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On-plant survival and inheritance of resistance to Cry1Ab toxin from *Bacillus thuringiensis* in a field-derived strain of European corn borer, *Ostrinia nubilalis*

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Abstract

BACKGROUND: The high dose plus refuge is one of the major components of the resistance management plan mandated for transgenic corn expressing Cry toxins from *Bacillus thuringiensis* Berliner (*Bt*) that targets the European corn borer, *Ostrinia nubilalis* (Hübner) (Lepidoptera: Crambidae). This strategy was based on assumptions such as functional recessive inheritance, which has not been previously tested for *O. nubilalis*. The authors used a field-derived resistant strain of *O. nubilalis* to define the nature of resistance to Cry1Ab toxin by examining the inheritance and on-plant survival of susceptible and resistant insects and their F_1 progeny.

RESULTS: The resistant strain exhibited >800-fold resistance to Cry1Ab. Resistance was primarily autosomal and controlled by more than one locus or multiple alleles at one locus. The degree of dominance *D* calculated on the basis of LC_{50} values was −0.45 ($h' = 0.27$), indicating that resistance was incompletely recessive. No survivors were found on vegetative-stage *Bt* corn, although both resistant larvae and their F_1 progeny were able to survive on reproductive corn 15 days after infestation.

CONCLUSIONS: A field derived *O. nubilalis* strain exhibited high levels of resistance to Cry1Ab and survived on transgenic corn by feeding on tissues with low Cry1Ab expression. The Cry1Ab resistance was primarily autosomal, incompletely recessive and polygenic. Tissue and on-plant survival data indicated that dominance varies depending on plant stage.

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

Keywords: Lepidoptera; inheritance; transgenic maize; Cry1Ab; high dose plus refuge; insecticide resistance management

1 INTRODUCTION

High dose plus refuge is one of the major components of the resistance management plan mandated for *Ostrinia nubilalis* (Hübner) (Lepidoptera: Crambidae) in areas cultivated with genetically modified corn (*Zea mays* L.) expressing Cry toxins from *Bacillus thuringiensis* Berliner (*Bt*).[1] This strategy is more effective in delaying resistance evolution when the frequency of resistance alleles is low and resistance is functionally recessive.[2,3]

Dominance of resistance to *Bt* toxins in laboratory strains of *O. nubilalis* has been shown to increase as concentration decreases,[4] which can be particularly important for this pest because Cry1Ab expression levels vary between vegetative and reproductive stages, with high concentrations in leaves relative to other tissues such as pollen, silks, ear shank and kernels.[5–7] This variation in Cry1Ab expression levels between vegetative and reproductive corn tissues suggests the need for testing the assumption of functional recessive inheritance using corn plants during both corn growing stages.

For *O. nubilalis*, the functional recessive inheritance of resistance has only been tested using Cry1F-expressing corn plants.[8] Cry1Ab-resistant strains previously tested lack the ability to survive on corn plants,[9,10] either because they have moderate levels of resistance or because they have lost the ability to use corn plants as a food source after many generations feeding on artificial diet.[4,11] A recent study testing the survival of third-instar larvae

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of *O. nubilalis* showed that a Dipel-selected strain survived on vegetative- and reproductive-stage plants expressing Cry1Ab, but hybrid F_1 progeny were not tested.[12]

In 2001, Cry1Ab-resistant individuals of *O. nubilalis* were identified from a field collection from Kandiyohi, Minnesota, based on increased survival at a diagnostic Cry1Ab concentration.[13,14] The progeny derived from these survivors were further selected in the laboratory using leaf discs expressing Cry1Ab.[13] Because this strain was initiated with insects from the field, it may provide a more appropriate means to test assumptions related to resistance management strategies for Cry1Ab-expressing corn.

In the present study, a field-derived strain of *O. nubilalis* was used to define the nature of resistance to Cry1Ab toxin by examining the inheritance in progeny from reciprocal crosses with a susceptible strain. Inheritance patterns were examined in bioassays with Cry1Ab toxin applied to artifical diet to determine the degree of dominance of the resistance, possible sex linkage/maternal effects and the monogenic or polygenic nature of the resistance. In addition, the assumption of functional recessive resistance to Cry1Ab toxins was assessed by determining the survival of Cry1Ab-susceptible and Cry1Ab-resistant parental strains and the F_1 progeny from reciprocal crosses in greenhouse experiments with entire corn plants and in laboratory bioassays with reproductive corn tissues.

2 MATERIALS AND METHODS

2.1 Insects and rearing

The resistant *O. nubilalis* colony originated from a field collection of 126 diapausing larvae collected from non-*Bt* hybrids in Kandiyohi Co., Minnesota, in 2001. After diapause was terminated, 113 pupae were placed in rearing cages, and emerging adults were mass mated. Approximately 900 individuals from the F_1 progeny were used in diagnostic bioassays in which neonates were exposed to a concentration of Cry1Ab protoxin corresponding to the upper end of the 95% confidence interval of the LC_{99}.[13,14] After 7 days, 14 individual larvae that survived exposure to the diagnostic concentration were transferred to untreated diet, reared to adults and used to initiate the resistant strain. This strain was further selected at the F_8 generation by exposure to corn leaf discs cut from freshly excised corn leaves expressing Cry1Ab (MON810). Individuals that survived exposure to corn leaf discs after 4 days were then pooled to initiate another resistant strain that was further selected by exposure to the diagnostic Cry1Ab concentration for 7 days at each generation of rearing.[14] After generation 21, the resistant strain (SKY = selected Kandiyohi) was maintained by exposure to a Cry1Ab concentration that was approximately 20-fold in excess of the diagnostic concentration (1000 ng cm^{-2}, trypsin-activated Cry1Ab formulation). Rearing methods for *O. nubilalis* were based on those developed at the USDA-ARS Corn Insect Research Unit, Ames, Iowa.[11,15,16]

Two different susceptible strains were used for on-plant bioassays and for inheritance experiments. The KY (susceptible Kandiyohi) strain originated from the same field collection as the resistant SKY population but was never exposed to Cry1Ab. A second susceptible strain originated in 2005 from a field collection of ≈900 individuals from Iowa and Nebraska. The susceptible FIELD strain was reared for six generations in the absence of exposure to Cry1Ab before bioassays were conducted, and was determined to be susceptible on the basis of >99% mortality at the previously described diagnostic Cry1Ab concentration.[13,14]

2.2 *Bt* toxins

The toxin used in diagnostic assays and selections until generation 21 was a Cry1Ab protoxin (CellCap®) provided by Dow/Mycogen Co. (Indianapolis, IN). Because of limited availability of Cry1Ab protein, the source of toxin was changed to a purified trypsin-resistant core after 21 generations. Although the resistant insects were obtained from diagnostic bioassays with protoxin, survival of larvae on freshly excised corn leaves expressing truncated Cry1Ab (MON810) indicated that resistance alleles also conferred resistance to the activated toxin. This toxin was obtained either from *B. thuringiensis* subsp. *kurstaki*, provided by Monsanto Co. (St Louis, MO), or from *Escherichia coli* (Migula) (host strain JM103), provided by the *Bacillus* Genetic Stock Center. Cry1Ab was purified from *E. coli* by a modification of the method described by Lee *et al.*[17] Cry1Ab concentrations in different batches were standardized by SDS-PAGE/densitometry.[18]

2.3 Mass crosses and bioassays

To evaluate sex linkage and dominance, F_1 progeny from reciprocal mass crosses between resistant SKY and susceptible FIELD strains were tested. The sex of pupae was determined visually through observation of genitalia under a microscope.[19] Reciprocal crosses were made by mass mating the susceptible FIELD strain and the resistant strain (SKY) to produce two F_1 populations: $\text{FIELD}_{♂} \times \text{SKY}_{♀}$ and $\text{SKY}_{♂} \times \text{FIELD}_{♀}$. To estimate the number of loci influencing resistance, F_1 progeny from reciprocal crosses were backcrossed to the susceptible and resistant strain. The power of indirect tests for modes of inheritance is higher when the backcross progeny are originated from crosses between F_1 progeny and the parental strain which is more dissimilar in susceptibility to the toxicant.[20] Based on results of tests with F_1 progeny, which indicated that resistance was mostly recessive, males and females of each F_1 were backcrossed to resistant moths to produce four backcross populations ($A = [\text{FIELD}_{♂} \times \text{SKY}_{♀}]_{♂} \times \text{SKY}_{♀}$; $B = \text{SKY}_{♂} \times [\text{FIELD}_{♂} \times \text{SKY}_{♀}]_{♀}$; $C = [\text{SKY}_{♂} \times \text{FIELD}_{♀}]_{♂} \times \text{SKY}_{♀}$; $D = \text{SKY}_{♂} \times [\text{SKY}_{♂} \times \text{FIELD}_{♀}]_{♀}$). Because one of the methods used to estimate the number of loci also requires the phenotypic variance of backcross to both parental strains,[21] moths of the same sex from both reciprocal F_1 crosses were pooled and backcrossed with susceptible females and males to produce two backcross populations ($E = [\text{FIELD}_{♂} \times \text{SKY}_{♀} + \text{SKY}_{♂} \times \text{FIELD}_{♀}]_{♂} \times \text{FIELD}_{♀}$; $F = \text{FIELD}_{♂} \times [\text{FIELD}_{♂} \times \text{SKY}_{♀} + \text{SKY}_{♂} \times \text{FIELD}_{♀}]_{♀}$). For all crosses, 100 pupae of each strain/sex were pooled together in mating cages. Adults were allowed to emerge and mate, and eggs were collected to provide neonates for subsequent bioassays. Strains and crosses were tested for susceptibility to Cry1Ab in survival bioassays. Neonates (<24 h after eclosing) were exposed to Cry1Ab toxin on the surface of artificial diet.[14] Some concentrations used to test F_1 and backcross progeny were also tested in parental strains, which is necessary for evaluating the number of loci using direct and indirect tests to distinguish among modes of inheritance.[20]

2.4 Concentration–mortality curves

After mortality was corrected for control mortality,[22] the data from survival assays were analyzed by probit regression using POLO-PC to calculate the median lethal concentrations (LC_{50} values) and slopes.[23] Resistance ratios were estimated on the basis of intercepts and slopes of probit lines obtained for each strain or

cross. This method allows the confidence intervals for resistance ratios to be estimated.[24]

2.5 Dominance of resistance

Two methods were used to calculate dominance. Using Stone's method,[25] the degree of dominance D of Cry1Ab resistance in the F_1 populations was estimated through LC_{50} values for the parental strains and F_1 progeny. Stone's method estimates the degree of dominance as follows:

$$D = \frac{2X_2 - X_1 - X_3}{X_1 - X_3}$$

where X_1, X_2 and X_3 are the logarithms of the LC_{50} values for the resistant homozygotes, heterozygotes and susceptible homozygotes respectively. In addition, the effective dominance h at specific concentrations c, h_c, was calculated.[26,27]

2.6 Sex linkage and maternal effects

The maternal influence and the sex-linked nature of the resistance were examined by comparing observed larval concentration responses of the F_1 progeny and the four backcross populations originated from crosses between the F_1 progeny and the resistant strain. Concentration responses of F_1 progeny and the four backcross populations were compared on the basis of the slope and confidence intervals of median lethal concentrations obtained by probit analysis.

2.7 Number of loci

The data obtained in survival bioassays with backcross progeny from mass crosses were used to determine the number of loci by three different approaches including Lande's method,[21] the direct test for monogenic inheritance[20] and indirect tests of models with one, two, five and ten loci.[28]

2.7.1 Lande's method

This procedure assumes equal and additive effects of loci and uses genotypic and phenotypic variances to estimate the minimum number of independently segregating genes (n_E) contributing to a quantitative character between two divergent populations.[21] To calculate n_E, methods described in detail previously[28] were followed.

2.7.2 Direct test

The direct tests use chi-square values calculated from the observed and expected mortalities of the backcross population.[20] A chi-square value was calculated for each concentration and compared with a chi-square distribution with one degree of freedom. The null hypothesis of monogenic inheritance was rejected if this test indicated $P < 0.05$.

2.7.3 Indirect test

In the indirect tests of monogenic and polygenic models, the fit between observed mortality and mortality predicted by models with one, two, five and ten loci was tested. This follows the approach described previously.[4,28,29] The slopes of the concentration–mortality lines for the parental strains were calculated as the mean of the slopes experimentally determined for each parental strain. It was assumed that each locus had one allele conferring resistance (R) and the other conferring susceptibility (S). Equal and additive effects of loci in polygenic models were also assumed. The expected mortality for each concentration under each hypothesis was estimated as the cumulative probability from the appropriate tolerance distributions for each model (PROBNORM).[30] The average absolute difference between observed and expected mortalities was calculated as the mean of the absolute values of expected percentage mortality minus the observed percentage mortality for each concentration.[28]

2.8 Pollen and silk bioassays

2.8.1 Pollen and silk sources

Pollen and silk bioassays were conducted using three *Bt* events and their non-*Bt* isolines: *Bt*11 (N4242 YG), MON810 (Pioneer 38G17 *Bt*) and Event 176 (Mycogen 2657 *Bt*)[31] with the resistant SKY strain and the susceptible KY strains. The pollen was suspended in a 0.5 µg ml^{-1} Triton X-100 solution and dispensed onto the surface of 1 mL of artificial diet in Bio-CV-128 bioassay trays and allowed to air dry. Pollen concentration was estimated to be ≈11 023 grains cm^{-2} or 7.35 mg cm^{-2}. A single neonate was transferred to each well and allowed to feed for 7 days, after which weight and mortality were recorded. Trays were held at 27 °C, 80% RH and a scotophase of 0 : 24 h light : dark cycle.

Unpollinated silk was stripped from ears, wrapped in 8 mm diameter bundles and inserted through a seam cut into the side of a plastic drinking straw. The silk-filled tubes were cut into ≈2 cm segments. One end of each silk tube was immersed in water and placed in a 32-cell rearing tray (product no. 9074; Bio-Serv, Frenchtown, NJ). A single neonate was transferred to the dry end of the silk tube, and a vented, clear plastic laminate lid was applied to cover the cell. Larvae were allowed to feed for 7 days (27 °C, 80% RH and a scotophase of 0 : 24 h light : dark cycle), after which weight and mortality were recorded.

2.8.2 Data analysis

Larval weights obtained for pollen and silk were first tested for normality of data distribution using the Shapiro–Wilk test, and normal probability plots were constructed in SAS PROC UNIVARIATE.[30] To homogenize variances, weight values were square root transformed. Then, data were analyzed with a two-way ANOVA implemented in SAS PROC MIXED.[30] The two main factors were *O. nubilalis* type and *Bt* events. Treatment means were separated using least-squares means (LSMEANS) tests at a 5% significance level.[30] To facilitate comparisons and create a control for natural resistance on pollen and silk, data from the three isolines were united for comparisons with *Bt* events.

The survival of larvae on silk or pollen was treated as a binomial random variable. Then, the number of larvae alive after 7 days was considered to be the number of successes in a series of trials where each larva was tested independently. The probability of success (π) was the number of larvae alive divided by the number of larvae tested on pollen ($n = 48$) and silk ($n = 32$). One-tailed z-tests for two independent proportions were used to determine whether the proportion of resistant larvae was statistically higher than the proportion of susceptible larvae surviving on the *Bt* event or isoline tested ($H_1: \pi_{\text{resistant}} - \pi_{\text{susceptible}} > 0$).[32]

2.9 On-plant assays

2.9.1 Plants and treatments

Greenhouse experiments were carried out with a MON810 hybrid (RX 634, Yield Guard, YG) expressing the Cry1Ab toxin and its isoline (RX 634) in the vegetative (V8-V9) and reproductive (R1-R2)

stages. These stages were chosen to represent those that coincide with infestations of the first and second *O. nubilalis* generations common to bivoltine populations that occur throughout most of the US corn belt.[33,34] Two factors were combined in factorial design: two corn hybrids and three *O. nubilalis* types – the susceptible KY strain, resistant KY strain and F_1 hybrid progeny. This combination yielded six overall treatments.

The experiments were carried out in a greenhouse at the University of Nebraska, Lincoln, Nebraska, in May–June 2005 (vegetative and reproductive plants) and June 2006 (reproductive plants). Seeds of Yield Guard hybrid RX 634 YG and its isoline RX 634 were planted in a substrate mixture composed of 50% soil, 30% peat moss and 20% sand in 18.9 L pots using three seeds per pot. Two weeks later, the plants were thinned to a single plant per pot, which were then watered daily and fertilized as needed until reaching the appropriate phenological stage for infestation. The vegetative- and reproductive-stage experiments were conducted separately. In each experiment, the six treatments were randomly assigned to six areas in the greenhouse, and 30 plants of each hybrid were utilized for corn borer infestations (10 plants treatment^{-1}). The greenhouse was divided into areas with a 2 m aisle separating them to reduce the risk of larvae moving among treatments. Plants were infested (30 neonates plant^{-1}) by placing a centrifuge tube containing the neonates one leaf below either the whorl (V8–V9 stage) or the primary ear (R1–R2 stage).[34,35] All plants were dissected 15 days after infestation to record survival, larval weight and instar. The injury to vegetative-stage plants was evaluated on the basis of leaf injury using the 1–9 Guthrie scale.[36]

2.9.2 *Cry1Ab expression*

Corn plants were tested for toxin expression with an enzyme-linked immunosorbent assay (ELISA). Leaf samples were taken from upper leaves when plants were at the V13–V14 stage, either during the dissections to evaluate plant injury and larval growth on plants infested during the vegetative stage or before infestation on plants infested during the reproductive stage. Samples were stored at −80 °C until quantification. The *Bt*-Cry1Ab/Cry1Ac ELISA kit was obtained from Abraxis (Warminster, PA) and stored at 4 °C before use.

2.9.3 *Data analysis*

The data were analyzed as a randomized complete block design with years (2005 and 2006) as block, using a two-way analysis of variance (ANOVA) implemented in SAS PROC MIXED employing the RANDOM statement,[30] with *O. nubilalis* types (unselected Kandiyohi, Cry1Ab selected Kandiyohi and hybrid F_1 progeny) and corn hybrids (MON810 and isoline) as the main factors. To homogenize variances, weight values were square root transformed, and treatment means were separated using LSMEANS tests at the 5% significance level.[30] The survival data were analyzed as binomial. To facilitate comparisons across *Bt* events, 95% confidence intervals (CIs) for binomial proportions were calculated using the modified Wald method.[37]

To estimate the dominance of European corn borer resistance to Cry1Ab-expressing plants (h_p), use was made of the quantitative values of three different traits obtained for resistant, hybrid F_1 progeny and susceptible types respectively, which were analyzed by the same methods as described previously.[26,27] The traits used in the calculation of effective dominance on plants were larval survival, percentage weight gain and the combination of the two traits. Because Cry1Ab toxins affect both growth and survival, these two components of fitness were combined by multiplying their phenotypic values.[8] For each European corn borer type, survival on Cry1Ab corn was estimated by adjusting for mortality observed on non-expressing plants,[22] and percentage weight gain was calculated relative to the larval weight of each type on isoline plants. For each *O. nubilalis* type, the frequency distribution of instars found on MON810 plants was compared against the frequency distribution of instars found on isoline plants using a chi-square test.

3 RESULTS

3.1 Concentration–mortality curves: maternal influence, sex linkage, resistance levels and dominance

The analysis of data obtained from reciprocal crosses between susceptible and resistant insects, as well as data obtained from the four backcross combinations between F1 progeny and resistant insects, indicated that resistance was primarily autosomal and recessive. The mortalities of *O. nubilalis* strains and crosses are presented in Table 1 and Fig. 1. The slopes of the concentration–mortality curves of FIELD♂ × SKY♀ and SKY♂ × FIELD♀ were 3.0 ± 0.4 and 1.6 ± 0.6 respectively. The hypothesis that these slopes are the same was rejected in POLO ($\chi^2 = 18.2$; df = 1; $P < 0.001$). The median lethal concentration for F_1 progeny of FIELD♂ × SKY♀ was twofold higher than the median lethal concentration of SKY♂ × FIELD♀.

Table 1. Responses to Cry1Ab of susceptible, resistant, F_1 and backcross larvae of *Ostrinia nubilalis*

Strain or cross	Generation	*n*	Slope ± SE	LC_{50} (95% FL)[a]	χ^2 (df)	RR[b]
FIELD (S)	F_6	446	3.5 ± 0.4	8 (7–10)	1.1 (4)	–
SKY (R)	F_{36}	559	2.5 ± 0.3	7376 (5284–9636)	9.6 (6)	815.4[d]
FIELD♂ × SKY♀	F_1	512	3.0 ± 0.4	71 (40–98)	10.6 (5)	7.2[d]
SKY♂ × FIELD♀	F_1	559	1.6 ± 0.6	32 (22–46)	12.0 (6)	3.9[d]
SKY × FIELD (pooled)	F_1	1024	2.2 ± 0.5	52 (38–70)	31.7[c] (5)	5.2[d]
FIELD × F_1 (pooled)	F_2	512	2.5 ± 0.2	15 (13–18)	1.6 (5)	2.0[d]
SKY × F_1 (pooled)	F_2	1640	1.3 ± 0.6	174 (68–408)	182.8[c] (9)	21.4[d]

[a] Units are ng Cry1Ab cm^{-2} diet, with 95% fiducial limits in parentheses.
[b] RR, resistance ratios estimated on the basis of intercepts and slopes of probit lines obtained for the strain or cross.[24]
[c] Indicates χ^2 value significant at $P = 0.05$.
[d] Ratios statistically higher than 1 at $P = 0.05$.

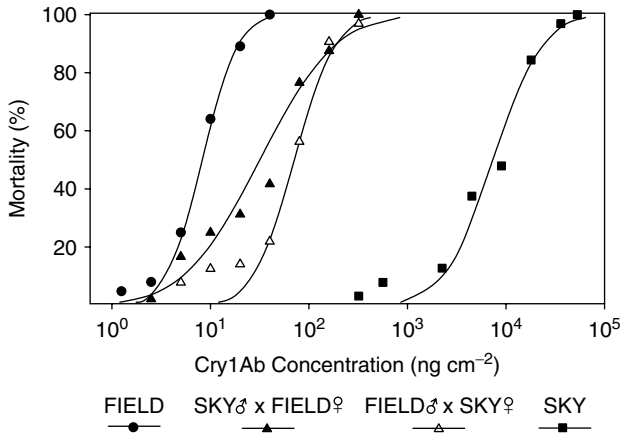

Figure 1. Concentration–mortality curves obtained with neonate larvae from susceptible and resistant parental strains and F_1 progeny of reciprocal crosses between parental strains.

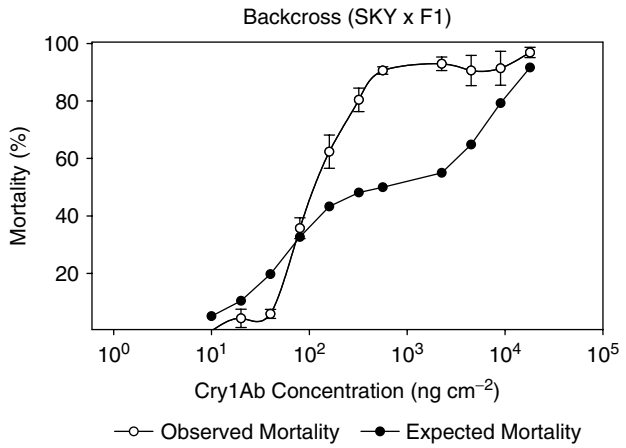

Figure 2. Expected concentration–mortality curve for monogenic inheritance and observed concentration–mortality curve obtained with neonate larvae from four backcross combinations between moths of F_1 progeny and the resistant parental strain (SKY). Expected % mortality at concentration $x = 0.5\times$ (% mortality of F_1 at x + % mortality of SKY at x), obtained from regression lines of parental strains.[20].

However, the LC_{90} of FIELD♂ × SKY♀ (188.5 ng cm^{-2}) was not significantly different from the LC_{90} of SKY♂ × FIELD♀ (200.9 ng cm^{-2}). Because the concentration–mortality curves of the four backcrosses exhibited a plateau response at 90% mortality (Fig. 2), only Cry1Ab concentrations causing percentage mortality between 0 and 90% were used to estimate the LC_{50} and to verify if there were maternal-related differences among the four backcrosses (Fig. S1 and Table S1, supplementary data). The concentration–mortality curves obtained for insects from the backcross with the least contribution of resistant females [D = SKY♂ × (SKY♂ × FIELD♀)♀] exhibited the lowest LC_{50} value [108.5 (70.3–166.5) ng cm^{-2}] but did not differ significantly from the backcross with the highest contribution of resistant females [A = (FIELD♂ × SKY♀)♂ × SKY♀] [LC_{50} = 140.6 (84.6–222.9) ng cm^{-2}]. The hypothesis that the slopes and intercepts were the same was not rejected in POLO ($\chi^2 = 6.8$; df = 6; $P = 0.340$), indicating that the four backcross populations exhibited similar responses to Cry1Ab.

Table 2. Effective dominance (h) of resistance in *Ostrinia nubilalis* larvae as function of Cry1Ab concentration

Concentration (ng cm^{-2})	Strain or cross	Survival (%)	Fitness[a]	h_c[b]
10 (5.5 times lower than diagnostic concentration)	KY	36.0	0.4	
	F_1	87.2	0.9	0.80
	SKY	100.0	1.0	
20 (2.8 times lower than diagnostic concentration)	KY	10.9	0.1	
	F_1	80.8	0.8	0.78
	SKY	100.0	1.0	
40 (1.4 times lower than diagnostic concentration)	KY	0.0	0.0	
	F_1	77.8	0.8	0.78
	SKY	100.0	1.0	
80 (1.4 times higher than diagnostic concentration)	KY	0.0	0.0	
	F_1	69.1	0.7	0.70
	SKY	98.9	1.0	
160 (2.8 times higher than diagnostic concentration)	KY	0.0	0.0	
	F_1	11.0	0.1	0.11
	SKY	98.9	1.0	
320 (5.5 times higher than diagnostic concentration)	KY	0.0	0.0	
	F_1	1.8	0.02	0.01
	SKY	97.3	1.0	

[a] The fitness of susceptible larvae exposed to each concentration was estimated as the survival of susceptible larvae divided by the survival of resistant larvae. The fitness of F_1 exposed to each dose was estimated as the survival of F_1 divided by the survival of resistant larvae.
[b] h_c was estimated as follows: $h = (w_{12} - w_{22})/(w_{11} - w_{22})$, where w_{11}, w_{12} and w_{22} are the fitness values at a particular Cry1Ab concentration for resistant, F_1 and susceptible larvae respectively. The fitness of resistant larvae exposed to Cry1Ab toxin was defined as 1.

The level of dominance is illustrated by concentration–mortality curves (Fig. 1), calculation of D using LC_{50} values and calculation of h at different concentrations (Table 2). The position of the concentration–mortality curves for the reciprocal crosses was closer to the susceptible parent than to the resistant parent, indicating that the inheritance of the resistance to Cry1Ab in SKY was incompletely recessive. The degree of dominance (D) of the resistance based on pooled F_1 data was −0.45 ($h' = 0.27$). Effective dominance calculations indicate that dominance increases as Cry1Ab concentration decreases. Indeed, resistance was virtually recessive at high concentration (320 ng cm^{-2}; $h = 0.01$) and partially dominant at low concentration (10 ng cm^{-2}; $h = 0.80$).

3.2 Number of genes influencing resistance

Results from three methods used to analyze backcross data indicated that resistance in the SKY strain is controlled by more than one locus or multiple alleles at one locus.

3.2.1 Lande's estimate of the minimum number of factors affecting resistance

This method uses the genotypic and phenotypic variances to estimate the minimum number of independently segregating

Table 3. Direct test for deviation between observed and expected mortality for the monogenic model (df = 1)

Concentration (ng cm^{-2})	Observed		Expected[a]			
	Dead	Alive	Dead	Alive	χ^2	*P*
20	6	122	12	116	3.57	0.0588
80	46	82	21	107	37.83	<0.0001[b]
320	103	25	65	63	46.20	<0.0001[b]
2250	119	9	72	56	70.46	<0.0001[b]
4500	116	12	88	40	28.81	<0.0001[b]
9000	117	11	95	33	20.44	<0.0001[b]

[a] Expected % mortality Y_x at each concentration x, calculated as $Y_x = 0.5 \times$ (% mortality of F_1 at x + % mortality of R × S (pooled) at x.
[b] Probability values indicating significant differences between the observed and expected mortality ($P<0.05$).

genes (n_E). The estimate of the minimum number of freely segregating effective factors (n_E) controlling Cry1Ab resistance in SKY was 2.6. This result is in agreement with the hypothesis of a small number of loci with major effects on Cry1Ab resistance (Table 1).

3.2.2 *Direct and indirect tests*

The direct tests indicated that resistance was not monogenic because in most of the concentrations tested the mortalities were statistically different from the expected (Table 3). The five-locus model represented the best fit and exhibited the lowest mean difference, with three out of six points deviating from the expected value. All other models indicated at least four points out of six deviating from the expected value (Table 4 and Fig. 3).

3.3 Pollen assays

The significant interaction between *O. nubilalis* type and event indicates that the growth inhibition caused by feeding on *Bt* pollen varied across the events ($F = 41.63$; df = 3, 565; $P < 0.0001$). Figure 4A shows the weight of susceptible and resistant individuals reared on pollen of both *Bt* and non-*Bt* events. No significant difference in growth was detected between susceptible and resistant individuals reared on MON810. The resistant strain developed better than the susceptible strain on *Bt*11 and Event 176. For *Bt*11, however, no significant differences in proportion of surviving larvae were detected between susceptible (0.89) and resistant larvae (0.98; $z = 1.32$, $P = 0.0934$) (Table 5). Nevertheless, the lowest proportion of surviving larvae was observed for the susceptible strain on Event 176 pollen (0.02), whereas all resistant larvae survived on Event 176 (1.0; $z = 46.49$, $P < 0.0001$) (Table 5).

3.4 Silk assays

The significant interaction between *O. nubilalis* type and event indicates that the growth inhibition caused by feeding on *Bt* silk varied across the events ($F = 8.35$; df = 3, 363; $P < 0.0001$). Figure 4B shows the weight of susceptible and resistant individuals reared on silk of both *Bt* and non-*Bt* events. Event 176 silk did not cause significant growth inhibition in either susceptible or resistant larvae, and no significant differences in proportion of surviving larvae were detected between susceptible (1.0) and resistant strains (0.94; $z = -0.69$, $P = 0.7549$) (Table 5). However, *Bt*11 and MON810 caused significantly higher growth inhibition in resistant individuals (Fig. 4B). A significantly higher proportion of susceptible larvae failed to survive on MON810 and *Bt*11 (0.0) compared with resistant larvae on MON810 (0.74; $z = 9.03$, $P < 0.0001$) or *Bt*11 (0.78; $z = 10.4$, $P < 0.0001$) (Table 5).

3.5 On-plant assays

3.5.1 *Cry1Ab expression*

The average concentrations of Cry1Ab in leaves of plants (V13–V14 stage) from experiments with vegetative- and reproductive-stage plants were 3.02 ± 0.26 and 3.96 ± 0.19 µg g^{-1} (fresh weight) respectively. These values were either higher or similar to those reported for MON810 plants grown in the greenhouse[38] and in the field.[6,7,39]

3.5.2 *Vegetative stage*

The results of vegetative-stage plant experiments are presented in Table 6 and Fig. 5. No survivors were detected on MON810 plants. On isoline plants, the resistant strain exhibited lower survival compared with the hybrid F_1 progeny and the susceptible strain, but the larval weight was not significantly different among European corn borer types ($F = 0.18$; df = 1, 174; 0.8387) (Fig. 5A). Virtually no leaf injury was detected on MON810 plants, whereas on isoline plants, the Guthrie injury ratings averaged 4.5, which

Table 4. Indirect tests for deviation between observed and expected mortality (df = 1) for monogenic and additive polygenic models

Cry1Ab concentration (ng cm^{-2})	Model							
	One locus		Two loci		Five loci		Ten loci	
	χ^2	*P*	X^2	*P*	χ^2	*P*	χ^2	*P*
20	39.2	<0.001[a]	54.5	<0.001[a]	3.5	0.061	2.3	0.132
80	8.4	0.004[a]	9.5	0.002[a]	0.1	0.822	13.9	<0.001[a]
320	45.9	<0.001[a]	46.1	<0.001[a]	18.6	<0.001[a]	11.0	<0.001[a]
2250	23.6	<0.001[a]	25.5	<0.001[a]	1.0	0.315	22.4	<0.001[a]
4500	0.3	0.600	0.6	0.483	59.7	<0.001[a]	355.7	<0.001[a]
9000	16.9	<0.001[a]	13.8	<0.001[a]	336.5	<0.001[a]	2608.7	<0.001[a]
Average mortality (%)	35.4		35.8		36.3		35.4	
Mean difference (%)	15.7		17.1		7.1		8.8	

[a] Probability values indicating significant differences between expected and observed mortality ($P < 0.05$).

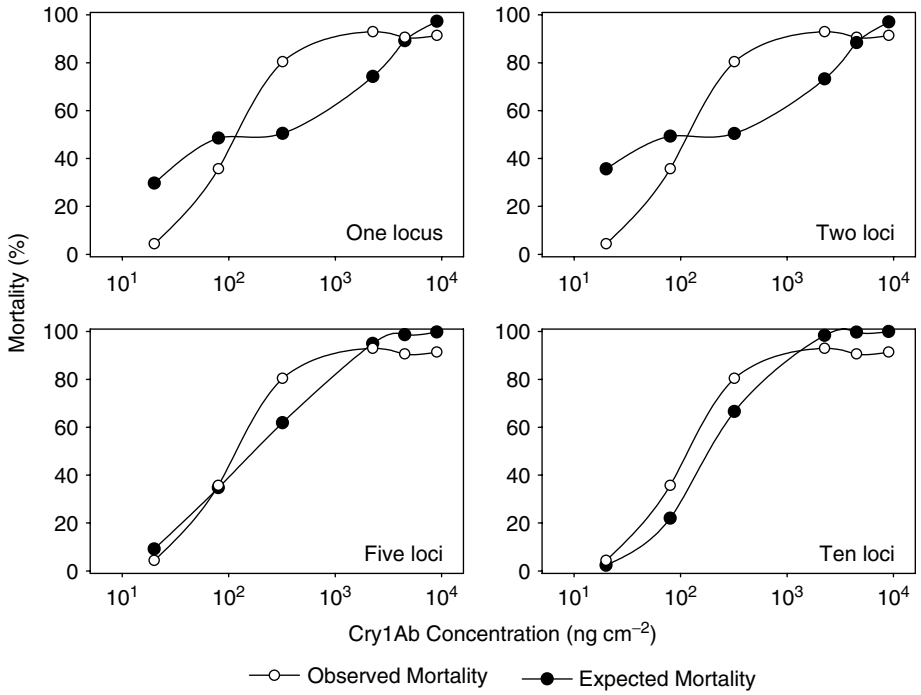

Figure 3. Observed versus expected mortality at each of the five Cry1Ab concentrations tested on neonate larvae from four backcross combinations between moths of F_1 progeny and the resistant parental strain (SKY).

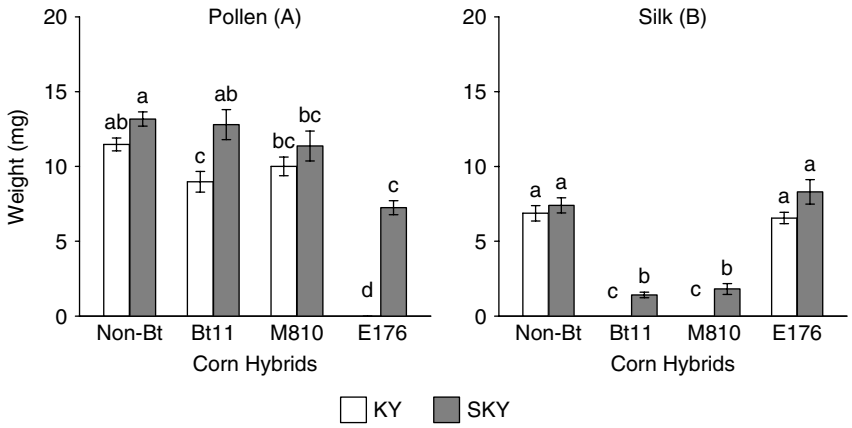

Figure 4. Mean weight (± SE) of larvae from SKY and control *Ostrinia nubilalis* strains ($n = 48$ pollen assays and $n = 32$ silk assays) on tissues derived from Cry1Ab-expressing events and combination of near-isoline plants. Treatment means were separated using least-squares means (LSMEANS) tests at a 5% significance level.

corresponds to plants exhibiting several leaves with shot-hole and elongated lesions. There was a significant *O. nubilalis* type by corn hybrid interaction ($F = 4.93$; df = 2, 174; $P < 0.0001$), which indicates differences among injury ratings caused by *O. nubilalis* types. Both susceptible and hybrid F_1 progeny caused more injury to isoline plants than the resistant strain (Table 6).

3.5.3 *Reproductive stage*

The results of reproductive-stage plant experiments are presented in Table 6 and Fig. 5. In contrast to results from vegetative-stage plants, survivors of all *O. nubilalis* types were detected on MON810 plants, although survival rates of susceptible larvae were minimal (0.3%) and not statistically higher than zero ($t = 1.45$; df = 19; $P = 0.081$) (Table 6). The resistant strain exhibited the highest survival rates on MON810 plants. In contrast, on isoline plants, the resistant strain showed lower survival compared with the hybrid F_1 progeny and the susceptible strain. The percentage mortalities of resistant, hybrid F_1 progeny and susceptible larvae feeding on MON810 were 43.5, 82.2 and 99.4% respectively (Table 7). The significant *O. nubilalis* type by corn hybrid interaction indicates that larval weights of *O. nubilalis* types were significantly different for each plant type ($F = 12.0$; df = 2, 354; $P < 0.0001$). In isoline plants, hybrid F_1 larvae exhibited the highest larval weight, followed by susceptible and resistant larvae (Fig. 5B). In MON810, hybrid F_1 progeny and resistant larvae exhibited similar larval weights, and the susceptible strain exhibited the lowest larval weight (Fig. 5B). The percentage reductions in weight of resistant, hybrid F_1 progeny and susceptible larvae feeding on MON810 were 32.6, 63.0 and 75.0% respectively (Table 7). Therefore, although resistant and hybrid F_1 larvae exhibited similar weights on MON810, hybrid

Table 5. Survival of two strains of European corn borer (F_{12}) from Kandiyohi County, Minnesota, after 7 days feeding on pollen and silk of *Bt* events

		Survival (%) (95% CI)[a]	
Corn event	Strain	Pollen	Silk
Non-*Bt*	KY	97.9 (94.1–99.3)	94.8 (88.4–97.8)
	SKY	95.8 (91.1–98.1)	94.1 (89.8–96.7)
*Bt*11	KY	89.1 (77.0–95.3)	0.0 (0.0–11.3)
	SKY	97.9 (89.1–99.6)	78.1 (61.2–88.9)
MON810	KY	97.9 (89.1–99.6)	0.0 (0.0–11.0)
	SKY	91.7 (80.4–96.7)	74.2 (56.8–86.3)
Event 176	KY	2.1 (0.4–10.9)	100.0 (87.9–100.0)
	SKY	100.0 (92.6–100.0)	93.5 (79.2–98.2)

[a] Confidence intervals estimated by the modified Wald method.[37]

Table 6. Injury and survival of larvae from European corn borer types released on vegetative and reproductive transgenic corn MON810 and MON810's isoline

			Survival (%) (95% CI)[a]	
Corn event	Strain or cross	Guthrie injury rating (1–9)	Vegetative	Reproductive
Isoline	KY	5.6 ± 0.6 a	12.7 (9.4–16.9)	56.7 (52.7–60.6)
	F_1	4.7 ± 0.6 a	11.0 (7.9–15.0)	61.3 (57.4–65.1)
	SKY	3.4 ± 0.4 b	2.7 (1.4–5.2)	33.3 (29.7–37.2)
MON810	KY	1.0 ± 0.0 c	0.0 (0.0–1.3)	0.3 (0.0–1.2)
	F_1	1.0 ± 0.0 c	0.0 (0.0–1.3)	11.0 (8.7–13.8)
	SKY	1.2 ± 0.1 c	0.0 (0.0–1.3)	18.3 (15.9–22.2)

[a] Confidence intervals estimated by the modified Wald method.[37]

F_1 larvae exhibited a twofold reduction in weight caused by feeding on transgenic plants compared with the reduction in weight of resistant larvae. Analysis of the frequency distribution of instars from these two *O. nubilalis* types grown on isoline and MON810 plants revealed similar trends. The frequency distribution of instars of resistant larvae found on MON810 plants was similar to the frequency distribution of instars found on isoline plants ($\chi^2 = 2.17$; df = 2; $P = 0.3383$). In contrast, for hybrid F_1 larvae, the frequency distribution of instars exhibited a significant departure from the expected frequency ($\chi^2 = 7.81$; df = 2; $P = 0.0151$), which indicates a major developmental delay of hybrid F_1 larvae feeding on MON810. Calculations of dominance of resistance using relative phenotypic values obtained for survival and weight and the combination of these two variables varied from 0.17 to 0.31, which indicates that resistance in SKY is incompletely recessive (Table 7).

4 DISCUSSION AND CONCLUSION

The present study indicates that the resistant SKY strain exhibited high levels of resistance to Cry1Ab and the ability to survive on transgenic corn by feeding on tissues of low Cry1Ab expression. The resistance is primarily autosomal and incompletely recessive, and cannot be explained by a single locus with two alleles. SKY was the first *O. nubilalis* strain exhibiting some degree of survival on reproductive-stage corn expressing Cry1Ab when neonates were used to infest plants.

The SKY strain exhibited an intermediate resistance level (815-fold) (Table 1 and Fig. 1) among those reported for *O. nubilalis* strains selected with Dipel (65-fold),[9] Cry1Ac (162-fold),[40] Cry1Ab (>1000-fold)[4] and Cry1F (3000-fold).[41] A maternal influence associated with inheritance of Cry1Ab resistance was also identified in analysis of concentration–mortality curves of reciprocal crosses between susceptible and resistant strains (Table 1 and Fig. 1). However, the similarities among concentration–mortality responses of the four backcross combinations [the hypothesis that the slopes

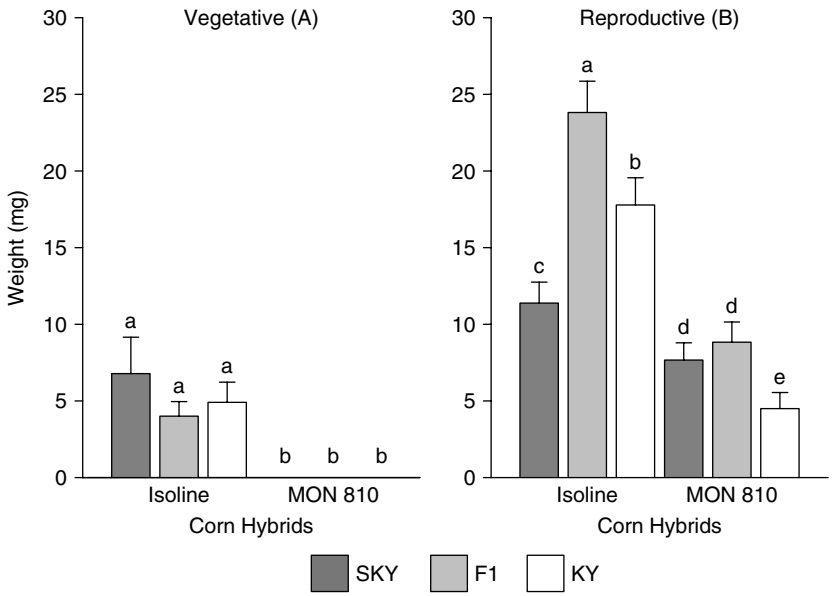

Figure 5. Larval weight of European corn borers types after 15 days feeding on vegetative and reproductive stages of the MON810 plant (RX 634 YG) and its isoline (RX 634): European corn borer types tested were susceptible to Cry1Ab (KS), resistant to Cry1Ab (SKY) and progeny from reciprocal crosses between susceptible and resistant parents (F_1). Error bars represent standard errors. Bars in a chart followed by the same letter are statistically similar (Fisher's protected LSD, at a 5% significance level).

Table 7. Phenotype values, relative fitness and effective dominance of *Ostrinia nubilalis* resistance to transgenic corn MON810 expressing Cry1Ab toxin during the reproductive stage

Strain or cross	Phenotype value[a]	Fitness[b]	h_p[c]
	Percentage of survival		
KY	0.6	0.0	
F_1	17.8	0.3	0.31
SKY	56.5	1.0	
	Percentage of weight gain		
KY	25.3	0.4	
F_1	37.0	0.5	0.28
SKY	67.4	1.0	
	Larval survival and weight gain combined		
KY	0.2	0.0	
F_1	6.6	0.2	0.17
SKY	38.1	1.0	

[a] Phenotype values for each trait are measures of genotype performance on MON810 relative to the performance of the same genotype on the isoline.
[b] Each fitness value is the phenotype value of the strain divided by the phenotype value of the fittest (i.e. resistant) strain.
[c] The dominance at each concentration was calculated as: (phenotype value of F_1 − phenotype value of susceptible)/(phenotype value of resistant − phenotype value of susceptible).

and intercepts were the same was not rejected ($\chi^2 = 6.8$; df = 6; $P = 0.340$)] suggesting that resistance was primarily autosomal (Fig. S1 and Table S1, supplementary data). Although it cannot be concluded that the resistance is autosomal, it appears unlikely that the maternal influences observed in this study contributed significantly to Cry1Ab resistance.

All methods used to analyze the number of genes involved in resistance on the basis of the response of backcross progeny indicate that more than one locus contributed to resistance in SKY. The estimate of the minimum number of freely segregating effective factors (n_E) controlling Cry1Ab resistance in SKY was 2.6, which is in agreement with the polygenic model of inheritance. Results of indirect tests indicate that resistance may be controlled by as many as five loci (Table 4 and Fig. 3). A previous study with two Cry1Ab-resistant *O. nubilalis* strains selected under laboratory conditions indicated that Cry1Ab resistance was polygenic in these strains.[4] It is important to mention that results from direct tests used to rule out monogenic inheritance do not consider other mutations at the same locus that might impact susceptibility and result in deviations from the model with a single locus and two alleles. In addition, results from polygenic models are approximate because one of the assumptions used to generate the polygenic models is that inheritance of resistance is additive, yet resistance was shown to be incompletely recessive in SKY. Therefore, the present results indicate that Cry1Ab resistance in the SKY strain is complex and cannot be explained by a single locus with two alleles.

The degree of dominance D calculated on the basis of LC_{50} values was −0.45 ($h' = 0.27$), indicating that resistance was incompletely recessive. The resistance ratios of FIELD♂ × SKY♀ and FIELD♂ × SKY♀ were only 7.2- and 3.9-fold respectively, in contrast to a resistance ratio of 815-fold in the resistant strain. The differences between the two reciprocal crosses were therefore small considering the magnitude of resistance in SKY (815-fold). More importantly, the calculations of effective dominance h_c indicated that dominance was dependent on toxin concentration, with resistance being nearly recessive at the concentration corresponding to 6 times the diagnostic concentration used in monitoring for resistance in field populations.

Greenhouse tests indicate that dominance may vary depending on plant stage. Vegetative-stage MON810 plants infested with SKY tended to exhibit higher Guthrie ratings (Table 6), although no survivors were found on this stage. However, larvae that survived on reproductive-stage corn were found feeding on silk, ear shanks, ear tips, kernels and pollen accumulated in leaf axils (Tables S2 and S3 supplementary data). A seasonal analysis of tissue-specific expression indicated that Cry1Ab levels in leaves of MON810 plants were not reduced during the reproductive stage,[6] which suggests that resistant insects likely used other plant parts with lower levels of Cry1Ab expression such as pollen, silk, kernels and ear shank.[5–7] In addition, results from pollen and silk assays corroborated on-plant assays, suggesting that resistant insects were able to survive on tissues with lower Cry1Ab expression. The results are consistent with the Cry1Ab expression profile of reproductive tissues of various Cry1Ab corn hybrids including Event 176, MON810 and *Bt*11.[5,42] Event 176 exhibits relatively high levels of Cry1Ab expression in pollen and poor expression in non-green tissues such as the pith and kernel.[42] MON810 and *Bt*11 were engineered with a promoter to express toxin throughout the plant, although at low levels in pollen.[5] Susceptible insects died on pollen of Event 176, whereas SKY exhibited high survival. In contrast, silk from MON810 and *Bt*11 caused 100% mortality to susceptible insects but very low mortality to SKY (Table 5). Similar results were obtained with Asian corn borer, *Ostrinia furnacalis* (Guenée) (Lepidoptera: Crambidae), which exhibited some ability to survive after 3 days feeding on silk and kernels of MON810 plants but no survival on leaf tissue.[7] Two factors may contribute to increased survival of *O. nubilalis* on reproductive-stage corn. The suitability of the corn plant as a larval host changes as the plant matures, favoring *O. nubilalis* larvae establishment and survival.[43] In addition, the second generation of *O. nubilalis* larvae are exposed to a mixture of leaves and reproductive tissues that may favor the survival of resistant insects that initiate feeding on low-Cry1Ab-expressing tissues.

On reproductive-stage isoline plants, the resistant and F_1 hybrid larvae exhibited the lowest and the highest fitness respectively (Table 6 and Fig. 5B). It is unclear whether the reduced fitness is a consequence of resistance or whether inbreeding associated with repeated selection may have adversely affected fitness. Importantly, this fitness deficit was not apparent in the F_1 progeny, which exhibited higher fitness than either parental strain. This hybrid vigor indicates genetic differences between resistant and susceptible strains that may have been caused by genetic drift or selection.[44] Similar results with on-plant studies were obtained with Cry1F-resistant *O. nubilalis*[8] and Cry1Ab-resistant *Diatraea saccharalis* (F.) (Lepidoptera: Crambidae).[45] In both studies, the F_1 hybrid larvae exhibited higher survival on isoline plants, although this pattern was not consistent for *D. saccharalis* among all isolines tested.[45] To account for genetic differences, fitness comparisons of *O. nubilalis* strains or crosses were based on the percentage reduction in weight and survival of larvae feeding on Cry1Ab-expressing plants in relation to these phenotypic characteristics for each type feeding on isolines.[8] Calculations of degree of dominance based on reduction in weight of surviving larvae and on larval survival and larval weight combined revealed that resistance was incompletely recessive, as estimates were between 0.17 and

0.31 (Table 7). The present findings are similar to those reported for F_1 hybrid progeny from Cry1Ab-resistant *D. saccharalis*, which exhibited higher survival than susceptible insects on Cry1Ab-expressing corn plants.[45] However, for Cry1F-resistant *O. nubilalis*, resistance was functionally recessive for both vegetative and reproductive stages.[8]

The present study is the first attempt to evaluate the assumption of functional recessive inheritance for resistance to Cry1Ab in *O. nubilalis*. These results indicate that, for vegatative-stage plants, the resistance identified in the SKY strain is functionally recessive. Therefore, progeny from the second generation, which may have completed development on transgenic plants, are unlikely to survive on high-Cry1Ab-expressing tissues of vegetative-stage plants. This does not preclude an increased frequency of Cry1Ab resistance alleles for the second generation, where the resistance does not appear to be functionally recessive. However, it should also be noted that survival was measured at 15 days after infestation and does not reflect survivorship to the adult stage. Because the development of the F_1 progeny was significantly delayed on the reproductive-stage plants, it is likely that a much higher proportion of larvae would be unable to complete development than reflected by the 15 day survival.

These results should be interpreted with caution because *O. nubilalis* genotypes were not evaluated throughout their life cycle. Therefore, to test the assumption of recessive inheritance fully, more studies are necessary to verify the number of fertile progeny produced by moths that complete development on transgenic plants. Other resistance-delaying effects not tested in the present study may ameliorate the risk of *O. nubilalis* resistance to Cry1Ab. Reproductive fitness costs and low survival of diapausing insects, as observed in overwintering *Leptinotarsa decemlineata* (Say) (Coleoptera: Chrysomelidae)[46] and *Pectinophora gossypiella* (Saunders) (Lepidoptera: Gelechiidae),[47] may counterbalance the effects of incomplete recessive inheritance observed for reproductive-stage plants.

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SUPPORTING INFORMATION

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

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Table S1. Responses to Cry1Ab of progeny from four backcross combinations obtained from F_1 progeny and resistant strain (SKY)

Backcross (F_2)	n[a]	Slope ± SE	LC_{50} (95% FL)[b]	χ^2 (df)
A = (FIELD♂ × SKY♀)♂ × SKY♀	234	2.3 ± 0.3	141 (84–223)	7.2 (3)
B = SKY♂ × (FIELD♂ × SKY♀)♀	234	2.5 ± 0.3	120 (49–223)	3.8 (3)
C = (SKY♂ × FIELD♀)♂ × SKY♀	266	2.0 ± 0.3	151 (118–196)	2.8 (4)
D = SKY♂ × (SKY♂ × FIELD♀)♀	266	2.2 ± 0.3	108 (70–167)	6.0 (4)

[a] Probit models were fit using lower Cry1Ab concentrations that caused mortality response of 20, 40, 80, 160, 320 and 563 ng cm^{-2}.

[b] Units are ng Cry1Ab cm^{-2} diet, with 95% fiducial limits in parentheses.

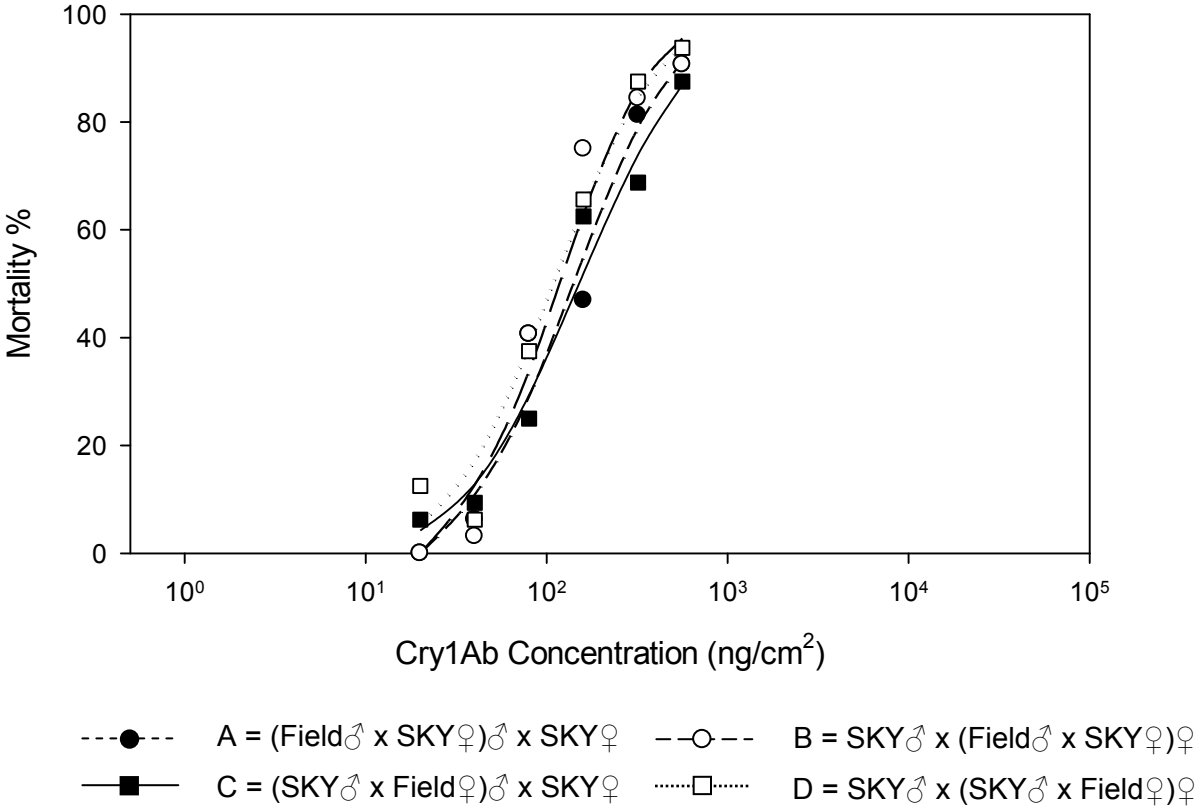

Figure S1. Concentration–mortality curves of neonate larvae originated from four backcross combinations obtained from F_1 progeny and the resistant strain (SKY).

Table S2. Distribution (%) of larvae from KY (susceptible), F_1 (KY × SKY) and SKY (resistant) among plant parts of negative isoline plants and *Bt* corn

	Isoline			MON810		
Plant part	KY	F_1	SKY	KY	F_1	SKY
Ear	51.5	52.9	39.0	0.0	31.8	55.5
Silk	13.0	17.2	50.3	100.0[a]	18.2	30.9
Leaf	24.8	22.7	10.7	0.0	43.9	13.6
Stalk	10.6	7.2	1.1	0.0	6.1	2.7

[a] Only two susceptible larvae survived on silk of *Bt* corn.

Table S3. Distribution (%) of larvae from KY (susceptible), F_1 (KY × SKY) and SKY (resistant) among leaf parts of negative isoline plants and *Bt* corn

	Isoline			MON810		
Leaf Part	KY	F_1	SKY	KY	F_1	SKY
Mid-rib	12.2	6.1	0.0	0.0	17.2	13.3
Blade	1.2	0.0	0.0	0.0	0.0	0.0
Collar	18.3	11.0	60.0	0.0	3.4	13.3
Axils[a]	19.5	34.1	5.0	0.0	69.0	13.3
Sheath	48.8	48.8	35.0	0.0	10.3	60.0

[a] Pollen was found accumulated in leaf axils.