

Many roads to resistance: how invertebrates adapt to Bt toxins

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Summary

The Cry family of *Bacillus thuringiensis* insecticidal and nematocidal proteins constitutes a valuable source of environmentally benign compounds for the control of insect pests and disease agents. An understanding of Cry toxin resistance at a molecular level will be critical to the long-term utility of this technology; it may also shed light on basic mechanisms used by other bacterial toxins that target specific organisms or cell types. Selection and cross-resistance studies have confirmed that genetic adaptation can elicit varying patterns of Cry toxin resistance, which has been associated with deficient protoxin activation by host proteases, and defective Cry toxin-binding cell surface molecules, such as cadherins, aminopeptidases and glycolipids. Recent work also suggests Cry toxin resistance may be induced in invertebrates as an active immune response. The use of model invertebrates, such as *Caenorhabditis elegans* and *Drosophila melanogaster*, as well as advances in insect genomics, are likely to accelerate efforts to clone Cry toxin resistance genes and come to a detailed and broad understanding of Cry toxin resistance. *BioEssays* 27:614–624, 2005. © 2005 Wiley Periodicals, Inc.

Introduction

Bacillus thuringiensis (Bt) is an invertebrate pathogen whose virulence is largely attributable to large crystalline inclusions produced during sporulation.⁽¹⁾ These inclusions are composed of pore-forming proteins known as crystal (Cry) toxins. Unlike chemical pesticides, Cry toxins are macromolecules that often affect only a narrow range of species, leaving vertebrates and most beneficial invertebrates unaffected. The Cry protein family provides a rich diversity of toxin variants with different spectra of activity.^(2,3) Given these desirable properties, Cry toxins are currently deployed on an enormous scale to combat insect pests (especially of corn and cotton) and disease vectors, like mosquitoes and blackflies. Transgenic

crops expressing insecticidal Cry toxin genes were planted on ~14 million ha worldwide in 2002.⁽⁴⁾ This kind of selective pressure makes the genetic adaptation of insect targets a real possibility. Two pest species (*Plutella xylostella* and *Trichoplusia ni*) have evolved Cry toxin resistance in the field,^(5–8) but more are likely to follow.

A molecular-level understanding of Cry toxin resistance will undoubtedly be important for ensuring lasting utility for Bt-based technologies already existing, as well as those that may emerge in the future. For example, the observation that Cry proteins can intoxicate animal parasitic nematodes⁽⁹⁾ may open the way to new medicinal applications for these molecules as anthelmintics. Knowledge of resistance mechanisms will throw much-needed light on the mode of action of Cry toxins at a molecular level, and will provide the opportunity to monitor resistance outbreaks at early stages in target invertebrate populations. Research into Cry toxin resistance also provides an opportunity to gain fundamental insights into a more general question about pathogenesis: how bacterial pore-forming toxins recognize and compromise their targets. This review will discuss the complexity of Cry toxin resistance, as interpreted from toxicological, biochemical and genetic studies, and will cover recent findings that have given us a better grasp on the diverse molecular resistance mechanisms available to invertebrate targets.

Simple models of cry toxin action and resistance mechanisms

Cry toxins constitute a family of proteins that are active against numerous invertebrate targets (Fig. 1). Cumulatively, Cry toxins are active against a wide spectrum of insects, including Lepidoptera (moths), Coleoptera (beetles), Diptera (mosquitoes and flies), and Hymenoptera (wasps and bees), and against nematodes.⁽²⁾ Of the >200 reported Cry sequences, those used in resistance studies and discussed in this review are shown, color-coded with respect to activity spectrum. It is clear that similar sequences tend to have similar target spectra. However, single Cry toxins, such as Cry1Ba, can affect a broad class of organisms, and very distantly related toxins, such as Cry1Aa and Cry2Aa, can have similar activity spectra. Structural studies have shown that Cry toxins that appear highly divergent by sequence analysis (e.g. Cry1Aa, Cry3Aa, Cry2Aa, ~20% amino acid identity in pairwise analyses) have remarkably similar three-domain structures^(10–12) (Fig. 1B).

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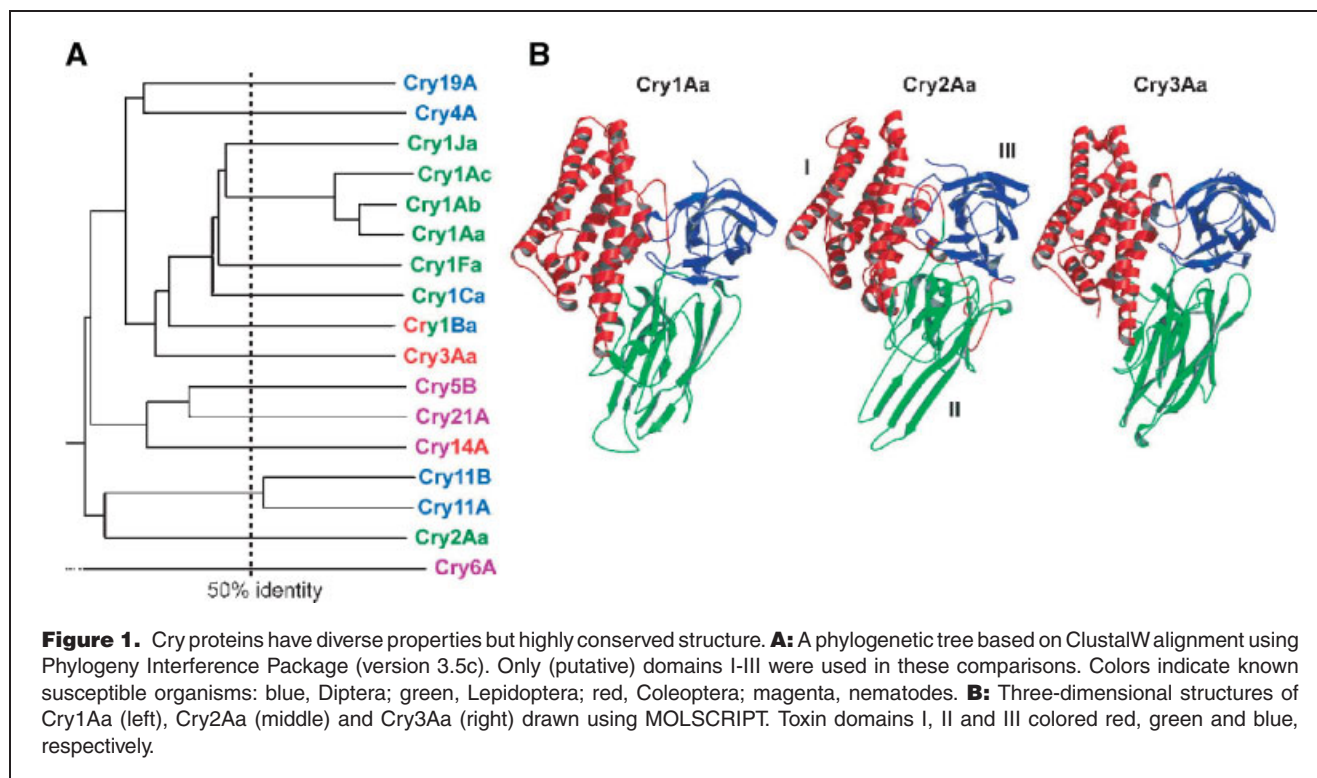
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The high degree of structural conservation among Cry toxins suggests that they possess a fundamental mechanism of action. Indeed, numerous physiological and biochemical studies with various toxins and target animals support the following two-phase mechanism of Cry toxin action: i) solubilization and proteolytic activation in the midgut, and ii) specific binding to intestinal cells and cytolytic pore formation⁽¹⁾ (Fig. 2). A previous review by Heckel⁽¹³⁾ summarized a number of distinct physiological scenarios that could allow an organism to overcome Cry toxin action assuming the above model. These scenarios include defective solubilization, deficient proteolytic activation, over-proteolysis (i.e. degradation of toxin), sequestration of toxin molecules by non-functional binding sites, defects in functional binding sites, defective pore formation and enhanced cellular repair. Some of these scenarios are illustrated in Fig. 2. For a given case of Cry toxin resistance in a typical insect pest, determining which of these (or other) resistance mechanisms has occurred, and defining the molecules that actually control them, can be difficult. Moreover, a comprehensive understanding of Cry toxin resistance mechanisms must account for the many known Cry toxins with their distinct spectra of target organisms. This potentially makes the study of resistance a complex, case-by-case effort. Fortunately, some relatively simple toxicological experiments suggest that, for the many known Cry toxins, there are a limited number of resistance mechanisms, but there are certainly more than one.

Selection and toxicological studies

The isolation of heritably resistant organisms is an important step for any rigorous demonstration of resistance mechanism. The common method of selecting resistant insect strains has been to rely on natural resistance alleles that are presumed to be rare in the starting population. In this method, a population reared on a toxin-laced diet is expected to become enriched in resistance alleles as it adapts to the selective pressure over multiple generations. This kind of method has been relied upon repeatedly to study resistance, with at least 60 resistant strains in at least 12 insect species reported.^(14,15) The advantage of this method is that it may select for alleles that are present in field populations; the potential difficulty of this method is that the genetic heterogeneity in these populations may lead to complex modes of resistance that are difficult to study. Furthermore, the starting population may, by chance, be devoid of resistance alleles.

Studies on lepidoptera

Because of their enormous economic importance, lepidopteran pests have been the subject of numerous selection studies. A few examples are shown in Table 1. These examples are meant to depict the range of Cry toxins used in selection studies, and the diversity of resistance levels and cross-resistance patterns that emerge from these selection experiments. Cross resistance here is defined to mean resistance to a toxin other than that to which the resistant strain was

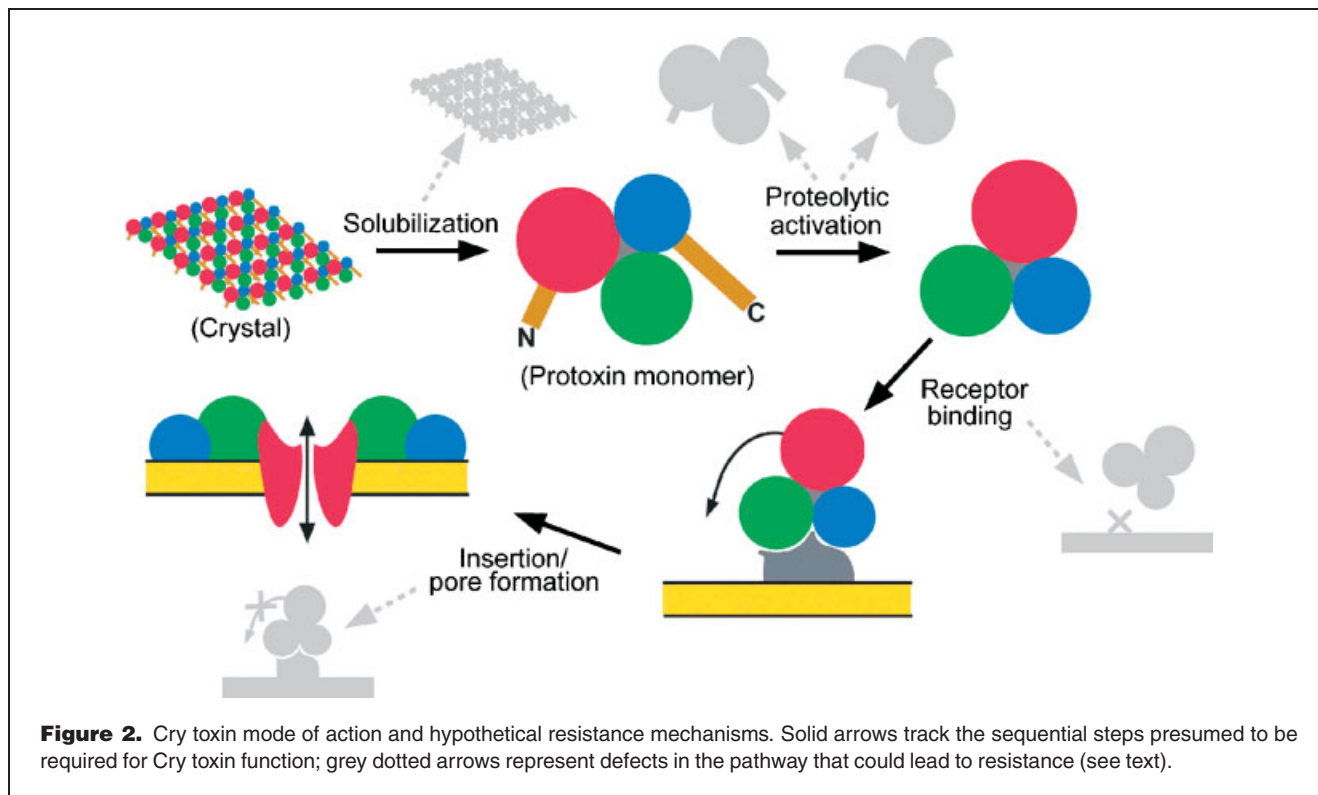


Table 1. Cry toxin resistance in selected lepidopteran strains

Strain	Cry toxin ^a									(ref)	
	Cry1Aa	Cry1Ab	Cry1Ac	Cry1Fa	Cry1Ja	Cry2Aa	Cry1Ca	Cry1B ^b	Btk ^c		Bte ^d
<i>H. virescens</i> YHD2	+	+	+	+		-	-	-			17
<i>H. virescens</i> CP73-3			>10,000			+	(+)	(+)			22
<i>P. gossypiella</i> AZP-R	+	+	50			-	-	(+)			16,18
<i>P. gossypiella</i> APHIS-98R	+	+	3100		(+)	-	-	-			16,73
<i>P. interpunctella</i> 198 ^f	+	+	>100			+	(+)	+		+	23
<i>P. xylostella</i> NO-QA	+	+	+	+	+	-	-	-	+	32	19,74
<i>P. xylostella</i> BCS-Cry1C-1	+	+	+	+	+	(+)	+	-			24
<i>P. xylostella</i> BCS-Cry1C-2	-	-	-	-	-	-	1090	504			24

+, cross resistance; (+), limited cross resistance; -, no or very little cross resistance.

^aToxin to which strain was selected is accompanied by fold-resistance measured to that toxin.

^bBoth Cry1Ba and Cry1Bb have been used in various cross-resistance studies.

^c*Bt kurstaki* contains Cry1Aa, Cry1Ab, Cry1Ac and Cry2A toxins.

^d*Bt entomocidus* contains Cry1Aa, Cry1Ab, Cry1C, and Cry1D toxins.

selected. Several strains shown represent cases for which some understanding of the resistance mechanism has been elucidated (and will be discussed below).

A general conclusion that we can draw from these studies is that in different selection systems, cross-resistance patterns vary considerably. To illustrate, in three Cry1Ac-selected strains (*Heliothis virescens* strain YHD2 and *Pectinophora gossypiella* strains AZP-R and APHIS-98R), cross resistance to Cry1Ab and Cry1Aa is observed, but there is very little cross resistance, if any, to Cry1Ca or Cry2Aa.^(16–18) The *Plutella xylostella* strain NO-QA, which was selected for resistance to a mixed formulation of Cry1A toxins, also exhibited no cross resistance to Cry1Ca or Cry2Aa.^(19,20) This condition of high-level (>500-fold) resistance to Cry1A toxins that does not lead to cross resistance to Cry1Ca is defined as ‘mode 1’ resistance.⁽²¹⁾ This description does not fit a *H. virescens* strain (CP73-3), which was also selected for resistance to Cry1Ac. This strain exhibited moderate, broad-spectrum resistance that included cross resistance to Cry2Aa and Cry1Ca.⁽²²⁾ A similar mode was observed in *Plodia interpunctella* strain 198^r, which was selected for resistance to a *Bt* strain expressing Cry1A, Cry1C and Cry1D proteins.⁽²³⁾ These observations suggest two basic types of resistance, one exhibiting high-level, narrow-spectrum resistance, and the other featuring moderate-level, broad-spectrum resistance.

Yet another, distinct type of resistance was found when a *P. xylostella* strain was selected for high-level resistance to Cry1Ca. In the case of strain BCS-Cry1C-1, a steady increase in resistance to Cry1Ca over ten generations, from zero to 740-fold, was accompanied by a dramatic increase in cross resistance to Cry1Ac, from zero to 47,500-fold.⁽²⁴⁾ This case therefore featured high-level resistance to both Cry1Ac and Cry1Ca toxins. In this study, it was found that the Cry1Ca

resistance could be genetically isolated from the Cry1Ac resistance by outcrossing, illustrated by the BCS-Cry1C-2 strain (see Table 1). It is worth noting that the loss of Cry1Ac resistance may have caused some loss in Cry1Ca resistance in this outcrossed strain. While this population had a history of exposure to Cry1A-containing *Bt* formulations, this fact could not explain how the Cry1Ac resistance levels increased so dramatically during selection with Cry1Ca. The observations are consistent with two genetically separable resistance mechanisms co-existing in the BCS-Cry1C-1 strain: one that simultaneously provided strong resistance to Cry1Ca and weak resistance to Cry1Ac, and one that simultaneously provided strong resistance to Cry1Ac and weak resistance to Cry1Ca.

Studies on other invertebrates

Selection for resistance in non-lepidopteran species has received significantly less attention, even though beetles, flies, mosquitoes, and nematodes constitute a formidable group of *Bt*-susceptible pests and disease agents. Table 2 outlines data from resistance studies in the beetles *Leptinotarsa decemlineata* and *Chrysomela scripta*, the mosquito *Culex quinquefasciatus*, and the nematode *Caenorhabditis elegans*. From this table, one sees that Cry toxins affecting these species are different from the ones that target lepidopterans and largely cluster separately on a phylogenetic tree (see Fig. 1A). However, striking structural similarities among diverse Cry toxins (see Fig. 1B) suggest there will be common threads in how they act and how resistance develops. More detailed studies focused on these species will help build a complete picture of Cry toxin resistance mechanisms and help elucidate which mechanisms are shared among different insect orders and between insects and nematodes.

Table 2. Cry toxin resistance in selected non-lepidopteran strains

Strain	Cry toxin ^a									(ref)
	Cry3Aa	Cry1Ba	Cry4 ^b	Cry11A	Cry11B	Cry19A	Cry5B	Cry14A	Cry6A	
<i>L. decemlineata</i> (no name)	+									26
<i>C. scripta</i> (no name)	>800									27
<i>C. quinquef.</i> <i>Cq4AB</i>	+	+								29,30
<i>C. quinquef.</i> <i>Cq4D</i>	>5000		+	+	(+)	–				29,30
<i>C. elegans</i> <i>bre-4(ye13)</i>							+	(+)	–	31,32
							>100			

+, cross resistance; (+), limited cross resistance; –, no or very little cross resistance.

^aToxin to which strain was selected is accompanied by fold-resistance measured to that toxin.

^bA mixture of Cry4A and Cry4B toxins.

In *L. decemlineata* and *C. scripta* strong resistance to Cry3Aa has been selected.^(25–27) In the case of *C. scripta*, strong cross resistance to Cry1Ba (400-fold) was reported,⁽²⁷⁾ suggesting that Cry3Aa and Cry1Ba require a common factor in this target organism. This is a significant observation, since Cry1Ba is unusual in its ability to intoxicate species from multiple insect orders.⁽²⁸⁾

In *C. quinquefasciatus*, selection for resistance to a Cry4A/Cry4B mixture (strain Cq4AB) led to moderate resistance to the selecting agent, very high cross resistance to Cry11A (>5000-fold) and limited cross resistance (9-fold) to Cry11B. In the same species, when Cry11A was used as the selecting agent (strain Cq4D), strong resistance was achieved, but similar to strain Cq4AB, cross resistance to Cry11B was limited.⁽²⁹⁾ These two strains point to a common resistance mechanism that essentially overcomes Cry11A function altogether, and only partially diminishes the toxicity of Cry11B. More recently it was shown that resistance to Cry19A is not observed in the Cq4AB and Cq4D strains.⁽³⁰⁾

The nematode *C. elegans*, which is susceptible to the Cry toxins Cry5B, Cry14A, Cry21A and Cry6A,⁽⁹⁾ is not considered a pest but is well suited for studies of Cry toxin resistance because of the genetic, genomic, and cell biological tools that are well developed in this organism. The hermaphroditic strain Bristol N2 was chemically mutagenized to generate Cry5B-resistance alleles.⁽³¹⁾ Fig. 3 illustrates a facile selection scheme in which Cry5B expression in the nematodes' bacterial diet discriminates resistant from susceptible animals. Selection for Cry5B resistance has allowed the isolation of more than 200 resistant lines, 45 of which were further characterized and found to be mutated at one of five possible loci. These Cry5B-resistant animals are called *bre* mutants (for *Bt* toxin *r*esistant), and the five loci are designated *bre-1* through *bre-5* (Table 3).^(31,32) *bre* mutants are resistant to Cry5B, are not strongly resistant to the unrelated toxin Cry6A,⁽³¹⁾ and show limited but significant cross resistance (19-fold) to Cry14A, a toxin that also targets Coleoptera.^(32,33) The selection of *bre* mutants was unique because it did not rely on naturally occurring resistance alleles, and strong selection in a forward genetic screen biased the system toward single-gene-mediated resistance mechanisms.

Taken together, these selection studies highlight the variety of systems in place to study resistance to Cry toxins with diverse specificities. Cross-resistance experiments underscore the complexity of Cry toxin resistance. On the one hand, it appears that high-level resistance is limited to closely related toxins in most cases; on the other hand, cases where broad-spectrum resistance occurs seem to correlate with only moderate levels of resistance overall. Many cases of "limited" cross resistance occur, where the degree of resistance to the selecting agent is much higher than is the cross resistance to a second toxin. While the objective to understand molecular mechanisms of resistance requires more sophisticated tools,

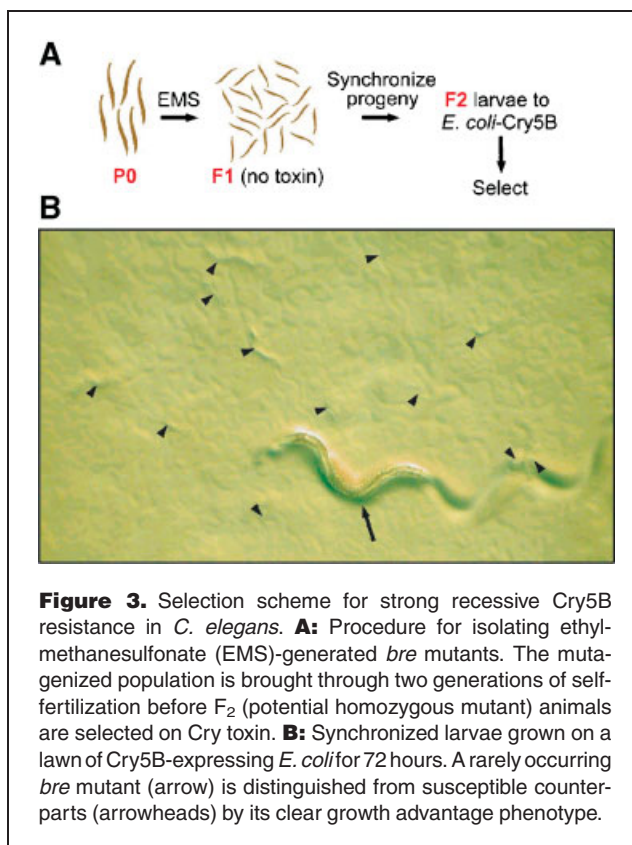


Figure 3. Selection scheme for strong recessive Cry5B resistance in *C. elegans*. **A:** Procedure for isolating ethylmethanesulfonate (EMS)-generated *bre* mutants. The mutagenized population is brought through two generations of self-fertilization before F₂ (potential homozygous mutant) animals are selected on Cry toxin. **B:** Synchronized larvae grown on a lawn of Cry5B-expressing *E. coli* for 72 hours. A rarely occurring *bre* mutant (arrow) is distinguished from susceptible counterparts (arrowheads) by its clear growth advantage phenotype.

these cross-resistance studies are powerful in their ability to categorize which toxins are overcome by common mechanisms. The observation that certain combinations of Cry toxins are unlikely to be resisted simultaneously is the basis for the commercial strategy of toxin stacking, whereby multiple toxins that do not elicit strong cross resistance to each other are co-expressed in the same crop. Under ideal conditions, the stacking approach would reduce the occurrence of field resistance exponentially.⁽³⁴⁾

Biochemical analysis of resistant strains

To say that Cry toxin resistance is complex overall is not to say that it cannot be reduced to rather simple molecular mechanisms. Over the last 15 years, biochemical assays have been established in order to study toxin–invertebrate interactions directly, in both susceptible and resistant strains. The ability to evaluate toxin-binding properties and to characterize the

Table 3. Cry5B resistant *C. elegans* mutants

Gene/locus	<i>bre-1</i>	<i>bre-2</i>	<i>bre-3</i>	<i>bre-4</i>	<i>bre-5</i>
Number of alleles	1	14	19	9	2
Reference allele	ye4	ye31	ye28	ye13	ye17

activity of native gut proteases, has enabled researchers to test some of the hypotheses about resistance that emerge from Cry toxin mode of action.

Changes in proteolytic processing

Protease-mediated activation is an early step in the Cry toxin pathway. In many cases, the C-terminal half of the protoxin is dispensable for full toxic activity, and it has recently been shown that specific N-terminal cleavage events are required for toxin function.^(35,36) Resistant insects have been analyzed for toxin activation defects in a number of ways. Midgut extract from the *P. interpunctella* strain 198^r was found to have significant defects in overall serine protease activity compared to the susceptible strain. This defect led to inappropriate processing of Cry1Ac to products of higher molecular weight than the expected 61 kDa form.⁽³⁷⁾ Further analysis showed that a specific proteolytic enzyme was not present in the resistant strain, and that this enzymatic phenotype cosegregated with the resistance phenotype in outcrossing experiments.⁽³⁸⁾ When 198^r was fed Cry1Ab that had been activated *in vitro*, its resistance factor fell 10-fold, suggesting that the resistance in this strain was partially a result of toxin activation defects.⁽³⁹⁾ The *H. virescens* CP73-3 strain, which also exhibits moderate but broad-spectrum resistance (including resistance to both Cry1Ab and Cry2Aa), was also reported to exhibit abnormal protease profiles by gel analysis.^(40,41) That these protease-defective strains both exhibit moderate, broad-spectrum resistance suggests that a common protease activity may be sufficient *in vivo* for the activation of diverse Cry toxins. It is speculated that a complete lack of gut protease activity would be lethal, and this may explain why protease defects have not been found to correlate with high-level resistance.

Binding site modifications

The first report of a clear biochemical change in a Cry toxin-resistant strain was the discovery that Cry1Ab resistance in a laboratory-selected strain of *P. interpunctella* (strain 343) corresponded to defects in a high-affinity Cry1Ab binding site (affinity was reduced 50-fold in the resistant strain).⁽⁴²⁾ The report also showed that, in this Cry1Ab-resistant strain, Cry1Ca (a toxin to which it was hyper-susceptible) actually bound with higher affinity to a distinct high-affinity site. An intriguing observation was that the high-affinity site targeted by Cry1Ab was an overlapping, low-affinity site for Cry1Ca. Above all, these results demonstrated a connection between receptor binding and resistance, but they also provided a glimpse at the complexity of Cry toxin interactions with target membranes, and presented a model whereby two toxins might functionally diversify by adapting to one of multiple alternative receptor sites.

The apparent complexity deepened with more extensive analysis of toxin binding in Cry1A-resistant strains in other

species. *P. xylostella* strain NO-QA was highly resistant to Cry1Aa, Cry1Ab and Cry1Ac. The failure of Cry1Ab and Cry1Ac to bind to vesicle preparations from this strain made a strong case for receptor defects as the cause of resistance; however, Cry1Aa binding was unaffected in this strain even though Cry1Aa resistance is high.⁽²⁰⁾ It would be difficult to argue that multiple resistance mechanisms were at work, since resistance to all three Cry1A toxins has been mapped to a single locus in this strain.^(43,44) When similar techniques were used to evaluate *P. gossypiella* strain AZP-R, which is also resistant to all three Cry1A toxins, it was found that the binding of Cry1Ab was clearly abolished, but Cry1Ac bound normally.⁽⁴⁵⁾ Similarly, *H. virescens* strain YHD2 exhibits resistance to Cry1Aa, Cry1Ab and Cry1Ac. In earlier work on this strain, when its resistance was ~10,000-fold, it was found that binding of Cry1Aa to vesicle preparations was abolished, but binding of Cry1Ab and Cry1Ac toxins was unchanged.⁽⁴⁶⁾ Later, when the resistance of YHD2 to Cry1Ac had further increased to ~73,000-fold, the binding of all three Cry1A toxins was completely abolished.⁽⁴⁷⁾

Results such as these have shown that binding defects are correlated with resistance, but they call on complex models to explain how a resistance allele can cause resistance to all three Cry1A toxins but abolish binding for only a subset of them. The data suggest that the ability to recognize and bind to the membrane is distinct from the ability to insert into the membrane as a pore-forming unit. For example, in a hypothetical receptor mutant that leads to resistance to CryX and CryY, CryX binding might be abolished altogether, while CryY binding occurs, but inappropriately. This 'inappropriate' binding fails to stimulate the conformational changes that are needed to drive the pore-forming reaction of CryY at the membrane. This idea is consistent with a co-receptor model where both receptor and co-receptor are required for toxicity. Loss of one of the co-receptors may or may not abolish binding, but will always abolish proper pore formation. Whatever the case, the data point to the existence of multiple cell surface determinants that mediate both binding and functional pore formation. The identification of functional cell surface determinants has been propelled by the biochemical and genetic characterization of receptor molecules, and is discussed below.

Receptor molecules that influence resistance

Even though the majority of cases of resistance are correlated with binding site defects, it has been difficult to define which receptor molecules are defective in any given case. Several Cry toxin-binding molecules have been identified and characterized by biochemical methods. These include aminopeptidase N (APN), alkaline phosphatase, cadherins, glycolipids and, in one case, a high molecular weight, highly glycosylated glycoprotein.^(1,48–52) More challenging has been the demonstration of the relevance of any of these receptor molecules in

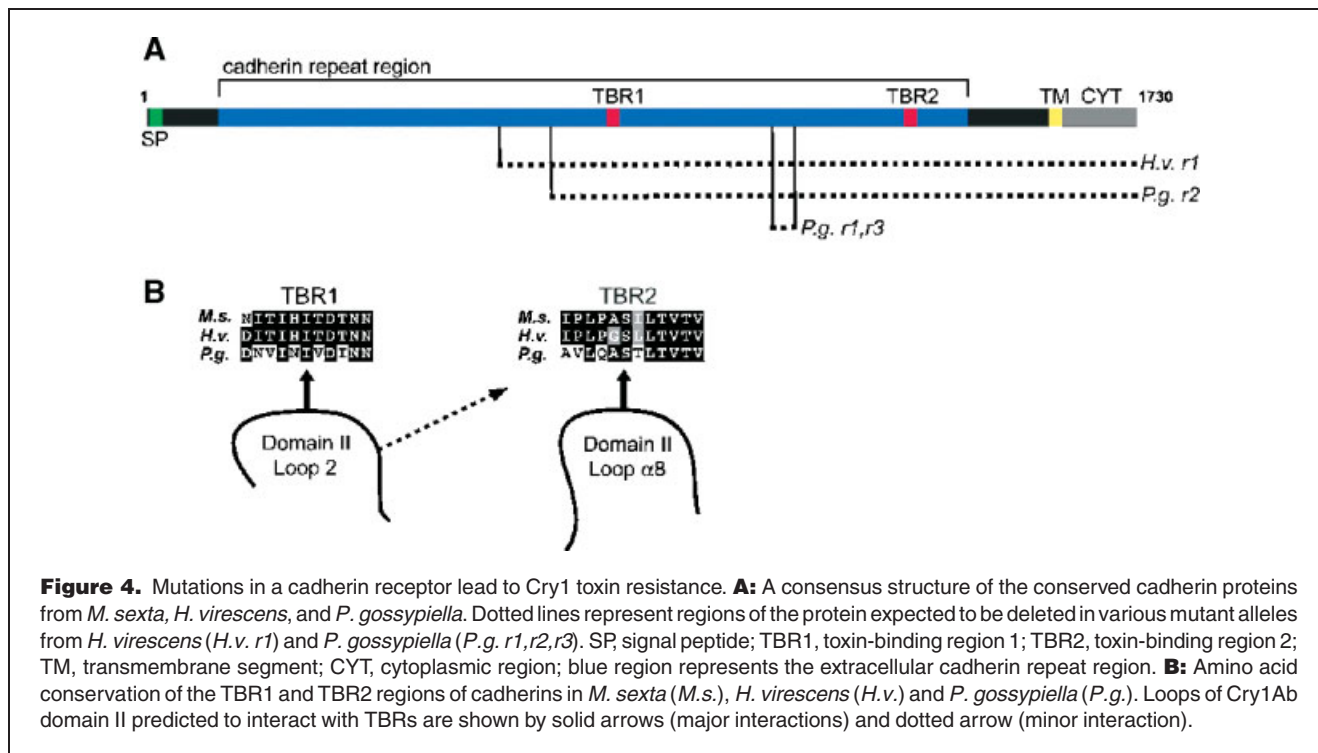


Figure 4. Mutations in a cadherin receptor lead to Cry1 toxin resistance. **A:** A consensus structure of the conserved cadherin proteins from *M. sexta*, *H. virescens*, and *P. gossypiella*. Dotted lines represent regions of the protein expected to be deleted in various mutant alleles from *H. virescens* (*H.v. r1*) and *P. gossypiella* (*P.g. r1,r2,r3*). SP, signal peptide; TBR1, toxin-binding region 1; TBR2, toxin-binding region 2; TM, transmembrane segment; CYT, cytoplasmic region; blue region represents the extracellular cadherin repeat region. **B:** Amino acid conservation of the TBR1 and TBR2 regions of cadherins in *M. sexta* (*M.s.*), *H. virescens* (*H.v.*) and *P. gossypiella* (*P.g.*). Loops of Cry1Ab domain II predicted to interact with TBRS are shown by solid arrows (major interactions) and dotted arrow (minor interaction).

vivo, especially by showing that their absence results in Cry toxin resistance in otherwise susceptible animals.

Aminopeptidases

Bhatnagar and co-workers showed that Cry1Ca binds to a specific APN in *Spodoptera litura*,⁽⁵³⁾ and that depletion of this receptor in *S. litura* by RNA interference gives rise to Cry1Ca resistance.⁽⁵⁴⁾ They found that animals injected with APN dsRNA exhibited 4-fold lower Cry1Ca-induced mortality, and even offspring of injected animals retained a significant level of resistance compared to control animals. The capacity for true genetic lesions in APN loci to mediate Cry toxin resistance is therefore a possibility, particularly with respect to the toxin Cry1Ca. The ability to generate insects with heritable Cry1Ca-resistance^(24,55,56) provides an opportunity to directly test the role of APN genes in resistance. Using a converse approach, the potential for an APN transgene to confer Cry toxin susceptibility to a normally resistant insect has been demonstrated.⁽⁵⁷⁾ *Drosophila melanogaster*, which is resistant to Cry1Ac, was transformed with a *M. sexta* gene encoding a Cry1Ac-binding APN. Transformants were now killed by Cry1Ac, proving that this single gene could modulate toxin susceptibility in vivo. It would be interesting to now investigate using genetics what other *Drosophila* genes, if any, contribute to Cry1Ac susceptibility in this transgenic strain.

Cadherins

The role of insect cadherin receptors in Cry toxin resistance has been established by biochemical and molecular genetic

means. Reported efforts to map Cry toxin resistance loci in lepidopteran insects have been limited to *H. virescens* (strain YHD2)⁽⁵⁸⁾ and *P. xylostella* (strain NO-QA).⁽⁴⁴⁾ Interestingly, both strains exhibit similar resistance patterns, with very high, recessive resistance to Cry1A toxins and very little cross resistance to Cry2Aa or Cry1Ca (see Table 1). For both strains, linkage analysis has revealed a single major resistance locus (designated *BtR-4* in YHD2 and *BtR-1* in NO-QA). The *H. virescens* *BtR-4* locus was found to be tightly linked to a cadherin gene encoding what is probably a high-affinity Cry1A receptor.⁽⁵⁹⁾ This class of receptor was first identified as a Cry1Ab-binding molecule in *Manduca sexta*.⁽⁵⁰⁾ It is now known that the *M. sexta* cadherin receptor binds to multiple Cry1A toxins via toxin domain II, and that this binding is likely mediated by two distinct polypeptide epitopes on the receptor, designated TBR1 and TBR2^(60,61) (Fig. 4). There are data to suggest that this cadherin receptor not only brings the toxin to the membrane but also facilitates a specific proteolytic cleavage step that triggers toxin oligomerization and efficient pore formation.⁽³⁶⁾ The cadherin gene corresponding to *BtR-4* encodes a protein (HevCaLP) that is 63% identical to the *M. sexta* cadherin receptor, and the gene is interrupted by a retrotransposon in YHD2 animals. This allele (*H.v. r1*, see Fig. 4A) is predicted to eliminate the toxin-binding regions on the truncated protein product. It has not been shown that YHD2 animals transformed with a normal copy of the *HevCaLP* gene are restored to higher susceptibility, though the ability of the *Bombyx mori* homologue to confer Cry

Cry1A toxins can bind to insect glycosphingolipids,⁽⁵¹⁾ and it was recently reported that Cry1Aa, Cry1Ab, and Cry1Ac each bind to *M. sexta* glycolipids in vitro.⁽⁶⁶⁾ All of these observations suggest that multiple Cry toxins function through carbohydrate receptors, and that loss of these receptors, in particular glycolipids, is an important resistance mechanism.

The few studies that have successfully identified the genes responsible for Cry toxin resistance implicate both protein and glycolipid molecules as important receptor classes. Direct biochemical studies demonstrate that Cry toxins possess both protein-binding and carbohydrate-binding properties, and that protein binding can occur via multiple regions of the toxin polypeptide. Cry toxins have therefore functionally adapted to multiple receptor types in various ways. Perhaps what little has been discovered about functional Cry toxin receptors through coupled genetic and biochemical analysis will give rise to more precise hypotheses about toxin mode of action and resistance mechanisms that can be tested in a broader context. Towards this end, a recent in vitro study has provided evidence for the sequential action of APN and cadherin, acting as co-receptors, in converting Cry1Ab protoxin into a pore-forming entity.⁽⁷⁰⁾

Induced resistance

Based on the evidence presented thus far, Cry toxin resistance appears to be consistently associated with preventing toxin association with target cells, by defects in activation or receptor binding. There is experimental support for the idea that invertebrate resistance can also be mediated by mechanisms downstream of initial toxin binding, as an active defense response. This finding emerged from two recent, independent studies. First, it was observed that resistance of flour moth (*Ephesia kuehniella*) to a Bt toxin-spore formulation could be physiologically induced by pre-exposure to a low dose of the toxin-spore mixture.⁽⁷¹⁾ The report showed that this induction was correlated with a heightened immune status, and that both resistance and the heightened immune status could be passed to offspring as a maternal effect, not necessarily as a genetic alteration, but as a primed defense response.

The induction of Cry toxin defense responses was analyzed in *C. elegans* by global transcriptional profiling.⁽⁷²⁾ It was found that two mitogen-activated protein kinase (MAPK) pathways, upregulated in response to Cry5B, control functional defense mechanisms. Loss of function of either pathway was found to give rise to strong Cry5B hypersensitivity. It is remarkable that one of these pathways, defective in the *sek-1* mutant, protects the animal against Cry5B, but not the control stressor cadmium chloride, suggesting that *C. elegans* mounts a defensive response that is specific to pore-forming toxins. Similar MAPK pathways had previously been shown to be important for innate immune functions in various animal systems. The mechanism by which these pathways defend against Cry toxins is not understood, and it is not clear whether

genetic alterations enhancing these pathways (e.g. by constitutive activation) play a significant role in resistant invertebrate populations.

Concluding remarks

The elucidation of Cry toxin-resistance mechanisms has only begun. It is clear that invertebrates are able to arrive at diverse solutions to the challenge of Cry toxin exposure, reminding us that Cry toxins, unlike conventional chemical pesticides, are drawn from nature and the interaction between toxin and invertebrate target has acquired a considerable level of complexity over evolutionary time. A complete understanding of Cry toxin resistance therefore requires a view of the toxin as both an antibiotic compound with distinct physico-chemical properties, and as a pathogenic virulence factor, to which target organisms are primed to respond through innate immune pathways. A complete understanding of the subject will thus require diverse methods and analytical systems.

Resistance gene identification is powerful since the resistance phenotype itself confers functional relevance to the discovered gene product. This approach to understanding resistance mechanisms has the capacity to confirm the importance of receptors or proteases implicated through biochemical studies, and to identify new factors, such as modulators of immune responses, that have escaped other methods of analysis. The greatest difficulty in resistance gene identification lies in establishing genetic tools in the major invertebrate groups targeted by Cry toxins. Thus far, *C. elegans* has been the only *Bt*-susceptible organism for which the resistance phenotype has been rapidly reduced to definitive gene identification without prior knowledge of candidate molecules. The development of linkage maps in common lepidopteran pests has required substantial effort, since at least four *Bt*-susceptible species for which linkage maps are described (*B. mori*, *H. virescens*, *P. xylostella* and *Helicoverpa armigera*) each have no fewer than 28 chromosomes. New genomics technologies are making it more feasible to generate the genetic and physical maps that are the bread and butter of positional gene cloning in eukaryotes. The recent whole-genome sequencing of *Anopheles gambiae*, and current genome sequencing efforts in other insects such as *B. mori* and the mosquito *Aedes aegypti* are an indication that these tools are rapidly emerging.

As our knowledge about resistance mechanisms expands, so will the potential to use them in ways that will substantially delay resistance in actual pest populations. Understanding resistance provides us with the tools for resistance allele monitoring, intelligent toxin stacking, and may contribute to the engineering of modified toxins that can 'outsmart' common resistance strategies. Finally, we cannot currently appreciate how Cry toxins will be applied in the future. Recent evidence that some Cry toxins affect animal parasitic nematodes⁽⁹⁾ may represent a first step towards utilizing these invertebrate-

active molecules in medicine to cure or stave off nematode diseases that affect billions of people. Whatever the application, the crisis of antibiotic resistance has taught us unequivocally that, in our efforts to control rapidly-multiplying organisms, we must anticipate and counter their evolutionary game plan before they can actually play their hand.

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