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Bruce E. Tabashnik
University of Arizona, brucet@ag.arizona.edu

Fangneng P. Huang
Louisiana State University Agricultural Center, fhuang@agcenter.lsu.edu

Mukti N. Ghimire
Louisiana State University Agricultural Center

B. Rogers Leonard
Lousiana State University Agricultural Center, rleonard@agcenter.lsu.edu

Blair D. Siegfried
University of Nebraska-Lincoln, bsiegfried1@ufl.edu

See next page for additional authors

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Authors
Bruce E. Tabashnik, Fangneng P. Huang, Mukti N. Ghimire, B. Rogers Leonard, Blair D. Siegfried, Murugesan Rangasamy, Yajun Yang, Yidong Wu, Linda J. Gahan, David G. Heckel, Alejandro Bravo, and Mario Soberón

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Efficacy of Genetically Modified *Bt* Toxins against Insects with Different Genetic Mechanisms of Resistance

Bruce E Tabashnik,[1] Fangneng Huang,[2] Mukti N Ghimire,[2]
B. Rogers Leonard,[2] Blair D Siegfried,[3] Murugesan Rangasamy,[3]
Yajun Yang,[4] Yidong Wu,[4] Linda J Gahan,[5] David G Heckel,[6]
Alejandra Bravo,[7] and Mario Soberón[7]

1. Department of Entomology, University of Arizona, Tucson, Arizona, USA
2. Department of Entomology, Louisiana State University Agricultural Center, Baton Rouge, Louisiana, USA
3. Department of Entomology, University of Nebraska, Lincoln, Nebraska, USA
4. Department of Entomology, College of Plant Protection, Nanjing Agricultural University, Nanjing, China
5. Department of Biological Sciences, Clemson University, Clemson, South Carolina, USA
6. Department of Entomology, Max Planck Institute for Chemical Ecology, Jena, Germany
7. Instituto de Biotecnología, Universidad Nacional Autónoma de México, Cuernavaca, Morelos, Mexico

Corresponding author – Bruce E. Tabashnik, email brucet@cals.arizona.edu

Abstract

Transgenic crops that produce *Bacillus thuringiensis* (*Bt*) toxins are grown widely for pest control,[1] but insect adaptation can reduce their efficacy.[2–6] The genetically modified *Bt* toxins Cry1AbMod and Cry1AcMod were designed to counter insect resistance to native *Bt* toxins Cry1Ab and Cry1Ac.[7] Previous results suggested that the modified toxins would be effective only if resistance was linked

with mutations in genes encoding toxin-binding cadherin proteins.[7] Here we report evidence from five major crop pests refuting this hypothesis. Relative to native toxins, the potency of modified toxins was >350-fold higher against resistant strains of *Plutella xylostella* and *Ostrinia nubilalis* in which resistance was not linked with cadherin mutations. Conversely, the modified toxins provided little or no advantage against some resistant strains of three other pests with altered cadherin. Independent of the presence of cadherin mutations, the relative potency of the modified toxins was generally higher against the most resistant strains.

The toxins produced by *Bt* kill some major insect pests but cause little or no harm to people and most other organisms.[8] *Bt* toxins have been used in sprays for decades and in transgenic plants since 1996 (ref. 6). Transgenic corn and cotton producing *Bt* toxins were planted on >58 million hectares worldwide in 2010 (ref. 1). The primary threat to the long-term efficacy of *Bt* toxins is the evolution of resistance by pests.[2–6] Many insects have been selected for resistance to *Bt* toxins in the laboratory, and some populations of at least eight crop pests have evolved resistance to *Bt* toxins outside of the laboratory, including two species resistant to *Bt* sprays and at least six species resistant to *Bt* crops.[2–6,9–13]

The most widely used *Bt* toxins are crystalline proteins in the Cry1A family, particularly Cry1Ab in transgenic *Bt* corn and Cry1Ac in transgenic *Bt* cotton, which kill some lepidopteran larvae.[3] Cry1A toxins bind to the extracellular domains of cadherin, aminopeptidase, and alkaline phosphatase in larval midgut membranes.[14,15] Disruption of *Bt* toxin binding to midgut receptors is the most common general mechanism of insect resistance.[9] Mutations in the genes encoding midgut cadherins that bind Cry1Ac are linked with resistance in at least three lepidopteran pests of cotton,[16–18] but such cadherin mutations are not the primary cause of many other cases of field- and laboratory-selected resistance.[9,19,20]

Although some aspects of the mode of action of *Bt* toxins remain unresolved, extensive evidence shows that after Cry1A protoxins are ingested by larvae, they are solubilized in the gut and cleaved by mid-gut proteases such as trypsin to yield activated 60-kD monomeric toxins that bind with membrane-associated receptors.[14,15] The signaling model suggests that after protease-activated monomeric toxins bind to cadherin, initiation of a magnesium-dependent signaling pathway causes cell death.[14,15] In contrast, a recent version of the pore formation model[21] proposes the following sequence of events: protease-activated monomers bind to glycosylphosphatidylinositol (GPI)-anchored proteins, including aminopeptidases and alkaline phosphatases. This interaction promotes binding of toxin monomers to cadherin, which facilitates protease cleavage of the N terminus of the toxin, including helix α-1 of domain I, inducing oligomerization of the toxin. The toxin oligomers bind with increased affinity to GPI-anchored receptors and create pores in the midgut membrane that cause osmotic shock and cell death.

According to the pore formation model, the binding of protease-activated toxin to cadherin is essential for removal of helix α-1, which in turn promotes oligomerization.[7] Therefore, we hypothesized that genetically modified Cry1Ab and Cry1Ac toxins (Cry1AbMod and Cry1AcMod) lacking helix α-1 could form oligomers without cadherin and kill insects in which cadherin was altered or absent. Consistent with this hypothesis, Cry1AbMod and Cry1AcMod formed oligomers capable of in vitro pore formation in the absence of cadherin, whereas Cry1Ab and Cry1Ac did not.[7,22] Moreover, the modified toxins killed larvae

with reduced susceptibility to native Cry1A toxins caused by RNA interference silencing of the cadherin gene in *Manduca sexta* and by naturally occurring deletion mutations in the cadherin gene of resistant *Pectinophora gossypiella*.[7] Although these results suggested the potential utility of modified toxins for countering cadherin-based resistance, it remained unclear if the modified toxins would be useful against cadherin-based resistance in other species or against resistance caused by other mutations. Here we used laboratory bioassays to compare responses to modified and native *Bt* toxins by 12 resistant and susceptible strains of five species of major crop pests (*P. xylostella*, *O. nubilalis*, *Diatraea saccharalis*, *Helicoverpa armigera*, and *Heliothis virescens*) with various genetic mechanisms of resistance (see Methods section and Supplementary Table 1).

Cry1AbMod and Cry1AcMod strikingly reduced resistance in the field-selected resistant strain (NO-QAGE) of *P. xylostella* (Figs. 1 and 2 and Supplementary Table 2). We calculated the resistance ratio as the concentration of toxin killing 50% of larvae (LC_{50}) for a resistant strain divided by the LC_{50} for a conspecific susceptible strain. For the resistant strain of *P. xylostella*, the resistance ratios were >21,000 for Cry1Ab, 3.1 for Cry1AbMod, >110,000 for Cry1Ac, and 4.8 for Cry1AcMod (Fig. 2). We measured the reduction in resistance ratio for the modified toxin relative to its native counterpart as the resistance ratio for the native toxin divided by the resistance ratio for the corresponding modified toxin. The resistance ratio was reduced by a factor of >6,900 for Cry1AbMod relative to Cry1Ab and >23,000 for Cry1AcMod relative to Cry1Ac (Supplementary Table 2).

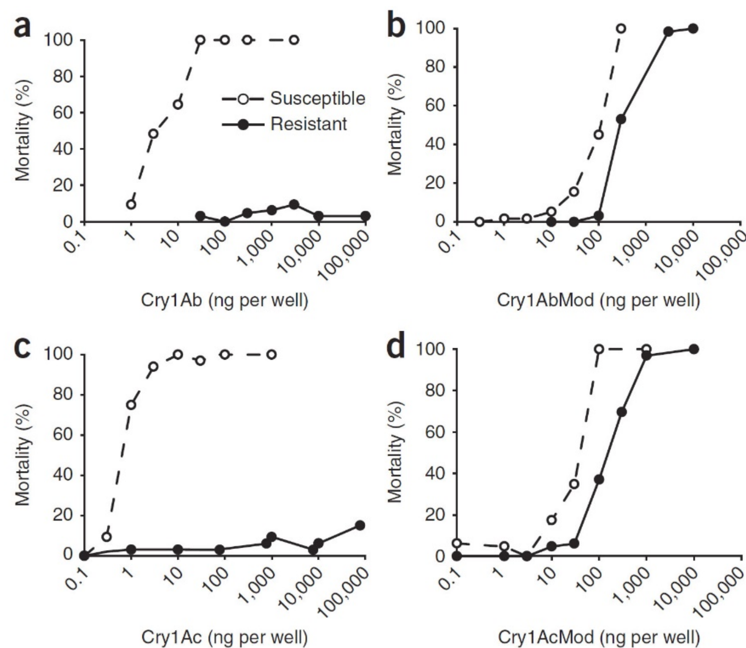

Figure 1. Responses of susceptible and resistant strains of *P. xylostella* to native and genetically modified *Bt* toxins: (a) Cry1Ab, (b) Cry1AbMod, (c) Cry1Ac, and (d) Cry1AcMod.

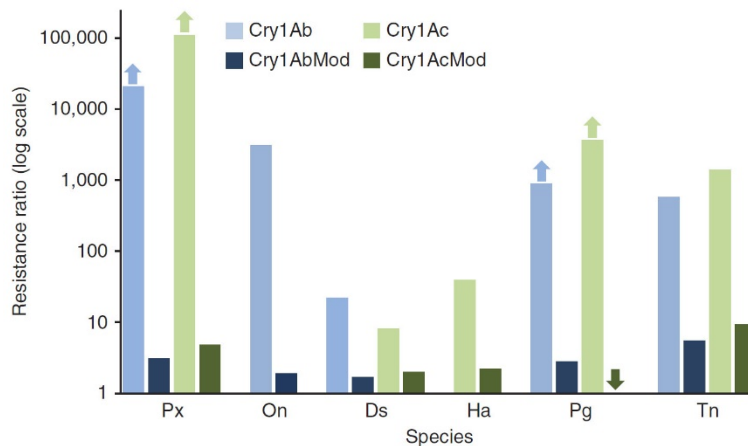

Figure 2. Resistance to native *Bt* toxins Cry1Ab and Cry1Ac (light bars) and genetically modified *Bt* toxins Cry1AbMod and Cry1AcMod (dark bars) in six species of insect pests. Data are reported here for *P. xylostella* (Px), *O. nubilalis* (On), *D. saccharalis* (Ds), and *H. armigera* (Ha) (Supplementary Table 2) and were reported previously for *P. gossypiella* (Pg)[7] and *T. ni* (Tn).[10] Resistance ratios are the concentration of toxin killing 50% of larvae (LC50) for each resistant strain divided by the LC50 for the conspecific susceptible strain. The arrows pointing up indicate resistance ratios higher than the top of the bar that cannot be estimated precisely because mortality of the resistant strains of Px and Pg against native toxins was so low that we could not accurately estimate LC_{50} values. The arrow pointing down indicates a resistance ratio <1 (0.41) for Cry1AcMod versus Pg.[7]

Results with laboratory-selected resistant strains of three other major crop pests (KS, *O. nubilalis*; Bt-RR, *D. saccharalis*; and SCD-r1, *H. armigera*) were qualitatively similar to those described above for *P. xylostella* (Fig. 2 and Supplementary Table 3). For each of these three strains, the resistance ratio was lower for modified toxins than for the corresponding native toxins (Fig. 2). The lower resistance ratios for modified toxins than for their native counterparts seen with the four resistant strains described above are similar to previously reported results with *P. gossypiella*[7] and *Trichoplusia ni*[10] (Fig. 2).

To better understand the reductions in resistance ratio for modified toxins relative to native toxins, we evaluated the potency of toxins, which is inversely related to the LC_{50} value.[23] We calculated the potency ratio of each modified toxin as the LC_{50} of a native toxin divided by the LC_{50} of the corresponding modified toxin. This analysis shows that the reductions in resistance ratio for modified toxins relative to native toxins occurred because modified toxins were more potent than native toxins against resistant strains in four of six cases and less potent than native toxins against susceptible strains in all cases (Fig. 3 and Supplementary Table 4). For example, against the resistant strain of *P. xylostella*, potency was >350-fold higher for Cry1AbMod than for Cry1Ab, and >540-fold higher for Cry1AcMod than for Cry1Ac. However, against the susceptible strain of *P. xylostella*, each modified toxin was less potent than the corresponding native toxin. Although Cry1AbMod was significantly more potent than Cry1Ab against the resistant strain of *D. saccharalis* ($P < 0.05$, Supplementary Table 3), we do not know if the observed 2.8-fold difference in potency

would substantially enhance control. In two exceptions to the trend that potency against resistant strains was higher for modified toxins than native toxins, Cry1AcMod was less potent than Cry1Ac against resistant strains of *H. armigera* and *D. saccharalis* (Fig. 3 and Supplementary Table 4).

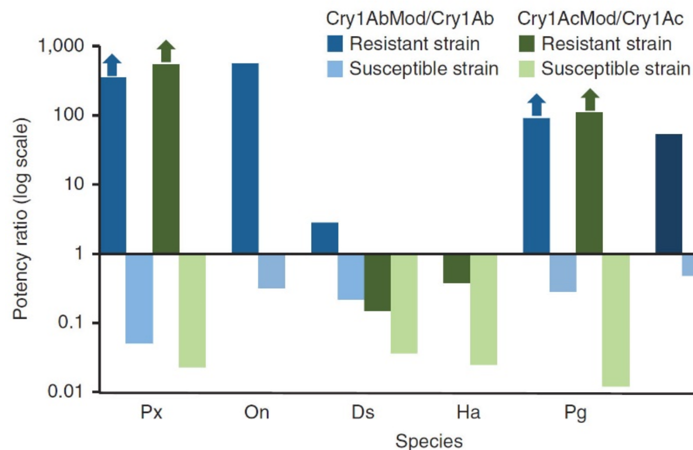

Figure 3. Potency of modified *Bt* toxins relative to native *Bt* toxins. Data are reported here for *P. xylostella* (Px), *O. nubilalis* (On), *D. saccharalis* (Ds), and *H. armigera* (Ha) (Supplementary Table 3) and were reported previously for *P. gossypiella* (Pg)[7] and *T. ni* (Tn).[10] Potency ratio is the LC_{50} of a native toxin divided by the LC_{50} of the corresponding modified toxin for a resistant strain (dark bars) or a susceptible strain (light bars). Values >1 indicate the modified toxin was more potent than the native toxin. Values <1 indicate the native toxin was more potent than the modified toxin. The arrows pointing up indicate potency ratios higher than the top of the bar that cannot be estimated precisely because mortality of the resistant strains of Px and Pg against native toxins was so low that we could not accurately estimate LC_{50} values.

In addition to the tests evaluating mortality described above, we examined growth inhibition in a susceptible strain and three laboratory-selected strains of *H. virescens* with different levels and mechanisms of resistance to Cry1Ac: the YFO strain had relatively low cadherin-based resistance, the YEE strain had higher resistance based on an ABC transporter mutation and the YHD3 strain had the highest level of resistance based on both cadherin and ABC transporter mutations.[24] We estimated resistance ratios for these strains based on the toxin concentration causing 50% larval growth inhibition (IC_{50}). Relative to Cry1Ac, Cry1AcMod reduced the resistance ratio by a factor of >990 for YHD3 and ~100 for YFO (Supplementary Table 5). YEE was highly resistant to both Cry1Ac and Cry1AcMod (Supplementary Table 5). Based on IC_{50} values and growth inhibition at the highest toxin concentration tested against each strain, Cry1AcMod was more potent than Cry1Ac against YHD3, but Cry1AcMod was less potent than Cry1Ac against YEE, YFO and the susceptible strain (Supplementary Tables 5–7).

The results here refute the hypothesis that Cry1AbMod and Cry1AcMod are more effective than native toxins if and only if resistance is caused by mutations in genes encoding

toxin-binding cadherin proteins. Cry1AcMod was not more effective than Cry1Ac against two strains in which resistance was caused primarily by a mutant cadherin allele: the SCD-r1 strain of *H. armigera*[18] and the YFO strain of *H. virescens*[24] (Fig. 2 and Supplementary Tables 4, 6 and 7). These results indicate that the modified toxins do not always effectively counter resistance caused by cadherin mutations. Furthermore, one or both of the modified toxins were more potent than their native counterparts against several strains in which resistance was conferred primarily or entirely by other mutations. Field-selected resistance in the NO-QAGE strain of *P. xylostella* and related strains is genetically linked with a mutation in a locus encoding an ABC transporter protein and not with the cadherin locus.[19,25] Results here confirm and extend the idea that resistance in NO-QAGE does not involve altered cadherin. Western blot analysis shows that an anti-cadherin antibody detected a 210-kD protein in NO-QAGE and in a susceptible strain of *P. xylostella* (Supplementary Fig. 1). In contrast, we found that in *P. gossypiella*, an anti-cadherin antibody detected a 180-kD protein in a susceptible strain, but not in a strain that has cadherin-based resistance (Supplementary Fig. 1).

For the resistant strain (KS) of *O. nubilalis*, analysis of its parent strain (SKY) shows that the resistance is associated with mutations in a gene encoding an aminopeptidase P, but not with mutations that alter cadherin or aminopeptidases N.[26,27] Similar to the results described above for NO-QAGE, blots with anti-cadherin antibody detected no difference between resistant and susceptible strains of *O. nubilalis* in the quantity or size of cadherin bands.[26] In the Bt-RR strain of *D. saccharalis*, the resistance is associated with reduced expression of three aminopeptidase N genes and cadherin, but not with mutations in the genes encoding these proteins.[28,29] In addition, previous work showed the efficacy of the modified toxins against a greenhouse-selected resistant strain of *T. ni*.[10] Recent results indicate that this resistance is associated with reduced transcription of an aminopeptidase (APN1) and is linked with an ABC transporter gene, but not with genes encoding either cadherin or APN1 (refs. 25, 30). Against the three resistant strains of *H. virescens*, Cry1AcMod was more potent than Cry1Ac against the highly resistant YHD3 strain with both cadherin and ABC transporter mutations, but not against the two other resistant strains that had either a cadherin mutation or an ABC transporter mutation.

Overall, in evaluations of mortality in six cases with paired resistant and susceptible strains reported here (Figs. 2 and 3) and in four cases reported previously,[7,10] the potency ratio of the modified toxin relative to its native counterpart was positively correlated with the resistance ratio for the native toxin (Spearman's rank correlation, $r_s = 0.97$, d.f. = 8, $P < 0.0001$; Methods section). A similar trend occurred in the evaluations of growth inhibition for three resistant strains of *H. virescens*: the potency of Cry1AcMod relative to Cry1Ac was highest for the most resistant strain, YHD3 (Supplementary Tables 5–7). Thus, the evaluations based on either mortality or growth inhibition indicate that the relative potency of the modified toxins was generally higher against the strains most resistant to native toxins, independent of the presence or absence of cadherin mutations. In one exception to this pattern, Cry1AcMod was not more potent than Cry1Ac against the YEE strain of *H. virescens*, which had a resistance ratio of >2,400 for Cry1Ac (Supplementary Tables 5–7). In sum, the modified toxins provided an alternative pathway to toxicity that was substantially more

potent than the natural pathway in most instances when the natural pathway was severely disrupted.

The higher potency of modified toxins compared to native toxins against several resistant strains, including results from 21-d bioassays with *P. gossypiella*,[7] indicates that stability is probably not lower for modified toxins than for native toxins. When *P. gossypiella* larvae were exposed to Cry1Ac for 11 d and then transferred to an untreated diet, they survived and pupated.[17] Thus, the >90-fold higher potency of modified toxins over native toxins against resistant *P. gossypiella* in 21-d bioassays implies sustained toxicity of the modified toxins. Moreover, banding patterns resulting from digestion with insect midgut juice or trypsin (Supplementary Fig. 2) and other traits are similar for modified and native toxins.[22]

Based on the results reported here and previously,[7,10] the potency of at least one modified toxin was higher than its native counterpart for six of nine resistant strains tested. These six resistant strains represent six species from four families of Lepidoptera (Crambidae, Gelechiidae, Noctuidae and Plutellidae) in which resistance evolved in the laboratory, greenhouse or field. Modified toxins were more potent than native toxins against these resistant strains, yet native toxins were more potent against all susceptible strains tested. Although we do not know if the modified toxins will be useful in the field, the results suggest that it might be worthwhile to test combinations of modified toxins with native Cry1A, Cry2 or Vip toxins.[31] To assess the joint use of modified and native toxins, it must be determined if they act independently, antagonistically, or synergistically.[23] Insects can probably adapt to modified *Bt* toxins used alone or in combination with other toxins. Nonetheless, along with other control tactics[32] and toxins that have been used less extensively than Cry1A toxins,[33] the modified toxins may broaden the options for managing some pests.

Methods

Toxins

We tested the protoxin form of four toxins: Cry1Ab, Cry1Ac, Cry1AbMod, and Cry1AcMod. Toxins were produced as described previously.[7] We tailored *cry1Ab* and *cry1Ac* genes to create the modified genes *cry1AbMod* and *cry1AcMod* using a three-step PCR process.[7] Based on the coding sequences, Cry1AbMod and Cry1AcMod proteins are expected to lack 56 amino acids at the N terminus compared with Cry1Ab and Cry1Ac. In addition to lacking all of the amino acids of helix α-1 of domain I, Cry1AbMod and Cry1AcMod lack four of the ten amino acids of helix α-2a (52-GAGF-55) and have two amino acid substitutions in helix α-2a (57-58VL changed to MA) to provide a methionine for translation. As expected, the Cry1AbMod and Cry1AcMod protoxins were ~125 kD, and Cry1Ab and Cry1Ac protoxins were ~130 kD.[7]

Insect strains

Supplementary Table 1 lists the resistant and susceptible insect strains of six species used in this study (*P. xylostella, O. nubilalis, D. saccharalis, H. armigera, H. virescens,* and *P. gossypiella*) and one species examined in previous work (*T. ni*[10]). All susceptible strains were reared in the laboratory without exposure to *Bt* toxins or other insecticides. The origins of each strain tested in this study are described below.

P. xylostella

We tested a resistant strain (NO-QAGE) and a susceptible strain (Geneva 88) of the global vegetable pest, *P. xylostella* (diamondback moth), which is the first insect that evolved resistance to *Bt* toxins in open field populations.[34,35] The susceptible strain (Geneva 88) originated in 1988 from a cabbage field near Geneva, New York.[36] The NO-QAGE strain was derived from a field population in Hawaii that evolved resistance to *Bt* sprays containing Cry1Ab, Cry1Ac, and other *Bt* toxins.[35] In this strain, resistance is associated with reduced toxin binding to larval midgut membranes, and a major gene confers resistance to at least five *Bt* toxins including Cry1Ab and Cry1Ac.[35,37] Complementation tests show that the genetic locus conferring resistance in NO-QAGE also confers resistance in at least three other field-selected strains of *P. xylostella* from the continental US and Asia.[37,38] Our group35 created the resistant strain used here (NO-QAGE) by crossing NO-QA, a field-selected resistant strain from Hawaii38, with the susceptible strain Geneva 88, followed by selection of the F3 progeny with Cry1Ac.

O. nubilalis

The resistant strain (KS) originated from a field collection of 126 diapausing larvae from non-*Bt* corn in Kandiyohi County, Minnesota, in 2001. Of these 126 larvae, 14 that survived exposure to a diagnostic Cry1Ab concentration were used to start the resistant strain SKY.[39,40] The resistant SKY insects were backcrossed with a susceptible strain (KY) that originated from the same collection, allowed to mate, and the progeny were selected with Cry1Ab.[40–43] The resistant survivors from this reselection were subjected to a second cycle of backcrossing, mating, and selection with Cry1Ab. The survivors were used to start the

KS strain. The susceptible strain (ELS) was established in 1993 from ~500 *O. nubilalis* larvae collected in the Lombardia region of northern Italy. In previous work, the ELS strain was called I[42], Europe-S[26,43] and Els[41].

D. saccharalis

The susceptible strain (Bt-SS) was established using larvae collected from corn fields near Winnsboro in northeastern Louisiana during 2004. A Bt-resistant strain (Bt-RR) was developed from a single isoline family using an F_2 screen.[44] Bt-RR larvae completed development on commercial Cry1Ab corn hybrids.[44] Before the current study, the Bt-RR strain was backcrossed three times with the Bt-SS strain and reselected for resistance with Cry1Ab corn leaf tissue in the F_2 generation after each backcross.

H. armigera

The susceptible strain of *H. armigera* (SCD) originated from the Cote D'Ivoire in the 1970s and was obtained from Bayer Crop Science in 2001. Yang et al. (ref. 18) created the resistant strain (SCD-r1) by introgressing a mutant cadherin allele (r1) from the resistant GYBT strain into the SCD strain by means of repeated backcrossing and selection. The GY strain was started in August 2001 with 300 larvae collected from late season *Bt* cotton in Gaoyang County, Hebei Province, China.[45] GYBT was derived from GY by 28 generations of selection with larvae exposed by diet surface overlay to activated Cry1Ac toxin.[45]

H. virescens

The susceptible strain (CNW) originated from field collections in North Carolina and was obtained in 1999 from the Department of Entomology Insectary at North Carolina State University. The resistant YHD2 strain was started with eggs collected from seven tobacco fields in Yadkin County, North Carolina, in 1988 and was the second replicate selected in the laboratory with Cry1Ac, which was obtained initially from *Bt* subspecies *kurstaki* strain HD73 (ref. 46). The resistant YHD3 strain was created by crossing YHD2 with the susceptible strain CNW and selecting with Cry1Ac24. YHD3 was homozygous for resistant alleles at two separate loci, one encoding a cadherin protein (*BtR-4*) and the other an ABC transporter protein (*BtR-6*). One group[24] used crosses with the CNW strain followed by marker-assisted selection to create two less resistant strains: a moderately resistant strain (YEE) that had only the ABC transporter resistance alleles and was reared on diet with 50 μg Cry1Ac per ml diet; and the least resistant strain (YFO), which had only the cadherin resistance alleles and could be reared on diet with at most 5 μg Cry1Ac per ml diet.

P. gossypiella

The susceptible APHIS-S strain of *P. gossypiella* was derived in 1997 from the APHIS strain reared at the USDA-APHIS Pink Bollworm Rearing Facility in Phoenix, Arizona.[32] The APHIS strain was started with insects collected from Arizona more than 30 years ago and had been infused yearly with wild individuals before the APHIS-S strain was started. The resistant AZP-R strain was started in 1997 by collecting individuals from ten cotton fields in Arizona and selecting their progeny with various concentrations of Cry1Ac in the diet.[47]

Bioassays

We used established bioassay techniques for each species. All bioassays were done in the laboratory with larvae tested individually on diet. We either put toxins on the surface of diet in wells of bioassay trays (diet surface overlay; *P. xylostella*, *O. nubilalis*, and *H. armigera*) or mixed toxins into diet (diet incorporation; *D. saccharalis* and *H. virescens*). All bioassays involved diet with a series of 5 to 8 toxin concentrations, including controls with no toxin added. The total number of larvae tested for each combination of insect strain and toxin ranged from 240 to 1,529 (Supplementary Table 1). We conducted replicates on two or more dates for 10 of the 12 strains tested in bioassays. We replicated bioassays with the CNW and YEE strains of *H. virescens* only on one date. Toxins and diet from two or more separate batches were tested on separate dates in bioassays with *P. xylostella, D. saccharalis*, and *H. armigera.* In nearly all bioassays, the experimenters did not know the identity of the toxins until after the results were recorded.

We cannot exclude the possibility that variation among species in bioassay methods (including differences in the age of larvae when bioassays started), affected the extent of differences between conspecific strains (resistance ratios) and between proteins within a strain (potency ratios). However, we suspect that such effects were relatively minor and did not alter qualitative conclusions. Moreover, in the comparisons among three resistant strains of *H. virescens* with different sets of mutations conferring resistance, bioassay methods were identical across strains, including age of larvae, method of exposure and environment. Summaries of the bioassay methods and relevant references for each species are provided below.

P. xylostella

We used diet surface overlay bioassays to test third instars, with one larva per well of 128-well plastic bioassay trays.[35] Fifty μl of water containing 0.005% Triton X-100 and an appropriate amount of *Bt* toxin were added to each well. Mortality was recorded after 6 d at 27°C, 14L:10D.

O. nubilalis

We used diet surface overlay bioassays to test neonates (< 24 h old) individually in 128-well trays.[48] Thirty μl of water containing 0.1% Triton X-100 and an appropriate amount of *Bt* toxin were added to each well. After 7 d at 27°C, 24 D, and 80% RH, larvae were scored as dead if they died or if they had not grown beyond the first instar and did not weigh > 0.1 mg.

D. saccharalis

We used diet incorporation bioassays to test neonates (< 24 h old).[49] Toxins were diluted with distilled water and mixed with diet. We added ~0.7 ml of diet into each well of a 32-well plate and put one neonate in each well. After 7 d at 28°C, 16L:8D, and 50% RH, larvae were scored as dead if they died or if they weighed ≤ 0.1 mg based on visual estimation.

H. armigera

We used diet surface overlay bioassays to test second instars that had been starved for 4 h.[18] Toxins were diluted with PBS (0.01 M, pH 7.4). We put 900 μl of liquid artificial diet in each well of a 24-well plate. After the diet cooled and solidified, 100 μl of PBS with an appropriate concentration of toxin was added to each well and allowed to air dry. We put one second instar in each well. After 5 d at 26 ± 1°C, 16L:8D and 60% RH, larvae were scored as dead if they died or if they weighed < 5 mg.

H. virescens

We used diet incorporation bioassays to test neonates (< 24 h old) individually in 2-ml plastic vials, with one gram of diet per vial.[50] After diet cooled to 50°C, toxin was mixed into diet with a Cuisinart Quick Prep motorized mixer. Larvae were weighed after 6 d at 27°C, 16L:8D, 60% RH.

Data analysis

For all species except *H. virescens*, we used probit analysis of mortality data to estimate the concentration of toxin killing 50% of larvae (LC_{50}) and its 95% fiducial limits (FL), as well as the slope of the concentration-mortality line and its standard error (s.e.m.). For *H. virescens*, we performed a parallel probit analysis of weight data to estimate the toxin concentration causing IC_{50}, 95% FL, slope and s.e.m. We estimated the IC_{50} as the toxin concentration in diet yielding larval weight that is 50% of the mean larval weight of the same strain reared on diet without toxin (control). Probit analysis was done with SAS[51] for *P. xylostella*, *H. virescens*, and *D. saccharalis*; POLO-PC[52] for *O. nubilalis*; and PoloPlus[53] for *H. armigera*.

In cases where insects were so resistant that the LC_{50} (or IC_{50}) could not be estimated from probit analysis because of low mortality (or low growth inhibition), we inferred that the LC_{50} (or IC_{50}) was greater than the highest concentration tested. For example, with resistant *P. xylostella*, the highest concentration of Cry1Ab tested (100,000 ng toxin per well) killed only 3.1% of larvae. Thus, we inferred that the LC_{50} was > 100,000 ng toxin per well.

We calculated the resistance ratio as the LC_{50} (or IC_{50}) for a resistant strain divided by the corresponding LC_{50} (or IC_{50}) for the conspecific susceptible strain. We calculated the reduction in resistance ratio for modified toxins as the resistance ratio for the native toxin divided by the resistance ratio for the corresponding modified toxin. Potency is inversely related to LC_{50} or IC_{50} (ref. 23). We calculated the potency ratio as the LC_{50} (or IC_{50}) of the native toxin divided by the LC_{50} (or IC_{50}) of the corresponding modified toxin. We considered values of LC_{50} (or IC_{50}) significantly different if their 95% FL did not overlap, which is a conservative criterion.[54]

For *H. virescens* we used analysis of variance (ANOVA)[51] to compare larval growth inhibition of Cry1AcMod versus Cry1Ac for each of three resistant strains and one susceptible strain. For the two resistant strains of *H. virescens* (YHD3 and YFO) that were tested in two independent trials on different dates (Trials 1 and 2, Supplementary Table 7) we compared the results between trials with two-way ANOVA. For YHD3, this showed significant effects of Trial and Trial X Toxin interaction, so we analyzed each trial separately with one-way ANOVA. For YFO, effects of Trial and Trial X Toxin interaction were not significant,

so we used two-way ANOVA to analyze data jointly from Trials 1 and 2. We used Spearman's rank correlation test (http://faculty.vassar.edu/lowry/VassarStats.html; accessed June 4, 2011) to evaluate the association between the resistance ratio for the modified toxin and the potency of the modified toxin relative to its native counterpart for the six cases studied here and four cases studied previously in which both the resistance ratio and potency ratio were calculated from mortality data (Supplementary Tables 2–4).

Preparation of BBMV

Midguts of fourth instar *P. xylostella* and *P. gossypiella* were dissected and used to prepare brush border membranes vesicles (BBMVs) by differential precipitation using $MgCl_2$ (ref. 55) and stored at −70°C until use.

Cadherin western blots

For detecting cadherin with anti-cadherin antibodies, BBMVs were prepared in the presence of 5 mM $CaCl_2$. Ten micrograms of BBMVs were separated by 10% SDS-PAGE and electrotransferred to PVDF membrane. For *P. xylostella*, an anti-cadherin antibody raised against *H. virescens* cadherin[56] (1:10,000) and secondary anti-rabbit (1:5,000) conjugated with horseradish peroxidase For *P. gossypiella*, membranes were incubated with an antibody (anti-CR8-11) that recognizes a portion of *P. gossypiell*a cadherin[57] (1:30,000) and secondary anti-rabbit (1:5,000) antibody conjugated with horseradish peroxidase.

Stability of Cry1Ab, Cry1AbMod, and Cry1Ab Mutant Y110E after digestion with insect midgut juice or trypsin

Cry1Ab mutant Y110E had a single amino acid change (tyrosine to glutamic acid) in position *d* of helix alpha-3 generated by site-directed mutagenesis.[58] We activated 20 μg of each toxin by incubation for 1 h at 37°C with 10 μg of midgut juice from *M. sexta* or 4 μg of trypsin. Phenylmethylsulfonyl fluoride (1 mM final concentration) was added to stop proteolysis. The samples were separated by 10% SDS-PAGE and stained with Coomasie blue. To obtain midgut juice, we dissected midgut tissue from fifth instar *M. sexta* larvae and separated the midgut juice from solid material by centrifugation followed by filtering through 0.22 μm filters. Total protein was determined by the Bradford assay and small aliquots of midgut juice were stored at −70°C until use.

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Supplementary Information

Efficacy of Genetically Modified Bt Toxins Against Insects with Different Genetic Mechanisms of Resistance

Bruce E. Tabashnik[1], Fangneng Huang[2], Mukti N. Ghimire[2], B. Rogers Leonard[2], Blair D. Siegfried[3], Murugesan Rangasamy[3], Yajun Yang[4], Yidong Wu[4], Linda J. Gahan[5], David G. Heckel[6], Alejandra Bravo[7] & Mario Soberón[7]

[1]Department of Entomology, University of Arizona, Tucson, Arizona, USA.
[2]Department of Entomology, Louisiana State University Agricultural Center, Baton Rouge, Louisiana, USA.
[3]Department of Entomology, University of Nebraska, Lincoln, Nebraska, USA.
[4]Department of Entomology, College of Plant Protection, Nanjing Agricultural University, Nanjing 210095, China.
[5]Department of Biological Sciences, Clemson University, Clemson, South Carolina, USA.
[6]Department of Entomology, Max Planck Institute for Chemical Ecology, Jena, Germany.
[7]Instituto de Biotecnología, Universidad Nacional Autónoma de México, Cuernavaca, Morelos, Mexico.
Address correspondence to B.E.T. (brucet@cals.arizona.edu).

This Supplementary Information contains:
Supplementary Tables 1-7
Supplementary Figures 1 and 2

Supplementary Table 1. Resistant and susceptible strains tested with modified and native Bt toxins.

Insect species	Strain name	Strain type[a]	Genetic basis of resistance	Reference(s)
P. xylostella	NO-QAGE	R	mutant ABC[b], not cadherin or APN[c]	19, 25, 35-38
	Geneva 88	S		
O. nubilalis	KS	R	mutant APP[d], not cadherin or APN	26, 27, 43
	ELS	S		
D. saccharalis	Bt-RR	R	reduced expression of cadherin	28, 29, 44
	Bt-SS	S	and three APNs	
H. armigera	SCD-r1	R	mutant cadherin	18
	SCD	S		
H. virescens	YFO	R	mutant cadherin	24
	YEE	R	mutant ABC	24
	YHD3	R	mutant ABC and cadherin	24
	CNW	S		
P. gossypiella[e]	AZP-R	R	mutant cadherin	17
	APHIS-S	S		
T. ni[f]	GipBtR	R	mutant ABC	10, 30
	GipS	S	reduced APN expression[g]	

[a]R: resistant, S: susceptible

[b]ABC transporter protein

[c] aminopeptidase N

[d] aminopeptidase P

[e]Bioassay results with modified and native toxins reported previously[7], new results from Western blots for cadherin detection are reported here (**Supplementary Figure 1**).

[f]Bioassay results with modified and native toxins reported previously[10].

[g]Complementation test results show that the mutation(s) in the Glen resistant strain of *T. ni* reducing APN expression are allelic with the mutation(s) in the GipBtR strain (P. Wang pers. comm).

Supplementary Table 2. Reduction in resistance ratio for modified toxins relative to native toxins against *Plutella xylostella*, based on mortality of resistant (R) and susceptible (S) strains.

Strain type [a]	Toxin	n	Slope (SE)	LC_{50} (95% FL) [b]	Resistance ratio (RR) [c]	Reduction in RR [d]
Plutella xylostella (concentration in ng toxin per well)						
R	Cry1Ab	440	NA [e]	>100,000 [f]	>21,000	
S	Cry1Ab	824	1.5 (0.1)	4.69 (3.7 – 20)		
R	Cry1AbMod	448	4.0 (0.5)	288 (250 – 340)	3.1	>6900
S	Cry1AbMod	816	1.1 (0.1)	92.7 (NA)		
R	Cry1Ac	384	NA	>77,500 [g]	>110,000	
S	Cry1Ac	416	2.4 (0.3)	0.713 (0.56 – 0.89)		
R	Cry1AcMod	448	2.2 (0.3)	144 (110 – 180)	4.8	>23,000
S	Cry1AcMod	416	1.1 (0.1)	30.4 (20 – 49)		

[a] R: resistant, S: susceptible; see **Supplementary Table 1** for strain details.

[b] Concentration killing 50% of larvae and its 95% fiducial limits in ng toxin per well

[c] LC_{50} of resistant strain divided by LC_{50} of susceptible strain

[d] Resistance ratio of native toxin divided by resistance ratio of its modified counterpart

[e] Not available, could not be estimated

[f] The highest concentration tested (100,000 ng toxin per well) killed only 3.1% of larvae.

[g] The highest concentration tested (77,500 ng toxin per well) killed only 15.6% of larvae.

Supplementary Table 3. Reduction in resistance ratio for modified toxins relative to native toxins against *Ostrinia nubilalis*, *Diatraea saccharalis,* and *Helicoverpa armigera,* based on mortality of resistant (R) and susceptible (S) strains.

Strain type [a]	Toxin	n	Slope (SE)	LC_{50} (95% FL) [b]	Resistance ratio (RR) [c]	Reduction in RR [d]
Ostrinia nubilalis (concentration in ng toxin per cm^2 diet)						
R	Cry1Ab	448	0.9 (0.2)	6640 (3200–21,000)	3100	
S	Cry1Ab	448	1.8 (0.2)	2.15 (0.48 – 14)		
R	Cry1AbMod	384	1.3 (0.1)	11.9 (8.3 – 16)	1.9	1600
S	Cry1AbMod	320	2.2 (0.3)	6.24 (3.6 – 13)		
Diatraea saccharalis (concentration in µg toxin per g diet)						
R	Cry1Ab	1042	1.4 (0.1)	13.7 (11 - 17)	22	
S	Cry1Ab	1516	1.1 (0.1)	0.627 (0.45 - 0.88)		
R	Cry1AbMod	1072	2.5 (0.2)	4.95 (4.2 - 5.9)	1.7	13
S	Cry1AbMod	916	3.1 (0.3)	2.89 (2.5 - 3.4)		
R	Cry1Ac	1529	1.5 (0.1)	0.387 (0.30 - 0.50)	8.2	
S	Cry1Ac	936	2.0 (0.2)	0.0474 (0.038 - 0.057)		
R	Cry1AcMod	1018	1.9 (0.3)	2.61 (1.9 - 3.6)	2.0	4.1
S	Cry1AcMod	1120	1.9 (0.2)	1.31 (0.99 - 1.7)		
Helicoverpa armigera (concentration in ng toxin per cm^2 diet)						
R	Cry1Ac	471	2.2 (0.2)	1940 (1120 – 2820)	39	
S	Cry1Ac	477	1.3 (0.1)	50 (8 – 110)		
R	Cry1AcMod	527	1.7 (0.1)	3650 (2280 – 5910)	2.2	18
S	Cry1AcMod	575	1.7 (0.1)	1660 (1310 – 2080)		

[a] R: resistant, S: susceptible; see **Supplementary Table 1** for strain details.

[b] Concentration killing 50% of larvae and its 95% fiducial limits

[c] LC_{50} of resistant strain divided by LC_{50} of susceptible strain

[d] Resistance ratio of native toxin divided by resistance ratio of its modified counterpart

Supplementary Table 4. Potency ratio of modified Bt toxins for resistant and susceptible strains of six major crop pests, calculated as the LC_{50} of a native toxin divided by the LC_{50} of the corresponding modified toxin. Values >1 indicate the modified toxin was more potent than the native toxin, which occurred for all resistant strains below except Cry1AcMod relative to Cry1Ac for *D. saccharalis* and *H. armigera.* Values <1 indicate the native toxin was more potent than modified toxin, which occurred for all susceptible strains.

Species	Toxin pair	Potency ratio	
		Resistant strain	Susceptible strain
P. xylostella	Cry1AbMod/Cry1Ab	>350	0.050
	Cry1AcMod/Cry1Ac	>540	0.023
O. nubilalis	Cry1AbMod/Cry1Ab	560	0.32
D. saccharalis	Cry1AbMod/Cry1Ab	2.8	0.22
	Cry1AcMod/Cry1Ac	0.15	0.036
H. armigera	Cry1AcMod/Cry1Ac	0.53	0.030
P. gossypiella[a]	Cry1AbMod/Cry1Ab	>91	0.28
	Cry1AcMod/Cry1Ac	>110	0.012
T. ni[b]	Cry1AbMod/Cry1Ab	53	0.48
	Cry1AcMod/Cry1Ac	11	0.069

[a] Potency ratio based on previously reported data[7].

[b] Potency ratio based on previously reported data[10].

Supplementary Table 5. Reduction in resistance ratio for Cry1AcMod relative to Cry1Ac against *Heliothis virescens*, based on growth inhibition of resistant (R) and susceptible (S) strains.

Strain[a]	Toxin	n	Slope (SE)	IC_{50} (95% FL)[b]	Resistance ratio (RR)[c]	Reduction in RR[d]
R: YHD3	Cry1Ac	312	NA[e]	>100[f]	>2400	
R: YEE	Cry1Ac	300	NA	>100[g]	>2400	
R: YFO	Cry1Ac	450	1.4 (0.3)	35.0 (19.3 – 85)	850	
S: CNW	Cry1Ac	360	0.5 (0.1)	0.041 (0.001 - 0.22)		
R: YHD3	Cry1AcMod	312	1.4 (0.5)	37.1 (13 – 220)	2.5	>990
R: YEE	Cry1AcMod	300	NA	>100[h]	>6.6	NA
R: YFO	Cry1AcMod	450	NA	>100[i]	>6.6	100[j]
S: CNW	Cry1AcMod	360	2.1 (0.5)	15.1 (9.6 – 51)		

[a] R: resistant, S: susceptible; YHD3 had cadherin and ABC transporter protein mutations, YEE had only the ABC transporter mutation, and YFO had only the cadherin mutation (Supplementary Table 1)
[b] Concentration causing 50% larval growth inhibition in µg toxin per ml diet (95% fiducial limits)
[c] IC_{50} of resistant strain divided by IC_{50} of susceptible strain
[d] Resistance ratio of Cry1Ac divided by resistance ratio of Cry1AcMod
[e] Not available, could not be estimated
[f] The highest concentration tested (100 µg Cry1Ac per ml diet) caused 7.4 to 15.5% growth inhibition of YHD3 (**Supplementary Table 7**).
[g] The highest concentration tested (100 µg Cry1Ac per ml diet) caused 18.0% growth inhibition of YEE (**Supplementary Table 7**).
[h] The highest concentration tested (100 µg Cry1AcMod per ml diet) caused 6.1% growth inhibition of YEE (**Supplementary Table 7**).
[i] The highest concentration tested (100 µg Cry1AcMod per ml diet) caused 48.6% growth inhibition of YFO (**Supplementary Table 7**).
[j] One hundred µg Cry1Ac per ml diet caused 48.6% growth inhibition, which is slightly less than 50% growth inhibition. If IC_{50} is estimated to be 100, the reduction in RR would be 130 (850/6.6). If the IC_{50} is estimated to be slightly higher than 100 µg Cry1Ac per ml, the reduction in RR would be slightly less than 130 (e.g., 850/7 = 121, 850/8 = 106). Because of this approximation, we rounded the reduction in RR to 100, using just one significant digit. This conservative approach may underestimate the reduction in RR.

Supplementary Table 6. Potency ratio for modified Bt toxins against *H. virescens*, calculated as the IC_{50} of Cry1Ac divided by the IC_{50} Cry1AcMod. The value of >2.7 for YHD3 indicates the modified toxin was more potent than native toxin against this highly resistant strain. The values <1 for YFO, YEE and CNW indicate the native toxin was more potent than modified toxin against these strains.

Strain [a]	Potency ratio
R: YHD3	>2.7
R: YEE	<1 [b]
R: YFO	<0.35
S: CNW	0.0027

[a] R: resistant, S: susceptible; see **Supplementary Table 1** for strain details.
[b] Although the potency ratio for YEE could not be estimated from IC_{50} values, Cry1AcMod was significantly less potent than Cry1Ac based on responses at the highest concentration tested (100 µg Cry1Ac per ml diet; **Supplementary Table 7**).

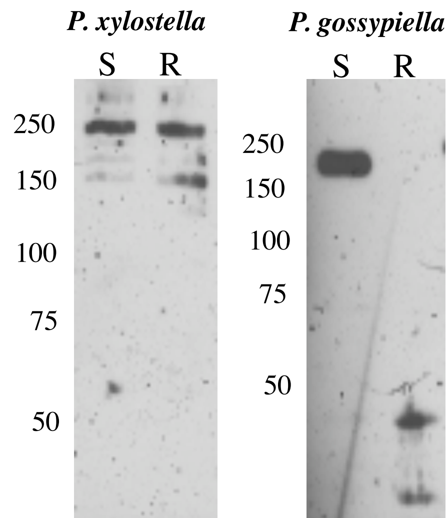
Supplementary Table 7. Larval growth inhibition of resistant (R) and susceptible strains of *H. virescens* caused by the highest concentrations tested of Cry1AcMod and Cry1Ac.

Strain	Type	Toxin	Concn. (μg/ml)	Trial[a]	Growth inhibition (%)[b]	P[c]
YHD3	R: cadherin + ABC	Cry1AcMod	100	1	85.9 ± 1.1	
		Cry1Ac	100	1	15.5 ± 2.2	<0.0001
		Cry1AcMod	100	2	64.7 ± 4.8	
		Cry1Ac	100	2	7.4 ± 10.1	<0.0001
YEE	R: ABC	Cry1AcMod	100	1	6.1 ± 1.8	
		Cry1Ac	100	1	18.0 ± 2.2	<0.0001
YFO	R: cadherin	Cry1AcMod	100	1+2	48.6 ± 2.2	
		Cry1Ac	100	1+2	82.6 ± 1.1	<0.0001
CNW	Susceptible	Cry1AcMod	10	1	34.2 ± 6.3	
		Cry1Ac	10	1	98.0 ± 0.3	<0.0001

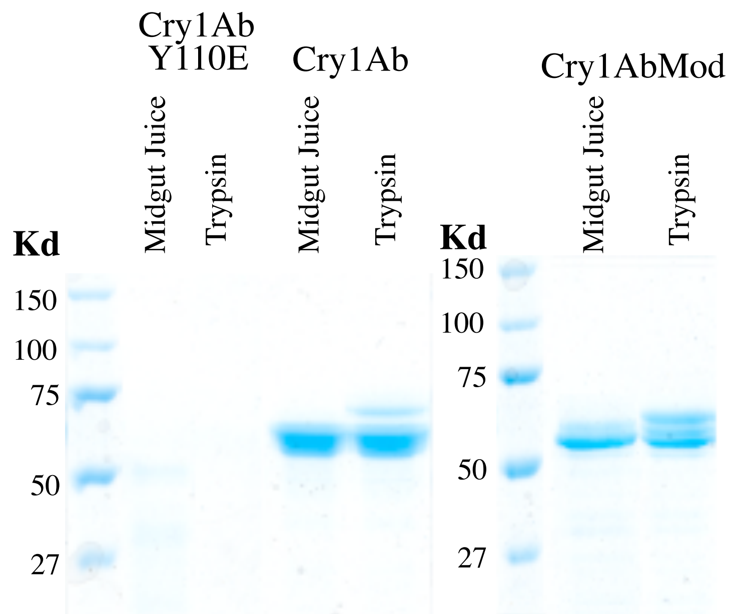
[a] Trial 1: n = 60 larvae per toxin per strain; Trial 2: n = 18 larvae per toxin per strain. For YHD3, we found a significant difference in growth inhibition between Trials 1 and 2 and thus analyzed data separately for each trial. For YFO, no significant difference occurred between Trials and thus we analyzed data jointly for Trials 1 and 2 (see **Online Methods** for details).

[b] Growth inhibition (%) = (1 - [larval weight on treated diet/mean larval weight on control diet]) X 100%, see Methods; values are means ± standard errors

[c]Probability that the difference in growth inhibition between Cry1AcMod and Cry1Ac occurred by chance, based on analysis of variance (ANOVA) (see **Online Methods** for details).



Supplementary Figure 1. Western blot of brush border membrane vesicles from susceptible (S) and resistant (R) strains of *P. xylostella* and *P. gosypiella* revealed with anti-cadherin antibody. In *P. xylostella,* a band of 210 Kd occurred in both the resistant NO-QAGE strain and the susceptible Geneva 88 strain. In contrast, results with *P. gossypiella* show a band of 180 Kd in the susceptible APHIS-S strain, but not in the resistant AZP-R strain that has cadherin mutations.



Supplementary Figure 2. Stability of Cry1Ab, Cry1AbMod, and Cry1Ab mutant Y110E after digestion with insect midgut juice or trypsin (see **Online Methods**). Cry1Ab and Cry1AbMod were similarly stable after digestion. Cry1Ab mutant Y110E was not stable.