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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*; GenBank 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.

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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

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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 Cry3A 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 100 mM CAPS, 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).

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