Supporting Information

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SI Materials and Methods

Percentage of Non–*Bacillus thuringiensis* (Bt) and Bt Cotton from 1997 to 2012. We used data from U.S. Department of Agriculture–Agricultural Marketing Service (1) to tabulate percentages of non–Bt and Bt cotton with one and two toxins in three states (Georgia, Arkansas, and Mississippi) and the United States globally from 1997 to 2012 (Fig. 1 and Fig. S1).

Toxin Concentration in Plants. We used ELISA (Qualiplate kit, Envirologix) to measure the Bt toxin concentrations in fresh terminal leaves of cotton plants (Cry1Ac in DP 448 B; Cry1Ac and Cry2Ab in DP 164 B2RF). Arbitrarily selected plants were sampled every 2 wk, between 38 DAP (presquaring stage) and 95 DAP (late fruiting stage). A single leaf was sampled per plant and a total of 10 leaves per cultivar were collected on any given date. Leaves were stored at −80 °C. At the time of analysis, a leaf punch sample (15–20 mg) was taken for each leaf by snapping the 1.5 mL Eppendorf tube cap down on the leaf. Extraction buffer (0.5 mL) was added to the tube and the plant tissue was grinded with a pestle. Sample extracts were diluted with the buffer solution at 1:11/1:51 and 1:201 for Cry1Ac and Cry2Ab, respectively, to bring assay results within the range of calibration. The optical density of Cry1Ac and Cry2Ab calibrators from purified toxin solution was measured using a spectrophotometer (microtiter plate reader) to establish the standard curve. The concentrations of Cry1Ac and Cry2Ab in samples ($\mu g \cdot g^{-1}$ fresh tissue) were calculated based on concentration levels from the standard curve (parts per billion).

Population Genetics Model. We simulated the evolution of *Helicoverpa zea* resistance to two-toxin cotton using a deterministic population genetic model with two unlinked autosomal loci, similar to models used by Gould (2), Alstad (3), Gould et al. (4) and Hamilton (5). Locus 1 affected responses to Cry1Ac and locus 2 affected responses to Cry2Ab. Each locus had two alleles: r_1 and r_2 conferring resistance and s_1 and s_2 susceptibility to Cry1Ac and Cry2Ab, respectively. We assumed random mating and initial gametic equilibrium. When we used the input parameters of Alstad (3) and Hamilton (5) in our model, our model's projections agreed with the projections from those two models.

We assumed that host plants either produced no Bt toxins (non–Bt refuge plants) or two Bt toxins (Cry1Ac and Cry2Ab). For *H. zea* and two-toxin cotton, this is an unrealistically optimistic scenario, because two-toxin cotton and Cry1Ac cotton overlapped for about 7 y in the United States (Fig. 1 and Fig. S1). The overlap with one- and two-toxin cotton would yield faster evolution of resistance to two-toxin cotton than the scenario we modeled with no one-toxin cotton (4, 6, 7). Thus, our assumption of no overlap between Cry1Ac cotton and two-toxin cotton favors overestimation of the time for resistance to two-toxin cotton. We assumed a 10% refuge of non–Bt host plants in most simulations (Fig. 5), but also examined effects of 10%, 25%, and 50% refuges under some conditions (Fig. 6).

The initial frequency of r_2 was 0.001 in all simulations, which represents an ideal condition based on the assumption of little or no previous exposure to Cry2Ab. We evaluated two assumptions for the initial frequency of r_1: 0.001 and 0.1. For r_1, an initial frequency of 0.001 is unrealistically optimistic for evaluating responses to two-toxin cotton in *H. zea*, because two-toxin cotton was first registered in 2002, 7 y after Cry1Ac cotton was registered (8). By 2009, the first year in which two-toxin cotton exceeded 50% of Bt cotton in the United States (Fig. 1), *H. zea* had been exposed to Cry1Ac cotton for more than a decade and evidence of field-evolved resistance to Cry1Ac had been reported for some *H. zea* populations in the southeastern United States (9, 10). Therefore, an initial frequency of 0.1 for r_1 is probably an underestimate for some populations, which would favor overestimation of the time to resistance.

The fitness of doubly susceptible homozygotes ($s_1s_1s_2s_2$) on non–Bt plants was 1. Because we did not detect significant fitness costs reducing survival on non–Bt cotton (Fig. 2), the fitness of all other genotypes on non–Bt plants was also 1 and we simulated three generations per year to correspond with the three generations per year *H. zea* develops on cotton in some areas of the southeastern United States (9, 10).

We used four sets of genotype-specific fitness parameters on two-toxin cotton (Table S3) corresponding to four sets of assumptions about the dominance of resistance and redundant killing: (*i*) completely recessive resistance ($h_p = 0$) and complete redundant killing [redundant killing factor (RKF) = 1] (ideal conditions), (*ii*) completely recessive resistance ($h_p = 0$) and partial redundant killing ($RKF = 0.64$, based on empirical data in Fig. 2), (*iii*) partially recessive resistance ($h_p = 0.25$) and complete redundant killing, and (*iv*) partially recessive resistance ($h_p = 0.25$) and partial redundant killing ($RKF = 0.64$).

For each generation, we simulated selection with a set of standard equations (11, 12), using the fitness parameters for each of the nine genotypes on two-toxin cotton (Table S3).

First, we calculated the mean fitness of each gamete i, based on the weighted mean fitness of each genotype containing gamete i:

$$\overline{w}_i = \sum_{j=1}^{4} x_j \; w_{ij},$$

where i is r_1r_2, r_1s_2, s_1r_2, or s_1s_2; j = 1–4 represents r_1r_2, r_1s_2, s_1r_2, and s_1s_2, respectively; x_j is the frequency of any gamete j; and w_{ij} is the fitness of the larval genotype containing gametes i and j. Next, we calculated the mean fitness of the pest population as the sum of mean fitnesses of the gametes, weighted by the frequencies of the gametes:

$$\overline{w} = \sum_{i=1}^{4} x_i \; \overline{w}_i,$$

where i = 1–4 represents r_1r_2, r_1s_2, s_1r_2, and s_1s_2, respectively, and x_i is the frequency of any gamete i.

Gametic disequilibrium (D), which is generated by directional selection, was calculated in each generation as:

$$D = (x_{r1r2} * x_{s1s2}) - (x_{r1s2} * x_{s1r2}).$$

We assumed the two loci segregated independently, which means the rate of recombination between loci during meiosis (c) was 0.5 (4, 12). With w_H representing the fitness of the double heterozygote ($r_1s_1r_2s_2$), we calculated the frequency of a gamete i after each generation of selection (x_i') as:

$$x_i' = \frac{x_i\overline{w}_i - cw_HD}{\overline{w}},$$

for the r_1r_2 and s_1s_2 gametes, and as:

$$x_i' = \frac{x_i\overline{w}_i + cw_HD}{\overline{w}},$$

for the r_1s_2 and s_1r_2 gametes.

At the end of each year, based on random mating, we calculated the frequency of each of the nine insect genotypes from the gamete frequencies. For example, the frequency of $r_1r_1r_2r_2$ was $(x_{r1r2})^2$, the frequency of $r_1r_1r_2s_2$ was $2(x_{r1r2})(x_{r1s2})$, and $r_1s_1r_2s_2$ was $2(x_{r1r2})(x_{s1s2})+2(x_{r1s2})(x_{s1r2})$.

We calculated fitness on two-toxin cotton as the sum of the fitness values of the nine genotypes on two-toxin cotton weighted by the proportion of each genotype in the population. The time to resistance was the number of years until population fitness on two-toxin cotton at the end of the year was ≥0.25.

If initial frequency is equal for *r1* and *r2*, and fitness on two-toxin cotton is 1 for doubly resistant homozygotes ($r_1r_1r_2r_2$) and 0 for all other genotypes, our resistance criterion is met when the frequency of r_1 and r_2 reaches 0.71, yielding a frequency of 0.25 for $r_1r_1r_2r_2$ and 0.25 fitness for the population. In this case, the resistance criterion used here takes longer to reach than the criterion applied in most previous studies. For example, the criterion used by Gould et al. (4), Onstad and Meinke (7), and Ives et al. (13) was a frequency of 0.5 for r_1 and r_2, which yields a frequency 0.063 for $r_1r_1r_2r_2$. Under most conditions, the increase in frequency of r_1 and r_2 from 0.5 to 0.71 occurs in one or a few generations, so this difference in criteria has relatively little impact when the frequency is equal for r_1 and r_2. However, with a higher initial frequency of r_1 (i.e., 0.1) than r_2 (0.001) and partial redundant killing, the criterion of ≥0.25 fitness can be met substantially before both r_1 and r_2 reach a frequency ≥0.5. For example, if fitness of $r_1r_1s_2s_2$ on two-toxin cotton is >0.25, a population fitness of ≥0.25 can be achieved with a high frequency of r_1, even if the frequency of r_2 remains low.

Toxin Concentration in Plants. The results in our study are similar to previous results in terms of the relative toxicity of Cry1Ac and Cry2Ab to *H. zea* and the concentrations of these toxins in Bt cotton plants. In diet bioassays with a susceptible strain, the toxin concentration causing 50% mortality (LC_{50}) of Cry2Ab relative to Cry1Ac was fivefold higher in our study and 20-fold higher in a previous study (14). The concentration of Cry2Ab relative to Cry1Ac in terminal leaves was 43-fold higher at the presquaring stage and 10-fold higher at the fruiting stage, when insects were first fed material from cotton plants in our study, and 33-fold higher for 75-d-old plants in a previous study (14).

The Cry1Ac concentrations measured in our study over the growing season in one- and two-toxin cotton are within the range measured in 13 commercial cultivars producing only Cry1Ac (15, 16). The two-toxin cotton cultivar used in our experiment (DP 164 B2RF) contains Bt genes from event MON 15985 (17). The concentrations of Cry2Ab measured in our study were equivalent or higher than concentrations reported for event MON 15985 (18, 19). Furthermore, similar to our results, levels of Cry2Ab in leaf samples of MON 15985 significantly declined to a mean of 16.7 $\mu g \cdot g^{-1}$ of fresh leaves 108 d after planting (18).

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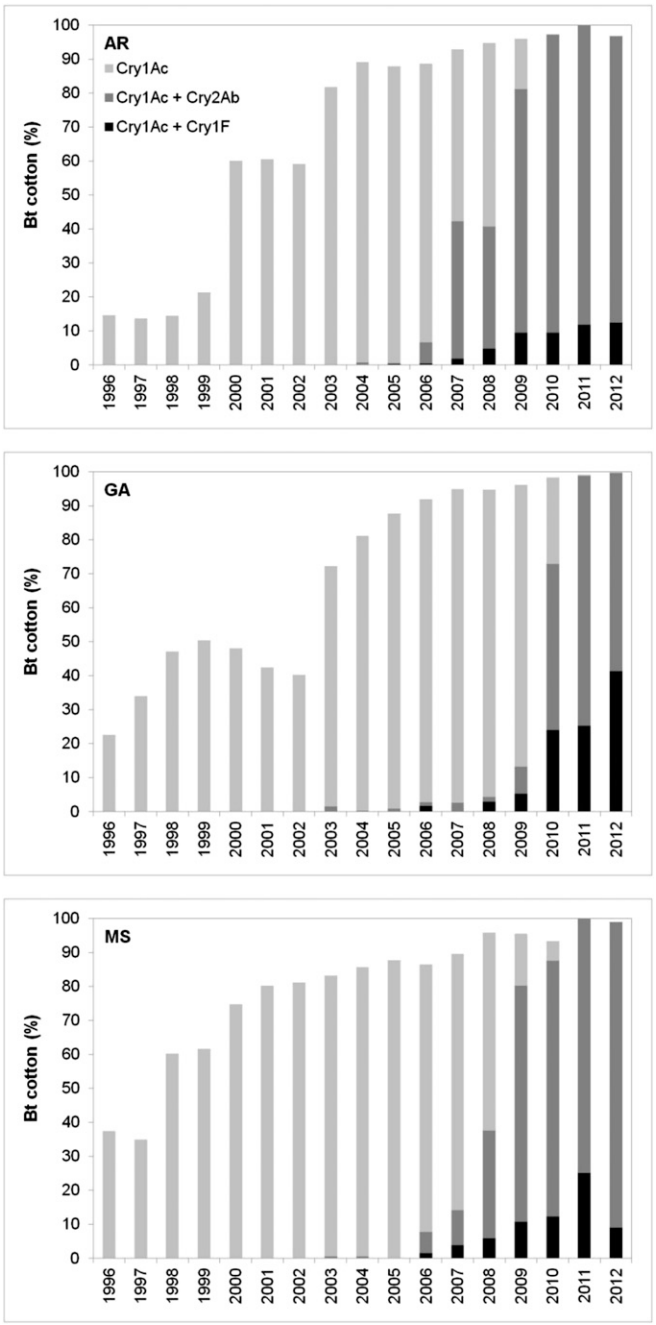

Fig. S1. Percentage of total hectares of upland cotton planted to Bt cotton from 1996 to 2012 in Arkansas (AR), Georgia (GA), and Mississippi (MS). The non–Bt cotton percentage is 100% minus the total height of each bar.

Table S1. Responses of *H. zea* to Cry1Ab toxin incorporated in diet

Strain	*N*	LC_{50} (μg·ml^{-1})	95% Fiducial limits		Slope
			Lower	Upper	
GA	288	63	25	110	1.2
GA-R	288	940	130	7,000	1.1

GA, field-derived strain from Georgia; GA-R, resistant strain derived from the GA strain and selected with Cry1Ac in the laboratory; *N*, number of larvae tested.

Table S2. Selection experiments followed by diet bioassays to assess cross-resistance between Cry1A and Cry2A toxins in eight species of lepidopteran pests

Species	Strain	Selected with	Cross-resistant to	Parameter	CRR	Ref.
Significant cross-resistance detected in individual studies						
Heliothis virescens	CP73-3	Cry1Ac	Cry2A	LC_{50}	53	1
	YHD2	Cry1Ac	Cry2A	LC_{50}	15	2
	KCBhyb	Cry2Aa	Cry1Ac	LC_{50}	188	3
	CXC	Cry2Aa	Cry1Ac	LC_{50}	289	3
Helicoverpa zea	Not named	Cry1Ac	Cry2Aa	LC_{50}	3.3	4
Pectinophora gossypiella	BX-R1	Cry2Ab	Cry1Ac	LC_{50}	420	5
	BX-R2	Cry2Ab	Cry1Ac	LC_{50}	21	5
Cross-resistance not significant in individual studies						
Diatracea saccharalis	Cry1Ab-RR	Cry1Ab	Cry2Ab	LC_{50}	0.51	6
Helicoverpa armigera	SP15	Cry2Ab	Cry1Ac	LC_{50}	1.54	7
	GYBT	Cry1Ac	Cry2Aa	LC_{50}	1.40	8
	BtR	Cry1Ac	Cry2Ab	IC_{50}	1.09	9
	LFR_{10}	Cry1Ac	Cry2Ab	IC_{50}	1.01	9
	Not named	Cry1Ac	Cry2Ab	LC_{50}	1.05	10
	BX	Cry1Ac	Cry2Ab	LC_{50}	1.4	11
	SCD-r1	Cry1Ac	Cry2Aa	LC_{50}	1.2	12
Helicoverpa punctigera	Hp4.13	Cry2Ab	Cry1Ac	LC_{50}	1.58	13
	Hp4.13	Cry2Ab	Cry1Ab	LC_{50}	0.32	13
Helicoverpa zea	AR	Cry1Ac	Cry2Aa	LC_{50}	1.55	14
	GA-R	Cry1Ac	Cry2Ab	LC_{50}	1.98	This study
Plutella xylostella	SZBT	Cry1Ac	Cry2Aa	LC_{50}	1.20	15
Trichoplusia ni	GLEN-Cry1Ac-BCS	Cry1Ac	Cry2Ab	IC_{50}	2.24	16

The cross-resistance ratio (CRR) is the LC_{50} (concentration killing 50%) or IC_{50} (concentration causing 50% inhibition of growth) of the toxin that was not used in selection for a selected strain divided by the LC_{50} or IC_{50} of the same toxin for an unselected control strain. CRR > 1 indicates that the selected strain was cross-resistant to the toxin not used in selection. For example, in the CP73-3 strain of *H. virescens*, selection with Cry1Ac increased the LC_{50} of Cry2A of the selected strain by 53-fold relative to the unselected strain, yielding CRR = 53. In one study not reported in the table (17), bioassays evaluated survival of *P. gossypiella* strains selected for resistance to Cry1Ac and unselected strains to individual concentrations of Cry2Aa. Comparisons of survival of the three selected strains to the survival of two unselected strains at a concentration of 1 μg of Cry2Aa per ml of diet showed significantly higher survival in the selected than unselected strains. Survival of the unselected strain at that concentration was 0, which precludes calculation of a resistance ratio.

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Table S3. Fitness of the nine insect genotypes on two-toxin Bt cotton producing Cry1Ac and Cry2Ab as a function of dominance (h_p) and *RKF*

Conditions		Genotype-specific fitness								
h_p	*RKF*	r_1r_1 r_2r_2	r_1r_1 r_2s_2	r_1r_1 s_2s_2	r_1s_1 r_2r_2	r_1s_1 r_2s_2	r_1s_1 s_2s_2	s_1s_1 r_2r_2	s_1s_1 r_2s_2	s_1s_1 s_2s_2
0	1	0.80	0.036	0.036	0.036	0.036	0.036	0.036	0.036	0.036
0	0.64	0.80	0.40	0.40	0.40	0.036	0.036	0.40	0.036	0.036
0.25	1	0.80	0.51	0.036	0.51	0.23	0.036	0.036	0.036	0.036
0.25	0.64	0.80	0.51	0.40	0.51	0.23	0.036	0.40	0.036	0.036

The fitness of the double homozygotes was fixed in all simulations, while we varied the fitness of the other seven genotypes depending on dominance (h_p) and the *RKF*, as explained below. *Fitness of $s_1s_1s_2s_2$*: The extensive field data of Jackson et al. (1) for susceptible populations of *H. zea* from North Carolina show that survival of susceptible *H. zea* on two-toxin Bt cotton (producing Cry1Ac and Cry2Ab) relative to non–Bt cotton was 3.6% (2). We defined fitness of $s_1s_1s_2s_2$ on non–Bt cotton as 1 and assumed that fitness on two-toxin cotton relative to non–Bt cotton is proportional to survival on two-toxin cotton relative to non–Bt cotton. Thus, we used 0.036 as the fitness of $s_1s_1s_2s_2$ on two-toxin cotton. *Fitness of $r_1r_1r_2r_2$*: The fitness of $r_1r_1r_2r_2$ on two-toxin Bt plants is sometimes set at 1 in modeling studies, indicating complete resistance (3–5). Here we set the fitness of $r_1r_1r_2r_2$ at 0.80 to account for incomplete resistance, which typically occurs for one-toxin plants (6) and is likely for two-toxin plants. Fitness of $r_1r_1r_2r_2$ of 0.8 rather than 1 tends to slightly slow evolution of resistance. *Fitness of the other seven genotypes*: The fitness of the seven genotypes other than the double homozygotes depended on dominance (h_p = 0 or 0.25) and redundant killing (*RKF* = 1 or 0.64), as explained below. To estimate the fitness of one genotype relative to another from empirical data, we assumed that the relative fitness of different genotypes on two-toxin plants is proportional to their relative survival on two-toxin plants. We also assumed that each locus contributed equally to fitness on two-toxin plants, so fitness was equal within the following pairs of genotypes: $r_1r_1r_2s_2$ and $r_1s_1r_2r_2$, $r_1r_1s_2s_2$ and $s_1s_1r_2r_2$, and $r_1s_1s_2s_2$ and $s_1s_1r_2s_2$. *Dominance*: Dominance (*h*) is calculated for a single resistance locus as:

$$h = (W_{rs} - W_{ss})/(W_{rr} - W_{ss}) \ , \qquad \textbf{[S1]}$$

where W_{ss}, W_{rs}, and W_{rr} are the fitnesses of *ss*, *rs*, and *rr*, respectively (7). Values of *h* vary from 0 for completely recessive resistance to 1 for completely dominant resistance. We extended this to two loci, with dominance of resistance to two-toxin plants defined as:

$$h_p = (W_{r1s1r2s2} - W_{s1s1s2s2})/(W_{r1r1r2r2} - W_{s1s1s2s2}). \qquad \textbf{[S2]}$$

Values of h_p vary from 0 for completely recessive resistance to 1 for completely dominant resistance. We rearranged Eq. **S2** to solve for the fitness of double heterozygotes:

$$W_{r1s1r2s2} = h_p(W_{r1r1r2r2} - W_{s1s1s2s2}) + W_{s1s1s2s2}. \qquad \textbf{[S3]}$$

Redundant killing. We define the *RKF* as:

$$RKF = 1 - (W_{r1r1s2s2} - W_{s1s1s2s2}), \qquad \textbf{[S4]}$$

where $W_{s1s1s2s2}$ and $W_{r1r1s2s2}$ are the fitnesses on two-toxin cotton of $s_1s_1s_2s_2$ and $r_1r_1s_2s_2$, respectively. The value of *RKF* varies from 0 for no redundant killing to 1 for complete redundant killing, which means the fitness on two-toxin cotton is equal for $r_1r_1s_2s_2$ and $s_1s_1s_2s_2$. Lack of complete redundant killing (*RKF* < 1) can be caused by any factors causing $W_{r1r1s2s2} > W_{s1s1s2s2}$. One such factor is cross-resistance to toxin 2 caused by selection with toxin 1. However, *RKF* < 1 can occur without cross-resistance. For example, this can happen when the concentration of toxin 2 declines seasonally so that it is not high enough to cause mortality. In this case, fitness is not affected by toxin 2 or locus 2, yielding $W_{r1r1} > W_{s1s1}$ and *RKF* < 1. *RKF* is most useful as an index of redundant killing when fitness of $s_1s_1s_2s_2$ is close to 0 and becomes less useful as $s_1s_1s_2s_2$ approaches 1. In our modeling, we assumed that fitness was the same for $r_1r_1s_2s_2$ and $s_1s_1r_2r_2$, but if these genotypes do not have equal fitness, *RKF* can be evaluated separately for each of the two genotypes. Although other formulas could be conceived for measuring the extent of redundant killing, Eq. **S4** for calculating *RKF* is particularly useful because it focuses on the extent of the increase in fitness of $r_1r_1s_2s_2$ and $s_1s_1r_2r_2$ relative to fitness of $s_1s_1s_2s_2$. As this key difference increases, resistance is expected to evolve faster. Relative to fitness of $s_1s_1s_2s_2$, increased fitness of $r_1s_1s_2s_2$ and $s_1s_1r_2s_2$ would also accelerate resistance evolution. This condition would also yield increased fitness of $r_1r_1s_2s_2$ and $s_1s_1r_2r_2$ relative to $s_1s_1s_2s_2$ and thus would be reflected in *RKF* based on Eq. **S4**. *Ideal and data-based assumptions about fitness*: Under ideal conditions, resistance is completely recessive (h_p = 0) and complete redundant killing occurs (*RKF* = 1), yielding a fitness advantage relative to doubly susceptible homozygotes ($s_1s_1s_2s_2$) only for doubly resistant homozygotes ($r_1r_1r_2r_2$). The experimental results here (Fig. 2) show that survival on two-toxin cotton was 11 times higher for the GA-R strain (6.7%) selected for resistance to Cry1Ac than for its parent strain GA (0.6%) (Fig. 2). We assumed that GA-R individuals were $r_1r_1s_2s_2$ and GA individuals were $s_1s_1s_2s_2$. Based on the assumptions described above that the fitness of $s_1s_1s_2s_2$ is 0.036 and the relative fitness of different genotypes on two-toxin plants is proportional to their relative survival on two-toxin plants, we estimated the fitness of $r_1r_1s_2s_2$ on two-toxin plants as 0.036 × (6.7%/0.06%) = 0.40. Applying Eq. **S4**, this yields *RKF* = 0.64. With h_p = 0 and *RKF* = 0.64, a fitness advantage relative to doubly susceptible homozygotes occurs for four genotypes other than $r_1r_1r_2r_2$: $r_1r_1s_2s_2$, $r_1r_1r_2s_2$, $s_1s_1r_2r_2$, and $r_1s_1r_2r_2$ (fitness = 0.40). Because GA apparently had some resistance alleles, we infer that the survival of $s_1s_1s_2s_2$ would be equal to or less than survival of GA, which would yield an equal or lower value of *RKF*. Because resistance evolves faster with less redundant killing (lower *RKF*) (Figs. 5 and 6), our assumption of *RKF* = 0.64 would favor accurate estimation or overestimation of the time for resistance to evolve. Based on our experimental data, *h* = 0.25 for resistance to Cry1Ac cotton (Fig. 2). We assumed that h_p was also 0.25 for two-toxin cotton. Applying Eq. **S3**, with h_p = 0.25, $W_{r1r1r2r2}$ = 0.8, and $W_{s1s1s2s2}$ = 0.036 yields $W_{r1s1r2s2}$ = 0.227 (rounded to 0.23). Our estimate of *h* = 0.25 on Cry1Ac cotton is lower than a previous estimate of *h* = 0.83 based on responses of *H. zea* to Cry1Ac in diet (8, 9). With h_p = 0.25, we calculated the fitness of the two genotypes with three resistance alleles (r1r1r2s2 and r1s1r2r2) as the mean fitness of the double heterozygotes (fitness = 0.227) and the doubly resistant homozygotes [(0.227 + 0.80)/2 = 0.514, rounded to 0.51].

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