Contamination of refuges by *Bacillus thuringiensis* toxin genes from transgenic maize

Charles F. Chilcutt*† and Bruce E. Tabashnik‡

*Department of Entomology, Texas A&M University, 10345 Agnes Street, Corpus Christi, TX 78406; and ‡Department of Entomology, University of Arizona, Tucson, AZ 85721

Communicated by William S. Bowers, University of Arizona, Tucson, AZ, January 23, 2004 (received for review December 11, 2003)

Transgenic crops producing insecticidal toxins from *Bacillus thuringiensis* (Bt) are widely used to control pests, but their benefits will be lost if pests evolve resistance. The mandated high-dose/refuge strategy for delaying pest resistance requires planting refuges of toxin-free crops near Bt crops to promote survival of susceptible pests. We report that pollen-mediated gene flow up to 31 m from Bt maize caused low to moderate Bt toxin levels in kernels of non-Bt maize refuge plants. Immunoassays of non-Bt maize sampled from the field showed that the mean concentration of Bt toxin Cry1Ab in kernels and the percentage of kernels with Cry1Ab decreased with distance from Bt maize. The highest Bt toxin concentration in pooled kernels of non-Bt maize plants was 45% of the mean concentration in kernels from adjacent Bt maize plants. Most previous work on gene flow from transgenic crops has emphasized potential effects of transgene movement on wild relatives of crops, landraces, and organic plantings, whereas implications for pest resistance have been largely ignored. Variable Bt toxin production in seeds of refuge plants undermines the high-dose/refuge strategy and could accelerate pest resistance to Bt crops. Thus, guidelines should be revised to reduce gene flow between Bt crops and refuge plants.

Genetically modified crops that produce insecticidal proteins from *Bacillus thuringiensis* (Bt) kill some key pests, but their usefulness will be cut short if pests adapt. Pests have not yet evolved resistance to Bt crops in the field (1). However, many have been selected for resistance in the laboratory, and diamondback moth (*Plutella xylostella*) has evolved resistance to Bt sprays in the field (1–3). To counter the threat of resistance, the U. S. Environmental Protection Agency has mandated the "high-dose/refuge strategy" requiring farmers to grow toxin-free crop refuges near Bt crops (ref. 4 and www.epa.gov/pesticides/biopesticides/pips/bt_brad.htm). The purpose of toxin-free refuges is to promote survival of susceptible pests. Ideally, rare resistant adults emerging from Bt plants mate with relatively abundant susceptible adults from refuges, and their heterozygous progeny are killed by a high dose of toxin from Bt plants. Models predict that resistance will be delayed substantially if these assumptions hold (4), but pollen-mediated gene flow from Bt crop plants to refuge plants could disrupt this strategy.

Initial concerns about gene flow from transgenic crops emphasized movement of transgenes to wild relatives of crops, landraces, and organic plantings (www.epa.gov/pesticides/biopesticides/pips/bt_brad.htm and refs. 5–8), whereas a recent report noted contamination of the U.S. seed supply of conventional maize, soybean, and canola by DNA sequences from transgenic varieties of the same crops (9). However, potential effects of transgene movement on pest resistance have been largely overlooked. Gene flow to refuges from Bt maize and Bt cotton, which grew on >14 million hectares worldwide in 2002,§ could cause Bt toxin production in seeds of refuge plants. Such refuge contamination could affect major pests of both crops that feed partially or primarily on seeds, including the cotton pest pink bollworm (*Pectinophora gossypiella*) (10), the corn earworm (*Helicoverpa zea*), and the European corn borer (*Ostrinia nubilalis*), which attacks corn ears as well as stalks (11).

Because outcrossing is higher in maize than cotton (12), contamination of refuges by Bt genes is more likely in maize. Thus, we focused here on maize, a primarily wind-pollinated plant with fertilization occurring at up to 200 m (13). To test the hypothesis that gene flow from Bt maize causes Bt toxin production in non-Bt maize refuges, we sampled kernels from ears of non-Bt maize along transects near Bt maize. In each of two experiments, ELISA tests of non-Bt maize sampled from the field showed that the mean concentration of Bt toxin Cry1Ab in kernels and the percentage of kernels with Cry1Ab decreased with distance from Bt maize. These results imply that pollen-mediated gene flow from Bt maize caused Bt toxin production in some kernels of non-Bt maize refuge plants.

Materials and Methods

Maize Plants. We used six pairs of commercial maize hybrids, with a transgenic Bt hybrid (B5405Bt, N6800Bt, N79-L3Bt, N83-Z8Bt, P31B13Bt, and T1866Bt) and a near-isogenic non-Bt hybrid (B5405, N6800, N79-P4, N83-N5, P3223, and T1866) in each pair. All six transgenic hybrids produced Bt toxin Cry1Ab. Four Bt hybrids originated from the Bt11 insertion event (B5405Bt, N6800Bt, N79-L3Bt, and N83-Z8Bt) and two Bt hybrids from the Mon810 insertion event (P31B13Bt and T1866Bt). Each commercial Bt hybrid had been produced by crossing a Bt line (either Bt11 or Mon810) with a conventional line and backcrossing resulting Cry1Ab-producing plants with the conventional line for several generations, finally selfing all Bt-producing plants to produce a line homozygous for the *cry1Ab* gene. Each backcrossed Bt line had been crossed with a second conventional line to produce hybrid seed for planting that was hemizygous for the *cry1Ab* gene. Plants grown from this hybrid seed should carry the *cry1Ab* gene in half of their eggs and pollen. Thus, commercial Bt plants fertilized with Bt pollen are expected to have the *cry1Ab* gene in 75% of their seeds (50% hemizygous and 25% homozygous).

Field Experiment Design. We conducted two field experiments at the Texas Agricultural Research Station (Corpus Christi) in 2002. Each of two replicate plots was divided into two main plots 44 rows (0.97 m per row) wide by 56 m long. Each main plot was divided into six subplots with 1.5 m between them. Each subplot was 8 m long by 44 rows wide with ≈40 ears per row. In experiment 1, the first eight rows of each subplot were planted with one of the six Bt hybrids, and the adjacent 36 rows were planted with its near-isogenic non-Bt counterpart. Experiment 2 tested the effect of a barren gap of 15 m on gene flow. Each of six subplots was planted with a random mixture of all six Bt hybrids in the first eight rows. After a barren gap of 15 m, the next 29 rows were planted with one of the six non-Bt hybrids. In both

Abbreviation: Bt, *Bacillus thuringiensis*.

†To whom correspondence should be addressed. E-mail: c-chilcutt@tamu.edu.

§James, C. (2002) *Global Status of Commercialized Transgenic Crops: 2002* (International Service for the Acquisition of Agri-biotech Applications, Ithaca, NY), ISAAA Brief no. 27, preview.

www.pnas.org/cgi/doi/10.1073/pnas.0400546101

experiments, non-Bt maize was planted north of Bt maize. The predominant wind direction was from the southeast (16 kph mean daily wind speed), which favored gene flow from Bt maize to non-Bt maize.

Maize Sampling. In both experiments, ears were sampled from two rows of each Bt subplot (rows −1 and −3; 1 and 3 m south of the non-Bt subplot). Rows sampled from each non-Bt subplot were 1–4, 8, 16, 24, and 32 (1–31 m north of the Bt subplot) in the first experiment; and 16, 24, and 32 in the second experiment. From each subplot, 20 ears per sampled row were harvested when all hybrids were <15% moisture. Each sample was threshed to remove kernels. From each sample, 30 randomly selected kernels were set aside for testing of individual kernels to estimate the percentage of kernels producing Cry1Ab. The remaining kernels in each sample were ground into a coarse powder to estimate the concentration of Cry1Ab, as described below.

Cry1Ab Tests. The concentration of Cry1Ab in each ground sample was determined by using kits (EnviroLogix, Portland, ME) to perform ELISA. Ground samples (2 g) were placed in an extraction/dilution buffer for 24 h. Next, 100 μl of each extract and controls (0, 0.5, 1.5, and 5 ppb Cry1Ab) were added to test wells of ELISA plates coated with antibodies raised against Cry1Ab toxin. The wells were covered with parafilm, and plates were placed on an orbital shaker at 200 rpm to incubate (as was done in all steps) for 15 min followed by the addition of 100 μl of Cry1Ab-enzyme conjugate and incubation for 1 h. Next, contents of the wells were shaken out and wells washed three times with buffer (PBS, pH 7.4/Tween 20 in deionized water), then 100 μl of substrate (horseradish peroxidase-labeled Cry1Ab antibody) was added and incubated for 30 min, followed by the addition of 100 μl of stop solution (1.0 M hydrochloric acid). The optical density of each well was then read by using an Opsys MR microplate reader (450-nm wavelength), and Cry1Ab concentration was calculated for each sample. Because each sample contained kernels pooled from 20 ears per sampled row, each estimate of Cry1Ab concentration represents a composite for the kernels in each sampled row.

To estimate the percentage of kernels containing Cry1Ab in each subplot, 30 seeds from each sample were germinated, and leaf punches from each seedling were tested for Cry1Ab with qualitative ELISA by Mid-West Seed Services (Brookings, SD).

Data Analysis. We used analysis of covariance to test for the effects of non-Bt hybrid, Bt pollen source (Mon810 or Bt11), and distance from Bt planting (covariate) on Cry1Ab concentration and percentage of kernels with Cry1Ab. In Experiment 1, non-Bt hybrid did not interact with the covariate, but Bt pollen source did. Therefore, mean Cry1Ab concentrations were pooled by Bt pollen source for all comparisons. In experiment 2, non-Bt hybrid did not interact with the covariate. Therefore, means for all hybrids were pooled.

Results

Production of Bt toxin Cry1Ab in non-Bt maize occurred at up to 31 m from Bt maize, the greatest distance we examined in two field experiments (Figs. 1–3). The minimum distance between Bt and non-Bt maize was 1 m in experiment 1 and 15 m in experiment 2. In both experiments, ELISA tests of non-Bt maize showed that the mean concentration of Bt toxin Cry1Ab in kernels and the percentage of kernels with Cry1Ab decreased with distance from Bt maize (Figs. 1–3).

Experiment 1. The concentration of Bt toxin in non-Bt maize was highest within 2 m of Bt maize and decreased rapidly with distance (Fig. 1). Consistent with previous reports (14), the concentration of Cry1Ab in kernels of Bt hybrids was higher for

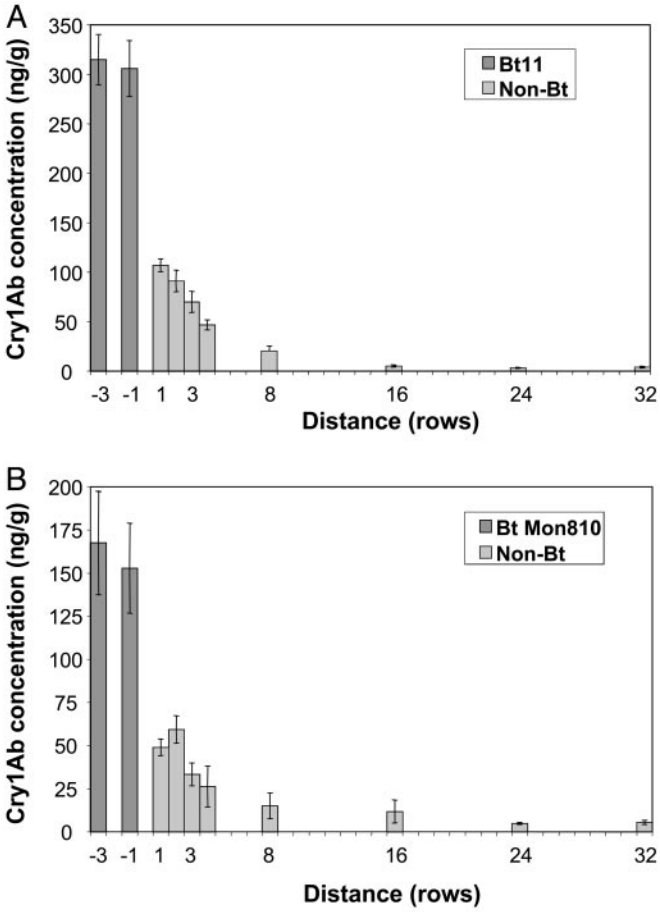

Fig. 1. Mean (± SE) concentration of Bt toxin Cry1Ab in non-Bt maize kernels as a function of distance from Bt maize in experiment 1. One row = 0.97 m. Rows −1 and −3 were Bt maize 1 and 3 m from non-Bt maize. (*A*) Two Bt11 event hybrids and paired non-Bt hybrids. Log(Cry1Ab concentration) = −0.07 + 45(1/distance), $r^2 = 0.94$, $F = 404$, $P < 0.0001$. (*B*) Four Bt Mon810 event hybrids and paired non-Bt hybrids. Log(Cry1Ab concentration) = 0.9 + 29 (1/distance), $r^2 = 0.84$, $F = 79$, $P < 0.0001$.

Bt11 plants (310 ± 18 ng/g) than for Bt Mon810 plants (160 ± 19 ng/g, Fig. 1). Maximum Bt toxin concentrations in kernels of non-Bt maize were 140 ng/g and 68 ng/g for refuge plants near Bt11 and Mon810 hybrids, respectively. These maximum toxin concentrations in non-Bt maize are 45% and 43% of the mean concentrations in the adjacent Bt11 and Mon810 plants, respectively. At 31 m from Bt maize, the mean concentration of Cry1Ab in kernels of non-Bt maize was 3.9 ± 1.2 ng/g for refuge plants near Bt11 hybrids and 5.5 ± 1.2 ng/g for refuge plants near Mon810 hybrids. These toxin concentrations in non-Bt maize are 1.2% and 3.5% of the mean concentrations in the adjacent Bt11 and Mon810 plants, respectively.

In Bt maize, the mean percentage of kernels with Cry1Ab was 74.5% (rows −1 and −3, Figs. 2 and 3*B*), consistent with the 75% expected from hemizygous parents (see *Materials and Methods*). Maximum percentages of kernels of non-Bt maize with Cry1Ab were 60% for refuge plants near Bt11 and 43% for refuge plants near Mon810 hybrids. These maximum percentages in non-Bt maize are 0.8 and 0.57, respectively, in proportion to the 75% mean Bt kernels in the Bt11 and Mon810 plants.

Experiment 2. With a barren gap of 15 m between the refuge and Bt maize, the maximum concentration of Cry1Ab in non-Bt maize was 87 ng/g and the maximum percentage of Bt kernels

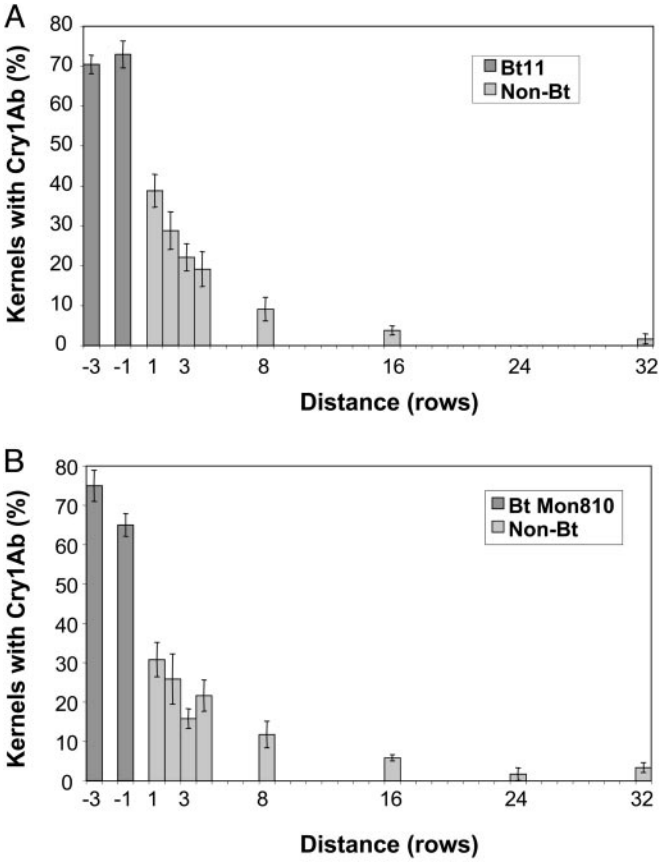

Fig. 2. Mean (±SE) percentage of Cry1Ab maize kernels in non-Bt maize ears as a function of distance from Bt maize in Experiment 1. Rows are labeled as in Fig. 1. (*A*) Two Bt11 event hybrids and paired non-Bt hybrids. Log(Cry1Ab %) = 0.02 + 33(1/distance), $r^2 = 0.81$, $F = 106$, $P < 0.0001$. (*B*) Four Bt Mon810 event hybrids and paired non-Bt hybrids. Log(Cry1Ab %) = 0.63 + 26(1/distance), $r^2 = 0.88$, $F = 103$, $P < 0.0001$.

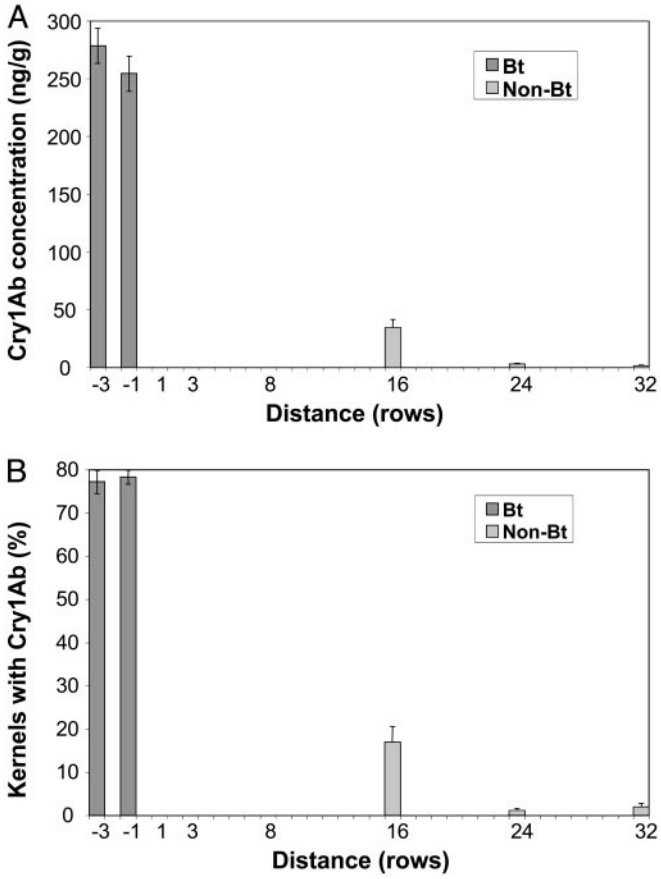

Fig. 3. Bt toxin Cry1Ab in non-Bt maize kernels as a function of distance from Bt maize in Experiment 2. Rows are labeled as in Fig. 1. Rows 1–15 were barren. Bt maize plants were a random mixture of two Bt11 hybrids and four Bt Mon810 hybrids. The non-Bt maize plants were the six nontransgenic counterparts of the aforementioned six Bt hybrids. (*A*) Mean (±SE) concentration of Cry1Ab. (*B*) Mean (±SE) percentage of Cry1Ab maize kernels.

in non-Bt maize was 43%. In proportion to values in the adjacent Bt maize, the maximum concentration of Cry1Ab in non-Bt maize was 0.33 and the maximum percentage of Bt kernels was 0.55. As in experiment 1, analysis of non-Bt maize showed that the mean concentration of Cry1Ab in kernels and the percentage of kernels with Cry1Ab decreased with distance from Bt maize (Fig. 3).

Discussion

The results show low to moderate levels of Bt toxin Cry1Ab in ears of non-Bt maize refuge plants within 31 m of Bt maize. Maximum concentrations of Bt toxin in non-Bt maize, which occurred within 2 m of Bt maize, were 43–45% of the mean concentrations in Bt maize. The mean concentration of Bt toxin Cry1Ab in kernels and the percentage of kernels with Cry1Ab decreased with distance from Bt maize, which implies that pollen-mediated transgene flow from Bt maize caused contamination of non-Bt maize refuge plants.

Results reported here and previously (15, 16) suggest that some pests are exposed to ears of non-Bt maize plants that have a mosaic of kernels with and without Bt toxin in various proportions. It will be important to quantify variation in Bt toxin concentration within and among ears, and to determine how pests respond to such variation. Pest responses might be affected by behavioral avoidance of toxin (17, 18) and other factors. On non-Bt refuge plants, vegetative tissues such as leaves and stalks do not produce Bt toxin, which means that pests feeding on these tissues escape exposure to Bt toxin as intended. However, this might enable some pests to survive initially on vegetative tissues, switching later to feeding on a mixture of kernels with and without Bt toxin. The net effect could be increased survival of heterozygous pests, caused by little or no exposure to Bt toxin for early instars and exposure to intermediate doses of Bt toxin for less susceptible later instars.

Even without gene flow between Bt and non-Bt maize, only 75% of the kernels of commercial Bt maize hybrids are expected to produce Bt toxin, because the *cry1Ab* gene is expected in only half of the pollen and eggs from hemizygous parents (see *Materials and Methods*). Our results and those of Sedlacek *et al.* (15) confirmed this expectation for Bt11 and Mon810 hybrids, the two main types of Bt maize. In contrast to non-Bt maize plants, vegetative tissues of Bt maize plants produce Bt toxin. Thus, pests attacking Bt maize plants generally will be exposed to Bt toxin in vegetative tissues such as ear-husks before they encounter kernels.

Gene flow from Bt maize to non-Bt maize could accelerate pest resistance two ways. First, if Bt toxin in refuge plants kills susceptible larvae, fewer susceptible adults will be produced, and the ability of refuges to delay resistance will be diminished. Second, if intermediate toxin levels kill susceptible larvae but allow survival of heterozygotes, the functional dominance of resistance will increase and resistance will evolve faster (4, 15, 16, 19, 20).

The extent of Bt gene flow into refuges depends on many factors, including refuge size, shape, and distance from the Bt crop; wind speed and direction; pollen longevity and settling rate; as well as similarity of Bt and non-Bt hybrids in maturation times and height of male and female flowers (12, 13, 21–24). Thus, various physical, ecological, and molecular methods of gene containment could reduce contamination of refuges by gene flow from Bt crops. In one previously reported experiment, the maximum Bt toxin expression in non-Bt maize kernels grown near Bt maize was 12% (15). In our experiments, non-Bt maize was planted downwind from Bt maize. Planting non-Bt maize upwind of Bt maize would reduce gene flow into refuges. Although increased gene flow from non-Bt could produce Bt maize with intermediate levels of toxin, this might be a less serious problem because, as noted above, production of Bt toxin in vegetative tissues of Bt maize would not be affected.

Although some hazards of transgene flow have been recognized (www.epa.gov/pesticides/biopesticides/pips/bt_brad.htm, refs. 5–9), movement of Bt genes into refuges had escaped attention. This is reflected in the current U.S. Environmental Protection Agency Biopesticide Registration Action Document for Bt crops (www.epa.gov/pesticides/biopesticides/pips/bt_brad.htm). Its environmental assessment notes that 200-m separation between different maize types is generally required for homogeneous seed production for Foundation Seed, yet its rules for resistance management allow refuge strips of non-Bt maize as narrow as 4 m (four rows) in Bt maize fields.

The potential for gene flow between Bt and non-Bt crop varieties also has been largely ignored in models analyzing effects of planting refuges at various spatial scales (4, 25–28). Gene flow from Bt crops to refuges could reduce the usefulness of refuge strips or rows within fields of Bt crops. Without molecular barriers or other barriers to gene flow, planting random mixtures of Bt and non-Bt seed could yield a high proportion of non-Bt plants with Bt toxin in their seeds, thereby limiting the benefits of refuge plants. Although seed mixtures have not been adopted widely in developed countries, they could be common in developing nations because of the small scale of farms and limited regulatory ability to implement refuges at larger spatial scales (29, 30).

In conclusion, results reported here indicate that the refuge strategy should be revised to reduce contamination of seeds in refuges by gene flow from Bt plants. Although close proximity between Bt plants and refuges increases the potential for mating between resistant and susceptible insects, it may also increase gene flow between Bt and non-Bt plants. Revised guidelines must consider these opposing influences in each crop–pest system to maximize the benefits of refuges for delaying pest resistance.

We thank Gary Odvody, Robert Smith, Yves Carrière, Mark Sisterson, and Martha Hunter for helpful suggestions and Carlos Correa and Leo Sanfilipo for technical assistance. Support was provided by Texas A&M University and the University of Arizona.

1. Tabashnik, B. E., Carrière, Y., Dennehy, T. J., Morin, S., Sisterson, M. S., Roush, R. T., Shelton, A. M. & Zhao, J-Z. (2003) *J. Econ. Entomol.* **96**, 1031–1038.
2. Ferré, F. & Van Rie, J. (2002) *Annu. Rev. Entomol.* **47,** 501–533.
3. Tabashnik, B. E. (1994) *Annu. Rev. Entomol.* **39,** 47–79.
4. Gould, F. (1998) *Annu. Rev. Entomol.* **43,** 701–726.
5. Snow, A. A. (2002) *Nat. Biotechnol.* **20,** 542.
6. Dale, P. J., Clarke, B. & Fontes, E. M. G. (2002) *Nat. Biotechnol.* **20,** 567–574.
7. Ellstrand, N. C. (2003) *Philos. Trans. R. Soc. London B* **358,** 1163–1169.
8. Hancock, J. F. (2003) *Bioscience* **53,** 512–519.
9. Mellon, M. & Rissler, J. (2004) *Gone to Seed: Transgenic Contaminants in the Traditional Seed Supply* (Union of Concerned Scientists, Cambridge, MA).
10. Ingram, W. R. (1994) in *Insect Pests of Cotton*, eds. Matthews, G. A. & Turnstall, J. P. (CABI, Wallingford, U.K.), pp. 107–148.
11. Burkness, E. C., Hutchison, W. D., Bolin, P. C., Bartels, D. W., Warnock, D. F. & Davis, D. W. (2001) *J. Econ. Entomol.* **94,** 197–203.
12. Llewellyn, D. & Fitt, G. (1996) *Mol. Breed.* **2,** 157–166.
13. Luna, V. S., Figueroa, M. J., Baltazar, M. R, Gomez, L. R., Townsend, R. & Schoper, J. B. (2001) *Crop Sci.* **41,** 1551–1557.
14. Mendelsohn, M., Kough, J., Zigfridais, V. & Matthews, K. (2003) *Nat. Biotechnol.* **21,** 1003–1009.
15. Sedlacek, J. D., Komaravalli, S. R., Hanley, A. M., Price, B. D. & Davis, P. M. (2001) *J. Econ. Entomol.* **94,** 586–592.
16. Horner, T. A., Dively, G. P. & Herbert, D. A. (2003) *J. Econ. Entomol.* **96,** 914–924.
17. Gould, F., Anderson, A., Landis, D. & Van Mellaert, H. (1991) *Entomol. Exp. Appl.* **58,** 199–210.
18. Dowd, P. F. (2001) *J. Econ. Entomol.* **94,** 1067–1074.
19. Tabashnik, B. E. & Croft, B. A. (1982) *Environ. Entomol.* **11,** 1137–1144.
20. Tabashnik, B. E., Gould, F. & Carrière, Y. (2004) *J. Evol. Biol.*, in press.
21. Morris, W. F., Kareiva, P. M. & Raymer, P. L. (1994) *Ecol. Appl.* **4,** 157–165.
22. Staniland, B. K., McVetty, P. B. E., Friesen, L. F., Yarrow, S., Freyssinet, F. & Freyssinet, M. (2000) *Can. J. Plant Sci.* **80,** 521–526.
23. Daniell, H. (2002) *Nat. Biotechnol.* **20,** 581–586.
24. Klein, E. K., Lavigne, C., Foueillassar, X., Gouyon, P.-H. & Larédo, C. (2003) *Ecol. Monogr.* **73,** 131–150.
25. Caprio, M. A. (2001) *J. Econ. Entomol.* **94,** 698–705.
26. Onstad, D. W. & Gould, F. (1998) *J. Econ. Entomol.* **91,** 585–593.
27. Onstad, D. W. & Guse, C. A. (1999) *J. Econ. Entomol.* **92,** 1256–1265.
28. Carrière, Y., Sisterson, M. S. & Tabashnik, B. E. (2004) in *Insect Pest Management: Field and Protected Crops*, eds. Horowitz, R. & Ishaaya, I. (Springer, Berlin), pp. 65–95.
29. Dirie, A. M., Cohen, M. B. & Gould, F. (2000) *Environ. Entomol.* **29,** 972–978.
30. Fitt, G., Andow, D. A., Carrière, Y., Moar, W. A., Schuler, T., Omoto, C., Kanya, J., Okech, M., Arama, P. & Maniania, N. K. (2004) in *Environmental Risk Assessment of Genetically Modified Organisms: A Case Study of Bt Maize in Kenya*, eds. Hilbeck, A. & Andow, D. A. (CABI, Wallingford, Oxon, U.K.), Vol. 1, in press.