Field-evolved resistance by western corn rootworm to multiple *Bacillus thuringiensis* toxins in transgenic maize

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Edited by Charles J. Arntzen, Arizona State University, Tempe, AZ, and approved January 27, 2014 (received for review September 12, 2013)

The widespread planting of crops genetically engineered to produce insecticidal toxins derived from the bacterium *Bacillus thuringiensis* (Bt) places intense selective pressure on pest populations to evolve resistance. Western corn rootworm is a key pest of maize, and in continuous maize fields it is often managed through planting of Bt maize. During 2009 and 2010, fields were identified in Iowa in which western corn rootworm imposed severe injury to maize producing Bt toxin Cry3Bb1. Subsequent bioassays revealed Cry3Bb1 resistance in these populations. Here, we report that, during 2011, injury to Bt maize in the field expanded to include mCry3A maize in addition to Cry3Bb1 maize and that laboratory analysis of western corn rootworm from these fields found resistance to Cry3Bb1 and mCry3A and cross-resistance between these toxins. Resistance to Bt maize has persisted in Iowa, with both the number of Bt fields identified with severe root injury and the ability western corn rootworm populations to survive on Cry3Bb1 maize increasing between 2009 and 2011. Additionally, Bt maize targeting western corn rootworm does not produce a high dose of Bt toxin, and the magnitude of resistance associated with feeding injury was less than that seen in a high-dose Bt crop. These first cases of resistance by western corn rootworm highlight the vulnerability of Bt maize to further evolution of resistance from this pest and, more broadly, point to the potential of insects to develop resistance rapidly when Bt crops do not achieve a high dose of Bt toxin.

evolution | resistance management | transgenic crops

The global area devoted to transgenic crops producing insecticidal toxins derived from the bacterium *Bacillus thuringiensis* (Bt) has increased rapidly over the past 15 y, with Bt crops covering more than 69 million hectares in 2012 (1). Most of this area was planted in Bt cotton and Bt maize (1). Benefits of Bt crops include effective management of target pests, decreased use of conventional insecticides, and reduced harm to nontarget organisms (2–5). However, the evolution of resistance could diminish these benefits. The western corn rootworm, *Diabrotica virgifera virgifera* LeConte (Coleoptera: Chrysomelidae), is a major pest of maize, with larval feeding on maize roots and associated management costs causing economic losses in excess of $1 billion per year (6). Through 2013, three Bt toxins have been used in transgenic maize for management of western corn rootworm: Cry3Bb1, mCry3A, and Cry34/35Ab1 (7).

In the United States and elsewhere, commercial registration of a Bt crop is accompanied by a resistance-management plan to delay the onset of pest resistance. Resistance management for Bt crops has focused on the refuge strategy, in which refuges of non-Bt crops allow the survival of Bt-susceptible insects, which may mate with resistant insects that survive on the Bt crop (8). To the extent that the heterozygous progeny from these matings have lower fitness on a Bt crop than their Bt-resistant parent, delays in resistance may be achieved, and these delays in resistance increase with the quantity of refuge (9). Additionally, refuges are far more effective in delaying resistance when Bt crops achieve a high dose of toxin against a target pest. High-dose Bt crops kill more than 99.99% of susceptible insects and render resistance a functionally recessive trait (9, 10). None of the currently commercialized Bt maize targeting the western corn rootworm is high dose, so the risk of resistance is increased (11, 12).

In 2003, Cry3Bb1 maize was registered by the United States Environmental Protection Agency (US EPA) for management of western corn rootworm larvae (7). In 2009, farmers in Iowa observed severe injury to Cry3Bb1 maize by larval western corn rootworm in the field, and subsequent laboratory assays revealed that this injury was associated with Cry3Bb1 resistance (13). More fields with Cry3Bb1 resistance were identified in 2010 (14), and research in fields identified in 2009 as harboring Cry3Bb1-resistant western corn rootworm found no difference in survival for this pest between non-Bt maize and Cry3Bb1 maize (11). Current threats to Bt maize include the spread of Bt-resistant western corn rootworm and the loss of additional Bt toxins through the presence of cross-resistance. In this paper we report that injury to Cry3Bb1 maize in the field has persisted through 2011 and expanded to include mCry3A maize. Analysis of western corn rootworm collected in 2011 revealed that (*i*) severe injury to Cry3Bb1 maize and mCry3A maize in the field was associated with resistance, and (*ii*) cross-resistance between Cry3Bb1 and mCry3A was present. These results demonstrate that insects can evolve resistance rapidly to Bt crops that are not high dose and raise concerns about the adequacy of current resistance-management strategies.

Significance

Crops genetically engineered to produce insecticidal toxins derived from the bacterium *Bacillus thuringiensis* (Bt) kill pest insects and reduce the use of conventional insecticides. However, the evolution of Bt resistance can diminishes these benefits. The western corn rootworm is a serious pest of maize and is managed with Bt maize. Beginning in 2009, western corn rootworm with resistance to maize producing the Bt toxin Cry3Bb1 imposed severe injury to Cry3Bb1 maize in Iowa. We show that cross-resistance exists between Cry3Bb1 maize and mCry3A maize and is associated with severe injury to Bt maize in farmers' fields. These results illustrate that Bt crops producing less than a high dose of toxin against target pests may select for resistance rapidly; consequently, current approaches for managing Bt resistance should be reexamined.

Author contributions: A.J.G. and J.L.P.-M. designed research; J.L.P.-M., E.H.C., M.W.D., A.M.H., D.A.I., and R.S.K. performed research; A.J.G. and J.L.P.-M. analyzed data; and A.J.G. and J.L.P.-M. wrote the paper.

Conflict of interest statement: A.J.G. has received research funding related to this project from Monsanto and has received funding not related to this project from AMVAC, Dow AgroSciences, DuPont-Pioneer, Monsanto, Syngenta, and Valent. A.J.G. has filed for a patent of the plant-based bioassay described in this article.

This article is a PNAS Direct Submission.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1317179111/-/DCSupplemental.

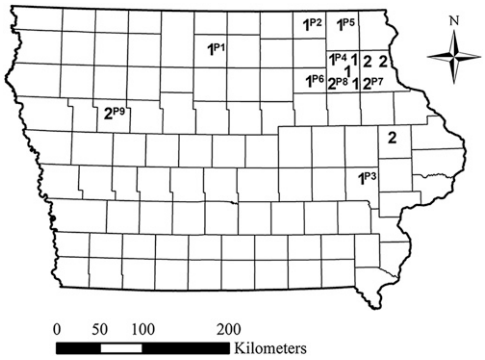

Fig. 1. Fields with more than one node of root injury to Bt maize in Iowa during 2011. Sections within a map are individual counties; a number within a county represents a single field with more than one node of root injury. The numeric value indicates the type of maize that was injured: 1, Cry3Bb1 maize; 2, mCry3A maize. Superscripted values correspond to fields used in bioassays (Table 1).

Results

In 2011, we identified 15 fields with severe injury to either Cry3Bb1 maize or mCry3A maize from larval western corn rootworm (Fig. 1), and we tested the progeny of western corn rootworms collected from nine of these fields with plant-based bioassays (Table 1). We found that feeding injury to Cry3Bb1 maize and mCry3A maize in the field was associated with resistance by the western corn rootworm to Cry3Bb1 maize and mCry3A maize. In laboratory bioassays of larval survival, there was a significant interaction between population type and maize type ($F = 19.17$; $df = 5,75$; $P < 0.0001$). For both Cry3Bb1 maize and mCry3A maize, survival of 2011 western corn rootworm populations did not differ between Bt maize and non-Bt maize (Fig. 2 *A* and *B*). In contrast, control populations of western corn rootworm, that had not experienced selection for resistance, displayed significantly lower survival on Bt maize than on non-Bt maize, and significantly lower survival on Bt maize than 2011 populations (Fig. 2 *A* and *B*). These results demonstrate resistance to mCry3A and Cry3Bb1 in 2011 populations. There was no significant difference in survival on Cry34/35Ab1 maize for 2011 populations and control populations, indicating an absence of resistance (Fig. 2*C*).

Multiple regression analysis showed a significant association among populations for survival on Cry3Bb1 maize and mCry3A maize, and this association was present regardless of whether the dependent variable was survival on Cry3Bb1 maize ($F = 72.9$; $df = 1,14$; $P < 0.0001$) or survival on mCry3A maize ($F = 79.2$; $df = 1,14$; $P < 0.0001$) (Fig. 3*A*). This result indicates the presence of cross-resistance between Cry3Bb1 maize and mCry3A maize. No other variables were significant in multiple regression models with either Cry3Bb1 maize or mCry3A maize as the dependent variable. When the dependent variable was survival on Cry34/35Ab1 maize, the only significant factor in the model was survival on the non-Bt near isoline ($F = 8.99$; $df = 1,15$; $P = 0.009$), and no significant effects were found for either Cry3Bb1 maize or mCry3A maize ($P > 0.10$), indicating an absence of cross-resistance between Cry34/35Ab1 and either mCry3A or Cry3Bb1 (Fig. 3 *B* and *C*).

Among larvae that were recovered from bioassays, the proportion of insects in the third (i.e., final) instar was not affected by an interaction between population type and maize type ($F = 1.03$; $df = 5,75$; $P = 0.4$) or by population type ($F = 1.87$; $df = 1,15$; $P = 0.19$). However, there was a significant effect of maize type ($F = 21.54$; $df = 5,75$; $P < 0.0001$), and for each of the pairs of Bt maize versus the non-Bt strain near isoline, the proportion of third-instar larvae was significantly lower ($P < 0.001$ in all cases) for the Bt hybrid than for the non-Bt strain near isoline: Cry3Bb1 maize = 0.53 ± 0.04 (mean ± SE), and non-Bt near isoline = 0.77 ± 0.03; mCry3A maize = 0.56 ± 0.03, and non-Bt near isoline = 0.75 ± 0.03; Cry34/35Ab1 maize = 0.15 ± 0.05, and non-Bt near isoline = 0.62 ± 0.03. This result indicates that larval development was delayed on Bt maize compared with non-Bt maize for both 2011 populations and for control populations.

In control bioassays that received neonate larvae but were checked by hand ($n = 20$), no pupae or teneral adults were found, indicating that larvae were not pupating within bioassay containers. For control cups that received late second- and early third-instar larvae and were checked by hand ($n = 20$), an average of 3.3 ± 0.74 pupae and 1.4 ± 0.74 teneral adults (newly molted but still in the soil) were found, indicating that pupae, if present, could be found by searching the soil by hand.

We conducted two meta-analyses of bioassay data for western corn rootworm on Cry3Bb1 maize. In the first meta-analysis we compared data on resistance in field populations from 2009 through 2011 and found a significant interaction between year and population type ($F = 4.33$; $df = 2,32$; $P = 0.02$), with population type defined as either control populations or populations from problem fields (i.e., fields where farmers complained of injury to Bt maize) (Fig. 4). Corrected survival on Cry3Bb1 maize was significantly greater for populations from problem fields in 2011 than in 2009, indicating that populations sampled from fields in 2011 were more resistant to Cry3Bb1 maize than the populations sampled in 2009 (Fig. 4). In contrast, corrected survival on Cry3Bb1 maize for control populations did not differ

Table 1. History of populations used in bioassays

Site	Type of maize evaluated for root injury	Root injury*	Field history			
			No. of years maize was planted†	No. of years Bt maize was planted‡		
				Cry3Bb1	mCry3A	Cry34/35Ab1
P1	Cry3Bb1	2.9 ± 0.2	9	4	0	0
P2	Cry3Bb1	2.6 ± 0.3	3	2	0	0
P3	Cry3Bb1	2.4 ± 0.4	9	4	0	0
P4	Cry3Bb1	2.2 ± 0.3	9	7	0	0
P5	Cry3Bb1	2.0 ± 0.6	9	5	0	0
P6§	Cry3Bb1	1.6 ± 0.7	6	3–5	0	1–3
P7	mCry3A	3.0 ± 0.0	4	1	1	1
P8	mCry3A	2.3 ± 0.4	2	0	1	0
P9	mCry3A	2.0 ± 0.5	4	2	1	0

*Mean ± SD for root injury on a node injury scale of 0–3 (34).

†Number of years maize was planted since 2003 (the year Bt maize was commercialized for western corn rootworm) or since the field was last planted in a crop other than maize, whichever is smaller.

‡Data on Bt maize exclude the first year maize was grown in a field because western corn rootworm larvae were not present to experience selection.

§Uncertainty regarding field history is reflected in the range of values.

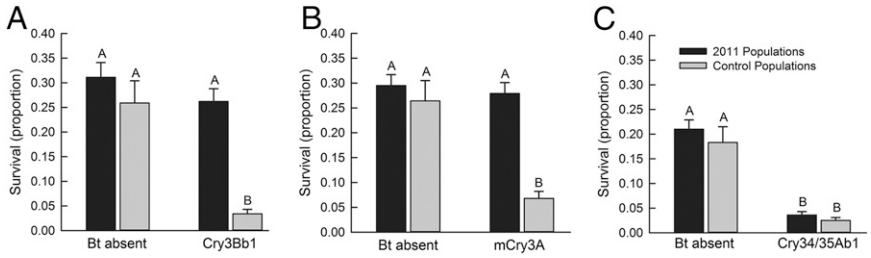

Fig. 2. Survival of western corn rootworm larvae on (*A*) Cry3Bb1 maize, (*B*) mCry3A maize, and (*C*) Cry34/35Ab1 maize. Control populations are described in *Methods*, and 2011 populations are described in Table 1. Bar heights represent the average survival among 2011 populations ($n = 9$) or control population ($n = 8$). Error bars indicate the SEM. In each figure "Bt absent" represents the non-Bt near isoline of Bt maize. Letters indicate pairwise differences within each graph.

between 2009 and 2011 (Fig. 4). In a second meta-analysis, we compared the magnitude of resistance to Cry3Bb1 maize by western corn rootworm with the resistance to Cry1Ac cotton by pink bollworm, *Pectinophora gossypiella* Saunders (Lepidoptera: Gelechiidae). The magnitude of resistance was determined by quantifying a resistance ratio, which was the quotient of survival for resistant individuals on Bt plants (e.g., Cry3Bb1 maize) divided by survival of susceptible individuals on Bt plants. For western corn rootworm with resistance to Cry3Bb1 maize, we found a resistance ratio of 6.15 (Table S1), whereas pink bollworm with resistance to Cry1Ac cotton had a resistance ratio of >99 (Table S2).

Discussion

Resistance to Cry3Bb1 maize in Iowa was found for populations of western corn rootworm sampled from fields during 2009 and 2010, with several of these fields displaying severe injury to Cry3Bb1 maize (13, 14). In 2011, the scope of severe injury to Bt maize expanded to include fields that contained either Cry3Bb1 maize or mCry3A maize (Fig. 1 and Table 1). Laboratory bioassays found that severe injury to Cry3Bb1 maize and mCry3A maize by western corn rootworm in 2011 was associated with resistance to both types of Bt maize (Fig. 2 *A* and *B*). An average of one node of root injury or greater was used as the threshold for classifying fields as having severe rootworm injury, because this criterion is used by the US EPA to classify single-toxin Bt maize as having greater-than-expected feeding injury and because injury to one node of roots (i.e., a ring of *ca*. 12 roots around the base of the plant) is associated with an average reduction in yield of 17% (15, 16). The total number of fields found with severe root injury has increased over time, with three fields identified in 2009 (13), seven fields in 2010 (14), and 15 fields in 2011 (Fig. 1), indicating that resistance has persisted, if not increased, with time. However, greater awareness among farmers of resistance and consequently greater effort in searching also could have contributed to this pattern. Additionally, survival on Cry3Bb1 maize for insects sampled from fields in 2011 was significantly greater than in 2009 (Fig. 4), again suggesting that resistance in the field has persisted, if not increased, over time. However, the data were obtained from only a subset of populations collected from fields where farmers complained of feeding injury to Bt maize; so the extent to which this pattern may have resulted from the nonrandom sampling of western corn rootworm populations across the landscape is currently unknown.

We found a significant correlation among populations for survival on Cry3Bb1 maize and mCry3A maize, indicating cross-resistance between mCry3A and Cry3Bb1 (Fig. 3*A*). In contrast, there was no evidence of cross-resistance between Cry34/35Ab1 maize and either Cry3Bb1 or mCry3A maize (Fig. 3 *B* and *C*). Classification of Cry toxins is based on phylogenetic similarity: toxins that share the same numeric value (e.g., Cry1A and Cry1F) have a more recent common ancestral gene (17, 18). Likewise, cross-resistance is found more often among toxins with the same numeric value than among toxins with different numeric values (19, 20). Consequently, the greater phylogenetic similarity of Cry3Bb1 and mCry3A may correspond to a greater similarity in the mode of action in these toxins as compared with Cry34/35Ab1. Both Cry3Bb1 and mCry3A belong to the three-domain Cry family, whereas Cry34/35Ab1 belongs to the binary-like family of Cry toxins (21). The structural similarity among three-domain toxins suggests potential similarities in binding sites in the insect midgut, whereas competitive binding analysis with Cry34/35Ab1 and mCry3A indicates at least some discordance in binding sites (21, 22). Proteins in the insect midgut involved with the mode of action for three-domain Bt toxins include alkaline phosphatase, aminopeptidase, and cadherin, and these proteins have been isolated from the midgut of the Chrysomelidae, the family of which western corn rootworm is a member (23, 24). Thus, alteration of certain Bt-binding sites may confer resistance to Cry3Bb1 and mCry3A simultaneously while not affecting susceptibility to Cry34/35Ab1.

The development of resistance to Cry3 maize (i.e., to Cry3Bb1 maize and mCry3A maize) and subsequent injury to Cry3 maize was associated with an average of 3.6 y of cultivation of Cry3 maize within fields (Table 1). Because western corn rootworm is univoltine (i.e., has one generation per year (6)), this period of cultivation translates to 3.6 generations of selection. Resistant populations could have resulted from independent evolution of resistance within each field, by pest dispersal from fields where resistance already was present, or both. Adult western corn rootworm often disperse less than 40 m/d, facilitating the evolution of resistance within an individual field; however, dispersal of greater than 1 km also has been documented, possibly enabling resistant genotypes to colonize new fields (25, 26). Resistance to Cry3Bb1 maize was documented in the northeastern quarter of Iowa during 2009 and 2010 (13, 14), raising the possibility that resistance alleles could have been locally prevalent in the vicinity of some of the fields sampled during 2011. However, laboratory selection generated Cry3Bb1-resistant strains after

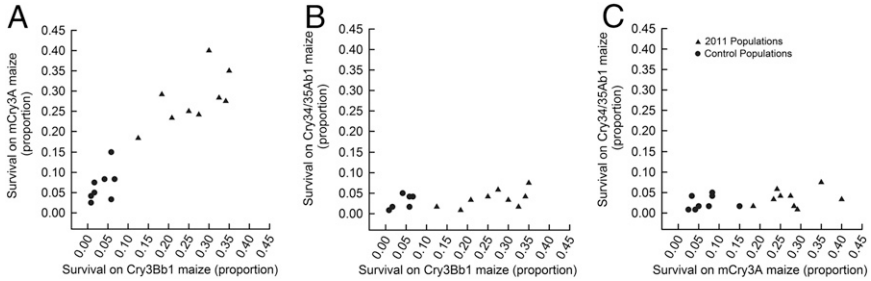

Fig. 3. Correlations among populations for survival on Bt maize. Type of Bt maize compared are (*A*) mCry3A maize vs. Cry3Bb1 maize, (*B*) Cry34/35Ab1 maize vs. Cry3Bb1 maize, and (*C*) Cry45/35Ab1 maize vs. mCry3A maize. Control populations are described in *Methods*, and 2011 populations are described in Table 1. For mCry3A maize vs. Cry3Bb1 maize, there was a significant correlation ($P < 0.0001$) among populations.

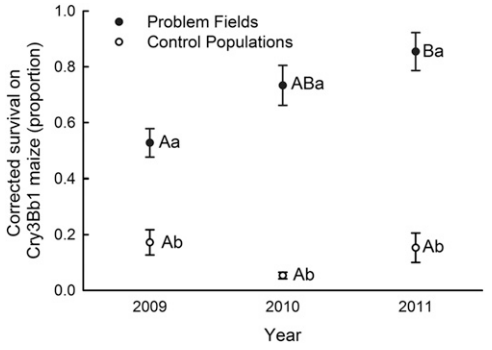

Fig. 4. Corrected survival on Cry3Bb1 maize between 2009 and 2011. Populations labeled as problem fields were from fields of farmers who complained of injury to Bt maize. Control populations were either from fields where injury to Bt maize was not observed (2009) or were western corn rootworm strains that were brought into the laboratory before the commercialization for Bt maize. Data for 2009 are from Gassmann et al. (13), data for 2010 are from Gassmann et al. (14), and data for 2011 are presented in this paper. Symbols represent sample means; error bars indicate the SEM. Capital letters denote pairwise differences among years within a class of populations (i.e., problem fields or control populations); lowercase letters provide a pairwise comparison between classes of populations (i.e., problem fields vs. control populations) within a year.

three generations, and previous studies found that Cry3Bb1-resistant populations in the field were associated with as few as three generations of selection (13, 14, 27). Thus, much of the Cry3 resistance found here likely either developed independently, or at least increased in magnitude, in each field. The one exception was field P8, where only one generation of selection occurred because the field had been planted to a nonmaize crop (i.e., a nonhost plant for rootworm) 2 y earlier (Table 1). The proportion of larvae surviving on mCry3A maize was significantly greater for western corn rootworm from P8 than from control populations (P8 = 0.40 ± 0.04; control populations = 0.07 ± 0.01; $F = 35.3$; df =1,7; $P = 0.0006$); however on the non-Bt near isoline, the survival of P8 (0.39 ± 0.12) and control populations (0.26 ± 0.04) did not differ ($F = 1.14$; df =1,7; $P = 0.32$). Consequently, the Cry3-resistant insects found in P8 likely evolved resistance elsewhere and then dispersed into that field.

Past research has found that none of the commercial Bt toxins targeting western corn rootworm achieve a high dose of toxin (11, 12). For all three Bt toxins studied here, the average survival for control populations of western corn rootworm on Bt maize exceeded 2% (Fig. 2). This finding is consistent with the lack of a high dose for Bt maize targeting western corn rootworm, because survival on high-dose Bt crops is expected to be less than 0.01% (11). The dose of toxin achieved by a Bt crop is important because it affects the functional dominance of resistance, and consequently, the rate of pest adaptation (9). The case of Bt resistance in western corn rootworm suggests that dose also matters because it affects the magnitude of resistance necessary to cause injury to Bt crops in the field. Resistance ratios measure the magnitude of resistance and are calculated as the quotient of survival for the resistant strain divided by survival of the susceptible strain, when both strains are challenged with a Bt crop. The resistance ratio for survival on Cry3Bb1 maize by resistant western corn rootworm was 6.15 (Table S1). In contrast, Cry1Ac cotton targeting the pink bollworm does achieve a high dose (28), and the resistance ratio for on-plant survival of resistant pink bollworm on Cry1Ac cotton was >99 (Table S2). Similarly, resistance ratios for diet-based bioassays with Bt toxin were 22 for western corn rootworm with resistance to Cry3Bb1 maize and 520–1,700 for pink bollworm with resistance to Cry1Ac cotton (27, 29). Thus, it appears that pest resistance to Bt crops that are not high dose may be characterized by resistance that (*i*) is less than the level of resistance necessary to injure high-dose crops and (*ii*) can evolve rapidly, in as few as three pest generations (13). However, differences in the mode of action between Cry1 and Cry3 toxins, in addition to biological differences between western corn rootworm and pink bollworm, may have contributed to this pattern.

Beginning in 2009, the US EPA approved Bt maize with a pyramid of two Bt toxins targeting the western corn rootworm, including Cry3Bb1 with Cry34/35Ab1 and mCry3A with Cry34/35Ab1 (30–32). Pyramiding of two Bt toxins delays resistance because individuals that harbor resistance alleles to one toxin are killed by a second toxin, with greater delays in resistance arising when the frequency of resistance alleles is low and there is an absence of cross-resistance between Bt toxins (33). Consequently, approval of these pyramids for management of western corn rootworm was accompanied by a reduction in non-Bt refuges to 5% (30–32). However, the presence of resistance to one toxin in a pyramid diminishes the effectiveness of a pyramid to delay resistance, and, coupled with reduced refuge size, may hasten the evolution of resistance. In light of resistance to Cry3Bb1 and mCry3A, the 5% refuge associated with current Bt pyramids targeting western corn rootworm may do little to delay resistance, and larger refuges should be considered as a tactic to delay resistance (12). Additionally, cultivation of Bt maize should be better integrated with other strategies for management of western corn rootworm, such as crop rotation, which will reduce selection for resistance and may help delay the further evolution of Bt resistance by this pest (13).

Methods

Field Visits. We visited fields between July 27 and September 7, 2011, in response to notification of rootworm injury to Bt maize by crop consultants, farmers, regional agronomists, and others in the agricultural community. At each field, the location was recorded using a global positioning system (GPS) (Legend HCX; Garmin). We sampled roots to measure rootworm injury and collected adult western corn rootworms ($n = 340 \pm 128$) for later use in bioassays. The relative abundance of the western corn rootworm and the northern corn rootworm *Diabrotica barberi* Smith and Lawrence was estimated by direct observation, and in all cases the western corn rootworm constituted at least 98% of the adults present in the field (Table S3).

We sampled a total of 12 roots from the interior portion of each field. Samples were taken at least 15 m from the field edge, along two transects spaced 15 m apart. Six roots were sampled along each transect with a space of 2 m between roots. For each field, the presence of rootworm-active Bt toxin in each maize plant sampled was verified based on ELISA (Envirologix). Rootworm injury was quantified based on the 0–3 node-injury scale (34), and any plants that did not test positive for Bt toxin were not evaluated for root injury (6 of 180 plants). For fields with an average root injury of greater than one node, we mapped the county within Iowa that contained the field by plotting GPS coordinates in Google Earth (Google, Inc.) and then manually transferring the location, accurate to the level of an individual county, to a map of Iowa (Iowa Department of Natural Resources, Iowa Geological Survey). An average root injury of one node was used as the threshold for classifying fields as having severe rootworm injury because this criterion is used by the US EPA to classify Cry3Bb1 maize as having greater-than-expected feeding injury (15). Furthermore, this injury is associated with an average reduction in yield of 17% (16).

Bioassays. Plant-based bioassays were conducted following Gassmann et al. (13, 14). Western corn rootworm adults, collected from fields where roots were sampled, were brought to the laboratory, and each population was held in an individual cage (18 × 18 × 18 cm; L × W × H) (Megaview Science) in a growth chamber at 25 °C and a light:dark ratio of 16 h:8 h. Cages contained maize leaf tissue and an artificial diet (western corn rootworm adult diet, productF9768B-M; Bio-Serv) as food for the rootworm adults and a water source provided by a 1.5% (wt/vol) agar solid. Adults were provided with an oviposition substrate that consisted of moist, finely sieved soil (<180 μm) placed in a Petri dish (diameter = 10 cm). Eggs obtained from each population were placed separately in 45-mL plastic cups containing moistened, sieved soil, were sealed in a plastic bag, and were placed in a cold room at 6 °C for at least 5 mo to break diapause. Eggs were held in a cold room until their removal for bioassays. For fields visited in 2011, we conducted bioassays on a total of nine populations, six from Cry3Bb1 fields and three from mCry3A fields, all with an average of more than one node of feeding injury from corn rootworm (Table 1).

We also evaluated eight control populations, obtained from the US Department of Agriculture's North Central Agricultural Research Laboratory (NCARL) in Brookings, SD. All control populations were diapausing strains of western corn rootworm that were brought into the laboratory before 2003, which is the year that Bt maize was commercialized for management of western corn rootworm (7). Thus, control populations represent field populations that never experienced selection for resistance to Bt maize. The year that control populations were collected and the sites of collection were (*i*) 1986, Moody County, SD; (*ii*) 1995, Brookings County, SD; (*iii*) 1995, Phelps County, NE; (*iv*) 1995, Potter County, SD; (*v*) 1996, York County, NE; (*vi*) 1999, Butler County, NE; (*vii*) 2000, Centre County, PA; and (*viii*) 2000, Finney County, KS. Eggs of control populations were sent from NCARL to Iowa State University in diapause and on arrival were held at 6 °C for later use in bioassays.

For both 2011 populations and control populations, neonate larvae were obtained for bioassays by removing eggs from 6 °C, washing eggs from the soil using a 250-μm screen, and then placing *ca*. 5,000 eggs atop moistened, sieved soil held in a Petri dish (diameter = 10 cm). Petri dishes with eggs were held in an environmental chamber at 25 °C for 2 wk, after which time larvae began hatching.

Bioassays used three transgenic maize hybrids, each of which contained a unique Bt toxin targeting western corn rootworm. These three Bt toxins represent all the commercialized Bt toxins that target western corn rootworm as of 2013 (7). The hybrid DKC 6169 (DeKalb Brand; Monsanto Company) produced Cry3Bb1; the hybrid 82H82 (Garst Brand; Syngenta) produced mCry3A; and the hybrid 2T789 (Mycogen Brand; Dow AgroSciences) produced Cry34/35Ab1. For each Bt hybrid, we also measured the survival of larval western corn rootworm on a near isogenic hybrid that lacked a gene for a rootworm-active Bt toxin but otherwise was genetically similar to its respective Bt hybrid. In the case of Cry3Bb1 maize, the non-Bt hybrid was DKC 6172 (DeKalb); for mCry3A maize the non-Bt hybrid was 82K79 (Garst); and for Cry34/35Ab1 maize the non-Bt hybrid was 2T777 (Mycogen). None of these seeds had been treated with insecticide or fungicide before their use in bioassays; however, as a precaution, all maize seeds were soaked for 1 h in a 10% bleach solution and stirred every 15 min to remove any potential traces of pesticide. Seeds then were rinsed 10 times with deionized water and allowed to dry for at least 24 h.

Maize plants used in bioassays were grown in a greenhouse (16 h:8 h light: dark cycle) in 1-L containers following Gassmann et al. (13, 14), with one plant per container. Once plants reached the V5–V6 stage [i.e., five- to six-leaf stage (35)], they were used in bioassays. For bioassays, plants first were trimmed to a height of 20 cm to allow them to fit within an incubator, and two to three leaves (trimmed to 8 cm long) were left on each plant. Recently hatched larvae (less than 1 d old) were removed from the soil surface within a Petri dish using a fine brush and then were placed at the base of a maize plant on a root that had been exposed by moving away a small amount of soil. Each plant received 12 neonate larvae. A barrier (Tree Tanglefoot; Tanglefoot Company) was placed around the inside edge at the top of each bioassay container to ensure that larvae could not move between containers. Containers with plants, soil, and larvae were placed in an incubator with light and humidity control (I41LL Percival Scientific) for 17 d (24.4 °C, 65% relative humidity, 16 h:8 h light:dark cycle), and plants were watered as needed. An incubation period of 17 d was chosen because it provided enough time for larvae on non-Bt maize to reach the third and final instar but was sufficiently short to ensure that larvae would not reach the pupal stadium (36). Maize plants remained in their original 1-L containers throughout the bioassay. Bioassays were conducted between March and September, 2012, and bioassays alternated between 2011 populations and control populations. For each combination of maize hybrid (e.g., Cry3Bb1 maize) and population, 10 bioassay containers were run for a total of 1,020 bioassay containers (17 populations × 6 maize hybrids × 10 containers) and 12,240 larvae.

After 17 d in an incubator, bioassay containers were removed, and the aboveground tissue of the maize plant was excised. The soil, containing roots and larvae, then was removed from the 1-L containers and placed on a Berlese funnel for 4 d to extract larvae from the soil. Rootworm larvae were collected in 15-mL glass vials containing 10 mL of 85% ethanol. Larval survival per bioassay container was calculated as the proportion of larvae recovered after 17 d divided by the number of neonate larvae placed into the container. A microscope (Leica MZ6) with digital camera and image analysis software (Motic Images Inc.) was used to measure the width of larval head capsules, and the larval instar then was determined based on the scale of Hammack et al. (37). For larvae recovered from each bioassay container, the proportion of larvae in the third and final instar was calculated.

To ensure that larvae were not pupating in bioassay containers, two types of control bioassays were run alongside bioassays measuring resistance. In the first type of control, bioassay containers that received neonate larvae from 2011 populations and control populations were treated identically to the other bioassays; however, instead of being placed on Berlese funnels, roots and soil were carefully searched by hand for larvae, pupae, and teneral adults. In the second type of control, 12 larvae in the late stage of the second instar and in the early stage of the third and final instar were placed into each bioassay container, and then the containers were placed in an incubator for 17 d, after which roots and soil were carefully searched by hand for larvae, pupae, and teneral adults. Larvae for this second control were from a non-diapausing strain of western corn rootworm and were reared to late second and early third instar on mats of maize seedlings (38). Instar was determined by visual inspection of head capsule width and body size using a dissection microscope. Both types of controls were run at the same time as bioassays with 2011 populations and control populations and used plants in containers that were identical to bioassays with 2011 populations and control populations. The average time that soil from an individual control bioassay was searched for western corn rootworm by an individual researcher was 47 ± 7 min. Twenty bioassays were run for each type of control for a total of 40 control bioassays.

Field History. For all 2011 populations tested in bioassays, a history of crops grown in the field from which they were collected was obtained for 2003–2011 (Table 1). A starting year of 2003 was chosen because this is the year Bt maize was commercialized for management of the western corn rootworm (7). We conducted interviews with crop consultants and farmers to determine the crop grown in a field each year (e.g., maize, soybeans, or alfalfa). For years in which maize was planted, follow-up questions were asked about whether the maize contained a Bt trait targeting the corn rootworm and the type or types Bt toxin present for management of corn rootworm (i.e., Cry3Bb1, mCry3A, Cry34/35Ab1, or Cry3Bb1 + Cry34/35Ab1).

Data Analysis. All data analyses were conducted using SAS Enterprise Guide 5.1 (39). Data on the proportion of larvae surviving per bioassay container and on the proportion of third-instar larvae were analyzed with a mixed-model ANOVA (PROC MIXED in SAS). Data were transformed by the arcsine of the square root to ensure normality of the residuals. Fixed effects in the model included maize type (Cry3Bb1 maize, non-Bt near isoline to Cry3Bb1 maize, mCry3A maize, non-Bt near isoline to mCry3A maize, Cry34/35Ab1 maize, and non-Bt near isoline to Cry34/35Ab1 maize) and population type (control populations vs. 2011 populations). Random factors in the analysis were population nested within population type and the interaction of maize type with population nested within population type.

Because there was a significant interaction between population type and maize type for survival, pairwise comparisons were made among 2011 populations and control populations within each pair of Bt maize and its non-Bt near isoline (e.g., Cry3Bb1 maize and its non-Bt near isoline). Pairwise comparisons were based on least-squares means and used the PDIFF option in PROC MIXED with a significance level of $P < 0.008$ based on a Dunn–Šidák correction for six pairwise comparisons (40). There also was a significant effect of maize type on the proportion of third-instar larvae, and pairwise comparisons were made between each of the three pairs of Bt and non-Bt hybrids, with a significance level adjusted at $P < 0.017$ for three pairwise comparisons. Additionally, a comparison was made between proportion survival of population P8 (Table 1) and control populations on both mCry3A maize and the non-Bt near isoline of mCry3A maize. The analysis was a mixed-model ANOVA with the fixed factor of population type and the random factor of population nested within population type.

Cross-resistance among the three types of Bt maize was tested with multiple regression (PROC REG) that analyzed the mean proportion survival for each of the 17 populations (nine 2011 populations and eight control populations) on all six of the maize hybrids. Survival on each type of Bt maize (i.e., Cry3Bb1, mCry3A, and Cry34/35Ab1) was coded as the dependent variable in a regression model that included survival on the remaining five maize hybrids as independent variables. Thus, in total, three multiple regression models were tested. For each model, independent variables were removed and added by running a procedure that used both forward and backward stepwise selection (SELECTION = STEPWISE option in PROC REG), following Sokal and Rohlf (40), with the criteria of $P < 0.05$ for an independent variable to enter the model and $P > 0.10$ for an independent variable to be eliminated from the model. However, for the Bt hybrid that was the dependent variable, the non-Bt near isoline was always retained in the model to factor out any differences in survival among populations that were unrelated to the presence of Cry toxins.

We conducted two meta-analyses on resistance of western corn rootworm to Cry3Bb1 maize. In the first analysis, we compared data on resistance to Cry3Bb1 maize in field populations from 2009 to 2011, and in the second analysis we compared the magnitude of resistance in western corn rootworm on Cry3Bb1 maize with the resistance of pink bollworm *P. gossypiella* Saunders (Lepidoptera: Gelechiidae) on Cry1Ac cotton.

For the first meta-analysis, data on resistance of western corn rootworm to Cry3Bb1 maize in the field were from Gassmann et al. (13) for 2009, Gassmann et al. (14) for 2010, and the data presented in this paper for 2011. Two classes of western corn rootworm populations were tested: (*i*) problem fields, which were fields visited in response to complaints by farmers of injury to Bt maize, and (*ii*) control populations, which were either diapausing laboratory strains brought into culture before 2003 or, in the case of Gassmann et al. (13), populations from fields that were not associated with observations of severe feeding injury to Bt maize. For every population within each year, corrected survival on Cry3Bb1 maize was calculated based on Abbott (41), as proportion survival on Cry3Bb1 maize divided by proportion survival on non-Bt maize. Data on mean corrected survival per population were analyzed with an ANOVA (PROC GLM) that included the factors of population type (problem fields vs. control populations) and year. Because a significant interaction between population type and year was present, pairwise comparisons were made between problem fields and control populations during each year (three pairwise comparisons) and among years for problem fields and control populations (six pairwise comparisons), with a significant level of $P = 0.0057$ based on a Dunn–Šidák correction for nine pairwise comparisons. Among problem fields, bioassays were conducted on a population from an individual field only once, with the exception of the field designated P1 in Gassmann et al. (13) and the same field designated S3 in Gassmann et al. (14). Thus, field S3 was excluded from the 2010 data to achieve a statistically independent sample. (However, inclusion of S3 does not alter the statistical significance of the results.) Six of the control populations tested in 2011 were the same as those tested in 2010. However, because there was no correlation in corrected survival for these populations between 2010 and 2011 ($r = 0.08$; df = 4; $P = 0.88$), data were treated as statistically independent and included in the analysis. (However, excluding data from 2010 does not alter the statistically significant interaction or the significant pairwise difference between problem fields from 2009 vs. 2011.)

For the second meta-analysis, we compared resistance ratios for western corn rootworm with resistance to Cry3Bb1 maize and pink bollworm with resistance to Cry1Ac cotton. We reviewed published data for survival of pink bollworm on Cry1Ac cotton (29, 42). Data for the survival of western corn rootworm on Cry3Bb1 maize were from this paper and from Gassmann et al. (13, 14). For both insects, resistance ratios were calculated following Tabashnik et al. (29) as the quotient of corrected survival for resistant insects on a Bt crop (i.e., Cry3Bb1 maize or Cry1Ac cotton) divided by corrected survival for susceptible insects on the same Bt crop. A detailed description of these calculations is provided in Tables S1 and S2.

ACKNOWLEDGMENTS. We thank Wade French of the US Department of Agriculture (USDA) Agricultural Research Service for providing the control populations, Dow AgroSciences, Monsanto, and Syngenta for providing maize seed, and R. Hellmich, S. Jakka, and R. Shrestha for comments on this paper. This research was supported by Biotechnology Risk Assessment Grant Program Competitive Grant 2012-33522-20010 and North Central Regional Integrated Pest Management Program Competitive Grant 2011-34103-30620 from the USDA National Institute of Food and Agriculture.

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