GLOBAL MECHANISMS OF BT RESISTANCE IN THE COTTON BOLLWORM, *HELICOPERA ZEA*: THE EXPECTED AND UNEXPECTED

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Abstract

Evolved resistance to genetically modified crops including economically important products such as cotton, corn, soybeans and many others has become rapidly and globally widespread over the last 20 years. There are now examples of important agricultural pests of crops expressing Bt (*Bacillus thuringiensis*) bacterial proteins (Cry1Ac, Cry1F, Cry2Ac) that have evolved resistance to these Bt-toxins. Increases in resistance have been reported with each field season in recent years to Cry1Ac in *Helicoverpa zea*. RNA-seq is a method to quantitatively measure total gene expression in an organism. This method was used to measure differences in global gene expression between a Bt-susceptible and a Bt-resistant strain of bollworm, where the differences in susceptibility to Cry1Ac +Cry1F toxin was 100-fold. We found gene expression differences that would be expected based on our current understanding of Bt mode of action and Bt resistance, including increased expression of proteases and reduced expression of Bt-interacting receptors in Bt-resistant bollworms. We have also found additional expression differences between the two strains of bollworm in genes that have not previously been thoroughly investigated, non-coding and immune system associated genes. This suggests that there are multiple different mechanisms influencing the development of Bt-resistance in addition to potential previously unrecognized pathways of resistance in this agricultural pest species. Also, it is important to consider how investigations into the genetic mechanisms of Bt-resistance will aide in understanding how resistance develops, and this could also provide better monitoring tools for resistance. Ideally this knowledge can then be applied to reduce the vulnerability of novel and next-generation integrated pest management technologies to pest resistance.

Introduction

Helicoverpa zea, commonly known as a bollworm, is one of the most prevalent insect pests on cotton in the United States (USDA 1925). During a growing season, the adult moths lay their eggs on the leaves of cotton plants, and the caterpillars can cause extensive damage to bolls leading to a reduction in the production of useable cotton (USDA 1925). In one growing season of cotton, there can also be multiple generations of *Helicoverpa zea* present in a given field or area (University of California 2019, Fleming et al. 2018). While *Helicoverpa zea* are largely prevalent on cotton, they are also known to feed on corn, sorghum, and hundreds of other cultivated and non-cultivated hosts (University of California 2019, Fleming et al. 2018).

First approved for commercial use in 1995 and subsequently planted in 1996 for the first time, transgenic cotton has been an effective means of controlling many lepidopteran pests including *Helicoverpa zea* (Fleming et al. 2018). The Cry-1Ac, Cry-1F, and other Cry protein producing genes isolated from *Bacillus thuringiensis* are among those successfully inserted into transgenic cotton; also included in transgenic cotton more recently are the Vip proteins (Palma et al. 2014, Fleming et al. 2018). One of the latest generations of transgenic cotton used commonly in agriculture to control *Helicoverpa zea* is Widestrike III, released in 2015; this Bt-transgenic cotton product expresses Cry1Ac, Cry1F, and Vip3A protein toxins (Fleming et al. 2018). Other commonly planted transgenic cotton strains include TwinLink Plus and Bollgard 3, which both express Cry proteins as well (Fleming et al. 2018). A major benefit of these protein toxins is that they are non-toxic to humans and other important insect predators such as birds which make this pest control technique much more advantageous than chemical insecticides (Qaim and De Janvry 2005, EPA 2002). Effective pest control in cotton cultivation is of the utmost importance in an industry that produces an

estimated 4.5 million tons of cotton annually in the United States alone (Statista 2018).

When a Bt toxin is ingested, the protein goes through multiple processes including solubilization, toxin activation, toxin binding to low-affinity sites, toxin binding to high-affinity sites, toxin insertion into the midgut epithelial membrane, and finally disruption of cell osmosis and septic infection due to gut bacteria entering through pores formed by the toxin. This process leads to death of the insect (Palma et al. 2014, Bravo et al. 2007). Ingested Bt protein interacts with specific protease enzymes (serine proteases) in order to achieve cleavage and activation; the subsequently cleaved protein toxin then binds to specific families of receptors found in the insect midgut (cadherins, alkaline phosphatases, and aminopeptidases) in order to initiate pore formation in the midgut epithelial membrane (Palma et al. 2014, Bravo et al. 2007). Transgenic Bt-crops are effective in the control of a myriad of pest species including lepidopterans, coleopterans, and others (Palma et al. 2014, Bravo et al. 2007). Unfortunately, in recent years resistance to Cry protein toxins in transgenic Bt crops has been detected worldwide in multiple pestiferous species.

A primary concern in pest management using transgenic crops is the development of resistance, whether this be to sprayable insecticides or transgenic insecticides. In several pest species world-wide, resistance to Bt-crops and Cry family protein toxins has been detected. Populations of fall armyworms (*Spodoptera frugiperda*) in Puerto Rico have been determined to be resistant to Cry1F in transgenic corn (Moar et al. 2008). Additional resistant fall armyworm populations have recently been discovered in North Carolina as well (Tabashnik et al. 2009, Huang et al. 2014). Another pest species, the maize stalk borer (*Busseola fusca*), has developed resistance to Cry1Ab in South Africa (van Rensburg et al. 2007). Other reports include resistance to Cry proteins (Cry3Bb, mCry3A, Cry1Ac) in the western corn rootworm (*Diabrotica v. virgifera*) and the pink bollworm (*Pectinophora gossypiella*) in the United States and India, respectively (Gassmann et al. 2011, Gassmann et al. 2014, Dhurua et al. 2011). There have also been several successfully lab-reared resistant strains for tobacco budworms (*Heliothis virescens*) at NC State University by Dr. Fred Gould; this strain is over 32000-fold resistant to multiple Bt toxins (Gould et al. 1997). The use of lab-reared colonies has proven effective in investigations into the mechanisms of resistance (Jackson et al. 2007). A resistant strain of the fall armyworm was also developed at NCSU demonstrating resistance to both Cry1A and Cry2A2 (Jackson et al. 2007). In regards to *Helicoverpa zea*, resistance has developed to Bt toxins expressed in transgenic cotton (Cry1Ac--(Tabashnik et al. 2013, Tabashnik et al. 2015) and Cry2Ab2--(Reisig et al. 2018) in addition to sweet corn, Cry1A.105, Cry1Ab, and Cry2Ab2--(Dively et al. 2016)) in the United States. Resistance to the latest generation of transgenic cotton, Widestrike III (Cry1Ac and Cry1F) has developed rapidly since 2015 (Yang et al. 2017, Fleming et al. 2018).

Studies of resistance have resulted in several different suggested mechanisms for resistance to Bt toxins. Bt-resistant *Heliothis virescens* and other lepidopteran species have been discovered that affect toxin activation in the midgut in addition to shifts in toxin binding ability to the midgut epithelial peritrophic membrane (Soberón et al. 2010, Pardo-Lopez et al. 2013). The most prevalent mutations discovered at present are changes in midgut proteases, midgut cadherin receptors, midgut GPI-anchored alkaline phosphatases and shifts in glycolipid synthesis (Soberón et al. 2010, Jurat-Fuentes et al. 2011, Pardo-Lopez et al. 2013). Alterations in ATP-binding cassette (ABC) transporters have also been implicated in practical resistance (Gahan et al. 2010, Atsumi et al. 2012). Most recently, Jin et al. (2018) discovered a dominant point mutation in a tetraspanin gene (TSPAN) associated with field-evolved resistance of the cotton bollworm, *Helicoverpa armigera*, to transgenic cotton in China. Resistance in *Helicoverpa* species has also been implicated to be influenced by a decrease in Cry1Ac activation by midgut proteases (Zhang et al. 2018). Aside from changes in transporters, enzymes, and receptor proteins, there is recent evidence that non-coding genetic elements are also involved in insecticide resistance. In populations of diamondback moth (*Plutella xylostella*) larvae resistant to chemical insecticides and Bt- endotoxins, altered expression levels of long non-coding RNAs (lncRNAs) have been discovered when comparing resistant to susceptible insects (Etebari et al. 2015). LncRNAs have also been determined to play a role in resistance to chlorantraniliprole in the diamondback moth, using high-throughput sequencing (Zhu et al. 2017). Further investigation into the role of lncRNAs in insecticide resistance is a novel area of research. A key function of lncRNAs is gene regulation via the suppression or activation of target genes. Due to these functions, further research into how these non-coding genes are involved in insecticide resistance is necessary (Dempsey and Cui 2016). Additionally, due to the mode of action of Cry1Ac and other Bt-protein toxins resulting in sepsis in the insect pest, an under investigated pathway of resistance in insects may be a bolstered immune system. There is potential for increased expression of immune genes allowing a resistant insect to become less vulnerable to the resulting bacterial infection when feeding on Bt protein toxins.

Due to the rapidly increasing levels of resistance of agricultural pests such as *Helicoverpa zea* to genetically modified

cotton, the purpose of our work was to identify at the genetic level, potential mechanisms for the development of resistance to *Bacillus thuringiensis* toxins in this lepidopteran species in order to aide in the development of solutions to pesticide resistance. The objectives of this experiment are to identify and analyze, using a shotgun-transcriptomics approach with RNA-Seq, individual genes, gene families, and non-coding genes that may be implicated in resistance development in *Helicoverpa zea.* This will be conducted by comparing gene expression levels of Bt-resistant neonate and Bt-susceptible neonate *Helicoverpa zea*.

Materials and Methods

Sample Collection and Preparation

In order to investigate the transcriptional profiles and differences in gene expression levels in *Helicoverpa zea*, we used lab-reared colonies of Bt-resistant (Wake Forest Strain: Cry1Ac +Cry1F; 100-fold resistant) *Helicoverpa zea* and obtained susceptible lab reared insects from Benzon Research Inc. Susceptibility was determined following the bioassay protocol outlined in Reisig et al. (2018). The insects were reared on the same diet in the same lab for at least two generations before the start of sampling. We created 5 resistant samples and 5 susceptible samples in order to have sufficient replicates. Each of these samples were constructed from 10 neonate *Helicoverpa zea* that were all un-fed and lab-reared within 24 h of emergence. For each sample, the 10 neonates were mechanically homogenized into one DNAse and RNAse-free tube.

RNA Extraction

Total RNA was extracted from each of the 10 samples using the RNeasy Mini Kit following manufacturer's protocol (Qiagen, Valencia, CA). RNA from each sample was analyzed for purity using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) by the NC State University Genomics Core Facility. Only those samples that exhibited sufficient purity (RNA Integrity Number >9.0) were used for subsequent sequencing.

RNA Sequencing

RNA-seq library preparation and sequencing were carried out by the NCSU Genomic Services Lab (Raleigh, NC, USA). RNA-Seq libraries were prepared using the TruSeq RNA Library Prep Kit v2 (Illumina, San Diego, CA, USA) as per the manufacturer's instructions to develop cDNA libraries for all samples using 500 µg of total RNA per sample. The NextSeq 500 System (Illumina, San Diego, CA, USA) on the paired-end setting with a read length of 2 x 150 bp was used for transcriptome sequencing. Using a High Output Flow Cell, we obtained a sequencing depth of >25 million reads per library. There were ten libraries constructed; 5 for resistant and 5 for susceptible. Raw reads were converted to fastq files with the SRA Toolkit v2.9.2 (Leinonen et al., 2011), and the FastQC tool v0.11.7 (Andrews 2010) was implemented for read quality assessment. For the Illumina sequencing, we required that the base qualities have a Phred score of >30 for the majority of the reads. Resulting Fastq files then proceeded to transcript assembly and quality control steps.

Transcript Assembly and Quality Control

Transcript assembly and quality control was performed by the NC State Bioinformatics Core (Raleigh, NC, USA). Raw reads were assembled with StringTie (Pertea et al. 2015) where 45224 primary transcripts were assembled using the reference genome of *Helicoverpa zea* which were assembled into transcript set 1. For those reads that did not align with StringTie, the program Trinity (Haas et al. 2013) was used to construct an alternate set of transcripts. For the 149108 transcripts assembled with Trinity, these were then processed through the Blobology program (Kumar et al. 2013) to ascertain the presence of possible contaminates. Only transcripts that were annotated as Lepidoptera were subsequently saved, this was 108867 transcripts. Ribosomal RNA transcripts were removed from the second transcript set as well. The remaining 108841 transcripts were then clustered using the Evigene program (Rago et al. 2016), resulting in 34059 transcripts for transcript set 1. Transcript set 1 and set 2 were then combined and clustered with Evigene which resulted in 26800 primary transcripts and 12095 alternates. These were then annotated through blobology to check for contaminates. Each fastq file for each replicate was then trimmed for adapter sequence and quality using the TrimmoMatic (Bolger et al. 2014) sequence trimmer. Using the *Helicoverpa zea* reference genome from NCBI, each trimmed replicate file was mapped to the reference genome using HiSat2 (Kim et al. 2015). Resulting mapped files were then input into StringTie in order to assemble the RNA-Seq alignments into potential transcripts. The transcript annotations for each replicate were merged into a single "expressed transcriptome" file, which was then used to guide gene boundaries when calculating differential expression values (log2 fold change) between the susceptible and resistant cultivars via CuffDiff (Trapnell et al. 2013). Threshold for log2 fold change in differential expression was ≥ 0.25 log 2 fold change. Results were then imported into the R statistical software platform (R core

team 2012) for quality control checks and visualization of results. The sequence of transcripts that were determined to be differentially expressed were extracted from the reference genome and used in a Blast search against insects to provide initial annotations. Quality control steps were conducted with FPKM, boxplots, MDS plot, PCA plot, normalization, heatmap, and volcano plot. All quality control steps were passed by all replicates. After all transcripts were assembled and quality control steps passed, 6098 transcripts were identified as differentially expressed in this experiment. Of these 6098, 3042 transcripts had higher expression in the susceptible strain with 267 being found only in this strain. The remaining 3056 had higher expression in the resistant strain with 323 being only expressed in the resistant strain. Blast2GO was implemented to functionally annotate open reading frame assignments (Götz et al. 2008). Gene ID and function was determined using BLASTx (E- value cut off 10^{-5}), using Lepidopterans taxonomy to filter results, running against the nr and swissprot databases (Götz et al. 2008).

Data Analysis and Figure Construction

Figures and tables for this paper were constructed using Microsoft Excel and PowerPoint (2018).

Results

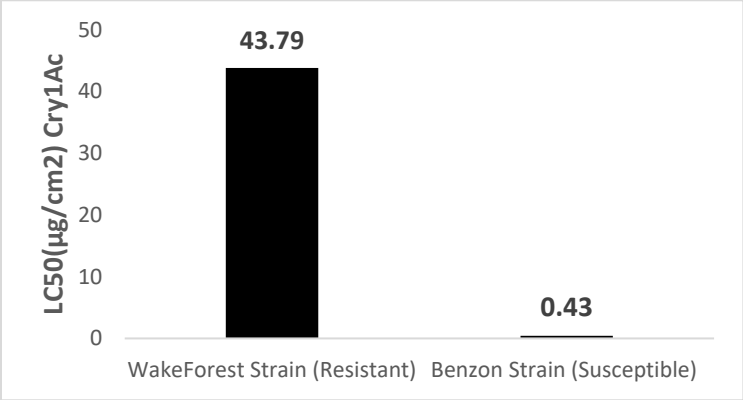

Figure 1. LC50 Resistant vs Susceptible.

Prior to performing RNAseq, Bt-susceptibility bioassays were conducted to determine level of resistance in two strains of the bollworm. The WakeForest (resistant) strain was found to have an LC50 of 43.79 µg/cm^2 for Cry1Ac (Unpublished data 2018) while the Benzon (susceptible) strain had an LC50 of 0.43 µg/cm^2 for Cry1Ac (Reisig et. al. 2018), indicating a fold difference in resistance of at least 100 (Fig.1).

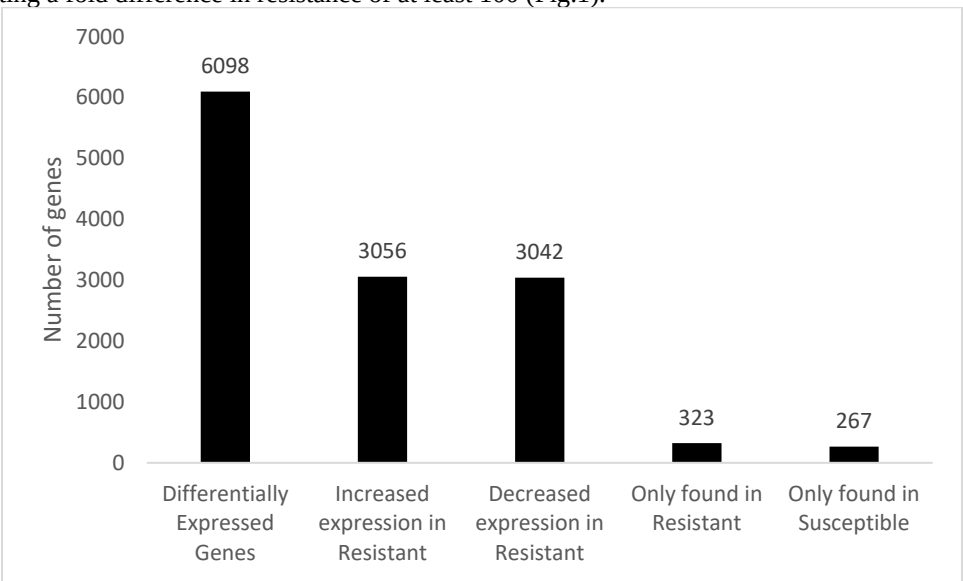

Figure 2. Differentially expressed genes in each strain of bollworm.

Conducting RNAseq resulted in the following gene expression results for the resistant and susceptible bollworm strains (also see Fig. 2). For both strains of the bollworm studied, there were 6098 differentially expressed genes. A differentially expressed gene is a particular gene that when compared to the control (the susceptible strain in this experiment) has either increased expression levels (up-regulated) or decreased expression levels (down-regulated) in comparison to the control. It was found that 3056 genes had increased expression in the resistant strain of bollworm while 3042 had reduced expression levels. In addition to these differentially expressed genes there were genes expressed that were ONLY found in either the resistant or susceptible strains, i.e., 323 genes expressed in resistant, and 267 only expressed in susceptible bollworms. Therefore, the resistant strain of bollworm had a higher number of genes with increased expression levels as well as a higher number of genes only found in this strain, in comparison to the control susceptible strain.

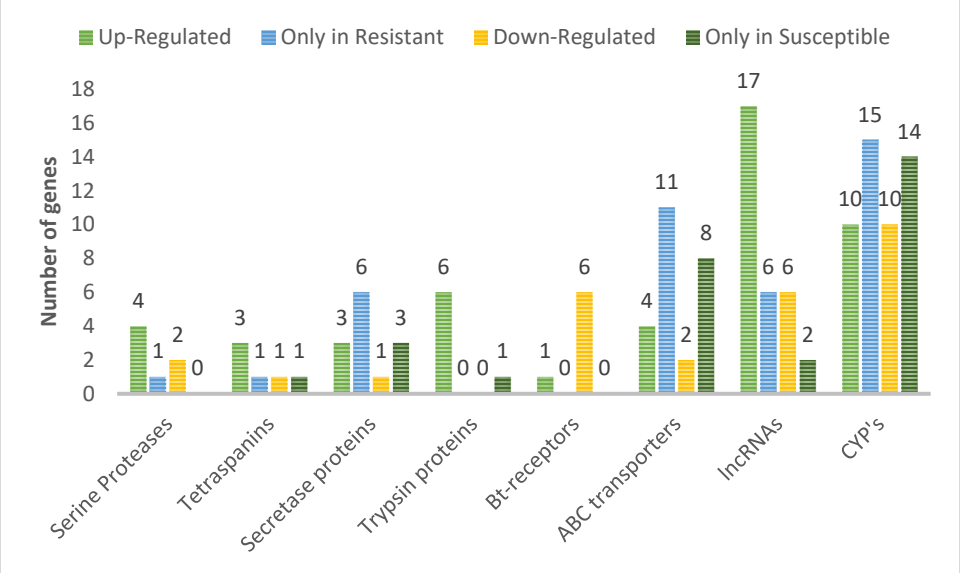

Figure 3. Resistance-associated genes categorized by protein family.

Fig. 3 depicts the number of differentially expressed genes (as well as genes only found in either strain) in gene families that have been recognized by previous research (see introduction) to be associated with Bt-resistance in lepidopterans. The number of genes for each family are as follows: serine proteases (4 up-regulated, 2 down-regulated, 1 only in resistant), tetraspanins (3 up-regulated, 1 down-regulated, 1 only in resistant, 1 only in susceptible), secretase proteins (3 up-regulated, 1 down-regulated, 6 only in resistant, 3 only in susceptible), trypsin proteins (6 up-regulated, 1 only in susceptible), Bt-receptors (1 up-regulated, 6 down-regulated), ABC transporters (4 up-regulated, 2 down-regulated, 11 only in resistant, 8 only in susceptible), long non-coding RNAs (lncRNAs) (17 up-regulated, 6 down-regulated, 6 only in resistant, 2 only in susceptible), cytochrome P450s (CYPs) (10 up-regulated, 10 down-regulated, 15 only in resistant, 14 only in susceptible). For all gene families, there was increased expression levels in the resistant strain of the bollworm with the exception of intestinal receptors (aminopeptidases, alkaline phosphatases, cadherins).

Table 1. Top differentially expressed resistance associated genes including general function and log2 fold change.

	Gene	Gene ID	General Function	Log2 Fold Change
Tetraspanins	Hzea.18477	Tetraspanin 1	Protein stabilization, cell signaling pathways, associated with Bt resistance	-4.92
	Hzea.11255	Tetraspanin 1	Protein stabilization, cell signaling pathways, associated with Btresistance	-3.37
Serine Proteases	Hzea.23538	Serine Protease 9-like	Protein cleavage	-5.13
	Hzea.24399	allergen Api m 6-like	Serine Type Endopeptidase	-6.86176
Secretase Proteins	Hzea.10453	Gamma-Secretase	Cleavage of transmembrane proteins	-4.7
	Hzea.30068	Beta-Secretase 1-like	Protein Cleavage	-3.04

Trypsin Proteins	Hzea.15497	Chymotrypsin 1-like	Intestinal protein cleavage	-4.44
	Hzea.4257	Trypsin 3A1-like	Intestinal protein cleavage	-5.31
Bt-Receptors	Hzea.14992	Alkaline Phosphatase 2	Intestinal receptor for Cry1Ac	3.77
	Hzea.17125	Alkaline Phosphatase 1 like	Intestinal receptor for Cry1Ac	6.26
	Hzea.11178	Mutant Cadherin (BtR)	Intestinal receptor for Cry1Ac	-2.93
Peptidases	Hzea.12825	Carboxypeptidase B like	Peptide cleavage	-3.04
	Hzea.7667	Carboxypeptidase B like	Peptide cleavage	2.03
Transporters	Hzea.29517	Multidrug Resistance protein 1 like	Efflux transporter	-2.22
	Hzea.3344	Multidrug Resistance protein 4 like	Efflux transporter	2.06
	Hzea.9148	ABC Transporter ABCC3	Transporter protein	-3.36
	Hzea.10318	ABC Transporter ABCC3	Transporter protein	-3.06
Non-coding genes	Hzea.12022	lncRNA	Various roles including transcriptional regulation	-4.87
	Hzea.2506	lncRNA	Various roles including transcriptional regulation	-4.65
Chitin	Hzea.21994	Chitin Synthase A & B	Chitin synthesis	-3.11
Stress response	Hzea.18297	Heat Shock Protein 12.2	Cellular stress response	-5.02
Metabolic genes	Hzea.9448	CYP337B2v2 & CYP337B1v1	Xenobiotic metabolism, associated with resistance to insecticides	-4.23
	Hzea.29377	CYP337B3v1	Xenobiotic metabolism, associated with resistance to insecticides	-4.1
Protease	Hzea.3274	E3 ubiquitin-protein ligase Siah1-like	Proteosome mediated protein degradation	-5.46839

Table 1 depicts for each category of resistance associated gene, the top 2 highest fold-change genes. Included in the table are gene id, general function, and magnitude of log2 fold change (negative=up-regulation in resistant, positive=down-regulation). The proteins with highest fold difference were in the serine protease group (Hzea.24399: -6.86176 log2 fold increase) and the Bt-receptor group (Hzea.17125: 6.26 log2 fold decrease).

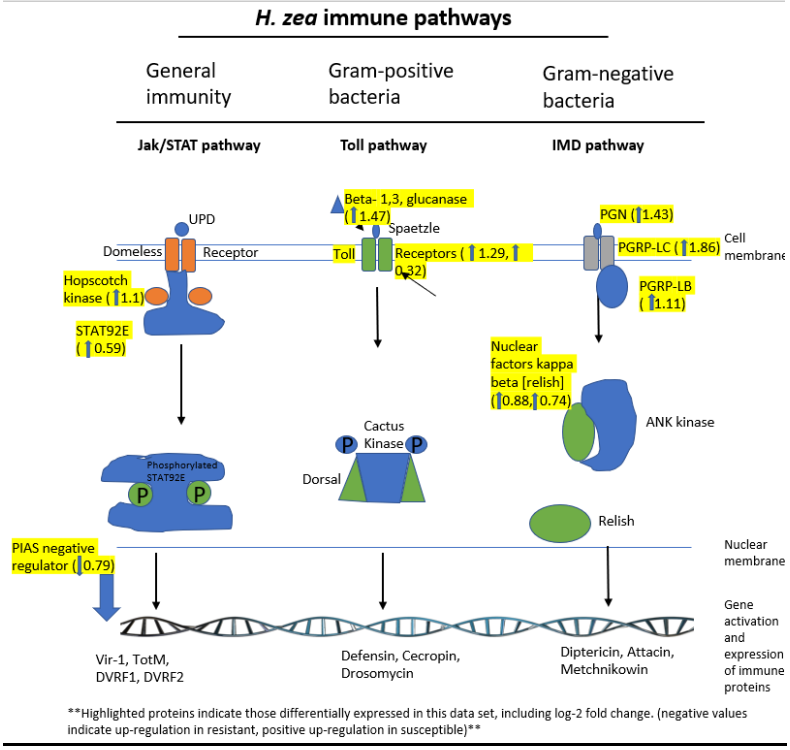

Figure 4. Generalized immunity pathways in *Helicoverpa zea*.

Fig. 4 displays 3 generalized immune pathways found in *Helicoverpa zea*, the Jak/STAT, Toll, and IMD pathways. Included in each pathways are receptors, regulators, activating protein kinases, and other pathway associated proteins. Highlighted in yellow are those proteins that were differentially expressed in this experiment, with the magnitude of fold change and direction (up- or down-) indicated as well. In the Jak/STAT pathway, 3 genes were differentially expressed: Hopscotch kinase (1.1 log2 fold increase), STAT92E (0.59 log2 fold increase), and PIAS (a negative regulator) (0.79 log2 fold decrease). In the Toll pathway 3 genes were differentially expressed: Beta-1,3, glucanase (1.47 log2 fold increase) and 2 Toll receptor proteins (1.29 and 0.32 log2 fold increase). In the IMD immunity pathway 5 genes were differentially expressed: PGN (1.43 log2 fold increase), PGRP-LC (1.86 log2 fold increase), PGRP-LB (1.11 log2 fold increase), and 2 NF-kappa-beta proteins (0.88 and 0.74 log2 fold increase). In each of these three pathways, the proteins involved in cell membrane receptors or protein activation were the main types of proteins observed to be differentially expressed.

Table 2. Top differentially expressed genes associated with 3 *Helicoverpa zea* immunity pathways.

Gene	Gene ID	General Function	Log2 Fold Change
Hzea.27139	Peptidoglycan (PGN) Recognition Protein C	IMD immune pathway, receptor	-1.86
Hzea.11065	Immunoglobulin binding protein	IMD immune pathway, binding	-3.41
Hzea.15446	Fas-binding factor 1	IMD immune pathway, binding	-3.75496
Hzea.28790	Beta- 1,3-glucanase protein	Pathogen recognition protein (Toll pathway)	-1.47469
Hzea.19146	Death associated protein kinase 1	Toll immune pathway	-0.678
Hzea.15107	Toll like receptor 3	Toll immune pathway, receptor	-1.29648
Hzea.8646	tyrosine kinase hopscotch	JAK/STAT pathway, activation	-1.10303
Hzea.17134	SH3 domain-containing kinase-binding protein 1	JAK/STAT pathway	-0.67685
Hzea.3426	E3 SUMO-protein ligase PIAS3	JAK/STAT pathway, negative regulation	0.79

Table 2 depicts the top three differentially expressed genes associated with each of the 3 immune pathways examined in this study. Included in the table is gene id, general function, and magnitude of log2 fold change (negative=up-regulation in resistant, positive=down-regulation). Of the three immunity pathways, the IMD pathway had the highest log2 fold increase as well as the highest number of genes recognized to be differentially expressed. In this pathway, the two highest log2 fold increase genes were Hzea.11065 (-3.41) and Hzea.15446 (-3.75496).

Discussion

Role of Proteases, Receptors, and Transporters in Resistance

Previous work surrounding Bt-resistance in *Helicoverpa zea* have identified several different mechanisms of resistance. Namely, changes in proteases (secretases, trypsins), midgut receptors (cadherins, aminopeptidases, alkaline phosphatases), transporters (ABC), and tetraspanins have all been implicated in Bt-resistance (Soberón et. al. 2010, Jurat-Fuentes et. al. 2011, Pardo-Lopez et al. 2013, Gahan et al. 2010, Atsumi et al. 2012, Jin et. al. 2018). In this experiment, through the use of RNAseq to analyze gene expression differences in resistant vs susceptible bollworms, we have found supporting evidence for these previous studies (Fig. 3, Table 1). This lends further support to these particular mechanisms of resistance and their role specifically in the bollworm. Some areas of interest would include confirming their relative contribution to resistance to the various Bt toxins and other potential proteins toxins in development, and what was the genetic mechanism for the establishment of these resistance mechanisms.

Role of Non-protein Coding Genetic Elements in Resistance

Outlined in the introduction section were the traits and functions of long non-coding RNAs. In this current study, there were 17 lncRNAs with increased expression levels in the resistant strain and only 2 lncRNAs with reduced expression in resistant bollworms (Fig. 3). Due to these significantly increased levels of lncRNAs being expressed in Bt-resistant *Helicoverpa zea*, the results suggest these lncRNAs are playing a role in resistance to Cry1Ac. This could be through the activation or suppression of other target genes known to be involved in Bt-resistance (for example, serine proteases, Bt-receptors, transporters, etc.) since activation or suppression are both major functions of long non-coding RNAs (Dempsey and Cui 2016). However, further investigation beyond RNAseq data will be necessary to confirm this hypothesis. Having a better *Helicoverpa zea* genome will be critical to this process, and with the data generated in this study, could for the first time bring “new light” into understanding the evolution of insecticide resistance like similar studies being conducted in humans to understand the regulation of xenobiotic metabolism.

Role of Insect Immunity in Resistance

When Bt-susceptible insects consume Cry1Ac protein toxins (and other toxins used in transgenic crops), the hypothesized mechanism of death is ultimately due to sepsis when gut bacteria enter the body of the insect (caused by pore formation due to Cry protein interactions). In this study we hypothesized that one potential mechanism for resistance to transgenic cotton stems from a bolstered immune system (in addition to the other previously recognized mechanisms of resistance just discussed). We propose that resistance is linked to either increased expression or activity of immune response proteins in *Helicoverpa zea*.

There are three major immune pathways found in the cotton bollworm: JAK/STAT, Toll, and IMD. The IMD immune response pathway primarily deals with responses to gram-negative bacteria. The major components (among others) are as follows: Peptidoglycan recognition proteins (PGRPs), Peptidoglycan binding proteins, Fas-associated death domains (FADD), DREDD, Relish, Transforming growth factor betas (TAK, TAB), Nuclear factors kappa beta, immunoglobulin binding proteins, Fas binding factors, caspases, and defensive proteins (Cao et. al. 2015, Liu et al. 2014). In this experiment, we found a number of genes involved in the IMD pathway with increased expression in the resistant strain: Fas-binding factor (-3.75 log2 fold change), immunoglobulin binding protein (-3.41 log2 fold change), Peptidoglycan recognition protein C (-1.86 log2 fold change), Peptidoglycan binding protein (-1.43 log2 fold change), Nuclear factor kappa betas (-0.88, -0.74 log2 fold change), and transforming growth factor betas (-0.54, -0.48 log2 fold change) (Fig. 4; Table 2).

The second immune pathway found in insects is the Toll pathway. The Toll pathway deals with immune responses to gram-positive bacteria, fungi, and developmental processes. There are some components of the Toll pathway that are shared with the IMD pathway, the major components of which are: Spatzle, Toll receptors, Beta-1,3, glucanases, Cactus proteins, Dorsal proteins, death associated protein kinases (DAP), and cecropin proteins (Cao et. al. 2015, Liu et. al. 2014). In this experiment, components of the Toll pathway have also been found to have increased expression in the resistant strain of the bollworm: Beta-1,3, glucanase (-1.47 log2 fold change), DAP kinases (-0.68 log2 fold

change), Toll protein (-0.33 log2 fold change), and Toll receptor (-1.30 log2 fold change) (Fig. 4; Table 2).

The third major immune pathway in insects, JAK/STAT, was also found to have differentially expressed components. The JAK/STAT pathway, instead of specializing to either gram-negative or positive bacteria, deals with the generalized immune response as well as some developmental processes. The major components of this pathway are Domeless receptors, Hopscotch kinases, STAT proteins, PIAS regulators, SOCS proteins, and defensive proteins (Cao et. al. 2015). In this study, the following components of JAK/STAT were differentially expressed: Hopscotch kinase (-1.1 log2 fold change), STAT5B (-0.59 log2 fold change), SH3 binding protein (-0.68 log2 fold change), and PIAS3 (a negative regulator) (0.79 log2 fold change) (Fig. 4; Table 2).

Of the three immune pathways, the IMD pathway had the greatest number of mRNA transcripts with increased expression in the resistant strain (10 transcripts) as well as the highest amount of fold change (Fas-binding factor (-3.75 log2 fold change), immunoglobulin binding protein (-3.41 log2 fold change), Peptidoglycan recognition protein C (-1.86 log2 fold change)) (Fig. 4; Table 2). This may be due to gram-negative bacteria commonly colonizing the gut-cavities of lepidopterans (Xia et. al. 2018). Due to exposure to gut-bacteria being the commonly recognized mechanism of death in insects exposed to Bt-transgenic crops, it is possible this explains why the IMD immune response pathway was the most affected (Fig. 4; Table 2). However, both gram-negative and gram-positive bacteria are found in gut-cavities of lepidopterans (Xia et. al. 2018). Overall, the increased expression in these three immune pathways may be aiding in resistance due to increased ability to fight bacterial infection. The insect gut-microbiome and immune system has been previously implicated in chemical insecticide resistance (chlorpyrifos); it is possible that similar interactions are occurring with Bt-resistance as well (Xia et. al. 2018). If increased insect immunity is a resistance pathway for Bt toxins, one outcome to resistance might be an insect resistant to naturally occurring infections.

Conclusions and Future Directions

In conclusion, in this study we have confirmed previous knowledge about Bt-resistance in *Helicoverpa zea* using RNAseq to compare gene expression levels. We found increased expression in proteases, transporters, tetraspanins, and secretases; in addition to decreased expression or changes in Cry1Ac interacting receptors in the resistant strain of the bollworm. In addition to these changes, additional genetic shifts were observed which have not been recognized for Bt resistance. These were increased expression of long non-coding RNAs and immune pathway associated genes. In considering these two previously under recognized mechanisms of Bt-resistance, more research is certainly needed to fully comprehend how these influence resistance to Bt-crops and the role of epigenetics in this process. This information is also vital as we likely move from Cry proteins to new toxins to “fight” Bt resistance.

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