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Increasing tolerance to Cry1Ac cotton from cotton bollworm, *Helicoverpa armigera*, was confirmed in Bt cotton farming area of China

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Abstract. 1. Changes in the frequency of Cry1Ac resistance genes and shifts in tolerance of cotton bollworm, *Helicoverpa armigera*, to the Cry1Ac toxin were assessed using bioassays of F_1 and F_2 offspring of isofemale lines from Anci County of Hebei Province (a multiple-crop system including corn, soybean, peanut, and Bt cotton) and Xiajin County of Shandong Province (an intensive Bt cotton planting area) in Northern China during 2002–2005.

2. A conservative analysis of the overall results indicated that there was a small increase in the frequency of major, non-recessive resistance genes over time.

3. The relative average development ratings [RADR – growth rate of a line on a Bt diet in proportion to the growth rate on a non-Bt (NBT) diet] of the bollworm larvae in F_1 tests increased significantly from year to year, indicating a gradual trend towards higher tolerance to Cry1Ac in the field populations.

4. There were also significant positive correlations between RADR of the lines in the F_1 generation and the RADR of their F_2 offspring, indicating that the tolerance was genetically based.

5. Quantitative genetic simulation analysis showed that resistance of *H. armigera* to Bt cotton in Xiajin could evolve to a high level in 11–15 years if no effective resistance management measures are carried out.

Key words. Cry1Ac, *Helicoverpa armigera*, Bt cotton, resistance frequency.

Introduction

Genetically modified cotton expressing the Cry1Ac toxin from the bacterium *Bacillus thuringiensis* (Bt) has been commercially cultivated in China since 1998. Bt cotton provides an efficient tool for controlling cotton bollworm, *Helicoverpa armigera* Hübner (Lepidoptera: Noctuidae), the major insect pest of cotton in many areas of China (Guo, 1997; Wu & Guo, 2005). This results in very positive economic returns to growers and reductions in insecticide use in cotton (Pray *et al.*, 2001; Wu & Guo, 2005). However, due to the continuous production of Bt toxin in engineered cotton plants and the widespread use of Bt cotton, the pest could evolve resistance and nullify the benefits of Bt cotton (Tabashnik *et al.*, 1990, 1992; Tabashnik, 1994; Gould *et al.*, 1995; Chaufaux *et al.*, 1997; Liang *et al.*, 2000). Although resistance to Bt crops in the field has not been documented, laboratory selection has produced Bt-resistant strains of many pests (Tabashnik *et al.*, 2003). Further, populations of diamondback moth (*Plutella xylostella*) and cabbage looper (*Trichoplusia ni*) have evolved resistance to Bt sprays (Janmaat & Myers, 2003; Tabashnik *et al.*, 2003).

The rate of resistance evolution in an insect population to a Bt crop depends on a number of factors, including pest population

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dynamics, initial frequency of resistance alleles in the pest population, genetic mode and stability of resistance, fitness of resistant individuals, temporal and spatial distribution of the insect pest on different host plants, and gene flow among different geographical populations (McGaughey & Whalon, 1992; Tabashnik, 1994; Alstad & Andow, 1995; Wu & Guo, 1997; Peck *et al.*, 1999; Wu *et al.*, 1999). Several deployment tactics designed to delay resistance have been proposed. The most promising resistance management strategy entails the use of plants with a high dose of toxin in combination with the maintenance of refuge crops that produce Bt-susceptible insects within the pest population. The refugia strategy is compulsively mandated in the USA and Australia, where cotton is planted on a large scale. In China, mixed plantings of cotton, corn, soybean, and peanut are common, and resistance management has solely relied on the refugia function of non-cotton crops within the cropping systems of smallholders (Wu *et al.*, 2002a). However, the sizes of these natural refugia in different areas are highly variable, and they may have low effectiveness in some situations, especially in intensive Bt cotton planting areas where the concentration of Bt toxin in the plants is not a high dose for the target pests.

To maintain the effectiveness of Bt cotton, it is necessary to monitor the increases in the frequency of resistance genes to Bt toxin with a method that is appropriate for the pest and specific Bt crop(s) (Storer *et al.*, 2003a,b; Wu & Guo, 2005). Specialised bioassays have been developed to estimate the frequency of Bt resistance (R) alleles (Gould *et al.*, 1997; Andow & Alstad, 1998), but these methods focus on detecting homozygous resistant individuals. Burd *et al.* (2003) developed a bioassay using F_1/F_2 generation larvae for estimating the frequency of major, non-recessive resistance alleles in heterozygous genotypes of *Helicoverpa zea*. Using this method, the authors carried out experiments to monitor the frequency of resistance alleles and any quantitative shifts in larval Cry1Ac tolerance of *H. armigera* from field areas in the Yellow River cotton-farming region of China.

In the two areas, Hebei and Shandong Provinces, commercialisation of Bt cotton began in 1998, and by 2000 Bt cotton had almost completely replaced non-transgenic cotton cultivars. Table 1 shows the history of Bt cotton deployment in the two locations sampled. Maize, cotton, peanut, and soybean are the main host crops of *H. armigera* in the two counties (data provided by the local governments). The average percentages of land planted to Bt cotton, corn, peanut, and soybean during 2000–2005 were 71.42, 23.94, 3.17, and 1.46% in Xiajin, and 9.52, 68.55, 12.37, and 9.55% in Anci respectively. In general, Xiajin is considered to be a Bt cotton and corn planting system, while Anci County is considered to have a multiple-crop farming system.

Materials and methods

Laboratory strains

To evaluate the level of susceptibility to Bt of cotton bollworm larvae from field populations, the authors compared their response to Cry1Ac toxin in artificial diet with the responses of two Bt-susceptible laboratory strains (SS1 and SS2) of cotton bollworm collected from Xinxiang of Henan Province in 1996 and Langfang, Hebei Province in 1998 respectively, which had been continuously cultured in the laboratory without exposure to Bt toxin. A substrain (LF-R) from a subset of the Langfang population selected more than 60 generations with Cry1Ac (Wu & Guo, 2004) and had 282–fold resistance in its F_{61} generation. This strain was used as a positive control. To obtain F_1 generation offspring of each of these laboratory strains, one virgin adult male and one virgin adult female were paired in a 500–ml clear glass cup. At least 100 single pairs were established from randomly selected individuals in each of these strains. A total of 64, 57, and 54 female moths (lines) of LF-R, SS1, and of SS2 respectively, laid sufficient fertile eggs to be used for the F_1 generation bioassay.

Table 1. The planting history of Bt cotton and other host crops of the bollworm during 1998–2005 in Anci County, Hebei Province and Xiajin County, Shandong Province.

Location	Year	Conventional cotton (%)	Bt cotton (%)	Maize (%)	Peanut (%)	Soybean (%)	Total planting area (ha)
Anci County	1998	1.62	0.81	73.55	10.31	13.71	34 890
	1999	1.47	1.47	73.52	10.27	13.27	34 580
	2000	0.00	7.22	67.97	11.90	12.90	32 870
	2001	0.00	13.14	66.46	10.35	10.03	34 420
	2002	0.00	11.46	67.33	11.15	10.05	33 817
	2003	0.00	5.25	71.23	14.81	8.70	30 800
	2004	0.00	10.53	68.22	13.32	7.93	31 013
	2005	0.00	9.53	70.11	12.68	7.68	31 886
Xiajin County	1998	35.02	8.75	46.46	4.71	5.05	39 601
	1999	3.83	34.44	46.75	10.65	4.33	36 933
	2000	0.00	71.41	21.98	4.16	2.44	46 400
	2001	0.00	64.11	31.07	2.96	1.85	54 067
	2002	0.00	69.36	25.07	3.84	1.72	50 266
	2003	0.00	74.30	21.56	2.92	1.22	54 733
	2004	0.00	76.65	21.23	1.77	0.35	56 533
	2005	0.00	72.69	22.75	3.35	1.20	55 667

Collection of insect strains

Anci County of Hebei Province and Xiajin County of Shandong Province, which are located about 400 km away from each other in the largest cotton-growing region of China (the Yellow River cotton region), were selected as sites for moth sampling in 2002 (Li *et al.*, 2004), 2003, 2004, and 2005.

During June to September 2003, a total of 170 adult female bollworms were collected from two light traps (250 W) at a site in Xiajin County. During August and September of 2004 in Xiajin County, a light trap equipped with a 1000 W searchlight was used to collect a total of 2675 female moths. By the same method, 680 female moths were captured at a site in Anci County in June and July of 2004; 989 female moths and 1345 female moths were collected in Anci County and in Xiajin County in 2005 respectively. Adult females were placed individually into 250–ml clear plastic cups that were then covered with gauze to provide a substrate for egg laying. Moths were kept at 28 ± 1 °C, 70–80% RH, and a photoperiod of LD 14:10 h. Eggs were collected on a daily basis. In Xiajin, a total of 71, 738, and 332 female moths (lines) in 2003, 2004, and 2005 respectively, laid sufficient fertile eggs to be used for the bioassay. In Anci, 279 and 253 female moths, respectively, in 2004 and 2005 laid sufficient fertile eggs.

Bioassay of F_1 generation on Bt and non-Bt diets

At larval hatching, 24–35 neonates from each female line were placed on a non-Bt (NBT) diet and 24–35 neonates were placed on Cry1Ac-containing diet. The composition of the control diet is described in Zhou *et al.* (1981). The concentration of Bt toxin was 1.0 μg per ml of diet. Cry1Ac was obtained as a gift from Mycogen Corp. (San Diego, CA) in a formulation of their product, MVPII (20%). To avoid degradation, Cry1Ac toxin was stored at –70 °C. The toxin was thoroughly mixed into the diet. The diet was placed into glass test tubes, and stored in a refrigerator. A single neonate was placed into each test tube with a fine brush and the test tubes were sealed with cotton plugs. The insects were kept at 28 ± 1 °C, 70–80% RH, and a photoperiod of LD 14:10 h. The bioassays for the lines from the two areas were carried out in each location. Larvae were scored for developmental stage after 6 days. Instar was determined based on head capsule and body size (Li *et al.*, 2004).

Bioassay of the F_2 generation on Bt and non-Bt diets

The lines of field population (about 20%) that performed at least 80% as well on Bt diet as they did on NBT diet were saved for testing the F_2 generation because it was hypothesised that F_1 lines that developed relatively well on the Bt diet carried a major resistance allele in the heterozygous form. For each saved line the individuals that had developed on the NBT diet were reared to adult emergence and mated in a mating cage. The next generation was bioassayed with the same method used for the F_1 lines. Ten lines from the Xiajin population were successfully mated in 2003 and their F_2 larvae were bioassayed on Cry1Ac and control diet. In 2004, 78 lines from the Anci population and 18 lines from the Xiajin population were successfully tested as F_2 larvae. Eighteen lines from the Anci population and 33 lines from the Xiajin population were successfully tested as F_2 larvae in 2005.

Data analysis

It is possible that larvae from some female lines grew well on the Bt diet due to environmental vigour effects and not because of genetically based resistance to the toxin. Because lines with high vigour are expected to perform better than the other lines on both the NBT diet and the Bt diet, vigour effects were controlled for by calculating the growth rate of a specific line on the Cry1Ac diet, relative to its growth rate on the control diet. More specifically, the relative average development rating (RADR) for a line was calculated as the average body length rating of larvae from that line reared on the Bt diet divided by the average rating of larvae from that line reared on the NBT diet. PROC UNIVARIATE was used to determine average ordinal ranking for each single female line on each of the diets. Data on the RADR of lines from Anci and Xiajin populations and laboratory strains were analysed using analysis of variance (ANOVA), and means for populations within these years were separated using the protected least significant difference (LSD) test. PROC CORR was used to determine if, for each of the two populations, there was a correlation between the average ordinal rankings of the single female lines on the two diets and if there was a correlation between the relative average rating of one parent and their offspring (SAS Institute 1988).

Quantitative genetic model

The genetic simulation tool QuCim can predict the outcome of random mating after a special selection schedule, when genetic information for the targeted traits is known (Wang *et al.*, 2003). Genetic parameters determine the change of resistance allele frequency in a population. The key parameters are heritability, selection intensity, initial resistance allele frequency, and others. First the required genetic parameters are estimated using field and laboratory data, and then these estimates are used to predict the change of resistance of the two field insect populations under different selection pressures.

In quantitative genetics, the heritability (h^2) is the degree of resemblance between relatives and is defined as the ratio of additive genetic variance (V_A) to phenotypic variance (V_P) (Falconer & Mackay, 1996):

$$h^2 = V_A/V_P. \quad (1)$$

The additive variance can be estimated from the covariance between parents and their offspring (Cov_{OP}), i.e.

$$V_A = 2Cov_{op}. \quad (2)$$

The change in the mean value for a trait in a population after selection is called the response to selection (R) (Falconer & Mackay, 1996), which is a function of heritability (h^2), selection intensity (i), and additive genetic variance (V_A),

$$R = ih\sqrt{V_A} = iV_A / \sqrt{V_P}. \quad (3)$$

Then the equation for selection intensity (i) would be:

$$i = R\sqrt{V_P} / V_A. \quad (4)$$

In this study, the response to selection was first estimated from the field population sampled in consecutive years, the additive variance was estimated from the correlation between parents and their offspring, i.e. eqn 2, and the phenotypic variance was estimated from the sampled field population. Then the selection intensity could be estimated. The selection intensity estimate will then be used in the computer simulation model to predict changes in the resistance allele frequency over time.

Results

Bioassays on F_1 generation test

A total of 57 female lines from the SS1 population were screened on the Cry1Ac diet in 2005. The distribution of RADR of 6-day-old larvae from all lines on Cry1Ac is presented in Fig. 1a. The relative ratings for most lines on Cry1Ac ranged from 0.28 to 0.32. The maximum RADR for any line was 0.34. The average relative rating was 0.30 for all the SS1 lines (Table 2). The distribution of RADR of 6-day-old larvae from 54 female lines of SS2 population on Cry1Ac is presented in Fig. 1b. The relative ratings for all lines on Cry1Ac ranged from 0.26 to 0.34, with the average relative rating for all lines being 0.29 (Table 2). The distribution of RADR of 6-day-old larvae from 64 female lines of LF-R population on Cry1Ac is presented in Fig. 1c. The relative ratings for all lines on Cry1Ac ranged from 0.80 to 1.00. In this case the average rating for all lines was 0.91 (Table 2).

A total of 234 female lines were screened on Cry1Ac diet in the Anci population in 2002, as is reported in Li *et al.* (2004). Figure 2a shows the distribution of RADR of 6-day larvae for the female lines. In general, the relative rating for all lines was 0.30 (Li *et al.*, 2004). A total of 279 female lines from the Anci population were screened on Cry1Ac diet in 2004. The distribution of RADR of 6-day-old larvae from all lines on Cry1Ac is presented in Fig. 2b. The relative ratings for most lines on Cry1Ac ranged from 0.4 to 0.5. The maximum RADR for any line was 0.63. The average relative development rating was 0.42 (Table 2). A total of 253 female lines from the Anci population were screened on Cry1Ac diet in 2005. The distribution of RADR of 6-day-old larvae from 253 female lines from the Anci population (all lines) on Cry1Ac in 2005 is presented in Fig. 2c. The relative ratings for most lines on Cry1Ac ranged from 0.4 to 0.6. The maximum RADR for any line was 0.72 and the average relative development rating for all lines was 0.50 (Table 2).

A total of 420 female lines from the Xiajin population were screened on the Cry1Ac diet in 2002. The distribution of RADR of 6-day-old larvae for all lines is presented in Fig. 3a. The relative ratings for most lines ranged from 0.3 to 0.4, with the average relative rating being 0.38 (for detailed results see Li *et al.*, 2004). Figure 3b shows the distribution of RADR of 6-day larvae on Cry1Ac for a total of 71 female lines from the Xiajin population in 2003. The mean relative rating of all lines combined on Cry1Ac was 0.50 (Table 2), and none of the 71 lines had a relative development rating above 0.80. The distribution of RADR of 6-day-old larvae from 738 female lines from the Xiajin population in 2004 is presented in Fig. 3c. The relative ratings for most lines ranged from 0.5 to 0.6, and the mean for all lines was 0.53 (Table 2). The distribution of RADR of 6-day-old larvae from 322 female lines from the Xiajin population in 2005 is presented in Fig. 3d. The relative ratings for most lines ranged from 0.5 to 0.7, and the mean of all lines was 0.55 (Table 2).

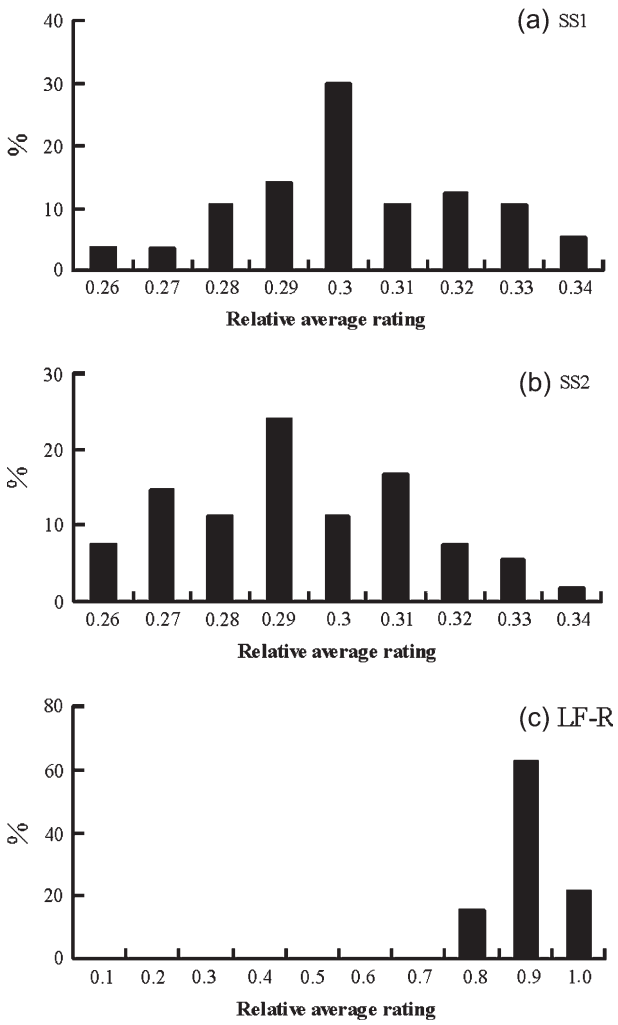

Fig. 1. Distribution of relative average development rating for 6-day-old larvae of *Helicoverpa armigera* F_1 generation female lines in SS1, SS2, and LF-R populations.

Compared with two Bt-susceptible strains, the RADR of the bollworm larvae in the F_1 test in Anci has increased significantly from year to year, but did not reach the level of resistant population (Table 2, $P = 0.0001$), and similar conclusions were obtained in the Xiajin population, but it is particularly notable that their mean was significantly higher than that for the Anci population

Table 2. Relative average development ratings for 6-day-old larvae of *Helicoverpa armigera* F_1 and F_2 generation female lines in Anci and Xiajin populations.

		Relative average development rating per line	
Location	Population	F_1 generation	F_2 generation
Anci	LF-R	0.91 ± 0.008 A	
	2005	0.50 ± 0.005 B a	0.53 ± 0.024 a
	2004	0.42 ± 0.005 C a	0.44 ± 0.009 a
	2002	0.30 ± 0.006 D a	0.26 ± 0.037 a
	SS1	0.30 ± 0.003 D	
	SS2	0.29 ± 0.003 D	
Xiajin	LF-R	0.91 ± 0.008 A	
	2005	0.56 ± 0.005 B b	0.61 ± 0.019 a
	2004	0.53 ± 0.003 B b	0.65 ± 0.019 a
	2003	0.50 ± 0.010 C b	0.66 ± 0.027 a
	2002	0.38 ± 0.006 D b	0.55 ± 0.020 a
	SS1	0.30 ± 0.003 E	
	SS2	0.29 ± 0.003 E	

Means (± SE) with different letters were statistically different ($P < 0.05$; LSD test). Capital letters in same column indicated the difference in different years in the same location. Small letters in same row indicated the difference between F_1 generation and F_2 generation in the same year. SS1, SS2 and LF-R represented two Bt-susceptible laboratory strains and a positive control respectively.

in same year ($P = 0.0001$), indicating a gradual trend towards higher level of tolerance to Cry1Ac in intense Bt cotton region.

There was a significant positive correlation between average rating for female lines on NBT diet and Cry1Ac diet in the Xiajin population in 2003–2005 ($P < 0.05$; Table 3), and similar conclusions were obtained in the Anci population in 2004–2005 ($P < 0.05$; Table 3) and in the SS1 and LF-R population in 2005 ($P < 0.05$; Table 3). These results indicated that the families with higher average ratings on the NBT diet also had higher average ratings on the Cry1Ac diet. Therefore, calculating the relative average rating is justified for removing effects of environmental vigour (see Burd *et al.*, 2003).

Bioassay on the F_2 generation test

A total of 71 female lines from Anci that were chosen based on not very high relative development rating in the F_1 generation were successfully screened in the F_2 generation in 2004 (Table 4). The means of RADR for all F_2 generation larvae was 0.44 (Table 2). A total of 18 female lines from Anci were successfully screened in the F_2 generation in 2005 (Table 4). The mean of RADR for F_2 generation larvae was 0.53 (Table 2). There was no significant difference between the means of RADR for the F_1 generation and that of the F_2 generation (Table 2).

A total of 10 female lines from Xiajin that developed more quickly than others in the F_1 generation were successfully screened in the F_2 generation in 2003 (Table 4). The mean of RADR for all F_2 generation larvae was 0.66, which was significantly higher than the RADR (0.50) in F_1 families ($P < 0.05$; Table 2). A total of 18 female lines from Xiajin were successfully screened in the F_2 generation in 2004 (Table 4). The RADR for all F_2 generation larvae was 0.65 (Table 2). It should be noted that there were two lines (#365, #1524) with relative average ratings that were substantially higher than that of other lines. The relative average ratings of the two lines reached about 0.8 respectively. The #365 family average ratings were 6.82 on NBT and 5.41 on Bt diet, and #1524 family average ratings were 6.56 on the NBT diet and 5.08 on the Bt diet. In the F_1 generation, their relative average ratings were 0.83 and 0.81 respectively. A total of 33 female lines from Xiajin were successfully screened in the F_2 generation in 2005 (Table 4). The RADR for all F_2 generation larvae was 0.61 (Table 2), and their mean was significantly higher than that for F_1 generation (Table 2, $P < 0.05$). There were three lines (#45, #276, #374) with relative average ratings that were substantially higher than that of other lines. The three lines of relative average rating reached about 0.8 respectively. The #45 family average ratings were 6.37 on NBT and 5.19 on Bt diet; #276 family average ratings were 6.26 on the NBT diet and 5.23 on the Bt diet; #374 family average ratings were 6.90 on the NBT diet and 5.52 on the Bt diet. In the F_1 generation, their relative average ratings

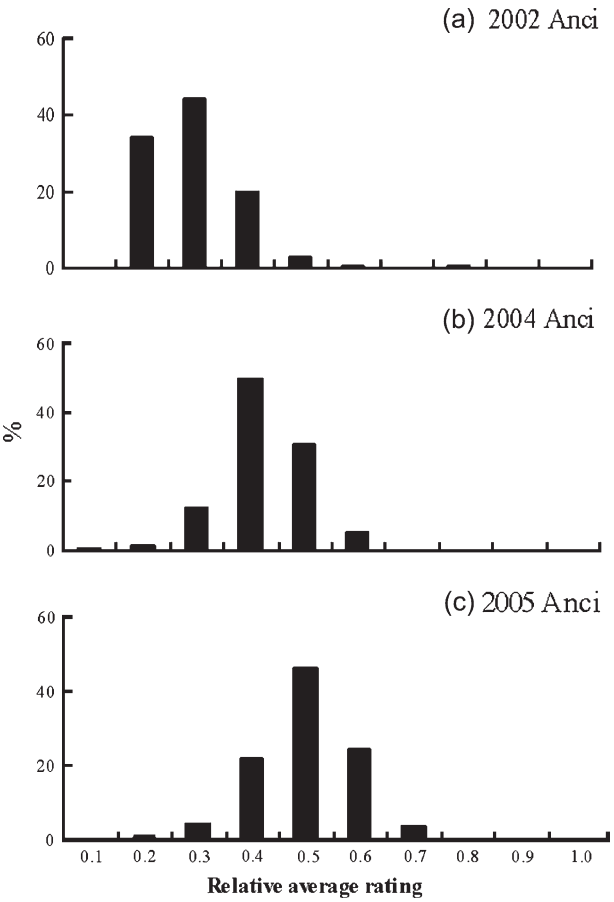

Fig. 2. Distribution of relative average development rating for 6-day-old larvae of *Helicoverpa armigera* F_1 generation female lines in the Anci population during 2002–2005.

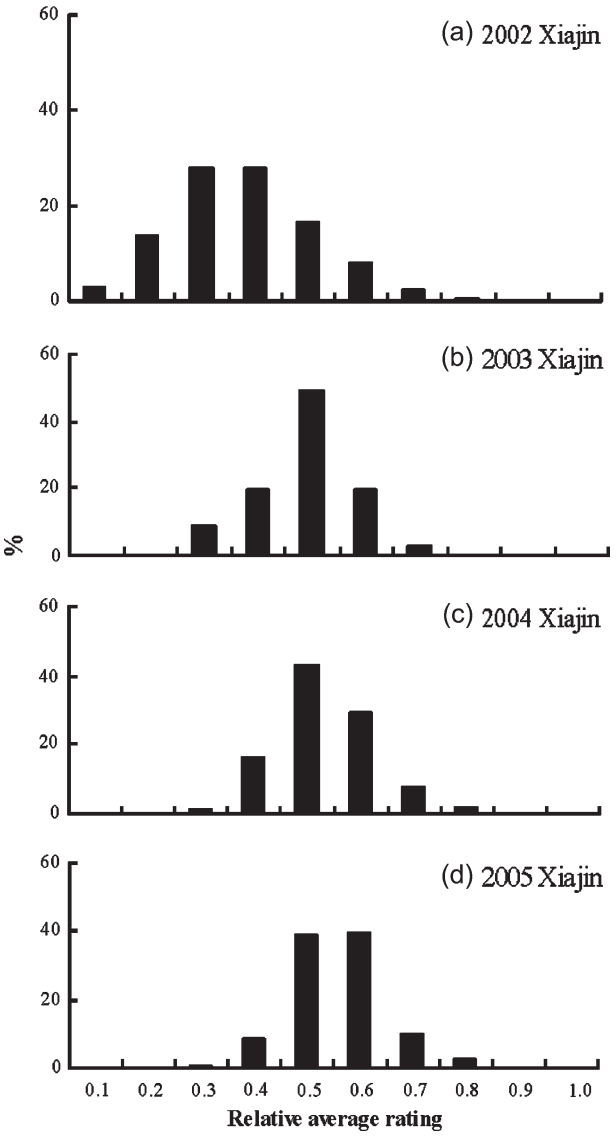

Fig. 3. Distribution of relative average development rating for 6-day-old larvae of *Helicoverpa armigera* F_1 generation female lines in the Xiajin population during 2002–2005.

were 0.80, 0.83, and 0.81 respectively. The means of RADR in the F_2 generation were significantly higher than that of F_1 generation during 2003–2004 in the Xiajin population (Table 2). This result indicated genetically based tolerance to the toxin.

The total sample size in the F_2 generation tests for the Anci and Xiajin populations between 2002 and 2005, when the previously published data of Li *et al.* (2004) are included is 99 and 83 isofemale lines respectively. There was a significant positive correlation between the relative developing rating for a line in the F_1 generation and the relative development rating of the offspring in the F_2 generation in the two populations ($P < 0.05$; Fig. 4), which indicates that a line that has a higher RADR in its F_1 generation will have a higher RADR of the offspring in F_2 generation. This is additional evidence of genetically based variation in response to Cry1Ac.

Table 3. Correlation for average development rating between development on NBT diet and on Cry1Ac diet in the F_1 generation.

Population	Year	Correlation coefficient	*P*-value
SS1	2005	0.76	0.0001
SS2	2005	0.10	0.4706
LF-R	2005	0.31	0.0132
Anci	2005	0.22	0.0005
	2004	0.21	0.0003
Xiajin	2005	0.25	0.0002
	2004	0.13	0.0005
	2003	0.20	0.0003

SS1, SS2, and LF-R represented two Bt-susceptible laboratory strains and a positive control respectively.

Simulation analysis of the bollworm tolerance to Bt cotton

The heritability of Anci and Xiajin populations were estimated using the RADR of F_1 and F_2 generations from 99 isofemale lines in Anci and 83 lines in Xiajin during 2002–2005. The values of Cov_{OP} were 0.004112 in Anci and 0.003397 in Xiajin. The values of V_P from the RADR of their F_1 generation were phenotypic variance for isofemale lines in the two provinces during 2002–2005. Dividing V_A by V_P gives a heritability of 0.43 for Anci and a heritability of 0.62 for Xiajin (Table 5).

Selection proportion of Anci and Xiajin were estimated by the selection reaction of the means of RADR for their F_1 generation between every 2 years for the two areas. First, the intensity of selection was calculated using eqn 4 and values of selection intensity (i) for given values of p were tabulated in Appendix table A of Falconer and Mackay (1996). The selection proportion in Anci for 2 years were 20% and 40% and in Xiajin for 3 years were 3.2%, 25%, and 31% respectively. Hence, the means of selection proportion for the two areas were found to be 30% in Anci and 20% in Xiajin (Table 6).

The effects of genes were estimated based on the laboratory and field strains. The mean RADR for the laboratory susceptible strain was 0.30 (Table 2), and the lowest value of RADR for the laboratory resistant strain was 0.8, then the lines with RADR of 0.3 and 0.8 during 2002–2005 in Anci and Xiajin were removed. Hence the means were 0.30 for susceptible homozygote, 0.53 for susceptible heterozygote, and 0.8 for resistance homozygote.

Key parameters used in the simulated dynamics of resistance allele frequency in field populations of Anci and Xiajin County are presented in Tables 5 and 6. Each year was used as selection cycle, where all the factors influencing resistance evolution were incorporated into every year, reflected by several important parameters, such as heritability, the initial frequency of resistance alleles, selection intensity *et al.* The initial frequency of the resistance alleles used in the model were the mean values calculated during 2002–2005 in each population – 0.000357 in

Table 4. Estimates of resistance (R) allele frequency from responses of cotton bollworm to 1.0 μg of Cry1Ac per ml of diet.

Location	Year	F_1 generation bioassay		F_2 generation bioassay		Estimated R allele frequency†
		Total	≥0.8*	Total	≥0.8*	
Anci	2005	253	0	18	0	0.00000
	2004	279	0	71	0	0.00000
	2002	234	1	8	1	0.00107
Xiajin	2005	322	8	33	3	0.00233
	2004	738	16	18	2	0.00068
	2003	71	0	10	0	0.00000
	2002	420	1	22	1	0.00059

*Moth is regarded as an individual with a resistance allele if the relative average development ratings of its F_1 line and F_2 were more than 0.8.
†Because each mated female carries two of her own alleles and two from her male counterpart (if she mated only once), with this in mind, screening the number of females on the Cry1Ac diet to characterise (4 × total number) genomes, estimated gene frequency for the resistance gene to Cry1Ac toxin would be the number of individual with resistance allele/(4 × total number).

Anci and 0.0009 in Xiajin. The QuCim modelling results showed that under the current planting systems in two Counties, it would take 15 years in Anci for resistance allele frequency to reach 0.5 and it would take 11 years in Xiajin (Fig. 5).

Discussion

Resistance allele frequencies can only increase over time if either heterozygotes are partially resistance of if there are resistant homozygotes in the population. The most probable carriers of Bt resistance genes in current field populations are expected to be heterozygotes (Gould, 1998), so the fitness of these heterozygotes compared with fitness of susceptible individuals will have a major impact on the rate of resistance evolution. In most cases resistance genes are partially or completely recessive under field conditions (Carrière *et al.*, 2004) so the fitness of heterozygotes is similar to that of susceptible individuals. Based on the assumption that most Bt resistance genes are phenotypically recessive, or are effectively recessive when challenged with high dose plants (Tabashnik *et al.*, 2002), most bioassays are designed to produce and test homozygote resistant larvae (Gould *et al.*, 1997; Andow & Alstad, 1998). This assumption does not hold for the bollworm, *Helicoverpa zea*, where resistance to Cry1Ac was inherited as a phenotypically dominant or partially dominant trait in field-derived strains (Burd *et al.*, 2000), and current cotton cultivars do not challenge *H. zea* with a high dose. When the predominant Bt cultivars produce a high dose of toxin the only potentially important resistance genes are major genes that provide high levels of resistance, but when crops only produce a moderate dose of toxin for a specific pest, major and minor resistance genes can contribute to the evolution of resistant pest populations (Gould, 1998). The selection conditions for *H. armigera* may be similar to those with *H. zea*, so testing major and minor resistance genes in heterozygous form is critical.

The bioassay in the present study was specifically designed to test the fitness of individuals with major and minor resistance genes and to detect resistance genes in the heterozygous form. Because the frequency of resistance genes in current *H. armigera* populations are expected to be low, none of the females collected in the present study are expected to carry resistance genes in homozygous form, and only half of the offspring from any of the F_1 lines should be carrying a resistance gene. Even if an initial female was carrying a major, dominant, resistance gene, the mean growth of her F_1 offspring on Bt diets should be less than that found on the NBT diet. Therefore, it was assumed that if the RADRs of any F_1 line and its F_2 were more than 0.8, it was carrying a major resistance gene. It is possible that some of these lines performed well because they contained multiple

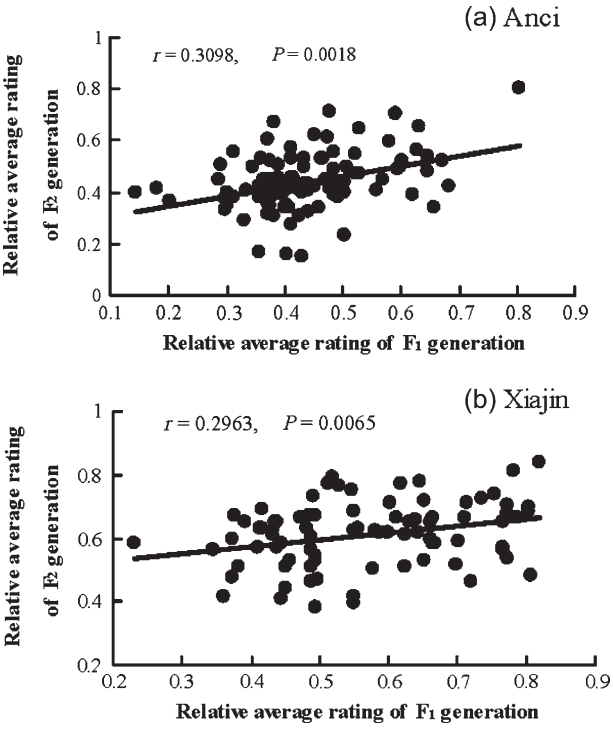

Fig. 4. Correlations between the relative average development rating for a line in the F_1 generation and the relative average development rating of the offspring in the F_2 generation in Anci and Xiajin populations during 2002–2005. *r* and *P*-values are those of Pearson correlations.

Table 5. Estimation of heritability by positive correlation between the RADR of one parent and their offspring in Anci and Xiajin populations.

Populations	Cov_{OP}	V_A	V_P	$h^2 = V_A/V_P$
Anci	0.004112	0.008224	0.018931	0.43
Xiajin	0.003397	0.006794	0.010974	0.62

resistance genes that each had very small effects on resistance but were much more common than major resistance genes in the *H. armigera* population.

Screening the offspring of 71 total females on the Cry1Ac diet in Xiajin in 2003 allowed 284 haploid genomes to be characterised, because each mated female carries two of her own alleles and two from her male counterpart (if she mated only once). In the F_1 screening from Xiajin, no line could be found that had a distinctly higher level of resistance than that of other lines. Based on the assumption that every resistance individual contained a resistance gene with a major effect, it was estimated that the frequency of major resistance genes was 0/284 or 0.00000 in Xiajin in 2003 (Table 4). In 2004, a similar result was obtained in the F_1 screening from Anci, but two lines were found with a distinctly higher level of resistance than that of any other lines, with a relative average rating reaching ≈ 0.8 in the F_1 generation in the Xiajin population. Based on the assumption that both of the two lines contained a resistance gene with a major effect, it was estimated that the resistance gene frequency was 2/2952 or 0.00068 in Xiajin in 2004 (Table 4). It should be noted that this estimate is conservative because other female lines from both areas that developed faster than average F_1 lines may have been carrying minor genes or major genes with lower levels of dominance. In 2005, a similar result was obtained in the F_1 screening from Anci as in 2004, but three lines were found with a distinctly higher level of resistance than that of any other lines, with relative average rating reaching ≈ 0.8 in the F_1 generation and F_2 generation in the Xiajin population in 2005. Based on the assumption that each of the three lines contained a resistance gene with a major effect, the resistance gene frequency was estimated as 3/1328 or 0.00233 in Xiajin in 2005 (Table 4).

The bioassays from 2002 through 2005 indicate that there is a trend toward more major resistant genes over time in Xiajin, even though the numbers are far too low for statistical inferences. The statistically significant increase in RADR of *H. armigera* over the years of the study also suggests a change in the tolerance of *H. armigera* field populations.

This apparent increase in tolerance that was found could be due to an increase in the frequency of minor resistance genes, but the possibility that the results were due to testing conditions being less stringent in each successive year cannot be ruled out. Because the F_1 results in 2002, 2004, and 2005 show that the Xiajin population is more tolerant of Cry1Ac than the Anci population, it is likely that this difference is genetically based. It is not surprising that the Xiajin population would have become more tolerant because more Bt cotton is grown in this region than in the region where Anci County is located. If more Bt cotton were planted in Xiajin, resistance would increase faster. The tolerance increase in the Anci population, a multiple crop planting system, may relate to immigration of cotton bollworm moths from Bt cotton intensive planting areas. The migration activities of the bollworm moths occur frequently in northern China, and it has been shown that the first generation moths in Anci mainly emigrate from other areas in recent years (Wu *et al.*, 2001, 2002b). Further, it is notable that in 2002, 2003, 2004, and 2005 selection on the F_1 lines in Xiajin County resulted in increased resistance of the F_2 larvae (Table 2). This strongly suggests that there is heritable variation for resistance in the Xiajin area. In contrast, selection on the F_1 lines from Anci in 2002, 2004, and 2005 did not lead to an increased resistance level (Table 2). Overall, these data highlight the need for careful monitoring of resistance levels in the Xiajin area.

Studies have identified several consistently important parameters that determine the rate at which cotton bollworm populations are expected to adapt to Bt cotton (Ru *et al.*, 2002; Kranthi & Kranthi, 2004). Because the model output depends on uncertain values for important parameter inputs, results of these models should not be interpreted as predictive of absolute time to resistance (Storer *et al.*, 2003b). The model in the present study incorporates the biology, genetics, and operations factors contributing to key parameters based on the successive monitoring results. The simulation analysis indicates that a natural refuge strategy is necessary for resistance management in an intensive

Table 6. Estimation of selection proportion of Anci and Xiajin populations by the response to selection from the field populations sampled in consecutive years.

Year	Mean RADR for F_1 generation	Realised response to selection (R)	Additive variance (V_A)	Standard error of phenotype ($\sqrt{V_P}$)	Selection intensity (i)	Selection proportion (P%)
Anci						
2005	0.50	0.08	0.008224	0.13829	1.40	20%
2004	0.42	0.12	0.008224	0.13829	0.94	40%
2002	0.30					
Xiajin						
2005	0.56	0.03	0.006794	0.137591	0.50	3.2%
2004	0.53	0.04	0.006794	0.137591	0.67	25%
2003	0.49	0.11	0.006794	0.137591	1.86	31%
2002	0.38					

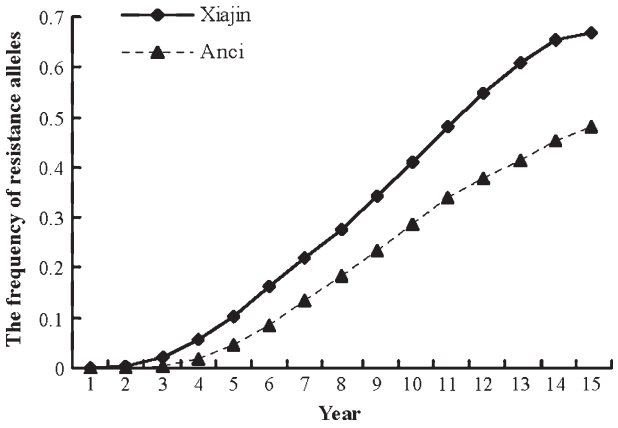

Fig. 5. Simulation dynamics of resistant evolution in Anci and Xiajin populations in the coming years in Anci and Xiajin agro-systems.

Bt cotton planting area like Xiajin. Because China does not commercialise other Bt crops, one planting system consisting of wheat, corn, soybean, and peanut can serve as effective refuges for all generations of *H. armigera* (Wu *et al*., 2002a, 2004). The release of transgenic cotton expressing two pyramided Bt genes in the near future will provide another option for resistance management of the pest.

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