Characterization of the Cry1Ah resistance in Asian corn Borer and its cross-resistance to other *Bacillus thuringiensis* toxins

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Transgenic crops producing insecticidal proteins are effective to manage lepidopteran pests. Development of insect-resistance is the major threat to *Bacillus thuringiensis* (Bt) crops such as Cry1Ah-Maize. Laboratory selection with Bt-Cry1Ah toxin incorporated in artificial diet, during 48 generations of Asian corn borer (ACB) *Ostrinia furnacalis* produced 200-fold resistance. This resistant colony ACB-AhR readily consumed and survived on Cry1Ah-expressing Bt-maize. Cross-resistance analysis showed high cross-resistance to Cry1F (664-fold), moderate cross-resistance to Cry1Ab (28.38-fold), Cry1Ac (22.11-fold) and no cross-resistance to Cry1le toxin. This ACB-AhR cross-resistant phenotype is different from ACB-Cry1Fa resistant population that showed no cross resistance to Cry1Ah, suggesting that different mechanisms of resistance were selected in these two populations. Bioassays of reciprocal F1 crosses progeny suggested autosomal inheritance of Cry1Ah resistance with no maternal effects. The dominance of resistance increased as concentration decreased. In Cry1Ah-maize tissues the progeny of reciprocal F1 crosses behaved as functionally recessive. Progenies analysis from backcrosses (F1 resistant strain) suggested polygenic contribution to Cry1Ah resistance in ACB-AhR. The use of multiple toxins is an imperative factor for delaying evolution of resistance to Cry1Ah-corn in ACB. However, the fact that ACB-AhR showed cross resistance to Cry1Fa indicates that selection of toxins for pyramided plants should be carefully done.

To improve insect pest management, genetic modified crop plants such as cotton and corn based on insecticidal proteins from *Bacillus thuringiensis* (Bt) are now the most widely used strategies1. Since failures of synthetic chemicals, microbial insecticides derived from Bt have proven ample potential for pest management2. Insecticidal proteins are extensively used both in sprays formulations and transgenic crops to control lepidopteran pests having negligible effects on non-target pests3–5. These Bt insecticidal proteins are valuable as they can bring season long protection against lepidopteran pests6.

Since worldwide commercialization of transgenic Bt crops in 1996, cry genes that codify for insecticidal proteins such as Cry1Ab, Cry1Ac, Cry1F, Cry1Ah, and Cry3Bb1 have been expressed in corn, cotton and soybean and grown extensively in several countries7. However, the efficacy of transgenic crops can be reduced by the continuous use of single trait products. Insect resistance is the major hazard to the enduring success of Bt crops8. Bt resistance in field conditions has been reported in different insect pests including *Ostrinia nubilalis*9, *Plutella xylostella*10, *Trichoplusia ni*11, *Spodoptera frugiperda*12, *Busseola fusca*13, *Diabrotica virgifera virgifera*14 and *Pectinophora gossypiella*15. In order to delay resistance evolution, implementation of suitable resistance management practices is necessary. The application of high-dose/refuge strategy and pyramiding of two or more toxins with different mode of action have been the foremost strategies used worldwide to delay evolution of resistance in different pests toward Bt crops16–18. The theory underlying the refuge strategy implies the planting of non-Bt host plants in close vicinity to Bt crops to assure that the rare resistant individuals selected on Bt crops will mate with susceptible individuals from nearby refuges of host plants without Bt toxins therefore generating

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heterozygous progenies that should be sensitive to the high dose concentration of the Bt toxin expressed in the transgenic plants, implying recessive inheritance. Multiple components are predicted to favor the success of the refuge strategy: recessive inheritance of resistance, low initial resistance allele frequency, pest movements and mating patterns, fitness costs and incomplete resistance. Moreover, the success of the strategy based on applying rotations of different toxins or toxin mixtures works best if the inheritance of resistance to each toxin is recessive.

Transgenic corn expressing the activated 65 kDa Cry1Ah was developed by Origen Seeds Ltd to target Asian corn borer (ACB). Knowledge of the genetic basis of resistance to Cry1Ah toxin is imperative for designing and refining managing tactics to minimize evolution of resistance in this pest. The present study offers insights into the mechanisms linked to resistance evolution. We describe here the results of the experiments that analyzed the mode of inheritance for Cry1Ah resistance in a laboratory selected strain resistant to Cry1Ah (ACB–AhR), evaluated maternal effects, sex linkage and effective dominance. In addition, backcross crosses were performed to estimate the number of loci affecting the inheritance. Also, the assumption of functional recessive resistance to Cry1Ah toxins was measured by determining the survival of Cry1Ah-resistant, susceptible parental strains and the F1 progenies from reciprocal crosses in experiments with Bt corn plants. Finally, cross resistance patterns among different Bt toxins were also analyzed. The implications of this study will be helpful for the future managing of ACB resistance to transgenic Cry1Ah-maize.

### Results

#### Selection of ACB for Cry1Ah resistance.

Bioassays using Cry1Ah toxin and Bt maize tissues revealed that ACB–AhR strain developed a very high level of resistance to Cry1Ah toxin after 48 generations of selection. The diet bioassays validated that larvae from the resistant strain had a higher level of resistance to Cry1Ah toxin compared with larvae from the susceptible strain. The LC50 values for the susceptible (ACB–BtS) and Cry1Ah-selected colonies (ACB–AhR) were significantly different showing LC50 values of 0.33 and 63.91 µg/g Cry1Ah toxin, respectively. The differences in LC50 values between ACB–BtS and ACB–AhR strains and the resulting resistance ratio of more than 193.67-fold validates that resistance to Cry1Ah toxin is attainable for this species.

#### Cross-resistance.

The LC50 values for Cry1Ab and Cry1Ac were significantly higher in the ACB–AhR strain compared to the ACB–BtS strain, i.e. bioassays with Cry1Ac led to a 22.11-fold resistance, indicating that the selected ACB–AhR strain is slightly cross-resistance to Cry1Ac. Similarly, a 28.38-fold resistance was observed when exposed to Cry1Ab toxins (Table 1). The highest level of cross-resistance was detected with Cry1F with a resistance ratio of up to 464 fold, whilst no cross-resistance was observed with Cry1Ie (Table 1).

#### Survival on plant tissues.

Neonate larvae of ACB–AhR, ACB–BtS strains and their F1 progeny displayed significantly lower rate of feeding on Bt maize tissues than on non-Bt maize tissues. The survival rates of ACB–AhR strain were significantly greater than ACB–BtS strain and F1 progenies when larvae were fed on Cry1Ah-expressing maize leaf tissue after 7 days (Table 2). In addition, ACB–AhR strain exhibited less survival rate on Bt kernel followed by Bt leaves, highest survival of resistant strain was perceived on silk tissues (Table 2). In contrast, in case of non-Bt leaves these strains did not differ significantly (F1 = 12.3; P = 0.0023). ACB–AhR strain showed more survival rate than susceptible strain when fed on non-Bt silk tissues (F1 = 3.52; P = 0.0689).

The larvae of all strains significantly differed when they were fed on kernel tissue of non-Bt maize (F1 = 25.2, P = 0.0002) (Table 2).

#### Quantification of Cry1Ah toxin.

Cry1Ah concentrations were determined in leaves, silk and kernels tissues of the Cry1Ah- maize. Kernel showed the highest concentration of Cry1Ah (Fig. 1). The concentration of Cry1Ah toxin in these plant tissues was 41.84, 57.10 and 78.66 ng/g dry weight, respectively (Table 1).

### Table 1. Susceptibility of ACB–AhR and ACB–BtS of *O. furnacalis* to 5 Bt toxins.

| Bt toxin | Strain | n | LC50 (95% FL) (µg/g) | Slope ± SE | χ² | df (χ²) | RRb |
|----------|--------|---|---------------------|------------|---|--------|------|
| Cry1Ab   | ACB–BtS| 768| 0.33 (0.27–0.39)    | 1.667 ± 0.120 | 5.11 | 12 | — |
|          | ACB–AhR | 1296 | 63.91 (54.77–74.38) | 1.292 ± 0.0865 | 13.30 | 22 | 193.67 |
| Cry1Ab   | ACB–BtS | 336 | 0.21 (0.16–0.27)    | 1.758 ± 0.192 | 1.82 | 5 | — |
|          | ACB–AhR | 336 | 5.96 (4.86–7.25)    | 1.929 ± 0.173 | 2.36 | 5 | 28.38 |
| Cry1Ac   | ACB–BtS | 336 | 0.28 (0.19–0.38)    | 1.774 ± 0.181 | 5.56 | 5 | — |
|          | ACB–AhR | 336 | 6.19 (5.05–7.42)    | 1.488 ± 0.125 | 7.31 | 10 | 22.11 |
| Cry1F    | ACB–BtS | 336 | 7.53 (6.17–9.14)    | 1.975 ± 0.174 | 3.76 | 5 | — |
|          | ACB–AhR | 672 | 7.29 (6.31–8.43)    | 1.826 ± 0.117 | 2.29 | 12 | 0.97 |
| Cry1F    | ACB–BtS | 336 | 0.39 (0.25–0.53)    | 1.313 ± 0.174 | 1.66 | 5 | — |
|          | ACB–AhR | 672 | 181.02 (150.84–222.54) | 1.457 ± 0.112 | 2.77 | 12 | 464.10 |

Table 1. Susceptibility of ACB–AhR and ACB–BtS of *O. furnacalis* to 5 Bt toxins. n, number of larvae tested. RRb, resistance ratio = LC50 of particular strain or cross divided by LC50 of susceptible strain. ACB–BtS is susceptible strain. ACB–AhR is the resistant strain of *Ostrinia furnacalis*. © 2018 Nature America Inc. All rights reserved.
Maternal effects and sex linkage. The maternal influence and the sex-linked nature of the resistance were examined by comparing observed larval concentration responses of the F1 progeny. The LC50 for the F1 progeny from reciprocal crosses to Cry1Ah was significantly greater than the LC50 for the susceptible parental strain and significantly less than the LC50 for the resistant parental strain (Table 3). Likewise, the mean slope of the concentration-mortality line did not differ between the reciprocal crosses. Thus, inheritance was autosomal; neither maternal effects nor sex-linkage were evident.

Estimation of the degree of dominance. Dominance estimations at different concentrations of Cry1Ah toxin revealed that the resistance was dominant in low concentration treatment but decreased if the concentration increased. The level of dominance is obtained by the calculation of effective dominance at different toxin concentrations, h varied with concentration, from dominant inheritance at low concentrations to recessive inheritance at high concentrations. For example, results presented partially dominance at 0.5 μg/g (h=0.79), and declined to incomplete-recessive by treatment concentrations of 50.0 μg/g (h=0.4) and complete recessive 250 μg/g (h=0).

Plant tissue bioassays showed that the h values were 0.13, 0.06 and 0 for Cry1Ah maize leaves, silks and kernel, respectively (Table 5), which indicated that the resistance was functionally recessive at leaves stage of maize plant development. At the silking stage of the plant, the dominance was virtually recessive but it was completely recessive at the kernel stage of the plant development.

Discussion

Bt maize, specifically the first generation of biotech Bt maize hybrids target both O. nubilalis and O. furnacalis. A few strains of O. nubilalis have developed resistance to the expressed protein in different events of Bt maize, Cry1Ac, Cry1Ab or Cry1F in laboratory-selection experiments. In this work we showed that the selection of O. furnacalis with Cry1Ah resulted in a high level of resistance to this toxin (200-fold for ACB-AhR). We showed

| Insect Strains | Leaf (%) | Silk (%) | Kernel (%) |
|----------------|----------|----------|------------|
|                | Cry1Ah-corn | Non-Bt | Cry1Ah-corn | Non-Bt | Cry1Ah-corn | Non-Bt |
| ACB-AhR        | 6.3 ± 1.2 a  | 80.6 ± 2.5 a* | 60.0 ± 2.9 a | 86.7 ± 3.3 a* | 2.7 ± 0.6 a | 79.3 ± 2.4 a* |
| ACB-BtS        | 0.7 ± 0.7 b  | 75.0 ± 3.2 a* | 0.0 | 80.0 ± 2.5 ab* | 0.0 | 60.0 ± 5.2 b* |
| S♀ × R♂        | 1.4 ± 0.7 b  | 78.5 ± 1.8 a* | 3.3 ± 0.8 b | 84.2 ± 1.7 a* | 1.3 ± 0.3 b | 52.7 ± 1.3 bc* |
| R♂ × S♀        | 0.7 ± 0.7 b  | 59.7 ± 3.0 b* | 0.0 | 68.3 ± 7.4 b* | 0.0 | 49.2 ± 1.2 c* |

Table 2. Survival of ACB-AhR, ACB-BtS strains of Ostrinia furnacalis and F1 progenies fed on Bt and non-Bt maize tissues. Data are means ± SE. The means within column followed with same letter are not significantly different (P<0.05) and the “*” indicate that Cry1Ah-corn and non-Bt corn are significantly different according to Fisher’s Protect LSD test.
that ACB-AhR could feed and survive on leaf, silk and kernel tissues of Bt-maize, although survival rates were low on leaves and kernels (Table 2). The low survival on Cry1Ah-leaves of ACB-AhR was unexpected since this tissue showed the lower Cry1Ah concentration (Fig. 1). It could be possible that other plant molecules present in leaves and kernels of Cry1Ah-maize contribute to the low survival rate observed in these tissues. In agreement with this, both ACB-BtS and ACB-AhR showed higher survival rates on non-Bt silk than on non-Bt leaves or kernels.

Table 3. Responses of F₁ progenies from reciprocal crosses between resistant and susceptible strains of Ostrinia furnacalis to Cry1Ah toxin. *n* number of larvae tested. RR, resistance ratio = LC₅₀ of strain or cross divided by LC₅₀ of susceptible strain. *ACB-BtS is susceptible strain of Ostrinia furnacalis. *Progeny of mass cross between resistant male and susceptible female.

Table 4. Effective of dominance (*h*) of resistance to Cry1Ah-selected Asian corn Borer. *Fitness of the susceptible parent and the reciprocal cross was estimated from the survival rate of the larvae at a specific treatment concentration divided by the survival rate of the resistant parent at the same concentration. *\( h = (\omega_{RS} - \omega_{SS})/\omega_{RR} - \omega_{SS} \) where \( \omega_{RS} \) is the fitness of the heterozygous offspring, \( \omega_{SS} \) is the fitness homozygous susceptible parent, \( \omega_{RR} \) is the fitness of homozygous resistant parent. \( h \) can vary from 0 (completely recessive resistance) to 1 (complete dominant resistance).

Table 5. Effective dominance values (*h*) of Cry1Ah resistance in ACB-AhR strain of the Ostrinia furnacalis based on Cry1Ah maize plant tissues bioassays. *Fitness of the susceptible parent and the reciprocal cross was estimated from the survival rate of the larvae at a specific treatment concentration divided by the survival rate of the resistant parent at the same concentration. *\( h \) can vary from 0 (completely recessive resistance) to 1 (complete dominant resistance) (see Materials and Methods).
In earlier findings, Cry1F-selected strain (ACB-FR) established a high level of resistance (1700-fold) and the larvae presented potential to survive and consume Cry1F toxin despite this resistance. Four strains of *O. nubilalis* that were exposed to Cry1Ab toxin for 10 generations developed low level of the resistance (2.0- to 10-fold) and caused reduced susceptibility to other toxins to which these selected strains were not exposed. However, high level of resistance to Cry1Ac with a peak of 162-fold resistance was observed in a Minnesota strain of *O. nubilalis* after only eight generations of selection.

Several factors are involved in resistance levels among strains. However, resistance levels are linked to the number of generations selected, concentrations of toxins consumed, selective agents itself, bioassay procedures and susceptibility among unselected strains. In the present study, the levels of resistance to Cry1Ah in laboratory-selected strains resulted in about 200-fold resistance to purified Cry1Ah toxin under moderate selection pressure. These are the highest levels of the resistance to a Cry1Ah toxin ever documented for *O. furnacalis*. Previously, different *O. furnacalis* resistant strains to different Cry1 toxins were selected and characterized, including 106-fold resistance to Cry1Ab, 40-fold and 113-fold resistance to Cry1Ab and Cry1Ac respectively, or 1700-fold resistance to Cry1F after 49 generations of selection. Factors contributing in increased tolerance to Bt crops may be the time exposure to Bt crops, resistance genes and genetic background of different populations.

The results from the analysis of F1 progenies from reciprocal crosses performed between resistant and susceptible strains suggest that resistance to Cry1Ah was autosomal inherited with no maternal effects. The values of the present study are consistent with nearly all previous findings with Bt resistance including resistance to Cry1F in *O. furnacalis*, or to Cry1Ab and Cry1Ac, Greenhouse-Derived Strain of *Trichoplusia ni* to Cry1Ac and *B. thuringiensis* subsp. *kurstaki*, *Helicoverpa armigera* to Cry1Ac and resistance to Cry1F maize in a strain of *S. frugiperda*. Usually, resistance to Bt toxins behave in agreement with autosomal mode of inheritance. In contrast, inheritance of Cry1Ab resistance in a field-derived strain of *O. nubilalis* and resistance to Cry1Ac in *P. xylostella* in leaf dips bioassay had proven maternal influence on the survival of F1 progenies. Also, sex linkage phenomenon was described to inheritance pattern of resistance to Cry3Bb1 in *Diatroica virgifera virgifera*. These dissimilarities in results are possibly related to use of different insects species.

The spectrum of cross-resistance occurs when the Bt toxins demonstrate similarities in their mechanisms of action. In the present study, selection for Cry1Ah resistance has resulted in slight cross-resistance to Cry1Ab and Cry1Ac with resistance ratios of 28.38 and 22.11-fold, respectively. Cross-resistance of Cry1Ah to Cry1Ab and Cry1Ac is shown in Table 6.

| Concentration (μg/g) | Actual mortality (%) | Expected mortality (%) | $\chi^2$ | $P$ |
|----------------------|----------------------|------------------------|--------|-----|
| RR♀ × F1♂ (SS♀ × RR♂) | 0.5 | 7.3 | 8.3 | 0.14 | 0.712 |
|                      | 2.5 | 59.6 | 28.1 | 6.23 | 0.013* |
|                      | 5.0 | 48.9 | 35.4 | 7.69 | 0.006* |
|                      | 12.5 | 63.5 | 47.9 | 9.39 | 0.002* |
|                      | 25.0 | 81.2 | 58.7 | 20.16 | $>0.001^*$ |
| $\sum \chi^2$       |       |       |       | 43.61 |     |
| F1♀ (SS♀ × RR♂) × RR♂ | 0.5 | 7.3 | 8.3 | 0.13 | 0.712 |
|                      | 2.5 | 19.8 | 28.1 | 3.29 | 0.069 |
|                      | 5.0 | 44.8 | 35.4 | 3.68 | 0.055 |
|                      | 12.5 | 59.4 | 47.9 | 5.05 | 0.025* |
|                      | 25.0 | 75.0 | 58.7 | 10.54 | $>0.001^*$ |
| $\sum \chi^2$       |       |       |       | 22.69 |     |
| RR♀ × F1♂ (RR♀ × SS♂) | 0.5 | 7.2 | 8.3 | 0.13 | 0.712 |
|                      | 2.5 | 26.0 | 24.3 | 0.15 | 0.692 |
|                      | 5.0 | 42.7 | 35.8 | 2.01 | 0.156 |
|                      | 12.5 | 64.6 | 47.9 | 10.68 | 0.001* |
|                      | 25.0 | 86.4 | 57.9 | 31.94 | $>0.001^*$ |
|                      | 62.5 | 95.8 | 79.5 | 15.69 | $>0.001^*$ |
| $\sum \chi^2$       |       |       |       | 60.63 |     |
| F1♀ (RR♀ × SS♂) × RR♂ | 0.5 | 7.3 | 8.3 | 0.13 | 0.712 |
|                      | 2.5 | 23.9 | 24.3 | 0.06 | 0.937 |
|                      | 5.0 | 38.5 | 35.8 | 0.32 | 0.570 |
|                      | 12.5 | 58.3 | 47.9 | 4.17 | 0.041* |
|                      | 25.0 | 81.2 | 57.9 | 21.32 | $>0.001^*$ |
|                      | 62.5 | 94.8 | 79.5 | 13.75 | $>0.001^*$ |
| $\sum \chi^2$       |       |       |       | 39.64 |     |

Table 6. Direct test for deviation between observed and expected mortality for a monogenic model (df = 1).
Asian corn borer (ACB-AcR) resistance was controlled by a single locus31. A field-derived strain of diamondback moth fit best to polygenic inheritance to Cry1Ac 48. Resistance to Cry1F in a population of O. furnacalis revealed contributions from two different genes 50. Besides, resistance to Cry1Ac toxin in H. armigera initially was monogenic, but showed better fit to polygenic control as resistance increased39.

ACB-AhR laboratory-selected strain showed increased survival on Cry1Ah expressing leaf and silk tissues, although the surviving level was low in kernel tissue of transgenic maize. The Cry1Ah resistance was primarily autosomal, no maternal effects and under polygenic control. Dominance varies depending on toxin concentrations but resistance to Cry1Ah in ACB-AhR was functionally recessive in Bt-maize. These results should be useful in dealing the development of resistance management strategies, and the mechanism of inheritance and cross-resistance will be valuable to minimize the negative effects of cross-resistance on the durability of Bt toxins. Pyramided Bt crops that express two or more toxins that protect against same insect pest is considered a key tactic deployed to delay evolution of pest resistance to succeed in reducing refuges. One of the important components favoring the durability of pyramided Bt crops is that these plants express Cry proteins that lack cross-resistance. Our results show that ACB–AhR has high cross resistance with Cry1Fa toxin, indicating that careful studies should be done to determine cross-resistance since the absence of cross-resistance between two toxins derived from a resistant strain selected against only one-toxin could be not enough to make a decision to develop pyramided Bt crops. This should be taken in account for future development of genetically modified pyramided Bt crops.

Materials and Methods

Ethics statement. For insect collection in maize field, no specific permits were required by authorities.

None of the species used in this study are endangered or protected.
Insect strains. Susceptible and resistant strains of *O. furnacalis* used in the present study were obtained from laboratory colonies. A susceptible strain of Asian corn borer (ACB-BtS) was originally collected from corn fields in Liaoning Province. This population was established in the laboratory by rearing on artificial diet without exposure to insecticides for 23 generations, at the Institute of Plant Protection (IPP), Chinese Academy of Agricultural Sciences (CAAS), Beijing. A Cry1Ah-resistant strain (ACB-AhR) was established by 90 pairs of male and female larvae, collected from the fields in the Shaanxi province located in summer maize region of central China. The resulting offspring were used to establish a laboratory strain that was maintained using standard rearing techniques under selection pressure with increasing concentrations of Cry1Ah protein incorporated into artificial diet for several generations. The concentrations initially was 0.05 μg/g (Cry1Ah toxin/diet), and was increased to 0.1 μg/g in the 2nd generation, 0.2 μg/g in the 3rd generation, 0.4 μg/g in the 4th generation, 0.8 μg/g in the 5th–7th generation, 2.5 μg/g in the 8th–10th generation, 4.0 μg/g in the 11th–13th generation, and 6.0 μg/g in the 14th–23rd generation. The resistance characteristics were tested at the 48th generation. Larvae were reared at 27 ± 1 °C, 70–80% relative humidity (RH), with a photoperiod of 16:8 h light: dark (L:D).

Bt toxins. Cry1Ah expressed in Bt subsp. *kurstaki* strain Cry1Ah (HD-73) was trypsin-activated and used for selection and bioassays. Trypsin-activated Cry1Ab, Cry1Ac and Cry1F (98% pure protein), were produced by Marianne P. Carey, Case Western Reserve University, USA. Cry1Ee was expressed as a recombinant protein in *E. coli* 

Quantification of Cry1Ah toxin expressed in Bt maize tissues. To determine the Bt proteins concentration in the corn leaves, silks and kernel tissues, 10 samples from each tissue were taken randomly. The leaf, silk and kernel tissues were diluted at a rate of 1:10 (milligram sample: microliter PBST buffer) and fully ground by mortar and pestle. The concentration of Cry1Ah in maize leaves, silks, and kernels was confirmed by enzyme-linked immunosorbent assay (ELISA) with Cry1Ah detection kits as per the protocol of manufacturer (Shanghai MLBIO Biotechnology Co. Ltd.).

Plant tissue bioassays. ACB neonate larvae were tested on the leaves, silks and kernels of transgenic maize (Bt-21) expressing Cry1Ah protein and a non-Bt control. Resistance of ACB-AhR to Bt transgenic maize was evaluated with leaves, silks and kernel to homozygous parental strains (ACB-BtS & ACB-AhR and F1 progenies). The F1 progeny, corn borer pupae were separated by gender, and 150 Cry1Ah-selected females were pooled with 150 control males, and 150 control females were pooled with 150 Cry1Ah-selected males in mating cages. The leaves were cut into small pieces with a pair of scissors and placed in individual wells of a 24-well rearing plate. One neonate larva was infested in each well, and then covered with a piece of moistened filter paper and lid. Plates were then incubated at 27 ± 1 °C, 70% RH and a photoperiod of 16:8 h (L:D). The number of surviving larvae was recorded either daily or every 2 days, and fresh tissue was provided when required. The bioassays were evaluated in terms of insect survival. Each strain was assayed with 120 larvae and replicated three times. Silk tissue from 10 plants of Bt and non-Bt were collected and were placed into a plastic container with a disc of wet filter paper at bottom. Ten larvae (<12 h after hatching) were placed inside each container using a fine brush, and then covered with a piece of moistened filter paper and lid. Kernels were collected from field and were fast frozen with liquid nitrogen. 15–20 grains of kernel from Bt and non-Bt plants were placed into a container and were infested with 10 larvae. All rearing trays and containers were kept in an incubator 27 ± 1 °C with a photoperiod of 16:8 h (L:D) and 80% RH. The number of surviving larvae were recorded either daily or every 2 days, and fresh tissues were provided when necessary.

Diet bioassay. We used survival bioassays to evaluate susceptibility to Cry1Ah of the susceptible strain, the resistant strain, and progeny of mass crosses. Bt test solutions were serial diluted in water; we tested 6 to 9 concentrations from 0.2–12.5 μg/g (toxin/diet) for ACB-BtS and 5–800 μg/g (toxin/diet) for ACB-AhR. Dilutions were added to an agar-free semi-artificial diet to form a testing medium 

Inheritance experiments. To evaluate sex linkage and dominance the influence of sex on Cry1Ah inheritance was tested by bioassays of F1 progeny from reciprocal mass crosses between resistant and susceptible strains. The power of indirect tests for modes of inheritance is higher when the backcross progeny are originated from crosses between F1 progeny and the parental strain which is more dissimilar in susceptibility to the toxin 

In the reciprocal mass cross between resistant and susceptible strains, 100 resistant male pupae were pooled with 100 susceptible male pupae in one cross. In another reciprocal cross, we pooled 100 resistant male pupae with 100 susceptible female pupae. The estimation of number of genes involved in inheritance of Cry1Ah resistance was carried in bioassays of backcross progenies. Males and females of each F1 were backcrossed to resistant parental strains to produce four backcross populations and tested for susceptibility as described above.
Statistical analysis. For bioassays with multiple concentrations, probit regression using POLO-PC (LeOra Software 1987) was used to estimate the LC50 with 95% fiducial limits (FL), as well as the slopes of the concentration-mortality lines and their standard errors, Chi-square (χ²) values and resistance ratio (RR). Resistance ratios were calculated by dividing the LC50 of a particular strain by the LC50 for the susceptible strain. The data of F1 reciprocal crosses between ACB-AHR and ACB-BIS were also analyzed with the equality and parallel tests using Polo Plus.

To calculate dominance, the responses of F1 progeny were compared with parental susceptible and resistant strains. Dominance of Cry1Ah resistance at the different toxin concentrations was measured as reported by Bourguet, et al.20 and methods adapted from Liu and Tabashnik 199754. The values of degree of dominance (h) range from 0 that indicates completely recessive to 1 that indicates a fully completely dominant resistance and h value of 0.5 defines co-dominant or additive trait. The characters used in the calculation of dominance were larval survival after 7 days of infestation and the combination of the two traits.

The backcross generation obtained from mating F1 progenies with parental resistant strains was verified to estimate the number of genes affecting resistance by using the approach described by Tabashnik, et al.10 and using the method reported by Lande55. The monogenic inheritance model was tested to compare observed and expected mortality of the backcross progeny at different Cry1Ah concentrations by using the Chi-square test50,56. Expected mortality in the backcross can be calculated directly from experimental data following the method as described previously52,56. The survival percentage of larvae fed on Cry1Ah-corn and non-Bt corn was arcsine transformed to assure the normality of data prior to statistical analysis. The means difference in each corn tissue of Bt and non-Bt were tested using Fisher’s protected least significant difference (SAS Institute, Cary, NC. 2009).

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Author Contributions

K.L.H. and M.Z.S. designed the experiment. M.Z.S. and Y.D.Q. performed the experiment. Z.Y.W. and K.L.H. provided the insect, reagents and materials. M.Z.S. drafted the manuscript. M.Z.S., Y.D.Q., A.B., M.S. analyzed data. A.B., M.S., and K.L.H. reviewed and edited the manuscript.

Additional Information

Competing Interests: The authors declare that they have no competing interests.

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