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Sek Yee Tan
University of Nebraska–Lincoln

Bonifacio F. Cayabyab
University of the Philippines, Los Baños

Edwin P. Alcantara
University of the Philippines, Los Baños

Fangneng Huang
Louisiana State University

Kanglai He
Chinese Academy of Agricultural Sciences

See next page for additional authors

Authors
Sek Yee Tan, Bonifacio F. Cayabyab, Edwin P. Alcantara, Fangneng Huang, Kanglai He, Kenneth Nickerson, and Blair Siegfried

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Sek Yee Tan,[1] Bonifacio F. Cayabyab,[2] Edwin P. Alcantara,[3] Fangneng Huang,[4] Kanglai He,[5] Kenneth W. Nickerson,[6] and Blair D. Siegfried[1]

1 Department of Entomology, University of Nebraska–Lincoln, Lincoln, NE 68583, USA
2 National Crop Protection Center, Crop Protection Cluster, Plant and Environmental Health Division, College of Agriculture, University of the Philippines, Los Baños, College Laguna 4031, Philippines
3 National Institute of Molecular Biology and Biotechnology, University of the Philippines, Los Baños, College Laguna 4031, Philippines
4 Department of Entomology, Louisiana State University Agricultural Center, Baton Rouge, LA 70803, USA
5 Institute of Plant Protection, Chinese Academy of Agricultural Sciences, No. 2, West Yuanmingyuan Road, Beijing 100193, China
6 School of Biological Sciences, University of Nebraska–Lincoln, E151 Beadle Center, Lincoln, NE 68588, USA

Corresponding author – Blair D. Siegfried, Dow AgroSciences LLC, 9330 Zionsvill Road, Indianapolis, Indiana, 46268, USA, fax: +1 (402) 472-4687, email: bsiegfried1@unl.edu

Abstract

The European (*Ostrinia nubilalis* Hübner) and Asian corn borers (*Ostrinia furnacalis* Guenée) are closely related and display similar sensitivity to Cry1 toxins. In this study, we compared the binding patterns of Cry1Ab and Cry1F toxins between both *Ostrinia* spp., as well as the expression of putative cadherin and aminopeptidase-N (APN)-like protein receptors. Additionally, cDNA sequences of these putative toxin receptors from both *Ostrinia* species were compared. Ligand blots for both species indicated a similar binding pattern for

Cry1Ab with the strongest immunoreactive band at 260 kDa in both species. In addition, similar expression of the putative cadherin- and APN-like protein receptors were observed at 260 and 135 kDa, respectively. A high degree of similarity (98% amino acid sequence identity) of cDNA sequences for both putative receptor sequences was observed. The Cry1F ligand blot revealed that *O. furnacalis* and *O. nubilalis* BBMV exhibited slightly different binding patterns, with strong binding to putative proteins at 150 and 140 kDa, respectively. Both proteins appeared to also bind Cry1Ab, although the signal intensity was much reduced with Cry1Ab. *O. furnacalis* showed an additional but weaker band at 210 kDa relative to the 150 kDa band. *Diatraea saccharalis* (Fabricius), which was used as an outgroup species, exhibited different binding patterns than either *Ostrinia* species, with both Cry1Ab and Cry1F toxins binding to a 210 kDa protein. These results support the previous experiments indicating that *O. nubilalis* and *O. furnacalis* share similar patterns of susceptibility to Cry toxins.

1. Introduction

The first Bt corn expressing a Cry1Ab toxin was introduced in the United States in 1996 and has been extremely effective against a number of target pests including the European corn borer, *Ostrinia nubilalis* (Hübner), one of the most destructive pests in the United States Corn Belt (ILSI/HESI, 1998; Mason, 1996). In recent years, different Cry toxins with unique target sites have been introduced as stacked/pyramided toxins with the assumption that these pyramided events will have increased durability relative to single toxin events (Zhao et al., 2005). Cry1Ab and Cry1F are expressed in both single and pyramided traits, while Vip3Aa20, Cry1A.105, and Cry2Ab2 are commercialized only as pyramided traits (USEPA, 2009). In addition to the United States, countries with significant acreage of Bt corn include Brazil, Argentina, Canada, South Africa, Uruguay, Spain, Honduras, Chile, Egypt, Romania, Slovakia, Czech Republic, Portugal and the Philippines (James, 2010).

A sibling species of *O. nubilalis*, the Asian corn borer, *Ostrinia furnacalis* (Guenée), is the most economically important corn stalk boring pest in Asia (Lewvanich, 1973; Mutuura and Monroe, 1970), and Bt corn expressing Cry1Ab (MON810) has been cultivated in the Philippines since early 2003 providing season-long crop protection against *O. furnacalis* and significantly increased yield (Gonzalez, 2002; James, 2003). In addition, the approval for the cultivation of stacked events that express Cry2Ab2 and Cry1A.105 was given in year 2009 (ISAAA, 2012) while Cry1Fa expressing events have been evaluated in field trials and are pending registration approval (Thompson et al., 2010). There is a strong likelihood that Bt corn introduction to control the Asian corn borer will be successful throughout its distribution.

Ostrinia furnacalis is morphologically and biologically very similar to *O. nubilalis* (Mutuura and Monroe, 1970). In general, *O. furnacalis* is considered the Asian sibling of *O. nubilalis* which originated from Europe and subsequently was introduced to the United States (Frolov et al., 2007). In Asia, *O. furnacalis* is distributed throughout India, Southeast Asia, China, Korea, Japan, Australia, New Guinea, Solomon Islands, and western Micronesia (Lewvanich, 1973; Mutuura and Monroe, 1970). Interestingly, both *Ostrinia* species are found in China (Zhou, 1988), with *O. nubilalis* being restricted to the Northwest while *O. furnacalis* is scattered throughout most regions of the country. Their host preferences are almost identical (Caasi-Lit, 2006; Nafus and Schreiner, 1991; Mason, 1996), with both exhibiting preference for maize.

Three putative receptors have been reported to be important for Cry toxin binding in Lepidoptera (Pigott and Ellar, 2007), including aminopeptidase N-(APN), cadherin- and alkaline phosphatase-like proteins. The APN is a class of enzymes that cleave neutral amino acids from the N-terminus of polypeptides and in cooperation with endopeptidases and carboxypeptidases, digest proteins derived from the insect's diet (Wang et al., 2005). Among the 4 isoforms, class 3 is made up of the largest group of Lepidopteran APN and has been reported to bind with Cry1Aa, Cry1Ab, Cry1Ac, Cry1Ba and Cry1Fa (reviewed by Pigott and Ellar, 2007). Cadherin is by far the most studied binding receptor of Bt toxins. A novel cadherin-like protein was first isolated from the midgut epithelium of Manduca sexta (Linnaeus) based upon its binding affinity to Cry1Ab (Vadlamudi et al., 1993). The cadherin protein is glycosylated and usually anchored to the membrane by a single transmembrane domain, while the APN is GPI-anchored (Pigott and Ellar, 2007). In contrast to APN and cadherin -like receptors, research on alkaline phosphatases as Cry toxin receptors is limited. Alkaline phosphatase is a GPI-anchored membrane glycoprotein and its involvement as a Cry1Ac receptor was described for both Heliothis virescens (Fabricius) (Jurat-Fuentes and Adang, 2004) and M. sexta (McNall and Adang, 2003).

Both *O. furnacalis* and *O. nubilalis* are similar in sensitivity to a variety of Cry1 toxins, thus suggesting shared toxin receptors and mechanisms of toxicity for the two species (Tan et al., 2011). The present study compared Cry1Ab and Cry1F toxin binding proteins between the two species as well as expression of the putative toxin receptors, specifically the cadherin- and APN-like receptors. In addition, the complete cDNAs of putative toxin receptors for both *Ostrinia* spp. were compared. These results provide increased understanding of the receptor binding patterns and mechanisms that are important to strategize insect resistance management of Bt toxins expressed in corn.

2. Materials and methods

2.1. Insects

A Bt-susceptible *O. nubilalis* colony maintained at the University of Nebraska-Lincoln (UNL) was established from a field collection of ca. 400 larvae that originated from northern Italy and has been maintained in the absence of selection for over 150 generations, using standard rearing techniques (Guthrie et al., 1965; Lewis and Lynch, 1969) as described by Siqueira et al. (2004b). About 400 late instar larvae of *O. furnacalis* were generously provided by the University of the Philippines, Los Baños in 2009. They were collected from Santiago, Lubao, Pampanga and mass reared in the laboratory up to 5 generations. The larvae were flash frozen and shipped on dry ice to UNL and then stored at –80°C.

In addition to the two *Ostrinia* species, a laboratory population of *Diatraea saccharalis* (Fabricius) was established from a field collection of >300 larvae from non-Bt corn fields near Winnsboro in Franklin Parish, LA during December 2008 and maintained in the laboratory as described by Tan et al. (2011). *D. saccharalis* was included in this study as an outgroup to compare toxin binding and expression with the two closely related corn borer species.

2.2. Brush border membrane vesicles preparation

Brush border membrane vesicles (BBMV) of *O. furnacalis*, *O. nubilalis* and *D. saccharalis* were prepared following the method described by Pereira et al. (2009). There were two to three batches of approximately 400 early fifth instar larvae of *O. furnacalis*, *O. nubilalis* and *D. saccharalis* prepared for this purpose. Larvae from each batch were placed on top of a chilled glass dissection plate. The last three abdominal segments, as well as the head and thorax of each larva was removed and subsequently, the midgut was pulled gently from the larval body. Gut contents were displaced by rolling a small glass culture tube over the length of the gut. Dissected gut tissue was quickly rinsed briefly in ice-cold MET buffer (300 mM mannitol, 17 mM Tris–HCl pH 7.5, 5 mM EGTA, protease inhibitor [Complete EDTA-free protease inhibitors cocktail]; Roche Applied Science, Indianapolis, IN), and flash frozen on dry ice or in liquid nitrogen. Guts were stored at –80°C before being processed by following the MgCl2 precipitation method (Wolfersberger et al., 1987). Briefly, gut tissue was homogenized on ice in a tight-fitting glass Dounce homogenizer in ice-cold MET buffer (10%, wt./vol.). The homogenate was diluted with an equal volume of ice-cold 24 mM MgCl2

and blended briefly with the homogenizer, and held on ice for 15 min before centrifugation at low-speed 2500g for 15 min at 4°C. This separated heavier cell debris, while the supernatant from the initial centrifugation was further centrifuged at 34,000g for 30 min at 4°C. The pellet was resuspended in MET buffer, held on ice for 15 min and centrifuged at 2500g and then at 34,000g for 30 min at 4°C. The pellet, which corresponded to the BBMV preparation, was resuspended in MET buffer and stored at –80°C.

The enzyme activity and protein concentration of the BBMV preparations were determined with alkaline phosphatase activity using p-nitrophenylphosphate as a substrate (Wako LabAssay™, Wako Pure Chemical Industries, Ltd., Osaka, Japan) and the dye binding method of Bradford (1976) using the Coomassie Plus Protein Assay (Pierce, Rockford, IL) with bovine serum albumin (BSA) as the standard, respectively. The relative enzyme activity of each sample (units of activity/lg protein) was determined. Alkaline phosphatase activity was used to determine the enrichment of the BBMV preparations. Activity was typically 3–5 times higher in BBMV preparations relative to the initial homogenates.

2.3. Bt toxin preparation

Cry toxins were prepared from fermentation of recombinant Escherichia coli strains transformed to express Cry1Ab (ECE53) obtained from the Bacillus Genetic Stock Center of The Ohio State University, Columbus, OH. The culture was grown at 37°C for 48 h in Luria–Bertani media. Protoxin was obtained from *E. coli* fermentation products following the method described by Lee et al. (1992). The solubilized protein was digested with (L-1-tosylamido-2-phenylethyl chloromethyl ketone)-treated trypsin from bovine pancreas (Sigma–Aldrich®) and insoluble material was removed by centrifugation. The protoxin preparations were dialyzed against 50 mM $NaCO_3$/$NaHCO_3$ buffer (pH 10.0) using a SnakeSkin™ pleated dialysis tubing, 10 k molecular weight cut off (Thermo Scientific, Rockford, IL). The Cry1Ab toxin preparation was quantified by densitometric quantification (Crespo et al., 2008) of the 60–65 kDa proteins after sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (Laemmli, 1970) and compared to a standard curve for bovine serum albumin. Cry1Ab endotoxin was stored at –80°C. Lyophilized Cry1F toxin (13.7% (weight) of active ingredient) was supplied by Dow AgroSciences LLC, Indianapolis, IN. Purified Cry1F toxin was produced by fermentation of recombinant *Pseudomonas fluorescens* Flügge (strain MR872), proteolytically activated, and chromatographically purified. The Cry1F toxin was solubilized with 50 mM $NaCO_3$/$NaHCO_3$ buffer (pH 10.0) for the ligand blots.

2.4. Antibodies

Cry1Ab/Cry1Ac monoclonal antiserum was provided by Monsanto Co. (St. Louis, MO). The cadherin antibody was synthesized from a 14-residue peptide (1018LPEIYAPDREPDT1032) deduced from the *O. nubilalis* cadherin-like gene (Flannagan et al., 2005). It was conjugated with a cysteine residue at the N-terminal region, used for antibody generation and purified using standard procedures (Sigma Genosys, The Woodlands, TX). The APN antibody (anti-APN-4) was derived from a peptide of 300 amino acid residues expressed in E. coli using the pET28a vector (Pereira et al., 2009). It contained the C-terminus of an APN isoform 4 gene isolated from *O. nubilalis* which exhibited high similarity with APN of M. sexta (Bravo et al., 2004) and Spodoptera exigua (Hübner) (Herrero et al., 2005) that are associated with the Bt toxin binding. The peptide was purified and dialyzed into phosphate buffered saline (PBS), concentrated and loaded onto a SDS–PAGE to check for purity. The purified protein was provided to Sigma Genosys (The Woodlands, TX) for polyclonal antibody generation and was purified using standard procedures. Lyophilized rabbit anti-Bt-Cry1F monoclonal antibody at 0.2 mg was obtained from Abraxis™ and diluted in PBS buffer to 1 mg/ml.

2.5. Ligand blots and immunoblottings

Because the BBMV prepared from *O. furnacalis* were from frozen specimens, we loaded lanes based on relative alkaline phosphatase activity of 30 units of enzyme activity from both *Ostrinia* spp. and *D. saccharalis* to ensure that the BBMV proteins were viable. Total protein loadings for these BBMVs were 97–98 lμg/lane for both *Ostrinia* spp. while the protein loading for *D. saccharalis* was approximately 50 μg/lane.

BBMV proteins were separated by SDS–PAGE (Laemmli, 1970) and electroblotted onto pure nitrocellulose membranes (Bio-Rad Inc., Hercules, CA) for 1 h at 80 V using a Mini Trans-Blot electrophoretic transfer cell (Bio-Rad, Hercules, CA), and blocked for 4 h at room temperature with Tris-buffered saline (TBS, pH 9.0) containing 4% (w/v) nonfat, dry milk powder as previously described to identify putative Cry toxin receptors from *O. nubilalis* (Hua et al., 2001; Flannagan et al., 2005; Siqueira et al., 2006) Membranes were washed once with TBS buffer containing 0.05% Tween-20 followed by two consecutive washes with TBS buffer. These three washes were the standard washing regime for all immunoblots. Subsequently, two nitrocellulose membranes were separately incubated with 0.12 μg/mL of activated Cry1Ab or Cry1F in TBS buffer containing 2% of milk powder (blocking buffer) overnight at 4°C and then washed. Membranes were separately

incubated in blocking buffer for 2 h with monoclonal rabbit anti-Cry1Ab or Anti-Cry1F (3:10,000), followed by polyclonal goat anti-rabbit conjugated with alkaline phosphatase (1:5000) (Sigma Aldrich®). After the last wash, colorimetric detection of bands on the nitrocellulose membranes were achieved by incubating in TBS buffer containing 5-Bromo-4-chloro-3-indolyl phosphate/Nitro blue tetrazolium (SIGMA FAST™ BCIP/NBT; Sigma Aldrich®) as the substrate. Western blot assays for the putative cadherin and APN-like proteins were performed as described for the ligand blotting, except that a rabbit anti-cadherin and anti-APN-4 serum (3:10,000) were used to incubate the blot at room temperature for 2 h.

2.6. Comparative cDNA sequences of the Bt toxin putative receptors

Complete cDNA sequences of the putative cadherin and APNlike receptors of both *Ostrinia* spp. were obtained from GenBank (Table 1). Nucleotide and translated amino acids sequences were aligned using Clustal X (Thompson et al., 1994) and subjected to pairwise sequence analyses with protein distance matrix using Bioedit, version 7.0.9.0 (Hall, 1999).

3. Results

3.1. Ligand blots

Ligand blots of Cry1Ab and Cry1F toxins, as well as immunoblots for putative cadherin and APN receptors are shown in Fig. 1. Table 2 lists the approximate molecular weight (kDa) of immunoreactive bands that are indicative of either toxin binding or putative receptors. Ligand blots of both *Ostrinia* spp. indicated similar binding to Cry1Ab toxin whereby similar and strong band signals were detected at 260 kDa. For *O. nubilalis*, two additional bands were detected with slightly increased intensity of the 140 kDa band compared to the 160 kDa band. In comparison, a faint band at 150 kDa was detected and both 140 and 160 kDa bands were absent in *O. furnacalis*.

The Cry1F ligand blot revealed that both *O. furnacalis* and *O. nubilalis* BBMV exhibited strong binding to proteins of 150 and 140 kDa, respectively. *O. furnacalis* showed an additional but weaker band at 210 kDa. The 150 kDa protein from *O. furnacalis* and the 140 kDa protein from *O. nubilalis* appeared to have affinity for both Cry1Ab and Cry1F, although signal intensity was much reduced for Cry1Ab relative to Cry1F.

In general, *D. saccharalis* exhibited a completely different pattern of binding relative to the two *Ostrinia* species with a single band greater than >210 kDa for both Cry1Ab and Cry1F toxins.

3.2. Immunodetection of putative toxin receptors

Immunoblots identified common immunoreactive bands for the putative cadherin (260 kDa) and APN (135 kDa)-like receptors for both *Ostrinia* spp. Two additional bands (150 and 170 kDa) were detected in the *O. nubilalis* lane for the cadherin blot. A very faint signal was observed at 170 kDa in the *O. furnacalis* lane but the 150 kDa band was not detected. In the APN blot, the 135 kDa bands were the most prominent and intense compared to 95 and 120 kDa proteins in both *Ostrinia* spp. No signal was detected for immunoblots from D. sachcaralis BBMV for either the cadherin or APN antisera.

Comparison of the Cry1Ab ligand and cadherin immunoblots suggests that a similar band at 260 kDa was observed in both *Ostrinia* species which may be indicative of a putative cadherin-like receptor for Cry1Ab binding.

3.3. Comparative cDNA of the Bt toxin putative receptors

Results of cDNA sequence analyses indicated that the putative cadherin and APN-like receptors are similar between *O. furnacalis* and *O. nubilalis* (Tables 3 and 4). Pairwise comparison of the putative cadherin-like receptors showed little variation between *O. furnacalis* and *O. nubilalis* (i.e. 1.16–1.42% and 1.84–2.98% for nucleotide and amino acid sequences, respectively). Similarly, variation of isoforms 1, 2, 3 and 4 APN-like receptors between the two species were low and ranged from 1.84–3.14% and 4.86–6.52% for nucleotide and amino acid sequences, respectively. Thus, intraspecific comparisons of both cadherin and APN -like receptors showed low variation for the nucleotide (<2%) and amino acid (<3%) sequences.

4. Discussion

The mode of action for Bt Cry proteins in Lepidopteran larvae involves solubilization of the crystal in the alkaline larval midgut, proteolytic processing of the protoxin by midgut proteases, binding of the Cry toxin to midgut receptors, and insertion of the toxin into the apical membrane to create ion channels (Bravo et al., 2007; Gill et al., 1992). Subsequently, the osmotic balance of the midgut epithelial cells

is disturbed, inducing cell lysis, gut paralysis and ultimately, mortality (Bravo et al., 2007; Gill et al., 1992). Alteration of one or more of these processes can change the toxicity of Bt to specific insects and lead to resistance (Ferré and Van Rie, 2002). Other resistant mechanisms reported involve processes resulting in cell death (apoptosis) in the midgut leading to autophagy, cell sloughing (shedding), and finally repair by cell replacement through differentiation from the stem cell pool (Hakim et al., 2010). Among these processes, the most frequent mechanism of resistance to Bt toxins in Lepidoptera involves binding site modification (Ferré and Van Rie, 2002). Among the binding proteins that have been identified in Lepidopteran larvae, cadherin and aminopeptidase N-like receptors are the most extensively studied and have been previously identified as Bt toxin binding proteins in *O. nubilalis* (Denolf et al., 1993; Hua et al., 2001; Flannagan et al., 2005; Siqueira et al., 2006; Khajuria et al., 2009, 2011). However, similar studies relative to *O. furnacalis* are lacking.

Our results showed that both *Ostrinia* species exhibited similar toxin binding as well as expression and sequence analysis of the cDNA of putative cadherin- and APN-like receptors. A 260 kDa band in the Cry1Ab ligand blot was observed for both species which is similar to the position of one of the major bands identified in the cadherin immunoblot and similar in molecular weight to previously reported *O. nubilalis* cadherin (Flannagan et al., 2005). Although it is not possible to conclusively identify this band as a cadherin based solely on similarity in molecular weight, these results suggests that a major receptor involved in Cry1Ab binding for both species is a cadherin-like protein. This conclusion is consistent with a previous report on the Cry1Ab binding site in *O. nubilalis* (Hua et al., 2001; Flannagan et al. 2005) in which a high molecular weight cadherin-like protein was observed to bind to Cry1Ab. Two additional bands (150 and 170 kDa) were detected from *O. nubilalis* but very faint in *O. furnacalis* lane. The sensitivity of the antibody, which was derived from the *O. nubilalis* cadherin like gene, may have allowed detection of degradation products of cadherin, as similarly observed in the proteolytic degradation of the M. sexta BT-R1 cadherin (Candas et al., 2002).

In our study, a second band of 140 kDa band was observed from *O. nubilalis*, suggesting that both the cadherin (260 kDa) and a second protein are acting as the primary receptors for Cry1Ab in *O. nubilalis*. The 140 kDa band was similar in position to the APN identified from the immunoblot in both *Ostrinia* species. *O. furnacalis* exhibited a similar pattern but stronger binding at 260 kDa compared to the second band which was slightly larger in size (150 kDa), suggesting a higher affinity between the Cry1Ab toxin and the cadherin-like protein.

Comparisons of Cry1Ab and Cry1F ligand blots indicate that there might be a shared receptor in both *Ostrinia* species with a molecular weight of 140 kDa for *O. nubilalis* and 150 kDa for *O. furnacalis* that binds to both the Cry1Ab and Cry1F proteins. The signal from the Cry1Ab ligand blot was significantly reduced relative to Cry1F in both species, suggesting that Cry1Ab and Cry1F bind differentially to the putative receptor. The binding patterns observed from the *O. nubilalis* Cry1Ab ligand blot and the cadherin immunoblot are comparable to those reported by Hua et al. (2001) and Flannagan et al. (2005), respectively which suggest that a high-molecular weight Cry1Ab binding protein corresponds to a cadherin-like protein. However, in contrast to results of Hua et al. (2001) who reported a role for cadherin binding to Cry1F in *O. nubilalis*, our results suggest that Cry1F does not bind to cadherin-like proteins in either species.

Binding of Cry1Ab and Cry1F to lower molecular weight proteins (140 and 150 kDa proteins for *O. nubilalis* and *O. furnacalis*, respectively) may be indicative of binding to APN-like proteins which and appear to be the main target receptors for Cry1F toxin in both *Ostrinia* species. The position of APN-like proteins identified from immunoblots is similar in molecular weight to those identified as the main Cry1F binding proteins from ligand blots, although the positions were not identical. However, there are a number of different APN genes that have been identified in both species, and it is possible that the sequence used to generate antiserum did not represent the most important of the various proteins that are involved in toxin bindings. It is clear however, based on pairwise comparisons of the cDNA of different isoforms of APN proteins in both *Ostrinia* species, that these proteins are highly similar. This sequence similarity supports the results from ligand blots and APN immunoblots, which indicate comparable toxin binding for the two species.

The present results also demonstrate that Cry1Ab and Cry1F have high affinity to different protein receptors, thus supporting the observation of low cross resistance levels from Cry1Ab to Cry1F in *O. furnacalis* and *O. nubilalis* (Siqueira et al., 2004a; Xu et al., 2010). Furthermore, it has been reported that Cry1Ab shares a low affinity receptor with Cry1F in *O. nubilalis* (Hua et al., 2001). The Cry1Ab resistance in *O. nubilalis* was reportedly caused by the reduction of cadherin levels in the insect midgut that significantly lowered the Cry1Ab binding (Siqueira et al., 2006). To date, studies examining the mode of resistance for *O. furnacalis* against Cry1Ab and Cry1F are lacking and represent an avenue for future research.

It should be noted that because the ligand blots were conducted under denaturing conditions, and as a consequence, our results may not

reflect the precise nature of toxin binding to intact BBMV proteins. However, Keeton et al. (1998) reported that toxin binding to BtR1 from *M. sexta* was unaffected by the conditions used to separate BBMV proteins. Moreover, Daniel et al. (2002) reported that denaturation of *M. sexta* aminopeptidase N results in exposure of binding epitopes that remain hidden under non-denaturing conditions.

Although *D. saccharalis* is a member of the family Crambidae, the binding patterns of both Cry1Ab and Cry1F ligand blots were distinctly different from the two *Ostrinia* species. A similar 210 kDa binding protein was observed in the *D. saccharalis* for both Cry1Ab and Cry1F ligand blots and may represent a cadherin-like receptor as described by Yang et al. (2011). However, there was no evidence of binding to putative amino peptidase-N isoforms, which had previously been reported to confer Cry1Ab resistance in this species (Yang et al., 2010). No evidence of cross-reactivity with the cadherin and aminopeptidase antsera was detected, thus further confirming that both *O. furnacalis* and *O. nubilalis* are closely related and distinct from *D. saccharalis*.

As a component of the resistance management and biosafety stewardship of Bt crops, knowledge concerning the sensitivity as well as specific mechanisms of toxicity will be useful to developing deployment and registration strategies for Bt crops. Both *Ostrinia* species demonstrated similar patterns of sensitivity to Cry1 toxins (Tan et al., 2011), and the current studies demonstrate similar patterns of binding to Cry1Ab and possibly Cry1F and similar expression and sequence homology of putative toxin receptors. The affinities of Cry1Ab and Cry1F to candidate receptors were different in both species, with Cry1F not binding at all to the 260 kDa protein but strongly binding to the 140–150 proteins. Although this result has been obtained with denatured proteins, it suggests that the two toxins are compatible as stacking partners or in rotation for transgenic maize cultivation for both *O. nubilalis* and *O. furnacalis*. Importantly, field populations of *O. nubilalis* in the United States have maintained their susceptibility since the first Bt corn introduction (Siegfried et al., 1995; Siegfried et al., 2007; Siegfried and Hellmich 2012) with Cry1Ab Bt corn registered in 1995 followed by Cry1Fa in 2001 (USEPA, 2009). The technology has a great potential to control the Asian corn borer, and based on results of the present investigation suggesting common target receptors, the information generated for resistance management for *O. nubilalis* may be leveraged for decisions regarding the use of transgenic corn against *O. furnacalis*.

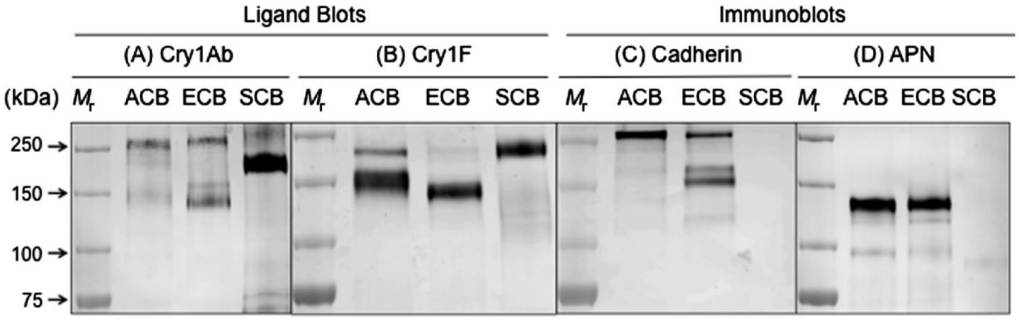

Fig. 1. Cry1Ab and Cry1F ligand blots, and immunoblotting of cadherin and aminopeptidase analyses using BBMV preparations from the guts of *O. furnacalis* (ACB), *O. nubilalis* (ECB) and *D. saccharalis* (SCB) 5th instar larvae. (A) Binding of Cry1Ab to BBMV proteins as detected by Cry1Ab polyclonall antibody. (B) Binding of Cry1F to BBMV proteins as detected by Cry1F polyclonal antibody. (C) Expression of cadherin-like protein as detected by antibody prepared from Cry1Ab binding region of cadherin DNA sequence originated from *O. nubilalis* (anti-OnBt-R1). (D) Expression of aminopeptidase N (APN)-like protein as detected by antibody prepared from partial aminopeptidase DNA sequence originated from *O. nubilalis* (anti-APN-4).

Table 1. GenBank source of the partial cDNA nucleotide sequences of putative cadherin- and aminopeptidase-N (APN)-like receptors of *O. furnacalis* and *O. nubilalis*.

Insect species	Putative receptor	Accession no.	Cited reference (direct submission[a] unless asterisked)
O. furnacalis	Cadherin	EU022587.1	Chang et al., 2007
	Cadherin	EF068248.1	Wang et al., 2006
	APN-1	GQ927480	Xu et al., 2009
	APN-1	GQ927479	Xu et al., 2009
	APN-1	EF190464	Wang et al., 2006
	APN-2	EU564811	Xu et al., 2008
	APN-2	EU826127	Xu et al., 2008
	APN-3	EU137839	Xu et al., 2007
	APN-3	EF538427	Xu et al., 2007
	APN-4	EU571948	Xu et al., 2008
	APN-4	EU826126	Xu et al., 2008
O. nubilalis	Cadherin	AY612336.1	Coates et al., 2005a*
	Cadherin	FJ011674.1	Bel et al., 2009*
	Cadherin	AX147201	Flannagan et al., 2001
	APN-1	EF103942	Coates et al., 2006
	APN-2	EF103943	Coates et al., 2006
	APN-3	EF103944	Coates et al., 2006
	APN-4	EF103945	Coates et al., 2006

[a] Directly submitted sequences are not cited in the current references.

Table 2. Approximate molecular weight (kDa) of the protein bands that bind to Cry1Ab and Cry1F toxins, as well as expression of putative cadherin and aminopeptidase-N (APN)-like receptors in *O. furnacalis, O. nubilalis* and *D. saccharalis*. The values were estimated from ligand blots and immunoblots of Fig. 1.

Insect species	Cry1Ab	Cry1F	Cadherin	APN
O. furnacalis	260, 150[a]	150, 210	260, 170[b], 150[b]	135, 120[a], 95[a]
O. nubilalis	260, 160, 140	140	260, 170, 150	135, 120[a], 95[a]
D. saccharalis	210	210	Not detected	Not detected

Bands with molecular weights followed by ([a]) exhibited reduced intensity in both ligand and immunoblots. Bands with molecular weights followed by ([b]) in *O. furnacalis* were very faint compared to correspondent bands in the cadherin immunoblot of *O. nubilalis*.

Table 3. The distance matrix of pairwise comparisons of complete cDNA of putative cadherin-like receptors between *O. furnacalis* and *O. nubilalis*[a] using nucleotide and translated amino acid sequences.

Accession numbers	*Ostrinia* spp.	*O. nubilalis* 1	*O. nubilalis* 2	*O. nubilalis* 3	*O. furnacalis* 1	*O. furnacalis* 2
Nucleotide sequences						
AY612336	*O. nubilalis* 1	0	0.0150	0.0039	0.0137	0.0142
FJ011674	*O. nubilalis* 2	0.0150	0	0.0133	0.0117	0.0116
AX14707	*O. nubilalis* 3	0.0039	0.0133	0	0.0129	0.0135
EF068248	*O. furnacalis* 1	0.0137	0.0117	0.0129	0	0.0109
EU022587	*O. furnacalis* 2	0.0142	0.0116	0.0135	0.0109	0
Translated amino acid sequences						
AY612336	*O. nubilalis* 1	0	0.0288	0.0102	0.0298	0.0236
FJ011674	*O. nubilalis* 2	0.0288	0	0.0184	0.0215	0.0184
AX14707	*O. nubilalis* 3	0.0102	0.0184	0	0.0235	0.0163
EF068248	*O. furnacalis* 1	0.0298	0.0215	0.0235	0	0.0184
EU022587	*O. furnacalis* 2	0.0236	0.0184	0.0163	0.0184	0

[a] Shaded values represent pairwise comparisons between *O. furnacalis* and *O. nubilalis*.

Table 4. The distance matrix of pairwise comparisons of complete cDNA of putative aminopeptidase-N (APN)-like receptors isoforms 1, 2, 3 and 4 between *O. furnacalis* and *O. nubilalis*[a] using nucleotide and translated amino acid sequences.

Accession numbers	*Ostrinia* spp.	Nucleotide sequences				Translated amino acid sequences			
		O. nubilalis 1	*O. furnacalis* 1	*O. furnacalis* 2	*O. furnacalis* 3	*O. nubilalis* 1	*O. furnacalis* 1	*O. furnacalis* 2	*O. furnacalis* 3
APN-1									
EF103942	*O. nubilalis* 1	*0*	*0.0200*	*0.0184*	*0.0247*	0	*0.0596*	*0.0486*	*0.0652*
GQ927480	*O. furnacalis* 1	*0.0200*	0	0.0086	0.0097	*0.0596*	0	0.0160	0.0269
GQ927479	*O. furnacalis* 2	*0.0184*	0.0086	0	0.0102	*0.0486*	0.0160	0	0.0214
EF190464	*O. furnacalis* 3	*0.0247*	0.0097	0.0102	0	*0.0652*	0.0269	0.0214	0
		O. nubilalis 1	*O. furnacalis* 1		*O. furnacalis* 2	*O. nubilalis* 1		*O. furnacalis* 1	*O. furnacalis* 2
APN-2									
EF103943	*O. nubilalis* 1	0	*0.0313*		*0.0314*	0		*0.0533*	*0.0533*
EU564811	*O. furnacalis* 1	*0.0313*	0		0.0145	*0.0533*		0	0
EU826127	*O. furnacalis* 2	*0.0314*	0.0145		0	*0.0533*		0	0
APN-3									
EF103944	*O. nubilalis* 1	0	*0.0259*		*0.0268*	0		*0.0584*	*0.0607*
EU137839	*O. furnacalis* 1	*0.0259*	0		0.0141	*0.0584*		0	0.0255
EF538427	*O. furnacalis* 2	*0.0268*	0.0141		0	*0.0607*		0.0255	0
APN-4									
EF103945	*O. nubilalis* 1	0	*0.0201*		*0.0219*	0		*0.0602*	*0.0626*
EU571938	*O. furnacalis* 1	*0.0201*	0		0.0118	*0.0602*		0	0.0166
EU826126	*O. furnacalis* 2	*0.0219*	0.0118		0	*0.0626*		0.0166	0

a. Italic values represent pairwise comparisons between *O. furnacalis* and *O. nubilalis*.

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