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Cross-Resistance of Cry1Ab-Selected *Ostrinia nubilalis* (Lepidoptera: Crambidae) to *Bacillus thuringiensis* δ-Endotoxins

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ABSTRACT Corn plants expressing the toxin from *Bacillus thuringiensis* (Berliner) have proven to be effective in controlling lepidopteran pests such as the European corn borer, *Ostrinia nubilalis* (Hübner) (Lepidoptera: Crambidae). Several Bt toxins are being tested and incorporated into crop genomes, although tests for cross-resistance among different toxins have been limited by a lack of resistant colonies. Four different colonies of *O. nubilalis* selected with full-length Cry1Ab incorporated into artificial diet developed significant levels of resistance (2.0- to 10-fold) within 10 generations. Additionally, selection with Cry1Ab resulted in decreased susceptibility to a number of other toxins to which the selected colonies were not previously exposed. Significantly, levels of resistance were highest to Cry1Ac with resistance ratios up to 51.0-fold. Low levels (less than five-fold) of cross-resistance were detected with Cry1F. In contrast, Cry9C susceptibility was unaffected by selection with Cry1Ab. These results indicate that the availability of multiple toxins could improve resistance management strategies, provided cross-resistance among toxins is not a factor.

KEY WORDS European corn borer, insecticide, growth inhibition, mortality, transgenic corn

THE USE OF BIOLOGICAL INSECTICIDES has undergone a major revival in recent years (Watkinson and Milner 1994). Products based on toxins from the soil microorganism *Bacillus thuringiensis* (Berliner) (Bt) have been developed for controlling a variety of pest species. Products derived from Bt are the most widely used microbial insecticides and have been shown to be highly toxic to certain insects with no known adverse effect to humans, beneficial insects, and other nontarget organisms. Gene transfer technology has permitted the insertion and expression of various Bt toxin genes in the plant genome (Chilton et al. 1993), thereby providing a new approach for pest control, and transgenic Bt crops were initially introduced into the market in United States in 1996 (Estruch et al. 1997). Bt genes encoding Cry1Ab, Cry9C, Cry1Ac, and Cry1F proteins in transgenic maize, *Zea mays* L. (referred to as Bt corn), have provided an effective means to control lepidopteran pests such as the European corn borer, *Ostrinia nubilalis* (Hübner) (Koziel et al. 1993, Fischoff 1996).

Although these altered plants provide an important pest control alternative to chemical insecticides, there is concern that their widespread use could lead to rapid evolution of resistance among target pest species. The diamondback moth, *Plutella xylostella* (L.), is the only lepidopteran species that has evolved resistance to Bt toxins in the field after repeated use of formulated Bt insecticides (Kirsch and Schmutterer 1988, Tabashnik et al. 1990, Kao and Cheng 1994, Tabashnik 1994). Resistance to Bt and its toxins also has been reported from selection studies in laboratory populations of several insect species (for reviews, see Bauer 1995, Schnepf et al. 1998, Frutos et al. 1999, Ferre and Van Rie 2002), suggesting that pest insects can develop resistance to Bt toxins.

Cry1Ab-resistant (>200-fold) *P. xylostella* were not cross-resistant to Cry1B and Cry1C (Ferre et al. 1991). Similarly, high level of resistance (>500-fold) in *Spodoptera littoralis* (Boisduval) to Cry1C (Muller et al. 1996) did not confer cross-resistance to Cry1F, and *P. xylostella* with >240-fold resistance to Cry1F were not cross-resistant to Cry1B (Tabashnik et al. 1994). If these resistance specificities (i.e., resistance to a single toxin) occur in field, resistance management strategies could incorporate alternating or stacking of multiple toxins that are unaffected by cross-resistance.

Despite many years of using Bt products in the field for control of *O. nubilalis*, baseline studies with field populations *O. nubilalis* provided no indication of evolved resistance to Cry1Ab or Cry1Ac (Siegfried et al. 1995, Marçon et al. 1999, Tabashnik et al. 2003). Nevertheless, *O. nubilalis* has evolved low levels of resistance (14-fold) to Cry1Ab after a relatively few generations of selection in the laboratory under chronic exposure (Chaufaux et al. 2001). Additionally, resistance of *O. nubilalis* to Bt toxins was reported from laboratory-selected colonies with the Bt formulation Dipel ES (73-fold) (Huang et al. 1997) and the

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MVP formulation of Cry1Ac expressed in *Pseudomonas fluorescens* Migula (162-fold) (Bolin et al. 1999) after seven and eight generations of selection, respectively. Resistance in the Dipel ES-selected strain was inherited as an incompletely dominant autosomal trait (Huang et al. 1999a) and was apparently related to decreased proteolytic activation of toxin in the midgut (Huang et al. 1999b). However, Dipel ES comprises five different toxins, and it is likely that multiple factors contributed to resistance.

Proposed resistance management strategies for Bt corn have included the potential deployment of multiple insecticides in mixtures or in sequence, provided different toxins can be identified that are not affected by cross-resistance. Identifying cross-resistance patterns is an important tool not only for identifying resistance mechanisms but also for determining the effect of a given resistance mechanism on other toxins (Scott 1990). To date, cross-resistance in laboratory-selected *O. nubilalis* colonies was reported by Bolin et al. (1999) where a Cry1Ac-selected strain showed a low level of cross-resistance to Cry1Ab, suggesting that the two toxins share a common resistance mechanism. Denolf et al. (1993) reported that Cry1Ab and Cry1Ac share a common receptor in *O. nubilalis*, although Cry1Ab seems to bind to a second receptor not shared with the Cry1Ac toxin (Hua et al. 2001). Therefore, Cry1Ac-selected strains would still show some susceptibility to Cry1Ab, which may explain the low level of cross-resistance to Cry1Ab observed in the Cry1Ac-selected strain.

The identification of novel Bt toxins for transgenic plants could provide an important resistance management tool provided cross-resistance does not affect efficacy of the novel toxin. The cross-resistance patterns of Cry1Ab-selected colonies of *O. nubilalis* would provide important information for potential replacement of Cry1Ab-corn events, as well insight into potential resistance mechanisms that may evolve in *O. nubilalis* populations. Therefore, the current study was conducted to assess cross-resistance patterns among various Bt toxins in Cry1Ab laboratory-selected colonies of *O. nubilalis.*

Materials and Methods

Insect Rearing. Rearing procedures for European corn borer were based on those developed at the USDA–ARS Corn Insects Research Unit, Ames, IA (Guthrie et al. 1965). Larvae were reared at 27 ± 0.7°C with a photoperiod of 24:0 (L:D) h and 80% RH on a wheat germ-based diet (Lewis and Lynch 1969). Insects were moved into mating cages as pupae where adults were maintained with 8-h scotophase at 18 ± 0.7°C and 16-h photophase at 27 ± 0.7°C with 80% RH. Cages were misted with water twice daily, and adult diet was provided to maximize egg production (Leahy and Andow 1994). Egg masses were collected and incubated within plastic petri dishes containing moistened filter paper until larval hatch.

***O. nubilalis* Strains.** Six strains of *O. nubilalis* designated Europe-S, Europe-R, Nebraska-S, Nebraska-R, Iowa-R, and RSTT-R were established in the laboratory. The European strains originated from a field collection of ≈500 individuals from the Lombardia region of northern Italy. This population was provided to the University of Nebraska after 20 generations of laboratory rearing and was divided into two subpopulations, one exposed throughout larval development to Cry1Ab toxin and the other reared in the absence of toxin (control). The same procedure was applied to Nebraska population. The Iowa-R strain was previously selected by chronic exposure to Cry1Ab (Lang et al. 1996) and provided by Pioneer Hi-Bred International (Johnston, IA). The RSTT-R strain resulted from a combination of individuals from both the Nebraska- (F_{10}) and Europe (F_{28})-selected strains. All the resistant strains were selected with full-length Cry1Ab toxin for at least 40 generations.

Selection. A fermentation paste of *B. thuringiensis* subspecies *kurstaki* strain HD1–9 containing ≈9.5% Cry1Ab toxin by weight was used as a source of Cry1Ab for the selection diet (Novartis Seeds, Research Triangle Park, NC). Selected strains were initially exposed throughout larval development to Cry1Ab in the rearing diet (0.2 μg/ml of diet). The toxin concentration was steadily increased in succeeding generations to target 70% mortality in the exposed insects. At each generation, five replicates of 300 neonates per rearing container were initiated for each generation for control strains, and five replicates of 1000 neonates were initiated for selected strains. Upon pupation, the total number of pupae in three randomly selected replicates of both control and selected strains were counted to estimate percentage of survival from neonate larvae to pupation. Larvae were maintained at 27°C, a photoperiod of 24:0 (L:D) h, and 80% RH. After pupation, insects were transferred to mating cages, and adults were maintained as described previously.

Bioassays. Bioassays were conducted with six different Bt toxins. Full-length Cry1Ab was purified from *B. thuringiensis kurstaki* strain HD1–9, which produces only Cry1Ab protein (Carlton and Gonzales 1985) (provided by Syngenta Seeds, Research Triangle Park, NC). The crystal protein preparation was obtained by density gradient centrifugation of fermentation products and contained ≈98% crystal protein as determined by phase contrast microscopy. Trypsin activated Cry1Ab and Cry1Ac were provided by the Monsanto Company (St. Louis, MO) and purified from solubilized full-length toxins, which were treated with bovine pancreatic trypsin until ≈90% was converted to the trypsin-resistant core protein. The Cry1 F protein was provide by Dow AgroSciences (Indianapolis, IN) and was produced through fermentation of recombinant *P. fluorescens*, strain MR872. At completion of fermentation, the *P. fluorescens* cell walls were enzymatically lysed, and the Cry1F crystals were separated and purified. After digestion to the trypsin-resistant core, the truncated Cry1F was purified by ion exchange chromatography. Cry9C toxin was provided by Agrevo Group (Gent, Belgium) and was produced

from a Cry-free *B. thuringiensis* ssp. Berliner strain 1715 transformed with plasmid pG19CK containing the Cry9C gene. After expression of the protoxin, active toxin was obtained by trypsin treatment. All toxin preparations were purified to >90% purity based on information provided by suppliers.

The bioassay method described by Marçon et al. (1999) was used to assess toxicity for all previously described toxins. Neonates (<24 h after eclosing) were exposed to purified Bt toxins overlaid onto the surface of artificial diet. The diet developed for *Heliothis virescens* (F.) (King et al. 1985) was used in all bioassays. One milliliter of diet was dispensed into each well of 128-wells trays (each well 16 mm in diameter, 16 mm in height; CD International, Pitman, NJ) and allowed to solidify. Seven concentrations of each toxin were prepared in 0.1% Triton X-100 nonionic detergent to obtain uniform spreading onto the diet surface. Each well was treated with 30 μl of the appropriate solution, and controls were treated with 0.1% Triton X-100 only. After the diet was completely dry, a single neonate (<24 h after eclosing) was placed in each well and then covered with a vented lid. Trays were held in a growth chamber for 7 d at 27°C, 24-h scotophase, and 80% RH. Mortality and larval weights were recorded after 7 d. Larvae that had not grown beyond first instar and weighing ≤0.1 mg were considered dead. Thus, the criterion for mortality used in this study accounted for both severe growth inhibition and death.

Statistical Analysis. Probit analysis (Finney 1971) was conducted using the POLO-PC statistical software (LeOra Software 1987). A likelihood ratio test for parallelism as well as resistance ratio (i.e., LC_{50} ratios) comparisons were performed according to Robertson and Preisler (1992). Resistance ratios were considered to be significant ($P < 0.05$) when the confidence limits did not include the value one. The EC_{50} values (the estimated concentration of Cry1Ab that would cause 50% growth inhibition) and 95% fiducial limits (FL) were determined by nonlinear regression using PROC NLIN (SAS Institute 1999) fitted to a probit model with numerical derivatives as described by Marçon et al. (1999). An F test ($P < 0.05$) was used to determine whether the parameters of the nonlinear probit model differed significantly between the control and selected strains.

Results

Estimates of mortality throughout selection experiments and across generations indicated that *O. nubilalis* reared in the presence of Bt-Cry1Ab protoxin exhibited consistently higher mortality (50–80%) than control strains (20–40%) (Fig. 1). The average percentage of mortality among the selected colonies varied between 58 and 63%, whereas the unselected Europe and Nebraska control strains average 27 and 30% mortality, respectively, across all generations. The mortality differences between control and selected strains indicated that selection pressure was applied through chronic exposure during larval development, although it was difficult to estimate the intensity of selection due to significant mortality in the unselected strains. Nevertheless, the intensity of selection increased with increasing Cry1Ab concentration in almost every generation (Fig. 1).

Concentration–mortality regression lines obtained by probit analysis and nonlinear regression of growth inhibition are presented in Tables 1 and 2, respectively. Increased tolerance to full-length Cry1Ab was observed among all the selected colonies (Table 1). LC_{50} values ranged from 3.65 ng/cm^2 (Europe-S) to 35.7 ng/cm^2 (Europe-R). Among the selected strains, Europe-R and RSTT-R exhibited the highest resistance compared with the unselected Europe-S strain. Lower levels of resistance were observed in the selected Nebraska-R and Iowa-R strains compared with the unselected Nebraska-S strain. EC_{50} values were generally lower than the corresponding LC_{50}, although a similar pattern of susceptibility was observed (Table 2). EC_{50} values for Cry1Ab protoxin ranged from 0.41 ng/cm^2 for the unselected Europe strain to 13.0 ng/cm^2 for the selected RSTT-R strain.

Although selections for all strains were conducted with full-length Cry1Ab, bioassays results suggest that cross-resistance exists to toxins for which the strains were not previously exposed. All selected strains tolerated consistently higher concentrations of Cry1Ac based on both growth inhibition and mortality (Table 1 and 2; Fig. 2). Additionally, increased tolerance was observed to Cry1F (Table 1; Fig. 2) in the Europe-R, RSTT-R, and Iowa-R strains.

Interestingly, trypsin activated-Cry1Ab toxin was generally more toxic to the control strains compared with full-length Cry1Ab and less toxic to selected strains, resulting in generally higher resistance levels for the truncated toxin. The selected Europe-R and RSTT-R strains showed strong resistance to trypsin-activated Cry1Ab and cross-resistance to Cry1Ac, low cross-resistance to Cry1F, and very low cross-resistance to Cry9C. Although the selected Iowa-R strain was not significantly different from the respective control for full-length Cry1Ab, significant resistance to truncated Cry1Ab, Cry1Ac, and Cry1F was observed. The selected Nebraska-R strain also showed cross-resistance to truncated Cry1Ab and Cry1Ac, although a lower magnitude of resistance was observed relative to the selected Europe-R and RSTT-R strains, and there was no indication of cross-resistance to Cry1 F and Cry9C. Growth inhibition cross-resistance data were generally comparable with mortality data (Table 2), although both the selected Europe-R and RSTT-R strains exhibited a slightly higher resistance level for Cry1Ac when mortality data were analyzed.

Discussion

Since Bt-resistance in insects was first reported by McGaughey (1985), there has been considerable effort to select insect populations for resistance to Bt in the laboratory. At least 16 major insect pests have been selected in >50 laboratory experiments (Tabashnik 1994). Our efforts to select for resistance to Cry1Ab in

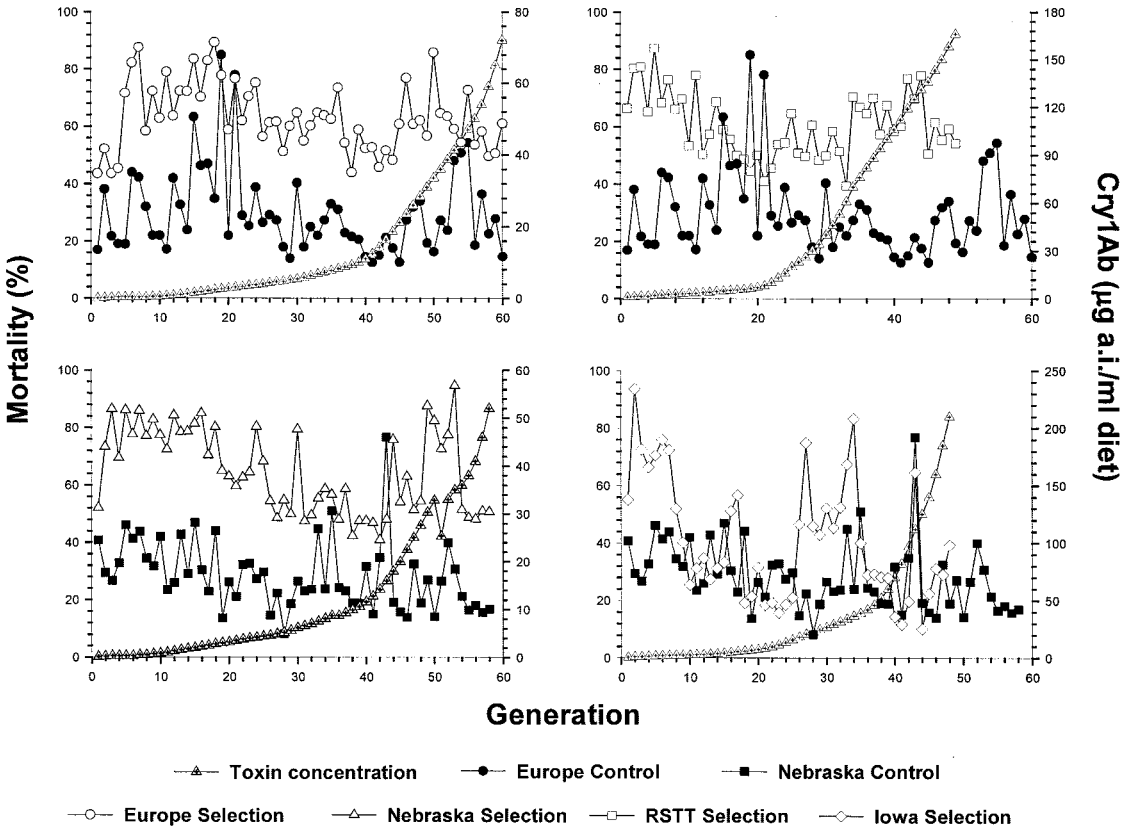

Fig. 1. Comparison of *O. nubilalis* mortality of selected strains on Cry1Ab-treated rearing diet versus the control strains maintained on normal rearing diet. $n = 900$ larvae reared in each control colony and 3,000 larvae were exposed to Cry1Ab incorporated into rearing diet at increasing concentrations. Mortality represents the percentage of neonates that did not pupate.

O. nubilalis have resulted in increased tolerance to Cry1Ab after relatively few generations of selection (Chaufaux et al. 2001). Bioassays of susceptibility indicate that *O. nubilalis* populations developed Cry1Ab resistance levels up to ≈10-fold at the time the bioassays were conducted. Despite the higher sensitivity of growth inhibition analysis to detect sublethal effects, the levels of resistance were similar among the strains and toxins at both LC_{50} and EC_{50} values. Although these bioassays were initiated to compare toxicity of Cry1Ab protoxin and other Bt toxins, trypsin-activated Cry1Ab was also tested in the study. Interestingly, the selected *O. nubilalis* strains exhibited higher levels of resistance to the activated Cry1Ab compared with the Cry1Ab protoxin. Differences in resistance levels suggest either differences in the receptor-binding interactions between protoxin and toxin or differences in proteolytic activation, detoxification, or both either of which could be linked to resistance. Activity of protease enzymes may account for the differences in toxicity between Cry1Ab toxin and Cry1Ab protoxin. Because bovine trypsin was used to activate the Cry1Ab protoxin, it is likely that the product of toxin cleavage is different from that which occurs through the activity of insect proteases. Oppert et al. (1994) observed a slightly larger Cry1Ac (63 kDa) activated with porcine trypsin compared with *Plodia interpunctella* (Hübner) gut extracts (61 kDa). Also, differences in processing of Cry1Ac by bovine trypsin and gut juice from *Choristoneura fumiferana* Clemens were previously reported by Milne and Kaplan (1993). These reports and results observed in the present work may suggest that differences in susceptibility between protoxin and trypsinized Cry1Ab are related to different properties or specificities of insect versus mammalian proteases (Miranda et al. 2001).

Results of this investigation are consistent with other *O. nubilalis* selection experiments (Huang et al. 1997, Bolin et al. 1999) in which exposure to Bt toxins has lead to increased levels of tolerance, although the levels of resistance to the selective agent (Cry1Ab protoxin) were generally lower than in the previous selection experiments. Lower levels of resistance in the present investigation may be related to the use of combined mortality and growth inhibition to estimate susceptibility. In both the Huang et al. (1997) and Bolin et al. (1999) studies, growth inhibition was not measured. That both the mortality and growth inhibition data provided similar resistance levels in the

Table 1. Susceptibility of European corn borer populations to Bt toxins

Toxin	Population	Gen[a]	*n*	Slope ± SE[b]	LC_{50} (95% FL)[c]	LC_{95} (95% FL)[c]	χ^{2d}	RR (95% FL)[ef]
Cry1Ab	Europe-S	69	336	4.98 ± 0.82a	3.65 (3.07–4.19)	7.80 (6.44–10.89)	2.15	—
	Europe-R	69	318	3.14 ± 0.30a	35.7 (28.4–46.0)	119 (81–247)	9.67	9.8 (8.0–11.9)
	RSTT-R	41	240	2.92 ± 0.40b	32.9 (26.8–39.4)	120.0 (89.3–193.0)	0.87	9.0 (7.1–11.5)
	Nebraska-S	52	335	4.02 ± 0.38a	12.14 (10.88–13.49)	31.15 (26.35–39.19)	2.14	—
	Nebraska-R	50	331	2.66 ± 0.40b	23.00 (14.25–31.61)	95.41 (62.12–245.87)	6.58	1.9 (1.5–2.5)
	Iowa-R	61	335	4.06 ± 0.45a	14.4 (12.75–16.07)	36.7 (30.95–46.79)	2.78	1.2 (1.0–1.4)
Trc1Ab	Europe-S	69	336	3.38 ± 0.35a	2.33 (1.86–2.91)	7.16 (5.21–12.18)	6.17	—
	Europe-R	69	336	4.87 ± 0.72a	90.6 (66.4–108.9)	197.4 (156.2–338.0)	7.54	38.9 (31.8–47.5)
	RSTT-R	41	336	2.37 ± 0.49b	74.8 (52.8–96.5)	370.3 (237.0–975.8)	1.41	32.1 (23.4–44.0)
	Nebraska-S	50	333	2.91 ± 0.39b	4.18 (3.29–5.05)	15.35 (11.83–22.86)	1.85	—
	Nebraska-R	50	240	4.43 ± 1.01a	37.17 (not available)	87.38 (not available)	3.61	8.9 (6.6–12.0)
	Iowa-R	61	336	3.86 ± 0.51a	40.7 (33.3–47.3)	108.6 (90.1–144.0)	3.81	9.7 (7.4–12.8)
Cry1Ac	Europe-S	69	336	3.98 ± 0.55a	12.98 (9.46–16.51)	33.62 (24.84–61.36)	6.51	—
	Europe-R	69	288	1.76 ± 0.20b	459 (336–661)	3970 (1818–23058)	6.84	35.4 (26.5–47.1)
	RSTT-R	41	240	2.30 ± 0.66b	683 (533–989)	3558 (1746–40874)	2.50	52.6 (36.4–76.0)
	Nebraska-S	50	288	3.04 ± 0.29a	17.43 (12.83–23.77)	60.69 (39.99–133.35)	7.09	—
	Nebraska-R	50	335	3.01 ± 0.45a	125.0 (82.33–167.0)	440.8 (299–1031)	6.37	7.2 (5.5–9.4)
	Iowa-R	61	336	2.69 ± 0.35a	93.9 (61.4–127.2)	384 (260–798)	6.35	5.4 (4.1–7.1)
Cry1F	Europe-S	69	336	4.24 ± 0.69a	7.45 (6.08–8.70)	18.23 (14.73–26.33)	4.47	—
	Europe-R	69	191	4.05 ± 0.53a	34.75 (29.63–40.19)	88.50 (71.37–123.37)	0.13	4.7 (3.7–5.9)
	RSTT-R	41	239	3.49 ± 0.48a	25.6 (20.7–30.5)	75.9 (59.8–110.2)	2.10	3.4 (2.7–4.4)
	Nebraska-S	50	336	3.03 ± 0.34a	7.92 (6.59–9.37)	27.6 (21.5–39.7)	2.53	—
	Nebraska-R	50	336	3.27 ± 0.47a	7.53 (5.85–9.14)	23.95 (18.68–35.37)	0.70	1.0 (0.7–1.3)
	Iowa-R	61	286	2.87 ± 0.28a	29.05 (24.72–34.16)	108.8 (84.1–155.3)	3.64	3.7 (2.9–4.7)
Cry9C	Europe-S	70	335	3.17 ± 0.33a	79.33 (62.51–97.21)	262.4 (195.9–425.1)	6.11	—
	Europe-R	69	288	3.52 ± 0.43a	162 (120–242)	476 (298–1558)	7.73	2.0 (1.6–2.5)
	RSTT-R	41	288	4.34 ± 0.68a	129 (109–149)	309 (248–450)	1.13	1.6 (1.3–2.0)
	Nebraska-S	52	335	3.53 ± 0.37a	49.3 (42.7–55.9)	144.1 (119.6–187.5)	2.16	—
	Nebraska-R	52	334	2.62 ± 0.28a	23.1 (19.4–26.9)	98.1 (75.4–143.5)	0.84	0.5 (0.4–0.6)
	Iowa-R	61	240	3.12 ± 0.40a	60.6 (38.8–83.9)	203.8 (134–533)	3.82	1.2 (1.0–1.5)

[a] Number of generations of selection with Cry1Ab protoxin.

[b] Values followed by the same letter within a column are not significantly different ($P \geq 0.05$). Significance of differences among slopes determined by likelihood ratio test of equality followed by pairwise comparisons with nonoverlapping fiducial limits (Robertson and Priesler 1992).

[c] Nanograms of Bt toxin/cm^2 of treated artificial diet surface.

[d] Chi square significant ($P < 0.05$).

[e] Resistance ratio; ratio of the LC_{50} values between selected colonies and the nonselected colony, calculated using the method of Robertson and Preisler (1992).

[f] Fiducial limits (95%) for the lethal concentration ratio. If the interval contained the value 1.0, the LC_{50} values were not significantly different.

Table 2. Growth inhibition of European corn borer populations to Bt toxins

Toxin	Population	Gen[a]	*n*	EC_{50} (95% FL)[b]	EC_{50} (95% FL)[b]	EC_{99} (95% FL)[b]	RR[c]
Cry1Ab	Europe-S	69	336	0.41 (0.00–0.53)	5.73 (2.95–10.26)	25.08 (6.96–54.76)	—
	Europe-R	70	336	2.44 (1.87–3.04)	47.21 (23.40–88.31)	248.61 (63.55–578.06)	6.0[d]
	RSTT-R	41	336	5.33 (3.19–8.25)	126.68 (34.00–378.76)	748.03 (59.26–2920.87)	13.0[d]
	Nebraska-S	50	336	2.00 (0.00–2.66)	19.37 (8.22–39.24)	69.24 (13.28–168.35)	—
	Nebraska-R	50	336	5.30 (3.05–8.51)	93.90 (24.66–281.70)	471 (38–1805)	2.7[d]
	Iowa-R	61	336	5.31 (3.72–7.41)	56.52 (21.71–122.69)	212.6 (34.2–558.5)	2.7[d]
Trc1Ab	Europe-S	70	336	0.17 (0.16–0.18)	1.10 (0.94–1.28)	3.13 (2.42–3.88)	—
	Europe-R	70	336	9.52 (8.56–10.58)	104.37 (75.68–139.34)	399.59 (231.57–595.65)	56.0[d]
	RSTT-R	41	336	10.29 (7.49–13.74)	159.22 (65.09–336.06)	739.29 (131.43–1925.83)	60.5[d]
	Nebraska-S	50	336	0.37 (0.34–0.39)	2.07 (1.94–2.21)	5.46 (4.75–6.20)	—
	Nebraska-R	50	336	4.18 (3.33–5.01)	51.40 (29.36–85.55)	209.8 (70.0–420.8)	11.3[d]
	Iowa-R	61	336	4.38 (3.30–5.45)	115.98 (59.55–214.94)	728.1 (196.7–1714.7)	11.8[d]
Cry1Ac	Europe-S	69	336	1.36 (1.26–1.44)	5.96 (5.70–6.24)	13.66 (12.26–15.13)	—
	Europe-R	69	336	8.71 (5.88–12.55)	96.61 (34.57–221.31)	372.16 (52.79–1037.38)	6.4[d]
	RSTT-R	41	336	14.35 (11.81–16.91)	1345 (858–2084)	17157 (7428–33415)	10.6[d]
	Nebraska-S	50	336	1.17 (1.09–1.24)	5.11 (4.65–5.63)	11.69 (9.70–13.74)	—
	Nebraska-R	50	336	11.34 (8.93–13.76)	151 (82–260)	644 (197–1347)	9.7[d]
	Iowa-R	61	336	13.87 (10.13–17.83)	253.6 (114.1–513.3)	1293 (268–3288)	11.9[d]
Cry1F	Europe-S	69	336	0.77 (0.66–0.88)	6.42 (4.42–9.12)	21.01 (10.14–34.60)	—
	Europe-R	70	336	3.84 (2.63–5.28)	66.80 (24.80–154.97)	331.09 (47.64–969.55)	5.0[d]
	RSTT-R	43	336	4.13 (2.47–6.34)	54.49 (16.41–146.78)	231.4 (23.84–778.66)	5.4[d]
	Nebraska-S	50	336	0.86 (0.81–0.90)	5.48 (4.72–6.33)	15.54 (11.96–19.29)	—
	Nebraska-R	52	336	0.30 (0.24–0.35)	3.32 (2.28–4.80)	12.84 (6.00–22.16)	0.4[d]
	Iowa-R	61	336	2.78 (2.48–3.08)	32.67 (23.55–44.22)	130.13 (72.61–200.07)	3.2[d]
Cry9C	Europe-S	69	336	23.77 (18.3–31.32)	952 (359–2158)	7535 (1211–21859)	—
	Europe-R	69	336	26.17 (20.81–32.76)	377.2 (188.2–676.2)	1683 (460–3610)	1.1
	RSTT-R	41	336	17.75 (13.22–23.32)	315 (133–648)	1576 (303–4012)	0.8
	Nebraska-S	50	336	27.83 (21.54–36.77)	1578 (556–3775)	15172 (2183–47185)	—
	Nebraska-R	50	336	26.66 (16.31–47.68)	2797 (376–14020)	37973 (830–274700)	1.0
	Iowa-R	61	336	19.80 (13.22–29.94)	315.8 (96.4–808.7)	1492 (158–4753)	0.7

[a] Number of generations of selection with Cry1Ab protoxin.
[b] Nanograms of Bt toxin/cm^2 of treated artificial diet surface.
[c] Resistance ratio; ratio of the LC_{50} values between selected colonies and the nonselected colony, calculated using the method of Robertson and Preisler (1992).
[d] Significant difference ($P < 0.05$) between control and selected populations based on F-test to determine differences among parameters of the nonlinear probit model.

current study suggest that these data reflect the actual resistance levels.

Huang et al. (1997) reported resistance to the commercial formulation Dipel ES up to 73-fold after three to seven generations. Because Dipel ES comprises multiple Bt endotoxins, including Cry1Aa, Cry1Ab, Cry1Ac, Cry2A, and Cry2B, it is unclear which component was the most important in the selection, or whether an additive effect of multiple toxins contributed to the overall resistance. Additionally, cross-resistance studies with strains selected with multiple toxins could be compromised because of the likelihood that multiple factors contribute to resistance. Unlike the results reported with Dipel selection, Bolin

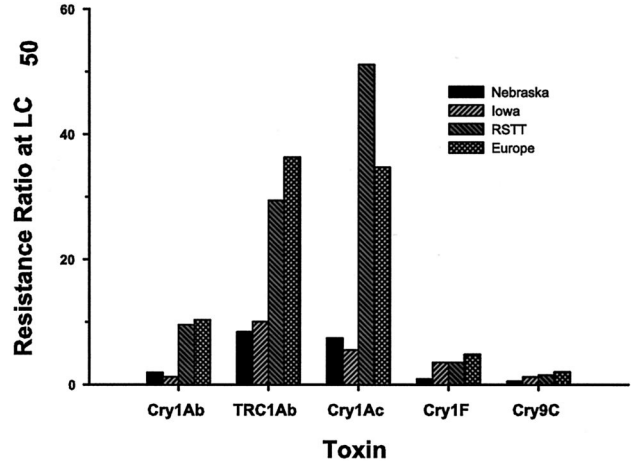

Fig. 2. Resistance ratios of selected strains of European corn borer for various Bt toxins.

et al. (1999) used a single toxin (Cry1Ac) to select for resistance and reported a resistance ratio up to 162-fold after eight generations of selection, although the resistance level declined rapidly in the absence of selection. Additionally, exposure to Cry1Ac in the former study was only for a short period during early larval development so that the intensity of selection was likely to be much higher than in the current study. The relatively low level of resistance observed in the Cry1Ab-selected colonies might be due to moderate selection pressure (≈60% overall mortality) that was achieved by chronic exposure throughout larval development.

The results of laboratory selections reported here are unlikely to reflect resistance mechanisms to evolve under field conditions because the conditions of exposure and intensity of selection are fundamentally different. Although these strains exhibit increased feeding on Cry1Ab expressing corn tissue, they do not complete development on transgenic plants (B.D.S.. unpublished). However, the spectrum of cross-resistance exhibited by these strains provides a preliminary assessment of potential resistance mechanisms and compatibility among different toxins. Apart from the low levels of cross-resistance to Cry1Ab in Cry1Ac selected *O. nubilalis* (Bolin et al. 1999), our results are the first report of strains selected with Cry1Ab showing cross-resistance to other toxins from Bt. In Cry1Ac-selected *O. nubilalis* strains, Bolin et al. (1999) reported low levels of cross-resistance to Cry1Ab. In contrast, the present results showed a high level of cross-resistance among Cry1Ab-resistant strains of *O. nubilalis* to Cry1Ac, which is not unexpected given that these two toxins share >85% similarity in amino acid sequence and 49–52% in the N-terminal region that is believed to be involved in the specificity of some Cry1 proteins (Höfte and Whiteley 1989, Chambers et al. 1991). Also, these data are consistent with previous studies (Wolfersberger 1990, Gould et al. 1992, Denolf et al. 1993) that suggest Cry1Ac and Cry1Ab share a common binding site.

In contrast to cross-resistance to Cry1Ac, only low levels of cross-resistance to Cry1F were observed among the Cry1Ab-selected strains. Chambers et al. (1991) described the Cry1F toxin as distinct from Cry1A toxins in both its spectrum of insecticidal activity against lepidopteran larvae and in its amino acid sequence. Ballester et al. (1999) reported that for *P. xylostella*, Cry1Ab, Cry1Ac, and Cry1F share a common binding site. It is possible that Cry1Ab, Cry1Ac, and Cry1F share at least one receptor in *O. nubilalis* (Hua et al. 2001), which might explain the pattern of cross-resistance. However, with such a low level of resistance, it is possible that Cry1F also binds to or has a higher affinity for a different receptor. Hua et al. (2001) suggested that contrary to the high-affinity binding site shared by Cry1F and Cry1Ab in *P. xylostella* (Granero et al. 1996), Cry1Ab and Cry1F bind with low affinity to a common receptor in *O. nubilalis.* Such a pattern of binding would support the low level of cross-resistance observed among the selected strains used in this investigation. High levels of cross-resistance to other Cry1 toxins have previously been reported in *P. xylostella* selected with Cry1F (Tabashnik 1994) and included a very high level of resistance to Cry1Ab. The relationship between the Cry1Ab and Cry1F toxins and potential cross-resistance warrants further consideration as the only two commercially available transgenic hybrids express either the Cry1F or Cry1Ab toxin.

No cross-resistance to Cry9C was observed among the Cry1Ab-selected strains. Zhao et al. (2001) observed low cross-resistance to Cry9C in some strains of *P. xylostella* selected with Cry1C. The lack of Cry1Ab cross-resistance to Cry9C agrees with previous binding analysis in *O. nubilalis* (Hua et al. 2001) in which Cry9C did not compete with Cry1Ab, suggesting that Cry9C binds to receptors that are different from those which bind Cry1Ab.

Bt corn expressing different toxins offers the potential for rotation or gene stacking in conjunction with the current high-dose refuge approach for managing resistance in transgenic plants. However, insecticide resistance management and mitigation procedures that involve multiple toxins would be compromised if cross-resistance exists. High levels of cross-resistance would enable insects to survive exposure to newly discovered toxins, which may not be phylogenetically related but compete for a common binding receptor.

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References Cited

Ballester, V., F. Granero, B. E. Tabashnik, T. Malvar, and J. Ferre. 1999. Integrative model for binding of *Bacillus thuringiensis* toxins in susceptible and resistant larvae of the diamondback moth, *Plutella xylostella.* Appl. Environ. Microbiol. 65: 1413–1419.

Bauer, L. S. 1995. Resistance: a threat to the insecticidal crystal proteins of *Bacillus thuringiensis.* Fla. Entomol 78: 414–443.

Bolin, P. C., W. D. Hutchison, and D. A. Andow. 1999. Long-term selection for resistance to *Bacillus thuringiensis* Cry1Ac endotoxin in a Minnesota population of European corn borer (Lepidoptera: Crambidae). J. Econ. Entomol. 92: 1021–1030.

Carlton, B. C., and J. M. Gonzales. 1985. Plasmids and delta-endotoxin production in different subspecies of *Bacillus thuringiensis,* pp. 246–252. *In* J. A. Hoch and P. Setlow [eds.], Molecular biology of microbial differentiation. American Society of Microbiology, Washington, DC.

Chambers, J. A., A. Jelen, M. P. Gilbert, C. S. Jany, T. B. Johnson, C. Gawron Burke, and C. G. Burke. 1991. Isolation and characterization of a novel insecticidal crystal

protein gene from *Bacillus thuringiensis* subsp. *aizawai.* J. Bacteriol. 173: 3966–3976.

Chaufaux, J., M. Seguin, J. J. Swanson, D. Bourguet, and B. D. Siegfried. 2001. Chronic exposure of the European corn borer (Lepidoptera : Crambidae) to CrylAb *Bacillus thuringiensis* toxin. J. Econ. Entomol. 94: 1564–1570.

Chilton, M., M. Koziel, T. C. Currier, and B. J. Miflin. 1993. Bt maize for control of European corn borer, pp. 370–377. *In* R. D. Lumsden and J. L. Vaughn [eds.], Pest management: biologically based technologies. American Chemical Society, Washington, DC.

Denolf, P., S. Jansens, M. Peferoen, D. Degheele, and J. Vanrie. 1993. Two different *Bacillus thuringiensis* delta-endotoxin receptors in the midgut brush-border membrane of the European corn corer, *Ostrinia nubilalis* (Hübner) (Lepidoptera, Pyralidae). Appl. Environ. Microbiol. 59: 1828–1837.

Estruch, J. J., N. B. Carozzi, N. Desai, N. B. Duck, G. W. Warren, and M. G. Koziel. 1997. Transgenic plants: an emerging approach to pest control. Nat. Biotechnol. 15: 137–141.

Ferre, J., and J. Van Rie. 2002. Biochemistry and genetics of insect resistance to *Bacillus thuringiensis.* Annu. Rev. Entomol. 47: 501–533.

Ferre, J., M. D. Real, J. Van Rie, S. Jansens, and M. Peferoen. 1991. Resistance to the *Bacillus thuringiensis* bioinsecticide in a field population of *Plutella xylostella* is due to a change in a midgut membrane receptor. Proc. Natl. Acad. Sci. U.S.A. 88: 5119–5123.

Finney, D. J. 1971. Probit analysis. Cambridge University Press, London, England.

Fischoff, D. A. 1996. Insect-resistant crop plants, pp. 214–227. *In* G. S. Presley [ed.], Biotechnology and integrated pest management. CAB, Wallingford, UK.

Frutos, R., C. Rang, and F. Royer. 1999. Managing insect resistance to plants producing *Bacillus thuringiensis* toxins. Crit. Rev. Biotechnol. 19: 227–276.

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. U.S.A. 89: 7986–7990.

Granero, F., V. Ballester, and J. Ferre. 1996. *Bacillus thuringiensis* crystal proteins CRY1Ab and CRY1Fa share a high affinity binding site in *Plutella xylostella* (L.). Biochem. Biophys. Res. Commun. 224: 779–783.

Guthrie, W. D., E. S. Raun, F. F. Dicke, G. R. Pesho, and S. W. Carter. 1965. Laboratory production of European corn borer egg masses. Iowa State J. Sci. 40: 665–683.

Höfte, H., and H. R. Whiteley. 1989. Insecticidal crystal proteins of *Bacillus thuringiensis.* Microbiol. Rev. 53: 242–255.

Hua, G., L. Masson, J. L. Jurat-Fuentes, G. Schwab, and M. J. Adang. 2001. Binding analyses of *Bacillus thuringiensis* Cry delta-endotoxins using brush border membrane vesicles of *Ostrinia nubilalis.* Appl. Environ. Microbiol. 67: 872–879.

Huang, F., L. L. Buschman, R. A. Higgins, and W. H. McGaughey. 1999a. Inheritance of resistance to *Bacillus thuringiensis* toxin (Dipel ES) in the European corn borer. Science (Wash DC) 284: 965–967.

Huang, F., K. Y. Zhu, L. L. Buschman, R. A. Higgins, and B. Oppert. 1999b. Comparison of midgut proteinases in *Bacillus thuringiensis*-susceptible and -resistant European corn borer, *Ostrinia nubilalis* (Lepidoptera: Pyralidae). Pestic. Biochem. Physiol. 65: 132–139.

Huang, F. N., R. A. Higgins, and L. L. Buschman. 1997. Baseline susceptibility and changes in susceptibility to *Bacillus thuringiensis* subsp. *kurstaki* under selection pressure in European corn borer (Lepidoptera: Pyralidae). J. Econ. Entomol. 90: 1137–1143.

Kao, C., and E. Cheng. 1994. The resistance and cross resistance of diamondback moth, *Plutella xylostella* to *Bacillus thuringiensis* in Taiwan. J. Agric. Res. China 43: 425–432.

King, E. G., G. G. Hartley, D. F. Martin, and M. L. Laster. 1985. Large-scale rearing of a sterile backcross of the tobacco budworm *Heliothis virescens* (Lepidoptera: Noctuidae). J. Econ. Entomol. 78: 1166–1172.

Kirsch, K., and H. Schmutterer. 1988. Low efficacy of a *Bacillus thuringiensis* (Berl.) formulation in controlling the diamondback moth, *Plutella xylostella* (L.), in the Philippines. J. Appl. Entomol. 105: 249–255.

Koziel, M. G., G. L. Beland, C. Bowman, N. B. Carozzi, R. Crenshaw, L. Crossland, J. Dawson, N. Desai, M. Hill, S. Kadwell, et al. 1993. Field performance of elite transgenic maize plants expressing an insecticidal protein derived from *Bacillus thuringiensis.* Biotechnology 11: 194–200.

Lang, B. A., D. J. Moellenbeck, D. J. Isenhour, and S. J. Wall. 1996. Evaluating resistance to CryIA(b) in European corn borer (Lepidoptera: Pyralidae) with artificial diet. Resist. Pestic. Manage. 8: 29–31.

Leahy, T. C., and D. A. Andow. 1994. Egg weight, fecundity, and longevity are increased by adult feeding in *Ostrinia nubilalis* (Lepidoptera, Pyralidae). Ann. Entomol. Soc. Am. 87: 342–349.

LeOra Software 1987. POLO-PC, a user's guide to probit and logit analysis. LeOra Software, Berkeley, CA.

Lewis, L. C., and R. E. Lynch. 1969. Rearing the European corn borer on corn leaf and wheat germ diets. Iowa State J. Sci. 44: 9–14.

Marçon, P.C.R. G., L. J. Young, K. L. Steffey, and B. D. Siegfried. 1999. Baseline susceptibility of European corn borer (Lepidoptera: Crambidae) to *Bacillus thuringiensis* toxins. J. Econ. Entomol. 92: 279–285.

McGaughey, W. H. 1985. Insect resistance to the biological insecticide *Bacillus thuringiensis.* Science (Wash DC) 229: 193–195.

Milne, R., and H. Kaplan. 1993. Purification and characterization of a trypsin-like digestive enzyme from spruce budworm (*Choristoneura fumiferana*) responsible for the activation of delta-endotoxin from *Bacillus thuringiensis.* Insect Biochem. Mol. Biol. 23: 663–673.

Miranda, R., F. Z. Zamudio, and A. Bravo. 2001. Processing of Cry1Ab delta-endotoxin from *Bacillus thuringiensis* by *Manduca sexta* and *Spodoptera frugiperda* midgut proteases: role in protoxin activation and toxin inactivation. Insect Biochem. Mol. Biol. 31: 1155–1163.

Muller, C. J., J. Chaufaux, C. Buisson, N. Gilois, V. Sanchis, and D. Lereclus. 1996. *Spodoptera littoralis* (Lepidoptera: Noctuidae) resistance to CryIC and cross-resistance to other *Bacillus thuringiensis* crystal toxins. J. Econ. Entomol. 89: 791–797.

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.

Robertson, J. L., and H. K. Preisler. 1992. Pesticide bioassays with arthropods. CRC, Boca Raton, FL.

SAS Institute 1999. SAS procedure guide computer program, version 8.0. SAS Institute, Cary, NC.

Schnepf, E., N. Crickmore, J. Van Rie, D. Lereclus, J. Baum, J. Feitelson, D. R. Zeigler, and D. H. Dean. 1998. *Bacillus thuringiensis* and its pesticidal crystal proteins. Microbiol. Mol. Biol. Rev. 62: 775–806.

Scott, J. G. 1990. Investigating mechanisms of insecticide resistance: methods, strategies, and pitfalls, pp. 39–57. *In* R. T. Roush and B. E. Tabashnik [eds.], Pesticide resistance in arthropods. Chapman & Hall, New York

Siegfried, B. D., P.C.R. G. Marcon, J. F. Witkowski, R. J. Wright, and G. W. Warren. 1995. Susceptibility of field populations of the European corn borer, *Ostrinia nubilalis* (Hübner) (Lepidoptera, Pyralidae), to *Bacillus thuringiensis* (Berliner). J. Agric. Entomol. 12: 267–273.

Tabashnik, B. E. 1994. Evolution of resistance to *Bacillus thuringiensis.* Annu. Rev. Entomol. 39: 47–79.

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.

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.

Tabashnik, B. E., Y. Carrière, T. J. Dennehy, S. Morin, M. S. 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.

Watkinson, I., and R. J. Milner. 1994. Global view of present and future markets for Bt products. Agric. Ecosyst. Environ. 49: 3–7.

Wolfersberger, M. G. 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. Experientia (Basel) 46: 475–477.

Zhao, J. Z., Y. X. Li, H. L. Collins, J. Cao, E. D. Earle, and A. M. Shelton. 2001. Different cross-resistance patterns in the diamondback moth (Lepidoptera: Plutellidae) resistant to *Bacillus thuringiensis* toxin Cry1C. J. Econ. Entomol. 94: 1547–1552.

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