Diverse genetic basis of field-evolved resistance to Bt cotton in cotton bollworm from China

Haonan Zhang[a,1], Wen Tian[a,1], Jing Zhao[a,1], Lin Jin[a], Jun Yang[a], Chunhui Liu[a], Yihua Yang[a], Shuwen Wu[a], Kongming Wu[b], Jinjie Cui[c], Bruce E. Tabashnik[d], and Yidong Wu[a,2]

[a]Key Laboratory of Integrated Management of Crop Diseases and Pests (Ministry of Education), College of Plant Protection, Nanjing Agricultural University, Nanjing 210095, China; [b]Institute of Plant Protection, Chinese Academy of Agricultural Sciences, Beijing 100193, China; [c]Institute of Cotton Research, Chinese Academy of Agricultural Sciences, Anyang 455000, China; and [d]Department of Entomology, University of Arizona, Tucson, AZ 85721

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Evolution of pest resistance reduces the efficacy of insecticidal proteins from *Bacillus thuringiensis* (Bt) used in sprays or in transgenic crops. Although several pests have evolved resistance to Bt crops in the field, information about the genetic basis of field-evolved resistance to Bt crops has been limited. In particular, laboratory-selected resistance to Bt toxin Cry1Ac based on recessive mutations in a gene encoding a toxin-binding cadherin protein has been identified in three major cotton pests, but previous work has not determined if such mutations are associated with field-selected resistance to Bt cotton. Here we show that the most common resistance alleles in field populations of cotton bollworm, *Helicoverpa armigera*, selected with Bt cotton in northern China, had recessive cadherin mutations, including the deletion mutation identified via laboratory selection. However, unlike all previously studied cadherin resistance alleles, one field-selected cadherin resistance allele conferred nonrecessive resistance. We also detected nonrecessive resistance that was not genetically linked with the cadherin locus. In field-selected populations, recessive cadherin alleles accounted for 75–84% of resistance alleles detected. However, most resistance alleles occurred in heterozygotes and 59–94% of resistant individuals carried at least one nonrecessive resistance allele. The results suggest that resistance management strategies must account for diverse resistance alleles in field-selected populations, including nonrecessive alleles.

F_1 screen | F_2 screen | dominant resistance

The toxins produced by *Bacillus thuringiensis* (Bt) kill some major insect pests, but cause little or no harm to people and most other organisms (1). Bt toxins have been used in insecticidal sprays for decades and in transgenic plants since 1996 (2). Farmers planted transgenic corn and cotton producing Bt toxins on more than 66 million hectares worldwide in 2011 (3). The primary threat to the long-term efficacy of Bt toxins is the evolution of resistance by pests (4, 5). Many insects have been selected for resistance to Bt toxins in the laboratory, and at least nine species of pests have evolved some degree of resistance to either Bt sprays or Bt crops in the field (4–18).

Understanding the genetic basis of insect resistance to Bt toxins is useful for managing pest resistance to Bt crops (4, 5, 18). Knowledge of the level of dominance of resistance is especially important because dominance affects the success of the refuge strategy, which is the most widely adopted approach for delaying pest resistance to Bt crops worldwide (5, 18). This strategy is based on the idea that refuges of non-Bt host plants near Bt crops provide susceptible insects to mate with resistant insects. Refuges are expected to delay resistance most effectively if resistance is inherited as a recessive trait, because the matings between homozygous-resistant and homozygous-susceptible adults produce heterozygous progeny that are killed by the Bt crop. Conversely, if resistance is not recessive and some of the heterozygous progeny survive on the Bt crop, refuges are expected to be less effective for delaying resistance. Dominance can be quantified with the parameter h, which varies from 0 for completely recessive resistance to 1 for completely dominant resistance (19). Because the term "dominant" sometimes implies complete dominance, hereafter we use the term "nonrecessive" to refer to resistance that differs substantially from completely recessive resistance.

Although field-evolved resistance to Bt crops has been documented in several pests, previous reports have not identified the gene or genes conferring resistance in any of these cases and the level of dominance of resistance has not been reported in most cases (8–14). Previous results with laboratory-selected strains indicate that mutations disrupting a cadherin protein that binds Cry1Ac in the larval midgut are tightly linked with recessive resistance to Cry1Ac in three major cotton pests: *Heliothis virescens*, *Pectinophora gossypiella*, and cotton bollworm, *Helicoverpa armigera* (20–22). Previous work, however, has not determined if such mutations are associated with field-evolved resistance to Bt cotton.

Here we tested the hypothesis that the genetic basis of resistance to Bt toxin Cry1Ac in *H. armigera* from China is similar in laboratory- and field-selected populations. In the laboratory-selected SCD-r1 strain of *H. armigera* derived from China, the recessive r_1 allele of the cadherin gene *Ha_BtR* has a deletion mutation causing a more than 400-fold increase in the concentration of Cry1Ac needed to kill 50% of larvae (LC_{50}) compared with the nearly isogenic susceptible SCD strain that lacks this allele (23). Bt cotton producing Cry1Ac was commercialized in China in 1997 and has accounted for more than 90% of cotton planted in northern China since 2004 (24), and planting of Bt cotton has been limited in northwestern China (14, 25). Previous analyses of 15 *H. armigera* field populations sampled in 2010 show significantly decreased susceptibility to Cry1Ac in northern China compared with northwestern China, including 2- to 16-fold resistance based on LC_{50} values (14).

Consistent with previous work (14), the results here show that in the field populations screened during 2009–2010, the frequency of alleles conferring resistance to Cry1Ac was three times higher in northern China than in northwestern China. The most common resistance alleles in field-selected populations carried recessive cadherin mutations similar to those identified via

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[1]H.Z., W.T., and J.Z. contributed equally to this work.

[2]To whom correspondence should be addressed. E-mail: wyd@njau.edu.cn.

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laboratory selection. However, laboratory selection did not detect the nonrecessive alleles that occurred in the majority of resistant individuals in field-selected populations.

Results

Resistance Allele Frequency in Field Populations. Consistent with previous data (14), results of the F_1 screen conducted here with 593 *H. armigera* males caught in the field during 2009–2010 show that the proportion of males in which we detected resistance to Cry1Ac was three times higher for field populations from northern China (0.16) than for a field population from northwestern China (0.057) (Fisher's exact test, one-tailed $P = 0.0037$) (Tables S1 and S2). Independent results from the F_2 screen of 363 field-derived isofemale lines also showed that the proportion of lines with resistance was three times higher for a field population from northern China (0.17) than for field populations from northwestern China (0.066) (Fisher's exact test, one-tailed $P = 0.0017$) (Tables S3–S5). The resistance allele frequency (with 95% confidence interval) estimated from the F_1 screen was three times higher for northern China (0.087, 0.070–0.107) than for northwestern China (0.029, 0.012–0.064) (Table S1). The resistance allele frequency estimated from the F_2 screen also was three times higher for northern China (0.052, 0.034–0.076) than for northwestern China (0.016, 0.0098–0.027) (Table S3).

Dominance, Cadherin Mutations, and Magnitude of Resistance Conferred by Alleles Isolated from Field Populations. ***Resistance alleles from the F_1 screen.*** Among the 56 lines generated by crossing survivors from the F_1 screen with the susceptible SCD strain, bioassay results at the diagnostic concentration of Cry1Ac (1 μg Cry1Ac per square centimeter of diet) show that for northern China, 84% (42 of 50) of the resistance alleles were recessive cadherin alleles (r_c) and 16% (8 of 50) were nonrecessive alleles at either the cadherin locus or another locus (R) (Fig. 1*A* and Table S6). In contrast, for northwestern China, all of the resistance alleles were recessive cadherin alleles (six of six) (Fig. 1*A* and Table S6). ***Resistance alleles from the F_2 screen.*** From 10 isofemale lines that yielded five or more survivors in the F_2 screen, we generated 10 resistant strains for further analysis (*Materials and Methods*). Sequencing of the cadherin gene revealed that three of these resistant strains from Anyang in northern China (AY9, AY16, and AY27) had the recessive cadherin resistance allele r_1 that was previously identified and characterized from the laboratory-selected SCD-r1 strain (23).

We conducted additional experiments with AY9 (as a control) and the seven resistant strains that lacked the r_1 allele (Table 1). Results from testing the progeny of crosses between these eight resistant strains and the susceptible SCD strain show that resistance was recessive in six strains (mean survival = 3.5%, range = 0–11%; mean $h = 0.072$, range = 0.0–0.26) and not recessive in two strains from northern China (survival = 58% for AY423 and 38% for AY441; $h = 0.64$ for AY423 and 0.66 for AY441) (Fig. 2 and Table S7). Taking into account the recessive resistance conferred by the r_1 allele in the two resistant strains from northern China (AY16 and AY27) that were not tested in crosses, resistance alleles were recessive in 75% (six of eight) of strains from northern China (Fig. 1*B*).

For seven of the eight resistant strains tested with crosses, survival of progeny was significantly higher for the cross with resistant strain SCD-r1 than for the cross with the susceptible strain SCD, which implies that mutations at the cadherin locus contributed to resistance to Cry1Ac in these seven strains (Fisher's exact test, $P < 0.0001$ for each strain) (Fig. 2 and Table S7). In the exceptional case, survival was not higher for the progeny of resistant strain AY423 from the cross with SCD-r1 (56%) than with SCD (58%) (Fisher's exact test, $P = 1$), which implies mutations at the cadherin locus did not confer resistance in this strain. This conclusion is also supported by the lower survival for the progeny from the cross between AY423 and SCD-r1 (56%) than for AY423 (90%) (Fisher's exact test, $P = 0.0004$) (Table S7). In addition, the index of commonality (C) with the cadherin locus was −0.03 for AY423, indicating it did not have resistance alleles at the cadherin locus (Table S7). We confirmed this conclusion with independent data showing that resistance in AY423 was not genetically linked with the cadherin locus (Figs. S1 and S2). In contrast to the results with AY423, the value of C ranged from 0.6 to 1.2 (mean = 1.0) for the other seven resistant strains, indicating they had resistance alleles at the cadherin locus (Table S7). Taking into account the two resistant strains with the r_1 allele that were not tested in crosses (AY16 and AY27), 8 of 10 strains had recessive cadherin resistance alleles; one strain (AY441) had one or more nonrecessive cadherin resistance alleles, and one strain (AY423) had nonrecessive resistance that did not involve the cadherin locus (Table 1).

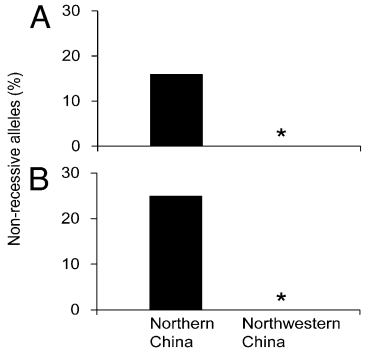

Fig. 1. Percentage of resistance alleles that were nonrecessive alleles in *H. armigera* from northern and northwestern China. (*A*) F_1 screen of field-collected males. (*B*) F_2 screen of field-mated females. Asterisks indicate 0% nonrecessive alleles in northwestern China.

Sequencing of the coding region of the cadherin gene revealed that four of the nine strains with cadherin-based resistance had severe disruptions in this gene, including three strains (AY9, AY16, and AY27) with the previously identified r_1 allele that has a deletion and premature stop codon and strain AY148 that has a different deletion and premature stop codon (Table 1). The five other strains with cadherin-based resistance (AY335, AY440, AY441, SC23, and SW34) had amino acid substitutions, but lacked deletions, insertions, and premature stop codons in the cadherin gene (Fig. S3). Three substitutions in the putative toxin-binding region (E1266L, R1268E, E1270V) occurred in all three larvae examined from the only strain with nonrecessive cadherin-based resistance (AY441), but not in any other strain.

We evaluated the magnitude of resistance to Cry1Ac based on survival at the diagnostic concentration and the resistance ratio, which is the LC_{50} for a strain divided by the LC_{50} for the susceptible SCD strain (Table 1 and Tables S7 and S8). The three most resistant strains were AY9 and SCD-r1 with the r_1 allele and AY423 with noncadherin resistance, which had resistance ratios of 530–1,000 and 88–92% survival at the diagnostic concentration (Table 1). AY148, which had a different premature stop codon than r_1, had 46% survival at the diagnostic concentration (resistance ratio not determined). For the five strains with cadherin-based resistance that did not have deletions or insertions (AY335, AY440, AY441, SC23, and SW34), the resistance ratio ranged from 31 to 95 and survival at the diagnostic concentration ranged from 42 to 60%.

Genotype Frequency and Expected Survival. To better understand how the frequency and dominance of the alleles described above affect evolution of resistance, we used the F_1 and F_2 screen

Table 1. Traits of resistance alleles in *H. armigera* isolated from F_2 screens of field populations from China and in a laboratory-selected strain

Strain	Recessive*	Cadherin-based†	Survival (%)‡	Resistance ratio§	Cadherin allele
Northern China (AY, Anyang)					
AY9	Yes	Yes	88	1,000	r_1: premature stop at 428G
AY16	Yes	Yes	ND	ND	r_1: premature stop at 428G
AY27	Yes	Yes	ND	ND	r_1: premature stop at 428G
AY148	Yes	Yes	46	ND	r_9: premature stop at 1260E¶
AY335	Yes	Yes	60	89	r_{10}: substitutions‖
AY423	No	No	90	660	Substitutions**
AY440	Yes	Yes	50	47	r_{11}: substitutions‖
AY441	No	Yes	58	95	r_{12}: substitutions‖
Northwestern China (SC, Shache; SW, Shawan)					
SC23	Yes	Yes	44	39	r_{13}: substitutions‖
SW34	Yes	Yes	42	31	r_{14}: substitutions‖
Laboratory-selected strain					
SCD-r1	Yes	Yes	92	530	r_1: premature stop at 428G

ND, no data available.
*Based on data from crosses (Fig. 2 and Table S7).
†Based on data from crosses, linkage analysis, and DNA sequences (Fig. 2, Figs. S1–S3 and Table S7).
‡Survival at the diagnostic concentration of Cry1Ac (1 μg Cry1Ac per square centimeter of diet).
§LC_{50} of Cry1Ac for the resistant strain divided by LC_{50} of Cry1Ac for the susceptible SCD strain (Table S8).
¶Intron between Exons 24 and 25 not spliced.
‖Amino acid substitutions, including consistent substitutions in the putative toxin-binding region, but no premature stop codons, deletions, or insertions compared with susceptible strain SCD (Fig. S3).
**Amino acid variation, but no consistent substitutions in the putative toxin-binding region.

results to estimate the frequency of the two classes of resistant genotypes detected in the field populations screened: r_cr_c, which includes any combination of two recessive cadherin resistance alleles (e.g., r_1r_1 or r_1r_9), and *Rx*, which entails at least one nonrecessive resistance allele (*R*) at any locus and any allele (*x*) at the same locus. We used two methods to estimate genotype frequencies: an indirect method (Table S9) based on the Hardy–Weinberg principle and a direct method (Tables S10 and S11) based on the genotypes of resistant individuals detected with the F_1 and F_2 screens. Because individuals with one *R* allele and a second resistance allele at the same locus were extremely rare, individuals with one *R* allele and one susceptible allele at the same locus (*Rs*) accounted for virtually all *Rx* individuals in the indirect method and all *Rx* individuals in the direct method.

For populations from northern China, results from both the F_1 and F_2 screens analyzed by both the indirect and direct methods show that even though nonrecessive alleles accounted for at most 25% of all resistance alleles detected (Fig. 1), individuals with at least one nonrecessive resistance allele (*Rx*) accounted for 59–94% of resistant individuals (Fig. 3 and Tables S9–S11). Based on the F_1 screen data for northern China, the estimated frequency of r_cr_c was 0.0053 from the indirect method and 0.018

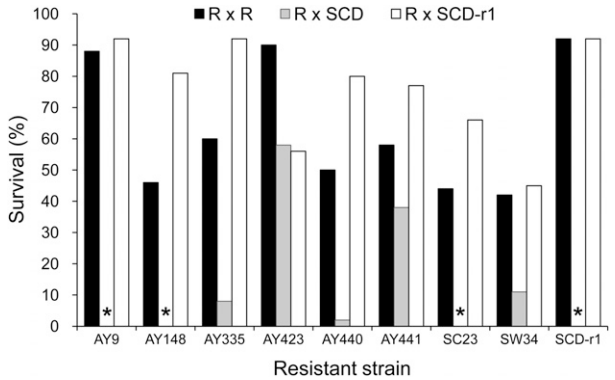

Fig. 2. Survival at the diagnostic concentration of Cry1Ac of nine resistant (R) strains (black bars; eight strains isolated from the F_2 screen and the laboratory-selected strain SCD-r1), and progeny from crosses between each resistant strain and either the susceptible SCD strain (gray bars) or the resistant SCD-r1 strain (white bars). Asterisks indicate 0% survival for progeny from crosses between the SCD strain and each of four resistant strains (AY9, AY148, SC23, and SCD-r1), indicating completely recessive resistance ($h = 0$) in these strains.

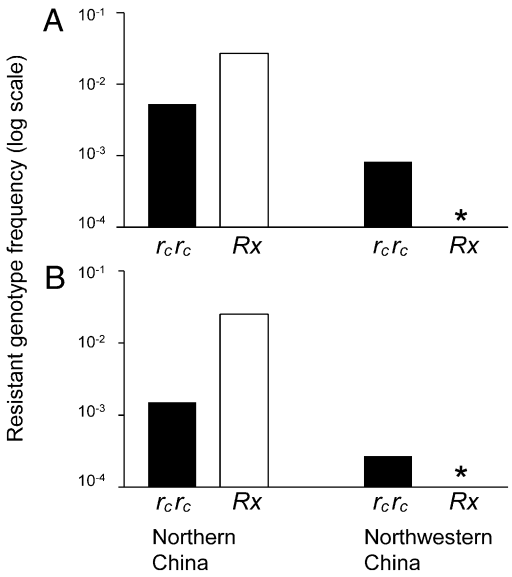

Fig. 3. Resistant genotype frequencies in *H. armigera* from northern and northwestern China estimated from allele frequencies using the indirect method (Table S9). The two classes of resistant genotypes depicted are r_cr_c (any two recessive cadherin alleles) and *Rx* (one nonrecessive resistance allele at any locus and any allele at the same locus). (*A*) F_1 screen of field-collected males. (*B*) F_2 screen of field-mated females. Asterisks indicate the frequency of *Rx* in northwestern China was zero.

from the direct method, compared with 0.027 for *Rx* from both methods. Thus, based on F_1 screen data for northern China, recessive cadherin alleles accounted for 84% of all resistance alleles detected, yet individuals with at least one nonrecessive resistance allele accounted for 59% (direct method) to 84% (indirect method) of individuals with resistant genotypes (Tables S9–S11). Results from the F_2 screen for northern China show a similar pattern, with an estimated frequency of 0.0015 for $r_c r_c$ and 0.025 for *Rx* from the indirect method (Table S9) and 0.0055 for $r_c r_c$ and 0.012 for *Rx* from the direct method (Tables S10 and S11). For these F_2 screen data, recessive cadherin alleles accounted for 75% of all resistance alleles detected, yet individuals with one nonrecessive resistance allele accounted for 69% (direct method) to 94% (indirect method) of individuals with resistant genotypes (Tables S9–S11).

In contrast to the results from northern China, no nonrecessive alleles were detected in populations from northwestern China (Tables S4–S6) and thus the estimated frequency of *Rx* was zero (Tables S9–S11). For northwestern China, the estimated frequency of $r_c r_c$ was 0.00082 from F_1 screen data and 0.00027 from F_2 screen data using the indirect method, compared with zero using the direct method for both F_1 and F_2 screen data (Tables S9–S11).

We calculated expected survival at the diagnostic concentration of Cry1Ac based on the estimated genotype frequencies (Tables S9–S11) and compared it with independent data for observed survival at the same concentration in previously reported bioassays for populations sampled in 2010 (14) (Table S12). For northern China, expected survival based on the mean survival calculated from genotype frequencies estimated from the F_1 and F_2 screen data were 2.2% for both the indirect and direct methods, which matched the observed survival of 2.2% (Table S12). For northwestern China, expected survival based on both F_1 and F_2 screen data using either the indirect or direct method matched the observed survival of 0% (Table S12).

Discussion

Together with previous results, the data reported here are unique in enabling a direct comparison of the genetic basis of laboratory-selected resistance and field-selected resistance to a Bt crop. The cadherin r_1 allele originally identified from a laboratory-selected strain derived from Gaoyang of northern China in 2001 (22, 23, 26) was the most common resistance allele detected in field-selected populations from northern China. This allele accounted for the resistance detected in 38% (three of eight) of resistant strains from Anyang of northern China (Table 1), which is about 300 km southwest of Gaoyang. Previous work showed that of 15 field populations from China tested in 2010, Anyang was the most resistant, with 2.6% survival at the diagnostic concentration and a resistance ratio of 16 for Cry1Ac activated toxin (14). The cadherin r_1 allele and other cadherin resistance alleles accounted for 88% (seven of eight) of the resistance alleles isolated from Anyang using the F_2 screen (Table 1), which supports the idea that laboratory-selected strains can be useful for finding loci that are important in field-evolved resistance to Bt crops. However, given the diversity of cadherin resistance alleles in field populations of *H. armigera* identified here (Table 1) and previously (26–28), DNA screening that focused solely on detection of the r_1 allele or even all cadherin alleles would underestimate the resistance allele frequency. This problem could be addressed by using F_1 and F_2 screens to detect any recessive resistance alleles at the cadherin locus, as well as nonrecessive resistance alleles at any loci, as done here and in previous studies (10, 28, 29).

Unlike the r_9 cadherin allele identified here and all 13 previously identified naturally occurring cadherin resistance alleles [eight in *H. armigera* (28), four in *P. gossypiella* (21, 30), and one in *H. virescens* (20)], five cadherin resistance alleles detected here (r_{10}–r_{14}) had amino acid substitutions but lacked insertions and deletions (Table 1). Although binding of Cry1Ac has not been examined in the strains with these five alleles, some artificially induced single amino acid substitutions in the toxin-binding region of *H. virescens* cadherin reduced binding of Cry1Ac (31).

Most of the resistance alleles identified here had recessive mutations at the cadherin locus, but the F_1 and F_2 screens detected resistance alleles that were not recessive, including one nonrecessive cadherin allele and another allele that is not at the cadherin locus ($h > 0.6$ for both) (Table 1 and Table S7). Based on results from crosses, mutations at the cadherin locus contribute to the nonrecessive resistance to Cry1Ac in the field-selected strain AY441 from northern China (Fig. 2 and Table S7). Further testing is required to determine if the three predicted amino acid substitutions in the putative toxin-binding region of cadherin in this strain (E1266L, R1268E, and E1270V) (Fig. S3) contribute to resistance. Data from crosses and linkage analysis show that the nonrecessive resistance in the AY423 strain was not conferred by alleles at the cadherin locus (Fig. 2, Figs. S1 and S2, and Table S7). Additional work is needed to determine if this resistance entails mutations in an ABC transporter gene or altered aminopeptidase expression, as reported from other insects (16, 17). Despite examples of nonrecessive, laboratory-selected resistance, and speculation about nonrecessive resistance in the field (4–6, 9, 13, 32–34), the data herein are unique in documenting nonrecessive resistance alleles identified from any insect population with field-selected resistance to a Bt crop.

Although the recessive alleles accounted for 75–84% of resistance alleles detected by the F_1 and F_2 screens, individuals with at least one nonrecessive resistance allele accounted for 59–94% of individuals with resistant genotypes. This difference in the relative importance of recessive alleles between allele and genotype frequency happened because most resistance alleles occurred in individuals heterozygous for resistance, which is typical in the early stages of resistance evolution (4, 5). Three traits of resistance alleles affect the rate at which their frequency increases in the field: dominance, magnitude (increased fitness on Bt cotton plants), and fitness costs (decreased fitness on non-Bt host plants) (35, 36). If the magnitude of resistance and fitness costs are similar in nonrecessive and recessive alleles, continued selection for resistance is expected to increase the frequency of the nonrecessive alleles faster than the recessive alleles (5, 36), thereby increasing the relative importance of the nonrecessive alleles. More data from plant bioassays are needed to fully assess the dominance, magnitude, and fitness costs of the resistance alleles detected herein. Meanwhile, the results from diet bioassays show that relative to the recessive r_1 cadherin allele, the magnitude of resistance in strain AY441 conferred by a nonrecessive cadherin allele was lower, whereas resistance in strain AY423 conferred by a nonrecessive allele not at the cadherin locus was similar (Table 1). Survival of 5–20% of susceptible *H. armigera* larvae on Bt cotton plants in the field occurs at the end of the growing season, when the concentration of Cry1Ac declines (37). In contrast, the diagnostic concentration of Cry1Ac in our diet bioassays killed 100% of more than 1,400 susceptible larvae (14), which suggests that survival at this concentration is a conservative criterion for evaluating resistance to Bt cotton. In addition, increased survival at the diagnostic concentration was associated with increased survival on Bt cotton plants (27). These results imply that the nonrecessive resistance seen here at the diagnostic concentration (Fig. 2 and Table S7) is likely to confer nonrecessive resistance to Bt cotton. A more conclusive test of this hypothesis could be performed by determining survival on Bt cotton for the heterozygous progeny from matings between resistant and susceptible parents (9). Nonetheless, the available evidence suggests that strategies for monitoring and managing resistance of *H. armigera* to Bt cotton in China should address the potential consequences of nonrecessive resistance.

Consistent with previous results (14), the results here from F_1 and F_2 screens of *H. armigera* collected from the field during 2009 and 2010 show a significantly higher Cry1Ac resistance allele frequency in populations from northern China, where Bt cotton has been planted intensively, than in populations from two areas of northwestern China, where Bt cotton has not been planted intensively. Baseline data show that susceptibility to Cry1Ac was not lower in northern China than in northwestern China before Bt cotton was commercialized (38), which implies that exposure to Bt cotton in northern China selected for decreased susceptibility to Cry1Ac. In addition, populations in northern China were not resistant to a different Bt toxin, Cry2Ab, which supports the conclusion that resistance to Cry1Ac in northern China represents a specific response to Bt cotton producing that toxin (14). Although *H. armigera* migrates over long distances (37, 39), any gene flow occurring between northern and northwestern China has not been sufficient to eliminate differences in resistance to Cry1Ac between the two regions.

Despite significant decreases in susceptibility to Cry1Ac in field populations of *H. armigera* from northern China reported here and previously (14), no field control failures have been reported. Two factors that could be reducing the negative impact of field-evolved resistance of *H. armigera* to Cry1Ac in northern China are the reduction in this pest's population density from 1992 to 2006 (24) and the continued application of more than 10 insecticide sprays per season on cotton (14, 40). Moreover, even the most resistant population examined in 2010 (Anyang) had only 2.6% survival at the diagnostic concentration of Cry1Ac (14). One factor delaying resistance in northern China may be the high percentage of host plants consisting of crops other than cotton that do not produce Bt toxins and thus may act as refuges for *H. armigera* (37).

For the many crop-pest combinations not examined here, additional work is needed to test the correspondence between the genetic basis of laboratory-selected resistance and field-selected resistance to Bt crops. The genes conferring field-evolved resistance to Bt crops have not been reported in other cases, but data are available on the dominance of such resistance for two other pests. Storer et al. (11) reported almost completely recessive resistance of *Spodoptera frugiperda* to Cry1F in Bt corn (h = 0.07–0.14) and Downes et al. (10, 41) found recessive "incipient" resistance of *Helicoverpa punctigera* to Cry2Ab in Bt cotton (h = 0.01). In both of these cases, laboratory-selected resistant strains have not been available for comparison. For *P. gossypiella*, field populations in the United States have remained susceptible to Bt cotton (42), but it will be intriguing to compare the genetic basis of laboratory-selected resistance with field-selected resistance of populations from India and China (12, 15).

The results here showing that nonrecessive alleles are important in field-evolved resistance to Bt cotton in *H. armigera* differ from results indicating a primary role of recessive mutations in an ABC transporter gene in field-evolved resistance to Cry1Ac selected by Bt sprays in two major vegetable pests, *Plutella xylostella* and *Trichoplusia ni* (16). We do not know if this difference in the genetic basis of field-evolved resistance between Bt toxins in sprays and in transgenic crops will be sustained as more cases are analyzed. Meanwhile, based on theoretical work (5, 36) and the relatively limited empirical data summarized above, we hypothesize that even when recessive resistance alleles are more common initially, nonrecessive alleles are likely to be more important in determining the trajectory of field-evolved resistance to Bt crops. Until this hypothesis is refuted, we recommend increased efforts to detect, characterize, and design strategies to counter resistance to Bt crops that is conferred by nonrecessive alleles.

Materials and Methods

Insect Strains. The susceptible SCD strain of *H. armigera* was started with insects from the Ivory Coast, Africa over 30 y ago and was maintained in the laboratory without exposure to insecticides or Bt toxins (23). The r_1 allele of the cadherin gene (*Ha_BtR*) was isolated from the resistant strain GYBT, which was started in August 2001 with 300 late instars collected from Bt cotton in Gaoyang County of Hebei Province of northern China and selected with Cry1Ac for 28 generations in the laboratory (22). The resistant strain SCD-r1 was created by introgressing r_1 from GYBT into SCD (23). Larvae were reared on an artificial diet and adults were maintained as described previously (14).

Bioassay. We tested second instars using a diet surface overlay bioassay with activated Cry1Ac toxin, as described previously (14). Previous work with the SCD-r1 strain showed that 1 μg Cry1Ac per square centimeter of diet (diagnostic concentration), yielded essentially 0% survival of larvae that were either homozygous-susceptible (*ss*) or had one r_1 resistance allele and one susceptible allele (r_1s), and close to 100% survival of homozygous-resistant larvae (r_1r_1) (22). In most bioassays, we used the diagnostic concentration. We used a series of concentrations to estimate the LC_{50} of Cry1Ac for each of nine strains (Table S8). We adjusted for control mortality (range = 4–8%) to estimate LC_{50} values, but not in the bioassays with only the diagnostic concentration, which had lower control mortality (<5%).

Field Collection of Moths. We collected *H. armigera* moths for the F_1 and F_2 screens (detailed below) with two light traps (250 W) separated by >200 m at each sample site in cotton fields from northern China in 2009 and northwestern China in 2010. Because we collected moths 1 y earlier in northern China than in northwestern China and Bt cotton was planted extensively in northern China, our comparisons may underestimate the difference in susceptibility to Cry1Ac between the two regions. Male moths for the F_1 screen were collected from three sites in northern China (Anci, Hebei Province in June; Anyang, Henan Province in June; Xiajin, Shandong Province in August) and from one site in northwestern China (Shawan, Xinjiang Uygur Autonomous Region in August). Female moths for the F_2 screen were collected from one site in northern China (Anyang in June) and two sites in northwestern China (Shache in July and Shawan in August, both in Xinjiang).

F_1 Screen. Field-caught male moths were crossed individually to homozygous-resistant female moths (r_1r_1) from the SCD-r1 strain (23). F_1 offspring (48 second instars per family) from 593 single-pair families were tested at the diagnostic concentration using the bioassay described above. Expected survival of the F_1 progeny in these bioassays depends on the genotype of the field-caught male (29): 0% for a susceptible homozygote (*ss*), 50% for individuals with one susceptible allele and either any recessive resistance allele at the cadherin locus (r_c) or a nonrecessive resistance allele at any locus (*R*) (genotypes r_cs_c or *Rs*), and 100% for individuals with the genotypes r_cr_c or *RR* (Table S1). We scored the genotypes of field-captured males depending on the survival of their F_1 progeny: *ss* if <30%, r_cs_c or *Rs* if 30–70%, and r_cr_c or *RR* if >70%.

We determined the dominance of the resistance alleles detected with the F_1 screen by crossing survivors of the F_1 screen with the susceptible SCD strain (s_cs_c) and testing the progeny at the diagnostic concentration. Expected survival of the progeny of these F_1 survivors × SCD crosses is 0% if the resistance allele is recessive (progeny are r_cs_c) and 50% if the allele is dominant (half of progeny are *Rs* and half are *ss*). We scored the allele as recessive if survival was <30% and not recessive (partially to completely dominant) if survival was ≥30%.

F_2 Screen. We established an isofemale line from each of 363 field-captured, field-mated females. The F_1 adults from each isofemale line mated among themselves to produce F_2 progeny. We used the bioassay described above to test 68–144 (96 in most cases) F_2 larvae from each isofemale line at the diagnostic concentration. The expected survival of F_2 progeny depends on the genotypes of the field-mated female and her mates. If the parents of an isofemale line had no resistance alleles, the expected survival of F_2 progeny is 0%. Assuming each field-collected female mated with a single male, and one of the two parents had a single recessive allele conferring resistance, the expected survival of F_2 progeny is 6.25% (1/16) (43). We scored the parents of isofemale lines as having at least one resistance allele if their survival was >3%. If survival was significantly >6.25%, we inferred that together the original female and male parents of the isofemale line either had two recessive resistance alleles or a single nonrecessive allele (Tables S4 and S5).

Characterizing Resistance Alleles from the F_2 Screen. To characterize the resistance alleles detected in the F_2 screen, we generated and tested resistant strains from each of 10 isofemale lines: eight from Anyang in northern China (AY9, AY16, AY27, AY148, AY335, AY423, AY440, AY441) and one each from Shache (SC23) and Shawan (SW34) in northwestern China. We started each of these 10 resistant strains by exposing 500–1,000 F_2 larvae from an isofemale line to the diagnostic concentration. Sequencing of the cadherin gene (see details below) in the survivors from this exposure identified the r_1 cadherin allele in three strains (AY9, AY16, and AY27). Because the effects of this allele were characterized previously (23, 26), we included only one of the three strains with the r_1 allele (AY9) as a control in subsequent experiments. For AY9 and the other seven resistant strains, we let the F_2 survivors of exposure to the diagnostic concentration mate with the susceptible SCD strain to avoid inbreeding effects. Adults from the resulting hybrid progeny (F_3) mated among themselves to produce F_4 progeny. Larvae of the F_4 generation and up to two additional generations were selected at 2–15 times the diagnostic concentration.

Dominance and role of cadherin locus. We crossed each of eight resistant strains isolated from the F_2 screen with the susceptible SCD strain and separately with the resistant SCD-r1 strain (Table S7). Forty-eight to 96 F_1 larvae from each cross were tested at the diagnostic concentration using the bioassay described above. We evaluated dominance in each resistant strain by comparing survival between the resistant strain, SCD, and the F_1 progeny from the cross between the resistant strain and SCD (Table S7). We calculated the dominance parameter h, which varies from 0 (completely recessive) to 1 (completely dominant) (Table S7) (19). We classified alleles with $h < 0.3$ as recessive and alleles with $h > 0.6$ as nonrecessive, which may underestimate the proportion of nonrecessive alleles because alleles with $h = 0.13$ and 0.26 were considered recessive. Resistance was not completely dominant in any strain, which enabled evaluation of the contribution of the cadherin locus using a complementation test for allelism (29, 44) (Table S7). We also tested for genetic linkage with the cadherin locus for the resistance in strain AY423 (Figs. S1 and S2).

Cadherin allele sequences. We prepared cDNA and conducted PCR amplification as described previously (28) to obtain full-length sequences of cadherin alleles from each of two to three final instar larvae from each of the 10 resistant strains isolated from the F_2 screen (Fig. S3).

Data Analysis. To estimate resistance allele frequencies from F_1 and F_2 screens, we used the frequentist approach (29). We used Fisher's exact test with one-tailed probability values to check if the frequency of lines in which resistance was detected was significantly higher in northern China than northwestern China.

Tables S1–S12 and Figs. S1–S3 include further details of the materials and methods.

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