12-9-2008

Increased Survival of Western Corn Rootworm on Transgenic Corn within Three Generations of On-Plant Greenhouse Selection

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Edited by May R. Berenbaum, University of Illinois at Urbana-Champaign, Urbana, IL, and approved October 16, 2008 (received for review June 10, 2008)

To delay evolution of insect resistance to transgenic crops producing *Bacillus thuringiensis* (Bt) toxins, nearby "refuges" of host plants not producing Bt toxins are required in many regions. Such refuges are expected to be most effective in slowing resistance when the toxin concentration in Bt crops is high enough to kill all or nearly all insects heterozygous for resistance. However, Bt corn, *Zea mays*, introduced recently does not meet this "high-dose" criterion for control of western corn rootworm (WCR), *Diabrotica virgifera virgifera*. A greenhouse method of rearing WCR on transgenic corn expressing the Cry3Bb1 protein was used in which approximately 25% of previously unexposed larvae survived relative to isoline survival (compared to 1–4% in the field). After three generations of full larval rearing on Bt corn (Constant-exposure colony), WCR larval survival was equivalent on Bt corn and isoline corn in greenhouse trials, and the LC_{50} was 22-fold greater for the Constant-exposure colony than for the Control colony in diet bioassays with Cry3Bb1 protein on artificial diet. After six generations of greenhouse selection, the ratio of larval recovery on Bt corn to isoline corn in the field was 11.7-fold greater for the Constant-exposure colony than the Control colony. Removal from selection for six generations did not decrease survival on Bt corn in the greenhouse. The results suggest that rapid response to selection is possible in the absence of mating with unexposed beetles, emphasizing the importance of effective refuges for resistance management.

Bacillus thuringiensis | toxicity assay | MON863 | reciprocal cross | resistance

A "high-dose/refuge strategy" is required in many areas as a means of delaying the evolution of resistance to crops expressing transgenic insecticidal proteins derived from the soil bacterium *Bacillus thuringiensis* (Bt) (Berliner) (1). This strategy involves an extremely high concentration of toxin (25 times the amount needed to kill 99% of the susceptible insects) to ensure that heterozygotes do not survive exposure in the Bt crop, thus making resistance functionally recessive (2). In addition, a nearby refuge is maintained where the pests do not encounter Bt toxin. It is expected that a large number of susceptible pests emerging from the refuge will mate with any resistant individuals emerging from the Bt field.

The duration of susceptibility of insect pests to Bt toxins depends on many factors, including dose of the toxin. Although most Bt toxins targeted toward lepidopteran pests meet the high-dose standard defined above, Cry1Ac targeted toward *Heliocoverpa zea* (Hübner) is not high-dose (2). This same protein does meet the high-dose standard in the context of targeting *Heliothis virescens* (Fabricius), a closely related Heliothine species often found within the same Bt cotton, *Gossypium hirsutum* L., fields. An increase in resistance alleles has been reported for several field populations of *H. zea*, but not in *H. virescens* or most other major lepidopteran pests targeted by Bt crops (3).

As in the case of Cry1Ac targeting *H. zea*, the Bt corn, *Zea mays* L., currently registered for control of corn rootworms (*Diabrotica* spp.) is not high-dose, but rather is considered low-to-moderate (4, 5). The western corn rootworm (WCR), *Diabrotica virgifera virgifera* LeConte (Coleoptera: Chrysomelidae), overwinters in the egg stage in the soil. Larvae emerge in spring and begin feeding on the roots of host plants, undergoing three instars before pupating. Although rootworm-targeting Bt corn provides good protection of grain yield, it is common to observe adult WCR emerging from all of the rootworm Bt products currently available. In contrast, emergence of most lepidopteran target pests from transgenic crops would not be expected. Beetle emergence from plots of Bt corn expressing Cry34Ab1+Cry35Ab1 proteins averaged 3.53% that of isoline plots (6). Emergence from transgenic plots of the modified Cry3A protein and the Cry3Bb1 protein, which are also currently registered for rootworm control, were similar (B.E.H., V. Kaster, H. York-Steiner, R. Kurtz, T. Clark, L. Meinke, D. Moellenbeck, W. French, and T. Vaughn, unpublished data). Clearly, none of the transgenic events currently registered for WCR control expose larvae to a level considered high-dose. It is not known what proportion of survivors of WCR-targeted Bt corn have a susceptible genotype through escaping lethal exposure to the toxin or what proportion, if any, are genetically resistant.

Here we report evolution of resistance to transgenic corn expressing the Cry3Bb1 protein within three generations of selection under greenhouse conditions allowing relatively high larval survival (25% versus 1–4% under field conditions). Four colonies were subjected to different regimes of exposure to Bt corn: exposure as neonates (Neonate-exposure colony), exposure as late instars (Late-exposure colony), constant exposure throughout larval development (Constant-exposure colony), and an unexposed control (Control colony). On-plant rearing conditions differed between colonies to achieve differing Bt exposures (see *Materials and Methods* below). After three generations of selection, the LC_{50} of the Constant-exposure colony was approximately 22-fold greater than the LC_{50} of the Control colony. After six generations, percent survival on Bt corn relative

Author contributions: B.D.S., T.W.S., and B.E.H. designed research; L.N.M., M.L.H., N.J.M., and T.A.S. performed research; M.R.E. analyzed data; and L.N.M., B.D.S., N.J.M., T.W.S., and B.E.H. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

See Commentary on page 19029.

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This article contains supporting information online at www.pnas.org/cgi/content/full/0805565105/DCSupplemental.

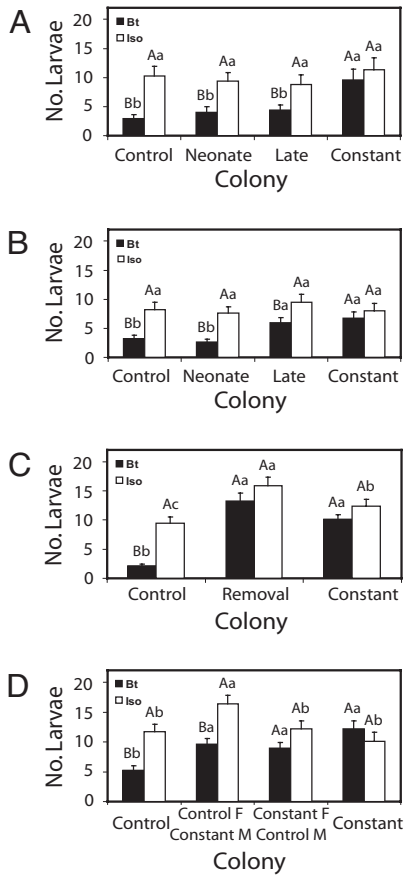

Fig. 1. Mean (± SEM) number of larvae from different laboratory colonies recovered during trials on Bt (black bars) and nontransgenic isoline (white bars) corn (*A*) in the greenhouse after three generations of selection and (*B*) in the greenhouse after six generations of selection. The Constant-exposure colony after six generations of selection was further tested after (*C*) six generations of removal from selection, and (*D*) reciprocal crosses with the unselected Control colony (F = female, M = male). Although untransformed data are shown, analyses were performed using square root ($x + 0.5$) transformed data. Bars with the same letters are not significantly different ($P \geq 0.05$). Capital letters indicate comparisons between isoline and Bt within colonies. Lowercase letters indicate comparisons between colonies within isoline or Bt corn.

to isoline corn was 11.7-fold greater in the field for the Constant-exposure colony than for the Control colony.

Results and Discussion

The WCR is an adaptively dynamic insect pest with documented resistance to chemical controls targeting larvae (7), chemical controls targeting adults (8), and crop rotation commonly used as a cultural control method (9). Interestingly, insecticide resistance in this pest may persist for many generations in the absence of selection (10). Here, we describe the evolution of resistance to a low-to-moderate dose Bt product under greenhouse conditions.

Greenhouse Experiments. When WCR survivors of Bt corn were selected and mated in the laboratory, resistance evolved in as few as three generations. In greenhouse experiments, the number of WCR larvae recovered from the Constant-exposure colony was equivalent from both Bt and isoline corn after three and six generations of selection (Fig. 1 *A* and *B*). Significantly more adults of Constant-exposure and Late-exposure colonies were recovered from Bt corn than adults of Control or Neonate-exposure colonies in generations 3 and 6 (supporting information (SI) Fig. S1 *A* and *B*).

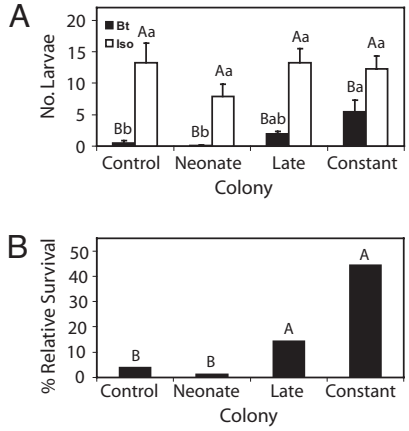

Fig. 2. (*A*) Mean (± SEM) number of larvae from different laboratory colonies recovered during trials on Bt (black bars) and nontransgenic isoline (white bars) corn in the field after six generations of selection. (*B*) Percentage of survivors on Bt corn relative to survivors on isoline corn from each laboratory colony in the field. Although untransformed data are shown, analyses were performed using square root ($x + 0.5$) transformed data (*A*) and rank transformed data (*B*). Bars with the same letters are not significantly different ($P \geq 0.05$). For (*A*) capital letters indicate comparisons between isoline and Bt within colonies and lowercase letters indicate comparisons between colonies within isoline or Bt corn.

Diet Bioassays. After three generations of selection, the resistance ratio of the Constant-exposure colony was 22.22 (Table S1). After six generations of selection, the resistance ratio of the Constant-exposure colony was still greater than that of the Control and Neonate-exposure colonies, but was only 4.14. The LC_{50} of the Constant-exposure colony did not decrease, but the ratio was lower because of the relatively high Control colony LC_{50} (5.75) (Table S1). Following removal from selection, the resistance ratio of the removal from selection colony (5.48) was higher than that of the Constant-exposure colony (2.85). Variation in response to Cry3Bb1 was reported among geographically distinct WCR populations tested before widespread commercial use of Bt corn (5), providing a basis for comparison. The LC_{50} values for the Control, Neonate-exposure, and Late-exposure colonies were in the range of those populations, but the LC_{50} value of the Constant-exposure colony was more than fivefold the average LC_{50} value previously reported (5).

Field Experiment. Because selection for resistance to Bt proteins under laboratory conditions has rarely resulted in strains capable of surviving on Bt crops in the field (11), we evaluated all colonies under field conditions. Significantly more WCR larvae from the Constant-exposure colony were recovered from Bt corn than from the Control or Neonate-exposure colonies (Fig. 2*A*). Larval survival on Bt corn relative to isoline corn in the field was significantly greater for the Constant-exposure colony (44.4%) than for the Control colony (3.79%) and the Neonate-exposure colony (1.15%), but not for the Late-exposure colony (14.4%) (Fig. 2*B*). The magnitude of the difference in survival on Bt relative to isoline between the Constant-exposure colony and the Control colony was 11.7-fold. There were no significant differences in the average dry weight of larvae recovered for any treatment, implying again (12) that once WCR establish on Cry3Bb1 Bt corn, growth is relatively normal, although adult emergence is generally delayed (6).

Although larval survival on Bt corn and isoline corn in the greenhouse was equivalent after three and six generations of

selection (Fig. 1 *A* and *B*), significantly more WCR larvae were recovered from isoline corn than from Bt corn in the field, even for the Constant-exposure colony (Fig. 2*A*). In attempting to compare greenhouse and field assays of WCR on Bt corn, it is important to note that survival of natural field populations of WCR on Bt corn relative to survival on isoline corn is in the range of 1–5%, depending on which of three registered products are evaluated. For plants expressing Cry3Bb1, survival relative to isoline was 1.34% when averaged across nine different fields in Missouri, Iowa, South Dakota, and Nebraska while actual survival on Bt plants was only 0.042% (B.E.H., T. Clark, L. Meinke, D. Moellenbeck, W. French, and T. Vaughn, unpublished data). Actual survival to adulthood on nontransgenic plants in the field will usually vary from 1 to 10% (13) and was 3.1% when averaged across the same nine fields. In greenhouse trials reported here and additional trials we have conducted with Cry3Bb1 plants, survival of unselected WCR larvae on Bt corn relative to survival on isoline was approximately 25% while actual survival was usually about 20% on isoline and 5% on Bt when infested with WCR eggs (actual survival on isoline corn may approach 50% when infested with neonate larvae). The current field experiment was terminated before adult emergence to prevent escape of resistant insects. Because larvae were recovered rather than adults, any mortality that might have occurred late in larval development or during pupation was not measured, although most WCR late instar larvae survive to the adult stage (14, 15).

Reciprocal Crosses. Performance of progeny from reciprocal crosses between the Constant-exposure colony and the Control colony on Bt corn in the greenhouse did not differ significantly from the Constant-exposure colony in terms of number of larvae recovered (Fig. 1*D*), average weight, and number of adults emerged (Fig. S1*D*). The percentage of larvae recovered from Bt relative to isoline corn was 44.3% for the Control colony, 58.8% for the cross of Constant-exposure males by Control females, 73.0% for the opposite reciprocal cross, and 120.2% for the Constant-exposure colony. These same percentages for adult emergence were 45.9%, 62.0%, 48.4%, and 77.3%, respectively.

In contrasts of the ratio of Bt survivors:isoline survivors (combining larval and adult data), there was no significant difference between the reciprocal crosses ($F = 0.24$; df = 1, 289; $P = 0.624$), indicating that no significant maternal effects were evident within the crosses. In the same analysis, nonrecessive effects were highly significant ($F = 23.7$; df = 1, 289; $P = 0.0001$), but no dominance effect was found ($F = 0.23$; df = 1, 289; $P = 0.634$). The dominance value (h) was 0.285 for larvae and 0.296 for adults. Both the linear contrasts and the calculations of h point to nonrecessive inheritance of resistance.

Regression of LC_{50} on relative survivorship (Bt :isoline) of larvae was significant ($F = 6.08$; df = 1, 9; $P = 0.0390$), yielding a regression equation of relative survival = 44.8226 + 1.199106*LC_{50} ($r^2 = 0.43$). However, when examining only the reciprocal crosses, the LC_{50} data from diet bioassays (Table S1) were characteristic of susceptible insects whereas the greenhouse results were characteristic of resistant insects (Fig. 1*D*, Fig. S1*D*). The cause for this difference remains unknown. One possible contributing reason for differences between on-plant and diet bioassays with reciprocal crosses could be the role of feeding behavior. Dramatic differences between the feeding behavior of WCR larvae on Bt corn and isoline corn suggest neonate larvae alter feeding behavior to reduce exposure to Bt proteins (16). For low-to-moderate dose toxins, any allele that confers even slight resistance is expected to be favored by natural selection (17). Genes with small effects are often common in populations and response to selection can be very rapid (18, 19). Root growing points are higher in total soluble protein compared to older root tissue; Cry34Ab1+Cry35Ab1 (20) and Cry3Bb1 proteins also follow this profile (T. Vaughn, personal communication). Interestingly, behavioral responses to toxins can prevent and even decrease the levels of physiological resistance in insect populations (21), an important result suggested by previous modeling work (22).

Removal from Selection. After six generations of greenhouse selection on Bt corn for the Constant-exposure colony and an additional six generations of removal from selection (i.e., rearing on nontransgenic plants), the number of larvae recovered from Bt corn (Fig. 1*C*) and the number of adults recovered (Fig. S1*C*) did not differ significantly from the numbers recovered from isoline corn. Finally, the LC_{50} of the Removal from selection colony remained higher than that of the Control colony (Table S1). It should be noted that the LC_{50} of the Constant-exposure colony and the Removal from selection colony were lower than the LC_{50} of the Constant-exposure colony from previous generations.

Parimi, *et al.* (10) evaluated laboratory and field strains of WCR for resistance to aldrin and methyl-parathion. As observed in greenhouse results with the Removal from selection colony on Bt corn, resistance to both aldrin and methyl-parathion was relatively stable in the absence of selection pressure. The chemical class to which aldrin belongs was banned from use in 1972. Since resistance had evolved before the 1972 ban (7), exposure of WCR to aldrin has been declining or absent for more than thirty years, yet resistance has remained high (10). Resistance to methyl-parathion was not documented until the mid-1990s (8), but resistance to this chemical also has persisted without additional selection pressure.

Genetic Evaluation. Neutral genetic diversity in each colony was tracked using 11 microsatellite markers. The estimated effective population sizes of the four colonies were comparable, with overlapping confidence intervals and on the order of 100 individuals. These population sizes were sufficiently small that significant changes in microsatellite allele frequencies were observed between generations within each colony with the exception of the Control colony between generations 13 and 14 and the Constant-exposure colony between generations 8 and 9 (after six generations of exposure to Bt). Although the changes in allele frequencies were significant, their magnitude was small; the largest value of F_{ST}, the proportion of genetic variation due to differences between samples, was 3.18% between the Control colony at generation 9 and the initial F1 population used to found the four colonies. However, the population sizes were not so small that they caused genetic diversity to be lost from the colonies during the course of the experiment. The mean expected heterozygosity (H_E) of the initial F1 between the wild type insects and the nondiapausing strain was 0.478. This measure of genetic diversity did not change significantly over time in any colony, nor did it differ between the parents and F1 of the reciprocal crosses between the Control and the Constant-exposure colonies. These results indicate that the biological differences observed between the colonies were due to the selection regime imposed and not stochastic genetic processes such as genetic drift or founder effects.

Research Implications. Results with the Neonate-exposure and Late-exposure colonies may simulate grassy weeds serving as alternate hosts near Bt corn (23) or a mixture of Bt and isoline corn as has been proposed as a refuge strategy by Pioneer®. Although we only have one colony per treatment, our data suggest that selection for resistance may be minimal when neonate larvae are exposed to Bt corn but development is completed on isoline corn (the Neonate-exposure colony was not significantly different from the Control colony for any parameter of resistance). However, in a scenario where initial development occurs on grassy weeds and the weeds are then sprayed with

herbicide, or in a seed-mix scenario where isoline food resources are significantly depleted forcing larvae to move (24), resistance might be expected to evolve, given that survival of the Late-exposure colony on Bt corn relative to isoline survival was significantly (3.8-fold) greater than the Control colony in the field.

Recently, Lefko, *et al.* (20) documented an increased WCR survivorship from F1 to F9 of 15.1- and 58.5-fold for populations from Rochelle, IL and York, NE, respectively, selected to survive on event DAS-59122–7 containing the Cry34Ab1+Cry35Ab1 proteins. Despite up to a 5,850% increase in survivorship, damage to DAS-59122–7 per 100 eggs from the York selected population in the greenhouse only increased 350% from F1 to F11 and Bt corn was still significantly less damaged than isoline roots. We did not collect damage ratings, but given similar root protection in the field to DAS-59122–7, we would expect damage increases to Bt corn to be similar to Lefko, *et al.* (20). Performance of each product in the field might be better, given differing selection intensities between the greenhouse and field, but this was not tested in either study. Lefko, *et al.* (20) did not evaluate their selected populations in the field, and we did not evaluate plant damage in the field or greenhouse, so direct comparison of field survivorship is not possible.

Although we have not included summaries of colony performance from generation to generation as in Lefko, *et al.*, we do have similar data. For the Constant-exposure colony, 1.5% of eggs survived Bt corn the first generation (F1) of rearing in the greenhouse. After six generations of selection on Bt corn, ≈4.1% of the eggs survived Bt corn to produce adults in greenhouse rearing. In a controlled greenhouse experiment, an average of 2.73 adults were produced per plant on Bt corn from 50 Constant-exposure eggs from generation 6 (Fig. S1*B*). Thus, adult production on Bt corn increased from 1.5% for F1 to 5.5% for F6, an increase of about 3.7-fold in six generations of selection. One reason that the increase in survival to the adult stage may not have increased as rapidly in the current study as the 58.5-fold increase found in Lefko, *et al.* (20) is that relative to isoline survival, we already had a high rate of survival on Bt corn under our greenhouse rearing conditions (approximately 25% relative to isoline compared to 1–4% relative to isoline survival in the field).

Taken together, our results suggest that rapid response to selection is possible in the absence of mating with unexposed beetles. These data emphasize the importance of effective refuges for resistance management, especially for low-to-moderate dose toxins.

Materials and Methods

Colony Development. Eggs from a feral WCR population collected near Dodge City, KS, in July 2002 by French Agricultural Research were purchased and used for an unrelated field experiment in Missouri in 2003. Beetles from the 2003 experiment were collected from susceptible corn, kept alive, and brought to the laboratory where they were mated with each other and resulting eggs overwintered at 8 °C. In April 2004, eggs were removed from cold storage, reared on isoline corn, and resulting adults were crossed reciprocally with a nondiapausing WCR strain (25) so that generation time could be reduced from 9 months (1 year in the field) to 2 months. The wild-type genes were introgressed because the nondiapausing colony has been maintained in the laboratory for more than 200 generations and has lost genetic variability (26). After combining eggs from the two reciprocal crosses, a total of 4,242 adults emerged that laid a total of 241,000 eggs. From these eggs, four separate colonies were established, each fed optimally as adults but differing in larval diet. Adults were held in the laboratory under 14:10 [L:D] photoperiod and 25 °C. Adults from all colonies were maintained in 30 × 30 × 30 cm cages (MegaView) and provided with artificial diet (27), fresh non-Bt corn leaves, and water. Oviposition substrate consisted of 1 cm moist 70 mesh (212 μm) sieved soil in Petri dishes with the surface scarified to promote oviposition and dishes were replaced weekly (twice weekly for the first year). Eggs were recovered by rinsing the soil through a 60 mesh sieve (250 μm) with water. The Control colony was reared on isoline corn (DKC 60–15), the Neonate-exposure colony was exposed to Bt corn (MON863, Monsanto Company, variety DKC 60–12) as neonate larvae but subsequently reared on isoline, the Late-exposure colony was reared on isoline corn for 1 week and then Bt corn from second instar to pupation, and the Constant-exposure colony was reared solely on Bt corn as larvae (except as described below).

For the Control colony, cohorts of 125 neonate larvae of hatching eggs covered by 1 cm of soil were transferred via a fine nylon artist's brush to seedling corn (approximately 45 seeds, 4 d after germination) in 15 cm × 10 cm oval containers (708 ml, The Glad Products Company) filled approximately 4 cm deep with a growth medium of 2:1 autoclaved soil and ProMix™ (Premier Horticulture Inc.). After 7 d, the living corn was cut at the soil surface, and the remaining contents transferred upside down to a 33 cm × 19 cm container (5.7 liters, Sterilite Corporation) with new growth medium (approximately 115 seeds, 4 d after germination) to allow larvae to complete development and pupate. The Neonate-exposure colony was reared identically to the Control colony, but the neonate larvae were first placed on a germinated Bt corn seedling without soil and then the seedling plus larvae were transferred to isoline corn. The Late-exposure colony was reared the same as the Control colony for the first week, but second instar larvae were removed from their first container (isoline corn) using modified Tullgren funnels and then transferred to a 15.5 liter pot with 2 Bt corn plants at approximately V6-V7 (28). Late-exposure colony larvae finished their development on Bt corn plants and pupated in the pots. Just before predicted adult emergence, one plant was cut at the base and the other corn plant was passed through a hole in insect netting, which was secured around the corn plant stalk with a cable tie and to the pot with a rubber band. Finally, the Constant-exposure colony was reared exclusively on Bt corn plants (except as described below). This involved large beds (1.2 m wide × 7.5 m long × 25 cm deep) of the same growth medium used above in which 294 kernels of Bt corn were planted. Each plant was infested with 200 eggs at approximately V3 stage during the first few generations with egg hatch at approximately V5–6. The number of eggs per plant was reduced in later generations to 100 with infestation at V1–2 and egg hatch approximately V4. Beds were covered with fine mesh screen to prevent adult escape 5–6 weeks following infestation, depending on temperature. Adults from all colonies were collected daily.

To ensure enough individuals to maintain the colony as well as conduct controlled greenhouse and field experiments in which eggs were removed from the colony, it was sometimes necessary to rear one generation on isoline corn before initiating another generation of selection on Bt. Thus, ''generation 6'' of the Constant-exposure colony refers to six generations of selection on Bt, but these generations were interspersed with three additional generations of increase on isoline corn. When comparing colonies, actual generation numbers were not the same for all colonies. The Figs., Table, and text refer to generations of selection, not total generations in culture. For the Constant-exposure colony, after generation 2, rather than putting eggs back onto Bt, all eggs were put onto isoline to increase the population size of the colony. Generations 4 and 5 were also increased on isoline, primarily in preparation for the field trial, which needed a large number of eggs that would not go back into the colony. The Neonate-exposure colony was not increased on isoline except in advance of the field experiment. The Late-exposure colony was increased after generation 3 and generation 5 on isoline corn.

Greenhouse Experiments. ***Standard procedures.*** WCR survival was evaluated on Bt (DKC 60–12) and isoline (DKC 60–15) corn in greenhouse trials. For each replication of each treatment, three pots were planted with two corn seeds each; two pots (3.8 liters) for larval recovery and one pot (19 liters) for adult recovery. Following germination, seedlings were thinned to one plant per pot. The same growth medium was used as for rearing. To prevent larval escape (23), drainage holes on all pots were fitted with 114-μm stainless steel mesh (TWP Inc.). Plants were watered as needed and fertilized approximately 6 wk after planting with 1.25 ml of Peters Professional® Multi Purpose 20–20-20 (The Scotts Company LLC).

Three weeks after planting, pots were infested with 50 WCR eggs suspended in 0.15% agar solution pipetted into a 2.5-cm hole in the soil. Holes were covered and the plants lightly watered. At infestation, a subsample of eggs was placed on moist filter paper in a Petri dish. The dish was placed near the pots and monitored for percent hatch and time to hatch.

Larvae were recovered from two sets of pots 1 and 2 wk following peak egg hatch. Recovery was accomplished by cutting plants near the soil surface, then emptying the pots into modified Tullgren funnels equipped with a 60 W light bulb. The root ball was carefully broken to encourage drying. Larvae were collected in attached pint jars filled with 2.5 cm water, and were subsequently transferred after 2 and 4 d to 95% ethanol. Larval dry weight was obtained after desiccation in an oven (Thelco model 16, GCA/Precision Scientific Co.).

The corn plant in adult emergence pots was passed through a hole in insect

netting, which was secured around the stalk with a cable tie and to the pot with a rubber band. Pots were checked for adults three times weekly until no adults were collected for two consecutive weeks. Recovered adults were stored in 95% ethanol until they could be sexed, counted, and dry weight taken as described for larvae. Greenhouse air temperature was recorded on an hourly basis (HOBO, model H08–001-02).

Generations 3 and 6. All four colonies were evaluated after generations 3 (August 2005) and 6 (June 2006) of selection of the Constant-exposure colony using the standard procedures described above. During the two experiments, hourly air temperatures in the greenhouse averaged 23.5 ± 0.09 °C SE (range 12.6–33.6 °C) and 26.4 ± 0.08 °C SE (range 18.3–42 °C), respectively. Soil temperatures likely did not vary as extensively. The larval recovery experiment was designed as a randomized complete block split-plot with the main plot being treatment and the subplot being recovery date. The adult recovery experiment was designed as a randomized complete block. There were at least 15 replications for each larval recovery time and adult emergence (25 replications for adult emergence at generation 3).

Reciprocal Crosses. Newly emerged adults from the Control (generation 13) and Constant-exposure colonies (generations 6 and 7 of Bt exposure) were placed in separate rearing cages (30 × 30 × 30 cm) (MegaView). Males were segregated for 10 days to reach sexual maturity before introduction to females. At least 100 virgin females from the Control colony were allowed to mate with males from the Constant-exposure colony and vice versa. Adults were maintained as described above.

Offspring of the reciprocal crosses, along with the Control and Constant-exposure colonies, were evaluated for growth and survival under standard greenhouse procedures (December 2006). Hourly air temperature averaged 22.9 ± 0.07 °C SE (range 9–38.3 °C). The larval recovery experiment was designed as a randomized complete block split-plot with the main plot being treatment and the subplot being larval recovery date with 20 replications. The adult recovery experiment was designed as a randomized complete block with 20 replications.

Removal from Selection. A subset of the Constant-exposure colony was removed from selection after six generations on Bt corn and reared on isoline for six generations. Larvae were evaluated for growth and survival using the standard greenhouse procedures described above along with larvae from the Control colony and the Constant-exposure colony (July 2007). Greenhouse air temperatures during this experiment averaged 27.7 ± 0.31 °C SE (range 17.1–39.7 °C). The larval recovery experiment was designed as a randomized complete block split-plot, with the main plot being treatment and the subplot being larval recovery date with 15 replications. The adult recovery experiment was designed as a randomized complete block with 15 replications.

Field Experiment. All colonies were evaluated on both Bt (variety DKC 60–12) and isoline (variety DKC 60–15) corn in field experiments at the Bradford Research and Extension Center of the University of Missouri near Columbia, MO in 2006. The experiment was designed as a randomized complete block with ten replications. Each replicate of each treatment consisted of a single plant infested with 500 viable eggs from one of the above colonies. To ensure adequate numbers of eggs, each colony was increased on isoline in time to lay eggs for the field experiment. Each infested plant was destructively sampled by putting the whole root ball with soil in an onion bag, which was then hung in a greenhouse with the cooling system turned off. Temperatures in such a greenhouse in late June in Missouri are often 50–65 °C. Under these conditions, larvae leave the hot and drying soil in search of a more suitable environment (12, 24). Larvae were captured in water pans below each root ball, and were transferred to 95% ethanol at least twice daily. Natural infestation by the southern corn rootworm, *D. undecimpunctata howardi* Barber, is possible in central Missouri, so the species of each rootworm larva was determined based on the presence or absence of urogomphi on the posterior margin of the anal plate (29). Most, but not all, southern corn rootworm larvae can be detected by this technique (12). The number of WCR larvae recovered and larval dry weight were recorded.

Diet Bioassays. Bioassays were conducted by exposing neonate larvae to increasing concentrations of Cry3Bb1 applied to artificial diet. The colonies were tested at generations 3 (July 2005) and 6 (June 2006). Offspring of reciprocal crosses (June and November 2006) and the colony removed from selection (August 2007) were also evaluated, along with the Control and Constant-exposure colonies. Each generation was increased on isoline corn before diet bioassay evaluations to separate genetic effects from other Cry3Bb1 effects. All bioassays were conducted as described by Siegfried, *et al.* (5).

Genetic Evaluation. Changes in genetic diversity of the colonies over time were tracked using 11 microsatellite loci (30). Samples were examined from the F1 generation used to initiate the four colonies and at various generations thereafter, as well as the parents and F1 generations of the reciprocal crosses between the Control and Constant-exposure colonies. Samples of the colonies were examined at the following generations: Control at generations 4, 10, 13, and 14; Neonate-exposure at generations 4 and 9; Late-exposure at generations 3 and 6; and Constant-exposure at approximately generations 8 and 9 (six generations of selection).

DNA was extracted from adult beetles using AquaPure Genomic DNA kits (Bio-Rad). The microsatellite loci were amplified by PCR in three multiplex reactions using multiplex PCR kits (Qiagen) according to the manufacturer's instructions in a 10 μl volume with 20 ng genomic DNA. One of the PCR primers for each microsatellite was labeled with a fluorescent dye that allowed the amplicons to be detected and sized using a Beckman-Coulter CEQ 8000 genetic analysis system (Beckman-Coulter). The number of individuals successfully analyzed from each sample ranged from 26 to 60.

Statistical Analysis. ***Greenhouse experiments.*** Although nontransformed data are shown in the figures, data from all experiments were square root ($x + 0.5$) transformed before analysis to meet the assumptions of the analysis (31). Larval recovery data were analyzed as a randomized complete block three way factorial design (four colonies, two corn types, and two larval recovery times) using PROC MIXED of the SAS statistical package (32). The model contained the main effect of colony, corn type, larval recovery date, and all possible interactions. Replications were included as the random variable. A separate analysis was done for number of larvae recovered and average larval weight. Adult emergence data were analyzed separately as a randomized complete block design using PROC MIXED. Since there was no interaction of colony × collection period (larval sample 1 and 2), the main effect of colony is presented in Figs. 1–2.

Reciprocal crosses were further analyzed to specifically test for maternal, nonrecessive, and dominance effects. For each replication of each collection period (1st larval, 2nd larval, and adult), the number of individuals recovered from Bt corn was divided by the number of individuals recovered from isoline corn to provide the colony's relative survival on Bt corn, adding 1 to the numerator and denominator to avoid division by zero. Because model assumptions were not initially met, ratios were log transformed (31). Data were analyzed as a randomized complete block design using PROC MIXED. Since there was no interaction of colony × collection period (larval sample 1, 2, and adult emergence), the main effect of colony is presented. Specific contrasts were made between the two reciprocal crosses to test for maternal effects. Since they were not significant, these were pooled in the contrasts that follow. Contrasts between the Control colony and the Constant-exposure colony were used to test for nonrecessive effects. Dominance effects were evaluated by contrasting the parental colonies and their reciprocal crosses. In addition, the dominance value (h) was calculated from the reciprocal cross larval recovery and adult recovery data, as suggested by Tabashnik, *et al.* (3). Dominance values of 0 indicate completely recessive resistance, while dominance values of 1 indicate completely dominant resistance.

Field experiment. The number of larvae recovered in the field (Fig. 2*A*) were analyzed as a randomized complete block design using PROC MIXED. The model contained the main effect of colony, corn type, and all possible interactions. In addition, within each replication, the ratio of larvae recovered from Bt corn:isoline corn was analyzed because the ratio represents relative survival on Bt corn and it controls for differences in egg hatch between strains (Fig. 2*B*). Because model assumptions were not met initially, the ratios were rank transformed (33) and analyzed as a randomized complete block design using PROC MIXED; however, untransformed data averaged across replications are presented. Only bioassays in which control mortality was <20% and which had at least three concentrations producing mortality >0 and <100% were subjected to further statistical analysis. Bioassays were conducted in duplicate on three different dates, depending on availability of eggs. Mortality data were analyzed by probit analysis using POLO-PC (34). Resistance ratios were calculated by dividing the LC_{50} of the selected colony by the LC_{50} of the accompanying Control colony.

A regression analysis was performed to determine the ability of LC_{50} data from diet bioassays to predict relative survival of insects on plants. Larval LC_{50} data were collected from each colony at generations 3 and 6 and from larvae of the reciprocal crosses. These data were paired with their respective relative survivals (number of individuals recovered from Bt corn/number of individuals recovered from isoline corn) for each colony at each generation. The regression was performed using PROC REG of the SAS statistical package.

Genetic evaluation. Genepop 4.0 (35) was used to calculate expected heterozygosities (H_E) as a measure of overall genetic diversity for each sample, F_{ST} which is the proportion of genetic variation due to differences between samples and a measure of allele frequency differences, and to perform exact tests of allele

frequency difference between samples. Differences in H_E between samples were tested using a Wilcoxon test for matched pairs. The effective population size of each colony was estimated with the pseudolikelihood method (36).

ACKNOWLEDGMENTS. We thank R. Bukowsky and J. Barry for technical assistance. We thank Fred Gould, Aaron Gassmann, Tom Coudron, and two anonymous reviewers for valuable suggestions on earlier drafts. Aaron Gassmann also assisted in calculations of maternal, nonrecessive, and dominance effects as well as the dominance value, *h*. This research was supported, in part, by USDA-ARS, Biotechnology Risk Assessment Award No. 2006–33522-17716, the University of Missouri Division of Plant Sciences, and by Monsanto Corporation.

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Supporting Information

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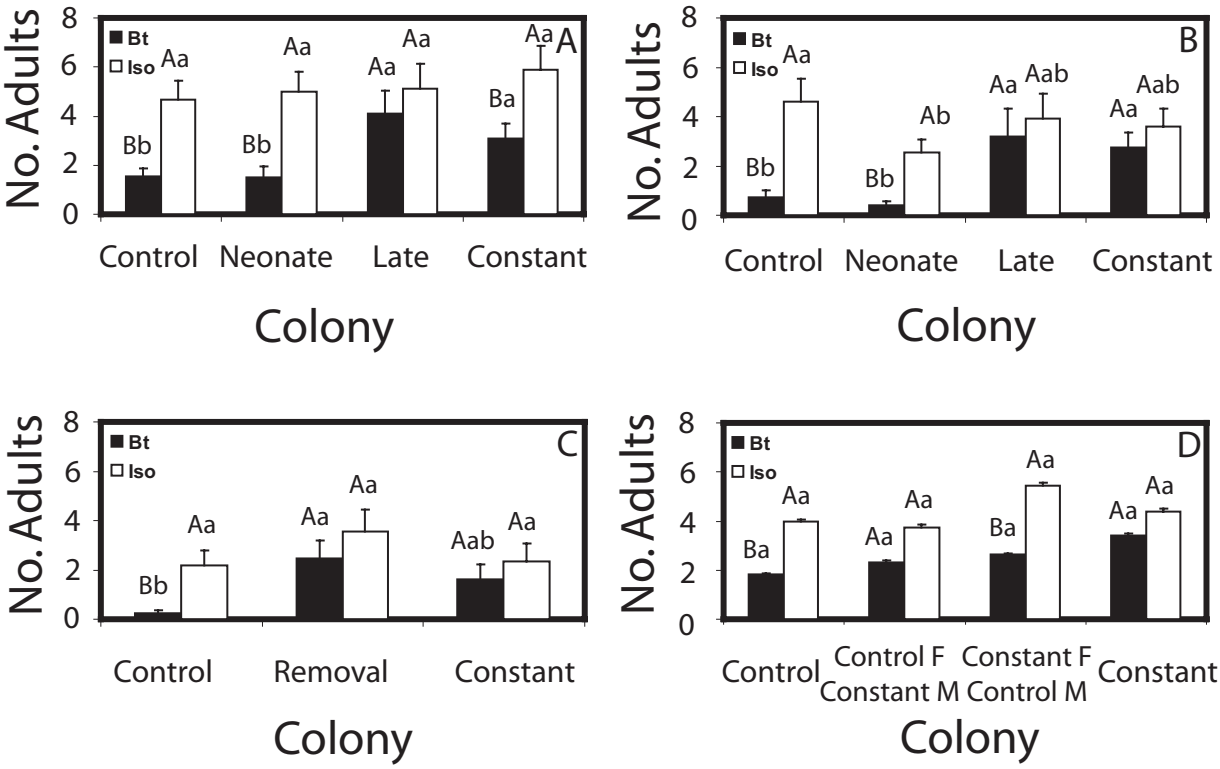

Fig. S1. Mean (± SEM) number of beetles from different laboratory colonies recovered during trials on Bt and nontransgenic isoline corn (*A*) in the greenhouse after three generations of selection and (*B*) in the greenhouse after six generations of selection. The Constant-exposure colony after six generations of selection was further tested after (*C*) six generations of removal from selection, and (*D*) reciprocal crosses with the unselected Control colony (F = female, M = male). Although untransformed data are shown, analyses were performed using square root (x + 0.5) transformed data. Bars with the same letters are not significantly different ($P \geq 0.05$). Capital letters indicate comparisons between isoline and Bt within colonies. Lowercase letters indicate comparisons between colonies within isoline or Bt corn.

Table S1. LC_{50} (95% confidence interval) of Cry3Bb1 toxin to neonate western corn rootworm larvae

Sample	Colony	*n*	LC_{50}	Slope	SE	Resistance Ratio*
Generation 3	Control	174	0.94 (0.45–1.46)	1.95	0.42	1
	Neonate	153	1.64 (0.25–4.10)	1.34	0.23	1.74
	Late	651	2.31 (0.75–4.68)	1.20	0.11	2.46
	Constant	80	20.89 (8.59–151.11)	1.00	0.32	22.22
Generation 6	Control	274	5.75 (2.45–12.19)	0.71	0.15	1
	Neonate	168	2.57 (0.40–7.38)	1.28	0.21	2.24
	Late	140	29.74†	—	—	5.17
	Constant	272	23.83 (13.08–49.60)	0.89	0.17	4.14
Reciprocal Crosses	Control♀×Constant♂	285	1.89 (0.15–4.88)	1.20	0.17	—
	Constant♀×Control♂	276	2.12 (0.43–4.67)	1.14	0.16	—
Removal from Selection	Control	212	1.51 (1.07–1.98)	2.89	0.52	1
	Removal	324	8.28 (5.17–12.52)	1.17	0.16	5.48
	Constant	319	4.30 (1.49–8.53)	0.75	0.15	2.85

n=number of individuals.

*Resistance ratios were calculated for each experiment by dividing the selected colony's LC_{50} by the control colony's LC_{50}.

†No confidence interval generated due to poor fit of data. Significant deviations from the probit regression model occurred only for Late in Generation 6; df = 3 in all cases except Constant in Generation 3 (df = 2).