

Estimating the frequency of Cry1F resistance in field populations of the European corn borer (Lepidoptera: Crambidae)

Blair D Siegfried,^{a*} Murugesan Rangasamy,^a Haichuan Wang,^a Terence Spencer,^a Chirakkal V Haridas,^b Brigitte Tenhumberg,^b Douglas V Sumerford^c and Nicholas P Storer^d



Abstract

BACKGROUND: Transgenic corn hybrids that express toxins from *Bacillus thuringiensis* (*Bt*) have suppressed European corn borer populations and reduced the pest status of this insect throughout much of the US corn belt. A major assumption of the high-dose/refuge strategy proposed for insect resistance management and *Bt* corn is that the frequency of resistance alleles is low so that resistant pests surviving exposure to *Bt* corn will be rare.

RESULTS: The frequency of resistance to the Cry1F *Bt* toxin was estimated using two different screening tools and compared with annual susceptibility monitoring based on diagnostic bioassays and LC₅₀ and EC₅₀ determinations. An F₁ screening approach where field-collected individuals were mated to a resistant laboratory strain and progeny were assayed to determine genotype revealed that resistance alleles could be recovered even during the first year of commercially available Cry1F corn (2003). Estimates of frequency from 2003–2005 and 2006–2008 indicated that, although allele frequency was higher than theoretical assumptions (0.0286 and 0.0253 respectively), there was no indication that the frequency was increasing. Similar estimates in 2008 and 2009 using an F₂ screening approach confirmed the presence of non-rare resistance alleles (frequency \approx 0.0093 and 0.0142 for 2008 and 2009, respectively). The results of both screening methods were in general agreement with the observed mortality in diagnostic bioassays and LC₅₀ and EC₅₀ determinations.

CONCLUSIONS: These results are consistent with previous modeling results, suggesting that the high-dose/refuge strategy that is in place for *Bt* corn may be effective in delaying resistance evolution even when a relatively high frequency of resistance alleles exists.

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Keywords: *Bt* resistance; allele frequencies; European corn borer; F₁ and F₂ screening; pair mating

1 INTRODUCTION

The European corn borer, *Ostrinia nubilalis* (Hübner) (Lepidoptera: Crambidae), is one of the most destructive pests of corn in the United States. Transgenic corn plants that express insecticidal proteins from *Bacillus thuringiensis* (Berliner) (*Bt*) have become an effective method of protecting corn plants from damage by *O. nubilalis* and have been widely adopted throughout the US corn belt.^{1–3} Transgenic corn hybrids expressing the Cry1Ab or Cry1F insecticidal proteins from *Bt* for control of *O. nubilalis* have been used commercially in the United States since 1996 and 2003 respectively. Approximately 65% of the total 37.3 million ha of US maize planted in 2011 was planted with *Bt* hybrids targeting European corn borer, corn rootworm (*Diabrotica* spp.) or both pests,² exceeding 70% in Iowa and South Dakota, with likely higher levels of adoption in some counties.

The most widely cited insect resistance management strategy to delay the evolution of resistance to *Bt* toxins involves the high-dose/refuge strategy.^{4–6} One of the key assumptions of this

strategy is that alleles conferring resistance to *Bt* toxins are low, and a frequency of $\leq 10^{-3}$ has commonly been used as a default when modeling the evolution of resistance to *Bt* toxins.⁷ However, the high-dose/refuge strategy may still be effective at higher frequencies, provided the mortality of heterozygotes is high.⁸ Carrière and Tabashnik⁹ report modeling results showing that, in theory, the refuge strategy can delay or even reverse resistance

* Correspondence to: Blair D Siegfried, Department of Entomology, University of Nebraska, Lincoln, NE 68583-0816, USA. E-mail: bsiegfried1@unl.edu

a Department of Entomology, University of Nebraska, Lincoln, NE, USA

b School of Biological Sciences, University of Nebraska, Lincoln, NE, USA

c USDA-ARS, Corn Insect and Crop Genetics Research Unit, Iowa State University, Ames, IA, USA

d Dow AgroSciences, Indianapolis, IN, USA

evolution with an initial resistance allele frequency as high as 0.3, especially if the resistance is associated with a high fitness cost.¹⁰

Resistance monitoring of European corn borer for Cry1Ab susceptibility has been in place since 1995 and has involved traditional LC₅₀ determinations as well as diagnostic concentration bioassays that involve exposure to a concentration approximating the upper end of the LC₉₉ derived from initial baseline assessment developed prior to release of the toxin.^{11,12} While the number of documented field control failures and reports of unexpected damage to *Bt* crops have increased in recent years,^{10,13–15} annual assessments of susceptibility to Cry1Ab have not revealed any significant change in susceptibility or identified populations that survive on Cry1Ab-expressing plants.^{11,16–18} The results of similar monitoring efforts for other Cry toxins have yet to be reported.

While resistance monitoring programs that involve LC₅₀ comparisons and measuring survival at diagnostic *Bt* concentrations have been useful for maintaining records of susceptibility for many *Bt* crops and toxins, accurate estimates of allele frequencies are difficult to obtain because most traditional bioassay methods would not detect recessive alleles in heterozygotes^{11,19} and reliable detection of allele frequencies less than about 10^{–2} is impractical. Andow and Alstad¹⁹ described a method referred to as the F₂ screen that offers the advantage of potentially detecting recessive resistance alleles in a heterozygous state. This methodology involves collecting a large number of individuals from the field and establishing single-female family lines. The offspring of each collected female are inbred within family lines, and the offspring of these matings (i.e. the F₂ of the collected generation) are then screened at a discriminating concentration of *Bt* toxin that distinguishes resistant and susceptible phenotypes. The purpose of the inbreeding process is to allow potentially heterozygous offspring of the collected females to mate with each other, generating a significant and easily detectable fraction of homozygous resistant offspring. Through back-calculation of the frequency of family lines containing a resistant allele, the frequency of the resistance allele in the sampled population can be estimated.

A second approach to estimating resistance allele frequencies involves what has been referred to as an F₁ screen.²⁰ As described by Gould *et al.*,²¹ estimates of resistance allele frequency can be obtained through single-pair matings of field-collected individuals (of unknown genotype) with individuals from a resistant laboratory colony, provided the resistance is recessive and conferred by a single genetic locus. Single-pair matings of the resistant strain (RR) with field-collected individuals will result in progeny (F₁) that are either 100% RS if the field-collected parent is SS, 100% RR if the field-collected parent is RR or a ratio of 1RR:1RS if the field-collected parent carries one resistant allele. Screening these progeny at a concentration of *Bt* that discriminates between RS and RR genotypes provides an efficient means of screening for rare resistance alleles. It should be noted that such an approach could also detect the presence of non-recessive resistance alleles at loci other than the locus that confers resistance in lab-selected strains.²²

Although a number of different attempts have been made to isolate Cry1Ab resistance in *O. nubilalis*, no major resistant alleles have ever been recovered either through laboratory selection experiments^{23–25} or by F₂ screening of field populations,^{26–29} strongly suggesting that the frequency of alleles conferring resistance to Cry1Ab-expressing plants is below 10^{–3} in all the populations examined to date. In contrast, major Cry1F resistance alleles in *O. nubilalis* have been identified through laboratory selection experiments.³⁰ Selection with Cry1F has yielded a

strain with high levels of resistance (>3000-fold) conferred by a single recessive and autosomal allele in only 20 generations, and greenhouse experiments with potted plants have shown the strain capable of developing on Cry1F-expressing plant tissue.³¹ Additionally, a field population with higher than expected levels of Cry1F resistance and with characteristics identical to the laboratory-selected population (e.g. autosomal, recessive and conferred by a single locus) was identified in 2004.¹⁶ Because the resistance in this strain was highly recessive, a simple reciprocal crossing experiment between individuals from the lab-selected colony and the strain derived from the resistant field population was performed. In 13 families tested, the F₁ progeny derived from each strain exhibited nearly 100% survival at a Cry1F concentration that was tenfold higher than the original diagnostic concentration, confirming that the resistance in the field collection was conferred by the same genetic locus as the lab-selected population (Siegfried BD, unpublished).

Based on the ease with which resistance can be selected for in the laboratory and the detection of resistance alleles at the same locus in at least one field population, the frequency of alleles that confer Cry1F resistance may be higher than that estimated for Cry1Ab and may be increasing among field populations. The following report describes the results of annual Cry1F monitoring from populations across the US corn belt and F₁ and F₂ screening experiments to document the allele frequency for Cry1F resistance in a representative population of *O. nubilalis*. Results of these tests show conclusively that resistance alleles can be detected in field populations, although these populations remain susceptible to Cry1F, suggesting that the high-dose/refuge strategy for *Bt* corn is robust enough to maintain susceptibility in *O. nubilalis* even at background resistance allele frequencies that are greater than previously anticipated.

2 MATERIALS AND METHODS

2.1 Annual resistance monitoring

2.1.1 Insects

Field collections consisted of either adults obtained from sweep net samples or diapausing larvae collected from non-*Bt* corn. A minimum population size of 50 larvae, 50 adults, 25 mated females or 25 egg masses has been considered a valid sample size.¹¹ Small population sizes have limited the number of insects that can be collected in some years, and fewer insects have been used to establish populations for bioassays in some populations. Sample sizes (mean and range) for all collections from 2002 to 2010 were 165 adult females (26–>500), 119 larvae (24–210) and 103 egg masses (11–338). Collection sites were chosen on the basis of information derived from sales and insecticide use records provided by the Agricultural Biotechnology Stewardship Technical Committee.¹¹ At least 12 collections were assayed from 2002 to 2011. Rearing procedures for *O. nubilalis* have been widely reported and are based on those developed at the USDA Lab in Ankeny, Iowa.^{32,33} Larvae were reared at 27 °C and 80% RH in 24 h light and on wheat-germ-based diet. At pupation, insects were moved to mating cages where adults were maintained with an 8 h scotophase at 18.3 °C and a 16 h photophase at 27 °C with an RH of 80%. Egg masses from the mated females were collected and held within plastic petri dishes provided with filter paper moistened with sterile water to prevent desiccation, and incubated at 27 °C until hatching.

2.1.2 Bioassays

Bioassay of neonate larvae involved exposure to Cry1F solutions applied to the surface of single wells of artificial diet.³⁴ The authors attempted to utilize progeny obtained directly from field-collected insects whenever possible. In some instances, insects from later generations were used, or in cases where adult females were collected from the field, their progeny (F_0) were assayed directly. Bioassays were performed in 128 well trays (each well 16 mm diameter \times 16 mm height; CD International, Pitman, NJ). Dilutions of *Bt* were prepared in 0.1% Triton-X 100 to obtain uniform spreading of *Bt* solution on the diet surface. Individual neonate larvae (less than 24 h after hatching) were placed in wells, and mortality and combined larval weight were recorded 7 days later. Control treatments consisted of wells treated with 0.1% Triton-X 100. In the recording of mortality, larvae that had not grown beyond first instar (i.e. ≤ 0.1 mg) were considered to be dead.

All bioassays were conducted with Cry1F toxin produced through fermentation of recombinant *Pseudomonas fluorescens* (strain MR872) which was partially purified, then digested with trypsin to form truncated Cry1F. The truncated Cry1F was further purified by chromatography (Q-sepharose). The purified truncated Cry1F was then desalted and freeze dried and provided by Dow AgroSciences, Indianapolis, Indiana (Lot No. 104550). In the initial 3 years of monitoring, Cry1F was provided in aqueous solution and stored at -80°C . From 2005 onwards, Cry1F was provided as lyophilized crystals and stored at -80°C to increase stability.

2.1.3 Diagnostic bioassays

A diagnostic concentration of Cry1F protein that approximated the upper end of the 95% confidence interval of the LC_{99} (60 ng cm^{-2}) derived from baseline data³⁵ was tested against each field population. Bioassays with diagnostic concentrations employed the same exposure techniques described previously. Egg masses collected during a given 24 h period were held in plastic petri dishes, provided with filter paper moistened with sterile water to prevent desiccation and incubated at 27°C until hatching. Neonate larvae were selected at random and placed in individual wells treated with the diagnostic Cry1F concentration as described previously. Approximately 672 individual larvae were sampled from each collection (three replicates of 112 larvae on two different dates) unless insufficient eggs were available for bioassay. The proportion of surviving larvae that were ≤ 0.1 mg was recorded after 7 days. When mortality was recorded, larvae that had not grown beyond first instar and weighed ≤ 1.0 mg were considered

to be dead. As a result, the criterion for mortality accounts for both severe growth inhibition and death.

2.2 F_1 screening

2.2.1 Field collections

O. nubilalis larvae and adults were obtained from the University of Nebraska Agricultural Research and Development Center, Saunders County, Nebraska, from 2003 to 2008. A summary of the collections is provided in Table 1. All field-collected larvae were obtained from non-*Bt* plants and reared on a wheat-germ-based diet in the laboratory at $20-28^\circ\text{C}$ and 80% RH with a 16:8 h L:D photoperiod. First-generation larvae were reared to pupation, sexed according to characters described by Heinrich³⁶ and used directly in F_1 screens. Second-generation larvae were reared to last instar and then transferred individually to microfuge tubes with vented lids and maintained in the dark at 10°C for at least 100 days to terminate diapause.³⁷ The larvae were then transferred to 128-well trays (CD International, Pitman, NJ) containing 1% agar as a moisture source and held at 30°C with a 16:8 h L:D photoperiod, and pupation occurred within the wells. Pupae were sexed and used in subsequent F_1 screens. In some years, adult males were collected from weedy vegetation at the Agricultural Research and Development Center, Saunders County, Nebraska, in aerial nets and caged with virgin females from the resistant laboratory colony (Table 1).

2.2.2 Lab strains

The Rmy1F strain was selected in the laboratory and was shown to exhibit greater than 3000-fold resistance to Cry1F in diet bioassays.³⁰ The resistance was shown to be inherited as a recessive, autosomal trait and conferred by a single genetic locus.³¹ Throughout the study period, this strain was in culture for 58–107 generations with repeated selection every 3–4 generations by exposure to a diagnostic concentration of Cry1F that discriminated between resistant and susceptible insects.¹⁶ In 2007, the authors also employed the H1F strain which was derived from a field collection from Hamilton County, Iowa, in 2004 and which exhibited higher than expected (i.e. $>1\%$) survival at a diagnostic concentration of Cry1F based on the upper end of the 95% confidence interval of the LC_{99} determined for susceptible populations.¹⁶ This strain was subsequently selected by exposure to leaf discs of Cry1F-expressing corn plants and by exposure to the diagnostic Cry1F concentration as previously described. The resistance identified in this strain was similar in magnitude to the Rmy1F strain, and was recessive and autosomal. Crossing

Table 1. Summary of *O. nubilalis* collections used in F_1 screening experiments from 2003 to 2008

Year	Stage collected	Resistant strain	Number of family lines	Number of lines screened	
				♀ Parent	♂ Parent
2003	Larvae	RMy1F	104	15	4
2004	Diapausing larvae	RMy1F	198	51	9
2005	Adult ♂	RMy1F	127	—	17
	Diapausing larvae		130	23	3
2006	Adult ♂	RMy1F	59	—	5
	Diapausing larvae		212	24	6
2007	Diapausing larvae	RMy1F	253	33	6
		H1F	116	9	1
2008	Adult ♂	RMy1F	27	—	0
	Diapausing larvae		50	11	3

with the Rmy1F strain previously confirmed that the resistance was conferred by a common genetic locus because progeny were as resistant as either parental strain. Rearing of both resistant strains was timed to synchronize availability of virgin adults with field-collected populations for pair mating experiments.

2.2.3 Single-pair mating

Field-collected insects were individually crossed to the homozygous resistant moths from the laboratory colonies previously described. Each pair was placed in a small mating cage ($6 \times 6 \times 10$ cm) made of polyvinylchloride³⁷ and provided with cotton wool saturated with adult diet (1.4% agar, 40% sucrose in w/v). The egg laying surface of the cages were at a 45° angle and covered with 0.6 cm screen upon which wax paper sheets were placed as an oviposition substrate. All cages were maintained with a 16:8 h L:D photoperiod at 80% RH. Egg masses were transferred to a small petri dish (6 cm diameter) containing a water-saturated filter paper as source of moisture and allowed to hatch.

2.3 F₂ screening

2.3.1 Field collections

The procedure in F₂ screening involved collection of mated females or males from field collections and establishment of isofemale lines, sib-mating of F₁ offspring in each isoline and screening of F₂ neonates for resistance against Cry1F toxin as described previously. In July and August 2008, mated female *O. nubilalis* adults were collected from weedy vegetation at the Agricultural Research and Development Center, Saunders County, Nebraska, using sweep nets. Light traps maintained on Iowa State University research farms in Story County, Iowa, were also used for both male and female *O. nubilalis* adults. Mated females were used directly to establish isofemale lines, and males were individually mated with lab susceptible females to establish single-female lines. In June 2009, a combined sample of 164 mated females was obtained from several counties in eastern Nebraska and western Iowa. The majority of females (122) were collected from weedy vegetation at the University of Nebraska Agricultural Research and Development Center, Saunders County, Nebraska. The remaining individuals were collected from various locations in Dakota County, Nebraska, and from Harrison, Ida, Cherokee and Plymouth counties in western Iowa. In general, *O. nubilalis* populations were very low in 2009, requiring samples to be pooled across collections.

Mated females were individually caged in oviposition cages as previously described, and eggs were collected and incubated until hatching. Larvae were reared on a wheat-germ-based diet using standard rearing methods^{32,33} at 27 ± 0.7 °C and 80% RH under a 24 h photophase. Rearing containers contained corrugated cardboard rings positioned above the rearing diet as pupation sites. The pupal rings were transferred into a mating cage and maintained under a 16 h photophase where the adults from a single family line eclosed and mated. F₂ egg masses were collected daily and incubated in petri dishes until hatching, as previously described.

2.4 Diagnostic bioassays

The same diagnostic assays described previously were used to evaluate the F₁ and F₂ family lines, except that neonates were exposed to 120 ng cm⁻², which corresponds to $2 \times$ the upper end of the 95% confidence interval of the LC₉₉ derived from baseline susceptibility of field populations,³⁵ to minimize the risk of false positives.

The F₁ screen assumes that the resistance is recessive, autosomal, is conferred by a single locus and is fixed within

the resistant lab strains, which the authors believe to be true for both the RMy1F and H11F strains. Therefore, the expected mortality at the diagnostic concentration is dependent on the genotype of the field-collected parent. If the field-collected parent is homozygous for susceptibility, the resulting progeny should all be heterozygotes, resulting in 100% mortality at the diagnostic concentration. However, if the field-collected parent carries one resistant allele, a 1:1 ratio of heterozygotes to resistant homozygotes will result, and approximately 50% mortality at the diagnostic bioassays is expected. If the parent is homozygous for resistance, all progeny will be resistant, and 100% survival at the diagnostic bioassay is expected. A minimum of 28 neonates per F₁ line were exposed, depending on the availability of eggs.

For F₂ screens, sib-mating of F₁ offspring within a single family line should result in 1 in 16 F₂ neonates (6.25%) being homozygous for resistance and capable of surviving exposure to the diagnostic concentration¹⁹ if either grandparent carried a recessive resistance allele, and 25% if both grandparents carried a single allele or if one grandparent was homozygous for resistance. For diagnostic assays of F₂ family lines, a minimum of 16 individual larvae were tested at the diagnostic concentration, although for most families the number of larvae tested was ≥ 48 .

2.5 Statistical analyses

For annual monitoring surveys, bioassays were conducted in triplicate on two different dates and included at least five *Bt* protein concentrations that produced a mortality of >0 but $<100\%$. Data were analyzed by probit analysis^{38,39} to determine lethal concentrations. Observed mortality was corrected for mortality in control treatments, and lethal concentrations with 95% fiducial limits were calculated. Larval weights were transformed to percentage growth inhibition relative to the controls, and these data were analyzed by non-linear regression.^{34,40}

To estimate resistance allele frequencies ($E[P_R]$) from the F₁ screening, equation (4) from Yue et al.⁴¹ was used, and 95% credible intervals for these estimates were obtained using equation (15) from Andow and Alstad.⁴² In instances where the survival of the F₁ line approached 100%, it was assumed that the field-collected individual represented two resistance alleles in the present calculations. To find the probability of a false negative (P_{NO}) in an F₁ screen, which is calculated on the basis of control mortality, the total number of F₁ larvae entering the screen and the actual (and unknown) number of resistant larvae, use was made of equation (5) from Wenes et al.⁴³

Resistance allele frequency from each F₂ screen was estimated using methods in Andow and Alstad¹⁹ for mated females, and their credible intervals were evaluated using Bayesian inference as described by Andow and Alstad.⁴² In experiments where male parents were mated to virgin females from a susceptible lab colony that was assumed to be homozygous susceptible, calculations were modified according to equation (5) from Stodola et al.²⁹ to estimate resistance allele frequencies and their credible intervals. The probability of a false negative (P_{NO}) in the F₂ screen was calculated using equation (4) from Stodola and Andow²⁸ and was based on the number of F₁ males and F₁ females, the number of F₂ offspring screened per isofemale line and the control mortality of F₂ larvae. Because of the conservative nature of the diagnostic concentration bioassays, a false positive rate of 0.1% was used to identify a family line that exhibited significant survival. A one-proportion z-test⁴⁴ was used to compare the frequencies of survivors within single family lines that scored positive for resistance relative to the expected frequencies if one parent

carried one allele (1/16 survival in the F_2 line). In instances where survival was significantly greater than 6.25%, the authors tested whether there was significant departure from 1/4 or 25%, the expected proportion for a family line originating from a resistant homozygote, and frequency calculations were adjusted for the presence of two resistance alleles in the isofemale line. In instances where survival was significantly lower than 6.25%, the authors tested whether there was significant departure from 3% as the cut-off for false positives as suggested by Zhang *et al.*²² Families not significantly greater than 3% were excluded from further analysis.

A χ^2 test of homogeneity was used to establish the significance of the difference in F_2 estimates from Iowa and Nebraska in 2008 and 2009. To test for differences in F_1 estimates, Fisher's exact test was used in order to account for the relatively small sample sizes. All calculations and statistic analyses were performed using MATLAB software.⁴⁵

3 RESULTS

3.1 Annual susceptibility monitoring

Results of annual assessments of Cry1F susceptibility among geographically distinct *O. nubilalis* populations, involving both diagnostic bioassays and concentration responses, are presented in supporting information Table S1 and Fig. 1. In general, both the LC_{50} and EC_{50} remained relatively consistent over the 10 year sampling period, although a general decline in susceptibility was noted from 2002 to 2004 (Fig. 1) and may have been associated with loss of activity during storage of the solubilized Cry1F toxin. After 2004, lyophilized crystals rather than solutions were stored at -80°C , and results have been generally consistent from year to year (Fig. 1a). Additionally, in most years that susceptibility determinations have taken place, there has been a consistent level of variation between the most susceptible and most tolerant populations based on LC_{50} and EC_{50} values (Fig. 1b). The only exception to this consistency was in 2004 when the Hamilton County, Iowa, population was identified, which exhibited elevated LC_{50} and EC_{50} values (Fig. 1). Confirmation that this population possesses resistance similar to that in the Cry1F laboratory-selected population has been previously described.¹⁶ It should be noted that this population was established from only 11 egg masses,¹⁶ such that the reduced susceptibility exhibited by this population could have been an artifact of small sample size.

Measurement of survival at a diagnostic Cry1F concentration corresponding to the upper end of the 95% confidence interval of the LC_{99} derived from baseline susceptibility was also carried out (Table S1), and here the Hamilton County, Iowa, population was identified with almost 50% survival. As previously described, this population originated from a small sample of egg masses with several generations of rearing prior to bioassay. As a consequence, the frequency of resistance could have resulted from a single egg mass with significant sib-mating such that a high proportion of resistant homozygotes were present in the population. In subsequent years of sampling from similar locations, survival at the diagnostic concentration did not exceed 0.1% (Table S1). Importantly, the mean percentage survival in 2011 (0.069) was less than that observed in 2002 (0.092), indicating that there was no overall increase in resistance frequency over the 10 years of sampling.

3.2 F_1 screening

A summary of the field collections and establishment of family lines for F_1 screening experiments is provided in Table 1. Of the total

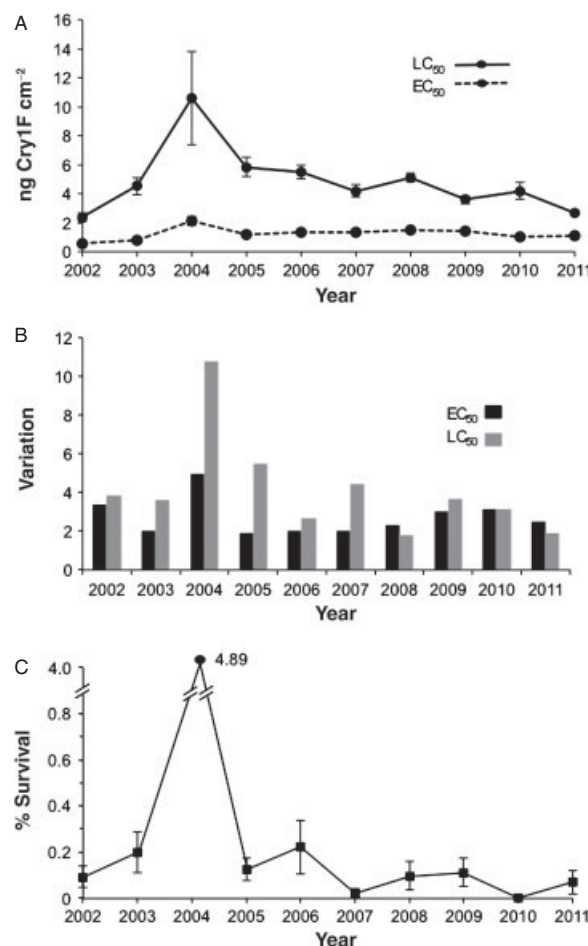


Figure 1. (A) Variation in Cry1F susceptibility, based on LC_{50} (lethal concentration causing 50% mortality) and EC_{50} (effective concentration causing 50% growth inhibition) for *O. nubilalis* populations. Error bars indicate the standard error of the mean of at least ten populations sampled per year. (B) Variability in response to Cry1F among *O. nubilalis* field populations from 2003 to 2011. Variation is expressed as the ratio of the highest to lowest LC_{50} and EC_{50} for each year of monitoring. (C) Mean percentage survival (based on observed mortality and absence of larval growth) of *O. nubilalis* populations exposed to a diagnostic Cry1F concentration. Error bars indicate the standard error of the mean of at least ten populations per year.

1276 single-pair matings established over six consecutive years from 2003 to 2008, only 220 family lines (17.2%) produced viable progeny that were available for subsequent diagnostic bioassays (Table 1). This low compatibility was more apparent when field-collected males were paired with a resistant female (7.5% success) relative to field-collected females mated to a resistant male (30%). In 2007, the field-derived H1F resistant strain, which was in culture for <30 generations, was utilized to pair with field-collected individuals to determine whether long-term rearing was affecting mating success. A similar low rate of successful matings (9%) was observed with this strain.

In spite of the low rate of successful matings, an average of 37 families per year were screened over six consecutive years, and in each year except 2008 at least one resistance allele was detected in the field collections. Of the ten resistance alleles (four homozygous and two heterozygous individuals) that were identified from 2003 to 2008, none of them was recovered from a field-collected male, reflecting the low rate of successful mating

Table 2. Summary of F_1 screening results and expected frequency estimates for *O. nubilalis* resistance to Cry1F

Year	Number of family lines selected	Individuals detected ^a		$E[P_R]$ (95% CI)
		RR	RS	
2003	19	1 (28; 100%)	1 (28; 46.4%)	0.0286 (0.0116–0.0516) ^b
2004	60	1 (28; 100%)	0	
2005	43	0	1 (47; 59.5%)	0.0253 (0.0083–0.0501) ^b
2006	35	1 (28; 100%)	0	
2007	49	1 (98; 98.9%)	0	
2008	14	0	0	

^a Numbers of family lines where RR or RS individuals were detected (number of individuals tested in diagnostic Cry1F bioassays; percentage survival).

^b Frequency estimates derived from pooled data for years 2003–2005 and 2006–2008.

^c Frequency estimates for 2003–2005 not significantly different from 2006–2008 (Fisher's exact test, $P > 0.05$).

between field-collected males and females from the laboratory colony. Resistant homozygotes were identified in four of the years sampled, based on nearly 100% survival at the diagnostic concentration and larval development comparable with control treatments (data not shown). Field-collected heterozygotes were identified on the basis of their offspring exhibiting approximately 50% survival and larval development comparable with the control, although only two individuals were identified. During the first year that Cry1F-expressing corn was commercially available (2003), both a resistant homozygote and heterozygote were identified.

To estimate resistance allele frequencies among the field populations with 95% confidence intervals, data were pooled across three consecutive years (2003–2005 and 2006–2008) in order to increase sample size and statistical power to those necessary to detect changes in frequency (Table 2). These results suggest that the resistance allele frequency was already quite high during the initial 3 years of commercial availability ($E[P_R] = 0.0286$ with 95% CI between 0.0116 and 0.0516) (Table 2). The frequency for 2006–2008 remained similar ($E[P_R] = 0.0253$ with 95% CI between 0.0083 and 0.0501) and was not significantly different from the first 3 years.

For the F_1 screens, control mortality did not exceed 10%, and at least 28 individuals were screened such that the calculated probability of false negative (P_{NO}) was calculated as 1.6×10^{-8} , suggesting a very high detection probability.

3.3 F_2 screening

Control mortality did not exceed 10% and, in all but one family, at least 48 F_2 individuals were screened per family line. In addition, the population of F_1 individuals that were used to initiate a family line was at least 50 individuals, and a 1:1 sex ratio was assumed. Therefore, the overall probability of a false negative (P_{NO}) was calculated as 7×10^{-5} , again indicating a very high detection probability.

3.3.1 2008

A total of 435 *O. nubilalis* males and females were collected from Nebraska and Iowa, and 272 (62.5%) produced viable offspring from F_1 sib-mating. Of the 127 field-collected males, 44%

Table 3. Actual and expected numbers of resistant individuals in the initial F_2 screens of isofemale lines that scored positive for an allele that conferred resistance

Isofemale line	Number tested	Larvae > 0.1 mg at day 7			Probability of fit
		Observed	Expected	Observed (%)	to expected ^a
2008					
IA11	172	24	10.75	13.95	<0.001 ^b
IA24	86	2	5.38	2.33	0.133
IA59	96	3	6.00	3.13	0.206
NE57	16	2	1.00	12.50	0.303
NE139	48	4	3.00	8.33	0.551
NE133	48	3	3.00	6.25	0.992
NE4	48	1	3.00	2.08	0.234
NE179	64	2	4.00	3.13	0.302
2009					
105	368	8	23.0	2.1	0.001 ^c
2	48	6	3.00	12.5	0.073
13	56	12	3.50	21.4	<0.001 ^d
11	168	2	10.5	1.2	0.007 ^c
30	72	4	4.5	5.5	0.780

^a Probability estimated using a one-sample z-test to test for significant departure from an expected frequency of 1/16 or 6.25%.

^b A one-proportion z-test for significant departure from an expected frequency of 1/4 or 25% was significant ($P < 0.01$) and the line was assumed to represent a single recessive allele.

^c A one-proportion z-test to test for significant departure from 3% was used to eliminate false positives. For both line 105 and line 11, observed survival was significantly less than 3%, and these lines were excluded from further analysis.

^d A one-proportion z-test for significant departure from an expected frequency of 1/4 or 25% was not significant ($P = 0.5352$), and the isofemale line was assumed to represent two recessive resistance alleles.

successfully established F_1 isolines when mated with laboratory susceptible females (data not shown). A minimum of 50 adults in each isolate were used in F_1 sib-mating, and at least 64 neonates in the F_2 generation were screened for Cry1F resistance. Of 268 isofemale lines successfully used in the F_2 screen in 2008, eight isolines exhibited survivorship that was significantly greater than 0.1%, with an overall average of 6.4% survival (Table 3). One of the lines (IA11) was significantly greater than 6.25% but significantly lower than 25%, which is the expected rate of survival for F_2 progeny originating from an isofemale line carrying two resistance alleles. This line was therefore included in the frequency calculation as representing a single resistance allele.

The calculated resistance allele frequency based on survivorship at the F_2 generation for Nebraska was 0.0135 (95% CI between 0.0050 and 0.0253), and for Iowa it was 0.0076 (95% CI between 0.0021 and 0.0165) (Table 4). None of the isolines originating from field-collected males mating with females from the susceptible laboratory colony produced survivors in the diagnostic bioassays. There was no statistically significant difference between the resistance frequencies obtained for the two collection sites (Table 4).

3.3.2 2009

In June 2009, a total of 164 mated females were collected in Nebraska and Iowa, and 53% of them produced isolines with viable eggs for F_2 screening. Out of 88 isolines screened, five had survivors with an average of 8.54% survival (Table 3). Two of the

Table 4. Summary of isolines established for F₂ screening of Cry1F resistance in field-collected *O. nubilalis* populations from Iowa and Nebraska

Year	Collection site	P ₁ lines started	F ₂ lines screened ^a	Number of F ₂ lines with 7 day survival ^b	E[P _R] (95% CI) ^c
2008	NE	127	111	5	0.0135 (0.0050–0.0253) ^d
	IA	183	158	3	0.0076 (0.0021–0.0165) ^d
	Total	310	259	8	0.0093 (0.0086–0.0321) ^e
2009	NE/IA	164	88	4	0.0142 (0.0046–0.0278) ^e

^a Each successful line was started with at least 40 progeny derived from the P₁ generation.^b Survivorship in individual families described in Table 2.^c Frequency estimates derived separately for Nebraska and Iowa in 2008 and also from pooled (Nebraska and Iowa) data for 2008 and 2009.^d Frequency estimate for Iowa in 2008 not significantly different from that in Nebraska (χ^2 test for homogeneity = 1.44, $P > 0.05$).^e Frequency estimates for 2008 not significantly different from 2009 for the pooled data (n^2 test for homogeneity = 0.443, $P > 0.05$).

isolines (105 and 11) exhibited significantly less than 3% survival and were excluded from further analysis.²¹ In contrast, line 13 exhibited significantly greater than 6.25% survival but was not significantly different from 25%, and was assumed to represent two recessive resistance alleles. The estimated Cry1F-resistant allele frequency for 2009 was 0.0142 (95% CI between 0.0046 and 0.0278), and the allele frequencies estimated during 2009 were not statistically significantly different from the overall frequency calculated in 2008 for both Nebraska and Iowa collections (0.093; 95% CI between 0.00086 and 0.0321) (Table 4).

4 DISCUSSION

The combined results for F₁ and F₂ screens of *O. nubilalis* field populations for Cry1F resistance indicate that such alleles can be readily detected from field populations by either screening approach. The implication of these results is that the frequency of Cry1F resistance among representative *O. nubilalis* populations in the midwestern United States may be higher than anticipated and, importantly, may have already been present at relatively high frequencies prior to the introduction of Cry1F-expressing corn plants. Again importantly, the frequency of alleles conferring Cry1F resistance identified by both screening methods does not appear to have increased over the 8 years of the study. These results are consistent with annual resistance monitoring of *O. nubilalis* populations, which utilizes diagnostic concentration bioassays and dose–response assays and which indicates a generally consistent level of susceptibility among field populations. This consistency across years, combined with the lack of changes in frequency estimates, suggests that the high-dose/refuge strategy is effective for maintaining Cry1F susceptibility in *O. nubilalis* even if the initial frequency of resistance alleles exceeds theoretical assumptions.

An additional consideration is the market penetration for Cry1F corn hybrids since commercial launch in 2003. Based on publicly available estimates of total acres of *Bt* corn planted,² and on private market penetration data for hybrids that express different *Bt* toxins for corn borer control (Storer NP, unpublished data), it is unlikely that Cry1F exceeded 20% of the total corn grown in Iowa and Nebraska until 2007. From 2008 to 2012, the ratio of the area of Cry1F corn to the area of corn that did not contain any toxins for corn borer control (which provides a measure of selection pressure for resistance) was approximately 1:1. As a consequence, the consistency in Cry1F susceptibility noted throughout the study may also be associated with moderate exposure to Cry1F corn. Since 2009, single-toxin Cry1F corn has begun to be replaced by pyramided-trait products that are expected to further reduce the potential for resistance development.²

The continued efficacy of Cry1F-expressing corn may also be associated with fitness costs and incomplete resistance.⁴⁵ Fitness costs are evident when homozygous resistant insects on a *Bt* plant have lower fitness than susceptible larvae on non-*Bt* plants.^{46,47} Pereira *et al.*⁴⁸ compared life-history traits and population growth rates of genotypes homozygous and heterozygous for resistance with susceptible homozygotes to Cry1F. Although the existence of weak and recessive to incompletely recessive fitness costs were indicated in the resistant strain when reared on artificial diet, resistant homozygotes exhibited clearly reduced fitness on *Bt* plants relative to susceptible insects on non-*Bt* plants.³¹ These results demonstrate the incomplete nature of the resistance, which may play a role in delaying resistance evolution and maintaining efficacy of Cry1F-expressing plants. Additionally, the lack of cross-resistance to Cry1Ab in Cry1F-resistant *O. nubilalis*³⁰ may also be an important factor in maintaining efficacy of Cry1F-expressing hybrids, because Cry1Ab is the other major Cry toxin deployed in *Bt* corn and has been combined with Cry1F in newly released pyramided-trait products where two toxins with independent modes of action are deployed in the same hybrids.

As noted previously, the frequency of resistance to Cry1F among field populations of *O. nubilalis* appears to have already been relatively high in unselected populations. Similar results have been reported for Cry2Ab and Vip3A in populations of *Helicoverpa armigera* and *H. punctigera* from Australia^{20,49} and for *Pectinophora gossypiella* from Arizona,⁵⁰ although recent estimates for *P. gossypiella* are much lower than the initial estimates.⁵¹ The reasons for these high initial frequencies remain unclear. Mahon *et al.*²⁰ suggest that some other selective agent provides an advantage against the toxin and/or there is a high rate of mutation, introducing resistance alleles that are not selected against. In the case of Cry1F and *O. nubilalis*, prior selection by Cry1Ab, which was first introduced in 1996 and occupied the majority of the *Bt* market from 1996 to 2003, is effective against Cry1F-resistant *O. nubilalis*,³⁰ and therefore prior selection by Cry1Ab should not have affected the frequency of Cry1F resistance. Interestingly, the mechanism of resistance to Cry1F remains elusive,⁵² and critical evaluation of the biochemical and physiological factors that confer resistance to Cry1F may provide further insight into its relatively high initial frequency and an apparent lack of response to selective pressures among field populations.

Both the F₁ and F₂ screens used in this study confirmed the existence of resistance alleles in representative field populations and allowed the calculation of frequency and confidence intervals. In addition, the calculated frequencies were similar between the two methods, although it should be noted that the resistance alleles identified were not confirmed to be identical. One

advantage offered by the F_2 screen is that it should recover any resistance allele that increases fitness of individuals in the presence of toxin, but frequency estimates obtained from the F_2 screen may be higher because it potentially detects resistance conferred by more than one locus.⁵³ In contrast, the F_1 screen can detect dominant alleles at any locus but is specific for recessive alleles at the same locus that confers resistance in the selected strain. In the present tests, the estimates obtained by the F_2 screen were slightly lower than those obtained in the F_1 screens.

It should be noted that the results of the F_1 screen may have been compromised by the relatively low rate of successful matings between the lab-selected resistant colonies and field-collected insects. The low rate of mating success reduced the efficiency of the technique relative to the F_2 screen, where nearly 60% of the family lines could be bioassayed in contrast to only 17% for the F_1 screen. The nature of the mating incompatibility between the resistant laboratory colony and feral populations remains uncertain and warrants further investigation. Clearly, behavioral and physiological differences could have arisen from long-term lab rearing, which would affect mating success and affect the efficiency of F_1 screening approaches. Importantly, frequency estimates obtained from the F_1 screen may have been artificially elevated if assortative mating, where the lab strain preferentially mates with field-collected individuals carrying a resistance allele, resulted in increased mating success. The unexpectedly high number of resistant homozygotes that were detected may be an indicator that assortative mating was taking place, resulting in inflated estimates of Cry1F resistance frequency. Higher frequencies of Cry2Ab resistance have previously been noted for F_1 screens when directly compared with F_2 screening methods in Australian populations of *H. armigera*;⁵³ however, the basis of the difference in frequencies between the methods could not be explained by either fitness costs or the possibility that alleles at linked loci may be homozygous lethal. Another factor affecting frequency estimates derived from F_1 screens is the potential to identify resistant individuals with non-recessive resistance alleles at loci other than the locus that confers resistance in lab-selected strains, which may result in overestimation of frequency.^{2,53} However, in the case of Cry1F resistance in *O. nubilalis*, if such alleles were frequent in field populations, the annual assessments of susceptibility would not be as consistent and changes in susceptibility might be expected.

Frequency estimates obtained using an F_2 screen would not be subject to similar issues of assortative mating or the detection of non-recessive resistance alleles. However, because the frequency estimates obtained by this method were similar to the estimates obtained from the F_1 screen, it seems unlikely that assortative mating inflated frequency estimates. In addition, because the percentage of families that were successfully screened was much higher for the F_2 screen, the cost per successful mating was much lower than for the F_1 screen. One complicating factor associated with the F_2 screen, especially when using diet bioassays, is the possibility of false positives. In at least two of the families that tested positive (i.e. survival significantly greater than 0.1%), the survival was significantly lower than the expected frequency of 1 in 16, and it is possible that the calculated frequency estimates were inflated by false positives or that some other incomplete resistance factor was identified. In another two families, survival was significantly greater than 6.25%, suggesting that either the parental female or her mate was homozygous for resistance or that some degree of dominance was associated with a different resistance mechanism. Similar heterogeneity in response of F_2 family lines of *H. armigera* screened for resistance to Cry2Ab was observed by Mahon et al.,⁵³

who suggested that the frequency of survival is heterogeneous and implies that the resistance mechanism may vary among isofemale lines.

Finally, it is clear that, while both screening methods provide an estimate of resistance allele frequencies, the results represent essentially a point estimate for one or at best two populations in a given year. The ability to screen multiple populations with either of these techniques is limited by the time and labor expenses necessary to obtain single family lines. In contrast, diagnostic bioassays, although more limited in sensitivity of detection, have identified resistant individuals among field populations and have provided a continuous record of susceptibility across geographically distinct collection sites across multiple years.^{11,12,16}

Surveys of *Bt* cornfields for *O. nubilalis* damage, as well as the recessive nature of resistance among selected populations, support the high-dose nature of *Bt* corn against this pest. Annual surveys of *O. nubilalis* susceptibility and repeated attempts to select for resistance to Cry1Ab protein suggest a major allele that confers resistance to Cry1Ab is rare among field populations. In contrast, laboratory selection with the Cry1F protein has isolated an *O. nubilalis* strain that exhibits high resistance conferred by a single, recessive genetic factor. The results from the present investigation confirm that the frequency of Cry1F resistance is relatively high, and that the frequency was already high prior to introduction of the technology. However, there is no indication that the frequency of this resistance has increased, suggesting that the high-dose/refuge strategy may be robust enough to delay resistance evolution even when the frequency of resistance exceeds theoretical estimates of initial allele frequency.

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SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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