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# Identification, cloning and expression of a Cry1Ab cadherin receptor from European corn borer, *Ostrinia nubilalis* (Hübner) (Lepidoptera: Crambidae)

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**Abstract:** Transgenic corn expressing the Cry1Ab toxin from *Bacillus thuringiensis* is highly toxic to European corn borer, *Ostrinia nubilalis*, larvae. A putative Cry1Ab receptor (OnBt-R<sub>1</sub>) molecule was cloned and sequenced from a cDNA library prepared from midgut tissue of *O. nubilalis* larvae. The 5.6 Kb gene is homologous with a number of cadherin genes identified as Cry1 binding proteins in other lepidopterans. Brush border membrane vesicles were prepared using dissected midguts from late instars. A 220-kDa protein was identified as a cadherin-like molecule, which bound to Cry1Ab toxin and cross-reacted with an anti-cadherin serum developed from recombinant expression of a partial *O. nubilalis* cadherin peptide. Two additional proteins of smaller size cross-reacted with the anti-cadherin serum indicating that Cry1Ab binds to multiple receptors or to different forms of the same protein. *Spodoptera frugiperda* (SF9) cells transfected with the OnBt-R<sub>1</sub> gene were shown to express the receptor molecule which caused functional susceptibility to Cry1Ab at concentrations as low as 0.1 µg/ml. These results in combination suggest strongly that a cadherin-like protein acts as receptor and is involved with Cry1Ab toxicity in *O. nubilalis*.

**Keywords:** Lepidoptera, *Bacillus thuringiensis*, Binding analysis, Cadherin-like protein, Midgut, Cry toxins

## 1. Introduction

*Bacillus thuringiensis* (Berliner) (Bt) is a Gram-positive, spore-forming bacterium that produces crystalline inclusion bodies during sporulation that contain insecticidal  $\delta$ -endotoxins. Lepidopteran insects are particularly susceptible to Cry1 toxins, which bind specifically to midgut receptors and are highly toxic after ingestion. Solubilization of the crystal releases a 130-kDa protoxin, which is activated by proteases in the insect midgut to form the truncated 65-kDa toxin. The target of the activated toxin is the apical (brush border) membrane of larval midgut cells (Bravo et al., 1992). Binding of the activated toxin to midgut-specific

receptors causes the toxin conformation change, which allow its insertion and formation of ion channels or pores in the midgut apical membrane, leading to osmotic imbalance of the insect gut (Gill et al., 1992; Knowles, 1994; Schnepf et al., 1998).

Cry1-binding proteins detected on ligand blots of insect brush border membrane vesicles (BBMV) have been identified as members of the aminopeptidase N and cadherin families although the relative role of the two putative receptor molecules in insects has yet to be conclusively determined. A 210-kDa cadherin-like glycoprotein has been identified as a Cry1Ab binding protein in BBMV prepared from the midguts of *Manduca sexta* larvae (Vadlamudi et al., 1993 and Vadl-



Pairwise sequence analyses with other cadherin-like proteins were performed with Genetics Computer Group (GCG, version 10, Madison, WI), using the 'GAP' function and default settings (gapweight = 8, lengthweight = 2). Multiple alignments were performed using ClustalX (gapweight = 15, lengthweight = 0.30) (Thompson et al., 1997), followed by a fine-tuning alignment of block sequences.

#### 2.4. Antibody production

A PCR fragment was generated containing the nucleotides that encode amino acids 958–1503 of the *O. nubilalis* cadherin gene. This fragment was subsequently cloned into the pET28 expression vector (Novagen) and transformed into BL21-De3 cells (Invitrogen). Transformed cells were grown at 37 °C to an OD<sub>600</sub> of 0.8, induced with IPTG (1 mM) and harvested after 14 h of growth at 16 °C. The suspension was centrifuged at 10,000g for 15 min and the supernatant was removed. The cell pellet was suspended in 1/25 volume lysis buffer (5 mM Imidazole, 500 mM NaCl, 20 mM Tris-HCl pH 7.9); 1 mg/ml lysozyme; Complete Protease Inhibitor (Roche). The protein was purified according to the His-Bind Kit protocol (Novagen), except Talon Metal Affinity Resin (Clontech) was substituted for the Ni<sup>2+</sup> resin provided in the kit. The purified protein was dialyzed into PBS and concentrated to 1 mg/ml. The concentrated protein was loaded onto an SDS-PAGE gel and silver stained according to the SilverQuest protocol (Invitrogen). The purity of the OnBt-R<sub>1</sub> fragment was estimated at >95% and 1 mg of protein was provided to Strategic Biosolutions for polyclonal antibody construction and protein A purification using standard procedures.

#### 2.5. Brush border membrane vesicles (BBMV) preparation

BBMV were prepared with gut tissue dissected from ice-chilled 5th stage larvae. Larvae were excised so that both the last three abdominal segments and the head plus thorax were removed, and the midguts were pulled gently from the carcass. A small glass culture tube was rolled over the length of the gut to displace the gut contents. Dissected gut tissue was transferred to a centrifuge tube containing ice-cold MET buffer [300 mM Mannitol, 17 mM Tris-HCl [pH 7.5], 5 mM EGTA, protease inhibitor (complete EDTA-free protease inhibitors, Roche), vigorously vortexed and briefly centrifuged for 5 min at 1000g to obtain the clean midguts. Guts were either frozen at -80 °C or processed immediately by the differential magnesium precipitation method of Wolfersberger et al. (1987). Briefly, gut tissues were homogenized on ice in a tight-fitting glass Dounce homogenizer in ice-cold MET buffer (10% weight/volume). The homogenate was diluted with

an equal volume of ice-cold 28 mM MgCl<sub>2</sub>, blended and held on ice for 15 min before centrifugation. A low-speed centrifugation (2500g for 15 min at 4 °C) was used to pellet heavier cell debris, and the supernatant from the initial centrifugation was further centrifuged at 30,000g for 30 min at 4 °C. The resulting pellet was resuspended in MET buffer and centrifuged again at 30,000g for 30 min at 4 °C. The resulting pellet which corresponded to the BBMV preparation was resuspended in HBS-N buffer (10 mM Hepes, pH 7.4, 150 mM NaCl), flash frozen in liquid nitrogen and stored at -80 °C. The protein concentration of the BBMV preparations was determined by the bicinchoninic acid method (Smith et al., 1985). Alkaline phosphatase activity was used as a marker enzyme to track purification of the BBMV preparation, and it was 8–12 times higher in BBMV preparations than in the initial homogenates (data not shown).

#### 2.6. Immunoblotting

Ligand blots of Cry1Ab binding to BBMV proteins were performed using the chemiluminescence Western Light™ kit (Tropix, Inc., Bedford, MA). Equal amounts (80 µg) of BBMV protein were separated by SDS-PAGE as described by Laemmli (1970), electroblotted onto polyvinylidene difluoride (PVDF) membrane (Bio-Rad Inc., Hercules, CA) for 90 min by a Mini Trans-Blot Electrophoretic transfer cell (Bio-Rad), and blocked for 2 h at room temperature with phosphate buffered saline (PBS) (pH 8.0) containing 5% non-fat dry milk powder, 5% glycerol, 0.5% Tween-20. The PVDF membrane was then incubated with activated Cry1Ab (250 ng/ml) in blocking buffer overnight at 4 °C and subsequently washed three times with blocking buffer. The blot was then incubated with polyclonal rabbit anti-Cry1Ab (1:2500) (provided by Monsanto Co., St. Louis, MO), washed three times, then incubated with goat anti-rabbit-AP (2nd antibody at 1:10,000) and washed three times with blocking buffer. The PVDF membrane was washed with assay buffer (Tropix Inc., Bedford, MA) and detection was performed with the CDP-Star® chemiluminescence Kit (Tropix Inc.) using a Fluor-S imager (Bio-rad).

Western blots for cadherin-like proteins were performed as described above, except that the anti-OnBt-R<sub>1</sub> serum previously described (1:3000 at room temperature for 1 h) was used. The membrane was washed as described above, and an anti-rabbit-AP serum (1:10,000 at room temperature for 1 h) was applied to the PVDF membrane. After repeated washings with assay buffer (Tropix Inc.), the membrane was incubated with CDP-Star for 5 min and the image captured as described above.

### 2.7. Expression of OnBt-R<sub>1</sub> in *Spodoptera frugiperda* Sf9 cells

*Spodoptera frugiperda* (Sf9) cells (ATCC 1711-CRL) were grown at 27 °C in Sf-900 II serum-free medium (Invitrogen). Full-length OnBt-R<sub>1</sub> cDNA (5494 bp) was cut from a pBlue-script/Bt-R<sub>1</sub> construct and cloned into plasmid pFastBac (Invitrogen) at different cloning sites downstream from the polyhe-

drin promoter. The plasmid was recombined into the bacmid backbone via DH101Bac *E. coli* cells in a Bac-Bac Expression system (Invitrogen). Bacmids (2 µg/100 µl Sf-900 medium) that harbored OnBt-R<sub>1</sub> were mixed with CellFectin (6µl/100 µl Sf900 medium) (Invitrogen), and incubated at room temperature for 30 min. The mixture was diluted with

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1  MGVERFFPAV LLVSLASAAL ANQRCSYIIA IPRPETPELP PIDYEGKSW S EQQLIPGPTR
61  EEVCMENFLP DQMIQVIYME EEIEGDVIIA KLNQGSNTP VLSIMSGQPR AQLGPEFRQN
121 EADGQWSLVI TQRQDYETAT MQSYVFSIQV EGESQAVLVA LEIVNIDNPN PILQVVSACV
181 IPEHGEARLT DCVYQVSDRD GEISTRFMFTF RVDSSRAADE SIFYMVG EYD PSDWFMNKM T
241 VGINSPLNFE TTQLHIFSVT ASDSLPNNHT VTMVQVENV ESRPPRWVEI FSVQQFDEKT
301 NQSFSLRAID GDTGINRAIN YTLIRDDADD FFSLEVI EDG AILHVTEIDR DKLERELFNL
361 TIVAYKSTDA SFATEAHIFI IVNDVNDQRP EPLHKEYSID IMEETPMTLN FNEEFGFHDR
421 DLGENAQYTV ELEDVFPFGA ASAFYIAPGS GYQRQTFIMG TINHTMLDYE DVIFQNI IIK
481 VKA VDMNNAS HVGEALVYVN LINWDELPI FEESYSASF KETVGAGFPV ATV LALDRDI
541 DDVVVHSLMG NAVDYLFIDE STGEIFVSM D DAFDYHRQNT L FVQVRADDT LGDGPHTVT
601 TQLVIELEDV NNTPTLRLP RSTPSVEENV PEGYEISREI TATDPDTSAY LWF EIDWDST
661 WATKQGRETN PTEYVCIVI ETIYPTEG NR GSAIGRLVVQ EIRDNV TIDF EEFEMLYLTV
721 RVRDLNTVIG DDYDEATFTI TIIDMNDNAP IFANGTLTQT MRVRELAASG T LIGSVLATD
781 IDGPLYNQVR YTIQPRNTP EGLVKIDFTT GQIEVDANEA IDADEPWRFY LYTTVIASDE
841 CSENRTECP PDSNYFEVPG DIEIEIIDTN NKVPEPLTEK FNTTVYVWEN ATSGDEVVQL
901 YSHDRDRDEL YHTVRYTMNF AVNPRLRDFF EVDLDTGRLE VHYPGDEKLD RDGDEPTHTI
961 FVNFIDNFFS DGDGRRNQDE VEIPVLLDV NDNAPEMPLP DELRFDVSEG AVAGVRVLPE
1021 IYAPDRDEPD TDNSRVGYGI LDLTITDRDI EVPDLFTMIS IENKTGELET AMDLRGYWGT
1081 YEIFIEAFDH GYPQQRSNET YTLVIRPYNF HHPVFVFPQP DSVIRLSRER ATEGGVLATA
1141 ANEFLEPIYA TDEDGLHAGS VTPHVQNEE AVQYFDITEV GAGENSGQLI LRQLFPEQIR
1201 QFRITIRATD GGTEPGPLWT DVTFSVVPV TQGDVPFSEN AATVAFPEGE EGLRESFELP
1261 QAEDLKNHLC EDDCQDIYR FIDGNNEGLF VLDQSSNVIS LAQELDREVA TSYTLHIAAS
1321 NSPDATGIPL QTSILVVTVN VREANPRPIF EQDLYTAGIS TLDSIGRELL TVRASHTEDD
1381 TITYTIDRAS MQLDSSLEAV RDSAFALHAT TGVLSLNMQP TASMHGMEFF DVIATDTASA
1441 IDTARVKVYL ISSQNRVTFI FDNQLETVEQ NRNFI AATFS TGFNMTCNID QVVPFSDSSG
1501 VAQDDTTEVR AHFIRDNVVP QAQEVEAVRS DTVLLRTIQL MLSTNSLVLQ DLVTGDTPTL
1561 GEESMQIA VY ALAALSAVLG FLCIVLLLAL CRTRALNRQ LQALSMTKYG SVDSGLNRAG
1621 LAPGTNKHAV EGSNPMWNEA IRAPDFDAIS DASGSDSLIG IEDMPQFRDD YFPPGDTDSS
1681 SGIVLHMGEA TDNKPVTTHG NNFGFKSTPY LPQPHPK

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**Figure 1.** Deduced amino acid sequence of *O. nubilalis* BT-R<sub>1</sub>. The protein sequence analysis was done by using the ISREC-ProfileScan server (<http://hits.isb-sib.ch/cgi-bin/PFSCAN>) and PROSITE (<http://us.expasy.org/prosite/>). The putative signal peptide sequence and TM spanning region are underlined and boxed, respectively. Full-black arrows denote predicted putative *N*-glycosylation sites. CR1–CR11 are cadherin repeats and MPR, the membrane-proximal region. The bolded sequence at the C-terminal sequence represents the intracellular domain.

0.8 ml Sf-900 medium and cells ( $10^6$ /ml in 35 mm well in culture plate) washed with Sf-900 medium once. The Bacmid/cell mixture was added to the well and incubated at room temperature for 5 h after which the medium was removed and 2 ml Sf-900 medium containing penicillin and streptomycin were added to the well. Ligand blot with anti-OnBt-R<sub>1</sub> was used to examine the expression 3–5 days after infection.

To determine if baculovirus infected cells were susceptible to the Cry1Ab toxin, cells infected with OnBt-R<sub>1</sub>-containing baculovirus were compared with non-infected cells and with cells infected with baculovirus containing an empty vector. The constructs were driven by a very late promoter and analyzed 3–5 days post-infection. Cells were washed once with PBS, and different concentrations of Cry1Ab were applied to the cells in PBS at different time intervals. The cells were examined by light microscopy to determine cell viability in the presence of toxin.

### 3. Results

#### 3.1. Identification and analysis of the *O. nubilalis* cadherin-like protein

Degenerate primers designed from the *M. sexta* cadherin Bt-R<sub>1</sub> (Vadlamudi et al., 1995) were used in to amplify a 280 base pair fragment from *O. nubilalis* midgut cDNA. Sequence analysis revealed the fragment was a Bt-R<sub>1</sub> homologue, and it was subsequently used to screen an *O. nubilalis* midgut cDNA library generating several positive clones. The largest inserts contained 5328 bp with an open reading frame of 5151 bp (Genbank Accession No. AX147201), and a polyadenylation signal in the 3'-UTR. Upon sequence analysis of the largest positive clones, all matched the protein molecule sequence in Fig. 1 consisting of 1717 amino acids which contains a transmembrane region (TM) of 23 amino acids. The extracellular domain comprises a signal sequence (SP) of 22 amino acid residues, 11 cadherin repeats (CR), and a membrane-proximal region (MPR) (Fig. 1). Fourteen putative *N*-glycosylation sites were identified in the protein sequence. The protein cytosolic domain is composed of 126 amino acid residues.

Phylogenetic analysis of the predicted OnBt-R<sub>1</sub> amino acid sequence, based on alignment with other lepidopteran cadherins identified as Cry1 receptors, revealed that this receptor shares 62–70% similarity and 56–63% identity (Table 1) with these other cadherin molecules. This analysis indicated that the OnBt-R<sub>1</sub> is most closely related to a cadherin from *Chilo suppressalis* (Family Pyralidae) and *P. gossypiella* (Family Gelechiidae) (Fig. 2) and most distant from the cadherin genes identified from five different noctuid species.

#### 3.2. Immunoblots

Immunoblots were performed to identify potential Cry1Ab binding proteins using both anti-Cry1Ab and anti-OnBt-R<sub>1</sub> serum. The Cry1Ab toxin bound to three proteins of approximately 220, 170, and 160 kD in size (Fig. 3, Lane 2). The anti-cadherin detected bands of similar molecular weight (Fig. 3, Lane 1) indicating that the proteins binding the Cry1Ab toxin are cadherin-like proteins. No bands were visible in the controls performed without Cry1Ab and anti-cadherin serum (data not shown).

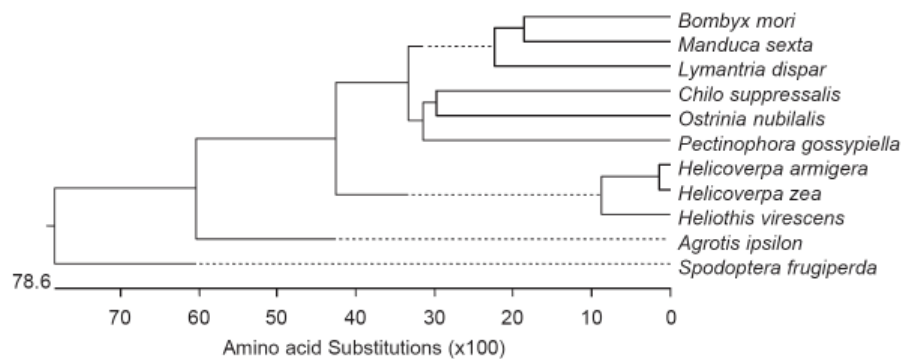
#### 3.3. Expression of OnBt-R<sub>1</sub>

Sf9 cells infected with the recombinant DNA carrying OnBt-R<sub>1</sub> cDNA produced a moderate amount a protein of approximately 190 kDa that reacted specifically with anti-OnBt-R<sub>1</sub> serum (Fig. 4). The slightly smaller size relative the major 220 kDa band observed in blots of native BBMV protein is likely explained by differences in post-translational modifications that could affect molecular weight of the expressed protein. There were several smaller molecular weight proteins in the supernatant obtained from cell lysates that cross-reacted with the antiserum indicating possible degradation of the full-length receptor or expression of peptide fragments (Fig. 4). Only the 190 kDa band was visible in membrane fractions of the cell lysate indicating that full-length OnBt-R<sub>1</sub> was expressed predominately within the cell membrane fraction. In control samples, there was a very faint 190 kDa band indicating that Sf9 cells might express small amount of a Bt-R<sub>1</sub> homologue.

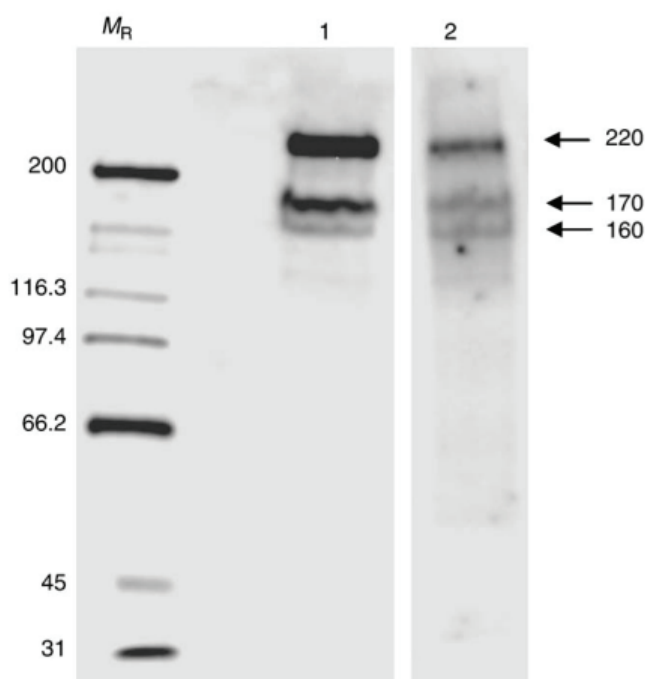
**Table 1.**

Pairwise comparison of *O. nubilalis* cadherin-like protein to cadherins from 10 other species of Lepidoptera

Species	Similarity (%)	Identity (%)	Accession no.
<i>Ostrinia nubilalis</i>	—	—	CAC 41165
<i>Lymantria dispar</i>	70.4	63.1	AAL 26896
<i>Bombyx mori</i>	70.6	64.6	BAA 77212
<i>Manduca sexta</i>	69.1	61.8	AAG 37912
<i>Heliothis virescens</i>	68.6	61.5	AAK 85198
<i>Helicoverpa zea</i>	68.1	60.7	CAC41166
<i>Helicoverpa armigera</i>	67.8	60.5	AAM 69351
<i>Chilo suppressalis</i>	66.4	59.4	AAM 78590
<i>Pectinophora gossypiella</i>	66.3	59.0	AAP 30715
<i>Spodoptera frugiperda</i>	63.0	53.3	CAC41167
<i>Agrotis ipsilon</i>	62.6	56.3	Under submission

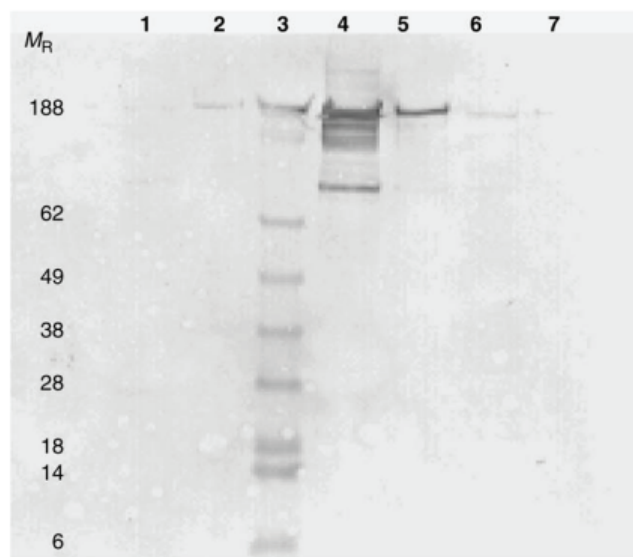


**Figure 2.** Phylogenetic tree of aligned Lepidoptera cadherin-like proteins identified as Cry1 receptors. The tree was performed with MegAlign (DNASar). Species correspondent GenBank accession numbers are in Table 1.



**Figure 3.** Binding of anti-cadherin (anti-OnBt-R1) (Lane 1) from *O. nubilalis* and Cry1Ab toxin (Lane 2) to BBMVs proteins.

Cells infected with pFast/Bac alone were not affected by Cry1Ab (Fig. 5c and e). However, 30 min after incubation with Cry1Ab, pFast/Bt-R1 infected cells started swelling and lysing even at concentrations as low as 0.1  $\mu\text{g/ml}$  Cry1Ab (Fig. 5d). At higher magnification, it was observed that the pFast/Bt-R1 infected cells were enlarged and more granulated as compared to the controls (data not shown). At higher concentrations (5  $\mu\text{g/ml}$ ), cell lysis was clearly evident and the cell contents (DNA-like material) were released (Fig. 5f). However, control cells did not swell or lyse in the presence of Cry1Ab even at elevated (5  $\mu\text{g/ml}$ ) concentrations.

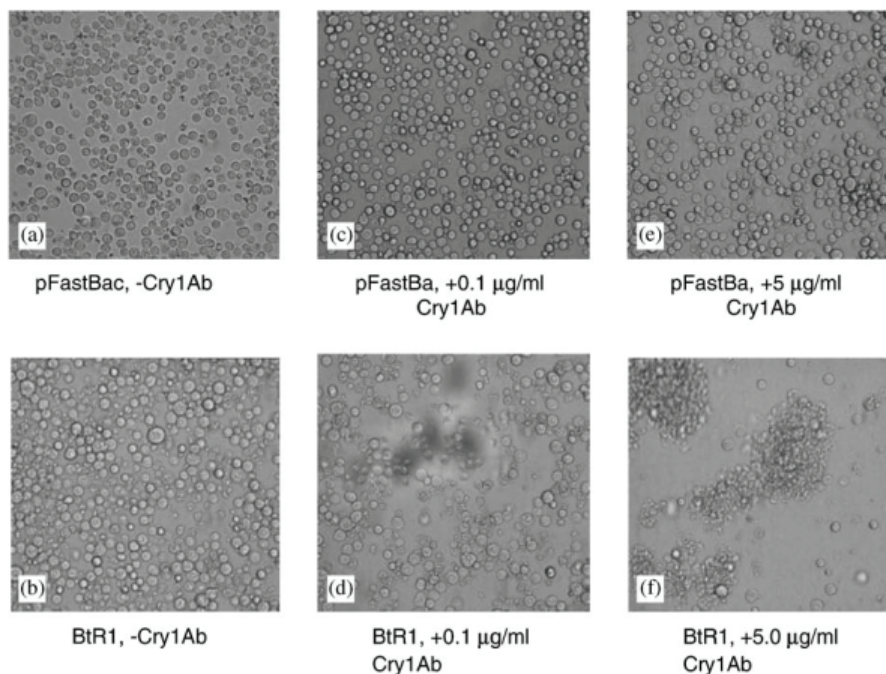


**Figure 4.** Western blot assay of Sf9 transfected cells: Sf9 cell were transfected with pFastBac or pFastBac/BtR1. Five days after transfection. Cells were lysed and centrifuged. The supernatants and pellets were subjected to Western blot (see Material and methods). (1) pFastBac/supernatant, (2) pFastBac/pellet, (3) protein marker, (4) pFastBac/BtR1 supernatant, (5) pFastBac/BtR1 pellet, (6) non-transfected supernatant, and (7) non-transfected cell pellet.

#### 4. Discussion

The relatively recent identification of receptors for Bt toxins has provided a greater understanding of their mechanism of toxicity. In addition to the aminopeptidase-like proteins that have been identified as receptors for Bt toxins (Sato, 2003), cadherin-like proteins have also been identified as Bt Cry1A receptors in a number of different lepidopterans (Zhuang and Gill, 2003). In the present work, a cadherin-like protein present in the BBMVs of *O. nubilalis* was identified as a receptor for the Cry1Ab toxin. These results confirm the involvement of a cadherin-like protein that binds to the Cry1Ab toxin





**Figure 5.** Effect of Cry1Ab on pFastBac and OnBt-R<sub>1</sub> transformed-Sf9 cells. (a,c,e) Mock transformed Sf9 cells incubated with 0, 0.1 and 5 µg/ml of Cry1Ab, respectively. (b,d,f) BtR1-transformed Sf9 cells incubated with 0, 0.1 and 5 µg/ml of Cry1Ab, respectively.

and confers susceptibility to Sf9 cells transfected with the OnBt-R1 gene. Results from immunoblot analysis suggest that Cry1Ab binds to three putative proteins from susceptible insects, and that the same three bands cross-react with an anti-OnBt-R1 serum suggesting that all three proteins belong to the cadherin-family of proteins. Denolf et al. (1993) suggested that Cry1Ab binds to two different gut receptors from *O. nubilalis*, while more recent efforts (Hua et al., 2001) suggested that Cry1Ab binds up to four different receptors. Although both aminopeptidases and cadherins have been identified as binding proteins for Cry1 Bt toxins in lepidopteran midgut apical membranes, it appears more likely that the Cry1Ab binding protein for *O. nubilalis* is a cadherin-like protein. The 220-kDa receptor identified in the present study is consistent with results from Hua et al. (2001) who reported a similar molecular weight protein from *O. nubilalis* midguts as a cadherin-like protein.

Cadherins constitute a large family of transmembrane glycoproteins responsible for cell adhesion and maintenance of the integrity of selective cell-cell interactions (Nollet et al., 2000). Although their specific functions in insects have not been fully resolved, they seem to play an important role in the binding of Cry1 toxins in a number of different species (Vadlamudi et al., 1993; Nagamatsu et al., 1999; Gahan et al., 2001; Hua et al., 2001). The *O. nubilalis* cadherin-like protein shows a relative high similarity and identity to other members of the cadherin superfamily in insects. Eleven CRs are present in the *O. nubilalis* cadherin and 14 *N*-glycosylation sites are distrib-

uted along the extracellular domains. The relatively high similarity to other lepidopteran cadherin-like proteins indicates that the *O. nubilalis* cadherin-like protein shares related structures, functions, and consequently specificity for Cry1 Bt toxins.

Expression of the OnBt-R1 in Sf9 cells provided strong evidence that this molecule not only binds Cry1Ab but is responsible for its toxicity since transfected cells were susceptible to toxin concentrations as low as 0.1 µg/ml while control (untransfected) cells exhibited no response. It has been suggested that Cry toxins bind to a specific receptor and are then inserted into the membrane to form a pore that alters membrane permeability. The consequence is lysis of the epithelial cells and death of the insect (Knowles, 1994). The present results suggest that a cadherin-like protein from *O. nubilalis* midgut tissue mediates both binding and insertion into the cell membrane.

Identification of the Cry1Ab binding protein in *O. nubilalis* is an important step in our understanding of potential resistance mechanisms that might evolve for transgenic corn which is currently comprised almost exclusively by Cry1Ab expressing hybrids. Further characterization of this receptor will facilitate our understanding of possible mutations in the receptor that could alter binding characteristics and confer resistance. A number of recent studies have been conducted to determine and characterize the binding region for lepidopteran cadherins (Nagamatsu et al., 1999; Gomez et al., 2001; Dorsch et al., 2002). Hua et al. (2004) determined that the cadherin domain 12 is critical for Cry1Ab binding in *M. sexta* which was the

minimum region necessary to confer *Drosophila* S2 cell susceptibility to Cry1Ab. Identification of the *O. nubilalis* cadherin-like protein and epitope mapping studies will eventually help to design molecular tools to detect resistance associated with altered binding to cadherin receptors. Additionally, future development of transgenic hybrids will be facilitated by the ability to predict cross-resistance among different Bt toxins based on a thorough characterization of receptor molecules. In general, such information should facilitate the rational implementation of management practices that employ transgenic corn hybrids for controlling European corn borer populations.

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