

Three cadherin alleles associated with resistance to *Bacillus thuringiensis* in pink bollworm

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Evolution of resistance by pests is the main threat to long-term insect control by transgenic crops that produce *Bacillus thuringiensis* (Bt) toxins. Because inheritance of resistance to the Bt toxins in transgenic crops is typically recessive, DNA-based screening for resistance alleles in heterozygotes is potentially much more efficient than detection of resistant homozygotes with bioassays. Such screening, however, requires knowledge of the resistance alleles in field populations of pests that are associated with survival on Bt crops. Here we report that field populations of pink bollworm (*Pectinophora gossypiella*), a major cotton pest, harbored three mutant alleles of a cadherin-encoding gene linked with resistance to Bt toxin Cry1Ac and survival on transgenic Bt cotton. Each of the three resistance alleles has a deletion expected to eliminate at least eight amino acids upstream of the putative toxin-binding region of the cadherin protein. Larvae with two resistance alleles in any combination were resistant, whereas those with one or none were susceptible to Cry1Ac. Together with previous evidence, the results reported here identify the cadherin gene as a leading target for DNA-based screening of resistance to Bt crops in lepidopteran pests.

Because of their low toxicity to vertebrates and most other nontarget organisms, insecticidal crystal proteins from *Bacillus thuringiensis* (Bt) are environmentally friendly alternatives to conventional insecticides (1). Bt toxins kill insects by binding to specific target sites and disrupting midgut membranes (1). Transgenic crops producing Bt toxins that kill lepidopteran larvae are grown on millions of hectares, but evolution of resistance by pests could cut short their success (2–5). So far, no field outbreaks of resistance have occurred in response to Bt crops (5, 6). However, field populations of the diamondback moth (*Plutella xylostella*) have evolved resistance to Bt sprays and laboratory strains of many pests have been selected for resistance to Bt toxins (2, 3).

Concerns about resistance to Bt crops led the U.S. Environmental Protection Agency to mandate the “refuge strategy” for delaying pest resistance (7). Thus, farmers growing cotton that produces Bt toxin Cry1Ac must plant refuges of non-Bt cotton to enable survival of susceptible pests. The refuge strategy is expected to be most effective when resistance to Bt cotton is inherited as a recessive trait. Ideally, rare homozygous resistant pests (with two resistance alleles) emerging from Bt cotton mate with relatively abundant homozygous susceptible pests (with no resistance alleles) from refuges. If resistance is recessive, Bt cotton kills heterozygous progeny (with only one resistance allele) produced by such matings. Models predict that under these conditions, refuges can delay resistance substantially (4, 8).

Tests of the refuge strategy have been problematic because of the difficulty of monitoring resistance in the field, especially when resistance is recessive and rare. Bioassays have been used to estimate resistance allele frequencies, but their failure to distinguish between heterozygotes and susceptible homozygotes usually necessitates huge sample sizes or multigenerational experiments to detect rare resistant homozygotes (9–11). DNA-based detection of resistance alleles in heterozygotes could increase efficiency >1,000-fold compared with conventional bioassays (11), yet such screening

requires knowledge of the molecular genetic basis of resistance to Bt toxins in field populations.

The most common type of lepidopteran resistance to Bt toxins (called “mode 1”) entails a high level of resistance to at least one Cry1A toxin, recessive inheritance, reduced binding of at least one Cry1A toxin, and little or no cross-resistance to Cry1C (12). Mode 1 resistance occurs in some strains of diamondback moth, Indian-meal moth (*Plodia interpunctella*), tobacco budworm (*Heliothis virescens*), and pink bollworm (*Pectinophora gossypiella*) (refs. 12 and 13, and J. González-Cabrera, B. Escriche, B.E.T., and J. Ferré unpublished data). The simplest explanation for mode 1 resistance is that modifications of target sites reduce or eliminate binding of Cry1A toxins in homozygous resistant individuals, but have little effect on susceptibility of heterozygous individuals to Cry1A toxins. Such modifications would also have little effect on susceptibility of resistant homozygotes to toxins such as Cry1C that attack target sites independent from Cry1A receptors (2, 3). The two major candidates for targets of Cry1A toxins are aminopeptidases and cadherins, both of which bind Cry1A toxins in Lepidoptera (14–18).

Genetic mapping experiments with the laboratory-selected YHD2 strain of *H. virescens*, a major cotton pest, showed tight linkage between resistance to Cry1Ac and a cadherin-encoding gene (called *BtR-4* or *HevCaLP*), but not to genes encoding aminopeptidases (19). Insertion of a retrotransposon disrupts *BtR-4* in the YHD2 strain, which has >10,000-fold resistance to Cry1Ac (19). Complementation tests using crosses between field-collected males and YHD2 females suggest that *BtR-4* resistance alleles occur in field populations (9, 19), but the resistance allele in the YHD2 strain has not yet been detected in the field or associated with survival on Bt cotton.

If mutations in cadherin genes are the primary basis of mode 1 resistance in the field, focusing on these genes could accelerate progress in resistance monitoring and management. Here we tested the hypothesis that mode 1 resistance to Bt toxin Cry1Ac is linked to the cadherin gene in pink bollworm, a worldwide cotton pest. We report that field-derived strains of pink bollworm harbored three cadherin alleles associated with resistance to Cry1Ac and survival on Bt cotton.

Materials and Methods

Insect Strains. We used six strains of pink bollworm: a susceptible strain (APHIS-S), four laboratory-selected Bt-resistant strains (AZP-R, SAF97-R, MOV97-R10, and TX01-R), and an unselected heterogeneous strain (TX01). Bioassays indicated that major alleles for Cry1Ac resistance were rare or absent in APHIS-S, which had been reared in the laboratory for >20 years without exposure to insecticides (20). AZP-R was started by pooling survivors of exposure to various concentrations of Cry1Ac in diet from 10

Abbreviation: Bt, *Bacillus thuringiensis*.

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strains derived in 1997 from Arizona cotton fields (20, 21). AZP-R was further selected by using 10 or 100 μg of Cry1Ac per ml diet, leading to 3,100-fold resistance to Cry1Ac (13). Two of the strains contributing individuals to AZP-R were SAF97 and MOV97, which were started in 1997 from individuals collected in Safford and Mohave Valley, respectively (20). The TX01 strain was derived from 24 individuals collected from Tornillo, Texas, in 2001. SAF97, MOV97, and TX01 were reared without exposure to Cry1Ac. These three strains had a high proportion of susceptible individuals, but also had some resistant individuals (ref. 20 and results reported here). A subset of each of these three heterogeneous strains was selected with 10 μg Cry1Ac per ml diet to produce the resistant strains SAF97-R, MOV97-R10 (22, 23), and TX01-R, respectively. All strains were reared in the laboratory on wheat germ diet (21).

RNA and Reverse Transcription-PCR of the Pink Bollworm Cadherin Gene. PCR with partially degenerate primers based on *H. virescens* *BtR-4* (19) amplified a product from APHIS-S genomic DNA containing two exons and an intron in the predicted positions. The exon sequence was identical to region 4,933–4,986 of the pink bollworm cadherin cDNA sequence (BT-R₂) from an unnamed susceptible strain (18). Based on the observed identity, we used the BT-R₂ sequence to design specific primers for isolating the coding sequence of the cadherin gene from our susceptible and resistant strains of pink bollworm. Specific primers divided the gene into three overlapping fragments: –106 (in the 5' untranslated region) to 1441, 1381–3645, and 3221 to the poly(A) tail (see Table 2, which is published as supporting information on the PNAS web site, www.pnas.org).

Total RNA was extracted with TRIzol reagent (GIBCO/BRL) according to the manufacturer's instructions and reverse transcribed with SuperScript II RNase H⁻ reverse transcriptase (Invitrogen). The cDNA fragments served as templates for subsequent PCR amplification with denaturation at 94°C for 3 min, followed by 35 cycles at 94°C for 1 min, at 55°C for 1 min, and at 72°C for 2 min. PCR products of the expected sizes were excised and purified by using the QIAquick gel extraction kit (Qiagen, Valencia, CA) and cloned by using the pGEM-T easy vector system (Promega). At least three clones for each fragment were fully sequenced in both strands. DNA sequence files were visualized by using CHROMAS version 1.45 (Technelysium Pty, Helensvale, Australia) and analyzed by using DNAMAN (Lynnon BioSoft, Montreal) software.

DNA Purification. Individual larvae or pupae were put on parafilm stretched over a hard surface. After grinding in 50 μl of cold lysis buffer (5 mM Tris-HCl, pH 8.0, containing 0.5 mM EDTA, 0.5% Nonidet P-40, and 1 mg/ml proteinase K), the extracts were incubated at 65°C for 30 min, and at 95°C for 10 min before PCR amplification (as described above).

Diet Bioassays. We used two types of diet bioassays: survival and growth bioassays. In both types, neonates were tested individually in the dark at 29°C ($\pm 2^\circ\text{C}$) and the source of Cry1Ac was MVP II (Dow Agrosiences), which is identical to holotype Cry1Ac in the active region of the toxin (23). In survival bioassays, larvae were fed diet with 10 μg of Cry1Ac per ml of diet for 21 days (20). Adjusted survival was calculated as survival of larvae on treated diet divided by survival of larvae on untreated diet $\times 100\%$. In growth bioassays, larvae were weighed after eating diet with 1 μg of Cry1Ac per ml for 11 days (13). The high concentration and long duration of the survival bioassay diagnose a high level of resistance (13, 20, 24). In the growth bioassay, resistant larvae grow faster than others, but the lower concentration and shorter duration enable recovery of most susceptible as well as resistant individuals, thus providing larger sample sizes and more powerful tests for analyzing inheritance (13).

For linkage analysis (see below), at the end of both bioassays,

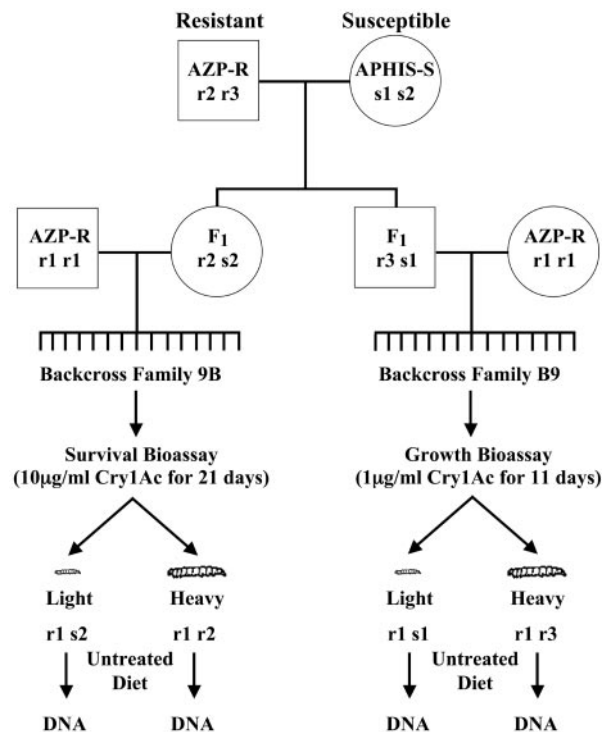


Fig. 1. Linkage analysis. DNA from two backcross families (9B and B9) was analyzed to test for linkage between the cadherin gene and resistance to Cry1Ac. A resistant male from AZP-R was crossed with a susceptible female from APHIS-S to create F₁ family B. A female from F₁ family B was backcrossed to a male from AZP-R family 9 to create backcross family 9B. One of her brothers was backcrossed to a female from AZP-R family 9 to create backcross family B9. After exposure to Cry1Ac in bioassays, survivors were weighed, reared to pupation on untreated diet, and frozen for DNA analysis. DNA screening of 62 backcross progeny (22 from family 9B and 40 from family B9) showed that all resistant (heavy) individuals were *rr*, whereas all susceptible (light) individuals were *rs*.

survivors were weighed and reared to pupation on untreated diet. Survivors (backcross progeny), their parents (F₁ and AZP-R), and grandparents (APHIS-S and AZP-R) were frozen individually in 95% ethanol at –70°C for DNA testing.

Linkage Analysis. To generate informative families of pink bollworm for biphasic linkage analysis (25), we conducted single-pair crosses (13). We crossed the susceptible APHIS-S strain with the resistant AZP-R strain to produce F₁ progeny and separately backcrossed male and female F₁ progeny to AZP-R (Fig. 1). Resistance of AZP-R to Cry1Ac is functionally recessive and controlled primarily by a major locus (13). Previous analysis of 696 larvae from 12 backcross families tested with the growth bioassay revealed a bimodal weight distribution consistent with the expected 1:1 ratio of resistant (*rr*) to susceptible (*rs*) larvae (13).

We screened the DNA of parents of the 12 backcross families to find families in which markers at the cadherin locus would enable a test for linkage. The linkage analysis reported here focuses on two such backcross families (9B and B9, Fig. 1). An AZP-R male and an APHIS-S female were crossed to produce F₁ family B. From F₁ family B, a female was backcrossed to an AZP-R male from family 9 to create family 9B. One of her brothers was backcrossed to an AZP-R female from family 9 to create family B9. For backcross family 9B, we screened DNA of survivors from the survival bioassay. Because crossing over occurs only in male Lepidoptera and the father of family 9B was resistant (*rr*), examining this family tested the hypothesis that the cadherin locus and genetic control of resistance to Cry1Ac are on the same chromosome. Backcross family B9, in which the

father was an F_1 (rs), was tested with the growth bioassay to determine how tightly resistance is linked to the cadherin locus. The larger sample size afforded by the growth bioassay was essential for detecting potential crossing over between the cadherin gene and resistance loci in the F_1 father.

Larvae weighing >10 mg in the survival bioassay or >20 mg in the growth bioassay were designated as heavy (putative rr), the others were designated as light (putative rs). To determine whether resistance was genetically linked with the cadherin locus, we tested the association between phenotype (heavy vs. light) and cadherin genotype.

Greenhouse Bioassays. We used greenhouse bioassays (20, 23, 26, 27) to assess the association between cadherin genotype and survival on cotton plants. Paper towels bearing eggs laid by AZP-R females in the laboratory were divided into pieces (1 cm²), each with 40 eggs. Each piece was randomly assigned to one of three treatments: control, Bt cotton, or non-Bt cotton. To estimate cadherin genotype frequencies in AZP-R before the greenhouse bioassays, control eggs were allowed to hatch and neonates were frozen for DNA analysis. To infest plants, we put the remaining eggs under the bracts of selected bolls. On May 14, 2002, we infested 100 bolls on 20 Bt cotton plants (Deltapine 33B) and 50 bolls on 10 non-Bt cotton plants (Deltapine 5415). Infested bolls were enclosed in screened cages to capture survivors that emerged from bolls. After 3 weeks, cages were checked every 2 days for individuals that emerged from bolls, which were allowed to pupate and were frozen for DNA analysis. After 7 weeks, bolls were checked for larvae and pupae. Individuals that reached the fourth instar by 7 weeks were scored as survivors. Survival percentage was estimated as the number of survivors divided by the number of entrance holes \times 100%. For individuals tested on plants, DNA was analyzed only from those that reached the pupal stage.

Results

Cadherin Alleles from Susceptible and Resistant Pink Bollworm. Cadherin cDNA isolated from the susceptible APHIS-S strain had 5,208 bp encoding a predicted protein of 1,735 aa (Fig. 2), which we call BtR (for Bt resistance, GenBank accession no. AY198374). Similar to other lepidopteran cadherins (17–19, 28), the proposed structure of BtR includes a putative membrane signal sequence, 11 extracellular cadherin repeats, a membrane-proximal extracellular domain, a transmembrane domain, and a cytoplasmic domain (Fig. 2). Amino acid identity of BtR to other lepidopteran cadherins is 60% for *Bombyx mori* (BtR175, GenBank accession no. BAA77212), 58% for *Manduca sexta* (BT-R₁, GenBank accession no. AF319973), and 55% for *H. virescens* (HvCaLP, GenBank accession no. AF367362).

Compared with BT-R₂, the cadherin previously reported for susceptible pink bollworm (18), the cDNA sequence of BtR from APHIS-S is 99% identical from nucleotides 1 to 5031, with no deletions or insertions, 31 nucleotide substitutions, and 12 amino acid substitutions. However, from nucleotide 5032 to the end of the coding sequence, amino acid identity is only 8%. In this region, amino acid identity with cadherins from *M. sexta*, *B. mori*, and *H. virescens* is 32–43% for BtR, but only 4–7% for BT-R₂, which might reflect a sequencing error in BT-R₂.

We identified three alleles ($r1$, $r2$, and $r3$) of the *BtR* gene in the resistant AZP-R strain, each with a unique major deletion (Fig. 2). The $r1$ allele lacks 24 bp (nucleotides 3386–3407 and 3413–3414) resulting in two amino acid substitutions (G1136R and T1137R) and omission of eight amino acids (1129–1135 and 1138). The $r2$ allele has a 202-bp deletion (2415–2616) creating a frame shift that introduces a premature stop codon after amino acid 805. The $r3$ allele has a 126-bp deletion (3302–3427) that eliminates 42 amino acids (1103–1144). No other insertions, deletions, or premature stop codons occurred in these three r

alleles. Moreover, we did not find any amino acid substitution that occurred in all three r alleles.

All individuals tested from AZP-R ($n > 150$) had two copies of r alleles ($r1r1$, $r1r2$, $r1r3$, $r2r2$, $r2r3$, or $r3r3$). Two other resistant Arizona strains tested had high frequencies of r alleles, with $r1$ and $r2$ in SAF97-R and $r1$ and $r3$ in MOV97-R10. In contrast, none of the three r alleles occurred in 50 individuals from APHIS-S.

Linkage Analysis. We tested for genetic linkage between *BtR* and resistance to Cry1Ac by using backcross ($F_1 \times$ AZP-R) families (13). The intron adjacent to the $r1$ deletion (between nucleotides encoding amino acids Leu-1143 and Glu-1144) served as a genetic marker. In the first 540 bp of this “marker” intron, each r allele in AZP-R has a unique pattern of 24 polymorphic sites ($mr1$, $mr2$, and $mr3$; Table 1), which facilitated efficient genotyping by direct sequencing of PCR products.

Screening DNA from the parents of 12 backcross families identified two informative families (9B and B9) in which various r and s alleles segregated (Fig. 1). The AZP-R grandfather was $r2r3$ and the APHIS-S grandmother had two alleles, designated $s1$ and $s2$, that differ from each other and from $r2$ and $r3$ in marker intron sequence ($ms1$ and $ms2$, Table 1). Alleles $r1$ and $s2$, which have the same marker intron sequence (Table 1), were distinguished by the 24-bp deletion in the coding region that occurred in $r1$ but not in $s2$. For family 9B, the AZP-R father was $r1r1$ and the F_1 mother was $r2s2$. For family B9, the AZP-R mother was $r1r1$ and the F_1 father was $r3s1$. As expected, the weight distribution of larvae fed diet with Cry1Ac was bimodal for both of these backcross families (Fig. 3).

To test for linkage, we determined the *BtR* genotype for 11 heavy (putative rr) and 11 light (putative rs) individuals from family 9B as well as 20 heavy and 20 light individuals from family B9. In family 9B, all 11 heavy progeny were $r1r2$ whereas all 11 light progeny were $r1s2$ ($\chi^2 = 22$, $df = 1$, $P < 0.0001$). Analogously, in family B9, all heavy progeny were $r1r3$ and all light progeny were $r1s1$ ($\chi^2 = 40$, $df = 1$, $P < 0.0001$). Because crossing over occurs only in males in Lepidoptera, the results from family 9B (in which the father was $r1r1$) indicate linkage between resistance and the chromosome carrying *BtR*. The results from family B9 (in which the father was $r3s1$) demonstrate tight linkage (no observed recombinants) between resistance and *BtR*.

Association Between Cadherin Alleles and Resistance in a Heterogeneous Strain from Texas. We used survival and growth bioassays and a selection experiment to determine whether $r1$, $r2$, or $r3$ were associated with Cry1Ac resistance in a heterogeneous strain (TX01) derived in 2001 from a cotton field in Texas. This heterogeneous strain had been reared in the laboratory for nine generations without exposure to Cry1Ac.

In survival bioassays (21 days on diet with 10 μ g Cry1Ac per ml diet), adjusted survival of TX01 was 14% ($n = 650$). We determined the *BtR* genotype for nine survivors from this diagnostic test. Five were $r3r3$ and four were $r1r3$. In addition, we tested neonates with growth bioassays (11 days on diet with 1 μ g Cry1Ac per ml), weighed them, and determined the *BtR* genotype of six heavy larvae (11–50 mg, mean = 29.5 mg) and 20 light larvae (<6 mg, mean = 1.1 mg). Of the six heavy larvae, four were $r1r3$ and two were $r3r3$. Of the 20 light larvae, 12 had one r allele (4 had $r1$, 8 had $r3$) and 8 had none.

We selected the TX-01 strain for resistance by feeding 540 neonates of the F_{10} generation on diet with 10 μ g Cry1Ac per ml for 21 days. Survivors of this single generation of selection were pooled and allowed to mate to begin the TX01-R strain. All 20 larvae tested from the first generation progeny of these survivors were rr : nine were $r1r1$, ten were $r1r3$, and one was $r3r3$. Consistent with recessive inheritance of resistance to Cry1Ac in pink bollworm (13, 20, 26, 24), these results show that in TX01

	← SIG	
BtR	MAGDACILVTVLLTFAATSVFGQETTSSRCYYMTDAIPREPDKPDDLPDLEWTTGGWTDWPLIPAEPRDDVCINGWYPLTSTSLGTIIHMEEEIEGDVAIA	100
r1	-----	100
r2	-----I--I-----A-----	100
r3	-----	100
BtR	KLNYDGSSTPEIQPMVIGSFNLLSPEIRNENGAWLYITNRQDYETPTMRRYTFDVRVPEDETRAARVSLSIENIDNDPIVRVLDACQVPELGEPRLTD	200
r1	-----	200
r2	-----	200
r3	-----	200
	→ CR1	
BtR	CVYQVSDDEDGRLSIEPMTFRLLTSDREDVQIFYVEPAHITGDWFNMQITIGILSALNFESNPLHIFQITALDSWPNNHTVTVMVQVQNVHRPFRMEIFA	300
r1	-----	300
r2	-----	300
r3	-----	300
	→ CR2	
BtR	VQQFDEMTEQQFQVRAIDGDTGIGKAIHYTLETDEEEDLFFIETLPGGHGDAIFSTAMIDVDRLRDVFRLSLVAYKYDNVSFATPTPVVIVNDINNKK	400
r1	-----	400
r2	-----Q-----	400
r3	-----	400
	→ CR3	
BtR	POPLQDEYTIISIMEETPLSLNFAELFGFYDEDLIYAQFLVEIQGENPPGVEQAFYIAPTAGFQNTFAIGTQDHRMLDYEDVPFQNIKLVKIATDRDNTN	500
r1	-----	500
r2	-----	500
r3	-----	500
	→ CR4	
BtR	FTGVAEVDNVLINWDEEPIFEEDQLVVKFKETVPKDYHVGRRAHADRDI GDSVVHSLGNANTFLRIDEETGDIYVAIDDAFDYHRQNEFNIVRAQDT	600
r1	-----	600
r2	-----	600
r3	-----	600
	→ CR5	
BtR	MSEPESRHTATAQLVIELEDVNTTPTLRLPRVSPSVEENVPEGFEINREITATDPDTTAYLQFEIDWDTSFATKQGRDTPNPIEFHGCVDIETIFPNPAD	700
r1	-----V-----	700
r2	-----	700
r3	-----	700
	→ CR6	
BtR	TREAVGRVVAKEIRHNVTIDFEEFEFLYLVTRVRLDHTDGRDYDESTFTIIIDMNDNWPWASGLFNQTF SIRERSSTGVVIGSVLATDIDGPLYNQV	800
r1	-----	800
r2	-----	800
r3	-----	800
BtR	RYTII PQEDTPEGLVQIHVFTGQITVDENGAIDADIPRWHLNYTVIASDKCSEENEENCPPDPVFWDTLGDNVINIVDINNKPAAADLSRFNETVYIYE	900
r1	-----R-----	900
r2	-----M*-----	806
r3	-----	900
	→ CR7	
BtR	NAPDFTNVVKIYSIDEDRDEIYHTVRYQINYAVNQRLRDFFAIDLDSGQVYVENTNNE LLDRDRGEDQHRIFINLIDNFYSEGDNRRNVNTTEVLVILLD	1000
r1	-----	1000
r3	-----	1000
	→ CR8	
BtR	ENDNAPELPTPEELSSISENLQEGITLDGESDVIYAPDIDEEDTPNSHVGYAILAMTVNTRDLDTVPRLLNMLSNNVTGFLQTAMPLRGYGTYDISI	1100
r1	-----V-----	1100
r3	-----V-----	1100
	→ CR9	
BtR	LAFDHGIPQQISHEVYELEIRPYNYNPPQVFPESGTLRLALERAVNNVLSLVNGDPLDRIQAI DDDGLDAGVTFDVI VGDADASNYFRVNDGGDNFG	1200
r1	-----RR-----L-----	1192
r3	-----	1158
	↑ “marker” intron → CR10	
BtR	TLLLTQALPEEGKEFEVTVIRATDGGTEPRSYSTDSTITVLFVPTLGDPFIQDNTYSVAFFEKEVGLTERFSLPHAEDEPKNKLCCTDDCHDIYYRIFGGVDY	1300
r1	-----	1292
r3	-----	1258
	→ CR11	
BtR	<u>EPFDLDPVTVNIFLKSELDRETTATHVVQVAASNSPTGGGIPLPGSLLTVTVTREADPRPVFEQR</u> LYTAGISTSDNINRELLTVRATHSENAQLTYTIE	1400
r1	-----	1392
r3	-----	1358
	→ MPR	
BtR	DGSMVDSTLEAVKDSAPHLNAQTGVLILRIQPTASMQGMFEFNVVIATDPDEKTDTAEVKVVYLISQNRVSVFI FLNDVETVESNRDFIAETFVGFNMTC	1500
r1	-----V-----	1492
r3	-----	1458
	→ TM	
BtR	NIDQVLPGTNDAGVIEAMAEVHAHFIQDNPVSADEELRSDTQLRSVQGVNQRLLVNLNLDVTVGSPDLGTAGVQIT IYVLAGLSAAILAFCLILL	1600
r1	-----	1592
r3	-----H-----	1558
	→ CYT	
BtR	ITFIVRTRALNRRLEALSMTKYGSVDSGLNRVGI AAPGTNKHAE GSNPIWNEQIKAPDFDAISDTSDES DLI GIEDLPQFKSDYFPPEDES SAHA AFS D	1700
r1	-----	1692
r3	-----R-----	1658
BtR	RTPRGNDAPIAHSSNNFGFNTSPFSAEFTNRRMRP	1735
r1	-----	1727
r3	-----	1693

Fig. 2. Amino acid sequence of BtR (from the susceptible APHS-S strain) and resistance alleles *r1*, *r2*, and *r3* deduced from cloning and sequencing cDNA. Identical residues are designated by dashes. Deleted amino acids are indicated by dots and gray background. An asterisk shows the premature stop codon in *r2*. Protein sequence analysis was done by using the Institut Suisse de Recherches Experimentales sur le Cancer (Lausanne, Switzerland) ProfileScan server (<http://hits.isb-sib.ch/cgi-bin/PFSCAN>) and the Simple Modular Architecture Research Tool (<http://smart.ox.ac.uk>). Horizontal arrows specify start sites of putative domains (33). SIG, signal peptide; CR, cadherin repeat; MPR, membrane-proximal region; TM, transmembrane domain; CYT, cytoplasmic domain. The vertical arrow indicates the position of the “marker” intron. Bold, underlined amino acids show the putative binding region (18).

Table 1. Polymorphic nucleotides in the “marker” intron used to identify cadherin alleles

Marker	31	92	143	183	196	200	221–224	284	380	381	383	408	419	429	434	440	447	461–463	488	513	520	530	538	540
mr1	A	A	C	G	T	A	. . .	G	C	A	T	C	T	A	T	C	A	. . .	G	G	C	C	T	G
mr2	C	G	C	G	G	A	. . .	G	A	G	T	C	C	C	C	T	T	AAC	A	A	T	T	G	C
mr3	C	G	A	A	G	G	. . .	A	C	G	G	T	C	C	C	C	T	AAC	A	A	C	C	T	G
ms1	C	G	C	A	G	G	TAAG	G	C	G	T	C	C	C	C	T	AAC	A	A	C	C	T	G	
ms2	A	A	C	G	T	A	. . .	G	C	A	T	C	T	A	T	C	A	. . .	G	G	C	C	T	G

This intron occurs between nucleotides encoding amino acids 1143 and 1144 (Fig. 2). The first 540 bp of the intron were amplified. PCR products were recovered from agarose gels using the QIAquick gel extraction kit (Qiagen) and sequenced. Diagnostic nucleotides are shown in boldface. Dots indicate deletions.

and TX01-R, larvae with two copies of *r* alleles were resistant, whereas those with one *r* allele or none were susceptible.

Greenhouse Bioassays. In greenhouse bioassays with the AZP-R strain, all six resistant genotypes (*r1r1*, *r1r2*, *r1r3*, *r2r2*, *r2r3*, and *r3r3*) were represented among survivors of Bt cotton (Fig. 4). The frequency of the three *r* alleles and the six *rr* genotypes did not vary significantly among a control group of neonates, survivors on Bt cotton, and survivors on non-Bt cotton (Fig. 4). Thus, we did not detect variation among alleles or among *rr* genotypes in survival on Bt cotton or on non-Bt cotton. Further, on either Bt cotton or non-Bt cotton, pupal weight and time to pupation did not vary significantly among the six *rr* genotypes (one-way ANOVA, *df* = 5, 34, *P* > 0.27 in each case). These results show that some individuals with any of the six *rr* genotypes can survive on Bt cotton and on non-Bt cotton, yet they do not exclude minor or moderate differences in performance among genotypes.

Survival was 4.7% (92 of 1,941) on Bt cotton and 10.9% (108 of 907) on non-Bt cotton, which yields an adjusted survival on Bt

cotton of 43.3% (4.7 divided by 10.9), similar to previous results (refs. 20, 26, and 27, but also see ref. 23).

Discussion

Several lines of evidence support the hypothesis that the *r1*, *r2*, and *r3* alleles of the pink bollworm cadherin gene *BtR* confer recessively inherited resistance to Bt toxin Cry1Ac. First, screening of four strains from Arizona showed that these alleles occurred at high frequency in three resistant strains but not in a susceptible strain. Of >150 individuals tested from the AZP-R strain, which has 3,100-fold resistance to Cry1Ac, all were *rr*

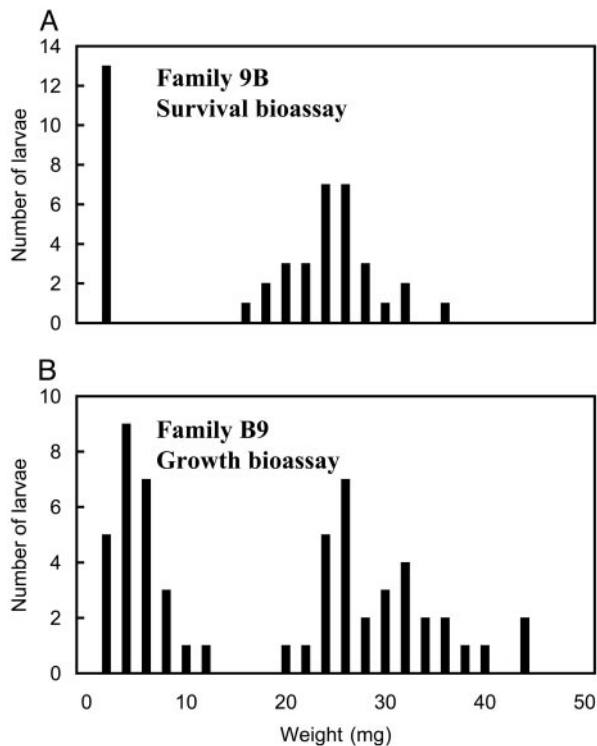


Fig. 3. Weight distribution of backcross larvae tested in bioassays. (A) Backcross family 9B was tested with the survival bioassay (10 μg of Cry1Ac per ml of diet for 21 days). Of 58 larvae tested, 30 were heavy (>10 mg), 13 were light, and 15 died. (B) Family B9 was tested with the growth bioassay (1 μg of Cry1Ac per ml of diet for 11 days). Of 60 larvae tested, 30 were heavy (>20 mg), 27 were light, and 3 died.

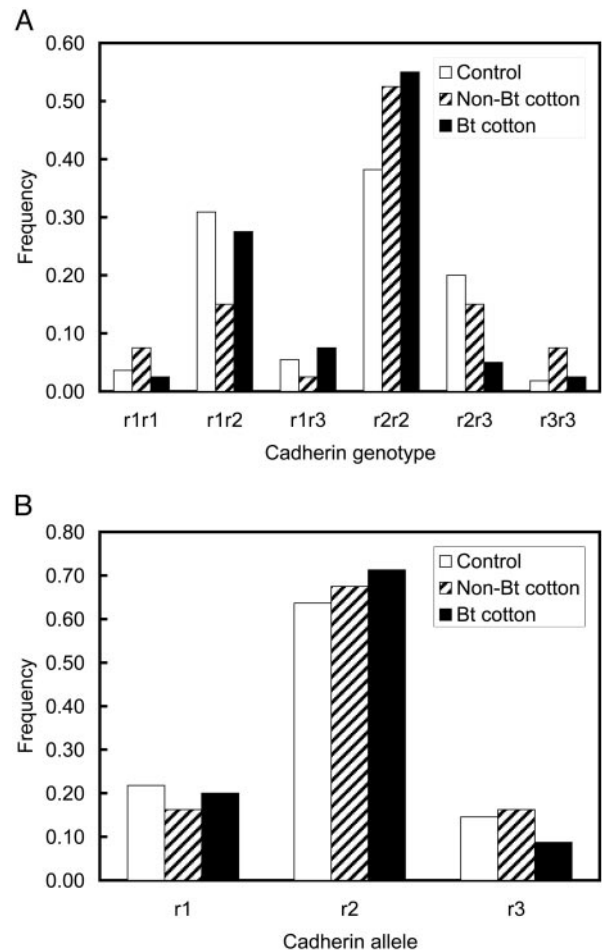


Fig. 4. Greenhouse bioassays with the resistant AZP-R strain of pink bollworm. (A) Cadherin genotype frequencies of larvae (number of individuals = 55 for control, 40 for survivors on non-Bt cotton, and 40 for survivors on Bt cotton). Control genotype frequencies did not deviate significantly from Hardy–Weinberg equilibrium ($\chi^2 = 0.58$, *df* = 5, *P* = 0.99). (B) Cadherin allele frequencies of larvae (number of alleles = 110 for control, 80 for survivors on non-Bt cotton, and 80 for survivors on Bt cotton).

(*r1r1*, *r1r2*, *r1r3*, *r2r2*, *r2r3*, or *r3r3*). Second, in tests of 62 backcross progeny from a genetic linkage analysis with AZP-R and the susceptible APHIS-S strain, all resistant larvae were *rr*, whereas all susceptible larvae were *rs*. Thus, the *BtR* locus is tightly linked with Cry1Ac resistance in the AZP-R strain. Third, of 35 larvae tested from the heterogeneous TX01 strain recently derived from the field in Texas, all 15 resistant larvae were *rr*, whereas all 20 susceptible larvae had either one *r* allele or none. Fourth, all 20 larvae tested from the resistant TX01-R strain, derived by a single selection of the TX01 strain, were *rr*.

Although the results described above demonstrate a strong association between resistance and the three *r* alleles, functional studies of the proteins encoded by the *r* alleles are needed to definitively determine their effect on susceptibility to Cry1Ac. Some additional evidence available now suggests, but does not prove, a causal connection. Each of the *r* alleles has a deletion in a gene encoding cadherin protein, which binds Cry1Ac in pink bollworm (18). All three deletions are upstream of the DNA encoding the putative toxin-binding region of cadherin (17, 18). The stop codon introduced by the 202-bp deletion in *r2* is expected to block production of the binding region. The predicted amino acid deletions associated with *r1* (8 aa) and *r3* (42 aa) are \approx 100 aa upstream of the putative binding region (Fig. 2).

We hypothesize that the deletions in *r1*, *r2*, and *r3* interfere with toxicity of Cry1Ac and thereby confer resistance in homozygous resistant individuals lacking the susceptible form of cadherin protein. This parallels the mechanism proposed for the YHD2 strain of *H. virescens*, in which disruption of a related cadherin gene is tightly linked with resistance to Cry1Ac (19). In *H. virescens* and pink bollworm, reduced binding of one or more Cry1A toxins to brush border membrane vesicles is associated with resistance (refs. 29 and 30, and J. González-Cabrera, B. Escriche, B.E.T., and J. Ferré, unpublished data).

The three pink bollworm resistance alleles identified here each harbor a unique deletion, whereas the resistance allele in the YHD2 strain of *H. virescens* has an insertion (19). Thus, at least four different mutations of the cadherin gene that prevent production of a full-length protein are associated with Cry1Ac resistance. This variation among resistance alleles differs from some well documented cases of insecticide resistance. For example, replacement of a single amino acid (alanine-302) encoded by the *Rdl* gene is associated with cyclodiene resistance in a wide range of insects (31), and a single overtranscribed resistance allele of the *DDT-R* gene occurs globally in *Drosophila melanogaster* (32). Because a limited number of Bt-resistant strains of pink bollworm and *H. virescens*

have been tested so far, we suspect that more extensive screening will uncover additional cadherin resistance alleles.

The four known cadherin resistance alleles are recessive, showing that production of some full-length protein in heterozygotes is sufficient for cadherin's role in the mode of action of Cry1Ac. Although many cadherins mediate cell-cell adhesion in vertebrates (33), their normal function in Lepidoptera is not known. Whereas mutational disruption of cadherins in humans is associated with cancer (33), fitness costs associated with Bt resistance in pink bollworm suggest that its cadherin resistance mutations reduce overwintering survival and survival on non-Bt cotton (22, 34).

Resistance to Cry1Ac in both the pink bollworm and the YHD2 strain of *H. virescens* fits the mode 1 profile and is linked to the cadherin locus. We hypothesize that cadherin mutations are also associated with mode 1 resistance in other lepidopteran pests, such as diamondback moth and Indianmeal moth. Conversely, other types of resistance (3, 12) are likely to involve other loci.

Although results reported here for pink bollworm and previously for *H. virescens* provide strong evidence that cadherin mutations are associated with Cry1Ac resistance, a crucial practical issue is whether such mutations enable survival on Bt cotton plants. For pink bollworm, putative *ss* and *rs* individuals had essentially no survival on Bt cotton plants in previous tests (20, 26, 27). In contrast, results reported here show that *rr* larvae from the AZP-R strain had 43.3% survival on Bt cotton relative to non-Bt cotton. All AZP-R individuals tested had two *r* alleles, and we did not detect variation in survival on Bt cotton among the six *rr* genotypes. Therefore, we cannot exclude the possibility that other genes, environmental factors, or both contributed to variation in survival of AZP-R on Bt cotton. More work is needed to elucidate these additional factors and their potential interactions with cadherin genotype. Nonetheless, our results show that in pink bollworm, cadherin alleles are associated with survival on Bt cotton as well as resistance to Cry1Ac in diet. Thus, the cadherin locus is a prime target for developing DNA-based screening of resistance to Bt crops in field populations of lepidopteran pests.

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