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Cis-mediated down-regulation of a trypsin gene associated with Bt resistance in cotton bollworm

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Transgenic plants producing insecticidal proteins from the bacterium *Bacillus thuringiensis* (Bt) are useful for pest control, but their efficacy is reduced when pests evolve resistance. Here we examined the mechanism of resistance to Bt toxin Cry1Ac in the laboratory-selected LF5 strain of the cotton bollworm, *Helicoverpa armigera*. This strain had 110-fold resistance to Cry1Ac protoxin and 39-fold resistance to Cry1Ac activated toxin. Evaluation of five trypsin genes revealed 99% reduced transcription of one trypsin gene (*HaTryR*) was associated with resistance. Silencing of this gene with RNA interference in susceptible larvae increased their survival on diets containing Cry1Ac. Bioassays of progeny from crosses revealed that resistance to Cry1Ac was genetically linked with *HaTryR*. We identified mutations in the promoter region of *HaTryR* in the resistant strain. In transfected insect cell lines, transcription was lower when driven by the resistant promoter compared with the susceptible promoter, implicating *cis*-mediated down-regulation of *HaTryR* transcription as a mechanism of resistance. The results suggest that *H. armigera* can adapt to Bt toxin Cry1Ac by decreased expression of trypsin. Because trypsin activation of protoxin is a critical step in toxicity, transgenic plants with activated toxins rather than protoxins might increase the durability of Bt crops.

The gram-positive bacterium *Bacillus thuringiensis* (Bt) produces a variety of crystalline (Cry) proteins that can kill certain insect pests, but have little or no toxicity to most non-target organisms[1,2]. In 2013, farmers planted transgenic crops producing Bt toxins on 76 million ha worldwide[3], but the evolution of resistance in some pest populations has reduced the efficacy of these Bt crops[4]. Previously reported mechanisms of resistance to Bt toxins include reduced binding of activated Bt toxins to midgut receptors and reduced conversion of Bt protoxins to activated toxins by insect midgut proteases such as trypsin[2,5–7].

In China, planting of transgenic cotton that produces Bt toxin Cry1Ac has helped to suppress the cotton bollworm, *Helicoverpa armigera*, and reduce insecticide use against this pest[8,9]. Although Bt cotton has remained useful against *H. armigera* in China, high levels of resistance to Cry1Ac have been selected in many laboratory strains of *H. armigera*, and significant increases in the frequency of resistant individuals in field populations have provided an early warning of resistance in northern China[10–13]. Various mechanisms of resistance to Cry1Ac have been identified previously in *H. armigera*, including qualitative changes in or reduced levels of the confirmed and putative midgut receptors cadherin, aminopeptidase, alkaline phosphatase, and ABCC2 proteins[14–17]. In addition, a serine protease from the midgut of a Cry1Ac-resistant strain of *H. armigera* from India had significantly reduced expression, resulting in improper processing of the protoxin[18]. Our previous work with ten laboratory-selected strains of *H. armigera* from China showed that total protease activity in the larval gut was negatively associated with resistance to Cry1Ac[19], which suggests that reduced protease activity may contribute to resistance in these strains.

Here we evaluated the role of five trypsin genes in resistance to Cry1Ac in one of the laboratory-selected strains of *H. armigera* from China. We found that transcription of one of the five genes, which we name *HaTryR*, was greatly reduced in the resistant strain. We also discovered that silencing of *HaTryR* with RNA interference (RNAi) increased survival of susceptible larvae exposed to Cry1Ac and that resistance to Cry1Ac was genetically linked with *HaTryR*. We identified mutations in *HaTryR* promoter elements associated with resistance that implicate *cis*-mediated down-regulation of *HaTryR* transcription as a mechanism of resistance in *H. armigera*.

Table 1 | Responses of *H. armigera* larvae from a resistant strain (LF5) and a susceptible strain (LF) to Cry1Ac protoxin and activated toxin

Strain	Form of Cry1Ac	LC_{50} (95% fiducial limits) (ng Cry1Ac per g diet)	Resistance ratio[a]
LF	protoxin	9.1 (1.7–20)	1.0
LF5	protoxin	980 (700–1300)	110
LF	activated toxin	7.5 (2.3–14)	1.0
LF5	activated toxin	290 (180–420)	39

[a]LC_{50} of the each strain divided by the LC_{50} of the susceptible LF strain

Results

Resistance to Cry1Ac protoxin and activated toxin. Bioassay results indicated that, relative to its unselected parent strain (LF), the laboratory-selected LF5 strain of *H. armigera* was resistant to Cry1Ac protoxin and Cry1Ac activated toxin (Table 1). We calculated the resistance ratios for LF5 as the concentration of Cry1Ac (ng Cry1Ac per g diet) killing 50% of larvae (LC_{50}) for LF5 divided by the LC_{50} for LF. The resistance ratios were 110 for protoxin and 39 for activated toxin (Table 1).

Abundance of five trypsin mRNAs in midguts of resistant and susceptible larvae. We compared the abundance of five trypsin mRNAs in the midguts of fifth instars between the resistant and susceptible strains. Quantitative real time PCR (qRT-PCR) showed 99% lower abundance of the mRNA for one trypsin gene, *HaTryR,* in the resistant strain compared to the susceptible strain ($P < 0.01$, Figure 1). For the other four trypsin genes, no significant difference occurred between the resistant and susceptible strains (Figure 1). These results suggest that decreased expression of *HaTryR* might contribute to resistance by decreasing the amount of activated toxin in the gut of resistant insects.

Effects of *HaTryR* dsRNA on susceptible larvae. For susceptible (LF) larvae, RNA interference (RNAi) by feeding droplets of *HaTryR* double-stranded RNA (dsRNA) significantly reduced the abundance of *HaTryR* mRNA in the midgut relative to larvae fed *GFP* dsRNA as a control (Figure 2A). When fed diet treated with Cry1Ac protoxin, susceptible larvae that had ingested *HaTryR* dsRNA had twice the survival of larvae that had ingested *GFP* dsRNA (Figure 2B). These data demonstrate that reducing the expression of *HaTryR* in susceptible insects decreased their susceptibility to Cry1Ac protoxin.

Mutations in the promoter of the resistant *HaTryR* allele. Cloning and sequencing of the genomic DNA of the promoter region of *HaTryR* in the resistant and susceptible strains revealed several insertions, deletions, and substitutions (Figure 3). These differences include four insertions (5 bp from -489 to -485, 1 bp at -447, 4 bp from -407 to -404, and 36 bp from -364 to -329), five deletions (4 bp from -359 to -355, 1 bp at -306, 3 bp from -283 to -281, 29 bp from -227 to -199, and 9 bp from -124 to -116) and 99 substitutions in the promoter region (-515 to -1), as well as two substitutions in the 5′ untranslated region (5′ UTR, +1 to +39) (Figure 3). The 36 bp insertion from -364 to -329 introduces three putative *cis*-acting regulatory elements: HLF, GATA-1 and CAP (Figure 3). The 29 bp deletion from -227 to -199 removes the putative YY1 element, whereas the 9 bp deletion from -124 to -116 omits the 5′ eight nucleotides of the putative C/EBP element. In addition, the T-392 A-382 substitution may disrupt the putative Ind element (Figure 3).

Activity of *HaTryR* promoters from susceptible and resistant insects in Sf9 cells. To determine if the mutations in the promoter region of the resistance allele reduce transcription of *HaTryR*, we compared the promoter activity of Sf9 cells transfected with the resistant or susceptible promoter-pYr-PromDetect construct. The activity of the resistant promoter was 7-fold lower than that of the susceptible promoter ($p < 0.01$, Figure 4). These data suggest that lower expression of *HaTryR* in the resistant insects was caused by the mutations in the promoter region of the resistant *HaTryR* allele.

Genetic linkage between *HaTryR* and Cry1A resistance. Sequencing of the *HaTryR* promoter region in larvae from each of two single-pair backcross families tested in bioassays shows genetic linkage between *HaTryR* and resistance to Cry1Ac (Table 2). In each of two backcross families (see Methods), the frequency of the putative *HaTryR* resistance allele was significantly higher than expected at random for larvae that survived exposure to Cry1Ac (Fisher's exact test, $P < 0.0001$, Table 2). By contrast, in larvae from each backcross family fed untreated diet, the observed frequency of the putative resistance allele did not differ from the 0.50 frequency expected under random assortment (Fisher's exact test, $P > 0.60$, Table 2).

Discussion

Evaluation of five trypsin genes in *H. armigera* larvae revealed 99% reduced transcription of one trypsin gene (*HaTryR*) in the laboratory-selected LF5 strain, which had 110-fold resistance to Cry1Ac protoxin relative to its unselected parent strain (LF). Reducing the expression of this gene with RNAi increased the survival of susceptible larvae exposed to Cry1Ac protoxin. These results indicate that reduced transcription of *HaTryR* in *H. armigera* larvae decreased their susceptibility to Cry1Ac. We identified mutations in the promoter region of *HaTryR* in the resistant strain and found that in Sf9 cells, transcription driven by the resistant *HaTryR* promoter was lower than transcription driven by the susceptible *HaTryR* promoter. These results suggest that *cis*-mediated down regulation of *HaTryR*

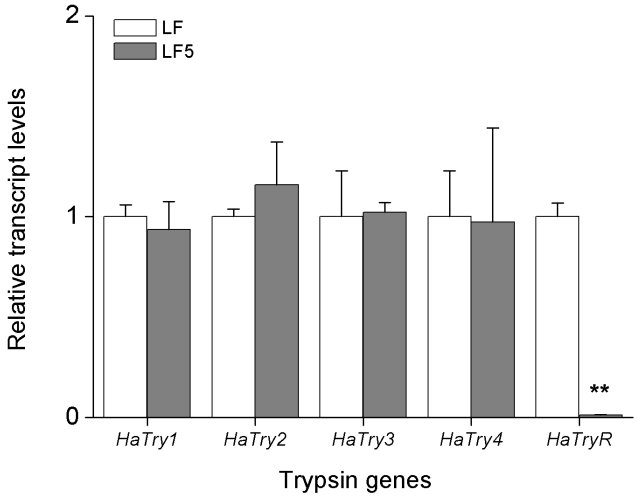

Figure 1 | Relative abundance of mRNA transcripts of five trypsin genes based on qPCR of resistant and susceptible *H. armigera* larvae. Error bars indicate SEM (n = 5 larvae per bar). Asterisks indicate that the expression of *HaTryR* was significantly lower in resistant larvae than in susceptible larvae (t- test, df = 4, t = −14.3, $P < 0.01$).

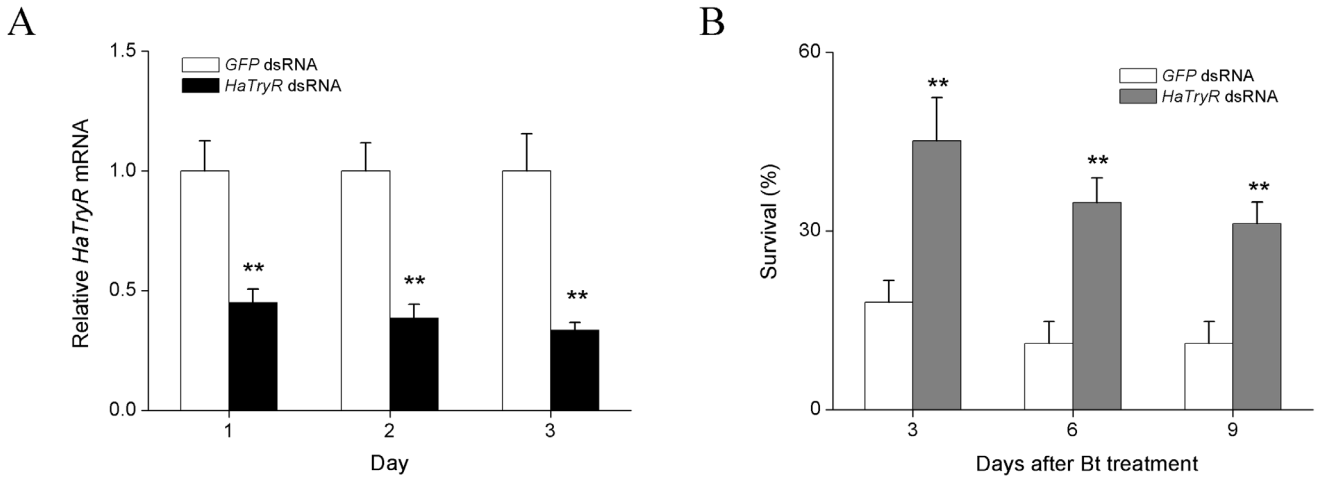

Figure 2 | Effects of *HaTryR* dsRNA on susceptible *H. armigera* larvae. A. Relative abundance of *HaTryR* mRNA in LF larvae fed *GFP* dsRNA (control) or *HaTryR* dsRNA at 1, 2, and 3 days after last ingestion of dsRNA. B. Survival (mean ± SE) of third instar LF larvae fed diet treated with 120 μg Cry1Ac protoxin per gram diet for larvae previously treated with either *GFP* dsRNA (control) or *HaTryR* dsRNA. Asterisks indicate significant differences between larvae treated *GFP* dsRNA and *HaTryR* dsRNA (t-tests, $P < 0.01$ in each of the six comparisons).

causes reduced transcription of this gene and contributes to resistance in the LF5 strain. It remains to be determined which of the several mutations we found in the promoter region contribute to this reduced transcription. As far as we know, this is the first report of *cis*-mediated down regulation of transcription associated with Bt resistance.

We also found that resistance to Cry1Ac was genetically linked with *HaTryR* in two backcross families. Because no crossing-over occurs in female Lepidoptera during oogenesis[20], the significant association between resistance to Cry1Ac and the *HaTryR* locus in family A (female F_1 X resistant male) indicates that one or more genes conferring resistance are on the same chromosome as *HaTryR*. Because crossing over does occur in male Lepidoptera during spermatogenesis[20], the significant association between resistance to Cry1Ac and the *HaTryR* locus in family B (male F_1 X resistant female) indicates that one or more genes conferring resistance are at or near the *HaTryR* locus. If the gene(s) conferring resistance are on the same chromosome as *HaTryR*, but not at the same locus, a stronger association is expected between resistance and the putative *HaTryR* allele in family A (no crossing-over) than in family B (crossing-over). This association, however, was not significantly stronger in family A (101 *rr*: 19 *rs*) than in family B (96 *rr*: 24 *rs*) ($P = 0.50$), which implies that resistance is tightly linked with the *HaTryR* locus. The finding that some *rs* survived on treated diet (19

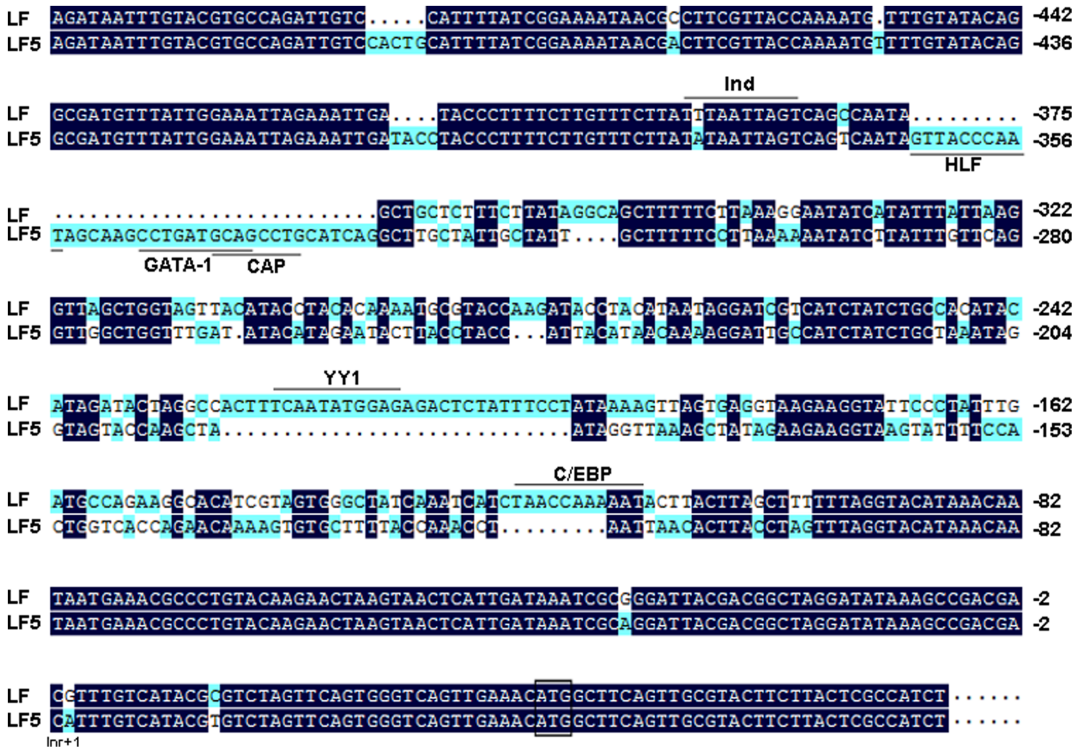

Figure 3 | Alignment of the 5′ flanking sequence of *HaTryR* from the midgut of Cry1Ac-susceptible (LF) and -resistant (LF5) *H. armigera* larvae. The putative *cis*-acting regulatory elements are shown with lines. Inr+1 indicates the predicted transcription start site (TSS). Start codon (ATG) is marked by a box.

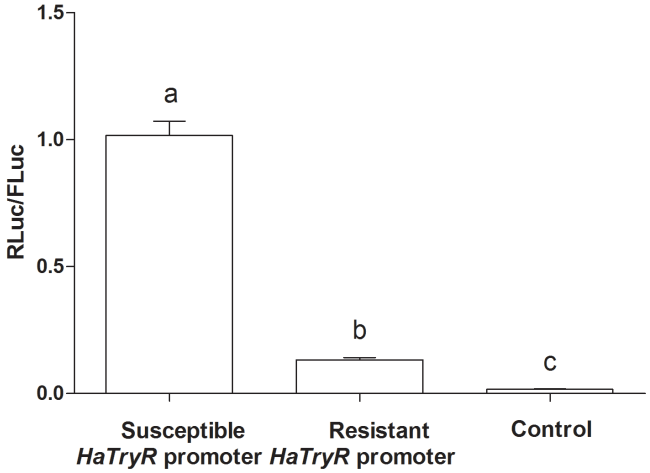

Figure 4 | Analysis Cry1Ac-susceptible and resistant *HaTryR* promoter sequences in transfected Sf9 cells. Promoter activities were calculated as the activity of sea pansy (*Renilla reniformis*) luciferase (Rluc) relative to the activity of firefly luciferase (Fluc). Sf9 cells transfected with pYr-PromDetect empty vector were used as a negative control (Control). Means with different letters were significantly different ($P < 0.05$, n=3).

larvae in family A and 24 larvae in family B) indicates that inheritance of resistance conferred by the *HaTryR* resistance allele is not completely recessive, one or more genes not linked with *HaTryR* contributed to resistance, or both.

The results here showing an association between reduced transcription of a trypsin gene and resistance to Cry1Ac in the laboratory-selected LF5 strain of *H. armigera* from China are similar to previous work showing an association between reduced transcription of a serine protease gene (HaSP2) and resistance to Cry1Ac in a laboratory-selected strain of *H. armigera* from India[18]. Reduced transcription of HaSP2 was associated with reduced activation of Cry1Ac protoxin[18]. The resistance ratio for the laboratory-selected Indian strain was 72 for Cry1Ac protoxin, but only 1.2 for Cry1Ac activated toxin, implying that reduced activation of Cry1Ac was responsible for all or nearly all of the resistance[18]. By contrast, the resistance ratio for LF5 was 110 for Cry1Ac protoxin and 39 for Cry1Ac activated toxin, indicating that a mechanism other than reduced activation of Cry1Ac contributes substantially to resistance in LF5.

Protease-mediated resistance was first demonstrated in a laboratory-selected strain of *Plodia interpunctella* selected for resistance to Bt subspecies *entomocidus*[6]. Reduction of protease activity has also been associated with resistance to Cry1A toxins in laboratory-selected strains of *Heliothis virescens*[21] and *Ostrinia nubilalis*[22,23]. In one resistant strain of *O. nubilalis*, the resistance ratio was 250 for Cry1Ab protoxin compared with 12 for Cry1Ab activated toxin[23]. In a field-selected resistant strain of *Plutella xylostella* from Malaysia, a substrain that was not exposed to Cry1Ac in the laboratory had a resistance ratio of 315 to Cry1Ac protoxin compared with 25 for activated toxin[24]. Selection with activated Cry1Ac in the laboratory boosted the resistance ratios to >15,000 for Cry1Ac protoxin versus 94 for Cry1Ac activated toxin[24].

In conjunction with results from several previous studies, the new data reported here show that in some cases, pest resistance is lower to Bt activated toxin than protoxin (but see[25] for a notable exception). In such cases, transgenic plants that produce activated toxins could be more durable than plants that produce protoxins or partially activated toxins. Implementing this idea in China would favor planting of the GK type of Bt cotton producing truncated Cry1Ac rather than Bt cotton that produces full-length Cry1Ac[26,27]. However, resistance management strategies for *H. armigera* and other pests must also consider mechanisms that confer resistance to activated toxins.

Table 2 | Genetic linkage between *HaTryR* and resistance to Cry1Ac. Treated diet contained 5 μg Cry1Ac protoxin per g of diet. Values of *P* are based on Fisher's exact test evaluating deviation from the outcome expected under random segregation (1 : 1 ratio of *rr* to *rs*)

Backcross family	Control diet			Treated diet		
	rr	*rs*	*P*	*rr*	*rs*	*P*
A: $F_{1♀} \times LF5_{♂}$	56	64	0.70	101	19	<0.0001
B: $F_{1♂} \times LF5_{♀}$	65	55	0.61	96	24	<0.0001

Methods

Insects and resistance selection. We used a Cry1Ac-susceptible strain of *H. armigera* (LF), which was started with insects collected from Langfang, Hebei Province, China in 1998, and had been reared in the laboratory for >15 years on artificial diet without exposure to Bt toxins[19]. We generated the LF5 strain by selecting the LF strain initially at 1 μg Cry1Ac protoxin per g diet for 38 generations. We then increased the selection concentration for LF5 to 5 μg Cry1Ac protoxin per g diet for 104 generations of selection.

Insect bioassays. Cry1Ac protoxin and activated toxin were kindly supplied by the Biotechnology Research Group, Institute of Plant Protection, Chinese Academy of Agricultural Science. For activation, Cry1Ac protoxin was incubated 2 h at 37°C with 1/25 ratio of bovine pancreas trypsin (Sigma), 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. *H. armigera* bioassays with Cry1Ac protoxin and activated toxin were performed with a diet incorporation procedure. Approximately 1–1.5 g artificial diets incorporated with various concentrations of Cry1Ac protoxin or toxin suspended in distilled water or equal volume of distilled water (control) was put in each well of 24-well plates. A single first instar larva was placed into each well, and 24 larvae were used for each treatment. Mortality was recorded after seven days, and all assays were replicated three times. Pooled data were subjected to statistical analysis; the concentration killing 50% of larvae (LC_{50}) was calculated by probit analysis with the computer program POLO (LeOra Software, Berkeley, California 1987).

Quantitative RT-PCR. Sequences encoding trypsin genes from *H. armigera* were retrieved from GenBank and were used compare gene expression in the Cry1Ac-susceptible and resistant strains: *HaTry1* (EU982841[28]), *HaTry2* (EU325549[29]), *HaTry3* (EU325548[29]), *HaTry4* (AY437836) and *HaTryR* (KF791044). Total RNA was extracted from the midgut of fifth instars of susceptible or resistant strains with TRIzol® reagent (Invitrogen) according to the manufacturer's instructions and was treated with DNase I (Takara) to remove any residual DNA, and was reverse-transcribed with SuperScript III RNase H^- reverse transcriptase (Invitrogen).

Quantitative real-time PCR (qPCR) amplification and analysis were carried out using the Applied Biosystems 7500 Fast Real-time PCR System. The transcriptional profiles of selected *H. armigera* trypsin genes were analyzed using the SYBR Premix Ex Taq™ (TaKaRa) system. Gene-specific primers designed for real-time PCR are listed in Table 3. *H. armigera* actin (GenBank accession no. X97615) and GADPH (GenBank accession no. JF417983) were used as controls. The PCR involved a 95°C step for 30 s followed by 40 cycles at 95°C for 5 s, 58°C for 15 s, and 68°C for 30 s. All reactions were run in triplicate with three independent biological replicates and monitoring the dissociation curve to control potential formation of primer dimers. PCR efficiency was determined by a series of 3-fold dilutions of cDNA from LF larvae. The absolute value of the slope (Ct value Vs Log) for each primer set was <0.1, and all amplification efficiencies were >99% when compared to the endogenous control. Data obtained were analyzed using the relative $2^{-\Delta\Delta CT}$ quantitation method to calculate transcript abundance, which was standardized to 1 for reactions with samples from susceptible larvae. We calculated percentage reduction in mRNA as the relative abundance of mRNA in susceptible larvae (1) minus the relative abundance in resistant larvae multiplied by 100%.

Cloning and sequence analysis of *HaTryR*. The 3′- rapid amplification of cDNA ends (RACE) and 5′-RACE were performed using 3′-full RACE Core Set Ver. 2.0 Kit and 5′-full RACE Kit (TaKaRa) to obtain the cDNA 3′ and 5′ ends of the *H. armigera* trypsin gene *HaTryR* (primers are listed in Table 3). All the amplified PCR products were recovered and cloned into the pMD20-T Vector (TaKaRa) for sequencing.

The NCBI/BLAST database and ClustalX[30] were used to analyse the homology of *H. armigera* trypsin genes. Multiple alignments and identity calculations were done with DNAMAN 6.0 (Lynnon Corporation).

RNAi-mediated gene silencing and survival assays. Primers were designed for *HaTryR* and green fluorescent protein (GFP) genes, and the T7 primer sequence was added to both forward and reverse primers (Table 3). The dsRNA for RNAi was prepared using PCR products as a template for *in vitro* transcription. *In vitro*

Table 3 | Primers used in this study

Primer name	Strand orientation	Sequence(5′→3′)	Application
GADPH	Forward	CATTGAAGGTCTGATGACCACTGT	Quantitative RT PCR
	Reverse	CAGAGGGTCCATCCACTGTCTT	
	Probe	CACGCCACCATTGCCACCCA	
Actin	Forward	GGTGCACTGGCGATATTGG	
	Reverse	CTTGGGTCTTGACAGCAATGC	
	Probe	AACCCCTTGGTCTGCCATGATAGCCTT	
HaTry1 (EU982841)	Forward	GGTGCACTGGCGATATTGG	
	Reverse	CTTGGGTCTTGACAGCAATGC	
	Probe	AACCCCTTGGTCTGCCATGATAGCCTT	
HaTry2 (EU325549)	Forward	GCCCCAAGGCTTCCAGAT	
	Reverse	CAGACGAAGTACCCCATCCA	
	Probe	CCCGATGGCTTGCCAGTTGTTCA	
HaTry3 (EU325548)	Forward	GGATTCGGAACTTGCAATGGT	
	Reverse	CGAACTGCTGGCCATTGTC	
	Probe	CCGGCAGTGCTCTGACCCGC	
HaTry4 (AY437836)	Forward	CCAGCTATGTGCTGCTCGTTAC	
	Reverse	CGCAGATAATGTTCTCAGTCACAA	
	Probe	TGACCCTGCCATGGCCCCA	
HaTryR (KF791044)	Forward	GGCTCCCATGGCTATGAG	
	Reverse	CGTCAGTGAGACACTCCTGGAAG	
	Probe	AACGCCCTGGTCGGAATCTCTTCCT	
5′-RACE-GSP-outer	Reverse	AATGTCGTTGGAGCGAGGGGT	RACE PCR
5′-RACE-GSP-inner	Reverse	CCTACCACGACTCCTCCAGCCAA	
3′-RACE-GSP-outer	Forward	TGCTGTACCTGCCTCCTGTCAA	
3′-RACE-GSP-inner	Forward	ACTCCGAAGATGTTACCGTTTC	
dsRNA-*GFP*	Forward	TAATACGACTCACTATAGTCCCA-ACACTTGTCACTAC	dsRNA amplification
	Reverse	TAATACGACTCACTATAGAAACTCAA-GAAGGACCATG	
dsRNA-*HaTryR*	Forward	TAATACGACTCACTATAGTTC-ATTCTCGCCATCTTG	
	Reverse	TAATACGACTCACTATAGCATC-TTCGGAGTCATCGTA	
CTD039 SP1	Reverse	TTCAAGAGCACTTAGAGACTTT	Promoter cloning
CTD039 SP2	Reverse	TTGAACATCACGCTTAACACTA	
CTD039 SP3	Reverse	AGGGCTTTCAACAGATACTTC	
activity -Bgl II	Forward	ATAGATCTAGATAATTTGTACGTGCCAGATTGTC	Construction of luciferase reporter plasmids
activity –Nhe I	Reverse	ATGCTAGCGTTTCAACTGACCCACTGAA	
Genetic linkage-F	Forward	AGATAATTTGTACGTGCCAGATTGTC	Genetic linkage analysis
Genetic linkage-R	Reverse	AAGATGGCGAGTAAGAAGTACGCAA	

transcription to yield dsRNA of *HaTryR* was performed with T7 RNA polymerase using the HiScribe RNAi™ T7 *In Vitro* Transcription Kit (New England Biolabs) according to the manufacturer.

In terms of dsRNA delivery to third instar of *H. armigera*, oral delivery was more suitable for our purpose. The technique is not invasive (no mortality), and the larvae were immediately subjected to subsequent toxicity analysis. The 3rd instar larvae of LF were individually placed into each well of 24-well plates to avoid cannibalism and starved for 12 h. Seventy-two larvae were then fed with a 2 μl drop of 2.5 μg dsRNA from either *HaTryR* or *GFP* (nonspecific dsRNA as control) respectively in delivery buffer (10 mM Tris-Cl, pH 7.5; 10 mM EDTA). After 2 h, droplet-fed larvae were placed back into a well of a 24-well plate provided of artificial diet. Oral delivery was repeated three more times, with a total of 10 μg dsRNA fed over a 2 d interval. After the 2nd feeding of dsRNA, droplet-fed larvae were placed back into a well of a 24-well plate containing artificial diet that either 120 μg/g Cry1Ac protoxin or no treatment. Survival was assessed after 0, 3, 6, and 9 days. The experiment was repeated twice. To evaluate knockdown, we checked the expression levels of *HaTryR* by RT-PCR (1, 2, 3 d) after the last oral delivery by qRT-PCR as described above.

Promoter cloning and sequences analysis. Genomic DNA of susceptible and resistant strains of *H. armigera* was extracted using Tiangen Genomic DNA kit (TianGen Biotech Co., Ltd, Beijing). Nested specific primers (SP1, SP2, SP3) were designed to the *HaTryR* genomic DNA sequence (Table 3). Arbitrary primers (AP1, AP2, AP3) were provided with the Universal Genome Walking kit (TaKaRa). Three-rounds of TAIL-PCR were performed according to the manufacturer's instructions. After TAIL-PCR, DNA products were amplified from AP3 and SP3 primers and were cloned into the PMD 20-T vector (TaKaRa). The cloned plasmids were sequenced and similarity analysis and alignment were by Sequencher (version 4.1.4; Gene Codes Corporation). The TRANSFAC database (v. 1.3; http://www.cbrc.jp/research/db/TFSEARCH.html) was used to search for transcription factor binding sites. A search for promoter elements was performed with TESS (http://www.cbil.upenn.edu/cgi-bin/tess/tess). The sequence upstream of the putative transcriptional start site was predicted by the Neural Network Promoter Prediction website (http://www.fruitfly.org/seq_tools/promoter.html).

Transient transfections and dual luciferase assays. We constructed two reporter plasmids encompassing −515 to +39 bp (545 bp) of the susceptible *HaTryR* promoter and −515 to +39 bp (545 bp) of the resistant promoter by PCR of genomic DNA from susceptible and resistant strains, respectively, using the primers in Table 3. Amplicons were cloned into the pMD-19T simple vector (Takara) following the manufacturer's instructions. The recombinant plasmid was excised with Bgl II and Nhe I, and ligated into a pYr-PromDetect vector harboring dual reporters to improve accuracy (YRBIO, China). We used simultaneous measurement of two individual reporter enzymes within a single construct to evaluate promoter activity[31]. Briefly, the activity of the "experimental" reporter (luciferase from the sea pansy, *Renilla reniformis*) is correlated with the effect of the specific promoter, while the activity of the "control" reporter (firefly luciferase) with an HSV-TK promoter provides an internal control that serves as the baseline response. All constructs used in this study were restriction mapped and sequenced to confirm their authenticity.

We seeded 1.5×10^5 Sf9 cells per well in 24-well plates and transfected with pYr-PromDetect constructed without or with a susceptible or resistant *HaTryR* promoter using Lipofectamine 2000 (Invitrogen) and Opti-MEM (Gibco). All transfections were carried out in triplicate. After 48 h incubation, we collected cells and calculated promoter activity as the activity of sea pansy luciferase relative to the activity of firefly luciferase.

Genetic Linkage Analysis. For linkage analysis of the resistant *HaTryR* promoter and Cry1Ac resistance, a single-pair cross was obtained from mating a susceptible male and resistant female for F_1 progeny, and two backcross families were from a single-pair cross of female F_1 progeny with resistant male (Backcross family A) and resistant female (Backcross family B). Backcross families were reared on an artificial diet

(non-Cry1Ac-selected) or treated with 5 μg/g of Cry1Ac protoxin for 10 days to select Cry1Ac-resistant individuals (Cry1Ac-selected) as described elsewhere[32]. Larval genomic DNA was prepared from individual larvae, and a 600-bp genomic DNA fragment including the *HaTryR* promoter was amplified by PCR and sequenced to determine the allele types (primers are listed in Table 3), and the results were analyzed by using χ^2 test.

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Author contributions

C.L. and K.W. designed the experiments; C.L. and Y.X. performed the experiments; C.L., Y.X., X.L., B.E.T., B.O. and K.W. performed the statistical analysis; and C.L., X.L., B.O., B.E.T. and K.W. wrote the manuscript. All authors read and approved the final manuscript.

Additional information

Competing financial interests: The authors declare competing interests. B.E.T. is coauthor of a patent on modified Bt toxins, "Suppression of Resistance in Insects to *Bacillus thuringiensis* Cry Toxins, Using Toxins that do not Require the Cadherin Receptor" (patent numbers: CA2690188A1, CN101730712A, EP2184293A2,EP2184293A4, EP2184293B1, WO2008150150A2, WO2008150150A3). Pioneer, Dow AgroSciences, Monsanto and Bayer CropScience did not provide funding to support this work, but they have funded other work by B.E.T.

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