A Single Point Mutation Resulting in Cadherin Mislocalization Underpins Resistance against Bacillus thuringiensis Toxin in Cotton Bollworm*¶

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Transgenic plants that produce Bacillus thuringiensis (Bt) crystalline (Cry) toxins are cultivated worldwide to control insect pests. Resistance to B. thuringiensis toxins threatens this technology, and although different resistance mechanisms have been identified, some have not been completely elucidated. To gain new insights into these mechanisms, we performed multiple back-crossing from a 3000-fold Cry1Ac-resistant Bt strain from cotton bollworm (Helicoverpa armigera), isolating a 516-fold Cry1Ac-resistant strain (96CAD). Cry1Ac resistance in 96CAD was tightly linked to a mutant cadherin allele (mHaCad) that contained 35 amino acid substitutions compared with HaCad from a susceptible strain (96S). We observed significantly reduced levels of the mHaCad protein on the surface of the midgut epithelium in 96CAD as compared with 96S. Expression of both cadherin alleles from 96CAD and 96S in insect cells and immunofluorescence localization in insect midgut tissue sections showed that the HaCAD protein from 96S localizes on the cell membrane, whereas the mutant 96CAD-mHaCad was retained in the endoplasmic reticulum (ER). Mapping of the mutations identified a D172G substitution mainly responsible for cadherin mislocalization. Our finding of a mutation affecting membrane receptor trafficking represents an unusual and previously unrecognized B. thuringiensis resistance mechanism.

Bacillus thuringiensis-based products and transgenic B. thuringiensis plants have been widely used for insect control, providing efficient protection of plants against insect attack (1). Insects ingest crystal toxins (named Cry) produced by B. thuringiensis. Cry toxins are activated by insect midgut proteases, and they bind to different insect proteins (receptors), localized on the apical brush border membranes (BBM) of their midgut cells. Bound toxins insert into the membrane, forming lytic pores, leading to cell breakdown and insect death (1, 2).

Different Cry toxin receptors have been described, such as cadherin (Cad), alkaline phosphatase (ALP), aminopeptidase-N (APN), ATP-binding cassette subfamily C member 2 (ABCC2) transporter, and glycoprotein among others (3–11). Their expression in the plasma membrane is a requisite to exert their function as Cry receptors. As membrane proteins, Cry receptors reach the plasma membrane from the endoplasmic reticulum (ER)-Golgi interface through the trafficking of coated vesicles. There are three major types of coated vesicles in the cells: COPII, COPI, and clathrin. The COPII facilitates anterograde vesicle budding from ER to Golgi, whereas COPI performs retrograde transport from Golgi to the ER. Clathrin-coated vesicles mediate traffic from the cell surface and between the trans-Golgi and endosomes. Specific sorting signals of transmembrane proteins are required for trafficking (12). In addition, glycosylation of cell surface proteins might be critical for localization in the apical membrane (13).

Insect pests, such as Helicoverpa armigera, Heliothis virescens, Pectinophora gossypiella, Plodia interpunctella, Ostrinia nubilalis, Plutella xylostella, and Spodoptera exigua, have evolved resistance to Cry toxins in laboratory conditions, whereas P. gossypiella, P. xylostella, Trichoplusia ni, and Spodoptera frugiperda evolved resistance in the field (14–20). In the case of H. armigera, resistance alleles have been detected in the field, but no practical resistance cases have been documented (21). Different mutations in receptor proteins have been linked to insect resistance to Cry toxins, such as early stop codons, mutations affecting Cry1A binding, and mutations resulting in reduced expression of the receptors (21–27).

In this work, we established a new H. armigera Cry1Ac-resistant strain 96CAD, nearly isogenic to susceptible strain 96S,
starting from a highly Cry1Ac-resistant strain BtR. The selected 96CAD strain showed 516-fold Cry1Ac resistance, and we show here that it is tightly linked to an mHaCad gene allele. Nevertheless, we show here that expression levels of mHaCad gene and binding of Cry1Ac to the mHaCad receptor was not affected. We found that the mutant mHaCad protein of 96CAD was not found in the apical BBM of midgut cells and show also that the mislocalization of mHaCad in the ER instead of the plasma membrane was associated with Cry1Ac resistance in 96CAD. The mutation responsible for the mislocalization of mHaCad in 96CAD was identified and narrowed to a single amino acid residue. Therefore, Cry1Ac resistance due to absence of the cadherin on the midgut epithelial membrane, as reported for H. armigera and other species, is accomplished here by an unusual mechanism. This is the first report showing that B. thuringiensis resistance could be caused by a mutation affecting intracellular protein trafficking.

### Results

**Mechanism of Resistance in the BtR Cry1Ac-resistant Strain**—The BtR strain was previously reported to be >3000-fold resistant to Cry1Ac, autosomal, incompletely recessive, and polygenic (28, 29). To identify the resistance mechanism in the BtR strain, we analyzed single-nucleotide polymorphism (SNP) markers for different insect gut protein genes, such as HaCad, HaAbcc, HaApn, and HaAlp, that have been shown to be involved in Cry1Ac toxicity in H. armigera (supplemental Table S1) (25, 30–32). Resistant and susceptible individuals were first crossed, producing F1 progeny. Crossing over within homologous chromosomes does not occur in female Lepidoptera. Single pairs of F1 males and BT females were crossed to produce mapping families that were named A–E (Table 1). Progeny from each cross were either reared on a diet without toxin or with 60 μg/ml Cry1Ac toxin. Through this strategy, survivors on the Cry1Ac diet were expected to be homozygous for the recessive resistance mutation, whereas survivors on the control diet were expected to be either homozygous or heterozygous. Analysis of 20 larvae of each family shows that part of the Cry1Ac resistance is linked to the HaCad gene, whereas no linkage was found with the HaAbcc (including Abcc1–4), HaApn (including Apn1–6), or HaAlp genes (including Alp1 and -2) (Table 1). These data suggest that one of the resistance mechanisms in the BtR strain is associated with HaCad.

**Transfer of Cry1A Resistance Allele into a Susceptible Background**—The Cry1Ac resistance was introgressed into the susceptible parent 96S strain by back-crossing for six generations (named BC1–BC6), to generate the nearly isogenic line to the susceptible strain 96S, named 96CAD (supplemental Fig. S1). The toxicity of Cry1Ac toxin against the 96CAD and 96S strains was analyzed (Table 2). Compared with 96S, the 96CAD exhibited high levels of Cry1Ac resistance (516-fold). These data indicate that one gene allele is enough to confer high resistance levels to Cry1Ac in the 96CAD strain and that the higher Cry1Ac resistance levels observed in the original BtR strain (3000-fold resistance to Cry1Ac) could be due to an additional mechanism because 96CAD is only 516-fold resistant to Cry1Ac (28, 29). However, we cannot rule out the possibility that the higher resistance level of the BtR strain is due to other epigenetic factors, because no additional H. armigera-resistant strain was isolated from the back-crosses.

We conducted reciprocal crosses between 96CAD and 96S and tested the susceptibility of F1 progeny to Cry1Ac toxin to evaluate inheritance of Cry1Ac resistance. The responses were similar in F1 progeny from the two reciprocal crosses (Table 2), indicating that inheritance of Cry1Ac resistance is autosomal (i.e. no sex linkage or maternal effects). Based on the LC50 values for the F1 relative to the parent strains (Table 2), we calculated the dominance parameter (\(h\)), which varies from 0 for completely recessive to 1 for completely dominant. For both reciprocal crosses, \(h\) was 0.01, indicating that resistance in 96CAD was a nearly completely recessive trait.

**Cadherin Linked to Cry1Ac Resistance in the 96CAD**—The sequence of genomic DNA from the larvae progeny after crossing five individual pairs of 96CAD female and 96S male moths showed tight genetic linkage of HaCad with resistance to Cry1Ac (28, 29). However, we cannot rule out the possibility that the higher resistance level of the BtR strain is due to other epigenetic factors, because no additional H. armigera-resistant strain was isolated from the back-crosses.

**Multiple Amino Acid Substitutions in the mHaCad from 96CAD Strain**—We sequenced the resistant allele from 96CAD and the corresponding susceptible allele from 96S. Both predicted proteins, the wild type (HaCad) from 96S and mutant gene allele (mHaCad) from 96CAD, have ER signal peptides, two potential acidic ER export motifs (di-acidic motif, D\(^{655}\)SF and D\(^{1666}\)SD), and four putative sorting signals (YXXΦ or LL

### Table 1

Genetic linkage between mutations in HaCad and resistance to Cry1Ac

| Family | HaCad (rr/rs) | HaABCC2 (rr/rs) | HaAPN1 (rr/rs) | HaALP1 (rr/rs) |
|--------|---------------|-----------------|---------------|---------------|
| A      | 16/4          | 12/8            | 11/9          | 8/12          |
| B      | 13/7          | 8/12            | 8/12          | 9/11          |
| C      | 14/6          | 10/10           | 13/7          | 14/6          |
| D      | 18/2          | 7/13            | 6/14          | 7/13          |
| E      | 15/5          | 12/8            | 12/8          | 11/9          |
| Total* | 76/24         | 49/51           | 50/50         | 49/51         |

* For the genetic linkage analysis, we used \(\chi^2\) tests. The observed genotype frequencies of HaCad on the diet treated with B. thuringiensis toxins differed significantly from the expected genotype frequencies on the untreated diet (\(\chi^2 = 15.5, df = 1, p < 0.001\)), whereas genotype frequencies of HaABCC2, HaAPN1, and HaALP1 on the diet treated with B. thuringiensis toxins have no difference from the expected genotype frequencies on the untreated diet (\(\chi^2 = 0.5, df = 1, p = 0.48\); \(\chi^2 = 0.1, df = 1, p = 0.8\); \(\chi^2 = 0, df = 1, p = 1\), respectively).
motif) of the cell surface (Y1562XX, L1598LX, Y1621XX, and Y1667XX). These proteins differ by 35 amino acid substitutions, located in the extracellular region of the protein. Although there was a substitution (F8L) in the signal peptide, the other predicted sorting signal sequences did not differ (supplemental Fig. S2).

**Cry1Ac Binding to Cadherin Fragments from 96S and 96CAD**—We first analyzed the binding of Cry1Ac to cadherin from both strains. Previous published work identified an *H. armigera* cadherin region that is involved in Cry1Ac binding (33). Thus, toxin-binding regions from HaCad and mHaCad were cloned and expressed in *Escherichia coli* cells. We decided to analyze the binding of Cry1Ac to the purified cadherin fragments by ELISA binding assays, because this technique is appropriate to analyze binding parameters with purified proteins. ELISA binding assays showed that Cry1Ac bound to both HaCad and mHaCad fragments similarly (supplemental Fig. S3). Scatchard plot analysis of the data from saturation-binding curves revealed similar apparent binding (Kd) affinities of Cry1Ac to both HaCad and mHaCad fragments of 0.35 ± 0.05 and 0.69 ± 0.13 nM, respectively (supplemental Fig. S3). In addition, we performed homologous binding competition analysis of biotinylated Cry1Ac toxin to both cadherin fragments in the presence of different concentrations of unlabeled Cry1Ac toxin as competitor. Supplemental Fig. S3 shows that the binding of Cry1Ac to both cadherin fragments is specific because unlabeled toxin competed efficiently for the binding of the biotin-labeled Cry1Ac. These results indicate that mHaCad from 96CAD is not affected in Cry1Ac binding.

**Transcription of the mHaCad Gene and Expression of mHaCad Protein in 96CAD Strain**—Analysis of cadherin gene expression by qRT-PCR showed that transcript levels were similar in both strains (supplemental Fig. S4). However, the cadherin protein levels in brush border membrane vesicles (BBMV) isolated from both strains, analyzed by Western blotting assays, showed lower levels of the 210-kDa mHaCad protein band in the BBMV isolated from 96CAD strain compared with the 96S strain (Fig. 1B), suggesting that mHaCad is not located in the apical brush border microvilli of the midgut cells. We also compared the expression of HaCad and mHaCad proteins in a total protein sample preparation from whole midgut tissue of 96S and 96CAD strains by Western blotting. The cadherin protein can be detected in both strains, although the abundance of expressed HaCad in 96S strain was significant higher than mHaCad in the 96CAD strain. These data may indicate that mHaCAD is less efficiently translated in the insect cells or that it is degraded in the midgut cells of the 96CAD strain (Fig. 1D).

**Localization of Cadherin in 96S and 96CAD**—To further analyze the location of mHaCad, we immunolocalized cadherin protein in isolated mature columnar midgut cells by analyzing the fluorescence of the rhodamine-labeled secondary antibody that recognized the first anti-HaCad antibody. Mature columnar cells are easily identified under light microscopy by their morphology, including their size and the presence of brush border microvilli on their apical surface (34). Fig. 2 shows that the fluorescence signal was stronger on the apical BBM of isolated midgut cells from 96S compared with 96CAD. In the negative

### Table 2

| Strain or cross | LC50* (95% FL)* | Resistance ratio* |
|----------------|----------------|------------------|
| 96S            | 0.031 (0.009–0.054) | 1.0             |
| 96CAD          | 16.0 (5.8–28)     | 516              |
| 96CAD female × 96S male | 0.154 (0.091–0.27) | 4.97*          |
| 96CAD male × 96S female | 0.124 (0.10–0.15) | 4.0*            |

* Units are μg of Cry1Ac per ml of diet.

### Table 3

| Cross          | Control diet | Cry1Ac-treated diet* |
|----------------|--------------|----------------------|
| 96S × 96CAD    | rr rs ss     | rr rs ss             |
| 96S × 96CAD    | 8 12 0       | 20 0 0               |
| 96S × 96CAD    | 15 8 0       | 20 0 0               |
| 96S × 96CAD    | 10 10 0      | 20 0 0               |
| 96S × 96CAD    | 13 7 0       | 20 0 0               |
| 96S × 96CAD    | 14 14 0      | 20 0 0               |
| Total          | 49 51 100    | 100 0 0              |

* Diet treated with 60 μg of Cry1Ac toxin/ml. These data are significant based on Fisher’s exact test, p < 0.0001 for each family.

* For the genetic linkage analysis, we used χ2 tests. The observed genotype frequencies of HaCad on diet treated with *B. thuringiensis* toxins differed significantly from the expected genotype frequencies on the untreated diet (χ2 = 65.8, df = 1, p < 0.001).

### Figure 1

**Analysis of cadherin expression in BBMV isolated from susceptible 96S strain and Cry1Ac-resistant 96CAD strain.** A, BBM proteins from 96S and 96CAD strains stained with Coomassie Blue. B, Western blotting detection of cadherin protein in BBMV samples using anti-HaCad antibody and β-actin protein using anti-actin antibody as a loading control. C, total protein samples from midgut tissue of 96S and 96CAD strains stained with Coomassie Blue. D, Western blotting detection of cadherin protein in total protein samples from midgut tissue as described under “Experimental Procedures.”

### Figure 2

**Localization of Cadherin in 96S and 96CAD**—Analysis of cadherin gene expression by qRT-PCR showed that transcript levels were similar in both strains (supplemental Fig. S4). However, the cadherin protein levels in brush border membrane vesicles (BBMV) isolated from both strains, analyzed by Western blotting assays, showed lower levels of the 210-kDa mHaCad protein band in the BBMV isolated from 96CAD strain compared with the 96S strain (Fig. 1B), suggesting that mHaCad is not located in the apical brush border microvilli of the midgut cells. We also compared the expression of HaCad and mHaCad proteins in a total protein sample preparation from whole midgut tissue of 96S and 96CAD strains by Western blotting. The cadherin protein can be detected in both strains, although the abundance of expressed HaCad in 96S strain was significant higher than mHaCad in the 96CAD strain. These data may indicate that mHaCAD is less efficiently translated in the insect cells or that it is degraded in the midgut cells of the 96CAD strain (Fig. 1D).

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control of 96S columnar cells without primary antibody incubation, no positive staining was observed (Fig. 2). We quantified the fluorescence of the BBM from 21 images randomly selected, including control, 96S, and 96CAD, by analyzing the pixels in the highest point of the red color histogram using ImageJ. Significant differences were found using Fisher’s exact test ($p < 0.0001$). Error bars, S.E; a, b, and c, significant difference.

The cellular localization of mHaCad-GFP and HaCad-GFP proteins in transfected Hi5 cells was determined. Laser confocal scanning microscope observations of the GFP fluorescence revealed that mHaCad-GFP co-localized with ER in contrast to HaCad-GFP, which did not co-localize with the ER marker (supplemental Fig. S6).

We then permeabilized the cells with 1% Triton X-100 before their incubation with anti-HaCad antibody and analyzed the red fluorescence of the secondary antibody coupled to rhodamine-fluorescent dye (TRITC). The merge figure showing the image of the colocalization of GFP from mHaCad-GFP and the detection of the mutated cadherin protein with anti-HaCad antibody suggested that its localization is in the cytoplasm, whereas HaCad localizes mainly on the surface of the cell (Fig. 4B).

Analysis of co-localization of cadherin with an ER marker (dsRed2–ER) revealed that mHaCad-GFP co-localized with ER in contrast to HaCad-GFP, which did not co-localize with the ER marker (supplemental Fig. S6).
Analysis of cell surface immunofluorescence performed in Hi5 cells without Triton X-100 permeabilization revealed no red fluorescence signal after incubation with anti-HaCad antibody on the cell membrane of the mHaCad-GFP-expressing cells, whereas HaCad-GFP was clearly localized on the cell membrane (Fig. 4C). Images shown in Fig. 4, A and B, clearly show that mHaCAD is expressed in the Hi5 cells and suggest that its location is mainly cytoplasmic. The only difference in Fig. 4C is that these cells were not permeabilized with Triton X-100; thus, antibody could only detect the protein that was on the surface of the cells.

We also analyzed whether activated Cry1Ac was able to bind to the surface of transfected cells expressing either one of the two proteins. Fig. 5 shows that only HaCad-GFP-transfected cells were able to bind Cry1Ac to their plasma membrane, in contrast with mHaCad-GFP-transfected cells that did not bind Cry1Ac toxin (Fig. 5). Previously, we showed that mHaCad from the 96CAD strain is not affected in Cry1Ac binding by ELISA binding assays of Cry1Ac to both HaCad and mHaCad fragments (supplemental Fig. S3). These data suggest that mHaCad is affected in its localization on the plasma membrane in the Hi5 cells.

Western blotting analysis with total protein from Hi5 cells transfected with HaCad-GFP, mHaCad-GFP, and control cells transfected with empty vector demonstrated that anti-HaCad antibody recognizes a single band of 210 kDa in the HaCad-GFP-transfected line but not in the control cells, suggesting that this antibody is highly specific (supplemental Fig. S7). Supplemental Fig. S7 also shows that the expressed HaCad-GFP was significantly more abundant than the mHaCad-GFP. These results may suggest that retention of mHaCad in ER could induce its degradation.

Identification of Amino Acid Residues Involved in Cadherin Cellular Localization—To identify the mutations in mHaCad responsible for the mislocalization of the protein, four fragment substitutions between mHaCad and HaCad genes were constructed (R1–R4) (Fig. 6A). The R4 construct, where the Met1–Pro450 fragment of mHaCad from 96CAD was replaced with the one from 96S, recovered cell surface localization, indicating that mutations in the N-terminal region are responsible for mislocalization of mHaCad (Fig. 6B). Interestingly, the R4 construct enhanced susceptibility of Hi5 cells to Cry1Ac, in contrast with mHaCad-transfected cells, indicating again that mislocalization of cadherin is involved in Cry1Ac resistance (supplemental Fig. S8). The N-terminal fragment of mHaCAD showed 12 different amino acid residues in comparison with HaCAD. We generated four additional fragment substitutions between the mHaCad and HaCad genes (R5–R8) (Fig. 7A). Analysis of these constructions showed that the D172G substitution was critical for the mislocalization of mHaCad. Constructs R7 and R8 both include the mutation D172G introduced into the wild type HaCad, and both resulted in a protein that localized into the cytoplasm and not on the cell surface (Fig. from the 96CAD strain is not affected in Cry1Ac binding by ELISA binding assays of Cry1Ac to both HaCad and mHaCad fragments (supplemental Fig. S3). These data suggest that mHaCad is affected in its localization on the plasma membrane in the Hi5 cells.

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revealed that resistance to the Cry1Ac toxin (36, 37). Also, field monitoring showed that when the substitution G172D was introduced into mutant mHaCad, the resultant protein (modified mHaCad-G172D) did not co-localize with ER in the Triton X-100-permeabilized cells (supplemental Fig. S9A) and was successfully located on the cell membrane when we analyzed the surface immunofluorescence in Hi5 cells without Triton X-100 permeabilization (supplemental Fig. S9B). In addition, Hi5 cells expressing the modified mHaCad-GFP-G172D recovered high susceptibility to Cry1Ac toxin in comparison with the cells transfected with mHaCad-GFP (supplemental Fig. S9B). In silico analysis suggested that D172G was not associated with glycosylation or phosphorylation. Additional experimental evidence is needed to determine whether this is the case.

Close examination of a structural model of H. armigera HaCad suggested that the DD172ND motif (colored in red) is located on a highly conserved loop on the surface of HaCad genes. In the case of the laboratory-selected Cry1Ac-resistant H. armigera BtR strain, we showed that resistance is linked to a polygenic resistance mechanism involving at least two major genes (28, 29). This work suggested that studies including linkage analyses were quite useful to analyze strains with multiple resistance mechanisms. In the case of the Ha BtR strain, a single mechanism of resistance does not explain all of the properties of Cry1Ac resistance of this strain. When the cadherin-resistant allele was put in a susceptible background (96CAD strain), it could account for some (516 resistance ratio in 96CAD) but not all of the resistance (3000 resistance ratio in BtR), implying the presence of an additional mechanism. Our preliminary genetic data showed that this additional resistance mechanism was not caused by mutations in other receptor genes, such as abcc, apn, or alp. A novel Cry1Ac resistance mechanism was recently reported in P. xylostella involving enhanced MAPK transcription that caused reduced transcription of ABC2C and ALP proteins (23). Whether the transcription levels of the different Cry1Ac receptors are changed in BtR remains to be determined. Regarding the mHaCad allele identified here, we showed that the mHaCad gene expression levels or the binding
of Cry1Ac to the toxin-binding fragment of mHaCad were not affected.

Our data indicate that amino acid substitution (D172G, missense mutation) correlated with the defects in cadherin trafficking from ER to the plasma membrane and may be responsible for resistance to Cry1Ac toxin in *H. armigera*. Although our data show that D172G is mainly responsible for the defect in cadherin trafficking because the reversal of a single mutation, G172D, in mHaCad restored its transport to plasma membrane and the toxicity of Cry1Ac to Hi5 cells (supplemental Fig. S9), we cannot rule out the possibility that other amino acid substitutions in mHaCad also contribute to the defect in cadherin trafficking from the ER to the plasma membrane in the larvae.

It was previously shown that ER-retained proteins, resulting from mutations, activate the stress responses that lead to their degradation by the proteasome and autophagy (38–41). The high expression of the mHaCad gene in Hi5 cells allowed us to determine that the mHaCAD protein was translated and accumulated in the ER (Fig. 6 and supplemental Fig. S7), indicating that the lack of mHaCAD in the plasma membrane is principally due to its defect in membrane sorting. Thus, we propose that the retention of mutant mHaCad in the ER may be causing its degradation. This hypothesis remains to be analyzed.

Still we have to determine how the D172G mutation affects cadherin trafficking to the plasma membrane. It is important to mention that similar mutations affecting protein trafficking due to specific single amino acid substitutions have been described before in other systems. For example, several diseases that result from mislocalization of cell surface proteins are due to substitutions of specific residues. In the case of the N-methyl-D-aspartate (NMDA) heterotetramer receptor, a single amino acid of membrane domain GluN1 regulates the surface delivery of the NMDA receptor, suggesting that unknown sorting signals of cell surface proteins might control protein trafficking (42). If ER retention proteins interact with cell surface proteins facing the interior side of the ER, the proteins may accumulate in the ER, inhibiting its trafficking toward the Golgi (43). Also, if cytoplasmic proteins interact with the cytoplasmic side of cell surface proteins, the sorting signals might be masked, and their transport to the plasma membrane might be reduced (43, 44). Because the D172G amino acid residue involved in mislocalization of the mHaCad of 96Cad is located in the luminal part of cadherin inside the ER, it is possible that some ER retention proteins might bind to this region and hinder the transportation of mHaCad toward the plasma membrane. This hypothesis also remains to be studied in the future.

The Cry1Ac resistance levels of 96Cad (516-fold) are similar to those of the *H. armigera* GYBT strain containing the HaCad<sup>11</sup> knockout allele (546-fold) (21). We still need to determine the fitness cost of this mHaCad allele mutation and compare it with the fitness cost of the HaCad<sup>11</sup> knockout allele to determine whether cadherin-resistant alleles affected in membrane localization are more likely to be selected under field conditions.

Because other Cry toxin receptors are also localized in the plasma membrane, it is possible that a similar mechanism of resistance can evolve, affecting the trafficking of other Cry receptors. Mislocalization of *B. thuringiensis* toxin receptors might also be involved in other resistance events characterized previously. For example, a cadherin deletion mutant of the intracellular domain was linked to Cry1Ac resistance in three field populations of *H. armigera* (26). Considering that the deletion resulted in loss of two putative ER-trafficking signals (D1658FD and D1668SD), it is possible that this deletion might affect cadherin surface localization. In the pink bollworm, two mutant alleles (r<sup>1</sup> and r<sup>3</sup>) of a PgCad gene are linked to resistance to Cry1Ac, and these alleles comprise deletions expected to eliminate at least eight amino acids upstream of the putative toxin-binding region of the Cad protein (45). In *Ostrinia nubilalis*, the mRNA levels of the OnCad gene and the affinity of OnCad protein to *B. thuringiensis* toxin in the resistant Europe-R strain were not affected, but it was reported that the protein levels on BBMV were severely reduced (46). It is possible that in the latter two cases, reduced cadherin receptor in BBMV could be due to an altered localization of this protein and its degradation.

Receptor trafficking to the plasma membrane is necessary for the toxicity of Cry toxins. In this work, amino acid substitutions in a cadherin receptor were shown to lead to retention of the membrane protein in the endoplasmic reticulum and shown to be associated with high resistance to Cry1Ac toxin in *H. armigera*, an important crop pest. Analysis of insect transfected cells with the cadherin gene allele showed that the mutated cadherin protein was retained in the endoplasmic reticulum and that a single amino acid substitution (D172G) was mainly responsible for this phenotype in Hi5 cells transfected with mHaCad from 96Cad. The present study proves that cadherin-based resistance mechanisms can occur even with a protein with a limited number of mutations that did not affect binding to the toxin and with normal mRNA levels. It is possible that membrane trafficking of receptors could represent a major mechanism of resistance that may affect not only cadherin but other receptor proteins as well. Thus, future resistance monitoring studies should take into account mutations affecting receptor trafficking.

**Experimental Procedures**

**Insects**—The *H. armigera* susceptible strain (96S) was maintained in the absence of toxin, and the Cry1Ac-resistant strain (BtR) was continually selected with Cry1Ac as reported previously (28, 29). Insects were reared on an artificial diet (47) in an insect chamber with a controlled environment 27 ± 1 °C, 75 ± 10% relative humidity, and a photoperiod of 14 h/10 h light/dark.

**Evaluation of Resistance Mechanism**—Linkage analysis was performed using SNP markers (using primers described in supplemental Table S1) for HaCad, HaAPN, HaABCC, and HaALP genes by direct sequencing of amplified PCR products.

**Isolation of 96Cad Nearly Isogenic Line**—Supplemental Fig. S1 and supplemental Table S2 show the strategy for back-crossing (BC) and family selection. Females from the BtR strain (rr) were crossed (mass mating, 50 pairs) with males from the 96S strain (ss) to produce F<sub>1</sub> progeny (rs). The F<sub>1</sub> males were crossed (mass mating, 50 pairs) with the 96S females in paper cups (350 ml) to produce BC1 progeny (rs + ss, 1:1). Then the BC1 males were back-crossed individually with the 96S females...
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(single pair mating). After allele-specific PCR detection of genotypes of the HaCad gene of all BC1 males, only single-pair families derived from the rs males were kept to produce BC2 progeny (rs + ss, 1:1). In the same way, single-pair families between the BC2 males with the rS allele and the 96S females were kept to produce BC3 progeny (rs + ss, 1:1). We continued the crosses in single-pair families similarly among BC3, BC4, and BC5 males with 96S females until BC6 progeny was obtained. Finally, males and females of BC6 were single pair-mated to produce BC6-F1 progeny and selected females and males with genotype rr that were single pair-mated to produce BC6-F2 progeny, which is the 96CAD line. Cry1Ac (60 µg/ml) was used to select the 96CAD strain.

Evaluation of Inheritance of Cry1Ac Resistance—Virgin males and females from 96S and 96CAD were used in reciprocal mass crosses: 50 male 96S × 50 male 96CAD and 50 female 96CAD × 50 male 96S in plastic crates (5 liters). Adults were allowed to mate, and eggs were transferred to ovoposition gauzes. Newly hatched F1 neonates were transferred to individual 24-well plates for bioassay.

Cloning and Purification of the Toxin-binding Region of HaCad—The toxin-binding region of the cadherin protein from H. armigera was described previously (33). Gene fragments of 1097 bp containing the toxin-binding region of HaCad from 96S and 96CAD strains were amplified using specific primers (supplemental Table S1) and cloned into the expression vector pET22b. E. coli BL21 DE3 cells transformed with these constructions were grown at 37 °C until A600 of 0.6, and protein expression was induced with 1 mM isopropyl-β-D-galactopyranoside for 5 h at 30 °C. Proteins in inclusion bodies were solubilized with 8 M urea, pH 8.5, and loaded into a nickel-nitriotriacetic acid-agarose column (Qiagen, Hilden, Germany), washed with PBS plus 35 mM imidazole, eluted with PBS plus 250 mM imidazole, and quantified by the Bradford method with bovine serum albumin (BSA) as a standard.

Binding Assays of Cry1Ac Toxin to Purified Cadherin Fragments Containing a Toxin-binding Region—The Cry1Ac binding to purified cadherin fragments was analyzed by ELISA binding assays. Briefly, purified cadherin fragments (1 μg/well) were immobilized on ELISA plates and incubated with Cry1Ac (0–10 nM), toxin was detected with polyclonal anti-Cry1Ac (1:15,000 dilution), followed by incubation with an anti-rabbit IgG-HRP and revealed with substrate solution (1 mg/ml o-phenylene-diamine, 0.05% H2O2, 100 mM citrate buffer, pH 5.0). The absorbance was measured at 490 nm using the Emax Precision microplate reader.

Western Blotting—BBMV of midgut tissue of fourth instar larvae were prepared as described (48) and stored at −80 °C until used. Protein concentration was quantified by the Bradford method with BSA as a standard. The total protein pool of midgut tissue was prepared by the TRIzol method using the dispersed midgut tissue from 10 larvae in the fourth instar.

BBMV from the 96CAD and 96S strains were diluted to 1 mg/ml in PBS (10 mM Na2HPO4, 2 mM KH2PO4, 2.7 mM KCl, 137 mM NaCl, pH 7.4). BBMV (10 µg) were separated on a 10% SDS-polyacrylamide gel and transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Hong Kong, China) for 65 min at 200 mA. The PVDF membrane was blocked 2 h at room temperature in PBSTM blocking buffer (PBS, 0.1% Tween 20, 5% skim milk powder), washed five times (5 min each) with PBST (PBS, 0.1% Tween 20, pH 7.5), and probed with anti-HaCad antibody (1:10,000, 2 h, at room temperature in PBSTM), followed by HRP-conjugated secondary antibody (1:20,000, 1.5 h) (ZSGB-BIO, Beijing, China), and developed using an ECL chemiluminescent detection kit (Transgen Biotech, Beijing, China). Anti-β-actin mouse monoclonal antibody (Sigma-Aldrich) was used (1:5000, 1.5 h) to detect actin as a loading control. The same method was used for detection of cadherin in the total protein sample of midgut tissue from 96S and 96CAD strains.

For detection of cadherin protein in the cell line, equal amounts of Hi5 cells transfected with control vector, HaCad-GFP, or mHaCad-GFP were cultured in 6-well cell plates. Cells were recovered and lysed with IP Pierce lysis buffer (Thermo Scientific, Waltham, MA) and subjected to Western blotting analysis as described above. Polyclonal rabbit anti-HaCad antibody was raised against a truncated HaCad fragment of 735 bp amplified with specific primers (supplemental Table S1) and expressed in E. coli DH5α.

Immunofluorescence Detection in Midgut Tissue Sections—Midgut tissue was dissected from fourth instar larvae, fixed with 4% paraformaldehyde, embedded in paraffin after dehydration, and cut in a microtome as described previously (49). The sections were mounted on silanized glass slides, deparaffinated, and rehydrated as described (49); pretreated to unmask antigen using 10 mM citrate buffer (pH 6.0) for 10 min at 98 °C; and blocked with 5% goat serum. Finally, tissue sections were incubated at 4 °C overnight with anti-HaCad antibody (1:500), followed by goat anti-rabbit fluorescence antibody coupled to TRITC. After three washes with PBS, slides were coverslipped using mounting solution, and the fluorescence was analyzed.

Real-time Quantitative Polymerase Chain Reaction—Each biological replicate consisted of a pool of 20 midguts dissected from fifth instar H. armigera larvae. All samples were quickly frozen in liquid nitrogen and stored at −80 °C or used immediately. Each experiment consisted of three biological replicates. Total RNA was extracted from sample homogenates using TRIzol reagent (Invitrogen). RNA purity was evaluated in a NanoDrop 3300 (ThermoFisher Scientific, Waltham, MA). Genomic DNA contamination was eliminated by treatment with 0.5 µl of

nylendiamine, 0.05% H2O2, 100 mM NaH2PO4, pH 5.0). The reaction was stopped by adding 50 µl of 1 M H2SO4, and the absorbance was measured at 490 nm using the Emax Precision microplate reader.
DNase in a total 10-μl volume (Fermentas, Waltham, MA). Total RNA (4 μg) was used for reverse transcription using the SuperScript III first strand synthesis kit for qRT-PCR (Invitrogen) in a final volume of 20 μl. The cDNA was diluted in nuclease-free water for immediate qRT-PCR analysis or stored at −20 °C. All qRT-PCR (TaqMan, Waltham, MA) reactions were done in a 20-μl final volume containing 2× Maxima probe/ROX qRT-PCR Master Mix (10 μl), 10 μmol of forward and reverse primers (0.6 μl each; supplemental Table S1), 10 μmol of probe (0.4 μl), template cDNA (2 μl), and nuclease-free water (6.4 μl). Reactions were performed in 96-well plates using the Applied Biosystems 7500 FAST qRT-PCR system (ABI7500 Fast) under the following conditions: 50 °C for 2 min, 95 °C for 10 min, and 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The H. armigera β-actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) genes were used as dual internal references (GenBank™ EU527017 for β-actin and JF417983 for GAPDH). Each reaction was run in triplicate, and a no-template control was included. The mean of the dual reference Ct value was used in normalization according to the 2−ΔΔCt method.

Construction of Fusion Proteins—The HaCad genes from 96S and 96CAD strains were amplified by RT-PCR using the total RNA extracted from the midgut tissue of the larvae and cloned into the plasmid pie2-EGFP-N1 as described previously (50) for expression of GFP fusion proteins (HaCad-GFP and mHaCad-GFP). Primers used are listed in supplemental Table S1. pDsRed2-ER (BD Biosciences) is a mammalian expression vector encoding a fusion protein consisting of Discosoma sp. GFP-red fluorescent (DsRed2); the ER targeting sequence of calreticulin fused to the 5’ end of DsRed2; and the ER retention sequence, KDEL, fused to the 3’ end of DsRed2. This fused gene was amplified (supplemental Table S1) and cloned into pie2-EGFP-N1 (HindIII and EcoRI sites) for its expression in insect cells.

Transfection of Hi5 Cells and Cry1Ac Toxicity—The T. ni BTI-Tn-5B1–4 (Hi5) cell line was kindly supplied by Dr. Peter Tijssen (University of Quebec). Cells were grown in Grace’s insect cell culture medium, supplemented with 10% fetal bovine serum, 100 μg/ml penicillin, and 100 μg/ml streptomycin (Life Technologies, Inc.) at 28 °C. For transfection, Hi5 cells were grown overnight in 96-well cell culture plates. The constructed plasmids were transfected into the cells at 0.5 μg/well using Cellfectin II (Life Technologies). After 24 h of transfection, cells were fixed with 4% paraformaldehyde (10 min), stained for 15 min with Hoechst 3342 (Sigma-Aldrich), and observed under the fluorescence microscope. The fluorescence of cells transfected with ER marker (DsRed2-ER) was used to recognize ER. Blue fluorescence represents nuclei stained by Hoechst 3342 (1 μg/ml; Sigma-Aldrich).

Construction of Mosaic Cadherin Gene by Substitution of Fragments between HaCad and mHaCad Genes—Mosaic cadherin proteins R1–R3 were constructed by amplifying PCR fragments from HaCad using primers Pst-FP, Hind-FP, and Sac-FP with HaCad-RP as reverse primer (supplemental Table S1) containing appropriate restriction sites and substituting these fragments in the plasmid pmHaCad-GFP previously digested with the corresponding restriction enzymes (see the legend to Fig. 6A for the description of restriction sites used in each construction). R4 was amplified using primers Sac-FP and HaCad-RP with mHaCad as a template and substituted into the pHaCad-GFP plasmid previously digested with the corresponding restriction enzymes.

For construction of R6–R8 (Fig. 7A), the Kpn-FP forward primer was used with the reverse primers 473-RP, 564-RP, and 960-RP to amplify different fragments from mHaCad in a first PCR. For the second PCR, the forward primers 473-FP, 564-FP, and 960-FP were used with Hind-RP reverse primer (supplemental Table S1) to amplify fragments from HaCad. A final PCR with these two overlapped PCR products as templates was performed in the presence of primers Kpn-FP and Hind-RP. The resulting PCR products were finally substituted into pHaCad-GFP plasmid previously digested with KpnI and HindIII. The construction R5 was amplified using primers Kpn-FP and Sac-RP with HaCad as a template and substituted into
pmHaCad-GFP plasmid previously digested with the corresponding restriction enzymes.

For construction of modified mHaCad-G172D, two PCR were done. The first reaction was performed using primers Kpn-FP and 516-RP with HaCad as a template, and the second was carried out with 516-FP and Hind-RP, using mHaCad as a template. Then an additional PCR was performed with the two overlapping PCR products as a template in the presence of primers Kpn-FP and Hind-RP. Finally, the resulting PCR product was substituted into the plasmid pmHaCad-GFP previously digested with KpnI and HindIII.

Homology Modeling of Cadherin—Homology models of the N-terminal region of wild-type and mutant H. armigera cadherin proteins were constructed by using the SWISS-MODEL server. The primary amino acid sequences of HaCad and mHaCad were used as targets, and a template search with Blast and HHBlits was performed against the SWISS-MODEL template library. The mouse N-cadherin ectodomain (Protein Data Bank entry 3Q2W) was used as a template. Models were built based on the target-template alignment using Promod-II, and coordinates that were conserved between target and template were contained in the final model. Molecular graphics were performed with PyMOL version 1.6.

Statistical Analysis—SPSS version 20.0 (SPSS Inc., Chicago, IL) was used to estimate the concentration of Cry1Ac toxin necessary to kill 50% of larvae (LC50) and its 95% fiducial limits. The dominance parameter h from the LC50 values was calculated as described previously (24, 51). For the genetic linkage analysis, we used χ2 tests to determine whether the observed genotype frequencies differed significantly from the expected genotype frequencies on the untreated diet and on the diet treated with Bt toxins. The percentage of swollen cells after Cry1Ac treatment of transfected cells was calculated from the data of three independent experiments.

Author Contributions—K. W., K. L., and Y. X. designed the study. Y. X., Q. D., R. H., S. P., and Y. Y. performed the experiments. K. W., K. L., Y. X., G. L., M. S., and A. B. analyzed data. K. W., K. L., Y. X., M. S., and A. B. wrote the paper. All authors discussed the results and provided comments on the paper.

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