FORUM

Examination of the F_2 Screen for Rare Resistance Alleles to *Bacillus thuringiensis* Toxins in the Diamondback Moth (Lepidoptera: Plutellidae)

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ABSTRACT A synthetic laboratory population of the diamondback moth, *Plutella xylostella* (L.), was used to test the F_2 screen developed for detecting the frequency of rare resistance alleles to Cry1Ac and Cry1C toxins of *Bacillus thuringiensis* (Bt). Of the 120 single-pair matings set up, 106 produced enough F_2 families for screening of Cry1Ac or Cry1C resistance alleles using both transgenic broccoli and an artificial diet overlay assay with a diagnostic dose. When using Bt broccoli plants as the F_2 screen method, only one F_2 family was detected for Cry1Ac resistance and no family was detected for Cry1C resistance. Six families were detected for either Cry1Ac or Cry1C resistance using the diet assay. The survivors in the diagnostic diet assay were crossed with the resistant individuals to confirm their resistance genotypes. Four F_2 families were confirmed to contain one copy of an allele resistant to Cry1Ac in the original single-pairs and four other F_2 families contained an allele resistant to Cry1C. Our results suggest that using transgenic plants expressing a high level of a Bt toxin in an F_2 screen may underestimate the frequency of resistance alleles with high false negatives, or fail to detect true resistance alleles. The diagnostic diet assay was a better F_2 screen method to detect alleles, especially for the Cry1Ac resistance with monogenic inheritance in the diamondback moth. The estimated probabilities of false positives and false negatives were 33 and 1%, respectively, for detecting Cry1Ac resistance at the allele frequency of 0.012 using the diagnostic diet assay. Careful validation of the screening method for each insect-crop system is necessary before the F_2 screen can be used to detect rare Bt resistance alleles in field populations.

KEY WORDS *Plutella xylostella*, *Bacillus thuringiensis*, resistance, transgenic, second-generation screen

PROTEINS PRODUCED BY a common bacterium, *Bacillus thuringiensis* (Bt), and expressed in transgenic plants to protect them from insect attack are revolutionizing agriculture (Roush and Shelton 1997). Three transgenic Bt insecticidal crops (corn, cotton, and potato) have been approved for commercial use in the United States since 1996, and the use of Bt corn and Bt cotton has increased dramatically. More than 20 million acres of Bt transgenic crops were planted in the United States in 1999 and 2000 (Carpenter and Gianessi 2001). With the increased area of Bt crops, there is concern that insects will evolve resistance to Bt proteins expressed in the transgenic plants (Gould 1998). Success of a high dose-refuge strategy, the only strategy currently used for resistance management of Bt transgenic crops, requires that the inheritance of resistance is mostly recessive and the frequency of resistant alleles is low (Andow et al. 1998). Because of limitations of both the dose-mortality analyses and diagnostic-dose method normally used for resistance monitoring (Roush and Miller 1986, Caprio et al. 2000), other approaches to detect rare resistance alleles have been proposed (Gould et al. 1997, Andow and Alstad 1998).

The F_2 screen was designed to estimate the frequency of rare resistance alleles in natural populations (Andow and Alstad 1998). This method involves collecting a large number of gravid individuals from the field and establishing single-female family lines. The F_1 offspring of each collected female are inbred within family lines. The F_2 offspring are then screened to detect any homozygous resistant individuals. In theory, $\approx 1/16$ of the F_2 larvae will be homozygous resistant (RR genotype) for families beginning with one resistance allele from the grandparents in the F_2 screen (Andow and Alstad 1998). Through back-calculation of the frequency of resistance-allele carrying family lines, the frequency of the resistance allele in the sampled population can be estimated. The F_2 screen has been used in the European corn borer (*Ostrinia nubilalis*) (Andow et al. 1998, 2000) and the rice stem borer (*Scirpophaga incertulas*) (Bentur et al. 2000) for detecting resistance to Bt transgenic crops. In neither case was an allele conferring a high level of resistance to Bt crops detected in isofemale lines up to

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188 (for *O. nubilalis*) or >450 (for *S. incertulas*), although low frequencies of 'partial resistance' alleles were detected in both insects (Andow et al. 2000, Bentur et al. 2000). It was suggested that in many cases, the F_2 screen accompanied by field screening could be very effective for detecting low frequencies of recessive and dominant resistance alleles, but there is a need to validate the F_2 screen by use of laboratory colonies with known frequencies of resistance alleles (EPA 2001a). More recently, the renewed registrations of Bt corn products for 7 yr are conditioned on the registrants carrying out appropriate programs, including to detect the emergence of insect resistance as early as possible (EPA 2001b). Several measures, including use of the F_2 screen, were recommended for resistance monitoring.

There have been no cases of control failures due to resistance to Bt transgenic plants in the field, but the diamondback moth, *Plutella xylostella* (L.), developed resistance to Bt toxins in foliar sprays under field conditions (Tabashnik et al. 1990, Shelton et al. 1993). Laboratory populations of Bt-resistant *P. xylostella* derived from different field populations after field exposures to different Bt toxins can also survive on transgenic crucifers expressing a high level of Cry1Ac (Metz et al. 1995, Ramachandran et al. 1998, Tang et al. 1999) and Cry1C (Zhao et al. 2000). An autosomal recessive gene in *P. xylostella* was shown to confer high levels of resistance to four Bt toxins: Cry1Aa, Cry1Ab, Cry1Ac, and Cry1F (Tabashnik et al. 1997). The Cry1C resistance was autosomally inherited and incompletely recessive when evaluated using a leaf dip assay, and recessive when using Cry1C transgenic broccoli (Zhao at el. 2000). In indirect tests of inheritance, the monogenic model provided a better fit than either the two loci or five loci model. However, it was shown that reduced binding was not the major mechanism of resistance to Cry1C, suggesting that there may be other mechanism(s) responsible for the Cry1C resistance. The resistance to Cry1C was inherited independently from resistance to Cry1Ab (Liu and Tabashnik 1997), which was controlled by the same gene for Cry1Ac resistance (Tabashnik et al. 1997); and it was not cross-resistant to Cry1A protoxins (Cry1Aa, Cry1Ab, Cry1Ac) (Zhao at el. 2001).

The objective of this research was to test the F_2 screen by using a synthetic population of *P. xylostella* with known low frequencies of Cry1Ac and Cry1C resistance alleles. We examined the F_2 screen using transgenic broccoli plants expressing a high level of either Cry1Ac or Cry1C and an artificial diet overlay assay of each toxin at a discriminating dose.

Materials and Methods

Transgenic Broccoli Expressing Cry1Ac or Cry1C Toxin. Cytoplasmic male sterile broccoli (*Brassica oleracea* ssp. *italica*) was transformed with a full length synthetic *cry1Ac* gene of Bt (Metz et al. 1995). Progeny were produced by pollinating transformed plants with 'Green Comet' hybrid broccoli. Toxin expression in the progeny was verified by screening the plants with *P. xylostella* neonates when plants were 4–5 wk old (Tang et al. 2001). Only those plants on which the neonates showed 0% survival were categorized as high expressing and were used as Bt plants in the tests. These Bt plants could also kill 100% of the neonates of RS F_1 heterozygotes (susceptible × Cry1Ac resistant strain) (Tang et al. 2001).

A synthetic truncated *cry1C* gene (1.9 kb long) was introduced into broccoli (Green Comet hybrid) by Cao et al. (1999). The Cry1C protein in the leaves of transgenic broccoli plants used (lines H12 and H14) was ≈ 0.4% of total soluble protein (Cao et al. 1999). These Bt plants also killed 100% of all instars of RS F_1 heterozygotes (susceptible × Cry1C resistant strain) (Zhao et al. 2000).

***Bacillus thuringiensis* Toxins.** Liquid formulations of Cry1Ac (MVP, 10%) and Cry1C (M-C, 15%) protoxins expressed in and encapsulated by transgenic *Pseudomonas fluorescens* (Mycogen, San Diego, CA) were used for bioassays.

Insects. Three strains of *P. xylostella* were used for crosses and to obtain a synthetic population with known allele frequencies for resistance to Cry1Ac and Cry1C. The susceptible Geneva 88 strain (S) was collected in 1988 from cabbage at the New York State Agricultural Experiment Station, Robbins Farm, Geneva, NY, and has been maintained on a wheat germ-casein artificial diet (Shelton et al. 1991) for >200 generations. While on diet, the strain was kept in an environmental chamber at 27 ± 1°C, 50 ± 2% RH, and a photoperiod of 16:8 (L:D) h. The Cry1Ac resistant strain (Cry1Ac-R) was collected in 1994 from crucifer fields in Loxahatchee, FL, after extensive field exposure to Bt formulations, and reared on Cry1Ac-expressing broccoli (Metz et al. 1995) for >75 generations. This strain was susceptible to Cry1C-expressing broccoli (Cao et al. 1999). The resistance to Cry1Ac in this strain was incompletely recessive inherited and probably controlled by a single allele (Tang et al. 1997). The Cry1C resistant strain (Cry1C-R) was originally collected in 1997 from a collard field in Lexington, SC, and, by using a cabbage leaf-dip bioassay, was determined to have a 31-fold resistance to Cry1C before the selection (Zhao et al. 2000). This population was selected for resistance to Cry1C protoxin using leaf dip assays for the initial 13 generations of selection. Leaves or plants of Cry1C-transgenic broccoli were used for selection in the next 13 generations. The Cry1C selected colony was then crossed to the S strain and then backcrossed to S strain. The offspring of the backcross were selected again using either Cry1C protoxin in leaf dip assays or Cry1C-transgenic plants for 10 generations. The resulting Cry1C resistant strain (Cry1C-R) was evaluated using a leaf dip assay and found to be 480-fold resistant to Cry1C protoxin, but susceptible to Cry1Ac (Zhao et al. 2001). The survival of the susceptible laboratory colony on nontransgenic broccoli (85% after 3 d for neonates) was not significantly different than the Cry1C-R strain (Zhao et al. 2001) or the Cry1Ac-R strain (J.-Z.Z., unpublished data).

Table 1. Detection of RR genotype of *P. xylostella* in populations with known resistant genotypes for Cry1Ac resistance using two bioassay methods

Strains or crosses	Expected genotypes of offspring[a]	Expected % mortality	χ^2 test for broccoli bioassay			χ^2 test for diet bioassay		
			% Mortality[b] (SEM)	χ^2	P	% Mortality[b] (SEM)	χ^2	P
S	SS	100	100 (0)	0.0	0.99	100 (0)	0.0	0.99
R	RR	0	12 (3)	4.1	<0.05	17 (4)	30.3	<0.01
R × S (F_1)	RS	100	100 (0)	0.0	0.99	100 (0)	0.0	0.99
RS × RS (F_2)	1/4 RR,1/2RS,1/4 SS	75	91 (2)	41.0	<0.01	89 (2)	16.1	<0.01
$(1/2RS+1/2SS)^2$ (BC × BC)	1/16RR,3/8RS,9/16SS	94	98 (1)	5.7	<0.05	98 (1)	5.7	<0.05

[a] Expected genotypes of offsprings if monogenic inheritance.
[b] Corrected mortality relative to control.

Bioassay Methods. We used two screening methods to detect resistant individuals of *P. xylostella* in different strains or in progeny of various crosses. Transgenic broccoli plants grown in the greenhouse were used in one of the bioassay methods and nontransgenic broccoli (Green Comet hybrid) was used as a control. Third or fourth expanded leaves from the top of 8- to 12-wk-old broccoli plants were individually put into 30-ml plastic cups (Polar Plastics, Winston-Salem, NC). Twenty to 50 *P. xylostella* neonates were transferred into each cup for different resistance genotypes and in different broccoli treatments. Cups were covered with lids and held at 27 ± 1°C, 50 ± 2% RH, and a photoperiod of 16:8 (L:D) h for 3 d to determine mortality.

The second screening method was an artificial diet overlay assay similar to Mascarenhas et al. (1998) and Siegfried et al. (2000). Liquid diet (5 ml per cup) (Shelton et al. 1991) was poured into 30-ml plastic cups and allowed to cool. Solutions of Cry1Ac or Cry1C protoxins were prepared in 0.1% Triton-X100 to obtain uniform spreading of the Bt solution on the diet surface and 0.2 ml of Cry1Ac or Cry1C solutions were applied to and distributed over the diet surface, in each cup. Control treatments consisted of diet in cups treated with 0.2 ml of 0.1% Triton-X100. Cups were allowed to air dry for 1.5–2 h before infesting with the same number of neonates as in Bt plants assays. Cups with neonates were held for 7 d under the same conditions as the broccoli assay before assessing mortality. Preliminary assays showed 100% mortality of neonates of Geneva 88 at 1.0 ng/ml of Cry1Ac or Cry1C. We used 10 ng/ml for Cry1Ac and 1.0 ng/ml for Cry1C as discriminating concentrations since they caused >99% mortality of the heterozygous RS genotype for Cry1Ac or Cry1C resistance and 100% mortality of the SS genotype (J. -Z. Z. and A. M. S., unpublished data).

Tests of Bioassay Methods to Detect RR Genotypes of Cry1Ac and Cry1C Resistance. To obtain the appropriate populations with different resistance genotypes, the following crosses were made: F_1, S (female) × Cry1Ac-R or Cry1C-R; backcross (BC), S (female) × F_1; and F_2, F_1 × F_1. At least 30 males and 30 females were used for each cross. The susceptible, resistant, F_1, F_2, and BC×BC strains or crosses for both the Cry1Ac and Cry1C resistance were used to validate the bioassay methods to be used for the F_2 screening. Neonates of each strain or cross were infested in cups with broccoli leaves or with treated diet. A total of 100–200 neonates (four or five replicates and 20–50 neonates per replicate) were used for most treatments of Bt broccoli and diet assays. There were 40 and 60 neonates for each broccoli and diet control with three or four replicates. Larvae that had not grown beyond first instar in the diet assay were considered to be dead. The corrected mortality was calculated using the formula of Abbott (1925).

F_2 Screen for Rare Resistance Alleles in a Synthetic Population. The synthetic population contained ≈3% each of Cry1Ac and Cry1C resistance alleles. It was created by releasing 30 moths of RS F_1 of Cry1Ac resistance (1:1 for male:female) and 60 moths of RS F_1 of Cry1C resistance into a cage containing 410 moths of S strain. The major reason for a higher number of RS genotype of Cry1C resistance was the uncertainty of monogenic or polygenic resistance (Zhao et al. 2000). The eggs from moths of the synthetic population, collected after 24 h, were put on artificial diet in

Table 2. Detection of RR genotype of *P. xylostella* in populations with known resistant genotypes for Cry1C resistance using two bioassay methods

Strains or crosses	Expected genotypes of offspring[a]	Expected % mortality	χ^2 test for broccoli bioassay			χ^2 test for diet bioassay		
			% Mortality[b] (±SEM)	χ^2	P	% Mortality[b] (±SEM)	χ^2	P
S	SS	100	100 (0)	0.0	0.99	100 (0)	0.0	0.99
R	RR	0	23 (3)	129.0	<0.01	12 (2)	20.6	<0.01
R × S (F_1)	RS	100	100 (0)	0.0	0.99	100 (0)	0.0	0.99
RS × RS (F_2)	1/4 RR,1/2RS,1/4SS	75	95 (1)	2.0	0.01	80 (2)	2.7	>0.05
$(1/2RS+1/2SS)^2$ (BC×BC)	1/16RR,3/8RS,9/16S	94	100 (0)	10.2	<0.01	98 (1)	4.8	<0.05

[a] Expected genotypes of offsprings if monogenic inheritance.
[b] Corrected mortality relative to control.

Table 3. F_2 screen for Cry1Ac resistance in the synthetic population

Pair no.	% survival on broccoli, 3 d		% survival in diet assay, 7 d	
	Cry1Ac broccoli	Control	Cry1Ac, 10 ng/ml	Control
21	1.3	40	3.8	43
34	0	70	0.6	73
43	0	65	3.1	60
86	0	73	1.9	78
92	0	83	1.3	85
99	0	65	5.0	75

n = 160 for all Bt treatments, n = 40 for all controls.

the same manner as Geneva 88. About 1,000 moths were used to produce eggs in each of two generations of the synthetic population. There were 120 single-pair matings (P_1) using the moths of the second generation of the synthetic population. Egg sheets of the F_1 progeny for each pair were collected every day for 4 d, and were pooled into the diet. The pupae of F_1 progeny for each pair were collected separately to get the F_1 moths. There were106 F_2 family lines generated by sib-mating 50–100 F_1 moths of each pair. Eggs laid on the first and second days were used for the F_2 screening, while eggs laid on the third day were used to obtain F_2 moths and F_3 lines for partial families. A total of 160 neonates was used for each Bt broccoli and diet treatment that included four replicates of each treatment and 40 neonates per replicate. There were 40 neonates in two replicates for each broccoli and diet assay control.

Confirmation of Resistance Alleles Detected in F_2 Screen. We used two separate methods to confirm the resistance alleles detected in the F_2 screen. This procedure was designed primarily to eliminate false positive results from the diet assays. The first method was to retest the F_3 generation derived from families that had survivors in the diet assay in the F_2 screen. These F_3 families were obtained by sib-mating each of the F_2 moths. The bioassay methods were the same as in the F_2 screen, but each treatment was related to either Cry1Ac or Cry1C resistance according to the survivorship in the F_2 screen.

The second method consisted of single pair mating of survivors at diagnostic doses of Cry1Ac or Cry1C protoxin in the diet assay of the F_2 screen with the corresponding Cry1Ac- or Cry1C-R strain. If there were more than one pair for the same F_2 family, the pair that produced the most eggs was used for the bioassay. The neonates from each single pair were tested using Bt broccoli and the diet assay as in the F_2 screen. We used 20–40 larvae in two replicates for each treatment because of the limited number of progeny from each single pair.

Table 4. F_2 screen for Cry1C resistance in the synthetic population

Pair no.	% survival on broccoli, 3 d		% survival in diet assay, 7 d	
	Cry1C broccoli	Control	Cry1C, 1.0 ng/ml	Control
35	0	65	0.6	63
39	0	53	0.6	75
73	0	60	1.3	70
89	0	75	0.6	75
90	0	78	1.3	85
104	0	40	0.6	60

n = 160 for all Bt treatments, n = 40 for all controls.

Statistical Analysis. Chi-square analysis (Zar 1996) was used to test if the observed mortality in different treatments fit the predicted mortality based on monogenic inheritance for validation of the bioassay methods. Estimated resistance allele frequencies for Cry1Ac and Cry1C resistance were calculated using equation 1 from Andow and Alstad (1998). The 95% confidence intervals (CI) of allele frequencies (Andow and Alstad 1999) and the probability of false negatives based on a binomial distribution were estimated in Microsoft Excel. SAS programs were used for analysis of variance (ANOVA) (SAS Institute 1985) for comparison of control mortality. Mortality data were transformed using arcsine (square root (p)) for proportion of mortality before each ANOVA was performed.

Results

Validation of Bioassay Methods to Detect RR Genotypes of Cry1Ac and Cry1C Resistance. The homozygous resistant genotype (RR) in five strains or crosses for Cry1Ac resistance were detected by using both Bt broccoli and the diagnostic diet assay. The observed mortality of Cry1A-R, F_2, and BC × BC was significantly higher than expected from a monogenic inheritance model (Table 1). The survivors of Cry1Ac-R on Bt broccoli after 3 d were reared on Bt broccoli expressing Cry1Ac, and 90% of them became pupae. The results for detecting the RR genotype of Cry1C resistance were similar to that of Cry1Ac resistance, but there were no survivors on Bt broccoli for the larvae of BC × BC progeny (Table 2). For the survivors of Cry1C-R on Bt broccoli after 3 d, 83.4% became pupae. Only the observed mortality of the F_2 for Cry1C resistance at the diagnostic diet assay was not significantly different from the expected value.

F_2 screen for Rare Resistance Alleles in a Synthetic Population. For the 106 F_2 families from the synthetic population tested with the F_2 screen method, one and six families produced survivor(s) on the Bt broccoli expressing Cry1Ac and the diagnostic Cry1Ac diet assay, respectively (Table 3). Cry1C resistance was detected in six other families by the diagnostic diet assay, but no resistance was detected using the Bt broccoli expressing Cry1C (Table 4). In four of the six F_2 families with survivors in the Cry1C diet assay, only one larva survived from the total 160 neonates tested (0.6% survival).

Confirmation of Resistance Alleles Detected in F_2 Screen. For the Cry1Ac resistance detected using the diet assay method in the F_2 screen, four of the six families (No. 21, 86, 92, 99) were confirmed to have a resistance allele in the F_3 screen using both Bt broccoli and the diet assay (Table 5). Further testing of the

Table 5. Tests for conformation of Cry1Ac resistance allele detected in F_2 screen

Pair no.	Generation or crosses	% RR genotype in offsprings[a]	% survival on broccoli, 3 d		% survival in diet assay, 7 d	
			Cry1Ac broccol	Control	Cry1Ac, 10 ng/m	Control
21	F_3	6.3	11	78	16	90
	Survivor × R	100	75	75	85	90
34	F_3	6.3	0	65	0	83
	Survivor × R	100	0	70	0	75
43	F_3	6.3	0	73	0	93
	Survivor × R	100	0	75	0	90
86	F_3	6.3	1.9	63	2.5	80
	Survivor × R	100	80	85	73	85
92	F_3	6.3	2.5	75	1.9	90
	Survivor × R	100	65	80	83	95
99	F_3	6.3	5.0	68	4.4	85
	Survivor × R	100	53	75	65	85

[a] Methods for F_3 were same as F_2 screen. The genotype of Survivor × R progeny should be 100% RR if the survivors in F_2 screen were RR genotype.

offspring of the single pair mating between survivors on the Cry1Ac diet assay in the F_2 screen with Cry1A-R (Survivor × R) using both the Bt broccoli and the diet assay also confirmed that the survivors in the F_2 screen were resistant individuals for the same four families (No. 21, 86, 92, 99) (Table 5). For two other of the six families (No. 34, 43), although Cry1Ac resistance was detected in the F_2 screen using the diet assay, there was no confirmation of the RR genotype in the F_3 screen and the offsprings of Survivor × R, thus indicating false positive results.

For the Cry1C resistance detected by the diet assay in the F_2 screen, there were also four of the six families (No. 73, 89, 90, 104) that were confirmed as having a resistance allele in the F_3 screen using the diet assay. The results of tests on the progeny of Survivor × R using the diet assay were the same as the F_3 screen (Table 6). In both the F_3 screen and tests on offspring of Survivor × R, using Bt broccoli expressing a high level of the Cry1C toxin did not allow detection of the Cry1C resistance in most cases for the four families, despite being confirmed by the diet assay.

Detection Ability and Accuracy in the F_2 Screen. Four single-pairs in the 106 pairs were confirmed as having one copy of an allele for Cry1Ac resistance. Four other single-pairs were confirmed to contain an allele for Cry1C resistance. The estimated allele frequency was 0.012 (0.004–0.023) for resistance to either toxin (Table 7). Using Bt broccoli as the F_2 screen method did not allow detection of most of the resistance and resulted in 75% (3/4) and 100% (4/4) false negatives for detection of Cry1Ac and Cry1C resistance, respectively (Table 7). When using the diet assay in the F_2 screen, there were two families of false positives (2/6, or 33%) for Cry1Ac or Cry1C resistance (Table 7). Based on the binomial distribution with the sample size of 160, the estimated probability of false negatives (number = 0) were 1% for Cry1Ac resistance (average probability = 4.5/160 in four confirmed families) and 22% for Cry1C resistance (average probability = 1.5/160 in four confirmed families) (Fig. 1). The probability of false negatives was <5% when the sample size ≥110 for Cry1Ac resistance and ≥320 for Cry1C resistance (Fig. 1).

Discussion

The strengths of the F_2 screen include the ability to detect recessive and partial recessive resistance alleles; the collection of genetic information from wild

Table 6. Tests for conformation of Cry1C resistance allele detected in F_2 screen

Pair no.	Generation or crosses	% RR genotype in offsprings[a]	% survival on broccoli, 3 d		% survival in diet assay, 7 d	
			Cry1C broccoli	Control	Cry1C, 1.0 ng/ml	Control
35	F_3	6.3	0	63	0	83
	Survivor × R	100	0	65	0	80
39	F_3	6.3	0	75	0	88
	Survivor × R	100	0	75	0	85
73	F_3	6.3	0	68	0.6	80
	Survivor × R	100	0	75	60	95
89	F_3	6.3	0	78	1.3	90
	Survivor × R	100	0	75	75	85
90	F_3	6.3	0	68	1.3	93
	Survivor × R	100	10	80	60	90
104	F_3	6.3	0	70	1.3	80
	Survivor × R	100	10	75	70	85

[a] Methods for F_3 were same as F_2 screen. The genotype of Survivor × R progeny should be 100% RR if the survivors in F_2 screen were RR genotype.

Table 7. Estimated resistance allele frequencies and detection error in F_2 screens (n = 160)

Resistance allele	No. of families confirmed for resistance	Estimated R allele frequency	95% CI	F_2 screen results on 106 families		
				Method	No. of families detected	Errors
Cry1Ac	4	0.012	0.004–0.023	Bt broccoli	1	−3/4
				Diet assay	6	+2/6, −0.01
Cry1C	4	0.012	0.004–0.023	Bt broccoli	0	− 4/4
				Diet assay	6	+2/6, −0.22

+, false positives; −, false negatives.

populations before resistant lab colonies are created; and the ability to apply rigorous statistical confidence intervals to estimates of allelic frequencies (Caprio et al. 2000). But in all published resistance monitoring studies for Bt crops, no major resistance allele has been detected in field populations of *O. nubilalis* or *S. incertulas* using Bt corn and Bt rice, respectively, as the screen method (Andow et al. 1998, 2000; Bentur et al. 2000). It is unclear whether such results are due to the lack of resistance alleles in the examined populations or a lack of sensitivity of the Bt plant screening methods.

The application of the F_2 screen requires a reliable screen method for the F_2 progeny. Using diet and plant screens provided the same qualitative result for major and partial resistance detection in *O. nubilalis* (Andow et al. 1998). However, in our studies we used a synthetic population of *P. xylostella* containing known allele frequencies for Cry1Ac and Cry1C resistance in the order of 10^{-2}, and compared the ability to detect resistance by the two screen methods. Using Bt broccoli plants as the F_2 screen method resulted in 75 and 100% false negatives for Cry1Ac and Cry1C resistance, respectively, while using the diagnostic diet assay resulted in 33% false positives for either toxin. These results indicate the importance of the methodology used to identify resistant individuals (RR genotype). The estimated allele frequency for resistance to either toxin (≈1%) primarily by the diet assay was similar to the infested allele frequency (≈3%) in the synthetic population. One of the reasons for the difference between the estimated and infested frequencies may be the delay of releasing RS moths into the S population. The crosses between the moths of the S population, which occurred before releasing the RS moths into the cage, may have produced more homozygous SS eggs than expected from random crosses in the synthetic population.

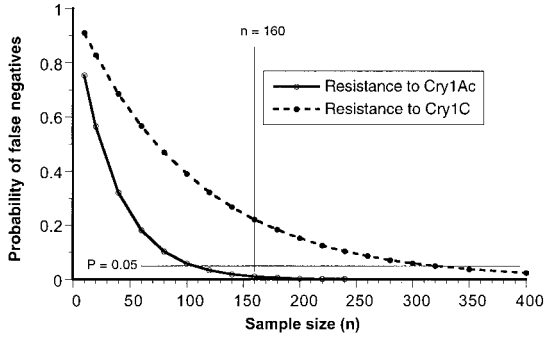

Fig. 1. Estimated probability of false negatives based on binomial distribution for detection of Cry1Ac or Cry1C resistance using a discriminating diet assay as the F_2 screen method.

We designed two independent experiments to eliminate the possible false positives of the F_2 screen by the diagnostic diet assay, and the results from the two experiments agreed when we used the diagnostic diet assay (Tables 5 and 6). Based on the results of the F_2 screen, there were only limited families that needed further tests to eliminate the possible false positives. The diagnostic diet assay could be used as an F_2 screen method especially for Cry1Ac resistance in *P. xylostella*, and further tests on the F_3 generation of the detected families could confirm the resistance alleles in each family. Our results showed that using transgenic plants expressing a high level of a Bt toxin as an F_2 screen method may underestimate the frequency of resistance alleles with high false negatives (for Cry1Ac), or fail to detect true resistance alleles (Cry1C) that were detected and confirmed by other methods. It was difficult to discover the possible false negatives when using this method alone in the F_2 screen.

Several factors may influence the ability to detect rare resistance alleles in the F_2 screen. First, there is a question of whether resistance is monogenic or polygenic inherited. The F_2 screen is designed to estimate monogenic resistance (Andow and Alstad 1998, Caprio et al. 2000). Resistance to Cry1Ac in *P. xylostella* was monogenic (Tang et al.1997). We were not sure if resistance to Cry1C was monogenic or polygenic (Zhao et al. 2000) before this study. The mortality of BC × BC offsprings was 100% on Cry1C broccoli (the expected mortality was 94% if monogenic inheritance) (Table 2) indicating that it was probably controlled by more than one gene. Both could be detected in our experiments using the diet assay as the F_2 screen method, but there were high false negatives and false positives for detecting Cry1C resistance (Table 7; Fig. 1). Second, there is a question of the F_2 screen methods. Our results indicated that the diagnostic diet assay method could detect the RR genotype for Cry1C resistance in the BC × BC population with a much lower survival than expected (1/16 RR genotype if monogenic inherited), but the Bt broccoli expressing Cry1C could not detect it at all (Table 2). One reason for the reduced detection abil-

Table 8. Comparison of control mortality for two screen methods in different tests

Populations	*n*	% mortality on broccoli control, 3 d			% mortality on diet control, 7 d		
		Min.	Max	Mean ± SEM[a]	Min.	Max	Mean ± SEM
Synthetic population	80	20.0	30.0	26.3 ± 2.4a	5.0	15.0	8.8 ± 2.4b
F_2 families[b]	1,200	30.0	70.0	48.6 ± 2.0a	10.0	57.5	26.3 ± 2.3b
F_3 families[c]	480	25.0	37.5	29.7 ± 1.6a	7.5	20.0	13.8 ± 1.5b

[a] Means within rows followed by different letters are significantly different ($P < 0.05$, Tukey's test).
[b] Data of 30 F_2 families (no. 1–30) were used.
[c] Data of 12 F_3 families as listed in Tables 5 and 6 were used.

ity using Bt broccoli plants may be that the level of Bt protein expressed is too high. Our selection experiences indicated that *P. xylostella* populations with a low level of Bt resistance cannot survive on Bt plants (Cao et al. 1999), but the same population can survive on the same line of Bt plants after a few more generations of selection using a Bt protoxin (Zhao et al. 2000, 2001). Third, mortality in the control and sample size in the F_2 screen can influence the ability to detect resistance alleles. The mortality on the broccoli control (3 d) was significantly higher than on the diet control (7 d) (Table 8). The control mortality for F_2 families either on broccoli or diet was much higher than the synthetic population from which the F_2 families were produced, indicating increased control mortality possibly because of inbreeding depression. In our screens of both the F_2 and F_3 generations, we used 160 neonates for each family and each screen method. The survival rates for most detected families were much lower than the expected rate of 1/16. In two F_2 families (No. 89, 104) with confirmed Cry1C resistance alleles, there was only one survivor (0.6%) from the 160 neonates tested in the diet assay (Tables 4 and 6). Such low survival rate was risky for false negatives if the sample size was not large and if the control mortality was high. The estimated probability of false negatives was <5% when the sample size was ≥110 for resistance to Cry1Ac and ≥320 for resistance to Cry1C (Fig. 1). No exact number of neonates were reported for the F_2 screen on *O. nubilalis* using Bt corn (Andow et al. 1998, 2000), but for *S. incertulas*, 37–51.6 F_2 larvae on average were tested and 38% (172/454) of the P_1 lines produced only one or two F_1 and F_2 families (Bentur et al. 2000). A small population of F_2 larvae for screening and a high control mortality (40–60% for *S. incertulas*) in combination would definitely decrease the detection ability for any screen method, and would increase the risk of false negative results, especially when the resistance allele frequency was low in the fields. We identified evident false positive or false negative results for both screening methods. Thus, careful validation of the screening method for each insect-crop system is necessary before the F_2 screen can be used to detect rare Bt resistance alleles in field populations.

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