Isolation of transcripts from *Diabrotica virgifera virgifera* LeConte responsive to the *Bacillus thuringiensis* toxin Cry3Bb1

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Abstract

Crystal (Cry) proteins derived from *Bacillus thuringiensis* (Bt) have been widely used as a method of insect pest management for several decades. In recent years, a transgenic corn expressing the Cry3Bb1 toxin has been successfully used for protection against corn rootworm larvae (Genus *Diabrotica*). The biological action of the Bt toxin in corn rootworms has not yet been clearly defined. Because development of resistance to Bt by corn rootworms will have huge economic and ecological costs, insight into larval response to Bt toxin is highly desirable. We identified 19 unique transcripts that are differentially expressed in *D. virgifera virgifera* larvae reared on corn transgenic for Cry3Bb1. Putative identities of these genes were consistent with impacts on metabolism and development. Analysis of highly modulated transcripts resulted in the characterization of genes coding for a member of a cysteine-rich secretory protein family and a glutamine-rich membrane protein. A third gene that was isolated encodes a nondescript 132 amino acid protein while a fourth highly modulated transcript could not be further characterized. Expression patterns of these four genes were strikingly different between susceptible and resistant western corn rootworm populations. These genes may provide useful targets for monitoring of Bt exposure patterns and resistance development in pest and non-target insect populations.

Introduction

Bacillus thuringiensis (Bt) crystalline protein toxins are used to effectively control many agricultural pests, including economically important leaf beetles in the family Chrysomelidae. A number of Bt proteins have been reported to kill beetle larvae (Hofte and Whiteley, 1989; Donovan et al., 1992; Lambert et al., 1992; Moellenbeck et al, 2001; Ellis et al., 2002; Baum et al., 2004; Masson et al., 2004; Schnepf et al., 2005). Currently, three active protein ingredients of corn (*Zea mays*) transgenic for a chrysomelid-specific toxin (Bt-corn) are registered for field use in the United States: Cry3Bb1, Cry34Ab1/Cry35Ab1 and mCry3A (US EPA, 2005a, 2005b, 2006). These transgenic plant protectants have proved very successful in enhancing crop production (Crowder et al., 2005). However, sustained economic success of this pest control technology depends on deployment of effective resistance management strategies. When larvae of the chrysomelid beetle *Diabrotica virgifera virgifera* LeConte (western corn rootworm, WCR) were continuously exposed to Cry3Bb1-corn, populations evolved resistance within a few generations (Meihls et al., 2008). Similarly, increased tolerance to Cry34/35Ab1-corn has been achieved through laboratory selection (Lefko et al., 2008). Analysis of physiological and molecular events associated with Bt- mediated larval death may provide insight into the mechanistic basis of resistance as well as tools for identifying resistant organisms in the field.

Bt protoxins are activated by insect gut proteases but the mode of action of Bt toxins remains unclear. Two alternative models have been postulated (reviewed in Soberon et al., 2009). In the more widely accepted pore-formation model (Knowles and Ellar, 1987; Bravo et al., 2004), Bt toxins are believed to initiate gut collapse by binding

to midgut receptor molecules. The alternative signaling model (Zhang et al., 2006) is based on the observation that signal transduction components are stimulated in insect cell culture following Bt interaction with receptors. Theoretically, resistance to Bt could result from interference at any stage of the toxicity pathway, including toxin processing. For example, Bt-resistance in lepidopteran insects was associated with defects in gut protease activities (Oppert et al., 1997; Li et al., 2004, Karumbaiah et al., 2007). Most reported examples of Bt resistance have been linked to cadherins or other potential receptors such as aminopeptidases and alkaline-phosphatases (reviewed in Bravo and Soberon, 2008). Interestingly, Khajuria et al. (2009) reported differential expression of genes that may confer both protease-mediated and receptor-mediated Bt resistance in *Ostrinia nubialis* (European corn borer).

In this paper, we show that *D. v. virgifera* larvae demonstrate altered transcriptional responses to transgenic corn (event MON 863, YieldGard Rootworm®, Monsanto Company) incorporating the Cry3Bb1 protoxin (USEPA, 2005a). Sequence characterization of three highly modulated transcripts led to the cloning of a novel protein in the superfamily of cysteine-rich secretory proteins, antigen 5, and pathogenesis-related 1 proteins (CAP), a glutamine-rich membrane protein and a putative protein of unknown function. Transcriptional profiles for these genes and a fourth uncharacterized transcript differ between Bt-susceptible and Bt-resistant strains and may have utility in insect resistance management.

Results and Discussion

Selection of Differentially Expressed Transcripts

WCR neonates were hatched and reared for 24 h on either a transgenic (Cry3Bb1) corn root diet or a root diet from the non-transgenic isoline before being processed for construction of a subtracted cDNA library. Approximately 400 cDNA clones were selected and sequenced from forward (enriched in genes positively responsive to Bt toxin) and reverse (enriched in genes negatively responsive to Bt toxin) subtracted cDNA libraries. Differential gene expression was validated with quantitative real-time RT-PCR (qRT-PCR) using cDNA specific primers for 19 unique sequences, nine of which were up-regulated and ten of which were down-regulated (Table 1).

Putative identities of genes with two-fold or greater change in relative expression suggested that a wide range of biological functions are potentially influenced by rearing on transgenic Bt corn. Synthesis of structural proteins (e.g., actin and cuticle protein) appeared to be upregulated for rootworms fed the Cry3Bb1 corn diet. Cuticle proteins are major components of insect larval integument (Hackman, 1984; Nakato et al., 1994). In Coleoptera and Lepidoptera, differentiation of epidermal cells to cuticle is regulated throughout development (Riddiford, 1993, Zhou and Riddiford, 2002, Minakuchi et al., 2009). Ecdysteroid-induced molting requires the absence of juvenile hormone (Riddiford, 1993). Interestingly, a juvenile hormone-inducible transcription factor, Krüppel homolog 1, also was upregulated (Table 1). Krüppel homolog 1 has been shown to be continuously expressed in beetle larval stages but absent in the pupal stage; its experimental suppression causes a precocious larval-pupal transition (Minakuchi et al., 2009). Induction of these genes following Bt ingestion may represent an inhibition of normal developmental activities due to intoxication. Cellulase enzyme expression was

downregulated, indicating a suppression of cellulosic digestive activity in Bt-exposed insects, which is also consistent with slowed development.

Severe suppression (>50-fold) was noted for three transcripts (RS18, RS200 and RS12) while 6-fold induction was noted for one transcript (FS218). Transcriptional response profiles for these genes were subsequently re-examined in an extended (48 h) Cry3Bb1 exposure experiment (Fig. 1). Suppression of transcripts RS18 and RS12 on Cry3Bb1-corn previously observed at 24 h was also observed after 48 h exposure. Induction of transcript FS218 for larvae reared on Cry3Bb1 corn increased 3-fold at 48 h compared to 24 h. Expression of RS200 for Cry3Bb1-exposed insects increased from 24 to 48 h, but remained lower than for isoline-reared controls.

Upregulated FS218 mRNA encodes a protein of the CRISP/Antigen-5/PR-1 superfamily. We used rapid amplification of cDNA ends (RACE) protocols to further characterize open reading frames (ORFs) for highly Cry3Bb1 responsive genes FS218, RS18, RS200 and RS12. Sequence extension by 5` and 3` RACE of the 203 bp FS218 transcript yielded an 893 nt mRNA sequence (GenBank no. EU189197). The ORF sequence starts with an ATG codon that follows a perfect rule for translation initiation and encodes a 253 aa polypeptide (Fig. 2). SignalP 3.0. (http://www.cbs.dtu.dk/services/SignalP/) predicted a signal peptide cleavage site between positions 19 and 20 (VKA-QT) indicating that the protein is likely to be directed into the endoplasmic reticulum and secreted into the extracellular space. ScanProsite (http://ca.expasy.org/tools/scanprosite/) identified a strong match to consensus sequence ^{179}GHYTAIVWART^{189}, the "Signature 1" motif of cysteine-rich secretory protein (CRISP) family members (Ellerman et al., 2006). We also identified sequence stretch

^{209}YLACNYGEGGNV^{219} (Fig. 2) as a second motif described as CRISP "Signature 2" by Ellerman et al. (2006). However, unlike CRISP family members, the FS218 protein contains only the N-terminal domain of CRISP and lacks the C-terminal domain, which is a characteristic of the CAP (CRISP/Antigen-5/PR-1) superfamily (Ellerman et al., 2006; Gibbs et al., 2008). The N-terminal region of CRISPs confers membrane-binding and protease activities (Ellerman et al., 2006; Cohen et al., 2008). CAPs have been implicated in extracellular endocrine or paracrine functions in regulation of the extracellular matrix during morphogenesis and cell-cell adhesions (Gibbs et. al., 2008) and have been isolated from insect, plant and fungus sources. While mammalian and reptilian CRISPs characteristically contain 16 cysteine residues allowing for eight possible disulfide bonds, CAPs contain fewer cysteine residues available for disulfide bonds. For example, only four disulfide bonds are present in the insect CAP proteins vespid venom allergen V5 (Henriksen et al., 2001) and red fire ant venom allergen "sol i 3" (Schmidt et al., 2003; Padavattan et al., 2008), while three probable disulfide bonds were found in a plant pathogenesis-related protein PR-1 (Fernandez et al., 1997) and only two disulfide bonds were predicted in fungal proteins (Sc7 and Sc14) from the basidomycete *Schizophyllum commune* (Schuren et al., 1993).The FS218 peptide sequence contains a total of 12 cysteine residues, allowing the formation of a maximum of 5 disulfide bonds, as predicted by DIpro 2.0 tool (available at http://scratch.proteomics.ics.uci.edu/). Possible disulfide bonds predicted for WCR protein were in the following descending order: Cys^{235}-Cys^{248}, Cys^{24}-Cys^{36}, Cys^{194}-Cys^{212}, Cys^{108}-Cys^{113}, and Cys^{43}-Cys^{49}. We thus characterize FS218 as member of the CAP (CRISP/Antigen-5/PR-1) superfamily, and have named it WCRCAP.

Down-regulated transcript RS18 encodes a glutamine- rich membrane protein. The 5′-RACE extension of the 287 bp RS18 cDNA fragment yielded a 1715 bp product (EU189199) with an ORF encoding a 571 aa polypeptide (Fig. 3). The topology prediction program SOSUI (http://bp.nuap.nagoya-u.ac.jp/sosui/ indicated that the protein sequence contains an N-terminal membrane anchorage sequence. A search of the Prosite profile library using the Motif Scan tool (http://myhits.isb-sib.ch/cgi-bin/motif_scan) identified a glutamine-rich region located in the protein sequence stretch between 192-565 aa (Fig. 3). Two coleopteran proteins, a 579 aa protein from *Tenebrio molitor* (BAA78480) and a 456 aa protein from *Anoplophora glabripennis* (ABQ65713) were found to have high homology using BlastP. The *T. molitor* protein was first detected as an induced protein following injection of a foreign body into the larval abdomen and was thus described as a putative 'encapsulation-relating protein" (Cho et al., 1999). Using the computational tools described above, we determined that the two related proteins possess glutamine-rich regions with N-terminal membrane anchorage sequences that are similar to the *D. v. virgifera* protein (data not shown).

A BLAST search with the full length *D. v. virgifera* mRNA sequence revealed homology to two EST sequences previously isolated from the *D. v. virgifera* midgut (Accession No. CN497699), and head (Accession No. EW771327). A homologous sequence (C0036848) was also reported from southern corn rootworm, *D. undecimpunctata* (Liu et al., 2004). Interestingly, soyacystatin N, a soybean cysteine protease inhibitor, was found to induce this *D. undecimpunctata* protein during early larval stages (Dr. Yilin Liu, personal communication). Thus, it appears that glutamine-

rich membrane proteins have functions beyond encapsulation in *Diabrotica* species and other coleopterans. For reference, we termed glutamine rich membrane protein WCRGRMP.

Two down-regulated genes have no known homologies. The 725 bp sequence of RS12 was extended in both directions to obtain a 2041 bp cDNA sequence (Accession No. GQ502198). Translation of the sequence revealed an ORF with a stop codon after 132 aa (data not shown). We were unable to identify the function of the hypothetical protein from its structure. Our attempts to further extend the RS200 transcript and characterize its ORF were not successful, and its function also remains unclear.

Developmental Regulation of Cry3Bb1-Responsive Genes

As Cry3Bb1-responsive genes described above were cloned from *D. v. virgifera* larvae, it was expected that each of these genes would be expressed in early developmental stages of insects reared on isoline corn. WCRCAP and WCRGRMP are maximally expressed at the 3rd instar developmental stage while RS12 and RS200 reach peak expression at the 2nd instar stage (Fig. 4). Although inter-individual variation in expression was moderately large, expression levels tended to drop off sharply at the pupal stage, indicating that these genes are also developmentally regulated. Because of high mortality associated with rearing on Cry3Bb1-corn, extended developmental profiles of these genes for susceptible larvae reared on Bt corn are not available.

Expression Patterns of Cry3Bb1-Resistant Rootworm

It is evident from the above findings that gene expression profiles of susceptible *D.v. virgifera* larvae are altered in response to Cry3Bb1 ingestion. Should similar transcriptional responses be expected for *D. v. virgifera* that evolve resistance to Bt-corn? We addressed this question with a laboratory Bt-resistant strain selected and maintained at the North Central Agricultural Research Laboratory (Oswald et al, personal communication). Neonates of the Cry3Bb1-resistant lines were transferred to transgenic corn or its isoline derivative and sub-sampled at first, second and third larval instar stages. Real-time RT-PCR for WCRCAP, WCRGRMP, RS12 and RS200 was performed using larval RNA isolated from individual specimens. Although relative expression of each gene changed with larval development, no significant difference was observed between expression patterns of WCR raised on Cry3Bb1or isoline corn diets (Fig. 5). Comparison of first instar data in Fig. 1 and Fig. 5 indicates that transcriptional regulation of these genes differs greatly between early instars of Cry3Bb1-susceptible and Cry3Bb1-resistant WCR. The similarity in transcriptional response between resistant WCR reared on Cry3Bb1 corn root and susceptible WCR raised on isoline corn root suggest that all four genes reside in metabolic pathways associated with the Cry3Bb1 toxicity response and are not responding to other possible trait differences between transgenic and isoline corn.

Bt-responsive gene expression changes were previously demonstrated in the midguts of Lepidoptera exposed to purified Bt toxins (Meunier et al., 2006; van Munster et al., 2007). Exposure of *Manduca sexta* and *Choristonneura fumiferana* to Cry1Ab induced differential expression of mRNA transcripts involved in a wide range of physiological functions including metabolism, immunity, and general stress response.

These previous studies analyzed gene expression changes using sublethal doses of toxin that was produced as protoxin in bacterial spores and enzymatically cleaved *in vitro*, while we have used whole transgenic plant sources for toxin delivery without the requirement of laboratory manipulation for protein activation or dose optimization. Bacterial formulations may contain debris and inactive toxin fractions in addition to active toxins which may induce a broader immune response.

In our study of gene expression responses to a Coleoptera-specific Bt toxin, we have identified Bt-responsive genes with putative functions in metabolism and morphogenesis. The key significance of these results is that we have demonstrated transcriptional responses that are consistent with Bt-mediated gene regulation in Bt-susceptible larvae and the apparent absence of such a regulatory response in resistant larvae. The characterized genes shed little light on the mechanism of toxicity; the observed transcriptional differences are consistent with Bt toxin-receptor interactions that either initiate pore formation or activate signaling pathways. Transcriptional differences could also be indirect consequences of a weakened gut epithelium causing susceptibility to enterobacteria-induced septicemia (Broderick et al., 2006). Certainly, further work, including functional characterization of two down-regulated genes, is warranted to assess possible regulatory pathways and the mechanisms by which resistant populations are able to overcome them.

The four specific gene products we isolated could be useful in field monitoring applications. For example, applications based on assays of transcriptional patterns at these target genes could be used to verify adequacy of insect resistance management plans, particularly plans that depend on the use of reserve plots that harbor unexposed,

susceptible populations. Detection of Bt-responsive transcriptional profiles in reserve populations putatively protected from Bt exposure could raise concerns about conformance to the resistance management models. Similarly, assays of transcriptional profiles for these genes in larvae sampled from Bt corn fields could provide a useful molecular method for early resistance detection, extending the range of mitigation options prior to economic failure. Finally, analysis of gene expression patterns at homologous genes in other coleopterans could be exploited as a monitoring tool for potential exposures and sub-lethal effects on non-target organisms.

Experimental procedures

Sample preparations

D. v. virgifera eggs, larvae and adults were obtained from the USDA North Central Agricultural Research Laboratory (NCARL) in-house cultures and from Crop Characteristics, Inc. (Farmington, MN). Cry3Bb1-resistant *D. v. virgifera* larvae were obtained from NCARL. All eggs and other developmental stages were kept at $25 \pm 1^{o}C$ with daily cycles of 16 h light and 8 h dark. Cry 3Bb1 corn seed and the isoline were obtained from Monsanto Co., St. Louis, MO, USA.

Exposure of *D. v. virgifera* neonates to the Cry3Bb1 Bt toxin was accomplished by placing ready-to-hatch eggs on pre-germinated mats of Bt corn root in small (300 cm^2) clear plastic trays. For controls, neonates were placed on similarly pre-germinated mats of isoline corn root. A total of 7500 eggs from a randomly mating population were divided among 6 trays, representing 3 biological replicates each for Bt and isoline corn.

Up to 100 neonates were removed at 24 h and at 48 h of exposure, after which larvae were flash frozen at -80^{o}C until RNA extraction. Pools of neonates were homogenized for 3 min using 3mm stainless steel beads with a Retsch MM300 mixing mill (Qiagen, Valencia, CA). Total RNA was isolated using Trizol reagent (Invitrogen) and treated with DNase I using Message Clean kit (GenHunter Corp, Nashville, TN).

For experiments with different developmental stages, either larval or adult *D. virgifera* fed on corn roots were sampled at various developmental stages and pooled into 3 replicates as follows: 1st instar (10/rep), 2nd instar (10/rep), 3rd instar (10/rep), pupae (3/rep), and adult (3/rep).

For experiments with Cry3Bb1-resistant *D. v. virgifera*, individual larvae were reared on Bt-mat or isoline-mat until sampled at first, second, and third instar stages, after which tissue was homogenized as described above. Total RNA extracted with Trizol reagent was precipitated with isopropanol in the presence of glycogen, dissolved in 45 ul of RNase-free water and treated with DNase I followed by heat inactivation of the enzyme as suggested by the manufacturer (Ambion, CA). The final volume of RNA isolated from each individual was 50 ul.

Construction of the Subtractive cDNA Library

Total RNA was isolated from pools of ~100 neonates that had been reared on Cry3Bb1 or isoline corn for 24 h. Total RNA extracted from *D. v. virgifera* in the Bt corn group was designated tester RNA, and that from the isoline control group was designated driver RNA. Synthesis of cDNA from 1 μg of total RNA was performed with the super SMART cDNA synthesis kit (Clontech, Palo Alto, CA) following the manufacturer's

instructions. The double-stranded cDNA obtained from 14 cycles of PCR amplification was used for PCR-Select cDNA subtraction according to the detailed manual provided with the kit manufacturer (Clontech). Subtracted cDNAs obtained from forward and reverse PCR-select hybridizations were used to construct the cDNA library by directly cloning them into PCR 2.1 vector with a PCR 2.1 TA Cloning Kit (Invitrogen, Carlsbad, CA), following the manufacturer's directions. Cloned products were sequenced using universal M13 primers and the sequences thus obtained were used to design gene specific primers.

Real-time quantitative PCR

Validation of differential expression data obtained from subtraction hybridization was performed using real-time RT-PCR (qRT-PCR). Primers that target specific transcripts were designed using PrimerQuest software (Integrated DNA Technologies, Coralville, Iowa). Total RNA was reverse transcribed with random hexamers using Superscript III First Strand cDNA kit (Invitrogen) according to the manufacturer's protocol, using 1µg as template. Real-time quantitative PCR amplifications were carried out with 1µl of 100-fold diluted cDNA on an ABI 7900 HT (Applied Biosystems, Foster City, CA) using a 30µL reaction at 58^{o} C for 40 cycles with the Sybr GreenER kit (Invitrogen) according to manufacturer's instructions. The 16S ribosomal RNA was used as an endogenous control for normalization in calculating the relative quantitative gene expression of target genes using the comparative C_T method ($2^{-\Delta\Delta}C_T$) described in User Bulletin #2 (ABI Prism 7700 Sequence Detection System, 10/2001 update).

For qRT-PCR gene expression analysis from individual resistant insects, 10 µl of total RNA prepared above was used in reverse transcription in a 20 µl reaction. PCR amplifications in 30 µl reaction volumes were carried out using 1 µl of cDNA as described above.

Sequencing of cDNA ends

Rapid amplification of cDNA ends (RACE) in 5` and 3` directions were carried by the SMART cDNA RACE kit (Clontech) or GeneRacer kit (Invitrogen) according to the manufacturer's instructions. For SMART-RACE, 2 µg of total RNA was reverse transcribed with PowerScript Reverse Transcriptase (Clontech) at 42 °C for 1.5 h to synthesize first strand 5` and 3`-RACE-Ready cDNA in the presence of 5` and 3` CDS primers respectively. The 5`-cDNA was tagged with SMART II oligo sequence by exploiting enzyme template switching at the upstream site. The RACE PCR product was amplified from 2 µl of cDNA using gene specific primer 1 (GSP1) and the 5′-RACE adaptor primer (UPM). Two µl of the diluted (50-fold) PCR product was used in a nested PCR with gene specific primer 2 (GSP2) and the nested adaptor primer (NUP). These two amplifications were performed with Advantage cDNA Polymerase Mix (Clontech) for 35 cycles of 95°C for 2 sec, 68°C for 4 min, followed by a final terminal extension step for 10 min at 68°C. The final PCR product was cloned into the PCR 2.1 TOPO TA vector (Invitrogen) and sequenced using vector and gene specific primers.

For the oligocapping method (GeneRacer kit, Invitrogen) of RNA Ligase-Mediated Rapid Amplification of 5` cDNA Ends (RLM-RACE), total RNA was dephosphorylated by calf intestine alkaline phosphatase and treated with tobacco acid

phosphatase to remove the 5` cap structure of mRNA, followed by ligation to an RNA oligonucleotide. After the cDNA was synthesized from oligo dT primers, an initial PCR was performed using the RACE outer gene-specific primers and the outer RNA adaptor primer in the kit. The RACE inner gene-specific primer and the inner RNA adaptor primer in the kit were used in the final PCR. PCR conditions were as follows: 5 cycles of 95°C for 2 sec, 72°C for 1 min; 5 cycles of 95°C for 2 sec, 70°C for 1 min: and 25 cycles of 95°C for 2 sec, 68°C for 1 min. Amplified fragments were cloned into TOPO vector for sequencing.

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Figure legends

Figure 1. Differential expression of transcripts in western corn rootworm neonates exposed to Cry3Bb1 and isoline corn. Open and solid columns represent level of gene expression in isoline and Cry3Bb1 corn, respectively. Note that relative gene expression for FS218 is represented on a linear scale while that of other genes is on a log scale.

Figure 2. FS218 mRNA sequence coding a member of CRISP/Antigen-5/PR-1 (CAP) superfamily (EU189197). Nucleotide sequences flanking the open reading frame also are shown. The ATG start codon follows a perfect -3/+4 rule for translation initiation. The N-terminal signal peptide is shown in italics. The CRISP motifs are shown in a box (Signature 1) and by highlight (Signature 2).

Figure 3. RS18 mRNA sequence coding a glutamine-rich membrane protein (EU189199). The upstream nucleotide sequence is shown in lower case and identifies the translation initiation site. The N-terminal transmembrane region (TM) is boxed. The glutamine-rich region is indicated in bold text.

Figure 4. Developmental expression pattern of *D. v. virgifera* genes WCRCAP (FS218), WCRGRMP (RS18), RS12 and RS200. Insects were reared on isoline corn. Average relative expression ± standard error over 3 pools (5 to 30) of whole animals is presented.

Figure 5. Expression profile of Cry3Bb1-responsive genes in Cry3Bb1-resistant larvae. Bars represent average fold changes ± standard error in 10 individual larvae exposed to

1 isoline or Cry3Bb1-corn roots. Open bars represent relative gene expression on isoline
2 corn while solid bars represent expression on transgenic corn.
3
4

1

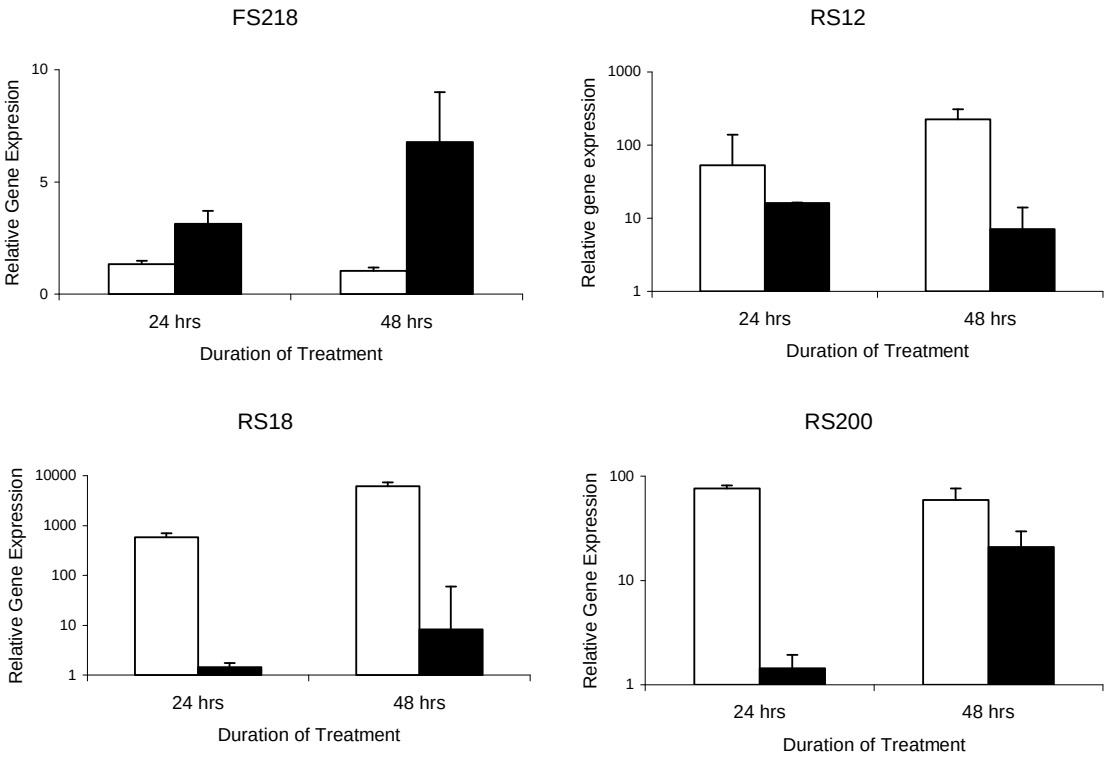
Table 1. Cry3Bb1-responsive transcripts isolated from *Diabrotica virgifera virgifera* . Genes isolated from first instar larvae through suppressive subtractive hybridization were evaluated by real-time RT-PCR for 2-fold or greater transcriptional differences following rearing on transgenic Cry3Bb1 or isoline corn.

Clone No.	Fold	Putative Identity/Homology	Accession No.	Reference
Induced genes				
FS218	6.0	CAP superfamily	EU189197	this study
FS206	2.2	Unknown	EW774849	GenBank submission only
FS212	2.1	Small calcium-binding mitochondrial carrier	GR979559	this study
FS220	2.1	Hypothetical protein XP_975918.1	GR367248	this study
FS25	2.0	Cuticle protein	GR367243	this study
FS98	2.0	Unknown	GR367244	this study
FS126	2.0	Esterase	GR367245	this study
FS227	2.0	Actin isoform	GR367250	this study
FS249	2.0	Kruppel-homolog 1	GR367252	this study
Repressed genes				
RS18	962.0	Glutamine rich membrane protein	EU189199	this study
RS200	119.0	Unknown	EU196367	this study
RS12	58.2	Hypothetical protein	GQ502198	this study
RS115	4.0	Fst protein	GR979560	this study
RS31	2.9	NADH dehydrogenase	EW775509	GenBank submission only
RS215	2.7	Hypothetical protein XP_966821	EW769490	GenBank submission only
RS11	2.5	Unknown	CN498204	Siegfried et. al, 2005
RS58	2.4	Celluase I	CN498554	Siegfried et. al, 2005
RS129	2.2	Hemolymph protein	EW770045	GenBank submission only
RS78	2.0	Unknown	GR367254	this study

2

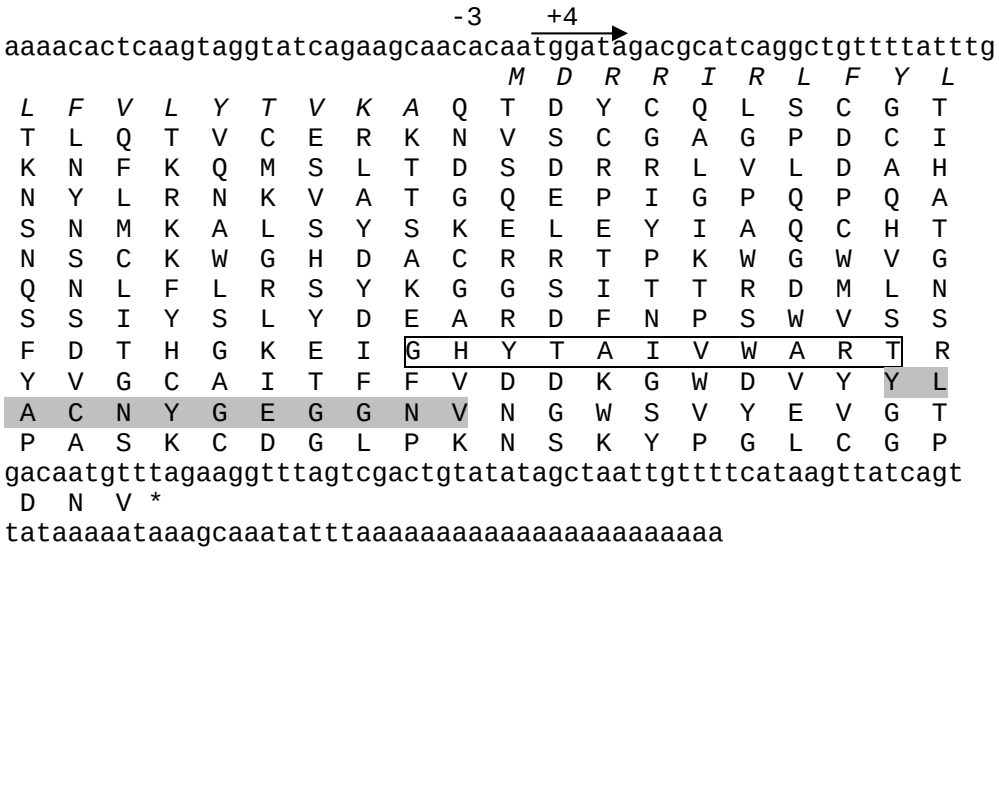
1 Figure 1

2


FS218
Relative Gene Expresion
10
5
0
24 hrs
48 hrs
Duration of Treatment
RS12
Relative gene expression
1000
100
10
1
24 hrs
48 hrs
Duration of Treatment
RS18
Relative Gene Expression
10000
1000
100
10
1
24 hrs
48 hrs
Duration of Treatment
RS200
Relative Gene Expression
100
10
1
24 hrs
48 hrs
Duration of Treatment

3

Figure 2


-3
+4
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M D R R I R L F Y L
L F V L Y T V K A Q T D Y C Q L S C G T
T L Q T V C E R K N V S C G A G P D C I
K N F K Q M S L T D S D R R L V L D A H
N Y L R N K V A T G Q E P I G P Q P Q A
S N M K A L S Y S K E L E Y I A Q C H T
N S C K W G H D A C R R T P K W G W V G
Q N L F L R S Y K G G S I T T R D M L N
S S I Y S L Y D E A R D F N P S W V S S
F D T H G K E I G H Y T A I V W A R T R
Y V G C A I T F F V D D K G W D V Y Y L
A C N Y G E G G N V N G W S V Y E V G T
P A S K C D G L P K N S K Y P G L C G P
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D N V *
tataaaaataaagcaaatatttaaaaaaaaaaaaaaaaaaaaaa

Figure 3

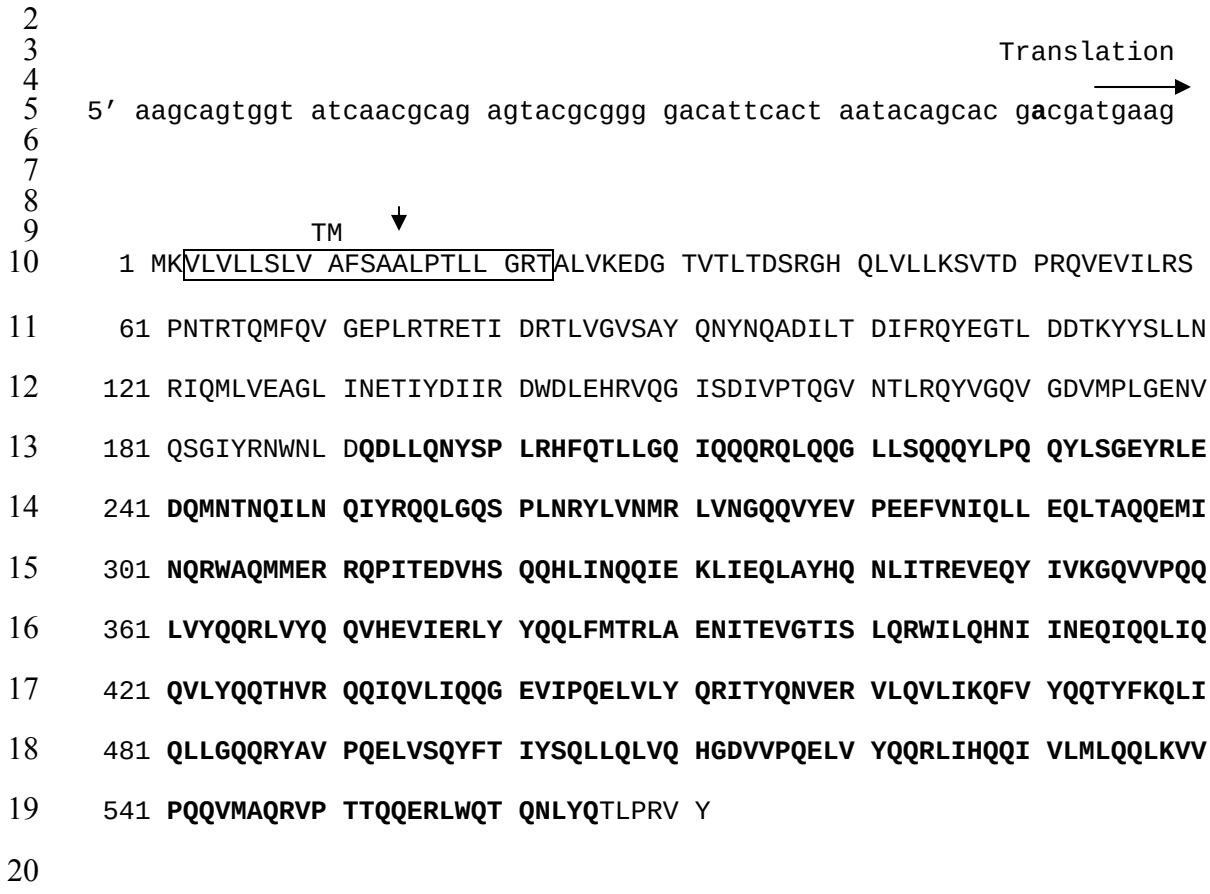
Translation
5′ aagcagtggt atcaacgcag agtacgcggg gacattcact aatacagcac gacgatgaag
TM
1 MKVLVLLSLV AFSAALPTLL GRTALVKEDG TVTLTDSRGH QLVLLKSVTD PRQVEVILRS
61 PNTRTQMFQV GEPLRTRETI DRTLVGVSAY QNYNQADILT DIFRQYEGTL DDTKYYSLLN
121 RIQMLVEAGL INETIYDIIR DWDLEHRVQG ISDIVPTQGV NTLRQYVGQV GDVMPLGENV
181 QSGIYRNWNL DQDLLQNYSP LRHFQTLLGQ IQQQRQLQQG LLSQQQYLPQ QYLSGEYRLE
241 DQMNTNQILN QIYRQQLGQS PLNRYLVNMR LVNGQQVYEV PEEFVNIQLL EQLTAQQEMI
301 NQRWAQMMER RQPITEDVHS QQHLINQQIE KLIEQLAYHQ NLITREVEQY IVKGQVVPQQ
361 LVYQQRLVYQ QVHEVIERLY YQQLFMTRLA ENITEVGTIS LQRWILQHNI INEQIQQLIQ
421 QVLYQQTHVR QQIQVLIQQG EVIPQELVLY QRITYQNVER VLQVLIKQFV YQQTYFKQLI
481 QLLGQQRYAV PQELVSQYFT IYSQLLQLVQ HGDVVPQELV YQQRLIHQQI VLMLQQLKVV
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Figure 4

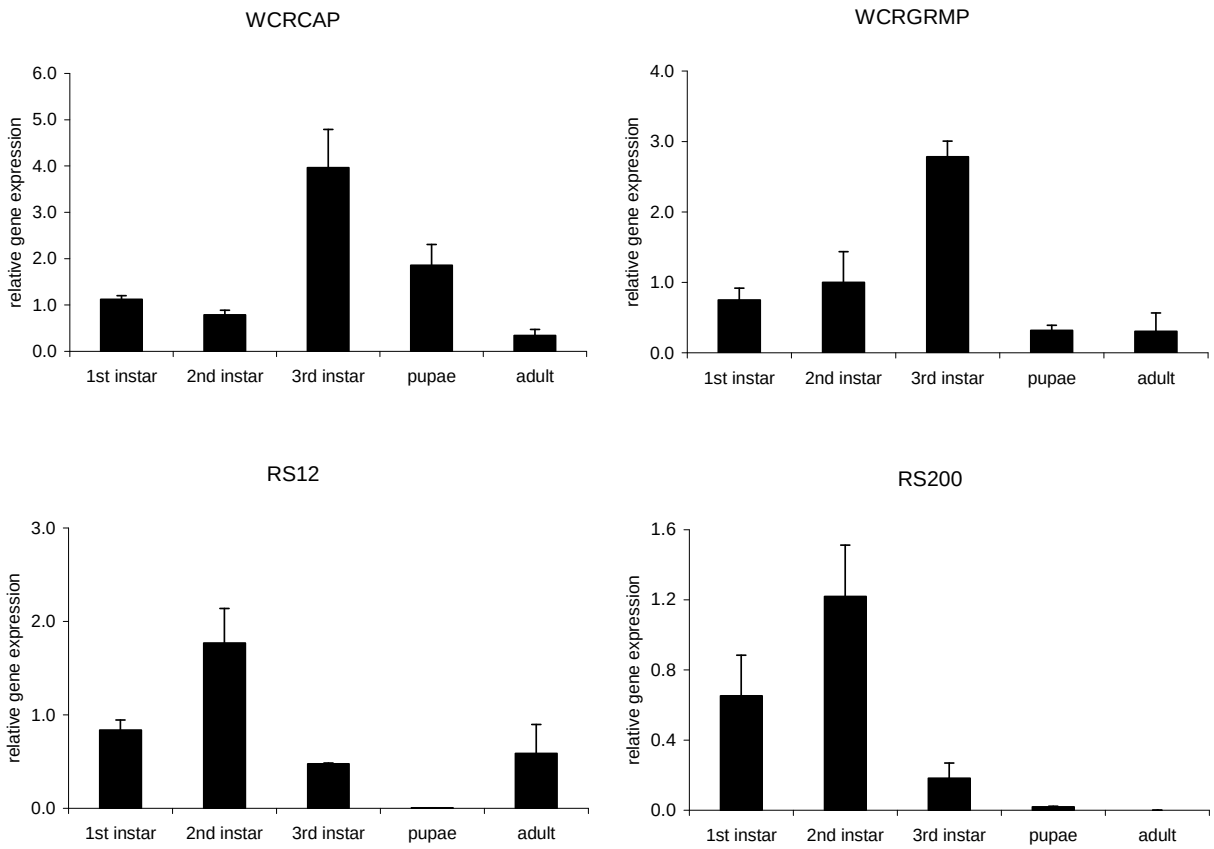
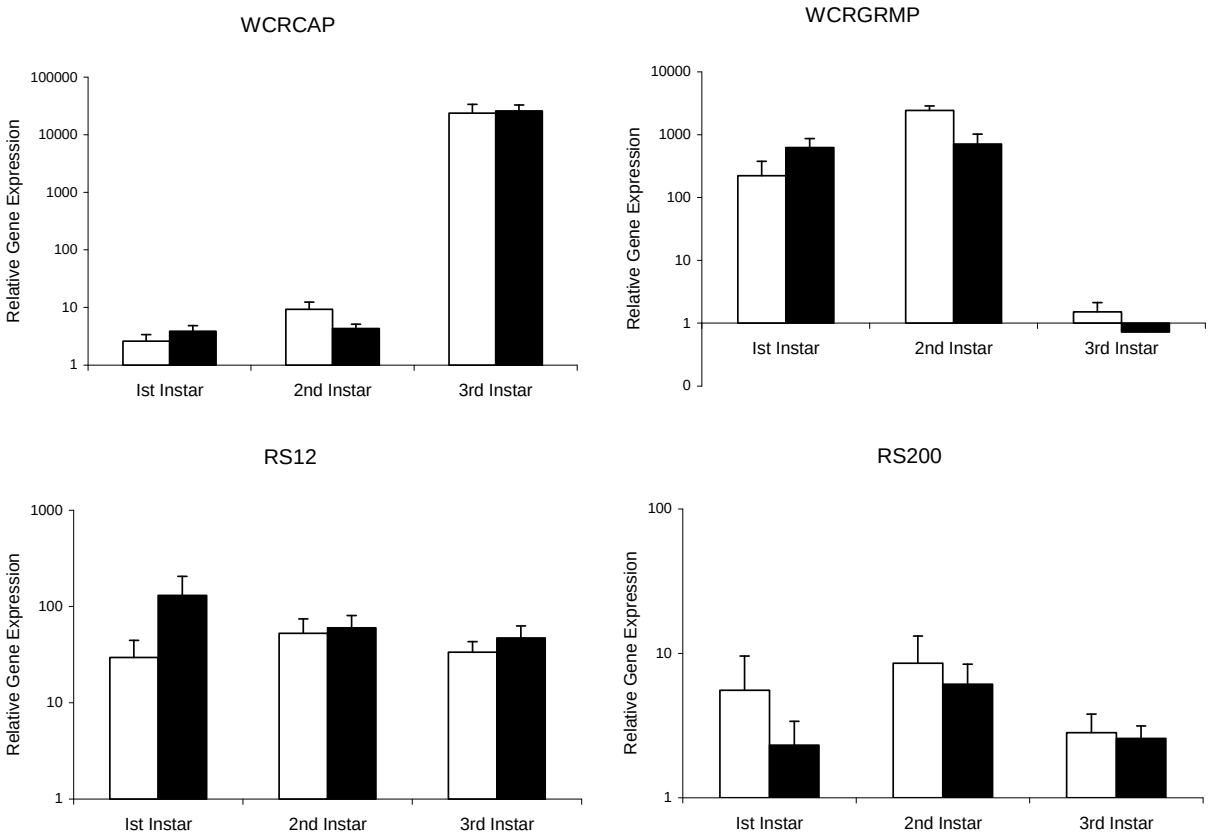

WCRCAP
relative gene expression
6.0
5.0
4.0
3.0
2.0
1.0
0.0
1st instar
2nd instar
3rd instar
pupae
adult
WCRGRMP
relative gene expression
4.0
3.0
2.0
1.0
0.0
1st instar
2nd instar
3rd instar
pupae
adult
RS12
relative gene expression
3.0
2.0
1.0
0.0
1st instar
2nd instar
3rd instar
pupae
adult
RS200
relative gene expression
1.6
1.2
0.8
0.4
0.0
1st instar
2nd instar
3rd instar
pupae
adult

Figure 5


WCRCAP
Relative Gene Expression
100000
10000
1000
100
10
1
Ist Instar
2nd Instar
3rd Instar
WCRGRMP
Relative Gene Expression
10000
1000
100
10
1
0
Ist Instar
2nd Instar
3rd Instar
RS12
Relative Gene Expression
1000
100
10
1
Ist Instar
2nd Instar
3rd Instar
RS200
Relative Gene Expression
100
10
1
Ist Instar
2nd Instar
3rd Instar