

**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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## 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 proteína Vip (Vegetative Insecticide Protein) cuja secreção é realizada durante fase de crescimento da bactéria é considerada como segunda geração de proteínas inseticidas em função desta não apresentar similaridade de sequências com todas as outras proteínas cristal (Cry), apresentando ainda maior espectro de controle de pragas. Uma das pragas alvo desta proteína 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 táticas de controle geradas através da biotecnologia. Dessa forma, estudos de caracterização da resistência envolvendo modo de ação e características genéticas envolvidas com resistência pode contribuir para melhorar estratégias de Manejo de Resistência 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 susceptível. No capítulo 2, abordamos as características de bio-ecologia da praga associado ao sistema de cultivo suportando o alto potencial adaptativo desta espécie para híbridos de milho expressando proteínas Bt no Brasil. No capítulo 3, estudos de proteômica mostrou que Vip-R1 e Vip-R2 quando comparado com SUS, não demonstraram diferenças para ativação da proteína nem ausência de ligação da proteína com receptor de membrana no intestino do inseto. Dados de transcriptoma descritos no capítulo 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 práticas de manejo de resistência para lagarta-do-cartucho e assim estender a durabilidade da tecnologia Viper<sup>®</sup> no campo.

Palavras-chave: *Spodoptera frugiperda*; Vip3A; Manejo de resistência de insetos; ligação proteína-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 maize 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-R1 and 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 maize 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

## 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 maize 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 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 gramineae, 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 ( $\delta$ -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  $\delta$ -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  $\delta$ -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  $\alpha$ -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- $\alpha$  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 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. interpunctella*. 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.

## 2 CONCLUSION

The fall armyworm (FAW), *S. frugiperda* (JE Smith, 1797) (Lepidoptera: Noctuidae), is the major maize 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 ( $\delta$ -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  $\delta$ -endotoxins (Estruch et al. 1996). Commercial hybrids expressing Vip3Aa20 (event MIR162) is available in Brazil under the brand Viptera<sup>®</sup>.

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 prerequisite 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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