

**MONITORING OF RESISTANCE DEVELOPMENT TO BT COTTON IN FIELD POPULATIONS OF  
*HELICOVERPA ARMIGERA* (LEPIDOPTERA: NOCTUIDAE)**

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**Abstract**

Evolution of resistance threatens the continuing success of transgenic crops expressing insecticidal proteins. One of the key factors for a successful resistance management is the timely implementation of monitoring program to detect early changes of resistance frequency in field populations and implementation of resistance management tactics. F<sub>1</sub> and F<sub>2</sub> screens, designed for accurately detecting rare resistance alleles, were used to estimate the frequencies of alleles conferring resistance to the Cry1Ac-expressing cotton in a field population of *Helicoverpa armigera*, which is closely related to the cotton bollworm and tobacco budworm in US. The potential mechanism for Bt resistance in *H. armigera* was associated with modified Bt receptor encoded by disrupted or truncated cadherin genes in the Bt-resistant strains. By using the F<sub>2</sub> and F<sub>1</sub> screening procedures, the resistance allele frequency in field population of *H. armigera* collected during 2007 in China was estimated to be 0.075 (95% CI: 0.053 - 0.100), which was 12-times greater than that estimated 9 years ago. This study provided valuable information for understanding field-evolved resistance in *H. armigera* after several years of intensive planting of Bt cotton expressing Cry1Ac. Our results also suggested that proactive tactics must be adopted to prevent further increase of resistance gene frequency.

**Introduction**

Since the first introduction in the late 1990s, transgenic cotton expressing *Bacillus thuringiensis* (Bt) insecticidal proteins has become the primary management strategy worldwide for controlling lepidopteran insects on cotton. Continuous and extensive adoption of Bt cotton allows insects to receive constant exposure to the insecticidal toxins, and consequently may hasten resistance evolution in pest populations. *Helicoverpa armigera* is a devastating pest on cotton in many Asian countries. In 1997, Bt cotton was first planted in China for managing *H. armigera*. Effective control of the insect by Bt cotton had prompted rapid increase of Bt cotton growing area since then. Laboratory selections conducted in China, Australia, and India demonstrated the capability of *H. armigera* to develop high levels of resistance to Cry1Ac toxin (Meng et al., 2004; James, 2007; Akhurst et al., 2003; Kranthi et al., 2000; Liang and Guo, 2000).

To ensure the durability of Bt cotton technology as an effective pest management tool, resistance monitoring is essential to provide information on early changes in resistance allele frequency in field populations. F<sub>2</sub> screen (Andow and Alstad, 1998) is a highly sensitive technique for detecting rare resistance alleles. The method involves collection of a large number of gravid females in order to establish isofemale lines from field populations. The F<sub>1</sub> adults from each isoline are allowed to sib-mate to produce F<sub>2</sub> progenies for screening resistant genotypes on Bt plants or on diet containing Bt toxins. Theoretically, if a field-collected gravid female carries a resistance allele, one in every 16 (6.25%) of its F<sub>2</sub> progeny, derived from sib-mating the F<sub>1</sub> offspring, should be homozygous for Bt resistance and be capable of surviving Bt treatment. Through back-calculation of the frequency of resistance-allele

carrying family lines, the frequency of the resistance allele in the sampled population can be estimated (Andow and Alstad, 1998 and 1999; Zhao et al., 2002).

In this study, we chose a cotton field in Qiuxian County (Hebei, China) for Bt resistance monitoring in *H. armigera*. This location has long history of Bt spray since 1991 and Bt cotton planting since 1998. During recent years, Bt cotton has been the predominant crop in this county, accounting for 62–73% of its total farmland area. The extensive planting of Bt cotton and the unique insect-crop associations in Qiuxian County make it an ideal sentinel area for monitoring Bt resistance.

During 1999, one year after Bt cotton was commercially planted in this county, an  $F_2$  screen was used to estimate the frequency of alleles conferring resistance to Bt cotton in field population of *H. armigera*. The resistance allele frequency was estimated to be 0.0058 (He et al., 2001). Considering the relatively high levels of resistance allele frequency detected in the early stage of Bt cotton use in field populations of *H. armigera* in Qiuxian County, we conducted another extensive search during 2003–2007 to determine if Bt resistance allele frequency in this major target pest had changed after several years of high adoption of this transgenic crop within this area.

### **Materials and Methods**

**Bt-susceptible and -resistant strains.** A Bt-susceptible strain was originally collected from non-cotton fields in 1991, and had been maintained on a meridic diet for >145 generations without exposure to any insecticides including Bt toxins. This strain was used for growth rate experiments and for verification of Cry1Ac protein expression in Bt cotton plants. A resistant strain was developed from a population originally collected from a cotton field in 1991. After being selected with Bt cotton leaves ( $R_{19}/33^B$  expressing Cry1Ac protein) (Meng et al., 2004) for 46 generations, the insect developed > 7000- fold resistance to Bt.

**Collection of female moths.** Collections of the second field generation of female *H. armigera* were performed each year by using two black light traps from 2003 to 2007 in Qiuxian County (Hebei, China). Field collected moths were individually placed in 250-ml-plastic cups (one moth/cup) covered with white cheesecloth for oviposition. A cotton pad moistened with 4% sugar solution was placed in each cup to provide moisture and food for the adults. All adults, eggs, and larvae were maintained at  $28\pm 1^\circ\text{C}$ , 70–80% RH, under a photoperiod of 14:10 h (L:D).

**Transgenic Bt cotton.** Xinmian 33<sup>B</sup> (NuCOTN33<sup>B</sup>, Bollgard<sup>®</sup>), a commercial variety expressing the Cry1Ac protein, was provided by Monsanto Far East Ltd (Beijing, China). The cotton was planted in pots (17 cm diameter, 15 cm high) that were maintained in a greenhouse. Each pot was planted with four to six cotton plants. To ensure that there was a sufficiently high level of Bt toxin expressed in the plants to kill all Bt-susceptible and -heterozygous genotypes of *H. armigera*, Bt Cry1Ac protein expression was verified by infesting the cotton plants with the susceptible strain as described by Meng et al. (2000). The cotton plants that caused 100% larval mortality were considered to be high Bt expressing and were subsequently used in the  $F_2$  screen for Bt resistance. A conventional non-Bt cotton variety, Sumian12, provided by Tai Cang Elite Seed Station (Jiangsu, China), was used as the control.

**$F_2$  Screen.** The  $F_2$  screening procedures used for detecting Bt resistance alleles in *H. armigera* were similar to the methods described by (He et al., 2001). The  $F_2$  screen included 1) collecting wild gravid females from cotton fields; 2) rearing  $F_1$  offspring for each isofemale line; 3) sib-mating  $F_1$  adults; 4) screening  $F_2$  neonates on intact Bt cotton plants; and 5) confirming resistance on Bt cotton plants.

To establish isofemale lines,  $F_1$  egg masses produced from each female were collected daily, and neonates from each line were reared on a meridic diet in a plastic Petri dish (5 x 1.5 cm).  $F_1$  adult males and females of each line were counted and placed in a large cage (23×23×30 cm, supplied with 4% sugar solution) for mass sib-mating. After one to two days, the adults of each line were transferred to plastic containers (23×16×15 cm) covered with white cheesecloth for oviposition. The  $F_2$  egg masses were collected daily. Neonates (<6 h old) were screened on Bt cotton plants for resistance.

In the  $F_2$  screen, more than 300  $F_2$  neonates of each isoline were released onto four Bt cotton plants in a pot in the greenhouse. To prevent larvae from escaping and moving to other pots, the pot was placed inside a cage (30×30×65 cm) covered with 0.125 mm nylon mesh. Larval mortality, body weight, and growth were recorded on the 5<sup>th</sup> d after inoculation. Larval instar was determined based on head capsule and body size. An isoline was considered to carry a

resistance allele if its surviving larvae had a body weight of  $\geq 0.8$  mg/larva and had developed to at least the second stadium (He et al., 2001). These growth and developmental rates on Bt cotton are similar to the rates of susceptible insects on non-Bt cotton. The surplus larvae not exposed to cotton plants from the potential lines carrying resistance alleles were reared on a meridic diet to the  $F_4$  generation. The  $F_4$  progenies of these lines, if available, were rescreened on Bt cotton plants for resistance verification. Larval mortality on non-Bt cotton was determined using the same procedures as used in the  $F_2$  screen.

**F<sub>1</sub> Screen.** An improved  $F_1$  screen method was used in this study. This method was originally developed by Gould et al. (1997). In brief, field-collected males (ss, rs, or rr genotype) were individually mated to virgin females ( $r_1r_1$ ) of homozygous resistance strain.  $F_1$  progeny were tested on Bt cotton leave using the same method described above. If the males carry homologous resistant alleles (rr), their progenies will survive on Bt cotton leave because they inherited an  $r_1$  allele from their mother and a field-derived resistance allele r from their father. If a male carries both r and s alleles, approximately 50% of their progenies will survive on Bt cotton. Based on this assumption, we can infer whether the male carried the resistance allele. Therefore,  $F_1$  survivors were considered resistant individuals if they reached the same growth rate of the resistant strain on Bt cotton leaf for 5 d.

**Statistical Analysis** The expected Bt resistance allele frequency in the field populations of *H. armigera* were estimated using the equations as described in Andow and Alstad (1998). The 95% confidence intervals of the estimations were calculated using equation 5 in Andow & Alstad (1999) if no resistance lines were detected, or equation 7 if resistance lines were detected. The probability of missing a major resistance allele if one had been present in a line ( $P_{No}$ ) was determined using the method as described in Stodola and Andow (2004). The estimated frequency was compared to previously published values (He et al., 2001) by using the method in Wenes et al. (2006).

## Results

**F<sub>2</sub> screen in 2003.** A total of 207 female moths were collected during 2003. The  $F_2$  screen for detecting Bt resistance alleles was successfully conducted for 105 lines. After feeding on Bt cotton for 5 d,  $F_2$  larvae from 12 lines survived in the  $F_2$  screen. Among the 12 lines, survivors from six lines reached a body weight of  $\geq 0.8$  mg and developed to the second stadium. These six lines were considered as potential positive lines for Bt resistance. Four lines survived and reached body weights  $\geq 0.8$  mg in  $F_4$  verification screen. Thus, these four lines were considered to be true positive lines for Bt resistance and the resistance allele frequency for the population collected during 2003 was estimated as 0.0119 with a 95% CI of 0.0039–0.0243 (Table 1, Figure 1).

**F<sub>2</sub> screen in 2004.** Only 49 females were collected during 2004 due to the poor weather conditions (heavy rains). Forty-two  $F_2$  lines were eventually screened on Bt cotton plants for resistance. For each line, an average of  $328 \pm 9$   $F_2$  neonates were screened for resistance to Bt cotton. Survivors in five lines reached the second stadium and had a body weight of  $\geq 0.8$  mg/larva. The  $F_4$  larvae from four lines met the criteria for resistance alleles as described above, and these lines were considered to carry resistance alleles. Thus, the expected frequency for resistance alleles for the population collected during 2004 was 0.0297 with a 95% CI of 0.0099–0.0606 (Table 1, Figure 1).

**F<sub>2</sub> screen in 2005.** A total of 337 females were collected during 2005.  $F_2$  neonates produced from 131 isofemale lines were screened for Bt resistance. The  $F_2$  screen showed that larvae from 13 lines were considered potential positive lines because the survivors developed to the second stadium and had body weights of  $\geq 0.8$  mg/larva.  $F_4$  verification screen showed that larval growth from seven lines met the criteria for Bt resistance, as described above, and were considered as true positive lines for Bt resistance. Based on the  $F_2$  and  $F_4$  screens, the frequency of Bt resistance alleles in the population collected in 2005 was estimated as 0.0154 with a 95% CI of 0.0067–0.0277 (Table 1, Figure 1).

**F<sub>1</sub> screening in 2006.** A total of 353 single-mating pairs were established from field-collected male moths, and only 127 pairs successfully laid sufficient fertile eggs to enable  $F_1$  screen. Approximately  $159.4 \pm 7.3$   $F_1$  neonates per single-pair family were assayed on tender Bt cotton leaves for 5 d. Among 122 surviving lines, 49 lines reached body weight  $\geq 0.6$  mg and developed beyond mid-2<sup>nd</sup> stadium, which were similar to those of resistant strain. These lines were considered as potential positive lines. The potential positive lines were re-screened, and 24 lines were confirmed as positive lines carrying resistant gene alleles. Estimated frequency of resistance alleles in 2006 was 0.094, with 95% confidence intervals ranging from 0.044 to 0.145 in the region (Table 1, Figure 1).

**F<sub>1</sub> screening in 2007.** A total of 374 males were collected and mated to YCR virgin females. Of the field collected males, 135 mating pairs successfully laid sufficient fertile eggs for F<sub>1</sub> screening. Larvae from 44 lines survived and reached the mid-second stadium and body weight  $\geq 0.6$  mg. These 44 lines were considered as potential positive lines. Confirmation test showed that 29 lines were true positive lines, which might carry gene alleles for resistance to Bt cotton. The expected resistance allele frequency in 2007 was 0.107 with a 95% CI between 0.055 and 0.159 (Table 1, Figure 1).

**F<sub>2</sub> screen in 2007.** A total of 320 females were collected during 2007, and 137 lines produced enough F<sub>2</sub> progeny for resistance screening. In the F<sub>2</sub> screen, 45 lines had survivors reached the criterion for potential positive lines that had a larval body weight of  $\geq 0.6$  mg/larva and developed into the 2<sup>nd</sup> stadium. The retests showed that 36 lines reached the criterion for resistant insects and were confirmed as true positive lines. The frequency of resistance alleles was estimated to be 0.075 with a 95% CI of 0.053 - 0.100 (Table 1, Figure 1).

Table 1. Field collection and screen (F<sub>2</sub> or F<sub>1</sub>) for resistant gene alleles to Bt cotton in field populations of *Helicoverpa armigera* in Qiuxian County (Hebei, China).

| Year | Screen method  | No. females collected | No. isolines screened | Survival lines after 5 d |                    |
|------|----------------|-----------------------|-----------------------|--------------------------|--------------------|
|      |                |                       |                       | Putative positives       | Verified positives |
| 2003 | F <sub>2</sub> | 207                   | 105                   | 12                       | 4                  |
| 2004 | F <sub>2</sub> | 49                    | 42                    | 6                        | 4                  |
| 2005 | F <sub>2</sub> | 337                   | 131                   | 50                       | 7                  |
| 2006 | F <sub>1</sub> | 353                   | 127                   | 49                       | 24                 |
| 2007 | F <sub>1</sub> | 374                   | 135                   | 44                       | 29                 |
| 2007 | F <sub>2</sub> | 320                   | 137                   | 45                       | 36                 |

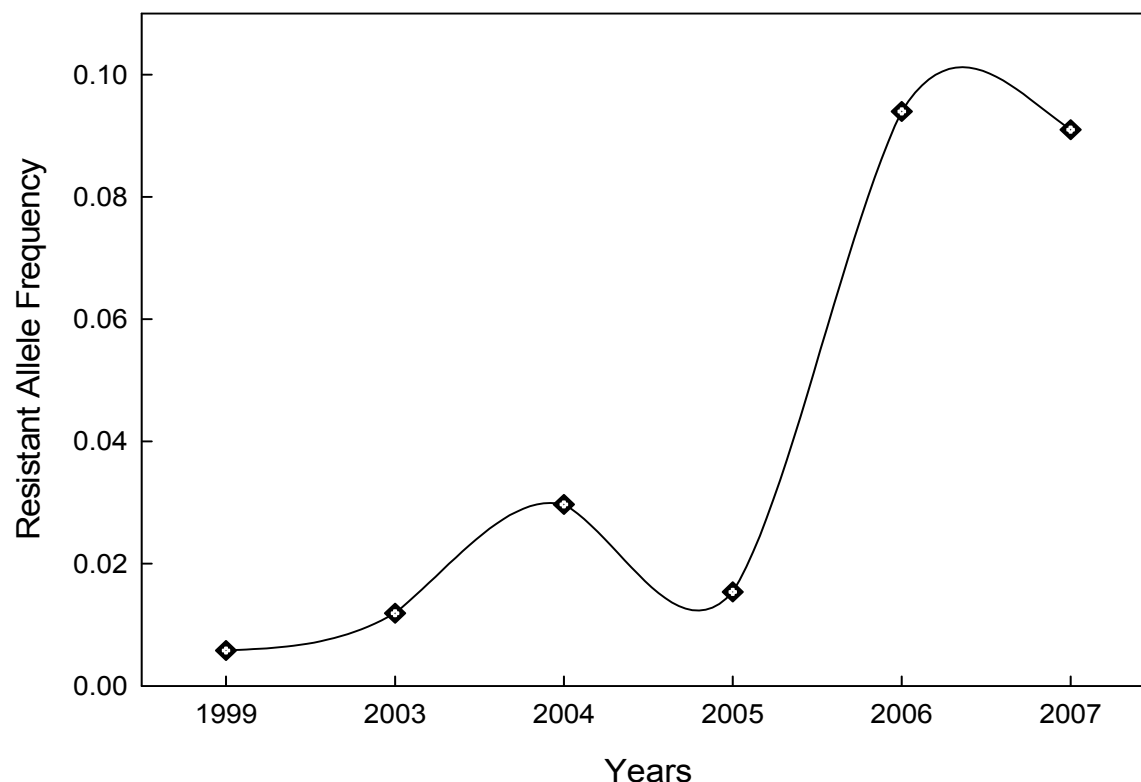


Fig. 1. Estimated frequencies of resistance alleles to Bt cotton in field population of *H. armigera* from 1999 to 2007 in Qiuxian County (Hebei, China).

### Discussion

Transgenic cotton which expresses Cry1Ac toxin has become the most important tool for managing the cotton bollworm in China and several other countries (James, 2007; Pray et al., 2002). Large-scale cultivation of Bt cotton could eventually lead to the evolution of resistance in *H. armigera*. Although significant fluctuation in the resistance allele frequencies was observed during 2003–2005, results show an increase in resistance allele frequency compared to that estimated for the populations collected during 1999 (0.0058). The  $F_1$  and  $F_2$  screens conducted in 2006 and 2007 revealed a >3-fold increase of resistant gene frequency comparing to the levels of 2003–2005, and >18-fold increase over the level of 1999, in the same population of *H. armigera*. These results suggest a potential risk of extensive plating of Bt cotton, which allows target insects to adapt and evolve resistance to Bt cotton.

The increase in resistance allele frequency in field populations of *H. armigera* in Qiuxian County detected in the current study corresponded to a significant increase in field survival of this lepidopterous pest in Bt cotton fields in that area. Field surveys showed that populations of *H. armigera* in Bt cotton fields increased 3 to 20-fold from 2003 to 2007 in this area (unpublished data). Both field sampling and laboratory  $F_2/F_1$  screens showed a significant increase in resistance levels in the field population of *H. armigera* from 1999 to 2005.

The relatively high resistance allele frequency to Bt cotton in field populations of *H. armigera* in Qiuxian County may be attributed to several factors. The initial resistant allele frequency (0.0058) estimated immediately after Bt cotton was commercially planted was relatively greater than that detected in other areas (Yang et al., 2007). Foliar applications of Bt microbial insecticide had been used to control *H. armigera* in this county since 1991 due to the

high levels of resistance that had developed in this pest to almost all chemical insecticides available at that time (He et al., 2001). The long history of the use of Bt as a microbial insecticide or incorporated into cotton plants for controlling *H. armigera* might play a key role in the increase of resistance allele frequency to Bt cotton in this area (Shen et al., 1998).

Although there was no evidence to show that *H. armigera* would outbreak soon in this area, field surveys indicated that moth density during the second field generation increased every year (unpublished data). Because of relatively high levels of resistance allele frequency already detected in the field populations of *H. armigera* in Qiuxian County, we believe it is time to introduce new Bt cotton varieties that express multi-Bt toxins to mitigate further increases in the Bt resistance allele frequency in the region. It is also important to integrate the transgenic Bt cotton technology with biological, chemical, and cultural practices to ensure continued success in managing this most devastating insect pest of cotton in Northern China. Our results also suggested that close monitoring of Bt resistance in *H. armigera* is needed to ensure the long-term success of Bt cotton technology as an effective pest management tool.

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