

SYNTHESIS

Evolutionary ecology of insect adaptation to Bt cropsYves Carrière,¹ David W. Crowder² and Bruce E. Tabashnik¹¹ Department of Entomology, University of Arizona, Tucson, AZ, USA² Department of Entomology, Washington State University, Pullman, WA, USA**Keywords**

Bacillus thuringiensis, fitness cost, host-plant resistance, incomplete resistance, population dynamics, resistance management, transgenic crops.

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Abstract

Transgenic crops producing *Bacillus thuringiensis* (Bt) toxins are used worldwide to control major pests of corn and cotton. Development of strategies to delay the evolution of pest resistance to Bt crops requires an understanding of factors affecting responses to natural selection, which include variation in survival on Bt crops, heritability of resistance, and fitness advantages associated with resistance mutations. The two main strategies adopted for delaying resistance are the refuge and pyramid strategies. Both can reduce heritability of resistance, but pyramids can also delay resistance by reducing genetic variation for resistance. Seasonal declines in the concentration of Bt toxins in transgenic cultivars, however, can increase the heritability of resistance. The fitness advantages associated with resistance mutations can be reduced by agronomic practices, including increasing refuge size, manipulating refuges to increase fitness costs, and manipulating Bt cultivars to reduce fitness of resistant individuals. Manipulating costs and fitness of resistant individuals on transgenic insecticidal crops may be especially important for thwarting evolution of resistance in haplodiploid and parthenogenetic pests. Field-evolved resistance to Bt crops in only five pests during the last 14 years suggests that the refuge strategy has successfully delayed resistance, but the accumulation of resistant pests could accelerate.

Introduction

Transgenic crops producing toxins from the soil bacterium *Bacillus thuringiensis* (Bt) are a major tool for controlling insect pests. Fourteen years after their commercialization, Bt corn and cotton are used in more than twenty developed and developing countries on six continents (James 2008). The predominant Bt toxins in Bt crops are crystalline (Cry) proteins that kill insects by binding to specific midgut membrane target sites and disrupting membrane integrity (Schnepf et al. 1998). Benefits of Bt crops vary temporally and spatially depending on pest abundance and crop traits, and sometimes include reduction in use of synthetic insecticides and increased yield (Cattaneo et al. 2006; Hunt et al. 2007; Buntin 2008; Showalter et al. 2009; Ma et al. 2009). Negative impacts on nontarget insects are generally lower for Bt crops than for broad-spectrum insecticides (Cattaneo et al. 2006; Romeis et al. 2006; Marvier et al. 2007; Wolfenbarger et al. 2008; Naranjo 2009). Yet, effects of Bt crops on population density of specific nontarget species can be negative, neutral, or positive (Bourguet et al. 2002; Dively 2005; Naranjo 2005; Sisterson et al. 2007a; Carrière et al.

2009a). Because insects are remarkably successful in adapting to toxins and other control tactics (Palumbi 2001; Onstad 2008), the evolution of resistance to Bt toxins by target pests threatens the continued efficacy of Bt crops (Tabashnik 1994; Gould 1998; Matten et al. 2008; Tabashnik and Carrière 2009).

Evolution of resistance to a Bt crop entails a genetically based decrease in susceptibility of a population to the Bt crop (National Research Council 1986; Tabashnik et al. 2009a). While most target pest populations remain susceptible to Bt crops, field-evolved resistance has been documented in some populations of five lepidopteran pests: cereal stem borer, *Busseola fusca*, in South Africa to Bt corn producing Cry1Ab (Kruger et al. 2009), fall armyworm, *Spodoptera frugiperda*, in Puerto Rico to Bt corn producing Cry1F (Matten et al. 2008), pink bollworm, *Pectinophora gossypiella*, in western India to Bt cotton producing Cry1Ac (Bagla 2010), cotton bollworm, *Helicoverpa zea*, in the southeastern United States to Bt cotton producing Cry1Ac and Cry2Ab (Luttrell et al. 2004; Tabashnik et al. 2008a, 2009a), and bollworm, *Helicoverpa punctigera*, in Australia to Bt cotton producing Cry1Ac and Cry2Ab (Downes et al. 2010). Field-evolved resistance

was reported to be associated with increased field damage by *B. fusca*, *S. frugiperda*, *P. gossypiella*, and *H. zea* (Matten et al. 2008; Kruger et al. 2009; Tabashnik et al. 2008b, 2009a; Bagla 2010). The widespread use of Bt crops and evidence of evolution of resistance by pests provide an exceptional opportunity to test hypotheses about how responses to selection are mediated by various genetic and ecological factors.

Below we review the effects of Bt crops on pest metapopulation dynamics, the conditions required for evolution of resistance to Bt crops, and how various factors affect evolution of resistance to Bt crops. We conclude by considering the implications of what has been learned about the evolutionary ecology of insect resistance to Bt crops and future research directions for enhancing strategies to delay insect resistance.

Effects of Bt crops on pest metapopulation dynamics

The evolution of resistance to Bt crops unfolds in metapopulations. Pest populations typically increase in fields of non-Bt crops or patches of wild hosts, which are source habitats, while fields of Bt crops are population sinks where populations decline. Gene flow between source and sink habitats allows for repeated colonization of Bt fields and provides opportunities for local adaptation. However, under some conditions, gene flow into Bt fields from non-Bt host plants can delay evolution of resistance (Comins 1977; Georghiou and Taylor 1977). In particular, the refuge strategy adopted widely to delay resistance to Bt crops is based on the idea that susceptible insects produced on non-Bt host plants near Bt crops will mate with resistant pests surviving on Bt crops (Gould 1998; Matten et al. 2008; Tabashnik and Carrière 2009).

Source-sink theory predicts that widespread use of Bt crops could reduce regional pest populations and the source potential of refuges (Gould 1998; Peck et al. 1999; Caprio 2001; Carrière et al. 2003; Sisterson et al. 2005). Field data from long-term monitoring of insect pest populations support these predictions for *Heliothis virescens* (Micinski et al. 2008), *Helicoverpa armigera* (Wu et al. 2008), *Helicoverpa zea* (Adamczyk and Hubbard 2006; Micinski et al. 2008; Storer et al. 2008), *Ostrinia nubilalis* (Storer et al. 2008; Hutchison et al., unpublished data), and *Pectinophora gossypiella* (Carrière et al. 2003). Furthermore, the source potential of refuges increased as refuge width and the isolation between refuges and Bt crops increased (Caprio et al. 2004) or the regional abundance of Bt crops decreased (Carrière et al. 2004a). The source potential of refuges likely increased in these cases because fewer eggs were laid on Bt crops and thus population growth was larger in refuges.

Consistent with these empirical findings, results from metapopulation simulation models indicate that pest population dynamics and resistance evolution are affected by the distribution and abundance of Bt fields and refuges, as well as the management of refuges from year to year (Peck et al. 1999; Caprio 2001; Storer et al. 2003; Sisterson et al. 2004, 2005). Nevertheless, the regional spread of resistance is initiated by a local increase in resistance frequency where Bt fields are abundant compared to refuges (Peck et al. 1999; Storer et al. 2003; Sisterson et al. 2004, 2005). Thus, while a metapopulation approach yields important insights for understanding the evolution of resistance at a regional scale, a focus on local populations is useful to evaluate conditions influencing the evolution of resistance to Bt crops. Indeed, simple population genetic models considering, in effect, a single Bt field and refuge have been central for development of the refuge strategy and have successfully accounted for field changes in resistance frequencies in several pest species (Gould 1998; Carrière and Tabashnik 2001; Onstad and Guse 2008; Tabashnik et al. 2008a; Crowder and Carrière 2009).

Conditions for evolution of resistance to Bt crops

Three conditions are necessary and sufficient for local evolution of resistance to Bt crops by natural selection (Endler 1986): (i) variation among individuals in survival on Bt crops, (ii) inheritance of survival on Bt crops, and (iii) fitness differences consistently associated with the variation in survival on Bt crops. In what follows we summarize information about these factors and how they affect evolution of resistance to Bt crops.

Genetic variation in resistance

The first widely planted Bt crop cultivars were corn producing Bt toxin Cry1Ab and cotton producing Bt toxin Cry1Ac (Tabashnik et al. 2009a). Throughout the growing season, these cultivars caused virtually 100% larval mortality for highly susceptible pests such as *Ostrinia nubilalis* and *Pectinophora gossypiella* (Tabashnik et al. 2000; Archer et al. 2001). For such highly susceptible pests, the concentration of Bt toxin in Bt plants is much higher than the concentration needed to kill essentially all susceptible larvae (Fig. 1). In such cases, mutations that confer small decreases in susceptibility to Bt toxin provide little or no fitness advantage. Instead, under these conditions, selection for increased survival on Bt plants typically favors single mutations with effects large enough to boost survival on Bt plants (Fig. 1A). In contrast, for target pests that are less susceptible to Cry1Ab and Cry1Ac such as *Helicoverpa armigera* and *Helicoverpa zea*, mortal-

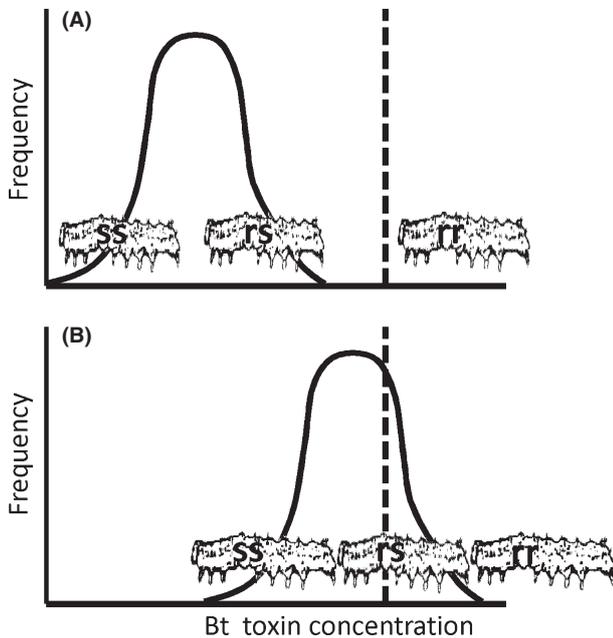


Figure 1 Distribution of insects from unexposed populations for which the given concentration of Bt toxin is the maximum tolerated. The dashed line shows the Bt toxin concentration produced by a Bt crop. The unexposed populations in (A) and (B) are composed almost entirely of susceptible (*ss*) and heterozygous (*rs*) individuals, while homozygous resistant (*rr*) individuals are extremely rare. (A) The insect population is highly susceptible. The toxin concentration in the Bt crop is higher than the concentration that kills virtually all larvae of the unexposed population. Mutations that cause major decreases in susceptibility are needed to boost survival on the Bt crop and thereby confer resistance. Survival is virtually 0% for both *ss* and *rs*, which yields recessive inheritance of resistance. (B) The unexposed population is inherently less susceptible and a substantial percentage can survive on the Bt crop. Mutations with small or moderate effects can boost survival on the Bt crop and thus confer resistance. Survival is greater for *rs* than *ss*, yielding non-recessive inheritance of resistance.

ity on Bt corn and cotton producing these toxins typically is lower than 95% and declines during the growing season (Storer et al. 2003; Showalter et al. 2009). Under these conditions, mutations conferring small or moderate decreases in susceptibility can boost survival on Bt crops, and thus polygenic resistance is more likely to evolve (Fig. 1B).

While the genetic basis of field-evolved resistance to Bt crops remains to be elucidated, genetic analysis of laboratory-selected resistance to Bt crops has usually identified single loci with major effects (Gahan et al. 2001; Morin et al. 2003; Yang et al. 2007; Pereira et al. 2008; Zhang et al. 2009). Based on these data and for simplicity, we will consider a theoretical framework in which resistance to each Bt toxin is controlled primarily by a single locus with two alleles, *r* for resistance and *s* for susceptibility.

Fitness costs occur when, in absence of Bt toxins, the fitness of individuals with *r* alleles is lower than the fitness of individuals without *r* alleles. Alleles conferring resistance to Bt often have negative pleiotropic effects that cause fitness costs, implying that *r* alleles will be maintained at a low frequency in populations not previously exposed to Bt crops (Gassmann et al. 2009). Fitness costs of resistance to Bt are usually recessive and involve a reduction in survival of about 25% in homozygous resistant (*rr*) individuals compared to homozygous susceptible (*ss*) individuals (Gassmann et al. 2009). Using this information, we can apply mutation-selection equilibrium theory to predict the *r* allele frequency in populations not exposed to Bt toxins. For a mutation with recessive effects, the frequency of a *r* allele at equilibrium is $q = (\mu/S)^{0.5}$, where μ is the mutation rate from the *s* to *r* allele and *S* is the selection coefficient (Futuyma 1979). Assuming μ ranges from 10^{-6} to 10^{-4} (Drake et al. 1998; Russell 2000), the initial *r* allele frequency is expected to range from 0.002 to 0.02, which is similar to the observed range for several lepidopteran pests targeted by Bt crops (Table 1).

Fitness costs are typically more prevalent and extensive in pests with high compared to low levels of Bt resistance (Gassmann et al. 2009). Also, in a selection experiment with *H. armigera*, a higher fitness cost was associated with increased levels of resistance within a single strain (Liang et al. 2008). In this experiment, resistance was quantified as a 'resistance ratio' calculated as the IC_{50} (concentration of Cry1Ac that inhibited larval growth by 50%) for the selected strain divided by the IC_{50} for an unselected strain. After 16, 34 and 87 generations of selection, the resistance ratios were 170, 210, and 2893, while costs affecting net replacement rate were respectively 29, 36, and 41%. The genetic basis of resistance also changed from primarily monogenic to polygenic during the course of selection. The spread of resistance alleles is expected to increase both the levels of resistance and fitness costs (Carrière et al. 1994; Carrière and Roff 1995). It is thus unclear whether the positive association between levels of Bt resistance and fitness costs, which was detected among pests and within the *H. armigera* strain, resulted from variation in resistance frequency or in costs associated with different resistance alleles. The latter factor, however, could have interesting implications for the evolution of resistance to Bt. As noted previously, for highly susceptible pests, *r* alleles with large effects are required to increase survival on Bt crops. Given that larger fitness costs could be associated with higher levels of resistance, larger fitness costs could be expected for *r* alleles with greater effects. In light of the mutation-selection balance that sets initial *r* allele frequency as described previously, *r* alleles with effects sufficiently large to confer survival on Bt crops could be rarer in highly susceptible pests than in less

Table 1. Estimated frequency of alleles conferring resistance to Bt crops in field populations of lepidopteran pests targeted by Bt corn or Bt cotton. Note that in most studies, frequency estimates were obtained after introduction of a Bt crop, which could have increased estimates of the resistance allele frequency. Insects were screened for resistance on Bt leaves or Bt crop plants unless noted otherwise.

Location	Crop	Toxin	1st year*	Years sampled†	Frequency	Confidence limits‡	Reference
Gelechiidae							
<i>Pectinophora gossypiella</i>							
AZ	Cotton	Cry1Ac	1996	1997	0.16§	0.05–0.26	Tabashnik et al. (2005a)
	Cotton	Cry1Ac	1996	1998	0.007§	0–0.02	Tabashnik et al. (2005a)
	Cotton	Cry1Ac	1996	2004	0.004§	0–0.01	Tabashnik et al. (2005a)
AZ, CA, TX	Cotton	Cry1Ac	1996	2001–05	0¶	0–0.0003	Tabashnik et al. (2006)
Noctuidae							
<i>Helicoverpa armigera</i>							
Australia	Cotton	Cry1Ac	1996	2002–06	0**	0–0.0009	Mahon et al. (2007)
Australia	Cotton	Cry2Ab	2004/05	2002–06	0.0033§	0.0017–0.0055	Mahon et al. (2007)
China	Cotton	Cry1Ac	1998	2006–07	0.094	0.044–0.145	Liu et al. (2008)
China	Cotton	Cry1Ac	1998	2003–05	0.0146	0.0084–0.0225	Xu et al. (2009)
<i>Heliothis virescens</i>							
LA, MS, NC, TX	Cotton	Cry1Ac	1996	1993	0.0015††	0.0003–0.0041	Gould et al. (1997)
LA, TX	Cotton	Cry1Ac	1996	1996–2002	0¶	0–0.0021	Gahan et al. (2007)
<i>Sesamia nonagrioides</i>							
Spain	Corn	Cry1Ab	1998	2004–05	0	0–0.0086	Andreadis et al. (2007)
Greece	Corn	Cry1Ab	–‡‡	2004–05	0	0–0.0097	Andreadis et al. (2007)
Pyralidae							
<i>Diatraea grandiosella</i>							
LA	Corn	Cry1Ab	1996	2005	0	0–0.0035	Huang et al. (2007a)
<i>Diatraea saccharalis</i>							
LA	Corn	Cry1Ab	1996	2004	0.0023	0.0003–0.0064	Huang et al. (2007b)
LA	Corn	Cry1Ab	1996	2005	0	0–0.0027	Huang et al. (2008a)
TX	Corn	Cry1Ab	1996	2007	0	0–0.0016	Huang et al. (2009)
<i>Ostrinia nubilalis</i>							
KS, TX	Corn	Cry1Ab	1996	2000–01	0	0–0.0044	Stodola et al. (2006)
France	Corn	Cry1Ab	–‡‡	1999–2000	0	0–0.00092	Bourguet et al. (2003)
MN, SD, IA	Corn	Cry1Ab	1996	1997–2000	0	0–0.00042	Bourguet et al. (2003)
KS, TX	Corn	Cry1Ab	1996	2000–01	0	0–0.0077	Bourguet et al. (2003)

*First year Bt crop was grown commercially in the location monitored.

†Year (s) insects were sampled to produce the frequency estimate.

‡The probability is 95% that the true mean is between the upper and lower limits.

§Insects were screened on diet with high Bt toxin concentration and related work showed that insects surviving this concentration also survive on Bt cotton.

¶Insects were screened for cadherin mutations associated with survival on Bt cotton (*P. gossypiella*) or high resistance to Cry1Ac in diet (*H. virescens*).

**Insects were tested on diet with Cry1Ac but not on Bt cotton.

††Insects were not tested on Bt cotton but resistance to Cry1Ac in diet was very high.

‡‡Bt corn was not commercialized in region sampled.

susceptible pests. Moreover, initial *r* allele frequency could be diminished by producing crops with higher toxin concentrations.

While the first Bt corn and cotton cultivars each produced one Bt toxin, some newer cultivars produce a 'pyramid' of two or more Bt toxins for the control of individual pest species (Tabashnik et al. 2009a). Furthermore, transgenic cultivars with two or more Bt toxins targeting individual pests are likely to become increasingly prevalent (Bravo and Soberón 2008; Matten et al. 2008). When resistance to each toxin is recessive and the genes

conferring resistance to the various toxins segregate independently, the use of multiple toxins can significantly reduce the number of resistant phenotypes in a pest population. For example, with a *r* allele frequency of 0.003 at each locus conferring resistance to one toxin (i.e., the average upper limit of the *r* allele frequency in *O. nubilalis*, Table 1), the expected frequency of individuals with homozygous resistance at all relevant loci would be 0.003^2 , 0.003^4 , and 0.003^6 , for crops producing 1, 2, and 3 toxins, respectively. Thus, with 4×10^6 insects per Bt field (Sisterson et al. 2005), the expected number of

individuals per field with homozygous resistance at the relevant loci would be 2360, 3.2×10^{-3} , and 1.4×10^{-8} , respectively. This suggests that genetic variation for resistance could be rare when two or more independently acting Bt toxins target the same pest and pest populations are small. In addition, fitness costs could be higher in pests that evolve resistance to crops producing many toxins, which could also contribute to the rarity of resistance alleles. The hypothesis of increased costs in insects resistant to crops with multiple toxins has not been evaluated, although it is supported by a study of the bacterium *Pseudomonas aeruginosa* where costs were greater in populations resistant to two antibiotics rather than one (Perron et al. 2007).

Although *r* allele frequency is low in populations of most pests (Table 1), genetic variation is often available

to confer resistance to Bt crops. Some populations of five lepidopteran species have evolved resistance to a Bt crop in the field (see above). In addition, strains of four pest species, including three lepidopterans and one coleopteran (corn rootworm, *Diabrotica virgifera virgifera*), have been selected in the laboratory or greenhouse for higher survival on commercial Bt cultivars (Table 2). Also, the diamondback moth, *Plutella xylostella*, has been selected for resistance to a noncommercial cultivar of Bt broccoli that produces Cry1Ac and Cry1C (Zhao et al. 2005). In sum, documented cases of resistance to commercial and noncommercial cultivars of Bt crops selected in the field, laboratory, and greenhouse include at least nine species of insect pests, eight Bt toxins, and three Bt crops.

For some pests, however, it may sometimes prove difficult to select for levels of resistance high enough to confer

Table 2. Survival on commercial Bt crops relative to survival on non-Bt crop counterparts for strains of four pest species selected in the laboratory or greenhouse for resistance to Bt toxins. Only studies reporting survival of lab- or greenhouse-selected strains from neonate to either pupa or adult are shown. Survival was measured in the greenhouse or using plants from the greenhouse unless noted otherwise. *Diatraea saccharalis*, *Helicoverpa armigera*, and *Pectinophora gossypiella* are lepidopterans; *Diabrotica virgifera virgifera* is a coleopteran.

Toxin(s), conditions	Insect species	Insect strain	Adjusted survival*	Reference
Corn				
Cry1Ab	<i>D. saccharalis</i>	B1F2	0.10	Huang et al. (2007b)
Cry34Ab/Cry35Ab	<i>D. v. virgifera</i>	Rochelle-S	0.18†	Lefko et al. (2008)
Cry34Ab/Cry35Ab	<i>D. v. virgifera</i>	York-S	0.19†	Lefko et al. (2008)
Cry3Bb, field tested	<i>D. v. virgifera</i>	Constant-exposure	0.44‡	Meihls et al. (2008)
Cry3Bb, field tested	<i>D. v. virgifera</i>	Late-exposure	0.14‡	Meihls et al. (2008)
Cry3Bb, field tested	<i>D. v. virgifera</i>	Neonate-exposure	0.015‡	Meihls et al. (2008)
Cotton				
Cry1Ac	<i>H. armigera</i>	Cry1Ac-sel	0.25	Fan et al. (2000)
Cry1Ac, 5 to 6-leaf stage	<i>H. armigera</i>	ISOC4	0.37–0.39	Bird and Akhurst (2004)
Cry1Ac, 15-leaf stage	<i>H. armigera</i>	ISOC4	0.91	Bird and Akhurst (2005)
Cry1Ac + Cry2Ab, pre-square	<i>H. armigera</i>	SP15	0.029	Mahon and Olsen (2009)
Cry1Ac + Cry2Ab, early square	<i>H. armigera</i>	SP15	0.044	Mahon and Olsen (2009)
Cry1Ac + Cry2Ab, fruiting	<i>H. armigera</i>	SP15	0.52	Mahon and Olsen (2009)
Cry1Ac + Cry2Ab, pre-square, field plants	<i>H. armigera</i>	SP15	0	Mahon and Olsen (2009)
Cry1Ac + Cry2Ab, fruiting, field plants	<i>H. armigera</i>	SP15	0.17	Mahon and Olsen (2009)
Cry1Ac	<i>P. gossypiella</i>	AZP-R	0.46	Liu et al. (2001)
Cry1Ac	<i>P. gossypiella</i>	AZP-R	0.43	Morin et al. (2003)
Cry1Ac	<i>P. gossypiella</i>	MOV97-R	1.10	Tabashnik et al. (2005b)
Cry1Ac	<i>P. gossypiella</i>	SAF97-R	1.50	Tabashnik et al. (2005b)
Cry1Ac	<i>P. gossypiella</i>	MOV97-H2, <i>r1r3</i> §	0.30	Carrière et al. (2006)
Cry1Ac	<i>P. gossypiella</i>	MOV97-H2, <i>r3r3</i> §	0.38	Carrière et al. (2006)
Cry1Ac	<i>P. gossypiella</i>	SAF97-H2, <i>r1r2</i> §	0.57	Carrière et al. (2006)
Cry1Ac	<i>P. gossypiella</i>	SAF97-H2, <i>r2r2</i> §	0.20	Carrière et al. (2006)

*Adjusted survival = survival of selected strain on Bt crop/survival of selected strain on non-Bt crop unless noted otherwise. Adjusted survival <1 indicates incomplete resistance (see text).

†Survival of the selected Rochelle-S strain on Bt corn (0.137)/survival of the unselected parent strain (Rochelle-US) on non-Bt corn (0.762). Survival of the selected York-S strain on Bt corn (0.156)/survival of the unselected parent strain (York-US) on non-Bt corn (0.827). If survival on non-Bt corn was lower for the selected strain (data not reported) than for the unselected strain, these calculations would underestimate adjusted survival on Bt corn for the selected strains.

‡Adjusted survival of an unselected strain on Bt corn was 0.038. Survival was significantly higher than 0.038 in the constant-exposure and late-exposure strains, but not in the neonate-exposure strain.

§Three cadherin alleles linked with resistance to Cry1Ac in *P. gossypiella* are denoted *r1*, *r2*, and *r3*. Adjusted survival is given for genotypes based on combinations of these alleles.

survival on Bt crops. For example, many unsuccessful attempts were made to select *O. nubilalis* for resistance to Cry1Ab corn in the laboratory, and only recent work suggests that this pest could achieve levels of resistance high enough for insects to survive to the adult stage on Bt corn (Pereira et al. 2008; Crespo et al. 2009). However, although survival of resistant larvae was high on Bt corn in these recent experiments, plants were dissected 15 days after infestation and survival to adulthood was not quantified. A lack of appropriate genetic variation or low frequency of recessive resistance alleles can constrain the evolution of resistance to insecticides (Peck et al. 1999; Brown and Hoffmann 2005; Labbé et al. 2007). There is a trend for the occurrence of resistance alleles to be lower in the Pyralidae than in Gelechiidae and Noctuidae, and so far no major alleles conferring resistance to Cry1Ab have been found in *O. nubilalis* despite many screening attempts (Table 1). Thus, results from selection experiments and available data on frequency of resistance alleles indicate that the evolution of resistance to Cry1Ab corn could be constrained in *O. nubilalis*.

Control measures aimed at keeping pest populations low could be envisaged to manage the evolution of resistance (e.g., Carrière et al. 2001) in pests where the initial frequency of *r* alleles is low (e.g., *O. nubilalis*) and resistance is recessive. Interestingly, Bt crops can lower regional pest population density, as summarized above. Regional declines in pest population density are influenced by pest movement, reproductive rate in refuges, and abundance, distribution, and toxicity of Bt crops (Carrière et al. 2003; Sisterson et al. 2007b). Spatially explicit data on the abundance and distribution of Bt crops coupled with information on pest life-history and response to Bt crops could be useful to design measures to reduce pest population densities regionally (Carrière et al. 2004a; Marvier et al. 2008).

Heritability of resistance

An important factor underlying the refuge strategy is that the dominance of resistance is reduced by increasing the dose of Bt toxins. When the concentration of a Bt toxin is low, some heterozygous (*rs*) individuals typically survive exposure to a Bt toxin, but when the concentration is high, only *rr* can survive (Tabashnik et al. 2004; Crespo et al. 2009). Accordingly, Bt toxin genes inserted in transgenic plants were modified to produce high concentrations of Bt toxins, and genetically transformed plants with high levels of Bt toxins were selected to produce commercial transgenic cultivars (Showalter et al. 2009).

Even when *r* alleles are present in insect populations in Bt fields, movement of insects from refuges to Bt fields can significantly reduce the heritability of resistance.

Resistance to crops that produce high concentrations of Bt toxins is recessive in highly susceptible pests, although this is not necessarily the case in pests less susceptible to Bt (Tabashnik et al. 2008a, 2009a). For refuges to be effective, the abundant susceptible insects produced on non-Bt host plants must mate with the rare resistant pests surviving on Bt crops. In such cases, when resistance is recessive, most hybrid offspring produced by resistant pests are killed by Bt crops. This reduces the heritability of resistance and delays the evolution of resistance (Gould 1998; Tabashnik and Carrière 2009). With time, however, movement of *rr* individuals from Bt fields to refuges can increase the frequency of *r* alleles in refuges (Sisterson et al. 2004), especially when fitness costs are absent (Carrière and Tabashnik 2001; Gould et al. 2006). Ultimately, some individuals with these *r* alleles will move back from refuges to Bt fields, which increases the heritability of resistance and accelerates the evolution of resistance (Comins 1977; Caprio and Tabashnik 1992; Sisterson et al. 2004).

The 'pyramid' strategy for delaying pest resistance is based on use of crops producing two or more distinct Bt toxins targeting individual pests. The pyramid strategy is expected to be most effective when resistance to each Bt toxin is recessive, fitness costs and refuges are present, and selection with any one of the Bt toxins does not cause cross-resistance to the others (Roush 1998; Zhao et al. 2005; Gould et al. 2006). Cross-resistance to Bt occurs when a genetically-based decrease in susceptibility to one toxin decreases susceptibility to other toxins (Tabashnik et al. 2009a,b). When resistance is recessive to both toxins in a pyramid, pests that bear two *r* alleles for survival to one toxin will nonetheless be killed unless they also bear two *r* alleles for survival to the second toxin. Thus when *r* alleles are rare, the only genotype with high survival on a crop that produces two or more Bt toxins is expected to be extremely rare. Accordingly, the refuge strategy is more effective for reducing the heritability of resistance when crops produce two or more Bt toxins than when crops produce a single Bt toxin (Gould 1998; Roush 1998).

Concentrations of Bt toxins in Bt corn and cotton typically decline as the growing season progresses, but seasonal changes in toxin concentration can vary among toxins and cultivars (Dutton et al. 2004; Nguyen and Ja 2009; Showalter et al. 2009). For example, in Bt cotton, Cry1Ac concentration usually decreases when plants start producing flowers and bolls (Showalter et al. 2009), while Cry2Ab concentration tends to spike in mid-season before declining (Adamczyk et al. 2001). Levels of a Bt vegetative insecticidal protein, Vip3A, are relatively stable throughout the season, although cotton plants producing Vip3A still lose some of their activity against *H. armigera* during

mid-season (Llewellyn et al. 2007). The reasons for the seasonal reduction in Bt concentration remain unclear, but could be related to mRNA instability, declining promoter activity, reduced nitrogen metabolism, lower overall protein production, and toxin interactions (Showalter et al. 2009).

The seasonal decline in toxin concentrations may increase the dominance of resistance and accelerate the evolution of resistance, especially in pests less susceptible to Bt toxins. For example, resistance to Cry1Ac cotton was recessive in *H. armigera* fed Bt cotton in the 5–6 leaf stage (Bird and Akhurst 2004), but became partially dominant on cotton in the 15 leaf stage, which had concentrations of Cry1Ac 75% lower than in 4-week-old plants (Bird and Akhurst 2005). However, a seasonal decline in toxin concentrations does not always increase the dominance of resistance. When *Diatraea saccharalis* larvae fed on each of seven commercial Cry1Ab corn cultivars in 2005 and 2006 (Wu et al. 2007), average survival for each insect genotype was lower on vegetative corn than on older, reproductive corn (2005: 0.5% vs. 1.5% for *ss*, 1.4% vs. 3.4% for *rs* and 9.6% vs. 24.5% for *rr*; 2006: 0.0% vs. 2.7% for *ss*, 3.5% vs. 6.8% for *rs*, and 14.3% vs. 18.1% for *rr*). Nevertheless, in 2005, the average dominance of resistance ($h = [\text{survival of } rs - \text{survival of } ss] / [\text{survival of } rr - \text{survival of } ss]$) was slightly higher on vegetative corn (0.099) than on reproductive corn (0.083). In 2006, the average dominance of resistance was higher than in 2005, and it was slightly higher on reproductive (0.27) than on vegetative (0.24) corn. Thus, the higher survival on reproductive corn relative to vegetative corn, which presumably reflects lower Cry1Ab concentration in reproductive corn (Wu et al. 2007), did not produce consistent or large increases in the dominance of resistance.

Seasonal declines in Bt toxin concentrations could also reduce success of the pyramid strategy. Mahon and Olsen (2009) measured seasonal changes in survival of a *H. armigera* strain highly resistant to Cry2Ab on cotton producing Cry1Ac and Cry2Ab. Survival of *rr* individuals was respectively 0, 2.5, and 8.5% on field-grown cotton in the pre-square, early square and fruiting stages, while survival of *ss* was 0, 0, and 1.6%. Survival of *rs* on pre-square and fruiting cotton was respectively 0 and 1.7% and did not differ significantly from *ss* (survival of *rs* on early square cotton was not measured), showing that resistance remained recessive on the different cotton stages. Mahon and Olsen (2009) did not measure the change in concentrations of Cry1Ac and Cry2Ab in cotton plants but proposed that increased survival of the Cry2Ab-resistant insects was likely due to a decline in the concentration of Cry1Ac. As the oldest cotton was tested soon after fruiting, it is also possible that survival of *rr* individuals and the

dominance of resistance could increase further on older cotton, or on cultivars where the concentration of the Bt toxins decline faster than in the experimental cultivar used. Accordingly, the seasonal decline in the concentration of one toxin in a pyramid (here Cry1Ac) could invalidate the fundamental assumption of the pyramid strategy (i.e., the killing of insects resistant to one toxin by another toxin), and thus accelerate evolution of resistance. It is noteworthy that *H. zea*, a pest in which seasonal changes in survival on Bt crops have been reported (Storer et al. 2003), has rapidly evolved resistance in the field to Cry1Ac and Cry2Ab produced by pyramided Bt cotton (Tabashnik et al. 2009a). There is a need to better evaluate and consider the consequences of seasonal declines in the concentrations of Bt toxins on the evolution of resistance to Bt crops (Brévault et al., unpublished data).

Selective advantage of resistance

Some field data suggest that refuges of non-Bt crops near Bt crops can delay the evolution of resistance (Tabashnik et al. 2008a, 2009a). More generally, in theory, the evolution of resistance can be delayed by any factors that reduce the selection coefficient between individuals with and without *r* alleles (Gould 1998; Carrière and Tabashnik 2001; Andow and Ives 2002; Crowder and Carrière 2009). Reducing the selection coefficient can be achieved through agronomic practices. For example, the selective advantage of resistance can be reduced by increasing refuge size, which increases the fitness of susceptible individuals relative to resistant individuals. Furthermore, the selective advantage of resistance can be reduced by implementing pest control practices such as pheromone mating disruption and elimination of crop residues containing insects in Bt fields, where resistant individuals are more abundant than susceptible individuals (Carrière et al. 2001; Andow and Ives 2002).

The selection coefficient between individuals with and without *r* alleles is also affected by environmental conditions and pest genetics, which interact to generate incomplete resistance and fitness costs. Incomplete resistance occurs when fitness of *rr* individuals is lower on Bt cultivars than on corresponding non-Bt cultivars (Carrière and Tabashnik 2001; Carrière et al. 2006; Crowder and Carrière 2009). Incomplete resistance reduces the selection coefficient favoring resistant individuals over susceptible individuals on Bt crops. On the other hand, fitness costs increase the selection coefficient favoring susceptible individuals in refuges. These factors influence the overall fitness of the *r* and *s* alleles across Bt fields and refuges (Carrière and Tabashnik 2001; Tabashnik et al. 2005a), which ultimately drives increases or decreases in the frequency of resistance (Fig. 2).

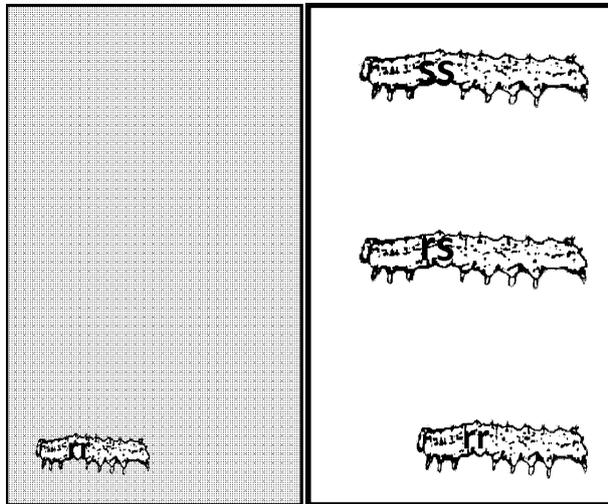


Figure 2 Fitness of resistance genotypes on a Bt crop (grey) and on a non-Bt host plant in a refuge (white). Fitness of each genotype in each habitat is proportional to the size of larvae in diagram. On the Bt crop, fitness of rr is 0.2 but rs and ss do not survive (resistance is recessive). In the refuge, fitness of ss and rs is 1 and fitness of rr is 0.8 (a recessive cost is present). On the Bt crop, the selection coefficient favoring rr over ss and rs is 0.2. In the refuge, the selection coefficient favoring ss and rs over rr is 0.2. Assuming that half of the larval population is exposed to each habitat, fitness of the r and s allele is equal and frequency of resistance is stable (see Tabashnik et al. 2005a, equation 6 for fitness calculation). However, if fitness of rr is 0.21 on the Bt crop, fitness of other genotypes is unchanged (thus smaller incomplete resistance), and half of the larval population is exposed to each habitat, fitness of r is higher than fitness of s and resistance frequency increases. On the other hand, if fitness of rr is 0.79 in the refuge, fitness of the other genotypes is unchanged (thus a higher cost), and half of the larval population is exposed to each habitat, fitness of r is smaller than fitness of s and resistance frequency declines. Thus, variation in costs and incomplete resistance has important effects on resistance evolution.

When r alleles are rare, which is typical before extensive exposure to Bt crops (Table 1), they are found primarily in rs individuals. Thus, in the initial stages of selection for resistance, nonrecessive costs expressed in rs individuals can delay the evolution of resistance more effectively than recessive costs that are only expressed in extremely rare rr individuals (Carrière and Tabashnik 2001). With nonrecessive costs, the fitness in refuges is higher for ss than rs individuals, which selects strongly against resistance (Carrière and Tabashnik 2001; Gould et al. 2006). Nonrecessive costs associated with Bt resistance were detected in 26% of fitness components assessed in 16 studies (Gassmann et al. 2009), suggesting that nonrecessive costs are more widespread than previously envisaged (Gould et al. 2006). Incomplete resistance, quantified as the fitness of rr on Bt crops divided by rr fitness on non-Bt crop counterparts, is common

and averaged 0.63 (range 0.11–1.5) in seven pests (Crowder and Carrière 2009; see also Table 2).

Given the powerful effects of fitness costs and incomplete resistance on resistance evolution, manipulation of refuges and Bt cultivars could enhance resistance management (Gassmann et al. 2009; Crowder and Carrière 2009). Fitness costs are affected by environmental conditions, including natural enemies, intraspecific competition, and host plant (Gassmann et al. 2009). Thus, using refuges of host plants that magnify costs or make them less recessive, or treating refuges with natural enemies that have similar effects, could delay resistance (Gassmann et al. 2009).

Factors affecting incomplete resistance have not been critically evaluated, but a reasonable hypothesis is that individuals that survive on Bt crops are not completely impervious to high concentrations of Bt toxins. This hypothesis is supported by studies of *H. armigera*, where survival of resistant individuals on Bt cotton relative to non-Bt cotton was lower on young cotton plants with high concentrations of Cry1Ac than on older plants with lower concentrations of Cry1Ac (Table 2; Bird and Akhurst 2004, 2005). This suggests that the production of high concentrations of Bt toxins in Bt crops could not only contribute in increasing costs, but also could reduce fitness of resistant individuals on Bt crops relative to non-Bt crops. Cultivars with two or more toxins could also enhance this effect because insects resistant to one toxin could be killed by the other toxin (Crowder and Carrière 2009). This hypothesis is supported by a recent study of a *H. armigera* strain highly resistant to Cry2Ab (Table 2; Mahon and Olsen 2009). In this strain, survival of resistant individuals on Bt cotton producing Cry1Ac and Cry2Ab relative to non-Bt cotton was lower on younger than older cotton plants (Table 2), presumably because the concentration of Cry1Ac diminished in older plants and thus Cry1Ac did not kill insects resistant to Cry2Ab. Production of toxins bypassing known resistance mechanisms (Soberón et al. 2007) or inducing negative cross-resistance could also be envisaged to reduce fitness of resistant individuals on Bt crops (Crowder and Carrière 2009).

Manipulation of costs and incomplete resistance will be achieved more easily if the genetic basis of resistance is similar across populations of a target pest. For example, if multiple resistance genes or alleles with different pleiotropic effects occur in a pest metapopulation, different strategies for modifying costs might be needed to efficiently delay resistance in different regions. So far the molecular basis of resistance in the five pests that evolved resistance to Bt crops in the field is unknown. Of the species selected for higher survival on Bt crops in the laboratory (Table 2), the molecular basis of resistance is

known in *H. armigera* and *P. gossypiella*. In both pests, resistance to Cry1Ac is linked with mutations in a gene encoding a cadherin protein that binds Cry1Ac (Morin et al. 2003; Yang et al. 2007). Three cadherin mutations were present in a *H. armigera* strain originating from the Hebei Province of China (Yang et al. 2007). A mutation in an aminopeptidase N gene also conferred high resistance to Cry1Ac in a *H. armigera* strain from the Henan Province of China (Zhang et al. 2009). In *P. gossypiella*, two mutant cadherin alleles denoted *r1* and *r3* occurred in western Arizona, whereas the mutant alleles *r1* and *r2* were found in eastern Arizona (Morin et al. 2003).

The effects of the cadherin and aminopeptidase N mutations on costs and incomplete resistance are unknown in *H. armigera*. However, these mutations could have different effects on costs and incomplete resistance, given that cadherin and aminopeptidase N proteins have different functions. In *P. gossypiella*, costs and incomplete resistance were largely similar in strains harboring different cadherin mutations or across genotypes when insects were reared on a single cotton cultivar (e.g., DP50 with or without Cry1Ac) (Carrière et al. 2005a, 2006). However, expression of costs and incomplete resistance sometimes changed markedly across different cultivars (Carrière et al. 2006). Because cadherin proteins may help to maintain integrity of the midgut membrane (Midboe et al. 2003; Carrière et al. 2009b), cadherin mutations may cause fitness costs by increasing permeability of the gut membrane to toxic phytochemicals (Carrière et al. 2004b). Each of the three cadherin mutations in *P. gossypiella* increased gut permeability to the cotton phytochemical gossypol, and costs were increased most by mutations inducing higher gut permeability (Williams 2009). Consistent expression of costs on particular cultivars, the pervasive presence of toxic phytochemicals in cotton, and the possibility that increased penetration of phytochemicals may magnify costs in individuals with cadherin mutations, indicate that refuges could be manipulated over a large scale by planting specific cultivars to enhance resistance management in *P. gossypiella*. More information on the consequences and distribution of molecular variation in resistance to Bt crops will be useful to improve our ability to manipulate costs and incomplete resistance.

So far the refuge strategy has been used to manage the evolution of resistance in diploid, sexually reproducing coleopterans and lepidopterans. However, recent developments in molecular biology and biotechnology suggest that future transgenic insecticidal crops could increasingly target haplodiploid and parthenogenic pests (Crowder and Carrière 2009). Simulation models have shown that as in diploid pests, refuges of nontransgenic host plants and recessive inheritance of resistance can delay resistance in haplodiploid and parthenogenic pests. However, con-

trary to results with diploid pests, fitness costs and incomplete resistance are required to significantly delay the evolution of resistance in haplodiploid and parthenogenic pests (Crowder and Carrière 2009).

In haplodiploid species, the evolution of resistance is often driven by selection for resistant males (Carrière 2003; Crowder et al. 2006; Crowder and Carrière 2009). The initial frequency of resistant haploid males is equal to the *r* allele frequency, while the frequency of diploid homozygous resistant females is lower (r^2). The majority of *r* alleles in females of a haplodiploid species are present in *rs* individuals, although all *r* alleles in males are found in *r* individuals. Thus, recessive costs in females have a lower influence on resistance evolution than nonrecessive costs, while costs expressed in males are not affected by dominance and are always important (Crowder and Carrière 2009). Accordingly, decreasing the fitness of *r* males generally has the largest effect on delaying the evolution of resistance, unless costs in females are large and nonrecessive. Similarly, incomplete resistance that lowers the fitness of *r* males on transgenic crops delays resistance more than incomplete resistance affecting *rr* females. In parthenogenic species, resistance can be delayed with refuges, fitness costs, and incomplete resistance, but incomplete resistance is more important than fitness costs (Crowder and Carrière 2009). Thus, in haplodiploid and parthenogenic pests, while refuges cannot appreciably delay resistance by reducing heritability, they can delay resistance considerably when resistance is incomplete and fitness costs are present.

Conclusion

Since their commercial introduction 14 years ago, Bt crops have achieved great success. The deployment of Bt crops has decreased the regional density of several pests of corn and cotton. Contrary to expectations of widespread field control failures based on worst-case scenarios, field-evolved resistance has been documented only in some populations of five pest species. While empirical evidence from the field is still limited, patterns within and across species are generally consistent with predictions from evolutionary theory (Tabashnik et al. 2008a, 2009a). For example, in *H. zea*, resistance to Cry1Ac in Bt cotton evolved faster in areas with lower refuge abundance, both within and among states in the southeastern USA (Tabashnik et al. 2008a, 2009a). Furthermore, comparisons across pest species suggest that recessive inheritance of resistance and abundant refuges of non-Bt host plants are two key factors that delay the evolution of resistance (Tabashnik et al. 2008a, 2009a).

Refuges have been used widely for delaying the evolution of resistance, but compliance with the refuge strategy

has varied in time and space. Compliance with refuge requirements was consistently high for Bt cotton in Arizona (Carrière et al. 2005b), but has declined for Bt corn in the USA (Jaffe 2009). In South Africa, the documented lack of compliance with the refuge strategy is suspected as a major factor contributing to rapid evolution of resistance to Bt corn in *B. fusca* (Kruger et al. 2009).

Even though few cases of resistance to Bt crops have been reported so far, a delay usually occurs between the introduction of a novel pesticide and the rise in the number of species that evolve resistance to the pesticide (Georghiou 1986). For example, for DDT used in cotton in the USA, the first case of resistance was documented more than 10 years after DDT was commercialized, followed by a rapid increase in the number of resistant species in the subsequent decade (Fig. 3). Bt cotton and DDT are similar in many ways with respect to resistance evolution (recessive inheritance of resistance, widespread adoption following commercialization, toxicity maintained for extended periods), but are different in other ways, including the much broader spectrum of insects killed by DDT and the lack of a refuge strategy for managing resistance to DDT. Nevertheless, the possibility remains that, as seen with DDT, the accumulation of pests resistant to Bt crops could accelerate. With increasing numbers of Bt cultivars commercialized, biotech companies are now in a better position to rapidly produce novel Bt cultivars that will delay the evolution of resistance, and biotech companies are already aggressively pursuing these technologies. Nevertheless, cautious resis-

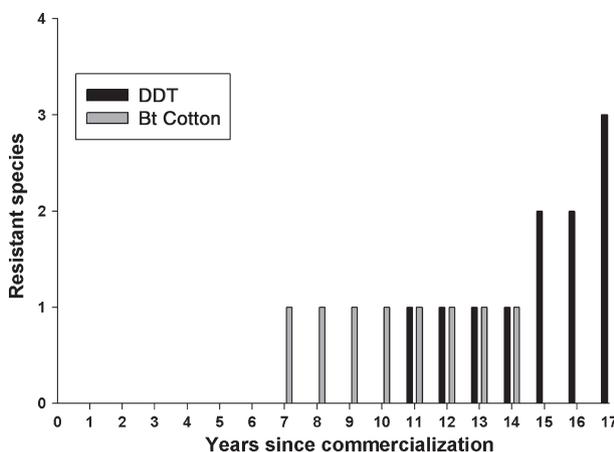


Figure 3 Cumulative number of species of cotton pests with documented field-evolved resistance to Bt cotton or DDT in years following their introduction in the US. Data on evolution of resistance to DDT are from APRD (2009). Some species categorized as evolving resistance to DDT between 1946 and 1963 in APRD (2009) were not included in the Figure (*Lygus hesperus* and *Spodoptera exigua*) because primary literature stated that populations tested for DDT resistance had not been collected in cotton.

tance management approaches involving refuges continue to be important (Jaffe 2009; Tabashnik et al. 2009a).

We see several research directions that could provide a stronger scientific basis for managing resistance to Bt crops. Although theory, small-scale experiments, and retrospective analysis of resistance monitoring data suggest that the refuge strategy has been useful (Gould 1998; Tabashnik et al. 2008a, 2009a), a more detailed analysis of the spatial distribution of Bt crops and refuges in conjunction with patterns of field-evolved resistance could help to more rigorously test the effects of refuges. The demonstrated ability of Bt crops to cause regional pest suppression suggests that better understanding of source-sink dynamics could also be useful for enhancing resistance management strategies.

New cultivars of Bt corn have been released to control both coleopteran pests such as corn rootworms (*Diabrotica spp.*) and lepidopteran pests (Tabashnik et al. 2009a). While refuges of non-Bt corn used to delay resistance in lepidopteran pests were often planted as far as 1 km from Bt corn fields, refuges for the new cultivars effective against rootworms and lepidopteran pests are planted in or adjacent to Bt corn fields in regions where the rootworms are important pests (US EPA 2008). This might reduce isolation of refuges from Bt crop fields for the most dispersive lepidopteran pests, which could enhance regional declines in their populations (Carrière et al. 2003; Hutchison et al., unpublished data). Spatially-explicit studies linking the distribution of Bt corn fields and refuges to variation in population density of lepidopteran pests in corn-dominated agroecosystems could yield better understanding of the source-sink dynamics of Bt crops.

Whereas most research on fitness costs associated with resistance to Bt toxins has been done in the laboratory and greenhouse, field research is needed to enhance our ability to manipulate costs (Gassmann et al. 2009). Furthermore, RNA interference and other novel techniques will offer increasing flexibility to reduce pest fitness on transgenic insecticidal crops (Crowder and Carrière 2009). Research is also needed to better understand the physiological basis of incomplete resistance to design transgenic cultivars that will increasingly delay resistance. Innovative research and incorporation of knowledge gained from observed patterns of field-evolved resistance into future resistance management strategies will help maximize the benefits of current and future transgenic crops.

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