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Transgenic plants expressing two *Bacillus thuringiensis* toxins delay insect resistance evolution

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Preventing insect pests from developing resistance to *Bacillus thuringiensis* (*Bt*) toxins produced by transgenic crops is a major challenge for agriculture. Theoretical models suggest that plants containing two dissimilar *Bt* toxin genes ('pyramided' plants) have the potential to delay resistance more effectively than single-toxin plants used sequentially or in mosaics. To test these predictions, we developed a unique model system consisting of *Bt* transgenic broccoli plants and the diamondback moth, *Plutella xylostella*. We conducted a greenhouse study using an artificial population of diamondback moths carrying genes for resistance to the *Bt* toxins Cry1Ac and Cry1C at frequencies of about 0.10 and 0.20, respectively. After 24 generations of selection, resistance to pyramided two-gene plants was significantly delayed as compared with resistance to single-gene plants deployed in mosaics, and to Cry1Ac toxin when it was the first used in a sequence. These results have important implications for the development and regulation of transgenic insecticidal plants.

Transgenic plants expressing insecticidal proteins from the bacterium *B. thuringiensis* (*Bt*) were first commercialized in 1996, and at least 16 companies have since been involved in developing *Bt* crops[1]. *Bt* had limited use as a foliar insecticide for over 40 years; in the last 7 years it has become a major insecticide because genes encoding *Bt* toxins have been engineered into important crops that were grown on 14.5 million hectares worldwide in 2002 (ref. 2). These crops have benefitted growers economically, reduced the use of other insecticides and, in the case of *Bt* corn, lowered the incidence of toxic fungal compounds (fumonisins) by reducing insect damage that makes the corn more susceptible to the fungi[3]. A recent study demonstrated that *Bt* cotton led to long-term regional pest suppression and reduced the need for insecticide sprays[4].

Although transgenic plants offer many unique opportunities for the management of pest populations, they also present new challenges, one of the main ones being the potential evolution of resistance. There are at least four possible ways in which plants with constitutive expression of *Bt* toxins can be used to delay resistance: (i) engineer plants to express toxin genes at a level at which not all susceptible individuals are killed; (ii) provide refuges for susceptible insects while engineering plants to express the genes at levels as high as possible within acceptable limits to avoid deleterious effects on yield, health or the environment; (iii) deploy different toxins individually in different varieties; and (iv) deploy plants expressing a mixture of different toxins. Among these options, the refuge–high dose (ii) and pyramiding (iv) strategies seem most promising[5–7]. A major difficulty for the refuge–high dose strategy is managing the insect population within the refuge to ensure that sufficient susceptible alleles will exist, while at the same time ensuring that damage to the refuge plants is minimized[7,8]. Until late in 2002, refuge–high dose was the only commercially available strategy for corn and cotton[9]. However, regulatory applications for pyramided cotton plants (Bollgard II) with two genes derived from *Bt* (*cry1Ac* and *cry2Ab2*) were approved for commercial use in Australia and the United States in 2002 (refs. 10,11). Few, if any, Cry1Ac-resistant pink bollworms, *Pectinophora gossypiella*, survived on Bollgard II[11,12], a result supporting the use of two *Bt* genes.

Plant breeders have considered and frequently endorsed the concept of using pyramided genes to delay the development of resistance in pest species, especially pathogens. Theoretical models suggest that varieties pyramiding two dissimilar insect toxin genes in the same plant have the potential to delay the development of resistance much more effectively than single-toxin plants used sequentially or in mosaics or seed mixtures, even with relatively small and more economically acceptable refuge sizes[6,7]. To test predictions of the models and to assess the effects of gene pyramiding on resistance management, we used a model system composed of broccoli plants transformed to express different Cry toxins (Cry1Ac, Cry1C) combined with four populations of diamondback moth, which carried resistance either to both, one or neither of the toxins. The objective of this study was to compare how quickly an insect population that contains a relatively high frequency of alleles for resistance to Cry1Ac and Cry1C evolves resistance to each or both toxins when exposed to plants that express both toxins simultaneously, sequentially or in mosaics.

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Table 1 Homozygous resistant genotype ratios in diamondback moth strains or cross progeny resistant to Cry1Ac and/or Cry1C

Strain or progeny from cross (female × male)	Expected homozygous resistant genotypes (%)[a]			Adjusted survival until pupation on different *Bt* plants (%)[b]			
	Cry1Ac-R	Cry1C-R	Cry1A/1C-R	Non-*Bt*	Cry1Ac	Cry1C	Two-gene
1. S (F_{289})	0	0	0	96	0	0	0
2. Cry1Ac-R (F_{12})	100	0	0	96	96	0	0
3. Cry1C-R (F_{17})	0	100	0	86	0	105	0
4. Cry1A/1C-R (F_{52})	100	100	100	88	109	111	100
5. S × Cry1Ac-R (F_{12})	0	0	0	98	0	0	0
6. S × Cry1C-R (F_{17})	0	0	0	92	0	0	0
7. S × Cry1A/1C-R (F_{52})	0	0	0	92	0	0	0
8. Cry1Ac-R (F_{12}) × Cry1C-R (F_{17})	0	0	0	88	0	0	0
9. Cry1A/1C-R (F_{52}) × Cry1Ac-R (F_{12})	100	0	0	90	102	0	0
10. Cry1C-R (F_{17}) × Cry1A/1C-R (F_{52})	0	100	0	88	0	102	0
11. Cry1A/1C-R × F_1 of (S × Cry1Ac-R)	50	0	0	80	50	0	0
12. Cry1A/1C-R × F_1 of (S × Cry1C-R)	0	50	0	82	0	49	0
13. (S × Cry1A/1C-R) → F_2	25	25	6.3	86	26	12	2.3
14. F_1 of (S × Cry1A/1C-R) × Cry1A/1C-R	50	50	25	92	45	32	21
15. F_1 of (Cry1Ac-R × Cry1C-R) × Cry1A/1C-R	50	50	25	92	46	30	24

[a]Expected genotypes of progeny if monogenic inheritance with two alleles for Cry1Ac or Cry1C resistance and no linkage between Cry1Ac- and Cry1C-resistance genes. [b]Adjusted survival on *Bt* plants (calculated as survival on *Bt* plants divided by survival on non-*Bt* plants, might be >100%) and survival on non-*Bt* plants. $n = 50$.

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RESULTS

Suitability of the model system

Preliminary trials on the interactions between four diamondback moth strains and the progeny from various crosses, and three types of *Bt* broccoli plants, proved that insect and plant genotypes used in the model system performed as expected (**Table 1**). For the three primary resistant (R) diamondback moth strains (Cry1Ac-R, Cry1C-R, Cry1A/1C-R), the one susceptible (S) primary strain and progeny from most of the 11 crosses from the four primary strains, the observed survival was consistent with that expected based on monogenic inheritance for Cry1Ac or Cry1C resistance and no linkage between Cry1Ac- and Cry1C-resistance genes. The results from strains 1–7 (**Table 1**) showed that there was high expression of one or two *Bt* toxins in the *Bt* broccoli plants, and that there was no cross-resistance between Cry1Ac and Cry1C resistance in the diamondback moth. The results from strains 14 and 15 showed that there was no linkage between the Cry1Ac- and Cry1C-resistance genes, because the estimated survival of the two-gene *Bt* broccoli for strain 14 should have been significantly higher than strain 15 if any linkage existed (recombination does not occur in female *Lepidoptera*)[13,14]. Crosses related to Cry1Ac-R, Cry1C-R and Cry1A/1C-R strains (strains 9–12 and 15) also showed allelic complementation for Cry1Ac or Cry1C resistance in the three strains (**Table 1**). The observed resistance to Cry1C in strains 13–15 was less than expected, suggesting that resistance was not simply inherited (see Discussion).

Population density in different treatments

After 12 generations of selection, there was a significantly ($P < 0.0001$) higher density of diamondback moth larvae and pupae on Cry1Ac broccoli plants in the mosaic and sequential treatments than on Cry1C plants or on two-gene plants (**Fig. 1**). Although technical problems prevented accurate density estimation in the first generation of these treatments, a preliminary cage test before the formal experiment (using similar treatments) found that initial surviving larval densities were about 0.2 larvae per plant on Cry1Ac plants, about 0.1 on Cry1C plants and zero on two-gene plants. Thus, all of the plants probably started with similar larval densities (0–0.2 larvae per plant), but there was already a trend toward an increase on the Cry1Ac plants even by the fourth generation (**Fig. 1**). In the sequential treatment, Cry1Ac plants were completely defoliated by diamondback moth larvae between generations 10 and 12, depending on replicate, because of the evolution of resistance, and were replaced by Cry1C plants thereafter. The insect density on Cry1C plants in the mosaic treatment was significantly ($P < 0.0001$) higher than on either Cry1C plants in the sequential treatment or two-gene plants in the pyramid treatment, at least from generation 9.

Resistance of diamondback moth in different treatments

We measured the resistance of diamondback moth in different treatments to Cry1Ac, Cry1C or both toxins by the survival of larvae on *Bt* broccoli plants expressing one or both toxins. The mean survival (± s.e.m.) of the artificial diamondback moth population before selection in cages was 0.53 ± 0.13% on Cry1Ac broccoli plants ($n = 750$) and 0.12 ± 0.02% on Cry1C plants ($n = 1{,}750$). After 18–24 generations of selection, the mean survival of larvae on Cry1Ac broccoli plants in the mosaic and sequential treatments was >70% (declining somewhat between generations 18 and 24 in the sequential treatment, perhaps because Cry1Ac plants were removed in generations 10–12) and significantly ($P < 0.0001$) higher than that in the pyramid treatment (<5%) (**Table 2**). The survival on Cry1C broccoli in the mosaic treatment was significantly ($P < 0.0001$) higher than that of the sequential and pyramid treatments. In one of the four replicates of the mosaic after 24 generations of selection, the survival on Cry1C broccoli was 12.1%. For the pyramid treatment, the survival was very low or zero on Cry1Ac, Cry1C or two-gene broccoli plants. The survival of larvae produced by moths from each cage and tested in the laboratory (**Table 2**) was consistent with the insect density data on *Bt* plants in the cages (**Fig. 1**).

DISCUSSION

The experimental data for the survival of the range of diamondback moth strains and crosses on the three genotypes of *Bt* broccoli showed that this model system is suitable for tests of the various two-toxin deployment strategies. The results from the cage tests indicate that pyramided two-gene plants can significantly delay resistance evolution to both toxins in a mosaic deployment, and to at least Cry1Ac when it is used initially in a sequential deployment. Although our population sizes were much smaller than those occurring in nature, the selection

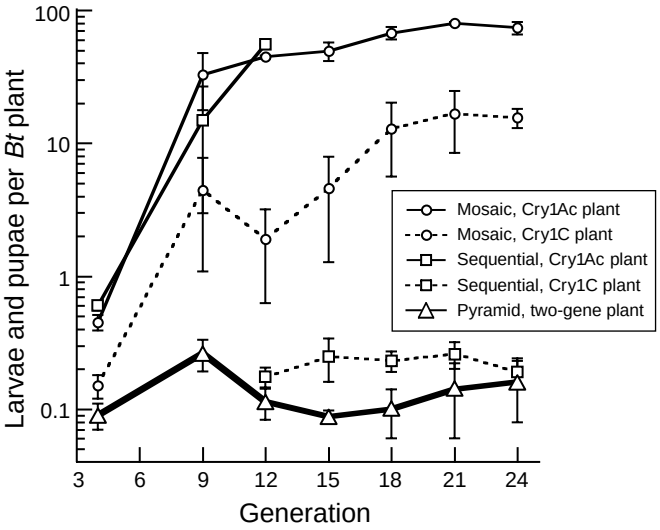

Figure 1 Populations of Cry1Ac-/Cry1C-resistant diamondback moths in cages with different *Bt* broccoli treatments. Mosaic, 40% Cry1Ac plants plus 40% Cry1C plants plus 20% refuge. Sequential, 80% Cry1Ac plants plus 20% refuge until control failures occurred; then plants were replaced by 80% Cry1C plants plus 20% refuge. Pyramid, 80% plants with pyramided expression of Cry1Ac and Cry1C plus 20% refuge of non-*Bt* broccoli. From generation 12 to 24, resistance to pyramided and sequential Cry1C plants was significantly less than to mosaic Cry1C plants or mosaic or sequential Cry1Ac plants ($P < 0.05$, HSD).

response to Cry1Ac in the pyramided treatments (**Table 2**) showed that our experiments had initial resistance frequencies sufficiently high to generate the effects expected in the field, that is, resistance can still evolve to pyramids[6,7]. Although *Bt* cotton with the pyramided two *Bt* genes is now in commercial use, total replacement of Cry1Ac gene varieties with two *Bt* gene varieties is not expected to occur for many years.

The mosaic was clearly inferior to the pyramiding strategy. Both population densities (**Fig. 1**) and the resistance frequencies (**Table 2**) were significantly higher on both Cry1Ac and Cry1C plants in the mosaic than in the pyramid treatments after 18 generations of selection (**Table 2**). This is consistent with models and experiments with other insecticides showing that mosaics select for resistance more quickly than either pyramid treatment (or mixtures, in the case of insecticides) or sequential deployment[6,7]. The responses to selection for resistance on the Cry1Ac plants in both the sequential and mosaic treatments were entirely consistent with results from models developed in 1998 (ref. 7). The models were run with an initial resistance allele frequency of 0.1 and it was assumed that all of the resistant homozygotes survived, but that heterozygotes and susceptible homozygotes died when on Cry1Ac plants. Under these conditions, the frequency of resistant homozygous larvae in a sequential treatment would reach 80–90% by generation 7. Population increases would begin by generation 4, with density increases of more than 100-fold by generation 12, limited only by larval food supply. In a mosaic treatment, resistance would be slightly slower to evolve, but the frequency of resistant homozygotes would still reach 80% by generation 12 and 95% before generation 18 (when resistance was first measured), again with rapid increases in larval numbers even from generation 4.

Farmers may plant crops in mosaic pattern when different products are available. Our experiments showed that allowing the concurrent release of cultivars with the two *Bt* genes in separate plants, each with one *Bt* gene, is not the best way to delay resistance. Even sequential release would result in control failure of at least one cultivar sooner than if pyramided varieties were used. The absence of a significant difference between the survival of insects on the two-gene plants in the pyramided and sequential strategy was due to the lack of resistance to Cry1C in both treatments, and not to the marked differences in resistance to Cry1Ac in the two treatments (**Table 2**). Thus, we believe the pyramided plants provided better resistance management than the sequential deployment for at least Cry1Ac. Although it is tempting to speculate that the absence of evolution of Cry1C resistance after 12 generations of selection in the sequence treatment may have something to do with the order of selection, the reverse sequence was not tested, so no conclusions can be drawn for this case.

Cry1C resistance evolved more slowly than Cry1Ac resistance in each of the three treatments. One reason for this might be the difference in the genetics of resistance to each toxin. Cry1A resistance is controlled by one autosomal recessive gene[15] located in linkage group 7 (ref. 14). Cry1C resistance is probably controlled by more than one autosomal recessive gene[16,17]. A recent mapping study indicates that the genes for Cry1C resistance in our colonies are located in two separate linkage groups (nos. 20 and 23), which are different from the group to which Cry1A resistance maps[18]. Modeling the resistance to Cry1C was limited by a lack of information on the survival value (that is, fitness) of these individual loci, and it was thus not possible to draw any meaningful conclusions about whether these results fit our models. The initial allele frequency of 20% for Cry1C resistance, although seemingly high, might have been too low to clearly demonstrate the resistance evolution on Cry1C broccoli plants in the sequential treatment within the time scale of our experiment.

A resistance management strategy is, in theory, most effective when the frequencies of resistance alleles are low, especially when below 0.01 (ref. 7). The initial allele frequency for most *Bt* crops and insect systems in field situations is much lower than what we used in the greenhouse cage tests, generally 0.001 or less[5]. Considering the relatively small population in a cage ($n = 400$ initially, $n \approx 2{,}000$ eggs laid after F_1

Table 2 Survival of Cry1Ac/Cry1C-resistant diamondback moth larvae from adults in cages on different *Bt* broccoli plants

Generation	Treatment	Mean survival (s.e.m.) (%)[a] on *Bt* plants[b]		
		Cry1Ac	Cry1C	2-gene
18	Pyramid	4.8 (2.9) B	0.25 (0.25) B	0.25 (0.25) B
	Mosaic	98 (0.98) A	6.1 (2.5) A	5.9 (2.8) A
	Sequential	87 (0.38) A	0.25 (0.25) B	0.25 (0.25) B
24[c]	Pyramid	4.1 (0.7) C	0 B	0 B
		[3.1, 3.2, 4, 6.1]	[0, 0, 0, 0]	[0, 0, 0, 0]
	Mosaic	94 (1.5) A	6.9 (1.9) A	3.9 (0.64) A
		[90, 95, 96, 96]	[3.1, 6.1, 6.3, 12]	[2.1, 4.1, 4.2, 5.1]
	Sequential	73 (2.0) B	0.25 (0.25) B	0.25 (0.25) B
		[69, 71, 74, 78]	[0, 0, 0, 1.0]	[0, 0, 0, 1.0]

[a]Adjusted survival was calculated as survival on *Bt* plants divided by survival on non-*Bt* plants. $n = 100$ for Cry1C or two-gene plants and 50 or 100 for Cry1Ac plants. [b]Within the same generation, means (± s.e.m.) within a column followed by the same letter are not significantly different ($P > 0.05$, HSD). [c]Survival data in square brackets are for each of the four replicates in each treatment.

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and before control failure), it would have been unwise to use initial resistance allele frequencies on the order of 10^{-3} (0.1%) or 10^{-2} (1%) because this would have resulted in a one in a million (10^{-6}) or one in ten thousand (10^{-4}) chance of having a homozygous resistant individual (RR) within the populations, even for Cry1A resistance controlled by one gene (and much less for Cry1C resistance controlled by two genes). Results from models[7] indicated that the benefits of pyramiding are much greater when initial frequencies of resistance alleles are low.

Since single-gene *Bt* plants were first grown commercially in 1996, they have had an overall positive impact on agriculture, human health and the environment by reducing the use of broader-spectrum foliar insecticides to control lepidopterous pests[3]. Although insect resistance resulting in control failures of any of the present *Bt* crops has not occurred[19], reducing the risk of resistance remains a top priority. The current resistance management strategy requires relatively large refuges in which susceptible alleles can be maintained. The maximum benefits to crop production, farm profitability and reduction of pesticide use would come from larger proportions of transgenic insecticidal crops, but long-term enjoyment of these benefits may only be feasible by limiting the percentage of the crops that are transgenic. The conflict between the economic costs of refuges and the need for resistance management may not be easily resolved with single-toxin strategies[7,8]. Modeling work[6,7] and the data generated from these experiments with the diamondback moth–*Bt* broccoli system suggest that stacking or pyramiding toxin genes that express toxins with different modes of action or binding characteristics at a 'high' dose offers a potential route for achieving longer delays in the development of resistance. We believe that industry should be encouraged to develop such plants for their increased durability for insect management and we suggest that the smaller refuge size required by pyramided toxin plants[6,7] may be an additional incentive for them to do so.

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METHODS

Insects. Four strains of diamondback moth were used. The susceptible Geneva 88 strain (S), the Cry1Ac-resistant strain (Cry1Ac-R) and the Cry1C-resistant strain (Cry1C-R), as reported previously[17], were used to develop an artificial population for the cage tests. The Cry1Ac-R and Cry1C-R strains survived on transgenic broccoli expressing Cry1Ac and Cry1C toxins of *Bt*, respectively, but did not show cross-resistance between Cry1Ac and Cry1C[20]. A multiply resistant strain (Cry1A/1C-R), resistant to both Cry1Ac and Cry1C toxins[16,20], was used to determine how the genes would interact on three plant genotypes.

An artificial population of diamondback moths was created by releasing 50 F_1 (S × Cry1Ac-R) moths and 100 F_1 (S × Cry1C-R) moths into a cage containing 100 moths of the S strain. For each strain, the total number of moths in the cage was 250, with a 1:1 ratio of female and male moths. After 24 h the eggs were collected from the cage and were put on artificial diet[21] for rearing of the F_1 larvae. About 1,000 F_1–F_3 moths were used to produce F_2–F_4 eggs of the artificial population. F_4 pupae were released in each cage as described below. The expected allele frequency in the artificial population was 0.10 for Cry1A resistance and 0.20 for Cry1C resistance (which is the same for each locus independently, for any locus contributing to Cry1C resistance). The mean survival rate (± s.e.m.) of F_4 neonates was 0.53 ± 0.13% (n = 750) on Cry1Ac broccoli plants, 0.12 ± 0.02% (n = 1,750) on Cry1C plants, and 75% (n = 100) on non-*Bt* plants was. Based on the adjusted survival on *Bt* plants relative to non-*Bt* plants (0.71% on Cry1Ac plants and 0.16% on Cry1C plants), the calculated initial allelic frequency was 8.4% for Cry1Ac resistance (square root of 0.71% for monogenic inheritance) and 20% for Cry1C resistance (fourth root of 0.16% for two-gene inheritance).

Transgenic broccoli plants expressing *Bt* toxins. Three types of transgenic broccoli (*Brassica oleracea* L.) plants producing high levels of Cry1Ac, Cry1C or both were used in the cage study[22–24]. The *cry1Ac* and *cry1C* progeny were verified by screening the plants with Cry1Ac-R or Cry1C-R diamondback moth neonates when plants were 4–5 weeks old[25]. Both *cry1Ac* and *cry1C* plants also killed 100% of the neonates of F_1 heterozygotes (S × Cry1Ac-R) or 100% of all instars of F_1 heterozygotes (S × Cry1C-R), respectively[17], indicating a high dose in terms of resistance management. Broccoli plants that expressed both Cry1Ac and Cry1C toxins were produced by sexual crosses between the two types of *Bt* transgenic broccoli and were characterized for *Bt* protein production and control of S, Cry1Ac-R and Cry1C-R diamondback moth strains[24]. ELISA analysis showed that Cry1Ac and Cry1C proteins were produced in the hybrids and in their F_1 progeny at levels comparable to those in the original single-gene parental lines (620–801 and 941–1,380 ng/g, respectively)[24].

Tests on suitability of the model system. There were 11 types of crosses using the 4 primary diamondback moth strains, resulting in 15 strains total (**Table 1**). Nontransgenic broccoli ('Green Comet' hybrid) plants were infested with the eggs from each strain or cross. The second instars were put on three types of *Bt* broccoli (Cry1Ac, Cry1C and Cry1Ac + Cry1C) leaf disks inside 30-ml plastic cups. Nontransgenic broccoli was used as a control. There was a total of 50 larvae in each treatment, either in five or ten replicates. Broccoli leaf disks were replaced 3 d after treatment and survival was determined until pupation at 27 ± 1°C. From previous studies[24,25], the survivors on *Bt* broccoli were expected to be homozygous for resistance to the corresponding toxin(s). To adjust for control mortality (4–20%), survival on *Bt* plants was divided by survival on non-*Bt* plants[26], so the adjusted survival might be >100% because of higher survival on *Bt* plants than on non-*Bt* plants.

Experimental design for the cage tests. All tests were conducted in greenhouses at Cornell University's New York State Agricultural Experiment Station (Geneva, New York, USA) under conditions similar to those previously reported[25]. The cages were made of nylon netting. Each cage was 1.8 m long × 0.9 m wide × 1.7 m high. Three treatments were included in the greenhouse cage tests: (i) pyramid, 80% plants with pyramided expression of Cry1Ac and Cry1C plus 20% refuge of non-*Bt* broccoli; (ii) mosaic, 40% Cry1Ac plants plus 40% Cry1C plants plus 20% refuge; and (iii) sequential, 80% Cry1Ac plants plus 20% refuge until control failures occurred, after which plants were replaced by 80% Cry1C plants plus 20% refuge. Control failures have often been defined as occurring when the frequency of resistance reaches >0.50 (ref. 6), but we continued sampling even after this level was reached (as for cross no. 13, **Table 1**). There were four replicates (cages) for each treatment and 25 plants total in each cage (20 *Bt* plants plus 5 non-*Bt* refuge plants). Inside each cage, a gap of at least 12 cm separated one type of broccoli plant from other type(s) and there was no overlap of leaves between different types of broccoli plants. Thus, larvae could not easily move between the different broccoli types. Four hundred F_4 pupae of the artificial population of diamondback moth were released into each cage. Preliminary tests indicated that about 15–25 d at 25–30 °C were needed to produce each generation in the cages. The non-*Bt* refuge plants were replaced each generation (about every 20 d) when most of the non-*Bt* plants were severely defoliated. The defoliated plant was cut at its base and placed onto the new replacement plant so larvae would not be lost. The three types of *Bt* plants were replaced about every 60 d (three insect generations) up to generation 12. The Cry1Ac plants in the mosaic treatment were replaced about every 30 d after generation 12 because of control failure in all replicates. In the sequential treatment, Cry1Ac plants were replaced by Cry1C plants in generations 10–12 after complete defoliation by diamondback moth larvae. No insecticide was used in the cages.

Data collection in cage tests. The numbers of diamondback moth larvae and pupae on each broccoli plant were counted when peak larval and pupal densities were reached in generations 4, 9, 12, 15, 18, 21 and 24. About 40 moths from generations 18 and 24 were collected from each cage and placed separately in oviposition cups in the laboratory to test the survival of the resulting larvae on *Bt* broccoli expressing either or both Cry1Ac and Cry1C toxins. The methods were similar to the tests on suitability of the model system described earlier, but with the following differences: there were 100 larvae for the treatments with expected survival <50% (ten replicates per treatment and ten larvae per replicate) and 50 larvae for the treatments with expected survival ≥ 50% (ten replicates per treatment and five larvae per replicate). Survival was determined after 3 d at 27 ± 1 °C. Control survival was 96–100% for the populations from each cage.

Statistical analysis. SAS programs were used for analysis of variance[27]. Data were transformed using the arcsine square-root value for proportion of survival, or the log $(x + 1)$ for insect density data before each analysis of variance was done. Treatment means were compared and separated by Tukey's studentized range test 'honestly significant difference' (HSD) at $P = 0.05$ (ref. 27).

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COMPETING INTERESTS STATEMENT
The authors declare that they have no competing financial interests.

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