MgCl2 24mM and incubated on ice for 15 min. Additional centrifugation was done at 4,500 rpm for 15 min at 4 °C. The supernatant was collected and centrifuged at 15,000 rpm for 40 min at 4 °C. After that, the supernatant was discarded and the pellet was resuspended in 500 µL of homogenization buffer and stored at -80 °C. The BBMV concentration was quantified by Bradfor method (Bradford 1976).

*Ligand-Blotting*

The ligand-blotting test was repeated three times and prepared according to Abdelkefi-Mesrati et al. (2011) using 20 µg of midgut extract and 40 nM protein (Vip3Aa20). The BBMVs were added with 10 µl Laemmli 4X sample buffer and incubated at 100 °C for 5 min. Samples were electrophoresed on SDS-PAGE gel (separation gel, 9%). After electrophoresis, the SDS-page gel was electro-transferred (under 100 V for 1 h) onto a nitrocellulose ECL membrane (GE Healthcare®) and subsequently subjected to blocking with 10 mL TBS (Tris-buffered saline (TBS) (1X) - 50 mM Tris-Cl, pH 7.5, 150 mM NaCl) containing 3% BSA (bovine serum albumin) and 0.01% Tween-20 under gentle stirring for 1 h. The membrane was then washed with TBST (Tris-buffered saline (TBS) (1X) - 50 mM Tris-Cl, pH 7.5, 150

mM NaCl, 0.1% Tween-20) for 10 min and then incubated for 2 h with Vip3Aa20 protein, which was diluted in TBST (15 µL protein/15 mL of TBST). After washing again with TBST (3 times for 10 minutes each), the membrane was incubated for 1 h in a solution containing the conjugated streptavidin/phosphatase at a dilution of 1:2000 (HRP) (GE Healthcare®) for specific binding to biotin. Then the membrane was washed again for 3 times with TBST and developed with 10 mL solution of NBT BCIP (Sigma®) according to instructions of the manufacturer. The reaction was subsequently stopped by adding water.

*Homologous and heterologous competition test*

Homologous and heterologous competition assays were performed in order to identify if resistance could be influenced by the reduction of binding affinity between protein and receptor under high concentration of homologous and heterologous proteins. If binding affinity is reduced in the resistant population, we would expect to see presence of band in the gel for treatments with high concentrations of competitior protein at the homologous competition assay while lack of band in high concentrations of protein at the heterologous competition assay. The tests were performed in two replicates according to Abdelkefi-Mesrati et al. (2011). Vip3Aa20 and Cry1Ab were activated as described in the protein activation section.

Activated Vip3Aa20 with the biotin label (100 ng) was incubated with unlabeled Vip3Aa20 for homologous competition and with unlabeled Cry1Ab for heterologous competition assay in an excess proportion of 50, 100, 500 and 1000-fold (protein labeled/protein unlabeled) and in the presence of 20 µg of BBMVs. Cry1Ab protein was selected for heterologous competition since it is present in commercial product combination with Vip3Aa20, as strategy to delay resistance for FAW.

The reactions were incubated for 1 h and 30 min at 28°C and mixed at 140 rpm. Thereafter, the sample was centrifuged at 13,000 rpm for 10 min at 4°C. BBMVs were washed twice with 500 µl of PBS, centrifuged at 13,000 rpm for 10 min at 4°C and dissolved in 20 µl of PBS. Samples were then incubated at 100 °C for 5 min along with 10 µl Laemmli 4X sample buffer and electrophoresed on SDS-PAGE gel (separation gel, 9%). After electrophoresis, SDS-page gel was electro-transferred (under 100 V for 1 hr) onto a PVDF membrane (GE Healthcare®) in a humid transfer cube - Mini Trans-Blot® Electrophoretic Transfer Cell (Bio-Rad©). After removed from the cassette, the membrane was washed in TBS buffer (50 mM de Tris and 150 mM of NaCl, pH 7,6) for 5 min. Specific binding was blocked through addition of 10 mL of TBS-T buffer (50 mM of Tris; 150 mM of NaCl, pH

7,6 and 0,1% Tween-20) added by 5% (weight/volume) of skim milk powder and kept under gentile mixing for 1 hr. After that, the membrane was washed two times in TBS-T buffer.

Membrane was then placed in Streptavidin-AP conjugate (Roche©) added by TBS-T buffer at a proportion 1: 2000 and kept under gentile mixing for 1 h at room temperature (22-25 °C). Then the membrane was rapidly immersed two times in TBS-T buffer and washed in excess in the same buffer for three times for 10 min each. Biotinylated protein was visualized through the addition of 10 mL substrate NBT/BCIP (Sigma-Aldrich©). The qualitative analyses were performed through the presence or absence of a band in the membrane for each concentration of the competitor added to the labeled Vip3Aa20. Absence of band in a given concentration represents a lack of protein-receptor binding influenced by the competitor protein.

### 3.3 Results

*Activation of VipAa20 by Vip-S2, Vip-R1 and Vip-R2 FAW in vitro*

Our first hypothesis was that FAW resistance to Vip3Aa20 is due to a lack of protein activation within the midgut. To test this hypothesis, we used gastric extracts from resistant and susceptible FAW to determine the presence of activated protein (62 kDa band) or unactivated protein (89 kDa) over time.

The results obtained from the qualitative analysis of the proteolytic reaction showed that midgut extracts can indeed activate Vip3Aa20, as we detected the presence of the 62 kDa activated protein band. However, there was no difference in the band profile between Vip-S2, Vip-R1 and Vip-R2. Vip3Aa20 appeared to reach complete activation when incubated with gastric extracts from the three different treatments after 120 min of incubation (Figure 3.1 A, 3.1 B and 3.1 C).

Kinetic analysis with the gastric fluid further showed no statistical difference between populations Vip-S2, Vip-R1 and VipR2 most of the times tested. A significant difference (F test - $P < 0.05$) was identified for the activation rate in the Vip-R1 population, as it activated less protein at 30 and 60 min when compared to the Vip-S2 and Vip-R2 colonies (Figure 3.2). No differences existed between the Vip-S2 colony and the highly resistant Vip-R2 colony. Overall, the processing of protoxin to toxin was similar in all tested populations. After 120 min, the band representing protoxin (89 kDa) almost disappeared after incubated with gastric fluid from Vip-S2, Vip-R1 and Vip-R2 (Figure 3.2). Thus, the outputs suggest that protein activation was not the limiting step for Vip3Aa20 resistance in either FAW resistant colony.

*Ligand-blotting analysis*

Our second hypothesis was that resistance was caused by a lack of protein-receptor binding in the insect midgut. Without binding, the protein would be unable to form pores and kill the insects. In this case, we would not expect bands representing potential Vip3Aa20-specific receptors in the Vip-R1 and VipR2 gel profiles. Ligand-blotting analysis showed an interaction between the biotin-labeled Vip3Aa20 protein and potential receptors present in the BBMVs of the three colonies tested (Vip-S2, Vip-R1 and Vip-R2). If proper binding occurred, we would expect bands at approximately 65 kDa which represents a potential receptor that has been demonstrated to interact with Vip3Aa20 in other researchs also (Bergamasco et al. 2013). Our data showed that all colonies generated bands of 65 kDa among other bands (approximately 30 kDa, 40 kDa, 43kDa 53 kDa, 90 kDa and 130 kDa) (Figure 3.3). Thus, resistance for the selected populations seems not to be related to protein-receptor binding.

*Homologous and heterologous competition tests*

Competition may lead to resistance due to reduced affinity between a protein and a binding site in a receptor. We performed suplementary studies evaluating homologous (Vip3Aa20/Vip3Aa20) and heterologous competition study (Vip3Aa20/Cry1Ab), using the most resistant population (Vip-R2) compared to our susceptible colony (Vip-S2).

The homologous competition test using labeled Vip3Aa20 in one concentration and unlabeled Vip3Aa20 in different concentrations demonstrated strong competition each other at 100 fold-change concentration of unlabeled protein; however competition was not seen at lower concentrations of unlabeled protein. Comparing Vip-R2 and Vip-S2, there was no difference in competition, suggesting that that affinity for the protein receptor was not reduced in the resistant colony (Figure 3.4).

Even not expected Cry1Ab to influence in FAW resistance to Vip3Aa20, we performed a supplementary assay for heterologous competition which demonstrated that the excess of unlabeled Cry1Ab was not able to induce competition against labeled Vip3Aa20, even at the highest concentration (1,000 fold-change). Furthermore, there was no difference among the Vip-R2 compared to the Vip-S2 colony (Figure 3.5). Resistance did not seem dependent on either reduced protein affinity or influenced by presence of Cry1Ab.

### 3.4 Discussion

Genetically modified crops containing Bt genes have been a widely adopted technology worldwide. Unfortunately, some of economic and environmental benefits have been lost because of the rapid evolution of pest resistance. Understanding the biochemical mechanisms of resistance may help identify possible gene candidates involved in Bt resistance. By comparing Vip3Aa20 resistant to a susceptible colony, our data did not support mechanisms involved with protein activation, protein-receptor binding or even a reduction of binding affinity. FAW resistance to Vip3Aa20, therefore, may involve other biochemical or molecular events described in the pore forming model (Bravo et al. 2004) or G-protein signaling pathway (Zhang et al. 2006).

The effectiveness of Bt crops is influenced by the specificity of each insecticidal protein, either Cry or Vip. Consequently, any changes in their mode of action could potentially result in insect resistance (Ferre and Van Rie 2002). The mode of action of Cry proteins has been recently characterized (Adang and Crickmore 2014). In general, insect mortality results from, first, an activation step in midgut fluids by serine proteases; second, favorable protein-receptor binding and pore formation; third, osmotic cell death; and fourth, the collapse of the intestinal barrier, facilitating resident gut bacteria invasion into the hemocele and septicemia.

The process for Vip is less understood compared to Cry proteins, but some similarities are expected. The proteolysis step to activate Vip3Aa20 is important, since, generally, only active toxins can bind to receptors and form pores. Research in other insects have reported that the insect midgut is highly rich in proteases, and protease alteration has been observed in insects resistant to Cry1A toxins (Oppert et al. 1997, Li et al. 2004). Our data show that FAW gastric extracts is able to activate Vip3Aa20 (Figure 4.1), most likely due to the presence of serine proteases. These results were consistent with Vip3Aa20 protoxin activation in *Manduca sexta* (Lee et al. 2003), and *Spodoptera exigua* (Caccia et al. 2014). Furthermore, all three of our FAW colonies tested (Vip-S2, Vip-R1 and Vip-R2) activated Vip3Aa20 at similar levels and rates, suggesting that FAW resistance is not due to an inability of protein activation. Similarly, Chakroun et al. (2016) showed no difference in the band profile and, hence, protein activation between susceptible and resistant populations of *H. armigera* to Vip3Aa, yet these populations significantly differed in processing rates. A similar difference in processing rate was seen in our study, but only at 30 and 60 mins, and in only in the Vip-R1 colony. This difference might be due to natural variability among strains as Vip-R1 and Vip-R2 came from different regions of Brazil (Goias State and Bahia State respectively)

(Bernardi et al. 2015). It is difficult to measure how much this difference is influencing resistance, as protein activation is only 1 factor in protein toxicity. Despite these differences, Vip3Aa20 protein was completely activated after 120 min of incubation in all colonies, and it is unlikely that serine-protease activity is a unique factor contributing for resistance (Ferre and Van Rie 2002, Ferre et al. 2008).

The binding between the Bt protein and the appropriate receptor is another important step resulting in insect death. Protein-receptor binding assays using biotin-labeled Bt proteins have been performed for Lepidoptera species including FAW (Sena et al. 2009, Chakroun et al. 2012, Chakroun and Ferre 2014). Our assay, based on ligand binding, evaluated whether or not biotinylated Vip3Aa20 toxin could bind to the putative receptors present in the BBMVs. We confirmed that Vip3Aa20 did indeed bind to receptors in the BBMVs, and, furthermore, binding occurred with no differences among our susceptible and resistant FAW populations. Our data is consistent with previous ligand blotting assays in *Chilo suppressalis* and *Sesamia inferens* (Han et al. 2014). In *Manduca sexta*, ligand-blotting analysis with Vip3A protein revealed bands with sizes of 80 and 110 kDa and for 120 and 210 kDa for Cry1Ab which represent potential midgut membrane proteins receptors (Lee et al. 2003). The same study demonstrated that Cry, and not Vip, was able to bind to N-aminopeptidase and cadherin receptors. Additional studies demonstrated that Vip3Aa interacted with midgut proteins of 55 kDa in *S. littoralis* (Abdelkefi-Mesrati et al. 2011a), 65 kDa in *Ephesia kuhniella* (Abdelkefi-Mesrati et al. 2011b), 65 kDa in *S. frugiperda*, *S. albula*, *S. cosmioides* and *S. eridania* (Bergamasco et al. 2013). Furthermore, studies performed with Prays oleae (Bernard) (Lepidoptera: Praydidae) and *Agrotis segetum* (Denis and Schiffermüller) (Lepidoptera: Noctuidae) showed that Vip3Aa interact with midgut protein of 65 kDa whereas Cry1Ac 210 kDa in *P. oleae* and 120 kDa in *A. segetum* (Ben Hamadou-Charfi et al. 2013).

Based on our estimated ligand binding sizes, and results from other studies, the candidate putative receptors for Vip3Aa20 in FAW may consist of alkaline phosphatases, N-aminopeptidases or ribosomal protein S2. The molecular weight of alkaline phosphatases is between 62 kDa and 68 kDa (Fernandez et al. 2006, Jurat-Fuentes and Adang 2006), aminopeptidases-N between 130 kDa and 150 kDa (Hua et al. 2004, Pacheco et al. 2009, Rajagopal et al. 2003) - but also available as homodimer form of 130 kDa, formed by 65 kDa monomers (Malik et al. 2006) - and ribosomal proteins at 66 kDa (Singh et al. 2010). Interestingly, ribosomal protein S2 was reported to strongly interact with Vip3Aa20 protein in the membrane region of FAW (Singh et al. 2010). Furthermore, silencing of this same ribosomal protein in *Spodoptera litura* reduces the efficacy of Vip3A (Singh et al. 2010). The

mechanisms for how the interaction between S2-protein and Vip3A could provoke the lysis of cells has not been explained and remains unknown.

In our competition assays Vip3Aa20 protein bound specifically to the BBMVs of Vip-S2 and Vip-R2 FAW populations, as labeled protein exhibited competition with 100X excess of the respective unlabeled protein. Additionally, there was no difference between Vip-R2 and Vip-S2 for binding affinity as both showed competition at the same concentration. Several studies involving binding assays with Cry proteins, and the reduced or absent protein-receptor binding of Cry toxins to BBMV, does not always reflect correlation with insect resistance. For example, a resistant strain of *P. gossypiella* exhibited typical resistance to Cry1Ab and Cry1Ac. However, the binding of Cry1Ac to the BBMV receptors of resistant *P. gossypiella* was not altered, although binding of Cry1Ab to the receptor was significantly reduced (Gonzalez-Cabrera et al. 2003). Likewise, *H. virescens* highly resistant to Cry1Aa, Cry1Ab and Cry1Ac did not show changes in the binding of Cry1Ab and Cry1Ac to the BBMV in the resistant strain (Lee et al. 1995).

Our supplementary heterologous competition assay was seen between unlabeled Cry1Ab and labeled Vip3Aa20 even in high excess of Cry1Ab (1000x), suggesting that these proteins do not share receptors in the BBMV in Vip-S2 nor in Vip-R2 strains. In other species, several studies have indicated that Vip and Cry proteins do not share binding sites including Vip3Aa20 and Cry1Ab in *D. saccharalis* (Davolos et al. 2015), and between Vip3Af, Cry1Ab and Cry1F in FAW (Lee et al. 2006, Sena et al. 2009, Liu et al. 2011, Gouffon et al. 2011, Ben Hamadou-Charfi et al. 2013, Chakroun and Ferré 2014, Jakka et al. 2015).

Our data show that, in resistant FAW, Vip3Aa20 is activated, and can bind to specific receptors; therefore resistance mechanisms must be downstream in the Vip toxic pathway. Other studies have shown additional and different mechanisms which may play a role for insect resistance to Bt proteins. For example studies involving mutations in genes responsible for ABCC2 transporter system have shown to be linked to resistance of several Lepidoptera pests to Cry1Ac and Cry2Ab (Guo et al. 2015, Park et al. 2014, Tay et al. 2015). Several hypotheses suggest a role for ABC transporters in Bt resistance (Gahan et al. 2010, Baxter et al. 2011, Atsumi et al. 2012). ABC transporters may be involved with Bt protein-receptor binding, the introduction of the Bt protein through the bilayer membrane, or work as a receptor totrigger downstream events (Vadlamudi et al. 1995). An additional hypothesis is that ABC transporters act through cell detoxification by reducing Bt protein in the cytosol (Russel et al. 2008). The hypothesis of cell detoxification through effluxing Bt protein from

the cytosol might be an option, as Vip3A was reported to be present mostly in the cellular membrane region but also in the cytoplasm when interacting with S2 ribosomal protein (66 kDa) at the cell membrane (Singh et al. 2010). In our study, we detected a 65 kDa protein which could indeed be S2 ribosomal protein involved in Vip3Aa20 inffluxing to the cytosol.

In summary, data from this study demonstrated that neither Vip3Aa20 activation nor protein binding were related to resistance in FAW. Resistant colonies did not show any significant difference when compared to a susceptible colony for all experiments. Other mechanisms may be the cause of resistance of FAW to Vip3Aa20, such as downstream events associated with pore forming or G-protein signaling pathway models. The downstream mechanisms of resistance to Vip toxins in FAW have not been described. However, the understanding of the physiological and molecular mechanisms involved with resistance of this species are fundamental for FAW resistance management in South America, and all of the Americas, as FAW is widespread across 2 continents. Further research to elucidate the causative mechanism of Vip3Aa20 resistance genes in susceptible and resistant populations of FAW will provide crucial information to improve approaches to resistance management.

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## Figures

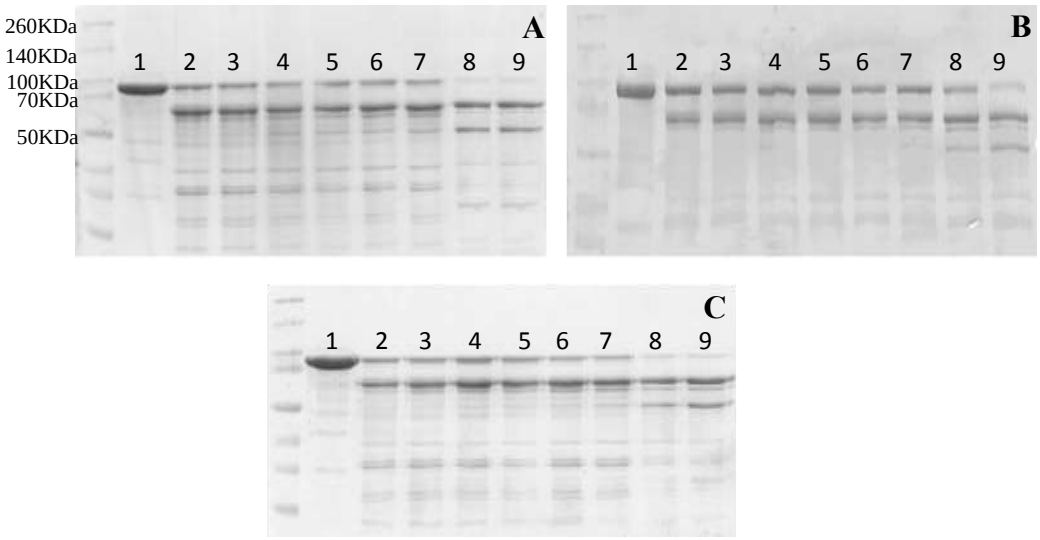


Figure 3.1. Proteolysis kinetics of Vip3Aa20 incubated with gastric fluid of FAW caterpillars. Incubations were performed at 30 ° C in 100 rpm. Columns are represented by time of protein activation. 1) Protein not incubated with gastric fluid; 2) 5 min; 3) 10 min; 4) 15 min; 5) 20 min; 6) 25 min; 7) 30 min; 8) 60 min; 9) 120 min. Samples of gastric fluid from Vip-S2 (A), Vip-R1 (B) and Vip-R2 (C) were used in the study.

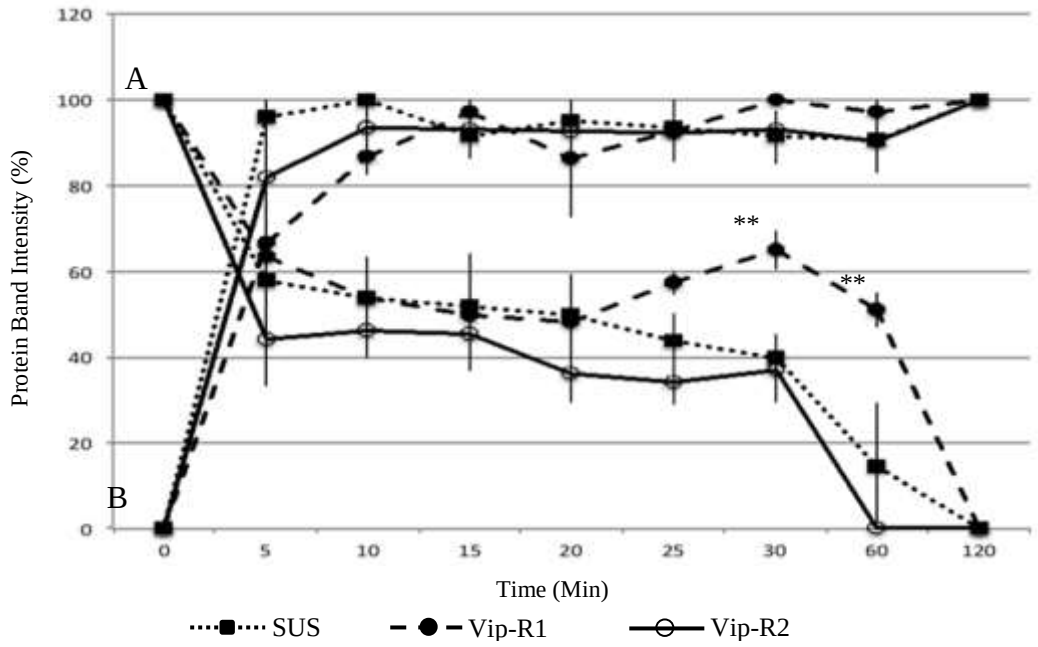


Figure 3.2. Graphic of kinetics of Vip3Aa20 proteolysis. Samples were incubated at different time intervals and the protoxin bands (89 kDa) and activated protein (62 kDa) were quantified by densitometry. The data points represent the mean of three replicates with the mean standard error indicated by error bars. Vip-S2 is represented by dot line; Vip-R1 is represented by dashed line; Vip-R2 is represented by the solid line. ** Statistically different. A: lines representing the 89 kDa protoxin; B: lines representing 62 kDa toxin.

Vip-S2 Vip-R1 Vip-R2

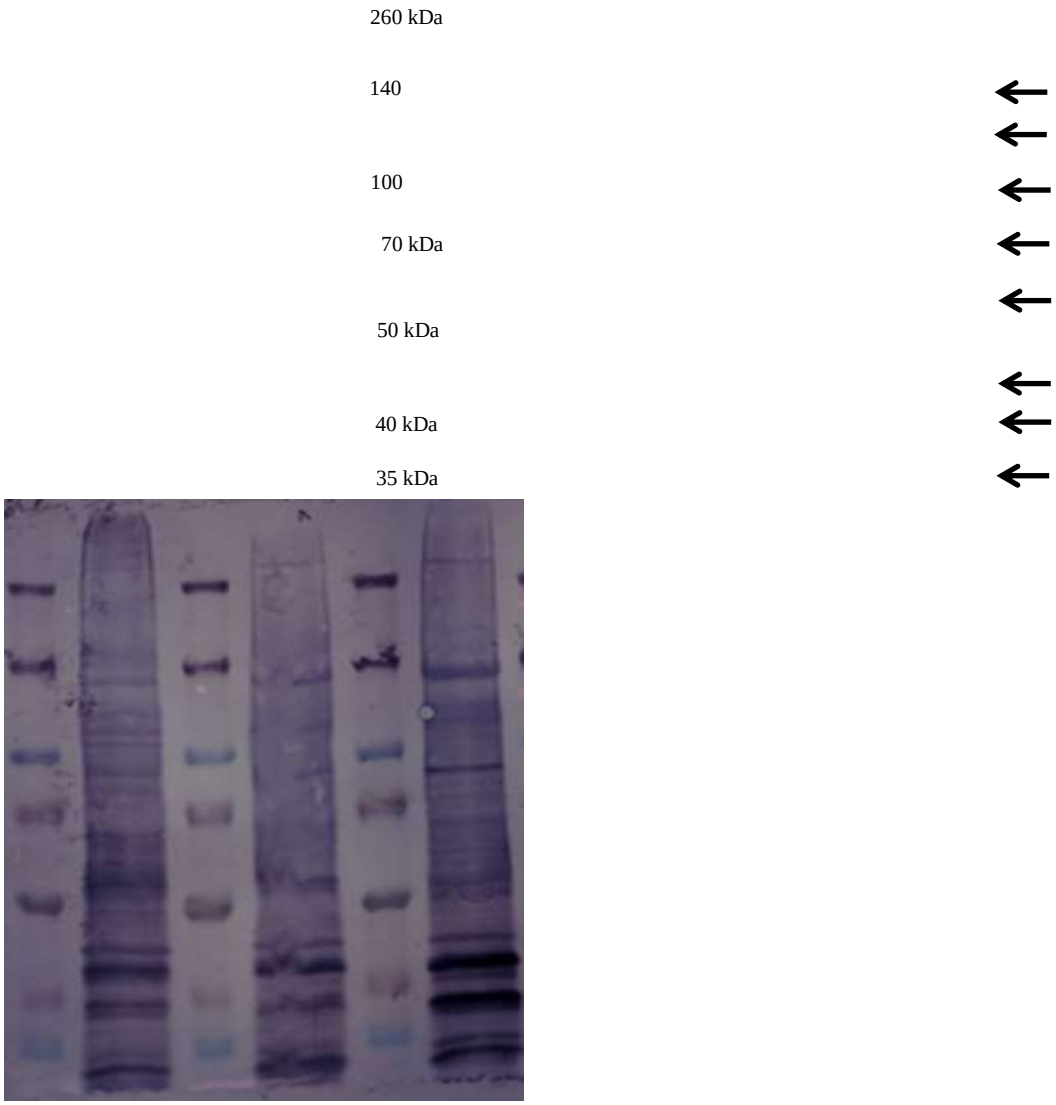


Figure 3.3. Binding of biotinylated Vip3Aa20 to FAW BBMV receptors for Vip-S2; Vip-R1 and Vip-R2. Arrows indicate FAW BBMV proteins recognized by activated Vip3Aa20 toxin for each population.

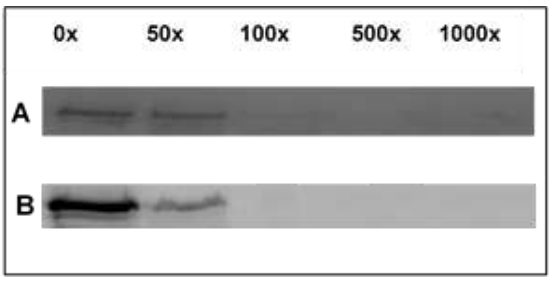

Figure 3.4. Homologous competition. Vip3Aa20 labeled and the competitor unlabeled Vip3Aa20 with excess of 50, 100, 500, 1000-fold concentration incubated with 20 µg de BBMVs from Vip-S2 (A) and Vip-R2 (B) FAW populations.

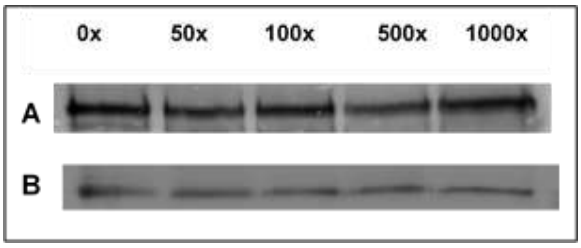


Figure 3.5. Heterologous competition. Vip3Aa20 labeled and the competitor unlabeled Cry1Ab with excess of 50, 100, 500, 1000-fold concentration, incubated with 20 µg de BBMVs from Vip-S2 (A) and Vip-R2 (B) FAW populations.

# 4 GENE EXPRESSION PROFILE OF FALL ARMYWORM (*Spodoptera frugiperda*) RESISTANT TO VIP3Aa20 BT PROTEIN


## Abstract

Genetically modified corn expressing Vip3Aa20 insecticidal protein from *Bacillus thuringiensis* Berliner (Bt) is a biotechnological option for the control of *Spodoptera frugiperda* (Fall armyworm – FAW) in some countries of South America (Argentina, Paraguay, Uruguay, Brazil and Colombia) as well as in the U.S. FAW has rapidly evolved resistance to Bt corn expressing other Cry proteins present in many countries of South America and remains a top concern for sustainable biotechnology control efforts. Alternatively, Vip3Aa20 protein also from Bt is commercially available in corn hybrids and remaining working well against FAW, however this species can adapt to this technology as well. Thus, characterization of Bt resistance in FAW can improve Insect Resistance Management (IRM) programs and extend the durability of control. In our study, we used a transcriptomic approach to compare gene expression profiles of FAW resistant to Vip3Aa20 to a susceptible colony. We included larvae that were both exposed and unexposed to Vip3Aa20, as well as across times after exposure. ABC transporter genes were down regulated in the resistant colony, independent of protein induction and/or time. We also found differential expression of genes in the G-protein signaling pathway (G-protein coupled receptor, adenylate cyclase and cAMP-specific 3',5'-cyclic phosphodiesterase). Our data will help understand mechanisms of resistance and develop best IRM practices in order to extend the durability of Vip3Aa20 to FAW.


Keywords: Vip3A; Insect resistance; Fall armyworm; Transcriptome; RNA-seq; ABC transporter

## 4.1 Introduction

The FAW is one of the most devastating insects in corn and other agronomic crops in most regions of the Americas (Blanco et al. 2016). Female FAW can lay more than 1,000 eggs and, in corn, larvae primarily feed in the whorl. When initial infestation occurs in early crop stages, the caterpillars feed on the base of the plant, reaching the growth meristem and reducing plant stand (Mendes et al. 2011). FAW reduces yield by 34 to 38% when larvae

attack leaves (Carvalho 1970) and can reach 100% when late instar larvae attack the crop seedlings and cut plants (Avila et al. 1997).

In tropical regions where agriculture is intensively produced, growing more than one cash crop within a year is a common practice. In Brazil, the emergence and adoption of the second crop season (corn planted immediately after soybean harvest within the same year) increases economic productivity for farmers, but also dramatically impacts dynamics of pest populations that feed on multiple hosts available year round. Thus, peaks of population density are seen throughout the year, driven by a phenomenon called the "green bridge", where the insects migrate from one crop to another in the same region (Horikoshi et al. 2016). Due to FAW polyphagy, this species largely benefits from the “green-bridge”, as the most important economic crops (e.g. corn, cotton and soybean) serve as FAW hosts, facilitating high infestations (Grützmacher et al. 2000, Horikoshi et al. 2016).

The most abundant technology used for FAW control is corn expressing toxic proteins derived from *Bacillus thuringiensis* (Bt-corn). Bt is a soil bacterium that produces endotoxins with a lethal effect on some species of insects. Bt crops are considered one of the most widely used insect control tools due to several advantages delivered by this technology (Walker et al. 2000). Bt corn adoption contributed to yield increases, regional pest suppression through widespread Bt hybrid adoption, reduction of insecticide use, and positive social impacts (NAS 2016).

Most Bt crops produce δ-endotoxins, or Cry toxins, which have been continuously used as the principal control for Lepidoptera. Recent cases of Lepidopteran resistance to Cry toxins (*Busseola fusca* in South Africa to Cry1Ab ( Kruger et al. 2009), FAW to Cry1F in Puerto Rico (Matten et al. 2008), *Pectinophora gossypiella* to Cry1Ac in India (Bagla 2010), *Helicoverpa zea* to Cry1Ac+Cry2Ab in United States (Luttrell and Luttrell 2004), *Helicoverpa punctigera* to Cry1Ac+Cry2Ab in Australia (Downes et al. 2010), and FAW in Brazil to Cry1F and Cry1Ab (Farias et al. 2014, Omoto et al. 2016) jeopardize the further utility of these active ingredients.

Vegetative insecticide proteins (Vips) are a different set of Bt toxins and appear to have a unique mode of action for insect control. For example, the Vip3Aa20 protein, registered for Lepidopteran control, shares no sequence homology with the known δ-endotoxins (Estruch et al. 1996, Estruch et al. 2001) and has a broad insecticidal spectrum (Estruch et al. 1996, Ferre and Van Rie 2002, Yu et al. 1997, Warren 1997). Although Vip3Aa20 shares some steps in the mode of action with Cry proteins, it utilizes a different molecular target and is distinct in

terms of pore forming (Lee et al. 2003) - the key step that leads to septicemia and insect death.

In Lepidoptera, two models describe how Cry toxins kill insects. The first is the pore forming model, where Cry protein is activated by proteases in the mid gut of insect, followed by binding to a primary receptor (e.g. cadherin), cleavage of helix α-1 and triggering protein oligomerization. The toxin oligomer then binds to a secondary receptor, such as aminopeptidase or alkaline phosphatase, which are anchored by a glycosylphosphatidylinositol anchor in the membrane. Finally, the toxin inserts itself into the membrane forming a pore that kills the insect cells (Bravo et al. 2004). Alternatively, the signal transduction model infers that, after protein processing and activation by proteases in larval mid gut, the toxin binds to cadherin receptors, triggering an intracellular cascade pathway that is mediated by G-protein activation, and then activates adenylyl cyclase. This signal then increases the levels of cyclic adenosine monophosphate (cAMP) which activates the protein kinase A cascade and leads to cell death (Zhang et al. 2006). These two distinct models proposed for Cry proteins share some initial steps, such as protein activation and protein interaction between cadherin receptor and activated protein, which is considered critical for toxicity (Soberón et al. 2009). Vip3Aa20 also shows some similarity to the pore-forming model, but it does not share the same receptor as Cry1Ab (Lee et al. 2003).

Insects can evolve in response to natural selection imposed by control methods, limiting their effectiveness and long-term viability (Hawthorne 1998). Over 500 species of insects have become resistant to conventional insecticides and they can also adapt to Bt toxin (Gut et al. 2002). Understanding the selection pressure imposed on insect populations in transgenic crops expressing Bt toxin requires investigations into the genetic as well as biochemical mechanisms of resistance, and, in turn, help establish a robust insect resistance management strategy. Most cases of Cry resistance are related to reduced or lack of Cry protein binding to cadherin as in *Helicoverpa armigera* - Cry1Ac (Xu et al. 2005), *Pectinophora gossypiella* - Cry1Ac and Cry1Ab (Morin et al. 2003), alkaline-phosphatase in *Heliothis virescens* - Cry1Ac and Cry2Aa (Gahan et al. 2001), and aminopeptidases in *Spodoptera exigua* - Cry1C (Herrero et al. 2005). Genes associated with reduced midgut protease activity were also implicated in resistance of *Plodia interpunctella* to Bt strain subsp. *entomocidus* (Oppert et al. 1997). Genes known as REPATs (Response to Pathogens) have been identified in response to Bt proteins (Navarro-Cerrilo et al. 2013). These genes renew damaged cell membranes caused by pathogen infection, and, when highly expressed, can protect against insect death by promoting membrane repair. α-REPAT proteins have been also found in FAW as defense

system (Rodriguez-Cabrera et al. 2008). More recently, several studies indicated ABC (ABC) transporters, especially ABCC2, are involved in Bt resistance. In *H. virescens*, mutations in this gene prevent binding of Cry1Ac protein to the receptor on the midgut membrane (Gahan et al. 2010). Loss of gene expression for this ABC transporter was responsible for resistance of *Plutella xylostella* to Cry1Ac protein (Baxter et al. 2011). A single mutation in ABCC2 was also linked to resistance of Bombyx mori to Cry1Ab (Atsumi et al. 2012). However, similar studies for FAW and Vip3Aa20 are lacking, specifically regarding pathways involved with cell death and resistance mechanisms in FAW.

Bt corn was first introduced in Brazil in 2008, and, in 2010, there were four 4 Cry proteins being expressed in commercial hybrids (Cry1Ab, Cry1F, Cry1A.105, Cry2Ab2) as well as one Vip (Vip3Aa20) (CTNBio 2016). In as little as 3 years, FAW had evolved resistance to all Cry proteins, even in stacked products that express more than 1 Cry protein. However, Vip3Aa20 remains an effective and important management tool, without evidence of field failures or FAW resistance. Yet the risk for resistance remains high, as Vip3Aa20 resistance alleles are low in frequency (0.0009, Bernardi et al. 2015), and a highly resistant colony was established using the F2 screen methodology (Bernadi et al. 2015, Bernardi et al. 2016). Understanding the mode of action and molecular mechanisms of FAW resistance to Vip3Aa20 before resistance evolves in the field would provide important information to develop resistance management practices and extend the durability of Vip technology.

The objective of this study was to determine potential pathways involved with the mode of action of Vip3Aa20 Bt protein against FAW and characterize genes potentially involved in resistance. We used high-throughput RNA sequencing and compared transcriptomic profiles of FAW colonies either susceptible or resistant (Bernardi et al. 2015, Bernardi et al. 2016) when exposed to Vip3Aa20. We tested two hypotheses which could be associated with mode of action pathways as well as resistance. Resistance of FAW to Vip3Aa20 is due to differential expression of genes 1) in the pore forming model (Bravo et al. 2004); or 2) in the G-protein signaling pathway model (Zhang et al. 2006). We also tested the hypothesis that the ABC transporter system is involved with resistance of Vip3Aa20 to FAW, as these proteins would be differentially expressed in the resistant colony. Understanding the molecular mechanisms of resistance of FAW to Vip3A20 will contribute to improved IRM tactics that protect Vip3Aa20's effectiveness and propose future strategies to extend its durability.

## 4.2 Material and Methods

*Susceptible and resistant FAW colony*

FAW resistant (Vip-R2) and susceptible (SUS) colonies were selected through F2 screen methodology (Andow and Alstad 1998) and reared in laboratory for 10 generations in order to be used in this study (Bernardi et al. 2015, Bernardi et al. 2016). The Vip-R2 colony has a high resistance ratio (RR>3200) compared to the SUS colony and are able to complete their entire life-cycle feeding on Viptera® corn tissue (Bernardi et al. 2016). Resistance alleles present in Vip-R2 were confirmed to reflect field resistance through F1 screen methodology (Horikoshi 2016).

*Experimental design and treatments*

SUS and Vip-R2 larvae of FAW (F10 generation) were reared on artificial diet (Kasten et al. 1978) and maintained under controlled conditions (temperature 25ºC ± 1ºC, humidity 70 ± 10% and light: dark 14:10 h). Once larvae reached the 4th instar stage, we exposed 10 larvae from each colony to Vip3Aa20 protein (concentration of ~2 x LC99 – 4000 ng/cm²), overlaid on artificial diet (Kasten et al. 1978). For the unexposed control, 10 larvae from each colony were fed only artificial diet (all treatments and control included 3 replications, for a total of 30 larvae). Separate samples for each treatment (exposed and unexposed) were used for midgut extraction at times zero, one, two, four and nine hours after protein exposure and frozen immediately after extraction. Midguts were kept in Eppendorf tubes stored at -80 °C. Extracted midguts were used for total RNA extraction using Kit RNeasy® Mini Kit from QIAGEN (Valencia, CA, USA). RNA quality was checked using Bioanalyser 2100 equipment (Agilent Technology Inc., CA, USA) according to manufacturer's instructions. We pooled midguts from the 1, 2, and 4 h collection points at equimolar concentrations for all samples (but maintained 3 replicates). Each treatment was coded with the initial letter referring to each treatments (S = SUS colony; R = Vip-R2 colony; P = larvae was exposed to protein; NP = larvae was not exposed to protein; 0 = RNA extracted with zero hour after starting the experiment; 124 = RNA extracted with 1, 2 and 4 hours after starting the experiment (RNA samples from 1, 2 and 4 hours treatments were pooled for library preparation and sequencing); 9 = RNA extracted with 9 hours after starting the experiment) (Table 4.1).

*RNA-seq libraries preparation and sequencing*

Library preparation and sequencing were performed by the Central Laboratory of High Performance in Technologies in Life Sciences (LACTAD) at UNICAMP (Campinas University, São Paulo, Brazil). Samples for sequencing of mRNA were prepared using the

mRNA-Seq Sample Prep Kit (Illumina, San Diego CA) as per the manufacturer's protocol. Library quantity and quality was estimated using an Agilent DNA 1000 kit on a 2100 Bioanalyzer, and then sequenced using an Illumina Hiseq 2500 platform as per the manufacturer's protocol, as paired-end (2 x 100pb). Twenty four samples (8 treatments, 3 replicates) were divided into 3 sequencing lanes (~300 million reads each lane), with expected coverage of 37.5 million reads per sample.

*Sequence trimming, de novo assembly and annotation*

Adaptor sequences were removed from reads by using Illumina bcl2fastq version 1.8.4 by the LACTAD facility. Afterwards, quality control and trimming were performed using the software bbmap/bbduk.sh with the following parameters: Phred quality score limit=32; No ambiguous nucleotide (N) = allowed; Length trimming: Reads less than 40 bases discarded. The high quality, trimmed data were used to assemble a de novo reference using Trinity software (versionTrinityrnaseq_r2013-02-25), with the following parameters: Minimum contig length = 200; Normalize maximum read coverage = 200. Afterwards, the reference was filtered using following approach: 1) All trimmed data were mapped to the reference using Bowtie software version 0.12.9 and RSEM; 2) Contigs with Reads Per Kilobase Million (RPKM) < 1 were removed using the software RStudio v1.0.136. Redundant sequences were then removed using CD-hit (version V4.6.6), with a threshold set for 0.95 sequence similarity.

*Differential gene expression analysis*

High quality trimmed data were imported to CLC Workbench (version 8.5.1) and mapped to the FAW annotated reference with the default mapping parameters (Maximum number of mismatches allowed = 2; Minimum length fraction = 0.8; Unspecific match limit = 10; Minimum paired distance = 10; Maximum paired distance = 250; Minimum exons coverage = 0.2; Minimum number of reads = 10; Minimum length of putative exons = 50).

Expression values were compared statistically using the bootstrapped receiver operating characteristic (bROC) algorithm, available as an integrated plug-in in CLC Bio genomics workbench. Expression values were transformed to log2 (E+1), where E is the original expression value. The expression data were normalized using median of M-values (MMV) method. Using gene expression data, we ran a pairwise comparison between SUS and Vip-R2, including protein exposure or not, across different times. Fold change values for gene expression were considered significant if P values were < 0.05.

*Weighted gene correlation network analysis (WGCNA)*

WGCNA is a systems biological method that describes the correlation patterns among genes across RNASeq samples. WGCNA can be used for finding clusters (modules) of highly correlated genes. Summarizing such clusters using module eigengenes and relating modules to one another or to external sample traits creates a network based gene screening method that can be used to identify potential pathways or even candidate genes associated to the trait (Langfelder and Horvath 2008). Outputs from the RNA-seq analysis (unique gene original expression value) from all treatments and replicates (total of 24) were used for the analysis. First we performed Principal Components Analysis (PCA) to visualize the sample-to-sample distance projected onto a 2D plane and to compare divergences and similarities based on gene expression values of the treatments. Original short-read count data was transformed to the homoscedastic form using Variance Stabilization Transformation (VST) in DESeq2 R package before submitted to WGCNA. A sample dendogram and heatmap analysis was also performed based on gene expression values for all samples in order to identify potential sample outliers for all parameters assessed. We then performed an analysis to identify signals of gene clustering which could potentially be associated to pathways involved in mechanisms of resistance as well as mode of action. This analysis was based on a gene co-expression network through the interaction patterns among genes. The analysis was done by correlation as a measure of co-expression using the unique gene reads generated in the RNA-seq analysis. Modules were then developed through hierarchical clustering, showing gene expression patterns across all samples for an individual group of genes. Thus, the differences in expression patterns of clusters should represent genes and pathways involved in Vip3A20 resistance.

## 4.3 Results

*Sequencing data, quality control and reference assembly*

The total number of reads generated in the Illumina Hiseq 2500 for the 8 treatments and 24 replicates was 1,118,935,706 reads of approximately 100pb, with average of 92.8% of bases ≥ Q30. All treatments and replications generated similar amounts of total sequencing except replicate 3 of the treatment RP9 which generated much less reads. All treatments with SUS colony produced total of 555,401,131 reads while Vip-R2 563,534,572. Protein exposure and control treatments generated 532,146,572 and 586,789,134 reads, respectively. Protein exposure at different times produced 233,791,936 and 298,354,636 reads for time 1,2,4-pooled hours (early stage) and 9 hours (late stage) respectively.

Low quality reads were removed through quality control (13.1%), thus only high quality reads was used for reference assembly and RNA-seq analysis (Table 4.2). High quality reads (972,478,618, or 86.9% of total reads) were submitted to Trinity for reference assembly generating 382,176 transcripts. Afterwards, the high quality trimmed data was mapped to this reference, filtering and selecting only transcripts with a RPKM > 1 (total of 55,009 transcripts). After eliminating redundant sequences, we generated a final reference with 35,592 contigs for subsequent RNA-seq analysis. Statistics on reference assembly is presented in the Table 4.3.

*Reference annotation and transcript mapping*

Assembled transcripts were annotated using the NCBI non-redundant database with minimum similarity and coverage parameters of 80% and 50% respectively. From 35,592 transcripts, 40.96% (14,579) had no annotation and 7.3% (2,595) were annotated to uncharacterized proteins. The remaining contigs (18,418 or 51.75%) with an annotation were used for differential gene expression analysis. The distribution of contig size is presented in Figure 4.1. The total reads mapping to the reference ranged from 83.51% to 99.01% among all replicates. The percentage of unique reads mapped ranged from 62.21% to 84.04%. (Table 4.4).

*PCA, dendogram and heat maps among treatments*

The PCA revealed two different clusters within SUS colony, separating replicates based on exposure or non-exposure to Vip3Aa20. Similarly, PCA generated different clusters between SUS and Vip-R2; however no clustering occurred with Vip-R2 tested across different times (0, 124-pooled and 9 hours after induction) nor based on presence or absence of Vip3Aa20, suggesting a lack of induced responses by the protein (Figure 4.2). Treatment RP9 (blue triangle 9 in the Figure 5.2) appeared as an outlier, likely due to replicate 3 which had much less sequence data compared to replicate 1 and 2 (Table 4.2). Nonetheless, the PCA showed strong clustering among the Vip-R2 and SUS strains and protein exposure seemed to only impact the SUS colony.

PCA data were supported by a dendogram and trait heatmaps. Based on these additional analyses, replicate 3 of RP9 (Vip-R, protein induced, time 9 hours) remained an outlier across samples (black bar in the Figure 4.3 A), thus we excluded this replicate for modules development. Similar to the PCA, SUS and Vip-R2 samples were categorized as different clusters (SUS green bars, Vip-R2 orange bar) (Figure 4.3 B). All samples exposed to

Vip3Aa20 clustered together, regardless of colony (protein exposed – pink bars; not exposed – light blue bars) (Figure 4.3 C). Samples from different times (0, 124-pooled and 9 h) clustered separately independently of SUS or Vip-R2 as well as protein induction or not (0 h – grey bars; 124-pooled – red bars; 9 h – yellow bars) (Figure 4.3 D).

*Modules development and candidate pathways and genes*

As previously mentioned, most cases of Bt resistance are related to reduced or lack of Cry protein binding to specific receptors. In the chapter 3, we demonstrated that resistance of FAW to Vip3Aa20 is not associated with protein activation or due to absence of protein-receptor binding; therefore resistance mechanisms must be downstream in the Vip toxic pathway. Other studies have shown additional and different mechanisms which may play a role for insect resistance to Bt proteins, such as REPAT genes as well as expression reduction of the ABC transporter system. Several hypotheses suggest a role for ABC transporters in Bt resistance (Gahan et al. 2010, Baxter et al. 2011, Atsumi et al. 2012). ABC transporters may be involved with Bt protein-receptor binding, the introduction of the Bt protein through the bilayer membrane, or work as a receptor to trigger downstream events (Vadlamudi et al. 1995). An additional hypothesis is that ABC transporters act through cell detoxification by reducing Bt protein in the cytosol (Russel et al. 2008). The hypothesis of cell detoxification through removing Bt protein from the cytosol might be an option as well, as Vip3A was reported to be present mostly in the cellular membrane region but also in the cytoplasm when interacting with S2 ribosomal protein (66 kDa) at the cell membrane (Singh et al. 2010). We used the modules development approach to narrow down the search for candidate genes associated with resistance as well as to detect pathways involved with Vip3Aa20 mode of action.

We detected 36 different modules potentially associated to FAW resistance or mode of action of Vip3Aa20 (Figure 4.4). Based on the module development analysis, we selected the three most representative modules. Module 1 includes genes with increased expression in SUS treatments, and decreased expression in Vip-R2 treatments (5,902 total transcripts, Figure 4.5 A). Alternatively, module 2 represents transcripts downregulated in all SUS samples, but upregulated in Vip-R2 samples (5,481 total transcripts, Figure 4.5 B). Module 3 included transcripts that were highly expressed in SUS samples exposed to Vip3Aa20, but had decreased expression in both SUS unexposed and all Vip-R2 samples (767 transcripts, Figure 4.5 C). These pathways provide a basis to investigate and identify genes and pathways potentially involved in resistance and Vip3Aa20 mode of action.

Based on these modules, we compared treatments to test the following hypotheses: *i*) differential gene expression between SUS and Vip-R2 across different times (SNP0 vs. RNP0, SNP9 vs. RNP9, SP124 vs. RP124 and SP9 vs. RP9) are associated with pore forming model; *ii*) differential gene expression between SUS and Vip-R are associated with G-protein signaling pathway; *iii*) FAW resistance to Vip3Aa20 is associated with ABC transporter system.

Comparison SNP0 vs. RNP0 showed a total of 6,184 genes differently expressed (2,789 up-and 3,678 down regulated), while SNP9 vs. RNP9 generated 3,824 genes (919 up-and 2,907 down regulated), a 38% reduction of differentiated gene expression from time zero to time 9.When treatments were exposed to protein, the comparison SP124 vs RP124 generated 7,592 differentially expressed genes (3,202 up-and 4,390 down regulated), while SP9 vs. RP9 generated total of 2,216 differentially expressed genes (1,364 up-and 853 down regulated). With the exception of SP9 vs. RP9, overall gene expression in Vip-R2 seems to represent more down regulated genes when compared to SUS. Comparatively, the SUS colony did not show much differences upon Vip3Aa20 exposure: SNP0 vs. SP124 generated 717 contigs (653 up-and 65 down regulated) while SNP9 vs SP9 generated 624 contigs (250 up-and 374 down regulated).

The list of differentially expressed genes for all comparisons was cross-referenced to the list of genes identified above modules to determine potential candidate genes associated to resistance. Comparing module 1 with SUS and Vip-R2 resulted in 305 genes in common (99.1% of common genes are downregulated). A similar comparison with module 2 resulted in 284 genes in common (97.81% upper regulated) and module 3 resulted in 30 genes in common (74.85% down regulated) (Table 4.5 A, Figure 4.6 A and B). Module 3 was also cross-referenced with differential genes expressed in all experiments comparing unexposed SUS to SUS exposed to Vip3Aa20 resulting in 92 genes in common (100% upper regulated) (Table 4.5 B, Figure 4.6 C).

Combining the differentially expressed genes with the list of genes in the modules, we identified some potential candidate genes which could be associated with FAW resistance to Vip3Aa20. We found 12 contigs associated to ABC transporter system that were down regulated in Vip-R2 for all replicates when SUS was compared to of Vip-R2. The log2 fold change ranged from -3.38 to -0.71 among all contigs (Table 4.6 A). Down regulation of those genes seems to be constitutive in the Vip-R2 colony as expression is not significantly changed over time nor with Vip3Aa20 exposure. The contigs were annotated to 6 different sub-families of ABC transporter proteins, mostly to sub-family G (Table 4.7).

As described above, G-protein signaling pathway is one model to describe Bt proteins' mode of action. It first involves G-protein coupled receptor binding to a hormone or peptide in order to trigger the pathway. The following step is the binding of α-subunit to adenylate cyclase, which is the first messenger for pathway signal transduction. Adenylate cyclase will then stimulate cAMP production which is the secondary messenger working as signal amplifier in the pathway. cAMP is also regulated by phosphodiesterase, which is regulated by kinase A protein cascade. One of our hypotheses was that resistance of FAW might be associated to deregulation of genes in this pathway. Our data showed that G-protein coupled receptors showed increased expression in the presence of the Vip3Aa20 protein in the SUS colony, but reduced in the Vip-R2 (Figure 4.8 A). For example four contigs annotated as G-protein coupled receptors Mth were down regulated in the Vip-R2 for all comparisons, with a log2 fold change ranging from -1.30 to -1.92. Exposure to Vip3A20 does not cause this gene to change expression in the Vip-R2 colony (Table 4.6 B). Additional genes related to the G-protein signaling pathway were differentially expressed among the SUS and Vip-R2 colonies. Expression of adenylyl cyclase-associated protein 2 was lower inVip-R2 compared to the SUS colony in the presence of Vip3Aa20 (124-pooled = -1.34 fold change; 9 h = -1.83 fold change), but there was no difference in expression between two strains when Vip3Aa20 is not offered (Table 4.6 C, Figure 4.8 B).

Additionally, we found adenylate cyclase type 2-like down regulated in the Vip-R2 colony exposed to Vip3A20 in the 124-pooled (-1.51 fold change) and the 9 h treatments (-1.30 fold change) confirming the influence of the previous compound on its production (Table 4.5 D, Figure 4.8 C). Adenylate cyclase catalyzes the formation of the signaling molecule cAMP in response to G-protein signaling pathway (Ding et al. 2004).

We also expected to find the cAMP gene down regulated in Vip-R2 as its production is stimulated by adenylate cyclase. Indeed the gene cAMP-specific 3',5'-cyclic phosphodiesterase was down regulated in the Vip-R2 colony exposed to Vip3Aa20 in the 124-pooled (-1.60 fold change) when compared to the SUS. However it was differentially expressed at 9 h regardless of the presence of Vip3Aa20 (Table 4.5 E, Figure 4.8. D). Interestingly, this gene appeared as upper regulated (1.76 fold change) in the SUS strain induced by protein compared to not induced at 124-pooled after induction (Table 4.5 G) suggesting that this compound somehow influences the activity of Vip3Aa20.

## 4.4 Discussion

FAW has a high tendency towards resistance evolution due to its biological and ecological characteristics, especially in Brazil where intensive agricultural production favors food abundance year-round and a large number of generations. In South America, mainly Brazil, Bt corn is the most widely used tactic of FAW control, which covers 80% of the total corn area (16 million hectare) in the first and second corn season (corn planted immediately after harvesting soybean within the same year) (Ceres 2015).The intense use of cash crops expressing similar Bt proteins has driven unmanageable selection pressure imposed by these technologies. However, four different Cry proteins commercially available through corn hybrids are not performing well against FAW (Farias et al. 2014, Omoto et al. 2016, Horikoshi et al. 2016). Only the Bt protein Vip3Aa20 remains effective against FAW; therefore understanding potential resistance mechanisms is imperative to retain trait durability. Based on differential gene expression analysis between SUS and Vip-R2 strains, our study identified potential pathways involved with mode of action of Vip3Aa20, and determined potential candidate genes associated with FAW resistance.

Our experimental design and analysis allowed inferences on candidate genes associated with the molecular pathway of Vip3Aa20, and FAW's resistance to this protein. We detected differential gene expression in the comparisons between SUS and Vip-R2 colonies, both with and without Vip3Aa20 exposure, providing evidence to support our hypotheses. Among all transcripts, our differential gene expression analyses suggested 12 genes potentially associated with resistance and 5 genes related to the protein's mode of action.

ABC transporter system has been reported as potential cause of at least seven cases of Lepidoptera species resistant to Bt crops (Gahan et al. 2010, Xiao et al. 2014). ABC proteins are usually located in the membrane and are responsible for importing or exporting substances through cellular membrane. We identified 12 contigs annotated as ABC transporters that were down regulated in the Vip-R2 colony when compared to SUS irrespective of Vip3A20 exposure. These ABC include 4 in sub-family G, 2 in sub-family D, 1 subfamily F, 1 sub-family A, 1 sub-family B, and three unclassified (i.e. no sub-family). ABC sub-family G member 1 (whose function is ABC 2 type transporter) in the contig TRINITY_DN80310_c1_g19_i1 was substantially downregulated, with a log2 fold change ranging from -3.02 to -3.48 among all experiments set (Table 4.7).

One hypothesis for ABC transporters' role suggested involvement Bt protein-receptor binding, introduction of the Bt protein through the bilayer membrane, or working as a receptor and triggering downstream events (Vadlamudi et al. 1995). However, we found

Vip3Aa20 binding to putative receptors that did not match the predicted size of known ABC transporters (150 kDa) in either the SUS or Vip-R2 colonies.

Another hypothesis is that ABC transporters act through cell detoxification by reducing the Bt protein in the cytosol (Russell et al. 2008). However, if ABC transporters were associated with detoxification, we might expect these genes to have increased expression in resistant colony instead of decreased, which our data show. In addition, detoxification processes carried out by ABC transporter system are mostly provided by the sub-family C (Russel et al. 2008), which was not differentially expressed based on our data. Also, if ABC transporter system was playing a detoxification role on FAW resistance to Vip3Aa20, resistance would more likely be dominant (Atsumi et al. 2012), however the resistance of Vip-R2 is recessive (Bernardi et al. 2016). Thus ABC transporter system is acting towards resistance in a different manner, potentially related to the G-protein signaling pathway.

Our differential gene expression analysis also detected five genes involved with G-protein signaling pathway, which is implicated in one of the mode of action models proposed for Cry proteins (Zhang et al. 2006). Four contigs, annotated as G-protein coupled receptor Mth, were down regulated in Vip-R2 after 1,2,4 h of protein exposure. The most down regulated gene was found in the contig TRINITY_DN71749_c1_g1_i1 (-1.92 fold change) (Figure 4.7). G-protein coupled receptors resemble on/off switches in the G-protein pathway. They are responsible for detecting molecules outside of the cell and activating cellular internal transduction of signal involved with the activation of G-protein signaling pathway. The signal transduction is first induced by the binding of the receptor to a hormone or even peptides (Trzaskowski et al. 2012). Thus, lack of induction by receptor binding will not activate the pathway.

The first messenger in the G-protein signaling pathway is adenylate cyclase, whose production is regulated by adenylyl cyclase-associated protein 2 thorough selective and non-covalent interaction. Adenylate cyclase has been described as a member of the signal cascade induced by Cry 1Ab toxin (Zhang et al. 2006). The gene expression of adenylyl cyclase-associated protein 2 was found to be down regulated for the Vip-R2 in 1,2,4 h as well as 9 h after Vip3Aa20 exposure. As expected based on the G-protein model, we also observed adenylate cyclase to be down regulated in the Vip-R2 at 1,2,4 h (-1.51 fold change) and 9 h (-1.30 fold change) after Vip3Aa20 exposure.

When subunit alpha of G-protein is not activated by the G-protein coupled receptor, adenylate cyclase is not induced to produce the second messenger (cAMP) in the signal cascade (Zhang et al. 2006). The production of cAMP is induced by the production of

adenylate cyclase, however the most important regulator for its production is cAMP-specific 3',5'-cyclic phosphodiesterase. This enzyme is one of the principal regulators of the G-protein signaling pathway. In our study we found cAMP-specific 3',5'-cyclic phosphodiesterase down regulated in the Vip-R2 strain after protein exposure at 1,2,4 hr (-1.6 fold change). The same gene was found as up regulated (1.76) in SUS Vip3Aa20-induced in the time 1,2,4 h compared to SUS colony not induced, corroborating the on/off switch. Our data suggests that 3',5'-cyclic AMP degradation is turned on in SUS enabling proper mode of action of Vip3Aa20, but turned off in Vip-R2. Further data supporting the down regulation of cAMP degradation was confirmed by the up regulation of the gene cyclic AMP-regulated protein in the Vip-R2 colony at 1,2,4 hr (1.21 fold change in exposed and 1.64 fold change in control, respectively). Studies with Cry1Ab exposed to cells containing cadherin receptors demonstrated that the Bt protein also stimulated cAMP production through induction of G-protein signaling pathway (Zhang et al. 2006). Our data are consistent with Vip3Aa20 killing FAW through the G-protein signaling pathway, which fits to the model proposed by Zhang et al. 2006.

Our data suggest that FAW resistance to Vip3Aa20 is related to changes in gene regulation of the ABC transporter system and the G-protein signaling pathway. In resistant FAW, the signal cascade has been impacted by the down regulation of ABC transporter system. ABC transporters (ABCC4 and ABCCG2) are known to play a role on cAMP and cGMP intracellular regulation (Li et al. 2007, Cheepala et al. 2013). ABCCG2 is highly expressed in small intestines (mouse) where it is expected to regulate cAMP in its tissue cells (Cheepala et al. 2013). Studies performed with gut epithelial cell lines of FAW demonstrated that ABCC4 transports cAMP across the membrane, and, when this gene is suppressed, concentration of cAMP is increased in the cytosol (Li et al. 2007). However the regulation of cAMP by ABC transporters in a certain tissues depends on both the amount of transporter and the number of different types of ABC transporters present (Cheepala et al. 2013). Studies in humans also demonstrated the ABC transporter is regulating cAMP through an efflux process. Alternatively, cAMP was also detected to be regulated by a second gene, phosphodiesterases, which was also down regulated in Vip-R2 (Sassi et al. 2008, Sassi et al. 2012). Resistance of FAW to Vip3Aa20 may be provided by the lack of cAMP effluxing from the cytosol by ABC transporters, as well as reduced activity of phosphodiesterase, which is not regulating the intracellular cAMP concentration (Figure 4.9). If cAMP is not removed from the cytosol, then it could deregulate the the G-protein signaling pathway, impacting Vip3A20's mode of action.

Based on these results, it appears that FAW resistance to Vip3Aa20 is associated with changes in the G-protein signaling pathway regulation, influenced by ABC transporter system. This pathway demonstrated to be involved with Vip3Aa20 mode of action in FAW is consistent with the Zhang model of Cry mode of action.

Additional studies including silencing candidate genes through RNAi or CRISP-Cas9 technologies would provide solid evidence that ABC transporters and the G-protein signaling pathway are involved in the mode of action of this protein as well as FAW resistance.

### 4.5 Conclusion

Resistance of FAW to Bt proteins in Brazil has forced farmers to return to traditional tactics of control, based on weekly insecticide applications that have larger environmental, social and economic implications in agricultural production. Research on Lepidopteran resistance to Bt crops has recently expanded and identified genetic mechanisms that potentially reduce susceptibility as well as propose models for the mode of action of Cry proteins. However, no models have been proposed for the mode of action of VIP, nor genes related to FAW resistance. In this study we demonstrated the use of transcriptome profiling of larval midguts combined with WGCNA analysis and identified genes related to ABC transporters and G-protein receptors that are associated with resistance. Further studies are needed that investigate additional steps involved with mode of action and to validate candidate genes of resistance.

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## Tables and Figures

Table 4.1. Treatment list for RNA extraction for experiment involving SUS and Vip-R2 colonies, protein induced and not and time of protein exposure.

| Treatment | Colony | Protein Induced (4000 ng/cm²) | Time feeding on diet (hr) | Larvae Number | Treatment Cod |
|---|---|---|---|---|---|
| 1 | Resistant | No | 0 | 30 | RNP0 |
| 2 | Resistant | No | 9 | 30 | RNP9 |
| 3 | Resistant | Yes | 9 | 30 | RP9 |
| 4 | Susceptible | No | 0 | 30 | SNP0 |
| 5 | Susceptible | No | 9 | 30 | SNP9 |
| 6 | Susceptible | Yes | 9 | 30 | SP9 |
| 7 | Resistant | Yes | 1,2,4 | 30 | RP124 |
| 8 | Susceptible | Yes | 1,2,4 | 30 | SP124 |

Table 4.2. Number of reads produced by high-throughput sequencing (Illumina HiSeq 2500) for each treatment and replicate.

| Treatment | Replicate | Number of Reads | % Bases >= Q30 |
|---|---|---|---|
| SNP0 | 1 | 40,036,642 | 93.56 |
| | 2 | 35,287,792 | 93.85 |
| | 3 | 53,850,570 | 93.93 |
| SNP9 | 1 | 66,762,402 | 93.87 |
| | 2 | 40,394,376 | 92.85 |
| | 3 | 60,333,294 | 93.83 |
| SP124 | 1 | 36,863,536 | 93.96 |
| | 2 | 36,879,996 | 93.83 |
| | 3 | 34,409,066 | 94.16 |
| SP9 | 1 | 28,062,584 | 94.11 |

| | | | |
|---|---|---|---|
| | 2 | 35,376,208 | 94.10 |
| | 3 | 87,144,668 | 94.26 |
| Sub total SUS | | 555,401,134 | |
| RNP0 | 1 | 40,781,432 | 94.18 |
| | 2 | 42,650,924 | 94.38 |
| | 3 | 48,893,022 | 94.21 |
| RNP9 | 1 | 53,615,152 | 93.87 |
| | 2 | 56,108,978 | 93.80 |
| | 3 | 48,074,550 | 94.20 |
| RP124 | 1 | 48,002,268 | 93.97 |
| | 2 | 48,888,308 | 94.05 |
| | 3 | 28,748,762 | 94.01 |
| RP9 | 1 | 92,600,328 | 94.14 |
| | 2 | 55,161,620 | 94.32 |
| | 3 | 9,228 | 70.16 |
| Sub total Vip-R2 | | 563,534,572 | |
| **Total** | | **1,118,935,706** | |

Table 4.3. Statistics on reference assembly performed with high quality data from SUS and Vip-R2.

| **Assembly Statistics** | **Value** |
|---|---|
| Total contigs | 35,592 |
| Total contigs annotated | 18,418 |
| Minimun lenght (pb) | 201 |
| Maximun lenght (pb) | 21,674 |
| Total of bases | 29,920,009 |
| Contigs > 1000 pb | 7,939 |
| Lenght average (pb) | 841 |
| N50 | 1,379 |

Table 4.4. High quality reads and statistics on RNA-seq yield and read mapping.

| Treat. | Rep. | Total Reads | High Quality Reads | Total Mapped Reads (%) | Reads Mapped in Pairs (%) | Uniquely Mapped Reads (%) |
|---|---|---|---|---|---|---|
| | 1 | 40,036,642 | 34,417,360 | 95.92 | 78.81 | 74.32 |
| SNP0 | 2 | 35,287,792 | 30,519,030 | 94.73 | 78.33 | 73.72 |
| | 3 | 53,850,570 | 46,660,194 | 95.49 | 79.98 | 75.07 |
| | 1 | 66,762,402 | 57,771,166 | 95.83 | 77.80 | 72.95 |
| SNP9 | 2 | 40,394,376 | 34,294,290 | 99.02 | 84.63 | 84.04 |
| | 3 | 60,333,294 | 52,162,736 | 95.97 | 79.84 | 75.57 |
| | 1 | 36,863,536 | 31,965,198 | 95.31 | 77.21 | 72.23 |
| SP124 | 2 | 36,879,996 | 31,885,784 | 95.60 | 77.27 | 72.55 |
| | 3 | 34,409,066 | 30,055,136 | 95.38 | 77.29 | 72.43 |
| | 1 | 28,062,584 | 24,479,234 | 95.37 | 78.62 | 73.46 |
| SP9 | 2 | 35,376,208 | 30,856,040 | 95.75 | 77.95 | 73.00 |
| | 3 | 87,144,668 | 76,312,278 | 96.10 | 80.73 | 76.19 |
| | 1 | 40,781,432 | 35,642,950 | 95.37 | 78.19 | 73.38 |
| RNP0 | 2 | 42,650,924 | 37,449,566 | 95.57 | 78.79 | 73.78 |
| | 3 | 48,893,022 | 42,760,762 | 95.90 | 77.27 | 72.50 |
| | 1 | 53,615,152 | 46,477,658 | 95.24 | 79.41 | 74.61 |
| RNP9 | 2 | 56,108,978 | 48,486,756 | 95.57 | 74.62 | 69.79 |
| | 3 | 48,074,550 | 41,980,598 | 95.66 | 77.71 | 72.92 |
| | 1 | 48,002,268 | 41,686,888 | 95.35 | 78.79 | 73.96 |
| RP124 | 2 | 48,888,308 | 42,557,880 | 95.15 | 78.49 | 73.69 |
| | 3 | 28,748,762 | 24,987,972 | 95.30 | 78.05 | 73.20 |
| | 1 | 92,600,328 | 80,754,978 | 95.19 | 79.18 | 74.09 |
| RP9 | 2 | 55,161,620 | 48,309,724 | 96.71 | 76.65 | 71.87 |
| | 3 | 9,228 | 4,440 | 83.51 | 65.81 | 62.21 |
| **Total** | | **1,118,935,706** | **972,478,618** | | | |

Table 4.5. Genes cross-referenced between modules and differently expressed in all experiments comparing SUS against Vip-R2 and SUS against SUS protein-exposure. Up – upper regulated genes; Dw – Down regulated genes. Mod 1: Module 1; Mod 2: Module 2; Mod 3: Module 3. Overall Exper.: Overall experiments.

A

| | **Genes in Common** | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **SNP0 vs RP0** | | | **SNP9 vs RP9** | | | **SP124 vs RP124** | | | **SP9 vs RP9** | | | **Overall Exper.** |
| | Total | Up % | Dw % | Total | Up % | Dw % | Total | Up % | Dw % | Total | Up % | Dw % | Total |
| **Mod 1** | 3,040 | 0.98 | 99.01 | 2,433 | 0.41 | 99.59 | 3,397 | 1.15 | 98.85 | 552 | 1.1 | 98.8 | 305 |
| **Mod 2** | 2,363 | 98.01 | 1.09 | 771 | 95.33 | 4.67 | 2732 | 98.25 | 1.75 | 1,139 | 99.65 | 0.35 | 284 |
| **Mod 3** | 155 | 35.5 | 65.5 | 91 | 25.3 | 74.7 | 437 | 13.5 | 86.5 | 121 | 27.3 | 72.7 | 30 |

B

| | **Genes in Common** | | | | | | |
|---|---|---|---|---|---|---|---|
| | **SNP0 vs. SP124** | | | **SNP9 vs. SP9** | | | **Overall Expr.** |
| | Total | Up % | Dw % | Total | Up % | Dw % | |
| **Mod 3** | 264 | 100 | 0 | 116 | 100 | 0 | 92 |

Table 4.6. Fold change on statistically differential gene expression for candidate genes associated to resistance of FAW to Vip3Aa20. Green box are represented by differential gene expression statistically different while red box are not for each experiment. Fold change based on gene expression values transformed to log2 (E+1).

| | Contigs | SP124vsRP124 | SNP0vsRNP0 | SNP9vs RNP9 | SP9vsRP9 |
|---|---|---|---|---|---|
| | **ABC Tranporter System** - Fold Change | | | | |
| A | TRINITY_DN70713_c22_g3_i2 | -1,47 | -1,31 | -1,65 | -1,24 |
| | TRINITY_DN81819_c2_g2_i2 | -1,52 | -1,39 | -1,53 | -2,01 |
| | TRINITY_DN81819_c2_g1_i1 | -1,31 | -1,36 | -1,31 | -2,14 |
| | TRINITY_DN79813_c1_g5_i1 | -2,61 | -1,92 | -2,44 | -1,57 |
| | TRINITY_DN80310_c1_g29_i1 | -2,36 | -2,29 | -1,97 | -1,71 |
| | TRINITY_DN82072_c1_g1_i1 | -1,86 | -1,05 | -0,61 | -1,57 |
| | TRINITY_DN77570_c1_g1_i1 | -1,70 | -0,71 | -1,89 | -0,76 |
| | TRINITY_DN80316_c0_g4_i1 | -1,89 | -1,76 | -2,07 | -0,02 |
| | TRINITY_DN81289_c1_g6_i3 | -2,02 | -1,97 | -2,10 | -2,14 |
| | TRINITY_DN80591_c1_g1_i2 | -2,63 | -2,73 | -2,35 | -1,77 |
| | TRINITY_DN80310_c1_g28_i1 | -3,04 | -3,00 | -2,87 | -1,35 |
| | TRINITY_DN80310_c1_g19_i1 | -3,02 | -3,26 | -3,48 | -1,97 |
| B | **G-protein coupled receptor Mth** | | | | |
| | TRINITY_DN79944_c2_g10_i1 | -1,30 | -0,16 | -0,06 | -0,90 |
| | TRINITY_DN79344_c1_g4_i9 | -1,64 | -0,45 | -0,10 | -0,93 |
| | TRINITY_DN79344_c1_g6_i1 | -1,47 | -0,38 | -0,11 | -1,17 |
| | TRINITY_DN71749_c1_g1_i1 | -1,92 | -1,20 | -1,52 | -1,12 |
| C | **Adenylyl cyclase-associated protein 2** | | | | |
| | TRINITY_DN64720_c1_g1_i2 | -1,34 | 0,08 | 0,23 | -1,83 |
| D | **Adenylate cyclase type 2-like** | | | | |
| | TRINITY_DN82135_c2_g3_i3 | -1,51 | -0,38 | -0,12 | -1,30 |
| E | **cAMP-specific 3',5'-cyclic phosphodiesterase** | | | | |
| | TRINITY_DN73414_c6_g5_i2 | -1,60 | 0,10 | 0,60 | 0,27 |
| F | **cyclic AMP-regulated protein** | | | | |
| | TRINITY_DN61457_c0_g1_i1 | 1,21 | 1,64 | 1,59 | -1,05 |

| | Contigs | SNP0vsSP124 | SNP9vsSP9 | RNP0vsRP124 | RNP9vsRP9 |
|---|---|---|---|---|---|
| G | **Adenylyl cyclase-associated protein 2 -** Fold Change | | | | |
| | TRINITY_DN73414_c6_g5_i2 | 1,76 | 0,56 | 0,07 | 0,19 |

Table 4.7. ABC transporter contigs identified as down regulated with its respective annotation and fold change range. (Fold change based on gene expression data transformed to log2 (E+1).

| **Contig Annotation** | **No. Haplotype** | **Fold Change Range ($log_2$)** |
|---|---|---|
| ABC transporter | 3 | 1.31 to 2.14 |
| ATP-binding cassette sub-family A member 1-like | 1 | 0.61 to 1.86 |
| ATP-binding cassette sub-family B member 8, mitochondrial | 1 | 0.71 to 1.89 |
| ATP-binding cassette sub-family D | 2 | 1.76 to 2.10 |
| ATP-binding cassette sub-family F member 1 | 1 | 2.35 to 2.73 |
| ATP-binding cassette sub-family G member 1 | 4 | 1.92 to 3.48 |

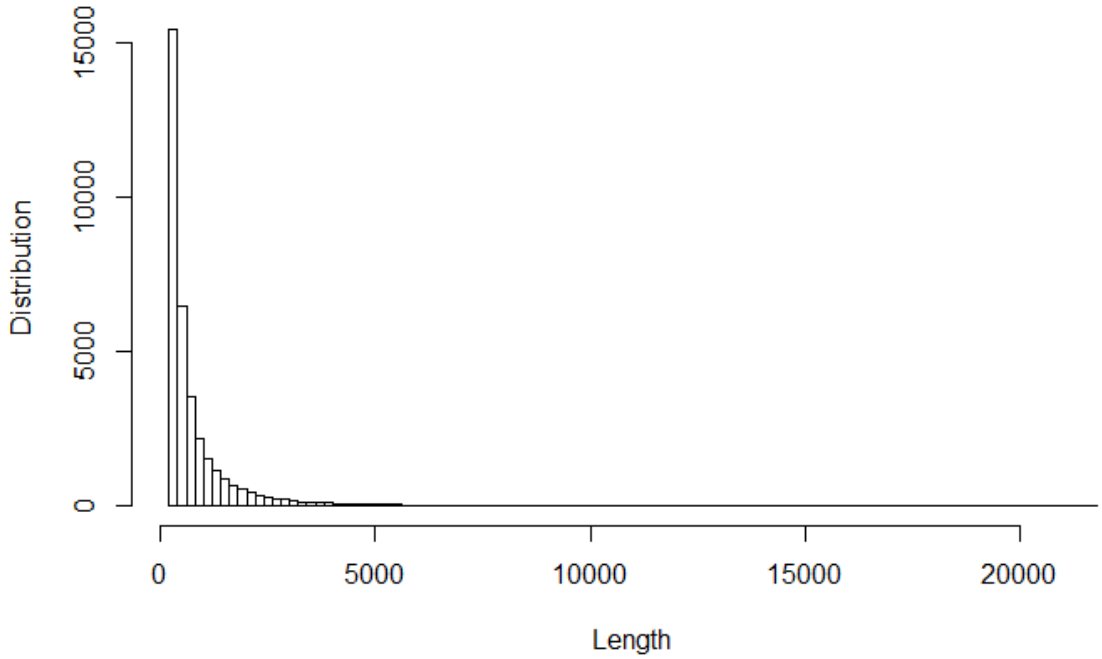



Figure 4.1. Size distribution of contigs from the reference assembly used for RNA-seq analysis.

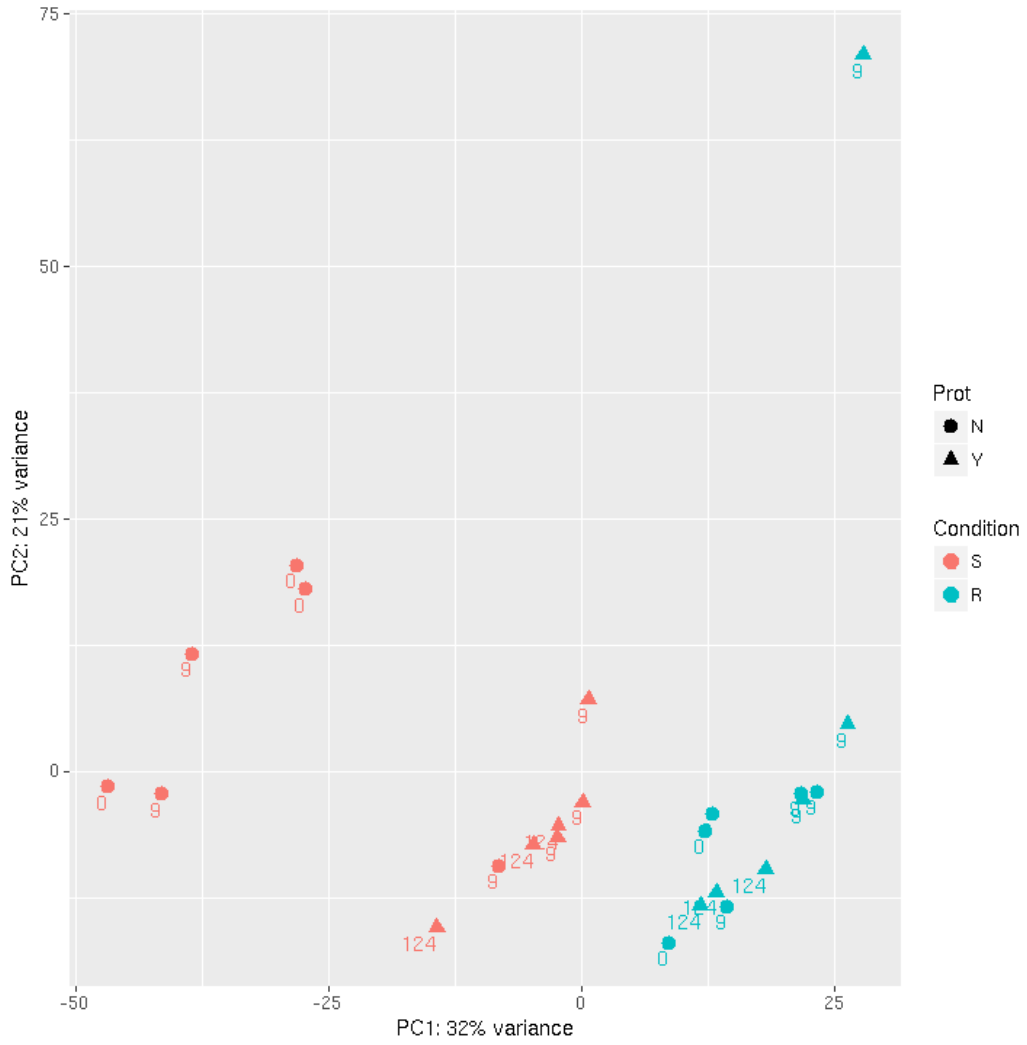



Figure 4.2. PCA plot using the rlog-transformed values of gene expression (unique gene reads). Data point format represents protein induced or not (bools – protein not induced; triangle – protein induced), and different colors represent SUS and Vip-R2 (Red – SUS; Blue – Vip-R2). PC1-axis is the direction that separates the data point the most. PC2-axis is the direction that separates the data de second most (orthogonal to the first direction).

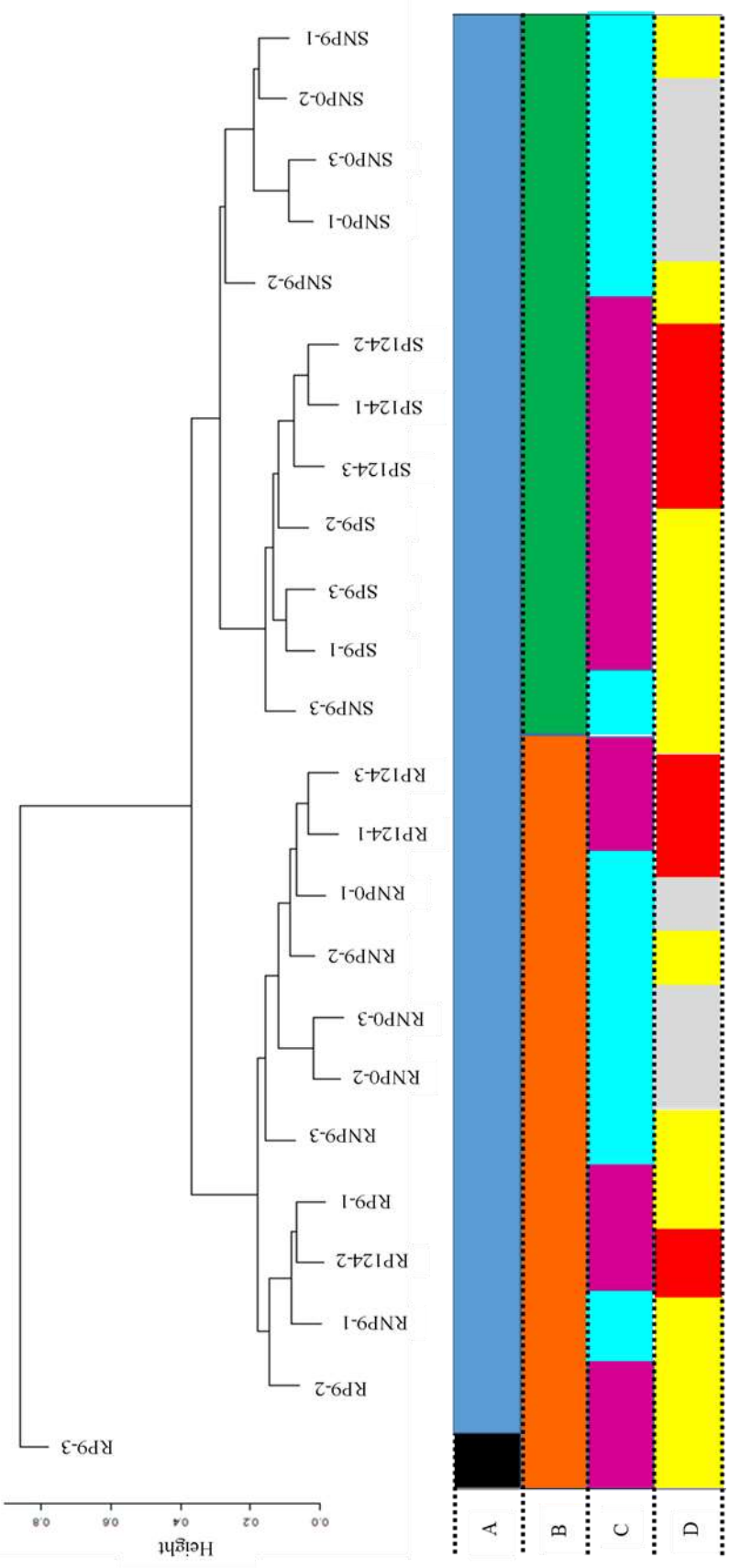



Figure 4.3. Sample dendrogram and trait heatmap (WGCNA output). A) Black bar represents the outlier sample. B) Orange bar represents all Vip-R2 samples while green bar represents SUS samples. C) Pink bars represent all samples induced with protein and light blue bars represents no protein induction. D) Grey bars represent gene expression profile at zero hours after experiment started; red bars represent samples for gene expression in the 1,2,4, hours after experiment started; yellow bars represent samples for gene expression at 9 hours after experiment started.

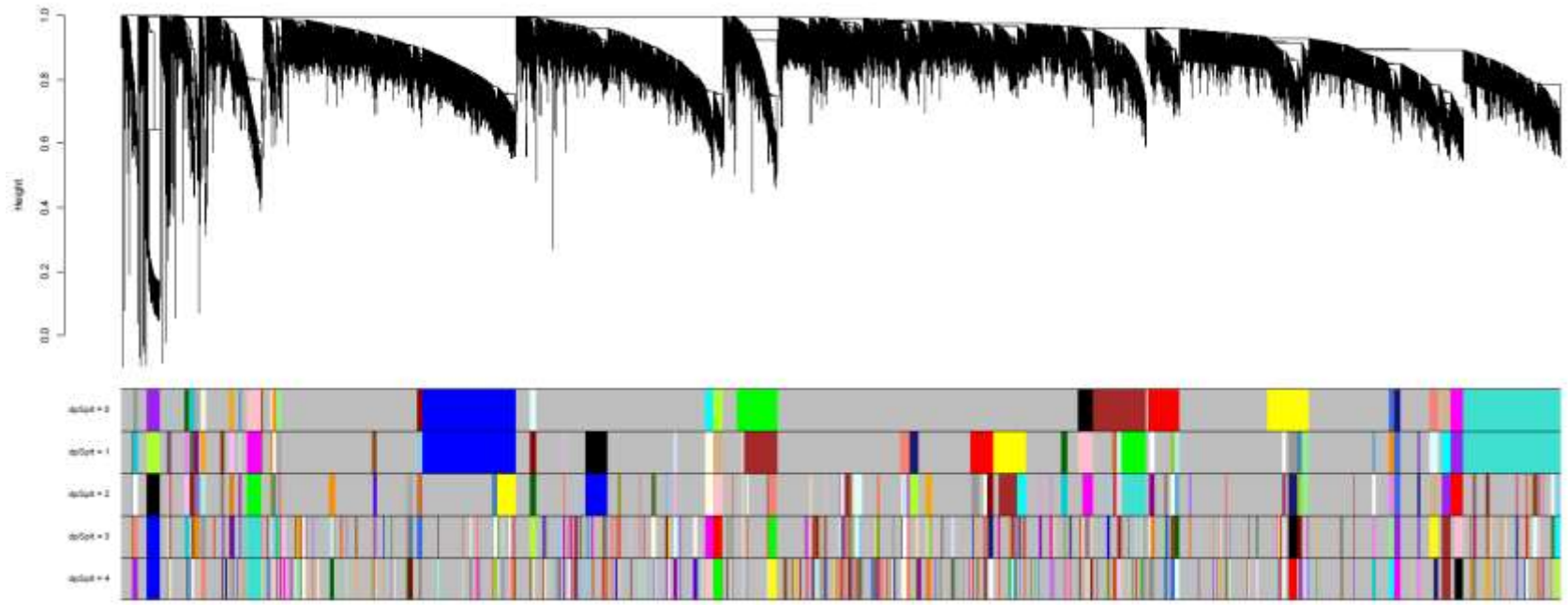



Figure 4.4. Hierarchical cluster tree of genes in the Vip-R2 network. Modules correspond to branches of the tree. The branches and module genes are assigned a color as can be seen from the color-bands underneath the tree.

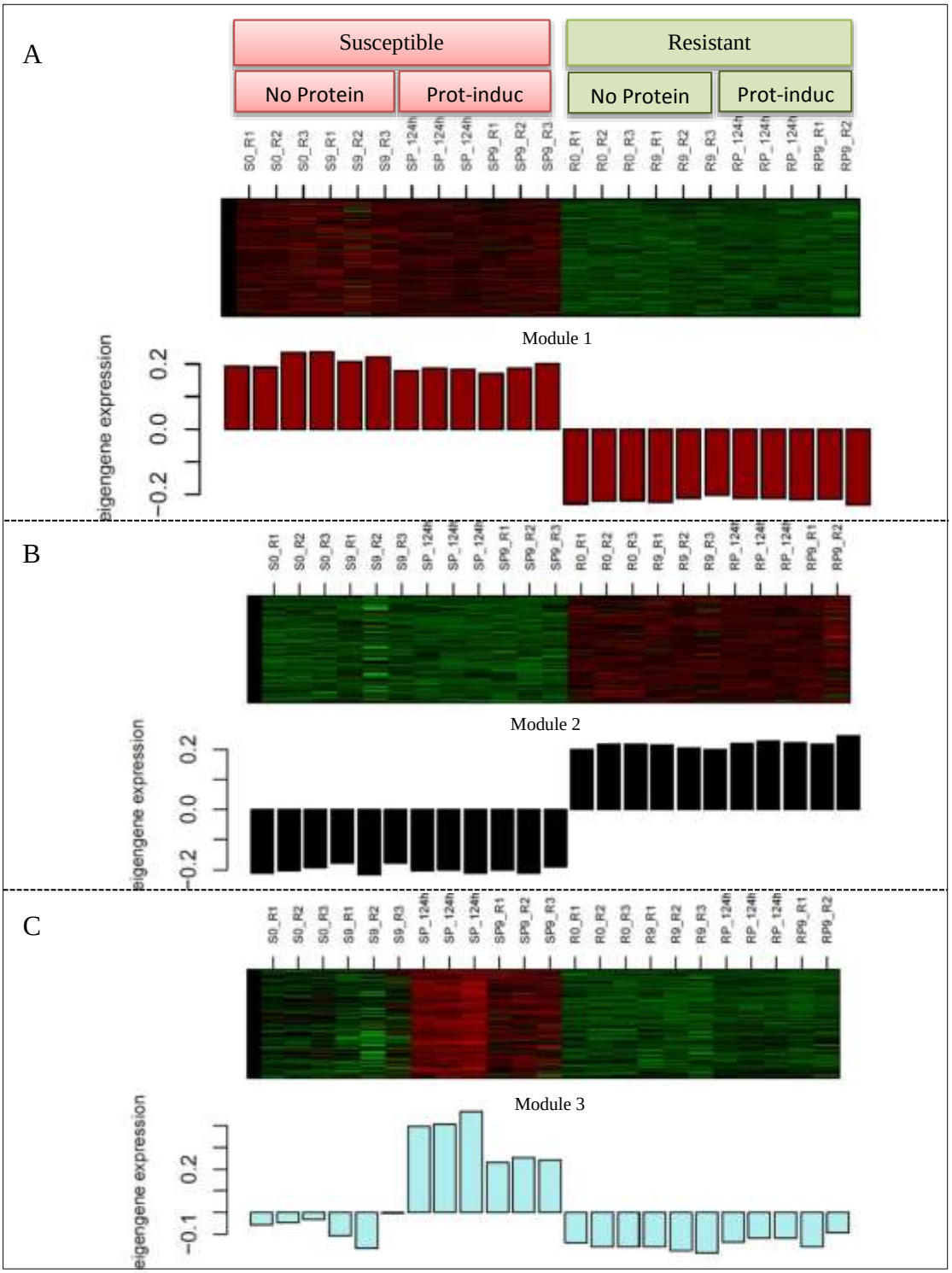



Figure 4.5. Selected modules associated to resistance of FAW to Vip3Aa20 (A: Module 1; B: Module 2; C: Module 3) and module associated to Vip3Aa20 mode of action to FAW (C: Module 3).

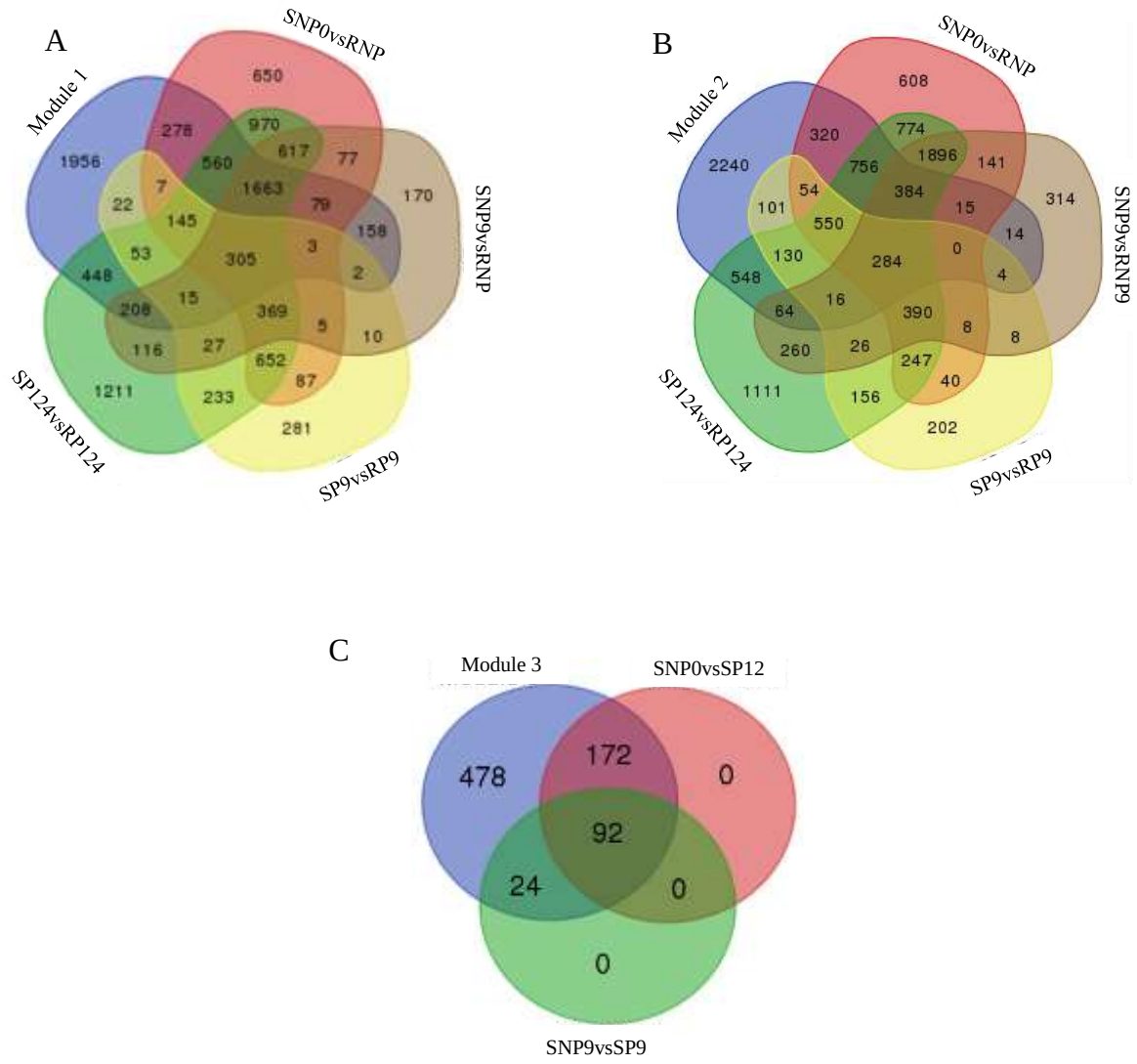



Figure 4.6. Venn diagram showing the common genes between modules and differential gene expression obtained from experiments. A) Comparison between genes present in the module 1 and experiments involving SUS and Vip-R2. B) Comparison between genes present in the module 2 and experiments involving SUS and Vip-R2. C) Comparison between genes present in the module 3 and experiments involving SUS and Vip-R2.

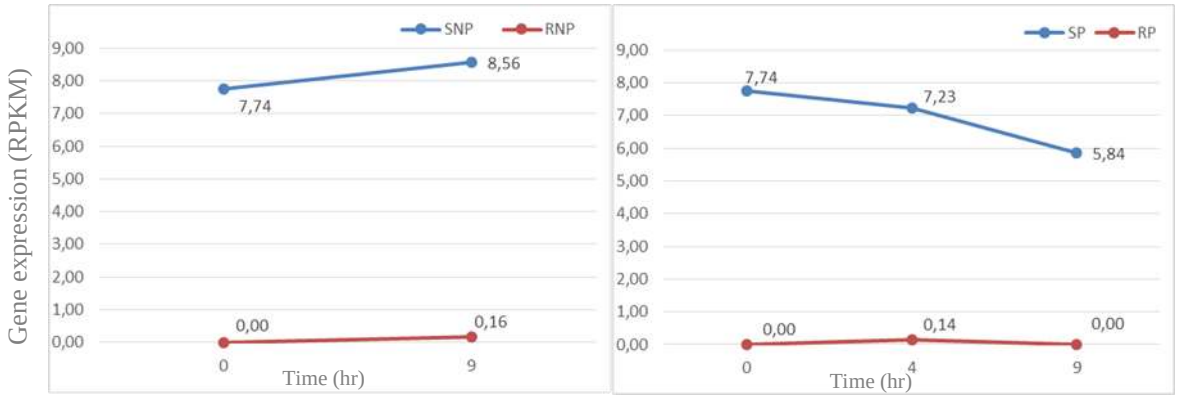
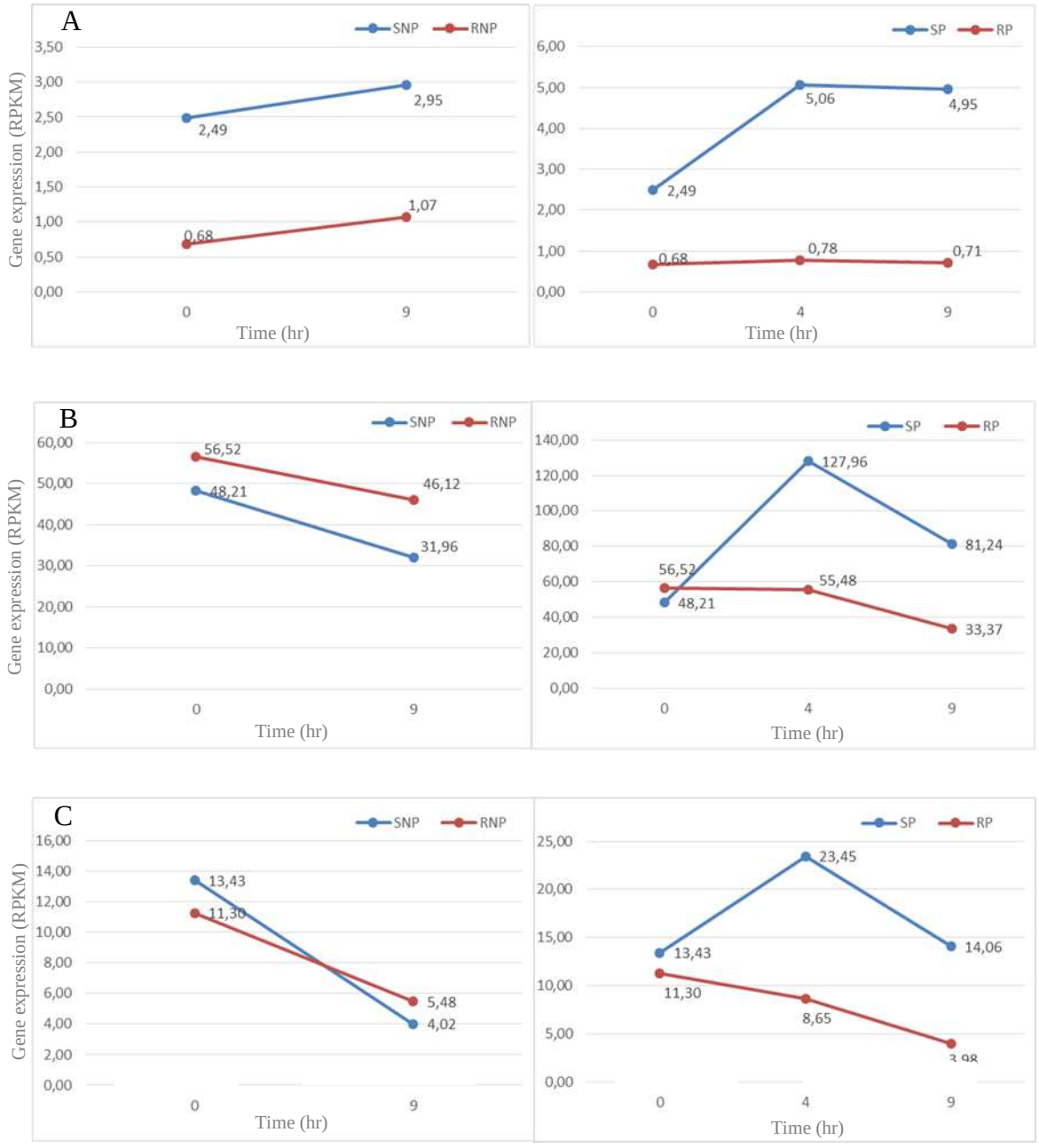



Figure 4.7. Differential gene expression between SUS and Vip-R2 for the gene ATP-binding cassette sub-family G in different times. Left side, not protein-induced; right side is protein induced. SNP: SUS; RNP: Vip-R2. Gene expression values is presented as transformed to log2 (E+1), where E is the original expression value.


A
SNP
RNP
Gene expression (RPKM)
3,50
3,00
2,50
2,00
1,50
1,00
0,50
0,00
2,49
2,95
0,68
1,07
0
9
Time (hr)
SP
RP
6,00
5,00
4,00
3,00
2,00
1,00
0,00
2,49
5,06
4,95
0,68
0,78
0,71
0
4
9
Time (hr)
B
SNP
RNP
60,00
50,00
40,00
30,00
20,00
10,00
0,00
56,52
46,12
48,21
31,96
0
9
Time (hr)
SP
RP
140,00
120,00
100,00
80,00
60,00
40,00
20,00
0,00
127,96
81,24
56,52
55,48
48,21
33,37
0
4
9
Time (hr)
C
SNP
RNP
16,00
14,00
12,00
10,00
8,00
6,00
4,00
2,00
0,00
13,43
11,30
5,48
4,02
0
9
Time (hr)
SP
RP
25,00
20,00
15,00
10,00
5,00
0,00
23,45
14,06
13,43
11,30
8,65
0
4
9
Time (hr)

continued

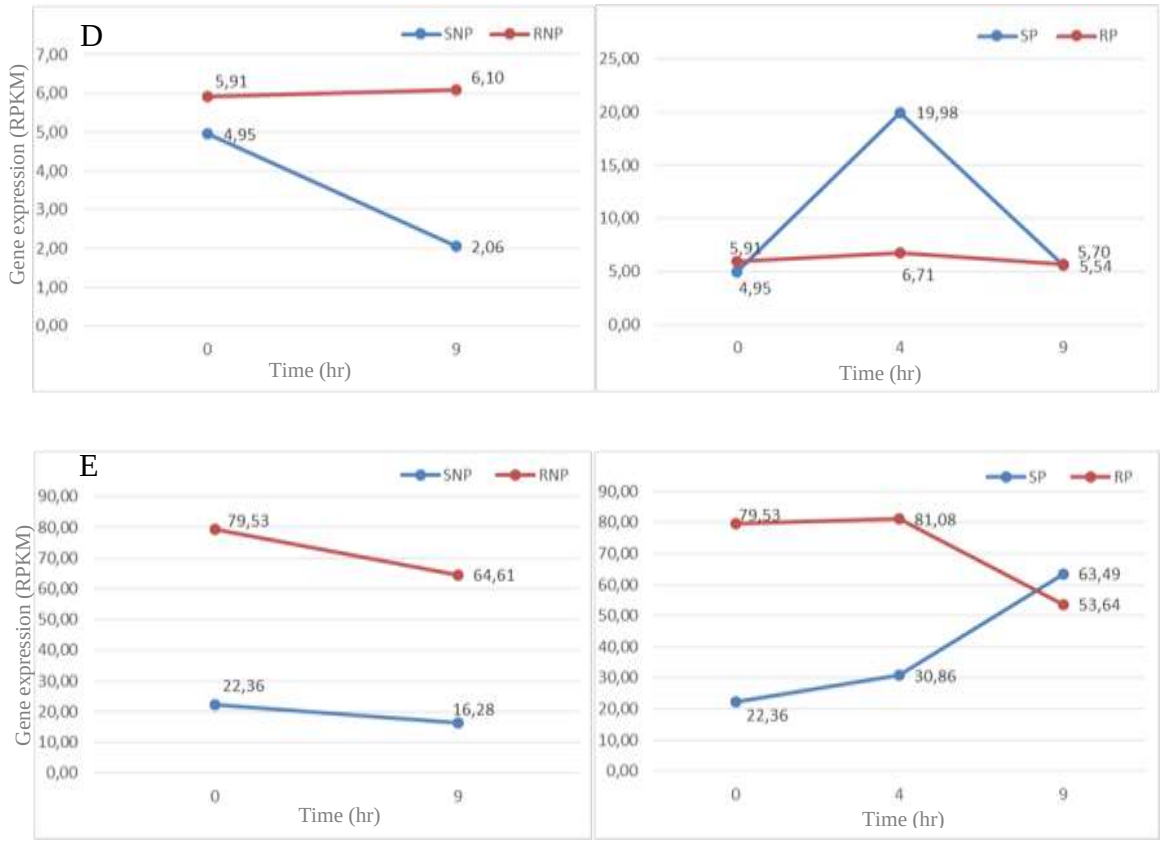



Figure 4.8. Differential gene expression between SUS and Vip-R2 for genes involved with G-protein signiling pathway. In the left side are not protein induced and the right side are protein induced. A) G-protein coupled receptor Mth; B) adenylyl cyclase-associated protein 2; C) Adenylate cyclase type 2-like.; D) cAMP-specific 3',5'-cyclic phosphodiesterase; E) cyclic AMP-regulated protein. SNP: SUS; RNP: Vip-R2. Gene expression values is presented as transformed to log2 (E+1), where E is the original expression value.

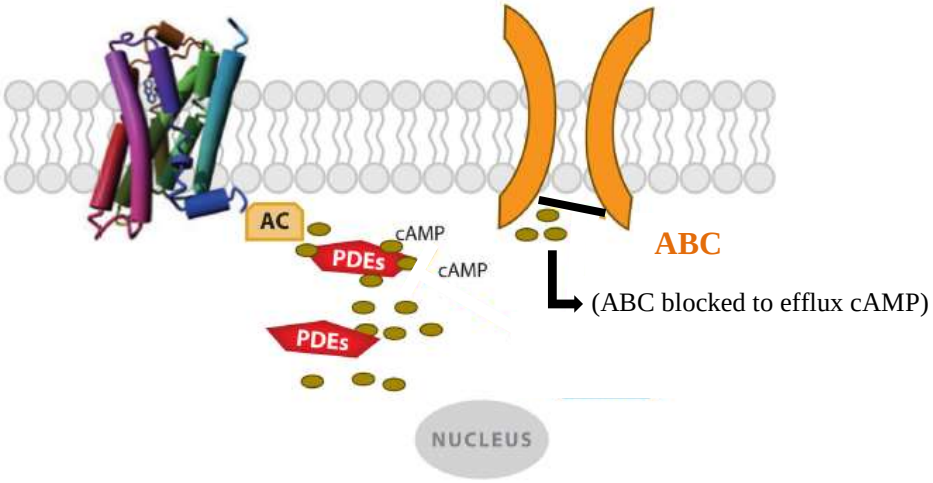



Figure 4.9. ABC transporter at the plasma membrane is not able to regulate the concentrations of intracellular cyclic nucleotides due to its down regulation in Vip-R2 colony (-3.25 fold change). In addition, cAMP-specific 3',5'-cyclic phosphodiesterase is not degrading cAMP due to its down regulation (-1.60 fold change) in Vip-R2 strain . Abbreviations: AC, adenylate cyclase; cAMP, cyclic adenosine monophosphate; PDE, phosphodiesterase. ABC, ABC transporter system. Figure adapted from Cheepala et al. 2013.

# 5 SUMMARY AND FUTURE WORK

The fall armyworm (FAW), *S. frugiperda* (JE Smith, 1797) (Lepidoptera: Noctuidae), is the major corn pest in Brazil and throughout South America (Blanco et al. 2016). In the last 4 years, Brazilian cotton and corn fields have experienced high FAW infestations causing large economic losses. Corn yield reductions caused by FAW can reach 34-38% (Carvalho 1970). When late instar larvae act as seedling cutworm, losses can reach up to 100% (Avila et al. 1997).

This pest has widely been controlled through the wide-scale adoption of transgenic corn hybrids expressing insecticide proteins derived from *Bacillus thuringiensis* (Bt) in South America (NAS 2016). The rapid adoption of genetically modified crops has been driven by various benefits provided by this technology, including effective insect control, reduced agricultural inputs (i.e. chemical pesticides) and positive economic impact for growers.

Most of the commercial hybrids in Brazil express Cry proteins, which produces inclusion bodies containing a number of proteins (δ-endotoxins) at the time of sporulation. In addition to Cry proteins, *B. thuringiensis* also produces a different class of proteins known as Vip (vegetative insecticidal protein). These proteins are produced by the bacteria during the vegetative stage of growth. Unlike delta-endotoxins, which are produced in the form of a protein crystal within the cell during sporulation, Vips are secreted into the nutrient growth medium (Estruch et al. 1996). Regarding sequence homology, Vips has no similarity with known δ-endotoxins (Estruch et al. 1996). Commercial hybrids expressing Vip3Aa20 (event MIR162) is available in Brazil under the brand Viptera®.

FAW can also develop resistance to Bt crops in response to the strong selection pressure that this technology imposes over field populations due to constitutive Bt protein expression throughout the crop's life cycle (Storer et al. 2012). Insect Resistance Management (IRM) strategies increase effectiveness when implemented proactively in the field. Thus, understanding the mechanisms of resistance before they occur in the field will contribute to improved risk assessment for resistance evolution as well as understand how rapid the resistance will evolve.

In chapter two, we discussed FAW resistance evolution to Bt toxins in Brazil within a framework of three interacting factors: *i*) Genetics; *ii*) Biology and ecology; and *iii*) Implementation of resistance management tactics. We suggest that these factors enabled FAW to overcome Bt crops in an unexpected and unprecedented period of time. Immense and rapid

reproduction, large scale dispersal, lack of fitness costs and high-doses, and poor refuge compliance have created a perfect storm that facilitated Bt resistance in FAW in Brazil. Further research and implementation on IRM strategies and would help understand the potential risk for resistance evolution before product launching and allow industry, academics and government agencies to propose and improve proactive resistance management strategies.

Extended sustainability requires understanding the biochemical as well as the genetics regarding the mode of action and mechanisms of resistance to improve (IRM). Thus the FAW resistant strain selected was used to characterize the mechanisms of resistance to Vip3Aa20. In chapter 3 we generated comparative proteomic data between susceptible and resistant strains and tested two hypotheses potentially associated with resistance of FAW to Vip3Aa20: 1) Resistance of FAW to Vip3Aa20 is given by reduced protein activation by the midgut extract through serine-proteases proteolysis activity; 2) Resistance is associated by the failure of protein binding to receptor, which is the immediate step after protein activation, and a pre-requisite for downstream mechanisms and insect cell death.

Several studies have associated resistance of insects to Cry proteins due to lack of receptor-protein binding. Thus, binding assays would demonstrate if the protein is not binding specifically to a putative receptor, which is the first step for the mode of action of Bt proteins. We demonstrated that neither Vip3Aa20 activation nor protein binding were the cause of resistance in FAW. Resistant colonies did not show any significant difference when compared to a susceptible colony, for all studies performed. Other mechanisms might be the cause of resistance of FAW to Vip3Aa20 in Brazil, such as downstream events associated to pore forming or G-protein signaling pathway models.

In chapter 4 we tried to identify the downstream events that might be associated with resistance. We used a transcriptomics approach to compare gene expression profiles of FAW resistant and susceptible to Vip3Aa20 to identify potential pathways associated with the proteins mode of action as well as candidate genes acting towards resistance. We detected four genes that were downregulated and one gene that was up-regulated that play a role in the G-protein signaling pathway regulation. Thus our hypothesis is that Vip3Aa20 is killing FAW through this pathway, consistent with the Zhang model of Cry mode of action (Zhang et al. 2006).

We also hypothesized that deregulation of G-protein pathway might be associated with the down regulation of ABC transporter system also detected in our study. One function of ABC transporters are to regulate intracellular cAMP and efflux to external regions (Li et al. 2007, Cheepala et al. 2013). Studies performed with gut epithelial cell lines of FAW

demonstrated that ABCC4 transports cAMP across the membrane, and when this gene is suppressed, concentration of cAMP is increased in the cytosol (Li et al. 2007). Thus, our hypothesis is that the resistance of FAW to Vip3Aa20 is provided by the lack of cAMP effluxing from the cytosol through ABC transporter, as well as reduced activity of phosphodiesterase, which is not regulating the intracellular cAMP concentration.

Additional studies involving silencing of those candidates genes through RNAi or CRISP-Cas9 technologies would bring more solid confirmation that G-protein signaling pathway is involved in the mode of action of this protein as well as resistance. We also encourage further research to better understand the relation between ABC transporter system and G-protein signaling pathway more deeply in insects.

In summary, proteomics allowed us to exclude the hypothesis that resistance of FAW to Vip3Aa20 was due to a lack of protein activation or protein-receptor binding while a transcriptomics approach allow us to validate the hypothesis that Vip3Aa20 acts in the insect midgut through the G-protein signaling pathway. Furthermore, we found strong evidence of ABC transporter system being involved with resistance likely through an interaction with G-protein signaling pathway. These results will allow us to characterize resistance of FAW to Vip3Aa20 using the resistant colony and enable an improved prediction of resistance evolution. Proteomics data will support the design of new products (chimeric protein-based) with a combination or different active domains to manage FAW resistance driven by lack of protein-receptor binding. Data on molecular mechanisms of resistance will help industry towards developing products with new mode of action as well as develop high-throughput molecular platform for resistance monitoring to detect early shifts of frequency of resistance allele and implement proactive resistance management.

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