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Fluorescent-based assays establish *Manduca sexta* Bt-R_{1a} cadherin as a receptor for multiple *Bacillus thuringiensis* Cry1A toxins in *Drosophila* S2 cells

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Abstract

A fluorescence-based approach was developed to analyze in vivo the function of *Manduca sexta* cadherin (Bt-R_1) as a Cry1 toxin receptor. We cloned a Bt-R_{1a} cDNA that differs from Bt-R_1 by 37 nucleotides and two amino acids and expressed it transiently in *Drosophila melanogaster* Schneider 2 (S2) cells. Cells expressing Bt-R_{1a} bound Cry1Aa, Cry1Ab, and Cry1Ac toxins on ligand blots, and in saturation binding assays. More Cry1Ab was bound relative to Cry1Aa and Cry1Ac, though each Cry1A toxin bound with high-affinity (Kd values from 1.7 to 3.3 nM). Using fluorescent microscopy and flow cytometry assays, we show that Cry1Aa, Cry1Ab and Cry1Ac, but not Cry1Ba, killed S2 cells expressing Bt-R_{1a} cadherin. These results demonstrate that *M. sexta* cadherin Bt-R_{1a} functions as a receptor for the Cry1A toxins in vivo and validates our cytotoxicity assay for future receptor studies.

Keywords: *Bacillus thuringiensis*; Bt-R_1; Cry toxin; *Drosophila* S2 cells; Cadherin; Cytotoxicity assay

1. Introduction

Cry toxins from *Bacillus thuringiensis* (Bt) have remarkable toxicity to certain insects. As active components of Bt spore—crystal formulations or insecticidal proteins in transgenic plants, Cry toxins provide effective control of lepidopteran pests on crops and forests (Shelton, 2002). Cry toxins are also components of larvicides such as Bt *israelensis* that are effective against mosquitoes that transmit disease.

Once Bt toxin crystals are ingested by a susceptible insect, they undergo various sequential steps that result in gut paralysis and insect death. Crystal proteins are solubilized and activated by midgut proteases to active Cry toxins, which bind to receptor proteins located on the midgut epithelium. After contact with receptors, toxins unfold and insert into the epithelial membrane (Aronson and Shai, 2001), causing osmotic cell lysis.

Cadherins, the subject of this study, are a class of Cry1 receptor proteins. The cadherin-like protein Bt-R_1 from *Manduca sexta* binds Cry1Aa, Cry1Ab and Cry1Ac toxins on ligand blots (Francis and Bulla, 1997). Purified membranes from COS cells expressing Bt-R_1 bound all three Cry1A toxins in binding assays and ligand blots (Keeton and Bulla, 1997). Furthermore, expression of Bt-R_1 on the surface of COS7 cells led to toxin-induced cell toxicity as monitored by immunofluorescence microscopy with fixed cells (Dorsch et al., 2002). Cadherin-like Bt-R_1 protein has been suggested to induce a conformational change in Cry1Ab that allows the formation of a pre-pore toxin oligomer (Gomez et al., 2002). In *Bombyx mori*, the cadherin-like protein BtR175 serves as a Cry1Aa receptor (Nagamatsu et al., 1998). Sf9 cells expressing BtR175 swell after exposure to Cry1Aa toxin, presumably due to formation of ion channels in cell membranes (Nagamatsu et al., 1999). When expressed in

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mammalian COS7 cells, BtR175 induced susceptibility to Cry1Aa (Tsuda et al., 2003). Involvement of a cadherin-superfamily gene disruption in resistance to Cry1Ac has been described for a laboratory resistant strain of *Heliothis virescens* (Gahan et al., 2001), although no cadherin-like proteins have been purified from this insect.

Cry toxins cause a cytotoxic response in some insect cell lines, and frequently cell swelling served as a marker of cytotoxicity (Himeno and Ihara, 1995; Knowles and Ellar, 1987; Kwa et al., 1998; McCarthy, 1994). Cell lines not susceptible to Cry1 toxins can be used to test receptor function in vivo. Recently, Tsuda et al. (2003) used tetrazolium salt reduction and release of lactate dehydrogenase activity to quantify Cry1Aa susceptibility of COS7 cells expressing BtR175 cadherin. To our knowledge, no in vivo measurements of the Cry1A-susceptibility of cultured cells expressing Bt-R_1 cadherin have been reported to date.

In this study, we test the hypothesis that Bt-R_1 cadherin is a functional receptor for Cry1Aa, Cry1Ab and Cry1Ac toxins using a fluorescence approach to monitor cytotoxicity in vivo. We cloned and expressed a modified Bt-R_1 protein (Bt-R_{1a}) in *Drosophila melanogaster* Schneider 2 (S2) cells, using co-expression with green fluorescent protein (GFP) as an indicator of percentage of cell transfection in a culture. Using the fluorochrome propidium iodide (PI) as cytotoxicity marker, we monitored susceptibility of S2 cells expressing Bt-R_{1a} to Bt Cry1 toxins. Our results demonstrate that Bt-R_{1a} cadherin functions as a Cry1A toxin receptor in vivo and authenticate the cell expression and cytotoxicity detection systems used for future Cry toxin receptor studies.

2. Materials and methods

2.1. Cloning of Bt-R_1 cadherin cDNA

M. sexta larvae were reared on artificial diet (Southland Products, Inc., Lake Village, AR) at 26 °C, 70% relative humidity with a photoperiod of 12:12 (light: dark) h. Midguts dissected from early 5th instar larvae were soaked in RNAlater (Ambion) for 1 h on ice, then total RNA prepared using RNeasy (Qiagen). First strand cDNA was synthesized from 5 μg RNA with oligo-dT(18) primer, 0.25 mM dNTPs and Superscript reverse transcriptase (Gibco-BRL), according to the manufacturer.

Two pairs of primers were designed from the Bt-R_1 sequence (GenBank AF319973):Ms-Cad/F1: 5′-GGCAGCAAAACCATCTGCAGCAACAAAATCATCTG; Ms-Cad/R1: 5′-GGTCGGGAAGACGAACTCAGGCGCGTAGTAGTTGA; Ms-Cad/F2: 5′-CGGGAGAGCTGGAGACCGCCATGGACCTCAAGGGA; Ms-Cad/R2: 5′-TTATCCGCGGGATCTTTCTGAACTGTCCGTTA-ACG. Primers were manufactured by Integrated DNA Technologies, Inc. (IDT). PCR products were amplified using synthesized cDNA as template to facilitate reconstruction of a full-length open reading frame. PCR was performed using the following procedure: 94 °C 5 min → 94 °C 30 s, starting at 70 °C annealing temperature was decreased one degree per cycle using the thermocycler touchdown feature until 56 °C (total of 15 cycles each for 30 s), 72 °C 4 min → 94 °C 30 s, 55 °C 30 s, 72 °C 4 min for 20 cycles → 72 °C 10 min → 4 °C. PCR products were cloned into pGEM-Teasy (Promega) and sequenced at the Molecular Genetics Instrumentation Facility (University of Georgia) in both forward and reverse directions to obtain the complete sequence of the 5′ and 3′ gene fragments. The 3′ cDNA of cloned cadherin was excised from pGEM-Teasy with *Nco* I and *Sac* II and cloned into pMECA (Genbank AF017063; Thompson and Parrott, 1998), and the resulting plasmid called pMECA-Cad1.9. The 5′ gene fragment was cloned upstream of pMECA-Cad1.9 to form pMECA-Cad. Finally, the full-length *BtR_{1a}* gene was reconstructed in the expression plasmid pIZT (Invitrogen) to obtain pIZT-Cad.

2.2. Preparation and labeling of Bt Cry toxins

Bt strains HD-37 and HD-73, producing Cry1Aa and Cry1Ac proteins, respectively, were obtained from the *Bacillus* Genetic Stock Center (Columbus, OH). An *Escherichia coli* strain producing Cry1Ab was provided by Dr. Luke Masson (Biotechnology Research Institute, Montreal, Canada). Growth of bacterial strains, purification and activation of Cry1A toxins was as previously described (Luo et al., 1999). Purified toxin samples (as determined by SDS-PAGE) were pooled, quantified by the Bradford protein assay (Bradford, 1976) using BSA as standard and stored at −80 °C until used.

Toxins (1 μg for ligand blots and specific binding assays or 10 μg for saturation binding assays) were radiolabeled with 0.5 mCi of $Na^{125}I$ (Amersham) using iodo-beads (Pierce) following the manufacturer's instructions. Specific activities were 30–40 μCi/μg (when labeling 1 μg of toxin) or 4–7 μCi/μg (when labeling 10 μg), based on input toxin.

2.3. Preparation of BBMV

Midguts were dissected from second day 5th instar larvae, and either immediately used to prepare total RNA or stored at −80 °C until used to prepare brush border membrane vesicles (BBMV). BBMV were prepared according to (Wolfersberger et al., 1987) and stored in 0.3 M mannitol, 5 mM EGTA, 17 mM Tris–Cl, pH 7.5,

at −80 °C until needed. Protein amount was determined according to (Bradford, 1976) using BSA as standard.

2.4. Specific binding of ^{125}I-Cry1A toxins to Bt-R_{1a} expressed on S2 cells

Specific ^{125}I-Cry1A binding to S2 cells transfected with pIZT or pIZT-Cad was measured using a membrane vesicle assay (Jurat-Fuentes and Adang, 2001) modified for insect cells. In these binding saturation assays, increasing amounts of ^{125}I-Cry1A toxins were incubated with 1×10^7 S2 cells (11 μg total protein as quantified using the method of Bradford, 1976) in 100 μl of binding buffer (137 mM NaCl, 2 mM KCl, 10 mM Na_2HPO_4, 1.7 mM KH_2PO_4, pH 7.5 plus 0.1% BSA) for 1 h at room temperature. Binding reactions were stopped by centrifugation and pellets washed once with 1 ml of ice-cold binding buffer. Radioactivity of the final pellets was counted in a Beckman model 4000 Gamma detector. Non-specific binding was measured as the amount of ^{125}I-Cry1A toxin bound in the presence of 1000 nM homologous unlabeled toxin in the binding reaction. Dissociation constants (K_{com}) and concentration of binding sites (B_{max}) for ^{125}I-Cry1A toxins were calculated using the KELL software (BIOSOFT, Cambridge, UK). Data shown are the means obtained from two experiments done at least in duplicate for each toxin.

2.5. Transient expression of Bt-R_{1a} in *Drosophila* S2 cells

Drosophila S2 (Invitrogen) cells were grown in serum-free insect cell media (Hyclone) and transfected as previously described (Banks et al., 2003). DNA for pIZT and pIZT-Cad plasmids was prepared using a Plasmid Maxi Kit (Qiagen). Approximately 1.5×10^6 S2 cells from a confluent culture were resuspended in 5 ml fresh media warmed to room temperature and allowed to adhere to 60 × 15 mm polystyrene dishes (Falcon) overnight. Plasmid transfection mixtures were prepared by mixing either 8 μg of pIZT or 20 μg of pIZT-Cad plasmid with 1 ml of serum-free insect medium (Hyclone) and 10 μl of Cellfectin reagent (Invitrogen). Four milliliters of medium containing 50 U of a penicillin-streptomycin cocktail per milliliter (Invitrogen) were added, and then the cells incubated at 25 °C for 2.5 days.

2.6. Immunoblot and ligand blot analysis

Drosophila S2 cells were seeded and transfected as described above. At 2.5 days post-transfection, aliquots of 1×10^7 cells were harvested by centrifugation at 2000 rpm for 2 min, and then washed three times with PBS (135 mM NaCl, 2 mM KCl, 10 mM Na_2HPO_4, 1.7 mM KH_2PO_4, pH 7.5). The cell pellets were resuspended in 200 μl SDS-PAGE sample buffer (Laemmli, 1970), heated in a 100 °C water bath for 10 min then centrifuged at 12,000 g for 5 min. A 20 μl aliquot of supernatant was separated by 8% SDS-PAGE and transferred to a PVDF filter (Millipore Corp., Bedford, MA). Filters were blocked with 5% skim milk in PBST (PBS + 0.1% Tween-20) at room temperature for 1 h, then probed with anti-BTR_1 antibody (kindly provided by R. Flannagan, Pioneer) in PBST + 0.1% skim milk for 2 h. After washing with PBST + 0.1% skim milk, filters were incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (Sigma) in PBST at room temperature for 1 h. BTR_1 protein was visualized using chemiluminescent substrate (ECL; Amersham Biosciences, Piscataway, NJ) and photographic film.

For ligand blotting, filters were blocked as for immunoblots, and then probed with 0.25 nM ^{125}I-Cry1A toxins for 1 h at room temperature. To demonstrate specificity of binding, 500-fold unlabeled homologous toxin was used as competitor. After washing with PBST + 0.1% skim milk, filters were exposed to X-ray film overnight.

2.7. Toxicity assays

S2 cells were transfected and incubated for 2.5 days, then 5×10^6 cells transferred to a 25 × 15 mm^2 tissue culture dish (Falcon). Fresh media with 20 μg/ml of Cry1 toxin (approximately 330 nM toxin concentration) was added and the cells incubated with gentle shaking for 2.5 h. Cells were stained with 4 μM (final concentration) of propidium iodide (PI) for 10 min, then immediately observed for GPF fluorescence and PI staining using a Leica DM *IRE*2 inverted fluorescent microscope.

For flow cytometry analyses, a FACSCalibur (Becton Dickinson) instrument was used. The results are the mean of three independent experiments done at least in triplicate. Treatments were conducted on two dishes of 3×10^6 cells, two samples of 10,000 cells from each dish being analyzed by flow cytometry. Cells were treated with toxin as for fluorescent microscopy, then gated for size determination by comparing forward scattered and side scattered light dual parameters. For GFP fluorescence, cells were excited with krypton-argon laser, and emission was monitored with a 530/30 nm band-pass filter. These GFP-gated cells were then examined for PI fluorescence by monitoring emission with a 585/42 nm band-pass filter.

A simple formula was developed to calculate the percentage of GFP positive cells in the pIZT-Cad cell populations killed by Cry1A toxins:

$$\%\ \text{GFP Dead Cells} = \left[\frac{\left(\text{GFP}^{+}\text{PI}^{+}\text{Cells}_{\text{Toxin}} + \text{GFP}^{-}\text{PI}^{+}\text{Cells}_{\text{Toxin}}\right) - \left(\text{GFP}^{+}\text{PI}^{+}\text{Cells}_{\text{control}} + \text{GFP}^{-}\text{PI}^{+}\text{Cells}_{\text{control}}\right)}{\text{GFP}^{+}\text{Cells}_{\text{Toxin}}}\right] \times 100$$

This formula accounts for the dead cells (PI-positive) in an untreated population, GFP positive dead cells that lost GFP due to cell leakage, and the observed transfection efficiency (GPF-positive) for an experiment.

3. Results

A cDNA encoding cadherin Bt-R_{1a} (AY094541) was cloned by PCR from *M. sexta* midgut cDNA. Bt-R_{1a} differs from Bt-R_1 (AF319973) by 37 nucleotides and 2 amino acid substitutions (His^{350} to Leu^{350} and Ala^{1189} to Glu^{1189}). The Bt-R_{1a} cDNA encodes a predicted 1717 amino acid pro-protein with 12 predicted cadherin repeats (Dorsch et al., 2002), a trans-endoplasmic reticulum signal peptide and a hydrophobic transmembrane domain.

The Bt-R_{1a} cDNA was transiently expressed in *Drosophila* S2 cells using the pIZT-Cad plasmid. Dual constitutive baculovirus promoters control Bt-R_{1a} and GFP-zeocin expression in pIZT-Cad. GFP provided a quick screening tool for visualizing transfected cells with an inverted fluorescent microscope. Typically, 40% to 50% of the transfected S2 cells in a culture dish showed strong GFP fluorescence at 2.5 days post-transfection. Expression of Bt-R_{1a} in S2 cells was detected by probing blots of total cell protein with anti-Bt-R_1 serum (Fig. 1). S2 cells transfected with pIZT-Cad expressed a 200-kDa Bt-R_{1a} protein that migrated slightly faster on SDS-PAGE than 210-kDa Bt-R_1 from *M. sexta* BBMV. A relatively minor expression protein product of 190-kDa in size, was also specific to pIZT-Cad transfected cells.

Blots of proteins from S2 cells transfected with pIZT-Cad were probed with ^{125}I-labeled Cry1Aa, Cry1Ab or Cry1Ac toxins (Fig. 1). ^{125}I-Cry1Aa and Cry1Ab bound 200- and 190-kDa forms of BtR_{1a} in S2 cells. In BBMV, these Cry1A toxins bound 210-kDa Bt-R_1 cadherin, plus a 120-kDa protein, most likely APN. The pattern for Cry1Ac binding was more complex revealing a high-degree of non-specific binding. ^{125}I-Cry1Ac bound 210-kDa Bt-R_1 in S2 cells and BBMV and the 120-kDa APN in BBMV (Knight et al., 1994; Sangadala et al., 1994). Binding of ^{125}I-Cry1A toxins to Bt-R_{1a} was specific as noted by elimination of binding in the presence of respective homologous unlabeled competitor.

Since Bt-R_1 is a high affinity binding protein for Cry1A toxins (Vadlamudi et al., 1995), we tested S2 cells expressing Bt-R_{1a} for the capacity to specifically bind ^{125}I-Cry1Aa, ^{125}I-Cry1Ab and ^{125}I-Cry1Ac. Control S2 cells transfected with pIZT bound 0.04% of input ^{125}I-Cry1Aa and 0.07% of input ^{125}I-Cry1Ac, while no specific binding of ^{125}I-Cry1Ab to these cells was detected. Under the same conditions, S2 cells transfected with pIZT-Cad bound 0.13%, 0.12%, and 1.8% of input ^{125}I-Cry1Aa, ^{125}I-Cry1Ab and ^{125}I-Cry1Ac, respectively. Quantitative parameters for ^{125}I-Cry1A binding to Bt-R_{1a} on S2 cells were calculated from saturation binding assays. In these assays, a constant

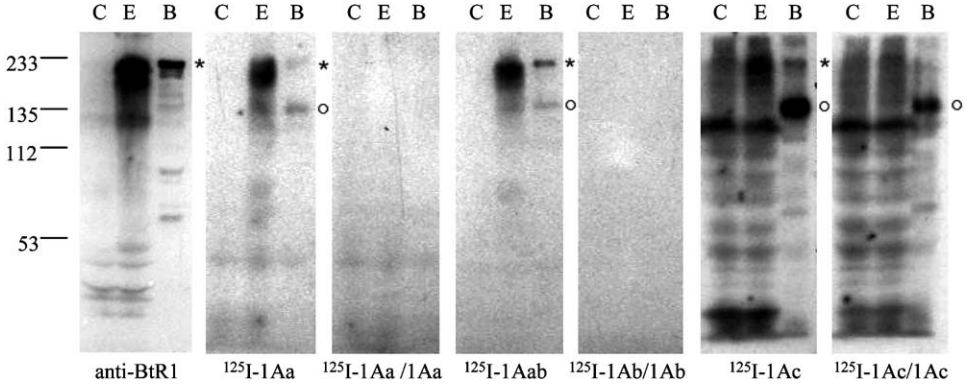

Fig. 1. Expression of Bt-R_{1a} in S2 cells transfected with pIZT-Cad and Cry1A toxin binding to expressed Bt-R_{1a} on blots. Cell proteins were separated by SDS-PAGE and then transferred to PVDF filters. After blocking, filters were probed with anti- Bt-R_1 sera or ^{125}I-labeled Cry1A toxins. Lane designations: C, S2 cells transfected with pIZT vector; E, cells transfected with pIZT-Cad; and B, *M. sexta* BBMV. (*) denotes Bt-R_1 cadherin protein and (○) denotes 120-kDa APN.

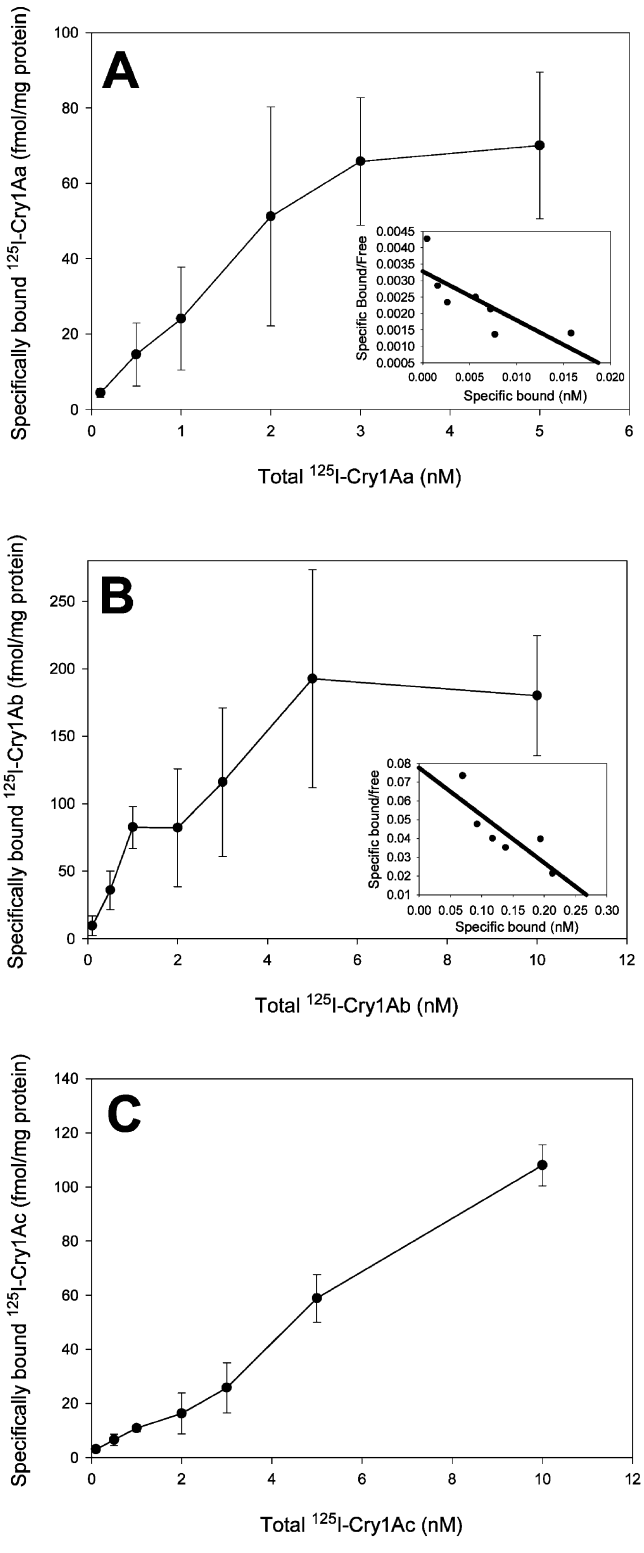

Fig. 2. Saturation of ^{125}I-Cry1A binding to S2 cells expressing Bt-R1$_a$. Cells were incubated with increasing ligand (^{125}I-Cry1A) concentrations for 1 h. Toxin binding was stopped by centrifugation. Data shown are specific binding values determined by subtraction of non-specific binding in the presence of 1000 nM unlabeled toxin. Bars depict the standard error of the mean calculated from five replicates. Scatchard plot transformation of data is shown in the inset.

concentration of S2 cells transfected with pIZT-Cad was incubated with increasing amounts of ^{125}I-Cry1A toxins in the presence or absence of excess homologous unlabeled toxin. As shown in Fig. 2, S2 cells expressing Bt-R_{1a} bound ^{125}I-Cry1Aa and ^{125}I-Cry1Ab toxins in a saturable manner. We observed high variation in ^{125}I-Cry1A toxin binding, with much of the variation occurring between experiments using different populations of transfected cells. Analysis by non-linear regression of the binding saturation data resulted in a binding affinity of $K_{com} = 1.7 \pm 0.5$ nM for ^{125}I-Cry1Aa and $K_{com} = 3.2 \pm 0.9$ nM for ^{125}I-Cry1Ab. Concentration of binding sites were $B_{max} = 59 \pm 13$ fmol/mg protein for ^{125}I-Cry1Aa and $B_{max} = 255 \pm 6$ fmol/mg protein for ^{125}I-Cry1Ab. As shown in Fig. 2C, we did not observe saturation of ^{125}I-Cry1Ac binding even at the highest ligand concentration tested. This observation agrees with the high levels of non-specific binding of this toxin observed in ligand blots of S2 cell proteins (Fig. 1). In saturation binding assays it is often difficult to discriminate between non-saturable binding and a low affinity binding site (Jones, 1982). Although we observed a change in the slope of the ^{125}I-Cry1Ac binding saturation curve at 2 nM of total ligand input, accurate quantitative parameters for binding could not be calculated for this potential binding site due to the non-saturable Cry1Ac binding behavior.

A functional toxin receptor on S2 cells, e.g. Bt-R_{1a}, should not only bind Cry1A toxins with high affinity, but should also catalyze toxin-induced cell death. This possibility was tested using GFP and PI as markers for transfected cells and for cell death, respectively. *Drosophila* S2 cells transfected with pIZT or pIZT-Cad plasmids produced an intense green fluorescence that was easily seen with an inverted fluorescent microscope (Fig. 3). Cytotoxicity was visualized with the inverted scope by adding PI to the culture dish. PI enters cells through damaged cytoplasmic membranes and binds to nucleic acids, producing a bright red fluorescence in damaged or dead cells. Cry1A and Cry1Ba toxins at 20 μg/ml were not cytotoxic to cells transfected with pIZT (Fig. 3). In contrast, Cry1Aa, Cry1Ab, and Cry1Ac, but not Cry1Ba were cytotoxic to S2 cells transfected with pIZT-Cad.

Flow cytometry provided a quantitative measure of the percentage of transfected cells (GFP-positive) and cytotoxic response (i.e. PI-positive). FACS was gated to count 10,000 cells for each sample for GFP and PI fluorescence. Fig. 4 shows representative scatter plots from a single experiment designed to test Cry1Ab toxicity to cells transfected with pIZT-Cad. Composite data are presented adjacent to the scatter diagrams. Similar experiments were conducted with Cry1Aa and Cry1Ac. The transfection protocol for S2 cells was efficient, since 56.2% of the pIZT and 41.5% of the pIZT-Cad S2 cells were GFP-positive (Fig. 4). For

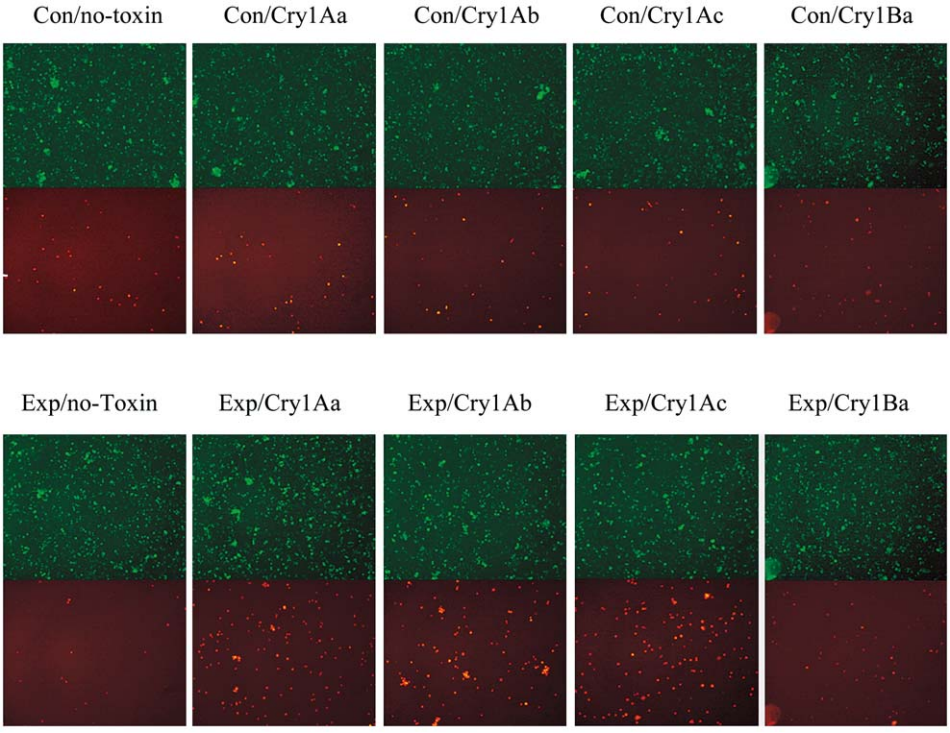

Fig. 3. Fluorescent images of transfected S2 cells and cytotoxicity of Cry1A toxins. S2 cells were transfected and treated with Cry1A toxins (20 μg/ml) or left untreated for 2 h. Cells were stained with propidium iodide (PI) and observed for GPF fluorescence and PI staining using a Leica DM *IRE*2 inverted fluorescent microscope. Upper panel: cells transfected with pIZT; lower panel: cells transfected with pIZT-Cad.

pIZT-transfected cells, the number of dead (PI-positive) cells did not increase after incubation with Cry1Aa, Cry1Ab, Cry1Ac or Cry1Ba treatments when compared to untreated cultures. In contrast, Cry1A cytotoxicity to pIZT-Cad cells was detected as a higher proportion of PI-positive (dead) cells, and slightly fewer GFP positive cells (Fig. 4). We explain these changes as due to pIZT-Cad S2 cells being permeabilized by Cry1A toxins, allowing PI to enter cells and causing some loss of GFP-associated fluorescence. Fig. 5 shows that each Cry1A toxin killed 12–14% of the pIZT-Cad transfected (GFP-positive) cells. Flow cytometry data were in agreement with fluorescent microscopy observations supporting the conclusion that Bt-R_{1a} cadherin

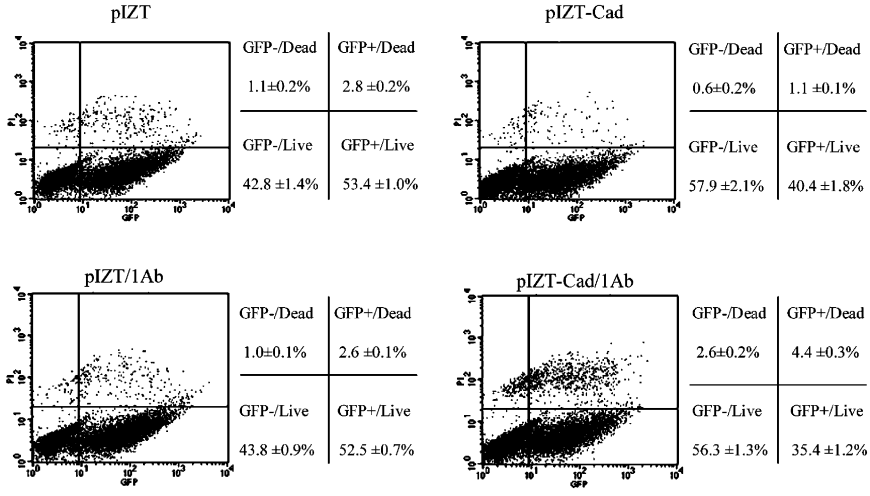

Fig. 4. Representative FACS scatter plots and quantification of Cry1Ab-mediated cytotoxicity to cells transfected with pIZT or pIZT-Cad. Transfected cells were treated as for Fig. 3, and then 10,000 cells per treatment were gated through a FACSCalibur (Becton Dickinson) flow cytometer. Each cell was gated for live (PI−) or dead (PI+), and GFP fluorescence (GFP+ or GFP−). The mean percentages of counted cells are presented adjacent to a representative scatter plot for a single experimental treatment. Error values are standard deviation of the mean.

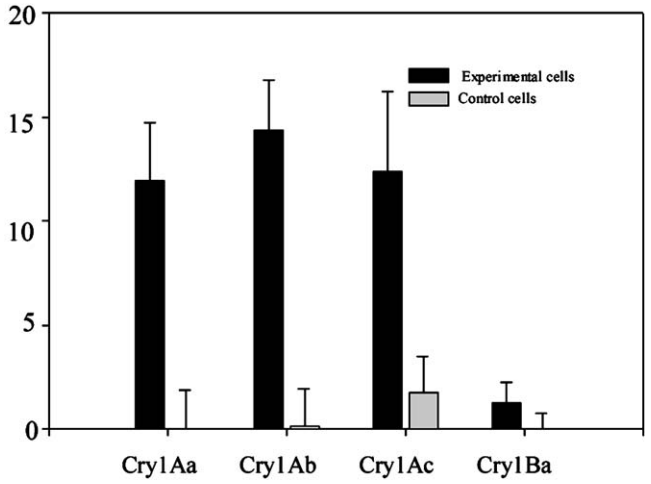

Fig. 5. Percentage of GFP+/PI+ (dead) S2 cells transfected with pIZT-Cad after treatment with various Cry1 toxins for 2 h. Transfected cells were treated as described for Fig. 3, and gated by FACS as in Fig. 4. The formula described in Materials and methods was then used to calculate percentages of GFP expressing cells that die (PI+) after toxin treatment. Values shown are the mean of three independent experiments with different transfected cell cultures conducted at least in triplicate. Bars denote standard deviation of the mean.

mediates toxicity of Cry1Aa, Cry1Ab and Cry1Ac toxins in vivo.

4. Discussion

We established functionality of Bt-R_{1a} cadherin from *M. sexta* as a receptor for Cry1Aa, Cry1Ab and Cry1Ac toxins in insect cells. This was accomplished using S2 cells and a dual-promoter plasmid for expressing GFP and Bt-R_{1a}. Transfected cells and Cry toxin-mediated cytotoxicity were easily visualized by fluorescent microscopy and cell events quantified by flow cytometry.

Bt-R_{1a} cDNA differs from Bt-R_1 by 37 nucleotides. While there is a possibility that Taq DNA polymerase introduced errors during PCR, it is more likely that Bt-R_1 and Bt-R_{1a} are alleles for the following reasons. Southern blot analyses of *M. sexta* genomic DNA revealed at least two homologues of Bt-R_1 (Franklin et al., 1997). Another sphingid, *B. mori*, has cadherin cDNAs that differ from BtR175 by 1–6 amino acids (Ikawa et al., 2000). *Pectinophora gossypiella*, a noctuid Lepidoptera, has three alleles each of which is disrupted in Bt-resistant larvae (Morin et al., 2003). It will be interesting to determine if multiple cadherins from allelic variations in Lepidoptera differ in binding of Bt toxins.

Insect and mammalian cell cultures recognized the signal and transmembrane sequences for Bt-R_1-type proteins correctly and expressed the heterologous proteins on their surfaces. However, Bt-R_1-type proteins expressed in S2, Sf9 cells and COS7 cells had apparent molecular sizes slightly smaller (200- versus 210-kDa) than Bt-R_1 in *M. sexta* BBMV (this study; Keeton and Bulla, 1997; Meng et al., 2001). The most likely explanation for this size difference is a deficiency in a glycosylation step in cultured cells relative to midgut epithelial cells. In agreement with previous reports that refute a role for glycosylation in Cry1A toxin binding to Bt-R_1 (Dorsch et al., 2002), this potential difference in glycosylation did not affect Cry1A toxin binding to Bt-R_{1a} in our ligand blot and binding saturation assays.

Previous authors (Keeton and Bulla, 1997) established Cry1Aa, Cry1Ab and Cry1Ac recognition of Bt-R_1 in cultured cells and BBMV. In our ligand blots, Bt-R_{1a} from S2 cells bound all three ^{125}I-Cry1A toxins, although the extent of binding was dependent on the toxin used. Recognition of Bt-R_{1a} in S2 cells was greatest for ^{125}I-Cry1Ab and ^{125}I-Cry1Aa whereas ^{125}I-Cry1Ac binding signal was weak. A similar pattern of recognition intensity was also observed in Bt-R_1 from *M. sexta* BBMV. Cry1A toxin recognition of both Bt-R_{1a} on S2 cells and BBMV was inhibited by homologous unlabeled toxin, demonstrating binding specificity. Binding of Cry1Ac toxin to additional S2 proteins on blots was not competed by excess unlabeled toxin, suggesting non-specificity. Reduced Cry1Ac binding to APN in BBMV was observed. Although the strength of Cry1Ab binding to Bt-R_{1a} on blots is consistent with its high affinity binding and receptor function for Cry1Ab in *M. sexta*, the denatured condition of blotted proteins is known to affect toxin recognition (Daniel et al., 2002).

Each Cry1A toxin bound to Bt-R_{1a} cadherin on S2 cells with high affinity under non-denaturing conditions in our binding saturation assays, in agreement with previous reports for Bt-R_1 expressed in Sf9 and COS7 cells (Dorsch et al., 2002; Vadlamudi et al., 1993). Interestingly, Cry1Ac binding was not saturated at the highest toxin concentration used in our assays. Since this toxin also bound non-specifically to several proteins on ligand blots of both control and experimental cells, we hypothesize the existence of a low affinity binding component in our binding assays. One possibility is interaction between Cry1Ac and S2 glycoproteins containing *N*-acetylgalactosamine. Analysis of the binding results at low ligand concentrations resulted in detection of a high affinity Cry1Ac binding site for cells expressing BTR_{1a}. As qualitatively detected in ligand blots, the highest binding to the cells was observed for Cry1Ab toxin, while Cry1Aa and Cry1Ac bound similar amounts of radiolabeled toxin. High variability in some of the binding measurements was observed when comparing results obtained from cells originated from different transfection events. We believe this disparity was a result of different levels of BTR_{1a} expression on the cells. Binding determinations

with the S2 cell line stably expressing Bt-R1a may yield more exact binding results.

The dual promoter expression plasmid with GFP has several advantages. The GFP marker provided facile detection of transfected cells and served as an internal control for each experimental treatment. A second advantage of dual promoter expression plasmids is that protein production correlates with the number of transfected cells and the amount of plasmid per cell. For example, in S2 cells a linear relationship was reported between the intensity of GFP fluorescence and the amount of human erythropoietin secreted (Shin and Cha, 2002). While the amounts of a large-sized protein (i.e. 200-kDa Bt-R_{1a}) will not equal a small-sized protein (30-kDa erythropoietin), the positive relationship between GFP and Bt-R_{1a} production should. Our results showing GFP and Bt-R_{1a} expression in S2 cells supports previous reports that baculovirus immediate early promoters function in S2 cells (Banks et al., 2003; Vanden Broeck et al., 1995). Therefore, GFP fluorescence serves as a quick screen for transfection and provides a baseline for comparing experiments. It is hoped that this correlation will apply to stably transformed S2 cell lines carrying a Bt-R_{1a} expression cassette.

The present method of PI staining detected Cry toxin cytoxicity to S2 cells expressing cadherin receptor. Upon Cry1Aa, Cry1Ab, and Cry1Ac exposure, the increased numbers of PI-positive cells in the pIZT-Cad transfected culture were readily visualized. We did not observe Cry1A-induced swelling of S2 cells as reported for Sf9 cells expressing BtR175 cadherin (Nagamatsu et al., 1999), nor did we observe cell lysis. The PI assay detected cytotoxic, i.e. necrotic, responses against a background of living cells. An alternative approach would be toxin treatment, cell fixation and inspection of cultures for damaged cells. This approach was taken by (Dorsch et al., 2002) with Bt-R_1 expressed in COS7 cells treated with Cry1Ab toxin. After fixing cells with paraformaldehyde, lysed cells that bound Cry1Ab were detected. The PI assay combined with a GFP marker has a significant advantage in that cells can be viewed directly by fluorescent microscopy, because cells carrying a receptor construct and responding to toxin fluoresce green and red.

Flow cytometry provided a method to quantify dead transfected cells as the ratio of PI-positive cells to total GFP-positive cells in a toxin-treated S2 cell culture. Cry1A toxins were cytotoxic to about 12–14% of the GFP-positive pIZT-Cad cells. The substantial amount of Bt-R_{1a} detected in transfected cells (Fig. 1) suggests that receptor was not limiting. Following are explanations that may justify the relatively low percentage of dead cells observed. One possibility is an adverse effect of the pH 6.4 cell culture medium on toxin-mediated cytotoxicity. For example, Cry1Ac is known to form smaller pores at pH 6.5 than at pH 10.5 (Binh Tran et al., 2001). This pH problem was solved for cultured insect cells using a 'lawn assay' where cells are suspended in an agarose gel and toxin in a high pH buffer applied to the surface (Gringorten et al., 1990). Unfortunately, this approach is not compatible with suspended cells and flow-cytometry. Another explanation is that components involved in toxin processing or cytoxicity are not present in the S2 cell system. For example, *M. sexta* midgut proteinases activate Cry1A protoxin differently than the commercial trypsin used in this study. Considering that midgut proteinase-activated toxin is more effective at pore formation in vitro (Miranda et al., 2001), this form may be more toxic to S2 cells expressing Bt-R_{1a}. Additionally, S2 cells may lack additional Cry1A-binding proteins necessary for optimal cytotoxicity. The 120-kDa APN of *M. sexta*, a well characterized Cry1Ac binding molecule (Sangadala et al., 2001; Sangadala et al., 1994), is a functional Cry1Ac receptor in transgenic *Drosophila* larvae (Gill and Ellar, 2002). This APN is also a component of lipid rafts, a membrane site where Cry1A toxins localize during the pore formation process (Zhuang et al., 2002). Assuming that combined action of both Bt-R_1 and APN may be necessary for optimal toxicity, expression of one of these molecules alone would result in limited cytotoxicity. Further investigation is needed to determine which of these factors limits Cry1A toxicity to S2 cells.

In some cultured cell types, loss of GFP-associated fluorescence has been considered a diagnostic indicator of cell death (Chu et al., 1999; Strebel et al., 2001). We observed evidence of this phenomenon, as a greater number (about 4%) of PI-positive GFP-negative cells were detected in pIZT-Cad transfected cells after Cry1A exposure. This phenomenon would under-estimate Cry1A toxin cytotoxicity by counting dead formerly GFP-positive cells as GFP-negative. We accounted for this underestimate of Cry1A-induced cytotoxicity in the formula to calculate the percentage of GFP-positive dead cells. Even without this adjustment PI staining was an excellent indicator of cytotoxicity and GFP fluorescence was retained in most dead S2 cells.

Overall, the S2 cell expression system combined with fluorescent markers proved to be an effective system for testing function of a known Bt receptor. This cell-based approach can serve as a platform for answering unresolved questions related to the nature of Bt toxin specificity and cellular interactions.

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References

Aronson, A.I., Shai, Y., 2001. Why *Bacillus thuringiensis* insecticidal toxins are so effective: unique features of their mode of action. FEMS Microbiol. Lett. 195, 1–8.

Banks, D., Hua, G., Adang, M.J., 2003. Cloning of a *Heliothis virescens* 110 kDa aminopeptidase *N* and expression in *Drosophila* S2 cells. Insect Biochem. Molec. Biol. 33, 499–508.

Binh Tran, L., Vachon, V., Schwartz, J.-L., Laprade, R., 2001. Differential effects of pH on the pore-forming properties of *Bacillus thuringiensis* insecticidal crystal toxins. Appl. Environ. Microbiol. 67, 4488–4494.

Bradford, M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Chu, Y.-W., Wang, R., Schmid, I., Sakamoto, K.M., 1999. Analysis with flow cytometry of green fluorescent protein expression in leukemic cells. Cytometry 36, 333–339.

Daniel, A., Sangadala, S., Dean, D.H., Adang, M.J., 2002. Denaturation of either *Manduca sexta* aminopeptidase *N* or *Bacillus thuringiensis* Cry1A toxins exposes epitopes hidden under non-denaturing conditions. Appl. Environ.Microbiol. 68, 2106–2112.

Dorsch, J.A., Candas, M., Griko, N., Maaty, W., Midboe, E., Vadlamudi, R., Bulla, L., 2002. Cry1A toxins of *Bacillus thuringiensis* bind specifically to a region adjacent to the membrane-proximal extracellular domain of BT-R_1 in *Manduca sexta*: involvement of a cadherin in the entomopathogenicity of *Bacillus thuringiensis*. Insect Biochem. Molec. Biol. 32, 1025–1036.

Francis, B.R., Bulla, Jr.L.A., 1997. Further characterization of BT-R_1, the cadherin-like receptor for Cry1Ab toxin in tobacco hornworm (*Manduca sexta*) midguts. Insect Biochem. Molec. Biol. 27, 541–550.

Franklin, S.E., Young, L., Watson, D., Cigan, A., Meyer, T., Bulla, Jr.L.A., 1997. Southern analysis of BT-R_1, the *Manduca sexta* gene encoding the receptor for the Cry1Ab toxin of *Bacillus thuringiensis*. Molec. Gen. Genet. 256, 517–524.

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

Gill, M., Ellar, D., 2002. Transgenic *Drosophila* reveals a functional in vivo receptor for the *Bacillus thuringiensis* toxin Cry1Ac1. Insect Molec. Biol. 11, 619–625.

Gomez, I., Sanchez, J., Miranda, R., Bravo, A., Soberon, M., 2002. 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.

Gringorten, J.L., Witt, D.P., Milne, R.E., Fast, P.G., Sohi, S.S., Van Frankenhuyzen, K., 1990. An in in vitro system for testing *Bacillus thuringiensis* toxins: the lawn assay. J. Invertbr. Pathol. 56, 237–242.

Himeno, M., Ihara, H., 1995. Mode of action of δ-endotoxin from *Bacillus thuringiensis* var. aizawai. In: Clark, J.M. (Ed.), Molecular Action of Insecticides on Ion Channels. American Chemical Society, Washington, DC, pp. 330–343.

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 mammalian cells. Biosci. Biotech. Biochem. 64, 2682–2685.

Jones, S.W., 1982. Identification of receptors in vitro. In: Eckelman, W.C. (Ed.), Receptor-binding Radiotracers. CRC Press, Inc., Boca Raton, FL, pp. 15–36.

Jurat-Fuentes, J.L., Adang, M.J., 2001. Importance of Cry1 δ-endotoxn domain II loops for binding specificity in *Heliothis virescens* (L.). Appl. Environ. Microbiol. 67, 323–329.

Keeton, T.P., Bulla, Jr.L.A., 1997. Ligand specificity and affinity of BT-R1, the *Bacillus thuringiensis* Cry1A toxin receptor from *Manduca sexta*, expressed in mammalian and insect cell cultures. Appl. Environ. Microbiol. 63, 3419–3425.

Knight, P.J.K., Crickmore, N., Ellar, D.J., 1994. The receptor for *Bacillus thuringiensis* CryIA(c) delta-endotoxin in the brush border membrane is aminopeptidase. N. Molec. Microbiol. 11, 429–436.

Knowles, B.H., Ellar, D.J., 1987. Colloid-osmotic lysis is a general feature of the mechanism of action of *Bacillus thuringiensis* delta-endotoxins with different insect specificities. Biochem. Biophys. Acta 924, 509–518.

Kwa, M.S.G., deMaagd, R.A., Stiekema, W.J., Vlak, J.M., Bosch, D., 1998. Toxicity and binding properties of the *Bacillus thuringiensis* delta-endotoxin Cry1C to cultured insect cells. J. Invertebr. Pathol. 71, 121–127.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685.

Luo, K., Banks, D., Adang, M.J., 1999. Toxicity, binding and permeability analyses of four *Bacillus thuringiensis* Cry1 δ-endotoxins by use of brush border membrane vesicles of *Spodoptera exigua* and *Spodoptera frugiperda*. Appl. Environ. Microbiol. 65, 457–464.

McCarthy, W.J., 1994. Cytolytic differences among lepidopteran cell lines exposed to toxins of *Bacillus thuringiensis* subsp. kurstaki (HD-263) and aizawai (HD-112): effect of aminosugars and *N*-glycosylation. In Vitro Cell. Dev. Biol. 30A, 690–695.

Meng, J., Candas, M., Keeton, T.P., Bulla, Jr.L.A., 2001. Expression in *Spodoptera frugiperda* (Sf21) insect cells of BT-R_1, a cadherin-related receptor from *Manduca sexta* for *Bacillus thuringiensis* Cry1Ab toxin. Protein Expr. Purif. 22, 141–147.

Miranda, R., Zamudio, F., Bravo, A., 2001. Processing of Cry1Ab δ-endotoxin from *Bacillus thuringiensis* by *Manduca sexta* and *Spodoptera frugiperda* midgut proteases: role in protoxin activation and toxin inactivation. Insect Biochem. Molec. Biol. 31, 1155–1163.

Morin, S., Biggs, R.W., Sisteron, M.S., Shriver, L., Ellers-Kirk, C., Higginson, D., Holley, D., Gahan, L.J., Heckel, D.G., Carriere, Y., Dennehy, T.J., Brown, J.K., Tabashnik, B.E., 2003. Three cadherin alleles associated with resistance to *Bacillus thuringiensis* in pink bollworm. Proc. Natl. Acad. Sci. USA 100, 5004–5009.

Nagamatsu, Y., Koike, T., Sasaki, K., Yoshimoto, A., 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.

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

Sangadala, S., Azadi, P., Carlson, R., Adang, M.J., 2001. Carbohydrate analyses of *Manduca sexta* aminopeptidase *N*, co-purifying neutral lipids and their functional interaction with *Bacillus thuringiensis* Cry1Ac toxin. Insect Biochem. Molec. Biol. 32, 97–107.

Sangadala, S., Walters, F., English, L.H., Adang, M.J., 1994. A mixture of *Manduca sexta* aminopeptidase and alkaline phosphatase enhances *Bacillus thuringiensis* insecticidal CryIA(c) toxin binding and $^{86}Rb^{+}$–K^{+} leakage in vitro. J. Biol. Chem. 269, 10088–10092.

Shelton, A.M., 2002. Economic, ecological, food safety, and social consequences of the deployment of Bt transgenic plants. Annu. Rev. Entomol. 47, 845–881.

Shin, H.S., Cha, H.J., 2002. Facile and statistical optimization of transfection conditions for secretion of foreign proteins from insect *Drosophila* S2 cells using green fluorescent reporter. Biotech. Prog. 18, 1187–1194.

Strebel, A., Harr, T., Bachmann, F., Wernli, M., Erb, P., 2001. Green fluorescent protein as a novel tool to measure apoptosis and necrosis. Cytometry 43, 126–133.

Thompson, J.M., Parrott, W.A., 1998. A size-based, blue/white selection multiple common and rare-cutter general cloning and transcription vector. BioTechniques 24, 922–927.

Tsuda, Y., Nakatani, F., Hashimoto, K., Ikawa, S., Matsura, C., Fukada, T., Sugimoto, K., Himeno, M., 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., Bulla, Jr.L.A., 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., Bulla, Jr.L.A., 1995. Cloning and expression of a receptor for an insecticidal toxin of *Bacillus thuringiensis*. J. Biol. Chem. 270, 5490–5494.

Vanden Broeck, J., Vulsteke, V., Huybrechts, R., De Loof, A., 1995. Characterization of a cloned locust tyramine receptor cDNA by functional expression in permanently transformed *Drosophila* S2 cells. J. Neurochem. 64, 2387–2395.

Wolfersberger, M.G., Luthy, P., Maurer, A., Parenti, P., Sacchi, V.F., Giordana, B., Hanozet, G.M., 1987. Preparation and partial characterization of amino acid transporting brush border membrane vesicles from the larval midgut of the cabbage butterfly (*Pieris brassicae*). Comp. Biochem. Physiol. 86A, 301–308.

Zhuang, M., Oltean, D.I., Gomez, I., Pullikuth, A.K., Soberon, M., Bravo, A., Gill, S.S., 2002. Heliothis virescens and *Manduca sexta* lipid rafts are involved in Cry1A toxin binding to the midgut epithelium and subsequent pore formation. J. Biol. Chem. 277, 13863–13872.