Research Article

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Reduced cadherin expression associated with resistance to Bt toxin Cry1Ac in pink bollworm

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Abstract

BACKGROUND: Better understanding of the molecular basis of resistance is needed to improve management of pest resistance to transgenic crops that produce insecticidal proteins from *Bacillus thuringiensis* (Bt). Here we analyzed resistance of the pink bollworm (*Pectinophora gossypiella*) to Bt toxin Cry1Ac, which is used widely in transgenic Bt cotton. Field-evolved practical resistance of pink bollworm to Cry1Ac is widespread in India, but not in China or the United States. Previous work with laboratory- and field-selected pink bollworm indicated that resistance to Cry1Ac is caused by changes in the amino acid sequence of a midgut cadherin protein (PgCad1) that binds Cry1Ac in susceptible larvae.

RESULTS: Relative to a susceptible strain, the laboratory-selected APHIS-R strain had 530-fold resistance to Cry1Ac with autosomal recessive inheritance. Unlike previous results, resistance in this strain was not consistently associated with insertions or deletions in the expected amino acid sequence of PgCad1. However, this resistance was associated with 79- to 190-fold reduced transcription of the *PgCad1* gene and markedly lower abundance of PgCad1 protein.

CONCLUSION: The ability of pink bollworm and other major pests to evolve resistance to Bt toxins via both qualitative and quantitative changes in receptor proteins demonstrates their remarkable adaptability and presents challenges for monitoring and managing resistance to Bt crops.
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Supporting information may be found in the online version of this article.

Keywords: *Bacillus thuringiensis*; Cry toxin; resistance; cotton; transcription

1 INTRODUCTION

Insecticidal *Bacillus thuringiensis* (Bt) proteins are used worldwide to control many important insect pests.[1,2] Whereas Bt toxins have been used in sprays for nearly a century, crops genetically engineered to produce Bt toxins have been cultivated since 1996 and were planted on over 100 million hectares in 2017.[1,3] These transgenic Bt crops provide many benefits including pest suppression, increased yields and farmer profits, reduced conventional insecticide use, decreased harm to non-target species, and enhanced biological control.[4–7] However, evolution of resistance to Bt crops by insect pests is reducing these benefits.[8]

Better understanding of the molecular mechanisms underlying resistance are needed to improve strategies for monitoring, delaying, and countering evolution of pest resistance to Bt crops. The most common mechanism conferring high levels of resistance to crystalline (Cry) toxins from Bt is reduced binding of toxins to larval midgut receptor proteins such as cadherin, aminopeptidase N, alkaline phosphatase, and ABC transporters.[2,9–15] Many mutations cause reduced binding of Bt toxins by disrupting the amino acid sequence of the receptors, often yielding truncated proteins.[2,9–15] Regulatory changes that decrease the abundance of receptor proteins by reducing transcription of the genes encoding them are also associated with resistance to Cry toxins, but have received less attention.[16–27]

Here we report the first evidence of reduced expression of a receptor protein associated with resistance to a Bt toxin in the pink bollworm, *Pectinophora gossypiella* (Saunders), one of the world's most destructive pests of cotton.[28] Pink bollworm is currently targeted by transgenic cotton producing either Bt toxin Cry1Ac alone in China or Cry1Ac + Cry2Ab toxins in India and elsewhere.[29,30] While this invasive pest has not evolved resistance to Cry1Ac in the United States and China,[8] widespread practical resistance to Bt cotton producing Cry1Ac and Cry1Ac + Cry2Ab has evolved rapidly in India,[30–34] the world leader in cotton production.

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The pink bollworm gene *PgCad1* encodes a cadherin protein that binds Cry1Ac in the midgut of susceptible larvae, which is necessary for toxicity.[35,36] Previous studies of lab- and field-selected pink bollworm from the United States, China, and India have shown that resistance to Cry1Ac is associated with disruptions in the amino acid sequence of *PgCad1* caused by insertion and deletion (*indel*) mutations in this gene, mis-splicing of messenger RNA (mRNA) for this gene, or both.[37–42] By contrast, in this study, we found that 530-fold resistance to Cry1Ac in a lab-selected strain of pink bollworm was associated with dramatically reduced transcription of *PgCad1* and abundance of PgCad1 protein.

2 MATERIALS AND METHODS

2.1 Insect strains and rearing

We used three strains of pink bollworm from Arizona: APHIS-S, AZP-R and APHIS-R. The APHIS-S strain originally obtained from the APHIS Pink Bollworm Rearing Facility in Phoenix, AZ, has been reared for more than 30 years without exposure to Bt toxins or other insecticides.[43,44] AZP-R, derived from 10 populations collected in Arizona cotton fields in 1997, has 1500-3100-fold resistance to Cry1Ac originally conferred by three cadherin alleles named r_1, r_2 and r_3.[37,45,46] Whereas all three cadherin alleles were initially present in AZP-R, only the *r2* allele currently exists in this strain due to fitness costs associated with the *r1* and *r3* alleles and continuous long-term laboratory rearing.[47] APHIS-R is a new strain derived by the mass selection of APHIS-S on diet containing a discriminating concentration of Cry1Ac (10 µg per mL diet) in October 2017. Survivors from the initial selection (~200 *rr* pupae) were pooled and maintained on laboratory diet in environmental chambers under standard rearing conditions (26 °C; 14:10 light:dark photoperiod). Beyond the initial selection in 2017, we selected APHIS-R two additional times on 10 µg Cry1Ac per mL diet corresponding to six generations between selections (i.e. once in April 2018 and again in October 2018).

2.2 *PgCad1* cloning and sequencing

2.2.1 *RNA extraction and cDNA synthesis*

We extracted total RNA from three pools of 10 midguts and from fourth instars of APHIS-R using TRI Reagent (Thermo Fisher Scientific, Waltham, MA, USA). Total RNA was treated with DNase I (Thermo Fisher Scientific) and cDNA was prepared using 1 µg of total RNA with both random hexamer primers and oligo-dT primers, with either a RETROscript® or a SuperScript™ IV First-Strand cDNA synthesis kits (Thermo Fisher Scientific) according to the manufacturer's instruction.

2.2.2 *Molecular cloning and DNA sequencing of* PgCad1 *cDNA*

Full-length *PgCad1* cDNA was PCR amplified using primers 52PgCad5 and 25PgCad3 (Table S1). PCR was performed using Platinum™ SuperFi™ Green PCR Master Mix with SuperFi™ GC Enhancer (Thermo Fisher Scientific), 0.5 µM of each sense and antisense primer, and 1 µL cDNA using a Biometra® TProfessional Gradient thermocycler (Biometra, Germany) at: 98 °C for 30 s (one cycle); 40 cycles of 98 °C for 10 s, 61.8 °C for 10 s and 72 °C for 3 min; then 72 °C for 5 min. PCR products were separated on an 1% agarose gel stained with SYBR Safe (Thermo Fisher Scientific). Bands were gel-purified using a Montage DNA Gel Extraction Kit (LSKGEL050; MilliporeSigma, Burlington, MA, USA) and ligated into pCR-XL-2-TOPO™ (Thermo Fisher Scientific). Plasmids were propagated in TOP10 OneShot™ OmniMax™ Chemically Competent *Escherichia coli* and purified using a QIAprep Spin MiniPrep kit with a QIAcube system (Qiagen, Hilden, Germany). Inserts were sequenced by Retrogen (San Diego, CA, USA) with primers: M13 reverse, T7, 52PgCad5, 89PgCad5, 70PgCad5, 72PgCad5, 76PgCad5, 78PgCad5, 79PgCad3, 81PgCad3, 85PgCad3, and 25PgCad3 (Table S1).

2.2.3 *PCR amplification and cloning of* PgCad1 *5′-untranslated region (UTR)*

Oligonucleotide primers 1PgCad5_UTR, 2PgCad5_UTR, 3PgCad3_UTR, and 4PgCad3_UTR (Table S1) were designed from the *PgCad1* 5′-UTR DNA sequence obtained from an assembled APHIS-S pink bollworm midgut transcriptome.[48] Primer pairs 1PgCad5_UTR + 3PgCad3_UTR and 2PgCad5_UTR + 4PgCad3_UTR were used in nested PCR to amplify the 5′-UTR products from a single female APHIS-S and APHIS-R larva at: 94 °C for 2 min (one cycle); 30 cycles of 94 °C for 30 s, 58.4 °C for 30 s and 72 °C for 30 s; then 72 °C for 10 min. PCR products were cloned into pCR®2.1-TOPO® TA cloning vector (Thermo Fisher Scientific) and propagated using TOP10 OneShot™ Chemically Competent *E. coli*. Plasmids were purified using a QIAcube system as before and inserts from 9–11 clones per individual were sequenced by Retrogen using M13 reverse or T7 vector primers.

2.2.4 PgCad1 *5′ genome walking*

Genomic DNA (gDNA) from two to three fourth instars and pools of ten APHIS-S and ten APHIS-R larval heads was extracted using the Gentra Puregene Tissue Kit (Qiagen) and subjected to DNA walking using the Universal GenomeWalker™ 2.0 kit (Takara Bio USA). We digested gDNA with *Stu* I and ligated it to the GenomeWalker™ Adaptor oligo according to the manufacturer's instructions. To amplify the 5′-promoter region of *PgCad1*, antisense gene specific primers (253PgCad3 and 254PgCad3 within exon 2 and exon 1 of *PgCad1*, respectively) were used with two provided adapter primers (AP1 and AP2) (Table S1). Two-rounds of touchdown PCR were performed according to the manufacturer's instructions: Primary PCR: 94 °C for 2 min (one cycle); 7 cycles of 94 °C for 25 s and 72 °C for 3 min; then 33 cycles of 94 °C for 25 s and 67 °C for 3 min; followed by 67 °C for 7 min after the final cycle; Nested PCR: 94 °C for 2 min (one cycle); five cycles of 94 °C for 25 s and 72 °C for 3 min; then 25 cycles of 94 °C for 25 s and 67 °C for 3 min; followed by 67 °C for 7 min. PCR products were cloned into pCR®2.1-TOPO® (Thermo Fisher Scientific) and plasmids were purified and sequenced as described above. Similarity analysis and alignments were done using ClustalW in Geneious® 10.2.5 (Biomatters, Newark, NJ, USA). We used SIGNAL SCAN (https://www-bimas.cit.nih.gov/molbio/signal/) to search the TRANSFAC® database (version 7.0) for putative transcription factor binding sites and promoter elements.[49]

2.3 Insect bioassays

We used 21-day diet bioassays to evaluate susceptibility to Cry1Ac.[38,43] Newly hatched neonates were placed individually in wells of bioassay trays (BIO-BA-128; C-D International, Pitman, NJ, USA) with approximately 1 g diet and wells were sealed with Pull N' Peel tab tray covers (BIO-CV-16; C-D International). MVP-II (Dow Agrosciences, San Diego, CA, USA), a liquid formulation containing a hybrid protoxin identical to the holotype Cry1Ac protoxin in its first 1067 of 1182 amino acids including the entire active toxin

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(domains I, II, and III)[34] was used as the source of Cry1Ac. We tested six concentrations of Cry1Ac ranging from 0–300 µg per mL diet. After 21 days at 26 °C (14:10 L:D photoperiod), we scored live fourth instar larvae, pupae, and adults as survivors.

2.4 Inheritance of resistance

We obtained and determined the sex of 140 APHIS-S pupae and 140 APHIS-R pupae and set up four mass crosses: 35 female APHIS-S X 35 male APHIS-S, 35 female APHIS-R X 35 male APHIS-R, 35 female APHIS-S X 35 male APHIS-R, and 35 female APHIS-R X 35 male APHIS-S in 350 mL paper cups. Adults were held at 26 °C (14:10 L:D photoperiod) and provided 10% sucrose. Eggs were collected on 12 cm^2 pieces of paper placed over meshed-screen lids. Eggs were collected every other day and pinned into diet cups containing approximately 150 mL untreated diet. We tested a total of 775 F_1 neonates including at least 32 neonates at each concentration for all four mass crosses.

To evaluate maternal effects and sex linkage of resistance in APHIS-R, we compared survival of F_1 progeny between the two reciprocal crosses between APHIS-S and APHIS-R.[38,45] To evaluate dominance, we compared survival of APHIS-R, APHIS-S, and their F_1 progeny.[38,50]

2.5 Transcript analysis

2.5.1 Endpoint reverse transcriptase PCR

We extracted total RNA from individual fourth instars (two males and two females) from each of three strains: APHIS-S, APHIS-R, and AZP-R. We used DNase I-treated RNA to make cDNA using a RETROscript® kit (Thermo Fisher Scientific). For amplification of a 417-bp fragment from the *PgCad1* cDNA, RT-PCR was performed using the gene-specific primers 54PgCad5 and 59PgCad3 (Table S1). In addition to DNase I treatment of the total RNA, to further eliminate the possibility of amplification of PCR products from contaminating gDNA, the primers used to amplify the *PgCad1* amplicon spanned from exon 21–24 (e.g., spans two introns). A 343-bp fragment of the pink bollworm ribosomal protein subunit 7 (RPS7) was amplified as an internal control using the gene-specific primers 1PgRPS75 and 2PgRPS73 (Table S1) (previously named RPS7F and RPS7R, respectively[51]). PCR was performed using 0.3 µL cDNA with 0.4 µM primer solutions and 0.025 units of ExTaq™ DNA Polymerase Premix (Takara Bio USA) per reaction. The DNA was initially denatured for 2 min at 94 °C, and the PCR amplification included 30 s annealing at 53.9 °C for *PgCad1* and 53.6 °C for *RPS7*, and a 1 min extension at 72 °C, with 25, 28, or 30 cycles for *PgCad1* and 23, 25, or 28 cycles for *RPS7*. RT-PCR products were separated on 1% agarose gels and stained with SYBR® Safe (Thermo Fisher Scientific).

2.5.2 Quantitative real-time RT-PCR

We used quantitative RT-PCR (qRT-PCR) according to MIQI guidelines[52,53] to validate gene-specific primers and to observe differences in transcript abundance in cDNA from APHIS-S, APHIS-R, AZP-R and between F_1 individuals from mass crosses. All qRT-PCR reactions were run in a BioRad CFX96™ thermocycler in 20 µL reactions containing 0.1 µM primer solutions and 1X KAPA™ SYBR® FAST qPCR Master Mix (MilliporeSigma). We used primers 245PgCad5 + 246PgCad3 (Table S1) to amplify a 91-bp *PgCad1* fragment and 3PgRPS75 + 5PgRPS73 (Table S1) to amplify a 146-bp *RPS7* fragment, respectively. Primers were validated and primer efficiencies were established using cDNA serial dilutions (total of 6 dilutions ranging from 1 to 1:10^6) from APHIS-S. Mean primer efficiencies (97.6% for *PgCad1* and 91.4% for *RPS7*) were obtained from three biological replicates each with three technical replicates from APHIS-S cDNA and were used for all subsequent qPCR experiments. PCR consisted of an initial 95 °C denaturation/KAPA activation step for 3 min, followed by 40 cycles of 95 °C denaturation for 3 s followed by 20 s annealing at 60 °C. Each reaction was run in triplicate and each experiment included three independent biological replicates run on separate 96-well plates. Each run included three replicates of no-template control (NTC) in which RNase-free water was substituted for the cDNA template. We also included cDNA from a single APHIS-S larva on each plate to normalize for differences between plates within the CFX Manager™ software package. Melt curve analysis was performed for each sample with single, sharp peaks for both genes. Normalized transcript levels for *PgCad1* were determined relative to *RPS7* controls using the $\Delta\Delta C_q$ method within the CFX Manager software.[54] Namely, C_q values were first corrected for the efficiencies of their respective primer pairs and then normalized to the *RPS7* housekeeping gene using the equation: $\Delta C_q = \Delta C_{q\,(PgCad1)} - \Delta C_{q\,(RPS7)}$. For each analysis, we also included three replicates of cDNA from a single APHIS-S fourth instar larva as a control calibrator to calculate $\Delta\Delta C_q$ relative to this internal standard, using the equation: $\Delta\Delta C_q = \Delta C_{q\,(\text{test samples})} - \Delta C_{q\,(\text{APHIS-S control calibrator})}$. Relative normalized expression values are calculated as $2^{-\Delta\Delta Cq}$. Treatment means were separated using Tukey's multiple comparison tests at the $\alpha = 0.05$ level.

2.6 Midgut protein analysis

2.6.1 Brush border membrane vesicle preparation

One hundred midguts from APHIS-R and APHIS-S fourth instar larvae were dissected and used for preparation of brush border membrane vesicle (BBMV) using differential centrifugation.[55,56] Final BBMV pellets were resuspended in ice-cold 0.02 M Tris–HCl pH 7.5, 0.15 M sodium chloride, 0.005 M EGTA, 1% CHAPS, 0.001 M PMSF, and the cOmplete™ mini EDTA-free protease inhibitor cocktail (MilliporeSigma). We also obtained midgut membrane precipitates from BBMV preparations (insoluble pellets obtained following homogenization, addition of 0.024 M $MgCl_2$, and centrifugation at 2500 *g*). Total protein concentrations of all preparations were determined using a Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific).

2.6.2 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblot analysis

For sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblots, equal amounts of total protein (45 µg) from APHIS-S and APHIS-R BBMV preparations were denatured in 1X NuPAGE™ LDS sample buffer (Thermo Fisher Scientific) at 70 °C for 10 min. Proteins were either stained with SimplyBlue™ Coomassie SafeStain (Thermo Fisher Scientific) or electroblotted onto a PVDF membrane using the iBlot™ 2 Dry Blotting System (Thermo Fisher Scientific). For immunoblotting, we used the iBind™ Western Device (Thermo Fisher Scientific) for serial incubations with primary (1:500 diluted α-rCR8-CR9 anti-PgCad1 rabbit antiserum[54]) and secondary antisera [1:2000 goat anti-rabbit IgG alkaline phosphatase (AP) conjugate (Bio-Rad) or 1:2000 diluted Amersham™ ECL™-horseradish peroxidase (HRP)-labeled anti-rabbit antibody (GE Healthcare Bio-Sciences, Pittsburg, PA, USA)]. Antibody binding was observed by either a color reaction catalyzed by AP or by chemiluminescence with HRP using a LAS-4000 imager (Fuji Film, Tokyo, Japan). Control

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samples included extracts from cultured *Tricoplusia ni* insect cells transfected with full-length *PgCad1*_s/pIB/V5-His-TOPO® or with untransfected *T. ni* cells. Novex™ Sharp Pre-stained protein standards (Thermo Fisher Scientific) were run simultaneously.

2.7 Data analysis

In diet bioassays, adjusted survival (%) was calculated as number of survivors on treated diet divided by survivors on untreated diet times 100%. Mortality values (%) corrected for background mortality (using samples from untreated diet) and LC_{50} values were calculated by PROC PROBIT (SAS version 9.4 build TS1M4). Non-overlapping 95% confidence intervals were used to establish significance. We estimated dominance (*h*), which varies from 0 for completely recessive resistance to 1 for completely dominant resistance as described.[50]

3 RESULTS

3.1 Inheritance of APHIS-R resistance to Cry1Ac

The concentration of toxin killing 50% of larvae (LC_{50}, in µg Cry1Ac per mL diet) was 530 times higher for APHIS-R (220) than APHIS-S (0.42) (Table 1). The LC_{50} values were similar for the progeny from the two reciprocal crosses between APHIS-R and APHIS-S (0.79 and 0.99) (Table 1), indicating autosomal inheritance of resistance to Cry1Ac in APHIS-R. At the diagnostic concentration of 10 µg Cry1Ac per mL diet, adjusted larval survival was 100% for APHIS-R versus 0% for APHIS-S and the F_1 progeny from the reciprocal crosses (Table 1), indicating completely recessive resistance ($h = 0$) at this concentration (Table 1).

Table 1. Responses to Cry1Ac of pink bollworm larvae from a resistant strain (APHIS-R), a susceptible strain (APHIS-S), and their F_1 progeny

Strain or cross*	N	LC_{50} (95% FL)†	RR‡	Survival (%)§
APHIS-S	192	0.42 (0.31–0.54)	1.0	0
♂APHIS-S X ♀APHIS-R	194	0.99 (NA)**	2.3	0
♀APHIS-S X ♂APHIS-R	193	0.79 (0.56–1.0)	1.9	0
APHIS-R	196	220 (64–280)	530	100

*For the two reciprocal crosses, the results are for the F_1 progeny.
†Concentration killing 50% in µg of Cry1Ac per mL diet with 95% fiducial limits in parentheses.
‡Resistance ratio, the LC_{50} of a strain divided by the LC_{50} of APHIS-S.
§Survival at 10 µg Cry1Ac per mL diet, adjusted for control mortality.
**Not available.

3.2 Cloning and cDNA sequencing of *PgCad1* from APHIS-R

We used PCR to amplify cDNA from three pools of RNA, each from 10 midguts of APHIS-R larvae. Using primers corresponding to full-length *PgCad1* from APHIS-S, we obtained one cDNA product of approximately 5.2 kb from two of the pools, and two cDNA products of approximately 5.2 and approximately 1.8 kb from the third pool (Fig. S1). Ten clones from three pools revealed that the band of approximately 5.2 kb consists of two different *PgCad1* transcript variants that differ by a single codon; one has 5208 bp and the other has 5205 bp (Fig. S2). The consensus sequence from the 5208 bp clones indicates that the full-length *PgCad1* from APHIS-R encodes a protein that is 99.8% identical (1732 of 1735 amino acids) to the wild-type PgCad1 protein from APHIS-S (Genbank accession number AAP30715.1). The three conservative amino acid substitutions in APHIS-R relative to APHIS-S are: I1100V, I1107T, and K1682R. We found no major deletions, insertions or other disruptive mutations in the approximately 5.2 kb *PgCad1* cDNA from APHIS-R. The nucleotide sequences for the *PgCad1-s* and *-r17* alleles reported in this paper are deposited in the GenBank public database with accession numbers MK922613 and MK922614, respectively.

The approximately 1.8 kb fragment consists of 1845 bp corresponding to *PgCad1* but has a 20-bp insertion in exon 4 and a 3383-bp deletion from within exon 6 to the beginning of exon 28 (Fig. S3). Using previous nomenclature for *PgCad1* alleles,[37,39] we name these mutations *r17A* and *r17B*, respectively, corresponding to the *r17* allele. These mutations introduce a premature stop codon after residue 174 that eliminates much of the cadherin protein (Fig. S4), including regions important for Cry1Ac binding.[56]

3.3 *PgCad1* transcript abundance

We used endpoint RT-PCR and qRT-PCR to assess the relative transcript abundance of *PgCad1* in APHIS-S and two Cry1Ac-resistant strains (APHIS-R and AZP-R) by testing four larvae individually from each of the three strains (total n = 12 larvae). Whereas endpoint RT-PCR amplified a single *RPS7* amplicon (control) in all larvae from the three strains, a 417-bp *PgCad1* band was observed in APHIS-S and AZP-R but not in APHIS-R (Fig. 1). These results suggest the *PgCad1* transcript was scarce in APHIS-R relative to APHIS-S and AZP-R.

Consistent with the endpoint RT-PCR results, qRT-PCR showed significantly less *PgCad1* transcript in APHIS-R than in APHIS-S and AZP-R (Fig. 2, Tukey's multiple comparison test, $P < 0.0001$ for each comparison). Based on results from four larvae per strain, the mean (±SE) normalized *PgCad1* transcript abundance in APHIS-R (0.005 ± 0.001) was 190-fold lower than in APHIS-S (0.968 ± 0.028) and 68-fold lower than in AZP-R (0.346 ± 0.012). The abundance of *PgCad1* transcript was also 2.8-fold lower in the resistant strain AZP-R relative to APHIS-S (Tukey's multiple comparison test, $P < 0.0001$).

We also compared *PgCad1* transcript abundance using qRT-PCR of cDNA from 18 larvae obtained from each of four mass crosses: APHIS-R X APHIS-R, APHIS-S X APHIS-S, APHIS-R females X APHIS-R males, and APHIS-S females X APHIS-R males (total n = 72 larvae, Fig. 3). Consistent with the results described above, normalized mean (±SE) *PgCad1* transcript abundance was 79-fold lower for APHIS-R (0.012 ± 0.001) than APHIS-S (0.974 ± 0.101) (Tukey's multiple comparisons test $P < 0.0001$, Fig. 3). *PgCad1* transcript abundance did not differ significantly between the reciprocal crosses (0.533 ± 0.064 and 0.536 ± 0.036, $P > 0.99$). *PgCad1* transcript abundance in the progeny from each reciprocal cross was significantly lower than in APHIS-S ($P < 0.0001$) and significantly higher than in APHIS-R ($P < 0.0001$) (Fig. 3).

3.4 PgCad1 protein expression

We used SDS-PAGE and immunoblot analysis to analyze PgCad1 protein expression in midgut BBMV preparations from APHIS-S and APHIS-R (Fig. 4). Using an anti-PgCad1 antibody to test APHIS-S, we detected the predicted approximately 200 kDa protein band in BBMV (Fig. 4(b) and (c), Lane 1), midgut precipitate (Fig. 4(b) and (c), Lane 6), and an extract from cultured *T. ni* insect cells transfected with full-length *PgCad1*_s/pIB/V5-His-TOPO® plasmid

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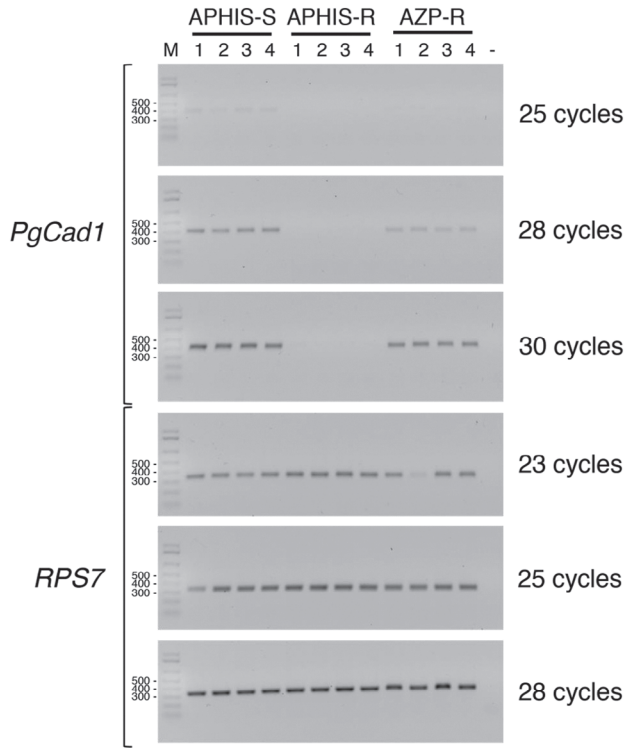

Figure 1. Endpoint RT-PCR of *PgCad1* and *RPS7* from the susceptible APHIS-S strain and two Cry1Ac-resistant strains (APHIS-R and AZP-R). cDNA from four individuals from APHIS-S, APHIS-R, and AZP-R was used as templates in endpoint RT-PCR to amplify the 417-bp *PgCad1* amplicon at 25, 28, and 30 cycles and the 343-bp *RPS7* amplicon at 23, 25, and 28 cycles. Lane M, 1 kb Plus DNA Ladder (Thermo Fisher Scientific); Lanes 1–4, Individuals 1–4 from APHIS-S, APHIS-R, and AZP-R; Lane '-', Negative RT-PCR control reaction without DNA template.

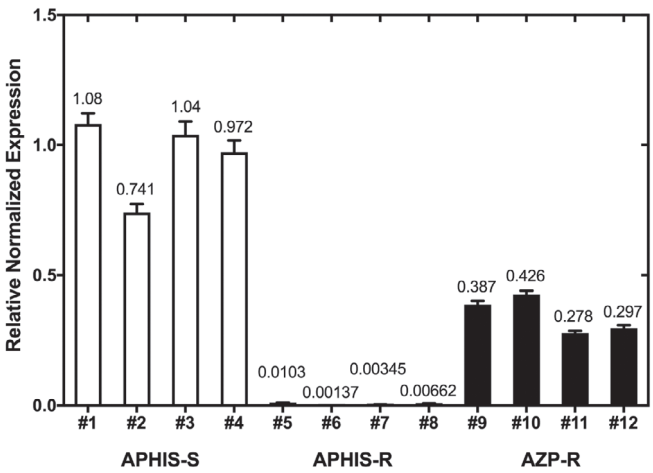

Figure 2. Quantitative real-time RT-PCR of *PgCad1* from susceptible (APHIS-S) and resistant (APHIS-R and AZP-R) strains of pink bollworm. Relative *PgCad1* transcript levels were determined for each of four individuals from each strain based on three technical and three biological replicates per individual (total of nine readings per individual). Numbers above each bar show the mean normalized relative transcript abundance for each individual and error bars show the standard error (SE).

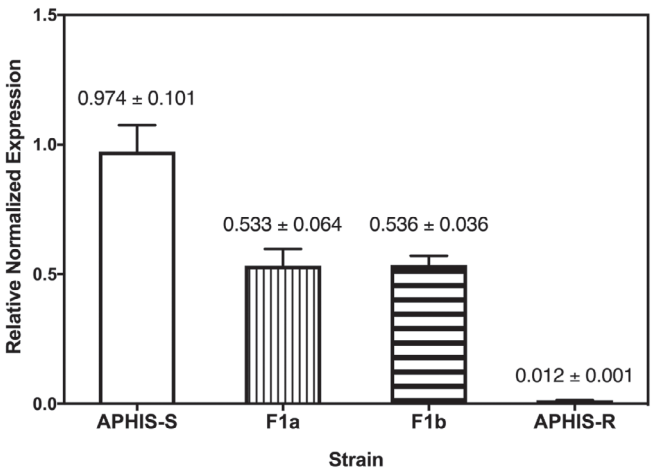

Figure 3. Relative *PgCad1* transcript levels in APHIS-S, APHIS-R, and the F_1 progeny from reciprocal crosses between the two strains determined with qRT-PCR. *PgCad1* transcript levels were normalized relative to *RPS7* and numbers above each bar show the mean transcript abundance with error bars showing the standard errors (SE).

DNA (Fig. 4(b) and (c), Lane 1). For APHIS-R, however, we did not detect the approximately 200 kDa protein band in BBMV (Fig. 4(b) and (c), Lane 4) or the midgut precipitate (Fig. 4(b) and (c), Lane 7), indicating the PgCad1 protein concentration was below the level of detection. The SDS-PAGE gel stained for protein shows equal loading of protein across the samples from both strains (Fig. 4(a)).

3.5 *PgCad1* promoter analysis

To test for differences between strains in the promoter region of *PgCad1*, we PCR amplified, cloned, and Sanger DNA sequenced approximately 227 bp upstream of the *PgCad1* coding sequence (CDS) using primers designed from the corresponding 5′-UTR sequence from the APHIS-S midgut transcriptome (comp13368_c0_seq1).[48] Although we found at least three different transcript variants, no consistent differences in the 5′-UTR occurred between APHIS-S and APHIS-R (Fig. S4). All three of the 5′-UTR variants we detected occurred in both strains. The predominant variant consists of 198 nucleotides from the nested primer 2PgCad5_UTR to the *PgCad1* start codon and occurred in 13 of the 20 clones (Fig. S4). Of the remaining variants, five clones lacked 3-bp 26 bp upstream of the *PgCad1* start codon and two clones had a 4-bp insertion 22 bp upstream of the start codon (Fig. S4).

To further assess the 5′-promoter region of *PgCad1*, we performed genome walking approximately 2 kb upstream of the *PgCad1* CDS. Although we found at least two common 5′-promoter sequences with differences in their predicted transcription factor sites (Tables S2 and S3), we did not find any consistent differences between APHIS-S and APHIS-R (Fig. S5). Whereas both common 5′-promoter sequences share 12 predicted transcription factor binding sites, their sequences differ and are predicted to have unique transcription factor binding sites. Namely, the 1806-bp sequence has two B_factor sites, a Bcd site, and two Zeste sites not shared by the 1832-bp sequence and the 1832-bp consensus promoter sequence has a unique AP-1 site, two Zeste sites, two B-factor sites, and a TBP factor site (Fig. S5).

4 DISCUSSION

The results reported here provide the first evidence of pink bollworm resistance to a Bt toxin associated with downregulation of a midgut receptor. Previous results from Cry1Ac-resistant pink bollworm implicate *indel* mutations in the *PgCad1* gene that alter transcript length and substantially disrupt the amino acid sequence of

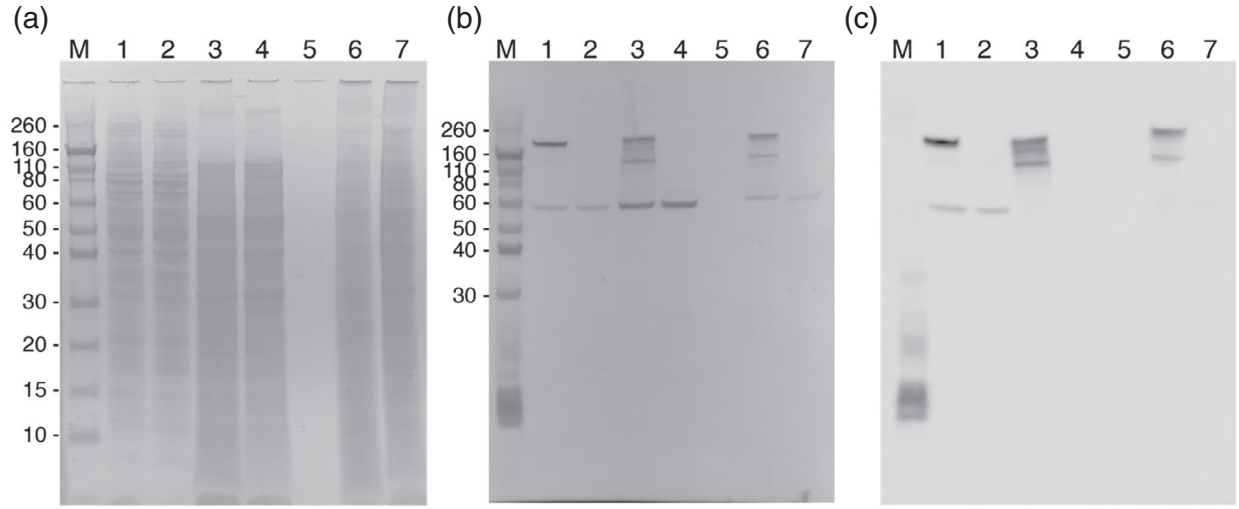

Figure 4. PgCad1 protein expression in pink bollworm midgut. Equal amounts of total protein (45 µg) were separated by SDS-PAGE and stained using Coomassie stain (a). Proteins separated by SDS-PAGE, were transferred to PVDF membranes, and immunoblots were probed with 1:500 diluted α-rCR8-CR9 anti-PgCad1 rabbit antiserum and secondary antisera [1:2000 goat anti-rabbit IgG alkaline phosphatase (AP) conjugate (b) or 1:2000 diluted horseradish peroxidase (HRP)-labeled anti-rabbit antibody (c)]. Lane M, Novex™ Sharp Pre-stained protein standards (Thermo Fisher Scientific); Lane 1, Cell extract from cultured *Tricoplusia ni* insect cells transfected with full-length *PgCad1*_s/pIB/V5-His-TOPO® plasmid DNA; Lane 2, Cell extract from untransfected *T. ni* cells; Lane 3, BBMV preparation from APHIS-S; Lane 4, BBMV preparation from APHIS-R; Lane 5, blank loading control; Lane 6, APHIS-S initial midgut precipitate; Lane 7, APHIS-R initial midgut precipitate.

the encoded cadherin protein.[37–42] Here we found that the predominant *PgCad1* transcript in the lab-selected APHIS-R strain, which has 530-fold resistance to Cry1Ac, is 99.8% identical to the wild-type sequence from the susceptible strain APHIS-S (Fig. S1). In contrast, the abundance of this full-length transcript and the corresponding protein are dramatically reduced in APHIS-R relative to APHIS-S (Figs. 1–4).

In addition to the full-length *PgCad1* transcript found in all three pools of APHIS-R midgut RNA samples (each from 10 larvae), we also discovered an internally deleted *PgCad1* cDNA that encodes a resistance-conferring truncated cadherin protein in one of the three pools (Fig. S2). This indicates most APHIS-R individuals had a downregulated full-length *PgCad1,* whereas a smaller proportion had both the downregulated full-length *PgCad1* and a transcript harboring an internal deletion. Both the end-point and qRT-PCR primers we used to assess transcript abundance are located within this deletion, which means that individuals harboring this mutation also likely contributed to the lower *PgCad1* transcript abundance observed in APHIS-R.

Another important result of this study is that *PgCad1* transcript abundance was significantly lower in the resistant strain AZP-R than in the susceptible strain APHIS-S (Fig. 2). Previous work shows that AZP-R harbors cadherin mutations that reduce Cry1Ac binding, toxin oligomerization, or both.[37,57] Based on qRT-PCR, the reduction of *PgCad1* transcript abundance relative to APHIS-S was only 2.8-fold in AZP-R compared with 79- to 190-fold in APHIS-R. Furthermore, because APHIS-R was derived from APHIS-S and these two strains have the same genetic background, it is likely that the markedly reduced transcription of *PgCad1* reflects selection for resistance to Cry1Ac in APHIS-R. By contrast, the much smaller reduction in *PgCad1* transcript abundance in AZP-R relative to APHIS-S could reflect an inherent difference between these two unrelated strains that did not arise from selection with Cry1Ac. Nonetheless, results from APHIS-R and AZP-R suggest that both qualitative and quantitative changes in cadherin protein may contribute to resistance within single strains of pink bollworm.

Although this study provides the first evidence of pink bollworm resistance to a Bt toxin associated with down-regulation of receptor expression, this phenomenon has been reported from several other major pests including *Aedes aegypti*, *Diatraea saccharalis*, *Helicoverpa armigera*, *Ostrinia furnacalis*, *Plutella xylostella*, *Spodoptera exigua*, and *Trichoplusia ni*.[16–27] Four of these examples showed that downregulation of cadherin was associated with Cry toxin resistance,[17,18,22,24] but only Yang *et al.*[22] showed no differences in cadherin cDNA sequence between a Cry1Abresistant and susceptible strain of *D. saccharalis*, implicating either *cis*- or *trans*-regulation of the transcript. Liu *et al.*[58] also reported that Cry1Ac resistance in a laboratory-selected strain of *H. armigera* involves *cis*-mutations in the promoter region of a trypsin gene (*HaTryR*) that down-regulates *HaTryR* transcription.

Because we did not find consistent differences between APHIS-R and APHIS-S in the sequence of the *PgCad1* promoter, we hypothesize that reduced transcription of this gene in APHIS-R is caused by one or more *trans*-acting regulatory factors that are neither within nor immediately upstream of this gene. Further experiments are needed to test this hypothesis. The *trans*-acting factor could be a transcription activator/repressor or its upstream protein regulator. For example, Cry1Ac resistance in *P. xylostella* is associated with the downregulation of a midgut *ALP* and two *ABCC* genes (*PxABCC2* and *PxABCC3*).[25] Like *PgCad1*, these two genes encode receptors of Cry1Ac. This down-regulation is mediated by a transcriptionally-activated upstream protein regulator from the mitogen-activated protein kinase (MAPK) signaling pathway (*PxMAP4K4*), which was also found on the same chromosome near the *PxABCC2* and *PxABCC3* genes.[25]

Alternatively, the *trans*-acting factor in pink bollworm could be a non-coding microRNA (miRNA) or long non-coding RNA (lncRNA) involved in post-transcriptional regulation of *PgCad1*. Studies in two species of *Ostrinia* have shown differences in miRNA profiles between resistant and susceptible strains that may influence Cry1Ab resistance.[59,60] Several Bt resistance genes, including those encoding cadherin, aminopeptidase N, ABC transporters,

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and various proteases, were predicted to be affected by these differentially expressed miRNAs.[59,60] Numerous differences in lncRNA profiles have also been found between susceptible and Cry1Ac-resistant strains of *P. xylostella*.[61,62]

In pink bollworm, the insertion of an intact 4739-bp chicken repeat 1 (CR1) retrotransposon named *CR1-1_Pg* generating the *r3* cadherin mutation disrupts both *PgCad1* and an intronic sense lncRNA named *PgCad1 lncRNA*.[63] *PgCad1 lncRNA* positively regulates transcription of *PgCad1* in the APHIS-S strain.[64] Feeding small interfering RNAs (siRNAs) targeting this lncRNA to APHIS-S larvae reduced *PgCad1* transcript abundance and decreased their susceptibility to Cry1Ac.[64] Although more experiments are needed to determine if this lncRNA plays a role in APHIS-R, these results provide experimental evidence that reducing *PgCad1* transcript abundance can lower susceptibility of pink bollworm to Cry1Ac.

Resistance to Cry1Ac and Cry2Ab in pink bollworm is associated with alternative splicing of cadherin (*PgCad1*) and an ABC transporter (*PgABCA2*), respectively.[30,39] However, we do not yet know what causes this alternative splicing. The new results reported here suggest that gene regulation as well as altered post-transcriptional mRNA splicing underlie many pink bollworm resistance phenotypes. More studies are needed to better understand how such processes are regulated and their role in resistance to Bt crops.

5 CONCLUSIONS

Previously studied Cry1Ac-resistant pink bollworm from India, China, and the US have indel mutations in the DNA sequence of *PgCad1*, altered splicing of *PgCad1* mRNA, or both that disrupt the amino acid sequence of this Cry1Ac-binding midgut protein.[37–42] Here we show for the first time that reduced cadherin expression is also associated with pink bollworm resistance to Cry1Ac. The ability of pink bollworm and other major pests to evolve resistance via both qualitative and quantitative changes in receptor proteins demonstrates their remarkable adaptability and presents challenges for monitoring and managing resistance to Bt crops.

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SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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