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Interaction of *Bacillus thuringiensis* Cry1 and Vip3A Proteins with *Spodoptera frugiperda* Midgut Binding Sites[▽]

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Vip3Aa, Vip3Af, Cry1Ab, and Cry1Fa were tested for their toxicities and binding interactions. Vip3A proteins were more toxic than Cry1 proteins. Binding assays showed independent specific binding sites for Cry1 and Vip3A proteins. Cry1Ab and Cry1Fa competed for the same binding sites, whereas Vip3Aa competed for those of Vip3Af.

Crops expressing *Bacillus thuringiensis* insecticidal proteins (Bt crops) are considered one of the most successful recent biotechnological achievements. The two main Bt crops commercially grown are maize and cotton. Almost all planted Bt maize expresses Cry1Ab for the control of stem borers, and more recently Cry1Fa maize has been introduced to control *Spodoptera* spp. Bt cotton has been engineered to express Cry1Ac. However, second-generation Bt cotton, which combines two insecticidal protein genes (such as *cry1Ab* and *vip3A* in VipCot cotton) that are active against lepidopteran species (3), has already been developed. The rationale behind this strategy is twofold: on the one hand, to widen the insecticidal spectrum, and on the other hand, for resistance management purposes (1). To achieve the second objective, a key requirement is that the two insecticidal proteins expressed in the plant differ in their modes of action, more particularly in their recognition of specific binding sites in the target insect (3).

The polyphagous *Spodoptera frugiperda*, a significant pest in the southern United States and a main pest in Central and South America, is quite tolerant to Cry1A proteins but susceptible to Cry1F and Vip3A (http://www.glfc.cfs.nrcan.gc.ca/bacillus). Given the lack of studies of the binding properties of Vip3A proteins in the *Spodoptera* genus and the important role that this family of proteins could play in the future in the control of such pests, the main goal of the present study was to determine the possible interactions at the binding site level of two Vip3A proteins (Vip3Aa and Vip3Af), Cry1Ab, and Cry1Fa in *S. frugiperda*.

B. thuringiensis strains EG7077 and EG11096 from EcoGen (Langhorne, PA) were used to express the proteins Cry1Ab and Cry1Fa, respectively. The genes encoding the Vip3Aa1 (NCBI accession no. AAC37036) and Vip3Af1 (NCBI accession no. CAI43275) proteins were kindly supplied by Bayer BioScience N.V. (Ghent, Belgium). Both *vip3A* genes had been modified to contain a His tag sequence at the C terminus of the protein to facilitate purification and were expressed in pMa5-8 (8) in *Escherichia coli* WK6.

Trypsin-activated, chromatography-purified Cry1 proteins were prepared as previously described (4). Expression of Vip3A proteins was induced with isopropyl-β-D-thiogalactopyranoside (IPTG), and cells were broken by lysozyme and sonication treatment. Vip3A proteins to be used in binding assays were further purified on a HiTrap Chelating HP column (GE Healthcare) and then treated with 1% bovine trypsin (Sigma-Aldrich) at 37°C for 1 h and purified by anion-exchange chromatography in a MonoQ 5/50 column as previously described for Cry proteins (4).

Vip3A and Cry1Fa proteins were labeled with biotin using the enhanced chemiluminescence protein biotinylation module kit (GE Healthcare), according to the manufacturer's instructions. Cry1Ab protein (26 μg) was labeled with 0.3 mCi $Na^{125}I$ using the chloramine-T method (9). Brush border membrane vesicles (BBMV) were prepared by the differential magnesium precipitation method from last-instar larvae (10).

Overlay bioassays in artificial diet were carried out in duplicate using seven concentrations of toxins and 16 neonatal larvae for each concentration. Assay plates were incubated for 7 days at 25°C ± 2°C, with a relative humidity of 65% ± 5% and a photoperiod of 16:8 (light/dark). The results, summarized in Table 1, showed that Vip3Aa and Vip3Af were highly toxic to *S. frugiperda*, whereas Cry1Fa exhibited moderate toxicity and Cry1Ab was the least active protein.

To determine the binding characteristics of the selected proteins to *S. frugiperda* BBMV, biotinylated Cry1Fa (50 ng) and Vip3Af (60 ng) were incubated for 1 h with 20 μg of BBMV in 0.1 ml of binding buffer (phosphate-buffered saline, pH 7.4, 0.1% bovine serum albumin) in the absence or presence of a 200-fold excess of unlabeled toxins. Binding was detected after electrophoresis and membrane transferring (5). Both Cry1Fa and Vip3Af bound specifically (Fig. 1), since an excess of the same unlabeled protein significantly reduced the binding of the labeled protein (Fig. 1, compare lane 5A with 1A and lane 1B with 2B). Cry1Fa recognizes the same sites as Cry1Ab since the latter significantly reduced the amount of bound labeled Cry1Fa (Fig. 1, lane 2A). In contrast, Cry1Fa binding was not reduced by either Vip3Aa or Vip3Af (Fig. 1, lanes 3A and 4A). While unlabeled Vip3Aa substantially reduced binding of la-

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TABLE 1. Toxicities of trypsin-activated Cry1 and trypsin-untreated Vip3A proteins to neonatal larvae of *S. frugiperda* (measured after 7 days)

Protein	LC_{50} (ng/cm^2) (FL_{95})[a]	Slope ± SE
Vip3Aa	49.3 (32.6–71.4)	1.53 ± 0.20
Vip3Af	21.0 (13.0–31.7)	1.17 ± 0.15
Cry1Ab	867 (539–1,215)	1.37 ± 0.24
Cry1Fa	170 (128–224)	2.1 ± 0.2

[a] FL_{95}, 95% fiducial limit; LC_{50}, 50% lethal concentration.

beled Vip3Af (Fig. 1, lane 3B), Cry1Ab and Cry1Fa did not compete for these sites (Fig. 1, lanes 4B and 5B). These results show that *S. frugiperda* has binding sites for Cry1Fa, shared by Cry1Ab, and independent binding sites shared by Vip3Af and Vip3Aa.

Binding experiments with ^{125}I-labeled Cry1Ab (1.3 nM) were performed using 7 μg of BBMV in a final volume of 0.1 ml binding buffer as described by Ferré et al. (2). The results showed that ^{125}I-labeled Cry1Ab binding was competed by Cry1Fa (Fig. 2) but not by any of the tested Vip3A proteins.

The long-term success of crops expressing *B. thuringiensis* insecticidal proteins will depend primarily on the abilities of insects to develop resistance against the insecticidal proteins. The present study shows that the four selected proteins bind specifically to *S. frugiperda* BBMV, with independent binding sites for the Cry1 (Cry1Ab and Cry1Fa) and Vip3A (Vip3Aa and Vip3Af) proteins. Heterologous competition between different Vip3A proteins had never been tested before. Because of their structural similarity, it is not surprising that Vip3Aa and Vip3Af shared binding sites. As far as we know, specific binding of Vip3A proteins has been shown only with Vip3Aa in *Manduca sexta*, *Ostrinia nubilalis*, *Heliothis virescens*, and *Helicoverpa zea* (6, 7). For the last two species, where binding competition experiments were performed, no competition was observed between Vip3Aa and Cry1Ac. Therefore, Vip3A and Cry1A proteins have independent binding sites in three noctuids (*H. virescens*, *H. zea*, and *S. frugiperda*) and probably also in other lepidopteran species. Considering that binding to specific receptors is a key step in the mode of action of *B. thuringiensis* insecticidal proteins, the odds of finding (high levels of) cross-resistance between Cry1A or Cry1F proteins and Vip3A proteins in the species described above are extremely low, and thus the strategy of combining these Cry1 proteins with Vip3A proteins is advisable.

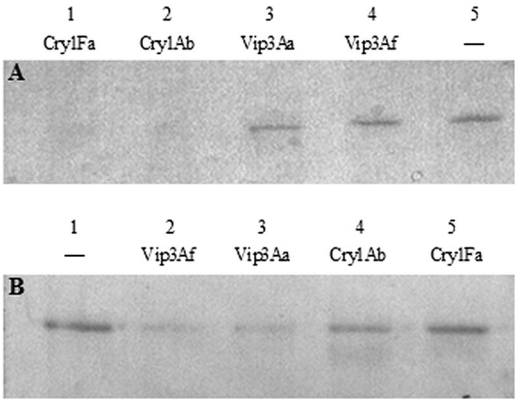

FIG. 1. Binding of biotinylated toxins Cry1Fa (A) and Vip3Af (B) to *S. frugiperda* BBMV in the absence of competitor (−) or in the presence of a 200-fold excess of competitor.

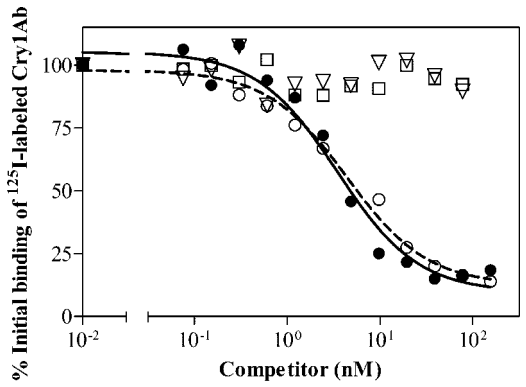

FIG. 2. Binding of ^{125}I-labeled Cry1Ab to *S. frugiperda* BBMV at increasing concentrations of unlabeled toxins Cry1Ab (●), Cry1Fa (○), Vip3Aa (□), and Vip3Af (▽). Each data point represents the mean of two replicates. Error bars have been omitted for clarity.

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