The Insecticidal Proteins of *Bacillus thuringiensis*

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I. Introduction

Bacillus thuringiensis (Bt) is a gram-positive, aerobic, endospore-forming bacterium belonging to morphological group I along with *Bacillus cereus*, *Bacillus anthracis*, and *Bacillus laterosporus* (Parry *et al.*, 1983). All these bacteria have endospores. Bt, however, is recognized by its parasporal body (known as the crystal) that is proteinaceous in nature and possesses insecticidal properties. These insecticidal proteins, synthesized during sporulation, are tightly packed by hydrophobic bonds and disulfide bridges. Various forms of true crystals have been observed using phase contrast microscope (Srinivas *et al.*, 1995; Jung *et al.*, 1995). The most common shape is a bipyramidal structure (Fig. 1). A Bt mutant defective in sporulation accumulates insecticidal proteins to form large crystal inclusion (Fig. 2) that remained encapsulated within the ghost

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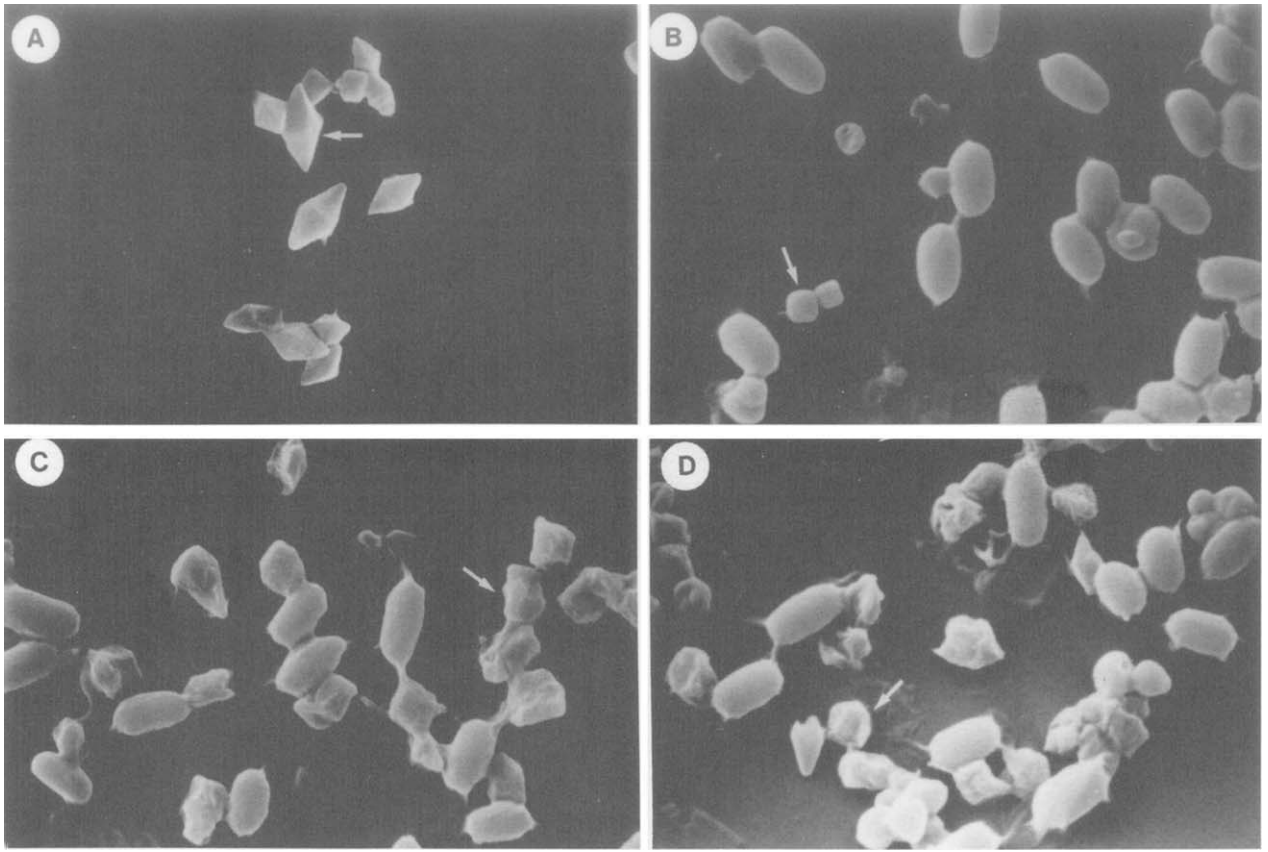


FIG. 1. Scanning electron micrograph of *Bacillus thuringiensis* crystals: (A) bipyramidal crystals produced by a lepidopteranactive strain; (B) spherical crystals produced by a mosquito-active strain; (C and D) irregularly shaped crystals produced by nontoxic strains (arrows indicate crystals). Reproduced with permission from Chilcote, C.N. and Wigley P.J. (1994). *Agric.* Eco systems *Environ.* **49**, 51–57.

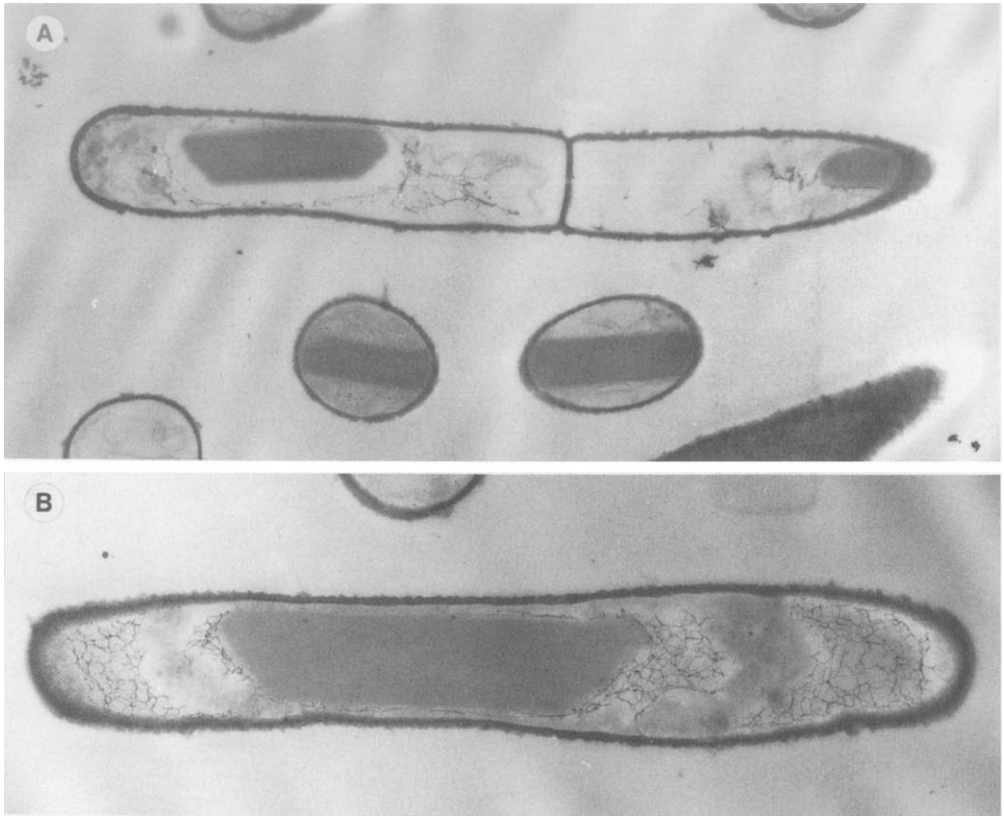
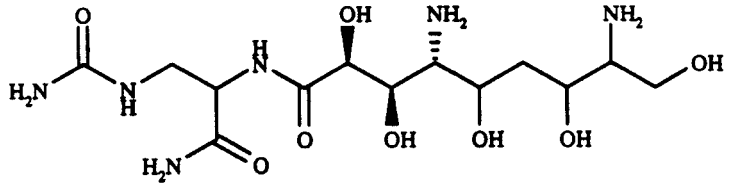


FIG. 2. Electron micrographs of a SpoOA mutant strain overproducing the CryIIIA crystal protein. Reproduced with permission from D. Lereclus, Institut Pasteur, Paris.

cell (Lereclus *et al.*, 1915). The first record on Bt goes back to 1901, when Ishiwata discovered a bacterium from diseased silkworm larvae that he named *Bacillus sotto* (Ishiwata, 1901). Between 1909 and 1912, Berliner (1915), working at a research station for grain processing in Berlin, investigated an infectious disease of the Mediterranean flour moth *(Ephestia kuehniella).* The infected insects were originally obtained from a mill in the district of Thueringen. In a detailed report, Berliner (1915) described a spore-forming bacterium as the causative agent and designated it as *B. thuringiensis.*

The first practical application of Bt was reported by Husz (1928) who isolated a Bt strain from *Ephestia* and tested it on European corn borer. This work eventually led to the first commercial product, Sporeine, which was produced in France in 1938 (Luthy *et al.*, 1982). The development of potent organic insecticides, however, prevented the interest for biological alternatives for pest control to some extent. The pioneering research of Steinhaus (1951) on Bt and a growing realization that organic insecticides are deleterious to the environment and human health spurred a renewed interest in Bt in the 1960s. This led to the introduction of viable Bt biopesticides like Thuricide and Dipel. For many years, the inclusion body protein and spores were generally recognized as the two essential ingredients for most of the insecticidal activity of *B. thuringiensis.* Scientists at the Sandoz company and Asano and Hori (1995) discovered in the supernatant of the *B. thuringiensis* a growth medium potency-enhancing factor, Kurstakolin (Fig. 3), which enhances the insecticidal activity of *B. thuringiensis* cellular preparations by 30%.

There are many subspecies and serotypes of Bt with a range of well-characterized insecticidal proteins or Bt toxins. Known Bt toxins kill subsets of insects among the Lepidoptera, Coleoptera, Diptera (Hofte and Whiteley, 1989), and nematodes (Feitelson *et al.*, 1992). The host range of Bt has expanded considerably in recent years due to extensive



$C_{13}H_{28}N_6O_8$

FIG. 3. Structure of Kurstakolin.

screening programs (Table I). By virtue of the lack of toxicity toward other species of animals, human beings, and plants, there is tremendous potential for exploiting Bt as a biological control agent (Jones and Khachatourians, 1995; Salama *et al.,* 1995; Bradley *et al.,* 1995).

Various aspects of Bt fermentation (Capalbo, 1995; Gangurde and Shethna, 1995), biology and genetics (Bulla *et al.,* 1978; Aronson, 1986), molecular biology (Hofte and Whiteley, 1989; Yoshisue *et al.,* 1995a; Dervyn *et al.,* 1995), mechanism of action (Gill *et al.,* 1992; Knowles, 1994), application as biopesticide (Gawron-Burke and Baum, 1991;

TABLE I

HOST RANGE OF *Bacillus thuringiensis*

		Susceptible families	
Order	Toxin	Family	Example
Insecta			
Lepidoptera	δ-Endotoxin	Most lepidopteran families susceptible examples	
		Spingidae	Hawkmoths
		Pieridae	Cabbage worms
		Lymantriidae	Tussock moths
		Tortricidae	Leafroller moths
		Noctuidae	Cutworms/armyworms
Diptera	δ-Endotoxin	Culicidae	Mosquitoes
		Simuliidae	Blackflies
		Anisopodidae	Gnats
		Chironomidae	Midges
		Psychodiae	Moth flies
		Sciaridae	Black fungus gnats
		Tipulidae	Craneflies
	Thiringiensin	Muscidae	Houseflies
		Calliphoridae	Blowflies
Coleoptera	δ-Endotoxin	Chrysomelidae	Leaf beetles
Phthiraptera		Philopteridae	Bird lice
		Trichodectidae	Mammalian lice
Arachnida			
Acari	Thuringiensin	Dermanyssidae	Animal mites
		Tetranychidae	Phytophagous mites
Nematoda			
Strongylida	?	Trichostrongylidae	Animal endoparasitic nematodes
Tylenchida	?	Tylenchidae	Phytophagous nematodes

Aronson, 1994, Pedersen *et al.*, 1995; Farrar and Ridgway, 1995; Yang *et al.*, 1995; Gibson *et al.*, 1995; Li *et al.*, 1995), and Bt transgenic plants (Peferoen, 1992; Kumar and Sharma, 1994) have been reviewed. Here, the classification and mode of action of Bt toxins are discussed. Strategies to screen new Bt strains/genes, expression of the toxin protein in transgenic microorganisms (Shin *et al.*, 1995), and plants and various resistance management strategies in agricultural systems are examined. The review puts emphasis on agricultural application of Bt.

II. Classification of Bt Toxins

A large number of Bt isolates are now available in laboratories around the world (Schnepf, 1995; Jung *et al.*, 1995; Burtseva *et al.*, 1995; Shin *et al.*, 1995). New strains are being added every year. Bt strains can be characterized by a number of techniques including serotyping, crystal serology, crystal morphology, protein profiles, peptide mapping, DNA probes, and insecticidal activity. De Barjac first attempted to classify Bt toxins based on flagellar (H) agglutination (De Barjac and Bonnefoi, 1962). Recently, the classification of Bt based on H antigen was revised (De Barjac and Franchon, 1990) (Table II). More than 40 H-serotypes are

TABLE II

CLASSIFICATION OF *Bacillus thuringiensis*

H-antigen	Variety	Toxicity[a]
1	*thuringiensis*	L,D
2	*finitimus*	
3a,3c	*alesti*	L
3a,3b,3c	*kurstaki*	L,D
3a,3d	*sumiyoshiensis*	
3a,3d,3e	*fukuokaensis*	D
4a,4b	*sotto*	L
4a,4c	*kenyae*	L,D
5a,5b	*galleriae*	L,C
5a,5c	*canadensis*	L
6	*entomocidus*	L
6a,6c	*oyamensis*	
7	*aizawai*	L,D
8a,8b	*morrisoni*	L,D,C
8a,8c	*ostriniae*	L
8b,8d	*nigeriensis*	

(*continues*)

TABLE II—*Continued*

H-antigen	Variety	Toxicity[a]
9	*tolworthi*	
L,D10a,10b	*darmstadiensis*	L,D
10a,10c	*londrina*11a,11b	
toumanoffi		
11a,11c	*kyushuensis*	L,D
12	*thompsoni*	L,D13
	pakistani	
14	*israelensis*	D
15	*dakota*	
16	*indiana*	
17	*tohokuensis*	
18a,18b	*kumamotoensis*	C
18a,18c	*yosoo*	
19	*tochigiensis*	
20a,20b	*yunnanensis*	L
20a,20c	*pondicheriensis*	L
21	*colmeri*	
22	*shandongiensis*	L
23	*japonensis*	C
24a,24b	*neoleonensis*	
24a,24c	*novosibirsk*	
25	*coreanensis*	
26	*silo*	
27	*mexicanensis*	L
28a,28b	*monterrey*	
28a,28c	*jegathesan*	D
29	amagiensis	
30	*medellin*	D
31	*toguchini*	
32	*cameroun*	
33	*leesis*	
34	*konkukian*	
35	*seoulensis*	
36	*malaysiensis*	D
37	*andalousiensis*	
38	*oswaldocruzi*	
39	*brasiliensis*	
40	*huazhongensis*	
41	*sooncheon*	
42	*jinghongiensis*	
43	*guiyangiensis*	
44	*higo*	
45	*roskildiensis*	

[a] *L, lepidopteran active; D, dipteran active; C, coleopteran active.*

now available and in many of these the array of Bt toxin genes present in isolates from a particular serovar are the same (Rabinovitch *et al.*, 1995). A notable exception is the presence of very different Bt toxin genes in subspecies morrisoni and tenebrionis within serotype 8a,b. Some of the serotypes are divided into subserotypes that can be differentiated by PCR (Bourque *et al.*, 1993; Brousseau *et al.*, 1993). However, a high level of sequence similarity among *B. anthransis, B. cereus, and B. Thuringiensis* does not permit construction of sequence-specific probes to be used in identification (Bourque *et al.*, 1994).

The most useful scheme for classification of Bt toxins is based primarily on homology of toxin gene sequences and the spectrum of insecticidal activity (Hofte and Whiteley, 1989; Ogiwara *et al.*, 1995). A large number of distinct Bt toxin genes have been cloned and sequenced since the first report published in 1981 (Schnepf and Whiteley, 1981). Hofte and Whiteley (1989) have classified 42 Bt genes into 14 distinct types and grouped them into four major classes. The classes are *cryI* (Lepidoptera specific), *cryII* (Lepidoptera and Diptera specific), *cryIII* (Coleoptera specific), and *cryIV* (Diptera specific). Many more Bt genes have since been sequenced and analyzed. Following the analysis of toxin domains of 29 distinct Bt toxin proteins, Feitelson *et al.* (1992) added two new major classes, *cryV* and *cryVI.* Several novel genes were also added within the previously defined classes (Table III). The nomenclature of Hofte and Whiteley (1989), based mainly on insecticidal activity, failed to accommodate genes that were highly homologous to known genes but with a different insecticidal spectrum. *cryIIA* and *IIB* were included in the Diptera-specific class because it is known that *cryIIB* is inactive against Diptera. *cryIC* is toxic to both Diptera and Lepidoptera (Smith and Ellar, 1994). Several genes with differing homology and bioactivity were named *cryV*, the next available Roman number in the original system (Gleave *et al.*, 1992, Tailor *et al.*, 1992).

Based on amino acid identity of full-length gene products, Crickmore *et al.* (1996) have introduced a systematic nomenclature for classifying the *cry* genes and their protein products. Most *cry* genes retain the name assigned by Hofte and Whiteley with a substitution of Arabic for Roman numerals (e.g., *cryl Aa*) to accommodate the newly discovered genes. Fifty genes comprising 16 homology groups are systematically arranged. Their dendrogram depicts the possible evolutionary relationships between the entire set of Bt toxins. Primary through quaternary ranks are based on 45, 75, and 95% level of sequence identity. Eighteen sets at the primary rank, CytA, CytB, and Cry1 through -16, are defined into 4 homology groups. Cry1, -3, -4, -7, -8, -9 and -10 form the largest group. Cry2 and Cry11 are the second group. The third group is Cry5, -12, -13 and -14. The fourth group is the two Cyt proteins. The Cry6, -15, and -16 consist of unique proteins.

TABLE III

Bacillus thuringiensis CRYSTAL PROTEIN GENES

Gene designation	Predicted M_r	Toxicity[a]
cryIA(a),(b),(c)	131–133	L
IB	137	L
IC	134	L
ID	133	L
IE	137	L
IF	134	L
IG	130	L
cryIIA	71	L,D
IIB	71	L
IIC	71	L
cryIIIA	73	C
IIIB	73	C
IIIC(a),(b)	73	C
cryIVA	134	D
IVB	128	D
IVC	77	D
IVD	72	D
cryV	80	L,C
Genes not yet cloned	130	?
	100	?
	40	?

[a] L, Lepidoptera; D, Diptera; C, Coleoptera; Based on Hofte and Whiteley (1989).

Crickmore *et al.* (1996) define *cry* as a gene from *B. thuringiensis* encoding a parasporal inclusion protein that exhibits pesticide activity or is homologous to a known *cry* gene.

1. The mnemonic cry shall remain for the crystal-forming pesticidal genes from B. thuringiensis. The cry gene nomenclature shall be distinguished at all ranks on the basis of comparative amino acid sequence identity of the full-length gene products.
2. The primary rank of the nomenclature shall be Arabic numbers. The *cry* genes whose products share less than 45% amino acid homology shall be characterized by different Arabic numbers.
3. The secondary rank shall be an uppercase letter. The *cry* genes of the same rank whose products show less than 75% homology shall be separated into different secondary ranks.
4. The tertiary rank shall be a lowercase letter without parentheses. The *cry* genes whose products share less than 95% homology shall be given different tertiary ranks.

5. The quaternary rank shall be allele numbers. The *cry* genes whose products differ in amino acid sequence, but are more than 95% identical to each other, shall be given separate quaternary ranks.

Crickmore *et al.* (1996) are the *B. thuringiensis cry* Gene Nomenclature Committee, a standing committee of the Bacillus Genetic Stock Center. They will assist workers in the field of *B. thuringiensis* genetics in assigning names of new *cry* genes and periodically review the literature of the *cry* genes.

III. Structure of Bt Toxin Proteins and Genes

Bt toxin genes are usually plasmid borne (Gonzalez *et al.*, 1995) but also chromosomally located (Carlson and Kolsto, 1993; Klier *et al.*, 1982; Kronstad *et al.*, 1983). The Bt toxin genes are encoded on plasmids of molecular weight 40–150 mDa (Carlton and Gonzalez, 1985; Jensen *et al.*, 1995). Most of the plasmids are of low copy number. In addition to the toxin-encoding plasmids, there are often several other cryptic plasmids of 4–150 mDa whose function is not clearly known. Many of the plasmid-encoded toxin genes are bordered by transposons and/or insertion sequences (Delecluse *et al.*, 1990). Dervyn *et al.* (1995) examined the transcriptional regulation of the *cryIVD* gene operon from *B. thuringiensis* subspecies *israelensis.*

Hofte and Whiteley (1989) compared sequences among a number of toxins with varying specificities and found five well-conserved regions designated blocks 1–5 (Fig. 4). Exceptions to this include CryIVC toxin of Bt subspecies israelensis and a novel toxin from subspecies *thompsoni* (Brown and Whiteley, 1992). Blocks 1 and 2 are very hydrophobic and are present as amphipathic α-helices with membrane-spanning potential. The protoxins designated CryIA–CryIG, CryIVA, and CryIVB contain 1100–1200 amino acids and the toxin is processed from within the amino half as shown in Fig. 4. The CryII, CryIII, and CryIVD protoxins are smaller, with processing to toxins as indicated. The carboxyl halves of the CryI, CryIVA, and CryIVB protoxins are also highly conserved except that there is a deletion of 26 amino acids in CryIA(b) protoxins.

On the basis of the conservation of the defined blocks, it was postulated that all of the Bt toxins probably have a three-dimensional conformation similar to that of a CryIIIA toxin reported by Li *et al.* (1991) (Fig. 5). According to this, the first 285 residues are present as a bundle of seven amphipathic α-helices, wherein six are arranged in a circle, and helix 5 is in the center (domain I). Residues 286–500 are organized as three β-sheets (domain II) and contribute to the toxin specificity. The remaining amino acids are also present as β-sheets and arranged like a

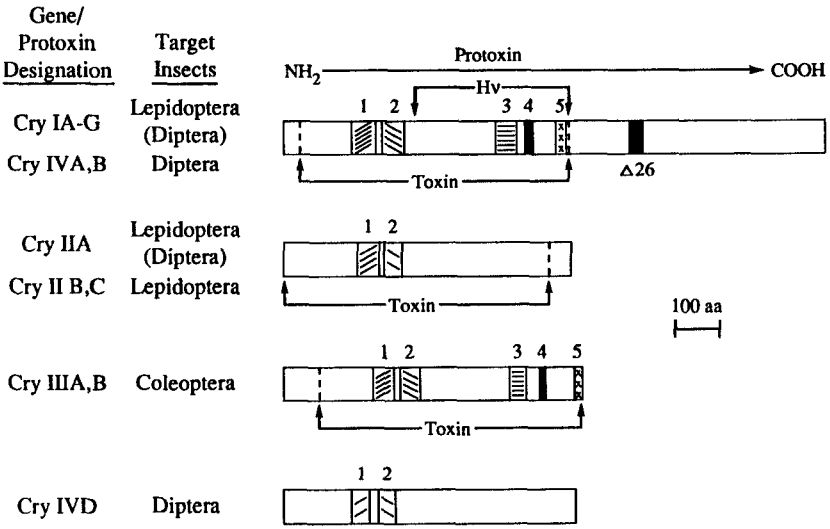


FIG. 4. General structural features of protoxins as deduced from gene sequences and other related data. Protoxins designated CryIA–CryIG, CryIVA, and CryIVB contain 1000–2000 amino acids, and the toxin is processed from within the amino half as shown. The CryII, CryIII, and CryIVD protoxins are smaller, with processing to toxins as indicated (not known for CryIVD). Regions marked 1–5 are highly conserved among the CryI, CryIII, CryIVA, and CryIVB toxins and less so (pramarily regions 1 and 2) for the CryII and CryIVD toxins. The carboxl halves of the CryI, CryIVA, and CryIVB protoxins are also extensively conserved. A major difference is the deletion of 26 amino acids (Δ26) in most of the CryIA(b) protoxins. Other portions of the toxins are more or less conserved within a particular class (i.e., those designated CryI or CryII, etc.) but not between these classes. Reproduced with permission from Dr. Aronson.

sandwich (domain III). All the three domains have specific functional roles. The first domain is required for toxicity, and domain II is important for specificity. Although the function of Domain III near the carboxyl end was not defined, it is speculated that it may have a role in the processing of protoxin (Aronson, 1994, Martens *et al.*, 1995) and channel-forming function (Chen *et al.*, 1993).

Wu and Aronson (1992) induced localized mutagenesis in central helix of domain I and found loss of toxicity but not the capacity to bind midgut membranes. A synthetic peptide of helix 5 could insert itself into membrane and form ion channels that confirmed the importance of this helix (Gazit and Shai, 1995). Single site mutations in the conserved alternating arginine region affect ionic channels formed by CryIA(a), a Bt toxin (Schwartz *et al.*, 1995). The assembly and organization of the α-5 and α-7 helices from the pore-forming domain of *B. thuringiensis* δ-endotoxin is relevant to a functional model for pore formation (Gazit and Shai, 1995). Similarly, a truncated peptide corresponding to the domain I of

CryIII β2 was shown to be sufficient for membrane channel activity and ion efflux from artificial membrane vesicles (Van Tersch *et al.*, 1994).

Mutations in domain I reduced the irreversible binding of toxin to BBMV (Chen *et al.*, 1995). The evidence that domain II is involved in specificity comes from the structural comparisons of CryIA toxins and construction of hybrid genes to analyze specificity domains (Schnepf *et al.*,1990; Ge *et al.*, 1991). Chen *et al.* (1993) concentrated on the highly conserved block 4 of domain III and used site-directed mutagenesis to substitute other amino acids for arginine. Studies with these mutant proteins revealed that domain III is not only involved in structural stability and integrity of the toxin protein but also in function as an ion channel. Wabiko and Yasuda (1995) investigated the location of toxic border and the requirement of the nontoxic domain for high-level *in vitro* production of active toxin from *B. thuringiensis* protoxin.

IV. Screening for New Insecticidal Proteins and Genes

As mentioned previously, the toxicity spectrum is being widened each year with the discovery of novel strains that are active against various organisms (Payne *et al.*, 1995; Hickle and Payne, 1995; Kawalek *et al.*, 1995). Following the early isolations of Bt from dead insect larvae, these bacteria have been found ubiquitously by using a novel enrichment technique that exploits unique germination properties of the spores (Martin and Travers, 1989) or by simply screening debris, such as soils, leaves, and dead larvae, for spore formers containing parasporal inclusions. An interesting example was the prevalence of isolates on the surfaces of leaves from various trees (Smith and Couche, 1991).

One of the most important aspects about establishing a Bt collection is to have a methodology with which one can rapidly and accurately characterize the strain, the toxin protein, and the gene. This is especially important if the differences among endotoxin genes, carried by a certain strain, are critical for its specificity and toxicity. The bioassay analysis is an exhaustive and time-consuming process because it is necessary to screen all the isolates in all of the target insects. Various methodologies have been described to simplify this process. The important approaches are

1. Southern blot analysis in search of homologous genes (Kronstad and Whiteley, 1986);
2. Reactivity to different monoclonal antibodies (Hofte and Whiteley, 1989); and
3. Electrophoretic analysis of PCR products using specific primers (Carozzi *et al.*, 1991).

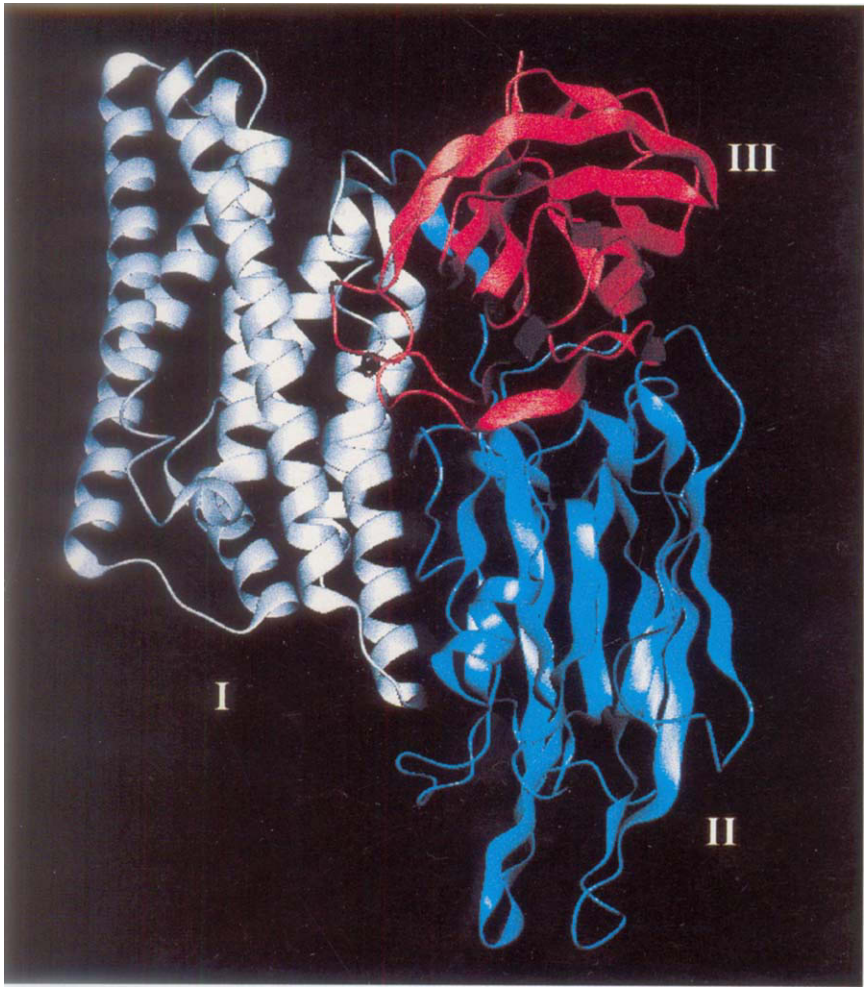


FIG. 5. A schematic ribbon diagram of the CryIIIA structure (Li *et al.*, 1991). Domain I, the putative membrane insertion domain, is a 7-helix bundle (left); domain II, the putative receptor binding region, is an assembly of three β-sheets (lower right); domain III is a β-sandwich in which the C terminus is buried (upper right).

Among the three approaches, PCR analysis is considered to be the best choice because it permits a rapid determination of the presence or absence of a sequence, it is highly sensitive, relatively fast, and can be used routinely. Carozzi *et al.* (1991) described the sequences of 12 PCR primers that can distinguish three major classes of Bt toxin genes (*cryI, cryIII,* and *cryIV*). However, it is important to delineate the genes in each subgroup because of the differential insect toxicity. PCR analysis of three *cryIA* subgroups was reported (Bourque *et al.,* 1993). This analysis did not allow for the identification of the remaining *cryI* gene subgroups. Thus, it is important to develop a complete PCR set of primers that allows the identification of all reported *cry* genes.

Bravo and co-workers (Ceron *et al.,* 1994) at the National University of Mexico designed four oligonucleotides that can be used to identify the strains that carry any of the *cryI* or *cryIII* genes. These primers were selected from a highly conserved region between *cryI*'s or *cryIII*'s genes by computer analysis using a Gene work 2 program that allows simultaneous alignment of several gene sequences. The primers were able to amplify a region ranging between 272 and 290 bp from all the *cryI* genes and between 688 and703 bp from all *cryIII* genes. Strains with unique PCR product profiles were then characterized by using additional gene-specific primers. A set of primers were designed that give a different molecular weight with each of the *cryI* and *cryIII* genes. These primers were selected from the highly variable region among all genes. They were designed to be used in mixtures of six to eight primers per reaction. By using these primers, all the *cryI* genes from *cryIA* to *cryIG*, including subdivisions of *cryIA* genes as well as all the *cryIII* genes from *cryIIIA* to *cryIIIE*, could be identified (Ceron *et al.*,1994). The important feature of this screening method is that with each gene a precise molecular weight product is expected. Genes that produce different-sized products may represent novel genes. Using a similar approach, Kalman *et al.* (1993) found a novel *cryIC* gene. One limitation of the methodology, however, is that new genes from a Bt collection cannot be identified if the gene in question does not have any of the primer sequences. In addition, PCR screening does not provide information of the specific target insect of the novel gene.

Bravo *et al.* (1992a) developed an immunocytochemical technique to identify proteins that may have potential toxicity toward selected insects. This was based on a clear correlation between binding of toxic protein to its specific receptor localized in the microvilli of the midgut cells and toxicity. By using this methodology, toxins that are highly toxic to *Diatraea grandiocella, Spodoptera frugiperda,* and *Rhopalosiphum maidis* (aphid) were found. The proteins to be tested should be

recognized by specific antibodies. They can also be labeled with biotin to be detected subsequently with streptavidin coupled to the peroxidase enzyme (Denolf *et al.*, 1993).

Another approach to identify a toxin protein is to analyze its effects on the permeability of brush border membrane vesicles. Changes in membrane permeability can be measured fluorometrically with a fluorescent dye sensitive to changes in membrane potential. Uemura *et al.* (1992) used membrane vesicles from *Bombyx mori* and found that toxic proteins were able to produce clear effect on ion transport, whereas nontoxic proteins did not do so. The novel toxins that are able to modify the permeability of the membranes from a selected larva are expected to have a higher potential of being toxic.

Entomopathogenic bacteria belonging to Bacillaceae and the immunological relationship between their insecticidal toxins are being studied by cloning the toxin gene next to a *Bacillus* promoter in *Escherichia coli*. Recombinants are first screened with degenerate nucleotides probes based on the DNA sequence of the δ-endotoxin gene. Recombinant plasmids from positive clones are transferred into toxin minus *Bacillus* strains and the protein produced is screened with antibodies directed against toxin from the wild-type *Bacilli* strain. This method can be adapted for screening large number of isolates with a wide variety of degenerate oligonucleotides.

V. Mechanism of Action

The target organ for Bt toxins is the insect midgut (Zimanyi *et al.*, 1995). The midgut of the lepidopteran larvae is a simple, tubular epithelium that dominates the internal architecture of the insect. The tissue is composed of two major cell types: a columnar cell with a microvillate apical border and a unique goblet cell, containing a large vacuolar cavity, linked to the apical surface by an elaborate and tortuous "valve" (Cioffi, 1979). The "K^+ pump" is located in the apical membrane of the goblet cell, pumping K^+ from the cytoplasm into the cavity and thence to the gut lumen via the valve. This electrogenic K^+ transport is the predominant feature of the larval lepidopteran gut. Disruption of the activity of K^+ pump as a result of toxin-induced pore formation in the plasma membrane of the columnar cells leads to osmotic imbalance. Another important feature of the midgut is that the pH of the lumenal fluid is about 12, which is essential for dissolving the crystalline Bt protoxins, usually soluble only above pH 9.5.

The crystalline protoxins are inactive. They are solubilized and then activated by gut trypsin like proteases (Milne and Kaplan, 1993, Tojo and Aizawa, 1983), which typically cleave some 500 amino acids from

the C terminus of 130-kDa protoxins and 28 amino acids from the N terminus, leaving a 65 to 55-kDa protease-resistant toxic active core comprising the N-terminal half of the protoxin (Hofte and Whiteley, 1989). The mature CryI A toxin is cleaved at the amino-terminal R2 arginine residue (Nagamotsu *et al.,* 1984) and the carboxyl-terminal K lysine residue (Bietlot *et al.,* 1989). A tightly bound 20-kilobase heterogeneous DNA fragment is involved in the proper proteolytic processing of protoxin (Bietlot *et al.,* 1993). The 70-kDa CryII, CryIII, and CryIVD proteins are naturally occurring truncated forms. The active toxins bind to specific receptors located on the apical brush border membrane of the columnar cells. Binding involves two steps, reversible (Hofmann and Luthy, 1986; Hofmann *et al.,* 1988a) and irreversible (Ihara *et al.,* 1993; Rajamohan *et al.,* 1995). The irreversible step is followed by the insertion of the toxin into the apical membrane. Various studies revealed that there are many different toxin-binding protein receptors (Gill *et al.,* 1992). Some of them were identified as 120 to 180-kDa glycoproteins (Garczynski *et al.,* 1991; Knowles *et al.,* 1991; Oddou *et al.,* 1991). In *Manduca sexta*, a 210-kDa membrane protein is the CryIAb receptor (Vadlomudi *et al.,* 1993, 1995). A 120-kDa aminopeptidase N has been reported as receptor for the Cry1 Ac toxin (Knight *et al.,* 1994; Sangadala *et al.,* 1994). *Bacillus thuringiensis* CryIAc δ-endotoxin-binding aminopeptidase in the *M. sexta* midgut has a glycosyl phosphatidylinositol anchor (Garczynski and Adang, 1995). After binding to the specific receptor, the toxin inserts irreversibly into the plasma membrane of the cell leading to lesion formation. Three models were proposed to explain the role of toxin receptor in pore formation (Knowles and Dow,1993). The first model envisages that the receptor is itself a transmembrane channel that is activated by the toxin in a manner analogous to the ligand gating mechanism employed by neurotransmitters. The second possibility is that the toxin and receptor together form a pore. The third model explains that the receptor catalyzes toxin association or insertion into the membrane and plays no further role in pore formation. The receptor may either simply act as a handle to which the toxin binds or induce a conformational change in the toxin, thus enabling it to insert into the membrane.

The formation of toxin-induced pores in the columnar cell apical membrane allows rapid fluxes of ions. Different studies revealed that the pores are K^+ selective (Sacchi *et al.*,1986), permeable to cations (Wolfersberger, 1989), permeable to anions (Hendrick *et al.,* 1989), or permeable to small solutes like sucrose, irrespective of the charge (Schwartz *et al.,* 1991a). Using a simple light scattering assay, Carroll and Ellar (1993) found that the midgut membrane permeability in the presence of CryIAc was altered for cations, anions, and neutral solutes, as well as for water. It appears

that the toxin forms or activates a relatively large aqueous channel in the membrane. The model proposed by Knowles and Dow (1993) placed emphasis on the cessation of the K^+ pump that leads to the swelling of columnar cells and osmotic lysis. The disruption of gut integrity results in the death of the insect from starvation or septicemia.

There seems to be a different mechanism of action with respect to CryIIA toxins.

Differences in the extent of solubilization may sometimes explain differences in the degree of toxicity among Cry proteins. Decreased solubility could be one potential mechanism for insect resistance (McGaughey and Whalon, 1992). English *et al.* (1994) compared the differences in solubility, binding to the brush border membrane, and ion channels formed by CryIIA and CryIAc toxins in *Helicoverpa zea.* The results showed unique attributes in the mode of action of CryIIA, which was less soluble than CryIAc and failed to bind to a saturable binding component on the midgut brush border membrane. In addition, voltage-dependent, nonselective channels were formed by this toxin in planar lipid bilayers. This behavior was reminiscent of several other channel-forming protein toxins of bacterial origin such as the *E.coli* active colicins, which have a strong voltage dependence. It was suggested that the unique mode of action of CryIIA may provide a useful tool in managing field resistance to Bt toxins.

Although the binding of the Cry toxins to receptors determines the insect specificity (Kronstad *et al.,* 1983; Van Rie *et al.,* 1990a), exceptions to correlation of binding and toxicity exist. CryIAc binds to ligand blots of *Spodoptera exigua* BBMV proteins without any toxicity to the insect larvae (Garczynski *et al.,* 1991). CryIAb is more toxic to gypsy moth than CryIAc but does not bind as well to receptors on BBMV (Wolfersberger, 1990). Irreversible binding and ion-channel function directly correlate to toxicity in gypsy moth, thus unraveling the "Wolfersberger paradox" (Liang *et al.,* 1995; Rajamohan *et al.,* 1995; Liebig *et al.,* 1995).

VI. Bt as a Biological Insecticide

Bacillus thuringiensis is the most popular biological control agent with a worldwide projected sales of about $90 million during 1995 (Lambert and Peferoen, 1992). Sixty-seven registered *B. thuringiensis* products have more than 450 uses and formulations (Dean and Adang, 1992; Rowe and Margoritis, 1987). *Bacillus thuringiensis* is the major pesticide against gypsy moth in forests (Twardus, 1989). *Bacillus thuringiensis* subsp. *israelensis* (BTI) is extensively used to control mosquitoes and blackflies (Becker and Margalit, 1993; de Barjac and Sotherland, 1990)

Bacillus thuringiensis subsp. *morrisoni* and BTI carry four genes that encode mosquito and blackfly active toxins: *cryIVA, cryIVB, cryIVC, and cryIVD.* BT also produces Cyt toxins that synergize the Cry toxins. *Bacillus thuringiensis* subsp. *jegathesan* encodes another potent mosquitocidal toxin immunologically related to CryIIA (Delecluse *et al.,* 1995). Thus, Bt plays an important role not only in agriculture and forestry but also in the area of human health (Haider *et al.,* 1986, 1987; Smith and Ellar, 1994; Orduz-Peralta *et al.,* 1992).

The Bt toxin normally accumulates during the stationary phase (Bechtel and Bulla, 1976) with exceptions (Krieg *et al.,* 1980). The earliest commercial production of Bt began in France in 1938 under the trade name Sporeine (Luthy *et al.,* 1982). During the 1960s, several industrial formulations of Bt were manufactured in the United States, France, Germany, and Soviet Union. The isolation of the highly potent *kurstaki* variety by Kurstak in 1962 and by Dulmage in 1967 (Dulmage,1970) provided a much-needed boost to the commercialization of Bt. The HD1 isolate of Dulmage is still the active ingredient in most Bt products used against caterpillar pests in agriculture, horticulture, and forestry. The discovery of new strains of Bt widened the toxicity spectrum of bioinsecticides. The use of conventional Bt insecticides, however, was found to have limitations like narrow specificity, short shelf life, low potency, lack of systemic activity, and the presence of viable spores (Lambert and Peferoen, 1992). These problems are now overcome by various approaches that utilize the tools of molecular biology and genetic engineering as well as conventional microbiological methods (Ben-Dov *et al.,* 1995).

A. Construction of Novel Bt Strains by Conjugation

The plasmid location of Bt toxin genes enabled the construction of novel Bt strains with microbial genetic approaches such as plasmid curing and conjugal transfer (Wiwat *et al.,* 1995; Battisti *et al.,* 1985). Conjugational transfer of native Bt plasmid between species of *Bacillus* is known to occur (Gonzalez *et al.,* 1982; Reddy *et al.,* 1987; Andrup *et al.,* 1995). Expression of transformed plasmid-coded genes was analyzed by genotyping of crystal proteins and flagellar antigenicity. This particular set of studies employed conjugational transfer of an entire toxic polypeptide coding gene located on native plasmids. On the other hand, more versatility of the conjugational transfer-mediated approach was demonstrated with a mobilizable plasmid bearing a cloned gene coding for a variant Bt gene (Klier *et al.,* 1983). Following the conjugational approach, scientists at Ecogen Corporation (USA) produced several bioinsecticides with broadened spectrum of toxicity (Gawron-Burke

and Baum, 1991). For instance, the product "Foil" is made from a strain that carries toxin genes active against European corn borer (Lepidoptera) and Colorado potato beetle (Coleoptera).

Using the conjugational approach, Bora *et al.* (1994) transferred the *cryIAa* gene of Bt into *Bacillus megaterium*, which resides in the cotton phyllosphere. Leaf bioassays of cotton plants, inoculated with a single spray of the transcipient, showed that there was protection to the cotton plants from *Helicoverpa armigera*. Enhanced production of insecticidal proteins occurs in *B. thuringiensis* strains carrying an additional crystal protein gene in their chromosomes (Kalman *et al.*, 1995).

The conjugational approach to create novel Bt strains has certain limitations. Not all the Bt toxin genes are located on transferable plasmids. Second, the toxin protein with useful insecticidal activity may be synthesized at low amounts. Plasmid incompatibility could also be a problem. A significant advantage to the conjugal transfer approach is the simplified registration process for the Bt product. The U.S. Environmental Protection Agency treats transconjugants in a similar manner as it treats wild-type Bt isolates.

Another interesting approach to expand the insecticidal host range of Bt is to make use of the *in vivo* genetic recombination property (Baum *et al.*, 1990). Lereclus *et al.* (1992) used insertion sequence IS232 to deliver *cryIIIA* gene into an isolate producing CryIAc toxin. Expression of the introduced gene did not alter the composition of the polypeptides normally produced by the strain. Novel Bt mutants, defective in sporulation but overproducers of toxin, have been isolated (Lereclus *et al.*, 1995). They can be used safely as a biopesticide in silkworm-rearing areas . These mutants were shown to achieve effective control of *H. armigera* in field-grown chick-pea (Satyanarayana, *et al.*, 1995).

B. Construction of Recombinant Bt Strains

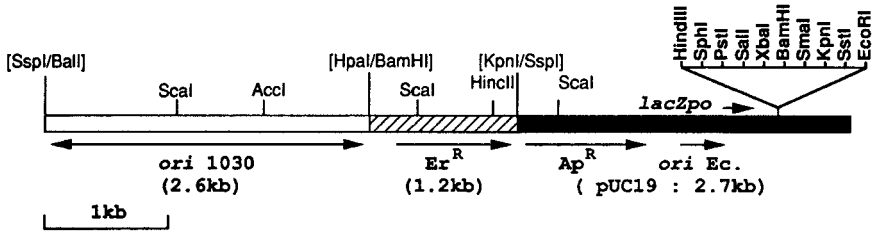
Development of novel cloning vectors for Bt has made possible the construction of improved Bt strains for use as microbial insecticides. The use of Bt as the host organism offers many advantages. Native Bt strains can stably maintain and efficiently express several homologous Bt toxin genes. The ability to maintain multiple Bt toxin genes in a single recipient broadens the insecticidal activity in an additive or synergistic manner. Multiple toxin genes with differing modes of action or receptor-binding properties may reduce the chances of insects developing resistance (Tabashnik, 1994).

An essential element in the successful engineering of Bt strains is the availability of suitable cloning vectors. A number of convenient shuttle vectors, functional in *E.coli* and *Bacillus* species, have been constructed

using replication origins from resident Bt plasmid (Baum *et al.,* 1990; Gawron-Burke and Baum, 1991). Considering the stability of resident Bt plasmids, shuttle vectors derived from resident plasmids might exhibit good segregational stability. Lereclus and Arantes (1992) selected a replication origin from a small cryptic plasmid of *Bacillus subtilis* (pHT1030) that exhibits excellent segregational stability. They constructed shuttle vectors (Lereclus *et al.,*1989; Arantes and Lereclus, 1991; Figure 6) to introduce new Bt toxin genes into Bt strains. For instance, *cryIIIA* gene from *Bacillus tenebrionis,* when introduced into Bt. *kurstaki* HD119, was highly expressed without affecting the level of expression of native *cry* genes (Gamel and Piot, 1992). Shin *et al.,* (1995) studied the distribution of cryV-type insecticidal protein genes in *B. thuringiensis* and cloned *cryV*-type genes from *B. thuringiensis* subsp. *kurstaki* and *B. thuringiensis* subsp. *entomocidus.* Wu and Federici (1995) improved production of the insecticidal CryIVD protein in *B. thuringiensis* using *cryIA(c)* promoters to express the gene for an associated 20-kDa protein. Ely (1995) constructed insecticidal proteins from *B. thuringiensis* δ-endotoxin and *Androctonus australis* neurotoxin AaHIT.

C. Construction of Transgenic Microbes

Cloned Bt toxin genes were introduced into a number of microbial hosts to create more stable and/or compatible agents for the toxin delivery. Monsanto scientists were the first to report the expression of the *cryIAb* gene in a root colonizing *Pseudomonas* at levels sufficient to kill lepidopteran larvae (Watrud *et al.,* 1985). The gene was later cloned into Tn5 and transposed into the chromosome of six corn root-colonizing



pHT304: 4±1 copies/chromosome
pHT315: 15±5 copies/chromosome
pHT370: 70±20 copies/chromosome

Fig. 6. Vectors for cloning genes in *B. thuringiensis* and *B. subtilis*; Gene 1991 108: 115-119 (Reproduced with permission from D. Lereclus, Institut Pasteur, Paris).

strains of *Pseudomonas fluorescens* and *Agrobacterium radiobacter* (Obukowicz *et al.*, 1986). Following this, many groups developed *Psuedomonas* strains carrying Bt toxin genes. The recombinant *Psuedomonas* is killed by a proprietary chemical treatment that crosslinks the bacterial cell wall to yield a nonviable encapsulated bacterium surrounding the crystal protein (Cell-Cap product of Mycogen; Gaertner *et al.*, 1993). Such a product is stable and safe for use in the environment.

An interesting example of a toxin gene in a foreign bacterium is the introduction of the *cryIAc* gene into the plant endophyte, *Clavibacter xyli* subsp. *cyanodontis* (Turner *et al.*, 1991). *Clavibacter* resides in the xylem of Bermuda grass *(Cynodon dactylon).* It also colonizes the vascular system of corn when artificially inoculated. The recombinant bacterium can be inoculated into the stems to establish an endogenous supply of the toxin for protection against European corn borer. Recently, *cryIAc* was introduced into the chromosome of *C. xyli* by using an integrative plasmid vector that facilitates homologous recombination between the vector and the bacterial chromosome (Lampel *et al.*, 1994). It is expected that this recombinant strain will show stability and *in planta* biological activity.

Introduction of Bt genes into root-nodulating bacteria, thereby providing protection to nodules from soil-dwelling pests, was accomplished by Nambiar *et al.* (1990). Recombinant *Bradyrhizobium* carrying *cryIVD* was produced and used to infect the roots of pigeon pea. The root nodule infestation by the larvae of the dipteran species, Rivella angulata, was reduced by 40%. Bezdicek *et al.* (1994) introduced the *cryIII* gene into *Rhizobium leguminosarum* and *R.meliloti* by using a broad host range vector, pRK311, containing *lacZ* promoter or *nifH* promoter. The recombinant rhizobia expressed the toxin in sufficient quantities within root nodules to significantly reduce feeding damage by the nodule-feeding insects, *Sitona lineatus* on *Pisum sativum* and *Sitona hispidulus* on *Medicago sativa.* The pRK311 plasmid remained stable in the rhizobia that were either free living or within nodules of the legumes. The engineered strains of *R.leguminosarum* were equally competitive with the wild-type strain. Udayasuriyan *et al.*, (1995) transferred an insecticidal protein gene of *B. thuringiensis* into plant-colonizing *Azospirillum* that may be used to control root-feeding insects. Mosquitocidal Bt toxin genes were also shuffled between *Bacillus sphaericus* and Bt subsp. *israelensis* to extend the host range of the bacteria for mosquito larvae (Bourgouin *et al.*, 1990; Bar *et al.*, 1991). The *cryIVA* gene of Bt subsp. *israelensis* was also introduced into various unicellular cyanobacteria with the intent of providing a more accessible source of the toxin for filter-feeding dipteran larvae (Angsuthanasombat and Panyim, 1989; Chungiatupornchai, 1990; Soltes-Rak *et al.*, 1993).

D. Baculoviruses as Bt Vectors

It was demonstrated that insects are susceptible to a wide variety of virus infections (King *et al.,* 1994). Many viruses are currently identified in insect hosts out of which baculoviruses carrying large, covalently closed, circular DNA genomes are useful as insect biocontrol agents. Two studies reported the insertion of Bt genes into the *Autographa californica* nuclear polyhedrosis virus genome (Martens *et al.,* 1990; Merryweather *et al.,* 1990). A full-length copy of the endotoxin coding sequence was inserted into the baculovirus genome in place of the polyhedrin gene-coding region. Martens *et al.* (1990) demonstrated that the protein produced in insect cells formed large crystals as inclusion bodies in the cytoplasm. When insect larvae were fed recombinant virus-infected cell extracts, the larvae perished. Merryweather *et al.* (1990) also cloned Bt gene upstream of the polyhedrin gene under the control of the AcNPV p10 promoter. When insects were given purified polyhedra in a bioassay, there was no effect on the larvae. It was possible that the protoxin produced by the virus remained as an intracellular protein and did not get processed and solubilized in the insect midgut for eventual toxicity. Addition of a suitable signal peptide sequence to the Bt toxin gene would facilitate secretion of the recombinant product from the cells into midgut and thereby improve its efficacy.

E. Insect-Tolerant Transgenic Crop Plants

An elegant, and perhaps the most effective delivery system for Bt toxins, is the transgenic plant (Stewart *et al.,* 1995). The major benefits of this system are economic, environmental, and qualitative. In addition to the reduced input costs to the farmer, the transgenic plants provide season-long protection independent of weather conditions, effective control of burrowing insects difficult to reach with sprays, and control at all of the stages of insect development. The important feature of such a system is that only insects eating the crop are exposed to the toxin. Genetic transformation of almost all the major crop species is now feasible with the development of an array of techniques ranging from the *Agrobacterium* approach to electric discharge-mediated particle acceleration procedure (Finch, 1994).

The first Bt-transgenic plants were made in 1987 (Barton *et al.,*1987; Fischhoff *et al.,* 1987; Vaeck *et al.,* 1987). The plants expressed full-length or truncated Bt toxin genes (*cryIA*) under the control of constitutive promoters. The expression of the toxin protein was very poor in the tobacco plants and the mortality of *M. sexta* larvae was only 20%. Truncated *cryIA* genes coding for the toxic N-terminal fragment provided better protection to the tobacco and tomato plants. When compared to

the plants transformed with full-length genes, the plants expressing truncated genes were more resistant to the larvae, and the highest reported level of toxin protein expression was about 0.02% of total leaf-soluble protein. Despite these low levels of expression, many of the plants were shown to be insecticidal to the larvae of *M. sexta*. However, many of the noctuid lepidopterans, which constitute a very serious group of insect pests, need higher amounts of Bt toxins for effective control. Gene truncation as well as the use of different promoters, enhancer sequences, and fusion proteins resulted in only limited improvement in Bt gene expression (Barton *et al.*, 1987; Carozzi *et al.*, 1992; Vaeck *et al.*, 1987).

In 1990, researchers at Monsanto made a significant advancement in the expression of Bt genes in plants (Perlak *et al.*, 1990). They noticed that Bt genes were excessively AT rich in comparison with normal plant genes. This bias in nucleotide composition of the DNA could have a number of deleterious consequences to gene expression because AT-rich regions in plants are often found in introns or have a regulatory role in determining polyadenylation. There are also instances in other eukaryotic systems in which AT-rich regions can signal rapid degradation of specific mRNAs. In addition, plants have a tendency to use G or C in the third base of redundant codons—A or T being rarer. Bt genes have the opposite tendency and because codon preference is thought to be linked to the abundance of the corresponding tRNAs, the overuse of rare codons would decrease the rate of synthesis of a Bt protein in plant cells.

Perlak *et al.* (1991) followed two approaches to modify the *cryIAb* and *cryIAc* genes. One approach included selective removal of DNA sequences predicted to inhibit efficient expression of Bt gene expression at both translational and mRNA levels by site-directed mutagenesis. These genes were termed partially modified (PM) genes. The other approach was to generate a synthetic gene with a fully modified (FM) nucleotide sequence, taking into account factors such as codon usage in higher plants, potential secondary structure of mRNA, and potential regulatory sequences. The PM-*cryIAb* gene is approximately 96% homologous to the native gene with a GC content of 41%, with the number of potential plant polyadenylation signal sequences (PPSS) reduced from 18 to 7 and the number of ATTTA sequences reduced from 13 to 7. The FM-*cryIAb* is approximately 79% homologous to the native gene, with a GC content of 49% and the number of PPSS reduced to 1 and all ATTTA sequences removed. The toxin protein levels in transgenic tobacco and tomato harboring these modified genes increased up to 100-fold over levels seen with the wild-type Bt gene in plants.

Perlak *et al.* (1990) made a gene construct in which the first 1359 nucleotides were derived from FM-*cryIAb* gene and the remaining se-

quence from *PM-cryIAc* gene. The variant gene was placed under the control of CaMV 35S promoter containing a duplicated enhancer region. Cotton-variety Coker 312 was transformed and the transgenic plants were shown to have total protection from *Trichoplusia ni* (Cabbage looper), *S. exigua,* and *H. zea* (cotton boll worm). The maximum level of toxin protein was 0.1% of total soluble protein.

The Monsanto group placed the FM-*cryIAc* gene under the control of *Arabidopsis thaliana* Rubisco small subunit promoter with its associated chloroplast transit peptide sequence (Wong *et al.,* 1992). Transgenic tobacco plants expressing this gene provided a 10- to 20-fold increase in *cryIAc* mRNA and protein compared to gene constructs in which CaMV 35S promoter with duplicated enhancer region was used to express the same gene. The toxin protein was localized in the chloroplast and in the tobacco plants that produce the Bt protein nearly 1% of the total leaf protein had the highest levels of Bt toxin proteins yet reported. The enhancement of Bt toxin protein levels in tissues in which Rubisco expression is highest may lead to very effective control of certain insect pests that feed on leaves and other green tissues.

Ciba Seeds, a subsidiary of Ciba Geigy Company, used microprojectile bombardment with two plasmids (pCIB4431 and pCIB3064) of a proprietary corn line to produce a commercial cultivar (*Federal Register* 60 *FR* 9656-9657 1995). Plasmid pCIB4431 contains two different tissue-specific promoters each fused individually to a copy of a synthetic *cryIAb* gene. The *cryIA(b)* gene encodes the first 648 amino acids, with an insecticidal-active (Koziel *et al.,* 1993) truncated product identical to that of the *cryIAb* gene of *B. thuringiensis* subsp. *kurstaki* strain HD-1 (Dulmage, 1970; Geiser *et al.,* 1986; Hofte and Whiteley, 1989). The truncated synthetic gene accommodates the preferred codon usage for maize (Murray *et al.,* 1989) that allows efficient expression of the *cryIAb* gene in plants (Perlak *et al.,* 1991; Koziel *et al.,* 1993). The modified gene has about 65% homology at the nucleotide level with the native gene and G+C content has been altered from 38 to 65%. The transgenic plant produces a protein that is identical to the first 648 amino acids of the full-length 1155-amino acid *CryIA(b)* protoxin that occurs in nature. This truncated protein contains the portion of the native protein that is responsible for its insecticidal activity. The first promoter is derived from the corn phosphoenolpyruvate carboxylase (PEPC) gene (Hudspeth and Grula, 1989). It promotes expression of *cryIAb* in green tissue. The second pollen-specific promoter used is derived from a maize calcium-dependent protein kinase (CDPK) gene (Estruch *et al.,* 1994). The combination of PEPC and pollen tissue-specific promoters provides high *cryIAb* gene expression in leaves and pollen, where it is

most effective in controlling European corn borer. PEPC intron 9 of the corn phosphoenolpyruvate carboxylase gene (Hudspeth and Grula, 1989) is located between the *cryIA(b)* structural gene and the 35S terminator. Its presence also increases the expression level of the *cryIA(b)* gene (Luehrsen and Walbot, 1991). The 3' untranslated termination sequences (CaMV) 35S from the cauliflower mosaic virus (CaMV) is present adjacent to the PEPC intron 9. Its function is to provide a polyadenylation site and it has been described previously (Rothstein *et al.*, 1987; Sanfacon *et al.*, 1991). The activity of the pollen-specific promoter, associated with its native CDPK structural gene in maize, is not modulated by calcium levels in the plant. Rather, the catalytic activity of the mature CDPK protein in maize is affected by calcium levels. Therefore, fusion of this promoter sequence to the *cryIAb* will not manifest in any changes in the calcium requirements of corn. High levels of CryIAb protein were obtained using both promoter configurations in the transgenic maize plants. Hybrid maize plants resulting from crosses of transgenic elite inbred plants with commercial inbred lines were evaluated for resistance to European corn borer *(Ostrinia nubilalis)* under field conditions. Plants expressing high levels of the insecticidal protein exhibited complete resistance to heavy infestations of the pest.

A similar approach was followed by Fujimoto *et al.* (1993) to enhance *cryIAb* gene expression in rice plants. Based on the codon usage of known rice genes, 66.6% of the codons in the coding region of the *cryIAb* gene were altered. The overall G+C content of the modified gene was 59.2%, whereas that of the original gene was 37.6%. The monocotyledons, including cereals, have higher G+C contents than those from dicots. The level of expression of the modified gene in transgenic rice was 0.05% of total soluble leaf protein. The plants were significantly resistant to two lepidopteran rice pests, leaf folder *(Cnaphalocrosis medinalis)* and stem borer *(Chilo suppressalis).*

Following the successful attempts to control lepidopteran insects by using FM-*cryIA* genes, synthetic *cryIII* genes were also made and expressed in tobacco and potato plants, primarily for the control of Colorado potato beetle (Coleoptera) (Sutton *et al.*, 1992; Perlak *et al.*, 1993). The Russet Burbank potatoes were protected from damage by all insect stages in the laboratory, and dramatic protection was discernible at multiple field locations (Perlak *et al.*, 1993).

Van der Salm *et al.* (1994) developed transgenic tobacco and tomato plants expressing two Bt genes, *cryIAb* and *cryIC,* specific toward lepidopteran insects. Both of the genes were partially modified to remove sequence motifs that affect mRNA stability in plant cells. The expression of a *cryIAb–cryIC* fusion gene resulted in protection against *S. exigua, Heliothis virescens,* and *M. sexta.* This study demonstrated the potential

of expressing translational fusions not only to broaden the insect resistance of transgenic plants, but also to simultaneously employ different gene classes in resistance management strategies (see Section VIII).

Recently, researchers at Calgene, in collaboration with Maliga and Svab (1993) (Waksman Institute), expressed a *cryIA* gene in tobacco chloroplasts using chloroplast transformation vectors and particle bombardment technique. The transplastomic tobacco expressed the Bt toxin at very high levels and achieved complete control of lepidopteran larvae (McBride *et al.,* 1995). The advantages of such a system are manyfold:

1. The Bt gene does not need any modification because the chloroplast transcriptional and translational apparatus are typically prokaryotic;
2. It is possible to have many copies of the Bt gene in each cell;
3. The expression of the gene will be high if driven by promoters like *rbcL* and *cab;* and
4. Because chloroplasts are maternally inherited, there is no risk of pollen transfer of the Bt gene to related plant species or weeds. The disadvantage of this approach lies in its tissue specificity. For instance, stem and fruit borers cannot be controlled following this method.

Most of the transgenic plants developed so far contained the Bt toxin genes under the control of the powerful, constitutively active 35S promoter. However, expression of the Bt toxin gene throughout the plant growth and development and in tissues in which it is not needed may encourage resistance development by the target insect (Harris, 1991). Kumar and Sharma (1994) reviewed alternative approaches like using wound and light-inducible promoters, tissue-specific promoters, and promoters responsive to chemical sprays being used in different laboratories.

VII. Resistance Development and Management Strategies

Resistance will eventually develop as a result of widespread use of any biopesticide. Resistance to *B. thuringiensis* endotoxins has already been developed in the laboratory (Tabashnik, 1994) that can be initiated by alteration of the target of insect–toxin interaction (Gould *et al.* 1992; MacIntosh *et al.,* 1991; Van Rie *et al.,* 1990b). In insect-tolerant transgenic plants, solubility and proteolytic processing are bypassed because only the toxin-soluble core of the Cry protein is produced. Transgenic plants with multiple genes coupled with other management strategies might slow resistance development. Although resistant mosquitoes have been selected with individual toxins such as CryIIA, due to the combination of four Cry toxins and the spore, mosquito resistance will be hard to evolve against BTI (Georghiou, 1994; Goldman *et al.,* 1986). Bt had been used as a biopesticide for more than two decades. Evolution

of resistance was presumed unlikely because of the lack of reports of substantial resistance development in open field populations (de Barjac, 1987). However, resistance to Bt was documented in field populations of diamondback moth (Tabashnik *et al.*, 1990, 1991; Rabindra *et al.*, 1995). These and many other reports confirmed doubts raised by the results of laboratory selection for resistance to Bt in several major pests (McGaughey, 1985; McGaughey and Beeman, 1988). Various aspects of insect's resistance to Bt viz. laboratory selection, resistance risk assessment, variation among conspecific populations, mechanisms, cross-resistance, genetics, stability, fitness costs, and management were recently reviewed (McGaughey, 1994; Tabashnik, 1994; Kennedy and Whalon, 1995). In this section, the mechanisms involved in resistance and strategies to manage its development are explored.

Intensive selection pressure on insect populations inevitably leads to the development of resistance. The resistance could be achieved by different mechanisms ranging from the point of protoxin ingestion to the insertion of toxin in the membrane. The factors affecting the binding of toxin to the receptor would result in selective resistance. On the other hand, those steps utilized by all the toxins viz. proteolysis of protoxins, conformational alterations, and membrane insertion may lead to cross-resistance. Studies revealed that midgut pH and the nature of proteases probably were not involved in achieving resistance (Kinsinger and McGaughey, 1979; Johnson *et al.*, 1990). Reduced binding of Bt toxin to the brush border membrane of the midgut epithelium was identified as a primary mechanism of resistance in *Plodia interpunctella* (Van Rie *et al.*, 1990c) and *Plodia xylostella* (Bravo *et al.*, 1992 a,b; Ferre *et al.*, 1991). Studies with radioactive-labeled CryIAb showed that a 50-fold reduction in binding was correlated with a 100-fold reduction in toxicity of CryIAb in a resistant versus a susceptible strain of *P. interpunctella* (Van Rie *et al.*, 1990c). A strain of *P. xylostella* from the Phillippines showed a 200-fold resistance to CryIAb and little or no binding of the toxin to the midgut epithelial membrane compared to a susceptible strain.

In contrast to the results for *P. interpunctella* and *P. xylostella*, two independent studies on *H. virescens* found no clear association between toxin binding and resistance to CryIAb or CryIAc (MacIntosh *et al.*, 1991; Gould *et al.*, 1992). The only evidence against involvement of the binding step in the mechanism of both resistance and specificity was presented by Wolfersberger (1990). He found that in *Lymantria dispar* there was a negative relationship between binding affinity and toxicity of two different Bt toxins toward a single strain of insect. That is, the more toxic protein is bound with less affinity than the less toxic one.

Wolfersberger's results are consistent with the idea that there could be differences in toxicity as well as differences in binding affinity.

An observation of considerable significance was that of resistance development in *P. interpunctella* to multiple toxins (McGaughey and Whalon, 1992). Selection of *P. interpunctella* colonies resistant to Bt isolates, known to contain multiple protoxins, resulted in the isolation of colonies resistant to several toxins (McGaughey and Johnson, 1993). The apparent frequency of such resistant colonies appears to be too high for two or more independent mutations, each altering a specific receptor. It is possible that resistance is due to the mutation of one locus affecting the ability of a variety of toxins as in the *H. virescens* colony with broad resistance (Gould *et al.,* 1992). It is also possible that these receptors may somehow interact or cluster, such that a single mutation affects the binding properties of several toxins (Aronson, 1994).

With the realization that insects can develop resistance to Bt, attention is now being focused on developing deployment strategies that might delay or prevent its evolution. Theoretically, resistance to conventionally sprayed Bt could develop slower and be narrower in scope and easier to manage than resistance to synthetic organic insecticides because Bt has a shorter residual period and much narrower spectrum of biological activity. Expression of Bt toxins in other bacteria or addition of ultraviolet blockers to formulations can extend the persistance of Bt, making it comparable to organic insecticides (Tabashnik, 1994). Expression of Bt in transgenic plants may continuously select pests intensively for resistance because insects are exposed to Bt even when they are not causing economic damage (Mallet and Porter, 1992). Various strategies were suggested to tackle the problem of resistance development and have been summarized by Whalon and McGaughey (1993). These tactics were patterned after those used or proposed for use in managing chemical insecticide resistance and typically involve variations of the following: (i) rotation or alteration of toxins, (ii) mixtures or sequences of toxins, (iii) provision of refuges, (iv) ultrahigh doses of toxin, and (v) temporal and spatial expression of Bt toxin genes in transgenic plants.

A. Rotations

Rotation or alteration of Bt toxins, insecticides, and cultural or biological control strategies is probably the simplest approach to resistance management. Success with this tactic depends on restoring susceptibility when selection pressure is discontinued or changed to another gene, toxin, or insecticide. However, rotations among toxins that confer cross-resistance to each other have limited value (Gould, 1988;

Gould *et al.,* 1992). Studies indicating considerable instability of resistance to Bt in *P. xylostella* (Hama *et al,* 1992) and *H. virescens* (Sims and Stone, 1991), and one case of negative cross-resistance in *P. interpunctella* (Van Rie *et al.,* 1990c), suggest that rotations might slow resistance development in certain situations. However, McGaughey and Beeman (1988) found that high levels of resistance in *P. interpunctella* were stable for long periods, and in such cases rotations may not be effective.

B. Mixtures of Toxins

Mixtures of toxins is also a relatively simple tactic that is possible in both conventional applications and transgenic plants. It is based on the idea that if resistance to each component in a mixture is rare, then individuals with resistance to all components will be exceedingly rare or absent. However, extensive cross-resistance among different Bt toxins may reduce the likelihood that mixtures will effectively control resistance (Gould *et al.,* 1992). Many field populations of *P. xylostella* evolved resistance to Bt formulations that contain mixtures of up to five toxins (Tabashnik *et al.,* 1990). In laboratory tests, *P. interpunctella* readily became resistant to a mixture of two Bt strains that contained at least six CryIA, CryIC, and CryII toxins (McGaughey and Johnson, 1992). Further research is needed to elucidate the patterns of response of different insect species to Bt mixtures before a suitable recommendation is made that assures prevention of resistance.

C. Refuges

Facilitating the survival of susceptible insects is one of the best approaches to slow resistance development. Results from modeling studies demonstrated that refuges and immigration of susceptible insects into pest populations can slow the evolution of resistance (Tabashnik, 1990). This was supported by the results from laboratory experiments on *H. virescens* and *P. xylostella* (Gould and Anderson, 1991; Schwartz *et al.,* 1991b). Spatial and temporal employment of refuges and factors affecting their efficacy need to be worked out at the field level. Spatial refuges facilitate random mating between susceptible and resistant adults and may limit movement of larvae between Bt-treated and untreated plants (Mallet and Porter, 1992). Spatial refuges can be provided among tissues within plants by ensuring tissue-specific expression of the Bt gene, among plants within fields by growing transgenic and nontransgenic plants in a defined ratio, or between fields in which neigh-

boring fields are sown with plant varieties differing in their susceptibility to a given insect.

D. Toxin Doses

There are two approaches dealing with high as well as low doses of Bt toxin application to circumvent resistance problems. The low-dose approach includes reduced rates and frequency of application, reduced thoroughness of application, and transgenic plants with low expression of toxin. This tactic aims to reduce populations only slightly or slow larval development to the point that the number of generations per year is reduced or natural enemies are more effective. However, this approach is not practical because farmers and pest managers prefer products that prevent any damage.

Denholm and Rowland (1992) advocated a high-dose strategy in conjunction with untreated refuges as a potential means of managing resistance development in transgenic plants. This approach maintains that constitutive and continuous expression of Bt toxins in transgenic plants may be sufficient to kill all of the heterozygotes in a population (McGaughey and Whalon, 1992). This approach is not possible with conventional Bt applications because foliar applications never cover the entire plant and do not persist long enough to achieve "continuous" expression of Bt (Whalon and McGaughey, 1993).

A high dose can be defined as that which consistently kills heterozygotes (Whalon and McGaughey, 1993). Determination of this dose is dependent on the genetics of resistance. It would be lowest in cases in which resistance is inherited recessively and highest in cases in which it is completely dominant. Because homozygous-resistant individuals are at a very low frequency early in the evolution of resistance and suitable refuges provide a continuous source of susceptible individuals, this tactic should be quite durable (Whalon and McGaughey, 1993).

An extremely high dose or ultrahigh dose is possible where target insects are very sensitive and Bt expression in transgenic plants is very high (1% of total protein). This dose is sufficiently high to kill even homozygous-resistant individuals. However, doubts persist because doses as high as 268 g/liter of a *B. thuringiensis* subsp. *kurstaki* formulation could not kill resistant individuals of *P. xylostella* (Tabashnik *et al.*, 1993). As discussed previously, binding affinity for toxins is a primary mechanism of resistance in *P. xylostella*. If binding affinity approaches zero, attempts to kill resistant insects with high doses may be futile (Tabashnik, 1994).

E. Gene Expression

Spatial, temporal, and inducible expression of Bt genes in transgenic plants is one of the features of management strategies. Continuous and constitutive expression of Bt genes results in significant selection pressure on pest populations. Tissue-specific (leaf, stem, root, boll, pod, or seed), stage-specific (vegetative or reproductive), and wound-specific promoters are now available that can be employed to rationalize Bt gene expression. Chemical sprays like that of salicylic acid can be used to induce Bt gene expression at will by using suitable promoters (Williams *et al.*, 1992). All these approaches need to be experimentally verified in a thorough manner.

Unfortunately, no transgenic plants have been experimentally evaluated with Bt-resistant insects. More work is needed to assess the role of behavior and other biological, ecological, and genetic factors in resistance development to Bt and to Bt transgenic plants.

VIII. Epilogue

Both chemical and microbial insecticides are currently used for insect control. Among chemical insecticides organophosphates (Counter, Dyfonate, Lorsban, Thimet, Parathion, and Penncap), pyrethroids (Ambush, Pounce, and Capture), carbamate (Furadan) and others (Asana XL) are used. Although organophosphates and pyrethroids can be effective, careful insect surveillance is required. Applications must be carefully timed to reach certain insect populations before the insects bore into the stalk and other plant organelles, and repeated applications are often necessary.

A class of insecticidal proteins, known as δ-endotoxins, are produced as parasporal crystals by *B. thuringiensis* in nature. These proteins are quite selective in their toxicity to specific organisms. The crystal proteins are typically produced as large protoxins. Following ingestion by a susceptible insect, the protoxin is solubilized in the alkaline insect gut, and then activated by digestive enzymes to yield a smaller protein. The activated protein binds to specific receptors in the insect midgut and brings about cell lysis by formation of pores. Cessation of feeding and death of the insects follow. These naturally occurring insecticidal proteins have been commercially produced and used as insecticides for decades. An extensive body of safety testing and experience supports their lack of toxicity to humans and animals and the absence of adverse effects on nontarget organisms and the environment.

Bacillus thuringiensis var. *kurstaki* (Btk) preparations are registered for use on corn, vegetables, cotton, deciduous nuts, and fruits. As crystalline powder formulations, Btk has been used commercially as an in-

secticide under the trade name Dipel. Availability of recombinant DNA technology has provided the opportunity of expressing these biocidal proteins in various organisms (Table IV). The production of insect control protein by various crop plants represents a potentially important new option in pest control and an attractive alternative to external application of insecticides. Transgenic plants producing the insecticidal proteins are quite effective in controlling various crop pests, even though only minute quantities are produced (Table V). Plants are being engineered to preferentially express the insect control protein in desired tissues, while minimizing its production in other plant tissues in which it is not needed for control of the target pest.

Transgenic plants hold great promise as an important new tool in integrated pest management programs. This technology allows the crop plant to deliver its own means of protection against insect attack. The expected result is a very specific and directed biological control method that is environmentally sound and that can be expected to reduce the need for manual and chemical inputs by the grower. Commercial bioinsecticide formulations are generally ineffective in controlling ECB on corn in which topical applications of the powder do not reach the inside of the plant tissue where the insects bore and feed (Bartels and Hutchison, 1995). Such transgenic crops provide farmers a means of controlling a serious insect pest that is not easily controlled by current chemical pesticides. Other advantages include: (i) reducing the risks associated with environmental spills or misapplication of chemical insecticides; (ii) eliminating unwanted effects on beneficial insect populations (which can be susceptible to conventional chemical applications)—these beneficial insects can, in turn, further reduce the reliance on chemical means of pest control; and (iii) reducing the consumption of fossil fuels required to deliver chemical inputs by machinery.

Because of the environmental pollution and associated toxicity with chemical insecticides, biological insect control has a bright future. Various mutant forms of insecticidal proteins with improved biological activity will be created in the future by fusing diverse domains (Hon'ee *et al.,* 1990) and *in vitro* mutagenesis of genes that codes for these biological agents (Aronson *et al.,* 1995; Rajamohan *et al.,* 1995).

Mosquitoes and blackflies are vectors of a multitude of diseases of man and animals through transmission of pathogenic viruses, bacteria, protozoa, and nematodes. At the molecular level, the processed toxin binds to a specific receptor molecule located on the plasma membrane of the susceptible insect midgut. This initial binding could account for the specificity of the toxin. After binding to the receptor, the toxin creates small pores in the gut membrane leading to colloidal–osmotic lysis

TABLE IV

BT GENES EXPRESSED IN VARIOUS ORGANISMS

Gene	Donor	Recipient plant	Institution
cry	Bt	Clavibacter	Crop genetics
cryIA(a)	Bt	Cranberry	University of Wisconsin
	Btk	Cotton	Agracetus
	Btt	Potato	ARS
cryIA(b)	Bt	Corn	Ciba-Geigy; Monsanto
		Tobacco	Rohm and Haas; Sandoz
	Btk	Corn	Ciba-Geigy; Northrup King
		Cotton	Monsanto; Northrup King
		Rapeseed	Agrigenetics
		Tobacco	Ciba-Geigy; North Carolina State University
		Tomato	Campbell; Monsanto; Northrup King; Rogers NK
	Btt	Corn	Northrup King
cryIA(c)	Bt	Corn	Monsanto
		Cotton	American Cyanamid; Miles; Monsanto
		Potato	Michigan State University
		Rapeseed	University of Georgia
	Btk	*Amelanchier laevis*	Dow
		Apple	University of California/Davis
		Brassica oleracea	Cornell University
		Clavibacter	Crop Genetics
		Corn	Crop Genetics; Monsanto
		Cotton	CalGene; Monsanto; Northrup King
		Poplar	University of Wisconsin
		Rapeseed	University of Chicago
		Spruce	University of Wisconsin
		Tobacco	CalGene
		Tomato	Agrigenetics; Campbell; Monsanto
		Walnut	ARS; University of California/ Davis
cryIB	Btt	Potato	ARS
cryIIA	Btk	Potato	Monsanto
cryIIIA	Bt	Eggplant	Rutgers University
		Potato	Monsanto
	Btk	Potato	ARS; Monsanto
	Btt	Eggplant	Rutgers University
		Potato	Frito-Lay; Monsanto

TABLE V

BT ENGINEERED CROPS[a]

Crop	Company/institution
Corn	Ciba-Geigy; DeKalb; Dow Elanco; Hunt-Wesson; Monsanto; Mycogen; North Carolina State University; Northrup King; Pioneer Hi-Bred; Rogers NK Seed
Rice	Louisiana State University
Cotton	Agracetus; American Cyanamid; Calgene; Delta and Pine Land; Miles; Monsanto
Apple	University of California
Potato	Frito-Lay; Michigan State University; Monsanto; U.S. Department of Agriculture
Tomato	Campbell; Monsanto; Rogers NK Seed; Sandoz
Eggplant	Rutgers University
Canola (oilseed rape)	AgriGenetics; University of Chicago; University of Georgia
Alfalfa	Mycogen
Walnut	U.S. Department of Agriculture
Tobacco	Agrigenetics; Calgene; Ciba-Geigy; North Carolina State University; Rohm and Haas
Poplar	University of Wisconsin
Spruce	University of Wisconsin
Cranberry	University of Wisconsin

[a] Since 1987, 14 crops and trees engineered to express the Bt toxin gene have been field tested in the United States by the companies and institutions shown in the table. Source: Applications and notifications submitted since 1987 to the U.S. Department of Agriculture to field test genetically engineered plants. Other Bt plants are under development, but have not reached the field test stage.

and kills the larvae rapidly. The receptor for an insecticidal protein of *B. thuringiensis* has been cloned (Vadlomude *et al.*, 1995).

Rajamohan *et al.*, (1995) and Chen *et al.*, (1995) studied the binding of the toxin to the receptor. They showed that the binding is a two-step process in which the irreversible binding is directly correlated to insect toxicity and not the initial binding. The amino acids of CryIAb toxin involved in the irreversible binding to the receptor are F37, and G374 of CryIAb toxin. Rajamohan *et al.*, (1994) also identified the amino acids (365–370) essential for the toxicity of another toxin, CryIAa, to *B. mori*. They also constructed several mutant toxins that increased toxicity, especially to gypsy moth (a forest pest insect) about 7–10 times more potent than the parental toxin. Hybrid wide-spectrum toxins, by switching the toxicity determining regions of different Cry toxins, may improve toxicity and yield a toxin with multiple insect specificity through protein engineering.

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