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The Insecticidal Crystal Proteins of *Bacillus thuringiensis*: Past, Present and Future Uses

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Introduction

Bacillus thuringiensis (Bt) is a Gram-positive, spore-forming bacterium, which produces a parasporal crystal during sporulation. These crystals are predominantly comprised of one or more proteins, called δ-endotoxins or insecticidal crystal proteins, known to possess insecticidal activity when ingested by certain insects. Numerous strains of Bt are currently known. Each strain produces differing numbers of δ-endotoxins with various insecticidal activities. A given δ-endotoxin is typically insecticidal towards a narrow spectrum of insect targets. Bt has been used since 1938 to produce an insecticidal spray for the control of certain insect pests. These sprays are known for their environmental safety and safety towards non-target insects as well as other animals. As agricultural practices demand ever-increasing safety for pest control practices, interest in Bt has increased. Originally, Bt was considered to be useful only against a few insect species in the order Lepidoptera, but expanded screening efforts have yielded several strains with a number of different activities, including activities against non-insect pests (Feitelson, Payne and Kim, 1992). As the spectrum of activity for Bt as a class of pest control agents increases, so too does its use. New technologies to aid strain discovery have hastened the identification of new strains harbouring novel activities. New technologies in the area of molecular biology are providing the means to construct strains of Bt with desired insecticidal

Abbreviations: AcMNPV, *Autographa californica* multiple nuclear polyhedrosis virus; AcNPV, *Autographa californica* nuclear polyhedrosis virus; Aq, *Agmenellum quadruplicatum*; BBMV, brush border membrane vesicle; Bt, *Bacillus thuringiensis*; CaMV, cauliflower mosaic virus; CPB, Colorado potato beetle; *Cxc*, *Clavibacter xyli* subsp. *cynodontis*; ECB, European corn borer; mRNA, messenger RNA; Pc, *Pseudomonas cepacia*; PCR, polymerase chain reaction; PEPC, phosphoenolpyruvate carboxylase; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; UV, ultraviolet.

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activities or to overcome or prevent resistance to these proteins by the target insect. Further, these new tools allow the introduction and expression of the δ-endotoxins in new host organisms, thereby increasing the possible uses for these insecticidal proteins. Their attributes of being direct, single gene products make them attractive for such engineering endeavours. This review focuses on the traditional uses of the δ-endotoxins as the active components of microbial sprays and the future uses of such sprays in light of new technologies and an increased understanding of the biology of these proteins. It also looks at the uses of these proteins in various new host organisms, and examines the issue of resistance management. The δ-endotoxins can be viewed as pesticidal proteins whose applications are broad, and rapidly expanding, and no longer limited to a particular use within the confines of one particular organism.

Traditional and current uses of Bt insecticides

Bacillus thuringiensis has been used to produce biological insecticides for foliar application for over 50 years. These products were, and are still, produced by fermentation of single Bt strains in crude, inexpensive media. As the Bt cells begin to exhaust the medium, they enter the sporulation phase of growth where one or more insecticidal proteins, or δ-endotoxins, are synthesized. The δ-endotoxins accumulate in morphologically distinct crystal(s) within the cells and are released into the medium with the spores when the cells ultimately lyse. Typically, the first step in the manufacturing process of a Bt bio-insecticide is to concentrate the fermentation solids (spores, δ-endotoxin crystals and unused particulate media components) by centrifugation. Sometimes tangential flow dialysis is also used to further concentrate the solids before spray drying. The dried material is then milled to a uniform size to produce a technical powder which can then be formulated in a variety of ways. The most common formulations are wettable powders or dispersible granules, but oil- and aqueous-flowable preparations have also been produced.

At least three major factors contribute to the effectiveness of Bt insecticides produced in this manner. First, a Bt strain must be obtained that produces δ-endotoxins which are effective on the target insects. Second, the strain must be able to produce large amounts of active δ-endotoxin during large-scale fermentations at a minimum cost. Third, a formulation that maximizes the longevity and effectiveness of the δ-endotoxins should be used.

Strain selection

The bacterial strains used for all of the early and some of the current Bt foliar insecticides are wild-type strains, that is, they were found in nature in the form in which they are used to produce the microbial spray. These strains have good activity on a variety of commercially important insects and they can be easily fermented and formulated. Current knowledge concerning the mechanism of action of Bt δ-endotoxins clearly indicates that the insecticidal

activity of a strain is determined by several factors. The number, type and amount of δ-endotoxin(s) produced are the primary determinants, and for many insects this alone can fully account for a strain's activity. However, for a few insects the spores and the δ-endotoxins appear to act in synergy, possibly due to septicaemia and the production of degradative enzymes and/or other toxins in the insect's gut (Heimpel and Angus, 1959). Most wild-type Bt isolates produce multiple δ-endotoxin proteins, each having a characteristic insecticidal activity spectrum. It is generally believed that the overall activity spectrum of a strain is due to additive and/or synergistic interactions of the individual δ-endotoxins present in their proportional amounts. Both the total amount of δ-endotoxin produced and the relative amount of each is largely determined by the copy number of each gene and the extent to which it is expressed. Increasing copy number of a single gene or the presence of multiple δ-endotoxin gene types can increase the amount of δ-endotoxin produced up to about 30% of the dry weight of the cell (Lilley, Ruffell and Somerville, 1980). Thus, the maximum amount of a single δ-endotoxin protein would be obtained from a strain expressing a single δ-endotoxin gene at its maximum biosynthetic capacity. However, the insecticidal spectrum of such a strain would be relatively narrow. In contrast, a strain producing several δ-endotoxins would have a broader insecticidal spectrum, but the activity contribution of each δ-endotoxin would be decreased relative to the proportional amount produced.

When the first Bt products were developed, little was known about the determinants of toxicity or the δ-endotoxin composition of the strains. Analysis of these strains shows that they have good biosynthetic capabilities in crude fermentation media, they have relatively broad insecticidal activity, and they express at least four different δ-endotoxins (*Table 1*). For example, the broad insecticidal activity spectra of HD1 (Dipel®, Abbott) is reflected in the insecticidal activities of the individuals δ-endotoxins (*Table 2*). In addition, high toxicity to a limited number of insects is predicted by the presence of three individual δ-endotoxins with good activity on the same insect.

For many target crops, such as cole crops, only a few of the pests listed in *Table 2* are of importance. In such cases, it should be possible to obtain a better performing bio-insecticide based upon a Bt strain that produces only those toxins with good activity on the target insects. The realization that closely related δ-endotoxins could have quite different insecticidal activities and the discovery that most δ-endotoxin genes are located on plasmids made it possible to begin to rationally construct strains with increased activity on specific pests. Knowing the insecticidal spectrum of an δ-endotoxin and its plasmid location, it is possible to use the classical genetic techniques of plasmid curing and conjugal plasmid transfer to eliminate unnecessary δ-endotoxins and/or introduce desirable ones. Bt strains present in several currently available bio-insecticides were constructed using these techniques (*Table 1*). Although these genetic manipulation techniques have the capability to increase potency and control specificity, they are limited by three major factors: (1) not all plasmids containing δ-endotoxin genes can be easily cured;

Table 1. Gene composition of several currently available Bt insecticides

		cry Gene type								
Product[a]	Company	IA(a)	IA(b)	IA(c)	IB	IC	ID	IIA	IIB	IIIA
Agree[a]	Ciba-Geigy	+		+		+	+			
Xentari[a]	Abbott	+	+			+	+			
Dipel[a]	Abbott	+	+	+				+	+	
Biobit[a]	Novo	+	+	+				+	+	
Javelin[a]	Sandoz	+	+	+				+	+	
Condor[a]	Ecogen	+	+	+				+	+	
Cutlass[a]	Ecogen	+	+	+				+	+	
Foil[a]	Ecogen			+						+
Novodor[a]	Novo Nordisk									+

Note: These data were generated at Ciba using gene specific primers in the polymerase chain reaction (Carozzi *et al.*, 1991). A positive PCR reaction only indicates the likely presence of the gene. It does not imply gene expression. Thus the gene may be present in the strain but the δ-endotoxin may not be present in the product. In some cases, the same gene type may be present on two different δ-endotoxin plasmids.
[a] Agree[a] (Burgess and Jarrett, 1985), Condor[a] (Gonzalez and Macaluso, 1992), Cutlass[a] (Gonzalez and Macaluso, 1992) and Foil[a] (Donovan *et al.*, 1991) were constructed using plasmid curing and conjugation techniques.

(2) not all δ-endotoxin plasmids can be transferred by conjugation; and (3) some plasmids contain more than one δ-endotoxin gene. Because of these limitations, it may not be possible to construct a strain containing only the desired δ-endotoxin genes using classical genetic techniques.

The tools of genetic engineering can be used to overcome all of the limitations of classical genetics. However, it is desirable to use both classical and molecular approaches together if possible. The classical techniques of plasmid transfer and curing are easier and quicker than recombinant procedures and can be complemented by molecular techniques when necessary. In addition to being a useful complement to the classical techniques of strain construction, molecular techniques have the potential to further optimize δ-endotoxin production in several ways. For example, they could be used to control gene dosage by altering plasmid copy number or by integrating the δ-endotoxin gene into an existing plasmid or the bacterial chromosome. Copy number mutations of a Bt vector have been reported (Arantes and Lereclus, 1991) which can to some extent control δ-endotoxin production. In addition, gene expression can also be controlled by altering the elements regulating transcription and translation (promoters and/or ribosome binding sites) or by increasing the mRNA stability of the message by modifying its 3′ end. Also, the δ-endotoxin itself could be engineered to have a broader insecticidal activity spectrum. Additionally, molecular techniques have the potential to further optimize and enhance δ-endotoxin production. As additional information on the determinants of specificity and toxicity are obtained, it may be possible to more rationally make directed changes in these parameters.

Regardless of the techniques used for rational strain construction, the first and perhaps the most difficult step is to determine the desired insecticidal activities and to identify δ-endotoxins with these properties. Multiple δ-endotoxin genes could be introduced into a strain for one or more of the following reasons: to increase toxicity on a particular pest, to broaden the

Table 2. Relative activities of δ-endotoxin gene types on selected insects[a]

Endotoxin	Px sen	Px res	Se	Sl	Tn	Hv	Hz	On	Ms	Ldis	Pb	Ld
cryIA(a)			–	–	+	++		++	+++		+++	
cryIA(b)	+++	–	–	–	+++	++	++	++	+++	+++	+++	
cryIA(c)	+++		–	–	+++	+++	+	++	+++	+++	+++	
cryIB	+++	+++		–		++		[++]	–		+++	
cryIC	++	++	[++]	++		–			++		+++	
cryID	–	–		–		≤+			+++		≤++	
cryIE	–	–	(++)	++		–			++			
cryIF	++		–			++		++				
cryIIA					++	++	++	++	+++	+++		
cryIIB					++	++	++	+		+++		
cryIIC												
cryIIIA												++
cryIIIB												+
cryIIIB2												++
cryIIIC(a)												++
cryIIIC(b)												+
cryIIID												(+++)
cryV								++				++

Px sen, *Plutella xylostella* (diamondback moth) sensitive to Dipel; Px res, *Plutella xylostella* (diamondback moth) resistant to Dipel; Se, *Spodoptera exigua* (beet armyworm); Sl, *Spodoptera litoralis* (cotton leafworm); Tn, *Trichoplusia ni* (cabbage looper); Hv, *Heliothis virescens* (tobacco budworm); Hz, *Helicoverpa zea* (cotton bollworm, corn earworm); On, *Ostrinia nubilalis* (corn borer); Ms, *Manduca sexta* (tobacco hornworm); Ldis, *Lymantria dispar* (gypsy moth); Pb, *Pieris brassicae* (European cabbage worm); Ld, *Leptinotarsa decemlineata* (Colorado potato beetle).

[a] Relative activities are estimates made from published values or from our unpublished data [data in brackets]. The activities are defined as follows: +++ = $LC_{50} \leq 10$ ng cm^{-2} or for Pb < 1 μg ml^{-1}; ++ = 10 ng cm$^{-2} \leq LC_{50} \leq 100$ ng cm^{-2} or for Pb 1 ≤ $LC_{50} \leq 10$ μg ml^{-1}; + = 100 ng cm$^{-2} \leq LC_{50} \leq 1000$ ng cm^{-2} or for Pb 10 ≤ $LC_{50} \leq 100$ μg ml^{-1}; – = $LC_{50} \geq 1000$ ng cm^{-2} or for Pb > 100 μg ml^{-1}.

Relative activities given in parentheses indicate the reported activity was not given in ng cm^{-2}. In these cases, the relative activity was estimated by comparison of the δ-endotoxin's activity on a second insect whose activity was reported elsewhere in ng cm^{-2}.

host range in cases where one δ-endotoxin cannot be identified with acceptable toxicity on multiple insect targets, or to manage resistance by including δ-endotoxins that bind to different sites in the insect midgut. Whatever the desired goal, the effectiveness of the strain will depend on the biosynthetic capacity of the cell, because introducing more toxins to broaden specificity will usually reduce the amount of each δ-endotoxin present.

The task of identifying δ-endotoxins with new or more potent activities typically involves screening wild-type strains of Bt. The presence of a new activity is obvious, but to detect the presence of a more potent δ-endotoxin, bioassays must be normalized to the amount of δ-endotoxin protein in the assay. To find genes encoding proteins that are more potent on insects known to be susceptible to Bt δ-endotoxins, it is necessary to isolate genetically the δ-endotoxin gene by classical or molecular methods so that activity per unit of δ-endotoxin protein can be compared with the specific activities of known δ-endotoxins.

Molecular probes such as gene-specific polymerase chain reactions (PCRs; Carozzi *et al.*, 1991) or hybridization with gene-specific DNA sequences (Gaetner, Sick and Schwab, 1993) are useful for the identification of known or related genes in strains with interesting insecticidal properties. A general

idea of the biological activity of a δ-endotoxin can be made if a known gene class is detected, but accurate prediction is not possible because specific activities of δ-endotoxins of a gene class can differ by as much as 10-fold on the same insect (Von Tersch *et al.*, 1991). Novel δ-endotoxins which cannot be identified using gene probes might be identified by biochemical or immunological means. Analysis of the solubilized proteins from crystal inclusion by SDS-PAGE can determine if proteins of an unexpected size are present. Differences of less than 1000 daltons can be resolved on such gels. Another method for finding unique proteins using antibodies directed against known δ-endotoxins was described by Geiser, Hartmann and Oddou (1993), which involves the binding of known δ-endotoxins to antibody affinity columns. A protein with insecticidal activity that does not bind may constitute a new δ-endotoxin class. After the genes of interest are identified, they must then be isolated by classical or molecular techniques and the activity spectrum and specific activities on individual insects determined. Finally, strains are constructed by combining the selected genes in a Bt strain suitable for fermentation.

Fermentation

The first Bt product was marketed in France in 1938. In 1960, there were five US companies marketing Bt bio-insecticides, but by 1979 only two remained. For a review of the historical development of commercial production of Bt bio-insecticides, the reader is referred to Rowe and Margaritis (1987). Both of these products were produced by growth of the bacterium in liquid medium in large mechanically agitated vessels sparged with air. This type of deep liquid fermentation is the preferred method of production for current Bt insecticides. The details of the fermentation, downstream processing and formulation of Bt products are generally kept as trade secrets. The development of a fermentation process is largely empirical and must be determined separately for each Bt strain selected. Unlike laboratory research where cost of media components, cell harvesting and sample processing are not the highest priority, the goal in commercial production is to make the maximum amount of active ingredient and final formulated product for the minimum cost. From the fermentation aspect, this necessitates the use of crude, inexpensive fermentation ingredients. These materials often contain a high proportion of insoluble protein and other materials. The insoluble protein is an inexpensive source of carbon and nitrogen and can be utilized because the Bt cells produce large amounts of extracellular enzymes in order to solubilize and degrade this material.

The suitability of these components depends on many factors. Clearly, the cost is important and the material must be readily available in large quantities whenever it is needed. In addition, the batch-to-batch uniformity of the fermentation substrates may be critical for development of an easily reproducible process. Since many crude protein sources are waste or by-products of other manufacturing processes, different production lots are not always of consistent nutritional value for fermentation. This inconsistency may result

from the fact that the fermentation substrates are not always handled, stored or even processed in the same manner, or because the starting materials from which the fermentation substrates are obtained are not always the same. Obtaining a consistent supply of any required inorganic nutrients, such as phosphate and divalent ions, may also be a problem because there are many crude sources of the materials which could be contaminated with unwanted inorganic poisons or inhibitors.

The development of an economical fermentation process involves a great deal of experimentation and must be specifically designed for a particular strain of Bt. Thus the production of a new bio-insecticide using a new Bt strain requires the development of a different fermentation process. This may only require minor changes in an existing process or the physiological properties of the new strain may be different enough to require major changes in the entire manufacturing process.

If strains are constructed by genetic manipulation, it should be possible to avoid the development of a different production process for each new Bt strain. Genetically manipulated strains could be constructed by introducing the desired δ-endotoxin genes into a 'standard' recipient strain. Such a recipient would be a variant of a strain for which the fermentation and formulation technology have already been developed. This variant could be developed using plasmid-curing techniques to remove those δ-endotoxin genes that are not desired in the new product. The recipient strain might be completely cured of all δ-endotoxin genes, or one or more δ-endotoxin genes from the original parent strain might be retained. The degree of plasmid curing would depend on whether any δ-endotoxin genes from the parent were desired in the final product and whether the desired genes were on plasmids that were free of undesirable genes. A genetically modified strain would then be constructed by introducing the desired δ-endotoxin genes into the plasmid-cured recipient strain using plasmid transformation, sexual conjugation or a variety of recombinant DNA techniques.

It should also be possible to increase δ-endotoxin production by genetically modifying the fermentation strain or the regulatory elements controlling δ-endotoxin gene expression. In fact, strain selection for increased δ-endotoxin production has been accomplished with Novodor® (Novo Nordisk). The strain in this product is a mutant *B. thuringiensis* var. *tenebrionis* obtained by gamma irradiation. It produces about twice as much δ-endotoxin as its parent (Gurtler and Petersen, 1991), apparently as a result of a regulatory change which results in the production of δ-endotoxin prior to sporulation. In addition, the results of Mettus and Macaluso (1990) suggest an additive increase in δ-endotoxin production in a strain where a δ-endotoxin gene is expressed by both its native sporulation dependent promoter and by a strongly expressed vegetative promoter. Donovan (1991) was also able to express a silent *cryIIB* gene by fusing it to the *cryIIIA* promoter. Furthermore, Gamel and Piot (1992) fused the *cryIC* promoter to the *cryIIIA* gene and introduced this construct into a strain already containing CryIA(a), CryIA(b), CryIA(c) and CryIIA proteins. They state that the introduction of the newly introduced *cryIIIA* gene did not interfere with expression of the

native genes. Together, these data show that δ-endotoxin expression can be regulated by placing δ-endotoxin genes under the control of promoters from either δ-endotoxin or non-endotoxin genes. This kind of regulatory change, together with changes in gene copy number and alterations at the ribosome binding site, should make it possible to further increase δ-endotoxin production.

Some other possibilities for increasing δ-endotoxin production include directing more of the cell's biosynthetic capacity to δ-endotoxin production or to prolong the time period allowed for biosynthesis. The major biosynthetic activity occurring during δ-endotoxin synthesis is the production of spores. This complex process consumes a substantial amount of biosynthetic capacity and for the most part spores are not required for insecticidal activity on many commercially important insects. The biosynthetic requirements for spore production might be diverted to δ-endotoxin production in strains where sporulation was aborted at an early stage. Spore-negative, δ-endotoxin-producing mutants of Bt have been reported (Johnson, 1981; Wakisaka *et al.*, 1982) and about as much or a little more δ-endotoxin was produced in the mutants as compared to their respective parental strains. Other spore-negative strains have been described where δ-endotoxin production was increased relative to the parental strains (Fitzjames, 1984; Herrnstadt and Gaertner, 1987). The greatest increase reported was about 1.5-fold better than the parental strain. Further improvements in this area might be realized as more information is obtained about the biosynthetic and regulatory relationships between δ-endotoxin synthesis and spore formation.

It might also be possible to isolate an overproducing mutant by extending the normal period for δ-endotoxin biosynthesis. Inhibiting or completely preventing cell lysis by elimination or inactivation of enzymes (Kingan and Ensign, 1968) responsible for cell wall degradation during sporulation could produce the desired phenotype.

Formulation and application

Bt bio-insecticides have been formulated in many ways since their introduction in 1938. Wettable powders, dispersible granules, dusts, microgranules, aqueous flowable liquids and oil-based flowable liquids that are dispersible in water have been developed. Early formulations were often difficult to apply and their performance was often poor or unreliable. Advances in application equipment and improvements in formulation technology have largely overcome application difficulties. However, different strains may well require different formulations because of differences in their respective fermentation media and their by-products. Also, batch-to-batch formulation problems can be encountered due to variable quality of the fermentation components.

Substantial improvements in the performance of Bt insecticides have been made through advances in formulation technology. Many new spreaders and stickers have been developed to help evenly distribute and bind the material over the leaf surface, which is often very waxy. Newer, more effective

additives are continually being developed and further improvements in this area can be expected in the future.

The second major task of a good formulation is to aid in the persistence of the active ingredient. It was recognized very early that Bt insecticides do not persist for very long in the environment (Gelernter, 1990). Possible causes for loss of activity include inactivation by ultraviolet (UV) light, heat, leaf exudates and pH (Pozsgay *et al.*, 1987; Dulmage and Aizawa, 1982). The most important factors have not been identified. Some of the factors are clearly environmental and others, like leaf surface chemistry, are plant-dependent. At high pH, particularly in the presence of reductant, δ-endotoxin crystals are solubilized and thus become more accessible to proteolytic inactivation. High leaf pH might be important on cotton where the leaf surface can be in the range of pH 8–10 (Andrews and Sikorowski, 1973).

Formulations of Bt insecticides have addressed these problems by either including additives to help prevent loss of the active ingredient or encapsulating the material in such a way that the active ingredient is protected from inactivation. The addition of UV protectants might effectively reduce irradiation damage. Perhaps inactivation due to heat could be controlled by including an additive, but no materials of this type have been described. Another approach to control the inactivating effects of UV light and/or leaf exudates would be to encapsulate the material. Much of the information on specific additives and formulations can be found in the patent literature and will not be reviewed here. Encapsulation can be accomplished chemically (McGuire and Shasha, 1992) or biologically (Barnes and Cummings, 1987). Chemical encapsulation involves a process in which the active ingredient is coated with some type of polymer, while biological encapsulation involves the expression of the δ-endotoxin genes in a microbe. For biological encapsulation, the intact microbe containing the δ-endotoxin protein is used as the active ingredient in the formulation. Unfortunately, there is a limited amount of information in the public domain on the effectiveness of any of the above formulation approaches. All are described as beneficial, but there are few data available that accurately quantify their protective effects. Formulation is an active but rather proprietary area of research that will undoubtedly increase the effectiveness of Bt insecticides in the future.

Most commercially available Bt insecticides can be tank-mixed with a variety of other agricultural chemicals (*1993 Crop Protection Chemicals Reference*, Chemical and Pharmaceutical Press, New York). These include other pesticides, herbicides and additives intended to promote insect feeding and thereby increase the effectiveness of the Bt insecticide. There is a good deal of information available on various positive interactions of Bt with other insecticides and feeding stimulants. Regardless of the effectiveness of any formulation or mix with beneficial agrochemicals, the benefits must be economical. To be useful, they must provide a benefit justified by the added price.

Spectrum of activity

The various insecticidal crystal proteins have been classified based upon their spectrum of activity and sequence homology. The classification put forth by Hofte and Whiteley (1989) placed the then known insecticidal crystal proteins into four major classes. Generally, the major classes are defined by the spectrum of activity with the CryI proteins active against Lepidoptera, CryII proteins active against both Lepidoptera and Diptera, CryIII proteins active against Coleoptera, and CryIV proteins active against Diptera. Subsequently, a crystal protein was identified with activity against both Lepidoptera and Coleoptera (Tailor *et al.*, 1992) and the authors suggested proteins with such activity be placed in a new class, the CryV proteins. This protein has activity against European corn borer (*Ostrinia nubilalis*; Lepidoptera) and Colorado potato beetle (*Leptinotarsa decemlineata*; Coleoptera). Another group (Schnepf *et al.*, 1992) discovered Bt crystal proteins with activity against nematodes. These proteins were placed into two groups based upon their structures and these two groups were proposed to be called CryV and CryVI, thus conflicting with the other authors. The nomenclature system is still evolving, but it is providing a needed clarity and consistency in the naming of the various δ-endotoxins. A δ-endotoxin with activity against a Hymenoptera has also been found (Payne *et al.*, 1992) and is yet unclassified. The relationship, both in terms of protein sequence homology and structural homology, of these new δ-endotoxins with the better known δ-endotoxins classified by Hofte and Whiteley (1989) remains unknown. As yet, it is unclear whether these new toxins have the same mode of action or whether they represent new modes of action as well as new pesticidal activities.

Within each major class, the δ-endotoxins are grouped according to sequence homology. The CryI proteins are typically produced as 130–140 kDa proteins which are proteolytically activated to produce proteins about 60–70 kDa. The active portion of the δ-endotoxin resides in the NH_2 terminal portion of the full-length molecule. Hofte and Whiteley classified the then known CryI proteins into six groups: IA(a), IA(b), IA(c), IB, IC and ID. Since then, proteins classified as CryIE, CryIF, CryIG and CryIX have been characterized. Other lepidopteran active genes have also been identified, but have not been classified as yet. *Table 3* lists various crystal proteins produced by *B. thuringiensis*. New coleopteran-active proteins have also been identified since this classification scheme was put forth. Originally, there was but one characterized protein active against Coleoptera and this was classified as CryIIIA. CryIIIA has activity against mealworm (*Tenebrio molitar*) and Colorado potato beetle (*Leptinotarsa decemlineata*). Subsequently, CryIIIB, CryIIIB2, CryIIIC, CryIIIC(b) and CryIIID δ-endotoxins have been identified with activity against Coleoptera.

The spectrum of insecticidal activity of an individual δ-endotoxin from Bt tends to be quite narrow, with a given δ-endotoxin being active against only a few (known) insects. Specificity is the result of the efficiency of the various steps involved in producing an active toxin protein and its subsequent interaction with the epithelial cells in the insect digestive tract. It is also likely

Table 3. Current list of Bt δ-endotoxins and their activities

Endotoxin/strain	Size (kDa)	Specificity	Reference
CryIA(a)	133	Lep	Schnepf, Wong and Whiteley (1985)
CryIA(b)	131	Lep	Wabiko, Raymond and Bulla (1986)
CryIA(c)	133	Lep	Adang *et al.* (1985)
CryIA(c) variants	133	Lep	Dardenne, Seurinck and Peferoen (1990); Von Tersch *et al.* (1991)
CryIB	138	Lep,Col	Brizzard and Whiteley (1988)
CryIC	135	Lep	Honee, van der Salm and Visser (1988)
CryIC(b)	134	Lep	Bosse, Masson and Brousseau (1990)
CryID	133	Lep	Hofte *et al.* (1990)
CryIE	133	Lep	Visser *et al.* (1990); Masson *et al.* (1992)
CryIF	134	Lep	Chambers *et al.* (1991)
CryIG	130		Smulevitch *et al.* (1991); Gleave, Hedges and Broadwell (1992)
CryIX	81	Lep	Gawron-Burke, Chambers and Gonzalez (1991)
PS81RR1	133	Lep	Payne and Sick (1990)
OS81GG	133	Lep	Payne, Sick and Thompson (1992)
81IA2	133	Lep	Payne and Sick (1991a)
81IB	132	Lep	Payne and Sick (1991a)
81IB2	135	Lep	Payne and Sick (1991a)
81IA	134	Lep	Payne and Sick (1991a)
PS81F[*n*][cryIE(a)]	133	Lep	Payne and Sick (1991b)
34 kDa crystal protein	34	Lep	Brown and Whiteley (1992)
PS158C2	47,37,34 and 32	Lep	Payne, Cummings and Cannon (1993)
CryIIA	71	Lep,Dip	Donovan *et al.* (1988)
CryIIB	71	Lep,Dip	Widner and Whiteley (1989)
CryIIC	110	Lep,Dip	Wu *et al.* (1991)
CryIIIA	73	Col	Herrnstadt *et al.* (1987)
CryIIIB	74	Col	Sick, Gaertner and Wong (1990)
CryIIIB2	74	Col	Donovan *et al.* (1992a)
CryIIIC[*v*]	74.4	Col	Donovan *et al.* (1993)
CryIIIC[*w*]	129	Col	Lambert *et al.* (1992a)
CryIIIC(b)	70	Col	Donovan, Rupar and Slaney (1992)
CryIIID	73	Col	Lambert *et al.* (1992b)
43F (resembles IIC)	74	Col	Sick and Gilroy (1991)
PS50C	130	Col	Foncerrada, Sick and Payne (1992)
PS40D1		Col	Hickle, Bradfisch and Payne (1992)
NCIMB40152	63–70,32 and 14	Col	Cidaria *et al.* (1991)
PS86A1	58 and 45	Col	Bradfisch, Michaels and Payne (1993)
PS86Q3	155,135, 98,62 and 58	Col	Bradfisch, Michaels and Payne (1993)

Continued over

Table 3. cont.

Endotoxin/strain	Size (kDa)	Specificity	Reference
Strain 14-4		Col	Carozzi *et al.* (1993)
Strain 14-5		Col	Carozzi *et al.* (1993)
Strain 14-8		Col	Carozzi *et al.* (1993)
Strain 14-10		Col	Carozzi *et al.* (1993)
CryIVA	134	Dip	Ward and Ellar (1987)
CryIVB	128	Dip	Chunjatpornchai *et al.* (1988)
CryIVC	78	Dip	Thorne *et al.* (1986)
CryIVD	72	Dip	Donovan, Dankocsik and Gilbert (1988)
PS71M3-1	135	Dip	Sick (1991)
PS71M3-2	78	Dip	Sick (1991)
CryV[a]	81	Col,Lep	Tailor *et al.* (1992)
PS140E2[b]	78,70 and 35	Hym	Payne *et al.* (1992a)
PS86Q3[b]	58–155	Hym	Payne *et al.* (1992a)
PS211B2[b]	27–175	Hym	Payne *et al.* (1992a)
PS17a(CryV[a])	156	Nem	Schnepf *et al.* (1992)
PS17b(CryV[a])	146	Nem	Schnepf *et al.* (1992)
PS33F2(CryV[a])	142	Nem	Schnepf *et al.* (1992)
PS63B(CryV[a])	91	Nem	Schnepf *et al.* (1992)
PS52A1(CryVI)	54	Nem	Schnepf *et al.* (1992)
PS69D1(CryVI)	45	Nem	Schnepf *et al.* (1992)
PS81F	133	Protozoa	Thompson and Gaertner (1991)

[a] Two groups have used the CryIIIC name.
[a] Two groups have proposed using CryV to describe a new class of δ-endotoxins.
[b] The name CryIE(a) has been proposed for this gene.
Lep, Lepidoptera active; Col, Coleoptera active; Dip, Diptera active; Hym, Hymenoptera active; Nem, Nematode active. The Hymenoptera active strains produce a variety of inclusion proteins ranging from about 27 to 155 kDa. The activity of the individual proteins and precursor–product relationships are unknown at present.

to be a result of the limited testing these proteins undergo to identify their activity. Typically, only a few pests of commercial significance are used to determine the activity of new strains or proteins. It is possible that a given δ-endotoxin has a broader spectrum of activity than is known, but other target organisms simply have not been tested.

To be insecticidal, a δ-endotoxin must first be ingested by the insect and proteolytically activated to form an active toxin. Activation of the insecticidal crystal proteins is a multistep process. After ingestion, the crystals must first be solubilized in the insect gut. The crystals are typically insoluble at neutral pH and soluble at alkaline pH. Once solubilized, the δ-endotoxins are activated by specific proteolytic cleavages. The proteases in the insect gut can play a role in specificity by determining where the protoxin is cleaved. The activated toxins are typically quite stable in the presence of trypsin or other proteases. Once activated, the δ-endotoxins bind to specific proteins present on the surface of the gut epithelial cells. These binding proteins have been called receptors by some researchers, but it is not known whether they perform the function of a true receptor, so others prefer these proteins simply to be called binding proteins. The interaction of activated δ-endotoxins and the cellular binding proteins can be monitored using iodinated δ-endotoxin and purified brush border membrane vesicles (Hofmann *et al.*, 1988a,b),

Western blots of the brush border membrane vesicle proteins probed with activated δ-endotoxin (Oddou, Hartmann and Geiser, 1991), or can be visualized with immunosectioning techniques (Bravo *et al.*, 1992; Bravo, Jansens and Peferoen, 1992). In all cases examined, binding occurs whenever there is toxicity. In most, but not all cases, toxicity results when there is binding. After binding, the δ-endotoxin apparently inserts itself, or part of itself, into the cell membrane, creating a pore that ultimately results in the osmotic rupture of the cell. It appears that solubilization, activation and binding are steps which play roles in determining specificity of a δ-endotoxin. It is not known whether membrane insertion, or perhaps subsequent steps, also provide levels of specificity. Presently, it is simplest to conjecture that the largest part of specificity is determined by the binding properties of an activated δ-endotoxin, since this is one of the better characterized aspects of the chain of events that results in toxicity. There have been recent reviews dealing with the mode of action of these protein toxins (Gill, Cowles and Pietrantonio, 1992; English and Slatin, 1992), and the reader is directed elsewhere for a more detailed description of the current knowledge concerning the mode of action of the δ-endotoxins.

In certain cases, a δ-endotoxin that is capable of activity against a particular insect is not active because one or more of the above steps are not properly carried out in the gut. Solubilizing and activating δ-endotoxins prior to insect feeding can sometimes result in a broader spectrum of activity. For instance, CryIB is active against the European corn borer (ECB: *Ostrinia nubilalis*; Lepidoptera) but the full-length protein is not active against the Colorado potato beetle (CPB: *Leptinotarsa decemlineata*; Coleoptera). If it is solubilized and activated with proteases, it is still active against ECB, but it is now also active against CPB (Bradley *et al.*, 1992). The gut of CPB apparently does not solubilize and/or activate CryIB. The gut environment of CPB has a more neutral pH than the gut environment of lepidopterans and therefore the solubilization of CryIB may not occur in CPB.

The proteolytic activation of a solubilized δ-endotoxin can play a role in determining its specificity. The δ-endotoxin from *B. thuringiensis* var. *aizawa*, originally called the IC1 gene, has been classified as a *cryIA(b)* gene based upon its homology with other known *cryIA(b)* genes. However, the IC1 protein has activity against both lepidopteran and dipteran insects depending upon how it is activated (Haider, Knowles and Ellar, 1986). This δ-endotoxin is processed differently in the yellowfever mosquito (*Aedes aegypti*: Diptera) than it is in European cabbage worm (*Pieris brassicae*; Lepidoptera). In *Aedes* gut juice, a 53 kDa active δ-endotoxin is obtained, whereas in *Pieris* gut juice, a 55 kDa activated toxin is obtained. IC1 differs from the HD-1 CryIA(b) by only four amino acids, so gross changes in the binding region do not seem to account for the difference in activity. The different cleavages possibly allow the activated molecules to fold differently, thereby exposing different regions capable of binding to the insect gut. The choice of specificity of the cleavage, in this case, appears to reside with the gut proteases of the different insects. This raises the possibility that other δ-endotoxins may carry cryptic activities and that their spectrum of activity

can be broadened by proper activation. An example of this is a *cryIIIC* gene described by Lambert *et al.* (1992a). This gene was isolated from Bt strain BTS137J and encodes a 129 kDa protein which is not active against any known insect unless it is proteolytically cleaved to produce an activated molecule prior to feeding. The activated 72 kDa protein has activity against Colorado potato beetle. If one supplies the proper proteolytic activity, the δ-endotoxin becomes toxic to at least one insect.

It is unclear what determines the spectrum of binding of the δ-endotoxin to the brush border of epithelial cells once it is properly activated. Some δ-endotoxins, such as CryIA(b), are active against a number of insects, while others appear to be active against only one (known) insect. The precise reasons for this are unclear at present. One possibility is that a single toxin, once activated, is able to bind gut proteins in a number of insects because of multiple binding sites within the binding domain. Domain II, postulated to be the binding domain for CryIIIA and likely for other δ-endotoxins also (Li, Carroll and Ellar, 1991), appears to be quite large and could possess more than one binding site. The argument for multiple binding sites on a single molecule would seem to be supported by the observation that some δ-endotoxins are active against multiple insects, sometimes even insects in different orders. The differential activity against the two orders, in some cases, results from differential cleavage of the same toxin in the gut juices from the different insects. The size of the region of a δ-endotoxin required for binding to the insect gut cell binding protein is unclear. Ge, Shivarova and Dean (1989) identified a region of CryIA(a) important for binding to the gut of the silkworm (*Bombyx mori*; Lepidoptera). This region lies between amino acids 332 and 450 of the δ-endotoxin, which is within Domain II as defined by Li, Carroll and Ellar (1991). CryIIA, which is toxic to both the lepidopteran tobacco hornworm (*Manduca sexta*) and the yellowfever mosquito (*Aedes aegypti*; Diptera), is 87% homologous with CryIIB, which has activity against only *Manduca* (Widner and Whiteley, 1989). By generating and characterizing hybrids between these two proteins, Widner and Whiteley (1990) showed that specificity towards *Aedes* larvae could be localized in 76 amino acids in the region between residues 307 and 382 of CryIIA. These two proteins differ by only 18 amino acids in this region, making it appear that relatively small changes in the proper region can drastically alter the spectrum of activity of a particular δ-endotoxin. In this case, the authors ruled out alternative processing in this region of the protein as an explanation for differential activity and concluded that differential binding was the cause of the different activity of the two proteins.

There may be stringent requirements for a specific interaction between the activated δ-endotoxin and the gut cell receptor proteins prior to cell lysis. Such requirements could influence the spectrum of activity. The orientation of the bound toxin relative to the cell membrane or the distance of the bound toxin from the cell surface could influence the efficiency with which the δ-endotoxin can insert itself into the membrane. The insect gut cell receptor, or binding protein, also plays a role in the relative activity of a given δ-endotoxin against a particular insect. Van Rie *et al.* (1989) have shown that

the differential activities of CryIA(a), CryIA(b) and CryIA(c) against tobacco budworm (*Heliothis virescens*: Lepidoptera) are not due to differences in the binding affinity of the activated δ-endotoxins to the target cell, but instead are a reflection of the relative concentrations of the different receptors. That is, all three activated δ-endotoxins bind their respective receptors with about the same affinity, but the more active δ-endotoxin appeared to have a greater number of receptor sites and hence the target cells could bind more toxin molecules. In contrast, tobacco hornworm (*Manduca sexta*: Lepidoptera) brush border membrane vesicles bind all three toxins with about the same affinity and appear to have the same number of receptors for each of the three δ-endotoxins. As one might expect, all three δ-endotoxins are about equally active against *Manduca*. Thus, the number of receptor molecules on the target cells can at least influence the activity of a δ-endotoxin and perhaps, below a critical concentration, determine whether or not a given δ-endotoxin can be active at all. Presently, it is not known how many δ-endotoxin molecules are needed to bring about cell lysis.

Another possibility to account for activity of a given δ-endotoxin against insects in different orders is that the toxin-binding proteins in a number of different insects are structurally related and hence are capable of binding the same δ-endotoxin. If a δ-endotoxin bound to receptors in different insects using the same binding region, the receptors would have to be very similar, at least in the region bound by the δ-endotoxin. In the case of CryIIA, the receptor in both Lepidoptera and Diptera would have to be similar enough to bind the same protein, although it seems that changes in a few amino acids, and perhaps their influence on the folding of the protein, also play an important role in the toxic action of this δ-endotoxin. The search for the δ-endotoxin-binding protein(s) in the insect gut is currently an active area with a number of laboratories close to cloning an insect gut Bt δ-endotoxin receptor. Once some of these receptors are characterized in terms of their sequence, one will be able to determine if a δ-endotoxin that is active against a number of insects is active because of its ability to bind multiple sites or because its particular binding site is present on a number of different receptor molecules whose sequence is conserved among a number of different insects, perhaps even in different orders of insects. If the latter is actually the case, however, one might anticipate that the δ-endotoxins would have a broader spectrum of activity than that observed, since their conserved targets would be likely to be present in a number of insects, especially closely related insects. It is somewhat surprising that some δ-endotoxins are not, in fact, active against closely related species, supporting the argument that the δ-endotoxin-receptor interaction is quite specific.

The role of the δ-endotoxin receptor in the insect gut is unknown, but it is conjectured to play an important role in the insect. This is based on the observation that in one of the cases where an insect developed resistance to a given δ-endotoxin, the insect had an increased susceptibility to another δ-endotoxin. Van Rie *et al.* (1990a,b) demonstrated that resistance to CryIA(b) in the Indian meal moth (*Plodia interpunctella*: Lepidoptera) was accompanied by an increased sensitivity to CryIC. The decreased susceptibil-

ity to CryIA(b) was accompanied by a decreased binding of CryIA(b) to brush border membrane vesicles from the resistant insects. Increased susceptibility to CryIC was accompanied by an increase in the number of binding sites for this δ-endotoxin. CryIA(b) and CryIC did not compete for the same binding sites in these insects. Such compensation might indicate a critical role for the receptor protein in the insect gut. However, in the case of resistance to CryIA(b) in the diamondback moth (*Plutella xylostella*; Lepidoptera), there was a decreased binding of CryIA(b), but there was not a difference in the binding of, or susceptibility to, CryIB or CryIC (Ferre *et al.*, 1991).

Finding and engineering new activities

Use of *B. thuringiensis* δ-endotoxins for insect control is increasing. With increased use, there has been an increased effort in finding new strains with improved or novel activities. The last few years have seen a dramatic increase in the number of known strains and genes from Bt having insecticidal activities. Further, Bt strains and proteins with activities against other classes of organisms, such as nematodes, have also been discovered. The first coleopteran active strain was reported in 1983 by Krieg *et al.* There are now a number of known strains with coleopteran activity. This is the result of expanded screening efforts as well as expanded strain isolation efforts. There are various strategies for identifying strains with a novel activity, but each ultimately rests upon the relevant biological assay.

New strains can be isolated, all of which can be screened against the target pest(s). This is, however, a labour-intensive approach with many of the same strains, or known strains, being tested repeatedly. The source material for strain isolation can be random samples, such as soil or grain dust from storage elevators. There have been a number of strategies developed to try to enrich a strain population for the desired activity. One can try to use a directed screen based on ecological factors, such as habitat of the insect target or isolation of strains from diseased target insects. Such ecological methods of directing a strain search seem to be reasonable methods for reducing the number of strains one must investigate to find a desired activity, although such methods do not guarantee finding the desired activity. However, such searches have proved useful, especially in light of the fact that the first Bt strain was isolated from diseased silkworms by Ishiwata in 1901. Various other approaches to reduce screening efforts include the use of genes with the desired activity as hybridization probes to identify strains with the same or related genes (Visser, 1989) or using oligonucleotide probes from known genes (Prefontaine *et al.*, 1987); the use of monoclonal antibodies specific for various δ-endotoxins and also somewhat predictive of insecticidal activity (Hofte *et al.*, 1988); and the use of the PCR to identify strains carrying genes the same as or related to a given gene (Carozzi *et al.*, 1991). Such screens can identify strains carrying genes related to known genes and they can also identify strains carrying genes not related to currently characterized genes. By identifying strains not related to known strains, one may be able to enrich for strains carrying a novel activity.

While the traditional screening approach, with its multiple variations and refinements, continues to bear fruit, other approaches for producing δ-endotoxins with novel activities are also possible. As more knowledge is gained as to how the δ-endotoxins function, it becomes increasingly likely that one can engineer a δ-endotoxin to have a new activity. The silk moth (*Bombyx mori*; Lepidoptera) specificity domain from CryIA(a) has been moved to CryIA(c), thus imparting a new insecticidal activity to the resulting chimeric protein (Ge, Shivarova and Dean, 1989). The chimeric protein with *Bombyx* activity is also capable of binding *Bombyx* brush border membrane vesicles (Lee *et al.*, 1992). Similar chimeric proteins were constructed to better define the regions of CryIA(c) associated with determining specificity towards various insects (Ge, 1991). The region between amino acids 332–450 appears responsible for activity against cabbage looper (*Trichoplusia ni*; Lepidoptera), while the region between amino acids 335–615 was identified as the tobacco budworm (*Heliothis virescens*; Lepidoptera) specificity region. Transfer of amino acids 450–612 from CryIA(c) to CryIA(a) results, surprisingly, in a protein with about 30-fold more activity against *Heliothis* than either parental δ-endotoxin. How broadly such engineering of improved δ-endotoxins can be applied remains to be seen, since the rules governing such improvement remain to be elucidated. The region of CryIIA that allows it to be toxic towards mosquitoes has been identified by moving a 76 amino acid region to CryIIB (Widner and Whiteley, 1990). Li, Carroll and Ellar (1991) have resolved the structure of the CryIIIA molecule and have determined that it is composed of three distinct domains. As regions of the molecule responsible for binding to the insect gut receptors are better defined, it may be possible to alter them in a directed manner. Epitope mutagenesis (Scott and Smith, 1990) could be used to change the binding characteristics of a given δ-endotoxin and perhaps also alter its spectrum of activity. Such an approach would be dependent upon the development of good strategies to select the desired binding, since the molecules are quite large and the number of potential mutants too large to screen in a strictly random fashion. However, the ability to isolate brush border membrane vesicles from the desired target insect could facilitate the search for altered molecules able to bind the desired insect gut proteins.

Sivasubramanian and Federici (1991) have demonstrated that fusions between a δ-endotoxin and a viral-derived protein from *Autographa californica* multiple nuclear polyhedrosis virus (AcMNPV) can produce proteins with novel activity. In this case, the authors claim that altering the binding of the δ-endotoxin indeed alters its spectrum of activity. A fusion between the AcMNPV gp-64 protein and CryIIIA showed signs of activity against cabbage looper (*Trichoplusia ni*; Lepidoptera), as judged by a slight increase in mortality relative to a plasmid control where insects were fed *Escherichia coli* strains mised into their diet. This same fusion appeared to cause a decrease in weight gain when fed to tobacco budworm (*Heliothis virescens*; Lepidoptera) neonate larvae. The role of the receptor, or cellular 'toxin-binding protein', in this toxicity is unknown, so how broadly this type of engineering can be applied is uncertain. Further, such chimeric proteins appear to be unstable, so

significant efforts in engineering stable fusion proteins may have to be made. More of these fusions will have to be attempted to ascertain the usefulness of this approach as compared with the random screening, or directed screening, of naturally occurring δ-endotoxins for new activities.

The steps of toxicity after binding are largely unknown. It is not known whether binding of the δ-endotoxin to the receptor results in a configurational change in the δ-endotoxin. If so, this could present another level of specificity. One can envision a δ-endotoxin binding to a cell surface protein in such a manner that a required configurational change does not occur properly and thus subsequent steps for toxicity are not possible. It is also possible that a specific interation between the cell receptor and the δ-endotoxin produces such a configurational change in the δ-endotoxin and this interaction could influence specificity. If such configurational changes do result from binding, the ability to engineer δ-endotoxins to alter specificity will be a more difficult task, since any changes in the δ-endotoxin which alter its binding ability could also deleteriously alter its ability to change configuration in response to binding.

It is unclear at this stage whether the receptor plays a passive role in events of toxicity or whether it plays an active role. If it plays a passive role, any epithelial cell surface protein may be able to serve as a receptor for an engineered δ-endotoxin, providing configurational changes mentioned above do not play a significant role in toxicity. The receptor may simply serve as an anchor near the cell surface, which allows a close interaction between the toxic region of the δ-endotoxin and the cell membrane. If the receptors play an active role in toxicity, such as triggering a cellular response, then specific receptors would have to be targeted. This would increase the difficulty of engineering a new host specificity onto an existing δ-endotoxin. When the insect cell's receptors are cloned and characterized, this issue will become less ambiguous. Until then, selection of new binding targets will have to rely on a more random approach.

Other host organisms

MICROORGANISMS

The first Bt δ-endotoxin gene was cloned and expressed in *Escherichia coli* in 1981 by Schnepf and Whiteley. Since then, many 'alternate hosts' have been transformed with δ-endotoxin genes. Examples of the micro-organisms other than *E. coli* that have been used as δ-endotoxin gene recipients are listed in *Table 4*. This list of alternate δ-endotoxin hosts can be grouped into four main categories: those used primarily to clone, or characterize specific δ-endotoxin genes; those used as fermentation hosts to increase production; those used to deliver the δ-endotoxin more effectively to the target insect pest; and insect pathogens which use δ-endotoxins to improve their effectiveness. This section will emphasize alternate hosts which have the potential of delivering the δ-endotoxin more effectively to the target insect and insect pathogens which use the δ-endotoxins to improve their effectiveness.

Although Bt is an effective microbial pesticide, a number of biological constraints limit its use. The Bt δ-endotoxin is short-lived on crops, thereby reducing its residual activity, necessitating the need for many applications during a growing season. Bt, as with other spray-on pesticides, is difficult to deliver to insect species which burrow into their host plant, hide under leaves or live primarily under the soil surface. In the case of aquatic insect control, maintaining the δ-endotoxin in the water at the level of insect feeding is difficult. Many of the problems and limitations of conventional Bt-based products are being addressed by molecular biologists using recombinant DNA technology. The isolation of δ-endotoxins has enabled scientists to clone and characterize the genes encoding these molecules. Each δ-endotoxin is encoded by a single gene; therefore, it can 'easily' be transferred to other Bts or to organisms such as plants, bacteria, fungi and algae. In the future, genetic engineering can be expected to result in the development of strains with improved potency, longer residual activity and broader host range, as well as novel delivery systems for δ-endotoxins, allowing better targeting and environmental persistence.

ALTERNATE DELIVERY IN EPIPHYTES AND ENDOPHYTES

Genetic engineering technology allows the use of micro-organisms that multiply on (epiphyte) or in (endophyte) plants to produce insecticidal proteins. This approach can overcome problems of insufficient coverage and persistence while providing season-long control.

One of the first attempts to improve the delivery and potency of δ-endotoxins against soil insect species was the work of Watrud *et al.* (1985) and Obukowicz *et al.* (1986a,b). The authors introduced a δ-endotoxin gene from *B. thuringiensis* var. *kurstaki* HD-1 into two corn root-colonizing strains of *Pseudomonas fluorescens*, using transposase deletion derivatives of the transposon Tn5 containing the δ-endotoxin gene. Their objective was to clone the δ-endotoxin gene into non-pathogenic soil microbes which showed a high degree of association with corn roots. The types of commercial products

Table 4. Micro-organisms that have been used as alternative hosts for cloning *B. thuringiensis* δ-endotoxin genes a

Bacillus cereus (Gonzalez, Brown and Carlton, 1982; Aronson and Beckman, 1987)
Bacillus subtilis (Shivakumar *et al.*, 1986; Earp and Ellar, 1988)
Bacillus sphaericus (Bar *et al.*, 1991)
Bacillus megaterium (Donovan *et al.*, 1988; Mettus and Macaluso, 1990)
Pseudomonas fluorescens (Obukowicz *et al.*, 1986a,b)
Pseudomonas cepacia (Stock *et al.*, 1990)
Caulobacter crescentus (Thanabalu *et al.*, 1992)
Agmellenum quadruplicatum (Angsuthanasombat and Panyim, 1989; Murphy and Stevens, 1992)
Rhizobium meliloti (Bezdicek, Quinn and Kahn, 1991)
Rhizobium leguminosarum (Bezdicek, Quinn and Kahn, 1991)
Clavibacter xyli subsp. *cynodontis* (Turner *et al.*, 1991)
Insect baculoviruses (Martens *et al.*, 1990; Merryweather *et al.*, 1990; Pang, Frutos and Federici, 1992)

envisioned to result from this type of work are pesticidal inocula that are applied to seeds or soil at the time of planting. Subsequent colonization of roots could then conceivably supply protection against soil-borne insect pests and minimize the need for multiple applications of pesticide during the growing season of the crop.

Since the practical application of this technology involved the release of a living recombinant bacteria into the field, many questions were raised as to its ecological impact. The general guidelines of the Federal Insecticide Fungicide Rodenticide Act were followed in assessing the ecological risk associated with releasing a living recombinant micro-organism. A number of studies were carried out to identify and monitor the engineered pseudomonad in the environment. Studies were designed to assess the genetic stability and likelihood of genetic exchange, in addition to the toxicity towards non-target species.

A native, genetically marked parental strain and its engineered derivative containing a chromosomal copy of the δ-endotoxin gene exhibited essentially identical survival profiles on the corn rhizoplane under growth chamber conditions. In those experiments, corn seeds had been treated with inocula and planted in non-sterile soil. The engineered and native isolates also showed virtually identical rapid decline survival profiles in sewage and surface water samples from various rivers and lakes. The only time persistence of the inocula was demonstrated was when water samples were filter sterilized prior to the addition of test inocula, suggesting that natural competitors, predators or parasites of microbes play an active role in limiting the persistence of introduced bacterial inocula. The case for genetic stability of the engineered pseudomonad and for lack of genetic exchange with other micro-organisms was based on the fact that the δ-endotoxin gene was inserted into the chromosome of *P. fluorescens* rather than being present on a plasmid. Chromosomal insertion of δ-endotoxin genes was preferred over plasmid insertion because chromosomal DNA is inherently more stable than plasmids and the *P. fluorescens* strain lacks indigenous plasmids and has demonstrated a limited capacity to accept plasmids from broad host-range incompatibility groups. The authors also postulated that if a plasmid could be introduced naturally into these isolates, the probability of transfer of the engineered trait from the chromosome to a second microbe would be very remote. Under optimal laboratory conditions, the estimated frequency of this transfer was 10^{-9}.

Toxicity assessments that were performed for engineered and native isolates of *P. fluorescens* included studies in non-target organisms such as mice, quail, fish, honeybees, earthworms and *Daphnia* spp. With the exception of target lepidopteran species such as *Manduca*, *Trichoplusia*, *Heliothis* and *Agrotis*, none of the studies demonstrated toxic effects resulting from the microbial treatments. The host range and relative pesticidal efficacy of the engineered *P. fluorescens* paralleled those of *B. thurinigiensis* var. *kurstaki*. Although initial results from this work appeared promising, anticipated field releases met with considerable opposition at the time, presumably due to the fact that the biocontrol product was a living recombinant organism.

Waalwijk, Dullemans and Maat (1991) developed another delivery system based on a grass root colonizing *Pseudomonas* to aid in the control of the root feeding dipteran *Tipula oleracea*. They cloned the cryIVB gene from *B. thuringiensis* var. *morrisoni* PG-14 and inserted it into *P. fluorescens* P1. To reduce the mobility of the introduced gene, Waalwijk *et al.* developed suicide vectors where integration is dependent upon recombination between homologous DNA sequences. These suicide vectors offer an improvement over the *Pseudomonas* constructs used by Obukowicz *et al.* (1986a,b) described above, because the Obukowicz strains would still allow mobilization of inserted genes by transposases provided by incoming plasmids. Waalwijk *et al.* tested 27 *P. fluorescens* isolates and found only one that hybridized with the recombinant DNA from strain P1. They suggested this indicated that the sequences flanking the *cryIVB* gene are relatively specific to the P1 strain and the probability that transgenic P1 cells would encounter these sequences in the field would be rather remote.

Stock *et al.* (1990) chose a strain of *Pseudomonas cepacia* (Pc) isolated from soil as a means of delivering a CryIA(c) protein to target insects. *Pseudomonas cepacia* 526 was found to colonize a variety of plant roots and leaves. The *cryIA(c)* gene from *B. thuringiensis* var. *kurstaki* HD-1 was cloned into a broad host range vector and the resulting plasmid conjugated into Pc526. Southern blot analysis confirmed the presence of the *cryIA(c)* gene in the transconjugants. The authors also noted that the Pc 526 colonies carrying the *cryIA(c)* gene were smaller than the wild-type Pc colonies and found that the Pc 526-Bt strain grew slower in liquid culture with a doubling time 2.2 to 2.5-fold longer than the wild-type Pc. They suggested that the introduction of the *cryIA(c)* gene had deleterious effects on Pc bacterial growth.

Western blot analysis demonstrated that the Pc 526-Bt strain produced a 78 kDa truncated version of the CryIA(c) protein that reacted weakly with HD-1 specific antiserum. The engineered strain was active against tobacco hornworm (*Manduca sexta*; Lepidoptera) at a concentration of 10^5–10^6 bacteria cm^{-2}. The establishment of Pc 526-Bt and its insecticidal activity were determined on axenically grown tobacco plants. The stability of the plasmid carrying the *cryIA(c)* gene was monitored because laboratory results suggested that the plasmid was unstable under non-selective conditions. Three days after tobacco leaves were inoculated with Pc 526-Bt and Pc 526, population estimates suggested that 10^8 bacteria per gram fresh weight remained and that the majority of that population was Pc 526 wild-type. The plasmid carrying the *cryIA(c)* gene was found in only 1.0% of the Pc 526-Bt population, confirming the observation that this plasmid is unstable under non-selective conditions. However, the proportion of Pc 526-Bt population expressing the *cryIA(c)* gene was large enough to protect the tobacco plants from *Manduca* feeding damage. Most larvae on plants treated with the Pc 526-Bt were killed, whereas most on the control plants survived.

These experiments estimate the number of leaf-colonizing Pc that would be required to protect a tobacco plant from *Manduca* feeding damage. However, since these data were generated on axenically grown tobacco, they may not

reflect the true field environment, where competition with other bacteria would be present. Further experiments in greenhouse environments and actual outdoor field trials will help address the question.

The plant endophyte *Clavibacter xyli* subsp. *cynodontis (Cxc)* was engineered to express a δ-endotoxin and subsequently introduced into corn plants (Dimock, Beach and Carlton, 1989; Kostka, Reeser and Miller, 1988) to protect against European corn borer (*Ostrinia nubilalis*; Lepidoptera). This work focused on genetically altering *Cxc* so that it contains the new DNA inserted into its chromosome. The inserted DNA contains the *cryIA(c)* gene from *B. thuringiensis* var. *kurstaki* HD-73 fused to a gene that confers kanamycin resistance, a tetracycline resistance gene, and an *E. coli* replicon that is not functional in the new host. The wild-type strain of *Cxc* described by Davis *et al.* (1984) is frequently found in nature colonizing the vascular system of Bermuda grass. In addition to Bermuda grass, the wild-type and engineered *Cxc* have been shown to colonize several important crop plants including maize (Kostka, Reeser and Miller, 1988). This organism is one of the only alternative hosts for Bt δ-endotoxins which has been approved for field testing.

Another example of using an endophyte to deliver a δ-endotoxin to soil-inhabiting insects is the work of Bezdicek, Quinn and Kahn (1991). They introduced the *cryIIIA* gene from *B. thuringiensis* var. *tenebrionis* into *Rhizobium meliloti* and *R. leguminosarum* BV. *vicaea* to protect alfalfa and pea from the feeding damage of two coleopteran insects, the clover root curculio (*Sitona hispidulus*) and the pea leaf weevil (*Sitona lineatus*), respectively. Two *cryIIIA* constructs were made that have NH_2-terminus-truncated forms of CryIIIA in the broad host range vector pRK311 and differ only in the promoters that drive expression of the *cryIIIA* gene. In one, expression relies on the lacZ promoter, which is expressed by *Rhizobium in vitro* and *in planta*, whereas the other construct uses the nifH promoter, which is active only when the *Rhizobium* are fixing nitrogen inside nodules. Western blot analysis showed that CryIIIA protein was produced *in vitro* and in nodules. Bioassays indicated that the CryIIIA protein expressed in rhizobia and nodules was toxic to Colorado potato beetle when fed recombinant *Rhizobium*-treated potato leaves and to larvae of *S. hispidulus* and *S. lineatus* feeding on alfalfa and pea nodules, respectively, containing the recombinant *Rhizobia*.

Other workers (Barnes and Cummings, 1987) have developed a different approach to using a pseudomonad as an alternative δ-endotoxin host. In an attempt to improve the foliar persistence of Bt insecticidal activity, they developed a delivery system based on a recombinant micro-organism that expresses a δ-endotoxin, but has been killed using heat and chemical treatment prior to field release. Because the organisms are dead, this product has the additional advantage of relative freedom from the environmental and safety concerns associated with outdoor testing or the release of living recombinant organisms. Using this system, researchers have produced lepidopteran active and coleopteran active δ-endotoxins in a non-pathogenic strain of *Pseudomonas fluorescens*. When transformed cells are grown in

submerged culture, the δ-endotoxin forms a typical δ-endotoxin-like crystal within the cell. However, unlike *Bacillus* cells, which normally burst at the end of the growth cycle releasing naked spores and crystals into the culture medium, the δ-endotoxin-producing *Pseudomonas* cells remain intact at the completion of the fermentation cycle. While still in the fermentation tank, the cells are chemically treated to kill the *Pseudomonas* cells and cause the bacterial cell wall to become more rigid through cross-linking of the cell wall components. The dead bacterial cell wall now serves as a protective 'micro-capsule' for the enclosed δ-endotoxin. Because this bacterium produces no spores, it avoids the release of living bacteria when the product is applied to crop plants in the field. In part due to the lack of viable spores and DNA, this delivery system should be particularly useful for recombinant δ-endotoxins.

In 1985, the EPA approved testing of this encapsulated product on small fields with a lepidopteran active δ-endotoxin, making it the first recombinant product approved for outdoor testing. Field experiments conducted during 1988 suggested that a foliar application of this engineered microbe protected cabbage from lepidopteran pests for 7 days, whereas insecticidal activity from traditional Bt sprays dissipated by 96 h post-application. This increase in persistence resulted in better insect control, and therefore higher yields. Similar tests have been conducted with engineered microbes targeted against the Colorado potato beetle (CPB) on potatoes. Results from these tests confirm that when equivalent toxin rates of the engineered microbe and its naturally occurring counterpart, *B. thuringiensis* var. *san diego*, are applied to potato foliage, the persistence conferred by the microbially encapsulated product resulted in superior CPB control. It was also shown that the protected δ-endotoxin could be applied at two-thirds the toxin rate and still maintain higher levels of CPB control than the full rate delivered by the *B. thuringiensis* var. *San diego* product.

The microbial encapsulation delivery system is said to offer a number of advantages over traditional Bt products (Gaertner, 1990; Gelernter, 1990): the longevity of insect control may decrease the number of applications, and therefore the cost, required for season-long insect control; a two-fold increase in foliar persistence results in better insect control, when applied at the same rate and same application schedule as naturally occurring Bts; higher persistence levels may allow reductions in the recommended field rate, while still maintaining excellent levels of insecticidal activity; and product shelf life is enhanced by the absence of living organisms in these preparations.

ALTERNATE HOSTS AS CONTROL AGENTS FOR AQUATIC INSECTS

One of the major factors limiting the duration of mosquito control following the application of Bt strains is the rapid sedimentation of spores and insecticidal crystals. Since most mosquito larvae feed at or near the water surface, the effective larvicidal activity of the toxins is limited to the short period of time prior to sedimentation. A potential approach to circumvent these problems is to engineer micro-organisms living in the upper layers of aquatic habitats to synthesize toxic proteins. Several workers have recognized

this and have transferred δ-endotoxin genes from *B. thuringiensis* var. *israelensis* into various aquatic insect food sources.

Thanabalu *et al.* (1992) cloned the 130 kDa (*cryIV*) toxin gene from *B. thuringiensis* var. *israelensis* into the broad host range plasmid pRK248 and expressed it in *Caulobacter crescentus* CB15. The recombinant *C. crescentus* cells were shown to be toxic to mosquito larvae. *Caulobacter* species are ubiquitous micro-organisms residing in the upper regions of aquatic environments. In the flagellate swarmer stage, *Caulobacter* species are motile, thus allowing distribution through the habitat. In the swarmer and stalked stages, they are capable of attachment to solid particles at or near the water surface. Thus *Caulobacter* species provide the potential for prolonged control by maintaining mosquitocidal toxins in larval feeding zones.

Angsuthanasombat and Panyim (1989) introduced the 130 kDa (*cryIV*) δ-endotoxin gene from *B. thuringiensis* var. *israelensis* into the cyanobacterium *Agmenellum quadruplicatum* (Aq) PR-6 by plasmid transformation. Lysates produced by sonication of recombinant Aq cells were shown to be toxic to mosquito larvae, but larvicidal activity of intact recombinant cyanobacteria was not demonstrated. The larvicidal activity of *E. coli* containing pAQPe388i was more effective than that of its Aq PR-6 counterpart, presumably due to the *E. coli* expressing higher amounts of undergraded δ-endotoxin.

INSECT BACULOVIRUSES AS ALTERNATE HOSTS

Baculoviruses are naturally occurring pathogens that infect some important lepidopteran and hymenopteran insects and are used in the control of these insects. A major drawback for a more widespread use of these viruses is their relatively slow insecticidal action. Baculoviruses produce a virion-occluding protein called polyhedrin late in the development of the virus. These viruses are capable of expressing foreign genes under the control of the polyhedrin promoter (Luckow and Summers, 1988). The polyhedrin gene is dispensable for virus replication, and the expression of foreign genes is based on the allelic replacement of the polyhedrin gene (Smith, Fraser and Summers, 1983). Genetically engineered variants of baculoviruses incorporating δ-endotoxin genes have been developed and are currently being evaluated to see if they are more effective control agents (Martens *et al.*, 1990; Merryweather *et al.*, 1990; Pang, Frutos and Federici, 1992).

Martens *et al.* (1990) introduced a *cryIA(b)* gene, isolated from *B. thuringiensis* var. *aizawai* 7.21, into the genome of the *Autographa californica* nuclear polyhedrosis virus (AcNPV). A transfer vector containing the β-galactosidase marker gene facilitated the screening of recombinants. All the β-galactosidase positive recombinant viruses analysed contained an insertion of the *cryIA(b)* gene. Recombinants were easily retrieved from infected *Spodoptera frugiperda* (Lepidoptera) cells, suggesting that the CryIA(b) δ-endotoxin is not toxic when produced within the cell. *Spodoptera* cells infected with recombinant ACNPV expressed the CryIA(b) protein at 5% of the total cell protein. CryIA(b) was found predominantly in the cytoplasm of

infected cells as large, crystalline inclusions, sometimes bipyramidal in shape. The ultrastructure is similar to that of native Bt δ-endotoxins. Although these crystalline inclusions have been shown in organisms other than native Bt strains (Shivakumar *et al.*, 1986; Oeda *et al.*, 1989), the observation of Martens *et al.* (1990) is the first report of crystal formation occurring in eukaryotic cells. The toxicity of CryIA(b) δ-endotoxin expressed by AcNPV recombinants was comparable with that of CryIA(b) expressed by a corresponding *E. coli* recombinant when tested against the European cabbageworm (*Pieris brassicae*; Lepidoptera).

Merryweather *et al.* (1990) inserted the *cryIA(c)* gene from *B. thuringiensis* var. *kurstaki* HD-73 into the genome of the baculovirus *Autographa californica* using two transfer vector systems. In the first, the δ-endotoxin gene was placed under the control of the polyhedrin gene promoter, thus giving a polyhedrin-negative recombinant virus, Ac(PH^-)Bt. In the second, the δ-endotoxin gene was inserted under the control of a copy of the AcMNPV p10 promoter positioned upstream of the polyhedrin gene to produce a polyhedrin-positive recombinant virus, Ac(PH^+)Bt. An analysis of the cells infected with each recombinant virus demonstrated that a large polypeptide of about 130 kDa was synthesized, which reacted with antiserum specific for the Bt HD-73 δ-endotoxin. Two additional peptides of 62 kDa and 44 kDa were also detected. The authors speculate that these smaller peptides were degradation products of the larger 130 kDa protein. The peak time of synthesis for the 130 kDa protein in insect cells was about 18 h post-infection.

When larvae of the cabbage looper (*Trichoplusia ni*, Lepidoptera) were exposed to a diet treated with cell extracts or virus particles from recombinant virus infected cells, the larvae refused to eat. This was attributed to the presence of δ-endotoxin, since feeding inhibition is a well-known characteristic of insects that ingest natural Bt δ-endotoxins. The Ac(PH^+)Bt virus had an LD_{50} value about two-fold higher than that of the unmodified AcMNPV. The authors contend that this was within known experimental variation of AcMNPV viruses and that the recombinant virus and the wild type were essentially of equal toxicity. They also suggested that trace amounts of δ-endotoxin in the virus preparation used in the bioassays may have affected the infection of cells in the midgut by the virus. Clearly more work needs to be done before any conclusions regarding the utility of these constructs are made.

A recombinant baculovirus containing a mosquitocidal *cryIVD* gene from *B. thuringiensis* var. *morrisoni* (Pg-14) fused with the lacZ gene from *E. coli* under control of the polyhedrin promoter was constructed by Pang, Frutos and Federici (1992). Strong expression of the chimeric protein was obtained in infected cells and in *Trichoplushia* larvae. In both, crystalline cuboidal inclusions were formed in the cell cytoplasm during late stages of infection. These inclusions were purified and shown by Western blot analysis to contain a protein that reacted with antibody raised against the CryIVD δ-endotoxin from *B. thuringiensis* var. *israelensis*. This chimeric protein, however, was not active against mosquito larvae.

The large protein capsules (polyhedra) containing the virions are the

infectious stages of baculoviruses as well as the most environmentally stable form of the virus. The application of polyhedra-negative viruses in the field is impractical, since naked virions are quickly inactivated (Bishop, 1989). Thus, recombinant viruses that have maintained the polyhedrin gene and therefore produce polyhedra are more desirable. Recombinants that have a δ-endotoxin gene inserted in another locus under the control of a promoter other than the polyhedrin promoter, for example the p10 gene, may be of more practical significance. The fact that δ-endotoxins produced in recombinant baculovirus-infected cells are biologically active provides a basis for a strategy in which Bt δ-endotoxins may be used to enhance the effectiveness of baculoviruses as microbial control agents.

Finally, an interesting approach to controlling Colorado potato beetle (CPB: *Leptinotarsa decemlineata*; Coleoptera) using an alternate host for δ-endotoxins is suggested by Bove and Hackett (1992). They propose engineering a δ-endotoxin into a spiroplasma (helical, wall-less bacteria in the Class Mollicutes) that is a commensal gut organism of CPB. Since no transformation system exists for introducing δ-endotoxin genes into the spiroplasma genome, their initial objectives are to develop such a system using either a native spiroplasma virus, such as SpV1 or *E. coli* plasmids. An *E. coli* plasmid containing a streptococcal transposon (Tn916) has already been used successfully as a vector system for *Mycoplasma* spp. (Dybvig and Cassell, 1987).

Other suitable hosts for expressing δ-endotoxins may be found in the groups of entomopathogenic fungi, protozoa and nematodes. Studies of the genetics and molecular biology of these groups, however, are in the very early stages of development and it is not likely that reports of inserting Bt genes into these organisms will appear in the near future.

Naturally occurring insect pathogens, including bacteria, fungi and viruses, occupy environmental niches in which they function as pest control agents; however, in most cases, this attribute cannot be readily adapted to crop protection. Clearly, there are major factors constraining the wider use of microbial pesticides. These include lack of environmental persistence, narrow host range, limited virulence and high production costs. Recombinant DNA technology will provide the tools for developing safe, efficient and cost-effective microbial control agents. It is now possible to combine the best traits of several different organisms into a single strain, precluding the need for complex mixtures of micro-organisms expressing δ-endotoxins which exhibit enhanced insecticidal activity and greater persistence in the environment.

Production of *Bacillus thuringiensis* insecticidal proteins in plants

The long history of efficacy and safety as microbial insecticides has placed the δ-endotoxins among the first genes of commercial interest to be engineered into plants. Since the δ-endotoxin is the product of a single gene, it is an ideal candidate for use in the rapidly progressing field of plant genetic engineering. Over the past decade, various Bt microbial sprays have been used to control insects on important crops such as corn, cotton, tomato and potato. Such

crops have the most to benefit from acquiring resistance to insect attack through genetic engineering. Insecticidal sprays have inherent disadvantages that make production of δ-endotoxins in plants attractive: farmers must spray their crops several times a season; the insecticide may be washed off by rains; and short residual time on the leaf, believed to be due to various environmental factors such as temperature, leaf pH and UV inactivation, may lead to efficacy problems. The creation of transgenic plants which effectively utilize the insecticidal potential of Bt was initially encumbered by technical difficulties. Over the past few years, however, a combination of advances in plant transformation and gene expression has made the production of effective transgenic Bt plants a viable possibility. It is expected that cotton, potato and corn varieties containing a Bt δ-endotoxin gene will be marketed within the next few years and varieties of genetically transformed vegetable crops containing a δ-endotoxin gene will follow not long after.

At present, the majority of transgenic crops containing Bt genes have been transformed with *cryI* genes which have activity against the lepidopterans, including the tobacco hornworm, tobacco budworm, tomato pinworm, corn earworm and European corn borer. More recently, the *cryIIIA* gene has been engineered into plants for protection against the coleopterans, including the Colorado potato beetle (Perlak *et al.*, 1993). Bt δ-endotoxin genes with activity against insects of the genus *Diabrotica*, such as corn rootworm, a major corn pest, will likely be produced in the next generation of transgenic plants.

First-generation transgenic Bt plants

The first published accounts of transgenic plants containing the Bt δ-endotoxin gene include Vaeck *et al.* (1987), Barton, Whiteley and Yang (1987), Adang *et al.* (1987), and Fischhoff *et al.* (1987). The first three groups published experiments using transgenic tobacco, whereas Fischhoff *et al.* published results on transgenic tomato transformed using *Agrobacterium*-mediated T-DNA transfer. Vaeck *et al.* used the 2′ T-DNA mannopine synthetase promoter and 3′ polyadenylation region of T-DNA gene 7 to drive the expression of the *cryIA(b)* gene from *B. thuringiensis* var. *berliner* 1715. The constructs tested in tobacco included the full-length gene coding for a 1155 amino acid protein, a fragment of the *cryIA(b)* gene coding for 610 NH_2-terminal amino acids which had previously been shown to be fully insecticidal (Hofte *et al.*, 1986), and two constructs with translational fusions between NH_2-terminal fragments of CryIA(b) and the neomycin phosphotransferase (neo) gene from transposon Tn5, which confers kanamycin resistance to plants. The authors anticipated that δ-endotoxin-neo fusion transformants selected on high levels of kanamycin would aid in the selection of transformants containing high levels of δ-endotoxin. Transgenic tobacco plants were obtained by leaf disk transformation of *Nicotiana tabacum* var. Petit Havana SR1. Tobacco transformed with the δ-endotoxin-neo fusions and the NH_2-terminal truncated δ-endotoxin gene constructs produced 75–100% mortality against tobacco hornworm (*Manduca sexta*; Lepidoptera)

larvae. The transformants containing the translational fusion constructs produced at a high frequency plants exhibiting 70–100% mortality against *Manduca*, whereas the unfused truncated endotoxin gene gave similar levels of toxicity but in a smaller percentage of transformants. This work also showed that plants transformed with the full-length endotoxin gene produced very little δ-endotoxin protein and were non-insecticidal. The levels of δ-endotoxin expressed in the highly insecticidal plants ranged from 2.6 to 190 ng CryIA(b) per mg soluble protein, or 0.0002–0.02% of total soluble protein. Further analysis of transformed tobacco showed that the plants expressing the highest levels of CryIA(b) contained five copies of the gene. Normal inheritance patterns of the Bt gene were confirmed in progeny plants (Vaeck, Reynaerts and Hofte, 1989).

Barton, Whiteley and Yang (1987) studied the expression of a NH_2-terminal fragment of the *cryIA(a)* gene from *B. thuringiensis* var. *kurstaki* HD-1 in transgenic tobacco. Transformants were obtained in *Nicotiana tabacum* cv. Havana 425 using an *Agrobacterium tumefaciens* binary vector system. They obtained insecticidal plants using constructs with either a full-length δ-endotoxin gene or a truncated δ-endotoxin gene encoding a 644 amino acid protein. Both δ-endotoxin genes were driven by a 425 bp cauliflower mosaic virus (CaMV) 35S promoter sequence, including the 5′ untranslated region of AMV RNA 4 and the nopaline synthetase 3′ polyadenlyation region. Tobacco calli expressing the full-length *cryIA(a)* gene at levels of 10–50 ng Bt per mg soluble protein became necrotic and died. None of the plants which were regenerated from the calli transformed with the full-length gene produced detectable levels of CryIA(a), mRNA or insecticidal activity, and many plants were found to contain deleted or broken *cryIA(a)* genes. Transgenic tobacco plants exhibiting the highest levels of toxicity contained 3–5 copies of the Bt gene. Northern blot analysis of transgenic plants showed hybridization of a δ-endotoxin-specific probe to a mRNA species shorter than the expected full-length transcript and also significant hybridization to distinct shorter fragments. The existence of fragmented Bt mRNA in transgenic plants was attributed to inefficient post-transcriptional processing or rapid turnover.

Adang *et al.* (1987) generated transgenic tobacco, *Nicotiana tabacum* cv. Xanthi, containing the full-length *cryIA(c)* gene from *B. thuringiensis* var. *kurstaki* HD-73 using a binary vector system and *Agrobacterium tumefaciens*. The 3.7 kb *cryIA(c)* gene was under control of the promoter and polyadenylation sequences of the T-DNA gene encoding the first step of mannopine synthesis (ORF 24). Northern blot analysis of transgenic plant RNA identified a 1.7 kb RNA corresponding to the 3′ end of the *cryIA* gene. At least one plant was identified with low levels of CryIA(c) protein (2 ng per mg soluble protein) and some degree of toxicity (53% mortality) towards tobacco hornworm (*Manduca sexta*; Lepidoptera).

Fischhoff *et al.* (1987) published the first transformation of tomato (*Lycopersicon esculentum* line VF36) via *Agrobacterium*-mediated T-DNA gene transfer with the *cryIA(b)* gene from *B. thuringiensis* var. *kurstaki* HD-1. Two truncated versions of the Bt gene were used: one encoded a protein of

646 amino acids and the other a protein of 725 amino acids. Each version was driven by the CaMV 35S promoter and the 3′ polyadenylation region from the nopaline synthetase gene. The findings of Vaeck *et al.* (1987) were strengthened by this study, which demonstrated that expression of the NH_2-terminal fragment of a δ-endotoxin produced plants with a high level of activity against tobacco hornworm (*Manduca sexta*), whereas plants containing a full-length δ-endotoxin gene did not confer 100% insecticidal activity. The study showed significant mortality and severe growth inhibition of *Heliocoverpa zea* (Lepidoptera) and *Heliothis virescens* (Lepidoptera) populations, which require higher levels of Bt δ-endotoxin for mortality. Progeny plants demonstrated the heritability patterns of a typical Mendelian dominant gene.

Transformation of dicots using *Agrobacterium* T-DNA was very successful in producing engineered plants containing Bt genes. There are published reports of δ-endotoxin expressing transgenic potato (Vaeck, Reynaerts and Hofte, 1989), cotton (Perlak *et al.*, 1990), tobacco and tomato (Vaeck, Reynaerts and Hofte, 1989; Honee, van der Salm and Visser, 1989). In 1989, Vaeck, Reynaerts and Hofte published results of studies on the transformation of tomato and potato plants with a portion of the *cryIA(b)* gene encoding the NH_2 terminal portion of the δ-endotoxin. Transgenic tomato plants exhibiting 100% mortality against tobacco hornworm (*Manduca sexta*) produced 60–80 ng CryIA(b) per gram of leaf tissue and potato plants with similar activity produced 90–150 ng CryIA(b) per gram of leaf tissue. In 1992, Jansens *et al.* demonstrated significant, although not complete, control of the American bollworm (*Helicoverpa armigera*; Lepidoptera) with transgenic tomatoes expressing a truncated CryIA(b) δ-endotoxin.

At least seven research groups had conducted transgenic field trials using plants expressing a δ-endotoxin gene by the end of 1990 (Goldberg and Tjaden, 1990), but the majority of results were not published. The first published report on the field performance of transgenic tomato plants expressing a δ-endotoxin gene was published in 1989 by Delannay *et al.* The authors demonstrated that transgenic tomato plants expressing the *cryIA(b)* gene were insecticidal against tobacco hornworm (*Manduca sexta*), but that higher levels of CryIA(b) expression would need to be achieved to fully control the agronomically important pests such as the tomato fruitworm (*Heliocoverpa zea*; Lepidoptera) and tomato pinworm (*Keiferia lycopersicella*; Lepidoptera). Their plants were homozygous progeny of the best T_0 plants in terms of expression of the transformed truncated *cryIA(b)* gene encoding a 725 amino acid protein derived from *B. thuringiensis* var. *kurstaki* HD-1.

The results of three Bt field trials were published in 1992 concerning transgenic tobacco (Warren *et al.*, 1992; Carozzi *et al.*, 1992; Hoffmann *et al.*, 1992) and cotton (Wilson *et al.*, 1992) using truncated δ-endotoxin genes. The test described by Warren *et al.* (1992) and Carozzi *et al.* (1992) consisted of six transgenic tobacco lines expressing a 645 amino acid truncated CryIA(b) protein from *B. thuringiensis* var. *kurstaki* HD-1 inserted at a single locus in the tobacco genome. The site of insertion contained a single copy of the

truncated *cryIA(b)* gene. This gene was under control of the CaMV 35S promoter and 35S 3′ polyadenylation sequences. Transgenic plants were generated using *Agrobacterium*-mediated leaf disk transformation of *Nicotiana tabacum* var. Havana 38. Both homozygous and hemizygous plants of each line were tested. Five of the six CryIA(b) lines tested provided economic control against both tobacco hornworm (*Manduca sexta*; Lepidoptera) and tobacco budworm (*Heliothis virescens*; Lepidoptera), an insect that is 40-fold less sensitive than *Manduca* to the CryIA(b) protein. During the course of a 14 week field test under extremely high insect pressure, it was demonstrated that hemizygous plants can express sufficient levels of CryIA(b) δ-endotoxin to protect tobacco against economic damage from tobacco hornworm and tobacco budworm. The authors observed that Bt δ-endotoxin levels increased throughout the course of plant development, with a substantial increase at the time of flowering. Levels at flowering ranged from 400 to 1000 ng CryIA(b) per gram fresh weight or up to 0.01% total soluble protein. Bt δ-endotoxin mRNA of the expected size was readily detected, but there were also distinct truncated RNA forms, perhaps resulting from incomplete transcripts or cleavage products.

Hoffmann *et al.* (1992) also reported on the field performance of transgenic tobacco plants (*Nicotiana tabacum* var. Xanthi) containing a translational fusion of the *cryIA(c)* gene with the neo gene (kanamycin resistance) from transposon Tn5. Transgenic plants contained a truncated *cryIA(c)* gene derived from *B. thuringiensis* var. *Kurstaki* HD-73 encoding a 612 amino acid NH_2-terminal fragment, with the translational fusion protein under control of the CaMV 35S promoter and the 3′ region of the tomato protease inhibitor I gene. The data from this study demonstrated control against artificial infestations of tomato fruitworm (*Heliocoverpa zea*; Lepidoptera). This *cryIA(c)*-neo fusion has also been transformed in potato, *Solanum tuberosum*, using an *Agrobacterium tumefaciens* binary vector system (Cheng *et al.*, 1992). Transgenic potato plants showed some activity against *Manduca sexta*, primarily limited to a reduction in feeding.

The results of the field tests of the first-generation transgenic tobacco plants were encouraging, but the studies concurred in concluding that the expression levels of Bt in plants were relatively low and mRNA analysis demonstrated the presence of substantial amounts of truncated transcripts. Clearly, the expression levels of Bt in crops such as tomato, cotton and corn would not be high enough to protect totally plants from the major pests of agronomic importance. Modifications to the gene and/or regulatory sequences were required to enhance expression.

Murray *et al.* (1991) examined the expression of *cryIA(b)*, *cryIA(c)* and *cryIIIA* genes in both transgenic tobacco and electroporated carrot protoplasts to study mRNA stability. The study examined full-length and truncated *cryIA(b)* and *cryIA(c)* genes from *B. thuringiensis* var. *kurstaki* and a full-length *cryIIIA* gene from *B. thuringiensis* var. *tenebrionis*. The *cryIA(b)* gene was under control of the CaMV 35S promoter and 3′ polyadenylation sequences from ORF 26 of the TR-DNA and the *cryIA(c)* gene was under control of the mannopine synthetase promoter. Northern analysis of

δ-endotoxin mRNA isolated from transgenic tobacco plants transformed with either full-length or truncated genes from both *cryIA(b)* and *cryIA(c)* showed hybridization only to truncated transcripts. Codon usage in the native δ-endotoxin genes is considerably different from that found in typical plant genes, which have a higher G + C content. Truncated δ-endotoxin gene transcripts in transgenic plants could result from a number of events relating to the high A + T content of these genes. These include premature transcriptional termination or polyadenylation in regions of high A + T content or inappropriate splicing or cleavage at sites recognized by the plant as splice sites. Instability of the mRNA could be the result of endonucleolytic or exonucleolytic degradation at specific sequences that destabilize the message during transcription or create pausing due to the formation of secondary structures. Instability of the mRNA could also be the result of inefficient translation due to codon usage differences between the Bt δ-endotoxin gene and plant genes. Northern blot analysis of *cryIA(b)* and *cryIA(c)* constructs electroporated into carrot protoplasts showed full-length non-degraded forms during the first 8 h after electroporation, but by 18 h the *cryIA* mRNA was degraded. The results of these transient expression experiments led the authors to conclude that the truncated transcripts observed in plants were the result of message instability rather than truncation due to premature transcriptional termination or polyadenylation. Using a series of 3′ deletion constructs in electroporated carrot cells, they demonstrated that deletion of sequences in the 3′ end of the *cryIA(b)* did not increase message stability, and the RNA instability was retained in the first 570 bases of the gene. The *cryIIIA* gene was also poorly expressed in electroporated carrot cells.

Second-generation transgenic Bt plants

Efforts to enhance Bt δ-endotoxin gene expression in second-generation transgenic plants include expressing δ-endotoxin genes with sequence modifications and/or new promoters, including tissue-specific promoters. Second-generation plants now include maize, which had, until recently, been particularly recalcitrant to genetic engineering. Recent advances in transformation have made it possible to introduce δ-endotoxin genes into maize.

To overcome problems presented by trying to express an A + T rich gene in an organism with a high G + C genomic content, several laboratories have made partially or completely modified *cryI* δ-endotoxin genes that have resulted in significant improvements in gene expression in cotton (Perlak *et al.*, 1990; Wilson *et al.*, 1992), tobacco, tomato (Barton and Miller, 1993) and maize (Koziel *et al.*, 1993). In 1990, Perlak *et al.* published reports on the performance of transgenic cotton plants, *Gossypium hirsutum* var. Coker 312, expressing modified forms of two δ-endotoxin genes. Constructs included genes expressing amino acids 1–612 of the CryIA(b) protein of *B. thuringiensis* var. *kurstaki* HD-1 and amino acids 1–640 or 1–615 of the *cryIA(c)* gene of *B. thuringiensis* var. *kurstaki* HD-73. Both genes were driven by a CaMV 35S promoter containing a duplicated enhancer region. To achieve these higher expression levels, the authors modified the DNA

sequence without changing the amino acid sequence. The regions targeted for sequence modification included those with potential roles as regulatory sequences, or sequences with predicted mRNA secondary structure. These modifications increased the levels of both CryIA(b) and CryIA(c) δ-endotoxins to 0.05–0.1% of the total soluble protein, or what the authors estimated to be a 100-fold increase in expression compared to the truncated wild-type gene. Insect bioassays using leaves from cotton plants containing the wild-type *cryIA(b)* gene failed to demonstrate consistent insect control, while the plants containing the highly modified *cryIA(b)* gene were toxic to the cabbage looper (*Trichoplusia ni*; Lepidoptera) and the 100-fold less sensitive beet armyworm (*Spodoptera exigua*; Lepidoptera). Whole plant assays performed to determine the ability of transgenic cotton plants to control cotton bollworm (*Helicoverpa zea*; Lepidoptera) showed an 80% protection of squares and bolls with the *cryIA(c)* gene and 70–75% protection with the *cryIA(b)* gene, which is 3 to 5-fold less active against *Helicoverpa*.

In a subsequent publication, Perlak *et al.* (1991) examined several versions of the modified *cryIA(b)* and *cryIA(c)* genes in both transgenic tobacco and tomato to determine more closely the increased expression associated with various sequence modifications. All *cryIA* genes were under the control of a CaMV 35S promoter with a duplicated enhancer region. Two versions of modifications were made – one was partially modified, while the second was fully modified. In neither case did sequence modifications alter the amino acid sequence of the *cryIA* genes. The partially modified *cryIA(b)* gene had 62 of 1743 bases changed to remove regions with potential plant polyadenylation signals and A + T rich regions. The fully modified *cryIA(b)* gene had 390 of 1845 bases changed to remove all ATTTA sequences, most potential plant polyadenylation sites, and regions of potential mRNA secondary structure. The fully modified *cryIA(b)* gene also replaced bacterial codons with plant-preferred codons. The partially modified *cryIA(b)* gene had 97% homology to the wild-type gene and a G + C content of 41%, compared with 37% in the wild-type *cryIA(b)* gene. The fully modified *cryIA(b)* had 79% homology with the wild-type gene with a G + C content increased to 49%. The majority of the transgenic tomato and tobacco plants expressing the partially modified *cryIA(b)* gene produced δ-endotoxin at levels of 20–200 ng CryIA(b) per mg total protein and 1–200 ng CryIA(b) per mg total protein, respectively. Over 10% of the plants containing fully modified *cryIA(c)* and *cryIA(b)* genes expressed between 600–2000 ng CryIA per mg of total protein. The most highly expressing transgenic plants containing the partially modified gene and the fully modified gene increased expression 10- and 100-fold, respectively, compared with the wild-type truncated gene. Levels of expression with the modified *cryIA* genes were now up to 0.2% of the total protein in the best plants.

Constructs containing different combinations of the changes were tested in transgenic tobacco to study the effects of particular sequences (Perlak *et al.*, 1991). Modifications in the 5′ one-third of the *cryIA(b)* gene were sufficient to produce expression levels comparable to the partially modified gene, whereas modifications to the 3′ half of the gene had no effect on wild-type

expression levels. The 5′ one-third of the *cryIA(b)* gene and nucleotides 246–283, which contain three potential polyadenylation signals, were identified as important, but no single region was identified as being critical to expression levels. Northern analysis of the modified *cryIA(b)* genes in transgenic plants indicated that, although the levels of mRNA were increased, the mRNA increase was not proportional to the level of protein increase. This observation led Perlak *et al.* to conclude that the problem associated with gene expression originated at the level of protein translation and not at the level of transcription.

Wilson *et al.* (1992) field tested three transgenic cotton lines containing a modified truncated *cryIA(b)* gene from *B. thuringiensis* var. *kurstaki* to examine resistance against the pink bollworm (*Pectinophora gossypiella*; Lepidoptera). Losses due to damage from the pink bollworm, as well as the expense of insecticides, make the pink bollworm one of the most important pests in cotton. Pink bollworm larvae entered the bolls of the transgenic lines as easily as the control lines. However, there was a 99.8% reduction in the number of live insects recovered from transgenic bolls and a 97% reduction in seed damage. Resistance to pink bollworm in the transgenic cotton lines was the same or greater than expected with insecticidal protection. The transgenic lines were highly resistant to cotton leaf perforator (*Bacculatrix thurberiella*; Lepidoptera), and sustained little damage due to the beet armyworm (*Spodoptera exigua*; Lepidoptera).

Barton and Miller (1993) constructed a synthetic *cryIA(a)* gene which resembled plant genes more closely, using a series of constructs with blocks of synthetic DNA representing 50 codons. Each of the constructs was studied to examine the effect of the modified block. Large increases (100-fold) in gene expression were seen with as little as 10% of the peptide coding region modified in the NH_2-terminal region.

Koziel *et al.* (1993) synthesized a truncated *cryIA(b)* gene encoding amino acids 1–648 of the *cryIA(b)* gene from *B. thuringiensis* var. *kurstaki* HD-1. This synthetic gene was completely redesigned to replace the bacterial codons with maize-preferred codons. The synthetic *cryIA(b)* gene had 65% homology with the wild-type gene and had a G + C content of 65%, compared with 37% for the native gene. The synthetic *cryIA(b)* gene, under control of various promoters, was introduced into immature embryos of an elite cultivar of maize using microprojectile bombardment. Two transgenic maize lines expressing this gene were field-tested in 1992. One line contained the synthetic *cryIA(b)* gene under control of the CaMV 35S promoter and 3′ polyadenylation sequences. The other line contained two versions of the synthetic *cryIA(b)* gene, one under control of the maize phosphoenolpyruvate carboxylase (PEPC) promoter, which is expressed in green tissues, and the other version under control of a maize pollen-specific promoter. Southern data and segregation ratios of progeny indicated a single site of insertion of the *cryIA(b)* gene with a few copies of the gene. Transgenic plants containing the two synthetic *cryIA(b)* genes under control of the PEPC and pollen-specific promoters produced over 1000 ng CryIA(b) per mg soluble protein at 7 weeks post-transplantation and up to 4000 ng CryIA(b) per mg soluble

protein in certain plants later in the season. CryIA(b) protein levels in certain plants with the CaMV 35S promoter were as high at week 7 as the levels in the PEPC and pollen promoter plants. Overall, the levels of CryIA(b) protein in the CaMV 35S promoter line were lower and much more variable (25–2000 ng CryIA(b) per mg soluble protein), both within a particular cross and among crosses with different genotypes, whereas the PEPC and pollen promoter expression levels were much less variable. Tissue-specific expression patterns were examined in pith, root, pollen/anther, and kernel tissues of the transgenic plants. The CaMV line had high levels of CryIA(b) in pith and root (25–4000 ng CryIA(b) per mg soluble protein), moderate levels in the kernel (25–280 ng CryIA(b) per mg soluble protein) and no detectable CryIA(b) in the pollen/anther. The PEPC and pollen promoter line had moderate levels in the pollen/anther (50–400 ng CryIA(b) per mg soluble protein), lower levels in root and pith (25–120 ng CryIA(b) per mg soluble protein) and very low levels in kernels (1–18 ng CryIA(b) per mg soluble protein). The results of the field test showed that the transgenic maize lines provided season-long protection from repeated heavy infestations of European corn borer (*Ostrinia nubilalis*; Lepidoptera), which totally devastated control plants.

Concerns that constitutive season-long expression of a δ-endotoxin gene in transgenic crops might lead to the development of resistance to δ-endotoxins in insects has led to the engineering of plants with δ-endotoxin genes expressed by tissue-specific or chemically inducible promoters. Williams *et al.* (1992) used a chemically induced promoter from tobacco to drive the expression of a truncated 645 amino acid *cryIA(b)* gene from *B. thuringiensis* var. *kurstaki* HD-1. The inducible promoter, PR-1a, is from a family of tobacco genes involved in systemic acquired resistance to pathogen infection. Chemically induced transgenic tobacco plants expressing the PR-1a/*cryIA(b)* gene were shown to provide resistance to tobacco hornworm (*Manduca sexta*; Lepidoptera). It is also expected that at least some future transgenic plants will be transformed with multiple δ-endotoxin genes that are known to bind different midgut receptors within the target insect to help deter the development of resistance.

In addition to using tissue-specific promoters for resistance management, several laboratories are looking at second-generation promoters to enhance expression of δ-endotoxin genes. A modified truncated *cryIA(c)* gene from *B. thuringiensis* var. *kurstaki* HD-73 under the control of the *Arabidopsis thaliana* ribulose-1,5-bisphosphate carboxylase small subunit promoter was tested in transgenic tobacco. According to Wong, Hironaka and Fischhoff (1992), the small subunit promoter was tested in transgenic tobacco. According to Wong, Hironaka and Fischhoff (1992), the small subunit promoter with its own 5′ untranslated leader and chloroplast transit peptide provided a 10 to 20-fold increase in CryIA(c) expression levels compared to the CaMV 35S promoter with a double enhancer. It was determined that the increase in expression was the result of a combined effect due to both the 5′ untranslated leader and the transit peptide. A similar increase in expression of the

CryIA(c) protein was observed when the 5′ untranslated leader and transit peptide were fused behind the CaMV 35S promoter.

The difficulty that has been encountered in expressing a Bt δ-endotoxin gene in plants has caused scientists to examine closely the factors that are important for optimal transgene expression in plants. The success of the modified synthetic *cryIA* genes has opened the door for studies on transcriptional and translational regulation of the *cryIA* genes, as well as other genes, in plants. In the future, we are likely to see more tissue-specific and inducible promoters replacing the constitutive promoters like the CaMV 35S promoter. Further refinement will bring focus to the role of 5′ untranslated regions, enhancers and 3′ polyadenylation signals.

Transgenic plants with insecticidal activity against coleopteran pests

Bacillus thuringiensis var. *tenebrionis* produces a δ-endotoxin (CryIIIA) with activity against coleopterans such as Colorado potato beetle (CPB; *Leptinotarsa decemlineata*). An engineered *cryIIIA* gene starting at amino acid 48 was introduced into tomato and potato plants using *Agrobacterium*-mediated transformation (Perlak *et al.*, 1988). The expression levels in plants transformed with the *cryIIIA* gene were very low, less than 0.001% of total soluble protein. Expression levels have been improved by engineering synthetic *cryIIIA* genes using the same strategy used for the synthetic *cryIA* genes. A modified version of the *cryIIIA* gene was engineered to remove potential polyadenylation sites and A + T rich regions, with a final G + C content of 49% as compared with 37% in the wild-type gene (Perlak *et al.*, 1993). The modified *cryIIIA* gene was introduced into Russet Burbank potato plants via *Agrobacterium*-mediated transformation. Expression of the *cryIIIA* gene was under control of the CaMV 35S promoter with a duplicated enhancer region. Expression levels of the CryIIIA protein were between 0.002 and 0.3% of total soluble protein. Tests conducted under both greenhouse and field conditions demonstrated complete control of neonate CPB larvae as well as reduced feeding of adults accompanied by reduced fecundity.

Several field tests of potato lines transformed with the *cryIIIA* gene of *B. thuringiensis* var. *tenebrionis* were conducted in 1992 (Boylan-Pett *et al.*, 1992; Sewell and Storch, 1992; Stoltz and Matteson, 1992). The transgenic potato plants had significantly lower defoliation ratings by the CPB than non-transformed plants. Descriptions of the constructs and expression levels were not published.

Efforts in many laboratories continue to produce new results on the expression levels of the truncated and synthetic δ-endotoxin genes in plants. Bt δ-endotoxin genes other than *cryIA* and *cryIIIA* are also being introduced into plants. Visser *et al.* (1992) have transformed tobacco with a partially modified *cryIC* gene for control of *Spodoptera* spp. insects. In the near future, a number of transgenic crops expressing a variety of δ-endotoxin genes (probably modified for high expression levels) will be available as part of the overall pest management system. Within the next 2–5 years, the first commercial crops are expected to be cotton and maize.

Insect resistance to Bt δ-endotoxins

The growing use of Bt microbial spray-on products together with reports of insect resistance to Bt δ-endotoxins have led to an increasing awareness of, and concern for, the possible development of insect resistance to Bt δ-endotoxins on a scale of economic significance. The concern is shared, to a greater or lesser degree, by academic and industrial researchers, governmental regulatory officials and environmental organizations (National Audubon Society, 1991; USDA, 1992; Fitt, 1992). In recognition of the need for further research and an industry-wide consideration of management strategies, a group of 13 US and European companies formed the Bt Management Working Group in 1988 and has since funded seven research projects in universities on several highly relevant topics.

The attention given to the potential for insect resistance to Bt δ-endotoxins in advance of their widespread use results from several factors. First, the experience with chemical insecticides to which over 500 insect species have become resistant (Georgiou and Taylor, 1986) suggests strongly that insects have a high capacity to evolve resistance in the absence of careful management of the agents supplying selection pressure. Second, the δ-endotoxins of Bt are viewed as a 'natural resource'. The loss or reduction of their usefulness for insect control by the development of insect resistance is perceived, somewhat romantically, as a greater loss than that of a synthetic chemical pesticide. This sentiment must be tempered by consideration of the fact that the number of novel Bt strains and δ-endotoxins identified and the number of species affected is growing exponentially as, mostly, industrial researchers screen, with ever greater precision and breadth, the tens of thousands of strains collected from all over the world. More new 'active ingredients' are likely to become available, faster and at lower cost, from these collections than from the laboratories of synthetic chemists. In addition, as noted above, the engineering of new insecticidal proteins based upon δ-endotoxins is a reality with steadily increasing potential. Are man-made Bt-based insecticides a 'natural resource'? Third, the creation of transgenic plants constitutively producing a δ-endotoxin is seen by some as a prophylactic use of an insecticide and, as such, counter to the current trend towards the managed use of insecticides in integrated pest management programmes.

Bt δ-endotoxins, however, are insecticides. The history and facts of insect resistance to insecticides is relevant (Roush and McKenzie, 1987). In the majority of cases, field resistance has been based upon alleles with major effects at one or two loci. Selection of such alleles has been under conditions of continuous high-intensity use of single insecticides. Polygenic resistance based upon additive alleles with minor effects has been rare. Most high-level resistance genes produce altered targets for, or increased metabolism of, insecticides. Critical parameters in the development of resistance include frequency of resistance alleles in the population, mode of inheritance and fitness of those alleles, number of generations per year, and influx of susceptible individuals into the population (Georgiou and Taylor, 1986).

Field resistance has been based upon alleles that generally do not confer large fitness disadvantages (Denholm and Rowland, 1992).

FIELD AND LABORATORY OCCURRENCES

Most of the insects resistant to Bt δ-endotoxins have been derived from laboratory selection experiments. The majority of these laboratory selections have begun with individuals brought in from field populations that had been treated previously with microbial Bt δ-endotoxins. Laboratory selection of resistance can provide important information about how a species can adapt to the stress of δ-endotoxins in its diet, but certain caveats must be kept in mind when considering the relevance to the potential for field resistance.

First, the pool of genetic variability in a laboratory population is a subset of that in field populations. Rare alleles capable of conferring high levels of resistance in the field could be absent from a laboratory population. There is usually infrequent, or no, immigration into the laboratory population once it is established. Selection occurs in a special environment less stressful than most field environments. Partial resistance alleles have a greater probability of becoming homozygous and a reduced likelihood of conferring a negative fitness. Laboratory seledction regimes can be controlled to allow the accumulation of recessive or additive alleles that produce polygenic resistance. Because most field insecticide treatments, at least on initial application, are high level, only highly resistant homozygous or heterozygous individuals survive. The partially resistant progenitors of a polygenic high-level resistance do not survive such selection stringency.

Indian meal moth (Plodia interpunctella)

The first report of significant insect resistance to Bt was made by McGaughey (1985). After noticing less susceptibility in laboratory colonies derived from Indian meal moth populations from grain bins treated with Bt relative to those not treated with Bt, he began selecting in one more resistant colony for increased resistance to Dipel®, a commercial formulation of strain HD-1, which produces the three CryIA proteins and CryIIA. The increase in resistance was rapid, with a 27-fold increase in just two generations and 97-fold after 15 generations. Other colonies were also successfully selected for increased resistance, although considerable variation was seen in the level of resistance among colonies. In one colony, with higher selection pressure, a 250-fold increase in resistance was achieved. Resistance was lost when selection was removed in the early generations but was maintained after populations had 'plateaued'. If this lack of reversion to susceptibility in the absence of selection was translatable to a field situation, rotation of different Bt δ-endotoxins to manage resistance to any one would be unlikely to be very effective. Inheritance of resistance was partially recessive and was estimated to involve only one or a few major loci (McGaughey and Beeman, 1988).

One Dipel®-resistant Plodia colony was compared with a susceptible colony in two-dose and LD_{50} assays with 57 strains of Bt known to be toxic to the

insect (McGaughey and Johnson, 1987). Twenty-one of the strains were active on the resistant colony and represented five of the eight serotypes tested. The toxicity was not a result of β-exotoxin. Examination of the midgut proteases from both Bt-susceptible and -resistant insects showed no difference in either the ability to activate δ-endotoxins or in total proteolytic activity on casein (Johnson *et al.*, 1990).

In further laboratory selection experiments involving a susceptible colony not previously exposed to Bt and a selected strain with a >250-fold decrease in susceptibility to Dipel®, McGaughey and Johnson (1992) were able to increase resistance of the susceptible colony 21-, 28- and 61-fold over 20 generations of selection to strains HD-198, HD-112 and HD-133, respectively. The rate of increase in resistance was slower than with Dipel®. Simultaneous selection of resistance to both Dipel® and HD-133 was effective (15×) but slower than to each strain alone. The Dipel® resistant colony developed an approximately 21-fold increased resistance to HD-133 in only 12 generations. These results are significant because they demonstrate the possibility of an insect population's relatively rapid acquisition of moderate resistance to several different endotoxin classes at once or sequentially. The HD-133 strain contains the CryIC and CryID proteins not present in the HD-1 (Dipel®) strain.

Van Rie *et al.* (1990a,b) determined the molecular basis for the most Dipel®-resistant colony. Brush border membrane vesicles from insects of the resistant colony were found to contain CryIA(b) binding proteins whose dissociation constants were approximately 50 times less than those of the unselected Dipel®-susceptible strain, while the number of binding proteins was unchanged. In contrast, the binding affinity for CryIC δ-endotoxin was about the same in the resistant and susceptible insects but the concentration of CryIC binding proteins was increased about three-fold in the resistant insects. This increase in CryIC binding proteins was reflected in an increased susceptibility of the Dipel®-resistant strain to CryIC. In the first case of a laboratory selected high level of Bt resistance, in a population of insects derived from one previously exposed to Bt in a 'field' situation, the mechanism of resistance seems to be an altered target site, one of the two most frequent mechanisms of high-level field resistance to chemical insecticides.

Tobacco budworm (Heliothis virescens)

Stone, Sims and Marrone (1989) reported selection of a laboratory strain of *Heliothis virescens* (Lepidoptera), resistant by about 24-fold versus the unselected population, *Pseudomonas fluorescens* expressing CryIA(b). The development of resistance to this level was rapid, just 7 generations, and then fluctuated between 13 and 20 × through generation 12. An interesting result was the marginal (4×) increase in resistance of the population to Dipel® and to the purified endotoxins from Dipel®. The difference was not the result of a differential resistance to the *Pseudomonas*. When the strain was further selected for five generations on Dipel® and, subsequently, seven generations

again on the recombinant Bt-expressing *Pseudomonas*, 75-fold resistance to the *Pseudomonas*, 71-fold resistance to purified CryIA(b), 57-fold resistance to Dipel® and 16-fold resistance to CryIA(c) had been achieved (Macintosh *et al.*, 1991).

The mode of inheritance of the resistance (Sims and Stone, 1991) was found to be several incompletely dominant autosomal factors. The resistance was unstable in the absence of selection for five generations, declining from 69 to 13 times that of the susceptible population. Resistance to CryIA(c) was, however, stable in the absence of selection (Marrone and MacIntosh, 1993).

The mechanism of resistance underlying the complex phenotype of this strain of *Heliothis* is also complex. Proteolytic composition of the gut juices and proteolytic activation of the endotoxins were not altered in the resistant strain (Marrone and MacIntosh, 1993). However, for CryIA(c) (resistance ratio = 16) the midgut binding protein affinity was reduced four-fold but the concentration of binding proteins increased four-fold, whereas for CryIA(b) (resistance ratio = 70) the binding affinity was reduced two-fold while the binding protein concentration increased six-fold. Also, in the resistant insects CryIA(b) was less effective in competing with CryIA(c) for the same binding protein than in the susceptible insects (MacIntosh *et al.*, 1991).

Another laboratory-selected Bt-resistant population of *H. virescens* was reported by Gould *et al.* (1992). This strain was selected at an average larval mortality of 75% per generation on purified trypsin-activated CryIA(c) toxin. Its resistance factor showed a slow increase to about 22× through generation 16 and then increased to 50× in generation 17. What is novel about this strain is its cross-tolerance not only to related δ-endotoxins [13-fold increase in resistance to CryIA(b); increased relative weight gain on CryIA(a)-containing diet], but to δ-endotoxins of different classes (53-fold increase in resistance to CryIIA; increased relative weight gain on CryIC- and CryIB-containing diet). However, it should be noted that CryIA(a), CryIB and CryIC are essentially inactive on *H. virescens*; differences in weight gain could only be detected at concentrations of at least 0.1% of diet (w/v). The significance of these results to the potential for the development of economically relevant broad-spectrum resistance to Bt δ-endotoxins is difficult to determine.

Neither the affinity of midgut membrane proteins binding CryIA(b) or CryIA(c), nor their concentration, was significantly different between the selected and control strains. The F_1 progenies were intermediate in resistance relative to the parents. The number of genes underlying the resistance phenotype was not determined. Gould *et al.* (1992) point out that at doses of CryIA(c) sufficient to kill 80–90% of the susceptible insects, heterozygotes would survive. This, of course, would lead to rapid fixation of resistance genes in the population and increase the proportion of homozygous-resistant individuals. A balanced consideration of the dose–response curves for the three populations (susceptible, resistant and F_1) leads to the observation that, at current levels of δ-endotoxin expression in transgenic plants (about 5–50 $\mu g\ g^{-1}$ fresh weight), the heterozygotes would not be likely to survive and many of the homozygotes, if formed, would perish. In fact, larvae from

the inbred resistant strain (CP73-3) die after feeding on moderately expressing (Bt approximately 0.01% of total leaf protein) CryIA(b) transformed tobacco plants (Warren, unpublished results).

Diamondback moth (Plutella xylostella)

The diamondback moth (*Plutella xylostella*; Lepidoptera) presents the first case of field-selected resistance to Bt (Tabashnik *et al.*, 1990). LC_{50} and LC_{95} laboratory determinations were made using insects from fields minimally and heavily treated with commercial formulations of Bt collected at two different times. Compared to untreated laboratory strains, the treated strains showed increases of 25- and 33-fold, respectively. The LC_{50} resistance ratio for a heavily treated field population increased by about two over a 1 year period. Further selection for nine generations in the laboratory for Bt resistance among three strains established from resistant field populations resulted in 5- to 7-fold increases in LC_{50}, which was an increase in resistance of 150- to 190-fold over unselected laboratory strains (Tabashnik, Finson and Johnson, 1991). Another five generations of selection led to a resistance ratio of 820. The LC_{50}s of Bt-treated field populations did not differ significantly over three generations. The resistance of field populations did not decline within a period of about 4 months in the absence of Bt treatments. As the authors point out, these studies do not address how fast and to what degree resistance can arise in untreated susceptible populations, but do suggest that high levels of resistance can develop quickly with stringent selection among moderately resistant populations. It should be pointed out that the moderate levels of resistance arose in populations repeatedly treated with the same set of sprayed-on δ-endotoxins in a relatively geographically isolated area.

In another case of field resistance to intense use of Bt sprays (Dipel®), the LC_{50} for CryIA(b) in the resistant insects was >200 times that of a susceptible laboratory strain, while the LC_{50}s for CryIB and CryIC, to which *Plutella* is also susceptible, were not significantly different (Ferre *et al.*, 1991; Van Rie, Van Mellaert and Peferoen, 1992). When these three toxins were radiolabelled and independently bound to midgut brush border membrane vesicles (BBMVs), CryIB and CryIC saturably bound to both susceptible and resistant insects. However, CryIA(b) bound only to susceptible insect BBMV. Competitive heterologous binding experiments showed that CryIA(b), CryIB and CryIC bind to independent proteins in the midgut membrane. Because Dipel® does not contain CryIB or CryIC, the resistance can be rationalized on the basis of the complete lack of binding of CryA(b). No data were reported on the toxicity or binding of CryIA(c), a component of Dipel® highly active on *Plutella*.

Plutella populations resistant to Bt have also been found in Florida, USA (Shelton *et al.*, 1993). These populations varied in their degree of resistance, relative to a susceptible laboratory population, to several Bt microbial products. It should be noted that neither the specific endotoxins present nor their concentrations were determined for the products, which makes the interpretation of the variation of product performance, LC_{50}s and resistance

ratios difficult. Nonetheless, a correlation was clearly established between a history of constant *Brassica* production, a high number of *Plutella* generations per year, repeated intense spraying of Bt and higher levels of resistance. Resistance ratios for the so-called 'kurstaki' products were from 321- to 461-fold, but for the 'aizawai' products these ratios varied from 3 to 4.1. The latter products have been in use for fewer years than the former. Neither the genetics nor mechanisms of resistance of these populations has been reported.

Almond moth (Cladra cautella)

McGaughey and Beeman (1988) reported only a seven-fold increase in resistance to strain HD-1 in a 10-year-old laboratory population subjected to selection for 21 generations.

Colorado potato beetle (Leptinotarsa decemlineata)

Another example of selection of increased resistance to Bt in a laboratory population derived from a field population previously treated with the same Bt strain was reported by Miller, Rahardja and Whalon (1990). After 10 generations of selection with a CryIIIA-containing product, 67-fold resistance was noted.

Significance of resistances identified to date

Several themes are evident in the cases of resistance cited above. First, most of the cases of field resistance, with the exception of three Florida populations of *Plutella*, involved moderate levels of resistance, which nontheless led to a reduction in the control of the treated insects. Second, the selection of high levels of resistance was rapid in the laboratory when starting with weakly to moderately resistant subpopulations from field populations previously treated with the same selective agent. Third, those previously treated populations were treated intensively over a period of time with a single Bt product, produced large numbers of generations per year and, in the cases of *Plodia* and Hawaiian and Philippine *Plutella*, saw little immigration. Fourth, laboratory selections initiated with previously untreated populations resulted in only moderate polygenic resistance. Fifth, high-level resistance derived from Bt-treated field populations was, in the only two cases known, *Plodia* and *Plutella*, correlated with target site modification, the elimination of gut membrane binding of at least one of the active δ-endotoxins. Finally, the only strain cross-resistant to more than one class of δ-endotoxin is only marginally so and incapable of survival on a constant diet of Bt or on a moderately expressing transgenic plant.

A couple of tentative conclusions from the studies on resistance to Bt so far are those already learned with chemical insecticides: (1) economically important field resistance develops under variable (the efficacy of Bt sprays declines with time) but continuous selection pressure with a single insecticide on many

generations with little or no immigration; (2) levels of resistance in field situations leading to commercially important control failure most likely result from one or two target site changes. The assumptions behind these conclusions are that a lack of control of some *Plodia* populations in grain storage bins resulted from the altered Bt receptor genes later selected in the laboratory and that the inheritance of resistance in the Philippine *Plutella* population is simple and correlated with the altered CryIA(b) binding. It will be interesting to see if the molecular and genetic analysis of the resistant *Plutella* populations from Florida and Hawaii result from simply inherited recessive genes encoding altered Bt receptors that no longer bind one or more of the active δ-endotoxins of the selective strain or reduce the number of such receptors in the midgut epithelium membranes.

An obvious conclusion from the above studies is that economically significant insect resistance to multiple component microbial Bt sprays *can* occur in field populations. However, the significance of these occurrences cannot be fully appreciated in the absence of definitive analyses of the mechanism of resistance, at the molecular level, the mode of inheritance of the resistance trait and co-segregation of the resistance and the altered molecular phenotypes. The relevance of results with laboratory selections within populations not previously exposed to Bt in decision making about resistance management for Bt products *in the field* is not obvious, beyond providing an indication of the genetic potential of the species. Of course, none of the results to date bear much on the issue of insect resistance to δ-endotoxin expressing transgenic plants.

Population genetics and resistance modelling

Much of the concern about the risk for widespread insect resistance to Bt products has arisen from the coincidence of three elements: (1) the predictions of population genetics models; (2) the occurrence of Bt-resistant insect populations; and (3) the creation of transgenic Bt expressing plants that deliver δ-endotoxins to insects in novel ways (McGaughey and Whalon, 1992). And much of the alarm arising from population genetics modelling derives from models incorporating transgenic plants, which are seen to be unique Bt pesticides, primarily because, in principle, they can challenge an insect population uniformly and stringently at all of its susceptible stages for all of its generations. Whether this uniqueness is, in balance, positive or negative depends on the model and, critically, on its assumptions.

Although population genetics models can be useful as heuristic tools for estimating the consequences of different patterns of insecticide use on the development of resistance (Gould, 1986, 1988, 1991), they involve numerous assumptions, most of which lack experimental validation (Tabashnik, 1986). Many of the known or suggested factors influencing the selection of resistance in field populations (Georgiou and Taylor, 1986) are not varied in the models most frequently used to predict resistance to Bt. Most of the models accommodate only one or, at most, two resistance (R) loci with only one R allele at each locus, and make normally unvalidated assumptions about

dominance and initial frequency of R alleles and about fitness of different genotypes. Tabashnik (1986) stated that resistance models needed experimental validation and, in particular, would benefit from baseline data on population susceptibility before selection and experimental estimates of biological parameters. With Bt, experimental validation of model parameters and assumptions has begun but results have not yet been published. The key parameters of resistance models that, together with experience with resistance to chemical insecticides, seem to be most in need of experimental attention are number of generations per year, intensity of selection, immigration of susceptible individuals (refugia), mode of inheritance of resistance alleles, number of selection agents and how they are applied in time and the particulars of species biology.

The priorities for future research in the nascent field of Bt resistance management seem obvious. First, for each insect species standardized assays must be adopted and used by all. Second, behavioural studies are needed on specific insect-crop combinations with +/–Bt transgenic versions of the crop where available. Third, baseline population susceptibilities must be established *before* Bt is used. These baselines should be determined by geographical location, insect species and selective agent used. The selective agent (transgenic plant or microbial spray) should be precisely characterized as to the nature and amount of endotoxins present. Domesticated laboratory cultures derived from geographically distant field populations are not relevant controls for monitoring the susceptibilities of field populations exposed to Bt. Fourth, a gut binding and toxicity (LC_{50}) database should be developed for each δ-endotoxin–insect combination. Many current Bt resistance data are uninterpretable because the actual active ingredient in a Bt product for a given insect species is unknown. Fifth, studies are needed to determine the effect on resistance development of multiple Bt δ-endotoxins, with different target sites used sequentially or simultaneously. The efficacy of multiple insecticide tactics is unresolved in general (Tabashnik, 1989). Finally, the most critical assumptions (e.g. initial R allele frequency) are in need of experimental validation.

Potential strategies for management of resistance to Bt

The body of experimental and modelling data suggests one seemingly obvious basic principle that can be applied to the use of Bt products as well as chemical insecticides: *Avoidance of fixation of resistance alleles*. The fixation of resistance alleles in homozygotes must be prevented. This can be done by adjusting the dose well above the LC_{50} of treated susceptible insects and maintaining it there. Of course, 'well above' cannot be determined precisely in advance of knowing what resistance alleles are in the populations to be treated and how they are inherited. So far, high-level Bt resistance genes from field populations have been recessive and, even in polygenic laboratory selected populations, additive. Dominant resistance genes are quite rare and have not yet been identified for Bt δ-endotoxins. In most cases, the 'high-dose' approach would eliminate heterozygotes. Bt is most effective on

early stages of susceptible insects. Consistent high doses applied to early stage instars are most easily achieved via transgenic plants. In practice, the 'high-dose' approach is probably not possible with spray-on products because applications are not continuous, persistence is relatively short and insects can escape treatment.

Another and much touted approach to avoiding homozygous resistant insects is to provide a source of susceptible insects for mating and maintaining resistance alleles in the homozygous state. Refugia of various sorts can supply susceptible insects. Refuges can occur outside the crop for polyphagous insects with alternate hosts or within the crop, either by design, through the planting of seed mixtures or susceptible border rows, or as a result of the choices made by individual growers. Another form of refuge, in the case of transgenic Bt crops, is the organ or tissue in which the Bt gene is not expressed but upon which the insect can feed without causing economic damage. Whether the tissue-specific or chemically controlled promoters that could condition such expression will be used will depend less on the availability of the technology than on the specifics of insect–crop interaction and the nature of the marketable product. Refugia used together with a consistent high dose might be a particularly powerful approach to delaying resistance for a large number of insect generations.

Fixation of resistance could also be potentially avoided through the use of more than one insecticide (>1 Bt δ-endotoxin or a Bt δ-endotoxin + a different, but equally effective, active principle). The efficacy of multiple active principles depends on the nature of the principles and how they are used. The effectiveness of simultaneous use derives from the multiplication of rare events, two rare events, where two loci and dominant resistance alleles are involved and four rare events are needed for resistant insect phenotype (where two loci and recessive resistance alleles are involved). Individuals resistant to two independently but simultaneously acting insecticides will have a very low probability of arising unless resistance alleles are present at a high frequency and the dose of the insecticide is such that it allows heterozygous individuals to survive. If multiple active principles are used in sequence, one at a time, their efficacy in delaying resistance will depend on the fitness of the resistance alleles and rapidity with which the population returns to susceptibility versus the frequency of alternating insecticides.

A potentially risky scenario might arise with microbial sprays applied to the same insect on the same crop in the same geographical area where transgenic plants are grown that express the same endotoxin delivered in the spray. In this situation, heterozygous individuals might arise in response to sprays, mate, emigrate and lay homozygous resistant eggs on transgenic crop plants that select stringently for high levels of resistance. Resistance would then be expected to develop relatively rapidly, depending on the frequency of resistance alleles in the population (F. Gould, pers. comm.). In this situation, multiple endotoxins with different sites of action (binding proteins) could be used, between microbe and plant, to manage resistance.

Conclusions regarding resistance

1. Field resistance to Bt has been very limited in occurrence and, in the two cases studied, was based upon altered δ-endotoxin binding. Insects resistant to one δ-endotoxin could be killed by another.
2. The significance of laboratory selections for what can happen in the field is a matter of speculation.
3. There is limited knowledge of baseline susceptibilities of various species from various geographical locations. Therefore, it is not easy to distinguish natural variation among susceptible populations from low to moderate resistance.
4. Knowledge of insect behaviour relevant to the population genetics models that raise concerns about the likelihood of insect resistance to Bt is limited.
5. Each crop–species–geographical location combination will present different challenges and opportunities for resistance management.
6. The management of resistance to a particular insect species will require consideration of the total picture of that species on all of its hosts and for all of the available control measures.
7. Bt δ-endotoxins remain a class of insecticides with many of the properties desired of modern insect control agents: relatively narrow spectrum of activity; non-toxic to mammals, birds, fish; usually inactive on arthropod species that prey on the pests susceptible to Bt; little environmental persistence; no bio-accumulation; stomach poisons consistent with IPM practices. They are large in number. Knowledge of their mode of action, with the increased potential for rational management that it brings, is preceding their widespread use. This is unprecedented for an insecticide.

Summary

Since the first use of *Bacilus thuringiensis* as a biological control agent for insects, a great deal of progress has been made. This progress continues with increasing speed. New Bt strains with improved insecticidal activity have been identified. New strains with new activities have also been identified, thereby increasing the spectrum of activity for microbial sprays as a whole. The current tools of molecular biology in concert with techniques of classical genetics, is making it possible to design and construct improved Bt strains for use as microbial sprays. As more novel activities are identified and more improved strains are constructed, the use of such microbial sprays in agriculture will increase. As the proteins responsible for the insecticidal activity, and their respective genes, become better characterized, more options will become available for their use. The ability to precisely introduce and express foreign genes in a variety of organisms presents the opportunity to deliver these proteins in a controlled manner. These new host organisms are also able to solve some of the problems inherent in spray formulations such as uneven application and the necessity for precise timing. New organisms, however, will not completely replace microbial sprays, since these

have a number of uses for which it is not feasible to engineer alternative hosts. The use of alternative hosts to express the insecticidal proteins from *B. thuringiensis* merely presents more options for insect control using a safe active ingredient.

As the mode of action of the δ-endotoxins is better understood and the structure of the proteins and their interactions with the insect gut cells is elucidated, the opportunity for engineering new activities will increase. This may allow the broadening of the spectrum of activity of a given δ-endotoxin or the engineering of completely novel activities. While such protein engineering possibilities are now in their infancy, the tools are in place to design and construct Bt strains with a desired collection of δ-endotoxins. This ability to design and engineer Bt strains with a desired spectrum of activity and a desired pattern of receptor recognition provides valuable tools both for insect control and for the management of resistance. Presently, the greatest concern about the rapidly increasing use of the Bt δ-endotoxins is that their widespread utilization will create a widespread insect resistance problem which will render them ineffective as a class of insect control compounds. Understanding the basis of resistance development, as well as its cost to the insect, will help develop strategies that prevent, or at least significantly delay, the development of resistance to a given δ-endotoxin. As more δ-endotoxins are discovered, and as more activities are likely to be engineered in the future, we will have even more tools for resistance management as well as for pest management.

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