Managing Insect Resistance to *Bacillus thuringiensis* Toxins

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Bacillus thuringiensis (*B.t.*) δ-endotoxins provide an alternative to chemical insecticides for controlling many species of pest insects. Recent biotechnological developments offer the promise of even greater use of *B.t.* toxins in genetically transformed pest-resistant crops. However, the discovery that insects can adapt to these toxins raises concerns about the long-term usefulness of *B.t.* toxins. Several methods for managing the development of resistance to *B.t.* toxins have been suggested, but none of these approaches offer clear advantages in all situations.

Insecticide resistance is a formidable complication of the use of chemical insecticides. Recently, several common species of pest insects have evolved resistance to *Bacillus thuringiensis* (*B.t.*) δ-endotoxins, indicating that biological pesticides can suffer the same fate. Although *B.t.* genes are currently used to transform plants in order to impart pest resistance in several major crops (*1–3*), the value of this approach could be seriously diminished by widespread development of resistance to *B.t.* toxins. Continued reliance on chemical insecticides might thus be necessary (*4*).

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B.t. in Pest Management

Bacillus thuringiensis is an aerobic, Gram-positive, spore-forming bacterium found commonly in the environment. It produces a number of insect toxins, the most distinctive of which are protein crystals formed during sporulation (*5*). These crystalline protein inclusions, or δ-endotoxins, are the principal active ingredients in *B.t.* formulations currently in use. The genes encod-

ing δ-endotoxin production have been cloned in other bacteria (6) and transferred into crop plants (7). This enables genetic improvements in the potency and host spectrum of *B.t.* strains (8) and development of crop varieties that produce *B.t.* toxins within their own tissues (1–3). The use of transgenic plants could overcome some of the stability problems associated with conventional *B.t.* application and improve control of pests that feed on plant parts that are difficult to treat by conventional methods (3, 9).

An advantage of *B.t.* toxins over chemical insecticides results from their specificity for pest insects. The toxins have no known detrimental effects on mammals or birds and are readily degraded in the environment. In addition, the limited range of activity of the toxins toward insects means that often a particular toxin will kill pest species but have no effect on predatory or predaceous species. This feature makes *B.t.* toxins highly desirable for use as components of integrated pest management (IPM) programs.

The site of *B.t.* toxin action is in the insect midgut, where it disrupts the cell membrane (10). In the bacterium, δ-endotoxins are synthesized as large protein molecules and crystallized as parasporal inclusions. In susceptible insects, these inclusions dissolve in the midgut, releasing protoxins that range in size from 27 to 140 kD and that are proteolytically converted into still smaller toxic polypeptides (11, 12). There is extensive variation in the size and structure of the inclusion proteins, the intermediate protoxins, and the active toxins that are presumed to relate to insect specificity.

Following activation, these toxins bind with high affinity to receptors (glycoproteins) on the midgut epithelium (13). After binding, the toxins generate pores in the cell membrane, disturbing cellular osmotic balance and causing the cells to swell and lyse through a process that has been termed "colloid-osmotic lysis" (12, 14–16).

Historically, *B.t.* strains have been classified on the basis of the flagellar or H-antigens of the vegetative cells into about 34 subspecies (17). However, this nomenclature system fails to consistently reflect the structure or vast diversity in insect specificity of the inclusion proteins. Recently, the crystal proteins and their genes have been classified based on their structure, antigenic properties, and activity spectrum into four major groups: CryI (Lepidoptera-specific), CryII (Lepidoptera- and Diptera-specific), CryIII (Coleoptera-specific), and CryIV (Diptera-specific) (12, 16). Each of these major groups has been further divided into several toxin types. At least 19 toxins have been described, and the list continues to grow (16). Many isolates produce several different Cry proteins (12). This heterogeneity in toxin production is responsible for some of the diversity in activity spectrum among strains.

The ability to solubilize and activate inclusion proteins influences the susceptibility of insects to *B.t.*, but the extent of that influence on host spectrum remains unresolved (18). In Lepidoptera, binding affinity of individual toxins to receptor sites on the midgut membrane accounts for the sensitivity of different insect species to various toxins (13, 14, 16, 19–22). However, binding site specificity may not be a simple system in which each toxin binds to a unique receptor (20). There appears to be a high degree of heterogeneity among binding sites in some species, suggesting that some sites may bind a single toxin whereas others may bind two or more toxins. Similarly, specific toxins may bind to more than one site in some insect species.

Insect Resistance to *B.t.*

Resistance to insecticides is a major agricultural and public health problem. Already more than 500 insect and mite species have acquired resistance (23). Microbial insecticides, similarly, induce resistance. Eight species have been selected for resistance to *B.t.* δ-endotoxins. The diamondback moth, *Plutella xylostella*, is notable as the only one having definitely evolved high levels of resistance in the field as a result of repeated use of *B.t.* (24). However, the Indianmeal moth, *Plodia interpunctella*, probably evolved low levels of resistance in stored grain treated with *B.t.* (25–27). Resistance in *Cadra cautella*, another pest of stored commodities, has been reported only from laboratory studies (26). Resistance in the tobacco budworm, *Heliothis virescens*, and the Colorado potato beetle, *Leptinotarsa decemlineata*, also has been reported only from laboratory selection experiments (28, 29), but resistance in these species is of great concern because of their economic significance. Resistance levels in two mosquito species (*Aedes aegypti* and *Culex quinquefasciatus*) and in the sunflower moth, *Homoeosoma electellum*, were relatively low (30).

The likelihood of insect resistance to the *B.t.* δ-endotoxins was considered for many years to be remote (31), in part because the mode of toxin action was thought to be very complex, involving multiple toxins and multiple target sites. Thus, a single change or mutation would be expected to have little effect (32). However, it is now recognized that rather than offering a mode of action so complex as to safeguard against resistance, the mode of action of *B.t.* provides many points at which behavioral or physiological changes might offer protection for the insects. Furthermore, transformation of plants with individual toxins would appear to bypass advantages offered by the mixtures of toxins normally produced in the bacteria.

Behavioral avoidance of formulated and purified *B.t.* toxins has been reported in Lepidoptera but has not been associated with any of the *B.t.* resistance cases reported (33). Avoidance may yet be observed as *B.t.* is used more extensively in either conventional or transgenic delivery systems where feeding choices are available (34).

Possible physiological mechanisms of resistance to *B.t.* δ-endotoxins include a change in gut pH or in enzymes that would affect dissolution and activation of the proteinaceous crystal. A study of protease activity in the midgut of resistant *Plodia interpunctella* revealed no obvious changes (35). However, this mechanism should not be ruled out in other species. So far, resistance has not been attributed to changes in the sensitivity of cells to pore formation or the capacity of insects to recover from effects of the toxins. Recovery from sublethal doses of *B.t.* is common among insects and may be subject to genetic variation.

In *Plodia interpunctella* and *Plutella xylostella*, resistance is due to a change in binding affinity of receptors or binding sites on the brush border membrane of the insect midgut (21, 22). This appears to be the same mechanism that is involved in host specificity of *B.t.* δ-endotoxins (19). Studies of iodinated δ-endotoxins and brush border membrane vesicles from the midguts of larvae demonstrated reduced binding affinity and decreased susceptibility that was specific for a toxin, CryIA(b), typical of *B.t.* subsp. *kurstaki*. CryIA(b) was a major constituent of the formulations used in selecting for resistance. Binding affinity and sensitivity to other types of toxins (CryIB and CryIC) remained high. Changes in binding have been found in resistant *Heliothis virescens*, but other mechanisms may also be involved in that species (36, 37). The mechanism of resistance in *Leptinotarsa decemlineata* is unknown at this time.

Resistance may be relatively specific to the toxin used in selection (21, 22, 36, 38). This specificity has led to speculation that resistance can be managed with the use of mixtures or sequences of unrelated toxins (39). However, cross-resistance among toxins occurs in some insect species. In *Heliothis virescens*, certain toxins bind to multiple receptors and some receptors bind multiple toxins (20). Indeed, a strain of *Heliothis virescens* selected for resistance to the CryIA(c) toxin typical of strain HD-73 (*B.t.* subsp. *kurstaki*) was cross-resistant to CryIA(a), CryIA(b), CryIB, CryIC, and CryIIA toxins (37). These findings provide

little encouragement for the multiple toxin approach to resistance management in *Heliothis virescens*. Many questions regarding the specificity or cross-reactivity of *B.t.* toxins and receptors must be answered before multiple toxin approaches can be recommended for managing resistance.

The capacity for resistance is widespread in some species. In *Plodia interpunctella*, resistance has been selected in colonies obtained from six grain storage sites in the midwestern United States (*26, 40*). Populations of *Plodia interpunctella* in the central United States have an approximate sevenfold range in susceptibility to *B.t.*, which suggests considerable genetic variation (*25*). *Leptinotarsa decemlineata* also exhibits a wide range in susceptibility among field populations (*41*). Resistance has already been reported in *Plutella xylostella* from Hawaii, the Philippines, and the continental United States (*24*).

In *Plodia interpunctella* and *Plutella xylostella*, resistance is partially recessive and apparently due to one or a few major loci (*25, 26, 42*). Resistance in *Heliothis virescens* may be partially recessive (*43*) but may involve multiple factors and be inherited as an additive trait (*37*). Resistance in *Leptinotarsa decemlineata* is due to one incompletely dominant gene and several genes that interact with it (*41*).

Resistance Management

Resistance management is an effort to delay or prevent adaptation in pest species. The adaptation may be to insecticides or to plant defense mechanisms. Resistance management may be viewed as management of a genetic resource, much the same as other renewable resources like forests, soil, water, and minerals are managed, except that the object of conservation is susceptibility genes or alleles. Resistance may be an inevitable consequence of insecticide use (*44*). Therefore, the goal of insect control policy should be to maximize the limited utility of insecticides.

Resistance management should be a strategy within the philosophy of IPM because it contributes to the ultimate IPM goal of implementing the best set of management tactics to hold pest populations below densities that could cause economic injury while minimizing socioeconomic and environmental impact resulting from the pesticide treatment. Resistance management within the context of IPM selects from four strategies: (i) diversification of mortality sources such that a pest is not selected by a single mortality mechanism, (ii) reduction of selection pressure for each major mortality mechanism, (iii) supply of susceptible individuals through maintenance of refuges or immigration, and (iv) estimation and prediction of progress toward resistance through the use of diagnostic tools, monitoring, and models.

Table 1. Tactics available for deploying insecticidal genes in plants.

Gene strategies	Single gene Multiple genes (pyramid or stacked) Chimeric genes
Gene promoter	Constitutive Tissue-specific Inducible (wound, phenology, elicitor)
Gene expression	High dose Low dose Mixtures
Field tactics	Uniform single gene Mixture of genes Gene rotation or sequence Mosaic planting Refuges (spatial, temporal)

Theories have been developed for managing insect resistance to *B.t.* (*3, 4, 39*), but there is little experimental data on their comparative utility. For conventionally sprayed *B.t.*, approaches used for managing resistance to chemical insecticides provide guidance. However, new strategies may be required to prevent insects from adapting to transgenic crops that express *B.t.* toxins (Table 1). Pests will probably adapt to *B.t.* regardless of how it is delivered; thus, a cautious approach to the use of *B.t.*-expressing plants is advised (*4, 45*).

Deployment of insect toxins through transgenic plants has much in common with pest control through conventional breeding to develop pest-resistant plants. Many species of insects, viruses, bacteria, fungi, and nematodes have adapted to insect- or disease-resistant cultivars (*46*). Although host-plant resistance is an accepted approach to protecting crops from insects, appropriate deployment strategies are needed in order to conserve the benefits of such resistant plants (*47, 48*).

Rotation or alternation of *B.t.* toxins with other toxins, insecticides, or cultural or biological control strategies can be used to manage resistance. The effectiveness of this approach depends upon restoration of susceptibility in pest populations when selection pressure is discontinued or changed to another gene, toxin, or organism. This approach assumes that there is some fitness cost (pleiotropy) associated with resistance and that reversion to a more susceptible condition will occur if selection pressure is reduced. Studies on *B.t.*-resistant *Plodia interpunctella*, *Plutella xylostella*, and *Heliothis virescens* have shown that in the early stages of selection, when resistance is still limited, resistance is relatively unstable and decreases when selection is discontinued (*26, 43, 49*). However, high levels of resistance in *Plodia interpunctella* were stable for long periods even in the absence of selective pressure (*26*).

Mixing toxins is another relatively simple approach that is possible in both conventional and transgenic plant deployment (*4*). With transgenic plants there are two ways to achieve a mixture: two or more seed lines can be engineered with different toxins and the seeds mixed before planting to produce a mixed population of plants or two or more *B.t.* toxin genes or other insecticidal proteins could be engineered into the same cultivar to produce a multiply (stacked or pyramid) transgenic plant (*50*).

In general, mixtures are likely to be more successful over time (durable) than is a single toxin, but use of mixtures is not necessarily better than sequential use of single toxins (*51, 52*). Simulation models have been used to compare sequential release of two single gene factors (seed rotation or alternation), random spatial mixtures of two single gene factors (mosaics or multilines), and addition of two resistance genes to a single cultivar (pyramid or stack) (*4, 47*). Durability varied depending upon the initial frequency of resistant alleles in the pest population, the manner of inheritance (dominant or recessive), and epistasis.

The durability of conventionally applied mixtures may depend upon equal persistence of the insecticides (*52*). If persistence differs, differential selection could occur, perhaps leading to accelerated resistance to both insecticides. For transgenic plants, the problem is one of differences in toxin expression rather than toxin persistence. Furthermore, this problem may be compounded in situations where crops are attacked by two or more pest species that differ in susceptibility to the toxins.

Cross-resistance among different *B.t.* toxins may reduce the likelihood that mixtures, rotations, or sequences of *B.t.* toxins will effectively delay resistance. Indeed, the evidence of binding site heterogeneity and cross-resistance in *Heliothis virescens* (*20, 37*) confirms that patterns of cross-resistance among *B.t.* toxins probably will differ among species of insects. These patterns must be defined before suitable mixtures or rotations can be recommended with any assurance of preventing or delaying resistance. Furthermore, studies on *Plutella xylostella* and *Plodia interpunctella* show that mixtures of *B.t.* toxins do not preclude the evolution of resistance and that they do not greatly improve durability (*40, 49*). *Plodia interpunctella* can evolve resistance to a variety of different *B.t.* toxins, both singly and in mixtures.

Theoretically, IPM helps delay resis-

Table 2. Recommendations of the U.S. Department of Agriculture Conference on *B.t.* Resistance.*

Monitor shifts in pest susceptibility following *B.t.* use.
Investigate risk of resistance in pest populations.
Experimentally validate resistance management strategies.
Integrate *B.t.* with other tactics in IPM programs.
Characterize cross-resistance patterns and mechanisms of *B.t.* toxins.
Assure an appropriate regulatory environment.
Establish a scientific advisory group to coordinate research and formulate resistance management strategies and regulations.

*This is a condensed list of the recommendations from (*58*).

tance by providing multiple sources of pest mortality (*45*), but it remains unclear whether pest adaptation to resistant cultivars is delayed by using additional biological or chemical controls (*53*). Furthermore, the use of both transgenic and conventionally applied *B.t.* to control the same pest population on a single crop or interbreeding populations on alternate hosts may exacerbate resistance development.

Developing and maintaining refuges to ensure the survival of susceptible insects may be the best tactic to manage resistance. Mathematical models have shown that refuges, which encourage survival of susceptible genotypes, immigration, or release of susceptible insects into pest populations greatly slow the evolution of resistance (*4, 44, 45, 47, 52, 54*). Natural refuges probably occur in crops with limited distribution that are attacked by pests with wide host ranges and expansive dispersal behavior, and additional refuges may not be necessary to enhance survival of susceptible pest genotypes. Additional refuges may be more beneficial where pests have restricted host ranges. The optimum spatial or temporal scale of refuges will almost certainly differ for each pest-host interaction. Mixtures of susceptible and transgenically resistant plants might be appropriate to control pest species that are not too mobile (*4, 47*). Tissue- or temporal-specific gene expression also might provide refuges in either space or time. For more mobile pest species, occasional rows, entire fields, or perhaps regions of untreated crops may be necessary to function as refuges.

Reduction in the rate of toxin application, reduced frequency of application, and use of transgenic plants that express low amounts of toxin are known collectively as "low dose" approaches. Low dose tactics that aim to reduce pest populations only slightly or slow pest larval development to the point that the number of generations per year is reduced probably serve to reduce selection pressure. However, from a practical standpoint, pest managers often prefer to eradicate all pests. Therefore, acceptance of low dose tactics may depend upon their effective incorporation into IPM programs. In instances where the pest population usually remains below the density that could cause economically important injury or where naturally occurring biological control agents could suppress a pest population partially controlled by *B.t.*, the low dosage tactic may be well accepted and successful.

A high dose is usually defined as one that consistently kills heterozygotes, the most abundant carriers of resistance. Such doses render the resistance trait functionally recessive and can result in slower progression of resistance (*54*). The toxin dose required is lowest in cases where resistance is recessive and highest where resistance is dominant. High dose approaches are not generally successful with conventional application because excessive cost, lack of uniform coverage, rapid degradation of the toxin deposits, and the inability to consistently target the most susceptible larval stages contribute to inconsistent control.

A high dose strategy in conjunction with untreated refuges has been advocated as a potential means of managing resistance development in transgenic plants (*45, 55*). Continuous expression of *B.t.* toxins in all tissues of transgenic plants could be sufficiently uniform and continuous to kill all heterozygotes. Refuges would then ensure a continuous influx of susceptible genotypes to mate with the relatively rare homozygous resistant individuals, and resistance could be effectively diluted or maintained at a very low level. If homozygous resistant individuals are at a very low frequency early in the evolution of resistance and untreated refuges provide a continuous source of susceptible individuals, this tactic could be quite effective. However, problems may arise when a single crop is attacked by more than one *B.t.*-susceptible pest with different susceptibilities or modes of inheritance of resistance.

An "ultrahigh dose" approach may be possible on occasion with very sensitive insects and optimal expression of *B.t.* in transgenic plants. Such a dose is defined as sufficient to kill both heterozygous and homozygous resistant insects. The transgenic plant essentially becomes a nonhost, and the pest cannot adapt. However, the ultrahigh dose tactic has never been successfully used.

The variety of *B.t.* expression patterns in transgenic plants offers added potential for resistance management. Constitutive expression in transgenic plants may cause great selection pressure on pest populations. Specific gene promoters can be used to limit exposure of pests to *B.t.* by causing the toxins to be expressed only in certain plant tissues (tissue-specific), at certain growth stages (temporal-specific), only in response to insect feeding (wound-specific), or only when induced by application of some elicitor (elicitor-specific) (*4, 39*). This approach seems particularly useful in certain crops. For example, toxins in potatoes need not be expressed in the edible tubers, and *B.t.* toxins in cotton could be directed to the economically important bolls. However, if tissue-specific promoters cause *B.t.* toxins to be expressed in high amounts in some tissues, and allow low amounts of expression in other tissues, resistance could be accelerated rather than delayed because insects would be selected differentially based upon the tissue utilized by different insect life stages. Any change in the stability, processing, or degradation of *B.t.* mRNA (*56*) through plant development could also provide a change in selection pressure and result in development of resistant insects.

Conclusions

Several major pest species, including *Heliothis virescens, Leptinotarsa decemlineata, Plodia interpunctella*, and *Plutella xylostella*, have already demonstrated the ability to adapt to *B.t.* in the laboratory. *Plutella xylostella* has evolved widespread resistance in the field. Thus, one can expect insect resistance to be a significant problem as *B.t.* use increases.

The possible tactics for resistance management include many options (Table 1). None offer clear advantages in all environments and with all pests except, perhaps, tactics that encourage survival or immigration of susceptible genotypes. Regardless of the approach used, resistance management becomes very complex where tactics must be coordinated against a pest on more than one crop or against more than one pest species. The new technologies and practices required for managing resistance will face challenges before being generally accepted (*57*).

Considerable controversy exists regarding how transgenic plants can or should be deployed to delay potential resistance development. Expression and dosage of gene products are functions of the various promoter, transcriptional, and translational factors associated with each resistance gene. The constitutively expressed gene may particularly promote resistance development, as it could lead to selection of all life stages of pests on all parts of the plants

throughout the entire growing season. Some researchers believe that a high dose delivered by transgenic plants could be used effectively in conjunction with refuges for susceptible insects without causing the pests to adapt. Combinations of B.t. with other mortality mechanisms and with refuges for susceptible insects are probably better approaches to managing resistance. However, it may be easier to obtain regulatory approval, and more profitable for industry, to use simpler strategies.

We urge development of a national research agenda with full government, university, and industry cooperation to develop, evaluate, and implement resistance management strategies for conventionally applied and transgenic B.t. toxins (Table 2). The most scientifically, environmentally, and sociologically acceptable pest suppression tools of this century and possibly the next are at stake. Given the slow pace of development of new insecticides and the environmental and social concerns about synthetic organic insecticides, the thoughtful management of B.t. toxins could be very beneficial.

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