University of São Paulo
Luiz de Queiroz College of Agriculture

Exploiting next generation sequencing techniques (NGS) to identify molecular markers for monitoring the resistance of *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae) to insecticides and Bt proteins

Antonio Rogério Bezerra do Nascimento

Thesis presented to obtain the degree of Doctor in Science.
Area: Entomology

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Antonio Rogerio Bezerra do Nascimento
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Advisor:
Prof. Dr. **CELSO OMOTO**

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RESUMO

Explorando técnicas de sequenciamento de próxima geração (NGS) para identificar marcadores moleculares para o monitoramento da resistência de *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae) a inseticidas e proteínas Bt

Técnicas de sequenciamento de DNA e RNA de próxima geração foram utilizadas para identificar marcadores moleculares associados à resistência de *Spodoptera frugiperda* (J.E. Smith) a inseticidas e proteínas de *Bacillus thuringiensis* Berliner (Bt). Para tanto, foram selecionadas linhagens de *S. frugiperda* resistentes a moléculas inseticidas (chlorpyrifos, lambda-cyhalothrin, lufenuron, teflubenzuron e spinosad) pertencentes a diferentes grupos químicos e ao milho YieldGard VT-PRO® que expressa proteínas Cry1A.105 e Cry2Ab2. Os resultados de expressão gênica entre as linhagens resistentes e suscetíveis aos inseticidas neurotóxicos chlorpyrifos e lambda-cyhalothrin, indicaram 935 genes associados à resistência a chlorpyrifos e 241 genes a lambda-cyhalothrin que foram diferencialmente expressos. A maior parte desses genes está relacionada a elevados nível de expressão de enzimas de detoxificação, principalmente das famílias *CYP3* e *CPY6*. Com relação ao inseticida teflubenzuron, o padrão de herança da resistência foi caracterizado como resistência autossômica, incompletamente recessiva e poligênica. Os resultados de expressão gênica entre as linhagens resistente e suscetível a teflubenzuron indicou 3.519 transcritos diferencialmente expressos, principalmente de enzimas de detoxificação dos grupos GSTs, UGTs, P450s, CEs, além de genes de transporte e regulação. Esse perfil de expressão gênica também foi identificando na linhagem resistente ao milho YieldGard VT-PRO®, o qual também demonstrou modificações nos níveis de expressão de outros grupos gênicos como caderina, aminopeptidases e alcalino-fosfatase. Por último, com a finalidade de identificarmos marcadores tipo SNP associados à resistência de *S. frugiperda* a inseticidas e proteínas Bt, o protocolo de genotyping by sequencing (GBS) foi utilizado para todas as linhagens resistentes mencionadas e a linhagem suscetível de referência. Foram recuperados 4.276 SNPs após os processos de filtragem, sendo identificados 53 locos polimórficos sob seleção estatisticamente significantes (FDR≤0,047), sendo que nenhum deles associado a regiões codificantes. No entanto, vários desses SNPs foram associados a regiões reguladoras do genoma. As análises utilizando DAPC resultou na formação de sete grupos, com a separação da linhagem suscetível de todas as linhagens resistentes. A linhagem resistente a chlorpyrifos apresentou um grupo exclusivo separado das demais linhagens resistentes, as quais permaneceram agrupadas. As análises de associação entre as linhagens suscetível e resistentes indicaram 17 locos associados a todas as linhagens resistentes, 114 locos associados à linhagem resistente a chlorpyrifos, 105 a lambda-cyhalothrin, 84 a lufenuron, 87 a teflubenzuron, 108 a spinosad e 62 ao milho YieldGard VT-PRO®. Dessa forma podemos concluir que os processos de resistência associados a inseticidas e toxinas Bt são decorrentes de um grande número de modificações moleculares em sítios específicos associados a detoxificação e processos de regulação. Portanto, a utilização de tecnologias que possibilitem a análise sistêmica e ampla desses fenômenos, como sequenciamento de nova geração, busca de marcadores moleculares em larga escala e estudos funcionais com diversos grupos de inseticidas devem ser a nova base de pesquisa para avançar o conhecimento dos processos adaptativos impulsionados pela evolução da resistência de insetos a inseticidas e proteínas Bt.

Palavras-chave: Transcritoma; Genotyping by sequencing; Manejo de resistência de insetos; Marcador molecular

ABSTRACT

Exploiting next generation sequencing techniques (NGS) to identify molecular markers for monitoring the resistance of *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae) to insecticides and *Bt* proteins

In this study we used Next-generation sequencing "NGS" for DNA and RNA sequencing to search for molecular markers associated with resistance of *Spodoptera frugiperda* (J.E. Smith) to insecticides and *Bacillus thuringiensis* Berliner (*Bt*) proteins. For this purpose, we selected *S. frugiperda* resistant strains to insecticides (chlorpyrifos, lambda-cyhalothrin, lufenuron, teflubenzuron and spinosad) belonging to different chemical groups and to the YieldGard VT-PRO® maize expressing Cry1A.105 and Cry2Ab2 proteins. The results of gene expression between resistant and susceptible strains of the neurotoxic insecticides chlorpyrifos and lambda-cyhalothrin demonstrated 935 differentially expressed genes associated with chlorpyrifos resistance and 241 differentially expressed genes associated with lambda-cyhalothrin. Most of these genes was related to high levels of expression in detoxification enzymes, especially the *CYP3* and *CPY6* families. Regarding to the insecticide teflubenzuron, the inheritance of resistance was characterized as autosomal, incompletely recessive and polygenic. The results of gene expression between resistant and susceptible strains of teflubenzuron indicated 3,519 differentially expressed transcripts, mainly detoxification enzymes from the GSTs, UGTs, P450s, CEs, as well as transport and regulation genes. This gene expression profile was also identified to YieldGard VT-PRO® resistant strain, which also demonstrated changes in the expression levels of other gene groups such as cadherin, aminopeptidases and alkaline phosphatase. Finally, to identify SNP markers associated with resistance of *S. frugiperda* to insecticides and Bt proteins, we used a genotyping by sequencing (GBS) protocol to all resistant strains and the susceptible strain. A total of 4,276 SNPs was recovered after filtering processes, where 53 polymorphic loci under selection were statistically significant (FDR≤0.047) and none of them was associated with coding regions. However, several of these SNPs were associated with regulatory regions of the genome. Analyses using DAPC resulted in the formation of seven clusters, with the susceptible line being separated from all resistant strains. The resistant strain to chlorpyrifos presented an exclusive cluster separated from the other resistant strains, which were grouped together. The association analyses between susceptible and resistant strains indicated 17 loci associated with all resistant strains, 114 loci associated with resistance to chlorpyrifos, 105 to lambda-cyhalothrin, 84 to lufenuron, 87 to teflubenzuron, 108 to spinosad and 62 to YieldGard VT-PRO® maize. Therefore, we can conclude that the resistance processes associated to insecticides and Bt toxins are due to a large number of molecular modifications at specific sites associated with detoxification and regulation processes. The use of technologies that allow for a systematic and comprehensive analyses of these phenomena, such as new-generation sequencing, large-scale molecular marker search, and functional studies with several insecticide groups should be the new research base to advance the knowledge on adaptive processes driven by the evolution of insect resistance to insecticides and Bt proteins.

Keywords: Transcriptome; Genotyping by sequencing; Insect resistance management; Molecular marker

1. INTRODUCTION

The fall armyworm, *Spodoptera frugiperda* (J.E. Smith, 1797) (Lepidoptera: Noctuidae) is a major pest of maize (Silva 2000; Valicente and Tuelher 2009). In Brazil, the damage to maize crops caused by this pest ranges from 20 to 100% (Cruz et al. 1999) and slightly less in other crops such as soybeans and cotton (Soares and Vieira 1998; Silva 2000). The high impact of *S. frugiperda* on crop production is a consequence of its wide biological plasticity and the intensive Brazilian production system. In the Brazilian Cerrado, intensive monoculture systems are used to produce mainly maize, soybeans, and cotton (Brannstrom et al. 2008). The intensive crop production throughout the year favors high population densities of *S. frugiperda* in some regions in Brazil.

Control tactics for *S. frugiperda* are based on the use of insecticides and transgenic crops that express the insecticidal protein from *Bacillus thuringiensis* (Bt crops). However, the number of cases of resistance to several insecticides and Bt toxins has increased. This has been related to the increase in selection pressure caused by the intensive use of insecticides and Bt crops, especially in maize.

In the USA, resistance of *S. frugiperda* to several insecticides has been identified (Yu, 1991; Yu and McCord, 2007; Yu et al., 2003). In Brazil, resistance cases were identified for pyrethroids (Carvalho et al., 2013; Diez-Rodriguez and Omoto, 2001), organophosphate (Carvalho et al., 2013), spinosins (Dourado, 2009), and the benzoylphenylurea group (Schmidt, 2002; Nascimento et al., 2015). In response to crop losses caused by *S. frugiperda* insecticide resistance, Bt maize varieties have been widely adopted, and nowadays Bt crops are the main control tactic for *S. frugiperda*. Besides the benefit to control, the use of Bt crops has also decreased the use of chemical insecticides and the risk to non-target organisms (Brookes and Barfoot 2012). However, the wide use of this technology has increased selection pressure, which has accelerated the development of resistance to Bt toxins in *S. frugiperda*, as confirmed for the toxins Cry1F (Farias et al., 2014) and Cry1Ab (Omoto, 2016), and for Bt maize VT-PRO expressing Cry2ab2 and Cry1A105 (Bernardi et al., 2015).

The development of new technologies to manage resistance of *S. frugiperda* is crucial to delay the evolution of resistance to insecticides and Bt (Head and Greenplate, 2012). Knowledge of population genetics (genetic diversity, gene flow, genetic drift, and frequency of resistant alleles) is important to assess the risk of resistance of new technologies (Flagel et al., 2015). Nonetheless, few genetic molecular markers have been developed to identify resistant alleles in *S. frugiperda.*

Molecular biology methods have been used to discover and characterize several resistance mechanisms in insects. Interesting examples are mutations in acetylcholinesterases (AChEs) that confer insensitivity to organophosphates and carbamates (Rasic et al., 2014), mutations in the voltage-dependent-sodium channel resulting in pyrethroid resistance (Saavedra-Rodriguez et al., 2007), and ABC transports that confer resistance to some Bt toxins (Gahan et al., 2010). In addition, studies have shown modifications in gene-expression patterns in response to insecticides, such as pyrethroids and organophosphates (Carvalho et al., 2013), diamides (Lin et al., 2013) and benzoylureas (Nascimento et al., 2016), and also in possible transposable elements involved in resistance processes (Rostant et al., 2012) .

Next-generation sequencing (NGS) provides new opportunities to discover genetic markers by using single-nucleotide polymorphisms (SNPs) (Davey et al., 2011). SNPs are point mutations that occur in alleles at a locus. SNPs tend to be biallelic mutations and usually occur in high densities within genomes. SNPs can be developed into molecular genetic markers, with low cost and minimal error during high-throughput genotyping screening. In addition, they can be rapidly developed and applied in the study of population genetics and in constructing gene maps. Recently, studies have identified SNPs to establish genetic markers for studying population genomics (Silva-Brandão et al., 2015) and phylogenetic evolution (McCormack et al., 2013), and to construct gene maps for non-model organisms (Flagel et al., 2015).

Another tool using NGS is genotyping by sequencing (GBS), which is based on the reduction of a complex genome by restriction enzymes, with a high capacity to discover SNPs at a low cost (Elshire et al., 2011; Sonah et al., 2013). With the development of these tools, important biological questions can be addressed, such as how to identify recombination breakpoints for linkage mapping or quantitative trait locus (QTL) mapping, to locate genome regions that differ among populations for quantitative genetic studies, to genotype large broods for marker-assisted selection, or to resolve the phylogeography of wild populations (Davey et al., 2011).

We proposed the use of RNA and DNA sequencing to identify molecular markers associated with resitance of *S. frugiperda* to insecticides and Bt proteins. Our objectives were: 1. To characterize the gene expression profile between resistant strains to the neurotoxic insecticides chlorpyrifos and lambda-cyhalothrin and susceptible strain; 2. To characterize the inheritance of resistance and gene differential expression between resistant and susceptible strains to teflubenzuron; 3. To perform transcriptome analysis between resistant and susceptible strains to Bt proteins, and 4. To explore GBS protocol to discovery SNPs associated to the resistance of *S. frugiperda* to insecticides and Bt proteins.

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2. MOLECULAR CHARACTERIZATION OF RESISTANCE OF *Spodoptera frugiperda* (LEPIDOPTERA: NOCTUIDAE) TO THE NEUROTOXIC INSECTICIDES LAMBDA CYHALOTHRIN AND CHLORPYRIFOS

ABSTRACT

Understanding the molecular mechanisms of insect resistance to insecticides can aid in designing new strategies for Insect Resistance Management (IRM) programs. In this study, we evaluated changes in gene expression levels in chlorpyrifos-resistant, lambda-cyhalothrin resistant, and susceptible strains of *Spodoptera frugiperda* (J.E. Smith) by using "Next-Generation Sequencing Technologies" (NGS). Fourth instars of *S. frugiperda* from resistant and susceptible strains were used for RNA extraction and cDNA sequencing. Paired-end reads were filtered based on a Phred score of 30 when aligned on the *S. frugiperda* draft genome. Differential gene expression was analyzed using the DeSeq2 package in R, allowing identification of 935 DEGs between the chlorpyrifos-resistant and susceptible strains, and 241 DEGs between lambda-cyhalothrin-resistant and susceptible strain, with a fold change > 2 and an FDR-adjusted p value of < 0.01. In both resistant strains, we observed overexpression of detoxification enzymes, mainly the *CYP3* and *CYP6* gene subfamilies, and genes associated with regulatory processes. Our results demonstrated that resistance to chlorpyrifos and lambda-cyhalothrin may be related to detoxification processes.

Keywords: Pyrethroids; Organophosphates; Detoxification; Cytochrome P450.

2.1. Introduction

Neurotoxic insecticides have been widely used to control agricultural and urban pests. Pyrethroids are a large class of synthetic insecticide analogs to pyrethrin, a substance present in the flowers of the pyrethrum daisy (*Tanacetum cinerariifolium*). Pyrethroids inhibit the deactivation and inactivation of sodium channels, resulting in prolonged opening of the sodium channels, which causes repetitive firing and depolarization of the nerve membrane and disrupts electrical signaling in the insect nervous system (Soderlund and Bloomquist 1989; Narahashi 1996; Soderlund 2005). Pyrethroids also induce autophagy and apoptosis in nerve cells (Park et al. 2015). A second group of insecticides, the organophosphates (OP), act on inhibition of acetylcholinesterase (AChE), an enzyme that catalyzes the hydrolysis of the neurotransmitting agent acetylcholine (ACh) (Fukuto 1990). Consequently, OP insecticides cause hyperexcitation of the insect nervous system (Spencer and O'brien 1957).

Prior to the advent of GMO "Genetically modified Organism" use, the control of the fall armyworm, *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae), was based on intensive application of chemical insecticides. Unfortunately, the indiscriminate application of

insecticides, mainly pyrethroids and organophosphates, contributed to the evolution of resistance of *S. frugiperda* to several compounds. High levels of resistance have been reported for several pyrethroid insecticides: lambda cyhalothrin, permethrin, cyhalothrin, tralomethrin, bifenthrin, and fluvalinate (Diez-Rodríguez and Omoto 2001; Carvalho et al. 2013) and the organophosphate insecticides malathion, chlorpyrifos, methyl parathion, diazinon, and sulprofos (Yu 1991; Yu 1992).

In several insect species, resistance to pyrethroids and organophosphates has been associated with mutations in genes coding target sites and/or with modifications in the expression profiles of genes for detoxification enzymes such as cytochrome P450, esterases, and glutathione S transferases. For example, in *S. frugiperda*, resistance associated with carbaryl was mainly due to enhanced oxidative metabolism (McCord and Yu 1987). This was also reported for pyrethroids and organophosphates (Carvalho et al. 2013) and benzoylureas (Nascimento et al. 2015).

Characterizing the molecular mechanisms that underlie insecticide resistance is crucial for identifying insecticide-resistance alleles and improving resistance-management strategies. In this study, we selected and characterized the resistance of *S. frugiperda* strains to the neurotoxic insecticides lambda-cyhalothrin and chlorpyrifos and used large-scale cDNA sequencing to compare the differential expression between resistant and susceptible strains.

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3. INHERITANCE, CROSS-RESISTANCE AND IDENTIFICATION OF MOLECULAR MARKERS ASSOCIATED WITH TEFLUBENZURON RESISTANCE IN *Spodoptera frugiperda* (LEPIDOPTERA: NOCTUIDAE)

ABSTRACT

The insecticide teflubenzuron acts by inhibiting chitin biosynthesis. This insecticide has been used to control the fall armyworm, *Spodoptera frugiperda* (J.E. Smith, 1797) (Lepidoptera: Noctuidae), and other lepidopteran pests. Knowledge of heritability features of resistance is highly important for the establishment of adequate and efficient resistance management strategies. Here, we selected a strain of *S. frugiperda* resistant to teflubenzuron, characterized the inheritance of resistance, cross-resistance to other chitin-synthesis inhibitors and developed a set of SNPs that can be used as a molecular marker in the future. The LC_{50} values (95% CI) were 641.47 (213.05 – 2748.81) $\mu g.mL^{-1}$ in the teflubenzuron-resistant (Tef-rr) and 0.47 (0.35 – 0.63) $\mu g.mL^{-1}$ in the susceptible strain (Sf-ss), based on a diet-overlay bioassay. The resistance ratio was ≈ 1,365-fold. Reciprocal crosses between Sf-ss and Tef-rr indicated that the inheritance of *S. frugiperda* resistance to teflubenzuron is autosomal and incompletely recessive. Low levels of cross-resistance was identified between teflubenzuron and other chitin-synthesis inhitors (lufenuron and novaluron). Backcrosses between heterozygous offspring with resistant parents revealed a polygenic effect. We identified a set of SNPs associated with genes for regulatory processes in the Tef-rr colony and in the offspring of the backcrosses. These results improved our knowledge of the inheritance of resistance of *S. frugiperda* to benzoylureas, and provided important information about possible genetic markers, which, in the future, can be an effective tool to aid in the management of teflubenzuron-resistant *S. frugiperda*.

Keywords: fall armyworm; heritability, chitin synthesis inhibitor; SNPs

3.1 Introduction

The evolution of resistance of insects to insecticides and Bt crops is of great concern to biologists, farmers, and the government. Strong selection pressure caused by numerous sprays of insecticides and wide adoption of Bt crops are responsible for increasing the frequency of resistance in many agroecosystems, including Brazil, especially in the successive crop systems used in the Cerrado region. Reports of phytosanitation problems associated with changes in pest susceptibility to control methods have heightened concern about the evolution of resistance in insects, especially in soybeans, maize, and cotton (Heckel 2003).

Spodoptera frugiperda (J.E. Smith, 1797) (Lepidoptera: Noctuidae) is a polyphagous species native to tropical regions of the Americas. The fall armyworm is a serious pest of several

economically important crops such as cotton (Santos 2011), soybeans (Moscard and Kastelic 1985), and maize (Silva 2000). Currently, Bt crops and insecticides are the main control methods for the fall armyworm.

Insecticides from the benzoylphenylurea group, which were introduced in the market in the early 1970s, have been successful in controlling several pest species due to their high insecticidal activity, making them suitable for use in Integrated Pest Management (IPM) programs (Beeman 1982). These insecticides inhibit chitin biosynthesis by interfering in the synthesis or deposition of chitin in the exoskeleton and other chitinized structures of insects (Merzendorfer 2003). Currently, compounds from the benzoylphenylurea group such as clorfluazurom, diflubenzuron, lufenuron, flufenoxurom, novaluron, triflumuron, and teflubenzuron are used to control insects in soybeans, cotton and maize crops (Agrofit 2018). The high selection pressure caused by this group of insecticides has decreased the susceptibility of *S. frugiperda* to benzoylphenylureas (Schmidt 2002), and has caused *S. frugiperda* to evolve resistance to lufenuron in populations in Goiás state, Brazil, with high resistance ratios and autosomal and polygenic inheritance of resistance (Nascimento et al. 2014).

Knowledge of the genetic basis of resistance is important for understanding, monitoring, and implementing proactive resistance-management strategies. In this study, we evaluated the genetic basis associated with the resistance of *S. frugiperda* to teflubenzuron. We also used a population-genomic approach to identify candidate SNPs that might be associated with selection caused by teflubenzuron.

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4. TRANSCRIPTOME AND COMPARATIVE ANALYSIS OF SUSCEPTIBLE AND TEFLUBENZURON-RESISTANT STRAINS OF *Spodoptera frugiperda* (LEPIDOPTERA: NOCTUIDAE)

ABSTRACT

The high selection pressure resulting from the widespread adoption of benzoylureas such as teflubenuzuron for the control of the fall armyworm, *Spodoptera frugiperda* (J.E. Smith), has been responsible for changes in the susceptibility of this species to chitin-synthesis inhibitor insecticides. We used cDNA sequencing to identify genes that showed differential expressions associated with resistance of this pest to teflubenzuron. We obtained approximately 250 million paired-end reads from Illumina Hiseq2500. *De novo* assembly resulted in 82,403 transcripts and 41,146 unigenes from Trinity. The transcript length distribution ranged from 301 to 26,723 bp with a mean length of 842.52 bp and an N50 of 1,086 bp. DEG analysis from DESeq2 identified 3,519 differentially expressed transcripts, based on an adjusted *p*-value $\leq$ 0.01 and log2 fold change $\geq$ 5. The resistant strain Tef-rr showed 991 down-regulated and 2,528 up-regulated transcripts compared to the susceptible strain Sf-ss. Through GO enrichment analysis of differentially expressed transcripts, we identified a large number of GO terms associated with regulation processes, mainly precatalytic spliceosome, catalytic step 2 spliceosome, GTP binding, transcription factor activity, and mRNA splicing via spliceosome. We identified 19 transcripts related to regulation of ecdysteroid hormones (ecdysteroid 22-kinase and ecdysone oxidase); and many ABC transport transcripts from the A, B, C, D and G families were more highly expressed in the resistant strain. Therefore, many detoxification enzymes such as GSTs, UGTs, P450s and CEs were up-regulated in the resistant strain. The large number of transcripts associated with detoxification processes demonstrated that this pathway is important for the evolution of resistance of *S. frugiperda* to teflubenzuron.

Keywords: Benzoylphenylureas; Detoxification Process; Regulation; Cytochrome P450

4.1. Introduction

The cuticle serves as the main barrier to protect insects. In addition to constituting the exoskeleton, the cuticle covers the digestive and respiratory systems, the reproductive organs, and some gland ducts (Andersen, 1979; Tunaz and Uygun 2003). Most of the cuticle is formed by proteins and chitin, a highly abundant polysaccharide in arthropods (Andersen, 1979). The specificity of the cuticular characteristics of insects constitutes an obviously desirable target for potentially selective insecticidal molecules (Beeman, 1982). Chitin-synthesis inhibitors (CSI) are chemically diverse compounds that affect the reproduction and development of chitin-synthesizing organisms (Merzendorfer 2003, 2012). These insecticides have been classified

according to their mode of action in several chemical groups, by the Insecticide Resistance Action Committee (IRAC). CSIs are divided into microbial-derived pyrimidine-nucleoside peptides, oxazolines, thiadiazines, and benzoylureas (BPUs, IRAC group 15) (Merzendorfer 2012). The benzoylureas are the most commonly used chitin-synthesis inhibitor insecticides. The efficiency of benzoylureas in controlling the population density of insect pests, together with their low toxicity in humans and other mammals, has stimulated studies on the effects of these compounds on the entomofauna associated with several agroecosystems, as well as updating their analogues, to maintain satisfactory levels of insect pest populations.

The mode of action of benzoylureas is not clear. Studies have shown that BPUs inhibit the incorporation of N-acetylglucosamine (GlcNAc), but their biochemical effects on enzymes, receptors, or intracellular organelles have not been determined (Matsumura 2010). Currently, the molecular mechanism of action of BPUs is thought to be associated with the sulfonylurea receptor (SUR), a type of ABC transporter subfamily C, which acts by altering vesicle trafficking and regulation of inward-rectifying potassium channels (Abo-Elghar et al. 2004)(Sun et al. 2015)(Bryan et al. 2006).

BPUs are currently used to control the fall armyworm *Spodoptera frugiperda* in Brazil. The high selection pressure resulting from the widespread adoption of BPUs such as lufenuron, novaluron, and teflubenuzuron to control this insect in maize, cotton, and soybean crops has modified the susceptibility of *S. frugiperda* populations to lufenuron (Nascimento et al. 2014; Schmidt 2002) and teflubenzuron (see Chapter 3). These studies showed that the fall armyworm has developed resistance to chitin-synthesis inhibitor insecticides.

Recently, the evolution of the Next-Generation Sequencing (NGS) sequencers has made it increasingly possible to perform low-cost transcripts, with high speed and a large amount of data (Hudson, 2008). Transcripts are used in a wide range of biological studies, and provide key information on the functioning and functional responses of organisms to diverse stimuli, for example allowing assessment to levels and profiles of gene expression in a comparative or non-comparative way (Hughes et al., 2009), identifying preserved orthologs for phylogenetic purposes (Hughes et al., 2009), and finding biomarkers for specific tissues and processes (Disset et al. 2009, Dunn et al. 2008), among others. The number of studies using these technologies to identify markers associated with resistance of insects to insecticides and *Bt* toxins has rapidly increased.

Here, we investigated modifications in the gene expression profile by comparing strains of *S. frugiperda* that are resistant or susceptible to teflubenzuron. The resistant strain was previously selected and characterized in the laboratory (see Chapter 3).

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5. TRANSCRIPTIONAL PROFILING ANALYSIS OF *Spodoptera frugiperda* (LEPIDOPTERA: NOCTUIDAE) RESISTANT TO YIELDGARD VT PRO® MAIZE

ABSTRACT

The wide adoption of genetically modified plants expressing the insecticide Bt has been the main control strategy for *Spodoptera frugiperda* (J.E. Smith) in Brazil. Although cases of resistance of the fall armyworm to Cry toxins have been increasing, very limited information is available for transcriptomic differences between resistant and susceptible strains to Bt toxins. In this study, we used RNA-seq to identify differential expression between resistant and susceptible strains of *S. frugiperda* to the commercial maize variety YieldGard VT PRO®, which expresses Cry1A.105 and Cry2Ab2 insecticidal proteins from *Bacillus thuringiensis* Berliner. Approximately 142 million paired-end reads were obtained from Illumina sequencing. *De novo* assembly resulted in 44,391 unigenes and 99,463 isoforms. DEG analysis showed that 19% of all unigenes were differentially expressed, with the FDR test ≤ 0.01 and relative expression > 5. A total of 10,281 transcripts were identified, with significant differences associated with several GOs and different metabolic pathways. Genes of aminopeptidntialtease were up-regulated in the VTPRO-resistant strain, while most of the carboxypeptidase and alkaline phosphatase genes were down-regulated. A large number of unigenes associated with detoxification processes, such as esterases and P450s, were identified as overexpressed in the *Bt*-resistant strain. Our results demonstrated a balance between regulation of detoxification processes and genes associated with the mode of action of the Bt toxin on resistant *S. frugiperda*.

Keywords: Bt proteins; Cry1A105; Cry2Ab2; fall armyworm; transcriptome

5.1. Introduction

Genetically modified plants expressing insecticidal proteins from *Bacillus thuringiensis* Berliner (Bt) have been used in the field since 1996. This modification has been an important tool to control insects and to reduce the amount of chemical insecticides used (Tabashnik et al. 2013). In recent years, the adoption of transgenic varieties in Brazil reached more than 93% of the field areas planted to maize, cotton, and soybeans (Celeres 2017).

Currently, the use of GMOs is the main control strategy for *Spodoptera frugiperda* (J.E. Smith) in Brazil (Okumura and de Cinque Mariano 2013; Waquil et al. 2013). The high selection pressure caused by the wide adoption of maize, cotton, and soybean varieties that express Cry toxins, and the current crop production system in Brazil with overlapping crops, have helped to increase the frequency of resistance of *S. frugiperda* to Cry toxins (Martinelli et al. 2007). A large number of commercial Bt maize and cotton varieties expressing Bt proteins

from the Cry1 family, such as Cry1F, Cry1A.105, Cry1Ac, and Cry1Ab, have been developed. Cases of fall armyworm resistance have already been reported for Cry1F (Farias et al. 2016), Cry1Ab (Omoto et al. 2016) and Cry1A105 and Cry2Ab2 (Bernardi et al. 2015). In addition, results for mortality have demonstrated cross-resistance between these proteins expressed in different Bt crops (Horikoshi et al. 2016).

The mode of action of Bt toxins against lepidopterans is well understood (Gill et al. 1992; Knowles 1994; Whalon and Wingerd 2003; Bravo et al. 2007). Nevertheless, the mechanism of resistance of insects to Bt toxins is less clear. Researchers list many possibilities for the mechanisms of resistance of lepidopterans to the Bt toxin (Heckel et al. 2007); currently, two hypotheses are accepted as mechanisms of resistance to Cry toxins. The sequential binding model (Bravo et al. 2004), which postulates that the high level of Cry resistance is due to modifications in binding with cadherins (Gahan et al. 2001; Horvath 2005; Zhao et al. 2010), aminopeptidases N (Zhang et al. 2009, Chang et al. 2008, Ingle et al. 2001) and/or ABC transport; and the signaling pathway model (Zhang et al. 2005; Zhang et al. 2006), which postulates that binding of Cry toxins caused by stimulation of the G protein and adenylyl cyclase increased cAMP levels and activation of protein kinase A, resulting in a cascade of signal transduction pathways that can either lead to cell death or protect cells from death. However, both hypotheses have gaps and doubtful aspects.

Therefore, it is necessary to increase efforts to clarify the molecular mechanisms of resistance of *S. frugiperda* to Cry toxins. We used next-generation sequencing (NGS) technologies to provide information about gene expression in susceptible and resistant strains of the fall armyworm to the commercial maize variety YieldGard VT PRO®, which expresses Cry1A105 and Cry2Ab2.

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6. ISCOVERY OF SNPs ASSOCIATED TO RESISTANCE OF *Spodoptera frugiperda* (LEPIDOPTERA: NOCTUIDAE) TO INSECTICIDES AND BT TOXINS

ABSTRACT

This study applied genotyping-by-sequencing protocol to discovery candidate SNPs markers associated with *S. frugiperda* resistant to insecticides and Bt toxins. All individual samples, from both resistant and susceptible strains, were characterized as corn strain. The SNP calling recovered 4276 SNPs after all filtering procedures. We detected 53 statistically significant polymorphic loci under selection (FDR≤ 0.047), none of them associated to coding regions. However, several of these SNPs were associated to regulatory regions of genome. The DAPC including resistant strains as *prior* information recovered seven clusters; the susceptible strain was distant from all resistant strains, Clo-RR strain sets an exclusive group, and the other strains clustered together. The association analyses between susceptible and resistant strains indicated 17 loci associated to all resistant strains, 114 loci significantly associated to Clo-RR, 105 to Lam-RR, 84 to Luf-RR, 87 to Tef-RR, 108 to Spi-RR and 62 significantly associated to VTPRO-RR. None these loci were associated with resistance mechanism previously described on the literature. Thus, these results support that the use of NGS contribute on insect resistance studies and help to find potentially new targets for management.

Keywords: Genotyping-by-sequencing; resistance mechanism; association analyses

6.1. Introduction

The evolution of insect resistance to insecticides is a contemporary example of evolutionary biology, especially when related to adaptive processes and natural selection (Oakeshott et al., 2003). Adaptation occurs when individuals of a population exhibit some characteristics with selective advantages in an environment with a certain selection pressure, but which of course will not be advantageous in other habitats without this pressure (Williams 1996). Thus, insect resistance can be characterized as an adaptive phenomenon due to the selective pressure promoted by controlling agents (insecticides and plants expressing Bt proteins from the entomopathogenic bacterium *Bacillus thuringiensis* (Berliner)), which promotes a selection of adapted phenotypes according to the genetic variability present in the population (Crow 1957, Georghiou 1972). In this context, resistance is defined as the development of an inherited ability of the organism to tolerate toxic doses that would be lethal to most individuals of the species (Croft and Vandebaan 1988). In a broader sense, resistance can be characterized as any inheritable change that leads to reduced susceptibility of some

individuals of a species (Tabashnik et al., 2014). According to the same author, approximately 546 species of arthropods present changes in susceptibility to some type of pesticide.

In the Brazilian scenario, especially when related to successive crop systems adopted in the Cerrado region, the reports of phytosanitary problems associated to changes in pest susceptibility to control methods have increased the concern with the evolution of resistance in insects, especially in the soybean, maize and cotton crops. The management of pest insects is complicated due to the rapid evolution of insect resistance to insecticides and genetically modified plants expressing Bt proteins (Bt plants), due to the continuous selection process that their populations are exposed (Heckel 2012). Therefore, the development of monitoring tools that allow the identification of susceptibility with accuracy, low cost and short time is necessary, aiming the delay of resistance evolution.

Spodoptera frugiperda has featured in the scenario of insect-pest in Brazil with strong adaptative capacity and resistance to several insecticides compounds (Yu 1991, Yu et al., 2003, Yu and McCord 2007). Resistance of *S. frugiperda* to insecticides was reported for pyrethroids (Diez-Rodriguez and Omoto 2001, Carvalho et al. 2013) and organophosphates (Carvalho et al., 2013), as well as reductions in susceptibility to benzophenylureas insecticides (Schmidt 2002, Nascimento et al 2014) and spinosyn (Golden and M. 2009). Several studies related resistance of insects to insecticides and Cry toxins to mutations on DNA sequences (Gahan et al 2001, Morin et al 2003), though there is still no vast literature associating adaptation of *S. frugiperda* to mutations.

Next-generation sequencing (NGS) technologies have been recently used for whole genome sequencing and for re-sequencing projects where the genomes of several specimens are sequenced to unravel large numbers of single nucleotide polymorphisms (SNPs) to explore within-species diversity, construct haplotype maps and performe genome-wide association studies (Nosil et al. 2012, Karina-Brandão et al. 2015).

The genotyping by sequencing (GBS) (Elshire et al. 2011; Sonah et al. 2013), has been a strong tool to identify the nucleotide diversity. This technology has revolutionized population genetics studies by the huge amount of genetic information that can be easily gathered for non-model genome (Davey et al. 2011). With high number of SNPs it is possible to estimate genetic variation and structure even at a relatively restricted geographic scale (Keller et al. 2012), host strains (Karina-Brandão et al. 2015, Karina-Brandão et al. 2018). Also genotyping-by-sequencing has been widely applied in population genetics studies of insects in recent years (Rasic et al. 2015, Silva-Brandão et al. 2015, Dussex et al. 2016, Lozier et al. 2016, Brunet et al. , Fouet et al. 2017, Ragland et al. 2017, Fritz et al. 2018, Silva-Brandão et al. 2018).

In this study we applied GBS to investigate the genetic variability of resistant strains of *S. frugiperda* to five classes of insecticides most used to its control in Brazil, and to Bt toxins, and of a susceptible lineage kept in laboratory. Our main objective was to identify SNPs putatively under selection and possibly associated to resistance of *S. frugiperda* to insecticides and Bt toxins.

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7. FINAL CONSIDERATIONS

The development of fast and efficient methods to detect the resistance of *S. frugiperda* to insecticidal molecules is crucial to implement Insect Resistance Management (RM) strategies in the field, mainly under tropical agrosystems. This thesis explored next generation sequencing, genotyping by sequencing, SNP calling and functional genomics to address which resistance mechanisms were associated to several insecticides and Bt proteins and establish a set of potential molecular markers to assist monitoring the resistance in the field. The direct link between one marker and the confirmation if an individual is resistant to a certain trait based on molecular technique is still a cherished aspiration, but results presented here will guide scientist on the insect genome, so they know where efforts must be put on.

Literature indicates resistance to pyrethroids and organophosphates associated with mutations in genes coding target sites and/or with modifications in the expression profiles of genes for detoxification enzymes such as cytochrome P450, esterases, and glutathione-S-transferases. On the Chapter 2, we showed that resistance to neurotoxic insecticides, such as lambda cyhalothrion and chlorpyrifos, are associated with overexpression of detoxification enzymes specially from *CYP3* and *CYP6* gene subfamilies.

On the other hand, resistance to teflubenzuron, a chitin-synthesis inhibitor, is more associated to regulatory process, mainly related to regulation of ecdysteroid hormones (ecdysteroid 22-kinase and ecdysone oxidase); and many ABC transport. Detoxification enzymes were also present but not the ones found on lambda cyhalotrin and clorpyrifos resistant strains. Resistance of *S. frugiperda* to teflubenzuron was characterized and cross- resistance to other benzoylureas was establish on Chapter 3, as a comparative transcriptome between teflubenzuron resistant strain and susceptible strain was presented on Chapter 4. Thus, comparing resistance to different groups of insecticides show us that regulatory process and detoxification enzymes are key players on *S. frugiperda*, however these two functional categories have a wide set of genes. We showed that each insecticide triggers a different set of detoxification gene family.

Resistance to Bt plant showed the same basal response to regulatory process and detoxification enzymes, plus cadherin receptors and membrane-associated glycosylated proteins such as aminopeptidase N (APN), alkaline phosphatase (ALP). Chapter 5 punctuates genes and pathways particularly to the resistance to Yieldgard VT-PRO®, hereby results show that resistance against insecticides and Bt plants has its differences and similarities.

Finally, Chapter 6 applied genotyping-by-sequencing protocol to discovery candidate SNPs markers associated with *S. frugiperda* resistant to chlorpyrifos, lambda-cyhalothrin, lufenuron, teflubenzuron and spinosad and to the YieldGard VT-PRO® event maize expressing Ccry1A.105 and Cry2Ab2 proteins. Results indicated a set of 17 loci in common among traits, and several loci specific to each insecticide and Yieldgard VT-PRO®. Summing up, results presented on all chapters put a number on how many molecular markers researchers should work to establish a link between field phenotyping individuals and molecular phenotyping individuals, and which are the most potentially genes, enzymes and regulatory process where these markers should be explored.

This thesis is a step forward on democratizing and strengthening the fields of genomics and transcriptomics to study agricultural pests, since literature using these technologies is still scarce in entomological studies, more specifically in the area of IRM. Although mechanisms of resistance will traditionally be related primarily to detoxification and mutation, research using deep sequencing technologies like ours has the power to open the horizons for identification of new resistance mechanisms, greatly expanding our views on the range of available options to manage insect resistance evolution to insecticides and Bt toxins. Thus, to identify reliable genetic markers and to identify new mechanisms of resistance, it is crucial to integrate methodologies at different molecular, genomic, transcriptional, proteomic, metabolomic and other levels. The increasement of knowledge on regulatory process, transposable elements, expression of specific isoforms, and/or post-transcriptional processes, as well as the collection of information on epigenetic mechanisms will be essential for future knowledge linking molecular studies to the evolution of insect resistance.