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Investigation of Cry3Bb1 resistance and intoxication in Western Corn Rootworm by RNA sequencing

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Abstract

The western corn rootworm (WCR) *Diabrotica virgifera virgifera* LeConte is a major pest of corn that has evolved resistance to transgenic maize that produces insecticidal Cry toxins. The specific mode of action of Cry3Bb1 and mechanism of resistance in WCR are unknown. This study compared gene expression between Cry3Bb1-susceptible and Cry3Bb1-resistant WCR neonates, in the presence and absence of Cry3Bb1. RNA-Seq data were analyzed to identify differentially expressed transcripts between strains of WCR, providing candidate transcripts for resistance to Cry3Bb1. Constitutive and Cry3Bb1-induced differences between strains caused the differential expression of 608 transcripts after 8 hr. Differentially expressed transcripts between strains included ABC transporters, proteases and α-amylases, which known to be receptors or activators of Cry toxins and involved in resistance to Cry toxins in other insects. The response to Cry3Bb1 treatment resulted in approximately 5,000 differentially expressed transcripts in the susceptible strain and included the same annotation categories found between strains but also included metalloproteases, cadherins and signaling proteins. None of these annotations were identified

in the response of the resistant strain to Cry3Bb1, which was represented by only 12 transcripts. Tissue-specific expression analysis of selected transcripts revealed that an α-amylase and a protease were expressed in the midgut, the target organ of Cry toxins. A protease inhibitor and two ABC transporters were expressed outside the midgut, suggesting a limited role in resistance. Numerous polymorphic sites were identified from the RNA-Seq data that showed allele frequency differences between the resistant and susceptible strains. Analysis of these polymorphisms in a larger set WCR strains suggested that the differences were due to genetic drift rather than being associated with resistance to Cry3Bb1. Polymorphisms identified in genes with known roles in resistance to Cry toxins did not appear to differ in frequency between resistant and susceptible strains.

Keywords: *Diabrotica*, genetically modified crops, resistance evolution

1 Introduction

The western corn rootworm (WCR) *Diabrotica virgifera virgifera* (LeConte) is a major pest of corn, *Zea mays* (L.), due to its invasiveness and its ability to adapt to synthetic insecticides and crop rotation with soya bean, *Glycine max* (L.) Merr (Ball & Weekman, 1963; Gray, Sappington, Miller, Moeser, & Bohn, 2009; Levine, Spencer, Isard, Onstad, & Gray, 2002; Meinke, Siegfried, Wright, & Chandler, 1998; Wright et al., 2000). In the United States, yield loss and management costs due to WCR are estimated in excess of U.S. $1 billion annually (Dun, Mitchell, & Agosti, 2010; Metcalf, 1986; Sappington, Siegfried, & Guillemaud, 2006; Tinsley, Estes, & Gray, 2013).

Different toxins from *Bacillus thuringiensis* Berliner with activity against WCR have been expressed in commercial corn hybrids, including Cry3Bb1, mCry3A, Cry34/35Ab1 and eCry3.1Ab (DiFonzo, 2017; Gassmann, 2016). Field-evolved resistance to Cry3Bb1, widespread in the U.S. Corn Belt (Gassmann, Petzold-Maxwell, Keweshan, & Dunbar, 2011, 2012; Gassmann et al., 2016; Ludwick et al., 2017; Schrader, Estes, Tinsley, Gassmann, & Gray, 2017; Wangila, Gassmann, Petzold-Maxwell, French, & Meinke, 2015), confers cross-resistance to mCry3A (Gassmann et al., 2014) and eCry3.1Ab (Jakka, Shrestha, & Gassmann, 2016; Zukoff et al., 2016).

The specific mode of action of Cry toxins in WCR has not been determined. However, it is believed that a general mode of action for *Bt* toxins, initially described in Lepidoptera, involves a series of steps disrupting midgut epithelial cells (Bravo, Gill, & Soberón, 2007; Gill, Cowles, & Pietrantonio, 1992; Usta, 2013). This mode of action is sometimes referred to as the "sequential binding model" (Jenkins, Lee, Valaitis, Curtiss, & Dean, 2000) or "pore formation model" (Zhang, Candas, Griko, Rose-Young, & Bulla, 2005). After ingestion, the crystal protein is solubilized and activated by gut proteases. The toxin binds to high-affinity receptors on the epithelial brush border of the midgut cells. These receptors oligomerize once bound to the

toxin. A second step of binding to other receptors of the midgut follows. This process leads to the insertion of the protein complex into the cell membrane, forming pores in the midgut epithelium. The resulting osmotic disruption of midgut tissue resulting from cell lysis leads to death of the larva by septicemia (Knowles, 1994; Knowles & Ellar, 1987; Usta, 2013; Van Rie, McGaughey, Johnson, Barnett, & Mellaert, 1990). Several proteins can thus be considered essential for proper functioning of the insecticidal properties of the toxin, including midgut proteases that are responsible for activation, and membrane-bound proteins that function in toxin binding, insertion and oligomerization.

Another model of cytotoxicity for *Bt* toxins was proposed by Zhang et al. (2005), Zhang, Candas, Griko, Taussig, and Bulla (2006) and involves an Mg2+-dependent cellular signaling pathway inducing apoptosis downstream of toxin binding to a receptor. This pathway includes binding to a membrane receptor, believed to be a cadherin, and this stimulates G protein and adenylyl cyclase, increasing cAMP levels and activating protein kinase A, which leads to cell death (Zhang et al., 2005, 2006).

The most abundant information available on *Bt* toxin resistance mechanisms comes from studies of Lepidoptera (Ferre & Van Rie, 2002; Heckel et al., 2007; Khajuria, Buschman, Chen, Siegfried, & Zhu, 2011; Khajuria et al., 2009; Lei et al., 2014). The resistance mechanisms most commonly reported from Lepidoptera involve disturbance of proteolytic toxin activation and toxin binding. Modification of these two critical steps in the Cry toxin mode of action can cause resistance. For instance, reduced proteolytic activity and absence of a necessary midgut protease for toxin activation resulted in slower and reduced activation of Cry protoxins in some colonies of *Plodia interpunctella* (Hübner) (Oppert, Kramer, Beeman, Johnson, & McGaughey, 1997; Oppert, Kramer, Johnson, Macintosh, & McGaughey, 1994; Oppert, Kramer, Johnson, Upton, & McGaughey, 1996). Alternatively, a change in protease activity was detected in Cry-resistant *Heliothis virescens* (Fabricius), promoting degradation of the toxin and reducing the amount in the midgut lumen for possible binding (Forcada, Alcácer, Garcerá, & Martínez, 1996). Other reported modifications include reduced binding by decreased expression of receptors such as aminopeptidase N and alkaline phosphatase (Herrero, Gechev, Bakker, Moar, & de Maagd, 2005; Herrero, Oppert, & Ferré, 2001; Jurat-Fuentes et al., 2011) or alteration of the Cry toxin binding sites of receptors, including cadherin, aminopeptidases N and P, and ABC transporters (Bel, Siqueira, Siegfried, Ferré, & Escriche, 2009; Ferre & Van Rie, 2002; Gahan, Pauchet, Vogel, & Heckel, 2010; Khajuria et al., 2009, 2011). The latter modifications resulted from point mutations, insertions or deletions in genes coding for receptors, eventually changing the sequence of their binding site for the toxin, but also from disturbance of post-binding events, such as membrane insertion. Hence, the two main phenomena

in the development of resistance to Cry toxins are variation in expression levels and sequence modification of the key proteins involved in the mode of action of Cry toxins.

In addition to WCR, two other coleopterans have evolved resistance to Cry toxins, at least under laboratory selection. The Colorado potato beetle *Leptinotarsa decemlineata* (Say) has been reported to be resistant to Cry3A after laboratory selection experiments (Loseva et al., 2002; Whalon, Miller, Hollingworth, Grafius, & Miller, 1993). Loseva et al. (2002) determined that the activity of midgut proteases of the Cry3Aa-resistant *L. decemlineata*, including aminopeptidase, was increased compared to the susceptible strain. These authors also observed that the resistant *L. decemlineata* expressed different protease isoforms relative to the susceptible strain and that the overall toxin binding was decreased in the resistant midgut. In a laboratory-selected strain of *Chrysomela tremula* Fabricius, Cry3A resistance was linked to a four base pair deletion in one of the intracellular domains of an *ABCB* transporter (Pauchet, Bretschneider, Augustin, & Heckel, 2016).

Other studies of molecules involved in the binding of Cry3 toxins in beetles provide complementary information. An "A Disintegrin and Metalloprotease," ADAM metalloprotease, was found to be involved in cleavage of Cry3Aa in *L. decemlineata* (Rausell, Ochoa-Campuzano, Martínez-Ramírez, Bravo, & Real, 2007). It was also described as a receptor of Cry3Aa in *L. decemlineata* (Ochoa-Campuzano, Real, Martínez-Ramírez, Bravo, & Rausell, 2007). A cadherin, a soluble α-amylase and a GPI-anchored alkaline phosphatase have been characterized as potential receptors for Cry3Aa in *Tenebrio molitor* Linnaeus (Bulushova et al., 2011; Oppert, Dowd, et al., 2012; Zúñiga-Navarrete, Gómez, Peña, Bravo, & Soberón, 2013). Differential expression of serine peptidases also could play a role in the efficacy of the protoxin as their expression was greatly reduced during Cry3Aa intoxication in susceptible *T. molitor* (Oppert, Martynov, & Elpidina, 2012).

Because the mechanism of Cry3Bb1 resistance in WCR is largely unknown, a general overview of changes in expression level between the susceptible and resistant insects could provide insight into the mechanism of resistance. RNA sequencing involves the identification of all transcripts specific to a physiological condition or developmental stage (Nagalakshmi, Waern, & Snyder, 2010; Wang, Gerstein, & Snyder, 2009). It can provide necessary information to compare expression levels between susceptible and resistant strains of WCR by providing a comprehensive collection of transcripts that are differentially expressed. RNA-seq provides precise measurements of transcript levels and isoform expression and also gives access to the transcript sequences and their potential variations between susceptible and resistant strains.

The similarity of three-dimensional structure among Cry1, 2, 3 and 4 toxins suggests a similar mode of action of these toxins and common receptor

proteins (Gómez et al., 2007; Griffitts & Aroian, 2005). The structural differences among these toxins are likely the source of toxin specificity among different species (Pigott & Ellar, 2007). Consequently, it is important to consider a wide range of possible resistance mechanisms to better understand resistance in WCR. To elucidate Cry3Bb1 resistance mechanisms in WCR, we examined changes in expression of transcripts encoding proteins involved in toxin activation and toxin binding. The objective of this work was to establish a list of differentially expressed transcripts between Cry3Bb1-susceptible and Cry3Bb1-resistant "Hopkinton" strains of WCR and to identify sequence polymorphisms that might be associated with the resistance to Cry3Bb1. In addition, the results provided information on transcripts potentially involved in the response to Cry3Bb1 in the two WCR strains.

2 Materials and Methods

2.1 RNA sequencing: sample collection and data analysis

2.1.1 Neonate rearing and collection of samples

Western corn rootworm neonates susceptible to Cry3Bb1 were obtained from eggs of a non-diapausing laboratory strain (Branson, 1976). The Cry3Bb1-resistant non-diapausing strain of WCR was initiated from a cross between the susceptible strain and a resistant population collected from the field in Hopkinton, Iowa, and maintained in the laboratory for over 30 generations (Gassmann et al., 2011; Ingber & Gassmann, 2015). The eggs were washed from the soil using tap water and a 60-mesh sieve. The washed eggs were placed into a Petri dish lined with moistened filter paper and stored in a growth chamber at 25°C until hatching.

Bt corn was grown from seeds expressing full-length Cry3Bb1 *Bt*-protein, Stone 6021VT3 (Monsanto Co., St. Louis, MO), along with a non-*Bt* near isoline (Stone 6021RR2). The two plant types were grown as described by Alouw and Miller (2015).

Because transcript expression is a dynamic process, we determined changes over time for both strains. We chose two early time points to minimize differences that resulted from the intoxication response of the susceptible strain. Moreover, susceptible neonates would die from a long exposure to Cry3Bb1. Neonates were <24 hr old and were transferred to germinated corn seeds maintained in Petri plates lined with moistened filter paper. For both strains, approximately 50–100 neonates were placed on non-*Bt* and Cry3Bb1 corn, respectively, in three independent replicates. The neonates were allowed to feed for 4–8 hr on either non-*Bt* corn or Cry3Bb1 corn. From the exposed larvae, three replicates of 30 neonate larvae were collected from corn roots for each treatment. RNA extraction was performed

using the RNeasy Mini Kit (Qiagen Inc., Valencia, CA) in RNase-free conditions and treated with DNase I (Qiagen Inc., Valencia, CA) following manufacturer's recommendations.

2.1.2 RNA sequencing: samples and data collection

A total of 24 neonate samples were sent to the University of Nebraska Medical Center DNA Sequencing Core Facility for RNA sequencing. The quality of total extracted RNAs was checked with an Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA). The RNA was then used for cDNA library construction that was sequenced using a HiSeq 2500 instrument (Illumina Inc., San Diego, CA) in 100-bp paired-end mode.

The paired-end reads were trimmed to remove low-quality reads and read portions using the Sickle program in paired-end mode (Joshi & Fass, 2011). The minimal quality threshold and minimum length (in base pairs) were both set to 20, which were the default values. The remaining trimmed reads were then aligned to a reference transcriptome (Eyun, Wang, & Benson, 2014) with the aligner Bowtie2 (Langmead & Salzberg, 2012). The data set was filtered to eliminate contigs of the transcriptome to which an average of <10 reads per sample was aligned.

2.1.3 Data analysis

A differential expression analysis was conducted to determine differences in transcript expression between the susceptible strain and the Hopkinton-resistant strain on non-*Bt* or *Bt* corn. This differential expression analysis was conducted with the R (R Development Core Team 2013) package DESeq2 (Love, Huber, & Anders, 2014). This program quantifies the effect of the different factors on the expression of transcripts, such as the effect of corn hybrid, WCR strain and their interaction. Data were analyzed separately by time point.

Full data set

The factors tested were as follows: (i) the effect of strain (susceptible versus resistant), considering the difference between strains across treatments as samples of larvae exposed to both non-*Bt* and Cry3Bb1 corn were included; (ii) the effect of treatment (non-*Bt* versus Cry3Bb1 corn, considering all samples and comparing Cry3Bb1 exposure to non-*Bt* exposure (control); and (iii) the interaction between the two factors measured in terms of transcripts for which there was an effect of the strain and of the treatment acting together on the transcription levels.

Subsets of samples: constitutive and Cry3Bb1-induced differences

Constitutive expression differences between the strains in the absence of toxin exposure and the differential responses of the strains to Cry3Bb1

exposure were investigated by analyzing subsets of the data. In the former case, only samples fed on non-*Bt* corn were considered. In the latter case, the data set was confined to samples fed on Cry3Bb1 corn. In all comparisons between strains, the fold change was calculated as the ratio of the expression level in Hopkinton neonates compared to the expression in susceptible neonates (Hopkinton/Susceptible), and all comparisons between treatments were calculated comparing the Cry3Bb1-treated samples to the non-*Bt* levels of expression (Cry3Bb1-treated/ non-*Bt*).

p-Values were corrected for multiple testing following the false discovery rate method of Benjamini and Hochberg (1995). Transcripts were considered significantly differentially expressed when adjusted *p*-value was ≤0.05 (FDR = 5%).

Annotations on sequence similarity and potential functions of the differentially expressed transcripts, using a BLASTx analysis of transcripts translated against the protein database and identification of protein domains with InterPro Scan, were determined by BLAST2GO (Conesa et al., 2005).

Single nucleotide polymorphism analysis

The program Freebayes (Garrison & Marth, 2012) v1.0.2-dirty was used to detect polymorphisms in transcript sequences. Freebayes is a Bayesian genetic variant detector able to model multiallelic loci from individuals with non-uniform copy numbers, across samples, from alignment data. The analysis with Freebayes used the aligned read data previously generated for gene expression analysis. A subset of polymorphisms that were likely to yield viable single nucleotide polymorphism (SNP) assays was chosen for further analysis. Only biallelic variants (SNPs and indels) with more than five supporting reads and with a minor allele frequency ≥0.2 and minimal quality score of 20 were conserved.

To identify polymorphisms with high levels of allele frequency differentiation between susceptible standard and resistant Hopkinton strains, *F*ST, the standardized variance in alleles between subpopulations (Weir & Cockerham, 1984; Wright, 1951), was computed. The hierfstat package (Goudet, 2005) for R (R Development Core Team 2013) was used to calculate *F*ST for each polymorphism. Because each sample in the RNA-Seq experiment consisted of a pool of individuals, hierfstat was run in a slightly non-standard way. Each sequence read supporting an allele was treated as a haploid individual, grouped by sample and nested within strain. The values of *F*ST therefore represented the variances in alleles between strains once the variance between samples within strains had been accounted for. Loci with unusually high levels of divergence between strains were identified as outliers in a box-and- whisker plot of *F*ST values, generated in R. Loci identified in this manner were selected for a genotyping study of susceptible and resistant WCR strains, and their transcripts of origin were annotated with BLAST2GO.

The selected polymorphisms were confirmed in a genotyping experiment using the MassArray technology (Gabriel, Ziaugra, & Tabbaa, 2001). Samples of individual WCR from the standard susceptible and resistant Hopkinton strains were genotyped. WCR from additional Cry3Bb1-resistant and Cry3Bb1-susceptible non-diapausing strains (Wangila & Meinke, 2016; Zukoff et al., 2016) was also included in the genotyping study (Method S1). The ability of the chosen loci to discriminate between resistant and susceptible strains was visually analyzed in a discriminant analysis of principal components (DAPC) (Jombart, Devillard, & Balloux, 2010) with the package adegenet (Jombart, 2008).

Additionally, transcripts that had previously been found to be differentially expressed and that had annotations indicating that they could be receptors of Cry3Bb1 were inspected for possible polymorphisms. Alignments of sequence reads against the transcript references were visualized with Tablet (Milne et al., 2013). Possible polymorphisms were noted, and the corresponding transcript sequences were translated with the online ExPASy tool (Gasteiger et al., 2003) to determine whether the mutations were synonymous or would modify the protein sequence.

2.2 Quantitative reverse transcriptase PCR

Quantitative reverse transcriptase PCR (qRT-PCR) was used to further investigate the expression patterns of selected transcripts identified as being of interest from the RNA-Seq study. The genes of interest were two ABC transporters, an α-amylase, a protease and a protease inhibitor that were differentially expressed between the strains in the RNA sequencing data analysis. Two qRT-PCR experiments were carried out. The first experiment measured gene expression of the susceptible and resistant Hopkinton neonate larvae after 8 hr of feeding on Cry3Bb1 or non-*Bt* corn to confirm the expression differences observed in the RNA-Seq experiment. The second experiment compared expression in the midgut (where Cry3Bb1 toxin acts) versus the rest of the body. To facilitate isolation of the midgut, resistant Hopkinton strain and susceptible strain larvae were reared to third instar before dissection (Method S1).

2.2.1 Material and sample collection

Neonates were collected as described for the RNA sequencing experiment. Larvae fed for 8 hr on Cry3Bb1 or non-*Bt* corn. Five independent replicates of 25 larvae were recovered per strain.

Midguts were dissected as described by Eyun et al. (2014). Third instar larvae, reared on non-*Bt* corn, were anaesthetized on ice before dissection. All material used for dissection was sprayed with RNaseZap® (Ambion Inc., Foster City, CA). Fifteen midguts were pooled in one tube, and the remaining

body parts were pooled in a different tube. Six replicates were prepared for each tissue type and strain. The tissues were flash-frozen in liquid nitrogen and held at –80°C before RNA extraction.

2.2.2 Primer design and qRT-PCR protocol

PCR primer design was performed using Primer3 (Rozen & Skaletsky, 1999). Default parameters were used, except for the melting temperature difference between primers was set to 2°C, and the length of the amplicons was set between 50 and 200 bases. Primersearch, a tool of EMBOSS (Rice, Longden, & Bleasby, 2000), was used to check for off-target priming within the transcriptome. The primers were synthesized by Sigma-Aldrich (Saint Louis, MO) (Table 1).

The qRT-PCR experiments were performed using an Applied Biosystems, Inc. 7500 FAST instrument, at the University of Illinois at Chicago College of Medicine, using Fast SYBR® Green Master Mix (Applied Biosystems,

Table 1. Primer names and sequences used for the qRT-PCR

Gene	*Primer name*	*Direction*	*Primer sequence*
Housekeeping genes			
β-Actin	Actin_F	Forward	TCCAGGCTGTACTCTCCTTG
	Actin_R	Reverse	CAAGTCCAAACGAAGGATTG
Elongation factor 1α	EF1_F	Forward	ACCAGATTTGATGGCTTTGG
	EF1_R	Reverse	CACCCAGAGGAGCTTCAGAC
Glyceraldehyde 3-phosphate dehydrogenase	GAPDH_F	Forward	TTGTGGTGAACACTCCGGTA
	GAPDH_R	Reverse	GGTCGCTACAAGGGATGTGT
Genes of interest			
ABC transporter 1	ABC1_F	Forward	TCTTCGCGGTACAGTGTTTG
	ABC1_R	Reverse	CACCGATATTAACAGAACT-GTGG
ABC transporter 3	ABC3_F	Forward	GGGGCCACTAGAACTTTTGC
	ABC3_R	Reverse	AACCCAACATTTCCACCAAA
ABC transporter 4	ABC4_F	Forward	AGAGCCATAGTTCGCCAAAA
	ABC4_R	Reverse	CTCCAGCGTCCATAACCAAT
α-Amylase	AMY1_F	Forward	TCGCTCAAGGAAGAAACACC
	AMY1_R	Reverse	ACCTGAACACCAGCAAATCC
Protease	Prot3_F	Forward	ATACCGCATGGGGCCTTTC
	Prot3_R	Reverse	TACAAGTTATGGCCACGGCA
Protease Inhibitor	Prin2_F	Forward	GGCAGATCCTCACTCACGTT
	Prin2_R	Reverse	CTCCGCCTTCATTCGTCCTT

Foster City, CA). The PCR cycle program consisted of an initial step of 3 min at 95°C, followed by 40 cycles of 10 s at 95°C, 30 s at 56°C and 30 s at 72°C for primer extension. Fluorescence was measured after each cycle. After a final step of 72°C for 5 min, a melting curve was taken from 65°C to 95°C by increments of 0.5°C for 5 s. The absence of genomic DNA contamination in the samples and primer dimers were confirmed by no reverse transcription negative controls (NRTC) and water (no template control; NTC). All reactions were carried out in a volume of 10 µl, with 2 µl of cDNA or negative control (NRTC and NTC), 0.5 µl of each primer at 10 µM, 5 µl of 2 X SYBR® Green PCR Master Mix and 2 µl of water.

2.2.3 Data analysis

The statistical analysis of the qPCR results was performed using the R (R Development Core Team 2013) package MCMC.qpcr (Matz, Wright, & Scott, 2013). The method uses a generalized linear mixed model (GLMM), allowing information from all genes to be considered to estimate fixed and random effects. GLMM offers the possibility to analyze complex designs by an ANOVA-type analysis. The model also considers efficiencies and their discrepancies between housekeeping genes and genes of interest. Prior to analysis, outliers of technical replicates were removed after visualization of the melt curve and amplification curve.

The analysis was performed over all the transcripts of interest and three housekeeping genes. The housekeeping genes selected for the analysis were β-actin, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and elongation factor 1-α (EF1) (Rodrigues et al., 2014). However, these genes were not specified as "control" genes as no assumption was made on their stability and the "naïve" model of the MCMC.qpcr package was used for analysis. The factors tested were the strain of the larvae and the plant type in the neonate exposure analysis. The analysis of the third instar larval samples tested the effect of strain and tissue on transcript expression.

A *p*-value calculated by the model was presented for each gene, indicating the significance of the factor effect on the gene expression. All *p*-values were corrected for multiple testing by applying the Benjamini–Hochberg method (1995), with a false discovery rate of 5%.

3 Results

3.1 Sequencing results

The paired-end sequencing of RNA extracted from Hopkinton and susceptible WCR neonates exposed to non-*Bt* and Cry3Bb1 corn for 4 and 8 hr yielded a total of 822,384,146 pairs of reads. On average, 93.98% of reads

for each sample mapped to the reference transcriptome (minimum: 92.63%, maximum: 95.21%).

3.2 Strain differences

3.2.1 Full data set strain effect

Significant strain effects in the full data set were observed for thirteen transcripts 4 hr after exposure. Five transcripts were expressed at higher levels in the Hopkinton strain compared to the susceptible strain, and eight transcripts were expressed at a lower level (Table S1). Six of the transcripts did not have a match with NCBI non-redundant protein sequence database in the BLASTx analysis. All the transcripts with significant BLASTx hits were expressed at a lower level in the Hopkinton strain. Putative functions for these transcripts included a zinc finger protein, a retrotransposon and a serine protease inhibitor.

After 8 hr of exposure, 608 transcripts showed a significant effect of strain on expression (Table S2). Among these transcripts, 359 were expressed at a higher level in Hopkinton and 249 at a lower level, compared to the susceptible strain. Inferred functions for these transcripts included immunity-related proteins, detoxification enzymes and transcription factors. Multiple differentially expressed transcripts were annotated as proteins previously characterized as putative receptors and protease activators of Cry toxins in other insects (Tables S2 and S5). Five sequences were identified as putative ABC transporters, of which three were more highly expressed in the susceptible strain. Five transcripts annotated as glycosyl hydrolases were all upregulated in the Hopkinton strain compared to the susceptible strain, across conditions, and two of these transcripts were annotated as α-amylases. Several different categories of proteases were found among the inferred functions of differentially expressed transcripts. Two transcripts were upregulated in the Hopkinton strain and were annotated as a cathepsin (cysteine protease) and a "venom protease-like" serine protease. The two other differentially expressed proteases were both downregulated in Hopkinton and were annotated as a trypsin-like serine protease and a putative lysosomal aspartic protease.

Two protease inhibitors were also observed to be part of the strain difference in the full data set analysis. A trypsin inhibitor was found to be expressed almost twofold higher in the susceptible strain compared to the Hopkinton strain. A second unique transcript with the same annotation was more abundant by a factor of 1.57 in Hopkinton.

3.2.2 Constitutive strain difference: strains on non-Bt corn

Some transcripts showed evidence of differential expression between strains when the data for larvae feeding on non-Cry3Bb1 corn were

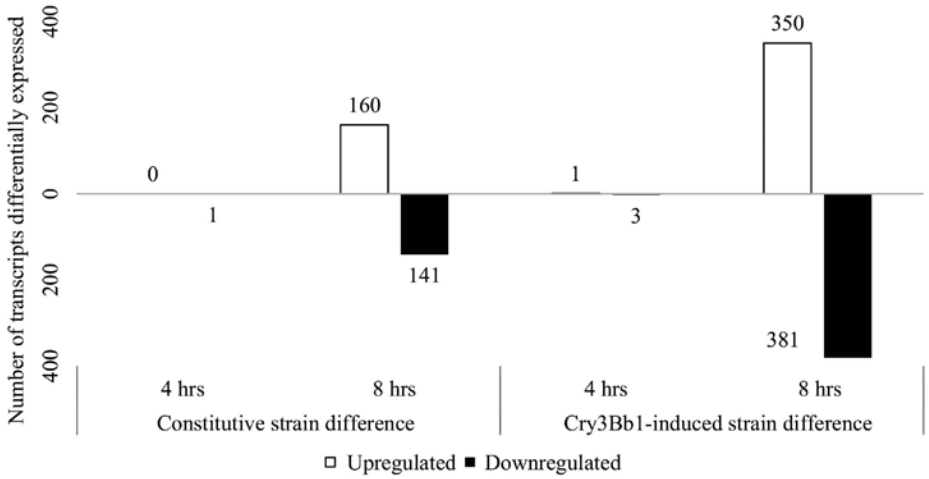

Figure 1. Number of differentially expressed transcripts due to constitutive difference between strains, in the absence of toxin exposure (left) and Cry3Bb1-induced strain difference (right), after 4 hr and 8 hr of exposure. The direction of differential expression was calculated using the level of expression in susceptible larvae as the baseline level ("Constitutive" = R-Iso/ S-Iso; "Cry3Bb1-induced" = R-Cry3Bb1/ S-Cry3Bb1). The upregulated transcripts are the transcripts with an expression level higher in the resistant strain than in the susceptible strain.

considered separately. Only one sequence was detected as significantly differentially expressed between strains after 4 hr feeding on non-*Bt* corn. This sequence, which could not be assigned a putative function, exhibited 2.1-fold greater expression in the susceptible strain relative to the Hopkinton strain.

After 8 hr, 160 transcripts were upregulated in Hopkinton and 141 downregulated, relative to the susceptible strain (Figure 1 and Table S3). Among these transcripts, one ABC transporter was differentially expressed and was downregulated in Hopkinton. Two glycosyl hydrolases were upregulated in Hopkinton (Figure 2). The three putative cysteine proteases, including a cathepsin common with the full data set analysis of strain differences, were upregulated in the Hopkinton strain, whereas a putative serine protease was downregulated. An aspartic endopeptidase, downregulated in the Hopkinton strain, was also common with the full data set strain effect.

As observed in the full data set analysis of strain differences, the expression of immunity-related transcripts was generally higher in Hopkinton than in the susceptible strain. However, the five protease inhibitors were all expressed at lower levels (1.65-fold to 2.6-fold change) in the Hopkinton strain relative to the susceptible strain.

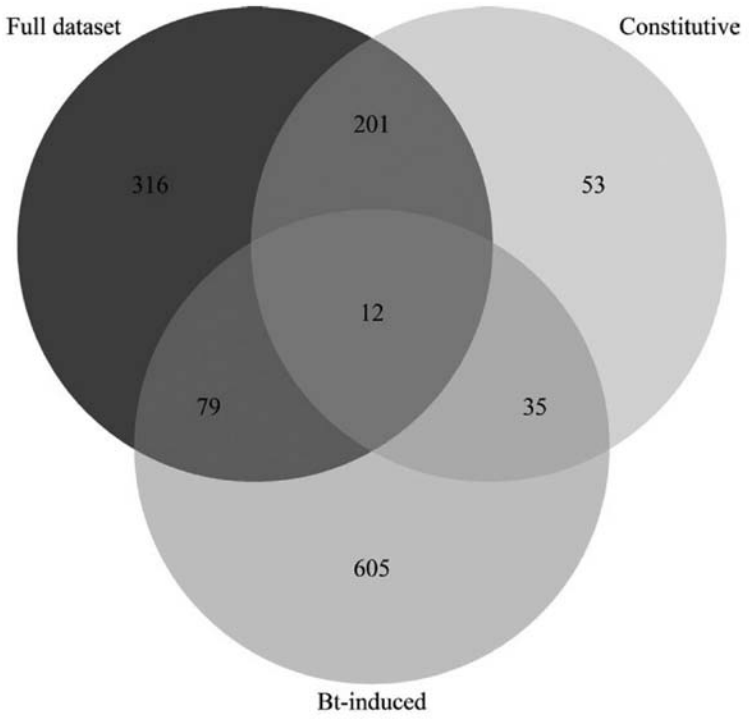

Figure 2. Number of differentially expressed transcripts different and common between the full data set analysis of strain difference, the constitutive strain difference (no Cry3Bb1 exposure) and Cry3Bb1-induced strain difference, at 8 hr.

3.2.3 Cry3Bb1-induced strain difference: differential expression between strains due to toxin exposure

The Cry3Bb1-induced strain difference after 4 hr of exposure indicated that one transcript coding for a zinc finger protein was upregulated in the resistant Hopkinton strain, whereas three other transcripts were downregulated compared to the susceptible strain. One was annotated as an armadillo repeat-containing protein, and the other two could not be annotated with BLAST2GO because no match was found with the non-redundant protein database.

After 8 hr of exposure to Cry3Bb1 corn, the Hopkinton strain exhibited increased expression of 350 transcripts and decreased expression of 381 others compared to the susceptible strain (Figure 1 and Tables S4 and S5). These transcripts included ABC transporters of different families; two were downregulated in the Hopkinton strain and 17 others were upregulated. However, none of these transcripts were identified as having a significant strain effect on their expression in the analysis of the full data set. Two cadherins, one up-and one downregulated in the Hopkinton strain, were also

observed. A putative aminopeptidase N was upregulated in Hopkinton when feeding on Cry3Bb1 corn. Five other metalloproteases were downregulated in the Hopkinton strain, and the 14 other differentially expressed proteases (of different types) were upregulated. The same amylase that was differentially expressed between strains in the full data set analysis was also upregulated in the analysis of Cry3Bb1-treated samples.

Transcripts encoding proteins related to immunity were consistently upregulated in Hopkinton in the Cry3Bb1-induced strain differences as well. These transcripts were annotated as macrophage mannose receptors, a toll-like receptor, a lysozyme precursor, a peptidoglycan-recognition protein, a macrophage migration inhibitory factor and a laccase-like multicopper oxidase. The range of expression changes from susceptible to Hopkinton strain was between 1.44-fold and 2.74-fold (Tables S2 and S4).

3.3 Response to Cry3Bb1

3.3.1 Full data set treatment effect and response of the susceptible strain to Cry3Bb1

When the full data set was analyzed for an effect of corn type (Cry3Bb1 vs. non-*Bt*) after 4 hr, 77 differentially expressed transcripts, 25 upregulated and 52 downregulated, were identified (Table S6). Among those 77 transcripts, 44 were not annotated. Among the 33 others, the annotations did not indicate any of the functions typically associated with *Bt* resistance in other insects (Table 2). However, other functions that are consistent with known mode of action of *Bt* toxins were identified, such as transport (two transcripts). The upregulated transcript encoding a transporter was annotated as α-tocopherol transfer protein, and the downregulated transcript in Cry3Bb1-exposed insects was a facilitated trehalose transporter. Many putative transcription factors were also differentially expressed between treatments. A protease inhibitor was identified and showed a twofold downregulation compared to its expression in untreated samples. One transcript annotated as a peritrophic matrix protein was more highly expressed in the Cry3Bb1-exposed samples.

After 8 hr, exposure to Cry3Bb1 corn resulted in 5,414 transcripts showing a significant effect of plant treatment in the full data set (Table S7). Among these transcripts, 45 ABC transporters were identified and 28 of these were downregulated in Cry3Bb1-exposed samples, a cadherin was downregulated in Cry3Bb1-treated samples and 21 metalloproteases were all upregulated in Cry3Bb1-exposed insects. Twenty-five transcripts encoded proteins likely to be involved in the G protein signaling pathway, all upregulated in insects feeding on Cry3Bb1 plants, except one annotated as a regulator of a cAMP-dependent kinase activity. Eleven peptidases were downregulated (aspartic, carboxy, serine, cysteine peptidases), and 24 were upregulated in

Table 2. Proteins previously involved in Cry toxin mode of action or resistance in insects. The literature cited is non-exhaustive

Protein	*Role in mode of action of Cry toxins*	*Modifications in resistance to Cry toxins*	*Insects*	*References*
ATP-binding cassette (ABC) transporter	Membrane insertion, receptor	Mutations, genetic link with resistance	*Ostrinia nubilalis, Diabrotica virgifera virgifera, Heliothis virescens, Chrysomela tremula*	Coates and Siegfried (2015), Flagel et al. (2015), Gahan et al. (2010), Pauchet et al. (2016)
ADAM proteases	Receptor, cleavage	—	*Leptinotarsa decemlineata*	Rausell et al. (2007)
Aminopeptidase N/P	Receptor	Mutations, change in expression levels	*Ostrinia nubilalis, Leptinotarsa decemlineata*	Khajuria et al. (2009, 2011), Loseva et al. (2002)
α-amylase	Receptor	—	*Tenebrio molitor, Galleria mellonella*	Bulushova et al. (2011)
Cadherin	Receptor	Mutations	*Ostrinia nubilalis*	Bel et al. (2009)
GPI-anchored alkaline phosphatase	Receptor	Reduced levels	Lepidoptera	Jurat-Fuentes et al. (2011)
Proteases	Protoxin activation	Change in expression levels	*Plodia interpunctella, Heliothis virescens*	Forcada et al. (1996), Oppert et al. (1994, 1996, 1997)
	Receptor	Decreased binding (binding assays)	*Plodia interpunctella, Spodoptera exigua* (Herrero et al. (2001, 2005)	

Cry3Bb1-exposed larvae compared to the non-exposed larvae (serine and cysteine peptidases). Ten transcripts encoding glycosyl hydrolases were upregulated, including an amylase, and 15 were downregulated in response to Cry3Bb1 exposure. Three transcripts encoded GPI-anchored adhesins, one of which was upregulated on Cry3Bb1, and two were downregulated in Cry3Bb1-treated samples.

The other differentially expressed transcripts with a significant treatment effect in the full data set analysis were assigned putative functions that were probably not directly related with toxin interaction but more likely part of a general response to Cry3Bb1 intoxication. For instance, seven differentially expressed protease inhibitors were upregulated in Cry3Bb1-exposed insects, as well as cell death-related and many immunity-related transcripts. Detoxification and stress responses were also noticeable from the upregulation of transcripts encoding cytochrome P450s, esterases,

glutathione-S- transferases, oxidases, peroxidases and heat-shock proteins (Table S7). The expression of putative transporters was also impacted by treatment, as 79 transcripts were upregulated and 45 downregulated in Cry3Bb1-exposed insects compared to the absence of toxin. Among these transcripts, several facilitated trehalose transporters were upregulated in Cry3Bb1-exposed larvae. Transcripts annotated as α-tocopherol transporters, already identified as different between treatments after 4 hr of exposure, were also upregulated in Cry3Bb1-treated samples after 8 hr. Transcripts encoding proteins with possible chitin-binding activity, likely components of the cuticle or the peritrophic matrix, were also differentially expressed (Table S7).

The response of the susceptible strain to Cry3Bb1 exhibited many common transcripts with the full data set treatment effect. After 4 hr, 13 transcripts were differentially expressed, including a downregulated trehalose transporter, a sodium channel, armadillo repeat-bearing proteins and a transcription regulator (Table S8). Nearly 80% (78%) of the transcripts that showed a significant effect of treatment in the full data set at 8 hr of exposure were common to those showing a significant effect of Cry3Bb1 exposure when the susceptible strain was analyzed alone (Figure 3 and Table S9).

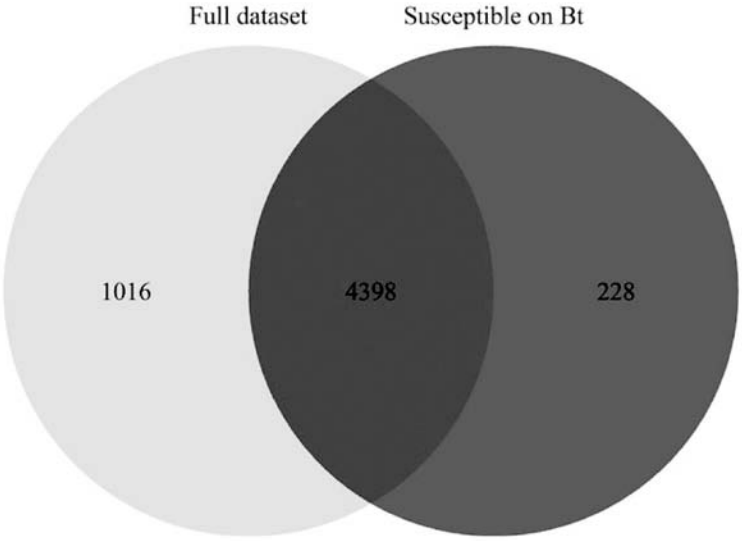

Figure 3. Number of differentially expressed transcripts different and common between the full data set analysis of the treatment effect and the response to Cry3Bb1 of the susceptible strain at 8 hr.

3.3.2 Response of the Hopkinton strain to Cry3Bb1 exposure

The response of the Hopkinton strain to the Cry3Bb1 toxin showed few differentially expressed transcripts between treatments, especially when compared to the response of the susceptible strain to Cry3Bb1. After 4 hr of exposure, no transcripts showed a significant response to *Bt* corn when the Hopkinton strain was analyzed alone. After 8 hr, five transcripts were upregulated in response to Cry3Bb1 exposure, including a transmembrane protein with a putative transporter function, a papilin-like protein (protease inhibitor), a cholinesterase (hydrolase), a Ras association domain-containing protein and a UDP-glucuronosyltransferase (Table S10).

3.4 Strain by treatment interactions

There were no significant effects of strain by treatment interaction on transcript expression after 4 hr of exposure. After 8 hr, 120 transcripts showed significant strain by treatment interaction effects. These transcripts included potential receptors such as ABC transporters, metalloproteases and glycosyl hydrolases. However, 43 transcripts were unannotated (Tables S5 and S11).

3.5 Complementary data on expression levels (8 hr) and location of expression by qRT-PCR

Five transcripts that showed significant strain effects in the RNA-Seq study were selected for further testing by qRT-PCR (Table 3). Of these, only the protease inhibitor showed a significant difference in expression between strains in the qRT-PCR study involving samples collected from neonate larvae. However, there was a contradiction between the results of the two experiments for this transcript as it was upregulated in the Hopkinton strain in the RNA-Seq study and upregulated in the susceptible strain in the qPCR study of neonate samples. None of the selected transcripts were affected by treatment alone or by the interaction of strain and treatment in the RNA-Seq results, but the qPCR results revealed that the expression of the two ABC transporters varied between Cry3Bb1-treated and untreated samples and that the ABC transporter identified as ABCC and the amylase were affected by the interaction between strain and treatment (Table 3). No significant effect of strain or treatment was observed for the expression of the aspartic protease in the qPCR results, also contradicting RNA-Seq results.

In the analysis of transcript expression location, two transcripts, the α-amylase and the protease, were expressed mostly in the midgut, whereas the two ABC transporters and the protease inhibitor were mostly expressed outside the midgut (Figure 4). The α-amylase was the only transcript significantly affected by the strain and was 4.35-fold more highly expressed in third instar larvae of the susceptible strain.

Table 3. Comparison of RNA-seq and qPCR results

Transcript	*Annotation*	*RNA-seq comparison*	*RNA-seq Fold changes*	*qPCR significant comparison*	*qPCR fold changes (R/S or Cry3Bb1/Iso)*
150540_c0_seq1	ABCG transporter	Susceptible vs. Resistant	1.5× up in S	Non-treated	1.60× up on
		Susceptible vs. Cry3Bb1	Cry3Bb1		
113542_c0_seq1	ABCC transporter	Susceptible vs. Resistant	1.97× in S	Non-treated vs. Cry3Bb1	1.85× up on Cry3Bb1
		Constitutive Susceptible vs. Resistant	1.76× in S	Interaction Strain by Treatment	2.25× up in S on isoline
133109_c0_seq1	α-Amylase	Susceptible vs. Resistant	1.51× up in R	Interaction Strain by Treatment	3.01× up in R on Cry3Bb1
		Cry3Bb1-induced Susceptible vs. Resistant	1.62× up in R		
146933_c0_seq1	Serine protease inhibitor	Susceptible vs. Resistant	1.57× up in R	Susceptible vs. Resistant	2.68× up in S
144462_c0_seq1	Aspartic protease	Susceptible vs. Resistant	1.63× up in S	No significant effect	
		Constitutive Susceptible vs. Resistant	1.63× up in S		

3.6 Sequence polymorphisms

The RNA-seq data contained 2,451 polymorphisms retained as significantly supported by the 24 samples after filtering. Of these 2,451 polymorphisms, 1,928 were tagged as SNPs, 202 were insertions, 73 were deletions, 174 were tagged as "Complex," and 74 were multiple nucleotide polymorphisms. The loci presenting more than two alleles were removed. A total of 2,256 biallelic polymorphisms were conserved for the subsequent analyses.

The resulting polymorphisms analyzed with hierfstat revealed that 124 loci had an outlier *F*ST value (Table S12). Of these, 51 were tested in the MassArray experiment. Thirty-four loci were confirmed as polymorphic and were used to study population differentiation. The DAPC showed that the loci separated Hopkinton from the other colonies but did not separate resistant populations from their susceptible counterparts (Figure S1).

The differentially expressed transcripts encoding ABC transporters, cadherins, aminopeptidase N and other sequences of interest were inspected to find possible polymorphisms that could interfere with Cry3Bb1 mode of action (Table S13). Some non-synonymous mutations were found in the

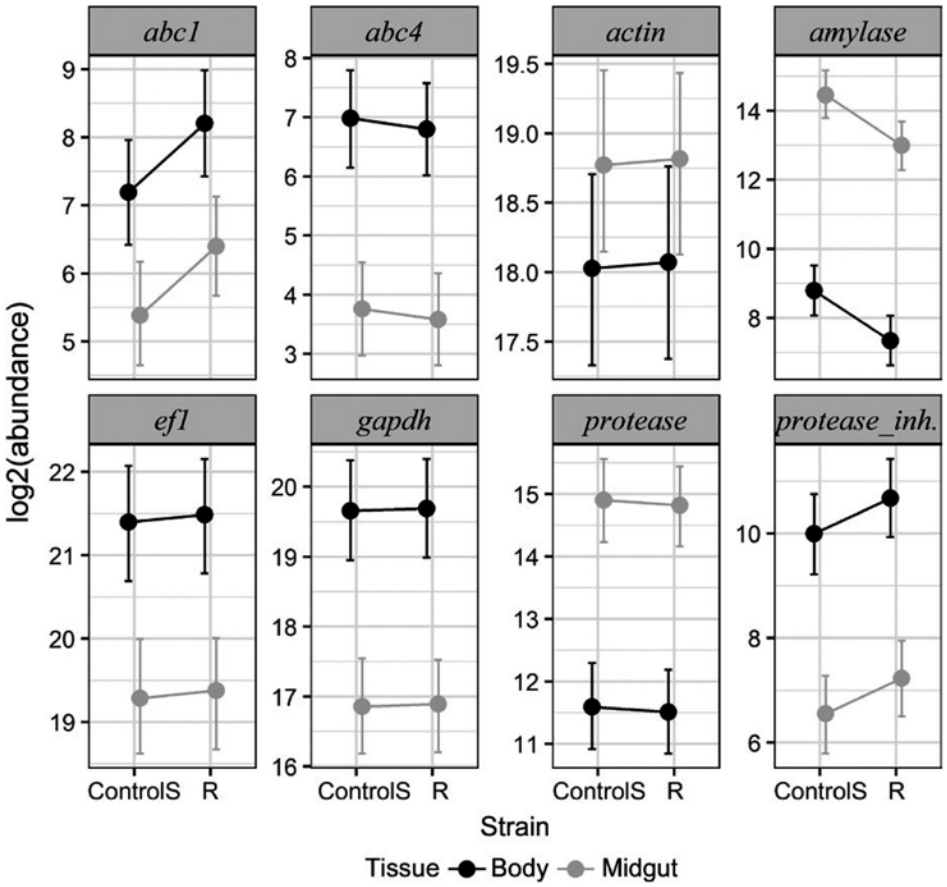

Figure 4. Expression of five transcripts of interest in third instar midgut versus the rest of the body.

predicted open reading frames of these transcripts encoding potential receptors of Cry3Bb1. However, there were no obvious differences between susceptible and Hopkinton samples in the frequencies of those polymorphisms.

4 Discussion

Two strains of western corn rootworm, susceptible and Hopkinton (Cry3Bb1-resistant), were exposed to Cry3Bb1 corn and non-*Bt* corn to obtain an overview of transcriptional changes and sequence variations between these strains and to compare the effects of exposure to non-*Bt* corn versus Cry3Bb1 corn. A primary objective of this work was to identify genes involved in resistance to Cry3Bb1 by western corn rootworm. Additionally, this work identifies potential transcripts involved in the response of neonates to Cry3Bb1 corn.

The differential expression comparison that provided the most useful information was the full data set analysis of strain effects after 8 hr of exposure, as it measured differences between strains across treatments (Table S2). The annotations of the differentially expressed transcripts in this comparison highlighted various transcript categories previously associated with *Bt* resistance and *Bt* mode of action in other insects, including an ABC transporter, an amylase and proteases (Bulushova et al., 2011; Gahan et al., 2010; Oppert et al., 1994) (Table 2). The possibility that the selected transcripts play a role in WCR resistance to Cry3Bb1 is hypothesized based on resistance mechanisms described in other insects, and their fitness costs previously evaluated by Heckel (1994) and reviewed by Gassmann, Carrière, and Tabashnik (2009). No fitness cost was detected for Cry3Bb1 resistance in the Hopkinton strain (Ingber & Gassmann, 2015), the strain studied here; hence, mechanisms involving a high fitness cost are unlikely.

A commonly proposed mechanism of resistance to *Bt* toxins is linked to altered toxin binding, which could be due to modified expression or modified coding sequence of the receptor(s) for Cry3Bb1. Several proteins have been characterized as potential receptors for Cry toxins; for instance, the ABC transporter family (Heckel, 2012). ABC transporters have been reported to be involved in resistance to Cry toxins in the Lepidopterans, *Heliothis virescens* (Fabricius), *Plutella xylostella* (Linnaeus), *Trichoplusia ni* (Hübner), *Bombyx mori* (Linnaeus), *Ostrinia nubilalis* (Hübner) and *Spodoptera exigua* (Hübner) resistant to Cry1A, Cry2A, Cry1Fa and Cry1Ca (Atsumi et al., 2012; Baxter et al., 2011; Coates & Siegfried, 2015; Gahan et al., 2010; Park et al., 2014; Tay et al., 2015). Mutations in the ABC transporter sequences in *H. virescens* and *B. mori* decreased binding of Cry toxins (Atsumi et al., 2012; Gahan et al., 2010) and *ABCC2* was found to be one of the genes present in a mapping study of the locus for resistance to Cry1Ac in *P. xylostella* and *T. ni* (Baxter et al., 2011) and in Cry1Fa-resistant *O. nubilalis* (Coates & Siegfried, 2015). A mapping study in WCR also identified a link between Cry3Bb1 resistance and a gene coding for *ABCB2* (Flagel et al., 2015), an ABC transporter of a different family than the families of ABC transporters identified in Lepidoptera. Moreover, an *ABCB2* has been genetically linked to Cry3A resistance and to its mode of action in another Chrysomelid, *Chysomela tremula* Fabricius (Pauchet et al., 2016).

The main hypothesis regarding the role of ABC transporters in resistance is that mutations changing the protein sequence or downregulation of the transcripts in the resistant strain cause a decrease in toxin binding. Downregulation of transcripts encoding ABC transporters was observed in multiple comparisons of our study between susceptible and Hopkinton strains (Tables S2–S4). However, the expression pattern of the two investigated ABC transporters showed that they were mostly expressed outside the midgut (Figure 4). Moreover, the qPCR results revealed that the expression of the ABCC

and ABCG transporters selected for qPCR comparison was not affected by strain differences (Table 3), which was the primary reason for their selection based on RNA-Seq results. Moreover, their location of expression suggests that if ABC transporters are involved in resistance to Cry3Bb1, they are unlikely to be encoded by the transcripts selected for our qPCR experiment.

The visual search for polymorphisms in differentially expressed transcripts annotated as ABC transporters revealed that multiple sequences exhibited nucleotide substitutions. However, these transcripts and polymorphisms would need to be investigated in another study to confirm the tissue location of the proteins, the allele frequencies of the observed mutations in susceptible and resistant strain and whether they interact with the toxin to confirm their role in resistance to Cry3Bb1 in WCR.

Glycosyl hydrolases, particularly α-amylases, also have been identified as potential Cry toxin receptors (Bulushova et al., 2011; Fernandez-Luna et al., 2010). A midgut α-amylase was identified as a potential receptor of Cry4Ba and Cry11Aa in the mosquito *Anopheles albimanus* Wiedemann (Fernandez-Luna et al., 2010), and as a functional receptor of Cry3A in the coleopteran *Tenebrio molitor* Linnaeus (Bulushova et al., 2011). However, the differentially expressed amylases identified in this study were more highly expressed in the resistant Hopkinton strain. The increase in amylase abundance in the Hopkinton strain may indicate toxin sequestration by an alternate binding protein. The α-amylase described as a receptor for Cry3A in *T. molitor* was soluble in the gut (Bulushova et al., 2011), which would imply no physical link between this amylase and the epithelial cells and would reinforce the possibility of its participation in resistance by avoiding membrane integration of the toxin and pore formation. The significant differential expression in our study of one of the α-amylases, characterized in the full data set strain effect and in the Cry3Bb1-induced difference, indicates involvement of the transcript in Cry3Bb1 exposure. The qRT-PCR results also revealed that the investigated α-amylase was expressed in the midgut and was sensitive to Cry3Bb1 exposure in Hopkinton neonates. Only synonymous mutations were detected in the transcript encoding the amylase investigated in the qRT-PCR experiments.

An aminopeptidase N, also a known receptor of Cry toxins (Jenkins et al., 2000; Knight, Crickmore, & Ellar, 1994), exhibited three nucleotide substitutions in the predicted open reading frame of the translated protein. If this protein interacts with Cry3Bb1, these sequence modifications could interfere with toxin binding. This transcript was also more highly expressed in Cry3Bb1-exposed Hopkinton neonates relative to Cry3Bb1-exposed susceptible neonates. Increased expression of an aminopeptidase N was also reported in the Cry3A-resistant strain of *L. decemlineata* (Loseva et al., 2002). However, reduced expression of aminopeptidase N genes was previously correlated with resistance to Cry1Ab in *O. nubilalis* (Coates, Sumerford,

Siegfried, Hellmich, & Abel, 2013) and to Cry1Ca in *S. exigua* (Herrero et al., 2005). Although the expression level of the aminopeptidase N in Hopkinton compared to the susceptible strain was consistent with the expression pattern observed in the Cry3A-resistant coleopteran *L. decemlineata*, it was not consistent with the pattern observed in Cry-resistant *O. nubilalis* and *S. exigua*.

A second hypothesis to explain resistance could involve altered protease levels. Proteases might contribute to resistance to Cry toxins in two different ways: (i) they could be involved in a reduced activation of the protoxin by reduced levels of transcripts encoding proteases in the resistant strain (Herrero et al., 2001; Oppert et al., 1997); (ii) they could be involved in cleavage of the functional toxin, which could lead to its inability to interact with the brush border cells of the resistant strain as proposed by Forcada et al. (1996) in *H. virescens*.

In Coleoptera, Cry3Aa resistance in *L. decemlineata* has been reported to involve the expression of different isoforms of proteases in the resistant strain and an increase in aminopeptidase activity in the midgut (Loseva et al., 2002). Aspartic, serine or cysteine proteases encoding transcripts were differentially expressed between Hopkinton and susceptible strains without a specific pattern of expression separating the different protease types (Table S2). In the full data set, two of the transcripts encoding proteases were more highly expressed in the susceptible strain. One of these transcripts, an aspartic protease, was also more highly expressed in the susceptible strain in the constitutive strain difference (Table 3, Tables S2 and S3). Decreased toxin activation could explain the relative absence of response to the toxin in the Hopkinton strain. A similar resistance mechanism mediated by reduced protease expression was proposed for the Indianmeal moth, *P. interpunctella*, where the protease necessary for the activation was lacking, resulting in the absence of toxicity of Cry1Ac (Oppert et al., 1997). It is assumed that a mechanism of resistance involving deficient proteolysis would confer high levels of cross-resistance to multiple Cry toxins (Heckel, 1994). Cross-resistance among Cry3Bb1, mCry3A and eCry3.1Ab has been observed in WCR (Gassmann et al., 2014; Jakka et al., 2016). However, this type of resistance may impose a fitness cost if the overall proteolytic activity of the midgut is also decreased. No fitness costs due to Cry3Bb1 resistance were observed in the Hopkinton strain (Ingber & Gassmann, 2015). Alternatively, two differentially expressed transcripts coding for serine proteases were more highly expressed in the Hopkinton strain in the full data set analysis when compared to susceptible larvae (Supplementary Table 2). The increased expression of protease-encoding transcripts could enhance toxin degradation and confer cross-resistance to other toxins (Forcada et al., 1996). Such cross-resistance is likely especially if the activation mechanism is common to different toxins along with low fitness costs (Heckel, 1994), which is in agreement

with the absence of fitness costs in the Hopkinton strain.

In the qRT-PCR study neonates, differential expression of the aspartic protease tested was not observed, and the protease inhibitor exhibited a different behavior than in the RNA-seq results. There were discrepancies between RNA-seq and qRT-PCR results for the five transcripts of interest. These transcripts were significantly different between strains in RNA-seq but were primarily selected based on their annotations. The low fold change (<2-fold; Table S2) may have contributed to the disagreement between RNA-Seq and qRT-PCR results (Gavery & Roberts, 2012; Gavery, Roberts, & White, 2013).

Some transcripts encoding immunity-related proteins had an increased expression in the Hopkinton strain in all strain-related comparisons (Tables S2–S4). These results suggest that the immunity level of the Hopkinton strain may be enhanced relative to the susceptible strain, even in the absence of exposure to Cry3Bb1. It should be noted that this difference was evident only after 8 hr of plant feeding, suggesting that food intake could play a role in this difference. The immune protein apolipophorin-III was previously reported to be upregulated in response to Cry3Ba exposure in *Tribolium castaneum* (Herbst) (Contreras, Rausell, & Real, 2013a,b). However, no apolipophorin was differentially expressed in the present RNA-seq results. Moreover, the immunity-related transcripts identified in this study are commonly found in the hemolymph, fat body cells and cuticle in insects (Dziarski & Gupta, 2006; Kang, Liu, Lundström, Gelius, & Steiner, 1998). Hence, it seems unlikely that these proteins are interacting directly with the toxin to reduce its toxicity.

The other result of this study was the detection of transcripts impacted by Cry3Bb1 exposure. There was no detectable response from the Hopkinton strain after 4 hr of exposure, and after 8 hr, only 12 transcripts were different between the two treatments in this strain. These results reflect the resistant status of the Hopkinton strain as it does not exhibit a stress response when exposed to Cry3Bb1 or to Cry3Bb1 expressing corn plants.

In susceptible insects, differential expression after 4 hr of feeding indicated that transcription factors, transposable elements, transporters and cell-adhesion were impacted by the exposure to Cry3Bb1. In addition, a transcript annotated as an attractin-like protein, which participates in the G protein-coupled receptor signaling pathway in mammals (Haqq et al., 2003; Yeo & Siddle, 2003), was upregulated after exposure. Because Cry toxin mode of action involves binding of the toxin to a membrane receptor, it may stimulate G protein and adenylyl cyclase, followed by an increase in cAMP levels and the activation of the protein kinase A, as proposed by Zhang et al. (2006). Zhang and collaborators (2005) also hypothesized that the receptor of Cry1Ab is a cadherin. Strain differences did not identify transcript encoding cadherins that were more highly expressed in the susceptible strain, but polymorphisms were detected in three transcripts encoding cadherins.

The role of these mutations would need to be confirmed as it is currently unknown whether the proteins encoded by the sequences investigated here interact with Cry3Bb1 in WCR. Cadherins may be involved in the mode of action in WCR, as the exposure to fragments of WCR cadherin and Cry3A or Cry3Bb1 in *Bt*-susceptible WCR, Colorado potato beetle and southern corn rootworm resulted in the enhanced activity of these toxins (Park, Abdullah, Taylor, Rahman, & Adang, 2009). The results observed here after 8 hr of exposure could support the involvement of the G protein pathway in the intoxication following Cry3Bb1 exposure, by increasing the expression of G protein-related transcripts such as regulators of the G protein, or cAMP-related factors and magnesium transporters. There was also an increase in an adenylate cyclase-related factor and other G protein-related transduction factors in the response to Cry3Bb1 of susceptible insects. However, based on the present results, it is unknown whether the G protein pathway is linked to the mode of action or a stress response in susceptible WCR.

Finally, transcripts coding for proteases were mainly increased during the exposure to Cry3Bb1, suggesting that they may have a role in intoxication. One of the main groups of transcripts showing a peptidase activity and being exclusively upregulated in Cry3Bb1-exposed samples was annotated as ADAM metalloproteases. This suggests that ADAM metalloproteases may not be involved in resistance, although the expression of these genes changes due to intoxication. These metalloproteases could be interesting for further study as they were characterized as receptors for Cry3Aa in *L. decemlineata* (Ochoa-Campuzano et al., 2007). They may represent possible receptors of Cry3Bb1 in susceptible WCR. It was also proposed that this metalloprotease was the Coleopteran counterpart of the Lepidopteran aminopeptidase N (Rausell et al., 2007).

Several polymorphisms in the RNA-Seq data appeared to separate the Hopkinton and standard susceptible strains and were therefore candidate genetic markers of the resistance to Cry3Bb1 in WCR. However, genotyping additional laboratory strains for these polymorphisms only separated Hopkinton from the other populations and did not separate resistant from susceptible populations in general. This suggests that the divergence at these loci between Hopkinton and the other laboratory strains was due to genetic drift. At the time these studies were conducted, the Hopkinton strain had been maintained in laboratory for 34 generations and reselected on Cry3Bb1 once a year or less. Similarly, Flagel et al. (2014, 2015) found that genetic markers within a QTL for resistance in the Hopkinton strain did not distinguish between other susceptible and resistant field populations of WCR.

In conclusion, our study forms the basis for future investigation of Cry3Bb1 resistance in WCR as it identified the differential expression of some proteases and potential receptors of Cry3Bb1, such as an α-amylase. However, further investigation of these candidate transcripts needs to be

made to confirm their role in the resistance mechanism. In particular, the ability of the proteins to bind or otherwise interact with the toxin molecule remains to be established. Although this study does not offer a definitive answer about the resistance mechanism, it is evident that differential expression is associated with resistance to Cry3Bb1 in WCR.

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