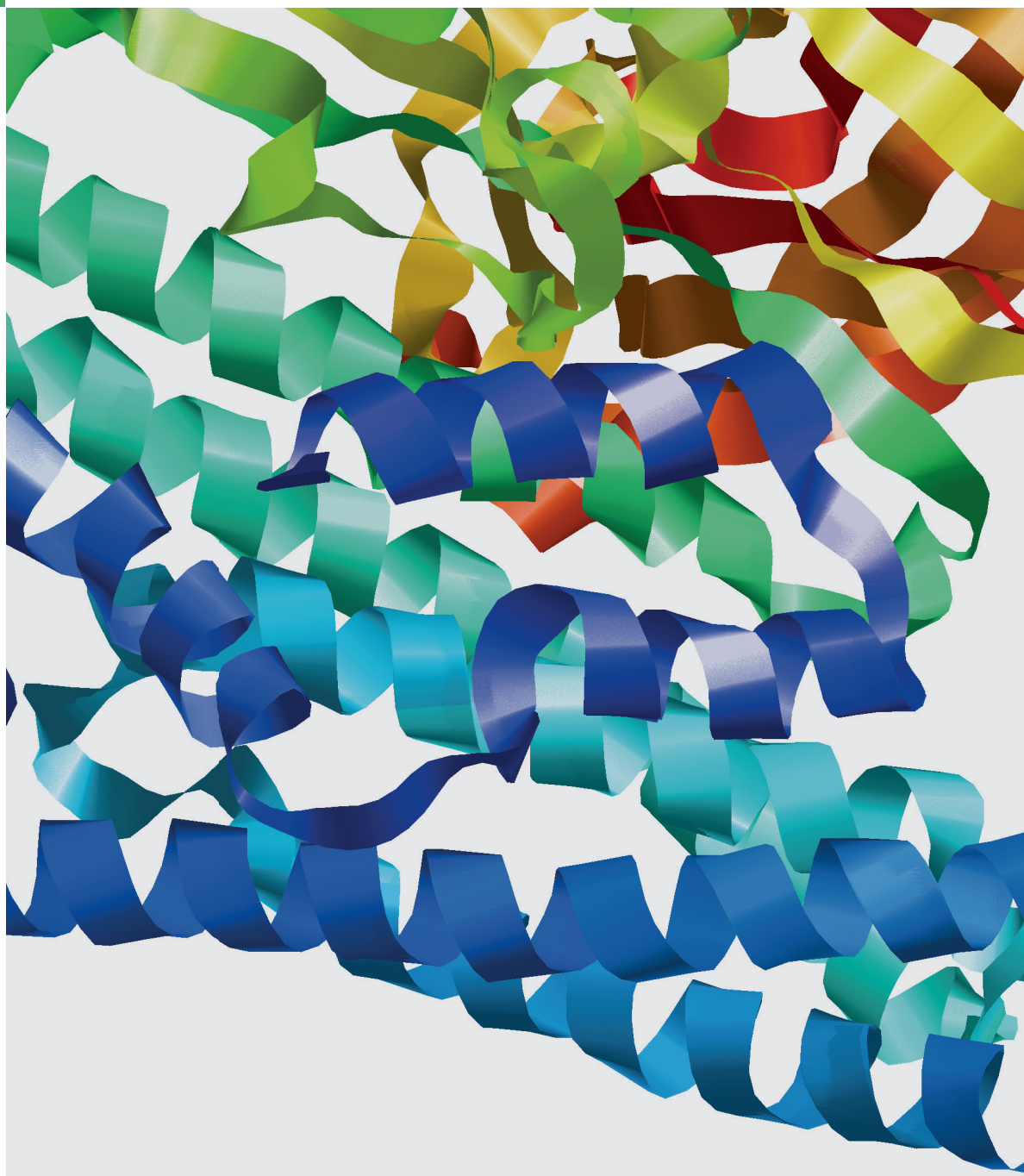
Bacillus thuringiensis toxins: their mode of action and the potential for interaction between them



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Bacillus thuringiensis toxins: Their mode of action and the potential interaction between them.

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Preface

Most insect resistant GM crops express one or more genes derived from the bacterium *Bacillus thuringiensis* (Bt). As part of the authorisation procedure for cultivation of such an insect resistant GM crop the potential effect of the GM crop on so-called non-target organisms (NTOs) is assessed. Often, the first step to assess potential effects on NTOs is to expose different NTOs to the Bt proteins produced by the GM crop. These experiments are usually performed by exposing a NTO to a single purified Bt protein.
Recently, the number of GM crops producing multiple Bt proteins is increasing. As proteins may influence each other, the mode of action and the effect of a Bt protein could potentially change due to the presence of other Bt proteins. In case of synergy, effects may occur at a lower dose than expected from experiments with single Bt proteins and it can be hypothesized that the specificity of the Bt proteins may change when multiple Bt proteins are present. If the presence of multiple Bt proteins influences their effect on NTOs, the current practice to study the effect on NTOs by exposing them to single Bt proteins may need to be adapted.
COGEM has commissioned two research projects to investigate whether the above mentioned concerns are justified and to develop guidelines for the risk assessment of GM crops producing multiple Bt proteins.
The present report '*Bacillus thuringiensis* toxins: their mode of action and the potential interaction between them' provides detailed information on the different groups of Bt proteins and describes the models suggested for their mode of action. In addition, different models that may be used to predict the effect of multiple toxins are explained. Moreover, literature on the combined effect of multiple Bt proteins, Bt protein cross-resistance and co-evolution was studied to investigate the likelihood of synergy occurring when multiple Bt proteins are produced by a GM crop. Finally, a check list is presented that can be used to decide if additional studies on the occurrence of synergy are needed to assess the risks of GM crops producing multiple Bt proteins on NTOs.
Overall, the present research report is an abundant source of valuable information for those involved in the risk assessment of GM crops producing multiple Bt proteins.

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Chair of the advisory committee

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Samenvatting

Bacillus thuringiensis (Bt) stammen produceren, in hun natuurlijke diversiteit, een rijk scala aan toxische eiwitten (Bt-toxinen). Algemeen wordt aangenomen dat ieder van die eiwitten slechts giftig is voor een beperkte taxonomische groep, meestal een groep binnen de insecten. Vanwege hun (vermeende) selectieve giftigheid voor plaagorganismen worden Bt genen gebruikt in genetisch gemodificeerde (GG) gewassen. Voordat GG gewassen toegelaten worden in de EU, moeten de mogelijke effecten op mens en milieu bepaald worden. Bij GG-gewassen met Bt genen is daarbij speciale aandacht voor de mogelijke effecten op niet-doelwitorganismen. Deze mogelijke effecten worden o.a. onderzocht door niet-doelwitorganismen in laboratoriumexperimenten bloot te stellen aan afzonderlijke Bt-eiwitten. De laatste jaren worden steeds meer gewassen ontwikkeld met meerdere Bt genen, en deze gewassen produceren dus een cocktail van Bt-toxinen. Om inzicht te verkrijgen of additionele gegevens noodzakelijk zijn om de potentiële effecten van dergelijke mengsels van Bt-toxinen te beoordelen, is door ECOSTAT in opdracht van de Nederlandse Commissie Genetische Modificatie (COGEM) een literatuurstudie verricht naar de eigenschappen van Bt-toxinen en de in de literatuur bekende gegevens over mengseltoxiciteit van Bt-toxinen.

De Bt-eiwitten kunnen worden verdeeld in twee hoofdgroepen, de kristalliserende eiwitten, die aanwezig zijn in Bt-sporen (Cry- en Cyt-toxinen) en de eiwitten die in de groeifase gemaakt worden (Vip-toxinen). De meeste toxinen worden door Bt gemaakt als protoxinen en worden pas toxisch na proteolyse.

De structuur van naamgeving is identiek voor de Cry-, Cyt en Vip-toxinen: voor elk begint de naam met Cry, Cyt of Vip, gevolgd door een getal, een hoofdletter, een kleine letter, en weer een getal (Hs. I-1, p. 27). De aminozuurvolgorde van de eiwitten vertoont meer overeenkomst als eerste getal, hoofd- en kleine letter gelijk zijn dan als alleen eerste getal en hoofdletter gelijk zijn, en is nog wat meer verschillend als alleen het eerste getal gelijk is. De volgorde van de getallen en letters is geen maat voor de verwantschap, maar is bepaald door de volgorde waarin de eiwitten beschreven zijn. Zowel de naam Cry als Vip wordt gebruikt voor meerdere groepen structureel verschillende toxinen.

De Cry-toxinen kunnen onderverdeeld worden in een aantal groepen, waarvan de grootste groep de groep van de 3-domein (3D) Cry-toxinen is. Deze toxinen hebben een vergelijkbare quaternaire eiwit structuur bestaande uit drie duidelijk gescheiden structurele domeinen (Hs. I-2, p. 35). 3D-Cry-toxinen maken vermoedelijk poriën in het celmembraan en zijn daardoor toxisch. De drie domeinen hebben verschillende functies bij de membraanperforatie. Domein II en III zorgen voor de binding van het toxine aan receptoren op het celmembraan. Hierdoor komt domein I dicht genoeg bij het membraan en ook bij andere aan het membraan gebonden Cry-toxinen, om poriën te vormen in het celmembraan, al dan niet als oligomeer met het Domein I van die andere Cry toxinen. Deze oligomeren kunnen niet alleen gevormd worden uit verbindingen tussen moleculen van hetzelfde type 3D-Cry-toxinen, maar soms ook door combinaties van verschillende typen 3D-Cry-toxinen.Voor Cry1Ab is dit proces uitgebreid onderzocht, wat geleid heeft tot twee verschillende modellen: Volgens het “sequential binding model” (Hs. I-2.2.2, p.

42) bindt het Cry-eiwit zich eerst aan een receptor, waardoor de eiwit conformatie verandert en vervolgens bindt het eiwit in die nieuwe conformatie aan een tweede receptor wat de mogelijkheid biedt om, al dan niet als oligomeer met andere Cry-eiwitten, een porie te vormen in het celmembraan. Volgens het ”signalling pathway model” (Hs. I-2.2.3, p. 44) vormt de Cry-toxine geen porie, maar start het een kettingreactie door zich te binden aan een receptor op het celmembraan, zodat die receptor een signaal aan de cel geeft waardoor een reactieketen gestart wordt, die uiteindelijk leidt tot de dood van de cel. Evaluatie van deze modellen leidt tot de conclusie dat het “sequential binding model” aannemelijker is voor Cry1Ab en andere 3D-Cry-toxinen waarbij de details van het bindingsproces afhankelijk kunnen zijn van het type 3D-Cry-toxine. (Hs. I-4, p. 56).

Het perforerende Domein I van de 3D-Cry-toxinen is opgebouwd uit 7 α-helices, α1 t/m α7, met helix α5 in het centrum en de overige 6 helices daaromheen gegroepeerd. Twee modellen worden beschreven voor de perforatie, het paraplu (umbrella) en het knipmes (penknife) model, en van die twee is het paraplumodel het aannemelijkst. In dat model prikt de centrale α5 helix samen met helix α4 door het celmembraan, terwijl de overige 5 helices in een parapluvorm over het celmembraan worden uitgespreid (Hs I-2.1, p 37 en Hs I-2.2.4, p. 46). Schattingen van het aantal domein I monomeren dat gezamenlijk als oligomeer een porie vormt, lopen uiteen van twee tot vier. De diameter van de porie is in de orde van 1.0 tot 2.6 nm. De poriën kunnen zich in twee toestanden bevinden, geopend of gesloten, en de kans om van de ene toestand naar de andere over te gaan hangt onder andere af van de omgevingsvariabelen, zoals de pH (Hs I-2.2.4, p. 46).

De andere groepen van Bt-toxinen zijn:

- Bin: Deze Cry-toxinen lijken in eiwitsequentie op het complementaire paar BinA en BinB van *B. sphaericus*. De giftigheid van deze toxinen is aanzienlijk groter in een mengsel met hun complementaire toxine. (Hs. I-5.1, p.60).
- Mtx: Deze groep van Cry-toxinen bestaat uit eiwitten die qua structuur lijken op het β-porie-vormende ε-toxine van *Clostridium perfringens*. Voor sommige Mtx-eiwitten is geen toxiciteit aangetoond, maar van enkele van deze toxinen is wel waargenomen dat ze de toxiciteit van andere (Mtx) toxinen kunnen vergroten (Hs. I-5.2, p. 62).
- Cyt: Deze toxinen veroorzaken in vitro cytolyse. In vivo zijn Cyt-proteinen vaak maar licht toxisch. Maar wanneer ze samen met andere Cry-toxinen voorkomen, kunnen ze elkaars toxische effect versterken. Vermoedelijk kunnen aan het celmembraan gebonden Cyt-toxinen dienen als receptoren voor Cry-toxinen, en vergroten ze daarmee de bindingskans voor Cry-toxinen (Hs I-5.5, p. 69).
- Vip: Dit zijn insectendodende eiwitten die in de groeifase van de bacterie worden gevormd. Er zijn 2 groepen van Vip-eiwitten: Die van Vip1 en Vip2, die ieder afzonderlijk slechts licht toxisch zijn maar elkaars toxiciteit versterken. En de groep van Vip3. Vip3 bindt zich aan andere membraanreceptoren dan de 3D-Cry-toxinen (Hs. I-5.6, p. 73).
- Niet-verwante Cry: Dit is een gemêleerde groep van Cry-toxinen met weinig overeenkomsten in eiwitsequentie. Enkele van deze Cry-toxinen kunnen optreden als bijbehorende complement voor een Bin-toxine (Cry34 met Bin-toxine Cry35)

of een Mtx-toxine (Cry37 met Mtx-toxine Cry23) (Hs. I-5.4, p. 67)

Insectenlarven nemen de Cry-toxinen meestal op via het voedsel, waarna de Cry-toxinen in de middendarm inwerken op de darmepitheelcellen, rechthoekige cellen met microvilli (Hs I-3.1, p. 49). Op het celmembraan van deze cellen zitten de receptoren voor de Cry-toxinen. Deze receptoren behoren meestal tot één van de volgende groepen: cadherin-achtige eiwitten (CAD), aminopeptidasen N (APN), alkalische fosfatasen (ALP) of glycolipiden (GL). In iedere insectensoort komen receptoren uit verschillende groepen voor, en vaak ook meerdere verschillende receptoren per groep. En ieder van die receptoren is weer specifiek voor een beperkte groep van Cry-toxinen (Hs. I-3.2, p. 51).

De opeenvolgende stappen in de vergiftiging door een Cry-toxine, beginnend bij de eiwit synthese en uiteindelijk leidend tot de dood van de larve, zijn te onderscheiden in 11 fasen. De fasen waarin de mogelijkheid voor synergetische processen tussen Cry-toxinen het grootst is, zijn de binding van het toxine aan de celmembraanreceptoren, de vorming van oligomeren en de membraanperforatie en poriewerking (Hs. I-6, p. 76).

In de database van de Natural Recources Canada zijn de toxiciteitsgegevens voor afzonderlijke Bt-toxinen verzameld. De toxiciteit wordt uitgedrukt in een breed scala van eenheden, waarvan sommige eenheden sterk afhankelijk zijn van de condities in het experiment (Hs. II-1, p. 83).

De toxiciteit van een mengsel van twee of meer toxinen die geen interactie vertonen kan met twee verschillende modellen beschreven worden. Als de toxinen op dezelfde wijze werken, wordt hun gezamenlijke toxiciteit voorspeld door het additieve model, en als alle toxinen geheel onafhankelijk van de andere toxinen hun toxische effect veroorzaken, wordt deze voorspeld door het onafhankelijk-effect model. Tussen beide modellen is maar een klein verschil in de voorspelde mengseltoxiciteit. Als de in het mengsel gecombineerde toxinen wel interactie vertonen, zal de toxiciteit van het mengsel afwijken van de door het “geen interactie model” voorspelde toxiciteit, bij synergisme is de toxiciteit hoger dan voorspeld, bij antagonisme lager. (Hs. II-2.1.1, p. 86).

Uit ongeveer 50 publicaties zijn gegevens verzameld over de toxiciteit van individuele Bt-toxinen en hun mengsels voor diverse soorten, 24 lepidoptera, 6 diptera en 2 nematoda, op één diptera en één nematoda soort na alle doelwit-organismen. De waargenomen mengseltoxiciteit is vergeleken met de door het additief model voorspelde toxiciteit van dat mengsel, waarbij de interactie is uitgedrukt in de Synergie Factor (SF), waarbij additief werkende toxinen leiden tot een SF van 1. In 47% van de 208 gegevens over het effect van de combinatie van twee toxinen op een soort of stam is geen synergie gevonden (SF<2). In 7% van de combinaties is antagonisme (SF<0,5) waargenomen, terwijl in 53% (zwakke) synergie (SF>2), en in 18% van de combinaties meer dan een zwakke synergie (SF>10) is gevonden. 53% van die gegevens met synergie heeft betrekking op resistente stammen, terwijl maar 20% van alle gegevens betrekking heeft op resistente stammen. Vrij sterke synergie (SF>20) is bijna alleen, en sterke synergie (SF>50) zelfs uitsluitend, gevonden als één van de Bt-toxinen behoorde tot één van de groepen Cyt-, Mxt- of Bin-toxinen en de onderzochte insectenstam hetzij resistent was

tegen of een relatief lage gevoeligheid had voor één van de andere Bt-toxinen (Hs. II-2.1.2, p. 89). Dit doet vermoeden dat de voornaamste oorzaak van synergie tussen Bt-toxinen gelegen is in het vermogen van de ene toxine om het resistentiemechanisme van het insect tegen de andere toxine te ondermijnen (Hs. II-2.1.3, p. 98).

Resistentie veroorzaakt door langdurige blootstelling aan de ene Bt-toxine kan soms ook leiden tot kruisresistentie tegen andere Bt-toxinen. In de verzamelde gegevens over kruisresistentie worden vaak ingewikkelde resistentiepatronen gevonden, hetgeen doet vermoeden dat selectie voor resistentie niet altijd zal leiden tot hetzelfde resistentiemechanisme (Hs. II-2.2, p. 118).

Bt-toxinen die in dezelfde Bt-stam zijn gevonden, zouden onder invloed van dezelfde selectiedruk geëvolueerd kunnen zijn. In dat geval zou hun toxisch werkingsmechanismen complementair kunnen zijn. Er is echter vooralsnog geen experimenteel bewijs om deze hypothese te ondersteunen (Hs. II-3, p. 132).

Alhoewel het werkingsmechanisme van sommige Bt-toxinen uitgebreid bestudeerd is, en data beschikbaar zijn over de gezamenlijke werking van bijna 80 Bt-toxine-paren, is er nog veel onbekend. Voor de risicoanalyse van gecombineerde Bt-toxinen zijn de belangrijkste lacunes in kennis het gebrek aan gegevens over interactie tussen Bt-toxinen in niet-doelwit organismen en over Mtx-Cry, Bin-Cry en Vip3 toxinen. Om in een risicoanalyse de toxiciteit voor verschillende soorten te kunnen vergelijken is het ook van het grootste belang dat deze toxiciteit in een vergelijkbare eenheid wordt uitgedrukt (Conc. & Recomm., p 139).

Op grond van de (onvolledige) kennis over de werkingsmechanismen van de verschillende typen Bt-toxinen en de waargenomen interacties in mengsels van die toxinen is de verwachte mogelijkheid van synergie bepaald voor de verschillende combinaties van Bt-toxine typen. Deze mogelijkheden worden in de tabel samengevat, waarbij met geen synergie een SF kleiner dan 2 bedoeld wordt en een SF tussen 2 en 10 als zwakke synergie wordt aangeduid.

	Waarschijnlijkheid van synergie tussen Bt-toxinen als de blootgestelde soort of stam		
Combinatie van Bt-toxinen	**voor beide zeer gevoelig is**	**voor één van beide niet gevoelig is, en een taxonomisch verwante[a] soort wel gevoelig is**	**voor één van beide niet gevoelig is, en taxonomisch verwante[a] soorten ook niet gevoelig zijn.**
3D-Cry / 3D-Cry	geen synergie	geen synergie of zwakke synergie	geen synergie of zwakke synergie
3D-Cry /{Bin-Cry, Mtx-Cry of Cyt}	geen synergie of zwakke synergie	mogelijk synergie	mogelijke synergie als 1: Synergie waargenomen is in een verwante soort (hoge waarschijnlijkheid) of 2: Een iets minder verwante soort gevoeliger is (lage waarschijnlijkheid)
{Bin-Cry, Mtx-Cry of Cyt} / {Bin-Cry, Mtx-Cry of Cyt}	geen synergie of zwakke synergie	mogelijk synergie	mogelijke synergie als 1: Synergie waargenomen is in een verwante soort (hoge waarschijnlijkheid) of 2: Een iets minder verwante soort gevoeliger is (lage waarschijnlijkheid)
Veronderstelde complementaire combinatie	synergie	synergie	synergie
{Niet geclassificeerd of groep 1/2} / {Bt toxin}	onbekend	onbekend	onbekend
Vip3 / 3D-Cry	waarschijnlijk hooguit zwakke synergie	onbekend	onbekend

[a]: Taxonomisch verwant moet in deze los geïnterpreteerd worden als zodanig verwant dat potentieel hetzelfde toxisch werkingsmechanisme verwacht kan worden.

Op basis van theoretische overwegingen hoe Bt-toxinen, gezien hun werkingsmechanisme, synergie zouden kunnen veroorzaken, en het waargenomen verband tussen de toxiciteit van enkele Bt-toxinen en hun mengsels, zijn aanbevelingen opgesteld ten behoeve van de beoordeling van GG planten gemodificeerd met meerdere genen coderend voor verschillende Bt-toxinen. Deze aanbevelingen kunnen worden samengevat als 1) Combinaties van 3D-Cry-toxinen zijn vermoedelijk niet of hooguit zwak synergistisch. 2) Bt-toxinen die samen een synergistisch paar vormen moeten samen getest en geëvalueerd worden. 3) Combinaties waarvan ook een Cyt-, Mtx-Cry- of Bin-Cry-toxine deel uitmaakt, kunnen sterk synergistisch zijn en moeten daarom nader onderzocht worden als

er geen aanvullende informatie aanwezig is. Dit geldt in het bijzonder als van sommige soorten bekend is dat ze zeer gevoelig zijn voor één van de gecombineerde toxinen terwijl andere verwante soorten veel minder gevoelig zijn. 4) 3D-Cry- en Vip3-toxine combinaties zijn vermoedelijk niet of hooguit zwak synergistisch, maar dit vermoeden is gebaseerd op een beperkte hoeveelheid informatie dus is wel enige aanvullende informatie nodig, speciaal over de effecten in resistente stammen en ongevoelige soorten. 5) Bij combinaties met Bt toxinen uit andere dan de hierboven genoemde groepen of met ongeklassificeerde Bt toxinen is altijd extra informatie nodig. 6) Als de complete verzameling van de gecombineerde Bt toxinen ook geproduceerd wordt door een natuurlijke Bt stam, dan verdient het aanbeveling om het effect van die stam te gebruiken bij de evaluatie van de GG plant waarin deze Bt toxinen zijn ingebouwd (Conc. & Recomm., p. 141).

Abstract

Natural *Bacillus thuringiensis* (Bt) strains can produce a wide range of toxic proteins (Bt toxins). It is generally assumed that the toxicity of each of these proteins is specific to a limited set of taxonomic groups, mostly an insect group. Because of their (assumed) selective toxicity to the targeted pest insects, Bt genes are used in the genetically modification (GM) of crops. Before these GM Bt crops are allowed on the EU market, an assessment is made of their potential risks for human health and the environment. One of the main effects considered in the environmental assessment of Bt crops is the potential effect of the GM crop on non target organisms. The potential effects are studied in laboratory experiments by exposing non-target organisms to each Bt toxin separately. Recently, crops have been developed producing more than one Bt protein. To evaluate whether additional data are necessary to assess the potential effects of such Bt toxin mixtures, ECOSTAT was commissioned by the Netherlands Commission on Genetic Modification (COGEM) to perform a literature study on the properties of Bt toxins and the data on Bt toxin mixture toxicity.

The Bt proteins can be divided in two main groups, the crystal forming proteins included in Bt spores (Cry and Cyt toxins) and the proteins produced in the vegetative state (Vip toxins). Most of these toxins are produced as protoxin, only becoming toxic after proteolysis.

The nomenclature of Cry, Cyt and Vip toxins is similar: a name starting with Cry, Cyt or Vip, followed by a number, a capital letter, a small letter and again a number (Ch I-1, p. 27). The amino-acid sequence of two of these proteins is more similar if they have the same first number, capital and small letter, than if they only share the first number and capital, and are even less similar if they only share the first number. The ordering of the numbers and letters is no indication of a relationship, but only has a historical background. The names Cry and Vip are each used for more than one group of structurally completely different toxins.

The Cry toxins can be divided in several groups, the largest of these is the group of 3-domain (3D) Cry toxins, sharing the same quaternary protein structure, consisting of three distinct structural domains (Ch. I-2, p. 35). 3D-Cry toxins are assumed to act by forming pores in the cell membrane of their target cells, the three domains having different functionalities in this process. Domain II and III are responsible for the binding to the target cell membrane, bringing Domain I close to the membrane and to the Domain I of other Cry monomers, allowing Domain I to form a pore through the cell membrane, whether first forming an oligomer with Cry monomers in the vicinity or not. These oligomers cannot only be formed by binding molecules of the same 3D-Cry toxin, but also by combining different types of 3D-Cry toxins. This process is extensively studied for one Cry type, Cry1Ab, leading to two distinct models: the sequential binding model (Ch. I-2.2.2, p. 42) assuming that the Cry molecule binds successively to two different receptors, the first facilitating a conformational change of the molecule and the second facilitating the changed protein to form oligomers and pores, and the signalling pathway model (Ch. I-2.2.3, p. 44) assuming that the Cry molecules do not form pores in the cell

membrane but, by binding to a membrane-bound receptor, initiate a chemical cascade in the cell leading eventually to cell death. Evaluation of these models has led to the conclusion that the sequential binding model for Cry1Ab is more likely for Cry1Ab and for other 3D-Cry toxins, with the proviso that the binding details might well be 3D-Cry type specific (Ch I-4, p. 56).

Two models, the umbrella and the penknife model, are described for the process of pore formation by Domain I of the 3D-Cry toxins, consisting of 7 α-helices, numbered α1 to α7, helix α5 in the centre, surrounded by the other 6 helices. The umbrella model, where the central α5 helix together with helix α4 penetrate the cell membrane and the other helices are spread out umbrella-wise on the cell membrane surface, seems to be the most realistic (Ch I-2.1, p 37 and Ch I-2.2.4, p. 46). Estimates of the number of monomers forming the oligomeric pores vary from two to four. The pore size is estimated to be between 1.0 and 2.6 nm in diameter and the pores can be in an open or closed state, alternating between these states in a frequency depending on environmental conditions like pH.

The other groups of Bt toxins are:

- Bin: Cry toxins sequentially resembling the BinA/BinB toxins from *B. sphaericus*. The toxicity of these toxins in combination with their conjugative complement is considerably larger than the toxicity of each of these toxins separately (Ch. I-5.1, p. 60).
- Mtx: A group of Cry proteins resembling β-pore forming toxins like the ε-toxin of *Clostridium perfringens*. Some Mtx proteins have no proven toxicity, and some of these apparently non-toxic Mtx proteins can enhance the toxicity of other (Mtx) toxins (Ch. I-5.2, p. 62).
- Cyt: Group of proteins showing in vitro a general cytolytic activity. Cyts are often only weakly toxic. Combined with some Cry toxins the toxic activity may be enhanced, supposedly because the Cyt protein, binding to the target cell membrane, functions as receptor for that Cry toxin (Ch. I-5.5, p. 69).
- Vip: Insecticidal protein formed in the vegetative phase. Two groups of Vip proteins can be distinguished: The group of Vip1 and Vip2, each separately only weakly toxic, enhancing each others toxicity strongly. And the group of Vip3 proteins. Vip3 binds to other cell membrane receptors than the Cry toxins (Ch. I-5.6, p. 73).
- Unrelated Cry: A diverse group with little sequence homology with the other Cry proteins. Some of these can act as conjugative toxin to a Bin toxin (Cry34 with Bin toxin Cry35) or an Mtx toxin (Cry37 with Mtx toxin Cry23) (Ch. I-5.4, p. 67).

In most cases Cry toxins enter their target organism, insect larvae, along with the food and target the midgut cells. The most important cell type lining the midgut of insect are columnar cells with an apical brush border of microvilli (Ch I-3.1, p. 49). On the midgut cell membrane receptors for Cry toxins are located, mainly belonging to the groups of cadherin-likes (CAD), aminopeptidase N (APN), alkaline phosphatases (ALP) and glycolipids (GL). In an insect species, receptors of several groups can be found, and each

receptor group often contains different receptors. Each of these receptors is specific for a limited number of Cry toxins (Ch. I-3.2, p. 51).

To evaluate which aspects of the route from synthesis of the protein to the death of the target could lead to potential synergism between two or more Bt toxins, this route was partitioned in 11 phases (Ch. I-6, p. 76). The most promising steps for the induction of synergy seem to be the binding to the cell membrane receptors, the formation of oligomers and the pore forming and functioning.

The toxicity of single Bt toxin can be found in the database of the Natural Recources Canada. The toxic dose is expressed in a wide variety of units, some of these units depending on the conditions in the experiment (Ch. II-1, p. 83).

The toxicity of a combination of two or more toxins which do not interact can be described by the additive model for combination toxicology if the mode of action of all these toxins is the same and with the independent effect model if the effect for each of these toxins occurs independently. The difference between the predicted dose-response curves for these two models is small. If the mixed toxins interact, the measured mixture toxicity will depart from the mixture toxicity predicted by the “no interaction model”. In case of synergism, the measured toxicity will be higher than the predicted one, in case of antagonism lower (Ch. II-2.1.1, p. 86).

Data on the combination of the single toxin toxicity and the combined toxicity are gathered from approximately 50 publications, giving data on a range of species, 24 Lepidoptera, 6 Diptera and 2 Nematoda, all target organism but for one Diptera and one Nematoda species. The observed combined toxicity is compared with the additive-model predicted combined toxicity, expressing the interaction as Synergy Factor (SF), SF being 1 for toxins with an additive mode of action. In 47% of the 208 data on the interactive effect of two toxins on a species strain, no synergy was observed (SF<2). Only 7% of these data showed antagonism (SF<0.5), whereas in 53% of the data (weak) synergism (SF>2) was observed, and in 18% of these combinations more than a weak synergy (SF>10). 53% of these synergistic data were observations on resistant strains, compared to 20% of all observed combinations. Considerable synergy (SF>20) was almost exclusively, and strong synergy (SF>50) even exclusively, observed if one of the toxins was either a Cyt toxin, Mtx toxin or Bin toxin and the insect strain tested was either resistant to or showed a very low sensitivity for the other Bt toxin (Ch. II-2.1.2, p. 89). This suggests that the main mechanism of synergy between Bt toxins is the removal of a resistance mechanism of the target insect (Ch. II-2.1.3, p. 98).

Resistance induced by one Bt toxin can result in cross-resistance to another Bt toxin. Data on cross-resistance were collected, leading to a complicated pattern of cross-resistances, suggesting that selection for resistance will not always select for the same resistance mechanism (Ch. II-2.2, p. 118).

Bt toxins isolated from the same Bt strain might have co-evolved, and co-evolution of Bt toxin genes might favour complementary mode of actions of these toxins. Evidence to

support that hypothesis is, however, as yet not available (Ch. II-3, p. 132).

Although the mode of action of some Bt toxins have been studied extensively, and data on interactions are available for almost 80 Bt toxin couples, much is still unknown. For risk assessment of stacked Bt toxins, the most important gaps in knowledge are the lack of data on synergy in less sensitive non-target species and the lack of data on Mtx-Cry, Bin-Cry and Vip3 toxins. For risk assessment, it is also of vital importance that the toxicity data should be expressed in a unit allowing comparison between species (Conc. & Recomm., p. 139).

The expected potential for synergy based on the (incomplete) knowledge on the mode of action of different types of Bt toxins and observed interaction in experiments with toxin mixtures, are summarised in a table, considering a SF below 2 as no synergy and a SF between 2 and 10 as weak synergy.

	Likelihood of synergy between Bt toxins if the exposed species or strain is		
Combination of Bt toxins	**sensitive to both.**	**not sensitive to one of the toxins, and a taxonomic related species[a] is sensitive.**	**not sensitive to one of the toxins, and taxonomic related species[a] are not sensitive either.**
3D-Cry / 3D-Cry	no synergy	no synergy or weak synergy	no synergy or weak synergy
3D-Cry /{Bin-Cry, Mtx-Cry or Cyt}	no synergy or weak synergy	potential synergy	potential synergy if 1: Synergy is observed in related species (high prob.) or 2: Some less related species are observed to be more sensitive (low prob.)
{Bin-Cry, Mtx-Cry or Cyt} / {Bin-Cry, Mtx-Cry or Cyt}	no synergy or weak synergy	potential synergy	potential synergy if 1: Synergy is observed in related species (high prob.) or 2: Some less related species are observed to be more sensitive (low prob.)
Assumed conjugative combination	synergy	synergy	synergy
{Unclassified or Group1/2} / {any Bt toxin}	unknown	unknown	unknown
Vip3 / 3D-Cry	probably at most weak synergy	unknown	unknown

[a]: Taxonomic related should be interpreted loosely, meaning related in a degree that potentially the same toxic mode of action may be expected.

The theoretical potentiality for synergism between Bt toxins, based on their known mode of action, together with the observed relationship between single toxin toxicity and the toxicity of toxin combinations, has resulted in recommendations for the assessment of the effect of GM plants modified with multiple Bt toxin genes. These recommendations can be summarized as 1) combinations of 3D-Cry toxins are probably not or only weakly synergistic. 2) Combinations of Bt toxins which are considered as synergistic couples should always be tested and evaluated together. 3) combinations whereof at least one of the Bt toxins is a Cyt, Bin-Cry of Mtx-Cry might be strongly synergistic and should be investigated further if no information is available on that particular combination, especially if some species are known to be highly sensitive to one of the combined toxins, where other related species only have a low sensitivity. 4) 3D-Cry and Vip3 toxin combi-

nations are probably not or only weakly synergistic. This conjecture is based on a limited data base, so some additional information may be needed, especially on the effects on resistant strains and non-sensitive species. 5) for combinations with Bt toxins from other than the above mentioned groups or with unclassified Bt toxins, more information is always needed. 6) If a natural type Bt strain produces the complete set of stacked Bt toxins, the effect of that strain should be used in evaluating the effect of the GM plant with these stacked Bt toxins (Conc. & Recomm., p. 141).

Introduction

The bacterial species *Bacillus thuringiensis* (Bt) can produce a wide variety of toxic proteins. It is generally assumed that most of these proteins are highly specific to a limited number of species, often insects. Most of these toxins are produced in the Bt spores, and these spores are used as pesticides for their insecticidal effect. To make plants resistant to insects, crops are genetically modified with Bt genes coding for these insecticidal proteins. Before these genetically modified (GM) Bt crops are allowed on the EU market, an assessment is made of their potential risks for human health and the environment. One of the main effects considered in the environmental assessment of Bt crops is the potential effect of the GM crop on non target organisms (NTO).

At present, a number of Bt crops have been approved for the EU market. Several crops may produce more than one Bt protein. These crops are often the result of conventional cross-breeding of crops with single Bt genes. Each so-called stacked event has to be assessed again for its potential risks. The question has arisen whether the evaluation of Bt crops with a single Bt gene insertion is sufficient to predict the potential risk of a Bt crops producing multiple Bt toxins with respect to potential interactions and synergy between these Bt proteins, and if not, how much additional information is needed.

To determine how the potential risks for NTOs of Bt crops producing several Bt toxins should be assessed, when the Bt crops producing each of these Bt toxins separately had been assessed as safe, the Netherlands Commission on Genetic Modification (COGEM) has commissioned a literature research into Bt toxins. This research should clarify the different scientific views on the mode of action of Bt toxins with emphasis on what the models for the mode of action can tell about their interaction. The research should lead to insight in the potential for interactions between Bt toxins, and how this insight can be used to assess the potential effect of Bt toxin combinations on non-target organisms exposed to GM crops producing several Bt toxins.

To address this research question, the following aspects are listed and/or evaluated in this report:

- An inventory of the toxic proteins produced by *Bacillus thuringiensis*.
- The knowledge on the structure and the mode of action of the different types of Bt toxins.
- The physiology of the insect gut and the Bt toxin receptors on the midgut cell membrane.
- The possibilities for interaction between Bt toxins consistent with their supposed mode of action.
- The observed toxicity of combinations of Bt toxins in relationship to the toxicity of each of these Bt toxins separately.
- The capacity of a Bt toxin to induce resistance also against another Bt toxin when it is inducing resistance against itself.
- The production of multiple Bt toxins in unmodified organisms.

Another literature research study performed by a combined group of the Biosafety and Biotechnology Unit of the Belgium Scientific Institute of Public Health, the Department of Crop Protection of the Ghent University, Plant Research International and the Great Lakes Forestry Centre of the Natural Resources Canada will focus on the ecotoxicological activity towards organisms and the assessment of effects on non-target organisms.

The report is divided in two parts:

- Part I deals with the individual Bt toxins, dividing the Bt toxins in five well-defined groups with a remainder of some uncategorised Bt toxins. The three dimensional structure of the proteins and the existing models for their mode of action are described for each Bt toxin group. For only one of the Bt toxin groups, the mode of action is well studied. For the other groups, far less data are available. For the well-studied Bt toxin group, the models deal with the interaction between the Bt toxin and the insect midgut cells. The life-cycle of the Bt toxin from production to the final toxic effect is divided in steps, and for each step it is evaluated whether interaction between co-occuring Bt toxins is possible.
- Part II deals with the effect of Bt toxins on individual animals, mainly insects, focussing on the effect of combinations of Bt toxins. The available data on combinations of Bt toxins in relationship to the toxicity of each of these toxins separately are listed and discussed. Data on cross-resistance against one Bt toxin induced by another Bt toxin are also listed and discussed. The potential effect of co-evolution of Bt toxins is discussed and some data on co-occurance of Bt toxins in natural Bt strains are given. The capacity of synergism between Bt toxins is evaluated by combining the observed combination toxicological effect of Bt toxin mixtures with the knowledge about their mode of action.

This study was started in October 2013 and finished in April 2014. The field of Bt toxins is fast developing. For instance, at the start of this study, the official Cry and Cyt list ([w1]) encompassed 755 different proteins, whereas at the end of this study, this list had grown with 3% to to 778 proteins. Manuscripts published during this study might have been missed if they have been published after the related part of this study has been finished.

Part I:

Bacillus thuringiensis toxin proteins: Nomenclature, Structure and mode of action.

I-1 Nomenclature of *Bacillus thuringiensis* toxins

Until 1998, each newly discovered Bt toxin was named according to its toxic function and some other not-systematically applied properties, like the strain from which it was isolated or the species group for which it was found to be toxic. In 1998, Crickmore *et al.* [30] introduced a new nomenclature for all Cry Bt proteins[1] based on sequence similarities. Their nomenclature also included the Cyt proteins[2]. The proteins named Cyt also meet the definition of Cry proteins[1], but for consistency with historical data, Crickmore *et al.* did not rename the Cyt proteins to a Cry name. The name of all Cry proteins starts with "Cry", followed by an arabic number (primary rank), a capital letter (secondary rank), a small letter (tertiary rank) and again a number (quaternary rank). Cry proteins with the same first number capital and small letter (for instance Cry1Aa1 and Cry1Aa2) are identical for at least 95% of their amino-acid sequence. Cry proteins with the same first number and capital letter (for instance Cry1Aa1 and CryAb1) have at least 78% of their amino-acid sequence in common. Cry proteins with the same first number (for instance Cry1Aa1 and Cry1Ba1) have at least 45% of their amino-acid sequence in common. The Cry proteins with the quaternary rank number "1" is considered to represent the holotype sequence for the complete set of all Cry protein with the same primary, secondary and tertiary rank.
This nomenclature is solely based on amino-acid sequences and does not necessary reflect a similar biological activity.
All newly discovered natural Bt crystal toxins are given a name according to this nomenclature. The complete list of the named Cry proteins can be found on the website of the Sussex university [w1].
VIP proteins (Vegetative Insecticidal Proteins) expressed and secreted in the vegetative state of *Bacillus thuringiensis* form another group of Bt toxic proteins. These proteins are named in a similar fashion, indicated as Vip followed by a numbering similar to the Cry protein [w1]. In section ***I-5.6 VIP proteins*** (p. 73) the Vip proteins are described more extensively.
Some Cry proteins are non-hemolytic but capable of preferentially killing cancer cells. These Cry proteins, produced by *B. thuringiensis* or related bacteria, are also named Parasporins. They are indicated as PS followed by a numbering similar to the Cry protein [w1]. The Parasporin proteins listed on [w1][3] are all Cry proteins, that is, these proteins can be indicated by their Cry-number or their PS number. Of these Parasporins, PS1, PS3 and PS6 are 3D-Cry proteins, PS4 probably belongs to the Mtx group [122]. PS2 and PS5 (Cry46 and Cry64) are not categorized in [172] or in a dendogram on [w1], but information in [88] and [166] allows to place these two also in the Mtx-group.

The most recent dendograms for the relation between the Bt proteins can be found on the

1 Cry protein according to the broad definition of Crickmore *et al*.[30]:
A parasporal inclusion (crystal) protein from *B. thuringiensis* that exhibits some experimentally verifiable toxic effect to a target organism, or any protein that has obvious sequence similarity to a known Cry protein.

2 Cyt denotes a parasporal inclusion (crystal) protein from *B. thuringiensis* that exhibits hemolytic activity, or any protein that has obvious sequence similarity to a known Cyt protein.

3 Parasporin list on [w1] per December 16, 2013.

Sussex university website [w1] In fig I-1 the dendogram per November 5, 2013 is given.

Several groups of Cry proteins can be distinguished based on (1) three-dimensional structure and (2) toxic mechanisms. By far the largest group is that of the 3 domain Cry proteins (3D). These proteins all have a very similar tertiary structure, which can be divided in three distinct sub-structures. In the section ***I-2 Three Domain Cry Proteins*** (p. 35) the 3D-Cry proteins are described more extensively.
The other Cry protein groups are

- The BIN-like proteins. These proteins have to be combined with a complementary protein to become synergistically toxic. The Cry proteins of type Cry35, Cry36 and Cry49 and proteins indicated as BinA and BinB belong to this group. In the section ***I-5.1 Binary Cry toxins and their conjugative toxins*** (p. 60) the Bin toxins are discussed.
- The Mtx-like group. Many of these proteins are toxic to mosquitoes (diptera), and three of them are parasporins. To this group belong the Cry proteins Cry15, Cry23, Cry33, Cry38, Cry45, Cry46, Cry51, Cry60, Cry64 and the non-toxic CryC35, CryNt32, a protein of 40 kDa, indicated as 40kDa, and the *B. sphaericus* proteins named Mtx2 and Mtx3. Cry23 is probably only active in combination with the small and unrelated Cry37. The Mxt proteins are discussed in the section ***I.5.2 The Mtx group*** (p. 62).
- The Cyt protein group, consisting of crystal Bt proteins with hemolytic activity. The Cry proteins of this type are named Cyt1, Cyt2 and Cyt3. In the section ***I-5.5 Cyt toxins*** (p. 69) the Cyt proteins are further described.
- A rest group, consisting of two subgroups, the group of Cry6, Cry22, and Cry37 and the group only consisting of Cry34. Cry34 is described in section ***I-5.1 Binary Cry toxins and their conjugative toxins*** (p. 60) as a conjugative toxin to the Bin toxin Cry35 and Cry37 is described in section ***I.5.2 The Mtx group*** (p. 62) as forming a conjugative pair with the Mtx protein Cry23. Cry6 and Cry22 are further described in the section ***I-5.4 The unrelated Cry toxins*** (p. 67).
- A group of Cry proteins without further information on their structure and functioning (Cry55, Cry61, Cry71 and Cry72), further described in the section ***I-5.4 The unrelated Cry toxins*** (p. 67).

All natural toxic crystal forming Bt proteins found in natural *Bacillus thuringiensis* strains are named according to the nomenclature of Crickmore *et al*. (1998), at least if the protein is submitted to the nomenclature committee. For modified proteins, this name-giving procedure is not followed. These proteins have a name indicating the modified protein combined with some code for the applied modification. In some modified proteins, the so called hybrid proteins, the domains of different natural occurring Cry proteins are combined, for instance Cry1A.105 is a 3-domain protein with domain I from Cry1Ab, domain II from Cry1Ac and domain III from Cry1F combined with a C-terminal region from Cry1Ac (record 43771 in [w2]).
Most *Bacillus thuringiensis* Cry proteins are not toxic in the initial form in which they are produced. This form is called a protoxin. After some transformations, often proteolytic cleavage of the N- and/or C-terminal of the protoxin, the remainder of the protoxin is

toxic. This toxic protein is indicated as (Cry) toxin or δ-endotoxin. Bt genes in GM plants often produce a shorter version of the protein than the *Bacillus thurigiensis* gene donor. This version is called the preactivated toxin. It is missing part of the C- and N-terminal fragments present in the bacterial protoxin, but still has to be activated by proteolytic cleavage of some terminal fragments, resulting in the same toxin as the toxin formed from the protoxin encoded by the Bt gene donor.

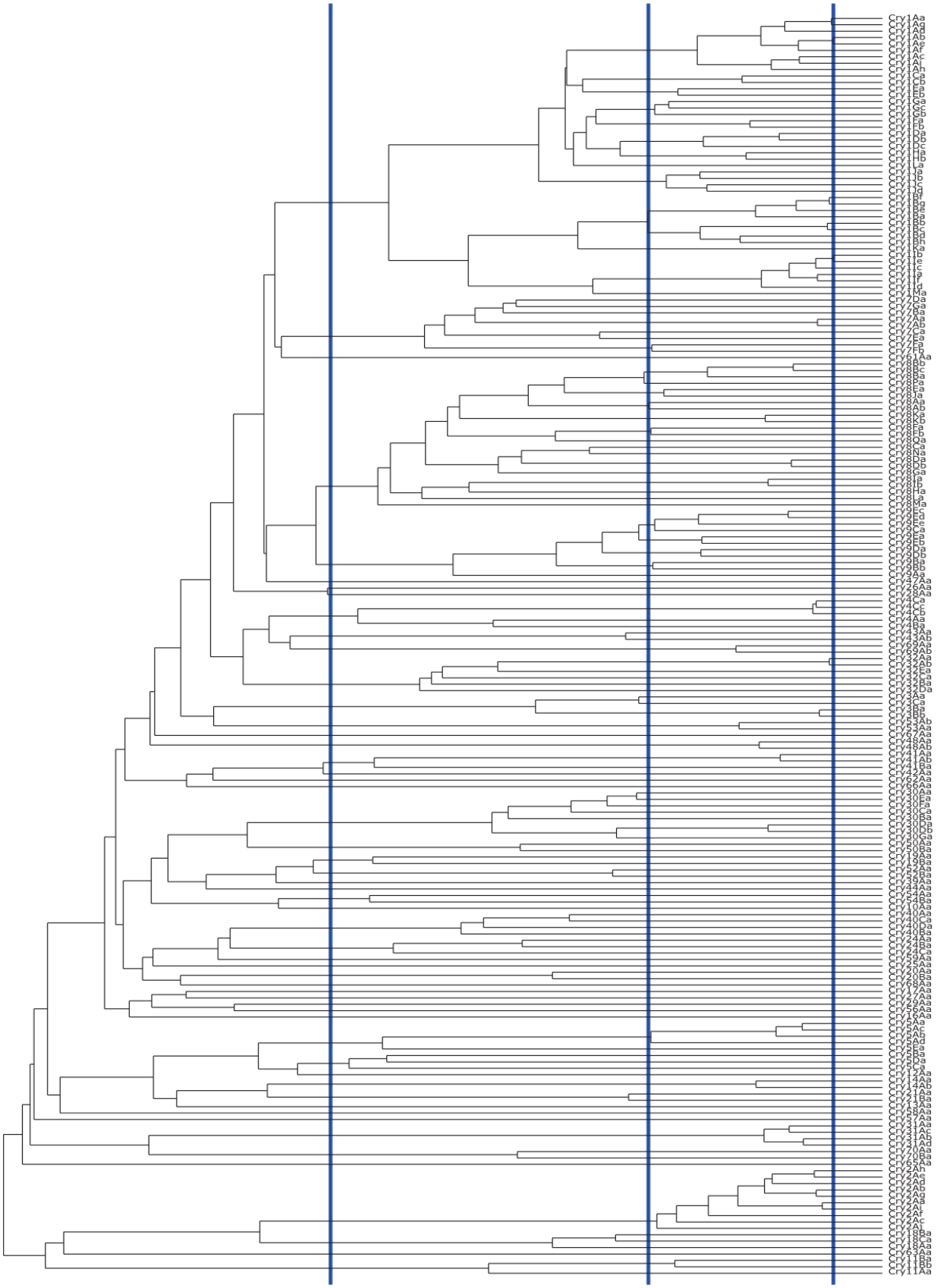
Cry1Aa
Cry1Aa
Cry1Ad
Cry1Ab
Cry1Ae
Cry1Af
Cry1Ac
Cry1Ai
Cry1Ah
Cry1Ca
Cry1Cb
Cry1Ea
Cry1Eb
Cry1Ga
Cry1Gc
Cry1Gb
Cry1Fa
Cry1Fb
Cry1Da
Cry1Db
Cry1Dc
Cry1Ha
Cry1Hb
Cry1La
Cry1Ja
Cry1Jb
Cry1Jc
Cry1Ia
Cry1Ba
Cry1Bg
Cry1Be
Cry1Bb
Cry1Bc
Cry1Bd
Cry1Bh
Cry1Ka
Cry1Ib
Cry1Ie
Cry1Ic
Cry1Ja
Cry1If
Cry1Id
Cry1Ma
Cry2Da
Cry2Ga
Cry2Ba
Cry2Aa
Cry2Ab
Cry2Ca
Cry2Ea
Cry2Fa
Cry61Aa
Cry8Bb
Cry8Bc
Cry8Ba
Cry8Ea
Cry8Ia
Cry8Aa
Cry8Ab
Cry8Kb
Cry8Ka
Cry8Fa
Cry8Fb
Cry8Qa
Cry8Na
Cry8Da
Cry8Db
Cry8Ga
Cry8Ib
Cry8Ha
Cry8La
Cry8Ma
Cry9Eg
Cry9Ed
Cry9Ee
Cry9Ca
Cry9Ea
Cry9Eb
Cry9Da
Cry9Db
Cry9Ba
Cry9Bb
Cry3Aa
Cry47Aa
Cry26Aa
Cry38Aa
Cry4Ca
Cry4Cc
Cry4Cb
Cry4Aa
Cry4Ba
Cry43Aa
Cry43Ab
Cry69Aa
Cry69Ab
Cry32Aa
Cry32Ab
Cry32Ea
Cry32Ca
Cry32Ba
Cry32Da
Cry3Aa
Cry3Ca
Cry3Ba
Cry3Bb
Cry53Ab
Cry53Aa
Cry67Aa
Cry48Aa
Cry48Ab
Cry41Aa
Cry41Ab
Cry41Ba
Cry42Aa
Cry62Aa
Cry56Aa
Cry30Ag
Cry30Ea
Cry30Fa
Cry30Ca
Cry30Ba
Cry30Da
Cry30Db
Cry30Ga
Cry50Aa
Cry50Ba
Cry19Aa
Cry19Ba
Cry52Aa
Cry55Ba
Cry55Aa
Cry34Aa
Cry54Aa
Cry54Ba
Cry10Aa
Cry40Aa
Cry40Ca
Cry40Da
Cry40Ba
Cry24Aa
Cry24Ba
Cry4Ba
Cry59Aa
Cry35Aa
Cry20Aa
Cry20Ba
Cry68Aa
Cry17Aa
Cry27Aa
Cry29Aa
Cry56Aa
Cry16Aa
Cry5Aa
Cry5Ac
Cry5Ab
Cry5Ad
Cry5Ea
Cry5Ba
Cry5Da
Cry5Ca
Cry12Aa
Cry14Aa
Cry14Ab
Cry21Aa
Cry21Ba
Cry13Aa
Cry18Aa
Cry57Aa
Cry31Aa
Cry31Ac
Cry31Ab
Cry31Ad
Cry70Aa
Cry70Ba
Cry65Aa
Cry2Ah
Cry2Ae
Cry2Ad
Cry2Ab
Cry2Ag
Cry2Aa
Cry2Ai
Cry2Af
Cry2Ac
Cry2Ak
Cry18Ba
Cry18Ca
Cry18Aa
Cry63Aa
Cry11Bb
Cry11Ba

Figure I-1.
The phylogram for the similarity between the 3D-Cry proteins based on the amino-acid sequences of the activated toxin. The blue lines demarcate the four levels of nomenclature rank and are drawn at a sequence homology (from left to right) of 45%. 78% and 95%.
Figure copied on November 5, 2013 from [w1], method described in [30].

In table I-1 a list is given of all sequenced primary Cry and Cyt protein groups listed in [w1], their category and, if known, the groups sensitive to the primary holotype (Cry*?* Aa1). The other Cry proteins from the same primary holotype group are in most cases, but not always, toxic to the same taxonomic groups and belong to the same morphological category. The toxicity classification in this table is mainly copied from that classification given by Frankenhuyzen *et al*. [43], summarizing the toxicity data given in the web-database of Frankenhuyzen [w8]. In chapter II-1 (p. 83) more information on this database is given and in Appendix D (p. 177) the toxicity data from that database on Cry1Ab are given as example. Note that some Cry toxins are not submitted to the Sussex University database ([w1]), and thus not given an official standardized name. An example of such a Cry toxin is the toxin named Cry14-4 isolated from a plasmid in a Bt strain, an Mtx class Cry toxin showing a sequence identity of 22% with Cry15Aa and 28% with Mtx3 [107]. These non-submitted toxins are no further discussed in this report.

Table I-1.
A list of all primary crystal and vegetative insecticidal *Bacillus thuringiensis* protein, listed per April 11th, 2013 on the Bt toxin nomenclature website ([w1]) and the Mtx *Bacillus sphaericus* proteins.
The category is according to [w1] and the specificity according to [43] unless otherwise indicated.

Primary type	Cate-gory[a]	Toxic specificity for species groups[b] tested and found sensitive	tested and found not sensitive	Max. Sec. Cat.	No. Tert. type	No. Quat. type
Cry1	3D	Lep, Dip (1A, 1B, 1C), Col (1B, 1I), ?Hem (1A)	Col (1A, 1J), Dip (1G, 1I)	N	52	258
Cry2	3D	Dip, Lep, Hem	Col, Coll, Hym, Iso, Neu, Orth, Thy	B	12	73
Cry3	3D	Col, Hem (3A), Hym (3A)	Coll (3A), Dip (3A), Lep (3A)	C	4	19
Cry4	3D	Dip	Lep (4B)	C	6	15
Cry5	3D	Nem, Hym (5A), Plat (5A)		E	8	13
Cry6	Group 1	Nem, Col (6A)[c]		B	2	4
Cry7	3D	Col (7A), Lep (7B)	Lep (7A), Col (7B), Dip (7B)	L	20	35
Cry8	3D	Col, Lep (8D)	Lep (8E, 8F, 8G)	T	28	49
Cry9	3D	Lep (9A, 9B, 9C, 9 E), ?Lep (9D), Col (9D)	Col (9B), Dip (9B, 9E)	G	15	36
Cry10	3D	Dip		A	2	5
Cry11	3D	Dip, Hem (11A)	Lep (11A)	B	3	8
Cry12	3D	Nem		A	1	1
Cry13	3D	Nem		A	1	1
Cry14	3D	Col, Nem		A	2	2
Cry15[d]	Mtx	Lep	Col, Dip	A	1	1
Cry16	3D	Dip		A	1	1
Cry17	3D		Dip	A	1	1
Cry18	3D	Col		C	3	3
Cry19	3D	Dip		C	3	3
Cry20	3D	Dip		B	3	4
Cry21	3D	Nem		F	6	8
Cry22	Group 1	Col, Hym (22A), Lep (22A)		B	4	7
Cry23[e]	Mtx	Col		A	1	1
Cry24	3D	Dip (24C)	Dip (24B)	C	3	3
Cry25	3D			A	1	1
Cry26	3D			A	1	1
Cry27	3D	Dip		A	1	1
Cry28	3D			A	1	2
Cry29	3D		Dip	B	2	2
Cry30	3D		Dip	G	8	11
Cry31 = PS1	3D	HLC	Dip, Bla, Iso, Neu, Orth	A	4	11

Primary type	Cate-gory[a]	Toxic specificity for species groups[b] tested and found sensitive	tested and found not sensitive	Max. Sec. Cat.	No. Tert. type	No. Quat. type
Cry32	3D	Dip (32B, 32C, 32 D), Lep (32A)	Dip (32A)	U	26	28
Cry33	Mtx		Dip, Lep	A	1	1
Cry34[f]	Group 2	Col	Lep	B	5	15
Cry35[f]	Bin	Col	Lep	B	3	7
Cry36[g]	Bin	Col		A	1	1
Cry37[e]	Group 1	Col		A	1	1
Cry38	Mtx		Col	A	1	1
Cry39	3D	Dip		A	1	1
Cry40	3D		Dip	D	4	4
Cry41 = PS3	3D	HCC	Dip, Bla, Iso, Lep, Orth	B	3	4
Cry42	3D	HCC	Dip, Bla, Iso, Lep, Orth	A	1	1
Cry43	3D	Col		C	6	7
Cry44	3D	Dip		A	1	1
Cry45[h]= PS4	Mtx	HCC	Dip, Bla, Hem, Iso, Lep, Orth	A	1	1
Cry46[m]= PS2	Mtx	HCC		A	2	3
Cry47	3D	Dip	Nem	A	1	1
Cry48[j]	3D	Dip	Col, Lep	A	2	5
Cry49[j]	Bin	Dip	Col, Lep	A	2	5
Cry50	3D			B	2	3
Cry51	Mtx	Lep		A	1	2
Cry52	3D			B	2	2
Cry53	3D			A	2	2
Cry54	3D	Dip[j], Lep[j]		B	3	4
Cry55	No info	Col, Nem		A	1	2
Cry56	3D			A	1	3
Cry57	3D			A	2	2
Cry58	3D			A	1	1
Cry59	3D			B	2	2
Cry60[k,l]	Mtx	Dip[l]		B	2	6
Cry61	No info			A	1	3
Cry62	3D			A	1	1
Cry63 = PS6	3D			A	1	1
Cry64[k]= PS5	Mtx			A	1	1
Cry65	3D			A	1	2
Cry66	3D			A	1	2
Cry67	3D			A	1	2
Cry68	3D			A	1	1
Cry69	3D			A	2	3
Cry70	3D			B	3	3
Cry71	No info			A	1	1
Cry72	No info			A	1	1
Cyt1	Cyt	Dip, Col (1A), ?Lep (1A)		D	5	13
Cyt2	Cyt	Dip (2A, 2B),	Lep (2A), Nem (2C),	B	5	24

Primary type	Cate-gory[a]	Toxic specificity for species groups[b] tested and found sensitive	tested and found not sensitive	Max. Sec. Cat.	No. Tert. type	No. Quat. type
		Col (2C), Siph (2C)	HCC (2C)			
Cyt3	No info			A	1	1
Vip1	Vip1/2			D	9	12
Vip2	Vip1/2			B	9	17
Vip3	Vip3			C	12	92
Vip4	??			A	1	1
Mtx1[n]	other					
Mtx2[n]	Mtx					
Mtx3[n]	Mtx					

[a]: The categories of Cry proteins distinguished are: 3 domain proteins (3D), proteins sequentially related to tne BinA/BinB toxins (Bin), proteins with a structural relationship to the Mtx2 and Mtx3 toxins, and in most cases toxic to mosquitoes (Mtx), proteins with hemolytic activity (Cyt) and two rest groups of unspecified, sequentially related Cry genes (Group 1 and Group 2). Of some Cry genes, no information is available (No info). Unless otherwise stated, the information is from [w1].

[b]: The taxonomic groups in this column are: Col: Coleoptera; Coll: Collembola; Dip: Diptera; Bla: Blattaria; Hem: Hemiptera; Hym: Hymenoptera; Iso: Isoptera; Lep: Lepidoptera; Neu: Neuroptera; Orth: Orthoptera; Siph: Siphonoptera; Thy: Thysanoptera; Plat: Platyhelminthes (Echinostomida); Nem: Nematoda (Rhabditida) ; HLC: Human Leukemic Cells; HCC: Human Cancer Cells. If the activity does not hold for all second categories, the second category is stated between brackets. In some cases, the activity is uncertain, and then the taxonomic group is preceded by "?". Unless otherwise stated, the data are from [43]. The distinction between active against Human Leukemic Cells or general Human Cancer Cells is from [172].

[c]: Cry6 acts according to [99] also against Coleoptera.

[d]: Cry15 is Mtx according to [w1] and [122] . In [172] Cry15 is listed as 3D, but that is probably a typing error.

[e]: Cry23 forms a conjugative pair with Cry37 [110].

[f]: Cry35 is conjugative toxin of Cry34 [110], [160].

[g]: Cry36 is sequentially close to Cry35. Its counterpart as Bin toxin is, as yet, unknown. Cry36 is in itself slightly toxic [110].

[h]: Cry45 belongs according to [122] to the Mtx group.

[i]: According to Jones *et al*. ([76], [77]) Cry48Aa and Cry49Aa produced by *B. sphaericus* are a conjugative toxin combination, highly specific toxic to *Culex quinquefasciatus*.

[j]: Cry54 acts against Diptera and Lepidoptera according to [44]

[k]: Cry60 and Cry64 are Mtx according to [88]

[l]: Cry60 acts according to [174] against Diptera.

[m]: Cry46 shows similarities with the Mtx group, this report, p. 65

[n]: Protein produced *Bacillus sphaericus* (*Lysinibacillus sphaericus*)

I-2 Three Domain Cry Proteins

I-2.1 The structure of 3-D Cry proteins

The 3D-Cry proteins form the largest and best studied group. The activated 3D-Cry toxins all have a similar three-dimensional structure, consisting of three separate domains. This structure was first described in 1991 [98]. Figure I-2 illustrates the three dimensional structure of the activated form of these toxic proteins. Many Cry proteins are produced as protoxin. The 3D-protoxin contains extra protein fragments at the N- and/ or at the C-terminal region of the 3D structure. The protoxin becomes a toxic Cry toxin by proteolytic cleavage of these fragments. Note that the Bt toxin genes cloned into plants often encode for the toxin with shorter extra protein fragments at the N- and/or C-terminal region. These shorter protoxins are called preactivated protoxins.
In their review, Rukmini *et al.* [152] give a schematic picture of the fragments proteolytic cleaved from Cry1, Cry2, Cry3 and Cry4, resp. (see fig. I-3). This scheme is a simplification and for each Cry group, one type is chosen as example.

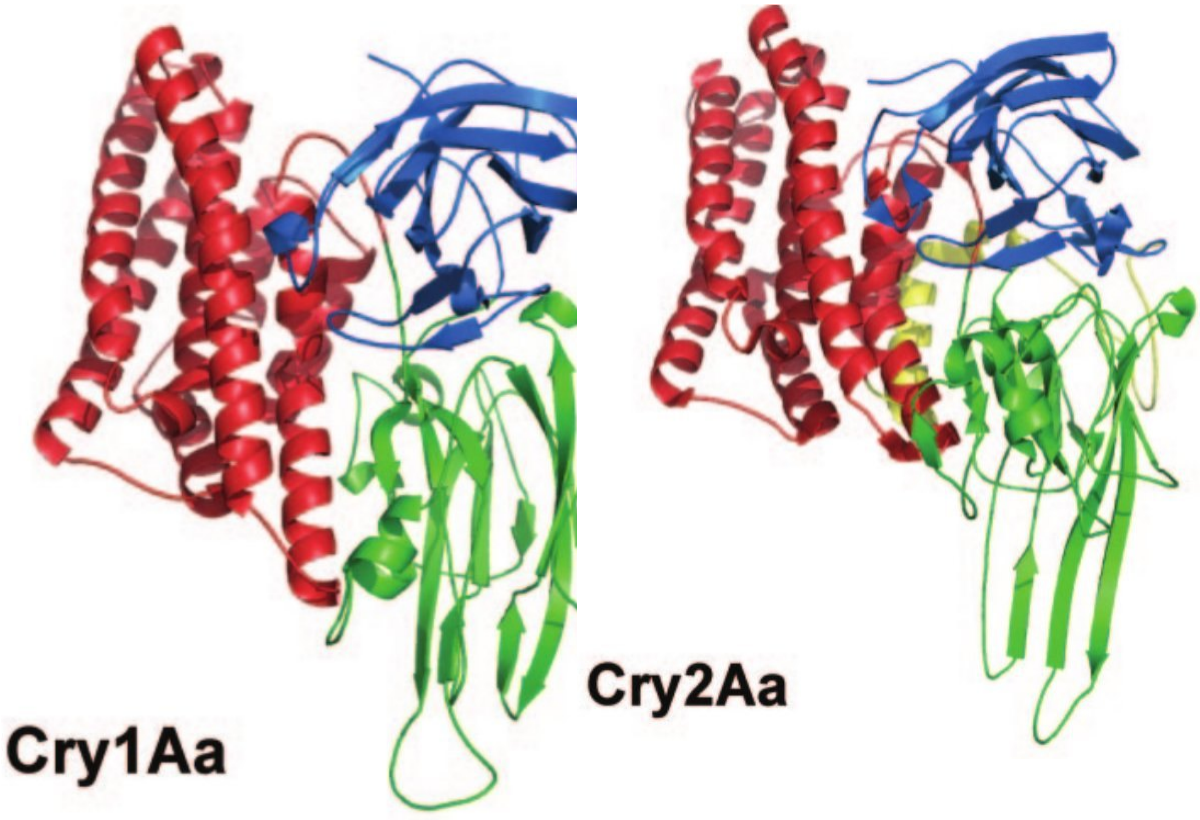

Figure I-2.
Crystal structure of activated Cry1Aa and Cry2Aa [136]. Domain I, consisting of 7 α-helices, in red. Domain II, consisting of 3 antiparallel β-plates in green, and Domain III forming a β-sandwich, in blue.
Note that the sequence identity of these two Cry proteins is less than 23% [136].

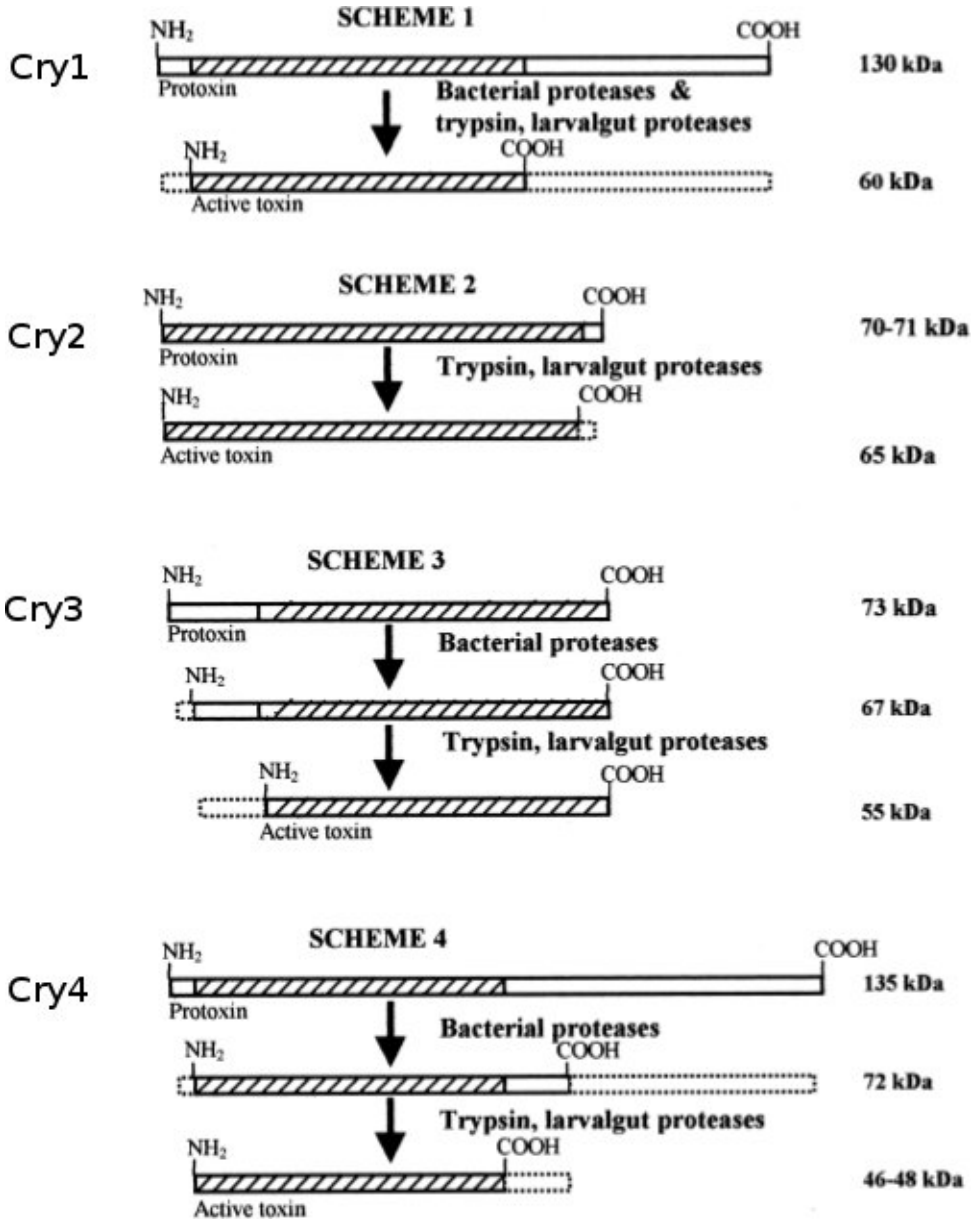

Figure I-3.
Various schemes for the activation of Cry1 to Cry4 protoxins according to Rukmini *et al.* [152]. The dotted regions are removed by cutting with proteases.

Domain I (also indicated as region Endotoxin_N [w3]) consists of 7 α-helices, helix α5 in the centre and the other 6 helices amphipathic surrounding α5 [110]. This structure is almost the same for all 3D-Cry proteins, and with that the most constant of the three domains. Helix α2 consists of two short helices, α2a and α2b, connected by a short bulging loop [56], [98]. Only the first of these two α2-helices, α2a, is packed against the

central helix α5 [98]. It is generally believed that part of the helices of domain I form the pores which permeate the cell membrane (e.g. [56], [98], [136], [159], [196]). The leaking of the cells is supposed to lead to cell death and eventually, to the death of the individual.
The hypothesis that domain I is pore-forming is supported by the observation that helices α3 to α7 are relatively large (over 30 Å) and are thus sufficiently long to span the hydrophobic cell membrane [136] and that the structure of domain I resembles the structure of the pore-forming domain of colicin A [128], [136]. Two different models for the mechanism of pore formation are proposed, the so called umbrella model [98] and the penknife model [66]. According to the umbrella model, the hydrophobic hairpin structure of helices α4 and α5 initiates pore formation and the rest of the domain I helices flatten out on the membrane in an "umbrella-like" molten globule state. This model is similar to the model proposed for the pore forming activity of domain I of Colicin A [96] and the Diphteria toxin [221]. According to the penknife model, the strongly hydrophobic helices α5 and α6, joined together by a loop, open in a penknife like fashion and inserts in to the membrane. The remainder of the Cry molecule would remain at the membrane surface or remain linked to the receptor [66]. Hogdman and Ellar [66] also proposed a penknife model for Colicin A. In both the penknife and the umbrella model, the central domain I helix α5 is one of the pore-forming helices. Both models are reviewed by Knowles [87] and illustrated by her (fig. I-4).
In contrast to these models, some authors claim that the complete toxin penetrates the membrane [123].

The hypothesis that the toxic effect of Cry proteins is due to the leakage caused by pores is challenged by Zhang *et al*. [223]. They assume that the binding and possible integration of the Cry protein in the cell membrane triggers a specific signalling pathway which, when started, leads to membrane blebbing (zeiosis), appearance of nuclear ghosts and cell swelling followed by cell lysis.
According to some models for the mechanism of Cry toxicity, helix α1 has to be cleaved before effectively becoming toxic [172]. This cleavage is observed for some Cry toxins (Cry1Ab, Cry1Ac) [49]. Other Cry toxins do not have the helices α1 and α2 (Cry4Ba) [15].

Domain II (also indicated as region Endotoxin_M [w3]) consists of three anti-parallel β-sheets, packed together to form a β -prism [98]. The first two sheets are each composed of 4 strands in a Greek key motif. These sheets are solvent exposed. The last (third) sheet is packed against domain I and interacts with that domain. This sheet has three β-strands in a greek-key-like motif and a short α-helix [136]. The structure of domain II is very variable. It is therefore believed that domain II plays a key factor in Cry toxin specificity.

Domain III (also indicated as delta_endotoxin_C [w3]) is structurally less variable than domain II, but more than domain I. It consists of a β- sandwich with two antiparallel β-sheets packed together with a "jelly roll" topology [136] Both sheets are composed of five strands, with the outer sheet towards the solvent and the inner sheet packed against domain II. Two long loops extend from domain III and interact with domain I [56].

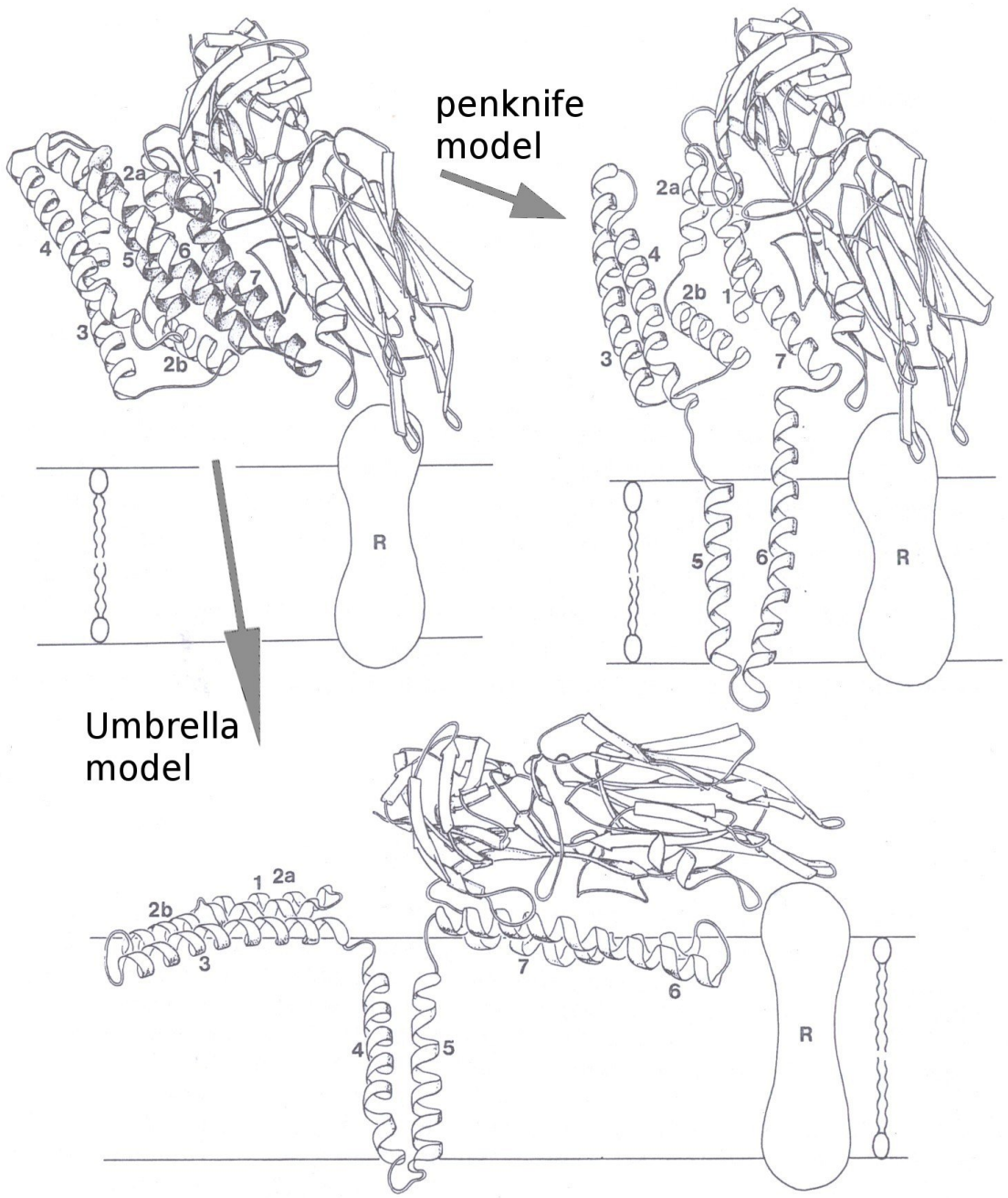

Figure I-4.
Two models for the insertion of Bt toxins into the insect cell membrane. The receptor in the insect cell membrane is indicated as R. The figure left above shows a possible orientation of the toxin as it binds to the receptor. Binding of receptor R to a site in domain II or III leads to a conformational change in domain I. Two models are proposed for the actual pore formation: (1) The penknife model [66]: Helices α5 and α6 flip into the cell membrane as an helical hairpin. (2) The umbrella model [98]: Helices α4 and α5 drop down into the cell membrane as an helical hairpin, and the other helices flatten out on the membrane surface, with their hydrophobic side towards the membrane. Adapted copy from the review of Knowles [87].

Most binding sites for receptors on the cell membrane are found in domain II and III. Pigott and Ellar [136] reviewed in 2007 the receptors for the binding sites on Cry proteins. These receptors are discussed in section ***I-3.2 The larval insect cell membrane receptors*** (p. 51).

Most known 3D-Cry proteins have been sequenced. The sequence of these proteins can be found in the protein database of the NCBI [w3]. The Cry nomenclature website [w1] links each sequenced Cry protein to the corresponding data record in the NCBI database. As first recognized by Höfte and Whiteley [67], the sequences of these 3D-protein show in most cases 5 blocks of conserved amino acid sequences within the toxin part of the Cry protoxin. In 1998, Schnepf *et al*. [159] gave an overview of the presence of the blocks with highly conserved sequences in 3D-Cry proteins. These blocks are graphically shown in fig. I-5.
Block 1 includes helix α5 of domain I.
Block 2 includes the last helix (α7) of domain I and the first β-strand of domain II.
Block 3 contains the last β-strand of domain II and the first of the buried strands in domain III.
Block 4 and 5 each contain a buried strand of domain III [159].

Some Cry toxins have a slightly different structure in domain I:
Cry4Ba: missing helices α1 and α2 in domain I [15].
Cry11Bb: Not only helix α2 but also helix α7 is interrupted by short loop, dividing the helix in α7a and α7b [59].
Cry30Ca: missing helices α1 and α2 in domain I [226].

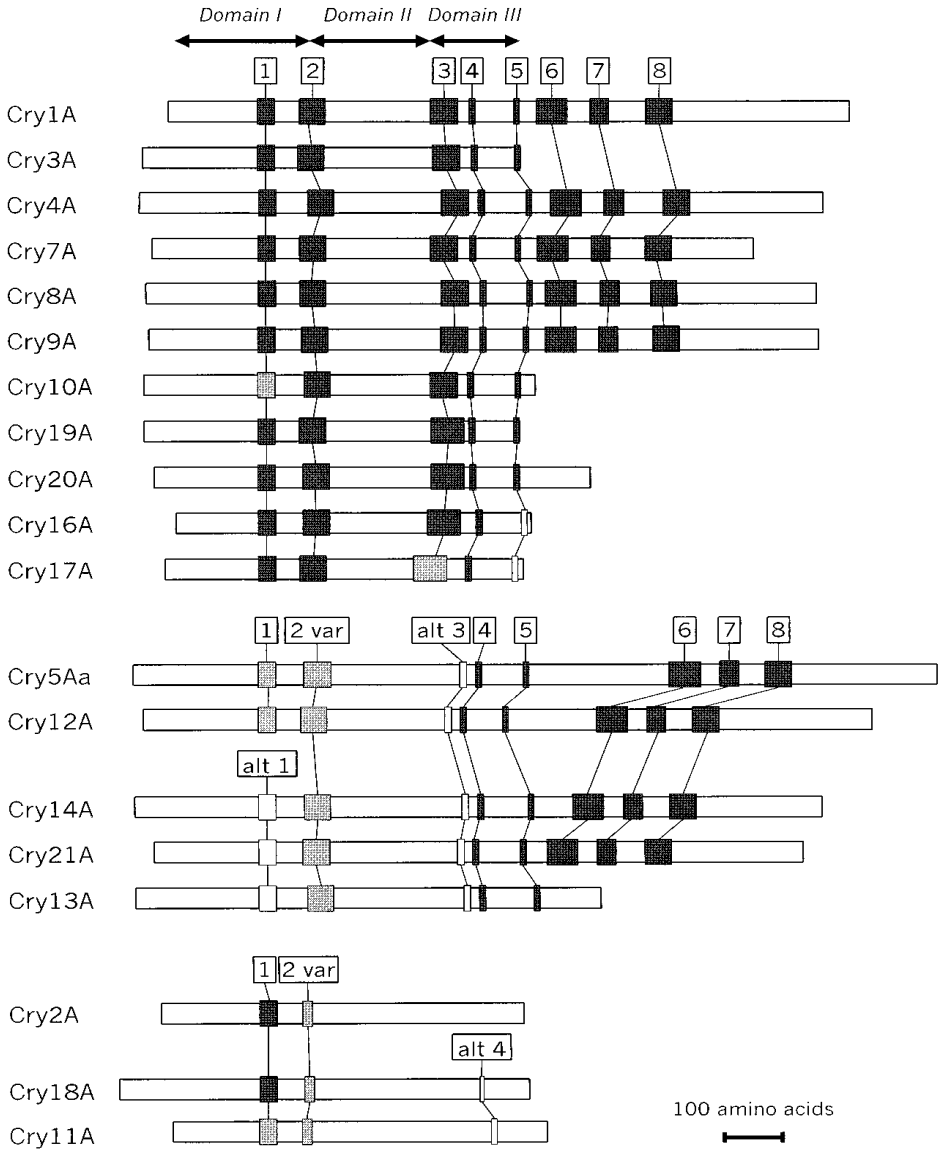

Figure I-5
Positions of conserved blocks among Cry proteins. The cartoon shows the sequence arrangement for each holotype toxin (e.g. Cry1Aa1) having at least one of the conserved blocks*.
Sequence blocks are shown as dark gray, light gray, or white to indicate high, moderate, or low degrees of homology, respectively, to the consensus sequence for each conserved block. The lengths of each protein and the conserved blocks within them are drawn to scale. (Copied from [159])

* Conserved blocks are defined as:
Highly conserved sequences conform to the consensus sequence at 75% or more of its positions.
Variant sequences (var) conform to the consensus sequence of the highly conserved group at 50 to 75% of the positions.
Alternate blocks (alt) are derived from groups of proteins having a consensus sequence over that sequence block that differs from the corresponding highly conserved sequence at more than half of its positions.
Non identical amino-acids are considered conserved if the fall within one of the following groups: a (A, G, S, T, or P); d (D, E, N, or Q); f (F, W, or Y); i (I, L, M, or V); and k (K or R))

I-2.2 Models for the toxic activity of 3D-Cry proteins.

Several models have been developed for the toxic mechanisms of the 3D-Cry proteins. The main 3 models are critically reviewed by Vachon et al. [191]. These models are (1) the classical model; (2) the sequential binding model; and (3) the signalling pathway model. Both the sequential binding model and the signalling pathway model are developed for Cry1Ab, and almost all experimental verification is performed with this Cry type. In some rare cases, the related Cry types Cry1Aa or Cry1Ac were used.

I-2.2.1 *The classical model*

The classical model can be considered as a simple predecessor of the sequential binding model which will be discussed later. The classical model can be split in 4 phases as described in fig. I-6. In phase I, the Cry protein is produced. This phase is typical for the formation of the Cry protein by *Bacillus thuringiensis*. If the protein is produced by a genetically modified (GM) plant, phase I will be different, because the plant is producing the protein instead of the bacterium. GM plants do not always produce the full length protoxin, but might produce a so-called preactivated toxin, in length somewhere between the protoxin and the activated toxin. In phase II, the Cry protoxin, or in case of GM plants the preactivated toxin, is dissolved in the insect gut, and proteases in the midgut cleave the Cry toxin from the appropriate N- and/or C-terminal fragments of the protoxin or preactivated toxin. Phase II might be slightly different for toxic Bt proteins produced by GM plants for a shorter N- and/or C-terminal fragment has to be cleaved proteolytically. Most investigation is done into the mechanism in phase III. In the classical model, this phase is assumed to be relatively simple. The Cry toxin binds to a receptor and then inserts into the cell membrane, forming a pore. Both binding and pore forming have been and still are investigated in detail, leading to the more extensive sequential binding model.
Phase IV simply is the result of pore formation: the membrane becomes more permeable due to the pores, the cell membrane does not function any more and the cell dies. And if too many gut cells of the insect die, the individual insect will die. According to Knowles [87] individual death follows cell death with a delay of several days, during which period the larva does not eat any more. The individual death results from starvation or from bacterial infection [87].

The classical model can be considered as a kind of basic frame for the other two models (sequential binding and signalling pathway). In these models, Phase I and II are the same, and in the sequential binding model also Phase IV.

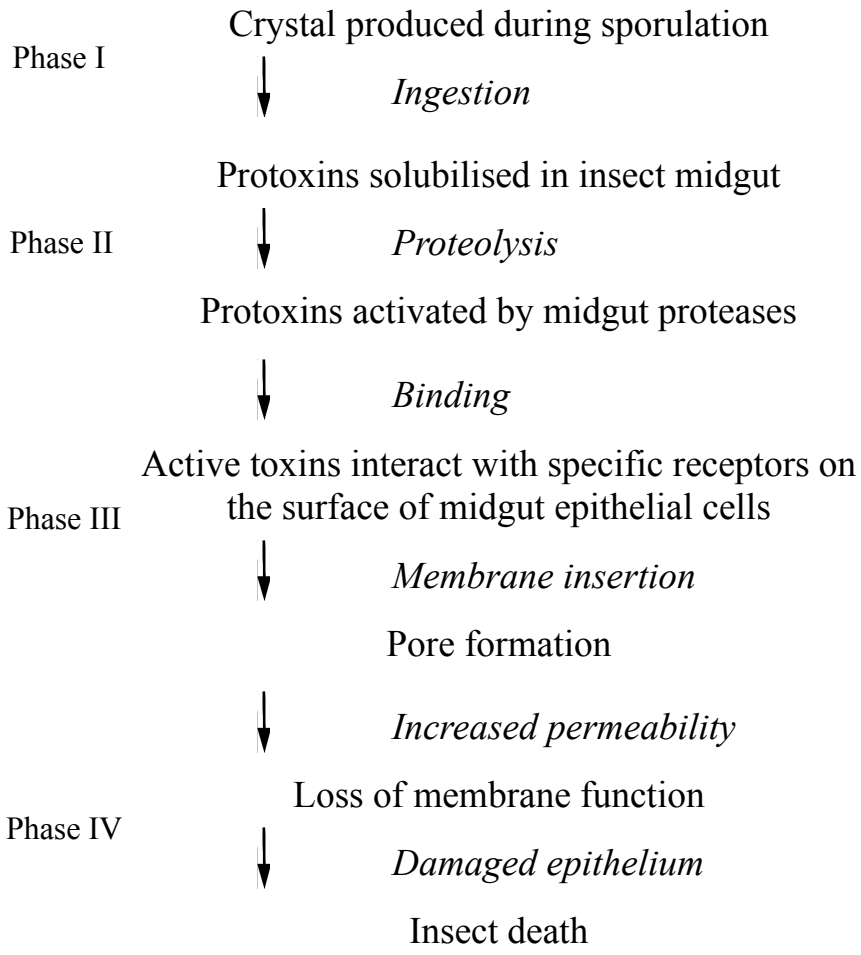

Figure I-6
The classical model. Schematic representation of the steps leading to insect death due to a 3D-cry protein, free after Vachon *et al*. [191].

I-2.2.2 *The sequential binding model*

The sequential binding model is developed by the groups of Mario Soberón and of Alejandra Bravo, both groups from the department of Molecular Microbiology (Microbiología Molecular) of the Institute for Biotechnology (Instituto de Biotecnología) from the National University of Mexico (Universidad Nacional Autónoma de México). The model is still under development, that is, the model is adapted to new observations, if necessary. Their model is described in [172] and most recently updated in [126]. This model addresses the sequence of events in phase III of the classical model, leading to a more detailed description. The model is based on the Cry1A proteins, and mostly on Cry1Ab.
Pardo-Lopez *et al*. [126] illustrated the sequential binding model schematically, see fig. I-7. In her review, she describes the model, illustrating each step in the model with references to experimental results. Most of these experiments are performed with Cry toxins from the Cry1-group.

The sequential binding model, as outlined in fig. I-7, has 6 steps. The first step is phase II of the classical model, and the other 5 steps are replacing the two steps of phase III.
In step 2, the (monomeric) Cry toxin binds either to an aminopeptidase N (APN) receptor [79] or to an alkaline phosphatase (ALP) receptor [1], [33], [84]. These receptors are relatively abundant on the cell surface. APN and ALP molecules themselves are anchored to the cell membrane surface by glycosylphosphatidylinsitol (GPI). The binding has a low affinity (K_d above 100 nM [126]). The ALP/APN binds to a binding site in Domain III of the Cry1 toxin. Note that each Cry type will only be able to bind to a limited number of APN or ALP variants. Some receptor types can bind different Cry types, others are highly specific. Most Cry types can bind to different receptor variants.
The main effect of the low-affinity APN/ALP-Cry binding is that the Cry toxin is now close to the membrane surface, and thereby has a higher probability to be close to the receptor-site of a cadherin or cadherin-like molecule (CAD). This is step 3 in the model. The CAD molecules are relatively rare but can bind to Cry toxin with a high affinity (Kd ≈ 1 nM [126]). Most CAD-like molecules consist of 4 parts, a small cytoplasmic domain (inside the cell), a transmembrane domain, a membrane proximal extracellular domain and an extending domain consisting of a number of repeats (CR repeats) [136].
While bound to CAD, the α1 helix is cleaved of domain I of the Cry toxin [49].
Then, the Cry toxin (without the α1 helix) forms oligomers (step 4). These oligomers are or become the pre-pores. In step 5, the Cry toxin binds again with ALP or APN receptors, but this time in the form of an oligomer. This binding has a high affinity (Kd<1 nM [126]) and enables the pre-pore to enter the membrane and form a pore (step 6). In [45] it is hypothesised that the binding to ALP or APN receptors in step 5 is reversible and not very receptor specific, leading to a high concentration of Cry oligomers near the ATP-binding cassette transporters (ABC transporters), a membrane system to facilitate the transport of small molecules through the membrane. Binding to an ABC-transporter protein would then facilitate membrane incorporation.
The oligomers forming the (pre-)pore deserve special interest. They can be formed from monomers from the same protein type (homo-oligomers) or from a combination of different monomer types (hetero-oligomers). If the Cry toxin-types combined in the oligomer are all toxic, the toxicity induced by the hetero-oligomer may be larger than that by each of the corresponding homo-oligomers. At least, that is one of the theoretical explanations for 3D-toxin synergism [126], [169]. On the other hand, non-toxic mutants, often with mutations in in helix α4, can be so-called dominant-negative (DN). That is, when mixed in equal amount with wild type Cry toxin, or in an even lower ratio of DN:Wt of 0.5:1 or 0.25:1, the toxicity of the wild type is severely reduced [19], [147]. It is assumed that the DN-mutant forms hetero-oligomers with the wild type, and that these hetero-oligomers are not toxic.

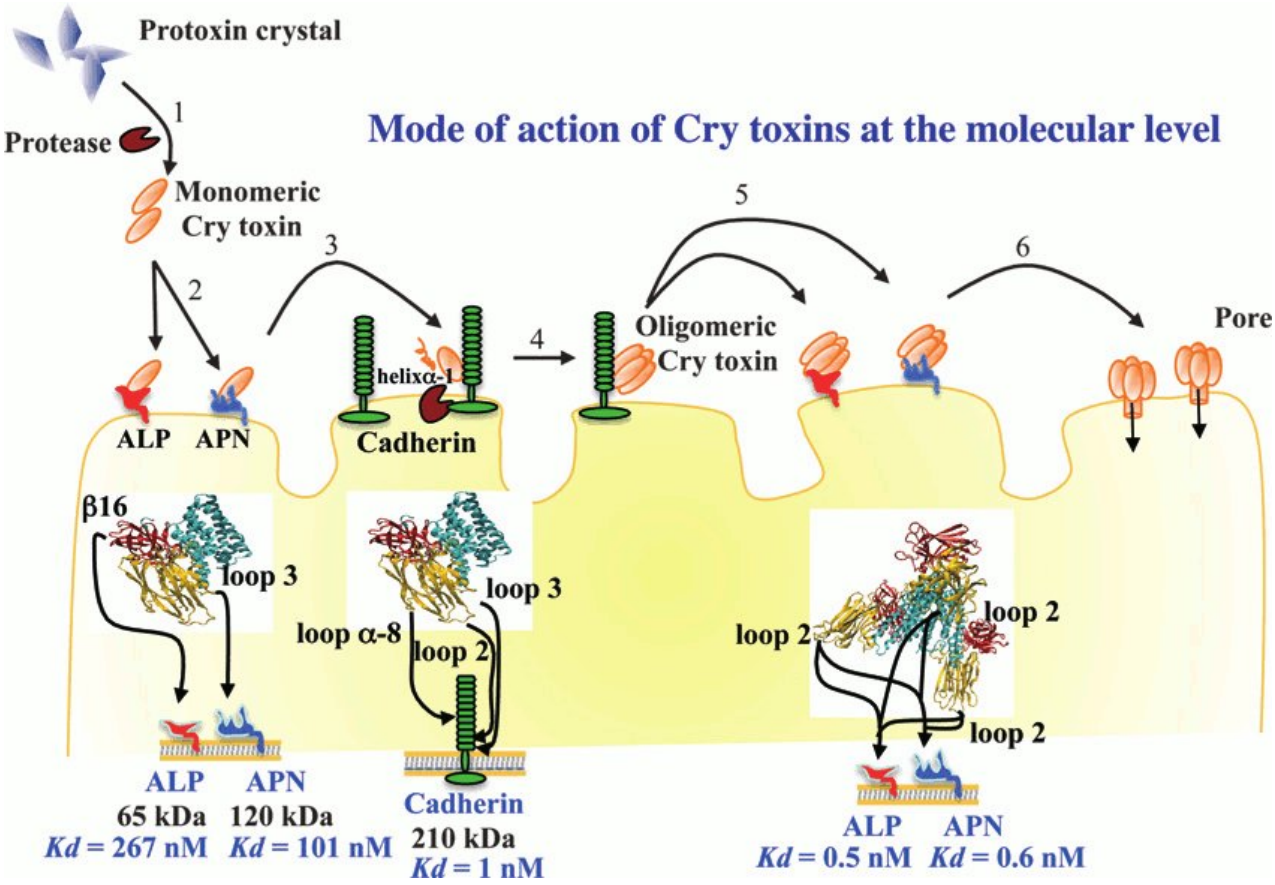

Figure I-7.
A schematic representation of the sequential binding model. The Cry protoxin is cleaved by proteases into monomeric Cry toxin molecules. These molecules bind loosely to ALP or APN receptors. This brings the Cry toxin in the proximity of the much rarer Cadherin-receptor. Cry toxin switch from being bound loosely to ALP/APN to being bound strongly to cadherin. When bound to cadherin, the Domain I α1 helix is cleaved from the rest of the Cry toxin. The Cry toxin monomer molecules without α1 oligomerize with each other, forming a pre-pore. The Cry toxin-oligomer (pre-pore) binds with high affinity to ALP or APN and then the pre-pore is able to penetrate the cell membrane.
(figure copied from [126])

I-2.2.3 *The signalling pathway model*

Zhang *et al*. [222], [223] have developed a completely different model for the mechanism of Cry toxin toxicity. Their model is based on experiments with Cry1Ab and cells from a culture started with ovarian cells from *Trichoplusia ni*. The cells of this culture (H5) do not have a cadherin receptor and are not sensitive to Cry1Ab toxin. A modified cell line (S5) had the cadherin receptor BT-R1. This cell line is sensitive to Cry1Ab toxin.
A schematic representation of the signalling pathway model is given in fig. I-8, copied

from Zhang *et al*. [223]. Their model gives an alternative to phase III and IV of the classical model. This model proposes the following steps in the toxic mechanism:

1. The Cry toxin binds to cadherin.
2. Cadherin signals to its coupled G-protein[4]. This signal depends on the presence of Mg^{2+}-ions.
3. Activated G-protein activates membrane bound Adenylyl Cyclase (AC).
4. Activated AC stimulates the production of cAMP.
5. cAMP activates Protein Kinase A (PKA)
6. The activated PKA induces cell blebbing followed by both cell swelling and cell death.

This cascade of events is based on the experiments described in [222] and [223], both performed with Cry1Ab toxin and cell lines S5 and H5 from *T. ni*. The main experimental results are:

- Cells without cadherin BT-R1 are not sensitive to Cry1Ab, cells with BT-R1 are sensitive.
- Binding of free Mg^{2+}-ions with EDTA[5] suppresses toxicity completely, adding extra Mg2+ restores part of the toxicity.
- Inhibiting G-protein activity with one inhibitor (NF449) decreases toxicity to about 50%. Another inhibitor (NF023) had no effect.
- Inhibiting AC decreases toxicity with about 50%.
- Increasing the amount of cAMP with the cAMP analog pCPT-cAMP increases toxicity. The level of toxicity reached is independent of whether AC is inhibited or not.
- Inhibiting PKA completely inhibits toxicity.
- After binding of Cry to BT-R1 and before the start of cell blebbing, EDTA (binding of free Mg^{2+}-ions) can stop the start of cell blebbing and prevent cell death.
- Even if blebbing has already started, cell swelling can still be stopped by bi- or trisaccharides which are sufficiently large to block the membrane channels. The saccharide raffinose (1.2-1.4 nm) stops cell swelling, sucrose (ca. 0.9 nm) reduces cell swelling and glucose (ca. 0.7 nm) has no effect on cell swelling. However, even if the cell swelling is stopped, the cells die.

4 G-proteins bind to the inner surface of the cell membrane, close to the receptors on the outer surface of that membrane. They play a central role in many signalling tasks [51].

5 EDTA chelating both Mg^{2+} and Ca^{2+} inhibits toxicity, EGTA, only chelating Ca^{2+} does not affect toxicity [222].

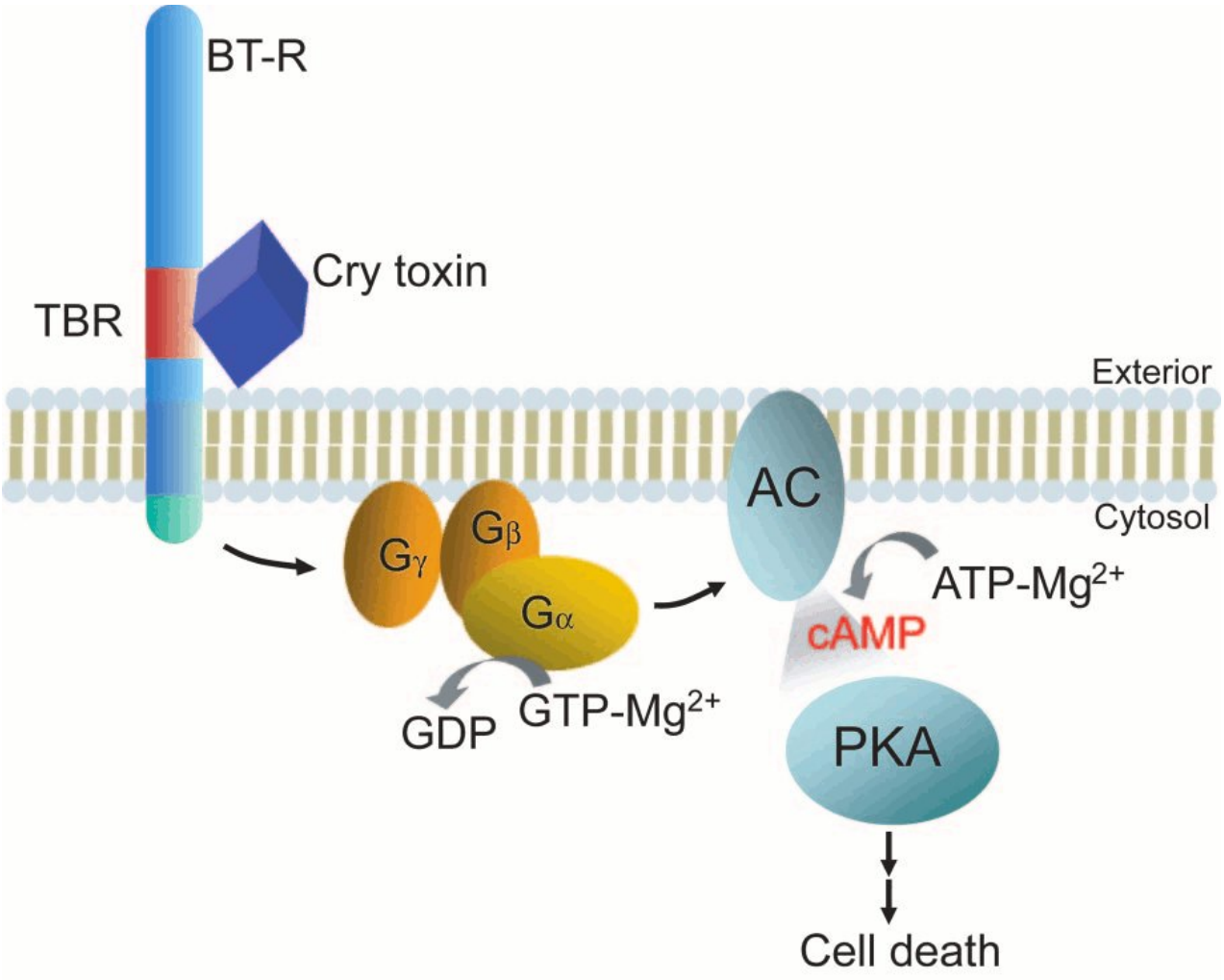

Figure I-8
A schematic representation of the signalling pathway model of Zhang et al. [223]. The Cry toxin binds to cadherin BT-R1, giving a signal to the G-protein coupled to that cadherin receptor. In the presence of Mg^{2+}-ions, G-protein is activated and in its turn activates Adenylyl Cyclase (AC), which promotes the production of cAMP. The cAMP activates Protein Kinase A (PKA), leading to cell death.
(figure copied from [223])

I-2.2.4 *Models for pore forming*

None of the three models explicitly describe the mechanism of pore forming (according to the signalling pathway model, pore-forming is irrelevant). As described earlier, two main models for pore forming exist, the umbrella model and the penknife model (p. 37) [87]. The umbrella model, in which helix α4 and α5 form the pore, is more widely accepted than the penknife model, in which helix α5 and α6 are the pore forming helices. This preference is based on several observations:

- Gazit *et al*. [48] measured and modelled the binding energy of helix fragments of

Cry3Aa in several membranes and concluded that the observed binding energies were compatible with the umbrella model. In their model, helices α4 and α5 are stuck through the membrane, helix α1 points away from the membrane, and the other helices are spread out on the membrane surface, helices α2, α3 and α6 in a more or less fixed configuration whereas helix α7 can rotate more freely at its connection with helix α6. For helix α2, the calculation of the exact position is complicated by the short loop between α2a and α2b, and the unbalance in the frequencies of charged amino-acid chains (more charged amino-acids near the C-terminus, α2b).

- Masson *et al.* [113] studied the effect of mutations in the helix α4 region of Cry1Aa on the toxicity and ion channel function in *Plutella xylostella*. They found that the binding of the non-toxic mutants was comparable to the binding of the wild type and the toxic mutant, but that the conductance in the ion channel induced by the non-toxic mutant was severely reduced compared to the toxic variants. Based on these observations, they proposed an extension of the umbrella model with a tetramere of Cry1Aa, in which the lumen of the pore is lined by the four helix α4 with each α4 pointing with 2 negatively charged amino-acid chains into the lumen (Glu129 and Asp136), and at the outside of the ring of helices α4 the four more hydrophobic helices α5. They assumed that the pores could grow over time by further oligomerization.
- Some Cry toxins have one or more proteolytic cleavage sites. These sites are found in the area before helix α4 and in the area after helix α5, but not in the helices α4 and α5 or their connecting loop [83]. In Cry4Aa, Cry4Ba [87], [83], Cry9Aa [83] and Cry48Aa [77], the cleavage site is between helices α5 and α6, so between the helices putatively involved in the penknife model.

The pores formed by Cry toxins are in most cases cation specific [5], [91], [108], [190], sometimes aspecific [21] or under specific conditions anion specific [161]. The anion or cation specificity may depend on the pH [161].
Estimations of the pore sizes vary from a radius of 0.5 nm to 1.3 nm. Cry1Ca induces in planar lipid bilayers at a pH of 8.0 pores with a radius between 1.0 and 1.3 nm [134], For Cry1Ac, the pore size in brush border membrane vesicles (BBMV) at pH 9.0 is estimated to be 1.2-1.3 nm [22]. Knowles and Ellar [85] estimated pore size radius to be between 0.5 and 1.0 nm. Raffinose, with a diameter of 1.14 nm [22], can block the pore channel of Cry1Ab in cultured cells [223], but can freely pass the pores of Cry1Ac in BBMV at pH=9.0 [22].
The pores are not always open. Depending on the conditions, the frequency and duration of the open periods differs [161]. The conductivity of these pores is also pH dependent [161]. Within one experiment, several conductance levels are found for the Cry toxin induced pores, ranging from about 10 to 250 pS in planar lipid bilayers to 85-420 pS in *Lymantria dispar* midgut epithelium fused on planar lipid bilayers [133], [134]. This might be due to pores of different sizes as a result of multimerization with different numbers of proteins, or to clusters of equally-sized pores, opening and closing simultaneously, in which case the individual pore conductance is probably in the order of 10 pS [134]. Peyronnet *et al*. [134] showed that the pores induced in their experiment by Cry1Ca are all of equal size with a radius between 1.0 and 1.3 nm. Given that they

observed up till 15 different conductance levels, they concluded that the pores occurred in synchronized clusters [134].
Ounjai *et al.* [124] have tried to analyse the three-dimensional structure of pores formed by Cry4Ba by electron crystallography. They used two different carbon support films in preparing the 2D crystal structure, one hydrophobic and the other negatively charged, leading to two different conformations of the trimeric pore structure (see fig. I-9). They suggest that these two conformations might coincide with the closed (the pinwheel-like shape) or the open (the propeller-like shape with an open circle in the middle) state of the pores. For the modified Cry1Abmod toxin (Cry1Ab without helix α1), Muñóz-Garay *et al*. [121] also found a trimeric propeller-like structure with a potential pore in the middle and with the same size as the propeller-like structure observed by Ounjai [124] (*a*=*b*=107 Å). Others found, however a four-fold symmetry for Cry4Ba pores [143]. The different Cry1A pores are described as dimers (Cry1Ab, Cry1Ac, [188]), trimers (Cry1Aa, Cry1Ab, Cry1Ac [121], [89]), and tetramers (Cry1Aa, Cry1Ab [49], [195]). Note that for the same Cry proteins, different oligomer sizes are mentioned. This shows that the understanding of the oligomerization process and the pore formations is by no means complete.

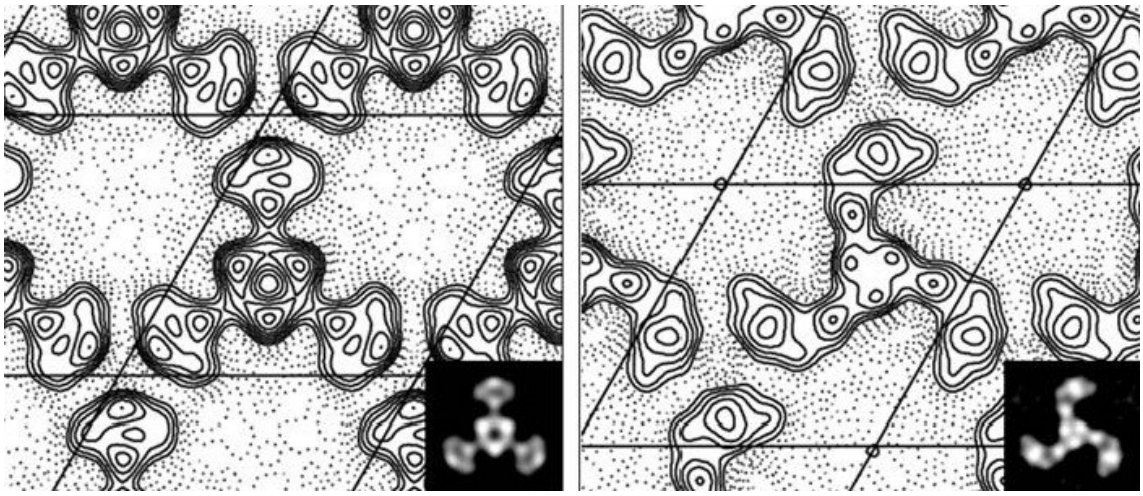

Figure I-9.
The trimeric pore structure of Cry4Ba as observed by electron crystallography with contour lines plotted 17 Å resolution. The left propeller-like configuration with (*a*=*b*=107 Å) was observed using a negatively charged carbon support, the right pinwheel-like structure (size *a*=*b*=112 Å) using a hydrophobic carbon support. Copied from Angsuthanasombat *et al*. [8].

I-3 The susceptible insect

The activity of Bt toxins is very specific. Most of them are toxic for the larval stage of some insect species. The toxin acts on the midgut of these larvae. During the larval stage the insect grows fast and has therefore a high food throughput. And the exposure of the insect to a toxin in the food is higher if the food consumption per unit of body mass is higher. Therefore, it is to be expected that larvae are more sensitive than adult insects, and that the first larval stages are more sensitive than the later ones.

I-3.1 Insect midgut.

In [87] the properties of the insect larval midgut relevant for the toxic mechanism of Cry toxins are described for three insect orders, Lepidoptera, Diptera and Coleoptera. The midgut is a simple tube made of one layer of cells resting on a basal membrane. In Lepidoptera larvae, the two most common cells types are: (1) columnar cells with an apical brush border of microvilli and (2) goblet cells containing a large cavity. In Diptera and Coleoptera larvae, the second cell type does not occur. These cells are graphically sketched in fig. I-10, copied from [87]. In table I-2 the chemical conditions in the gut of these three insect orders are listed.

Table I-2.
A summary of the midgut properties of three insect orders, relevant for the action of Cry toxins

Insect order	family	cell types	pH in gut lumen	K+:Na+ ratio in blood	protease type
Lepidoptera		Columnar and Goblet	high (up to 12)	high	
Diptera		Columnar	high	very low	
Coleoptera	Chrysomelidae	Columnar	neutral or slightly acid	high	cysteine and aspartate
	Scarabaeidae	Columnar	high	high	serine

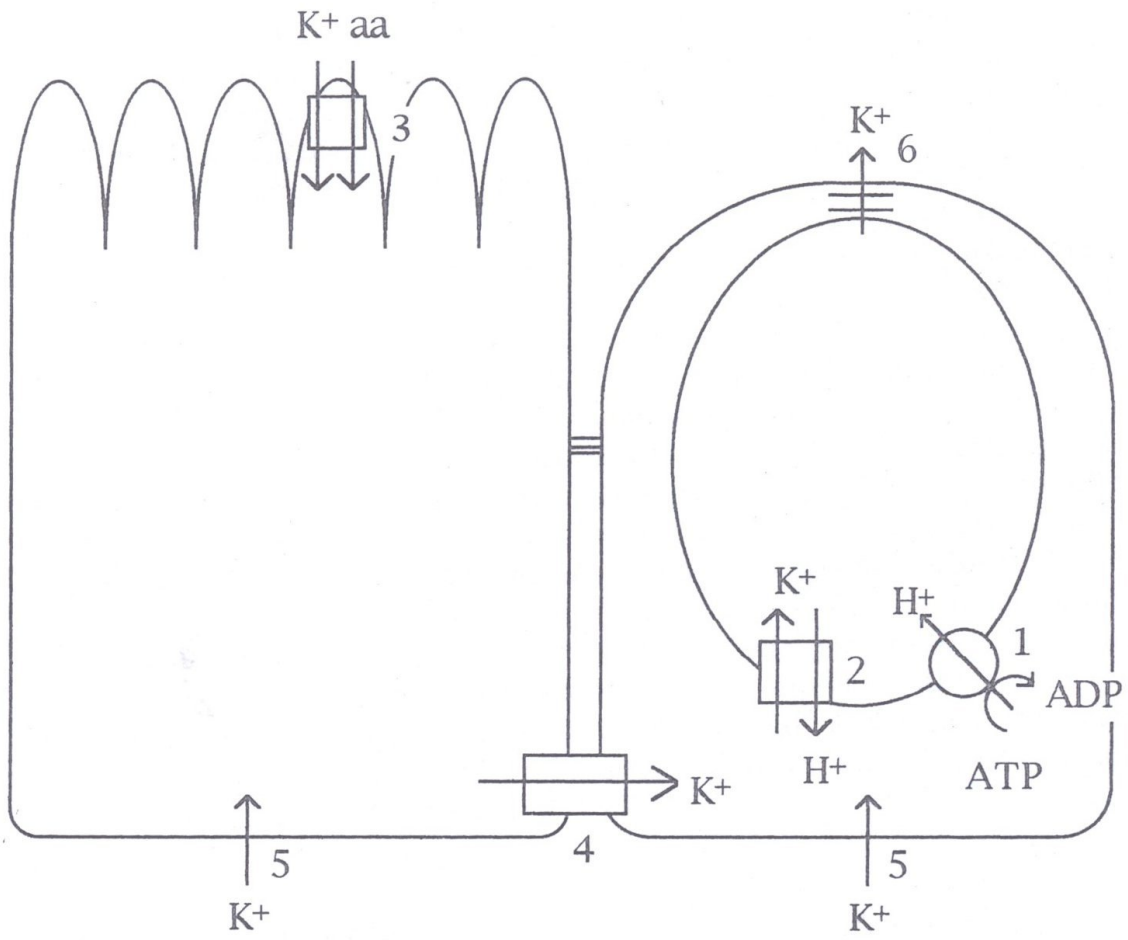

Figure I-10.
Schematic view of the two principle cell types of Lepidoptera larval midgut. Left: columnar cell (type 1) and right goblet cell (type 2). The apical (gut lumen) site is at the top of the diagram. The cells sit on a basement membrane and are connected by septate junctions and gap junctions. Major ion movements are indicated with arrows. The main pumps and channels are numbered as follows: (1) H^+-ATPase; (2) K^+/nH^+ exchanger; (3) K^+/amino acid co-transporter; (4) gap junction; (5) basal K^+ entry through channel and a pump; (6) K^+ leaving the goblet cavity through the apical valve. Copied from the review of Knowles [87].

Before the Cry (pro)toxin reaches the midgut epithelium, it has to pass the peritrophic membrane. The main function of this peritrophic membrane is probably to protect the midgut cell against mechanical damage from sharp food particles. Another of its functions is to protect the midgut against toxic substances, Some Cry (pro)toxins bind to the peritrophic membrane (for instance Cry1Ab), others not (for instance Cry1Ac) [87]. The peritrophic membrane also prevents the passage of Cry aggregates, but dissolved monomeric Cry proteins can pass that membrane [87]. At the anterior of the midgut, many insect larvae have so called caeca, stubby pointed tubes/bladder-like pounches, that increase the surface of the midgut. Some Cry receptors are located in these caeca (for

instance the Cadherin binding Cry11Aa in the midgut of *Aedae aegypti* [25]. Figure I-11 gives a schematic representation of the insect gut.

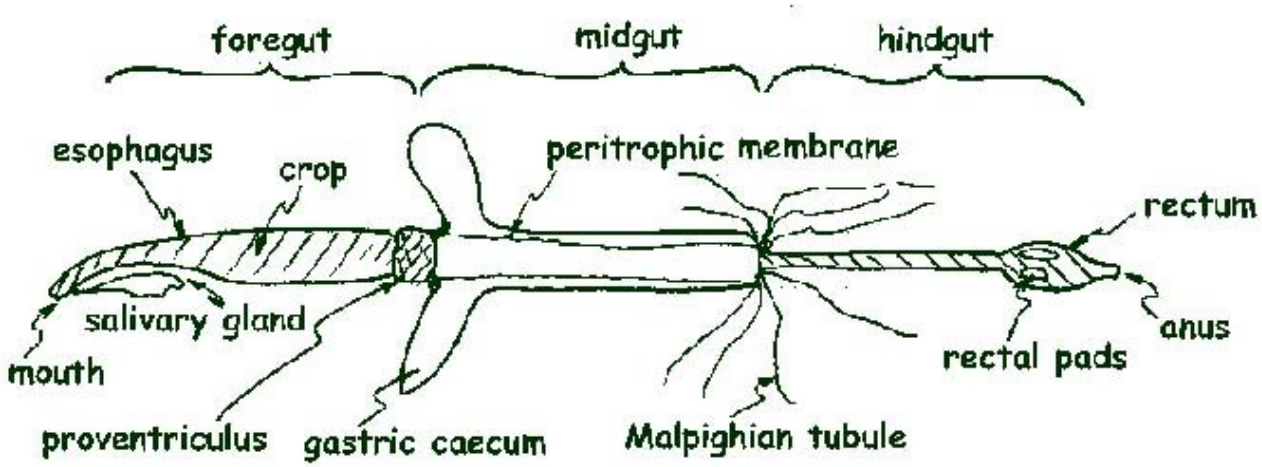

Figure I-11.
A generalized schematic picture of the insect gut.
Copied from [w4].

I-3.2 The larval insect cell membrane receptors.

Pigott and Ellar [136] have summarized the information on Cry toxin receptors in insect midgut cell membranes. Several types of molecules can function as receptors. Pigott and Ellar distinguish five groups:

- Aminopeptidase N (APN)
- Alkaline phosphatase (ALP)
- Cadherin or cadherin-like (CAD)
- Glycolipids (GL)
- Other receptors

Some of these receptor types play a role in the models describing the mode of action of the Cry toxin (APN and ALP in the sequential binding model, CAD in both the sequential binding and the signalling pathway model). Note that in most studied insect species at least one receptor was found, and often more than one. The receptors within some of these groups are classified in classes, and within one insect species, receptors of different classes from the same group may be present. Receptors of one group, for instance CAD, differ between different species and sometimes even within different strains of a species. A specific receptor may be able to bind to one or more specific Cry toxins, and each specific Cry toxin may be able to bind to one or more specific receptors.

I-3.2.1 *Aminopeptidase N*

APNs are enzymes that cleave neutral amino acids from the N-terminus of polypeptides [136]. Some APNs can also bind some Cry toxins. In insects, APN is attached to the membrane by a glycosylphosphatidylinositol (GPI) anchor.
Most APNs have several binding sites for glycans (carbohydrates). These sites can be distinguished in the N-linked and the O-linked glycolysation sites. N-linked glycans attach to the nitrogen of the amino acid site chain of asparagine or arginine. O-linked glycans attach to the hydroxy oxygen of the amino acid site chain of serine, threonine or tyrosine. For the interaction with Cry toxins, the N- and O-linked glycolysation sites, which can be linked to the carbohydrate N-acetylgalactosamine (GalNAc), are most important. At least some Cry toxins (Cry1Ac) are able to bind to GalNac.
Based on sequence homology, five classes of insect APNs can be distinguished [136]. Some insect species have APNs from different classes and an APN class may occur in multiple insect species from different orders. Some APN can bind some Cry toxins, and the APN-Cry combination is in most cases highly specific. For instance, a class 1 APN from *Manduca sexta* binds at one site[6] with Cry1Aa, Cry1Ab and Cry1Ac, and at a second site only with Cry1Ac. GalNAc inhibits the binding of Cry1Ac to both sites, but has no effect on the binding of Cry1Aa and Cry1Ab to the APN. [112]. This suggests that GalNAc binds to the Cry1Ac binding site[7]. Only a small fraction of all possible APN-Cry combinations is tested. In 2007 Pigott and Ellar [136] have collected the available data on these binding combinations for APNs of these 5 classes in 12 Lepidoptera species with 10 Cry types, 8 of which are Cry1-types. They distinguished whether the APN was produced by the insect or by some other organism/cell line in which the DNA coding for the APN was cloned. They also distinguished whether the APN was in its natural folding or denaturated. The tabular representation of the APN-Cry combinations tested is given in fig. I-12.

I-3.2.2 *Alkaline phosphatase*

ALP is attached to the membrane by a GPI anchor, like APN [136]. By Pigott and Ellar [136] three ALPs binding Cry toxins are described, glycoproteins ranging from 65 to 68 kDa.

1. In *Manduca sexta* (Lep): Cry1Ac binds to a 65 kDa ALP
2. In *Heliothis virescens* (Lep): Cry1Ac binds to 68 kDa ALP (HvALP)[8]
3. In *Aedes aegypti* (Dip): Cry11Aa binds to 65 kDa ALP

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6 Possibly, Cry1Aa, Cry1Ab and Cry1Ac do not bind to exactly the same site, but to neighbouring sites where one bound Cry molecule stereometrically hinders the binding of another molecule to the neighbouring site.

7 The hypothesis that GalNAc binds to the binding site of Cry1Ac is also supported by the finding that GalNAc pretreatment of Cry1Ac inhibits the binding of Cry1Ac to the peritrophic membrane [62].

8 In a Cry1Ac resistant *H. virens* strain, the ALP expression was suppressed. Cry1Ac binds to a terminal GalNAc on HvALP [79].

I-3.2.3 *Cadherin or cadherin-like molecules*

Cadherins form a large group of glycoproteins. For the binding of Cry proteins, a novel, cadherin-like protein was first isolated in 1993 from the midgut epithelium of *Manduca sexta*. This CAD binds to Cry1Ab, but not to Cry3Aa and Cry11Aa [192].
Most of these insect CAD-like molecules consist of 4 parts, a small cytoplasmic domain (inside the cell), a transmembrane domain, a membrane proximal extracellular domain (MPED) and an extending domain consisting of a number of repeats (CR). These repeats are numbered, starting with the CR furthest away from the membrane. So the CR with the highest number is closest to the membrane [136]. These CAD have been found in Lepidoptera on the apical membrane of the midgut columnar epithelian cells. The expression varies with the developmental larval stage, in *M. sexta* increasing progressively from the first to the fifth instar [117]. Although the number of CAD-receptors per membrane surface increases with increasing larval stage, the larger midgut diameter leads to a smaller fraction of the toxin molecules coming within the attraction zone of the receptors and therewith to a lower sensitivity to Cry toxins [55]. Pigott and Ellar [136] reported 12 CADs in Lepidoptera species, some of which are reported to bind to Cry toxins. The best studied Cry-binding CADs discussed by them are:

1. In *Manduca sexta*: Cry1Aa, Cry1Ab and Cry1Ac bind to BT-R1, a 210 kDa CAD with 12 CR; Cry3Aa and Cry11Aa do not bind to BT-R1.
2. In *Bombyx mori*: Cry1Aa binds to BtR175, a 175 kDa CAD with 9 CR. Cry1Ab and Cry1Ac bind, but with lower affinity, to BtR175. A few variants to BtR175 are reported with the same effect.
3. In *Heliothis virenscens*: Cry1Aa, Cry1Ab and Cry1Ac bind to HevCaLP, a CAD 75% identical to BtR175. In *H. virenscens* HevCaLP is necessary for binding to the BBMV of Cry1Aa, but not of Cry1Ab or Cry1Ac.
   Cry1Aa, Cry1Ab and Cry1Ac are toxic for *Drosophila* S2 cells expressing HevCaLP, Cry1Fa is not toxic to these cells.
4. In *Ostrini nubilalis*: Cry1Ab binds to OnBt-R1, a 220 kDa CAD [42].
5. In *Helicoverpa armigera*: Cry1Ac binds to a fragment of the *H. armigera* CAD produced by recombinant *E. coli* [197].
6. In *Pectinophora gossypiella*: Cry1Ac toxicity depends on CAD [119].

Since then, CAD-like proteins binding to Cry toxins are also discovered in other insect orders. Some examples are:

7. In *Aedes aegypti* (Dip): Cry11Aa, Cry11Ab and Cry4Aa bind to a 250 kDa CAD with 12 CR (binding to fragment CR7-12). Cry4Ba does not bind to this CAD [25].
8. In *Diabrotica virgifera virgifera* (Col): Cry3Aa and Cry3Bb bind to CAD fragment CR8-10 of a CAD with 10 CR. This fragment synergizes the toxicity of Cry3Aa and Cry3Bb to *Leptinotarsa decemlineata* [127], [156].

I-3.2.4 *Glycolipids*

Although already in 1986 a glypolipid was found to be able to bind to a Cry protein [32], this class of chemicals was not further investigated until recently when it was discovered

that Cry-resistance in *Caenorhabditis elegans* often correlates with a loss of glycolipids [54].
It was found that a *Manduca sexta* glycolipid binds with Cry1Aa, Cry1Ab and Cry1Ac [54]. And in *Plutella xylostella*, it was found that a Cry1Ac resistant strain had a considerable lower (order of 50%) neutral glycolipid levels than the sensitive strain [90].

I-3.2.5 *Other receptors*

A glycoconjugate, BTR-270, isolated from *Lymantria dispar* binds with high affinity to Cry1Aa and Cry1Ab and with low affinity to Cry1Ac. It does not bind to Cry1Ca, Cry2Aa, Cry2Ba and Cry3Aa [193].
P252 is a Cry1Aa, Cry1Ab, and Cry1Ac receptor isolated from the BBMV of *Bombyx mori* [68]. It shows strong similarities, and might even be identical, to a protein named chlorophyllid A-binding protein (ChBP), also isolated from the *B. mori* midgut [136].

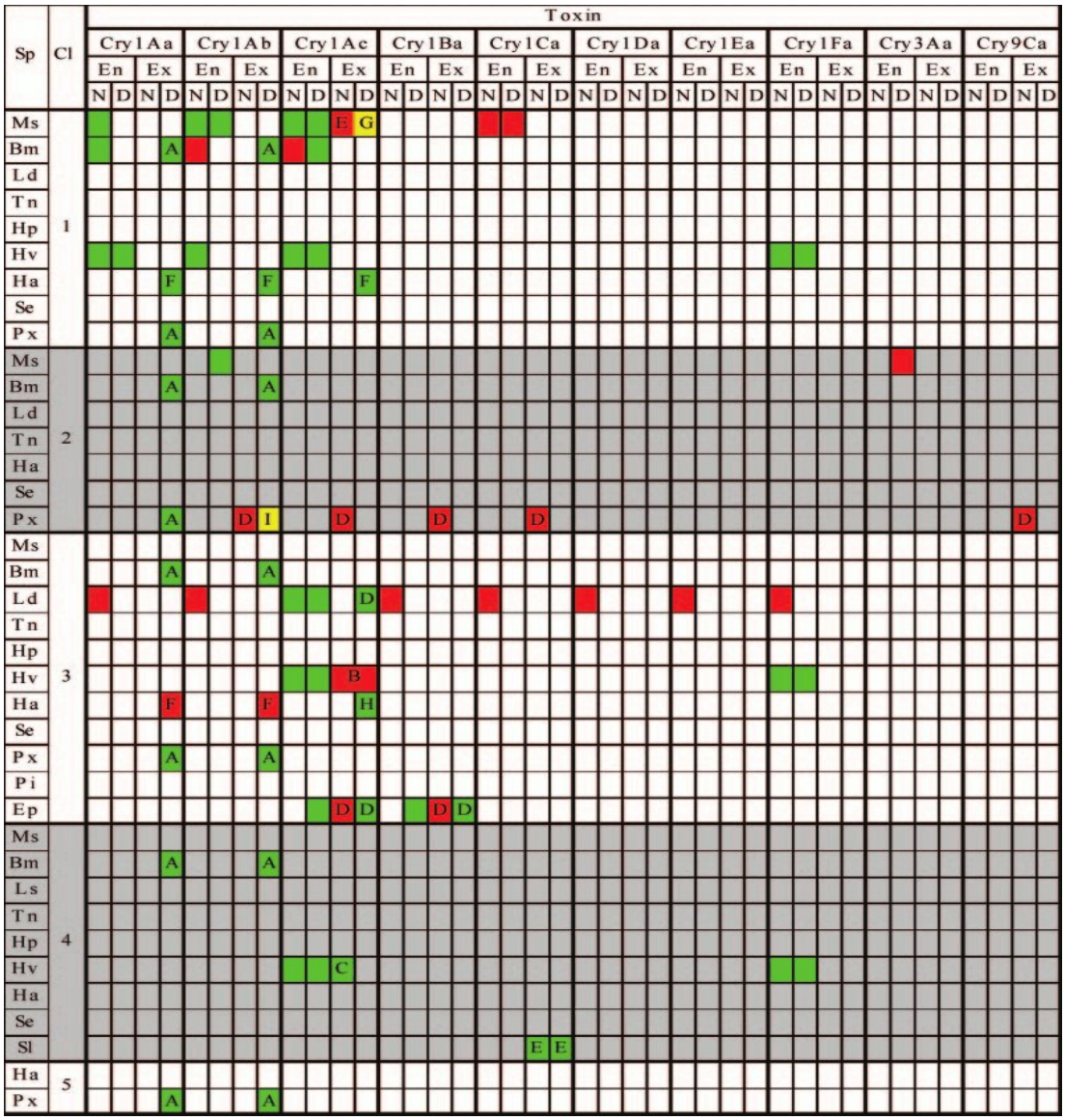


Figure I-12.
Summary given in [136] of the reported binding between Cry toxins and endogeneous (En) or exogeneous (Ex) produced, non denaturated (N) or denaturated (D) APNs. Based on sequence homologies, five classes (cl) of APN are distinguished. The name of the insect (all Lepidoptera) species is abbreviated. Bm: *Bombyx mori*; Ep: *Epiphyas postvittana*; Ha: *Helicoverpa armigera*; Hp: *Helicoverpa punctigera* ; Hv: *Heliothis virescens*; Ld: *Lymantria dispar*; Ls: Ms: *Manduca sexta*; Pi: *Plodia interpunctella* ; Px: *Plutella xylostella*; Se: *Spodoptera exigua*; Sl: *Spodoptera litura*; Tn: *Trichoplusia ni* . If an insect species is not listed for an APN class, this class was not (yet) disovered in that species.
Green: binding; red: no binding; yellow: conflicting reports; white/grey: no data.
APN expressed exogeneously by: A: *E. coli*; B: in vitro translation; C: S2 cells*; D: Sf9 cells*; E: Sf21 cells*; F: *T.ni* cells; G: *Drosophila* or Sf21 cells*; H: *E. coli* or *T. ni* cells; I: *E. coli* of Sf9* cells.
Figure copied from [136].

*: For the explanation of the cells types, see [136].

I-4 Evaluation of the models for 3D-Cry toxin action considering the available data

The sequential binding model and the signalling pathway model both describe the phase starting with the binding of the Cry toxin to the epithelial cell membrane until the process leading to cell death. Both models are based on experiments with the Cry1Ab toxin. And both models involve one or more essential binding steps between the toxin and cell membrane proteins (APN, ALP or CAD-like). Cry toxins are supposed to be highly specific, and one of the main factors causing this specificity is assumed to be the binding of the Cry toxin to cell membrane proteins. Therefore, the experimental results for Cry1Ab might not be representative for all other 3D-Cry toxins and the consequences of binding for the process leading to cell death might depend on the specific Cry toxin properties.

I-4.1 Signalling pathway model

The signalling pathway model is based on experiments with cell cultures of non-target cells which are not susceptible to Cry1Ab (H5 cells) and on modified cells from that culture, producing the cell membrane CAD-like protein BT-R1 (S5 cells) sensitive to Cry1Ab. Extrapolation to other cell types might be incorrect, especially since the main processes in this model are the signalling pathways in the cell. This model was published by Zhang *et al*. in 2005 [222] and 2006 [223]. After these papers almost no new research papers have appeared testing the details of the model steps or testing the model in another experimental cell system. The model is discussed in several reviews [73], [136], [171], [172], [191] where it is often concluded that there could be an intracellular response, but that the present data cannot support or contradict this hypothesis [171]. Only in one review the signalling pathway model was presented as the favourite one, repudiating the sequential binding model with the remark that “no direct evidence has been provided for such a mechanism [assembly of lytic pores in the plasma membrane by forming oligomers] in either living cells or an insect.”. That review was written by the group which has developed the signalling pathway model [73].
Sometimes, a signalling process leading to cell death is combined with a sequential binding model [80]. To be able to better judge the value of the signalling pathway model, experiments with natural target cells, that is epithelial midgut cells from susceptible and resistant insects, should be performed. One of the most interesting aspects which should be verified in these natural cell systems is that the S5 cells exposed to Cry1Ab first start to bleb, and only afterwards start with (osmotic) swelling (see fig I-13). And that the blebbing can be prevented if EDTA is added to chelate Mg^{2+}-ions. Adding a surplus of Mg2+, on the other hand, promotes the blebbing process in S5 cells.

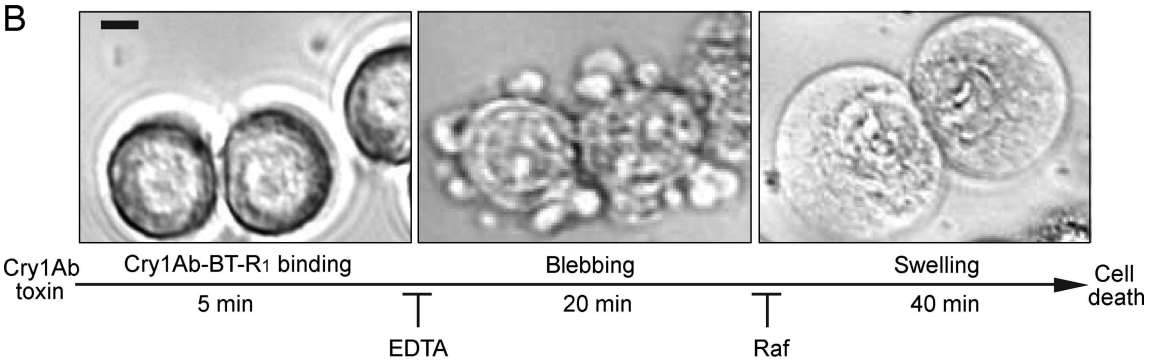


Figure I-13.
Cytological changes associated with the progression of the Cry1Ab toxin-induced cell death as viewed by a phase-contrast microscope. The arrow indicates the relative time for each stage. toxin-binding, membrane blebbing, and cell swelling. Beneath the time line it is indicated up till what moment (1) cell death can be blocked by EDTA, (2) cell swelling, but not cell death, can be blocked by raffinose. Scale of bar: 15 μm.
Figure copied from Zhang *et al*. [223].

I-4.2 Sequential binding model

The experimental foundation for the sequential binding model is for a large part based on research on artificial membranes or isolated membranes of the target insect cells. In these experiments, intracellular signalling pathways cannot be observed. The key feature of this model is the sequence of binding events, and the details of that sequence might be typical for the investigated Cry toxin, Cry1Ab. It is likely that other 3D-Cry toxins will follow other binding patterns, for other 3D-Cry toxins may have different binding sites. For instance, the Cry2Ab and CryAe binding site on the BBMV of *Helicoverpa zea* differs from the binding site of Cry1Ab on that BBMV [52].
One of the most interesting aspects in the sequential binding model is the role of the CAD-like receptor. The presence of the cadherin BT-R1 receptor greatly enhances the toxicity of Cry1Ab. However, if Cry1Ab is missing the first helix (α1) of domain I, the presence of BT-R1 becomes irrelevant for the toxicity of Cry1Ab (Cry1AbMod) [170]. For Cry4Ba it is observed that it is missing the helices α1 and α2 [15], which are lost by proteolyse during the crystalisation. This fits the observation that Cry4Ba does not depend on membrane-bound cadherin, facilitating the cleavage of helix α1, for its toxicity to *Aedes aegypti*, or at least, that this cadherin is not rate limiting [149]. Note however that according to [7] activated Cry4Ba is split in two fragments, of ca. 17 and ca. 47 kDa, resp., one containing the helices α1 to α5 and the other starting with the domain I helix α6 and also containing the domains II and III.
A further remarkable observation is that cloned CAD CR-fragments often synergize with Cry toxins: The BT-R1 fragment CR12-MPED of a cadherin from *Manduca sexta* (Lep) synergizes with Cry1Ab [24]. The toxin binding region (TBR) fragment of the *Helicoverpa armigera* cadherin Hacad1 synergizes with Cry1Ac against *H. armigera* and

Spodoptera exigua [129]. The fragment CR8-10 of a cadherin from *Diabrotica undecimpunctata howardi* (Col) synergizes with Cry3Aa and Cry3Bb [127]. The CR11-MPED fragment of the cadherin AgCad1 from *Anopheles gambiae* (Dip) synergizes with Cry4Ba[9] [46]. And the CR7-10 fragment of the cadherin from *Aedes aegypti* at an 10-fold excess increases the toxicity of Cry11Aa with a factor 2.7 and at a 25-fold excess with a factor 3.5, whereas the effect of Cry4Ba is only increased by this cadherin when the CAD is applied in a 25-fold excess and then only with a factor of 2.5 [149]. Neither of these CAD-fragments was bound to the membrane of the exposed cells, so the synergistic effect of these fragments could not be attributed to binding. As also remarked by Chen *et al.* [24], the primary expectation is that a surplus of free floating Cry receptors, like cadherin fragments, will bind the Cry toxins, and thereby inhibit their toxicity. Clearly, something happens with the Cry protein when bound to CAD. According to the sequential binding model, that something is the cleavage of helix α1 from the toxin, enabling a conformational change necessary for pore formation.
Some observations are, however, diametrically opposed to those described above. For Cry1Ab, Dorsch *et al*. [36] found in *Manduca sexta* that a free CR11 fragment of *M. sexta* CAD BT-R1 decreased the toxicity for *M. sexta*[10]. For Cry1Ac in *Helicoverpa armiga*, it is found that free CAD-fragments reduce its toxicity [106].
Note that the cadherin receptor also plays an essential role in the signalling pathway model. And that membrane free cadherin promoting toxicity, is highly unlikely under the signalling pathway model, because in that model the cadherin is assumed to give a signal to the cell with its intracellular or transmembrane domain if a Cry toxin is bound to the extracellular domain of the cadherin.

I-4.3 Summary of the model evaluation

Both models are limited in their applicability to Cry1Ab and very related Cry proteins. However, although the binding pattern may vary between Cry toxins, there is some indication that cadherin binding is also essential for the toxicity of the Cry4 protein group, an observation in agreement with the sequential binding model. A general conclusion might be that

1. Binding of the Cry toxin to the cell membrane is essential, and therefore the specificity of the binding is essential.
2. Binding might lead to conformational changes, and these changes might be necessary for toxicity.
3. Cry toxin binding to cell membrane proteins might follow a sequential cascade of "binding" → "conformational change" → "binding" ….
4. Perforation of the cell membrane by pores or the starting of a signalling pathway by protein-toxin interaction may lead to a weakening of the cell membrane structure (damage of the cytoskeleton?) resulting in cell blebbing.

9 Note that Gang et al. [46] used a mutated Cry4Ba protein, which was stabilized against proteolysis by removing a cleavage point between the helices α5 and α6, leading to a decrease in cytolysis in vitro but an increase in toxicity in vivo [7].

10 In [24] it is speculated that the difference in the effect of a CAD fragment on the toxicity of Cry1Ab might be due to the protein being unfolded at the synergistic effect and folded at the antagonistic effect.

The two models do not necessarily exclude each other, the 3D-Cry protein might form a pore and start a signalling pathway, either by its binding to CAD or by forming a membrane pore as also suggested in [80].

I-5 Not 3D-Cry toxins and other Bt toxins

I-5.1 Binary Cry toxins and their conjugative toxins

Three of the Cry proteins fall into the class of the so-called binary (Bin) toxins, together with BinA and BinB produced by *Bacillus sphaericus*[11]. These Bin Cry proteins are Cry35, Cry36 and Cry49 [30], [76], [w1] (see fig. I-14). Cry35 and Cry36 are produced by *B. thuringiensis* strains, Cry49 by *B. sphaericus*. These toxins are classified in the same group because of their sequence homology. The first described Bin toxins, BinA and BinB, are separately only slightly toxic but, when applied together, they increase each others toxicity considerably. For the other Bin toxins, it is therefore assumed that they will also have a conjugative toxin so that, when applied separately the Bin toxin and its conjugative are not or only slightly toxic but, when applied together with their conjugative toxin, the combined toxicity of the conjugative pair will be much higher. The conjugative toxin for Cry35 is Cry34 [38], for Cry49 it is Cry48 [76]. The conjugative toxin for Cry36 is as yet unknown. Due to sequence similarity with Cry35, Cry49 and the *B. sphaericus* proteins BinA and BinB, Cry36 is classified as Bin toxin [w1].
Not all known conjugative pairs involve a Bin toxin. The combination Cry23-Cry37 is also conjugative [110], but neither toxin belongs to the Bin group, Cry23 belongs to the Mtx group and Cry37 is classified in table I-1 as belonging to group 1 together with Cry6 and Cry22. This conjugative acting pair is described in section *I.5.2.2 The conjugative pair Cry23-Cry37* (p. 64).

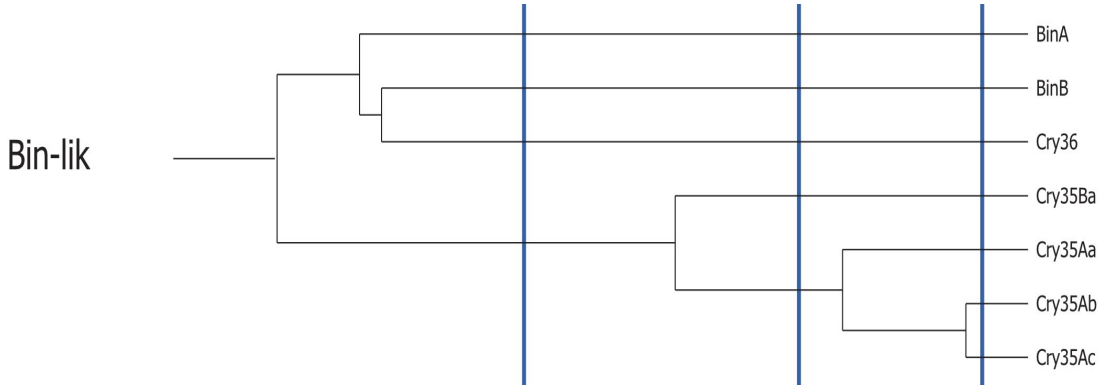


Figure I-14.
The phylogram for the similarity between the BIN-like Cry proteins based on their amino-acid sequences. The blue lines demarcate the four levels of nomenclature rank and are drawn at a sequence homology (from left to right) of 45%. 78% and 95%.
Figure copied on November 5, 2013 from [w1], method described in [30].

11 *Bacillus sphaericus* is renamed to *Lysinibacillus sphaericus*. In this report, the old name, *Bacillus sphaericus*, will be used.

I-5.1.1 *The conjugative pair Cry35-Cry34*

In 2001 a new Bt toxin effective against *Diabrotica virgifera virgifera* was discovered which appeared to consist in activated form out of two proteins of 14 and 44 kDa, respectively. These two proteins were coded for in one operon [118]. In 2002, Ellis *et al*. [38] further described these proteins after isolating Cry pairs from three *B. thuringiensis* strains, naming the discovered Cry pairs Cry34Aa1 / Cry35Aa1 ; Cry34Ab1 / Cry35Ab1 and Cry34Ac1 / Cry35Ac1. The Cry35A proteins were found to be 44 kDa with sequence homology with Bin insecticidal proteins from *B. sphaericus*. The Cry34A proteins were only 14 kDa and no sequence homology with any known bacterial protein was observed [38].
Alone, Cry34 appeared to be slightly toxic to *D. virgifera virgifera*, and Cry35 was not-toxic. Together, these two Cry genes were more toxic than predicted by the additive model [63].
Masson *et al*. [114] have investigated the pore-forming properties of Cry34 and Cry35 in Planar Lipid Bilayers using Cry34Ab1 and Cry35Ab1 and the two toxins combined in the crystals produced by the bacterial strain (PS). They observed that the 44 kDa Cry35 was reduced by proteolysis to a stable 40 kDa protein. In their tests for pore-forming, they both tested the complete 44 kDa Cry35 as the 40 kDa fragment of Cry35 (Cry35-40). The latter appeared to be more effective in pore formation.
At a pH of 5.5, Cry34, Cry35, Cry35-40, the combination of Cry34 and Cry35-40 (Cry34/35-40) and the bacterial strain crystals PS could induce pores in planar lipid bilayers. The conductance was highest for the PS, Cry34/35-40 and Cry35. At the end of the experiment, about ⅔ of the Cry34-pores, ½ of the Cry35-pores and between ⅓ to ½ of the PS and Cry35-40 pores were closed again. Of the Cry34/35-40-pores, only about 10% was closed at the end of the experiment. Not in all experiments, successful pores were formed. The success ratio was highest for PS (about 60%), going down in the order Cry34/35-40 (about 45%), Cry35-40 (about 30%) to less than 20% for Cry35 and Cry34 alone. This suggests that the combination of Cry35 where Cry35 is in its truncated version (or in the natural combination PS) is somewhat better in in preforming, inducing larger pores which remain longer effective [114].
At a pH of 9.0, none of the Cry proteins entered the planar lipid bilayer to a considerable degree. The Cry proteins could, however, destabilize the membrane [114]. Note that the target organism of this conjugative combination, the Coleoptera *D. virgifera virgifera*, has an acid midgut lumen and that at an acid pH of 5.5 the pore forming capacity of these Cry proteins was higher.
The Cry34/Cry35 combination binds to another binding site on the midgut BBMV of *D. virgifera virgifera* than the three other Cry proteins active against Coleoptera, Cry3Aa, Cry6Aa and Cry8Ba [99]. Two of these other Cry proteins, Cry3Aa and Cry8Aa are 3D-Cry proteins, Cry6Aa has only a small sequence homology with Cry37 and Cry22 [w1]. Cry35 binds stronger and more specific to the BBMV if Cry34 is also present [99].

I-5.1.2 *The conjugative pair Cry48-Cry49*

The conjugative pair Cry48-Cry49 is not produced in nature by *Bacillus thuringiensis* but by *B. sphaericus*, at least, up till now none of the *B. thurigiensis* strains isolated and analysed for toxin production was found to produce these proteins. The protein sequence of Cry49Aa shows homology to the *B. thuringiensis* proteins Cry35 and Cry36 (20% and 34%, resp.) and to the *B. sphaericus* Bin proteins BinA and BinB (30%) [76]. Cry48 is a 3-D protein.
Alone, Cry48 and Cry49 are not toxic to the mosquito *Culex quinquefasciatus*. Together they are highly toxic, with probably an optimal ratio for toxicity of 1:1 [76]. The combination is not found toxic for Coleoptera larvae or other tested Diptera larvae, including the related mosquitoes *Aedes aegypti* and *Anopheles gambiae* [77]. There is some indication that Cry49 might synergize Cry4Aa in its toxicity against *C. quinquefasciatus* [77]. This aspect is discussed further in chapter *II-2.1.2 The data combinations of Bt toxins* on p. 96.
Both Cry48 and Cry49 are proteolytic cleaved by proteases in the midgut juice of the target insect. For Cry49, no difference was found between the cleavage by *C. quinquefasciatus*, *A. aegypti*, trypsin, chymotrypsin or proteinase K. Cry48 was cleaved differently by *C. quinquefasciatus* midgut juice, resulting in fragments of 60 kDa and 46 kDa [77]. The difference in proteolytic cleavage between *C. quinquefasciatus* and *A. aegypti* midgut juice cannot explain the difference in toxicity of the Cry48/Cry49 combination, for *A. aegypti* larvae are also insensitive to the *C. quinquefasciatus* midgut juice pretreated Cry48/Cry49 combination [77].

I.5.2 The Mtx group

The Mtx group is a rather diverse group of toxins which are structurally related to the ε-toxin of *Clostridium perfringens*, (pro)aerolysin of *Aeromonas hydrophila* and lectin of *Laetiporus sulphureus*, all being β-pore forming toxins (PFT) [14]. It also contains a set of proteins without known toxicity.
In fig. I-15 the sequence similarity dendogram for the Mtx-group of is given. The group is named after the Mtx proteins from *Bacillus sphaericus*, but only Mtx2 and Mtx3 fall within the Mtx group.

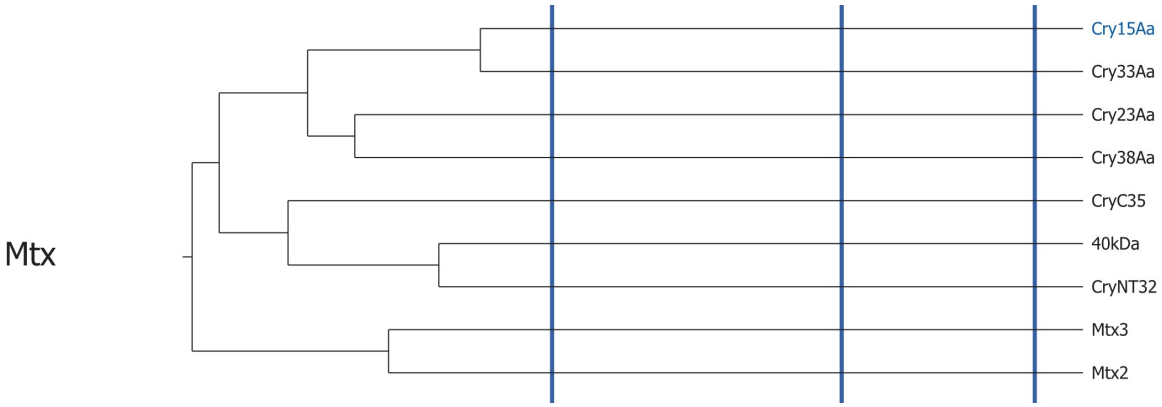


Figure I-15
The phylogram for the similarity based on amino-acid sequences between the Cry proteins and other proteins belonging to the Mtx group. The blue lines demarcate the four levels of nomenclature rank and are drawn at a sequence homology (from left to right) of 45%. 78% and 95%.
Figure copied on November 5, 2013 from [w1], method described in [30].

The third toxin with name Mtx, Mtx1, is an about 100 kDa ADP-ribosyltransferase with a completely different structure, related to the structure of the apoptosis-inducing toxin Piersin-1 from the Lepidoptera *Piersi rapae* (see fig. I-16) [20]. The N-terminal of this protein consists of an appr. 27 kDa ADP-ribosyltransferase, whereas the appr. 70 kDa C-terminal consists of 4 domains with a putative binding function [20]. The similarity between the three Mtx proteins is that they are all three produced in the vegetative stage by *B. sphaericus* and are active against mosquitoes.

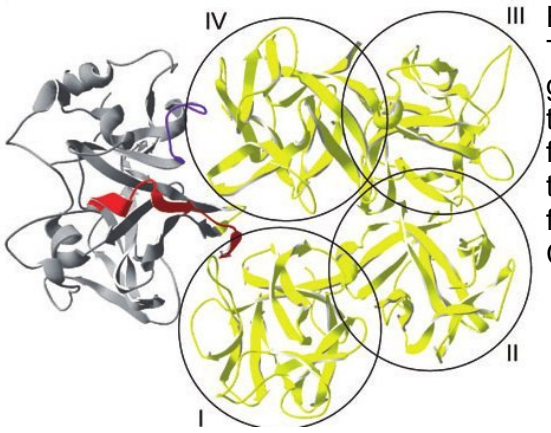


Figure I-16.
The three-dimensional structure of Mtx1. The grey with red part is the appr. 27 kDa N-terminal with a ADP-ribosyltransferase function, the four green domains are the C-terminal domains with a putative binding function.
Copied from [20].

I.5.2.1 *Mtx2 and Mtx3*

Mtx2 and Mtx3 are *B. sphaericus* proteins of 31.8 and 35.8 kDa resp. having 26 and 23% sequence homology with ε-toxin of *Clostridium perfringens*, respectively [186], [14], [104] making their three-dimensional structure somewhat related to the structure of the Cry23-Cry37 conjugative pair.

I.5.2.2 *The conjugative pair Cry23-Cry37*

Cry23 is a 29 kDa protein, classified in the Mtx group. Cry37 is a 13-14 kDa protein with some sequence homology with Cry6 and Cry22 [w1]. The three-dimensional structure of the association of Cry23 and Cry37 is described in [110]. The structure, consisting mainly of β-strands, has some analogy with proaerolysin (see fig. I-17). The structure of Cry23 suggests that it is able to form channels in a membrane. Cry37 putatively facilitate the binding of the Cry37-Cry23 complex to the cell membrane [110].
Toxicity data on the Cry23-Cry37 complex are given in the patent application of Donovan *et al*. [35] and more recently by Contreras *et al*. [28]. They both only tested the combination of Cry23 and Cry37, or at least only published the results of tests with the combined proteins.

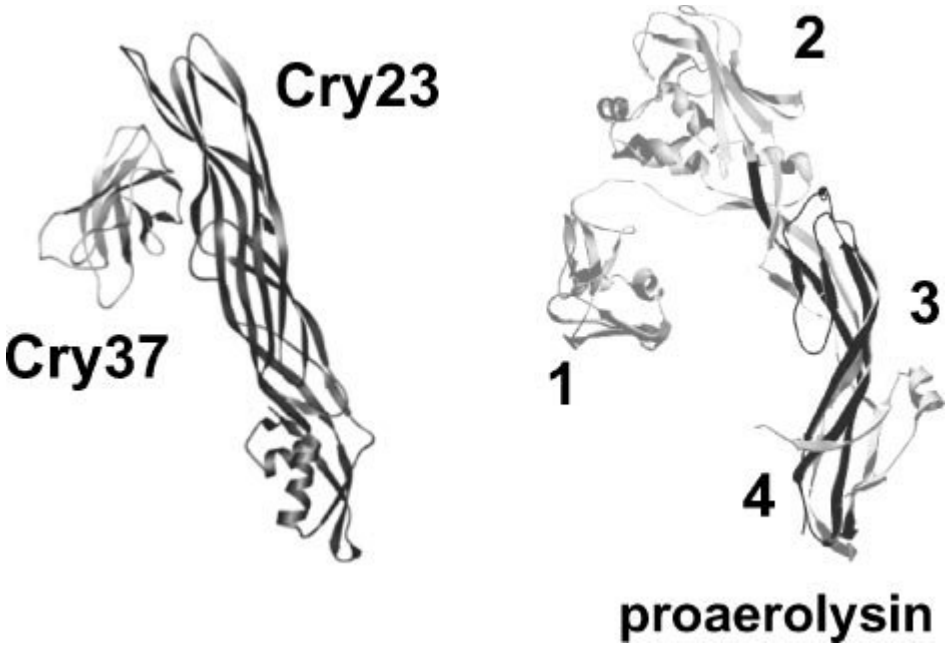


Figure I-17
The three-dimensional structure of the association between Cry23 and Cry37 in comparison to the toxin proaerolysin.
Figure copied from [110].

I-5.2.3 *The parasporins Cry45, Cry46 and Cry64*

Cry46 is a parasporin, active against human cancer cells. For incorporation in plants, it is probably of no interest. Note however, that Cry46 (PS2) shows homology to the crystal structure of the ε-toxin of *Clostridium perfringens* and to aerolysin of *Aeromonas hydrophila* [14] (see fig. I-18), and that Mtx2 has a 26% sequence homology with that ε-toxin [186], [14]. Therefore, Cry46 might also be considered a member of the Mtx group. In [88] Cry46 is listed as related to hydralysin, and according to the hydralysin analysis in [166], hydralysin is related to Mtx2, Cry15, Cry45 and Cry46. The parasporin Cry45 (PS4) also belongs to the Mtx group, just as the parasporin Cry64 (PS5) [88].

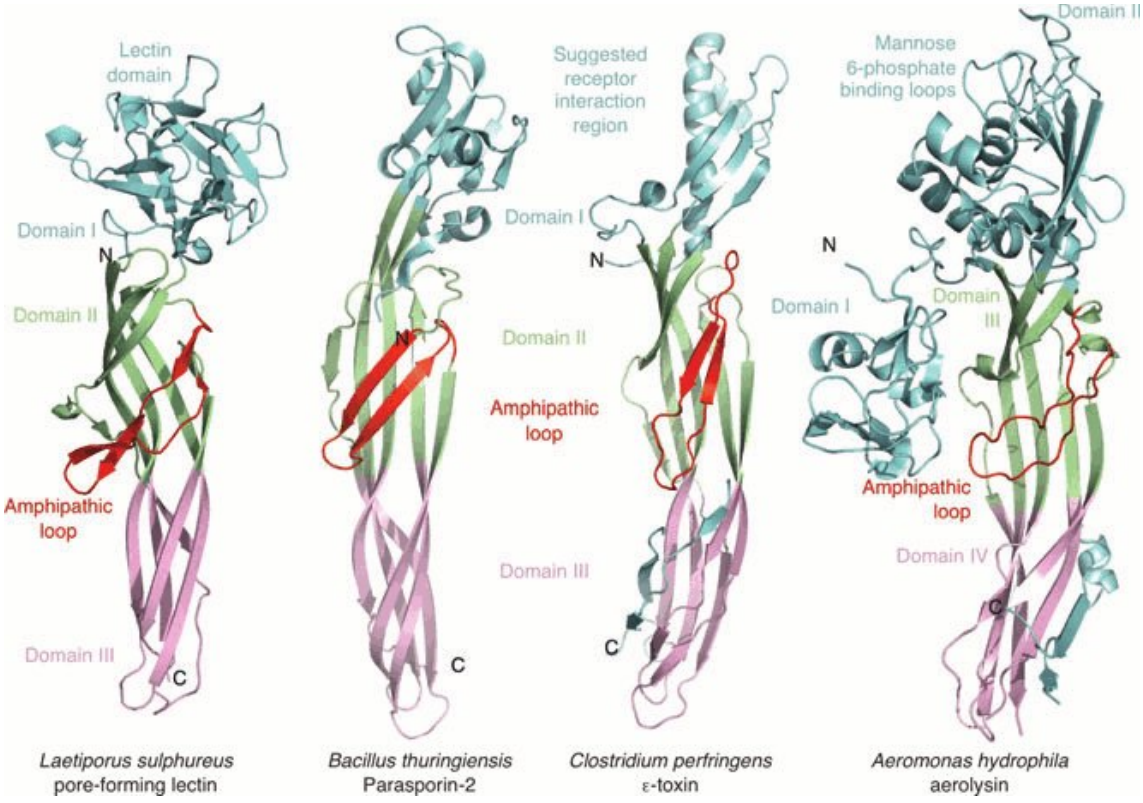


Figure I-18.
The 3-dimensional structure of Cry46 (Parasporin-2) in comparison to some other toxins.
Figure copied from [14]

I.5.2.4 *Other Mtx proteins*

According to de Maagd *et al*. [110], the activity of some other Mtx-class Cry proteins against some, but not all species, can also be enhanced by some other proteins. For instance, the activity of Cry15Aa against *Cydia pomonella*, but not against *Manduca sexta*, is enhanced by a 40 kDa protein (not classified as Cry). The activity of Cry33Aa is influenced by a protein named CryNT32, and CryNT32 has some sequence similarity

with the 40kDa protein promoting the activity of Cry15Aa [110]. The sequence-related 40 kDa protein and CryNt32 have both a small sequence similarity with the sequence-related pair Cry15 and Cry33 (fig. I-15).

Other Mtx-Cry are

- Cry38, with no known activity, found inactive against the coleoptera *Diabrotica virgifera* [w8].
- Cry51, found active against the Lepidoptera *Bombyx mori* [w8].
- Cry60 [88]. Both known variants of this Cry, Cry60Aa and Cry60Ba, are found toxic for *Culex quinquefasciatus*. [174].

Some Mtx proteins have no known toxic activity, such as CryNT32 and the 40 kDa protein included in the Mtx protein dendogram (Figure I-15), a protein indicated as 2D42 [2], [w9] and Cry14-4 [107]. For CryNt32 and 40kDa, it is known that they can sometimes enhance the activity of other proteins, namely of Cry33 and Cry15, respectively.

I-5.3 Some general remarks about in conjugative combination acting Cry

The Cry toxins showing synergistic action with a specific conjugative toxin are a very diverse group. The Cry proteins belonging to the Bin-group have as known complementary toxin a protein from the Bin-group (BinA and BinB), a completely unrelated Cry protein (Cry35 with Cry34), and a Cry protein from the 3D-group (Cry49 with Cry48). For Cry49 it has been suggested that it might be able to synergize at least one other member of the 3D-Cry toxin group, namely Cry4Aa.
Many Cry proteins belonging to the Mtx group are toxic on their own, but their toxicity to some species can be enhanced by a complementary protein, often also belonging to the Mtx group. This complementary protein might either be toxic or not when applied alone. At least one Mtx Cry protein (Cry23) seems to form a conjugative pair with a non-Mtx-group protein, namely with Cry37, a protein slightly sequentially related to Cry6 and Cry 22 (Group 1 in Table I-1, p. 32), although the structure of these three proteins is very different.

Little is known about the mode of action of these conjugative combinations. For the combination Cry34-Cry35 it is suggested that the combination forms more effective pores than each of the single Cry toxins. For the combination Cry23-Cry37, it is suggested that the, for toxicity essential, three-dimensional structure is only obtained by forming a dimeric complex with a similar structure as proaerolysin, the protoxin of aerolysin. For the combination Cry48-Cry49, nothing is known about their mode of conjugative action, a regrettable lack in knowledge since it concerns a conjugative stimulation of a Cry toxin belonging to the largest Cry group, the 3D-Cry proteins.

I-5.4 The unrelated Cry toxins

Some Cry proteins cannot be grouped obviously based on their amino-acid sequence. These Cry proteins are:

- Cry6, Cry22 and Cry37 which are slightly related based on sequence homology, but have a completely different structure. Cry37 forms a conjugative pair with the Mtx Cry toxin Cry23 and is described in the section ***I.5.2.2 The conjugative pair Cry23-Cry37*** (p. 64).
- Cry 34 which has no sequence homologies to any known bacterial toxin. Cry34 forms a conjugative pair with Cry35 and is described in the section ***I-5.1.1 The conjugative pair Cry35-Cry34*** (p. 61).
- Cry55, active against Nematoda and Coleoptera
- Cry61, Cry71 and Cry72 without any available information.

I-5.4.1 *Cry6*

Cry6A, a toxin of approx. 54 kDa, is active against Nematoda and Coleoptera. Research shows that it does not share its binding site to the *Diabrotica virgifera virgifera* LeConte BBMV with the conjugative pair Cry34-Cry35 [99].
In its toxicity to the nematode *Meloidogyne incognita* , Cry6Aa showed synergism with Cry55Aa, increasing the toxicity of the combination with a factor 5 [130].

I-5.4.2 *Cry22*

Cry22 has an unique 6-domain structure, with the domains arranged in a row (see fig. I-19) [153]. According to [w2] Cry22Aa1 consists of about three times as many animo-acids as Cry22Aa2, Cry22Aa3 and Cry22Ab (about 2170 versus about 720).

Figure I-19.
The three dimensional structure of Cry22.
Figure copied from [153].

I-5.4.3 *Cry55*

In its toxicity to the nematode *Meloidogyne incognita* , Cry55Aa showed synergism with Cry6Aa, increasing the toxicity of the combination with a factor 5 [130].
In some natural *B. thuringiensis* strains, the genes for Cry55 and Cry6 are situated together on a plasmid [58].
No sequence homology of Cry55 with other proteins is described in literature. A BLAST search [w10] showed Cry55 to have a 26% sequence homology to a yet unnamed protein of the nematicidal Bt strain DB27 (CDN39471.1), where its gene is located on a plasmid [70], [w3].

I-5.5 Cyt toxins

The Cyt toxins are a smaller group of toxins with crystal forming protoxins. This group was given a distinct name because the Cyt toxins possess a general cytolytic activity in vitro. They can cytolyse insect cell lines and also various mammalian cells and erythrocytes (Haemolysis) [187]. In vivo, they operate selectively against (dipteran) insects [110]. In their revision of the nomenclature of crystal *Bacillus thuringiensis* pesticidal proteins, Crickmore *et al*. [30] have retained their distinctive name.

I-5.5.1 *The structure of Cyt toxins*

Cyt toxins are about 25 kDa proteins. The three dimensional structure of the activated toxin consists of only one domain with both α-helices and β-strands. In the protein sequence, the α-helices and β-strands are mixed (β0[12] - β1 - α1- (β2- β3) [13]- α2 - β4 – α3- α4[14]- α5- β5 - β6 - β7 - α6 - β8) [26], [27], [100]. In the three dimensional structure, the β-strands are positioned in the center, flanked on both sites with α-helices, α1 and α2 on one site and α3 - α6 on the other site, see fig. I-20 for the three-dimensional structures. The three dimensional structure of Cyt1Aa with its extra β-sheets hairpin (β2- β3) strongly resembles the structure of volvatoxin A2 (VVA2), a fungal PFT from *Volvariella volvacea*, although Cyt1Aa and VVA2 have only 20% homology between their amino acid sequences [27], [102]. Some authors distinguish two separate domains in this structure, the N-terminal domain, rich in helix structures, and the C-terminal domain with the β-strands β5, β6 and β7 [148], others see it as only one domain. The α-helix rich N-terminal domain is assumed to be responsible for oligomerization and the three β-strands in the C-terminal domain for pore formation [148].
This toxin structure of one large domain (or two smaller domains) is the result of proteolytic cleavage of the Cyt protoxin, a dimer of the toxin with small N- and C-terminal fragments which are cleaved at activation. After activation, Cyt2Aa toxins can easily form dimers again, but Cyt1Aa, lacking the β0 strain and Cyt2Ba with only part of the β0 strain, do not dimerize spontaneously, leading to the conclusion that the β0 is probably essential for dimer forming [27].

12 Strain β0 is cleaved out in activation of Cyt1Aa [27], partly cleaved out in activation of Cyt2Ba, loosing its β-strain configuration [26], but not in activation of Cry2Aa [27].
13 The hairpin connecting β2-β3 is specific for Cyt1Aa [27].
14 Short helix α4 in Cyt1Aa [27], but not in Cyt2Ba [26].

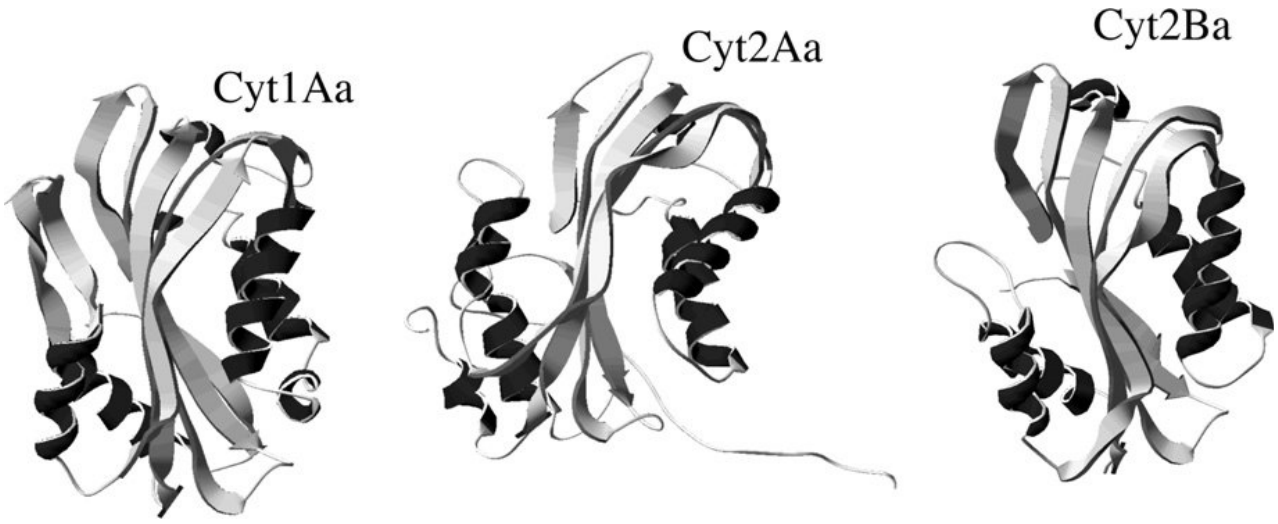


Figure I-20.
The three-dimensional structure of activated Cyt toxins.
Figure copied from [173]

I-5.5.2 *Mode of action of Cyt toxins*

Two models for the mode of action of Cyt toxins are proposed, the pore model and the detergent model. These models are schematically represented in fig. I-21.

I-5.5.2.1 *Pore model*

According to the pore model, the Cyt toxins aggregate in oligomeres. These oligomers are supposed to form beta-barrel pores with the beta sheets formed by β5, β6 and β7 transecting the membrane [100], [139]. These pores are cation selective channels [86]. The other elements of the Cyt toxin are spread out in an umbrella like fashion on the membrane surface, the beta-barrel being the handle of the umbrella [18].
It appears that even if the extra-membrane parts of the Cyt toxin are proteolytical cleaved, the pore function remains intact [37].
Some Cyt toxins with mutations in the N-terminal domain are not toxic, and when mixed with the wild type Cyt toxin, decrease the toxicity of the wild type considerable. This so-called dominant-negative effect is attributed to the formation of hetero-oligomers between mutant and wild type which are unable to form effective pores [148].

I-5.5.2.2 *The detergent model*

Butko [18] formulated an alternative for the pore forming model, the detergent model

[18], [111]. In that model, the Cyt toxin aggregates on the membrane and causes large non-specific defects in the membrane lipid structure. This model is a variant to the "carpet model" describing the effect of anti-microbial peptides [163]. It is speculated that the mechanism of Cyt toxicity might be the pore model at low Cyt concentration and the detergent model at higher Cyt levels [18].

I-5.5.2.3 *Oligomerization*

The α-helices are assumed to be active in the oligomerization, and in the specificity of the Cyt toxin [142]. It is assumed that the oligomerization occurs before the membrane insertion, at least for Cyt2Aa [140].

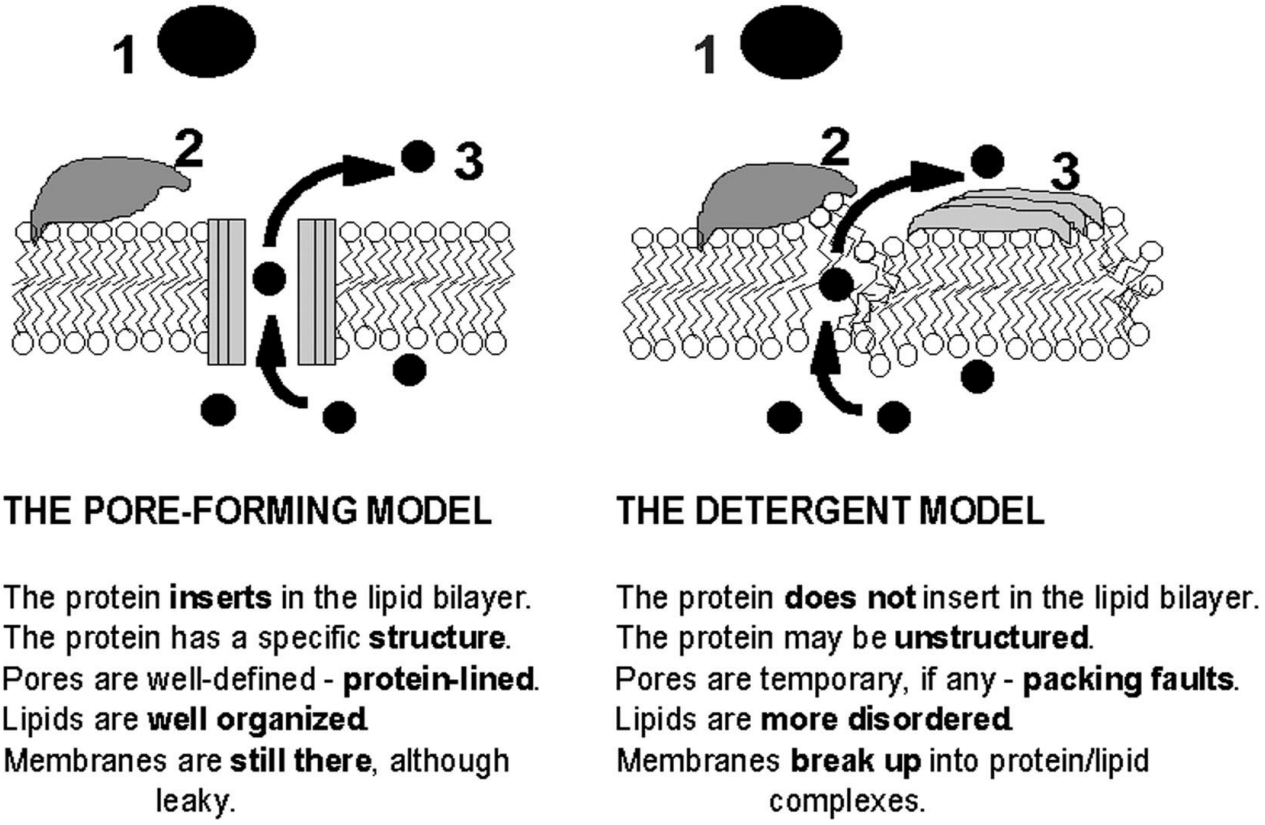


Figure I-21
The two models for the mechanism of the Cyt toxin toxicity. Left the pore forming model and right the detergent-mode model. The toxin molecule (1) approaches the membrane and binds to the membrane lipids, depicted as open circles with a zigzag tail (2). Then, in the pore forming model, the toxin molecules aggregate in a well defined structure and form a pore through the membrane. In the detergent model, the toxin molecules spread out over the membrane surface, fragmenting the membrane in toxin-lipid complexes (3). Intra- and extracellular molecules leak through the membrane, either through the well defined pores or through the faults in the lipid structure of the membrane.
Figure copied from [18]

I-5.5.3 *Interaction with Cry toxins*

Cyt toxins often synergize with Cry toxins, especially with Cry4 and Cry11 against Diptera (mosquitoes) larvae (e.g. [131], [185], [199], [207]), but Cyt toxins are also observed to synergize with Cry3Aa against larvae of a Coleoptera insect [40]. Between the Cry1A-group and Cyt1Aa no synergism is observed against larvae of some Lepidoptera species [116] and sometimes even antagonism, as between Cyt1Aa and Cry1Ac in cells and larvae of *Trichoplusia ni* [145].
It is assumed that the synergism between Cyt and Cry toxins is due to the Cyt protein binding to the cell membrane and there forming a receptor for the Cry toxin [131]. This receptor could, like cadherin, promote the oligomerization and pre-pore formation of the Cry toxin [132]. The antagonism between Cyt1Aa and Cry1Ac in *T. ni* also fits this hypothesis, since Cyt1Aa has no effect on *T. ni* , so it might be that Cyt1Aa does not bind to *T. ni* cells [145]. In that case, Cyt1Aa might compete with the cell membrane receptors for Cry1Ac, and thus decrease the amount of Cry1Ac bound to *T. ni* cell receptors.

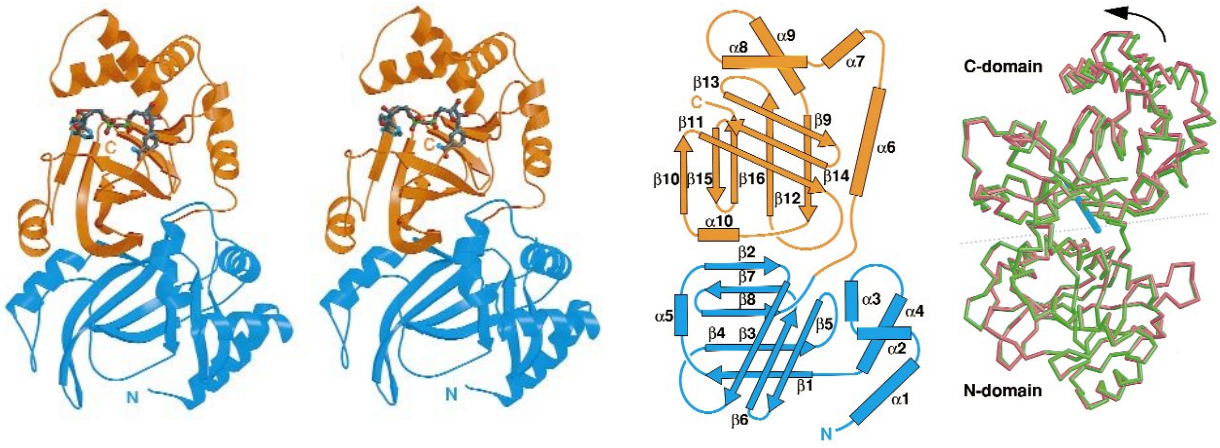
I-5.5.4 *Remarks on Cyt mode of action*

Experiments to assess the mode of action of Cyt and Cry toxins are performed in artificial membranes (Planar Lipid Bilayers), isolated membranes or at best cell lines, but not in intact target organisms. Given the experimental complexity of tests in target organisms on effects of membrane-toxin interactions, it cannot be expected that such experiments are performed. However, for Cyt toxins, that might pose a problem because activated Cyt proteins act cytolytic against all kinds of cells which are insensitive to Cyt proteins in their natural setting in the organism. Therefore, there is some important difference between the activity of Cyt toxins in vivo and in vitro. Thus, it might be that the observed mechanisms in vitro do not occur in vivo.

I-5.6 VIP proteins

Some *Bacillus thuringiensis* can also produce toxins during vegetative growth. These toxins are named Vegetative Insecticidal Proteins, or VIPs. The nomenclature of the Vip proteins is just as that of the Cry proteins, based on the sequence homology. The Vip proteins are divided in 3 main groups[15]. The Vip amino-acid sequences have no homology to the other Bt toxin amino-acid sequences [167]. The Vip1 and Vip2 groups form together a conjugative toxin pair [167]. The Vip3 group proteins do not need a conjugative protein for expressing toxicity.

I-5.6.1 *Vip1-Vip2 conjugative toxins*



a b c

Figure I-22.
The three dimensional structure of activated Vip2Aa. (a): The general folding in 2 domains. Blue: The N-domain; Orange: C-domain. The bound NAD with black bonds, nitrogen blue, oxygen red and phosphorus green. (b) Schematic folding pattern showing the homology between the domains and the secondary structure nomenclature. (c) Superposition of the open state of Vip2 (green) and the closed state (red). In the closed state the NAD binding cleft is closed.
Figures copied from [60]

In the combination Vip1-Vip2 the Vip1 protein binds to the cell membrane (according to [167] as a 100 kDa multimer), enabling the Vip2 protein to enter the cytoplasm. Vip1Ac is about 66kDa [97] and Vip2Ac about 45 kDa [97], Vip2Aa about 52 kDa [167]. Vip2 is an enzymatic ADP-ribosyltransferase, which transfers an ADP-ribose group to a target protein, releasing nicotinamide [167] (see fig. I-23). This enzymatic process is necessary in cell processes, like DNA replication and repair, but can also be used for malignant transformations and apoptosis [w7]. The three-dimensional structure of Vip2Aa, a toxin from *Bacillus cereus*, is determined by Han *et al*. [60]. This structure consists of two α/β domains, called the N- and the C-domain, with strong structural resemblance, although the sequence homology is only about 22% (see fig I-22). The C-domain has a cleft where NAD can bind, and the N-domain can become a Vip1 interaction component [60]. The folding also resembles that of some classical conjugative toxins, like the *Clostridium*

15 Vip protein Vip4Aa1 is given a separate class. The sequence of this protein is relatively close to the Vip1 sequences.

botulinum toxin C2 and the *C. perfrigens* toxin iota [167].
Vip1 has sequence homology in the order of 30% with the binding components of some other conjugative toxins, like C2-II of *Clostridium botulinum* toxin C2, Ib of the *C. perfringens* toxin iota, and PA of the *Bacillus anthracis* toxin antrax [167]. Vip1Ac oligomers are found to form slightly anion selective channels in planar lipid bilayers [97]. The homologous toxins PA and C2-II form oligomers in target cell membranes and planar lipid bilayers. These oligomers are probably heptamers [11], [97], forming a 14-stranded-β-barrel. The channels formed by Vip1Ac have a conductance of 350 pS. At high salinity, also a conductance of 700 pS is measured, probably indicating the formation of two neighbouring Vip1-channels [97]. These channels putatively form a gateway for the conjugative corresponding Vip2 toxin to enter the cytoplasma where it targets the cytoskeleton by ADP-ribosylation of actin [97].
The Vip1/Vip2 combinations can act selectively against Coleoptera species [60] and Hemiptera [154].

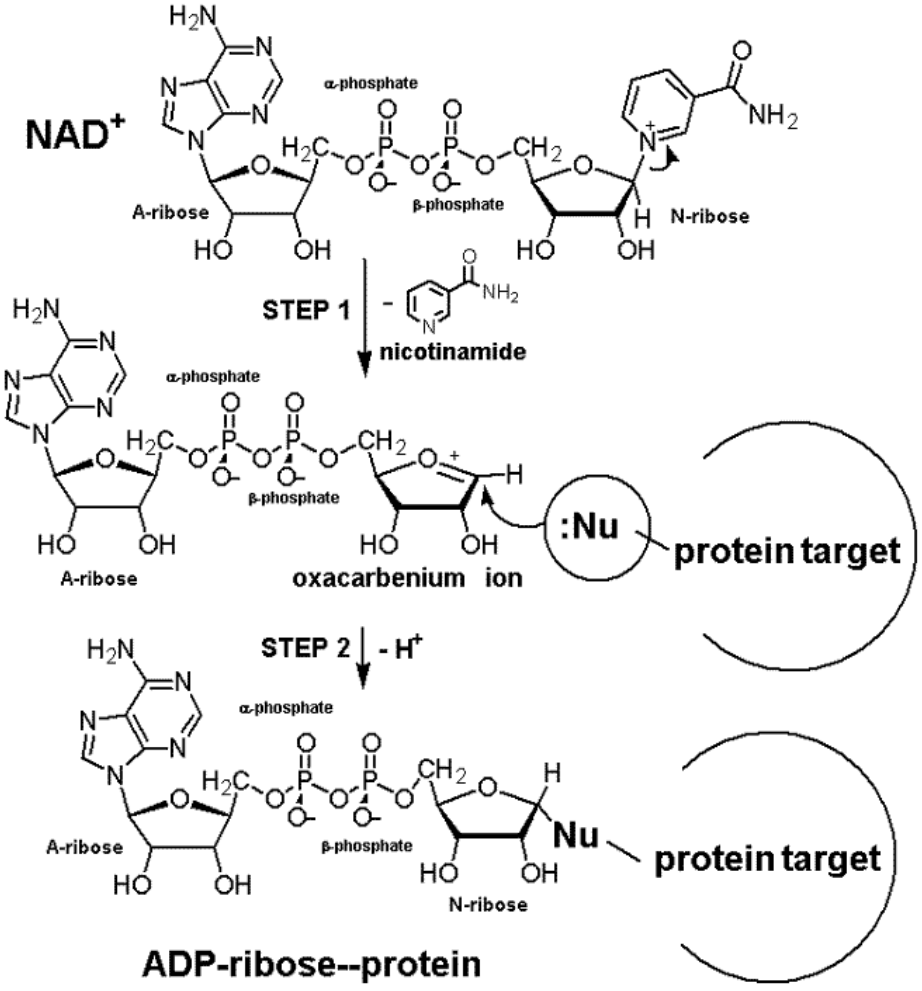


Figure I-23.
Schematic reaction mechanism in ADP-ribosyltransferase
Figure copied from [w7].

I-5.6.2 *Vip3 toxins*

Vip3 proteins act without the need for a conjugative protein. Vip proteins are secreted by the producing bacterium as a protoxin of about 88 kDa for Vip3A (91 kBa for VipBa [144]) which is proteolytically activated by its host gut juice to an about 62 kDa active toxin. In vitro the protoxin can also be proteolytically cleaved by trypsin [95]. The midgut juice of *Ostrinia nubilalis* , a Lepidoptera species not sensitive to Vip3A, also cleaves the protoxin to an active functional toxin, so the insensitivity of *O. nubilalis* is not due to a deviating composition of proteases in the midgut [217].
In the activation of an inactive protoxin to an active toxin, the mode of action of Vip3A resembles the mode of action of Cry1A proteins. However, activated Vip3A did not bind to an Cry1Ab binding APN protein isolated from *Manduca sexta* BBMV[95]. Neither did Vip3A bind to a cadherin fragment which strongly binds Cry1Ab. In *Heliothis virescens* and *Helicoverpa zea* Vip3A binds on the brush border membrane to another receptor than Cry1Ac [95], and Cry2Ae binds to still another binding site in *H. zea* [52]. On BBMV of *Spodoptera frugiperda*, Vip3Aa and Vip3Af compete for the same binding site, whereas Cry1Ab and Cry1Fa use another binding site for which these Cry1 toxins compete [162]. In *Agrotis segetum* BBMV Vip3Aa16 binds to a 65 kDa putative receptor whereas Cry1Ac binds to a 120 kDa receptor [61].
Activated Vip3A forms pores in planar lipid bilayers with a conductivity of about 310 pS under symmetrical conditions. These pores are predominately in an open state. Under similar conditions, the Cry1Ab induced pores show more conductance states, the main conductance state being about 730 pS [94]. The three-dimensional structure of Vip3A is unknown, but the amino-acid sequence does not indicate a three domain structure or a putative α-helix bundle able to form an α-pore [94].
In a test with Cry1Ia10 and Vip3Aa in four different *Spodoptera* species (*S. albula*, *S. erdania*, *S. frugiperda*, *S. latifascia*), for 3 out of 4 species the toxins seemed to act not-synergistic or at most very weakly synergistic. Only for *S. eridania* an antagonistic effect was observed, leaving open the possibility that Cry1Ia10 and Vip3Aa share the receptor in this species [12].
It can be concluded that the Vip3 and Cry1 proteins use different membrane receptors and form a different kind of pores. Therefore, it is not to be expected that resistance against Cry1 toxins will be coupled with resistance to Vip3 toxins.
Vip3 acts selectively against Lepidoptera. For some Lepidoptera, it is investigated whether resistance against a Cry1 toxin influences the sensitivity to Vip3 toxins. No such cross-resistance for Vip3A was found for *Trichoplusia ni* resistant to Cry1Ac [39]. Neither showed *Heliothis virescens* resistant for Vip3A any resistance to Cry1Ab or Cry1Ac [135] or *H. virescens* resistant to Cry1Ac any resistance to Vip3A [74].
The histopathology of Vip3Aa16 infected larvae of *A. segetum* shows vacuolization of the cytoplasm, brush border membrane lysis, vesicle formation in the goblet cells and disintegration of the apical membrane [61].

I-6 Can the mode of action tell something about syn- and antagonism?

The route of the toxin functioning of the 3D-Cry proteins can be partitioned in several successive phases, which can be classified as “exposure”, “uptake”, “chemical modification and internal distribution” and “biological effects”.

A. EXPOSURE

1. The production of the Cry protein by the *Bacillus thuringiensis* bacteria, or by the GM plants modified to produce Bt proteins.

B. UPTAKE

2. The entrance of the protoxin into the insect. The models don't discuss this part, and it will differ between different insect species and modes of application of the toxicant.
3. The transport to the midgut area. This part is not well described. Part of the solubilization of the protoxin crystals may occur in this phase.
4. The passing of the peritrophic membrane. In most models this step is not mentioned. Some indication exists that some (pro)toxins can be glued to this membrane [62]. Note that bacteria are too large to pass this membrane, thus the production of the toxicants may be a way to pass and then destroy that membrane so that the insect cell content becomes available for *Bacillus thuringiensis*.

C. CHEMICAL MODIFICATION AND INTERNAL DISTRIBUTION

5. The proteolytic cleavage of the N- and C-terminal from the protoxin. Note that the Cry protein produced by a GM plant (preactivated toxin) might be shorter than the Bt-produced protoxin, but after proteolytic cleavage the remaining amino-acid sequence is the same. The conformation of the toxin, stripped of these two terminal fragments, might change.
6. The binding of the toxin to the cell membrane. This part of the toxic process is extensively described, but far from completely understood. The binding might occur in several steps. For this step, several distinct, but not always contradictory, models are developed:
   a. binding promotes further cleavage of the toxin
   b. binding enables special conformational changes
   c. binding starts a signalling pathway in the cell
7. After cleavage of part of the toxin and/or conformational changes, the monomeric toxins form oligomers. These oligomers might be formed of combinations of the same type of monomers (homo-oligomers) or by combinations of different types of (3D-Cry) monomers (hetero-oligomers). Some models don't assume this step, and in some other models, oligomerization only occurs after penetration of the cell membrane.

8. The toxin-oligomers enter the cell membrane (or the toxin monomers enter the cell membrane and there form oligomers). These oligomeres form pores in the cell membrane. The signalling pathway model does not assume this step.

D. BIOLOGICAL EFFECTS

9. As result of the signalling pathway or because of damages by the pores, the membrane cytoskeleton disfunction and the cell starts blebbing. This step is not universally recognized, but mainly part of the signalling-pathway model.
10. If the pores formed by the toxin-oligomeres are open, the cells swell osmotic, leading to cell lysis. The effectiveness of the pores depends, among others on the size (diameter, conductivity) of the pores and on the frequency and the duration of its open phase.
11. As result of massive cell death, the insect stops eating and dies after some days, either from hunger or because of increased susceptibility to infections.

Note that although these 11 phases are formulated for 3D-Cry proteins, most of these phases are equally relevant to the other Cry, Cyt and Vip proteins. The phases 6, 7, 8 and 9 (binding, oligomerization, pore formation and blebbing) might have to be slightly adapted for these proteins.

For each of these 11 phases, the potential interactions between different Bt proteins will be discussed shortly without pretence to completeness. Some of these potential interactions will be studied in more depth in the next chapters.

Phase 1: The synthesis of the Cry proteins might influence each other. For instance, the genes for two Cry toxins might both be encoded in a single operon. The interactions in the production of the Cry proteins will not be discussed further in this report.

Phase 2: Most Cry proteins enter the insect with its food. Different Cry proteins can be present in the same food source. The ratio of these Cry proteins may depend on the specific properties of the food source. The different Cry proteins might be present as separate molecules, heteromeres or in a crystal form.

Phase 3: After being eaten and before entering the midgut, the crystal Cry proteins might be solubilized, making them vulnerable for proteolysis. This process may be pH dependent or depend on other conditions. The solved Cry proteins might, in turn, alter the conditions and thereby alter the solubilization of other Cry proteins.

Phase 4: The peritrophic membrane protects the midgut epithelium against mechanical damage from sharp food particles. Some Cry proteins might bind to this membrane. The binding of one of the Cry proteins might either decrease the probability for binding of the other Cry proteins by occupying binding sites, or form a new binding site for the other Cry proteins.

Phase 5: The proteolytic cleavage of the N- and C-terminal fragments of the Cry protoxin

might either occur before or after passing the peritrophic membrane. This process is enzyme catalysed. If both Cry protoxins depend on the same enzymes, the rate of cleavage might depend on the total Cry protoxin concentration, and not just for each Cry protoxin on its own concentration.

Phase 6: This step is one of the most interesting ones. If both Cry toxins bind to the same receptor, they will decrease the number of available binding sites for each other. If they bind to different binding sites, their binding rates may be independent. For some toxins (the Cyt toxins) it is suggested that their binding to the cell membrane forms a new binding site for other Cry toxins. If the Cry toxin undergoes some conformational change when bound to a membrane receptor, Cry toxins might influence each others conformational change if the distance between the binding sites of the different Cry toxins is small.
In addition, if the toxins bind to different receptors, and if the signalling pathway model is correct, this might lead to the starting of two different signalling pathways, with potential interactions between these pathways.

Phase 7: The presence of several different Cry toxins might lead to the formation of hetero-oligomers (oligomers consisting of different monomer types). It is hypothesized that the conductivity of the hetero-oligomer pores can be much higher or much lower than the average of the conductivity of the pores of the corresponding homo-oligomers. Note that this phase is deemed to be irrelevant in the signalling pathway model.

Phase 8: The ability of the toxin to enter the cell membrane, especially if this membrane penetration is only occurring after the oligomers are formed, may depend on the form of the molecule, and therefore on whether an homo- or hetero-oligomer tries to enter the cell membrane.
Note that this phase does not occur in the signalling pathway model.

Phase 9: The blebbing of the cell membrane (forming of weak spots in the membrane where the membrane starts bulbing) is an essential step in the signalling pathway model. It is the result of membrane cytoskeleton disfunctioning. It could also be envisaged to occur resulting from pores through that membrane cytoskeleton. The process is not well understood. Different signalling pathways might lead to more or less effect on the cytoskeleton, as also the occurrence of heteromeric pores might damage the cytoskeleton more or less than the corresponding homo-oligomers.

Phase 10: The Cry toxin pores are not continually opened. The period (frequency and duration) of the open phase may depend on the oligomer properties, for instance whether it is a homo-oligomer or a hetero-oligomer. It is sometimes assumed that pores occur in clusters, and there is some indication that the opening and closing of pores in a cluster is synchronized. In this way, an effective hetero-oligomer might also influence the overall permeability of the neighbouring homo-oligomer pores.

Phase 11: The period between the massive cell death of midgut epithelial cells and the death of the individual will depend on other environmental conditions. The Cry toxins

have by then performed their function. Whether the midgut cell death was caused by one Cry toxin or by a cocktail of Cry toxins is probably not relevant.

Interactions between different Cry, Cyt and Vip toxins are expected to occur predominantly in phase 6 (competition for binding sites, opening binding sites for another toxin by changing the conformation of the binding site due to binding, …), phase 7 (forming of homo- or hetero-oligomeres with consequences for the pore forming capacity of the oligomer) and phase 8 (the ability to enter the cell membrane and form effective pores may depend on the oligomers (homo- or hetero-) or on the presence of neighbouring other pores). The preceding chapters have focussed on the mode of action in these phases. In phase 1 (the production of the toxins) interaction might also occur, but that interaction is outside the scope of this report.

The above evaluation indicates whether and in which phases of the action of a Bt toxin, active interaction between different Bt toxins, either positive, neutral of negative, might occur. Unfortunately, in most cases the available data do not allow to predict whether a potential mode of synergistic interaction will actually occur.

In the second part of this report (p. 81) experimentally observed effects of combinations of Bt toxins are discussed. The experimental observation of interaction (positive, negative or neutral) is in most cases not combined with an experimentally based mechanistic explanation for that interaction.

I-7 Cry genes in GMO plants in Europe

Several genetically modified (GMO) plants are allowed in the EU. These plants are listed in the EU register of authorised GMO [w6]. This list is summarized in Appendix C (p. 176).

The crops in which genes from *Bacillus thuringiensis* have been introduced:

- cotton
- maize
- soybean

The Bt genes incorporated in these plants are:

- Cry1Ab
- Cry1Ac
- Cry1A.105 (hybrid of Cry1Ab (dom I), Cry1Ac (Dom II) and Cry1F (Dom III)
- Cry1F
- Cry2Ab (Cry2Ab2)
- Cry2Ae
- Cry3A (eCry3.1Ab and mCry3A)
- Cry3Bb1
- Cry34Ab1
- Cry35Ab1
- Vip3Aa2

Part II:

The effect of Bt toxins on the toxicity of other Bt toxins

II-1 Toxicity of single Bt toxins

The toxicity of individual Bt proteins has been extensively investigated. In 2009, Frankenhuyzen [43] has summarized all available data on the toxicity of Bt proteins included in the nomenclature list of Bt toxins [w1], and in the database on the website of the Natural Resources Canada, Canadian Forest Service [w8] (almost) all single toxicity data are compiled and made publicly available[16]. The database contains 1938 single Cry, Cyt or Vip toxicity data distributed over 184 different species. As example, searching this database for Cry1Ab (using Cry01Ab* in the search field), 191 records are found. Six of these records are on the toxicity of other Cry toxins to Cry1Ab resistant/susceptible strains, the other 185 records are toxicity data for Cry1Ab on 54 different species (see Appendix D, p. 177). Cry1Ab is one of the best studied Bt toxins. It is assumed to act selectively against Lepidoptera species, and 51 of the 54 species for which toxicity data are listed are Lepidoptera, 48 of these Lepidoptera species being target species. The non-lepidoptera in this list are a Diptera (*Aedes aegypti*), a Coleoptera (*Chrysoperla carnea*) and a Hemiptera (*Acyrthosiphon pisum*). Of the 187 data, 25 are only qualitative ((highly) active/inactive), the other data records contain an ED50 value (or ED50 larger or smaller than some value)[17]. These ED50 values are, however, not easily comparable. They can be expressed in

1. ng/larva with forced feeding of the larva,
2. ng/larva with the assumption of complete digestion of the food item with the toxin,
3. ng/cm^2 with the toxin applied to the food surface in the given quantity,
4. ng/cm^2 with the toxin applied to the food (leaf) surface by dipping the leaf in a toxin solution leading supposedly to the given concentration per cm^2 leaf surface,
5. cells/cm^2 with the cells being cells from toxin producing bacteria, applied to the food surface in the given quantity.
6. ng/ml with the toxin applied to the food (leaf) surface by dipping the leaf in a toxin solution with the given toxin concentration,
7. ng/ml with the toxin mixed through the food
8. ng/ml with toxin solved in water, larva living in that water.

The observed effect can be

1. Mortality
2. Growth
3. Growth inhibition
4. Weight
5. Frass (solid excreta) production

The large number of units for the toxic dose makes it difficult to compare the results of different experiments. And for some of the dosing methods, comparison of experiments

16 Per 2014-01-27, this database was last updated on 2010-12-16. Some publications from the first half of 2010 are not included (for instance [47], [164]), and some earlier publications are missing (for instance [75]), or only part of the data are included (for instance [115]).

17 In the complete database, 750 of the 1938 unique records are only qualitative data.

using the same dosing method is also difficult. The actual uptake for doses expressed in ng/cm^2 of a leaf surface will depend on the leaf thickness and dry-weight per mg of leaf. And if the dose is applied with the leaf dip method, and expressed in ng/ml in the water in which the leaf is dipped, the actual dose will in addition also depend on the water quantity clinging to the dipped-in leaf. If the dose is expressed in the number of cells producing the toxin, the actual dose will depend on the expression of the toxin-genes, and the condition of the toxin-producing cells.

For risk assessment of crop with Bt genes, the effect on so-called non-target organism (NTO) is considered an important criterion. Whether or not a species is classified as a non-target organism may depend on the objective of the toxin used, so being a target organism or not is not a species property but a property of the species-toxin purpose combination. If data are available on the toxicity of a toxin (combination) for a species, these toxicity data hold whether that species is a target organism or not given the considered application use of that toxin. As a workable general purpose definition of the concept "Non-target organism", in this report a species will be considered a non-target organism if it is not considered a pest organism for any agricultural crop or as medical health problem for human or any human life stock[18]. According to this definition, being an NTO or not does not depend on the specific toxin, nor on the intended toxin use.

The list below shows that the amount of data on non-target species is only limited. For only three species belonging to a different family than the target species data on the toxicity of Cry1Ab are given in the toxicity database [w8]. Scanning that complete database for the 10 non-target species which were used in any of the papers cited in this report (see Appendix B, p. 172), the hymenoptera *Apis mellifera* and *Nasonia vitripennis*, the Lepidoptera *Bombyx mori*, *Cacyreus marshalli* and *Danaus plexippus*, the coleoptera *Coleomegilla maculata* and *Hippodamia convergens*, the diptera *Chironomus tepperi*, the neuroptera *Chrysoperla carnea* and the nematode *Caenorhabditis elegans* only 95 data on toxicity appeared, more than half of them for the silkworm *Bombyx mori* (see table II-1). And of the 30 genera of Ladybirds listed on the Netherlands page of Wikipedia on Ladybirds [w11], only for one genus toxicity data are found in the Cry toxin toxicity database [w8] (the toxicity of Cry2Aa for *Hippodamia convergens,* Cry2Aa was found inactive).

18 The terms "crop" and "life stock" should both be used in their broadest possible way, including e.g. forestry and fish kept in aquaculture.

Table II-1.
The number of toxicity data available in the website of Natural Resources Canada, Canadian Forest Service [w8] on 10 non-target species. The species are selected for being used in some of the references used for this report. For only one of these 10 genera, data were available on other species from that genus[19].

species	number of toxicity data	class or phylum	order	family	popular name
Apis mellifera	2	Insecta	Hymenoptera	Apidae	honeybee
Bombyx mori	49	Insecta	Lepidoptera	Bombycidae	China silkworm
Cacyreus marshalli	12	Insecta	Lepidoptera	Lycaenidae	geranium bronze
Caenorhabditis elegans	9	Nematoda	Rhabditida	Rhabditidae	roundworm C. elegans
Chironomus (tepperi and riparius)	8	Insecta	Diptera	Chironomidae	rice midge and harlequin fly
Chrysoperla carnea	0	Insecta	Neuroptera	Chrysopidae	common green lacewing
Coleomegilla maculata	0	Insecta	Coleoptera	Coccinellidae	twelvespotted ladybird beetle
Danaus plexippus	13	Insecta	Lepidoptera	Nymphalidae	monarch butterfly
Hippodamia convergens	1	Insecta	Coleoptera	Coccinellidae	ladybird beetle
Nasonia vitripennis	1	Insecta	Hymenoptera	Pteromalidae	parasitic wasp

19 *Chironomus riparius*

II-2 The influence of Bt toxins on the toxicity of other Bt toxin

Most *Bacillus thuringiensis* strains, and also most *B. sphaericus* strains, produce a mixture of toxins. Transgenic plants, first developed by incorporating the gene for a single Bt toxin, increasingly are developed to produce more than one Bt toxin. To predict the toxicity of the mixture of Bt proteins, data on the interaction between Bt proteins are collected. This interaction can occur in two separate manners

1. If an organism is exposed to two or more toxins, the effect of each of the toxins in the toxin combination might be enhanced, inhibited or not influenced by the presence of the other toxins.
2. The (former) presence of a toxin in the environment might have led to the selection for resistance to that toxin in (target) organisms. The toxicity of another toxin to these (target) organisms might be influenced by the resistance of these organisms to the first toxin.

The first type of interaction is a matter of combination toxicity, the second of cross-resistance.
Note that the term "interaction" between two proteins is here used for the effect of the presence of both proteins in the the same organism at the same time or successively in the same organism or successively in a parent organism and its progeny. In other words, two proteins together in an organism (and its offspring), either at the same time or successively, are always interacting. That interaction can also be neutral if the proteins do not affect the effectiveness of each others mode of action.

II-2.1 Combination toxicology of Bt toxins

II-2.1.1 *The models*

Several models exist to predict the effect of two or more toxins together from the toxicity data for each of the toxins separately. Two of these models can be considered as null-models, describing the effect of the combination if the toxins do not interact. These models are:

1. The additive model.
   In this model, the toxins are considered to act in the same way on the organism. To correct for different toxicity levels of the separate toxins, for each toxin the amount is expressed in so called Toxic Units (TU), the dose divided by the toxicity parameter for the toxin. A common choice for that toxicity parameter is the LD50 or LC50, the dose or concentration leading to the mortality of 50% of the exposed individuals, or more generally, the ED50 or EC50, the dose or concentration leading to an effect of 50% on the observed toxicity parameter. The additive model predicts that the toxicity parameter, ED50(mixture) of a mixture of

toxins, T1, T2, … Tk in a ratio of r_1:r_2: … : r_k can be expressed as

$$\frac{1}{\mathrm{ED50(mixture)}} = \frac{r_1/R}{\mathrm{ED50(T1)}} + \frac{r_2/R}{\mathrm{ED50(T2)}} + \ldots + \frac{r_k/R}{\mathrm{ED50(T}k\mathrm{)}} \qquad [1]$$

with R=r_1+ r_2+....+r_k.
This additive model is, among others, described by Tabashnik [175].

2. The independent effect model.
   This model assumes that the toxins act completely independently. For the effect parameter mortality this implies that, if the probability that an individual dies of a given dose of T1 is p_1 and of a given dose of T2 is p_2, the probability that an individual surviving the dose of T1 (the expected fraction of individuals surviving that dose of T1 is (1-p_1)) will die of that given dose of T2 is p_2, independent of the dose of T1. The independent effect model is also knowns as the additive response model and as the additive effect model.

Although the theoretical difference between both "neutral-interaction" models is large (the additive model is based on the assumption that the toxins have a similar mode of action, whereas the independent effect model is based on the assumption that the toxins act completely independent), the difference between the resulting dose-response relationships is not very large (see fig. II-1). In most experimental cases, it will not be possible to differentiate between the two models. Note that the main difference is that for intermediate toxic levels, the additive model often predicts a higher toxicity than the independent effect model.

Experiments with toxin mixtures combined with simultaneous similar experiments with the separate toxins are used to evaluate whether the toxins interact, and if they do, whether that interaction is synergistic or antagonistic. In experiments where LD/ED50 values are estimated, the additive model is used in most cases to predict the effect of the toxin mixture. In experiments with toxicity data at only one to at most three doses, the independent effect model is often used, because the data set is too small to estimate ED50. In neither case, the authors made this choice based on the theoretical assumptions underlining these two models.

Using the additive model, the interaction of the toxins is often described by the Synergy Factor (SF), the ratio of the predicted ED50 value (using equation [1]) and the observed ED50 value for the mixture. In the model, a SF of 1 implies no interaction (the additive model is correct), an SF (sufficiently) larger than 1 implies synergy, a SF (sufficiently) smaller than 1 antagonistic action.

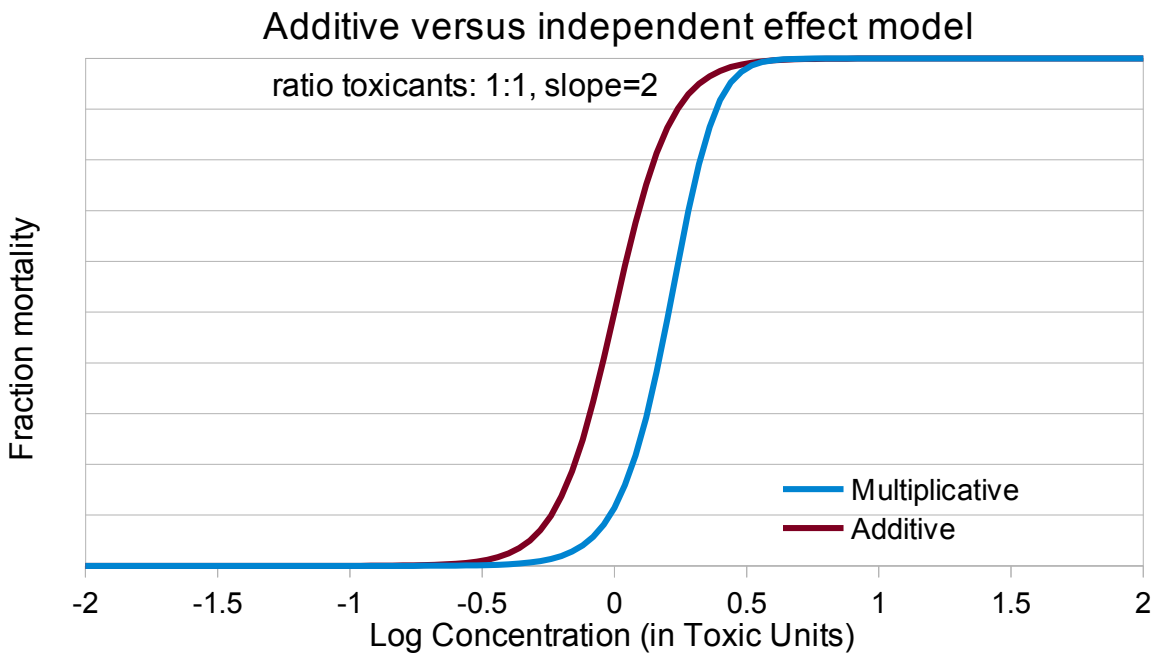


Figure II-1.
Example of the theoretical expected concentration-response relationship for the combined toxicity of two toxins in equal toxicity ratio if the model for the combined activity is additive or independent. The figure is based on a log-logistic concentration-response relationship.

For the independent effect model, the ED50 for the mixture can also be calculated, although that is more complicated and the calculation depends on the dose-response relationship. If the response is mortality, and $f_i(c_{Ti})$ is the probability that an individual survives exposure to a toxin Ti in the concentration c_{Ti}, the expected ED50 for a mixture of toxins with ratio of r_1:r_2: … :r_k with R=r_1+ r_2+....+r_k can be calculated by solving the equation

$$f_1\left(\frac{r_1}{R}*\mathrm{ED50}(\mathrm{mixture})\right)*f_2\left(\frac{r_2}{R}*\mathrm{ED50}(\mathrm{mixture})\right)*...*f_k\left(\frac{r_k}{R}*\mathrm{ED50}(\mathrm{mixture})\right)=0.5 \quad [2]$$

Then, just as with the additive model, the Synergy Factor for the independent effect model, SFm, can be calculated as the ratio of the predicted ED50 value (using equation [2]) and the observed ED50 value for the mixture.
Note that the independent effect model uses less strict assumptions about the interaction (the action of the toxins is completely independent) than the additive model (the dose response relationship for the toxins and the mixture of the toxins is the same if the dose of the toxins (mixture) is expressed in their TU's), but that for the calculation of the synergy factor, less assumptions are necessary for the additive model (only the ED50 have to be estimated) than for the independent effect model (the complete dose-response relationship has to be estimated).

In most cases, an S-shaped curve is assumed for the dose-response relationship, $f_i(c)$, decreasing from 1 to 0. For such functions, the ED50, that is the value of c for which $f_i(c)=0.5$, can be estimated fairly accurately, and this estimate depends only marginally on the exact shape of the function. For function values closer to 0 or 1, the dependency of the estimate of c on the shape of the function increases. And since the exact shape is unknown, the error in the estimate increases even more than indicated by the increased confidence interval (CI) for that estimate. For the increase in the CI as c is estimated for function values closer to 1 or 0 is only due to the decreasing of the slope of the chosen function at increasing distance from the ED50, where the function value is 0.5.
Given these practical considerations, the SF will be calculated using the additive model as null-model.

As argued above, experiments to asses the toxicity of single toxins and their mixtures, will not allow to distinguish between the two null-models, although the basic assumptions of these two models differ fundamentally. For cross-resistance experiments, these two sets of assumptions lead to completely different predictions. Theoretically, two toxins are assumed to interact according to the additive model, if they both have exactly the same mode of action. Therefore, if the target organism is selected to be resistant against the first toxin, it will likely also become resistant against the second toxin, for the mode of action of these toxins is the same. On the other hand, if two toxins interact according to the independent effect model, the action of the first toxin on the target organism is completely independent of the action of the second toxin. Therefore, if the target organisms is selected to be resistant against the first toxin, the susceptibility to the second toxin will probably not be influenced by this selection.
So if no cross-resistance is observed, the toxins probably don't use the same mode of action, and thus do not interact according to the additive model. In that case, the synergy factor could better be estimated using the independent effect model, which would lead to a slightly larger estimated synergy factor (SFm>SF).

II-2.1.2 *The data combinations of Bt toxins*

For data to be useful to assess the synergy between Bt toxins, the data on the individual toxins and on the toxin mixture should be gathered using the same method. In that case, the problem due to differences in toxin application methods, leading to all kind of different units for expressing toxicity, vanishes: the compared experiments use the same units and the SF is dimensionless, being expressed as ratio of two values with the same dimension.

A second important point is that the toxin dose should be expressed in some way in the amount of toxin, not in the number of cells or spores containing that toxin. If the bioassay experiment is performed with cells of (cloned) bacterial strains producing either the first, the second toxin or both together, dosing information on the number of cells does not give information on the amount of toxin dosed, for the expression of the toxin-gene may depend on the configuration of the cell. Only if information is available on the amount of

toxin produced per cell in each of these bacterial strains, these combination toxicity experiments can be used to assess the synergy.

To find such data, the literature was scanned. This scan was helped by the fact that in the field of Bt toxin-research, Tabashnik [175] is considered as the key reference for the additive interaction model. All papers citing [175] were scanned for useful data. That yielded 46 publications giving ED50 values for both the single toxins and their combination and 4 additional papers with sufficient survival data on individual toxins and combinations to classify the combination as to its interaction. These data are summarized in four tables (II-6, II-7, II-8, II-9), one centred around the Cry1 toxins, the second around the Cry4/Cry11 toxin complex, the third giving information on the alleged conjugative couple Cry48-Cry49 and the fourth around the nematocidal toxins. Each toxin combination, with all its available data, is only listed in one of these tables. Unless otherwise indicated, all given SF values are recalculated from the ED50 values given in the papers. These extensive tables are summarised in shorter tables (II-3, II-4, II-5) where the SF factor is classified in 6 groups: Antagonistic, also indicates as negative synergism, with an SF below 0.5, neutral, with an SF between 0.5 and 2; weak synergistic with an SF between 2 and 10; synergistic with an SF between 10 and 50, strong synergistic with an SF between 50 and 250 and extremely synergistic with an SF above 250[20].

The data on the toxin group centred around Cry1 are exclusively on Lepidoptera. These Lepidoptera are all target species. Note that the two Diptera in the Cry1-group table (tables II-3 and II-6), *Culex quinquefasciatus* and *Chironomus tepperi*, only appear in the combination Vip3Aa with Cyt2Aa3. This combination is a bit of an outlier in this table, only added to this table because other combinations of Vip3Aa are with Cry toxins of the Cry1-group. Cyt2A also appears in the table for the Cry4-Cry11 group, so the combination Vip3Aa with Cyt2Aa3 could equally well have been included in the Cry4-Cry11 group table.

The data on the toxin group centred around Cry4 and Cry11 (tables II-4 and II-7) are exclusively on Diptera, all midges within the infraorder of the Culicomorpha (suborder Nematocera). The species for which data are available are almost all target species, only the rice midge *Chironomus tepperi* is a non-target species.
On the combination of Cry49 with Cry48 and Cry4 (tables II-4 and II-8), only data on the Diptera *Culex quinquefasciatus* are available.
On the combination of Cry5, Cry6, Cry21 and Cry55 toxins (tables II-5 and II-9), data are only available on two species, both Nematoda, one target species (*Meloidogyne incognita*) and the other a non-target laboratory favourite (*Caenorhabditis elegans*).

20 If for a toxin combination several data are available for one species and these data do not fall within the same colour group, the following strategy is used: Within an experiment (that is one mixture ratio within one paper, the number of data is reduced to one by the following strategy: If data are available for mortality and some sub-lethal variable, only the value for mortality is used, if the LD50 is given for several exposure periods, the longest exposure period is used. Data from several experiments are combined using the strategy that in case of two data, the geometric mean is used to determine the colour group, and for more than 2 data, the median is used. However, if the data clearly break-up in two separate groups, the species is listed twice with different colours.

The size distribution of the observed SF values is given in table II-2 and figure II-2. Almost half of all tested toxin pairs are not synergistic to the tested species strain, and only 18% of the data indicate (strong) synergism, 53% of these data are observations on resistant strains.

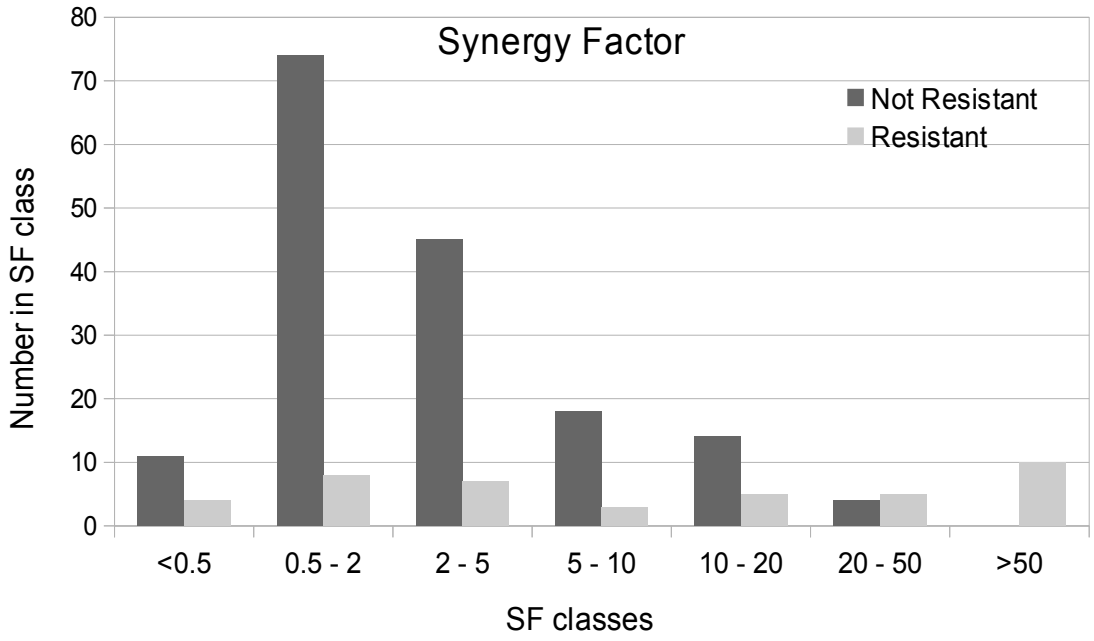


Figure II-2
Size distribution of the 208 collected SF values for species-toxin pair combinations (per paper only one SF for a species-toxin pair, see note table II-2), 167 SF values for non resistant species strains and 42 values for resistant species strains.

Table II-2.
The size distribution of SF values for two-toxin combinations[a]. Per paper, for each species-toxin pair combination, the measured SFs were combined to one SF-value[b]. Between brackets the number of data for resistant strains, and the percent of the data relating to resistant strains (no percent between brackets means no resistant strains in that class).

Toxin classes	number of data	Percent of observations with SF in class				
		SF<2	2≤SF<5	5≤SF<10	10≤SF<50	SF>50
Lepidoptera						
3D-Cry / 3D-Cry	55 (0)	65%	24%	7%	4%	0%
3D-Cry / Cyt	9 (1)	67%	11%	0%	11%	11% (100%)
3D-Cry / Vip3	5 (0)	40%	40%	20%	0%	0%
Lepidoptera & Diptera						
Cyt / Vip3	5 (0)	60%	40%	0%	0%	0%
Diptera						
3D-Cry / 3D-Cry	27 (1)	41%	37% (10%)	11%	11%	0%
3D-Cry / Cyt	37 (15)	22% (38%)	27% (20%)	19% (43%)	24% (44%)	8% (100%)
3D-Cry / Mtx1	12 (6)	67% (38%)	25% (67%)	0%	8% (100%)	0%
3D-Cry / Mtx2	12 (6)	33% (50%)	8%	8%	42% (60%)	8% (100%)
3D-Cry / Bin	14 (4)	36% (20%)	14%	14%	29% (50%)	7% (100%)
Cyt / Bin (=Bs BinA-BinB)	10 (3)	10%	30%	20%	10%	30% (100%)
Mtx1 / Cyt, Cyt+3D-Cry or Bin	7 (3)	43% (100%)	29% (100%)	29%	0%	0%
Mtx2 / Mtx1, Cyt, Cyt+3D-Cry or Bin	8 (3)	50% (50%)	12%	0%	25%	12% (100%)
Nematodes						
All combinations	7 (0)	71%	29%	0%	0%	0%
All species, all combinations	**208 (42)**	**47% (12%)**	**25% (13%)**	**10% (14%)**	**13% (36%)**	**5% (100%)**

[a]: If synergy is only assessed qualitatively, the observation is classified according to the following rules: no syn/anta: class SF<2; syn: class 2≤SF<5; syn with SF>*x* or SF<*x*: size class for *x*.

[b]: If several SF values for one species are given in a paper, these SF values are combined using the following rules: 1) If the SF is given for several well described strains of the same species, these SF values were treated as SF values from different species*. 2) If SFs are given for more than one toxicity endpoint, the SF with endpoint mortality is used. 3) If SFs are given for several exposure periods (mostly 24 and 48 hours), the SF for the longest exposure period is used. 4) Of the remaining SF values, the median is used, taking the geometric mean of the two central values in case of an even number of data**.

*: The nine distinct *Helicoverpa armigera* strains tested in [17] are treated as one species.

**: If one of these calculated SF values deviated strongly from the SF value in the original paper, that value was not used.

Looking at table II-3, it seems that no combination of toxins exists which is generally synergistic or antagonistic for all Lepidoptera species. In some species synergism seems to be absent (*Epinotia aporema*, *Sesamia inferens*), in others synergy seems to be common (*Chilo suppressalis*). However, these data are based on one or at most two papers, and the Synergy Factors might be correlated within one set of experiments. All SFs are less than 10, except for an SF of more than 400 for the combination of Cry1Ac and Cyt1A1 in a highly Cry1Ac resistant *Plutella xylostella* strain and 11 for a, compared to a sensitive strain, slightly resistant *P. xylostella* strain[21]. In addition a SF slightly over 20 for *Helicoverpa armigera* for the combination of Cry1Ac and Cry1F was reported in an ill-documented paper from 1998 [23], whereas others could not repeat this result and found no synergy in a similar experiment in 2008 [72].

For the toxins tested with Diptera (see table II-4), synergy seems to be more frequent. A considerable part of these data are gathered by one research group, the group of Margaret Wirth (Department of Entomology, University of California), using the same (selected) *C. quinquefasciatus* and *Aedes aegypti* strains.
The alleged conjugative combination Cry48-Cry49 is only tested on one species, the Diptera *C. quinquefasciatus* with a test enabling the estimation of the SF (see also table II-4). In tests with Cry48 and Cry49 producing Bt strains, either single or in combination, no toxicity was observed for one Coleoptera[22] and three Lepidoptera[23] species at dosing levels which led to 100% mortality if Bt strains selected for these target organisms would have been used [77].

Only six toxins are tested with at most two Nematoda species, three of which are Cry21 type toxins (see table II-5). The combination of Cry6Aa and Cry55Aa seems to act somewhat synergistic in *Meloidogyne incognita* and the combination of CryFa and CryHa is just weakly synergistic for *Caenorhabditis elegans*.

These tables show that resistance to a toxin often, but not always leads to synergy of that toxin with some other toxin:

- Compared to the susceptible *Culex quinquefasciatus* strain, in the *C. quinquefasciatus* strain resistant to the spores of *B. sphaericus* producing a BinA/BinB combination, the synergy is stronger between that spore and the single toxins Cry11Aa, Mtx2, Cyt1Ab and Cyt2Ba, and slightly stronger with Cry4Aa, and also stronger with the toxin mixture of Mtx2 with Cyt1Aa, and slightly stronger with the mixtures Mtx1 with Cyt1Aa, and Mtx1 with Mtx2. Note that the resistance to the Bs spore does not lead to cross-resistance to the Cyt toxins Cyt1Ab and Cyt2Ba [201], [204], [209], nor to Mtx1 or Mtx2 [209]. And that synergy between Mtx1 and Cyt1Aa and between Mtx2 and Cyt1Aa is lacking in both the Bs spore resistant *C. quinquefasciatus* strain and the susceptible strain. If anything, the SF is slightly less in the resistant strain, resulting is antagonism in that resistant strain between Mtx1 and Cyt1Aa.

21 The Resistance Ratio for the highly resistant strain (compared to the sensitive ROTH strain) was 1165 for Cry1Ac and 1818 for Cyt1A1, and the RR for the field derived, slightly resistant strain was 44 and 53, respectively.
22 Coleoptera *Anthonomus grandis*.
23 Lepidoptera *Anticarsia gemmatalis*, *Spodoptera frugiperda* and *Plutella xylostella*.

- In *C. quinquefasciatus* resistant to the combination of Cry4Aa and Cry4Ba, Cry4Aa either in combination with Cry4Ba or not, synergizes stronger with Mtx2 and with Mtx1 than in a comparable susceptible *C. quinquefasciatus* strain, and the combination of Cry4Aa and Cry4Ba also synergizes stronger with Cyt1Aa in this resistant *C. quinquefasciatus* strain than in the susceptible *C. quinquefasciatus* strain. The synergy between Mtx2 and Cry4Aa (plus Cry4Ba) is considerably larger than between Mtx1 and these Cry toxins. Resistance to Cry4Aa and Cry4Ba does not seem to influence the interaction between Cry4Ba and either Mtx1 or Mtx2, nor between Cry2Aa and Cyt1Aa in *C. quinque-fasciatus*, both *C. quinquefasciatus* strains being not sensitive to Cry4Ba [212] and Cry2Aa [204] and having a similar sensitivity to Cyt1Aa [204], and in neither strains synergy was observed between Cry4Ba and Mtx1 or Mtx2 or between Cry2Aa and Cyt1Aa.
- In *C. quinquefasciatus* resistant to Cry11Aa, the synergy between Cry11Aa and Cyt1Aa is enhanced compared to synergy in the susceptible *C. quinquefasciatus* strain. However, this resistance does not seem to influence the interaction between Cry11Ba and Cyt1Aa in *C. quinquefasciatus*. although the toxicity of Cry11Ba is decreased with a factor of about 10 by the resistance induced by Cry11Aa [200]. For this combination no synergy was observed in either of the *C. quinquefasciatus* strains.
- In neither the *C. quinquefasciatus* strain resistant to the combination of Cry4Aa, Cry4Ba, Cry11Aa and Cyt1Aa nor in the susceptible strain, synergy was observed between Cry11Ba and Cyt1Aa, nor between either Mtx1 or Mtx2 and the mixture of Cry4Aa, Cry4Ba, Cry11Aa and Cyt1Aa, that is the combination against which the resistant strain was resistant, so this resistance does not seem to affect the magnitude of the synergy. Some caution is needed in using the data of the mixtures of Mtx1 or Mtx2 with other toxins. For the susceptible strain Wirth *et al*. used in their 2014 paper ([212]) the data on Mtx1 and Mtx2 toxicity from their 2007 paper ([209]).
- In *C. quinquefasciatus* resistance against Cyt1Aa alone or against the combination of Cyt1Aa and Cry11Aa does not affect the synergy between Cyt1Aa and Cry11Aa. Resistance against Cyt1Aa does not induce cross-resistance against Cry11Aa [208].
- In *C. quinquefasciatus* resistant to the *B. thuringiensis* subsp. *jegathesan*, a strain producing seven toxins, among which Cry11B, Cry19A and Cyt2Bb1, as well as in *C. quinquefasciatus* resistant to Cry11B alone, the synergy between Cyt1Aa and Cry11A is strongly enhanced and the synergy between Cyt1Aa and Cry11B slightly. Note that resistance induced by Cry11B either as single toxin or in the combination of Bt subsp. *jegathesan*, led to a stronger resistance to Cry11A than to Cry11B.
- In *Plutella xylostella* selected for resistance against Cry1Ac[24], the synergy between Cry1Ac and Cyt1Aa is extremely strong and considerably stronger than in an unselected *P. xylostella* strain and in a susceptible *P. xylostella* strain. The sensitivity to Cry1Ac of the unselected strain is about a factor 40 less than the sensitive of the susceptible strain, and in the unselected strain synergy between

24 RR for resistant strain compared to susceptible (ROTH) strain is 1165.

Cry1Ac and Cyt1Aa is observed, whereas in the susceptible strain no synergy is observed.

The tables also show that strong synergy (SF>50), or even synergy with a SF>20, between 2 toxins only occurs in resistant strains and only if one of the toxins is either a Cyt toxin or Mtx2. The only exceptions to this rule[25] are the conjugative pair Cry48Aa/Cry49Aa and the synergy between Mtx1 and Mtx2 and between Bs spores (BinA/BinB) and Cyt1A in *Aedes aegypti* and between Cry4Ba and Cyt2A in *Culex quinquefasciatus* (and almost in *A. aegypti*) or between Cry4Ba and two or more other toxins in *C. quinquefasciatus*[26]. For the combination of Mtx1 and Mtx2 it can be remarked that Mtx1 is a much weaker toxin for *A. aegypti* than for the simultaneously tested *C. quinquefasciatus*. For the combination of BinA/BinB with Cyt1A, it holds similarly that the BinA/BinB producing Bs spores are much less toxic to *A. aegypti* than to *C. quinquefasciatus*. Although neither the combination Mtx1 and Mtx2 nor the combination Bs spores with Cyt1A showed synergy in *C. quinquefasciatus*, the mixture toxicity was still (slightly) higher for *C. quinquefasciatus* than for *A. aegypti* [151], [202], [201], [204], [207]. For the effect of the combination of Bs spores with Cyt1A on *A. aegypti* it can further be remarked that synergy is only observed after 24 hours. After 48 hours, the effect of the Bs spores separately is considerably larger (factor 10 to 20) than after 24 hours[27], so the synergistic effect of Cyt1A on the toxicity of the Bs spores seems primarily to be a speeding up of the toxic effect, not an enlargement of the final effect. For the combination of Cry4Ba with Cyt2A it can be remarked that Cry4Ba is only a weak toxin for *C. quinquefasciatus* in comparison to its toxicity to *Chironomus tepperi*. So maybe the Diptera with high synergy can be considered as resistant against these toxins. Although *A. aegypti* is about equally sensitive to Cry4Ba as *C. tepperi*, the 250 kDa CAD receptor of *A. aegypti*, which binds to Cry4Aa, Cry11Aa and Cry11Ab does not bind to Cry4Ba [25]. This could explain the higher synergy between Cry4Ba and Cyt2A in *A. aegypti* to Cry4Ba: The Cyt2A substitutes the function of the non-functioning CAD receptor. Note that the explanation might also be more trivial. In [141] three cloned bacterial strains are used to produce the toxins, one for Cry4Ba, one for Cyt2A and a third for the combination of Cry4Ba and Cyt2A. The toxins cloned in the bacteria producing both might be somehow more effective than the toxins cloned in bacteria producing only one toxin. And although it is claimed that the dosing is in the quantity of the isolated toxins, the toxin dosing might be bacterial strain dependent.
In *Chironomus tepperi* synergy is only observed between Cry4A and Cyt1A, and not between Cry4B or Cry11A and Cyt1A. When the sensitivity of *C. tepperi* is compared to the sensitivity of the susceptible strain of *C. quinquefasciatus*[28], it appears that *C. tepperi*

25 The combination Cry1Ac and Cry1F was also found to have a SF slightly over 20 for *Helicoverpa armigera* by Chakrabarti *et al.* in 1998 [23], but Ibargutxi *et al.* [72] could not repeat this synergistic effect in 2008, nor could they explain the difference.

26 These combinations are “Cry4Aa + Cry11Aa” or “Cry4Aa + Cry11Aa + Mtx2” or “Cry4Aa + Cry11Aa + Mtx1”[212]. Note that the calculated SF is based on assumed toxin ratio's, for not all ratio's were given in that paper.

27 In [201] the LC50 of *A. aegypti* for Bs spores is stated to be about 3-times higher after 48 hours than after 24 hours, but this must either be the result of a large control mortality, invalidating the experiment, or some typing error, for instance a decimal error in copying the data.

28 Note that LC50 values are better comparable between Diptera species than between

is relatively sensitive to Cry4B, with an 48h LC50 of 0.95 mg/l [69] compared to >200 mg/l for *C. quinquefasciatus* (CqS) [209], [212] and relatively resistant to Cry4A with an LC50 of approximately 200 mg/l [69] versus an LC50 value for *C. quinquefasciatus* (CqS) of 4 to 5 mg/l [207], [212]. The sensitivity of these two species to Cry11A and Cyt1Aa is in the same order (0.56 mg/l and 31 mg/l for *C. tepperi* [69] and 1.2 mg./l and 12 to 31 mg/l for *C. quinquefasciatus* [13], [209], [202], [212]). The synergy pattern in *C. tepperi* supports therefore the hypothesis that (positive) synergy is correlated to resistance, or a low susceptibility. And combining this observation with the pattern of CAD-binding, it might be hypothesized that (binding to) Cyt can act in a similar way on 3D-Cry as binding to a CAD receptor, that is, according to the sequential binding model, facilitating the cleavage of the α1 helix from domain I of the 3D-Cry [132].
As described in chapter *I-5.5.3 Interaction with Cry toxins* (p. 72), it is speculated that the Cyt toxin enhances the 3D-Cry toxicity by binding to the cell membrane and there functioning as a receptor for the 3D-Cry toxin [131], acting in a similar way as a CAD receptor. If insensitivity to a Cyt toxin is caused by the inability of the Cyt toxin to bind to the cell membrane, and if the unbound Cyt toxin then can still bind to free 3D-Cry toxins, the binding of Cyt toxin to 3D-Cry toxin could prevent the binding of 3D-Cry toxin to the cell membrane and thus lower the toxicity of the 3D-Cry toxin, and then the 3D-Cry and Cyt toxin would be antagonistic. That is, provided that Cyt toxin can only function as CAD, facilitating the cleavage of the α1 helix, if it is bound to the cell membrane. That could explain the antagonistic effect of Cyt1A on the Cry1Ac toxicity in *Trichoplusia ni* [145]. It can, however, not explain the antagonistic effect of Cyt1A and other toxins than 3D-Cry ones, such as between Cyt1A and Bs spores producing the BinA/BinB complex in *C. quinquefasciatus* in [202]. A year later, that combination was found weakly synergistic for *C. quinquefasciatus* in one of the other studies of Wirth et al. [204]. In the latter study, the sensitivity for the Bs spores was a factor 50 less than in the first study, so the difference in estimated synergy might be the result of an (accidental) low and high estimated toxicity for one of the combined toxins.

Given the hypothesis that some toxins, like Cyt and Mtx2, synergize other toxins if the target organism is resistant against that second toxin or not very sensitive to it, the categorizing of the combination Cry48Aa with Cry49Aa as a conjugative pair might be questioned. Maybe Cry49Aa, as a Bin protein, belongs with Cyt1, Cyt2 and Mtx2 to the category of the synergistic toxins, and is just considered as a couple with Cry48Aa because the organism tested (*C. quinquefasciatus*) is not very sensitive to Cry48Aa. Note, however, that for some very related species, the culicidae diptera *Aedes aegypti* and *Anopheles gambiae* , which are even less sensitive to Cry48, no synergy was observed between Cry48 and Cry49[29] [77].

The theory that, within a species or a group of related species, a relatively low sensitivity leads to a higher probability of synergy, is, however, not always supported by the data. In

Lepidoptera species because the toxin dose for these Diptera larvae is given as the concentration in the medium in which the Diptera are living, whereas the toxin dose in Lepidoptera species is expressed in all kind of units, depending on the exposure method.

29 It is reported that In *Aedes aegypti* and *Anopheles gambiae* no toxicity was observed for either Cry48, Cry49 or the combination of these two proteins at the (highest) dose tested [77].

testing nine different field derived *Helicoverpa armigera* strains for sensitivity to Cry1Ac, Cry2Ab and a combination of these toxins, the toxicity of the single Cry toxins (expressed in the expected toxicity of the combination) differed by factor 31, but the SF did not correlate with this expected toxicity at all[30] [17]. Not that in that study no synergy was observed (median SF 1.5), so without synergy no relation between toxicity and synergy should be expected. And two rice stem borers, *Chilo suppressalis* (Crambidae) and *Sesamia inferens* (Noctuidae) were tested for their sensitivity to Cry1Aa, Cry1Ab, Cry1Ac, Cry1Ba, Cry1Ca and mixtures of two of these toxins [47][31]. For three out of these five toxins, *C. suppressalis* was more sensitive than *S. inferens*[32], but for all tested toxin combinations, the estimated SF was larger for *C. suppressalis*. In some of the comparable tests with *C. suppressalis* much lower EC50 values were found for some of these Bt toxins [3], [93][33], especially for Cry1Ab and Cry1Ac, suggesting that the strain used in the combination experiment [47] was not the most sensitive one for these toxins.

In the above, SF values between 0.5 and 2 are not considered as indication of positive or negative synergism. This margin used in this report is larger than that used in most papers on synergy research. The used margin is no more than an educated guess, based on ecotoxicological experience that a difference in toxicity value of a factor 2 is in most cases irrelevant. Analysis of the variance in a subset of the data on synergy, consisting of six independently measured SF factors in different generations of the same strain, indicates that this margin is rather too small than too large. The SF values in this subset are from studies with the same, unselected *C. quinquefasciatus* strain, tested in six out of 44 succeeding generations for the synergy of Cry11Aa and Cyt1Aa [208]. The observed SF factors were 2.51; 4.65; 8.63; 4.61; 6.60; 5.96. If these SF values are assumed to be drawn from a log-normal distribution, the estimate for the mean SF is 5.66, and of the median 5.13, with a 95% confidence interval ranging from 30% to 280% of that mean, that is from 32% to 310% of the median. SF factors between 2 and 5 indicate the possibility of slight synergism, and between 5 and 10 weak synergism is likely. The SF range from 2 to 10 will be indicated as weakly synergistic. Even if synergism really exists if such an SF is observed, the synergy is too small for a considerable impact on the toxicity of the mixture.

Almost no data are available on synergy in non-target organisms. Only for the rice midge *Chironomus tepperi* and the laboratory test species *Caenorhabditis elegans* some data on synergy are available, between all combinations of Cry4Aa, Cry4Ba, Cry11Aa and Cyt1A, and between Cry5Aa and Cry6Aa, respectively. Non-target species are, however, not fundamentally different from target species, therefore it may be expected that observations about synergy in target organisms also hold for non-target organisms. Only if a specific Bt toxin is evolved in a *B. thuringiensis* strain which is entomopathogenic to a

30 r=-0.15

31 In this test, the toxin was dosed in the same way for both species, mixed through artificial food. Therefore, the measured sensitivity of these two Lepidoptera can be compared.

32 For these 3 toxins, Cry1Aa, Cry1Ab and Cry1Ba, the sensitivity of *C. suppressalis* was a factor 3.6, 5.6 and 47, resp. larger than the sensitive of *S. inferens*.

33 In all these tests, the toxin was mixed through (artificial) diet, toxin dose expressed in ng/ml diet. Note that the two papers with higher Cry toxicity ([3] and [93]) the toxin was activated with trypsin.

(set of) specific species, the relationship between that toxin and that (set of) species might be considered as special. The important point in the question of the usability of data from one species for another is therefore not whether humans consider a species as a target or not, but whether the bacteria in which the toxin was developed had a special relationship with one of these species, potentially making the toxicity species specific.

The data in the tables II-3 to II-9 are collected in a literature search for all data allowing the calculation of the SF for the combination of two or more Bt toxins, and this search was extended to combinations with the Bs toxins Mtx1, Mtx2, Mtx3[34], BinA, BinB and Bs spores, without any additional selection criterion, such as species group or observed synergy. It cannot be excluded, however, that some experiments without significant synergistic (or antagonistic) effects might have been missed because experiments without statistical significant effects are less often published.

II-2.1.3 *Some theoretical considerations on synergism*.

Synergistic mechanisms between toxins can roughly be classified in two groups:

1. Toxin A breaks down some barrier for the effective operation of toxin B. For instance, toxin A, a detergent, increases the solubility of toxin B, making toxin B available in a higher dose to the target organism. Or toxin A weakens a membrane that had prevented toxin B coming near its target site.
2. Molecules of toxin A and toxin B form some kind of complex, which is more toxic than each of the separate toxins individually. Such a mechanism is, for instance, suggested for some 3D-Cry combinations which individually form homo-oligomeres making pores in the target cell membrane, and together form hetero-oligomeres which are more effective in making membrane pores ([147], [169]).

Some mechanisms can either be classified in the first group, or in the second, depending on the circumstances. For instance, if substance A is not toxic in the form in which it is applied, but is a protoxin that has to be (enzymatically) transformed into a toxin to become toxic, and substance B stimulates that transformation, this can be considered in two ways: a) breaking down a barrier in a resistant strain which does not produce the enzyme promoting that transformation whereas the “wild type” target produces such an enzyme b) a (temporary) complex between (pro)toxin A and (enzyme) B, leading to an increased toxicity of A.

The results on the synergy testing suggest that the main cause of synergy between Bt toxins is the removal or at least suppression of resistance. For if synergy is (mainly) due to a combination of different toxin molecules forming a more active complex, the synergy would not depend on the resistance of the target organism.
In part I, two important potential mechanisms that might lead to synergy, were described:

1. Some Bt toxins, notably the Cyt toxins, might, by binding to the cell membrane, form an additional binding site for other Bt toxins. As one of the potential

34 Combinations with Mtx3 were not found.

mechanisms of resistance is the changing of the binding site, so that the toxin can no longer bind to the cell membrane and becomes harmless, a complementary toxin which produces a new binding site might work synergistic, especially in resistant target organisms.

2. The formation of heteromeres by two toxins, which are more effective than each of the separate homomeres. This phenomenon is described by Rodriguez-Almazán [147] and by Soberón [169]. In both cases, it is found that two non-toxic mutants of Cry1Ab can, together, become toxic again. The toxicity of the mixture of both mutants remains in both cases less than the toxicity of the wild type Cry toxins. The mutant, mixed with the wild type, decreases the toxicity of the wild type Cry1Ab. Rodriguez-Almazán [147] also investigated the antagonistic effect of the mutant on some other Cry toxins. Their mutant antagonized the toxicity of Cry1Aa, Cry1Ac and Cry1Fa, but did not affect the toxicity of Cry1Ca, Cry1D and Cry1Ea. This result suggested that ineffective Cry toxins can synergize each other up till the effectiveness of a well-evolved wild type. Up till now, there is no evidence that well developed, wild type Cry toxins can form together hetero-oligomers which are more effective than the homo-oligomers formed by the single toxins.

Table II-3.
Classification of the Synergy Factor between Cry1, Cry2, Vip3Aa proteins, also giving the synergy of these proteins with Cry9, Cyt1, Cyt2 and HD1 spores. **Blue**: SF<0.5; **Yellow**: 0.5≤SF<2; **Grey**: 2≤SF<5, **Grey**: 5≤SF<10; **Red**: 10≤SF<50; **Dark red with yellow symbols**: 50≤SF<250; [redacted]: 250≤SF.
Most of the species in this table are Lepidoptera, the species abbreviations are (for the non-lepidoptera, the class is given between brackets): Cf: *Choristoneura fumiferana*, Co: *Choristoneura occidentalis*, Cpa: *Chilo partellus*, Cpi: *Choristoneura pinus*, Cs-l: *Chilo suppressalis*, Cq: *Culex quinquefasciatus* (Dip), Ct: *Chironomus tepperi* (Dip), Ea: *Epinotia aporema*, Ei: *Earias insulana*, Ek: *Ephestia kuehniella*, Ev: *Earias vittella*, Ha: *Helicoverpa armigera*, Ld: *Lymantria dispar*, Md: *Malacosoma disstria*, Ol: *Orygia leucostigma*, Pc: *Perileucoptera coffeella*, Pi: *Plodia interpunctella*, Pg: *Pectinophora gossypiella*, Px: *Plutella xylostella*, Sa-l: *Spodoptera albula*, Ser: *Spodoptera erdania*, Sex: *Spodoptera exigua*, Sf: *Spodoptera frugiperda*, Sla: *Spodoptera latifascia*, Si: *Sesamia inferens*, Tn: *Trichoplusia ni*. For Px, data are given for a resistant strain (PxR) and (two)sensitive strains (PxS). Complete dataset is given in table II-6.

Table II-3a: Synergy factor between two (group of) toxins.

	Cry1					Cry2	Dipel	Vip3Aa
	A			B	C			
	Aa	Ab	Ac/Ac1	Ba	C/Ca	Aa		
Cry1Ab	Ld Cpa, Si, Ea Cs-l							
Cry1Ac	Cpa, Ea Ek, Ld	Ea, Ha, Ld, Si Cpa, Cs-l						
Cry1Ba		Si Cs-l	Si, Pc, Cs-l					
Cry1C/ Cy1Ca	Cs-l , Si Ha , Sex	Si Cs-l	Si Cs-l					
Cry1Ca				Ea				
Cry1Da				Ea	Ea			
Cry1Fa			Ha, Ei, Ha					
Cryla10								Ser Sa-l , Sla, Sf
Cry2Aa	Ek		Ha, Ek Ev					
Cry2Ab/ 2Ab2			Ha, Ei, Pg					
Cry9Ca	Cf	Cf						Px
HD1 spore	Pi , Px	Pi , Px	Pi , Px		Px	Px		
HD1 spore coat	Pi	Pi	Pi					
Cyt1A1			Tn, Pg PxS, [redacted]				Px	
Cyt2Aa3								Ha, Cq, Ct Cs-l, Sex

Table II-3b

Synergy factor between three or more toxins

Toxin 1	Toxin 2	Toxin 3	Synergy Factor
Cry1Aa	Cry1Ab	Cry1Ac	Ol , Cf, Co, Cpi, Ld, Md

Table II-4
Synergy factor between Cry4, Cry10, Cry11, Mtx1, Mtx2 and Cyt proteins, also giving the synergy of these proteins with Cry2, Cry29 and Cry30 . **Blue**: SF<0.5; **Yellow**: 0.5≤SF<2; **Grey**: 2≤SF<5, **Grey**: 5≤SF<10; **Red**: 10≤SF<50; **Dark red with yellow symbols**: 50≤SF<250; [redacted]: 250≤SF.
The species in the table are all Diptera, the species abbreviations are: Aa: *Aedes aegypti*, Ag: *Anopheles gambiae*, As: *Anopheles stephensi*, Cp: *Culex pipiens*, Cq: *Culex quinquefasciatus* , Ct: *Chironomus tepperi*. Several sensitive and resistant Cq strains were used, indicated as CqS or CqR*no*, the explanation of the resistant strain code is given in the notes to the table. Complete dataset is given in tables II-7 and II-8.

Table II-4a: Synergy factor between two (group of) toxins.

	B. sphaericus producing BinA/BinB	Cry4		Cry11		Cry48Aa	Mtx1	Mtx2	Cyt1Aa
		Aa	Ba	Aa	Ba				
Cry2Aa									CqS, CqR2
Cry4Aa	Aa, CqS CqR1								
Cry4Ba		Ct Aa. Ag, As, CqR2, CqS, Cq, Cp,							
Cry4Aa + Cry4Ba	Aa, CqS, CqR1						CqS, CqR2	CqS CqR2	CqS CqR2
Cry10Aa									Aa
Cry11Aa	CqS, Aa CqR1	Ct Aa, Cp, As	Aa, As, Cp, Ct,						
Cry4Aa + Cry11Aa									Aa
Cry4Aa + Cry4Ba + Cry11Aa	Aa CqS, CqR1						CqS CqR4	CqS CqR4	CqS CqR4
Cry4Aa + Cry11Aa + 20kDa[c]									Aa
Cry29Aa					As, Cp Aa				
Cry30Aa					Aa, As, Cp				
Mtx1	CqS, CqR1	CqS CqR2	CqS, CqR2	CqS, CqR3					
Mtx2	CqS CqR1	CqS CqR2	CqS CqR2	CqS CqR3			Cq, Aa		

	B. sphaericus producing BinA/BinB	Cry4		Cry11		Cry48Aa	Mtx1	Mtx2	Cyt1Aa
		Aa	Ba	Aa	Ba				
Cyt1A	CqS/CqS Aa/Aa CqR1	Ct , Aa	Ct, Aal	Ct Aal, Aa, CqS, CqR6, CqR7 CqR3, CqR8, CqR9	CqS, CqR3, CqR5, CqR2, CqR9, CqR8, CqR4		CqR1 CqS	CqR1 CqS	
Cry4Aa + Cry4Ba + Cry11Aa + Cyt1A							CqS CqR5	CqS, CqR5	
Cyt2A or Cyt2B	Aa, CqS CqR1		Aa, Cq						
Cry49Aa		Cq				Cq			

Table II-4b

Synergy factor between three or more toxins

Toxin 1	Toxin 2	Toxin 3	Toxin 4	Synergy Factor
Cry4Aa	Cry4Ba	Cry11Aa		Aa, As, Cp,
Cry4Aa	Cry4Ba	Mtx1		CqS, CqR2
Cry4Aa	Cry4Ba	Mtx2		CqS, CqR2
Cry4Aa	Cry4Ba	Mtx1	Cry11Aa	CqS
Cry4Aa	Cry4Ba	Mtx2	Cry11Aa	CqS
Cry4Ba	Cry11Aa	Cyt1Aa		Aal
Bs (BinA/BinB)	Cry4A	Cyt1Aa		Aa, CqS,
Bs (BinA/BinB)	Cry11A	Cyt1Aa		Aa, CqS, CqR1
Bs (BinA/BinB)	Cry4A + Cry4B	Cyt1Aa		Aa, CqS, CqR1
Bs (BinA/BinB)	Cry4A + Cry4B + Cry11A	Cyt1Aa		Aa, CqS, CqR1
Bs (BinA/BinB)	Mtx1	Cyt1Aa		CqS, CqR1
Bs (BinA/BinB)	Mtx2	Cyt1Aa		CqS, CqR1
Bs (BinA/BinB)	Mtx1	Mtx2		CqS, CqR1
Cry11Bb	Cry29Aa	Cry30Aa		As, Cp, Aa

CqR1: Cq resistant to *Bacillus sphaericus* (see [207], [209], [201], [204]) This Bs strain (2362) only produces the BinA/BinB Bin toxin pair. This Cq strain is selected for resistance against Bs 2362 since 1992.

CqR2: Cq resistant to Cry4Aa and Cry4Ba. The Cq strain was selected since 1991 (in [199] for approx. 27 generations). (see [199], [212], [200])

CqR3: Cq resistant to Cry11Aa. In [199], [212] and [208], the Cq strain was selected since 1991 (in [199] for approx. 27 generations), in [208] Cq was selected for approx. 48 generations. (see [199], [212], [200], [208])

CqR4: Cq resistant to Cry4Aa, Cry4Ba and Cry11Aa. The Cq strain was selected since 1991 (in [199] for approx. 27 generations). (see [199], [212], [200])

CqR5: Cq resistant to Cry4Aa, Cry4Ba, Cry11Aa and Cyt1A. The Cq strain was selected since 1991. (see [212], [200])

CqR6 Cq resistant to Cyt1A, selected for approx. 48 generations (see [208])

CqR7 Cq resistant to Cry11Aa and Cyt1A, selected for approx. 48 generations (see [208])

CqR8 Cq resistant to *Bacillus thuringiensis* subsp. *jegathesan.* This Bt produces 7 toxins, including Cry11B, Cry19A and Cyt2Bb1. Selection during approx. 40 generations [206].

CqR9 Cq resistant to Cry11B. Selection during approx. 40 generations. (see [206])

Table II-5
Synergy factor between Cry6 and Cry5 and Cry55 proteins. **Blue**: SF<0.5; **Yellow**: 0.5≤SF<2; **Grey**: 2≤SF<5, **Grey**: 5≤SF<10; **Red**: 10≤SF<50; **Dark red with yellow symbols**: 50≤SF<250; [redacted]: 250≤SF.
The species in the table are both Nematoda (Secernentea), the species abbreviations are: Ce: *Caenorhabditis elegans*, Mi: *Meloidogyne incognita*.
Complete dataset is given in table II-9.

Table II-5a: Synergy factor between two (group of) toxins.

	Cry5Ba/ Cry5Ba2	Cry21Fa	Cry21Ga	Cry55Aa
Cry5Ba/ Cry5Ba2				**Mi**
Cry6Aa/ Cry6Aa2	**Ce**, **Mi**			**Mi**
Cry21Ga		**Ce**		
Cry21Ha		**Ce**	**Ce**	

Table II-5b: Synergy factor between three or more toxins

Toxin 1	**Toxin 2**	**Toxin 3**	**Synergy Factor**
Cry5Ba	Cry6Aa	Cry55Aa	**Mi**

Table II-6
Synergy factor between Cry1, Cry2, Vip3Aa proteins, also giving the synergy of these proteins with Cry9, Cyt1, Cyt2 and HD1 spores. The calculated synergy factor (SF) is based on EC50 values, with effect mortality, unless otherwise stated (G: growth, F: Frass production, E: Emergence, C: Mortality in cell culture). Each line is one test, giving: *species*: *SF* (*ratio*) [*Effect if not mortality*/*test duration if test of several durations are given in one reference*] *reference*. For the ratio between the two proteins (values between brackets), first the column protein, then the row protein (w/w) is given.
Most of the species in this table are Lepidoptera, the species abbreviations are (for the non-lepidoptera, the class is given between brackets): Cf: *Choristoneura fumiferana*, Co: *Choristoneura occidentalis*, Cpa: *Chilo partellus*, Cpi: *Choristoneura pinus*, Cs-l: *Chilo suppressalis*, Cq: *Culex quinquefasciatus* (Dip), Ct: *Chironomus tepperi* (Dip), Ea: *Epinotia aporema*, Ei: *Earias insulana*, Ek: *Ephestia kuehniella*, Ev: *Earias vittella*, Ha: *Helicoverpa armigera*, Ld: *Lymantria dispar*, Md: *Malacosoma disstria*, Ol: *Orygia leucostigma*, Pc: *Perileucoptera coffeella*, Pi: *Plodia interpunctella*, Pg: *Pectinophora gossypiella*, Px: *Plutella xylostella*, Sa-l: *Spodoptera albula*, Ser: *Spodoptera erdania*, Sex: *Spodoptera exigua*, Sf: *Spodoptera frugiperda*, Sla: *Spodoptera latifascia*, Si: *Sesamia inferens*, Tn: *Trichoplusia ni*.

Table II-6a: Synergy factor between two (group of) toxins.

	Cry1					Cry2	Vip3Aa
	A			B	C		
	Aa	Ab	Ac/Ac1	Ba	C/Ca	Aa	
Cry1Ab	Cpa: 1.2 (1:1) [164] Cs-l: 4.9 (1:1) [47] Ea: 0.8 (1:1) [92] Ld: 0.3 (1:1) G [92] Si: 1.6 (1:1) [47]						

	Cry1					Cry2	Vip3Aa
	A			B	C		
	Aa	Ab	Ac/Ac1	Ba	C/Ca	Aa	
Cry1Ac	Cpa: 1.5 (1:1) [164] Ea: 0.5 (1:1) [155] Ek: 0.9 (2:1) [189] Ek: 2.6 (1:1) [189] Ek: 1.5 (1:2) [189] Ld: 3.8 (1:1) G [92] Ld: 7.3 (1:2) G [92] Ld: 4.9 (1:4) G [92] Ld: 4.1 (1:6) G [92] Ld: 4.6 (1:8) G [92] Ld: 2.7 (1:12) G [92]	Cpa: 2.9 (1:1) [164] Cpa: 5.2 (1:2) [164] Cpa: 4.0 (1:4) [164] Cpa: 3.4 (1:6) [164] Cpa: 2.0 (1:8) [164] Cpa: 3.5 (1:10) [164] Cpa: 3.9 (1:12) [164] Cs-l: 2.5 (1:1) [47] Ea: 0.74 (1:1) [155] Ha: ± 1 [23] Ld: 1.5 (1:1) G [92] Si: 0.7 (1:1) [47]					
Cry1Ba		Cs-l: 11 (1:1) [47] Si: 0.3 (1:1) [47]	Cs-l: 4.5 (1:1) [47] Pc: 0.9 (4:21) [57] Si: 0.2 (1:1) [47]				
Cry1C/ Cry1Ca	Cs-l: 1.6 (1:1) [47] Ha: 1.5 (1:5) [215] Ha: 2.7 (1:1) [215] Ha: 1.4 (5:1) [215] Sex: 2.0 (1:5) [215] Sex: 4.0 (1:1) [215] Sex: 2.3 (5:1) [215] Si: 0.1 (1:1) [47]	Cs-l: 4.4 (1:1) [47] Si: 0.2 (1:1) [47]	Cs-l: 3.0 (1:1) [47] Si: 0.6 (1:1) [47]				
Cry1Ca				Ea: 1.0 (1:1) [155]			
Cry1Da				Ea: 1.4 (1:1) [155]	Ea: 0.82 (1:1) [155]		

	Cry1					Cry2	Vip3Aa
	A			B	C		
	Aa	Ab	Ac/Ac1	Ba	C/Ca	Aa	
Cry1Fa			Ha: 1.0 (4:1) [72] Ha: 0.7 (1:1) [72] Ha: 0.6 (1:4) [72] Ha: 1.4 (4:1) G [72] Ha: 1.7 (1:1) G [72] Ha: 0.7 (1:4) G [72] Ha: 24 (2:1) [23] Ha: 26 (1:1) [23] Ha: 12 (1:2) [23] Ei: 1.1 (4:1) [72] Ei: 0.9 (1:1) [72] Ei: 1.1 (1:4) [72] Ei: 0.8 (4:1) G [72] Ei: 1.0 (1:1) G [72] Ei: 1.1 (1:4) G [72]			
Cry1Ia10							Sf: 6.4 (1:1) [12] Sa-I: 4.2 (1:1) [12] Sla: 4.1 (1:1) [12] Ser: 0.3 (1:1) [12]
Cry2Aa	Ek: 0.8 (2:1) [189] Ek: 1.3 (1:1) [189] Ek: 1.1 (1:2) [189]		Ha: 0.38 (1:3) [101] Ha: 0.52 (1:2) [101] Ha: 0.75 (1:1) [101] Ha: ± 1 [23] Ev: 6.9 (1:2) [220] Ev: 3.0 (1:1) [220] Ev: 13.7 (2:1) [220] Ek: 0.9 (2:1) [189] Ek: 1.1 (1:1) [189] Ek: 0.3 (1:2) [189]				

	Cry1					Cry2	Vip3Aa
	Aa	A Ab	Ac/Ac1	B Ba	C C/Ca	Aa	
Cry2Ab/ 2Ab2			Ha(B1): 0.9 (1:1) [17] Ha(B2): 1.4 (1:1) [17] Ha(B3): 0.8 (1:1) [17] Ha(Ca1): 0.7 (1:1) [17] Ha(Ca2): 2.4 (1:1) [17] Ha(Ca3): 2.1 (1:1) [17] Ha(Ca4): 1.4 (1:1) [17] Ha(Ca5): 1.5 (1:1) [17] Ha(Ch1): 0.6 (1:1) [17] Ha(B1): 0.5 (1:1) G [17] Ha(B2): 1.1 (1:1) G [17] Ha(B3): 0.6 (1:1) G [17] Ha(Ca1): 0.6 (1:1) G [17] Ha(Ca2): 1.0 (1:1) G [17] Ha(Ca3): 1.0 (1:1) G [17] Ha(Ca4): 1.5 (1:1) G [17] Ha(Ca5): 1.0 (1:1) G [17] Ha(Ch1): 0.3 (1:1) G [17] Ha: 1.6 (4:1) [72] Ha: 1.9 (1:1) [72] Ha: 3.1 (1:4) [72] Ha: 0.7 (4:1) G [72] Ha: 1.1 (1:1) G [72] Ha: 1.3 (1:4) G [72] Ei: 0.9 (4:1) [72] Ei: 0.9 (1:1) [72] Ei: 1.0 (1:4) [72] Ei: 1.0 (4:1) G [72] Ei: 1.1 (1:1) G [72] Ei: 1.1 (1:4) G [72] Pg: no syn/anta[g] [183]				
Cry9Ca	Cf: 1.2 (2:1)F [125]	Cf: 0.8 (2:1)F [125] Cf: 1.2 (1:1)F [125] Cf: 0.7 (1:2)F [125]					Px: 1.5 (1:1) [34][c]
HD1 spore	Pi: 9.8 (1:1) E [75] Pi: 4.6 (1:9) E [75] Px: 9.0 (1:1) [184]	Pi: 6.0 (1:1) E [75] Pi: 2.9 (1:9) E [75] Px: 4.9 (1:1) [184]	Pi: 7.9 (1:1) E [75] Pi: 1.8 (1:9) E [75] Px: 5.3 (1:1) [184]		Px: 1.8 (1:1) [184]	Px: 1.3 (1:1) [184]	
HD1 spore coat	Pi: 3.7 (1:1) E [75]	Pi: 2.7 (1:1) E [75]	Pi: 3.5 (1:1) E [75]				

	Cry1					Cry2	Vip3Aa
	A			B	C		
	Aa	Ab	Ac/Ac1	Ba	C/Ca	Aa	
Cyt1Aa	Px: No Syn/Anta [116][d]	Px: No Syn/Anta [116][d]	Px(Unsel): 11 (1:1) [157][f] Px(Sel): 453 (1:1) [157][f] Px(Roth): 2.0 (1:1) [157][f] Pg: No Syn/Anta [116][e] Px: No Syn/Anta [116][d]			Px: No Syn/Anta [116][d]	
Cyt1A1			Tn: 0.5 (99:1) C [145] Tn: 0.6 (199:1) C [145] Tn: 0.4 (399:1) C [145] Tn: 0.3 (799:1) C [145] Tn: 0.1 (ca 18:1)[a] [145] Tn: 0.2 (ca 150:1)[b] [145]				
Cyt2Aa3							Cs-l: 3.3 (6:1) [218] Sex: 4.3 (6:1) [218] Ha: 1.0 (6:1) [218] Cq: 0.6 (6:1) [218] Ct: 1.1 (6:1) [218]

Table II-6b

Synergy factor between three or more toxins

Toxin 1	**Toxin 2**	**Toxin 3**	**Toxin 4**	**Synergy Factor**
Cry1Aa	Cry1Ab	Cry1Ac		Cf: 0.9 (ca. 1:4:2.4) [175]
Cry1Aa	Cry1Ab	Cry1Ac		Co: 0.8 (ca. 1:4:2.4) [175]
Cry1Aa	Cry1Ab	Cry1Ac		Cpi: 1.4 (ca. 1:4:2.4) [175]
Cry1Aa	Cry1Ab	Cry1Ac		Ld: 1.3 (ca. 1:4:2.4) [175]
Cry1Aa	Cry1Ab	Cry1Ac		Md: 1.1 (ca. 1:4:2.4) [175]
Cry1Aa	Cry1Ab	Cry1Ac		Ol: 0.4 (ca. 1:4:2.4) [175]

[a]: Ratio not fixed, fixed Cyt1A1 dose (1.0 µg/cm^2 diet). Ratio given is ratio in mixture at LC50 for Cry1Ac.
[b]: Ratio not fixed, fixed Cyt1A1 dose (0.1 µg/cm^2 diet). Ratio given is ratio in mixture at LC50 for Cry1Ac.
[c]: A chimeric protein of Cry9Ca and Vip3Aa7 has a SF of 4.8
[d]: Experiment with limited number of doses, not allowing EC50 estimation. Cry1Aa/Cry1Ab/Cry1Ac/Cry2Aa toxin dosed in combination as Dipel, a formulated version of the HD-1 strain of *B. thuringiensis* subsp. *kurstaki*. No synergy or antagonism observed, both in a Px strain susceptible and in a strain resistant

to Dipel [116], [177]

[e]: Experiment with limited number of doses, not allowing EC50 estimation. No synergy or antagonism observed.

[f]: Three strains of Px were used, a known susceptible strain (Roth) and a Cry1Ac-selected (Sel) and an unselected (Unsel) strain starting from the same parent strain.

[g]: Limited number of doses tested, both in a susceptible and in a resistant Pg strain.

Table II-7.
Synergy factor between Cry4, Cry10, Cry11, Mtx1, Mtx2 and Cyt proteins, also giving the synergy of these proteins with Cry2, Cry29 and Cry30. The calculated synergy factor (SF) is based on EC50 values, with effect mortality, unless otherwise stated (G: growth, F: Frass production, E: Emergence, C: Mortality in cell culture). Each line is one test, giving: *species*: *SF* (*ratio*) [*Effect if not mortality*/*test duration if test of several durations are given in one reference*] *reference*. For the ratio between the two proteins (values between brackets), first the column protein, then the row protein (w/w) is given.
The species in the table are all Diptera, the species abbreviations are: Aa: *Aedes aegypti*, Ag: *Anopheles gambiae*, As: *Anopheles stephensi*, Cp: *Culex pipiens*, Cq: *Culex quinquefasciatus*, Ct: *Chironomus tepperi*. Several sensitive and resistant Cq strains were used, indicated as CqS or CqR*no*, the explanation of the resistant strain code is given in the notes below the table.

Table II-7a: Synergy factor between two (group of) toxins.

	B. sphaericus producing BinA/BinB	Cry4 Aa	Cry4 Ba	Cry11 Aa	Cry11 Ba	Mtx1	Mtx2	Cyt1Aa
Cry2Aa								CqS: 1.9 (1:1) [13] CqR2: 0.9 (1:1) [13]
Cry4Aa	Aa: 2.4 (1:3) 1d [207][n] Aa: 1.9 (1:3) 2d [207][n] CqS: 4.9 (1:3) 1d [207][n] CqS: 16 (1:3) 2d [207][n] CqR1: 22 (1:3) 1d [207][n] CqR1: 27 (1:3) 2d [207][n]							

	B. sphaericus producing BinA/BinB	Cry4 Aa	Cry4 Ba	Cry11 Aa	Cry11 Ba	Mtx1	Mtx2	Cyt1Aa
Cry4Ba		Aa: 4.4 (1:2) [6] Aa: 4.0 (1:1) [6] Aa: 2.7 (2:1) [6] Aa: 4.4 (1:1) [137] Aa: 6.1 (1:1) [31] Ag: 2.4 (1:2) [6] Ag: 2.4 (1:1) [6] Ag: 2.2 (2:1) [6] As: 2.1 (1:1) [137] As: 3.4 (1:1) [31] Cp: 14 (1:1) [137] Cp: 13 (1:1) [31] Cq: 13 (1:2) [6] Cq: 10 (1:1) [6] Cq: 8.6 (2:1) [6] CqS: 6.7 (1:1) [212] CqR2: 3.2 (1:1) [212] Ct: 1.2 (1:4) [69]						
Cry4Aa + Cry4Ba	Aa: 2.6 (1:3) 1d [207][n] Aa: 4.0 (1:3) 2d [207][n] CqS: 1.3 (1:3) 1d [207][n] CqS: 5.2 (1:3 2d [207][n] CqR1: 0.9 (1:3) 1d [207][n] CqR1: 15 (1:3) 2d [207][n]					CqS: 2.3 (1:1) [212][d] CqR2: 12 (1:1) [212][d]	CqS: 19 (1:1) [212][d] CqR2: 129 (1:1) [212][d]	CqS: 7.2 (1:3) [199] CqR2: 70 (3:1) [199]
Cry10Aa								Aa: 13 (1:1)[a] [65]
Cry11Aa	Aa: 13 (1:10) 1d [207][n] Aa: 7.6 (1:10) 2d [207][n] CqS: 2.3 (1:10) 1d [207][n] CqS: 2.4 (1:10) 2d [207][n] CqR1: 5.6 (1:10) 1d [207][n] CqR1: 79 (1:10) 2d [207][n]	Aa: 3.9 (2:1) [137] As: 5.4 (3:1) [137] Cp: 2.4 (1:1) [137] Ct: 0.9 (1:4) [69]	Aa: 0.9 (1:1) [137] As: 1.3 (1:32) [137] Cp: 1.7 (99:1) [137] Ct: 0.7 (1:4) [69]					
Cry4Aa + Cry11Aa								Aa: 2.6 (1:1) [81][f]

	B. sphaericus producing BinA/BinB	Cry4 Aa	Cry4 Ba	Cry11 Aa	Cry11 Ba	Mtx1	Mtx2	Cyt1Aa
Cry4Aa + Cry4Ba + Cry11Aa	Aa: 0.5 (1:3) 1d [207][n] Aa: 0.5 (1:3) 2d [207][n] CqS: 0.9 (1:3) 1d [207][n] CqS: 1.6 (1:3 2d [207][n] CqR1: 1.2 (1:3) 1d [207][n] CqR1: 1.8 (1:3) 2d [207][n]					CqS: 0.8 (1:1) [212][d] CqR4: 3.9 (1:1) [212][d]	CqS: 0.6 (1:1) [212][d] CqR4: 21 (1:1) [212][d]	CqS: 2.7 (1:3) [199] CqR4: 35 (3:1) [199]
Cry4Aa + Cry11Aa + 20kDa[c]								Aa: 4.7 (1:1) [82][c]
Cry29Aa					Aa: 4.4 (1:20) [78] As: 0.7 (1:20) [78] Cp: 1.3 (1:20) [78]			
Cry30Aa					Aa: 1.0 (1:20) [78] As: 1.0 (1:20) [78] Cp: 0.8 (1:20) [78]			
Mtx1	CqS: 9.8 (3:1) [209] CqR1: 3.1 (3:1) [209]	CqS: 0.4 (1:1) [212][d] CqR2: 3.9 (1:1) [212][d]	CqS: 0.3 (1:1) [212][d] CqR2: 0.3 (1:1) [212][d]	CqS: 1.5 (3:1) [209] CqS: 0.6 (1:1) [212][d] CqR3: 0.9 (3:1) [209] CqR3: 0.7 (1:1) [212][d]				
Mtx2	CqS: 11 (3:1) [209] CqR1: 148 (3:1) [209]	CqS: 17 (1:1) [212][d] CqR2: 36 (1:1) [212][d]	CqS: 1.0 (1:1) [212][d,h] CqR2: <0.3 (1:1) [212][d]	CqS: 9.9 (3:1) [209] CqS: 2.2 (1:1) [212][d] CqR3: 26 (3:1) [209] CqR3: <0.5 (1:1) [212][d]		Aa: 23 (?:?) [151][j] Cq: 1.2 (?:?) [151][j]		

	B. sphaericus producing BinA/BinB	Cry4		Cry11		Mtx1	Mtx2	Cyt1Aa
		Aa	Ba	Aa	Ba			
Cyt1A	Aa: 60 (10:1) 1d [204][m] Aa: 15 (3:1) 1d [204] Aa: 7.7 (10:1) 2d [204] Aa: 2.6 (3:1) 2d [204] Aa: 6.1 (1:1) [81][f] Aa: 2.8 (3:1) 1d [201][p] Aa: 17 (10:1) 1d [201][p] Aa: 25 (20:1) 1d [201][p] Aa: 24 (50:1) 1d [201][p] Aa: 2.1 (10:1) 2d [201][q] CqS: 2.8 (10:1) 1d [204] CqS: 3.6 (3:1) 1d [204] CqS: 3.6 (10:1) 2d [204] CqS: 23 (3:1) 2d [204] CqS 0.3 (10:1) [202] CqS 0.3 (5:1) [202] CqS 0.6 (3:1) [202] CqS 0.4 (1:1) [202] CqS 0.1 (1:3) [202] CqS 0.2 (1:5) [202] CqS 0.1 (1:10) [202] CqR1: 190 (10:1) 1d [204] CqR1: 88 (3:1) 1d [204][m] CqR1: 580 (10:1) 2d [204] CqR1: 950 (3:1) 2d [204] CqR1: 109 (10:1) [202] CqR1: 136 (5:1) [202] CqR1: 60 (3:1) [202] CqR1: 86 (1:1) [202] CqR1: 25 (1:3) [202] CqR1: 25 (1:5) [202] CqR1: 7.5 (1:10) [202]	Aa: 6.7 (1:1) [81] Ct: 2.2 (4:1) [69]	Ct: 0.5 (4:1) [69] Aal: Syn [41][g]	Aa: 7.0 (1:1) [131][i] Aa: 12 (2:1) [131][i] Aa: 22 (5:1) [131][i] Aa: 7.3 (10:1) [213] Aa: 5.6 (3:1) [213] Aa: 4.7 (1:1) [213] Aa: 4.5 (1:3) [213] Aa: 4.0 (1:10 [213] Aa: >5 (1:1) [81][f] CqS: 2.5 (3:1) [208] CqS: 4.6 (3:1) [208] CqS: 8.6 (3:1) [208] CqS: 4.6 (3:1) [208] CqS: 6.6 (3:1) [208] CqS: 6.0 (3:1) [208] CqS: 3.0 (1:3) [199] CqS: 19 (3:1) [206] CqR3: 43 (3:1) [208] CqR3: 18 (1:3) [199] CqR6: 6.4 (3:1) [208] CqR7: 5.4 (3:1) [208] CqR8: 95 (3:1) [206] CqR9: 76 (3:1) [206] Ct: 0.5 (4:1) [69] Aal: Syn [41][g]	CqS: 0.8 (3:1) [200][e] CqS: 1.9 (3:1) [206] CqR2: 4.8 (3:1) [200][e] CqR3: 1.0 (3:1) [200][e] CqR4: 11 (3:1) [200][e] CqR5: 0.7 (3:1) [200] CqR8: 5.2 (3:1) [206] CqR9: 4.2 (3:1) [206]	CqS: 0.7 (1:1) [209] CqR1: 0.2 (1:1) [209] Cq: Syn, SF<9 [216][k]	CqS: 2.3 (1:1) [209] CqR1: 1.1 (1:1) [209]	
Cry4Aa + Cry4Ba + Cry11Aa + Cyt1A						CqS: 1.6 (1:1) [212][d] CqR5: 2.3 (1:1) [212]	CqS: 0.8 (1:1) [212][d] CqR5: 0.9 (1:1) [212]	

	B. sphaericus producing BinA/BinB	Cry4 Aa	Cry4 Ba	Cry11 Aa	Cry11 Ba	Mtx1	Mtx2	Cyt1Aa
Cyt2A or Cyt2B	Aa: 4.5 (10:1) 1d [204][m] Aa: 3.1 (3:1) 1d [204] Aa: 2.1 (10:1) 2d [204] Aa: 3.6 (3:1) 2d [204] CqS: 2.2 (10:1) 1d [204] CqS: 3.8 (3:1) 1d [204] CqS: 0.2[b] (10:1) 2d [204][m] CqS: 11 (3:1) 2d [204] CqR1: 26 (10:1) 1d [204] CqR1: 20 (3:1) 1d [204][m] CqR1: 195 (10:1) 2d [204] CqR1: 287 (3:1) 2d [204]		Aa: 33 (1:2) [141][o] Cq: 19 (1:2) [141][o]					

Table II-7b

Synergy factor between three or more toxins

Toxin 1	**Toxin 2**	**Toxin 3**	**Toxin 4**	**Synergy Factor**
Cry4Aa	Cry4Ba	Cry11Aa		Aa: 2.8 (1:1:12) [137]; Cp-d: 5.1 (2:2:1) [137]; As: 2.9 (1:1:64) [137]; CqS: 377 (1:1:1)[l] [212]
Cry4Aa	Cry4Ba	Mtx1		CqS: 2.6 (1:1:2)[l] [212]; CqR2: 12 (1:1:2)[l] [212]
Cry4Aa	Cry4Ba	Mtx2		CqS: 51 (1:1:2)[l] [212]; CqR2: 135 (1:1:2)[l] [212]
Cry4Aa	Cry4Ba	Mtx1	Cry11Aa	CqS: 24 (1:1:3:1)[l] [212]
Cry4Aa	Cry4Ba	Mtx2	Cry11Aa	CqS: 120 (1:1:3:1)[l] [212]
Cry4Ba	Cry11Aa	Cyt1Aa		AaI: Syn [41][g]
Bs (BinA/BinB)	Cry4A	Cyt1Aa		Aa: 19 (8:1:1) 1d [207]; Aa: 13 (8:1:1) 2d [207]; CqS: 4.8 (8:1:1) 1d [207]; CqS: 5.2 (8:1:1) 2d [207]; CqR1: 92 (8:1:1) 1d [207]; CqR1: 1530 (8:1:1) 2d [207]
Bs (BinA/BinB)	Cry11A	Cyt1Aa		Aa: 23 (8:1:1) 1d [207]; Aa: 17 (8:1:1) 2d [207]; CqS: 1.5 (8:1:1) 1d [207]; CqS: 1.1 (8:1:1) 2d [207]; CqR1: 28 (8:1:1) 1d [207]; CqR1: 78 (8:1:1) 2d [207]
Bs (BinA/BinB)	Cry4A + Cry4B	Cyt1Aa		Aa: 20 (8:1:1) 1d [207]; Aa: 14 (8:1:1) 2d [207]; CqS: 2.2 (8:1:1) 1d [207]; CqS: 4.0 (8:1:1) 2d [207]; CqR1: 4.8 (8:1:1) 1d [207]; CqR1: 32 (8:1:1) 2d

Toxin 1	Toxin 2	Toxin 3	Toxin 4	Synergy Factor
Bs (BinA/BinB)	Cry4A + Cry4B + Cry11A	Cyt1Aa		Aa: 4.2 (8:1:1) 1d [207]; Aa: 3.7 (8:1:1) 1d [207]; CqS: 1.4 (8:1:1) 1d [207]; CqS: 2.1 (8:1:1) 2d [207]; CqR1: 1.6 (8:1:1) 1d [207]; CqR1: 4.0 (8:1:1) 2d [207]
Bs (BinA/BinB)	Mtx1	Cyt1Aa		CqS: 0.9 (8:1:1) [209]; CqR1: 2.9 (8:1:1) [209]
Bs (BinA/BinB)	Mtx2	Cyt1Aa		CqS: 0.6 (8:1:1) [209]; CqR1: 44 (8:1:1) [209]
Bs (BinA/BinB)	Mtx1	Mtx2		CqS: 5.6 (8:1:1) [209]; CqR1: 12 (8:1:1) [209]
Cry11Bb	Cry29Aa	Cry30Aa		Aa: 3.2 (1:10:10) [78]; Cp: 1.1 (1:10:10) [78]; As: 1.1 (1:10:10) [78]

[a]: Cry10Aa is tested with Cyt1Aa both as spore complex as in a pure crystal form. In both tests, the SF is 13.

[b]: Calculated SF factor deviates with factor 1000 from factor given in [204].

[c]: The toxins are applied in the cells of the bacteria producing the toxins, the calculated SF is based on the assumption that the amount of the combination [Cry4Aa, Cry11Aa and p20] and of [Cyt1Aa] in the cells producing both groups is equal to the amount produced in cells producing only one of these group. 20kDa is a regulatory protein, promoting the production of the Cry proteins.

[d]: The data on the toxicity of Mtx1 and Mtx2 in the 2014 publication of Wirth *et al*. [212] are the same as in their 2007 publication [209].

[e]: The data on the toxicity of Cyt1A in the 1998 publication of Wirth *et al*. [200] are the same as in their 1997 publication [199].

[f]: Toxins produced in cloned *E. coli*., In *E. coli* producing Cyt1Aa also a 20kDa promoter was cloned, ratio based on cells instead of toxin weight.

[g]: Experiment with limited number of doses, not allowing EC50 estimation. Synergy observed.

[h]: SF factor given in paper [212] is 1.0, recalculation gave 0.1. According to Wirth (personal communication), the correct value is 1.0.

[i]: SF factor copied from paper [131] after adjustment for the normalisation of the sum of the ratios to 1, because these ratios were not standardized in the paper (Bravo, personal communications).

[j]: Mtx1 and Mtx2 expressed singly or together in *E. col*i. Expression of Mtx1 in *E.coli* with or without also Mtx2 about equal, expression of Mtx2 in *E.coli* with also Mtx1 much lower than in *E. coli* without Mtx1. For calculation SF, assumed expression in combination: equal for Mtx1, 0.5 times expression in *E.coli* with only Mtx2 for Mtx2.

[k]: According to the English abstract of a non-read paper in Chinese, the SF value between Cyt1Aa and Mtx1 is high in *C. quinquefasciatus* [216]. The data in the abstract show that SF<9.

[l]: Ratio between Cry4Aa, Cry4Ba and Cry11Aa not given in paper, calculated SF based on the assumption that this ratio is 1:1:1.

[m]: The calculated SF factor deviates from the factor given in the original publication [204]. M. Wirth is contacted about these deviations and will look into them.

[n]: The data on the toxicity of the Bs spores in the 2004 pubication of Wirth *et al*. [207] are the same as in their 2001 publication [204].

[o]: The Bt toxins were dosed as purified inclusions from cloned *E. coli* bacteria producing Cry4Ba or Cyt2A or the combination of these two toxins. The ratio for the combination is only approximately 1:2.

[p]: Cyt1A applied as spore crystals, LC50 for Bs spore was factor 3 higher after 48h than after 24 hours, suggesting a substantial control mortality between 24 and 48 hours. Or, if the LC50 values after 24h and 48h are compared with the LC50 values of *A. aegypti* to Bs spores in [204], a error of a factor 100 in the reported LC50(48h) for Bs spores.

[q]: Cyt1A was applied as technical purified crystals. Density of larva per ml water 10 times higher than in other experiments in [201], LC50(48h) for Bs spores almost factor 70 lower than in other experiment in [201].

CqR1:	Cq resistant to *Bacillus sphaericus* (see [207], [209], [201], [204]) This Bs strain (2362) only produces the BinA/BinB Bin toxin pair. This Cq strain is selected for resistance against Bs 2362 since 1992.
CqR2:	Cq resistant to Cry4Aa and Cry4Ba. The Cq strain was selected since 1991 (in [199] for approx. 27 generations). (see [199], [212], [200])
CqR3:	Cq resistant to Cry11Aa. In [199], [212] and [208], the Cq strain was selected since 1991 (in [199] for approx. 27 generations), in [208] Cq was selected for approx. 48 generations. (see [199], [212], [200], [208])
CqR4:	Cq resistant to Cry4Aa, Cry4Ba and Cry11Aa. The Cq strain was selected since 1991 (in [199] for approx. 27 generations). (see [199], [212], [200])
CqR5:	Cq resistant to Cry4Aa, Cry4Ba, Cry11Aa and Cyt1A. The Cq strain was selected since 1991. (see [212], [200])
CqR6	Cq resistant to Cyt1A, selected for approx. 48 generations (see [208])
CqR7	Cq resistant to Cry11Aa and Cyt1A, selected for approx. 48 generations (see [208])
CqR8	Cq resistant to *Bacillus thuringiensis* subsp. *jegathesan.* This Bt produces 7 toxins, including Cry11B, Cry19A and Cyt2Bb1. Selection during approx. 40 generations [206].
CqR9	Cq resistant to Cry11B. Selection during approx. 40 generations. (see [206])

Table II-8.
Synergy factor between the alleged conjugative couple of the Cry48 and Cry49 proteins. The calculated synergy factor (SF) is based on EC50 values, with effect mortality after 48 hours. Each line is one test, giving: *species*: *SF* (*ratio*) *reference*. Each line is one test, giving: species: SF (ratio) reference. For the ratio between the two proteins (values between brackets), first the column protein, then the row protein (w/w) is given.
The only species in the table is the Diptera *Culex quinquefasciatus* (Cq).

	Cry49Aa
Cry4Aa	Cq: Slight (1:3) [77][a]
Cry48Aa	Cq: >31 (1:0.25) [76] Cq: >150 (1:2.5) [76] Cq: >42 (1:25) [76]

[a]: No LD50 is measured for the mixture. At 150 µg/ml of Cry4Aa, the mortality is 81% if 50 µg/ml Cry49Aa is added and without 47% for Cry4Aa alone. Cry49Aa alone is not toxic up till 200 µg/ml [77].

Table II-9.
Synergy factor between Cry6 and Cry5 and Cry55 proteins. The calculated synergy factor (SF) is based on EC50 values, with effect mortality. Each line is one test, giving: *species*: *SF* (*ratio*) *reference*. Each line is one test, giving: species: SF (ratio) reference. For the ratio between the two proteins (values between brackets), first the column protein, then the row protein (w/w) is given.
The species in the table are both Nematoda (Secernentea), the species abbreviations are: Ce: *Caenorhabditis elegans*, Mi: *Meloidogyne incognita*.

Table II-9a: Synergy factor between two (group of) toxins.

	Cry5Ba/ Cry5Ba2	Cry21Fa	Cry21Ga	Cry55Aa
Cry5Ba/ Cry5Ba2				Mi: 1.1 (1:1) [130]
Cry6Aa/ Cry6Aa2	Ce: 1.7 (4:1) [219] Ce: 1.9 (2:1) [219] Ce: 1.5 (1:1) [219] Ce: 1.6 (1:2) [219] Ce: 2.7 (1:4) [219] Mi: 1.9 (1:1) [130]			Mi: 3.5 (1:5) [130] Mi: 3.9 (1:2) [130] Mi: 5.0 (1:1) [130] Mi: 3.6 (2:1) [130] Mi: 2.4 (5:1) [130]
Cry1Ga		Ce: no syn/anta [71]		
Cry1Ha		Ce: 2.6 (2:1) [71] Ce: 2.0 (1:1) [71] Ce: 2.4 (1:2) [71]	Ce: no syn/anta [71]	

Table II-9b: Synergy factor between three or more toxins

Toxin 1	**Toxin 2**	**Toxin 3**	**Toxin 4**	**Synergy Factor**
Cry5Ba	Cry6Aa	Cry55Aa		Mi: 1.3 (1:1:1) [130]

II-2.2 Cross-Resistance of Bt toxins

A species strain will often become resistant to a toxin if individuals of that strain are exposed to a toxin during several generations. For a pesticide to remain effective, the development of resistance has to be prevented as much as possible. Based on the assumption that resistance has some fitness costs, two policies are followed:

1. Switching between different toxins, hoping that resistance against the first toxin is lost during the period that the second toxin is used.
2. The usage of several toxins together, hoping that it is to difficult to develop resistance to the toxin cocktail.

Both methods can only be effective if resistance against one of the toxins does not lead to resistance to the other toxins used. Resistance induced by one toxin (A) to another toxin (B) is called cross-resistance.
The resistance rate (RR) of a strain is calculated as the ED50 in the resistant strain divided by the ED50 in a susceptible reference strain. The higher the RR, the more resistant the strain. RR values of over 5000 are reported, for instance for a *Chrysomela scripta* strain resistant to Cry3A [40] and a *Spodoptera frugiperda* strain resistant to Cry1F [194]. To determine the Cross Resistant Ratio (CRR) the RR for the toxin is divided by the RR for the toxin which had induced the resistance. An CRR of 1 implies that the strain has become equally resistant to that toxin as to the toxin used to select for the resistance.
When several Bt toxin genes are incorporated in a crop species strain (an event), the intention can be the prevention of development of resistance against each of these toxins, or at least of resistance to all of the toxins in the toxin cocktail. Therefore, the cross resistance is often investigated and preferably toxin mixtures are used without any cross-resistance.
The literature is scanned for the term “Cross-resistance” in combination with *Bacillus thuringiensis*, leading to a limited dataset on cross-resistance without any claim to completeness. These data are listed in tables II-10, II-11, II-12 and II-14. Note that studies from which the cross-resistance ratio can be estimated are scarce because to assess the cross-resistance ratio, the sensitivity has to be compared of two strains, both descending from the same parent strain, one of these selected for resistance against a toxin and the other not. For both Diptera and Coleoptera, data on only one species are available, Culex *quinquefasciatus* and *Chrysomela scripta*, respectively. For Lepidoptera, data on 10 species are available, all target species, and half of these species belong to the family of Noctuidae.

The information in the tables suggests for the Lepidoptera toxins (tables II-10 and II-14) that:

1. Resistance against Cry1Ab induces resistance against Cry1Ac, Cry1C, Cry1D and sometimes also against Cry1F.
2. Resistance against Cry1Ac induces resistance against Cry1Aa, Cry1Ab and Cry1F, and not against Cry1Ba, Cry1C, Cry1D, Cry2Ab and Vip2Aa and sometimes against Cry2Aa.
3. Resistance against Cry1C does not induce resistance against Cry1Bb, Cry9Aa or

Cry9C. It often goes hand in hand with resistance against Cry1Aa, Cry1Ab, Cry1Ac, Cry1D, Cry1F and Cry1J. The results of resistance tests with a *P. xylostella* strain selected to be resistant to Cry1C and susceptible for Cry1Ac shows that resistance to Cry1C is not necessarily combined with resistance to Cry1Aa, Cry1Ab, Cry1D, Cry1F and Cry1J. This suggests that resistance to Cry1C does not have to use the same mechanism as resistance against these toxins with potential cross-resistance to Cry1C.

4. Resistance against Cry1D does not induce resistance against Cry1Ab and Cry1C, but does induce resistance against Cry1Ac.
5. Resistance against Cry2Ab induces resistance against Cry1Ac and Cry2Aa

This shows a rather complicated pattern, suggesting that resistance against most of the Cry toxins can develop in different ways. For instance, it suggests three potential resistance mechanisms against Cry1Ac. One shared with Cry1D but not with Cry1Ab, one shared with Cry1Ab but not with Cry1D and a third shared with both Cry1Ab and Cry1D. Given the complicated mode of action of Cry toxins, involving many separate sequential steps, the existence of several resistance mechanisms, disrupting the action of varying groups of Cry proteins, is to be expected.

For *C. quinquefasciatus* table II-11 suggests that

1. The resistance against Cry11Ba induces resistance against Cry11Aa and vice versa, and resistance against each of these Cry11 toxins induces cross-resistance against the combination of Cry4Aa and Cry4Ba and vice versa.
2. Resistance against Mtx1 is not induced by resistance against the Bin toxins BinA/BinB produced by *B.sphaericus*.

For the Coleoptera, only one dataset is available (table II-12). These data indicate that resistance against Cry3A can also lead to resistance against Cyt1B, but not against Cyt1A.

The observed complicated cross-resistance pattern makes a simple additive or independent interaction in the combination toxicology of two Cry toxins very unlikely. If no cross-resistance exists, absence of interaction would lead to a independent effect model, whereas an cross-resistance of approximately 1 suggests the additive model in absence of interaction. The complicated cross-resistance pattern does not necessarily imply that the toxins have a positive or negative interaction. For instance, if the toxic action of both toxins involve two receptors with sequential binding, both toxins might share the first receptor, leading to an additive effect for that receptor, whereas they might use different independent receptors as second receptor, leading to an independent effect for the second receptor[35].

Only for a limited number of pairs, information is available both on synergy and cross-resistance. These data are shown in table II-13. Only in one case, data are available on cross-resistance and synergy for the same species and toxin combination: In *Helicoverpa armigera* Cry1Ac and Cry2Ab do not show synergy, and resistance to Cry1Ac does not

35 Note that even if it would be known that the additive model is incorrect, the size of the positive or negative interaction would still be expressed as observed interaction compared to the interaction predicted by the additive model.

lead to cross-resistance to Cry2Ab. The absence of cross-resistance suggests that the mode of action of the Cry toxins differs, whereas absence of synergy is something like the default option. Most combinations of mode of action will lead to an absence of synergy, only very special combinations may induce synergy. And since the SF of all toxin combinations for which also CRR are available varies between 0.2 and 4.5, so hardly deviating from the neutral SF of 1, differences in the effect on cross-resistance between synergistic combinations and neutral combinations cannot be observed. Therefore, the available experimental information on cross-resistance cannot be used to evaluate the potential relationship between cross-resistance and synergy. Theoretically, it seems likely that strong cross-resistance points to a similar mode of action impeding the action of both toxins with the same mechanism, and with a similar mode of action the additive model for interaction, that is an absence of synergy, becomes more likely.

Table II-10.
Cross resistance within the group of Cry1, Cry2 and Cry9 toxins and between these toxin group and Vip3 toxin. The Cross-resistance ratio is the ratio of the RR for the given toxin and the resistance ratio for the toxin which has induced the resistance. If RR<2, no (substantial) resistance is observed, so no cross-resistance exists (NCR). The species is marked **Blue**: CRR<0.02 or NCR; **Yellow**: 0.02≤CRR<0.2; **Red**: 0.2≤CRR<2; **Dark red with yellow symbols**: 2≤CRR.
Each line is one test, giving: *species*: C*RR* (RR) *reference*.
Data on the Ledidoptera species Ha: *Helicoverpa armigera*[a], Hv: *Heliothis virescens*, On: *Ostrinia nubilalis*[b], Pg: *Pectinophora gossypiella*, Pi: *Plodia interpunctella*, Px: *Plutella xylostella*[c], Sex: *Spodoptera exigua,* Sli: *Spodoptera littoralis*
Complete dataset is given in table II-14.

	Insect strain resistant to:					
Tested with	Cry1Ab	Cry1Ac	Cry1C/Cry1Ca	Cry1Da	Cry2Ab	Dipel[d]
Cry1Aa		**Px: 0.34** (13) [50]	**Px: 0.36** (400) [225] **PxS: 0.008** (3.9) [225] **Sli: >0.57** (>80) [120]			**Pi: 0.09** (6.5) [115]
Cry1Ab		**Hv: 0.8** (13) [53] **Px: 0.04** (3.4) [158] **Px: 0.45** (18) [50]	**Px: 0.55** (600) [225] **PxS: NCR** [225] **Px: 1.1** (2.5) [158] **Sli: 0.02** (2.5) [120]	**Px: NCR** [158]		**Pi: 3.4** (250) [115]
Cry1Ac	**OnE: 3.6** (35) [168] **OnN: 3.8** (7.2) [168] **OnR1: >0.8** (>550) [29] **Px: 7.9** (40) [158]		**Px: 40** (44000) [225] **PxS: NCR** [225] **Px: 1.9** (4.2) [158]	**Px: 0.76** (2.3) [158]	**Pg: 1.9** (120) [182] **Pg: 0.24** (3.3) [182]	**Pi: 38** (2800) [115]
Cry1Ba		**Px: NCR** [165]				**Pi: 0.18** (13) [115]
Cry1Bb			**Px: 0.002** (2.3) [225] **PxS: 0.009** (4.7) [225]			
Cry1C/ Cry1Ca	**Px: 0.46** (2.3) [158] **Sex: na** (>20) [64]	**Px: 0.03** (2.4) [158] **Px: NCR** [165]		**Px: NCR** [158]		**Pi: 0.03** (2.1) [115]
Cry1Ac + Cry1C			**Px: 13** (14000) [225] **PxS: 0.005** (2.4) [225]			
Cry1Da	**Px: 0.47** (2.4) [158] **Sex: <0.86** (26) [64]	**Px: 0.03** (2.7) [158]	**Px: NCR** [225] **PxS: NCR** [225] **Px: 1.0** (2.2) [158] **Sli: 0.38** (3.9) [120] **Sli: 0.05** (7.1) [120]			

	Insect strain resistant to:					
Tested with	Cry1Ab	Cry1Ac	Cry1C/Cry1Ca	Cry1Da	Cry2Ab	Dipel[d]
Cry1Ea			**Sli: 0.74** (7.6) [120] **Sli: 0.25** (34) [120]			
Cry1F	**OnE: 0.5** (4.7) [168] **OnN: NCR** [168] **OnR1: 0.008** (5.8) [29] **Sex: na** (>8) [64]	**Px: >0.40** (>16) [50]	**Sli: NCR** [120] **Px: 7.2** (7900) [225] **PxS: NCR** [225]			
Cry1Ja			**Px: 12** (13000) [225] **PxS: 0.006** (2.9) [225]			
Dipel[d]		**Px: >0.07** (2.7) [50]				
Cry2Aa		**Px: NCR** [50] **Hv: 3** (53) [53]			**Pg: 0.9** (13) [182]	**Pi: 0.08** (5.5) [168]
Cry2Ab		**HaR1: NCR** [109]				
Cry9Aa			**Px: 0.003** (2.9) [225] **PxS: NCR** [225]			
Cry9C/ Cry9Ca	**OnE: 0.2** (2.0) [168] **OnN: NCR** [168]		**Px: 0.003** (3.5) [225] **PxS: NCR** [225]			
Vip3A		**Hv: NCR** [74]				

[a]: HaR1 is a resistant strain from *Helicoverpa armigera*, selected for 75 generations from the susceptible strain used in the comparison [109].
[b]: OnE: European On strain, OnN: On strain from Nebraska; OnR1: Strain selected for 26 generation, compared with not-selected strain derived from the same colony.
[c]: PxS: Strain of Px selected to be sensitive to Cry1Ac.
[d]: The combination indicated as “Dipel” can be applied in several formulations: As Dipel, as spores of *B. thuringiensis* subs *kurstaki*, or as HD-1 spore.

Table II-11.
Cross resistance within the group of Cry4, Cry11 and BinA/BinB and between these toxin group and Cry19A and Mtx1. The Resistance Ratio (RR) is the ratio of the ED50 in the susceptible strain and the ED50 in the resistant strain. The Cross-resistance ratio is the ratio of the RR for the given toxin and the resistance ratio for the toxin which has induced the resistance. If RR<2, no (substantial) resistance is observed, so no cross-resistance exists (NCR). The CRR is marked **Blue**: CRR<0.02 or NCR; **Yellow**: 0.02≤CRR<0.2; **Red**: 0.2≤CRR<2; **Dark red with yellow symbols**: 2≤CRR.
Each line is one test, giving: [*CRR*] (*RR*) *reference*. The CRR is only given if the control strain descends from the same parent strain as the resistant strain. All data are on the Diptera *Culex quinquefasciatus*.
In **Bold**: the susceptible and resistant strain descend from the same parent strain.

	Culex quinquefasciatus resistant to:						
Tested with	*B. sphaericus* (BinA/BinB)	Cry4Aa +Cry4Ba	Cry11Aa	Cry4Aa + Cry4Ba + Cry11Aa	Cry4Aa + Cry4Ba + Cry11Aa + Cyt1Aa	Cry11Ba	*B. thuringiensis*, subsp. *jegathesan*[a]
Bt *jegathesan*[a]		**0.06** (**2.5**) [200]	**0.5** (**3.3**) [200]	**0.06** (**3.9**) [200]	(3.5) [200]	**0.39** (**2.55**) [206]	(4.1) [206]
Bs (BinA/ BinB)	(>80000) [198]						
Bt *israelensis*[b]		**NCR** (**1.5**) [200]	**0.5** (**3.5**) [200]	**0.07** (**4.7**) [200]	(6.3) [200]	**NCR** (**1.35**) [206]	**NRC** (**0.93**) [206]
Cry4Aa + Cry4Ba	(0.60) [203]	(40) [200]				**4.2** (**28**) [206]	**2.5** (**10**) [206]
Cry4Aa + Cyt1Aa		(1.9) [210]	**1.8** (**12**) [210] **0.28** (**1.9**) [210]	(2.9) [210]	(2.1) [210]	(71) [210] (1.9) [210]	(10) [210] (1.4) [210]
Cry11Aa	(0.64) [203]	**0.13** (**5.1**) [200] (6.6) [210] (20) [210]	(7.4) [200] (6.8) [210] (15) [210]	**0.10** (**7.1**) [200] (8.2) [210] (11) [210]	(25) [200] (8.5) [210] (52) [210]	**2.7** (**18**) [206] (13) [210]	**5.1** (**21**) [206] (29) [210]
Cry4Aa + Cry4Ba + Cry11Aa	(0.96) [203]			68 [200]		**2.2** (**15**) [206]	**1.2** (**5.0**) [206]
Cry4Aa + Cry4Ba + Cry11Aa + Cyt1Aa	(1.8) [203]						
Cry11Aa + Cyt1Aa		(3.3) [210] (3.7) [210]	**1.3** (**8.5**) [210] **0.75** (**5.1**) [210]	(14) [210] (7.4) [210]	(84) [210] (13) [210]	(9.8) [210] (3.9) [210]	(8.4) [210] (12) [210]
Cry11Ba		**0.24** (**9.7**) [200]	**1.2** (**9.2**) [200]	**0.82** (**56**) [200]	(3.0) [200]	(6.6) [206]	**1.2** (**5.1**) [206]
Cry11Ba + Cyt1Aa		**NCR** (**1.6**) [200]	**1.0** (**7.1**) [200]	**0.05** (**3.7**) [200]	(3.3) [200]		
Cyt1Aa		**NCR** (**0.85**) [200]	**NCR** (**0.91**) [200]	**NCR** (**1.1**) [200]	(8.2) [200]	**0.55** (**3.6**) [206]	**NCR** (**2.0**) [206]
Cry19A		(1.5) [205]	(2.0) [205]	(3.0) [205]	(1.2) [205]	**0.53** (**3.5**) [206]	**0.78** (**3.2**) [206]
Mtx1	**NCR** (**1.7**) [198] **NCR** (**1.3**) [198]						

[a]: *B. thuringiensis*, subsp. *jegathesan* produces 7 toxins, including Cry11B, Cry19A and Cyt2Bb1. This resistant strain was selected during approx. 40 generations.
[b]: *B. thuringiensis*, subsp. *israelensis* produces Cry4A, Cry4B, Cry10A, Cry11A and Cyt1A

Table II-12.
Cross resistance within between Cry3 and some Cyt toxins. The Resistance Ratio (RR) is the ratio of the ED50 in the susceptible strain and the ED50 in the resistant strain. The Cross-resistance ratio is the ratio of the RR for the given toxin and the resistance ratio for the toxin which has induced the resistance. If RR<2, no (substantial) resistance is observed, so no cross-resistance exists (NCR). The CRR is marked **Blue**: CRR<0.02 or NCR; **Yellow**: 0.02≤CRR<0.2; **Red**: 0.2≤CRR<2; **Dark red with yellow symbols**: 2≤CRR.
Each line is one test, giving: *CRR* (*RR*). All data are on the Coleoptera *Chrysomela scripta* from [40].
The susceptible and resistant strain descend from the same parent strain.

	Chrysomela scripta resistant to
	Cry3A
Cry3A	**(>5000)**
Cyt1A	**0.006 (2.9)**
Cyt1B	**0.80 (400)**

Table II-13.
Synergy and cross-resistance for combinations of Cry toxins.
In the rows “synergy” the species with its synergy factor (SF) is listed (a range if more values are available) and in the rows “cross-resistant with resistance” and “resistance leads to cross-resistance of” the species with its cross-resistance ratio (CRR). A SF of 1 implies no synergism or antagonism, a CRR of 1 implies equal resistance as to the toxin which had induced the resistance. If the CRR is small or the Resistance Ratio (RR) <2, that is no (substantial) resistance is observed, no cross-resistance exists (NCR).
Data on the Ledidoptera species Cpa: *Chilo partellus*, Cs-l: *Chilo suppressalis*, Ea: *Epinotia aporema*, Ei: *Earias insulana*, Ek: *Ephestia kuehniella*, Ev: *Earias vittella*, Ha: *Helicoverpa armigera*, Hv: *Heliothis virescens*, Ld: *Lymantria dispar*, On: *Ostrinia nubilalis, Pc: Perileucoptera coffeella*, Pg: *Pectinophora gossypiella*, Px: *Plutella xylostella*[a], Sex: *Spodoptera exigua*, Sli: *Spodoptera littoralis.*

		Cry1Ac	Cry1Ba	Cry1Ca	Cry1Da	Cry1Fa	Cry2Aa	Cry2Ab
Cry1Aa	synergistic with	**Cpa: 1.5 Ea: 0.5 Ek: 0.9-2.6 Ld: 2.7-7.3**		**Cs-l: 1.6 Si: 0.1 Ha: 1.4-2.7 Sex: 2.0-4.0**				
	cross-resistant with resistance to	**Px: 0.34**		**Px: 0.36 PxS: 0.008 Sli: >0.57**				
Cry1Ab	synergistic with	**Ea: 0.7 ; Si: 0.7 Ha: ±1 Ld: 1.5 Cpa: 2.0-5.2 Cs-l: 2.5**		**Si: 0.2 Cs-l: 4.4**				
	cross-resistant with resistance to	**Hv: 0.8 Px: 0.04 Px: 0.45**		**Px: 0.55 PxS: NCR Px: 1.1 Sli: 0.02**				
	resistance leads to cross-resistance of	On: 0.8--3.8 Px: 7.9		Px: 0.46 Sex: ? (RR=20)				
Cry1Ac	synergistic with		**Si: 0.2 Pc: 0.9 Cs-l: 4.5**	**Si: 0.6 Cs-l: 3.0**		**Ha: 0.6-1.7 Ei: 0.8-1.1**	**Ha: 0.4-0.8; 12-26 Ek: 0.3-1.1 Ev: 3.0-14**	**Ha: 0.3-2.4 Ei: 0.9-1.1**
	cross-resistant with resistance to			**Px: 40 PxS: NCR Px: 1.9**				Pg: 0.2-1.9
	resistance leads to cross-resistance of		**Px: NCR**	Px: NCR (0.03)		**Px: >0.40**	**Px: NCR Hv: 3**	**Ha: NCR**
Cry1Ca	synergistic with				**Ea: 0.8**			
	cross-resistant with resistance to				**Px: NCR**			
	resistance leads to cross-resistance of				Px: NCR-1.0 PxS: NCR Sli: 0.05-0.4			

[a]: PxS: Strain of Px selected to be sensitive to Cry1Ac.

Table II-14.
Cross resistance within the group of Cry1, Cry2 and Cry9 toxins and between these toxin group and Vip3 toxin. The Resistance Ratio (RR) is the ratio of the ED50 in the susceptible strain and the ED50 in the resistant strain.
Each line is one test, giving: *species*: *RR* (*ratio*) *reference* [*T* (if toxin is trypsin activated)/*D/H/K* as source of Dipel-toxins[e]].
Data on the Ledidoptera species Ds: *Diatraea saccharalis*[a], Ha: *Helicoverpa armigera*[b], Hv: *Heliothis virescens*, On: *Ostrinia nubilalis*[c], Pg: *Pectinophora gossypiella*, Pi: *Plodia interpunctella*, Px: *Plutella xylostella*[d], Sex: *Spodoptera exigua*, Sf: *Spodoptera frugiperda*, Sli: *Spodoptera littoralis.*
In **Bold**: the susceptible and resistant strain descend from the same parent strain.

	Insect strain resistant to:						
Tested with	Cry1Ab	Cry1Ac	Cry1C/Cry1Ca	Cry1Da	Cry1F/Cry1Fa	Cry2Ab	Dipel[e]
Cry1Aa	DsR1: 190 [224] DsR2: 290 [224] DsR3: 100 [224] DsR4: 160 [224] DsR5: 76 [224] DsR6: 94 [224] DsR7: 71 [224] Ds: >80 [214]	**Px: 13** [50]	**Px: 400** [225] **PxS: 3.9** [225] Px: 3.1 [158] **Sli: >80** [120]				**Pi: 6.5** [115] H
Cry1Ab	DsR1: >500 [224] DsR2: >500 [224] DsR3: >500 [224] DsR4: 130 [224] DsR5: 120 [224] DsR6: 120 [224] DsR7: >500 [224] **OnE: 9.9** [168] **OnE: 39** [168] T **OnN: 1.9** [168] **OnN: 8.9** [168] T **OnR1: 680** [29] T OnR2: 5400 [29] T OnR2: 3300 [29] **Px: 5.0** [158] **Sex: >30** [64]	**Hv: 13** [53] **Px: 3.4** [158] **Px: 18** [50]	**Px: 600** [225] **PxS: 1.4** [225] **Px: 2.5** [158] Px: 19 [105] **Sli: 2.5** [120]	**Px: 1.5** [158]	Sf: >160 [194]		**Pi: 250** [115] H

	Insect strain resistant to:						
Tested with	Cry1Ab	Cry1Ac	Cry1C/Cry1Ca	Cry1Da	Cry1F/Cry1Fa	Cry2Ab	Dipel[e]
Cry1Ac	DsR1: 270 [224] DsR2: 30 [224] DsR3: 62 [224] DsR4: 250 [224] DsR5: 41 [224] DsR6: 75 [224] DsR7: 38 [224] Ds: 45 [214] **OnE: 35** [168] **OnN: 7.2** [168] **OnR1: >550** [29] T OnR2: >2800 [29] T OnR2: >37000 [29] **Px: 40** [158]	**HaR1: 3000** G [109] HaR2: 250 G [109] **Hv: >1000** [74] Hv: 210 [74] Hv: 320 [74] Pg: 980 [182] **Px: 88** [158] **Px: 39** [50]	**Px: 44000** [225] **PxS: 1.9** [225] **Px: 4.2** [158] Px: 20 [105]	**Px: 2.3** [158]	Sf: >290 [194]	**Pg: 120** [182] **Pg: 3.3** [182]	**Pi: 2800** [115] H
Cry1Ba		**Px: 1.3** [165]			Sf: >130 G [194]		**Pi: 13** [115] H Px: 5.4 [178] D
Cry1Bb			**Px: 2.3** [225] **PxS: 4.7** [225]				Px: 5.8 [179] D
Cry1Bh					On: 0.6 [103]		
Cry1C/ Cry1Ca	**Px: 2.3** [158] **Sex: >20** [64]	**Px: 2.4** [158] **Px: 2.0** [165]	**Px: 1100** [225] **PxS: 500** [225] **Px: 2.2** [158] Px: 11 [105] Px: 17 [105] **Sli: 10** [120] **Sli: >140** [120]	**Px: 1.8** [158]			**Pi: 2.1** [115] H Px: 1.2 [176] D
Cry1Ac + Cry1C			**Px: 14000** [225] **PxS: 2.4** [225]				
H04 (hyb. Cry1Ab & Cry1Ca)							Px: >500 [179] D

	Insect strain resistant to:						
Tested with	Cry1Ab	Cry1Ac	Cry1C/Cry1Ca	Cry1Da	Cry1F/Cry1Fa	Cry2Ab	Dipel[e]
Cry1Da	**Px: 2.4** [158] **Sex: 26** [64]	**Px: 2.7** [158]	**Px: 1.5** [225] **PxS: 1.0** [225] **Px: 2.2** [158] **Sli: 3.9** [120] **Sli: 7.1** [120]	**Px: 3.1** [158]			Px: 3.2 [179] D
Cry1Ea			**Sli: 7.6** [120] **Sli: 34** [120]				
Cry1F	DsR1: 6.9 [224] DsR2: 1.5 [224] DsR3: 4.9 [224] DsR4: 5.2 [224] DsR5: 3.9 [224] DsR6: 2.4 [224] DsR7: 5.2 [224] **OnE: 4.7** [168] **OnN: 1.0** [168] **OnR1: 5.8** [29] T **Sex: >8** [64]	**Px: >16** [50]	**Px: 7900** [225] **PxS: 1.7** [225] Px: 7.4 [105] **Sli: 0.8** [120] **Sli: 1.0** [120]		On: >3000 [103] Sf: >290 [194]		Px: >250 [178] D
Cry1A.105 (Hyb. Cry1Ab, Cry1Ac & Cry1F)	Ds: 4.1 [214]						
Cry1Ia							Px: 2.5 [179] D
Cry1Ja			**Px: 13000** [225] **PxS: 2.9 [225]** Px: 0.9 [105]				Px: >140 [179] D
Dipel[e]		**Px: 2.7** [50] K					**Pi: 73** [115] H
Cry2Aa		**Hv: 53** [53] **Px: 1.2** [50]	Pg: 2.9 [182]			**Pg: 13** [182]	**Pi: 5.5** [115] H
Cry2Ab	Ds: 0.5 [214]	**HaR1: 1.1** G [109] HaR2: 1.0 G [109]	Pg: 1.9 [182]			**Pg: 64** [182] **Pg: 14** [182]	
Cry9Aa			**Px: 2.9** [225] **PxS: 1.5** [225]				

	Insect strain resistant to:						
Tested with	Cry1Ab	Cry1Ac	Cry1C/Cry1Ca	Cry1Da	Cry1F/Cry1Fa	Cry2Ab	Dipel[e]
Cry9C/ Cry9Ca	**OnE: 2.0** [168] **OnN: 0.5** [168]		**Px: 3.5** [225] **PxS: 1.4** [225] Px: 2.1 [105]				
Vip3A		**Hv: 1.2** [74] Hv: 1.0 [74] Hv: 1.2 [74]			Sf: 1.3 [194]		

[a]: DsR1 is a *D. saccharalis* strain known to be resistant to Cry1Ab, Ds strains DsR2 to DsR7 were developed from 8 single pair families possessing resistance alleles to Cry1Ab Maize [224].

[b]: HaR1 and HaR2 are two resistant strains from *Helicoverpa armiger*a, HaR1 is selected for 75 generations from the susceptible strain used in the comparison, HaR2 is selected from a field strain [109].

[c]: OnE: European On strain, OnN: On strain from Nebraska
OnR1: Strain selected for 26 generation, compared with resistant strain derived from the same colony, OnR2 selected for 55 generations, susceptible strain from other source.

[d]: PxS: Strain of Px selected to be sensitive to Cry1Ac.

[e]: The combination indicated as “Dipel” can be applied in several formulations: As Dipel, indicated with D, as spores of *B. thuringiensis* subs *kurstaki*, indicated with K, as HD-1 spore, indicated as H.

II-3 Co-evolution of toxins.

Many Bt Cry toxins are isolated from bacterial strains producing also some other toxins. Cry toxins are very complex proteins, and therefore it is assumed that they are evolved under some selection pressure favouring the toxic properties of these proteins. Similarly, it is assumed that if two or more of these complex proteins are evolved in the same bacterial strain, selection pressure favours their co-occurrence. Therefore, most investigations on interaction between the toxicological properties of Cry proteins are performed on couples of Cry proteins produced by the same bacterial strain. Examples are:

- A Bt subsp *israelensis* strain produces Cry4Aa, Cry4Ba, Cry10Aa, Cry11Aa, Cyt1A and Cyt2A, and the interaction between these toxins is described in several publications of Margaret Wirth [199], [211].
- A Bt subsp. *jegathesan* strain produces Cry11B and Cry19A, and the interaction between these toxins is also studied by the group of Margaret Wirth [206].
- A Bt subsp. *kurstaki* strain produces Cry1Aa, Cry1Ab, Cry1Ac, Cry1I, Cry2A and Vip3A. The interaction of combinations of these toxins is frequently studied, see for instance, [12], [17], [92], [101], [164], [189], [220].
- Cry5Ba, Cry6Aa and Cry55Aa are found together is a Bt strain, and their interaction is investigated [130], [219].

There is little experimental evidence that Cry toxins which are not naturally co-occurring in the same bacterial strain show less synergy than Cry toxins which are co-occurring in bacterial strains, simply because the effect of couples of not-co-occurring Cry toxins is almost not investigated. The suggestion that the probability of synergy is slightly higher for co-evolved and therefore co-occurring Cry toxins is, however, not unreasonable.

On the website of the Sussex university [w1] all classified Cry, Cyt and Vip proteins are listed with information on the strain from which that protein was isolated. For each bacterial strain in that list, all sequenced Cry proteins isolated from that strain are included in that list. Each Cry protein isolated from a bacterial strain, even if it is identical to an already described Cry protein, will be given an unique number. In case of identity, it will be given a new quaternary number in the Cry nomenclature. So each newly isolated Cry protein will be included in the list as separate Cry protein. The Cry (including Cyt) and Vip list from that website [w1] is used to make a list of all bacterial strains known to produce at least two different Cry, Cyt or Vip proteins (see Appendix E, p. 183). In table II-15 these data are summarized. For each Cry, Cyt and Vip protein which has been observed to be produced in a strain also producing at least one other Cry, Cyt or Vip protein, it has been listed which other Cry, Cyt and/or Vip protein have been isolated from it. For most Cry proteins, only the first rank number is given (e.g. Cry7). For some well documented Cry proteins with many secondary (and tertiary) variations, the second rank code (e.g. Cry4A) or even third rank code (e.g. Cry1Aa) number is also given.

Studying table II-15 it is notable that the coupled toxins are often active against the same taxonomic group. However, this cannot be considered as an indication that coupled toxins

are usually active against the same taxonomic group. It could just as well purely be the consequence of the fact that the activity of the less studied toxins of the coupled toxins are only tested for the taxonomic group known to be sensitive to the more studied partners in that couple.

Unfortunately, the list from the Sussex University website [w1] is not complete. Isolated Cry proteins with sufficiently strong sequence relationship to an already documented Cry to get the same secondary or even tertiary rank code in the nomenclature system are sometimes not submitted to the nomenclature committee. For instance, in a study on Bt Cry proteins in Bt strains isolated from samples from Nashville, Tennessee, Rolle [150] found combinations of several lepidopteran-active Cry1 proteins with the dipteran-active Cry4, Cry10 and Cry11 proteins, showing the combination of toxins active against different target groups in the same bacterial strain. These Cry isolates are not (yet) incorporated in the official Cry list.

More on theoretical grounds than based on experimental evidence, the following hypothesis can be formulated:
If the Bt toxins are co-produced by some natural bacterial strain, and assuming that the proteins have co-evolved and the toxin property is advantageous for the bacterial strain, it seems likely that these toxins either

(1) act against different taxonomic groups (perform different tasks for the bacteria)
(2) do not induce cross-resistance against each other (are more effective against the target group because it is more difficult for the target to become resistant) or
(3) are synergistic to each other (increase the activity more than producing an extra quantity of only one of these toxins would do).

Note that the hypothesis of co-evolution of Cry genes is less probable for Cry genes located on plasmids.

Table II-15.
List of Cry, Cyt or Vip protein which have been isolated from a strain also producing other Cry, Cyt or Vip proteins. For each protein in the first column, the list is given of proteins which have been isolated from a strain which also produced that first column protein. Data based on the protein lists on the Sussex University website [w1][36]. For more details, see Appendix E (p. 183).

Protein	Cate-gory[a]	active against[b]	Isolated from the same strain for at least one of the protein variations
Cry1Aa	3D	lep, dip, ?hem	Cry1Ab; Cry1Ac; Cry1Ia; Cry2Aa; Cry2Ab; Cry2Ac; Cry24B; Cry30B/C; Cry39; Cry50; Cry58; Vip3Aa
Cry1Ab	3D	lep, dip, ?hem	Cry1Aa; Cry1Ac; Cry1Da; Cry1I; Cry2Aa; Cry2Ab; Cry9; Vip3Aa
Cry1Ac	3D	lep, dip, ?hem	Cry1Aa; Cry1Ab; Cry1C; Cry1E; Cry1Ia; Cry2Aa; Cry2Ab; Cry8; Vip3Aa
Cry1A•[c]	3D	lep, dip, ?hem	Cry1E; Cry2Ah; Cry1Ca; Cry1Fa
Cry1B	3D	lep, dip, col	Cry1C; Cry1F; Cry1G; Cry1H; Cry1J; Cry1K; Vip3Aa
Cry1C	3D	lep, dip	Cry1Ac; Cry1Ad; Cry1B; Cry1F; Vip3Aa
Cry1D	3D	lep	Cry1Ab; Cry1F; Cry1G; Cry1H; Cry1I; Cry2Ab
Cry1E	3D	lep	Cry1Ac; Cry1Ah; Cry8
Cry1F	3D	lep	Cry1Ad; Cry1B; Cry1C; Cry1D; Cry1G; Cry2Ab
Cry1G	3D	lep	Cry1B; Cry2Ac
Cry1H	3D	lep	Cry1B; Cry1D; Cry1K
Cry1I	3D	lep, col	Cry1Aa; Cry1Ab; Cry1Ac; Cry1D; Cry2Aa; Cry2Ab; Cry9; Vip3Aa
Cry1J	3D	lep	Cry1B
Cry1K	3D	lep	Cry1B; Cry1H
Cry2Aa	3D	dip, lep, hem	Cry1Aa; Cry1Ab; Cry1Ac; Cry1Ia; Cry2Ab; Cry24B; Cry30C; Cry50; Vip3Aa
Cry2Ab	3D	dip, lep, hem	Cry1Aa; Cry1Ac; Cry1D; Cry1F; Cry1Ia; Cry2Aa; Cry2Ac; Cry9; Vip3Aa
Cry2Ac	3D	dip, lep, hem	Cry1Ab; Cry1G; Cry2Ab; Cry2Ad
Cry2A•[d]	3D	dip, lep, hem	Cry1Ai; Cry2Ac
Cry3	3D	col, (A) hem, hym	Cyt2B
Cry4A	3D	dip	Cry4B; Cry10; Cry11A; Cry60; Cyt1; Cyt2
Cry4B	3D	dip	Cry4A; Cry10; Cry11A; Cry60; Cyt1; Cyt2
Cry4C	3D	dip	Cry30D/E/F/G; Cry40; Cry50; Cry52; Cry53; Cry54; Cry55; Cry56; Cry68; Cry69; Cry70; Cry71; Cry72; Cyt1D; Cyt2A
Cry5	3D	nem, (A) hym, plat	Cry6; Cry55; Cry65; Cyt2
Cry6	G1	nem, (A) col	Cry5; Cry55
Cry7	3D	(A) col, (B) lep	Cry33; Cry46; Cry61; Cry67; NT32KD
Cry8	3D	col, (D) lep	Cry9
Cry9	3D	(A,B,C,E,?D) lep, (D) col	Cry1Ab; Cry1I; Cry2Ab; Cry8
Cry10A	3D	dip	Cry4A; Cry4B; Cry11A; Cry60; Cyt1; Cyt2
Cry11A	3D	dip, hem	Cry4A; Cry4B; Cry10; Cry60; Cyt1; Cyt2
Cry11B	3D	dip	Cry19; Cry24; Cry25; Cry29; Cry30A/C; Cry60; Cyt1A; Cyt2B
Cry15	Mtx	lep	40kDa
Cry16	3D	dip	Cry17
Cry17	3D		Cry16
Cry19	3D	dip	Cry11B; Cry24; Cry25; Cry27; Cry30C; Cry60; Cyt2B
Cry20	3D	dip	Cyt2
Cry24	3D	(C) dip	Cry1Aa; Cry2Aa; Cry11B; Cry19; Cry25; Cry30C; Cry50; Cry60; Cyt2B

36 Lists downloaded from Sussex University website on February 16, 2014.

Protein	Cate-gory[a]	active against[b]	Isolated from the same strain for at least one of the protein variations
Cry25	3D		Cry11B; Cry19; Cry24; Cry30C; Cry60; Cyt2B
Cry27	3D	dip	Cry19
Cry29	3D		Cry11B; Cry30A; Cyt1A; Cyt2B
Cry30	3D		Cry1Aa; Cry2Aa; Cry4C; Cry11B; Cry19; Cry24B; Cry25; Cry29; Cry40; Cry50; Cry52; Cry53; Cry54; Cry55; Cry56; Cry58; Cry60; Cry68; Cry69; Cry70; Cry71; Cry72; Cyt1; Cyt2
Cry31	3D	HLC	Cry63
Cry33	Mtx		Cry7; Cry46; NT32KD
Cry34	G2	col	Cry35
Cry35	bin	col	Cry34
Cry39	3D	dip	Cry40
Cry40	3D		Cry4C; Cry30D; Cry39; Cry52; Cry53; Cry55
Cry41	3D	HCC	Cry42; Cry46; Cry66
Cry42	3D	HCC	Cry41; Cry46
Cry46	Mtx	HCC	Cry7; Cry33; Cry41; Cry42; NT32KD
Cry48	3D	dip	Cry49
Cry49	bin	dip	Cry48
Cry50	3D		Cry4C; Cry30C/E/G; Cry54
Cry52	3D		Cry4C; Cry30D; Cry40; Cry53; Cry55; Cry59
Cry53	3D		Cry4C; Cry30D/F; Cry40; Cry52; Cry54; Cry55; Cry68; Cry69; Cry70; Cyt1D; Cyt2A
Cry54	3D	dip, lep	Cry4C; Cry30E/F/G; Cry50; Cry53; Cry68; Cry69; Cry70; Cyt1D; Cyt2A
Cry55	??	col, nem	Cry4C; Cry5; Cry6; Cry30D; Cry40; Cry52; Cry53
Cry56	3D		Cry4C; Cry30E/G; Cry69; Cry70; Cry71; Cry72; Cyt1D
Cry58	3D		Cry1Aa; Cry30B
Cry59	3D		Cry52
Cry60	Mtx	dip	Cry4A; Cry4B; Cry10; Cry11A; Cry11B; Cry24; Cry25; Cry30C; Cyt1; Cyt2
Cry61	3D		Cry7; Cry67
Cry63	3D		Cry31
Cry65	3D		Cry5
Cry66	3D		Cry41
Cry67	3D		Cry7; Cry61
Cry68	3D		Cry4C; Cry30F; Cry53; Cry54; Cry69; Cry70; Cyt1D; Cyt2A
Cry69	3D		Cry4C; Cry30F/G; Cry50; Cry70; Cry71; Cry72; Cyt1D
Cry70	3D		Cry4C; Cry30F/G; Cry50; Cry69; Cry71; Cry72; Cyt1D
Cry71	??		Cry4C; Cry30G; Cry50; Cry69; Cry70; Cry72; Cyt1D
Cry72	??		Cry4C; Cry30G; Cry50; Cry69; Cry70; Cry71; Cyt1D
Cyt1	cyt	dip, (A) col, ?lep	Cry4A; Cry4B; Cry4C; Cry10; Cry11A; Cry11B; Cry29; Cry30A/F/G; Cry50; Cry60; Cry69; Cry70; Cry71; Cry72; Cyt2
Cyt2	cyt	(A,B) dip, (C) col, siph	Cry3; Cry4A; Cry4B; Cry5; Cry10; Cry11A; Cry11B; Cry19; Cry20; Cry24; Cry25; Cry29; Cry30A/C/F; Cry60; Cyt1
NT32KD	NT		Cry7; Cry33; Cry46
vip3Aa	vip		Cry1Aa; Cry1Ab; Cry1Ac; Cry1B; Cry1C; Cry1I; Cry2Aa; Cry2Ab
40kDa	NT		Cry15

[a]: The categories are described in part I, see also Table I-1 (p. 32). NT: not toxic.

[b]: The protein is observed to be toxic for the listed taxonomic groups. The taxonomic groups in this column are: col: Coleoptera; dip: Diptera; hem: Hemiptera; hym: Hymenoptera; lep: Lepidoptera; siph: Siphonoptera; plat: Platyhelminthes (Echinostomida); nem: Nematoda (Rhabditida) ; HLC: Human Leukemic Cells; HCC: Human Cancer Cells. If the activity does not hold for all second categories, the second category is stated between brackets before the taxonomic group. In some cases, the activity is

uncertain, and then the taxonomic group is preceded by “?”.

[c]: Cry1A•: the other Cry1A protein (Cry1Ad to Cry1Ai)

[d]: Cry2A•: the other Cry2A protein (Cry2Ad to Cry2Ak)

Conclusion and Recommendations

Prediction of synergy?

Can synergy between Bt toxins be predicted based on knowledge of their mode of action and synergy data of other Bt toxin combinations. The short answer to that question is: No. But it is possible to order Bt toxin (and Bs-toxin) combinations in risk categories? These risk categories should indicate which combinations might be synergistic to each other, and for which combinations strong synergy is less likely? In this classification, antagonism means an SF less than 0.5, no synergy an SF between 0.5 and 2, weak synergy an SF between 2 and 10, synergy an SF between 10 and 50 and strong synergy an SF above 50. The ordering in risk categories gives

- In mixtures of 3D-Cry toxins, (strong) synergy is not very likely.

- (Strong) synergy is very likely if the two toxins are known as a conjugative combination or assumed to be a conjugative combination. These combinations are:
  - BinA and BinB (Bin-group, *B. sphaericus* toxins)
  - Cry34 and Cry35 (Group 2-Cry and Bin-Cry)
  - Cry49 and Cry48 (Mtx-Cry and 3D-Cry)
  - Cry23 and Cry37 (Mtx-Cry and Group 1-Cry)
  - Vip1 and Vip2 (Vip)

- (Strong) synergy is more likely if one of the toxins is either
  - a toxin from the Cyt group.
  - a protein from the Mtx-group (Mtx2, Mtx3, Cry15, Cry23, Cry33, Cry38, Cry45, Cry46, Cry51, Cry60, Cry64, CryC35, CryNT32, and a further unnamed protein 40kDa; For CryC35, CryNT32, and 40kDa, no toxicity is reported [w1])[37]
  - a Bin-like toxin[38] (BinA, BinB, Cry35, Cry36, Cry49)

  If one of the toxins belongs to any of the classes listed above, strong synergy is more likely if the species under investigation is less sensitive to any of the individual toxins, especially if related species are much more sensitive to some of these individual toxins.

- For the completely unclassified Cry proteins (Cry55, Cry61, Cry71 and Cry72) and Cry proteins classified in the diffuse classes of Group 1 and 2[39] (Cry6, Cry22,

37 Note that Mtx1 does not belong to the Mtx-group. And that not all Mtx-like proteins are included in this list of generally acknowledged Mtx proteins (see e.g. 2D42 [2] and Cry14-4 [107]).

38 Of the Bin-like toxins, only the BinA/BinB combination was found in the collected data set on synergistic interaction. This combination seems to be slightly synergistic to susceptible *C. quinquefasciatus* in combinations with Cry4Aa, Cry4Ba and/or Cry11Aa, and strong synergistic in some of these combinations to *C. quinquefasciatus* resistant to BinA/BinB.

39 Cry6, Cry22 and Cry37 are Group 1 Cry proteins, Group 2 only contains Cry protein Cry34.

Cry34[40] and Cry37[41]), the available information is too scarce for even a tentative a priori estimate on the probability of synergy.

- Vip3 toxin seems to have a different binding mechanism than 3D-toxins, showing no cross-resistance. The data do not suggest (strong) synergy between Vip3 and 3D-Cry toxins. However, combination toxicity data are only available for relatively sensitive target species.

Weak synergy ($2 \leq SF < 10$) can occur between any two Bt toxins, and cannot be predicted. For Bt toxin combinations with no expected synergistic effect (combinations of 3D-Cry), weak synergism is probably slightly more likely in species with a low sensitivity compared to other related species, than in species with a high sensitivity to the individual toxins. Note that if the category "weak synergy" is split in two, "very weak synergy" with ($2 \leq SF < 5$) and "slightly weak synergy" ($5 \leq SF < 10$), this conclusion holds for both subcategories. The occurrence of either type of weak synergy cannot be predicted, but the occurrence of "very weak synergy" is more likely than that of "slightly weak synergy".

Toxins isolated from the same bacterial strain, or very related to a toxin couple isolated from the same bacterial strain, could well have some complementary mode of action. Synergy is, however, by no means the only form of effective complementarity.

The likelihood of synergy is summarized in table C1.

40 For Cry34, a synergistic effect with Cry35 is known.
41 For Cry37, a synergistic effect with Cry23 is known.

Table C1.
The potentiality of synergy for different combinations of Bt toxin types.

	Likelihood of synergy between Bt toxins if the exposed species or strain is		
Combination of Bt toxins	**sensitive to both.**	**not sensitive to one of the toxins, and a taxonomic related species[a] is sensitive.**	**not sensitive to one of the toxins, and taxonomic related species[a] are not sensitive either.**
3D-Cry / 3D-Cry	no synergy	no synergy or weak synergy	no synergy or weak synergy
3D-Cry /{Bin-Cry, Mtx-Cry or Cyt}	no synergy or weak synergy	potential synergy	potential synergy if 1: Synergy is observed in related species (high prob.) or 2: Some less related species are observed to be more sensitive (low prob.)
{Bin-Cry, Mtx-Cry or Cyt} / {Bin-Cry, Mtx-Cry or Cyt}	no synergy or weak synergy	potential synergy	potential synergy if 1: Synergy is observed in related species (high prob.) or 2: Some less related species are observed to be more sensitive (low prob.)
Assumed conjugative combination	synergy	synergy	synergy
{Unclassified or Group1/2} / {any Bt toxin}	unknown	unknown	unknown
Vip3 / 3D-Cry	probably at most weak synergy	unknown	unknown

[a]: Taxonomic related should be interpreted loosely, meaning related in a degree that potentially the same toxic mode of action may be expected.

The main lacks in our knowledge of Bt toxins.

At first sight a considerable amount of data is available on Bt toxin toxicity ([w8]). Unfortunately, a considerable fraction of these data cannot be used for interspecies comparison or for the comparison of the toxicity of different toxins or toxin combinations. For these comparisons the effective dose has to be compared between the experiments. Such a comparison is only possible if the dose in all experiments to be compared can be translated to the same unit, that unit measuring the quantity of either

- toxin weight per individual (larva) weight
- toxin weight per individual (larva), combined with individual weight.
- toxin weight per dry weight of food
- toxin weight per volume or weight of water if the exposed individual (larva) is housed in water.
- toxin weight per volume air if individual (larva) is exposed to the toxin by air (not relevant for Bt toxins).

The practise to express the dose of Bt toxin in all kind of different units does not help. And the fact that some of these units are experiment specific[42] and cannot be translated to any of the universal quantities listed above, makes comparison between the sensitivity of species and between the toxicity of toxins for the same species nearly impossible. To enable the comparison of species specific sensitivities to Bt toxins and of toxicities of Bt toxins for a species, it is of vital importance to develop a rigorous protocol enabling the comparison of the exposure between experiments. If technical restrictions prohibit the toxin addition in fixed doses per larva or per mg food, the actual dose per larva or per mg food should be measured and reported. The current lack of useful data on Bt toxin toxicity is serious impediment for the use of synergy data on taxonomically related species to predict potential synergy in the species under consideration.

Little data are available on the combination toxicology of Bt toxins for non-target species. Since the main difference between target and non-target species is whether people want to kill them or not, there is no a priori reason to assume that the sensitivity of target and non-target species differs, or that their reaction to combinations of toxicants depends on being target species or not. However, sensitivities differ between species, and Bt toxins used for incorporation in GM plants are selected by the criterion that they should be toxic to the intended target species, and preferably less toxic to other species. Therefore, it may be expected that the sensitivity of the target species is at the high sensitivity side of the species sensitivity distribution for that Bt toxin. In most cases, the combination of Bt genes stacked in a GM plant is not selected for their potency for synergism, but to broaden the range of targeted species or to be able to affect target species resistant to one of the stacked toxins, or preferably prevent the development of resistance by attacking the target species in different ways, hampering the development of resistance. If synergy is not the reason to combine toxins in GM crops, no a priori reason exists why synergism should be weaker or stronger in target organisms than in non-target organisms. If the (observed) lower sensitivity of a non-target organism is due to a similar mechanism as induced resistance in a target organism, and if induced resistance can be broken by a synergistic interaction between two Bt proteins, the same interaction might lead to synergism of the Bt toxins in that non-target organism. This reasoning is purely theoretically based. To corroborate these theoretical considerations, more experimental

42 Examples of experiment specific dosing units are:
- µg toxin per leaf surface area, the concentration per dry-weight unit depends on the leaf thickness and the leaf dry-weight percentage.
- µg toxin per ml of the water in which a leaf is dipped, the concentration per dry-weight unit depends on leaf water adsorption, leaf thickness and the leaf dry-weight percentage.
- number of cells of a toxin producing clone, the concentration per cell depends on the expression of the toxin gene and the general condition of the toxin producing cells.

evidence is needed on:

- the mechanism making individuals of susceptible species resistant.
- which steps in the mode of action of a Bt toxin cannot function within individuals of a not-susceptible related species.
- the interaction between Bt toxins in non-sensitive species which are taxonomically related to a sensitive species observed to show strong synergy in resistant strains and low synergy in its unselected wild type strains.

Synergy seems to be enhanced in resistant strains, However, this relation is only shown for a limited number of resistant strains and Bt toxin combinations. More research on the relationship between resistance and synergy between Bt toxins is needed, and especially between 3D-Cry toxins.

Research on the mode of action of 3D-Cry toxins has focussed on Cry1A toxins. To be able to use the information on the mode of action of toxins to predict interaction, that is synergy and cross-resistance, more information on similarities and differences between the mode of action of the different 3D-Cry toxins is needed.

Information on the mode of action of Bin-Cry and Mtx-Cry toxins is almost completely lacking, although for Mtx-Cry toxins, some information can be obtained by the comparison with structural similar toxins like the ε-toxin of *Clostridium perfringens*. Toxins of these two toxin groups might be able to enhance the toxicity of some 3D-Cry toxins, and therefore more information on the mode of action of these toxin groups would be helpful to understand synergy.

Information on the interaction between Vip3 and the diverse groups of Cry toxins is scarce, suggesting that Vip3 and 3D-Cry binds to different receptors and therefore do not induce cross-resistance, and that Vip3 and 3D-Cry do not synergize each other. More information on this interaction is needed to corroborate the suggestion that 3D-Cry and Vip3 do not interact.

Information on the interaction between Vip1-Vip2 toxin couples and any other Bt toxin is lacking, but as long as Vip1-Vip2 toxin couples are not used in GM plants, this deficiency is not problematic for GM plant risk assessment.

Recommendations.

The observations on the potential synergy of Bt toxins lead to recommendations with respect to the need for additional information for stacked Bt plants to assess effects on non-target organisms. To determine if, -and if yes- which additional information is needed, the following check list can be used. This check list often asks to consider the availability of knowledge on taxonomic related species. The term “taxonomic related species” should be interpreted loosely, meaning related in a degree that potentially the same toxic mode of action may be expected.

1. Is anything known about the synergy between the combined toxins?

2. Are the combined toxins considered to form a conjugative combination (BinA and BinB; Cry34 and Cry35; Cry23 and Cry37; Cry49 and Cry48; Vip1 and Vip2)?
3. Is anything known about synergy between any of the combined toxins and some other Bt toxin or related toxins?
4. Is anything known about cross-resistance against one of the combined toxins resulting from resistance against one of the other toxins in the combination?
5. Do the Bt toxins act mainly against the same taxonomic group? And if not, are the Bt toxins also toxic to the target group of the other Bt toxins?
6. Does any of the Bt toxins belong to one of the following groups?
    - Cyt (Cyt1, Cyt2, Cyt3)
    - Mtx (Mtx2, Mtx3, Cry15, Cry23, Cry33, Cry38, Cry45, Cry46, Cry51, Cry60, Cry64, CryC35, CryNT32 and a further unnamed protein 40kDa (for CryC35 and 40kDa, no toxicity is reported)
    - Bin (BinA, BinB, Cry35, Cry36, Cry49)
7. Is any of the Bt toxins not classified or does any of the Bt toxins belong to the diffuse Group 1 or Group 2 (Cry6, Cry22, Cry34, Cry37, Cry55, Cry61, Cry71, Cry72)?
8. Is any of the Bt toxins a Vip3 toxin?
9. Is a conjugative couple of Vip1 and Vip2 combined with other Bt toxins?
10. Are the Bt toxin genes originally cloned from the same Bt strain? Or, if the Bt genes are cloned from different strains, does any of these strains also encode for Cry proteins related to another of these Bt toxins?

Ad 1. If information on synergy between the combined toxins is available, use it.

Ad 2. If the combined toxins are assumed to form a conjugative combination, use data on the combined effect of these toxins in the risk assessment.

Ad 3. If information on the interaction between the first of two combined toxins and some other toxin is available then this information might be used if that other toxin is structurally related to the second toxin. More detailed,

a) if that other toxin is from the same group (3D, Mtx, Cyt, ..) as the second toxin, the synergy between the first toxin and the other toxin can be used as indication of the synergy between the two combined toxins. If the first toxin and the other toxin are synergistic and the second toxin is less toxic than the other toxin, it should be considered that the synergy between the combined toxins might be somewhat larger than the synergy between the first toxin and the other toxin.
b) If that other toxin belongs to another group than the second toxin of the combination, and the other toxin is either Cyt, Mtx or Bin, and the second toxin also belongs to one of these groups, strong synergy between the first toxin and the other toxin indicates that synergy between the combined toxins is not unlikely.
c) If neither that other toxin nor the second toxin belong to any of the groups Cyt, Mtx or Bin (in that case, most likely both the second and the other toxin are 3D-Cry toxins), the interaction between the first toxin and the other toxin cannot be used as indication for the interaction between the combined toxins.

Ad 4. If insects, resistant to one toxin, show considerable cross-resistance to another toxin, these two toxins probably use (partly) the same mode of action (e.g. the same receptors), and in that case synergy is less likely because using the same mode of action leads to an additive combination toxicology model.

Ad 5. If each of the combined toxins act exclusively against very different taxonomic groups, synergy is less likely[43].

Ad 6. If any of the combined toxins belong to any of the groups Cyt, Mxt or Bin, and no information as meant under point 1, 2 and 3 is available, additional information on synergy is needed.

Ad 7. Information about the unclassified Cry and Group 1 and 2 Cry is mostly lacking. Only information on the combinations Cry34 with Cry35 and Cry37 with Cry23 is available, and these combinations are strongly synergistic. For all other Bt toxin combinations, additional information on synergy is needed.

Ad 8. Information on the interaction with Vip3 toxins, although scarce, suggests that Vip3 and 3D-Cry toxins do not interact in sensitive target species. Some additional information on interaction in resistant species strains and non-sensitive species is needed. For combinations of a Vip3 toxin with Bt toxins from other groups (not 3D-Cry toxins), additional information on synergy is also needed.

Ad 9. Information on the interaction between the conjugative couple Vip1-Vip2 and any other Bt toxin is completely lacking, so additional information is needed.

Ad 10. If the combined toxins are cloned from the same bacterial strain, or toxins very related to these combined toxins, that is with the same primary and secondary indication in their nomenclature, are isolated from the same bacterial strain, information on the toxicity of that bacterial strain should be given.

Note that if weak synergy (SF<10) is deemed to be unacceptable in the risk analysis of combined, potentially interacting Bt toxins, the Bt toxin combination will always have to be tested to assess whether the actual synergy factor is acceptable. Neither the theory on the mode of (inter)action of Bt toxins, nor the available data on Bt toxin interaction warrant a prediction of an SF less than 2 (that is, no synergy) even if no known synergy enhancing factors are present.

43 Note that this recommendation is not based on experimental data, for the synergy between Bt toxins active against different taxonomic group has not been tested. The recommendation is based on the consideration that for synergy to occur both toxins have to be somehow active in the target organism. And if a species is completely uninfluenced by a toxin, there is no interaction to be promoted by the other toxin.

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APPENDICES

Appendix A

List of abbreviations.

3D	3 domain: Group of Cry toxins with a similar 3 domain structure.
ABC trans-porters	ATP-Binding Cassette transporters
ADP	Adenosine DiPhosphate
AgCad1	*Anopheles gambiae* cadherin
ALP	ALkaline Phosphatase
APN	AminoPeptidase N
BBMV	Brush Border Membrane Vesicles
Bs	*Bacillus sphaericus*. *B. sphaericus* has been renamed to *Lysinibacillus sphaericus*. In this report, the old name, *Bacillus sphaericus*, is used.
Bt	*Bacillus thuringiensis*
BT-R1	*Manduca sexta* cadherin
BtR175	*Bombyx mori* cadherin
CAD	Cadherin
Col	Coleoptera
CRno	Repeat fragment in the extending domain of cadherin. The number indicate the position of the repeat, starting with 1 for the repeat most distant from the membrane.
CRR	Cross-Resistance Ratio: the CRR for a toxin is the ratio between the Resistance Ratio (RR) for that toxin divided by the RR for the toxin for which the resistant strain is known to be resistant.
Dip	Diptera
DN	Dominant Negative: A non-toxic Cry mutant which decrease the toxicity of its wild type parent Cry type more than can be explained by binding-site competition.
ED50	Toxin dose leading to an effect of 50%, for instance a 50% growth inhibition or 50% mortality.
EDTA	EthyleneDiamineTetraAcetic acid
GalNac	N-acetylGalactosamine
GL	GlycoLipids
GM	Genetically Modified
GMO	Genetically Modified Organism
GPI	GlycosylPhosphatidylInsitol
Hacad1	*Helicoverpa armigera* cadherin
HevCaLP	*Heliothis virenscens* cadherin
HvALP	*Heliothis virescens* alkaline phosphatase
kDa	kilo Dalton; 1Da = 1 u (unified atomic mass unit)

LD50	Toxin dose leading to the mortality of 50% of the individuals exposed to that dose.
Lep	Lepidoptera
MPED	Membrane Proximal Extracellular Domain of a cadherin
NAD+	Nicotinamide Adenine Dinucleotide
NTO	Non target organism
OnBt-R1	*Ostrini nubilalis* cadherin
PFT	Pore Forming Toxin
PS	ParaSporin
RR	Resistance Ratio: the ratio between the EC50 in the resistant strain and in the susceptible strain. An RR of 1 indicates no resistance, the larger RR, the more resistance.
SF	Synergy Factor, the ratio between the predicted EC50 (based on the ED50 of the individual toxins using the)additive model and the observed ED50 for the mixture of the toxins
SFm	Synergy Factor, the ratio between the predicted EC50 (based on the ED50 of the individual toxins using the independent effect model) and the observed ED50 for the mixture of the toxins
TBR	Toxic Binding Region
TU	Toxic Units:The dose or (concentration) of the toxin expressed in the ED50 (the TU at the ED50 is 1)
Wt	Wild type

Appendix B

List of species with the abbreviation used for that species.[44]

target or not[a]	Abbr.	species	phy-lum[b]	class[c]	order	family	common name
TO	Aa	*Aedes aegypti*[45]	Arth	Insecta	Diptera	Culicidae	yellow fever mosquito
TO	Aal	*Anopheles albimanus*	Arth	Insecta	Diptera	Culicidae	malaria mosquito
TO	Ac	*Argyrotaenia citrana*	Arth	Insecta	Lepidoptera	Tortricidae	orange tortrix
TO	Ad	*Alphitobius diaperinus*	Arth	Insecta	Coleoptera	Tenebrionidae	lesser mealworm
TO	Ae	*Acantholyda erythrocephala*	Arth	Insecta	Hymenoptera	Pamphiliidae	pine fales webworm
TO	Af	*Actebia fennica*	Arth	Insecta	Lepidoptera	Noctuidae	black army cutworm
TO	Ag	*Anopheles gambiae*[46]	Arth	Insecta	Diptera	Culicidae	malaria mosquito (Africa)
TO	Ag-c	*Anthonomus grandis*	Arth	Insecta	Coleoptera	Curculionidae	cotton pest/boll weevil
TO	Ag-h	*Aphis gossypii*	Arth	Insecta	Hemiptera	Aphididae	cotton aphis
TO	Ag-l	*Anticarsia gemmatalis*	Arth	Insecta	Lepidoptera	Erebidae	velvetbean caterpillar/moth
TO	Ai	*Agrotis ipsilon*	Arth	Insecta	Lepidoptera	Noctuidae	dark sword-grass/black cutworm
NTO	Am	*Apis mellifera*	Arth	Insecta	Hymenoptera	Apidae	honeybee
TO	Ap	*Acyrthosiphon pisum*	Arth	Insecta	Hemiptera	Aphididae	pea aphid
TO	Aq	*Anopheles quadrimaculatus*	Arth	Insecta	Diptera	Culicidae	malaria mosquito
TO	As-d	*Anopheles stephensi*	Arth	Insecta	Diptera	Culicidae	malaria mosquito (India)
TO	As-l	*Agrotis segetum*	Arth	Insecta	Lepidoptera	Noctuidae	turnip moth
TO	Bf	*Busseola fusca*	Arth	Insecta	Lepidoptera	Noctuidae	maize stalk borer
NTO	Bm	*Bombyx mori*	Arth	Insecta	Lepidoptera	Bombycidae	China silkworm
TO	Cb	*Colaphellus bowringi*	Arth	Insecta	Coleoptera	Chrysomelidae	cabbage leaf beetle
NTO	Cc	*Chrysoperla carnea*	Arth	Insecta	Neuroptera	Chrysopidae	common green lacewing
NTO	Ce	*Caenorhabditis elegans*	Nem	Secern	Rhabditida	Rhabditidae	roundworm C. elegans
TO	Cf	*Choristoneura fumiferana*	Arth	Insecta	Lepidoptera	Tortricidae	eastern spruce budworm
NTO	Cm-c	*Coleomegilla maculata*	Arth	Insecta	Coleoptera	Coccinellidae	twelvespotted ladybird beetle
NTO	Cma	*Cacyreus marshalli*	Arth	Insecta	Lepidoptera	Lycaenidae	geranium bronze
TO	Cme	*Cnaphalocrocis medinalis*	Arth	Insecta	Lepidoptera	Crambidae	rice leafroller

44 Not all species in this list are mentioned in the report, but all species are used in experiments with Cry, Cyt or Vip proteins.

45 *Aedes aegypti* is now officially named *Stegomyia aegypti.*

46 *Anopheles gambiae* is also used as name to indicate a complex of at least 7 morphologically indistinguishable species.

target or not[a]	Abbr.	species	phy-lum[b]	class[c]	order	family	common name
TO	Co	*Choristoneura occidentalis*	Arth	Insecta	Lepidoptera	Tortricidae	western spruce budworm
TO	Cp	*Culex pipiens*	Arth	Insecta	Diptera	Culicidae	common house mosquito
TO	Cpa	*Chilo partellus*	Arth	Insecta	Lepidoptera	Crambidae	maize stem borer
TO	Cpi	*Choristoneura pinus*	Arth	Insecta	Lepidoptera	Tortricidae	jack pine budworm
TO	Cpo	*Cydia pomonella*	Arth	Insecta	Lepidoptera	Tortricidae	codling moth
TO	Cq-c	*Crioceris quatuordecim-punctata*	Arth	Insecta	Coleoptera	Chrysomelidae	asparagus beetle
TO	Cq	*Culex quinquefasciatus*	Arth	Insecta	Diptera	Culicidae	southern house mosquito
TO	Cr	*Choristoneura rosaceana*	Arth	Insecta	Lepidoptera	Tortricidae	oblique banded leaf roller/rosaceous leaf roller
TO	Cs-c	*Chrysomela scripta*	Arth	Insecta	Coleoptera	Chrysomelidae	cotton leaf beetle
TO	Cs-l	*Chilo suppressalis*	Arth	Insecta	Lepidoptera	Crambidae	rice stem borer
NTO	Ct	*Chironomus tepperi*	Arth	Insecta	Diptera	Chironomidae	rice midge
TO	Dg	*Diatraea grandiosella*	Arth	Insecta	Lepidoptera	Crambidae	southwestern corn borer
TO	Dp-h	*Diprion pini*	Arth	Insecta	Hymenoptera	Diprionidae	common pine sawfly
NTO	Dp-l	*Danaus plexippus*	Arth	Insecta	Lepidoptera	Nymphalidae	monarch butterfly
TO	Ds-hy	*Diprion similis*	Arth	Insecta	Hymenoptera	Diprionidae	pine sawfly (type of)
TO	Ds	*Diatraea saccharalis*	Arth	Insecta	Lepidoptera	Crambidae	sugarcane borer
TO	Du	*Diabrotica undecimpunctata howardi*	Arth	Insecta	Coleoptera	Chrysomelidae	southern corn rootworm (SCR)
TO	Dv	*Diabrotica virgifera virgifera*	Arth	Insecta	Coleoptera	Chrysomelidae	western corn rootworm (WCR)
TO	Ea	*Epinotia aporema*	Arth	Insecta	Lepidoptera	Tortricidae	(soy)bean shoot borer
TO	Ei	*Earias insulana*	Arth	Insecta	Lepidoptera	Nolidae	Egyptian stemborer / Egyptian / cotton spotted bollworm
TO	Ek	*Ephestia kuehniella*	Arth	Insecta	Lepidoptera	Pyralidae	Indian/Mediterranean floor moth / Mill Moth
TO	Ep	*Epiphyas postvittana*	Arth	Insecta	Lepidoptera	Tortricidae	light brown apple moth
TO	Es	*Eldana saccharina*	Arth	Insecta	Lepidoptera	Pyralidae	sugarcane borer
TO	Ev	*Earias vittella*	Arth	Insecta	Lepidoptera	Nolidae	spotted bollworm
TO	Ha	*Helicoverpa armigera*	Arth	Insecta	Lepidoptera	Noctuidae	cotton bollworm/corn earworm/Old World (African) bollworm/scarce bordered straw
NTO	Hc	*Hippodamia convergens*	Arth	Insecta	Coleoptera	Coccinellidae	ladybird beetle
TO	Hp	*Helicoverpa punctigera*	Arth	Insecta	Lepidoptera	Noctuidae	native budworm/Australian bollworm
TO	Hv	*Heliothis virescens*	Arth	Insecta	Lepidoptera	Noctuidae	tabacco budworm

target or not[a]	Abbr.	species	phylum[b]	class[c]	order	family	common name
TO	Hz	*Helicoverpa zea*	Arth	Insecta	Lepidoptera	Noctuidae	corn earworm moth
TO	Ld-c	*Leptinotarsa decemlineata*	Arth	Insecta	Coleoptera	Chrysomelidae	Colorado potato beetle
TO	Ld	*Lymantria dispar*	Arth	Insecta	Lepidoptera	Lymantriidae	gypsy moth
TO	Mb	*Mamestra brassicae*	Arth	Insecta	Lepidoptera	Noctuidae	cabbage moth
TO	Md	*Malacosoma disstria*	Arth	Insecta	Lepidoptera	Lasiocampidae	forest tent caterpillar
TO	Mh	*Meloidogyne hapla*	Nem	Secern	Tylenchida	Heteroderidae	northern root-knot nematode
TO	Mi	*Meloidogyne incognita*	Nem	Secern	Tylenchida	Heteroderidae	cotton (of southern) root-knot nematode
TO	Mp	*Myzus persicae*	Arth	Insecta	Hemiptera	Aphididae	green peach aphid
TO	Ms	*Manduca sexta*	Arth	Insecta	Lepidoptera	Sphingidae	tobacco hornworm/goliath worm
TO	Ns	*Neodiprion sertifer*	Arth	Insecta	Hymenoptera	Diprionidae	European pine sawfly
NTO	Nv	*Nasonia vitripennis*	Arth	Insecta	Hymenoptera	Pteromalidae	parasitic wasp
TO	Ol	*Orygia leucostigma*	Arth	Insecta	Lepidoptera	Lymantriidae	white-marked tussock moth
TO	On	*Ostrinia nubilalis*	Arth	Insecta	Lepidoptera	Crambidae	European corn worm/European corn borer/European high-flyer
TO	Pa	*Pikonema alaskensis*	Arth	Insecta	Hymenoptera	Tenthredinidae	yellowheaded spruce sawfly
TO	Pb-c	*Phaedon brassicae*	Arth	Insecta	Coleoptera	Chrysomelidae	daikon leaf beetle
TO	Pb-l	*Pieris brassicae*	Arth	Insecta	Lepidoptera	Pieridae	large white/cabbage butterfly/cabbage white
TO	Pc	*Perileucoptera coffeella*[47]	Arth	Insecta	Lepidoptera	Lyonetiidae	coffee leaf miner
TO	Pg	*Pectinophora gossypiella*	Arth	Insecta	Lepidoptera	Gelechiidae	pink bollworm
TO	Pi	*Plodia interpunctella*	Arth	Insecta	Lepidoptera	Pyralidae	Indian meal moth
TO	Pr	*Pieris rapae*	Arth	Insecta	Lepidoptera	Pieridae	small white/small cabbage white
TO	Px	*Plutella xylostella*	Arth	Insecta	Lepidoptera	Plutellidae	diamondback moth
TO	Sa-d	*Stegomyia albopictus*[48]	Arth	Insecta	Diptera	Culicidae	tiger mosquito/forest mosquito
TO	Sa-l	*Spodoptera albula*	Arth	Insecta	Lepidoptera	Noctuidae	gray-streaked armyworm moth/unbarred spodoptera moth
TO	Ser	*Spodoptera erdania*	Arth	Insecta	Lepidoptera	Noctuidae	southern armyworm
TO	Sex	*Spodoptera exigua*	Arth	Insecta	Lepidoptera	Noctuidae	beet armyworm/small mottled willow moth
TO	Sf	*Spodoptera frugiperda*	Arth	Insecta	Lepidoptera	Noctuidae	fall armyworm

47 *Perileucoptera coffeella* is also named *Leucoptera coffeella*.
48 *Stegomyia albopictus* was formerly named *Aedes albopictus.*

target or not[a]	Abbr.	species	phy-lum[b]	class[c]	order	family	common name
TO	Si	*Sesamia inferens*	Arth	Insecta	Lepidoptera	Noctuidae	(Asiatic) Pink [rice/stem/rice stem] borer
TO	Sla	*Spodoptera latifascia*[49]	Arth	Insecta	Lepidoptera	Noctuidae	lateral lined armyworm/garden armyworm/velvet armyworm
TO	Sli	*Spodoptera littoralis*	Arth	Insecta	Lepidoptera	Noctuidae	cotton leafworm/tobacco cutworm
TO	Tc	*Tribolium castaneum*	Arth	Insecta	Coleoptera	Tenebrionidae	red flour beetle
TO	Tm	*Tenebrio molitor*	Arth	Insecta	Coleoptera	Tenebrionidae	mealworm beetle
TO	Tn	*Trichoplusia ni*	Arth	Insecta	Lepidoptera	Noctuidae	cabbage looper

a: Target or not: TO: Target Organism; NTO: Non-target Organism
b: Phylum: Arth=Arthropoda; Nem=Nematoda
c: Class: Secern=Secernentea

49 *Spodoptera cosmioides* used to be considered a separate species, but is now considered as subspecies of *Spodoptera latifascia*.

Appendix C

A list of the GM plants allowed on the European Market (date March 17, 2014).

Event	Company	plant	gene	gene 2	gene 3	gene 4, 5, 6
DAS-81419-2	DOW	soybean	Cry1Ac	Cry1F		
MON87701	Monsanto	soybean	Cry1Ac			
MON15985	Monsanto	cotton	Cry1Ac	Cry2Ab2		
T304-40	Bayer	cotton	Cry1Ab			
GHB119	Bayer	cotton	Cry2Ae			
MON1445xMON531	Monsanto	cotton	Cry1Ac			
281-24-236 x 3006-210-23 (DAS-21Ø23-5 x DAS-24236-5)	DOW	cotton	Cry1Ac	Cry1F		
MON810	Monsanto	maize	Cry1Ab			
5307 (SYN-Ø53Ø7-1)	Syngenta	maize	eCry3.1Ab (Cry3A)			
MIR604	Syngenta	maize	mCry3A (Cry3A)			
MIR162	Syngenta	maize	Vip3Aa20			
MON89034	Monsanto	maize	Cry1A.105	Cry2Ab2		
MON88017	Monsanto	maize	Cry3Bb1			
59122 (DAS-59122-7)	DOW	maize	Cry34Ab1	Cry35Ab1		
1507 (DAS-Ø15Ø7)	DuPont	maize	Cry1F			
MON863	Monsanto	maize	Cry3Bb1			
Bt11	Syngenta	maize	Cry1Ab			
Plant crosses with many Cry genes						
MON89034x1507xMON88017x59122	Monsanto	maize	Cry1A.105	Cry1F	Cry2Ab2	Cry3Bb1, Cry34Ab, Cry35Ab
Bt11xMIR162xMIR604x1507x5307	Syngenta	maize	Cry1Ab	Cry1F	eCry3.1Ab (Cry3A)	mCry3A (Cry3A), Vip3Aa2
Bt11x59122xMIR604x1507	Syngenta	maize	Cry1Ab	Cry1F	mCry3A (Cry3A)	Cry34Ab1, Cry35Ab1
1507x59122xMON810	Monsanto, Mycogen/ DuPont, Dow	maize	Cry1Ab	Cry1F	Cry34Ab1	Cry35Ab1
MON863xMON810	Monsanto	maize	Cry1Ab	Cry3Bb1		

Appendix D

The toxicity data of Cry1Ab from the Canadian Forest Service website [w8] (at January 22, 2014).

Gene	Genus	Species	Assay Type	Instar Age	Insect Strain	ED50	Units	Para-meter	Quali-tative	Reference
cry01Ab	*Acyrthosiphon*	*pisum*	Free ingestion	nymph		>500000	ng/ml	mortality		Porcar
cry01Ab	*Argyrotaenia*	*citrana*	Diet surface	neonate		3.5	ng/cm2	mortality		Knight
cry01Ab	*Bombyx*	*mori*	Diet surface	L3		61(48-76)	ng/ml	mortality		Kim
cry01Ab	*Bombyx*	*mori*	Diet surface	L3		20(15-22)	ng/ml	mortality		Kim
cry01Ab	*Bombyx*	*mori*	Diet surface	L3		342(306-453)	ng/ml	mortality		Kim
cry01Ab	*Bombyx*	*mori*	Diet surface	L3		220(194-248)	ng/ml	mortality		Kim
cry01Ab	*Busseola*	*fusca*	Diet surface	neonate		0.01(0.007-0.02)	ng/cm2	mortality		Tounou
cry01Ab	*Cacyreus*	*marshalli*	Leaf dip	L3				growth inhibition	active	Herrero
cry01Ab	*Chilo*	*suppressalis*	Diet incorporation	neonate		1960(390-4960)	ng/ml	mortality		Xue
cry01Ab	*Chilo*	*suppressalis*	Diet incorporation	10d		6.5(4.8-8.2)	ng/ml	mortality		Alcantara
cry01Ab	*Choristoneura*	*rosaceana*	Diet surface	neonate		1.7	ng/cm2	mortality		Knight
cry01Ab	*Chrysoperla*	*carnea*	Diet incorporation	L1				mortality	not active	Romeis
cry01Ab	*Cnaphalocrocis*	*medinalis*	Leaf dip	L2	Nanjing (China)	7050(5150-9650)	ng/ml	mortality		Han
cry01Ab	*Cnaphalocrocis*	*medinalis*	Leaf dip	L2		98(40-130)	ng/cm2	mortality		Karim
cry01Ab	*Cnaphalocrocis*	*medinalis*							active	Ye
cry01Ab	*Cnaphalocrocis*	*medinalis*	Leaf dip	L2	Nanhui (China)	220(90-530)	ng/ml	mortality		Han
cry01Ab	*Conopomorpha*	*cramerella*	Diet surface	L3-L4		19.9(14.3-25.8)	ng/cm2	mortality		Santoso
cry01Ab	*Cydia*	*pomonella*	Diet incorporation	neonate		25(14-38)	ng/ml	mortality		Boncheva
cry01Ab	*Cydia*	*pomonella*	Diet incorporation	neonate		78(28-124)	ng/ml	mortality		Boncheva
cry01Ab	*Cydia*	*pomonella*	Diet surface	neonate		29200(10700-233000)	ng/ml	mortality	active	Rang
cry01Ab	*Danaus*	*plexippus*	Diet incorporation	L4		>100	ng/ml	mortality		Hellmich
cry01Ab	*Danaus*	*plexippus*	Diet incorporation	L3		9.6(6.0-15)	ng/ml	growth		Hellmich
cry01Ab	*Danaus*	*plexippus*	Diet incorporation	L3		35.1(30-106)	ng/ml	mortality		Hellmich
cry01Ab	*Danaus*	*plexippus*	Diet incorporation	L1		0.8(0.6-0.9)	ng/ml	growth		Hellmich
cry01Ab	*Danaus*	*plexippus*	Diet incorporation	L1		3.3(2.2-4.8)	ng/ml	mortality		Hellmich
cry01Ab	*Danaus*	*plexippus*	Diet incorporation	L4		18.3(9.4-40)	ng/ml	growth		Hellmich
cry01Ab	*Diatraea*	*grandiosella*	Diet incorporation	neonate		0.14(0.11-0.17)	ng/ml	mortality		Huang
cry01Ab	*Diatraea*	*saccharalis*	Diet incorporation	neonate		0.38(0.28-0.54)	ng/ml	mortality		Huang
cry01Ab	*Earias*	*vittella*	Leaf surface	neonate		0.447(0.306-0.662)	ng/cm2	mortality	active	Kranthi
cry01Ab	*Eldana*	*saccharina*	Diet surface	neonate				mortality	active	Tounou
cry01Ab	*Epinotia*	*aporema*	Diet incorporation	neonate		550(410-730)	ng/ml	mortality		Sauka

Gene	Genus	Species	Assay Type	Instar Age	Insect Strain	ED50	Units	Para-meter	Quali-tative	Reference
cry01Ab	*Helicoverpa*	*armigera*	Diet incorporation	neonate		1550(470-3270)	ng/ml	mortality		Chakrabarti
cry01Ab	*Helicoverpa*	*armigera*	Diet surface	neonate		54.2(16.2-194.2)X10**6	cells/cm2	mortality		Padidam
cry01Ab	*Helicoverpa*	*armigera*	Diet surface	neonate		660(430-810)	ng/cm2	mortality		Mandaokar
cry01Ab	*Helicoverpa*	*zea*	Diet incorporation	neonate		3450	ng/ml	mortality		Luttrell
cry01Ab	*Helicoverpa*	*zea*	Force feed	L4		89(27-152)	ng/larva	mortality		Karim
cry01Ab	*Helicoverpa*	*zea*	Diet surface	L1		74(52-114)	ng/cm2	mortality		Karim
cry01Ab	*Helicoverpa*	*zea*	Diet surface	neonate		1690(1300-2430)	ng/cm2	mortality		Chambers
cry01Ab	*Heliothis*	*virescens*	Diet surface	L1		1.5(0.8-2.3)	ng/cm2	mortality		Jurat-Fuentes
cry01Ab	*Heliothis*	*virescens*	Diet incorporation	neonate		200	ng/ml	mortality		Luttrell
cry01Ab	*Heliothis*	*virescens*	Diet surface	L2		<0.15X10**7	cells/cm2	weight		Honee
cry01Ab	*Heliothis*	*virescens*	Diet surface	neonate		68(57-82)	ng/cm2	mortality		Chambers
cry01Ab	*Heliothis*	*virescens*	Diet surface	L2		<0.025X10**7	cells/cm2	weight		Visser
cry01Ab	*Lobesia*	*botrana*	Diet incorporation	neonate		1400	ng/ml	mortality		de Escudero
cry01Ab	*Lymantria*	*dispar*	Diet surface	L1		1.08(0.35-3.41)	ng/cm2	mortality		Wolfersberger
cry01Ab	*Mamestra*	*brassicae*	Diet surface	L2		12.5X10**7	cells/cm2	weight		Visser
cry01Ab	*Mamestra*	*configurata*	Diet surface	L2		1410(240-3190)	ng/cm2	mortality		Masson
cry01Ab	*Mamestra*	*configurata*	Force feed	L2		29.8(15-46)	ng/larva	mortality		Masson
cry01Ab	*Marasmia*	*patnalis*	Leaf dip	L2		93(61-150)	ng/cm2	mortality		Karim
cry01Ab	*Maruca*	*vitrata*	Diet incorporation	L2		207(156-272)	ng/ml	mortality		Srinivasan
cry01Ab	*Ostrinia*	*furnacalis*	Diet incorporation	neonate		230(93-520)	ng/ml	mortality		Xue
cry01Ab	*Ostrinia*	*nubilalis*	Diet surface	neonate		27(22-35)	ng/cm2	mortality		Chambers
cry01Ab	*Ostrinia*	*nubilalis*	Diet incorporation	neonate	Dipel	944000(408000-4130000)	ng/ml	mortality		Li
cry01Ab	*Ostrinia*	*nubilalis*	Diet incorporation	neonate		4600(2790-7070)	ng/ml	mortality		Li
cry01Ab	*Ostrinia*	*nubilalis*	Diet incorporation	neonate		0.03(0.02-0.04)	ng/ml	mortality		Huang
cry01Ab	*Ostrinia*	*nubilalis*	Diet incorporation	L1		290(120-590)	ng/ml	mortality		Hua
cry01Ab	*Pandemis*	*pyrusana*	Diet surface	neonate		12.6(3.4-69.7)	ng/cm2	mortality		Knight
cry01Ab	*Pectinophora*	*gossypiella*	Diet incorporation	neonate				mortality	active	Tabashnik
cry01Ab	*Pectinophora*	*gossypiella*	Diet incorporation	L1		116(85-165)	ng/mg	mortality		Karim
cry01Ab	*Pieris*	*brassicae*	Leaf surface	L2				mortality	active	Visser
cry01Ab	*Platynota*	*stultana*	Diet surface	neonate		0.8(0.01-0.9)	ng/cm2	mortality		Knight
cry01Ab	*Plutella*	*xylostella*	Diet surface	L3		3.9(3.3-4.7)	ng/cm2	mortality	active	Tabashnik
cry01Ab	*Plutella*	*xylostella*	Leaf surface	neonate		1450(1150-1780)	ng/cm2	mortality		Mandaokar
cry01Ab	*Plutella*	*xylostella*	Leaf dip	L3		14000(490-90000)	ng/ml	mortality		Tabashnik
cry01Ab	*Plutella*	*xylostella*	Leaf dip	L3	Dipel	>10.6X10**6	ng/ml	mortality		Tabashnik
cry01Ab	*Pseudoplusia*	*includens*	Diet incorporation	neonate		670	ng/ml	mortality		Luttrell
cry01Ab	*Sesamia*	*calamistis*	Diet surface	neonate		0.1(0.06-0.38)	ng/cm2	mortality		Tounou
cry01Ab	*Spodoptera*	*exigua*	Diet incorporation	neonate		3180	ng/ml	mortality		Luttrell
cry01Ab	*Spodoptera*	*exigua*	Diet incorporation	neonate		107000(75000-187000)	ng/ml	mortality		Moar

Gene	Genus	Species	Assay Type	Instar Age	Insect Strain	ED50	Units	Para-meter	Quali-tative	Reference
cry01Ab	*Spodoptera*	*exigua*	Diet surface	L2		68X10**7	cells/cm2	weight		Honee
cry01Ab	*Spodoptera*	*exigua*	Diet surface	neonate		3880(3400-4480)	ng/cm2	mortality		Chambers
cry01Ab	*Spodoptera*	*exigua*	Diet surface	L2		5.6X10**7	cells/cm2	weight		Visser
cry01Ab	*Spodoptera*	*exigua*	Diet surface	L1		601(214-1014)	ng/cm2	mortality		De Maagd
cry01Ab	*Spodoptera*	*exigua*	Diet surface	neonate	HOL	150(77-262)	ng/cm2	mortality		Hernandez-Martinez
cry01Ab	*Spodoptera*	*exigua*	Diet surface	neonate	FRA	971(303-2251)	ng/cm2	mortality		Hernandez-Martinez
cry01Ab	*Spodoptera*	*frugiperda*	Diet surface	neonate		867(539-1215)	ng/cm2	mortality		Sena
cry01Ab	*Spodoptera*	*frugiperda*	Diet incorporation	neonate		95890	ng/ml	mortality		Luttrell
cry01Ab	*Spodoptera*	*littoralis*	Diet surface	L2		7243(2000-25900)	ng/cm2	mortality		Muller-Cohn
cry01Ab	*Spodoptera*	*littoralis*	Diet surface	L2	cry01C	17900(8900-51300)	ng/cm2	mortality		Muller-Cohn
cry01Ab	*Thaumetopoea*	*pityocampa*	Leaf dip	L1		895(694-1687)	ng/ml	mortality		Rausell
cry01Ab	*Trichoplusia*	*ni*	Diet incorporation	neonate		1230(900-1820)	ng/ml	mortality		Moar
cry01Ab	*Trichoplusia*	*ni*	Diet surface	neonate		3.4(2.3-4.0)	ng/cm2	mortality	active	Iracheta
cry01Ab1	*Spodoptera*	*exigua*	Diet incorporation	L3		>100000	ng/ml	mortality		De Maagd
cry01Ab10	*Agrotis*	*ipsilon*	Diet incorporation	neonate		>80000	ng/ml	mortality		Macintosh
cry01Ab10	*Helicoverpa*	*zea*	Diet incorporation	neonate		33000(15400-117800)	ng/ml	mortality		Macintosh
cry01Ab10	*Heliothis*	*virescens*	Diet incorporation	neonate		1700(1020-3190)	ng/ml	mortality		Macintosh
cry01Ab10	*Manduca*	*sexta*	Diet surface	neonate				mortality	active	Fischhoff
cry01Ab10	*Manduca*	*sexta*	Diet incorporation	neonate		40(20-120)	ng/ml	mortality		Macintosh
cry01Ab10	*Ostrinia*	*nubilalis*	Diet incorporation	neonate		3600(1760-6890)	ng/ml	mortality		Macintosh
cry01Ab10	*Spodoptera*	*exigua*	Diet incorporation	neonate		34000(26300-44300)	ng/ml	mortality		Macintosh
cry01Ab10	*Trichoplusia*	*ni*	Diet incorporation	neonate		190(100-390)	ng/ml	mortality		Macintosh
cry01Ab2	*Helicoverpa*	*armigera*	Diet incorporation	neonate		>16000	ng/ml	mortality	not active	Avilla
cry01Ab2	*Heliothis*	*virescens*	Diet incorporation	neonate	cry01Ac	>10**6	ng/ml	mortality		Lee
cry01Ab2	*Heliothis*	*virescens*	Diet incorporation	neonate		360(238-516)	ng/ml	mortality		Lee
cry01Ab2	*Lymantria*	*dispar*	Diet surface	neonate		37(28-45)	ng/cm2	mortality		Lee
cry01Ab2	*Lymantria*	*dispar*	Force feed	L4		205(157-267)	ng/larva	weight		Lee
cry01Ab2	*Lymantria*	*dispar*	Force feed	L4		214(136-301)	ng/larva	weight		Lee
cry01Ab2	*Lymantria*	*dispar*	Force feed	L4		157(128-193)	ng/larva	weight		Liang
cry01Ab2	*Manduca*	*sexta*		neonate		7.5(4.5-10.0)	ng/cm2			Lee
cry01Ab2	*Trichoplusia*	*ni*	Diet incorporation					mortality+ weight	Highly active	Thorne
cry01Ab3	*Helicoverpa*	*armigera*	Diet surface	L1	ANO2	1185(837-2055)	ng/cm2	mortality		Liao
cry01Ab3	*Helicoverpa*	*armigera*	Diet surface	L1	ANO2	692(458-1071)	ng/cm2	mortality		Liao
cry01Ab3	*Helicoverpa*	*armigera*	Diet incorporation	neonate		>16000	ng/ml	mortality	not active	Avilla
cry01Ab3	*Helicoverpa*	*punctigera*	Diet surface	L1		241(24-520)	ng/cm2	mortality		Liao
cry01Ab3	*Helicoverpa*	*punctigera*	Diet surface	L1		355(165-501)	ng/cm2	mortality		Liao
cry01Ab3	*Heliothis*	*virescens*	Diet surface	L1		1600(800-2400)	ng/cm2	mortality		Lee

Gene	Genus	Species	Assay Type	Instar Age	Insect Strain	ED50	Units	Para-meter	Quali-tative	Reference
cry01Ab3	*Trichoplusia*	*ni*	Diet surface	L1		2000(1600-4000)	ng/cm2	mortality		Lee
cry01Ab4	*Bombyx*	*mori*	Diet incorporation	L3		5100	ng/ml	mortality		Kondo
cry01Ab5	*Bombyx*	*mori*	Force feed	L5				mortality	not active	Nakatani
cry01Ab5	*Bombyx*	*mori*	Force feed	L4		>1000	ng/larva	mortality		Pujiastuti
cry01Ab5	*Heliothis*	*virescens*	Diet surface	neonate		10.0	ng/cm2	mortality		Hofte
cry01Ab5	*Heliothis*	*virescens*	Diet incorporation	neonate		1120(700-1640)	ng/ml	mortality		Gould
cry01Ab5	*Heliothis*	*virescens*	Diet incorporation	neonate	cry01Ac	14510(10200-20720)	ng/ml	mortality		Gould
cry01Ab5	*Heliothis*	*virescens*	Diet surface	neonate		43(27-68)	ng/cm2	mortality		Schnepf
cry01Ab5	*Mamestra*	*brassicae*	Diet surface	L1		162	ng/cm2	mortality		Hofte
cry01Ab5	*Manduca*	*sexta*	Diet surface	L1		6	ng/cm2	mortality		Hofte
cry01Ab5	*Manduca*	*sexta*	Diet surface	neonate		20(15-26)	ng/cm2	mortality		Schnepf
cry01Ab5	*Manduca*	*sexta*	Diet surface	L1		6.8(3.7-12.6)	ng/cm2	mortality		Hofmann
cry01Ab5	*Manduca*	*sexta*	Diet surface	L1		8.6	ng/cm2	mortality		Hofte
cry01Ab5	*Manduca*	*sexta*	Diet surface	L1		5	ng/cm2	mortality		Hofte
cry01Ab5	*Ostrinia*	*nubilalis*	Diet surface	neonate		50(26-95)	ng/cm2	mortality		Denolf
cry01Ab5	*Ostrinia*	*nubilalis*	Diet surface	neonate		50(31-82)	ng/cm2	mortality		Denolf
cry01Ab5	*Perileucoptera*	*coffeella*	Leaf dip	neonate		>1000000		mortality	not active	Filho
cry01Ab5	*Pieris*	*brassicae*	Leaf surface	L1		900(600-1400)	ng/ml	mortality		Hofmann
cry01Ab5	*Pieris*	*brassicae*	Leaf surface	L3		1.6	ng/larva	mortality		Hofte
cry01Ab5	*Pieris*	*brassicae*	Leaf surface	L3		1.5	ng/larva	mortality		Hofte
cry01Ab5	*Pieris*	*brassicae*	Leaf dip	L3		700	ng/ml	mortality		Hofte
cry01Ab5	*Plodia*	*interpunctella*	Diet surface	L3	HD-133+HD-1	11800(5800-75300)	ng/larva	mortality		McGaughey
cry01Ab5	*Plodia*	*interpunctella*	Diet surface	L3	HD-198	1300(1000-1600)	ng/larva	mortality		McGaughey
cry01Ab5	*Plodia*	*interpunctella*	Diet surface	L3	HD-133	10600(5500-32500)	ng/larva	mortality		McGaughey
cry01Ab5	*Plodia*	*interpunctella*	Diet surface	L3	HD-112	1100(900-1400)	ng/larva	mortality		McGaughey
cry01Ab5	*Plodia*	*interpunctella*	Diet surface	L3	Dipel	12300(8400-20600)	ng/larva	mortality		McGaughey
cry01Ab5	*Plodia*	*interpunctella*	Diet surface	L3		50(40-60)	ng/larva	mortality		McGaughey
cry01Ab5	*Plodia*	*interpunctella*	Diet surface	L2-L3		120(80-180)	ng/larva	mortality		Van Rie
cry01Ab5	*Plodia*	*interpunctella*	Diet surface	L2-L3	Dipel	>12800	ng/larva	mortality		Van Rie
cry01Ab5	*Plodia*	*interpunctella*	Diet surface	L2-L3		30(20-50)	ng/larva	mortality		Van Rie
cry01Ab5	*Plodia*	*interpunctella*	Diet surface	L2-L3	Dipel	26300	ng/larva	mortality		Van Rie
cry01Ab5	*Plutella*	*xylostella*	Diet surface	L3	Dipel	>1350	ng/cm2	mortality		Ferre
cry01Ab5	*Plutella*	*xylostella*	Diet surface	L3		6.7(2.8-16.1)	ng/cm2	mortality		Ferre
cry01Ab5	*Spodoptera*	*frugiperda*	Diet surface	neonate		>2000	ng/cm2	mortality		Aranda
cry01Ab5	*Spodoptera*	*littoralis*	Diet surface	L1		>1350	ng/cm2	mortality		Hofte
cry01Ab5	*Spodoptera*	*litura*	Diet incorporation	L3		6650(5020-9130)	ng/ml	mortality		Pujiastuti
cry01Ab5	*Thaumetopoea*	*pityocampa*	Leaf dip	neonate		895(694-1687)	ng/ml	mortality		Rausell
cry01Ab5	*Trichoplusia*	*ni*	Diet surface	neonate		480(380-600)	ng/cm2	mortality		Estada

Gene	Genus	Species	Assay Type	Instar Age	Insect Strain	ED50	Units	Para-meter	Quali-tative	Reference
cry01Ab6	*Actebia*	*fennica*	Force feed	L5		>3500	ng/larva	frass		Van Frankenhuyzen
cry01Ab6	*Actebia*	*fennica*	Force feed	L5		>2269	ng/larva	frass		Van Frankenhuyzen
cry01Ab6	*Choristoneura*	*fumiferana*	Force feed	L6		20.4(16.0-25.3)	ng/larva	frass		Van Frankenhuyzen
cry01Ab6	*Choristoneura*	*fumiferana*	Force feed	L6		13.2(10.0-17.2)	ng/larva	frass		Van Frankenhuyzen
cry01Ab6	*Choristoneura*	*occidentalis*	Force feed	L6		10.8(5.5-19.6)	ng/larva	frass		Van Frankenhuyzen
cry01Ab6	*Choristoneura*	*pinus pinus*	Force feed	L6		17.3(11.7-24.9)	ng/larva	frass		Van Frankenhuyzen
cry01Ab6	*Chrysoperla*	*carnea*	Diet incorporation	L1,L2,L3				mortality	active	Hilbeck
cry01Ab6	*Elasmolpalpus*	*lignosellus*	Diet incorporation	neonate		100000(31000-1123000	ng/ml	mortality		Moar
cry01Ab6	*Lymantria*	*dispar*	Force feed	L4		46.8(34.7-62.8)	ng/larva	frass		Van Frankenhuyzen
cry01Ab6	*Lymantria*	*dispar*	Force feed	L4		22.0(16.6-28.9)	ng/larva	frass		Van Frankenhuyzen
cry01Ab6	*Malacosoma*	*disstria*	Force feed	L5		25.5(15.1-39.6)	ng/larva	frass		Van Frankenhuyzen
cry01Ab6	*Orgyia*	*leucostigma*	Force feed	L4		25.0(15.1-40.1)	ng/larva	frass		Van Frankenhuyzen
cry01Ab6	*Plutella*	*xylostella*	Diet surface	L3		15(5-30)	ng/cm2	mortality		Granero
cry01Ab6	*Plutella*	*xylostella*	Leaf dip			600(400-1200)	ng/ml	mortality		Tang
cry01Ab6	*Spodoptera*	*exigua*	Diet incorporation	neonate	cry01C	5.86X10**6	ng/ml	mortality		Moar
cry01Ab6	*Spodoptera*	*exigua*	Diet incorporation	neonate		83000(54000-150000)	ng/ml	mortality		Moar
cry01Ab6	*Spodoptera*	*exigua*	Diet incorporation	neonate		63200(39300-112000)	ng/ml	mortality		Moar
cry01Ab6	*Trichoplusia*	*ni*	Diet incorporation	neonate		720(550-910)	ng/ml	mortality		Moar
cry01Ab7	*Aedes*	*aegypti*	Free ingestion	L2				mortality	active	Haider
cry01Ab7	*Aedes*	*aegypti*	Free ingestion	L2				mortality	active	Haider
cry01Ab7	*Aedes*	*aegypti*	Free ingestion	L2				mortality	active	Haider
cry01Ab7	*Aedes*	*aegypti*	Free ingestion	L2				mortality	active	Haider
cry01Ab7	*Pieris*	*brassicae*	Leaf disk	L3				mortality	not active	Haider
cry01Ab7	*Pieris*	*brassicae*	Leaf disk	L3				mortality	active	Haider
cry01Ab7	*Pieris*	*brassicae*	Leaf disk	L3				mortality	active	Haider
cry01Ab7	*Pieris*	*brassicae*	Leaf disk	L3				mortality	active	Haider
cry01Ab8	*Bombyx*	*mori*	Diet incorporation	L4		175800	ng/ml	mortality		Ihara
cry01Ab8	*Bombyx*	*mori*	Diet incorporation	L4		74000	ng/ml	mortality		Ihara
cry01Ab8	*Bombyx*	*mori*	Diet incorporation	L4		27800	ng/ml	mortality		Ihara
cry01Ab8	*Plutella*	*xylostella*	Leaf dip	L3		1100	ng/ml	mortality		Oeda
cry01Ab8	*Plutella*	*xylostella*	Leaf dip	L3		200-2000	ng/ml	mortality		Nakamura
cry01Ab8	*Spodoptera*	*litura*	Diet incorporation	L4		0.3X10**6	ng/ml	mortality		Nakamura
cry01Ab9	*Heliothis*	*virescens*	Diet incorporation	neonate		824(40-1400)	ng/ml	mortality		Rajamohan
cry01Ab9	*Lymantria*	*dispar*	Force feed	L4		2358(1758-2780)	ng/larva	weight		Lee
cry01Ab9	*Lymantria*	*dispar*	Force feed	L4		2650(2000-3050)	ng/larva	weight		Rajamohan
cry01Ab9	*Lymantria*	*dispar*	Diet surface	neonate		290(210-350)	ng/cm2	mortality		Rajamohan
cry01Ab9	*Lymantria*	*dispar*	Diet surface	neonate		375(280-435)	ng/cm2	mortality		Lee
cry01Ab9	*Manduca*	*sexta*	Diet surface	neonate		15(10-25)	ng/cm2	mortality		Rajamohan

Gene	Genus	Species	Assay Type	Instar Age	Insect Strain	ED50	Units	Para-meter	Quali-tative	Reference
cry01Ab9	*Manduca*	*sexta*		neonate		19.5(15-27)	ng/cm2			Lee
cry01Ab9	*Manduca*	*sexta*	Diet surface	neonate		62(36-91)	ng/cm2	mortality		Rajamohan
cry01Ab9	*Manduca*	*sexta*	Diet surface	neonate		154(90-230)	ng/cm2	mortality		Chen

Appendix E

List of Bacillus strains producing more than one Cry, Cyt or Vip toxin or related protein.

List based on the toxin lists on the website of the Sussex University [w1] (at February 16, 2014). Bacillus strains from a known serovar (subspecies) of which at least one other strain produces two or more toxins, are also listed.

species	strain	serovar	protein
B. sphaericus[a]	Bs IAB59		Cry48Aa, Cry49Aa
B. sphaericus[a]	Bs 47-6B		Cry48Aa2, Cry49Aa2
B. sphaericus[a]	Bs NHA15b		Cry48Aa3, Cry49Aa3
B. sphaericus[a]	Bs LP1G		Cry48Ab, Cry49Ab1
B. sphaericus[a]	Bs 2173		Cry48Ab2, Cry49Aa4
B. thuringiensis	Bt aizawai IPL7	*aizawai*	Cry1Aa3, Cry1Ab8
B. thuringiensis	Bt aizawai IC1	*aizawai*	Cry1Ab7
B. thuringiensis	Bt aizawai HD133	*aizawai*	Cry1Ab9, Vip3Aa
B. thuringiensis	Bt aizawai PS81I	*aizawai*	Cry1Ad1, Cry1Ca3, Cry1Fa2
B. thuringiensis	Bt aizawai 7.29	*aizawai*	Cry1Ca2, Cry1Ca5
B. thuringiensis	Bt aizawai HD68	*aizawai*	Cry1Da1
B. thuringiensis	Bt aizawai PS81A2	*aizawai*	Cry1Eb1
B. thuringiensis	Bt aizawai EG6346	*aizawai*	Cry1Fa1
B. thuringiensis	Bt aizawai BUN1-14	*aizawai*	Cry30Db1
B. thuringiensis	Bt aizawai	*aizawai*	Cry39Aa1, Cry40Aa1
B. thuringiensis	Bt aizawai SSK-10	*aizawai*	Cry9Ea1
B. thuringiensis	Bt alesti	*alesti*	Cry1Ae1, Cry1Ah2
B. thuringiensis	BGSC 4AW1	*andalousiensis*	Cry41Ba2, Cry66Aa2
B. thuringiensis	Bt berliner 1715	*berliner*	Cry1Ab1, Cry1Ab5
B. thuringiensis	Bt cameroun 273B	*cameroun*	CryC35, CryC53
B. thuringiensis	Bt dakota HD511	*dakota*	Cry7Ab1
B. thuringiensis	Bt dakota	*dakota*	Cry7Kb1, Cry7La1, Cry33Aa1, Cry46Aa, NT32KD
B. thuringiensis	Bt darmstadiensis PS17	*darmstadiensis*	Cry5Ab1, Cyt2Aa2
B. thuringiensis	Bt darmstadiensis73E10	*darmstadiensis*	Cyt2Aa2
B. thuringiensis	Bt entomocidus HD110	*entomocidus*	Cry1Ba2, Cry1Ca4, Vip3Aa37
B. thuringiensis	Bt entomocidus HD9	*entomocidus*	Cry1Ba4
B. thuringiensis	Bt entomocidus 60.5	*entomocidus*	Cry1Ca1
B. thuringiensis	Bt entomocidus BP465	*entomocidus*	Cry1Ib1
B. thuringiensis	Bt entomocidus	*entomocidus*	Cry1Aa4, Cry30Ba1, Cry58Aa1
B. thuringiensis	Bt entomocidus INA288	*entomocidus*	Cry44Aa
B. thuringiensis	Bt finitimus B-1166	*finitimus*	Cry26Aa1
B. thuringiensis	Bt finitimus B-1161	*finitimus*	Cry28Aa1
B. thuringiensis	Bt finitimus	*finitimus*	Cry28Aa2
B. thuringiensis	Bt fukuokaensis	*fukuokaensis*	Cry20Aa1, Cyt2Ba3
B. thuringiensis	Bt galleriae HD29	*galleriae*	Cry1Cb1
B. thuringiensis	Bt galleriae PGSI245	*galleriae*	Cry7Aa1
B. thuringiensis	Bt galleriae	*galleriae*	Cry8Da1, Cry9Aa1, Cry9Ba1, Cry9Ec1, Cry9-like
B. thuringiensis	Bt HD29	*gellarie*	Cry2Ac11, Cry2Ad5
B. thuringiensis	Bt israelensis ONR-60A	*israelensis*	Cry10Aa2

species	strain	serovar	protein
B. thuringiensis	Bt israelensis	*israelensis*	Cry4Aa1, Cry4Aa3, Cry4Ba2, Cry4Ba3, Cry4Ba5, Cry10Aa1, Cry10Aa3, Cry11Aa1, Cry11Aa2, Cry11Aa3, Cry60Aa2, Cry60Ba2, Cyt1Aa1, Cyt1Aa2, Cyt1Aa5, Cyt1Ca1, Cyt2Ba9
B. thuringiensis	Bt israelensis HD522	*israelensis*	Cry4Aa2, Cry4Ba4
B. thuringiensis	Bti BRC-LLP29	*israelensis*	Cry4Aa4, Cry10Aa4, Cry11Aa4
B. thuringiensis	Bt israelensis 4Q2-72	*israelensis*	Cry4Ba1
B. thuringiensis	Bt israelensis 4Q2	*israelensis*	Cyt2Ba1
B. thuringiensis	Bti HD 567	*israelensis*	Cyt2Ba10
B. thuringiensis	Bti HD 522	*israelensis*	Cyt2Ba11
B. thuringiensis	Bti INTA H41-1	*israelensis*	Cyt2Ba12
B. thuringiensis	Bti IPS82	*israelensis*	Cyt2Ba14
B. thuringiensis	Bt israelensis PG14	*israelensis*	Cyt2Ba2
B. thuringiensis	Bt japonensis Buibui	*japonensis*	Cry8Ca1
B. thuringiensis	Bt japonensis	*japonensis*	Cry9Bb1, Cry9Da2
B. thuringiensis	Bt japonensis N141	*japonensis*	Cry9Da1
B. thuringiensis	Bt jegathesan 367	*jegathesan*	Cry11Ba1; Cry19Aa1; Cry24Aa1; Cry25Aa1; Cry30Ca2; Cry60Aa1; Cry60Ba; Cyt2Bb1
B. thuringiensis	Bt kenyae K3	*kenyae*	Cry1Ab33
B. thuringiensis	Bt kenyae HD549	*kenyae*	Cry1Ac17, Cry2Aa4
B. thuringiensis	Bt kenyae	*kenyae*	Cry1Ac2, Cry1Ea2, Cry8Ka2
B. thuringiensis	Bt kenyae 4F1	*kenyae*	Cry1Ea1
B. thuringiensis	Bt kenyae PS81F	*kenyae*	Cry1Ea3
B. thuringiensis	Bt kenyae LBIT-147	*kenyae*	Cry1Ea4
B. thuringiensis	Bt kumamotoensis 867	*kumamotoensis*	Cry7Ab2
B. thuringiensis	Bt kumamotoensis	*kumamotoensis*	Cry8Aa1, Cry8Ba1
B. thuringiensis	Bt kurstaki HD1	*kurstaki*	Cry1Aa1, Cry1Ab3, Cry1Ab4, Cry1Ab10, Cry1Ac13, Cry1Ia3, Cry2Aa2, Cry2Ab1, Cry2Ab2, Vip3Aa33
B. thuringiensis	Bt kurstaki HD-1-02	*kurstaki*	Cry1Aa10
B. thuringiensis	Bt kurstaki	*kurstaki*	Cry1Aa11, Cry1Ab2, Cry1Ia1, Cry1Ia2, Cry2Aa1
B. thuringiensis	Bt kurstaki NRD-12	*kurstaki*	Cry1Aa6, Cry1Ab6, Cry1Ac6
B. thuringiensis	Bt kurstaki S93	*kurstaki*	Cry1Ab12
B. thuringiensis	DOR BT-1	*kurstaki*	Cry1Ab26
B. thuringiensis	Bt kurstaki HD73	*kurstaki*	Cry1Ac1, Cry1Ac7, Cry1Ac8
B. thuringiensis	Bt kurstaki YBT-1520	*kurstaki*	Cry1Ac10
B. thuringiensis	Bt kurstaki PS85A1	*kurstaki*	Cry1Ac4
B. thuringiensis	Bt kurstaki PS81GG	*kurstaki*	Cry1Ac5
B. thuringiensis	Bt kurstaki BNS3	*kurstaki*	Cry1Ia11
B. thuringiensis	Bt kurstaki S101	*kurstaki*	Cry1Ia6
B. thuringiensis	Bt kurstaki K1	*kurstaki*	Cry1La1
B. thuringiensis	Btk MnD	*kurstaki*	Cry2Ab25
B. thuringiensis	Bt kurstaki BtI109P	*kurstaki*	Cry3Ca1
B. thuringiensis	Bt kurstaki DP1019	*kurstaki*	Cry9Db1, Cry9Ed1
B. thuringiensis	Bt medellin	*medellin*	Cry11Bb1, Cry29Aa1, Cry30Aa1, Cyt1Ab1, Cyt2Bc1
B. thuringiensis	Bt morrisoni	*morrisoni*	Cry1Bc1, Cry1Fb3
B. thuringiensis	Bt morrisoni INA67	*morrisoni*	Cry1Fb2
B. thuringiensis	Bt morrisoni BF190	*morrisoni*	Cry1Hb1, Cry1Ka1
B. thuringiensis	Bt morrisoni EG2158	*morrisoni*	Cry3Aa5
B. thuringiensis	Bt morrisoni PG14	*morrisoni*	Cyt1Aa3, Cyt1Aa4
B. thuringiensis	Bt morrisoni HD12	*morrisoni*	Cyt2Ba4

species	strain	serovar	protein
B. thuringiensis	Bt morrisoni HD518	*morrisoni*	Cyt2Ba5
B. thuringiensis	Bt sotto PS80JJ1	*sotto*	Cry14Aa1
B. thuringiensis	Bt sotto	*sotto*	Cry1Aa2, Cry1Aa13, Cry2Aa3, Cry24Ba1, Cry30Ca1, Cry50Aa1
B. thuringiensis	Bt tenebrionis Mm2	*tenebrionis*	Cry3Aa11
B. thuringiensis	Bt tenebrionis	*tenebrionis*	Cry3Aa2, Cry3Aa4, Cry3Aa6, Cry3Aa12, Cyt2Ba6
B. thuringiensis	Bt thompsoni	*thompsoni*	Cry15Aa1, 40kDa
B. thuringiensis	BGSC 4Y1	*tochigiensis*	Cry7Ea3, Cry7Fa2, Cry61Aa3, Cry67Aa2
B. thuringiensis	Bt tolworthi 43F	*tolworthi*	Cry3Ba1
B. thuringiensis	Bt tolworthi	*tolworthi*	Cry9Ca1
B. thuringiensis	Bt wuhanensis HD525	*wuhanensis*	Cry1Bd1, Cry1Gb1
B. thuringiensis	Bt wuhanensis	*wuhanensis*	Cry1Ga2, Cry2Ac6
B. thuringiensis	Bt higo		Cry19Ba1, Cry27Aa1
B. thuringiensis	Bt Ly30		Cry1Aa12, Cry1Ac14, Cry1Ia9, Cry2Ab5
B. thuringiensis	Bt INTA Mol-12[c]		Cry1Aa15, Cry1Ac21
B. thuringiensis	Bt PS9-C12		Cry1Aa17, Cry2Ab17, Cry1Aa18
B. thuringiensis	Bt ly4a3		Cry1Ab-like, Cry1I-like
B. thuringiensis	Bt A20		Cry1Ab11, Cry1Ac12
B. thuringiensis	Bt B-Hm-16		Cry1Ab15, Cry9Ea2
B. thuringiensis	Bt WB9		Cry1Ab17, Cry2Ac4
B. thuringiensis	Bt HD12		Cry1Ab24, Cry1Da3, Cry1Ib11, Cry1Id2
B. thuringiensis	Bt C-33		Cry1Ac19, Cry1Ca11
B. thuringiensis	Bt 146-158-01		Cry1Ac24, Cry2Aa13
B. thuringiensis	INTA TA24-6		Cry1Ac29, Vip3Aa34
B. thuringiensis	Bt S6		Cry1Ah3, Cry1Ea9
B. thuringiensis	Bt SC6H8		Cry1Ai2, Cry2Ah1
B. thuringiensis	Bt EG5847		Cry1Bb1, Cry1Ja1
B. thuringiensis	Bt WBT-2		Cry1Bb2, Cry1Hb2, Cry1Ka2
B. thuringiensis	Bt 087		Cry1Cb3, Cry1Fb7
B. thuringiensis	Bt BTS00349A		Cry1Db1, Cry1Fb1
B. thuringiensis	Bt B-Pr-88		Cry1Db2, Cry1Fb5, Cry1Gb2, Cry2Ab4
B. thuringiensis	Bt JC291		Cry1Dc1, Cry1H-like
B. thuringiensis	Bt AB88		Cry1Ia4, Vip3Aa1
B. thuringiensis	Bt BF-4		Cry1Ib4, Cry2Ab15
B. thuringiensis	Bt T03B001		Cry1Ie2, Cry9Da4, Cry9Ee1, Cry9Aa4
B. thuringiensis	Bt SSy77		Cry2Aa15, Cry2Ab26
B. thuringiensis	Bt CMBL-BT1		Cry2Ab11, Cry2Ac8
B. thuringiensis	Bt ywc5-4		Cry2Ab13, Cry9Ea4
B. thuringiensis	Bt LTS-7		Cry2Ab28, Cry9Ea9
B. thuringiensis	Bt WZ-7		Cry2Ab6, Vip3Aa24
B. thuringiensis	Bt CMBL-BT2		Cry2Ac9, Cry2Ad4
B. thuringiensis	Bt B0195		Cry31Aa3, Cry31Ab1
B. thuringiensis	Bt M019[d]		Cry31Aa6, Cry31Ad1, Cry63Aa1
B. thuringiensis	Bt PS80JJ1		Cry34Aa1, Cry35Aa1
B. thuringiensis	Bt EG5899		Cry34Aa2, Cry35Aa2
B. thuringiensis	Bt PS69Q		Cry34Aa3, Cry35Aa3
B. thuringiensis	Bt PS185GG		Cry34Aa4, Cry35Aa4
B. thuringiensis	Bt PS149B1		Cry34Ab1, Cry35Ab1
B. thuringiensis	Bt PS167H2		Cry34Ac1, Cry35Ac1
B. thuringiensis	Bt EG9444		Cry34Ac2, Cry35Ab2
B. thuringiensis	Bt KR1369		Cry34Ac3, Cry35Ab3

species	strain	serovar	protein
B. thuringiensis	Bt EG4851		Cry34Ba1, Cry35Ba1
B. thuringiensis	Bt PS201L3		Cry34Ba2, Cry35Ba2
B. thuringiensis	Bt PS201HH2		Cry34Ba3, Cry35Ba3
B. thuringiensis	Bt A1462		Cry41Aa1, Cry41Ab1, Cry42Aa1, Cry46Aa2
B. thuringiensis	Sbt021[e]		Cry41Ba1, Cry66Aa1
B. thuringiensis	Bt LDC-9		Cry4A-like, Cry4Ba-like, Cry10A-like, Cyt1Aa-like, Cyt2Ba-like, Cry11Aa-like
B. thuringiensis	Bt Y41		Cry4Ca1, Cry30Da1, Cry40Ca1, Cry52Aa1, Cry53Aa1, Cry55Aa2
B. thuringiensis	Bt HS18-1[f]		Cry4Cb1, Cry30Ga1, Cry56Aa3, Cry69Ab1, Cry70Aa1, Cry71Aa1, Cry72Aa1, Cyt1Da2
B. thuringiensis	Bt Ywc2-8		Cry4Cb2, Cry30Ea2, Cry56Aa1
B. thuringiensis	Bt S2160-1		Cry4Cb3, Cry30Ea1, Cry30Ga2, Cry50Ba1, Cry54Ba1
B. thuringiensis	Bt MC28		Cry4Cc1, Cry30Fa1, Cry53Ab1, Cry54Aa1, Cry54Ab1, Cry68Aa1, Cry69Aa1, Cry69Aa2, Cry70Ba1, Cyt1Da1, Cyt2Aa3
B. thuringiensis	Bt BM59-2		Cry52Ba1, Cry59Ba1
B. thuringiensis	YBT 1518		Cry5Ba2, Cry6Aa2, Cry55Aa1
B. thuringiensis	Sbt003[g]		Cry5Ca1, Cry5Da1, Cry5Ea1, Cry65Aa1
B. thuringiensis	Bt T13001		Cry5Ca2, Cry5Da2, Cry5Ea2
B. thuringiensis	Bt IBL 4222		Cry60Aa3, Cry60Ba3
B. thuringiensis	Bt HQ122		Cry7Ab4, Cry7Ab6
B. thuringiensis	Sbt009[h]		Cry7Ea1, Cry7Fa1, Cry61Aa1, Cry67Aa1
B. thuringiensis	Bt 185		Cry8Ea1, Cry8Fa1, Cry8Ha1
B. thuringiensis	Bt F4		Cry8Ib1, Cry8La1
B. thuringiensis	Bt ST8[i]		Cry8Kb1, Cry8Pa1, Cry8Qa1
B. thuringiensis	Bt INTA Fr7-4[j]		Cry8Kb3, Cry8Pa3, Cry8Qa2
B. thuringiensis	Bt SC5 (D2)		Cry9Da3, Cry9Aa3
B. thuringiensis	Bt LLP29		Cyt1Aa6, Cyt2Ba15
Clostridium bifermentans	Cb malaysia CH18		Cry16Aa1, Cry17Aa1
Paenibacillus lentimorbus	P. lentimorbus semadara		Cry43Aa1, Cry43Ba1, Cry43-like
Paenibacillus popilliae	P. popilliae		Cry18Aa1, Cry18Ba1, Cry18Ca1

[a]: *B sphaericus* is renamed to *Lysinibacillus sphaericus*.
[b]: Strains Bt jegathesan 367 and Bt jegathesan combined [174].
[c]: Strains Bt INTA Mol-12 and INTA Mol-12 are combined.
[d]: Strains M019 and MO19 are combined.
[e]: Strains Sbt021 and SBt 021 are combined.
[f]: Strains Bt hs18-1, Bt Hs18-1, Bt HS18-1 and hs18-1 are combined
[g]: Strains with code Sbt003 and SBt 003 are combined.
[h]: Strains Sbt009 and SBt 009 are combined.
[i]: Strains Bt ST8 and ST8 are combined.
[j]: Strains Bt INTA Fr7-4 and INTA Fr7-4 are combined.