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A novel cadherin-like gene from western corn rootworm, *Diabrotica virgifera virgifera* (Coleoptera: Chrysomelidae), larval midgut tissue

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Abstract

A *cadherin-like* gene associated with larval midgut tissues was cloned from western corn rootworm (*Diabrotica virgifera virgifera*: Coleoptera), an economically important agricultural pest in North America and Europe and the primary target pest species for corn hybrids expressing Cry3 toxins from *Bacillus thuringiensis* (Bt). The full-length cDNA (5371 bp in length) encodes an open reading frame for a 1688 amino acid polypeptide. The putative protein has similar architecture to cadherin-like proteins isolated from lepidopteran midguts that have been shown to bind to Cry1 Bt toxins and have been implicated in Bt resistance. The *D. v. virgifera cadherin-like* gene is expressed primarily in the larval midgut and regulated during development, with high levels of expression observed in all instars and adults but not pupae. The corresponding genomic sequence spans more than 90 kb and is interspersed with 30 large introns. The genomic organization of the *cadherin-like* gene for this coleopteran species bears strong resemblance to lepidopteran cadherins suggesting a common molecular basis for susceptibility to Cry3 toxins in Coleoptera.

Keywords: cadherin, *Diabrotica*, Bt receptor, insect midgut, exon, intron.

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Introduction

The western corn rootworm, *Diabrotica virgifera virgifera* LeConte (Coleoptera: Chrysomelidae), is arguably the single most important pest of field corn, *Zea mays* L. (Levine & Oloumi-Sadeghi 1991), throughout much of the US Corn Belt and has recently become a major concern in Europe. Managing corn rootworm populations to minimize risk of economic loss has proven extremely difficult, as the insect has demonstrated an apparently unlimited capacity to evolve resistance to small molecule insecticides (Meinke *et al*., 1998; Parimi *et al*., 2003; Siegfried *et al*., 2004) and cultural control practices such as crop rotation (Levine *et al*., 2002).

Current alternative management technologies include use of transgenic crops that express insecticidal proteins toxic to corn rootworm larvae (Moellenbeck *et al*., 2001; Ellis *et al*., 2002). The first transgenic corn hybrids expressing δ-endotoxins from *Bacillus thuringiensis* (Bt) became commercially available in 2003 (Environmental Protection Agency, 2003). This technology represents a significant departure from traditional crop protection chemistry in that Bt toxins act on receptors in the insect midgut rather than the insect nervous system. While Bt corn offers a potentially powerful new tool for rootworm management, there are concerns that widespread adoption of such plants will rapidly lead to Bt toxin resistance and subsequent failure of the control strategy.

Within Lepidoptera, the generally accepted mode of action for Bt toxins involves solubilization of the crystal protein, releasing a 130 kDa protoxin that is activated by proteases in the insect midgut to form a truncated 65 kDa toxin. The target of the activated toxin is the apical (brush border) membrane of larval midgut cells (Bravo *et al*., 2004). Binding of the activated toxin to midgut specific

receptors causes a conformational change in the toxin, which allows its insertion into the membrane and formation of ion channels or pores, leading to osmotic imbalance of the insect gut (Schnepf *et al*., 1998).

In vitro binding assays have implicated cadherin-like proteins as one of the most likely Bt receptor molecules in the midgut membranes of insects. A 210 kDa cadherin-like glycoprotein was identified as a Cry1Ab binding protein in brush border membrane vesicles prepared from midguts of *Manduca sexta* larvae (Vadlamudi *et al*., 1993, 1995). In *Bombyx mori*, a 175 kDa cadherin-like protein was identified as a Cry1Aa binding protein (Nagamatsu *et al*., 1998a,b; Tsuda *et al*., 2003). The cadherin-like protein Bt-R_1 (*M. sexta*) binds to Cry1A toxins with high affinity (Gómez *et al*., 2002a,b). Two specific polypeptide regions of Bt-R_1, bind to domain II of Cry1A toxins (Gómez *et al*., 2003). The proposed model for Bt toxin receptors consists of one to 12 tandem repeats of homologous extracellular domains called cadherin repeats (CR), a signal peptide that targets the protein to the extracellular matrix, a membrane-proximal extracellular domain, a transmembrane domain, and a cytoplasmic domain (Dorsch *et al*., 2002).

Expression of a recombinant cadherin-like protein in resistant *B. mori* cells made them sensitive to Cry1Aa, providing evidence that cadherin-like proteins play a role in Bt toxicity (Nagamatsu *et al*., 1999). Gahan *et al.* (2001) reported that Cry1Ac resistance in the tobacco budworm, *Heliothis virescens*, was tightly linked to a cadherin-encoding gene. More recently, Morin *et al*. (2003) reported three different cadherin alleles from the pink bollworm, *Pectinophora gossypiella*, that were linked with resistance to Cry1Ac and survival on transgenic cotton that expresses Cry1Ac.

The δ-endotoxins from Bt that comprise the class *Cry3* genes from *B. thuringiensis* subsp. *tenebrionis*, *san diego*, *morrisoni*, *tolworthl* and *gallariae* encode for the Cry3A, Cry3B and Cry3C group of proteins, which exhibit coleopteran specific activity (Krieg *et al*., 1983; Hernstadt *et al*., 1986, 1987; Sick *et al*., 1990). In contrast to lepidopterans, little is known regarding specific target sites for Cry3 toxins in the coleopteran midgut. However, recent work with the Colorado potato beetle, *Leptinotarsa decemlineata*, has shown that pore formation activity of Cry3 toxins is dependent on toxin interaction with the brush border membranes followed by oligomeric pre-pore structure formation, similar to observations of Cry1A toxicity in Lepidoptera (Rausell *et al*., 2004). This general mechanism of toxin activation through membrane insertion suggests a common mode of action among different Cry toxin families.

In this paper, we describe cloning of the first coleopteran cadherin-like gene from the western corn rootworm, *Diabrotica virgifera virgifera*. We describe its expression pattern during development and in different tissues and assess its polypeptide and genomic architecture relative to other insect *cadherin-like* proteins.

Results

Cloning of cadherin-like cDNA in Diabrotica virgifera virgifera

Oligonucleotide primers designed from a previously isolated *D. v. virgifera* cadherin EST (Siegfried *et al*., 2005) were used to clone the full-length cadherin-like cDNA resulting in the isolation of a 5371 nt transcript (GenBank accession no. EF531715). When translated, the open-reading frame consisted of 1688 amino acids with a predicted molecular mass of 191 kDa and a calculated pI of 4.44 (Fig. 1). Several 5′-RACE and RLM-RACE experiments were carried out but no 5′ untranslated region longer than that shown in Fig. 2 was obtained, suggesting that the adenine residue 135 bp upstream of the ATG start codon is the transcription start site (designated as position +1 in Fig. 2). Analysis of the predicted protein sequence revealed 10 highly conserved cadherin-like repeats, 27 putative N-glycosylation sites, a membrane proximal region (aa: 1329–1569), a transmembrane region (aa: 1570–1593) and a cytosolic region (aa: 1594–1688) (Fig. 1). A signal peptide cleavage site was located at the N-terminus (aa: 22–23).

To confirm the functionality of the translation initiation site, the full-length coding sequence was expressed in Sf21 insect cell culture using baculovirus cloning and expression systems. Recombinantly expressed protein was identified by Western blotting with an antibody raised against the WCR cadherin-like peptide (Fig. 3). The higher than expected molecular weight indicates substantial post-translational modification (e.g. glycosylation).

Identification of putative Bt-binding regions

Using previously identified Bt toxin binding regions TBR1 (NITIHITDTNN) and TBR2 (SILTVTVT) reported for *M. sexta* as a guide (Gómez *et al.,* 2002a) we identified conserved binding motifs located near amino acid positions 850 (TBR1) and 1320 (TBR2) in various lepidopteran cadherin-like proteins (Fig. 4). Importantly, pattern searches of the *D. v. virgifera* cadherin sequence revealed two similar potential toxin binding regions: ^{833}DISIYVIDTNN^{843} and ^{1311}SSLNVTVN^{1318} (Fig. 4).

We tested the conservation of putative TBR1 and TBR2 sites in the related Mexican corn rootworm (MCR) *D. v. zeae* by cloning and sequencing a 1592 bp cDNA fragment (GenBank accession no. EF541348) using primers derived from the WCR *cadherin-like* cDNA sequence. As expected, the MCR cDNA has high sequence identity (99%) with WCR over the analysed region (base positions 2631–4222). The deduced 530 aa cadherin-like protein also is 99% identical in the analysed region (positions 824–1353) and contains TBR1 and TBR2 motifs, which are 100% identical to those from WCR.

Midgut specific expression of cadherin-like *protein*

The expression of the *cadherin-like* gene was examined using Northern blot analysis among the various developmental

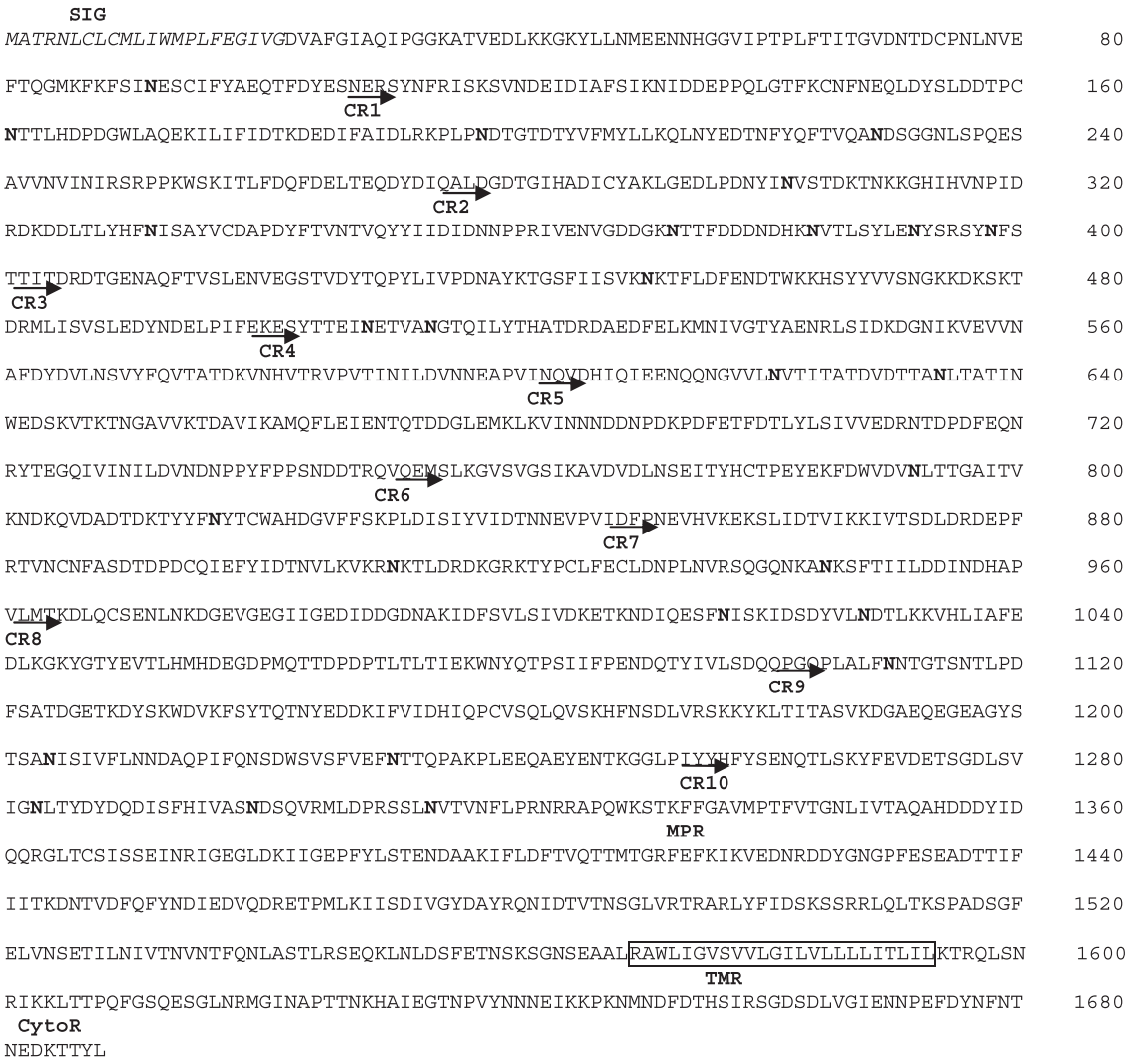

Figure 1. Deduced amino acid sequence of the *Diabrotica virgifera virgifera* cadherin-like protein. The putative signal peptide (SIG) is in italic fonts. Cadherin repeats (CR) are indicated by arrows. MPR, membrane proximal region; TMR, transmembrane repeats; and CytoR, cytosolic region. Bold denotes predicted putative *N*-glycosylation sites.

stages of *D. v. virgifera* (first, second, third instar, pupae and adults). The transcript is most abundant in first, second and third instars but appears to be downregulated in pupae before being upregulated again in adults (Fig. 5A). Analysis of the *cadherin-like* RNA in larval tissues indicates that expression is limited to midgut tissues as it was not detected in integument or fat body (Fig. 5B).

Phylogenetic analysis

Although the deduced *D. v. virgifera* aa sequences shares common architectural themes with lepidopteran cadherin-like proteins that have been identified as putative Bt toxin receptors, phylogenetic analysis revealed that this putative receptor has only 22–25% sequence identity with Lepidopteran cadherins (Fig. 6). As expected, the predicted protein was most closely related to another coleopteran cadherin-like protein identified from *Tribolium castaneum* (red flour beetle) with 30% identity.

Characterization of genomic structure

By genome walking along WCR *cadherin-like* gene, we have identified 30 introns, the four smallest of which were fully sequenced: introns 6 (655 bp), 11 (1990 bp), 28 (2207 bp) and 29 (1821 bp) (GenBank accession no. EF541349). Most of the other introns were very long and were progressively cloned and sequenced. Between 3 and 5 kb of sequence information was obtained for each of the other introns. BLAST analysis of the WCR intron sequences against sequence databases did not reveal significant homology with any known sequence. The intron sequences


```
        -1041 atataaaata aaatacaggg tgctctatta aaaaaaaaaa acaaaagttt
                                                          BRCZ3
-991 ggtctgccac tattttatcg aacatcctgt aacattctag ctaattttgt aatgtgaagc
-931 tcacagttag ctacaatttt tgttattaag tattatcgct atctattact ataacggatc
                          FTZ
-871 tacggagctt caccccacta atagttaatc accctgtata atggcagtta aatattgcat
                                                      FTZ
-811 tgttagctag ttctaagcta tcctgaaaat ttcaggtgtc taggtagcct agaactattt
                                                                  HD
-751 ttaaaatgga ttacaaaatt tgcggagtcc gtgacaccaa ccaagccaag tatataaaaa
          KR                                                     CF2II
-691 gattttaaat agtcagctat ctggatcttt gagtgtcccg agaaaatcct tattaccctg
                                    ZESTE                   HSF
-631 gactaatgct gaacgtgagc cttcgagacc acataacaaa ccaacaaatc agacaaagat
-571 caggagttca agacgtcatc gaaagacact taagtggaat gggcagggcc cttaataggc
                                FTZ
-511 cgggaactta atagtcaggg aacttaatag tcaataccac gccacgggcc acggacattc
-451 cacctcaatg ggcgccttgc cttagcttga acacaagatg gacggtggac atgacgaatc
-391 atggatcgga ggagcagaaa ctataaaaga agtcgatgac gtccaccgac tagatggacc
-331 gacgacataa aaagagtacc gagaaactgg ctacaagcgg tccagtgtag agaacactgg
-271 aaataattga gggaggccta tgtctagcag tgtacgtgat tggctgattg atgaggggaa
                                                    PRD
-211 tcttaacctg cggtttatct ggatttatat tatgcatcgc ctcagtaaat aaaattgaag
                                                BRCZ4
-151 ataatcagaa tatttgttta atacaattat taattttgaa catatccaca aaatactctt
                         PAX6          DFD
 -91 tattatctta tcgagttttt atcgatataa tcacttttata ctgcataaat taatattggc
                                PAX6                     FTZ
 -31 actggttatc gacatttttg tgtcatcact tAGAGCATAT CGAAAGATAA TAAAGTTAAT
                                     +1 Transcription start
 +30 CACAGGTATA AAACTTTTTT CATGAAATCT GTAAAATATT TAGCACTAAA CTATTTGTTT
 +90 TTGTGAGAGT TACAAGTGAG AGGGTGTTTT GTAAATGATG TGGATTGTTT GATACCCTTA
+120 AAAAACATCA Agtaagtata tg...intron1... attt  tttcttttag AAATGGCTAC
                                                          Start codon
```

Figure 2. An approximately 1 kb section of the upstream regulatory genomic sequence of the *cadherin-like* gene. The exon sequence is shown in upper case. The transcription start site is designated as position +1. Potential transcription factor binding sites, as revealed by the MatInspector computer program, are underlined. Only a portion of the first intron (with splice donor and acceptor GT-AG nucleotides in bold) is shown. The translation start codon ATG is boxed.

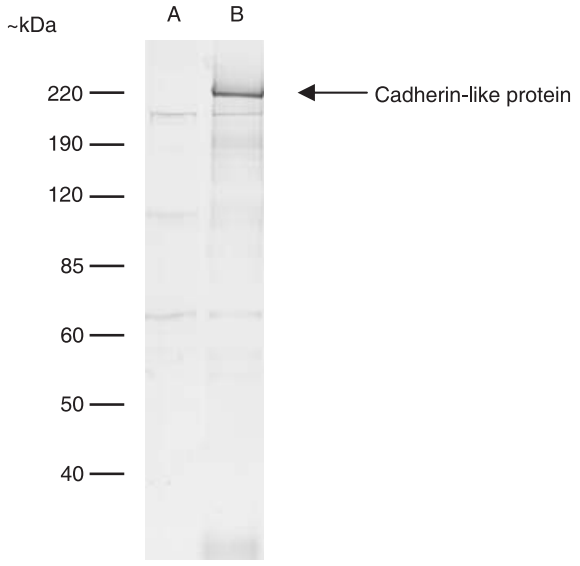

Figure 3. Expression of full-length cadherin-like protein in sf21 cell culture. The transiently expressed protein was immunoblotted with polyclonal antibody for the cadherin-like protein. Lane A: extracts from cells transfected with control vector only. B: extracts from cells transfected with recombinant vector.

delineated 31 exons that ranged from 57 (exon 1) to 414 bp (exon 24). The first exon is a noncoding exon and is designated exon-1 in Table 1. The translation start site maps to the third base of exon 1 and the protein translation stop codon is located at the 95 bp of exon 30. All of the exon–intron boundary sequences conform to the consensus splice donor (GT) and acceptor (AG) sites (Table 1). With very few exceptions, conserved sequences of GTAAG(T/A) were observed at the 3′ splice donor sites and (A/C)T_n(A/C)AG or (A/C)T_nAG sequences was identified at the 5′ splice acceptor sites (Table 1).

To identify the putative regulatory region within the 5′-flanking DNA, a 2371 bp genomic DNA fragment upstream from exon 1 was sequenced. As shown in Fig. 2, results of analyses with the MatInspector revealed several transcription factor binding sites within the 1 kb upstream of 5′-flanking region. These include four FTZ and two PAX6 sites. The fushi tarzu (Ftz) homeoprotein targets the majority of the genes in *Drosophila* (Liang & Biggin, 1998; Bowler *et al.* 2006). Interestingly, mouse R-cadherin was shown to be regulated by Pax6 transcription factor (Andrews & Mastick,

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                    Position (aa)
                                                    TBR1
> Manduca       855-PPDPTYWETEGNITIHITDTNNKVPQAETTKFDTVVYIYENA
> Bombyx        856-PDDPTYWETPGQVVIQIIDTNNKIPQPETDQFKAVVYIYEDA
> Heliothis     861-PPDPNYFNTPGDITIHITDTNNRVPRVEEDKFEEIVYIYEGA
> Helicoverpa   861-PPDPTYFETPGEITIHITDTNNKVPQVEDDKFEATVYIYEGA
> Lymantria     860-PPDPTYWETEGLISIYIIDTNNKIPQAEIELFSTTVYVWENA
> Pectinophora 861-PPDPVFWDTLGDNVINIVDINNKVPAADLSRFNETVYIYENA
> Chilo         853-PPDPMHFNTTGYIQIEILDTNNKVPRGTLGPIQVGGAHQREI
> Ostrinia      847-PPDPNYFEVPGDIEIEIIDTNNKVPEPLTEKFNTTVYVWENA
> WCR           822-AHDGVFFSKPLDISIYVIDTNNEVPVIDFPNEVHVKEKSLID
> MCR            ?-  AHDGVFFSKPLDISIYVIDTNNEVPVIDFPNEVHVKEKSLID

                                                    TBR2
> Manduca       1322-VAASNSPD-GGIPLPASILTVTVTVREADPRPVFMRELYTAGIST
> Bombyx        1321-IAASNSPT-GGIALT-STITITVNVREADPQPYFVRDLYTAGIST
> Heliothis     1329-IGASDSPSP-AAVLQASTLTVTVNVREANPRPVFQSALYTAGIST
> Helicoverpa   1329-IGASDTPN-PAAVLQASTLTVTVNVREANPRPVFQRALYTAGISA
> Lymantria     1325-VAASNSPD-SVNALPSNTLTVTVNVREANPRPMFTSEEYMAGIST
> Pectinophora 1330-VAASNSPTGGGIPLPGSLLTVTVTVREADPRPVFEQRLYTAGIST
> Chilo         1317-VAASNSPSATGTPLDGTTLTVTINVVEEDPRPVFERELYTAGISV
> Ostrinia      1318-IAASNSPDATGIPLQTSILVVTINVREANPRPIFEQDLYTAVIST
> WCR            1296-IVASNDSQVRMLDPRSSLNVTVNFLPRNRRAPQWKSTKFFGAVM
> MCR               ?-IVASNDSQVRMLDPRSSLNVTVNFLPRNRRAPQWKSTKFFGAVM
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Figure 4. Amino acid alignment of putative Bt toxin binding regions (TBRs). Known *Manduca sexta* cadherin-like protein motifs TBR1 and TBR2 were used to identify similar domains in cadherin-like proteins from other lepidopteran species, e.g. *Bombyx mori*, *Heliothis virescens*, *Helicoverpa armigera*, *Lymantria dispar*, *Pectinophora gossypiella*, *Chilo suppressalis*, *Ostrinia nubilalis.* This alignment was then used to identify TBR motifs in *Diabrotica* cadherin-like proteins from western and Mexican corn rootworms cloned in this study.

Table 1. Genomic organization of the *cadherin-like* gene (GenBank accession EF541349). Exon sizes are shown. All intron–exon junctions followed the GT-AG rule for RNA splicing. The 5′ acceptor and the 3′ donor splice sites are shown. Exon sequences are shown in upper case. The size of the coding region is indicated in parentheses for the first and last exon

Exon		Intron/exon junction	
No.	Size (bp)	5′-Acceptor	3′-Donor
–1	160		ACATCAA**gt**aagtat
1	57 (55)	gtttttattttttctttt**ag**AAATGGCTACGAGAAATCTA	TTTGAGG**gt**aagtat
2	165	tgcctacatttactttttt**ag**GCATAGTTGGCGACGTCGCC	ACTGATT**gt**aagtaa
3	224	aactaactgaatatttttc**ag**GTCCTAACTTGAATGTTGAA	CTTTAAT**gt**aagtac
4	107	ttcattatttatatttttt**ag**GAACAATTAGACTATAGTCTT	CACTAAG**gt**tagaaa
5	138	attttaaattaatctttttt**ag**GATGAAGATATATTCGCAAT	AGCAAAT**gt**aagaaa
6	123	tttatttttttttatttttt**ag**GATTCTGGAGGTAATCTTTC	CGAACAA**gt**aagtta
7	82	ttcatttacgatttttttgc**ag**GATTACGATATACAAGCTTT	TTACCAG**gt**gagtaa
8	332	atcattttatatttttttt**ag**ACAATTACATAAACGTCAGT	AGATACG**gt**aagtag
9	180	acatttttctattaattttt**ag**GGCGAAAACGCGCAATTCAC	CTATTAT**gt**aagaaa
10	197	attgaaataaaatatttttt**ag**GTTGTTTCCAATGGAAAAAA	AACTGAA**gt**aagtat
11	211	tacatatatttttctttttt**ag**AATGAATATAGTTGGAACTT	AAATCAA**gt**aagaaa
12	179	gacgctgtcatttatttttt**ag**GTGGACCATATACAAATCGA	CTATGCA**gt**aagtct
13	182	tatatttatattatgtttt**ag**ATTTTTAGAGATAGAAAATA	ACAGAAG**gt**aagtaa
14	166	ttaatttttcatttttatt**ag**GTCAAATTGTCATCAATATC	ATTGCAC**gt**aagtat
15	160	tatgtagtagcttatttttt**ag**GCCCGAATATGAAAAGTTT	GTTAGAC**gt**aagtat
16	133	tctacatttattatatttt**ag**ATCTCAATCTATGTCATCGA	AGAGATG**gt**aagaac
17	171	actttttttttaatatttc**ag**AGCCGTTTCGTACTGTAAAC	CCTTTAA**gt**aagtgt
18	122	agctaaaatttaatttttc**ag**ATGTTCGATCCCAAGGACAA	GAATAAG**gt**atgtgc
19	237	acctagttatttttttttt**ag**GACGGCGAAGTAGGAGAGGG	TTTACAC**gt**aagttt
20	132	cagtatgactatttgttttt**ag**ATGCATGACGAAGGAGATCC	CCTATCG**gt**aagaaa
21	142	ataatattaaactatttttt**ag**GATCAACAACCTGGTCAACC	AATTATG**gt**aagttg
22	104	tgcgttgatttatatttttt**ag**AAGATGATAAAATTTTCGTG	GTATAAG**gt**aatatt
23	414	taatctttgctcaattttc**ag**CTAACTATTACAGCCAGTGT	TGTTAAT**gt**aagtac
24	102	aattattttgttgatttttt**ag**TTTCTTCCACGTAACCGCA	CGCTCAA**gt**aagtat
25	229	atatatatttttttacttcc**ag**GCTCATGACGACGATTATAT	GGCAATG**gt**aagaat
26	110	tttacgaatactgtatttt**ag**GTCCATTTGAAAGTGAAGCT	AACACCG**gt**atgtat
27	194	tataaaaactactttttcc**ag**ATGTTAAAAATAATATCCGA	TATTAAA**gt**tagtaa
28	201	ttttggcttaatatatttttc**ag**CATAGTGACCAATGTGAACA	CCAGACA**gt**aagtaa
29	180	aaacaaaaataaaaaatttc**ag**GTTAAGTAACAGAATTAAAA	CTCACAG**gt**aagttt
30	239 (94)	ttattttcgtaatatttttt**ag**CATAAGAAGCGGTGATTCTG	

2003). The sequence GCATATC at position +3 closely resembles a consensus initiator element (Inr) (YYANWYY, where Y; C or T, and W; A or T). No canonical TATA box is present immediate upstream of transcription start site although a highly AT-enriched sequence is present around the expected –40 bp region. This phenomenon is common in house-keeping genes and several cadherin genes lacking the TATA box have been previously reported (Behrens *et al.*,

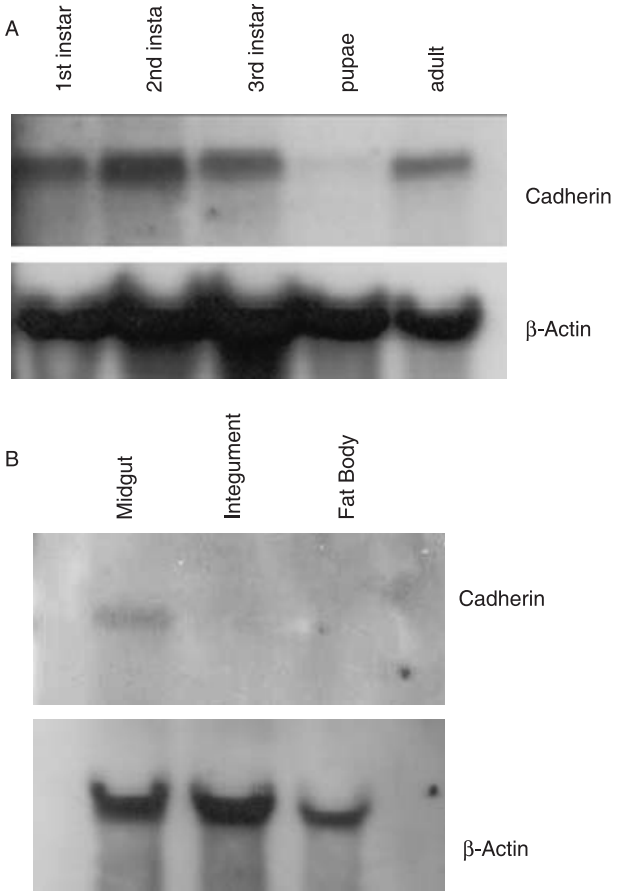

Figure 5. Northern blot analysis of *Diabrotica virgifera virgifera* RNA. Blots were probed with a DIG-labelled cadherin probe generated from the cadherin sequence and a *Diabrotica virgifera virgifera* actin for standardized loading. Total RNA (10 μg) was loaded in each lane. (A) RNA isolated from first, second, third instars, pupae and adults. (B) RNA isolated from midgut, integument and fat body.

Table 2. Similarities in the domain structure of lepidopteran (*Bombyx mori*) and coleopteran (*Diabrotica virgifera*) cadherin-like proteins. The location of cadherin repeats (CR) in multiple exons, exons encoding signal peptide (SIG) and transmembrane (TM) regions are indicated

Exon No.	*Bombyx mori**		*Diabrotica virgifera virgifera*	
	Position	Function	Position	Function
–1	1–83		1–160	
1	84–175	SIG	161–217	SIG
2	176–244		218–382	
3	245–403		383–606	
4	404–521		607–711	CR1
5	522–669		712–849	CR1
6	670–906	CR1	850–972	
7	907–1105	CR1, CR2	973–1054	CR2
8	1106–1395	CR2	1055–1386	CR2
9	1396–1569	CR3	1387–1566	CR3
10	1570–1763	CR3	1567–1763	CR3, CR4
11	1764–1881	CR4	1764–1974	CR4
12	1882–1983	CR4	1975–2153	CR5
13	1984–2153		2154–2335	CR5
14	2154–2332	CR5	2336–2501	CR5
15	2333–2507	CR5	2502–2661	CR6
16	2508–2709	CR6	2662–2794	CR6, TBR1
17	2710–2851	CR6, TBR1	2795–2965	CR7
18	2852–3043	CR7	2966–3077	CR7, CR8
19	3044–3165	CR7	3078–3324	CR8
20	3166–3384	CR8	3325–3456	
21	3385–3510	CR8	3457–3598	CR9
22	3511–3631	CR9	3599–3702	CR9
23	3632–3729	CR9	3703–4116	CR9, CR10, TBR2
24	3730–3862	CR9	4117–4218	CR10
25	3863–3967		4219–4447	
26	3968–4134	CR10	4448–4557	
27	4135–4233	CR10, TBR2	4558–4751	
28	4234–4426		4752–4952	TM
29	4427–4536		4953–5132	
30	4537–4694		5133–5371	
31	4695–4898	TM		
32	4899–5063			
33	5064–5166			
34	5167–5471			

**B. mori* data were extrapolated from the published report (Bel & Escriche, 2006).

1991; Faraldo & Cano 1993; Silos *et al.*, 1996; Whyte *et al.*, 1999).

Table 2 provides a comparative genomic overview of an exemplar lepidopteran *cadherin-like* gene (from the silkworm *Bombyx mori*) and the WCR *cadherin-like* gene described here. In contrast to the 31 exons and 30 introns we identified for WCR, lepidopteran *cadherin-like* genes are composed of 35 exons and 34 introns. Our genomic sequence information suggests that the *D. v. virgifera cadherin-like* gene spans over 90 kb, much larger than the reported sizes for lepidopteran *cadherin-like* genes (20–40 kb).

Discussion

Cadherins represent a large and diverse family of glycoproteins that are present in vertebrates and invertebrates and are essential for a number of cellular processes. These proteins are involved in mediating cell adhesion and play a fundamental part in normal development by participating in the maintenance of proper cell–cell contacts in vertebrates (Gumbiner, 2005) and invertebrates such as *Drosophila* (Godt & Tepass, 1998). At present, cadherin proteins identified from insect midguts have not been extensively studied and a function other than as receptors for Bt toxins has not been definitively assigned. In Lepidoptera, cadherins are not only specific receptors for the toxins, but also facilitate a post binding specific proteolytic cleavage step that induces toxin oligomerization and pore formation (Griffitts & Aroian, 2005).

Here, we have characterized the full cDNA sequence, the location of intron–exon boundaries and the 5′-untranslated region, including the promoter. To our knowledge, this is the first report of cloning of a *cadherin-like* gene from a Coleoptera. Despite relatively low sequence identity with lepidopetran receptors, the putative *D. virgifera* receptor

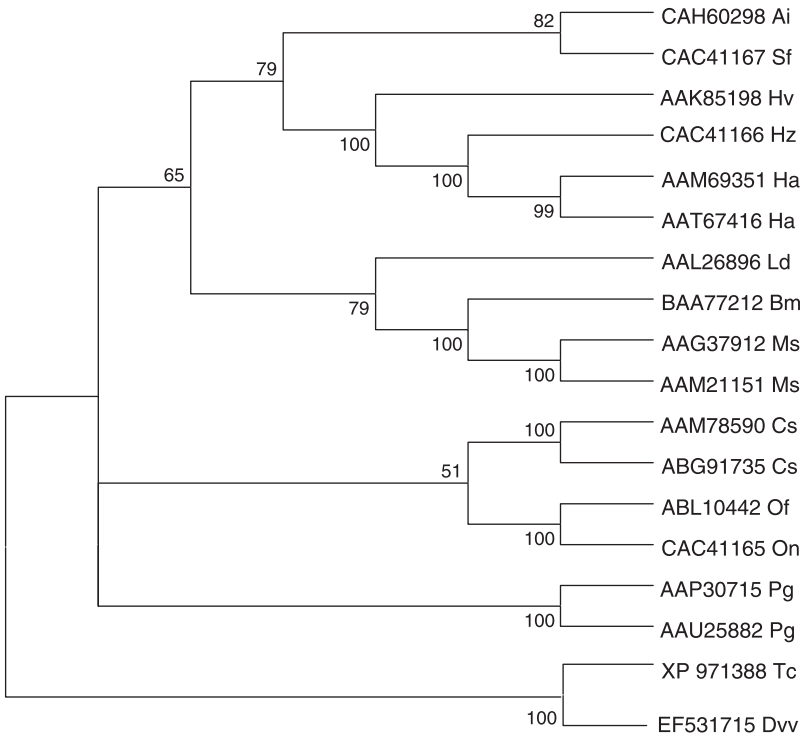

Figure 6. Unrooted neighbour-joining tree of aligned cDNA sequences for *Diabrotica virgifera virgifera* (Dvv) and Lepidoptera cadherin-like proteins identified as Cry1 receptors, along with a predicted cadherin sequence for *Triboleum castaneum*. Bootstrap support values for interior nodes are provided. The distance analysis was performed with MEGA version 3.1 (Kumar *et al.*, 2004). GenBank accession numbers are displayed within the tree. Pg, *P. gossypiella*; Ld, *L. dispar*; Ms, *M. sexta*; Bm, *B. mori*; Hv, *H. virescens*; Ha, *H. armigera*; Hz, *H. zea*; Dvv, *D. v. virgifera*; Ai, *A. ipsilon*; Sf, *S. frugiperda*; Cs, *C. suppressalis*; On, *O. nubilalis*; Of, *O. funarcalis;* Tc, *T. castaneum*.

shares striking similarities in protein architecture and genomic composition. Importantly, we have identified putative Bt-binding regions that are similar in amino acid sequence to those identified in lepidopteran cadherin-like proteins. As with other insects, expression of the protein is predominantly in midgut tissue. We observed expression of the gene during both larval and adult developmental stages. Because corn rootworms are susceptible to the Cry toxins only during larval stages (Nowatzki *et al.*, 2006), differences in the gut environment between adults and larvae may be responsible for larval specificity of the toxin. Finally, we found that the gene is remarkably well conserved between western and Mexican corn rootworms, including 100% amino acid homology within the putative Bt-binding regions.

Recently, a report was published by Bel & Escriche (2006) that discusses the genomic structure of *cadherin-like* genes in lepidopteran species. A priori this difference observed between Coleoptera and Lepidoptera *cadherin-like* genes may be expected considering the observed size heterogeneity even among the lepidopteran genes from *O. nubilalis*, *H. armigera* and *B. mori* (19.6 kb, 20.0 kb and 41.8 kb, respectively). Although intron size differences are large, the gene organization is very similar for these two orders of insects. For example, exon–intron organization, the number of exons, exon sizes, and the presence of non-coding first exons are all very similar. Homogeneity was also observed in the distribution of functional motifs such as amino terminal signal peptides (SIG), cadherin repeats (CR), predicted Bt toxin binding sites (TBR1 and TBR2) and carboxy terminal transmembrane coding regions (TM). Without exception, all of the cadherin repeats are encoded by multiple exons that span intron boundaries. Considering these structural similarities, it is highly likely that coleopteran and lepidopteran Bt-related *cadherin-like* genes share evolutionary conserved genomic and functional structure. Exploitation of this evolutionary conservation will be of great utility in interpreting molecular mechanisms of Bt toxicity and resistance in Coleoptera.

Experimental procedures

Full-length cDNA cloning

Total RNA was isolated from excised midguts of second instar *D. v. virgifera* using TRIzol Reagent (Invitrogen Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. Gene-specific primers (CLP-1: AATCGTCGTCATGAGCTTGAGCGGTGAC, and CLP-2: TCCACTGAGGAGCTCTGCGGTTACGTGG) were designed from a *D. v. virgifera* EST sequence (Siegfried *et al.*, 2005) and used in 5′-RACE with the SMART RACE kit (Clontech, Palo Alto, CA) according to the manufacturer's instructions. Two micrograms of total RNA was reverse transcribed with BD PowerScript Reverse Transcriptase at 42 °C for 1.5 h to synthesize first strand 5′-RACE-Ready cDNA in the presence of 5′ CDS primer and the SMART II oligo (Clontech). The 5′-RACE polymerase chain reaction (PCR) product was amplified from 2 μl of cDNA by PCR using CLP1 and the 5′-RACE adaptor primer (UPM). Two μl of the diluted (50-fold) PCR product was used in a nested PCR with CLP2 and the nested adaptor primer (NUP). These two amplifications were performed with 10 μM CLP1 (or CLP2) and UPM (or NUP), 10 mM

of each dNTP and 1 × Advantage cDNA Polymerase Mix (Clontech). PCR was performed with 35 cycles of 95 °C for 2 s, 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. The transcription start point was independently determined using the oligocapping method of RNA Ligase-Mediated Rapid Amplification of 5′ cDNA Ends (RLM-RACE) in the GeneRacer kit (Invitrogen). 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 primer 5′-TTCTCTTGTGCCAGCCATCCGTCGGGATC and the outer RNA adaptor primer in the kit. The RACE inner gene-specific primer 5′-TTGTGCCAGCCATCCGTCGGGATCATGCA and the inner RNA adaptor primer in the kit were used in the final PCR. PCR conditions were as follows: five cycles of 95 °C for 2 s, 72 °C for 1 min; five cycles of 95 °C for 2 s, 70 °C for 1 min: and 25 cycles of 95 °C for 2 s, 68 °C for 1 min. Amplified fragments were cloned in TOPO vector for sequencing.

Expression of cadherin-like *protein in insect cell culture*

Two micrograms total RNA that was isolated from WCR midgut tissue was subjected to reverse transcription with 18-mer oligo-dT for 1 h at 55 °C in a 20 μl reaction using Superscript III Reverse Transcriptase (Invitrogen) according to the manufacturer's protocol. The full-length *cadherin-like* mRNA was PCR amplified with forward (5′-GTAAATGATGTGGATTGTTTGATACCCT) and reverse (5′-ATCTATTAAGACCAACTTAAGCTAGACATAAG) primers using Advantage 2 PCR Polymerase Mix (Clontech). The purified DNA was polished with *Pfu* polymerase (Stratagene, La Jolla, CA) and directionally cloned into the Invitrogen's Gateway Entry vector pENTR/D-TOPO. The entire cloned fragment was sequenced to confirm the integrity of the cDNA sequence before transferring into the Gateway Destination vector pDEST8 by *in-vitro* recombination using Clonase II (Invitrogen). The pDEST8-*cadherin-like* cDNA construct was then used to transform *Escherichia coli* Bac10 hosts for the generation of recombinant baculovirus constructs to be used for transfection, infection and expression in Sf21 cells (Gateway Baculovirus Expression System, Invitrogen). For expression analysis, aliquots of cells were pelleted and lysed in 1 × SDS sample buffer and separated on 4–10% Tricine SDS–PAGE gels (Invitrogen). Proteins on the gel were transferred to polyvinylidene difluoride membrane and stained with an affinity-purified polyclonal antibody (diluted 1 : 5000) that was raised in rabbit against the carboxy terminal 22 amino acid peptide (GIENNPEFDYNFNTNEDKTTYL). Blots were washed and developed with the WesternBreeze kit for anti-rabbit antibody (Invitrogen) according to the supplied protocol.

Amplification of Mexican corn rootworm cadherin-like *cDNA*

The MCR total RNA was isolated from adult gut tissues using Trizol-Plus (Invitrogen). Five micrograms of RNA was reverse transcribed in a 100 μl reaction using SMART cDNA Amplification Kit (Clontech) according to the product insert. One microlitre of the reaction was used as a template for a 50 μl PCR that included forward (5′-TACTTGTTGGGCTCATGATGGTGTG) and reverse primers (5′-AATCGTCGTCATGAGCTTGAGCGGTGAC) designed from the WCR *cadherin-like* cDNA sequence, 10 mM each dNTP and 1 × Advantage cDNA Polymerase Mix (Clontech). PCR cycling parameters were: 94 °C for 1 min, followed by 40 cycles of 94 °C for 30 s, 60 °C for 30 s, and 68 °C for 3 min, with a final incubation for 10 min at 72 °C. The PCR product was cloned in a TOPO vector and sequenced.

Northern analysis

Standard rearing techniques (Jackson, 1986) were used to obtain 50–100 mg of first, second, and third instars, pupae and adult WCR which were frozen at –80 °C until needed. Fresh total RNA was obtained as described above and used for Northern blotting analysis. A DIG-labelled cDNA probe was designed from the *cadherin-like* sequence to produce a 722 bp fragment using the forward primer 5′-ACACACCATGCAATACTAC-3′ and reverse primer 5′-GTGCTAAAATTATACGATCTCG-3′. The specificity of the *Diabrotica cadherin-like* cDNA probe was verified by sequencing. The DIG-labelled actin control probe was amplified from a *D. v. virgifera* cDNA mix using the forward primer: 5′-TCAGGGTGTGATGGTAGG-3′ and reverse primer: 5′-CTCTTTCTGCTGTGGTGGTG-3′ to generate a 560 bp fragment. The probes were generated by DIG PCR labelling using the DIG PCR labelling kit (Roche Applied Science, Indianapolis, IN, USA). Samples of total RNA (7.5 μg) were separated by denaturing electrophoresis on a 1.2% agarose/formaldehyde gel. The RNAs were transferred overnight on to positively charged Nylon membranes (Roche Applied Science) by capillary blotting using DEPC-treated 20 × SSC transfer buffer (3.0 M NaCl, 0.3 M sodium citrate, pH 7.0) and irreversibly fixed in a baking oven at 80°C for 2 h. The blot was prehybridized for 1 h at 50°C in 5 ml of hybridization solution (DIG Easy hyb, Roche Applied Science). After adding the denatured probe to a fresh hybridization solution, the membrane was hybridized overnight at 50°C with continuous shaking. The blot was rinsed three times in 2 × SSC, 0.1% SDS at room temperature and three times with 0.5 × SSC, 0.1% SDS at 68°C to eliminate nonspecific hybridizations. The membrane was washed briefly in washing buffer (maleate buffer, 0.3% Tween-20), blocked for 1 h in 0.5 × blocking buffer (Roche Applied Science) and then incubated with anti-DIG at 1 : 15 000 dilution in blocking buffer for 45 min. The membrane was washed four times (15 min each) with washing buffer, briefly washed with detection buffer (Roche Applied Science) for 5 min, and incubated with the chemiluminescent substrate for alkaline phosphatase (CSPD, Tropix Inc., Bedford, MA, USA) for 5 min. The blot was exposed to chemiluminescence Biomax film (Kodak, Rochester, NY) at room temperature for 25 min. The membrane was stripped and reprobed with *D. v. virgifera* DIG-labelled actin probe (560 bp) to standardize loadings.

Genome walking

The cDNA sequence information was used as a starting point to characterize the genomic structure of the *cadherin-like* gene. Exon–intron boundaries were determined by cloning and sequencing of PCR amplified products using exon-specific primers. RNA splicing junctions were defined by comparing genomic and corresponding cDNA sequences. For this, four genomic libraries were generated from adult WCR genomic DNA that had been cut with different restriction enzymes (*Eco*RV, *Pvu*II, *Stu*I and *Dra*I) and ligated to an adaptor oligo using the Genome Walking kit (Clontech). These ligated DNA pools were then used as templates for two rounds of PCR. Primary and nested PCRs were performed using kit primers complementary to the adaptor sequences and gene-specific sense and anti-sense primers that were designed based on the cloned cDNA sequence. Each PCR mixture contained 2.5 μM of

dNTPs, 1 × Advantage buffer and 1.25 units of Advantage Polymerase Mix (Clontech) in a 50 μl reaction. For the nested PCRs, 1 μl of 50-fold diluted primary PCR product was used as a template. Amplicons derived from each set of primers were directly sequenced or subcloned into the TOPO vector and then sequenced.

Sequence analysis

DNA sequencing was performed with a 3730XL Genetic Analyzer (Applied Biosystems). Sequence editing was done with Sequencher software (Gene Codes, Ann Arbor, MI). Sequence searches, alignments and other sequence analyses were performed using NCBI BLAST programs. Putative transcription factor sites were identified by the MatInspector program (http://www.genomatix.de). The ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) was used for deducing polypeptide sequences. Protein domains were predicted using the Simple Modular Architecture Research Tool (SMART) (http://smart.embl-heidelberg.de) and MotifScan (http://myhits.isb-sib.ch/cgi-bin/motif_scan). Phylogenetic analyses were performed with AlignX (Vector NTI-Invitrogen Life Technology).

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References

Andrews, G.L. and Mastik, G.S. (2003). R-cadherin is a pax6-regulated, growth-promoting cue for pioneer axons. *J Neurosci* **23**: 9873–9880.

Behrens, J., Lowrick, O., Klein-Hitpass, L. and Birchmeier, W. (1991) The E-cadherin promoter: functional analysis of a G.C.-rich region and an epithelial cell-specific palindromic regulatory element. *Proc Natl Acad Sci USA* **88**: 11495–11499.

Bel, Y. and Escriche, B. (2006) Common genomic structure for the Lepidopteran cadherin-like genes. *Gene* **381**: 71–80.

Bowler, T., Kosman, D., Licht, J.D. and Pick, L. (2006). Computational identification of Ftz/Ftz-F1 downstream target genes. *Dev Biol* **299**: 78–90.

Bravo, A., Gomez, I., Conde, J., Munoz-Garay, C., Sanchez J, Miranda, R., *et al*. (2004) Oligomerization triggers binding of a *Bacillus thuringiensis* Cry1Ab pore-forming toxin to aminopeptidase N receptor leading to insertion into membrane microdomains. *Biochim Biophys Acta* **1667**: 38–46.

Dorsch, J.A., Candas, M., Griko, N.B., Maaty, W.S.A, Midboe, E.G., Vadlamudi, R.K., *et al*. (2002) Cry1A toxins of Bacillus thurigiensis bind specifically to a region adjacent to the membrane-proximal extracellular domain of BT-R1 in Manduca sexta: involvement of a cadherin in the entomopathogenicity of Bacillus thurigiensis. *Insect Biochem Mol Biol* **32**: 1025–1036.

Ellis, R.T., Stockhoff, B.A., Stamp, L., Schnepf, H.E., Schwab, G.E., Knuth, M., *et al*. (2002) Novel *Bacillus thuringiensis* binary insecticidal crystal proteins active on western corn rootworm, *Diabrotica virgifera virgifera* LeConte. *Appl Environ Microbiol* **68**: 1137–1145.

Environmental Protection Agency (2003) *Bacillus thuringiencis* Cry3Bb1 Protein and the genetic material necessary for its production (cector ZMIR13L) in event MON863 Corn Fact Sheet. EPA Publication Number 730-F-03-01.

Faraldo, M.L. and Cano, A. (1993) The 5′ flanking sequences of the mouse P-cadherin gene. Homologies to 5′ sequences of the E-cadherin gene and identification of a first 215 base-pair intron. *J Mol Biol* **231**: 935–941.

Gahan, L.J., Gould, F. and Heckel, D.G. (2001) Identification of a gene associated with Bt resistance in *Heliothis virescens*. *Science* **293**: 857–860.

Godt, D. and Tepass, U. (1998) *Drosophila* oocyte localization is mediated by differential cadherin-based adhesion. *Nature* **395**: 387–391.

Gómez, I., Miranda-Rios, J., Ridiño-Piñera, E., Oltean, D.I., Gill, S.S., Bravo, A., *et al*. (2002a) Hydropathic complementarity determines interaction of epitope 869HITDTNNK876 in *Manduca sexta* Bt-R_1 receptor with loop 2 of domain II of *Bacillus thuringiensis* Cry1A toxins. *J Biol Chem* **277**: 3037–30143.

Gómez, I., Sánchez, J., Miranda, R., Bravo, A. and Soberón, M. (2002b) Cadherin-like receptor binding facilitates proteolytic cleavage of helix α-1 in domain I and oligomer pre-pore formation of *Bacillus thuringiensis* Cry1Ab toxin. *FEBS Lett* **513**: 242–246.

Gómez, I., Dean, D.H., Bravo, A. and Soberón, M. (2003) Molecular basis for *Bacillus thuringiensis* Cry1Ab toxin specifity: two structural determinants in the *Manduca sexta* Bt-R_1 receptor interact with loops α-8 and 2 in domain II of Cry1Ab toxin. *Biochemistry* **42**: 10482–10489.

Griffitts, J.S. and Aroian, R.V. (2005) Many roads to resistance: how invertebrates adapt to Bt toxins. *BioEssays* **27**: 614–624.

Gumbiner, B.M. (2005) Regulation of cadherin-mediated adhesion in morphogenesis. *Nat Rev Cell Mol Biol* **6**: 622–634.

Hernstadt, C., Soares, G.C., Wilcox, E.R. and Edwards, D.L. (1986) A new strain of *Bacillus thuringiensis* with activity against coleopteran insects. *Bio/Technology* **4**: 305–308.

Hernstadt, C., Gilroy, T.E., Solesk, D.A., Bennet, B.D. and Gautner, F.H. (1987) Nucleotide sequence and deduced amino acid sequence of a coleopteran active delta-endotoxin gene from *Bacillus thuringiensis* subp. *san diego*. *Gene* **57**: 81–116.

Jackson, J.J. (1986) Rearing and handling of *Diabrotica virgifera* and *Diabrotica undecimpunctata howardi*. In *Methods for the Study of Pest Diabrotica* (Krysan, J.L. and Miller, T.A., eds), pp. 25–47. Springer, New York.

Krieg, A., Huger, A.M., Langenbruch, G.A. and Schenetter, W. (1983) *Bacillus thuringiensis* var. *tenebrionis*: ein neuer, gegenüber Laven von Coleopteren wirksamer Pathotype, Z. Angew. *Entomology* **96**: 500–508.

Kumar, S., Tamura, K. and Nei, M. (2004) MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. *Brief Bioinform* **5**: 150–163.

Levine, E. and Oloumi-Sadeghi, H. (1991) Management of diabroticite rootworms in corn. *Annu Rev Entomol* **36**: 229–255.

Levine, E., Spencer, J.L., Isard, S.A., Onstad, D.W. and Gray, M.E. (2002) Adaptation of the western corn rootworm to crop rotation: evolution of a new strain in response to a management practice. *Am Entomol* **48**: 94–107.

Liang, Z. and Biggin, M.D. (1998). Eve and ftz regulate a wide array of genes in blastoderm embryos: the selector homeoproteins directly or indirectly regulate most genes in *Drosophila. Development* **125**: 4471–4482.

Meinke, L.J., Siegfried, D., Wright, R.J. and Chandler, L.D. (1998) Adult susceptibility of Nebraska western corn rootworm (Coleoptera: Chrysomelidae) populations to selected insecticides. *J Econ Entomol* **91**: 594–600.

Moellenbeck, D.J., Peters, M.L., Bing, J.W., Rouse, J.R., Higgins, L.S., *et al.* (2001)Insecticidal proteins from Bacillus thuringiensis protect corn from corn rootworms. *Nat Biotechnol* **19**: 668–672.

Morin, S., Biggs, R.W., Sisterson, M.S., Shriver, L., Ellers-Kirk, C., *et al.* (2003)Three cadherin alleles associated with resistance to *Bacillus thuringiensis* in pink bollworm. *Proc Natl Acad Sci USA* **100**: 5004–5009.

Nagamatsu, Y., Toda, S., Yamaguchi, F., Ogo, M., Kogure, M., Nakamura, M., *et al.* (1998a) Identification of Bombyx mori midgut receptor for Bacillus thuringiensis insecticidal CryIA(a) toxin. *Biosci Biotechnol Biochem* **62**: 718–726.

Nagamatsu, Y., Toda, S., Koike, T., Miyoshi, Y., Shigematsu, S. and Kogure, M. (1998b) Cloning, sequencing, and expression of the Bombyx mori receptor for Bacillus thuringiensis insecticidal CryIA(a) toxin.cell-specific activity. *Biosci Biotechnol Biochem* **62**: 727–734.

Nagamatsu, Y., Koike, T., Sasaki, K., Yoshimoto, A. and Furukawa, Y. (1999) The cadherin-like protein is essential to specificity determination and cytotoxic action of the Bacillus *thuringiensis* insecticidal CryIAa toxin. *FEBS Lett* **460**: 385–390.

Nowatzki, T.M., Zhou, X., Meinke, L.J., Vaughn, T. and Siegfried, B.D. (2006) Effect of Bacillus thuringiensis cry3Bb1 protein on the feeding behavior and longevity of adult western corn rootworms (Coleoptera: Chrysomelidae). *J Econ Entomol* **99**: 927–930.

Parimi, S., Scharf, M.E., Meinke, L.J., Chandler, L.D. and Siegfried, B.D. (2003) Inheritance of methyl-parathion resistance in Nebraska western corn rootworm populations (Coleoptera: Chrysomelidae). *J Econ Entomol* **96**: 131–136.

Rausell, C., Garcia-Robles, I., Sanchez, J., Munoz-Garay, C., Martinez-Ramirez, A.C., Real, M.D., *et al.* (2004) Role of toxin activation on binding and pore formation activity of the *Bacillus thuringiensis* Cry3 toxins in membranes of *Leptinotarsa decemlineata* (Say). *Biochim Biophys Acta* **1660**: 99–105.

Schnepf, E., Crickmore, N., Van Rie, J., Lereclus, D., Baum, J., Feitelson, J., *et al.* (1998) *Bacillus thuringiensis* and its pesticidal crystal proteins. *Microbiol Mol Biol Rev* **62**: 775–806.

Sick, A., Gaertner, F. and Wong, A. (1990) Nucleotide sequence of a coleopteran-active toxin gene from a new isolate of *Bacillus thurigiensis* subp. tolworthi. *Nucleic Acids Res* **18**: 1305.

Siegfried, B.D., Meinke, L.J., Parimi, S., Scharf, M.E., Nowatzki, T.J., Zhou, X., *et al.* (2004) Monitoring western corn rootworm (Coleoptera: Chrysomelidae) susceptibility to carbaryl and cucurbitacin baits in the areawide management pilot program. *J Econ Entomol* **97**: 1726–1733.

Siegfried, B.D., Waterfield, N. and ffrench-Constant, R.H. (2005) Expressed sequence tags from *Diabrotica virgifera virgifera* midgut identify a coleopteran cadherin and a diversity of cathepsins. *Insect Mol Biol* **14**: 137–143.

Silos, S.A., Tamai, K., Li, K., Kivirikko, S., Kouba, D., Christiano, A.M., *et al.* (1996) Cloning of the gene for human pemphigus vulgaris antigen (desmoglein 3), a desmosomal cadherin. Characterization of the promoter region and identification of a keratinocyte-specific cis-element. *J Biol Chem* **271**: 17504–17511.

Tsuda, Y., Nakatani, F., Hashimoto, K., Ikawa, S., Matsuura, C., Fukada, T., *et al.* (2003) Cytotoxic activity of *Bacillus thuringiensis* Cry proteins on mammalian cells transfected with cadherin-like Cry receptor gene of *Bombyx mori* (silkworm). *Biochem J* **369**: 697–703.

Vadlamudi, R.K., Ji, T.H. and Bulla, L.A. Jr (1993). A specific binding protein from *Manduca sexta* for the insecticidal toxin of *Bacillus thuringiensis* subsp. berliner. *J Biol Chem* **268**: 12334–12340.

Vadlamudi, R.K., Weber, E., Ji, I., Ji, T.H. and Bulla, L.A. Jr (1995) Cloning and expression of a receptor for an insecticidal toxin of *Bacillus thuringiensis. J Biol Chem* **270**: 5490–5494.

Whyte, D.A., Li, C., Thomson, R.B., Nix, S.L., Zanjani, R., Karp, S.L., *et al.* (1999) Ksp-cadherin gene promoter. I. Characterization and renal epithelial cell-specific activity. *Am J Physiol* **277**: F587–F598.

