Inheritance, Stability, and Lack-of-Fitness Costs of Field-Selected Resistance to *Bacillus thuringiensis* in Diamondback Moth (Lepidoptera: Plutellidae) from Florida

JULIET D. TANG, SMADAR GILBOA,[1,2] RICHARD T. ROUSH,[1,3] AND ANTHONY M. SHELTON

Department of Entomology, Cornell University, New York State Agricultural Experiment Station, Geneva, NY 14456

J. Econ. Entomol. 90(3): 732–741 (1997)

ABSTRACT A colony of *Plutella xylostella* (L.), established from crucifer fields in Florida, was used to investigate resistance to *Bacillus thuringiensis* Berliner subsp. *kurstaki*. From an initial level of >1,500-fold, resistance fell within 3 generations in the absence of selection to ≈300-fold compared with susceptible larvae. Unlike previous cases of resistance to *B. thuringiensis* in *P. xylostella*, resistance in our Florida colony was stable at ≈300-fold without additional selection in the laboratory. High levels of resistance (>1,000-fold) recurred after a single exposure to *B. thuringiensis* subsp. *kurstaki* in the 4th generation. High levels of resistance did not recur after a 2nd selection in the 8th generation. Cage studies and genetic analysis of F_1 larvae and backcross progeny, where the resistant parents were characterized by stable levels of resistance, showed that resistance was an incompletely recessive, autosomal trait probably controlled by a single allele that did not confer detectable levels of reduced fitness in the absence of exposure to *B. thuringiensis*. As one of the few studies to demonstrate stable resistance to *B. thuringiensis* subsp. *kurstaki* from insects that were collected from the field and not subject to further selection in the laboratory, our results clearly emphasize the need to develop specific resistance management strategies for *B. thuringiensis* before there is widespread evolution of resistance.

KEY WORDS *Plutella xylostella*, *Bacillus thuringiensis*, transgenic plants, insecticide, resistance management

THE CONCEPT OF resistance management arose when it became clear that pests were evolving resistance faster than pesticide companies and integrated pest management (IPM) programs could adjust (Metcalf 1980). Fundamental to most discussions about specific resistance management plans are questions about the inheritance of resistance (especially dominance under field conditions), cross-resistance, and the fitness of resistant individuals in the absence of selection (e.g., Roush and McKenzie 1987).

Resistance to the delta endotoxins of *Bacillus thuringiensis* Berliner is of particular interest, because the endotoxins are environmentally safe and are now being used in transgenic plants. The type of resistance that evolves to *B. thuringiensis* can be quite distinct, depending upon the species, selecting regime, or geographical origin of the founder colony (Heckel 1994, Ferré et al. 1995). Laboratory selection of *Heliothis virescens* (F.) has produced the most varied types of resistance which appear to differ in inheritance, patterns of cross-resistance to other insecticidal crystal proteins, or underlying biochemical mechanisms (Stone et al. 1989; MacIntosh et al. 1991; Sims and Stone 1991; Gould et al. 1992, 1995). The founding colony in each case, however, was as susceptible as the control colony, suggesting that previous exposure in the field to *B. thuringiensis* was minimal.

Once field exposure has occurred and low levels of resistance are detected, there appear to be fewer differences in the type of resistance that evolves among colonies that are further selected in the laboratory. This seems consistent with the pattern that has been observed for most insecticides (Roush and McKenzie 1987). In *Plodia interpunctella* (Hübner), resistance to *B. thuringiensis* was incompletely recessive and did not cause detectable levels of reduced fitness in all of 5 laboratory-selected colonies (McGaughey and Beeman 1988). Before selection, these colonies showed low levels of resistance to *B. thuringiensis* from field exposure in grain bins (McGaughey 1985a, b). Differ-

[1]Department of Entomology, Cornell University, Ithaca, NY 14853.

[2]Current address: Department of Biology and Microbiology, California State University, Los Angeles, 5151 State University Drive, Los Angeles, CA 90032.

[3]Current address: Department of Crop Protection, Waite Institute, PMB 1, Glen Osmond, South Australia 5064, Australia.

0022-0493/97/0732–0741$02.00/0

ences in the rate at which resistance evolved and the varying degrees of recessiveness in each selected colony suggested, however, that polymorphic genotypes were present (McGaughey and Beeman 1988).

Field exposure to *B. thuringiensis* also has led to several cases of resistance in *Plutella xylostella* (L.). Detailed accounts of resistance come primarily from colonies that were collected in Hawaii (Tabashnik et al. 1990), Japan (Hama et al. 1992), and Florida (Shelton et al. 1993). (A CryIA(b)-resistant colony from the Philippines also has been described from 2 separate field collections [Ferré et al. 1991, Ballester et al. 1994].) The Hawaiian colony had developed low levels of resistance in the field, and subcolonies were selected in the laboratory to increase resistance further (Tabashnik et al. 1991). The Japanese colony evolved high levels of resistance from exposure in the greenhouse (Hama et al. 1992), and the Floridian colony evolved high levels of resistance from exposure in the field (Shelton et al. 1993).

Like *P. interpunctella*, cases of resistance in the different colonies of *P. xylostella* display an overall similarity. In the Hawaiian (selected) and Japanese colonies, resistance was an autosomal, recessive trait, primarily controlled by 1 or few genes (Hama et al. 1992, Tabashnik et al. 1992) whose frequency steadily declined in the absence of selection (Hama et al. 1992, Tabashnik et al. 1994a). With continued intensive selection, however, resistance appeared to be more stable in the colony from Hawaii (Tabashnik et al. 1995). In the Hawaiian and the Floridian colonies, resistance to *B. thuringiensis* subsp. *kurstaki* was attributed to a dramatic decrease in the toxicity of CryIA(a), CryIA(b), and CryIA(c) (Tabashnik et al. 1994a, Tang et al. 1996) with little cross-resistance to other toxin families such as CryIB (Tabashnik et al. 1994b, Tang et al. 1996), CryIC (Tabashnik et al. 1994a, Tang et al. 1996), and CryID (Tang et al. 1996). Resistance to HD-1 spore also was found in the Floridian colony (Tang et al. 1996) and cross-resistance with CryIF in the Hawaiian colony (Tabashnik et al. 1994b).

Improved understanding of the genetics of resistance will enhance our ability to design and justify recommended resistance management programs. Effective dominance of resistance is very important (Roush 1994), and the number of genes involved in resistance is relevant to addressing whether single gene models are adequate to describe resistance to *B. thuringiensis*. Whether the costs of resistance to fitness are important in the design of a resistance management plan is debatable (Roush 1989), but such costs will help to delay resistance.

Having documented some of the toxicological and biochemical aspects of resistance for our colony from Florida (Tang et al. 1996), we now report on its genetic basis. Our data show that, similar to Hawaiian (Tabashnik et al. 1992) and Japanese (Hama et al. 1992) colonies of *P. xylostella*, resistance was an incompletely recessive autosomal trait. However, less ambiguously than earlier studies, we found that resistance appeared to be caused by a single gene. In addition, other important differences are described, including the degree of recessiveness, the inherent stability of the trait, the lack of apparent fitness costs.

Materials and Methods

Insects. The susceptible colony was collected from cabbage at the New York State Agricultural Experiment Station Robbins Farm, Geneva, NY, in 1988 and reared continuously on a wheat germ–casein artificial diet (Shelton et al. 1991). Rearing conditions were 27 ± 1°C, 35 ± 2% RH, and photoperiod of 16:8 (L:D) h. The resistant colony (known as Loxa A) was collected from commercial kolhrabi fields in Loxahatchee, FL, in 1992 (Shelton et al. 1993). Loxa A larvae were reared on oilseed rape, *Brassica napus* subsp. *oleifera* ('Dwarf Essex'), in the greenhouse at 24–33°C, a photoperiod of 16:8 (L:D) h, and 20–80% RH. Compared with the Geneva colony, Loxa A larvae exhibited a >1,500-fold level of resistance to *B. thuringiensis* subsp. *kurstaki* in the 2nd generation after the colony was brought into the laboratory from the field (Shelton et al. 1993). Before testing, larvae were reared on rape plants in the greenhouse unless stated otherwise.

Leaf Dip Bioassays. Toxicity of Javelin WG (wettable granules, 6.4% (AI), Sandoz, Des Plaines, IL, Lot No. 7300960) was measured using a cabbage leaf dip bioassay similar to that used previously (Shelton et al. 1993). Javelin is a commercial formulation of *B. thuringiensis* subsp. *kurstaki* that contains spore and the genes for CryIA(a), CryIA(b), CryIA(c), CryIIA, and CryIIB (Koziel et al. 1993). On each day, 6–8 concentrations were prepared from a 3.16× dilution series including a control with 5 leaf disks (75 mm diameter, dipped for 10 s and allowed to air-dry) per concentration and 5 second instars (0.2–0.4 mg per larva) placed per leaf disk. Mortality was determined after 96 h at 27°C and tests were replicated on 2 different days unless indicated otherwise. Where resistance ratios were calculated, both the Geneva and Loxa A colonies were tested concurrently. For bioassays of backcross larvae, mortality at or near 50% was of particular interest, so we deviated from the 10- and 31.6-ppm concentrations normally used and instead tested 8, 20, and 40 ppm Javelin. Control mortality for all leaf dip bioassays was <4%.

Transgenic Leaf Bioassays. Leaf bioassays of broccoli, *Brassica oleracea* subsp. *italica*, that had been transformed to express the CryIA(c) protein of *B. thuringiensis* (Metz et al. 1995), were used to determine how stable resistance was in greenhouse cage studies. We used transgenic leaf assays instead of leaf dip bioassays because they required less preparation, the leaves produced only

CryIA(c) (Metz et al. 1995), and the level of CryIA(c) expressed in the broccoli leaves reliably killed all susceptible and heterozygous larvae but few resistant larvae (Metz et al. 1995). Equal numbers of leaf disks (75 mm in diameter) were cut from transgenic broccoli and 'Green Comet' broccoli, as the controls. Six 2nd instars were transferred onto each leaf disk in a petri dish (100 mm diameter), and 18 larvae were tested per leaf type per cage. Mortality was scored after 72 h at 27°C.

Selection. The effects of a single selection on resistance after the Loxa A colony was brought in from the field was examined by exposing 2nd instars (n = 1,289) in the 4th generation for 96 h to cabbage leaves dipped for 10 s in 316-ppm Javelin. Survivors of this colony, hereafter called Loxa A Selected, were transferred to rape plants. We obtained 114 adults to continue the colony.

The effects of a 2nd selection on resistance was examined by taking part of the Loxa A Selected colony and reselecting it 4 generations later (the 8th generation after the colony was brought in from the field). As in the 1st selection, 2nd instars ($n \geq$1,000) were exposed for 96 h to leaves dipped in 316-ppm Javelin, and >100 adults were collected for egg production. In the following generation, resistance to Javelin was evaluated with a leaf dip bioassay and data were compared with 8th generation larvae that were not selected a second time.

Crosses. To perform mating crosses for the inheritance and fitness studies, pupae were collected and placed individually into 28.4-ml cups. Sex was determined within 24 h of adult eclosion and reciprocal mass matings were set up. Crosses and strains used for the inheritance study were F_1 (Heterozygous resistant progeny) = Geneva (generation 96) × Loxa A Selected (generation 8), and backcross progeny = F_1 × Loxa A (generation 9), where the generation number represents the number of generations since the colony was taken from the field. Because there was no evidence for sex linkage from our data (see *Results*) or that of others (Hama et al. 1992, Tabashnik et al. 1992, Martinez-Ramirez et al. 1995), offspring from the reciprocal crosses were pooled unless stated otherwise.

Resistance Stability. The stability of resistance to *B. thuringiensis* was monitored with a leaf dip bioassay. The LC_{50} was estimated for the Loxa A colony in the 2nd, 3rd, 4th, 6th, 7th, and 10th generations after the colony was brought in from the field. We did the same for the Loxa A Selected strain, monitoring resistance in the 5th, 7th, 8th, 11th, and 19th generations after the colony was brought in from the field.

Fitness of Resistance Allele(s). To evaluate more directly whether the resistance allele(s) conferred any major fitness disadvantages, resistant and susceptible moths were crossed to ensure that there was variation for susceptibility. F_1 eggs from each reciprocal cross (Geneva [generation 100+] × Loxa A Selected [generation 15+]) were placed in separate cages (50 by 50 by 50 cm, 2 cages per cross) in the greenhouse on Green Comet broccoli, grown in 14.8-cm standard pots (9 pots per cage). Hybridized populations were allowed to cycle for 5 generations and new plants were introduced periodically as needed. To test the level of resistance for each cross type, 20 pupae were removed randomly from each cage every generation and set up in Plexiglas cylinder cages (13 cm diameter, 15 cm high) provisioned with 10% sugar water. After adults eclosed, eggs were collected and transferred to broccoli plants in the greenhouse. When larvae reached 2nd instar, they were used in leaf bioassays with transgenic broccoli.

Analysis of Leaf Dip Bioassay Data. To estimate parameters of dose–mortality regression lines for each leaf dip bioassay, data from the replicates were pooled and analyzed with probit models using the POLO program (Russell et al. 1977). The resistance ratio (LC_{50} Loxa A/LC_{50} Geneva) was then calculated to assess changes in the levels of resistance to Javelin over time.

Analysis of Transgenic Leaf Bioassay Data. Multivariate repeated measures analysis in SAS PROC GLM (Littel et al. 1993) was used to determine whether changes occurred over time in the level of mortality on CryIA(c)-expressing or normal broccoli in cages initially seeded with F_1 eggs from each cross type. Our model statement was G2–G6 = CROSS LEAF CROSS*LEAF/NOUNI, which resulted in multivariate analysis of the within-subjects effects of generation (generations 2 through 6), generation by F_1 cross type, generation by leaf type, and generation by cross by leaf. The between-subject effects analyzed were cross, leaf, and cross by leaf.

Analysis of Inheritance. Analysis of the inheritance of resistance used a series of statistical tests which have been previously outlined (Preisler et al. 1990, Tabashnik 1991, Tabashnik et al. 1992). Following Preisler et al. (1990), the degree of dominance (D) for resistance was calculated using the pooled data for the reciprocal F_1 crosses.

Because our F_1 data showed that resistance was incompletely recessive, we backcrossed the F_1 to Loxa A. The chi-square goodness-of-fit test (Sokal and Rohlf 1981) was used to determine how well the backcross mortality data, observed at each concentration (df = 1), fit mortality predicted by each model of inheritance. For the direct test of monogenic inheritance, calculations of expected mortality for the backcross offspring were based on experimental data. Assuming that the Geneva parents were homozygous-susceptible (SS), the Loxa A parents were homozygous-resistant (RR), and F_1 progeny were RS, progeny from the F_1 × Loxa A backcross should have been 50% RS and 50% RR. Because mortality of each of these genotypes had been determined empirically, direct calculations of the expected proportion dead at concentration c equaled 0.5 × (proportion of F_1 dead at c + proportion of Loxa A dead at c).

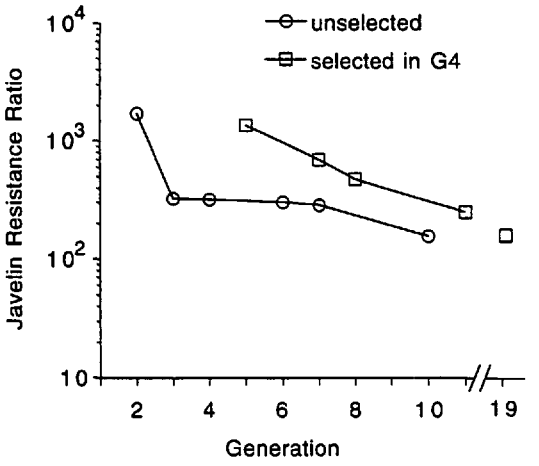

Fig. 1. Changes in the Javelin resistance ratio (=LC_{50} of resistant Loxa A colony/LC_{50} of Geneva colony) in the absence or presence of a single selection at 316 ppm in the 4th generation. LC_{50} values were estimated from POLO probit analysis of mortality data in leaf dip bioassays of 2nd-instar *P. xylostella*. Data from the 2nd generation were originally published in Shelton et al. (1993).

For indirect tests of monogenic and polygenic inheritance, several assumptions were made based on the mortality of the parental genotype classes (i.e., heterozygous at all loci or resistant at all loci). We assumed that the slope of each probit regression line describing each of the offspring genotypes was 1.859, or the mean of the experimentally determined F_1 slope (2.092) and the Loxa A slope (1.626). The standard deviation of the tolerance distribution was then equal to 0.5379, or the inverse of the slope (Finney 1971). The LC_{50} of backcross offspring heterozygous- or homozygous-resistant at all loci was considered to be the same as the parents, which was, respectively, 1.1 and 190.7 ppm Javelin. Assuming equal and additive effects of loci on a logarithmic scale for polygenic models, LC_{50} values for all possible intermediate genotypes (e.g., heterozygous at all loci but 1, heterozygous at all loci but 2, and so forth) were calculated as the antilog of values that were equidistant on a logarithmic scale bound by log(1.106) and log(190.7) (Tabashnik 1991, Tabashnik et al. 1992). Because the logarithm of the tolerance was assumed to be distributed normally (Finney 1971), the proportion dead at any given concentration for each genotype class was calculated as

$$PD = NORMSDIST[(LOG10[CONCENTRATION] - LOG10[LC_{50}])/0.5379], \quad (1)$$

where PD is the proportion dead (formula modified from an EXCEL spreadsheet). The proportion of the backcross progeny (PP) represented by each genotype class was described by the corresponding coefficient of the binomial expression appropriate for the number of genes being tested (coefficients were computed using the Pascal triangle). Finally, the expected mortality at each concentration for the backcross progeny was calculated by summing across all genotype classes, the product of N*PP*PD for each genotype class represented (Tabashnik 1991, Tabashnik et al. 1992), where N was the number of larvae tested. The chi-square goodness-of-fit test (Sokal and Rohlf 1981) was used to determine how well the observed data fit mortality predicted by each indirect model of inheritance.

Results

Resistance Stability and Reselection. After the Loxa A colony was brought in from the field, resistance to Javelin in leaf dip bioassays was monitored over time. A plot of the Javelin resistance ratio (Fig. 1) showed that the Loxa A colony was extremely resistant to *B. t. kurstaki* in the 2nd generation (resistance ratio >1,500), and that in the absence of selection, such high levels of resistance were unstable, declining significantly (based on nonoverlap of the 95% FL of the LC_{50}) from the 2nd generation to the 3rd generation (Table 1). A single selection of Loxa A at 316 ppm (≈30 times that needed to kill >99% of our susceptible strain) in the 4th generation resulted in only 40–60% mortality at 96 h and led to a resurgence of the resistance ratio to >1,000-fold (generation 5 of "selected in G4," Fig. 1). Comparison of the Javelin LC_{50} values for the 4th generation unselected

Table 1. Probit regression analysis of mortality data to Javelin in leaf dip bioassays at different generations after the Loxa A colony was brought in from the field

Generation	n^a	Slope ± SE	LC_{50} (95% FL), ppm	LC_{90} (95% FL), ppm	χ^{2b}
2	314	1.29 ± 0.29	513 (322–963)	5,047 (2,074–41,620)	2.1
3	290	0.91 ± 0.16	162 (88–284)	4,185 (1,696–22,752)	3.4
4	265	0.89 ± 0.13	96 (55–164)	2,636 (1,140–10,647)	1.1
6	237	1.17 ± 0.14	121 (82–174)	1,529 (875–3,548)	3.8
7	291	0.92 ± 0.11	115 (35–283)	2,863 (894–48,776)	8.1
10	290	1.30 ± 0.14	47 (24–80)	456 (241–1,329)	4.7

[a] Number of larvae tested in 2-d replicates.

[b] In each case, the χ^2 value from the goodness-of-fit test was less than the tabular value (df = 5 for generation 2 and df = 4 for all other generations; $P = 0.05$), indicating that the data fit the probit model.

Table 2. Probit regression analysis of mortality data to Javelin after the Loxa A colony was selected with Javelin in the 4th generation

Generation[a]	n^b	Slope ± SE	LC_{50} (95% FL) ppm	LC_{90} (95% FL) ppm	χ^{2c}
5	276	1.58 ± 0.24	681 (474–1,151)	4,404 (2,200–14,722)	0.7
7	99	1.35 ± 0.24	278 (103–826)	2,484 (834–90,043)	6.0
8	124	1.63 ± 0.25	191 (48–637)	1,171 (415–90,674)	6.4
11	264	1.39 ± 0.15	99 (61–162)	832 (436–2,440)	4.2
19	—	—	≈100[d]	—	—

[a] Number of generations after the colony was brought in from the field.
[b] Number of larvae tested in 2-d replicates except for generations 7 and 8, where only a 1-d replicate was performed.
[c] In each case, the χ^2 value from the goodness-of-fit test was less than the tabular value (df = 4 for generations 5 and 11 and df = 3 for generations 7 and 8; $P = 0.05$), indicating that the data fit the probit model.
[d] LC_{50} was estimated based on mortality at 100 ppm (48 of 100 dead) and 200 ppm Javelin (391 of 593 dead).

(Table 1, 96 ppm) with the 5th generation Loxa A Selected strain (Table 2, 681 ppm) showed there was a significant increase in LC_{50} because there was no overlap of the 95% FL.

As with the unselected Loxa A strain, these very high levels of resistance in the Loxa A Selected strain were not stable and again declined to ≈500-fold after 3 generations (Fig. 1). Thereafter, resistance in both the Loxa A Selected (generation 8–19) and unselected (generations 3–10) strains remained relatively stable (Tables 1 and 2) with resistance ratios of ≈150–300-fold (Fig. 1). Although the LC_{50} in the 10th generation of the unselected strain was significantly lower than LC_{50} values obtained for generations 2, 3, and 6, it was not significantly different from values obtained for the 4th and 7th generations (Table 1).

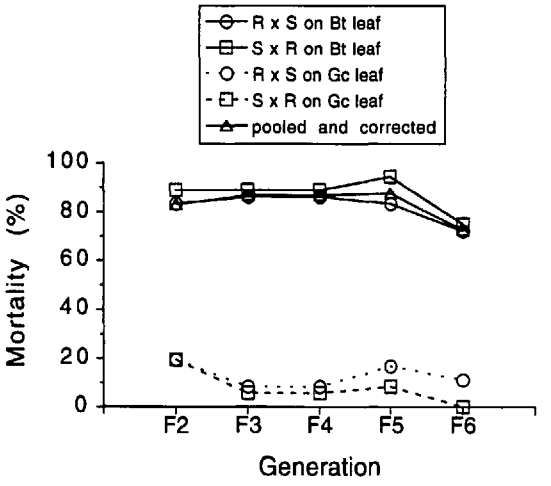

Fig. 2. Stability of resistance to CryIA(c) in hybridized (susceptible × resistant) populations of diamondback moth, as measured by mortality of 2nd instars on transgenic broccoli leaves. Cages were seeded with F_1 eggs from R × S (Loxa A female by Geneva male) or S × R (Loxa A male by Geneva female) crosses. Larvae from each generation were tested on CryIA(c)-expressing (Bt) and Green Comet (Gc) broccoli. Points for the pooled and corrected line (Δ) were calculated by pooling the mortality of the 2 crosses on Bt broccoli and correcting it for mortality on the Gc controls using equation 5 of Rosenheim and Hoy (1989).

Following the 4th generation selection with a 2nd selection in the 8th generation did not produce a significant increase in LC_{50} despite a near repeat of the overall mortality (40–70% at 96 h). The LC_{50} of 8th generation Loxa A Selected larvae was 191 ppm Javelin (Table 2). After a 2nd selection in the 8th generation, the LC_{50} of 9th generation larvae was determined to be 146 ppm (n = 380; slope ± SE, 0.90 ± 0.11; 95% FL of the LC_{50}, 84–236 ppm). Overlap of the 95% FL indicated that there was no significant effect of this 2nd selection.

Fitness of Resistance Allele(s). Resistance also appeared to be relatively stable in populations segregating for resistant and susceptible alleles over 6 generations on Green Comet broccoli in greenhouse cages (Fig. 2). Because we used transgenic leaves in our bioassays, only resistant homozygotes should have survived the assay. If resistance caused reduced fitness, then mortality should have increased over time (caused by a declining frequency of resistance, especially of resistant homozygotes), but this did not occur. Assuming that resistance was caused by a single gene (see *Genetic Basis of Resistance*) and that all of the 25% homozygous offspring survived, one would expect 75% mortality in each bioassay. A plot of the data, which was pooled for the 2 crosses and corrected for control mortality (Fig. 2, open triangles), showed that mortality in the bioassay ranged from 72.5 to 87.5% with an average value of 83.3%. Consistent with an earlier experiment (see Roush 1994), the observed mortalities tend to be slightly >75%. This could be caused by lack of homozygosity for resistance in the Loxa A Selected strain at the time the crosses were made, or because, on occasion, the transgenic plants can kill some homozygous larvae (Roush 1994).

For the cages from the R × S (resistant female by susceptible male) cross, percentage larval mortality of each cross type on CryIA(c)-expressing broccoli showed relatively few differences among F_2, F_3, F_4, F_5, and F_6 offspring (uncorrected values ranged from 72.2 to 86.1% mortality). For the S × R cage (resistant male by susceptible female), mortality on CryIA(c)-expressing broccoli was relative-

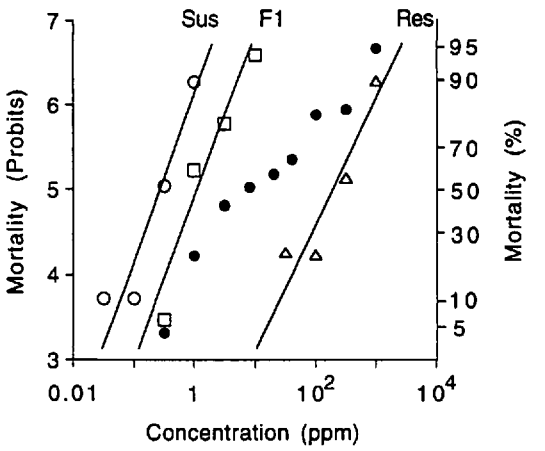

Fig. 3. Mortality responses to Javelin of susceptible (○), resistant (△), F_1 (□), and backcross larvae (●) in leaf dip bioassays. Larvae were from the following colonies or crosses: susceptible, Geneva, F1 = Geneva × Loxa A cross; resistant, Loxa A, backcross = F_1 × Loxa A cross. Regression lines were estimated from POLO probit analysis. The number of larvae tested was 60 for each backcross data point. F_1 and Loxa A regression data in this figure were used for chi-square analysis in Table 3.

ly constant (88.9%) with some deviation at F_5 and F_6, where mortality rose to 94.4%, then fell to 75.0%. Control mortality ranged from 8.3 to 19.4% for the R × S cages and from 0.0 to 19.4% for the S × R cages. Repeated measures analysis of this data showed that these deviations were not significant because there was no effect of generation (F = 0.7751, df = 3, P = 0.61), generation by cross (F = 0.0560, df = 3, P = 0.98), generation by leaf (F = 0.6944, df = 3, P = 0.64), or generation by cross by leaf (F = 0.2372, df = 3, P = 0.87). Thus, the relative stability of resistance in hybridized populations suggests that resistance was not causing detectable reductions in fitness. For the between-subject factors, only leaf was significant (F = 835.16, df = 1, P = 0.0001), which was expected because mortality on the transgenic leaf should have been significantly higher than mortality on the control leaf. The lack of differences in the reciprocal crosses is evidence that resistance in the Loxa A colony was an autosomal trait.

Genetic Basis of Resistance. The proximity of the dose–mortality regression lines of the Geneva and F_1 larvae indicates that resistance was an incompletely recessive trait (Fig. 3). Comparison of the Geneva and F_1 probit lines showed that, although they shared the same slope, they did not share the same y-intercept and hence were different lines. The degree of dominance, D, was −0.67.

To test the hypothesis that resistance was caused by a single locus, concentration–mortality responses of the backcross progeny to Javelin in leaf dip bioassays also were determined. Of the 9 concentrations evaluated (Fig. 3, closed circle), mortality deviated from expected only at 100 ppm (P = 0.005, df = 1; Table 3), indicating that a single-gene model provided an acceptable fit of the data to 8 of the 9 points evaluated.

Table 3. Direct test of monogenic inheritance for resistance to Javelin in Loxa A larvae by comparing expected and observed mortality of the backcross progeny

Concn, ppm	n^a	Observed no. deaths[b]	Expected no. deaths[c]	χ^2 (df = 1)	$P > \chi^2$
0.316	60	2	3.8	0.93	0.33
1	60	12	13.9	0.34	0.56
3.16	60	25	24.5	0.01	0.90
8	60	31	29.3	0.19	0.66
20	60	35	31.5	0.80	0.37
40	60	39	34.0	1.67	0.20
100	60	50	39.7	7.86	0.005*
316	60	51	49.2	0.37	0.54
1,000	60	58	56.4	0.78	0.38

*, Observed mortality significantly deviated from the model prediction (i.e., $P \leq 0.05$).

[a] Number of larvae tested.

[b] Backcross larvae were progeny of F_1 × Loxa A.

[c] Expected number of larvae dead at a given dose = $N \times 0.5$ (proportion F_1 larvae that die + proportion Loxa A larvae that die).

Using the indirect method of testing inheritance, we found that, compared with the 2-loci and 5-loci models, the 1-locus model provided the best fit to the observed data (Table 4). In the 1-locus model, the only concentration that deviated from the model prediction was, again, 100 ppm (χ^2 test, P = 0.003, df = 1). In the 2-loci model, deviations were significant at 2 of 9 concentrations tested (3.16 and 8 ppm), and in the 5-loci model, deviations were significant at 5 of 9 concentrations (1, 3.16, 8, 316, and 1,000 ppm) (Fig. 4).

Discussion

Our results indicate that resistance to both Javelin and transgenic plants expressing CryIA(c) in the Loxa A colony is most likely caused by a single autosomal gene expressed as an incompletely recessive trait. The allele or alleles conferring resis-

Table 4. Indirect tests for monogenic and polygenic inheritance of resistance to Javelin in Loxa A larvae by χ^2 analysis (df = 1)

Concn, ppm	Genetic model[a]					
	1 locus		2 loci		5 loci	
	χ^2	P	χ^2	P	χ^2	P
0.316	1.66	0.2	0.06	0.81	2.65	0.10
1	0.38	0.54	3.13	0.08	22.74	<0.001*
3.16	0.13	0.72	8.83	0.003*	22.14	<0.001*
8	0.42	0.52	3.71	0.05*	6.07	0.014*
20	1.21	0.27	0.17	0.68	0.02	0.88
40	2.38	0.12	0.13	0.72	1.60	0.21
100	8.82	0.003*	0.53	0.47	0.77	0.38
316	0.18	0.67	2.80	0.09	24.3	<0.001*
1,000	0.20	0.66	0.30	0.58	8.19	0.004*

*, Observed mortality significantly deviated from the model prediction (i.e., $P \leq 0.05$).

[a] Models assume equal and additive effects of loci.

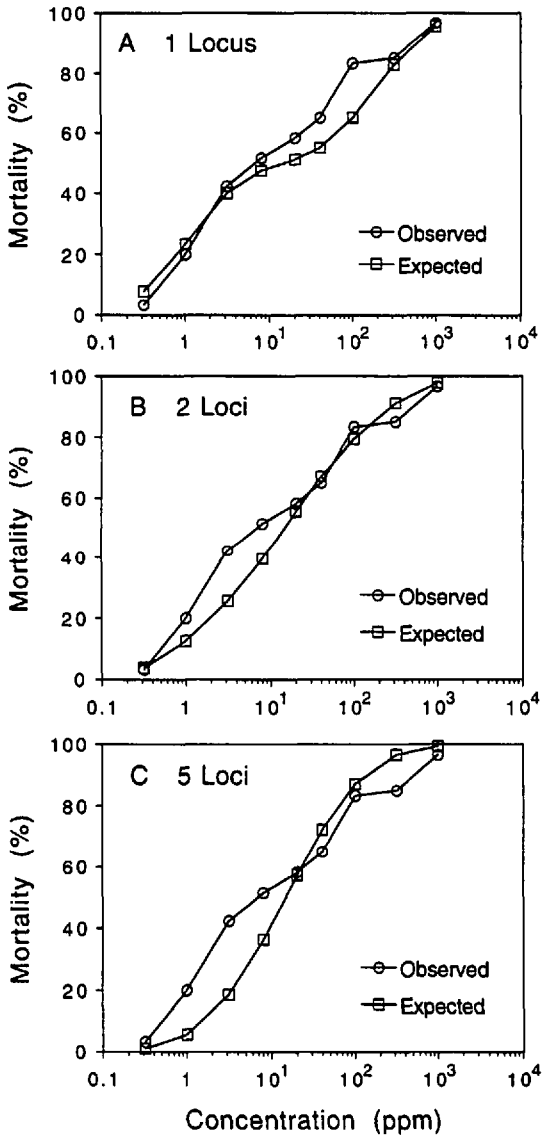

Fig. 4. Comparison of observed and expected mortality for larvae from the backcross F_1 × Loxa A, when the model for inheritance of resistance to Javelin was based on 1, 2, or 5 loci. The expected mortality given by each model was estimated by taking the average slope of F_1 and Loxa A regression lines (1.859), and setting it equal to the slopes of the regression lines for each of the various genotypes generated by the backcross. The results of chi-square analysis are shown in Table 4.

tance appear to be fairly stable in our colony and do not seem to cause any detectable reductions in fitness. Because resistance before and after selections at ≈50% mortality always stabilized ≈300-fold, we estimate that the frequency of the resistant allele(s) in the original field population was high.

Summarizing what is known about resistance to *B. thuringiensis* subsp. *kurstaki* in *P. xylostella*, we find that cases of resistance tend to show an overall similarity. Resistance in the Loxa A colony, like colonies from Hawaii (Tabashnik et al. 1992) and Japan (Hama et al. 1992), was caused by one or few autosomal genes, expressed as a recessive trait. The functional basis of resistance also appeared to be very similar. Both the Loxa A (Tang et al. 1996) and the Hawaiian (Tabashnik et al. 1993) colonies showed high levels of tolerance in feeding assays to the insecticidal crystal proteins CryIA(a), CryIA(b), and CryIA(c). When binding of CryIA(b) and CryIA(c) was investigated in the Loxa A (Tang et al. 1996) and Hawaiian (Tabashnik et al. 1994a) colonies, respectively, this feeding tolerance was attributed to dramatically reduced binding of the toxin to the midgut receptor. These similarities lead one to suspect that at least the same major locus is involved in all 3 colonies.

There are, however, important differences. There is some uncertainty in the exact number of genes involved. Of the 3 colonies investigated (Loxa A, Hawaiian, and Japanese), data for Loxa A seemed consistently to fit the monogenic model of inheritance best. This conclusion was based on the ≈75% mortality observed in transgenic leaf assays for insects taken from greenhouse cages segregating for resistant and susceptible alleles over time, and on direct and indirect tests of backcross mortality data to Javelin (1 deviation of 9 concentrations tested in both cases). Direct tests of the monogenic model for the Hawaiian (NO-Q) colony showed 1 deviation of 5 concentrations tested, and indirect tests showed 2 deviations of 5 in both the 1- and 2-loci models, leading to the conclusion that resistance was caused by 1 or few loci (Tabashnik et al. 1992). Without data for indirect tests of 1 or more loci for the Japanese (ROO) colony, we can conclude only that resistance was caused by 1 or few loci because the direct test of the monogenic model showed 7 deviations of 36 points plotted (9 concentrations tested) (Hama et al. 1992).

Other differences were the degree of recessiveness and the level of stability of resistance in the colonies. The trait was incompletely recessive in the Loxa A and Japanese (ROO) colonies ($D = -0.67$ and -0.74, respectively) (Hama et al. 1992), but was more nearly completely recessive in the Hawaiian (NO-Q) colony (Tabashnik et al. 1992). In the absence of selection, resistance in the Loxa A colony decreased from generations 2 to 3, then stabilized at ≈300-fold from generations 3 to 10. In Hawaiian subcolonies (NO-P, NO-Q, and NO-R) (Tabashnik et al. 1992) and Japanese colonies (RO and ROO) (Hama et al. 1992), resistance did not stabilize but continued to decline. Within 13 or fewer generations from the time selection were stopped, resistance fell to within 10-fold of the level found for susceptible larvae. More prolonged selection of NO-Q (subcolony called NO-Y) produced different results when selections were stopped. Resistance dropped in NO-Y and then stabilized at moderate levels of resistance, and in 1 of 6 isofemale lines started from

NO-Y, resistance remained high (Tabashnik et al. 1995).

These differences between our study and those of others may be caused by a combination of the intensity of selection, and at least for the inheritance studies, differences in the formulation of *B. thuringiensis* used. For example, Tabashnik and colleagues used Dipel 2× in their studies (Tabashnik et al. 1992, 1995), whereas we used Javelin. In our experience, Javelin tends to show higher resistance levels that Dipel 2× when tested on the same colony (Shelton et al. 1993), increasing the separation between resistant and susceptible strains and improving the discrimination between genotypes.

Although we do not know why resistance in the unselected Loxa A colony showed an initial 3.2-fold drop from generations 2 to 3 (LC_{50} dropped from 513 to 162 ppm), it is possible that a 2nd gene, conferring very high levels of resistance, was involved. Inheritance of this factor was demonstrated when we were able to reselect for similar high levels of resistance early, after the colony was brought in from the field. Any other information regarding the genetic basis of this factor, however, was not obtained because of its instability. Its disappearance within 1 or 2 generations from the time selection was removed also would explain why we were able to detect the presence of only one gene responsible for the 300-fold, stable level of resistance. If this 2nd gene conferring very high levels of resistance were eventually lost from the colony, then a 2nd selection would not produce an increase in resistance, similar to what we observed in our 8th generation selection. Additional selections, however, would verify whether the factor was indeed lost.

In the unselected Loxa A colony, the 300-fold level of resistance proved to be fairly stable between generations 3 through 10. This suggests that the major gene for resistance was inherently stable and did not cause significant reductions in fitness. Another possible explanation, however, was that resistance had become fixed in the original field population such that the colony collected was, in fact, completely homozygous for resistance (see Tabashnik et al. 1995). Therefore, it was important for us to examine the stability of resistance in subcolonies started from F_1 individuals (resistant × susceptible progeny). These greenhouse studies showed that, even in hybridized populations, resistance was still stable in the F_2 through F_6 generations and, therefore, free from antagonistic pleiotropic effects. Reduced fitness of resistant individuals, however, was determined to be the cause of instability in a subcolony of the Hawaiian NO-Q colony (Groeters et al. 1994).

Given these similarities and differences, do we still believe our earlier suspicion that the same major locus for resistance is involved in these geographically distinct colonies of *P. xylostella*? Although the uncertainty in the number of genes involved could be caused by imprecision of the leaf dip bioassay, it is conceivable that different alleles at the same locus were responsible for the differences in recessiveness and stability, and that depending upon the allele, varying degrees of recessiveness were conferred. It is tempting to envision several different DNA base changes that could dramatically reduce toxin binding to the midgut receptor and still have varying effects on the natural function of the midgut receptor in the insect; thus, the differences in fitness. The presence of antagonistic pleiotropic effects would depend upon how severely impaired the natural function was or to what degree other factors in the midgut could compensate for the impairment.

The most important implication of this data, however, is that 1, if not more, allele conferring high levels of resistance (300-fold) to the CryIA toxin already exists at high frequencies in field populations of *P. xylostella* in Florida, a major crucifer-growing area in the United States. This situation occurred after the frequent use of foliar sprays (Shelton et al. 1993), but also forewarns of problems to come if transgenic plants expressing single toxins of *B. thuringiensis* are introduced. Even if the types of toxins expressed by the plants were changed, we would expect resistance to evolve for each family of *B. thuringiensis* toxins as they were deployed.

Work with our susceptible and Loxa A diamondback moth colonies on transgenic broccoli (Metz et al. 1995) has already demonstrated that the level of expression of CryIA(c) in the plant is equivalent to that found in a high-dose strategy (Tabashnik and Croft 1982) (i.e., one that kills all homozygous-susceptible and heterozygous larvae but does not kill homozygous-resistant larvae). Although high dose strategies can slow resistance, they are only successful when resistance is still vary rare (alleles exist in the population mainly in the heterozygous state) and when there is immigration or preservation of susceptible larvae (Tabashnik and Croft 1982, Roush 1994). Simulations have shown that if any of these conditions are violated, a high-dose strategy may, in fact, accelerate rather than retard resistance (Tabashnik and Croft 1982). Clearly, when transgenic plants expressing single *B. thuringiensis* toxins are deployed, strategies that maintain refuges or use other methods of maintaining susceptibility must continue to be part of our regular resistance management practice.

Acknowledgments

We thank J. Cooley and B. Mitchell for technical assistance, T. D. Metz and E. D. Earle for providing the CryIA(c)-expressing broccoli, and Monsanto for providing the CryIA(c) gene used in the broccoli transformations. We are grateful for the financial support received from the Bt Management Working Group, USDA NRI Grant No. 91-37302-6199, and from BARD—The United States–Israel Binational Agricultural

Research and Development Fund (Postdoctoral Fellowship Award No. FI-0184-94).

References Cited

Ballester, V., B. Escriche, J. L. Ménsua, G. W. Riethmacher, and J. Ferré. 1994. Lack of cross resistance to other *Bacillus thuringiensis* crystal proteins in a population of *Plutella xylostella* highly resistant to CryIA(b). Biocontrol Sci. Technol. 4: 437–443.

Ferré, 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.

Ferré, J., B. Escriche, Y. Bel, and J. Van Rie. 1995. Biochemistry and genetics of insect resistance to *Bacillus thuringiensis* insecticidal crystal proteins. FEMS Microbiol. Lett. 132: 1–7.

Finney, D. J. 1971. Probit analysis, 3rd ed. Cambridge University Press, Cambridge.

Gould, F., A. Martinez-Ramirez, A. Anderson, J. Ferré, 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.

Groeters, F. R., B. E. Tabashnik, N. Finson, and M. W. Johnson. 1994. Fitness costs of resistance to *Bacillus thuringiensis* in the diamondback moth (*Plutella xylostella*). Evolution 48: 197–201.

Hama, H., K. Suzuki, and H. Tanaka. 1992. Inheritance and stability of resistance to *Bacillus thuringiensis* formulations in the diamondback moth, *Plutella xylostella* (Linnaeus) (Lepidoptera: Yponomeutidae). Appl. Entomol. Zool. 27: 355–362.

Heckel, D. G. 1994. The complex genetic basis of resistance to *Bacillus thuringiensis* toxin in insects. Biocontrol Sci. Technol. 4: 405–417.

Koziel, M. G., N. B. Carozzi, T. C. Currier, G. W. Warren, and S. V. Evola. 1993. The insecticidal crystal protein of *Bacillus thuringiensis*: past, present, and future uses. Biotechnol. Genet. Eng. Rev. 11: 171–228.

Littel, R. C., R. J. Freund, and P. C. Spector. 1993. SAS system for linear models, 3rd ed. SAS Institute, Cary, NC.

MacIntosh, S. C., T. B. Stone, R. S. Jokerst, and R. L. Fuchs. 1991. Binding of *Bacillus thuringiensis* proteins to a laboratory-selected line of *Heliothis virescens*. Proc. Natl. Acad. Sci. U.S.A. 88: 8930–8933.

Martinez-Ramirez, A. C., B. Escriche, M. D. Real, F. J. Silva, and J. Ferré. 1995. Inheritance of resistance to a *Bacillus thuringiensis* toxin in a field population of diamondback moth (*Plutella xylostella*). Pestic. Sci. 43: 115–120.

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

1985b. Evaluation of *Bacillus thuringiensis* for controlling Indianmeal moths (Lepidoptera: Pyralidae) in farm grain bins and elevator silos. J. Econ. Entomol. 78: 1089–1094.

McGaughey, W. H., and R. W. Beeman. 1988. Resistance to *Bacillus thuringiensis* in colonies of Indianmeal moth and almond moth (Lepidoptera: Pyralidae). J. Econ. Entomol. 81: 28–33.

Metcalf, R. L. 1980. Changing role of insecticides in crop protection. Annu. Rev. Entomol. 25: 219–256.

Metz, T. D., R. T. Roush, J. D. Tang, A. M. Shelton, and E. D. Earle. 1995. Transgenic broccoli expressing a *Bacillus thuringiensis* insecticidal crystal protein: implications for pest resistance management strategies. Mol. Breeding 1: 309–317.

Preisler, H. K., M. A. Hoy, and J. L. Robertson. 1990. Statistical analysis of modes of inheritance for pesticide resistance. J. Econ. Entomol. 83: 1649–1655.

Rosenheim J. A., and M. A. Hoy. 1989. Confidence intervals for the Abbott's formula correction of bioassay data for control response. J. Econ. Entomol. 82: 331–335.

Roush, R. T. 1989. Designing resistance management programs: how can you choose? Pestic. Sci. 26: 423–441.

1994. Managing pests and resistance to *Bacillus thuringiensis*: can transgenic crops be better than sprays? Biocontrol Sci. Technol. 4: 501–516.

Roush, R. T., and J. A. McKenzie. 1987. Ecological genetics of insecticide and acaricide resistance. Annu. Rev. Entomol. 32: 361–380.

Russell, R. M., J. L. Robertson, and N. E. Savin. 1977. POLO: a new computer program for probit analysis. Bull. Entomol. Soc. Am. 23: 209–213.

Shelton, A. M., R. J. Cooley, M. K. Kroening, W. T. Wilsey, and S. D. Eigenbrode. 1991. Comparative analysis of two rearing procedures for diamondback moth (Lepidoptera: Plutellidae). J. Entomol. Sci. 26: 17–26.

Shelton, A. M., J. L. Robertson, J. D. Tang, C. Perez, S. D. Eigenbrode, H. K. Preisler, W. T. Wilsey, and R. J. Cooley. 1993. Resistance of diamondback moth (Lepidoptera: Plutellidae) to *Bacillus thuringiensis* subspecies in the field. J. Econ. Entomol. 86: 697–705.

Sims, S. R., and T. B. Stone. 1991. Genetic basis of tobacco budworm resistance to an engineered *Pseudomonas fluorescens* expressing the δ-endotoxin of *Bacillus thuringiensis kurstaki*. J. Invertebr. Pathol. 57: 206–210.

Sokal, R. R., and R. L. Rohlf. 1981. Biometry, 2nd ed. Freeman, New York.

Stone, T. B., S. R. Sims, and P. G. Marrone. 1989. Selection of tobacco budworm for resistance to a genetically engineered *Pseudomonas fluorescens* containing the δ-endotoxin of *Bacillus thuringiensis* subsp. *kurstaki*. J. Invertebr. Pathol. 53: 228–234.

Tabashnik, B. E. 1991. Determining the mode of inheritance of pesticide resistance with backcross experiments. J. Econ. Entomol. 84: 703–712.

Tabashnik, B. E., and B. A. Croft. 1982. Managing pesticide resistance in crop–arthropod complexes: interactions between biological and operational factors. Environ. Entomol. 11: 1137–1144.

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, and M. W. Johnson. 1991. Managing resistance to *Bacillus thuringiensis*:

lessons from the diamondback moth (Lepidoptera: Plutellidae). J. Econ. Entomol. 81: 49–55.

Tabashnik, B. E., J. M. Schwartz, N. Finson, and M. W. Johnson. 1992. Inheritance of resistance to *Bacillus thuringiensis* in diamondback moth (Lepidoptera: Plutellidae). J. Econ. Entomol. 85: 1046–1055.

Tabashnik, B. E., N. Finson, M. W. Johnson, and W. J. Moar. 1993. Resistance to toxins from *Bacillus thuringiensis* subsp. *kurstaki* causes minimal cross resistance to *B. thuringiensis* subsp. *aizawai* in the diamondback moth (Lepidoptera: Plutellidae). Appl. Environ. Microbiol. 59: 1332–1335.

Tabashnik, B. E., N. Finson, F. R. Groeters, W. J. Moar, M. W. Johnson, K. Luo, and M. J. Adang. 1994a. Reversal of resistance to *Bacillus thuringiensis* in *Plutella xylostella*. Proc. Natl. Acad. Sci. U.S.A. 91: 4120–4124.

Tabashnik, B. E., N. Finson, M. W. Johnson, and D. G. Heckel. 1994b. Cross-resistance to *Bacillus thuringiensis* toxin CryIF in the diamondback moth (*Plutella xylostella*). Appl. Environ. Microbiol. 60: 4627–4629.

Tabashnik, B. E., N. Finson, M. W. Johnson, and D. G. Heckel. 1995. Prolonged selection affects stability of resistance to *Bacillus thuringiensis* in diamondback moth (Lepidoptera: Plutellidae). J. Econ. Entomol. 88: 219–224.

Tang, J. D., A. M. Shelton, J. Van Rie, S. De Roeck, W. J. Moar, R. T. Roush, and M. Peferoen. 1996. Toxicity of *Bacillus thuringiensis* spore and crystal protein to the resistant diamondback moth (*Plutella xylostella*). Appl. Environ. Microbiol. 62: 564–569.

Received for publication 9 February 1996; accepted 7 November 1996.