

Estimated Frequency of Nonrecessive *Bt* Resistance Genes in Bollworm, *Helicoverpa zea* (Boddie) (Lepidoptera: Noctuidae) in Eastern North Carolina

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ABSTRACT In summer 2000, adult female bollworm moths, *Helicoverpa zea* (Boddie), were collected from light-traps at four locations near the Tidewater Research Station, Plymouth, NC. Female moths were allowed to lay eggs, and at hatch, 72 larvae from each female were screened for growth rate on normal artificial diet and on diets containing 5.0 μg of either Cry1Ac or Cry2Aa *Bt* toxin per milliliter of diet. The growth rate bioassays were performed to isolate nonrecessive *Bt* resistance genes present in field populations of bollworm. We found one individual out of 583 screened that appeared to carry a major gene for resistance to Cry1Ac. Assuming four alleles per individual, the gene frequency is 1/2332 or 0.00043. Other females appeared to have minor genes for Cry1Ac resistance or major genes with lower levels of dominance. We also found one individual out of 646 screened that appeared to carry a major gene for resistance to Cry2Aa. The gene frequency for Cry2Aa resistance was estimated at 1/2584 or 0.00039. Again, other females seemed to carry additional minor resistance genes. Along with other results that indicate partially dominant inheritance of Cry1Ac resistance in bollworm, these allele frequency estimates are important for determining the rate of resistance evolution in *H. zea* to specific *Bt* toxins.

KEY WORDS *Bacillus thuringiensis*, *Bt* resistance, gene frequency, bollworm

TRANSGENIC COTTONS, SPECIFICALLY those containing the *Bt* endotoxin, have been widely planted in recent years. These *Bt* cottons provide an efficient method of pest management in areas where heliothines (*Heliothis virescens* [F.], tobacco budworm, and *Helicoverpa zea* [Boddie], bollworm) are key target pests. However, many researchers are concerned that the utility of this method may be short-lived because of pest evolution of resistance to transgenic *Bt* cottons (Tabashnik 1994, Kennedy and Whalon 1995, Gould 1998). To be successful in controlling *Bt* resistance evolution in heliothine pests of cotton, the cotton plants must express a high dose of toxin that kills susceptible and partially resistant insect pests. Recent laboratory and field studies have shown that this is not the case for the bollworm (Burd et al. 1999, Lambert et al. 1996, 1997, Mahaffey et al. 1994, 1995). Also, recent laboratory studies have shown that resistance to the Cry1Ac endotoxin for the bollworm may be inherited as a dominant or partially dominant trait

(Burd et al. 2000); this poses another risk to the sustainability of *Bt* cotton. It is important that we understand the ecological and genetic traits of the *Bt* cotton/bollworm interaction so that appropriate management strategies for the deployment and sustainability of *Bt* cotton can be developed. Determining these parameters will allow for the conceptualization and implementation of refuge strategies that take full advantage of this new technology.

This current study focuses on the assumption that the initial gene frequency for *Bt* resistance in bollworm is extremely low. Based on lab experiments from 1999, we were able to screen the progeny of field-collected female bollworm moths on a dose of Cry1Ac and a dose of Cry2Aa toxins that discriminated between resistant and susceptible groups of larvae. This screening process led to estimates of frequencies of resistance alleles in bollworm populations in eastern North Carolina.

Materials and Methods

Collection of Insect Strains. In August–October 2000, a total of 2155 adult female bollworm moths were collected from light-traps at four locations in eastern North Carolina near the Tidewater Research Station,

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Plymouth, NC. Adult females were placed individually into a 237-ml clear plastic cup and covered with cheesecloth to provide a substrate for egg laying. Moths were kept at 27–30°C, 55–60% RH and a photoperiod of 14:10 L:D hours. Cheesecloths were monitored daily for the presence of eggs. A total of 646 female moths (families) laid sufficient fertile eggs (≥ 72) to be used for the bioassays. Progeny from the first 63 female families bioassayed were placed on a lower concentration of Cry1Ac diet than the remaining females used (2.5 versus 5.0 μg of toxin/ml of diet) and were not included in the overall analysis for the Cry1Ac families. Therefore, only 583 female families were used for the analysis of Cry1Ac resistance.

Bioassays on *Bt* and Non-*Bt* Diets. Seventy-two neonates (0–12 h old) from each female line were placed onto each of three different diets (24 on each diet). These diets were non-*Bt* (NBT) (see Gould et al. 1995 for details), Cry1Ac-containing diet, and Cry2Aa-containing diet. The concentration of both *Bt* toxins was 5.0 μg /ml of diet. Previous bioassays allowed us to determine this discriminating dose (A.D.B., unpublished data). Originally, the toxin concentration for the Cry1Ac diet was 2.5 μg /ml of diet that was based on lab studies of moths collected in 1999. It was determined that this concentration was not high enough to separate out phenotypes based on our moths collected in 2000. There seemed to be very little effect on tested neonates and survival was always near 100% which is why the dose was raised to 5.0 μg /ml. Cry2Aa was purified from the NRD-12 isolate of *Bacillus thuringiensis* subsp. *kurstaki* and was expressed in a recombinant *Escherichia coli* strain (Moar et al. 1994). Cry1Ac was obtained as a gift from Mycogen Corp. in a formulation of their product, MVP II. The diet was poured into 24-well plastic bioassay plates (Corrigan and Company Inc., Jacksonville, FL) and was then stored in the refrigerator before use. A single neonate was placed into each well using a fine hair paintbrush, and plates were heat-sealed with Mylar film. Needle holes in the Mylar film above each well provided air exchange. Larvae were scored based on developmental stage after seven days. Instar was determined based on head capsule and body size (Neunzig 1969). All growth stage values were converted to an ordinal ranking system where 1 = first instar, 2 = early second, 3 = mid second, 4 = late second, 5 = early third, 6 = mid third, 7 = late third, 8 = early fourth, 9 = mid fourth, 10 = late fourth and 11 = early fifth.

Selection of Survivors. Survivors from the original bioassays were used for subsequent selection experiments. Any families that performed just as well on either *Bt* diet as they did on NBT diet were saved and rescreened. It was assumed that families performing as well on either or both of the *Bt* diets as on NBT diet were carriers of at least one resistance allele. These families were mass mated among themselves and selected on the appropriate *Bt* diet (5.0 μg /ml) the next generation. Several families that had no survivors on either *Bt* diet but performed well on NBT diet were also saved. These were used as control families for

subsequent tests. F_1 crosses were made between these control families and survivors from selected families reared on *Bt* diet to determine possible inheritance of resistance. A single family from Cry1Ac bioassays that had $\approx 50\%$ of its larvae as large as the control larvae (family #68) was saved and emerging adults were used in single pair matings in the next generation. The following matings were used for this family: 68♀ \times 68♂ (RS \times RS), control♀ \times 68♂ (SS \times RS) and 68♀ \times control♂ (RS \times SS). A control♀ \times control♂ cross (SS \times SS) was made for comparison to other crosses. Neonates from successful matings were placed on Cry1Ac diet containing 5.0 μg /ml of toxin. These larvae were weighed after 10 days.

Data Analysis. PROC UNIVARIATE was used to determine average ordinal ranking for each single female family on each of the three diets (SAS Institute 1990). PROC CORR was also used to determine if there was a correlation between instar size on any combination of two of the three diets. It is possible that some families that were larger on *Bt* diet attained this size because of vigor effects and not necessarily resistance to the toxin; therefore, we would expect these families to perform very well on NBT diet and also on the *Bt* diet. To control for these vigor effects, average ratings for all families on either *Bt* diet were standardized according to the highest rated family on NBT diet. All correlation tests used the Pearson correlation coefficient to determine significance at the $\alpha = 0.05$ level. Although original *P* values are still given in Table 1, a separate test according to Rice (1989) was used to enhance power and to dismiss the possibility of incorrectly rejecting one or more true null hypotheses. The test used was the sequential Bonferroni technique described by Holm (1979). The only significant correlation that would have changed was the average corrected ratings for families on Cry2Aa versus average rating for families on NBT. Therefore, no inferences will be made for this comparison based on the lack of significance. Mean larval weights from various selection experiments were separated using Fisher Least Significant Differences test with PROC GLM in SAS (SAS Institute 1990).

Results

Bioassays. A total of 583 female families were screened on Cry1Ac diet. The average rating for all larvae on Cry1Ac diet was ≈ 4.0 (Fig. 1a), which corresponds to a late second instar. Bioassays on Cry1Ac revealed one family with most surviving individuals as large as control larvae. The average rating of this single family was 7.4, corresponding to a late third to early fourth instar. Ten out of twenty-four larvae from this family survived on the *Bt* diet. The controls (NBT) for this family ranged from late third to mid fourth instar with a mean rating of 8.25. Survivorship for the family was 83% on control diet.

The average rating for all larvae on Cry2Aa diet was ≈ 3.0 (Fig. 1b), which corresponds to a mid second instar. Bioassays from Cry2Aa families also revealed one family with most surviving individuals as large as

Table 1. Correlation of selected traits among bollworm families selected on each of three diets (Cry1Ac, Cry2Aa, and NBT)

Comparison	r	P ^a
Average Ratings for Families on Cry1Ac vs Average Ratings for Families on Cry2Aa	0.38	<0.01
Average Rating for Families on Cry1Ac vs Average Rating for Families on NBT	0.12	0.055
Average Rating for Families on Cry2Aa vs Average Rating for Families on NBT	-0.01	0.89
% Survival for Families on Cry1Ac vs % Survival for Families on Cry2Aa	0.52	<0.01
% Survival for Families on Cry1Ac vs % Survival for Families on NBT	-0.12	0.06
% Survival for Families on Cry2Aa vs % Survival for Families on NBT	0.01	0.90
% Survival for Families on Cry1Ac vs Average Rating for Families on Cry1Ac	0.34	<0.01
% Survival for Families on Cry2Aa vs Average Rating for Families on Cry2Aa	0.13	<0.01
% Survival for Families on NBT vs Average Rating for Families on NBT	0.04	0.46
Average Corrected Ratings for Families on Cry1Ac vs Average Corrected Ratings for Families on Cry2Aa	0.39	<0.01
Average Corrected Ratings for Families on Cry1Ac vs Average Rating for Families on NBT	-0.02	0.75
Average Corrected Ratings for Families on Cry2Aa vs Average Rating for Families on NBT	-0.14	0.02

^a Probability > |r| under H₀; Rho = 0. See note in Data Analysis section concerning adjustment of P-values to increase power.

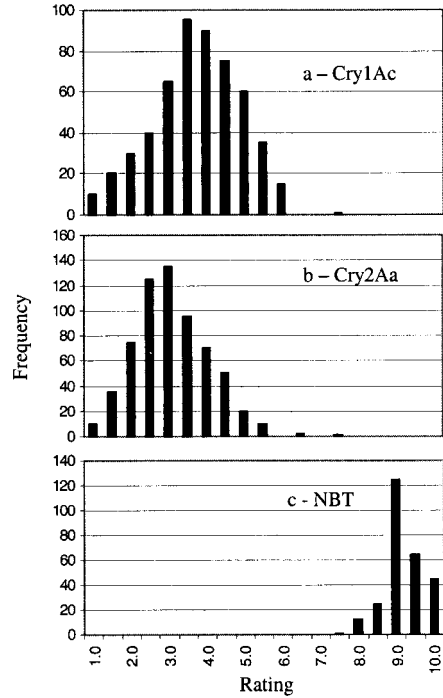


Fig. 1. Distribution of average rating for 7-d old larvae of *H. zea* female families on Cry1Ac, Cry2Aa, and NBT diets. Mean ratings are ≈4.0, 3.0 and 9.0, which corresponds to a late second instar, mid second instar and mid fourth instar, respectively.

controls. The average rating for this family was 6.5, which is between a mid third and late third instar. Nineteen out of 24 larvae from this family survived on the Cry2Aa diets and individual larvae ranged from early second instar to mid fourth instar. The controls (NBT) for this family ranged from late third to mid fourth instar with a mean rating of 8.55 and survival of 83%. The average rating on NBT diet was ≈9.0 (Fig. 1c), which corresponds to a mid fourth instar.

There was a significant correlation between instar ranking of female families on Cry1Ac and Cry2Aa diets (Table 1; Fig. 2) indicating that families with larger than average individuals on one diet had larger than average individuals on the other diet as well. There was no correlation between average ratings when comparing Cry1Ac or Cry2Aa versus NBT.

There was also a significant positive correlation for percentage seventh day survival of families on Cry1Ac diet and Cry2Aa diet (Table 1) indicating those families with higher survival on one *Bt* diet had higher survival on the other *Bt* diet. There was positive correlation between percentage seventh day survival and average rating for families on Cry1Ac and Cry2Aa (Table 1) showing that as average family rating increased so did percentage seventh day survival for families on both of these *Bt* diets. There was no correlation between percent seventh day survival and average rating for families on NBT diet.

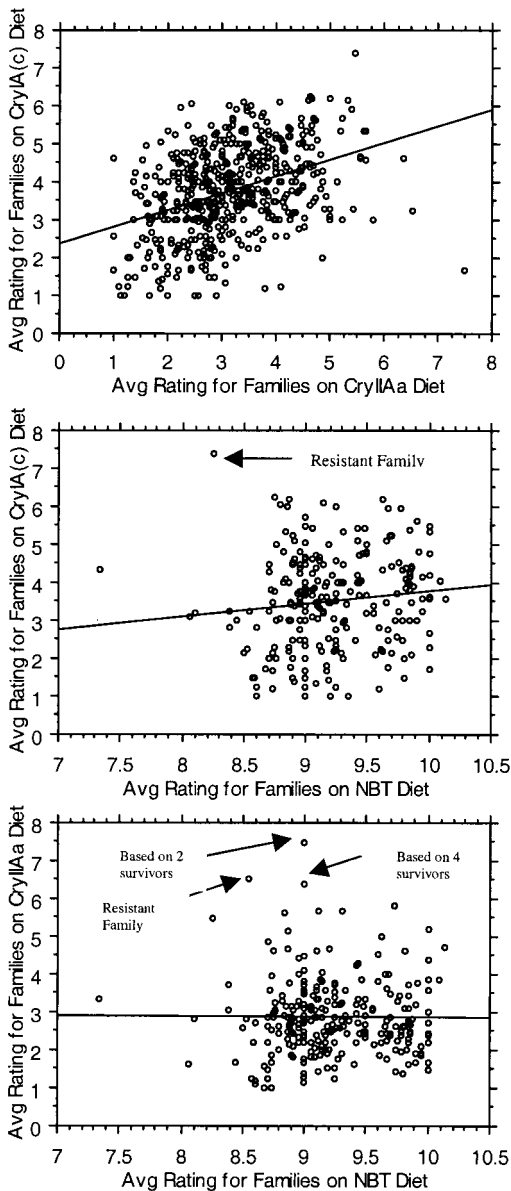


Fig. 2. Correlation graphs for all comparisons among Cry1Ac, Cry2Aa, and NBT families with average rating plotted for each of the families tested.

The positive correlation between the two *Bt* diets remained when examining ratings corrected for "vigor" (Table 1). Average corrected ratings for Cry2Aa families were negatively correlated with average uncorrected rating on NBT diet, but were not correlated on diet containing Cry1Ac.

Selection Experiments. The average larval weight for the 68♀ × 68♂ cross (RS × RS) was 27.56 mg with survival of 71%. The average weights for the 68♀ × control♂ (RS × SS) and the control♀ × 68♂ (SS × RS) crosses were 16.74 mg and 12.53 mg, respectively,

with survival of 49% and 60%, respectively. All control larvae once again died on the 5.0 µg/ml Cry1Ac diet.

The largest and smallest individuals that survived on the Cry1Ac diet from the reciprocal crosses were saved and used to test for heritability. Large females were grouped with large males (four successful single pairs) and likewise small females with small males (two successful single pairs) using single pairs matings. Larvae from the large individuals weighed 24.67 mg (with 86% survival) while larvae from small individuals weighed 1.88 mg (with 15% survival).

Individual survivors from the single family that performed very well on Cry2Aa were saved and mass mated with one another. Progeny from these individuals were placed on Cry2Aa diet containing 40.0 and 200 µg of toxin per milliliter of diet. Percentage seventh day survival on these two doses was 57% and 54%, respectively. Because of a limited supply of Cry2Aa toxin, no other inheritance experiments were performed with this line, which is currently being selected on 20.0 µg/ml of Cry2Aa.

Discussion

Based on our lab studies from 1999 (Burd et al. 2000), we concluded that inheritance for resistance to Cry1Ac toxin was dominant or incompletely dominant and may have been because of a single allele. Therefore, individuals that were heterozygous for resistance alleles were likely to survive when screened on a discriminating dose of this toxin in artificial diet. Statistically, the most probable carrier for a resistance gene in current field populations would be a heterozygote. Also the most probable mating would occur between heterozygote individuals and homozygote susceptible individuals. With this in mind, the likely offspring from this cross would be one-half heterozygote and one-half homozygote susceptible. From this, we assumed that if a screened line had 50% of the individuals that were the same size as their NBT counterparts then this would be considered a resistant line.

Screening 583 total females on Cry1Ac diet allowed us to characterize 2332 alleles because each mated female carries two of her own alleles and two from her male counterpart. With this in mind, our estimated gene frequency for the resistance gene to Cry1Ac toxin in family #68 would be 1/2332 or 0.00043 (95% confidence limits are 0.00001 to 0.00239). It should be noted that this estimate is conservative. As seen in Fig. 1, many female families had higher than average growth on Cry1Ac diet (i.e., the entire family reached third instar but was not quite as large as the controls). This may be a result of the presence of minor genes for Cry1Ac resistance or major genes with lower levels of dominance. In either case, this would serve to increase our estimate of the initial gene frequency for Cry1Ac resistance. Family #68, which produced large larvae on Cry1Ac diet, had a total of 10 out of 24 individuals survive. This is consistent with the 50% that we expected. Also, all survivors were consistent in size with their NBT counterparts.

Because 646 female families were screened on Cry2Aa diet, the number of alleles we actually tested was 2584. Therefore, our estimated gene frequency for resistance to Cry2Aa toxin would be $1/2584$ or 0.00039 (95% confidence limits are 0.00001 to 0.00216). Again, this is a conservative estimate based on the fact that other female families performed above average and may have been carrying minor genes or major genes with lower levels of dominance. This would also increase our estimate of initial gene frequency for Cry2Aa resistance. The one family that survived from the Cry2Aa bioassays had 19 out of 24 survivors. This is greater than the 50% that we expected, however, six of these individuals were mid second stage and smaller. Therefore, only 13 out of 24 individuals were as large as the control counterparts. It should be noted that Figs. 1 and 2 show Cry2Aa families that had average ratings of 7.5 and 6.4; but these ratings are based on survival of only two and four individuals, respectively, out of 24.

It is widely known that field populations of bollworms and tobacco budworms may often mate multiple times (Hendricks et al. 1970, Lamunyon 2000). Therefore, it is possible that the total number of alleles screened was much higher than our original estimates. However, according to Hendricks et al. (1970), in most cases females mated only once and in their specific case over 66% of the females carried a single spermatophore. Regardless of the number of times a female has mated, if sperm precedence is present then genetic material from only one male will contribute to offspring. Lamunyon (2000) suggests that even though female budworms mate multiply times, a single male will typically gain sperm precedence. Therefore, the possibility exists that more than four alleles were being characterized per mated female in our study. However, based on the research mentioned above, it is most likely that a large proportion of the females mated only once or that sperm precedence would still allow for only two alleles from the male to be contributed to offspring.

As seen in Fig. 2, individuals screened on NBT diet from both resistant families (one from Cry1Ac and one from Cry2Aa bioassays) were in the lowest 5% of their respective groups when examining average ratings. This suggests that a fitness cost may be present for individuals carrying major resistance genes.

Positive correlations between percentage seventh day survival and average rating for families on both *Bt* diets suggests that individuals carrying resistance genes will grow larger and have higher rates of survival when selected on their appropriate diet. Although many families had average ratings close to the chosen resistant families, especially for Cry2Aa bioassays, their survival was typically much lower than that of the chosen resistant line. Only families that had at least 50% survival were considered carriers of a major resistance gene.

According to correlation analyses from the original ratings and from the corrected ratings, vigor effects did not seem to play a role in the above average performance of some families on either *Bt* diet. If there

were strong positive correlations between ratings on NBT diet and ratings on either *Bt* diet (which would suggest vigor effects), then it would be difficult to attribute higher ratings of our larger sized families to resistance alone. Because this was not observed in our trials, we may conclude that genetically based resistance played the major role for *Bt* families attaining the largest sizes. It should also be noted that there was a positive correlation (for both corrected and uncorrected data) between families on Cry1Ac diet and those on Cry2Aa diet. These data, along with survival data, suggests that if a family performed better on one of the *Bt* diets then it performed better on the other *Bt* diet as well. This suggests low levels of cross-resistance are present in field populations of bollworms.

Based on our selection experiments with survivors from family #68, it appears that inheritance of resistance to Cry1Ac is incompletely dominant. Assuming our survivors from the original screen of family #68 were heterozygous for resistance, then matings between these survivors would produce 1:2:1 ratio of homozygous resistant:heterozygous resistant:homozygous susceptible. Matings between line #68 and a control individual would produce 50% heterozygotes and 50% homozygous susceptibles. Our results were consistent with expected survivorship if the resistance was inherited as a partially dominant gene. Also, subsequent matings with large \times large individuals and small \times small individuals obtained from the reciprocal crosses between line #68 and the control strain revealed that large \times large individuals weighed significantly more than small \times small individuals. The large \times large group was most likely the result of a cross between two heterozygotes and therefore weight and survival were consistent with that of the original cross of line #68 with itself. The small \times small group was presumed to be homozygous susceptible and therefore would all die when screened on Cry1Ac diet. Although there was limited survival, distribution of log weights was consistent with that of other individuals assumed to be susceptible.

Results from selection experiments and reciprocal cross experiments reported in this paper are consistent with those reported previously (Burd et al. 2000). Both of these studies indicated that resistance to Cry1Ac is inherited as a dominant or incompletely dominant trait. Along with the results from our current gene frequency estimates, this allows us to quantify certain parameters that are typically estimated from theory when modeling evolution of *Bt* resistance in field populations. If the alleles for resistance are as dominant and high as indicated by our results, then properly structured refuge systems become critically important for transgenic *Bt* cotton technology to be sustainable. It should be noted that our estimates of gene frequency and our finding of dominance is dependent upon the discriminating dose of toxin used in this current study. The dominance would correctly be characterized as functional dominance based on the dose used. However, based on previous work (Burd et al., unpublished data) it was determined that resistance to Cry1Ac was also dominantly inherited at a

dose of 100 $\mu\text{g}/\text{ml}$. Greenhouse tests also revealed that individuals originally characterized as resistant through lab bioassays readily survived on *Bt* cotton plants. Therefore, based on all data collected, it appears that the dominance may very well be genetically based dominance because heterozygotes could survive on transgenic cotton plants which were expressing the CryIAc toxin at much higher levels than the discriminating dose used in the current study.

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