Selection for Resistance to mCry3A-Expressing Transgenic Corn in Western Corn Rootworm

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ABSTRACT To investigate the development of resistance to mCry3A, a laboratory colony of the western corn rootworm, *Diabrotica virgifera virgifera* LeConte, was established from field survivors of mCry3A-expressing (MIR604) corn, *Zea mays* L. Feral adults emerging from MIR604 (selected) and isoline (control) field plots were collected and returned to the laboratory. Progeny of each colony was reared one generation on isoline corn and then crossed reciprocally with a nondiapausing colony. The resulting nondiapausing progeny were then reared on greenhouse corn in accordance with the wild type parent's origin (on MIR604 or isoline corn). After four, seven, and 10 total generations of selection, the resistance ratio of the selected colony was 0.5, 4.3, and 15.4 in terms of lethal concentration $(LC)_{50}$ values in toxicity assays, with the latter two LC_{50} values being significant. After seven generations of selection in total, selected and control colonies were screened on MIR604 and isoline corn under field conditions. There was a significant colony × corn pedigree interaction in terms of plant damage. There was no significant difference in damage between MIR604 and isoline corn, whereas this difference was significant for the control colony. After 14 generations of selection, a seedling bioassay was performed. Again, there was a significant colony × corn pedigree interaction, this time in terms of the number of larvae recovered. There was no significant difference in the number of larvae recovered from MIR604 and isoline corn for the selected colony, whereas this difference was significant for the control colony, although larval size was greater on isoline corn for both colonies. Resistance has developed in western corn rootworm laboratory colonies to all Bt proteins currently registered for corn rootworm management, which emphasizes the importance of adhering to resistance management plans for maintaining product efficacy.

KEY WORDS *Diabrotica virgifera virgifera*, *Bacillus thuringiensis*, MIR604, toxicity assay

The United States is the largest producer of corn, *Zea mays* L., in the world, producing 307 million tons of corn or 37% of the world's total corn production in 2008 (FAOSTAT 2010). The primary pest of corn in the United States and parts of Europe is the western corn rootworm, *Diabrotica virgifera virgifera* LeConte. Although western corn rootworm adults at high densities can cause reduced grain set by feeding on corn silks before pollination (Culy et al. 1992), the root-feeding larvae are the most damaging life stage. Larval western corn rootworm feeding can negatively affect corn plants by decreasing shoot biomass accumulation (Spike and Tollefson 1991), decreasing yield (Kahler et al. 1985, Schaafsma et al. 1993), changing the element composition of plants and grain (Kahler et al. 1985), reducing leaf CO_2 assimilation (Hou et al. 1997, Riedell and Reese 1999), and reducing the plant's photosynthetic rate (Godfrey et al. 1993, Urias-Lopez et al. 2000). The removal of root tissue can also leave plants susceptible to lodging, which further reduces yield, in part due to the difficulty of harvesting lodged plants (Levine and Oloumi-Sadeghi 1991).

Management options for western corn rootworm larvae include insecticidal seed treatments, granular and liquid soil insecticides, usually applied at planting time, crop rotation, and transgenic Bt hybrids. Western corn rootworm has a history of overcoming control measures. Resistance to the organochlorine insecticide aldrin was documented nearly 50 yr ago (Ball and Weekman 1963). Resistance to adult control measures such as methyl-parathion (organophosphate) and carbaryl (carbamate) insecticides also has been documented (Meinke et al. 1998). Crop rotation has become ineffective in an ever increasing region of the Corn Belt centered in north central Illinois and Indiana; resistance to crop rotation in the western corn rootworm is attributed to a loss of ovipositional fidelity to corn (Levine et al. 2002, Gray et al. 2009).

Corn plants expressing *Bacillus thuringiensis* Berliner (Bt) insecticidal proteins toxic to western corn

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rootworm were first registered for commercial sale in 2003. By 2009, 85% of corn planted in the United States was a biotech variety (transformed for herbicide tolerance, insect tolerance, or both). Of the 85%, nearly half (46%) were stacked varieties containing both herbicide tolerance and insect tolerance traits (NASS 2010). Although several Bt proteins are effective against rootworm larvae, only three—Cry3Bb1, Cry34/35Ab1, and mCry3A—are approved for use in transgenic corn plants in the United States (http://www.epa.gov/oppbppd1/biopesticides/pips/pip_list.htm). Although Bt proteins targeted toward rootworms have been primarily deployed as a single protein per corn variety, recently, a pyramid including Cry3Bb1 and Cry34/Cry35Ab1 was registered for commercial sale.

Insecticidal proteins for control of several lepidopteran larvae are expressed in plant tissues at a high dose (25× the lethal concentration $[LC]_{99}$). Such a high dose of protein is thought to make resistance functionally recessive and is used in conjunction with a refuge (corn plants not expressing protein) to supply susceptible insects; this forms the high dose/refuge strategy of insect resistance management (IRM) used for many pests (EPA 1998). Success of the high-dose/refuge strategy is aided if several factors are present. These include 1) the protein is expressed in the plant tissues at a high dose, 2) resistance is inherited recessively, 3) random mating occurs, 4) initial resistance alleles are rare, and 5) fitness costs are associated with the resistance. For western corn rootworm, none of the individual proteins nor the pyramid of Cry3Bb1 and Cry34/35Ab1 are expressed at high dose (EPA 2002; Storer et al. 2006; Hibbard et al. 2010a,b), there is evidence of nonrandom mating (Kang and Krupke 2009), and initial resistance allele frequencies may be much higher than initially assumed (Onstad and Meinke 2010).

Laboratory derived resistance to transgenic corn expressing Cry34/35Ab1 (Lefko et al. 2008) and Cry3Bb1 (Meihls et al. 2008) proteins has been identified for western corn rootworm, although resistance derived from field selection has not been formally documented in the refereed literature at this point for any rootworm-active protein. Understanding of the speed and strength of resistance that can develop in the absence of mating with unselected individuals could help delay resistance development in the field, in part by reinforcing the importance of a refuge to provide susceptible individuals. The goals of this research were to determine 1) whether western corn rootworm could develop resistance to mCry3A; 2) if so, how many generations of selection would be required to observe resistance; and 3) whether western corn rootworm resistant to mCry3A in the greenhouse survive on mCry3A-expressing (event MIR604) corn plants under field conditions at a higher rate than the unselected control colony relative to their survival on isoline.

Materials and Methods

Colony Development. Initial wild-type adult collection was conducted at three central Missouri sites in summer 2006. Additional information on adult collection methods can be found in Hibbard et al. (2010a). Adults were maintained in separate cages based on the corn phenotype (mCry3A or isoline) they were recovered from. Adults of each colony were treated the same and were maintained in 30- by 30- by 30-cm cages (MegaView, Taichung, Taiwan), provided with artificial diet (Jackson 1985), fresh non-Bt corn leaves, and water. Adults were held in the laboratory under 25°C and a photoperiod of 14:10 [L:D] h. The oviposition substrate provided consisted of 1 cm of moist soil which had been sieved through a 70 mesh (212-μm) screen. Soil was contained in petri dishes with the surface scarified to promote oviposition; dishes were replaced weekly.

Eggs recovered from field-collected adults were stored at room temperature for 2 wk and then overwintered in a refrigerator at ≈7°C for ≈6 mo to synchronize hatch (Fisher 1989). Eggs were then removed from cold storage, and both egg types were reared on isoline corn as larvae for one generation. Recovered adults were crossed reciprocally with a nondiapausing western corn rootworm strain (Branson 1976) so that generation time could be reduced from ≈9 mo (1 yr in the field) to ≈2.5 mo. Wild-type genes were introgressed because the nondiapausing colony has been maintained in the laboratory for >200 generations and has lost genetic variability (Kyung et al. 2007). Reciprocal crosses resulted in 104,000 (control) and 110,000 (selected) eggs; these eggs were used to continue selection under greenhouse conditions.

Larvae of control and selected colonies were reared under similar conditions. Two rearing methods were used, depending on available space at the time of egg collection. The first method involved large beds (1.2 m in width by 7.5 m in length by 25 cm in depth) of a growth medium of 2:1 autoclaved soil and ProMix (Premier Horticulture Inc., Quakertown, PA) in which isoline or MIR604 corn was planted. Each MIR604 plant was initially infested with ≈200 eggs at ≈V2 stage (Ritchie et al. 1992) during the first few generations with egg hatch at ≈V4–5. The number of eggs per plant was reduced in later generations to 100 eggs per plant. Isoline plants were infested with 50 eggs. Beds were covered with fine mesh screen to prevent adult escape 5–6 wk after infestation, depending on temperature. In the second method, eggs were placed in 15- by 10-cm oval containers (708 ml; The Glad Products Company, Oakland, CA) along with ≈45 corn seeds (MIR604 corn for the selected colony and isoline corn for the control colony). Containers were then filled ≈4 cm deep with the same growth medium mentioned above. After 21 d, the living corn was cut at the soil surface, and the remaining contents transferred upside down to a 33- by 19-cm container (5.7 liters; Sterilite Corporation, Clinton, SC) with new

growth medium and ≈115 germinated seeds (4 d after germination) to allow larvae to complete development and pupate. For rearing and on-plant bioassays Garst Brand 8446 CB/LL was used as the isoline and Garst Brand 84D47CB/LL/RW was used for MIR604.

The selected colony was reared exclusively on MIR604 corn plants (except as described below), whereas the control colony was reared exclusively on isoline corn plants. Adults from all colonies were collected six times per week and maintained as described above. However, to ensure enough individuals were available 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 of the selected colony on isoline corn before initiating another generation of selection. Thus, "seven generations of selection" refers to seven generations of selection on MIR604, but these generations may have been interspersed with additional generations of increase on isoline corn. The selected colony was increased on isoline corn after one and six generations of selection on MIR604 corn. Before being evaluated in the field (generation 6), the selected colony was increased on isoline corn to ensure enough eggs for the experiment. This colony also was reared on isoline corn after the 12th generation of selection. The tables and figures as well as the text refer to generations of selection, not total generations in culture.

Greenhouse Experiment. Western corn rootworm survival was evaluated on MIR604 and isoline corn in a greenhouse trial. For each replication of each treatment, three pots were planted with two corn seeds each. Pots for larval recovery were smaller, 3.3 liters, whereas pots for adult emergence were larger, 9.9 liters. After germination, seedlings were thinned to one plant per pot. The same growth medium was used as for rearing. To prevent larval escape, drainage holes on all pots were fitted with 114-μm stainless steel mesh (TWP Inc., Berkley, CA) as we have done in other greenhouse studies (Clark and Hibbard 2004).

Three weeks after planting, pots were infested with 50 western corn rootworm eggs suspended in 0.15% agar (CAS 9002-18-0, USB Corporation, Cleveland, OH) solution and applied via a pipetter into a 2.5-cm hole in the soil. After infestation, holes were covered and the plants lightly watered. A subsample of eggs was placed on moistened filter paper in a petri dish; the dish was placed near the pots and monitored for percentage of hatch and time to hatch. Larvae were recovered from two sets of pots one and 2 wk after peak egg hatch. Larval recovery was accomplished using modified Tullgren funnels equipped with a 60-W incandescent light bulb. Plants were cut near their base and after transfer to the funnel, the root ball was gently broken up to encourage drying. Larvae were collected in attached 473-ml jars filled with 2.5 cm of water and subsequently transferred after 2 and 4 d to 95% ethanol. Larvae were counted, head capsule width measured, and larval dry weight (scale model AB135-S FACT, Mettler Toledo Inc., Columbus, OH) was obtained after desiccation in an oven (Thelco model 16, GCA/Precision Scientific Co., Chicago, IL).

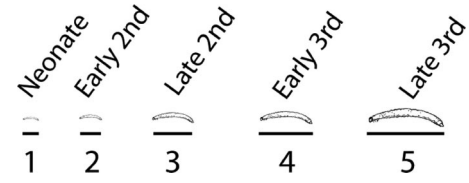

Fig. 1. Larval size scale used during the 2009 field experiment. Length was 2, 3, 5, 7, and 10 mm for 1–5, respectively.

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. Plants were watered as needed and fertilized ≈6 wk after planting with 1.25 ml of Peters Professional Multi Purpose 20–20-20 (The Scotts Company LLC, Marysville, OH). 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 and head capsule width recorded as described for larvae. Greenhouse air temperature was recorded on an hourly basis from each experiment (model H08–001-02, HOBO, Bourne, MA). Hourly air temperatures in the greenhouse averaged 27.9 ± 0.10°C (SEM; range, 19.8–36.6°C). Soil temperatures probably did not vary as extensively as the air temperature that was recorded. There were 15 replications for each larval recovery time and adult emergence.

Field Experiment. Both control and selected colonies were evaluated on both MIR604 and isoline corn in field experiments at the University of Missouri Bradford Research and Extension Center (Mexico silt loam comprised of 15% sand, 60% silt, and 25% clay). The experiment was designed as a randomized complete block with 20 replications. Each replicate of each treatment consisted of a single plant 1.52 m from any other plant and infested with 500 viable eggs from one of the above-mentioned colonies. To ensure adequate numbers of eggs, the selected 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 (Hibbard et al. 2004, 2005). Peak daily temperatures in such a greenhouse in late June in Missouri are often 50°C or more. Under these conditions, larvae leave the hot and drying soil in search of a more suitable environment. Larvae were captured in water pans below each root ball and most treatments were transferred to 95% ethanol at least twice daily. During collection, all larvae were counted and larval size was measured on a scale of 1–5, corresponding to 2, 3, 5, 7, and 10 mm in length (half-scale units also were recorded) (Fig. 1). Insects from the selected colony on MIR604 corn were not transferred to ethanol and weighed or head capsule width taken because the larvae were kept alive and used to initiate

another colony. Larvae of all other treatment combinations were counted, weighed, and head capsule width was recorded as described previously. After larval recovery, corn roots were removed from their onion bags, soaked and washed, and rated using the 0–3 scale (Oleson et al. 2005). Due to the possibility of releasing resistant individuals, multiple factors were considered to ensure survival to the adult stage did not occur, including 1) the experiment was terminated when larvae were at the late second-instar or early third-instar stage. Because 90% of weight gain occurs during the third instar for this insect, any larvae left in the field would need a host plant to complete development, 2) all plants within 10 m of the plots were killed on the day the experiment was terminated. Senescing corn and weeds are extremely poor hosts (Olmer and Hibbard 2008) and western corn rootworm larvae can only move three or four adjacent plants down the row or across one row of corn (Hibbard et al. 2004), 3) if any larvae survived, the nondiapausing trait is dominant and eggs of the nondiapausing strain hatch within 14 d at 25°C, so eggs would hatch in August or September when corn and other grasses are nonhosts (Hibbard et al. 2008, Chege et al. 2005), and 4) we applied liquid chlorpyrifos just after sampling.

Seedling Bioassays. After 14 generations of selection, an assay modified after Nowatzki et al. (2008) was conducted. Oviposition dishes were collected weekly from adult chambers and washed through a fine mesh to recover eggs. A target infestation level of 333 eggs per container (15- by 10-cm oval containers, The Glad Products Company, Oakland, CA) were calibrated in 0.15% agar covered with 20 ml of tap water and ≈2 cm of autoclaved soil (2:1 and ProMix, Premier Horticulture Inc., Quakertown, PA) and sat at room temperature. One week after egg collection from adult chambers, ≈15 g corn of the appropriate corn type was placed on top of the original 2 cm of soil. The seed was covered with ≈4 cm autoclaved soil and 80 ml of tap water was added, and the containers were placed into growth chambers (model I36LL, Percival Scientific, Perry, IA) at 25°C and a photoperiod of 14:10 l(L:D) h. Approximately 17 d after peak egg hatch, above ground corn leaf tissue was cut from the container and they were placed in Tullgren funnels to recover the larvae and were treated as described for the greenhouse experiment.

Toxicity Bioassays. To eliminate the effects of feeding behavior potentially associated with increased survival on MIR604 corn, toxicity bioassays were conducted by exposing neonate larvae to increasing concentrations of mCry3A surface applied to artificial diet (Pleau et al. 2002). Both colonies were tested at generations four, seven, and 10 of selection by Custom BioProducts (Maxwell, IA). All bioassays were conducted similar to those described by Siegfried et al. (2005).

Statistical Analysis. *Greenhouse Experiment.* Larval data were analyzed as a randomized complete block three way factorial design (two colonies × two corn pedigrees × two larval recovery times) by using PROC MIXED of the SAS statistical package (SAS Institute 2004). The model contained the main effect of colony, corn pedigree, larval recovery time, and all possible interactions. Because the effect of colony and corn were of primary interest, not the effect of larval recovery time, larval recovery time is not presented in larval figures. Replications were included as the random variable, all other variables were fixed. A separate analysis was done for number of larvae recovered, larval head capsule width, and average larval weight. Adult emergence data were analyzed separately as a randomized complete block two-way factorial design (two colonies × two corn pedigrees) by using PROC MIXED. The adult model contained the effect of colony, corn pedigree, and all possible interactions. Replications were included as the random variable, all other variables were fixed. Although nontransformed data are shown in the figures, data were $\log_{10}(x + 1)$ transformed before analysis to meet the assumptions of the analysis (Snedecor and Cochran 1989). Beyond the standard analysis of variance (ANOVA), we preplanned comparisons between colonies within corn pedigree and within corn pedigree between colonies. This was done with the LSMEAN output from PROC MIXED (least significant difference [LSD] technique).

Field Experiment. Larval number, size, and damage data were analyzed as a randomized complete block two way factorial design (two colonies × two corn pedigrees) by using PROC MIXED. The model contained the main effects of colony, corn pedigree, and all possible interactions. Replications were included as the random variable, all other variables were fixed. A separate analysis was done for number of larvae recovered, larval size, and root damage. Data were transformed $\log_{10}(x + 1)$ before analysis to meet the assumptions of normality. Beyond the standard ANOVA, we preplanned comparisons between colonies within corn pedigree and within corn pedigree between colonies. This was done with the LSMEAN output from PROC MIXED (LSD technique).

Seedling Bioassays. Larval number, head capsule width, and average dry weight were analyzed as a randomized complete block two way factorial design (two colonies × two corn pedigrees) using PROC MIXED. The model contained the effect of colony, corn pedigree, and all possible interactions. Larval number, head capsule width, and dry weight were transformed $\log_{10}(x + 1)$ before analysis to meet the assumptions of normality. Beyond the standard ANOVA, we preplanned comparisons between colonies within corn pedigree and within corn pedigree between colonies. This was done with the LSMEAN output from PROC MIXED (LSD technique).

Toxicity Bioassays. Larval survival data were analyzed as a randomized complete block by using PROC PROBIT. At generations four, seven, and 10, the experiment was replicated four, six, and seven times, respectively.

Table 1. Analysis of variance for greenhouse data after four generations of selection

Analysis	Effect	df	*F*	*P*
Larval no.	Colony	1,42	5.86	0.0198
	Corn	1,42	6.36	0.0156
	Corn × colony	1,42	1.10	0.3004
	Time	1,56	0.57	0.4548
	Time × colony	1,56	0.01	0.9314
	Corn × time	1,56	0.46	0.5011
	Corn × time × colony	1,56	0.21	0.6509
Larval head capsule	Colony	1,42	4.50	0.0399
	Corn	1,42	0.79	0.3797
	Corn × colony	1,42	1.06	0.3090
	Time	1,49	13.73	0.0005
	Time × colony	1,49	0.81	0.3719
	Corn × time	1,49	0.09	0.7664
	Corn × time × colony	1,49	0.35	0.5587
Larval wt	Colony	1,42	4.40	0.0419
	Corn	1,42	0.42	0.5192
	Corn × colony	1,42	2.31	0.1357
	Time	1,49	24.90	<0.0001
	Time × colony	1,49	0.01	0.9046
	Corn × time	1,49	0.00	0.9844
	Corn × time × colony	1,49	0.91	0.3459
Adult no.	Colony	1,42	0.78	0.4828
	Corn	1,42	4.69	0.0361
	Corn × colony	1,42	0.49	0.4884
Adult head capsule	Colony	1,19	0.04	0.8453
	Corn	1,19	0.10	0.7594
	Corn × colony	1,19	0.15	0.7069
Adult wt	Colony	1,19	0.70	0.4118
	Corn	1,19	1.71	0.2066
	Corn × colony	1,19	0.49	0.4909

Results

Greenhouse Experiments. After four generations of on-plant selection the interaction of colony × corn pedigree was not significant for any measurement, suggesting each colony performed similarly on isoline and MIR604 (Table 1). The selected and control colonies had similar numbers of larvae recovered from MIR604 corn (Fig. 2A), supporting this suggestion. Because percentage of hatch was similar between the two colonies for this trial with the selected colony being 68 ± 5.2% and the control colony being 69 ± 6.4%, any differences in this greenhouse study are not due to egg hatch. However, comparisons between colonies within corn pedigree and within corn pedigree between colonies show that the number of larvae recovered from MIR604 and isoline corn was not significantly different for the selected colony, and the control colony had significantly more larvae recovered from isoline corn than from MIR604 corn (Fig. 2A). Within a colony (selected or control), larvae recovered from MIR604 corn did not differ in head capsule width or dry weight compared with those recovered from isoline corn (i.e., the larvae were in the same stadium) (Fig. 2B and C; Table 1). However, larvae of the control colony recovered from MIR604 corn weighed significantly less than larvae of the selected colony recovered from MIR604 corn. The number of adults recovered from MIR604 and isoline corn was not significantly different for the selected colony, but significantly more adults were recovered from isoline corn than MIR604 corn for the control colony

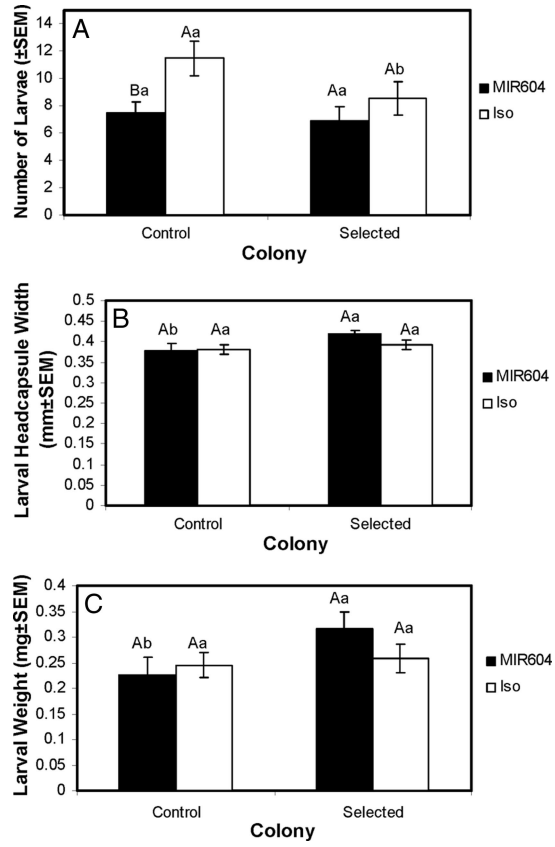

Fig. 2. Mean number (A), head capsule width (B), and size (C) of larvae recovered from laboratory colonies during trials on MIR604 and nontransgenic isoline corn in the greenhouse after four generations of selection. Although untransformed data are shown, analyses were performed using square root $(x + 0.5)$-transformed data (A and B) and log $[\log 10(x + 0.1)]$-transformed data (C). Bars with the same letters are not significantly different ($P = 0.05$). Uppercase letters indicate comparisons between isoline and MIR604 within colonies, and lowercase letters indicate comparisons between colonies within isoline or MIR604 corn.

(Fig. 3A; Table 1). Again, however, the interaction of colony × corn pedigree was not significant, suggesting the effects of corn were similar with each colony, and the selected and control colonies had similar numbers of adults recovered from MIR604 corn. Adults of control and selected colonies did not differ significantly in head capsule width or weight in any of the treatment combinations (Fig. 3B and C; Table 1).

Field Experiment. After seven generations of on-plant selection, the interaction of colony × corn pedigree was not significant for larval recovery when screened on MIR604 and isoline corn under field conditions, suggesting the effect of corn was similar with each colony (Table 2). There was no significant difference between the selected and control colonies in number of larvae recovered from MIR604 plants (Fig. 4A), supporting this suggestion. However, comparisons between colonies within corn pedigree and

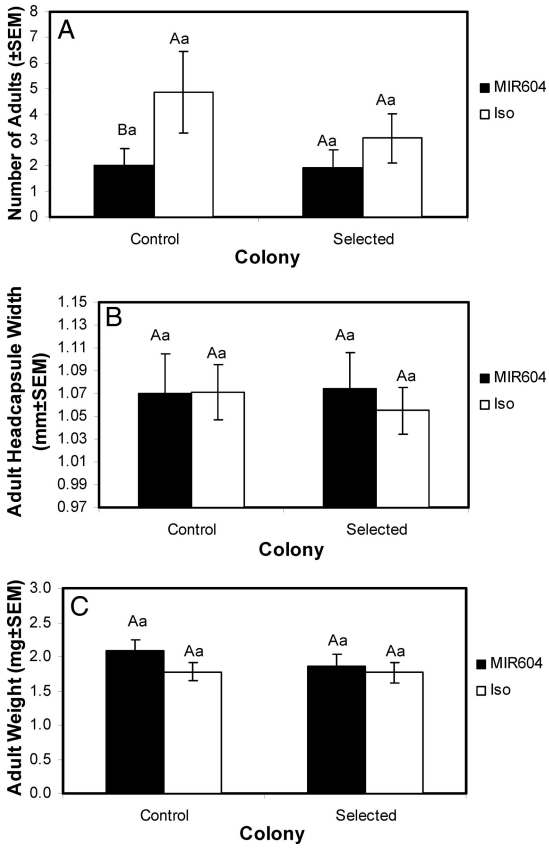

Fig. 3. Mean number (A), head capsule width (B), and size (C) of adults recovered from laboratory colonies during trials on MIR604 and nontransgenic isoline corn in the greenhouse after four generations of selection. Although untransformed data are shown, analyses were performed using square root ($x + 0.5$)-transformed data. Bars with the same letters are not significantly different ($P = 0.05$). Uppercase letters indicate comparisons between isoline and MIR604 within colonies, and lowercase letters indicate comparisons between colonies within isoline or MIR604 corn.

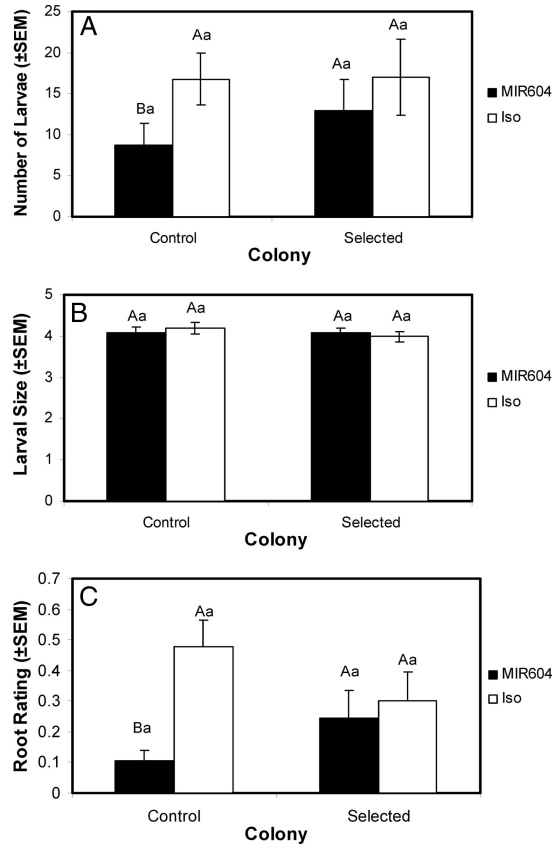

Fig. 4. Mean number (A) and size (B) of larvae recovered from laboratory colonies during trials on MIR604 and nontransgenic isoline corn in the field after seven generations of selection. Mean root damage rating (C) of corn plants in trials on MIR604 and nontransgenic isoline corn in the field after seven generations of selection. Although untransformed data are shown, analyses were performed using square root ($x + 0.5$)-transformed data (A and B) and log [log10($x + 0.1$)]-transformed data (C). Bars with the same letters are not significantly different ($P = 0.05$). Uppercase letters indicate comparisons between isoline and MIR604 within colonies, and lowercase letters indicate comparisons between colonies within isoline or MIR604 corn.

within corn pedigree between colonies done with the *t*-test output from PROC MIXED indicated that the number of larvae recovered from MIR604 and isoline corn was not significantly different for the selected colony, but significantly more larvae were recovered from isoline corn than MIR604 corn for the control colony (Fig. 4A). Interestingly, larval size did not differ for any of the treatment combinations (Fig. 4B; Table 2). When damage to roots in the field was analyzed, the interaction of colony × corn pedigree was significant (Table 2), suggesting that the effect of corn pedigree was different between the two colonies. In fact, there was no significant difference in damage to isoline and MIR604 roots from the selected colony, but this difference was significant for the control colony (Fig. 4C). Egg hatch of the selected colony was 70% in this trial and egg hatch of the control colony was 81%, which may help explain greater damage to isoline corn in the control colony and reduced damage to both corn lines for the selected colony.

Table 2. Analysis of variance for field data after seven generations of selection

Analysis	Effect	df	*F*	*P*
Larval no.	Colony	1,57	2.75	0.1027
	Corn	1,57	7.03	0.0104
	Corn × colony	1,57	0.36	0.5525
Size	Colony	1,50	0.39	0.5364
	Corn	1,50	0.18	0.6765
	Corn × colony	1,50	0.45	0.5060
Root damage	Colony	1,51	0.08	0.7805
	Corn	1,51	20.33	<0.0001
	Corn × colony	1,51	4.93	0.0309

Seedling Bioassays. Significantly more western corn rootworm larvae were recovered from isoline corn

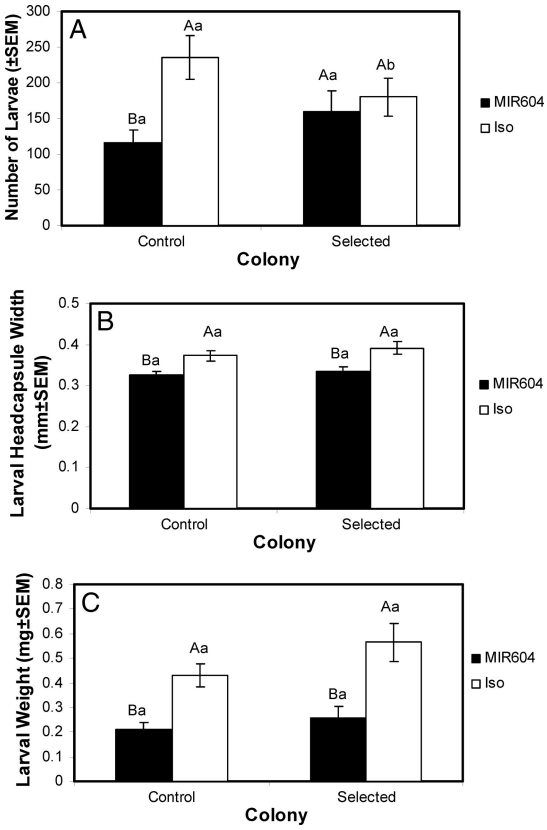

Fig. 5. Mean number (A), head capsule width (B), and size (C) of larvae recovered from laboratory colonies during trials on MIR604 and nontransgenic isoline corn in the greenhouse after 14 generations of selection. Although untransformed data are shown, analyses were performed using log [log10($x + 0.1$)]-transformed data. Bars with the same letters are not significantly different ($P = 0.05$). Uppercase letters indicate comparisons between isoline and MIR604 within colonies, and lowercase letters indicate comparisons between colonies within isoline or MIR604 corn.

than from MIR604 for the control colony, but this difference was not significant for the selected colony (Fig. 5A; Table 3). Although the difference between the number of larvae recovered from the selected and control colonies from MIR604 was not significant at the 5% level ($P = 0.0591$), the interaction of colony × corn pedigree was highly significant (Table 3), indicating that the effect of corn pedigrees on larval recovery was significantly different in terms of the number of larvae recovered. In addition to the ratios of larvae recovered from MIR604 to isoline being different for the two colonies, it should be noted that significantly fewer larvae were recovered from isoline for the selected colony than the control colony (Fig. 5A). Unfortunately, we do not have egg hatch data for this experiment so it is equally possible that egg hatch was lower for the selected colony or that there were fitness costs and this colony performed poorer on isoline corn. For both larval head capsule width and average dry weight, the size of larvae recovered was significantly smaller for larvae recovered from MIR604 than from larvae recovered from isoline corn, and there was no significant difference between the two colonies in terms of the size of larvae recovered from MIR604 (Fig. 5B and C; Table 3). The average dry weight of larvae recovered from isoline corn was significantly less for the control colony than the selected colony (Fig. 5B; Table 3), but this could be due to significantly more larvae feeding on the same amount of corn.

Table 3. Analysis of variance for laboratory data after 14 generations of selection

Analysis	Effect	df	*F*	*P*
Larval no.	Colony	1,7	0.08	0.7874
	Corn	1,14	31.51	<0.0001
	Corn × colony	1,14	13.01	0.0029
Larval head capsule	Colony	1,7	0.69	0.4337
	Corn	1,14	46.4	<0.0001
	Corn × colony	1,14	0.63	0.4415
Larval wt	Colony	1,7	2.63	0.1488
	Corn	1,14	88.15	<0.0001
	Corn × colony	1,14	1.62	0.2242

Table 4. LC_{50} data of mCry3A after one generation of field selection and multiple generations of greenhouse selection on mCry3A-expressing corn

Generation	Treatment	*N*	LC_{50} ($\mu g/cm^2$)	Resistance ratio
4	Control	4	0.55 (0.32–0.92)	
	Selected	4	0.26 (0.09–0.54)	0.5
7	Control	6	1.30 (0.87–2.00)	
	Selected	6	5.54 (2.36–26.43)	4.3
10	Control	7	0.73 (0.51–1.05)	
	Selected	7	11.25 (5.07–32.69)	15.4

Toxicity Bioassays. Four generations of on-plant selection did not significantly increase the selected colony's LC_{50} compared with the control colony (Table 4). Larvae of the selected colony screened after 7 and 10 generations of selection had significantly higher LC_{50} values than the paired control colony. The resistance ratios of the selected colony (LC_{50} of selected colony/LC_{50} of control colony) after 7 and 10 generations of selection were 4.3 and 15.4, respectively (Table 4).

Discussion

The western corn rootworm has proven to be highly adaptive, developing resistance to several classes of insecticides (Ball and Weekman 1963, Meinke et al. 1998) and crop rotation (Levine et al. 2002). Laboratory colonies with resistance to Cry34/35Ab1 (Lefko et al. 2008), Cry3Bb1 (Meihls et al. 2008) and the current work with mCry3A (Figs. 4 and 5; Table 4) suggest continued adaptability of this pest insect. For mCry3A, significant increases in resistance ratios were seen after seven and 10 total generations of selection (4.3- and 15.4-fold, respectively) in terms of LC_{50} values in toxicity assays (Table 4). After seven generations of selection in total, selected and control colonies were screened on MIR604 and isoline corn

under field conditions, and there was a significant colony × corn pedigree interaction for plant damage (Table 2). There was no significant difference in damage between MIR604 and isoline corn in the field, but this difference was significant for the control colony (Fig. 4C). After 14 generations of selection, a seedling bioassay was performed similar to Nowatzki et al. (2008). Again, there was a significant colony × corn pedigree interaction, this time for the number of larvae recovered (Table 3). There was no significant difference in the number of larvae recovered from MIR604 and isoline corn for the selected colony, but this difference was significant for the control colony (Fig. 5A).

After one generation of selection on MIR604 in the field, subsequent survival on MIR604 did not differ between the selected and control colony in greenhouse assays (Hibbard et al. 2010a). After three additional generations of selection on MIR604 in the greenhouse, LC_{50} values for mCry3A in diet bioassays were not significantly different between the selected and the control colony (Table 4). The colony × corn pedigree interaction also was not significant for larval recovery in greenhouse bioassays this same generation (Table 1) or field studies after seven total generations of selection (Table 2). Apparently, resistance to mCry3A developed more slowly than resistance to Cry3Bb1 under similar selection scheme and bioassays (Tables 1–4; Meihls et al. 2008). Even after 14 generations, resistance was not complete in that the selected colony still grew slower on MIR604 than on isoline corn in the seedling assay (Fig. 5B and C), though there were no differences in larval size on MIR604 or isoline corn from either colony in field studies (Fig. 4B; Table 2).

Because survivorship of unselected insects on MIR604 in the field was 5.12% relative to isoline corn (Hibbard et al. 2010a), the maximum field resistance ratio possible (100% survival on MIR604/5.12% initial survival on MIR604) is 19.53-fold, much lower than is possible for high dose Bt products and most insecticides, where resistance ratios in the hundreds or thousands are not uncommon (see Pereira et al. 2008a,b for an example of 3000-fold resistance to Cry1F). As noted, the maximum resistance ratio documented for the selected colony in the current study was 15.4 for LC_{50} data, which is not really comparable with field survivorship. For example, the strain of the European corn borer, *Ostrinia nubilalis* (Hübner), that had a 3,000-fold increase in LC_{50} data to Cry1F had just a few insects survive Cry1F plants and their F1 progeny had fitness close to zero (Pereira et al. 2008a,b). The dose situation with Cry1F and the European corn borer and mCry3A and the western corn rootworm are obviously dramatically different. Protein dose should be considered when making comparisons of resistance ratios of a species to different products or between species. Full field resistance to low-dose products will have much lower resistance ratios than maximum resistance ratios to high-dose products.

Since the introduction of Bt crops, field-evolved resistance has been documented in four pest species; *Spodoptera frugiperda* (J.E. Smith) to Cry1F in Bt corn (Storer et al. 2010), *Busseola fusca* (Fuller) to Cry1Ab in Bt corn (Van Rensburg 1999), *Pectinophora gossypiella* (Saunders) resistance to Cry1Ac in India (http://www.monsantoindia.com/monsanto/layout/pressreleases/mmb_pressrelease.asp), and *Helicoverpa zea* (Boddie) to Cry1Ac and Cry2Ab in Bt cotton (Tabashnik et al. 2009). No field failures have yet be documented for *H. zea*, causing some to question whether field resistance has evolved (Moar et al. 2008). Toxicity of Cry1Ab differs between prominent lepidopteran pests. Cry1Ac is not a high dose for *H. zea* but is high dose for tobacco budworm, *Heliothis virescens* (F.), and resistance to Cry1Ac has developed in *H. zea* populations but not *H. virescens* populations (Ali and Luttrell 2007). Similar to Cry1Ac targeting *H. zea*, mCry3A-, Cry3Bb1-, and Cry34/35Ab1-expressing transgenic corn for western corn rootworm management are not expressed in corn tissue at a high dose (EPA 2002; Storer et al. 2006; Hibbard et al. 2010a,b). It is possible that the low dose of these products has allowed selection for resistance to Cry3Bb1 (Meihls et al. 2008), Cry34/35Ab1 (Lefko et al. 2008), and mCry3A in the current study. However, it is also possible that having a low dose product may help slow resistance development. Models simulating adaptation to low-dose products have assumed that many of the individuals that survive have susceptible phenotypes (EPA Scientific Advisory Panel 2002). Hibbard et al. (2010a) documented that this assumption is likely correct for mCry3A. Having susceptible western corn rootworm genotypes emerging within a mCry3A block and available to mate with any western corn rootworm beetles that may be carrying resistance alleles also could be one factor delaying resistance to mCry3A in the field.

Understanding the biology of an organism is important in designing effective IRM strategies. Lepidopteran IRM often involves the high-dose/refuge strategy, which so far has been effective in delaying resistance in most lepidopteran species. For western corn rootworm, many of the assumptions that contribute to the success of the high-dose/refuge strategy are not fulfilled. Western corn rootworm has been shown to mate in a nonrandom manner (Kang and Krupke 2009), initial resistance alleles may be present at a higher frequency than initially assumed (Onstad and Meinke 2010), and the protein expressed in corn roots is not expressed in a high dose for western corn rootworm (EPA 2002; Storer et al. (2006); Hibbard et al. 2010a,b). Our current data and the work of Lefko et al. (2008), Meihls et al. (2008), and Meihls (2010) demonstrate that when western corn rootworm selected on Bt corn mate with one another, survival on Bt corn relative to isoline corn increases in a relatively short amount of time. Overall, recent results stress the importance of developing and adhering to resistance management plans for all corn rootworm Bt products.

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