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Bacillus thuringiensis as a Specific, Safe, and Effective Tool for Insect Pest Control

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Bacillus thuringiensis (Bt) was first described by Berliner [10] when he isolated a *Bacillus* species from the Mediterranean flour moth, *Anagasta kuehniella*, and named it after the province Thuringia in Germany where the infected moth was found. Although this was the first description under the name *B. thuringiensis*, it was not the first isolation. In 1901, a Japanese biologist, Ishiwata Shigetane, discovered a previously undescribed bacterium as the causative agent of a disease afflicting silkworms. Bt was originally considered a risk for silkworm rearing but it has become the heart of microbial insect control. The earliest commercial production began in France in 1938, under the name Sporeine [72]. A resurgence of interest in Bt has been attributed to Edward Steinhaus [105], who obtained a culture in 1942 and attracted attention to the potential of Bt through his subsequent studies. In 1956, T. Angus [3] demonstrated that the crystalline protein inclusions formed in the course of sporulation were responsible for the insecticidal action of Bt. By the early 1980's, Gonzalez *et al.* [48] revealed that the genes coding for crystal proteins were localized on transmissible plasmids, using a plasmid curing technique, and Schnepf and Whiteley [103] first cloned and characterized the genes coding for crystal proteins that had toxicity to larvae of the tobacco hornworm, from plasmid DNA of Bt subsp. *kurstaki* HD-1. This first cloning was followed quickly by the cloning of many other *cry* genes and eventually led to the development of Bt transgenic plants. In the 1980s, several scientists successively demonstrated that plants can be genetically engineered, and finally, Bt cotton reached the market in 1996 [104].

Keywords: *Bacillus thuringiensis*, biopesticide, *cry* gene, Cry protein

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The insecticidal bacterium Bt is a Gram-positive bacterium that produces proteinaceous inclusions during sporulation [53]. These inclusions can be distinguished as distinctively shaped crystals by phase-contrast microscopy. The inclusions are composed of proteins known as crystal proteins, Cry proteins, or δ-endotoxins, which are highly toxic to a wide variety of important agricultural and health-related insect pests as well as other invertebrates. Because of their high specificity and their safety for the environment, crystal proteins are a valuable alternative to chemical pesticides for control of insect pests in agriculture and forestry and in the home. It has been proposed that the rational use of Bt toxins will provide a variety of alternatives for insect control and for overcoming the problem of insect resistance to pesticides.

CLASSIFICATION OF BT

Bt seems to be indigenous to diverse environments. Strains have been isolated worldwide from many habitats, including soil, insects, stored-product dust, and deciduous and coniferous leaves [11, 26, 91]. Bt belongs to the Bacillaceae family and is closely related to *Bacillus cereus* [60, 75]. The only notable phenotypic difference between the two species is the production of one or more insecticidal crystals. However, there are many crystal-producing Bt strains without known insecticidal activities. Additionally, many acrystalliferous strains have been described and can be easily obtained by chemical mutagenesis or by plasmid curing, resulting in cells phenotypically indistinguishable from *B. cereus*. Furthermore, recent studies of a series of molecular markers in a large set of strains have indicated that *B. thuringiensis* and *B. cereus* should be considered as one species [19].

The primary classification of Bt strains are based on their serotypes according to their H flagella antigenic

determinants [33, 34]. To date, up to 69 different serotypes and 13 sub-antigenic groups, giving 82 serovars, have been defined and ranked as subspecies [73]. Although serotyping only reflects one characteristic of the species, it is the most common classification method used throughout the world. However, the flagella serotyping has limitations, proving unreliable as a predictor of insecticidal activity, even though the technique has greatly aided classification of isolates. The serotype Bt subsp. *morrisoni* (H8a8b), for example, includes isolates active against lepidopteran, coleopteran, and dipteran insects, and even includes isolates that are not active [97]. Furthermore, we have raised H-antisera against 27 serovars and isolated several new strains having innate *cry* genes that were not known previously from the same serotype strain [74, 84]. These results also demonstrated that H-serotyping might not be enough to represent the molecular characteristics of a strain.

BT CRY PROTEINS AND THEIR DIVERSITY

Individual Cry toxin has a defined spectrum of insecticidal activity, usually restricted to a few species in one particular order of Lepidoptera (butterflies and moths), Diptera (flies and mosquitoes), Coleoptera (beetles and weevils), Hymenoptera (wasps and bees), and nematodes, respectively [36, 89]. A few toxins have an activity spectrum that spans two or three insect orders. For example, Cry1Ba is most notably active against the larvae of moths, flies, and beetles [119]. The combination of toxins in a given strain, therefore, defines the activity spectrum of that strain.

Most δ-endotoxins are encoded by *cry* genes. The toxins were originally classified into four classes according to their amino acid sequence homology and insecticidal specificities [53]. CryI toxins are toxic to lepidopterans; CryIIs are toxic to lepidopterans and dipterans; CryIIIs are toxic to coleopterans; CryIVs are toxic to dipterans. Two additional classes, CryV and CryVI, were added for the nematode-active toxins [41]. Currently, the toxins are classified only on the basis of amino acid sequence homology, where each protoxin acquired a name consisting of the mnemonic Cry (or Cyt) and four hierarchical ranks consisting of numbers (*e.g.*, Cry25Aa1), depending on its place in a phylogenetic tree [31]. Thus, proteins with less then 45% sequence identity differ in primary rank (Cry1, Cry2, *etc.*), and 78% and 95% identity constitute the borders for secondary and tertiary rank, respectively. This system replaces the old nomenclature that used Roman numerals. The similarity in amino acid sequences of all known Cry proteins was taken as the only criterion for distributing them into classes and subclasses. Some classes, such as Cry6 and Cry15, do not show any significant homology with the rest of the Cry proteins. Occurring as a different group than that of Cry toxins, Cyt toxins have cytolytic activity. These toxins are in the 25–28 kDa mass range and, based on amino acid sequence, are not related to Cry proteins. Interestingly, *cry*-like genes were found in the anaerobic bacterium *Clostridium bifermentans*. The gene products, Cry16A and Cry17A, showed a remarkable mosquitocidal activity, and are the first reported cases of secreted or excreted mosquitocidal toxins derived from an anaerobic bacterium [5].

The main difference between the protoxin and the active toxin sequences is the large carboxyl-terminal end contained in the protoxin sequence. This fragment is highly conserved among some of the protoxin sequences. The putative function of this long carboxyl-terminal segment is to aid Cry proteins as they form an ordered crystalline array. Since most of the cysteine residues are located in this fraction of the protoxin, it has been suggested that an alkaline and reducing condition required for the solubility of these proteins is related to disulfide bridge formation within the protoxin fragment [29]. For example, Bt subsp. *finitimus* produces two inclusions, the first associated with the exosporium membrane and the second outside the exosporium. The various protoxin monomers comprising these crystalline inclusions are linked by intrachain disulfide bridges formed among the conserved cysteine residues localized in the C-terminal half of the protoxins. These disulfide linkages play a key role in both forming and stabilizing the crystalline structure [99]. However, this C-terminal fragment is not found in some toxins (Cry2, Cry3, Cry6, Cry10, and Cry11) or is very small in other protoxins (Cry1I and Cry13A); Cry1Ia and Cry1Ib have only 75 amino acid residues, and Cry13A has 111 residues [16].

MODE OF ACTION OF BT CRY PROTEINS

The *cry* genes code for proteins with a range of molecular masses from 50 to 140 kDa. Upon ingestion by the susceptible insect larvae, protoxins are solubilized and proteolytically digested to release the toxic fragments [53]. During this proteolytic activation, peptides are removed from both amino- and carboxyl-terminal ends of the protoxins. For the 130 to 140 kDa protoxins, the carboxyl-terminal proteolytic activation removes half of the molecule, resulting in an active toxin fragment of 60 to 70 kDa. A generally accepted model for Cry toxin action is that it is a multistage process. First, the activated toxin binds to receptors located on the apical microvillus membrane of epithelial midgut cells [17, 55]. After the toxin binds the receptor, it is thought that there is a change in the toxin's conformation, allowing toxin insertion into the membrane. Oligomerization of the toxin follows, and this oligomer then forms a pore that leads to osmotic cell lysis [87, 101].

The tertiary structures of six crystal proteins, Cry3Aa, Cry1Aa, Cyt2A, Cry2Aa, Cry3Bb, and Cry4Ba, have been solved by X-ray crystallography [15, 43, 51, 81, 82, 95].

An analysis in the accompanying review demonstrates that Cry3A and Cry1Aa show about 36% amino acid sequence identity [31]. This similarity is reflected in their three-dimensional structures. However, Cyt2A shows less than 20% amino acid sequence identity with those of Cry1Aa and Cry3A, and a similar alignment score would be obtained if the Cyt2A sequence were randomized. The Cyt toxins, unlike the Cry proteins, are able to lyse a wide range of cell types *in vitro* [53]. The overall structures of typical Cry proteins (*e.g.*, Cry3A, Cry1A, and Cry4B) possess three domains. Domain I consists of a bundle of seven antiparallel a-helices, in which helix 5 is encircled by the remaining helices. Domain II consists of three antiparallel β-sheets arranged in a so-called β-prism fold. Domain III consists of two twisted, antiparallel β-sheets forming a β-sandwich. Domain II, especially the highly variable loops in its apex, is involved in specific receptor binding as shown by mutagenesis studies [102]. Domain III was found to be involved in the role of recognition during receptor binding and thus in insect specificity [4, 76]. Following binding, at least part of Domain I inserts into the membrane in an oligomer to form an aqueous pore with other toxin molecules. Domain I resembles other pore-forming or membrane-translocation domains of bacterial toxins, and membrane entry might start by insertion of a hydrophobic two-helix hairpin. In an umbrella-like model, based on mutational and biophysical studies, a-helices four and five of several toxin molecules in an oligomer make up the pore, with the rest of the protein spreading over the membrane surface [44].

Activated Cry toxins have two known functions: receptor binding and ion channel formation. The activated toxin readily binds to specific receptors on the apical brush border of the midgut microvilli of susceptible insects. Receptor binding is a key factor in specificity. Two different insect proteins have been identified as receptors for Cry toxins: the 120-kDa aminopeptidase N (APN), also called Cry1Ac toxin-binding protein, purified from brush border vesicles of *Manduca sexta*, *Heliothis virescens*, and *Lymantria dispar* [45, 67, 114], and the 210-kDa cadherin-like glycoprotein, called Cry1Ab toxin-binding protein, purified from *M. sexta* midgut membranes [112]. Insect glycolipids were additionally suggested as a receptor in nematodes [50]. Specific binding involves two steps, one reversible and the other irreversible. Recent data suggest that toxicity correlates with irreversible binding [85]. Irreversible binding might be related to insertion of the toxin into the membrane but could also reflect a tighter interaction of the toxin with the receptor.

ISOLATION OF NEW BT *CRY* GENES

Intercellular crystal inclusions of Bt are comprised of one or more related insecticidal crystal proteins encoded by *cry* and *cyt* genes located mainly on plasmids [102]. There have been over 140 types of Cry proteins and 9 types of Cyt proteins discovered in Bt and several other microorganisms (*e.g.*, *Clostridium bifermentans*, *Paenibacillus popiliae*, *P. lentimorbus*, *B. sphaericus*), and more than 380 *cry* genes have been cloned and sequenced [32]. Finding a new *cry* gene from a natural Bt strain that has a good insecticidal potential is not an easy process, because Bt strains typically harbor between one to six *cry* genes, some of which are known to be cryptic [92]. Several techniques like Southern hybridization, chromatography, and DNA microarray, including the PCR-based method, which is one of the prevalent techniques, have been utilized for analyzing toxin contents or isolating new *cry* genes [9, 58, 79, 92]. In addition, even domain swapping and shuffling strategies have been applied for enlarging the host spectrum or increasing the toxicity of Cry proteins [66]. Screening by hybridization with known probes or degenerate oligonucleotides designed from N-terminal protein sequences is a common method used to find *cry* genes [38]. However, this method is labor intensive, and for large-scale screening of novel strains, another modified hybridization technique using a cocktail of *cry* gene sequences as a probe was reported by Beard *et al.* [7]. PCR-based approaches, using primers designed from conserved regions of known genes, have been widely used. Since Carozzi *et al.* [21] used PCR screening to predict the insecticidal activity of previously uncharacterized Bt strains, PCR-hybridization [62], PCR-RFLP (restriction fragment length polymorphism) [70], E-PCR (exclusive-PCR) [61], *etc.*, have been developed as modified PCR methods and have been used to clone new *cry* genes. Generally, however, these PCR approaches had a limitation in that their PCR products could not be directly used for expression, since the primers in the PCR reaction would be designed to amplify the conserved regions in part of the domain III and partial C-terminal regions.

On the other hand, to facilitate isolation of the intact toxic domain, we have designed two primer sets that enable us to detect the active region of all possible *cry1*-type genes, and have identified five new genes from two Korean isolates, Bt K1 and 2385-1 [84]. Although various novel *cry* genes have been expressed in the Bt Cry$^-$B strain to evaluate the insecticidal activity of these genes [23, 116], the expression of novel *cry* genes in Bt cells requires us to clone the full-length sequence of about 3.6 kb containing the complete coding region of the target gene, which is a very tedious and time-consuming process. Recently, we have described the expression of the N-terminal toxic region of crystal proteins using recombinant baculoviruses that produce recombinant polyhedra including Cry protein [24, 59]. The toxin proteins expressed using this approach have been used for bioassays of lepidopteran larvae.

PCS System for Cloning Bt Plasmids

Bt has long been noted for its numerous and diverse plasmids [49]. Bt subsp. *kurstaki*, for example, had up to 17 plasmids in sizes ranging from 2 to 250 kb [78]. Larger plasmids have predominantly been the focus of study, since most *cry* genes are located on these plasmids [20, 69]. Besides the Cry protein gene-bearing plasmids, various Bt strains harbor a complex array of cryptic plasmids. It has been very difficult to analyze these plasmids as a single fragment using traditional restriction endonuclease digestion/ligation, not only because of the rarity of unique restriction sites, but also for unknown reasons [2]. Most of the smaller Bt plasmids are still referred to as cryptic plasmids, since no striking functions have been attributed to them. To date, over 11 small plasmids from six Bt strains have been sequenced and reported, and these include pGI1, pGI2, pGI3 from subsp. *thuringiensis* H1.1 [2], pHD2 from subsp. *kurstaki* HD1 [93], pTX14-1, pTX14-2, pTX14-3 from subsp. *israelensis* [2], pHT1030 from subsp. *thuringiensis* LM2 [77], pUIBI-1 from subsp. *entomocidus* LBIT-113 [86], and pBMB9741 and pBMB2062 from Bt YBT-1520. As larger plasmids, the 127-kb pBtoxis

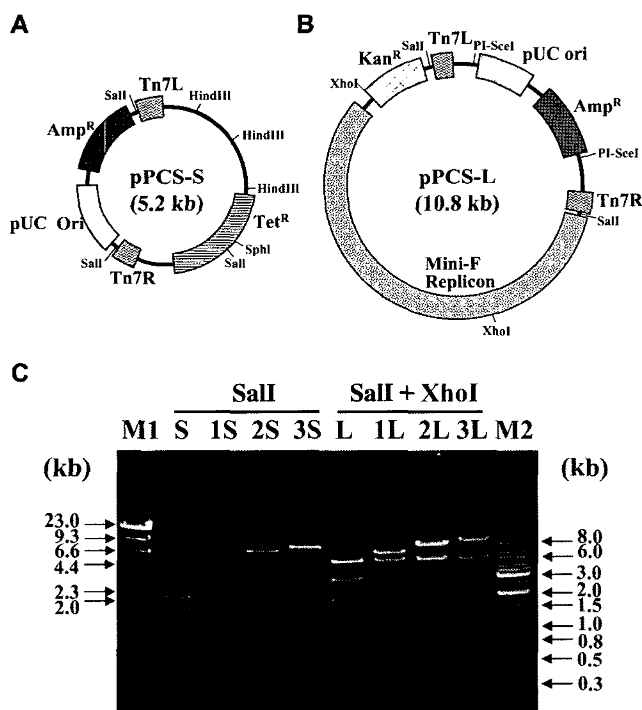

Fig. 1. PCS systems (**A** and **B**) and the transposed plasmids (C) of Bt subsp. *israelensis* by PCS.
A, pPCS-S; **B**, pPCS-L. Amp^R and Tet^R indicate ampicillin and tetracycline resistant genes, respectively. Tn7L and Tn7R indicate left (L) and right (R) ends of Tn7. pUC-ori is an *E. coli* replication origin. **C**: Lanes M1, Lambda DNA digested with HindIII; M2, 1-kb DNA ladder (Fermentas); S, pPCS-S; L, pPCS-L; S1, 2, 3, or L1, 2, 3, represent the Bt subsp. *israelensis* plasmid DNA captured by pPCS-S or pPCS-L, respectively.

from subsp. *israelensis* [12] and pBMB67 from Bt YBT-1520 [25] have also been presented and analyzed.

We have developed an easy, simple, and convenient system based on Tn7 transposition in order to clone circular DNAs in *Escherichia coli* cells and a designated plasmid capture system (PCS system; Fig. 1) [28]. The principle of the PCS system is that donor DNA containing an *E. coli* origin of replication for amplification and an antibiotic resistant gene for selection, located between a Tn7 left (L) and right (R) end, can be inserted into target circular DNA molecules by a transposition reaction using TnsABC* transposase, and the reacted DNA can be cloned and amplified in *E. coli*. Using this system, we were able to clone three small plasmids from subsp. *israelensis* (Fig. 1C) [27], and the PCS also was verified in the experiment by cloning bracovirus circular DNA genome segments [28]. This PCS might be applicable for cloning and analyzing larger sized plasmids from Bt.

Development of Bt Biopesticides

Insecticidal Bt products were first commercialized in France in the late 1930s [72]. For over 60 years, Bt has been one of the most consistent and significant biopesticides for use on crops as an insecticidal spray, containing a mixture of spores and the insecticidal crystals. By 1995, 182 Bt-based products were registered by the U.S. Environmental Protection Agency (EPA), but in 1999 Bt formulations constituted less than two percent of the total sales of all insecticides and represented around 80% of all biopesticides sold [22, 40]. The use of Bt has increased as insect pests have become resistant to chemical insecticides. Bt sprays are estimated to bring in U.S. $8 billion per annum. At one time, Bt sprays constituted $100 million in annual sales, but with the advent of transgenic plants engineered with the insecticidal *cry* gene, sales have decreased to $40 million. Half of current sales are used in Canadian forests to control the gypsy moth, spruce budworm, and other lepidopteran pests. The OECD (Organization for Economic Cooperation and Development) predicts that the biopesticide may grow to 20% of the world's pesticide market by 2020 [117].

Bt sprays are used sporadically and typically over small areas. Sprayable Bt formulations have penetrated cotton, fruit and vegetable, aquatic, and other insecticide markets. New Bt formulations have consistently made gains in a limited number of fruit and specialty vegetable markets over the last number of years. Sprays are also chosen by organic farmers to meet guidelines for using strictly nonsynthetic materials. An additional use of Bt is in the protection of stored commodities from pest infestation.

In the early 1950s, Steinhaus [105] began to experiment with Bt, produced Bt, and the agent Thuricide was soon

available. The name Thuricide has survived a maze of industrial transformations and is a product of Valent Biosciences today. As recounted by Beegle and Yamamoto [8], the early products have several problems. Standardization was based on spore count rather than potency, the products often contained a heat-tolerant exotoxin, and most were based on subsp. *thuringiensis* and were of low potency. The isolates of Kurstak and Dulmage were serotyped by de Barjac and Lemille [35] and designated subsp. *kurstaki* HD-1. They became the basis for products that were competitive with chemical insecticides in performance and cost, and before long, all of the Bt companies that produced Bt were producing subsp. *kurstaki*. It remains, by far, the greatest commercial success of microbial control, in the strictest sense of the word. Much of Bt's commercial success prior to the introduction of transgenic plants was in forestry. According to Lewis *et al.* [80], its use against the spruce budworm and gypsy moth in North American forests accounts for 60% or more of world sales. However, other varieties, such as the Coleoptera-active Bt subsp. *tenebrionis*, discovered by Krieg *et al.* [68], and the Diptera-active subsp. *israelensis* isolated by Goldberg and Margalit [47], have come to be used extensively for the control of larvae of pest and vector black flies and mosquitoes around the world, providing both medical and environmental benefit [71]. An example of an outstanding success in cooperation between industry and a governmental organization to achieve those benefits is the Onchocerciasis Control Programme of the World Health Organization (WHO), wherein Bt subsp. *israelensis* applications comprise up to 50% of all insecticide applications [52].

The relevant works on the screening and isolation of new Bt strains have been performed and finally resulted in the production of commercial products (Table 1) [39]. Among them, we isolated a Bt strain designated NT0423, belonging to Bt subsp. *aizawai*, an isolate from the soil of sericultural farms [64]. The manufactured article, using Bt strain NT0423, (named "Tobaggi" and produced from Dongbu Hannong Chemicals) is one of the registered Bt biopesticides in Korea (Fig. 2). This strain had at least five known crystal protein genes, *cry1Aa*, *cry1Ab*, *cry1C*, *cry1D*, and *cry2A*, and one new gene, *cry1Af1* (Genbank

Table 1. Bt-based biopesticide active ingredients and products[a].

Bt subspecies	Strain	Product name	Company	Target insect[b]
kurstaki				
	-[c]	Foray	Valent Bioscience Co.	Lepidopteran
	-	Biobit	Valent Bioscience Co.	Lepidopteran
	BMP123	BMP123	Becker Microbial Products, Inc.	Lepidopteran
	EG2348	Condor	Ecogen Inc.	Lepidopteran
	EG2371	Cutlass	Ecogen Inc.	Lepidopteran
	ABTS-351	Dipel	Valent Bioscience Co.	Lepidopteran
	EG7481	Crymax	Ecogen Inc.	Lepidopteran
	EG7826	Lepinox	Ecogen Inc.	Lepidopteran
	SA-11	Javelin	Certis USA	Lepidopteran
	SA-12	Thuricide	Certis USA	Lepidopteran
aizawai				
	GC-91	Agree	Certis USA	DBM, AW
	NB200	Florbac	Valent Bioscience Co.	DBM, AW
	ABTS	XenTari	Valent Bioscience Co.	DBM, AW
	NT0423	Tobaggi[d]	Dongbu Hannong Chemicals	DBM, AW
	GB413	Solbichae[d]	Green Biotech Co.	DBM, AW
israelensis				
	-	Gnatrol	Valent Bioscience Co.	Mushroom fly
	-	Bactimos	Valent Bioscience Co.	Mosquito & BF
	AM-65-52	VectoBac	Valent Bioscience Co.	Mosquito & BF
	SA3A	Teknar	Valent Bioscience Co.	Mosquito & BF
	BMP 144	BMP	Becker Microbial Products, Inc.	Mosquito & BF
tenebrionis				
	NB-176	Novodor	Valent Bioscience Co.	CPB, ELB

[a]The information about Bt strains and product names is from EPA-registered ingredients and products list of biopesticides (http://www.epa.gov/pesticides/biopesticides/product_lists/bppd_products_by_AI.pdf).
[b]DBM, diamond back moth; AW, armyworm; BF, black fly; CPB, colorado potato beetle; ELB, elm leaf beetle.
[c]Unidentified strain.
[d]Two products are produced by a Korean company.

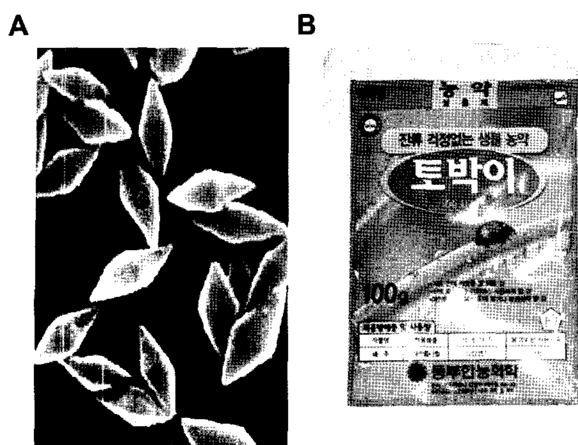

Fig. 2. Crystal proteins (**A**) and a biopesticide product (**B**) of Bt subsp. *kurstaki* NT0423.
A. C indicates crystal inclusions by scanning electronic microscopy. **B.** The commercialized product name is "Tobaggi" from Dongbu Hannong Chemicals.

Accession No. U82003). It has dual toxicity against lepidopteran larvae-like *Plutella xylostella*, *Spodoptera exigua*, and *Hyphantria cunea*, and dipteran larvae-like *Culex pipiens* and *Musca domestica* [63]. The developmental procedure for the Bt NT0423 product might be a typical example of Bt sequential research for Bt biopesticides.

BT *CRY* GENE AS A SYNTHETIC GENE FOR TRANSGENIC CROPS

Much of the technology developed to study the structure and function of the Cry proteins has provided the foundation for the genetic engineering of this class of biopesticides. The crystal protein genes have been used as models for optimizing gene expression in transgenic plants. In 1987, several reports demonstrated that insecticidal crystal protein genes from Bt could be introduced and expressed in the tissues of tobacco and tomatoes, resulting in pest-resistance transgenic plants [1, 6, 113]. In the U.S.A., the six transgenic crops (canola, corn, cotton, papaya, squash, and soybean) planted in 2003 produced an additional 2.4 million tons of food and fiber and increased farm income by US $1.9 billion. These transgenic crops also reduced the use of pesticides by 21,000 tons [30]. The use of Bt cotton has resulted in substantial decreases in insecticide use in developed and developing countries, and also increases in yield and profitability [104]. For example, it was documented that Indian farmers benefited from a 70% reduction in insecticide applied in cotton fields, resulting in a saving of US $30 per hectare in insecticide costs and an increase of 80–89% in harvested cotton yield [98]. In a survey conducted in China in 1999, a dramatic reduction in pesticide applications in Bt cotton fields was also reported, and the proportion of farmers reporting pesticide poisoning symptoms was reduced from 22% to 4.7% [57].

Transgenic maize plants resistant to corn rootworm were first commercialized in the U.S.A. in 2003. According to the EPA registered ingredients and products list of biopesticides, there are three Bt transgenic crops registered: corn, cotton, and potato (Table 2) [39]. The event MON863 was developed using a synthetic variant of the wild-type *cry*3Bb1 gene from Bt subsp. *kumamotoensis* that encodes a protein with eight times more insecticidal activity than the wild-type [115]. Many rice varieties have been transformed with genes encoding various Bt Cry proteins and have been shown to be resistant to one or more lepidopteran pests of rice [111]. Field trials of Bt rice commenced in China in 1998 and in India in 2001, but no Bt rice or other transgenic rice varieties have yet been released for commercialization [54].

Table 2. Bt *cry* gene-based transgenic crops[a].

Crop	Cry protein	Product name	Company	Target insect[b]
Corn	Cry1F	Herculex	Mycogen Seeds	ECB, CEW, FAW
		Pioneer	Pioneer Hi-bred	ECB, CEW, FAW
	Modified Cry1F	Mycogen	Mycogen Seeds	ECB, CEW, FAW
	Cry34Ab1 & Cry35Ab1	Herculex	Mycogen Seeds	CRW
		Pioneer	Pioneer Hi-bred	CRW
	Cry3Bb	Yieldgard	Monsanto Co.	CRW
	Cry3Bb1	MON 88017	Monsanto Co.	CRW
	Cry1Ab	MON 88017 X MON 810	Monsanto Co.	ECB, CEW, FAW, CRW
	Cry1Ab	Northrup king	Northrup king	ECB, CEW, FAW, CRW
Cotton	Cry2Ab	Bollgard	Monsanto Co.	CBW, TBW, PBW
Potato	Cry3A	New leaf	Monsanto Co.	CPB

[a]The information about crystal proteins and product names is adapted from EPA-registered ingredients and products list of biopesticides (http://www.epa.gov/pesticides/biopesticides/product_lists/bppd_products_by_AI.pdf).
[b]ECB, european corn borer; CEW, corn earworm; FAW, fall armyworm; CRW, corn rootworm; CBW, cotton bollworm; TBW, tobacco budworm; PBW, pink bollworm; CPB, Colorado potato beetle.

The most widely used method of plant transformation is the *Ti* plasmid of the bacterium *Agrobacterium tumefaciens*-mediated tools [56, 118]. Initially, many crop plants such as the major monocot species could not be transformed by this technology, and alternative methods were proposed, such as polyethyleneglycol-mediated transfer, microinjection, protoplast and intact cell electroporation, particle bombardment, *etc.* The analysis of *cry* gene sequences revealed that the A+T content represents about 60% of the total nucleotide content of the protein-encoding region. This value is 10% higher than that of typical plant genes. In plants, the A+T rich DNA segments could provide signals deleterious to transgene expression in plants, such as splice sites, poly(A) addition sites, ATTTA sequences, mRNA degradation signals, and transcription termination sites [107]. Therefore, when the *Bacillus* sequences were extensively modified, with synonymous codons to reduce or eliminate the potentially deleterious sequences and generate a codon bias more like that of a plant, expression improved dramatically. For a trial to establish transgenic apples in Korea, a new *cry* gene, *cry1-44*, which has toxicities to *Plutella xylostella*, *Spodoptera exigua*, *Hoshina longicellana*, and *Grapholita molesta*, was cloned from the Bt K-1 strain [83] and mutated. To construct a synthetic *cry1-44* gene for transgenic apples, multisite-directed mutagenesis methods were used and various mutant Cry44 proteins were expressed in insect cells and assayed for action against insect pests of apple trees.

RECOMBINANT VIRAL INSECTICIDE WITH BT CRY PROTEIN

Baculovirus is one of the safe biopesticide agents for integrated pest management; however, its use in the agricultural field has been very limited because of its slow

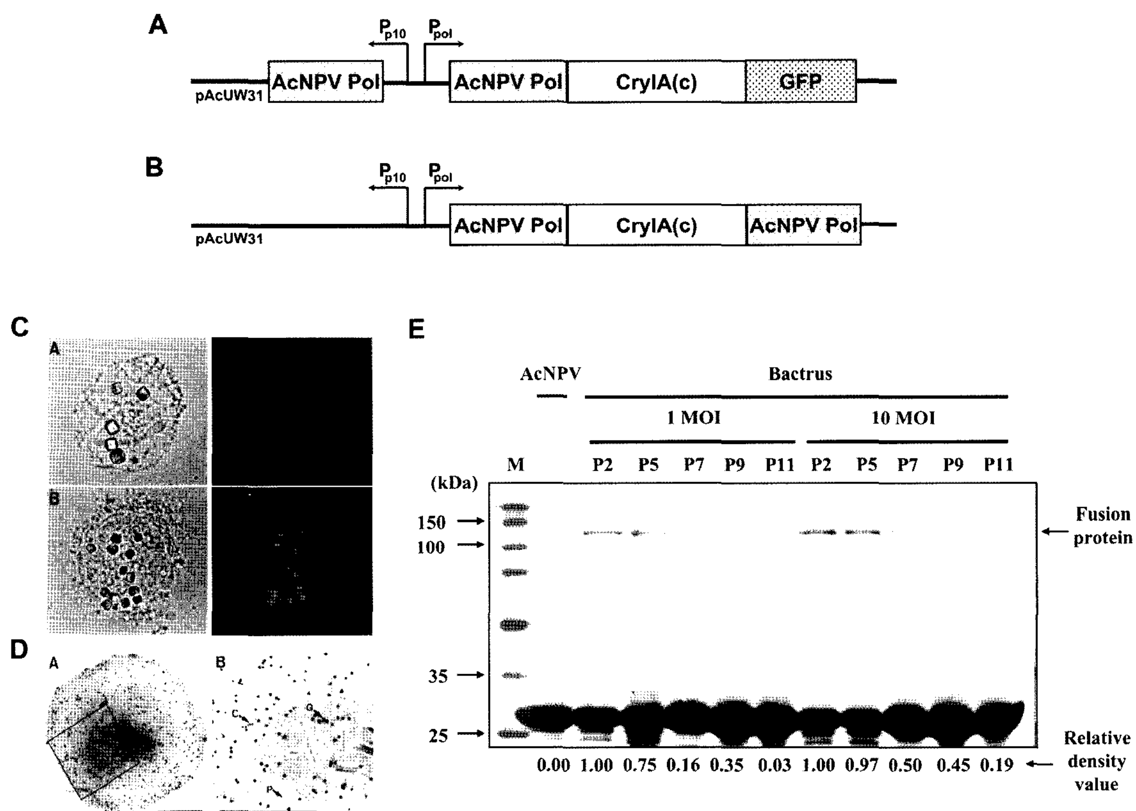

Fig. 3. Transfer vectors for the recombinant baculovirus ColorBtrus (**A**) and Bactrus (**B**), and the recombinant polyhedra of ColorBtrus (**C** and **D**) and Bactrus (**E**).

A and **B**: The 1.84 kb of PCR-amplified *cry1Ac* toxic fragment was cloned. pColorBtrus carries a wild-type AcMNPV polyhedrin gene under the control of the p10 (Pp10) promoter, as well as a polyhedrin-Cry1Ac-GFP fusion gene under the control of the polyhedrin (Ppol) promoter. pBactrus only carries a polyhedrin-Cry1Ac-GFP fusion gene under Ppol. The arrows show the direction of transcription. **C**: Small A, the polyhedra of wild-type, and small B, the recombinant polyhedra by ColorBtrus. Microscopy by light was placed at left side and by fluorescence at right side. **D**: Immunogold labeling of recombinant polyhedra. P, Anti-polyhedrin; G, Anti-GFP; C, Anti-crystal protein toxin antibodies were observed by transmission electron microscopy. **E**: The polyhedra produced by Bactrus at 1 and 10 MOI along serial passage. Passage numbers are indicated as "pn," p for passage and n for number. Lane M: protein molecular weight marker; p2–p11: Bactrus with different passage number. The relative density values at the bottom of the lanes were determined by densitometry scanning of the gel.

speed of action [14, 37, 88]. Depending on the baculovirus strain and target insect species, it can take from several days to weeks before the infected insect stops feeding. Many approaches have been developed to produce baculoviruses that improve killing speed or decrease effective feeding times. For examples, the expression of insect-specific toxins or other proteins like juvenile hormone esterase, prothoracicotropic hormone, or diuretic hormone, which are predicted to have noxious effects on the physiology of the host insect, have been studied [13]. Among these approaches, the expression of insect-specific neurotoxins, including mite toxin and scorpion toxins, showed the most significant increase in the pathogenicity of baculovirus [13, 18, 106]. There were several reports on the improvement of viral insecticidal activity by expressing the Bt Cry protein in baculovirus [90, 100]. Although the recombinant baculoviruses produced a large amount of Bt Cry protein, their pathogenicity was not enhanced in hemolymph or fat bodies, and the insecticidal mechanism of the Bt Cry protein has not yet been considered.

Recently, an alternative approach (named "recombinant polyhedra technology") has been developed to engineer baculoviruses, whereby the foreign protein is actually incorporated into the viral occlusion bodies [59]. This approach allows an insecticidal protein to be delivered to the gut of the insect, as well as being produced within its internal body. Through this recombinant polyhedra technology, the occlusion bodies incorporating the insecticidal Bt Cry1Ac toxin protein were able to be produced; the recombinant virus was named "ColorBtrus" (Figs. 3A, 3C and 3D) [24]. Co-expression of native polyhedrin and a polyhedrin-Cry1Ac-GFP fusion protein gave rise to occlusion bodies that had incorporated Cry1Ac and GFP, and contained occluded viruses. These occlusion bodies contained approximately 10 ng Cry1Ac toxins per 1.5×10^6 polyhedra. Both the infectivity and speed of action of ColorBtrus occlusion bodies are dramatically enhanced compared with the wild-type virus. It seems like our approach was successful because the toxin was delivered to the insect gut, its normal active site; on the other hand, the previous Bt-toxin viruses have been produced in infected cells within the insect, not in the midgut.

Two main advantageous features of ColorBtrus virus are its dual mode of action, killing either by Cry1Ac toxicity or by viral pathogenesis, and its much faster speed of killing as compared with the previous recombinant baculoviruses. The occlusion bodies of ColorBtrus should be toxic to all species susceptible to Cry1Ac toxin, including species not susceptible to *Autographa californica* nucleopolyhedrovirus (AcMNPV). Conversely, insects susceptible to AcMNPV but insensitive to Cry1Ac can be killed by viral replication. This property should be contributed to delayed resistance by the insect to Cry1Ac, resulting in the prevention of the few individuals within the population who have acquired Cry1Ac resistance from surviving and passing on their Cry1Ac resistance genes to progeny. In addition, this strategy might be applied to other Bt toxins or other gut active toxins with different modes of action, and the alternate use of these kinds of recombinant viruses may further reduce the emergence of resistance. In species sensitive to the toxin, the improvement in the insecticidal properties using the virus is remarkable; the LD_{50} of ColorBtrus is approximately two orders of magnitude lower than AcMNPV, and the ST_{50} of infected insects is reduced to slightly more than 1 day. To our knowledge, no other recombinant baculovirus capable of causing host mortality within 1 day post infection has been described [14]. Furthermore, infected insects cease to feed, consistent with pathology due to Cry1Ac [46, 53]. Thus, ColorBtrus represents a novel recombinant insecticide that combines positive attributes of both baculovirus and Cry1Ac toxin. The technology used can easily be applied to the expression of other Bt or gut active toxins and/or to the engineering of other baculoviruses.

In spite of this improvement in recombinant polyhedra technology, this recombinant has the trait of expressing stable recombinant protein, which might cause problems in the environment owing to the development of genetically modified organisms. Another type of Bt recombinant baculovirus, Bactrus, was constructed by the insertion of the Bt *cry* gene between two polyhedron genes of AcMNPV under the control of the polyhedron promoter (Fig. 3B) [65]. This recombinant virus expressed a decreased amount of recombinant proteins with serial passages, according to homologous recombination between two polyhedron genes out of the *cry* gene (Fig. 3E). This character of Bactrus, which can be reversed back to wild-type AcMNPV, might avoid the feasible problems encountered in genetically modified organisms.

FUTURE PROSPECTS

The use of Bt spray as an insecticide has several disadvantages; 1) Bt spray cannot be applied uniformly to all parts of the plant, 2) it cannot be delivered to pests that are inside plant tissues, and 3) Bt is susceptible to rapid degradation by UV light and removal by water runoff. Therefore, multiple applications are required to provide extended pest protection [102]. Moreover, since McGaughey reported resistance to Bt in Indianmeal moth populations [94], resistance to Bt sprays has also evolved in greenhouse populations of the cabbage looper and in field populations of the diamondback moth [42, 108]. Transgenic crops with Bt *cry* genes might overcome these kinds of disadvantages. This feature eliminates difficulties in targeting pests that burrow into plant tissues, as well as the labor and expenses associated with applying sprays. At present, field-evolved

resistance to Bt crops has not been documented. Tabashnik *et al.* [109] reported long-term resistance levels in transgenic Bt crop fields. Generally, it has been a concern that an increase in the wide planting of Bt crops might lead to rapid evolution of resistance to Bt toxins by pests [108]. However, bioassay results show no net increase from 1997 to 2004 in the mean frequency of pink bollworm resistance to Bt toxin [109]. This delay in resistance can be explained by the presence of refuges of cotton without Bt toxin, recessive inheritance of resistance, incomplete resistance, and fitness costs associated with resistance.

Basically, the advantages of Bt and Bt crops apparently includes 1) no harmful effects on vertebrates and humans, or the ecological environment, 2) low impacts on non-target organisms, and 3) a narrow spectrum of primarily leaf-feeding lepidopteran targets. These merits, including the short field life of the spray, are still this biopesticide's greatest challenge in the pesticide market [117]. For the sustainable use of Bt, it is imperative that there be 1) collections of Bt isolates, crystal proteins, and strains of related species, 2) investigations into the persistence of crystal proteins and possible long-term effects on non-target organisms and the environment, 3) development of improved resistance management strategies, and 4) genetic engineering of Bt genes into the plastid genomes of transgenic crops [96]. Environmentally safe-insect control strategies based on the Bts and their insecticidal crystal proteins are going to increase in the future, especially with the wide adoption of transgenic crops. In conclusion, the discovery of new toxins and new ways of presenting the toxin to the target insects, which includes the development of recombinant microorganisms and proteomic technology, could be adapted to the study of Bt crystal proteins; additionally, interaction studies between Bt and target insects involving modes of action of Bt Cry proteins and resistance mechanisms should be carried out, all of which are fundamental studies that will allow for improvement of existing Bt application strategies and the ability to design alternative options.

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