Theor Appl Genet (2003) 106:1225–1233
DOI 10.1007/s00122-002-1172-1

D. Bourguet · J. Chaufaux · M. Séguin · C. Buisson
J. L. Hinton · T. J. Stodola · P. Porter · G. Cronholm
L. L. Buschman · D. A. Andow

Frequency of alleles conferring resistance to Bt maize in French and US corn belt populations of the European corn borer, Ostrinia nubilalis

Received: 15 April 2002 / Accepted: 16 September 2002 / Published online: 10 December 2002

Abstract Farmers, industry, governments and environmental groups agree that it would be useful to manage transgenic crops producing insecticidal proteins to delay the evolution of resistance in target pests. The main strategy proposed for delaying resistance to *Bacillus thuringiensis* (*Bt*) toxins in transgenic crops is the high-dose/refuge strategy. This strategy is based on the unverified assumption that resistance alleles are initially rare ($<10^{-3}$). We used an F_2 screen on >1,200 isofemale lines of *Ostrinia nubilalis* Hübner (Lepidoptera: Crambidae) collected in France and the US corn belt during 1999–2001. In none of the isofemale lines did we detect alleles conferring resistance to *Bt* maize producing the Cry1Ab toxin. A Bayesian analysis of the data indicates that the frequency of resistance alleles in France was $<9.20 \times 10^{-4}$ with 95% probability, and a detection probability of >80%. In the northern US corn belt, the frequency of resistance to *Bt* maize was $<4.23 \times 10^{-4}$ with 95% probability, and a detection probability of >90%. Only 95 lines have been screened from the southern US corn belt, so these data are still inconclusive. These results suggest that resistance is probably rare enough in France and the northern US corn belt for the high-dose plus refuge strategy to delay resistance to *Bt* maize.

Keywords Transgenic crops · F_2 screen · *Ostrinia nubilalis* · Resistance management · Population genetics · Mutations

Communicated by D. Hoisington

D. Bourguet (✉) · J. Chaufaux · M. Séguin · C. Buisson
Unité de Recherches de Lutte biologique, INRA La Minière,
78 285 Guyancourt, France
e-mail: bourguet@jouy.inra.fr
Tel.: +33-1-30-83-36-55, Fax: +33-1-30-43-80-97

J. L. Hinton · T. J. Stodola · D. A. Andow
Department of Entomology, University of Minnesota, St. Paul,
MN 55108, USA

T. J. Stodola
Minnesota Center for Community Genetics,
University of Minnesota, USA

P. Porter · G. Cronholm
Department of Entomology, Texas A & M University,
College Station, TX, USA

L. L. Buschman
Department of Entomology, Kansas State University, Garden City,
KS 62846, USA

Introduction

Populations can adapt to a new environment by selection on standing genetic variation or by selection on new mutations (Orr and Betancourt 2001). When standing adaptive genetic variation exists, populations can respond immediately to sudden environmental change. This has been dramatically exemplified by the numerous failures of synthetic chemical insecticides against pest species (Georghiou and Lagunes-Tejeda 1991). Such alleles can occur in natural populations prior to any insecticide treatments via recurrent mutation (ffrench-Constant 1994; Andreev et al. 1999) or migration events (Raymond et al. 1991; Guillemaud et al. 1996).

The frequency of adaptive insecticide resistance alleles in unselected pest populations is currently a central question for the husbandry of genetically engineered crops producing *Bacillus thuringiensis* toxins (*Bt* crops) (Gould 1998). The rapid and extensive commercialisation of these *Bt* crops has magnified the risk that targeted insect pest species will rapidly adapt to this class of toxins (Gould 1998; Wolfenbarger and Phifer 2000). Globally, the estimated area planted to *Bt* crops for 2000 was 8.3 million hectares (James 2000) with 6.3 million hectares (76%) in the USA (NASS 2000). The risk of resistance led to the development of theoretical models to evaluate strategies to delay the evolution of resistance to *Bt* toxins in the target pests (e.g. Mallet and Porter 1992; Caprio 1998; Onstad and Gould 1998; Roush 1998; Peck et al. 1999). Among them the most-widely accepted strategy is the high-dose/refuge strategy (Alstad and Andow 1995), which is currently being implemented in

North America (Andow 2001). One of the key assumptions of the high-dose/refuge strategy is that alleles conferring resistance to *Bt* toxins are rare, i.e. a frequency $<10^{-3}$ (Roush 1994). Recently this value has been taken as a default when modelling the evolution of resistance to *Bt* toxins (e.g. Hurley et al. 1997; Onstad and Guse 1999).

Theoretically, the frequency of resistance alleles in unselected populations is expected to be low (Roush and McKenzie 1987). If the resistance alleles carry fitness costs in the absence of the toxin (Roush and McKenzie 1987; Coustau et al. 2000), they will equilibrate at a mutation-selection balance determined by the mutation rate (u), selection coefficient (s) and dominance (h) of the cost (Crow and Kimura 1970). Because for most mutations dominance is larger when the selection coefficient is smaller (Crow and Kimura 1970), the equilibrium frequency is largely determined by heterozygous effects and is approximately u/hs (Crow and Kimura 1970). If the per locus mutation rate is 10^{-6}–10^{-5}, the selection coefficient is 0.01–0.1 and dominance is sufficiently large, the equilibrium frequency will vary from 10^{-5} to 5×10^{-3}.

If instead the resistance alleles are selectively neutral in the absence of toxin (McKenzie 1996), and forward and back mutation rates are the same, they will drift to a stationary distribution of frequencies determined by the effective population size (equation 13.26, Wright 1969). Although it is possible that the frequency of resistance to some toxins could be quite high, such toxins are never commercialized, because pre-commercialization evaluations would indicate that the toxin had poor efficacy. Assuming that commercialization proceeds only when the frequency of resistant individuals is <0.01, for effective population sizes ranging from 10^3 to 10^6, the probability that a resistance allele has a frequency <0.001 is calculated to range from 0.36 to 0.71.

Measuring low frequencies of a recessive allele in field populations can be a significant logistical challenge. When the frequency of an allele conferring recessive resistance is $<10^{-3}$, more than 10^6 field-collected individuals must be screened to detect resistant individuals. Moreover, because resistance may be determined by several genetic mechanisms, a detection method should be able to detect any resistance mechanism. Because multigenic resistance is thought to evolve considerably more slowly than single-gene resistance (Crow and Kimura 1970), the method may focus on single-gene resistance. The F_2 screen used in this study improves the efficiency of measuring rare recessive alleles in natural populations, because 250 F_2 isofemale lines provide results similar to screening 10^6 naturally occurring phenotypes (Andow and Alstad 1998). Indeed, Schneider (1999) and Andow and Alstad (1999) determined that when progeny of about 750 isofemale lines are screened for susceptibility, it is possible to conclude with 95% confidence that the frequency of *Bt* resistance alleles is $<10^{-3}$. Moreover, this method allows us to calculate independently the type II statistical error, which is the probability that we have inadvertently failed to detect a resistance allele.

To-date, the assumption that resistance is rare has received little empirical support. Indeed, the few estimates on lepidopteran pest species suggest rather a high initial frequency of *Bt* resistance alleles (Gould et al. 1997; Tabashnik et al. 1997, 2000; but see Bentur et al. 2000 and below). The most dominant *Bt* crop world-wide is *Bt* maize, which produces either Cry1Ab, Cry1Ac, Cry1F or Cry9C. These toxins are active against the European corn borer (ECB), *Ostrinia nubilalis* (Hübner), which is the major lepidopteran pest of corn in North America and Europe (Kratigger 1997; Steffey et al. 1999). Andow et al. (1998, 2000) found no individuals of European corn borer (*Ostrinia nubilalis*) with alleles conferring resistance to *Bt* maize in about 280 lines from field populations of the US corn belt.

To ascertain that the frequency of major *Bt* resistance alleles in field populations of ECB are <0.001 we performed two intensive screenings in Southern France in 1999 and 2000, and additional screenings in the US during 1999–2001. Our data indicate that the frequency of *Bt* resistance alleles may be $<10^{-3}$ both in France and northern US and therefore sufficiently rare for the high-dose/refuge strategy to significantly delay resistance to transgenic *Bt* maize.

Materials and methods

F_2 screen

The F_2 screen is a four-step process (Andow et al. 1998). It can be conducted by: (1) sampling mated adult females from natural populations and establishing isofemale lines, (2) rearing and sib-mating F_1 progeny in each isofemale line, (3) screening F_2 neonates to evaluate susceptibility to *Bt* toxin, and (4) statistical analysis of the data. By sib-mating the F_1 generation, 1/16 of the F_2 larvae are expected to be homozygous for any resistance alleles that a field-collected female (or her mate) carried. Because each female carries at least four haplotypes (two of her own and two from her mate), each isofemale line enables the characterization of at least four alleles.

Moth collection and sib-mating

France

We collected two sets of *O. nubilalis* adults from the field. Sampling took place around Muret, which is 20 km south from Toulouse (Region Midi-Pyrénées). Moths were captured with insect nets during the 1st flight of 1999 and 2000. The first set was collected between 14 June to 19 June 1999 and the second set was collected a year later between 13 June and 15 June 2000. Each female was caged either alone or with one field-collected male. The purpose of caging females with males was to ensure mating of the few females that might not have already mated in the field. Fertile females produced one to five egg masses within the first 5 days after caging. Offspring from each isofemale line were reared on a maize flour-wheat germ-agar diet described by Poitout and Buès (1970) and modified by Gahukar (1975). This diet contains fumidil for controlling *Nosema* infection and benzoic acid as a preservative. For each line, the number of F_1 males and females which were sib-mated were recorded.

Table 1 The Bolin scale (Bolin 1998) is a modified Guthrie 1–9 corn damage scale (Guthrie et al. 1960). The scale allows more accurate and precise ratings of damage caused by first instar European corn borers on whorl-stage transgenic corn

Rating	Description
1	No feeding at all
1.5	Pinholes only, ≤2 leaves
1.75	Pinholes only, >2 leaves
2	≤2 shotholes on ≤2 leaves, no elongate lesions
2.5	>2 shotholes on ≤2 leaves, no elongate lesions
3	Shotholes on ≥3 leaves, no elongate lesions
4	Any elongate lesion/irregular lesion (<2.5 cm), but ≤2 leaves
5	>2 leaves with elongate/irregular lesions (<2.5 cm)
6	≤2 leaves with >2.5 cm long lesions
6.5	>2 leaves with >2.5 cm long lesions, but still <½ of the leaves on the plant
7	Long lesions (>2.5 cm) on ½ to 2/3 of the leaves on the plant
8	Long lesions on 2/3 of the leaves on the plant
9	Most leaves with long lesions (>2/3 of the plant); plant has a shredded appearance

United States

Adults were field collected at blacklight traps during the summers of 1999 and 2000 from nine localities. Samples of second flight corn borers were collected near Lamberton, Minn. (6 to 24 August 1999 and 11 to 28 August 2000), Rosemount, Minn. (17 to 20 August 1999 and 26 July to 15 August 2000), Becker, Minn. (25 to 26 August 1999), Beresford, S.D. (27 August 1999), Edmonson, Tex. (26 to 29 July 2000 and 17 to 26 July 2001), Garden City, Kan. (28 July to 4 August 2000 and 26 to 30 July 2001), and from a univoltine flight near Willmar, Minn. (30 June to 20 July 2000). The moths were shipped or carried to the lab and females were isolated in individual cages with a supply of water and adult diet (Leahy and Andow 1994). A female that had not yet laid an egg mass after 3 days were given a male.

Testing F_2 larvae

France

Egg masses produced by the sib-mated F_1 females were placed in plastic boxes with moist filter paper until emergence. For each isofemale line 118.36 ± 48.78 (1999) and 70.34 ± 45.46 (2000) F_2 neonates were tested for their susceptibility to the Cry1Ab toxin. During 1999, screenings were performed on a maize flour-wheat germ-agar diet surface-treated with 100 ng/cm^2 of Cry1Ab. Such a dose is about 5-times higher than the LC_{95} of several susceptible strains (J. C. unpublished results; Siegfried et al. 1995). Cry1Ab was obtained from the 407$^-$ sigK (spo$^-$) *cry1Ab B. thuringiensis* strain. This strain was a derivative of the wild-type *B. thuringiensis* strain 407 isolated by Lereclus et al. (1989) which expressed the *cry1Ab* gene described by Sanchis et al. (1998) without producing any spores. Consequently any differences in susceptibility among lines could be attributed to the Cry1Ab toxin. During 2000, screenings were performed directly on *Bt* maize (the Elgina hybrid expressing the Mon 810 transformation event and therefore producing a truncated Cry1Ab toxin). F_2 neonates were inoculated on leaves of at least two different *Bt* maize plants. Diets (1999) and *Bt* maize leaves (2000) were evaluated for surviving larvae at 5 days after inoculation. All lines with survivors were scored as potential positives, and these lines were propagated into the F_3 generation for potential re-testing. Whenever possible retained lines were re-screened using larvae from the F_3 or F_4 generation on treated diets (1999) and on *Bt* maize (2000).

United States

Newly laid F_2 egg masses were incubated at 25 °C until the head capsules blackened. At this stage, eggs will hatch within ½ a day. These egg masses were used to infest *Bt* maize plants to screen the neonates for resistance to the Cry1Ab toxin. For each isofemale line, 3,097 ± 2,328 (1999), 1,526 ± 877 (2000), and 2,579 ± 1,774 (2001) F_2 eggs were used to infest two different *Bt*-maize plants (Pioneer 36F30, expressing the Mon 810 transformation event, minimum 6-weeks old/4-leaf stage). Hatch rates were estimated after 2 days, and the plant was examined for signs of feeding damage after 7 days. Damaged plants were tested for expression of Cry1Ab toxin. Isofemale lines producing leaf damage ≥2.0 on the Bolin scale (Table 1, Bolin 1998) with holes ≥2 mm in diameter were considered to be potentially partially resistant, and these lines were re-screened on *Bt* maize during the F_3 or F_4 generation. The Bolin scale was designed to quantify more accurately and precisely damage caused by first instar European corn borers on whorl-stage *Bt* maize. Lines that produced damage ≥2.0 upon re-screening were considered partially resistant.

Statistical analysis

Expected allele frequencies were calculated using equation 1 from Andow and Alstad (1998). The 95% credibility intervals were calculated from equation 5 for major resistance alleles and from equation 7 for partial resistance alleles from Andow and Alstad (1999). Data were pooled when appropriate by sequentially re-calculating the prior distribution as described in Andow et al. (2000). For each line, detection probabilities were computed using the algorithm in Andow and Alstad (1998) that has been corrected (T. J. S. and D. A. A., unpublished).

Results

France

1999 families

Of the 750 females collected, 478 (64%) produced F_1 families. Of these 478 isofemale lines, 381 (80%) produced enough offspring to enable production of the sib-mated F_1. The other lines were lost because F_0 females did not produce enough eggs or because F_1 larvae died before pupation. In this latter case mortality was due to the development of insect pathogens (viruses or bacteria) or molds in the diet. Finally we were able to complete F_2 screening on 328 lines (43.7% of collected females). Over these 328 isofemale lines, F_1 family size was 16.63 ± 8.67 males and 14.14 ± 7.47 females. An average of 10.47 ± 6.93 F_2 neonates larvae per female were tested for their susceptibility to Cry1Ab. Mortality on untreated diet was below 5%. Seven lines (#24, #90,

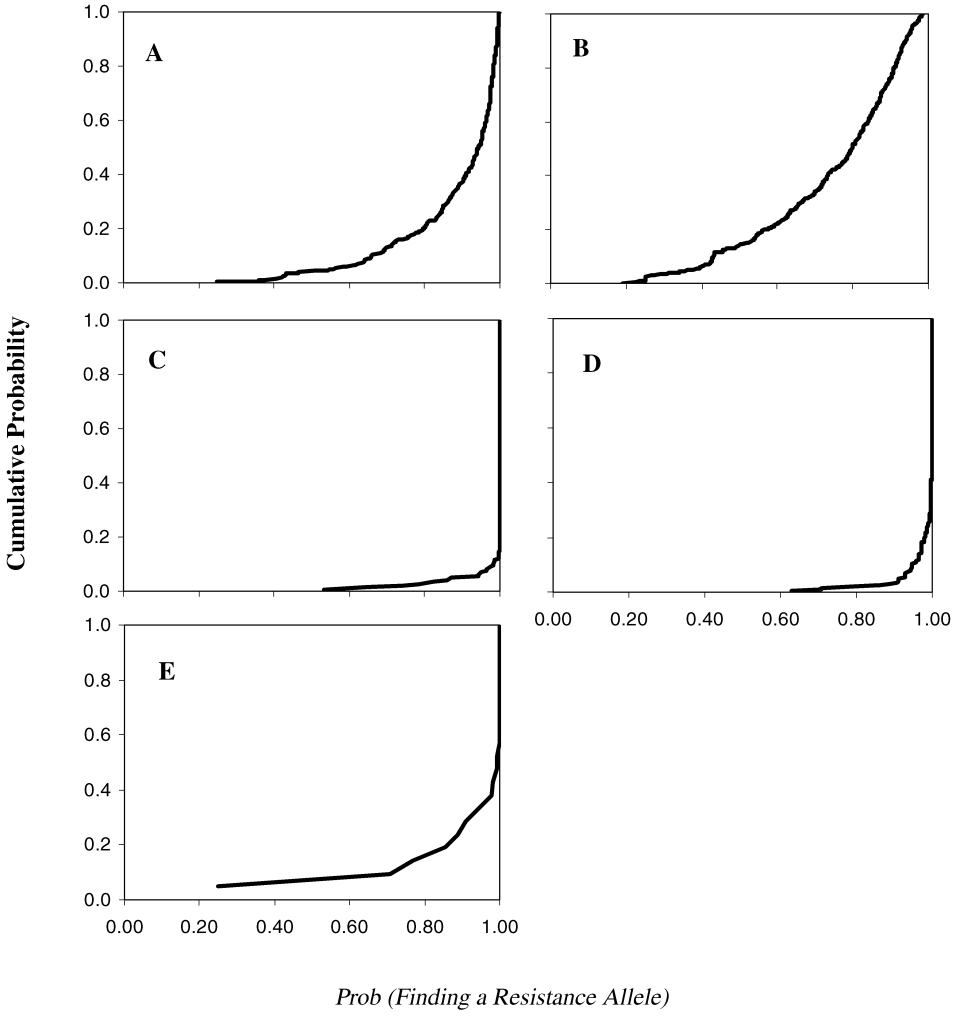

Fig. 1A–E Cumulative probabilities of detecting a resistance allele in an isofemale line if the line actually had a resistance allele. *A*-1999, France; *B*-2000, France; *C*-1999, USA; *D*-2000, USA; *E*-2001, USA

#164, #189, #238, #337 and #432) had F_2 larvae that survived 5 days on the treated diet, but none of these survivors reached pupation. Untreated larvae of two of these putative resistant lines (#24 and #164) laid eggs and were re-tested for Cry1Ab susceptibility at generation F_3 with 170 larvae (line #24) and F_4 using 114 larvae (line #164) on treated diet. None of them survived to the 2nd instar.

These results suggest that there were no major resistance alleles to Cry1Ab toxin among the isofemale lines we screened. The probability that we could detect a resistance allele in each of these family is shown in Fig. 1A. Approximately 48% of the lines had a detection of >95% and about 20% had a detection <80%. The experiment-wise detection probability was 88%. This result is comparable to the probability detection previously obtained by Andow et al. (2000).

2000 families

Of the 919 females collected, 629 (68%) produced F_1 progeny. This ratio was slightly higher than the one obtained from 1999 (64%) (2-tailed Fisher's exact test, $p < 0.05$). We did not find any difference between females caged alone or with one male (2-tailed Fisher's exact test, $p = 0.76$): 197 (68%) of the 291 females caged alone produced F_1 progeny and 432 (69%) of the 628 females caged with one male produced F_1 progeny. This suggests that most if not all the females captured either had already mated or were unwilling to mate or oviposit. Of the 629 lines, 603 (96%) produced enough offspring to enable production of the sib-mated F_1. The other lines were lost because F_0 females did not produce enough eggs or because F_1 larvae died before pupation. In this latter case mortality was due to the development of insect pathogens (viruses or bacteria) or molds in the diet. We were able to complete F_2 screening on 483 lines (52.6% of collected females). Over these 483 isofemale lines, F_1 family size was 10.78 ± 5.00 males and 8.49 ± 4.30 females. An average of 10.17 ± 9.11 F_2 neonatal larvae per female were tested on *Bt* maize producing Cry1Ab. Although none of the 483 lines fed extensively on *Bt* maize, five lines (#24, #46, #123, #173 and #311) had at least one larva still alive after 5 days and were considered putative resistant lines. These larvae were fed

2 more days on *Bt* maize and survivors were transferred to untreated diet. From these survivors a single larva reached pupation in lines #46, #123 and #173 and only the one from line #123 emerged as an adult female. This female was crossed with an untreated male from the same line. If the female was homozygous for an allele conferring major resistance to *Bt* maize, then her progeny would be expected to be either resistant homozygotes if the untreated male was also a resistant homozygote, 50% heterozygous/50% resistant homozygotes if this male was heterozygous, or 100% heterozygous if it was a susceptible homozygote. Very few larvae were obtained from this cross and only six offspring were tested on *Bt* maize. None of them survived after 4 days. The remaining untreated offspring from this cross did not yield any adults.

From untreated larvae we sib-mated the second generation of the five putative resistant lines (#24, #46, #123, #173 and #311) and of one susceptible line (#400). Only line #173 and the susceptible line provided F_3 larvae. These larvae were reared on untreated diet and the resulting adults were sib-mated to get F_4 larvae. These latter were tested for their susceptibility to *Bt* maize and for three doses of Cry1Ab on the diet (Table 2). None of the larvae survived on *Bt* maize after 5 days of exposure. However, compared with the susceptible line, larvae from line #173 showed a mortality level significantly lower at two of the three Cry1Ab doses tested (Table 2).

Although we detected putative resistant lines we did not identify any major resistance alleles in the second set of isofemale lines. The probability that we could detect a resistance allele in each of these families is shown in Fig. 1B. Overall we had lower detection probabilities compared with 1999: only 7.5% of the lines had a detection of >95% and approximately 49% of the lines had a detection of <80%. The experiment-wise detection probability was 0.74.

*Frequency of the major **Bt** resistance allele*

We observed $S = 0$ (the number of isofemale lines with a major *Bt* resistance) over the two sets of families we screened, giving estimates for major allele frequencies (Table 3). If we combine the two sets, $S = 0$ and $N = 811$ (total number of lines evaluated; 328 lines for 1999 and 483 lines for 2000), so $E[q] = 3.08 \times 10^{-4}$ with a 95% CI of $(0, 9.20 \times 10^{-4})$. In other words, the frequency of resistance is $<9.20 \times 10^{-4}$ with 95% probability. The detection probability associated with this estimate is 80%.

*Frequency of the partial **Bt** resistance allele*

In the first set performed in 1999 we had no evidence for partial resistance alleles. In the second set of lines the decreased mortality level observed in line #173 suggests that this line may have had an allele that conferred partial resistance to Cry1Ab. Assuming that the lines that were not re-tested did not have partial resistance, the results imply that $S = 1$ and $N = 811$. The expected frequency of alleles conferring partial resistance is $E[q] = 6.15 \times 10^{-4}$, with a 95% CI of $(7.46 \times 10^{-5}, 1.71 \times 10^{-3})$.

Table 2 Comparative mortality levels of F_4 larvae from isofemale line #173 (a putative resistant line) and line #400 (a susceptible line) from the 2000 families collected at Muret, France. The number of larvae tested is indicated in parentheses. Mortality levels were recorded after 5 days of exposure at different Cry1Ab doses and on *Bt* maize, and corrected for control mortality with Abbott's method

Dose of Cry1Ab	Isofemale lines		P[a]
	#400	#173	
11 ng/cm^2	84% (29)	2% (46)	***
58 ng/cm^2	96% (30)	80% (46)	*
289 ng/cm^2	100% (30)	98% (50)	NS
Bt maize	100% (12)	100% (700)	NS

[a] Type I error, 2-tailed Fisher's exact test: *** = $p < 0.0001$, * = $p < 0.05$, NS = non-significant

United States

1999 families

Two hundred and forty nine females were collected, 175 (70%) of these laid fertile eggs, 165 lines (94%) produced enough pupae to be sib-mated, and 146 lines (58.6% of collected females) were screened. Corn borer densities were much lower than normal during 1999 throughout the United States, making it difficult to acquire large numbers of adults. The 146 screened lines averaged 65.84 ± 38.15 F_1 males and 72.73 ± 42.56 F_1 females, with 42.58 ± 32.01 neonates per F_1 female screened in each isofemale line. Holes 2 mm or larger were observed on plants from two lines (#172 and #242) over two generations of testing. This was considered potential partial resistance. In no cases did a line produce larvae that survived to the second instar on *Bt* maize. This suggests that there were no major resistance alleles to the Cry1Ab toxin among these isofemale lines. The probability that we could detect a resistance allele if present in a line is shown in Fig. 1C. Approximately 93% of the lines screened had a >95% detection probability, and there was an experiment-wise detection probability of 98.6%. The precision in these results exceeds previously published work.

2000 families

Six hundred and forty five females were collected over the various field sites. Of these only 286 lines (44%) produced viable eggs, 285 lines were sib-mated, and 259 (40.2% of collected females) were screened on *Bt*-maize. Populations continued to be very low in

Table 3 Expected resistance allele frequency (E[q] for major resistance alleles and E[$q_{partial}$] for partial resistance alleles), with Bayesian 95% credibility intervals and experiment-wise detection probabilities for all natural populations of European corn borer studied in France and in the USA

Location	Year	Isofemale lines	E [q] (95% CI)	E[$q_{partial}$] (95% CI)	Experiment-wise detection probability
France					
Muret	1999	328	0.00076 (0–0.0022)	0.00076 (0–0.0022)	0.880
	2000	483	0.00050 (0–0.0015)	0.0010 (0.0001–0.0029)	0.741
USA					
Minnesota (Minn.)					
Becker	1999	7	0.0278 (0–0.078)	0.0278 (0–0.078)	0.965
Lamberton	1999	126	0.0020 (0–0.0058)	0.0040 (0.001–0.012)	0.993
	2000	36	0.0066 (0–0.019)	0.0066 (0–0.019)	0.968
Rosemount	1999	4	0.0417 (0–0.113)	0.0417 (0–0.113)	1.000
	2000	129	0.0019 (0–0.0057)	0.010 (0.003–0.019)	0.986
Willmar	2000	12	0.0179 (0–0.051)	0.0179 (0–0.051)	0.987
South Dakota (S.D.)					
Beresford	1999	9	0.0227 (0–0.065)	0.0450 (0.006–0.111)	0.898
Kansas (Kan.)					
Garden City	2000	56	0.0043 (0–0.013)	0.0043 (0–0.013)	0.993
	2001	6	0.0313 (0–0.087)	0.0625 (0.009–0.145)	0.933
Texas (Tex.)					
Edmonson	2000	18	0.0125 (0–0.036)	0.0250 (0.003–0.065)	0.957
	2001	15	0.0147 (0–0.042)	0.0147 (0–0.042)	0.911

northern United States, hampering collections. The F_1 families of the screened lines had a mean of 26.28 ± 14.43 males and 29.20 ± 17.17 females, and 52.26 ± 30.03 F_2 neonates per F_1 female were screened per isofemale line. Five lines caused damage ≥2.0 on the Bolin scale for two generations, indicating potential partial resistance. No second instar larvae were recovered, indicating that there were no major resistance alleles in these isofemale lines. Figure 1D shows the probability that we would detect a resistant allele if one were present in a line. Approximately 72% of the lines screened had a >95% detection probability, and the experiment-wise detection probability was 98.3%, which was similar to our 1999 results.

2001 families

One hundred and forty nine females were collected from field sites near Garden City and Edmunson. Of these, 27 lines (18%) producted viable eggs, 26 were sibmated, and 21 (14% of collected females) were screened on *Bt*-maize. The F_1 families averaged 21.32 ± 7.52 males and 20.60 ± 7.15 females, and 118.14 ± 65.27 F_2 larvae were screened per F_1 female. One line (#97) caused damage ≥2.0 on the Bolin scale for two generations indicating potential partial resistance. However no second instar larvae were recovered, indicating no major resistance alleles were present in these isofemale lines. The probability of detecting a resistance allele if one were present in the line is plotted in Fig. 1E. On average the experiment-wise detection probability was 91.7%.

*Frequency of the major **Bt** resistance allele*

We observed $S = 0$ (the number of isofemale lines with a major *Bt* resistance) over all of the samples we screened, giving several estimates of major allele frequencies (Table 3) for various localities. If we combine the Rosemount populations over years, $N = 133$, so E[q] = 1.8×10^{-3} with a 95% CI of (0, 5.5×10^{-3}). In other words, the frequency of resistance is $<5.5 \times 10^{-3}$ with 95% probability with a detection probability of 98.6%. Similarly, if we combine the Lamberton and Beresford data, because they are in the same geographic area of southwestern Minnesota and south eastern South Dakota, $N = 171$, so E[q] = 1.4×10^{-3} with a 95% CI of (0, 4.3×10^{-3}). In other words, the frequency of resistance is $<4.3 \times 10^{-3}$ with 95% probability with a detection probability of 98.3%. Finally, the Willmar and Becker sites are close together in central Minnesota, so $N = 19$, and E[q] = 1.2×10^{-2} with a 95% CI of (0, 3.5×10^{-2}). In other words, the frequency of resistance is $<3.5 \times 10^{-2}$ with 95% probability with a detection probability of 97.9%.

*Frequency of the partial **Bt** resistance allele*

During 1999, we confirmed partial resistance in one line from Lamberton and one line from Beresford, and during 2000, we found partial resistance in four lines from Rosemount and one line from Edmonson (Table 3). Combining the Rosemount populations over years, $N = 133$ and $S = 4$, so E[q] = 9.3×10^{-3} with a 95% CI of (3.1×10^{-3}, 1.9×10^{-2}). Combining the Lamberton and Beresford data, $N = 171$ and $S = 2$, so E[q] = 4.3×10^{-3}

with a 95% CI of (9.0×10^{-4}, 1.0×10^{-2}). Finally, combining the Willmar and Becker data, $N = 19$ and $S = 0$, so $E[q] = 1.2 \times 10^{-2}$ with a 95% CI of (0, 3.5×10^{-2}). Partial resistance alleles appear to be relatively common throughout the United States.

Discussion

Farmers, industry, governments, and environmental groups agree that it would be useful to manage transgenic crops producing insecticidal proteins to delay the evolution of resistance in target pests (Andow 2001). In the United States, the Environmental Protection Agency regulations required *Bt* maize to be grown with a 20% non-Bt maize refuge as part of an insect resistance management (IRM) strategy to delay the appearance of resistance in the European corn borer (EPA 1998). In Europe, the Scientific Committee on Plants has also recommended the use of non-*Bt* maize refuges (SCP 1999). This strategy, called the high-dose/refuge strategy (Alstad and Andow 1995), relies on three key assumptions: (1) *Bt* maize must be very toxic so that the gain of fitness conferred by resistance alleles is recessive (Tabashnik and Croft 1982; Bourguet et al. 2000a); (2) resistance alleles must be sufficiently rare (Roush 1998); and (3) refuges must be interspersed among the *Bt* maize fields so that susceptible and resistant insects will intermate (Comins 1977). Assumption (3) may be met by a combination of interplanting of the refuge within ½ mile of any *Bt* maize and by the scale of dispersal of the European corn borer (Andow 2001). Assumption (1) cannot be scientifically evaluated until resistance is recovered. Here we have evaluated assumption (2), that resistance is initially rare in *O. nubilalis*.

Although alleles conferring partial resistance are segregating in most populations studied, none of the isofemale lines were able survive on *Bt* maize. Based on the present data the frequency of resistance alleles in France was less than 9.20×10^{-4} with 95% probability and with a detection probability of 80%. Bourguet et al. (2000b) observed little genetic differentiation among populations of European corn borer from northern to southern France which may therefore constitute a single large panmictic unit. Consequently, the observed frequency of resistance may be characteristic of the entire *O. nubilalis* population in France. More extensive studies on the differentiation of European corn borer populations throughout Europe have not been done, so it is not yet possible to make inferences on the frequency of resistance in other parts of Europe.

The geographic distribution of the ECB in North America is extensive, but studies on its population genetic structure have not yet been published. In the absence of published data we grouped several of the US samples collected in nearby geographical areas together as potential panmictic units. The Becker and Willmar, Minn. samples can be combined into one central Minnesota sample, the Lamberton, Minn. and Beresford, S.D. populations can be combined into one southwestern Minnesota sample, and the Rosemount, Minn. sample can be combined with results on 91 isofemale lines from LeSeuer, Minn. (Andow et al. 1998) into a southeastern Minnesota sample. The Kansas and Texas samples are over 450 km apart, so these could be treated as samples from separate populations. The central Minnesota sample gives a frequency estimate of $<3.5 \times 10^{-2}$ with 95% probability. The southwestern Minnesota sample gives a frequency estimate of $<4.3 \times 10^{-3}$ with 95% probability. The southeastern Minnesota sample gives a frequency estimate of $<5.5 \times 10^{-3}$ with 95% probability. The Texas sample gives a frequency estimate of $<2.1 \times 10^{-2}$ with 95% probability, and the Kansas sample gives an estimate of $<1.2 \times 10^{-2}$ with 95% probability. If instead, we consider the entire corn belt region north of central Iowa as a single population of European corn borer, we would combine all of the Minnesota and South Dakota samples along with the 91 lines from LeSeuer, Minn. (Andow et al. 1998), and the 175 lines from Ames, Iowa (Andow et al. 2000). The frequency estimate for the northern corn belt was $<4.23 \times 10^{-4}$ with 95% probability and with a detection probability of 90%. And if one would consider the Kansas and Texas populations to be part of a single southern population, then the frequency estimate for this population of 95 lines would be $<7.7 \times 10^{-3}$ with a detection probability of 97%.

These results differ from the high initial frequencies found in *Heliothis virescens* (Gould et al. 1997) and *Pectinophora gossypiella* (Tabashnik et al. 2000). Our estimates on *O. nubilalis* suggest that *Bt* resistance is probably rare enough in France and in the northern US corn belt for the high-dose plus refuge strategy to delay resistance. However, uncertainties associated with implementation may affect its success. Will farmers plant large enough refuges close enough to *Bt* maize? In the United States, the available data are both encouraging and discouraging. The National Corn Growers Association, Biotechnology (website: http://www.ncga.com/biotechnology/main/index.html), reported that 70% of USA growers are complying with the refuge guidelines, and only 30% are not complying. If the compliance rate can be increased and resistance is recessive, then IRM for *Bt* maize has a high probability of success in northern USA.

Acknowledgements We thank M. Gohar, F. Gould, D. Heckel, D. Lereclus, C. Nielsen-LeRoux, B. Tabashnik and one anonymous reviewer for comments on this manuscript. We also thank Marie-Thérèse Bethenod, Magali Calabre, Marc Delos and Gaël Magnanon for helping in sampling and in carrying out the screens in France, and Justin Duris, Mark Galatowicz, Faith Hassinger, Beth Peterson, Jacob Roark, Scott Smith and Eleanore Wesserte for helping in sampling and carrying out the Minnesota screens. We finally thank Vincent Sanchis for providing the *B. thuringiensis* strain, Michael Catangui for helping us locate a sampling site in South Dakota and Bruce Potter and Lee French for helping with the blacklight traps at Lamberton, Minnesota. This work was supported by the INRA AIP "Organismes Génétiquement Modifés et Environnement" and by the MENRT A.O. "Impact des OGM" to D.B., and USDA-NRI Grant 99-35302-7937 to D.A.A. and W. D. Hutchison.

References

Alstad DN, Andow DA (1995) Managing the evolution of insect resistance to transgenic plants. Science 268:1894–1896

Andreev D, Kreitman M, Phillips TW, Beeman RW, ffrench-Constant RH (1999) Multiple origins of cyclodiene insecticide resistance in *Tribolium castaneum* (Coleoptera: Tenebrionidae). J Mol Evol 48:615–624

Andow DA (2001) Resisting resistance to *Bt* corn. In: Letourneau DK, Burrows BE (eds) Genetically engineered organisms: assessing environmental and human health effects. CRC Press, Boca Raton, Florida, pp 99–124

Andow DA, Alstad DN (1998) F2 screen for rare resistance alleles. J Econ Entomol 9:572–578

Andow DA, Alstad DN (1999) Credibility interval for rare resistance allele frequencies. J Econ Entomol 92:755–758

Andow DA, Alstad DN, Pang Y-H, Bolin PC, Hutchinson WD (1998) Using an F2 screen to search for resistance alleles to *Bacillus thuringiensis* toxin in European corn borer (Lepidoptera: Crambidae). J Econ Entomol 91:579–584

Andow DA, Olson DM, Hellmich II RL, Alstad DN, Hutchinson WD (2000) Frequency of resistance alleles to *Bacillus thuringiensis* toxin in an Iowa population of European corn borer. J Econ Entomol 93:26–30

Bolin PC (1998) Durability of *Bacillus thuringiensis* transgenic corn for management of the European corn borer (Lepidoptera: Crambidae). PhD thesis, University of Minnesota, St. Paul, Minnesota

Bourguet D, Génissel A, Raymond M (2000a) Insecticide resistance and dominance levels. J Econ Entomol 93:1588–1595

Bourguet D, Bethenod M-T, Pasteur N, Viard F (2000b) Gene flow in the European corn borer *Ostrinia nubilalis*: implications for the sustainability of transgenic insecticidal maize. Proc R Soc Lond B 267:117–122

Bentur JS, Andow DA, Cohen MB, Romena AM, Gould F (2000) Frequency of alleles conferring resistance to a *Bacillus thuringiensis* toxin in a Philippines population of *Scirpophaga incertulas* (Lepidoptera: Pyralidae). J Econ Entomol 93:1515–1521

Caprio MA (1998) Evaluating resistance management strategies for multiple toxins in the presence of external refuges. J Econ Entomol 91:1021–1031

Comins HN (1977) The management of pesticide resistance. J Theor Biol 65:399–420

Coustau C, Chevillon C, ffrench-constant RH (2000) Resistance to xenobiotics and parasites: can we count the cost? Trends Ecol Evol 15:378–383

Crow JF, Kimura M (1970) An introduction to population genetics theory. Harper and Row, New York

Environmental Protection Agency (EPA) (1998) The Environmental Agency's White Paper on *Bt* Plant-Resistance Management, 14 January 1998. US EPA, Washington, District of Columbia

ffrench-Constant RH (1994) The molecular and population genetics of cyclodiene resistance. Insect Biochem Mol Biol 24:335–345

Gahukar RT (1975) Nouvelles techniques adoptées pour l'élevage d'*Ostrinia nubilalis* Hübner sur milieu artificiel. Ann Zool Ecol Anim 7:491–498

Georghiou GP, Lagunes-Tejeda A (1991) The occurrence of resistance to pesticides in arthropods. Food and Agriculture Organization, Rome

Gould F (1998) Sustainability of transgenic insecticidal cultivars: integrating pest genetics and ecology. Annu Rev Entomol 43:701–726

Gould F, Anderson A, Jones A, Sumerford D, Heckel DG, Lopez J, Micinski S, Leonard R, Laster M (1997) Initial frequency of alleles for resistance to *Bacillus thuringiensis* toxins in field populations of *Heliothis virescens*. Proc Natl Acad Sci USA 94:3519–3523

Guillemaud T, Rooker S, Pasteur N, Raymond M (1996) Testing the unique amplification event and the worldwide migration hypothesis of insecticide resistance genes with sequence data. Heredity 77:535–543

Guthrie WD, Dicke FF, Neiswander CR (1960) Leaf sheath feeding resistance to the European corn borer in eight inbred lines of dent corn. Ohio Agric Exp Stn Res Bull 860

Hurley TM, Babcock BA, Hellmich II RL (1997) Biotechnology and pest resistance: an economic assessment of refuges. 97-W, Center for Agricultural and Rural Development, Ames, Iowa

James C (2000) Global status of commercialized transgenic crops: 2000. ISAAA Briefs No. 21: Preview. ISAAA, Ithaca, New York

Krattiger AF (1997) Insect resistance in crops: a case study of *Bacillus thuringiensis* (Bt) and its transfer to developing countries. ISAAA Briefs No. 2. ISAAA, Ithaca, New York

Leahy TC, Andow DA (1994) Egg weight, frcundity, and longevity are increased by adult feeding in Ostrinia nubilalis (Lepidoptera: Pyralidae). Ann Entomol Soc Am 87:342–349

Lereclus D, Arantes O, Chaufaux J, Lecadet MM (1989) Transformation and expression of a cloned delta-endotoxin gene in *Bacillus thuringiensis*. FEMS Microbiol Lett 60:211–218

McKenzie JA (1996) Ecological and evolutionary aspects of insecticide resistance. R.G. Landes, Georgetown, Texas

Mallet J, Porter P (1992) Preventing insect adaptation to insect-resistant crops: are seed mixtures or refugia the best strategy? Proc Roy Soc Lond B 250:165–169

National Agricultural Statistical Service (NASS) (2000) Prospective plantings, March 31, 2000. NASS, Agricultural Statistics Board, USDA, Washington, District of Columbia

Onstad DW, Gould F (1998) Modelling the dynamics of adaptation to transgenic maize by European corn borer (Lepidoptera: Pyralidae). J Econ Entomol 91:585–593

Onstad DW, Guse CA (1999) Economic analysis of transgenic maize and nontransgenic refuges for managing European corn borer (Lepidoptera: Pyralidae). J Econ Entomol 92:1256–1265

Orr HA, Betancourt AJ (2001) Haldane's sieve and adaptation from the standing genetic variation. Genetics 157:875–884

Peck SL, Gould F, Ellner SP (1999) Spread of resistance in spatially extended regions of transgenic cotton: implications for management of *Heliothis virescens*. J Econ Entomol 92:1–16

Poitout S, Buès R (1970) Elevage de plusieurs espèces de Lépidoptères Noctuidae sur milieu artificiel riche et sur milieu artificiel simplifié. Ann Zool Ecol Anim 2:79–91

Raymond M, Callaghan A, Fort P, Pasteur N (1991) Worldwide migration of amplified insecticide resistance genes in mosquitoes. Nature 350:151–153

Roush RT (1994) Managing pests and their resistance to *Bacillus thuringiensis*: can transgenic crops be better than sprays? Biocontrol Sci Tech 4:501–516

Roush RT (1998) Two-toxin strategies for management of insecticidal transgenic crops: can pyramiding succeed where pesticide mixtures have not? Phil Trans R Soc Lond B 353:1777–1786

Roush RT, McKenzie JA (1987) Ecological genetics of insecticide and acaracide resistance. Annu Rev Entomol 32:361–380

Sanchis V, Lereclus D, Menou G, Chaufaux J, Lecadet MM (1988) Multiplicity of delta-endotoxin genes with different insecticidal specificities in *Bacillus thuringiensis*. Mol Microbiol 2:393–404

Scientific Committee on Plants (SCP) (1999) Opinion of the SCP on *Bt* – Resistance monitoring opinion expressed on 4 March 1999

Schneider JC (1999) Confidence interval for Bayesian estimates of resistance allele frequencies. J Econ Entomol 92:755

Siegfried BD, Marçon PCRG, Witkowski JF, Wright RJ, Warren GW (1995) Susceptibility of field populations of the European corn borer, *Ostrinia nubilalis* (Hübner) (Lepidoptera: Pyralidae), to *Bacillus thuringiensis* (Berliner). J Agric Entomol 12:267–273

Steffey KL, Rice ME, All J, Andow DA, Gray ME, Van Duyn JW (eds) (1999) Handbook of corn insects. Entomological Society of America, Lanham, Maryland

Tabashnik BE, Croft BA (1982) Managing pesticide resistance in crop-arthropod complexes: interactions between biological and operational factors. Environ Entomol 11:1137–1144

Tabashnik BE, Liu Y-B, Finson N, Masson L, Heckel DG (1997) One gene in diamondback moth confers resistance to four *Bacillus thuringiensis* toxins. Proc Natl Acad Sci USA 94:1640–1644

Tabashnik BE, Patin AL, Dennehy TJ, Liu Y-B, Carrière Y, Sims MA, Antilla L (2000) Frequency of resistance to *Bacillus thuringiensis* in field populations of pink bollworm. Proc Natl Acad Sci USA 97:12,980–12,984

Wolfenbarger LL, Phifer PR (2000) Biotechnology and ecology – The ecological risks and benefits of genetically engineered plants. Science 290:2088–2093

Wright S (1969) Evolution and the genetics of populations, volume 2. The theory of gene frequencies. University Chicago Press, Chicago