APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Apr. 1997, p. 1453–1459 Vol. 63, No. 4
0099-2240/97/$04.00+0

Determination of Binding of *Bacillus thuringiensis* δ-Endotoxin Receptors to Rice Stem Borer Midguts

MI KYONG LEE,[1] R. M. AGUDA,[2] M. B. COHEN,[2] F. L. GOULD,[3] AND D. H. DEAN[1]*

Department of Biochemistry, The Ohio State University, Columbus, Ohio 43210[1]; Entomology and Plant Pathology Division, International Rice Research Institute, Manila, Philippines[2]; and Department of Entomology, North Carolina State University, Raleigh, North Carolina 27695[3]

Received 20 August 1996/Accepted 22 January 1997

Insecticidal activity and receptor binding properties of *Bacillus thuringiensis* toxins to yellow and striped rice stem borers (*Sciropophaga incertulas* and *Chilo suppresalis*, respectively) were investigated. Yellow stem borer (YSB) was susceptible to Cry1Aa, Cry1Ac, Cry2A, and Cry1C toxins with similar toxicities. To striped stem borer (SSB), Cry1Ac, Cry2A, and Cry1C were more toxic than Cry1Aa toxin. Binding assays were performed with ^{125}I-labeled toxins (Cry1Aa, Cry1Ac, Cry2A, and Cry1C) and brush border membrane vesicles (BBMV) prepared from YSB and SSB midguts. Both Cry1Aa and Cry1Ac toxins showed saturable, high-affinity binding to YSB BBMV. Cry2A and Cry1C toxins bound to YSB BBMV with relatively low binding affinity but with high binding site concentration. To SSB, both Cry1Aa and Cry1Ac exhibited high binding affinity, although these toxins are less toxic than Cry1C and Cry2A. Cry1C and Cry2A toxins bound to SSB BBMV with relatively low binding affinity but with high binding site concentration. Heterologous competition binding assays were performed to investigate the binding site cross-reactivity. The results showed that Cry1Aa and Cry1Ac recognize the same binding site, which is different from the Cry2A or Cry1C binding site in YSB and SSB. These data suggest that development of multitoxin systems in transgenic rice with toxin combinations which recognize different binding sites may be useful in implementing deployment strategies that decrease the rate of pest adaptation to *B. thuringiensis* toxin-expressing rice varieties.

Bacillus thuringiensis, a gram-positive, spore-forming bacterium, produces insecticidal crystal proteins called δ-endotoxins during sporulation. *B. thuringiensis* δ-endotoxins have been used as an alternative to chemical pesticides for managing insect pests. These proteins are toxic to a number of insect larvae in the orders Lepidoptera, Diptera, and Coleoptera (15, 50). *B. thuringiensis* toxin genes are currently being transferred to crop plant genomes to overcome field degradation problems of conventional *B. thuringiensis* applications and to improve the efficiency of *B. thuringiensis* toxins (2, 8, 10, 32, 45). However, the potential for insect resistance to *B. thuringiensis* toxins raises concern about their long-term effectiveness (27, 39, 41). Recently, high levels of insect resistance have been observed in *Plutella xylostella* from the field selection (1a, 7, 17, 38) and in *Plodia interpunctella*, *Heliothis virescens*, *Spodoptera exigua*, *Trichoplusia ni*, *Culex quinquefasciatus*, and *Leptinotarsa decemlineata* from the laboratory selection (6, 13, 20, 23, 25, 26, 28, 30, 33, 47).

Understanding the mechanisms of resistance to *B. thuringiensis* toxins could be helpful for the management of rapid onset of insect resistance. The mechanism of resistance can be related to disruption of the steps involved in the mode of *B. thuringiensis* toxin action such as solubilization, activation of protoxins to toxin, binding to the receptors, and pore formation (14, 15, 19, 37, 48). The resistance is often related to a change in receptor binding properties on the brush border membrane vesicles (BBMV) of the insect midgut (3, 7, 30, 42, 47). However, other studies showed no or minor changes in overall receptor binding properties in the laboratory- and field-selected resistant strains (13, 23, 24, 28).

* Corresponding author. Mailing address: Department of Biochemistry, The Ohio State University, 484 W. 12th Ave., Columbus, OH 43210. Phone: (614) 292-8829. Fax: (614) 292-3206. E-mail: dean.10@osu.edu.

Previous studies have found a narrow range of cross-resistance (1a, 6, 7, 40, 42–44). Cry1A type of *B. thuringiensis* toxins showed cross-resistance to Cry1F but not to Cry1B, Cry1C, or Cry1D (7, 40). These studies suggest the possibility that resistance can be managed with the use of toxin mixtures. The expression of modified *cry1C-cry1Ab* fusion genes in plants resulted in protection against lepidopteran insect species, suggesting the potential of broadening the insect resistance of transgenic plants and employing different gene classes as a resistance management strategy (46). In vitro competition binding studies demonstrated that Cry1B and Cry1C toxins recognized binding sites different from those recognized by Cry1Ab toxin (7, 47). However, a broad range of cross-resistance among *B. thuringiensis* toxins has also been observed (13, 28). These studies raise questions about the multiple-toxin approach to resistance management. Therefore, the specificity or cross-reactivity of *B. thuringiensis* toxins and receptors must be considered cautiously before applying this information in developing multiple-toxin approaches for managing resistance.

The yellow stem borer (YSB), *Sciropophaga incertulas*, and striped stem borer (SSB), *Chilo suppresalis*, are major pests of rice in Asia and Europe (31). Only moderate levels of stem borer resistance have been achieved by conventional plant breeding, and *B. thuringiensis* sprays are not effective for stem borer control because larvae bore into the plant shortly after hatching. Consequently, there has been much interest in the genetic engineering of rice with *B. thuringiensis* toxin genes (10, 16, 54). In this study, we examined the toxicity of four *B. thuringiensis* toxins (Cry1Aa, Cry1Ac, Cry2A, and Cry1C) against YSB and SSB and investigated the receptor binding properties of these toxins to the BBMV from YSB and SSB midguts and the relationship between binding sites of each toxin. Understanding the receptor binding properties of different δ-endotoxins can guide the development of rice plants which produce combinations of toxins that bind to different

TABLE 1. Insect toxicity and BBMV binding of toxins

Toxin	YSB			SSB		
	LC_{50}[a]	K_{com}[b]	B_{max}[c]	LC_{50}	K_{com}	B_{max}
Cry1Aa	1.32 ± 1.19	0.75 ± 0.07	1.10 ± 0.09	1,422.40 ± 621.53	0.26 ± 0.02	0.70 ± 0.09
Cry1Ac	0.33 ± 0.18	0.53 ± 0.06	3.97 ± 0.47	237.81 ± 165.57	0.46 ± 0.05	4.04 ± 0.53
Cry2A	5.45 ± 3.50	19.90 ± 2.4	7.75 ± 1.1	67.21 ± 40.04	50.60 ± 5.4	24.63 ± 3.3
Cry1C	0.85 ± 0.38	43.3 ± 5.4	20.69 ± 2.4	74.26 ± 43.18	35.0 ± 4.1	20.34 ± 2.7

[a] LC_{50} are expressed in nanograms per milliliter of diet (means ± standard errors).
[b] K_{com} values represent binding affinity calculated from the homologous competition assays and are expressed in nanomolar concentrations.
[c] B_{max} values represent binding site concentrations and are expressed in picomoles per milligram of BBMV protein. Values are the means of at least three experiments.

receptors. Such plants can be an important component of resistance management strategies for *B. thuringiensis*-transgenic rice.

MATERIALS AND METHODS

Purification and activation of recombinant *B. thuringiensis* δ-endotoxins. The *B. thuringiensis* δ-endotoxin genes *cry1Aa* and *cry1Ac* from strains HD-1 and HD-244, respectively, were subcloned into pKK 223-3. The *cry2A* gene cloned into pTZ 18R was supplied by T. Yamamoto (Sandoz). These proteins were purified from *Escherichia coli* as described by Lee et al. (19). Purified Cry1C toxin was a gift from Roger Frutos (BIOTROP-IGEPAM, CIRAD, Montpellier, France). The crystal protein was solubilized in solubilization buffer, i.e., 50 mM sodium carbonate buffer (pH 9.5) containing 10 mM dithiothreitol, at 37°C for 2 h. The solubilized protoxins were dialyzed against 50 mM sodium carbonate buffer (pH 9.5) to remove dithiothreitol and digested with trypsin at a trypsin/protoxin ratio of 1:20 (by mass) at 37°C for 2 h. Trypsin-activated Cry1Aa, Cry1Ac, Cry1C, and Cry2A toxins were used for bioassays and binding assays. Concentrations of the proteins were measured with Coomassie protein assay reagent (Pierce). Protoxins and activated toxins were examined on a sodium dodecyl sulfate–12% polyacrylamide gel electrophoresis gel (18).

Insect bioassays. YSB and SSB adults were collected from rice fields in the vicinity of the International Rice Research Institute (Laguna Province, Philippines) and brought to a greenhouse to oviposit on rice plants. The egg masses were surface sterilized and placed in scintillation vials containing artificial diet. The preparation of the YSB diet was described in detail by Aguda et al. (1). The modified southwestern corn borer diet (4) was used for SSB. Approximately 24 h after egg hatch, larvae were collected and introduced into vials with artificial diet containing toxin. The trypsin-activated Cry1Aa, Cry1Ac, Cry1C, and Cry2A toxins were mixed into the liquid insect diet at a temperature below 60°C. Six YSB larvae or 10 SSB larvae were used per vial. Larval mortality was recorded after 4 days of incubation at 27°C. Nine toxin concentrations were used for each toxin. For each toxin concentration, eight replicate vials of YSB or five replicate vials of SSB were prepared, resulting in 432 YSB or 450 SSB larvae assayed per toxin. An additional 24 vials of YSB or 10 vials of SSB were set up as controls, containing no toxin. The entire bioassay procedure was repeated on 6 days for YSB and 7 days for SSB, during June and July 1996. All four toxins were tested on each bioassay day. A different field collection of insects was used for each bioassay; thus, the larvae tested were always the progeny of field-collected adults. Egg masses were obtained from 100 to 200 females on each bioassay day. The effect of day was significant for SSB ($F = 4.91$, df = 4, $P = 0.004$) but not for YSB ($F = 2.11$, df = 4, $P = 0.106$). The high variability in 50% lethal concentrations (LC_{50}) among days may be attributable to high genetic variability in the insects collected from the field for the bioassays.

The data were analyzed by probit analysis (35) using POLO-PC (21a). Data from days on which one or more toxins did not fit the probit model ($\chi^2 > 14.07$, df = 7) were discarded, leaving a total of five bioassay days for each species. LC_{50} were log-transformed (because variances were proportional to means) and subjected to analysis of variance by use of the SAS package (36).

Preparation of BBMV. Midguts were dissected from last-instar larvae and kept on liquid nitrogen until use. BBMV were prepared by the differential magnesium precipitation method of Wolfersberger et al. (52). The BBMV were resuspended in the buffer (8 mM $NaHPO_4$, 2 mM KH_2PO_4, 150 mM NaCl [pH 7.4]), and the concentration of total protein concentration was measured by Coomassie protein assay reagent (Pierce).

Iodination of toxins. The activated toxins were iodinated with IODO-BEAD as described in the manufacturer's instructions (Pierce). One millicurie of $Na^{125}I$ (Amersham) solution, diluted in 50 mM sodium carbonate buffer (pH 9.5), was added to a vial which contained one IODO-BEAD (Pierce), and the vial was incubated for 5 min at room temperature. Twenty-five micrograms of toxin in sodium carbonate buffer was added to the reaction vial. After a 15-min incubation, the reaction was stopped by removing the labeled toxin solution from the IODO-BEAD. The reaction mixture was applied to a 2-ml Excellulose column (Pierce) which was equilibrated with 10 ml of sodium carbonate buffer to remove free iodine and possible degradation products. Specific activities of toxins were measured by trichloroacetic acid precipitation. The specific activities of Cry1Aa, Cry1Ac, Cry2A, and Cry1C were 0.74, 0.83, 0.73, and 0.46 μCi/μg, respectively. The stability and purity of labeled toxins were examined by sodium dodecyl sulfate–12% polyacrylamide gel electrophoresis and autoradiography.

Binding assays. BBMV were incubated with ^{125}I-labeled toxins in 100 μl of binding buffer (8 mM $NaHPO_4$, 2 mM KH_2PO_4, 150 mM NaCl [pH 7.4]) containing 0.1% bovine serum albumin. After 1 h of incubation at room temperature, the sample was centrifuged in a Fisher microcentrifuge for 10 min at 13,500 × *g* to separate bound from free toxin. The pellet containing the bound toxin was washed three times with binding buffer, and radioactivity in the resulting pellet was counted in a gamma counter (Beckman). For homologous and heterologous competition experiments, a fixed concentration of the labeled ligand (1 nM) was incubated with BBMV (50 μg/ml) in the presence of unlabeled competitors (from 0.5 to 1000 nM). Binding data were analyzed by use of the LIGAND computer program (29), which is a nonlinear and curve-fitting program. This computer program calculates the bound concentration of ligand as a function of the total concentration of ligand and give initial estimates of the affinity (K_d) and the binding site concentration (B_{max}). The computer adjusts the values (K_d and B_{max}) and nonspecific binding by an iteration process. Binding affinity (K_{com}) and binding site concentration (B_{max}) were calculated from the homologous competition assays. Each value was the mean of three independent experiments. K_{com} represents binding affinity (K_d) calculated from homologous competition assays. K_{com} is calculated by the same equation as that for K_d in earlier competition studies (14, 19, 48, 49).

RESULTS

All four toxins exhibited high toxicity to YSB, with LC_{50} of less than 5.45 ng/ml of diet (Table 1). The mean LC_{50} of these toxins did not differ significantly (least significant difference test, $P > 0.05$). SSB larvae were less susceptible to these toxins than YSB, with LC_{50} ranging from 67 to 1,422 ng/ml of diet. Cry1Ac, Cry2A, and Cry1C were more toxic than Cry1Aa to SSB ($P < 0.05$). There are large standard errors in estimates of the LC_{50}. Because SSB and YSB are difficult to maintain in culture, we used a new collection of insects from the field for each bioassay day. Analysis of variance indicated that the effect of day was significant for SSB ($F = 3.98$, df = 4, $P = 0.028$) but not for YSB ($F = 2.61$, df = 4, $P = 0.089$). It is likely that genetic variation in the field-collected insects explains in part the high variability in LC_{50} estimates. Probit analysis using POLO-PC indicated that the data fit a probit model and that the slopes, although low (0.3 to 0.6), were significant. The mortality for control larvae was consistently low (<10%).

We performed homologous competition assays with ^{125}I-labeled Cry1Aa, Cry1Ac, Cry2A, and Cry1C toxins in both YSB and SSB BBMV. From homologous competition experiments, the binding affinity (K_{com}) and binding site concentration (B_{max}) were calculated by use of the LIGAND computer program (Table 1) (29). Recently, it has been shown that the use of the term K_d as a binding constant for binding assays with *B. thuringiensis* toxins and BBMV is not appropriate since binding of the toxins to the receptors on BBMV includes both reversible and irreversible binding steps (22). Therefore, in this report, we use K_{com} to differentiate the K_d from the reversible kinetic studies as suggested by Wu and Dean (53). Cry1Aa and

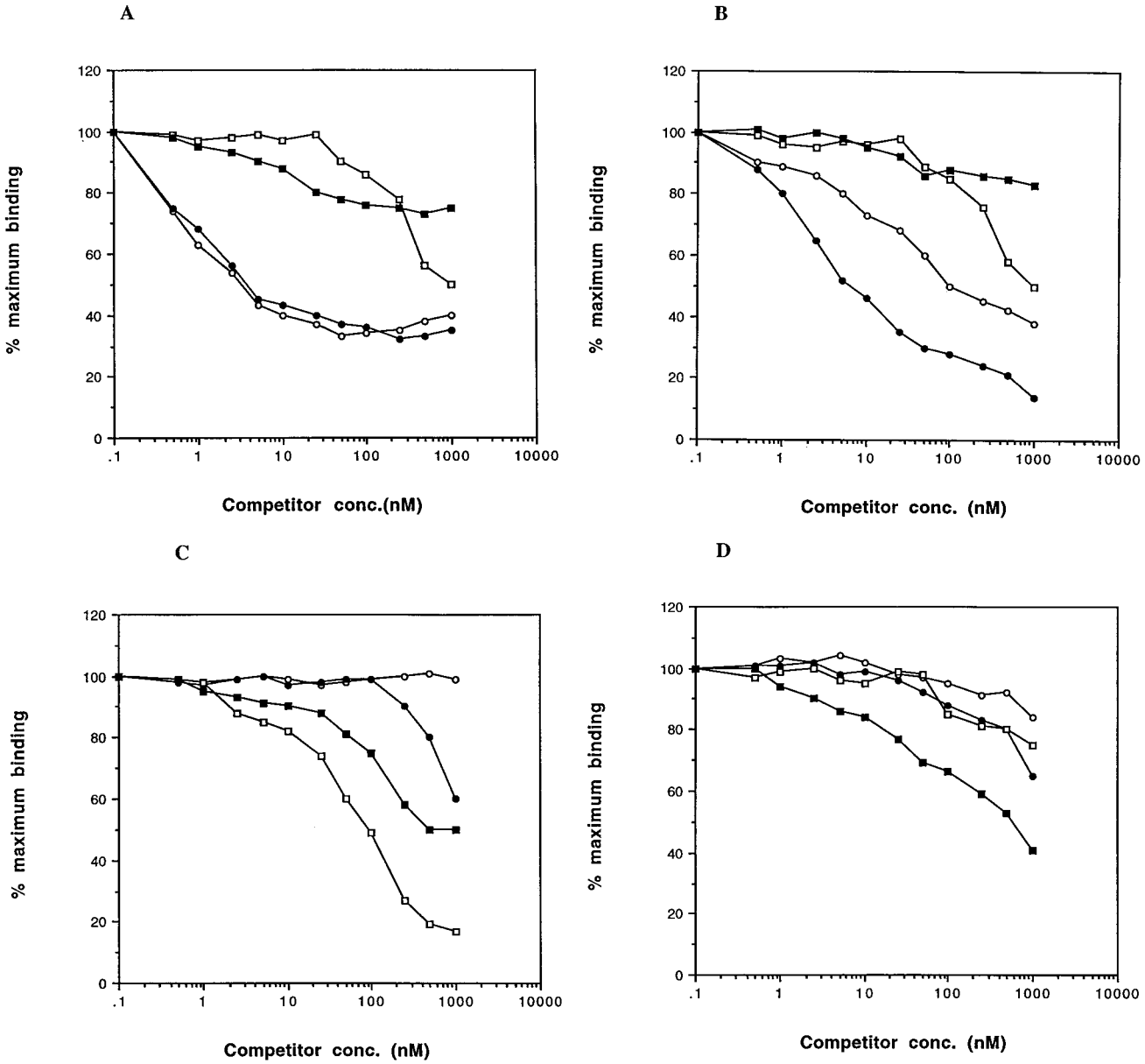

FIG. 1. Competition binding of labeled and unlabeled *B. thuringiensis* toxins to YSB BBMV. Vesicles (50 μg/ml) were incubated with labeled toxin in the presence of increasing concentrations of unlabeled Cry1Aa (○), Cry1Ac (●), Cry2A (□), and Cry1C (■) toxins. Competition binding of labeled Cry1Aa (A), Cry1Ac (B), Cry2A (C), and Cry1C (D) toxins and unlabeled competitor toxins was measured. Binding is expressed as the percentage of the amount bound upon incubation with labeled toxin alone. On YSB BBMV, these amounts were 1,528, 2,992, 3,678, and 2,318 cpm for labeled Cry1Aa, Cry1Ac, Cry2A, and Cry1C, respectively. Data were analyzed with the LIGAND computer program. Each data point is the mean of three experiments.

Cry1Ac toxins demonstrated specific, high binding affinities, with K_{com} values of 0.75 and 0.53 nM, respectively, for YSB BBMV (Fig. 1A and B; Table 1). These toxins also bound to SSB BBMV with high binding affinities (K_{com}) of 0.26 and 0.46 nM, respectively, although Cry1Aa and Cry1Ac showed much less toxicity to SSB than to YSB (Fig. 2A and B; Table 1). The binding site concentration (B_{max}) of Cry1Ac is about four times higher than that of Cry1Aa on both YSB and SSB BBMV. Cry2A also bound to YSB and SSB BBMV specifically but with relatively low binding affinities of 19.9 and 50.6 nM, respectively (Fig. 1C and 2C; Table 1). Cry1C protein bound to both YSB and SSB BBMV with relatively lower binding affinities, 43.3 and 35 nM, respectively (Fig. 1D and 2D; Table 1), than those of Cry1A type toxins. However, the binding site concentrations of Cry2A and Cry1C were higher than those of Cry1Aa and Cry1Ac in both YSB and SSB (Table 1).

Heterologous competition binding experiments were performed with labeled and unlabeled Cry1Aa, Cry1Ac, Cry2A, and Cry1C toxins. Unlabeled Cry1Ac competed for the binding sites of labeled Cry1Aa with about the same affinity as that of Cry1Aa on YSB (Fig. 1A). In SSB, Cry1Ac competed for the binding of labeled Cry1Aa with somewhat lower efficiency than that of unlabeled Cry1Aa (Fig. 2A). Unlabeled Cry1Aa also competed for the binding sites of labeled Cry1Ac in both insects but with low efficiency (Fig. 1B and 2B). These experiments demonstrated that Cry1Aa and Cry1Ac toxins recog-

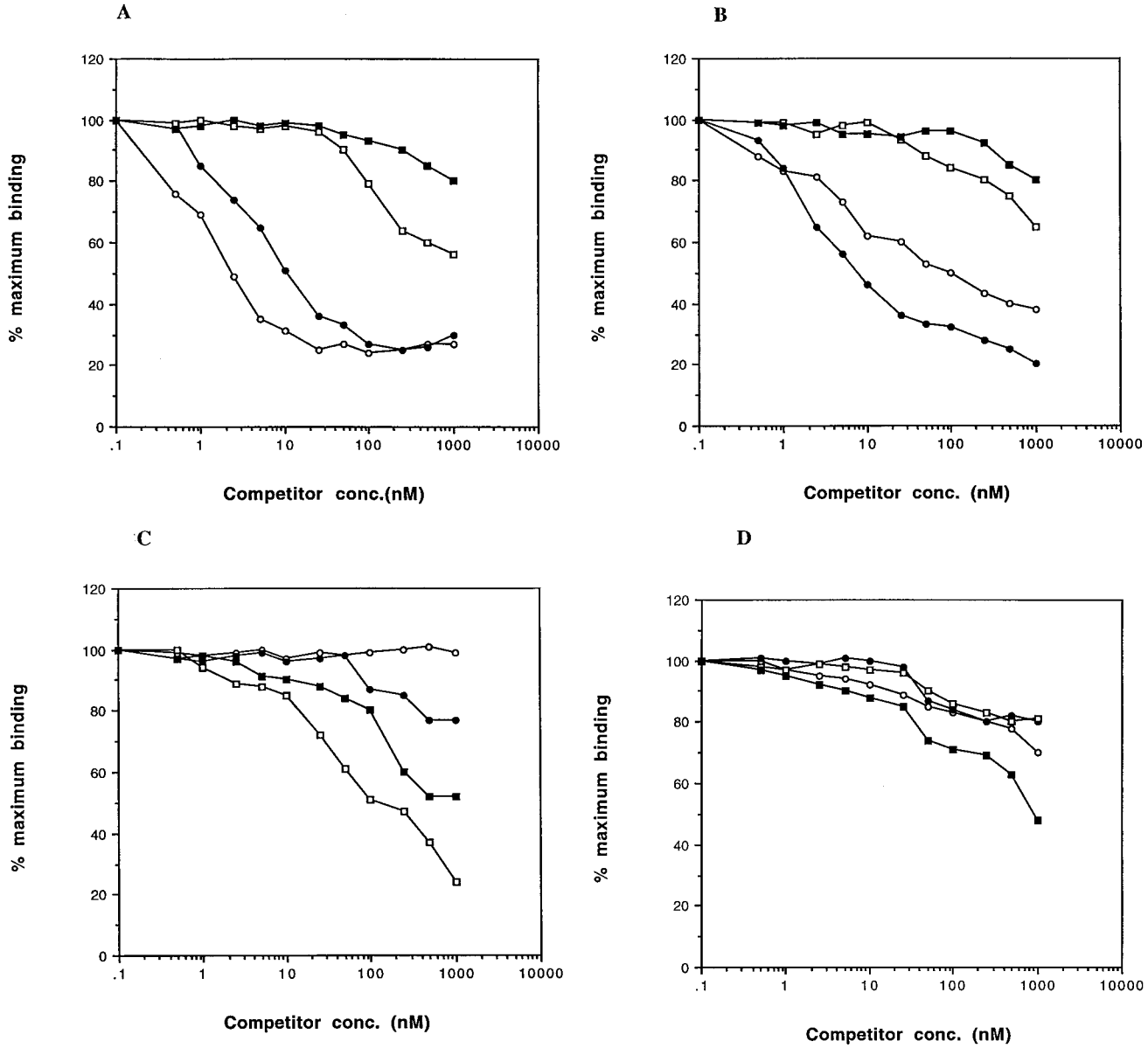

FIG. 2. Competition binding of labeled and unlabeled *B. thuringiensis* toxins to SSB BBMV. (A) Vesicles (50 μg/ml) were incubated with labeled toxin in the presence of increasing concentrations of unlabeled Cry1Aa (○), Cry1Ac (●), Cry2A (□), and Cry1C (■) toxins. Competition binding of labeled Cry1Aa (A), Cry1Ac (B), Cry2A (C), and Cry1C (D) toxins and unlabeled competitor toxins was measured. Binding is expressed as the percentage of the amount bound upon incubation with labeled toxin alone. On SSB BBMV, these amounts were 1,662, 2,879, 3,496, and 2,154 cpm for labeled Cry1Aa, Cry1Ac, Cry2A, and Cry1C, respectively. Data were analyzed with the LIGAND computer program. Each data point is the mean of three experiments.

nize at least some of the same binding sites. These results are in good agreement with those of a recent study (9). Cry2A showed marginal competition for the binding of labeled Cry1Aa only at high concentrations of unlabeled Cry2A in both YSB and SSB BBMV (Fig. 1A and 2A). Similar competition binding results were observed with labeled Cry1Ac and unlabeled Cry2A (Fig. 1B and 2B). Cry1Aa did not compete for the binding of labeled Cry2A protein in the BBMV of either insect. Cry1Ac showed partial competition for the binding of labeled Cry2A only at high concentrations of competitor in both insect species (Fig. 1C and 2C). Cry1C did not show significant competition for the binding of either labeled Cry1Aa or labeled Cry1Ac in both YSB and SSB midguts (Fig. 1A, 1B, 2A, and 2B). Also, Cry1Aa and Cry1Ac showed a very limited degree of competition for the binding sites of labeled Cry1C in both insects, and this competition was apparent only at high concentrations of competitor (Fig. 1D and 2D). Cry1C competed for the binding of labeled Cry2A protein at high competitor concentrations (Fig. 1C and 2C). Cry2A showed marginal competition for the binding of labeled Cry1C in both insects (Fig. 1D and 2D).

DISCUSSION

Extensive research efforts are currently being directed toward the development of commercially useful transgenic rice. One class of genes being introduced into the rice plant for insect control are *B. thuringiensis* toxin genes. Several rice lines

have now been transformed with single *B. thuringiensis* toxin genes (10, 16, 54). While these plants have been shown to be effective against YSB, SSB, and the leaffolder (*Cnaphalocrosis medinalis*), combining multiple *B. thuringiensis* toxins in rice will probably provide more sustainable control of these pests. Understanding the biochemical mechanisms of *B. thuringiensis* toxin activity can help to guide the selection of effective toxin combinations. This report assessed the role of receptors on midgut epithelial cell membranes of YSB and SSB as determinants of the insecticidal specificity of a variety of *B. thuringiensis* toxins (Cry1Aa, Cry1Ac, Cry2A, and Cry1C). The cross-reactivity in receptor binding between different toxins was examined as a means of selecting *B. thuringiensis* toxins that would be less likely to have cross-resistance in the field. Binding assays with resistant strains demonstrated the correlation between the lack of cross-resistance and the lack of change in binding properties (7, 42, 47).

It should be emphasized that the LC_{50} (Table 1) have large standard errors, despite the great care that we took to optimize our bioassay procedures. We used the progeny of field-collected moths in our bioassays, which probably contributed to the low slopes and high LC_{50} variability that we observed. To compensate for the presumed variability of the insects, we used large sample sizes and repeated the bioassays on 5 days. These and other measures aimed at reducing the variability of our bioassay results with stem borers have met with only limited success. Laboratories in other countries (e.g., Pakistan, Thailand, and China) have encountered similar problems with these insects. Binding studies were also conducted on BBMV prepared from field-collected insect larvae obtained over several days from rice fields in the vicinity of the International Rice Research Institute.

Our competition binding assays sometimes demonstrated a positive correlation between binding and toxicity, but this was not always observed. Some of the toxin binding assays require special comments. Cry2A and Cry1C proteins, which are almost as toxic to YSB as Cry1Aa and Cry1Ac are, have lower binding affinity than that of Cry1Aa and Cry1Ac. Also, Cry1Aa, which is less toxic than Cry2A and Cry1C to SSB, showed higher binding affinity than that of Cry2A and Cry1C. The binding affinities of Cry1Aa and Cry1Ac toxins to SSB were comparable to those to YSB, although these toxins were less active against SSB (Table 1). This discrepancy could be explained as follows. First, even though binding affinities of Cry2A and Cry1C proteins are lower than those of Cry1Aa and Cry1Ac toxins, the binding site concentrations are higher than those of Cry1Aa and Cry1Ac. Presumably, a higher binding site concentration may compensate for the lower binding affinity. Second, the mechanism of pore formation by *B. thuringiensis* toxin is considered to be a two-step process: initial binding of *B. thuringiensis* toxin to specific receptors is followed by irreversible binding by integration of toxin into the membrane.

A recent study demonstrated that the irreversible binding step was a determinant for toxicity of *B. thuringiensis* toxin to *Lymantria dispar* larvae (22). Furthermore, it has been observed that some mutant *B. thuringiensis* toxins do not alter initial binding (i.e., no changes in K_d) but do alter irreversible binding phenomena, causing significant loss of toxicity to *Manduca sexta* larvae (34). Therefore, it is possible that late binding events such as integration of toxins into the membrane, efficiency of oligomerization, and pore formation may be important determinants of insecticidal activity. Presumably, initial high-affinity binding of Cry1Aa to SSB BBMV could fail to cause high toxicity because of the low efficiency of later steps in pore formation. Wolfersberger (51) suggested that the inverse relationship between toxicity and binding affinity in *Lymantria dispar* may be due to differences in the efficiency of late binding events. While the information described above may account for low toxicity of toxins with high binding affinity, this information does not explain the similar and high toxicities of Cry2A and Cry1C to YSB and SSB, given their low binding affinities. Even though the binding affinities of Cry2A and Cry1C are comparatively lower than those of Cry1Aa and Cry1Ac, these binding affinities could presumably be strong enough to result in tight binding to the membrane receptor, integration into the membrane, and formation of a pore with the same or higher efficiency as that of Cry1A types of protein. Several reports also demonstrated high binding affinity of specific toxins to nonsusceptible insects and low binding affinity to susceptible insects (7, 11, 51).

In the current study, heterologous competition assays were performed to investigate the cross-reactivity of toxins and receptors in YSB and SSB. The binding data showed that for both YSB and SSB, Cry1Ac protein competed for the binding of labeled Cry1Aa protein with high affinity (Fig. 1A and 2A). Also, Cry1Aa protein competed for the binding of labeled Cry1Ac protein with high affinity in both YSB and SSB (Fig. 1B and 2B). These heterologous binding data demonstrated that Cry1Aa and Cry1Ac recognize at least some of the same binding sites on YSB and SSB midgut epithelial membranes. Another possibility is that Cry1Aa and Cry1Ac toxins recognize different receptors which are in juxtaposition and, therefore, the binding of one toxin might structurally hinder binding of another toxin. A recent report has demonstrated that determination of the receptor binding site relationship by either heterologous competition or ligand blotting alone is not consistent and may not be conclusive (21). Incomplete inhibition between Cry2A and Cry1A toxins demonstrated that Cry2A has one or more binding sites in YSB and SSB BBMV which cannot be efficiently recognized by Cry1Aa and Cry1Ac. Heterologous competition between Cry1C and Cry1A toxins in both insects demonstrated that Cry1Aa and Cry1Ac do not efficiently recognize one or more binding sites of the Cry1C protein. Cry1C demonstrated competition for the binding of labeled Cry2A in both YSB and SSB only at a high concentration of competitor (Fig. 1C and 2C). Cry2A did not seem to compete for the binding of labeled Cry1C toxin in either insect (Fig. 1D and 2D).

The Cry2A binding assay results require special comment. An earlier study on the mode of action of Cry2A in *Helicoverpa zea* indicated that Cry2A does not show saturable binding to BBMV and does not inhibit subsequent binding of labeled Cry1Ac. In contrast, Cry1Ac inhibited the nonsaturable binding of labeled Cry2A (5). However, in this study, Cry2A showed specific binding to rice stem borer BBMV and Cry1Ac did not show competition with Cry2A (Fig. 1C). Additionally, it was demonstrated that Cry2A formed voltage-dependent and not highly cation selective channels in planar lipid bilayers, unlike Cry1Ac toxin (5). These results suggest the unique mode of action of Cry2A toxin.

Several strategies such as rotation of toxins and use of multiple toxins for the management of insect resistance to *B. thuringiensis* toxins have been proposed. The *B. thuringiensis* toxin genes used in this study, *cry1Aa*, *cry1Ac*, *cry2A*, and *cry1C*, might be incorporated into transgenic rice because of their high potency to YSB and SSB. For the multiple-toxin approach, Cry1Aa and Cry1Ac together do not appear to be good choices as a dual set of toxins for controlling rice stem borers in transgenic plants because they compete for the same binding site and are likely to result in cross-adaptation. Cry1C might be used in combination with the Cry1A type of toxins due to their lack of cross-reactivity in receptor binding. Cry2A protein has

very different amino acid sequence and a possibly different mode of action from that of Cry1A toxins and could be used in combination with Cry1A proteins. Although our results suggest that Cry1Aa or Cry1Ac be combined with Cry2A and Cry1C in a multitoxin transgenic rice, we recognize that broad-based resistance to these toxins is possible (13). Furthermore, theoretical genetic studies have demonstrated that if multitoxin high-expression crops are planted without a refuge of non-*B. thuringiensis*-expressing hosts of the target pests, then the pests are likely to rapidly evolve separate adaptations to each toxin (12).

ACKNOWLEDGMENTS

We thank Daniel R. Zeigler for his critical review of the manuscript.

This research was supported by a grant from the Rockefeller Foundation to D.H.D.

REFERENCES

1. **Aguda, R. M., et al.** Unpublished data.
1a. **Ballester, V., B. Escriche, J. L. Mensua, G. W. Riethmacher, and J. Ferre.** 1994. Lack of cross resistance to other *Bacillus thuringiensis* crystal proteins in a population of *P. xylostella* highly resistant to CryIA(b). Biocontrol Sci. Technol. **4:**437–443.
2. **Barton, K. A., H. R. Whiteley, and N. S. Yang.** 1987. *Bacillus thuringiensis* δ-endotoxin expressed in transgenic *Nicotiana tabacum* provides resistance to lepidopteran insects. Plant Physiol. **85:**1103–1109.
3. **Bravo, A., S. Jansens, and M. Peferoen.** 1992. Immunocytochemical localization of *Bacillus thuringiensis* insecticidal crystal proteins in intoxicated insects. J. Invertebr. Pathol. **60:**237–246.
4. **Davis, F. M.** 1976. Production and handling of eggs of southwestern corn borer for host plant resistance studies. Miss. Agric. For. Exp. Stn. Tech. Bull. **74:**11.
5. **English, L., H. L. Robins, M. A. Von Tersch, C. A. Kulesza, D. Ave, D. Coyle, C. S. Jany, and S. Slatin.** 1994. Mode of action of CryIIA: a *Bacillus thuringiensis* delta-endotoxin. J. Insect Biochem. Mol. Biol. **24:**1025–1035.
6. **Estada, U., and J. Ferre.** 1994. Binding of insecticidal crystal proteins of *Bacillus thuringiensis* to the midgut brush border of the cabbage looper, *Trichoplusia ni* (Hubner) (Lepidoptera: Noctuidae), and selection for resistance to one of the crystal proteins. Appl. Environ. Microbiol. **60:**3840–3846.
7. **Ferre, J., M. D. Real, J. Van Rie, S. Jansens, and M. Perferoen.** 1991. Resistance to *Bacillus thuringiensis* in a field population of *Plutella xylostella* is due to a change in a midgut membrane receptor. Proc. Natl. Acad. Sci. USA **88:**5119–5123.
8. **Fischhoff, D. A., K. S. Bowdish, F. J. Perlak, P. G. Marrone, S. M. McCormick, J. G. Niedermeyer, S. G. Rogers, and R. T. Fraley.** 1987. Insect tolerant transgenic tomato plants. Bio/Technology **5:**807–813.
9. **Fiuza, L.-M., C. Nielsin-Leroux, E. Goze, R. Frutos, and J.-F. Charles.** 1996. Binding of *Bacillus thuringiensis* Cry1 toxins to the midgut brush border membrane vesicles of *Chilo suppressalis* (Lepidoptera: Pyralidae): evidence of shared binding sites. Appl. Environ. Microbiol. **62:**1544–1549.
10. **Fujimoto, H., K. Itoh, M. Yamamoto, J. Kyozuka, and K. Shimamoto.** 1993. Insect resistant rice generated by introduction of a modified δ-endotoxin gene of *Bacillus thuringiensis*. Bio/Technology **11:**1151–1155.
11. **Garczynski, S. F., J. W. Crim, and M. J. Adang.** 1991. Identification of putative insect brush border membrane-binding molecules specific to *Bacillus thuringiensis* δ-endotoxin by protein blot analysis. Appl. Environ. Microbiol. **57:**2816–2820.
12. **Gould, F.** 1991. Evolutionary potential of crop pests. Am. Sci. **79:**496–507.
13. **Gould, F., A. Martinez-Ramirez, A. Anderson, J. Ferre, F. J. Silva, and W. J. Moar.** 1992. Broad-spectrum resistance to *Bacillus thuringiensis* toxins in *Heliothis virescens*. Proc. Natl. Acad. Sci. USA **89:**7986–7990.
14. **Hofmann, C., P. Luthy, R. Hutter, and V. Pliska.** 1988. Binding of the delta-endotoxin from *Bacillus thuringiensis* to brush border membrane vesicles of the cabbage butterfly (*Pieris brasicca*). Eur. J. Biochem. **173:**85–91.
15. **Hofte, H., and H. R. Whiteley.** 1989. Insecticidal crystal proteins of *Bacillus thuringiensis*. Microbiol. Rev. **53:**242–255.
16. **International Rice Research Institute.** 1996. Rice Genetics III. Proceedings of the Third International Rice Genetics Symposium, 16 to 20 October 1995. IRRI, Manila, Philippines.
17. **Kirsch, K., and H. Schmutterer.** 1988. Low efficacy of *Bacillus thuringiensis* (Berl.) formulation in controlling the diamondback moth, *Plutella xylostella* (L.), in the Philippines. J. Appl. Entomol. **105:**249–255.
18. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
19. **Lee, M. K., R. E. Milne, A. Z. Ge, and D. H. Dean.** 1992. Location of *Bombyx mori* receptor binding region of a *Bacillus thuringiensis* δ-endotoxin. J. Biol. Chem. **167:**3115–3121.
20. **Lee, M. K., F. Rajamohan, F. Gould, and D. H. Dean.** 1995. Resistance to *Bacillus thuringiensis* Cry1A δ-endotoxins in a laboratory-selected *Heliothis virescens* strain is related to receptor alteration. Appl. Environ. Microbiol. **61:**3836–3842.
21. **Lee, M. K., and D. H. Dean.** 1996. Inconsistencies in determining *Bacillus thuringiensis* toxin binding sites relationship by comparing competition assays with ligand blotting. Biochem. Biophys. Res. Commun. **220:**575–580.
21a. **Le Ora Software.** 1987. POLO-PC: a user's guide to probit or logit analysis. LeOra Software, Berkeley, Calif.
22. **Liang, Y., S. S. Patel, and D. H. Dean.** 1995. Irreversible binding kinetics of *Bacillus thuringiensis* Cry1A δ-endotoxins to gypsy moth brush border membrane vesicles is directly correlated to toxicity. J. Biol. Chem. **270:**24719–24724.
23. **MacIntosh, S. C., T. B. Stone, R. S. Jokerst, and R. L. Fuchs.** 1991. Binding of *Bacillus thuringiensis* proteins to a laboratory-selected line of *Heliothis virescens*. Proc. Natl. Acad. Sci. USA **88:**8930–8933.
24. **Masson, L., A. Mazza, R. Brousseau, and B. Tabashnik.** 1995. Kinetics of *Bacillus thuringiensis* toxin binding with brush border membrane vesicles from susceptible and resistant larvae of *Plutella xylostella*. J. Biol. Chem. **270:**11887–11896.
25. **McGaughey, W. H., and D. E. Johnson.** 1994. Influence of crystal protein composition of *Bacillus thuringiensis* strains on cross-resistance in Indianmeal moth (Lepidoptera: Pyralidae). J. Econ. Entomol. **87:**535–540.
26. **McGaughey, W. H.** 1985. Insect resistance to the biological insecticide *Bacillus thuringiensis*. Science **229:**193–195.
27. **McGaughey, W. H., and M. E. Whalon.** 1992. Managing insect resistance to *Bacillus thuringiensis* toxins. Science **258:**1451–1455.
28. **Moar, W. J., M. Pusztai-Carey, H. Van Faassen, D. Bosch, R. Frutos, C. Rang, K. Luo, and M. J. Adang.** 1995. Development of *Bacillus thuringiensis* CryIC resistance by *Spodoptera exigua* (Hubner) (Lepidoptera: Noctuidae). Appl. Environ. Microbiol. **61:**2086–2092.
29. **Munson, P. J., and D. Rodbard.** 1980. LIGAND: a versatile computerized approach for characterization of ligand-binding systems. Anal. Biochem. **107:**220–239.
30. **Nielsen-Leroux, C., J. F. Charles, I. Thiery, and G. P. Georghiou.** 1995. Resistance in a laboratory population of *Culex quinquefasciatus* (Diptera: Culicidae) to *Bacillus sphaericus* binary toxin is due to a change in the receptor on midgut brush-border membranes. Eur. J. Biochem. **228:**206–210.
31. **Pathak, M. D., and Z. R. Khan.** 1994. Insect pests of rice. IRRI, Manila, The Philippines.
32. **Perlak, F. J., T. B. Stone, Y. M. Muskopf, L. J. Petersen, G. B. Parker, S. L. McPherson, J. Wyman, S. Love, G. Reed, D. Biever, and D. A. Fischhoff.** 1993. Genetically improved potatoes: protection from damage by Colorado potato beetles. Plant Mol. Biol. **22:**313–321.
33. **Rahardja, U., and M. E. Whaton.** 1995. Inheritance of resistance to *Bacillus thuringiensis* subsp. *tenebrionis* CryIIIA delta-endotoxin in Colorado potato beetle (Coleoptera: Chrysomelidae). J. Econ. Entomol. **88:**21–26.
34. **Rajamohan, F., E. Alcantara, M. K. Lee, X. J. Chen, A. Curtiss, and D. H. Dean.** 1994. Single amino acid changes in domain II of *Bacillus thuringiensis* by Cry1Ab δ-endotoxin affect irreversible binding to *Manduca sexta* midgut membrane vesicles. J. Bacteriol. **177:**2276–2282.
35. **Raymond, M.** 1985. Presentation d' um logrammed d'analysis log probit pour microordinnateur. Cah. ORSTOM Ser. Entomol. Med. Parasitol. **22:**117–121.
36. **SAS Institute.** 1985. SAS user's guide: statistics, 5th ed. SAS Institute, Cary, N.C.
37. **Schwartz, J. L., L. Garneau, L. Masson, and R. Brousseau.** 1991. Early response of cultured lepidopteran cells to exposure to δ-endotoxin from *Bacillus thuringiensis*: involvement of calcium and anionic channels. Biochim. Biophys. Acta **1065:**250–260.
38. **Shelton, A. M., J. L. Robertson, J. D. Tang, C. Perez, S. D. Eigenbrode, H. K. Preisler, W. T. Wilsey, and R. J. Cooley.** 1993. Resistance of diamondback moth (Lepidoptera: Plutellidae) to *Bacillus thuringiensis* subspecies in the field. J. Econ. Entomol. **86:**697–705.
39. **Tabashnik, B. E., N. L. Cushing, N. Finson, and M. W. Johnson.** 1990. Field development of resistance to *Bacillus thuringiensis* in diamondback moth (Lepidoptera: Plutellidae). J. Econ. Entomol. **83:**1671–1676.
40. **Tabashnik, B. E., N. Finson, M. W. Johnson, and D. G. Heckel.** 1994. Cross-resistance to *Bacillus thuringiensis* toxin CryIF in the diamondback moth (*Plutella xylostella*). Appl. Environ. Microbiol. **60:**4627–4629.
41. **Tabashnik, B. E.** 1994. Evolution of resistance to *Bacillus thuringiensis*. Annu. Rev. Entomol. **39:**47–79.
42. **Tabashnik, B. E., N. Finson, F. R. Groeters, W. J. Moar, M. W. Johnson, K. Luo, and M. J. Adang.** 1994. Reversal of resistance to *Bacillus thuringiensis* in *Plutella xylostella*. Proc. Natl. Acad. Sci. USA **91:**4120–4124.
43. **Tabashnik, B. E., N. Finson, M. W. Johnson, and W. J. Moar.** 1993. Resistance to toxins from *Bacillus thuringiensis* subsp. *kurstaki* causes minimal cross resistance to *B. thuringiensis* subsp. *aizawai* in the diamondback moth (Lepidoptera: Plutellidae). Appl. Environ. Microbiol. **59:**1332–1335.
44. **Tang, J. D., A. M. Shelton, J. Van Rie, S. D. Roeck, W. J. Moar, R. T. Roush, and M. Peferoen.** 1996. Toxicity of *Bacillus thuringiensis* spore and crystal

protein to resistant diamondback moth (*Plutella xylostella*). Appl. Environ. Microbiol. **62:**564–569.

45. **Vaeck, M., A. Reynaerts, H. Hofte, S. Jansens, M. De Beuckeleer, C. Dean, M. Zabeau, M. Van Montagu, and J. Leemans.** 1987. Transgenic plants protected from insect attack. Nature (London) **327:**33–37.
46. **Van der Salm, T., D. Bosch, G. Honee, L. Feng, E. Munsterman, P. Bakker, W. J. Stiekema, and B. Visser.** 1994. Insect resistance of transgenic plants that express modified *Bacillus thuringiensis cryIA(b)* and *cryIC* genes: a resistance management strategy. Plant Mol. Biol. **26:**51–59.
47. **Van Rie, J., W. H. McGaughey, D. E. Johnson, B. D. Barnett, and H. Van Mellaert.** 1990. Mechanism of insect resistance to the microbial insecticide *Bacillus thuringiensis*. Science **247:**72–74.
48. **Van Rie, J., S. Jansens, H. Hofte, D. Degheele, and H. Van Mellaert.** 1989. Specificity of *Bacillus thuringiensis* δ-endotoxins. Importance of specific receptors on the brush border membrane of the midgut of target insects. Eur. J. Biochem. **186:**239–247.
49. **Van Rie, J., S. Jansens, H. Hofte, D. Degheele, and H. Van Mellaert.** 1990. Receptors on the brush border membrane of the insect midgut as determinants of the specificity of *Bacillus thuringiensis* δ-endotoxins. Appl. Environ. Microbiol. **56:**1378–1385.
50. **Whiteley, H. R., and H. E. Schnepf.** 1986. The molecular biology of parasporal crystal body formation in *B. thuringiensis*. Annu. Rev. Microbiol. **40:** 549–576.
51. **Wolfersberger, M.** 1990. The toxicity of two *Bacillus thuringiensis* delta-endotoxins to gypsy moth larvae is inversely related to the affinity of binding sites on midgut brush border membranes for the toxins. Experentia **46:**475–477.
52. **Wolfersberger, M., P. Luthy, A. Maurer, P. Parenti, V. F. Sacchi, B. Giordana, and G. M. Hanozet.** 1987. Preparation of brush border membrane vesicles (BBMV) from larval lepidopteran midgut. Comp. Biochem. Physiol. **86A:**301–308.
53. **Wu, S.-J., and D. H. Dean.** 1996. Functional significance of loops in the receptor binding domain of *Bacillus thuringiensis* CryIIIA δ-endotoxin. J. Mol. Biol. **255:**628–640.
54. **Wunn, J. A., A. Kloti, P. K. Burkhardt, G. C. Ghosh Bisws, K. Launis, V. A. Inglesias, and I. Potrykus.** 1996. Transgenic indica rice breeding line IR58 expressing a synthetic *cryIA(b)* gene from *Bacillus thuringiensis* provides effective insect pest control. Bio/Technology **14:**171–176.