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# DNA Screening Reveals Pink Bollworm Resistance to Bt Cotton Remains Rare After a Decade of Exposure

BRUCE E. TABASHNIK,[1] JEFFREY A. FABRICK,[2] SCOTTIE HENDERSON,[3] ROBERT W. BIGGS, CHRISTINE M. YAFUSO, MEGAN E. NYBOER, NANCY M. MANHARDT, LAURA A. COUGHLIN, JAMES SOLLOME, YVES CARRIÈRE, TIMOTHY J. DENNEHY, AND SHAI MORIN[4]

Department of Entomology, University of Arizona, Tucson, AZ 85721

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**ABSTRACT** Transgenic crops producing toxins from the bacterium *Bacillus thuringiensis* (Bt) kill insect pests and can reduce reliance on insecticide sprays. Although Bt cotton (*Gossypium hirsutum* L.) and Bt corn (*Zea mays* L.) covered 26 million ha worldwide in 2005, their success could be cut short by evolution of pest resistance. Monitoring the early phases of pest resistance to Bt crops is crucial, but it has been extremely difficult because bioassays usually cannot detect heterozygotes harboring one allele for resistance. We report here monitoring of resistance to Bt cotton with DNA-based screening, which detects single resistance alleles in heterozygotes. We used polymerase chain reaction primers that specifically amplify three mutant alleles of a cadherin gene linked with resistance to Bt cotton in pink bollworm, *Pectinophora gossypiella* (Saunders), a major pest. We screened DNA of 5,571 insects derived from 59 cotton fields in Arizona, California, and Texas during 2001–2005. No resistance alleles were detected despite a decade of exposure to Bt cotton. In conjunction with data from bioassays and field efficacy tests, the results reported here contradict predictions of rapid pest resistance to Bt crops.

**KEY WORDS** transgenic crops, resistance management, *Bacillus thuringiensis*, molecular screening

To reduce reliance on insecticide sprays, crops have been genetically modified to produce toxins from *Bacillus thuringiensis* (Bt) that kill some key insect pests. First grown on a large scale in 1996, transgenic Bt crops covered 26 million ha globally in 2005 (James 2005), representing one of the fastest adoptions of new technology in the history of agriculture. Evolution of resistance by target pests is considered the primary threat to the continued success of Bt crops (Gould 1998, U.S. EPA 2001). In particular, worst-case scenarios yielded predictions that pests would evolve resistance to Bt crops in as few as 3 yr (Harris 1991, Gould et al. 1997, Roush 1997). Although resistance to Bt crops in the field has not been reported yet, laboratory selection has generated Bt-resistant strains of many pests (Tabashnik 1994, Ferré and Van Rie 2002, Tabashnik et al. 2003). Furthermore, resistance to Bt sprays has evolved in field populations of diamondback moth, *Plutella xylostella* (L.), and greenhouse populations of cabbage looper, *Trichoplusia ni* (Hübner) (Tabashnik 1994, Ferré and Van Rie 2002, Janmaat and Meyers 2003, Tabashnik et al. 2003).

Although several approaches have been proposed for delaying pest resistance to Bt crops, the refuge strategy has been the primary tactic implemented (Gould 1998, U.S. EPA 2001, Shelton et al. 2002, Zhao et al. 2005). In the United States and most other nations where Bt crops are planted, farmers must grow refuges of crop plants that do not make Bt toxins to promote survival of susceptible pests. Ideally, resistance is recessive and most of the rare homozygous resistant (*rr*) pests surviving on a Bt crop mate with the relatively abundant homozygous susceptible (*ss*) pests from refuges, producing heterozygous (*rs*) offspring that are killed by the Bt crop. Results from models and short-term, small-scale experiments suggest that under such conditions, refuges can delay resistance substantially (Liu and Tabashnik 1997, Gould 1998, Shelton et al. 2000, Carrière et al. 2004), yet long-term, large-scale field tests of the refuge strategy are difficult.

Problems in tracking the frequency of resistance (*r*) alleles in field populations arise from the scarcity of resistant individuals and the limitations of bioassays, which have been the primary tool for Bt resistance monitoring. Bioassays often require months of work to rear and test live insects from each field population. Furthermore, with typical recessive inheritance of Bt

[1] Corresponding author, e-mail: brucet@ag.arizona.edu.
[2] Current address: USDA–ARS, U.S. Arid Land Agricultural Research Center, 21881 N. Cardon Lane, Maricopa, AZ 85239.
[3] Current address: Interdisciplinary Arts and Sciences, University of Washington, Tacoma WA 98402.
[4] Current address: Faculty of Agriculture, Hebrew University of Jerusalem, Rehovot 76100, Israel.

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resistance, bioassays do not distinguish between *rs* and *ss* individuals and thus rely on detection of extremely rare *rr* individuals. In principle, DNA-based screening for *r* alleles can overcome these limitations. DNA screening can be conducted with properly preserved insects of any life stage, thereby greatly reducing labor, time, and cost. DNA-based detection of *r* alleles in *rs* individuals can increase efficiency relative to bioassay detection of *rr* individuals roughly 100- to 1,000-fold as *r* allele frequency varies from 0.01 to 0.001 (Tabashnik 1997). Thus, DNA-based screening could be especially useful in the crucial early stages of resistance evolution when most *r* alleles occur in *rs* individuals.

Here, we used DNA-based screening for long-term, large-scale monitoring of Bt resistance in pink bollworm, *Pectinophora gossypiella* (Saunders), a major pest that has experienced field selection for resistance to Bt cotton (*Gossypium hirsutum* L.) since 1996 (Henneberry and Naranjo 1998, Tabashnik et al. 2005a). In Arizona, pink bollworm larvae feed almost exclusively on cotton, and transgenic cotton producing Bt toxin Cry1Ac has accounted for more than half of the cotton acreage statewide since 1997 (Carrière et al. 2005). In light of pink bollworm's rapid evolution of resistance to Bt cotton in response to laboratory selection (Tabashnik et al. 2000, 2005b) and extensive exposure to Bt cotton in the field (Carrière et al. 2005), we have tracked resistance in field populations by using bioassays (Tabashnik et al. 2005a). However, because of the limitations of bioassays, we developed and implemented a powerful DNA-based screening method.

In laboratory-selected strains of pink bollworm and at least two other major lepidopteran pests of cotton, mutations in a cadherin gene are tightly linked with recessive resistance to Cry1Ac (Gahan et al. 2001, Morin et al. 2003, Xu et al. 2005). In several laboratory-selected strains of pink bollworm, three mutant alleles (*r1*, *r2*, and *r3*) of a cadherin gene (*BtR*) are associated with resistance to Cry1Ac and survival on Bt cotton (Morin et al. 2003, 2004; Tabashnik et al. 2004, 2005b). Each *r* allele has a deletion predicted to eliminate at least eight amino acids upstream of the putative Cry1Ac-binding region of cadherin protein (Morin et al. 2003). We previously developed a polymerase chain reaction (PCR)-based method for detecting the *r1*, *r2*, and *r3* alleles in pink bollworm (Morin et al. 2004). We isolated, cloned and sequenced the genomic region spanning the mutation in each *r* allele and designed allele-specific PCR primers for each region. The method can detect any of the three *r* alleles in a single heterozygote (*r1s*, *r2s*, or *r3s*) pooled with DNA from the equivalent of 19 susceptible (*ss*) individuals (Morin et al. 2004).

Here, we used the PCR method to screen for the three *r* alleles in pink bollworm sampled from 59 cotton fields during 2001–2005. As far as we know, this is the first report of molecular screening of insect field populations for alleles associated with survival on a Bt crop. We detected 97% of *r* alleles in blind positive controls, yet no *r* alleles were detected in 5,571 field-derived insects. In conjunction with previously reported results from bioassays and field efficacy tests (Dennehy et al. 2004, Tabashnik et al. 2005a), the results reported here provide strong evidence that pink bollworm resistance to Cry1Ac remains rare after a decade of exposure to Bt cotton. The results contradict predictions from worst-case scenarios and suggest that the refuge strategy may be helping to delay pest resistance to Bt crops.

## Materials and Methods

**Field Sampling.** Rather than sampling fields randomly, we tried to increase the probability of finding resistance (*r*) alleles by sampling fields that had relatively high levels of pink bollworm infestation in areas with relatively high abundance of Bt cotton. This nonrandom sampling approach favored overestimation of *r* allele frequency. The sampled insects probably represent <0.01% of the total of the field populations studied. Although the statewide average in Arizona for the percentage of cotton accounted for by Bt cotton was <70% during the study period, it ranged widely among years and regions, with some sampled regions having consistently >80% Bt cotton (Carrière et al. 2005; unpublished data).

Cotton bolls were sampled from 19 cotton fields (18 fields in Arizona and one field in California) from 2001 to 2005 as described by Dennehy et al. (2004). At each site, 300–2,000 bolls were collected from non-Bt cotton fields near Bt cotton fields. Bolls were taken to the University of Arizona Extension Arthropod Resistance Management Laboratory in Tucson, AZ. We obtained pink bollworm by collecting fourth instars that exited bolls and by opening bolls and removing larvae found inside. In 2001 and 2002, individuals screened with PCR were from the F1 to F8 generations of laboratory-reared progeny derived from field-collected insects. From 2003 to 2005, insects from bolls tested with PCR were obtained directly from field-collected bolls.

Pink bollworm males were collected in sticky traps baited with female sex pheromone (Tabashnik et al. 1999) in 40 cotton fields (36 fields in Arizona, three fields in California, and one field in Texas) from 2003 to 2005. At each site, several traps were placed around the perimeter of a cotton field, collected after 1–2 d, and brought to the laboratory. Live males that showed normal movement of appendages were removed from traps by using wooden toothpicks. A new toothpick was used for each male to avoid cross-contamination.

**DNA Preparation and PCR.** Insects collected from bolls and traps were stored in ethanol at −20°C. DNA was extracted using DNAzol (Tabashnik et al. 2005b), and PCR was done as described by Morin et al. (2004). The maximum number of individuals tested per pool was five for samples from 2001 to 2003 and 11 for samples from 2004 to 2005. Including controls and tests of field-sampled pink bollworm for each of the three *r* alleles, >4,000 PCR reactions were done.

**Statistical Analysis.** The probability of detecting no *r* alleles in a sample of *N* individuals was calculated as $(1 - [F \times D])^{2N \times A}$, where *F* is the frequency of

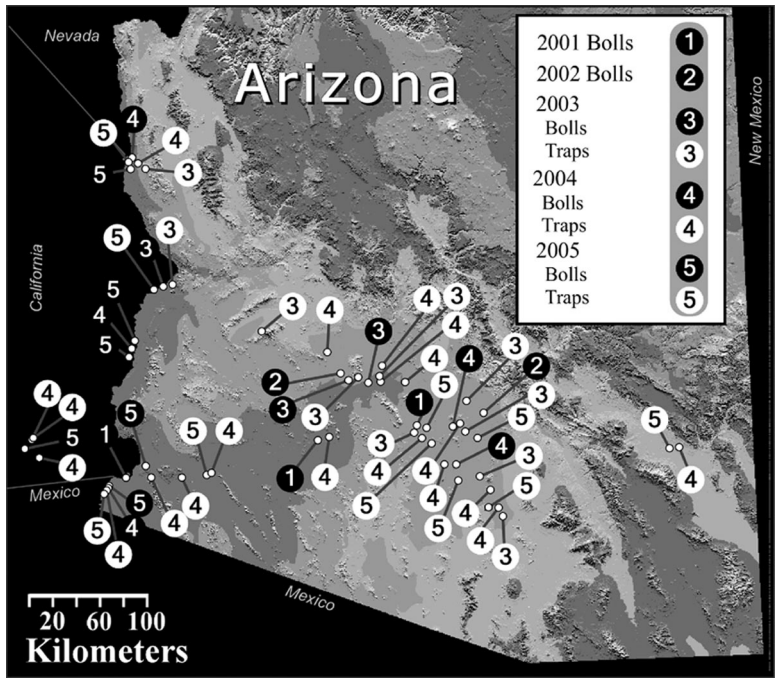


**Fig. 1.** Cotton fields in Arizona and California where pink bollworm were collected for DNA-based screening for resistance to Bt toxin Cry1Ac. The collection site near Ft. Hancock, TX, sampled in 2004 is not shown on the map.

resistance alleles, *D* is the probability of detecting an *r* allele present in screened individuals (0.97, based on the data from blind controls), 2*N* is the number of alleles screened, and *A* is the probability of amplifiable cadherin DNA occurring in field-sampled insects (estimated as 0.986, based on the proportion of positive results for amplification of a conserved sequence in 835 insects tested individually). We assumed that the probability of an *r* allele occurring was an independent event at each cadherin allele screened. For example, with an *r* allele frequency of 0.001, the probability of detecting no *r* alleles in the sample of 5,571 individuals (11,142 alleles) is $0.000023 = (1 - [0.001 \times 0.97])^{11,142 \times 0.986}$. Analogously, with an *r* allele frequency of 0.0003, the probability of detecting no *r* alleles in the sample of 5,571 individuals is $0.041 = (1 - [0.0003 \times 0.97])^{11,142 \times 0.986}$.

To compare DNA screening that detects *r* alleles in *rs* individuals with bioassays that detect only *rr* individuals, we used the following approach: Assuming Hardy–Weinberg equilibrium (Hartl and Clark 1989), the frequency of *rr* is the square of the frequency of the *r* alleles. For example, if the estimated frequency of *r* alleles is <0.0003, the estimated frequency of *rr* is <1 in 10 million ($0.0003^2 = 0.9 \times 10^{-7}$).

## Results

We obtained pink bollworm for DNA screening from 59 cotton fields in Arizona, California, and Texas by collecting infested cotton bolls or by catching males in pheromone-baited traps (Fig. 1; Table 1). Field-derived insects were preserved and screened with PCR (see Materials and Methods). Although the PCR method can detect single *r* alleles in pools of 20 individuals (Morin et al. 2004), we tested insects in

**Table 1. Pink bollworm screened for Bt resistance alleles by using PCR**

| Yr | Sampling method[a] | Sites | Individuals screened[b] |
|---|---|---|---|
| Arizona | | | |
| 2001 | Bolls | 3 | 300 |
| 2002 | Bolls | 2 | 94 |
| 2003 | Bolls | 3 | 150 |
| | Traps | 10 | 1,296 |
| 2004 | Bolls | 4 | 400 |
| | Traps | 16 | 1,595 |
| 2005 | Bolls | 3 | 150 |
| | Traps | 10 | 500 |
| 2001–2005 totals | | 51 | 4,485 |
| California | | | |
| 2004 | Bolls | 1 | 100 |
| | Traps | 3 | 789 |
| 2005 | Bolls | 3 | 150 |
| 2004–2005 totals | | 7 | 1,039 |
| Texas | | | |
| 2004 | Traps | 1 | 47 |
| All sites | | 59 | 5,571 |

[a] Individuals were sampled from cotton bolls or from traps baited with sex pheromone.

[b] Individuals tested in pools of at most 11 individuals for all three *r* alleles (*r1*, *r2*, and *r3*); mean *n* = 94 individuals tested per site (range 41–456).

pools of 11 or fewer to minimize chances of missing *r* alleles.

To determine whether the screening method was performing properly, we used an extensive series of positive and negative controls (Morin et al. 2004). To check the quality of DNA in field samples, we tested for amplification of a conserved region of the cadherin gene that occurs in all known susceptible and resistant alleles (Morin et al. 2004). We checked all pools by using this approach, and >99% tested positive. Because as few as one amplifiable allele from a pool of insects could yield a positive result for this control reaction, we also tested a subset of insects individually from each of the 59 field samples. Of the 835 individuals tested, 98.6% were positive.

In addition to standard positive controls for each of the three *r* alleles in all tests, we included "blind" positive controls as follows: Two researchers analyzed each field sample. One researcher prepared DNA and added individuals with one or two *r* alleles from laboratory-selected resistant strains in zero to three (usually one) of the pools tested from each field site. The other researcher performed PCR and did not know which, if any, of the pools contained these blind positive controls. The detection rate for blind positive controls was 97% (97/100).

We detected no *r* alleles in 5,571 pink bollworm sampled from 59 cotton fields in Arizona, California, and Texas from 2001 to 2005. Based on results described above showing 98.6% successful amplification of cadherin DNA and a detection rate of 97% for *r* alleles, the estimated probability is <0.0001 that the frequency of *r* alleles in the field was equal to or >0.001 (see Materials and Methods). The estimated probability is <0.05 that the frequency of *r* alleles in the field was equal to or >0.0003.

## Discussion

In conjunction with data from bioassays and field efficacy tests (Dennehy et al. 2004, Tabashnik et al. 2005a), the DNA screening results reported here provide strong evidence that resistance to Bt cotton was rare in pink bollworm despite a decade of exposure to Bt cotton. For pink bollworm, DNA screening detects single *r* alleles in heterozygotes, whereas bioassays detect only resistant homozygotes. Thus, detecting no *r* alleles in DNA screening of 5,571 individuals is roughly equivalent to finding no resistant individuals with two copies of the screened *r* alleles in bioassays of 10 million individuals (see Materials and Methods).

It is important to consider potential underestimation of *r* allele frequency based on DNA screening. DNA screening based solely on males caught in pheromone traps could cause underestimation if the probability of capture in traps was lower for *rr* or *rs* males than for *ss* males. However, tests conducted in large cages (64 $m^3$) in the field refuted this hypothesis for pink bollworm (Carrière et al. 2006). Furthermore, DNA screening of pink bollworm from bolls, which was independent of males caught in traps, also detected no *r* alleles ($n$ = 1,344; Table 1).

If alleles other than cadherin mutants *r1*, *r2*, and *r3* confer pink bollworm resistance to Bt cotton, the results of our DNA screening could underestimate the frequency of resistance. For example, resistance to Cry1Ac in some strains of diamondback moth is not linked with cadherin (Baxter et al. 2005). However, in four laboratory-selected Cry1Ac-resistant strains of pink bollworm tested so far, all resistant individuals screened have two copies of the known *r* alleles (i.e., *r1r1*, *r2r2*, *r3r3*, *r1r2*, *r1r3*, or *r2r3*), and no other resistant alleles have been detected (Morin et al. 2003; Tabashnik et al. 2004, 2005b). Although the presence of additional resistance alleles at the cadherin locus or other loci cannot be excluded, such alleles seem to be more rare than the three known resistance alleles.

In some years, the resistance allele frequency estimated from bioassays was somewhat higher than the estimate based on DNA screening. For Arizona cotton fields from 2001 to 2005, the mean yearly estimated resistance allele frequency from bioassays is 0.024 (range 0–0.075; 95% confidence interval 0–0.062). The bioassay data for 2001–2004 were reported previously (Tabashnik et al. 2005a). In 2005, bioassays detected no resistant individuals in Arizona or California ($n$ = 5,381 larvae from 15 sites; T.J.D., unpublished data). The difference in estimates between bioassays and DNA screening in some years could reflect overestimation by bioassays, underestimation by DNA screening, or both.

Resistance allele frequency was estimated from bioassays by assuming that all survivors of a diagnostic concentration of Cry1Ac in diet (10 $\mu$g of Cry1Ac per milliliter diet) were *rr*. However, because of environmental variation, some survivors may not have been genetically resistant. For example, in 2001 we observed 31 survivors from Arizona at the diagnostic concentration in bioassays (Tabashnik et al. 2005a), yet selection with Cry1Ac in diet did not yield a resistant strain. This lack of response to selection suggests that the survival was not heritable and thus the 2001 resistance allele frequency was overestimated from bioassays. In contrast, with strains derived from Arizona cotton fields in 1997, as few as three generations of selection with Cry1Ac produced strains capable of surviving on Bt cotton (Tabashnik et al. 2000, 2005b). Although stringent measures were used to keep pink bollworm strains separate and contamination of field-derived strains by resistant laboratory strains is unlikely, we cannot exclude the possibility that such contamination inflated some estimates of resistance allele frequency based on bioassays during 2001–2004. This could not have been a problem with the bioassay results in 1997, however, because no sources of contamination were available then. Also, we can infer that no such contamination affected bioassay results in 1999, 2000, and 2005 when no resistant individuals were detected in field-derived strains (total $n$ = 11,400 larvae tested at the diagnostic concentration).

The hypothesis that resistance alleles identified from laboratory-selected strains are important in field-evolved resistance can be tested only after resistance

evolves in the field. Meanwhile, data reported here show that the three resistance alleles consistently associated with resistance to Bt cotton in laboratory-selected strains of pink bollworm were rare in the field after a decade of extensive Bt cotton use. These results and related findings with pink bollworm and other insects (Wu et al. 2002; Bourguet et al. 2003; Burd et al. 2003; Carrière et al. 2003; Tabashnik et al. 2003, 2005a; Dennehy et al. 2004; Farinós et al. 2004; Stodola et al. 2006) contradict the worst-case predictions that pest resistance to Bt crops would evolve in as few as 3 yr (Harris 1991, Gould et al. 1997, Roush 1997). The most pessimistic predictions do not incorporate factors that seem to be delaying pest resistance to Bt crops, particularly large refuges of non-Bt crops that enable survival of susceptible insects, recessive inheritance of resistance, incomplete resistance, and fitness costs associated with resistance (Tabashnik et al. 2003, 2005a). Modeling results that do incorporate realistic estimates of these factors for pink bollworm suggest that rapid increases in resistance frequency will not occur (Carrière and Tabashnik 2001, Tabashnik et al. 2005a). Despite the success of Bt crops for their first decade, we think resistance remains a threat and caution against underestimating the adaptive capabilities of insect pests.

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