

ANALYSIS

Testing *Bt* refuge strategies in the field

Fred Gould

In 1999, an estimated 24% of corn and 5% of cotton grown worldwide contained a transgene for an insectical protein derived from the bacterium *Bacillus thuringiensis* (*Bt*). The prevalence of these so-called “*Bt* crops” has raised concerns about the possible widespread emergence of *Bt* resistant pests, which could ruin the utility of *Bt* crops. In 1996, the Environmental Protection Agency (EPA; Washington, DC) and industry moved to circumvent this danger by requiring farmers to plant a certain percentage of their cotton acreage in non-*Bt*-producing cultivars. This would provide pests a refuge where they could feed on plants lacking toxins, thereby maintaining *Bt* susceptible resistance alleles within the insect population. Since that ruling, there have been calls for increases in *Bt* refuge sizes by scientific panels by the EPA², industry³, and the Union of Concerned Scientists⁴ (Washington, DC), as well as two independent groups led by extension entomologists working directly with corn and cotton farmers⁵. However, critics have argued that before going forward with such increases, field tests are needed to prove that refuge strategies are effective. Now in this issue, Shelton et al.⁶ have moved us one step forward in addressing this concern, by reporting the results of controlled field tests to evaluate the impact of pest refuges on the evolution of insects’ resistance to transgenic insecticidal crops.

The premise for pest refuges is based on population genetic theory, which predicts toxin-susceptible insects produced in pest refuges will mate with toxin-resistant insects that survive on the transgenic insecticidal crops, thereby diluting the alleles for resistance and prolonging the pest population’s susceptibility. The 1996 EPA-approved plan required that cotton farmers plant 4% of their acreage in non-*Bt*-producing cultivars, and not treat this acreage with any insecticides that kill *Bt*-targeted pests, to ensure their survival in the refuge. Farmers who did not want to risk the potential crop damage in the 4% untreated refuge were offered the option of planting at least 20% non-*Bt* cotton that could be sprayed. The logic here was that conventional pesticides are expected to kill about 80% of the pests, therefore the 20% and 4% refuges should preserve an equivalent number of surviving *Bt*-susceptible insects.

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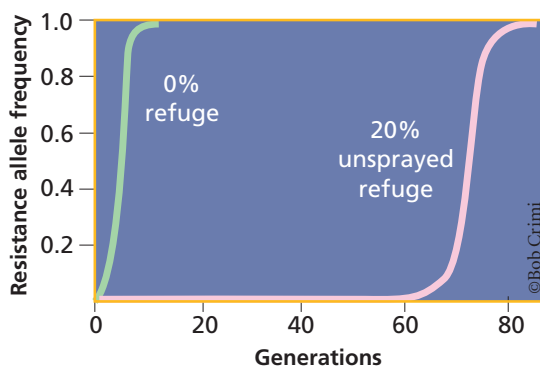


Figure 1. Theoretical expectations regarding the rate of *Bt* toxin resistance evolution in the presence or absence of a refuge when the transgenic plants produce a high dose of toxin that kills 99.9% of susceptible insects and 99% of heterozygotes. Note that at low resistance allele frequencies, the rate of adaption is much slower when there is a refuge (the resistance allele frequency in generation 0 is 0.001).

As illustrated in Fig. 1, refuges are expected to be very effective if the transgenic insecticidal crops produce a “high dose”—at least 25-fold the amount of toxin needed to kill susceptible insects. In fact, the EPA science advisory committee concluded that “a refuge/high dose approach must be employed within the current understanding of the technology”².

Unfortunately, the only way to directly test the refuge/high dose theory is to use it on a wide scale in some areas and not in others; economic and ethical issues, however, make this impossible. Even small scale field tests have faced major obstacles because they generally involve the release of pest insects with resistance alleles. Shelton et al. have circumvented this problem by developing *Bt*-producing broccoli, and using it for field tests of the diamondback moth in upstate NY where the moth cannot survive the winter.

Shelton et al. planted broccoli in plots with various types of refuges. They released insects with a known frequency of resistance alleles and examined how the refuges impacted both resistance allele frequency and pest population density. Unfortunately, Shelton et al. needed to release insects with a high frequency of resistance alleles (0.12 and 0.80) for practical reasons, even though they realized that the refuge/high dose approach relies on initially low resistance allele frequencies. This problem made it impossible for Shelton et al. to test the refuge/high dose strategy as a whole, but they were able to examine some aspects of the refuge component of the strategy.

One question addressed by Shelton et al. was how far away from the *Bt* plants must the non-*Bt* refuge plants be grown? One extreme

is to plant a mixture of seeds, with *Bt* and non-*Bt* plants side by side. A criticism of this approach is that larvae which begin feeding on non-*Bt* plants might move to, and feed on *Bt* plants, thereby diminishing the number of larvae completely escaping the selective impacts of the toxin. Shelton et al.’s field data indicate that this is a legitimate concern for the diamondback moth. While this would not be a problem for cotton pests, such as the pink bollworm, that don’t generally move between plants, it could affect other target insects such as the tobacco budworm and European corn borer. Fortunately, the current and

proposed refuge recommendations for these pests do not endorse seed mixtures.

Shelton et al. also tested whether insects would evolve resistance more rapidly when a refuge was sprayed than when it was unsprayed. In the 27 day period between the time that insect releases ended and the field measurement of resistance allele frequency was begun, insects from 20% sprayed refuge treatment and 20% unsprayed refuge treatment showed no difference in changes in allele frequency. (The initial frequency was ~85%, and first field measure was ~76%, based on the assumption that only larvae homozygous for resistance survive). The second field measurement indicated that the frequency of alleles in the sprayed refuge had increased by about 2%, but in the unsprayed refuge the frequency had decreased by about 12%. Between the second and third measurement the frequencies in both 20% refuge treatments had risen 0.5%. The heterogeneity in response to the two refuges over time is unexplained, but the overall difference of 10% is certainly in line with theoretical expectations.

Shelton et al. also found higher numbers of insects on *Bt* plants in the sprayed refuge treatments which they attribute to lower *Bt* susceptibility of these populations. Unfortunately, differences in susceptibility do not really explain the differences in densities because in period 1, when the mortality of larvae from the sprayed and unsprayed treatments are equivalent, there are already differences in the densities. The results from this experiment are also hard to interpret because Shelton et al. do not report on the efficacy of the spraying.

Although there are obvious limitations to these studies, they do confirm, in the field, that the mixed seed approach could be problematic and that an unsprayed refuge results in more rapid adaptation than an equal size sprayed refuge. Although scientists may not be surprised by these results, field studies like this one are essential for developing public confi-

dence in resistance management techniques.

1. James, C. *Global Review of Commercialized Transgenic Crops*. (ISAAA, Ithaca, NY, 1999).
2. E.P.A. 1998. Scientific Advisory Panel, Subpanel on *Bacillus thuringiensis* (Bt) plant-pesticides and resistance management, February 9-10, 1998. Docket No. OPPTS-00231.
3. ILSI. *An evaluation of insect resistance management in field corn: a science-based framework for risk*

assessment and risk management. (ILSI International Life Sciences Institute, November 23, 1999).

4. Rissler, J. and M. Mellon. *Now or never: Serious new plans to save a natural pest control*. (Union of Concerned Scientists, Cambridge MA., 1998).
5. E.P.A. and U.S.D.A. 1999. E.P.A. and USDA position paper on insect resistance management in Bt crops. http://www.epa.gov/pesticides/biopesticides/otherdocs/bt_position_paper_618.htm
6. Shelton et al. *Nat. Biotechnol.* **18**, 339–342 (2000).

From genome to cellular phenotype—a role for metabolic flux analysis?

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More than 20 genomes have been completely sequenced, and the first draft of the complete human genome is expected this year¹. This barrage of information should eventually lead to a better understanding of cellular physiology, which in turn may then be exploited to advance human health, agricultural production, and industrial fermentation. If the physiology of the organism under study is relatively well understood, such as for *Escherichia coli* or yeast, the compilation of this sequence data to reconstruct metabolic pathways may seem a reasonable challenge. However, if there is only limited biochemical information available, such as for *Treponema pallidum* (the causative agent of syphilis), a partial list of putative enzymes is unlikely to allow metabolic networks and cellular phenotypes to be established. Realistically, a list of genes alone is not enough to understand the pathophysiology or manipulate the metabolism of *T. pallidum*. Thus, a robust method of converting a list of putative enzymes into a set of metabolic pathways could be a powerful bioinformatic tool. In this issue, Schuster et al.² discuss a means of defining pathways rigorously and show how to express their constitutive activities as the sum of “elementary flux modes”, each of which represents a minimal sequence of steps that can in principle operate independently of any others.

The path from genome to phenotype involves multiple steps (Fig. 1). Although considerable efforts have been devoted to improving the early steps, we are only beginning to get a handle on the later ones. Sequencing the

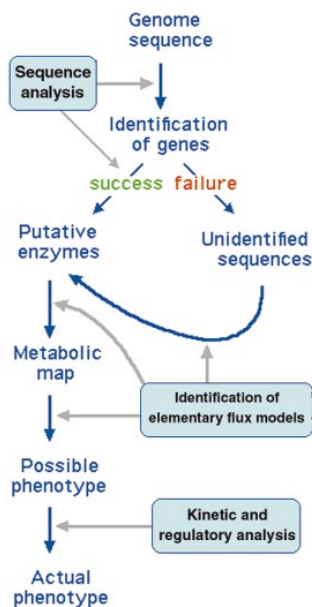


Figure 1. The necessary steps to convert a genome sequence into a phenotypic description include not only the early stages of identifying the genes, but also later ones that have received less attention: assembling the enzyme-catalyzed reactions into a metabolic map, determining what kinds of metabolic activity are stoichiometrically possible, and finally, determining which of these are kinetically possible. Identification of elementary flux modes is especially useful for defining the stoichiometric structure of the map, but it can also aid in identifying the roles of orphan genes remaining after bioinformatics studies have permitted the identification of other genes by comparison with the sequences of known enzymes.

genome is now just a matter of time and money, and the identification of gene function is becoming easier as a result of advances in comparative sequence analysis³. However, the increasing number of orphan sequences will require considerable effort to understand. It is thus not surprising that the development of computational algorithms to predict protein function from amino acid sequences is now a

core aim of bioinformatics⁴. However, even for *Caenorhabditis elegans*, with a much more compact and nonrepetitive genome than the human, as many as half of the proteins may be incorrectly identified¹—an observation that argues strongly for the need for an independent approach to locate errors in the results obtained from sequence analysis. In any case, it would be a serious mistake to conclude that the work ends with a complete list of the enzymes active in a given organism. Predicting a phenotype requires that the metabolic transformations are stoichiometrically, thermodynamically, and kinetically possible. This problem has often been regarded as if it were trivial, but it is not.

Elementary flux modes can be determined mathematically from the list of reactions in the system, without any kinetic information. Any conceivable set of fluxes becomes a weighted sum of the elementary modes. Glycolysis and the pentose phosphate pathway, for example, can be regarded in the simplest case as the sum of seven elementary modes. This may seem very abstract and only of interest to the theoretical biologist, without any practical implications for biotechnology. Indeed, why should a biotechnologist care whether metabolic pathways are rigorously defined or not?

To understand better why this is important, consider the following case. An enzyme appears to have a key role in controlling a flow of metabolites that we wish to suppress, so as to kill a harmful organism, or to increase the yield of a commercially valuable end product by decreasing a wasteful flow of intermediates into another pathway. How certain can we be that inhibiting the target enzyme or deleting its gene will actually affect the flux in the expected way? If there are no metabolic branchpoints, and if there are no other enzymes that catalyze the reaction, then such treatments should inhibit or eliminate the pathway. However, in reality, metabolic systems achieve robustness as a result of extensive redundancy⁵, and there are often several isozymes that catalyze the same reaction. An inhibitor may still work, even if isozymes are

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