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**The structure/function of new insecticidal proteins and
regulatory challenges for commercialization**

William Moar, Colin Berry and Kenneth Narva

Genetically modified crops produced by biotechnology methods have provided grower benefits since 1995 including improved protection of crop yield, reduced input costs, and a reduced reliance on chemical pesticides (Klumper and Qaim, 2014). These benefits have driven annual increases in worldwide adoption of GM crops, with the largest number of hectares being grown in the Americas (ISAAA 2014). In 2014, the majority of global biotech crops were planted to soybean (90.7 million hectares), maize (55.2 million hectares) and cotton (25.1 million hectares). Herbicide tolerance and insect resistance traits are by far the most widely commercialized biotech traits. Of the 181.5 MM hectares of biotech crops grown in 2014, approximately 43% (79 MM hectares) contained insect resistance traits alone or stacked in combination with herbicide tolerance traits (ISAAA 2014).

Among insect resistance traits most commercial events are based on 3-domain crystalline (Cry) or vegetative insecticidal proteins (VIPs) from *Bacillus thuringiensis* (Bt) (Table 1). The long-term success of these traits has depended on the use of insect resistant management (IRM) strategies to delay insect resistance (Gould 1998). Today there are several examples of insect pest populations that have evolved resistance to one or more Bt traits due to multiple generations of selection arising from deployment of these crops (Carriere et al. 2016). Field-evolved resistance to Bt proteins in crops such as maize and cotton requires new tools to manage the affected insect populations and continue to derive benefits from these Bt crops. One approach to counter insect resistance to single traits is to combine (pyramid) two or more proteins with differences in their mechanisms of action (MOA) that are effective against the target pest(s). For

example, SmartStax® maize was the first pyramided Bt crop offering protection using two distinct mechanisms of action (Cry3Bb1 and Cry34Ab1/Cry35Ab1) against the western corn rootworm, *Diabrotica virgifera virgifera*.

Table 1. Commercially registered *Bacillus thuringiensis* 3-domain and VIP insecticidal proteins

Developer	Event Name	OECD Unique Identifier	Bt Protein(s)	Pest Spectrum	Year Approved (Cultivation - USA)	Non-IR Genes ¹
Syngenta	176	SYN-EV176-9	Cry1Ab	Lepidoptera	1995	<i>pat</i>
Monsanto	MON 810	MON-00810-6	Cry1Ab	Lepidoptera	1996	<i>nptII</i>
Syngenta	Bt11	SYN-BT011-1	Cry1Ab	Lepidoptera	1996	<i>pat</i>
Dekalb Genetics Corporation	DBT418	DKB-89614-9	Cry1Ac	Lepidoptera	1997	<i>bar</i>
Aventis CropScience	CBH-351 ²	ACS-ZM004-3	Cry9C	Lepidoptera	1998	<i>pat</i>
Dow AgroSciences DuPont Pioneer	TC1507	DAS-01507-1	Cry1Fa	Lepidoptera	2001	<i>pat</i>
Monsanto	MON863	MON-00863-5	Cry3Bb1	Coleoptera	2003	<i>nptII</i>
Dow AgroSciences DuPont Pioneer	DAS-59122-7	DAS-59122-7	Cry34Ab1 Cry35Ab1	Coleoptera	2005	<i>pat</i>
Monsanto	MON88017	MON-88017-3	Cry3Bb1	Coleoptera	2005	<i>cp4 epsps</i>
Syngenta	MIR604	SYN-IR604-5	mCry3A	Coleoptera	2007	<i>pmi</i>
Monsanto	MON89034	MON-89034-3	Cry1A.105 Cry2Ab	Lepidoptera	2008	
Syngenta	MIR162	SYN-IR162-4	Vip3Aa20	Lepidoptera	2010	<i>pmi</i>
Syngenta	5307	SYN-05307-1	eCry3.1Ab	Coleoptera	2012	<i>pmi</i>

¹Non-IR (insect resistance) Genes *pat*: a selectable marker which confers tolerance to the herbicide glufosinate ammonium in plant tissue. *nptII*: a selectable marker which confers the ability to metabolize the antibiotics neomycin and kanamycin in plant tissue. *bar*: a selectable marker that confers tolerance to the herbicide glufosinate ammonium in plant tissue. *cp4 epsps*: a selectable marker confers tolerance to the herbicide glyphosate in plant tissue. *pmi*: a selectable marker that confers the ability to utilize mannose as a carbon source in plant tissue.

² Approved for environmental release and use as animal feed only.

Classes of proteins that are not cross resistant to currently commercialized insect resistance traits, and control other pests not controlled by current products, are needed. Table 2 depicts some of the non-3-domain insecticidal proteins currently in various stages of trait development.

Table 2. Non-3 domain insecticidal proteins in commercial development pipelines

Developer	Protein name	Protein structure family	Source	Pest spectrum	Reference
Monsanto	Cry51Aa2.834_16	Beta pore forming protein	<i>B. thuringiensis</i>	Hemiptera	Gowda et al. 2016
Monsanto	TIC2463	Beta pore forming protein	<i>B. thuringiensis</i>	Coleoptera	US20150274786
Dow AgroSciences	Cry6Aa	Alpha helical pore forming proteins	<i>B. thuringiensis</i>	Coleoptera	Dementiev et al. 2016
Bayer Crop Sciences	GNIP1Aa	membrane attack complex/perforin (MACPF) superfamily	<i>Chromobacterium piscinae</i>	Coleoptera	This issue
DuPont Pioneer	PIP-72Aa	unknown	<i>Pseudomonas chlororaphis</i>	Coleoptera	WO2015/038,734
DuPont Pioneer	AfIP-1A, AfIP-B	AfIP-1A: Agerolysin PFAM	<i>Alkaligenes faecalis</i>	Coleoptera	US20140033361
Monsanto	TIC 3670	Beta-pore forming protein	<i>Brevibacillus laterosporus</i>	Coleoptera	US20160319302

The 3-domain group of insecticidal Cry proteins has been the subject of extensive study over many years, including the first structure that was published in 1991 (Li et al. 1991). In contrast, our knowledge of non-3-domain toxins is far less advanced. Understanding of the mechanisms of action of these new families of insecticidal proteins will be greatly facilitated by elucidation of their structures. Knowledge of structure and function may allow toxin modification to modulate and retarget their activity, help to delay resistance development to existing traits, and also contribute to predictions of their specificity (target pests and non-target species) that can be validated through experimental testing, and when history of safe use (HOSU) information is

limited, as was for many of the examples in Tables 1-2. Recent advances in this field have increased the number of non-3-domain protein structures available, thus improving our understanding of the relationship between structure and function, resulting in a more knowledgeable prediction of activity. Nonetheless, major challenges remain.

This Journal of Invertebrate Pathology Special Issue is primarily a compilation of manuscripts from two meetings of the Society for Invertebrate Pathology (SIP) that aimed to assess the current state of the art in structure, function and commercial development of non-3-domain proteins. Papers arising from these meetings are presented here to make them available to a wider audience and to suggest directions for further research to advance the field. Papers are derived from a symposium at the 2014, 47th Annual SIP meeting in Mainz, Germany organized by Ken Narva and Colin Berry: “Structure and Function of Novel Insecticidal Toxins”, followed by a complementary workshop at the 2015 International Congress on Invertebrate Pathology and Microbial Control, and the 48th Annual SIP meeting in Vancouver, British Columbia, Canada organized by William Moar and Ken Narva: “Regulatory Considerations for the Commercialization of New Insecticidal Proteins”. An overview of the presentations is shown in Table 3, below.

Table 3. Structure/function presentations at the 2014 and 2015 SIP conferences.

<p>2014 SIP Conference Symposium: Structure and Function of Novel Insecticidal Toxins Organizers/Moderators: Ken Narva and Colin Berry</p>
<p>1. Structural and biophysical characterization of Cry34Ab1 and Cry35Ab1 Matthew S. Kelker, Colin Berry, Matthew D. Baker, Steven L. Evans, Reetal Pai, David McCaskill, Joshua C. Russell, Nick X. Wang, J.W. Pflugrath, Cheng Yang, Matthew Wade, Tim J. Wess, Kenneth E. Narva</p>
<p>2. Structure/function studies of Cry5B via alanine scanning mutagenesis Jillian Sesar; Melanie Miller, Yan Hu., Raffi V. Aroian</p>
<p>3. Insights into the structures of non-3-domain toxins through structural modelling Colin Berry</p>
<p>4. Novel MTX Toxins for Insect Control Yong Yin</p>
<p>5. Insecticidal toxins from <i>Photorhabdus luminescens</i> and <i>asymbiotica</i>, targeting the actin cytoskeleton and GTP-binding proteins Thomas Jank, Alexander E. Lang, Klaus Aktories</p>
<p>6. Molecular basis of parasporin-2 action toward cancer cells Sakae Kitada, Yusuke Yoshida, Yoshimi Ozaki, Hirioyasu Shimada</p>
<p>2015 SIP Conference Bacteria Division Workshop: Regulatory Considerations for the Commercialization of New Insecticidal Proteins Organizers/Moderators: William Moar and Ken Narva</p>
<p>1. Current insights on Bt insecticidal protein specificity and future direction Juan Luis Jurat-Fuentes, Neil Crickmore</p>
<p>2. Proteins 101: structure, function, and evolution Joe Jez</p>
<p>3. Protein sequences, structures and functions: rules for divergence and rules for conservation Adam Godzik</p>
<p>4. Modelling of insecticidal toxins and their potential interactions: Challenges and aspirations Colin Berry, Neil Crickmore</p>
<p>5. Safety considerations derived from Cry34/35Ab1 structure and function Kenneth E. Narva, Nick Storer, Rod Herman</p>
<p>6. Case study of a novel CRW insecticidal protein from <i>Chromobacterium</i> sp. Kimberly Sampson</p>
<p>7. Biochemical characterization of parasporin-4 and effects of the pro-parasporin-4 diet on the health of mice Shiro Okumura, Hironori Koga, Kuniya, Inouye, Eiichi Mizuki</p>
<p>8. Considerations for the safety assessment of novel insect control proteins: a regulatory perspective Phil MacDonald</p>
<p>9. Domain-based specificity of insecticidal β-pore forming proteins supports the overall safety assessment William J. Moar, Jeff Haas, Artem Evdokimov, Jim Baum, Andre Silvanovich, Yong Yin, Dave Bowen, Kevin Glenn, Adam Evans</p>

The goal of the 2014 symposium was to discuss new information on the structure and function of new insecticidal proteins while the 2015 workshop built on the 2014 symposium and discussed how knowledge of the structure/functions of new insecticidal proteins can address various topics (primarily non-target safety) required for regulatory approval. Since the 2014 symposium and 2015 workshop, a symposium entitled “Novel Insecticidal Agents and Next Gen Approaches for Insect Control” was held at the 2016 International Congress of Entomology Conference in Orlando, Florida representing the next in a series of ongoing, global scientific discussions on new insecticidal proteins, whose purpose was to share the state of the art of the technology, promote further research, and to assess and promote safe uses of the technology. Given the increasing number of insect resistance traits with elucidated protein structures we anticipate this area of research to be actively discussed in future meetings such as SIP.

Since the 2014 SIP Symposium, peer-reviewed manuscripts have been published demonstrating 1) numerous new insecticidal proteins are being developed to control insect pests and 2) their structures have been elucidated, and integrating this structural information with biochemical and bioinformatic analyses can enable testing and identification of structural and functional domains responsible for toxicity and specificity (Carriere et al. 2015; Dementiev et al. 2016; Gowda et al. 2016; Kelker et al. 2014; Xu et al. 2015). New information on insecticidal protein structure and function is being used to select candidates for crop improvement based on predictions of target pest specificity and non-target organism safety, and is important in designing effective pyramids for resistance management. Structure/function information is being further exploited to engineer proteins with improved attributes for broader pest specificity and increased potency while maintaining safety to other species.

In this Special Issue, there are 11 manuscripts that represent presentations from the 2014 symposium, the 2015 workshop, or relevant topics such as specificity and hazard (Table 4)

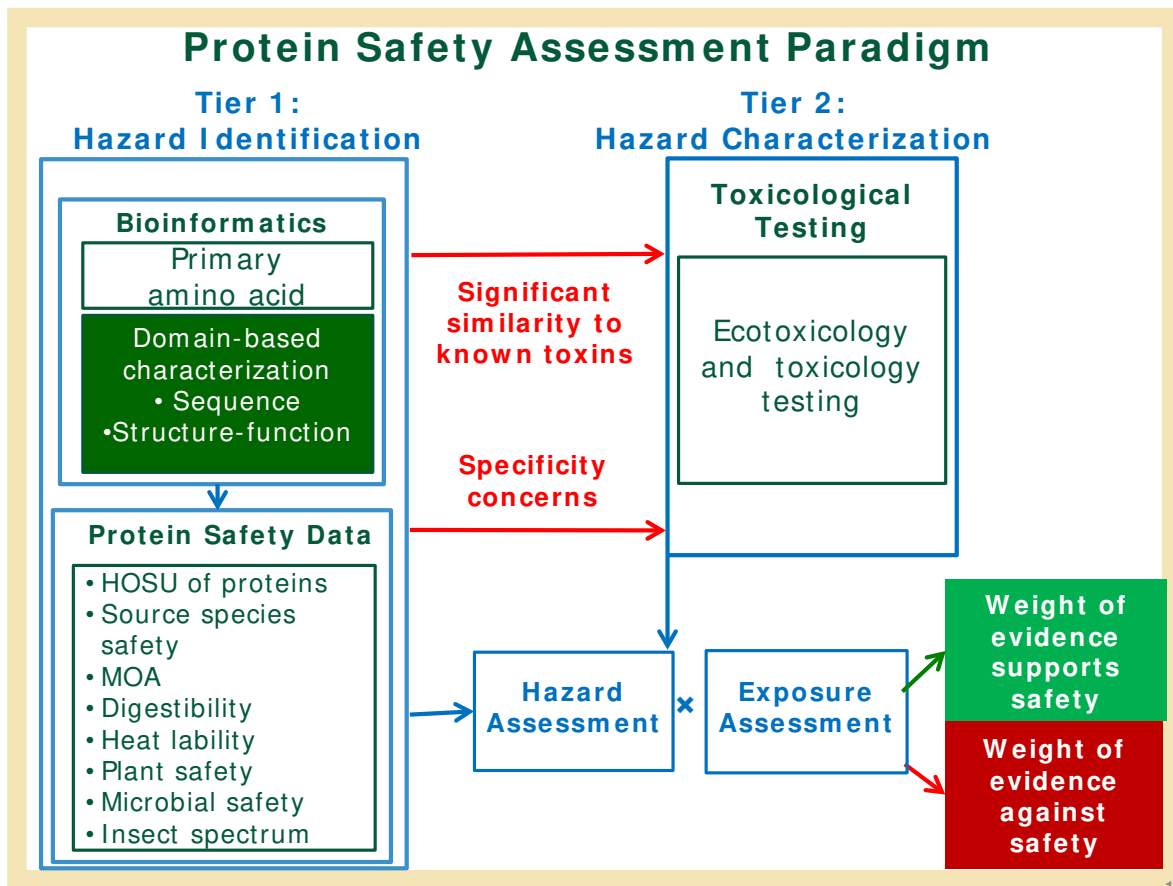
Table 4. Articles contained in this Special Issue of Journal of Invertebrate Pathology

Specificity determinants for Cry insecticidal proteins: insights from their mode of action. Jurat-Fuentes and Crickmore
Revisiting Protein Structure, Function, and Evolution in the Genomic Era. Jez
Structural classification of insecticidal proteins - towards an <i>in silico</i> characterization of novel toxins. Berry and Crickmore
The use of structural modelling to infer structure and function in biocontrol agents. Berry and Board
Safety considerations derived from Cry34Ab1/Cry35Ab1 structure and function. Narva <i>et al.</i>
Discovery of a novel insecticidal protein from <i>Chromobacterium piscinae</i>, with activity against Western Corn Rootworm, <i>Diabrotica virgifera virgifera</i>. Sampson <i>et al.</i>
Parasporins 1 and 2: their structure and activity. Akiba and Okumura
The sequence, structural, and functional diversity with a protein family and implications for specificity and safety: The case for ETX_MTX2 insecticidal proteins. Moar <i>et al.</i>
Insecticidal spectrum and mode of action of the <i>Bacillus thuringiensis</i> Vip3Ca insecticidal protein. Gomis-Cebolla <i>et al.</i>
Use of Species Sensitivity Distributions to Characterize Hazard for Insecticidal Traits. Boeckman and Layton
Glycan region of GPI anchored-protein is required for cytotoxic oligomerization of an anticancer parasporin-2, Cry46Aa1 protein, from <i>Bacillus thuringiensis</i> strain A1547. Abe <i>et al.</i>

One of the major conclusions of the 2015 workshop was that bioinformatics can take advantage of sequence, structural, and functional information, to characterize each protein domain individually, as well as whole proteins, to help inform the tiered approach to hazard identification for the protein safety regulatory assessment (Fig 1.). The basis for this tiered approach is the long known understanding that protein function is derived from the tertiary structure of protein domains, with each domain responsible for different aspects of protein

function. The various domains have different impacts on species/cell receptor specificity, and therefore can impact biosafety (positively or negatively) in environmental or agricultural uses. For insecticidal 3-domain Cry and aerolysin-like beta pore-forming proteins, some structural/functional domains are involved principally in forming the pore or in oligomerization, while other domains are demonstrated sites of specific cell receptor binding conferring specificity. Therefore, including differences and similarities in the receptor binding domain (that can be elucidated using bioinformatics) into the tiered safety assessment paradigm should increase our ability to predict safety in insecticidal proteins new to GM crops.

Fig. 1. **Hazard Assessment of New Insecticidal Proteins with the addition of Domain-based Characterization bioinformatics**



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