A Spodoptera exigua cadherin serves as a putative receptor for Bacillus thuringiensis Cry1Ca toxin and shows differential enhancement to Cry1Ca and Cry1Ac toxicity

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Abstract

Crystal toxin Cry1Ca from Bacillus thuringiensis (Bt) has an insecticidal spectrum encompassing lepidopteran insects that are tolerant to current commercially used Bt crops expressing Cry1A toxins, and may use as a potential bioinsecticide. The mode of action of Cry1A is fairly well understood. However, whether Cry1Ca interacts with the same receptor proteins as Cry1A remains unproven. In the present paper, we firstly cloned a cadherin-like gene SeCad1b from S. exigua (relatively susceptible to Cry1Ca). SeCad1b was highly expressed in the larval gut, but hardly detected in fat body, Malpighian tubules and remaining carcass. Secondly, we bacterially expressed truncated cadherin rSeCad1bp and its interspecific homologue rHaBtRp from H. armigera (more sensitive to Cry1Ac) containing the putative toxin binding regions. Competitive binding assays showed that both Cry1Ca and Cry1Ac could respectively bind to rSeCad1bp and rHaBtRp, and they did not compete with each other. Thirdly, Cry1Ca ingestion killed the larvae and decreased the weight of surviving larvae. Dietary introduction of SeCad1b-double-stranded RNA (dsRNA) reduced approximately 80% of the target mRNA, and partially alleviated the negative effect of Cry1Ca on larval survival and growth. Lastly, rSeCad1bp and rHaBtRp differentially enhanced the negative effects of Cry1Ca and Cry1Ac on the larval mortalities and growth of S. exigua and H. armigera. Thus, we provide the first lines of evidence to suggest that SeCad1b from S. exigua is a functional receptor of Cry1Ca.

Key words: S. exigua, H. armigera, cadherin receptor, crystal toxin, enhancement
*Bacillus thuringiensis* (Bt) produces crystal proteins (Cry toxins) that show specific insecticidal activity against insect pests from Lepidoptera, Diptera and Coleoptera (1). The Bt formulations are often sprayed and the Cry toxins are commonly expressed in transgenic plants for insect control in agriculture (2). For example, the transgenic cottons have been widely planted, and reached 3.8 million hectares in China in 2007 and 58.4 million hectares world-wide in 2011(3).

The beet armyworm *Spodoptera exigua* is a highly polyphagous herbivore and caused little economic damage to cotton previously. In contrast, the cotton bollworm *Helicoverpa armigera* is one of the major pests of cotton crops. *S. exigua* larvae are relatively resistant to Cry1A and susceptible to Cry1Ca. Conversely, *H. armigera* larvae are more sensitive to Cry1Ac rather than Cry1Ca (4). Since the transgenic cottons commercialized in China only produced Cry1Ac, *S. exigua* has become a major economic pest of cotton across a wide distribution in China a couple of years since commercialization of Bt cottons (5). Comparative studies on the interaction between the receptor proteins and specific Cry toxins will aid in the development of novel Bt biopesticides with increased efficacy, and in establishing resistance management strategies.

Cry1Ac and Cry1Ca are produced as crystal inclusion bodies. They must be proteolytically hydrolyzed by proteinases in susceptible caterpillars to produce active toxins. The molecular mechanism that mediates the insecticidal activity of activated Cry1A has been dominated by two models (2, 6, 7). The first is the sequential binding...
model: the activated toxin goes through complex sequential binding events with different receptor proteins in target caterpillar midgut cells, and eventually resulting in membrane pore formation. Cry1A initially binds to glycosylphosphatidylinositol (GPI)-anchored proteins such as aminopeptidase-N and alkaline phosphatase (7). This binding event concentrates the toxin in the microvilli membrane where it then binds to cadherin receptor. Binding to cadherin leads to further proteolytic cleavage and oligomer formation. Oligomers then bind to the GPI-anchored proteins, and finally insert into the membrane to form pore (8). The presence of such pores allows ions to pass freely through the plasma membrane, leading to destruction of the membrane potential, cell swelling, cell lysis, and the death of the host larvae (7). The second model is named the signaling pathway model. According to this model, cytotoxicity is mediated by the specific binding of Bt toxins to their cadherin receptors. This activates Mg$^{2+}$-dependent adenylyl cyclase/protein kinase A signaling pathways that lead to necrotic cell death (9, 10). Moreover, a combination of above two models has also been proposed (11).

In either of the models, insect cadherin is a Bt toxin-binding receptor. Up to now, insect cadherins have been proven or suggested to interact with Cry toxins in at least six lepidopteran (2), two dipteran (12-14), and two coleopteran species (15, 16). A toxin-binding cadherin receptor is composed of five domains: signal peptide (SP), 8-12 cadherin repeats (CRs), membrane-proximal extracellular domain (MPED), transmembrane region (TM) and internal cytoplasmic domain (IC) (17). In *Manduca sexta* cadherin, MsBtR1, three Cry1A-binding sites are indicated in CR7 (18, 19),
CR11 (20) and CR12 (21). Cadherin fragments containing the critical toxin-binding region enhanced the activities of Cry toxins in some lepidopteran (22-24), coleopteran (16, 25) and dipteran (13, 26) insects. However, cadherin fragments have also been reported to reduce Cry1A toxicity to some lepidopterans (20, 27). Moreover, mutations in lepidopteran cadherin genes were genetically linked to the resistance to Cry toxins in Heliothis virescens (28, 29), H. armigera (30-33), and Pectinophora gossypiella (34). Furthermore, down regulation of a cadherin gene was also associated with the resistance to Cry1Ab in Diatraea saccharalis (35). Notably, cadherin is a critical receptor for Cry toxins that mediates intoxication.

In contrast to the Cry1A model, the mechanism of action of Cry1Ca has not been identified. It seems aminopeptidase N is an important receptor of Cry1Ca. A class 4-aminopeptidase N (AAK69605) from Spodoptera littoralis was proven to be specific Cry1Ca receptors by RNA interference silencing in vivo (36) and in Sf21 cells (37), by ligand blot interaction and immunoprecipitation in Sf21 cells (38). Moreover, an S. exigua Cry1Ca-resistant strain lacked expression of its class 1-aminopeptidase N (AAP44964) (39). However, in vitro binding assays revealed that all the three Cry1Ca structural domains interacted with S. littoralis brush-border membrane vesicles (BBMV) (40). Considering the findings that α1-helix of Cry1A-domain I interacts with a cadherin-like receptor at the initial step of the binding process (41), the observed binding of Cry1Ca-domain I to BBMV (40) let us hypothesize that a cadherin-like-protein is the receptor of Cry1Ca (4).

In the present paper, we firstly cloned a cadherin-like gene SeCad1b from S.
exigua. Bacterially expressed truncated cadherin rSeCad1bp and its interspecific homologue rHaBtRp from H. armigera could respectively bind to Cry1Ca and Cry1Ac. Cry1Ca and Cry1Ac could not compete with each other when binding to rSeCad1bp and rHaBtRp. Dietary introduction of SeCad1b-double-stranded RNA (dsRNA) reduced approximately 80% of the target gene expression, and partially alleviated the negative effect of Cry1Ca on larval survival and growth. In contrast, rSeCad1bp and rHaBtRp differentially enhanced the negative effects of Cry1Ca and Cry1Ac on the larval mortalities and growth of S. exigua and H. armigera. Thus, our results support the hypothesis that SeCad1b is a functional receptor of Cry1Ca.

MATERIALS AND METHODS

Rearing of insects

H. armigera and S. exigua larvae were routinely reared on an artificial diet (27.5 g wheat-germ powder, 7.5 g soybean flour, 2.0 g yeast, 2.5 g agar, 93 ml water, 0.2 g Wesson salt mixture, 0.7 g vitamin C, 0.41 mg B1, 0.82 mg B2, 0.41 mg B6, 0.01 mg B12, 0.04 mg biotin, 1.63 mg nicotinamide, 0.41 mg folic acid, 1.63 mg calcium pantothenate, 32.64 mg inositol and 0.2 g citric acid) the same as that described previously (42), in an insectary under controlled temperature (28±1 °C), photoperiod (14h light/10h dark) and relative humidity (70-80%), without exposure to any Bt toxin.

Molecular cloning and phylogenetic analysis

According to the sequence of a cadherin-like gene (ADV17664.2) in NCBI,
specific primers were designed (Supplement material A) and RT-PCR was performed using the cDNA template from S. exigua larval midgut. To characterize the full sequence, total RNA from the fourth-instar larval midguts was extracted with the SV total RNA isolation system (Promega, Madison, WI, USA) according to the manufacturer’s instructions. Using the SMART™ RACE cDNA amplification kit (Clontech, Takara Bio Inc., Dalian, China), the 5'- and 3'-RACE Ready cDNA was synthesized following the manufacturer’s instructions, primed by oligo (dT) primer and the SMART II A oligonucleotide. Antisense and sense gene-specific primers (Supplement material A) corresponding to the 5′-end and 3′-end of the RT-PCR obtained fragment and the universal primers in the SMART™ RACE kit were used to amplify the 5′-end and the 3′-end. The PCR products were subsequently ligated into pGEM-Teasy vector (Promega, Madison, WI, USA) and sequenced. After full-length cDNA was obtained, we designed a pair of primers to verify the full-length (Supplement material A). The reaction systems and the thermal cycling conditions were available from the authors upon request. The resulting sequence, SeCad1b, was submitted to GenBank (JQ365181.1). Protein analysis was done by ISREC Profile server (http://hits.isb-sib.ch/cgi-bin/PFSCAN).

The cadherin proteins from 18 lepidopteran insect species were used to construct the maximum-likelihood (ML) trees using RAxML version 7.26 (43) with the best-fitting model (WAG+γ) after estimated by ProTest (44). The reliability of ML tree topology was evaluated by bootstrapping sampling of 1000 replicates.

Expression and purification of rSeCad1bp and rHaBtRp
Similar method described previously (22) was used. Briefly, DNAs encoding *S.
exigua* cadherin region from a.a. 877 to a.a. 1480 (named rSeCad1bp) and *H.
armigera* corresponding fragment from a.a. 870 to a.a. 1467 (named rHaBtRp) were
amplified from their larval midgut cDNA templates by RT-PCR using PrimeSTAR HS
DNA Polymerase (Takara Bio Inc., Dalian, China), with forward and the reverse
primers containing BamHI and XhoI restriction sites in the 5′-ends respectively
(Supplement material A). The PCR products were purified with AxyPrep™ DNA Gel
Extraction Kit (Axygen Scientific, Silicon Valley, California, USA), double digested
with BamHI and XhoI FastDigest restriction enzymes (Fermentas, Thermo Fisher
Scientific, USA) for 10 min at 37 °C, and ligated into the previously digested pET-30a
(+) vector (Novagen, Madison, WI) to generate the pET-rSeCad1bp and
pET-rHaBtRp plasmids, respectively. The coding sequences and clone orientations
were confirmed by sequencing.

For expression, the constructed recombinant plasmids were transformed into
*Escherichia coli* strain BL21 (DE3) (Stratagene, La Jolla, CA). Positive clones were
selected at 37 °C in LB medium supplemented with 50 μg/ml kanamycin, and
cultured until the absorbance reached 0.5-0.8 at 600 nm. rSeCad1bp and rHaBtRp
were induced by adding isopropyl β-D-thiogalactoside (IPTG) to a final concentration
of 0.8 mM. The BL21 *E. coli* cultures were then grown for an additional 6 h at 37 °C,
200 rpm. *E. coli* cells were harvested by centrifugation at 8000 g for 20 min (4 °C),
and then resuspended and lysed in isolation buffer (2 M urea, 20 mM Tris-HCl, 0.5 M
NaCl, 2% Triton X-100, pH 7.4) by sonication, and the inclusion bodies were isolated
from the crude cell lysate by centrifugation at 12000 g for 15 min (4 °C).

The recombinant proteins were produced as inclusion bodies and were isolated and solubilized in binding buffer (8 M urea, 20 mM Tris-HCl, 0.5 M NaCl, 5 mM imidazole, pH 7.4) at 200 rpm constant shaking rate for 1 h (28 °C). The protein fragments with His-tag were purified by a nickel-nitrilotriacetic acid (Ni-NTA) affinity column (HisTrap HP column; GE Healthacre) and eluted from the column by 500 mM imidazole. His-tag was removed by enterokinase (Genscript Biology Company, Nanjing, China). The cleaved proteins were refolded by a gradient of decreasing concentration of urea, desalting with dialysis bag (molecular-weight-cutoff: 8000-14000, Sigma) against 10 mM Tris-HCl (pH 7.4) overnight at 4 °C. Total protein content of each sample was determined by the Bradford method using bovine serum albumin (BSA, Sigma) as the standard protein (45). The purified proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Toxin dot-blot binding assays

Cry1Ac and Cry1Ca were activated toxins, and were obtained from Envirologix Inc. in the USA. Their purities and molecular weights were confirmed by SDS-PAGE and their quantities were determined by Bradford’s method. Activated Cry1Ac and Cry1Ca were biotinylated with EZ-Link Sulfo-NHS-LC-Biotinylation Kit (Pierce, FL, USA) similar to the method described by Atsumi et al. (46).

For competition binding assays, purified rSeCad1bp and rHaBtRp peptides (4 μg) were respectively dotted onto a nitrocellulose filters. After blocking in PBS-T buffer with 3% BSA, rSeCad1bp and rHaBtRp were respectively probed with a mixture of a
biotinylated toxin (for rSeCad1bp, Cry1Ca 0.4 µg/mL, Cry1Ac 2 µg/mL; for rHaBtRp, Cry1Ca 2 µg/mL, Cry1Ac 0.4 µg/mL) and either of the unlabeled Cry1Ca and Cry1Ac at the weight ratio of 1:0, 1:50 and 1:500. Filter was bathed in toxin-contained solution for 3 h at room temperature. After washing away the unbound toxins, the presence of biotinylated one was determined by streptavidin-horseradish peroxidase, followed by SuperSignal west pico chemiluminescent substrate (Pierce, FL, USA), as described by the manufacturers.

**Preparation of bacterially expressed dsRNA**

The dsRNA-expressing vector pET-2P was constructed from the plasmid pET-30a(+), which contained T7 promoter and T7 terminator (47). A 489 bp fragment of *SeCad1b* gene encoding a.a. 1117–a.a. 1278 and a 414 bp fragment of enhanced green fluorescent protein gene *egfp* (control) were amplified by RT-PCR respectively from *S. exigua* larval midgut’s cDNA and PUB-nls-EGFP (Provided by Dr. Handler, USDA) using specific primers (Supplement material A). The products were individually cloned into the plasmid pET-2P within EcoRI sites to generate the recombinant plasmids of pET2p-rSeCad1b and pET2p-egfp. *E. coli* HT115 (DE3) competent cells lacking RNase III were prepared using standard CaCl₂ methodology and were transformed with pET2p-rSeCad1b or pET2p-egfp. Individual colonies of HT115 (DE3) were inoculated into LB medium supplemented with 50 µg/ml kanamycin and 12.5 µg/ml tetracycline and cultured with shaking (250 rpm/min) at 37 °C overnight. Cells were induced to express dsRNA by adding 0.4 mM IPTG and incubated for an additional 4 h at 37 °C. The expressed dsRNA was extracted and
confirmed by electrophoresis on 1% agarose gel (data not shown). Bacteria cells were centrifuged at 5000 g for 10 min, and resuspended in 0.05 M PBS (pH 7.4) at the ratio of 10:1 (concentration of 10×), and then used for bioassay.

dsRNA ingestion assay

Our preliminary experiments revealed that ingestion of dsRNA-expressed bacteria could release intact dsRNA. A surface contamination bioassay was conducted to test the impact of dietary SeCad1b-dsRNA on S. exigua larval sensitivity to Cry1Ca by a two-stage feeding protocol. At the first stage, 100 μl of suspension of the HT115 clone expressed SeCad1b-dsRNA or egfp-dsRNA (approximately 50 μg of dsRNA according to our preliminary experiment), or 0.05 M PBS (pH 7.4) solution (negative control) was overlaid onto a 24-well plate that had been filled with 1 ml of artificial diet. After drying, a newly-eclosed S. exigua neonate was placed in each well of the plate and 24 first instars were bioassayed. The diets were changed with new overlaid ones on each day and the larvae were fed for 4 days. At the second stage, the larvae previously feeding the PBS-, egfp-dsRNA- and SeCad1b-dsRNA-contained diets were respectively transferred to newly prepared PBS- and Cry1Ca (1 μg/cm²)-contained diets. The larvae were taken to corresponding new overlaid diets on each day. The mortalities were measured and the body masses of the survivors were weighed 4 days after the initiation of the second stage. The larvae were considered dead if they could not move their legs and body after one leg was touched with a fine needle. This experiment was replicated 5 times and a total of 120 larvae were used for each treatment.
Quantitative real-time PCR (qPCR)

Three groups of samples were prepared and each group was repeated in triplicate. The first group was derived from the first (1st)-, second (2nd)-, third (3rd)- and fifth (5th)- instar larvae, male and female pupae (MP and FP) and sexually mature male and female adults (MA and FA) feeding normal diet for the analysis of temporal expression patterns of SeCad1b. The second was derived from foregut (FG), midgut (MG), hindgut (HG), fat body (FB), Malpighian tubules (MT), and remaining carcass (RC) of the fifth-instar larvae feeding normal diet for the analysis of tissue specific expression of SeCad1b. The third was from the midgut of the larvae exposed to dsRNA for four days to evaluate the RNA interference effect. Total RNA of each sample was extracted using SV Total RNA Isolation System Kit (Promega, USA). Purified RNA was subjected to DNase I to remove any residual DNA according to the manufacturer’s instructions. The mRNA abundance of the putative SeCad1b gene in each template of the treated larvae was estimated by qPCR using SYBR Premix Ex Taq™ (Perfect Real Time) (Takara Bio Inc., Dalian, China) and ABI 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) according to the manufacturer’s instruction. An RT negative control (without reverse transcriptase) and a non-template negative control were included for each primer set to confirm the absence of genomic DNA and to check for primer-dimer or contamination in the reactions, respectively. Housekeeping genes GAPDH and EF were selected as internal controls, and the primers of SeCad1b, GAPDH and EF genes were designed with Beacon Designer 7 software (Premier Biosoft International, Palo Alto, CA, USA).
(Supplement material A). Each 20 μl qPCR reaction system contained 50 ng cDNA as template. The following standard PCR protocol was used: denaturing at 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s and 60 °C for 34 s. After amplification, the melting curves were determined by heating the sample up to 95 °C for 15 s, followed by cooling down to 60 °C for 1 min, and heating the samples to 95 °C for 15 s.

The generation of specific PCR products was confirmed by gel electrophoresis. Each primer pair was tested with a logarithmic dilution of a cDNA mixture to generate a linear standard curve (crossing point CP plotted vs. log of template concentration), which was used to calculate the primer pair efficiency. All experiments were repeated in triplicate. Data were analyzed by the $2^{-\Delta\Delta Ct}$ method (48), using the geometric mean of GAPDH and EF for normalization according to the strategy described previously (48, 49).

**Enhancement of rSeCad1bp and rHaBtRp to the toxicity of Cry1Ac and Cry1Ca**

The enhancement of rSeCad1bp and rHaBtRp to the toxicity of Cry1Ac and Cry1Ca to second-instar of *S. exigua* and *H. armigera* was determined by the surface contamination bioassay. Cry1Ac (0.125 μg/cm² for *S. exigua*; 0.01 μg/cm² for *H. armigera*) or Cry1Ca (0.035 μg/cm² for *S. exigua*; 2.0 μg/cm² for *H. armigera*) was mixed respectively with rSeCad1bp or rHaBtRp at toxin:peptide molar ratio of 1:50 in 0.05 M PBS (pH 7.4). The maximum dose of rSeCad1bp or rHaBtRp alone in 0.05 M PBS was used as a negative control. One hundred μl of each of the test solutions was overlaid onto a 24-well plate that had been filled with 1 ml of artificial diet. After drying, one second instar, starved for 4 h, was placed in each well of the plate and 24
larvae were bioassayed. This entire experiment was repeated in triplicate and a total of 72 larvae were used for each treatment. The larvae were taken to new overlaid diets on each day and the mortalities were measured and the body masses of the survivors were weighed after 6 days.

Statistics

Mortality (%), larval weight and the q-PCR data were given as mean ± SE. The arcsine-square-root transformation mortality data, larval weight and the q-PCR data were analyzed by ANOVA followed by the Tukey-Kramer test. All the statistical analyses were performed using SPSS for Windows (SPSS, Chicago, IL, USA).

RESULTS

Cloning and sequence analysis of SeCad1b

A single cDNA containing the entire coding sequence was obtained and confirmed. The cDNA consists of 5597 bp (JQ365181.1), shares high degree of nucleotide identities in 5’-UTR (94.9% and 97.8%), ORF (98.5% and 98.3%) and 3’-UTR (96.3% and 95.6%) with the corresponding regions in recently published cadherin-like cDNAs ADV17664.2 and AEB97395.1 from S. exigua, respectively (24). We considered these three sequences as allelic variants, and named them SeCad1a (ADV17664.2), 1b (JQ365181.1) and 1c (AEB97395.1) respectively. Similarly, BtR175 from Bombyx mori had three allelic variants that differ at one, five, or six amino acid positions (50-52). SeCad1b has an open reading frame of 5220 bp that encodes a 1739-amino acid protein (Supplement material B). The predicted protein
sequence has a calculated molecular mass of 194352 Da and an isoelectric point of 4.47. Similar to other Bt cadherin receptors (17, 28, 30, 52-54), SeCad1b consists of SP at its N-terminus, eleven CRs, MPED, TM, and IC. Twenty-one putative calcium-binding sequences (DXNDN or DXD) are distributed throughout the extracellular domain. Integrin recognition sequences, RGD (55) and LDV (56), are located in CR2 and CR8, respectively (Supplement material B).

In a comparison of the full-length lepidopteran, coleopteran and dipteran cadherin-like sequences, identity is higher within orders. SeCad1b shares the amino acid identities from about 50% to 76% with the potential cadherin receptor proteins from eighteen lepidopteran species listed in Figure 1A, has 27% and 24% amino acid identities with AgCad1 (XP_312086.5) and AdCad (EFR21126) respectively from two dipterans Anopheles gambiae and Anopheles darlingi, and shows 14%-16% amino acid identities with those from three coleopterans (Tenebrio molitor, ABL86001.2, TmCad1; Tribolium castaneum, XP_971388, TcCad1; Diabrotica virgifera virgifera, AAV88529, DvvCad).

The evolutionary relationship of the cadherin receptor proteins derived from eighteen lepidopteran species was evaluated using unrooted phylogenetic tree (Figure 1A). Those from 8 Noctuidae species (except that from T. ni) clustered together, and those from 4 Pyralidae species clustered together with 94% bootstrap support. Among the cadherins from 9 Noctuidae species, three allelic variants (SeCad1a, b and c) from S. exigua were first joined together and then clustered together with SlCad from...
congenus species *S. litura*, with 100% bootstrap support. Two other pairs of cadherins from congenus species (SiCad and SnCad, HaBr and HvCad) were also clustered together respectively, with 100% bootstrap support. Similarly, out of 4 cadherins from Pyralidae insect species 2 from genus *Ostrinia* species were joined together with 100% bootstrap support (Figure 1A).

**Stage- and tissue-specific expression profile**

We determined the stage-specific expression profile of SeCad1b during the development stages. The gene was highly expressed in the third and fifth instar larvae. However, it was hardly detected in male and female pupae, and male and female adults (Figure 1B).

Tissue specific expression of SeCad1b was also tested. SeCad1b was highly abundant in the midgut, and then in the foregut and hindgut. In contrast, SeCad1b was hardly detectable in other tissues such as fat body, Malpighian tubules, and remaining carcass (Figure 1C).

**Competition assays in ligand blot**

Bacterially expressed cadherin fragments were purified (Supplement material C). Competition experiments were done by incubating the labeled toxin in the presence or the absence of the unlabeled ones. From the profile of homologous and heterologous competitive binding, it was found that both Cry1Ca and Cry1Ac could respectively bind to rSeCad1bp and rHaBrRp. Moreover, both Cry1Ca and Cry1Ac did not compete with each other when binding to rSeCad1bp and rHaBrRp (Figure 2).

**RNAi knockdown of SeCad1 results in decreased susceptibility to Cry1Ca**
Bacterially-expressed dsRNAs were dietarily introduced into *S. exigua* neonates (first instars). After continuous ingestion *SeCad1b*-dsRNA for 4 days, the amount of *SeCad1b* transcript in the larvae was reduced by approximately 80%, comparing to those in the larvae feeding normal or *egfp*-dsRNA-contained diet, with the former significantly lower than the latter two (Figure 3A).

Feeding normal diet, *egfp*-dsRNA-overlaid and *SeCad1b*-dsRNA-contained diets did not kill larvae. When the larvae previously feeding normal and *egfp*-dsRNA-overlaid diets were transferred to Cry1Ca-overlaid diet, 81% and 84% of them were dead. When the larvae previously feeding *SeCad1b*-dsRNA-contained diet were exposed to Cry1Ca, the mortality was 52% (Figure 3B). ANOVA analysis revealed that the larvae subsequently feeding *SeCad1b*-dsRNA- and Cry1Ca-contained diets had significantly lower mortality than those first feeding normal or *egfp*-dsRNA-overlaid diets and then feeding Cry1Ca-contained diet ($F_{2,6}=32.3, P<0.01$). Thus, reduction of *SeCad1b* transcript increased larval tolerance to Cry1Ca.

The mean weights of the surviving larvae after experiment were also measured. The average weights of larvae feeding *egfp*-dsRNA-overlaid and *SeCad1b*-dsRNA-contained diets reduced by 2.2% and 14.3% respectively, comparing to those feeding normal diet. When the larvae previously feeding normal and *egfp*-dsRNA-overlaid diets were transferred to Cry1Ca-overlaid diet, their average weights were decreased 50.6% and 48.4% respectively. When the larvae previously feeding *SeCad1b*-dsRNA-contained diet was exposed to Cry1Ca, the
weight was reduced 24.8% (Figure 3C). One-way ANOVA showed that the weights of larvae subsequently feeding normal and Cry1Ca-overlaid diets, or feeding egfp-dsRNA- and Cry1Ca-overlaid diets were greatly lower than that of larvae subsequently feeding SeCad1b-dsRNA- and Cry1Ca-overlaid diets ($F_{5,12}=4.32$, $P<0.05$). Therefore, the weight reduction by Cry1Ca exposure can partially alleviate by RNA interference-mediated knockdown of SeCad1 gene.

Enhancement of insecticidal activity of toxins by rSeCad1bp and rHaBtRp

To test the enhancement of bacterially-expressed truncated cadherins to the toxicities of Cry toxins, the diet overlaid with the truncated cadherin was used to feed the larvae. The mortalities and the larval weights were measured.

For *S. exigua* larvae, 0.035 µg/cm² of Cry1Ca caused approximately 8% of larval mortality. When the larvae were exposed to Cry1Ca and rSeCad1bp (rather than rHaBtRp), the larval mortality greatly increased. At the concentration of 0.125 µg/cm², Cry1Ac caused little larval mortality. When the larvae were exposed to both Cry1Ac and cadherin fragment (either rSeCad1bp or rHaBtRp), the mortalities were significantly increased (Figure 4A). At the tested concentration, Cry1Ca exposure significantly reduced larval weight. When the larvae were exposed to Cry1Ca and rSeCad1bp (but not rHaBtRp), the larval weight was further decreased. Cry1Ac at the concentration of 0.125 µg/cm² did not affect the larval weight. When the larvae were exposed to both Cry1Ac and rHaBtRp (but not rSeCad1bp), the larval weight was dramatically reduced (Supplement material DA).

For *H. armigera*, both Cry1Ca (2.0 µg/cm²) and Cry1Ac (0.01 µg/cm²) could kill
The presence of rSeCad1bp and rHaBtRp respectively enhanced the toxicity of Cry1Ca and Cry1Ac and killed more larvae (Figure 4B). Regarding the larval weight, Cry1Ca alone showed significant negative influence, in contrast to Cry1Ac alone. Moreover, the presence of rSeCad1bp but not rHaBtRp caused more weight loss for Cry1Ca-ingested larvae. Similarly, the presence of rHaBtRp but not rSeCad1bp resulted in more weight reduction for Cry1Ac-ingested larvae (Supplement material DB).

Thus, rSeCad1bp showed higher enhancement of Cry1Ca activity, whereas rHaBtRp exhibited higher enhancement of Cry1Ac toxicity in both S. exigua and H. armigera.

**DISCUSSION**

In this report, we provide four lines of evidence to support a hypothesis that SeCad1b is a functional Cry1Ca receptor in S. exigua. The first line of evidence was that SeCad1b showed typical protein domains associated with the functional Cry toxin receptors. SeCad1b consists of SP, 11 CRs, MPED, TM, and IC domains, similar to other functional Cry toxin receptors (28, 30, 52-54). Moreover, SeCad1b was highly expressed in the third and fifth instar larvae, but hardly detected in pupae and adults. Furthermore, SeCad1b was highly abundant in the gut, but hardly detected in fat body, Malpighian tubules, and remaining carcass. The structural features and expression profile indicate that SeCad1b may act as a Cry1Ca receptor.

The second line of evidence was that both Cry1Ca and Cry1Ac could bind...
rSeCad1bp and rHaBtRp. Homologous competitive binding assays revealed that both
Cry1Ca and Cry1Ac could respectively bind to rSeCad1bp and rHaBtRp, and
heterologous competitive binding experiment showed that Cry1Ca and Cry1Ac did
not compete with each other. These results indicate that Cry1Ca does not share the
same binding sites with Cry1Ac toxin in both rSeCad1bp and rHaBtRp. It has long
been known that larvae of *Spodoptera* spp. are relatively resistant to Cry1A toxins but
highly susceptible to Cry1Ca. Conversely, *H. armigera* larvae are more sensitive to
Cry1Ac rather than Cry1Ca (4). An interesting question arises: are the differences in
cadherin binding sites response for, at least partially, the susceptibility differences of
Cry1Ca and Cry1Ac toxins to *Spodoptera* spp. and *H. armigera* larvae? This deserves
further research in the future.

The third line of evidence was the most important. Cry1Ca ingestion killed
approximately 80% of the larvae and significantly decreased weight of surviving
larvae. Dietary introduction of *SeCad1b*-dsRNA reduced approximately 80% of the
target gene, and decreased the mortality from approximately 80% in Cry1Ca-ingested
larvae to about 60% in Cry1Ca and *SeCad1b*-dsRNA exposed larvae. Moreover,
ingestion of *SeCad1b*-dsRNA partially alleviated the negative effect of Cry1Ca on
larval growth. Similarly, RNA interference knockdown of cadherin receptors led to
susceptibility decrease to Cry toxins in lepidopteran (35, 41) and coleopteran (15)
insect species. Moreover, the Cry1Ab resistance in *D. saccharalis* is likely associated
with the reduction in expression of cadherin gene *DsCAD1* (35). Comparably, when
6th instar larvae of *S. littoralis* were released on castor leaf discs coated with 6 μg of
Cry1C, 61% of the larvae were killed and all the surviving larvae failed to pupate. In contrast, when the larvae were injected 4 μg of dsRNA of aminopeptidase N receptor before Cry1C exposure, the transcript level of target gene slapn in midgut reduced by 95%, only 15% of the treated larvae were killed and 70% of the surviving treated larvae successfully pupated. Moreover, the gene silencing was retained during the insect’s moulting and development and transmitted to the subsequent generation albeit with a reduced effect (36). The results obtained in the present paper and have reported by Rajagopal et al. (2002) suggested that SeCad1b is a receptor of Cry1Ca, but it is less important than another Cry1Ca receptor aminopeptidase N.

Fourthly, cadherin receptor fragments containing the critical toxin-binding regions were able to enhance Cry toxin activity to lepidopteran (22-24), coleopteran (16, 25) and dipteran (13, 26) insects. Recently, it was reported that the cadherin fragment (CR10-MPED) significantly enhanced the toxicity of Cry1B and Cry1C to S. exigua larvae (24). Consistent with these results, we found that the toxicity of Cry1Ca and Cry1Ac was increased by rSeCad1bp and rHaBtRp respectively, in both S. exigua and H. armigera larvae.

Therefore, we provide the first lines of evidence in the present paper to suggest that SeCad1b from S. exigua is a functional receptor of Cry1Ca. Similarly, cadherins in seven lepidopteran, two dipteran and two coleopteran species have been confirmed or suggested to be receptors for other Cry toxins (2, 12-16).

In addition, we found that rSeCad1bp showed higher enhancement of Cry1Ca activity, whereas rHaBtRp exhibited higher enhancement of Cry1Ac activity, in both
S. exigua and H. armigera larvae. Similarly, SfCad fragment from S. frugiperda greatly increase Cry1Fa toxicity to S. frugiperda larvae than MsBtR1 fragment from M. sexta (57). Our data support the hypothesis proposed previously by Chen et al. (2007) and Peng et al. (2010), that the binding of cadherin peptide to Cry toxin is a prerequisite for the peptide’s enhancement effect (22, 54).

However, both SfCad and MsBtR1 fragments bound Cry1Fa with high affinity and shared a common binding site on Cry1Fa toxin, but the former cadherin fragment exhibit more enhancement effect than the latter in S. frugiperda larvae (57). Similarly, both a soluble HaBtR fragment from H. armigera (27) and a HaBtR fragment produced in inclusion body (54) showed high affinity to Cry1Ac. However, the soluble HaBtR fragment reduces Cry1Ac insecticidal toxicity (27) whereas the HaBtR fragment produced in inclusion body enhances Cry1Ac insecticidal toxicity (54) to H. armigera larvae. These results suggested that other synergistic mechanisms (for example, soluble vs. inclusion body) rather than the cadherin binding affinity may also influence the synergistic ability of cadherin peptides (57). Our enhancement results of rSeCad1bp and rHaBtRp to different Cry toxins in S. exigua and H. armigera provide an excellent system to comparatively address the mechanisms. This finding deserves further research in the future.

Taken together, a cadherin-like protein SeCad1b from S. exigua was found to bind Cry1Ca. Reducing SeCad1b transcript level in actively feeding caterpillar overcame the negative effect on larval survival and growth caused by Cry1Ca toxicity. Moreover, cadherin SeCad1b and HaBtR could deferentially enhance the toxicity of
Cry toxins to *S. exigua* and *H. armigera* larvae. It was suggested that SeCad1b is a functional receptor of Cry1Ca.

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**REFERENCES**

12: 1407-1416.
insecticidal activity of *Bacillus thuringiensis* Cry1A toxins by fragments of a toxin-binding cadherin correlates with oligomer formation. Peptides **30**:583-588.


26
277:46849-46851.
37. Agrawal N, Malhotra P, Bhatnagar RK. 2004. siRNA-directed silencing of
320:428-434.
insect cell-expressed aminopeptidase N of Spodoptera litura with insecticidal
crystal protein Cry1C. Appl. Environ. Microbiol. 68.
thuringiensis Cry1Ca-resistant Spodoptera exigua lacks expression of one of four
Aminopeptidase N genes. BMC Genom. 6:96.
role of Bacillus thuringiensis Cry1C and Cry1E separate structural domains in the
interaction with Spodoptera littoralis gut epithelial cells. J. Biol. Chem.
279:15779-15786.
Engineering modified Bt toxins to counter insect resistance. Science
318:1640-1642.
42. Xiao K, Shen K, Zhong J-F, G.-Q. L. 2010. Effects of dietary sodium on
performance, flight and compensation strategies in the cotton bollworm (Hubner)
(Lepidoptera: Noctuidae). Front. Zool. 7:11.
43. Stamatakis A. 2006. RAxML-VI-HPC: maximum likelihood-based phylogenetic
analyses with thousands of taxa and mixed models. Bioinformatics
22:2688-2690.
45. Bradford MM. 1976. A rapid and sensitive method for the quantitation of
microgram quantities of protein utilizing the principle of protein-dye binding.
46. Atsumi S, Mizuno E, Hara H, Nakanishi K, Kitami M, Miura N, Tabunoki H,
Watanabe A, Sato R. 2005. Location of the Bombyx mori aminopeptidase N type
1 binding site on Bacillus thuringiensis Cry1Aa toxin. Appl. Environ. Microbiol.
71:3966-3977.
47. Lu D, Yang J, Li G-Q. 2010. Preliminary study on techniques for bacterial
expression of dsRNA from proline hydrogenase gene in Leptinotarsa
real-time RT’PCR. Nucleic Acids Res. 29:e45.
49. Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A,
Speleman F. 2002. Accurate normalization of real-time quantitative RT-PCR data
by geometric averaging of multiple internal control genes. Gen. Biol.
3:research0034.
50. Ikawa S, Tsuda Y, Fukada T, Sugimoto K, Himeno M. 2000. cDNA cloning of
the Cry1Aa receptor variants from Bombyx mori and their expression in


Abbreviations: The abbreviations used are: Bt, Bacillus thuringiensis; Cry, crystal protein; GPI, glycosylphosphatidylinositol; SP, signal peptide; CR, cadherin repeats; MPED, membrane-proximal extracellular domain; TM, transmembrane region; IC, internal cytoplasmic domain; IPTG, isopropyl β-D-thiogalactoside; BSA, bovine serum albumin; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride membrane; NC, nitrocellulose filter; PBS, phosphate buffered saline;
(TnCad, AEA29692), *Pectinophora gossypiella* (PgBtR, AAP30715), *Plutella xylostella* (PxCad, ABU41413), *Diatraea saccharalis* (DsCad1, AFI81418), *Chilo suppressalis* (CsCad, AAM78590) and *Danaus plexippus* (DpCad, EHJ65251).

Numbers on interior branches are bootstrap values. B. Temporal expression patterns of SeCad1b from cDNA templates derived from the third- (3rd) and fifth- (5th) instar larvae, male and female pupae (MP and FP) and sexually mature male and female adults (MA and FA). C. Tissue specific expression of SeCad1b from cDNA templates derived from foregut (FG), midgut (MG), hindgut (HG), fat body (FB), Malpighian tubules (MT), and remaining carcass (RC) of the fifth-instar larvae. The mean ± SE was calculated to measure the relative transcript levels by the $2^{-\Delta\Delta Ct}$ method, using the geometric mean of *GAPDH* and *EF* genes for normalization.

**FIGURE 2.** Competition binding assays of Cry1Ac and Cry1Ca with rSeCad1bp and rHaBtRp. rSeCad1bp (4 µg) and rHaBtRp (4 µg) were respectively probed with a mixture of a biotinylated toxin (for rSeCad1bp, Cry1Ca 0.4 µg/mL, Cry1Ac 2 µg/mL; for rHaBtRp, Cry1Ca 2 µg/mL, Cry1Ac 0.4 µg/mL) and either of the unlabeled Cry1Ca and Cry1Ac at the weight ratio of 1:0, 1:50 and 1:500.

**FIGURE 3.** Negative effects of dsRNAs and Cry1Ca alone and in a mixture to the survival and growth of *Spodoptera exigua* larvae. (A) The expression of SeCad1b after dietary ingestion of SeCad1b-dsRNA. The relative amount of SeCad1b transcript in controls either feeding normal diet or on egfp-dsRNA-overlaid diet, and in larvae feeding SeCad1b-dsRNA-overlaid diet was compared, normalized to the expression of *GAPDH* and *EF*. (B) Mortalities (%) and (C) weight (mg) of *S. exigua* larvae feeding normal diet, or feeding diet overlaid by egfp-dsRNA, SeCad1b-dsRNA, Cry1Ca, Cry1Ca+egfp-dsRNA and Cry1Ca+SeCad1b-dsRNA. Bars that do not share the same lowercase or uppercase letter are significantly different at $P = 0.05$ or $P = 0.01$.

**FIGURE 4.** Enhancement of cadherin fragment rSeCad1bp and rHaBtRp to the negative effect of Bt toxins on the survival of *Spodoptera exigua* (A) and *Helicoverpa armigera* larvae (B). The larvae respectively feed normal diet, or feed diet overlaid by rHaBtRp, rSeCad1bp, Cry1Ca, Cry1Ca+rHaBtRp, Cry1Ca+rSeCad1bp, Cry1Ac, Cry1Ac+rHaBtRp or Cry1Ac+rSeCad1bp. Bars that do not share the same lowercase or uppercase letter are significantly different at $P = 0.05$ or $P = 0.01$.

**SUPPLEMENT MATERIAL** SeCad1b clone and characterization in *Spodoptera exigua*. A. Nucleotide primers used to obtain SeCad1b cDNA by 5'- and 3'-RACE, generate rSeCad1bp and rHaBtRp peptides, synthesize dsRNA, and perform qRT-PCR. B. Amino acid sequence and domain structure of SeCad1b from *Spodoptera exigua*. C. Overexpression and purification of rSeCad1bp and rHaBtRp. D. Enhancement of cadherin fragment rSeCad1bp and rHaBtRp to the negative effect of Bt toxins on the growth of *Spodoptera exigua* and *Helicoverpa armigera* larvae.
Fig 1
Fig 2

<table>
<thead>
<tr>
<th>rSeCad1bp</th>
<th>biotinylated Cry1Ca</th>
<th>biotinylated Cry1Ac</th>
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Toxin ratio: 1:0, 1:50, 1:500
Fig 4

A

Mortality (%)

rHaBtRp   rSeCadhp   Cry1Ca   Cry1Ac
-   -   -   -
+   +   +   +
-   -   +   +
-   -   -   -

B

Mortality (%)

rHaBtRp   rSeCadhp   Cry1Ca   Cry1Ac
-   -   -   -
+   +   +   +
-   -   +   +
-   -   -   -