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Binding analyses of Cry1Ab and Cry1Ac with membrane vesicles from *Bacillus thuringiensis*-resistant and -susceptible *Ostrinia nubilalis*

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Abstract

The binding properties of *Bacillus thuringiensis* toxins to brush border membrane vesicles of Dipel-resistant and -susceptible *Ostrinia nubilalis* larvae were compared using ligand-toxin immunoblot analysis, surface plasmon resonance (SPR), and radiolabeled toxin binding assays. In ligand-toxin immunoblot analysis, the number of Cry1Ab or Cry1Ac toxin binding proteins and the relative toxin binding intensity were similar in vesicles from resistant and susceptible larvae. Surface plasmon resonance with immobilized activated Cry1Ab toxin indicated that there were no significant differences in binding with fluid-phase vesicles from resistant and susceptible larvae. Homologous competition assays with radiolabeled Cry1Ab and Cry1Ac toxin and vesicles from resistant and susceptible larvae resulted in similar toxin dissociation constants and binding site concentrations. Heterologous competition binding assays indicated that Cry1Ab and Cry1Ac completely competed for binding, thus they share binding sites in the epithelium of the larval midguts of *O. nubilalis*. Overall, the binding analyses indicate that resistance to Cry1Ab and Cry1Ac in this Bt-resistant strain of *O. nubilalis* is not associated with a loss of toxin binding.

Keywords: *Bacillus thuringiensis*; Cry1Ab; Cry1Ac; Surface plasmon resonance; European corn borer; *Ostrinia nubilalis*; Resistance mechanisms

Economically important crop pests have been controlled effectively with *Bacillus thuringiensis* (Bt) transgenic technology. The success of Bt crops will be impacted if Bt-resistant pests are selected under the high doses of plant-produced Bt. Research has characterized two major types of Bt resistance mechanisms in insects [1]. In most cases, high-level resistance to Bt toxins has been attributed to altered toxin binding to receptors in the brush border membrane of insect midguts [2–8]. Resistance to Bt also has been associated with altered gut proteinases, including decreased activation of Bt protoxin by reduced activities of gut proteinases [9–11], increased degradation of active toxin prior to or post-membrane binding [12], or increased rate of cell repair or replacement in the brush border membrane [13].

Our previous studies in *Ostrinia nubilalis* larvae demonstrated that the activity of soluble trypsin-like proteinases was significantly reduced in Dipel-resistant larvae relative to susceptible larvae, which resulted in reduced protoxin activation [14]. These results were

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similar to those in an entomocidus-resistant strain of *Plodia interpunctella* that lacked a major trypsin-like proteinase associated with Bt-resistance [9,10]. A reduction of Cry1Ab binding also was reported in the entomocidus-resistant *P. interpunctella* strain, but this reduction only partially contributed to resistance [15]. In addition to reduced proteinase activity and toxin binding, other factors associated with enhanced oxidative metabolism were involved [16].

Although reduced protoxin activation has been proposed as a potential resistance factor in Bt-resistant *O. nubilalis* [14], the binding of Cry toxins to brush border membrane vesicles (BBMVs) from the resistant strain has not been studied. Therefore, multiple approaches were used to evaluate the binding properties of Cry toxins to brush border membranes of the resistant and susceptible strains of *O. nubilalis*. Results from ligand-toxin immunoblot analysis, surface plasmon resonance (SPR), and radiolabeled toxin binding assays using BBMVs from Bt-resistant and -susceptible *O. nubilalis* indicated that resistance is not associated with a loss of toxin binding.

Materials and methods

Insect strains. A susceptible strain of *O. nubilalis* was established from egg masses collected from cornfields near St. John, Kansas during 1995 and has been reared on artificial diet for over 45 generations [17]. A Bt-resistant strain (KS-SC) was selected from this susceptible strain for more than 41 generations by exposing neonates to a diet containing *B. thuringiensis* subsp. *kurstaki* HD-1 (Dipel, Abbott Laboratories, Chicago, IL) using doses that induced 80–95% mortality. A susceptible strain of *P. interpunctella* (688s), which was collected from grain storage on a farm in Riley County, Kansas [18], and continuously reared on untreated cracked-wheat diet in the laboratory for more than 15 years, was also used in this study to compare the binding specificity of Cry1Ca.

Bacillus thuringiensis toxins. For ligand-toxin immunoblot and SPR analyses, Cry1Ab, Cry1Ac, and Cry1Ca protoxins were produced from recombinant *Escherichia coli* ECE54, ECE53, and ECE125, which harbor *cry1Ab*, *cry1Ac*, and *cry1Ca* genes, respectively. Recombinant *E. coli* strains were provided by *Bacillus* Genetic Stock Center, Ohio State University, and partially purified as previously described [19]. For radioligand binding assays, Cry1Ab and Cry1Ac protoxins were obtained from recombinant *B. thuringiensis* EG7077 and EG11070 strains, respectively (Ecogen, Langhorn, PA). Each *B. thuringiensis* strain was grown at 29 °C for 48 h in CCY medium [20] supplemented with 10 μg/ml tetracycline for the EG7077 strain and 3 μg/ml chloramphenicol for the EG11070 strain. Spores and crystals were collected by centrifugation at 9700*g* at 4 °C for 10 min. Pellets were washed four times with 1 M NaCl, 10 mM EDTA, and resuspended in 10 mM KCl. For all binding experiments in this study, Cry1Ab, Cry1Ac, and Cry1Ca protein inclusions were suspended in 50 mM sodium carbonate buffer, pH 10.5, containing 10 mM dithiothreitol. Protoxins were trypsin-activated (trypsin type XI: from bovine pancreas, Sigma Chemical, St. Louis, MO) at 37 °C, 2 h (1 mg trypsin per 10 mg protoxin). Activated toxins were purified by anion-exchange chromatography [7].

BBMV preparation. Brush border membrane vesicles (BBMVs) were prepared from midguts obtained from fifth instars [21]. The BBMV pellet was resuspended in Hepes-buffered saline (HBS) (10 mM Hepes, pH 7.4, 150 mM NaCl, and 3.4 mM EDTA), frozen in aliquots in liquid nitrogen, and stored at −80 °C. BBMVs were used directly in ligand-toxin immunoblot assays, or for SPR, BBMVs were sonicated to create uniform vesicles of less than 0.5 μm [22]. Sonication was performed on ice at 100 W and 20 kHz three times, each for 30 s. Leucine aminopeptidase activities of crude homogenate and BBMV preparation were determined as previously described [14]. To prepare BBMVs for radioligand binding analysis, fifth-instar larvae were collected, frozen in liquid nitrogen, preserved in dry ice, and sent to the University of Valencia, Spain. Upon arrival, larvae were dissected and BBMVs were prepared as above.

Protein concentration in the preparations of Bt toxins and BBMVs was measured by the method of Bradford [23] using the Coomassie plus protein assay (Pierce, Rockford, IL), with bovine serum albumin (BSA) as a protein standard.

Ligand blotting. Ligand blot analysis was performed with Bt toxins and BBMVs from Bt-resistant and -susceptible larvae of *O. nubilalis*, to compare the number and relative molecular mass of binding proteins as well as the relative toxin binding intensity. Briefly, 20 μg of BBMV protein from resistant or susceptible larvae was separated in a 7% Tris–acetate gel (Invitrogen, San Diego, CA) under denaturing conditions and transferred to a polyvinylidene difluoride Q membrane (PVDF) (Millipore, 0.45 μm, Fisher Scientific, Pittsburgh, PA) in Nupage transfer buffer (Invitrogen). The PVDF membrane was cut into strips and blocked with 3% (w/v) BSA in phosphate-buffered saline (PBS) buffer, pH 7.4 [24], at room temperature with agitation for 2 h. Membrane strips were incubated with 16.7 nM of trypsin-activated Cry1Ab, Cry1Ac, or Cry1Ca toxins in PBS buffer containing 0.1% BSA (w/v) and 0.3% (v/v) Tween 20 at room temperature with agitation for 2 h. Western blot analysis was performed using rat polyclonal antiserum against Cry1Ac (1:5000), cross-reactive to Cry1Ab and Cry1Ca, and goat anti-rat IRDye 800 (LI-COR, Lincoln, NE) (1:5000) to visualize bound Cry toxins. BBMVs prepared from a Bt-susceptible strain (688s) of *P. interpunctella* larvae and Cry1Ca were included as a control for specific binding.

Toxin labeling and radioligand binding assays. Trypsin-activated Cry1Ab and Cry1Ac were labeled with ^{125}I by the method of chloramine T [25]. The specific radioactivity of ^{125}I-Cry1Ab and Cry1Ac was 3.38 and 2.39 mCi/mg, respectively. To determine specific binding and specify a suitable concentration of BBMVs for competition assays, a series of concentrations (0–0.5 μg/μl) of BBMV protein from resistant or susceptible larvae of *O. nubilalis* were incubated with either 0.56 nM ^{125}I-Cry1Ab or 0.20 nM ^{125}I-Cry1Ac, respectively, in 100 μl PBS buffer supplemented with 0.1% BSA at room temperature for 60 min. Toxin bound to BBMVs was separated from free toxins in the suspension by centrifugation at 16,100*g* at 4 °C for 10 min. The pellet was washed twice with 500 μl ice-cold PBS buffer containing 0.1% BSA. The radioactivity remaining in the pellet was measured with a 1282 Compugamma CS Universal gamma counter (LKB–Wallac Pharmacia, Turku, Finland) and considered total binding. An approximately 1000-fold excess of unlabeled toxin was used to determine non-specific binding. Specific binding was estimated by subtracting the non-specific binding from total binding.

For Cry1Ab homologous competition assays, increasing amounts of unlabeled Cry1Ab toxin were added into the binding reaction suspensions (100 μl) containing the labeled Cry1Ab toxin and an appropriate concentration of BBMVs from the resistant or susceptible larvae, as determined in previous experiments. The reaction mixtures were incubated at room temperature for 60 min, and the proportion of bound labeled ligand was determined for each reaction. Cry1Ac homologous competition assays were also performed in the same way. For reciprocal heterologous competition assays, increasing amounts of unlabeled Cry1Ab (or Cry1Ac) toxin were added into the binding reactions containing the labeled Cry1Ac (or Cry1Ab) toxin and an appropriate concentration of BBMVs from the susceptible larvae, and the proportion of bound labeled ligand was determined for each reaction. The concentration of BBMV protein was 0.05 μg/μl from the

resistant larvae or 0.02 μg/μl from the susceptible larvae in the competition assays for labeled Cry1Ac, and 0.05 μg/μl from both resistant and susceptible larvae for labeled Cry1Ab. Radioligand binding experiments were independently performed at least twice.

The estimation of dissociation constants (K_d) and binding site concentrations (R_t) were performed with the LIGAND software [26]. Statistical tests (*t* test) and charts were performed using GraphPad Prism version 3.02 for Windows (GraphPad Software, San Diego, CA, www.graphpad.com). According to the heterologous competition experiments, Cry1Ab and Cry1Ac share all binding sites in the midgut of *O. nubilalis*. Based on this result, the R_t values were estimated for each strain from the analysis of the combined data from the homologous competition experiments with ^{125}I-Cry1Ab and ^{125}I-Cry1Ac.

Surface plasmon resonance assays. SPR methods were adapted from Masson et al. [22], using a BIAcore 3000 and carboxymethylated dextran (CM5) sensor chips (BIAcore, Uppsala, Sweden). Carboxyl groups of the CM-dextran chains of the sensor chip surface were activated by exposure to a mixture of 0.1 M NHS (*N*-hydroxysuccinimide) and 0.1 M EDC (*N*-ethyl-*N*′-[3-dimethyl-aminopropyl]carbodiimide hydrochloride) (1:1, v/v). The reactive succinimidyl ester groups were covalently attached to the free amino group of the N terminal residue and the solvent-facing lysine or arginine residues of immobilized proteins. Immobilized Cry1Ab, Cry1Ca, and BSA measured approximately 3000 resonance units (RU), equivalent to approximately 3 ng protein. HBS buffer (BIAcore) was used as running and diluting buffers for all vesicle experiments. After each injection of BBMVs, surface regeneration was performed by injecting two separate 1 min pulses of 5 μl of regeneration buffer (1% Zwittergent 3–14) per minute, followed with one injection of 0.5% sodium dodecyl sulfate solution.

BBMVs were diluted to 0.3 μg/μl with HBS buffer containing 0.1 μg/μl of BSA, and injected over immobilized Cry or BSA protein surfaces at a flow rate of 10 μl/min for 120 s. Twenty microliters of diluted BBMVs prepared from susceptible larvae of *O. nubilalis* was injected over the surfaces of Cry1Ab, Cry1Ca, and BSA to determine specific binding responses. Twenty microliters of diluted BBMVs from resistant and susceptible larvae was sequentially injected over immobilized Cry1Ab to compare binding of BBMVs to the Cry protein. The injections of BBMVs from the susceptible strain were repeated twice over the chip containing Cry1Ab, Cry1Ca, and BSA. Comparison injections of BBMVs from resistant and susceptible strains were repeated three times over a chip containing Cry1Ab. The binding responses of vesicle proteins to the immobilized protein surfaces were automatically recorded in real time. These data were used to analyze the binding nature (specific or non-specific binding) and relative binding levels of BBMVs to immobilized proteins.

Results

Ligand blotting

Cry1Ab and Cry1Ac bound to BBMV proteins from *O. nubilalis* (Figs. 1A and B). Binding of Cry1Ab and Cry1Ac was mostly to a BBMV protein of ~145 kDa. Cry1Ab and Cry1Ac also bound to proteins with molecular masses of ~126, 220, and >300 kDa. In addition, Cry1Ac bound to a protein with a molecular mass of ~185 kDa, but for Cry1Ab, the binding signal to the protein was weak (not marked). There were no significant differences in the binding patterns or intensity of either Cry1Ab or Cry1Ac to BBMVs from Bt-resistant or -susceptible larvae of *O. nubilalis*. Cry1Ca did not bind to BBMVs from either the Dipel-resistant or -susceptible strains of *O. nubilalis*, but it bound to two different proteins in BBMVs from a Cry1Ca-susceptible strain (688s) of *P. interpunctella* (Figs. 1C and D).

Binding of radiolabeled toxins

BBMVs from both resistant and susceptible larvae of *O. nubilalis* demonstrated specific binding with either ^{125}I-Cry1Ab or ^{125}I-Cry1Ac toxin. Specific binding of both toxins was obtained with increasing concentrations of BBMV proteins (Fig. 2). The maximum specific binding of ^{125}I-Cry1Ab to BBMVs from resistant and susceptible larvae was 16.7% and 21.4% of the total input radioactivity, respectively. Similarly, maximum specific binding of ^{125}I-Cry1Ac to BBMVs from the resistant

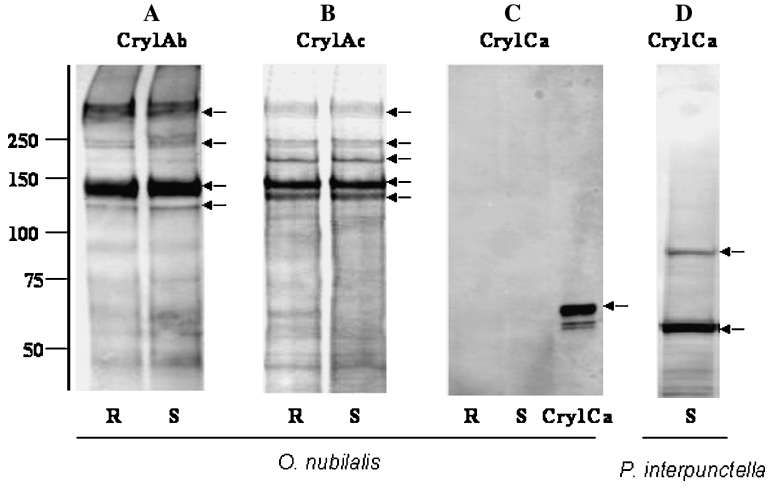

Fig. 1. Binding of activated forms of Cry1Ab (A), Cry1Ac (B), and Cry1Ca (C) to BBMVs from Bt-resistant (R) and -susceptible (S) strains of *O. nubilalis*, and of Cry1Ca to BBMVs from Bt-susceptible *P. interpunctella* larvae (D). Arrows indicate toxin-binding proteins. Lane Cry1Ca (C) was purified trypsin-activated Cry1Ca used as a positive control for activated Cry1Ca. Molecular mass markers are indicated at the left in kDa.

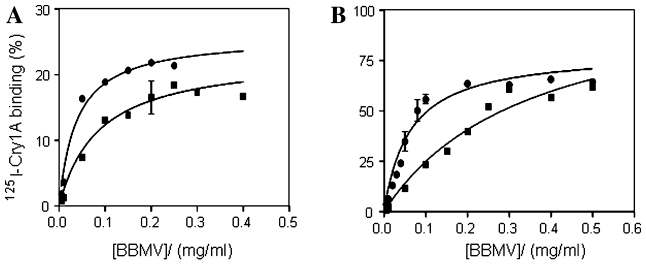

Fig. 2. Specific binding of ^{125}I-Cry1Ab (A) and ^{125}I-Cry1Ac (B) as a function of BBMV protein concentration in susceptible (●) and resistant larvae (■) of *O. nubilalis*.

and susceptible larvae was 61.9% and 64.5%, respectively.

Homologous competition experiments showed similar binding curves of either ^{125}I-Cry1Ab or ^{125}I-Cry1Ac to BBMVs from the resistant and susceptible larvae (Fig. 3). The estimated dissociation constants (K_d) of Cry1Ab were 7.9 ± 2.9 nM for the resistant larvae, and 6.0 ± 1.4 nM for the susceptible larvae (Table 1). The K_d values of Cry1Ac were 3.3 ± 0.9 and 2.3 ± 0.9 nM for the resistant and susceptible strains, respectively. There were no significant differences ($P > 0.05$) in the K_d values of either toxin for BBMVs from resistant and susceptible larvae. The R_t value for the resistant strain was 13.1 ± 7.2 pmol/mg of vesicle protein, and 42.6 ± 24.3 pmol/mg for the susceptible strain (Table 1).

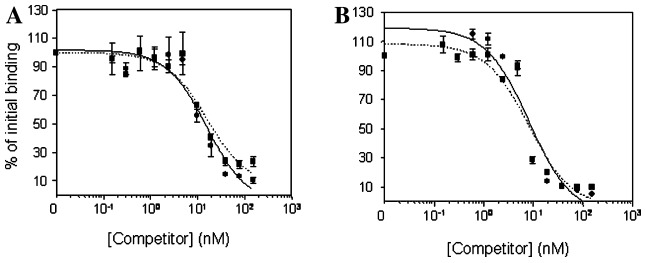

Fig. 3. Binding of ^{125}I-Cry1Ab (A) and ^{125}I-Cry1Ac (B) to BBMVs from susceptible (●, solid line) and resistant (■, dashed line) larvae of *O. nubilalis* at different concentrations of non-labeled homologous competitor.

Table 1
Binding parameters of *B. thuringiensis* toxins with BBMVs from susceptible and resistant larvae of *O. nubilalis*

Insects	Toxin	K_d ± SD (nM)[a]	R_t ± SD (pmol/mg)[b]
Susceptible	Cry1Ab	6.0 ± 1.4	42.6 ± 24.3
	Cry1Ac	2.3 ± 0.9	
Resistant	Cry1Ab	7.9 ± 2.9	13.1 ± 7.2
	Cry1Ac	3.3 ± 0.9	

[a] Values obtained from homologous competition.
[b] R_t is expressed as pmol of binding sites per milligram of total vesicle protein.

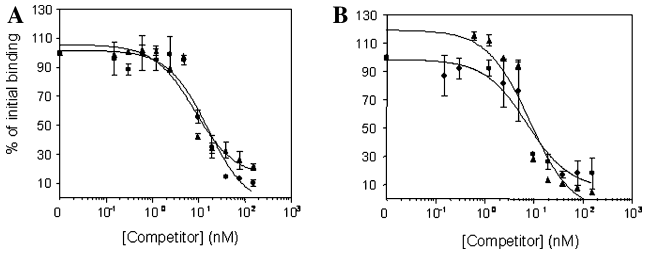

Fig. 4. Binding of ^{125}I-Cry1Ab (A) and ^{125}I-Cry1Ac (B) to BBMVs from susceptible larvae of *O. nubilalis* at different concentrations of non-labeled competitor. Cry1Ab (●), Cry1Ac (▲). For comparison, each chart includes homologous competitor data.

There were also no significant differences ($P > 0.05$) in the R_t values between the resistant and susceptible strains. These data suggest that the resistant and susceptible strains of *O. nubilalis* have a similar binding affinity and binding capacity for either Cry1Ab or Cry1Ac. Reciprocal heterologous competition experiments demonstrated that Cry1Ab and Cry1Ac toxins were able to completely compete for binding to BBMVs from the susceptible larvae (Fig. 4).

Surface plasmon resonance

SPR demonstrated significant differences in the binding responses of fluid-phase BBMVs from susceptible larvae of *O. nubilalis* to immobilized Cry1Ab, Cry1Ca, and BSA (Fig. 5A). For Cry1Ca and BSA surfaces, there was a small amount of non-specific binding of 44 ± 27 and 52 ± 38 (mean ± SD) RU, respectively. In contrast, susceptible *O. nubilalis* BBMV injected over the Cry1Ab surface showed a slow and continuous increase in signal, indicating a specific binding event. At the end of injection, the signal stabilized at a significantly higher level (257 ± 38 RU) relative to Cry1Ca and BSA. This interaction was specific and stable for binding of BBMVs to immobilized Cry1Ab.

The same amounts of BBMVs from Bt-resistant and -susceptible larvae of *O. nubilalis* were sequentially

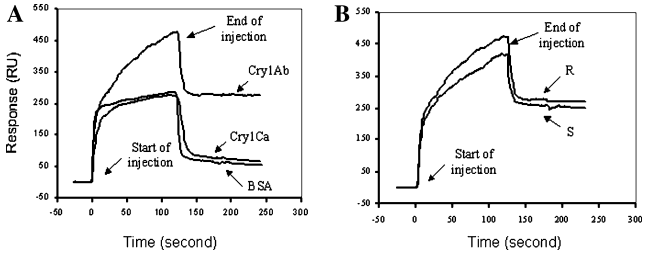

Fig. 5. Real-time binding responses (raw data) of BBMVs from *O. nubilalis* larvae to immobilized trypsin-activated Bt toxins. BBMV preparations from susceptible larvae were injected over the chip surface containing Cry1Ab, Cry1Ca, and BSA (negative control) (A). BBMV preparations from resistant (R) and susceptible (S) larvae were injected over a Cry1Ab surface (B).

injected over the same Cry1Ab surface (Fig. 5B). The binding response patterns were similar and there was no significant difference ($P > 0.05$) in the final stabilized signal levels, i.e., 264 ± 35 RU for BBMVs from resistant larvae, and 260 ± 51 RU for BBMVs from susceptible larvae.

Discussion

Bt toxin binding to BBMVs from Bt-resistant and -susceptible *O. nubilalis* larvae was compared by three different methods. Results from assays using ligand-toxin immunoassays, SPR, and radiolabeled toxins indicated that Cry1Ab and Cry1Ac toxin binding was similar in BBMVs from both strains. In ligand-toxin immunoblot and SPR assays, Cry1Ca did not bind to BBMVs from either resistant or susceptible *O. nubilalis*, yet Cry1Ca did interact with two proteins in BBMVs from a Bt-susceptible strain (688s) of *P. interpunctella*. These binding patterns correlate with the observations that Cry1Ca is not toxic to *O. nubilalis* (Li et al., unpublished) but is toxic to *P. interpunctella* [27]. The basic patterns of Cry1Ab and Cry1Ac binding proteins in the BBMVs of *O. nubilalis* larvae in this study were similar to those detected by Hua et al. [24], although there were some differences in the molecular masses of binding proteins in the two studies. These differences may be due to different gel systems and protein markers or protein degradation during vesicle preparation.

The Bt-resistant strain of *O. nubilalis* in the present study has relatively high levels of resistance to Cry1Aa, Cry1Ab, Cry1Ac, and Cry2Aa toxins that are present in Dipel, the commercial formulation used for the selection (Li et al., unpublished). It has been proposed that Cry2Aa has a different mode of action from the Cry1A toxins, and there are very few instances of shared binding sites of Cry2Aa and Cry1A toxins in BBMVs [28,29]. The resistance and cross-resistance patterns in Dipel-resistant *O. nubilalis* suggest a mechanism of resistance other than binding site alteration, and reduced soluble trypsin-like proteinase activity has been associated with reduced Cry1Ab protoxin activation in these insects [14].

The broad-spectrum resistance found in this Bt-resistant *O. nubilalis* strain is similar to that reported in entomocidus-resistant *P. interpunctella*, resistant to entomocidus component toxins Cry1Aa, Cry1Ab, and Cry1C, but also cross-resistant to Cry1Ac, Cry1B, and Cry2A [27]. Resistance to Bt in these insects is supported by multi-factoral resistance mechanisms [9,10,15,16]. As well, *Heliothis virescens* selected with Cry1Ac were cross-resistant to Cry1Aa, Cry1Ab, Cry2Aa, Cry1B, and Cry1C [30]. No differences were found in the binding of Cry1Ab or Cry1Ac toxin in Cry1Ac-resistant *H. virescens*, but differences in proteinase activities have been associated with resistance in several Bt-resistant *H. virescens* strains [11,13].

Different Cry1A toxins appear to share common binding sites in the midgut epithelium of larval lepidopteran insects [6,15,31–33]. Similarly, our results demonstrate that Cry1Ab and Cry1Ac compete for the same binding sites in *O. nubilalis* and are in agreement with other previously published studies with this insect [24,34]. There are examples where the alteration of a common binding site is sufficient to confer resistance (or cross-resistance) to all toxins sharing the altered binding site [4,7,35]. It is for this reason that this type of information is valuable for resistance management purposes, although mechanisms of resistance other than altered binding may present non-predictable cross-resistance patterns.

While reduced proteinase activity confers resistance to Cry1Ab and Cry1Ac in this Bt-resistant strain of *O. nubilalis*, other resistance factors may also be involved. Therefore, other studies are needed on resistance factors in Dipel-resistant *O. nubilalis*, such as physiological comparisons in the pore formation and the rate of repair or replacement of impaired brush border membrane cells, as well as genomic and proteomic approaches to compare multiple alterations that may affect toxin mode of action.

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