RESEARCH ARTICLE

A Toxin-Binding Alkaline Phosphatase Fragment Synergizes Bt Toxin Cry1Ac against Susceptible and Resistant *Helicoverpa armigera*

Wenbo Chen1, Chenxi Liu1☯, Yutao Xiao1, Dandan Zhang1, Yongdong Zhang1, Xianchun Li2, Bruce E. Tabashnik2, Kongming Wu1*  

1 The State Key Laboratory for Biology of Plant Disease and Insect Pests, Institute of Plant Protection, Chinese Academy of Agricultural Sciences, West Yuanmingyuan Road, Beijing, 100193, China,  
2 Department of Entomology, University of Arizona, Tucson, AZ, 85721, United States of America  

☯ These authors contributed equally to this work.  
* kmwu@ippcaas.cn

Abstract

Evolution of resistance by insects threatens the continued success of pest control using insecticidal crystal (Cry) proteins from the bacterium *Bacillus thuringiensis* (Bt) in sprays and transgenic plants. In this study, laboratory selection with Cry1Ac yielded five strains of cotton bollworm, *Helicoverpa armigera*, with resistance ratios at the median lethal concentration (LC50) of activated Cry1Ac ranging from 22 to 1700. Reduced activity and reduced transcription of an alkaline phosphatase protein that binds Cry1Ac was associated with resistance to Cry1Ac in the four most resistant strains. A Cry1Ac-binding fragment of alkaline phosphatase from *H. armigera* (HaALP1f) was not toxic by itself, but it increased mortality caused by Cry1Ac in a susceptible strain and in all five resistant strains. Although synergism of Bt toxins against susceptible insects by toxin-binding fragments of cadherin and aminopeptidase N has been reported previously, the results here provide the first evidence of synergism of a Bt toxin by any toxin-binding peptide against resistant insects.

Introduction

The insecticidal proteins of *Bacillus thuringiensis* (Bt) kill some major insect pests, but are harmless to vertebrates and most other organisms [1–3]. In 2013, farmers planted genetically modified corn and cotton producing Bt toxins on 76 million hectares worldwide [4]. Widespread use of Bt toxins in sprays and transgenic crops has caused field-evolved resistance to Bt toxins in some pests, which entails a genetically based decrease in susceptibility [5, 6]. Some
degree of field-evolved resistance has been reported in two pest species exposed to Bt sprays and in nine pest species exposed to Bt crops [7–21].

The most widely used Bt proteins are crystalline (Cry) toxins, particularly Cry1Ab in Bt corn and Cry1Ac in Bt cotton that kill lepidopteran larvae [2, 6]. A key step in the mode of action of Cry1A toxins is binding of activated toxin to midgut membrane proteins [3]. Mutations that interfere with this step can confer resistance to Bt toxins [3, 22–24]. Insect midgut proteins that bind Cry toxins and are considered receptors for these toxins include cadherin, aminopeptidase N (APN), and alkaline phosphatase (ALP) [3, 24]. Disruption or reduced expression of the genes encoding these toxin-binding proteins is a common mechanism of resistance to Bt toxins in Lepidoptera [3, 22–31].

In many cases, toxin-binding fragments of cadherin or APN are not toxic alone, but they interact with Bt toxins either as synergists that increase mortality [32–46] or as antagonists that decrease mortality [45, 47–51]. Previous work, however, has not examined if ALP fragments synergize or antagonize Bt toxins. Moreover, many researchers have proposed that synergistic fragments of Bt toxin receptors might be useful for delaying or countering resistance, yet previous work has tested these fragments only against susceptible strains of pests [32–51].

Here we tested for synergism of Cry1Ac by an ALP fragment in susceptible and resistant strains of the cotton bollworm, Helicoverpa armigera, the most serious pest of cotton in China [52]. Bt cotton producing Cry1Ac was introduced to China in 1997 and has achieved great success against H. armigera [53, 54], yet monitoring has provided an early warning of field-evolved resistance to Cry1Ac in this pest [16, 30, 31, 55]. Previous work showed that Cry1Ac binds to two ALPs from H. armigera called HaALP1 and HaALP2 [56]. We analyzed the relationship between ALP and resistance to Cry1Ac in five laboratory-selected strains of H. armigera derived from a common susceptible strain from China. The results show that reduced activity and transcription of ALP were associated with resistance to Cry1Ac in the four most resistant strains studied here. We also discovered that a fragment of HaALP1, which we name HaALP1f, synergized Cry1Ac against larvae from a susceptible strain and all five resistant strains.

Results

Resistance associated with activity and transcription of ALP, but not with activity of APN

Relative to a susceptible strain (96S), laboratory selection yielded resistance ratios based on the median lethal concentration (LC50) of activated Cry1Ac ranging from 22 to 1700 in five strains of H. armigera (Table 1). Reduced ALP activity and transcription were associated with resistance to Cry1Ac in the four most resistant strains (LF10, LF30, LF60, and LF120), but not in

| Strain   | LC50 (95%fiducial limits) (μg Cry1Ac per ml diet) | Resistance ratio* |
|----------|-------------------------------------------------|-------------------|
| 96S (susceptible) | 0.013 (0.007–0.021) | 1.0 |
| LF5 (resistant)   | 0.286 (0.18–0.42)   | 22   |
| LF10 (resistant)  | 0.624 (0.45–0.86)   | 48   |
| LF30 (resistant)  | 3.80 (2.1–5.5)      | 280  |
| LF60 (resistant)  | 9.15 (5.8–13)       | 700  |
| LF120 (resistant) | 22.1 (16–31)        | 1700 |

* LC50 of each strain divided by the LC50 of the susceptible strain 96S.
the least resistant of the five selected strains (LF5) (Figs 1 and 2). ALP activity and transcription did not differ significantly between LF5 and the susceptible strain, but LF10, LF30, LF60 and LF120 had significantly lower ALP activity and transcription than the susceptible strain (Figs 1 and 2). As expected, ALP activity and ALP transcription were positively associated across all observations for the six susceptible and resistant strains of *H. armigera* (linear regression, $F_{1, 34} = 58.6, R^2 = 0.63, P < 0.0001$). The resistance ratio for Cry1Ac activated toxin (log-transformed) was negatively associated with mean ALP activity across the six susceptible and resistant strains (linear regression, $F_{1, 4} = 35.1, R^2 = 0.90, P = 0.004$). APN activity did not vary significantly among the six strains (Fig 3).

### Production and characterization of *H. armigera* ALP fragment (HaALP1f)

Using PCR amplification, we cloned a previously described 780 bp cDNA fragment of the gene from *H. armigera* encoding HaALP1 [56]. The peptide encoded by this cDNA fragment, referred to here as HaALP1f, is predicted to have 260 amino acid residues (192A-T451) and a molecular weight of 30 kDa. SDS-PAGE analysis of the total extracts of *Escherichia coli* cells transformed with the his-tagged HaALP1f-pET28a+ construct revealed a protein band of the expected size (about 35 kDa, indicated by a black arrowhead in Fig 4A. This band was not only the strongest protein band, but also the only inducible band by IPTG (compared lane 1 and 2 in Fig 4A). This band was present in the supernatant (lane 3 in Fig 4A) and the pelleted inclusion bodies (lane 4 in Fig 4A). After purification with Ni-affinity column that captures his-tagged proteins, the 35–37 kDa band was the only visible band on the gel (lane 5 in Fig 4A). Western blot hybridization of the Ni column-purified proteins from IPTG-induced HaALP1f-expressing *E. coli* cells with the anti-his antibody showed that the visible 35–37 kDa band was strongly hybridized with the anti-his antibody, confirming that this band represented the his-tagged HaALP1f (Fig 4B). Western blot also revealed a weak positive band of around 74 kD (Fig 4B), suggesting that a small portion of the heterologously expressed HaALP1f existed as a homodimer.

### Cry1Ac binding to HaALP1f

Ligand blot analysis revealed that the lysates of *E. coli* cells expressed with his-tagged HaALP1f had a protein of the same size with the his-tagged HaALP1f (35–37 kDa) that bound to the activated Cry1Ac (lane 2 in Fig 5). And the intensity of the Cry1Ac-binding protein band was much stronger in the Ni column-purified proteins from IPTG-induced ALP-expressing *E. coli* cells (lane 3 in Fig 5) than in the crude lysates of *E. coli* cells expressed with his-tagged HaALP1f. By contrast, the lysates of control *E. coli* cells transformed with the empty pET28a + vector did not have a protein capable of binding to the activated Cry1Ac (Lane 1 in Fig 5). In addition, overlay plot of the surface plasmon resonance (SPR) sensorgrams between different concentrations of the purified HaALP1f and immobilized activated Cry1Ac also showed HaALP1f-activated Cry1Ac binding interaction (Fig 6). A 1:1 binding stoichiometry produced the following apparent rate constants of the bimolecular interaction: $k_a = 3.27 \times 10^5 \text{M}^{-1}\text{s}^{-1}$ and $k_d = 2.48 \times 10^{-3} \text{s}^{-1}$, $K_D = 7.58$ nM.

### HaALP1f synergizes Cry1Ac against susceptible and resistant strains

We tested for synergism in a series of bioassays that examined mortality caused by Cry1Ac activated toxin alone, HaALP1f alone, and combinations of various concentrations of Cry1Ac activated toxin and HaALP1f. For each of the six susceptible and resistant strains of *H. armigera*, one or more combinations of HaALP1f and Cry1Ac caused significantly higher mortality than Cry1Ac alone (Figs 7 and 8). For each of the six strains, HaALP1f was not toxic by itself, as
Fig 1. ALP activity in BBMV from susceptible and resistant *H. armigera* larvae. Different letters above the error bars indicate significant differences between means (\( P < 0.05 \)).

doi:10.1371/journal.pone.0126288.g001

Fig 2. Relative ALP transcription detected by qRT-PCR in susceptible and resistant *H. armigera* larvae. Different letters above the error bars indicate significant differences between means (\( P < 0.05 \)).

doi:10.1371/journal.pone.0126288.g002
Fig 3. APN activity in BBMV from susceptible and resistant \textit{H. armigera} larvae. The same letter (a) above the standard error bars indicates no significant differences in mean APN activity among strains (P > 0.05).

doi:10.1371/journal.pone.0126288.g003

Fig 4. Detection of HaALP1f expressed in \textit{E. coli}. (A) SDS-PAGE separation of the protein extracts from HaALP1f-expressing \textit{E. coli} cells. M: Molecular weight markers; Lane 1: total extract from non-induced HaALP1f-expressing \textit{E. coli} cells; Lane 2: total extract from IPTG-induced HaALP1f-expressing \textit{E. coli} cells; Lane 3: the supernatant (from 20 min centrifugation at 25,000xg) of total extract from IPTG-induced HaALP1f-expressing \textit{E. coli} cells; Lane 4: The pelleted inclusion body (from 20 min centrifugation at 25,000xg) of total extract from IPTG-induced HaALP1f-expressing \textit{E. coli} cells; Lane 5: Purified HaALP1f from the pelleted inclusion body from IPTG-induced HaALP1f-expressing \textit{E. coli} cells by using Ni-affinity column. (B) Detection of purified HaALP1f by Western blot.

doi:10.1371/journal.pone.0126288.g004
indicated by the lack of significant difference in mortality between HaALP1f in PBS buffer alone and the control with only the PBS buffer (6 pairwise comparisons, \( P > 0.05 \) in each comparison, Fig 7). In addition, with the data from all six strains pooled, mortality did not differ significantly between the PBS buffer with HaALP1f (mean = 10.9\%, 95\% confidence interval = 8.6 to 13.2) and the PBS buffer without HaALP1f (mean = 8.6\%, 95\% confidence interval = 5.8 to 11.4\%); t-test, \( t = 1.36, df = 34, P = 0.18 \). Therefore, the significantly increased
mortality seen with the combination of HaALP1f and Cry1Ac was caused by synergism, rather than independent toxicity of HaALP1f.

For each strain, we tested a fixed concentration of Cry1Ac that by itself caused 23 to 48% mortality (mean = 35%), with the concentration ranging from 37 ng/cm² for the susceptible strain to 1500 ng/cm² for the most resistant strain (LF120) (Fig 7). For each strain, we tested HaALP1f in combination with the fixed concentration of Cry1Ac in ratios of HaALP1f:Cry1Ac of 1:1, 5:1, 15:1 or 50:1 by weight. For all six strains, the 50:1 ratio of HaALP1f:Cry1Ac significantly increased mortality compared with Cry1Ac alone, with a mean increase in mortality of 26% (range = 6.9 to 38%) (Fig 7).

For all strains except LF10, ratios of 15:1 and 5:1 HaALP1f:Cry1Ac also significantly increased mortality compared with Cry1Ac alone (Fig 7). At the 1:1 ratio of HaALP1f:Cry1Ac, mortality with the mixture was significantly higher than with Cry1Ac alone only for the most resistant strain (LF120) (Fig 7). For all strains, the increase in mortality was significantly higher for HaALP1f:Cry1Ac at 50:1 compared with 1:1 (U test, P = 0.02). The increase in mortality caused by adding HaALP1f to Cry1Ac did not differ significantly between the susceptible strain (mean = 25%, SE = 6) and the most resistant strain (LF120, mean = 18%, SE = 6; paired t-test, t = 1.7, df = 3, P = 0.18).

For the susceptible strain, we also examined the effects of a 15:1 ratio of HaALP1f:Cry1Ac across a series of five concentrations of Cry1Ac activated toxin (Fig 8). The larval mortality was significantly higher for the mixture of HaALP1f and Cry1Ac than for Cry1Ac alone at each of
the five concentrations of Cry1Ac (Fig 8). The LC50 (95% FL) of Cry1Ac was 29 (23.4 to 34.6) ng/cm² without HaALP1f and 19 (13.3 to 22.7) ng/cm², representing a significant, 1.6-fold synergy of Cry1Ac by HaALP1f.

Discussion

The results here considered together with previous data indicate that several mechanisms are associated with resistance to Cry1Ac in lab- and field-selected populations of *H. armigera*, including reduced ALP activity and transcription, reduced APN activity, reduced conversion of protoxin to toxin, down-regulation of trypsin, mutations disrupting the extracellular and
intracellular domains of cadherin, a mutation disrupting the ATP-binding cassette protein ABCC2, and mutations in unidentified genes [28–31, 57–65]. In the four most resistant strains of *H. armigera* studied here (LF10, LF30, LF60 and LF120), resistance to activated Cry1Ac toxin ranged from 48- to 1700-fold relative to a susceptible strain, and was associated with reduced ALP activity and transcription (Table 1, Figs 1 and 2).

The association between resistance to Cry1Ac and reduced ALP activity and transcription in these four strains is similar to results reported previously for resistant strains of three species of moths in the family Noctuidae, including the BtR strain of *H. armigera* [29]. The BtR strain was derived from the same susceptible strain we studied here (96S), selected in the laboratory with Cry1Ac protoxin in diet, and had >2900-fold resistance to Cry1Ac protoxin relative to 96S [29]. Relative to 96S, ALP activity was reduced by 2.3-fold in BtR [29] and 4.1-fold in LF120 (Fig 1); ALP transcription was reduced by 1.6-fold in BtR [29] and 2.9-fold in LF120 (Fig 2). In addition to reduced ALP activity, BtR also had reduced APN activity compared with 96S [29]. In contrast, previous data showed that expression of cadherin and APN was similar in LF120 and 96S [66]. Consistent with this previous result, APN activity did not vary significantly among the six strains analyzed here, including LF120 and 96S (Fig 3). However, we cannot exclude the possibility that several single amino acid differences found between LF120 and 96S in the cadherin and APN genes contribute to resistance [66]. The least resistant of the five selected strains studied here (LF5) had 22-fold resistance to Cry1Ac activated toxin and it did not differ from 96S in either ALP activity or transcription (Figs 1 and 2). Previous work identified cis-mediated down-regulation of trypsin as a mechanism of resistance in LF5 [64], mis-splicing of the ABCC2 gene as a mechanism of resistance in LF60 [65], and significantly reduced chemotrypsin-like activity in LF10 and LF30 relative to LF5 [63].
In a previous study, HaALP expressed in Sf9 cells bound activated Cry1Ac, but this binding depended on N-linked oligosaccharides [56]. Although proteins expressed in E. coli cells are usually not glycosylated, our two independent experiments using ligand blotting and SPR analysis confirmed that the HaALP1f expressed in E. coli bound activated Cry1Ac. Furthermore, at least three recent papers demonstrate binding of E. coli-expressed ALP to Cry toxins: binding of MsALP from M. sexta to Cry1Aa, Cry1Ab and Cry1Ac [67], binding of Aa-mALP from Aedes aegypti to Cry4Ba [68], and binding of a truncated ALP fragment (AgALP1t) from Anopheles gambiae to Cry11Ba [69]. The apparent affinity (K_d) measured by SPR is 7.58 nM for HaALP1f-Cry1Ac binding in our study compared with 4 μM for MsALP-Cry1Ac binding reported for Manduca sexta [67]. This comparison shows that the binding affinity was 527-fold greater in our study.

The results here indicate that a fragment of ALP from H. armigera (HaALP1f) synergized Cry1Ac against a susceptible strain and all five resistant strains of H. armigera tested here. As far as we know, these results provide the first evidence that any ALP fragment synergizes a Bt toxin and the first evidence that any toxin-binding peptide synergizes a Bt toxin against a resistant strain. It remains to be determined if HaALP1f or other ALP fragments synergize Bt toxins against other strains of H. armigera or other pests. In future research aimed to determine the potential usefulness of toxin-binding fragments for managing resistance, it will be essential to test for synergism in resistant strains of pests.

Materials and Methods
Insect strains and rearing
We used six strains of H. armigera: one susceptible strain (96S) and five Cry1Ac-resistant strains (LF5, LF10, LF30, LF60 and LF120) [70]. The 96S strain was started with 20 pairs of adults collected from Xinxiang, Henan Province, China in 1996, and had been reared in the laboratory for >15 years on artificial diet without exposure to Bt toxins [71, 72]. The five resistant strains originated from the LF strain, which was started with 200 larvae collected from cotton fields in Langfang, Hebei Province, China in 1998 [73]. No permits were required because all collections were made in China under the auspices of the Chinese Ministry of Agriculture. Larvae from all strains were reared on diet. Rearing, selection, and bioassays were conducted at 27±2°C, photoperiod 14L:10D, and 75±10% relative humidity.

Selection
We selected each of the five resistant strains with MVIPII (Dow AgroSciences), a commercial formulation of CryAc protoxin [74–76] incorporated in diet at concentrations that yielded about 20% survival of neonates to the pupal stage [77]. First, we generated the LF5 strain by selecting the LF strain initially at 1 μg Cry1Ac protoxin per g diet for 38 generations (Table 2).

| Strain | Parent strain | Year started | Selection concentration(μg per g diet) |
|--------|---------------|--------------|--------------------------------------|
| LF5    | LF*           | 2002         | 5                                    |
| LF10   | LF5           | 2003         | 10                                   |
| LF30   | LF10          | 2007         | 30                                   |
| LF60   | LF30          | 2008         | 60                                   |
| LF120  | LF60          | 2008         | 120                                  |

*Started in 1998 with 200 larvae collected from Langfang, Hebei Province, China.
2002, we increased the selection concentration for LF5 to 5 μg Cry1Ac protoxin per g diet. We maintained that concentration for 104 generations of selection for LF5. In 2003, we started the LF10 strain with a subset of LF5 and selected at 10 μg Cry1Ac protoxin per g diet. We used analogous methods to start the LF30, LF60, and LF120 strains (Table 2).

**Bioassays**

For each of the six strains of *H. armigera*, we used diet incorporation bioassays to determine the LC₅₀ of Cry1Ac and diet overlay bioassays to determine the toxicity of Cry1Ac with and without HaALP1f. We used the activated form of Cry1Ac in all bioassays. To obtain activated Cry1Ac toxin, Cry1Ac protoxin was incubated 2 h at 37°C with a 25:1 ratio of trypsin (Sigma) to protoxin, and the soluble trypsinized toxin was purified by a Superdex 200 HR 10/30 column (Amersham Biosciences) on a fast protein liquid chromatography (FPLC) system. Cry1Ac protoxin was extracted and purified from the HD73 strain of *B. thuringiensis* subsp. *kurstaki* by the Biotechnology Group in Institute of Plant Protection, Chinese Academy of Agricultural Sciences, Beijing, China.

In all bioassays, we put one first instar in each well of a 24-well plate, with 24 first instars in each replicate and three replicates per treatment (total n = 72 per treatment). Larvae were considered dead if they died or did not reach third instar after 7 days.

**Diet incorporation bioassays to determine LC₅₀ of Cry1Ac.** We used diet incorporation bioassays to determine the LC₅₀ of activated Cry1Ac toxin for each of the six strains. Various concentrations of Cry1Ac activated toxin were added and thoroughly mixed with diet to obtain the desired concentrations. After mixing, the diet solidified and we put pieces of solid diet (1 mg) into each well of a 24-well plate.

**Diet overlay bioassays to determine effects of HaALP1f on toxicity of Cry1Ac.** We used diet overlay bioassays to test the activated form of Cry1Ac with and without HaALP1f. Cry1Ac, purified HaALP1f, or both were diluted in PBS buffer, and incubated at 4°C for 1 h. We poured 1.5 ml liquid diet into each well of a 24-well plate. After the diet solidified, 100 μl samples of the appropriate treatment materials (see below) were applied to the diet surface of each well and allowed to air dry.

Treatments consisted of activated Cry1Ac either alone (37 ng/cm² for 96S, 99 ng/cm² for LF5 and LF10, 620 ng/cm² for LF30 and LF60, 1500 ng/cm² for LF120) or with HaALP1f at four ratios of HaALP1f:Cry1Ac by weight (1:1, 5:1, 15:1 and 50:1). We also tested the susceptible 96S strain at a series of concentrations of Cry1Ac activated toxin (19 to 75 ng/cm²) either alone or in combination with HaALP1f at a fixed ratio of 15:1 HaALP1f:Cry1Ac by weight. We used three controls: untreated diet, diet treated only with PBS buffer, and diet treated only with HaALP1f in PBS buffer. In the treatments with only HaALP1f in PBS buffer, we tested the highest concentration of HaALP1f used in evaluating synergy for each strain, which ranged from 1870 ng/cm² for 96S to 74,600 ng/cm² for LF120.

**Brush border membrane vesicles (BBMV) and Aminopeptidase N (APN) Activity**

We isolated BBMV by differential centrifugation [78], assessed by SDS-PAGE and kept them at -80°C until used. We measured BBMV protein concentration [79] using bovine serum albumin (BSA) (TransGene) as the standard.

The activity of APN, a marker enzyme for lepidopteran BBMV, was assayed by using leucine p-nitroanilide as the substrate [29]. For each strain, APN activity in the BBMV preparations was enriched six to eight-fold relative to the initial midgut homogenates. Three micrograms protein of each samples was used for assays. Enzymatic activities were monitored for 3 min as
changes in optical density (OD) at 410 nm wavelength at room temperature in a microplate reader (BioTek). We calculated the maximum initial velocity (Vmax) using the Gen5 Data Analysis Software. For each of three independent preparations of BBMV, we measured APN activity three times for each of three samples in Oct. 2014.

Alkaline phosphatase (ALP) activity

We determined ALP activity using a commercial kit (Alkaline phosphatase, Hou-Bio, P. R. China) as described by Jurat-Fuentes et al. (2011) [29]. Five μg protein of each samples was used for assays. Enzymatic activities were monitored for 2–3 min as changes in optical density (OD) at 405 nm wavelength at room temperature in a microplate reader (BioTek). We calculated the maximum initial velocity (Vmax) using the Gen5 Data Analysis Software. For each of three independent preparations of BBMV, we measured ALP activity three times for each of six samples.

ALP RNA determination by quantitative real-time (qRT-PCR)

1) RNA preparation and cDNA synthesis. Total RNA was extracted from midguts of 5th instar H. armigera larvae using Trizol reagent (Invitrogen) according to manufacturer’s instructions. Total RNA was treated with DNase I (TaKaRa) to remove residual genomic DNA contamination. The integrity of total RNA was verified on a 1% agarose gel. Two μg RNA for each sample was reverse-transcribed with Quantscript RT Kit (TianGen, China) according to the manufacturer’s instructions. We used first strand cDNA as a template for qRT-PCR, with primers and reaction conditions as described previously for H. armigera [29].

2) Primer design. Oligonucleotide primers were designed using Primer 3 (http://frodo.wi.mit.edu/primer3/). Primers were made to amplify a 128 bp conserved region between the HaALP1 (accession no. EU729322) and HaALP2 (accession no. EU729323) isoforms. TaqMan probes (Invitrogen) were labeled at the 5’ end by the reporter dye FAM and at the 3’ end by the quencher dye TAMRA. Forward primer 5’ ATA GGC GTA GAC GGC ACG G 3’, reverse primer 5’ CGA GTC GTC GTC ACA ATA CCG 3’, and 5’-FAM CGC CGA GGA GAC TGT CAA GCC GCT T3’-TAMRA were used for HaALP fragment amplification. As endogenous control, we amplified a 184 bp fragment of H. armigera actin (accession no. X97615) with forward primer 5’ CAC AGA TCA TGT TCG AGA CGT TCA A 3’, reverse primer 5’- GCC AAG TCC AGA CGC AGG AT-3’ and 5’-FAM CCG CCA TGT ACG TCG CCA TCC AGG 3’-TAMARA.

3) Real-time PCR reactions and data analysis. We performed qRT-PCR in triplicate for each of at least three independent biological samples using methods similar to Jurat-Fuentes et al. 2011 [29]. Reactions for each H. armigera sample (25 μl) consisted of 12.5 μl of Premix Ex Taq (2×) (TaKaRa), 0.5 μl of Rox Reference DyeII (50×), probe (0.2 μM), primers (0.4 μM), 1 μl of sample cDNA and 8.5 μl sterilized ultrapure water.

Amplification conditions were an initial denaturation at 95°C for 2 min followed by 40 cycles of 95°C for 15 s, and a single step for annealing and extension was done at 60°C for 60 s. We used a relative quantitation method \(2^{-ΔΔCT}\) [80] to evaluate quantitative variation. Transcript amounts were standardized to 1 with the sample from susceptible larvae containing the highest transcript levels from the three biological replicate reactions performed.

Cloning, expression and purification of HaALP1f

A 780 bp cDNA fragment of the gene encoding HaALP1 [56] was cloned and expressed in E. coli as a His-tag recombinant protein. Total RNA was extracted and treated with DNase I (TaKaRa) as described above, then reverse-transcribed with SuperScript III RNase H
reverse-transcriptase (Invitrogen). The cDNA fragments were used as a template for PCR amplification using primers ALP-F (5′-CGGGATCCGGAAGACGGCGAACCGCACCTG-3′ BamHI) and ALP-R (5′-CGCTCGAGAGTGCGATAGTTGCTCAAGGGT-3′ XhoI). The PCR products were purified with DNA purification system Kit (Biomed) and cloned into the pMD 19-T simple vector (TaKaRa) following the manufacturer’s instructions. The recombinant plasmid was excised with BamHI and XhoI, subcloned into the His-tagged expression vector pET28a+ (Novagen), and transfected into E. coli BL21 (DE3) cells (Transgen, China). The transformants were cultured overnight with constant agitation at 37°C in 5 ml of Luria-Bertani (tryptone 1.0% (w/v), yeast extract 0.5% (w/v) and NaCl 1.0% (w/v), supplemented with 20 mg/L Kanamycin). The following morning, 4 ml of this overnight culture were used to inoculate 400 ml of LB broth plus 20 mg/L kanamycin in a 1000 ml flask. This culture was allowed to grow at 37°C with constant agitation until reaching an OD of 0.7 at 600nm, and expression was induced with 0.2 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) for 5 h at 37°C with constant agitation.

For purification of the expressed fragment (HaALP1f), pellets of cultured E. coli BL21 cells transformed with the ALP fragment-pET28a+ construct were collected by centrifugation at 3000×g, 4°C for 20 min, washed with 50 ml of ice-cold PBS buffer, re-suspended in 30 ml of PBS buffer, and sonicated for 15 min on ice. After 25,000×g centrifugation for 20 min at 4°C, the expressed HaALP1f fragment as inclusion bodies were solubilized with 15 ml of 8M urea in PBS buffer. The solubilized fragments were subjected to affinity purification using Ni-Sepharose beads (Amersham Biosciences). The HaALP1f fragments were eluted with 15 ml of 500 mM imidazole and dialyzed against PBS buffer. The purified ALP fragments were separated by 10% SDS-PAGE.

Detection of HaALP1f and binding of Cry1Ac to HaALP1f

Western blot analysis was used to detect expression of HaALP1f. Purified HaALP1f was separated in a 10% SDS-PAGE gel and transferred to a polyvinylidene difluoride (PVDF) membrane filter. At room temperature, the PVDF membrane was blocked for 2 h in 25 ml of blocking buffer (PBS buffer, 5% skim milk powder, pH 7.4), hybridized with 2.5 μl of anti-His-tag monoclonal antibody (Cal Bio, China) (1:10,000) for 1 h, and washed five times (5 min each) with 25 ml of PBST (PBS buffer, 0.1% Tween-20, pH 7.4). After washing, the PVDF membrane was probed with 1.5 μl of HRP-conjugated secondary antibody (ZSGB-BIO, China) (1:20,000) for 1 h at room temperature. The resultant his-tagged HaALP1f fragment peptide-antibody complex on the PVDF membrane was visualized using the Super ECL Plus Detection Kit (Applygen, China).

We used ligand blot analysis to detect binding of Cry1Ac to purified HaALP1f. As in Western blot analysis, this analysis was performed at room temperature and purified HaALP1f was separated in a 10% SDS-PAGE gel and transferred to a PVDF membrane. The PVDF membrane was blocked for 2 h with 25 ml blocking buffer (PBS buffer, 5% skim milk powder, pH 7.4), incubated in 25 ml of PBST buffer (PBS buffer, 0.1% Tween-20, pH 7.4) containing 3 μg of activated Cry1Ac toxin for 1.5 h, and washed. After washing five times (as described above), the PVDF membrane was incubated with 2.5 μl of polyclonal anti-Cry1Ac antibody (1:10,000) for 1.5 h at room temperature in PBST buffer. After washing again, the PVDF membrane was probed with 1.5 μl of an HRP-conjugated secondary antibody (1:20,000) for 1 h, and visualized as described above.

SPR experiment was performed on a BIAcore3000 machine (Biocore AB). Cry1Ac toxin in 10mM sodium acetate, pH 4.0, was immobilized on a CM5 sensor chip by amine coupling method (Biacore AB). The flow buffer HBS (10 Mm HEPES, 150mM NaCl, 0.005% Tween20
(v/v), pH 7.4) was used at a flow rate of 40 μl/min. Multiple concentrations (31.25 nM, 62.5 nM, 125 nM, 250 nM, 500 nM) of HaALP1f was injected across the flow cell containing the Cry1Ac and one blank flow cell containing ethanolamine as a blocking agent. Surfaces were regenerated with a 30-second injection of 5 mM NaOH at a flow rate of 40 μl/min. Signal responses from the blank flow cells were subtracted from all response curves and data were locally fitted using BIAevaluation Ver. 4.1 (Biacore AB). The curves were fitted to a simple 1:1 Langmuir binding model (A+B ⇋ AB) to obtain apparent rate constants.

**Statistical analyses**

We used POLO [81] to estimate the concentration of Cry1Ac activated toxin killing 50% (LC50) and its 95% fiducial limits, as well as the slope of the concentration-mortality line and its standard error (SE). We calculated resistance ratio as the LC50 of a strain divided by the LC50 of the susceptible strain (96S).

We used analysis of variance (ANOVA) with Duncan’s multiple range test for multiple comparisons (P < 0.05) to evaluate a) variation among strains in relative ALP transcription; b) variation within strains in mortality among each of the eight controls and treatments in diet overlay bioassays; and c) variation among strains in ALP activity and transcription, and d) variation among strains in APN activity. We used linear regression to assess the relationship between ALP activity and both resistance ratio (log-transformed) and ALP transcription [82].

We tested for synergism using what Tabashnik [83] described as "perhaps the simplest approach," by comparing mortality caused by Cry1Ac alone with mortality caused by sublethal concentrations of HaALP1f. The controls with only HaALP1f and PBS buffer showed that HaALP1f at the highest concentrations tested in combination with Cry1Ac did not cause mortality (Results). In this case, assuming no synergism, adding HaALP1f to Cry1Ac is not expected to increase mortality. Thus, significantly greater mortality caused by combinations of Cry1Ac and HaALP1f relative to Cry1Ac alone indicates synergism [83]. This approach has been applied previously to evaluate synergism between Cry toxins [84], between Cry and Cyt toxins [85], and between Cry toxins and fragments of cadherin-binding proteins [46]. For each of the four ratios of HaALP1f to Cry1Ac (1:1, 5:1, 15:1 and 50:1) tested against each strain, we used ANOVA with Duncan’s multiple range test for multiple comparisons (P < 0.05) to determine if the mean mortality with the combination of Cry1Ac and HaALP1f was significantly greater than the mean mortality with Cry1Ac alone (Fig 7). For the susceptible 96S strain, we also examined the effects of a 15:1 ratio of HaALP1f:Cry1Ac across a series of five concentrations of Cry1Ac activated toxin (Fig 8). In addition to comparing mortality of the combination of Cry1Ac and HaALP1f versus Cry1Ac alone at each concentration as described above, we used POLO [81] to estimate the LC50 of Cry1Ac with and without HaALP1f. We used these data to test for synergism by determining if the LC50 with HaALP1f was significantly greater than the LC50 without HaALP1f, as indicated by non-overlap of the 95% fiducial limits.

**Supporting Information**

S1 Table. Data for Figs 1 and 3 APN & ALP activity.
(XLSX)

S2 Table. Data for Fig 2 ALP transcription.
(XLS)

S3 Table. Data for Fig 6 Cry1Ac binding to HaALP1f.
(XLSX)
S4 Table. Data for Fig 7 Synergism of Cry1Ac by HaALP1f vs. susceptible & resistant strains.
(XLS)

S5 Table. Data for Fig 8 Synergism of Cry1Ac by HaALP1f vs. susceptible strain.
(XLSX)

S6 Table. Data for enrichment of APN activity in BBMV.
(XLS)

Acknowledgments
We thank Brenda Oppert and Yidong Wu for comments that improved the paper.

Author Contributions
Conceived and designed the experiments: KW WC CL. Performed the experiments: WC CL.
Analyzed the data: WC CL YZ DZ YX XL BET KW. Wrote the paper: BET WC CL XL KW.

References
1. Mendelsohn M, Kough J, Vaituzis Z, Matthews K (2003) Are Bt crops safe? Nat Biotechnol 21: 1003–1009. PMID:12949561
2. Sanahuja G, Banakar R, Twyman RM, Capell T, Christou P (2011) Bacillus thuringiensis: a century of research, development and commercial applications. Plant Biotechnol J 9: 283–300. doi:10.1111/j.1467-7652.2011.00595.x PMID:21375687
3. Pardo-López L, Soberón M, Bravo A (2013) Bacillus thuringiensis insecticidal three-domain Cry toxins: mode of action, insect resistance and consequences for crop protection. FEMS Microbiol Rev 37: 3–22. doi:10.1111/j.1574-6976.2012.00341.x PMID:22540421
4. James C (2013) Global Status of Commercialized Biotech/GM Crops: 2013. ISAAA Brief No. 46. ISAAA: Ithaca, NY.
5. Tabashnik BE (1994) Evolution of resistance to Bacillus thuringiensis. Annu Rev Entomol 39: 47–79.
6. Tabashnik BE, Van Rensburg JBJ, Carrière Y (2009) Field-evolved insect resistance to Bt crops: definition, theory, and data. J Econ Entomol 102: 2011–2025. PMID:20069826
7. Tabashnik BE, Cushing NL, Finson N, Johnson MW (1990) Field development of resistance to Bacillus thuringiensis in diamondback moth (Lepidoptera: Plutellidae). J Econ Entomol 83: 1671–1676.
8. Janmaat AF, Myers J (2003) Rapid evolution and the cost of resistance to Bacillus thuringiensis in greenhouse populations of cabbage loopers, Trichoplusia ni. Proc Roy Soc London B 270: 2263–2270.
9. Luttrell RG, Ali I, Allen KC, Young SYIII, Szalanski AL, Williams K, et al. (2004) Resistance to Bt in Arkansas populations of cotton bollworm, pp. 1373–1383. In D. A. Richter (ed.), Proceedings, 2004 Belt-wide Cotton Conferences, 5–9 January 2004, San Antonio, TX, National Cotton Council of America, Memphis, TN.
10. Van Rensburg J (2007) First report of field resistance by stem borer, Busseola fusca (Fuller) to Bt-transgenic maize. South African Journal of Plant and Soil 24: 147–151.
11. Tabashnik BE, Gassmann AJ, Crowder DW, Carrière Y (2008) Insect resistance to Bt crops: evidence versus theory. Nat Biotechnol 26: 199–202. doi:10.1038/nbt1382 PMID:18259177
12. Downes S, Parker T, Mahon R (2010) Incipient resistance of Helicoverpa punctigera to the Cry2Ab Bt toxin in Bollgard II cotton. PLoS ONE 5: e12567. doi:10.1371/journal.pone.0012567 PMID:20830203
13. Storer NP, Babcock JM, Schlenz M, Meade T, Thompson GD, Bing JW, et al. (2010) Discovery and characterization of field resistance to Bt maize: Spodoptera frugiperda (Lepidoptera: Noctuidae) in Puerto Rico. J Econ Entomol 103: 1031–1038. PMID:20857709
14. Dhurua S, Gujar GT (2011) Field-evolved resistance to Bt toxin Cry1Ac in the pink bollworm, Pectinophora gossypiella (Saunders) (Lepidoptera: Gelechiidae), from India. Pest Manage Sci 67: 898–903. doi:10.1002/ps.2127 PMID:21438121
15. Gassmann AJ, Petzold-Maxwell JL, Keweshan RS, Dunbar MW (2011) Field-evolved resistance to Bt maize by western corn rootworm. PLoS ONE 6: e22629. doi: 10.1371/journal.pone.0022629 PMID: 21829470

16. Zhang H, Yin W, Zhao J, Jin L, Yang Y, Wu S, et al. (2011) Early warning of cotton bollworm resistance associated with intensive planting of Bt cotton in China. PLoS ONE 6: e22874. doi: 10.1371/journal.pone.0022874 PMID: 21857961

17. Alcantara E, Estrada A, Alpuerto V, Head G (2011) Monitoring Cry1Ab susceptibility in Asian corn borer (Lepidoptera: Crambidae) on Bt corn in the Philippines. Crop Prot 30: 554–559.

18. Alvi AHK, Sayyed AH, Naeem M, Ali M (2012) Field Evolved Resistance to Bt maize in cotton bollworm from China. PLoS ONE 7(10): e29975. doi:10.1371/journal.pone.0029975 PMID: 22238687

19. Tabashnik BE, Brévault T, Carru CC (2001) Identification of a gene associated with Bt resistance in Heliothis virescens. Science 293: 857–860. PMID: 1148008

20. Ferré J, Van Rie J (2002) Biochemistry and genetics of insect resistance to Bacillus thuringiensis. Annu Rev Entomol 47: 501–533. PMID: 11729083

21. Heckel DG, Gahan LJ, Baxter SW, Zhao JZ, Shelton AM, Gould F, et al. (2007) The diversity of Bt resistance genes in species of Lepidoptera. J Invert Pathol 95: 192–197.

22. Wu Y (2014) Detection and mechanisms of resistance evolved in insects to Cry toxins from Bacillus thuringiensis. Adv Insect Physiol 47: 297–342.

23. Morin S, Biggs RW, Sisterson MS, Shriver L, Ellers-Kirk C, Higginson D, et al. (2003) Three cadherin alleles associated with resistance to Bacillus thuringiensis in pink bollworm. Proc Natl Acad Sci USA 100: 5004–5009. PMID: 12695565

24. Gahan LJ, Gould F, Heckel DG (2001) Identification of a gene associated with Bt resistance in Heliothis virescens. Science 293: 857–860. PMID: 1148008

25. Herrero S, Gechev T, Bakker P, Moar WJ, de Maagd RA (2005) Bacillus thuringiensis Cry1Ca-resistant Spodoptera exigua lacks expression of one of four aminopeptidase N genes. BMC Genom 6: 96.

26. Xu X, Yu L, Wu Y (2005) Disruption of a cadherin gene associated with resistance to Cry1Ac δ-endotoxin of Bacillus thuringiensis in Helicoverpa armigera. Appl Environ Microbiol 71: 948–954. PMID: 15691952

27. Jurat-Fuentes JL, Karumbaiah L, Jakka SRK, Ning C, Liu C, Wu K, et al. (2011) Reduced levels of membrane-bound alkaline phosphatase are common to lepidopteran strains resistant to Cry toxins from Bacillus thuringiensis. PLoS ONE 6(3): e17606. doi:10.1371/journal.pone.0017606 PMID: 21930253

28. Chen J, Hua G, Jurat-Fuentes JL, Abdullah MA, Adang MJ (2007) Synergism of Bacillus thuringiensis toxins by a fragment of a toxin-binding cadherin. Proc Natl Acad Sci USA 104: 13901–13906. PMID: 17724346

29. Hua G, Zhan R, Abdullah MA, Adang MJ (2008) Anoplophora chinensis cadherin AgCad1 binds the Cry4Ba toxin of Bacillus thuringiensis israelensis and a fragment of AgCAD1 synergizes toxicity. Biochem 47: 5101–5110.

30. Abdullah MAF, Moussa S, Taylor MD, Adang MJ (2009) Manduca sexta (Lepidoptera: Sphingidae) cadherin fragments function as synergists for Cry1A and Cry1C Bacillus thuringiensis toxins against noctuid moths Helicoverpa zea, Agrotis ipsilon and Spodoptera exigua. Pest Manag Sci 65:1097–1103. PMID: 20021198

31. Pacheco S, Gómez I, Gill SS, Bravo A, Sobrón M (2009) Enhancement of insecticidal activity of Bacillus thuringiensis Cry1A toxin by fragment of a toxin-binding cadherin correlates with oligomer formation. Peptides 30: 583–588. doi:10.1016/j.peptides.2008.08.006 PMID: 18778475
36. Park Y, Abdullah MAF, Taylor MD, Rahman K, Adang MJ (2009) Enhancement of Bacillus thuringiensis Cry3Aa and Cry3Bb toxicities to coleopteran larvae by a toxin-binding fragment of an insect cadherin. Appl Environ Microbiol 75: 3086–3092. doi: 10.1128/AEM.00268-09 PMID: 19329664

37. Park Y, Hua G, Abdullah MAF, Rahman K, Adang MJ (2009) Cadherin fragments from Anopheles gambiae synergize Bacillus thuringiensis Cry4Ba’s toxicity against Aedes aegypti larvae. Appl Environ Microbiol 75: 7280–7282. doi: 10.1128/AEM.01870-09 PMID: 19801487

38. Peng D, Xu X, Ruan L, Yu Z, Sun M (2010) Enhancing Cry1Ac toxicity by expression of the Helicoverpa armigera cadherin fragment in Bacillus thuringiensis. Res Microbiol 161: 383–389. doi: 10.1016/j.resmic.2010.04.004 PMID: 20438837

39. Peng D, Xu X, Ye W, Yu Z, Sun M (2010) Helicoverpa armigera cadherin fragment enhances Cry1Ac insecticidal activity by facilitating toxin-oligomer formation. Appl Microbiol Biotechnol 85: 1033–1040. doi: 10.1007/s00253-009-1242-1 PMID: 19652967

40. Zhang R, Hua G, Urbauer JL, Adang MJ (2010) Synergetic and inhibitory effects of aminopeptidase peptides on mosquito Anopheles gambiae. Biochem 49: 8512–8519. doi: 10.1021/bi1009908 PMID: 20809561

41. Gao Y, Jurat-Fuentes JL, Oppert B, Fabrick JA, Liu C, Gao J, et al. (2011) Increased toxicity of Bacillus thuringiensis Cry3Aa against Cricceris quatuordecimpunctata, Phaedon brassicae and Colaphellus bowringi by a Tenebrio molitor cadherin fragment. Pest Manag Sci 67: 1076–1081. doi: 10.1002/ps.2149 PMID: 21495115

42. Rahman K, Abdullah M, Ambati S, Taylor MD, Adang MJ (2012) Differential protection of Cry1Fa toxin against Spodoptera frugiperda larval gut proteases by cadherin orthologs correlated with increased synergism. Appl Environ Micro 78: 354–362. doi: 10.1128/AEM.06212-11 PMID: 22081566

43. Lu Q, Zhang Y, Cao G, Zhang L, Liang G, Lu Y, et al. (2012) A fragment of cadherin-like protein enhances Bacillus thuringiensis Cry1B and Cry1C toxicity to Spodoptera exigua (Lepidoptera: Noctuidae). J Integrative Agriculture 11: 628–638.

44. Rodríguez-Almazán C, Reyes EZ, Zúñiga-Navarrete F, Muñoz-Garay C, Gómez I, Evans AM, et al. (2012) Cadherin binding is not a limiting step for Bacillus thuringiensis subsp. israelensis Cry4Ba toxicity to Aedes aegypti larvae. Biochem J 443: 711–717. doi: 10.1042/BJ20111578 PMID: 22329749

45. Hua G, Zhang Q, Zhang R, Abdullah AM, Linser PJ, Adang MJ (2013) AgCad2 cadherin in Anopheles gambiae larvae is a putative receptor of Cry11Ba toxin of Bacillus thuringiensis subsp. jegathesan. Insect Biochem Mol Biol 43:153–161. doi: 10.1016/j.ibmb.2012.11.007 PMID: 23231770

46. Hua G, Park Y, Adang MJ (2014) Cadherin AdCad1 in Alphitobius diaperinus larvae is a receptor of Cry3Bb toxin from Bacillus thuringiensis. Insect Biochem Mol Biol 45: 11–17. doi: 10.1016/j.ibmb.2013.10.007 PMID: 24225445

47. Dorsch JA, Candas M, Griko NB, Maaty WSA, Midboe EG, Vadlamudi RK, et al. (2002) Cry1A toxins of Bacillus thuringiensis bind specifically to a region adjacent to the membrane-proximal extracellular domain of BT-R1 in Manduca sexta: involvement of a cadherin in the entomopathogenicity of Bacillus thuringiensis. Insect Biochem Mol Biol 32: 1025–1036. PMID: 12213239

48. Xie R, Zhuang M, Ross LS, Gómez I, Otteen DI, Bravo A, et al. (2005) Single amino acid mutations in the cadherin receptor from Heliothis virescens affect its toxin binding ability to Cry1A toxins. J Biol Chem 280: 8416–8425. PMID: 15572369

49. Zhang R, Hua G, Andacht TM, Adang MJ (2008) A 106-kDa aminopeptidase is a putative receptor for Bacillus thuringiensis Cry11Ba toxin in the mosquito Anopheles gambiae. Biochem 47:11263–11272. doi: 10.1021/bi801181g PMID: 18826260

50. Hua G, Zhang Q, Bayyareddy K, Adang MJ (2009) Anopheles gambiae alkaline phosphatase is a functional receptor of Bacillus thuringiensis jegathesan Cry11Ba toxin. Biochem 48:9785–9793. doi: 10.1021/bi9014538 PMID: 19747003

51. Liu C, Wu K, Wu Y, Gao Y, Ning C, Oppert B (2009) Reduction of Bacillus thuringiensis Cry1Ac toxicity against Helicoverpa armigera by a soluble toxin-binding cadherin fragment. J Insect Physiol 55: 686–693. doi: 10.1016/j.jinsphys.2009.05.001 PMID: 19446599

52. Wu K, Guo Y (2005) The evolution of cotton pest management practices in China. Annu Rev Entomol 50: 31–52. PMID: 15355239

53. Wu K, Lu Y, Feng H, Jing Y, Zhao J (2008) Suppression of cotton bollworm in multiple crops in China in areas with BT toxin-containing cotton. Science 321: 1676–1678. doi: 10.1126/science.1160550 PMID: 18801998

54. Lu Y, Wu K, Jiang Y, Guo Y, Desneux N (2012) Widespread adoption of BT cotton and insecticide decrease promotes biocontrol services. Nature 487:362–365. doi: 10.1038/nature11153 PMID: 22722864
55. Jin L, Zhang H, Lu Y, Yang Y, Wu K, Tabashnik BE, et al. (2014) Large-scale test of the natural refuge strategy for delaying insect resistance to transgenic Bt crops. Nat Biotechnol doi: 10.1038/nbt.3100

56. Ning C, Wu K, Liu C, Gao Y, Jurat-Fuentes JL, Gao X (2010) Characterization of a Cry1Ac toxin-binding alkaline phosphatase in the midgut from Helicoverpa armigera (Hübner) larvae. J Insect Physiol 56: 666–672. doi: 10.1016/j.jinphys.2010.02.003 PMID: 20170658

57. Sarjan M, Ma G, Rahaman M, Schmidt O (2009) Resistance against Bacillus thuringiensis endotoxins in a laboratory population of Helicoverpa armigera is based on an elevated immune status. Journal ILMU DASAR 10: 77–84.

58. Caccia S, Hernandez-Rodriguez CS, Mahon RJ, Downes S, James W, Bautsoens N, et al. (2010) Binding site alteration is responsible for field-isolated resistance to Bacillus thuringiensis Cry2A insecticidal proteins in two Helicoverpa species. PLoS ONE 5: e9975. doi: 10.1371/journal.pone.0009975 PMID: 20376312

59. Ma G, Roberts H, Sarjan M, Featherstone N, Lahnstein J, Akhurst R, et al. (2005) Is the mature endotoxin Cry1Ac from Bacillus thuringiensis inactivated by a coagulation reaction in the gut lumen of resistant Helicoverpa armigera larvae? Insect Biochem Mol Biol 35: 729–739. PMID: 15894190

60. Zhang S, Cheng H, Gao Y, Wang G, Liang G, Wu K (2009) Mutation of an aminopeptidase N gene is associated with Helicoverpa armigera resistance to Bacillus thuringiensis Cry1Ac toxin. Insect Biochem Mol Biol 39: 421–429. doi: 10.1016/j.ibmb.2009.04.003 PMID: 19376227

61. Gunning RV, Dang HT, Kemp FC, Nicholson IC, Moores GD (2005) New resistance mechanism in Helicoverpa armigera threatens transgenic crops expressing Bacillus thuringiensis Cry1Ac Toxin. Appl Environ Micro 71: 2558–2563. PMID: 15870346

62. Rajagopal R, Arora N, Sivakumar S, Rao NGV, Nimbalkar SA, Bhatnagar RK (2009) Resistance of Helicoverpa armigera to Cry1Ac toxin from Bacillus thuringiensis is due to improper processing of the protoxin. Biochem J 419: 309–316. doi: 10.1042/BJ20081152 PMID: 19146482

63. Cao G, Zhang L, Liang G, Li X, Wu K (2013) Involvement of nonbinding site proteinases in the development of resistance of Helicoverpa armigera (Lepidoptera: Noctuidae) to Cry1Ac. J Econ Entomol 106: 2514–2521. PMID: 24498753

64. Liu C, Xiao Y, Li X, Oppert B, Tabashink BE, Wu K (2014) Cis-mediated down-regulation of a trypsin gene associated with Bt resistance in cotton bollworm. Sci Rep 4: 7219. doi: 10.1038/srep07219 PMID: 25427690

65. Xiao Y, Zhang T, Liu C, Heckel DG, Li X, Tabashink BE, et al. (2014) Mis-splicing of the ABCC2 gene linked with Bt toxin resistance in Helicoverpa armigera. Sci Rep 4: 6184. doi: 10.1038/srep06184 PMID: 25154974

66. Li Y, Wu J, Liu C, Yuan X, Wu K, Liang G, et al. (2012) Comparative analysis of Cry1Ac toxin oligomerization and pore formation between Bt-susceptible and Bt-resistant Helicoverpa armigera larvae. J Integrative Agriculture 11:101–108.

67. Flores-Escobar B, Rodríguez-Magadan H, Bravo A, Soberón M, Gómez I (2013) Differential role of Manduca sexta aminopeptidase-N and alkaline phosphatase in the mode of action of Cry1Aa, Cry1Ab, and Cry1Ac toxins from Bacillus thuringiensis. Appl Environ Microbiol 79: 4543–4550. doi: 10.1128/AEM.01662-13 PMID: 23686267

68. Thammasitirong A, Dechklar M, Leetachewa S, Pootanakit K, Angsuthanasombat C (2011) Aedes aegypti membrane-bound alkaline phosphatase expressed in Escherichia coli retains high-affinity binding for Bacillus thuringiensis Cry4Ba toxin. Appl Environ Microbiol 77: 6836–6840. doi: 10.1128/AEM.05775-11 PMID: 21856837

69. Hua G, Zhang R, Rayberrdedy K, Adang MJ (2009) Anopheles gambiae alkaline phosphatase is a functional receptor of Bacillus thuringiensis jegathesan Cry11Ba toxin. Biochemistry 48: 9785–9793. doi: 10.1021/bi9014538 PMID: 19747003

70. Cao G, Feng H, Guo F, Wu K, Li X, Liang G, et al. (2014) Quantitative analysis of fitness costs associated with the development of resistance to the Bt toxin Cry1Ac in Helicoverpa armigera. Sci Rep 4: 5629. doi: 10.1038/srep05629 PMID: 25005122

71. Liang G, Tan W, Guo Y (1999) An improvement in the technique of artificial rearing cotton bollworm. Plant Protect 25: 15–17.

72. Liang G, Wu K, Yu H, Li K, Feng X, Guo YY (2008) Changes of inheritance mode and fitness in Helicoverpa armigera (Hübnner) (Lepidoptera: Noctuidae) along with its resistance evolution to Cry1Ac toxin. J Invertebr Pathol 97: 142–149. PMID: 17950749

73. Cao G, Zhang L, Liang G, Li X, Wu K (2013) Involvement of nonbinding site proteinases in the development of resistance of Helicoverpa armigera (Lepidoptera: Noctuidae) to Cry1Ac. J Econ Entomol 106: 2514–2521. PMID: 24498753
74. Tabashnik BE, Liu YB, Dennehy TJ, Sims MA, Sisterson MS, Biggs RW, et al. (2002) Inheritance of resistance to Bt toxin Cry1Ac in a field-derived strain of pink bollworm (Lepidoptera: Gelechiidae). J Econ Entomol 95: 1018–1026. PMID: 12403429
75. Gilroy TE, Wilcox ER (1992) Hybrid Bacillus thuringiensis gene, plasmid, and transformed Pseudomonas fluorescens. U.S. Patent 5,128,130.
76. Adang MJ, Staver MJ, Rocheleau TA, Leighton J, Barker RF, Thompson DV (1985) Characterized full-length and truncated plasmid clones of the crystal protein of Bacillus thuringiensis subsp. kurstaki HD-73 and their toxicity to Manduca sexta. Gene 36:289–300. PMID: 3000881
77. Liang G, Tan W, Guo Y (2000) Studies on the resistance screening and cross-resistance of cotton bollworm to Bacillus thuringiensis (Berliner). Sci Agric Sinica 33: 46–53.
78. Wolfersberger M, Luethy P, Maurer A, Parenti P, Sacchi FV, Giordana B, et al. (1987) Preparation and partial characterization of amino acid transporting brush border membrane vesicles from the larval midgut of the cabbage butterfly (Pieris brassicae). Comp Biochem Physiol 86:301–308.
79. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248–254. PMID: 942051
80. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2−ΔΔCT method. Methods 25: 402–408. PMID: 11846609
81. Russell RM, Robertson JL, Savin NE (1977) POLO: a new computer program for probit analysis. Rev Entomol Soc Am 23: 209–215.
82. SAS Institute, 1998. SAS/STAT User’s Guide, Release 6.03 Edition. SAS Institute, Cary, NC. PMID: 15597549
83. Tabashnik BE (1992) Evaluation of synergism among Bacillus thuringiensis toxins. Appl Environ Micro 58: 3343–3346. PMID: 1444368
84. Tabashnik BE, Fabrick JA, Unnithan GC, Yelich AJ, Masson L, Zhang J, et al. (2013) Efficacy of genetically modified Bt toxins alone and in combinations against pink bollworm resistant to Cry1Ac and Cry2Ab. PLoS ONE 8 (11): e80496. doi: 10.1371/journal.pone.0080496 PMID: 24244692
85. Fernández-Luna MT, Tabashnik BE, Lanz-Mendoza H, Bravo A, Soberón M, Miranda-Ríos J (2010) Single concentration tests show synergism among Bacillus thuringiensis subsp. israelensis toxins against the malaria vector mosquito Anopheles albimanus. J Invertebr Pathol 104: 231–233. doi: 10.1016/j.jip.2010.03.007 PMID: 20361977