B. thuringiensis – identification, biology and uses

Vincent Sanchis-Borja

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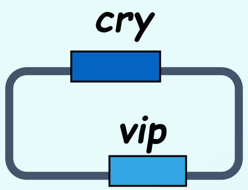
HuPlant(Cost Action 16110) training School

The *Bacillus cereus sensu lato* group

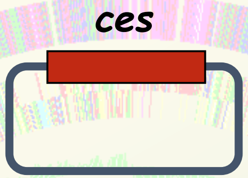
Gram^{+}, sporulating, low GC% (35%) bacteria

Set of common genes represent ~ 75% of the genome (~ 4 Mb)



***Bacillus thuringiensis* specific genes**

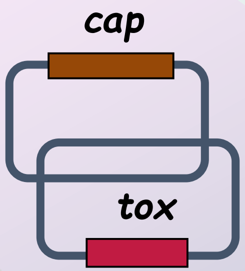
A biopesticide agent



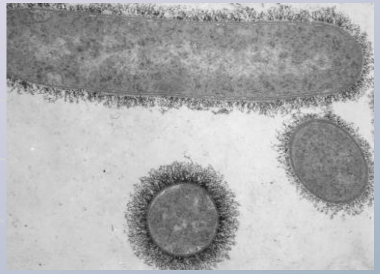
***Bacillus cereus* specific genes**

A food borne pathogen

A typical *B. cereus s. l.* genome contains ≈5500 protein coding genes



***Bacillus anthracis* specific genes**



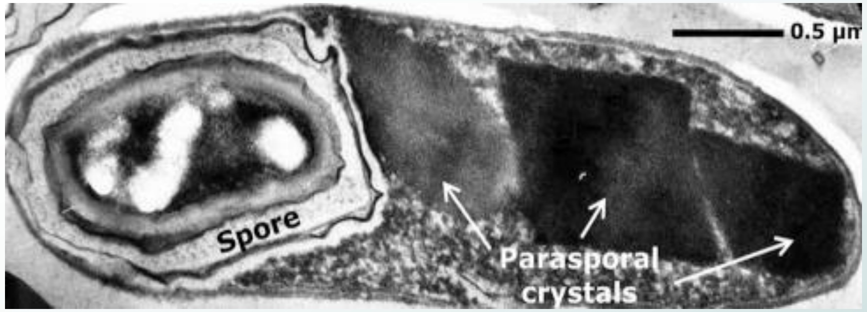
A bioterrorism agent

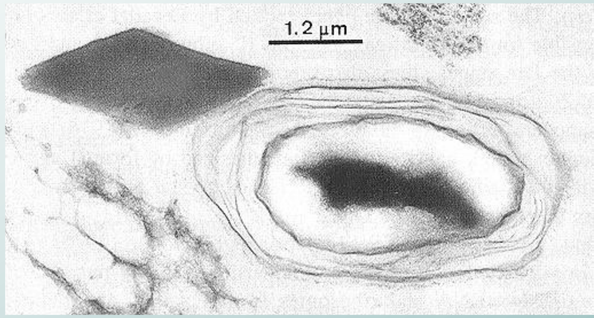
Harbors a diverse range of plasmids that vary in number and in size (2–200kb)

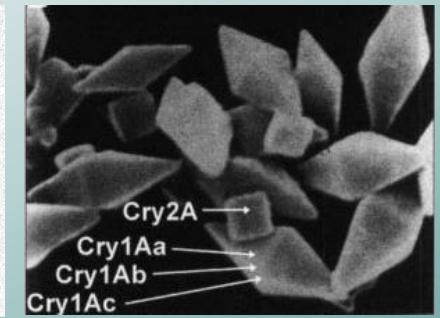
The specific pathogen properties of these bacteria are due to plasmid

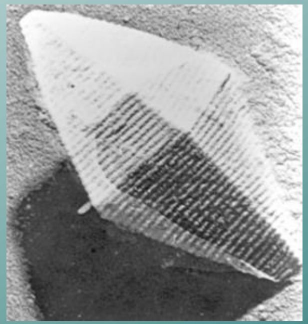
What is *Bacillus thuringiensis* ?

- Common soil bacterium
- Present in nature in a variety of forms (strains)
- Characterized by the production of crystal inclusions containing proteins (Cry toxins) that are toxic to insects
- Isolates frequently produce crystals containing multiple Cry proteins.
- Commonly used for commercial agriculture, including organic farming
- Extremely well-known toxin in terms of human health & environmental safety



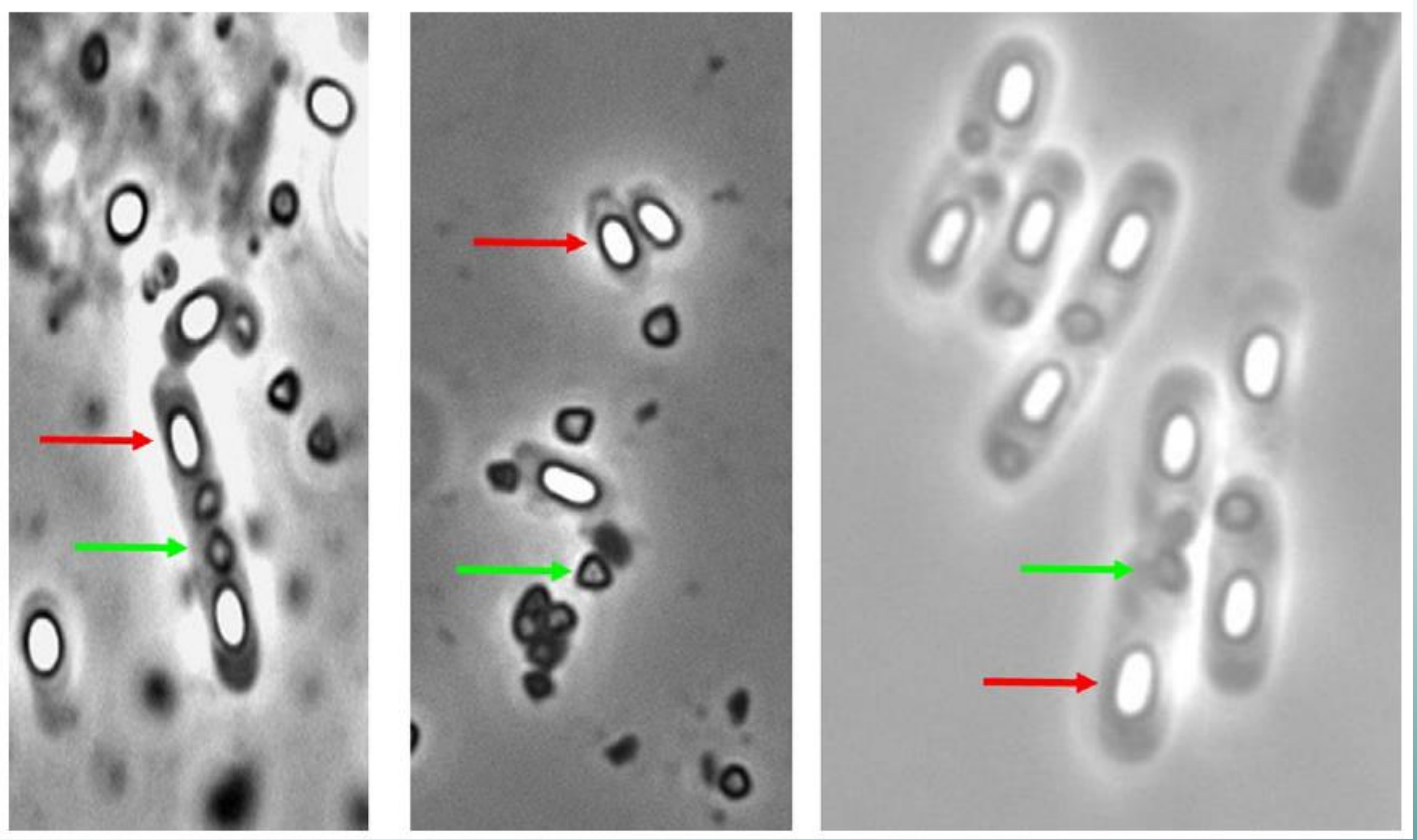






Free spores & crystals in technical powder

Identification of *Bacillus thuringiensis* strains



Photomicrographs of *Bacillus thuringiensis* strains viewed by phase-contrast microscopy showing the parasporal crystals of insecticidal toxins (green arrows) or spores (red arrows)

Bacillus thuringiensis (Bt) historical background

- **1901** : Discovered in silkworm by the Japanese bacteriologist Ishiwata “Sottokin”.
- **1911** : A new isolation by Berliner on *Ephestia kuehniella* (Zeller) larvae from Thuringe (Germany)

•**1938** : First commercial preparation (Sporéine) by Libec Laboratories in France.

•**1956 :** development of an industrial process known as submerged fermentation, by the Pacific Yeast Product Company, which allowed production of Bt on a large scale.

•**1960 :** First commercial use in the United State.

•**1977 :** Discovery of *Bacillus thuringiensis* var. *israelensis* toxic to flies by Goldberg and Margalit

•**1981 :** first cloning of a *Cry* gene

•**1983 :** discovery, of *Bacillus thuringiensis* var. *tenebrionis* toxic to beetles by Krieg

•**1985 :** First insect resistant transgenic plant

•**1990 :** strain and cry gene isolation: several tens of thousands of isolates yielding over 250 distinct Cry proteins

•**1995:** First Bt transgenic plant commercialised in USA

•**2017:** 100 millions hectares of biotech crops with insect resistance Bt genes were planted all over the world, grown by up to 17 million farmers globally in 2017

Insecticidal activity of *Bacillus thuringiensis* strains

- Bt is a highly heteromorphous comprising a very large number of strains distributed in more than 70 serotypes, including various *subspecies: kurstaki, tenebrionis, israelensis…* with various pathogenic activities against insect larvae from different orders

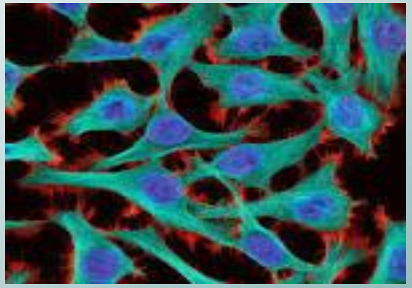
lepidoptera

coleoptera

diptera (mosquitoes)

or against nematodes

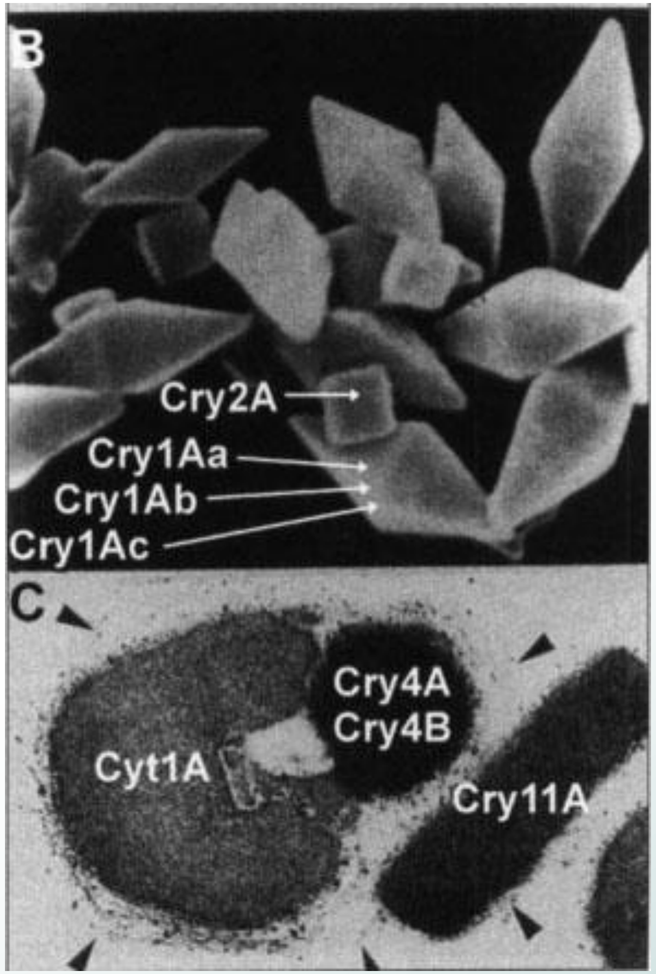
or against cancer cells



or with yet unknown activity

Crystal toxin description

Bt isolates frequently produce crystals containing multiple Cry proteins.



Federici, et al., 1998

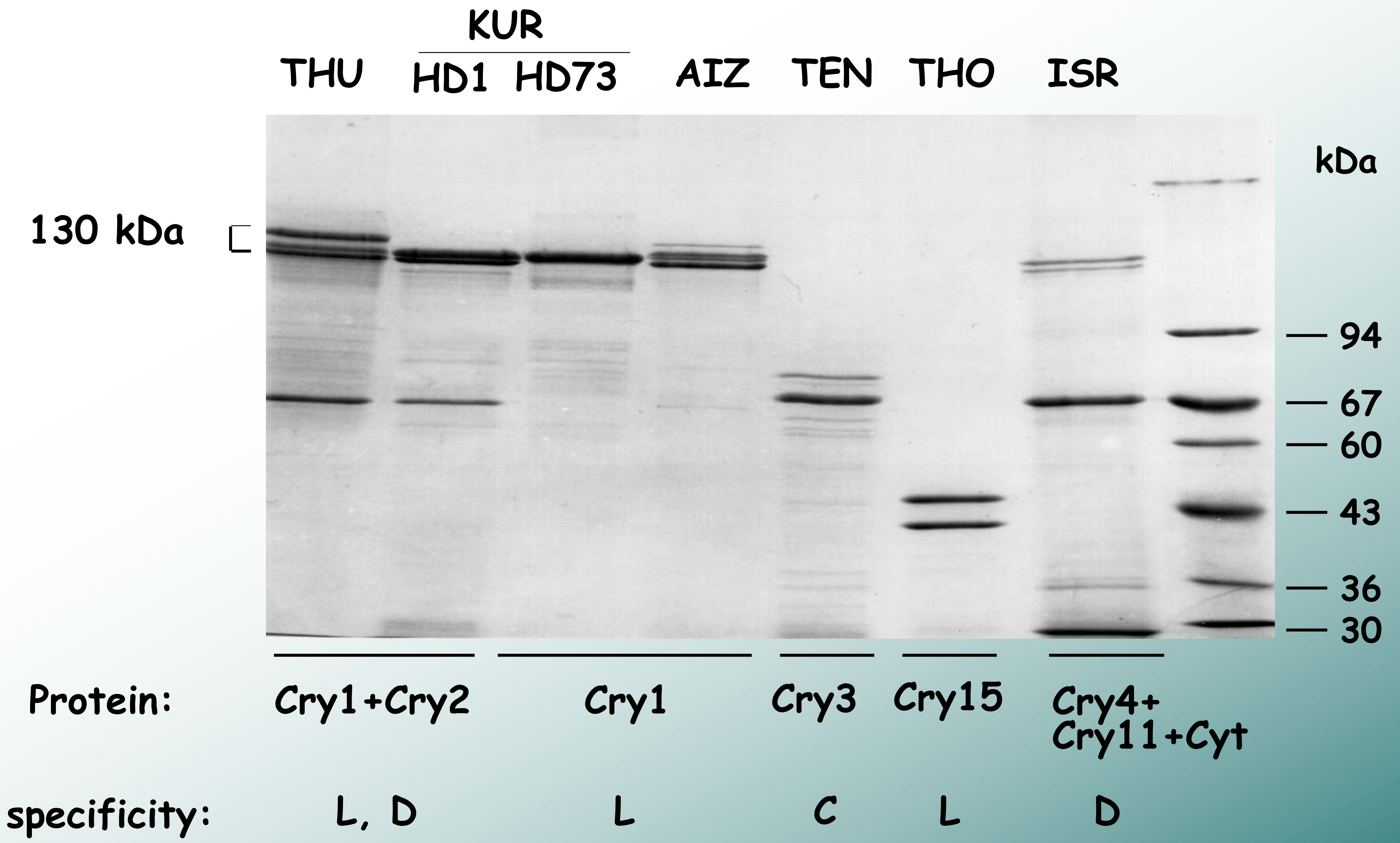
B. thuringiensis subsp. *Kurstaki*

Bipyramidal crystals of Cry1Aa, Cry1Ab, and Cry1Ac
and
Cuboidal crystal of Cry2A.

B. thuringiensis subsp. *israelensis*

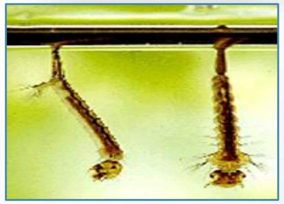
Large semispherical inclusion of Cyt1Aa,
and
Dense spherical body of Cry4Aa and Cry4Ba
and.
Bar-shaped body of Cry11Aa

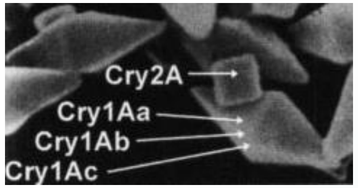
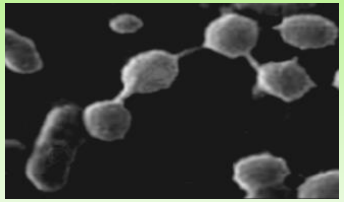
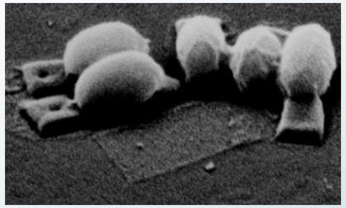
Crystal protein content analysis



Crystal toxins classification



Gene	Crystal shape	Protein size (kDa)	Insecticidal activity
cry1 (A,B,… and a,b,…)		130-138	Lepidoptera
cry2		69-71	Lepidoptera and Diptera
cry3		73-74	Coleoptera
Cry4, cry11		73-134	Diptera

Hofte and Whiteley, 1989

Classification of the Cry toxins

Bt toxicity is due to the Cry proteins

These Cry toxins consist in a very large protein family. There are classified as follows:

Rank 1: 74 classes: Cry1, 2…, 74

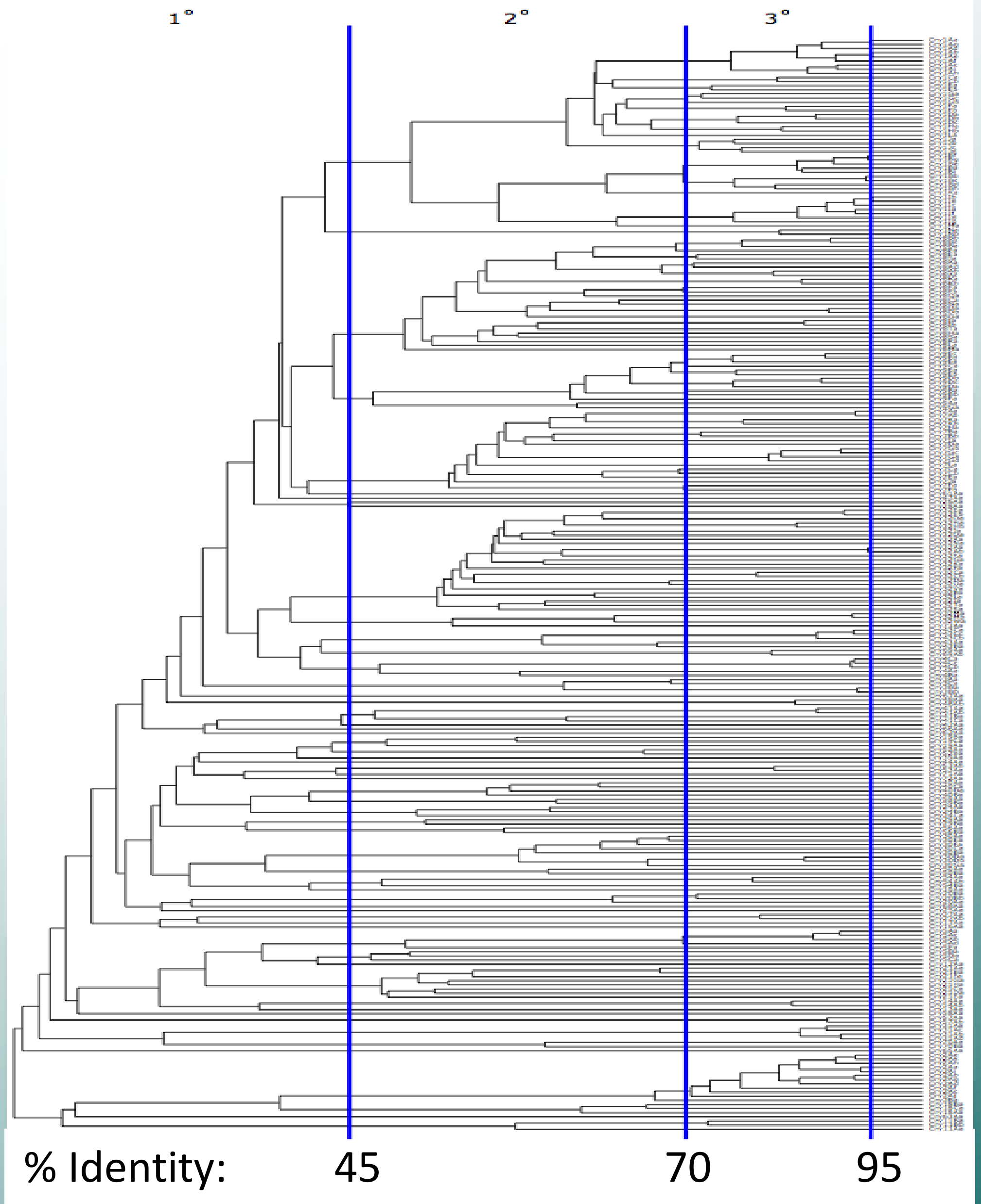
Rank 2: ~ 300 subclasses: Cry1A, B, C…

Rank 3: > 500 alleles: Cry1Aa, b, c, d…

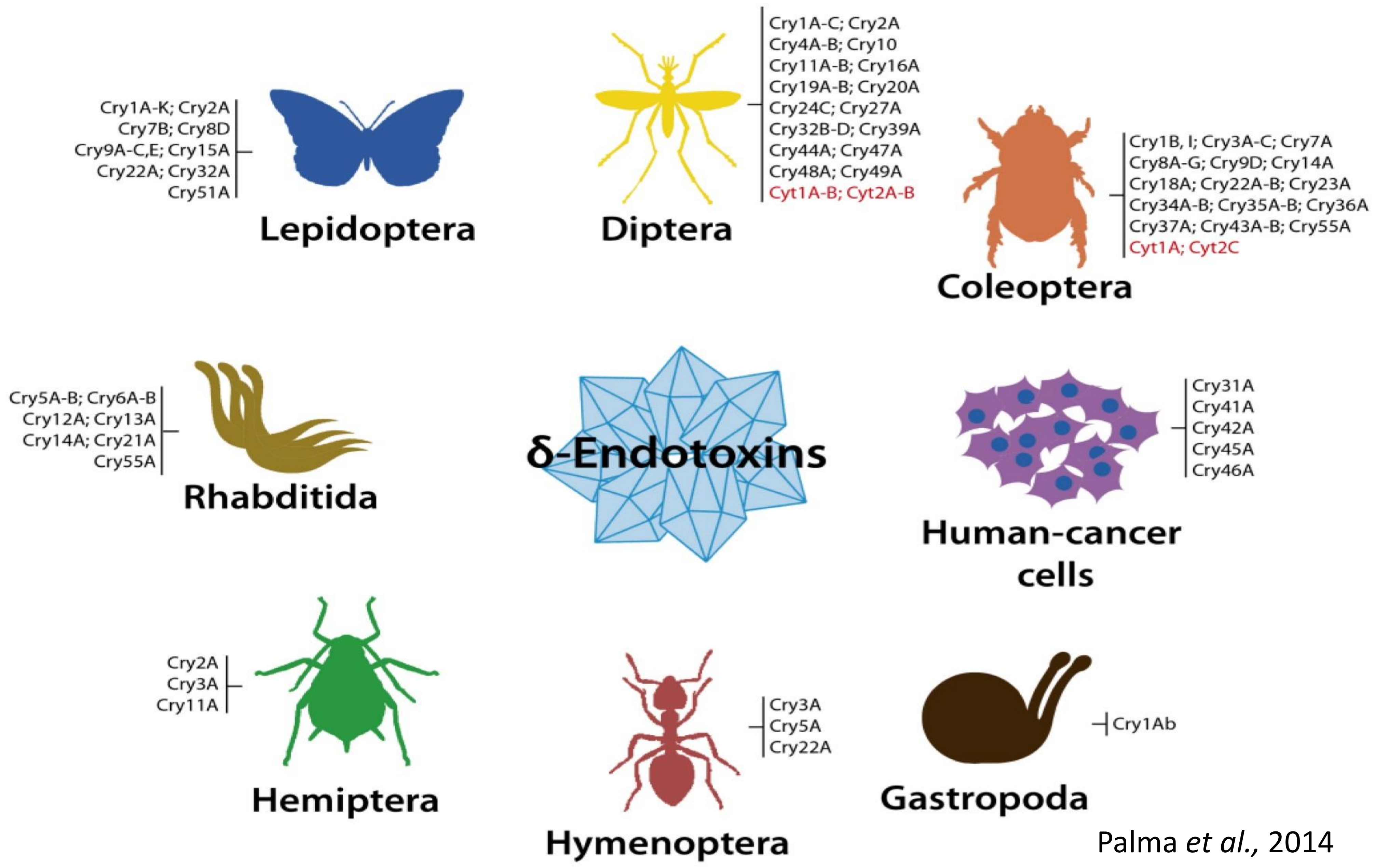
Together, these toxins allow to kill:

- ✓ Various insect larvae (lepidoptera, Cry1, 2…; coleoptera, Cry3, 8…; mosquitoes, Cry4, 11…)
- ✓ Nematodes (Cry5, 6…)
- ✓ Cancer cells (Cry41, 45…)

dendrogram describing the relatedness of the toxins which share the common three-domain

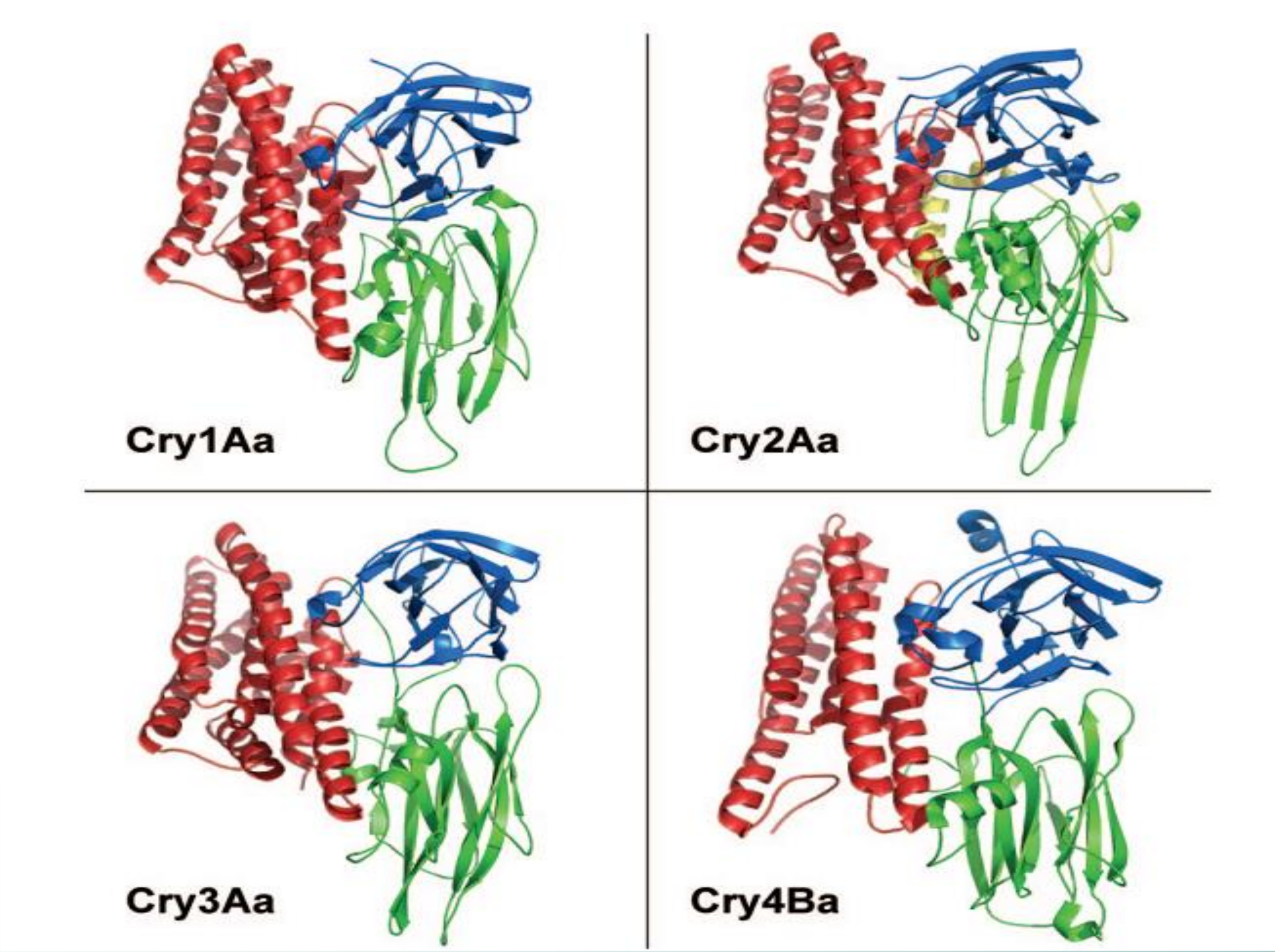


Summarized view showing the known host spectrum of Bt Cry toxins



Palma *et al.*, 2014

Crystal toxin structure: 3 domains structure



Craig R. Pigott, and David J. Ellar Microbiol. Mol. Biol. Rev. 2007

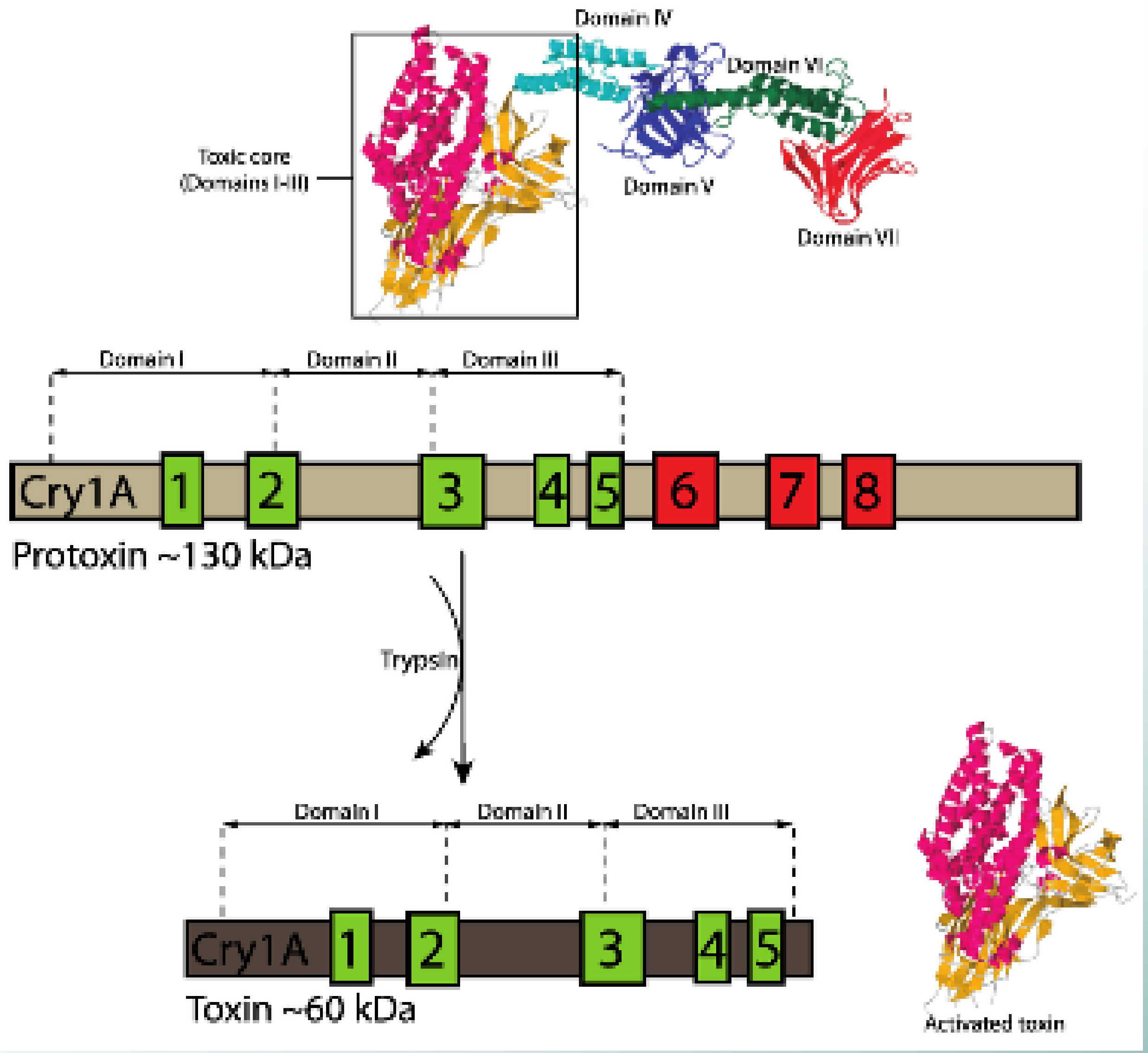
Domain I seven helix bundles

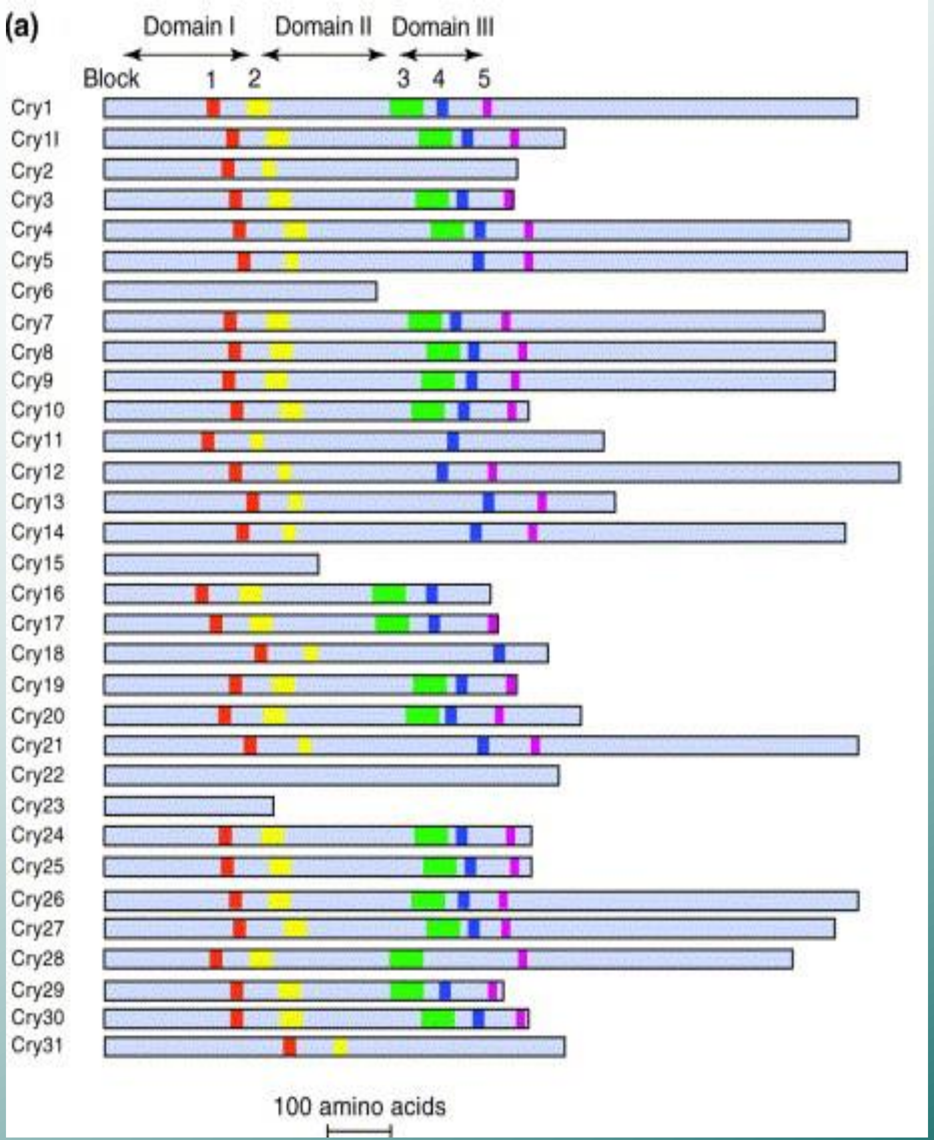
Domain II triple anti-parallel β sheets

Domain III β-sheet sandwich

- The long, hydrophobic and amphipathic α helices of Domain I is equipped for transmembrane pore formation.
- The β sheet structure of domain II and domain III are involved in receptor binding and specificity determination.

Crystal gene structure: 5 conserved domains

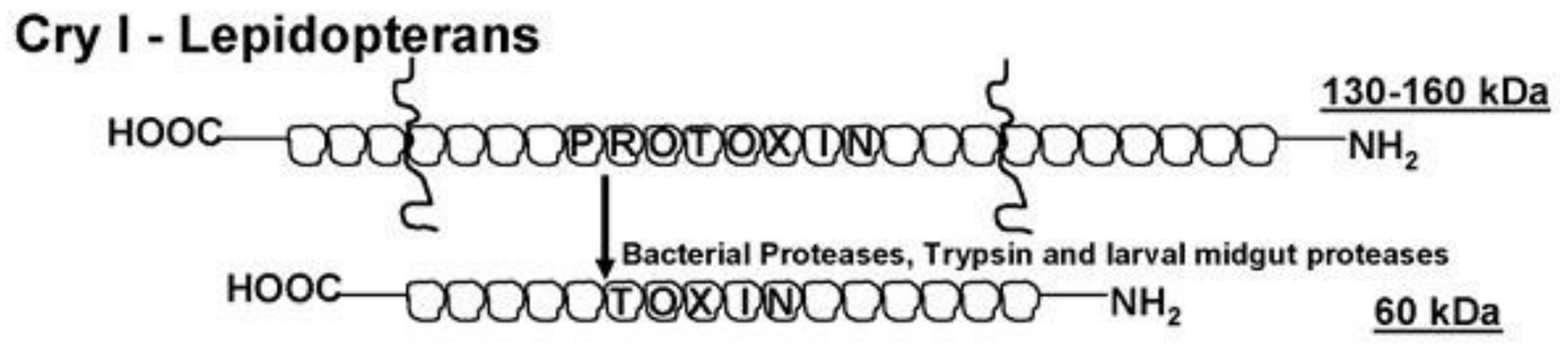




Mode of action of the Cry toxins

- Most Cry toxins are synthesized as inactive protoxins.

- Conversion of the protoxin (e.g., 130 kDa) into the active toxin (e.g., 68 kDa) requires the combination of a slightly alkaline pH (7.5-8) and the action of a specific protease(s) found in the insect gut
:



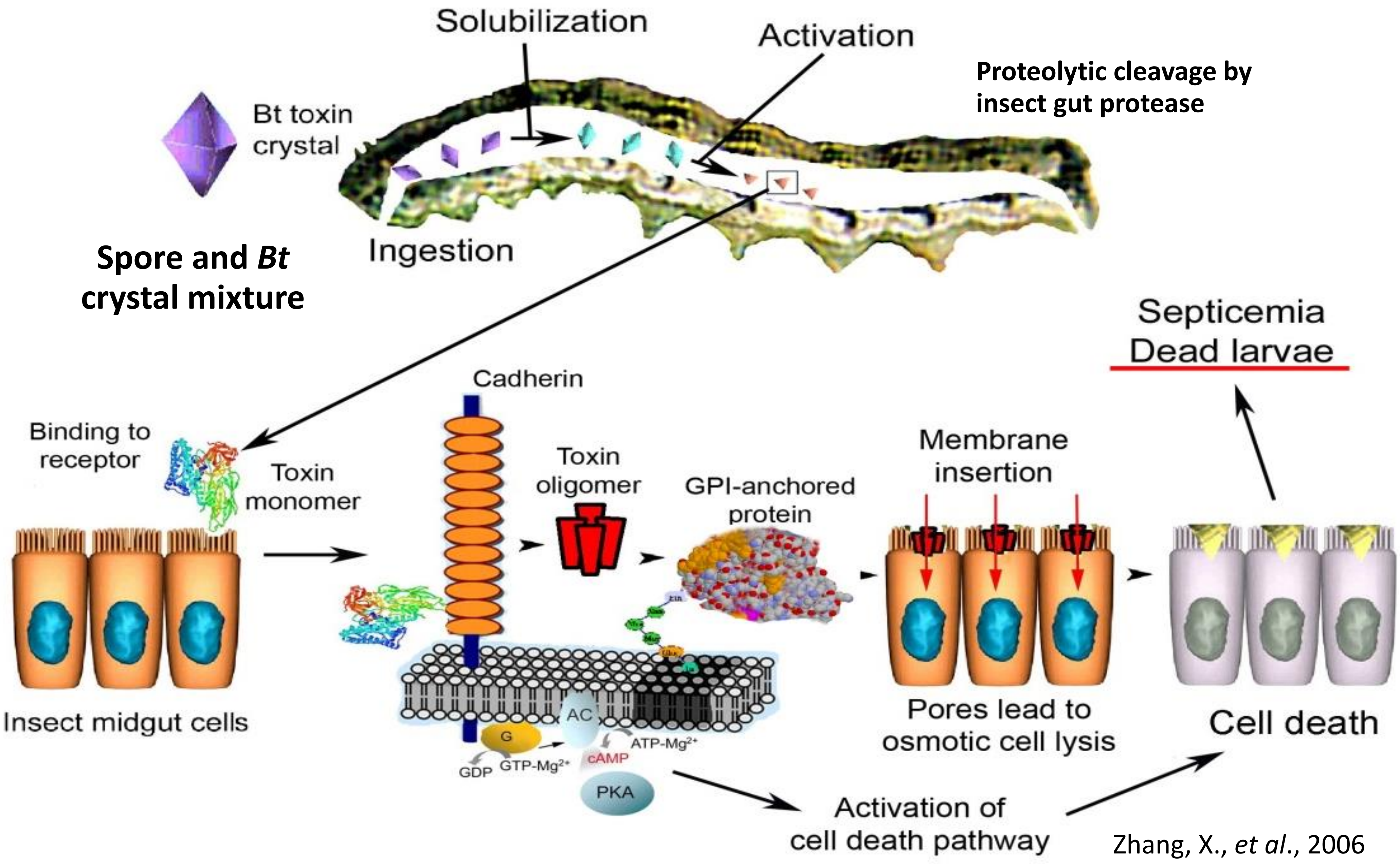
Brar *et al.*, 2007

- The active toxin binds to protein receptors on the insect gut epithelial cell membrane

- The toxin forms an ion channel between the cell cytoplasm and the external environment, leading to loss of cellular ATP gut wall to break down, allowing spores and normal gut bacteria to enter the body.

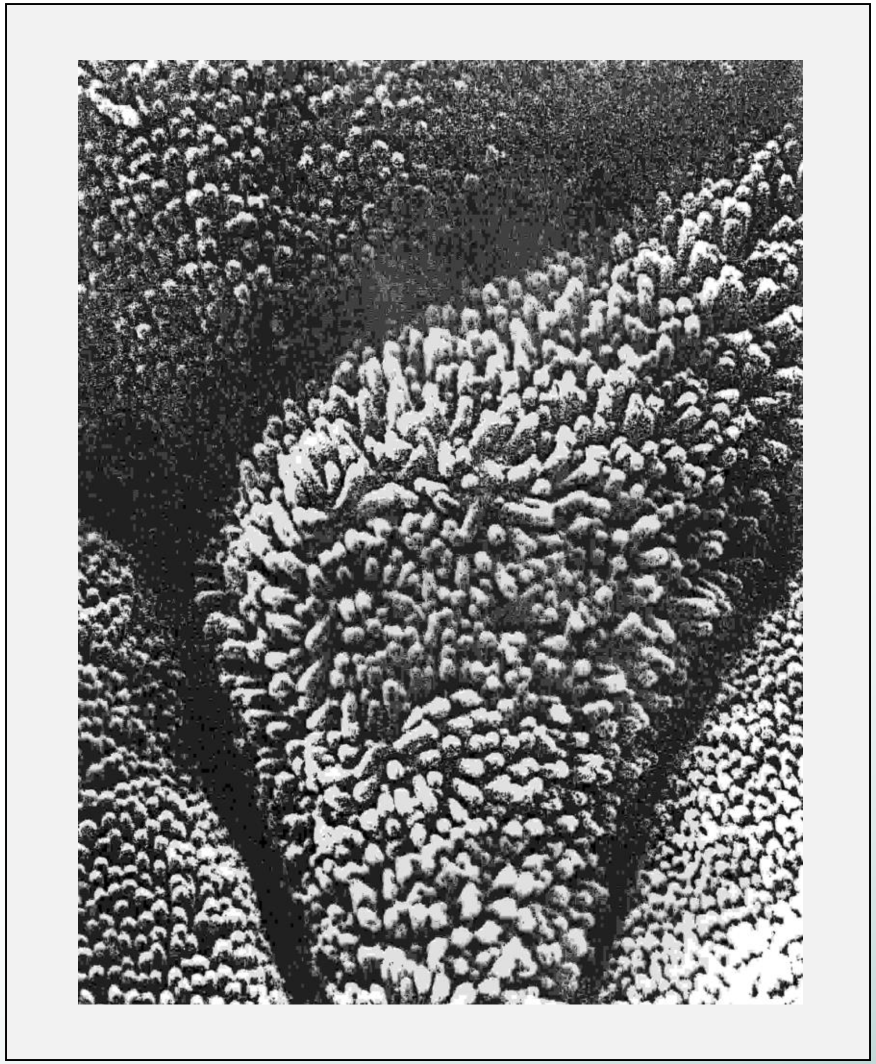
. - The insect dies as spores and gut bacteria proliferate in the body.

Schematic mode of action of the Cry toxins

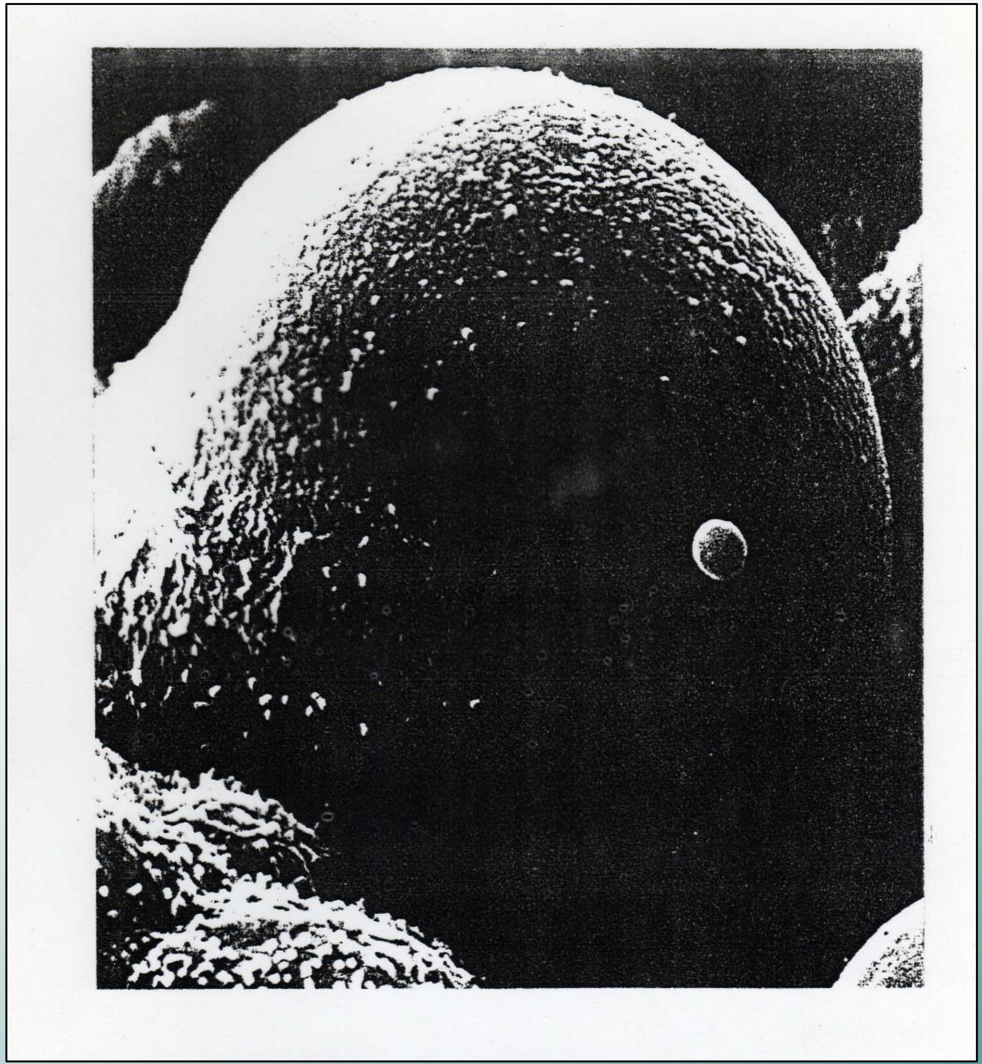


Zhang, X., *et al*., 2006

Effect of insecticidal toxins on epithelial cells

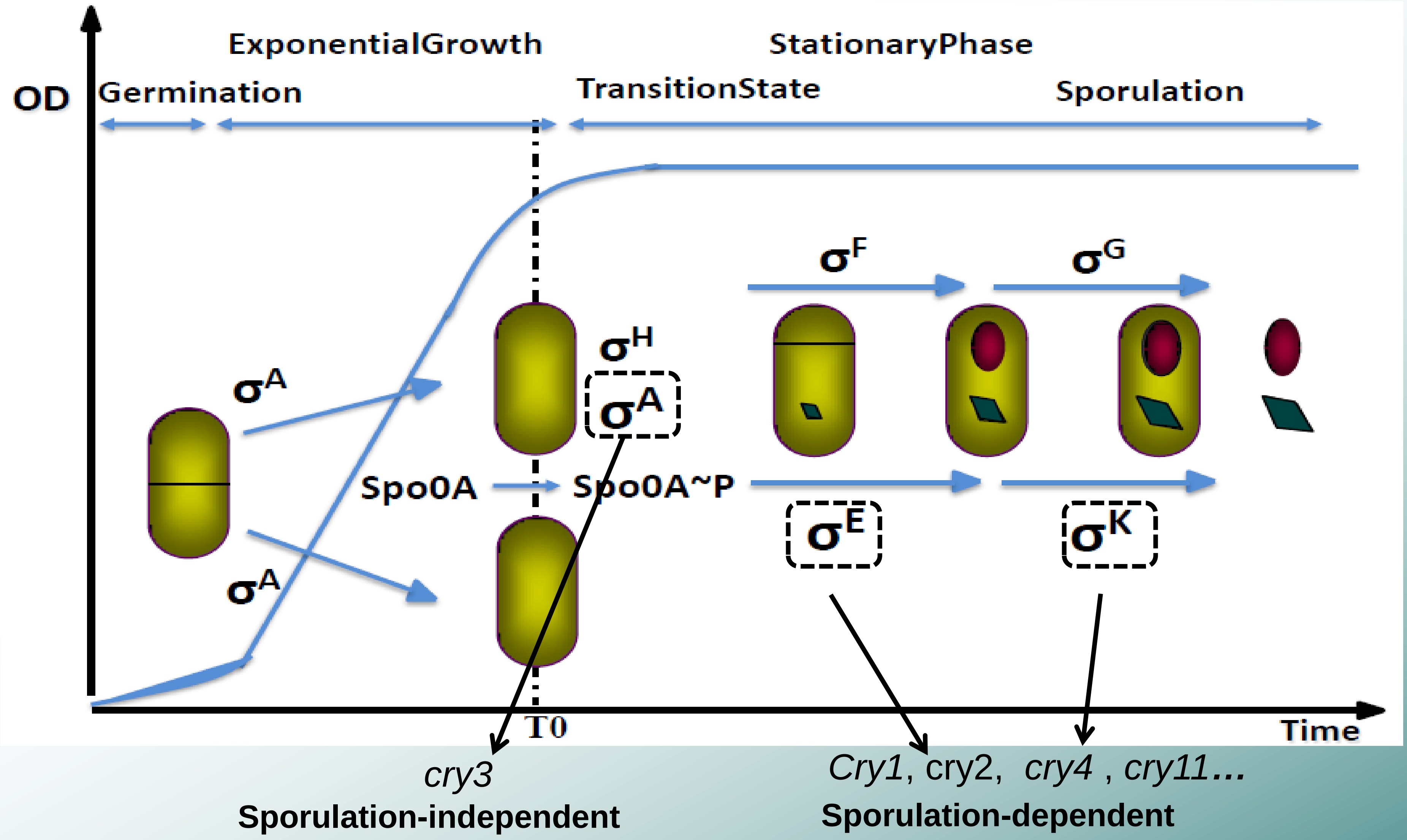


Appical Microvilli
(control)



Swelling of cells and lysis
(2 hours post intoxication))

Regulation of *cry* gene expression



cry1 (lepidopteran), cry4 and cry11 (dipteran) and *cry2* (dipteran and lepidopteran) genes are controlled by sporulation sigma factors whereas *cry3* (coleopteran) is not

Post-transcriptional regulation

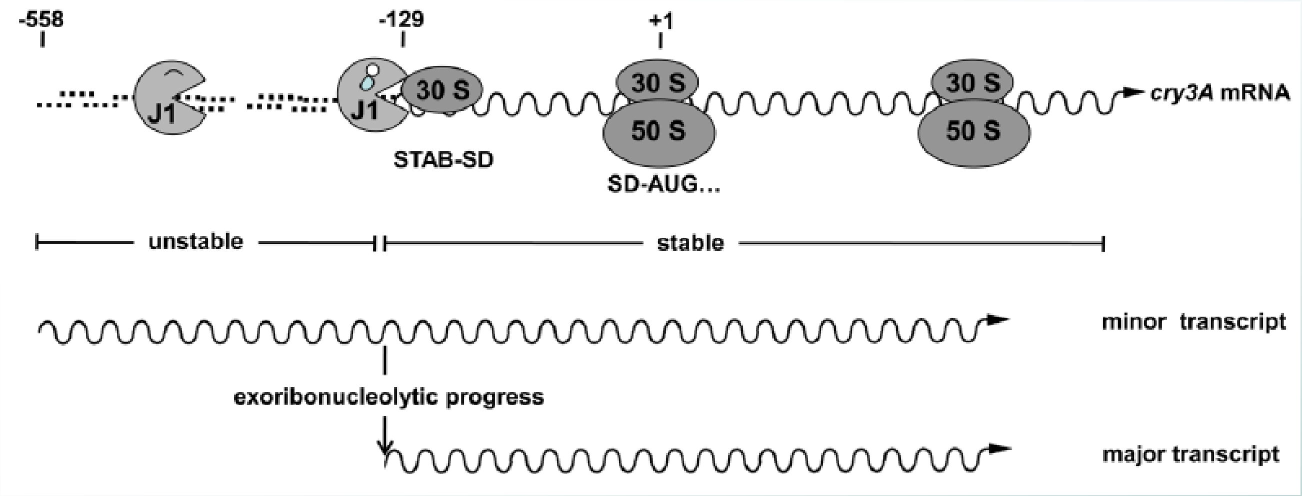
- The half life of general mRNA is about 2-3 min (Nilsson *et al.*, 1984).

- *cry1* mRNAs have half lives of about 10 min (Glatron and Rapaport, 1972).

- Terminator of most *cry1* genes contain large inverted repeats which are capable to form stem loop and protect the mRNA from 3’ ribonuclease degrading enzymes.

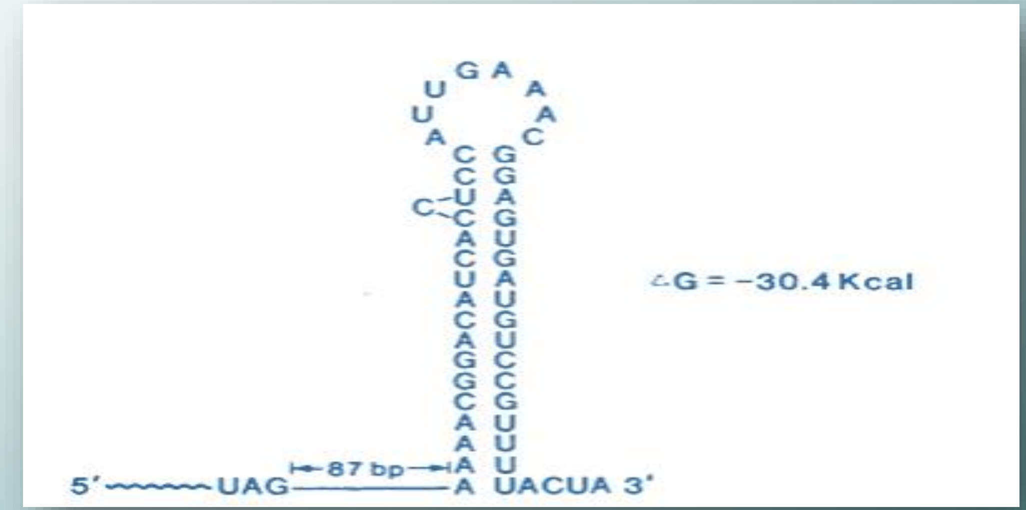
- A Shine Dalgarno sequence (STAB-SD) in the 5' untranslated region acts as a 5 ’ mRNA stabilizer.

- These characteristics increase *cry* mRNA stability.



The STAB-SD sequence in cry3A mRNA. The 30S ribosomal subunit can bind to the STAB-SD sequence mRNA and block the further 5 ’-3 ’ exoribonucleolytic progress of RNase J1.

Agaisse and Lereclus 1996

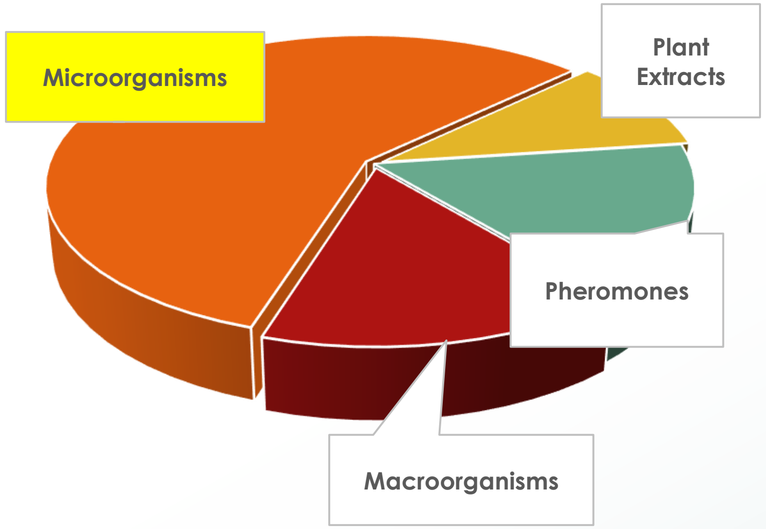


The secondary structure at the 3’ end of *cry1Aa* gene mRNA from *B. thuringiensis* subsp. *kurstaki* HD1

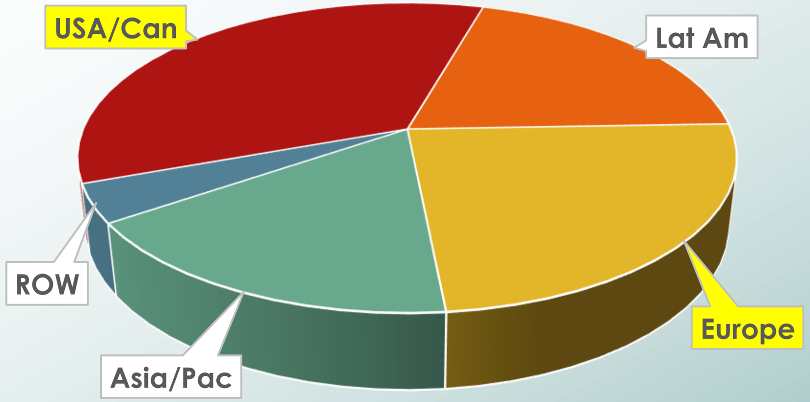
Wong and Chang, 1986

Biocontrol Market stats

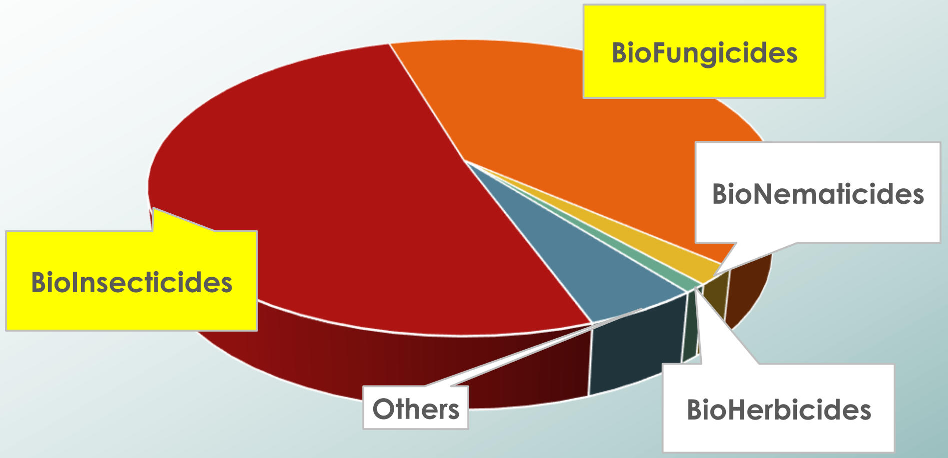
2015 Biocontrol Market



2015 Biopesticide Market



2015 Biopesticide Market



Source: DUNHAM TRIMMER international Bio Intelligence

B. thuringiensis and its uses as a biological control agent

- Health Authorities
- Individual use
- Farmers
- Distributors

- Mosquitoes Control

- Viticulture

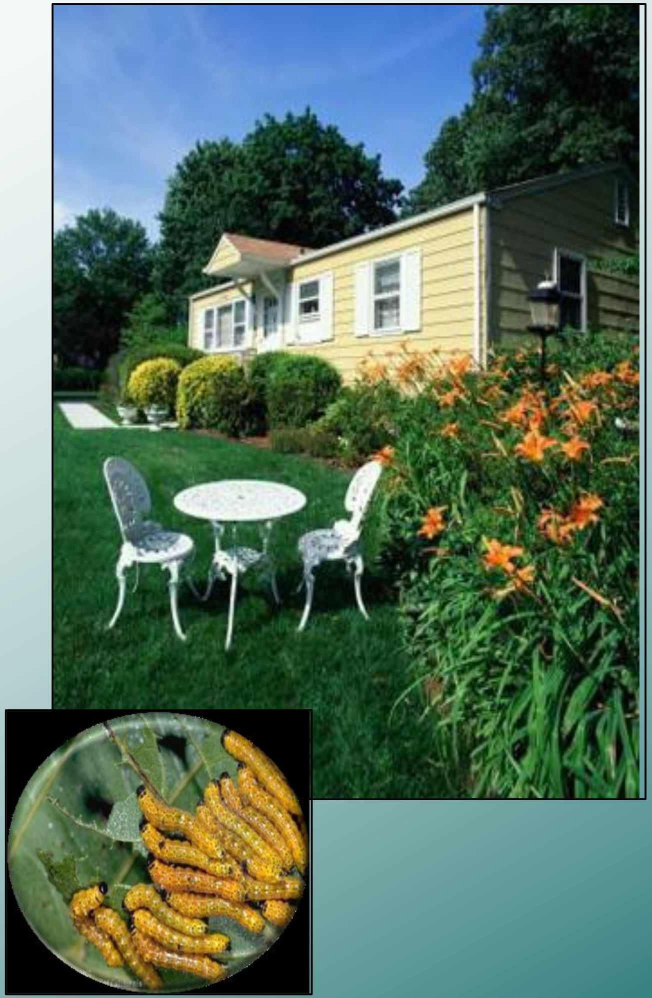
- Forestry

- Orchard

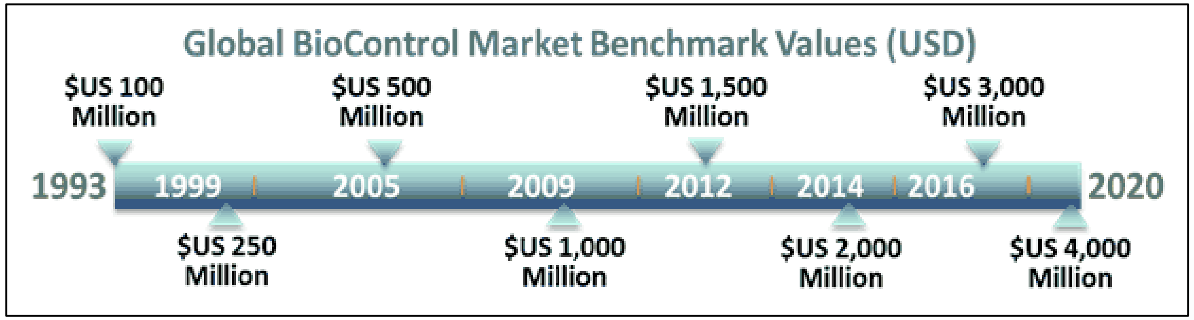
- Cereals

- Vegetables

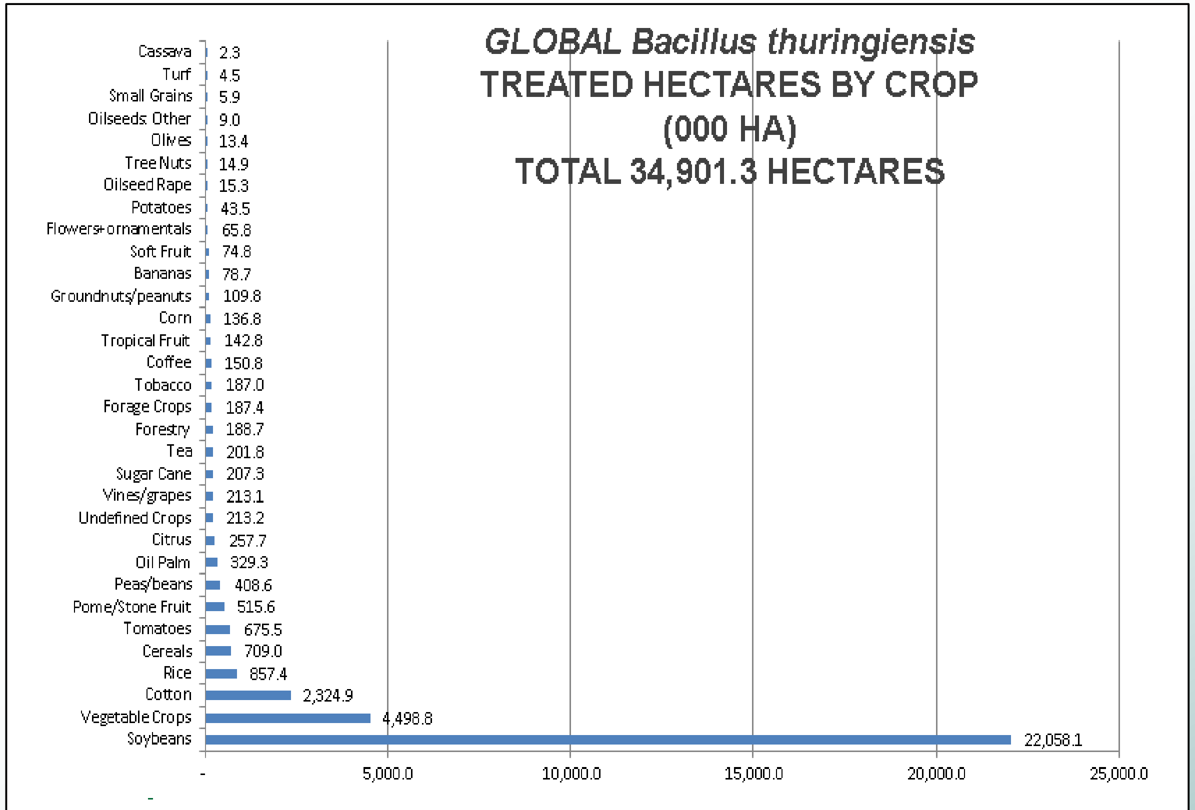

Outdoor residential areas



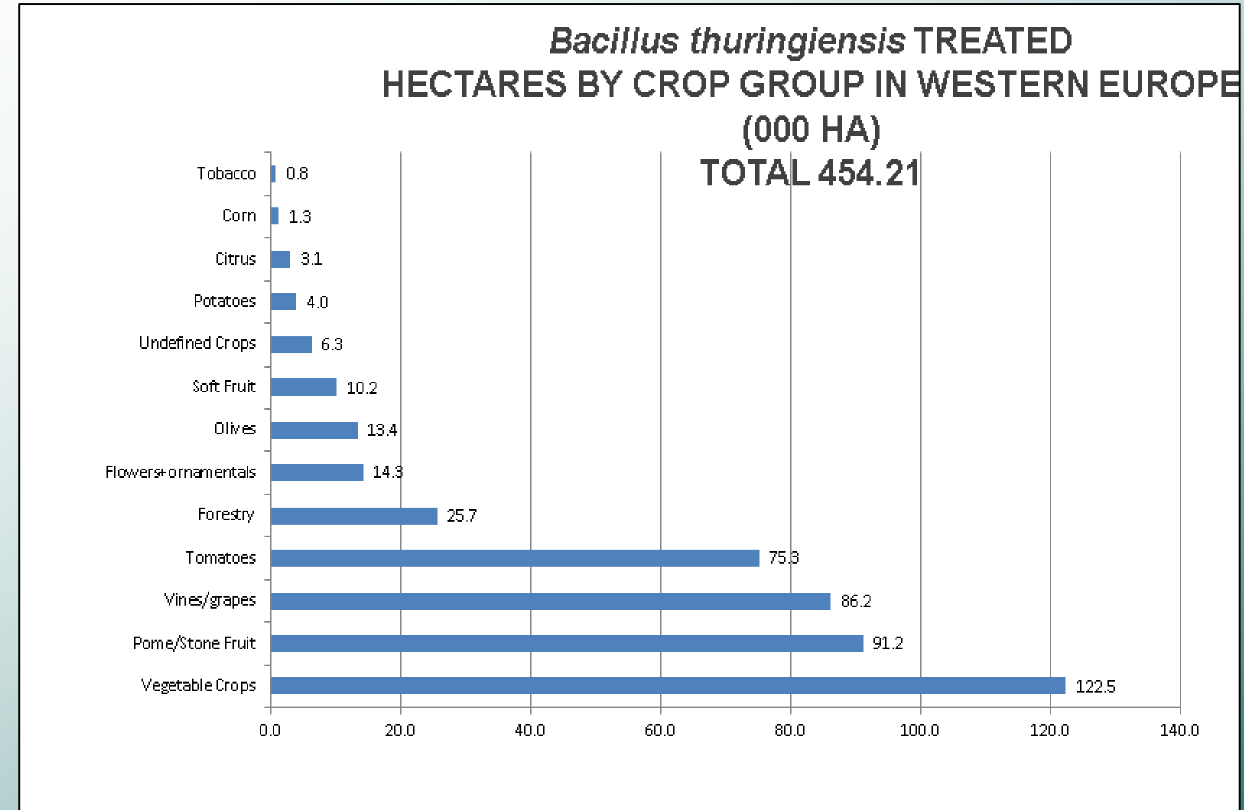
Current market for Bt pesticides



Biocontrol products today hold just 5% to 6% of the total crop protection market of approximately $50 billion in value worldwide, and 60% of all biopesticides use consists of Bt-based products.



Source: GfK I-Map Sigma Query Jan 2016



Source: GfK I-Map Sigma Query Jan 2016

Advantages of *Bt* Bioinsecticides

- Because for the toxin to be effective it has to be ingested, this limits the susceptibility of none target insects and other animals to this insecticide.

- Highly-specific compared to many synthetic insecticides -> Btk, Bta: Lepidopteran larvae only

- Safe to users, livestock, wildlife mammals have acidic gut

- Highly compatible with Integrated Risk Management (IPM)

- No effect on beneficial insects

- No toxic residues

Limitations of *Bt* Bioinsecticides

- High specificity = narrow spectrum -> ineffective against many key pests (i.e., aphids, mites, thrips, etc)

- *B. thuringiensis* toxin can only kill a susceptible insect during a specific developmental stage.

- Most effect against young larvae -> Proper timing of sprays is critical L1-L2 best; L3-maybe; L4-L5-too late. No effect on eggs, pupae, adults

- Sensitive to environmental conditions : Solar UV radiation (spray late in day, use sunscreen) Alkaline water (high pH)

- No contact activity -> Must be ingested to be effective Good spray coverage is critical to success

- Insects that attack plant roots are less likely to ingest a *B. thuringiensis* toxin that has been sprayed on the surface of a host plant.

Some desirable features of new genetically engineered and improved *Bt* products

- ***Broadened host range or optimized activity on a desired target insect***

To maximize market size or to target a selected niche

- ***Increased persistance***

To reduce the need for regular applications

- ***Improved potency***

To achieve the desired effect in a cost effective manner

- ***Sporulation deficient mutants***

To minimize possible unforeseen environmental effects arising from the dissemination of large amounts of viable spores

- ***Recombinant strains free of non-*****Bt** ***DNA or antibiotic resistance genes***

To facilitate regulatory approval for environmental release and/or registration as a biopesticide

Development of safer and more effective Bt-Based biopesticides

Objectives :

- Design new biopesticides and validate the relevance of these products and their performance, while ensuring better security of use for farmers, consumers and the environment.

- Improve Bt strains used as biopesticides by increasing their efficiency and persistence in the environment while avoiding the spread of viable spores in the environment

APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Feb. 1997, p. 779–784 Vol. 63, No. 2
0099-2240/97/$04.00+0

A Recombinase-Mediated System for Elimination of Antibiotic Resistance Gene Markers from Genetically Engineered *Bacillus thuringiensis* Strains

VINCENT SANCHIS,[1,2*] HERVÉ AGAISSE,[1] JOSETTE CHAUFAUX,[2] AND DIDIER LERECLUS[1,2]
Unité de

ELSEVIER

Journal of Biotechnology 48 (1996) 81–96

Construction of new insecticidal *Bacillus thuringiensis* recombinant strains by using the sporulation non-dependent expression system of *cryIIIA* and a site specific recombination vector

Vincent Sanchis[a,b,*], Hervé Agaisse[a], Josette Chaufaux[b], Didier Lereclus[a,b]
[a] *Unité de Biochimie Microbienne, Institut Pasteur, 28 rue du Dr. Roux, 75724 Paris Cedex 15, France*
[b] *Station de Recherches de Lutte Biologique, INRA, La Minière, 78285 Guyancourt Cedex, France*

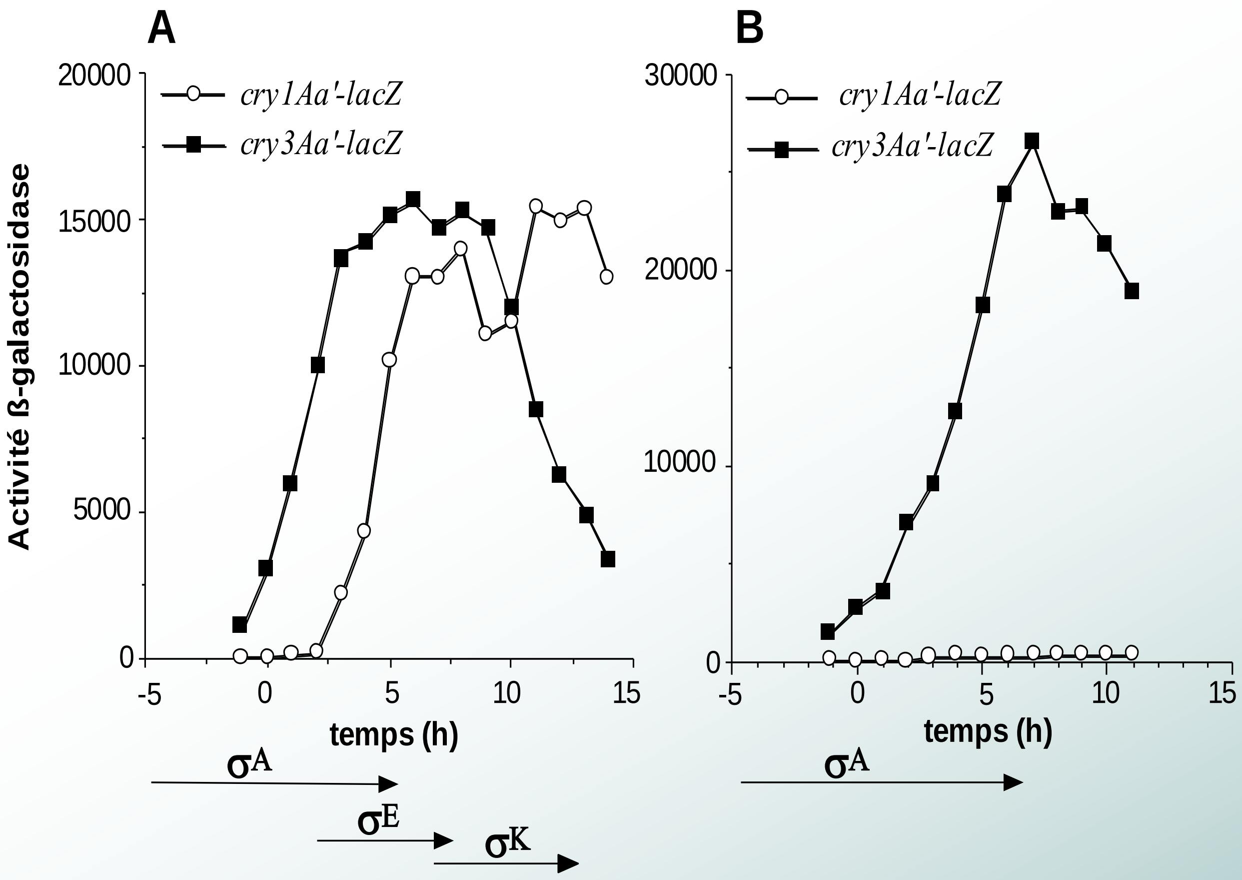
APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Sept. 1999, p. 4032–4039 Vol. 65, No. 9
0099-2240/99/$04.00+0

Development and Field Performance of a Broad-Spectrum Nonviable Asporogenic Recombinant Strain of *Bacillus thuringiensis* with Greater Potency and UV Resistance

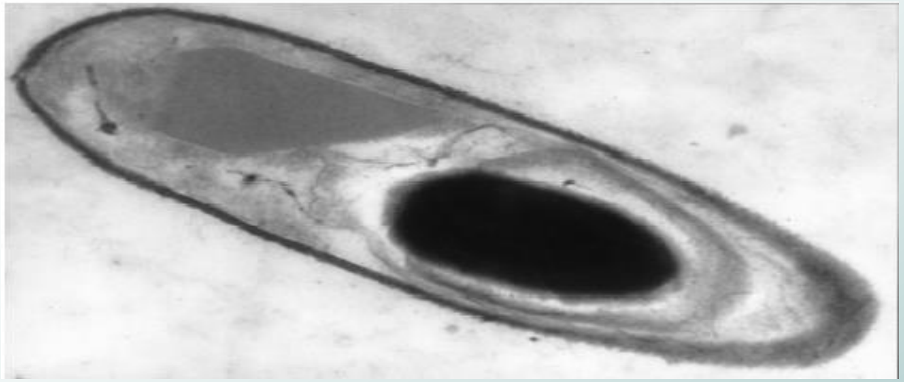
VINCENT SANCHIS,[1,2*] MICHEL GOHAR,[3†] JOSETTE CHAUFAUX,[2] OLIVIA ARANTES,[1‡] ALAIN MEIER,[4] HERVÉ AGAISSE,[1,2] JANE CAYLEY,[5] AND DIDIER LERECLUS[1,2]
Unité de Biochimie Microbienne[1] and Laboratoire des Fermentations, Unité de Physiologie Cellulaire,[4] Institut Pasteur, Centre National de la Recherche Scientifique, 75724 Paris Cedex 15, Station de Recherches de Lutte Biologique, Institut National de la Recherche Agronomique, La Minière, 78285 Guyancourt,[2] and AgrEvo Prodetech, 13367 Marseille Cedex 11,[3] France, and Hoechst Schering AgrEvo GmbH, Hoechst Works, D-65926 Frankfurt am Main, Germany[5]

Construction of an asporogenic non viable of *B. thuringiensis* strain

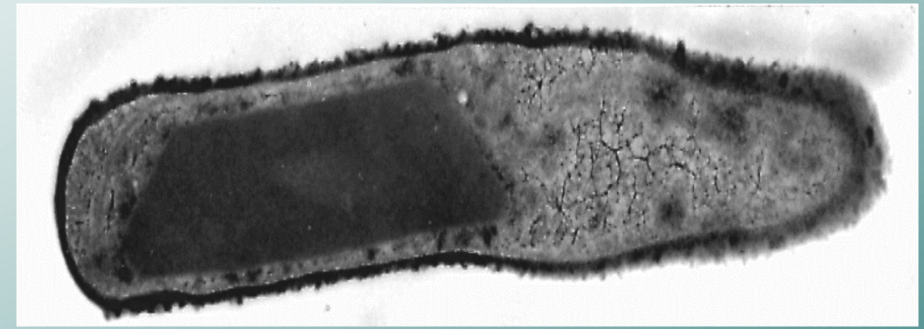
Expression of cry1A'lacZ and cry3A'lacZ transcriptional fusions in two different genetic backgrounds: WT (A) and Δ*spoOA* (B)



The transcription of the cry3A gene is independent of the regulatory genes involved in the sporulation process



Longitudinal section of Bt WT producing large amounts of Cry1A at the end of sporulation before cell lysis.



Longitudinal section of a Bt ΔspoOA strain producing large amounts of Cry3A during the stationary phase.

Necessary steps for obtaining insect-resistant transgenic plants

- Introduction of cry genes into plant cells

  => Transformation by Agrobacterium or direct gene transfer in the plant cell (transformation of protoplasts, particle guns)

- Expression of cry genes in the host plant

  => genes with Eukaryotic structure (synthetic genes)

- Integration of cry genes into the host genome

  => Stable and transmissible expression to daughter cells of the transgene

- Selection and regeneration of whole plants from genetically modified cells

  => Efficient transfer, use of selection markers, obtaining an entire organism from selected transformants

Construction of a transgenic tobacco transformed with the *cry1C* gene

On the left, the untransformed control. On the right is tobacco transformed with a cry1C gene modified for expression in plants. In both cases 40 larvae of *S. littoralis* were deposited on the leaves. Damage observed after 72 hours. (Photo: J. Tourneur, INRA)

Commercialized transgenic plants resistant to insects

Cotton: 1st commercialised in 1996

- in *2017*: 14 countries;

  18 million hectares IR and 5.2 million hectares IR/HT

  77% of the global cotton area

  12,8% of the global biotech crop area

Maize: 1st commercialised in 1996

- in *2017*: 14 countries

  5.3 million hectares IR and 48.1 million hectares stacked IR/HT

  28,6 % of the global maize area

  28,1 % of the global biotech crop area

Soybean: 1st commercialised in 1996

- in *2017*: 9 countries

  24.4 million million hectares stacked IR/HT

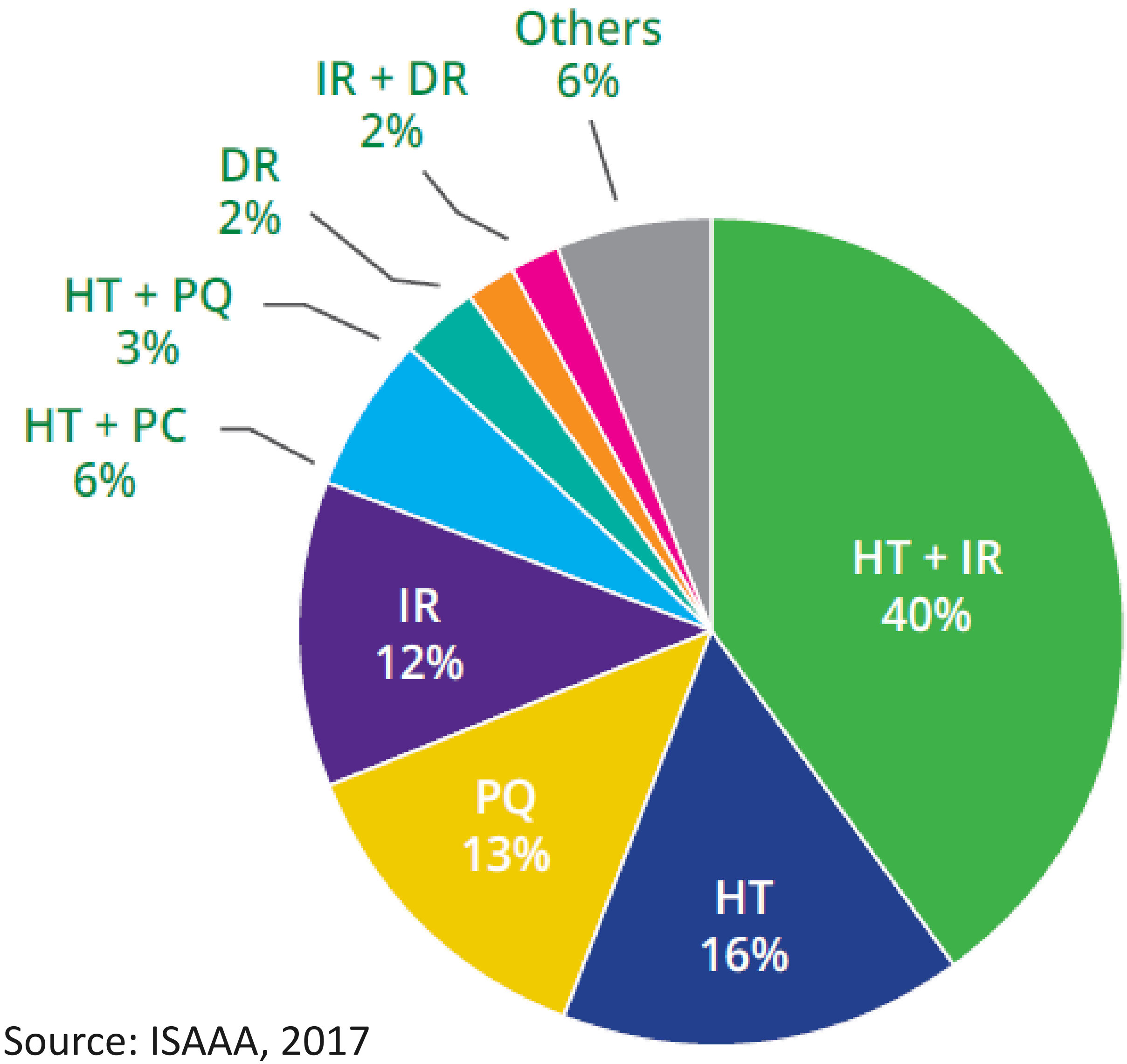
  20 % of the global soybean area

  12,8% of the global biotech crop area

Potato: first commercialised in 1996, withdrawn in 2001

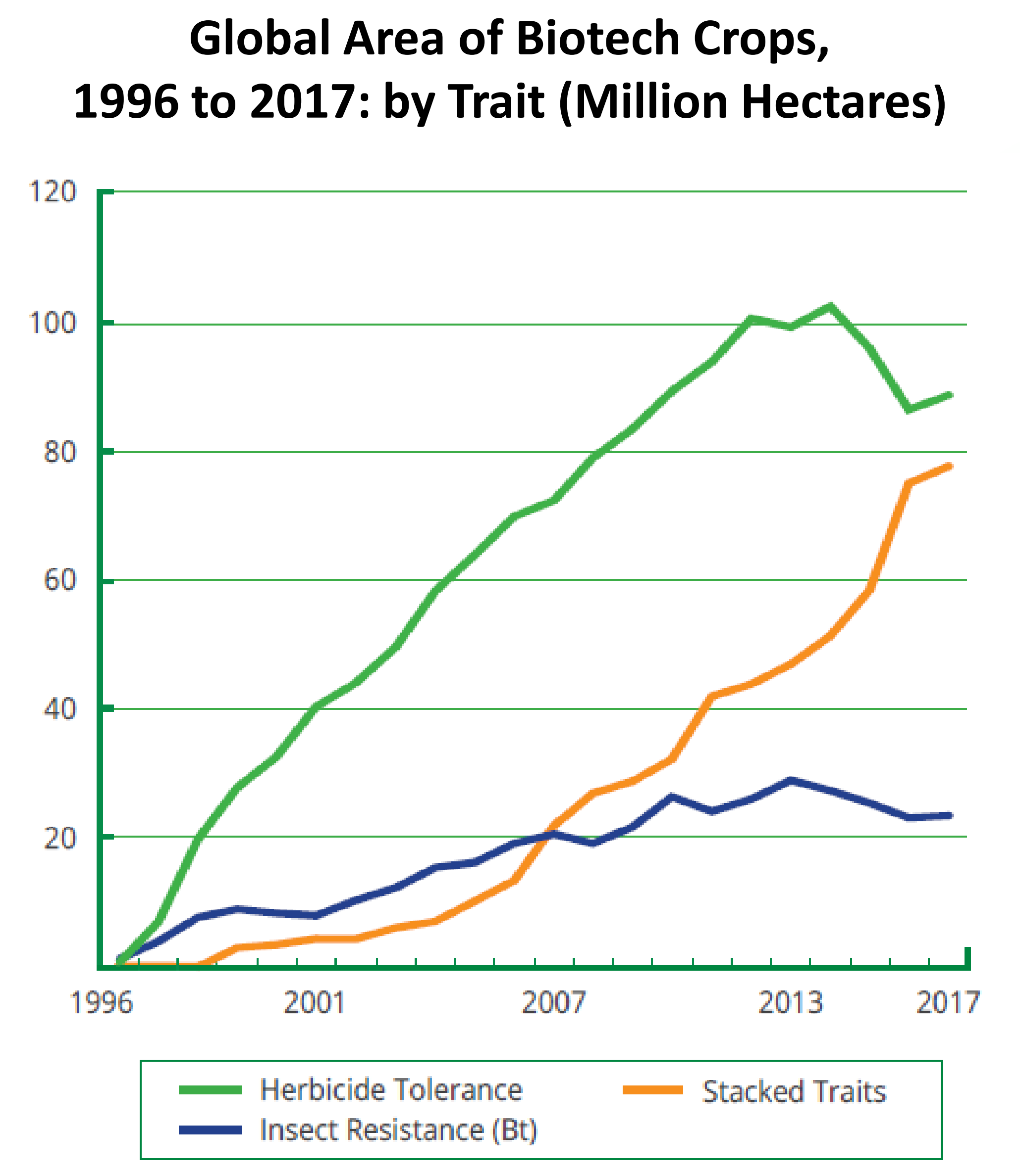
Distribution of traits of approved GM events

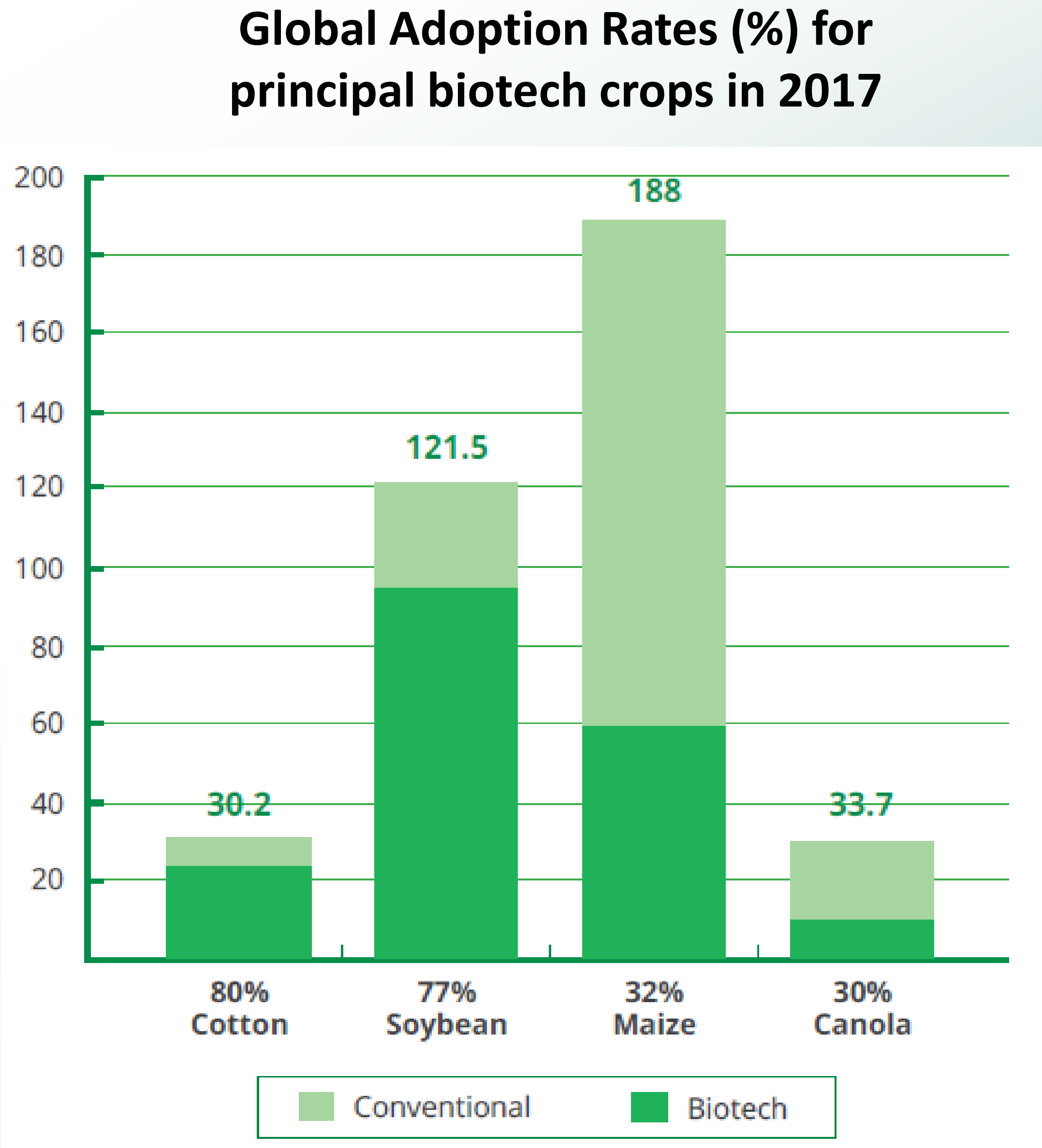
HT - Herbicide Tolerance;
IR - Insect Resistance;
DR - Disease Resistance;
PC -Pollination Control;
PQ - Modified Product Quality:



Source: ISAAA, 2017

Distribution of biotech crops by crop and by trait (million hectares)





Source: ISAAA, 2017

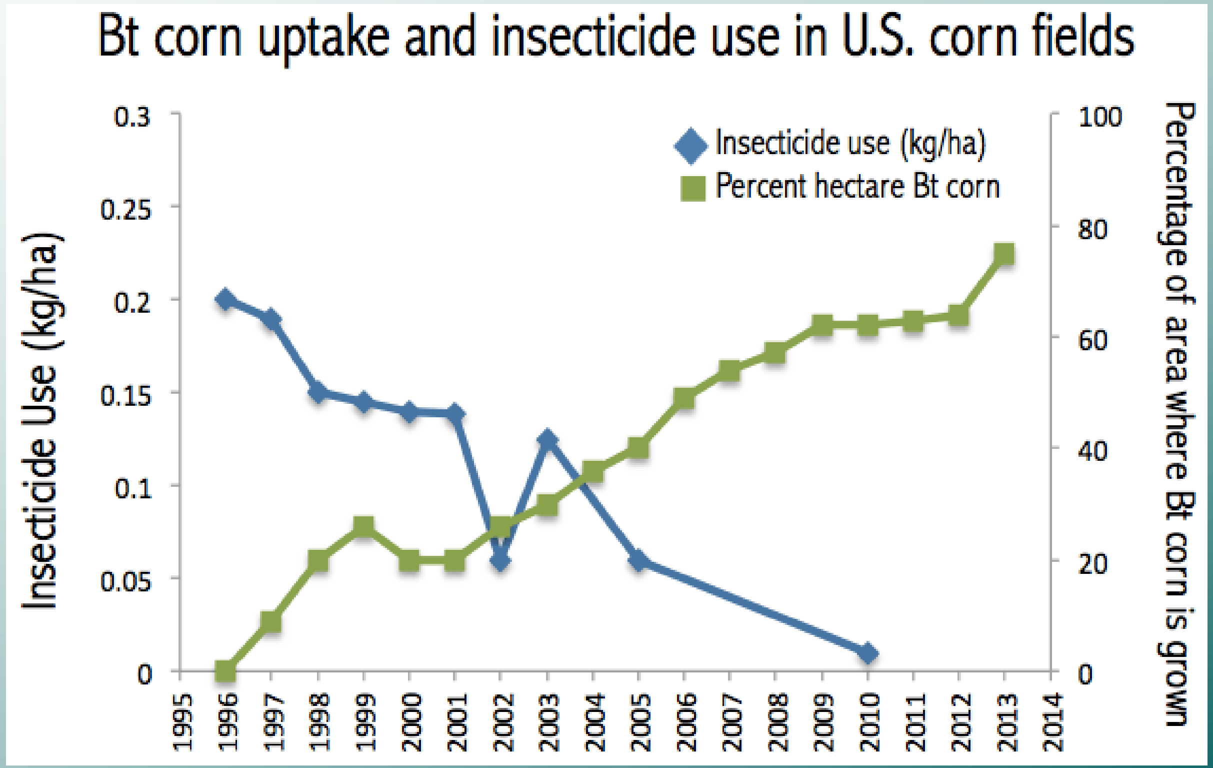
The cultivation of Bt plants is widely spread

Global Adoption Rates (%) for principal Bt crops in 2017

Bt Plants	% USA	% World
Corn	87	29
Cotton	88	77
Soybean	0*	20

* In the US only Herbicide tolerant soybean is grown

In USA, in the period of 1995-2010, the amount of pesticides used per acre of corn decreased by 99%, while insecticide use on cotton crops reduced approx. 95%.



Global crop protection market in 2017

US$M	Herbicides	Insecticides	Fungicides	Others	Biotech	Total
North America	5,772	2,287	2,298	442	12,398	23,197
Europe	5,312	1,672	4,412	396	10	11,622
Japan	1,100	1,089	880	62	0	3,131
Australia	562	196	150	22	35	965
Industrial Countries	**12,566**	**5,244**	**7,740**	**922**	**12,443**	**38,915**
Latin America	5,317	3,048	4,054	461	3,664	16,364
Rest of Far East	3,612	2,751	2,648	401	398	9,810
Rest of World	1,692	1,966	1,238	215	676	5,787
Developing Countries	**10,441**	**7,765**	**7,940**	**1,077**	**4,738**	**31,961**
Total	**23,007**	**13,009**	**15,680**	**1,999**	**17,181**	**70,876**

Source: Cropnosis Agrochemical Service, 2017

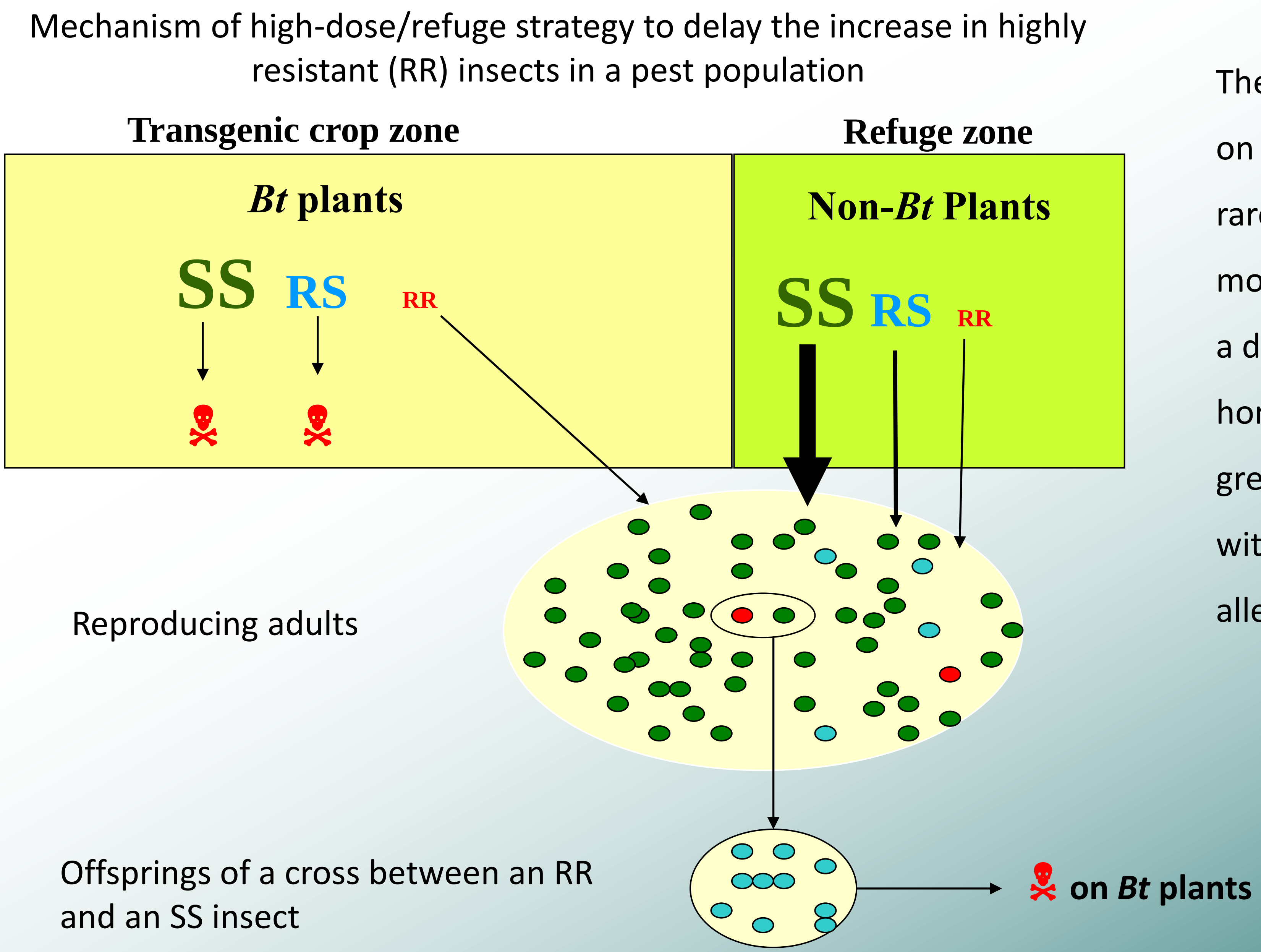
Managing the risks of insect resistance associated with the use of transgenic *Bt* crops

How to cope with insect resistance to Bt toxins?

Several strategies for decreasing the rate at which insects adapt to *Bt* toxins produced in transgenic plants have been proposed and implemented . These strategies include:

- 1) engineering plants to produce *Bt* toxins only in the tissues that are prone to insect attack;
- 2) using rotations (in which transgenics may be alternated in time with non-transgenics), or mosaics (in which mixtures of transgenic and non-transgenic plants are grown together);
- 3) creating refugia in which a portion of a field may be planted with non-transgenics, or the “high dose-refuge” (HDR) strategy ;
- 4) developing resistance monitoring programmes.

Schematic representation of the "high dose-refuge" (HDR) strategy



The success of the HDR strategy depends on resistance being a rare and recessive trait and the genetically modified plants producing a dose of toxin sufficient to kill all homozygous susceptible individuals (SS-green) and all heterozygous individuals with for both resistance and susceptibility alleles (RS-blue)

Thank you for your attention