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

Piracicaba
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Antonio Rogerio Bezerra do Nascimento
Bachelor in Biological Sciences

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Prof. Dr. **CELSO OMOTO**

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1. Transcritoma 2. Genotipagem por sequenciamento 3. Manejo da resistência de insetos 4. Marcador molecular I. Título

DEDICATION

I would like to dedicate this work to:

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My mon Helena Maria do Nascimento Silva and my brother Manoel Rodrigo Bezerra do Nascimento, the symbol of love and giving, who encouraged and supported me.

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SUMMARY

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; Genotipagem por sequenciamento; 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

FIGURE LIST

TABLE LIST

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.

2.2. Materials and Methods

2.2.1. Insects

The susceptible strain Sf-sus of *S. frugiperda* has been maintained at the Laboratory of Arthropod Resistance to Control Tactics, Department of Entomology and Acarology, Luiz de Queiroz College of Agriculture (Escola Superior de Agricultura “Luiz de Queiroz” - ESALQ/USP), Piracicaba, São Paulo state, Brazil, without selection pressure from insecticides for more than 20 years. Strains resistant to lambda-cyhalothrin and chlorpyrifos were selected from insects collected in maize fields in Paraná (PR) and Bahia (BA) states by using F_2 screening methods (Andow and Alstad 1998). At the laboratory, the armyworms were maintained on an artificial diet based on beans, wheat germ, soy protein, yeast, and casein (Kasten et al. 1978). During development, the armyworms were maintained in a climate-controlled chamber at 25 ± 1 °C, 60 ± 10% RH and 14:10 h (L: D).

2.2.2. F2 screen

A total of 72 and 86 two-parent isofamilies from PR and BA, respectively, were established from the field-collected insects. Bioassays were performed using topical application of 1 μL of insecticide dissolved in acetone, applied on the dorsal pronotum of fourth-instar larvae with an operated micro-applicator. Chlorpyrifos (99% pure, Dow AgroSciences) and lambda-cyhalothrin (87.39% pure, Syngenta) were used. The initial concentrations for selection were 1000 and 56 $\mu g.i.a.ml^{-1}$ of chlorpyrifos and lambda-cyhalothrin, respectively. The armyworm larvae used in the bioassay were maintained in climate chamber at 25 ± 1 °C, 60 ± 10% RH and 14:10 h (L: D) photoperiod. After 24 h the surviving larvae were transferred to the artificial diet to complete their life cycle. These surviving insects were considered the parental resistant strain. In each generation, if 50% or more of the insects survived to adulthood, we conducted another selection procedure with increasing concentrations of the insecticides.

2.2.3. Characterization of *S. frugiperda* resistance to chlorpyrifos and lambda-cyhalothrin

For the toxicological characterization of the susceptible (Sf-ss), chlorpyrifos-resistant (Clo-rr), and lambda-cyhalothrin-resistant (Lam-rr) strains, 8 to 12 logarithmically spaced insecticide concentrations were tested for each strain, with a topical application. The different concentrations were applied to the dorsal pronotum of fourth-instar larvae. The control treatment consisted of acetone only. The bioassay plates containing the larvae were stored in a climate chamber at a temperature of 25 ± 2 °C, relative humidity of 60 ± 10% and photophase of 14 h. The experimental design was completely randomized, with five replicates (12 larvae/replicate), totaling 60 larvae tested per concentration. The mortality was assessed after 24 h. Larvae that showed no perceptible movement after being probed with a brush were considered dead.

The mortality data of the strains were subjected to Probit analysis (Finney 1971), using the software POLO PC (Leora software 1987). The resistance ratio was estimated by dividing the LC_{50} of the resistant strain by the LC_{50} of the susceptible strain. Tests for parallelism and equality of regression constants were conducted.

2.2.4. RNA extraction

The Lam-rr (resistant to lambda-cyhalothrin), Clo-rr (resistant to chlorpyrifos) and Sf-ss (susceptible) strains were used to compare the gene expression profiles between the resistant and susceptible strains. Each RNA library was prepared from 50 mg of tissue from fourth-instar larvae, in three replicates.

A Direct-zol™ RNA mini-prep kit (Zymo Research®, Irvine, CA, USA) was used for the RNA isolation, following the manufacturer's description. Four larvae were placed in 1.5 mL microtubes and 700 μL of Trizol™ Reagent (Invitrogen®, Carlsbad, CA, USA) was added for mechanical maceration of the tissues. The homogenate was centrifuged at 16,000 *g* for 5 min at room temperature (22 °C). The supernatant was transferred to a new microtube and 700 μL of 95% ethanol was added. The solution was transferred to filter columns and centrifuged at 16,000 *g* for 30 s. 400 μL of RNA wash buffer, 5 μL of DNAse I (6 $U.\mu L^{-1}$) and 75 μL of DNA digestion buffer were added to the membrane and the mix was incubated at room temperature for 15 min. Next, 400 μL of Direct-zol™ Rna PreWash (Zymo Research®, Irvine, CA, USA) was added and centrifuged at 16,000 *g* for 30 s. Finally, 700 μL of Wash Buffer RNA was centrifuged at 16,000 *g* for 2 min or until the wash buffer was completely removed, and the samples were resuspended in 50 μL of DNA/RNA-free water and stored in an ultra-freezer at –80 °C until evaluation of concentration and quality.

Total RNA samples were sent for purity and integrity evaluation at the Central Laboratory of High-Performance Technologies in Life Sciences (LACTAD / UNICAMP). Then, the samples were used for library preparation and sequencing on the Illumina Hiseq2500® platform. For the sequencing of the cDNA libraries, the paired-end protocol was used, giving reads of approximately 100 bp.

2.2.5. Alignment and Re-annotation

RNA reads obtained from sequencing on the Illumina HiSeq2500 platform were assessed for quality using FastQC v0.11.5 (Andrews 2010). Illumina adapter sequences and low-quality reads (Phred quality score <20 bp) were trimmed with Trimmomatic v0.36 (Bolger et al. 2014), followed by alignment against the draft *S. frugiperda* reference genome (Gouin et al. 2017), using Bowtie 2 (Langmead and Salzberg 2012).

To maximize the functional identification, the transcripts were annotated using BLASTX search (Altschul et al., 1998), against NR-NCBI (non-redundant). Enzyme

classification (EC) codes and the annotation of metabolic pathways KEGG (Kyoto Encyclopedia of Genes and Genomes) (Kanehisa et al. 2007) were generated with Blast2GO (Conesa et al. 2005) with an E-value cut-off set to 10^{-3}. Gene open reading frames (ORFs) were predicted using Transdecoder v2.0.1, and the ORFs were blasted against the GO (Gene Ontology), EggNOG (Powell et al. 2011), and UniProt databases, with an E-value cut-off set to 10^{-5}.

2.2.6. Identification of DEGs

Differentially expressed gene (DEG) between strains of *S. frugiperda* resistant and susceptible to lambda-cyhalothrin and chlorpyrifos were determined based on expression abundances in the susceptible and each resistant strain. The calculation of relative abundance was obtained by aligning reads against the reference transcriptome, using RSEM v.1.1.17 (Li and Dewey 2011), in order to estimate the expression abundance of genes and isoforms by FPKM (Fragments Per Kilobase of exon per Million mapped fragments).

The differential expression analysis used the DESeq2 package (Love et al. 2014). The data for expression-abundance estimation were normalized using correction factors based on the effective size of the libraries. The candidate transcripts, associated with the resistance mechanisms of *S. frugiperda,* were selected if they showed a significant difference of FDR $\leq$ 0.01 and a fold change > 2. The GO terms of these transcripts were analyzed for statistically significant enrichment, using TopGO for Biological Process, Metabolic Process, and Molecular Process terms.

2.2.7. Variant analysis

Paired-end reads were used to identify Single Nucleotide Polymorphisms (SNPs). The reads were aligned against the transcripts from the reference genome (Gouin et al. 2017) using BWA software (Li and Durbin 2010). Indels were not included, because alternative splicing prevents reliable indel discovery. SNPs were called using the SAMtools (Li 2011) and FreeBayes (Garrison and Marth 2012). Using the vcftools, SNPs were called only for positions with a minimal mapping quality of 20 and coverage of 25. SNPs were annotated using SnpEff (Cingolani et al. 2012).

2.3 Results and Discussion

The results of the concentration-response lines for the insecticides chlorpyrifos and lambda-cyhalothrin are shown in Fig. 1. The selected strains (Clo-rr and Lam-rr) showed a significant difference in their responses compared to the susceptible strain (Sf-ss).

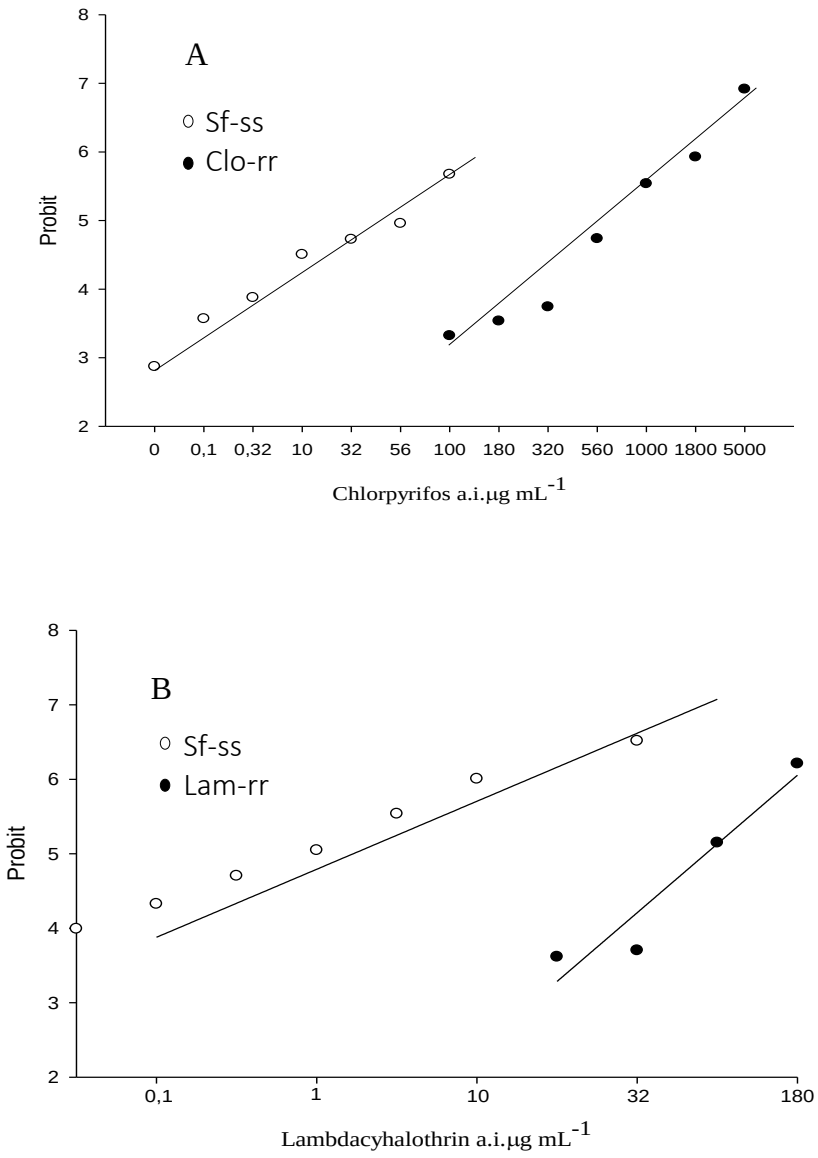


Figure 1. Concentration-probit log of susceptible and resistant strains of *S. frugiperda* to the insecticide chlorpyrifos (A) and lambda cyhalothrin (B)

The mortality response of the chlorpyrifos-resistant strain (Clo-rr) showed an LC_{50} of 854.41 (617.35 – 1236.96) $\mu g.mL^{-1}$, and the susceptible strain (Sf-ss) showed an LC_{50} of 33.64 (8.70 – 71.36) $\mu g.mL^{-1}$ chlorpyrifos, with a resistance ratio of 25.4, which indicates a

moderate level of resistance. The lambda-cyhalothrin-resistant strain Lam-rr showed an LC_{50} of 76.18 (49.34 – 107.21) μg.mL^{-1} and the susceptible strain of 0.35 (0.09 – 0.86) μg.mL^{-1} lambda-cyhalothrin, with high resistance ratio of 217 (Table 1).

Table 1. Concentration – mortality of susceptible and resistant strains of *S. frugiperda* to chlorpyrifos and lambda cyhalothrin.

Strain (insecticide)	**n**	**Slope (±SE)**	**LC_{50} (CI 95%)**	χ^2	**df**	**RR**
Sf-ss (Lambda-cyhalothrin)	468	0,63 ± 0,07	0,35 (0,09 – 0,86)	13,80	5	-
Lam-rr (Lambda-cyhalothrin)	504	2,92 ± 0,23	76,18 (49,34 – 107,21)	8,78	3	217,65
Sf-ss (Chlorpyrifos)	488	1,81 ± 0,28	33,64 (8,70 – 71,36)	5,29	7	-
Clo-rr (Chlorpyrifos)	742	2,05 ± 0,13	854,41 (617,35 – 1236,96)	16,29	5	25,40

1. *LC_{50}values followed by the same letter in the columns do not differ significantly for the confidence intervals (95%). The significance of the confidence intervals was determined by the likelihood ratio test, followed by multiple comparisons.
2. **df = degrees of freedom.
3. ***Resistance ratio (RR) = LC_{50} of resistant strain/LC_{50} of susceptible strain.

Re-annotation of *S. frugiperda* genome

The fourth-instar larvae of the chlorpyrifos- and lambda-cyhalothrin-resistant and susceptible strains of *S. frugiperda* were used for RNA sequencing. Three biological replicates for each strain were used to construct cDNA libraries. Each library was sequenced on the Illumina Hiseq 2500 platform, using paired-end 100 base-pair reads (Appendix A).

Re-annotation of genes from the draft genome increased the number of genes identified (Fig. 2). A total of 19,600 transcripts were annotated using NR-NCBI; 11,902 in KOG; 6,801 in GO; and 6,381 in KEGG. The total number of all annotated transcripts was 19,621, that is, 8,579 more than reported by (Gouin et al. 2017).

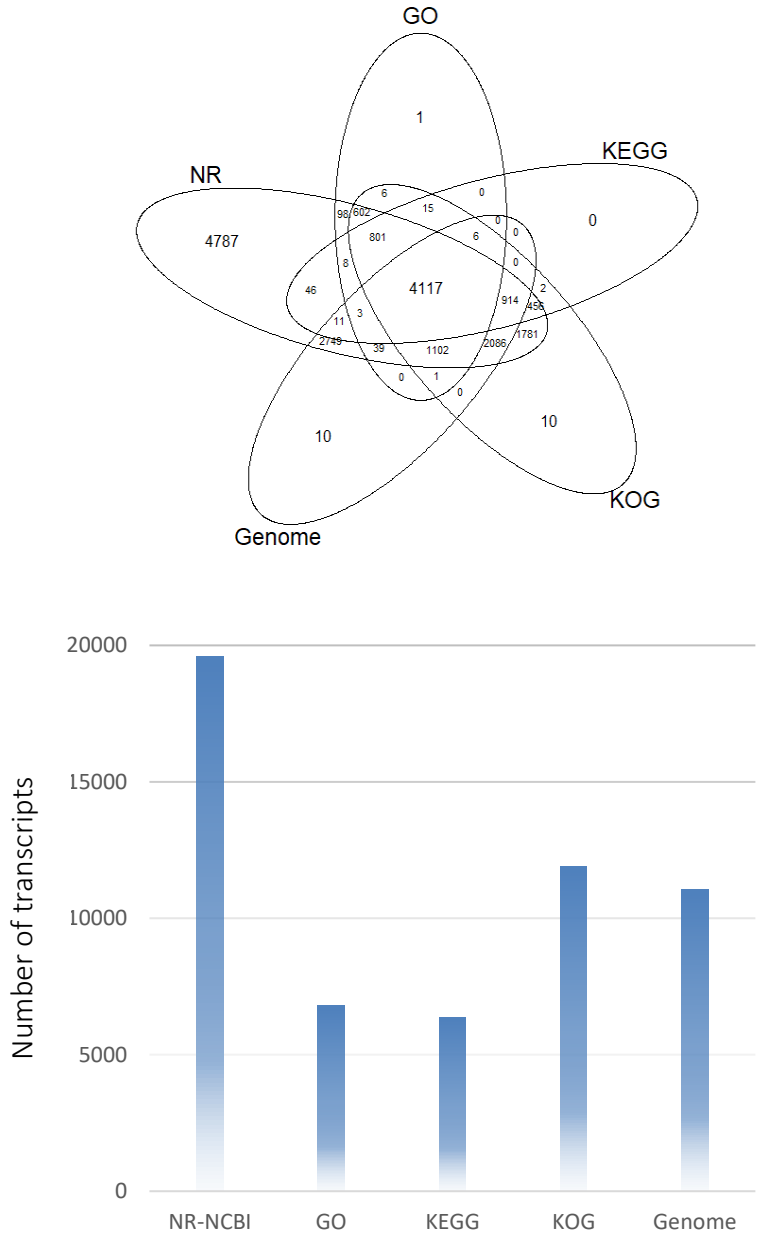


Figure 2. Veen diagram and distribution of re-annotation of the number of transcripts obtained from draft genome of *S. frugiperda*

DEG in chlorpyrifos-resistant and susceptible strains

We performed a comparative analysis between transcriptomes of the chlorpyrifos-resistant and susceptible *S. frugiperda* strains. We identified 935 differentially expressed genes (DEGs) in Clo-rr compared to Sf-ss, which included 511 up-regulated and 424 down-regulated genes (Fig. 3) (Appendix B and D).

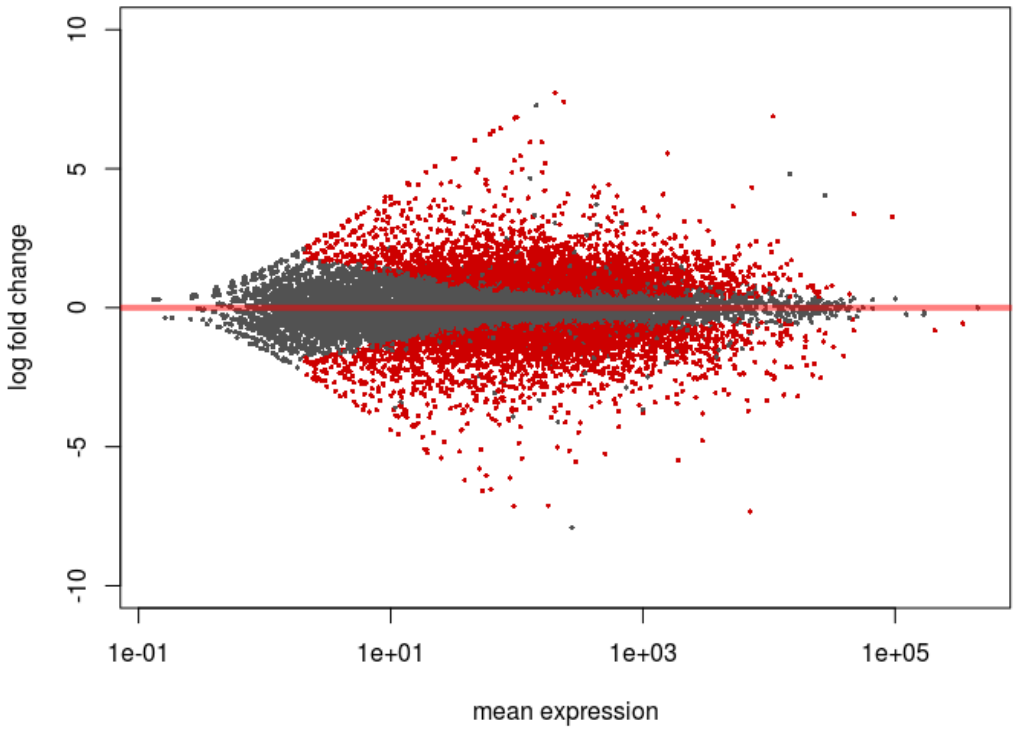
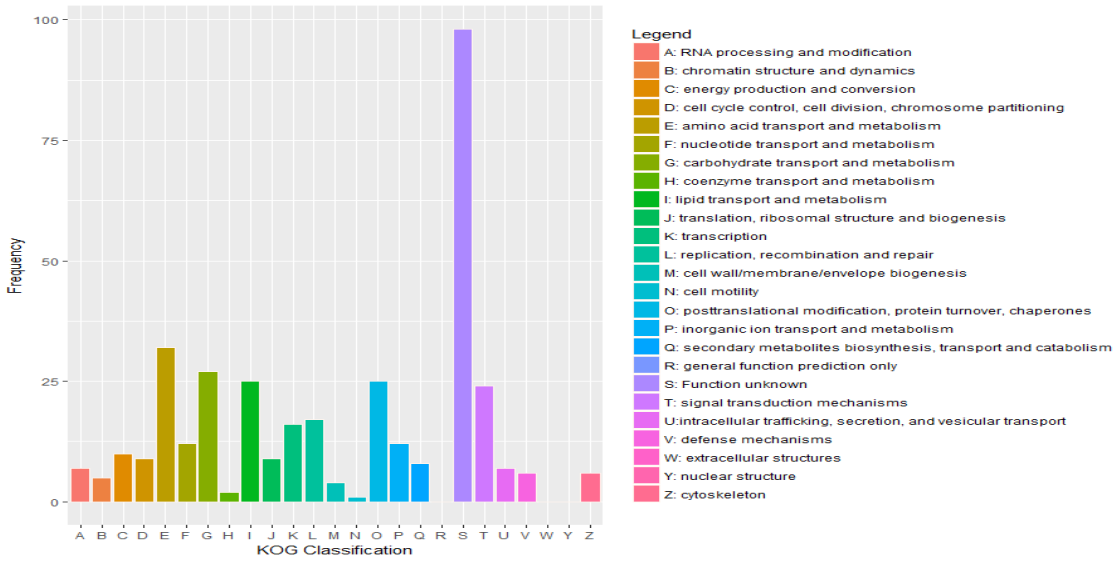


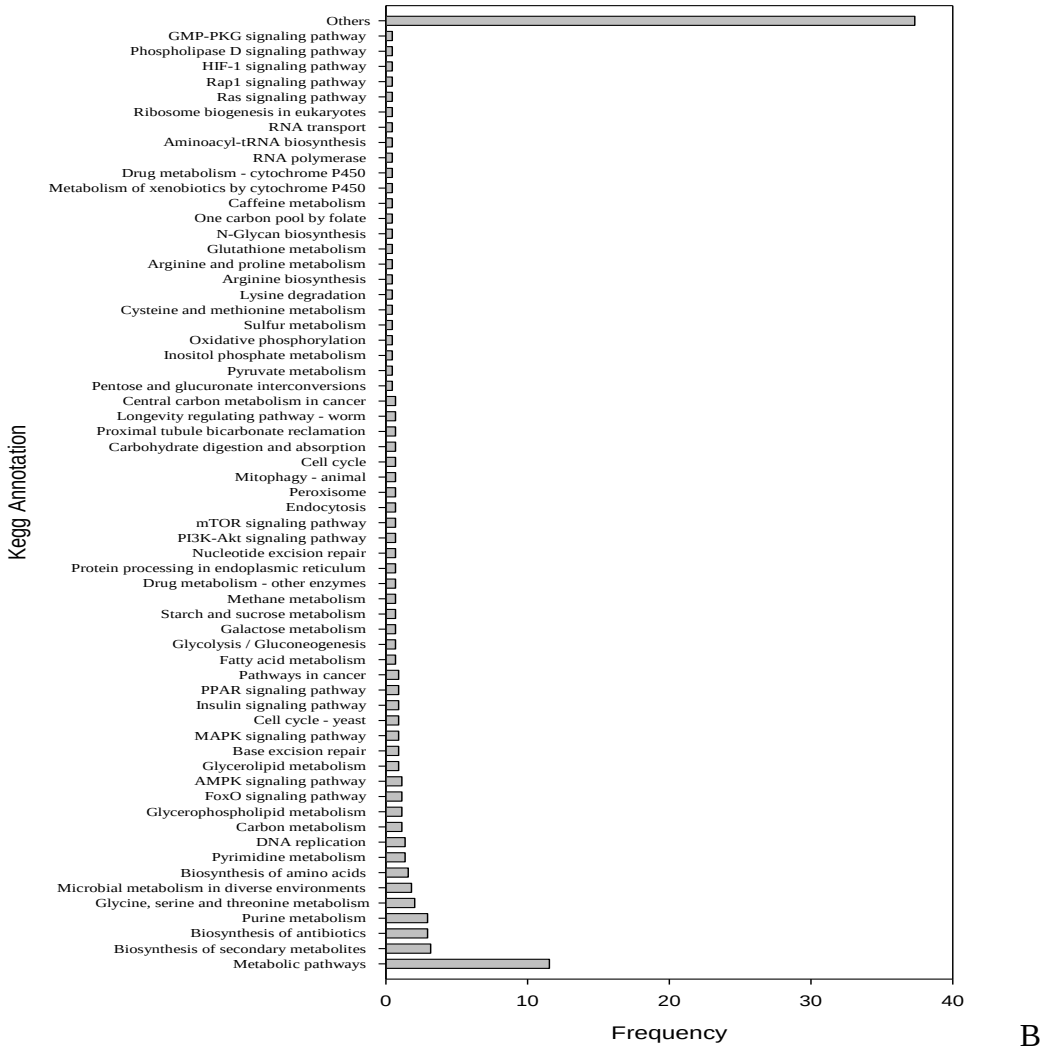
Figure 3. MA plot shows the log2 fold change by the mean of FPKM normalized counts between susceptible and resistant strain of *S. frugiperda* to chlorpyrifos. Red point are genes with adjusted FDR < 0.01

The analysis of function of DEGs in *S. frugiperda* showed that the functions of amino acid, lipid, and carbohydrate transport were frequent in the differentially expressed genes. In addition, functions associated with regulatory processes with post-transcriptional and transduction signals were highly frequent in that set of genes (Fig. 4A). KEGG pathway analysis showed the abundance of metabolism-pathway and biosynthesis of secondary metabolites functions (Fig. 4B).

GO term enrichment showed that the regulation function was significantly present in DEGs of *S. frugiperda* resistant to chlorpyrifos, followed by ion-gated channel activity, insecticide metabolic processes, and transport functions (Table 2).



A



B

Figure 4. Distribution of DEGs between resistant and susceptible strains of *S. frugiperda* to chlorpyrifos annotated by KOG databases (A) and KEGG (B)

Table 2. Ten the best Gene Ontology (GO) enrichment of DEGs between resistant and susceptible strains of *S. frugiperda* to chlorpyrifos

GO.ID	Term	Annotated	Significant	Expected	weight
Celular Component					
GO:0000932	P-body	16	0	0.59	0.0041
GO:0030496	midbody	30	1	1.1	0.0043
GO:0005741	mitochondrial outer membrane	30	0	1.1	0.0045
GO:0000328	fungal-type vacuole lumen	4	0	0.15	0.0055
GO:0044446	intracellular organelle part	2110	70	77.56	0.006
GO:0031514	motile cilium	15	0	0.55	0.0117
GO:0031519	PcG protein complex	18	2	0.66	0.0133
GO:0070451	cell hair	4	0	0.15	0.0135
GO:0008232	activator ecdysone receptor complex	3	0	0.11	0.017
GO:0005940	septin ring	8	0	0.29	0.0171
Mollecular Function					
GO:0022839	ion gated channel activity	64	1	3.11	0.00072
GO:0004843	thiol-dependent ubiquitin-specific prote...	18	0	0.87	0.00074
GO:0004884	ecdysteroid hormone receptor activity	6	0	0.29	0.00212
GO:0019905	syntaxin binding	4	0	0.19	0.00226
GO:0003735	structural constituent of ribosome	172	2	8.36	0.00356
GO:0005344	oxygen transporter activity	7	0	0.34	0.00833
GO:0015299	solute:proton antiporter activity	17	0	0.83	0.00942
GO:0042578	phosphoric ester hydrolase activity	248	8	12.05	0.01091
GO:0031418	L-ascorbic acid binding	7	0	0.34	0.01135
GO:0046920	alpha-(1->3)-fucosyltransferase activity	11	0	0.53	0.01361
Biological Process					
GO:0035076	ecdysone receptor-mediated signaling pat...	9	0	0.42	0.0001
GO:1901069	guanosine-containing compound catabolic ...	88	1	4.09	0.00036
GO:0046039	GTP metabolic process	99	1	4.6	0.00058
GO:0016579	protein deubiquitination	26	0	1.21	0.00064
GO:0007312	oocyte nucleus migration involved in ooc...	9	0	0.42	0.00071
GO:0034765	regulation of ion transmembrane transpor...	72	0	3.35	0.0011
GO:0007349	cellularization	72	1	3.35	0.00126
GO:0006412	translation	428	9	19.89	0.00265
GO:0017143	insecticide metabolic process	12	0	0.56	0.00269
GO:0006464	cellular protein modification process	1154	31	53.63	0.00286

Detoxification of insecticides is an important adaptation of resistant strains to insecticides and toxins. We evaluated the gene-expression profile of four main groups of detoxification enzymes: cytochrome P450 (Fig. 5), glutathione S-transferase (Fig. 6), UDP-glycosyltransferase (Fig. 7), and esterases (Fig. 8), related to resistance of insects to pesticides.

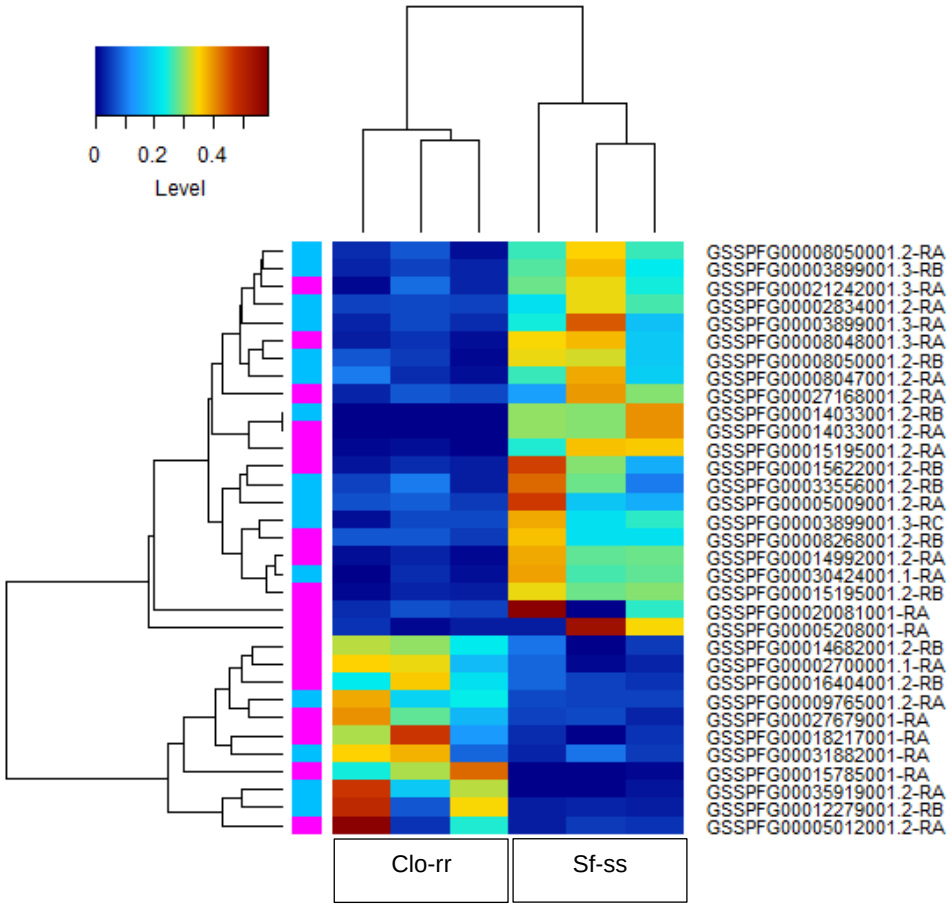


Figure 5. Heatmap of cytochrome P450 that are differentially expressed in susceptible and resistant *S. frugiperda* strains to chlorpyrifos

Some *CYP* gene subfamilies were predominantly overexpressed in the Clo-rr, including *CYP333B3, CYP367A6, CYP340AA1, CYP6AB14*, and *CYP6A2*. Many studies have found a high level of P450 gene expression for insect resistance to insecticides. The *CYP3*, *CYP6*, and *CYP9* gene families mainly participate in the metabolism of xenobiotic compounds (Nelson et al. 1996, Zhang et al. 2016).

Our results suggested that GST and UGT enzymes were not responsible for the resistance of the fall armyworm to this OP. Ten GSTs were differentially expressed between the Sf-ss and Clo-rr strains, but only one gene, associated with glutathione S-transferase epsilon 14, was overexpressed in Clo-rr. The same occurred with UGT: we identified 14 subfamilies of *UGT* genes in DEGs, but only the *UGT39B4* subfamily was overexpressed in Clo-rr.

Our DEG results showed 6 carboxylesterase genes with FDR < 0.01 and a log2 fold change of 2.02 – 3.22 times. Three of these genes (GSSPFG00010082001.3-RA, GSSPFG00020288001.5-RA, GSSPFG00000131001.3-RA) were overexpressed by around

3.22- and 2.96-fold in the Clo-rr strain. This family consists of enzyme hydrolysis esters of short-chain fatty acids. Many studies have associated the high activation of esterases in populations of *Aedes aegypti* (Poupardin et al. 2014) and *Aedes albopictus* (Grigoraki et al. 2015) with resistance to temephos OP.

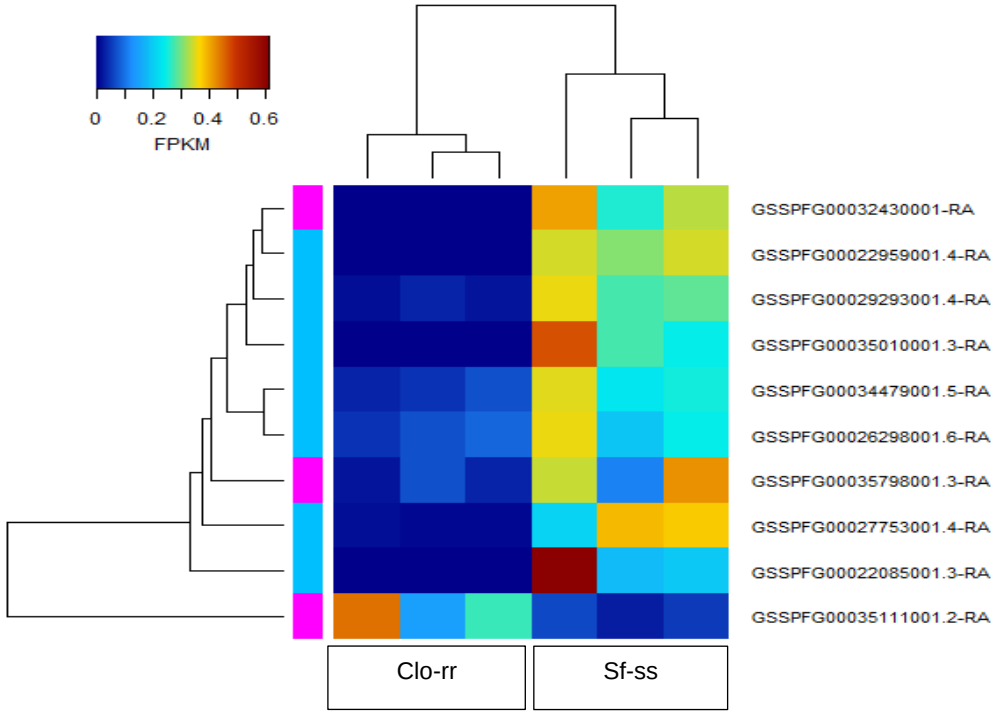


Figure 6. Heatmap of Glutathione S-transferase that are differentially expressed in susceptible and resistant *S. frugiperda* strains to chlorpyrifos

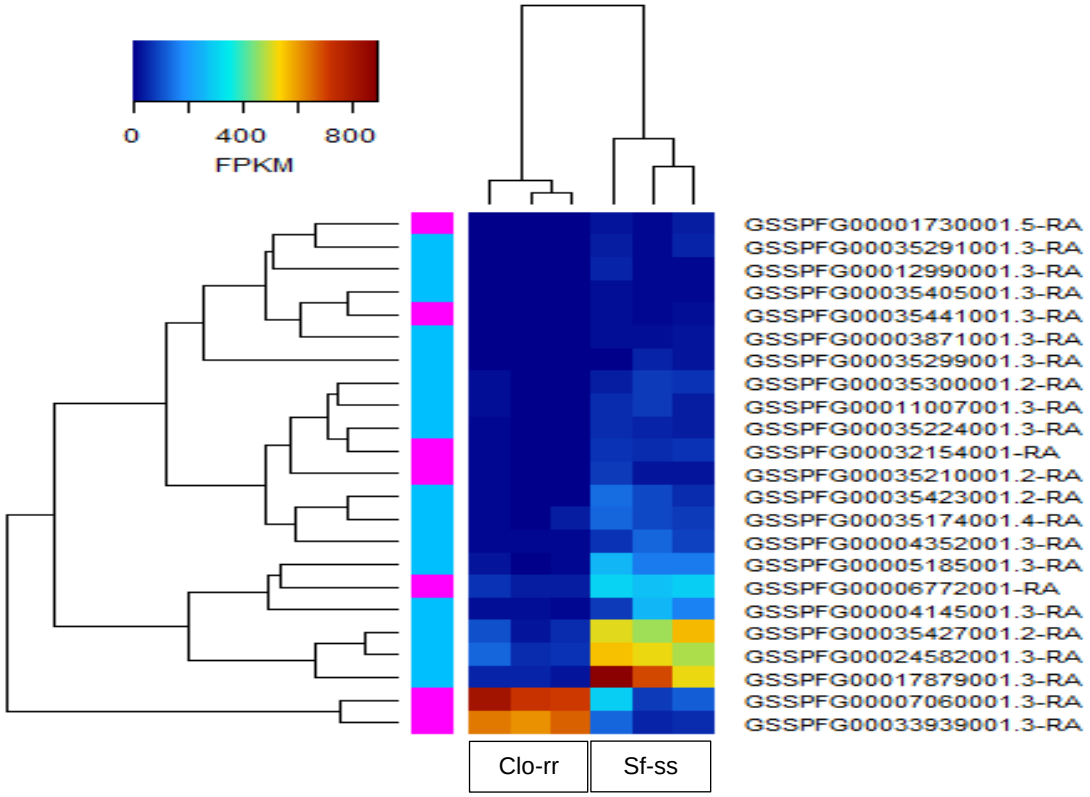


Figure 7. Heatmap of UDP-glicosyltransferase that are differentially expressed in susceptible and resistant *S. frugiperda* strains to chlorpyrifos

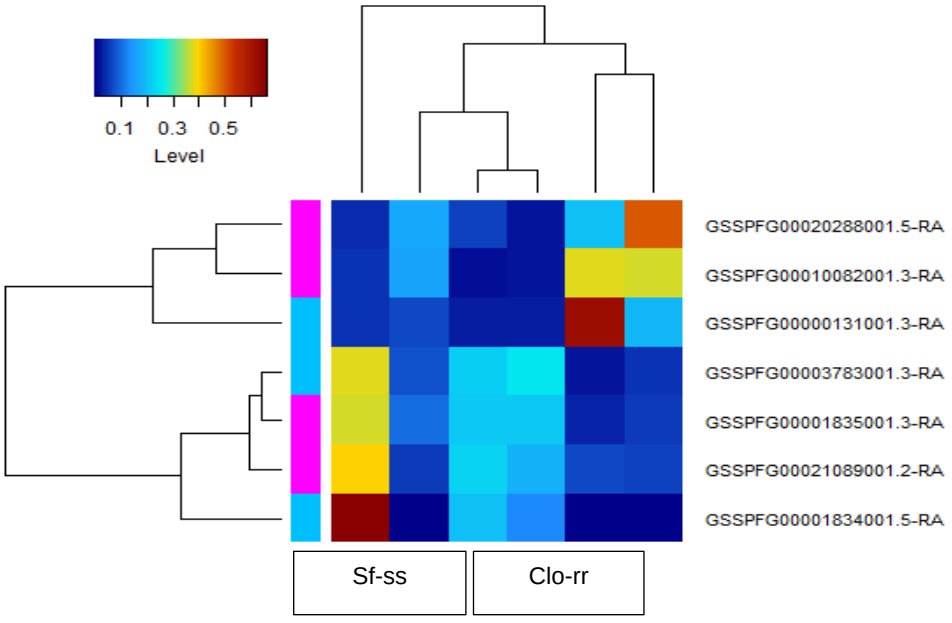


Figure 8. Heatmap of carboxylesterases that are differentially expressed in susceptible and resistant *S. frugiperda* strains to chlorpyrifos

OP resistance has been hypothesized to occur by three mechanisms. The first involves mutations in acetylcholinesterase genes. AChE molecules are targeted by OP

insecticides. This enzyme is responsible for hydrolyzing the neurotransmitter acetylcholine at the nerve synapse; without hydrolyzation of the neurotransmitter, the neurons fire repeatedly, ultimately leading to death by exhaustion (Gunning and Moores 2001). Mutations in AChE reduce the sensitivity to the insecticide. The second suggested mechanism involves a high level of acetylcholinesterase CCE from genetic regulation and/or duplication of CCE responsible for hydrolyzing the insecticide. The third involves structural mutations in certain carboxylesterases that improve their kinetics for insecticide hydrolysis (Oakeshott et al. 2005).

We identified 38 SNPs in acetylcholinesterase genes of *S. frugiperda*; however, all were annotated with synonymous variation. Probably these targets are essential for survival, and therefore only a few conserved point mutations can be tolerated, decreasing insecticide sensitivity while maintaining normal protein function (Misra et al. 2013). However, we also identified 35 non-synonymous variations (Table 3). Several of the SNPs were identified in genes for regulation mechanisms, which were overexpressed in Clo-rr. These mutations can influence the increase in detoxification of xenobiotic compounds in resistant strains. In Phase I and Phase II of detoxification processes, enzymes can be transcriptionally activated in a constitutive manner, through mutations in either cis-acting elements or trans-acting factors, conferring pesticide resistance. Metabolic resistance can also arise due to mutations that increase the catalytic activity of these detoxification enzymes (Misra et al. 2013)

Table 3. Non-synonymous variations found in DEGs between susceptible and resistant strains *of S. frugiperda* to chlorpyrifos

Gene	Position	Reference	Alternative	Aminoacid	Description
GSSPFG00004750001-RA	404	T	C	Ser82Pro	uncharacterized protein LOC110374347 isoform X1
GSSPFG00004750001-RA	421	C	A	His87Gln	uncharacterized protein LOC110374347 isoform X1
GSSPFG00034837001-RA	1228	A	C	Thr410Pro	ankyrin repeat domain-containing protein 29-like
GSSPFG00032735001-RA	432	C	T	Ala88Val	lipase member H-like
GSSPFG00009193001-RA	1408	G	A	Gly470Arg	Retrovirus-related Pol polyprotein from transposon 17.6
GSSPFG00009193001-RA	764	C	G	Thr255Ser	Retrovirus-related Pol polyprotein from transposon 17.6va
GSSPFG00009193001-RA	737	C	T	Ser246Phe	Retrovirus-related Pol polyprotein from transposon 17.6
GSSPFG00009193001-RA	1445	C	G	Ala482Gly	Retrovirus-related Pol polyprotein from transposon 17.6
GSSPFG00009193001-RA	445	A	G	Thr149Ala	Retrovirus-related Pol polyprotein from transposon 17.6
GSSPFG00022404001.1-RA	1024	A	G	Thr342Ala	uncharacterized protein LOC110370032 isoform X1
GSSPFG00031936001-RA	161	A	T	Tyr54Phe	repressed by EFG1 protein 1-like isoform X2
GSSPFG00031936001-RA	710	A	T	Tyr237Phe	repressed by EFG1 protein 1-like isoform X2
GSSPFG00031936001-RA	12	C	G	Phe4Leu	repressed by EFG1 protein 1-like isoform X2
GSSPFG00001505001.1-RA	568	A	T	Thr190Ser	inositol oxygenase-like
GSSPFG00001505001.1-RA	586	A	C	Lys196Gln	inositol oxygenase-like
GSSPFG00001505001.1-RA	94	G	A	Val32Ile	inositol oxygenase-like
GSSPFG00001505001.1-RA	23	C	T	Pro8Leu	inositol oxygenase-like
GSSPFG00016195001-RA	1109	T	G	Tyr9Asp	polyubiquitin-C isoform X1
GSSPFG00007452001.1-RA	920	T	A	Phe307Tyr	carboxypeptidase B-like
GSSPFG00026173001.1-RA	119	C	G	Thr40Ser	peroxidase-like isoform X1
GSSPFG00026173001.1-RA	805	C	T	Pro269Ser	peroxidase-like isoform X1
GSSPFG00026173001.1-RA	2223	T	G	Phe741Leu	peroxidase-like isoform X1
GSSPFG00032739001-RA	952	C	G	Thr284Ser	sequestosome-1-like isoform X1
GSSPFG00032739001-RA	1045	A	C	Gln315Pro	sequestosome-1-like isoform X1
GSSPFG00032739001-RA	804	C	G	Pro235Ala	sequestosome-1-like isoform X1
GSSPFG00001835001.3-RA	1097	G	T	Glu365Asp	carboxylesterase CXE3
GSSPFG00009772001.1-RA	226	G	A	Asp76Asn	Alcohol dehydrogenase
GSSPFG00021966001.1-RA	415	C	A	Leu139Ile	argininosuccinate lyase
GSSPFG00021966001.1-RA	409	G	A	Ala137Thr	argininosuccinate lyase
GSSPFG00021966001.1-RA	1216	G	A	Val406Ile	argininosuccinate lyase
GSSPFG00013620001-RA	112	C	T	Ser3Phe	nose resistant to fluoxetine 6-like
GSSPFG00030424001.1-RA	1246	C	G	Pro416Ala	cytochrome P450 6k1-like
GSSPFG00032463001-RA	766	A	G	Ile256Val	UPF0704 protein C6orf165 homolog

DEG in lambda cyhalothrin-resistant and susceptible strains

Comparative analysis between the transcriptomes of the lambda cyhalothrin-resistant and susceptible *S. frugiperda* strains with FDR < 0.01 and log2 fold change > 2 showed 241

differentially expressed genes (Fig. 9 and Appendix C and D), including 139 up-regulated and 102 down-regulated genes in Lam-rr.

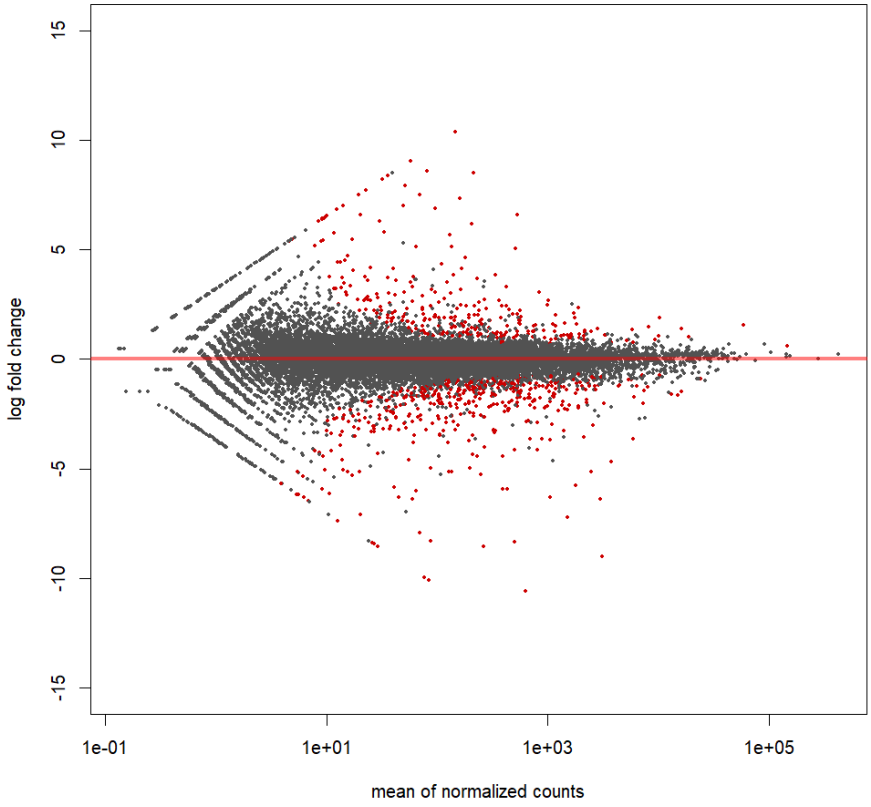
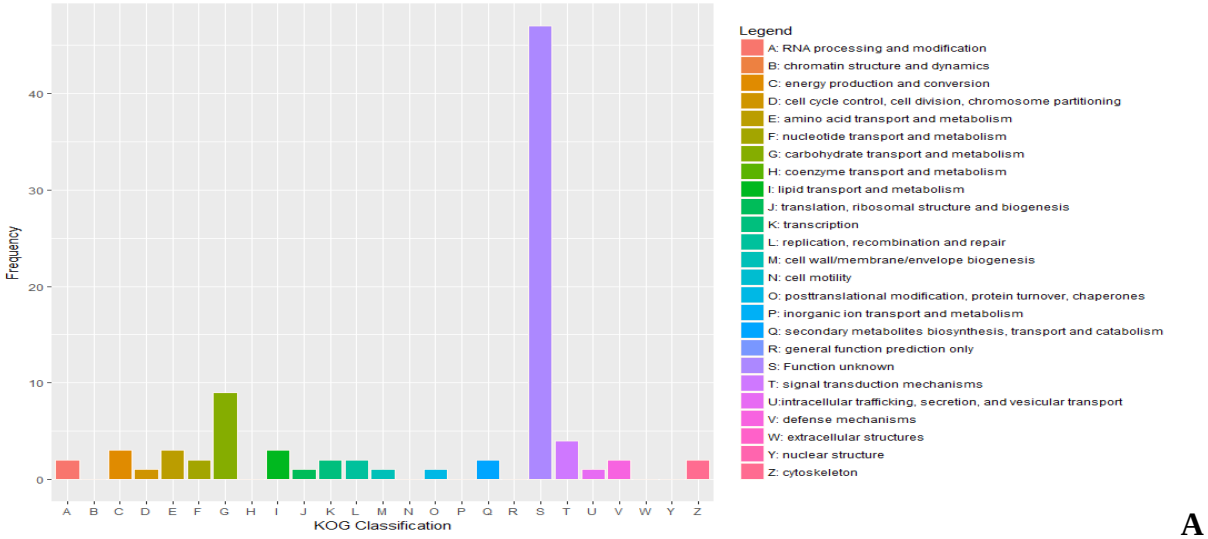


Figure 9. MA plot shows the log2 fold changes by mean of FPKM normalized counts between susceptible and resistant strain of *S. frugiperda* to Lambda cihalothrin. Red point are genes with adjusted FDR < 0.01

The Eukaryotic Orthologous Groups annotation indicated that around 10% of DE genes were related to metabolism and carbohydrate transport. The categories of signal-transduction mechanism, energy production and conversion, lipid transport, and metabolism comprised less than 5% of the genes (Fig. 10).

The cythocrome P450 subfamilies *CYP6AE44*, *CY321A8*, *CYP321A10*, and *CYP321A7* were overexpressed in the Lam-rr strain, showing log2 fold changes of 2.01 – 3.61 times (Fig. 11A). Although present in the Clo-rr strain, *CYP3* and *CYP6* also showed a relationship to the resistance of *S. frugiperda* to pyrethroids. In mosquitoes, when there is high selection pressure caused by pyrethroids, the overexpression of these families is reported in strains resistant to permethrin (Djouaka et al. 2008; Müller et al. 2008) and deltamethrin (Stevenson et al. 2011). Recently, a study described the relationship of a high frequency of subfamily *CYP*3 to failures of deltamethrin and fenvalerate to control *Helicoverpa armigera* (Hübner) in Brazil (Durigan et al. 2017); and another study found overpression of *CPY6* in

Australian populations (Ranasinghe et al. 1998). Although several studies have demonstrated the importance of *Kdr* mutations for the resistance of insects to pyrethroids, our results emphasize the possible efficiency of the P450 families *CYP3* and *CYP6* in conferring insect resistance to pyrethroids, and in the future these may serve as molecular markers to monitor the evolution of resistance to this insecticide.



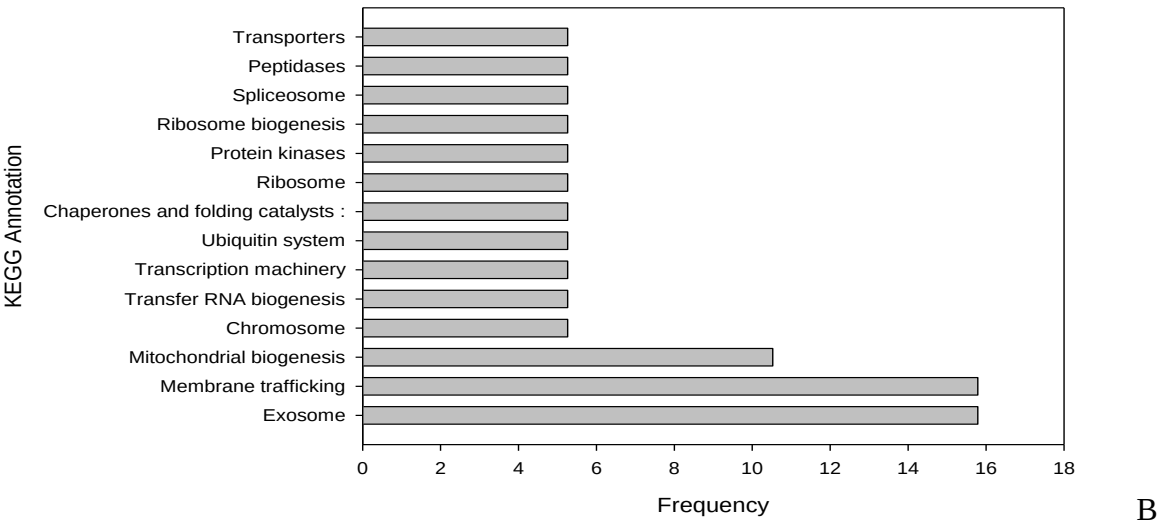


Figure 10. Distribution of DEGs between resistant and susceptible strains of S. frugiperda to lambda cyhalothrin annotated by KOG databases (A) and KEGG (B)

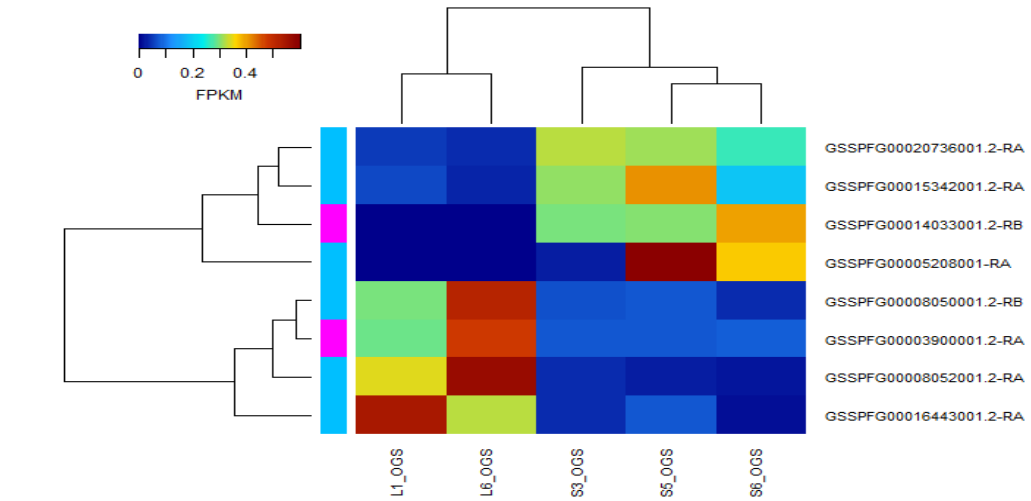
We identified four glutathione S-transferase genes in differentially expressed genes (Fig. 11B). The glutathione S-transferase epsilon 9 (GSSPFG00026804001.5-RA) and glutathione S-transferase sigma 2 (GSSPFG00003077001.5-RA) were overexpressed in Lam-rr. Studies have reported the overexpression of glutathione S-transferases épsilon and sigma

associated with detoxification of endogenous compounds and pesticides (Reddy et al. 2011, Li et al. 2015)

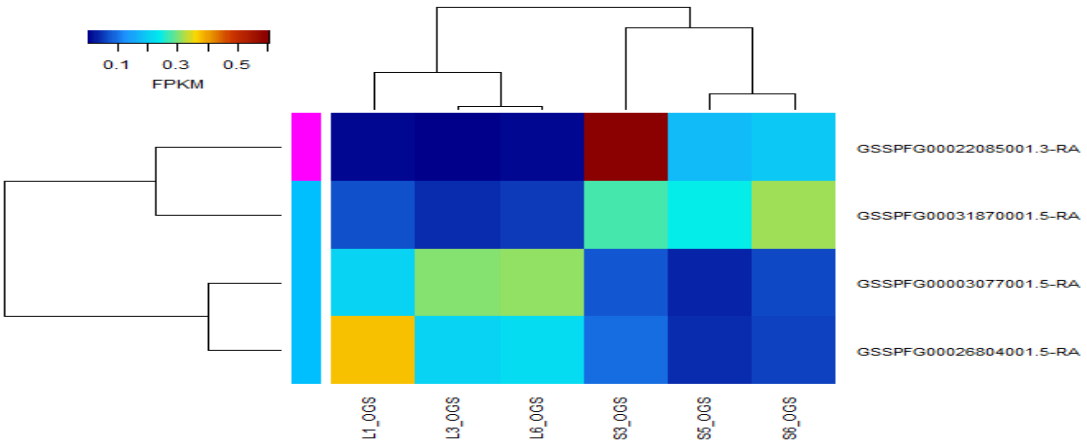
Table 4. Ten the best Gene Ontology (GO) enrichment of DEGs between resistant and susceptible strains *of S. frugiperda* to lambda cihalothrin

GO.ID	Term	Annotated	Significant	Expected	weight
Celular Component					
GO:0030496	midbody	30	0	0.17	0.0049
GO:0000932	P-body	16	0	0.09	0.0056
GO:0000328	fungal-type vacuole lumen	4	0	0.02	0.006
GO:0005741	mitochondrial outer membrane	30	0	0.17	0.0069
GO:0005730	nucleolus	104	1	0.57	0.0074
GO:0044446	intracellular organelle part	2110	8	11.66	0.008
GO:0031514	motile cilium	15	0	0.08	0.014
GO:0070451	cell hair	4	0	0.02	0.0151
GO:0031519	PcG protein complex	18	0	0.1	0.016
GO:0005871	kinesin complex	41	0	0.23	0.0172
Mollecular Function					
GO:0022839	ion gated channel activity	64	0	0.68	0.00078
GO:0004843	thiol-dependent ubiquitin-specific prote...	18	0	0.19	0.00124
GO:0019905	syntaxin binding	4	0	0.04	0.00246
GO:0004884	ecdysteroid hormone receptor activity	6	0	0.06	0.00267
GO:0004558	alpha-1,4-glucosidase activity	20	0	0.21	0.00385
GO:0005344	oxygen transporter activity	7	0	0.07	0.01025
GO:0042578	phosphoric ester hydrolase activity	248	2	2.62	0.01144
GO:0015299	solute:proton antiporter activity	17	0	0.18	0.01167
GO:0031418	L-ascorbic acid binding	7	0	0.07	0.01279
GO:0003735	structural constituent of ribosome	172	2	1.82	0.01417
Biological Process					
GO:0035076	ecdysone receptor-mediated signaling pat	9	0	0.09	0.00014
GO:1901069	guanosine-containing compound catabolic	88	0	0.87	0.00045
GO:0046039	GTP metabolic process	99	0	0.98	0.00072
GO:0016579	protein deubiquitination	26	0	0.26	0.00098
GO:0007312	oocyte nucleus migration involved in ooc...	9	0	0.09	0.00101
GO:0046580	negative regulation of Ras protein signa...	16	0	0.16	0.00141
GO:0017143	insecticide metabolic process	12	0	0.12	0.00269
GO:0007349	cellularization	72	0	0.71	0.00282
GO:0006259	DNA metabolic process	505	16	5	0.00293
GO:0031503	protein complex localization	22	0	0.22	0.00294

A



B



C

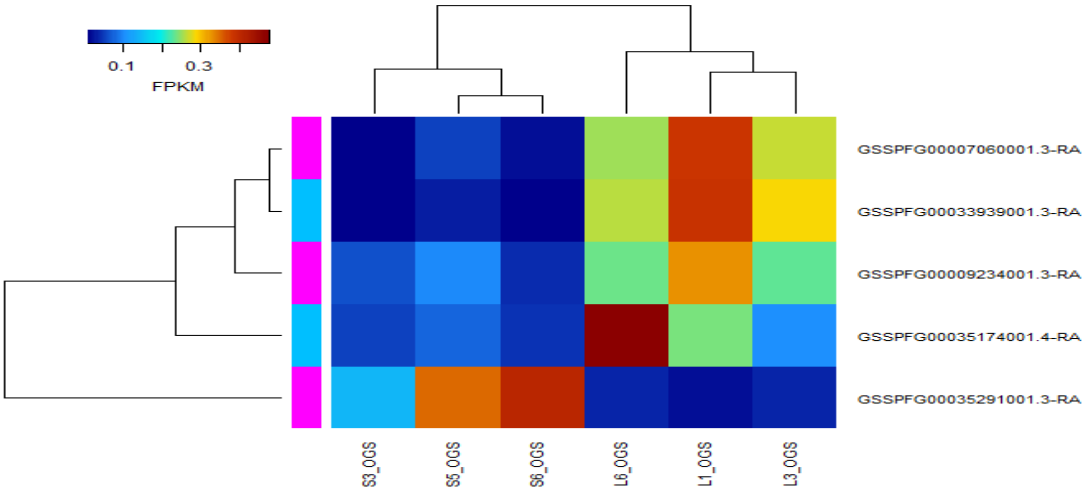


Figure 11. Heatmap of cytochrome P450 (A) Glutathione S-transferase (B) and UDP-glycosyltransferase (C) that are differentially expressed in susceptible and resistant *S. frugiperda* strains lambda cyhalothrin

The overexpression of UDP-glycosyltransferase genes showed a close relationship to the resistance of *S. frugiperda* to lambda cyhalothrin (Fig. 11C). We identified four genes that were up-regulated in the resistant strain: the UDP-glycosyltransferase subfamilies *UGT2B7* (GSSPFG00009234001.3-RA), *UGT39B4* (GSSPFG00033939001.3-RA, GSSPFG00007060001.3-RA), and UDP-glucuronosyltransferase 1-6-like (GSSPFG00035174001.4-RA).

We identified 5 SNPs on DEGs of *S. frugiperda* with non-synonymous variation. All SNPs were related to detoxification enzymes. These results strengthen our hypothesis that cytochrome P450 and UGTs, mainly *CYP3and UGT39B4*, may have strong relation with the resistance of *S. frugiperda* to lambda cihalothrin.

Table 5. Non-synonymous variations found in DEGs between susceptible and resistant strains of *S. frugiperda* to lambda cihalothrin

Gene	Position	Refence	Alternative	Amino	Description
GSSPFG00008052001.2-RA	478	G	A	Glu160Ly	cytochrome P450 CYP321A10
GSSPFG00007060001.3-RA	589	T	G	Phe197Val	UDP-glycosyltransferase 39B4
GSSPFG00035376001.5-RA	208	G	C	Val70Leu	microsomal glutathione transferase
GSSPFG00035376001.5-RA	14	T	C	Val5Ala	microsomal glutathione transferase
GSSPFG00035174001.4-RA	123	T	A	Asp41Glu	UDP-glucuronosyltransferase 1-6-like

Data generated in this study showed 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, presented overexpression of detoxification enzymes, mainly the *CYP3* and *CYP6* gene subfamilies, and genes associated with regulation processes. Our results demonstrated that resistance to chlorpyrifos and lambda-cyhalothrin may be related to detoxification processes.

2.3. Conclusions

- None synominous mutations was identified in acetylcholinesterases;
- Twenty-two cytochrome P450 genes was overexpressed in resistant strain to chlorpyrifos;
- Four cytochromes P450, two UDP-glucuronosyltransferase and four glutathione transferases were overexpressed in strain resistant to lambda-cyhalothrin.

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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.

3.1. Material and Methods

3.1.1. Insects

The susceptible *S. frugiperda* strain (Sf-ss) has been maintained in the Arthropod Resistance Laboratory (ESALQ, Brazil) without insecticides for more than 20 years. The resistant strain (Tef-rr) was selected from field-collected larvae feeding on maize in Mato Grosso state, Brazil, during the 2014–2015 crop season.

3.1.2. Insecticides

Commercial formulations of insecticides used in the bioassays were Teflubenzuron (Nomolt® 150, teflubenzuron 150 g/L, Basf S.A., São Paulo, Brazil), lufenuron (Match®, 50 g/L, Syngenta, Basel, Switzerland) and novaluron (Rimon Supra®, 100 g/L, Syngenta, Basel, Switzerland).

3.1.3. Selection of teflubenzuron-resistant *S. frugiperda* strain

A strain of *S. frugiperda* resistant to teflubenzuron was selected from a parental population composed of approximately 1000 larvae collected in the field. Selection followed the F2 screen method (Andow and Alstad 1998). The procedure was begun by isolating a mating couple from the parental population (P). The progeny of each couple is considered an isofamily line (F1). Finally, progeny from sib-mating in the F1 generation, the F2 generation, is used in bioassays. The surface-treatment diet was used to select the Tef-rr strain. The artificial diet based on bean, wheat germ, and casein proposed by Kasten et al. (1978) was poured into 24-well acrylic plates (Costar®, Corming®). Teflubenzuron was diluted in water, followed by addition of Triton X-100 surfactant at 0.1%, and a volume of 30 µL/well was applied on the surface of the diet. The control was distilled water with surfactant applied to the diet. Third-instar larvae of *S. frugiperda* were placed individually in each well after the diet surface had dried. Larvae fed on the treated substrate for five days, in a climate-controlled chamber at temperature 25 ± 2 °C, relative humidity 60 ± 10% and photophase of 14 h. After five days, surviving *S. frugiperda* larvae were collected and transferred to plastic cups (100 mL) containing 50 mL of the artificial diet and sealed with a round acrylic plate until pupation.

3.1.4. Characterization of *S. frugiperda* resistance to teflubenzuron

Toxicological characterization of *S. frugiperda* strains was done with 5 to 12 logarithmically spaced teflubenzuron concentrations. The different concentrations were applied to the diet surface with the aid of an automatic micropipette (30 µL per well). The control treatment consisted of distilled water and surfactant only. Each well was infested with one early third-instar *S. frugiperda* larva. The bioassay plates were stored in a climate-

comtrolled chamber at a temperature of 25 ± 2 °C, relative humidity of 60 ± 10% and photophase of 14 h. The mortality was assessed after five days.

The mortality data for the susceptible and resistant strains were subjected to Probit analysis (Finney 1949, 1971). The resistance ratio of the strains was estimated by dividing the LC_{50} of the Tef-rr strain by the LC_{50} of the Sf-ss strain. Tests were conducted for parallelism and equality of regression constants.

3.1.5. Genetic crosses

After the adults emerged, reciprocal crosses of ♂ Tef-rr × ♀ Sf-ss and ♀ Tef-rr × ♂ Sf-ss were performed. The adults were kept in cylindrical PVC cages (20 cm height × 15 cm diameter) that were covered inside with paper (oviposition substrate) and closed at the top with Petri dishes. Approximately ten couples were paired in each cage (20 adults in total). The adults were provided a 10% honey solution, which was replaced every 48 h. The progeny of the reciprocal crosses (F_1 generation) were reared on an artificial diet (Kasten et al. 1978) until the third larval instar. Subsequently, third-instar larvae from the ♂Tef-rr and ♀Sf-ss parental strains and the heterozygotes from the reciprocal crosses were subjected to bioassays using the ingestion method, in which the surface of the artificial diet was treated with different concentrations of teflubenzuron to determine the concentration-response curves, according to the methodology described above.

The dominance level was obtained from equation [1] (Bourguet et al. 2000), where $M_{SS,}M_{RR}$ and M_{RS} are the mortalities of the Sf-ss, Tef-rr and heterozygous strains, respectively, exposed to different concentrations of teflubenzuron. D values close to zero (D = 0) represent completely recessive inheritance, and values close to 1 (D = 1) represent completely dominant resistance. Also, dominance level was estimated by Stone (1968) [2].

Equation [1]

$$D = \frac{M_{RS} - M_{SS}}{M_{RR} - M_{SS}}$$

Equation [2]

$$D = \frac{(2X_F - X_R - X_S)}{(X_R - X_S)}$$

3.1.6. Number of genes associated with teflubenzuron resistance in *S. frugiperda*

The method proposed by Tsukamoto (1983) and Roush and Daly (1990), was used to calculate the number of genes associated with teflubenzuron resistance in *S. frugiperda*. The F_1 strains (heterozygous) were backcrossed with individuals from the resistant strain Tef-rr. The bioassays were performed following the same procedures described above.

The possibility of monogenic inheritance was calculated using the chi-squared test [3] (Sokal and Rohlf, 1995).

Equantion [3]

$$\chi^2 = \frac{(Ni - pni)^2}{pqni}$$

Equantion [4]

$$p = \frac{a+b}{2}$$

Where Ni is the mortality observed at concentration i; p is the expected mortality calculated from the Mendelian model (Georghiou 1969)[4]. Where is the mortality in the parental strain used, is the mortality of the heterozygote derived from the reciprocal crosses, ni is the number of individual tested and $q = 1 - p$.

The hypothesis of monogenic inheritance is rejected when the calculated chi-square ≥ tabulated chi-square value, with 1 degree of freedom.

3.1.7. Cross-resistance

The same surface-treatment diet bioassay method as above was used to determine the toxicity of the various insecticides. The mortality of the Tef-rr strain was assessed four days after treatment for teflubenzuron, lufenuron, and novaluron. Those larvae that did not respond to stimulation with a fine brush, or had deformed bodies were considered to be dead. LC_{50} values were estimated using the POLO software (LeOra Software, Petaluma, CA, USA).

3.1.8. DNA Extraction

Extraction of DNA was performed with the modified CTAB method (Doyle and Doyle 1990). Briefly, 50 mg of tissue from individual *S. frugiperda* larvae was macerated in 650 µL of extraction buffer containing 2% Cetyltrimethyl ammonium bromide (CTAB), 1.4 M NaCl, 100 mM tris(hydroxymethyl)aminomethane (Tris-HCl) at pH 8.0, 20 mM ethylenediaminetetraaceticacid (EDTA) at pH 8.0, 1% polyvinylpyrrolidone, 0.2% β-mercaptoethanol, and 20 µL proteinase K (0.1 µg·mL^{-1}). Macerated samples were incubated at 55 °C for 1 h, followed by addition of 650 µL of chloroform:isoamyl alcohol (24:1) and mixed until emulsion. Samples were centrifuged (14,000 *g* × 5 min × 4 °C) and then the supernatant was collected. We then added 200 µL of the same extraction buffer, including β-mercaptoethanol proteinase K and one volume of chloroform:isoamyl alcohol (24:1). The emulsion was thoroughly vortexed, centrifuged (14,000 *g* × 5 min × 4 °C), and the supernatant collected; this process was repeated 3 times. Samples were combined with 650 µL of cold isopropanol and incubated at –20 °C overnight before centrifugation (14,000 *g* × 5 min × 4 °C). The DNA was washed with 1 mL of 70% ethanol twice. The pellet was dried at room temperature and resuspended in 40 µL TE and Rnase A (10 µg.m L^{-1}) and stored at –20 °C until further analysis.

3.1.9. WGS Library preparation

We sequenced eight pools of DNA: Parental strains (susceptible and resistant) and offspring from both cohorts (Supplemental Material 1). Each pool contained nine insects. Whole genomes were sequenced using Miseq (Illumina, Inc., San Diego, CA, USA) at the Molecular and Cellular Imaging Center at the Ohio State University. Briefly, the DNA was sheared into ~300–400 bp fragments in an ultrasonicator and used to create sequencing libraries with an NEBNext Ultra DNA library prep kit (New England Biolabs) according to the manufacturer's instructions. Each pool was individually barcoded.

3.1.10. SNP calling

Qualities of short reads were evaluated using FastQC (Andrews 2010) and filtered using BBmap software (http://jgi.doe.gov/data-and-tools/bbtools). Nucleotides with a Phred quality score < 30 were excluded from the subsequent analyses.

To identify SNPs, we aligned the paired-end reads obtained from the eight DNA pools with the reference genome available (INRA, INRIA, IRISA, GenOuest), using Bwa software (Li and Durbin 2010). Alignment files were converted to SAM/BAM files using SAMtools (Li 2011). We then used Picard (https://broadinstitute.github.io/picard/) to optimize the BAM files and remove duplicate reads. SNP calling was performed by applying freebayes (https://arxiv.org/abs/1207.3907) (Garrison et al. 2012). The programs Vcftools (Danecek et al. 2011; Danecek et al. 2011) and vcffilter (Müller et al. 2017) were used to filter only SNPs with a quality score > 20 and depth > 10.

3.1.11. Estimating population genetic parameters

Nucleotide diversity was estimated for each SNP within strains, using PoPoopulation (Kofler et al. 2011a). Fst calculations and an exact test were performed for all variations across the Tef-rr and Sf-rr genomes, using PoPoolation2 software (Kofler et al. 2011b). The heterozygosity was evaluated by the method proposed by (Bunin 2000). The set of SNPs that showed an FST $\geq$ 0.15 and a *p*-value by exact test $\leq$ 0.01 was annotated using SnpEff (Cingolani et al. 2012).

3.2. Results

3.2.1. Characterization of teflubenzuron resistance of *S. frugiperda*

We tested 33 isofamilies with the F2 screening method, with a diagnostic concentration of teflubenzuron of 10 µg.mL^{-1}. We identified 11 positive families with evidence of resistance, and further selected them to establish the teflubenzuron-resistant strain (Tef-rr). The success of the selection can be observed from the concentration-response curve of the teflubenzuron-resistant strains (Fig. 12). The LC_{50} values were 0.47 (0.35 – 0.63) and 641.47 (213.05 – 2748.81) µg.mL^{-1} for Sf-ss (susceptible) and Tef-rr (resistant) respectively, which provided a resistance ratio of ≈ 1,365 fold. Both Sf-ss and Tef-rr showed no evidence of distortion at $\chi^2 > 0.01$ ($P = 0.02$) and ($P = 0.03$) respectively (Table 6), indicating a good fit to the probit model.

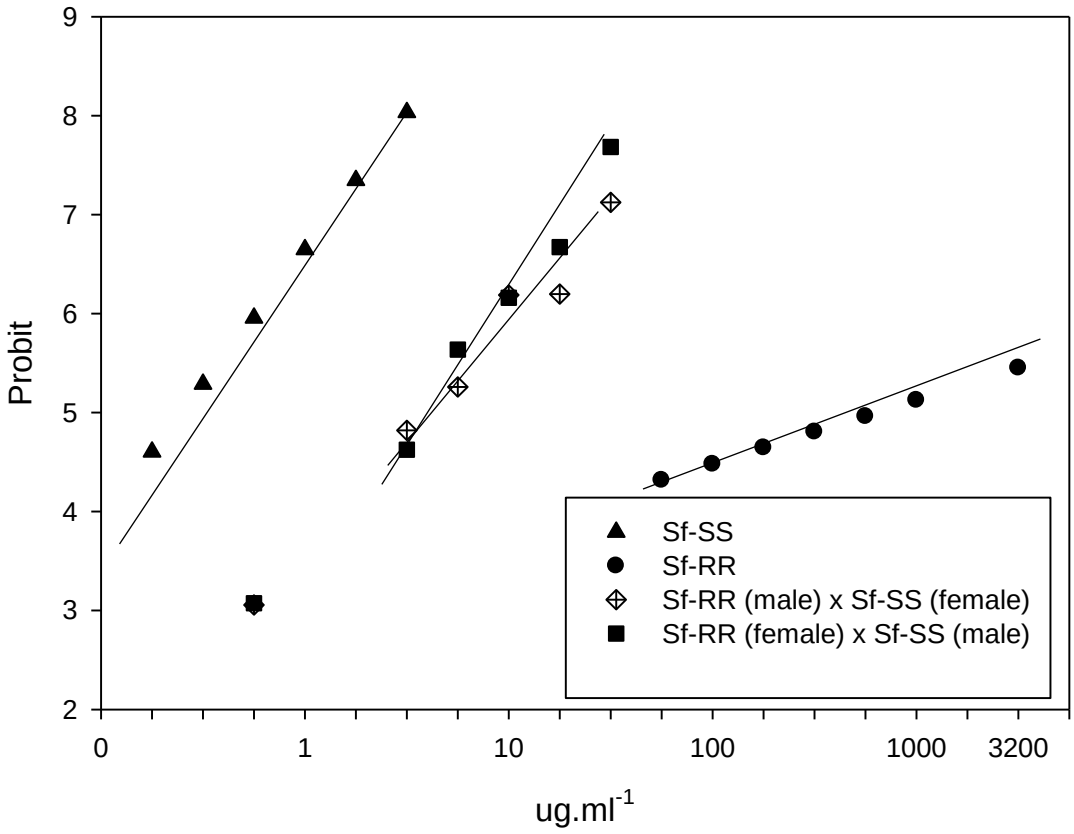

Figure 12. Log concentration–probit of susceptible (Sf-ss) and resistant (Tef-rr) *S. frugiperda* strains and of progenies of reciprocal crosses between susceptible and resistant strains

Table 6. Concentration – mortality to teflubenzuron of susceptible (Sf-ss) and resistant (Tef-rr) *S. frugiperda* strains and progenies of reciprocal crosses between Sf-ss and Tef-rr strains

Strains	n	Slope±SE	LC_{50}(95% CI) * (µg AI mL^{-1})	χ^2	Df**	RR***
Sf-ss	715	3.07 ± 0.24	0.47(0.35 – 0.63)[a]	11.630	4	-
Tef-rr	840	0.64 ± 0.09	641.47 (213.05 – 2748.81)[c]	10.590	4	1,364.83
Tef-rr♂ vs Sf-ss♀	936	2.05 ± 0.14	4.88 (3.58 – 6.31)[b]	8.054	5	10.38
Tef-rr ♀ vs Sf-ss ♂	983	2.29 ± 0.17	3.94 (3.13 – 4.78)[b]	7.604	6	8.38

4. *LC_{50}values followed by the same letter in the columns do not differ significantly for the confidence intervals (95%). The significance of the confidence intervals was determined by the likelihood ratio test, followed by multiple comparisons.
5. **df = degrees of freedom.
6. ***Resistance ratio (RR) = LC_{50} of resistant strain/LC_{50} of susceptible strain.

3.2.2. Inheritance of resistance

Bioassays from the two reciprocal crosses showed no significant differences, since there was an overlap of 95% CI of the LC_{50} values (Table 6). The LC_{50} values were 4.88 (3.58 – 6.31) and 3.94 (3.13 – 4.78) with offspring from crosses ♂ Tef-rr × ♀ Sf-ss and ♀ Tef-rr × ♂ Sf-ss, respectively. Therefore, the hypothesis of parallelism was not rejected (P = 0.247, df = 1). The overlap of the confidence intervals indicated that inheritance of teflubenzuron resistance of *S. frugiperda* is autosomal, and not related to maternal effects or sex linkage.

The Stone's (1968) method to estimate dominance values for the reciprocal crosses of the offspring (Tef-rr♂ × Sf-ss♀ and Tef-rr♀ × Sf-ss♂) were 0.32 and 0.29, respectively. The dominance level evaluated using the Bourguet-Genissel-Raymond method showed a decrease in dominance level with increasing teflubenzuron concentration (Fig. 13). Both results indicated an incompletely recessive inheritance.

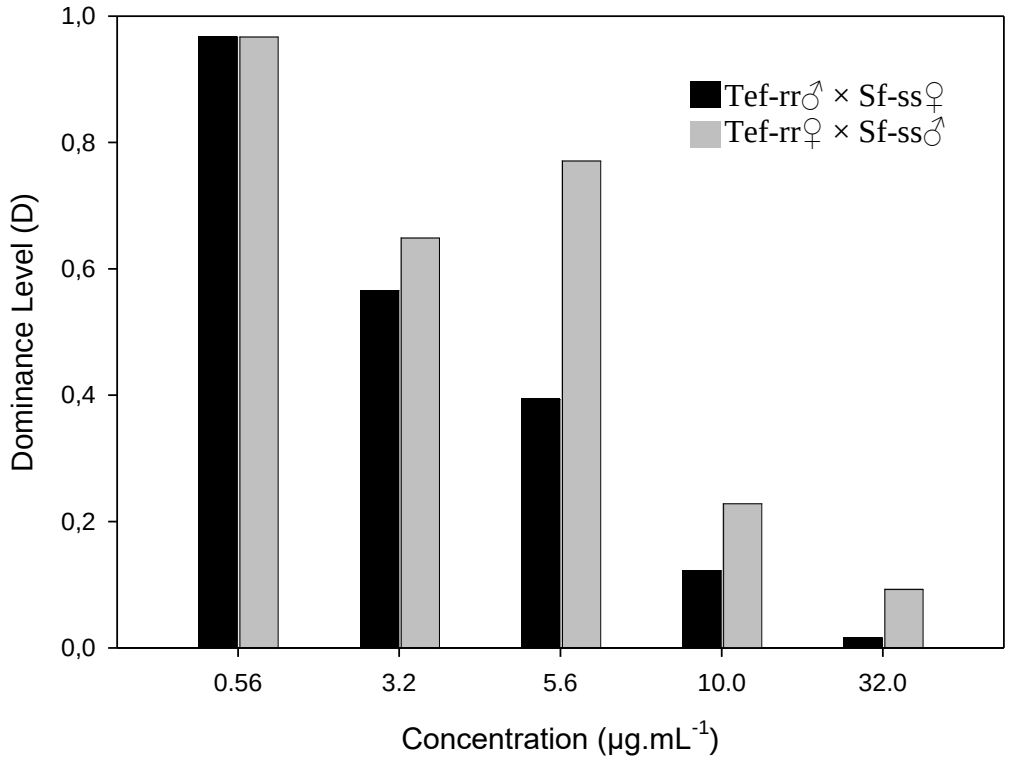

Figure 13. Level of dominance of *S. frugiperda* resistance as a function of teflubeunzuron concentration

We analyzed the number of genes associated with *S. frugiperda* resistance to teflubenzuron. The direct hypotheses test of monogenic inheritance based on responses F_1 × Tef-rr backcross was significant ($P < 0.01$) for concentrations between 1 and 10 $\mu g.mL^{-1}$, rejecting the monogenic hypothesis. On the other hand, concentrations higher than 32 $\mu g.mL^{-1}$ showed no significant difference between the observed and expected mortality (Table 7).

Table 7. Chi-square analysis of the mortality data from backcross between the progeny of reciprocal cross (Tef-rr♂ x Sf-ss♀) and Tef-rr *S. frugiperda* strain (F1 progeny) exposed to different concentrations of teflubenzuron

Concentration µg AI mL^{-1}	**Expected mortality**	**Observed mortality**	**χ^2 (df = 1)**	***P***
1	1.042	5.042	18.47	< 0.00001*
3.2	21.677	9.574	8.10	0.0044*
10	44.167	22.222	14.06	0.0001*
32	49.167	48.958	0.001	0.9674
100	64.600	68.750	0.72	0.3951
320	70.795	78.125	2.49	0.1142
1000	80.530	82.291	0.19	0.6629

3.2.3. Cross-resistance

Both susceptible and resistant strains were tested for cross-resistance to lufenuron and novaluron (Table 8). These results demonstrated low levels of cross-resistance between teflubenzuron-resistant strain (Tef-rr) and lufenuron and novaluron. The LC_{50} (CI%) values for Tef-rr exposed to lufenuron and novaluron were 28.01 (14.00 – 45.05) and 26.53 (21.96 – 31.70) $\mu g.mL^{-1}$ respectively.

Table 8. Cross-resistance of *S. frugiperda* resistant strain (Tef-rr) to benzoilfeniureas

Strains	Insecticide	n	Slope±SE	LC_{50}(95% CI) (µg AI mL^{-1})	χ^2	df[a]	RR[b]
Sf-ss	Teflubenzuron	715	3.07 ± 0.24	0.47(0.35 – 0.63)	11.63	4	-
	Lufenuron	963	1.99 ± 3.13	0.23 (0.17 – 0.29)	11.94	4	-
	Novaluron	696	2.60 ± 0.30	0.35 (0.23 – 1.73)	9.33	3	-
Tef-rr	Teflubenzuron	840	0.64 ± 0.09	641.47 (213.05 – 2748.81)[c]	10.59	4	1,364.83
	Lufenuron	739	2.36 ± 0.16	28.01 (14.00 – 45.05)	40.65	5	121.75
	Novaluron	659	2.05 ± 0.24	26.53 (21.96 – 31.70)	2.51	4	75,8

a df = degrees of freedom.

[b] Resistance ratio (RR) = LC_{50} of resistant strain/LC_{50} of susceptible strain.

3.2.4. Population genomic comparison of resistant and susceptible strains of *S. frugiperda*

The sequencing of four pooled WGS libraries generated 60.5 million high-quality paired-end reads after adaptor and quality trimming. The mean length was 229 bp and the maximum length was 300 bp, resulting in a mean fragment length of 429 bp (Table 9). We obtained annotation of a large number of SNPs, 43% of which were responsible for the same functional modification of the genome (Fig. 14). We identified 724 variants between Tef-rr and Sf-ss with FST ≥ 0.15 and p-value from the exact test ≤ 0.01. The distribution of the annotated SNP regions in the GO database showed that a large number of SNPs were found in genes for expression regulation (17.2%) and nucleic-acid binding (50.96%) (Fig. 15).

Table 9. Summary of Miseq sequence using pooled *S.frugiperda* strains resistant and susceptible to teflubenzuron

Raw total sequences	60.529,881
Reads mapped	60.478,739
Average length	229 bp
Maximum length	300 bp
Insert size average	429,7

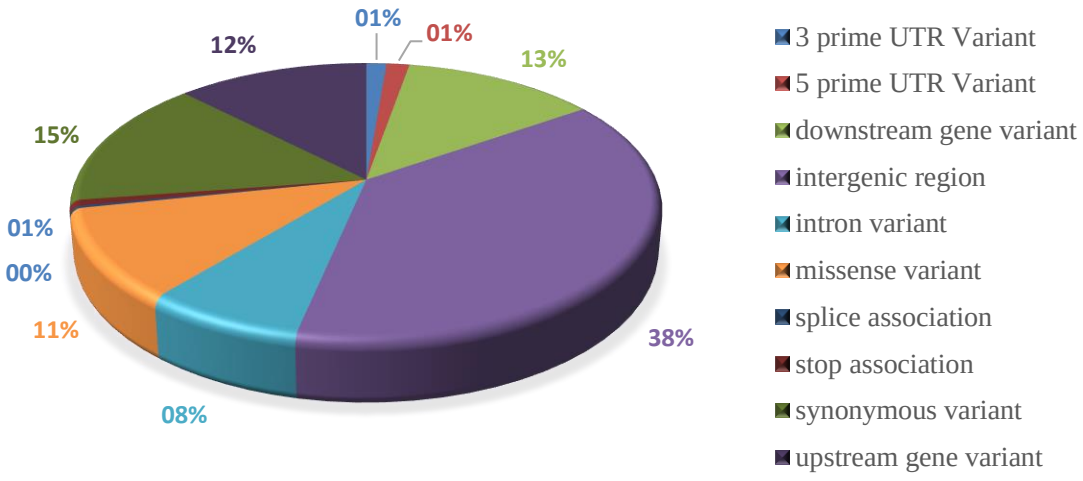

Figure 14. Distribution of the annotated SNP regions

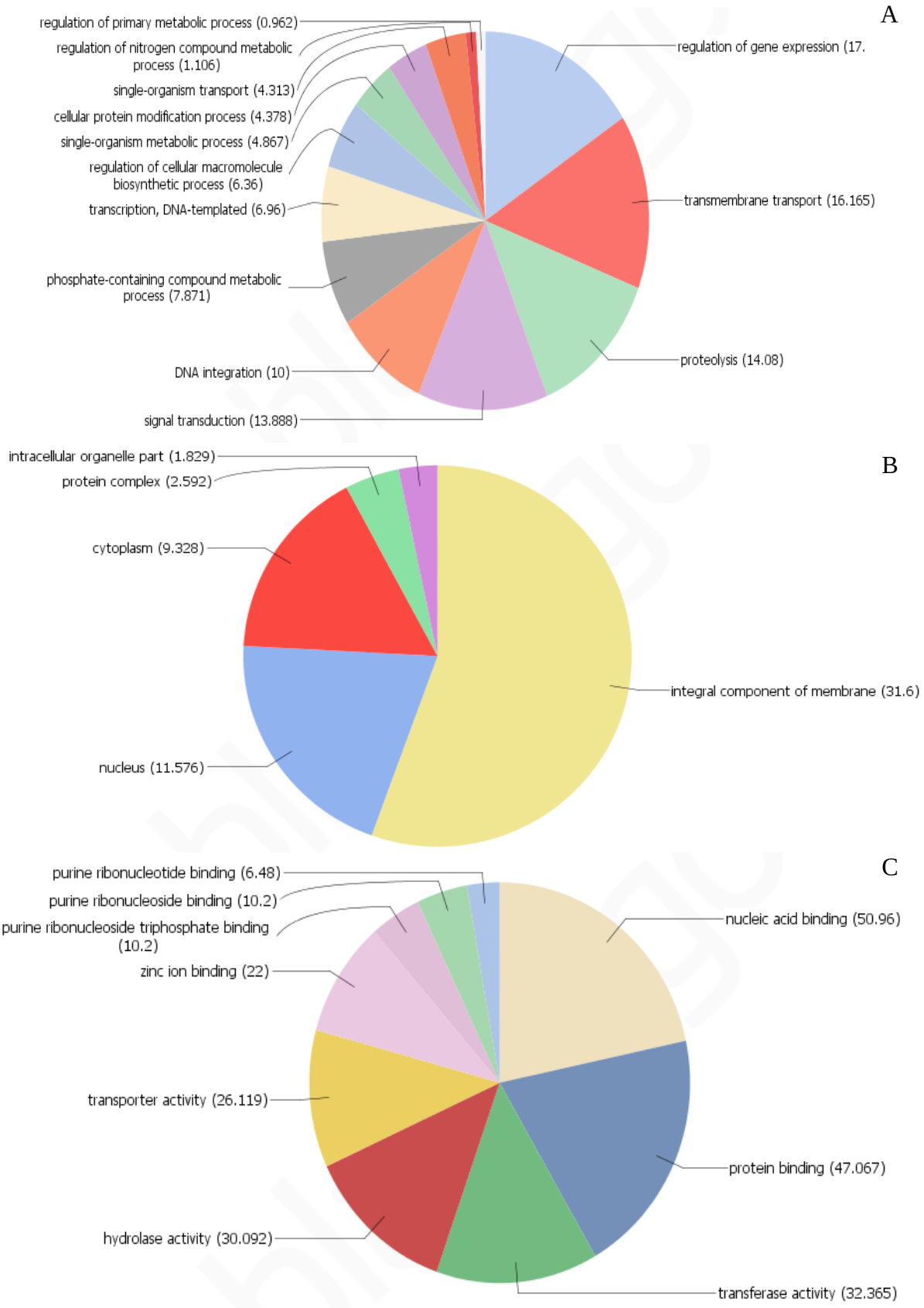

Figure 15. Distribution of the annotated SNP regions in three GO categories (A – biological process, B – cellular component and C -molecular function)

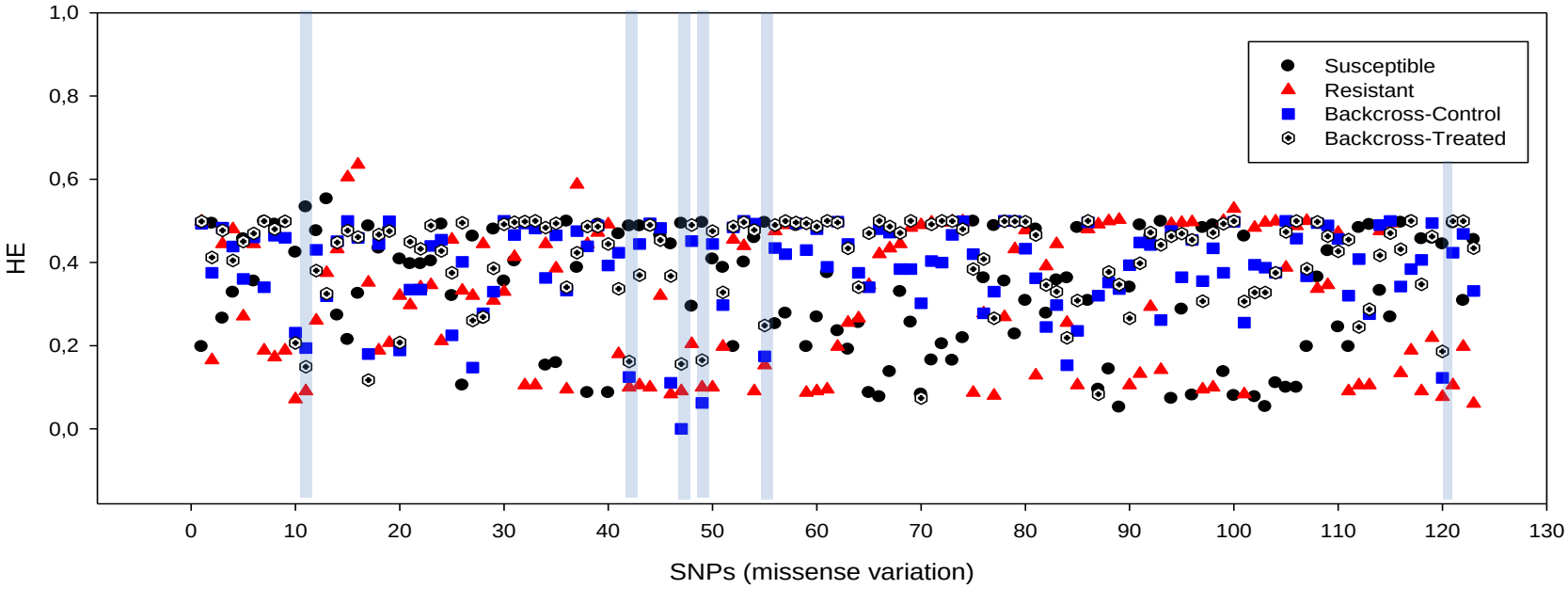

Figure 16. Heterozygozityy results for the resistance, susceptible and two backcrosses (treated and control) strains of *S. frugiperda*. All variations presented FST > 0.15 and exact test ≤0.01. SNPs with blue highlight where heterozygosity results of backcrosses have behavior similar to parental resistant.

3.3. Discussion

High selection pressure caused by the intense use of insecticides has led to the evolution of *S. frugiperda* resistance. The widespread use of benzoylphenylurea insecticides has accelerated the evolution of resistance in Brazil. For example, a lufenuron-resistant strain of *S. frugiperda* was identified and found to show a high level of resistance (915 times) (Nascimento et al 2014). In the present study, a strain resistant to teflubenzuron, an insecticide from the benzoylphenylurea group, was selected from a field population collected in Mato Grosso state, Brazil. We determined the genetic inheritance of the resistance of *S. frugiperda* to teflubenzuron, and observed a high level of resistance to this insecticide (≈ 1,365 fold).

Our results from heterozygous lines showed overlapping confidence intervals, indicating that the resistance of *S. frugiperda* to teflubenzuron is autosomally inherited. This result indicated that genes related to resistance are probably located in autosomal genes, excluding sex-linked inheritance and maternal effects. This pattern of genetic inheritance has been widely found in lepidopteran species resistant to insecticides and Bt toxins, e.g., Dipel resistance (Huang 1999) and Cry1Ab resistance in *Ostrinia nubilalis* (Crambidae) (Alves et al. 2006).

Understanding of teflubenzuron resistance inheritance showed that, at lower concentrations, it assumes incompletely dominant features, but in higher concentrations the resistance of *S. frugiperda* to teflubenzuron is incompletely recessive. The higher concentration is close to the concentration currently used in the field. In resistance management, the level of dominance is a variable feature, resulting not only from the genetic background but also from the interaction between phenotypes and environmental conditions (Bourguet et al. 2000). The level of dominance is one of the most important features for successful IRM (Lenormand and Raymond 1998), since the frequency of resistant insects could be related to the level of dominance. When this inheritance is recessive, the evolution of resistance is delayed, because the phenotype is present only in homozygotes and the alleles that confer resistance are rare (ffrench-Constant 2013). However, the use of concentrations lower than the level recommended for field use helps to maintain heterozygous individuals in the system and increases the frequency of the resistant alleles in the population. With continued selection pressure, the degree of individual resistance should increase rapidly, with a concomitant increase in the likelihood of heterozygote mating that produces homozygotes.

We tested the standard monogenic inheritance model by comparing the observed and expected mortality of the offspting of the backcrosses. There was a significant deviation

between the observed and expected mortalities for three concentrations. The results suggest that more than one gene influenced teflubenzuron resistance in these fall armyworms. This result agrees with the findings of Nascimento et al. (2014), who identified polygenic inheritance of *S. frugiperda* resistance to lufenuron.

The results for cross-resistance showed that teflubenzuron-resistant insects possessed low-cross resistance to lufenuron (121.75-fold) and novaluron (75.8-fold). This result may be related to strong selection of insects with overexpression of the detoxification genes, such as cytochrome P450 (CYP), glutathione S-transferases (GSTs), UDP-Glucosyltransferases (UGTs), and esterases (CCEs) (Nascimento et al. 2015). These genes are largely associated with detoxification of xenobiotic compounds in several lepidopteran species. Therefore, selection of these genes within these superfamilies may be responsible for the evolution of resistance to different insecticide compounds within the same IRAC group.

We used population genomic methods to identify SNP markers associated with resistance of *S. frugiperda* to teflubenzuron. Sequences from resistant and susceptible strains were aligned against the draft genome of *S. frugiperda* (Gouin et al. 2017). Interestingly, functional annotation of the SNP regions showed that 62% of the candidate SNPs have a linked function or are present in the same network. However, we did not find any SNPs associated with reported mechanisms of resistance to benzoylphenylureas, such as CYP, GSTs, UGTs and CCEs Nascimento et al. 2015), or with mutation in chitin synthases (Douris et al. 2016). The overrepresentation of functionally correlated SNPs may indicate regulation of many pathways in a larger picture, not just known genes**.** The distribution of GO categories showed that a larger number of SNPs were associated with regulation of gene expression and binding, which supports the hypotheses of gene regulation as a mechanism of resistance.

Our data indicated that several candidate SNPs showed signals of strong positive selection. Thus, a large proportion of the variation does affect the fitness of the organisms and is subject to Darwinian selection. Directional selection tends to eliminate the variation within populations, and to either increase or decrease the variation between species (Nielsen 2005). Positive selection is a type of selection associated with the strong selection caused by insecticides in agroecosystems. We reported several variations reducing SNPs for fixation; this may indicate the former presence of a hitchhiking effect associated with SNPs. In this case, the frequency of SNPs that are not associated with insecticide selection regions or with mutations, but are linked to them by physical proximity on the chromosome, changes (Kreitman 2001; Kasai 2004). Yan (Yan et al. 1998) reported this effect in *Aedes aegypti* resistant to OP.

Here, we selected a teflubenzuron-resistant strain of *S. frugiperda,* with a resistance ratio of 1,369-fold. The inheritance of resistance is autosomal and incompletely recessive. We also demonstrated cross-resistance among teflubenzuron, lufenorun and novaluron. A group of SNPs were selected within the teflubenzuron-resistant strain. These SNPs indicated that the resistance mechanism is a dense and intricate network, in which many regulatory genes are important. Taken together, these results help to understand the mechanism of resistance to benzoylphenylureas and to support the development of management strategies, particularly considering cross-resistance between insecticides. The group of molecular markers defined here may be refined for monitoring protocols in the future, which would help the implementation of appropriate IRM strategies in a growing season.

3.4. Conclusions

- The resistance ratio of the resistant strain to teflubenzuron was ≈ 1,364-fold;
- Inheritance of resistance was autosomal, incompletely recessive and polygenic;
- Teflubenzuron sresistanttrain showed cross resistance to lufenuron (121.75-fold) and novaluron (75.8-fold)
- There are large number of SNPs fixed in resistant strain to 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 ≤ 0.01 and log2 fold change ≥ 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).

4.2. Material and Methods

4.2.1. Insects

The susceptible *S. frugiperda* strain (Sf-sus) has been maintained at the Laboratory of Arthropod Resistance to Control Tactics, Department of Entomology and Acarology, Luiz de Queiroz College of Agriculture (Escola Superior de Agricultura "Luiz de Queiroz" - ESALQ/USP), Piracicaba, São Paulo state, Brazil, without selection pressure from insecticides for more than 20 years. The *S. frugiperda* strain resistant to teflubenzuron (Tef-rr) was selected from insects collected on maize in Mato Grosso state, using the F2 screening method (Nascimento et al. 2018). Thr tef-rr strain was maintained on an artificial diet based on beans, wheat germ, soy protein, yeast, and casein (Kasten et al. 1978). During all development stages, *S. frugiperda* was maintained at 25 ± 1 °C, 60 ± 10% RH and 14:10 h (L: D).

4.2.2. RNA extraction

The tef-rr and Sf-ss strains were used to compare gene expression profiles between these resistant and susceptible strains. Each RNA library was prepared from 50 mg of tissue from third-instar larvae, in three replicates.

A Direct-zol™ RNA mini-prep kit (ZymoResearch®) was used for RNA isolation, following the manufacturer's protocol. Four larvae were placed in 1.5 mL microtubes and 700 µL of Trizol™ Reagent (Invitrogen®) was added for mechanical maceration of the tissue. The homogenate was centrifuged at 16,000 *g* for 5 min at room temperature. The supernatant was transferred to a new microtube, and then 700 µL of 95% ethanol was added. The solution was transferred to filter columns and centrifuged at 16,000 *g* for 30 s. RNA wash buffer (400 µL), 5 µL of DNAse I (6 U.µL) and 75 µL of DNA digestion buffer were added to the membrane, which was incubated at room temperature for 15 min. Next, 400 µL of Direct-zol Rna PreWash was added. Samples were centrifuged at 16,000 *g* for 30 s. Finally, 700 µL of wash buffer RNA was added and the samples were centrifuged at 16,000 *g* for 2 min. The samples

were resuspended in 50 μL of DNA/RNA-free water and stored in an ultra-freezer at –80 °C until the evaluation of concentration and quality.

Total RNA samples were sent for evaluation of purity and integrity to the Central Laboratory of High-Performance Technologies in Life Sciences (LACTAD / UNICAMP), followed by library preparation and sequencing on the Illumina Hiseq2500 platform. For the sequencing of the cDNA libraries, the paired-end protocol was used, giving readings of approximately 100 bp.

4.2.3. *De novo* assembly

The RNA reads obtained were assessed for quality using FastQC v0.11.5 (Andrews 2010). Illumina adapter sequences and low-quality reads (Phred quality score <20 bp) were trimmed with Trimmomatic v0.36 (Lohse and Usadel 2014). *De novo* assembly was conducted using the program Trinity v2.0.6 (Haas et al. 2013) with a default k-mer size of 25 bp and a minimum transcript length of 200 bp. To maximize the effectiveness of the *de novo* assembly, we digitally normalized raw reads using the *normalization_in_silico.* This procedure reduces the number of duplicate reads, facilitating construction of the *de novo* assembly. In order to obtain a high-quality assembly, we evaluated the number of paired-end reads detected in the transcriptome, using Bowtie 2 (Langmead and Salzberg 2012).

4.2.4. Functional annotation

The transcripts were identified and annotated using a BLASTX search (Altschul et al., 1998) against the NR-NCBI database (non-redundant). Enzyme classification (EC) codes and annotation of the metabolic pathways in KEGG (Kyoto Encyclopedia of Genes and Genomes) (Kanehisa et al. 2007) were generated with Blast2GO (Conesa et al. 2005), with an E-value cut-off set to 10^{-3}. Gene open reading frames (ORFs) were predicted using Transdecoder v2.0.1, and ORFs were blasted against the GO (Gene Ontology), EggNOG (Powell et al. 2011), and UniProt databases, with an E-value cut-off set to 10^{-5}.

4.2.5. Identification of differentially expressed genes

Differentially expressed genes (DEGs) between the teflubenzuron-resistant and susceptible strains of *S. frugiperda* were determined based on gene expression abundance in each strain. The relative abundance was calculated by aligning reads against the reference transcriptome, using RSEM v.1.1.17 (Li and Dewey 2011), in order to estimate the expression abundance of genes and isoforms by FPKM (Fragments Per Kilobase of exon per Million mapped fragments).

Differential expression analysis was performed using the DESeq2 package (Love et al. 2014). First, data were normalized using correction factors based on the effective size of the libraries. The candidate transcripts associated with the resistance mechanisms of *S. frugiperda* were selected, based on a significant difference FDR ≤ 0.01 and fold change > 5. The GO terms of the transcripts with FDR ≤ 0.01 and fold change > 5 were analyzed for statistically significant enrichment, using TopGO for biological process, metabolic process, and molecular process terms.

4.3. Results

4.3.1. *De novo* assembly of the reference transcriptome

The reference transcriptome was prepared from six cDNA libraries. The sequencing and assembly are summarized in Table 10. We obtained approximately 250 million raw reads. After removing adaptor sequences and duplicate reads, we used a total of approximately 60 million filtered reads, containing around 694 million nucleotides (Table 10). In total, 82,403 transcripts and 41,146 unigenes were obtained from the *de novo* assembly. Transcript lengths ranged from 301 to 26,723 bp, with a mean length of 842.52 bp (Fig. 17) and N50 of 1,086 bp.

Table 10. Summary statistics of the de novo assembly of the *S. frugiperda* transcriptome

Strains	**Raw reads *** **(Paired-end)**	**Filtered reads** **(Paired-end)**
Susceptible (Sf-Sus)	86.093.577	81.092.057
Teflubenzuron Resistance (Tef-rr)	60.941.126	57.173.159
Assembly		
Total of genes		33,174
Total of transcripts		82,403
GC%		41.21
Total assembled bases		69.426.223
Min. length of transcripts		301
Max. length of transcripts		26,723
N50		1,086

* Raw Reads were obtained from cDNA sequencing in Illumina Hiseq2500 platform.

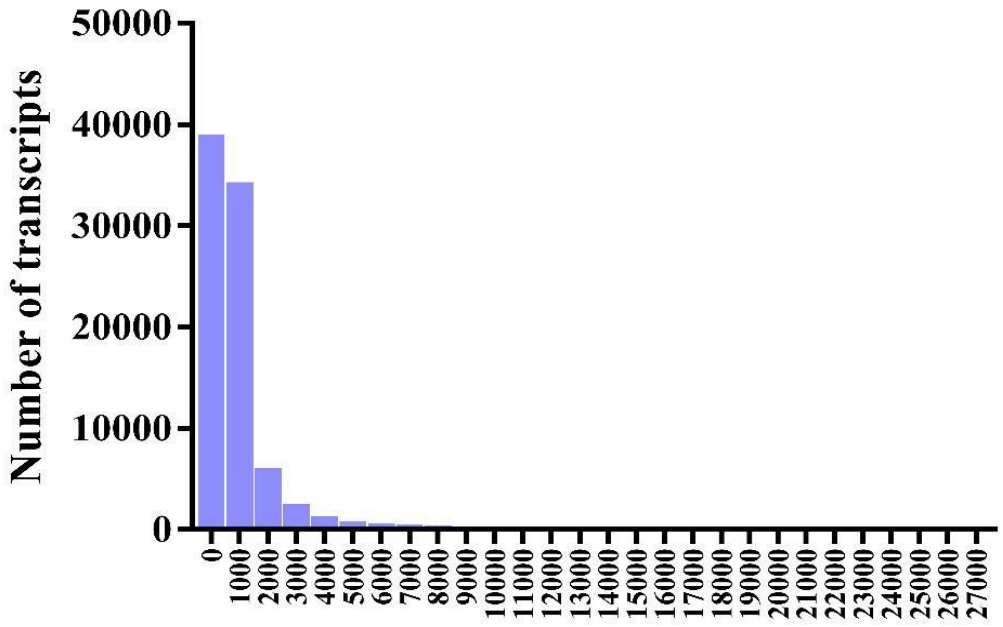

Figure 17. Length distribution of assembled transcripts

4.3.2. Functional Annotation

The results of the functional annotation of the 82,403 transcripts in several databases are shown in Table 11. The non-redundant-NCBI database provided the highest number of

annotated transcripts, with a total of 46,554 (56.49%) having an e-value less than 10^{-3}. Comparisons to other databases gave annotated transcripts totaling 39,961 (48.49%), 23,179 (41.61%), 27,850 (33.79%), 33,623 (40.80%), 40,366 (48.98%), and 29,450 (35.73%) transcripts in the swiss-prot, GO, Pfam, KOG, EggNOG, and Kegg databases respectively (Table 11).

Table 11. Transcript annotation rate in several databases

Database	**Number of transcripts**	**Percentage (%)**
Blast Nr	46,554	56.49
Swiss-Prot	39,961	48.49
GO	23,179	41.61
Pfam	27,850	33.79
KOG	33,623	40.80
Eggnog	40,366	48.98
Kegg	29,450	35.73

Annotated transcripts had the highest homology with lepidopteran species, e.g. *Bombyx mori* (Lepidoptera: Bombycidae) (14.44%) and *Amyelois transitella* (Lepidoptera: Pyralidae) (14.44%), followed by *Papilio xuthus* (Lepidoptera: Papilionidae) (7%), *Pectinophora gossypiella* (Lepidoptera: Gelechiidae) (6%), and *Papulio machaon* (Lepidoptera: Papilionidae) (6%). Representatives of the genus *Spodoptera* (Lepidoptera: Noctuidae) matched 7.13% of the best hits, divided among *S. litura*, *S. exigua*, *S. frugiperda*, and *S. littoralis* (Fig. 18).

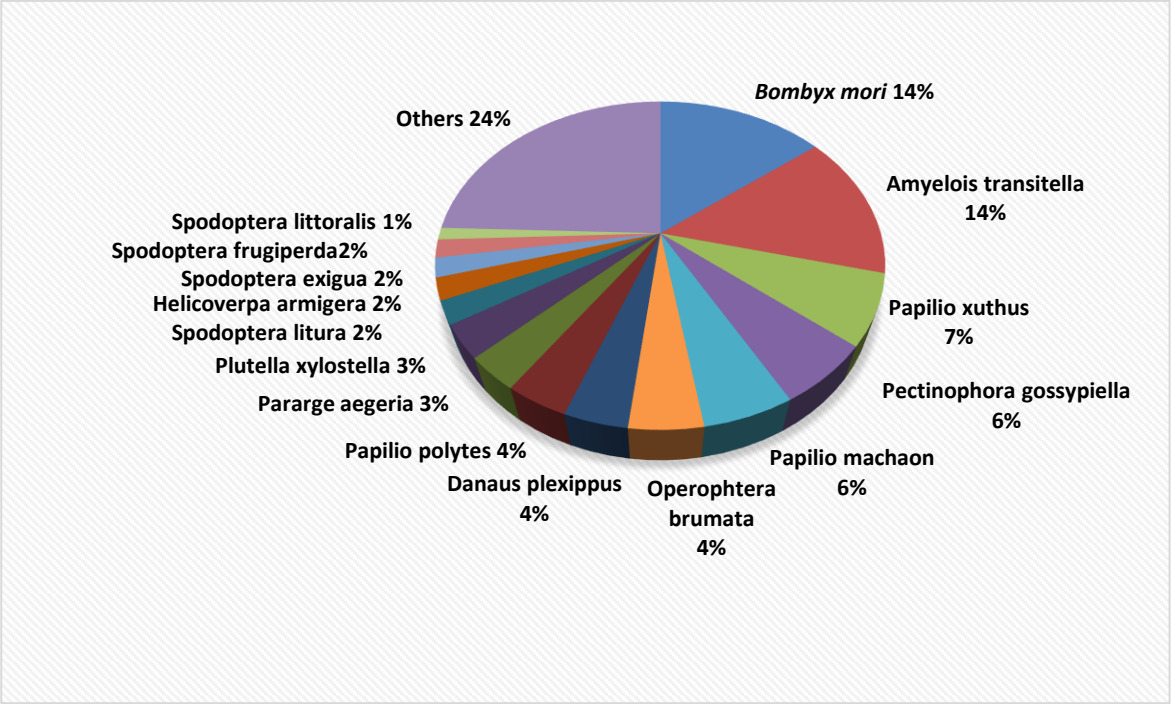

Figure 18. The most frequent species distribution of the BlastX hits. (NCBI-nr)

4.3.3. Differential Gene Expression Analyses

To understand the molecular mechanisms of *S. frugiperda* resistance to teflubenzuron, we compared the gene expression between the Sf-ss and Tef-rr strains (Appendix E). We identified 3,519 differentially expressed transcripts, based on the adjusted *p-value* ≤ 0.01 and log2 fold change ≥ 5. Tef-rr showed 991 down-regulated and 2,528 up-regulated transcripts compared to Sf-ss.

A total of 2,578 transcripts (73.25% of all differentially expressed transcripts) were assigned to 22 KOG categories (Fig. 19). The largest category was post-translational modification, protein turnover, and chaperones (10.47% of DEGs); followed by signal transduction mechanisms (9.23% of DEGs), lipid transport, and metabolism (9.07% of DEGs); and translation, ribosomal structure, and biogenesis (7.40% of DEGs). Categories with lower numbers of transcripts were cell wall/membrane/envelope biogenesis (0.85% of DEGs), nuclear structure (0.85% of DEGs), extracellular structures (1.04% of DEGs), and defense mechanisms (1.16% of DEGs).

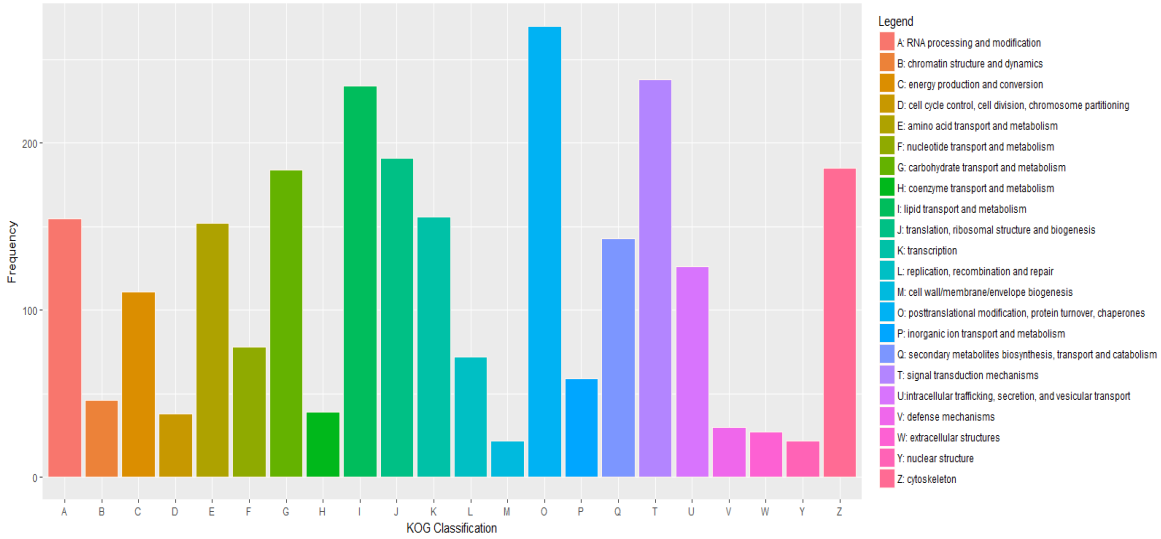

Figure 19. KOG (Eukaryotic Orthologous Groups) functional classification of the DEGs of resistant and susceptible strains of the *S. frugiperda* to teflubenzuron

GO enrichment analysis was performed for the differentially expressed transcripts. Table 3 shows the ten best GO terms for cellular components, molecular functions, and biological processes. All significant GO terms had a large number of annotated transcripts. We identified a large number of GO terms associated with regulation, mainly precatalytic spliceosome (GO:0071011), catalytic step 2 spliceosome (GO:0071013), GTP binding (GO:0005525), transcription factor activity (GO:0003700), and mRNA splicing, via spliceosome (GO:0000398) (Table 12).

We identified 19 transcripts related to regulation of ecdysteroid hormones (ecdysteroid 22-kinase and ecdysone oxidase); of these, 14 transcripts were up-regulated in the resistant strain. A large number of ABC transport transcripts from A, B, C, D and G families were more highly expressed in the resistant strain (Table 13). The most commonly reported detoxification enzymes were GSTs, UGTs, P450s, and CEs. Many of these were up-regulated in the resistant strain. The overall detoxification pattern of gene expression compared between Sf-ss and Tef-rr is shown on the hierarchical clustering heatmap (Fig. 20 and 21).

Table 12. Gene Ontology (GO) enrichment of DEGs

GO.ID	Term	Annotated	Significant	Expected	*P-value*
Cellular Component					
GO:0071011	precatalytic spliceosome	539	35	39.71	< 1e-30
GO:0071013	catalytic step 2 spliceosome	469	29	34.55	< 1e-30
GO:0005634	nucleus	4857	320	357.83	< 1e-30
GO:0000139	Golgi membrane	202	21	14.88	< 1e-30
GO:0005654	nucleoplasm	1211	66	89.22	< 1e-30
GO:0005912	adherens junction	299	25	22.03	< 1e-30
GO:0043234	protein complex	4743	301	349.43	< 1e-30
GO:0005938	cell cortex	492	45	36.25	< 1e-30
GO:0008091	spectrin	103	14	7.59	< 1e-30
GO:0045172	germline ring canal	88	5	6.48	< 1e-30
Molecular Function					
GO:0005525	GTP binding	278	19	22.15	< 1e-30
GO:0003735	structural constituent of ribosome	436	42	34.73	< 1e-30
GO:0005515	protein binding	3603	240	287.02	< 1e-30
GO:0003700	transcription factor activity	535	39	42.62	< 1e-30
GO:0032550	purine ribonucleoside binding	422	24	33.62	< 1e-30
GO:0005524	ATP binding	142	6	11.31	< 1e-30
GO:0003729	mRNA binding	610	55	48.59	< 1e-30
GO:0008017	microtubule binding	287	26	22.86	< 1e-30
GO:0003779	actin binding	557	53	44.37	< 1e-30
GO:0003677	DNA binding	737	37	58.71	< 1e-30
Biological Process					
GO:0008152	metabolic process	13518	1027	1054.57	<1e-30
GO:0007298	border follicle cell migration	343	27	26.76	<1e-30
GO:0071822	protein complex subunit organization	1540	108	120.14	<1e-30
GO:0000398	mRNA splicing, via spliceosome	824	52	64.28	<1e-30
GO:0048477	oogenesis	1419	98	110.7	<1e-30
GO:0002064	epithelial cell development	990	62	77.23	<1e-30
GO:0007095	mitotic G2 DNA damage checkpoint	242	10	18.88	<1e-30
GO:0007052	mitotic spindle organization	569	38	44.39	<1e-30
GO:0019226	transmission of nerve impulse	867	51	67.64	<1e-30
GO:0000022	mitotic spindle elongation	240	16	18.72	<1e-30

Table 13. Relative expression levels of several transcripts associated to resistance mechanism of *S. frugiperda* to teflubenzuron

Transcript	log2 FoldChange	FDR	Expression	Description
Ecdysteroid regulation				
DN16745_c0_g1_i2	-7.648	<0.001	Dow-regulated	Ecdysteroid 22-kinase
DN17217_c1_g1_i6	8.445	<0.001	Up-regulated	Ecdysteroid 22-kinase
DN19438_c1_g2_i1	6.147	0.002	Up-regulated	ecdysteroid 22-kinase
DN19811_c0_g1_i11	-7.070	0.001	Dow-regulated	Ecdysteroid 22-kinase
DN19811_c0_g1_i2	-7.076	<0.001	Dow-regulated	Ecdysteroid 22-kinase
DN19811_c0_g1_i3	8.819	<0.001	Up-regulated	Ecdysteroid 22-kinase
DN20408_c1_g1_i1	7.528	0.012	Up-regulated	ecdysteroid 22-kinase
DN20408_c1_g1_i8	10.821	0.001	Up-regulated	ecdysteroid 22-kinase
DN22009_c1_g1_i3	7.253	0.001	Up-regulated	Ecdysteroid 22-kinase
DN22009_c1_g1_i7	10.188	0.003	Up-regulated	Ecdysteroid 22-kinase
DN22116_c1_g1_i1	8.127	<0.001	Up-regulated	Ecdysteroid 22-kinase
DN22116_c1_g1_i3	8.444	0.014	Up-regulated	Ecdysteroid 22-kinase
DN24134_c0_g1_i5	9.985	<0.001	Up-regulated	ecdysteroid-regulated 16 kDa
DN24134_c0_g1_i6	9.583	<0.001	Up-regulated	ecdysteroid-regulated 16 kDa
DN24134_c0_g1_i7	11.323	<0.001	Up-regulated	Ecdysteroid-regulated 16 kDa
DN25899_c0_g1_i5	9.035	<0.001	Up-regulated	Ecdysteroid 22-kinase
DN26872_c0_g1_i2	-6.380	<0.001	Dow-regulated	ecdysone oxidase
DN26872_c0_g1_i7	-7.405	<0.001	Dow-regulated	ecdysone oxidase
DN27694_c0_g1_i11	7.193	<0.001	Up-regulated	Ecdysteroid 22-kinase
DN28284_c2_g2_i5	8.629	<0.001	Up-regulated	ecdysteroid 22-kinase
ABC Transporter				
DN18055_c0_g1_i1	-6.560	<0.001	Dow-regulated	ABCC1
DN21021_c1_g1_i3	-5.573	0.017	Dow-regulated	ABCD2
DN17508_c0_g1_i1	-5.545	0.016	Dow-regulated	ABCC Sur X1
DN29385_c4_g1_i2	-3.353	<0.001	Dow-regulated	ABC G member 4
DN27518_c2_g2_i2	-3.162	0.002	Dow-regulated	ABC C member 4
DN29021_c3_g1_i3	5.004	0.005	Up-regulated	ABC transporter white del-I119 mutant
DN22892_c0_g1_i5	5.407	0.019	Up-regulated	ABCC member Sur X1
DN28749_c2_g2_i2	5.888	0.009	Up-regulated	ABC C
DN21928_c0_g1_i1	6.053	0.004	Up-regulated	ABC Transporter sub-family A ABCA2
DN18055_c0_g1_i7	6.123	0.001	Up-regulated	ABC B member 1
DN16809_c0_g1_i3	6.402	0.013	Up-regulated	ABC B member 1
DN14711_c0_g1_i2	6.666	0.003	Up-regulated	ABC C member 4
DN16809_c0_g1_i1	6.868	0.001	Up-regulated	ABC B member 1
DN15075_c0_g1_i3	6.924	<0.001	Up-regulated	ABC B member 1
DN27678_c0_g1_i8	7.867	0.001	Up-regulated	ABC D member 2
DN14362_c0_g1_i1	7.875	0.002	Up-regulated	ABC C member 2
DN29385_c4_g1_i3	8.032	0.012	Up-regulated	ABC G member 4
DN29624_c3_g1_i6	10.565	0.002	Up-regulated	ABC C member 2

Continue

Transcript	log2 FoldChange	FDR	Expression	Description
Tegument				
DN11042_c0_g1_i1	7.997	<0.001	Up-regulated	chitinase 7
DN16087_c0_g1_i3	-6.577	<0.001	Dow-regulated	Cuticular hypothetical 12
DN16396_c0_g1_i2	-7.598	<0.001	Dow-regulated	TPA: cuticle
DN17403_c0_g3_i2	10.456	0.002	Up-regulated	cuticular glycine-rich 13
DN17656_c2_g1_i6	8.716	<0.001	Up-regulated	chitin binding
DN17805_c0_g2_i5	9.946	<0.001	Up-regulated	cuticle isoform B-like
DN18689_c0_g1_i4	7.450	0.010	Up-regulated	cuticle isoform B-like
DN20159_c1_g1_i3	-5.130	0.016	Dow-regulated	chitin binding domain 3
DN20159_c1_g1_i5	10.324	0.002	Up-regulated	chitin binding domain 3
DN20520_c0_g1_i1	5.334	<0.001	Up-regulated	cuticular RR-2 motif 127
DN20562_c0_g1_i1	9.762	0.001	Up-regulated	cuticular RR-1 motif 3 precursor
DN20834_c0_g1_i3	9.370	<0.001	Up-regulated	cuticle 2-
DN21272_c3_g1_i3	-8.784	<0.001	Dow-regulated	cuticle CPH45
DN21451_c1_g1_i3	5.606	0.009	Up-regulated	endocuticle structural glyco bd-5-like
DN21700_c0_g1_i3	-8.217	<0.001	Dow-regulated	cuticular hypothetical 11 precursor
DN21782_c0_g1_i5	5.149	<0.001	Up-regulated	chitin binding
DN22503_c3_g1_i2	-13.878	<0.001	Dow-regulated	cuticle 4
DN22624_c0_g1_i3	11.468	<0.001	Up-regulated	cuticular RR-2 motif 59
DN22768_c0_g2_i4	-6.484	<0.001	Dow-regulated	cuticular RR-1 motif 16 X1
DN23582_c0_g1_i12	5.282	<0.001	Up-regulated	chitin binding
DN23582_c0_g1_i2	11.737	<0.001	Up-regulated	chitin binding
DN23941_c0_g1_i4	-9.194	<0.001	Dow-regulated	cuticular RR-1 motif 33 X1
DN24455_c0_g1_i3	9.335	0.005	Up-regulated	cuticular RR-1 motif 21 precursor
DN26858_c1_g1_i3	-6.573	<0.001	Dow-regulated	endochitinase A-like
DN26858_c1_g1_i8	6.903	0.015	Up-regulated	endochitinase A-like
DN27920_c0_g1_i1	5.835	<0.001	Up-regulated	cuticle 1
DN27920_c0_g1_i3	9.472	<0.001	Up-regulated	cuticle 1
Sulfotransferase				
DN21145_c2_g2_i3	6.712	0.008	Up-regulated	sulfotransferase 1C4-like
DN22563_c1_g1_i2	7.803	<0.001	Up-regulated	sulfotransferase 1C4-like X3
DN23725_c0_g1_i3	8.273	<0.001	Up-regulated	sulfotransferase 1C4

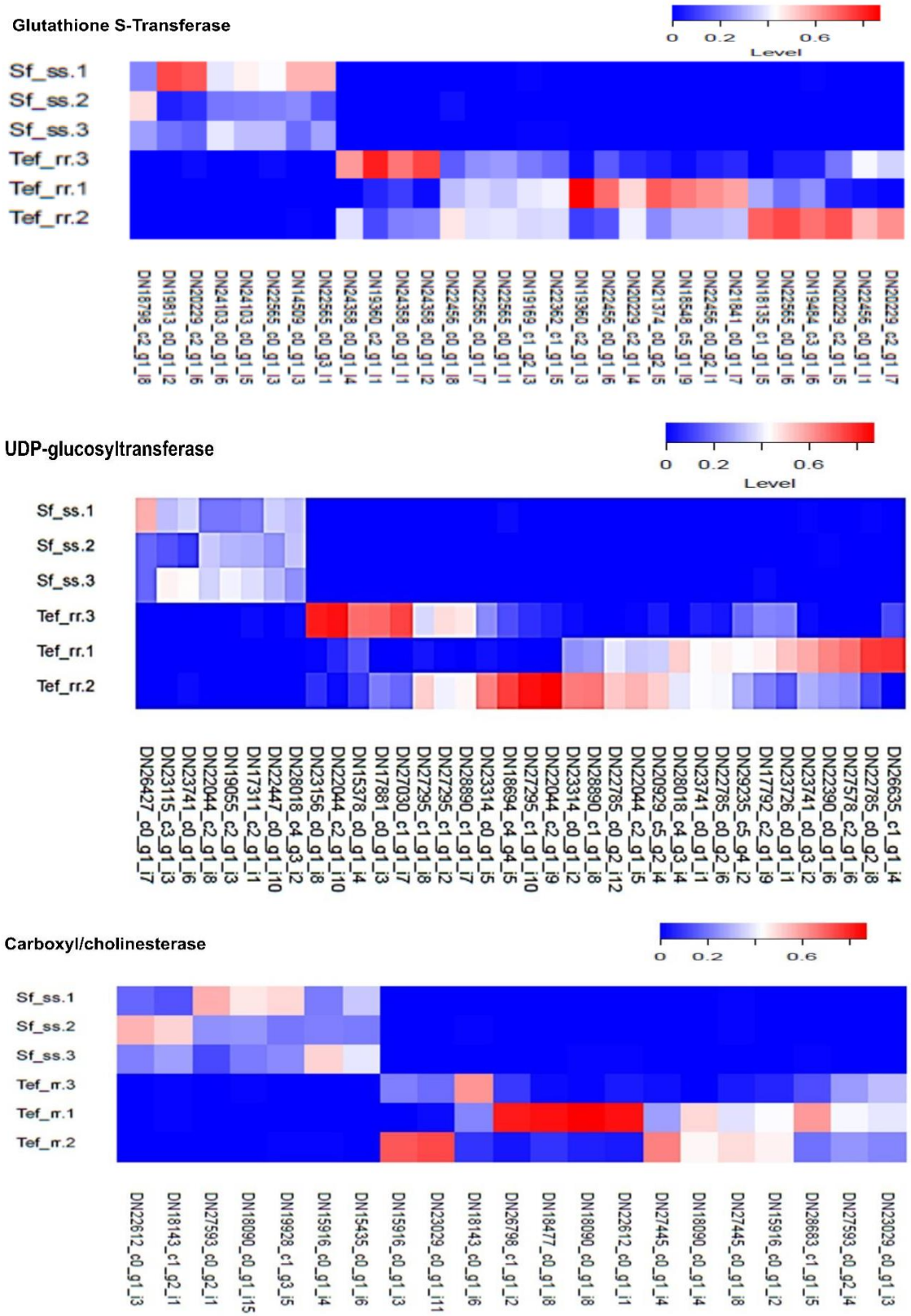

Figure 20. Heatmap of gene expression values depicting clustering of transcripts associates with enzymes of detoxification between susceptible and resistant strains of *S. frugiperda* to teflubenzuron based on the expression of mRNAs for a set of significant isoforms (Padj < 0.01 and fold change > 5).

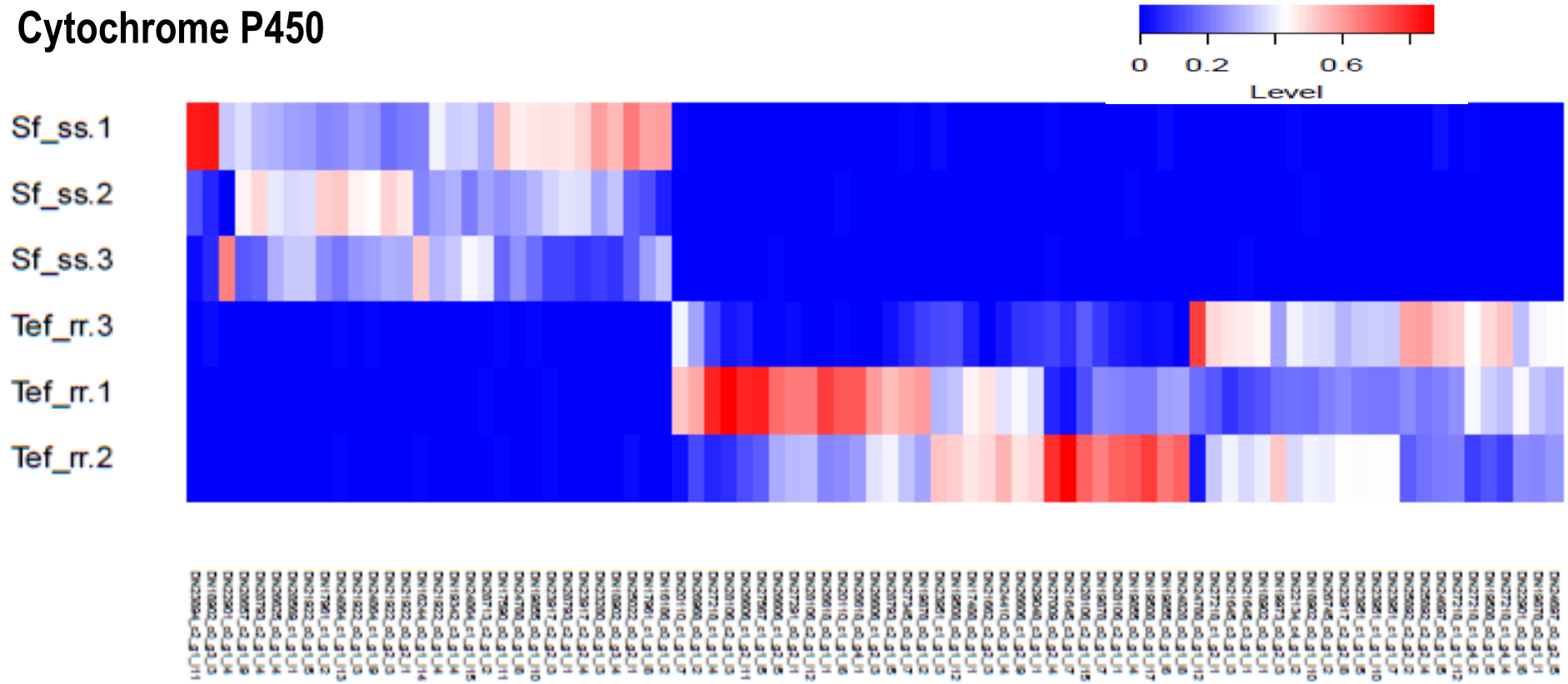

Figure 21. Heatmap of gene expression values depicting clustering of transcripts associates with cytochrome P450 between susceptible and resistant strains of *S. frugiperda* to teflubenzuron based on the expression of mRNAs for a set of significant isoforms (Padj < 0.01 and fold change > 5).

4.4. Discussion

The rapid evolution of resistance of *S. frugiperda* to insecticides has been of great concern to famers and researchers in Brazil and across the Americas. Benzoylurea insecticides are effective against fall armyworm, but changes in its susceptibility have been observed. Here we used a strain of *S. frugiperda* that is resistant to teflubenzuron, an important insecticide in the benzoylureas group, to understand the molecular mechanism of resistance. We obtained over 82 thousand transcripts, 56% of which were annotated, and most of which were up-regulated in the resistant strain. Especially notable was the up-regulation of transcripts annotated as ABC transporters, detoxification enzymes, and post-translational modification functions.

Insect resistance to insecticides is a biologically complex phenomenon related to adaptive processes. Currently, the main mechanisms thought to be associated with resistance development are mutations in the target sites of insecticides, alterations in detoxification and metabolism of insecticides, and tegumental changes that limit insecticide penetration (Georghiou 1972). At present, resistance to teflubenzuron or to other insecticides with cross-resistance with teflubenzuron has been observed in six different lepidopterans and one homopteran. The first case of resistance was observed in 1988, in the diamondback moth *Plutella xylostella* (L.) (Lepidoptera: Plutellidae) (Perng et al. 1988); and the most recent case in 2015, in the fall armyworm (Farias et al. 2015). Although resistance has been associated with increased metabolic detoxification, the molecular mechanism of action of benzoylphenylureas in lepidopterans is presently merely speculative, and our results showed that regulatory processes may be an important adaptation of *S. frugiperda* to survive this insecticide pressure.

We identified 3,519 differentially expressed transcripts between Tef-rr and Sf-ss, with a large number of them up-regulated in the resistant strain. These results are similar to those reported for lufenuron-susceptible and resistant strains of *S. frugiperda* (Nascimento et al. 2015) and for chlorantraniliprole-susceptible and resistant strains of *P. xylostella* (Lin et al. 2013; Kakumani et al. 2014).

We observed a high number of ABC transporters up-regulated in the resistant strain, including two sulfonylurea receptor genes (*SUR* genes). In mammals, sulfotransferase is responsible for hormone regulation and xenobiotic detoxification. The SULTs family catalyzes the transfer of the sulfuryl group from 3’-phosphoadenosine 5’-phosphosulfate to most hydroxyl-containing compounds (Maiti et al. 2004). In lepidopterans, SULTs can be

involved in the metabolism of juvenile hormone ecdysteroids and in detoxification of insecticides (Slade and Wilkinson 1974; Yamamoto and Liu 2015; Nascimento et al. 2015). The *SUR* gene, an atypical ABC transporter of subfamily C8/9 (Inagaki et al. 1995; Wilkens 2015), acts as a transporter in vesicles carrying N-acetylglucosamine to chitin synthase (Abo-Elghar et al. 2004). However, the two SUR gene isoforms that we obtained showed low fold-change values. The sulfotransferases were the detoxification group with the fewest transcripts identified. All transcripts associated with sulfotransferase 1C4 were up-regulated in Tef-rr. Thus, our results showed that sulfotransferases and ABC transporters related to it are not associated with resistance of *S. frugiperda* to teflubeunzuron. Other ABC transport families were identified, probably helping to transport compounds from detoxification processes.

Detoxification metabolism is highly important for teflubenzuron resistance, based on our results. Enzymes coded by these genes are responsible for degradation, detoxification, and/or sequestration of xenobiotics. The higher detoxification activities in the Tef-rr compared to the Sf-ss strain provide evidence that detoxification enzymes such as cytochrome P450s, glutathione S-transferases (GSTs), uridine diphosphate-glycosyl transferases (UGTs), and carboxyl-esterases (COEs) have been key for the resistance of *S. frugiperda* to teflubenzuron. Our study identified 175 differentially expressed transcripts related to detoxification enzymes ($p < 0.01$ and fold change > 5).

P450 monooxygenases are one of the main classes of enzymes associated with lepidopteran resistance to insecticides, such as pyrethroids (Joußen et al. 2012), organophosphates, carbamates, neonicotinoids (Cichón et al. 2013), benzoylureas (Nascimento et al. 2015), and diamides. However, recent studies have shown that *CYP*12A4 has no role in the detoxification of lufenuron in a resistant strain of *Drosophila melanogaster* Meigen. Our results demonstrated that 54 P450 transcripts were up-regulated in the teflubenzuron-resistant strain, similarly to a lufenuron-resistant strain of *S. frugiperda* (Nascimento et al. 2015). Many P450 families such as *CYP3*, *CYP4*, *CYP6*, and *CYP9* have been suggested as one of the mechanisms involved in lepidopteran resistance to insecticides (Pittendrigh 1997; Ranasinghe and Hobbs 1998; Yang et al. 2006). Our results indicated overexpression of genes *CYP3*, *CYP4*, and *CYP6* in the Tef-rr strain.

UGTs were highly expressed in the Tef-rr strain. Overexpression of UGTs was also observed in lufenuron-resistant *S. frugiperda* (Nascimento et al. 2015). UDP-glycosyltransferases (UGTs) are a multigene family that has shown importance in regulation of endobiotics and detoxification of xenobiotics by catalyzing the conjugation of a range of diverse lipophilic compounds, using sugar to produce glycosides (Ahn et al. 2011). UDP-

glycosyltransferases might have an important role in regulating the ecdysteroid titer in *S. frugiperda*. The hypothesis is that glucose conjugation by UGTs is a mechanism for ecdysteroid inactivation (O'Reilly and Miller 1989), which may be associated with overexpression of the ecdysteroid 22-kinase that regulates ecdysteroid inactivation by phosphorylation.

Notably, we observed that several functional classifications within differentially expressed transcripts included both pre- and post-transcriptional processes. Thus, modifications across several levels of biological processes may be responsible for the evolution of resistance to teflubenzeron in *S. frugiperda*. As an example of a pre-transcriptional process, we observed several transcripts associated with mRNA splicing activities. mRNA splicing removes non-coding introns from newly synthesized pre-mRNA by a two-step transesterification reaction. Intron excision is catalyzed by the spliceosome, a large and dynamic ribonucleoprotein particle (RNP) that assembles on each intron (Will and Luhrmann 2010; Plaschka et al. 2017). Our results revealed that differentially expressed genes were associated with both the first and second steps of splicing activities. Our data are similar to results for spinosad resistance in *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae), which also appears to be related to splicing regulation (Berger et al. 2016). Berger and co-workers analyzed resistant and susceptible strains of *T. absoluta*, and identified genes with the intrinsic capacity to generate transcript diversity of the gene nAChR via alternative splicing, an exon skipping event-mediated resistance.

The post-translational modification function was also abundant in DEG. This protein category is related to chemical changes that proteins may undergo after translation, such as acetylation, glutathionylation, glycosylation, hydroxylation, methylation, oxidation, and phosphorylation (Weis 2001). Post-translational modifications are also related to detoxification and modifications of an insect's cuticle, which have been shown to have an important role in the resistance of *S. frugiperda* to the benzoylphenylureas group, due to their mode of action on the insect cuticle (Nascimento et al. 2015).

The mode of action of benzoylphenylureas, which includes lufenuron, diflubenzuron, and teflubenzuron, is related to the chitin synthesis pathway. However, no effect on the deposition of UDP-GlcNAc and chitin synthase, for example, was observed (Mayer et al. 1981). Therefore, how benzoylphenylureas acts on chitin synthesis is not yet determined. Resistance of *Tetranychus urticae* Koch to etoxazole, an acaricide that inhibits chitin synthesis, was associated with mutations in conserved regions of chitin synthase gene (Van Leeuwen et al. 2012; Douris et al. 2016). We did not detect mutations in this gene or

significant changes in expression of this enzyme in the fall armyworm. However, we observed up-regulation of chitinase (DN11042_c0_g1_i1) and endochitinase A-like (DN26858_c1_g1_i8) in our data. We also observed overexpression of genes related to chitin binding, specifically cuticle isoform B-like, cuticular RR-1 and RR-2, and cuticle 1 and 2. Cuticular proteins and chitin are the most abundant compounds in the insect tegument, and these proteins are fundamental to cuticle architecture. The largest family of cuticular proteins, Rebers and Riddiford (R&R), is subdivided into 2 famlies: RR-1 and RR-2. RR-1 is associated with soft regions of the cuticle (Suderman et al. 2006) such as intersegmental membranes; whereas RR-2 is associated with hard regions such as sclerites and head capsules (Vannini and Willis 2016). Although some data suggest that genes involved in chitin metabolism, modification and degradation are not the targets of resistance of *S. frugiperda* to benzoylphenylureas (Nascimento et al. 2015), changes in the expression profile of these genes may be associated with a compensatory mechanism in strains resistant to benzoylphenylureas. Insects treated with benzoylphenylureas have shown a disorganized cuticle architecture (Ker 1977; Ker 1978; Gangishetti et al. 2008).

The gene expression profiles of the teflubenzuron-resistant and susceptible strains of *S. frugiperda* suggest that resistance is a phenomenon regulated by several genes, associated with several pathways, which include detoxification enzymes, pre- and/or post-transcription processes, and chitin synthesis. It is feasible that the strong selection pressure caused by insecticides has selected multiple genes that are responsible for adaptations to xenobiotics. Together with classical detoxification genes, adaptation processes provide a set of genes responsible for the regulation pathway, potentially associated with fitness costs to the resistant strains. We cannot exclude the possibility of an epistatic process, operating together with detoxification and regulatory processes. Therefore, additional studies are needed to improve understanding of the evolution of resistance at the molecular level, using a systemic approach. Advances in sequencing technologies can expand the knowledge of the processes that lead to the resistance of insects to insecticides.

We obtained approximately 250 million paired-end reads which resulted in 82,403 transcripts and 41,146 unigenes from De novo assembly. DEG analysis identified 3,519 differentially expressed transcripts, based on an adjusted p-value ≤ 0.01 and log2 fold change ≥ 5. The resistant strain Tef-rr showed 991 down-regulated and 2,528 up-regulated transcripts compared to the susceptible strain Sf-ss. Process as precatalytic spliceosome, catalytic step 2 spliceosome, GTP binding, transcription factor activity, and mRNA splicing via spliceosome were identified within 10 best in GO enchment. 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.

4.5. Conclusions

- 3,519 differentially expressed transcripts were identified with 991 overexpressed and 2,528 up-regulated in Tef-rr
- Large number of GO terms differentially expressed were associated with regulatory processes, mainly precatalytic spliceosome, catalytic step 2 spliceosome, GTP binding, transcription factor activity, and mRNA splicing via spliceosome;
- Many detoxification enzymes such as GSTs, UGTs, P450s and CEs were up-regulated in the resistant strain.

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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.

5.2. Material and methods

5.2.1. Selection of *S. frugiperda* resistant to VT-PRO

A strain resistant to Yieldgard VT-PRO® maize (event MON-89034) was maintained in the laboratory under selection pressure for 32 generations. The strain was selected from individuals collected from maize crops in Bahia state (Bernardi et al. 2015). The selection was made after offering leaves of VT-PRO maize to stage V6 larvae.

For bioassays, the artificial diet proposed by Kasten et al. (1978) was loaded onto bioassay trays (BIO-BA-128, CD International Inc., Pitman, NJ, USA) containing 128 cells (1

mL per cell). To prepare the test concentrations, the Cry1A105 and Cry2Ab2 proteins were diluted in buffer (50 mM CAPS, pH 11, 2 mM DTT), and 0.1% of Triton X-100 surfactant was added to this solution. We tested 5–8 concentrations of Cry2Ab2 and Cry1A.105. After the drying period, one *S. frugiperda* neonate (0–24 h old) was added per cell, with the aid of a fine brush. After the infestation, the trays were sealed with self-adhesive plastic sheets (BIO-CV-16, CD International, Inc.) that allowed gas exchange with the external environment. The bioassay trays were kept in a climate-conditioned room at 27 ± 1 °C, relative humidity 60 ± 10% and photophase 14 h. Mortality and weight of surviving larvae were evaluated after 7 days. Larvae that did not survive past the first instar were also considered dead.

Mortality data from the Sf-ss (susceptible) and VTPro-Res (resistant to VT-PRO) strains were submitted to Probit analysis (Finney, 1978) using the POLO PC program (Leora Software, 1987). The resistance ratio between the strains was estimated by dividing the LC_{50} of the VTPro-Res strain by the LC_{50} of the Sf-ss strain.

5.2.2. De novo assembly and DEG analysis

Steps of transcriptome assembly, annotation, and DEGs analysis can be observed in chapter 4.

5.3. Results

5.3.1. Read assembly

Six cDNA libraries representing VT-PRO-susceptible and resistant strains of *S. frugiperda* were sequenced, using the Illumina 2500 Hiseq platform. A total of 72,333,606 and 69,879,988 paired-end reads were obtained from Illumina sequencing of the Sf-ss and VTPro-Res strains, respectively. Around 68,761,715 and 66,861,758 filtered reads were obtained after removing the adaptor, selected for quality and size for both strains. Approximately 146 million nucleotides were used to construct the transcripts (Table 14).

Table 14. Summary of the de novo assembly of the transcriptome strains of *S. frugiperda*

Strains	**Raw reads *** **(Paired-end)**	**Filtered reads** **(Paired-end)**
Susceptible (Sf-Sus)	72.333.606	68.761.715
Resistant (VTPro-Res)	69.879.988	66.861.758

Total of Transcripts	192.170
%GC	39,54
Total assembled bases	146.290.747
Average	761,26
N50	1143
After filter	
Total of unigenes	44,391
Total of transcripts	99,463
%GC	40.68
Total assembled bases	77.262.665
Min. length of transcripts	201
Max. length of transcripts	25,508
N50	1,214

* Raw Reads were obtained from cDNA sequencing in Illumina Hiseq2500 platform.

In total, 192,170 transcripts were obtained from the *de novo* assembly. The transcripts had a mean length of 761,26 nt and an N50 of 1,143 nt. A large number of reads (94.71%) were aligned in the *de novo* assembly. After removing transcripts with low expression, a final reference transcriptome was obtained, with 44,391 unigenes, resulting in 99,463 transcripts. Transcript lengths ranged from 201 to 25,508 nt with a mean length of 776.80 nt (Fig. 22) and an N50 of 1,214 nt.

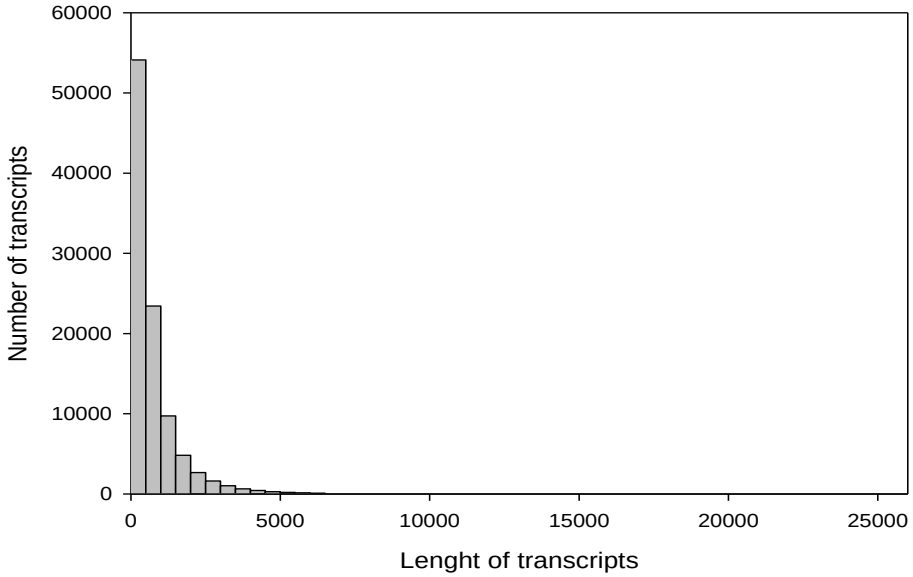

Figure 22. Length distribution of the transcripts on reference transcriptome of *S. frugiperda* susceptible and resistant to the VT-PRO event.

5.3.2. Functional Annotation

We annotated the transcripts against the nr-NCBI, swiss-prot, GO, Pfam, KOG, EggNOG, and KEGG databases. In the distribution of alignments by species against the Nr-NCBI database, 52.02% of all transcripts were mapped (1e-5 cut-off threshold) (Appendix D). Eleven of the top-hip alignments showed high similarity to lepidopterans. We obtained 64.4% of alignments with representatives of *Helicoverpa armigera* (L.) (Lepidoptera: Noctuidae), followed by *Amyelois transitella* (Walker) (Lepidoptera: Pyralidae) (2.82%), and *Bombyx mori* L. (Lepidoptera: Bombycidae) (2.61%). Among representatives of the *Spodoptera* complex, we obtained ≈ 6.5% identifications, which were represented by *S. litura* (Fabricius) (Lepidoptera: Noctuidae) (2.42%), *S. exigua* (Hübner) (Lepidoptera: Noctuidae) (2.26%), and *S. frugiperda* (1.96%) (Fig. 23).

The functional annotation by Gene Ontology of the reference transcripts resulted in the identification of 48 functional categories, divided into cellular component, molecular function, and biological processes. A total of 32,601 were identified by comparison against the GO database (Fig. 24), with a large number of GO terms associated with molecular functions of binding processes and catalytic processes. In biological processes, a large number of GO terms were associated with metabolic processes and cellular processes.

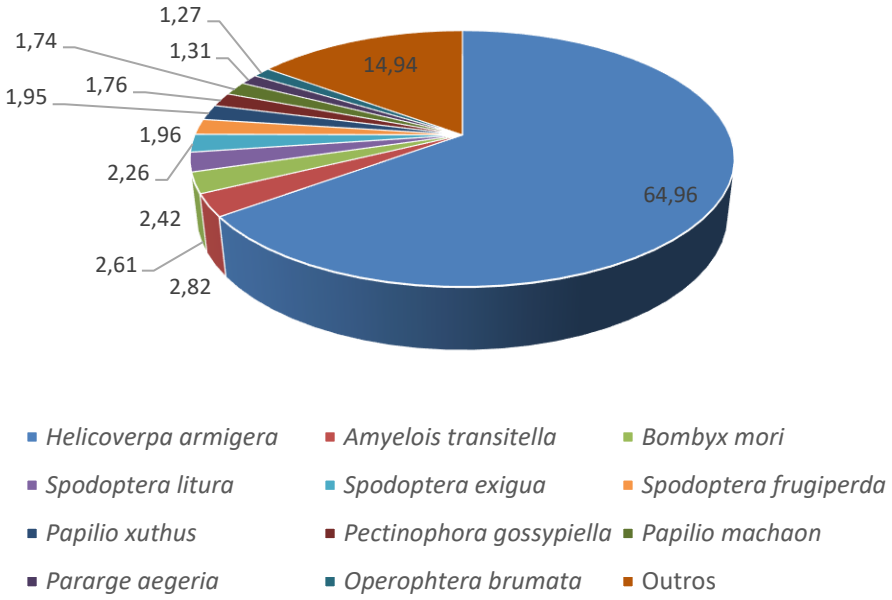

Figure 23. Percentage of the top hits by species from blastx results of searches against NR database (NCBI)

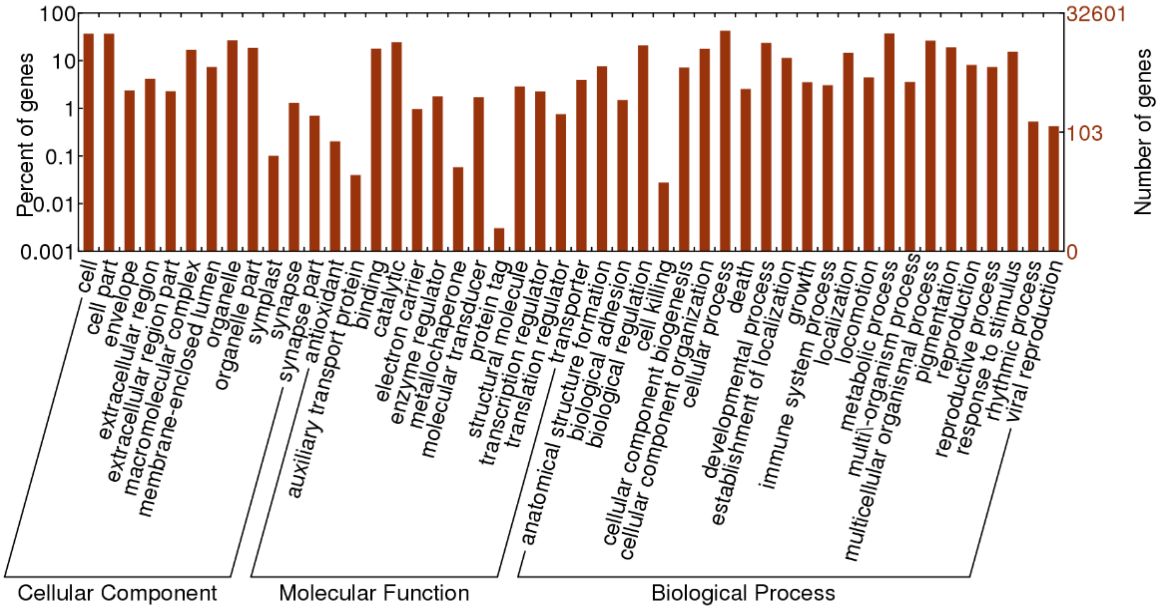

Figure 24. Gene ontology in transcriptome of *S. frugiperda*

5.3.3. Differentially expressed gene

Differential expression analyses between VT-PRO-susceptible and resistant *S. frugiperda* strains identified 10,281 transcripts with relative expression ≥ 5 and FDR ≤ 0.01

(Appendix F). This number corresponds to 19% of all transcripts (Fig. 25). Transcripts associated with several GOs and different metabolic pathways were obtained.

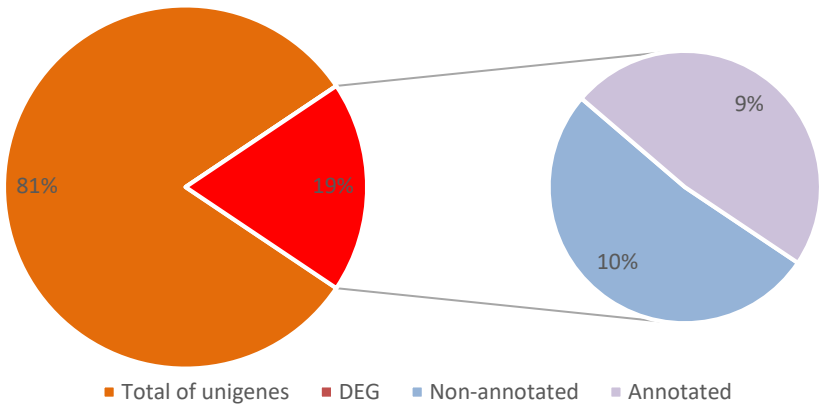

Figure 25. Distribution of differentially expressed genes of *Spodoptera frugiperda*

We performed the GO enrichment analysis on differentially expressed transcripts. Table 2 shows the ten best GO terms for cellular component, molecular function, and biological process. We identified a large number of GO terms associated with spliceosome (GO:0071011), (GO:0071011), (GO:0071013) in the cellular component category, GTP binding (GO:0005525), and structural constituent of ribosome (GO:0005525) in molecular function, and metabolism processes (GO:0008152) in biological process (Table 15).

The hundred best GO terms obtained by the enrichment analysis using TopGO were summarized with REVIGO. This analysis showed that 75.11% of unigenes annotated into a molecular function were associated with regulation of mRNA processes, mainly mRNA binding (Fig. 26).

The prediction function and classification of differentially expressed ORFs were searched against the eukaryote database (KOG). A total of 3,621 unigenes (35.15% of all the Differentially Expressed Genes) were assigned to 22 KOG categories (Fig. 27). The largest categories were signal transduction mechanisms (6.84 and 8.91%), post-translational modification (7.15 and 7.96%), and carbohydrate transport and metabolism (7.53 and 7.04%). Low numbers of KOGs were found for down-regulation of extracellular structures (0.61%) and cell motility (0.08%), and for up-regulation of nuclear structure and cell motility (0.08%).

Functional identification of differentially expressed unigenes showed a large number of transcripts associated with the mode of action of Bt toxins in lepidopterans. Genes of aminopeptidase were up-regulated in the VTPro-Res strain, with a log2 fold change of 4.29.

Table 15. Gene Ontology (GO) enrichment of DEUs

GO.ID	Term	Annotated	Significant	Expected	*P-value*
Celular Component					
GO:0071011	precatalytic spliceosome	539	35	39.71	< 1e-30
GO:0071013	catalytic step 2 spliceosome	469	29	34.55	< 1e-30
GO:0005634	nucleus	4857	320	357.83	< 1e-30
GO:0000139	Golgi membrane	202	21	14.88	< 1e-30
GO:0005654	nucleoplasm	1211	66	89.22	< 1e-30
GO:0005912	adherens junction	299	25	22.03	< 1e-30
GO:0043234	protein complex	4743	301	349.43	< 1e-30
GO:0005938	cell cortex	492	45	36.25	< 1e-30
GO:0008091	spectrin	103	14	7.59	< 1e-30
GO:0045172	germline ring canal	88	5	6.48	< 1e-30
Mollecular Function					
GO:0005525	GTP binding	278	19	22.15	< 1e-30
GO:0003735	structural constituent of ribosome	436	42	34.73	< 1e-30
GO:0005515	protein binding	3603	240	287.02	< 1e-30
GO:0003700	transcription factor activity	535	39	42.62	< 1e-30
GO:0032550	purine ribonucleoside binding	422	24	33.62	< 1e-30
GO:0005524	ATP binding	142	6	11.31	< 1e-30
GO:0003729	mRNA binding	610	55	48.59	< 1e-30
GO:0008017	microtubule binding	287	26	22.86	< 1e-30
GO:0003779	actin binding	557	53	44.37	< 1e-30
GO:0003677	DNA binding	737	37	58.71	< 1e-30
Biological Process					
GO:0008152	metabolic process	13518	1027	1054.57	<1e-30
GO:0007298	border follicle cell migration	343	27	26.76	<1e-30
GO:0071822	protein complex subunit organization	1540	108	120.14	<1e-30
GO:0000398	mRNA splicing, via spliceosome	824	52	64.28	<1e-30
GO:0048477	oogenesis	1419	98	110.7	<1e-30
GO:0002064	epithelial cell development	990	62	77.23	<1e-30
GO:0007095	mitotic G2 DNA damage checkpoint	242	10	18.88	<1e-30
GO:0007052	mitotic spindle organization	569	38	44.39	<1e-30
GO:0019226	transmission of nerve impulse	867	51	67.64	<1e-30
GO:0000022	mitotic spindle elongation	240	16	18.72	<1e-30

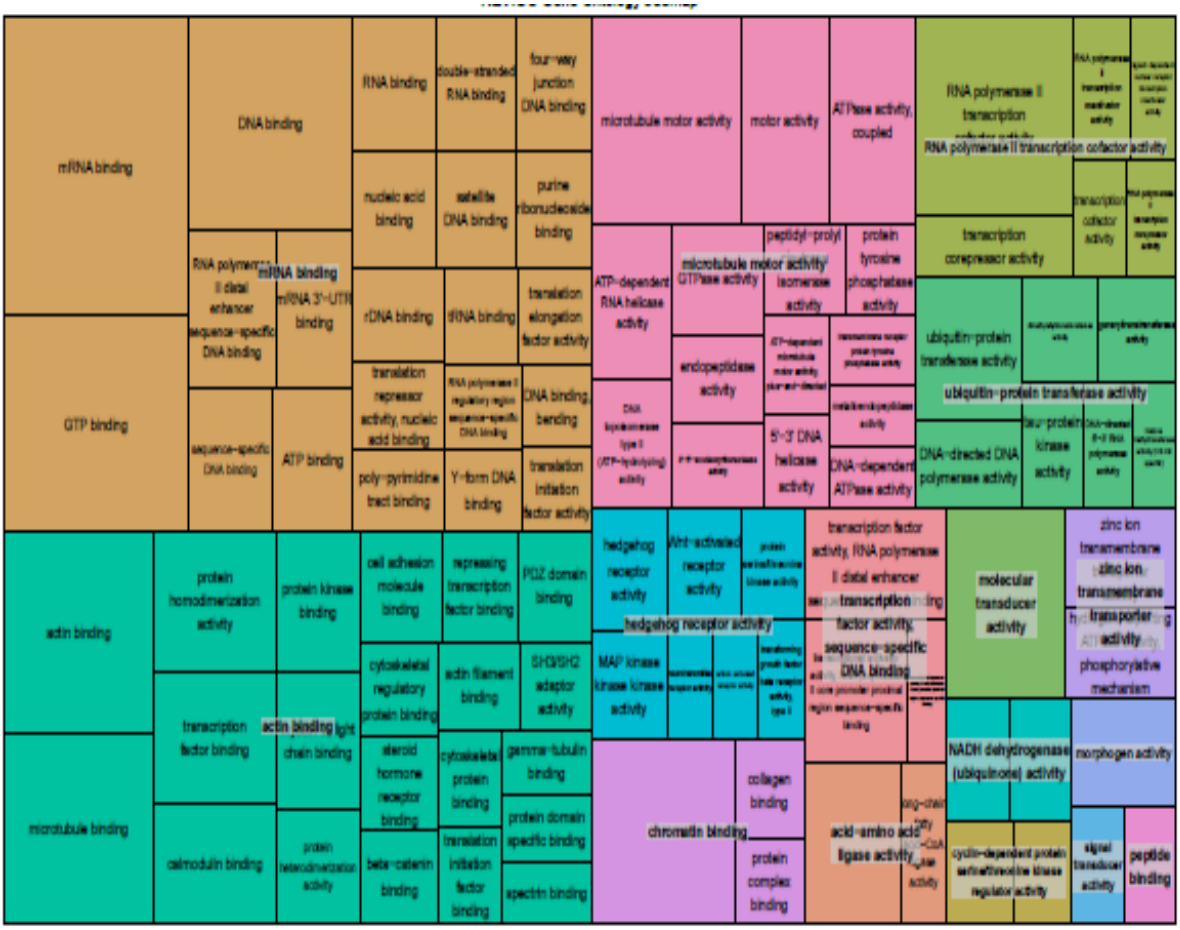

Figure 26. Gene Ontology (GO) annotation of molecular function category summarized using REVIGO of the 100 best GO terms by enrichment analysis in DEG transcripts

A: RNA processing and modification; B: chromatin structure and dynamics; C: energy production and conversion; D: cell cycle control, cell division, chromosome; partitioning; E: amino acid transport and metabolism; F: nucleotide transport and metabolism; G: carbohydrate transport and metabolism; H: coenzyme transport and metabolism; I: lipid transport and metabolism; J: translation, ribosomal structure and biogenesis; K: transcription; L: replication, recombination and repair; M: cell wall/membrane/envelope biogenesis; N: cell motility; O: posttranslational modification, protein turnover, chaperones; P: inorganic ion transport and metabolism; Q: secondary metabolites biosynthesis, transport and catabolism; R: general function prediction only; S: Function unknown; T: signal transduction mechanisms; U:intracellular trafficking, secretion, and vesicular transport; V: defense mechanisms; W: extracellular structures; Y: nuclear structure; Z: cytoskeleton

Figure 27. KOG Classification of differentially expressed unigenes of *S. frugiperda* strains resistant and susceptible to VTPRO

Most of the carboxypeptidase and alkaline phosphatase unigenes were down-regulated in the resistant strain (Fig. 28A). We identified 30 unigenes classified as Mucin; of these, 11 transcripts were overexpressed in the VTPro-Res strain and 19 transcripts were down-regulated in the same strain, with a log 2 fold change of into 6.48 and –5.89 (Fig. 28B).

Increases in the expression level were detected for 5 unigenes in the VTPro-Res strain associated with ABC subfamilies G8, C2 and A2, with a log2 fold change of around 1.82 – 4.95. However, the majority of the DEUs associated with ABC transporter genes were down-regulated in resistant larvae of *S. frugiperda*, such as unigenes associated with ABC family F (32636_c2_g2), ABC family B (30479_c0_g1; 28418_c0_g1; 23443_c2_g1), and ABC family G (31702_c0_g1; 19767_c0_g1; 31342_c1_g1; 29997_c0_g1; 32275_c1_g1) (Fig. 29).

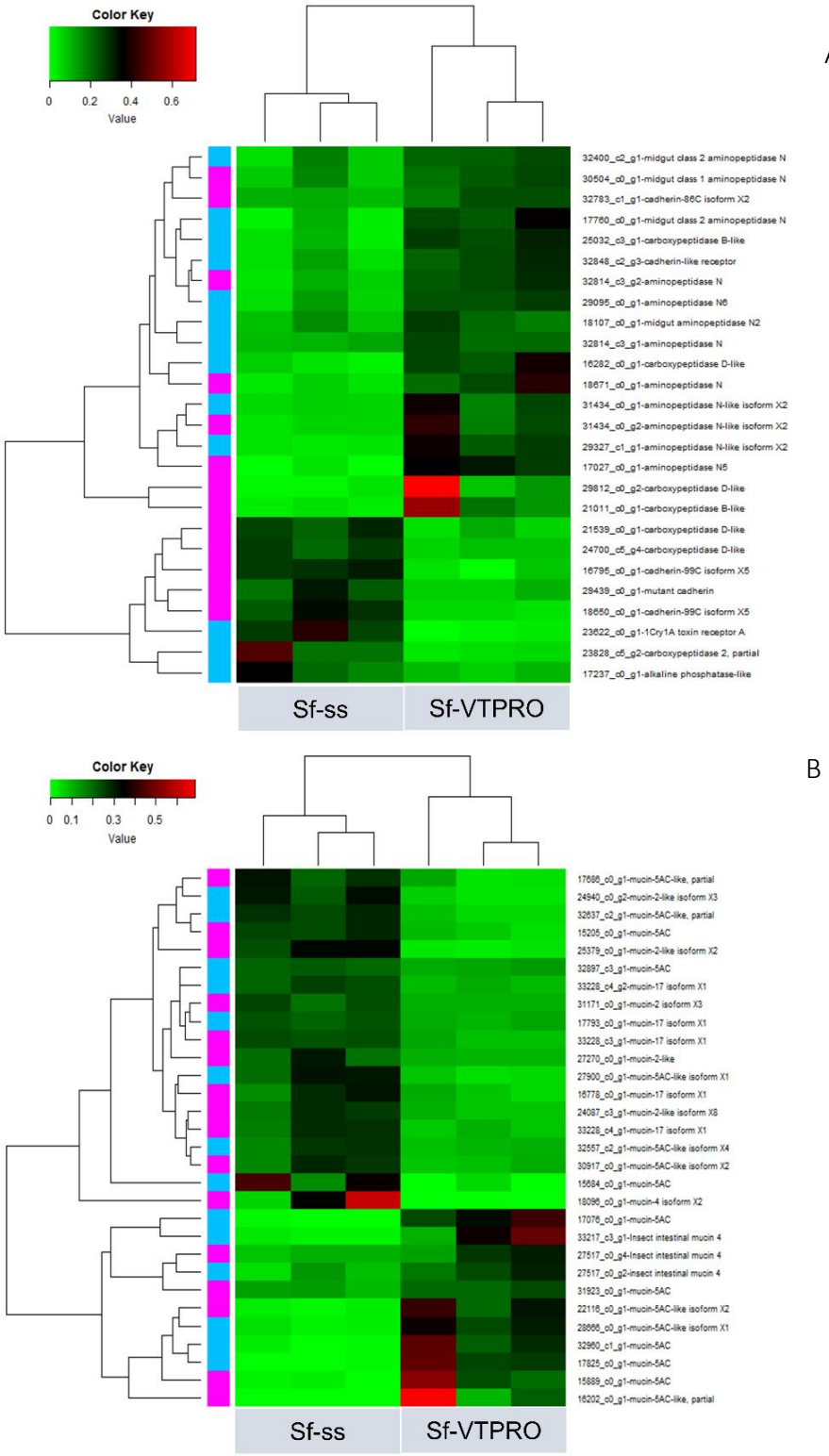

Figure 28. Heatmap of unigenes related to the mode of action of Bt toxins in lepiodptera (A) and mucins (B)

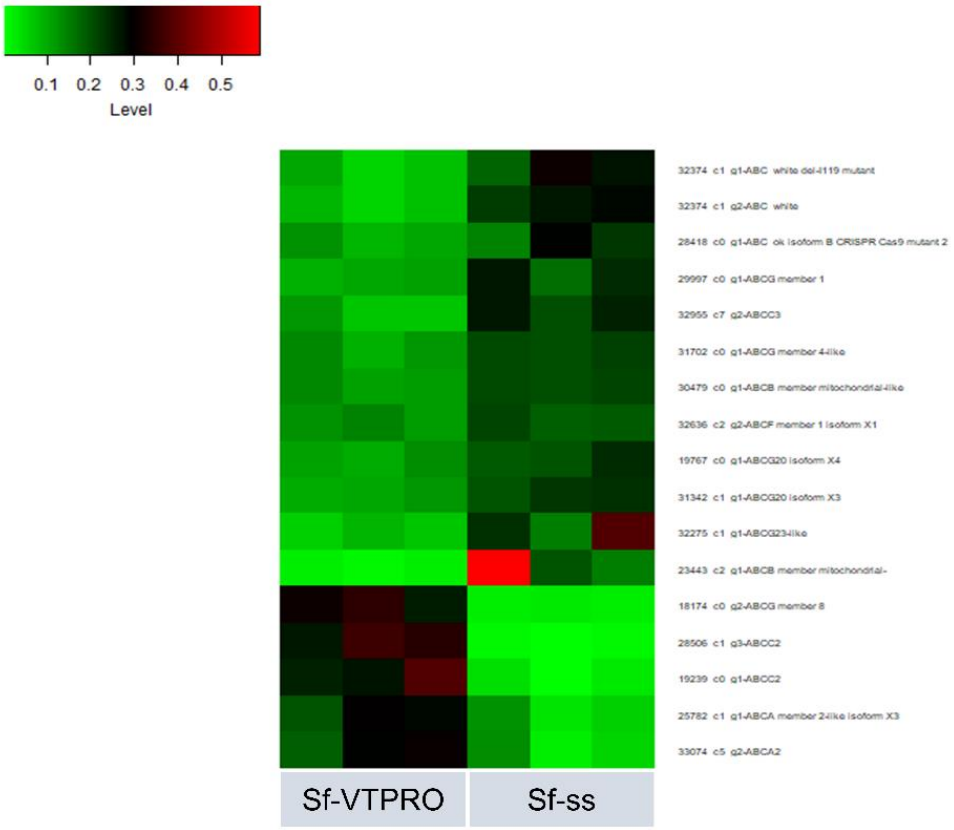

Figure 29. Heatmap of unigenes which shown functional identification with ABC transporter

A large number of unigenes associated with detoxification processes were identified in the DE analyses. We found a large number of up-regulated unigenes in the VTPro-Res strain, e.g. esterases (Fig. 30A) and P450s (Fig. 31). Most GSTs were down-regulated (Fig. 30B).

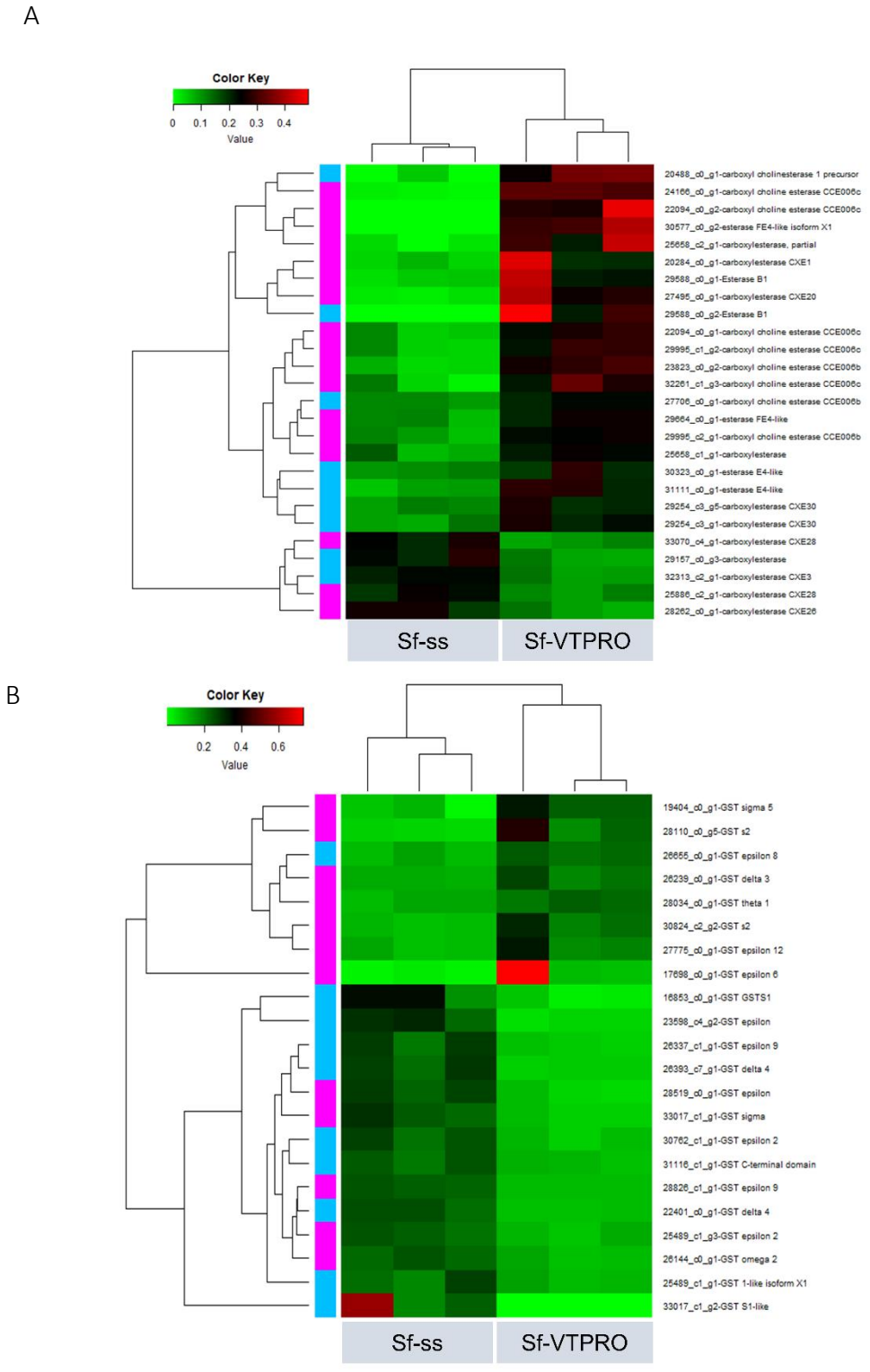

Figure 30. Heatmap of unigenes which shown functional identification with esterases (A) and GST's (B).

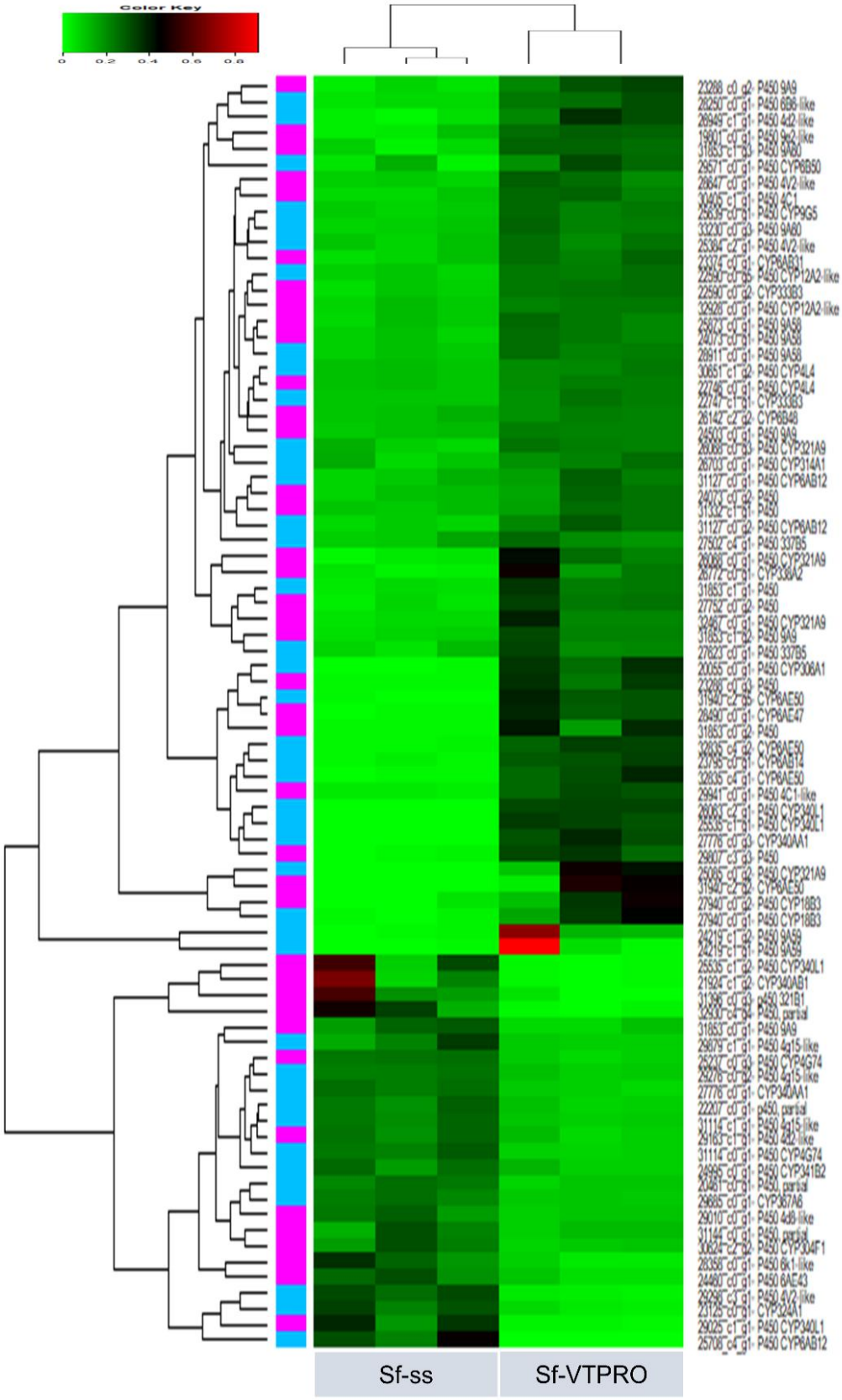

Figure 31. Heatmap of unigenes which shown functional identification with P450's

5.4. Discussion

Although the evolution of insect resistance to Cry toxins is critical to agriculture, the mechanisms of resistance to Cry toxins remain unclear. Modifications in the gene expression profile between resistant and susceptible insect strains to Cry toxins, mainly ABC transporter (Xu et al. 2015) and receptor genes, have been hypothetically associated with resistance processes. In this study, a resistant (VTPro-Res) and a susceptible strain (Sf-ss) to a VT-PRO event, which expressed Cry1A.105 and Cry2ab2, were investigated using robust RNA-Seq to analyze the modifications in gene expression of *S. frugiperda*.

Due to the decreasing cost and increasing acceptance of RNA-seq approaches, this technology has been increasingly used to identify the possible mechanisms of resistance in insects (Lin et al. 2013; Nascimento et al. 2015). In this study, three biological replicates of Sf-ss (susceptible strain) and VTPro-Res (resistant strain) were sequenced, using Illumina Hiseq 2500. Around 136.7 million high-quality reads were obtained, generating more than 44 thousand unigenes and 99,463 transcripts, with a mean length of 761.26 nt. These results will aid in the determination of the complete genome of *S. frugiperda* (Gouin et al. 2017). The transcripts were identified and annotated using public databases, including nr-NCBI, uniport, Pfam, GO, KOG, and KEGG. This information was crucial to identify possible candidate genes related to resistance of *S. frugiperda* to Yieldgard VT-PRO® event. Our DEG analyses revealed a large number of unigenes with alterations in the expression profile, with a total of 10,286 genes showing FDR < 0.01.

Up-regulated genes were more numerous among the KOG categories. DEG analyses indicated that the Cry1Ac-resistant strain of *Plutella xylostella* L. has more up-regulated than down-regulated unigenes (Cingolani et al. 2012; Lei et al. 2013), as identified in lufenuron-resistant *S. frugiperda* (Nascimento et al. 2015). The wider distribution of functional categories in DEG indicated that the resistance might result from several regulatory processes caused by strong selection pressure. These results agree with those found by Bernardi, who characterized the resistance of *S. frugiperda* to VT-PRO by heritability assays, which showed that the inheritance of resistance is polygenic (Bernardi et al. 2017). A large number of transcripts were associated with metabolic processes (GO:0008152) and with the KOG classification “carbohydrate transport and metabolism”, which may be related to the high fitness cost associated with resistance to a VT-PRO event (Bernardi et al. 2017).

Although the mode of action of Cry toxins is widely studied, the remaining gaps in knowledge impede understanding of the precise mechanism of resistance of insects to Bt

toxins. Currently, two hypotheses are accepted as models for the mode of action of *cry* toxins: the sequential binding model (Bravo et al. 2004), and the signaling pathway model (Zhang et al. 2005; Zhang et al. 2006).

Our results showed that cadherin receptors (CAD) are down-regulated in the resistant strain VTPro-Res. In both models, the cadherin receptor proved to be of crucial importance to the interaction between toxin and cell. The binding of the monomeric Cry toxin to the cadherin receptor causes an additional proteolytic activation of the toxin, where helix α1 of domain I is cleaved (Soberón et al. 2000; Gómez et al. 2002). However, the importance of CAD to the mechanism of action of Cry toxins is still under investigation. Several studies have reported down-regulation of the cadherin receptor, e.g: Cry1Ab resistance in *Diatraea saccharalis* (Fabricius) (Lepidoptera: Crambidae) (Yang et al. 2011), and Cry1Ac resistance in *Helicoverpa armigera* (L.) (Lepidoptera*:* Noctuidae) and *Ostrinia furnacalis* Guenée (Lepidoptera: Cramnidae) (Jin et al. 2014). Studies also report mutations of the CAD receptor in lepidopterans resistant to Cry toxins, such as *Pectinophora gossypiella* (Saunders) (Lepidoptera: Gelechiidae) (Soberón et al. 2007), *O. nubilalis* (Jin et al. 2014; Bel et al. 2008), *Heliothis virescens* (Fabricius) (Lepidoptera*:* Noctuidae) (Gahan et al. 2001), and *H. armigera* (Yang et al. 2006). Our hypothesis is that a low level of transcription of CAD genes, resulting in the disruption of the sequential binding model proposed by Bravo et al (2004), is one of the mechanisms responsible for VT-PRO resistance in *S. frugiperda*.

In the next step in the sequential binding mode of action (Bravo et al. 2004), toxin oligomers bind to the soluble ectodomains of membrane-associated glycosylated proteins such as aminopeptidase N (APN), alkaline phosphatase (ALP), or the BTR-270 glycoprotein. These proteins are GPI-anchored and enriched in lipid rafts, and disruption of lipid rafts by cholesterol depletion reduces pore formation by Cry toxins (Gahan et al. 2001). The low expression of ALP observed here may result in reduced Cry toxin binding to the brush border membrane in the resistant strain (Jurat-Fuentes et al. 2011).

The transcriptome in *S. frugiperda* resistant to VT-PRO events showed overexpression of the ABCC2 unigenes. This result is the opposite of those found by others. Investigating the midgut transcriptome in *Plutella xylostella* L. (Lepidoptera: Plutellidae), Lei and colleagues observed down-regulation of the *ABCC2* unigenes (Lei et al. 2013). The same pattern was observed for *O. furnacalis* resistant to Cry1Ab and Cry1Ac (Zhang et al. 2017) and for *P. xylostella* diamondback moths resistant to Cry1Ac (Guo et al. 2015). Multi-functional characteristics have been attributed to these genes, usually associated with regulation of lipid metabolism; therefore, down-regulation of ABCG might be linked to a higher fitness cost of

resistance of *S. frugiperda* to VT-PRO events. Although the ABC transporter has been reported as an important receptor in the interaction between the Bt toxin and midgut microvilli of insects, it might be involved in facilitating the Cry pre-pore formation in the lipid bilayer membrane (Heckel 2012). Although the function of the ABC transporter in the mode of action of Cry toxins is under discussion, some studies have reported that mutations in ABCC2 were responsible for the resistance of *H. armigera* to the Cry2Ab toxin (Tay et al. 2015), *H. virescens* to Cry1Ac (Gahan et al. 2010), and *S. exigua* (Lepidoptera: Noctuidae) to a biopesticide based on Cry1Ca (Park et al. 2014).

Detoxification processes have been not associated with resistance to Cry toxins. However, overexpression of ABCC2 and detoxification enzymes such as cytochrome P450 (CYP), acetylcholinesterases (CCE), and UDP-glycosyltransferases (UGT) related to resistance to insecticide compounds (Carvalho et al. 2013; Nascimento et al. 2015; Liu et al. 2016) might be supporting the mechanism of resistance to Cry toxins, helping to inactivate and excrete xenobiotics.

Our RNA-seq between VTPRO-resistant and susceptible strains of *S. frugiperda* showed that the mechanism of resistance to Cry toxins may be the result of processes of genetic regulation involving receptors, signaling, and detoxification enzymes. These results illustrate the importance of systematic studies to better understand the processes related to the evolution of resistance in insects. The use of high-performance tools including genomic, transcriptomic, proteomic, and metabolomic assays is essential to understand the adaptive processes of insects in agroecosystems.

5.5. Conclusions

- Aminopeptidase genes were up-regulated in the VTPRO-resistant strain;
- Most of the carboxypeptidase and alkaline phosphatase genes were down-regulated in the VTPRO - resistant strain;
- Fifty-five cytochromes P450 and twenty-one esterases were overexpressed in resistant strain.

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6. DISCOVERY 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.

6.2. Material and Methods

6.2.1. Insects

A total of 70 individuals of *S. frugiperda* were collected from corn-fields on several regions of Brazil (Tab 1). Field collected populations were selected against insecticides and Bt toxins using protocols described earlier in last chapters.

Table 16. Resistant strains of *Spodoptera frugiperda* to several insecticides compounds and Bt toxins

Strain	Compound	Group	Sampling area
Sus	-	-	-
Clo-RR	Chlorpyrifos	Organophosphate	Luis Eduardo Magalhães, BA
Lam-RR	Lambda-cihalothrin	Pyrethroid	Correntina, PR
Tef-RR	Teflubenzuron	Benzoylureas	Sapezal, MT
Luf-RR	Lufenuron	Benzoylureas	Montevideu, GO
Spi-RR	Spinosad	Spinosin	Luis Eduardo Magalhães, BA
VTPRO-RR	Cry1A105 + Cry2Ab2	*Bacillus thuringiensis*	Luis Eduardo Magalhães, BA

6.2.2. DNA extraction and GBS library preparation

DNA was extracted using the modified CTAB method (Doyle and Doyle 1990). Briefly, 50 mg larval tissue of individual *S. frugiperda* was macerated in 650 uL of extraction buffer containing 2% Cetyltrimethyl ammonium bromide (CTAB), 1.4M of NaCl, 100 mM tris(hydroxymethyl)aminomethane (Tris-HCl) at pH 8.0, 20 mM ethylene diamine tetra acetic acid (EDTA) at pH 8.0, 1% polyvinylpyrrolidone, 0.2% β-Mercaptoethanol, and 20 µL

proteinase K (0.1 ug·mL-1). Samples were incubated at 55 °C for 1 h, followed by addition of 650 uL of Chloroform:Isoamyl Alcohol (24:1) and mixed until the formation of an emulsion. Samples were centrifuged (14,000 g x 5 min x 4°C) for supernatant collection. After, 200 uL of the same extraction buffer above except for β-Mercaptoethanol and proteinase K was added, and one volume of Chloroform:Isoamyl Alcohol (24:1). Emulsion was vortexed and centrifuged (14,000 g x 5 min x 4°C) for supernatant collection; this process was repeated 3 times. Following, 650 uL of cold isopropanol was added to the samples, and the mixture was incubated at -20 °C overnight before centrifugation (14,000 g x 5 min x 4°C). The DNA was washed with 1 mL 70% ethanol twice. The pellet was dried in room temperature and resuspended in 40 µL TE and RNAse A (10 ug.mL-1).

An extra purification step was used to guarantee integrated and pure DNA. A aliquot of 10 ul of diluted DNA was used. It was added 50 ul of PB buffer (Qiagen®) to the aliquot and the solution was mixed by pipetting and transfered to filter plate (AcroPrep advance 96 well filter plates, PALL® Life Science). The product was centrifuged for 2 minutes at 3000 g. Elution was discarded and 250 ul PE buffer (Qiagen®) was added at the membrane, and centrifuged for 1 minute at 3000 g; this step was repeated twice. Finally, 30 µL TE was added and centrifuged for 1 minute at 3000 g to elute of genomic DNA. Final DNA concentration was measured using Qubit™ dsDNA High Sensitive Assay Kit (ThermoFisher Scientific®), following manufactory instructions, and stored at -20°C.

Genotyping-by-sequencing libraries were prepared using standard protocol described by Elshire et al (2011) with modifications at the Molecular and Cellular Imaging Center, The Ohio State University (Ohio Agricultural Research and Development Center (OARDC), Wooster, United State). Genomic DNA from individual samples was digested with *Mse*I restriction enzyme (New England Biolabs, Ipswich, MA, USA). We used 2 spacers (internal adapter) with 3 to 7 bp, one on each side of sequence flanking the restriction site and the external barcode on the 3’ of its top stand (Appendix). Library amplicons was sequenced in an Illumina HiSeq 2500 platform using the 125 paired end protocol.

6.2.3. Strain identification

Spodoptera frugiperda present two host strains (corn and rice-strain). To certificate that all individuals of our strains were of the same host strain, all samples were genotyped Thus, each sample was identified using length polymorphism associated to the mitochondrial COI gene, making use of restriction enzymes *MspI*. Primers proposed by Levy et al. (2002)

were used. PCR reaction were made in total volume of 25 μL volume reaction containing 1x GoTaq buffer (Promega®), 2 mM MgCl 2, 0.01 mM dNTP, 0.001 μM primers, 1 U Taq DNA polymerase (Promega®) and 100 ng of Total DNA. Conditions for thermocycling were 1 min incubation at 100 ° C, followed by 30 cycles of 1 min denaturation at 94 ° C, 1 min annealing at 58 ° C and extension of 7 min at 72 ° C. With final moment of 7 min of 72 ° C (Juarez et al. 2012). Amplicons were visualized on 2% agarose gel stained with ethidium bromide with estimated molecular weight by 100 bp markers (Promega®). Thereafter, PCR products were purified, and digested using the *Msp*I enzyme. Samples were digested by incubating at 37 °C for 30 minutes, after which the bands were separated and identified from agarose gel (2%).

Demultiplexing and SNP calling

Paired end reads obtained from sequencing Illumina HiSeq2500® platform were assessed for quality using FastQC v0.11.5 (Andrews 2010). Samples demultiplexing and SNP calling were performed using the software Stacks (Catchen et al. 2013). Raw reads were demultiplexed and cleaned using process_radtags script. Next, sequences were alignned against the draft genome of *S. frugiperda* (Gouin et al. 2017) as reference, using Bwa (Li and Durbin 2010). The pstacks was used to compare all .bam files from each sample. It was estabelish a set of loci and SNPs at each locus using a maximum likelihood framework (Hohenlohe et al. 2010). Next, cstacks was used to bulit a catalog with a set of concensus loci. sstacks matched each sample against the catalog in order to define the allelic state at each locus.

6.2.4. Outlier detection

The software Bayescan 2.1 (Foll and Gaggiotti 2008) was used to detected loci putatively under selection using the Bayesian likelihood method via reversible-jump Monte Carlo Markov chain (MCMC). Generally, such Bayesian approaches assume that allele frequencies within populations follow a Dirichlet distribution, estimating the probability that each locus is subject to selection using a Bayesian method. The difference in allele frequency between this common gene pool and each subpopulation was measured by a subpopulation specific F_{ST} coefficient. We run analysis under default parameters. Loci were considered to be candidates under selection if FDR < 0.047.

6.2.5. Population Genetics

Populations package in Stacks was used to computing a number of population genetics statistics as well as to create all input files necessary to others analysis. Defaut paramentrs were used, with especific values of r = 0.50, p = 4, and min_maf = 0.05. When necessary, the data was converted using PGDSpider (Lischer and Excoffier 2011).

A discriminant analysis of principal components (DAPC) (Jombart et al. 2010) was used to give a visual evaluation of the genetic structure of strains of *S. frugiperda*, using the package adegenet (Jombart 2008). Adegenet was also used to compute contributions of each allele to the clustering pattern, with fixed threshold = 0.001.

Additionally, the package NetviewP was used to estimated population structure using a network-based approach (Neuditschko et al. 2012), using Nearest Neighbors k-NN=5. Input files were created with the package *population* (Stacks), and the genetic-distance matrix o all samples was computed with Plink 1.7 (Purcell et al. 2007).

6.2.6. Association analyses

To investigate candidate SNPs involved on resistance mechanisms we applied a standard case/control association analyses between resistant strains (cases) and susceptible strain (control) available on the package Plink 1.7 (Purcell et al. 2007). Inputs files *.ped and *.map were created directly from the package *population* (Stacks).

6.2.7. Genome annotation

Loci putatively under selection and significant in the association test were mapped and alignned against the draft genome of *S. frugiperda* (Gouin et al. 2017) as referenc, using BWA (Li and Durbin 2010). The functionl annotations of those loci were conducted with the software SnpEff (Cingolani et al. 2012).

6.3. Results and discussion

All individual samples evaluated were characterized as corn strain according to the *MspI* site in COI.

The number of mapped reads were 2.4 million per sample, with 89% of them mapped to the reference genome. The SNP calling from stacks recovered 4276 SNPs after all filtering procedures using the package *population*.

We detected 53 statistically significant polymorphic loci under selection based on False Discovery Rate ≤ 0.047 (Fig. 1), all of them with $\alpha > 1.03$ and $P > 0.68$. Among these, 48 loci had log10 values of PO above 1.5, indicating strong selection, and 18 loci presented decisive selection. Jeffreys (1961), proposed a scale classify selection where log10PO > 0.5 substantial selection; log10PO > 1 strong selection; $\log_{10}PO > 1.5$, very strong and $\log_{10}PO > 2$ decisive selection.

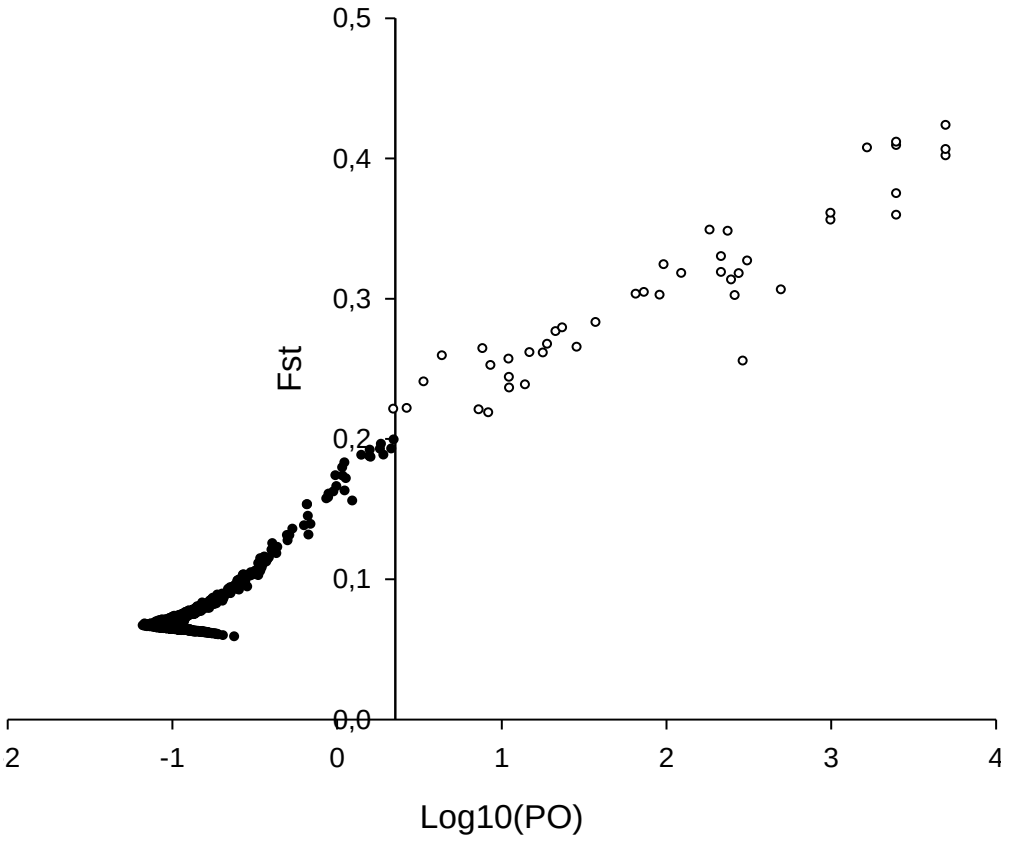

Figure 32. F_{ST} plotted against the log10(PO). The vertical line shows the threshold PO used for identifying outlier loci.

Annotation of variants under selection indicated 20 SNPs associated with intergenic regions and 34 loci related to functional regions of the genome (Tab 2). Most of the SNPs were from upstream gene variant (12), downstream gene variant (7) and intron variant (10). None of the SNPs putatively under selection was associated with coding regions. This result

strengthens the need for increasing studies on regulatory regions of the genome in entomology. High number of SNPs on upstream and downstream genes variant may be indicative that the selective pressure occurs on promoter regions, although many variation might indeed be functionally neutral (McGillivray et al. 2018). Regulatory sequences might be located outside the targeted regions, however, more than 90% the polymorphisms functionally validated are located in cis-regulatory regions (Rockman and Wray 2002).

The DAPC including resistant strains as *prior* information resulted on a visual assessment between group structures, which recovered seven clusters (Fig. 2). The susceptible strain is distant from all resistant strains (Figs. 2 and 3), Clo-RR strain sets an exclusive group, while the other strains cluster together (Spi-RR, Luf-RR, Tef-RR VTPRO-RR and Lam-RR). This pattern is also present when the density of individuals and function discriminant plot were plotted (Fig. 3). The distance between Sf-ss and the other strains can be associated to the laboratory conditions. Sf-ss has been maintained under laboratory rearing conditions for many years, which would result in genetic drift. On the other hand, resistant strains were collected from the field more recently. Many types of genetic change can occur during long period in laboratory or control conditions, such as change in allele frequencies and loss of genetic variation due to genetic drift, usually associated with founder effect and inbreeding depression (Frankham and Loebel 1992).

The resistant strain Clo-RR found apart from the other strains can be related to differences on the level of resistance among them. While Clo-RR presented resistance ratio ≈ 27-fold, all other resistant strains showed resistance ratio greater than 200-fold. Organophosphates are known to cause strong selection pressure, however the widespread adoption of Bt plants to control *S. frugiperda* has been reducing the spray of the first-generation insecticides, as chlorpyrifos. The resistance ratio of these insecticides is still high in *S. frugiperda* (Carvalho et al. 2013), which is related to the strong fitness cost associated to resistance to organophosphates (Djogbénou et al. 2010) (Shi et al. 2004).

The same pattern can be observed on the network built based on Netview analysis (Fig. 4). All individuals from Sus and Clo-RR strains grouped as two isolated clusters, and a third complex structure was established with all but two remaining individuals from VTPRO-RR and one individual from Lam-RR strains, which clustered isolated from the main clusters.

Tab 2. List of SNPs putatively under selection, according to Bayescan results.

Table 17. List of SNPs putatively under selection, according to Bayescan results

SNP ID	Locate	Description
7618_3	upstream_gene_variant	gamma-aminobutyric acid type B receptor subunit 1-
51391_51	upstream_gene_variant	pancreatic triacylglycerol lipase-like
103444_81	upstream_gene_variant	reverse transcriptase
119438_77	upstream_gene_variant	fatty acyl reductase FAR12
143976_45	upstream_gene_variant	uncharacterized protein LOC110379650
198343_50	upstream_gene_variant	uncharacterized protein LOC110383219 isoform X2
205425_83	upstream_gene_variant	uncharacterized protein LOC110375326
236991_75	upstream_gene_variant	-
236990_69	upstream_gene_variant	-
241653_69	upstream_gene_variant	-
271547_74	upstream_gene_variant	-
315457_20	upstream_gene_variant	Calcium-binding mitochondrial carrier protein SCaMC-2
4929_21	intron_variant	mitochondrial enolase superfamily member 1-like
236467_89	intron_variant	1-acyl-sn-glycerol-3-phosphate acyltransferase alpha-like
236469_51	intron_variant	1-acyl-sn-glycerol-3-phosphate acyltransferase alpha-like
236473_65	intron_variant	1-acyl-sn-glycerol-3-phosphate acyltransferase alpha-like
236473_35	intron_variant	1-acyl-sn-glycerol-3-phosphate acyltransferase alpha-like
308859_66	intron_variant	heparan-alpha-glucosaminide N-acetyltransferase-like
308860_13	intron_variant	heparan-alpha-glucosaminide N-acetyltransferase-like
315027_61	intron_variant	polycomb protein l(1)G0020
315027_22	intron_variant	polycomb protein l(1)G0020
315087_9	intron_variant	GTPase-activating protein CdGAPr
50568_78	synonymous_variant	uncharacterized protein LOC110380377 isoform X6
198353_82	synonymous_variant	uncharacterized protein LOC110383219 isoform X2
21251_88	3_prime_UTR_variant	-
205796_20	5_prime_UTR_variant	-
2715_11	downstream_gene_variant	armadillo repeat-containing gudu
236626_87	downstream_gene_variant	-
241476_68	downstream_gene_variant	Catenin alpha, partial
279723_12	downstream_gene_variant	pyridoxal phosphate phosphatase PHOSPHO2-like
291765_58	downstream_gene_variant	zinc transporter ZIP13 homolog
291766_35	downstream_gene_variant	zinc transporter ZIP13 homolog
291767_20	downstream_gene_variant	zinc transporter ZIP13 homolog

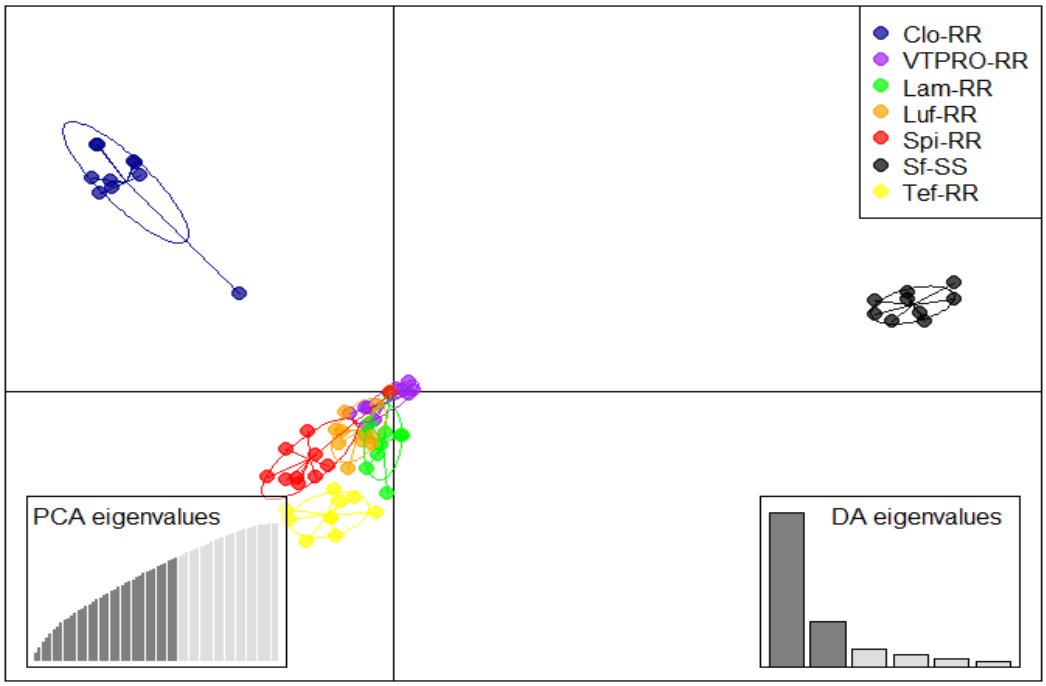

Figure 33. Discriminant analysis of principal components (DAPC) of samples by strains. In scatterplot clusters are shown by diffenret colours and inertia ellipses, dots represent individuals

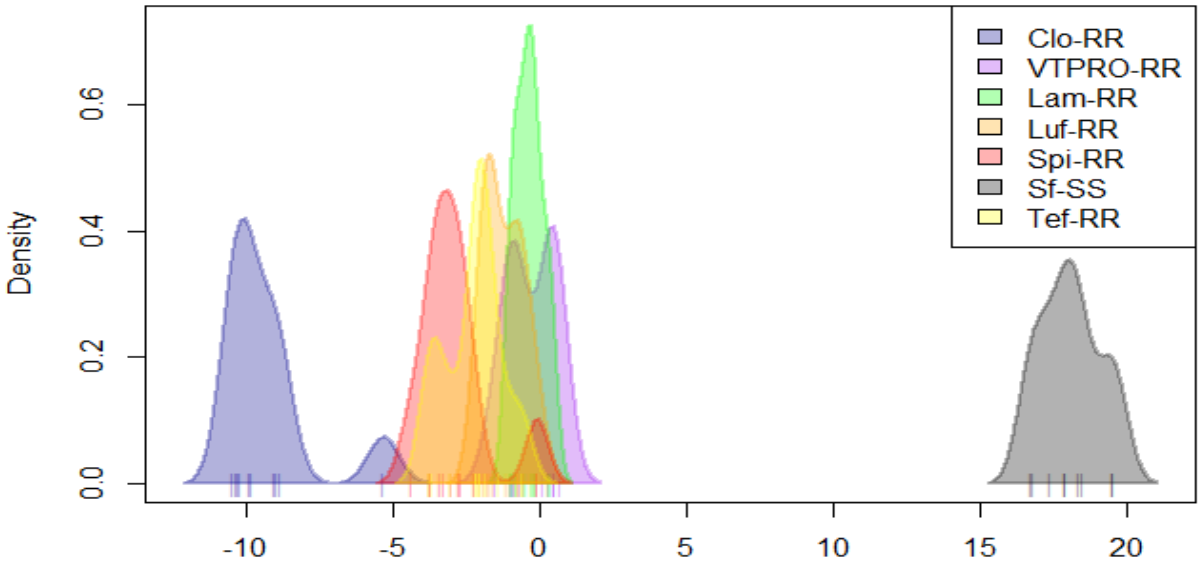

Figure 34. Discriminant analysis of principal components (DAPC) the best discrimination of individuals into pre-defined groups.

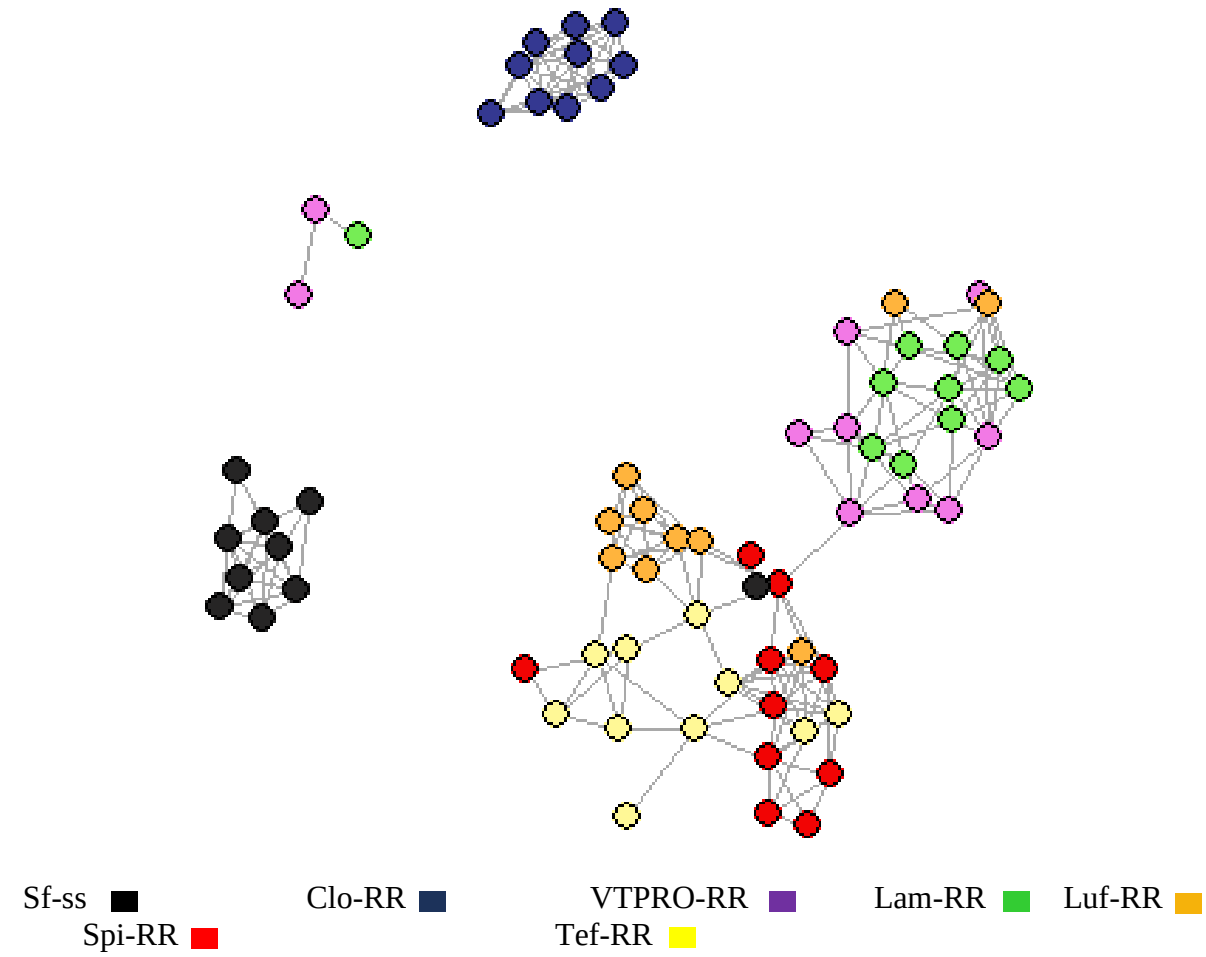

Figure 34. . Network of resistant and susceptible strains of *S. frugiperda* to insecticides and Bt toxins, at k =8, based on 4276 SNPs

Seventy-one loci were above the threshold of 0.001 fixed to estimate the contributions of alleles to the clustering pattern (Fig. 5). None of those loci were putatively under selection according to the Bayescan analysis. Loci putatively under selection are not responsible for the cluster pattern we found, as found for field populations of *S. frugiperda* (Silva-Brandão et al. 2018). The annotation of these loci recovered intergenic regions (36), intron variants (11), upstream variants (20), downstream variants (2), 5 prime UTR variants (1) and missense variant (1). KOG classification showed loci associated to RNA processing and modification (2), energy production and conversion (1) and lipid transport and metabolism (1).

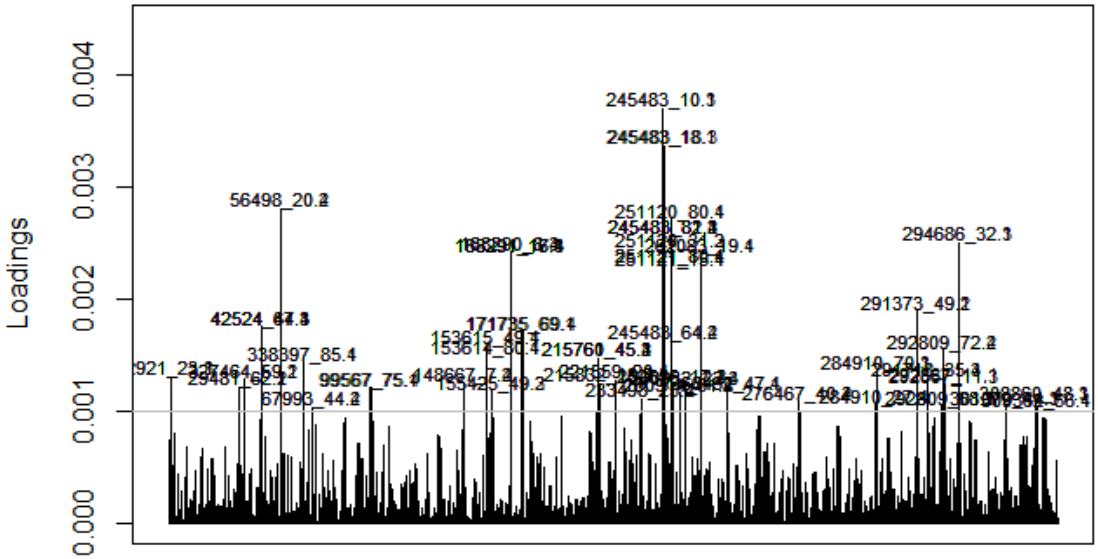

Figure 36. Loci contributions to clustering pattern above the threshold of 0.001.

The association analyses between the susceptible and resistant strains indicated 114 loci significantly associated to Clo-RR, 105 loci associated to Lam-RR, 84 loci to Luf-RR, 87 loci to Tef-RR, 108 loci to Spi-RR and 62 significantly loci associated to VTPRO-RR ($P < 0.01$). The venn diagram indicated that 17 loci are associated to all resistant strains (Tab 3).

Table 18. Significant SNPs on association test between susceptible and all resistant strain (p < 0.01)

SNP	Locate	Description
2921_23	UP	nuclear inhibitor of phosphatase 1
40143_48	UP	nucleolar 8
50561_33	UP	uncharacterized protein LOC110380377 isoform X6
50561_7	UP	uncharacterized protein LOC110380377 isoform X6
77488_71	UP	centrosomal of 120 kDa-like
117228_19	IN	cyclic AMP response element-binding A
148688_25	IN	PREDICTED: protein IMPACT-like
205791_47	UP	uncharacterized protein LOC107451535
226307_72	DW	Uncharacterized protein OBRU01_07656
263222_15	MI	Ankyrin repeat domain
280150_33	IN	androgen-dependent TFPI-regulating -like
301089_52	IN	hypothetical protein g.10399
301089_40	IN	hypothetical protein g.10399
303995_51	SY	hypothetical protein g.7842, partial
308859_47	IN	heparan-alpha-glucosaminide N-acetyltransferase-like
308859_66	IN	heparan-alpha-glucosaminide N-acetyltransferase-like

Here, we discuss only on exclusive loci associated to each strain. Most exclusive SNPs were located on intergenic regions, following by upstreams and downstream variations in all resistant strains. We obtained less than 20 SNPs in each strains on exon and introns regions (Fig 6). A large number of SNPs were associated with coding and regulators regions of the genome; however, none were associated with mechanisms of resistance previously reported in insects. Tables 4-7 lists the annotation of exclusive variants, considering association test with $p < 0.01$.

Mutations on resistant strains to insecticides and Bt varieties are considered one of the main mechanisms found in insects (Gahan et al 2001, Morin et al 2003). Many targets and pathways are associated to resistance to insecticides, such as pyrethroids, organophosphate, benzoylureas, spinosins and Bt toxins, and most of the research has been developed only on those targets. Here we found mutations beyond the expected genes, pathways and targets. These results are expected with the use of GBS since this technique allow a broader research for SNPs. The use of GBS for insect resistant research is opening a new set of genes, pathways and potential targets to be investigated, which may be critical to understand insect resistance and rapid adaptation. We argue that although a large body of research is being done on insect resistant traits, many questions are still open, and this may be caused by a narrowed view focusing mostly on detoxification. Therefore, our results support the use of deep genome sequencing to complete insect resistance studies and potentially find new targets for management.

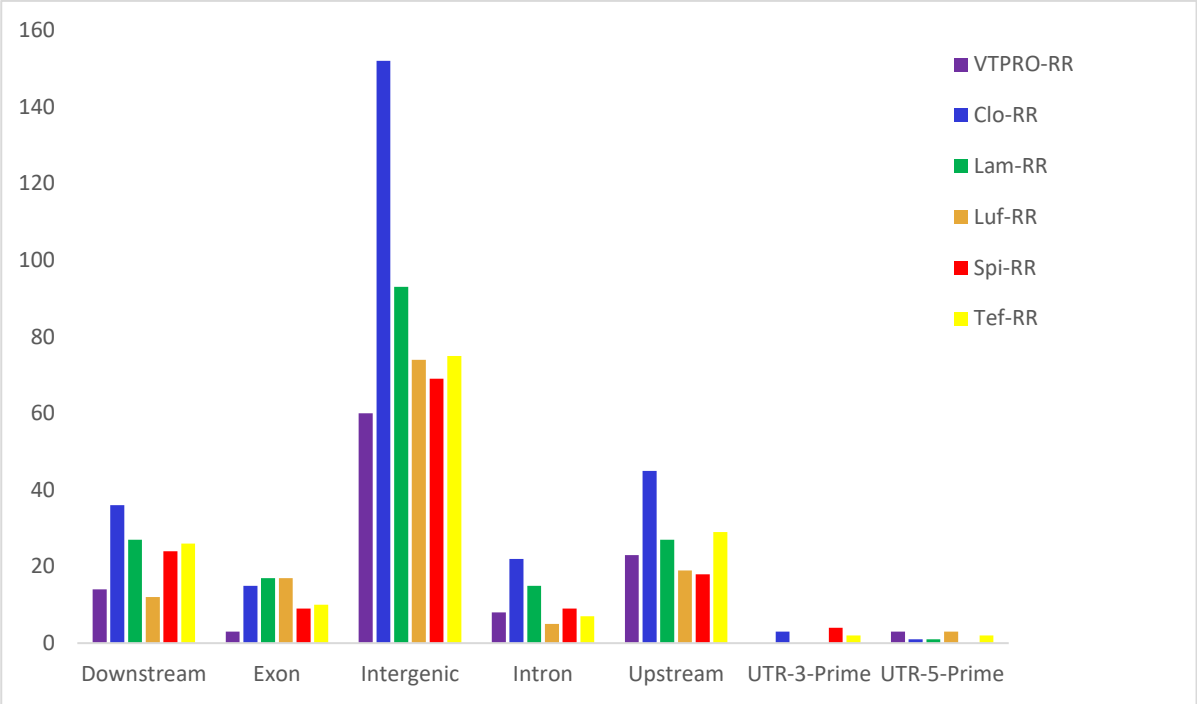

Figure 37. Number of exclusive SNPs by region which presented $p < 0.05$ by pairwise association test between resistant and susceptible strains.

Table 19. List of exclusive significant SNPs on association between susceptible and resistant strain to spinosad ($p < 0.01$)

SNP	Locate	Description
67912_78	IN	uncharacterized protein LOC110383125 isoform X3
90505_37	IN	UPF0183 CG7083
175942_79	IN	ectonucleoside triphosphate diphosphohydrolase 5 iso X1
236473_65	IN	1-acyl-sn-glycerol-3-phosphate acyltransferase alpha-like
295705_48	IN	Uncharacterized protein LOC110381109, partial
308860_52	IN	heparan-alpha-glucosaminide N-acetyltransferase-like
315107_40	IN	SID1 transmembrane family member 1-like
23842_85	MI	gag-pol poly
23845_81	MI	gag-pol poly
24332_51	MI	hypothetical protein g.46121
263222_45	MI	Ankyrin repeat domain
50574_10	SY	uncharacterized protein LOC110380377 isoform X6
221559_84	SY	reverse transcriptase
247710_81	SY	NADH dehydrogenase subunit 3 (mitochondrion)
263229_73	SY	Ankyrin repeat domain
276459_67	SY	protein LOC105396011

Table 20. List of exclusive significant SNPs on association between susceptible and resistant strain to lufenuron ($p < 0.01$).

SNP	Locate	Description
287782_6	5UTR**	retrotransposon-like family member retr-1
89200_79	IN	fatty alcohol acetyltransferase
91698_43	IN	hypothetical protein
278512_24	IN	rho GDP-dissociation inhibitor 1 isoform X2
301089_33	IN	hypothetical protein g.10399
80189_74	MI	protein LOC105841383
80189_34	MI	protein LOC105841383
148570_55	MI	endonuclease and reverse transcriptase
149218_88	MI	hypothetical protein, partial
277221_70	MI	protein LOC106710629
80189_43	SY	protein LOC105841383
149218_6	SY	hypothetical protein, partial
744572_35	SY	DNA replication licensing factor Mcm2
188871_18	SY	hemicentin-1-like isoform X1
263204_21	SY	protein LOC105842936
276456_25	SY	protein LOC105396011
276459_19	SY	protein LOC105396011
276465_43	SY	protein LOC105396011

Table 21. List of exclusive significant SNPs on association between susceptible and resistant strain to Lambda cyhalotrin ($p < 0.01$).

SNP	Locate	Description
5109_28	5UTR	hypothetical protein HELRODRAFT_164187
117226_18	IN	cyclic AMP response element-binding A
131077_49	IN	galactokinase-like
136648_15	IN	fatty acid synthase-like
211215_59	IN	unknown, partial
278943_82	IN	serine protease snake-like
300741_24	IN	uncharacterized protein LOC110381853
301089_34	IN	hypothetical protein g.10399
302230_32	IN	uncharacterized protein LOC110381914
308859_6	IN	heparan-alpha-glucosaminide N-acetyltransferase-like
308859_10	IN	heparan-alpha-glucosaminide N-acetyltransferase-like
24159_15	MI	Uncharacterized protein, partial
148722_32	MI	PREDICTED: protein IMPACT-like
148722_22	MI	PREDICTED: protein IMPACT-like
744572_31	MI	DNA replication licensing factor Mcm2
198353_72	MI	uncharacterized protein LOC110383219 isoform X2
198355_10	MI	uncharacterized protein LOC110383219 isoform X2
276452_84	MI	PREDICTED: uncharacterized protein LOC105396011
198353_55	start_lost	uncharacterized protein LOC110383219 isoform X2
102893_9	stop_gained	uncharacterized protein LOC110381211
23838_70	SY	gag-pol poly
50563_40	SY	uncharacterized protein LOC110380377 isoform X6
744572_29	SY	DNA replication licensing factor Mcm2
744572_47	SY	DNA replication licensing factor Mcm2
223211_72	SY	PREDICTED: uncharacterized protein LOC106131092

Table 22. List of exclusive significant SNPs on association between susceptible and resistant strain to chrlopirifos ($p < 0.01$).

SNP	Locate	Description
272716_50	3UTR	retrovirus-related Pol poly from transposon isoform X4
5488_62	IN	aminopeptidase N5
5488_21	IN	aminopeptidase N5
29483_35	IN	ATP-dependent RNA helicase DHX57
29482_11	IN	ATP-dependent RNA helicase DHX57
67912_10	IN	uncharacterized protein LOC110383125 isoform X3
96604_84	IN	Pancreatic lipase-related 2
117226_9	IN	cyclic AMP response element-binding A
136490_84	IN	octopamine receptor Oamb isoform X2
161510_89	IN	CD109 antigen-like
182810_81	IN	calcium-dependent secretion activator isoform X15
213442_49	IN	UHRF1-binding protein 1-like isoform X1
261751_5	IN	peroxisomal N(1)-acetyl-spermine spermidine oxidase-like isoform X3
287981_6	IN	luciferin 4-monooxygenase-like
302226_20	IN	uncharacterized protein LOC110381914
308859_50	IN	heparan-alpha-glucosaminide N-acetyltransferase-like
308860_45	IN	heparan-alpha-glucosaminide N-acetyltransferase-like
24333_56	MI	hypothetical protein g.46121
223214_51	MI	PREDICTED: uncharacterized protein LOC106131092
275996_45	MI	RING finger 17
277211_4	MI	PREDICTED: uncharacterized protein LOC106710629
23838_9	SY	gag-pol poly
48298_12	SY	uncharacterized protein LOC110372877
148690_61	SY	PREDICTED: protein IMPACT-like
188871_25	SY	hemicentin-1-like isoform X1
188871_13	SY	hemicentin-1-like isoform X1
188871_8	SY	hemicentin-1-like isoform X1
263226_80	SY	Ankyrin repeat domain
275996_29	SY	RING finger 17
276472_3	SY	PREDICTED: uncharacterized protein LOC105396011
293257_38	SY	uncharacterized protein LOC110372466

Table 23. List of exclusive significant SNPs on association between susceptible and resistant strain to YieldGard VT-Pro ($p < 0.01$).

SNP	Locate	Description
309359_51	5UTR	nuclease HARBI1
37445_12	5UTR	RNA-directed DNA polymerase from mobile element jockey-like
198353_19	5UTR	uncharacterized protein LOC110383219 isoform X2
5105_33	DW	hypothetical protein HELRODRAFT_164187
301106_51	DW	rRNA 2 -O-methyltransferase fibrillarin
301105_71	DW	rRNA 2 -O-methyltransferase fibrillarin
291767_20	DW	zinc transporter ZIP13 homolog
154204_13	IN	charged multivesicular body 4
269107_62	IN	glucosidase II alpha-subunit
296370_66	IN	protein GPR107
272895_73	MI	skin secretory xP2-like
198353_70	MI	uncharacterized protein LOC110383219 isoform X2
130682_49	UP	cytochrome P450 9A58
130682_21	UP	cytochrome P450 9A58
130682_30	UP	cytochrome P450 9A58
302789_41	UP	G2 mitotic-specific cyclin-B isoform X1
324177_54	UP	GTP-binding 1
24608_42	UP	GTP-binding 1
56590_20	UP	odorant receptor 10a-like
223229_67	UP	PREDICTED: uncharacterized protein LOC106131092
163157_57	UP	tick transposon
163159_50	UP	tick transposon
21461_58	UP	uncharacterized protein LOC110370955 isoform X1
158027_40	UP	uncharacterized protein LOC110373219 isoform X1
294685_75	UP	uncharacterized protein LOC110374949
294685_30	UP	uncharacterized protein LOC110374949
143976_27	UP	uncharacterized protein LOC110379650

Table 24. List of exclusive significant SNPs on association between susceptible and resistant strain to teflubenzuron ($p < 0.01$).

SNP	Locate	Description
280388_55	3UTR	pol poly
198356_46	3UTR	uncharacterized protein LOC110383219 isoform X2
299499_84	DW	alanine aminotransferase 1
299499_38	DW	alanine aminotransferase 1
299499_29	DW	alanine aminotransferase 1
299499_25	DW	alanine aminotransferase 1
222183_37	DW	apoptosis-stimulating of p53 protein 1 isoform X2
276280_9	DW	armadillo repeat containing 3
180182_8	DW	CDKN2A-interacting -like
304966_87	DW	cytoplasmic FMR1-interacting -
295157_37	DW	piggyBac transposable element-derived 4-like isoform X5
260936_70	DW	PREDICTED: uncharacterized protein LOC106106354
291766_35	DW	zinc transporter ZIP13 homolog
44860_66	IN	2-oxoglutarate mitochondrial
5277_66	IN	aminopeptidase N-like
148667_7	IN	PREDICTED: protein IMPACT-like
90500_77	IN	UPF0183 CG7083
276467_46	MI	PREDICTED: uncharacterized protein LOC105396011
223211_26	MI	PREDICTED: uncharacterized protein LOC106131092
277221_43	MI	PREDICTED: uncharacterized protein LOC106710629
198363_73	MI	uncharacterized protein LOC110383203
198356_22	MI	uncharacterized protein LOC110383219 isoform X2
265549_25	SY	ATP-dependent DNA helicase PIF1-like
223211_27	SY	PREDICTED: uncharacterized protein LOC106131092
411271_18	SY	uncharacterized protein LOC110370955 isoform X2
263748_45	SY	uncharacterized protein LOC110383380, partial
263748_23	SY	uncharacterized protein LOC110383380, partial
263219_65	UP	Ankyrin repeat domain
26404_63	UP	cell division cycle 2 like-1 isoform X1
271713_69	UP	endonuclease-reverse transcriptase
262512_11	UP	fatty acid synthase-
16058_52	UP	H(+) Cl(-) exchange transporter 5 isoform X1
171735_23	UP	histidine-rich glyco -
171735_18	UP	histidine-rich glyco -
150252_48	UP	MBF2
51392_56	UP	pancreatic triacylglycerol lipase-like
223258_65	UP	PREDICTED: uncharacterized protein LOC106131092
49203_12	UP	putative alcohol dehydrogenase
288182_55	UP	retinaldehyde-binding 1-like
288182_32	UP	retinaldehyde-binding 1-like
176197_57	UP	T-box transcription factor TBX6-like
182870_72	UP	tick transposon
143976_45	UP	uncharacterized protein LOC110379650
198356_89	UP	uncharacterized protein LOC110383219 isoform X2
315639_67	UP	uncharacterized protein LOC110384665

- UP= Upstream variation
- DW= Downstream variation
- IN = Intro variant
- MI = Missense variant
- SY = Synonymous variant

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.

6.4. Conclusions

- 53 SNPS were statistically significant under selection based on False Discovery Rate ≤ 0.047;
- DAPC analyses formed seven clusters
- The susceptible strain is genetically different froma all resistant strain and form a separated cluster;
- Clo-RR strain sets an exclusive group, separate from all other resistant strains;
- Resistant strains have 17 loci in commom;
- Many loci were specific for each insecticide and Yieldgard VT-PRO®.

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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.

APPENDICES

APPENDIX A

Raw reads from Illumina HiSeq 2500 sequencer on strains of *S. frugiperda.*

Library	**Nº of reads**	**% Bases >=Q30**
SUS 1	56.629.084	85,00
SUS 2	58.848.022	84,92
SUS 3	56.710.048	84,70
λ-RES 1	35.959.892	84,93
λ-RES 2	38.466.264	84,16
λ-RES 3	52.101.284	84,88
CLO-RES 1	32.830.634	84,14
CLO-RES 2	52.679.582	84,58
CLO-RES 3	44.061.574	84,63

APPENDIX B

List of DEG with fold change > 2 and PDR < 0.01 between susceptible and resistant strains to chlorpyrifos

Transcript	LFC	Description
GSSPFG00009648001-RA	5.13	15-hydroxyprostaglandin dehydrogenase [NAD(+)]-like
GSSPFG00033214001-RA	2.17	17-beta-hydroxysteroid dehydrogenase 14-like
GSSPFG00033213001-RA	2.44	17-beta-hydroxysteroid dehydrogenase 14-like
GSSPFG00010018001-RA	-2.04	23 kDa integral membrane -like
GSSPFG00021151001-RA	3.19	27 kDa glyco -like isoform X2
GSSPFG00019071001-RA	2.19	28S ribosomal mitochondrial
GSSPFG00025461001-RA	-2.97	2-amino-3-ketobutyrate coenzyme A mitochondrial
GSSPFG00014050001-RA	2.00	39S ribosomal mitochondrial
GSSPFG00017980001-RA	2.17	39S ribosomal mitochondrial
GSSPFG00002230001-RA	2.07	3-hydroxyacyl-CoA dehydrogenase type-2-like
GSSPFG00023535001-RA	-2.12	3-phosphoinositide-dependent kinase 1 isoform X1
GSSPFG00007142001-RA	2.66	5-methylcytosine rRNA methyltransferase NSUN4
GSSPFG00005328001-RA	2.26	A disintegrin and metallo ase with thrombospondin motifs 16-like
GSSPFG00007212001-RA	-2.85	abhydrolase domain-containing 2
GSSPFG00010463001-RA	2.66	abnormal spindle
GSSPFG00005170001.1-RA	-2.84	acetyltransferase ACT16
GSSPFG00028769001-RA	-2.05	acid phosphatase type 7-like isoform X1
GSSPFG00034814001-RA	3.69	acidic fibroblast growth factor intracellular-binding protein
GSSPFG00017921001-RA	2.06	actin-related protein 8
GSSPFG00018854001-RA	-2.36	activating transcription factor of chaperone isoform X2
GSSPFG00020527001-RA	2.30	acyl- dehydrogenase family member 9
GSSPFG00029310001-RA	-5.11	acyl- desaturase
GSSPFG00028093001-RA	-2.80	adenosine deaminase CECR1-like
GSSPFG00029161001-RA	-2.52	adenosine deaminase CECR1-like
GSSPFG00029148001-RA	2.20	adenosine deaminase-related growth factor
GSSPFG00016916001-RA	3.10	adenylate cyclase
GSSPFG00012255001-RA	-2.02	Adenylate cyclase type 2
GSSPFG00028660001.4-RA	-2.07	adenylate cyclase-like
GSSPFG00002626001.1-RA	-2.31	adipose triglyceride lipase
GSSPFG00017055001-RA	6.37	AF165427_1ecdysis-triggering hormone precursor
GSSPFG00034100001.3-RA	-5.15	AF261971_1trypsin precursor 9
GSSPFG00031647001-RA	2.64	aladin-like
GSSPFG00018417001-RA	2.01	alanine--glyoxylate aminotransferase 2-like
GSSPFG00009772001.1-RA	2.05	Alcohol dehydrogenase
GSSPFG00009774001.1-RA	10.88	alcohol dehydrogenase
GSSPFG00026420001-RA	7.14	aldo-keto reductase AKR2E4-like
GSSPFG00012908001.3-RA	6.62	alkaline C-like
GSSPFG00031872001-RA	2.82	allergen Api m 6-like
GSSPFG00010046001-RA	-2.30	alpha-L-fucoside fucohydrolase
GSSPFG00025544001-RA	-2.23	alpha-tocopherol transfer -
GSSPFG00018152001.1-RA	-3.07	alpha-tocopherol transfer -like
GSSPFG00009746001-RA	-2.12	altered inheritance of mitochondria 3-like isoform X1
GSSPFG00016631001.1-RA	-2.42	amidophosphoribosyltransferase-like
GSSPFG00027029001.1-RA	-2.60	amidophosphoribosyltransferase-like isoform X1
GSSPFG00018671001-RA	2.80	aminoacylase 1
GSSPFG00009316001-RA	-2.08	angiopoietin-related 2 isoform X1
GSSPFG00024169001-RA	-2.12	angiotensin-converting enzyme
GSSPFG00027012001-RA	-3.51	ankyrin repeat domain-containing 29-like
GSSPFG00034837001-RA	-3.87	ankyrin repeat domain-containing protein 29-like
GSSPFG00035122001.3-RA	2.03	antennal esterase CXE11

Continue

Transcript	LFC	Description
GSSPFG00004132001.2-RA	3.23	antennal esterase CXE14
GSSPFG00035354001.3-RA	3.25	antennal esterase CXE14
GSSPFG00002447001-RA	7.48	antennal esterase CXE14
GSSPFG00030553001.3-RA	3.79	antennal esterase CXE19
GSSPFG00004135001-RA	2.51	antennal esterase CXE4
GSSPFG00016664001.1-RA	2.51	antichymotrypsin-1-like isoform X9
GSSPFG00013898001-RA	2.54	apolipophorins isoform X2
GSSPFG00027050001.2-RA	3.56	apterous-like isoform X1
GSSPFG00034711001-RA	-2.56	apyrase-like
GSSPFG00028821001-RA	-2.06	arf-GAP with coiled- ANK repeat and PH domain-containing 2-like
GSSPFG00020438001-RA	-2.75	arf-GAP with dual PH domain-containing 1-like isoform X2
GSSPFG00030927001-RA	2.34	arginine N-methyltransferase 7 isoform X3
GSSPFG00007572001-RA	2.31	arginine N-methyltransferase 7 isoform X4
GSSPFG00021966001.1-RA	2.16	argininosuccinate lyase
GSSPFG00028748001-RA	-2.83	arrestin domain-containing 17-like
GSSPFG00031110001.1-RA	-2.48	arrestin domain-containing 17-like
GSSPFG00029361001-RA	-2.24	arrestin domain-containing protein 17-like
GSSPFG00029362001-RA	-3.91	arrestin domain-containing protein 2-like
GSSPFG00033995001-RA	3.16	arylsulfatase B-like
GSSPFG00016626001-RA	2.31	aspartate--tRNA mitochondrial
GSSPFG00002772001.1-RA	-2.12	atlastin-like
GSSPFG00033272001-RA	-8.71	ATP synthase subunit mitochondrial
GSSPFG00020552001-RA	2.04	ATPase family AAA domain-containing 5
GSSPFG00014490001-RA	2.51	aurora kinase B
GSSPFG00026451001-RA	-2.42	autophagy-related 2 homolog A
GSSPFG00011367001-RA	-2.37	autophagy-related 2 homolog A
GSSPFG00005530001.3-RA	-2.08	autophagy-related 2 homolog A
GSSPFG00019895001.3-RA	-2.06	autophagy-related 2 homolog A
GSSPFG00010270001.4-RA	-2.81	beclin 1-associated autophagy-related key regulator
GSSPFG00000179001.3-RA	-2.46	beta-1,3-glucan binding
GSSPFG00019060001.5-RA	-2.43	beta-1,3-glucan binding
GSSPFG00002369001-RA	-2.31	bifunctional purine biosynthesis PURH
GSSPFG00034091001-RA	-2.28	bifunctional purine biosynthesis PURH
GSSPFG00008539001.2-RA	-2.58	bile salt-activated lipase
GSSPFG00020484001-RA	-2.03	BTB POZ domain-containing 9 isoform X1
GSSPFG00028281001-RA	2.14	C1A cysteine protease precursor
GSSPFG00013180001-RA	-2.05	C2 domain-containing 5 isoform X2
GSSPFG00012681001.3-RA	-2.11	cadherin
GSSPFG00003442001-RA	-2.47	cadherin-like protein
GSSPFG00008811001.1-RA	-2.42	cadherin-like protein
GSSPFG00013984001-RA	-2.00	cadherin-like receptor
GSSPFG00026155001.1-RA	-2.47	calcium-independent phospholipase A2-gamma-like
GSSPFG00010737001-RA	-2.31	calphotin-like
GSSPFG00002621001.1-RA	2.15	calumenin-B
GSSPFG00023182001-RA	-2.84	cAMP-responsive element-binding -like 2 isoform X3
GSSPFG00029006001-RA	4.80	carbonyl reductase [NADPH] 1-like
GSSPFG00001834001.5-RA	8.28	carboxyl choline esterase CCE016d
GSSPFG00010082001.3-RA	-3.23	carboxylesterase CXE23
GSSPFG00020288001.5-RA	-2.99	carboxylesterase CXE23
GSSPFG00000131001.3-RA	-2.96	carboxylesterase CXE23
GSSPFG00021089001.2-RA	2.15	carboxylesterase CXE28
GSSPFG00001835001.3-RA	2.02	carboxylesterase CXE3
GSSPFG00003783001.3-RA	2.57	carboxylesterase CXE3

Continue

Transcript	LFC	Description
GSSPFG00007452001.1-RA	-2.09	carboxypeptidase B-like
GSSPFG00007122001.1-RA	-2.40	carboxypeptidase D-like
GSSPFG00014016001.1-RA	-3.05	carboxypeptidase inhibitor
GSSPFG00007075001-RA	-3.26	carboxypeptidase N subunit 2-
GSSPFG00030544001-RA	-2.07	cathepsin L ase
GSSPFG00033789001-RA	2.17	CD109 antigen isoform X1
GSSPFG00027059001.1-RA	2.66	cell division cycle 20 homolog
GSSPFG00027362001.3-RA	2.16	chemosensory 4
GSSPFG00035277001.3-RA	-2.84	chemosensory CSP4
GSSPFG00006521001.3-RA	-2.02	chemosensory CSP7
GSSPFG00016748001-RA	-2.17	chitin binding
GSSPFG00022444001-RA	-2.33	chitin synthase
GSSPFG00021091001.3-RA	2.09	cholinesterase 1-like
GSSPFG00000426001-RA	2.90	chromobox homolog 5-like
GSSPFG00013442001.1-RA	2.24	chromosome-associated kinesin KIF4 isoform X1
GSSPFG00005604001.2-RA	3.46	chymotrypsin-2 isoform X1
GSSPFG00019810001.3-RA	-2.28	chymotrypsin-like elastase family member 2A
GSSPFG00023392001-RA	-2.06	chymotrypsin-like elastase family member 2A
GSSPFG00030120001.3-RA	2.37	chymotrypsin-like serine protease
GSSPFG00030121001.3-RA	4.11	chymotrypsin-like serine protease
GSSPFG00035484001.3-RA	2.00	chymotrypsin-like serine protease precursor
GSSPFG00034352001.3-RA	3.25	chymotrypsin-like serine protease precursor
GSSPFG00008227001.3-RA	3.73	chymotrypsin-like serine protease precursor
GSSPFG00000363001-RA	5.93	cilia- and flagella-associated 206-like
GSSPFG00008199001-RA	2.89	cilia- and flagella-associated 36
GSSPFG00009943001.1-RA	3.28	circadian clock-controlled -like
GSSPFG00031860001-RA	-2.53	cis, cis-muconate transporter protein
GSSPFG00022161001.1-RA	2.29	citron Rho-interacting kinase-like
GSSPFG00009987001.2-RA	2.62	claret segregational
GSSPFG00029737001-RA	2.65	clavesin-2-like
GSSPFG00001418001-RA	2.19	Cleavage and polyadenylation specificity factor subunit 1
GSSPFG00019124001-RA	-2.90	clotting factor B-
GSSPFG00015806001-RA	3.49	CLUMA_CG017006, isoform A
GSSPFG00017831001-RA	-2.02	C-Maf-inducing -like
GSSPFG00026948001.3-RA	-3.21	cobatoxin B
GSSPFG00029532001.1-RA	2.23	condensin complex subunit 1
GSSPFG00018164001-RA	2.30	condensin complex subunit 2
GSSPFG00025174001-RA	-3.86	cuticle 8-like
GSSPFG00014609001-RA	-2.34	cuticle -like
GSSPFG00006935001.1-RA	-2.41	cuticle protein 63-like
GSSPFG00025933001-RA	-3.86	Cuticular 4
GSSPFG00012550001.1-RA	2.06	cyclin-dependent kinase 1
GSSPFG00015803001-RA	2.80	cyclin-dependent kinases regulatory subunit-like
GSSPFG00013787001-RA	-2.51	cystathionine beta-synthase
GSSPFG00030545001-RA	-2.13	cysteine ase
GSSPFG00034252001-RA	-3.70	cysteine dioxygenase type 1
GSSPFG00012309001-RA	-3.17	cysteine dioxygenase type 1
GSSPFG00005134001-RA	-4.18	cytochrome b5-like
GSSPFG00003819001-RA	-2.90	cytochrome b5-like
GSSPFG00002700001.1-RA	-2.70	cytochrome CYP333B3
GSSPFG00015785001-RA	-5.50	cytochrome CYP340AA1
GSSPFG00031882001-RA	-2.52	cytochrome CYP340AA1
GSSPFG00020081001-RA	2.53	cytochrome CYP340AA1
GSSPFG00018217001-RA	-3.08	cytochrome CYP367A6
GSSPFG00012279001.2-RB	-3.45	cytochrome CYP6AB14

Continue

Transcript	LFC	Description
GSSPFG00005012001.2-RA	-2.96	cytochrome CYP6AB14
GSSPFG00008268001.2-RB	2.10	cytochrome P450
GSSPFG00033556001.2-RB	2.16	Cytochrome P450
GSSPFG00014682001.2-RB	-2.13	cytochrome P450 4C1-like
GSSPFG00005208001-RA	3.45	cytochrome P450 4C1-like
GSSPFG00027679001-RA	-2.64	cytochrome P450 4V2-like
GSSPFG00027168001.2-RA	2.26	cytochrome P450 4V2-like
GSSPFG00009765001.2-RA	-2.23	cytochrome P450 6a2-like
GSSPFG00003899001.3-RC	2.64	cytochrome P450 6AE43
GSSPFG00003899001.3-RA	2.67	cytochrome P450 6AE43
GSSPFG00003899001.3-RB	2.85	cytochrome P450 6AE43
GSSPFG00005009001.2-RA	2.08	cytochrome P450 6B6-like
GSSPFG00021242001.3-RA	2.73	cytochrome P450 6k1-like
GSSPFG00030424001.1-RA	4.04	cytochrome P450 6k1-like
GSSPFG00008050001.2-RB	2.68	cytochrome P450 CY321A8
GSSPFG00008050001.2-RA	2.84	cytochrome P450 CY321A8
GSSPFG00002834001.2-RA	2.24	cytochrome P450 CYP307A1
GSSPFG00008048001.3-RA	3.44	cytochrome P450 CYP321A7
GSSPFG00014992001.2-RA	4.34	cytochrome P450 CYP321A7
GSSPFG00008047001.2-RA	2.40	cytochrome P450 CYP321A9
GSSPFG00015622001.2-RB	3.43	cytochrome P450 CYP321B1
GSSPFG00015195001.2-RB	3.71	cytochrome P450 CYP321B1
GSSPFG00015195001.2-RA	5.48	cytochrome P450 CYP321B1
GSSPFG00035919001.2-RA	-4.37	cytochrome P450 CYP340L1
GSSPFG00014033001.2-RA	8.95	cytochrome P450 CYP340L1
GSSPFG00014033001.2-RB	8.95	cytochrome P450 CYP340L1
GSSPFG00016404001.2-RB	-2.10	cytochrome P450 CYP49A1
GSSPFG00022722001.1-RA	-2.87	cytoplasmic dynein 2 heavy chain 1
GSSPFG00024594001-RA	2.16	cytosolic 10-formyltetrahydrofolate dehydrogenase
GSSPFG00034107001.1-RA	2.29	D2-like isoform X1
GSSPFG00017138001-RA	-2.77	D-amino-acid oxidase
GSSPFG00003268001-RA	2.34	DDB1- and CUL4-associated factor 13
GSSPFG00006856001-RA	2.33	deglycase DJ-1-like
GSSPFG00020771001-RA	2.32	deoxyuridine 5 -triphosphate nucleotidohydrolase
GSSPFG00018767001.3-RA	-8.27	diapausin precursor
GSSPFG00009264001.3-RA	-3.95	diapausin precursor
GSSPFG00014147001.3-RA	-3.49	diapausin precursor
GSSPFG00000813001.3-RA	-3.43	diapausin precursor
GSSPFG00021577001.3-RA	-3.41	diapausin precursor
GSSPFG00021578001-RA	-3.36	diapausin precursor
GSSPFG00009267001.2-RA	-2.92	diapausin precursor
GSSPFG00000812001.3-RA	-2.36	diapausin precursor
GSSPFG00032088001-RA	2.09	disks large-associated 5
GSSPFG00031159001-RA	2.07	disulfide-isomerase A3
GSSPFG00001314001-RA	2.09	DNA ligase 1
GSSPFG00017902001-RA	2.29	DNA polymerase epsilon subunit 2
GSSPFG00034614001-RA	2.84	DNA polymerase epsilon subunit 3
GSSPFG00006172001-RA	4.41	DNA polymerase epsilon subunit 4
GSSPFG00019092001-RA	2.00	DNA primase large subunit
GSSPFG00022518001-RA	2.11	DNA primase small subunit isoform X2
GSSPFG00003122001-RA	2.24	DNA replication licensing factor Mcm5
GSSPFG00011196001-RA	2.63	DNA-directed RNA polymerases and III subunit RPABC3
GSSPFG00015522001-RA	2.24	DNA-directed RNA polymerases I, II, and III subunit RPABC4
GSSPFG00005194001-RA	2.03	dolichyl-diphosphooligosaccharide-- glycosyltransferase subunit 1

Continue

Transcript	LFC	Description
GSSPFG00000876001-RA	2.33	Dol-P-Glc:Glc(2)Man(9) c(2)-PP-Dol alpha-1,2-glucosyltransferase
GSSPFG00000376001-RA	2.55	Dol-P-Glc:Glc(2)Man(9) c(2)-PP-Dol alpha-1,2-glucosyltransferase
GSSPFG00020765001-RA	3.64	dynein intermediate chain ciliary isoform X2
GSSPFG00014756001-RA	-2.08	E3 ubiquitin- ligase AMFR-like
GSSPFG00005179001-RA	-2.34	early endosome antigen 1
GSSPFG00000519001-RA	-5.90	ecdysteroid regulated
GSSPFG00032727001.4-RA	-2.43	ecdysteroid-regulated 16 kDa
GSSPFG00022972001-RA	-6.72	ecdysteroid-regulated 16 kDa -like
GSSPFG00030496001.3-RA	-3.42	ejaculatory bulb-specific 3-like
GSSPFG00014982001-RA	3.70	elongation of very long chain fatty acids AAEL008004-like
GSSPFG00019503001-RA	2.88	elongation of very long chain fatty acids AAEL008004-like isoform X2
GSSPFG00014977001-RA	-2.23	elongation of very long chain fatty acids AAEL008004-like isoform X3
GSSPFG00018893001-RA	-2.07	elongation of very long chain fatty acids AAEL008004-like isoform X3
GSSPFG00015129001-RA	6.32	endocuticle structural glyco bd-5-like
GSSPFG00016923001-RA	2.20	endocuticle structural glycoprotein SgAbd-5-like
GSSPFG00001457001.1-RA	2.56	endocuticle structural glycoprotein SgAbd-5-like isoform X1
GSSPFG00021712001-RA	2.68	endocuticle structural glycoprotein SgAbd-5-like isoform X1
GSSPFG00022717001-RA	-9.06	endonuclease-reverse transcriptase
GSSPFG00034846001-RA	-5.13	endonuclease-reverse transcriptase
GSSPFG00015456001-RA	-4.06	endonuclease-reverse transcriptase
GSSPFG00017681001-RA	-3.63	endonuclease-reverse transcriptase
GSSPFG00010996001-RA	9.75	endonuclease-reverse transcriptase
GSSPFG00010014001-RA	10.52	endonuclease-reverse transcriptase
GSSPFG00027217001-RA	10.52	endonuclease-reverse transcriptase
GSSPFG00002128001-RA	10.52	endonuclease-reverse transcriptase
GSSPFG00022606001-RA	10.76	endonuclease-reverse transcriptase
GSSPFG00018008001-RA	2.43	enhancer of rudimentary homolog
GSSPFG00029279001.3-RA	-5.76	epididymal secretory E1
GSSPFG00021762001.3-RA	-3.34	epididymal secretory E1
GSSPFG00020976001.4-RA	-2.62	epididymal secretory E1
GSSPFG00010179001.4-RA	-4.90	esterase FE4-like
GSSPFG00025860001-RA	2.17	estrogen sulfotransferase-like
GSSPFG00003799001-RA	2.53	estrogen sulfotransferase-like
GSSPFG00004492001.1-RA	-2.61	eukaryotic translation initiation factor 2-alpha kinase-like isoform X1
GSSPFG00022000001.3-RA	-2.26	eukaryotic translation initiation factor 4E binding
GSSPFG00006596001-RA	-5.25	Excitatory amino acid transporter 3
GSSPFG00018493001-RA	-2.33	exonuclease GOR-like isoform X1
GSSPFG00034561001-RA	2.15	exosome complex component
GSSPFG00032738001-RA	2.20	exosome complex component RRP42
GSSPFG00019703001.1-RA	2.86	facilitated trehalose transporter Tret1-2 homolog
GSSPFG00028909001-RA	-2.33	facilitated trehalose transporter Tret1-like
GSSPFG00032327001.1-RA	4.76	facilitated trehalose transporter Tret1-like
GSSPFG00000602001-RA	-3.17	farnesoate epoxidase-like isoform X1
GSSPFG00020555001.2-RA	-2.67	farnesoate epoxidase-like isoform X1
GSSPFG00009481001-RA	2.27	fatty acid synthase-like
GSSPFG00020132001-RA	2.78	fatty acid synthase-like
GSSPFG00034790001-RA	2.12	fatty acyl reductase
GSSPFG00012337001-RA	2.28	fatty acyl reductase
GSSPFG00009405001-RA	5.56	fatty acyl- reductase CG5065
GSSPFG00021279001-RA	-3.20	fatty acyl reductase FAR6
GSSPFG00019501001-RA	-2.29	fatty alcohol acetyltransferase
GSSPFG00032967001.1-RA	-2.14	fatty alcohol acetyltransferase
GSSPFG00032968001.1-RA	-2.14	fatty alcohol acetyltransferase
GSSPFG00004182001-RA	-3.14	F-box only 32 isoform X1
GSSPFG00031858001-RA	-2.06	f-box only 7-like isoform 1

Continue

Transcript	LFC	Description
GSSPFG00027636001-RA	-2.26	fibrillin-2-like isoform X1
GSSPFG00007524001-RA	2.64	fibroin heavy chain-like
GSSPFG00022872001-RA	4.58	Fibulin 1
GSSPFG00031717001-RA	2.40	filamin-A isoform X1
GSSPFG00025035001-RA	2.16	FK506-binding 5-like
GSSPFG00020553001-RA	2.98	Flagellar FliJ protein
GSSPFG00002054001-RA	2.20	flap endonuclease 1 isoform X1
GSSPFG00004035001-RA	2.43	flap endonuclease 1 isoform X1
GSSPFG00016704001-RA	2.80	flotillin-2 isoform X3
GSSPFG00033731001-RA	-2.14	forkhead box
GSSPFG00004994001-RA	-2.38	G- coupled receptor Mth2-like isoform X2
GSSPFG00023583001-RA	-2.27	G- coupled receptor Mth2-like isoform X4
GSSPFG00004992001-RA	3.58	G- coupled receptor Mth2-like isoform X4
GSSPFG00029622001-RA	2.92	G2 mitotic-specific cyclin-B isoform X1
GSSPFG00001375001-RA	3.57	G2 mitotic-specific cyclin-B isoform X1
GSSPFG00019523001-RA	2.00	G2 mitotic-specific cyclin-B3 isoform X1
GSSPFG00009630001-RA	-2.63	gastric triacylglycerol lipase-like
GSSPFG00022015001-RA	-3.48	glucose dehydrogenase [quinone]
GSSPFG00003676001-RA	-3.09	glucose dehydrogenase [quinone]-like
GSSPFG00003675001-RA	-2.51	glucose dehydrogenase [quinone]-like
GSSPFG00000931001-RA	2.38	glucose dehydrogenase [quinone]-like
GSSPFG00028043001-RA	4.02	glucose dehydrogenase [quinone]-like
GSSPFG00027352001-RA	4.27	glucose dehydrogenase [quinone]-like
GSSPFG00001362001-RA	4.96	glucose dehydrogenase [quinone]-like
GSSPFG00008550001-RA	6.83	glucose dehydrogenase [quinone]-like
GSSPFG00007661001-RA	-2.02	glucose dehydrogenase [FAD, quinone]-like
GSSPFG00033197001-RA	-3.00	glutaminase liver mitochondrial-like isoform X2
GSSPFG00028314001-RA	-2.39	glutamyl-tRNA(Gln) amidotransferase subunit mitochondrial
GSSPFG00035111001.2-RA	-2.45	glutathione S-transferase epsilon 14
GSSPFG00035798001.3-RA	3.04	glutathione S-transferase epsilon 6
GSSPFG00035010001.3-RA	5.90	glutathione S-transferase epsilon 6
GSSPFG00027753001.4-RA	5.01	glutathione S-transferase epsilon 9
GSSPFG00022959001.4-RA	5.73	glutathione S-transferase s1
GSSPFG00022085001.3-RA	7.25	glutathione S-transferase s1
GSSPFG00032430001-RA	8.61	glutathione S-transferase s1
GSSPFG00026298001.6-RA	2.01	glutathione S-transferase sigma 5
GSSPFG00034479001.5-RA	2.51	glutathione S-transferase sigma 5
GSSPFG00029293001.4-RA	3.73	glutathione S-transferase-like
GSSPFG00027902001-RA	-3.27	glycerol kinase-like isoform X3
GSSPFG00009146001-RA	-3.21	glycerol kinase-like isoform X4
GSSPFG00013506001-RA	2.14	glyoxylate reductase hydroxypyruvate reductase-like
GSSPFG00009814001-RA	2.73	GMP reductase 1-like
GSSPFG00016836001.1-RA	-2.30	golgin subfamily A member 1
GSSPFG00012063001-RA	-2.40	golgin subfamily A member 6 22
GSSPFG00003960001-RA	-3.04	group XV phospholipase A2-like
GSSPFG00022562001-RA	-2.81	group XV phospholipase A2-like
GSSPFG00021034001-RA	-2.71	grpE mitochondrial
GSSPFG00029054001-RA	-2.57	gustatory receptor
GSSPFG00000405001.5-RA	-3.18	heat shock
GSSPFG00026860001-RA	-2.41	hemicentin-1-like isoform X1
GSSPFG00007500001.2-RA	-4.21	hemolin
GSSPFG00008668001.3-RA	-2.95	hemolymph ase 19
GSSPFG00017707001-RA	-2.74	hemolymph ase 19
GSSPFG00033538001-RA	2.34	high-affinity Na+-dependent glutamate transporter

Continue

Transcript	LFC	Description
GSSPFG00009577001-RA	-3.51	histidine decarboxylase isoform X4
GSSPFG00017063001-RA	2.58	histidine triad nucleotide-binding 1-like
GSSPFG00016113001.3-RA	2.26	histone 2A variant
GSSPFG00007545001.4-RB	-4.51	homeobox extradenticle isoform X2
GSSPFG00006392001-RA	2.64	homocysteine S-methyltransferase 1-like
GSSPFG00016822001-RA	-5.02	hypothetical protein
GSSPFG00028052001-RA	-2.54	hypothetical protein
GSSPFG00001905001-RA	6.02	hypothetical protein
GSSPFG00010377001-RA	-8.45	hypothetical protein AB894_15325, partial
GSSPFG00006992001-RA	-5.13	hypothetical protein AB894_15325, partial
GSSPFG00028108001-RA	-5.13	hypothetical protein AB894_15325, partial
GSSPFG00022430001-RA	5.17	hypothetical protein AB894_15325, partial
GSSPFG00001547001-RA	5.07	hypothetical protein AB894_15365, partial
GSSPFG00030179001-RA	-2.71	hypothetical protein ALC56_06702, partial
GSSPFG00002776001-RA	-2.52	hypothetical protein B5V51_11470
GSSPFG00015662001-RA	-2.05	hypothetical protein B5V51_12790
GSSPFG00028175001-RA	-2.60	hypothetical protein B5V51_12827, partial
GSSPFG00004566001-RA	2.36	hypothetical protein B5V51_13147
GSSPFG00010779001-RA	2.44	hypothetical protein B5V51_13558
GSSPFG00026461001-RA	2.55	hypothetical protein B5V51_1404
GSSPFG00019342001-RA	2.21	hypothetical protein B5V51_14335
GSSPFG00002290001-RA	3.46	hypothetical protein B5V51_14476
GSSPFG00023879001-RA	2.14	hypothetical protein B5V51_4033
GSSPFG00022658001-RA	-3.05	hypothetical protein B5V51_4234
GSSPFG00005146001-RA	2.74	hypothetical protein B5V51_4611
GSSPFG00000069001-RA	2.35	hypothetical protein B5V51_5595, partial
GSSPFG00013363001-RA	-6.76	hypothetical protein B5V51_6519, partial
GSSPFG00017504001-RA	-3.02	hypothetical protein B5V51_746
GSSPFG00000817001-RA	5.26	hypothetical protein g.18358, partial
GSSPFG00034706001-RA	-6.02	hypothetical protein g.7559
GSSPFG00032824001-RA	-2.97	hypothetical protein KGM_05173
GSSPFG00028815001-RA	-5.41	hypothetical protein KGM_12914
GSSPFG00001944001-RA	2.47	hypothetical protein KGM_20847
GSSPFG00017215001-RA	-4.12	hypothetical protein N594_01555
GSSPFG00016486001-RA	-2.34	hypothetical protein RR46_00448
GSSPFG00006944001.1-RA	-2.68	hypothetical protein RR46_04746
GSSPFG00003245001-RA	-3.32	hypothetical protein RR46_09774
GSSPFG00029043001-RA	-3.90	hypothetical protein, partial
GSSPFG00026626001-RA	2.59	hypothetical protein, partial
GSSPFG00010762001-RA	3.18	hypothetical protein, partial
GSSPFG00012008001.1-RA	2.82	ileal sodium bile acid cotransporter-like isoform X1
GSSPFG00026567001.2-RA	-5.30	Immune-related Hdd1
GSSPFG00034840001-RA	3.98	importin subunit alpha-1-like
GSSPFG00028007001-RA	2.58	importin subunit alpha-3
GSSPFG00005643001-RA	-5.11	indole-3-acetaldehyde oxidase-like
GSSPFG00030014001-RA	-2.40	inducible metallo ase inhibitor -like isoform X1
GSSPFG00030011001-RA	-3.66	inducible metallo ase inhibitor -like isoform X2
GSSPFG00030012001-RA	-2.47	inducible metallo ase inhibitor -like isoform X2
GSSPFG00025578001-RA	-4.06	inducible metalloproteinase inhibitor protein-like isoform X1
GSSPFG00001505001.1-RA	-2.17	inositol oxygenase-like
GSSPFG00029070001-RA	-2.01	inositol-trisphosphate 3-kinase homolog isoform X3
GSSPFG00028418001-RA	-3.75	Insect intestinal mucin 3
GSSPFG00020037001-RA	-2.99	insulin receptor substrate 1
GSSPFG00020036001-RA	-2.48	insulin receptor substrate-1
GSSPFG00006526001-RA	-3.48	insulin-like growth factor-binding complex acid labile subunit isoform X3

Continue

Transcript	LFC	Description
GSSPFG00004610001-RA	-2.49	insulin-like receptor
GSSPFG00021315001-RA	2.64	integumentary mucin C.1-like
GSSPFG00034251001-RA	-2.75	intestinal mucin 8
GSSPFG00012308001-RA	-2.30	intestinal mucin 8
GSSPFG00012306001-RA	-2.23	intestinal mucin 8
GSSPFG00031954001-RA	2.36	isoforms D E-like
GSSPFG00030385001-RA	2.79	juvenile hormone diol kinase
GSSPFG00030172001-RA	-2.75	keratin-associated 19-2-like
GSSPFG00019729001.1-RA	2.53	kinesin KIF20B
GSSPFG00001949001.2-RA	2.60	kinesin KIF23
GSSPFG00005966001.4-RA	2.23	kinetochore NDC80 homolog
GSSPFG00003814001-RA	3.04	Kinetochore-associated 1
GSSPFG00027434001-RA	-3.58	lachesin-like isoform X2
GSSPFG00024020001-RA	3.18	lactoylglutathione lyase
GSSPFG00030309001-RA	2.18	large neutral amino acids transporter small subunit 2
GSSPFG00000476001-RA	6.94	larval cuticle 16 17-like
GSSPFG00000475001-RA	3.32	larval cuticle 1-like
GSSPFG00018123001-RA	2.31	larval cuticle LCP-14-like
GSSPFG00010503001-RA	4.34	larval cuticle LCP-14-like
GSSPFG00000471001-RA	4.34	larval cuticle LCP-17-like
GSSPFG00010501001-RA	3.70	larval cuticle LCP-17-like isoform X2
GSSPFG00019763001-RA	8.97	larval/pupal rigid cuticle protein 66-like
GSSPFG00010944001-RA	-2.09	L-dopachrome tautomerase yellow-f2-like
GSSPFG00013851001.4-RA	-2.08	lebocin 1
GSSPFG00035421001.3-RA	-2.27	lebocin 2
GSSPFG00032633001-RA	4.28	leucine-rich repeat and calponin homology domain-containing 3 isoform X3
GSSPFG00002785001-RA	-2.61	leucine-rich repeat-containing 24-like
GSSPFG00023160001.2-RA	-4.34	leucine-rich repeat-containing 57 isoform X2
GSSPFG00009603001.3-RA	-4.05	leucine-rich repeat-containing 57 isoform X2
GSSPFG00004187001-RA	-5.01	lin-28 homolog isoform X2
GSSPFG00004411001-RA	-3.37	lipase 3-like
GSSPFG00032729001.1-RA	-2.07	lipase member H-A-like
GSSPFG00015354001-RA	-3.93	lipase member H-like
GSSPFG00032735001-RA	-3.65	lipase member H-like
GSSPFG00033974001.1-RA	4.48	lipase member I-like
GSSPFG00020830001.1-RA	-2.64	lipophorin receptor
GSSPFG00032730001-RA	-2.07	lipoprotein lipase-like
GSSPFG00025428001-RA	-3.11	LLP homolog
GSSPFG00002321001.3-RA	2.17	low affinity immunoglobulin epsilon Fc receptor isoform X1
GSSPFG00026057001.1-RA	-7.74	low density lipo receptor adapter 1-like
GSSPFG00023696001-RA	2.62	low density lipo receptor isoform X1
GSSPFG00014567001.1-RA	-2.99	LOW QUALITY PROTEIN: uncharacterized protein LOC110378735
GSSPFG00031327001-RA	-2.58	L-threonine 3- mitochondrial
GSSPFG00009835001-RA	-2.47	LTV1 homolog isoform X1
GSSPFG00021596001-RA	-2.04	LTV1 homolog isoform X2
GSSPFG00014701001.1-RA	2.30	luciferin 4-monooxygenase-like
GSSPFG00001619001.1-RA	3.35	luciferin 4-monooxygenase-like
GSSPFG00025204001-RA	4.69	luciferin 4-monooxygenase-like
GSSPFG00034708001-RA	5.10	luciferin 4-monooxygenase-like
GSSPFG00009247001-RA	5.37	luciferin 4-monooxygenase-like
GSSPFG00001618001.1-RA	4.00	luciferin 4-monooxygenase-like isoform X2
GSSPFG00010221001.1-RA	2.67	lymphokine-activated killer T-cell-originated kinase-like
GSSPFG00029927001-RA	-3.45	lysosomal thioesterase PPT2 homolog
GSSPFG00014909001.3-RA	-2.64	lysozyme 2

Continue

Transcript	LFC	Description
GSSPFG00013328001.3-RA	-4.69	lysozyme 3
GSSPFG00004890001.2-RA	-2.06	male-specific protein
GSSPFG00004454001-RA	2.28	maltase 2-like
GSSPFG00004452001-RA	-2.64	maltase A1-like
GSSPFG00009826001-RA	-2.54	MAP kinase-interacting serine threonine- kinase 1-like isoform X1
GSSPFG00031687001-RA	2.24	methionine aminopeptidase 1 isoform X1
GSSPFG00028217001-RA	-3.69	mitochondrial fission 1
GSSPFG00007720001-RA	3.30	mitotic spindle assembly checkpoint MAD2A
GSSPFG00016496001-RA	-2.80	monocarboxylate transporter 9-like
GSSPFG00031557001-RA	2.30	monocarboxylate transporter 9-like
GSSPFG00022466001-RA	-3.36	msta-like isoform X1
GSSPFG00030580001-RA	2.21	msta-like isoform X1
GSSPFG00026146001-RA	-2.20	mucin-4 isoform X2
GSSPFG00011375001.1-RA	-2.18	multifunctional ADE2
GSSPFG00023233001-RA	3.44	multiple inositol polyphosphate phosphatase 1-like
GSSPFG00012209001-RA	2.10	muscle M-line assembly unc-89-like
GSSPFG00030770001-RA	2.12	mutant cadherin
GSSPFG00021448001-RA	-2.44	mutS homolog 4-like
GSSPFG00004425001-RA	-3.35	myoneurin-like isoform X1
GSSPFG00013168001-RA	-2.71	myosin-VIIa isoform X1
GSSPFG00034476001-RA	-5.83	myrosinase 1-like
GSSPFG00015774001-RA	-5.83	myrosinase 1-like
GSSPFG00024840001-RA	-3.34	myrosinase 1-like
GSSPFG00015120001.1-RA	-3.22	myrosinase 1-like isoform X1
GSSPFG00030408001-RA	-3.81	NAD kinase mitochondrial
GSSPFG00013658001-RA	-2.27	NAD kinase mitochondrial
GSSPFG00022362001-RA	-2.19	NEDD8 ultimate buster 1-like isoform X2
GSSPFG00008361001-RA	-2.36	negative elongation factor E
GSSPFG00017505001-RA	-2.76	neuropathy target esterase sws isoform X2
GSSPFG00015450001.1-RA	-5.04	neuropeptide SIFamide receptor
GSSPFG00010558001-RA	-2.35	neutral ceramidase
GSSPFG00006248001-RA	-5.81	nitrogen permease regulator 2
GSSPFG00018496001.1-RA	2.67	nose resistant to fluoxetine 6-like
GSSPFG00013620001-RA	3.59	nose resistant to fluoxetine 6-like
GSSPFG00000144001-RA	-3.23	nuclear pore complex NUP62-like
GSSPFG00025607001.1-RA	-2.82	nuclear protein 1
GSSPFG00019843001-RA	-2.42	nuclease HARBI1
GSSPFG00018678001-RA	2.93	nuclease HARBI1
GSSPFG00027083001-RA	2.01	nucleoplasmin isoform X1
GSSPFG00019614001-RA	2.74	nucleoporin NDC1
GSSPFG00013411001-RA	2.79	nucleoporin NDC1
GSSPFG00020929001.3-RA	-4.68	odorant binding 13
GSSPFG00003295001.3-RA	-2.04	odorant binding 17
GSSPFG00023814001.3-RA	3.43	odorant binding 8
GSSPFG00002072001-RA	-2.50	ommochrome-binding -like
GSSPFG00002619001.1-RA	3.75	organic cation carnitine transporter 7-like
GSSPFG00013603001-RA	-3.13	organic cation transporter -like
GSSPFG00000276001-RA	-2.73	organic cation transporter -like
GSSPFG00017353001-RA	2.09	organic cation transporter -like
GSSPFG00022137001-RA	2.86	organic cation transporter -like
GSSPFG00023336001-RA	-2.25	organic cation transporter -like isoform X1
GSSPFG00016320001-RA	2.49	organic cation transporter protein-like
GSSPFG00009936001.1-RA	2.51	origin recognition complex subunit 1
GSSPFG00033317001-RA	2.27	origin recognition complex subunit 3
GSSPFG00016551001-RA	3.81	origin recognition complex subunit 4

Continue

Transcript	LFC	Description
GSSPFG00013724001-RA	-3.29	ornithine mitochondrial
GSSPFG00016363001-RA	2.87	pachytene checkpoint 2 homolog
GSSPFG00004434001-RA	6.68	paired box and transposase domain containing
GSSPFG00031859001-RA	6.87	paired box protein and transposase domain containing protein
GSSPFG00029907001-RA	-3.25	pancreatic lipase-related 2
GSSPFG00033398001-RA	-2.97	pancreatic lipase-related 2
GSSPFG00024194001-RA	-2.96	pancreatic lipase-related 2-like
GSSPFG00024523001.1-RA	-3.10	pancreatic triacylglycerol lipase-like isoform X2
GSSPFG00028318001-RA	2.10	pentatricopeptide repeat-containing mitochondrial-like
GSSPFG00006083001.5-RA	3.06	peptidoglycan-recognition LB-like
GSSPFG00029435001-RA	2.35	Peptidyl-prolyl cis-trans isomerase
GSSPFG00008031001-RA	-6.30	peritrophin type-A domain 2
GSSPFG00027822001.1-RA	-3.16	peritrophin type-A domain 2
GSSPFG00023203001-RA	3.36	peritrophin-1-like precursor
GSSPFG00026173001.1-RA	-2.07	peroxidase-like isoform X1
GSSPFG00023805001.3-RA	2.28	pheromone binding 4
GSSPFG00025372001.3-RA	2.36	pheromone binding 4
GSSPFG00019459001-RA	-2.18	phosphatidate phosphatase
GSSPFG00022882001-RA	-2.53	phosphatidate phosphatase LPIN2
GSSPFG00010839001-RA	-3.45	phosphoenolpyruvate carboxykinase [GTP]-like
GSSPFG00015150001-RA	-2.13	phosphoenolpyruvate carboxykinase [GTP]-like
GSSPFG00005275001-RA	-2.75	phospholipase A1 member A-like isoform X1
GSSPFG00001442001-RA	-6.77	phospholipase B-like 2
GSSPFG00031115001.1-RA	-2.18	phosphoribosyl pyrophosphate synthetase
GSSPFG00008431001-RA	-2.03	phosphoribosylformylglycinamidine synthase
GSSPFG00031628001-RA	-2.18	phosphoserine aminotransferase
GSSPFG00031211001-RA	-2.62	phosphoserine phosphatase isoform X2
GSSPFG00011351001-RA	-2.14	pickpocket 28-like
GSSPFG00011995001-RA	2.77	POC1 centriolar protein homolog A
GSSPFG00028781001-RA	-3.14	poly [ADP-ribose] polymerase
GSSPFG00035987001.2-RA	-3.68	polycalin
GSSPFG00003808001.4-RA	-2.21	polyhomeotic 2
GSSPFG00016195001-RA	-2.13	polyubiquitin-C isoform X1
GSSPFG00025865001-RA	3.84	PR domain zinc finger 1 isoform X1
GSSPFG00005760001-RA	-4.06	predicted protein
GSSPFG00032680001-RA	2.81	PREDICTED: gelsolin-like
GSSPFG00031260001-RA	4.60	PREDICTED: uncharacterized protein K02A2.6-like
GSSPFG00012108001-RA	-4.64	PREDICTED: uncharacterized protein LOC101943458 isoform X2
GSSPFG00027209001-RA	6.42	PREDICTED: uncharacterized protein LOC103569118
GSSPFG00024850001-RA	5.41	PREDICTED: uncharacterized protein LOC105384457
GSSPFG00000523001-RA	-4.43	PREDICTED: uncharacterized protein LOC105386897
GSSPFG00024290001-RA	5.97	PREDICTED: uncharacterized protein LOC105393267
GSSPFG00022122001-RA	-2.63	PREDICTED: uncharacterized protein LOC106106200
GSSPFG00028816001-RA	-4.10	PREDICTED: uncharacterized protein LOC106132060
GSSPFG00033033001-RA	-8.34	PREDICTED: uncharacterized protein LOC106134628
GSSPFG00025507001-RA	5.05	PREDICTED: uncharacterized protein LOC106135043
GSSPFG00032466001-RA	-2.34	PREDICTED: uncharacterized protein LOC106136491
GSSPFG00025710001-RA	-4.07	PREDICTED: uncharacterized protein LOC106136949 isoform X2
GSSPFG00001039001-RA	-2.43	PREDICTED: uncharacterized protein LOC106138911 isoform X1
GSSPFG00019785001.1-RA	2.37	PREDICTED: uncharacterized protein LOC106140213
GSSPFG00005330001-RA	3.25	PREDICTED: uncharacterized protein LOC106140333
GSSPFG00025374001-RA	-3.32	PREDICTED: uncharacterized protein LOC106141232
GSSPFG00001071001-RA	-3.35	PREDICTED: uncharacterized protein LOC106141703
GSSPFG00027760001-RA	-4.04	PREDICTED: uncharacterized protein LOC106142604

Continue

Transcript	LFC	Description
GSSPFG00032866001-RA	2.27	PREDICTED: uncharacterized protein LOC106143377
GSSPFG00015111001-RA	8.19	PREDICTED: uncharacterized protein LOC106708082
GSSPFG00030306001-RA	-2.62	PREDICTED: uncharacterized protein LOC106709162
GSSPFG00019794001-RA	2.55	PREDICTED: uncharacterized protein LOC106709162
GSSPFG00001099001-RA	8.05	PREDICTED: uncharacterized protein LOC106716864
GSSPFG00028732001-RA	4.63	PREDICTED: uncharacterized protein LOC106719438, partial
GSSPFG00019212001-RA	5.15	PREDICTED: uncharacterized protein LOC108742303 isoform X1
GSSPFG00034293001-RA	2.08	prefoldin subunit 3
GSSPFG00009331001.1-RA	-2.84	probable 4-coumarate-- ligase 1
GSSPFG00033874001.1-RA	-2.78	probable 4-methylmuconolactone transporter
GSSPFG00019144001.1-RA	-3.81	probable alpha-ketoglutarate-dependent hypophosphite dioxygenase
GSSPFG00002255001.1-RA	-2.60	probable bifunctional methylenetetrahydrofolate dehydrogenase cyclohydrolase 2 isoform X2
GSSPFG00023199001-RA	2.99	probable chitinase 3 precursor
GSSPFG00007792001-RA	2.18	probable DNA replication complex GINS PSF2
GSSPFG00028417001-RA	-3.68	probable endochitinase
GSSPFG00010031001-RA	2.29	probable H ACA ribonucleo complex subunit 1
GSSPFG00014008001-RA	2.76	probable histone-binding Caf1
GSSPFG00024789001-RA	-2.51	probable hydroxyacid-oxoacid mitochondrial
GSSPFG00016642001-RA	-2.20	probable phospholipid-transporting ATPase IF isoform X2
GSSPFG00019329001-RA	2.26	probable ribosome production factor 1
GSSPFG00007885001-RA	-2.88	probable serine/threonine-protein kinase fhkE isoform X1
GSSPFG00006614001-RA	-2.39	probable sulfite mitochondrial isoform X1
GSSPFG00018888001-RA	-2.50	probable sulfite mitochondrial isoform X3
GSSPFG00020549001-RA	-2.12	probable sulfite mitochondrial isoform X3
GSSPFG00005259001-RA	2.14	prostaglandin reductase 1-like
GSSPFG00030065001-RA	3.15	prostatic acid phosphatase-like
GSSPFG00033346001-RA	-2.72	protein GDAP2 homolog isoform X2
GSSPFG00020029001-RA	2.27	protein msta
GSSPFG00012232001.1-RA	2.17	protein nessun dorma isoform X1
GSSPFG00018644001-RA	2.36	protein obstructor-E-like
GSSPFG00013477001.1-RA	2.88	protein Spindly
GSSPFG00004897001-RA	-5.08	protein takeout
GSSPFG00031710001-RA	2.20	protein zwilch isoform X1
GSSPFG00005477001-RA	-2.11	proton-coupled amino acid transporter CG1139
GSSPFG00011264001-RA	-2.93	proton-coupled folate transporter-like
GSSPFG00024881001-RA	-2.24	proton-coupled folate transporter-like
GSSPFG00009647001-RA	-2.22	proton-coupled folate transporter-like
GSSPFG00007623001-RA	-2.07	proton-coupled folate transporter-like
GSSPFG00020557001-RA	-2.71	purine nucleoside phosphorylase-like isoform X2
GSSPFG00018555001-RA	-2.48	putative aminopeptidase W07G4.4
GSSPFG00008922001.1-RA	-2.53	putative breast carcinoma amplified sequence
GSSPFG00012223001.1-RA	-2.69	putative defense protein 3
GSSPFG00019276001-RA	4.57	putative fatty acyl-CoA reductase CG5065
GSSPFG00025002001-RA	4.31	pyruvate kinase-like isoform X3
GSSPFG00005651001-RA	-2.12	rab-related isoform X1
GSSPFG00031050001.1-RA	2.94	regulator of cytokinesis 1-like
GSSPFG00008092001-RA	-3.38	relaxin receptor 2-like
GSSPFG00008097001.2-RA	-3.83	REPAT23
GSSPFG00008095001.2-RA	2.40	REPAT23
GSSPFG00010387001.2-RA	2.46	REPAT23
GSSPFG00018300001-RA	2.53	REPAT23
GSSPFG00033663001-RA	-4.49	REPAT25
GSSPFG00010392001-RA	-6.65	REPAT35
GSSPFG00008100001.2-RA	2.14	REPAT38
GSSPFG00010389001.2-RA	9.74	REPAT38

Continue

Transcript	LFC	Description
GSSPFG00025091001.1-RA	2.21	replication factor C subunit 4
GSSPFG00031936001-RA	-2.21	repressed by EFG1 protein 1-like isoform X2
GSSPFG00001276001-RA	3.74	retinaldehyde-binding 1-like
GSSPFG00033049001-RA	4.63	Retrotransposable element Tf2 protein type 1
GSSPFG00014414001-RA	2.89	retrotransposon-like family member retr-1
GSSPFG00032615001-RA	2.93	Retrovirus-related Pol
GSSPFG00003370001-RA	-4.02	Retrovirus-related Pol poly from transposon
GSSPFG00000141001-RA	-3.90	Retrovirus-related Pol poly from transposon
GSSPFG00013081001-RA	2.04	Retrovirus-related Pol poly from transposon
GSSPFG00017132001-RA	-2.78	retrovirus-related Pol poly from transposon isoform X4
GSSPFG00016407001-RA	5.56	Retrovirus-related Pol poly from transposon TNT 1-94
GSSPFG00009193001-RA	-3.46	Retrovirus-related Pol polyprotein from transposon 17.6
GSSPFG00029259001-RA	-4.17	reverse transcriptase
GSSPFG00032593001-RA	2.29	reverse transcriptase
GSSPFG00012046001-RA	3.71	reverse transcriptase
GSSPFG00021546001-RA	-2.56	Rhomboid-related 3
GSSPFG00026349001-RA	-3.28	ribonuclease H1
GSSPFG00028114001-RA	-3.41	ribonuclease H1-like
GSSPFG00034609001-RA	-8.24	ribosomal S6 kinase alpha-5-like isoform X1
GSSPFG00018747001-RA	-2.41	RNA helicase Mov10l1 isoform X1
GSSPFG00025596001-RA	-2.51	RNA helicase Mov10l1-like
GSSPFG00029225001-RA	-2.29	RNA helicase Mov10l1-like
GSSPFG00004349001-RA	2.85	RNA-binding motif X-linked 2
GSSPFG00005010001-RA	-3.69	RNA-directed DNA polymerase from mobile element jockey-like isoform X1
GSSPFG00034316001-RA	2.14	ruvB-like 2
GSSPFG00010067001-RA	2.59	ruvB-like 2
GSSPFG00030383001-RA	2.90	sarcoplasmic calcium-binding s and IV-like
GSSPFG00010735001-RA	7.53	secretory carrier-associated membrane 1
GSSPFG00019560001-RA	-2.94	senecionine N-oxygenase isoform X1
GSSPFG00017742001-RA	-2.30	senescence-specific cysteine protease SAG39-like
GSSPFG00032739001-RA	-2.01	sequestosome-1-like isoform X1
GSSPFG00005732001-RA	-2.27	serine cytosolic isoform X1
GSSPFG00007332001-RA	2.10	serine protease
GSSPFG00028284001.3-RA	2.14	serine protease
GSSPFG00012841001.3-RA	3.97	serine protease
GSSPFG00035027001.3-RA	6.96	serine protease
GSSPFG00012891001.3-RA	2.22	serine protease 33
GSSPFG00012890001.4-RA	2.30	serine protease 33
GSSPFG00008228001.2-RA	5.68	serine protease 37
GSSPFG00025855001-RA	-2.94	serine protease easter-like
GSSPFG00021515001-RA	-2.23	serine protease inhibitor 10
GSSPFG00033438001-RA	-3.65	serine protease inhibitor 6
GSSPFG00026736001.1-RA	2.30	serine protease inhibitor dipetalogastin
GSSPFG00028801001-RA	2.13	serine protease snake-like
GSSPFG00010428001-RA	2.21	serine threonine- kinase nek2
GSSPFG00000762001.1-RA	3.00	serine threonine- kinase polo isoform X1
GSSPFG00026457001-RA	-2.10	serine-rich adhesin for platelets-like
GSSPFG00032448001-RA	-2.08	serine-rich adhesin for platelets-like
GSSPFG00019750001-RA	2.47	serpin B5-like
GSSPFG00032320001-RA	2.46	SET and MYND domain-containing 4
GSSPFG00010704001-RA	2.46	SET and MYND domain-containing protein 4
GSSPFG00019214001-RA	4.74	sex peptide receptor-like
GSSPFG00035978001.2-RA	-2.27	sid 1
GSSPFG00031837001-RA	2.21	sine oculis-binding homolog isoform X2

Continue

Transcript	LFC	Description
GSSPFG00025000001-RA	-2.10	single-stranded DNA-binding 3 isoform X2
GSSPFG00023887001.4-RA	-4.20	small heat shock
GSSPFG00032498001.3-RA	-2.11	small heat shock
GSSPFG00016896001-RA	2.00	small integral membrane 12
GSSPFG00024184001-RA	-5.94	Sodium channel Nach
GSSPFG00006549001-RA	-6.87	sodium channel Nach-like
GSSPFG00024291001-RA	-3.18	sodium potassium calcium exchanger 3-like
GSSPFG00010033001-RA	2.11	sodium potassium-transporting ATPase subunit beta-2-like isoform X1
GSSPFG00025934001-RA	-2.67	sodium-coupled monocarboxylate transporter 1
GSSPFG00005409001-RA	-2.61	sodium-independent sulfate anion transporter-like
GSSPFG00011250001-RA	-2.76	soluble guanylate cyclase 89Db-like
GSSPFG00010213001-RA	2.99	solute carrier family 23 member 2 isoform X1
GSSPFG00029271001.1-RA	-3.40	solute carrier family 25 member 38-A-like isoform X1
GSSPFG00028553001.1-RA	2.06	solute carrier family 35 member B1 homolog isoform X1
GSSPFG00007140001.1-RA	5.36	solute carrier family 46 member 3-like
GSSPFG00032594001-RA	2.37	solute carrier family facilitated glucose transporter member 6-like
GSSPFG00001443001.1-RA	3.70	solute carrier family facilitated glucose transporter member 6-like
GSSPFG00030610001-RA	5.03	soma ferritin-like
GSSPFG00024234001.1-RA	-2.54	sorbitol dehydrogenase-like
GSSPFG00006990001-RA	-2.08	sortilin-related receptor isoform X4
GSSPFG00018735001-RA	2.09	spermatogenesis-associated 5
GSSPFG00034559001-RA	-2.95	sphingomyelin phosphodiesterase isoform X1
GSSPFG00030590001.1-RA	-3.18	sphingomyelin synthase-related 1-like isoform X1
GSSPFG00003020001-RA	-3.40	stabilizer of axonemal microtubules 2
GSSPFG00007929001-RA	-2.25	sulfate bicarbonate oxalate exchanger sat-1
GSSPFG00000378001.1-RA	-2.44	suppressor of tumorigenicity 14 homolog
GSSPFG00010230001.1-RA	4.11	synaptic vesicle glyco 2B
GSSPFG00012298001-RA	-2.41	synaptic vesicle glyco 2B-like
GSSPFG00032470001-RA	-3.86	TBC1 domain family member 12-like
GSSPFG00010050001-RA	-2.43	TBC1 domain family member 15 isoform X3
GSSPFG00034205001-RA	-3.39	T-complex-associated testis-expressed protein 1-like
GSSPFG00035684001.3-RA	-2.00	toll-interacting B-like
GSSPFG00019760001-RA	-3.80	TPA: putative cuticle protein
GSSPFG00030978001-RA	5.32	transcription factor
GSSPFG00004348001-RA	-2.91	transcription initiation factor TFIID subunit 13
GSSPFG00006067001-RA	2.31	transcription termination factor 2-like
GSSPFG00024980001.1-RA	2.37	transcriptional regulator ATRX-like
GSSPFG00001372001-RA	-3.37	translin-associated factor X-interacting 1-like isoform X1
GSSPFG00026671001.4-RA	-4.32	transmembrane protease serine 9-like
GSSPFG00008272001-RA	2.60	transporter svop-1
GSSPFG00017794001.1-RA	-2.33	transporter SVOPL
GSSPFG00008890001-RA	-3.64	transposase
GSSPFG00012485001-RA	-2.33	trehalase-2
GSSPFG00023406001.1-RA	-2.52	trifunctional purine biosynthetic protein adenosine-3 isoform X1
GSSPFG00005917001-RA	2.16	trimethyllysine mitochondrial
GSSPFG00006278001-RA	2.44	tRNA (uracil-5-)-methyltransferase-like A
GSSPFG00021809001.2-RA	4.69	trypsin CFT-1-like
GSSPFG00013282001.6-RA	2.48	trypsin T2a
GSSPFG00013283001.6-RB	3.92	trypsin T2a
GSSPFG00001201001.2-RA	2.16	trypsin T6
GSSPFG00008043001.5-RA	4.40	trypsin-like serine protease
GSSPFG00033734001-RA	-2.34	tryptophan 2,3-
GSSPFG00002209001-RA	-3.12	tryptophan 2,3-dioxygenase
GSSPFG00017398001-RA	-2.86	tryptophan 2,3-dioxygenase-like
GSSPFG00018576001-RA	-2.12	t-SNARE domain-containing 1

Continue

Transcript	LFC	Description
GSSPFG00026125001-RA	-2.80	turtle isoform X2
GSSPFG00001573001-RA	-4.16	tyrosine- phosphatase cdcA-like
GSSPFG00010355001-RA	-4.59	tyrosine- phosphatase corkscrew-like
GSSPFG00004175001-RA	2.03	U1 small nuclear ribonucleo A
GSSPFG00022946001.1-RA	2.07	U6 snRNA-associated Sm LSm3
GSSPFG00028899001-RA	2.26	ubiquinone biosynthesis monooxygenase mitochondrial
GSSPFG00035174001.4-RA	2.61	UDP-glucuronosyltransferase 1-6-like
GSSPFG00011007001.3-RA	2.64	UDP-glucuronosyltransferase 2B7-like
GSSPFG00024582001.3-RA	2.64	UDP-glycosyltransferase 33B13
GSSPFG00006772001-RA	2.61	UDP-glycosyltransferase 33B14
GSSPFG00003871001.3-RA	2.22	UDP-glycosyltransferase 33F4
GSSPFG00035300001.2-RA	2.81	UDP-glycosyltransferase 33F4
GSSPFG00035427001.2-RA	3.04	UDP-glycosyltransferase 33F4
GSSPFG00012990001.3-RA	3.61	UDP-glycosyltransferase 33F5
GSSPFG00035291001.3-RA	6.13	UDP-glycosyltransferase 33F5
GSSPFG00035441001.3-RA	6.99	UDP-glycosyltransferase 33F5
GSSPFG00032154001-RA	3.55	UDP-glycosyltransferase 33V3
GSSPFG00033939001.3-RA	-3.18	UDP-glycosyltransferase 39B4
GSSPFG00007060001.3-RA	-2.30	UDP-glycosyltransferase 39B4
GSSPFG00035210001.2-RA	2.85	UDP-glycosyltransferase 40F3
GSSPFG00035224001.3-RA	3.59	UDP-glycosyltransferase 40F3
GSSPFG00005185001.3-RA	3.61	UDP-glycosyltransferase 40F3
GSSPFG00035423001.2-RA	3.58	UDP-glycosyltransferase 40F5
GSSPFG00017879001.3-RA	4.03	UDP-glycosyltransferase 40F5
GSSPFG00035405001.3-RA	3.28	UDP-glycosyltransferase 40M3
GSSPFG00035299001.3-RA	3.92	UDP-glycosyltransferase 40R3
GSSPFG00004352001.3-RA	3.01	UDP-glycosyltransferase 40R4
GSSPFG00004145001.3-RA	3.06	UDP-glycosyltransferase 40R4
GSSPFG00001730001.5-RA	4.57	UDP-glycosyltransferase 43A2
GSSPFG00020652001-RA	3.10	UNC93-like protein
GSSPFG00010609001-RA	3.32	UNC93-like protein
GSSPFG00001679001-RA	-2.96	uncharacterized LOC101743290 precursor
GSSPFG00000520001-RA	-2.43	uncharacterized LOC101743290 precursor
GSSPFG00015981001-RA	-3.42	uncharacterized protein C14orf119 isoform X1
GSSPFG00031907001-RA	3.84	uncharacterized protein LOC101737697
GSSPFG00014454001-RA	4.05	uncharacterized protein LOC105841553
GSSPFG00034591001-RA	-3.29	uncharacterized protein LOC105842057
GSSPFG00010362001-RA	-2.45	uncharacterized protein LOC105842936
GSSPFG00000584001-RA	-5.74	uncharacterized protein LOC110369721
GSSPFG00031376001-RA	2.70	uncharacterized protein LOC110369733
GSSPFG00010932001.1-RA	2.35	uncharacterized protein LOC110369879
GSSPFG00022404001.1-RA	-2.25	uncharacterized protein LOC110370032 isoform X1
GSSPFG00014949001-RA	-2.70	uncharacterized protein LOC110370085
GSSPFG00031662001-RA	-2.37	uncharacterized protein LOC110370368
GSSPFG00027443001-RA	-2.19	uncharacterized protein LOC110370514
GSSPFG00030139001-RA	-2.18	uncharacterized protein LOC110370714
GSSPFG00034423001-RA	-4.35	uncharacterized protein LOC110370772
GSSPFG00004211001-RA	-4.57	uncharacterized protein LOC110370869
GSSPFG00023195001-RA	-3.15	uncharacterized protein LOC110370967
GSSPFG00004794001-RA	-2.87	uncharacterized protein LOC110371158
GSSPFG00019391001-RA	2.53	uncharacterized protein LOC110371825
GSSPFG00021534001-RA	-2.96	uncharacterized protein LOC110371830
GSSPFG00026558001-RA	2.67	uncharacterized protein LOC110372231 isoform X1
GSSPFG00004981001-RA	-3.78	uncharacterized protein LOC110372414

Continue

Transcript	LFC	Description
GSSPFG00011890001.1-RA	-4.04	uncharacterized protein LOC110372548 isoform X1
GSSPFG00003137001-RA	-2.06	uncharacterized protein LOC110372651
GSSPFG00032310001-RA	-4.14	uncharacterized protein LOC110372871
GSSPFG00011728001-RA	2.98	uncharacterized protein LOC110373313 isoform X1
GSSPFG00000834001-RA	-7.17	uncharacterized protein LOC110373377
GSSPFG00017188001-RA	-2.02	uncharacterized protein LOC110373407 isoform X2
GSSPFG00035416001.3-RA	-7.05	uncharacterized protein LOC110373446
GSSPFG00027451001.3-RA	-3.34	uncharacterized protein LOC110373446
GSSPFG00027450001.3-RA	-3.46	uncharacterized protein LOC110373453
GSSPFG00035032001.3-RA	-2.37	uncharacterized protein LOC110373453
GSSPFG00003535001-RA	-2.18	uncharacterized protein LOC110373801
GSSPFG00015871001-RA	2.27	uncharacterized protein LOC110374069
GSSPFG00006493001-RA	-2.31	uncharacterized protein LOC110374078
GSSPFG00032987001-RA	2.22	uncharacterized protein LOC110374147
GSSPFG00004750001-RA	-3.95	uncharacterized protein LOC110374347 isoform X1
GSSPFG00004390001-RA	-3.20	uncharacterized protein LOC110374795
GSSPFG00028178001-RA	-3.36	uncharacterized protein LOC110374899
GSSPFG00027900001-RA	-2.41	uncharacterized protein LOC110374982
GSSPFG00010055001-RA	-4.89	uncharacterized protein LOC110375196
GSSPFG00011761001-RA	-2.18	uncharacterized protein LOC110375526
GSSPFG00034195001-RA	2.44	uncharacterized protein LOC110375836 isoform X2
GSSPFG00018367001-RA	6.39	uncharacterized protein LOC110375937
GSSPFG00005339001-RA	-3.88	uncharacterized protein LOC110376622
GSSPFG00013684001-RA	-2.79	uncharacterized protein LOC110376622
GSSPFG00021162001-RA	2.53	uncharacterized protein LOC110376764
GSSPFG00015011001-RA	2.20	uncharacterized protein LOC110376945
GSSPFG00029617001-RA	-3.24	uncharacterized protein LOC110377027
GSSPFG00009513001-RA	3.68	uncharacterized protein LOC110377251
GSSPFG00030045001-RA	4.20	uncharacterized protein LOC110377251
GSSPFG00004511001-RA	3.00	uncharacterized protein LOC110377859
GSSPFG00030711001-RA	3.01	uncharacterized protein LOC110377859
GSSPFG00007878001-RA	-2.01	uncharacterized protein LOC110377877
GSSPFG00011517001-RA	-2.50	uncharacterized protein LOC110378079 isoform X2
GSSPFG00002528001-RA	-2.17	uncharacterized protein LOC110378206 isoform X5
GSSPFG00014728001-RA	2.26	uncharacterized protein LOC110378285
GSSPFG00023843001-RA	4.11	uncharacterized protein LOC110378318
GSSPFG00024642001-RA	-2.68	uncharacterized protein LOC110378667
GSSPFG00015766001-RA	2.50	uncharacterized protein LOC110379113
GSSPFG00004996001.1-RA	2.20	uncharacterized protein LOC110379517 isoform X1
GSSPFG00008403001-RA	-5.25	uncharacterized protein LOC110379629 isoform X2
GSSPFG00001154001-RA	-3.05	uncharacterized protein LOC110379638
GSSPFG00019287001-RA	2.19	uncharacterized protein LOC110379767
GSSPFG00005032001-RA	3.52	uncharacterized protein LOC110379975
GSSPFG00004632001.1-RA	-2.79	uncharacterized protein LOC110380036
GSSPFG00027823001.1-RA	-3.63	uncharacterized protein LOC110380230
GSSPFG00032062001-RA	-2.90	uncharacterized protein LOC110380435
GSSPFG00007676001-RA	-2.17	uncharacterized protein LOC110380654
GSSPFG00010771001.1-RA	-3.63	uncharacterized protein LOC110380742
GSSPFG00027084001-RA	-2.07	uncharacterized protein LOC110380777 isoform X1
GSSPFG00031000001-RA	4.61	uncharacterized protein LOC110381032
GSSPFG00034791001-RA	-2.29	uncharacterized protein LOC110381391
GSSPFG00003584001-RA	-2.16	uncharacterized protein LOC110381914
GSSPFG00007151001-RA	2.52	uncharacterized protein LOC110382040
GSSPFG00035794001.1-RA	-3.89	uncharacterized protein LOC110382259
GSSPFG00032316001-RA	2.34	uncharacterized protein LOC110382548

Continue

Transcript	LFC	Description
GSSPFG00021378001-RA	-2.02	uncharacterized protein LOC110382661
GSSPFG00032710001-RA	-3.90	uncharacterized protein LOC110382803
GSSPFG00033129001-RA	-3.10	uncharacterized protein LOC110383153
GSSPFG00000681001-RA	2.08	uncharacterized protein LOC110383153
GSSPFG00021054001-RA	2.80	uncharacterized protein LOC110383323
GSSPFG00011797001-RA	3.89	uncharacterized protein LOC110383391
GSSPFG00025216001-RA	-3.17	uncharacterized protein LOC110383732 isoform X2
GSSPFG00021306001.1-RA	-2.04	uncharacterized protein LOC110383837
GSSPFG00008377001-RA	-2.47	uncharacterized protein LOC110383860
GSSPFG00008954001-RA	-2.88	uncharacterized protein LOC110384015
GSSPFG00019586001-RA	-2.39	uncharacterized protein LOC110384111
GSSPFG00014786001-RA	-7.77	uncharacterized protein LOC110384139
GSSPFG00017800001-RA	2.12	uncharacterized protein LOC110384242
GSSPFG00027890001-RA	-2.93	uncharacterized protein LOC110384273
GSSPFG00021458001.4-RA	2.67	uncharacterized protein LOC110384589
GSSPFG00030767001-RA	-3.02	uncharacterized protein LOC110384665
GSSPFG00025868001-RA	-5.14	uncharacterized protein LOC110385670
GSSPFG00012936001-RA	-6.13	Uncharacterized protein OBRU01_04723
GSSPFG00010616001-RA	-5.56	Uncharacterized protein OBRU01_14834
GSSPFG00007211001-RA	-3.58	Uncharacterized protein OBRU01_18464, partial
GSSPFG00022905001-RA	-3.58	Uncharacterized protein OBRU01_18464, partial
GSSPFG00006042001-RA	-3.32	Uncharacterized protein OBRU01_20570
GSSPFG00025795001-RA	2.17	Uncharacterized protein OBRU01_23748, partial
GSSPFG00029884001-RA	-3.11	Uncharacterized protein, partial
GSSPFG00021886001-RA	2.64	Uncharacterized protein, partial
GSSPFG00000119001-RA	5.59	Uncharacterized protein, partial
GSSPFG00015999001-RA	-3.36	unknown unsecreted protein
GSSPFG00016311001-RA	-2.62	unnamed protein product, partial
GSSPFG00000684001-RA	2.36	UPF0160 isoform X1
GSSPFG00032226001.1-RA	-2.12	UPF0183 CG7083
GSSPFG00032463001-RA	5.92	UPF0704 protein C6orf165 homolog
GSSPFG00033052001-RA	-3.95	uricase
GSSPFG00027683001-RA	-3.49	uricase
GSSPFG00011382001-RA	-2.18	uridine phosphorylase 1 isoform X1
GSSPFG00016701001-RA	2.41	vanin 3 isoform X3
GSSPFG00027556001-RA	2.25	venom allergen 5-like
GSSPFG00008056001.3-RA	-3.56	venom carboxylesterase-6
GSSPFG00024879001-RA	4.23	very high density lipo
GSSPFG00013173001-RA	5.59	very high density lipo
GSSPFG00022858001-RA	4.43	very high density lipoprotein
GSSPFG00022859001-RA	5.93	very high density lipoprotein
GSSPFG00018652001.2-RA	-2.31	virescein precursor
GSSPFG00000066001-RA	3.12	V-type proton ATPase subunit e 2-like isoform X1
GSSPFG00032324001-RA	-2.34	WD repeat domain phosphoinositide-interacting 4-like isoform X1
GSSPFG00000651001-RA	-2.09	WD repeat domain phosphoinositide-interacting 4-like isoform X1
GSSPFG00008383001-RA	-2.14	WD repeat-containing 35
GSSPFG00016495001-RA	5.41	WD repeat-containing 78-like
GSSPFG00023082001-RA	-2.73	yippee-like CG15309 isoform X3
GSSPFG00009000001-RA	-2.30	zinc finger 235-like
GSSPFG00003724001.1-RA	-2.42	zinc finger 41-like
GSSPFG00001120001-RA	-2.63	zinc finger CCHC domain-containing 24-like
GSSPFG00008449001-RA	5.45	Zinc finger DNA binding
GSSPFG00009413001-RA	3.68	zinc transporter ZIP1-like
GSSPFG00025576001-RA	-8.16	zonadhesin-like isoform X1
GSSPFG00020182001-RA	-4.71	zonadhesin-like isoform X1

APPENDIX C

List of DEG with fold change > 2 and PDR < 0.01 between susceptible and resistant strains to lambda cihalothrin

Transcript	LDC	Description
GSSPFG00026557001-RA	-2.05	17-beta-hydroxysteroid dehydrogenase 14-like
GSSPFG00035969001.2-RA	-2.48	AF261981_1trypsin precursor
GSSPFG00002539001-RA	-2.02	aldehyde oxidase AOX3
GSSPFG00026420001-RA	7.21	aldo-keto reductase AKR2E4-like
GSSPFG00007381001-RA	-2.08	aldose reductase-like
GSSPFG00034368001-RA	-2.43	alpha-tocopherol transfer -like
GSSPFG00002447001-RA	7.55	antennal esterase CXE14
GSSPFG00027050001.2-RA	3.41	apterous-like isoform X1
GSSPFG00033272001-RA	-9.85	ATP synthase subunit mitochondrial
GSSPFG00019868001-RA	-7.43	ATP-dependent RNA helicase dbp2-like isoform X1
GSSPFG00000108001-RA	-2.28	beta-N-acetylglucosaminidase 2
GSSPFG00008539001.2-RA	-2.98	bile salt-activated lipase
GSSPFG00023741001-RA	2.28	bromodomain-containing DDB_G0280777-like isoform X2
GSSPFG00010737001-RA	-2.46	calphotin-like
GSSPFG00015571001.3-RA	-3.25	carboxyl choline esterase CCE006c
GSSPFG00016748001-RA	-2.98	chitin binding
GSSPFG00018941001-RA	-2.28	chitinase
GSSPFG00034354001.3-RA	2.70	chymotrypsin 2
GSSPFG00030121001.3-RA	2.11	chymotrypsin-like serine protease
GSSPFG00034357001.5-RA	4.53	chymotrypsin-like serine protease precursor
GSSPFG00000363001-RA	6.00	cilia- and flagella-associated 206-like
GSSPFG00008199001-RA	3.64	cilia- and flagella-associated 36
GSSPFG00015806001-RA	4.02	CLUMA_CG017006, isoform A
GSSPFG00000469001-RA	2.43	cuticular glycine-rich 21 isoform X1
GSSPFG00005208001-RA	8.10	cytochrome P450 4C1-like
GSSPFG00003900001.2-RA	-2.01	cytochrome P450 6AE44
GSSPFG00008050001.2-RB	-2.35	cytochrome P450 CY321A8
GSSPFG00008052001.2-RA	-3.61	cytochrome P450 CYP321A10
GSSPFG00016443001.2-RA	-3.16	cytochrome P450 CYP321A7
GSSPFG00015342001.2-RA	2.40	cytochrome P450 CYP340L1
GSSPFG00020736001.2-RA	2.41	cytochrome P450 CYP340L1
GSSPFG00014033001.2-RB	9.02	cytochrome P450 CYP340L1
GSSPFG00006856001-RA	2.32	deglycase DJ-1-like
GSSPFG00004193001-RA	-8.82	deoxynucleotidyltransferase terminal-interacting 2
GSSPFG00018767001.3-RA	-6.20	diapausin precursor
GSSPFG00014147001.3-RA	-3.89	diapausin precursor
GSSPFG00021877001.3-RA	-2.40	diapausin precursor
GSSPFG00021161001-RA	2.09	DNA-directed RNA polymerase III subunit RPC4
GSSPFG00018283001-RA	-2.53	dynamin-like 120 kDa mitochondrial isoform X4
GSSPFG00020765001-RA	6.57	dynein intermediate chain ciliary isoform X2
GSSPFG00014982001-RA	-2.14	elongation of very long chain fatty acids AAEL008004-like
GSSPFG00015129001-RA	9.51	endocuticle structural glyco bd-5-like
GSSPFG00016923001-RA	2.41	endocuticle structural glycoprotein SgAbd-5-like
GSSPFG00001457001.1-RA	3.73	endocuticle structural glycoprotein SgAbd-5-like isoform X1
GSSPFG00029134001-RA	-6.11	endonuclease-reverse transcriptase
GSSPFG00026914001-RA	-6.11	endonuclease-reverse transcriptase
GSSPFG00002821001-RA	-6.11	endonuclease-reverse transcriptase
GSSPFG00010996001-RA	-2.87	endonuclease-reverse transcriptase
GSSPFG00024745001-RA	2.23	endonuclease-reverse transcriptase
GSSPFG00020802001-RA	2.61	endonuclease-reverse transcriptase
GSSPFG00031996001-RA	3.00	endonuclease-reverse transcriptase
GSSPFG00028332001-RA	3.69	endonuclease-reverse transcriptase

Continue

Transcript	LDC	Description
GSSPFG00010759001-RA	4.28	endonuclease-reverse transcriptase
GSSPFG00023822001-RA	5.20	endonuclease-reverse transcriptase
GSSPFG00022606001-RA	6.13	endonuclease-reverse transcriptase
GSSPFG00010014001-RA	10.59	endonuclease-reverse transcriptase
GSSPFG00027217001-RA	10.59	endonuclease-reverse transcriptase
GSSPFG00002128001-RA	10.59	endonuclease-reverse transcriptase
GSSPFG00029279001.3-RA	-6.44	epididymal secretory E1
GSSPFG00021762001.3-RA	-4.61	epididymal secretory E1
GSSPFG00006596001-RA	-6.10	Excitatory amino acid transporter 3
GSSPFG00030893001-RA	-3.86	FAD synthase-like
GSSPFG00019278001-RA	2.08	fatty acyl- reductase CG5065 isoform X1
GSSPFG00019277001-RA	3.59	fatty acyl- reductase CG5065 isoform X6
GSSPFG00025591001-RA	4.69	fatty acyl- reductase CG5065 isoform X6
GSSPFG00016704001-RA	4.30	flotillin-2 isoform X3
GSSPFG00011317001-RA	-7.93	glucose dehydrogenase [quinone]-like
GSSPFG00005765001-RA	-2.84	glucose dehydrogenase [quinone]-like
GSSPFG00031870001.5-RA	2.38	glutathione S-transferase epsilon 14
GSSPFG00026804001.5-RA	-2.01	glutathione S-transferase epsilon 9
GSSPFG00022085001.3-RA	5.65	glutathione S-transferase s1
GSSPFG00003077001.5-RA	-2.15	glutathione S-transferase s2
GSSPFG00012065001-RA	5.68	glycine-rich DOT1-like
GSSPFG00014831001-RA	-4.16	hemocyte protease-3
GSSPFG00032512001.2-RA	-3.45	hemocyte protease-3
GSSPFG00017707001-RA	-2.26	hemolymph ase 19
GSSPFG00010762001-RA	2.92	hypothetical protein
GSSPFG00022430001-RA	4.13	hypothetical protein AB894_15325
GSSPFG00031522001-RA	-3.29	hypothetical protein AB894_15350
GSSPFG00016590001-RA	2.27	hypothetical protein AB894_15350
GSSPFG00014912001-RA	-3.55	hypothetical protein B5V51_11822
GSSPFG00004566001-RA	2.56	hypothetical protein B5V51_13147
GSSPFG00014911001-RA	-2.05	hypothetical protein B5V51_3476
GSSPFG00000069001-RA	2.38	hypothetical protein B5V51_5595
GSSPFG00000817001-RA	4.12	hypothetical protein g.18358
GSSPFG00033078001-RA	8.47	hypothetical protein g.4183
GSSPFG00032434001-RA	-2.00	hypothetical protein RR46_04775
GSSPFG00026567001.2-RA	-2.66	Immune-related Hdd1
GSSPFG00031848001-RA	-5.18	Insect intestinal mucin 4
GSSPFG00018848001-RA	-2.58	integral membrane GPR155 isoform X2
GSSPFG00019867001-RA	-2.07	integral membrane GPR155 isoform X2
GSSPFG00022293001.1-RA	-3.28	juvenile hormone binding 2
GSSPFG00029067001-RA	2.80	juvenile hormone binding an-0921 precursor
GSSPFG00001833001.3-RA	-6.78	juvenile hormone esterase-like
GSSPFG00003780001.2-RA	-5.90	juvenile hormone esterase-like
GSSPFG00001832001.3-RA	-4.40	juvenile hormone esterase-like
GSSPFG00003779001.5-RA	-4.08	juvenile hormone esterase-like
GSSPFG00033737001-RA	2.79	kazrin isoform X1
GSSPFG00016260001-RA	-2.55	lactoylglutathione lyase
GSSPFG00032633001-RA	3.13	leucine-rich repeat and calponin homology domain-containing 3 isoform X3
GSSPFG00032729001.1-RA	-2.43	lipase member H-A-like
GSSPFG00012130001-RA	-2.86	LOW QUALITY PROTEIN: vitellogenin-like
GSSPFG00030954001-RA	-7.30	lysozyme-like
GSSPFG00034319001.3-RA	4.44	macrophage mannose receptor 1-like
GSSPFG00002256001-RA	-2.10	membrane alanyl aminopeptidase-like isoform X1

GSSPFG00002598001-RA	-2.12	membrane alanyl aminopeptidase-like isoform X2

Continue

Transcript	LDC	Description
GSSPFG00035376001.5-RA	-2.76	microsomal glutathione transferase
GSSPFG00028217001-RA	-2.55	mitochondrial fission 1
GSSPFG00014715001-RA	4.60	mitochondrial ribosomal L33
GSSPFG00034035001.4-RA	-6.06	multidrug resistance-associated 4-like isoform X1
GSSPFG00030334001-RA	-2.93	multiple inositol polyphosphate phosphatase 1-like
GSSPFG00013168001-RA	-3.13	myosin-VIIa isoform X1
GSSPFG00024840001-RA	-2.15	myrosinase 1-like
GSSPFG00015120001.1-RA	-2.29	myrosinase 1-like isoform X1
GSSPFG00007963001-RA	-2.28	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit mitochondrial
GSSPFG00019843001-RA	-4.09	nuclease HARBI1
GSSPFG00018678001-RA	3.85	nuclease HARBI1
GSSPFG00002619001.1-RA	3.36	organic cation carnitine transporter 7-like
GSSPFG00013603001-RA	-3.45	organic cation transporter -like
GSSPFG00019753001-RA	2.48	ovalbumin-related X-like
GSSPFG00004434001-RA	7.01	paired box and transposase domain containing
GSSPFG00031859001-RA	7.18	paired box protein and transposase domain containing protein
GSSPFG00001090001-RA	-8.85	pancreatic lipase 3
GSSPFG00001089001-RA	-2.01	pancreatic lipase 3
GSSPFG00010173001-RA	-4.59	Pancreatic lipase-related 2
GSSPFG00032884001-RA	-2.02	pancreatic triacylglycerol lipase-like
GSSPFG00031103001-RA	2.59	pancreatic triacylglycerol lipase-like
GSSPFG00012791001-RA	-2.58	peptide mitochondrial-like
GSSPFG00022903001-RA	-6.05	peroxidase-like
GSSPFG00007595001-RA	-5.89	peroxidase-like
GSSPFG00005824001-RA	6.53	pollen-specific leucine-rich repeat extensin 2
GSSPFG00035987001.2-RA	-3.43	polycalin
GSSPFG00000423001-RA	-5.17	PREDICTED: mucin-2-like
GSSPFG00031260001-RA	8.43	PREDICTED: uncharacterized protein K02A2.6-like
GSSPFG00027209001-RA	6.50	PREDICTED: uncharacterized protein LOC103569118
GSSPFG00032910001-RA	-2.00	PREDICTED: uncharacterized protein LOC105392606
GSSPFG00015302001-RA	2.36	PREDICTED: uncharacterized protein LOC105397991
GSSPFG00010123001-RA	-5.24	PREDICTED: uncharacterized protein LOC106106200
GSSPFG00021282001-RA	-3.12	PREDICTED: uncharacterized protein LOC106106354
GSSPFG00015632001-RA	-2.99	PREDICTED: uncharacterized protein LOC106106354
GSSPFG00025507001-RA	6.99	PREDICTED: uncharacterized protein LOC106135043
GSSPFG00015111001-RA	5.81	PREDICTED: uncharacterized protein LOC106708082
GSSPFG00001099001-RA	8.13	PREDICTED: uncharacterized protein LOC106716864
GSSPFG00028732001-RA	6.59	PREDICTED: uncharacterized protein LOC106719438
GSSPFG00019212001-RA	5.90	PREDICTED: uncharacterized protein LOC108742303 isoform X1
GSSPFG00034882001-RA	-4.15	PREDICTED: uncharacterized protein LOC109487234
GSSPFG00031528001-RA	-5.82	PREDICTED: uncharacterized protein LOC109612963
GSSPFG00000187001-RA	-2.21	prion-like-(Q N-rich) domain-bearing 25
GSSPFG00028417001-RA	-3.43	probable endochitinase
GSSPFG00008707001-RA	-4.41	probable palmitoyltransferase ZDHHC8
GSSPFG00014036001-RA	-3.42	probable pseudouridine-5 -phosphatase isoform X1
GSSPFG00011921001-RA	-2.61	pro-resilin-like isoform X1
GSSPFG00017519001-RA	2.57	protease inhibitor 6
GSSPFG00034273001.1-RA	3.19	protein odr-4 homolog
GSSPFG00008335001-RA	6.20	pyrroline-5-carboxylate reductase-like isoform X2
GSSPFG00035982001.2-RA	-3.16	REPAT15
GSSPFG00035970001.2-RA	-2.01	REPAT15
GSSPFG00010386001.2-RA	-2.61	REPAT24
GSSPFG00033663001-RA	-2.64	REPAT25
GSSPFG00010392001-RA	-8.70	REPAT35
GSSPFG00010389001.2-RA	2.73	REPAT38

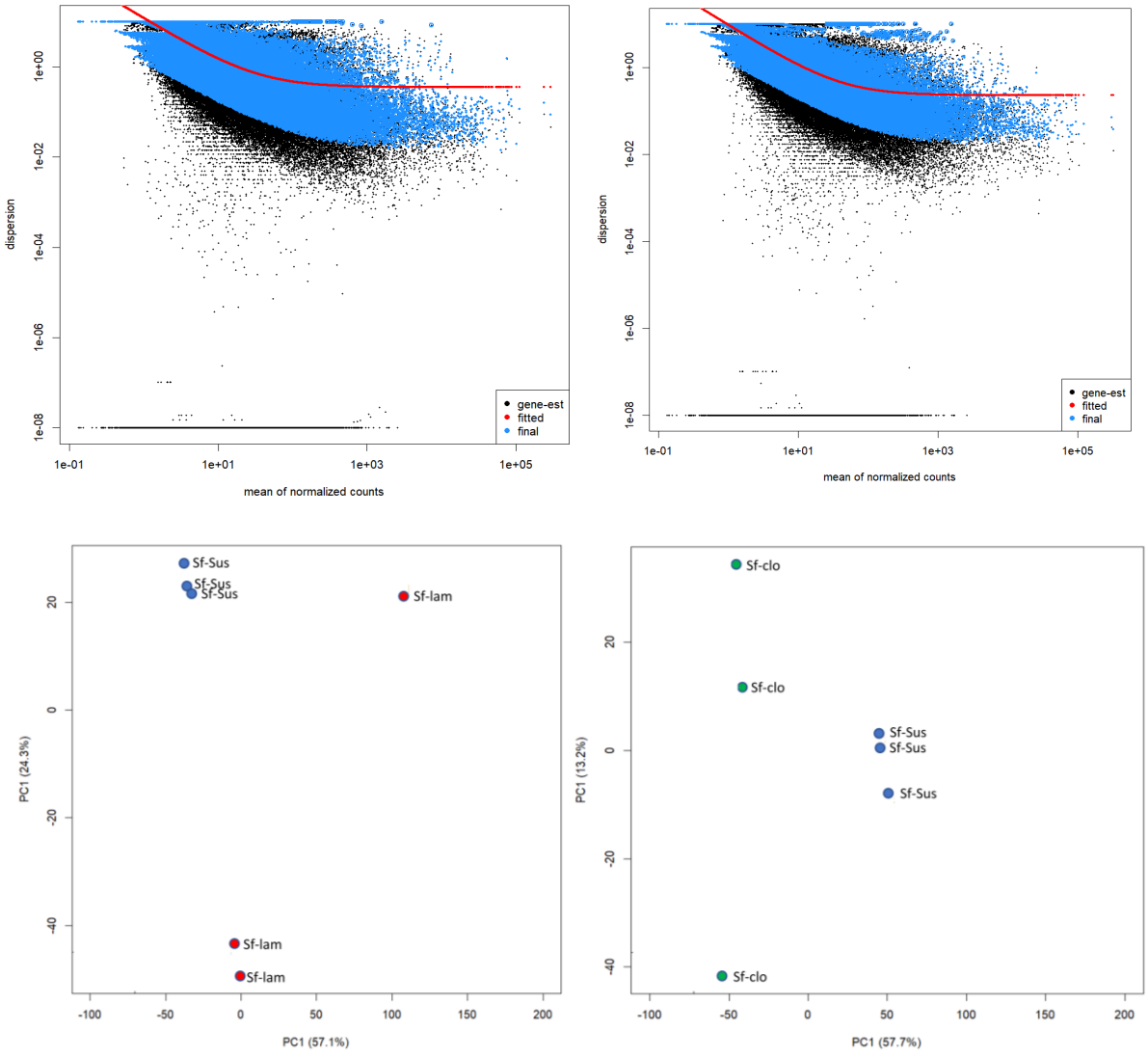
Continue

Transcript	LDC	Description
GSSPFG00013455001-RA	-3.29	REPAT39
GSSPFG00032034001-RA	-2.74	REPAT40
GSSPFG00008105001.2-RA	-2.27	REPAT43
GSSPFG00008104001.2-RA	-2.28	REPAT46
GSSPFG00030247001-RA	2.92	repetitive proline-rich cell wall 1-like
GSSPFG00019548001-RA	-3.28	Retrovirus-related Pol
GSSPFG00032615001-RA	3.51	Retrovirus-related Pol
GSSPFG00012046001-RA	3.22	reverse transcriptase
GSSPFG00034609001-RA	-7.13	ribosomal S6 kinase alpha-5-like isoform X1
GSSPFG00029806001-RA	-7.10	RL9_SPOFR ame: Full=60S ribosomal L9
GSSPFG00005010001-RA	-5.20	RNA-directed DNA polymerase from mobile element jockey-like isoform X1
GSSPFG00013006001-RA	-2.87	rRNA-processing UTP23 homolog
GSSPFG00007555001-RA	3.86	secretory carrier-associated membrane 1
GSSPFG00010735001-RA	7.60	secretory carrier-associated membrane 1
GSSPFG00012841001.3-RA	5.23	serine protease
GSSPFG00013450001.3-RA	-2.86	serine protease 19
GSSPFG00012891001.3-RA	2.41	serine protease 33
GSSPFG00012874001-RA	6.94	serine protease 33
GSSPFG00012890001.4-RA	7.44	serine protease 33
GSSPFG00019214001-RA	3.60	sex peptide receptor-like
GSSPFG00023887001.4-RA	-3.60	small heat shock
GSSPFG00024291001-RA	-2.11	sodium potassium calcium exchanger 3-like
GSSPFG00010230001.1-RA	2.86	synaptic vesicle glyco 2B
GSSPFG00029260001-RA	-2.19	synaptic vesicle glyco 2C-like
GSSPFG00007340001-RA	-3.25	tigger transposable element-derived 2-like
GSSPFG00008272001-RA	2.33	transporter svop-1
GSSPFG00032515001.3-RA	-3.63	trypsin-like protease
GSSPFG00006582001-RA	-2.06	ubiquitin-fold modifier-conjugating enzyme 1
GSSPFG00035174001.4-RA	-2.23	UDP-glucuronosyltransferase 1-6-like
GSSPFG00009234001.3-RA	-2.19	UDP-glucuronosyltransferase 2B7-like
GSSPFG00035291001.3-RA	3.11	UDP-glycosyltransferase 33F5
GSSPFG00033939001.3-RA	-4.13	UDP-glycosyltransferase 39B4
GSSPFG00007060001.3-RA	-3.39	UDP-glycosyltransferase 39B4
GSSPFG00000119001-RA	5.65	Uncharacterized protein
GSSPFG00016766001-RA	2.19	uncharacterized protein LOC110370179
GSSPFG00012374001-RA	2.71	uncharacterized protein LOC110370179
GSSPFG00004211001-RA	-7.00	uncharacterized protein LOC110370869
GSSPFG00028708001.1-RA	-2.27	uncharacterized protein LOC110373229 isoform X2
GSSPFG00035416001.3-RA	-4.81	uncharacterized protein LOC110373446
GSSPFG00027451001.3-RA	-2.50	uncharacterized protein LOC110373446
GSSPFG00027450001.3-RA	-3.26	uncharacterized protein LOC110373453
GSSPFG00004750001-RA	-2.76	uncharacterized protein LOC110374347 isoform X1
GSSPFG00020750001-RA	-2.81	uncharacterized protein LOC110375478
GSSPFG00018247001.2-RA	-8.23	uncharacterized protein LOC110376065
GSSPFG00017245001.1-RA	-2.71	uncharacterized protein LOC110377335
GSSPFG00012458001-RA	2.00	uncharacterized protein LOC110377515
GSSPFG00023843001-RA	3.04	uncharacterized protein LOC110378318
GSSPFG00034653001-RA	3.85	uncharacterized protein LOC110378389
GSSPFG00031000001-RA	6.49	uncharacterized protein LOC110381032
GSSPFG00018578001-RA	-6.00	uncharacterized protein LOC110381835
GSSPFG00032710001-RA	-2.59	uncharacterized protein LOC110382803
GSSPFG00011376001-RA	2.33	uncharacterized protein LOC110384109
GSSPFG00019586001-RA	-2.70	uncharacterized protein LOC110384111
GSSPFG00014786001-RA	-7.17	uncharacterized protein LOC110384139

Continue

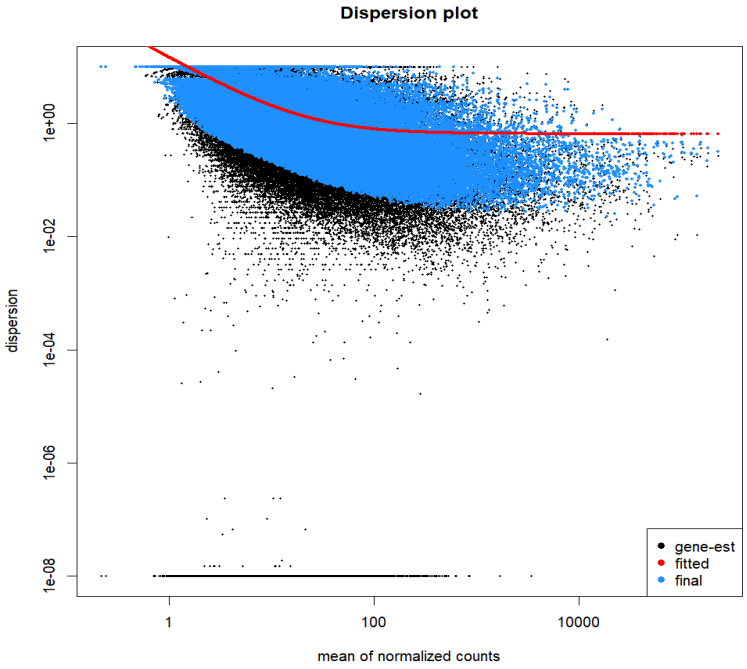
Transcript	LDC	Description
GSSPFG00004412001-RA	2.32	uncharacterized protein LOC110384189 isoform X2
GSSPFG00012146001-RA	-2.07	Uncharacterized protein OBRU01_07656
GSSPFG00032846001-RA	2.51	Uncharacterized protein OBRU01_15509
GSSPFG00032463001-RA	6.99	UPF0704 protein C6orf165 homolog
GSSPFG00011536001-RA	-3.58	vitellogenin-like
GSSPFG00018976001-RA	-4.98	xanthine dehydrogenase-like

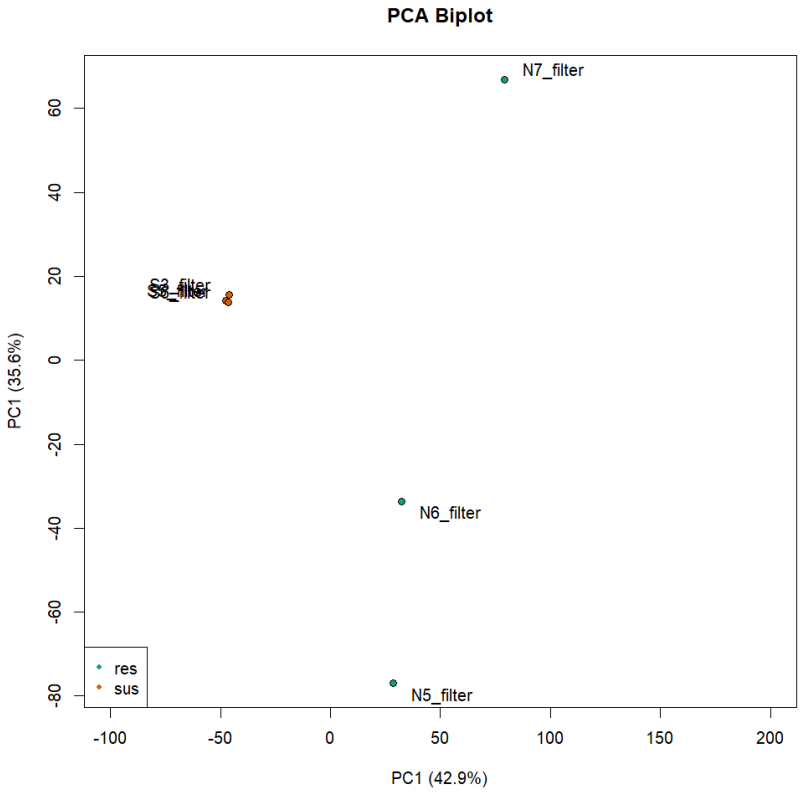
APPENDIX D



Dispesion plot and fit estimated of transcripts (A)Deseq2 Sf-ss x Clo-rr (B) Deseq2 Sf-ss x Lam-rr (C) PCA analysis from Deseq2

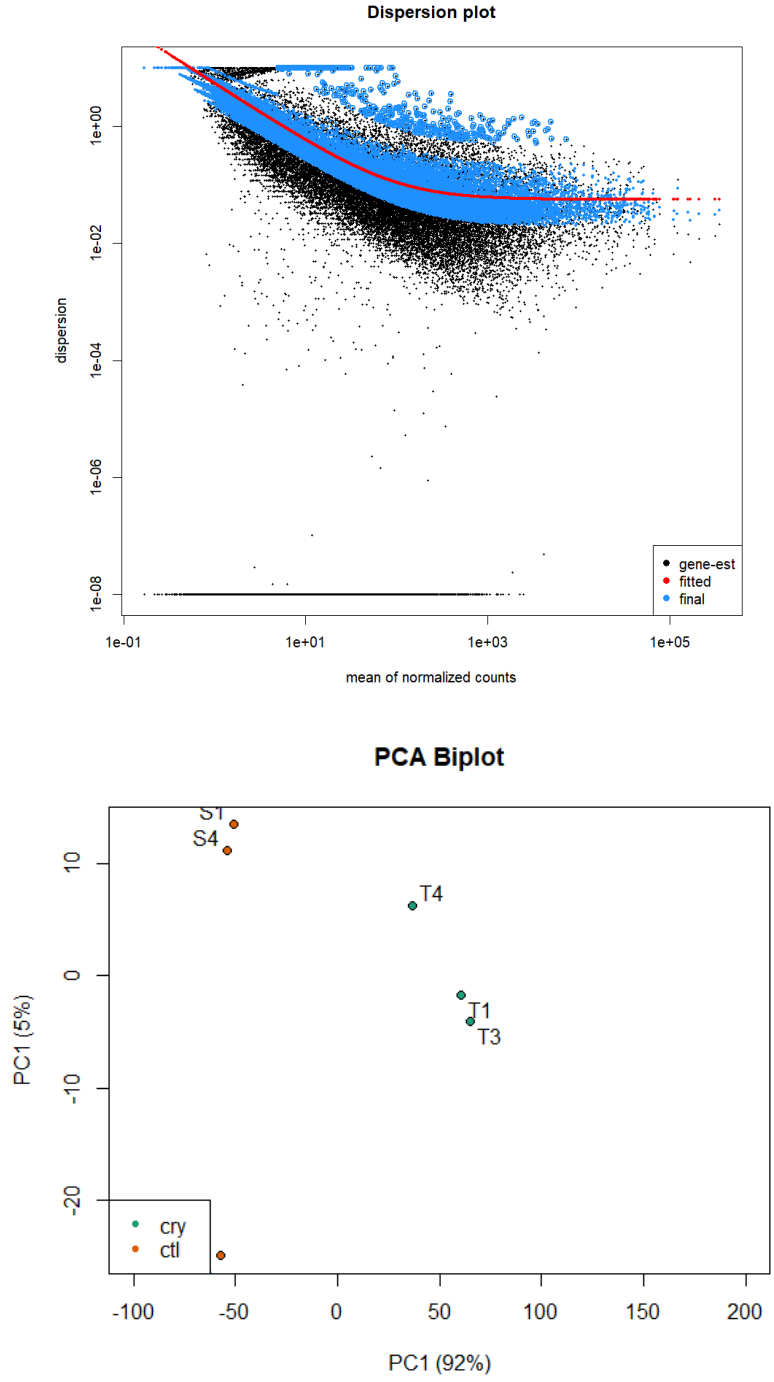
APPENDIX E





Dispesion plot and fit estimated of transcripts from Deseq2 Sf-ss x Tef-rr (A) PCA analysis (B)

APPENDIX F



Dispesion plot and fit estimated of transcripts from Deseq2 Sf-ss x VTPRO-rr (A) PCA analysis (B)