The midgut cadherin-like gene is not associated with resistance to *Bacillus thuringiensis* toxin Cry1Ac in *Plutella xylostella* (L.)

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**Abstract**

The Gram-positive bacterium *Bacillus thuringiensis* (Bt) produces Cry toxins that have been used to control important agricultural pests. Evolution of resistance in target pests threatens the effectiveness of these toxins when used either in sprayed biopesticides or in Bt transgenic crops. Although alterations of the midgut cadherin-like receptor can lead to Bt Cry toxin resistance in many insects, whether the cadherin gene is involved in Cry1Ac resistance of *Plutella xylostella* (L.) remains unclear. Here, we present experimental evidence that resistance to Cry1Ac or Bt var. *kurstaki* (Btk) in *P. xylostella* is not due to alterations of the cadherin gene. The bona fide *P. xylostella* cadherin cDNA sequence was cloned and analyzed, and comparisons of the cadherin cDNA sequence among susceptible and resistant *P. xylostella* strains confirmed that Cry1Ac resistance was independent of mutations in this gene. In addition, real-time quantitative PCR (qPCR) indicated that cadherin transcript levels did not significantly differ among susceptible and resistant *P. xylostella* strains. RNA interference (RNAi)-mediated suppression of cadherin gene expression did not affect larval susceptibility to Cry1Ac toxin. Furthermore, genetic linkage assays using four cadherin gDNA allelic biomarkers confirmed that the cadherin gene is not linked to resistance against Cry1Ac in *P. xylostella*. Taken together, our findings demonstrate that Cry1Ac resistance of *P. xylostella* is independent of the cadherin gene.

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**Keywords:** Bacillus thuringiensis, *Plutella xylostella*, Cadherin-like gene, Cry toxin receptor, Cry1Ac resistance

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proteins with one or more EC/CR-like domains (domains defined as being non-consecutive or lacking the conserved calcium-binding motifs of protocadherin) are designated as cadherin-like proteins (Hulpiau and van Roy, 2009). Despite their diversity and unique functions, the cadherin-like genes received little attention from insect toxicologists until the first cadherin-like gene was identified to be a midgut functional receptor of Bt Cry1A toxin and was subsequently cloned in Manduca sexta (Vadlamudi et al., 1993, 1995). Since then, midgut cadherin-like genes involved in insect Bt resistance have been cloned in diverse insects from the Lepidoptera, Coleoptera, and Diptera. These cadherin-like proteins share common structural characteristics including an extracellular domain (generally containing 9–12 cadherin repeats), a membrane-proximal extracellular domain (MPD), a transmembrane domain (TMD), and a cytoplasmic domain (CPD) (Bel and Escriche, 2006; Pigott and Ellar, 2007). To date, insect midgut cadherins have been reported to interact with Bt Cry toxins in at least eight lepidopteran insects, two dipteran insects, and four coleopteran insects (Bravo et al., 2011; Yang et al., 2011; Contreras et al., 2013; Park and Kim, 2013; Ren et al., 2013; Hua et al., 2014).

The diamondback moth, Plutella xylostella (L.), is notorious for its ability to develop resistance to many insecticides, and the total management cost for this pest controlling is estimated to be US $4–5 billion annually (Furlong et al., 2013). Moreover, its ability to develop resistance to many insecticides, and the total management cost for this pest controlling is estimated to be US $4–5 billion annually (Furlong et al., 2013). In 2013; Park and Kim, 2013; Ren et al., 2013; Hua et al., 2014). The DBM1Ac-S strain was kept unselected while the DBM1Ac-R and SZ-R strains have been kept under constant selection with a Cry1Ac toxin solution, which regularly kills 50–70% of the larvae on sprayed cabbage leaves. The near-isogenic NIL-R strain was constructed when this study carried out, and it was generated by multiple (six times) backcrossing between DBM1Ac-S and DBM1Ac-R and with selection of offspring as for DBM1Ac-R (Zhu et al., 2014). At the time of this study, the resistance ratios (resistant larvae LC50 value divided by susceptible larvae LC50 value) of DBM1Ac-R (LC50 = 3.052.33 μg/ml), NIL-R (LC50 = 3.401.51 μg/ml), and SZ-R (LC50 = 563.06 μg/ml) to Cry1Ac protoxin were about 3500-, 4000-, and 450-fold compared to the DBM1Ac-S (LC50 = 0.86 μg/ml), respectively, and the resistance ratio of SH-R (LC50 = 1.323.18 μg/ml) to the Btk formulation was about 1900-fold compared to the DBM1Ac-S (LC50 = 0.70 μg/ml). All strains were reared on JingFeng No. 1 cabbage (Brassica oleracea var. capitata) without exposure to any Bt toxins or chemical pesticides at 25 °C, 65% RH, and a 16D:8L photoperiod. Adults were fed with a 10% sucrose solution.

2.2. Cry1Ac toxin preparation and bioassays

Cry1Ac protoxin was extracted and purified from Bt kurstaki strain HD-73 as previously described (Perera et al., 2009). Both purified Cry1Ac protoxin and trypsin-activated toxin were quantified by densitometry as described elsewhere (Crespo et al., 2008). The purified Cry1Ac toxin was solubilized in 50 mM Na2CO3 (pH 9.6) and stored at −20 °C until used.

The toxicity of the Cry1Ac toxin or Btk formulation was determined with 72-h bioassays using larvae from five strains of P. xylostella and a leaf-dip method as described elsewhere (Yang et al., 2009). Ten third-instar P. xylostella larvae were tested for each of seven toxin concentrations, and bioassays were replicated four times. Mortality data were corrected using Abbott's formula (Abbott, 1925), and experiments in which control mortality exceeded 10% were discarded and repeated. The LC50 values were calculated by Probit analysis (Finney, 1971).

2.3. CDNA synthesis and gDNA isolation

Fourth-instar larvae from different P. xylostella strains were anesthetized on ice for about 15 min before the midgut tissues were dissected in sterile DEPC (diethyl pyrocarbonate)-treated distilled water containing 0.7% NaCl. Total RNA was extracted from a pool of dissected midguts from fourth-instar larvae for each P. xylostella strain using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The integrity of the RNA was determined using 1% TBE agarose gel electrophoresis, and the RNA was then quantified with a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). For gene cloning, the first-strand cDNA was prepared using 5 μg of total RNA and the PrimeScript™ II 1st strand CDNA Synthesis Kit (TaKaRa, Dalian, China) following manufacturer's recommendations. For qPCR analysis, the first-strand cDNA was prepared using 1 μg of total RNA with the PrimeScript RT kit (containing gDNA Eraser, Perfect Real Time) (TaKaRa, Dalian, China) following the manufacturer's instructions. The synthesized first-strand cDNA was immediately stored at −20 °C until used.

Genomic DNA (gDNA) was prepared from fourth-instar larvae of DBM1Ac-S and NIL-R strains using a TIANamp Genomic DNA Kit (TIANGEN, Beijing, China) with the following slight modification in the pretreatment of samples: the individuals were first well homogenized with an electric pestle in 1.5-ml centrifuge tubes before gDNA was extracted according to the manufacturer’s instructions.
2.4. Gene cloning and sequencing

The cadherin cDNA was PCR amplified using the two strategies described elsewhere (Bel et al., 2009). Briefly, for the analysis of length polymorphisms (in 2% agarose gels) and based on our corrected sequence (GenBank: KM370099), cadherin cDNA was first amplified by the designed full-length primer pairs with five overlapping fragments (Table 1). In addition, the occurrence of large inversions or deletions was tested in a second amplification strategy by amplifying the whole cadherin cDNA with a specific full-length primer pair, followed by nested PCR amplifications using the same full-length primer pairs with five overlapping fragments as described above.

The PCR reactions (25 µl total volume) contained 18.5 µl of double-distilled H₂O (ddH₂O), 2.5 µl of 10 × LA Taq Buffer, 2 µl of dNTP Mix, 4 µM of each specific primer, 1 ng of first-strand cDNA template, and 0.2 µl LA Taq HS polymerase (TaKaRa, Dalian, China). Reactions were then performed in an S1000™ Thermal Cycler (BioRad, USA) with the following parameters: one cycle of 94°C for 6 min; followed by 35 cycles of 94°C for 30 s, 50°C for 45 s, and 72°C for 5 min; and a final cycle of 72°C for 15 min. The nested PCR parameters were as follows: one cycle of 94°C for 6 min; followed by 35 cycles of 94°C for 30 s, 55°C for 45 s, and 72°C for 1.5 min; and a final cycle of 72°C for 10 min.

Isolated gDNA from untreated larvae and larvae that survived exposure to Cry1Ac was used to genotype the cadherin gene by PCR amplification as described elsewhere (Zhang et al., 2012b). Briefly, four genomic cadherin cDNA fragments harboring a single intron were designed based on the corrected full-length cadherin gDNA sequence (Diamondback moth Genome Database, Gene ID: Px012847). These genotyping primers and their corresponding covering regions and PCR product sizes are listed in Table 1. The PCR amplification reactions (25 µl) contained 2.5 µl 10 × LA Taq Buffer, 2 µl of dNTPs, 4 µM of each primer, 1 µl of genomic DNA template, and 0.2 µl LA Taq HS polymerase (TaKaRa, Dalian, China). Reactions were performed for 35 cycles of 6 min at 94°C, 30 s at 58°C, and 45 s at 72°C, followed by a final extension at 72°C for 10 min. The PCR amplified fragments were then sequenced to determine the cadherin genotypes.

All of the cloning primers for each gene were designed with Primer Premier 5.0 software (Premier Biosoft, Canada). Amplicons of the expected size were excised from 1.5% to 2.5% agarose gels, purified using the Gel Mini Purification Kit (Generay, Shanghai, China), and subcloned into the pEASY-T1 (Transgen, Beijing, China) or pMD18-T vectors (TaKaRa, Dalian, China) before transformation into Escherichia coli TOP10 competent cells (Transgen, Beijing, China) for sequencing.

2.5. In silico gene sequence analysis

Gene sequences were aligned with DNAMAN 7.0 (Lynnon BioSoft, USA). The nucleotide sequence-similarity analyses were performed with the BLAST tool at the NCBI website (http://blast.ncbi.nlm.nih.gov/). The deduced protein sequence, calculated isolectric point (pI) and molecular weight (Mw) were obtained with the ExPaSy translate tool Translate (http://web.expasy.org/translate/) and the ExPaSy proteomics tool Compute pI/Mw (http://ca.expasy.org/tools/pi_tool.html) from the Swiss Institute of Bioinformatics, respectively. The N-terminal signal peptide

Table 1

<table>
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<tr>
<th>Purpose</th>
<th>Primer name</th>
<th>Primer sequence (5’-3’)</th>
<th>PCR product size (bp)</th>
<th>Positions (bp)</th>
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<tr>
<td></td>
<td>Cad-F3</td>
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<td>1045</td>
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<td></td>
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<td>40–5227</td>
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<td></td>
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<td>40–5227</td>
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<td>309–476</td>
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<td>qCad-R</td>
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<td>4146–4579</td>
</tr>
<tr>
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<td>TCCGATCTGACGCTCTCTCTCTT</td>
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</table>

² The forward and reverse primers used to synthesize dsRNA template have a T7 RNA polymerase promoter sequence (5’-TAATACGACTCACTATAGGGAGA-3’) appended to their 5’ and 3’ ends.

² The second PCR product size is the size of the corresponding cadherin gene allele of each gDNA fragment.

Positions corresponding to the corrected full-length cDNA sequence (GenBank: KM370099) of the P. xylostella cadherin gene.
2.6. Phylogenetic tree construction

Cadherin sequences with complete open reading frames (ORFs) were statistically significant. 323 were used to determine whether differences between treatments. VAs with Holm–Sidak’s tests (overall significance level = 0.05). Four technical replicates and three biological replicates were used for each treatment. One-way ANOVA was determined using the SignalP 4.0 server (http://www.cbs.dtu. dk/services/SignalP/). The transmembrane region and membrane topology was analyzed with Phobius software (http://phobius.sbc.su.se). The protein-specific motif was searched and analyzed using Myhits software (http://myhits.isb-sib.ch/cgi-bin/motif_scan) and Prosite software (http://www.expasy.ch/prosite/). The presence of N- and O-glycosylation sites on the predicted protein sequence was determined using the NetNGlyc 1.0 and NetO Glyc 4.0 server (http://www.cbs.dtu.dk/services/NetNGlyc/ and http://www.cbs.dtu.dk/services/NetO Glyc/), respectively.

2.7. qPCR analysis

The expression pattern of cadherin was analyzed by qPCR using midgut tissues of fourth-instar larvae from all of the Cry1Ac-resistant strains. A specific primer set of the cadherin gene was designed in the CDNA region without alternative splicing and was used in PCR reactions (25 μl), which contained 9.5 μl of ddH₂O, 12.5 μl of 2 × SuperReal Premix Plus (TIANGEN), 7.5 μM of each specific primer, 11 μl of first-strand CDNA template, and 0.5 μl 50 × ROX Reference Dye (TIANGEN). PCR reactions were performed using an initial denaturation for 15 min at 95 °C followed by 40 cycles of denaturation at 95 °C for 15 s, annealing for 30 s at 50 °C, and extension for 32 s at 72 °C. Melting curve analysis, an automatic dissociation step cycle was added. Reactions were performed in an ABI 7500 Real-Time PCR system (Applied Biosystems, USA) with data collection at stage 2, step 3 in each cycle of the PCR reaction. Amplification efficiencies and linear correlation between CDNA template and the quantities of PCR product generated by the gene-specific primers were calculated from the disso- ciation curve of quadruplicate replicates using five 2-fold serial dilutions (1:1, 1:2, 1:4, 1:8, and 1:16). Only results with single peaks in melting curve analyses, 95–100% primer amplification efficiencies, and >95% correlation coefficients were used for subsequent data analysis. Negative control reactions included ddH₂O instead of CDNA template, which resulted in no amplified products (data not shown). The amplified fragments were sequenced to confirm that potential expression differences were not due to sequence mutations in the targeted genes. Relative quantification was performed using the 2^−ΔΔCt method (Livak and Schmittgen, 2001), and the expression level was normalized to the ribosomal L32 gene (GenBank: AB180441) as validated elsewhere (Bautista et al., 2008; You et al., 2013). Four technical replicates and three biological replicates were used for each treatment. One-way ANOVA with Holm–Sidak’s tests (overall significance level = 0.05) were used to determine whether differences between treatments were statistically significant.

2.8. dsRNA synthesis and RNAi assays

Cadherin gene expression was silenced by injection of dsRNA into early third-instar P. xylostella larvae. Specific primers containing a T7 promoter sequence at the 5′ end to generate dsRNA targeting the corrected sequences of cadherin (GenBank: KM377009) or EGFP (GenBank: KC896843) were designed (Table 1) using the Primer Premier 5.0 software (Premier Biosoftware, Canada). After amplification,primer PCR products (480 bp for dsCad and 469 bp for dsEGFP) were used as template for in vitro transcription reactions to generate dsRNAs using the T7 Ribomax™ Express RNAi System (Promega, Madison, WI, USA). After the synthesized dsRNAs were suspended in injection buffer [10 mM Tris–HCl (pH 7.0); 1 mM EDTA], the preparation was subjected to 1% agarose gel electrophoresis, and the dsRNA was quantified spectrophotometrically before it was microinjected into larvae. To avoid the detection of transcriptome changes resulting from exposure to the toxin, none of the larvae were exposed to Cry1Ac toxin before dsRNA microinjection. The detection of silencing effect and the quantities of dsRNA injected were optimized in preliminary experiments of our previous study with the cadherin gene (Yang et al., 2009).

The early third-instar larvae were microinjected with dsRNA with the aid of an SZX10 microscope (Olympus, Tokyo, Japan). The Nanoliter 2000 microinjection system (World Precision Instruments Inc., Sarasota, FL, USA) with sterilized fine glass capillary microinjection needles pulled by P-97 micropipette puller (Sutter Instrument, Novato, CA, USA) was used to deliver 70 nanoliters of injection buffer or dsRNAs (300 ng) into the hemocoel of early third-instar DBM1Ac-S P. xylostella larvae. The volume of sample microinjected into each larva was determined to result in <20% larval mortality 5 days post-injection (data not shown). Larvae were starved for 6 h and anesthetized for 30 min on ice before microinjection. More than 30 larvae were injected for each treatment, and three independent experiments performed. Injected larvae were allowed to recover for about 3 h at room temperature and were then returned to normal rearing conditions for the subsequent qPCR assays and bioassays.

RNAi effectiveness was assessed by qPCR 48 h post-injection using CDNA prepared from isolated total midgut RNA, and the qPCR conditions were as described above. Leaf-dip bioassays were performed for 72 h using larvae at 48 h after dsRNA injection and Cry1Ac protoxin concentrations that approximated the LC₅₀ (1 μg/ml) and LC₉₀ (2 μg/ml) values for non-injected DBM1Ac-S larvae. Bioassays were performed with 40 larvae per RNAi treatment and toxin concentration, and each bioassay was replicated three times. Mortality in control treatments was <10%, and bioassay data processing was as described above. One-way ANOVAs with Holm–Sidak’s tests (overall significance level = 0.05) were used to assess the differences between qPCR and bioassay treatments.

2.9. Genetic linkage analysis

The near-isogenic NIL-R (resistant) and DBM1Ac-S (susceptible) strains were used for linkage analysis as described elsewhere (Tiewsiri and Wang, 2011). A single-pair cross was conducted between a male NIL-R and a female DBM1Ac-S to generate an F₁ progeny. A diagnostic Cry1Ac toxin dose killing 100% of the F₁ (heterozygous) larvae was determined in bioassays as described above. Reciprocal crosses between an F₁ and NIL-R moths were made to generate two backcross families (backcross family a and b). The progeny from each backcross family (total of 40 larvae per family) were reared on a control diet (cabbage without Cry1Ac toxin) or an experimental diet (cabbage with 20 μg/ml of Cry1Ac toxin) to determine whether differences between treatments were statistically significant.
3. Results

3.1. Resistance to Cry1Ac toxin or Btk formulation in *P. xylostella* is independent of cadherin gene mutation

The cadherin sequences deposited in GenBank (EF541176) and two *P. xylostella* genome databases (DBM-DB, http://iae.fafu.edu.cn/DBM/search.php; GenBank ID: Px012847; KONAGAbase, http://dbm.dna.affrc.go.jp/px/; GenBank ID: PXUG_V1_000947) (Jouraku et al., 2013; You et al., 2013), were analyzed to determine whether these cadherin sequences were falsely annotated with respect to ATG start codon position and coding sequence (CDS). We then used the cadherin unigene sequence information in our transcriptome (Xie et al., 2012) and the characteristic of eukaryotic sequence surrounding ATG start codon (known as Kozak sequence) to preliminarily correct the whole CDS region of cadherin gene. We first used specific primers (Table 1) to clone the preliminarily corrected full-length *P. xylostella* cadherin cDNA sequence using the susceptible *DBM1*Ac-S larvae. Our large-scale sequencing efforts (30 clones from two independent cDNA batches), together with analysis of all the cadherin sequences in diverse *P. xylostella* strains worldwide from the GenBank and *P. xylostella* genome databases, found a 10-bp fragment (5’-GACTGCTTA-3’) that was incorrectly sequenced (5’-CAACCTCAT-3’) in the reference cadherin cDNA sequence (GenBank: EF541176), and this incorrect sequence would change the corresponding aa sequence from 969 NYL to 969 YAY. The final corrected *P. xylostella* cadherin cDNA sequence was deposited in GenBank under accession number KM370099. The bona fide *P. xylostella* cadherin cDNA has an open reading frame (ORF) of 5160 bp, which encodes a 1719-aa residue with a predicted molecular weight of 191.63 kDa and a predicted isoelectric point of 4.29. The deduced aa sequence of the *P. xylostella* cadherin exhibits features characteristic of known lepidopteran cadherin genes (Pigott and Ellar, 2007) (Fig. 1A). It contains a 25-aa N-terminal signal peptide, 12 cadherin repeats (from 1 to 12), followed by a membrane-proximal region (MPR), a 28-aa transmembrane domain (TMD), and a 128-aa cytoplasmic domain (CPD). Ten of the 12 cadherin repeats are from 79- to 135-aa long as determined by a search with Myhits and Prosite software with E-values ≤2e−5, but cadherin repeat 1 contains 46-aa with an E-value of 0.27, and cadherin repeat 12 contains 111-aa with an E-value of 0.028. Additionally, the cadherin protein sequence also contains 10 putative N-glycosylation sites, however, no O-glycosylation site was found.

Phylogenetic analysis was conducted by constructing a neighbor-joining (NJ) tree based on multiple alignments of 43 putative and identified full-length cadherin sequences with more than 1000 aa from three insect orders (Fig. 2). The unrooted tree showed that the identified lepidopteran cadherins including the *P. xylostella* cadherin gene (GenBank: KM370099) are more evolutionarily conserved and are clearly grouped into one cluster. Moreover, the *P. xylostella* cadherin gene shares high CDS sequence identity (>99%) with only one cadherin gene, which was PxCad10 (GenBank ID: P012847) from DBM-DB, and further sequence analysis showed that these two genes are the same cadherin gene, which suggested that this cadherin gene is a single-copy gene in the genome. The cadherins from both coleopteran and dipteran insects are also evolutionarily conserved within these insect orders. Interestingly, we find lepidopteran cadherin orthologous genes in coleopteran insects and one dipteran insect *Anopheles gambiae* (AgCad1, GenBank: XM_312086), while there is no lepidopteran cadherin orthologous gene in other dipteran insects. In addition, all of the putative cadherins with >1000 aa and complete ORFs found in the DBM-DB (except P012847) share relatively low aa identities with the *P. xylostella* cadherin gene (GenBank: KM370099) and group into another independent cluster.

The final corrected full-length *P. xylostella* cadherin cDNA sequence was then cloned to determine whether putative alterations were associated with resistance to Cry1Ac or Btk in our *P. xylostella* strains. Extensive sequencing of full-length cadherin amplicons from larval midgut tissues of all the susceptible and resistant strains (30 clones from two independent cDNA batches for each of the strains) failed to detect any non-synonymous mutations associated with the resistant phenotype (data not shown). However, we detected a very rare transcript (5%) with a 6-bp deletion (GGCGCT) in exon 31 that can lead to deletion of two aa (AL) in

Fig. 1. Schematic structure of the *P. xylostella* cadherin protein and sequence variation in the cadherin transmembrane domain from diverse *P. xylostella* strains. (A) The common structural characteristics of the deduced *P. xylostella* cadherin protein sequence include an N-terminal signaling peptide, an extracellular domain containing 12 cadherin repeats, a membrane-proximal extracellular domain (MPD), a transmembrane domain (TMD), and a cytoplasmic domain (CPD). (B) Alternative splicing within exon 31 of the cadherin cDNA sequence leads to the deletion of two amino acids (AL) in the transmembrane domain (TMD) of diverse Bt-susceptible and Bt-resistant strains of *P. xylostella*. GenBank accession numbers or Gene IDs of these cadherin protein sequences from these *P. xylostella* strains are BIS (ABU41413), Fuzhou-S (DBM-DB, Px012847), PKS (KONAGAbase, PXS_G1_000947), Waita (AA98024), Waita/Cowra hybrid (AA98023), Phil-S (AB63546), SBT (AB63545), and BIR (EF569598).
3.2. The overall transcript levels of cadherin gene do not differ between Cry1Ac-susceptible and -resistant strains. We designed a cadherin primer set for qPCR analysis in the current study (Table 1). Although we found that the expression level of cadherin was numerically lower in DBM1Ac-R and NIL-R than in DBM1Ac-S, the overall difference among the five strains was not statistically significant (P > 0.05; Holm–Sidak’s test; n = 3) (Fig. 3). This finding is consistent with a deep analysis of our previous transcriptome data for DBM1Ac-R and SZ-R in this study (Table 2) (Lei et al., 2014).

3.3. Silencing of cadherin expression does not affect the Cry1Ac susceptibility of P. xylostella larvae

To further test whether the cadherin-like protein can function as a Cry1Ac receptor in P. xylostella, we used RNA interference (RNAi) to silence its expression by injecting gene-specific double-stranded RNA (dsPxCad) into the early third-instar DBM1Ac-S larvae. The dsPxCad sequence was complementary to an internal region (nucleotides 4146–4579) of the cadherin mRNA. Relative cadherin expression levels in control and experimental larvae (non-injected, injected with buffer, or with dsEGFP targeting the EGFP gene) were determined 48 h post-injection (Fig. 4A). Injection of dsPxCad into larvae significantly reduced cadherin transcript levels by about 80% relative to controls (P < 0.05; Holm–Sidak’s test; n = 3). Subsequent bioassays performed at 48 h post-injection demonstrated that silencing of cadherin gene expression did not significantly reduce larval susceptibility to Cry1Ac protoxin (P > 0.05; Holm–Sidak’s test; n = 3) (Fig. 4B). These results suggest that the cadherin-like protein is not a Cry1Ac receptor in P. xylostella.

Fig. 2. Phylogenetic relationship of cadherin genes in three insect orders. The neighbor-joining (NJ) consensus tree was generated by Clustal W alignment of the deduced amino acid sequences of the screened cadherin genes from different insect species available in the GenBank database or the cadherin genes searched by annotation keyword “cadherin” in the DBM-DB using MEGA 5.0 software (Tamura et al., 2011). The cadherin genes from the DBM-DB with <1000 aa or partial coding sequence were not used for the tree construction. GenBank accession numbers or Gene IDs are indicated in parentheses. The corrected P. xylostella cadherin gene (KM370099) is marked by a solid black diamond. Abbreviations: 1. Lepidoptera (Bm, Bombyx mori; Ha, Helicoverpa armigera; Hv, Heliothis virescens; Hz, Helicoverpa zea; Of, Ostrinia furnacalis; On, Ostrinia nubilalis; Tn, Trichoplusia ni; Se, Spodoptera exigua; Dp, Danaus plexippus; Ds, Diatraea saccharalis; Sl, Spodoptera litura; Px, Plutella xylostella; Al, Agrafis iphile; Cs, Chilo suppressalis; DS, Diatraea saccharalis; Li, Lymantria dispar; Mse, Manduca sexta; Msep, Myrtillinae separate; Pg, Pectinophora gossypiella; Sl, Spodoptera frugiperda; Sl, Sesamia inferens; Sz, Sesamia nonagrioides); 2. Coleoptera (Tc, Tribolium castaneum; Tm, Tenebrio molitor; Dv, Diabrotica virgifera virgifera); 3. Diptera (Dm, Drosophila melanogaster; Ag, Anopheles gambiae; Aa, Aedes aegypti; Md, Musca domestica).

The putative cadherin transmembrane region with tandem repeat of three AL aa, and this transcript is a common alternative splicing isoform coexisting with full-length transcript in some heterozygous individuals of all strains. Interestingly, transmembrane region prediction showed that this two-aa deletion (AL) cannot greatly affect the transmembrane characteristics of this cadherin protein. Furthermore, sequence analysis showed that this kind of alternative splicing within exon 31 of the cadherin sequence can occur in diverse Bt-susceptible and Bt-resistant P. xylostella strains (Fig. 1B), which indicated that this alternative splicing isoform is not correlated with Bt resistance in P. xylostella. Moreover, sequencing of the full-length cadherin amplicons (10 clones each from three individual midgut samples) from NIL-R larvae surviving exposure to 10,000 µg/ml of Cry1Ac protoxin and from untreated larvae failed to detect a significant frequency change of the alternative splicing isoform (the frequency change was only about 1.7% in treated larvae) or any other mutations associated with survival of this treatment, which further confirmed that Cry1Ac resistance in P. xylostella is independent of mutations in the cadherin gene.
3.4. The cadherin gene is not linked to Cry1Ac resistance in P. xylostella

To further determine whether the cadherin gene is genetically linked to Cry1Ac resistance in our resistant strains, we first identified different cadherin gene alleles using four specific cadherin gDNA primer pairs. These primers can amplify genomic DNA fragments (about 400–600 bp) containing only one intron in each fragment (Supplementary data), and intron size polymorphisms can make these primers amplify gene alleles with different intron sizes (Table 1, Fig. 5A). Given that these alleles can only be detected in some of both DBM1Ac-S and NIL-R individuals (<5%), we inferred that the cadherin gene was not linked to Cry1Ac resistance in our P. xylostella resistant strains. To test this inference in the current study, we used the four primer sets as allelic markers to genotype the cadherin gene alleles in P. xylostella individuals for genetic linkage analysis.

A single-pair cross between a male from the near-isogenic NIL-R (resistant) and a female from the DBM1Ac-S (susceptible) strains was used to generate F1 progeny, then reciprocal F2 backcross families including backcross family a and b were generated by single-pair backcrossing F1 male with NIL-R female selected with or without a lethal dose of Cry1Ac toxin, respectively. The larvae from both backcross families exhibited a survival rate of approximately 50% with Cry1Ac selection, which statistically fits the predicted survival rate for monogenic recessive inheritance of the Cry1Ac resistance in P. xylostella. If the cadherin gene was linked to Cry1Ac resistance, half of the unselected backcross progeny and all the Cry1Ac-selected progeny should inherit a Bt resistance allele, however, we did not detect segregation patterns of known polymorphic sites among progeny. Genotyping of the unselected and Cry1Ac-selected larvae from backcross family a (Fig. 5B) showed that the cadherin genotype remained the same in both backcross groups and that the corresponding gene alleles were not detected in all of the DBM1Ac-S and NIL-R individuals. Similar results were also obtained with backcross family b (data not shown). Since that only backcross individuals homozygous for Cry1Ac resistance allele could survive the Cry1Ac selection, we can conclude that these cadherin alleles are not associated with survival of Cry1Ac exposure and there is no association between cadherin genotype and Cry1Ac resistance phenotype. Therefore, the cadherin gene is independent of the resistance to Cry1Ac in P. xylostella.

4. Discussion

The mode of action of Bt Cry toxins has been extensively studied and is relatively well-established: the toxins sequentially interact with different midgut receptors, resulting in the formation of membrane pores and insect death (Pardo-López et al., 2013; Gómez et al., 2014). Hence, high levels of insect resistance to Bt Cry toxins are always associated with alterations in receptor genes and the disruption or decrease or toxin–receptor interaction (Ferré and Van Rie, 2002). Among the diverse receptors of Bt Cry toxin, cadherin has been considered as the first high-affinity protein that binds with Bt Cry toxins in the insect midgut. This important role of cadherin has been further demonstrated by the restoration of susceptibility in highly resistant insects with modified Cry1Ac that bypasses the cadherin receptor (Sobrón et al., 2007; Tabashnik et al., 2011).

The molecular mechanisms of lepidopteran resistance to Bt Cry toxins are more diverse and complex than previously thought (Heckel et al., 2007). In Lepidoptera, the most common type of resistance to Bt Cry toxins, which has been designated Mode 1 resistance, is characterized by a >500-fold resistance to at least one Cry1A toxin, recessive inheritance, reduced binding of at least one Cry1A toxin to midgut receptors, and lack of cross-resistance to Cry1C toxin (Tabashnik et al., 1998). Mode 1 resistance, which has been reported in laboratory-selected strains of three lepidopterans (Heliothis virescens, Pectinophora gossypiella, and Helicoverpa zea),

Table 2

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<th>Length (bp)</th>
<th>Top hit*</th>
<th>FDR-valuec</th>
<th>FCd</th>
<th>Expression</th>
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* The unigenes were obtained by searching against our P. xylostella midgut transcriptome (Xie et al., 2012) with the full-length cDNA sequence of cadherin query, and all of the perfectly matched unigenes are listed here. The expression levels of these unigenes were derived from our original RNA-Seq libraries before filtration under the arbitrary threshold (FDR value < 0.001 and the absolute expression value log2 ratio ≥ 1) in our RNA-Seq study conducted in 2012 and published recently (Lei et al., 2014).

b The unigenes were annotated with the BlastX tool in the NCBI database in 2012.

c FDR value, the False Discovery Rate value when performing Gene Ontology (GO) functional enrichment analysis using Blast2GO software with Fisher’s exact test.

d FC, Fold Changes of gene expression [log2 (MK or GK-RPKM/MM-RPKM)]. MM: DBM1Ac-S; MK: DBM1Ac-R; GK: GK-R.

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Fig. 4. Silencing of cadherin gene expression and the effect of silencing on susceptibility to Cry1Ac. (A) Effect of injection with buffer, dsEGFP, or dsPxCad on the relative expression of the cadherin gene when compared to the control non-injected P. xylostella larvae. Values are the means and standard errors (SEM) from three biological replicates tested in four technical repeats. Values with the same letter are not significantly different (P < 0.05; Holm–Sidak’s test; n = 3). Both quantitative determinations (the bar chart) and qualitative determinations (the agarose gel of PCR products) are shown. (B) Susceptibility to two concentrations of Cry1Ac protoxin in P. xylostella larvae injected with buffer or dsRNA targeting EGFP (dsEGFP) or cadherin (dsPxCad). Values are the means and standard errors (SEM) from three biological replicates tested in four technical repeats. Within each concentration, values with the same letter are not significantly different (P > 0.05; Holm–Sidak’s test; n = 3).

Fig. 5. Analysis of the linkage between resistance to Cry1Ac and the cadherin gene in P. xylostella. (A) Genomic structure of the P. xylostella cadherin gene as predicted from its corrected full-length gDNA sequence (DBM-DB, Gene ID: Pox12847) and cDNA sequence in this study (GenBank: KM370099). Based on intron length polymorphism, four genomic PCR fragments of the cadherin gene (gDNA fragment 1, 2, 3, and 4) with only one allele of different sizes, which can be used as allelic biomarkers for subsequent genetic linkage analysis. (B) gDNA samples from individuals in the two backcross family groups were used in PCR assays with primers detecting cadherin alleles in four genomic regions (1, 2, 3, and 4) as described in Table 1 and the Materials and Methods. These regions were selected based on the method used in a previous analysis of the linkage between cadherin and Cry1Ac resistance in T. ni (Zhang et al., 2012b). To detect the two-band or one-band isoform patterns for cadherin, the PCR products were resolved by 2% agarose gel electrophoresis. The molecular size markers in (B) are (from top to bottom band): 600, 500, 400, 300, 200, and 100 bp, respectively. The expected sizes of genomic amplicons in (B) are 481 bp for fragment 1, 582 bp for fragment 2, 446 bp for fragment 3, and 369 bp for fragment 4. No alternative alleles were detected among all the larvae tested.

amnigera) is caused by mutations in a midgut cadherin-like protein (Gahan et al., 2001; Morin et al., 2003; Xu et al., 2005). Laboratory-selected insect resistance to Bt Cry toxins, however, does not represent the mechanism of resistance that has evolved in the field (Tabashnik et al., 2003). Thus far, field- or greenhouse-evolved Bt resistance has been documented only in P. xylostella (Tabashnik et al., 1990) and Trichoplusia ni (Wang et al., 2007). Mode 1 resistance to Cry1Ac toxin in a greenhouse-evolved resistant T. ni strain has been recently determined to be linked to an ABCC2 gene mutation (Baxter et al., 2011) and the concomitant down-regulation of an aminopeptidase N (APN1) (Tiewsiri and Wang, 2011). Further study excluded the involvement of the cadherin gene in this type of resistance to Cry1Ac toxin in T. ni (Zhang et al., 2012b). Similarly, the field-evolved Cry1Ac resistance in P. xylostella exhibiting typical Mode 1 resistance has been attributed to the same mutated ABCC2 gene (Baxter et al., 2011), but the role of the cadherin gene

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Sequence variation in the insect midgut cadherin gene has been documented. Resistance to Cry1Ac in the P. xylostella strains DBM1Ac-R (Tang et al., 1996) and NIL-R (Zhu et al., 2014) in the current study fits the Mode 1 resistance. Consistent with previous genetic mapping data (Baxter et al., 2005), our results determined that different cadherin isoforms expressed in the midgut cadherin gene are not the genetic basis for Mode 1 resistance in P. xylostella. However, this evidence does not exclude the possibility that the resistance resulted from an alteration in cadherin gene expression. Then, our subsequent qPCR analysis demonstrated that the current transcript levels of the cadherin gene did not significantly differ between susceptible and resistant strains, which is nearly coincident with our previous preliminary qPCR data for DBM1Ac-R (Yang et al., 2012) and a deep analysis of our recent transcriptome data for DBM1Ac-R and SZ-R (Lei et al., 2014). Moreover, our RNAi experiment confirmed that this binding is not required for Cry1Ac toxicity, just as it is not required for toxicity in T. ni (Zhang et al., 2012b). Consequently, the detailed experimental evidence in this study demonstrated that the Mode 1 resistance in our P. xylostella strains is independent of the midgut cadherin gene. Nonetheless, different P. xylostella strains may have different Bt resistance mechanisms (Baxter et al., 2005). As the cadherin protein can bind Cry1Ac toxin (Higuchi et al., 2007), or may be involved in Cry1Ac toxin activation process, a cadherin knockout mutation may still have the potential to cause Cry1Ac resistance in other P. xylostella strains. Hence, further study is needed to see whether the cadherin can be involved in Cry1Ac resistance in other P. xylostella strains.

Sequence variation in the insect midgut cadherin gene has been documented. In addition to resulting from mutation, such sequence variation could result from alternative splicing of its mRNA precursor. Cadherin sequence variation resulting from alternative splicing has been reported in Ostrinia nubilalis (Bel et al., 2009) and T. ni (Zhang et al., 2013). More recently, the alternative splicing of the midgut cadherin gene was reported to be associated with field-evolved resistance in Pectinophora gossypiella to Cry1Ac produced by Bt cotton (Fabrick et al., 2014). In this study, we also found an alternative splicing isoform of the P. xylostella midgut cadherin gene, which results in deletion of two amino acids (AL) in the cadherin transmembrane domain. This alternative splicing isoform, however, was only found in some heterozygous individuals from all of our Cry1Ac-susceptible and -resistant P. xylostella strains at a low frequency. Moreover, our cadherin sequence analysis also showed that this alternative splicing isoform occurs in diverse Bt-susceptible Bt-resistant P. xylostella strains worldwide. Therefore, it follows that Cry1Ac resistance in P. xylostella cannot be attributed to this alternative splicing isoform. When individuals of the near-isogenic resistant strain NIL-R were selected by exposing them to a high dose of Cry1Ac, the frequency of this cadherin splicing isoform did not significantly change, providing further evidence that this cadherin splicing isoform does not contribute to Cry1Ac resistance in P. xylostella.

Insect midgut cadherin represents a unique member of the cadherin superfamily, and there may be several midgut cadherin genes involved in toxicity of different Bt Cry toxins despite their low sequence identities. For example, two midgut cadherins sharing only 14% amino acid identity separately function as receptors of Cry4Ba and Cry11Ba in A. gambiae (Hua et al., 2008, 2013). In addition to a midgut cadherin receptor of Cry3Ba toxin, a second midgut cadherin was also found in the RNA-Seq database of Alphitobius diaperinus (Hua et al., 2014). Previous study determined that the midgut cadherin in the P. xylostella genome is a single-copy gene (Baxter et al., 2005), which is also reflected by phylogenetic analysis in the current study (Fig. 2). Although this midgut cadherin gene (GenBank: KM370099), which shares high sequence identity with other lepidopteran cadherins, was not associated with Cry1Ac resistance, we cannot exclude the possibility that other cadherin genes in the P. xylostella genome sharing low amino acid identity with this midgut cadherin gene (GenBank: KM370099) can serve as functional receptors of Bt Cry toxins. As a matter of fact, our previous midgut tissue-specific transcription data showed that there were vast cadherin unigenes expressed in the P. xylostella larval midgut (Xie et al., 2012). Therefore, further study is needed to determine whether these cadherins are involved in Cry1Ac resistance in P. xylostella.

In this study, we cloned and obtained the bona fide cDNA sequence of the midgut cadherin gene in P. xylostella, and determined that no sequence mutations were involved in Cry1Ac resistance. Expression of the midgut cadherin gene at the transcript level did not differ among susceptible and resistant strains of P. xylostella. Moreover, an RNAi experiment failed to detect any correlation between reduced cadherin transcript levels and susceptibility to Cry1Ac toxin. Genetic linkage analysis showed no correlation between cadherin alleles and Cry1Ac resistance in P. xylostella. In summary, the results from this study demonstrate that Cry1Ac resistance in P. xylostella is independent of the mutations and transcript levels of the midgut cadherin gene in all of our tested resistant strains. By determining that this gene is not involved in resistance, our findings help clarify the complex molecular mechanism of Cry1Ac resistance in P. xylostella.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jip.2015.01.004.

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