Research Article

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# Evidence of field-evolved resistance to Cry1Ac-expressing *Bt* cotton in *Helicoverpa armigera* (Lepidoptera: Noctuidae) in northern China

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## Abstract

**BACKGROUND: Evolution of resistance threatens the continued success of transgenic crops expressing insecticidal proteins. One of the key factors for successful resistance management is the timely implementation of monitoring programmes to detect early changes of resistance allele frequency in field populations. $F_1/F_2$ screen, dose–response bioassays and field survey were used to monitor resistance to the Cry1Ac-expressing cotton in a field population of *Helicoverpa armigera* (Hübner), the primary target of transgenic *Bt* cotton in China.**

**RESULTS: Field survey showed an increased trend of egg populations of *H. armigera* on *Bt* cotton in the Qiuxian area from 2003 to 2007. By using the $F_2$ screening procedure, the resistance allele frequency in the Qiuxian (Hebei, China) population of *H. armigera* collected during 2007 was estimated to be 0.075 (95% CI: 0.053–0.100), which was 12 times greater than that estimated 9 years ago. Dose–response bioassay with the field population collected from the same area showed a significant resistance level (11-fold) to Cry1Ac toxin compared to a laboratory susceptible strain.**

**CONCLUSION: This study documented a case of field-evolved resistance in *H. armigera* after several years of intensive planting of *Bt* cotton. Proactive tactics must be adopted to prevent further increase of resistance gene frequency in the Qiuxian region.**


**Keywords:** resistance allele frequency; transgenic *Bt* cotton; *Helicoverpa armigera*; $F_1$ screen; $F_2$ screen; dose–response bioassay; resistance monitoring

## 1 INTRODUCTION

Transgenic cotton expressing *Bacillus thuringiensis* Berliner insecticidal protein (*Bt* cotton) provides a safe and effective method for controlling lepidopteran pests of cotton throughout the world. However, like conventional chemicals, transgenic plants are not immune to the risk of resistance development in target insects. The benefit of transgenic *Bt* cotton technology might be short-lived if proactive tactics are not taken to minimise the risk of resistance development. Field control failures or reduced control efficacies of transgenic *Bt* corn due to resistance development have been documented in two cases, the fall armyworm, *Spodoptera frugiperda* (Smith), to Cry1F corn in Puerto Rico in 2006[1] and the African stem borer, *Busseola fusca* (Full.), to Cry1Ab corn in South Africa in 2007.[2] In addition, laboratory selection has produced *Bt*-resistant strains of many pests,[3] including resistance to *Bt* toxin[4–6] and to *Bt* cotton[7] in *Helicoverpa armigera* (Hübner). Furthermore, field populations of diamondback moth, *Plutella xylostella* (L.), and a greenhouse population of cabbage looper, *Trichoplusia ni* (Hübner), have developed resistance to *Bt* sprays,[8,9] and field populations of *H. armigera* developed a relatively high resistance frequency in China.[10,11] These examples demonstrated that the target insects of *Bt* plants are capable of developing resistance to either or both insecticidal crystal proteins (ICP) and *Bt* crops.

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To ensure a successful resistance management programme, a cost-effective monitoring technique is essential to accurately detect rare resistance gene alleles in field insect populations.[12,13] Several methods have been used or suggested for detecting resistance to *Bt* crops.[14] Among these, the $F_2$ screen and $F_1$ screen methods have been proved to be effective and sensitive methods for detecting rare resistant alleles at the early stage of resistance evolution.[15–17] The $F_2$ screen includes screening the $F_2$ progeny of iso-line families developed from wild mated females or pair-matings of wild collected males and females,[17] while the $F_1$ screen involves screening the $F_1$ offspring derived from single-pair crosses between field-collected individuals and a laboratory strain with known resistance.[16] The $F_1$ screen method has been used only for *H. virscenas* and sugarcane borer, *Diatraea saccharalis* F., in the USA[16,18] and for *H. armigera* in China,[10,19] while the $F_2$ screen was more frequently used to estimate the frequency of alleles conferring resistance to *Bt* in several field crop insects.[11,17,20–26] Compared to the $F_1$ and $F_2$ screening procedures, dose–response bioassay is a relatively inexpensive and simple method to determine relative susceptibility to insecticides. This method is usually used for confirming the presence of resistance in insect populations or for verifying if a field control failure is due to resistance development.[14] However, the dose–response method is not sensitive enough to detect resistance at low allele frequencies (e.g. $P < 10^{-3}$). This method may not detect recessive resistance alleles until their frequencies are too high for countermeasures to be effective. Information generated from the dose–response method, such as reports of poor field control, would not be timely in managing resistant populations for *Bt* crops. Consequently, the dose–response method, when used alone, has not been recommended for monitoring resistance to *Bt* crops.[16,17,27]

In this paper, we first present a 5-year field survey that showed an increased trend of egg populations of *H. armigera* on *Bt* cotton in Qiuxian area from 2003 to 2007. Second, we show the results of an $F_2$ screen for detecting *Bt* resistance alleles in *H. armigera* populations collected in Qiuxian County, Heibei Province in northern China during the 2007 cotton growing season, and compare and analyse the results from $F_1$ screen and $F_2$ screen methods in 2007. Third, we provide the results of dose–mortality bioassays and demonstrate a measurable level of resistance development in field populations of *H. armigera* in this area. Finally, we present and discuss a significant shift of *Bt* resistance allele frequency in *H. armigera* in this region during the last nine years. Results of the multiple-year monitoring suggested that proactive tactics must be adopted in this region to prevent further increase of *Bt* resistance gene frequency in *H. armigera*.

## 2 MATERIALS AND METHODS

### 2.1 Laboratory *Bt*-susceptible and resistant strains of *Helicoverpa armigera*

A susceptible strain (YCS) of *H. armigera*, collected originally in July 1991 from a cotton field near Yanshi City (latitude 34.73 °N and longitude 112.77 °E) in Henan, China, had been reared for 160 generations on artificial diet without exposure to any insecticides or *Bt* toxins. In this study, the susceptible strain was used for verifying the presence of Cry1Ac toxin in *Bt* cotton plants and as a reference for determining the relative susceptibility of field insect populations.

The resistant strain (YCR) was developed from a field population collected originally from the same location as the YCS strain. The colony was first reared on a *Bt*-free diet for 53 generations, and was thereafter subjected to continuous selection with *Bt* cotton leaves (R19/33B expressing Cry1Ac toxin). After being selected for an additional 42 and 88 generations, the colony demonstrated ≈1700-fold and ≈7000-fold resistance to Cry1Ac toxin.[7,10] The results of a genetic study demonstrated that the resistance in the YCR strain of *H. armigera* was controlled by an autosomal, incompletely recessive gene.[28]

### 2.2 Field survey and insect collections for $F_2$ screen and dose–response bioassays

*Helicoverpa armigera* moths were collected during 2007 in Qiuxian County, Hebei, China, where Cry1Ac-expressing *Bt* cotton had been commercially planted since 1998.[24] The area of *Bt* cotton had increased every year since 1998. By 2001, conventional cotton had been completely replaced by *Bt* cotton, which accounted for 74% of the total cropping land in the region (unpublished data). The resistance allele frequency in the population of *H. armigera* collected during 1999 in this county was estimated at 0.0058 (95% CI, 0–0.0187) by using an $F_2$ screen.[24]

Field surveys for egg production of second, third and fourth generations of *H. armigera* on *Bt* cotton plants were conducted yearly from 2003 to 2007. During the growing season from June to September, egg densities of second, third and fourth generations were surveyed each year from 2003 to 2007 by adopting a diagonal sampling method. Twenty cotton plants were selected as a sample and 5 samples per 100 $m^2$ were examined for egg density of each generation. The survey was conducted at 3-day intervals, and the numbers of eggs per 100 plants were recorded for each generation.

To conduct $F_2$ screening in 2007, a field population of *H. armigera* was collected from 16 June to 20 June in Qiuxian County area. Mated moths of the second field generation were collected from two light traps, placed >2 km apart. Each trap covered a large open area of Cry1Ac cotton. The field-collected gravid females were reared separately in the laboratory to produce $F_2$ isofemale lines for screening *Bt* resistance alleles. $F_2$ progenies from each iso-female line were screened with *Bt* cotton leaves using the method described by Liu *et al.*[10] A large number (c.a. 250) of field-collected moths were reared separately to produce $F_1$ larvae for the dose–response bioassays.

### 2.3 *Bt* insecticide protein

Cry1Ac protoxin, supplied by Monsanto Company (St Louis, MO, USA), was a lyophilised (freeze-dried) formulation of MVP containing 21% Cry1Ac protoxin of *kurstaki* isolate of *B. thuringiensis*. The protoxin was encapsulated through transgenic *Pseudomonas fluorescens* Migula (Mycogen Corporation, San Diego, CA, USA). Distilled water was used to dissolve the Cry1Ac, and seven concentrations were prepared for determining the susceptibility of the field-collected population and the YCS strain.

### 2.4 Source of transgenic *Bt* cotton used for $F_2$ screen

The *Bt* cotton used for $F_2$ screen was Xinmian33$^B$ (NuCOTN33$^B$, Bollgard, a commercial variety expressing *Bt* Cry1Ac protein purchased from Monsanto Far East Ltd (Beijing, China). Cotton leaves collected from the plants at the seedling stage (6- to 7-weeks-old) grown in a clear-roofed greenhouse were used in the $F_2$ screen for identifying *Bt* resistance alleles. Expression of Cry1Ac toxin in *Bt* cotton was verified by examining the larval mortality of the YCS strain as described by Meng *et al.*[29] Only those plants that produced sufficient levels of Cry1Ac toxin to kill all susceptible



  

*H. armigera* were used in the $F_2$ screen. The non-*Bt* conventional cotton, SM-12, was provided by Tai Cang Elite seed station (Jiangsu Province, China) and was used as control.

### 2.5 $F_2$ screen for identifying resistance alleles

The $F_2$ screening procedures for detecting *Bt* resistance alleles in *H. armigera* involve (1) collecting mated adult females of *H. armigera* from cotton fields to establish iso-female lines in the laboratory; (2) rearing $F_1$ progeny and sib-mating $F_1$ adults in each iso-female line to develop $F_2$ iso-female lines; (3) screening $F_2$ neonates on *Bt* cotton leaves; and (4) confirming resistance on *Bt* cotton leaves.[17]

Female adults of *H. armigera* were collected before sunrise and placed individually in clear plastic cups (250 mL) covered with white gauzes for oviposition. A moistened cotton pledget with 4% sugar solution was put in each cup to feed the adults. To establish iso-female lines, $F_1$ egg masses from each female were collected daily and were sanitised by soaking eggs in 5% formaldehyde solution for 3 min. To avoid potential multiple matings in the field-collected females, the adult females were anatomised after oviposition to confirm that they contained only one spermatophore in their *bursa copulatrix*. The $F_1$ progeny derived from females mated only once were used. All larvae, adults and eggs were held at $28 \pm 1\,^{\circ}C$, 70–80% RH, and a photoperiod of 14 : 10 h light : dark. $F_1$ neonates of each line were reared on artificial diet[30] in plastic Petri dishes (5 × 1.5 cm). Pupae from the same line were kept in a large cage (23 × 23 × 30 cm). After emergence, $F_1$ male and female moths of each line were supplied with 4% sugar solution as supplemental nutrition and were allowed to carry out mass sib-mating within the cage. After 1–2 d in the adult mating cages, the adults of each line were transferred to a plastic container (23 × 16 × 15 cm) covered with white cheesecloth for collecting egg masses. F2 egg masses for each iso-line were collected daily. Neonates (<6-h-old) were screened on *Bt* cotton leaves by using the procedures described by Liu *et al*.[10] After 5 d feeding on *Bt* cotton, survivors were weighed and scored for developmental stages. If the survivors grew and developed at the same rate as the resistant strain (reached ≥0.6 mg body weight and at least mid-second instar), the line was considered as a potential positive line and its wild female parent was considered to carry a major resistance allele.[10]

To minimise false positive lines, the survivors of the potential positive lines were reared on artificial diet, and the $F_4$ progenies were re-screened on *Bt* cotton leaves to verify if the lines carried alleles for resistance to *Bt* cotton. The procedures for the resistance confirmation were the same as used in the $F_2$ screen described above. Larval mortality on non-*Bt* conventional cotton was determined using the same procedures as used in the $F_2$ screen.

Two statistical calculations were used to analyse results from an $F_2$ screen and to estimate the expected *Bt* resistance allele frequency in the field population of *H. armigera* with 95% credibility interval.[17,31] The probability of missing a major resistance allele that was not detected in an iso-line if one had been present ($P_{NO}$) were computed using the method described by Stodola and Andow.[32]

Resistance allele frequencies estimated from 1999–2007 were statistically analysed using SAS Proc GLM and Proc Reg procedures.[33] Mean separation was conducted using SAS Proc Means/LSD or Ls means separation programmes at $P < 0.05$.

### 2.6 Susceptibility to Cry1Ac toxin in a field population of *Helicoverpa armigera* collected in 2007

To determine the relative susceptibilities of field populations of *H. armigera* to Cry1Ac toxin after several years of extensively planting of transgenic *Bt* cotton, female adults were collected from two locations (traps) of *Bt* cotton field in Qiuxian. Approximately 250 mated females were collected from traps and allocated in five boxes (23 × 16 × 15 cm) covered with white gauze. A moistened cotton pledget saturated with 4% sugar solution was placed in each box to provide food and moisture for the adults. The moths were transferred to the laboratory for oviposition, and $F_1$ egg masses were collected daily. The egg masses were sanitised by soaking the eggs in 5% formaldehyde solution for 3 min. All adults and eggs were held at 28 °C, 70–80% RH and 14 : 10 h light : dark photoperiod. Approximately 350 neonates were used for a dose bioassay to determine the susceptibility to Cry1Ac toxin.

Neonates (<6-h-old) derived from the field population and the YCS susceptible strain were assayed on wheat germ diet containing Cry1Ac at 0 (control), 0.3125, 0.625, 1.25, 2.5, 5, 10, 20 μg $mL^{-1}$ diet. The artificial diet used for bioassays was a modified version of Shen and Wu[30] based on the recipe of Brewer.[34] Diet mixed with Cry1Ac was dispensed into 30-mL cups. One neonate was transferred into each cup with a fine brush. Each treatment (concentration) included four replicates, and each replicate consisted of 10 individually reared insects. Treated larvae were maintained at $28 \pm 1\,^{\circ}C$, 70–80% RH and 14 : 10 h light : dark photoperiod. Larval mortality was recorded after 5 d. $LC_{50}$ values were estimated by using POLO software (LeOra Software 2002). Resistance ratios (RRs) were calculated based on the $LC_{50}$ value of the field population divided by that of the susceptible laboratory strain. Resistance levels were classified based on the Shen method[30] as susceptible if RR = 3- to 5-fold; minor resistance if RR = 3–5; low resistance level if RR = 5.1- to 10-fold; medium resistance level if RR = 10.1- to 40-fold; high resistance level if RR = 40.1- to 160-fold; and extremely high resistance level if RR > 160-fold.

## 3 RESULTS

### 3.1 Egg populations of *Helicoverpa armigera* on *Bt* cotton

Field survey showed that there was an obviously increased trend of egg populations of *H. armigera* on *Bt* cotton in Qiuxian area from 2003 to 2007 (Fig. 1). Between 2003 and 2007, the number of eggs in 100 *Bt* plants increased from 18 to 314 (or 1600%) for the second generation, and from 46 to 138 (200%) for the third generation, and from 38 to 192 (or 400%) for the fourth generation. In 2007, the egg density of the fourth generation was not higher than that of previous year (2006). Relatively low temperature and higher precipitation during the season in 2007 might have contributed to the decrease in the egg density.

### 3.2 *Bt* resistance allele frequency for the *Helicoverpa armigera* population collected during 2007 crop growing season

A total of 320 females were collected during 2007, and 146 (~46%) female moths laid fertile eggs. Among these, 137 lines (43%) produced enough $F_2$ progeny for resistance screening. Among the 137 screened lines, an average of 18.1 ± 0.8 $F_1$ males and 15.8 ± 0.7 $F_1$ females were obtained for each iso-line. An average of 105.1 ± 1.45 $F_2$ neonates per line was screened for *Bt* resistance. In the $F_2$ screen, neonates of 30 $F_2$ iso-lines died after feeding on *Bt* cotton leaves for 5 d. Among the 107 survival lines in the $F_2$ screen (78.1%), 45 lines had survivors that reached the criterion[10]



  

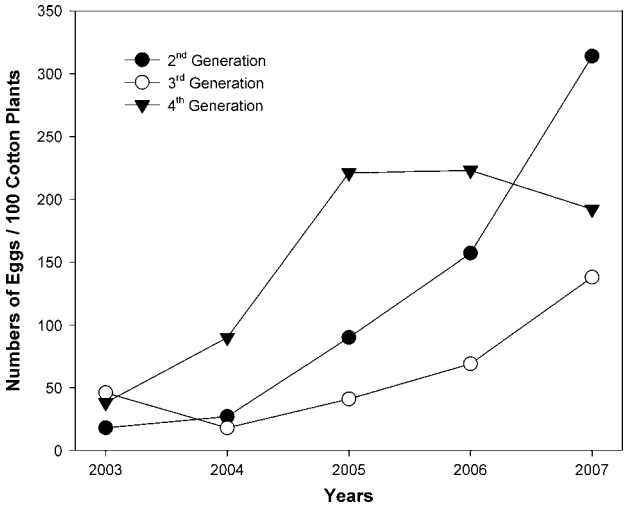


**Figure 1.** Egg densities of second, third and fourth generations of *Helicoverpa armigera* during growing season from 2003 to 2007 in Qiuxian County, northern China. The data were collected by Qiuxian County Plant Protection Station.

for potential positive lines with a larval body weight of $\geq$0.6 mg larva$^{-1}$ and developed into the second instar. Larval mortality of *H. armigera* on conventional non-*Bt* cotton larvae was 13.6%.

To confirm if the potential positive lines identified in the $F_2$ screen actually possessed resistance alleles, $F_2$ larvae of these potential positive lines were reared in diet for further tests. Thirty-six of the 45 potential positive lines identified in the $F_2$ screen produced enough $F_4$ neonates for resistance confirmation. The other nine lines were lost due to pathogenic infection and diet contamination. In the resistance confirmation, approximately 120 $F_4$ neonates for each line were re-tested for survival on *Bt* cotton plants. The re-tests showed that the survival of all 36 lines reached the criterion for resistant insects and were confirmed as true positive lines. Thus, the frequency of resistance alleles was estimated to be 0.075 with a 95% CI of 0.053–0.100. More than 70% lines of the $F_2$ screen had a detection probability >90% (Fig 2). The average experiment-wise detection probability was 0.938.

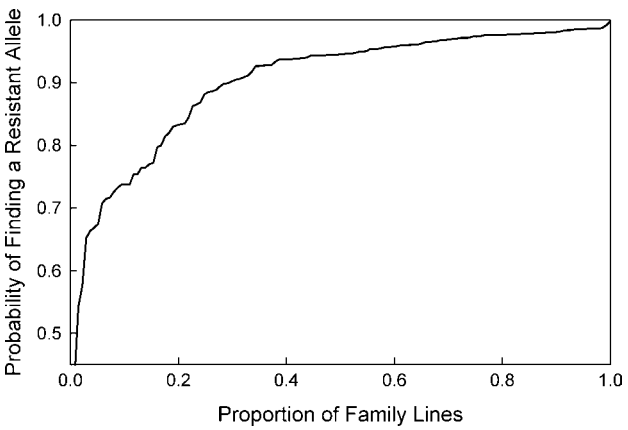


**Figure 2.** The cumulative probability density function (PDF) of detecting a resistance allele if one actually existed, e.g. 1 – probability of a false negative ($P_{NO}$).

### 3.3 Susceptibility to Cry1Ac toxin in the field population of *Helicoverpa armigera* collected from Qiuxian area during 2007

The $LC_{50}$ for the field population of *H. armigera* was 2.23 μg mL$^{-1}$ with a 95% CI of 1.72–2.86 μg mL$^{-1}$, while it was 0.20 μg mL$^{-1}$ with a 95% CI of 0.16–0.24 μg mL$^{-1}$ for the susceptible strain (Table 1). The slope of the dose–response was 1.6 ± 0.2 for the field population and 2.5 ± 0.3 for the susceptible strain. The 11-fold difference in the values of the $LC_{50}$ between the field and susceptible populations was significant based on the non-overlapping of the 95% CIs.

## 4 DISCUSSION

The monitoring programme for resistance to *Bt* cotton in *H. armigera* was initiated in Qiuxian County region during 1999, one year after *Bt* cotton was commercially planted in this region. During the nine-year (1999–2007) monitoring, resistance allele frequency to Cry1Ac cotton in field populations of *H. armigera* was determined using $F_2/F_1$ screening procedures (Table 2).[10,11,24] The initial estimated resistance allele frequency for the population of *H. armigera* collected during 1999 was 0.0058.[24] The resistance allele frequencies for the populations collected during 2003–2005 ranged from 0.012 to 0.03,[11] slightly but consistently greater than that estimated in 1999. Compared to the previous year estimation, the *Bt* resistance allele frequency increased considerably (6-fold) in 2006, but remained relatively unchanged during the 2006–2007 seasons.[10] Both the $F_1$[10] and $F_2$ screening methods were used to examine *Bt* resistance alleles for the populations collected during 2007. The estimated resistance allele frequency for the 2007 populations was 0.107 with a 95% CI of 0.065–0.137 based on the $F_1$ screen[10] and was 0.075 with a 95% CI of 0.053–0.1 based on the $F_2$ screen in this study. The resistant allele frequencies estimated using two different methods were similar based on the large overlapping of their 95% CIs. Our nine-year (1999–2007) monitoring programme demonstrated that there was a significant increase in *Bt* resistance allele frequency for the Qiuxian population of *H. armigera* after the commercialization of transgenic *Bt* cotton in this area.

Dose–response bioassay showed that the population of *H. armigera* collected during 2007 was 11-fold less susceptible to Cry1Ac toxin than the susceptible strain. A field population with >10-fold resistance to insecticide is normally considered as a sign of resistance development in the field,[35] and the result from the dose–response bioassay indicated that a substantial resistance level to Cry1Ac toxin had developed in the field population of *H. armigera* in Qiuxian County. More importantly, a field survey demonstrated that the egg population of *H. armigera* on *Bt* cotton plants increased considerably during 2003–2007 in this area, suggesting that field resistance in *H. armigera* had involved after several years of intensive planting of *Bt* cotton in this area. Based on our knowledge, this was the first documented report that a significant shift of *Bt* resistance allele frequency occurred in a target insect population of *Bt* cotton after several years of intensive planting of transgenic *Bt* cotton in an area. Field resistance to *Bt* cotton was also suspected in *Helicoverpa zea* (Boddie) in the southern region of the United States.[36] However, the conclusion regarding field-evolved resistance in *H. zea* is questionable because field control failures or reduced efficacies of *Bt* cotton due to resistance development have not been reported in this area.[37]

The $F_1$ screen and $F_2$ screen have been reported to be efficient ways to detect rare recessive alleles for field insect



  

**Table 1.** Dose response (5d mortality) of neonates of field-collected population and laboratory susceptible strain of *Helicoverpa armigera* to Bt Cry1Ac toxin incorporated into artificial diet

| Population | *n* | $LC_{50}$. (μg $mL^{-1}$) | $RR_{LC50}$[b] | 95% CI Lower | 95% CI Upper | $LC_{90}$ (μg $mL^{-1}$) | $RR_{LC90}$ | Slope (± SE) |
|---|---|---|---|---|---|---|---|---|
| Laboratory susceptible strain[a] | 320 | 0.195 | 1 | 0.158 | 0.241 | 0.644 | 1 | 2.471 (± 0.317) |
| Field-collected population | 320 | 2.233 | 11.451 | 1.716 | 2.860 | 13.666 | 12.2 | 1.629 (± 0.163) |

[a] The baseline data of susceptible strain were contributed by Dr Zhou Xiaomei (unpublished data).
[b] RR: $LC_{50}$ of the field population divided by the $LC_{50}$ of the susceptible strain.

**Table 2.** Estimated frequencies of resistance alleles to *Bt* cotton with 95% CIs in field population of *Helicoverpa armigera* from 1999 to 2007, in Qiuxian County (Hebei, China)

| Year, and reference | Screening method | Screening material | No. lines screened | No. positive lines | Frequency (95% CI) |
|---|---|---|---|---|---|
| 1999[24] | $F_2$ | *Bt*-cotton plants | 128 | 2 | 0.0058 (0–0.0187) |
| 2003[11] | $F_2$ | *Bt*-cotton plants | 105 | 4 | 0.0119 (0.0039–0.0243) |
| 2004[11] | $F_2$ | *Bt*-cotton plants | 42 | 4 | 0.0297 (0.0099–0.0606) |
| 2005[11] | $F_2$ | *Bt*-cotton plants | 131 | 7 | 0.0154 (0.0067–0.0277) |
| 2006[10] | $F_1$ | *Bt*-cotton leaves | 127 | 24 | 0.094 (0.044–0.145) |
| 2007[10] | $F_1$ | *Bt*-cotton leaves | 135 | 29 | 0.107 (0.055–0.159) |
| 2007 | $F_2$ | *Bt*-cotton leaves | 137 | 36 | 0.075 (0.053–0.100) |

populations.[14,16,17] The major advantages of these two methods include the ability to detect recessive and partial recessive alleles, the collection of genetic information from wild populations, and the ability to apply rigorous statistical procedure to estimate allelic frequencies.[38] The application of the two methods requires a reliable diagnostic technique that can identify the phenotypic resistance in the $F_1$ or $F_2$ progeny. To minimise environmental influence in *Bt* resistance screening, in the current study we used the fully expanded *Bt* cotton leaves as the screening materials instead of whole *Bt* cotton plants as used in our previous screenings. A similar method has been successfully used for screening *Bt* resistance alleles in the sugarcane borer, *Diatraea saccharalis* (F.).[39] We noted that the use of *Bt* cotton leaves for screening *Bt* resistance in *H. armigera* could significantly reduce the space required and cost (e.g. material and labour) for the screening. The use of leaf tissues as the screening materials also minimised the impact of any variation in *Bt* protein expression levels that can occur within whole plants.[39]

During 2007, a total of 135 $F_1$ iso-lines of *H. armigera* developed by mating resistant females with field-collected males were successfully examined for *Bt* resistance using the $F_1$ screen.[10] The resistance allele frequency estimated based on the $F_1$ screen was 0.107 with a 95% CI of 0.055–0.159. The current study using the $F_2$ screening procedures identified 36 out of 137 lines carrying resistance alleles. The *Bt* resistance frequency based on the $F_2$ screen was 0.075 with a 95% CI of 0.053–0.1. The similar results of the $F_1$ and $F_2$ screen suggest that both methods are effective in detecting *Bt* resistance alleles. Cost is an important consideration of a resistance monitoring model. Compared to the $F_2$ screen, the $F_1$ screening method should greatly reduce costs associated with screening *Bt* resistance alleles and thus facilitate monitoring of *Bt* resistance in *H. armigera*.

The $F_2$ screen in this study revealed a slightly lower frequency (0.075) than that (0.107) detected in the $F_1$ screen.[10] The difference observed in the $F_2$ screen might be associated with the loss of nine potentially positive lines during the resistance verification, possibly due to pathogenic infection, while such a loss did not occur in the $F_1$ screen. Therefore, we considered that the allele frequency from $F_2$ screen might be conservative.

Several factors could be associated with the significant increase of *Bt* resistance allele frequency in *H. armigera* in the Qiuxian area. Prior to the introduction of *Bt* cotton varieties, *Bt* sprays had been a main tool for controlling *H. armigera* in Qiuxian County, especially during the 1990s.[24] In 1998, *Bt* cotton expressing Cry1Ac protein was first planted in this region to control this devastating cotton pest.[24] Since then, the *Bt* cotton growing area has rapidly expanded in this area, and reached 100% *Bt* cotton in 2001 (unpublished data). The long-term and large-scale adoption of *Bt* products and the use of single-toxin (Cry1Ac) expressed cotton likely applied a heavy selection pressure on the target insects and prompted rapid resistance development in this region. The second major reason for the rapid increase in *Bt* resistance frequency might be the use of non-high dose expressed *Bt* cotton varieties. Several studies have shown that current commercial *Bt* cotton varieties do not produce a high dose against *H. armigera*.[11] This non-high dose expression of *Bt* toxins could increase the survival of the individuals carrying resistance alleles, which might allow resistant allele(s) to accumulate in the field populations. In addition, lack of refuges could be another factor that played an important role for the resistance development in this area. Although many other host plants of *H. armigera* (such as soybean, peanut, corn) could act as potential natural refuges to provide susceptible populations,[40] the area of such refuge crops in this region was very limited.[9] Studies have also shown that these crops provided susceptible refuge populations that were not as effective as non-*Bt* cotton.[41]

In spite of no evidence to show that there would soon be an outbreak of *H. armigera* in this region, our results provided a precaution that the resistance in the primary target, *H. armigera*, to *Bt* cotton poses a serious threat to the long-term success of transgenic *Bt* cotton in the northern cotton region of China. Effective management plans must be developed and implemented to prevent or minimise further resistance development to *Bt* cotton



  

in *H. armigera* in the region. These resistance management plans may include use of the high-dose-refuge strategy as used in the United States, dual/multi-*Bt* toxin-producing cotton,[42] and other non-*Bt* control tactics such as biological, chemical, and cultural practices.

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