Can interactions between *Bt* proteins be predicted and how should effects on non-target organisms of GM crops with multiple *Bt* proteins be assessed?

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Can interactions between *Bt* proteins be predicted and how should effects on non-target organisms of GM crops with multiple *Bt* Proteins be assessed?

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Colophon

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Foreword

The risk assessment of genetically modified plants that have been equipped with more than one introduced gene presents a special challenge for risk assessment, especially if the possibility exists that the different gene products interact with each other. In the case of plants expressing several *Bacillus thuringiensis* toxic proteins this possibility is no theory. The science of combination toxicology learns us that non-linear interactions between toxicants acting at the same time can give rise to extremely complicated phenotypic effects, if one toxicant affects the action of another in a positive or negative way. Important issues in the analysis are similarities in the mode of action and linearity of the dose-response. In addition, the target species upon which the toxicants act might also determine whether effects are additive, synergistic or antagonistic. In this extremely complicated field, a combination of knowledge is necessary from molecular toxicology, ecotoxicology, plant science and risk assessment. The present report, together with the accompanying report by ECOΣTAT aims to provide this combination of knowledge and answers the questions mentioned above. These reports, in conjunction with discussions at a workshop organized 15 October 2014 will be a valuable contribution towards a practical approach for the Netherlands Commission on Genetic Modification's (COGEM's) risk assessment strategy of genetically modified plants expressing more than one toxicant.

Prof. dr. N.M. van Straalen
Chair of the advisory committee

Preface authors

This report has been drafted as a response to a call of the Netherlands Commission on Genetic Modification (COGEM) to address the questions on how *Bacillus thuringiensis* (*Bt*) proteins can influence each other and whether *Bt* protein interactions affect organisms not targeted by genetically modified (GM) crops differently than the separate *Bt* proteins. During a first consultation with the advisory committee, it became clear that these issues were raised to address open questions encountered in the evaluation of impacts of GM crops expressing multiple *Bt* proteins on non-target organisms (invertebrates). First, COGEM would like to know whether interactions between *Bt* proteins can be predicted and if there are arguments to state whether interactions will occur or not. Second, COGEM would like to know to what extent studies on interactions, either conducted with target organisms or non-target organisms, are relevant for risk/safety assessment of GM crops. In particular, COGEM questioned whether data on combination toxicity of *Bt* proteins in target organisms can be extrapolated to non-target organisms. Further, the question was raised what has to be done in the risk/safety assessment if interactions have been shown to occur.

Answering the research questions can be done from different angles. A parallel project, also commissioned by COGEM and performed by EcoΣtat, addresses the questions from a biochemical and a toxicological perspective (EcoΣtat, 2014). For detailed information on the molecular mechanisms of *Bt* protein interaction and models to assess such interactions, we therefore refer to this project. The angle from which we looked at the topic is eco-toxicology. More in particular, we looked into the question on how *Bt* proteins may affect each other at the level of their eco-toxicological activity towards invertebrates. The theme for addressing the research questions is the risk/safety assessment of GM crops. Especially those type of *Bt* proteins were considered that are relevant to address the research question on how effects on non-target invertebrates of GM crops expressing several *Bt* proteins can be assessed.

The intent of this report is to review the main body of available information that provides feedback to the questions of COGEM, to identify strengths and gaps in research on *Bt* protein interactions and to give recommendations for the development of guidance for the evaluation of potential adverse effects of GM crops containing multiple *Bt* proteins on non-target invertebrates. We hope we succeeded in this attempt.

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We would like to acknowledge Ulrich Ehlers (Federal Office of Consumer Protection and Food Safety (BVL), Germany), Lucia Roda Ghisleri (Ministerio de Agricultura, Alimentación y Medio Ambiente (MAGRAMA), Spain), Heidi Mitchell (Office of the Gene Technology Regulator (OGTR), Australia), Kazuyuki Suwabe (Ministery of Agriculture, Forestry and Fisheries (MAFF), Japan), Laura Esther Tovar Castillo, Rosa Ines González Torres and Sol Ortiz Garcia (Executive Secretary of Inter-Secretarial Commission of Biosafety of Genetically Modified Organisms (CIBIOGEM), Mexico), John Kough (Environmental Protection Agency (EPA), USA) for their feedback on risk/safety assessment of crops expressing multiple *Bt* proteins.

Verder willen we 'het Begeleidingscomité' bedanken voor hun constructieve commentaar naar de inhoud toe. Het was een fijne ervaring om samen aan dit project te werken.

Contents

Summary

Context

Genes expressing *Bacillus thuringiensis* (*Bt*) toxins have been incorporated into genetically modified (GM) plants to render these resistant to certain insect pests. Of particular interest have been the genes encoding Cry (Crystal) proteins, but also the gene encoding the vegetative insecticidal protein Vip3Aa has been incorporated into crop plants. Over the last decennium, GM events have been crossed through traditional breeding, resulting in stacked GM events expressing several *Bt* insect resistance genes. Experiments demonstrate that interactions between two or more toxins can either enhance or decrease their activity. It is thus possible that interactions between *Bt* proteins produced by GM plants occur and thereby influence their effect on non-target invertebrates compared to GM plants expressing just a single *Bt* gene.

This report has been drafted as a response to a call of the Netherlands Commission of Genetic Modification (COGEM) to address two main questions: (1) can interactions between *Bt* proteins be predicted and (2) to what extent are studies on interactions relevant for risk/safety assessment of GM crops. The questions were tackled from an eco-toxicological angle, in particular taking into account those types of information that are relevant for risk/safety assessment of GM crops. Answering the questions was done by reviewing and considering the current knowledge on the specificity of *Bt* proteins, on known interactions between *Bt* proteins and the methods to assess these interactions, and available guidance for risk/safety assessment of GM crops combining multiple *Bt* proteins. Also the information reviewed in a parallel project addressing the same questions, but from a biochemical and toxicological perspective (EcoΣtat, 2014), was taken into account when formulating conclusions.

Specificity of *Bt* proteins

Published data on invertebrate activity of *Bt* proteins from *B. thuringiensis* are incorporated into the *Bt* Toxin Specificity Database (http://www.glfc.cfs.nrcan.gc.ca/bacillus). To date, 158 of the 329 known holotype toxins have been tested against 252 species distributed across 95 families in 25 orders, eight classes and five phyla. Activity spectra of the tested toxins are summarised by species or family in supplementary tables S1 and S2. Of the 158 proteins tested, 30 were reported to have no pesticidal activity, 59 were active against Lepidoptera, 42 against Diptera, 40 against Coleoptera, 10 against Hemiptera, four against Hymenoptera, and one against Orthoptera. Reports of toxicity to Trichoptera, Neuroptera and Siphonaptera were not substantiated. Twelve proteins were reported to have activity outside the phylum Arthropoda against five orders in the phyla Platyhelminthes and Nematoda.

Based on non-parametric distributions of 50% lethal concentration (LC_{50}s) estimates pooled across all proteins and all taxa (n = 262), *B. thuringiensis* pesticidal proteins can be classified as having high toxicity when active in the 0.01 – 0.10 µg/ml range (below 25% percentile; Diptera-active proteins), medium toxicity when active in the 0.10 – 10 µg/ml range (Lepidoptera, Diptera and Coleoptera-active proteins), and low toxicity when LC_{50}s are in the 10 – 1000 µg/ml range (above 75% percentile; Coleoptera- and Nematoda-active proteins).

Activity outside the order of primary specificity (hereafter referred to as cross-activity) was reported for 28 proteins affecting 75 taxa and was substantiated by reasonable evidence (mortality estimates) for 21 proteins and 59 taxa. Cross-activities occur in 16 families across the three classes of pesticidal proteins (Cry, Cyt and Vip). Within the phylum Arthropoda, cross-activities were substantiated for 12 proteins (Cry1Ca, 1Ia, 4Aa, 8Da, 10Aa, 11Aa, 30Fa, 30Ga, 51Aa, 54Aa, 56Aa, Vip1A/2A) affecting species across two orders, five proteins (Cry1Ac, 1Ba, 2Aa, 3Aa, Cyt1Aa) affecting three orders, and one protein (Cyt1Ba) affecting four orders, all within the class Insecta. Testing of insecticidal proteins against species in other arthropod classes and other phyla has not produced conclusive evidence of lethal activity outside the class Insecta. Cross-phylum activity was substantiated for three insecticidal proteins (Cry1Ab, 2Ab, 3Bb), which had sublethal effects on nematode growth and reproduction at very high dose levels.

Cross-toxicity (LC_{50}s) was quantified for 18 proteins involving 19 species (Table S3). Compared to toxicity ranges established for Diptera-, Coleoptera-, Lepidoptera- and Nematoda-active proteins, 16 cross-activities are in the low- (10 – 1000 µg/ml), seven in the medium- (0.10 – 10) and two in the high-toxicity range (0.01 – 0.10 µg/ml). Insecticidal activities of proteins outside the suite of orders that is normally affected (i.e., Diptera, Coleoptera and Lepidoptera) are mostly in the low-toxicity range. This is the case for toxicity of Cry1Ab, 1Ac, 2Aa, 3Aa, 4Aa, 11Aa and Cyt1Aa to Hemiptera (aphids), of Cry51Aa to Hemiptera (*Lygus* spp.), and of Cyt1Ba to Hymenoptera (sawflies). The exception is high toxicity of Cry3Aa to fire ants (Hymenoptera). Activities that are within the suite of orders normally affected but outside a protein's primary order affinity are often in the low- or medium-toxicity range of corresponding reference proteins. This group includes dipteran toxicity of Cry1Ac, lepidopteran toxicity of Cry8Da and coleopteran toxicity of Cry1Ba, 1Ia, 10Aa, Cyt1Aa, and Cyt1Ba. Dipteran toxicity of Cry1Ba, 1Ca, and Cyt1Ba is of less interest from a non-target safety perspective because those toxicities occur at high dose levels compared to Diptera-active reference proteins.

Interactions among *Bt* proteins

Fifty *in vivo* laboratory tests were found on interactions between *Bt* proteins occurring in invertebrate pest species, covering 24 different *Bt* proteins (Cry, Cyt and Vip). The majority of studies have been conducted on dipteran and lepidopteran insect pests and a few studies

on coleopteran pest species and nematodes. Tables S5 and S6 provide a comprehensive overview of the studies done on dipteran and lepidopteran species. In contrast to the EcoΣtat project, in this project the type of interactions observed, are presented as described in the source papers and no other interpretation was given to the data.

Two mathematical models, the dose/concentration addition and independent action model, have been proposed for assessing interactions in mixtures of toxins. Interaction studies carried out in a risk/safety assessment context of GM plants rather used empirical approaches, than one of the two predictive models. In theory, model selection in a specific situation depends on (knowledge about) the toxins' mode of action. However, as it may be very difficult, and sometimes impossible, to determine the mode of action of a toxin on the basis of existing information, in practice models are often chosen without profound knowledge of the exact mode of action of the toxins involved. Results of studies may also depend on the concentration ratio in which they are combined, the method of production of the *Bt* proteins and their solubility. Clearly, the model and method by which the effect is calculated may affect the outcome of the studies and therefore care was taken to compare results from several studies.

Up till now, the study of interactions between *Bt* proteins has mainly focussed on three-domain Cry proteins and Cyt proteins. The interactions among *Bt* proteins with the same primary specificity that are supported by reasonable evidence are so far limited to those interactions among the Cry (Cry4Aa, Cry4Ba, Cry10Aa, Cry11Aa) and Cyt (Cyt1Aa) proteins acting against mosquitoes (Diptera) from *B. thuringiensis* subsp. *israelensis*. Among the proteins (Cry/Cry and Cyt/Cry combinations) from this *B. thuringiensis* subsp. clear synergistic effects have been observed. Further, one can say that synergistic or antagonistic interactions are likely to occur when a Cyt protein is present. The few data or the contradictory results for the interactions among other dipteran-active *Bt* proteins besides those from subsp. *israelensis*, and for Lepidoptera-, Coleoptera- and nematode-active *Bt* proteins make it hard to draw conclusions on which type of interactions (antagonistic, additive or synergistic) that might occur among other *Bt* proteins than those of subsp. *israelensis* with the same primary order specificity. For Cry proteins having a different primary order activity (i.e. a Cry protein with Coleoptera activity and a Cry protein with Lepidoptera activity), available studies show that interactions among these proteins are not likely to occur.

A particular observation made is that *Bt* toxin combinations can be synergistic for one insect species, but be neutral or antagonistic for another species belonging to the same order. That the type of interaction is insect species-specific, seems to be a common phenomenon among *Bt* protein interactions sharing a same primary order specificity, independent of the *Bt* (Cry, Cyt or Vip) proteins involved. However, further research is needed to substantiate this observation, which is drawn from the little information yet available.

Can interactions between *Bt* proteins be predicted?

On the basis of the currently available reliable knowledge on interactions between *Bt* proteins (i.e., their specificity and mode of action of *Bt* proteins), and not considering the binary proteins for which it is known they interact, we conclude that:

- synergistic or antagonistic interactions are likely to occur when a Cyt protein is present;
- specificity of the *Bt* proteins (including primary order and cross-order specificity), is a useful tool in predicting if interactions might occur;
- if the specificities of *Bt* proteins do not overlap, the possibility that interactions will occur is unlikely;
- for *Bt* proteins with the same specificity, the current knowledge of interactions between *Bt* proteins is not sufficient to make a prediction; and
- the preliminary observation that interactions among *Bt* protein combinations can be synergistic for one insect species, but be neutral or antagonistic for another, complicates the predictability of the occurrence of interactions.

To what extent are studies on *Bt* protein interactions relevant for risk/safety assessment?

The relevance of *in vivo* laboratory studies assessing *Bt* protein interactions in target pest species for risk/safety assessment, as a source of information to assess potential impacts on non-target invertebrates, seems to be little. Given the indications that the occurrence of interactions (additive, synergism or antagonism) is insect species-dependent, they will reveal that synergistic interactions between the *Bt* proteins do or do not occur in the target pests, but will not necessarily reflect whether this will also be the case for non-target invertebrates of the same order as the target pest tested.

In the risk/safety assessment of stacked GM events, testing the potential impact of *Bt* protein interactions on non-target invertebrates has been considered. On the basis of the reviewed data, additional testing of stacked GM events was only considered relevant if they contain *Bt* proteins with overlapping specificity(ies), as their combined presence may lead to changes in activity. For *Bt* proteins with non-overlapping specificities, the *in vivo* laboratory studies done with the single events could still be of value in the risk/safety assessment of the stacked GM event. Moreover, as it is not expected that *Bt* protein interactions would enlarge specificity to more species, testing of impacts could be restricted to those species that fall within the order(s) affected by the *Bt* proteins. For the species that fall outside the order specificity(ies) of the *Bt* proteins, the *in vivo* laboratory studies done with the single events could still apply in the risk/safety assessment of the stacked GM event.

Samenvatting

Context

De genen die *Bacillus thuringiensis* (*Bt*) toxines tot expressie brengen, zijn in genetisch gewijzigde (GG) planten ingebracht om deze resistent te maken tegen bepaalde schadelijke insecten. Voornamelijk de genen die coderen voor Cry-("Crystal") proteïnen, maar ook het gen dat het vegetatieve insectendodend proteïne Vip3Aa codeert, werden reeds geïncorporeerd in gewassen. In het laatste decennium, worden steeds meer GG-events gekruist via traditionele teelttechnieken, resulterend in GG-planten die verscheidene *Bt*-insectresistentiegenen tot expressie brengen (ook "stacked" GG-events genoemd). Studies tonen aan dat interacties tussen twee of meer toxines hun activiteit kunnen verlagen of verhogen. Het is dus mogelijk dat er interacties tussen *Bt*-proteïnen geproduceerd door GG-planten plaatsvinden en dat daardoor hun effect op niet-doelwitinvertebraten verandert ten opzichte van GG-planten die slechts één *Bt*-gen tot expressie brengen.

Dit rapport werd geschreven als een antwoord op een oproep van de Nederlandse Commissie Genetische Modificatie (COGEM) om twee hoofdonderzoeksvragen op te lossen: (1) kunnen interacties tussen *Bt*-proteïnen voorspeld worden en (2) in welke mate zijn studies naar interacties relevant voor de risicobeoordeling van GG-gewassen. De vragen werden behandeld vanuit een ecotoxicologische invalshoek, waarbij voornamelijk rekening gehouden werd met informatie die relevant is voor de risicobeoordeling van GG-planten. Bij het beantwoorden van de vragen werd rekening gehouden met de huidige kennis van de specificiteit van *Bt*-proteïnen, van de interacties tussen *Bt*-proteïnen, inclusief de methodes om deze interacties te evalueren, en van de beschikbare richtsnoeren voor de risicobeoordeling van GG-gewassen die verscheidene *Bt*-proteïnen combineren. Ook de informatie bijeengebracht in een parallel project dat dezelfde vragen behandelt, maar dan vanuit een biochemisch en toxicologisch perspectief (EcoΣtat, 2014), werd in rekening gebracht bij het formuleren van de conclusies.

Specificiteit van *Bt*-proteïnen

De gepubliceerde data over de activiteit van *Bt*-proteïnen afkomstig van *B. thuringiensis* ten opzichte van insecten zijn opgenomen in de databank over de specificiteit van *Bt*-toxines (http://www.glfc.cfs.nrcan.gc.ca/bacillus). Tot op heden, werden 158 van de bekende 329 holotype toxines getest tegen 252 soorten verspreid over 95 families in 25 orden, acht klassen en vijf fyla. De activiteitsspectra van de geteste toxines zijn samengevat per soort of per familie in de aanvullende tabellen S1 en S2. Van de 158 geteste proteïnen, werd van 30 gerapporteerd dat ze geen pesticide-activiteit bezitten. 59 proteïnen zijn actief tegen Lepidoptera, 42 tegen Diptera, 40 tegen Coleoptera, 10 tegen Hemiptera, vier tegen Hymenoptera en één tegen Orthoptera. De rapporten over toxiciteit tegen Trichoptera, Neuroptera en Siphonaptera waren niet onderbouwd. Over twaalf proteïnen werd

gerapporteerd dat ze activiteit hebben buiten het fylum van de Arthropoda, namelijk tegen vijf orden in de fyla Platyhelminthes en Nematoda.

Gebaseerd op de non-parametrische distributies van de schattingen van de 50% lethale concentraties (LC_{50}s), samengevoegd voor alle proteïnen en alle taxa (n = 262), kunnen *Bt*-proteïnen geklasseerd worden als hoog toxisch wanneer ze actief zijn tussen 0.01 – 0.10 µg/ml (beneden de 25% percentiel; Diptera-actieve proteïnen), als middelmatig toxisch wanneer ze actief zijn tussen 0.10 – 10 µg/ml (Lepidoptera-, Diptera- en Coleoptera-actieve proteïnen), en als laag toxisch wanneer de LC_{50}-waarden tussen 10 – 1000 µg/ml liggen (boven de 75% percentiel; Coleoptera- en Nematoda-actieve proteïnen).

Activiteit buiten de orde van hoofdspecificiteit werd gerapporteerd voor 28 proteïnen die 75 taxa beïnvloeden en was redelijk onderbouwd (sterftecijfers) voor 21 proteïnen en 59 taxa. Deze kruisreactiviteit komt voor in 16 families bij alle drie klassen van *Bt* proteïnen (Cry, Cyt en Vip). Binnen het fylum Arthropoda werd de hoofdspecificiteitoverschrijdende werking onderbouwd voor 12 proteïnen (Cry1Ca, 1Ia, 4Aa, 8Da, 10Aa, 11Aa, 30Fa, 30Ga, 51Aa, 54Aa, 56Aa, Vip1A/2A) die species over twee orden beïnvloeden, voor vijf proteïnen (Cry1Ac, 1Ba, 2Aa, 3Aa, Cyt1Aa) die drie orden beïnvloeden, en voor één proteïne (Cyt1Ba) dat vier orden beïnvloedt, allen binnen de klasse Insecta. Het testen van de insectendodende proteïnen buiten de klasse van de Insecta (tegen soorten in andere Arthropoda klassen en andere fyla) heeft geen sluitend bewijs opgeleverd voor een letale activiteit. Activiteit tegen soorten uit verschillende fyla is onderbouwd voor drie insectendodende proteïnen (Cry1Ab, 2Ab, 3Bb), die subletale effecten hebben op de groei en de reproductie van nematoden bij zeer hoge dosissen.

De hoofdspecificiteitoverschrijdende toxiciteit (op basis van LC_{50}-waarden) werd gekwantificeerd voor 18 proteïnen en 19 soorten (Tabel S3). In vergelijking met de toxiciteitsniveaus bepaald voor Diptera-, Coleoptera-, Lepidoptera- en Nematoda-actieve proteïnen, bevinden 16 hoofdspecificiteitoverschrijdende activiteiten zich in het lage (10 – 1000 µg/ml), zeven in het middelhoge (0.10 – 10) en twee in het hoge toxiciteitsniveau (0.01 – 0.10 µg/ml). De activiteiten van de proteïnen buiten de reeks van orden die gewoonlijk beïnvloed worden (namelijk Diptera, Coleoptera en Lepidoptera), vallen meestal in het lage toxiciteitsniveau. Dit is het geval voor de toxiciteit van Cry1Ab, 1Ac, 2Aa, 3Aa, 4Aa, 11Aa en Cyt1Aa tegen Hemiptera (bladluizen), van Cry51Aa tegen Hemiptera (*Lygus* spp.) en van Cyt1Ba tegen Hymenoptera (bladwespen). Een uitzondering is de hoge toxiciteit van Cry3Aa tegen vuurmieren (Hymenoptera). De activiteiten binnen de reeks van orden die gewoonlijk beïnvloed worden, maar die wel buiten de hoofdspecificiteit van het proteïne ressorteren, vallen vaak in het lage- of middelhoge-toxiciteitsniveau van de overeenkomstige referentieproteïnen. Deze groep omvat Diptera-toxiciteit van Cry1Ac, Lepidoptera-toxiciteit van Cry8Da en Coleoptera-toxiciteit van Cry1Ba, 1Ia, 10Aa, Cyt1Aa en Cyt1Ba. Diptera-toxiciteit van Cry1Ba, 1Ca en Cyt1Ba zijn van minder belang vanuit het veiligheidsperspectief

van niet-doelwitorganismen, omdat die bij hoge dosissen voorkomen in vergelijking met de Diptera-actieve referentieproteïnen.

Interacties tussen *Bt*-proteïnen

Vijftig *in vivo* laboratoriumproeven met in het totaal 24 verschillende *Bt*-proteïnen (Cry, Cyt en Vip), die de interacties tussen de proteïnen in ongewervelde schadelijke soorten testen, werden gevonden. Het merendeel van de studies werd uitgevoerd met schadelijke Diptera en Lepidoptera en een aantal studies met schadelijke Coleoptera en nematoden. De tabellen S5 en S6 geven een overzicht van de studies gedaan met Diptera en Lepidoptera. In tegenstelling tot het EcoΣtat project, werd in dit project het type van interactie dat geobserveerd werd, weergegeven zoals beschreven in de bronnen en werd er geen ander interpretatie gegeven aan de data.

Twee wiskundige modellen, de dosisadditie- of concentratie-additiebenadering en de onafhankelijke-werkingsbenadering, worden vooropgesteld voor de evaluatie van interacties in mengsels van toxines. De studies naar interacties uitgevoerd in de context van de risicobeoordeling van GG-planten, maken i.p.v. één van de twee voorspellende modellen eerder gebruik van een empirische benadering. In theorie hangt de selectie van het model in een specifieke situatie af van (de kennis over) de werkingswijze van het toxine. Echter, daar het zeer moeilijk kan zijn, en soms zelfs onmogelijk, om de werkingswijze van een toxine te bepalen op basis van bestaande informatie, wordt in de praktijk een model vaak gekozen zonder grondige kennis van de exacte werkingswijze van de betrokken toxines. De resultaten van de studies kunnen ook afhangen van de concentraties waarin de toxines gecombineerd worden, de methode van productie van de *Bt*-proteïnen en hun oplosbaarheid. Het model en de methode waarmee het effect is berekend, kan de uitkomst van de studies dus beïnvloeden en daarom werden de resultaten van verscheidene studies met de nodige voorzichtigheid vergeleken.

Tot op heden heeft de studie van interacties tussen *Bt*-proteïnen zich voornamelijk gericht op 3-domein Cry-proteïnen en Cyt-proteïnen. De interacties tussen *Bt*-proteïnen met dezelfde specificiteit die ondersteund worden door voldoende bewijsmateriaal zijn tot nog toe beperkt tot de interacties tussen de Cry- (Cry4Aa, Cry4Ba, Cry10Aa, Cry11Aa) en Cyt- (Cyt1Aa) proteïnen van *B. thuringiensis* subsp. *israelensis*, allen actief tegen muggen (Diptera). Tussen de proteïnen (Cry/Cry en Cyt/Cry combinaties) van deze *B. thuringiensis* ondersoort werden duidelijk synergetische effecten waargenomen. Verder kan men zeggen dat synergetische of antagonistische interacties waarschijnlijk zullen optreden wanneer een Cyt-proteïne aanwezig is. De schaarse gegevens of de tegenstrijdige onderzoeksresultaten van de interacties tussen andere Diptera-actieve *Bt*-proteïnen dan die afkomstig van de subsp. *israelensis*, en Lepidoptera-, Coleoptera- en nematode-actieve *Bt*-proteïnen maken het moeilijk om conclusies te trekken over het type van interacties (antagonistische, additieve of synergetische) dat zou kunnen optreden tussen *Bt*-proteïnen met dezelfde

ordespecificiteit. Voor Cry-proteïnen met een verschillende orde-activiteit (namelijk een Cry-proteïne met Coleoptera-activiteit en een Cry-proteïne met Lepidoptera-activiteit), tonen de beschikbare studies aan dat interacties tussen deze proteïnen onwaarschijnlijk zijn.

Een aparte observatie is dat combinaties van *Bt*-toxines synergetisch zijn voor één bepaalde insectensoort, maar neutraal of antagonistisch voor een soort van dezelfde orde. Dat het type van interactie soort-specifiek is, is blijkbaar een algemeen fenomeen voor interacties tussen *Bt*-proteïnen die eenzelfde hoofdordespecificiteit delen en is onafhankelijk van het betrokken *Bt*-proteïne (Cry, Cyt of Vip). Echter, verder onderzoek is nodig om deze observatie die gebaseerd is op de nog maar schaarse informatie, te onderbouwen.

Kunnen interacties tussen *Bt*-proteïnen voorspeld worden?

Op basis van de huidig beschikbare en betrouwbare kennis van interacties tussen *Bt*-proteïnen (m.b.t. hun specificiteit en hun werkingswijze), en de binaire proteïnen waarvan men weet dat ze interageren, buiten beschouwing latend, besluiten we dat:

- synergetische of antagonistische interacties waarschijnlijk optreden wanneer een Cyt-proteïne aanwezig is;
- de specificiteit van de *Bt*-proteïnen (inclusief hoofdorderspecificiteit en kruisreactiviteit), een handig hulpmiddel is om te voorspellen of er interacties kunnen optreden;
- als de specificiteit van *Bt*-proteïnen niet overlapt, het onwaarschijnlijk is dat interacties zullen optreden;
- voor *Bt*-proteïnen met dezelfde specificiteit, de huidige kennis van interacties tussen *Bt*-proteïnen niet voldoende is om een voorspelling te maken; en
- de preliminaire observatie dat een interactie tussen *Bt*-proteïnen synergetisch kan zijn bij één insectensoort, maar neutraal of antagonistisch bij een ander, de voorspelling van het optreden van interacties bemoeilijkt.

In welke mate zijn studies naar interacties tussen *Bt*-proteïnen relevant voor de risicobeoordeling?

De relevantie van *in vivo* laboratoriumproeven die interacties tussen *Bt*-proteïnen in schadelijke soorten evalueren, als een bron van informatie om de potentiële impact op niet-doelwitinvertebraten te evalueren in de risicobeoordeling, lijkt klein. Daar er aanwijzingen zijn dat het optreden van interacties (additieve, synergetische of antagonistische) soort-afhankelijk is, zullen de studies met de schadelijke soort onthullen dat synergetische interacties tussen *Bt*-proteïnen al dan niet gebeuren in het doelwitorganisme, maar niet noodzakelijkerwijs in niet-doelwitinvertebraten van dezelfde orde als het doelwitorganisme.

In de risicobeoordeling van “stacked” GG-events, wordt het testen van de potentiële impact van de interacties tussen *Bt*-proteïnen op niet-doelwitinvertebraten in overweging genomen. Afgaande op de gepubliceerde data, werd het extra testen van “stacked” GG-events enkel relevant bevonden wanneer deze *Bt*-proteïnen een overlappende specificiteit hebben. In dit geval, zou de gecombineerde aanwezigheid van de toxines kunnen leiden tot een verandering in activiteit. Voor *Bt*-proteïnen met een niet-overlappende specificiteit, kunnen de *in vivo* laboratoriumonderzoeken gedaan met de events waaruit het “stacked” GG-event bestaat (ook “single” events genoemd) nog steeds gelden in de risicobeoordeling van het “stacked” GG-event. Bovendien, aangezien het niet verwacht wordt dat interacties tussen *Bt*-proteïnen de specificiteit verruimen naar meer species, kan het testen van de effecten beperkt worden tot die soorten die binnen de orde(n) vallen waarop de *Bt*-proteïnen effect hebben. Voor de soorten die buiten de ordespecificiteit(en) van de *Bt*-proteïnen vallen, kunnen de *in vivo* laboratoriumproeven uitgevoerd met de single events nog steeds gelden in de risicobeoordeling van het “stacked” GG-event.

1. Introduction

Bacillus thuringiensis (*Bt*) is a Gram-positive bacterium that is known to produce different type of toxins with pesticidal activity almost exclusively against larval stages of different insect orders. Sporulating cells of *B. thuringiensis* synthesise parasporal inclusions comprising one or more pesticidal proteins, commonly referred to as δ-endotoxins. The Cry (Crystal) and Cyt (Cytolytic) toxins are formed upon sporulation in crystals (Crickmore et al., 1998). During the growth stage another type of toxin can be synthesised: the vegetative insecticidal proteins, abbreviated as VIPs (Estruch et al., 1996). These three types of *Bt* toxins, further referred to as *Bt* proteins, are covered in this report.

Bt proteins are known to have pesticidal activity and have been used to control pests in forestry (van Frankenhuyzen, 1993) and agriculture (Sanchis and Bourguet, 2008), either in microbial sprays or after incorporation into crops through genetic modification, or to control arthropodborne human and animal diseases (Guillet et al., 1990). Cry toxins mainly act against insect pests, but are also known to be active against Nematoda; Cyt toxins and Vip proteins target Insecta (see 2.2.3.1.). There are many different Cry, Cyt and Vip proteins with different specificity to certain insect taxa. This great diversity is likely to have developed through sequence divergence and subsequent swapping of domains within the toxin genes (de Maagd et al., 2001). Most of the reported δ-endotoxins are active against lepidopteran (moths and butterflies), dipteran (flies and mosquitoes) and coleopteran (beetles) insect species (van Frankenhuyzen, 2009; 2013). When insects ingest δ-endotoxins, the toxins become activated by enzymes in their digestive tract. In a susceptible host, the active δ-endotoxins selectively bind to receptors located in the midgut of susceptible species. The exact mode of action of the activated *Bt* proteins is still under research, but in all models proposed the binding step is considered critical to result in toxicity (EcoΣtat, 2014). Activated three-domain Cry toxins bind to glycoprotein or glycolipid receptors on the insect midgut epithelium microvillar membrane (Pigot and Ellar, 2007), while the Cyt proteins appear to bind to lipids in the microvillar membrane (Li et al., 1996). Similar to the Cry and Cyt proteins, Vip proteins bind to insect midgut receptors, apparently different from those of Cry proteins (Sena et al., 2009 and references therein). Once bound to the receptors, the gut is perforated, causing the insect to die within 48 to 120 hours due to cell lysis and septicaemia (Broderick et al., 2006). The mode of action of these toxins may be similar for other targets than insects. For example, glycolipids have been documented to be receptors for Cry for the nematode *Caenorhabditis elegans* (Griffitts et al., 2005).

Genes expressing *Bt* toxins have been incorporated into genetically modified (GM) plants to render these resistant to certain insect pests. Of particular interest have been the Cry proteins, but also the vegetative insecticidal protein Vip3Aa has been incorporated into crop plants. The focus of this report will, given the research questions of COGEM, therefore be on the insecticidal activity of the *Bt* proteins and how interactions between *Bt* proteins can

influence this activity. Genes expressing Cyt proteins are currently not present in commercially available GM crops. The Cyt proteins, however, are taken into account in this report, as they can provide insight in interactions between *Bt* toxins.

Before a GM plant is allowed for commercial cultivation, potential adverse effects of the GM plant towards organisms it does not envisage, termed non-target organisms, need to be assessed (EFSA, 2010a,b). In particular, potential impacts on those species that provide ecosystem services, such as biological control or pollination, are evaluated. Over the last decennium, GM plants have been crossed through traditional breeding, resulting in plants expressing several *Bt* insect resistance genes. Experiments, both *in vitro* and *in vivo*, demonstrate interactions between two or more δ-endotoxins influencing their activity. It is thus possible that interactions between *Bt* proteins produced by GM plants occur and thereby influence their effect on non-target organisms compared to GM plants expressing just a single *Bt* protein gene. Cultivation of GM plants with combined *Bt* insect-control traits may, therefore, require additional assessment. In the EU, some guidelines on how potential interactions between *Bt* toxins and their effects on non-target organisms can be evaluated exist. The EFSA guidelines (EFSA, 2010a,b) specify that the environmental risk assessment of GM plants with combined traits *shall focus on the characterisation and potential consequences of issues related to potential synergistic, additive and antagonistic effects resulting from the combination of the events* and that *in order to confirm the absence of these potential effects, the potential impact on target organisms should be assessed*. However, despite this guidance, some questions remain.

The overall aim of the project was to answer two research questions:
(1) **Can interactions between *Bt* proteins be predicted?** A sub-question raised during the course of the project was on the role of cross-activity in predicting interactions.
(2) **To what extent are studies on interactions relevant for risk/safety assessment of GM crops?** In particular, COGEM would like to know whether the results of studies with target organisms can be extrapolated to non-target organisms. Subsequently, the question was raised what has to be done in the risk/safety assessment if interactions have been shown to occur.

To answer the first question, we tried to gain insight in the potential interactions between *Bt* proteins, especially those present in GM crops currently authorised for cultivation worldwide. This was done by considering the current knowledge on their specificity, i.e. their primary insect order specificity and cross-order activity (Chapter 2), the methods to assess toxin interactions (Chapter 3) and by reviewing the information on known interactions between *Bt* proteins and how interactions between *Bt* proteins may affect their activity (Chapter 4). A detailed description of the *Bt* protein activity at the molecular level was not be given, as this forms part of another project commissioned by COGEM.

To answer the second question information was gathered on the experience with the evaluation of potential adverse effects on non-target invertebrates of GM crops containing several *Bt* proteins. Methods on how interactions between *Bt* proteins have been examined up till now, and available guidance for the evaluation of potential effects on non-target organisms of GM crops containing several *Bt* proteins are addressed (Chapter 5).

Finally, considering all information collected, a reply to the research questions was formulated (Chapter 6) and gaps in the knowledge of *Bt* protein interactions that could be relevant for risk/safety assessment were identified.

2. Activity spectrum of *Bt* proteins

2.1. Classification of *Bt* proteins

Bacillus thuringiensis, or *Bt*, and its role as an insect pathogen were discovered at the beginning of the 20th century, almost simultaneously in Japan and in Germany (from where the name *thuringiensis* stems). Insecticidal formulations based on spore/crystal-mixtures of *Bt* where already in use in the 1920s, but diversified from the 1950s onwards (Beegle and Yamamoto, 1992). The first transgenic crops expressing one or several *Bt* proteins for insect pest control have been on the market since 1996 (de Maagd et al., 2001). With time, the number of known different proteins, insecticidal or not, produced in the parasporal crystals of *Bt* or soluble in the growth medium, produced by *Bt* or by (mostly) related bacterial species has grown. This has resulted in an overwhelming array of diverse structures and specificities, of which only a fraction has been utilised or more or less thoroughly characterised in terms of activity spectrum, protein structure and mode of action (de Maagd et al., 2003). The rules for nomenclature and classification of the *Bt* (and related) insecticidal proteins, as well as the phylogeny and a regularly updated list of cloned and sequenced protein toxin genes can be found in the *Bt* toxin nomenclature website (http://www.Btnomenclature.info/).

The original classification for a, at the time limited number of known *Bt* crystal proteins, distinguished just two types: Cry (Crystal) and Cyt (Cytolytic – having *in vitro* haemolytic activity). Further classification of Cry proteins, with Roman numerals and further subclassification with Capitals and lower case letters between parentheses (for example: CryIA(b), CryIIIA(a)) was foremost based on the observed biological activity spectrum: CryI – lepidopterans; CryII – lepidoperans and dipterans, CryIII – coleopterans, CryIV – dipterans (Höfte and Whiteley, 1989). As the number and diversity of *Bt* toxins grew through discovery, it became apparent that such a classification was untenable and it was replaced by one based exclusively on amino acid sequence similarity (Crickmore et al., 1998). Roman numerals where replaced by Arabic numerals (CryI became Cry1), although care should be taken in interpreting older literature: the numeral replacement is not always from Roman to the corresponding Arabic (for example CryIIIA(a) became Cry3Aa, but CryIIIC became Cry7Aa). The proper translation from old into new name can be found in the holotype toxins list on the nomenclature website (http://www.Btnomenclature.info/). The classification of new Cry proteins into 4 ranks and subranks (for example: Cry1Aa1) is based on their position in the phylogenetic tree of these proteins, and depends on the degree of sequence similarity with already known proteins. At the time of conception of the new nomenclature, proteins with the same primary rank (such as Cry1's) had more than 45% sequence identity, with the same secondary rank (such as Cry1A's) had more than 78% sequence identity, and with the same tertiary rank (such Cry1Aa's) had more than 95% identity, while the fourth rank is optional and used to distinguish proteins with minor sequence differences.

While the rank-based nomenclature is helpful for instantly recognising the level of similarity between different proteins, Cry toxin nomenclature is confounded by the fact that having the same general name (Cry) does not necessarily imply even generally similar protein structure or mode of action. Thus Cry is *"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"* (Crickmore et al., 1998). As a result, the known Cry proteins comprise at least five different major homology groups with, as far as is known, essentially different protein structures and modes of action: the three-domain Cry proteins, which is by far the largest group, the binary toxins including Cry34/35, the MTX-like proteins including Cry15, and the small subclasses Cry6 and Cry22. Three-domain Cry proteins comprise the largest and oldest group studied, and are so named because of their active protein moiety consisting of three structural domains, which has either been experimentally determined or inferred from homology with the proteins of which the structure is known. Currently there are approximately 270 different "holotypes" of this class known. Binary toxins are so called because they require two protein components, encoded by separate genes for their insecticidal activity. They are found not only in *B. thuringiensis* crystals, but also in the mosquitocidal *Lysibacillus* (formerly *Bacillus*) *sphaericus*. The MTX are so called because they have mosquitocidal activity. Cyt proteins are defined as crystal proteins with more general i*n vitro* cytolytic activity (de Maagd et al., 2003). Besides crystal proteins, *B. thuringiensis* and related species also produce a number of soluble insecticidal proteins during vegetative growth (as opposed to sporulative growth, when parasporal crystals are formed): Vegetative Insecticidal Proteins Vip1/Vip2 (acting as a binary toxin) and Vip3 (Estruch et al., 1996; Warren, 1997). The latter, currently consists of 13 holotypes (http://www.Btnomenclature.info/).

2.2. Specificity and cross-order activity of *Bt* proteins

2.2.1. Introduction

Pesticidal proteins produced by *B. thuringiensis* Berliner are widely used in pest control applications. A key feature that makes *Bt* proteins attractive for pest management applications is their high degree of specificity. Specificity of a toxin protein is defined as the range of species or taxa that it affects (its activity spectrum). Host range specificity was initially recognised as toxicity of a subspecies or strain that was restricted to a specific insect order, in particular Lepidoptera (subsp. *kurstaki*, *aizawai*), Coleoptera (*tenebrionis*) or Diptera (*israelensis*). The link between host range and presence of specific crystal proteins was established as individual toxins from cloned protein genes became available for testing, which lead to recognition of toxin families with strong affinity for either Lepidoptera (Cry1), Coleoptera (Cry3), Diptera (Cry4) or Lepidoptera and Diptera (Cry2) (Höfte and Whiteley, 1989), which hereafter will be considered as the orders of primary specificity. Within this primary order affinity, *Bt* proteins display a unique spectrum of insecticidal activities that is

manifested in the degree of toxicity towards different species, genera or families (Estruch et al., 1996; Warren, 1997; van Frankenhuyzen, 2009). However, order-specific proteins can also display activity outside their order of primary specificity. For example, various Lepidoptera-, Coleoptera- and Diptera-active proteins have been reported to affect species in other orders (e.g., Haider et al., 1986; Tailor et al., 1992; Bradley et al., 1995; Walters and English, 1995; Omolo et al., 1997; Zhong et al., 2000; Bulla and Candas, 2004; Porcar et al., 2009; de Souza Aguiar et al., 2012) and even other phyla (Höss et al., 2008, 2011, 2013).

The purpose of this chapter is to examine current knowledge on the specificity of *Bt* proteins and the extent of activities outside their orders of primary specificity, that is toxicity to species in other orders or even other classes or phyla (hereafter referred to as cross-activity). The chapter is an updated compilation of two previous reviews (van Frankenhuyzen, 2009, 2013).

2.2.2. General considerations and restrictions

This review is limited to toxin proteins that are listed in the *Bt* Toxin Nomenclature Database (Crickmore, N., Zeigler, D.R., Schnepf, E., Van Rie, J., Lereclus, D., Baum, J, Bravo, A., Dean, D.H., *Bacillus thuringiensis* toxin nomenclature, http://www.Btnomenclature.info/; December 2013) and for which bioassay data are available. At the time of compilation, 158 of the 329 listed proteins had been tested for pesticidal activity against a total of 252 species distributed across 95 families in 25 orders, eight classes and five phyla. Bioassay data for the remaining 171 proteins are either not available or were missed.

Data on toxicity to target pest insects were obtained from the *Bt* Toxin Specificity Database (van Frankenhuyzen, K., Nystrom, C., http://www.glfc.cfs.nrcan.gc.ca/bacillus; December 2013). The database summarises published toxicity data by protein, gene or target species, and is restricted to spore-free preparations of crystals or (pro)toxins obtained through expression of cloned genes or purification from single-gene strains, which were tested individually (with the exception of binary toxins). Extracted data were supplemented with data from publications post 2010, when the database was last updated. Data on toxicity to non-target species were obtained from the Nontarget Effects of *Bt* Crops Database (Marvier et al.; http://delphi.nceas.ucsb.edu/*Bt*crops; December 2013), which is limited to a subset of proteins used in transgenic crops (Cry1Ab, Cry1Ac, Cry1F, Cry2Ab, Cry3Aa, Cry3Bb, and Cry9Ca). The database was last updated in 2006 and extracted data were supplemented with information from relevant papers published after 2006.

For the purpose of this review, activity of a particular protein was inferred from mortality in laboratory bioassays with the toxin protein, or in laboratory and greenhouse tests with insect-resistant transgenic plants expressing *Bt* proteins. Bioassays were included in this review only when they were conducted with individual toxin proteins purified from the parental strain or produced in recombinant systems. Two types of transgenic plant studies were included, those involving exposure of the test species to toxin protein expressed in

leaves, pollen or other tissues, and those involving exposure via prey (in the case of predator species) or hosts (in the case of parasitoid species) reared on transgenic plant tissues.

Reported activities were summarised by test species for each tertiary-rank holotype protein, using a binary response (active or not active) based on mortality, with the exception of activity against Nematoda (see below). The mortality response was considered for all life stages, bioassay method, and crystal protein preparation (crystal, protoxin or activated toxin). Proteins were scored as 'not active' when they did not evoke a mortality response at the highest concentration tested, and 'possibly active' in case of conflicting data or when mortality was reported but not supported by actual data. Activity against Nematoda included sublethal responses, in particular inhibition of growth and reproduction, which are metrics that are commonly used as endpoints in bioassays with nematodes. Specificity was evaluated across families and orders by rolling up the species into the higher ranking taxa. A toxin was considered active against a family or order when it affected at least one species in that family or order and not active when none of the species tested were affected.

2.2.3. Specificity

2.2.3.1. Activity profiles

Current knowledge of specificity is restricted by the range of toxins tested and the range of species used in those tests. The majority (~80%) of the 158 proteins was tested against 10 species or less that were distributed across five or fewer families restricted to one or two orders (Fig. 1). Only 14 proteins, 13 Lepidoptera-active (belonging to the Cry1, Cry2, Cry9 and Vip3 superfamilies) and one Coleoptera-active (Cry3Aa), were tested against 25 species or more (Fig. 1, top). The two proteins with the best characterised activity profiles (Cry1Ab, Cry1Ac) are used in transgenic crops and were tested against ~100 species. Supplementary Table S1 provides a comprehensive overview of the 158 proteins and 252 species that have been tested.

Pesticidal activity of the 158 proteins is summarised at the family level in Table S2. Of the 86 proteins tested against Lepidoptera, 59 affected at least one species in at least one of 20 families. Lepidopteran activity has been reported in the Cry1, 2, 7, 8, 9, 15, 22, 30, 32, 51, 54, 56 and 59 families, as well as in the Cyt1 and Vip3 families. The Noctuidae, Plutellidae and Pyralidae are the most commonly tested families (Fig. 2, top). Of the 72 proteins tested against Diptera, 42 were active across seven families. Dipteran activity has been reported for 21 Cry and two Cyt families, with Culicidae being the most frequently tested taxon (Fig. 2, middle). Of the 65 proteins tested against Coleoptera, 40 had activity across eight families. Coleopteran activity has been reported to occur in 17 Cry, two Cyt and one Vip family and has been most commonly demonstrated in the Chrysomelidae and Scarabidae (Fig. 2, bottom). Hemipteran toxicity has been reported for 10 of the 14 tested proteins in the Cry1, 2, 3, 4, 11, 51, Cyt1 and Vip1A/2A families. Most reports of hemipteran toxicity

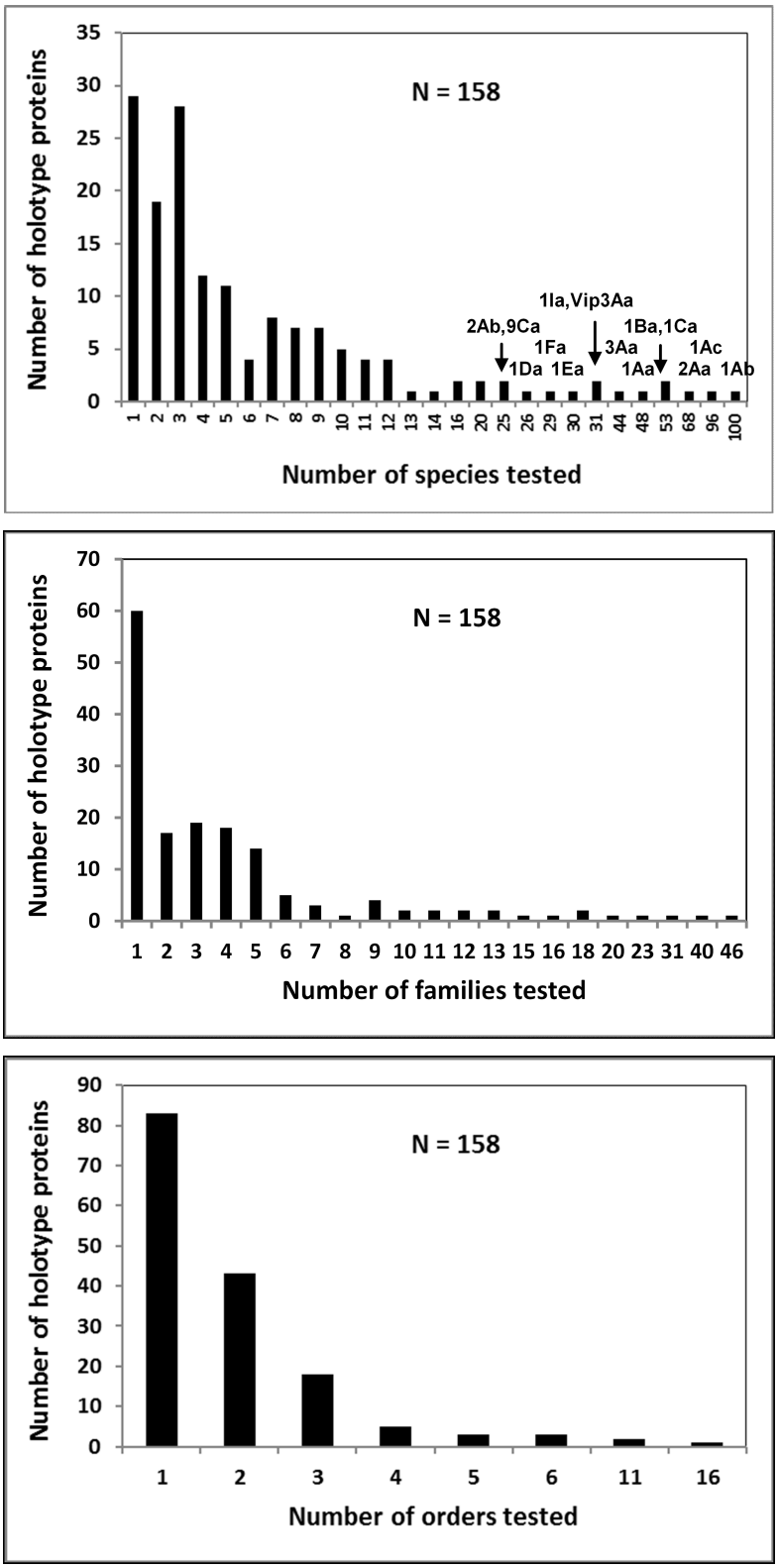

Figure 1. Distribution of the number of *B. thuringiensis* pesticidal proteins (Cry, Cyt and Vip) as a function of the number of species (top), families (middle) and orders (bottom) they were tested against. The number of orders was tallied across phyla (Arthropoda, Platyhelminthes, Nematoda); the arthropod subclass of Acari was counted as one order.

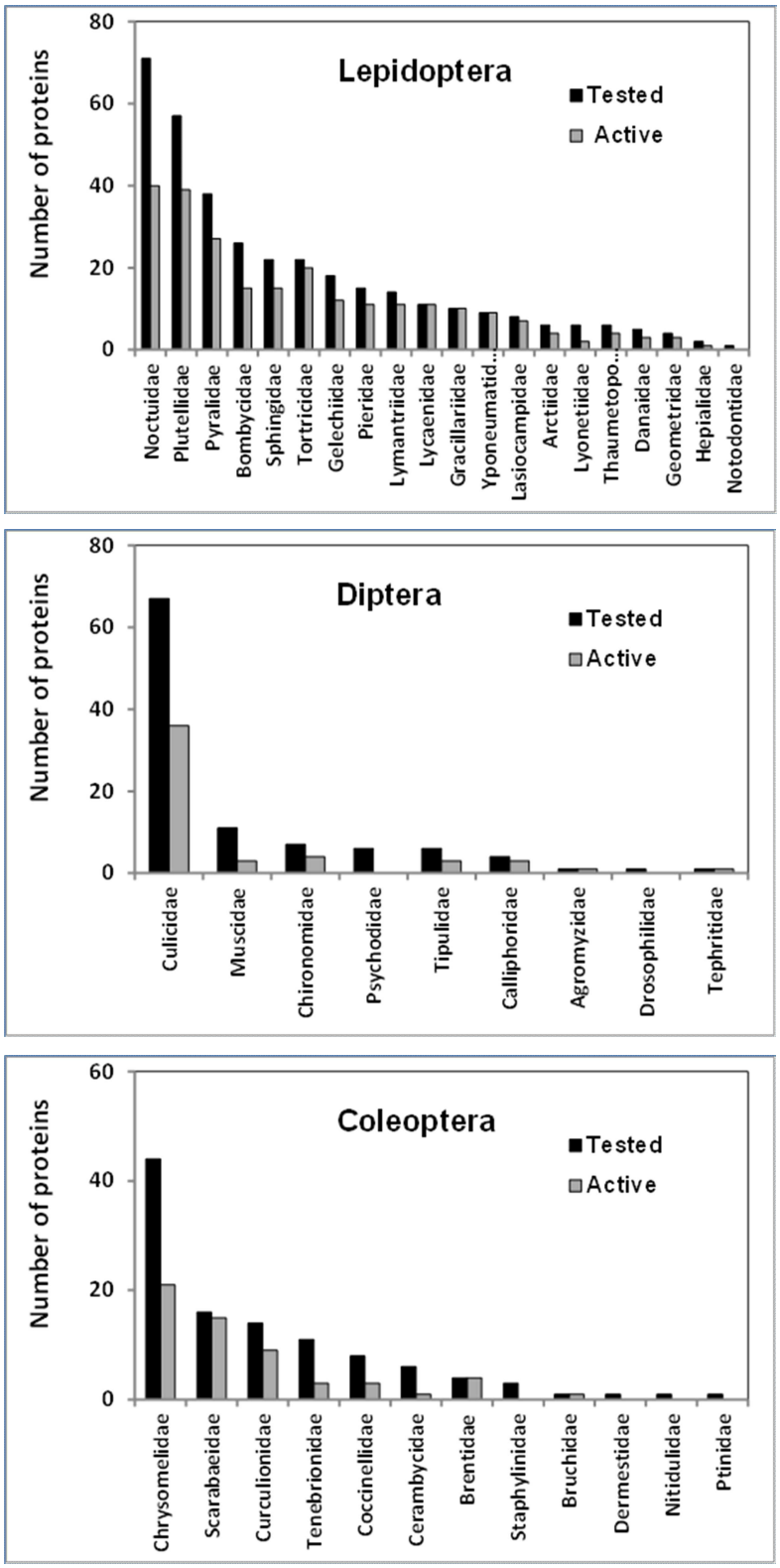

Figure 2. Distribution of the number of *B. thuringiensis* pesticidal proteins (Cry, Cyt and Vip) that were tested and the number that was found active across families of Lepidoptera (top), Diptera (middle) and Coleoptera (bottom).

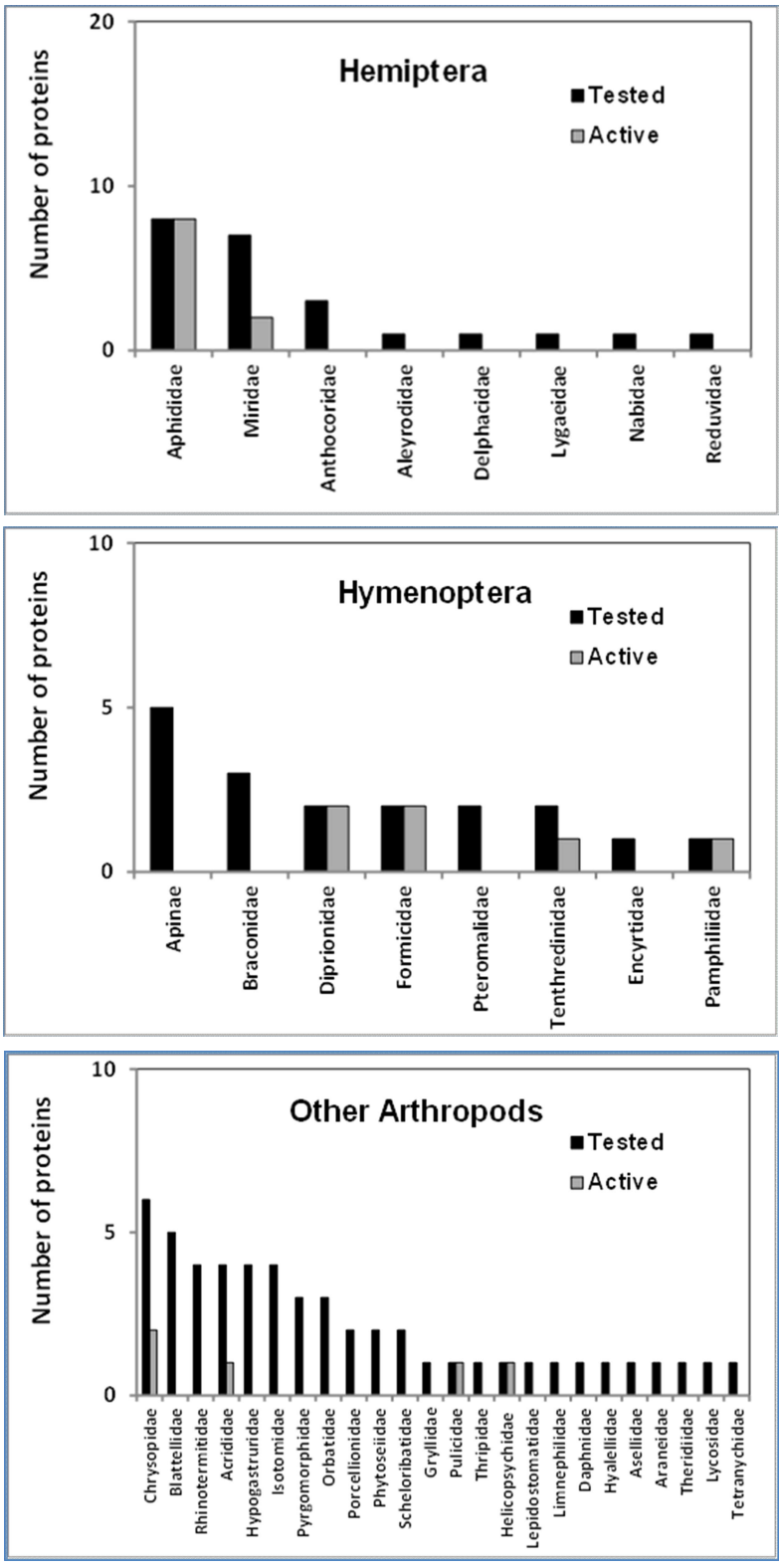

Figure 3. Distribution of the number of *B. thuringiensis* pesticidal proteins (Cry, Cyt and Vip) that was tested and the number that was found active across families of Hemiptera (top), Hymenoptera (middle) and other Arthropoda (bottom).

pertain to aphids (Aphididae) (Fig. 3, top). Nine proteins have been tested for hymenopteran activity, which is to date limited to toxicity of Cry3Aa, 5Ac, 22Aa and Cyt1Ba to sawflies or ants (Fig. 3, middle). Toxicity outside these major insect orders has been reported for four proteins (Cry1Ab, 2Aa, 7a and Cyt2Ca) in four other insect orders (Trichoptera, Neuroptera, Orthoptera, and Siphonaptera, respectively) despite testing of 14

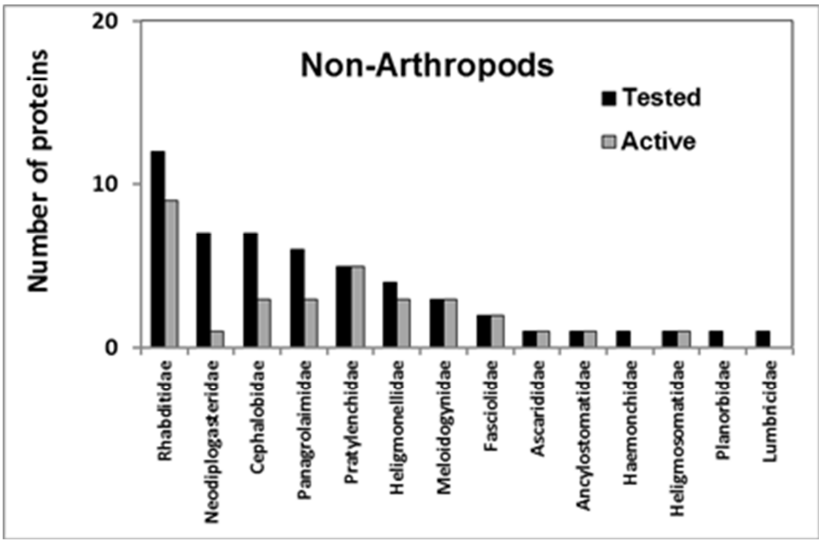

Figure 4. Distribution of the number of *B. thuringiensis* pesticidal proteins (Cry, Cyt and Vip) that was tested and the number that was found active across families outside the phylum Arthropoda (bottom).

proteins against species distributed across 24 families in 13 arthropod orders (Fig. 3, bottom). Activity outside the phylum Arthropoda was tested with 14 proteins against species across 14 families, seven orders and four phyla, and toxicity was reported for nine of those (5Aa, 5Ab, 5Ba, 6Aa, 6Ba, 12Aa, 14Aa, 21Aa, 55Aa) against five orders in the phyla Platyhelminthes and Nematoda (Fig. 4). Nematode activity was reported for three additional proteins (Cry1Ab, 2Ab, 3Bb), but only in terms of inhibited growth and reproduction (not mortality).

2.2.3.2. No pesticidal activity

Of the 158 proteins for which bioassay data are readily available, 30 were reported to have no pesticidal activity (Fig. 5). Eleven of those are individually tested proteins belonging to the Vip1/2, Cry34/35, and Cry48/49 families, which are active as binary toxins only (Warren, 1997; Baum et al., 2004; Jones et al., 2008). Fourteen proteins (Cry1Ga, 1Ha, 5Ad, 17Aa, 24Ba, 29Aa, 30Aa, 30Ba, 30Ca, 33Aa, 38Aa, 40Aa, 40Ba and Vip3Ad) were tested against four species or less, which means that lack of their toxicity is likely attributable to

limited testing. The remaining five proteins are so-called parasporins belonging to the Cry31A, 41A, 45A, 46A and 63A families, which have no known insecticidal activity but are toxic to human cancer cells. None of the parasporins were tested as purified or recombinant proteins. Rather, their lack of insecticidal activity was inferred from screening of parental strains against five (Cry31Ad, Cry63Aa), 11 (Cry 41Aa, Cry45Aa) or 13 (Cry31Aa) species from as many as six arthropod orders (Lepidoptera, Diptera, Isoptera, Orthoptera, Blattodea, Acari) (Mizuki et al., 1999; Okumura et al., 2005; Hayakawa et al., 2007; Jung et al., 2007; Nagamatsu et al., 2010). It should be noted that none of the parasporin-carrying strains

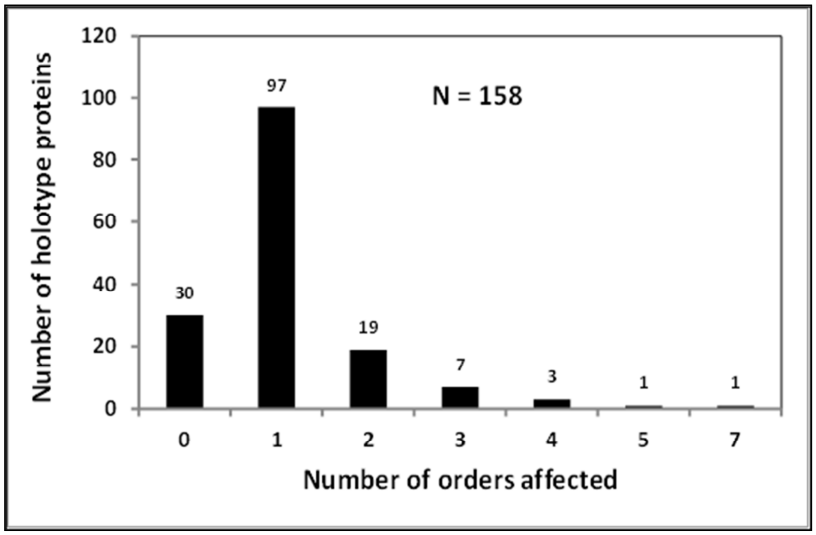

Figure 5. Distribution of the number of *B. thuringiensis* pesticidal proteins (Cry, Cyt and Vip) as a function of the number of orders (bottom) that was affected.

were tested against other orders that are known to be affected by *Bt* proteins, most notably Coleoptera and rhabditid nematodes. It is therefore likely that expanded screening will eventually reveal pesticidal activity of parasporins.

2.2.3.3. Single-order proteins

The majority (97 or 61%) of the tested toxin proteins were reported to affect species within one order only (Fig. 5). Of those, 44 are active within the order of Lepidoptera, 23 within Coleoptera, and 25 within Diptera. Other insect orders affected by single-activity proteins are Hymenoptera (Cry5Ac) and Orthoptera (Cry7Ca). Unequivocal proof of hymenopteran toxicity of Cry5Ac is lacking, however. Its toxicity to the sawfly *Diprion pini* was inferred from bioassays with a 75-kDa protein that was 'highly probable to correspond to the Cry5Ac toxin' (Garcia-Robles et al., 2001), while bioassay data against the pharaoh ant *Monomorium pharaonis* were shown for the parental strain and for unspecified toxin

proteins from that strain (see Table 6 and 7 in Payne et al., 1997). Recombinant Cry7Ca was toxic to larval *Locusta migratoria* (Orthoptera) with an LC_{50} of 10 µg/ml (Wu et al., 2011) but pesticidal activity outside that order has not been determined. Three proteins were reported to have activity restricted to one order within the phylum Nematoda (Cry6Ba, 12Aa, and 21Aa). Activity of another putative nematode toxin, Cry13Aa, has not been established unequivocally: the gene is known from nematode-active strains (Narva et al., 1995; Kotze et al., 2005) but tests of purified protein or cloned gene product against nematodes have not been published to date.

2.2.3.4. Toxicity profiles

Levels of primary-order toxicity are shown in more detail in Figure 6 for the most commonly tested toxin families and taxa within the three insect orders that are mainly targeted by *Bt* pesticidal proteins. Median LC_{50} values for Cry1 and Vip3 proteins across the four most commonly tested lepidopteran families range between 0.007 and 0.04 µg/cm^2 in diet-surface assays and between 0.8 and 10 µg/ml in diet-incorporation assays (Fig. 6, top; for more information on assays, see 3.2). Average toxicity of dipteran proteins varies from 0.07 µg/ml for the most active proteins (Cry11) against the most susceptible genus (*Aedes*) to 2 µg/ml for the least active protein (Cyt1A) against the most refractory genus (*Culex*) (Fig. 6, middle). The most frequently tested coleopteran protein, Cry3Aa, ranges in toxicity between 0.04 µg/cm^2 against *Leptinotarsa* and 6.5 µg/cm^2 against *Diabrotica* (Fig. 6, bottom).

Reference toxicity ranges were constructed for proteins with well-established primary order affinities. Non-parametric distributions of published LC_{50} values for Diptera-, Coleoptera-, Lepidoptera- and Nematoda-active proteins were obtained by using the box plot function in Minitab16 statistical software and the 25-75% percentile of those distributions were used to define toxicity ranges (Table 1; van Frankenhuyzen, 2013). Non-parametric distributions were obtained by pooling bioassay data from the Toxin Specificity Database within protein families (as indicated in Table 1), and across species as follows. Cry4, Cry11 and Cyt1: all Culicidae (*Aedes aegypti*, *Anopheles stephensi*, *Anopheles gambiae*, *Anopheles albimanus*, *Culex pipiens*, *Culex quinquefasciatus*); Cry3: all Chrysomelidae (*Diabrotica*, *Leptinotarsa*, *Pyrrhalta, Chrysomela* spp.); Cry 1: 46 Lepidoptera species belonging to 12 families; Vip3A: 10 Lepidoptera species belonging to three families. Lepidopteran and coleopteran assays were constricted by assay type (diet-incorporation and diet-surface layering). Both methods are commonly used in lepidopteran assays, but the majority of coleopteran toxicity values are derived from diet-surface assays.

In general, dipteran toxins are the most active with median LC_{50} values of 1 µg/ml or less and a 75% percentile below 10 µg/ml. Lepidopteran toxins are active in the range of a few µg/ml with a 75% percentile of 15 - 20 µg/ml, while nematicidal proteins are active above

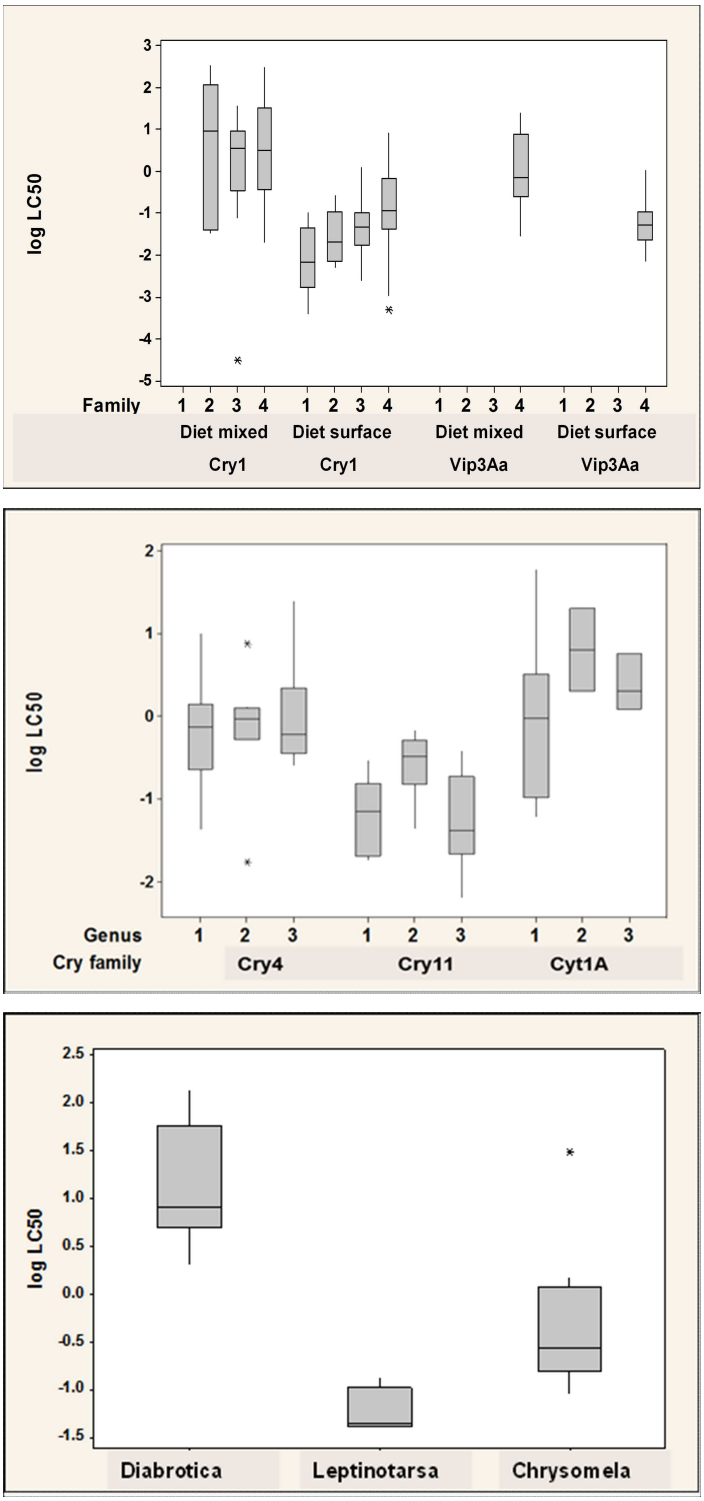

Figure 6. Distribution of 50% lethal concentration (LC_{50}) estimates for Lepidoptera-active Cry1 and Vip3A proteins averaged across families (1= Plutellidae; 2 = Sphingidae; 3 = Pyralidae; 4 = Noctuidae) (top); for Diptera-active Cry4, Cry11 and Cyt1A proteins averaged across genera (1 = *Aedes*; 2 = *Anopheles*; 3 = *Culex*) (middle); and for the Coleoptera-active Cry3Aa averaged across three genera (bottom). LC_{50} estimates are expressed in µg/ml (Diet mixed) or µg/cm^2 (Diet surface) in top panel, µg/ml in middle panel, and µg/cm^2 in bottom panel.

that range. Coleopteran toxins are on average less active than lepidopteran proteins and have a wider toxicity range. By applying the consistent 1:30 ratio of lepidopteran median LC_{50} values in diet-surface: diet-incorporation assays (deduced from the data in Table 1) to coleopteran diet-surface LC_{50}s, coleopteran toxins are expected to be active in the 5 µg/ml range if they were tested using diet-incorporation methods.

Table 1. Toxicity ranges of Cry4, Cry11 and Cyt1 proteins to Diptera (DIP); various proteins to Nematoda (NEM); Cry3 proteins to Coleoptera (COL) and Cry1 and Vip3 proteins to Lepidoptera (LEP) when layered on diet surface (Surface) or incorporated into diet (Mixed). Toxicity ranges are characterised by median values and 25% and 75% percentiles of distributions of published LC_{50}s (in µg/ml or µg/cm^2).

Protein [1]	Order	Number of LC_{50}s	Method	Median	25%	75%	Unit
Cry4	DIP	31		0.90	0.40	1.30	µg/ml
Cry11	DIP	31		0.06	0.02	0.25	µg/ml
Cyt1	DIP	17		1.20	0.70	6.00	µg/ml
	NEM	9		25	15	125.0	µg/ml
Cry3	COL	13	Surface	0.18	0.06	20.0	µg/cm^2
Cry1	LEP	237	Surface	0.07	0.01	0.25	µg/cm^2
Cry1	LEP	160	Mixed	2.00	0.20	15.0	µg/ml
Vip3	LEP	17	Surface	0.08	0.03	0.30	µg/cm^2
Vip3	LEP	8	Mixed	2.5	0.35	20	µg/ml
All	**All**	**262**		**1.2**	**0.12**	**10.0**	**µg/ml**

[1] Proteins were pooled by primary rank; data included in the pooling pertain to the following proteins:
DIP - Cry4: 4Aa, 4Ba; Cry11: 11Aa, 11Ba, 11Bb; Cyt1: 1Aa, 1Ab.
NEM - Cry5Aa, 5Ab, 5Ba, 6Aa, 55Aa
COL - Cry3: 3Aa, 3Ba, 3Bb
LEP - Cry1: 1Aa, 1Ab, 1Ac, 1Ad, 1Ae, 1Ba, 1Bb, 1Ca, 1Cb, 1Da, 1Db, 1Ea, 1Eb, 1Fa, 1Ia, 1Ie, 1Ja, 1Jb, 1Ka; Vip3: Vip3Aa
All: above listed proteins combined

Based on the 25-75% percentile of the distribution of µg/ml LC_{50} values pooled across all proteins and all taxa (n = 262 assays, Table 1), *Bt* pesticidal proteins can be classified as

having high toxicity when active in the 0.01 - 0.10 µg/ml range (below 25% percentile), medium toxicity when active in the 0.10 - 10 µg/ml range, and low toxicity when LC_{50}s are in the 10 - 1000 µg/ml range (above 75% percentile).

2.2.4. Cross-activity

Considering both qualitative and quantitative reports, 31 of the 158 *Bt* proteins tested to date were active across more than one order (Fig. 5). That number includes the dually-active (Lepidoptera, Diptera) Cry2Ag (Liang et al., 2011), the nematode-active Cry6Aa, which affects nematode species in three orders (Narva et al., 1993; Guo et al., 2008), and Cry5Ba affecting nematodes across four orders (Guo et al., 2008; Hu et al., 2010; Urban et al., 2013; Wang et al., 2012b). Because these cross-order activities are within these proteins' primary specificities, they do not fall under the definition of cross-activity. Seventeen of the 28 remaining cross-active proteins affected species across two orders, seven across three orders, two across four orders, and one across five or over seven orders (Fig. 5). Which orders were reported as being affected by proteins that displayed activity outside their orders or primary specificity, is summarised in a compilation of both qualitative and quantitative reports in Figure 7. Lack of quantitative data is indicated throughout the text by the comment 'bioassay data not reported'. Species outside the order of primary affinity for which quantitative toxicity data were published are referred to in the text without further comment, as those data are presented in supplementary Table S3, while Table S4 lists the studies that reported no toxicity to species outside the orders of primary affinity. Figure 7 also reports for each of the proteins with cross-order activity the number of species that were tested and their distribution across the various taxa.

2.2.4.1. Across two orders

Seventeen of the 28 cross-active proteins were reported to affect species across two orders (Fig. 7, top). Fourteen of those were active across two insect orders, and three had activity in both Insecta and Nematoda. Two of the proteins affecting two insect orders are well-known Lepidoptera-active proteins: Cry1Ca affects several Diptera (*A. aegypti, A. gambiae, C. quinquefasciatus*; Smith et al., 1996), while Cry1Ia is toxic to two Coleoptera (*Leptinotarsa decemlineata, Anthonomus grandis*; Naimov et al., 2001; Ruiz de Escudero et al., 2006; Martins et al., 2008). Two other proteins are active against Coleoptera and Lepidoptera: the Coleoptera-active Cry8Da affects *Plutella xylostella* (Lepidoptera; Asano et al., 2003), while Cry22Ab affects some Lepidoptera (*Trichoplusia ni*, *P. xylostella*; Mettus and Baum, 2000; bioassay data not reported). Coleoptera-active proteins can affect other orders as well: Cry22Aa is toxic to *A. grandis* (Isaac et al., 2001; bioassay data not reported) and pharaoh ants (Hymenoptera: *M. pharaonis;* bioassay data not reported; Payne et al., 1997), while Cyt2Ca was reported to affect the cat flea (Siphonaptera: *Ctenocephalides felis*; bioassay data not reported; Rupar et al., 2000). Two Diptera toxins, Cry4Aa and Cry11Aa, affect aphids (Hemiptera: *Acyrthosiphon pisum*, Porcar et al., 2009; *Macrosiphum euphorbiae*, Walters and English, 1995), while a third, Cry10Aa, was recently demonstrated

PHYLUM	ART							NEM		
CLASS	INS						ARA	CHR		
ORDER	COL	LEP	DIP	HEM	HYM	SIP	ACA	RHA	TYL	DIP
Cry1Ca		50	3							
Cry1Ia	7	21	2				1			
Cry3Bb	8				1			1		
Cry4Aa			7	1						
Cry8Da	4	3								
Cry10Aa	1	2	5							
Cry11Aa		1	9	2						
Cry14Aa	1							5		1
Cry22Aa	2				1					
Cry22Ab	4	8								
Cry30Fa		2	1							
Cry30Ga		1	1							
Cry54Aa		2	1							
Cry55Aa	1								2	
Cry56Aa		2	1							
Cyt2Ca	3					1				
Vip1A/2A	5	7	1	1						

PHYLUM	ART									MOL	PLA	NEM		
CLASS	INS							HEX	ARA	GAS	TRE	CHR		
ORDER	COL	LEP	DIP	HEM	HYM	NEU	BLA	CO	ACA		ECH	RHA	DIP	TYL
Cry1Ac	7	61	2	8	8	2	1	2	3	1		1		
Cry1Ba	7	42	3											
Cry2Ab	1	17	4	1		1						1		
Cry3Aa	33	3	2	3	1			2						
Cry5Aa											1	4	1	1
Cry5Ab											1	1		1
Cry51Aa	3	6		2										

PHYLUM	ART												ANN	NEM										
CLASS	INS											HEX	CRU			ARA		CLIT	CHR					
ORDER	COL	LEP	DIP	HEM	HYM	ISO	NEU	ORT	BLA	THY	TRI	CO	AMP	IS	CLA	ACA	AR	HAP	ASC	RHA	DIP	TYL		
Cry1Ab	7	56	2	9	3		1			3	4	2	1	2	1	3	3	2		1				
Cry2Aa	5	37	9	5	4	1	2	1	1			2		1										
Cyt1Aa	1	3	10	1																				
Cyt1Ba	4	1	1	1	4																			

Figure 7. Distribution of pesticidal activities of *B. thuringiensis* pesticidal proteins that affect species across two (top), three (middle) or more (bottom) orders. The number of species that was tested within each order is indicated inside the corresponding box, and is identified in supplementary Table S3 and S4. No shading (white) means that none of the species tested was affected. Grey shading means that at least one of the species tested was affected. Light grey indicates cross-activities that are substantiated (published estimates of LC_{50} or mortality response to a well-defined dose). Dark grey indicates cross-activities that are not unequivocally established (lack of quantitative data or conflicting data). **Phyla**: ANN = Annelida; ART = Arthropoda; MOL = Mollusca; NEM = Nematoda; PLA = Platyhelminthes. **Classes**: ARA = Arachnida; CHR = Chromadorea; CLIT = Clitellata; CRU = Crustacea; GAS = Gastropoda; Hex = Hexapoda; INS = Insecta; TRE = Trematoda. **Orders**: ACA = Acari; AMP = Amphipoda; AR = Araneae; ASC = Ascarida; BLA = Blattodea; CLA = Cladocera; CO = Collembola; COL = Coleoptera; DIP (INS) = Diptera; DIP (NEM) = Diplogasterida; ECH = Echinostomida; HAP = Haplotaxida; HEM = Hemiptera; HYM = Hymenoptera; IS = Isopoda; ISO = Isoptera; NEU = Neuroptera; LEP = Lepidoptera; ORT = Orthoptera; RHA = Rhabditida; SIP = Siphonaptera; THY = Thysanoptera; TRI = Trichoptera; TYL = Tylenchida.

to be toxic to cotton boll weevil (*A. grandis*; de Souza Aguiar et al., 2012). Proteins, other than the known dually active Cry2, with toxicity to both Lepidoptera and Diptera include Cry30Fa (*A. aegypti* and *P. xylostella*; Tan et al., 2010), Cry30Ga (*A. aegypti* and *P. xylostella*; Zhu et al., 2010a), Cry54Aa (*A. aegypti* and *Spodoptera exigua*, *Helicoverpa armigera*; Tan et al., 2009), and Cry56Aa (*A. aegypti* and *P. xylostella*, *H. armigera*; Zhu et al., 2010b). The last of the 14 proteins affecting two insect orders is the binary Vip1A/Vip2A toxin, which is toxic to corn rootworms (*Diabrotica virgifera*; Warren, 1997) and cotton aphid (Hemiptera: *Aphis gossypii*; Sampurna and Maiti, 2011). The remaining three proteins were reported to affect species in both Insecta and Nematoda. More specifically, coleopteran activity has been cited for Cry14Aa (*Diabrotica* spp.; Payne and Narva, 1994; bioassay data not reported) and Cry55Aa (flea beetles; Bradfisch et al., 1999; bioassay data not reported), proteins which are known to affect nematodes (Payne et al., 1996; Wei et al., 2003; Guo et al., 2008; Peng et al., 2011), while recent studies revealed activity of the well-known coleopteran Cry3Bb toxin to a free-living soil nematode (Höss et al., 2011, 2013).

2.2.4.2. Across three orders

Seven of the 28 cross-active proteins were reported to have activity in three orders (Fig. 7, middle). The Lepidoptera-active Cry1Ac is toxic to tsetse flies (Diptera: *Glossina morsitans*; Omolo et al., 1997) and an aphid (Li et al., 2011). The Lepidoptera-active Cry1Ba affects several Coleoptera, including *L. decemlineata, Chrysomela scripta*, *Hypothenemus hampei* (Bradley et al., 1995; Federici and Bauer, 1998; Zhong et al., 2000; Naimov et al., 2001; Lopez-Pazos et al., 2009) and *A. grandis* (Martins et al., 2006; bioassay data not reported), as well as some Diptera (*Lucilia cuprina*; Heath et al., 2004; bioassay data not reported; *Musca domestica,* Zhong et al., 2000). Besides its dual activity against Lepidoptera and some Diptera, Cry2Ab affects nematodes (Höss et al., 2013). The Coleoptera-active Cry3Aa was reported to have activity to Hemiptera and Hymenoptera. Laboratory experiments with high doses revealed toxicity to two aphid species (*M. euphorbiae* and *A. pisum*; Walters and English, 1995; Porcar et al., 2009; Li et al., 2011). Hymenopteran toxicity to the imported red fire ant *Solenopsis invicta* was reported by Bulla and Candas (2004), with preliminary indication that the LC_{50} could be as low as 70 ng/ml. Cry5Aa and Cry5Ab are toxic to nematodes in two orders (Phylum: Nematoda; Narva et al., 1991; Sick et al., 1994) and were reported to affect the liver fluke *Fasciola hepatica* (phylum: Platyhelminthes; Narva et al., 1991). However, those tests failed to conclusively demonstrate liver fluke toxicity because results were confounded by high control mortality. Cry51Aa is toxic to the plant bugs *Lygus hesperus* and *Lygus lineolaris* (Hemiptera), as well as to *L. decemlineata* (Coleoptera) (Baum et al., 2012). Lepidopteran activity of this protein needs to be confirmed: Cry51Aa2 was not toxic to several Lepidoptera (*Heliothis virescens*, *Agrotis ipsilon*, *Spodoptera frugiperda*, *Ostrinia nubilalis*, *Helicoverpa zea*) (Baum et al., 2012), but Cry51Aa1, which differs by seven amino acids, was toxic to *Bombyx mori* (Huang et al., 2007; bioassay data not reported).

2.2.4.3. Across four or more orders

Cry2Aa and Cyt1Aa were reported to affect species in four orders (Fig. 7, bottom). In addition to its known dipteran and lepidopteran activities, Cry2Aa is toxic to *M. euphorbiae* (Hemiptera; Walters and English, 1995). Hilbeck et al. (1999) reported low levels of mortality in the lacewing *Chrysoperla carnea* (Neuroptera) when reared on Cry2Aa-fed *Spodoptera littoralis* larvae, but other studies showed no effect of high Cry2Aa concentrations on either *C. carnea* (Sims, 1997) or *Chrysoperla sinica* (Wang et al., 2012a). The Diptera-active Cyt1Aa is toxic to Coleoptera (*C. scripta*; Federici and Bauer, 1998), Hemiptera (*A. pisum*; Porcar et al., 2009), and possibly Lepidoptera (*P. xylostella*; Sayyed et al., 2001). However, Cyt1Aa lacked toxicity to *P. xylostella* in the studies by Meyer et al. (2001), a discrepancy that remains unresolved.

The two remaining proteins (Cry1Ab, Cyt1Ba) were reported to be active across more than four orders. Because of its widespread use in transgenic crops, Cry1Ab has been tested against the broadest range of species, spanning three phyla and six classes, resulting in reports of possible activity in one nematode order (Höss et al., 2008) and six insect orders (Fig. 7). The only cross-order activity within the class Insecta that has been substantiated, however, is toxicity to Hemiptera (*A. pisum*; Porcar et al., 2009). Cross-activity in the other orders was not established unequivocally. Its dipteran activity was inferred from Haider et al. (1986), who reported *A. aegypti* toxicity of a purified *aizawai* crystal protein but failed to demonstrate that the activity was due to Cry1Ab. Subsequent work with the *cry1Ab7* gene cloned from that strain, demonstrated toxicity to lepidopteran and dipteran cell lines, but did not include *in vivo* tests (Haider and Ellar, 1987). Cry1Ab at a high dose affected survival of *Cheilomenes sexmaculatus* (Coleoptera; Dhillon and Sharma, 2009), and of *Adalia bipunctata* when fed toxin-coated prey (Schmidt et al., 2009), but subsequent studies did not confirm toxicity to *A. bipunctata* (Porcar et al., 2010; Álvarez-Alfageme et al., 2011) or other coccinellids (Pilcher et al., 1997; Lundgren and Wiedenmann, 2004; Bai et al., 2006; Álvarez-Alfageme et al., 2008). Likewise, reports of Cry1Ab toxicity to *C. carnea* (Neuroptera; Hilbeck et al., 1998, 1999; Dutton et al., 2002) are at odds with other studies showing no effects (Pilcher et al., 1997; Rodrigo-Simón et al., 2006), even at very high dose levels (Romeis et al., 2004). Effects of Cry1Ab-corn material on survival of the caddisfly *Helicopsyche borealis* (Trichoptera; Rosi-Marshall et al., 2007) cannot be unequivocally attributed to Cry1Ab toxicity as the study did not include an isogenic control to eliminate other plant-derived factors (Beachy et al., 2008).

Cyt1Ba has possibly the most diverse insecticidal activity spectrum of all *Bt* proteins reported to date. It has been reported to be toxic to the leaf-mining fly *Lyriomyza trifolii* (Diptera; Payne et al., 1995); the tarnished plant bug *L. hesperus* (Hemiptera; Stockhoff and Conlan, 1998); several Coleoptera including *D. virgifera* and *Hypera postica,* as well as *A. grandis* and *Lissorhotrus aryzophilus* (bioassay data not reported) (Payne et al., 1995; Bradfisch et al., 1998); at least one Lepidoptera (*Choristoneura fumiferana*; van

Frankenhuyzen and Tonon, 2013); and several species of sawflies (Hymenoptera; van Frankenhuyzen and Tonon, 2013). Hemipteran toxicity needs to be confirmed, as Wellman-Desbiens and Côté (2005) failed to demonstrate Cyt1Ba toxicity against this pest and provided evidence that solubilisation buffer may have contributed to the toxicity reported by Stockhoff and Conlan (1998).

2.2.4.4. Reported versus substantiated

Using mortality estimates as reasonable but minimum evidence for cross-activity, there is insufficient evidence (i.e. qualitative data only) in the following cases: toxicity of Cry5Aa and Cry5Ab to Platyhelminthes, Cry14Aa to Coleoptera, Cry22Aa to Coleoptera and Hymenoptera, Cry22Ab to Lepidoptera, Cry51Aa to Lepidoptera, Cry55Aa to Coleoptera, and Cyt2Ca to Siphonaptera. In addition, quantitative data contradicted by other studies present enough uncertainty to indicate lack of evidence for unequivocal cross-toxicity, which is the case for Cry1Ab and Coleoptera, Diptera, Neuroptera and Trichoptera; Cry2Aa and Neuroptera; Cyt1Aa and Lepidoptera; and Cyt1Ba and Hemiptera. Excluding those cases reduces the number of cross-activities from 28 proteins affecting 75 high-ranking taxa to 21 proteins affecting 59 taxa, as indicated by light-grey shading in Figure 7.

2.2.5. Biological significance of cross-activities

Quantitative estimates of toxicity (LC_{50}s) to species outside orders of primary affinity are available for 18 proteins and 19 species (Table S3). Plotting toxicity values outside primary order specificities with toxicity ranges of the reference proteins from Table 1 shows how cross-activities vary widely across proteins and taxa (Fig. 8; updated from van Frankenhuyzen, 2013). Cry30Fa, 30Ga, 54Aa and 56Aa were not included in the figure as their primary specificities are not clear. Sixteen of the reported activities (LC_{50}s; but EC_{50}s for nematode activity of Cry1Ab, Cry2Ab, and Cry3Bb) group within or above those of nematode-active proteins in the low-toxicity range, seven group with the majority of insect-active proteins in the medium-toxicity range, and two fall in the high-toxicity category bench-marked by Diptera-active Cry11 proteins.

The first type of cross-activity that is of interest for ecological safety of *Bt* pesticidal proteins are activities in orders outside the suite of orders that are normally affected, such as toxicity of Lepidoptera-, Diptera- and Coleoptera-active proteins to Hemiptera, Hymenoptera and Nematoda. Hemipteran toxicity has been substantiated for nine proteins (Cry1Ab, 1Ac, 2Aa, 3Aa, 4Aa, 11Aa, 51Aa, Cyt1Aa, Vip1A/2A) and has been quantified in four of those (Table S3). Cry4Aa and Cry11Aa have low toxicity to aphids with LC_{50} estimates ranging between 125 and 500 µg/ml. Qualitative data indicate a similar level of toxicity of Cry1Ab, 1Ac, 2Aa, 3Aa and Cyt1Aa, with doses of 100-500 µg/ml resulting in mortalities between 10 and 100%. Toxicity of Cry51Aa to *Lygus* bugs is up to 6-fold higher than aphid toxicity of other crystal proteins, but still falls in the low-toxicity range (Fig. 8). In contrast, the Vip1A/2A binary toxin is 2- to 3-orders of magnitude more toxic to aphids than most

aphid-active crystal proteins and falls well within the range of medium toxicity. Hymenopteran activity is confined to two proteins: Cyt1Ba has low toxicity to sawflies and Cry3Aa has high toxicity to fire ants. Three insect-active proteins (Cry1Ab, 2Ab, and 3Bb) affected a soil-dwelling nematode but they affected growth and reproduction, not survival, at dose levels that were two orders of magnitude higher than is typical for nematode-active proteins (Table 2).

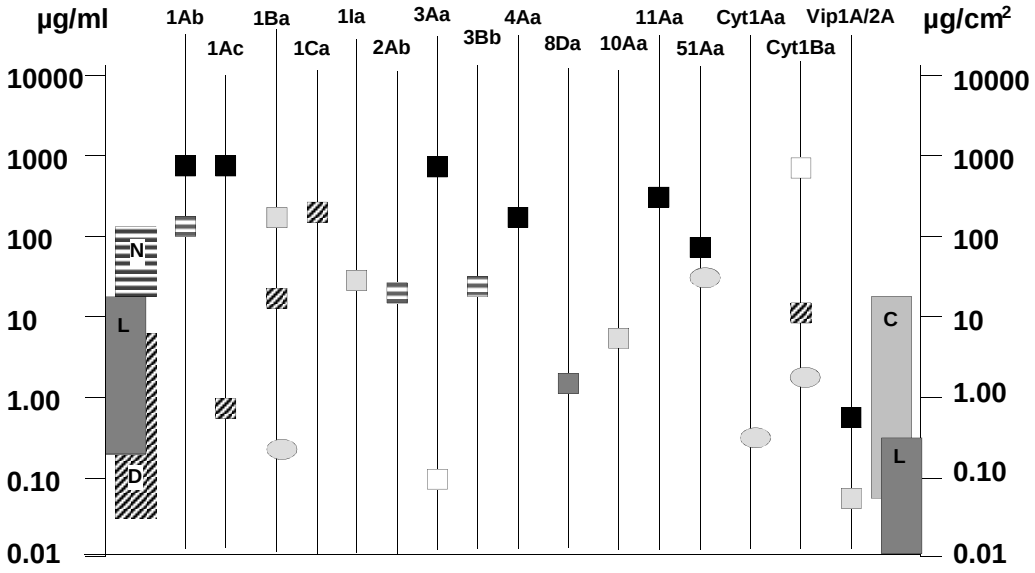

Figure 8. Comparison of toxicities outside orders of primary specificity of 16 cross-active proteins with reference toxicity ranges of predominantly Lepidoptera (L)-, Coleoptera (C)-, Diptera (D)-, and Nematoda (N)-active proteins (from Table 1). Reported LC_{50} values are presented as squares (estimates expressed in µg/ml, left Y-axis) or ovals (estimates expressed in µg/cm^2, right Y-axis). Hatching inside the symbols corresponds to orders indicated by hatching of the bars that represent toxicity ranges of the reference proteins. Additional order activities: Hemiptera (black squares) and Hymenoptera (open squares). Positions of symbols are approximate; exact values can be found in Table S3.

The second type of cross-activity that is of interest from an ecological safety perspective includes activities that are within the suite of orders normally affected (Lepidoptera, Coleoptera and Diptera) but outside a protein's primary order affinity, especially when that activity occurs at levels that are within the toxicity range of corresponding reference proteins. This group includes dipteran toxicity of Cry1Ac, which is within the expected toxicity range of Diptera-active proteins, lepidopteran toxicity of Cry8Da within the range of Lepidoptera-active reference proteins, and coleopteran toxicities of lepidopteran (Cry1Ba, Cry1Ia) and dipteran (Cry10Aa, Cyt1Aa, Cyt1Ba) toxins within the range of Coleoptera-active proteins. The high toxicity of Vip1A/2A to corn rootworm makes it the most active

coleopteran toxin known to date, having an LC_{50} that is ~200-fold lower than the (inferred) median value of Coleoptera-active proteins in diet-incorporation assays. Dipteran toxicity of Cry1Ba, Cry1Ca, and Cyt1Ba are of less interest from a non-target safety perspective because those toxicities occur at high dose levels compared to Diptera-active reference proteins.

2.2.6. Discussion

The past few decades have revealed an astounding diversity of *Bt* crystal protein genes encoding proteins that are pesticidal to a broad array of taxa. Available data undoubtedly underestimate that diversity because characterisation of biological activity lags far behind gene discovery. The specificity picture is fragmentary at best, considering that: (i) more than half (171) of the 329 holotype proteins have not been tested at all; (ii) the majority of the 158 tested proteins were bio-assayed against a limited number of species (10 or less, Fig. 1), and (iii) species and toxins tested are not equally distributed across protein families and taxa (Table S1).

Host specificity of *B. thuringiensis* is a much treasured feature that makes it attractive for environmentally acceptable pest control applications. Specificity was initially recognised as biological activity of strains that was limited to specific insect orders, in particular Lepidoptera, Coleoptera and Diptera. The first classification of crystal proteins (Höfte and Whiteley, 1989) reflected that order specificity and included only one rank family (Cry2) with dual specificity. Since then, testing has revealed cross-order activity in at least 21 toxin proteins distributed over 16 primary rank families and across all three protein classes (Cry, Cyt and Vip). Cross-toxicities that are supported by reasonable evidence are so far limited to the class Insecta in the phylum Arthropoda. Testing of insecticidal proteins against species in other arthropod classes and other phyla (Table S2, Fig. 7) has not produced conclusive evidence of lethal activity outside the class Insecta.

Lack of cross-activity in the remaining protein families needs to be interpreted with caution, because lack of presence is not proof of absence. It is presently unknown to what extent absence of cross-activity is the result of insufficient testing. It is clear that the majority of the proteins have not been tested extensively across high-ranking taxa (Fig. 1). Only 36% of the 97 proteins with reported ‘single-order’ activity (Fig. 5) were actually tested against species outside their primary order, illustrating how selection of tests species is biased towards the order in which activity was initially reported. Such bias obviously limits the evaluation of cross-order activities and it is likely that more cross-activities will be uncovered as testing is expanded. This notion is supported by the observation that 41% of the 75 proteins that were tested against species in two orders or more (Fig. 1, bottom) were reported to have cross-activity (Fig. 5). The number of reported (suspected) cross-activities (28) suggests that cross-activity may be more common than suggested by the number of cases substantiated to date (21). For example, many of the suspected cross-activities were reported in patents without supporting data but are presumably based on evidence locked

up in proprietary company files. Notable examples are the frequently cited but unsubstantiated coleopteran activity of several nematode-active proteins (Cry6A, Cry14A and Cry 55A).

Positive evidence of cross-activity needs to be interpreted with caution as well. Although supported by mortality data in the case of 21 proteins and 32 species (Table S3), the evidence is not strong in most cases, considering that (i) only a little more than half (58%) of the toxicity data reported in Table S3 involve actual LC_{50} estimates, (ii) very few of those include statistics that allow rigorous evaluation of dose-mortality regressions, and (iii) most reports are based on single studies that have yet to be confirmed by work in other laboratories. The only cross-activities that have been validated by independent studies are toxicity of Cry1B, Cry1Ia and Cyt1Ba to Coleoptera and toxicity of Cry3Aa and Cry11Aa to Hemiptera (Table S3). All other cross-activities need to be viewed with caution until they are confirmed through additional testing.

The increase in number of crystal protein families that display activity across a broad range of taxa, from nematodes to insects, gives rise to the question if order specificity is still a functional concept. Cross-activities of *Bt* pesticidal proteins have potential implications for ecological (non-target) safety of transgenic crops, an issue that has become the focus for intense scientific, pubic and regulatory debate (Wolfenbarger and Phifer, 2000). The degree of concern depends on the level of toxicity relative to toxicities of proteins within their orders of primary specificity. This was evaluated by plotting quantitative toxicity estimates (LC_{50}s) for cross-active proteins with their normal (target) toxicity range (Fig. 8).

Almost two-thirds of the quantified cross-activities are presumably of limited biological significance because they involve toxicities that are low (>10 µg/ml) compared to toxicity ranges of the so-called order-specific reference proteins (Fig. 8). For example, numerous studies showing no adverse effects of Cry proteins in transgenic crops on aphids (e.g., Kalushkov and Nedved, 2005; Ramirez-Romero et al., 2008; Zhang et al., 2008; Digilio et al., 2012) are in line with their low inherent toxicity in laboratory assays.

Effects of the insect-active Cry1Ab, Cry2Ab and Cry3Bb proteins on *C. elegans* (Höss et al., 2008; 2011; 2013) are the first substantiated cases of cross-phylum activity. Although the activities plot within the range of Nematoda-active proteins in Figure 8, the reported activities were based on inhibition of growth and reproduction, rather than mortality which typically requires a 100-fold higher dose as shown by data for Cry5Ba in Table 2. Because estimates of the EC_{50}s for inhibition of growth and reproduction were about two orders of magnitude greater than such EC_{50}s of known Nematoda-active proteins (Table 2; comparison within *C. elegans*), the reported activities are not likely to have any ecological significance. This is supported by studies showing no effect of transgenic crops in nematode abundance, growth, reproduction or community structure (Saxena and Stotzky, 2001; Griffiths et al., 2007; Höss et al., 2011).

One-third of the reported cross-activities can be classified as being of medium or high toxicity and fall within the range of toxicities exhibited by order-specific proteins commonly used in pest control applications. These activities are of potential biological interest, but their development for pest control applications (such as Cry10Aa for control of boll weevil) would benefit from additional non-target effect studies. Cross-activities in the high-toxicity

Table 2. Concentrations (µg/ml) of various proteins causing 50% inhibition of reproduction, growth or survival in three species of Nematoda (citation in parentheses)

Protein	*C. elegans*			*M. hapla*	*M. incognita*
	Reproduction	Growth	Survival	Survival	Survival
Cry1Ab	54 (2)	225 (2)			
Cry2Ab	23 (4)				
Cry3Bb	8 (3,4)	22 (3)			
Cry5Aa			10 (5)		
Cry5Ab			32 (5)		
Cry5Ba	0.066 (7) [1]	0.042 (8)	6.7 (4) [2]	18 (1)	146 (6)
Cry6Aa	0.230 (7) [1]			24 (6)	383 (6)
Cry14Aa	0.016 (7) [1]				
Cry21Aa	0.047 (7) [1]				
Cry55Aa				23 (1)	103 (6)

(1) Guo et al., 2008; (2) Höss et al., 2008; (3) Höss et al., 2011; (4) Höss et al., 2013; (5) Narva et al., 1991; (6) Peng et al., 2011; (7) Wei et al., 2003[1]; (8) Wang et al., 2012a

[1] Wei et al. (2003) reported EC_{50} values in ng/µl in the text but in ng/ml in their Figure 3C; the correct unit is ng/ml (see reference 4)

[2] Reported in that reference as a personal communication by R. Aroian

category flag a need for studies to address possible ecological concerns. For example, because Cry3Aa is highly toxic to fire ants, it may be desirable to investigate direct and indirect effects of Cry3A crops on ants. Although transgene applications of Vip1A/2A to combat corn rootworm are not being considered, such applications could need to take into account possible effects on plant-sucking insects.

In conclusion, expanded testing of *Bt* pesticidal proteins is revealing activities outside their range of primary specificity. Cross-activities are not uncommon, having been substantiated for ~16% of the 158 proteins tested to date, and may be more prevalent

considering that one-third of proteins that were tested against species in two or more orders were confirmed to be cross-active. Substantiated cross-activities are to date primarily limited to the class Insecta, with 12 proteins affecting species across two orders, five proteins affecting three orders and one protein affecting four orders. Cross-phylum activity is known for three insecticidal proteins that also affect nematodes, albeit at a sublethal level. One-third of the cross-activities occurred at toxicity levels that are well within or even below the toxicity range of order-specific proteins, indicating a potential for effects on non-target species outside their normal host range. Most cross-activities have yet to be confirmed through independent testing, but the few that have been validated firmly establish the notion that *Bt* proteins are not as order-specific as was conventionally believed to be the case. This requires attention in the design and regulatory approval of their use in pest control applications.

3. Methods to study interactions among *Bt* proteins

3.1. Introduction

The approach usually followed to test the hypothesis that the potency/toxicity of insecticidal proteins when combined is unchanged and can be predicted from that of the component proteins alone (or in other words whether the combined effects of the component proteins in the mixture are merely additive), is to test for the lack of a synergistic/antagonistic interaction between the component proteins in a sensitive species. Experiments to investigate the interactions among *Bt* proteins (and between *Bt* proteins and other toxins) are primarily laboratory feeding bioassays, although *in vitro* bioassays have been used as well. To test for interactions, a model is used defining what no interactive effects (a null model) are. If such a model enables prediction of the toxicity of a mixture directly from the toxicities of the components and their relative proportions in the mixture, interaction is absent. Making use of such models, methods have been developed to test interactions between *Bt* proteins. Alternatively, empirical methods without model selection can be used to test for interactions. The main purpose of this chapter is to describe existing models to test for interactions, the methods based on these models and the empirical methods to assess interactions among *Bt* proteins.

3.2. General experimental design of *in vivo* bioassays

Choice of study species. In the case of insects (Lepidoptera, Coleoptera, Diptera), the tests conducted used one or, occasionally, more sensitive species that are easily reared in a laboratory setting. In all cases, except for two (*B. mori* and *Chironomus tepperi*), the sensitive species chosen were the target pest organisms for the tested proteins or close relatives. In some studies with *Bt* proteins acting against the same insect order, the response of (a) highly sensitive species was compared with that of (a) less sensitive species (Greenplate et al., 2003; Hunter, 2006; Xue et al., 2005; Ibargutxi et al., 2008). For one study with nematodes, a target species (*Meloidogyne incognita*) was selected (Peng et al., 2011), whereas in another a surrogate non-target species was used (*C. elegans*) (Höss et al., 2013).

Methods of exposure. Interaction experiments with terrestrial insects (lepidopteran or coleopteran larvae) were basically feeding bioassays. These were mostly done by contaminating an artificial diet for the sensitive species with pure (microbially produced) proteins or purified parasporal inclusions. In some studies, sporulated *Bt* cultures were used to prepare the test toxins (e.g., Wirth et al., 1997, 2004; Xue et al., 2005); interestingly, spores have been reported to synergise Cry-toxicity in certain insects, but not in others (see Wirth et al., 1997 and references therein, Liu et al., 1998). Greenplate et al. (2003) used plant tissue that was lyophilised and powdered to expose test organisms in a feeding bioassay. Dilutions of proteins (or plant tissue powder samples) were either incorporated

into a solid (gelled) diet ("diet-incorporation"), which results in an LC_{50} (median lethal concentration) estimate expressed in unit protein per ml diet) or applied to the surface of a solid diet ("diet-surface contamination"), which results in an LC_{50} estimate expressed in unit protein per unit surface area). The choice for diet incorporation versus surface contamination exposure may depend on the behaviour of the test insect and the quantity of test protein required (which can be substantially lower for the latter method) (Liao et al., 2002). Meyer et al. (2001) used fresh cabbage leaf disks dipped in concentrations of lyophilised *Bt* powders and a commercial *Bt*-formulation (Dipel) for one of the tested species (*P. xylostella*). For terrestrial insects, neonate larvae or larvae of later instars were then placed on the diets for (usually individual) exposure. Larvae of mosquitoes were exposed in deionised/dechlorinated water or in buffer, occasionally supplemented with food materials, like yeast extract (Delécluse et al., 1993; Poncet et al., 1995). In experiments with nematodes, juveniles were directly exposed in multiwell plates to aqueous dilutions of proteins, to which antimicrobials were added to prevent microorganism growth (Peng et al., 2011) or which were supplemented with a bacterial suspension as food for the nematodes (Höss et al., 2013).

The *Bt* protein mixtures tested mainly consisted of two proteins but a number of studies involved three or more proteins (Table S5 and S6). Depending on the approach and bioassay design (see 3.3), proteins in the mixture were tested at one, a few or multiple concentrations (in the latter case allowing the calculation of LC or EC values) and in a single or in multiple protein concentration ratios. The tested concentration ratios either reflected the expected ratio of concentrations expressed in the transgenic event (e.g. Ibargutxi et al., 2008), or were based on an empirical approach assuming that interaction effects may differ depending on the ratio of concentrations of the different proteins in the mixture.

Depending on the study, negative controls (diet only, diet + buffer, or less frequently diet + heat-inactivated *Bt* protein for terrestrial species; water or buffer only for aquatic mosquito larvae) were usually also included in the bioassay design. Some studies also used specific positive controls (e.g. exposure of known highly susceptible species to the test proteins in Ibargutxi et al., 2008).

Measured endpoints. In most cases, survival (or mortality) of exposed immatures was assessed; in some cases, also growth (or growth inhibition) of the immatures was monitored (by measuring body weight and/or determining developmental stage reached at the end of the test). When LC or EC values are calculated for experiments using artificial diets, they are usually expressed as µg(ng)/ml diet or µg(ng)/g diet for diet incorporation studies, and as $\mu g/cm^2$ diet for diet-surface contamination studies. For aquatic test organisms, like nematodes and mosquito larvae, these values are expressed as µg(ng)/ml. In a study by van Frankenhuyzen et al. (1991), the failure to produce frass (solid excreta) by exposed larvae was used as an endpoint. Test duration differed strongly depending on the study, ranging from a single day (in all studies with mosquito larvae), up to a period of several days when a

certain level of mortality or a certain developmental stage is reached, or even spanning the full extent of larval and pupal development of the test organism (e.g. Tabashnik et al., 2013).

3.3. Methods and data analysis

The choice of an experimental method and the analysis of the results of experiments assessing interaction effects among *Bt* proteins are dependent on whether the activity spectrum of the component proteins of the mixture overlaps or not. When the activity spectra of the proteins do overlap, different methods have been proposed to assess and analyse interaction effects depending on whether the mode of action (or sensu stricto, the molecular target site) of the involved proteins is similar or dissimilar. In the former case, the "similar joint action" (dose/concentration addition) model is most often used (see 3.3.1), whereas in the latter case, the "independent joint action" (response addition) model is theoretically considered to be more appropriate (see 3.3.2; Finney, 1971; SCHER, SCCS, SCENHIR, 2012). The respective models analyse the dataset for significant interactions, i.e. deviations from the null hypothesis of concentration or response addition. Interaction occurs when the combined effect of two or more toxins is stronger (synergistic) or weaker (antagonistic) than would be expected on the basis of dose/concentration addition or response addition, respectively. However, there is considerable confusion and discussion in the literature on the appropriateness of these models for analysing datasets of mixture toxicity. This is primarily related to the fact that the definitions of "mode of action" and "site of action" are not unequivocal (Cedergreen et al., 2008). Alternatively, some studies have used empirical approaches to evaluate interactions among *Bt* proteins with overlapping activity spectra (see 3.3.3). Also for proteins with non-overlapping activity spectra, several empirical approaches have been proposed. A non-overlapping activity spectrum implies that no organisms are known which are sensitive to all of the component proteins in the mixture (e.g. mixture combining Lepidoptera- and Coleoptera-active Cry proteins).

3.3.1. Overlapping activity spectra: Methods based on similar joint action model

When the different component toxins in a mixture have similar effects, so that one component can be substituted as a constant proportion of another, the null model is called similar joint action, often also named dose/concentration addition, simple similar action or Loewe additivity. This model is generally considered to be most appropriate for testing interactions among "chemically similar poisons such as *B. thuringiensis* toxins" (Tabashnik, 1992) or more precisely toxins with the same mode of action, binding to the same molecular receptor. The principle behind the model is that the toxins differ only in potency, can be regarded as dilutions of each other and are assumed not to interact with each other at the biochemical target site. In the similar joint action model, it is also assumed that the dose/concentration-response lines for the different components of the mixture are parallel (Finney, 1971; SCHER, SCCS, SCENHIR, 2012).

Under the dose/concentration addition (similar joint action) model, the overall principle is that dose/concentrations of single components are added after being multiplied by a scaling factor that accounts for differences in the potency of the individual toxins. The mixture dose/concentration (D_m) is the sum of the adjusted doses/concentrations (aD_i) of the individual components D_i:

$$D_m = \sum_{i=1}^{n} aD_i$$

In other words, the null hypothesis is that the effect of the mixture (in terms of mortality or another endpoint) can be predicted by the sum of the toxicities and their relative proportions in the mixture. This also means that toxicity can be expected if the summed dose/concentration is high enough to exceed the threshold of toxicity of the mixture, even when the concentration level of each individual toxin is below its own effect threshold (i.e. below its own no observed effect concentration) (SCHER, SCCS, SCENHIR, 2012 and references therein).

A method to assess interactions among *Bt* toxins based on the similar joint action model was developed and described in detail by Tabashnik (1992). This method is based on LC_{50}/LD_{50} values as an indicator of effect, but can make use of other median endpoints as well (e.g. growth inhibition parameters in Lee et al., 1996; Ibargutxi et al., 2008). The use of LC or EC values requires testing of several concentrations.

The expected (theoretical) toxicity of a mixture (m) of two toxins a and b, as indicated by an LC_{50} value, can be calculated as:

$$LC_{50(m)} = [r_a/LC_{50(a)} + r_b/LC_{50(b)}]^{-1}$$

in which r_a and r_b are the relative proportions of toxins a and b in the mixture.

For a three-component mixture this would be:

$$LC_{50(m)} = [r_a/LC_{50(a)} + r_b/LC_{50(b)} + r_c/LC_{50(c)}]^{-1}$$

Thus, the expected LC_{50} value of the mixture is the harmonic mean of the intrinsic LC_{50} values of the components weighted by their proportions in the mixture. In most studies, also a synergistic factor (SF) is calculated by dividing the expected toxicity $LC_{50(m)}$ by the observed (experimental) toxicity of the mixture in the bioassay.

As a statistical approach, 95% confidence intervals (CI) of the observed LC values can be used and compared to expected values. If the expected value is the same as the observed value, then the effect is simply additive and SF = 1. If the expected LC is greater than the upper limit of the 95% CI for the observed value, then there is synergism and SF > 1. If the expected LC is lower than the lower limit of the 95% CI for the observed value, then there is antagonism and SF < 1 (Poncet et al., 1995).

This approach was mainly followed by workers studying the toxicity of mixtures of different Cry proteins from 1992 on (Table S5 and S6), but also of Cry and Cyt and of Vip and Cyt protein mixtures (Wirth et al., 1997; Del Rincón-Castro et al., 1999; Sayyed et al., 2001; Pérez et al., 2005; Yu et al., 2012).

A slightly different approach to that proposed by Tabashnik (1992), but based on the same principle of concentration addition, was recently used by Höss et al. (2013). To test if the joint effect of the different toxins in a mixture can be regarded as concentration-additive, concentrations of the single proteins in the mixture can be transformed to LC_{50}- or EC_{50}-based toxic units (TU). The individual TUs for each toxin can be calculated by dividing its concentration in the mixture by its LC_{50} or EC_{50} value. This gives a dimensionless figure expressing the fraction of toxicity in the mixture that is expected based on its single toxicity. Summing up the TUs of all proteins in the mixture, results in the total toxicity of the mixture. In case of a mixture (m) of two toxins a and b, for which LC_{50} values have been determined, this will be:

$$TU_m = \frac{c_a}{LC_{50(a)}} + \frac{c_b}{LC_{50(b)}}$$

in which c_a and c_b are the concentrations of toxins a and b in the mixture.

Under the concentration addition model, there will be a 50% effect (mortality or inhibition) if the sum of TUs of the mixture equals 1. A higher TU, necessary to cause a 50% effect, would mean a less-than-additive effect or antagonistic effect; a lower TU, a more-than-additive effect or synergistic effect.

3.3.2. Overlapping activity spectra: Methods based on independent joint action model

Independent joint action (or response addition, simple dissimilar action, effects addition, Bliss independence) occurs if toxins act independently from each other, usually through different modes of action that do not interact physically, chemically or biologically (Finney, 1971; SCHER, SCCS, SCENHIR, 2012). Here, the effects of a mixture can be estimated directly from the probability of responses to the individual components (response addition) or the sum of biological responses (effects addition). Thus, the joint action of a mixture is explained from their effects on the test organisms, rather than from their concentrations, like in the concentration addition model described above.

The toxicity of a mixture in terms of probability of an individual being affected (survival or mortality, growth or growth arrestment) can be expressed as:

$P_m = 1 – (1-p_1).(1-p_2)…(1-p_n)$

with P_m being the response to the mixture and p_1, p_2, … p_n being the responses due to exposure to the individual components C_1, C_2, … C_n when present in a specified concentration.

The equation can also be written as:

$$E(C_m) = 1 - \prod_{i=1}^{n}(1 - E(C_i))$$

where $E(C_m)$ is the combined effect at the mixture concentration and $E(C_i)$ is the effect of the individual mixture component i applied at concentration C_i. Effects are expressed as fractions of a maximum possible effect, so 0% < E < 100% or 0 < E < 1 (Finney, 1971; SCHER, SCCS, SCENHIR, 2012).

Based on above theory, a practical formula used to determine expected mortality caused by a mixture of two toxins a and b is:

$E = O_a + O_b(1 - O_a)$

where E is the percentage of mortality expected, O_a is the observed percentage of mortality caused by toxin a and O_b is the observed percentage of mortality caused by toxin b. Observed and expected mortalities of the mixture can be analysed using an appropriate statistical test.

Alternatively, the null hypothesis of the independent joint action model can also be formulated so that the proportion of test individuals (e.g. exposed larvae) surviving exposure to a mixture of toxins is the product of the proportions of individuals that survive exposure to each of the toxins separately (Finney, 1971). For instance, with two toxins a and b:

$S_{(ab)EXP} = S_{(a)OBS} \times S_{(b)OBS}$

where $S_{(ab)EXP}$ is the proportion of the test population expected to survive exposure to a combinations of toxins a and b, $S_{(a)OBS}$ is the observed proportion of individuals surviving exposure to toxin a and $S_{(b)OBS}$ is the observed proportion of individuals surviving exposure to toxin b. Expected mortality for test individuals exposed to the combination of a and b can be calculated as $(1- S_{(ab)EXP})x100$ (%). Expected numbers of dead and live individuals can simply be calculated by multiplying the expected mortality and survival rates, respectively, by the sample size used when each toxin is tested separately. Observed survival data can be adjusted for mortality occurring in the negative control. A statistical test (e.g. Fisher's exact test) can be used to determine if a significant difference occurs between the observed and expected numbers of dead and live individuals. For instance, when more of the individuals exposed to the mixture die than expected, deviation of the null hypothesis is referred to as

"synergism". The same approach can be used to test for independent joint action among more than two toxins (Fernández-Luna et al., 2010; Tabashnik et al., 2013).

In the independent joint action model, the test organisms can be exposed to a single or to a few concentrations of each component of the mixture and the mixture itself. Thus, it does not require to perform more extensive bioassays with a series of concentrations needed to calculate LC values. However, if more concentrations are combined, deviations from the null hypothesis of response addition/independent action may be different depending on the concentration set tested, complicating interpretation of the laboratory findings in terms of consistency. Tabashnik et al. (2013) therefore proposed to pool the data across the different concentration sets for each combination of Cry proteins they tested.

The independent joint action model was chosen by workers studying interactions among *Bt* toxins and other insecticidal agents, like entomopathogenic viruses (McVay et al., 1977) or chemical insecticides (Salama et al., 1984), but it has also been used to assess synergism/antagonism between *Bt* spores and *Bt* toxins (Liu et al., 1998), between Cry and Cyt proteins (Fernández-Luna et al., 2010) and even among Cry proteins with overlapping activity spectra (Greenplate et al., 2003; Fernández-Luna et al., 2010; Tabashnik et al., 2013).

3.3.3. Empirical (no model) approaches for overlapping or non-overlapping activity spectra

A first used empirical approach to test for synergism/antagonism of mixtures of **two toxins with overlapping activity spectra** is simple in design. First, a range of concentrations is tested to determine the dose-response relationship for each toxin separately. Next, a mixture containing enough of the first toxin to kill 10 to 50% of the (target) insect population and a sublethal amount of the second toxin is tested. The same dose of the first toxin that is included in the mixture is then tested alone in a simultaneous experiment. Significantly greater mortality caused by the mixture than by the first toxin alone (e.g. as analysed by ANOVA) is evidence for synergism. A number of mainly earlier studies have used this simple approach to examine synergism among *Bt* toxins and between *Bt* toxins and protease inhibitors (Wu and Chang, 1985; Yu et al., 1987).

Raybould et al. (2010) assessed interactions among **two proteins considered to have non-overlapping spectra of activity**, the first being Lepidoptera-active (L-Cry), whereas the second is Coleoptera-active (C-Cry). In the first bioassay with a lepidopteran pest species L-Cry was considered to be the "toxin", whereas C-Cry was deemed the "non-toxin" (and vice versa for the second bioassay with a coleopteran pest species*)*. The hypothesis for the first bioassay was that in the absence of significant interactions, the expected effect of the L-Cry + C-Cry mixture on a lepidopteran pest species is identical to the effect of L-Cry alone; a similar hypothesis was set for the bioassay with the coleopteran pest species.
Each diet-incorporation bioassay in Raybould et al. (2010) comprised eight treatments, including three negative controls. In the mixture treatments, two concentrations of the

toxin were chosen intending to yield 30 and 70% mortality of the targeted/sensitive test organism, combined with a concentration of the non-toxin expected to kill 90% of the other test organism. Effects of protein mixtures on the test organisms versus those of component proteins alone and of the non-toxin proteins on the activity of the toxin proteins were tested using analysis of variance (ANOVA), with terms for group, toxin concentration, presence or absence of the non-toxin, and the interaction between the latter two factors. As the interactions between the factor toxin concentration and the presence/absence of the non-toxin were never significant, the effect tested was that of the non-toxin on the potency of the toxin averaged over the two toxin concentrations.

A similar simpler approach to test for interactions among Cry proteins with non-overlapping activity spectra was followed by Herman and Storer (2004). Neonate larvae of target pest species were exposed to artificial diets treated with either L-Cry alone, the binary toxin C-Cry alone, a combination of both, or a buffer control. Mortality and body weight (growth inhibition) were measured. A single concentration was chosen for each protein intended to produce a moderate level of growth inhibition. The data were subjected to statistical analysis: two-way ANOVA was used to test for significant interaction effects on larval growth inhibition between the binary toxin C-Cry and L-Cry.

Hunter (2006) performed interaction experiments with **three toxins with overlapping and non-overlapping spectra**: the Lepidoptera-active proteins L-Cry1 and L-Cry2, and the Coleoptera-active protein C-Cry. In these diet-incorporation bioassays, two sensitive lepidopteran species and a coleopteran species were exposed to the involved (pure) proteins alone or in combination; buffer and water controls were also included. Both lepidopteran species were exposed to a range of concentrations (six) of the expected toxins (L-Cry1 and L-Cry2), alone and in combination, spiked or not with a single concentration of the expected non-toxin (C-Cry); the C-Cry spike alone was also tested. Likewise, the coleopteran species was exposed to a range of concentrations of C-Cry alone, C-Cry combined with a spike of L-Cry1, a spike of L-Cry2, or a mixed spike of L-Cry1 + L-Cry2 (1:1), and also to the non-toxin spikes alone. In all cases, the concentration of the spikes was 30 µg/ml diet. This choice is not explained, but we assume that, like in Raybould et al. (2010), this concentration was selected as it was expected to produce high mortality in the organism susceptible to the spike (e.g. 30 µg of C-Cry per ml diet would kill 100% of coleopteran larvae). LC_{50} values and MIC_{50} (median molt inhibitory concentration) values were calculated based on observed mortality and growth arrestment (i.e., not developing beyond the first instar), respectively. The 95% confidence intervals were used to investigate interaction effects: overlap of 95% confidence intervals among treatments would indicate comparable activity, and thus in the case of the comparison of protein mixtures versus single proteins, a lack of a significant interaction effect. Fisher's exact tests were also used to compare responses between the spiked protein groups and their respective buffer control groups.

To assess the potential interaction within a quadruple stacked GM event, containing **six different Cry proteins with overlapping and non-overlapping spectra**, Levine et al. (2008) separately assessed the potential interactions among the Lepidoptera-active Cry proteins and concurrently the potential for interactions between the Lepidoptera- and the Coleoptera-active Cry proteins. To assess whether the activity of the combined Lepidoptera-active proteins is altered by the presence of the Coleoptera-active proteins, the biological activity of the stacked GM event (plant material) to a sensitive lepidopteran species was compared with the sub-stack only containing the Lepidoptera-active Cry proteins. The hypothesis of no interaction was tested by statistically comparing the observed GI_{50} values of the quadruple stack and the sub-stack.

3.4. Discussion

Two mathematical models, dose/concentration addition and independent action, have been proposed for assessing mixtures of toxins. In theory, model selection in a specific situation depends on (knowledge about) the toxins' mode of action. However, there is considerable confusion and discussion in the literature on the appropriateness of these models for analysing datasets of mixture toxicity. This is primarily related to the fact that the definitions of "mode of action" and "site of action" are not unequivocal (Cedergreen et al., 2008; Syberg et al., 2009). Indeed, it may be very difficult, and sometimes impossible, to determine the mode of action of a toxin, as it may depend, among other factors, on the concentration used and the organism exposed. As a result, in studies of mixture toxicity of *Bt* proteins predictive models are often chosen without knowledge on the exact mode of action of the toxins involved in the assessment. Interestingly, one study (Liao et al., 2002) used both models to predict interactions among two Cry proteins and found that these yielded similar predictions.

A review of studies in the peer-reviewed literature indicates that the method described by Tabashnik (1992), based on the dose/concentration addition model, is most widely used for studying interactions among *Bt* proteins. Syberg et al. (2009) concluded that there is substantial empirical evidence that this model provides reliable predictions both for chemicals with similar and dissimilar action and that it should therefore be preferred as a general reference model for the risk assessment of chemical mixtures. As a practical drawback, use of the dose/concentration addition model requires testing of multiple concentrations in order to calculate LC or EC values. In contrast, different concentrations are not necessarily tested in the independent action model: some studies using this model tested interactions among two or more *Bt* toxins at a single concentration only (e.g. Fernández-Luna et al*.,* 2010). The latter approach may, however, yield incomplete insight in the mixture toxicity, as Tabashnik et al. (2013) found that combinations of toxins tested at different concentrations yielded a different outcome in terms of deviation from independent action.

Clearly, the efficacy and interaction among different toxins produced by transgenic plants will depend on their relative concentrations expressed in the plants. From a perspective of risk assessment of GM plants combining several *Bt* proteins, it may thus be sufficient to test expected expression levels of the component proteins involved in the mixture and use the independent action to predict mixture toxicity. The consideration that for the risk assessment of GM plants ecologically relevant concentrations should be tested may also explain why in the context of applications for the cultivation of these plants, empirical approaches are mostly used, rather than the above mentioned predictive models. Obviously, whatever method is used to test interactions among *Bt* proteins in the laboratory, uncertainty will remain whether the responses of target organisms to combinations of toxins in plants would match the responses observed in diet bioassays (Tabashnik et al*.,* 2013).

4. Interactions among *Bt* proteins

While studying the effectiveness of the bio-insecticide *B. thuringiensis* subsp. *israelensis* against mosquitoes in the 1980s, it was discovered that the *Bt* proteins in the parasporal crystals enhance each other's activity. Subsequently, the contribution of *Bt* proteins to the activity of other insecticidal commercial formulations containing *B. thuringiensis*, i.e. subsp. *kurstaki* HD-1 or NRD-12 and subsp. *morrisoni* PG-14, was studied and also revealed interactive effects among *Bt* toxins. As a result of these findings, later on, several studies on interactions between *Bt* proteins have been conducted in order to find solutions to prevent or to overcome resistance development in insects or to broaden the activity spectrum of existing *B. thuringiensis* pesticides. Indeed, combining genes encoding for *Bt* proteins which differ in their mode of action, receptor binding and sequence homology might prevent or delay resistance development to a particular insect.

An interaction describes the combined effect of two or more proteins or chemicals. The previous chapter has discussed two null models which define the situation of "no interaction". If the action of the combination of the *Bt* proteins is greater than expected on the basis of that of the null model, this is called synergism; if the effect of the combined *Bt* proteins is less than expected, one speaks of antagonism. In case the activity of the combined *Bt* proteins is the sum of the single *Bt* proteins, the interaction is termed additive.

In this chapter, we summarise the current information on known interactions between *Bt* proteins based on *in vivo* laboratory bioassays (see 3.2) and on how these interactions between *Bt* toxins may affect their activity. All bioassays were considered regardless of the method used for analysis of interactions and the measurement endpoint(s) considered (see 3.3). Besides synergism between *Bt* proteins (as purified protein, as protein present in crystal inclusions or in spore/crystal powders), synergism has also been found between *Bt* spores (free of crystal proteins) and Cry proteins (e.g. Tang et al., 1996), and between *Bt* proteins and *Bt* spores (containing crystal proteins) or spore coat proteins (e.g., Johnson and McCaughey et al., 1996; Johnson et al., 1998). The latter two types of studies were not considered as they do not help much to get insight in *Bt* protein interactions.

Studies on *Bt* protein interactions are limited to studies with target pest species, except for *B. mori* and *C. tepperi*. Studies with species that were reared in the lab to become resistant to a *Bt* protein or commercial *Bt* formulation are not reported. Species that have gained resistance to a certain *Bt* toxin have changed characteristics, such as alterations in toxin activation or reduced affinity of the receptor for the toxin (Pardo-López et al., 2013), and may thus influence the level of synergism observed.

The results, in particular the type of interactions observed, are presented as described in the papers and no other interpretation was given to the data. At the time of compilation, 50 studies had been found, covering 24 different *Bt* proteins (Cry, Cyt and Vip proteins). Much

information on interactions between *Bt* proteins comes from work done on *Bt* strains affecting mosquito *Aedes*, *Anopheles* and *Culex* species being vectors of human diseases (see Chapter 4.1). In the context of plant protection, interactions have been studied between *Bt* proteins present in commercial formulations active against herbivores, and between *Bt* proteins combined in transgenic plants. The majority of studies have been conducted on lepidopteran insect pests, in particular *H. armigera* (Chapter 4.2), and a few studies on coleopteran pest species (Chapter 4.3) and nematodes (Chapter 4.4). Tables S5 and S6 provide a comprehensive overview of the studies done on dipteran and lepidopteran species.

4.1. Interactions among *Bt* proteins affecting dipteran activity

Interactions among Cyt and Cry proteins

Wu and Chang (1985) were the first to observe that when proteins from *B. thuringiensis* subsp. *israelensis* crystals were mixed, the activity of some combinations was greater than expected from the activity of the individual proteins. They found that the toxicity (mortality rate) of mixtures of the Cyt1Aa and 65-kDa (Cry10Aa or Cry11Aa) proteins or mixtures of Cyt1Aa and the 130-kDa proteins (Cry4Aa and Cry4Ba) proteins of *B. thuringiensis* subsp. *israelensis* fed to *A. aegypti* larvae was greater than expected on the basis of their individual toxicities. By comparing LC_{50} values, a synergistic relationship between the Cyt1Aa and 130-kDa (Cry4Aa and Cry4Ba) proteins was also observed by Chilcott and Ellar (1988), but in contrast to Wu and Chang (1985), they reported that Cyt1Aa did not interact synergistically with the 65-kDa protein against *A. aegypti* larvae. Reanalysis of the data of Chilcott and Ellar (1988) by Tabashnik (1992), however, reaffirmed synergism between the Cyt1Aa protein and the 65-kDa (Cry10Aa or Cry11Aa) protein and confirmed the synergism between the Cyt1Aa protein and 130-kDa (Cry4Aa and Cry4Ba) proteins against *A. aegypti*. By comparing the observed LC_{50} values with the expected LC_{50} values, it was shown that mixtures of the Cyt1Aa and 65-kDa proteins had a 4-fold increase in activity and there was a 10-fold positive synergism between the Cyt1Aa and 130-kDa proteins.

Wu et al. (1994) confirmed synergism between Cyt1Aa and Cry11Aa against *A. aegypti* larvae and found 4-5 times higher toxicity (LC_{50}) for the combination than for either of the proteins alone. By using purified inclusions containing Cyt1Aa and Cry11Aa proteins, Chang et al. (1993) demonstrated that this combination of proteins also has a synergistic effect to *C. quinquefasciatus.* Comparing LC_{50} and LC_{95} values, one and a half time to a two-fold increase in activity was observed compared to the activity of Cry11Aa. Using sporulated toxin powders, enhanced activity of Cyt1Aa-Cry11Aa (1:3 ratio) against *C. quinquefasciatus* (SF = 3) was confirmed by Wirth et al. (1997). A synergistic effect to *C. quinquefasciatus* was also observed for the Cyt1Aa-Cry4Aa-Cry4Ba (SF = 7.2) and Cyt1A-Cry4Aa-Cry4Ba-Cry11Aa (SF = 2.7) toxin powder combinations. Apart from *C. quinquefasciatus*, a synergistic effect (decreased LC_{50}) of Cyt1Aa and Cry11Aa was also demonstrated in *A. albimanus* by

Fernández-Luna et al. (2010). Crickmore et al. (1995) were the first to synthesise the Cry4Aa and Cry4Ba protein separately. They showed that Cyt1Aa synergised the toxicity (LC_{50}) of Cry4Aa and Cry4Ba 10- to 15-fold, respectively, and of Cry11Aa 2-fold over the expected value, and that a combination of the four proteins resulted in an approximately 6-fold enhancement in toxicity against *A. aegypti*. Also in *A. albimanus* a synergistic effect (decreased LC_{50}) of Cyt1Aa and Cry4Ba was observed (Fernández-Luna et al., 2010). Khasdan et al. (2001) showed that Cyt1Aa (with P20 that asks as a chaperone for expression in *Escherichia coli*) synergises the activity of Cry4Aa and Cry11Aa against *A. aegypti* larvae: an SF of 25.9 and 34, respectively, was obtained. A lower SF value (of 3) was displayed when Cyt1Aa was combined with both Cry4Aa and Cry11Aa. Further, Hernández-Soto et al. (2009) observed synergism between Cyt1A and Cry10Aa pure crystals against *A. aegypti* larvae (SF = 12.6). In conclusion, the Cyt1Aa protein, which has low mosquitocidal activity (Delécluse et al., 1991), is particular because it synergises the other four proteins Cry4Aa, Cry4Ba, Cry10Aa and Cry11Aa from *B. thuringiensis* subsp. *israelensis*. It was demonstrated that Cyt1Aa protein synergises Cry11Aa toxicity by functioning as a receptor molecule: membrane-bound Cyt1Aa enhances binding of Cry11Aa (Pérez et al., 2005).

Possible synergism between the toxins of *B. thuringiensis* subsp. *israelensis* Cry4Aa, Cry4Ba, Cry11A and Cyt1A in two-component mixtures against *C. tepperi* larvae was assessed by Hughes et al. (2005). In contrast to the studies with mosquitoes, mixtures of Cyt1Aa-Cry11Aa (SF = 0.5), Cyt1Aa-Cry4Ba (SF = 0.5) and Cry4Ba-Cry11Aa (SF = 0.7) were mildly antagonistic. Synergy was only detected in the Cyt1Aa-Cry4Aa (SF = 2.2) mixture.

Cyt1Aa from *B. thuringiensis* subsp. *israelensis* also synergises heterologous mosquitocidal toxins. A synergistic effect as observed for *B. thuringiensis* subsp. *israelensis* was demonstrated by Yu et al. (1987) for a Cyt1Aa and a Cry11Aa protein, with low toxicity to *A. aegypti*, isolated from the PG-14 isolate of *B. thuringiensis* subsp. *morrisoni* to *A. aegypti* larvae. Depending on the dosage, the addition of the Cyt1Aa protein resulted in a moderate enhancement (5.7-8.8 times at concentrations of 0.1 to 1 µg/ml) of the toxicity (LD_{50}) of Cry11Aa, and in a marked increase (140 times) when 10 µg/ml was added. Similarly, when the sublethal dose of Cry11Aa was increased from 0.64 ng/ml to 0.08 µg/ml in the assay system of the Cyt1Aa protein, the toxicity of Cyt1Aa was enhanced from 1.8 to more than 300 times. Wu et al. (1994) confirmed synergism between Cyt1Aa and Cry11Aa of *B. thuringiensis* subsp. *morrisoni* PG-14 against *A. aegypti* larvae and found as for *B. thuringiensis* subsp. *israelensis* 4-5 times higher toxicity (LC_{50}) for the combination than for the proteins alone.

However, Cyt1Aa did not synergise activity of Cry11Ba from *B. thuringiensis* subsp. *jegathesan* against *C. quinquefasciatus* to Cry11Ba that has high toxicity to *C. quinquefasciatus* (Wirth et al., 1998). An antagonistic factor of 0.8 was calculated for the LC_{50} value obtained at a ratio of 1:3 (Cyt1Aa:Cry11Ba) calculated at 24h. Later Wirth et al.

(2004) found a low synergistic effect using the same experimental conditions for the LC_{50} values (SF = 1.9), but not for the LC_{95} values (SF = 0.8).

Co-expression of Cyt2Aa from *B. thuringiensis* subsp. *darmstadiensis*, a *Bt* subspecies toxic to dipteran insects, and Cry4Ba from *B. thuringiensis* subsp. *israelensis* revealed synergism of the proteins against *A. aegypti* and *C. quinquefasciatus* larvae (Promdonkoy et al., 2005). The synergism factors were 33.4 for *A. aegypti* and 18.5 for *C. quinquefasciatus*.

Interactions among Cry proteins

Besides synergism between Cyt and Cry proteins, interactions have also been observed between all possible combinations of Cry4Aa, Cry4Ba and Cry11Aa of *B. thuringiensis* subsp. *israelensis*. Mixtures of Cry4Aa and Cry4Ba protein inclusions were 5-fold more toxic (LC_{50}) than Cry4Aa and 130-fold than Cry4Ba to *C. quinquefasciatus* (Angsuthanasombat et al., 1992). Synergism, but less, was also found for Cry4Aa and Cry4Ba when tested against *A. aegypti* (2- to 6-fold) and *A. gambiae* (2- to 3-fold) larvae. Delécluse et al. (1993) found that the activity of inclusions (LC_{50} and LC_{90}) containing both Cry4Aa and Cry4Ba was higher than that of the single-peptide inclusions to larvae of *A. aegypti*, *A. stephensi* and *C. pipiens*. Poncet et al. (1995) confirmed the results of Delécluse et al. (1993). The SFs based on comparing LC_{50} values for Cry4Aa and Cry4Ba for *A. aegypti, A. stephensi* and *C. pipiens* were 4.4, 2.1 and 14 respectively.

There was moderate, but significant synergy between Cry4Aa and Cry11Aa against the three mosquito species with factors ranging from 2.4 (*C. pipiens*) to 5.4 for *A. stephensi* (Poncet et al., 1995). While synergy was found between Cry4Aa and the Cry4Ba toxins and the Cry4Aa and the Cry11Aa toxins, a simple additive effect was observed between Cry4Ba and Cry11Aa against *A. aegypti* and *A. stephensi* (0.9 and 1.3, respectively) and a slight synergistic effect against *C. pipiens* (SF ≤ 2). The observed LC_{50} and LC_{90} values for the three-component mixtures were about threefold lower than the expected values, assuming a global synergism among the Cry4Aa, Cry4Ba and Cry11Aa toxins against the three mosquito species tested. The results of Poncet et al. (1995) on Cry4Ba and Cry11Aa are in contrast to those of Tabashnik (1992) who concluded that the Cry4Ba and Cry11Aa toxins are weakly antagonistic against *A. aegypti* larvae: about triple the LC_{50} expected in the absence of synergism, was measured. Crickmore et al. (1995), however, confirmed the results by Poncet et al. (1995), by reporting that the activity of pure Cry4Ba and Cry11Aa proteins is enhanced 2-fold and Cry4Aa-Cry4Ba-Cry11Aa activity 3-fold over the expected LC_{50} values of the mixture against *A. aegypti*.

The target spectrum of Cry48/49Aa binary toxin present in certain *L. sphaericus* strains is limited to *Culex* so far, and the binary toxin does not affect *Aedes* and *Anopheles* mosquitoes. Cry49Aa not only improves Cry48Aa activity, but also weakly synergises (1.7-fold) the related three-domain toxin Cry4Aa in its activity to *C. quinquefasciatus* when

administered in a 1:3 ratio at 200 µg/ml (Jones et al., 2008). However, synergism was not evident at other combined concentrations of 20 or 2 µg/ml.

The Cry29A and the Cry30A protein from *B. thuringiensis* subsp. *medellin*, are nontoxic to *A. aegypti*, *C. pipiens* and *A. stephensi* alone or combined (Juárez-Pérez et al., 2003). Cry29A synergised the toxicity of Cry11Bb against *A. aegypti* by a 4-fold factor, but not against *C. pipiens* (SF = 1.2) and *A. stephensi* (SF = 0.6). The Cry30A protein did not affect the activity of Cry11Bb against any of the three mosquitoes species and the combination of Cry29A-Cry30A-Cry1Bb only showed a synergistic effect against *A. aegypti*.

Interactions among Cyt and Vip proteins

The Cyt2Aa3 activity from *B. thuringiensis* subsp. *sichuansis* is affected by Vip3Aa29, a protein with activity against lepidopteran species. The Cyt2Aa3 protein exhibits toxicity against the Diptera *C. quinquefasciatus* and *C. tepperi*. The combination of Vip3Aa29 and Cyt2Aa3 exerted an additive effect to *C. tepperi* and some antagonistic effect to *C. quinquefasciatus* (Yu et al., 2012).

4.2. Interactions among *Bt* proteins affecting lepidopteran activity

Interactions among Cry proteins

The Cry1Aa, Cry1Ab and Cry1Ac proteins produced by *B. thuringiensis* subsp. *kurstaki* HD-1 were tested for their interactions with each other to determine the contribution of each Cry1A protein to the *in vivo* toxicity of HD-1 crystal against lepidopteran species. By comparing the toxicity (50% frass failure dose) of the individual Cry1A proteins with that of HD-1 crystals (containing Cry1Aa, Cry1Ab and Cry1Ac, but also Cry2Aa and Cry2Ab), van Frankenhuyzen et al. (1991) observed that the toxicity of HD-1 crystals was similar to the expected toxicity of the combined Cry1A proteins towards the lepidopteran larvae of *Choristoneura occidentalis, C. fumiferana* and *Malacosoma disstria*. HD-1 crystals were, however, shown to be 2-fold more toxic to *Choristoneura pinus* and *Orygia leucostigma* and greatly more toxic to *Lymantria dispar* (28-fold) compared to Cry1Ac. Synergistic effects of Cry1A toxins were suggested. Tabashnik (1992) re-evaluated the data of van Frankenhuyzen et al. (1991) using a different method to calculate the expected toxicity and revealed no synergism of the Cry1A proteins against *O. leucostigma* and *L. dispar.*

In additional bio-assays with *L. dispar* comparing 50% growth inhibition dosages (ID_{50}) of purified Cry1A proteins, synergism was observed with a mixture of Cry1Aa and Cry1Ac (3 to 7 times the expected toxicity depending on the protein ratio used), a weak synergistic or additive effect with Cry1Ab and Cry1Ac (SF = 1.5), and an antagonistic effect (one-third of the expected toxicity) with a mixture Cry1Aa and Cry1Ab (Lee et al., 1996). These results may explain the no positive synergism among the three Cry1A proteins by Tabashnik (1992): the synergistic effect of Cry1Aa-Cry1Ac might be masked by the antagonistic effect of

Cry1Aa-Cry1Ab. The observation that Cry1Aa and Cry1Ac bind to different membrane vesicles in *L. dispar*, while Cry1Aa and Cry1Ab to the same membrane vesicle, may explain different type of interactions between these Cry proteins. With the Cry1Ab-Cry1Ac mixture less synergistic effect was expected than with the Cry1Aa-Cry1Ac mixture, since the pore-forming activity of Cry1Ab was not that high as that of Cry1Aa (Lee et al., 1996). In assays with *B. mori,* Lee et al. (1996) did not observe synergistic effects with any of the three Cry1A toxin combinations tested against *L. dispar* (data not reported). This lack of synergism was postulated to be due to the presence of a high-affinity binding site for the Cry1Aa protein and the lack of such a binding site for Cry1Ac.

Chakrabarti et al. (1998) did not find significant alternation in toxicity when the combinations of Cry1Ab and Cry1Ac were used against *H. armigera* compared to the individual proteins. Sharma et al. (2010) showed that combining Cry1Ac with Cry1Ab led to an increase in toxicity towards the spotted maize stem borer, *Chilo partellus*. Depending on the ratio of Cry1A proteins used, a synergistic factor of 2 to 5 was obtained. The increase in toxicity was shown to come from an increase in toxin binding to the midgut. Toxin mixtures (1:1) of Cry1Aa with Cry1Ab or Cry1Ac showed an additive or slightly synergistic effect (SF of 1.2-1.5).

In the research to prevent or delay resistance development to lepidopteran cotton pest species, in particular to *H. armigera* (cotton bollworm), Cry proteins known to have at least some toxicity to this species were used in combination. No significant alternation in the toxicity (ID_{50}) towards *H. armigera* larvae was observed by Chakrabarti et al. (1998) when the combinations of Cry1Ac and Cry2Aa were used. Bioassays conducted with Cry1Ac, isolated from *B. thuringiensis* subsp. *kurstaki* HD73, and Cry2Aa, isolated from *B. thuringiensis* subsp. *kurstaki* HD-1, however, showed a small but significant antagonistic interaction based on LC_{50} values (SF = 0.3 to 0.7) between these *Bt* proteins against *H. armigera* (Liao et al., 2002). Further, the body weight reduction caused by Cry1Ac and Cry2Aa was less than for Cry1Ac alone but greater than for Cry2Aa alone at the ratios tested at 16 ng/cm^2. Against *Earias vitella* larvae, Yunus et al. (2011) reported synergism for Cry1Ac and Cry2Aa (isolated from *B. thuringiensis* HD73 and *Bt* strain MR 1.7, respectively) in the Cry ratios tested. The toxicity (LC_{50}) of the mixture was to 1.6- to 125-fold higher, the degree of increase depending on the ratio of Cry proteins tested and whether the Cry1Ac or Cry2Aa was used for comparison.

The Cry1Ac and Cry2Ab proteins, both derived from *B. thuringiensis* subsp. *kurstaki* interacted synergistically in the mixtures tested against *H. armigera* (Ibargutxi et al., 2008). The synergism factor increased with increasing relative proportions of Cry2Ab. However, an additive effect of Cry1Ac and Cry2Ab2 to *H. armigera* was found by Brévault et al. (2009) via comparing a.o. the expected LC_{50} and IC_{50} (growth inhibition concentration) with the observed ones. No explanation for the differing results with those of Ibargutxi et al. (2008) was provided. Against *Erias insulana*, Cry1Ac and Cry2Ab had an additive effect in all

proportions tested (Ibargutxi et al., 2008), and an additive interactive effect was also found for Cry1Ac and Cry2Ab (both present in MON15985) against *H. zea*, *H. virescens* and *S. frugiperda* larvae by calculating the expected population response (Greenplate et al., 2003).

Chakrabarti et al. (1998) reported on the synergism between Cry1Ac and Cry1Fa, the latter derived from *B. thuringiensis* subsp. *aizawai,* against *H. armigera*. Depending on the ratio of the Cry proteins, the observed toxicity (ID_{50}) of the mixture was 12- to 26-fold times higher than the expected toxicity. As an explanation for the observed synergism, it was suggested that the formation of a hetero-oligomer may have better insertion ability than a homo-oligomer complex, or that the binding of Cry1Ac and Cry1Fa to different receptors increases toxicity. However, according to Ibargutxi et al. (2008) combined Cry1Ac and Cry1Fa showed an additive interaction in all proportions analysed for *H. armigera*, as determined by mean LC_{50} values and larval growth inhibition studies. These differences in results by Chakrabarti et al. (1998) may be explained by the use of different types of Cry1Fa toxins or to differences in the *Bt* strains used in the production of Cry1Fa. Also against *E. insulana* (spotted bollworm), Ibargutxi et al. (2008) found an additive interaction in all proportions analysed for the Cry1Ac and Cry1Fa combination. Adamczyk and Gore (2004) found that when *S. exigua* and *S. frugiperda* were fed cotton leaves containing Cry1Ac and Cry1Fa (281-24-236 x 3006-210-23) this did not significantly reduce larval survival compared to *Spodoptera* species fed Cry1Fa leaf material.

A study of Xue et al. (2005) revealed that the Cry1Ca toxin could enhance the toxicity of Cry1Aa against *H. armigera* and that Cry1Aa toxin could enhance the toxicity of Cry1Ca against *S. exigua*. A ratio of 1:1 of Cry1Aa and Cry1Ca was most toxic against both species: the synergistic effects of Cry1Aa and Cry1Ca toxins were more evident in *S. exigua* (SF = 4) than in *H. armigera* (SF = 2.7).

Cry1Ac, from *B. thuringiensis* subsp. *kurstaki* HD-73, is toxic to *H. armigera* (LC_{50} = 1.6 x 10^{-8} cells/ml) and has a relatively high level of toxicity against *Pectinophora gossypiella* (LC_{50} = 0.27 x 10^{-8} cells/ml), but not against *S. littoralis*. In contrast Cry1Ca, from *B. thuringiensis* subsp. *aizawai* 4J4, displays some toxicity against *S. littoralis* (LC_{50} = 9.8 x 10^{-8} cells/ml), but not against *P. gossypiella* and *H. armigera*. By combining Cry1Ac and Cry1Ca, no toxicity to *H.armigera*, a similar level of toxicity against *P. gossypiella* as that of Cry1Ac (LC_{50} = 0.4 x 10^{-8} cells/ml), and more toxicity against *S. littoralis* compared to Cry1Ca (LC_{50} = 0.12 x 10^{-8} cells/ml) was found (Khasdan et al., 2007). In the context of the evaluation of interactions between Cry proteins combined in GM maize crops several *in vivo* bioassays with sensitive insects, in particular with *O. nubilalis*, have been carried out. Binning (2009) determined that maize leaf tissue containing Cry1Ab and Cry1Fa (MON810 x TC1507) does not have a synergistic or antagonistic effect on larval mortality and weight of *O. nubilalis*, *S. frugiperda*, *H. zea* and *Diatraea grandiosella*. Levine et al. (2008) found no interaction effects between Cry1Fa (TC1507), Cry1A.105 and Cry2Ab2 (the latter two are both present in MON89034) on

O. nubilalis, by demonstrating that the observed toxicity (ID_{50}) of MON89034 x TC1507 x NK603 maize leaf tissue did not differ significantly from the predicted ID_{50}.

Besides interactions between Lepidoptera-active proteins also potential interactions between Lepidoptera-active and Coleoptera-active proteins have been studied. Herman and Storer (2004) did not demonstrate a potential interaction between Cry1Fa (TC1507) and the Coleoptera-active Cry34/35Ab1 (59122) on *O. nubilalis*. Hunter (2006) showed that the activity (LC_{50} and MIC_{50} - Molt Inhibitory Concentration) of Cry1A.105 and Cry2Ab2, both present in MON89034, was not altered by the presence of Cry3Bb1 against *O. nubilalis*. *Vice versa*, it was shown that the activity of Cry3Bb1 was not altered by addition of Cry1A.105 and Cry2Ab2 against *L. decemlineata*. Neither the activity (growth inhibition) of Cry1A.105, Cry2Ab2 and Cry1Fa against *O. nubilalis* was affected by Cry3Bb1 and Cry34/35Ab1 (Levine et al., 2008). Further, no effect of Cry3A, which is toxic to certain species in the coleopteran family of Chrysomelidae, was detected on the activity (% mortality) of Cry1Ab on *O. nubilalis* larvae (Raybould et al., 2010).

Further, studies have been done to pest insect species of other crops and trees, including rice, bean, coffee and spruce. To evaluate interactions among *Bt* protoxins in rice stem borer larvae of *Sesamia inferens* and *C. suppressalis*, toxicity assays were performed with mixtures of Cry1Aa-Cry1Ab, Cry1Aa-Cry1Ca, Cry1Ab-Cry1Ac, Cry1Ab-Cry1Ba, Cry1Ab-Cry1Ca Cry1Ac-Cry1Ca, and Cry1Ac-Cry1Ba at 1:1 (w/w) ratios (Gao et al., 2010). All protoxin mixtures demonstrated significant synergistic toxicity activity against *C. suppressalis*, with values of 1.6- to 11-fold higher toxicity than the theoretical additive effect. In contrast, all but one of the *Bt* protoxin mixtures were antagonistic in toxicity to *S. inferens*.
In order to control the bean shoot borer, *Epinotia aporema*, the toxicity of six different Cry protoxins (Cry1Aa, Cry1Ab, Cry1Ac, Cry1Ba, Cry1Ca and Cry1Da) were tested in combination (Sauka et al., 2007). The SF calculated for different Cry1A protoxin mixtures Cry1Aa-Cry1Ab, Cry1Aa-Cry1Ac, Cry1Ab-Cry1Ac, Cry1Aa-Cry1Ab-Cry1Ac and Cry1Ca-Cry1Da was smaller than 1, suggesting an antagonistic effect. The Cry1Ba-Cry1Ca and Cry1Ba-Cry1Ca-Cry1Da mixtures showed a SF close to 1, indicating an additive effect. The Cry1Ba-Cry1Da mixture had a SF bigger than 1, suggesting a possible synergism between these toxins.

Cry1Ac and Cry1Ba were shown to act additive when delivered to the coffee leaf miner *Perileucoptera coffeella* larvae (Guerreiro-Filho et al., 1998). The observed LC_{50} resembled the expected LC_{50} of the mixture.

Frass failure bioassays with mixtures of an altered variant of Cry9Ca, from *B. thuringiensis* subsp. *tolworthi*, and one of the two Cry1A toxins produced by HD-1 (Cry1Aa and Cry1Ab) showed neither synergism nor antagonism against the spruce budworm, *C. fumiferana* at the ratios tested (Pang et al., 2002).

The crystals of *B. thuringiensis* subsp. *kurstaki* strain BNS3 are composed of three kinds of Cry proteins: Cry1Aa, Cry1Ac and Cry2Aa. Testing three different ratios, the activity (LC_{50}) between Cry1Ac and Cry2Aa was shown to be antagonistic against *Ephestia kuehniella* larvae (SF = 0.21-0.56), a pest in stored food products. Cry1Aa that is not individually active, acted synergistically with Cry1Ac and Cry2Aa (Tounsi et al., 2005).

Another approach to counter resistance is to engineer *Bt* toxins to make them more effective against pests that are resistant to the previously deployed toxins. Combinations of a modified Cry1Ab (Cry1AbMod lacking 56 amino acids at the N-terminus) with the native Cry1Ac and Cry2Ab have been tested. The net results (observed versus expected % mortality) across all combinations tested, showed significant synergism between Cry1AbMod and Cry2Ab against susceptible *P. gossypiella* (mean increase of 12% mortality), whereas the other combinations of toxins (Cry1AMod + Cry1Ac, Cry2Ab + Cry1Ac or Cry1AbMod + Cry1Ac + Cry2Ab) did not show consistent synergism or antagonism (Tabashnik et al., 2013).

Interactions among Cyt and Cry proteins

Cyt1Aa, originally known to be toxic only to mosquitoes and related dipterans (see 2.2.4.3), has been shown to also interact with Cry proteins affecting insects outside the order of the Diptera (i.e. Lepidoptera). Cyt1Aa was shown to antagonise activity of Cry1Ac from *B. thuringiensis* subsp. *kurstaki* towards the cabbage looper, *T. ni* (Del Rincón-Castro et al., 1999). Cyt1Aa exhibited no toxicity to *T. ni*, but increased the LC_{50} of Cry1Ac 6.5 to 8-fold over the expected value when 0.1 and 1 µg of Cyt1Aa was mixed to the diet, respectively. The antagonistic effect was also evident by joint action analysis (SF = 0.3 to 0.6). Similarly, Meyer et al. (2001) reported that Cyt1Aa lowered mortality of another lepidopteran pest species: the diamondback moth, *P. xylostella*, but only slightly (in 8 out of 12 trials), when the Cyt1Aa protein was added to HD-1 powder or Dipel, a formulated version of the HD-1 strain of *B. thuringiensis* subsp. *kurstaki*, which contains Cry1Aa, Cry1Ab, Cry1Ac, Cry2Aa, spores, and other materials from the HD-1 strain. In contrast, purified Cyt1Aa and Cry1Ac crystals (1:1) showed a synergistic interaction (SF = 2) against a Malaysian *P. xylostella* population (Sayyed et al., 2001) and an insecticide-susceptible population (SF = 11). The discrepancy in observed results between Meyer et al. (2001) and Sayyed et al. (2001) was believed to be mainly due to the variation in the susceptibilities of different populations of *P. xylostella* to the *Bt* toxins. Further, Cyt1Aa showed to have no effect on mortality of *P. gossypiella* caused by MVPII, a liquid formulation containing Cry1Ac (Meyer et al., 2001).

Cyt1Aa with Cry1Ac (and P20, acting as a chaperone for expression in *E. coli*) had a comparable level of toxicity than Cry1Ac alone against *H. armigera* and *P. gossypiella*. A clone expressing *cry1Ca* in addition to *cry1Ac, p20 and cyt1Aa* exhibited a 16-fold enhanced toxicity to *H. armigera* (LC_{50} of 0.16×10^{-8} cells/ml compared to LC_{50} of 2.51×10^{-8} cells/ml), suggesting a synergistic interaction between Cry1Ca and Cyt1Aa. Cyt1Aa failed to raise the

toxicity of these Cry1Ac and Cry1Ca toxins against *P. gossypiella* and *S. littoralis*, but significantly enhanced toxicity against *H. armigera* (Khasdan et al., 2007).

Interactions among Cyt or Cry and Vip proteins

In the development of improving the toxicity of the *B. thuringiensis* subsp. *kurstaki* strain YBT1520, including *cry1Aa*, *cry1Ab*, *cry1Ac* and *cry2Aa,* to more lepidopteran pest species, Vip3Aa7 has been reported to enhance the toxicity (LC_{50}) of the *B. thuringiensis* strain against *S. exigua* up to 10-fold, but not the toxicity to *H. armigera* (Zhu et al., 2006). A similar toxicity against *H. armigera* was retained compared to YBT1520.

The Vip3Aa29 protein with high activity against lepidopteran insects synergises with Cyt2Aa3, from *B. thuringiensis* subsp. *sichuansis* strain MC28. The Cyt2Aa3 protein exhibits toxicity against Diptera, but not against Lepidoptera. Co-expression of Vip3Aa29 and Cyt2Aa3 increased the effect on the Lepidoptera *C. suppressalis* (SF = 3.3) and *S. exigua* (SF = 4.3), respectively, and an additive effect was found against *H. armigera* (Yu et al., 2012).

Cry1Ia10 isolated from *B. thuringiensis* subsp. *thuringiensis* T01-328 and Vip3Aa isolated from *B. thuringiensis* subsp. *kurstaki* HD-1 were tested in combination (Bergamasco et al., 2013). A synergistic effect of Vip3Aa and Cry1Ia10 in *Spodoptera frugiperda*, *Spodoptera albula*, *Spodoptera cosmioides* larvae was observed when they were combined. However, in *Spodoptera eridania* an antagonistic effect was observed. A competition of the two *Bt* proteins for the same receptor in the latter *Spodoptera* species was proposed as explanation for the observed antagonism.

4.3. Interactions among *Bt* proteins affecting coleopteran activity

The Cry34Ab1 protein, active against *Diabrotica undecimpunctata howardi* (Southern corn rootworm), is synergised by Cry35Ab1, which alone does not show activity to *D. undecimpunctata*. At a 9/1 mass ratio of Cry34Ab1 to Cry35Ab1 protein, potency (based on growth inhibition) was increased over 8-fold compared with that observed with Cry34Ab1 alone (Herman et al., 2002).

Herman and Storer (2004) did not observe a potential interaction between Cry34/35Ab (59122) and the Lepidoptera-active Cry1Fa (TC1507) on *D. undecimpunctata howardi* (southern corn rootworm). Hunter (2006) showed that the activity of Cry3Bb, both present in MON89034, was not altered by the presence of Cry1A.105 and Cry2Ab against *L. decemlineata.*

An effect (increased mortality) of Cry1Ab, which is toxic to certain Lepidoptera, on Cry3A activity on *L. decemlineata* was found at 72 h, but disappeared at 96 h (Raybould et al., 2010). These findings indicate that Cry1Ab may be associated with a more rapid effect of

Cry3A on *L. decemlineata*, but not with a change in the proportion of *L. decemlineata* surviving to the end of the experiment.

4.4. Interactions among *Bt* proteins affecting nematode activity

Some *B. thuringiensis* Cry proteins are highly toxic to nematodes. In order to select the best toxin combination for management of *M. incognita*, combinations of Cry6Aa, Cry5Ba and Cry55Aa were tested (Peng et al., 2011). The results showed that a combination of Cry6Aa and Cry55Aa showed significant synergistic toxicity against *M. incognita* juveniles (up to 5-fold for a 1:1 ratio), as well as the Cry6Aa and Cry5Ba combination (SF = 2). However, the Cry5Ba-Cry55Aa and Cry6Aa-Cry5Ba-Cry55Aa toxin mixtures showed no significant synergistic effect in toxicity towards *M. incognita*.

Höss et al. (2013) showed that a combination of Cry1A.105, Cry2Ab2 and Cry3Bb1 proteins, having all three sublethal activity to *C. elegans*, showed a decreased (2 to 4-fold) sublethal toxicity (inhibitory effect on reproduction) then when administered separately.

4.5. Discussion

In the last 30 years, interactions between *Bt* proteins present in parasporal inclusions of *B. thuringiensis* subsp. have been studied, as well as interactions between heterologous *Bt* proteins. The phenomenon of interactions has been reported most extensively for *B. thuringiensis* subsp. *israelensis* and subsp. *kurstaki* parasporal inclusions. *B. thuringiensis* subsp. *israelensis* produces four Cry toxins (Cry4Aa, Cry4Ba, Cry10Aa, and Cry11Aa) and two Cyt toxins (Cyt1Aa and Cyt2Ba). The mosquitocidal activity of a *B. thuringiensis* subsp. *israelensis* against *Culex*, *Aedes* and *Anopheles* species is not just an additive effect of each toxin, but a complex synergistic interaction among them. The Cry4Aa, Cry4Ba, Cry10Aa and Cry11Aa interact mutually, enhancing each other activity compared to the single proteins, and Cyt1Aa enhances the activity of the four Cry toxins Cry4Aa, Cry4Ba, Cry10Aa and Cry11Aa. Cyt1Aa of *B. thuringiensis* subsp. *israelensis* also synergises three-domain Diptera-active Cry proteins from other *B. thuringiensis* subsp. with low toxicity to a certain mosquito species. The implication of these results is that toxins that are highly toxic to a certain mosquito species or have high binding affinity, gain little of no value from assisted binding by Cyt1A. So far, no studies with Cyt2Ba from *B. thuringiensis* subsp. *israelensis* have been conducted. *B. thuringiensis* subsp. *kurstaki* produces four Cry toxins (Cry1Aa, Cry1Ab, Cry1Ac and Cry2Aa) and between the three Cry1A toxins, and Cry1Ac and Cry2Aa different types of interactions have been reported.

The interactions among the *Bt* proteins originating from one particular *Bt* species that are supported by reasonable evidence are so far limited to Cry/Cry and Cyt/Cry interactions in mosquitoes mentioned in the former paragraph. A clear synergistic effect has been

observed. Although quite some work has been done on interactions between the Cry proteins of *B*. *thuringiensis* subsp. *kurstaki* against Lepidoptera, contradicting results make it hard to draw conclusions. A particular observation for studies among Cry proteins affecting Lepidoptera-activity is that Cry1A toxin combinations can be synergistic for one insect species, but be neutral or antagonistic for another. For example, for the Cry1Ab-Cry1Ac combination an antagonistic interaction was found against *L. dispar* (Lee et al., 1996), no effect against *H. armigera* (Chakrabarti et al., 1998) and *B. mori* (Lee et al., 1996) and a synergistic effect against *C. partellus* (Sharma et al., 2010). That the type of interaction is insect-specific, seems to be a common phenomenon for interactions among *Bt* protein sharing a same primary order specificity, independent of the *Bt* (Cry, Cyt or Vip) proteins involved.

Interactions between heterologous *Bt* proteins have been studied in the context of resistance management or activity spectrum enlargement of *B. thuringiensis* pesticides. Particularly for the cotton pest *H. armigera*, different combinations of two Cry proteins known to act against lepidopteran pests have been tested. Noteworthy is that different type of interactions have been observed in *H. armigera* for the same Cry protein combination tested by different research groups. For example, for the Cry1Ac-Cry1F combination both a synergistic and an additive effect were observed (Chakrabarti et al., 1998; Ibargutxi et al., 2008). Differences could be attributed to the type of Cry protein used, the strain used for production of the Cry protein or the variation in the susceptibilities of different insect populations.

Furthermore, it was discovered that Cyt proteins, mostly found in *Bt* strains active against Diptera, do not only affect dipteracidal Cry proteins, but may also affect *Bt* proteins active against insects outside the order of Diptera. For example, Cyt1Aa synergises Cry4Aa and Cry3A activity against *C. quinquefasciatus* and the coleopteran *C. scripta*, respectively.

Moreover and on a different level, some combinations of Cry proteins can be affected in their toxicity properties depending on the concentration ratio in which they are combined. For example, for the combination of Cry1Aa and Cry1Ca highest toxicities against *S. exigua* and *H. armigera* were observed at a ratio of 1:1 (Xue et al., 2005). Results of such studies may also depend on the means by which the synergistic effect is calculated (see 3.3) and the method of production of the *Bt* proteins and their solubility. For instance, in some cases, toxins can have different toxicity when produced in *E. coli* (van Frankenhuyzen et al., 1991; Khasdan et al., 2007). Therefore, it remains difficult to extrapolate the results of studies not done in a risk/safety assessment context to what might occur in GM plants in agricultural fields. In a risk/safety assessment context, only studies for which the equivalence in biological activity of the *Bt* protein produced in the microbe to the one produced in the GM plant is proven, would be accepted.

The mechanisms behind the interactions among *Bt* proteins is still not clearly understood and different hypotheses have been proposed to explain the molecular mechanisms. For the

occurrence of synergism, several hypotheses have been formulated. Summarising those hypotheses, a first hypothesis is that there is a correlation between synergism and increase in toxin binding, i.e. the formation of a hetero-oligomer may have better insertion ability than a homo-oligomer complex (Chakrabarti et al., 1998), or the use of a partner synergising protein, such as Cyt1A, may stimulate binding (Pérez et al., 2005; Sharma et al., 2010). The presence of a toxin mix might thus enhance toxicity by preventing non-productive binding. Another possibility is that the *Bt* proteins do not share the same high affinity binding sites: the individual pores made by different toxins may act cooperatively and show higher toxicity than the individual pores (Chakrabarti et al., 1998; Lee et al., 1996; Xue et al., 2005). In other words, in this case, competition for the same binding site might explain the absence of any potential synergistic interaction. Based on binding assays among *Bt* proteins, a common binding site in the midgut of lepidopteran species has been proposed for several Cry proteins (Hernández and Ferré, 2005; Hernández-Rodríguez et al., 2008; 2013). However, the presence or absence of such a common binding site *per se* does not necessarily allow to predict the type of interaction to be expected. For example, the common binding site for the toxins belonging to the Cry2A family (o.a. Cry2Aa and Cry2Ab) which is not shared by Cry1Ac (Hernández-Rodríguez et al., 2008), does not explain the no effect or small antagonistic interaction observed against *H. armigera* by Cry1Ac and Cry2Aa (Chakrabarti et al., 1998; Liao et al*.,* 2002).

5. Assessing impacts of multiple *Bt* proteins on non-target organisms

5.1. *Bt* proteins in authorised GM plants

Presently the most important worldwide approved *Bt* crops are maize, cotton and potato, but also for soybean, eggplant and tomato, few events expressing a *Bt* gene have been authorised (ISAAA GM Approval Database; http://www.isaaa.org/gmapprovaldatabase/default.asp and Table 5). The introduced proteins (or modifications thereof) originating from *B. thuringiensis* species are Cry1Ab, Cry1Ac, Cry1Fa, Cry2Ab, Cry2Ae, Cry3Aa, Cry3Bb, Cry34/35Ab, Cry9Ca and Vip3Aa, their use varying among crop and envisaged pest control. *Bt* corn producing Cry1 (Cry1A.105, Cry1Ab, Cry1Fa, or Cry1Ac) or Cry2Ab targets lepidopteran pests like *O. nubilalis* (European corn borer); *Bt* maize producing Cry3 (Cry3Aa, Cry3Bb, Cry3.1Ab or the binary Cry34/35Ab proteins) targets coleopteran pests (*Diabrotica* spp.). The Cry9Ca protein and more recently Vip3Aa have been used as alternatives to control lepidopteran pests in maize. The majority of *Bt* cotton expresses a *cry1* gene (*cry1Ab*, *cry1Ac*, *cry1Ab-Ac, cry1Ca* or *cry1Fa*) for the control of lepidopteran pests including *H. armigera* (cotton bollworm) and *P. gossypiella* (pink bollworm). One cotton event produces Vip3Aa (COT102), one event Cry2Ab (MON15985) and another one Cry2Ae (GHB119) to control lepidopteran pests. In all *Bt* potato Cry3Aa is introduced to manage coleopteran insects, in particular *L. decemlineata* (Colorado potato beetle).

First generation *Bt* crops generally produce one *Bt* toxin. Second generation *Bt* crops produce multiple Cry toxins acting against species of the same order, thereby reducing the possible development of insect resistance and/or to control a wider spectrum of pests. A second generation *Bt* cotton, Bollgard II (MON15985), produces Cry2Ab besides Cry1Ac and has definitely replaced the first generation Bollgard I cotton (MON531) when resistance in *P. gossypiella* was detected. The *Bt* corn MON89034, expressing the *cry2Ab* gene besides the *cry1A.105* gene, provides improved protection against *H. zea* (corn earworm) compared to MON810, solely expressing *cry1Ab* (Monsanto, 2009). Further, a trend observed during recent years is to cross existing *Bt* crops by conventional breeding to obtain crops with multiple Cry toxins controlling both coleopteran and lepidopteran pest insects. For example *Bt* maize MON89034 x TC1507 x MON88017 x 59122 expresses a series of toxins including the Cry34Ab/Cry35Ab binary toxin and Cry3Bb to control coleopteran pests, and also Cry1A.105, Cry2Ab and Cry1Fa for the control of lepidopteran pests. Gene stacking in crops will probably continue by combining existing events, with the introduction of novel *Bt* genes identified in novel *B. thuringiensis* isolates or by introducing novel *cry* genes engineered to improve insecticidal activities against important pest insects.

Table 5. *Bt* proteins present in authorised single GM crop events worldwide (according to information available in ISAAA GM Approval Database)

	Cry1Ab	Cry1Ac	Cry1Ab-Ac	Cry1A.105	Cry1Ca	Cry1Fa	Cry2Ab	Cry2Ae	Cry3Aa	Cry3.1Ab	Cry3Bb	Cry34/35Ab	Cry9Ca	Vip3Aa
maize														
4114						X						X		
5307										X				
59122												X		
Bt10, Bt11, Bt176	X													
CBH-351													X	
DAS-06275-8						X								
DBT418		X												
MIR162														X
MIR604									X					
MON801, MON802, MON809, MON810	X													
MON863, MON88017											X			
MON89034				X			X							
TC1507, TC6275						X								
cotton														
281-24-236						X								
3006-210-23		X												
31707, 31803, 31807, 31808, 42317		X												
BNLA-61		X												
COT102														X
COT67B	X													
Event1		X												
GHB119								X						
GFM-Cry1A			X											
GK12			X											
MLS9124					X									
MON531, MON757, MON1076		X												
MON15985		X					X							
T303-3, T304-40	X													
potato														
1210 amk									X					
2904/1 kqs									X					
BT06, BT12, BT10, BT16, BT17, BT18, BT23									X					
HLMT15-15, HLMT15-3, HLMT15-46														
ATBT04-27, ATBT04-30, ATBT04-31, ATBT04-36, ATBT04-6,									X					
RBMT15-101, RBMT21-129, RMBT21-350, RBMT21-350, RBMT22-82, RMBT22-186, RMBT22-238, RMBT22-262									X					
SEMT15-02, SEMT15-07, SEMT15-15									X					
SPBT02-5, SPBT02-7									X					

Table 5 (*continued*).

	Cry1Ab	Cry1Ac	Cry1Ab-Ac	Cry1A.105	Cry1Ca	Cry1Fa	Cry2Ab	Cry2Ae	Cry3Aa	Cry3.1Ab	Cry3Bb	Cry34/35Ab	Cry9Ca	Vip3Aa
soybean														
DAS-81419		X				X								
MON87701		X												
eggplant														
Event EE1		X												
tomato														
5354		X												
poplar														
12, 741		X												

In GM crops, up till now mainly *Bt* proteins naturally occurring in *B. thuringiensis*, but adapted for expression in GM plants, have been introduced. Genes encoding three-domain toxins (Cry1Ab, Cry1Ac, Cry1Ca, Cry1Fa, Cry2Ab, Cry2Ae, Cry3Aa, Cry3Bb, and Cry9Ca), binary toxins (Cry34/35) and vegetative insecticidal proteins (Vip3Aa) have been used. Often these *Bt* proteins have been slightly modified in their DNA sequence to have increased activity (Pardo-López et al., 2009). Also chimeric *Bt* proteins combining domains of different *Bt* proteins have been created *de novo* through domain swapping and introduced into GM crops. Domain swapping was explored in the mid 1990's to create new combinations among the existing domains of natural proteins to generate chimeric Cry proteins with broader spectrum of activity or with increased toxicity (de Maagd et al., 1999). Some examples of chimeric *Bt* proteins composed of portions of different Cry proteins and occurring in GM crops are Cry1A.105 and Cry3.1Ab (Table 5). The Cry1A.105 protein, active against lepidopteran pest species, is a modified version of the *Bt* Cry1A protein consisting of domains I and II and the C-terminal region of the Cry1Ac protein, and domain III of Cry1Fa (US EPA, 2010b). The amino acid sequence identity of Cry1A.105 with Cry1Ac, Cry1Ab and Cry1Fa proteins is 93,6%, 90.0% and 76,7%, respectively. Cry3.1Ab is a chimeric Cry3A-Cry1Ab protein having activity against coleopteran insects (Walters et al., 2010). The gene Cry3.1Ab consists of a fusion between the 5' end (domain I, domain II and 15 AA of domain III) of a modified *cry3A* gene and the 3' end (domain III and variable region 6) of a synthetic *cry1Ab* gene (FSANZ, 2012). It must be noted that also *Bt* proteins having the same name as those naturally found in *B. thuringiensis* may be synthetic proteins combining parts of different *cry* genes. For example, the *cry1Ac* gene in the GM cotton event MON15985 active core part is from the *cry1Ac1* gene, while the carboxy-terminal portion of the protein is derived from the *cry1Ca3* and *cry1Ab1* gene (OGTR, 2003).

5.2. Guidance for assessing impacts of multiple *Bt* proteins on non-target organisms

In GM crops *Bt* proteins may be combined through two approaches, resulting in so-called GM stacked events: by conventional breeding where two or more parental events expressing *Bt* gene(s) are combined or via molecular stacking of traits where several *Bt* genes are simultaneously or consecutively transformed into a recipient crop. As the risk/safety assessment of re-transformed events will follow the scenario of a single GM event, the focus of this chapter will be on the environmental risk/safety assessment of impacts on non-target organisms of stacks obtained through conventional breeding and meant for commercial cultivation. However, the information provided is also of relevance for stacks obtained through transformation.

Bt proteins combined in a GM stacked event might interact and give rise to environmental concerns, in particular the combination may pose a risk to non-target organisms and the ecosystem services (e.g., pest control, pollination, soil nutrient cycling) they provide. The interactions of most concern in risk/safety assessment are synergism and additivity, while antagonism is more an issue relevant for product efficacy.

Information on how to assess impacts on invertebrate non-target organisms in case multiple *Bt* proteins are present in a GM crop is rare. Few publications allude that GM crops combining events with transgene protein products that have a similar and potentially synergistic type of mode of action need special attention in the environmental risk/safety assessment as the combined presence of toxins might result in a changed effect on target or non-target organisms (EuropaBio, 2005; De Schrijver et al., 2007; Wolt et al., 2010). In the EU, some guidelines on how potential interactions between *Bt* toxins and their effects on non-target organisms can be evaluated, exist. The EFSA guidelines on environmental risk assessment (EFSA, 2010a) specify that the evaluation of GM plants with combined biocidal compounds "*shall focus on the characterisation and potential consequences of issues related to potential synergistic, additive and antagonistic effects resulting from the combination of GM events*" and that "*in order to confirm the absence of these potential effects, the potential impact on target organisms should be assessed*". The Scientific Opinion on the Assessment of Potential Impacts of GM plants on Non-Target Organisms (EFSA, 2010b) further specifies that "*Applicants shall perform studies (or provide existing data) with combined administration of proteins when the genetic modification results in the expression of two or more proteins in the GM plant. In planta tests with the stacked event shall be included in tier 1 studies.*" These *in planta* tests are not meant for testing unintended effects of the combined traits on non-target organisms, but to test unintended effects of the genetic modification (e.g. insertional effect) on non-target organisms.

By looking at the environmental risk/safety assessments done by several countries and by sending around a short questionnaire (Box 1), more information related to the

environmental risk/safety assessment of impacts on non-target invertebrates of GM crops containing several *Bt* proteins was collected from risk assessment bodies in countries within the EU that have carried out environmental risk assessments on mandate of EFSA, but also from outside the EU. One of the outcomes of this questionnaire is that in the EU (BE (WIV-ISP), DE (BVL), ES (MAGRAMA)) and in other parts of the world (Australia (OGTR), Japan (MAFF), Mexico (CIBIOGEM) and USA (US EPA)) some country-specific strategies exist, although guidance documents or consolidated approaches are lacking. Only in Japan, a note by the Committee of Impact Assessment on Biological Diversity on how to address the environmental risk/safety assessment of GM stacked events has been published (available only in Japanese on http://www.bch.biodic.go.jp/).

Box 1 – Questionnaire on risk/safety assessment of crops expressing several *Bt* proteins

This questionnaire is part of the COGEM project to address specific questions related to the environmental risk assessment of GM crops containing several *Bt* proteins for commercial release. The questionnaire focusses on, but is not limited to those GM crops combined through conventional breeding of single GM events and containing several *Bt* proteins. The questionnaire was sent around to EU countries that have been involved in the evaluation of an environmental risk assessment on mandate of EFSA (BE, ES, UK, DE) and to several countries outside of the EU (Australia, Canada, Japan, Mexico and USA). Answers were received from six of the contacted bodies within the above mentioned countries. The purpose of the questionnaire was to (1) obtain information on how interactions are evaluated and (2) to understand the approaches taken to identify and assess potential risks to NTOs.

The questions raised we're the following:

1. Does your country have guidelines on how to evaluate interactions between Cry proteins?
2. Does your country have guidelines on how to evaluate if these interactions impact non-target organisms?
3. If you do not have any guidance document, do you request specific data requirements (to be provided in the dossiers) to evaluate the two points mentioned above? If so, could you list these?

Among the countries contacted there seems to be a common understanding that when *Bt* proteins are combined in a GM crop, this warrants consideration of any potential interaction of the combined traits that might lead to an adverse effect. However, the approach taken on how to consider the issue of interactions among *Bt* proteins differs from country to country and does not necessarily result in a risk/safety assessment of the GM stacked event. Most countries contacted would first evaluate whether interactions would occur between the *Bt* proteins present in the GM crop. If it is determined that no

interactions occur (see below on how this is done), the impact on the non-target invertebrates is evaluated on the basis of the information available in the environmental risk/safety assessment of the parental events and further testing to see whether the presence of multiple *Bt* proteins would impact non-target invertebrates is not considered necessary. In other words, the data on impacts on non-target invertebrates of the parental events are considered as the starting point for the environmental risk/safety assessment of GM events combined by crossing and are bridged to the GM stacked event (see also Pilacinski et al., 2011). If there is interaction, the strategies to follow in order to perform an environmental risk/safety assessment differ slightly, but in general one would likely require additional information, sometimes including additional testing of invertebrate species (see below). One country, though, considers that tests with non-target invertebrate species should be provided even in the absence of indications of such interactions (with target species).

Besides information on the general approach for assessing impacts on organisms of combined *Bt* proteins, the outcome of the questionnaire also provided information on data requirements for GM crops combining *Bt* proteins. The assessment of whether interactions occur is often done on a case-by-case basis depending on the *Bt* proteins combined and the information provided in the cultivation application or available in literature. Different types of information are considered, including protein activity specificity; protein cross-activity; information on the sequence homology with known *Bt* proteins; molecular targets/the receptor sites; efficacy bioassays and efficacy field trials; bioassays studying interactions among *Bt* proteins with either the same or similar protein combinations. In other words, most countries do not have a prescriptive set of data they would require to assess if interactions occur. However, for some countries a bioassay determining interactions among the *Bt* proteins in a sensitive species, most often the target pest, is a standard requirement to be provided in an application for cultivation of a GM crop when specific evidence points to interaction between the proteins.

If synergism between the *Bt* proteins has been established, a potential risk to invertebrate non-target organisms is identified and an estimation of the risk is made. Here again, countries tend not to be prescriptive about exactly what they require to come to a risk conclusion. The information available on the environmental risk/safety assessment of the parental events and the interaction could be sufficient to come to a risk conclusion, while in other cases additional information would be requested, including laboratory toxicity studies with invertebrate non-target organisms or field studies testing the impact on abundance or variety of non-target invertebrate populations in GM crop fields, as compared to non-GM plants. Both types of tests can help to conclude whether a synergistic effect occurring between the *Bt* proteins results in significant toxicity for non-target invertebrates.

6. Conclusions for risk/safety assessment

6.1. Can *Bt* protein interactions be predicted?

Interactions between *Bt* proteins have been studied in the context of resistance management, activity spectrum enlargement of *B. thuringiensis* pesticides and more recently in the context of risk/safety assessment of GM crops. The main focus has been on interactions between Lepidoptera-active *Bt* proteins and between *Bt* proteins active against mosquitoes (Diptera). Interaction studies among *Bt* proteins with coleopteran and nematode-activity have also been conducted, but they are limited. In answering the research question on whether *Bt* proteins can influence each other activity: the answer is clearly 'yes'.

The mechanisms behind the interactions among *Bt* proteins are still not clearly understood and different hypotheses have been proposed to explain the molecular mechanisms (EcoΣtat, 2014). Up till now, the study of interactions between *Bt* proteins has mainly focussed on three-domain Cry proteins and Cyt proteins. The phenomenon of interactions has been reported most extensively for the *Bt* proteins present in two *B. thuringiensis* subsp.: the Diptera-active *Bt* proteins of *B. thuringiensis* subsp. *israelensis*, producing Cry4Aa, Cry4Ba, Cry10Aa, Cry11Aa and two Cyt toxins (Cyt1Aa and Cyt2Ba), and subsp. *kurstaki* parasporal inclusions containing Cry1Aa, Cry1Ab, Cry1Ac, Cry2Aa and Cry2Ab proteins active against Lepidoptera. Taking into account the contradicting results on interactions for the five Lepidoptera-active Cry proteins, to our opinion, reliable scientific knowledge exists mostly for Cry/Cry and Cyt/Cry interactions in mosquitoes. Given that the targets of currently authorised GM crops are Coleoptera and/or Lepidoptera pest species, information on interactions in mosquitoes is of little direct relevance for risk/safety assessment, but is still useful to draw some conclusions.

On the basis of the currently available reliable knowledge of interaction studies with target organisms and the mode of action of *Bt* proteins (i.e. binding to receptors), and not considering the binary proteins for which it is known they interact, one can say that interactions are likely to occur when a Cyt protein is present. Genes expressing Cyt proteins are currently not present in commercially available GM crops. One can also postulate that the *Bt* proteins co-occurring in *B. thuringiensis* subsp. *israelensis* express a global synergism, but there is insufficient evidence to state that global synergism among *Bt* proteins is a common phenomenon in other *B. thuringiensis* species. Information on whether *Bt* proteins originating from one particular *B. thuringiensis* species generally interact synergistically could be useful information for risk/safety assessment in that it would aid in predicting interactions. For the Cry1 family active against Lepidoptera, several studies to assess interactions among these toxins (Cry1Aa, Cry1Ab, Cry1Ac, Cry1Ba, Cry1Ca, Cry1Da, and Cry1Fa) have been conducted (see Table S6). From these data it seems that the type of *Bt* protein interaction (additivity, synergism or antagonism) occurring, differs and is insect

species-specific. That the type of interaction among *Bt* proteins depends on the insect species tested, even seems to be a common phenomenon for *Bt* proteins sharing same primary order specificity, independent of the *Bt* (Cry, Cyt or Vip) proteins involved. One publication even suggests differences in susceptibility at the population level within an insect species (Meyer et al., 2001). However, further research is needed to substantiate this assumption which is drawn from the little yet available information.

GM crops, not only combine *Bt* proteins originating from one specific *B. thuringiensis* species and active against one insect order, but also *Bt* proteins originating from different *B. thuringiensis* species and not necessarily acting against the same insect order. Available studies with Cry proteins having a different primary order activity show that synergism among these proteins is not likely to occur. When Cry34/35Ab was combined with Cry1Fa, Cry3Bb with Cry1A.105 and Cry2Ab, Cry3A with Cry1Ab, and Cry3Bb and Cry34/35Ab with Cry1A.105, Cry1F and Cry2Ab, no change in activity towards a lepidopteran or coleopteran pest species was found (Herman and Storer, 2004; Hunter, 2006; Levine et al., 2008; Raybould et al., 2010). Additional evidence that these interactions are not likely to occur comes from specificity data of the individual Cry proteins. The Cry1 and Cry2 family act specifically against Lepidoptera, while the Cry3 family affects Coleoptera. The reason for this distinction in activity lies, among other reasons, within the molecular structure of the *Bt* proteins, the type of receptors they bind to and in the fact that Lepidoptera- and Coleoptera-active *Bt* proteins need different pHs to be activated in the insect mid-gut.

In conclusion, to the research question on whether interactions between *Bt* proteins can be predicted, there are two answers. The answer is ‘yes’, when it comes to interactions between the Lepidoptera- (Cry1Ab, Cry1Ac, Cry1Fa, Cry2Ab, Cry2Ae) and Coleoptera-active (Cry3Aa, Cry3Bb, Cry34/35Ab) three-domain Cry proteins present in currently authorised GM crops. Different types of information, including the outcomes of interaction studies with these Cry proteins, the specificity of the Cry proteins and their mode of action have been taken into account to come to this conclusion. For new *Bt* protein combinations in GM plants for which no interaction studies have been done, we postulate that on the basis of the order specificity of the *Bt* proteins, including primary order and cross-order activity, one can predict if interactions might occur. If the activities do not overlap, it is unlikely that interactions will occur. This assumption can, however, only been made if sufficient information is available on the specificity of the *Bt* proteins introduced in the GM plant. What is known on the specificity of *Bt* proteins currently present in GM plants is discussed below.

The answer is ‘no’ when it comes to interactions between *Bt* proteins with the same primary order activity. The current knowledge of interactions between *Bt* proteins is not sufficient to make such a prediction. Further, the preliminary observation that interactions among *Bt* protein combinations can be synergistic for one insect species, but be neutral or antagonistic for another, complicates the predictability of the occurrence of interactions.

Sub-question: What is known on the specificity of *Bt* proteins present in GM plants?

For the *Bt* proteins present in authorised GM plants (Cry1Ab, Cry1Ac, Cry1Ca, Cry1Fa, Cry2Ab, Cry2Ae, Cry3Aa, Cry3Bb, Cry34/35Ab, Cry9Ca and Vip3Aa), primary order activity is limited to Lepidoptera, Coleoptera and Diptera (Table S1 and S2). The Cry1A, Cry1C, Cry1F, Cry2A and Cry9C proteins are known to be active against Lepidoptera; Cry3 proteins to Coleoptera; and Cry2Ab has dual primary order affinity: it is active against Lepidoptera and Diptera. The chimeric proteins present in GM plants, Cry1A.105, Cry1Ab-Ac and Cry3.1Ab, have novel bioactivities compared to their parental *Bt* protein, but they still fall within the range of the primary order specificity of the parental *Bt* protein. Cry1A.105 and Cry1b-Ac their activity spectrum is limited to Lepidoptera, Cry3.1Ab is active against Coleoptera.

The activity of Cry proteins which was initially considered to be limited to a specific insect order (primary order activity), can also exceed that order (cross-activity). This cross-order activity occurs among all three protein classes (Cry, Cyt and Vip) and has been detected mostly within the Insecta, and in a few cases involves Nematoda. Cross-activity studies on several insect orders have been done for Cry1Ab, Cry1Ac, Cry2Ab, Cry3Aa and Cry3Bb (Fig. 7), but not for the other *Bt* proteins present in GM plants. Cross-order toxicities for the Lepidoptera-active Cry1Ab, Cry1Ac and Cry2Ab that are supported by reasonable evidence, are the following: the Cry1Ab protein has been shown to be toxic to an aphid species (Hemiptera: *A. pisum*; Porcar et al., 2009) and the nematode *C. elegans* (Höss et al., 2008); Cry1Ac is toxic to tsetse flies (Diptera: *G. morsitans*; Omolo et al., 1997) and an aphid (Hemiptera: *A. pisum*; Li et al. 2011) and Cry2Ab affects nematodes (Höss et al., 2013). The Coleoptera-active Cry3Aa was reported to have activity against to two aphid species (Hemiptera: *M. euphorbiae* and *A. pisum*; Walters and English, 1995; Porcar et al., 2009; Li et al., 2011) and to the imported red fire ant (Hymenoptera: *S. invicta*; Bulla and Candas, 2004), while Cry3Bb has been reported to affect Nematoda (Höss et al., 2011, 2013). The only cross-activity mentioned above that has been validated by independent studies is the toxicity of Cry3Aa to Hemiptera. The other cross-activities still need to be confirmed through additional testing and therefore are best viewed with caution in the context of environmental risk/safety assessment.

From a risk/safety assessment perspective, apart from the primary order activity, it is relevant to look at cross-activities that fall outside the protein's primary order affinity, in particular when that activity occurs at levels that are within the lethal toxicity range of corresponding reference proteins. For the *Bt* proteins Cry1Ab, Cry1Ac, Cry2Ab, Cry3Aa and Cry3Bb, quantitative estimates of toxicity to species outside orders of primary affinity are available, except for the cross-activity of Cry3Aa to Diptera. Cross-activity of Cry1Ab, Cry1Ac and Cry3Aa to Hemiptera groups in the low-toxicity range ($LC_{50s} > 10$ µg/ml). Qualitative data indicate a similar level of toxicity for these three proteins with doses of 360-500 µg/ml resulting in mortalities between 40 and 100%. On the other hand, Cry1Ac cross-activity to

Diptera falls within the high-toxicity range (LC_{50s} = 0.01 - 0.10 µg/ml) of Diptera-active proteins (LC_{50s} of 0.5-0.7 µg/ml). Cross-activity of Cry1Ab, Cry2Ab and Cry3Bb to Nematoda occurred at dose levels that were two orders of magnitude above those typical for nematode-active proteins and thus not likely to have any biological significance. This assumption is supported by studies showing no effect of transgenic crops on nematode abundance, growth, reproduction or community structure (Saxena and Stotzky, 2001; Griffiths et al., 2007; Höss et al., 2011).

There is evidence that even low-toxicity proteins can affect (target) pests in terms of sublethal effects when expressed in transgenic plants, as is the case for Cry5Ba expressed in tomato roots against root-knot nematodes (Li et al., 2008) and Cry51Aa expressed in cotton against *Lygus* bugs (Baum et al., 2012). This means that even cross-activities in the low-toxicity category could have implications for susceptible non-target species that may merit further investigation.

6.2. To what extent are studies on *Bt* protein interactions relevant for risk/safety assessment?

In the risk/safety assessment of GM plants containing several *Bt* proteins, the potential impact of the combined presence of *Bt* proteins on non-target invertebrates is taken into account. A logic first step in the risk/safety assessment would thus be to determine if interactions between the *Bt* proteins actually occur. While in some jurisdictions, *in vivo* laboratory studies with sensitive species, often the target pest, is a standard requirement to see whether interactions occur, this is not the case in other jurisdictions. Taking into account the information reviewed in the above chapters and in the EcoΣtat project, we will discuss here to what extent *in vivo* laboratory studies with target invertebrates assessing *Bt* protein interactions are relevant for risk/safety assessment.

Due to the lack of evidence that *Bt* proteins with a different primary activity would interact, *in vivo* bioassays with the target pests and testing interactions between *Bt* proteins that have a clear different order specificity (i.e. primary and cross-order activity) are considered less relevant for risk/safety assessment. In case of insufficient knowledge of the activity spectrum of one of the *Bt* proteins, such as in the case for the binary protein Cry34/35Ab, or in case of limited studies on interactions, such as in the case of VIP proteins, and thus on the interactions that might occur, testing would have priority until the necessary knowledge has been obtained.

As there are indications that *Bt* proteins with the same primary order activity interact, *in vivo* bioassays testing interactions in target pest species between *Bt* proteins that have a same specificity would be more relevant for risk/safety assessment to determine if interactions between the *Bt* proteins would occur.

In answering the question whether interaction studies with target pest species, reveal something on the occurrence of interactions in other species, we want to note the following: Given the indications that the occurrence of interactions (additivity, synergism or antagonism) is insect species-dependent, even if reliable studies are available demonstrating no interactions between the *Bt* proteins active against species within the envisaged insect order, uncertainty that the *Bt* proteins may have a changed activity (antagonistic or synergistic) to some species within the target order will remain. In other words, extrapolating results on interactions from studies done with one species to another species within the same order would currently not be recommendable.

In summary, the relevance of *in vivo* laboratory studies assessing *Bt* protein interactions in target pest species for risk/safety assessment, as a source of information to assess potential impacts on non-target invertebrates, seems to be little. Tests with *Bt* proteins having overlapping specificity will reveal that synergistic interactions between the *Bt* proteins do or do not occur in the target pests, but do not necessarily reflect if this will also be the case for non-target invertebrates of the same order. *In vivo* bioassays testing interactions between *Bt* proteins with different specificity can, however, add to the weight of evidence that interactions will not to occur in non-target invertebrates.

Follow-up question: What has to be done in the risk/safety assessment?

In the risk/safety assessment of GM crops combining multiple *Bt* proteins, it is useful to evaluate whether interactions between the *Bt* proteins occur and if these interactions might impact non-target invertebrates. Assessing whether interactions occur is often done on a case-by-case basis considering different types of information such as the *Bt* protein activity spectra; sequence homology between the *Bt* proteins; the *Bt* protein receptor sites; efficacy bioassays and field trials. Bioassays studying interactions among the *Bt* proteins in target pest species, with either the same or similar *Bt* protein combinations as present in the GM plant are also taken into account. If synergism between the *Bt* proteins has been established, a potential risk to invertebrate non-target organisms is identified and an estimation of the risk is made. In a few cases, studies testing the combined effect in non-target organisms are then requested.

Here we propose an approach on how to assess the impact on non-target invertebrates of GM plants combining several *Bt* proteins, starting from the information on the specificity of the *Bt* proteins present in the GM plant and the conclusions drawn, although some preliminary, from the literature review on interaction studies (Fig. 9). If the specificity of the *Bt* proteins does not overlap, synergism is not expected and in this case *in vivo* bioassays with non-target invertebrates done in the context of the risk/safety assessment of the single event, could apply in the risk/safety assessment of the stacked GM event. Also in the case there is sufficient information available to claim that the *Bt* proteins with overlapping specificity will not interact, studies done with the single *Bt* proteins to assess the impact on non-target invertebrates can be used in the risk/safety assessment of the stacked GM event.

If there are indications of synergy or if synergy has been established, further testing using non-target invertebrates has been considered. The question can be raised if the same set of non-target invertebrates tested in the risk/safety assessment of the single GM event should be re-tested. The literature review does not reveal scientific evidence that points in the direction that for example two interacting Coleoptera-active Cry proteins would become active outside the order of Coleoptera. Therefore, testing the same range of non-target invertebrates as done for the single event would not really aid to come to a risk/safety assessment conclusion. If there are indications for synergistic interactions between the Cry proteins, testing non-target invertebrate species that fall within the specificity of the *Bt* proteins (primary and cross-order activity) would therefore be more reasonable than

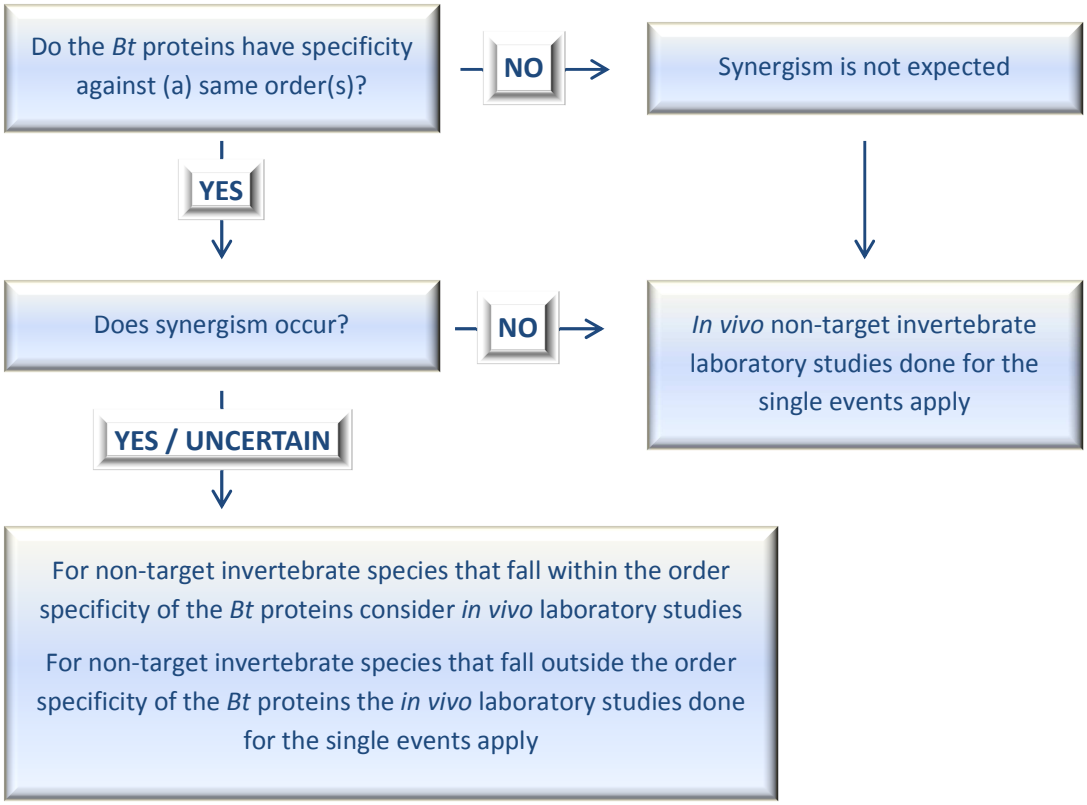

Figure 9. Schematic overview of how to evaluate impacts of GM plants combining several *Bt* proteins (Cry and/or Vip proteins) on non-target organisms.

testing the same range of non-target invertebrates as done for the single events. Depending on the protection goals specified in the problem formulation, it can be considered to test non-target invertebrates falling within the *Bt* protein order specificity that are valued in the agro-ecosystem. For example, coccinellids in case the target pest is a coleopteran. For the

species that fall outside the order specificity of the *Bt* proteins the *in vivo* laboratory studies done with the single events could still apply.

As an alternative for this approach or in addition, the uncertainty that the *Bt* proteins may have an increased effect on non-target invertebrates can be taken into account in the risk/safety assessment of the stacked GM event.

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Tables S1-6

Table S1. Supplementary data summarising activity of *B. thuringiensis* pesticidal proteins at the species level

Phyla: PLA = Platyhelminthes; ART = Arthropoda; NEM = Nematoda; MOL = Mollusca; ANN = Annelida
Classes: INS = Insecta; Hex = Hexapoda; CRU = Crustacea; ARA = Arachnida; TRE = Trematoda; CHR = Chromadorea; GAS = Gastropoda; CLIT = Clitellata
Orders: COL = Coleoptera; LEP = Lepidoptera; DIP = Diptera; HEM = Hemiptera; HYM = Hymenoptera; ISO = Isoptera; NEU = Neuroptera; SIP = Siphonaptera; ORT = Orthoptera; BLA = Blattodea; THY = Thysanoptera; TRI = Trichoptera; CO = Collembola; AMP = Amphipoda; IS = Isopoda; CLA = Cladocera; ACA = Acari; AR = Araneae; ECH = Echinostomida; ASC = Ascaridida; RHA = Rhabditida; DIP = Diplogasterida; TYL = Tylenchida; HAP = Haplotaxida
0 = none of the species tested were affected; 1 = at least one species was affected; 2 = conflicting or unsubstantiated data.

Phylum	Class	Order	Family	Genus	Species	No. toxins tested
ART	INS	Lepidoptera	Arctiidae	*Hyphantria*	*cunea*	4
ART	INS	Lepidoptera	Arctiidae	*Spilosoma*	*virginica*	1
ART	INS	Lepidoptera	Arctiidae	*Diacrisia*	*obliqua*	2
ART	INS	Lepidoptera	Bombycidae	*Bombyx*	*mori*	26
ART	INS	Lepidoptera	Danaidae	*Danaus*	*plexippus*	5
ART	INS	Lepidoptera	Gelechiidae	*Pectinophora*	*gossypiella*	16
ART	INS	Lepidoptera	Gelechiidae	*Phthorimaea*	*operculella*	5
ART	INS	Lepidoptera	Gelechiidae	*Tecia*	*solanivora*	1
ART	INS	Lepidoptera	Geometridae	*Lambdina*	*fiscellaria*	4
ART	INS	Lepidoptera	Gracillariidae	*Conopomorpha*	*cramerella*	10
ART	INS	Lepidoptera	Hepialidae	*Wiseana*	*cervinata*	2
ART	INS	Lepidoptera	Hepialidae	*Wiseana*	*copularis*	1
ART	INS	Lepidoptera	Hepialidae	*Wiseana*	*jocosa*	1
ART	INS	Lepidoptera	Lasiocampidae	*Malacosoma*	*disstria*	8
ART	INS	Lepidoptera	Lycaenidae	*Cacyreus*	*marshalli*	11
ART	INS	Lepidoptera	Lymantriidae	*Lymantria*	*dispar*	14
ART	INS	Lepidoptera	Lymantriidae	*Orgyia*	*leucostigma*	7
ART	INS	Lepidoptera	Lyonetiidae	*Perileucoptera*	*coffeella*	6
ART	INS	Lepidoptera	Noctuidae	*Anticarsia*	*gemmatalis*	7
ART	INS	Lepidoptera	Noctuidae	*Earias*	*vittella*	5
ART	INS	Lepidoptera	Noctuidae	*Earias*	*insulana*	8
ART	INS	Lepidoptera	Noctuidae	*Agrotis*	*ipsilon*	27
ART	INS	Lepidoptera	Noctuidae	*Agrotis*	*segetum*	3
ART	INS	Lepidoptera	Noctuidae	*Chrysodeixis*	*chalcites*	3
ART	INS	Lepidoptera	Noctuidae	*Actebia*	*fennica*	10
ART	INS	Lepidoptera	Noctuidae	*Busseola*	*fusca*	4
ART	INS	Lepidoptera	Noctuidae	*Helicoverpa*	*zea*	29
ART	INS	Lepidoptera	Noctuidae	*Helicoverpa*	*punctigera*	9
ART	INS	Lepidoptera	Noctuidae	*Helicoverpa*	*armigera*	40
ART	INS	Lepidoptera	Noctuidae	*Heliothis*	*virescens*	27
ART	INS	Lepidoptera	Noctuidae	*Mamestra*	*brassicae*	13
ART	INS	Lepidoptera	Noctuidae	*Mamestra*	*configurata*	3
ART	INS	Lepidoptera	Noctuidae	*Pseudoplusia*	*includens*	8
ART	INS	Lepidoptera	Noctuidae	*Spodoptera*	*exigua*	41
ART	INS	Lepidoptera	Noctuidae	*Spodoptera*	*frugiperda*	34
ART	INS	Lepidoptera	Noctuidae	*Spodoptera*	*littoralis*	15
ART	INS	Lepidoptera	Noctuidae	*Spodoptera*	*exempta*	2
ART	INS	Lepidoptera	Noctuidae	*Spodoptera*	*litura*	15
ART	INS	Lepidoptera	Noctuidae	*Spodoptera*	*albula*	2
ART	INS	Lepidoptera	Noctuidae	*Spodoptera*	*cosmioides*	2
ART	INS	Lepidoptera	Noctuidae	*Spodoptera*	*eridania*	2
ART	INS	Lepidoptera	Noctuidae	*Trichoplusia*	*ni*	32
ART	INS	Lepidoptera	Noctuidae	*Rachiplusia*	*nu*	1
ART	INS	Lepidoptera	Noctuidae	*Sesamia*	*calamistis*	4
ART	INS	Lepidoptera	Noctuidae	*Sesamia*	*inferens*	5
ART	INS	Lepidoptera	Noctuidae	*Mythimna*	*unipunctata*	1
ART	INS	Lepidoptera	Notodontidae	*Clostera*	*anachoreta*	1
ART	INS	Lepidoptera	Pieridae	*Pieris*	*brassicae*	11
ART	INS	Lepidoptera	Pieridae	*Pieris*	*rapae*	6
ART	INS	Lepidoptera	Plutellidae	*Plutella*	*xylostella*	57
ART	INS	Lepidoptera	Pyralidae	*Chilo*	*suppressalis*	10
ART	INS	Lepidoptera	Pyralidae	*Ostrinia*	*nubilalis*	31
ART	INS	Lepidoptera	Pyralidae	*Ostrinia*	*furnacalis*	8
ART	INS	Lepidoptera	Pyralidae	*Ephestia*	*kuehniella*	4
ART	INS	Lepidoptera	Pyralidae	*Plodia*	*interpunctella*	4
ART	INS	Lepidoptera	Pyralidae	*Galleria*	*mellonella*	1
ART	INS	Lepidoptera	Pyralidae	*Cnaphalocrocis*	*medinalis*	4
ART	INS	Lepidoptera	Pyralidae	*Crocidolomia*	*binotalis*	2
ART	INS	Lepidoptera	Pyralidae	*Diatraea*	*saccharalis*	6
ART	INS	Lepidoptera	Pyralidae	*Diatraea*	*grandiosella*	3
ART	INS	Lepidoptera	Pyralidae	*Eldana*	*saccharina*	4
ART	INS	Lepidoptera	Pyralidae	*Elasmolpalpus*	*lignosellus*	5
ART	INS	Lepidoptera	Pyralidae	*Hellula*	*undalis*	2
ART	INS	Lepidoptera	Pyralidae	*Sciropophaga*	*incertulas*	5
ART	INS	Lepidoptera	Pyralidae	*Maruca*	*vitrata*	5
ART	INS	Lepidoptera	Pyralidae	*Marasmia*	*patnalis*	4
ART	INS	Lepidoptera	Sphingidae	*Manduca*	*sexta*	22
ART	INS	Lepidoptera	Thaumetopoeidae	*Thaumetopoea*	*pityocampa*	6
ART	INS	Lepidoptera	Tortricidae	*Choristoneura*	*fumiferana*	10
ART	INS	Lepidoptera	Tortricidae	*Choristoneura*	*occidentalis*	7
ART	INS	Lepidoptera	Tortricidae	*Choristoneura*	*pinus pinus*	3
ART	INS	Lepidoptera	Tortricidae	*Choristoneura*	*rosaceana*	8
ART	INS	Lepidoptera	Tortricidae	*Argyrotaenia*	*citrana*	8
ART	INS	Lepidoptera	Tortricidae	*Ctenopsuestis*	*obliquana*	2
ART	INS	Lepidoptera	Tortricidae	*Cydia*	*pomonella*	11
ART	INS	Lepidoptera	Tortricidae	*Epiphyas*	*postvittana*	4
ART	INS	Lepidoptera	Tortricidae	*Planotortrix*	*octo*	2
ART	INS	Lepidoptera	Tortricidae	*Lobesia*	*botrana*	10
ART	INS	Lepidoptera	Tortricidae	*Leguminivora*	*glycinivorella*	1
ART	INS	Lepidoptera	Tortricidae	*Epinotia*	*aporema*	7
ART	INS	Lepidoptera	Tortricidae	*Platynota*	*stultana*	8
ART	INS	Lepidoptera	Tortricidae	*Pandemis*	*pyrusana*	8
ART	INS	Lepidoptera	Tortricidae	*Thaumatotibia*	*leucotreta*	6
ART	INS	Lepidoptera	Yponeumatidae	*Prays*	*olea*	9
ART	INS	Diptera	Culicidae	*Aedes*	*aegypti*	50
ART	INS	Diptera	Culicidae	*Aedes*	*triseriatus*	1
ART	INS	Diptera	Culicidae	*Anopheles*	*stephensi*	28
ART	INS	Diptera	Culicidae	*Anopheles*	*gambiae*	10
ART	INS	Diptera	Culicidae	*Anopheles*	*quadrimaculatus*	1
ART	INS	Diptera	Culicidae	*Anopheles*	*albimanus*	3
ART	INS	Diptera	Culicidae	*Culex*	*pipiens*	32
ART	INS	Diptera	Culicidae	*Culex*	*fatigans*	1
ART	INS	Diptera	Culicidae	*Culex*	*pervigilans*	1
ART	INS	Diptera	Culicidae	*Culex*	*quinquefasciatus*	24
ART	INS	Diptera	Culicidae	*Culex*	*tritaeniorhynchus*	1
ART	INS	Diptera	Chironomidae	*Chironomus*	*tepperi*	7
ART	INS	Diptera	Tipulidae	*Tipula*	*paludosa*	4
ART	INS	Diptera	Tipulidae	*Tipula*	*oleracae*	3
ART	INS	Diptera	Tipulidae	*Tipula*	*abdominalis*	1
ART	INS	Diptera	Calliphoridae	*Lucilia*	*cuprina*	4
ART	INS	Diptera	Calliphoridae	*Lucilia*	*sericata*	2
ART	INS	Diptera	Calliphoridae	*Calliphora*	*stygia*	2
ART	INS	Diptera	Muscidae	*Glossina*	*mortisans*	1
ART	INS	Diptera	Muscidae	*Musca*	*domestica*	10
ART	INS	Diptera	Drosophilidae	*Drosophila*	*melanogaster*	1
ART	INS	Diptera	Tephritidae	*Ceratitis*	*capitata*	1
ART	INS	Diptera	Psychodidae	*Clogmia*	*albipunctata*	6
ART	INS	Diptera	Agromyzidae	*Liriomyza*	*trifolii*	1
ART	INS	Coleoptera	Bruchidae	*Callosobruchus*	*maculatus*	1
ART	INS	Coleoptera	Brentidae	*Cylas*	*puncticollis*	4
ART	INS	Coleoptera	Brentidae	*Cylas*	*brunneus*	4
ART	INS	Coleoptera	Cerambycidae	*Apriona*	*germini*	1
ART	INS	Coleoptera	Cerambycidae	*Anoplophora*	*glabripennis*	5
ART	INS	Coleoptera	Chrysomelidae	*Colaphellus*	*bowringi*	4
ART	INS	Coleoptera	Chrysomelidae	*Diabrotica*	*undecimpunctata*	23
ART	INS	Coleoptera	Chrysomelidae	*Diabrotica*	*virgifera virgifera*	19
ART	INS	Coleoptera	Chrysomelidae	*Diabrotica*	*longicornis*	1
ART	INS	Coleoptera	Chrysomelidae	*Diabrotica*	*balteata*	1
ART	INS	Coleoptera	Chrysomelidae	*Chrysomela*	*scripta*	3
ART	INS	Coleoptera	Chrysomelidae	*Leptinotarsa*	*decemlineata*	19
ART	INS	Coleoptera	Chrysomelidae	*Haltica*	*tombacina*	1
ART	INS	Coleoptera	Chrysomelidae	*Agelastica*	*coerulae*	3
ART	INS	Coleoptera	Chrysomelidae	*Agelastica*	*alni*	1
ART	INS	Coleoptera	Chrysomelidae	*Oulema*	*melanopus*	2
ART	INS	Coleoptera	Chrysomelidae	*Phaedon*	*brassicae*	2
ART	INS	Coleoptera	Chrysomelidae	*Phaedon*	*cochleariae*	2
ART	INS	Coleoptera	Chrysomelidae	*Plagiodera*	*versicolora*	1
ART	INS	Coleoptera	Chrysomelidae	*Phyllotreta*	*armoraciae*	1
ART	INS	Coleoptera	Chrysomelidae	*Phyllotreta*	*cruciferae*	1
ART	INS	Coleoptera	Chrysomelidae	*Pyrrhalta*	*aenescens*	2
ART	INS	Coleoptera	Chrysomelidae	*Pyrrhalta*	*luteola*	1
ART	INS	Coleoptera	Coccinellidae	*Hippodamia*	*convergens*	2
ART	INS	Coleoptera	Coccinellidae	*Coleomegilla*	*maculata*	4
ART	INS	Coleoptera	Coccinellidae	*Adalia*	*bipunctata*	3
ART	INS	Coleoptera	Coccinellidae	*Henosepilachna*	*vigintioctomaculata*	1
ART	INS	Coleoptera	Coccinellidae	*Stethorus*	*punctillim*	1
ART	INS	Coleoptera	Coccinellidae	*Cryptolaemus*	*montrouzieri*	2
ART	INS	Coleoptera	Coccinellidae	*Cheilomenes*	*sexmaculatus*	2
ART	INS	Coleoptera	Coccinellidae	*Propylea*	*japonica*	1
ART	INS	Coleoptera	Curculionidae	*Anthonomus*	*grandis*	14
ART	INS	Coleoptera	Curculionidae	*Asymmathetes*	*vulcanorum*	3
ART	INS	Coleoptera	Curculionidae	*Hypera*	*brunneipennis*	1
ART	INS	Coleoptera	Curculionidae	*Hypera*	*postica*	2
ART	INS	Coleoptera	Curculionidae	*Otiorhynchus*	*sulcatus*	1
ART	INS	Coleoptera	Curculionidae	*Hypothenemus*	*hampei*	2
ART	INS	Coleoptera	Curculionidae	*Lissorhoptrus*	*oryzophilus*	1
ART	INS	Coleoptera	Curculionidae	*Myllocerus*	*undecimpustulatus*	1
ART	INS	Coleoptera	Dermestidae	*Attagenus*	*unicolor*	1
ART	INS	Coleoptera	Nitidulidae	*Carpophilus*	*hemipterus*	1
ART	INS	Coleoptera	Ptinidae	*Gibbium*	*psylliodes*	1
ART	INS	Coleoptera	Tenebrionidae	*Tenebrio*	*molitor*	9
ART	INS	Coleoptera	Tenebrionidae	*Tribolium*	*castaneum*	6
ART	INS	Coleoptera	Scarabaeidae	*Anomala*	*corpulenta*	4
ART	INS	Coleoptera	Scarabaeidae	*Anomala*	*cuprea*	5
ART	INS	Coleoptera	Scarabaeidae	*Anomala*	*exoleta*	1
ART	INS	Coleoptera	Scarabaeidae	*Anomala*	*orientalis*	1
ART	INS	Coleoptera	Scarabaeidae	*Amphimallon*	*solstitiale*	1
ART	INS	Coleoptera	Scarabaeidae	*Cotinis*	*spp*	2
ART	INS	Coleoptera	Scarabaeidae	*Cyclocephala*	*borealis*	1
ART	INS	Coleoptera	Scarabaeidae	*Cyclocephala*	*pasadenae*	1
ART	INS	Coleoptera	Scarabaeidae	*Holotrichia*	*oblita*	4
ART	INS	Coleoptera	Scarabaeidae	*Holotrichia*	*parallela*	4
ART	INS	Coleoptera	Scarabaeidae	*Melolontha*	*melolontha*	2
ART	INS	Coleoptera	Scarabaeidae	*Popillia*	*japonica*	7
ART	INS	Coleoptera	Staphylinidae	*Atheta*	*coriaria*	3
ART	INS	Neuroptera	Chrysopidae	*Chrysopa*	*pallens*	1
ART	INS	Neuroptera	Chrysopidae	*Chrysoperla*	*sinica*	1
ART	INS	Neuroptera	Chrysopidae	*Chrysoperla*	*carnea*	6
ART	INS	Trichoptera	Lepidostomatidae	*Lepidostoma*	*spp.*	1
ART	INS	Trichoptera	Lepidostomatidae	*Lepidostoma*	*liba*	1
ART	INS	Trichoptera	Helicopsychidae	*Helicopsyche*	*borealis*	1
ART	INS	Trichoptera	Limnephilidae	*Pycnopsyche*	*scabripennis*	1
ART	INS	Hymenoptera	Diprionidae	*Diprion*	*pini*	1
ART	INS	Hymenoptera	Diprionidae	*Diprion*	*similis*	1
ART	INS	Hymenoptera	Diprionidae	*Diprion*	*sertifer*	1
ART	INS	Hymenoptera	Pamphiliidae	*Acantholyda*	*erythrocephala*	1
ART	INS	Hymenoptera	Tenthredinidae	*Pikonema*	*alaskensis*	1
ART	INS	Hymenoptera	Tenthredinidae	*Athalia*	*rosea*	1
ART	INS	Hymenoptera	Apinae	*Apis*	*mellifera*	4
ART	INS	Hymenoptera	Apinae	*Apis*	*cerana cerana*	1
ART	INS	Hymenoptera	Apinae	*Bombys*	*occidentalis*	1
ART	INS	Hymenoptera	Braconidae	*Macrocentrus*	*ancylivorus*	1
ART	INS	Hymenoptera	Braconidae	*Meteorus*	*pulchricornis*	1
ART	INS	Hymenoptera	Braconidae	*Cotesia*	*marginiventris*	1
ART	INS	Hymenoptera	Braconidae	*Cotesia*	*flavipes*	1
ART	INS	Hymenoptera	Braconidae	*Cotesia*	*plutella*	1
ART	INS	Hymenoptera	Braconidae	*Diaeretiella*	*rapae*	1
ART	INS	Hymenoptera	Braconidae	*Microplitis*	*mediator*	1
ART	INS	Hymenoptera	Encyrtidae	*Copidosoma*	*floridanum*	1
ART	INS	Hymenoptera	Pteromalidae	*Nasonia*	*vitripennis*	2
ART	INS	Hymenoptera	Formicidae	*Solenopsis*	*invicta*	1
ART	INS	Hymenoptera	Formicidae	*Monomorium*	*pharaonis*	1
ART	INS	Hemiptera	Lygaeidae	*Oncopeltus*	*fasciatus*	1
ART	INS	Hemiptera	Miridae	*Lygus*	*hesperus*	6
ART	INS	Hemiptera	Miridae	*Lygus*	*lineolaris*	1
ART	INS	Hemiptera	Miridae	*Cytorhinus*	*lividipennis*	1
ART	INS	Hemiptera	Anthocoridae	*Orius*	*majusculus*	1
ART	INS	Hemiptera	Anthocoridae	*Orius*	*insidiosis*	1
ART	INS	Hemiptera	Anthocoridae	*Orius*	*albidipennis*	3
ART	INS	Hemiptera	Anthocoridae	*Orius*	*sauteri*	1
ART	INS	Hemiptera	Reduviidae	*Zelus*	*renardii*	1
ART	INS	Hemiptera	Nabidae	*Nabis*	*sp*	1
ART	INS	Hemiptera	Delphacidae	*Nilaparvata*	*lugens*	1
ART	INS	Hemiptera	Aleyrodidae	*Bemisia*	*tabaci*	1
ART	INS	Hemiptera	Aphididae	*Macrosiphum*	*euphorbiae*	4
ART	INS	Hemiptera	Aphididae	*Rhopalosiphum*	*padi*	2
ART	INS	Hemiptera	Aphididae	*Myzus*	*persicae*	3
ART	INS	Hemiptera	Aphididae	*Sitobion*	*avenae*	1
ART	INS	Hemiptera	Aphididae	*Acyrthosiphon*	*pisum*	6
ART	INS	Hemiptera	Aphididae	*Aphis*	*gossypii*	2
ART	INS	Isoptera	Rhinotermitidae	*Reticulitermes*	*flavipes*	4
ART	INS	Blattodea	Blattellidae	*Blattella*	*germanica*	5
ART	INS	Orthoptera	Gryllidae	*Acheta*	*domesticus*	1
ART	INS	Orthoptera	Gryllidae	*Gryllus*	*bimaculatus*	0
ART	INS	Orthoptera	Pyrgomorphidae	*Atractomorpha*	*bedeli*	3
ART	INS	Orthoptera	Acrididae	*Locusta*	*migratoria*	4
ART	INS	Siphonaptera	Pulicidae	*Ctenocephalides*	*felis*	1
ART	INS	Thysanoptera	Thripidae	*Frankliniella*	*tenuicornis*	1
ART	INS	Thysanoptera	Thripidae	*Stenchaetothrips*	*biformis*	1
ART	INS	Thysanoptera	Thripidae	*Anaphothrips*	*obscurus*	1
ART	HEX	Collembola	Hypogastruridae	*Xenylla*	*grisea*	4
ART	HEX	Collembola	Isotomidae	*Folsomia*	*candida*	4
ART	CRU	Cladocera	Daphnidae	*Daphnia*	*magma*	1
ART	CRU	Amphipoda	Hyalellidae	*Hyalella*	*azteca*	1
ART	CRU	Isopoda	Asellidae	*Caecidotia*	*communis*	1
ART	CRU	Isopoda	Porcellionidae	*Porcellio*	*scaber*	2
ART	ARA	Araneae	Araneidae	*Araneus*	*diadematus*	1
ART	ARA	Araneae	Theridiidae	*Theridon*	*impressum*	1
ART	ARA	Araneae	Lycosidae	*Pirata*	*subpiraticus*	1
ART	ARA	Trombidiformes	Tetranychidae	*Tetranychus*	*urticae*	2
ART	ARA	Sarcoptiformes	Orbatidae	*Oppia*	*nitens*	2
ART	ARA	Mesostigmata	Phytoseiidae	*Noeseiulus*	*cucumeris*	1
ART	ARA	Mesostigmata	Phytoseiidae	*Euseius*	*concordis*	1
ART	ARA	Mesostigmata	Phytoseiidae	*Euseius*	*californicus*	1
ART	ARA	Sarcoptiformes	Scheloribatidae	*Scheloribates*	*praeincisus*	1
PLA	TRE	Echinostomida	Fasciolidae	*Fasciola*	*hepatica*	2
NEM	CHR	Rhabditida	Cephalobidae	*Acrobeloides*	*spp.*	7
NEM	CHR	Rhabditida	Rhabditidae	*Caenorhabditis*	*elegans*	12
NEM	CHR	Rhabditida	Rhabditidae	*Distolabrellus*	*veechi*	7
NEM	CHR	Rhabditida	Panagrolaimidae	*Panagrellus*	*redivivus*	6
NEM	CHR	Rhabditida	Ancylostomatidae	*Ancylostoma*	*ceylanicum*	1
NEM	CHR	Rhabditida	Heligmonellidae	*Nippostrongylus*	*brasiliensis*	4
NEM	CHR	Rhabditida	Haemonchidae	*Haemonchus*	*contortus*	1
NEM	CHR	Rhabditida	Heligmosomatidae	*Heligmosomoides*	*bakeri*	1
NEM	CHR	Ascaridida	Ascarididae	*Ascaris*	*suum*	1
NEM	CHR	Diplogasterida	Neodiplogasteridae	*Pristionchus*	*pacificus*	7
NEM	CHR	Tylenchida	Pratylenchidae	*Pratylenchus*	*spp.*	5
NEM	CHR	Tylenchida	Meloidogynidae	*Meloidogyne*	*hapla*	3
NEM	CHR	Tylenchida	Meloidogynidae	*Meloidogyne*	*incognita*	2
MOL	GAS		Planorbidae	*Gyraulus*	*spp*	1
ANN	CLIT	Haplotaxida	Lumbricidae	*Eisenia*	*fetida*	1
ANN	CLIT	Haplotaxida	Lumbricidae	*Lumbricus*	*terrestris*	1

No. species tested — total 252

329	total
171	not tested
158	tested

Table S2. Distribution of pesticidal activities of *B. thuringiensis* proteins by families

Phyla: PLA = Platyhelminthes; ART = Arthropoda; NEM = Nematoda; MOL = Mollusca; ANN = Annelida
Classes: INS = Insecta; Hex = Hexapoda; CRU = Crustacea; ARA = Arachnida; TRE = Trematoda; CHR = Chromadorea; GAS = Gastropoda; CLIT = Clitellata
Orders: COL = Coleoptera; LEP = Lepidoptera; DIP = Diptera; HEM = Hemiptera; HYM = Hymenoptera; ISO = Isoptera; NEU = Neuroptera; SIP = Siphonaptera; ORT = Orthoptera; BLA = Blattodea; THY = Thysanoptera; TRI = Trichoptera; CO = Collembola; AMP = Amphipoda; IS = Isopoda; CLA = Cladocera; ACA = Acari; AR = Araneae; ECH = Echinostomida; ASC = Ascaridida; RHA = Rhabditida; DIP = Diplogasterida; TYL = Tylenchida; HAP = Haplotaxida
0 = none of the species tested were affected; 1 = at least one species was affected; 2 = conflicting or unsubstantiated data.

B. thuringiensis proteins

Lepidoptera

Phylum	Class	Order	Family	1Aa	1Ab	1Ac	1Ad	1Ae	1Ah	1Ba	1Bb	1Bd	1Be	1Bf	1Ca	1Cb	1Da	1Db	1Ea	1Eb	1Fa	1Ga	1Gb	1Gc	1Ha	1Ia	1Ib	1Id	1Ie	1If	1Ja	1Jb	1Jc	1Ka	2Aa	2Ab	2Ac	2Ae	2Af	2Ag	3Aa	4Ba	4Cb	7Aa	7Ba	8Da	8Ea	8Fa	8Ga	9Aa	9Bb	9Ca	9Da	9Eb	9Ec	9Ee	10Aa	11Aa	15Aa	22Ab	30Fa	30Ga	31Aa	31Ad	32Aa	33Aa	34/35	41Aa	45Aa	48/49A	51Aa	52Ba	54Aa	56Aa	59Ba	63Aa	cyt1Aa	cyt1Ba	cyt2Aa	Vip1A/2A	Vip1Ac	Vip1Ca	Vip2Ac	Vip3Aa	Vip3Ad	Vip3Ae	Vip3Af	Vip3Ag	Vip3Ca
ART	INS	LEP	Arctiidae	1		1				1					1											0								0																																																									
			Bombycidae	1	2	2				0					1		1	1	1		1					1	0	1						0	1											0						1			1								0	0	0	0		0	0		2					0								2					
			Danaidae		1	1															0																															1																																	0					
			Gelechiidae	1	1	1				1	0				2		2		0		0	0			0						1				1	1														1		1																									0							1					
			Geometridae							0					1		1		1																																																																							
			Gracillariidae	1	1	1				1					1	1	1		1		1					1																																																																	
			Hepialidae							1																																								0																																									
			Lasiocampidae	1	1	1				1					1		1		1																															0																																									
			Lycaenidae	1	1	1				1					1		1		1		1										1				1																	1																																								
			Lymantriidae	1	1	1				2	1				1		1		0		1										1				1	1														0		0																																							
			Lyonetiidae	0	0	1				1					0				0																																																																								
			Noctuidae	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1		1	0		1	0	1			0	1	1	1	1	0	1	1	1	1	1	1	0			0		0	0	0	0	1	1	1	0	0	0	0	0			2	0		0	0	0		0			0	0	0	1	1	1	0			0	0	0	0	0	1	0	1	1	1	1
			Notodontidae																																						0																																																	
			Pieridae	1	1	1				1					1		0		1							1					1			1	0	1				1											0																																	0					
			Plutellidae	2	1	1	1		1	1		1	1	1	1		2		0		1		1	1		1	1	1	1	1	1	1	1	0	1	1	0	0		1			0		1	1	0	0	0	1	0	1	0	1	1	1				2	1	1	0	0	1	0		0	0	0		0		1		0	2							1					
			Pyralidae	1	1	1			1	1	1		1	1	1		0		0		1			1		1			1	1	1	1	1		1	1	1	1												1	0	1	0	1	1	1				0							0				0								0	0				1				0	0
			Sphingidae	1	1	1				2					2		1		1							0					1				1	1	1					0		0							1	1						0	1																		0		0	0				1					
			Thaumetopoeidae	1	1	1				1					0				0																																																																								
			Tortricidae	1	1	1	1			1					1	0	1		1	1	1					1			1		0				1	1														1		1							1																			1						1				1	
			Yponeumatidae	1	1	1									1	1					1					1					1				1																																																							

Coleoptera

Phylum	Class	Order	Family	1Aa	1Ab	1Ac	1Ba	1Fa	1Ia	1Ib	1Id	1Ie	1Jb	2Aa	2Ab	3Aa	3Ba	3Bb	3Ca	7Aa	7Ab	8Aa	8Ba	8Bb	8Bc	8Ca	8Da	8Db	8Ea	8Fa	8Ga	8Ka	9Bb	9Da	9Eb	9Ec	9Ee	10Aa	14Aa	15Aa	18Aa	22Aa	22Ab	22Ba	23Aa	34Aa	34Ab	34Ba	35Aa	35Ab	35Ba	34/35	36Aa	37Aa	38Aa	43Aa	43Ba	48/49A	51Aa	55Aa	cyt1Aa	cyt1Ba	cyt2Ca	Vip1A/2A	Vip1Ac	Vip1Ca	Vip2Aa	Vip2Ac	Vip3Aa
ART	INS	COL	Brentidae													1		1	1	1																																																	
			Bruchidae													1																																																					
			Cerambycidae	0			0									1		0		0						0																																											
			Chrysomelidae		0	0	1		1	0	0	0	0	0		1	1	1	1	1	0	1		1	1		0						0	0	0		0		2	0		0	1			0	1	0	0	0	0	1	1		0				1	2	1	1	1	1			0		0
			Coccinellidae		2	0		0						0	0	0		2			1																																																
			Curculionidae			0	1		1					0		1				0												1	0					1				2	2	1														0				1							
			Dermestidae													0																																																					
			Nitidulidae													0																																																					
			Ptinidae													0																																																					
			Scarabaeidae													1					0	1	1			1	1	1	1	1	1			1							1				1									1		1	1												
			Staphylinidae		0											0		0																																																			
			Tenebrionidae						0							1													0	0	0														1									1										0	0	0		0	

Diptera

Phylum	Class	Order	Family	1Ab	1Ac	1Ba	1Bc	1Bd	1Ca	1Gb	1Ia	2Aa	2Ab	2Ac	2Ag	3Aa	4Aa	4Ba	4Cb	9Bb	9Ec	10Aa	11Aa	11Ba	11Bb	15Aa	16Aa	17Aa	19Aa	19Ba	20Aa	24Ba	24Ca	27Aa	29Aa	30Aa	30Ba	30Ca	30Fa	30Ga	31Aa	31Ad	32Aa	32Ba	32Ca	32Da	33Aa	39Aa	40Aa	40Ba	41Aa	44Aa	45Aa	47Aa	48Aa	49Aa	48/49A	52Ba	54Aa	56Aa	59Ba	60Aa	60Ba	63Aa	69Aa	cyt1Aa	cyt1Ab	cyt1Ba	cyt2Aa	cyt2Ba	cyt2Bc	Vip1A/2A	Vip1Ca	Vip2Ac	Vip3Aa
ART	INS	DIP	Agromyzidae																																																																	1							
			Calliphoridae			1																																																1												1			0						
			Chironomidae														0	1				0	1																																											1			1						0
			Culicidae	2	0	0			1		0	1	2	0	1	0	1	1	1	0	0	1	1	1	1	0	1	0	1	1	1	0	1	1	0	0	0	0	1	1	0	0	0	1	1	1	0	1	0	0	0	1	0		0	0	1	1	1	1	0	1	1	0	1	1	1		1	1	1	0	0	0	0
			Drosophilidae									0																																																															
			Muscidae		1	1	1	0		0		0				0				0																					0										0		0																						
			Psychodidae																																						0	0					0				0		0											0											
			Tipulidae	0							0						0	1					1																																											1									
			Tephritidae																																																															1									

Hemiptera

Phylum	Class	Order	Family	1Ab	1Ac	1Bf	1Gc	1Ja	2Aa	2Ab	3Aa	4Aa	11Aa	51Aa	cyt1Aa	cyt1Ba	Vip1A/2A
ART	INS	HEM	Anthocoridae	0	0					0							
			Aleyrodidae						0								
			Aphididae	1	1				1		1	1	1		1		1
			Delphacidae	0													
			Lygaeidae						0								
			Miridae	0		0	0	0	0					1		2	
			Nabidae		0												
			Reduvidae		0												

Hymenoptera

Phylum	Class	Order	Family	1Ab	1Ac	1Ah	2Aa	3Aa	3Bb	5Ac	22Aa	cyt1Ba
ART	INS	HYM	Apinae	0	0	0	0		0			
			Braconidae	0	0		0					
			Encyrtidae		0							
			Diprionidae							1		1
			Formicidae					1			2	
			Pamphiliidae									1
			Pteromalidae		0		0					
			Tenthredinidae		0							1

Other Arthropods

Phylum	Class	Order	Family	1Aa	1Ab	1Ac	1Bf	1Ia	2Aa	2Ab	3Aa	7Ca	30Ga	31Aa	41Aa	45Aa	cyt2Ca
ART	INS	BLA	Blattellidae			0			0					0	0	0	
ART	INS	ISO	Rhinotermitidae						0					0	0	0	
ART	INS	NEU	Chrysopidae	0	2	0	0		2	0							
ART	INS	ORT	Acrididae									1		0	0	0	
			Gryllidae						0								
			Pyrgomorphidae											0	0	0	
ART	INS	SIP	Pulicidae														2
			Thripidae		0												
ART	INS	TRI	Helicopsychidae		2												
			Lepidostomatidae		0												
			Limnephilidae		0												
ART	HEX	CO	Hypogastruridae		0	0			0		0						
			Isotomidae		0	0			0		0						
			Daphnidae		0												
ART	CRU	AMP	Hyalellidae		0												
ART	CRU	IS	Asellidae		0												
			Porcellionidae		0				0								
ART	ARA	AR	Araneidae		0												
			Theridiidae		0												
			Lycosidae		0												
ART	ARA	ACA	Orbatidae		0	0								0			
			Phytoseiidae		0	0											
			Scheloribatidae			0		0									
			Tetranychidae		0												

Non-Arthropods

Phylum	Class	Order	Family	1Ab	1Ac	2Ab	3Bb	5Aa	5Ab	5Ad	5Ba	6Aa	6Ba	12Aa	14Aa	21Aa	55Aa
PLA	TRE	ECH	Fasciolidae					2	2								
NEM	CHR	ASC	Ascarididae								1						
NEM	CHR	DIP	Neodiplogasteridae					0			1	0	0	0	0	0	
NEM	CHR	RHA	Ancylostomatidae								1						
			Cephalobidae					0			1	1	0	0	0	1	
			Haemonchidae							0							
			Heligmonellidae								1	0			1	1	
			Heligmosomatidae								1						
			Panagrolaimidae					0			1	1	0	0	1		
			Rhabditidae	1	0	1	1	2	1		1	1	0	0	1	1	
NEM	CHR	TYL	Meloidogynidae								1	1					1
			Pratylenchidae					1	1			1	1	1			
MOL	GAS		Planorbidae		0												
ANN	CLIT	HAP	Lumbricidae	0													

Table S3. Supplementary data summarising toxicity reported in the literature for *B. thuringiensis* pesticidal proteins outside the order of their primary specificity

LC_{50} = concentration causing 50% mortality.COL = Coleoptera; LEP = Lepidoptera; DIP = Diptera; HEM = Hemiptera; HYM = Hymenoptera; NEU = Neuroptera; TRI = Trichoptera; NEM = Nematoda (phylum); PLA = Platyhelminthes (phylum).

Bioassay methods are indicated by numbers (1 = forced ingestion (droplet feeding, force-feeding); 2a = diet incorporation; 2b = ingestion of transgenic plant tissue (pollen, seed, leaves); 2c = prey or hosts fed with transgenic tissue; 3 = free ingestion (aquatic); 4 = leaf dip; 5a = diet surface; 5b = leaf surface; 5c = toxin-coated prey; na = not available.

Toxin	Order	Genus	Species	Method	LC_{50}	Dose	Units	% mortality	Comment	Reference
Cry1Ab	COL	*Cheilomenes*	*sexmaculatus*	2a		1000	µg/ml	40	control: 10% mortality	Dhillon and Sharma 2009
		Adalia	*bipunctata*	5c		25	µg/ml	49	control: 17% mortality	Schmidt et al. 2009
	DIP	*Aedes*	*aegypti*	3	25-50		µg/ml			Haider et al. 1986
	HEM	*Acyrthosiphon*	*pisum*	2a		500	µg/ml	40	activated toxin	Porcar et al. 2009
	NEU	*Chrysoperla*	*carnea*	2a		100	µg/ml	57	control: 30% mortality	Hilbeck et al. 1998
		Chrysoperla	*carnea*	2c		na		66	control: 25% mortality	Hilbeck et al. 1999
		Chrysoperla	*carnea*	2c		0.72	µg/g	82	control: 44% mortality	Dutton et al. 2002
	TRI	*Helicospyche*	*borealis*	2b		na		43	control: 19% mortality	Rosi-Marshall et al. 2007
	NEM	*Caenorhabditis*	*elegans*	3	54		µg/ml		reproduction inhibition	Höss et al. 2008
		Caenorhabditis	*elegans*	3	225		µg/ml		growth inhibition	Höss et al. 2008
Cry1Ac	DIP	*Glossina*	*morsitans*	2a	0.7		µg/ml		activated toxin	Omolo et al.1997
	HEM	*Acyrthosiphon*	*pisum*	2a		500	µg/ml	71	protoxin	Li et al. 2011
Cry1Ba	DIP	*Musca*	*domestica*	2a	20.0		µg/ml			Zhong et al. 2000
	COL	*Chrysomela*	*scripta*	4	150		µg/ml			Zhong et al. 2000
		Chrysomela	*scripta*	5b	0.3		µg/cm^2			Federici and Bauer 1998
		Chrysomela	*scripta*	5b	0.2		µg/cm^2			Bradley et al. 1995
		Leptinotarsa	*decemlineata*	4	142		µg/ml			Naimov et al. 2001
		Hypothenemus	*hampei*	5a		5.0	µg/cm^2	44		Lopez-Pazos et al. 2009
Cry1Ca	DIP	*Aedes*	*aegypti*	3	141		µg/ml			Smith et al. 1996
		Anopheles	*gambiae*	3	283		µg/ml			Smith et al. 1996
		Culex	*quinquefasciatus*	3	126		µg/ml			Smith et al. 1996
Cry1Ia	COL	*Leptinotarsa*	*decemlineata*	4	33.7		µg/ml			Naimov et al. 2001
		Leptinotarsa	*decemlineata*	4	10.0		µg/ml			Ruiz de Escudero et al. 2006
		Anthonomus	*grandis*	2a	21.5		µg/ml			Martins et al. 2008
Cry2Aa	HEM	*Macrosiphum*	*euphorbiae*	2a		200	µg/ml	93	crystals	Walters and English 1995
	NEU	*Chrysoperla*	*carnea*	2c		na		47	control: 25% mortality	Hilbeck et al. 1999
Cry2Ab	NEM	*Caenorhabditis*	*elegans*	3	23.0		µg/ml		reproduction inhibition	Höss et al. 2013
Cry3Aa	HEM	*Acyrthosiphon*	*pisum*	2a		500	µg/ml	40	protoxin	Porcar et al. 2009
		Acyrthosiphon	*pisum*	2a		500	µg/ml	60	activated toxin	Porcar et al. 2009
		Acyrthosiphon	*pisum*	2a		500	µg/ml	71	protoxin	Li et al. 2011
		Macrosiphum	*euphorbiae*	2a		360	µg/ml	100	crystals	Walters and English 1995
	HYM	*Solenopsis*	*invicta*	2a	0.07		µg/ml		activated toxin	Bulla et al. 2004
		Solenopsis	*invicta*	2a		200	µg/ml	95	activated toxin	Bulla et al. 2004
Toxin	**Order**	**Genus**	**Species**	**Method**	**LC_{50}**	**Dose**	**Units**	**% mortality**	**Comment**	**Reference**
Cry3Bb	NEM	*Caenorhabditis*	*elegans*	3	8.0		µg/ml		reproduction inhibition	Höss et al. 2011, 2013
		Caenorhabditis	*elegans*	3	23.0		µg/ml		growth inhibition	Höss et al. 2011
Cry4Aa	HEM	*Acyrthosiphon*	*pisum*	2a		125	µg/ml	40	protoxin	Porcar et al. 2009
		Acyrthosiphon	*pisum*	2a		125	µg/ml	90	activated toxin	Porcar et al. 2009
Cry8Da	LEP	*Plutella*	*xylostella*	4		2.0	µg/ml	40		Asano et al. 2003
Cry10Aa	COL	*Antohnomus*	*grandis*	2a	7.1		µg/ml			de Souza Aguiar et al. 2012
Cry11Aa	HEM	*Acyrthosiphon*	*pisum*	2a	125-500		µg/ml		protoxin	Porcar et al. 2009
		Acyrthosiphon	*pisum*	2a		125	µg/ml	35	activated toxin	Porcar et al. 2009
		Macrosiphum	*euphorbiae*	2a		350	µg/ml	100	crystals	Walters and English 1995
Cry30Fa	LEP	*Plutella*	*xylostella*	2a	153		µg/ml			Tan et al. 2010
	DIP	*Aedes*	*aegypti*	3	6.5		µg/ml			Tan et al. 2010
Cry30Ga	DIP	*Aedes*	*aegypti*	3	7.1		µg/ml			Zhu et al. 2010a
	LEP	*Plutella*	*xylostella*	4	6.8		µg/ml			Zhu et al. 2010a
Cry51Aa	HEM	*Lygus*	*hesperus*	2a		250	µg/ml	60	crystals	Baum et al. 2012
		Lygus	*hesperus*	2a	72.9		µg/ml		protoxin	Baum et al. 2012
		Lygus	*lineolaris*	2a		200	µg/ml	52	crystals	Baum et al. 2012
	COL	*Leptinotarsa*	*decemlineata*	5a	26.0		µg/cm^2			Baum et al. 2012
Cry54Aa	DIP	*Aedes*	*aegypti*	3	9.0		µg/ml			Tan et al. 2009
	LEP	*Spodoptera*	*exigua*	2a	5.1		µg/ml			Tan et al. 2009
		Helicoverpa	*armigera*	2a	13.6		µg/ml			Tan et al. 2009
Cry56Aa	DIP	*Aedes*	*aegypti*	3	0.15		µg/ml			Zhu et al. 2010b
	LEP	*Plutella*	*xylostella*	2a	16.8		µg/ml			Zhu et al. 2010b
		Helicoverpa	*armigera*	2a	44.2		µg/ml			Zhu et al. 2010b
Cyt1Aa	LEP	*Plutella*	*xylostella*	4	2.4		µg/ml			Sayyed et al. 2001
	COL	*Chrysomela*	*scripta*	5b	0.25		µg/cm^2			Federici and Bauer 1998
	HEM	*Acyrthosiphon*	*pisum*	2a		125	µg/ml	10	protoxin	Porcar et al. 2009
Cyt1Ba	COL	*Hypera*	*postica*	5a		0.50	µg/cm^2	57-91		Bradfisch et al. 1998
		Diabrotica	*virgifera*	5a		4.50	µg/cm^2	90		Payne et al. 1995
	LEP	*Choristoneura*	*fumiferana*	1		0.12	µg/larva		27% mortality	van Frankenhuyzen and Tonon 2013
	DIP	*Lyriomyza*	*trifoli*	2a	10.0		µg/ml			Payne et al. 1995
	HEM	*Lygus*	*hesperus*	2a		4.5	µg/ml		82% mortality	Stockhoff and Conlan 1998
	HYM	*Diprion*	*similis*	1	500		µg/ml			van Frankenhuyzen and Tonon 2013
		Pikonema	*alaskensis*	1		500	µg/ml	71		van Frankenhuyzen and Tonon 2013
		Acantholyda	*erythrocephala*	1		500	µg/ml	54		van Frankenhuyzen and Tonon 2013
		Neodiprion	*sertifer*	1		500	µg/ml	21		van Frankenhuyzen and Tonon 2013
Vip1A/2A	COL	*Diabrotica*	*virgifera*	2a	0.02/0.04		µg/ml			Warren 1997
	HEM	*Aphis*	*gossypii*	2a	0.6		µg/ml			Sampurna and Maiti 2011

Table S4. Supplementary data that support lack of cross-activity of *B. thuringiensis* pesticidal proteins in the high-ranking taxa listed in Fig. 4

(ART = Anthropoda; ANN = Annelida; MOL = Mollusca), classes (INS = Insecta; HEX = Hexapoda; CRU = Crustacea; ARA = Arachnida; GAS = Gastropoda; CLIT = Clitellata) and orders (abbreviations as in Table S2; ISO = Isoptera; ORT = Orthoptera; BLA = Blattodea; THY = Thysanoptera; CO = Collembola; AMP = Amphipoda; IS = Isopoda; CLAD = Cladocera; ACA = Acari; AR = Araneae; HAP = Haplotaxida). Bioassay methods are indicated by numbers as in Table S3. na = not available.
References not listed in the References section of the report or in Supplementary References can be found in van Frankenhuyzen, K., Nystrom, C. (http://www.glfc.cfs.nrcan.gc.ca/bacillus) or in Marvier et al. (http://delphi.nceas.ucsb.edu/btcrops)

Toxin	Phylum	Class	Order	Family	Genus	Species	Method	Dose	Unit	Reference
1Ab	ART	INS	COL	Coccinellidae	*Coleomegilla*	*maculata*	2b	2.75	µg/g	Pilcher et al. 1997
					Coleomegilla	*maculata*	2b	na		Lundgren and Wiedenmann 2004
					Propylea	*japonica*	2c	na		Bai et al. 2006
					Oulema	*melanops*	2b	40	µg/g	Meissle et al. 2012
					Adalia	*bipunctata*	2c	4.7	µg/g	Alvarez-Alfageme et al. 2011
					Adalia	*bipunctata*	2a	45	µg/ml	Alvarez-Alfageme et al. 2011
					Stethorus	*punctillum*	2c	na		Alvarez-Alfageme et al. 2008
					Adalia	*bipunctata*	2a	50	µg/ml	Porcar et al. 2010
					Cryptolaemus	*montrouzieri*	2a	50	µg/ml	Porcar et al. 2010
				Staphylinidae	*Atheta*	*coriaria*	2a	50	µg/ml	Garcia et al. 2010
			DIP	Tipulidae	*Tipula*	*abdominalis*	2b	na		Jensen et al. 2010
			HEM	Aphididae	*Rhopalosiphum*	*padi*	2b	na		Lumbierres et al. 2004
					Rhopalosiphum	*padi*	2b	na		Lozzia et al. 1998
					Rhopalosiphum	*padi*	2b	3.4	µg/g	Dutton et al. 2002
					Sitobion	*avenae*	2b	0.2	µg/g	Ramirez-Romero et al. 2008
				Delphacidae	*Nilaparvata*	*lugens*	2b	na		Bernal et al. 2002
				Miridae	*Cyrtorhinus*	*lividipennis*	2c	na		Bernal et al. 2002
				Anthocoridae	*Orius*	*majusculus*	2c	na		Zwahlen et al. 2000
					Orius	*majusculus*	2b	na		Pons et al. 2004
					Orius	*majusculus*	2b	6	µg/g	Lumbierres et al. 2012
					Orius	*majusculus*	2c	3	µg/g	Lumbierres et al. 2012
					Orius	*insidiosis*	2b	2.75	µg/g	Pilcher et al. 1997
					Orius	*insidiosis*	2b	4.5	µg/g	Al-Deeb et al. 2001
					Orius	*albidipennis*	2c	na		Gonzalez-Zamora et al. 2007
			HYM	Braconidae	*Cotesia*	*marginiventris*	2c	0.62	µg/g	Vojtech et al. 2005
					Cotesia	*flavipes*	2c	na		Prutz and Dettner 2004
				Apidae	*Apis*	*mellifera*	2b	na		Novartis 2000
					Apis	*mellifera*	2b	7	µg/g	Hanley et al. 2003
			NEU	Chrysopidae	*Chrysoperla*	*carnea*	2b	2.75	µg/g	Pilcher et al. 1997
					Chrysoperla	*carnea*	2c	na		Lozzia et al. 1998
					Chrysoperla	*carnea*	2a	1000	µg/ml	Romeis et al. 2004
					Chrysoperla	*carnea*	2c	2.5	µg/g	Dutton et al. 2002
					Chrysoperla	*carnea*	2c	na		Rodrigo-Simón et al. 2006
			THY	Thripidae	*Frankliniella*	*tenuicornis*	2b	3.77	µg/g	Obrist et al. 2005
					Stenchaetothrips	*biformis*	2b	1.4	µg/g	Akhtar et al. 2010
			TRI	Lepidostomatidae	*Lepidostoma*	*spp*	2b	na		Jensen et al. 2010
					Lepidostoma	*liba*	2b	na		Chambers et al. 2007
				Limnephilidae	*Pycnopsyche*	*scabripennis*	2b	na		Jensen et al. 2010
		HEX	CO	Isotomidae	*Folsomia*	*candida*	2a	na		Sims and Martin 1997
					Folsomia	*candida*	2b	2.4	µg/g	Clark and Coats 2006
				Hypogastruridae	*Xenylla*	*grisea*	2a	na		Sims and Martin 1997
		CRU	AMP	Hyalellidae	*Hyalella*	*azteca*	2b	na		Chambers et al. 2007
			IS	Asellidae	*Caecidotea*	*communis*	2b	na		Jensen et al. 2010
				Porcellionidae	*Porcella*	*scaber*	2b	na		Escher et al. 2000
			CLAD	Daphniidae	*Daphnia*	*magna*	2b	na		MRID
		ARA	ACA	Tetranychidae	*Tetranychus*	*urticae*	2b	0.95	µg/g	Lozzia et al. 2000
					Tetranychus	*urticae*	2b	3.4	µg/g	Dutton et al. 2002
				Orbatidae	*Oppia*	*nitens*	2b	na		Yu et al. 1997
				Phytoseiidae	*Noeseiulus*	*cucumeris*	2c	5	µg/g	Obrist et al. 2006
		ARA	AR	Araneidae	*Araneus*	*diadematus*	2b	2.6	µg/g	Ludy and Lang 2006
				Lycosidae	*Pirata*	*subpiraticus*	2c	0.8	µg/g	Chen et al. 2009
	MOL	GAS		Planorbidae	*Gyraulus*	*sp.*	2b	na		Chambers et al. 2007
	ANN	CLIT	HAP	Lumbricidae	*Eisenia*	*fetida*	2a	2.4	µg/g	Clark and Coats 2006
					Lumbricus	*terrestris*	2a	ns		Saxena and Stotsky 2001
					Lumbricus	*terrestris*	2a	15	µg/g	Zwahlen et al. 2003
1Ac	ART	INS	COL	Coccinellidae	*Coleomegilla*	*maculata*	2a	100	µg/ml	Li et al. 2011
					Cheilomenes	*sexmaculatus*	2a	1000	µg/ml	Dhillon and Sharma 2009
					Propylea	*japonica*	2c	0.009	µg/g	Zhang et al. 2006
					Hippodamia	*convergens*	2a	20	µg/ml	Sims 1995
				Curculionidae	*Anthonomus*	*grandis*	2a	100	µg/ml	Sims 1995
				Chrysomelidae	*Leptinotarsa*	*decemlineata*	2a	100	µg/ml	Sims 1995
					Diabrotica	*undecimpunctata*	2a	100	µg/ml	Sims 1995
			DIP	Culicidae	*Aedes*	*aegypti*	2a	100	µg/ml	Sims 1995
			HEM	Aphididae	*Myzus*	*persicae*	2b	na		Schuler et al. 2001
					Myzus	*persicae*	2a	100	µg/ml	Sims 1995
					Aphis	*gossypii*	2b	na		Zhang et al. 2008
					Macrosiphum	*euphorbiae*	2a	400	µg/ml	Walters and English 1995
				Nabidae	*Nabis*	*sp*	2c	na		Ponsard et al. 2002
				Reduviidae	*Zelus*	*tenardii*	2c	na		Ponsard et al. 2002
				Anthocoridae	*Orius*	*sauteri*	2c	na		Zhang et al. 2008
					Orius	*albidipennis*	2a	1000	µg/ml	Gonzalez-Zamora et al. 2007
			HYM	Braconidae	*Cotesia*	*plutella*	2c	na		Schuler et al. 2003, 2004
					Diaeretiella	*rapae*	2c	na		Schuler et al. 2001
					Microplitis	*mediator*	2a	500	µg/ml	Liu et al. 2005
				Encyrtidae	*Copidosoma*	*floridanum*	2c	na		Baur and Boethel 2003
				Pteromalidae	*Nasonia*	*vitripennis*	2a	20	µg/ml	Sims 1995
				Tenthredinidae	*Athalia*	*rosae*	2b	7.5	µg/g	Howald et al. 2003
				Apidae	*Apis*	*mellifera*	2a	20	µg/ml	Sims 1995
					Bombus	*occidentalis*	2b	0.01	µg/g	Morandin and Winston 2003
			NEU	Chrysopidae	*Chrysoperla*	*carnea*	2c	0.285	µg/g	Lawo et al. 2010
					Chrysoperla	*carnea*	1	4	µg/larva	Rodrigo-Simón et al. 2006
					Chrysoperla	*carnea*	2c	na		Rodrigo-Simón et al. 2006
					Chrysoperla	*carnea*	2a	20	µg/ml	Sims 1995
					Chrysopa	*pallens*	2c	na		Guo et al. 2008
			BLA	Blattellidae	*Blatella*	*germanica*	2a	100	µg/ml	Sims 1995
		HEX	CO	Isotomidae	*Folsomia*	*candida*	2a	na		Sims and Martin 1997
				Hypogastruridae	*Xenylla*	*grisea*	2a	na		Sims and Martin 1997
		ARA	ACA	Orbatidae	*Oppia*	*nitens*	2b	na		Yu et al. 1997
				Scheloribatidae	*Scheloribates*	*praeincisus*	2b	na		Oliveira et al. 2007
				Phytoseiidae	*Euseius*	*californicus*	2b	na		de Castro et al. 2012
1Ba	ART	INS	COL	Chrysomelidae	*Phaedon*	*cochleriae*	4	2000	µg/ml	Zhong et al. 2000
				Cerambycidae	*Anoplophora*	*glabripennis*	1	na		D'Amico et al. 2004
			DIP	Culicidae	*Aedes*	*aegypti*	3	2000	µg/ml	Zhong et al. 2000
1Ia	ART	INS	COL	Chrysomelidae	*Agelastica*	*coerulae*	4	1900	µg/ml	Shin et al. 1995
					Phaedon	*brassicae*	4	1900	µg/ml	Shin et al. 1995
					Diabrotica		5a	na		Kostichka et al. 1996
				Tenebrionidae	*Tenebrio*	*molitor*	2a	na		Gleave et al. 1993
			DIP	Tipulidae	*Tipula*	*oleracae*	4	100	µg/ml	Ruiz de Escudero et al. 2006
				Culicidae	*Culex*	*pervigilans*	3	2000	µg/ml	Gleave et al. 1993
	ART	ARA	ACA	Phytoseiidae	*Euseius*	*concordis*	2a	6	µg/ml	de Castro et al. 2012
2Aa	ART	INS	COL	Chrysomelidae	*Leptinotarsa*	*decemlineata*	5a	9	µg/cm²	Sims 1997
					Diabrotica	*undecimpunctata*	5a	9	µg/cm²	Sims 1997
					Diabrotica	*virgifera*	5a	15	µg/cm²	Sims 1997
				Coccinellidae	*Hippodamia*	*convergens*	2a	50	µg/ml	Sims 1997
				Curculionidae	*Anthonomus*	*grandis*	2a	100	µg/ml	Sims 1997
			HEM	Aphididae	*Rhopalosiphum*	*padi*	2a	200	µg/ml	Sims 1997
				Aleyrodidae	*Bemisia*	*tabaci*	2a	200	µg/ml	Sims 1997
				Lygaeidae	*Oncopeltus*	*fasciatus*	2a	200	µg/ml	Sims 1997
				Miridae	*Lygus*	*hesperus*	2a	500	µg/ml	Baum et al. 2012
			HYM	Apidae	*Apis*	*mellifera*	2a	50	µg/ml	Sims 1997
				Braconidae	*Macrocentrus*	*ancylivorus*	2a	200	µg/ml	Sims 1997
				Pteromalidae	*Nasonia*	*vitripennis*	2a	50	µg/ml	Sims 1997
				Braconidae	*Meteorus*	*pulchricornis*	2a	200	µg/ml	Sims 1997
			ISO	Rhinotermitidae	*Reticulitermes*	*flavipes*	2a	200	µg/ml	Sims 1997
			NEU	Chrysopidae	*Chrysoperla*	*sinica*	2b	33.5	µg/g	Wang et al. 2012
					Chrysoperla	*sinica*	2a	300	µg/g	Wang et al. 2012
					Chrysoperla	*carnea*	2a	50	µg/ml	Sims 1997
			ORT	Gryllidae	*Acheta*	*domesticus*	2a	100	µg/ml	Sims 1997
			BLA	Blatellidae	*Blatella*	*germanica*	2a	100	µg/ml	Sims 1997
		HEX	CO	Isotomidae	*Folsomia*	*candida*	2a	na		Sims and Martin 1997
					Folsomia	*candida*	2a	200	µg/ml	Sims 1997
				Hypogastruridae	*Xenylla*	*grisea*	2a	na		Sims and Martin 1997
					Xenylla	*grisea*	2a	200	µg/ml	Sims 1997
		CRU	IS	Porcellionidae	*Porcella*	*scaber*	2a	200	µg/ml	Sims 1997
2Ab	ART	INS	COL	Coccinellidae	*Coleomegilla*	*maculata*	2a	400	µg/g	Li et al. 2011
			HEM	Anthocoridae	*Orius*	*albidipennis*	2c	na		Gonzalez-Zamora et al. 2007
			NEU	Chrysopidae	*Chrysoperla*	*carnea*	2c	na		Rodrigo-Simón et al. 2006
3Aa	ART	INS	LEP	Notodontidae	*Clostera*	*anachoreta*	2b	13	µg/g	Zhang et al. 2011
				Noctuidae	*Spodoptera*	*exigua*	2a	na		Hernstadt et al. 1986
					Trichoplusia	*ni*	2a	na		Hernstadt et al. 1986
			DIP	Culicidae	*Aedes*	*aegypti*	2a	na		Hernstadt et al. 1986
				Muscidae	*Musca*	*domestica*	2a	na		Hernstadt et al. 1986
			HEM	Aphididae	*Macrosiphum*	*euphorbiae*	2b	na		Ashouri et al. 2001
					Myzus	*persicae*	2b	na		Kalushkov and Nedved 2005
		HEX	CO	Isotomidae	*Folsomia*	*candida*	2a	na		Sims and Martin 1997
				Hypogastruridae	*Xenylla*	*grisea*	2a	na		Sims and Martin 1997
8Da	ART	INS	LEP	Noctuidae	*Spodoptera*	*litura*	4	2	µg/ml	Asano et al. 2003
				Bombycidae	*Bombyx*	*mori*	4	2	µg/ml	Asano et al. 2003
11Aa	ART	INS	LEP	Sphingidae	*Manduca*	*sexta*	5a	1	µg/cm²	Feldmann et al. 1995
22Aa	ART	INS	COL	Chrysomelidae	*Diabrotica*	*virgifera*	5a	na		Mettus and Baum 2000
22Ab	ART	INS	LEP	Noctuidae	*Heliothis*	*virescens*	na			Mettus and Baum 2000
					Helicoverpa	*zea*	na			Mettus and Baum 2000
					Spodoptera	*exigua*	na			Mettus and Baum 2000
					Spodoptera	*frugiperda*	na			Mettus and Baum 2000
				Pyralidae	*Ostrinia*	*nubilalis*	na			Mettus and Baum 2000
			COL	Chrysomelidae	*Leptinotarsa*	*decemlineata*	na			Mettus and Baum 2000
					Diabrotica	*undecimpunctata*	na			Mettus and Baum 2000
51Aa	ART	INS	LEP	Noctuidae	*Heliothis*	*virescens*	5a	26	µg/cm²	Baum et al. 2012
					Helicoverpa	*zea*	5a	26	µg/cm²	Baum et al. 2012
					Agrotis	*ipsilon*	5a	26	µg/cm²	Baum et al. 2012
					Spodoptera	*frugiperda*	5a	26	µg/cm²	Baum et al. 2012
				Pyralidae	*Ostrinia*	*nubilalis*	5a	26	µg/cm²	Baum et al. 2012
			COL	Chrysomelidae	*Diabrotica*	*virgifera*	5a	26	µg/cm²	Baum et al. 2012
Vip1A/2A	ART	INS	COL	Chrysomelidae	*Leptinotarsa*	*decemlineata*	2a	na		Warren 1997
				Tenebrionidae	*Tenebrio*	*molitor*	2a	na		Warren 1997
			LEP	Pyralidae	*Ostrinia*	*nubilalis*	2a	na		Warren 1997
				Noctuidae	*Agrotis*	*ipsilon*	2a	na		Warren 1997
					Spodoptera	*exigua*	2a	na		Warren 1997
					Spodoptera	*frugiperda*	2a	na		Warren 1997
					Heliothis	*virescens*	2a	na		Warren 1997
					Helicoverpa	*zea*	2a	na		Warren 1997
				Sphingidae	*Manduca*	*sexta*	2a	na		Warren 1997
			DIP	Culicidae	*Culex*	*pipiens*	3	na		Warren 1997
Cyt1Aa	ART	INS	LEP	Sphingidae	*Manduca*	*sexta*	1	na		Koni et al. 1994
				Gelechiidae	*Pectinophora*	*gossypiella*	2a	1.6	µg/ml	Meyer et al. 2001
				Plutellidae	*Plutella*	*xylostella*	4	40	µg/ml	Meyer et al. 2001

Table S5. Overview of studies assessing interactions among *B. thuringiensis* proteins affecting dipteran activity

reference	*Bt* proteins	species	endpoints	model	interaction	factor [#]
Angsuthanasombat et al., 1992	Cry4Aa-Cry4Ba	*C. quinquefasciatus*	LC_{50}	-	S	5- to 130-fold
		A. aegypti			S	2- to 6-fold
		A. gambiae			S	2- to 3-fold
Chang et al., 1993	Cyt1Aa-Cry11Aa	*C. quinquefasciatus*	LC_{50}, LC_{95}	-	S	1.5-fold
Chilcott and Ellar, 1988	Cyt1Aa-Cry4Aa-Cry4Ba	*A. aegypti*	LC_{50}	-	S	SF= 10*
	Cyt1Aa-Cry11Aa	*A. aegypti*			AD*	SF= 4*
Crickmore et al., 1995	Cyt1Aa-Cry4Aa	*A. aegypti*	LC_{50}	SJA	S	SF= 10
	Cyt1Aa-Cry4Ba	*A. aegypti*			S	SF= 15
	Cyt1Aa-Cry11Aa	*A. aegypti*			S	SF= 2
	Cyt1Aa-Cry4Aa-Cry4Ba-Cry11Aa	*A. aegypti*			S	SF= 6
	Cry4Ba-Cry11Aa	*A. aegypti*			S	SF= 2
	Cry4Aa-Cry4Ba-Cry11Aa	*A. aegypti*			S	SF= 3
Delécluse et al., 1993	Cry4Aa-Cry4Ba	*A. aegypti ; A. stephensi ; C. pipiens*	LC_{50}, LC_{90}	SJA	S	ND
Fernández-Luna et al., 2010	Cyt1Aa-Cry11Aa	*A. albimanus*	LC_{50}	IJA	S	ND
	Cyt1Aa-Cry4Ba	*A. albimanus*			S	ND
	Cyt1Aa-Cry4Ba-Cry11Aa	*A. albimanus*			S	ND
Hernández-Soto et al., 2009	Cyt1Aa-Cry10Aa	*A. aegypti*	LC_{50}	SJA	S	SF= 12.6
Hughes et al., 2005	Cyt1Aa-Cry4Aa	*C. tepperi*	LC_{50}	SJA	S	SF= 2.2
	Cyt1Aa-Cry4Ba				AN	SF= 0.5
	Cyt1Aa-Cry11Aa				AN	SF= 0.5
	Cry4Aa-Cry4Ba				AD	SF= 1.2
	Cry4Aa-Cry11Aa				AD	SF= 0.9
	Cry4Ba-Cry11Aa				AN	SF= 0.7
Jones et al., 2008	Cry4Aa-Cry49Aa	*C. quinquefasciatus*	mortality	-	S	1.7-fold (at 200 µg/ml)
					NE	- (at 20 µg/ml)
					NE	- (at 2 µg/ml)

reference	*Bt* proteins	species	endpoints	model	interaction	factor [#]
Juárez-Pérez et al., 2007	Cry29A-CryBb11	*A. aegypti*	LC_{50}	SJA	S	SF= 4.2
		C. pipiens			AD	SF= 1.2
		A. stephensi			AN	SF= 0.6
	Cry30A-CryB11	*A. aegypti*			AD	SF= 1
		C. pipiens			AD	SF= 0.8
		A. stephensi			AD	SF= 0.9
	Cry29A-Cry30A-Cry11Bb	*A. aegypti*			S	SF= 3.1
		C. pipiens			AD	SF= 1
		A. stephensi			AD	SF= 1
Khasdan et al., 2001	Cyt1Aa-Cry4Aa-P20	*A. aegypti*	LC_{50}	SJA	S	SF= 25.9
	Cyt1Aa-Cry11Aa-P20				S	SF= 34.4
	Cyt1Aa-Cry4Aa-Cry11Aa-P20				S	SF= 3.0
Poncet et al., 1995	Cry4Aa-Cry4Ba	*A. aegypti ; C. pipiens; A. stephensi*	LC_{50}, LC_{90}	SJA	S	SF= 4.4; 14; 2.1
	Cry4Aa-Cry11Aa	*A. aegypti ; C. pipiens; A. stephensi*			S	SF= 3.9; 2.4; 5.4
	Cry4Ba-Cry11Aa	*A. aegypti ; A. stephensi*			AD	SF= 0.9; 1.3
		C. pipiens			S	SF= 2.1
	Cry4Aa-Cry4Ba-Cry11Aa	*A. aegypti ; C. pipiens; A. stephensi*			S	SF= 2.7; 3.5; 2.9
Promdonkoy et al., 2005	Cyt2A2-Cry4Ba	*A. aegypti*	LC_{50}	SJA	S	SF= 33.4
		C. quinquefasciatus			S	SF= 18.5
Wirth et al., 1997	Cyt1Aa-Cry11Aa	*C. quinquefasciatus*	LC_{50}	SJA	S	SF= 3
	Cyt1Aa-Cry4Aa-Cry4Ba				S	SF= 7.2
	Cyt1Aa-Cry4Aa-Cry4Ba-Cry11Aa				S	SF= 2.7
Wirth et al., 1998	Cyt1Aa-Cry11Ba	*C. quinquefasciatus*	LC_{50}	SJA	A	SF= 0.8
Wirth et al., 2004	Cyt1Aa-Cry11Ba	*C. quinquefasciatus*	LC_{50}, LC_{90}	SJA	S	SF_{LC50} = 1.9
						SF_{LC90} = 0.8
Wu and Chang, 1985	Cyt1Aa-Cry11Aa or Cry10A	*A. aegypti*	mortality rate at 16h	-	S	ND
	Cyt1Aa-Cry4Aa-Cry4Ba	*A. aegypti*			S	ND
Wu et al., 1994	Cyt1Aa-Cry11Aa	*A. aegypti*	LC_{50}	-	S	4- to 5-fold

reference	*Bt* proteins	species	endpoints	model	interaction	factor [#]
Yu et al., 1987	Cyt1Aa-Cry11Aa	*A. aegypti*	LD_{50}	-	S	1.8- to 300-fold
Yu et al., 2012	Cyt2Aa-Vip3Aa	*C. quinquefasciatus*	LC_{50}, IC_{50}	SJA	AN	SF= 0.6
		C. tepperi			AD	SF= 1.1

[#] Factors for LC_{90} values are not reported, unless values result in different type of interaction; the *Bt* protein(s) to which the LC_{50} value of the mixture compared with to determine the x-fold factor is (are) are underlined. Reported factors have been round off to one digit after the comma.

*Reanalysis by Tabashnik revealed synergism.

SF = Synergy Factor; S = synergism; AD = additive; AN = antagonism; IJA = Independent Joint Action model; SJA = Similar Joint Action model; LC_{50} = 50% Lethal Concentration; LC_{90} = 90% Lethal Concentration; LD_{50} = 50% Lethal Dose; IC_{50} = 50% growth Inhibition Concentration; ND = Not Determined; NE = No Effect observed.

Table S6. Overview of studies assessing interactions among *B. thuringiensis* proteins affecting lepidopteran activity

reference	*Bt* proteins	species	endpoints	model	interaction	factor [#]
Adamczyk and Gore, 2004	Cry1Ac-Cry1Fa	*S. exigua; S. frugiperda*	mortality	-	NE	-
Bergamasco et al., 2013	Cry1Ia-Vip3Aa	*S. frugiperda*	LC_{50}, LC_{90}	-	S	4.3 to 12.3-fold
		S. albula			S	2.6 to 10.2-fold
		S. cosmioides			S	9.73 to 2.6-fold
		S. eridania			AN	2.10 to 3.7-fold
Binning, 2009	Cry1Ab-Cry1Fa	*O. nubilalis; S. frugiperd; H. zea; D. grandiosella*		EM	NE	-
Brévault et al., 2009	Cry1Ac-Cry2Ab	*H. armigera*	LC_{50} & IC_{50}	SJA	AD	ND
Chakrabarti et al., 1998	Cry1Ac-Cry1Ab	*H. armigera*	ID_{50}	SJA	NE	ND
	Cry1Ac-Cry1Fa				S	SF= 12.3 to 26.3
	Cry1Ac-Cry2Aa				NE	ND
Del Rincón-Castro et al., 1999	Cyt1Aa-Cry1Ac	*T. ni*	LC_{50}	-	AN	6.5- to 7.9-fold
			LC_{50}	SJA	AN	SF= 0.3 to 0.6
Gao et al., 2010	Cry1Aa-Cry1Ab	*C. suppressalis*	LC_{50}	SJA	S	SF= 4.9
		S. inferens			S	SF= 1.6
	Cry1Aa-Cry1Ca	*C. suppressalis*			S	SF= 1.6
		S. inferens			AN	SF= 0.1
	Cry1Ab-Cry1Ac	*C. suppressalis*			S	SF= 2.6
		S. inferens			AN	SF= 0.7
	Cry1Ab-Cry1Ba	*C. suppressalis*			S	SF= 11.0
		S. inferens			AN	SF= 0.3
	Cry1Ab-Cry1Ca	*C. suppressalis*			S	SF= 4.4
		S. inferens			AN	SF= 0.2
	Cry1Ac-Cry1Ca	*C. suppressalis*			S	SF= 3.1
		S. inferens			AN	SF= 0.6
	Cry1Ac-Cry1Ba	*C. suppressalis*			S	SF= 4.5
		S. inferens			AN	SF= 0.2

reference	*Bt* proteins	species	endpoints	model	interaction	factor [#]
Greenplate et al., 2003		*H. zea*	population response	IJA	AD	$X^2_{test} < X^2_{tabular}$
		H. virescens			AD	$X^2_{test} < X^2_{tabular}$
		S. frugiperda			AD	$X^2_{test} < X^2_{tabular}$
Guerreiro-Filho et al., 1998	Cry1Ac-Cry1Ba	*P. coffeella*	LC_{50}	SJA	AD	SF= 0.9
Herman and Storer, 2004	Cry1Fa-Cry34/35Ab1	*O. nubilalis*	mortality & weight	-	NE	-
Hunter, 2006	Cry1A.105-Cry2Ab2-Cry3Bb1	*O. nubilalis*	LC_{50} & MIC_{50}	-	NE	-
Ibargutxi et al., 2008	Cry1Ac-Cry1Fa	*H. armigera*	LC_{50} & ID_{50}	SJA	AD	SF_{LC50}= 0.7 to 1.0 SF_{ID50}= 1.4 to 1.7
		E. insulana			AD	SF_{LC50}= 0.9 to 1.1 SF_{ID50}= 0.8 to 1.1
	Cry1Ac-Cry2Ab	*H. armigera*	LC_{50} & ID_{50}	SJA	S	SF_{LC50}= 1.6 to 3.1 SF_{ID50}= 0.7-1.4
		E. insulana			AD	SF_{LC50}= 0.9-1.0 SF_{ID50}= 1.0-1.2
Khasdan et al., 2007	Cry1Ac-Cry1Ca	*H. armigera*	LC_{50}, LC_{90}	-	NE	-
	Cry1Ac	*P. gossypiella*			NE	-
	Cry1Ca	*S. littoralis*			S	82-fold
	Cry1Ac-Cyt1Aa-P20	*H. armigera*			NE	-
		P. gossypiella			NE	-
	Cry1Ac-Cry1Ca-Cyt1Aa-P20	*H. armigera*			S	16-fold
		P. gossypiella			NE	-
		S. littoralis			NE	-
Lee et al., 1996	Cry1Aa-Cry1Ac	*L. dispar*	ID_{50}	SJA	S	SF= 2.7 to 7.3
	Cry1Ab-Cry1Ac				AD	SF= 1.5
	Cry1Aa-Cry1Ab				AN	SF= 0.3

reference	*Bt* proteins	species	endpoints	model	interaction	factor [#]
Levine et al., 2008	Cry1Ab-Cry1A.105-Cry2Ab	*O. nubilalis*	ID_{50}	SJA	AD	SF= 1.1
	Cry1Ab-Cry1A.105-Cry2Ab-Cry34/35Ab-Cry3Bb		ID_{50}	-	NE	-
Liao et al., 2002	Cry1Ac-Cry2Aa	*H. armigera*	LC_{50}	SJA	AN	SF_{LC50}= 0.4-0.7
				IJA	AN	SF_{LC50}= 0.3-0.6
Meyer et al., 2001	Cyt1Aa- HD-1 or Dipel *	*P. xylostella*	mortality		AN	ND
	Cyt1Aa – MVPII*	*P. gossypiella*			NE	-
Pang et al., 2002	Cry1Aa-Cry9Ca	*C. fumiferana*	FFD_{50}	SJA	AD	SF= 1.2
	Cry1Ab-Cry9Ca				AD	SF= 0.7-1.2
Raybould et al., 2010	Cry1Ab-mCry3A	*L. decemlineata*	mortality	EM	NE	-
Sayyed et al., 2001	Cyt1Aa-Cry1Ac	*P. xylostella*	LC_{50}	SJA		
		- unselected population			S	SF= 2
		- insecticide susceptible population			S	SF= 11
Sauka et al., 2007	Cry1Aa-Cry1Ab	*E. aporema*	LC_{50}	SJA	AN	SF= 0.8
	Cry1Aa-Cry1Ac				AN	SF= 0.5
	Cry1Ab-Cry1Ac				AN	SF= 0.7
	Cry1Aa-Cry1Ab-Cry1Ac				AN	SF= 0.7
	Cry1Ba-Cry1Ca				AD	SF= 1.0
	Cry1Ba-Cry1Da				S	SF= 1.4
	Cry1Ca-Cry1Da				AN	SF= 0.8
	Cry1Ba-Cry1Ca-Cry1Da				AD	SF= 1.0
Sharma et al., 2010	Cry1Ab-Cry1Ac	*C. partellus*	LC_{50}	SJA	S	SF= 2.0 to 5.2
	Cry1Aa-Cry1Ab				AD-S	SF= 1.2
	Cry1Aa-Cry1Ac				AD-S	SF= 1.5
Tabashnik et al., 2013	Cry1AbMod-Cry2Ab	*P. gossypiella*	mortality	IJA	S	2-25%
	Cry1AbMod-Cry1Ac				NE	-6 - 13%
	Cry1Ac-Cry2Ab				NE	0%
	Cry1AbMod-Cry1Ac-Cry2Ab				NE	-3 - 1%

reference	*Bt* proteins	species	endpoints	model	interaction	factor [#]
Tounsi et al., 2005	Cry1Ac-Cry2Aa	*E. kuehniella*	LC_{50}	SJA	AN	SF= 0.2 to 0.6
	Cry1Aa-Cry1Ac				S	ND
	Cry1Aa-Cry2Aa				S	ND
van Frankenhuyzen et al., 1991	Cry1Aa-Cry1Ab-Cry1Ac	*C. occidentalis; C. fumiferana; M. disstria*	FFD_{50}		NE	-
		C. pinus; O. leucostigma			AD[#]	SF= 1
		L. dispar			AD[#]	SF= 1
Xue et al., 2005	Cry1Aa-Cry1Ca	*H. armigera*		SJA	S	SF= 1.4 to 2.7
		S. exigua			S	SF= 2 to 4
Yu et al., 2012	Vip3Aa29-Cyt2Aa3	*H. armigera*	LC_{50} & IC_{50}	SJA	AD	SF= 1.0
		C. suppressalis			AN	SF= 3.3
		S. exigua			S	SF= 4.3
Yunus et al., 2011	Cry1Ac-Cry2Aa	*E. vitella*	LC_{50}	-	S	1.6- to 125-fold
Zhu et al., 2006	YBT1520-Vip3Aa	*H. armigera*	LC_{50}	-	NE	-
		S. exigua			S	10-fold

[#] Factors for LC_{90} values are not reported, unless values result in different type of interaction; the *Bt* protein(s) to which the LC_{50} value of the mixture compared with to determine the x-fold factor is (are) are underlined; Reported factors have been round off to one digit after the comma.

*Dipel and HD-1 contain (Cry1Aa, Cry1Ab, Cry1Ac, Cry2A); MVPII contains Cry1Ac. LC_{50} = 50% Lethal Concentration; LC_{90} = 90% Lethal Concentration; LD_{50} = 50% Lethal Dose; IC_{50} = 50% growth Inhibition Concentration; ID_{50} = 50% growth Inhibition Dose; FFD_{50} = 50% Frass Failure Dose; IJA = Independent Joint Action model; SJA = Similar Joint Action model; EM = Empirical Model; ND = Not Determined; NE = No Effect observed; SF = Synergy Factor; S = synergism; AD = additive; AN = antagonism.