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Broad-spectrum resistance to *Bacillus thuringiensis* toxins in *Heliothis virescens*

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ABSTRACT **Evolution of pest resistance to insecticidal proteins produced by *Bacillus thuringiensis* (*Bt*) would decrease our ability to control agricultural pests with genetically engineered crops designed to express genes coding for these proteins. Previous genetic and biochemical analyses of insect strains with resistance to *Bt* toxins indicate that (*i*) resistance is restricted to single groups of related *Bt* toxins, (*ii*) decreased toxin sensitivity is associated with changes in *Bt*-toxin binding to sites in brush-border membrane vesicles of the larval midgut, and (*iii*) resistance is inherited as a partially or fully recessive trait. If these three characteristics were common to all resistant insects, specific crop-variety deployment strategies could significantly diminish problems associated with resistance in field populations of pests. We present data on *Bt*-toxin resistance in *Heliothis virescens*, a major agricultural pest targeted for control with *Bt*-toxin-producing crops. A laboratory strain of *H. virescens* developed resistance in response to selection with the *Bt* toxin CryIA(c). In contrast to other cases of Bt-toxin resistance, this *H. virescens* strain exhibits cross-resistance to *Bt* toxins that differ significantly in structure and activity. Furthermore, the resistance in this strain is not accompanied by significant changes in toxin binding, and resistance is inherited as an additive trait when larvae are treated with high doses of CryIA(c) toxin. These findings have important implications for *Bt*-toxin-based pest control.**

One of the few microbes that has been used successfully in agricultural insect pest control is *Bacillus thuringiensis* (*Bt*). Liquid and powder formulations of this bacterium hold a small but growing share of the pest-control-agent market (1). Genes from *Bt* that code for production of a set of insecticidal proteins are being cloned and transferred to a number of crop plants (2–4). Expression of *Bt* genes in crop plants is appealing because the toxicity of the proteins coded for by these genes is restricted to specific groups of insects (5) and because plants producing these toxins are not expected to interfere with the action of natural enemies of pests (e.g., refs. 6 and 7).

Many toxic proteins with varying degrees of homology in amino acid sequence have been isolated from worldwide collections of *Bt* strains (8). At least nine distinct proteins have been characterized that are toxic to lepidopteran caterpillars (8). Toxicity of these proteins is generally related to their ability to bind to receptors in the larval midgut, and it has been demonstrated that within a single insect, there may be different receptors for different *Bt* toxins (9–11).

Excitement over the potential of engineered plants with *Bt* genes has been tempered by laboratory and field work which indicates that pest insects have the capacity to adapt to *Bt* and its toxic proteins (34). However, studies of insect resistance to *Bt* toxins have found that resistance is toxin-specific. Indian meal moths that were >100-fold resistant to the *Bt* toxin CryIA(b) were not at all resistant to CryIC (12). Diamondback moths that were >200-fold resistant to CryIA(b) were not resistant to CryIB or CryIC (13). A strain of *Heliothis virescens* selected with CryIA(b) and a mixture of CryIA and CryIIA proteins in an HD-1 strain of *Bt* was significantly resistant to only some strains of *Bt* (14). Restriction of resistance to a single or highly related group of toxins may be explained by studies indicating that the biochemical basis for resistance includes changes in receptors in the insect midgut (12, 13, 15).

While high levels of resistance are cause for concern, the specificity of resistance has led to a belief that as insects become resistant to one *Bt* toxin, that toxin could be successfully replaced by a different *Bt* toxin (16). Additionally, genetic analyses of *Bt* resistance have demonstrated that the resistance is usually inherited as a mostly recessive trait (14, 15, 17–20). The rate at which such recessive traits become established in a population can be significantly decreased by the proper use of *Bt*-toxin-producing plants, so these genetic results have prompted the development of specific "resistance management" strategies (21, 22).

We report here on *Bt*-toxin resistance in a strain of *H. virescens*, a pest of cotton, soybean, tobacco, tomato, and other agricultural crops. The resistance in our strain of *H. virescens* is not toxin-specific, does not appear to be related to changes in midgut receptors, and is not inherited as a recessive trait when larvae are exposed to high doses of *Bt* toxin.

EXPERIMENTAL PROCEDURES

Design of Selection Experiment. As part of a larger study on insect resistance to *Bt* toxins, a strain of *H. virescens* was selected for survival on an artificial diet containing CryIA(c). The selected strain and the control strain were initiated from a sample of a field population collected in July 1988. Precautions, which are described below, were taken to avoid genetic bottlenecks during the initiation of the strains.

The individually laid eggs of *H. virescens* were collected from three tobacco fields near Carpenter, North Carolina, over a period of 4 days. A total of 700 neonates from these collection sites were reared in the laboratory on artificial diet, in individual 25-ml cups (23). Adults that emerged from this F_0 generation were divided equally among 25 oviposition containers. After *ca.* 5 days of oviposition, 2 moths from each of the oviposition containers were checked for disease. Seventy F_1 larvae from each container were reared on artificial diet (i.e., a total of 1750 larvae). The first 50 larvae to pupate from each set of 70 were placed in oviposition

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Abbreviations: *Bt*, *Bacillus thuringiensis*; BBMV, brush-border membrane vesicle.

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containers. This rearing regime was continued through the F_3 generation to permit the field strain to adapt to laboratory conditions (see ref. 24).

The selection experiment was begun in the F_4 generation, using an equal number of larvae from each of the initially isolated groups. A temporally staggered control strain was developed over time so that neonate larvae were always available for testing. The total number of larvae reared per generation in this control strain was generally between 400 and 720. Larvae from the selected strain, CP73-3, were placed on a diet containing purified toxin from *Bt* strain HD-73, which only produces CryIA(c). Selection was carried out in each generation unless the vigor of the colony was judged to be low or egg production was insufficient. The CryIA(c) protein concentration was adjusted to between 0.15 and 0.4 μg per ml of diet, depending on the tolerance of the colony and the quality of the lot of toxin being used. Percent survival was recorded after an average of 5.4 days, and larvae were then transferred to normal diet to complete their development.

Preparation of *Bt* Toxins. The CryIA(c) toxin was purified from HD-73 with techniques described by Hofte *et al.* (25). The *Bt* toxins tested in the cross-resistance study were CryIA(a), CryIA(b), CryIB, CryIC, and CryIIA. The CryIA(a) and CryIA(b) toxins were cloned and produced as 133-kDa and 130-kDa proteins in a recombinant *Escherichia coli* system described by Hofte *et al.* (25, 26). CryIB was cloned from *Bt* var. *entomocidus* HD-110. Its sequence is identical to that published by Brizzard and Whiteley (27). CryIC was also cloned from HD-110. It differed slightly from the CryIC gene isolated by Honee *et al.* (28) as described by Ferre *et al.* (13). These proteins were truncated to their toxic form by trypsin digestion (26). M. Peferoen and B. Lambert (Plant Genetic Systems, Ghent, Belgium) provided these proteins as a gift. The 65-kDa CryIIA protein was cloned and produced in *E. coli* by the method of Moar *et al.* (29). Its nucleotide sequence is identical to that of other cloned CryIIA genes (29). Purity of CryI and CryII proteins was judged to be >80%, based on SDS/PAGE (see ref. 29).

Testing Resistance and Cross-Resistance. Degree of resistance to CryIA(c) was first tested after four generations of selection (30–40 days per generation). Tests for resistance to other toxins were conducted between generations 13 and 15 of selection. Probit analysis (30) of larval survival over a range of doses (three to six) was used for assessing degree of resistance, except in the first tests with CryIA(c), where a single dose was used, and in tests with toxins that caused <50% mortality of the control strain, even at relatively high concentrations (50 μg/ml of diet). For these toxins, growth rates on diets containing a range of toxin concentrations were used as a measure of resistance.

Progeny from reciprocal crosses of the selected strain and the control strain were tested as neonates on five concentrations of CryIA(c). These crosses were replicated twice. The tolerance of these F_1 progeny was compared with that of simultaneously tested progeny of the two parental lines.

Receptor Binding of CryIA(c) and CryIA(b). The affinity of CryIA(c) and CryIA(b) for membrane receptors in the midgut, as well as the concentration of these receptors, was studied to determine whether the two strains differed in toxin-binding properties. Midguts of fifth-instar larvae of the two *H. virescens* strains were examined using techniques described by Ferre *et al.* (13). Brush-border membrane vesicles (BBMVs) were prepared from a total of 1.5 g and 1.7 g (wet weight) of midgut material from the control and selected lines, respectively. The final yield was 2 mg of vesicle protein per gram of midgut tissue, as determined by using the Bradford (31) assay reagents from Bio-Rad. When CryIA(c) was tested, the apparent dissociation constant (K_d) of the receptor–ligand complex and the binding-site concentration (R_t) in the BBMV preparations were determined from competition experiments (12) using duplicate samples of BBMVs (100 μg/ml), 6 nM labeled CryIA(c), and a range of concentrations of unlabeled CryIA(c) in 100 μl (final volume) of phosphate-buffered saline (8 mM Na_2HPO_4/2 mM KH_2PO_4/150 mM NaCl, pH 7.4) with 0.1% bovine serum albumin. After a 90-min incubation at room temperature, the reaction mixtures were filtered through glass-fiber filters. The radioactivity retained in the filters was measured in an LKB 1282 γ counter. When CryIA(b) was used, the concentration of BBMV protein was 80 μg/ml and that of labeled CryIA(b) was 10 nM.

Binding data were then analyzed with the LIGAND computer program (32), which calculates the bound concentration of labeled ligand as a function of the total concentration of labeled and unlabeled ligand. Through an iterative process, the program adjusts the estimated initial values of K_d, R_t, and nonspecific binding until the curve approximates the experimental curve as closely as possible. A *t* test was used to determine whether the mean values of the binding characteristics of the two insect strains were significantly different.

RESULTS

The *H. virescens* strain selected with CryIA(c) toxin was challenged on toxic diet for 17 of the 22 generations of the experiment (up to 26 August 1991). The average mortality per generation was 74.4%, and the average number of pupae per selected generation was 125.4. The effective population size could not be computed, because it was not possible to estimate the variance in number of eggs laid per female.

After four generations of selection, survival of the control and selected strains had begun to diverge, with 9.3% survival of the control and 22.0% survival of the selected strain after 6 days on 0.40-μg/ml CryIA(c). In generation 6, survival after 7 days was 2.5% for controls and 15% for the selected strain. Tests conducted subsequent to generation 6 involved multiple toxin concentrations, and the results are summarized in Fig. 1. After 10 generations, the ratio of the LC_{50} of the selected strain to the LC_{50} of the control strain (resistance ratio) was about 10. The resistance ratio of the selected and control strains increased to 50 after 17 generations of selection.

Cross-Resistance. The LC_{50} of the selected strain on CryIA(b) was 13 times higher than that of the control strain (Table 1). For CryIIA, the LC_{50} of the selected strain was 53 times higher than that of the control (Table 1). When larvae of the selected and control strains were reared on varying concentrations of Cry-

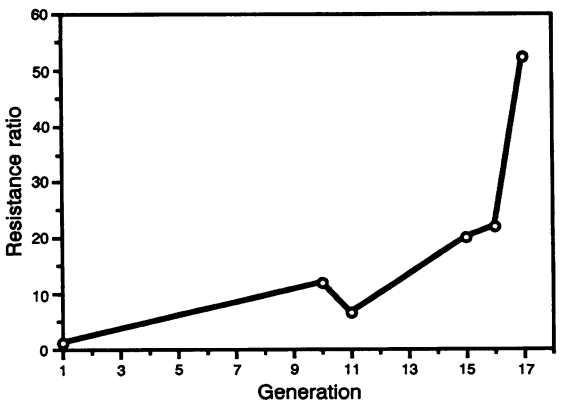

FIG. 1. The CryIA(c) resistance ratio of the *H. virescens* strain selected on CryIA(c) and the control strain as a function of the number of generations of selection. The resistance ratio is calculated based on the LC_{50} values of the two strains exposed to CryIA(c) as neonates.

Table 1. Resistance of control and selected *H. virescens* strains to *Bt* toxins

Toxin	Strain	LC_{50}, μg/ml	Lower 95% C.I.	Upper 95% C.I.	Slope
CryIA(b)	Control	1.12	0.70	1.64	1.38
	Selected	14.51	10.20	20.72	1.18
CryIIA	Control	0.30	0.10	0.61	0.91
	Selected	15.84	12.42	20.05	1.23

C.I., confidence interval.

IA(a), CryIB, and CryIC, the selected strain always grew significantly faster (Fig. 2).

Genetic Crosses. When F_1 reciprocal crosses were tested on CryIA(c), their LC_{50} values and the slopes of their toxin-concentration response lines were almost identical (Fig. 3*A*), eliminating the possibility of sex linkage. Compared with the parental strains, slopes for the hybrids were more shallow. At low CryIA(c) concentrations, survival of the hybrids was similar to that of the control strain (Fig. 3*A*), or at least more similar to survival of the selected strain (Fig. 3*B*). At higher CryIA(c) concentrations, the mortality of the hybrids was intermediate between those of the selected and control strains (Fig. 3).

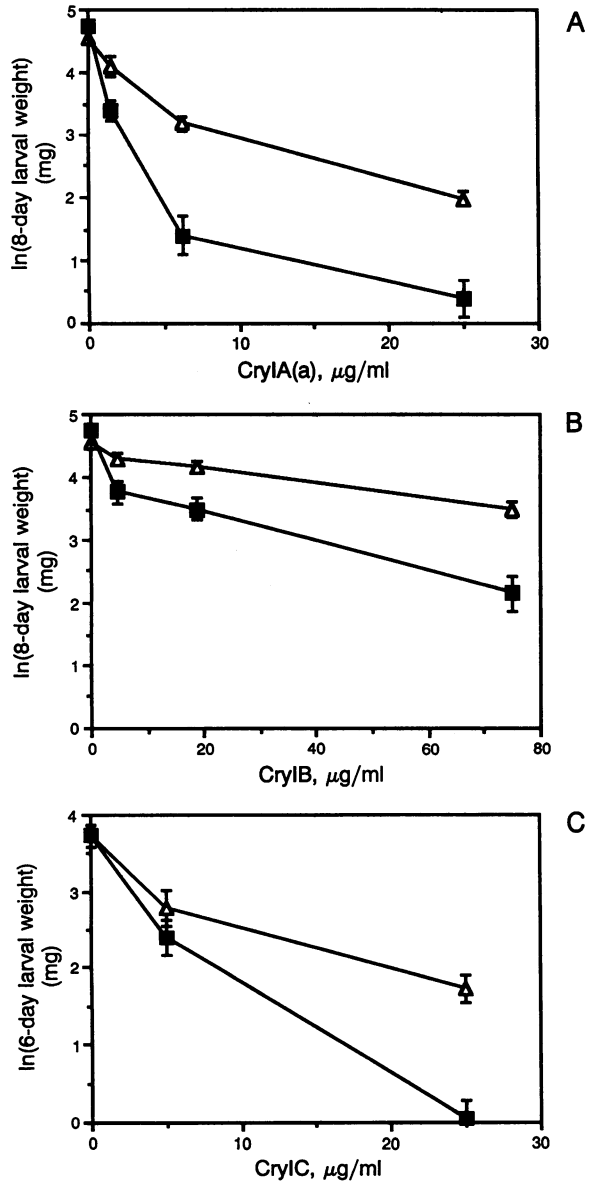

FIG. 2. Growth of larvae of the control (■) and selected (△) *H. virescens* strains exposed to various concentrations of CryIA(a) for 8 days (*A*), CryIB for 8 days (*B*), or CryIC for 6 days (*C*).

Table 2. Concentration of binding sites (R_t) and apparent equilibrium dissociation constant (K_d) of *Bt* crystal proteins incubated with BBMVs of *H. virescens*

Toxin	Strain	K_d, nM	R_t, pmol/mg of vesicle protein	R_t/K_d
CryIA(c)	Control	0.95 ± 0.15	3.40 ± 0.24	3.57
	Selected	0.76 ± 0.17	2.90 ± 0.40	3.80
CryIA(b)	Control	2.60 ± 1.20	3.30 ± 1.80	1.27
	Selected	1.10 ± 0.40	1.00 ± 0.20	0.91

K_d and R_t values (mean ± SE) were calculated from homologous competition experiments.

Binding Studies. The K_d and R_t of each strain for CryIA(c) and CryIA(b) were calculated from three separate competition experiments per toxin (Fig. 4). The means and standard errors of the calculated K_d and R_t values for the control and selected strains are presented in Table 2. Neither the apparent K_d nor the R_t differ at $\alpha = 0.05$ for either toxin. Values of the R_t/K_d coefficient [which estimates the overall binding affinity of the vesicles for the CryIA(c) and CryIA(b) proteins] appear very similar for both strains.

DISCUSSION

Results of this study indicate that selection of *H. virescens* with a single type of *Bt* toxin can lead to broad-spectrum *Bt* resistance. The finding of cross-resistance between CryIA(c) and CryIA(b) is not surprising, because these two toxins are similar in structure and toxicity. Of all the evidence regarding cross-resistance, that between CryIA(c) and CryIIA was the least expected. The amino acid sequence of CryIIA is very different from that of CryIA(c) (8, 29). Furthermore, recent studies on the mode of action of CryIIA in *Helicoverpa zea*[¶] indicate that in contrast to previously studied *Bt* toxins, CryIIA does not show saturable binding to BBMVs. When CryIIA was incubated with BBMVs, it did not inhibit subsequent binding of CryIA(c), but when CryIA(c) was incubated with BBMVs, it inhibited the nonsaturable binding of CryII(a). Additionally, CryIIA did not lead to the voltage-independent cation-selective channels in planar lipid bilayers, characteristic of CryIA(c).[¶]

Binding studies with CryIA(c) and CryIA(b) indicated that the resistance was not due to changes in BBMV-saturable binding characteristics. The existence of strong cross-resistance to CryIIA, which does not show saturable binding, would not have been expected if the resistance were only due to saturable binding changes. Our results contrast with those of MacIntosh *et al.* (15), who found that an *H. virescens* strain selected with CryIA(b), and with an HD-1 strain that produces multiple toxins (14, 15), had a lower affinity for CryIA(b) and more receptors per mg of protein than a susceptible strain. We found no significant differences in CryIA(b) binding characteristics of our strains, but the trend in our data was for the resistant strain to have higher affinity and fewer receptors than the susceptible strain. This information, as well as the cross-resistance data, suggest that the mechanism of resistance in our strain is different from that of the strain tested by MacIntosh *et al.* (15). The resistant strain tested by MacIntosh *et al.* (15) had 71-fold resistance to CryIA(b) and only 16-fold resistance to CryIA(c), whereas our strain was more resistant to CryIA(c) than to CryIA(b).

[¶]English, L., Robbins, H. L. & Slatin, S., First International Conference on Molecular Biology of *Bacillus thuringiensis*, eds. Aronson, A., Bulla, L., Carlton, B. & Rapaport, G., July 26–29, 1991, San Francisco, p. 19 (abstr.).

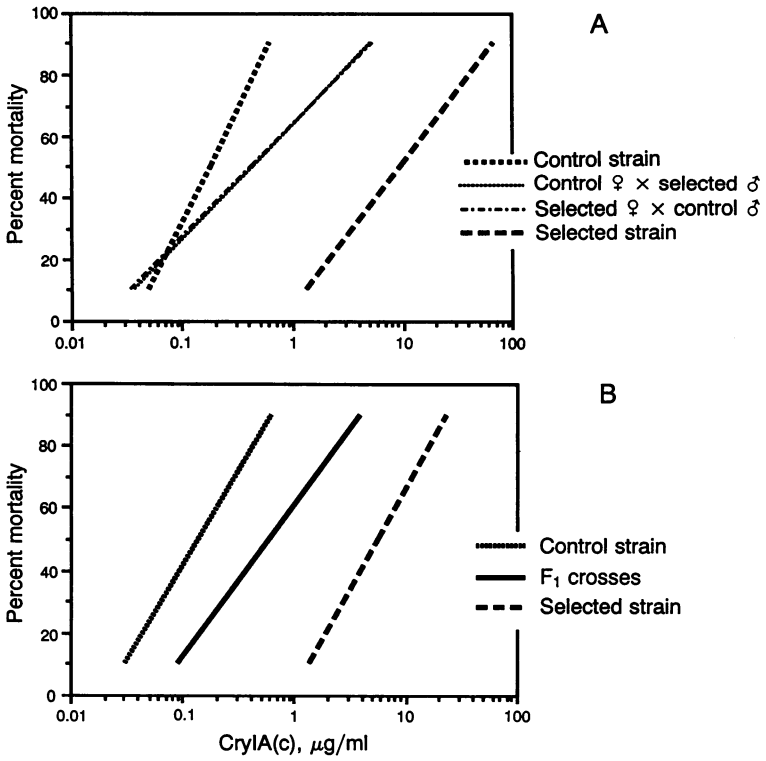

FIG. 3. Percent mortality of the control and selected strains as well as progeny from crosses as a function of the CryIA(c) concentration (note $\log_{10}$ scale) in their diet. (*A*) First cross with reciprocal crosses tested separately. Mortality lines for the progeny of the reciprocal crosses are nearly identical. (*B*) Second cross with reciprocals tested together.

Results of the genetic crosses indicate that at toxin concentrations that kill only 10–20% of the susceptible line, resistance is at least partially recessive. However, at higher concentrations that kill 80–90% of the susceptible strain, resistance is inherited as an additive trait. If plants are engineered to produce enough toxin to kill most of the susceptible larvae and are planted over large areas, response to selection would be rapid if larvae with such intermediate *Bt*-toxin tolerance were present (21). We have not yet determined the number of loci involved in resistance.

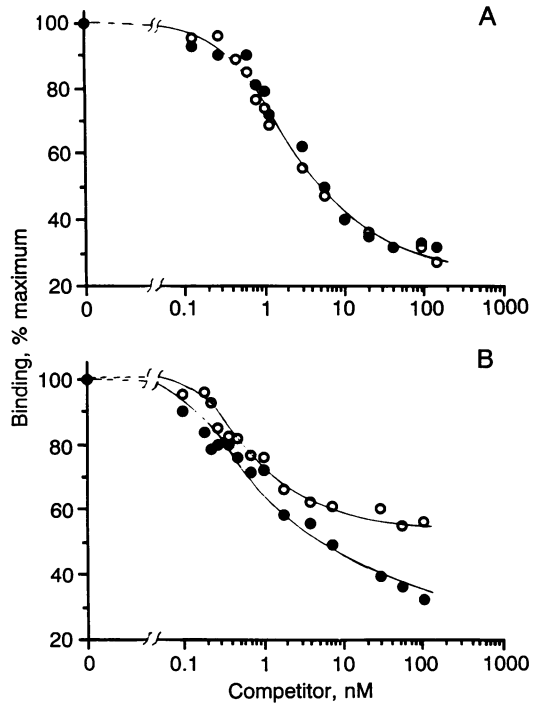

FIG. 4. (*A*) Binding of ^{125}I-labeled CryIA(c) to BBMVs at various concentrations of nonlabeled CryIA(c) competitor. (*B*) Binding of ^{125}I-labeled CryIA(b) at various concentrations of nonlabeled CryIA(b). ●, Susceptible strain; ○, resistant strain.

Previous studies of *Bt*-toxin resistance found that resistance was restricted to a single type of toxin. This led to a hypothesis that it would be possible to replace one *Bt* toxin with another as insects adapted to specific toxins (12, 16). Our findings clearly indicate that selection with a single *Bt* toxin could lead to broadly based resistance that would preclude control of an insect population with any *Bt* product.

There are now two cases in which resistance to *Bt* has been clearly shown to be restricted to a single class of *Bt* toxins (12, 13) and one case in which resistance is not specific (this study). More studies of cross-resistance patterns and of the mechanisms of resistance will be needed before any generalizations can be made. Until then, we cannot assume that discoveries of new *Bt* toxins with unique biochemical properties will decrease the consequences of misusing *Bt*.

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1. Gelernter, W. D. (1990) in *Managing Resistance to Agrochemicals*, ACS Symposium Series 421 (Am. Chem. Soc., Washington).
2. Gasser, C. S. & Fraley, R. T. (1989) *Science* **244**, 1293–1299.
3. Meeusen, R. L. & Warren, G. (1989) *Annu. Rev. Entomol.* **34**, 373–381.

4. Perlak, F. J., Fuchs, R. L., Dean, D. A., McPherson, S. L. & Fischhoff, D. A. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 3324–3328.
5. Van Rie, J. (1991) *Trends Biotechnol.* **9,** 177–179.
6. Johnson, M. T. & Gould, F. (1992) *Environ. Entomol.* **21,** 586–597.
7. Thoms, E. M. & Watson, T. F. (1986) *J. Invert. Pathol.* **47,** 178–183.
8. Hofte, H. & Whiteley, H. R. (1989) *Microbiol. Rev.* **53,** 242–255.
9. Hoffman, C., Luthy, P., Hutter, P. & Pliska, V. (1988) *J. Biochem. (Tokyo)* **173,** 85–91.
10. Van Rie, J., Jansens, S., Hofte, H., Degheele, D. & Van Mellaert, H. (1989) *Eur. J. Biochem.* **186,** 239–247.
11. Wolfersberger, M. G. (1990) *Experientia* **46,** 475–477.
12. Van Rie, J., McGaughey, W. H., Johnson, D. E., Barnett, B. D. & Van Mellaert, H. (1990) *Science* **247,** 72–74.
13. Ferre, J., Real, M. D., Van Rie, J., Jansens, S. & Peferoen, M. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 5119–5123.
14. Marrone, P. G. & MacIntosh, S. C. in *Bacillus thuringiensis: An Environmental Biopesticide*, eds. Entwistle, P. F., Cory, J. S., Bailey, M. J. & Higgs, S. R. (Wiley, New York), in press.
15. MacIntosh, S. C., Stone, T. B., Jokerst, R. S. & Fuchs, R. L. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 8930–8933.
16. Cutler, K. (1991) *Ag. Biotech. News* Jan./Feb., p. 7.
17. McGaughey, W. H. & Beeman, R. W. (1988) *J. Econ. Entomol.* **81,** 28–33.
18. Sims, S. R. & Stone, T. B. (1991) *J. Invert. Pathol.* **57,** 206–210.
19. Martinez-Ramirez, A. C., Escriche, B., Real, M. D., Silva, F. J. & Ferre, J., *Pestic. Sci.*, in press.
20. Tabashnik, B. E., Schwartz, J. M., Finson, N. & Johnson, M. W. (1992) *J. Econ. Entomol.* **85,** 1046–1055.
21. Gould, F. (1986) *Environ. Entomol.* **15,** 1–10.
22. Gould, F. (1988) *BioScience* **38,** 26–33.
23. Burton, R. L. (1970) *J. Econ. Entomol.* **63,** 1969–1970.
24. Service, P. M. & Rose, H. A. (1985) *Evolution* **39,** 943–945.
25. Hofte, H., de Greve, H., Seurinck, J., Jansens, S., Mahillon, J., Ampe, C., Vandekerckhove, J., Vanderbruggen, H., van Montagu, M., Zabeau, M. & Vaeck, M. (1986) *Eur. J. Biochem.* **161,** 273–280.
26. Hofte, H., Van Rie, J., Jansens, S., Van Houtven, A., Vanderbruggen, H. & Vaeck, M. (1988) *Appl. Environ. Microbiol.* **54,** 2010–2017.
27. Brizzard, B. L. & Whiteley, H. R. (1988) *Nucleic Acids Res.* **16,** 4168–4169.
28. Honee, G., van der Salm, T. & Visser, B. (1988) *Nucleic Acids Res.* **16,** 6240.
29. Moar, W. J. (1990) Ph.D. dissertation (Univ. of California, Riverside).
30. SAS Institute (1985) *SAS Users Guide: Statistics* (SAS Inst., Cary, NC).
31. Bradford, M. M. (1976) *Anal. Biochem.* **72,** 248–254.
32. Munson, P. J. & Rodbard, D. (1980) *Anal. Biochem.* **107,** 220–239.
33. Tabashnik, B. E., Cushing, N. L., Finson, N. & Johnson, M. W. (1990) *J. Econ. Entomol.* **83,** 1671–1676.