Production and Characterization of *Bacillus thuringiensis* Cry1Ac-Resistant Cotton Bollworm *Helicoverpa zea* (Boddie)[▽]

Konasale J. Anilkumar,¹ Ana Rodrigo-Simón,² Juan Ferré,² Marianne Pusztai-Carey,³
Sakuntala Sivasupramaniam,⁴ and William J. Moar^{1*}

Department of Entomology and Plant Pathology, Auburn University, Auburn, Alabama 36849¹; Department of Genetics, University of Valencia, Dr. Moliner 50, 46100 Burjassot (Valencia), Spain²; Department of Biochemistry, Case Western Reserve University, 10900 Euclid Avenue, Cleveland, Ohio 44106-4935³; and Monsanto Company, 700 Chesterfield Parkway North, St. Louis, Missouri 63017⁴

Received 15 July 2007/Accepted 6 November 2007

Laboratory-selected *Bacillus thuringiensis*-resistant colonies are important tools for elucidating *B. thuringiensis* resistance mechanisms. However, cotton bollworm, *Helicoverpa zea*, a target pest of transgenic corn and cotton expressing *B. thuringiensis* Cry1Ac (Bt corn and cotton), has proven difficult to select for stable resistance. Two populations of *H. zea* (AR and MR), resistant to the *B. thuringiensis* protein found in all commercial Bt cotton varieties (Cry1Ac), were established by selection with Cry1Ac activated toxin (AR) or MVP II (MR). Cry1Ac toxin reflects the form ingested by *H. zea* when feeding on Bt cotton, whereas MVP II is a Cry1Ac formulation used for resistance selection and monitoring. The resistance ratio (RR) for AR exceeded 100-fold after 11 generations and has been maintained at this level for nine generations. This is the first report of stable Cry1Ac resistance in *H. zea*. MR crashed after 11 generations, reaching only an RR of 12. AR was only partially cross-resistant to MVP II, suggesting that MVP II does not have the same Cry1Ac selection pressure as Cry1Ac toxin against *H. zea* and that proteases may be involved with resistance. AR was highly cross-resistant to Cry1Ab toxin but only slightly cross-resistant to Cry1Ab expressing corn leaf powder. AR was not cross-resistant to Cry2Aa2, Cry2Ab2-expressing corn leaf powder, Vip3A, and cypermethrin. Toxin-binding assays showed no significant differences, indicating that resistance was not linked to a reduction in binding. These results aid in understanding why this pest has not evolved *B. thuringiensis* resistance, and highlight the need to choose carefully the form of *B. thuringiensis* protein used in experiments.

Transgenic cotton expressing *Bacillus thuringiensis* Cry1Ac (Bt cotton) has been used commercially in the United States since 1995 (10), and the area under Bt cotton production has steadily increased over that period (22). Bt cotton provides excellent control of many lepidopteran pests of cotton and thereby exerts tremendous selection pressure for resistance. Concerns regarding resistance to Bt cotton and Bt corn have led the U.S. Environmental Protection Agency to mandate Insect Resistance Management (IRM) strategies for all target pests of Bt crops (11). Perhaps partly because of these IRM strategies, there has yet to be a case of field resistance to Bt cotton after 10 years of intense cultivation (4).

In the United States, tobacco budworm, *Heliothis virescens* F., pink bollworm, *Pectinophora gossypiella* (Saunders), and cotton bollworm, *Helicoverpa zea* (Boddie) are the three major target pests of Bt cotton. Although current Bt varieties express a high dose of Cry1Ac against *H. virescens* and *P. gossypiella*, it is still not sufficient to kill all *H. zea* (21). In particular, high *H. zea* population pressure and varied expression of Cry1Ac in different cotton tissues associated with plant age and stress can result in increased *H. zea* larval survival (1, 19, 21). *H. zea* is highly polyphagous and can be a major pest in field corn and is

the key pest in sweet corn in many areas. Therefore, *H. zea* also is exposed to Cry1Ab in Cry1Ab-expressing Bt corn, which is similar in structure (>90% amino acid similarity) (8) and mode of action to Cry1Ac. Cross-resistance to Cry1Ab has been reported in populations of *Helicoverpa armigera* (2), *H. virescens* (17), *H. zea* (33), *P. gossypiella* (45, 46, 48), and *Trichoplusia ni* (51). These factors increase the likelihood of resistance development to Bt cotton by *H. zea* (6, 33).

Cry1Ac resistance in Bt cotton pests such as *H. virescens* (17, 18) and *P. gossypiella* (45, 46, 48) is relatively well studied, using populations selected in the laboratory with MVP II (a commercial formulation containing Cry1Ac protoxin inclusion bodies encapsulated in *Pseudomonas fluorescens* cells), and these results have helped formulate nationwide IRM strategies. However, these IRM strategies may not be optimal for *H. zea* because *B. thuringiensis* resistance mechanisms, and other factors can be different in different insect species (14, 20). Therefore, it is of great interest to establish a Cry1Ac-resistant *H. zea* population to examine mechanisms of *B. thuringiensis* resistance, patterns of cross-resistance and other parameters in this insect.

Several attempts at selecting for Cry1Ac resistance in *H. zea* using MVP II have been met with limited success (R. E. Jackson [U.S. Department of Agriculture Agricultural Research Service, Stoneville, MS], personal communication; W. J. Moar, unpublished data). Possible reasons for this limited success include (i) the fitness costs involved with resistance to the Cry1Ac protoxin or other compounds in MVP II or (ii) the

* Corresponding author. Mailing address: 301 Funchess Hall, Department of Entomology and Plant Pathology, Auburn University, Auburn, AL 36849. Phone: (334) 844-2560. Fax: (334) 844-5005. E-mail: moarwil@auburn.edu.

[▽] Published ahead of print on 16 November 2007.

allele frequency for MVP II resistance being very low (6). Furthermore, resistance selection using MVP II may not adequately reflect Cry1Ac resistance selection to *H. zea* in planta because although Bt cotton expresses full-length solubilized Cry1Ac protoxin (39), it is at least partially activated to toxin by plant proteases immediately upon plant cell disruption. This observation is similar to Cry1Fa in cotton, where the full-length protoxin is expressed, but only activated toxin is recovered from plant tissue (16). Besides the use of MVP II, other Cry1Ac forms or preparations have also been used for resistance selection, including *E. coli* containing Cry1Ac inclusion bodies, *B. thuringiensis* Cry1Ac protoxin crystals with spores, and Cry1Ac activated toxin (2, 28, 33, 53). However, it is not known whether there are differences between these different Cry1Ac forms in terms of resistance selection as it pertains to Bt cotton. Insect susceptibility to *B. thuringiensis* proteins may vary with the form of *B. thuringiensis* protein ingested, especially in *B. thuringiensis*-tolerant species, and the form of the *B. thuringiensis* protein used may have a dramatic impact on the resulting *B. thuringiensis* resistance mechanism(s) (26, 34, 38).

We report here for the first time that moderately high and stable resistance to Cry1Ac toxin has been attained in *H. zea*, and this resistance has at least been partly characterized. This strain has differential susceptibilities to various forms of Cry1Ac and Cry1Ab, is still susceptible to cypermethrin and, unlike in most Cry1Ac-resistant insects, resistance does not appear to be due to alterations in receptor binding (14, 20).

MATERIALS AND METHODS

Insect strains. A laboratory susceptible colony of *H. zea* (SC) was established in September 2004 from a laboratory colony from Monsanto (Union City, TN). The culture at Monsanto is annually infused with insects collected from corn. Insects were reared on pinto bean-based artificial diet at $27 \pm 1^\circ\text{C}$ with a photoperiod of 14:10 h (light-dark) (35).

Bt proteins and pyrethroids. An *Escherichia coli* strain expressing Cry1Ac protoxin from *B. thuringiensis* subsp. *kurstaki* strain HD-1 (provided by L. Mason, Biotechnology Research Institute, National Research Council, Montreal, Quebec, Canada) was cultured, and the activated toxin was prepared as indicated elsewhere (34, 40). Cry2Aa2 protoxin was prepared as described by Moar et al. (34). Cry1Aa (*B. thuringiensis* EG1273), Cry1Ab (*B. thuringiensis* EG7077), and Cry1Ac (EG11070) clones were provided by Ecogen, Inc. (Langhorne, PA), and were used to prepare trypsin-activated toxins as described by Estela et al. (12). MVP II and lyophilized corn leaf powder containing Cry1Ab (229.55 $\mu\text{g/g}$) and Cry2Ab2 (6 mg/g) were supplied by Monsanto (St. Louis, MO). MVP II is a formulated, freeze-dried powder containing 19.1% Cry1Ac protoxin inclusion bodies encapsulated in *P. fluorescens*. Vip3A (100% active, salt-free) was supplied by Syngenta (Greensboro, NC). A representative pyrethroid, cypermethrin, cyano(3-phenoxyphenyl) methyl 3-(2,2-dichloroethenyl)-2,2-dimethylcyclopropanecarboxylate (92% active ingredient PL86-172) was supplied by FMC Corp. (Philadelphia, PA).

Selection experiments. Selection experiments were initiated after four generations of rearing SC and generating baseline susceptibility values for Cry1Ac toxin and MVP II.

Two strains of *H. zea* were selected for Cry1Ac resistance on artificial diet (MR and AR, discussed below). *B. thuringiensis* protein concentrations were prepared in distilled water and mixed thoroughly 20:80 (wt/wt) with artificial diet when the diet temperature was $<60^\circ\text{C}$ and poured onto selection trays. Microtiter plates (96 and 384 wells) were used as selection arenas. Typically at least 2,000 neonates were used for selection for each of the first three generations followed by at least 1,000 neonates for most subsequent selections. Individual neonates were exposed to MVP II (MR) or Cry1Ac toxin (AR) for 7 days; only larvae that molted (based on larval head capsule size) were selected and reared to pupation on diet containing no *B. thuringiensis* protein.

AR was selected at 50 (generation 1), 80 (generations 2 and 3), and 200 (generations 4 and 5) μg of Cry1Ac activated toxin/g. After five generations, selection concentration was increased to 500 $\mu\text{g/g}$ and was not increased further

due to limited supply of toxin; for every 60 g of diet, 30 mg of activated toxin was required. AR was selected for resistance every generation; currently, this strain is under its 25th generation of selection. Preliminary experiments showed that MVP II was two- to threefold less toxic than Cry1Ac toxin. Therefore, MR was selected at 100 (generation 1), 200 (generation 2), 500 (generations 3 to 5), and 1,000 (generations 6 to 8) μg of Cry1Ac in MVP II/g of diet. Selection of MR could not be continued beyond eight generations due to suboptimal larval number (reduced hatching percent), ultimately leading to the loss of the strain after generation 11.

Testing resistance. At selected generations, diet incorporation bioassays were conducted concurrently for SC and resistant strains to determine resistance levels. Five to seven concentrations of *B. thuringiensis* compounds were incorporated into artificial diet as described above and assayed against neonates (0 to 16 h old). Each *B. thuringiensis* compound-diet concentration was poured into 16 to 32 wells of a 128-well CD International bioassay tray (CD International, Pitman, NJ). One "active" neonate was loaded per well and covered with ventilated covers (CD International), and the bioassay trays were incubated at $27 \pm 1^\circ\text{C}$ and 60% relative humidity with a photoperiod of 14:10 h (light-dark). Assays were rated after 7 days; dead and first-instar larvae were considered as dead (3). Bioassays were replicated at least three times.

Cross-resistance to MVP II. Initial LC_{50} (i.e., concentration required to kill 50% of the population) values generated for SC against Cry1Ac activated toxin and MVP II at generation 0 indicated a 2.9-fold increase for MVP II (Table 1). Based on these observations, similarly higher 50% lethal concentration (LC_{50}) values for AR were expected when tested against MVP II compared to 1Ac toxin. To test this assumption, bioassays were conducted with AR using MVP II after 7, 11, and 16 generations of selection and concurrently with Cry1Ac toxin. After the LC_{50} values were calculated from probit analysis, ratios of the LC_{50} values for MVP II and activated Cry1Ac toxin were generated for AR and SC (Table 2).

Cross-resistance to other *B. thuringiensis* proteins and cypermethrin. Tests for cross-resistance to other Cry proteins (Cry1Ab toxin, Cry1Ab-corn powder, Cry2Aa2, and Cry2Ab2-corn powder), Vip3A, and cypermethrin were conducted between generations 15 and 20 of selection (resistance ratio [RR] of ~ 100 -fold). The expression level of Cry1Ab and Cry2Ab2 in corn tissue was too low to obtain sufficient mortality; therefore, growth rates on diets containing a range of concentrations were used. The mean larval weight was recorded, and the percent weight loss (compared to the untreated control) in different concentrations of *B. thuringiensis* proteins was calculated considering mean larval weight in the untreated control to be 100%.

AR and SC larvae were reared to third instar (8.32 ± 1.29 mg) on untreated diet diluted with 20% water and treated topically on the thoracic terga with 0.5 μl of acetone only (control) or 0.5 μl of acetone with a range of cypermethrin concentrations (49). Twelve larvae were tested per concentration; treated larvae were transferred to 24-well bioassay trays containing diet. In addition, 10 AR larvae (weight, 7.83 ± 1.46 mg) from Cry1Ac selection (500 $\mu\text{g/g}$ of diet) were treated at 1.99 ng/mg body weight. Mortality was assessed after 24 h. All treatments were replicated three times, and each replication consisted of a total of seven concentrations and a control except as described above. Lethal doses were calculated by using probit analysis (Polo Plus [LeOra Software, Berkeley, CA]) and adjusted for body weight.

Labeling of Cry1Ac and Cry1Aa toxins. Cry1Aa and Cry1Ac toxins used for binding experiments were obtained from recombinant *B. thuringiensis* strains EG1273 and EG11070, respectively. Both toxins were activated with trypsin, dialyzed overnight, and purified by anion-exchange chromatography in a MonoQ HR 5/50 column using an ÄKTA Explorer 100 system (GE Healthcare, Uppsala, Sweden) with a 30-ml gradient of 20 mM Tris-HCl (pH 8.6) to 20 mM Tris-HCl (pH 8.6) and 1 M NaCl, as described by Estela et al. (12). Sample purity was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the protein concentration was determined by densitometric analysis using bovine serum albumin as a standard.

Labeling of Cry1Aa and Cry1Ac was performed by incubating 20 μg of toxin with 0.30 mCi of [^{125}I]NaI (Nucliber, Madrid, Spain) using chloramine-T (50). Toxins were labeled twice to have relatively freshly labeled toxins throughout the study. The specific activities obtained for Cry1Aa and Cry1Ac were, respectively, 2.3 and 47 mCi/mg (first labeling) and 0.6 and 1.4 mCi/mg (second labeling).

BBMV preparation and binding assays. Fifth-instar AR and SC larvae were dissected in MET buffer (250 mM mannitol, 17 mM Tris-HCl, 5 mM EGTA [pH 7.5]), and midguts were removed and frozen at -80°C . Frozen midguts were shipped on dry ice to the University of Valencia. Brush border membrane vesicles (BBMV) were prepared by the differential magnesium precipitation method (52), frozen in liquid nitrogen, and stored at -80°C until used. BBMV protein concentrations were determined according to the method of Bradford (5).

TABLE 1. Resistance development in *H. zea* when selected using activated toxin and MVP II

Strain	G ^a	Total no. of insects tested ^b	LC ₅₀ (95% FL) ^c	Slope (mean ± SE)	RR ^d
Activated toxin-resistant strain (AR)					
SC ^e		800	9.13 (5.83–12.53)	1.61 ± 0.41	
AR	4	384	107.64 (75.37–155.6)	1.42 ± 0.3	12.12
SC		480	8.89 (5.71–13.72)	1.71 ± 0.41	
AR	7	222	321.22 (251.27–371)	1.89 ± 0.13	35.91
SC		384	8.94 (6.37–15.27)	1.93 ± 0.31	
AR	11	175	1,450 (690–2,392)	1.42 ± 0.47	122.67
SC		384	11.82 (7.01–19.24)	1.76 ± 0.42	
AR	16	72	— ^f		>100
SC		384	13.90 (9.11–21.44)	2.41 ± 0.51	
AR	19	192	1,390 (743–12,017)	1.39 ± 0.46	92.69
SC		192	15.00 (9.90–22.45)	2.31 ± 0.52	
MVP II-resistant strain (MR)					
SC	0	1,120	26.13 (16.34–35.62)	1.73 ± 0.24	
MR	4	384	384.3 (282.31–568.12)	1.79 ± 0.27	16.61
SC		640	23.13 (16.34–35.62)	1.73 ± 0.24	
MR	7	672	298.40 (155.16–455.5)	1.67 ± 0.41	12.01
SC		1,120	24.84 (13.48–41.89)	2.47 ± 0.57	
MR	9–11		— ^g		

^a G, generations of *H. zea* continuously selected with *B. thuringiensis*.

^b One to five replicates with one to seven concentrations.

^c LC₅₀ values are presented in micrograms of *B. thuringiensis* protein per gram of diet. FL, fiducial limits.

^d The LC₅₀ for AR divided by the LC₅₀ for SC.

^e SC, susceptible colony.

^f 47% survivors at 1.5 mg/g.

^g No selection due to reduced larval number; the resistant strain crashed after 11 generations.

Binding experiments were performed as previously described (12). A fixed amount of ¹²⁵I-labeled toxins and BBMV (0.05 mg/ml) was incubated for 1 h at room temperature with increasing the concentrations of unlabeled homologous toxin in a 0.1-ml final volume of binding buffer (PBS–0.1% BSA; 1 mM KH₂PO₄, 10 mM Na₂HPO₄, 137 mM NaCl, 2.7 mM KCl [pH 7.4], 0.1% bovine serum albumin). After 10 min of centrifugation at 16,000 × g, the pellets were washed twice in binding buffer. The final radioactivity remaining in the BBMV pellets was measured in a 1282 Compugamma CS gamma counter (LKB Pharmacia). Experiments were replicated four times with SC and twice with each AR batch. *N*-Acetylgalactosamine (GalNAc) was obtained from Sigma (St. Louis, MO). Cry1Ac binding in the presence of the GalNAc inhibitor was performed as described above, but with a preincubation of ¹²⁵I-labeled Cry1Ac with GalNAc for 45 min at room temperature prior to the start of the assay with the addition of the BBMV. Experiments were replicated three times.

Data analysis. Bioassay data were analyzed by probit analysis (15) using Polo Plus. LC₅₀ values with nonoverlapping 95% fiducial limits were considered as significantly different. RR values were calculated by dividing the LC₅₀ values for AR or MR by that of SC. The percent weight loss data for AR and SC in Cry1Ab and Cry2Ab cross-resistance studies was subjected to paired *t* tests using SPSS (44). A chi-square test was conducted to test for significant differences between

ratios of MVP II and Cry1Ac activated toxin LC₅₀ values for both AR and SC. Binding results were analyzed by using the LIGAND computer program (37).

RESULTS

Selection response in AR and MR. There was a significant increase in resistance after four generations of selection using Cry1Ac activated toxin (AR) and MVP II (MR) compared to the SC (Table 1). During the first seven generations, the rate of resistance evolution was three times faster in AR than in MR. The rate of resistance evolution in AR increased with an increase in selection pressure, and 12-, 36-, and 123-fold resistances were observed after 4, 7, and 11 generations of selection, respectively (Table 1). Resistance (based on mortality) in AR did not increase further, as the selection concentration was not increased above 500 µg/g of diet due to limited toxin availability; however, there has been an increase in the num-

TABLE 2. Cross-resistance of AR to MVP II

Strain	G ^a	Total no. of insects tested ^b	LC ₅₀ (95% FL) ^c	Slope (mean ± SE)	RR ^d	MVP II/Cry1Ac ratio ^e
AR	7 (36)	192	117.79 (63.38–175.45)	1.81 ± 0.34	4.7	0.37
SC ^f		1,120	24.84 (13.48–41.89)	2.47 ± 0.57		2.78
AR	11 (123)	576	197.10 (134.1–333.51)	1.36 ± 0.29	7.9	0.14
SC		576	24.94 (13.86–44.87)	2.57 ± 0.37		2.11
AR	16 (>100)	448	397.93 (245.87–749.86)	1.56 ± 0.42	10.3	0.28
SC		448	38.53 (24.87–55.56)	2.41 ± 0.53		2.76

^a G, generations of *H. zea* continuously selected with Cry1Ac activated toxin; values in parentheses indicate the RR to Cry1Ac-activated toxin.

^b One to five replicates with one to seven concentrations.

^c LC₅₀ values are presented in micrograms of Cry1Ac in MVP II per gram of diet. FL, fiducial limits.

^d The LC₅₀ for AR divided by the LC₅₀ for SC.

^e The ratio of LC₅₀ values for MVP II divided by the LC₅₀ values for Cry1Ac activated toxin (data from Table 1).

^f SC, susceptible colony.

TABLE 3. Cross-resistance of AR to other *B. thuringiensis* proteins and cypermethrin

Strain	G ^a	Compound	Total no. of insects tested ^b	LC ₅₀ (95% FL) ^c	Slope (mean ± SE)	RR ^d
AR	19 (93)	Cry1Ab	200	— ^h		ND ^e
SC ^f			200	133.33 (98.42–261.41)	1.82 ± 0.55	
AR	15 (>100)	Vip3A	512	22.29 (15.18–31.07)	2.59 ± 0.49	0.94
SC			512	23.73 (16.82–33.80)	2.24 ± 0.35	
AR	16 (>100)	Cry2Aa2	672	101.83 (72.60–167.39)	2.83 ± 0.51	1.55
SC			672	65.70 (46.27–109.34)	1.89 ± 0.27	
AR	16 (>100)	Cypermethrin	288	1.70 (1.11–2.61) ^g	2.08 ± 0.40	1.85
SC			288	0.92 (0.61–1.33) ^g	2.55 ± 0.52	

^a G, generations of *H. zea* continuously selected with *B. thuringiensis* protein; values in parentheses indicate the RR when bioassays were conducted.

^b Three to five replicates with five to seven concentrations.

^c LC₅₀ values are in micrograms of *B. thuringiensis* protein per gram of diet. FL, fiducial limits.

^d The LC₅₀ for AR divided by the LC₅₀ for SC.

^e ND, not determined because the LC₅₀ for AR could not be obtained.

^f SC, susceptible colony.

^g Lethal dose (ng/mg [body weight]).

^h 8.44% mortality at 400 µg/g of diet.

ber of large (third-instar) larvae in subsequent generations (K. J. Anilkumar, unpublished data). Resistance in MR did not increase above 17-fold, even after selecting at higher concentrations for three additional generations (Table 1). Selection in MR could not be continued beyond 8 generations due to reduced larval numbers (lower-percentage egg hatch), ultimately leading to loss of the strain after 11 generations.

Cross-resistance of AR to MVP II. The ratio of LC₅₀ values for MVP II to that of Cry1Ac activated toxin for both strains indicated significant ($\chi^2 = 6.16$, $P = 0.01$, $df = 1$) differences (Table 2). Based on these ratios MVP II was more toxic to AR than expected (it should be less toxic, as observed in SC), resulting in only partial cross-resistance (Table 2).

Cross-resistance of AR to other *B. thuringiensis* proteins and cypermethrin. There was significant cross-resistance to Cry1Ab activated toxin (Table 3). AR larvae lost significantly ($t = 14.70$, $P = 0.045$) less (11%) weight compared to SC at the highest concentration of Cry1Ab-expressing corn powder (3.84 µg/g) able to be incorporated in the diet (Fig. 1a). There was no cross-resistance to Cry2Aa2 protoxin inclusion bodies, Cry2Ab2-expressing corn powder (Fig. 1b, $t = -0.385$, $P = 0.72$), Vip3A, and cypermethrin (Table 3). AR was also tested with cypermethrin while being reared on diet containing 500 µg/g of Cry1Ac activated toxin. The results (45.8% ± 5.9% mortality at 1.99 ng/mg of body weight) were not different when AR was reared on a regular diet (containing no Cry1Ac).

Binding of ¹²⁵I-labeled Cry1A toxins to BBMV. Binding of ¹²⁵I-labeled Cry1Ac to BBMV from AR and SC did not show significant differences even when AR was at its highest resistance ratio. As shown in Fig. 2a, homologous competition curves followed a similar pattern with BBMV from both strains. Binding parameters (the dissociation constant [K_d] and the concentration of binding sites [R_t]) obtained from the competition experiments were not significantly different (t tests, $P > 0.05$; Table 4). Binding of Cry1Aa was tested because this toxin shares binding sites with Cry1Ac (24), and it has been shown that, in some resistant strains, alteration of a Cry1A common binding site may be observed when no differences with Cry1Ac are detected due to contribution of other binding sites (29, 43). In our case, binding of ¹²⁵I-labeled Cry1Aa did

not show significant differences between SC and AR (Fig. 2b, Table 4).

To differentiate between binding of Cry1Ac that takes place solely through domain II from binding that requires domain III, GalNAc was used as a diagnostic tool, since this sugar inhibits binding of Cry1Ac through domain III to GalNAc residues in the membrane. Preincubation of ¹²⁵I-labeled Cry1Ac with GalNAc prior to BBMV resulted in partial inhi-

FIG. 1. Toxicity of Cry1Ab (a) and Cry2Ab2 (b) expressing corn leaf powder to susceptible (SC) and Cry1Ac-resistant (AR) *H. zea*. Values are expressed as the percent weight loss relative to the corresponding controls at different concentrations. The data represent the means of four replications, and error bars show the standard deviation. The asterisk indicates significant differences in Student t tests ($t = 14.70$, $P = 0.045$).

FIG. 2. Binding of ^{125}I -labeled Cry1Ac (A) and ^{125}I -labeled Cry1Aa (B) to BBMVs from susceptible (SC) (●, solid line) and Cry1Ac-resistant (AR at the 11th generation of selection) *H. zea* (▲, broken line) at increasing concentrations of unlabeled homologous competitor. The data represent the means of four experiments for SC and two experiments for AR, and the error bars indicate the standard error of the mean.

bition of binding ($\sim 34\%$); however, this inhibition was similar in both strains (Fig. 3).

DISCUSSION

Since the advent of transgenic Bt crops, determining the most appropriate form of a *B. thuringiensis* protein for resistance selection has been an issue for debate. There is a fine balance between what forms of the protein(s) are (i) expressed in plants, (ii) present in insects upon ingestion, (iii) available for testing, and (iv) relatively toxic to the target insects (requiring large quantities of protein if susceptibility is low). Historically, and in some cases currently, truncated *B. thuringiensis* proteins are expressed within transgenic plants, in other cases, full-length protoxins are expressed. However, recent reports by

FIG. 3. Percent binding of ^{125}I -labeled Cry1Ac to BBMVs from susceptible (SC) and Cry1Ac-resistant (AR) *H. zea* in the absence (SC and AR) or presence (SC+GN and AR+GN) of 25 mM GalNAc. The data represent the means of three experiments, and error bars indicate the standard error of the mean.

Gao et al. (16) and Li et al. (31) demonstrate that what the insect actually ingests may be different than what is originally expressed in the plant. As a result, researchers are faced with the dilemma of choosing the most appropriate form of the protein while facing potential logistical constraints. Choosing is not an easy task because insects can vary in their susceptibility to the various forms of *B. thuringiensis* proteins (26).

Our results demonstrate that Cry1Ac-resistant *H. zea* can be selected and maintained using Cry1Ac activated toxin in the laboratory. The laboratory strain originating from Monsanto has had annual infusions of *H. zea* collected from corn and therefore should have higher genetic variability (and therefore higher *B. thuringiensis*-resistant allele frequency) than laboratory colonies with no infusion of field-derived insects. Our initial LC_{50} values for SC of 9 $\mu\text{g/g}$ of diet (Cry1Ac toxin) and 26 $\mu\text{g/g}$ of diet (MVP II) are significantly higher than those reported by Luttrell et al. (33) for Cry1Ac toxin (0.02 $\mu\text{g/g}$ of diet for colony 9103Z) and Ali et al. (3) for MVP II (2.08 $\mu\text{g/g}$ of diet), respectively, for their laboratory *H. zea* colony that has had no infusion of field insects for at least 10 years. If we compare the LC_{50} of AR at generation 19 to 9103Z, we would observe an RR of $\sim 69,500$. Although other variables such as bioassay methodology and host strain need to be considered, these results suggest that *H. zea* can be selected to have tremendous differences in Cry1Ac susceptibility relative to a highly homogeneous laboratory colony. Higher levels of resistance in AR were not sought due to the naturally high tolerance to Cry1Ac and the cannibalistic nature of *H. zea*, both resulting in the need for relatively large quantities of Cry1Ac activated toxin to rear these insects individually. The availability of appropriate selection materials, especially purified protein and plant material, is still a major constraint for producing resistant colonies, especially for insects that have a relatively high tolerance to *B. thuringiensis* proteins such as *H. zea*.

Resistance development in AR was relatively quick compared to reports for other insects (2, 18). Possible reasons for this relatively rapid rate of resistance evolution include the selection of only larvae that had molted, thereby eliminating a higher percentage of susceptible insects in each generation (3); the use of Cry1Ac activated toxin; and a relatively high initial Cry1Ac toxin resistance allele frequency (6). A relatively rapid rate of resistance evolution was also observed in *Spodoptera exigua* and another strain of *H. zea* selected using Cry1C and Cry1Ac activated toxins, respectively (33, 35).

TABLE 4. Dissociation constants (K_d) and concentration of binding sites (R_t) for binding of Cry1A proteins to BBMVs from *H. zea*^a

<i>B. thuringiensis</i> toxin	Sample (generation of selection) ^b	$K_d \pm \text{SD}$ (nM)	$R_t \pm \text{SD}$ (pmol/mg) ^c
Cry1Ac	SC	1.1 ± 0.1	17.0 ± 0.9
	AR (4)	0.50 ± 0.3	22.3 ± 4.9
	AR (7)	0.4 ± 0.1	28 ± 15
	AR (11)	2.9 ± 0.1	49 ± 3
Cry1Aa	SC	3.2 ± 0.4	2.7 ± 0.7
	AR (7)	3.8 ± 0.1	4.1 ± 0.7
	AR (11)	3.2 ± 0.3	5.0 ± 1.2

^a Values are the means of two replicates for resistant insects and four replicates for the SC strain (using two independently labeled Cry1Ac and Cry1Aa batches).

^b SC, susceptible colony; AR, Cry1Ac-resistant colony.

^c Expressed as pmol per milligram of total vesicle protein.

The loss of MR after achieving only 17-fold resistance contrasts with reports for *H. virescens* (17, 18) and *P. gossypiella* (46, 48). However, our current results with *H. zea* agree with previous unpublished observations by at least two different laboratories. Furthermore, concurrent selection with the same parental colony (SC) resulting in moderately high and stable resistance to Cry1Ac toxin but not to MVP II further validates prior reports. Only partial cross-resistance in AR to MVP II suggests further that MVP II may not be the most effective Cry1Ac selection agent against *H. zea* considering that the Cry1Ac toxin fragment in MVP II is identical to the Cry1Ac toxin used in selection (8). This view is based on the following assumptions: (i) Cry1Ac protoxin (as in MVP II) is not the only or primary form of Cry1Ac ingested by *H. zea* when feeding on Bt cotton and (ii) the genes necessary to develop resistance to the Cry1Ac protoxin inclusion bodies (as in MVP II) were as high in the population as that for Cry1Ac activated toxin (6).

There was a 2.1- to 2.8-fold difference in toxicity between Cry1Ac activated toxin and MVP II for SC, and an ~2-fold difference would be expected after cleavage of Cry1Ac from ~130 to ~65 kDa (35). Therefore, *H. zea* (SC) does not appear to have difficulty converting protoxin to toxin, although potential difficulties could have been masked by the increased toxicity of other compounds in MVP II (sublethal toxicity to heat-treated MVP II was observed at the highest rate of MVP II tested in MR selection studies [W. J. Moar, unpublished data]). MVP II is used to determine *H. zea* susceptibility in field populations as part of the Environmental Protection Agency-mandated *B. thuringiensis* resistance monitoring program. Although the precise form and ratio of Cry1Ac toxin and protoxin in Bt cotton is uncertain, results presented here suggest that the specific methodologies used for determining *H. zea* susceptibility to Cry1Ac in the monitoring program should carefully consider the form of Cry1Ac protein used. Our results suggest that, if relatively low levels of field resistance were to evolve comparable to that which developed in AR, monitoring bioassays using MVP II might not be able to identify these resistant individuals.

Cross-resistance studies are invaluable for determining suitable insecticidal compounds for pyramiding with Cry1Ac, as well as to help determine possible resistance mechanisms. Current resistance management theory promotes the sequential or simultaneous use of different insecticidal compounds provided that cross-resistance does not occur among these different toxins (41). As also reported for other Cry1Ac-resistant insects (2, 17, 33, 45, 46, 48, 51), AR was cross-resistant to Cry1Ab. This is not unexpected because Cry1Ab and Cry1Ac toxins share >90% amino acid homology (8). However, this cross-resistance is unlikely to be related to changes in binding affinity of Cry1A toxins because no binding differences were observed between SC and AR (24). Only slight cross-resistance to Cry1Ab corn leaf powder indicates either a possible interaction of Cry protein with leaf secondary metabolites or that most or all of the Cry1Ab was only partially activated and that AR may have difficulty in proteolytically cleaving the protoxin. The fact that corn leaf material was immediately freeze-dried after harvesting and then ground into powder suggests that plant proteases might have been unable to degrade or activate Cry1Ab until after ingestion, indirectly implicating proteolysis as a potential resistance mechanism. Cry1Ab protoxin activation in

corn is further supported from a recent study that showed that corn extract partially activated Cry1Ab protoxin, suggesting that Cry1Ab protoxin is partially activated by proteases in Bt corn (31).

Lack of cross-resistance to Cry2Aa2 and Cry2Ab2 was probably due to differences in the amino acid sequence and mode of action between Cry1Ac and Cry2A (8, 9). Cry1Ac-resistant *H. virescens* (strain YHD2), *P. gossypiella*, and *H. armigera* have shown no detectable cross-resistance to Cry2A proteins (2, 17, 46, 48). Therefore, our results also confirm that the use of Cry2Ab2 pyramided with Cry1Ac (as occurs in Bollgard II) should be a viable approach for managing potential resistance to Cry1Ac. There was also no cross-resistance to Vip3A in AR. This would be expected because this protein does not share any sequence homology with Cry1Ac and is known to bind to separate receptors (13, 30, 54). Therefore, these results suggest that Vip3A would also be a valuable asset in pyramiding *B. thuringiensis* proteins for delaying *B. thuringiensis* resistance development in *H. zea* (as occurs in VipCot).

Cypermethrin was tested for cross-resistance in AR because growers often spray Bt cotton with pyrethroids when high *H. zea* populations exist, and pyrethroid oversprays are currently recommended to mitigate *H. zea* resistance to Bt cotton (21). AR was tested with cypermethrin both on untreated diet and on Cry1Ac-treated diet. The primary reasoning behind the use of Cry1Ac-treated diet was to more realistically simulate pyrethroid exposure to a potentially *B. thuringiensis*-resistant *H. zea* larva feeding on Bt cotton. Because no cross-resistance to cypermethrin was observed for larvae feeding either on untreated or on Cry1Ac-treated diet, these results suggest that pyrethroids can continue to be used when necessary and probably have been a valuable Bt cotton IRM practice since the introduction of Bt cotton in 1996.

The narrow spectrum of *B. thuringiensis* resistance suggests an alteration in the binding site of Cry1Ac (14). However, in contrast to other Cry1Ac resistant insects, we did not detect any significant reduction in binding. Lack of Cry1Ac binding has been reported in some Cry1Ac-resistant populations of *H. virescens* (23), *P. gossypiella* (36), *H. armigera* (2), *P. xylostella* (42, 47), and *T. ni* (51). Because Cry1Ac is known to bind to GalNAc residues of glycosylated membrane proteins (27), we tried to dissect Cry1Ac binding using GalNAc as an inhibitor, thus discriminating between GalNAc-dependent and GalNAc-independent binding (12). Again, we could not find any binding difference between these strains. Another way to look for binding alterations is to use different Cry1A toxins known to bind to a common receptor. Cry1Aa, Cry1Ab, and Cry1Ac share binding sites in *H. zea* (24). Although Cry1Aa has low toxicity to *H. zea*, this toxin was used in binding analyses as a diagnostic tool because it has been shown that in *H. virescens* and *Ostrinia nubilalis*, resistant insects that showed reduced or no binding of Cry1Aa to the Cry1A common receptor, it still could bind Cry1Ac (29, 43). Similar to Cry1Ac, there were no significant differences in Cry1Aa binding in terms of either dissociation constants (K_d) or concentration of binding sites (R_t) for Cry1Aa among the samples. Therefore, reduction in binding does not seem to be the mechanism of resistance in AR, in spite of the narrow spectrum of cross-resistance observed. The fact that total cross-resistance does not even extend to protoxin forms of Cry1Ac (MVP II) and Cry1Ab (Bt

corn powder) might be indicative of a differential activation of protoxin in the insect midgut, as opposed to in vitro bovine-trypsin activation (25, 38). Alternatively, the C-terminal end of the protoxin may protect the active toxin from the degradative action of midgut proteases, resulting in a higher yield of the fully active toxin (7, 32).

Results from the present study demonstrate that broad assumptions cannot be made that all target pests will respond in the same manner to a particular *B. thuringiensis* (protein or formulation). Because AR represents just a single strain, additional selections against geographically distinct *H. zea* populations are recommended to determine potential different resistance characteristics, as has been demonstrated for *H. virescens* (17, 18). Although AR is currently only ~100-fold resistant to Cry1Ac, we feel that this level of resistance is appropriate for characterization because (i) *H. zea* is 10- to 40-fold less susceptible to Cry1Ac than *H. virescens* or *P. gossypiella*; (ii) a lower level of resistance necessary to survive on Bt cotton might be expected, and lower levels of *B. thuringiensis* resistance not resulting in total survivorship on Bt cotton might be appropriate for initiating alternative control strategies; and (iii) higher levels of resistance are difficult to achieve due to logistical constraints. We have shown that *H. zea* does react differently to Cry1Ac activated toxin and MVP II than other cotton pests, and therefore this information can be used to more adequately adopt cotton IRM strategies for all target pests. Possible implications could include the following: if *H. zea* has difficulty evolving resistance to full-length or mature forms of *B. thuringiensis* proteins (as suggested for MVP II), proteins could be designed appropriately; and if resistance is not primarily due to binding differences, other potential resistance mechanisms should be explored. Our results also show that Cry1Ac-resistant *H. zea* is susceptible to Cry2Ab2 (found in Bollgard II), Vip3A (found in VipCot), and pyrethroids such as cypermethrin. These results show that the cotton-growing community has many alternative control methods to help delay the evolution of Cry1Ac (and other *B. thuringiensis* proteins) resistance for the future.

ACKNOWLEDGMENTS

We thank Nancy Adams, Monsanto Co., Union City, TN, for providing *H. zea*; Monsanto Co. for providing Cry1Ab and Cry2Ab2; Syngenta for providing Vip3A; and G. Head, Monsanto Co., St. Louis, MO, for review of the manuscript.

This research was supported by the U.S. Department of Agriculture, Cotton Incorporated, and the Spanish Ministry of Education and Science (projects AGL2003-09282-C03-01 and AGL2006-11914).

REFERENCES

- Adamczyk, J. J., Jr., D. D. Hardee, L. C. Adams, and D. V. Sumerford. 2001. Correlating differences in larval survival and development of bollworm (Lepidoptera: Noctuidae) and fall armyworm (Lepidoptera: Noctuidae) to differential expression of Cry1A(c) delta-endotoxin in various plant parts among commercial cultivars of transgenic *Bacillus thuringiensis* cotton. *J. Econ. Entomol.* **94**:284–290.
- Akhurst, R. J., W. James, L. J. Bird, and C. Beard. 2003. Resistance to the Cry1Ac delta-endotoxin of *Bacillus thuringiensis* in the cotton bollworm, *Helicoverpa armigera* (Lepidoptera: Noctuidae). *J. Econ. Entomol.* **96**:1290–1299.
- Ali, M. I., R. G. Luttrell, and S. Y. Young III. 2006. Susceptibilities of *Helicoverpa zea* and *Heliothis virescens* (Lepidoptera: Noctuidae) populations to Cry1Ac insecticidal protein. *J. Econ. Entomol.* **99**:164–175.
- Bates, S. L., J. Z. Zhao, R. T. Roush, and A. M. Shelton. 2005. Insect resistance management in GM crops: past, present and future. *Nat. Biotechnol.* **23**:57–62.
- Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**:248–254.
- Burd, A. D., F. Gould, J. R. Bradley, J. W. Van Duyn, and W. J. Moar. 2003. Estimated frequency of nonrecessive *Bacillus thuringiensis* resistance genes in bollworm, *Helicoverpa zea* (Boddie) (Lepidoptera: Noctuidae) in eastern North Carolina. *J. Econ. Entomol.* **96**:137–142.
- Choma, C. T., W. K. Surewicz, P. R. Carey, M. Pozsgay, T. Raynor, and H. Kaplan. 1990. Unusual proteolysis of the protoxin and toxin from *Bacillus thuringiensis*. *Eur. J. Biochem.* **189**:523–527.
- Crickmore, N., D. R. Zeigler, J. Feitelson, E. Schnepf, J. V. Rie, D. Lereclus, J. Baum, and D. H. Dean. 1998. Revision of the nomenclature for the *Bacillus thuringiensis* pesticidal crystal proteins. *Microbiol. Mol. Biol. Rev.* **62**:807–813.
- English, L., H. L. Robbins, M. A. Von Tersch, C. A. Kulesza, D. Ave, D. Coyle, C. S. Jany, and S. L. Slatin. 1994. Mode of action of CryIIA: a *Bacillus thuringiensis* delta-endotoxin. *Insect Biochem. Mol. Biol.* **24**:1025–1035.
- Environmental Protection Agency. 1998. The Environmental Protection Agency's white paper on Bt plant-pesticide resistance management. Publication 739-S-98-001. U.S. Environmental Protection Agency, Washington, DC.
- Environmental Protection Agency. 2001. Biopesticides registration action document: *Bacillus thuringiensis* plant incorporated protectants. U.S. Environmental Protection Agency, Washington, DC. http://www.epa.gov/pesticides/biopesticides/pips/bt_brad.htm.
- Estela, A., B. Escriche, and J. Ferré. 2004. Interaction of *Bacillus thuringiensis* toxins with larval midgut binding sites of *Helicoverpa armigera* (Lepidoptera: Noctuidae). *Appl. Environ. Microbiol.* **70**:1378–1384.
- Estruch, J. J., G. W. Warren, M. A. Mullins, G. J. Nye, J. A. Craig, and M. C. Kozel. 1996. Vip3A, a novel *Bacillus thuringiensis* vegetative insecticidal protein with a wide spectrum of activities against lepidopteran insects. *Proc. Natl. Acad. Sci. USA* **93**:5389–5394.
- Ferre, J., and J. Van Rie. 2002. Biochemistry and genetics of insect resistance to *Bacillus thuringiensis*. *Annu. Rev. Entomol.* **47**:501–533.
- Finney, D. J. 1971. Probit analysis. Cambridge University Press, London, England.
- Gao, Y., K. J. Fencil, X. Xu, D. A. Schwedler, J. R. Gilbert, and R. A. Herman. 2006. Purification and characterization of a chimeric CryIF delta-endotoxin expressed in transgenic cotton plants. *J. Agric. Food Chem.* **54**: 829–835.
- 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.
- Gould, F., A. Anderson, A. Reynolds, L. Bumgarner, and W. Moar. 1995. Selection and genetic analysis of a *Heliothis virescens* (Lepidoptera: Noctuidae) strain with high levels of resistance to *Bacillus thuringiensis* toxins. *J. Econ. Entomol.* **88**:1545–1559.
- Greenplate, J. T. 1999. Quantification of *Bacillus thuringiensis* insect control protein Cry1Ac over time in Bollgard cotton fruit and terminals. *J. Econ. Entomol.* **92**:1377–1383.
- Griffitts, J. S., and R. V. Aroian. 2005. Many roads to resistance: how invertebrates adapt to Bt toxins. *Bioessays* **27**:614–624.
- Jackson, R. E., J. R. Bradley, Jr., J. W. Van Duyn, and F. Gould. 2004. Comparative production of *Helicoverpa zea* (Lepidoptera: Noctuidae) from transgenic cotton expressing either one or two *Bacillus thuringiensis* proteins with or without insecticide oversprays. *J. Econ. Entomol.* **97**:1719–1725.
- James, C. 2006. Global status of commercialized biotech/GM crops: 2006. ISAAA brief no. 35. ISAAA, Ithaca, NY.
- Jurat-Fuentes, J. L., F. L. Gould, and M. J. Adang. 2002. Altered glycosylation of 63- and 68-kilodalton microvillar proteins in *Heliothis virescens* correlates with reduced Cry1 toxin binding, decreased pore formation, and increased resistance to *Bacillus thuringiensis* Cry1 toxins. *Appl. Environ. Microbiol.* **68**:5711–5717.
- Karim, S., S. Riazuddin, F. Gould, and D. H. Dean. 2000. Determination of receptor binding properties of *Bacillus thuringiensis* delta-endotoxins to cotton bollworm (*Helicoverpa zea*) and pink bollworm (*Pectinophora gossypiella*) midgut brush border membrane vesicles. *Pestic. Biochem. Physiol.* **67**:198–216.
- Karumbaiah, L., B. S. Oppert, J. L. Jurat-Fuentes, and M. J. Adang. 2007. Analysis of midgut proteinases from *Bacillus thuringiensis*-susceptible and -resistant *Heliothis virescens* (Lepidoptera: Noctuidae). *Comp. Biochem. Physiol.* **146**:139–146.
- Keller, M., B. Sneh, N. Strizhov, E. Prudovsky, A. Regev, C. Koncz, J. Schell, and A. Zilberstein. 1996. Digestion of delta-endotoxin by gut proteases may explain reduced sensitivity of advanced instar larvae of *Spodoptera littoralis* to CryIC. *Insect Biochem. Mol. Biol.* **26**:365–373.
- Knowles, B. H., P. J. K. Knight, and D. J. Ellar. 1991. N-acetyl galactosamine is part of the receptor in insect gut epithelia that recognizes an insecticidal protein from *Bacillus thuringiensis*. *Proc. R. Soc. London* **245**:31–35.
- Kranthi, K. R., S. Kranthi, S. Ali, and S. K. Banerjee. 2000. Resistance to "Cry1Ac delta-endotoxin of *Bacillus thuringiensis*" in a laboratory-selected strain of *Helicoverpa armigera* (Hubner). *Curr. Sci.* **78**:1001–1004.

29. Lee, M., F. Rajamohan, F. Gould, and D. H. Dean. 1995. Resistance to *Bacillus thuringiensis* CryIA delta-endotoxins in a laboratory-selected *Heliothis virescens* strain is related to receptor alteration. *Appl. Environ. Microbiol.* **61**:3836–3842.
30. Lee, M. K., P. Miles, and J. S. Chen. 2006. Brush border membrane binding properties of *Bacillus thuringiensis* Vip3A toxin to *Heliothis virescens* and *Helicoverpa zea* midguts. *Biochem. Biophys. Res. Commun.* **339**:1043–1047.
31. Li, H., L. L. Buschman, F. Huang, K. Y. Zhu, B. Bonning, and B. Oppert. Dipel-selected *Ostrinia nubilalis* larvae are not to resistant transgenic corn expressing *Bacillus thuringiensis* Cry1Ab. *J. Econ. Entomol.*, in press.
32. Lightwood, D. J., D. J. Ellar, and P. Jarrett. 2000. Role of proteolysis in determining potency of *Bacillus thuringiensis* Cry1Ac δ -endotoxin. *Appl. Environ. Microbiol.* **66**:5174–5181.
33. Luttrell, R. G., L. Wan, and K. Knighten. 1999. Variation in susceptibility of noctuid (Lepidoptera) larvae attacking cotton and soybean to purified endotoxin proteins and commercial formulations of *Bacillus thuringiensis*. *J. Econ. Entomol.* **92**:21–32.
34. Moar, W. J., J. T. Trumble, R. H. Hice, and P. A. Backman. 1994. Insecticidal activity of the CryIIA protein from the NRD-12 isolate of *Bacillus thuringiensis* subsp. *kurstaki* expressed in *Escherichia coli* and *Bacillus thuringiensis* and in a leaf-colonizing strain of *Bacillus cereus*. *Appl. Environ. Microbiol.* **60**:896–902.
35. Moar, W. J., M. Puszta-Carey, H. V. 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.
36. Morin, S., R. W. Biggs, M. S. Sisterson, L. Shriver, C. Ellers-Kirk, D. Higginson, D. Holley, L. J. Gahan, D. G. Heckel, Y. Carriere, T. J. Dennehy, J. K. Brown, and B. E. Tabashnik. 2003. Three cadherin alleles associated with resistance to *Bacillus thuringiensis* in pink bollworm. *Proc. Natl. Acad. Sci. USA* **100**:5004–5009.
37. Munson, P. J., and D. Rodbard. 1980. LIGAND: a versatile computerized approach for characterization of ligand-binding systems. *Anal. Biochem.* **107**:220–239.
38. Oppert, B., K. J. Kramer, D. E. Johnson, S. C. MacIntosh, and W. H. McGaughey. 1994. Altered protoxin activation by midgut enzymes from a *Bacillus thuringiensis* resistant strain of *Plodia interpunctella*. *Biochem. Biophys. Res. Commun.* **198**:940–947.
39. Perlak, F. J., W. R. Deaton, T. A. Armstrong, R. I. Fuchs, S. R. Sims, J. T. Greenplate, and D. A. Fishhoff. 1990. Insect resistant cotton plants. *Bio/Technology* **8**:939–943.
40. Puszta-Carey, M., P. Carey, T. Lessard, and M. Yaguchi. October 1994. Isolation, quantitation and purification of insecticidal proteins from *Bacillus thuringiensis*. U.S. patent 5,356,788.
41. Roush, R. T. 1998. Two-toxin strategies for management of insecticidal transgenic crops: can pyramiding succeed where pesticide mixtures have not? *Philos. Trans. R. Soc. London* **353**:1777–1786.
42. Sayyed, A. H., R. Haward, S. Herrero, J. Ferré, and D. J. Wright. 2000. Genetic and biochemical approach for characterization of resistance to *Bacillus thuringiensis* toxin Cry1Ac in a field population of the diamondback moth, *Plutella xylostella*. *Appl. Environ. Microbiol.* **66**:1509–1516.
43. Siqueira, H. A. A., J. González-Cabrera, J. Ferré, R. Flannagan, and B. D. Siegfried. 2006. Analyses of Cry1Ab binding in resistant and susceptible strains of the European corn borer, *Ostrinia nubilalis* (Hubner) (Lepidoptera: Crambidae). *Appl. Environ. Microbiol.* **72**:5318–5324.
44. SPSS. 2006. SPSS base 14.0 for Windows user's guide. SPSS, Inc., Chicago, IL.
45. Tabashnik, B. E., Y. Carriere, T. J. Dennehy, S. Morin, M. A. Sisterson, R. T. Roush, A. M. Shelton, and J. Z. Zhao. 2003. Insect resistance to transgenic Bt crops: lessons from the laboratory and field. *J. Econ. Entomol.* **96**:1031–1038.
46. Tabashnik, B. E., T. J. Dennehy, M. A. Sims, K. Larkin, G. P. Head, W. J. Moar, and Y. Carriere. 2002. Control of resistant pink bollworm (*Pectinophora gossypiella*) by transgenic cotton that produces *Bacillus thuringiensis* toxin Cry2Ab. *Appl. Environ. Microbiol.* **68**:3790–3794.
47. Tabashnik, B. E., Y. B. Liu, T. Malvar, D. G. Heckel, L. Masson, V. Ballester, F. Granero, J. L. Mensua, and J. Ferre. 1997. Global variation in the genetic and biochemical basis of diamondback moth resistance to *Bacillus thuringiensis*. *Proc. Natl. Acad. Sci. USA* **94**:12780–12785.
48. Tabashnik, B. E., Y. Liu, R. A. de Maagd, and T. J. Dennehy. 2000. Cross-resistance of pink bollworm (*Pectinophora gossypiella*) to *Bacillus thuringiensis* toxins. *Appl. Environ. Microbiol.* **66**:4582–4584.
49. Usmani, K. A., and C. O. Knowles. 2001. Toxicity of pyrethroids and effect of synergists to larval and adult *Helicoverpa zea*, *Spodoptera frugiperda*, and *Agrotis ipsilon* (Lepidoptera: Noctuidae). *J. Econ. Entomol.* **94**:868–873.
50. 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.
51. Wang, P., J.-Z. Zhao, A. Rodrigo-Simón, W. Kain, A. F. Janmaat, A. M. Shleton, J. Ferré, and J. Myers. 2007. Mechanism of resistance to *Bacillus thuringiensis* toxin Cry1Ac in greenhouse population of cabbage looper, *Trichoplusia ni*. *Appl. Environ. Microbiol.* **73**:1199–1207.
52. Wolfersberger, M. G., P. Luethy, P. Maurer, P. Parenti, V. F. Sacchi, B. Giordana, and G. M. Hanozet. 1987. Preparation and partial characterization of amino acid transporting brush border membrane vesicles from the larval midgut of the cabbage butterfly (*Pieris brassicae*). *Comp. Biochem. Physiol.* **86A**:301–308.
53. Xu, X., L. Yu, and Y. Wu. 2005. Disruption of a cadherin gene associated with resistance to Cry1Ac delta-endotoxin of *Bacillus thuringiensis* in *Helicoverpa armigera*. *Appl. Environ. Microbiol.* **71**:948–954.
54. Yu, C., M. A. Mullins, G. W. Warren, M. G. Koziel, and J. J. Estruch. 1997. The *Bacillus thuringiensis* vegetative insecticidal protein Vip3A lyses midgut epithelium cells of susceptible insects. *Appl. Environ. Microbiol.* **63**:532–536.