

genetic difference from the comparator (parent line, sibling line or null segregant). In fact, the plants that are transformed are not homozygous at every locus. Many generations of back-crossing and/or selective crossing are needed to produce a viable commercial line from the original transgenic plant. After approval, the event is usually crossed into many diverse varieties of the same crop. The genetic diversity will include expression of various allergens. We do agree that measuring endogenous allergenicity might provide useful information if the introduced gene is a transcription factor or is intended to alter the expression of

an allergen. We simply pointed out the magnitude of natural variability and urge that additional data are needed to establish criteria that would be useful. Statistically significant differences are much less important than biologically meaningful differences. Overall, we think that we have given a balanced view that can improve the assessment compared with some current regulatory demands.

COMPETING INTERESTS STATEMENT

The authors declare competing financial interests: details accompany the full-text HTML version of the paper at <http://www.nature.com/naturebiotechnology/>

Field-evolved resistance to *Bt* toxins

To the Editor:

An article by Tabashnik *et al.*¹ in your February issue states that, for the first time, the frequency of resistant alleles has increased substantially and that there is field-evolved *Bacillus thuringiensis* toxin (*Bt*) resistance in bollworm, *Helicoverpa zea* (Boddie), in the United States because of the extensive use of *Bt* cotton. Tabashnik *et al.* base their conclusions on two publications by Randy Luttrell's laboratory in which Cry1Ac toxicity to numerous *H. zea* populations was evaluated: Luttrell *et al.*² (before introduction of *Bt* cotton) and Ali *et al.*³ (after introduction of *Bt* cotton).

We emphatically disagree with the conclusions of Tabashnik *et al.* that the data published in these two articles demonstrate field-evolved resistance in *H. zea* for four reasons: first, the definition of *Bt* resistance used by Tabashnik *et al.* is purely laboratory based, whereas field efficacy and larval survival on plant tissues are the ultimate criteria for contextualizing laboratory-based estimates of resistance, and no change in *Bt* cotton efficacy has been documented during the past decade; second, larval samples should not be collected from *Bt* crops because they will not be representative of the population as a whole, especially for highly mobile insects such as *H. zea*; third, the data from Luttrell's laboratory on which Tabashnik *et al.* base their conclusions have been evaluated using LC₅₀ (median lethal dose; 50%) values to

measure resistance, which introduces artifacts into the analysis; and fourth, the baseline comparator used to assess variability in these laboratory assays is not representative of field susceptibility; when a more appropriate comparator colony is employed, results from Luttrell's laboratory bioassays indicate no change in susceptibility. We discuss each of these aspects in turn below.

Pest resistance can be defined in two very different ways, either based on laboratory conditions, or based on real-world, field conditions. Laboratory resistance is defined as a statistically significant, genetically mediated reduction in sensitivity of the target organism to the controlling agent, relative to a susceptible laboratory strain. In this setting, resistance is typically

observed as an increase in population LC₅₀, or as enhanced growth and/or survival at a discriminating concentration, compared with a susceptible colony. Although laboratory-based estimates of resistance are essential in proactive resistance management programs to achieve early warning of reduced larval susceptibility and thus potential resistance problems, proof of resistance ultimately must rest on field efficacy. Laboratory tests are unable to accurately predict or interpret the impact that any given frequency and/or intensity of resistance will have on pest (or population) survival and fitness in a field environment. Field resistance can be defined as a genetically mediated increase in the

ability of a target pest to feed and complete development on one or more commercial line(s) of *Bt* cotton under field conditions. This definition incorporates the potential for incomplete resistance (e.g., increased feeding but delayed or incomplete development to adult) and fitness costs. In the case of *H. zea*, not only have there been no reports of widespread control failures, as confirmed in Tabashnik *et al.*, but also there has been no documented change in efficacy of *Bt* cotton anywhere in the US Cotton Belt. Although control of *H. zea* by *Bt* cotton has never been complete (http://www.epa.gov/scipoly/sap/meetings/2000/october/brad4_irm.pdf), no further increase in survival has been observed since *Bt* cotton was introduced in 1996. The most recent monitoring data from 2007 parallel results from previous years⁴. In other words, no real-world field data support the laboratory-based conclusions made by Tabashnik *et al.* regarding a mounting resistance problem with *H. zea*. In contrast to *H. zea* in *Bt* cotton, fall armyworm, *Spodoptera frugiperda*, has developed resistance to Cry1F *Bt* corn in Puerto Rico (A. Reynolds, Entomological Society of America Annual Meeting, December 2007). In this case, there was a change in field performance resulting in field failures, and subsequently it was demonstrated that *S. frugiperda* showed no mortality at the highest concentration of Cry1F tested in laboratory bioassays. As a result, there was an immediate voluntary discontinuation of commercial cultivation of Cry1F *Bt* corn in Puerto Rico.

With regard to choosing the most appropriate method for measuring changes in susceptibility, the US Environmental Protection Agency (EPA) mandated annual surveys of *H. zea* susceptibility to Cry1Ac across all cotton production regions as part of the overall post-commercial monitoring process. The susceptibility monitoring protocol was developed and accepted by experts from EPA, academia, extension and industry. The methods involved collecting insects from non-*Bt* crops, rearing them to subsequent generations (F₁–F₂), conducting neonate bioassays using Cry1Ac (MVP II) at diagnostic concentrations, including the use of 'failure to molt into second instars' as the definition of mortality, and comparing results to a susceptible laboratory colony and previous years' results. (Note that Luttrell *et al.*² used Cry1Ac toxin for conducting bioassays, which has significantly different toxicity against *H. zea* compared to MVP II (ref. 5), which was used for official resistance monitoring and by Ali *et al.*³.) These procedures are similar to those used



in monitoring *Bt* resistance for European corn borer, *Ostrinia nubilalis*⁶. The detection of a significant increase in survival using diagnostic concentrations indicates the possible presence of resistant alleles, but population-level field resistance is confirmed only if the results can be repeated, resistance is shown to be heritable and those insects can survive on *Bt* plants. The sole use of LC₅₀ values (as in Luttrell *et al.*² and Ali *et al.*³) for measuring resistance has severe limitations, which is why the EPA-mandated monitoring program recommends diagnostic concentrations in most cases.

First, estimating LC₅₀ values requires using a range of concentrations resulting in 0–100% mortality; typically these concentrations are based on the susceptible population, not on tolerant or resistant populations. If no concentration results in >50% mortality, the LC₅₀ will be a poor extrapolation. For example, in Ali *et al.*³, the highest concentration used was 150 µg/ml, and yet there were four colonies in which the estimated LC₅₀ far exceeded 150 µg/ml. (Note the tremendous variability in 95% confidence interval for LC₅₀ values or for resistance ratios (RRs) for these four colonies, especially F3704 in 2004.) Second, the LC₅₀ value is an estimate with inherent variability, and comparisons between LC₅₀ values must take this uncertainty into account (hence the importance of statistically valid 95% CI). Third, measures of mortality (dead only) in laboratory bioassays tend to overestimate resistance because these assays are typically short in duration and allow insects to survive with minimal feeding. For this reason, the failure to molt to 4th instars has been considered as the criterion for mortality in a 21-day assay for *Pectinophora gossypiella* (<http://cals.arizona.edu/pubs/crops/az1437/section3.pdf>). Fourth, the use of the median response is not likely to be the most relevant measure as the response of susceptible field populations far exceeds 50% mortality. And fifth, discriminating concentrations are much more efficient for detecting low frequencies of resistance because all individuals are tested at the appropriate concentration⁶.

Another aspect that is critical in determining field-evolved resistance is the comparison of field populations to a stable, vigorous laboratory population that exhibits as many of the attributes of field populations as possible in the absence of selection (a susceptible population). Determining the appropriate laboratory colony to use is not trivial, especially for *H. zea*. Before the release of *Bt* cotton in the United States, several studies assessed *H. zea* susceptibility

to Cry1Ac and observed high variability. MacIntosh *et al.*⁷ reported an LC₅₀ value of 10.0 µg Cry1Ac toxin/ml for their laboratory colony. Stone and Sims⁸ reported 16-fold variation in LC₅₀ values to Cry1Ac toxin among field-collected *H. zea* populations ranging from 0.45 µg/ml to 7.39 µg/ml. Luttrell *et al.*² reported results from four laboratory colonies tested in 1992–1993 using Cry1Ac toxin, and found LC₅₀ values ranging from 0.02 µg/ml to 8.82 µg/ml, representing 441-fold variation. The lowest LC₅₀ value came from a Mississippi State University laboratory colony that had been maintained in isolated culture for 20 years with no exposure to external genetic material. The highest LC₅₀ value was detected in a laboratory colony from the United States Department of Agriculture, Agricultural Research Service (USDA-ARS; Washington, DC) in Stoneville, Mississippi (the same colony used for official EPA monitoring from 1996–2005). A similar high level of variation in LC₅₀ values was measured by Luttrell *et al.*² for seven field-collected colonies ranging from 0.02–5.97 µg/ml, representing a 299-fold variation. As a result, one is left with a dilemma: which laboratory colony best represents natural susceptibility and is the most appropriate for use in monitoring programs? The two highly inbred strains described by Luttrell *et al.*² and Ali *et al.*³ were perhaps not the best comparators upon which to draw conclusions concerning *Bt*

resistance in field populations. One colony bioassayed by Ali *et al.*³ that may be suitable as a comparator is the MonLab colony (LC₅₀ (µg/ml) values (95% CI) for 2003 and 2004 were 32.2 (11.4–575) and 19.8 (9.8–46.6), respectively). This colony has received annual infusions of field-collected insects, and the LC₅₀ using Cry1Ac toxin has not significantly changed from 1990–2004 (refs. 3,5,7). However, because of the variation in susceptible colonies throughout the Cotton Belt, perhaps either additional susceptible colonies from representative geographic locations should be used as comparators, or if a putative tolerant and/or resistant population is observed against one susceptible colony, this population can be tested using standardized methods using other susceptible colonies representing these various geographic locations.

When the data in Ali *et al.*³ are reexamined in this light, they do not support a conclusion of *Bt* resistance. In 2002, only two laboratory colonies were used and both had similar LC₅₀ values. Only one colony collected from non-*Bt* crops had an RR > 10. However, an RR of 13 is trivial in relation to the 300- to 440-fold variation among populations described in Luttrell *et al.*². Furthermore, had another laboratory colony such as MonLab been used as a comparator, the resistance ratio would have been about 1 (Table 1). In 2003 and 2004, MonLab was included as a laboratory colony. Using

Table 1 Generation of resistance ratios (RR) for field-collected *H. zea* using UALab and MonLab as laboratory reference colonies^a

Year/ collection number	RR (95% confidence intervals) (UALab) ^b	RR (95% confidence intervals) (MonLab) ^{c,d}
2002		
F1802	13.1 (8.32–20.5)	1.15 (0.78–1.82)
2003		
F0803	19.8 (7.79–50.4)	1.02 (0.76–6.42)
F1103	30.5 (7.45–125)	2.35 (0.89–33.6)
F1303	20.3 (10.1–40.8)	1.57 (0.91–3.80)
F2103	10.6 (7.41–15.1)	0.82 (0.65–1.06)
F3003	25.5 (10.2–63.9)	1.98 (0.97–8.79)
F3303	18.6 (10.9–31.9)	1.44 (0.96–2.60)
2004		
F1804	14.7 (8.07–26.8)	2.24 (1.36–4.17)
F2004	20.8 (11.2–38.8)	3.17 (1.17–6.23)
F3404	11.4 (3.77–34.6)	1.74 (0.69–7.40)
F3704	578 (91–3663)	88.0 (4.60–185)
F3804	10.4 (5.58–19.40)	1.59 (0.93–3.02)

^aResistance ratios (RR) defined as LC₅₀ of field-collected colony divided by LC₅₀ of reference colony. ^bData from ref. 3. ^cRR (95% confidence interval) calculated using MonLab LC₅₀ values from 2003 (32.15 µg/ml) and 2004 (19.83 µg/ml)³. MonLab LC₅₀ value used for 2002 calculation was estimated by taking the mean of 2003 and 2004 LC₅₀ values. ^d95% confidence interval calculated by taking lower and upper 95% confidence intervals of field-collected colony LC₅₀ divided by LC₅₀ of reference colony.

MonLab instead of UALab, none of the eight colonies in 2003 and only one of 13 colonies in 2004 collected from non-*Bt* crops (F3704; collected on July 2004 from Pickens (Desha County), AR, USA) had a RR > 10 (Table 1). Therefore, 'resistance' from 2002 was not repeatable in 2003. F3704 was sent to Auburn University and USDA-ARS in Ames, Iowa for resistance confirmation. At Auburn, F3704 was confirmed as being highly resistant to Cry1Ac toxin but, as has been observed for other *Bt*-resistant populations of *H. zea*, went extinct due to fitness costs^{5,9}. F3704 also went extinct in both the Luttrell and USDA-ARS laboratories⁹. In addition, elevated bioassay responses in field collections from Pickens (Desha County) have not been observed since 2004; if resistance truly is "field evolved," should we not have observed some change or 'shift' in efficacy in this region up to and including 2007? Therefore, even based on the definition chosen by Tabashnik *et al.* and ignoring the efficacy of commercial *Bt* cotton plants, field-evolved resistance to *Bt* cotton has not yet been detected. Furthermore, similar high levels of variability (299- to 456-fold) in responses to Cry1Ac were observed among laboratory and field-collected *H. zea* populations in 1992–1993 and in 2004 (refs. 2,3).

Collectively, reexamining these data suggests that large genetic variation in Cry1Ac-susceptibility has always been present within *H. zea* populations (at least by 1992–1993, before Bollgard commercialization), and there is no evidence to suggest that there has been a significant shift in susceptibility since the introduction of *Bt* cotton. Other comparable events have occurred that should give us caution in using these data to conclude widespread *Bt* resistance has evolved in *H. zea*. For example, Tabashnik *et al.*¹⁰ reported that alleles for Cry1Ac resistance in *P. gossypiella* were present in surprisingly high frequencies in 1997 in *Bt* cotton fields in Arizona. However, since 1997, not only has resistance to *Bt* cotton by *P. gossypiella* not occurred in the field, laboratory-based estimates of the *Bt* resistance allele frequency in *P. gossypiella* actually have decreased. Such counterintuitive outcomes of laboratory-based resistance monitoring underscore the critical necessity to require results of field tests as the ultimate validation of resistance claims. Similarly, the range of responses to Cry1Ac currently reported in *H. zea* populations remains comparable to that when it was originally measured, and no observable change in *Bt* cotton efficacy has occurred. The primary difference in this

case is that *H. zea*'s response to Cry1Ac is, and always has been, highly variable among populations, probably reflecting an inherent tolerance to *Bt* proteins and its highly polyphagous nature and annual migratory behavior. Consequently, based upon the historical and current results, it is premature to conclude that field-evolved resistance to *Bt* cotton has arisen in *H. zea*, as Randy Luttrell has noted himself (<http://agfax.com/news/2008/02/btresist0208.htm>).

Public scientists and the agricultural industry must continue to be vigilant and monitor for potential changes in susceptibility to *Bt* proteins. Even so, it is important to be cautious in interpreting laboratory data, particularly where comparisons are made among very complex and variable sets of data, conducted during different time periods, by multiple researchers, in different laboratories, using different susceptible colonies and with unique protein sources.

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1. Tabashnik, B.E., Gassmann, A.J., Crowder, D.W. & Carrière, Y. *Nat. Biotechnol.* **26**, 199–202 (2008).
2. Luttrell, R.G., Wan, L. & Knighten, K.J. *Ecol. Entomol.* **92**, 21–32 (1999).
3. Ali, M.I., Luttrell, R.G. & Young, S.Y.J. *Ecol. Entomol.* **99**, 164–175 (2006).
4. Ali, M. I. *et al.* Presentation at the Beltwide Cotton Conference, Nashville, TN, Jan. 10–11, 2008.
5. Anilkumar, K.J. *et al. Appl. Environ. Microbiol.* **74**, 462–469 (2008).
6. Siegfried, B.D. *et al. Am. Entomol.* **53**, 208–214 (2007).
7. MacIntosh, S.C. *et al. J. Invertebr. Pathol.* **56**, 258–266 (1990).
8. Stone, T.B. & Sims, S.R. *J. Econ. Entomol.* **86**, 989–

994 (1993).

9. Anilkumar, K.J. *et al. J. Econ. Entomol.* **101**, 1421–1431 (2008).
10. Tabashnik, B.E. *et al. J. Econ. Entomol.* **96**, 1031–1038 (2003).

Bruce E Tabashnik, Aaron J Gassman, David W Crowder & Yves Carrière reply:

We welcome the opportunity to confirm one of the main conclusions of our paper¹: some field populations of a major cotton pest, *Helicoverpa zea*, evolved resistance to Cry1Ac, the *Bacillus thuringiensis* toxin (*Bt*) in first-generation transgenic *Bt* cotton (also called Bollgard cotton). This conclusion is based on extensive resistance monitoring data for 1992 to 2006 from five papers by Randall Luttrell and his collaborators^{2–6}, including crucial information about field efficacy and larval survival on *Bt* cotton plants from three papers not cited by William Moar *et al.* above. These data show that the field-evolved resistance documented with laboratory diet bioassays (see Table 1 below) is associated with increased survival on *Bt* cotton leaves (Fig. 1) and control problems in the field^{2–6}.

The primary goal of monitoring insect resistance to *Bt* crops is not to document field failures, but rather to detect resistance in field populations soon enough to enable proactive management of resistance. Thus, the US Environmental Protection Agency (EPA) mandates monitoring to provide "an important early warning sign" of resistance in field populations⁷. Evolution of resistance is defined as a heritable decrease in a population's susceptibility to a toxin^{8,9}. Susceptibility is typically measured in laboratory bioassays testing the progeny of field-sampled insects for responses to the toxin. Such bioassays document resistance if one or more populations with a history of exposure to the toxin in the field are significantly less susceptible than conspecific populations that have had less exposure⁹. Because resistant individuals are most likely to be found in the field surviving on *Bt* crops, sampling insects from *Bt* crops is an essential component of resistance monitoring.

In their rigorous resistance monitoring program, Luttrell and collaborators^{2–6} appropriately sampled *H. zea* larvae from *Bt* cotton and *Bt* corn, as well as from various non-*Bt* plants (Table 1). By sampling *H. zea* from *Bt* cotton fields with high boll damage and testing their progeny, Luttrell and collaborators^{2–6} showed that reduced field efficacy was associated with increased larval survival on toxin-treated diet and on *Bt* cotton leaves.

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