PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Two–toxin strategies for management of insecticidal transgenic crops: can pyramiding succeed where pesticide mixtures have not?

R. T. Roush

Phil. Trans. R. Soc. Lond. B 1998 **353**, 1777-1786
doi: 10.1098/rstb.1998.0330

THE ROYAL SOCIETY

Two-toxin strategies for management of insecticidal transgenic crops: can pyramiding succeed where pesticide mixtures have not?

R. T. Roush

Department of Crop Protection, University of Adelaide, Waite Campus, PMB1, Glen Osmond, South Australia 5064, Australia (rroush@waite.adelaide.edu.au)

Transgenic insect-resistant crops that express toxins from *Bacillus thuringiensis* (Bt) offer significant advantages to pest management, but are at risk of losing these advantages to the evolution of resistance in the targeted insect pests. All commercially available cultivars of these crops carry only a single Bt gene, and are particularly at risk where the targeted insect pests are not highly sensitive to the Bt toxin used. Under such circumstances, the most prudent method of avoiding resistance is to ensure that a large proportion of the pest population develops on non-transgenic 'refuge' hosts, generally of the crop itself. This has generated recommendations that 20% or more of the cotton and maize in any given area should be non-transgenic. This may be costly in terms of yields and may encourage further reliance on and resistance to pesticides. The use of two or more toxins in the same variety (pyramiding) can reduce the amount of refuge required to delay resistance for an extended period. Cross-resistance among the toxins appears to have been overestimated as a potential risk to the use of pyramids (and pesticide mixtures) because cross-resistance is at least as important when toxicants are used independently. Far more critical is that there should be nearly 100% mortality of susceptible insects on the transgenic crops. The past failures of pesticide mixtures to manage resistance provide important lessons for the most efficacious deployment of multiple toxins in transgenic crops.

Keywords: *Bacillus thuringiensis*; insect resistance; *Helicoverpa*; cotton; corn borer; maize

1. INTRODUCTION

For more than 50 years, the breeding of crop cultivars that suffered reduced losses to insects has played a major role in pest management research (Painter 1951). However, in spite of significant successes, particularly against pests that attack crops in a single plant genus or family (Maxwell & Jennings 1980), progress on classical 'host-plant resistance' is often slow and the overall impact on pest management has been limited. Molecular genetic engineering offers the potential to introduce new insect resistance traits across species barriers, as first demonstrated in parallel studies on tobacco in 1987 using genes from the bacterium *Bacillus thuringiensis* (Bt) and cowpea (Schuler *et al*. 1998).

The most successful of these transgenic crops, and the only ones that have been commercially released, have been those using the crystal protein endotoxin (*cry*) genes from Bt, especially those that produce Cry 1A toxins. After their commercial introduction in 1996, transgenic Cry 1A cotton and maize were planted on one million and three million hectares ($1\,ha = 10^4\,m^2$), respectively, in 1997 (Tabashnik *et al*., this issue). Transgenic potatoes producing a Cry 3A toxin for control of the Colorado potato beetle (*Leptinotarsa decemlineata*) are very effective (Feldman & Stone 1997), but were grown on only 10 000 hectares in 1997 (Tabashnik *et al*., this issue), largely due to strong competition from new insecticides (especially imidacloprid) that control a wider range of pests.

Bt transgenic crops can significantly reduce the use of insecticides, increasing the abundance of non-target and beneficial species in crops (e.g. Fitt *et al*. 1994), and reducing the need for insecticidal sprays even for pests not targeted by the transgenics (e.g. Feldman & Stone 1997). In the case of cotton, actual reductions in use have been in the range of 50–60% (Roush & Shelton 1997). The reduction of insecticide sprays in crops may have particular significance in the tropics. There is considerable evidence that the use of such sprays is a major selective force for insecticide resistance in pests of medical importance, particularly mosquitoes (Georghiou 1990), which although not targeted by agricultural sprays are nonetheless in the fields at the times the sprays are made. Key factors among these cross-ecosystem problems are the impacts of agricultural use of pyrethroid insecticides that affect the control of *Anopheles* mosquitoes by insecticide-impregnated bednets. The malaria transmitted by these mosquitoes causes more than one million deaths per year, mostly of African children (Curtis *et al*., this issue). At least some of this pyrethroid use is on crops currently targeted for Bt transgenic technology, including cotton.

Therefore Bt transgenic crops offer a number of benefits for the environment and human health, but these benefits are at risk due to the potential for resistance (Tabashnik *et al*., this issue). Bt sprays have been used for decades, but they are of such short persistence when applied in the field that they generally have poor efficacy

against most insects and therefore have had limited use (Roush 1994). One exception is the diamondback moth, *Plutella xylostella*, which was intrinsically quite susceptible to Bt toxins, and suffered considerable Bt use when resistance had evolved to other insecticides. The diamondback moth has now evolved resistance to Bt sprays in many if not most areas of the tropics (Tabashnik 1994*a*; Perez & Shelton 1997; Tabashnik *et al.*, this issue). Bt transgenic crops significantly increase the efficacy of the Cry toxins compared to Bt sprays, and therefore the potential for resistance, perhaps primarily because transgenic crops are more likely to be used than Bt sprays (Roush 1994).

Owing to the current commercial significance of Bt crops, the urgency of resistance management plans for their deployment, and the depth of public concern about the future of Bt, this paper will concentrate on Bt transgenic crops. However, the general principles applied to Bt crops will also be relevant to other kinds of insecticidal transgenic crops.

2. RESISTANCE MANAGEMENT TACTICS FOR BT CROPS

Before developing the main topic of this paper—an exploration of the use of toxin stacking or pyramiding—it will help to put pyramiding in context with an overview of tactics that have been proposed for resistance management for Bt transgenic crops. There are at least eight possible types of tactics to slow selection by transgenic plants, some of which are mutually exclusive: (i) express toxin genes only moderately strongly, so that not all susceptible individuals are killed; (ii) modify the expression of the genes in each plant, such that they are expressed only as needed to protect the crop through tissue-specific, temporal-specific or inducible promoters; (iii) express the toxins to as high as is agronomically acceptable; (iv) deploy different toxins individually in different varieties, simultaneously; (v) deploy toxins sequentially, i.e. reserve toxins until previous ones fail; (vi) deploy plants with a mixture of toxins; (vii) leave non-transgenic crop and non-crop host-plants as 'refuges' for susceptible insects; and (viii) deploy the crops as part of an overall integrated pest management (IPM) programme that combines multiple tactics for control.

Owing to the lack of technological feasibility, poor efficacy of resistance management and impracticality of pest control, low and variable expression ((i) and (ii) from the list above) have been essentially abandoned as viable deployment options, at least for the near term, in favour of (iii) high expression (Roush 1996, 1997*a*; Gould 1998). The success of high expression depends critically on the use of (vii) a refuge (Roush 1994; Gould 1998). The need for refuges provides a strong incentive for integrating transgenic crops within a more general IPM programme (viii), in some cases potentially using transgenic crops as a means of regulating pest population growth to support other non-insecticidal tactics (Roush 1997*a*). As will be discussed in § 5, pyramiding or stacking multiple toxins in the same plants (vi) appears to be a much more effective strategy than deploying them individually ((iv) or (v)).

Currently, all cultivars of insecticidal transgenic crops that are available commercially carry only a single Bt gene. However, single-toxin crops are particularly at risk where the targeted insect pests are not highly sensitive to the Bt toxin used (Roush 1997*b*; Gould & Tabashnik 1998). As will be discussed in § 4, the most prudent method of avoiding resistance under such circumstances is to ensure that a large proportion of the pest population develops on non-transgenic 'refuge' hosts, generally of the crop itself. This has generated recommendations that 20% or more of cotton and maize should be non-transgenic in Australia (R. T. Roush, unpublished results) and North America (Gould & Tabashnik 1998; Andow & Hutchison 1998). Such high refuges may cost in terms of yields, encourage further reliance on pesticides, and exacerbate resistance to the pesticides used. Pyramids offer the potential for superior delays in resistance with smaller and more acceptable refuge sizes (Roush 1997*b*).

3. SIMULATION MODELLING

(a) *Underlying assumptions*

This paper will make extensive use of simple computer simulation models. It would be preferable to make decisions for resistance management on the basis of realistic experiments in the field, but these would take years and considerable expense (both in money and delays of the environmental and health benefits). Simulation models are our best tools in the foreseeable future.

Specialized versions of the same basic simulation model were developed for one or two toxins in the plants, to which the insect population could respond to by up to three resistance loci. Except as noted, both toxins used in a pyramided plant were assumed to be at risk for the development of resistance. Initially, two loci in the insects were specific to toxins A and B, respectively, but in later simulations (described in § 5f) a third locus was added that could confer resistance to both toxins. Except as noted, the initial frequency of resistance alleles at each locus was 10^{-3}, following the conclusions of Gould *et al.* (1997) for a cotton bollworm, *Heliothis virescens*. This assumption is not an endorsement of the estimate of Gould *et al.* (1997); to the contrary, their data seem inconsistent even with the predictions given within their paper for single-gene inheritance of the resistance studied. Nonetheless, because resistance management is more effective when the frequency of resistance is lower (Roush 1994, 1997*a*), an assumption of 10^{-3} should be conservative.

To be even more conservative, the models assumed that there are no fitness-costs to resistance. If fitness-costs do exist, pyramiding strategies will work even better compared to sequential deployment (Gould 1994, 1998), as can also be true for pesticide mixtures (Roush 1989). These simulations also assumed that resistant homozygotes were unaffected by the plants, and except as noted, that susceptible larvae always died when they fed on transgenic plants. Except as noted, the simulations all assumed that 10% of eggs in the population are laid on non-transgenic hosts. Implicitly, eggs laid on non-transgenic hosts survived equally well as resistant homozygous insects on transgenic hosts. Population growth was density independent. The models also assumed that selective toxin exposure occurred only for larvae, as would be true for lepidopteran pests on Bt transgenic crops. The

Cry 3A transgenic Bt potato plants mentioned above, control both adult and larval Colorado potato beetles (Feldman & Stone 1997). Single-locus models developed for selection on both adults and larvae of Colorado potato beetles (Roush 1996; Gould *et al*. 1994) essentially give the same results as discussed here. The extremely high sensitivity of potato beetles to Cry 3A toxin encourages optimism about resistance management for that species (Roush 1994).

Mating was assumed to be random throughout the populations (implying that transgenic and non-transgenic hosts are close enough to one another for moths to freely exchange) and, in the absence of selection, the frequencies of the genotypes are based on the Hardy–Weinberg expression (where p represents the frequency of the resistance allele (R) and q the susceptible allele (S), p^2 gives the frequency of RR homozygotes, $2pq$ for RS heterozygotes, and q^2 for SS homozygotes).

It is further assumed that larvae do not move between transgenic and non-transgenic hosts, i.e. that there are neither seed mixtures nor row mixtures of transgenic and non-transgenic hosts that allow interplant dispersal. Significant exchange of larvae between transgenic and non-transgenic plants, which has been observed in cotton (R. T. Roush and G. P. Fitt, unpublished results), maize (P. Davis and R. T. Roush, unpublished results), rice (Bennett *et al*. 1997) and broccoli (Shelton *et al*. 1998), can accelerate resistance in theory (Mallet & Porter 1992; Tabashnik 1994*b*; Roush 1996) and has done so in experiments with diamondback moth (Shelton *et al*. 1998). Seed mixtures can also allow significant damage to the crop, at least in cotton (R. T. Roush and G. P. Fitt, unpublished results).

(b) *Modelling format*

All versions of the simulation model used the same deterministic general format. Populations distribute their eggs in Hardy–Weinberg proportions at random across refuge and transgenic habitats, with larvae suffering mortality based on their genotypes. Where some percentage of the population escapes exposure to the toxins, the model simply sets that fraction of the population aside from a selection routine. In the absence of the third locus with cross-resistance, linkage between loci was followed through frequencies of all ten possible unique two-locus genotypes (including coupling and repulsion). Recombination (r) between the loci was adjusted from 0.01 to 0.5. Except as noted, the time until resistance evolves is measured as the number of (non-overlapping) generations until the frequency of the resistance allele exceeds 50%. This is a convenient measure of resistance, which is independent of assumptions on population growth. At the level of changes in frequency of the resistance allele, the models were checked against similar models (e.g. Mani 1985; Mallet & Porter 1992; Tabashnik 1994*b*) and gave the same results for the same parameter values. The model also tracks population density on both transgenic and non-transgenic host plants.

4. SINGLE-TOXIN BT TRANSGENIC CROPS

The mortality of heterozygous (RS) insects is the strongest influence on the rate of evolution of resistance for insecticidal crops that produce only a single Bt toxin, provided that there is a significant refuge to produce moths that can mate with resistant survivors and ensure that most of their offspring will be heterozygous or susceptible (Roush 1997*a*,*b*). When there is only one resistance locus in the insects and the mortality of heterozygous individuals exposed to transgenic crops exceeds 95%, resistance can be delayed for more than 40 generations even when resistance is initially as common as 10^{-3} and only 10% of the pest population develops on refuge hosts (figure 1). This is the basis of the 'high-expression' or 'high-kill' strategy for resistance management. However, when the mortality of heterozygotes is less than 90%, one needs a refuge of more than 20% to delay resistance for more than 20 generations, and greater than 10% even if the initial resistance frequency is 10^{-6} (Mallet & Porter 1992; Roush 1994, 1997*a*,*b*). Not even the survival of 10% of the susceptible individuals on transgenic plants can do much to delay resistance (figure 1). Where the survival of susceptibles does appear to cause a delay of resistance (i.e. at 80% mortality of RS in figure 1), it is only because resistance is effectively very recessive.

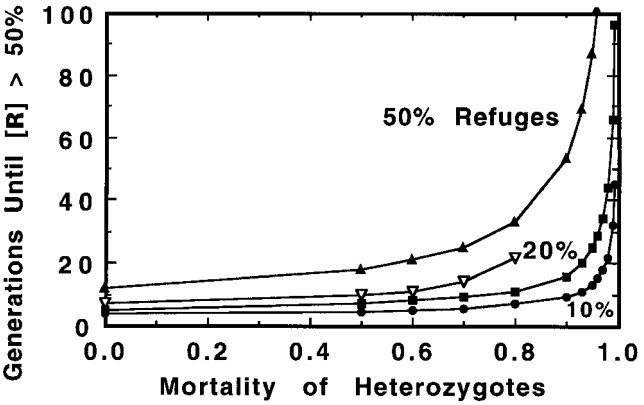

Figure 1. Effect of mortality of RS heterozygous larvae and proportion of eggs that developed on non-transgenic refuge hosts on the evolution of resistance. As discussed in the text, results are from a simulation model with the following assumptions: a single locus, random mating, no selective mortality of resistant homozygous larvae, that some fraction of the population escapes exposure (refuges of 10% (filled circles), 20% (filled squares and open triangles) or 50% (filled triangles)), and initial frequencies of resistance allele of 10^{-3}. For all of the filled symbols, it was assumed that all susceptible insects died when they fed on transgenic plants; the curve with the open symbols represents a case in which 10% of the susceptibles on transgenic plants survived when there was a 20% refuge.

Although 100% mortality of Bt-resistant heterozygotes has been observed for *Heliothis virescens* (Gould *et al*. 1997) and the diamondback moth (Roush 1994; Metz *et al*. 1995), it seems unlikely that all species targeted by some or all current single-toxin Bt cultivars are as well controlled, especially *Helicoverpa* species on maize and cotton (Roush 1997*b*; Andow & Hutchison 1998; Gould 1998; Gould & Tabashnik 1998). In such cases, the only way to delay resistance for single-toxin plants is with very large refuges (figure 1). Pyramids offer a means to delay resistance with practically acceptable refuge sizes (Roush 1997*b*), as described next.

5. TWO TOXINS: PYRAMIDING

(a) *Options for two-toxin deployment*

Given two different insecticidal toxins that are believed not to share cross-resistance (i.e. there is no one mechanism in the pests that can confer resistance to both), there are three general choices as to how the toxins could be deployed: (i) individually but simultaneously in different varieties, i.e. as a mosaic (either as a seed mix within the same field or in neighbouring fields); (ii) sequentially (one after another in an evolutionary race with the pests); or (iii) they could be stacked in the same variety, i.e. pyramided (see § 2). Previous studies have shown that sequential deployment is always at least as good as, and often superior to, mosaics for insecticides and toxins (Roush 1989, 1997*a*,*b*). Given that the development of toxin cultivars is a commercial exercise, it seems unlikely that withholding resistance genes, especially when developed by competing companies, will be seen as consistent with a free market. However, because sequential deployment is a better option than mosaics, pyramids will be compared with sequential deployment in the following discussion.

In contrast to single-toxin 'high-kill' strategies, pyramiding relies on the idea that each toxin is used individually in a way that would kill all insects susceptible to that toxin, and in so doing, kills insects that are resistant to the companion toxin (Roush 1997*a*). This is 'redundant killing' in the sense that most of the population, which is susceptible to both toxins, is killed twice (Comins 1986; Gould 1986*a*,*b*). The extent to which individuals that are resistant to one toxin are killed by the other is central to the effectiveness of the pyramiding strategy.

(b) *Candidate toxins for pyramiding*

Ideally, the two toxins should be as unrelated as possible, such as a Bt toxin and a digestive inhibitor, to minimize the chance that a single gene in the pest species could confer resistance to both factors. As a practical matter, the only toxins that will likely be available for pyramiding in at least the next five years will be Bt Cry proteins. Although there is a wide range of alternative toxins under development, including inhibitors of digestive enzymes (proteinase and amylase inhibitors), lectins, chitinases and peroxidases (Carozzi & Koziel 1997; Schuler *et al*. 1998), few have yet proven to be effective against the same pests for which Bt toxins are being successfully deployed. For example, although an alpha-amylase inhibitor in seed-targeted expression is very effective for the control of pea weevils (Schroeder *et al*. 1995), no complementary Bt toxin is known. The more promising candidates that might complement Bt toxins include cholesterol oxidase against *Helicoverpa* and *Heliothis* species (Purcell 1997), and proteinase inhibitors against rice stem borers (Bennett *et al*. 1997). Although often more effective in tobacco than other crops, non-Bt toxins typically cause only a 30–80% delay in development rather than a practically significant increase in mortality (Carozzi & Koziel 1997; Schuler *et al*. 1998). Other newer candidates, such as *Helicoverpa* stunt virus (Schuler *et al*. 1998) and toxins from *Photorhabdus luminescens*, are probably at least five to ten years from commercial release. More than ten years were required to develop Bt transgenic crops using toxin genes that were already well characterized by 1986.

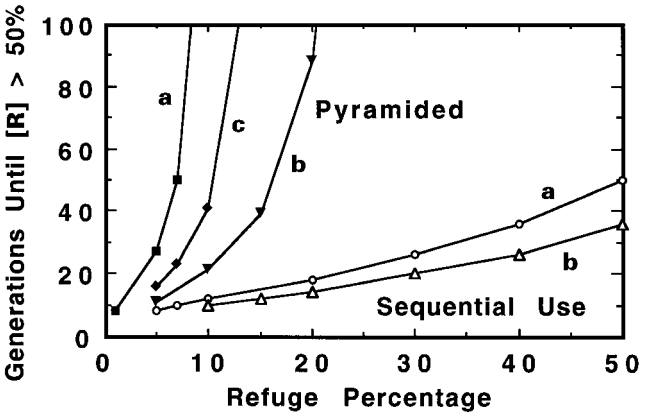

Figure 2. The evolution of resistance with the sequential deployment of two toxins compared with the use of the toxins jointly in a pyramided variety, for a range of percentages of the population in refuges. For comparison, it was assumed that there was 70% mortality of RS heterozygotes for each toxin (curve 'a'), 50% mortality of RS heterozygotes for each toxin (curve 'b'), or 50% mortality of RS for one toxin and 30% for the other (curve 'c'). The values shown for 'sequential' curves are twice the number of generations required for resistance to evolve to just one of the toxins when used alone, as if cultivars bearing one and then the other of the toxins were sequentially deployed.

Even for Bt toxins, probably no more than two with effectiveness against any given pest will be useful and available in the next five years. Given that decreased binding has proven to be a common mechanism of resistance, the appropriate toxins should at least have different binding sites. Further, they should not use toxins that have already shown significant levels of cross-resistance in strains observed to date, such as between Cry 1A, Cry 1F and Cry 1J (Tabashnik *et al*. 1997*a*, this issue). However, at least two good toxins seem to be available for most key pests, such as Cry1A and Cry 9C for corn borers (Roush 1997*b*).

(c) *Refuge needs for one- and two-toxin plants without cross-resistance*

Even though individuals with resistance to two toxicants may be very rare initially, a refuge is still necessary for pyramiding to be effective in delaying resistance (Curtis 1985; Gould 1986*a*,*b*; lower left of figure 2, refuge less than 5%). However, when selection can respond only at separate toxin-specific loci in the insects, the refuge for a similar delay of resistance can be much smaller when toxins are pyramided than if they are sequentially deployed (Roush 1997*b*). As long as at least 50% of the heterozygotes are killed when they feed on transgenic plants, a pyramid with even a 10% refuge can delay resistance for longer than if the two toxins are sequentially deployed with a 30–40% refuge (figure 2, compare pyramid curves 'a' and 'b' with sequential curves 'a' and 'b'). If the control of heterozygotes is less than 50% for both toxins, essentially neither strategy will be effective for delaying resistance (e.g. see lower left of figure 1).

As with insecticide mixtures (Mani 1985), pyramids are most effective when at least one of the resistances is

mostly recessive (Gould 1986*a*,*b*). If resistance to at least one toxin confers no more than 30% survival in a pyramid, even a 10% refuge can support a significant delay of resistance (figure 2, compare pyramid curves 'b' and 'c'). However, as with single toxins, it is still desirable for the expression of both toxins to be as high as is agronomically acceptable, both for the control of heterozygotes and, as will be seen in the next subsection, for the control of susceptible homozygotes.

Even though 100% of heterozygous *H. virescens* and diamondback moths have been killed in experiments on current Bt cultivars, current cultivars do not even kill 100% of susceptible larvae among *Helicoverpa* species. Based on extrapolations of diamondback moths (§ 5d), one might expect that plants that kill around 95% of susceptible homozygotes (in the range of *Helicoverpa* species on current Bt cotton cultivars) would likely result in 50–70% mortality of heterozygotes. However, a key feature of the pyramiding strategy is that only one of the types of heterozygotes needs to have such high mortality; pyramiding two or more toxins into a cultivar increases the chance that at least one will be especially favourable to resistance management.

Because pyramids can reduce the need for large refuges, they provide a way to use Bt genes in cotton without relying on maize and other crops as refuges for *Helicoverpa* species (Roush 1997*b*). This is especially important in light of the expected releases of Bt cotton and maize into the USA and Mexico, South America, Africa, China and India in the same regions with the same *Cry 1A* gene.

(d) *Effect of mortality of susceptible homozygotes on pyramids*

The success of pyramids in the absence of cross-resistance is less dependent on high mortalities of heterozygotes than a single toxin. Whereas the single toxin requires large refuges whenever mortalities of heterozygotes are less than 90% (figure 1), pyramids can still be effective with relatively small refuges of 10% even for heterozygous mortalities of 30–50% (figure 2). However, in contrast to plants with single toxins (figure 1), the survival of susceptible insects on transgenic plants has a major effect on the durability of pyramids (Roush 1994; figure 3). Whereas the high-kill strategy aims to control heterozygotes directly, the pyramiding strategy aims to do so by killing the individuals resistant to one toxin with a second toxin. The requirement to exceed 95% kill to achieve significant benefits still applies, but in this case, it is the mortality of susceptible homozygotes that matters (figure 3).

In considering what would be reasonable parameter values for these simulations, I used the example of Bt-resistant diamondback moths from Hawaii and Florida, for which concentration mortality data are provided by Tabashnik *et al.* (1992) and Tang *et al.* (1997). Resistance in these strains appears to be primarily due to a single major gene (Tabashnik *et al.* 1997*b*; Tang *et al.* 1997), so the F1 larvae used in bio-assays are presumed to be heterozygotes. In these populations, Bt concentrations that cause 99%, 95% and 80% mortality of SS homozygotes cause only about 10%, 70% and 50% mortality of F1 larvae, respectively. For the sake of simulations shown in figure 3,

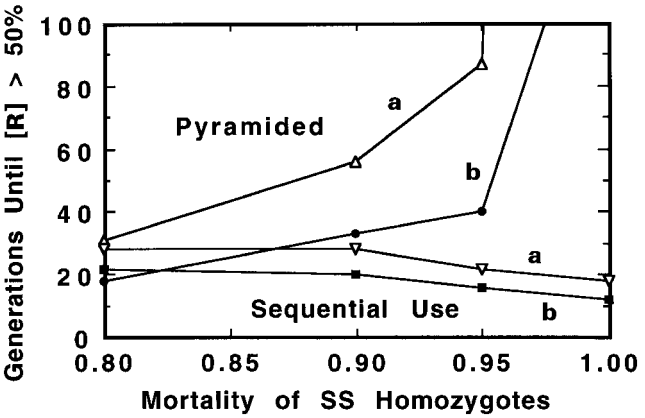

Figure 3. Influence of mortality of susceptible (SS) homozygotes on the evolution of resistance to transgenic crops for one toxin used alone (toxin A or B) or two toxins 'pyramided' in the same plant (A plus B), assuming that there is no cross-resistance. For simplicity, it is assumed that: (i) the mortalities of the single heterozygotes ($R_aS_aS_bS_b$ or $S_aS_aR_bS_b$) would be 70% when tested against just the one toxin to which they are resistant at SS mortalities of 90–100%, and 50% when SS mortality is 80%; (ii) mortalities of the homozygous susceptible genotypes are the same for both genes; and (iii) 20% (curve 'a') or 10% (curve 'b') of the eggs in each generation are laid on non-transgenic hosts. To more easily make comparisons across one- and two-toxin strategies, the mortalities for susceptible homozygotes are given in terms of what would be observed if the larvae were exposed to just one toxin at a time.

I assumed, for simplicity, that the mortality of RS heterozygotes was 70% for cases of susceptible mortality ranging from 90 to 100%, but to conservatively avoid making resistance excessively recessive, RS mortality was 50% when SS mortality dropped to 80%.

For single-toxin plants, the initial frequency of resistance alleles is less important to durability than the mortality of heterozygotes (Roush 1994, 1997*a*,*b*). However, pyramids are considerably more effective when resistance frequencies are low, provided that susceptible homozygotes are all killed by each of the toxins used separately (figure 4, compare curves labelled 'a' for sequential introductions and pyramids). However, even a lower initial frequency of the resistance allele does not significantly help in cases where transgenic plants cause only the poor mortality of susceptible homozygotes. For example, where the mortality of susceptible homozygotes and heterozygotes is only 80% and 50%, respectively (the point at 80% mortality on curve 'b' in figure 3), even with an initial allele frequency of 10^{-5}, the pyramiding strategy is slightly worse than a sequential release strategy, with resistance in 32 and 35 generations, respectively. As in figure 3, not much is lost by pyramiding; on the other hand, much can be gained from pyramiding if the mortality of susceptible insects is consistently greater than 95%, especially if the 'pyramided' varieties are released while initial resistance allele frequencies are still low (figure 4).

(e) *Effect of seed-line purity on pyramids*

As already seen, the pyramiding strategy relies on the high mortality of susceptible insects for each of the toxins used in the pyramids. However, because of practical

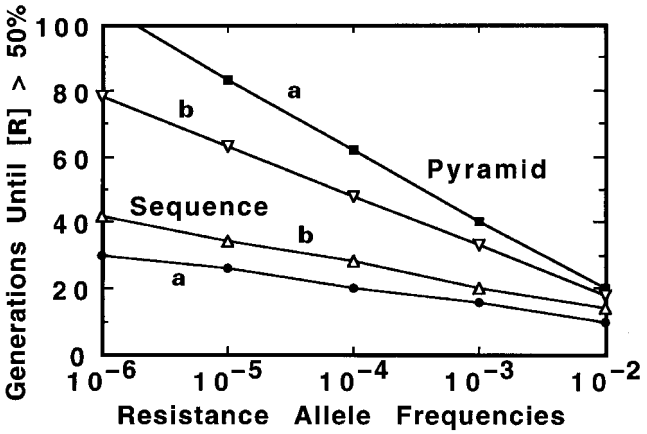

Figure 4. Effect of initial allele frequencies on the evolution of resistance for sequential and pyramided deployment of two toxins. A 10% refuge is assumed, with either 70% mortality of RS heterozygotes and 100% mortality of SS homozygotes (curve 'a'), or 70% for RS and 90% for SS for each toxin (curve 'b'). For comparative purposes, the curves labelled 'b' are an expansion across a wider initial frequency of the resistance allele of the points given for curve 'b' at 90% mortality of SS homozygotes (and an initial frequency of 10^{-3}) in figure 3.

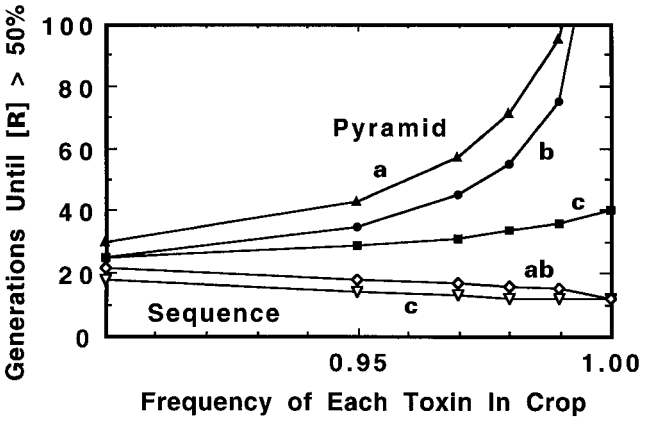

Figure 5. Effect of seed-line impurity on durability of pyramids compared with the sequential use of the same toxins. Mortality of heterozygotes is 30% and 100% for susceptible homozygotes for each toxin for curves 'a' and 'b', but 5% of susceptible homozygotes survive for the curves labelled 'c'. For pyramids, either 100% of the crop carries toxin A, but the frequency of B is varied across a range of 90–100% (curve 'a'), or both toxins are allowed to vary in frequency from 90 to 100%, with their joint occurrence assumed to be random (curves 'b' and 'c'). For sequential introductions, both cultivars have the same levels of seed purity.

problems in incorporating two genes into the same cultivar, there may be some impurity of seed lines, such that some plants carry only one or the other of the toxin genes. For example, when the frequency of each toxin in the crop is 95% and segregating is random, the frequency of plants with both A and B is 0.9025, that of plants with A or B only is 0.095 (0.0475 each), and that of plants with neither A nor B is 0.0025. This would provide considerable opportunity for selection for resistance to either A or B without protection from the other toxin, and faster resistance (figure 5). Seed producers indicate that at least 97% purity is expected, which should ensure considerable benefits from pyramiding, but the increase in durability from improved purity would seem to justify the extra effort whenever there is 100% mortality of susceptible insects from each of the toxins deployed alone (figure 5, curves 'a' and 'b'). For example, when both toxins are present at a frequency of only 99%, resistance evolves in 75 generations, only half that when both toxins are represented in every plant (160 generations). On the other hand, if even 5% of the susceptible insects survive each toxin, there is still an advantage to pyramiding, but comparatively little benefit from increased seed purity (figure 5, 'c' curves).

(f) *Effect of linkage between resistance loci on pyramids*

Close chromosomal linkage between two resistance loci decreases the benefits of a mixture (Mani 1985). However, in the absence of other factors that limit the effectiveness of a pyramid, not even close linkages reduce the durability of pyramids to that of sequential introductions (figure 6).

(g) *Cross-resistance among Bt toxins*

Because the Bt Cry endotoxins are at least superficially similar to one another, it is possible that resistance genes will evolve that can overcome both Bt genes to be used in the pyramid, even if they have different binding sites. It is

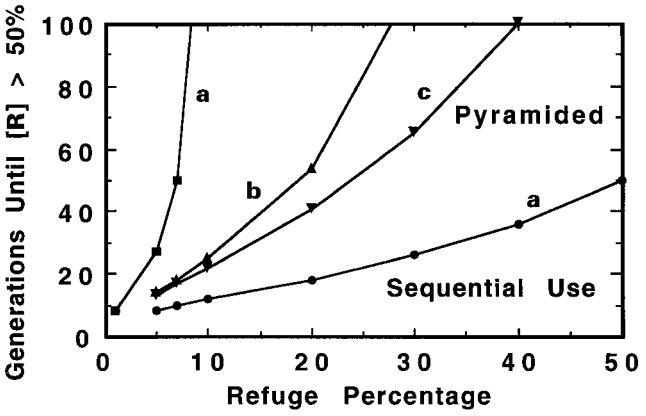

Figure 6. Effect of chromosomal linkage on evolution of resistance for pyramids and sequential introduction of the same toxins. Curve 'a' assumes no linkage and is the same as 'a' in figure 2; curve 'b' is same as 'a' but with recombination reduced to 10% from 50%. In 'c', recombination is only 1%.

widely assumed that such a resistance mechanism would be degradative and have a dominant expression, even though no such genes have yet been described (as discussed below). Strictly speaking, it is not dominance *per se* (the resemblance to resistant parents) that is important, but the extent to which heterozygotes survive. Thus, whether degradative or dominant, any genes that provide greater than 10% survival on the transgenic plants would cause resistance rather quickly (figure 1), and the pyramiding strategy will be much less effective than expected when compared with the simulation results given so far. However, the more important question is whether it would be better to sequentially deploy the genes given the possibility of cross-resistance, i.e. what is the relative effect of cross-resistance on both options? For example, a cross-resistance gene conferring 30% survival in heterozygotes with a high initial frequency would cause the rapid failure of a pyramid, but it would also do the same

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

for a sequential deployment strategy (as will be subsequently illustrated).

Simulation modelling can be used to identify the characteristics of cross-resistance genes and the conditions of toxin expression under which sequential deployment would be more effective than pyramiding. To model cross-resistance, an additional locus was added to the model described to this point. One resistance locus continued to confer resistance to toxin A, and one to toxin B, with the survival of the pyramid being the product of survival values for each of the toxins when used alone. The third locus (C) confers resistance to both toxins, and is added to the survival conferred by the first two loci, up to a maximum of 100% survival. In considering the literature on pesticide resistance mechanisms in the broadest sense, it is hard to imagine any gene could confer cross-resistance without also conferring resistance to each Bt toxins when used alone.

If the cross-resistance gene, R_c, has fitnesses and initial gene frequencies greater than or equal to those of the specific genes, R_a and R_b, selection for resistance to any one toxin deployed alone occurs just about as quickly for pyramids. This is simply because the cross-resistance gene has the same advantages for resistance to the single toxins as do the specific resistance genes. For example, let us assume that all of the resistance genes have the same initial frequency (10^{-3}), a refuge of 10%, and the same fitnesses for all similar genotypes in the presence of the appropriate toxins. Specifically, assume that the susceptible S_aS_a and S_cS_c homozygotes all die when feeding on plants with toxin A, and all S_bS_b and S_cS_c die on plants with toxin B; but only 70% of R_aS_a and R_cS_c heterozygotes die when feeding on plants with toxin A and 70% of R_bS_b and R_cS_c on plants with toxin B. Simulations show that when the toxins are pyramided, the frequency of R_c reaches 64% after six generations of selection, but that the frequencies of R_a and R_b are essentially unchanged. In contrast, if only toxin A is deployed, frequencies of both R_a and R_c reach 50% after seven generations (figure 7, lower pair of curves, second pair of points from the left). If the R_cS_c heterozygotes suffer only 60% mortality, simulations predict that the frequency of R_c would exceed 50% in only five generations. However, the frequency of R_c would also reach 59% by six generations if either toxin A or B was deployed alone (figure 7, lowest left pair of points).

When the R_cS_c heterozygotes suffer 80% mortality and the R_aS_a and R_bS_b heterozygotes only 70%, the initial use of toxin A alone selects for the R_a allele ('R_a (seq)' in figure 8) a little more rapidly than the R_c allele ('R_c (seq)'), but R_c is still strongly selected when both resistance alleles are rare. The rate of increase of R_c slows after the frequency of R_a exceeds 10%, owing to the reduced average contribution of R_c to survival, but then increases again when selection is reintroduced by the switch to toxin B. However, because R_c is already so common from the selection with toxin A, resistance to toxin B by R_c appears by generation 8, even though the toxin B-specific resistance allele R_b is still uncommon. Thus, there is widespread resistance to both toxins by generation 8. If the two genes were pyramided ('pyr' in figure 8), cross-resistance does cause the pyramid to fail first (the increase shown is almost entirely to R_c), but only a generation earlier (figure 8).

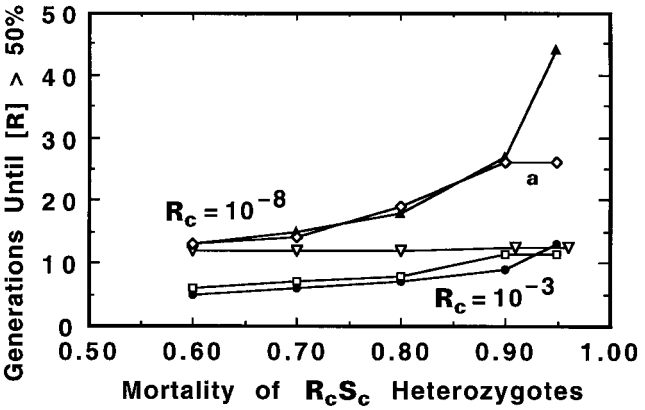

Figure 7. Influence of a gene for cross-resistance, R_c, on the durability of pyramided (filled symbols) and sequentially introduced toxins (open symbols). For a refuge of 10%, R_aS_a and R_bS_b mortality is 70%, and S_aS_a and S_bS_b is 100%, except in curve 'a', where R_aS_a and R_bS_b mortality is 95%. The initial frequency for R_a and R_b is 10^{-3} for toxins A and B, but the initial frequency for R_c is either 10^{-3} (lower pair of curves) or 10^{-8} (upper pair of curves and flat line of open triangles).

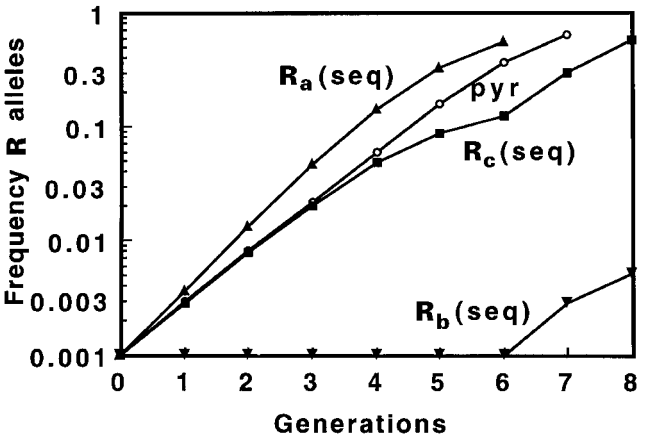

Figure 8. Example of change in allele frequencies over time for a gene conferring cross-resistance and two alleles causing more specific resistance; a more detailed look at the case when the mortality of R_cS_c is 80% and the initial frequency for all resistance alleles, R_a, R_b and R_c, is 10^{-3} (as for the third pair of points from the left in the bottom curve of figure 7). 'pyr' stands for pyramid, 'seq' for sequence.

These results should not be surprising. The cross-resistance gene has selective advantages no different than any other single gene without regard to whether there are one or two toxin genes present, and any gene conferring more than 10% survival in heterozygotes will cause resistance quickly in the absence of the benefits of pyramiding (figure 1). For sake of reference, in the absence of cross-resistance, sequential use under these assumptions would be expected to last for 12 generations and pyramids for 160 (results outlined in figures 2 and 3). In the presence of the cross-resistance gene, all-use strategies fail quickly; there is no advantage to pyramids, but little loss either. In the absence of cross-resistance, there is a ten-fold advantage to pyramiding.

These simulations imply that if broad cross-resistance genes exist, they must have frequencies and/or fitnesses lower than those of the Bt-resistance genes that have

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

already been described. Otherwise, such broad cross-resistance genes should already have been encountered, but they have not. In at least four intensively studied cases of Bt resistance in the diamondback moth, resistance does not extend to Cry 1C (Tabashnik *et al*. 1997*b*, this issue; Tang *et al*. 1996), which would have been the best candidate to pyramid with Cry 1A. Although there were early reports of broad cross-resistance in *H. virescens* (Gould *et al*. 1992), subsequent studies have shown that the major locus for resistance to Cry 1A did not confer a significant effect on resistance to Cry 2A (Heckel *et al*. 1996).

To explore this further, it was assumed that the major cross-resistance locus had an initial frequency of 10^{-8}, but all else remained the same (figure 7, except for curve 'a'). Here again, there was little difference between pyramiding and sequential release or else pyramiding held the advantage (figure 7). When the initial frequency of R_c is 10^{-8}, the pyramiding curve is the same whether the mortalities of R_aS_a and R_bS_b are 70% or 95% (curve 'a'), because resistance for the pyramid is driven entirely by the survival of R_cS_c. When the initial frequency of R_c is 10^{-8} and mortality of R_cS_c is greater than 60%, R_c never reaches 50% frequency in sequential use, so the time for resistance is a flat 12 generations. Note the flattening of the 'a' sequences curve (just above the 'a' itself). In this case, the survival and initial frequency of R_cS_c are so low that it never significantly contributes to resistance in the sequence; resistance is simply due to R_a and R_b.

Because the characteristics, fitnesses and frequencies of cross-resistance genes cannot yet be fully anticipated, it may not be possible to completely discount their impacts on pyramided varieties. However, the conditions under which cross-resistance is most likely to cause failures of pyramids faster than sequential deployment, seem to be (i) when the mortalities of resistance heterozygotes for toxins A and B are sufficiently high enough (95% or higher) for there to be an appreciable delay of resistance even when the toxins are deployed alone (as in figure 1), and simultaneously (ii) where pyramids would not be strongly favoured even in the absence of cross-resistance, particularly when there is survival of susceptible homozygotes to the toxins when they are deployed alone (as in figure 3).

(h) *Differences with pesticide mixtures*

Contrary to the popular myth, there is no good experimental evidence that insecticide mixtures help to manage resistance (Tabashnik 1989). How is it that pyramiding of transgenic plants can succeed where pesticide mixtures have failed? Pesticide mixtures, in effect, too often occupy those regions of 'parameter space' where two-toxin strategies are no better than single-toxin strategies (as shown in the lower left-hand area of figure 3). As a result of incomplete coverage and residue decay, the mortality of susceptible homozygotes is rarely consistently high enough for pesticide mixtures to be effective. As an illustration from laboratory experiments, selection against the Indian meal moth with mixtures of toxins at concentrations that initially allowed 19% of the insects to survive, and rarely killed more than 75% of the selected line, produced resistance fairly rapidly, with little delay compared with the use of individual toxins (McGaughey & Johnson 1992), just as would be predicted from these models. Achieving the high mortality of susceptible homozygotes is a key problem for pyramids, but at least the current cultivars meet or come close to this standard for the targeted pests. Another problem is that it seems unlikely that the mortality of heterozygotes is high in the field for most cases of pesticide resistance, where resistance is so often dominant in the field (Roush & Daly 1990). Yet another key difference is that whereas there are relatively low economic and environmental costs to pyramids, the use of pesticide mixtures requires higher pesticide application costs and increased risks for the environment, especially in terms of effects on natural enemies of pests (Tabashnik 1989).

6. CONCLUSIONS

The pyramiding of toxin genes offers what appears to be the most effective way to manage resistance to Bt and other insecticidal transgenic toxins. Obtaining consistently high mortality of susceptible homozygotes is a major limitation to the durability of pyramids, but it is a factor that can readily and easily be tested before any prospective release. A major limitation for single-toxin plants, low mortality of heterozygous insects, cannot be easily tested before release, because it requires anticipating all manner of resistance alleles that may occur at low frequency in the field. Cross-resistance has long been a concern for the use of pesticide mixtures and pyramids, but this paper suggests that the risks have been greatly overestimated. If cross-resistance occurs, pyramids seem unlikely to do much worse than sequential releases of the same toxin genes; on the other hand, in the absence of cross-resistance, pyramids may cause a great delay of resistance.

Pyramids have the potential to greatly reduce refuge requirements for successful resistance management from perhaps 30–40% down to perhaps 10% (figure 2). However, small refuges remain risky (as when mortalities of heterozygotes are lower than expected, e.g. case 'b' in figure 2). The more prudent way to deploy transgenic crops remains to keep refuges as large as is economically feasible. To prevent economic losses to these refuges, other non-insecticidal control techniques (e.g. pheromone disruption of mating, classically bred resistance, suppression of overwintering stages of insects through stalk or soil disruption, and crop rotation) should be used to manage population growth across the entire system of transgenic and non-transgenic plants (Roush 1997*a*).

The motto of the Royal Society is '*Nullius in verba*', which can be translated as 'take nobody's word for it' and expressed the determination of early Fellows of the Society to verify all statements with an appeal to facts. In this case, I encourage interested readers to further investigate the points raised with additional modelling and experiments. It is widely thought that Bt transgenic crops are at risk from the rapid evolution of resistance. It is therefore important for the scientific community to rapidly come to a consensus about the best tactics for resistance management and to lobby for their implementation.

For the sake of brevity, I have often cited reviews rather than their original papers, but I thank T. J. Higgins, T. N. Hanzlik,

R. Wu, M. Peferoen & J. Van Rie, and G. P. Fitt for information on their work on alpha-amylase inhibitors in peas, *Helicoverpa* stunt virus, proteinase inhibitors in rice, Bt toxins on corn borers, and Bt expression in transgenic cotton, respectively. I also thank B. Tabashnik, F. Gould, D. Heckel, F. Perlak, G. Fitt and M. Caprio for discussions and correspondence on topics discussed here, and A. M. Shelton, J. D. Tang, P. Davis and G. P. Fitt for research collaborations.

REFERENCES

Andow, D. A. & Hutchison, W. D. 1998 Bt-corn resistance management. In *Now or never: serious new plans to save a natural pest control* (ed. M. Mellon & J. Rissler), pp. 19–66. Cambridge, MA: Union of Concerned Scientists.

Bennett, J., Cohen, M. B., Katiyar, S. K., Ghareyazie, B. & Khush, G. S. 1997 Enhancing insect resistance in rice through biotechnology. In *Advances in insect control: the role of transgenic plants* (ed. N. Carozzi & M. Koziel), pp 75–93. London: Taylor and Francis.

Carozzi, N. & Koziel, M. 1997 *Advances in insect control: the role of transgenic plants* London: Taylor and Francis.

Comins, H. 1986 Tactics for resistance management using multiple pesticides. *Agric. Ecosyst. Environ.* **16**, 129–148.

Curtis, C. F. 1985 Theoretical models of the use of insecticide mixtures for the management of resistance. *Bull. Entomol. Res.* **75**, 259–265.

Feldman, J. & Stone, T. 1997 The development of a comprehensive resistance management plan for potatoes expressing the Cry 3A endotoxin. In *Advances in insect control: the role of transgenic plants* (ed. N. Carozzi & M. Koziel), pp. 49–61. London: Taylor and Francis.

Fitt, G. P., Mares, C. L. & Llewellyn, D. J. 1994 Field evaluation and potential ecological impact of transgenic cottons (*Gossypium hirsutum*) in Australia. *Biocontrol Sci. Technol.* **4**, 535–548.

Georghiou, G. P. 1990 The effect of agrochemicals on vector populations. In *Pesticide resistance in arthropods* (ed. R. T. Roush & B. E. Tabashnik), pp. 183–202. New York: Chapman & Hall.

Gould, F. 1994 Potential and problems with high-dose strategies for pesticidal crops. *Biocontrol Sci. Technol.* **4**, 451–461.

Gould, F. 1986*a* Simulation models for predicting durability of insect-resistant germplasm: a deterministic diploid, two locus model. *Environ. Entomol.* **15**, 1–10.

Gould, F. 1986*b* Simulation models for predicting durability of insect-resistant germplasm: Hessian fly (Diptera: Cecidomyiidae)-resistant winter wheat. *Environ. Entomol.* **15**, 11–23.

Gould, F. 1998 Sustainability of transgenic insecticidal cultivars: integrating pest genetics and ecology. *A. Rev. Entomol.* **43**, 701–726.

Gould, F. &. Tabashnik, B. E. 1998 Bt-cotton resistance management. In *Now or never: serious new plans to save a natural pest control* (ed. M. Mellon & J. Rissler), pp. 67–105. Cambridge, MA: Union of Concerned Scientists.

Gould, F., Martinez-Ramirez, A., Anderson, A., Ferre, J., Silva, F. J. & Moar, W. F. 1992 Broad-spectrum resistance to *Bacillus thuringiensis* toxins in *Heliothis virescens*. *Proc. Natn. Acad. Sci. USA* **89**, 7986–7988.

Gould, F., Follet, P., Nault, B. & Kennedy, G. G. 1994 Resistance management strategies for transgenic potato plants In *Advances in potato pest biology and management* (ed. G. W. Zehnder, M. L. Powelson, R. K. Jansson & K. V. Raman), pp. 255–277. St Paul, MN: American Phytopathological Society Press.

Gould, F., Anderson, A., Jones, A., Sumerford, D., Heckel, D. G., Lopez, J., Micinski, S., Leonard, R. & Laster, M. 1997 Initial frequency of alleles for resistance to *Bacillus thuringiensis* toxins in field populations of *Heliothis virescens*. *Proc. Natn. Acad. Sci. USA* **94**, 3519–3523.

Heckel, D. G., Gahan, L. C., Gould, F. & Tabashnik, B. E. 1996 Mapping major and minor loci conferring resistance to Bt toxins in Lepidoptera. In *Proceedings of the second Pacific Rim conference on biotechnology of* Bacillus thuringiensis *and its impact to the environment, Nov 4–8*, pp. 468–480. Chiang Mai, Thailand: Entomology and Zoology Assocation.

McGaughey, W. H. & Johnson, D. E. 1992 Indian meal moth (Lepidoptera: Pyralidae) resistance to different strains and mixtures of *Bacillus thuringiensis*. *J. Econ. Entomol.* **85**, 1594–1600.

Mallet, J. & Porter, P. 1992 Preventing insect adaptation to insect-resistant crops: are seed mixtures or refugia the best strategy? *Proc. R. Soc. Lond.* B **250**, 165–169.

Mani, G. S. 1985 Evolution of resistance in the presence of two insecticides. *Genetics* **109**, 761–783.

Maxwell, F. G. & Jennings, P.R. (eds) 1980 *Breeding plants resistant to insects*. New York: Wiley.

Metz, T. D., Roush, R. T., Tang, J. D., Shelton, A. M. & Earle, E. D. 1995 Transgenic broccoli expressing a *Bacillus thuringiensis* insecticidal crystal protein: implications for pest resistance management strategies. *Mol. Breeding* **1**, 309–317.

Painter, R. H. 1951 *Insect resistance in crop plants*. New York: Macmillan.

Perez, C. J. & Shelton, A. M. 1997 Resistance of *Plutella xylostella* (Lepidoptera: Plutellidae) to *Bacillus thuringiensis* Berliner in Central America. *J. Econ. Entomol.* **90**, 87–93.

Purcell, J. P. 1997 Cholesterol oxidase for the control of boll weevil. In *Advances in insect control: the role of transgenic plants* (ed. N. Carozzi & M. Koziel), pp. 95–108. London: Taylor and Francis.

Roush, R. T. 1989 Designing resistance management programs: how can you choose? *Pestic. Sci.* **26**, 423–441.

Roush, R. T. 1994 Managing pests and their resistance to *Bacillus thuringiensis*: can transgenic crops be better than sprays? *Biocontrol Sci. Technol.* **4**, 501–516.

Roush, R. T. 1996 Can we slow adaptation by pests to insect transgenic crops? In *Biotechnology and integrated pest management* (ed. G. Persley), pp. 242–263. London: CABI.

Roush, R. T. 1997*a* Managing resistance to transgenic crops. In *Advances in insect control: the role of transgenic plants* (ed. N. Carozzi & M. Koziel), pp. 271–294. London: Taylor and Francis.

Roush, R. T. 1997*b* Bt-transgenic crops: just another pretty insecticide or a chance for a new start in resistance management? *Pestic. Sci.* **51**, 328–334.

Roush, R. T. & Daly, J. C. 1990 The role of population genetics in resistance research and management. In *Pesticide resistance in arthropods* (ed. R. T. Roush & B. E. Tabashnik), pp. 97–152. New York: Chapman & Hall.

Roush, R. T. & Shelton, A. M. 1997 Assessing the odds; the emergence of resistance to Bt transgenic plants. *Nature Biotech.* **15**, 816–817.

Schroeder, H. E., Gollasch, S., Moore, A., Tabe, L. M., Craig, S., Hardie, D. C., Chrispeels, M. J., Spencer, D. & Higgins, T. J. V. 1995 Bean alpha-amylase inhibitor confers resistance to the pea weevil (*Bruchus pisorum*) in transgenic peas (*Pisum sativum* L.). *Plant Physiol.* **107**, 1233–1239.

Schuler, T. H., Poppy, G. M., Kerry, B. R. & Denholm, I. 1998 Insect-resistant transgenic plants. *Trends Biotechnol.* **16**, 169–175.

Shelton, A. M., Tang, J. D., Earle, E. D. & Roush, R. T. 1998 Can we manage resistance to Bt-engineered plants? Results of greenhouse and field tests. In *Proceedings of the Sixth Australian Applied Entomological Research Conference, Brisbane, Australia, October 1998.* (In the press.)

Tabashnik, B. E. 1989 Managing resistance with multiple pesticide tactics: theory, evidence, and recommendations. *J. Econ. Entomol.* **82**, 1263–1269.

Tabashnik, B. E. 1994*a* Evolution of resistance to *Bacillus thuringiensis*. *A. Rev. Entomol*. **39**, 47–79.

Tabashnik, B. E. 1994*b* Delaying insect adaptation to transgenic crops: seed mixtures and refugia reconsidered. *Proc. R. Soc. Lond*. B **255**, 7–12.

Tabashnik, B. E., Schwartz, J. M., Finson, N. & Johnson, M. W. 1992 Inheritance of resistance to *Bacillus thuringiensis* in diamondback moth (Lepidoptera: Plutellidae). *J. Econ. Entomol*. **85**, 1046–1055.

Tabashnik, B. E., Liu, Y.-B., Finson, N., Masson, L. & Heckel, D. G. 1997*a* One gene in diamondback moth confers resistance to four *Bacillus thuringiensis* toxins. *Proc. Natn. Acad. Sci. USA* **94**, 1640–1644.

Tabashnik, B. E., Liu, Y.-B, Malvar, T., Heckel, D. G., Masson, L., Ballester, V., Granero, F., Ménsua, J. L. & Ferré, J. 1997*b* Global variation in the genetic and biochemical basis of diamondback moth resistance to *Bacillus thuringiensis*. *Proc. Natn. Acad. Sci. USA* **94**, 12 780–12 785.

Tang, J. D., Shelton, A. M., Van Rie, J., De Roeck, S., Moar, W. J., Roush, R. T. & Peferoen, M. 1996 Toxicity of *Bacillus thuringiensis* spore and crystal protein to the resistant diamondback moth (*Plutella xylsotella*). *Appl. Environ. Microbiol*. **62**, 564–569.

Tang, J. D., Gilboa, S., Roush, R. T. & Shelton, A. M. 1997 Inheritance, stability, and fitness of resistance to *Bacillus thuringiensis* in a field colony of *Plutella xylostella* (L.) (Lepidoptera: Plutellidae) from Florida. *J. Econ. Entomol*. **90**, 732–741.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES