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Insect resistance to *Bacillus thuringiensis*: uniform or diverse?

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Resistance to the insecticidal proteins produced by the soil bacterium *Bacillus thuringiensis* (Bt) has been documented in more than a dozen species of insect. Nearly all of these cases have been produced primarily by selection in the laboratory, but one pest, the diamondback moth (*Plutella xylostella*), has evolved resistance in open-field populations. Insect resistance to Bt has immediate and widespread significance because of increasing reliance on Bt toxins in genetically engineered crops and conventional sprays. Furthermore, intense interest in Bt provides an opportunity to examine the extent to which evolutionary pathways to resistance vary among and within species of insect. One mode of resistance to Bt is characterized by more than 500-fold resistance to at least one CrylA toxin, recessive inheritance, little or no cross-resistance to CrylC, and reduced binding of at least one CrylA toxin. Analysis of resistance to Bt in the diamondback moth and two other species of moths suggests that although this particular mode of resistance may be the most common, it is not the only means by which insects can attain resistance to Bt.

Keywords: allelism; *Bacillus thuringiensis*; diamondback moth; evolution; genetic variation; resistance

1. INTRODUCTION

Widespread insecticide resistance and rising concerns about environmental hazards have spurred the search for alternatives to conventional insecticides. Thus, insecticides derived from the common bacterium *Bacillus thuringiensis* (Bt) are becoming increasingly important for pest management (Entwistle *et al*. 1993).

Bt is a natural pathogen of some pests. Insecticidal proteins produced by Bt are extremely toxic to certain pests, but cause little or no harm to people, wildlife, and most beneficial insects. Therefore, compared with many conventional insecticides, Bt-based insecticides pose less risk to the environment and are more compatible with biological control.

Bt has been used in sprays for more than 30 years, but recent breakthroughs in biotechnology have greatly enhanced the role of Bt in agriculture. Through genetic engineering, scientists have transferred genes encoding Bt toxins into crop plants. In effect, these transgenic plants produce their own environmentally benign insecticide.

Three transgenic crops that produce Bt toxins were grown commercially in the USA during 1997: nearly 3 Mha of Bt maize, 1 Mha of Bt cotton, and 10 kha of Bt potato (Wadman 1997; Mellon & Rissler 1998). These large plantings represent a huge increase in use of Bt, but only a tiny portion of the potential world market for Bt crops. At least 15 other Bt crops—including apple, broccoli, poplar, rice, tomato, and walnut—have been approved for field testing by the US Department of Agriculture (Mellon & Rissler 1998). Despite some problems, Bt crops have generally performed well, and in most cases, have greatly reduced the use of conventional insecticides.

Foliar sprays of Bt containing toxins, spores, and other materials were used for decades without any reports of pest resistance from the field. This led some to wonder whether insects could evolve resistance to Bt. With laboratory-selected resistance to Bt demonstrated in many pests and field-evolved resistance to Bt documented in the diamondback moth (*Plutella xylostella*), adaptation by pests is now considered the biggest threat to the long-term success of Bt.

Several recent reviews cover the general topics of evolution and management of resistance to Bt (Ferré *et al*. 1995; Gould 1998; McGaughey & Whalon 1992; Tabashnik 1994). Here we ask, 'how much does the genetic basis of resistance to Bt vary among populations of moths?' The following sections provide a brief overview of the biology of Bt and resistance to Bt, a summary of variation in the genetic basis of resistance to Bt among three widely separated populations of diamondback moth, and comparisons with other Bt-resistant strains of diamondback moth and other moths.

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Finally, we consider the implications of the aforementioned results for prolonging the efficacy of Bt through resistance management.

2. OVERVIEW OF BT AND RESISTANCE TO BT

During sporulation, Bt produces crystalline inclusions composed of proteins called δ-endotoxins. Because they occur in crystals, these proteins are referred to as Cry toxins (Crickmore *et al*. 1998). To kill insects, Bt crystals must be ingested. In the alkaline insect midgut, crystals dissolve into protoxins, which are cleaved by proteases into active toxins (Gill *et al*. 1992). Toxins bind to and form pores in the brush border of midgut membranes; this makes cells swell and lyse, eventually causing death (Gill *et al*. 1992).

X-ray crystallography has revealed the three-dimensional structure of toxins Cry3A (Li *et al*. 1991), which kills beetles, and Cry1Aa (Grochulski *et al*. 1995), which kills moth larvae. Each toxin has three domains. Current thinking is that the α-helices of domain I are critical in pore formation and the loops at the ends of the β-sheets of domain II are essential for binding (Grochulski *et al*. 1995).

The only well-characterized mechanism of resistance to Bt is reduced binding of toxin to midgut membranes (Ferré *et al*. 1991, 1995; Van Rie *et al*. 1990). Nonetheless, this is not the only mechanism, as several cases of resistance to Bt are not associated with reduced binding of toxin (Ferré *et al*. 1995; Oppert *et al*. 1997; Tabashnik 1994).

Although Bt is sometimes mistakenly considered a singular entity, thousands of strains of Bt are housed in various collections maintained by industry, governments, and academia. Each strain has a characteristic set of toxins. Generally, toxins with related amino-acid sequences kill related insects. For example, Cry1 toxins kill larvae of some species of moths, whereas Cry3 toxins are lethal to certain beetles.

The number of reported DNA sequences for Bt toxin genes grew from four in 1985 to 135 in 1997 (Crickmore *et al*. 1998). This rapid growth has prompted the establishment of a new system of nomenclature and a web site to track the latest genes (Crickmore *et al*. 1998). The expansion includes gene variants that differ by less than 5% of their amino-acid sequence (e.g. six different forms of Cry1Aa), as well as discovery of major new types of Bt toxin genes.

If there are so many Bt toxin genes, with more being discovered every year, why not just switch to a new toxin when resistance occurs? Two factors limit the potential for the toxin-switching strategy. First, a relatively small subset of toxins kill any particular pest. For example, Cry1C but not Cry1A toxins are highly effective against the beet armyworm, *Spodoptera exigua* (Moar *et al*. 1995). Second, selection with one toxin or set of toxins can produce cross-resistance to others (Gould *et al*. 1992; McGaughey & Johnson 1992; Moar *et al*. 1995; Tabashnik *et al*. 1996). So, despite the abundance and diversity of Bt toxins, resistance remains a serious concern.

Laboratory selection experiments show that many pests, including targets of Bt crops, can readily evolve resistance to Bt (Tabashnik 1994). However, the diamondback moth offers the only opportunities now to study patterns of evolution of resistance to Bt in open-field populations. Damage inflicted and control costs for this cosmopolitan pest of cabbage and related vegetables exceed one billion US dollars annually (Talekar & Shelton 1993). Larvae of the diamondback moth eat the foliage of cruciferous vegetables and, if susceptible, are killed by Bt toxins. Field-evolved resistance to Bt has been documented in populations from the USA (Florida, Hawaii and New York), Central America (Costa Rica, Guatemala, Honduras and Nicaragua), and Asia (China, Japan, Malaysia, the Philippines and Thailand) (Perez & Shelton 1997; Tabashnik 1994; Wright *et al*. 1997; Zhao *et al*. 1993).

3. VARIATION IN BT RESISTANCE AMONG DIAMONDBACK MOTH POPULATIONS

How similar is the genetic basis of resistance to Bt in different populations of diamondback moth? Expectations depend on the frequency and type of resistance-conferring mutations (McKenzie & Batterham 1994). If mutations conferring resistance to Bt are exceedingly rare, a single mutational event might occur and the resulting resistance-conferring allele might subsequently spread worldwide by migration. This is the scenario proposed by Raymond *et al*. (1991) for one type of mosquito resistance to organophosphates. If resistance-conferring mutations are highly constrained (ffrench-Constant *et al*. 1993), yet somewhat more common, similar or identical mutations might arise independently in many populations. If mutations conferring resistance to Bt are neither exceedingly rare nor highly constrained, populations might differ in the loci at which resistance-conferring mutations occur. In this last scenario, variation among populations might occur in key traits such as the spectrum of resistance and cross-resistance, dominance, and the mechanism of resistance.

To test these ideas, a team of scientists from five research groups in three countries compared Bt-resistant strains of diamondback moth derived from Hawaii (NO-QA), Pennsylvania (PEN), and the Philippines (PHI). Here we provide highlights of experimental analyses of cross-resistance, dominance of resistance, genetic correlations among resistances to different toxins within each strain, interstrain complementation tests for allelism, and binding of toxins to their target sites in the midgut. Results reported previously (Tabashnik *et al*. 1997*a*,*b*), and summarized here, suggest that one pair of populations shares a locus at which similar or identical mutations can confer resistance to at least four Bt toxins. In contrast, resistance to Bt in a third population involves a different mutation at the shared locus. Further, each population has at least two independently segregating genes that can affect resistance to Bt.

(a) *Methods*

Three Bt-resistant strains were isolated from field populations that had been treated extensively with foliar applications of commercial formulations of the Bt subspecies *kurstaki* containing Cry1A toxins and other materials (Tabashnik *et al*. 1997*a*,*b*). Before comparisons were done, each strain was subjected to selection with Bt in the laboratory to further reduce the frequency of susceptible

individuals. Aside from binding data, which are summarized briefly, the results described here are from bioassays in which groups of third-instar larvae were exposed to cabbage-leaf discs that had been dipped in distilled-water dilutions of CrylAa, CrylAb, CrylAc, CrylC, CrylF, CrylJ, or distilled water only as a control. The susceptible LAB-P strain was included as a control in all bioassays. Strains were tested side by side to avoid potentially confounding effects of environmental differences between laboratories.

(b) *Spectrum of resistance and cross-resistance*

Responses of each resistant strain to six Cryl toxins revealed a pattern that emerged in all of our tests: the NO-QA strain from Hawaii and the PEN strain from Pennsylvania were similar, but the PHI strain from the Philippines was different. Both NO-QA and PEN were extremely resistant to CrylAa, CrylAb, and CrylAc, susceptible to CrylC, and cross-resistant to CrylF and CrylJ. Like NO-QA and PEN, PHI was resistant to the CrylA toxins and susceptible to CrylC. However, unlike the other two strains, PHI showed no cross-resistance to CrylF or CrylJ.

(c) *Dominance*

Before reviewing the experimental results, we shall digress briefly here to define dominance and explain its significance for resistance management. The simplest genetic basis for resistance would be a single locus with one allele (R) for resistance and another for susceptibility (S). Although we know that resistance to Bt involves more than two alleles at one locus, alleles with major effects do exist (Heckel *et al*. 1997; Tabashnik *et al*. 1995, 1997*a*,*b*; Tang *et al*. 1997), and the simplest model is a reasonable starting point.

Because alleles for Bt resistance are rare initially (Gould *et al*. 1997), individuals homozygous for resistance to Bt (RR) are exceedingly rare initially, occurring at about the square of the frequency of the resistance allele (Tabashnik 1997). Thus, the response of heterozygotes (RS) to Bt determines the initial course of evolution of resistance. If Bt kills heterozygotes, the resistance is termed recessive. If heterozygotes survive exposure to Bt, the resistance is called dominant.

Several resistance-management strategies, including the popular 'refuge–high dose' strategy, work best if resistance is recessive. A refuge is an area in which a portion of the pest population is not exposed to Bt. Hence, refuges enable survival of susceptible individuals. The idea behind the refuge–high dose strategy is that the very rare homozygous resistant (RR) adults mate with the much more abundant homozygous susceptible (SS) adults emerging from the refuge, and these matings generate heterozygous (RS) offspring. If resistance is recessive (heterozygotes are killed by Bt) and other assumptions of the strategy are valid, this approach can substantially postpone the evolution of resistance.

To evaluate dominance, we crossed each resistant strain with the susceptible LAB-P strain. In all cases, we paired a single virgin male from one strain with a single virgin female from another strain. The progeny from each single-pair family were reared and tested separately from all other families.

As with the spectrum of resistance, NO-QA and PEN were alike in terms of dominance, but PHI was different. For NO-QA and PEN, resistance to CrylAa, CrylAb, CrylAc and CrylF was partly to completely recessive. PHI showed recessive inheritance of resistance to CrylAb, but its resistance to CrylAa and CrylAc was not recessive. Mortality caused by CrylAc to the 16 F_1 families from PHI × LAB-P ranged from 21 to 90% with a mean of 64%; this result suggests control by one or more semidominant mutations.

We were surprised to find evidence for dominant resistance to CrylAa in PHI. Mortality caused by CrylAa ranged from 0 to 10% (mean 4.6%) in 5 of the 16 single-pair F_1 families derived from PHI × LAB-P. These results show that PHI harboured at least one dominant mutation conferring resistance to CrylAa. In contrast, mean mortality caused by CrylAa averaged 76% in F_1 families from NO-QA × LAB-P and 85% in F_1 families from PEN × LAB-P. None of the 14 F_1 families from PEN × LAB-P had mortality less than 20%. One of the 13 F_1 families from NO-QA × LAB-P had 11% mortality and two others had lower than expected mortality. Unexpectedly low mortality in response to CrylAa in some F_1 families from NO-QA × LAB-P suggests that at least one non-recessive mutation conferring resistance to CrylAa also occurred in NO-QA.

(d) *Genetic correlations and numbers of loci influencing resistance within each strain*

To determine whether single genes in NO-QA could confer resistance to more than one toxin, we generated hybrids by crossing NO-QA × LAB-P, reared the hybrids for one or more generations, then selected them with a single toxin. We found that by selecting with CrylAa, we could generate cross-resistance to CrylAb, CrylAc, and CrylF. Analogously, selection with either CrylAb or CrylAc immediately produced cross-resistance to each of the other three toxins in this set. These data support the hypothesis that the NO-QA strain harbours a mutation conferring resistance to CrylAa, CrylAb, CrylAc, and CrylF.

Although the selection approach described above is a compelling way to test for the effects of a single locus on resistance to several toxins, we needed a more efficient approach to enable comparisons to be made among all three resistant strains. Thus, we used experiments in which the progeny from each single-pair were split so that groups of siblings from each family were tested with either CrylAa, CrylAb, CrylAc, or CrylF.

As with our earlier comparisons, results of the split-brood experiments showed that NO-QA and PEN were alike, yet PHI was different. Strong genetic correlations (mean $r = 0.80$) were evident between all six pairwise combinations of CrylAa, CrylAb, CrylAc, and CrylF for NO-QA and PEN, but not for PHI. The observed genetic correlations are consistent with the hypothesis that in NO-QA and PEN a single mutation can confer resistance to all four toxins. In contrast, resistance to CrylF in PHI was not correlated with resistance to the other toxins. Further, pairwise correlations between the CrylA toxins were either weak (CrylAa–CrylAc and CrylAb–CrylAc) or not significant (CrylAa–CrylAb) in PHI. Therefore, the evidence suggests that NO-QA and PEN

have one or more multitoxin resistance mutations that are rare or absent in PHI.

The data suggest that NO-QA and PEN each harbour a multitoxin resistance gene that can confer resistance to at least four toxins, but they also show that each of these strains has at least two independently segregating resistance genes. As noted above, some hybrid F_1 families from NO-QA × LAB-P had unexpectedly low mortality in response to CrylAa. Likewise, some hybrid F_1 families from PEN × LAB-P had patterns of mortality that cannot be explained by allelic variation at a single locus. Overall, these patterns are reflected in significant toxin × family interactions in mortality in the hybrid F_1 families from NO-QA × LAB-P, PEN × LAB-P, and PHI × LAB-P. These interactions indicate that at least two independently segregating loci influence resistance within each strain.

(e) *Interstrain complementation tests for allelism*

We used interstrain complementation tests to determine whether the mutations conferring resistance in different strains were alleles at a shared locus. If resistance is recessive and controlled by the same locus in two different strains, hybrid progeny from crosses between the two strains will receive one allele for resistance from their father and another from their mother. Although these resistance alleles will not necessarily be identical, they will be at the same locus. Thus, lacking an allele for susceptibility at the resistance locus, the hybrid progeny will be resistant.

An alternative hypothesis is that resistance is controlled by locus 1 in resistant strain A and by independently segregating locus 2 in resistant strain B. If so, at locus 1, hybrid progeny from a cross between strains A and B will receive an allele for resistance from their strain A parent and an allele for susceptibility from their strain B parent. The converse will be true for locus 2. If resistance is recessive and epistasis is absent, such doubly heterozygous progeny will be susceptible. Thus, the shared-locus hypothesis predicts that hybrid progeny from interstrain crosses will be resistant and the independent-locus hypothesis predicts that they will be susceptible.

Our results support the shared-locus hypothesis. Hybrid progeny from NO-QA × PEN were resistant to CrylAa, CrylAb, CrylAc, and CrylF. Hybrid progeny from PHI × NO-QA and PHI × PEN were resistant to CrylAb. CrylAb was the only toxin for which PHI showed recessive inheritance of resistance and thus the only toxin for which the test for allelism was informative for PHI.

(f) *Conclusions from comparisons among Bt-resistant strains of diamondback moth*

The complementation data show that all three strains share a resistance locus. We call this gene 'BtR-1' of the diamondback moth. As described above, mutations at this locus in each strain confer resistance that is recessive. Biochemical assays described elsewhere show that this resistance is associated with reduced binding (Tabashnik *et al*. 1997*b*). The genetic correlation analyses show that the NO-QA and PEN strains have at least one multitoxin resistance allele at this locus that can confer resistance to CrylAa, CrylAb, CrylAc, and CrylF. Given that these four toxins share a binding site in the diamondback moth (Ballester *et al*. 1994; Granero *et al*. 1996), the simplest interpretation is that, in NO-QA and PEN, one mutation can alter binding of all four toxins.

In contrast, the PHI strain has one or more alleles at the BtR-1 locus that confer resistance to CrylAb but not to CrylAa or CrylF. These data imply that an alternative mutation at the BtR-1 locus alters binding of CrylAb, but not other toxins. Results from the Bt-resistant SERD3 strain of diamondback moth from Malaysia also show reduced binding of CrylAb with no change in binding of CrylAa or CrylAc (Wright *et al*. 1997).

In summary, NO-QA and PEN share a major resistance locus at which at least one multitoxin resistance mutation occurs. These two strains are also similar in their spectrum of resistance and cross-resistance, dominance of resistance, and mechanism of resistance. The PHI strain has a different allele for resistance at the shared locus and a narrower spectrum of resistance, with no cross-resistance to CrylF or to CrylJ. Compared with resistance in NO-QA and PEN, resistance to CrylAb in PHI shows the same dominance and mechanism, but resistance to CrylAa and CrylAc does not.

The multitoxin resistance alleles at locus BtR-1 in NO-QA and PEN are similar but not necessarily identical. We suspect that they arose independently because of the geographical isolation between the source locations in Hawaii and Pennsylvania and the recent appearance of resistance to Bt. The PHI strain contains an allele at the BtR-1 locus that is different from that of NO-QA or PEN and thus must have arisen independently.

Thorough analyses of resistance to Bt in the Loxa A strain of diamondback moth, which was derived from a resistant field population in Florida (Tang *et al*. 1996, 1997), show similarities with the resistance to Bt in the strains from Hawaii and Pennsylvania described above. Although CrylF and CrylJ have not yet been tested against Loxa A, this strain from Florida is extremely resistant to CrylAa, CrylAb, and CrylAc, yet, like the other strains, it is susceptible to CrylC. Resistance to CrylAc in Loxa A is recessive and probably controlled by a single locus (Tang *et al*. 1997). Resistance to CrylAb in Loxa A is associated with reduced binding (Tang *et al*. 1996). We do not know whether a single locus confers resistance to several toxins in Loxa A or whether the mutation or mutations conferring resistance to CrylA toxins in Loxa A are allelic with those in NO-QA and PEN.

4. COMPARISONS WITH RESISTANCE TO BT IN OTHER MOTHS

Comparisons with the tobacco budworm (*Heliothis virescens*) and the Indianmeal moth (*Plodia interpunctella*), two species in which resistance to Bt has been studied intensively, reveal striking parallels with the diamondback moth. First, at least one strain of each species exhibits a similar type of resistance to CrylA toxins. We'll call this 'mode 1' of resistance to Bt, which is characterized by extremely high resistance (over 500-fold) to at least one CrylA toxin, recessive inheritance, little or no cross-resistance to CrylC, and reduced binding of at least one CrylA toxin. Mode 1 resistance has been reported for

the NO-QA, PEN, and Loxa A strains of diamondback moth (Tabashnik *et al*. 1994, 1996, 1997*a*,*b*; Tang *et al*. 1996, 1997), the PHI strain of diamondback moth against CrylAb (Tabashnik *et al*. 1997*b*), the YHD2 strain of tobacco budworm (Gould *et al*. 1995; Heckel *et al*. 1997; Lee *et al*. 1995), and the 343R strain of Indianmeal moth (McGaughey 1985; Van Rie *et al*. 1990).

Second, at least one strain of each of these three moth species showed resistance to Bt that differs from mode 1. Resistance to CrylAa and CrylAc in the PHI strain of diamondback moth was not recessive and not associated with reduced binding (Tabashnik *et al*. 1997*b*). Resistance in the SEL and CP73 strains of tobacco budworm was less than 100-fold, not recessive, and not associated with reduced binding (Gould *et al*. 1992; MacIntosh *et al*. 1991; Sims & Stone 1991). Oppert *et al*. (1997) reported evidence for altered protease activity as a mechanism of resistance in Indianmeal moth strains 133-r and 198-r. These two strains, which had been selected with Bt subspecies *aizawai* and *entomocidus*, respectively, both showed resistance to CrylB and CrylC as well as to CrylA toxins (McGaughey & Johnson 1992, 1994). Other examples of resistance also show traits different from mode 1 resistance, such as resistance to CrylC in the diamondback moth (Liu & Tabashnik 1997), *Spodoptera exigua* (Moar *et al*. 1995), and *Spodoptera littoralis* (Chaufaux *et al*. 1997).

5. IMPLICATIONS FOR RESISTANCE MANAGEMENT

Because a particular pest can have more than one mode of resistance, characterization of one or a few resistant strains may not be sufficient for understanding resistance to Bt in a species. Therefore, in designing resistance-management strategies, it is unwise to assume that populations of a given pest can evolve only one type of resistance to Bt. For example, despite the finding of many cases of recessive resistance to Bt toxins in the diamondback moth, dominant resistance to CrylAa in the PHI strain violates one of the key assumptions of the refuge–high dose strategy.

In the light of this variation in resistance to Bt within species, how should we proceed? As resistance to Bt begins to evolve in the field in pests other than the diamondback moth, it will be critical to determine if mode 1 resistance is predominant. If so, it might be reasonable to implement strategies that are likely to be especially effective against this type of resistance. If diverse modes are found in other pests, as in the diamondback moth, or if a different type of resistance is most common, rethinking of strategies may be needed. In the meantime, increasing the spatial and temporal refuges from exposure to Bt should delay most, if not all, types of resistance.

In any case, admission of ignorance is important because current efforts at resistance management rely heavily on computer simulations and laboratory experiments, with few or no rigorous tests of tactics in the field. Careful tracking of resistance episodes in the field, either in conjunction with either designed experiments or the natural experiments that are proceeding on millions of hectares, can help to test predictions about evolution of resistance. Such tests are essential for enhancing the credibility of resistance management and sustaining the efficacy of benign insecticides such as Bt toxins.

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