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Potential of the *Bacillus thuringiensis* Toxin Reservoir for the Control of *Lobesia botrana* (Lepidoptera: Tortricidae), a Major Pest of Grape Plants▽

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The potential of *Bacillus thuringiensis* Cry proteins to control the grape pest *Lobesia botrana* was explored by testing first-instar larvae with Cry proteins belonging to the Cry1, Cry2, and Cry9 groups selected for their documented activities against Lepidoptera. Cry9Ca, a toxin from *B. thuringiensis*, was the protein most toxic to *L. botrana* larvae, followed in decreasing order by Cry2Ab, Cry1Ab, Cry2Aa, and Cry1Ia7, with 50% lethal concentration values of 0.09, 0.1, 1.4, 3.2, and 8.5 μg/ml of diet, respectively. In contrast, Cry1Fa and Cry1JA were not active at the assayed concentration (100 μg/ml). In vitro binding and competition experiments showed that none of the toxins tested (Cry1Ia, Cry2Aa, Cry2Ab, and Cry9C) shared binding sites with Cry1Ab. We conclude that either Cry1Ia or Cry9C could be used in combination with Cry1Ab to control this pest, either as the active components of *B. thuringiensis* sprays or expressed together in transgenic plants.

Most *Bacillus thuringiensis* strains that have been identified contain a mixture of up to eight different Cry proteins (26), which provides a great diversity of insecticidal toxins (2, 14), representing an important toxin reservoir for the development of numerous pest control products.

The grape moth (*Lobesia botrana*) is the most important component of the lepidopteran pest complex attacking vineyards in Europe (4, 27, 28). This pest has been successfully controlled using certain *B. thuringiensis* strains (3, 34). However, the effects of single Cry proteins against this pest have not been tested systematically, with the exception of a single study (30) that reported Cry1Ab to be the most active of a series of Cry1 proteins tested.

Reduced binding of toxin to the insect midgut target sites represents a well-known mechanism of resistance to *B. thuringiensis* toxins (7). Alterations and modifications in certain insect gut molecules that could be associated with resistance to *B. thuringiensis* toxins have been also reported (9, 16, 17, 20, 38).

In the present study, we have evaluated the potential of *B. thuringiensis* toxins for the control of *L. botrana*. We determined the insecticidal activities of some of the most common lepidopteran-active Cry proteins (selected from the Cry1, Cry2, and Cry9 classes) and investigated their binding site relationships to determine their suitability for combined use in pest management programs.

For this, seven single Cry toxins, purified from recombinant *B. thuringiensis* strains, were tested. Strains EG7077 (Cry1Ab), Cry1Ja (EG11096), and Cry1Fa (EG7279) were obtained from Ecogen Inc. (Langhorne, PA) and EG7543 (Cry2Aa) and EG7699 (Cry2Ab) from Monsanto Co. (Chersterfield, MO), and these strains were grown in casein hydrolysate-yeast extract medium (36), supplemented with the appropriate antibiotic (3 μg/ml of chloramphenicol for EG11069, EG7543, and EG7699; 10 μg/ml of tetracycline for EG7077; and 4 μg/ml of chloramphenicol for EG7279), and further activated by trypsin treatment, as described previously (6). Trypsin-activated proteins used for binding assays were purified by fast protein liquid chromatography (Pharmacia, Uppsala, Sweden). Cry1Ia7 (Cry1Ia) was produced in recombinant BL21(DE3) *Escherichia coli* cultures. Cells were grown overnight in LB with kanamycin (50 μg/ml) at 37°C and used to inoculate 750 ml of 2× TY (tryptone-yeast extract) culture medium (23). The culture was grown at 37°C until the optical density at 600 nm was 0.5 to 0.6 and then incubated at 25°C for 45 min. Isopropyl-β-D-thiogalactopyranoside (IPTG) (1 mM) was added and the incubation continued for 2 h at 25°C. Cold phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.5) (0.5 volume) was added to the culture, and cells were recovered by centrifugation (16,000 × *g*, 15 min). The pellet was resuspended in cold PBS (1/10 volume), centrifuged, and stored at −80°C. Cells were thawed on ice with a 1/33 volume of cold binding buffer (40 mM imidazole, 4 M NaCl, 160 mM Tris-HCl, pH 7.9; Novagen, Darmstadt, Germany) and sonicated for 60 s in 15-s pulses. Protein was expressed with a histidine residue, and purification was performed using an affinity nickel column (His Bind purification kit; Novagen). Finally, the buffer was changed to carbonate (50 mM $NaCO_3$, 100 mM NaCl, pH 11.3) with a Sephadex G-25 prepacked column (Amersham Biosciences, Uppsala, Sweden) and the protein solution stored at 4°C until use. Cry1Ia was trypsin activated for binding assays but used as a protoxin for insect bioassays. Purified and activated Cry9Ca (the Lys mutant) (22) was obtained from Bayer CropScience (Gent, Belgium). The protein concentration was determined as described by Bradford (1), using bovine serum albumin (BSA) as a standard. Strain HD-1, obtained from the

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Bacillus Genetic Stock Center (BGSC, OH), was used as a standard in bioassays for activity comparisons. Spore-crystal suspensions were obtained, and the crystals were purified as described previously (33) and administrated to larvae without any previous treatment.

The toxicities of individual purified toxins and HD-1 were determined by incorporating the proteins into an artificial diet (3.9% wheat germ, 3.25% brewer's yeast, 0.1% corn oil, 0.1% nipagin, 2.5% agar, 3.25% corn flour, 0.32% ascorbic acid, and 0.1% benzoic acid [wt/vol]) (25). Initially, the differential susceptibilities of *L. botrana* larvae to individual Cry toxins at a relatively high concentration (100 μg of toxin/ml of diet) were tested. In a second experiment, bioassays involving a series of five toxin concentrations of each Cry protein, ranging from 0.012 to 30 μg of toxin/ml of diet, were used to determine the concentration-response relationship. For this, a solidified diet containing the appropriate toxin was offered individually to *L. botrana* larvae. Twenty-five neonate larvae were tested for each toxin concentration as well as for the control. The whole bioassay was performed three times. Larvae in the bioassays were maintained under constant conditions (22 ± 1°C, 65% ± 5% relative humidity, and a 16:8 [light/dark {h}]) photoperiod). Mortality was recorded after 5 days. Control experiments were performed under the same conditions but without any Cry protein in the diet. Concentration-mortality data were subjected to probit analysis (8), and the 50% lethal concentration (LC_{50}) values were calculated using the POLO-PC program (24).

Brush border membrane vesicles (BBMV) of *L. botrana* were prepared as described previously (33). Activated and purified Cry1Ab was labeled with ^{125}I by the chloramine T method (37), and the specific activity of the radio-iodinated toxin was analyzed by a sandwich enzyme-linked immunosorbent assay (37). The specific activity for ^{125}I-Cry1Ab was 2.9 mCi/mg. Cry1Ia was trypsin activated, and Cry1Ab and Cry9Ca were trypsinized and further purified by fast protein liquid chromatography (as performed for radio iodination). Each protein was then biotinylated using a kit, following the manufacturer's instructions (Amersham Biosciences, Uppsala, Sweden). Binding experiments with *L. botrana* BBMV and ^{125}I-Cry1Ab were performed as described previously (6). The appropriate conditions for time of incubation, concentration of labeled toxin, and concentration of BBMV were determined in preliminary tests using the grape moth. These conditions were 1.2 ng of ^{125}I-Cry1Ab, 0.15 mg/ml BBMV, and 1 h incubation at room temperature in a binding buffer (PBS [1 mM KH_2PO_4, 10 mM Na_2HPO_4, 137 mM NaCl, 2.7 mM KCl, pH 7.4], 0.1% BSA; final volume, 0.1 ml). Competition experiments were performed by increasing concentrations of nonlabeled Cry1Ab, Cry1Ia, Cry2Aa, Cry2Ab, and Cry9Ca. ^{125}I-Cry1Ab bound to the BBMV after the assay was measured using a gamma counter (Compugamma 1282; LKB). All the experiments were performed at least twice. Quantitative analyses for obtaining the dissociation constants and the binding site concentrations were performed with the LIGAND program (29), using data from the homologous-competition experiment. Graphical representations and curve fittings were plotted using Graphpad Prism version 4.0 for Windows (Graphpad Software, San Diego, CA). Binding assays with biotinylated toxins were carried out with a binding buffer (PBS, 0.1% BSA; final volume, 0.1 ml) by incubating the biotinylated protein with the appropriate amount of BBMV (25 μg) for 1 h at room temperature. The amount of biotinylated protein was 30 ng of Cry1Ab, 140 ng of Cry1Ia, and 20 ng of Cry9Ca. The same binding conditions described for Cry1Ab were used for competition binding assays, but a ≥400-fold excess of unlabeled protein was also included. Toxin bound to the BBMV after incubation was recovered by centrifugation at 11,000 × *g* for 10 min at 4°C, followed by two washes with 0.5 ml of cold binding buffer as described elsewhere (11). The final pellet was suspended with 10 μl electrophoresis sample buffer (21) and boiled for 10 min, and the proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels. Control lanes with biotinylated toxins were run using 8 to 10 ng of Cry1Ab, Cry1I, and Cry9C. Proteins were electrotransferred onto a nitrocellulose membrane (Hybond ECL; Amersham Biosciences) and blocked with a 3% enhanced-chemiluminescence blocking agent (Amersham Biosciences), 0.1% BSA, and 0.1% Tween 20 in PBS. The membrane was incubated for 1 h with streptavidin conjugated to alkaline phosphatase (Roche Diagnosis, IN) and washed three times with 0.1% BSA, 0.1% Tween 20 in PBS in order to detect the amount of biotinylated toxin bound to the BBMV. The final detection was carried out with a nitroblue tetrazolium-BCIP (5-bromo-4-chloro-3-indolylphosphate) solution (Roche Diagnosis), following the manufacturer's instructions.

TABLE 1. Toxicity and relative potencies of the active Cry proteins to neonate *Lobesia botrana* larvae[a]

Cry protein	Regression value ± SE		LC_{50} (μg/ml)	Goodness of fit value		Relative potency	95% CI	
	Slope	a^b		χ^2	df		Lower	Upper
Cry1Ab	1.9 ± 0.2	4.7 ± 0.1	1.4	0.3	3	1		
Cry1Ia	2.9 ± 0.2	2.3 ± 0.2	8.5	0.7	3	0.2	0.1	0.3
Cry2Aa	2.6 ± 0.3	3.7 ± 0.3	3.2	1.8	3	0.4	0.3	0.6
Cry2Ab	1.7 ± 0.2	6.3 ± 0.2	0.1	1.3	3	9.0	6.3	12.9
Cry9Ca	2.6 ± 0.3	7.7 ± 0.3	0.09	0.1	3	16.1	11.6	22.3
HD-1[c]	1.9 ± 0.2	5.7 ± 0.1	0.44	0.9	3	3.3	2.3	4.7

[a] Parameters were obtained from the POLO-PC program (24). The χ^2 value was not significant ($P > 0.05$) by a goodness of fit test for each regression. Slopes could not be fitted in parallel. The relative potencies were calculated with respect to that for Cry1Ab, and the values were expressed as the ratio of the LC_{50} value for each Cry protein to the LC_{50} value for Cry1Ab (31).

[b] *a*, intercept of the regression line.

[c] HD-1 means the entire content of the Cry proteins obtained from the *B. thuringiensis kurstaki* HD-1 strain.

Cry1Fa did not have any effect on mortality or larval growth at the concentration of 100 μg/ml, whereas Cry1Ja caused less than 10% mortality, but a reduction in the size of larvae was observed. Low toxicities for Cry1Fa and Cry1Ja were previously reported by Herrero et al. (10, 11) with *Cacyreus marshalli* and *L. botrana*. In contrast, at this high concentration, all the other five Cry toxins tested (Cry1Ab, Cry1Ia, Cry2Aa, Cry2Ab, and Cry9C) resulted in the mortality of all the treated larvae. The most insecticidal toxins were Cry9C, Cry2Ab, and Cry1Ab, with LC_{50} values of 0.09, 0.1, and 1.4 μg/ml, respectively, followed by Cry2Aa and Cry1Ia (3.2 and 8.5 μg/ml, respectively) (Table 1). The only previous study with *L. botrana* involved assays with a limited number of Cry1 toxins: Cry1Aa, Cry1Ab, Cry1Ac, Cry1B, Cry1C, Cry1D, and

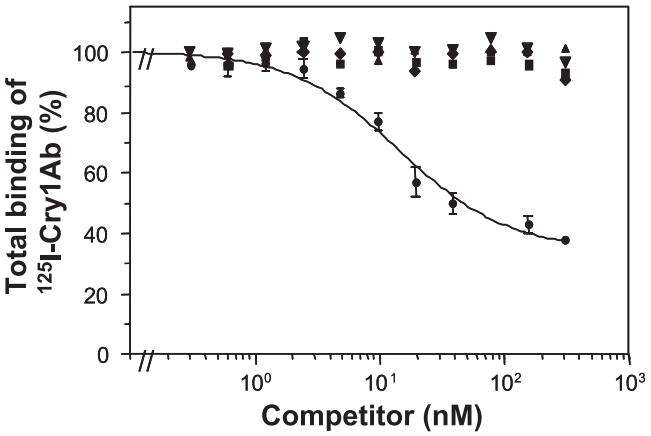

FIG. 1. Binding of ^{125}I-Cry1Ab to *L. botrana* BBMV at increasing concentrations of unlabeled Cry1Ab (●), Cry1Ia (◆), Cry2Aa (▼), Cry2Ab (▲), and Cry9Ca (■). Experiments were repeated three times, and error bars represent the standard errors of the means.

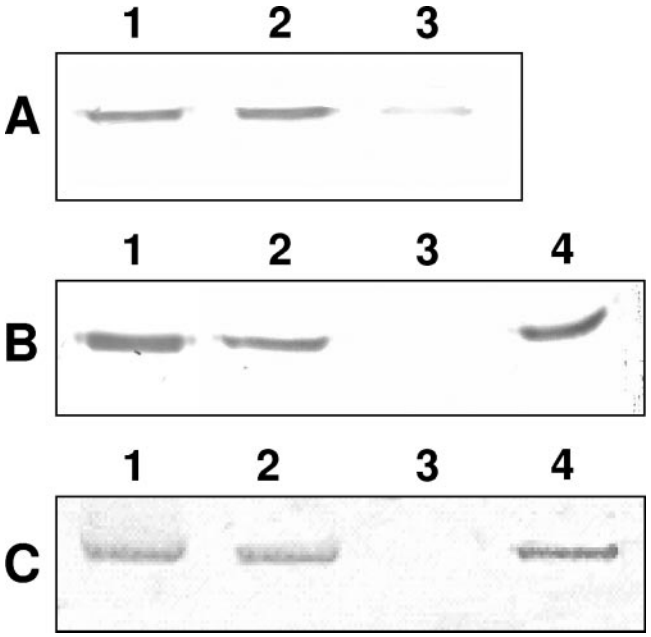

FIG. 2. Binding of biotinylated toxins to *Lobesia botrana* BBMV. (A) Biotinylated Cry1Ab (lane 1), binding of biotin-Cry1Ab alone (lane 2), and binding of biotin-Cry1Ab in the presence of an excess of unlabeled Cry1Ab (lane 3). (B) Biotinylated Cry1Ia (lane 1), binding of biotin-Cry1Ia alone (lane 2), and binding of biotin-Cry1Ia in the presence of an excess of unlabeled Cry1Ia (lane 3) or unlabeled Cry1Ab (lane 4). (C) Biotinylated Cry9Ca (lane 1), binding of biotin-Cry9Ca alone (lane 2), and binding of biotin-Cry9Ca in the presence of an excess of unlabeled Cry9Ca (lane 3) or Cry1Ab (lane 4).

Cry1E (30). That study reported that Cry1Ab was the protein most active against the grape moth, whereas the results for the present work revealed that *L. botrana* was 9- and 16-fold more sensitive to the Cry2Ab and Cry9C proteins, respectively, than to Cry1Ab (Table 1). Our experimental results are in agreement with the observation that commercial *B. thuringiensis* insecticides containing Cry1Ab protein are toxic to the grape moth (32, 34). Other proteins present in commercial strains whose crystal compositions include Cry1Aa, Cry1Ac, Cry1D (30), and Cry2Aa could contribute to the total activity of these strains.

Radio-iodinated Cry1Ab bound to *L. botrana* BBMV in vitro, and its binding was specific (Fig. 1). Quantitative analysis of the homologous competition data gave a dissociation constant (± standard error of the mean) of 6.5 ± 1.5 nM and a binding site concentration (± standard error of the mean) of 8 ± 2 pmol/mg for Cry1Ab binding sites. Heterologous competition experiments with excesses of unlabeled Cry1Ia, Cry2Aa, Cry2Ab, and Cry9Ca showed that none of them shared common binding sites with ^{125}I-Cry1Ab (Fig. 1). Biotinylated Cry1Ia, biotinylated Cry9Ca, and also biotin-labeled Cry1Ab, used as a control, bound to *L. botrana* BBMV, and their binding was specific (Fig. 2, lanes 1 to 3). Nonlabeled Cry1Ab used as a competitor in the assays did not displace biotin-Cry1Ia (Fig. 2B, lane 4) or biotin-Cry9Ca (Fig. 2C, lane 4). In vitro binding experiments were performed to determine whether the assayed Cry proteins could be used either as substitutes for the most frequently used proteins in pest management programs or in combination with Cry proteins produced by *B. thuringiensis* strains or other recombinant microorganisms. Our competition experiments with labeled Cry1Ab showed that Cry1Ia, Cry2Aa, Cry2Ab, and Cry9Ca did not share Cry1Ab binding sites. Similarly, Cry1Ab was shown not to share binding sites with biotinylated Cry1Ia and Cry9Ca in *L. botrana* BBMV. The fact that Cry1Ia did not share common binding sites with Cry1A toxins has recently been reported for the cotton pest *Earias insulana* (13). Cry2A toxin-specific binding could not be demonstrated in this study by using the same conditions as those used for Cry1A toxins, but this is not surprising, as these toxins seem to have different modes of action (5, 18, 19).

To date, *L. botrana* control has been performed using some commercial products based on the HD-1 strain (e.g., Dipel; Abbot Laboratories, Chicago) or other strains belonging to serovars *kurstaki* or *aizawai* (Delfin and Xentari, respectively), which have been found to be effective against *L. botrana* under laboratory or field conditions (15, 32, 34). The activities of these strains have been associated with a group of proteins belonging to the Cry1 and Cry2 classes (35). Our results with the HD-1 strain indicated an LC_{50} value lower than those for the Cry1Ab and Cry2Aa toxins, probably due to a synergic action among the proteins that constitute the crystal (12, 39). No commercial strain used for *L. botrana* control contains all of the most active proteins, Cry1Ab, Cry2Ab, and Cry9C. In addition, Cry1Ab and Cry9C do not share binding sites. Consequently, this protein combination in a particular strain is likely to prove highly effective as a bioinsecticide for use in the control of the grape berry moth, together or in combination with Cry1Ab. Moreover, Cry9Ca is of particular interest because of its high potency against the grape berry moth and its broad host range, which includes other important pests, like *Ostrinia nubilalis* and *Agrotis segetum*, that are difficult to control with current *B. thruringiensis*-based products (22).

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