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RNAi induced knockdown of a cadherin-like protein (EF531715) does not affect toxicity of Cry34/35Ab1 or Cry3Aa to *Diabrotica virgifera virgifera* larvae (Coleoptera: Chrysomelidae)

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Abstract

The western corn rootworm (WCR), *Diabrotica virgifera virgifera* LeConte, is an important maize pest throughout most of the U.S. Corn Belt. *Bacillus thuringiensis* (Bt) insecticidal proteins including modified Cry3Aa and Cry34/35Ab1 have been expressed in transgenic maize to protect against WCR feeding damage. To date, there is limited information regarding the WCR midgut target sites for these proteins. In this study, we examined whether a cadherin-like gene from *Diabrotica virgifera virgifera* (*DvvCad*; Gen-Bank accession # EF531715) associated with WCR larval midgut tissue is necessary for Cry3Aa or Cry34/ 35Ab1 toxicity. Experiments were designed to examine the sensitivity of WCR to trypsin activated Cry3Aa and Cry34/35Ab1 after oral feeding of the *DvvCad* dsRNA to knockdown gene expression. Quantitative real-time PCR confirmed that *DvvCad* mRNA transcript levels were reduced in larvae treated with cadherin dsRNA. Relative cadherin expression by immunoblot analysis and nano-liquid chromatography–mass spectrometry (nanoLC-MS) of WCR neonate brush border membrane vesicle (BBMV) preparations exposed to *DvvCad* dsRNA confirmed reduced cadherin expression when compared to BBMV from untreated larvae. However, the larval mortality and growth inhibition of WCR neonates exposed to cadherin dsRNA for two days followed by feeding exposure to either Cry3Aa or Cry34/35Ab1 for four days was not significantly different to that observed in insects exposed to either Cry3Aa or Cry34/35Ab1 alone. In combination, these results suggest that cadherin is unlikely to be involved in the toxicity of Cry3Aa or Cry34/35Ab1 to WCR.

Keywords: *Diabrotica virgifera virgifera,* Cry34Ab1, Cry35Ab1, Cry34/35Ab1, Cry3Aa, Cadherin, RNAi

1. Introduction

Corn rootworms of the genus *Diabrotica* are important pests of maize that negatively impact grain production. Immature rootworm larvae cause severe root damage that disrupts water and nutrient uptake by maize plants and weakens the structural support provided by roots such that plants become lodged during strong wind and rain events, resulting in reduced harvest efficiency. Adult rootworms feed on maize silk during pollen shed, which may result in poorly filled ears when densities are high (Krysan, 1986). Among the different species of corn rootworms, western (*Diabrotica virgifera virgifera* LeConte) and northern (*Diabrotica barberi* Smith & Lawrence) corn rootworms are the most significant economic pests throughout the U.S. Corn Belt (Gray et al., 2009). Annual losses from reduced yield and control expenditures have been estimated to exceed $1 billion (Gray et al., 2009; Metcalf, 1983; Sappington et al., 2006). Corn rootworm control measures include crop rotation, soil insecticides and seed treatment to control root-feeding larvae, and foliar applications that target ovipositing females (Levine and Oloumisadeghi, 1991; van Rozen and Ester, 2010).

Transgenic maize events that express toxins from *Bacillus thuringiensis* (Bt) that are resistant to feeding damage by rootworm larvae have been available since 2003 and include MON863 and MON88017 (which express Cry3Bb1), MIR604 (expresses a modified Cry3Aa engineered to contain a protease cleavage site resulting in greater toxicity to corn rootworms), event 5307 (expresses eCry3.1Ab, an engineered protein representing a variable-region exchange of a lepidopteran-active protein, Cry1Ab, with a Cry3A region) and DAS-59122-7 (expresses the binary Cry34/35Ab1 toxin) (Carroll et al., 1997; USEPA, 2014; Walters et al., 2010).

DAS-59122-7 was commercialized in maize hybrids either as a single trait, Herculex RW® and Optimum® AcreMax® RW®, or in breeding stacks with Cry3Bb1 (MON88017) as SmartStax® or mCry3Aa, Agrisure® 3122. The dual WCR trait events expressing two Bt proteins for the purpose of insect resistance management were more recently deregulated and show superior insect control compared to single trait events (Hibbard et al., 2011; Hitchon et al., 2015; Prasifka et al., 2013). Pyramided maize events expressing two Bt Cry proteins with different modes of action are predicted to dramatically delay insect resistance evolution (Carriere et al., 2015; Storer et al., 2012), provided that there is no prior resistance to one of the stacked traits (Tabashnik and Gould, 2012).

Maize events that express Cry34/35Ab1 have been demonstrated to control populations that have evolved field resistance to mCry3Aa and Cry3Bb (Gassmann et al., 2011, 2014) and there is no apparent cross-resistance between Cry34/35Ab1 and either Cry3Bb1, mCry3A or eCry3.1Ab (Wangila et al., 2015; Zukoff et al., 2016). In view of the growing number of reports of field evolved resistance in WCR (Gassmann, 2012; Gassmann et al., 2011, 2014; Wangila et al., 2015), it is important to better understand their mode of action, and one approach is identification of binding proteins as candidate receptors.

Cadherins are a class of proteins known to be involved in Bt Cry protein binding and toxicity to insects in the orders Lepidoptera, Diptera and Coleoptera (Pardo-Lopez et al., 2013; Pigott and Ellar, 2007). Epithelial cadherin has long been recognized for its involvement in cell-to-cell adhesion that mediates many facets of tissue morphogenesis in vertebrates (Gumbiner, 2005; Halbleib and Nelson, 2006). In insects such as *Manduca sexta*, it is believed to play an important role in larval midgut epithelial organization during rapid cell proliferation and tissue growth (Midboe et al., 2003). In relation to Bt toxicity, insect cadherins have been reported to interact with 3-domain Bt Cry proteins in coleopteran insects. For example, Cry3Aa has been demonstrated to interact with cadherin in *Tenebrio molitor*, TmCad1. This TmCad1 was shown to be a functional receptor of Cry3Aa when the sensitivity of the larvae towards Cry3Aa was reduced with successful RNA interference (RNAi) of the cadherin gene by injection of TmCad1 dsRNA into the larvae (Fabrick et al., 2009). In a similarly designed experiment in *Tribolium castaneum*, the cadherin (TcCad1) and sodium solute symporter (TcSSS) which contains cadherin repeat fragments, were identified as putative binding proteins of Cry3Ba in ligand blots using brush border membrane vesicle preparations. The susceptibility of *T. castaneum* to Cry3Ba was reduced when both of these targets were down regulated through RNAi-mediated knockdown, which is indicative of the involvement of these binding proteins in the toxicity of Cry3Ba (Contreras et al., 2013).

It has also been recently reported that a 185 kDa cadherin (AdCad1) from larvae of the lesser mealworm (*Alphitobius diaperinus*) is a receptor for the Cry3Bb toxin (Hua et al., 2014). In this experiment, the susceptibility of *A. diaperinus* was reduced through RNAi-mediated knockdown of the cadherin gene and the toxicity of the Cry3B protein was restored after feeding the insect with a cadherin repeats (CR9), which is one of the components in the AdCad1. Further, Cry7Ab3 has been reported to bind with a putative

cadherin-like protein in *Henosepilachna vigintioctomaculata* (Coccinellidae), through binding analyses with ligand blots the cadherin-like protein was identified through matrix assisted laser desorption-time of flight-mass spectrometry (MALDI-TOF-MS) (Song et al., 2012). Although it was shown that the coccinellid was sensitive to Cry7Ab3 and histopathological examination of the midgut epithelium revealed extensive damage, it is still unknown whether the cadherin is involved in the toxicity.

In the current study, a *D. v. virgifera* cadherin (*Dvv*Cad) (Sayed et al., 2007) (accession number EF531715) was tested for its involvement in the toxicity of Cry3Aa and Cry34/35Ab1. The experimental design used here involved RNAi suppression of the cadherin mRNA and protein levels through feeding WCR with *DvvCad* dsRNA followed by *in vitro* diet bioassay exposure to Cry34/ 35Ab1 or Cry3Aa. The results of these experiments indicate that RNAi of cadherin in WCR had no effect on Cry34/35Ab1 or Cry3Aa toxicity suggesting that receptors other than cadherin mediate toxicity of these proteins.

2. Material and methods

2.1. Cry34Ab1, truncated Cry35Ab1 and truncated Cry3Aa preparations

Expression constructs encoding amino acid residues 1–124 of Cry34Ab1, 1–354 of truncated (tr) Cry35Ab1, and 1–644 of Cry3Aa were transformed into a Dow AgroSciences proprietary *Pseudomonas fluorescens* expression strain (Squires et al., 2004). The seed culture for the Cry34Ab1 expression strain was grown overnight in Luria Broth media containing 15 mg/ml tetracycline, while trCry35Ab1 and Cry3Aa expression strains were grown overnight in M9 minimal media containing 1% glucose. Methods for purification of Cry34/Cry35Ab1 are described by Kelker et al. (2014). Final Cry34Ab1 and trCry35Ab1 samples were filtered through a 0.22 μm filter and applied to a Superdex 75 26/90 column pre-equilibrated in 20 mM sodium citrate pH 3.3.

Inclusion bodies (IB) of from *P. fluorescens* cells transformed to express the native Cry3Aa protein from *Bacillus thuringiensis tenebrionis* were isolated using high-pressure cell lysis with a 16,000 psi microfluidizer processor (Microfluidics, Westwood, MA) with lysis buffer (50 mM Tris, 200 mM NaCl, 10% glycerol, 0.5% Triton X-100, 20 mM EDTA, 4 mM Benzamidine, 1 mM DTT, pH 7.5). The lysate was centrifuged at 14,000 *g* for 40 min at 4 °C. The pellet was washed with lysis buffer three times and re-suspended in 10 mM EDTA. The IB paste was stored at −80 °C until trypsin digestion to release the activated Cry3Aa toxin. Approximately 5 ml of the IB paste was diluted in 100mMCAPS, pH 10.5 and a mixture of 1:15 of TPCK-treated trypsin (Sigma, St. Louis, MO): protein (w/w) was prepared. The mixture was incubated with gentle agitation at 21 °C for 16 h and centrifuged at 23,000*g* for 25 min at 4 °C. The supernatant was collected and diluted with 10 mM CAPS, pH 10.5. The trypsin activated Cry3Aa core was purified using ion exchange chromatography with a HiTrap Q HP column (GE Healthcare, Pittsburg, PA), pre-equilibrated in 50 mM CAPS, pH 10.5, and gradient elution with 50 mM CAPS, pH 10.5 + 1 M NaCl. Fractions containing the toxic core protein (activated protein) were concentrated with 10 kDa MWCO Amicon concentrators, centrifuged at 5000 *g* for 10 min and buffer exchanged into 10 mM CAPS, pH 10. Complete activation or truncation was confirmed by SDS-PAGE analysis. The molecular mass of the full-length Cry3Aa was ≈73 kDa, and the trypsin core was ≈55 kDa, respectively. The cleavage site between amino acid residue 159 and 160 characteristic of the trypsinized Cry3Aa toxin (Carroll et al., 1997) was confirmed by Edman N-terminal sequencing PPSQ-33A (Shimadzu, Kyoto, Japan). Complete amino acid sequences for the full-length and trypsin core are described by Narva et al. (2013).

Protein concentrations of the 14 kDa Cry34Ab1, 40 kDA tr-Cry35Ab1 and 60 kDa trCry3Aa were analyzed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Laemmli, 1970), following a densitometric quantification method (Crespo et al., 2008). Proteins were stored at –80 °C until use.

2.2. Cloning of Diabrotica virgifera virgifera cadherin

Three putative cadherin genes were identified in WCR midgut transcriptome databases from both University of Nebraska (Eyun et al., 2014) and Dow AgroSciences LLC (data not shown). One of the cadherin genes exhibited high similarity (score = 130, E-value = 1e-102) with a cadherin gene from *Tenebrio molitor* (accession number DQ988044.2) (Fabrick et al., 2009). All *Dvv*Cad peptides identified in BBMV preparations by mass spectrometry exhibited high similarity with the sequence for this cadherin homolog from *T. molitor*, but no peptide matches to the other two putative cadherin proteins were detected. The *D. v. virgifera* cadherin gene was amplified by qRT-PCR and cloned into the pIZT-V5 vector (Invitrogen™). Plasmid templates used for PCR included the forward: DuCadFor 5′ CTCGA-GATGGCTACGAGAAATCTATG 3′ (with an Xho I site added) and reverse: DUCadHisRev 5′ CTCGAGTTAGTGATGATGGTGGTGATGGAGA-TATGTAGTTTTATCCTC 3′ (with an Xho I site and 6X-His tag added) primers. PCR reactions yielded the expected 5094 bp product. PCR fragments were gel purified in a 1% agarose gel and visualized with SYBR® green dye (Promega Corp., Madison, WI). The excised products were ligated into pCR-Blunt II-TOPO (Invitrogen, Grand Island, NY). Ten clones were grown in selective medium and minipreps were performed using the Machery-Nagel Nucleospin kit (Machery-Nagel, Bethlehem, PA). The plasmids were digested with EcoRI to confirm the presence of the insert and positive clones were sequenced. One clone having the expected sequence was chosen for further subcloning. The cadherin gene was excised as an XhoI fragment and ligated into pBAC(–) derived from pBAC-5 (Novagen, Hornsby, Australia), with all tag sequences removed from the multiple cloning sites, which had been linearized with XhoI and treated with alkaline phosphatase. Ten colonies were picked for plasmid isolation and digested with BamHI to confirm the presence and orientation of the insert. Four clones with the correct insert were pooled and treated with the Endotoxin Removal Kit (Mo-Bio, Carlsbad, CA) as directed by the manufacturer.

Generation of recombinant baculovirus: pBAC(–)/*Diabrotica* CadHis was diluted to 0.1 μg/μl in endotoxin-free TE buffer. Sf9 cells were seeded into a 12-well plate at 5×10^5 cells/well and incubated for 1 h at 27 °C. A mixture of 0.5 ml media (Sf900 II SFM) (Invitrogen, Grand Island, NY), 2.5 μl flashBAC DNA and 2.5 μl pBAC(–)/*Diabrotica* CadHis (0.1 μg/μl) was prepared and mixed gently. Two μl CellFectin II (Invitrogen, Grand Island, NY) was added and incubated at room temperature for 20 min. Media was removed from cells and the transfection mix was added and rocked gently at 27 °C for 5 days. An additional 0.5 ml fresh media was added after the first 5 h of incubation. After 5 days, the media was removed by centrifugation at 2000 *g* for 5 min in a picofuge (Stratagene, La Jolla, CA). The media containing virus was transferred to a clean tube and stored at 4 °C. For amplification of the virus, Sf9 cells were seeded into a 15 ml shake culture at 1×10^6 cells/ml. Transfection media (0.5 ml) was added and the flask was incubated at 27 °C/130 rpm for 72 h. After 72 h the cells were pelleted at 830 *g* for 5 min and the media was removed, filtered through a 0.2 μm filter and stored at 4 °C (P1 virus stock). Titer of a 0.5 ml sample of the amplified virus stock was determined. The expression of the amplified virus stock was scaled up in High Five cells and the recombinant protein was purified.

2.3. WCR cadherin and green fluorescent protein (GFP) dsRNA synthesis

All PCR products were sequenced and further confirmed by blast search against the NCBI non-redundant database. Using purified PCR products as template, the cadherin or GFP dsRNAs were respectively synthesized with gene specific primers flanked with T7 at 5′ end: T7+AAGAACAGGCTGAGTATGA and T7+CATAACTGCTCCAAAG for Cadherin, and T7+GGGAGGTGATGCTACATACGGAA and T7+GGGTTGTTTGTCTGCCGTGAT for GFP, by using the MEGAscript T7 kit (Life Technologies, Grand Island, NY) following manufacturer's instructions. Synthesized dsRNAs were purified using an RNeasy Mini Kit from Qiagen (Valencia, CA) following manufacturer's instructions. All dsRNA preparations were quantified using a Nanodrop 1000 spectrophotometer (Thermo Scientific, Waltham, MA) at 260 nm and analyzed by gel electrophoresis to determine purity. Purified dsRNA products were aliquoted and stored at –80 °C until use.

2.4. RNAi of WCR cadherin and Bt protein exposure by diet-based bioassay

Non-diapausing WCR eggs (Crop Characteristics Inc., Farmington, MN) were incubated at 28 °C in soil for 10 days. Eggs were washed from soil with water, surface sterilized with 10% formaldehyde for 3 min and triple rinsed with sterile water (Pleau et al., 2002; Stevo and Cagan, 2012). Eggs were hatched and larvae maintained on a Dow AgroSciences proprietary WCR diet until bioassay.

A 2-stage diet bioassay was conducted in 24-well cell culture plates with each well containing 1.5 ml of larval diet. Test aliquots of dsRNA or Bt protein at 80 μl/well were pipetted onto the diet surface and dried at room temperature in laminar flow hoods. WCR neonates were transferred to treated artificial diet and the insects were enclosed in the bioassay arena with Breathe Easy® gas permeable sealing membrane (USA Scientific, Orlando FL).

In Stage-1, neonates <24-hour after hatching were exposed to diet treated with either water, *Dvv*Cad dsRNA or GFP dsRNA both at 500 ng/cm^2 for two days. In Stage-2 of the bioassay, survivors from the Stage-1 exposure were transferred with a camel hairbrush to a different bioassay plate following the treatment combinations listed in Table 1. Twenty randomly chosen insects from each Stage-1 treatment were exposed to diet treated with 15 μg/cm^2 of Cry34Ab1 and 15 μg/cm^2 trCy35Ab1 (Cry34/tr35Ab1) in 20 mM sodium citrate, pH 3.5 or water with five insects/well and four bioassay wells/treatment. Similar Stage-2 bioassays were conducted with 1000 μg/cm^2 trCry3Aa protein after exposure to *Dvv*Cad dsRNA. Buffer controls consisted of diet treated with 10mM CAPS, pH 10. During Stage-2, bioassay plates were held under controlled environmental conditions (28 °C, 24-h scotophase, 60–80% RH) for 4 days. Eight and nine replications were performed for Cry34/tr35Ab1 and trCry3Aa respectively.

Table 1. Exposure of *D. virgifera virgifera* in 2-Stage bioassays comprising the treatment combinations of dsRNA of a putative cadherin and *Bacillus thuringiensis* (Bt) proteins. Bt and buffer tested were 15 + 15 (or 30 total) μg/cm^2 Cry34/tr35Ab1 and 20 mM sodium citrate, pH 3.5, or 1000 μg/cm^2 trCry3Aa and 10 mM CAPS, pH 10.

Treatment in Stage-1	Treatment in Stage-2	Treatment type
*Dvv*Cad dsRNA	Bt	Test sample
*Dvv*Cad dsRNA	Water	Negative control
GFP dsRNA	Bt	Test sample
GFP dsRNA	Water	Negative control
Water	Bt	Positive control
Water	Buffer	Negative control

From four replications of each Bt bioassay, ten (Stage-1) and >3 (Stage-2) surviving WCR larvae were collected to determine *DvvCad* mRNA expression at the end of Stage-1 and Stage-2 exposures. Live insects were flash frozen and stored at –80 °C for quantitative real-time PCR (qRTPCR).

At the end of Stage-2 exposures, the total number of insects exposed to each treatment, the number of dead insects, and the weight of surviving insects were recorded. Percent mortality and percent growth inhibition were calculated for each treatment. Growth inhibition (GI) was calculated as follows: GI = [1 – (TWIT/ TNIT)/ (TWIBC/TNIBC)], where TWIT is the total weight of insects in the treatment, TNIT is the total number of insects in the treatment, TWIBC is the total weight of insects in the buffer control, and TNIBC is the total number of insects in the buffer control. Control mortality did not exceed 10%.

An initial analysis using studentized residuals was used to confirm the assumptions of normality. Analyses of variances with PROC GLIMMIX and multiple pair wise comparisons with Tukey's Kramer HSD (SAS-Institute, 2011) were used to detect significant differences (α = 0.05) in larval mortality, growth inhibition, and relative *DvvCad* transcript levels between treatments at the end of Stage-1 (2 d) and Stage-2 (6 d) exposures.

2.5. WCR cadherin knockdown measurement by quantitative real-time PCR (qRT-PCR)

Cadherin mRNA expression on batches of larvae collected during the bioassays were evaluated by qRT-PCR. Total RNA was isolated using RNeasy kit (Qiagen, Valencia, CA) and 1 µg RNA was used to synthesize cDNA using a Quantitech reverse transcription kit (Qiagen, Valencia, CA). cDNAs were diluted 50× with nuclease freewater and used as template for qRT-PCR. Reactions for qRT-PCR included 2 µl (50 ng/µl) of cDNA template, 10 µl of SYBR Green mix (Applied Biosystems, Carlsbad, CA), 0.25 µl forward and reverse primers, and 7.5 µl of nuclease freewater, for a total volume of 20 µl. Reactions were set up in a Micro Amp 96-Well tray (Applied Biosystems, Carlsbad, CA) in duplicate. Actin mRNA was used as endogenous control with the primers, Actin-F (5′–TCCAGGCTGTCTCTCCTTG–3′) and Actin-R (5′–CAAGTCCAAACGAAGGATTG–3′). qRT-PCR was performed on 7500 Fast qRT-PCR system from Applied Biosystems (Carlsbad, CA). Relative quantification (RQ) was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

2.6. Mass insect exposure and brush border membrane vesicle (BBMV) preparation

To determine the effect of dsRNA exposure on protein expression, approximately 1000–1500 WCR neonates were exposed to 500 ng/cm^2 *DvvCad* dsRNA, 500 ng/cm^2 GFP dsRNA or water for two days followed by exposure to untreated diet to allow insect feeding for four additional days. The diet was treated with 900 µl of test material applied to the diet surface of a Petri plate (8.6 cm in diameter). WCR neonates (<24 h after hatching) were used in Stage-1 exposure. Approximately 1000–1500 and 50–100 larvae per petri plate were used in Stage-1 and Stage-2 exposure respectively. All bioassay preparations were performed under sterile conditions in a laminar flow hood and held under controlled environmental conditions. Larvae were collected at the end of Stage-1 exposure and Stage-2 bioassay respectively, flash frozen on dry ice and stored at –80 °C. Bioassays were replicated two or three times and whole body larvae were pooled for each brush border membrane vesicle (BBMV) preparation. There were two batches of BBMV prepared for both immunoblot and MS analyses.

Pooled whole larval bodies were individually processed to prepare BBMV using the MgCl$_2$ precipitation method (Wolfersberger et

al., 1987). The final BBMV pellet was resuspended in 50% diluted ice-cold homogenization buffer (0.3 M Mannitol, 17mMTris–HCl, pH 7.5). The protein concentrations of these BBMV preparations were determined using Coomassie Blue (Pierce, Rockford, IL) with bovine serum albumin (BSA) as the standard (Bradford, 1976). Protein concentrations ranged from 2 to 6 mg/ml. BBMV preparations were flash frozen in liquid nitrogen and stored at –80 °C in 30 µl aliquots until use.

2.7. WCR cadherin knockdown measured by immunoblot

Western blots were used to assess *DvvCad* knockdown in BBMV preparations from larvae collected at the end of Stage-1 and Stage-2 exposure using a 3–8% NuPAGE Tris-Acetate gel with 30 µg of BBMV protein per lane. Samples were run in duplicate and one set was used for western blot and the other was stained with coomassie brilliant blue. Band intensity and pattern in the coomassie-stained gel were comparable between lanes (results not shown) indicating that the lanes were loaded with equal protein. For western blots, the gel was transblotted to nitrocellulose using iBlot nitrocellulose mini stacks for 7 min at 5 V (Invitrogen, Carlsbad, CA), then blocked for 1 h in WesternBreeze blocker/diluents (Invitrogen, Carlsbad, CA). The blot was then incubated for 2 h in 1:3000 Anti-cadherin antibody in blocker/diluent. The cadherin affinity-purified polyclonal antibody was raised in rabbit against the carboxy terminal 14 amino acid peptide DYNFNTNEDKTTYL (GenScript Corp., Piscataway, NJ). The blot was washed twice for 15 min in wash solution then incubated in 1:5000 goat anti-Rabbit IgG-HRP (Bio-Rad Laboratories, Hercules, CA) in blocker/diluent for 1 h at room temperature. The blot was then washed twice for 15 min in wash solution, developed with Amersham ECL western blotting detection reagents (GE Healthcare, Buckinghamshire, UK) for 1 min.

2.8. WCR cadherin knockdown measured by mass spectrometry

Aliquots of frozen BBMV from each of the treatments (water, GFP dsRNA and *Dvv*Cad dsRNA) in Stage-1 and Stage-2 exposure were extracted and digested for mass spectrometry using filter assisted sample preparation (FASP) with minor modifications (Wisniewski et al., 2009). Briefly, thawed aliquots (approx. 200 µg total protein each) were diluted with an equal volume of 4% SDS prepared in 100 mM triethylammonium bicarbonate (TEAB) pH 8.2 and 100 mM dithiothreitol (DTT). The samples were incubated at 90 °C for 5 min to extract, denature and reduce membrane bound proteins. After cooling to room temperature, the samples were transferred to a 10 kDa molecular weight cutoff spin filter concentrator (Thermo Scientific, Rockford, IL). The SDS was diluted with 10 volumes of 8 M urea freshly prepared in 100 mM TEAB. After centrifugation at 14,000 *g* to remove SDS, the samples were alkylated in the spin filter with 50 mM iodoacetamide for 20 min in the dark. Excess iodoacetamide was quenched with 100 mM DTT in 8 M urea. Three additional centrifugations with fresh aliquots of 8 M urea in 100 mM TEAB were used to complete the removal of SDS from the samples. The resulting samples (approx. 20 µl) were diluted with 10 µl of LysC/trypsin (Promega Corporation, Madison WI) prepared in 100 mM TEAB to give approximately 5.3 M urea. Initial digestion relying on LysC activity was carried out at 37 °C for 2 h, according to the manufacturer's directions. Samples were diluted with 100 mM TEAB to give a final concentration of urea of approximately 0.8 M. Digestion continued for 14 h at 37 °C. Digested peptides were collected after centrifugation through the filter, and the filter rinsed with two aliquots of 500 mM NaCl in 100 mM TEAB. The combined filtrates were acidified with formic acid, and the peptides were desalted by adsorption and elution from a C18 spin column (Harvard Apparatus part 74–4601, Holliston MA) with 80% acetonitrile in 0.1% formic acid.

The resulting peptide samples were concentrated *in vacuo* and analyzed using a chip based trap and nanoLC separation (Eksigent ChiPLC) interfaced to a Thermo QExactive mass spectrometer. A top 12 data dependent MS2 acquisition was used with: 70k resolution for MS1; automatic gain control MS1 =1E06; max IT MS1 =2 msec; and 17.5k resolution for MS2; automatic gain control MS2 = 5E04; max IT = 80 msec. Triplicate injections of each digest were carried out.

MS/MS mass spectra were analyzed using the following software protocol. The acquired raw files were converted into MS1 and MS2 files using RawExtract 1.9.9.2 (McDonald et al., 2004). The MS/ MS spectra in the MS2 files were searched with the ProLuCID algorithm(Xu et al., 2006) against a protein database generated by 6- frame translation of WCR RNASeq data generated at Dow Agro- Sciences. In order to accurately estimate peptide probabilities and false discovery rates, a target-decoy database containing the reversed sequences of all the proteins appended to the target database was used (Peng et al., 2003). Tandem mass spectra were matched to sequences using the ProLuCID algorithm with 100 ppm peptide mass tolerance. The search space included all fully- and half-tryptic peptide candidates that fell within the mass tolerance window with no miscleavage constraint. Search parameters included a static modification of carbamidomethylation at cysteine (57.02146 amu). The validity of peptide spectrum matches (PSMs) was assessed in DTASelect2 (Cociorva et al., 2007; Tabb et al., 2002) using two SEQUEST (Eng et al., 1994) defined parameters, the cross-correlation score (XCorr), and normalized difference in crosscorrelation scores (DeltaCN). The search results were grouped by charge state (+1, +2, +3, and greater than +3) and tryptic status (fully tryptic, half-tryptic), resulting in 6 distinct sub-groups. In each one of these sub-groups, the distribution of Xcorr, DeltaCN, and DeltaMass values for (a) direct and (b) decoy database PSMs was obtained, then the direct and decoy subsets were separated by discriminant analysis. Full separation of the direct and decoy PSM subsets is not generally possible; therefore, peptide match probabilities were calculated based on a nonparametric fit of the direct and decoy score distributions. A peptide confidence threshold was dynamically set and only peptides with delta mass less than 5 ppm were accepted to achieve protein level false discovery rate below 1%. After this last filtering step, we estimated that the protein and peptide false discovery rates were below 1% and 0.1%, respectively. The software tools mentioned above, including RawXtractor1.9.9.2, ProLuCID and DTASelect2 were downloaded from http://fields. scripps.edu/downloads.php.

Label free peptide quantification for *Dvv*Cad was carried out using Skyline (MacLean et al., 2010) to extract the MS1 peak areas of peptides identified using ProLuCID. A spectral library of peptides with retention times identified from the BBMV analyses was created using a pepXML file generated from the DTASelect results and mzML files (Kessner et al., 2008) of the raw data. This spectral library was used to identify high resolution accurate mass chromatographic peaks with MS1 isotopic dot product matches to the theoretical isotopic composition >0.8 and retention times within 4 min of the spectral library entry (Schilling et al., 2012). The peak areas were normalized based on the total amount of protein present in the original digests, and based on total MS1 signal for each sample. Relative amounts of *Dvv*Cad were calculated as the average of the measured peptide peak areas. Six peptides for *Dvv*Cad which were reliably detected were used for protein abundance quantifications across all samples: "AVDVDLNSEITYHCTPEYEK", "DGEVGEGIIGEDIDDGDNAK", "DLQCSENLNKDGEVGEGIIGEDIDDGDNAK", "GLTCSISSEINRIGEGLDK", "IIGEPFYLSTENDAAK", and "MNIVGTYAENR".

3. Results and discussion

In this study, we evaluated whether *DvvCad* (EF531715) (Sayed et al., 2007) is involved in the toxicity of Cry34/35Ab1 or Cry3Aa to WCR first instar larvae. Our experimental design was to use dsRNA to knockdown *Dvv*Cad expression in neonate larvae during Stage-1 exposure prior to diet-based feeding of Cry34/tr35Ab1 or activated Cry3Aa insecticidal proteins during Stage-2 of the bioassay. We hypothesized that suppression of *Dvv*Cad and reduction in insect susceptibility to these Bt proteins would indicate that *Dvv*Cad plays a central role in Bt protein toxicity. Suppression *Dvv*Cad transcript levels after exposure to *Dvv*Cad dsRNA was confirmed through qRT-PCR. Additionally, the protein level of *Dvv*Cad was significantly reduced after exposure to *Dvv*Cad dsRNA based on immunoblotting and high-resolution mass spectrometry of *Dvv*Cad from BBMV proteins prepared from dsRNA exposed and control larvae.

3.1. Down-regulation of western corn rootworm cadherin mRNA and protein

Results of expression analysis by qRT-PCR (Figure 1) confirmed down regulation of the WCR putative cadherin mRNA in larvae collected immediately after exposure to dsRNA (Stage-1) and after exposure with Cry34/tr35Ab1 and trCry3Aa (Stage-2). Normalized relative *DvvCad* transcript in WCR larvae fed with the *Dvv*Cad dsRNA (<0.1 relative transcript level) was significantly reduced by >90% relative to larvae exposed to GFP dsRNA and water (1–1.2 relative transcript level), at the end of Stage-1 (day two) and Stage- 2 (day six) exposure.

To determine the reduction in *Dvv*Cad protein generated by RNAi, the 2-Stage bioassay was repeated using a larger experimental format to obtain increased numbers of neonate larvae exposed to dsRNA for subsequent proteomic analysis. BBMV samples were analyzed by immunoblot and mass spectrometry (Figure 2). Both methods revealed that *Dvv*Cad protein (MW ca. 191 kDa) was significantly reduced in both Stage-1 and Stage-2 samples of BBMV prepared from WCR larvae exposed to *Dvv*Cad dsRNA, with >90% reduction in cadherin protein determined by mass spectrometry analyses. Larvae collected at the end of Stage-2 exposure (6 days
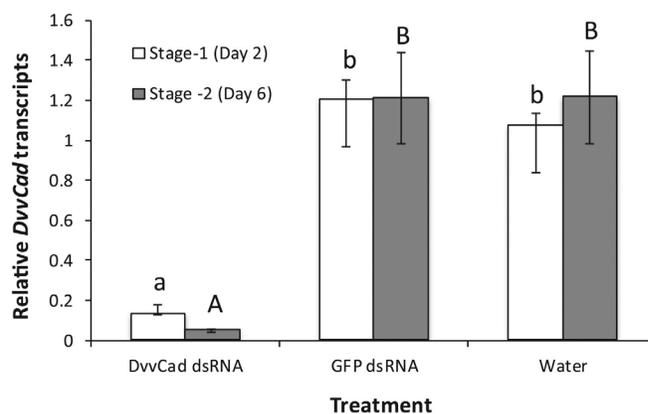

Figure 1. Normalized relative *DvvCad* transcript level of *D. virgifera virgifera* larvae collected at the end of Stage-1 and Stage-2 exposure with Cry34/tr35Ab1 and trCry3Aa, at day-two and day-six after insect infestation. Lower case letters (Stage 1) and capital letters (Stage 2) indicate mean values of relative *DvvCad* transcripts after *DvvCad* dsRNA treatment were significantly reduced when compared to GFP dsRNA and water treatments, according to Tukey-Kramer HSD test (*P* > 0.05). The error bars denote the standard error of each mean relative transcript expression.
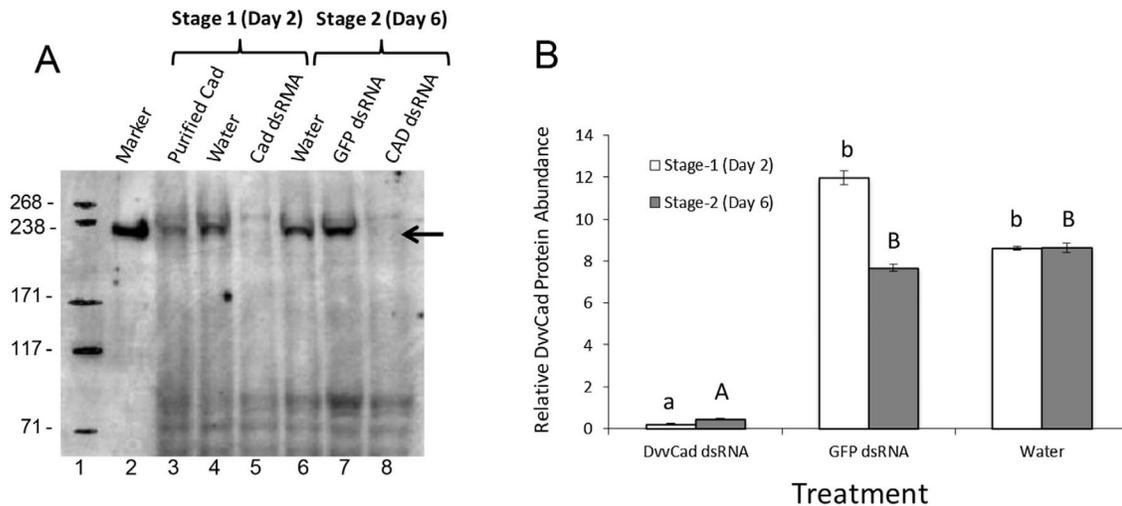
Figure 2. RNAi down regulation of *DvvCad* (EF# EF531715) protein detected by immunoblot (A) and mass spectrometry (B). Each sample of BBMV was prepared from WCR whole body larvae and collected at the end of Stage-1 and Stage-2 exposure, which corresponded to 2 and 6 days after infestation. **(A)** Lane 1, HiMark Standards; lane 2, 20 ng purified *DvvCad*; lanes 3–5, 30 µg BBMV prepared from larvae that were exposed to water, GFP dsRNA, or *DvvCad* dsRNA, respectively, in Stage-1 exposure; lanes 6–8, 30 µg BBMV prepared from larvae that were exposed to water, GFP dsRNA or *DvvCad* dsRNA, respectively, in Stage-1 exposure, and subsequently transferred to untreated diet in Stage-2 exposure. Arrow indicates the location of 191 kDa *Dvv*Cad protein. **(B)** Mean values of normalized relative protein expression based on six measured peptides in the *Dvv*Cad protein, measured using triplicate injections across all BBMV samples collected at the end of Stage-1 exposure and Stage-2 bioassay. Lower case letters (Stage 1 and capital letters (Stage 2)) indicate mean values of relative *Dvv*Cad protein abundance of *DvvCad* dsRNA treatment were significantly reduced compared to GFP dsRNA and water treatments, according to Tukey-Kramer HSD test ($P > 0.05$). The error bars denote the standard error of each mean relative protein expression.

post initiation of the experiment) also exhibited reduced amounts of *Dvv*Cad protein even though they received no further dsRNA treatment after the 2-day exposure in Stage-1, indicating a residual effect from the *DvvCad* dsRNA that lasted throughout Stage-2 exposure to the Bt proteins.

3.2. Effect of WCR cadherin down-regulation on the susceptibility of western corn rootworm to Cry34/35Ab1 and Cry3Aa

Negative control mortality in the Stage-2 exposure was consistently <10% (Figure 3). GFP dsRNA did not affect the sensitivity of the *D.*

virgifera virgifera larvae fed with either Cry34/tr35Ab1 or trCry3Aa Bt proteins. The sensitivity of WCR to these Bt proteins was significantly higher than the negative controls (combinations of water and buffer in the 2-Stage bioassay). Both Bt toxins provided a higher percentage of larval growth inhibition (approximately 60% for Cry34/tr35Ab1 and 40% for trCry3Aa) compared to the negative control. However, neither toxin treatment yielded greater than 10–20% larval mortality at the end of Stage-2 such that larval growth inhibition provided a more reliable indicator of toxicity than mortality. One possible explanation is that sensitivity of larger larvae to toxin on diet in Stage-2 was reduced due to prior feeding activity in Stage-1.
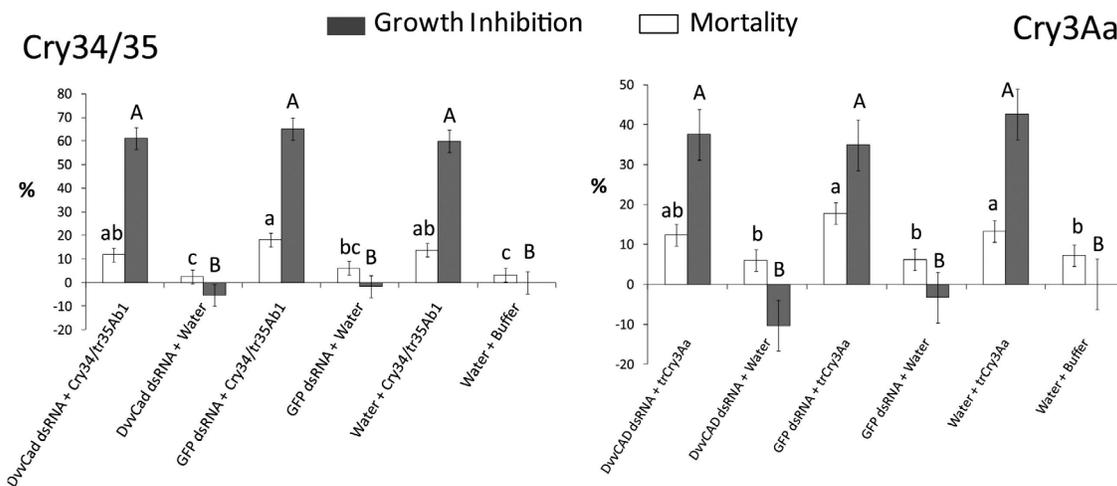

Figure 3. Mean percent mortality and growth inhibition of WCR larvae in 2-stage-bioassays with *DvvCad* and GFP dsRNAs at 500 ng/cm^2 in the Stage-1 exposure, combined with 15 + 15 (or 30) µg/cm^2 of Cry34/trCry35Ab1 **(A)** and 1000 µg/cm^2 of trCry3Aa **(B)** exposure in Stage-2. Negative controls comprised of exposure to combinations of GFP dsRNA or water in Stage-1 exposure, and water or buffer in Stage-2 exposure. The positive control was a combination of water and the respective Bt protein. Columns followed by the same letters and casing within each figure were not significantly different according to Tukey-Kramer HSD test ($P > 0.05$). The error bars denote the standard error of the mean percent mortality or growth inhibition among the treatments.

Although trCry3Aa was tested at the highest possible concentration (1000 µg/cm^2), WCR larvae exhibited lower growth inhibition (40%) compared with larvae exposed to a sublethal concentration of Cry34/tr35Ab1 at 30 µg/cm^2 (60%). A similar pattern of relative sensitivity to these Bt proteins was observed by Li et al. (2013), although in this study neonates were exposed to toxin without prior feeding resulting in higher mortality compared to those observed in the present study.

Results of Stage-2 involving exposure to 30 µg/cm^2 of Cry34/tr35Ab1 or 1000 µg/cm^2 of trCry3Aa after 48 h exposure to *DvvCad* dsRNA is shown in Figure 3. In all cases, the prior exposure to *DvvCad* dsRNA did not affect toxicity of either protein suggesting that *DvvCad* is unlikely to be involved in Cry34/35Ab1 or Cry3Aa toxicity in WCR. Results from the current study showed that both mRNA transcript and protein levels of the *DvvCad* were significantly reduced two days after initial exposure to treated diet with reduced expression and protein abundance sustained throughout the 4-day exposure to Bt toxins (Stage-2) and in the absence of the *DvvCad* dsRNA (Figs. 2 and 3). The rapid cadherin down regulation within the initial 2-day-exposure in Stage-1 suggests that a rapid turnover rate of the *DvvCad* is consistent with its role in supporting regeneration or expansion of the midgut epithelial cells for larval growth, as has been reported for *M. sexta* (Midboe et al., 2003). The sustained and high level of *DvvCad* mRNA and protein suppression strongly suggests that the RNAi response from the initial exposure was maintained throughout exposure to the Bt toxin.

A synergistic effect of a WCR cadherin toxin-binding fragment in combination with Cry3Aa or Cry3Bb was reported by Park et al. (2009). Chymotrypsin activated Cry3Aa and Cry3Bb specifically bound to a WCR cadherin fragment CR8-10 in ELISA plate assays. The authors further demonstrated that unprocessed Cry3Aa and Cry3Bb crystal toxicity against the southern corn rootworm (SCR) increased by 3-fold and 8-fold, respectively when tested as protein mixtures at a 1:10 ratio of Cry protein to cadherin fragment CR8-10. However, since the authors reported that Cry3Aa crystals were not active against WCR, only Cry3Bb with and without CR8-10 was bioassayed, resulting in a 13-fold increase in lethality against WCR when combined. This report of increased Cry3Aa potency on SCR is interesting as it suggests a role for cadherin as a possible Cry3Aa receptor. However, our results from the 2-stage bioassay of trCry3Aa and Cry34/35Ab1 in WCR larvae in which cadherin expression was reduced by >90% after exposure to *DvvCad* dsRNA indicate that the WCR cadherin protein is not critical to the mode of action of Cry3Aa or Cry34/35Ab1. Although Cry34/35Ab1 binds weakly to recombinant WCR cadherin under denaturing conditions (data not shown) and cadherin may be important within the context of the Bt toxin sequential binding model (Bravo et al., 2007), it does not appear to be functionally involved in the toxicity of Cry34/35Ab1 or trCry3Aa to western corn rootworm.

Identification of the WCR Bt receptor(s) involved in the mode of action of Cry34/35Ab1 is important for understanding the potential for cross resistance of new technologies aimed at maintaining the durability of corn rootworm resistance traits. To date laboratory selection and maintenance of Cry34/35Ab1 resistance in WCR has been difficult to achieve (Alves et al., 2013; Lefko et al., 2008). This suggests that resistance determinants for Cry34/35Ab1 may be unique compared to three domain Bt proteins. Identification of WCR midgut proteins that bind Cry34/35Ab1 combined with receptor validation by RNAi knockdown or genetic characterization of resistant strains will help to understand the likelihood for field-evolved resistance to Cry34/35Ab1 traits.

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