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BREEDING AND GENETICS

Genetic Basis for Variability of Cry1Ac Expression Among Commercial Transgenic *Bacillus thuringiensis* (Bt) Cotton Cultivars in the United States

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ABSTRACT

The amount of Cry1Ac δ-endotoxin in transgenic *Bacillus thuringiensis* Berliner (Bt) or Bollgard cotton varies among commercial cultivars. These differences in expression have been correlated with survival levels in Lepidoptera, indicating that all Bollgard cultivars do not provide the same level of control. The objective of this study was to determine if differences in overall expression among commercial cultivars of Bollgard cotton were under simple genetic control. These findings could influence the way breeders select cultivars by evaluating for efficacy in insect control in addition to agronomic traits. Two sets of crosses were made in the greenhouse with cultivars that express the endotoxin at high and low levels. The parents and F_1 and F_2 generations were planted in the field. The amount of Cry1Ac was quantified using a commercial ELISA kit. Variances within the two F_2 breeding populations were highly significant because of genetic segregation for Cry1Ac expression. Using the modified Castle-Wright formula, the estimation of the number of contributing genes in both breeding populations was small. These data show that genetic background has a major effect on Cry1Ac expression. Because backcrossing is the primary method used by commercial cotton breeders, the selection and use of donor and/or recurrent parents that will result in a high level of Cry1Ac expression is crucial.

Since 1996, transgenic cotton plants containing the Cry1Ac δ-endotoxin from the soil bacterium, *Bacillus thuringiensis* Berliner (Bt) (Bollgard, Monsanto Co., St. Louis, MO), have been used as a tool to selectively manage lepidopteran pests. Growers and researchers have noted that many lepidopteran pests are not adequately controlled with this technology (Fitt et al., 1994; Bacheler and Mott, 1997; Smith, 1998; Fitt, 1998). Although this technology is highly effective against the tobacco budworm [*Heliothis virescens* (F.)], and the pink bollworm [*Pectinophora gossypiella* (Saunders)] (Williams, 2000), supplemental foliar insecticide applications have been used in some Bollgard fields to control fall armyworms [*Spodoptera frugiperda* (J. E. Smith)], beet armyworms [*Spodoptera exigua* (Hübner)], corn earworms, [*Helicoverpa zea* (Boddie)], and the Old World pests, *Helicoverpa armigera* (Hübner) and *Helicoverpa punctigera* (Wallengren) (Bacheler and Mott, 1997; Roof and DuRant, 1997; Fitt, 1998; Smith, 1998; Burd et al., 1999).

All cultivars and plant structures of Bollgard cotton do not provide the same level of lepidopteran control. Overall levels of expression among Bollgard cultivars have been correlated to survival levels in various lepidopteran pests that are intrinsically tolerant to Cry1Ac (Adamczyk et al., 2001). In particular, differences in larval survival of corn earworms and larval development of fall armyworms were correlated to differential expression of Cry1Ac in various plant structures among commercial cultivars of Bollgard cotton (Adamczyk et al., 2001). Profiling season-long expression of Cry1Ac in Bollgard cultivars has also shown that Cry1Ac levels decrease as the plant ages (Fitt, 1998; Sachs et al., 1998; Greenplate et al., 2000; Adamczyk et al., 2001). Holt (1998) correlated this decline in Bollgard cultivars to increased survival of *H. armigera*. Furthermore, season-long differences in expression among cultivars can vary as much as 2-fold throughout the season (Adamczyk et al., 2001), and plant structures, such as terminal leaves, express more Cry1Ac compared with certain flower structures (Greenplate, 1999; Greenplate et al., 2000; Adamczyk et al., 2001; Gore et al., 2001). Therefore, control strategies may be further compromised by the differential expression of Cry1Ac among plant structures and cultivars that create a spatial source for survivors to develop. Factors that

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influence the level of expressed Cry1Ac among Bollgard cultivars are still not fully understood, but the location of the gene insertion and genetic background have been implicated (Sachs et al., 1998).

Strategies for management of insect resistance to Cry1Ac in Bollgard cotton are now mandated by the Environmental Protection Agency (EPA) in the United States. This strategy involves planting a refuge of non-Bt cotton as an environment for susceptible moths to mate. In addition, transgenic plants must express Cry proteins more than 25-times the LD_{99} of a given lepidopteran pest so that survivors are rare and heterozygous individuals are rendered functionally recessive (i.e. high-dose strategy) (Gould, 1998). Currently, only the tobacco budworm and pink bollworm are managed using this high-dose strategy with Bollgard cotton in the United States (MacIntosh et al., 1990). Cotton pests, such as the corn earworm, are only sub-lethally controlled with Bollgard technology (Jenkins et al., 1992). Consequently, expression levels in Bollgard cultivars must be properly measured prior to commercialization to ensure that the level of Cry1Ac provides a high-dose to adequately control certain lepidopteran pests.

The objective of this research was to determine if differences in expression among Bollgard cultivars are under genetic control. These studies are necessary to determine if breeding and selection of transgenic crops can be based on differential expression of plant-insect resistance in addition to agronomic traits.

MATERIALS AND METHODS

Crosses. To determine if Cry1Ac expression differences among Bollgard cultivars are under genetic control, crosses were made from plants derived from distantly related backgrounds. 'Stoneville 4691B' (Stoneville Pedigree Seed Co., Collierville, TN) and a 'Paymaster 1218BR' (Delta and Pine Land Co.; Scott, MS) were chosen to cross with two other cultivars (NuCOTN 33B and DP 458BR; Delta and Pine Land Co.). Both NuCOTN 33B and DP 458BR express significantly more Cry1Ac in all plant structures than other cultivars grown in the mid-south of the United States, including ST 4691B and PM 1218BR (Adamczyk and Sumerford, 2001; Figure 1). NuCOTN 33B and DP 458BR were developed using the same recurrent parent (cv. DP 5415). Reciprocal crosses were made in the greenhouse between DP 458BR x PM 1218BR plants and NuCOTN 33B x ST 4691B plants. Seeds produced from the F_1 crosses were grown in the greenhouse and F_2 seed were harvested.

Parents, F_1 crosses, and the resulting F_2 generations for both of the above mentioned breeding populations were planted on 1 May 2002 in field plots located in Stoneville, MS. Breeding populations were planted adjacent to one another in the same field, which maintained identical growing conditions. Single row plots (5.0 m) were arranged in a randomized complete block design with 6 populations (i.e. P_1, P_2, F_1 (♀ x ♂), F_1 (♂ x ♀), F_2 (♀ x ♂), F_2 (♂ x ♀)) and 4 replications. The F_2 crosses had 3 plots in each block (i.e. extra levels of replication in anticipation that they would have the highest variances), while the parents and F_1 crosses only had one level of replication (i.e. single plot) per block. All plots were maintained according to local management practices.

Prior to the current investigation, Adamczyk and Sumerford (2001) investigated the within-season and site (i.e. different location and soil types) affect on Cry1Ac expression in eight Bollgard commercial cultivars. Seasonal and site variability was detected for date of sampling (F = 3.86; P = 0.008), site (F = 1.69; P = 0.207), and their interaction (F = 2.23; P = 0.075). The variation among the eight cultivars was greater (F = 77.19; $P < 0.001$), and the only significant interaction was cultivar x sampling date (F= 2.39; $P < 0.001$). The two cultivars, NuCOTN 33B and DP 458BR, whose recurrent parent was DP 5415, had about 1.5- to 2.0-fold higher Cry1Ac expression than the other six cultivars (Figure 1). Using variance component analysis, the single de-

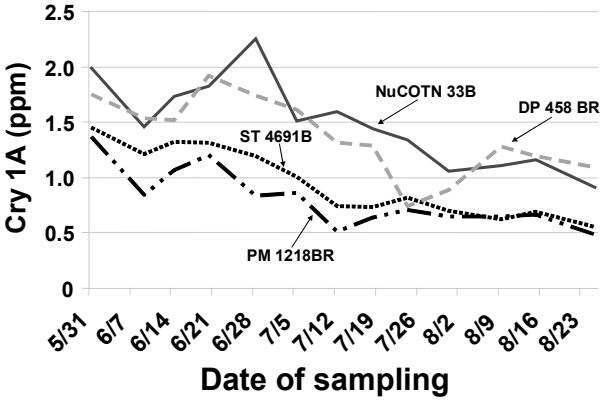

Figure 1. The amount of Cry1Ac produced in selected Bollgard cultivars throughout the growing season in Mississippi (adapted from Journal of Insect Science 1.13 at http://insectscience.org/1.13).

gree of freedom comparison of these two cultivars (NuCOTN 33B and DP 458BR) versus the other six cultivars accounted for 100% of the total variance of the cultivar differences. Based on these results (Adamczyk and Sumerford, 2001), additional site or location replications was not deemed necessary (i.e. cost prohibitive and time constraints) in these experiments; however, expression of Cry1Ac among the two breeding populations was measured at different crop growth stages throughout the 2002 season.

Quantification of CryAc1 δ-endotoxin. Levels of Cry1Ac were quantified using a commercially available kit (Envirologic, Inc., Portland, ME) and the method described by Adamczyk and Sumerford (2001). For each breeding population, all plots within a block were sampled at various times and at various crop growth stages (Table 1). Five to eight healthy plants from each plot were selected for Cry1Ac quantification. Terminal leaves were harvested from an individual plant per plot, placed in plastic bags, and transported to the laboratory in a cooler with ice. Within 1 h, three subsamples were excised from each leaf using a standard 6.0 mm diameter paper ticket punch. Samples were weighed to accurately determine the initial amount of leaf tissue (ca. 12-17 mg) and placed into 2.0 ml tubes containing 6.4 mm stainless-steel ball bearings (BioSpec Products, Inc., Bartlesville, OK). Cry1Ac extraction buffer (1.5 ml) (EnviroLogic, Inc., Portland, ME) was then added to the tube. The tissue was then homogenized for 30 s using a mini-beadbeater-8 (BioSpec Products, Inc., Bartlesville, OK). The tubes were then centrifuged at 10,000 rpm for 2 min and a 1:11 dilution (using Cry1A extraction buffer). A commercial quantification plate kit then was used to quantify the amount of Cry1Ac present for each cultivar (EnviroLogic, Inc., Portland, ME). For all dates, samples were plotted against a standard curve with Cry1Ab calibrators supplied in the kit. A simple conversion was used to express values as "Cry1Ac" as dictated by the kit protocol. The amount of Cry1Ac was expressed as parts per million (ppm) after accounting for the proper dilution factors.

Statistics. Because the F_2 lines had three plots in each block and every plot had measurements taken from several plants, an initial analysis was performed to estimate these components of variance pooled across all genotypes as follows:

$$\text{Total Variance} = \sigma^2_{\text{Plant}} + \sigma^2_{\text{Plot}} + \sigma^2_{(\text{Block*Genotype})}$$

For the NuCOTN 33B x ST 4691B cross, total variance = 4.39 + 0.46 + 0; and for the DP 458BR x PM 1218BR cross, total variance = 3.06 + 0.32 + 0.

For both breeding populations, PROC MIXED (version 8.2; SAS Institute, Cary, NC) was used to estimate these components of variance based on restricted maximum likelihood optimization (Littell et. al., 1996). In this analysis, genotype was a fixed effect and the random effects were block, block*genotype and plot (genotype block). In further analysis, variances were compared between genotypes. Based on the results of this preliminary analysis, the residual or σ^2_{Plant} was used for this comparison. The plot and block*genotype components of variance were ignored.

Table 1. Date of sampling and plant growth stage for the NuCOTN 33B x ST 4691B and DP 458BR x PM 1218BR breeding populations

Breeding population and replicate (block)	Date of sampling	Crop stage[z]
NuCOTN 33B x ST 4691B		
1	25 June 2002	2nd week of squaring
2	26 June 2002	2nd week of squaring
3	9 July 2002	2nd week of bloom
4	12 July 2002	2nd week of bloom
DP 458BR x PM 1218BR		
1	16 June 2002	1st week of squaring
2	24 June 2002	2nd week of squaring
3	2 July 2002	1st week of bloom
4	25 July 2002	Mid-bloom

[z] Breeding populations were planted as separate but adjacent experiments on 1 May 2002.

Table 2. Mean Cry1Ac expression and variation within each generation for the NuCOTN 33B x ST 4691B and DP 458BR x PM 1218BR breeding populations

Generation	No. plants tested	Mean Cry1Ac (ppm)	Variance
NuCOTN 33B x ST 4691B			
NuCOTN 33B	**20**	**9.15**	**1.7798**
ST 4691B	**19**	**2.54**	**0.9635**
NuCOTN 33B x ST 4691B F_1	**18**	**5.80**	**0.3479**
ST 4691B x NuCOTN 33B F_1	**20**	**5.40**	**1.2154**
NuCOTN 33B x ST4691B F_2	**78**	**6.00**	**6.1238**
ST 4691B x NuCOTN 33B F_2	**67**	**6.21**	**7.2813**
DP 458BR x PM 1218BR			
DP 458BR	**18**	**7.86**	**2.0927**
PM 1218BR	**19**	**3.04**	**1.5939**
DP 458BR x PM 1218BR F_1	**20**	**4.04**	**1.3818**
PM 1218BR x DP 458BR F_1	**20**	**4.20**	**1.3401**
DP 458BR x PM 1218BR F_2	**74**	**3.96**	**4.5795**
PM 1218BR x DP 458BR F_2	**78**	**4.69**	**3.8257**

The variance (σ^2_{Plant}) for each genotype in each breeding population is provided in Table 2. The variance of the two parental populations and the reciprocal F_1 populations were used to estimate environmental variability. The variance of the segregating reciprocal F_2 populations contained a genetic component, as well as an environmental component. The variance of the parents and the F_1 populations were combined to estimate the environmental variability. The variance of the F_2 populations was combined to estimate the environmental plus genetic variability. Based on chi-square test for homogeneity of variance, the variances that were combined were not significantly different with one exception.

Using one variance for the F_2 populations and another for the parents and F_1 populations combined, tests for dominance, epistasis, and inbreeding were performed based on *F*-test on linear functions of the genotypes means as described below using PROC MIXED (Version 8.2; SAS Institute, Cary, NC).

The estimation of non-additive gene action was characterized as dominance, epistasis, and inbreeding depression. The mean of the parents would be equal to the F_1 and F_2 means if gene action was just additive. Significant deviation from the expected was defined as dominance. If the only non-additive gene action was dominance, then the expected value of [(mean of P_1 plus the mean of P_2) + 2 x (mean of the F_1)/4] should equal the F_2 mean. Significant deviation of the mean of ¼ P_1 + ¼ P_2 + ½ F_1 from the mean of the F_2 was classified as epistasis. Inbreeding depression was conditioned by a decrease of heterozoygosity from 100% in the F_1 to 50% in the F_2 and significant inbreeding depression was an indication of dominance gene action.

Estimation on the number of major genes conferring expression differences was calculated using a modified Castle-Wright formula of Cockerham (1986). The variance/covariance matrix for variance (generations) was determined using PROC MIXED (SAS Institute, Cary, NC).

RESULTS AND DISCUSSION

The mean expression of Cry1Ac δ-endotoxin was different between the breeding populations of NuCOTN 33B x ST 4691B and DP 458BR x PM 1218BR ($F = 72.90$, $P < 0.0001$; F=31.87, P < 0.0001, respectively) (Table 2). Cry1Ac expression for NuCOTN 33B was 360% higher than for ST 4691B ($P < 0.001$), and expression for DP 458BR was 259% higher than for PM 1218BR ($P < 0.001$). These results are consistent with those previously reported by Adamczyk and Sumerford (2001) (Figure 1). The pooled within plot variance for the non-segregating populations of parents was 1.112 and 1.513 for the NuCOTN 33B x ST 4691B and DP 458BR x PM 1218BR breeding populations, respectively. The

Table 3. Generation mean comparison for the NuCOTN 33B x ST 4691B and DP 458BR x PM 1218BR breeding populations

Gene action	Differences	F-Value	Probability
NuCOTN 33B x ST 4691B			
Parents	**6.620**	**383.38**	**<0.001**
Reciprocal, F_1	**0.406**	**1.39**	**0.242**
Reciprocal, F_2	**-0.205**	**0.23**	**0.630**
Dominance, F_1	**-0.243**	**1.02**	**0.317**
Dominance, F_2	**0.261**	**0.23**	**0.651**
Inbreeding, F_1 vs F_2	**-0.503**	**0.86**	**0.397**
Epistasis	**-1.529**	**0.53**	**0.506**
DP 458BR x PM 1218BR			
Parents	**4.817**	**141.13**	**<0.001**
Reciprocal, F_1	**-0.158**	**0.03**	**0.685**
Reciprocal, F_2	**-0.735**	**5.06**	**0.026**
Dominance, F_1	**-1.334**	**22.58**	**<0.001**
Dominance, F_2	**-1.126**	**1.70**	**0.240**
Inbreeding, F_1 vs F_2	**-0.207**	**0.06**	**0.818**
Epistasis	**1.838**	**0.29**	**0.610**

F_2 variances for the breeding populations were 6.526 and 4.052, respectively. The larger, highly significant variances within the F_2 populations were due to the genetic segregation for Cry1Ac expression.

To further evaluate the significant genetic variation, generation mean analyses were calculated (Table 3). In the NuCOTN 33B x ST 4691B breeding population, the only significant comparisons were between the parents. Reciprocal differences or tests for non-additivity of gene action were not detected. The regression of mean Cry1Ac expression on the expected linear additive model produced a correlation of $r = 0.99$ for the NuCOTN 33B x ST 4691B, and a correlation of $r = 0.91$ for DP 458BR X PM 1218BR generation means. The results of the test for parental means versus F_2 was highly significant indicating negative dominance for Cry1Ac expression in their cross population (Table 3). The reciprocal difference between F_2 populations also was significant at $P = 0.03$.

Using the modified Castle-Wright formula by Cockerham (1986), an estimation of the number of major genes affecting the Cry1Ac levels for both breeding populations was low. Number of genes for NuCOTN 33B X ST 4691B was 0.98 and $\sigma^2 = 0.69$, and for DP 458BR X PM 1218 was 1.07 and $\sigma^2 = 0.278$. The assumptions for this analysis were (1) all genes have equal effects, (2) there was no linkage among genes, (3) there was no non-additive gene action, and (4) all plus genetic effects were from one parent. If any of the assumptions are not valid, the number of genes estimated is underestimated. In view of the small number of genes estimated, the only assumption showing caution in interpretation was the detection of dominance in the DP 458BR x PM 1218BR breeding population.

This study reinforces previous studies (Adamczyk and Sumerford, 2001) that show that genetic background has a major effect on Cry1Ac expression in Bollgard cultivars. As previously stated, both NuCOTN 33B and DP 458BR are derived from the same parental background (DP 5415), and the same number of major genes affecting the expression level across the two breeding populations was calculated even when sampling across the two breeding populations was conducted over different times in the season and during different crop growth stages. In a previous study (Adamczyk and Sumerford, 2001), the cultivar by environment interaction between two high expression parents (NuCOTN 33B and DP 458BR) and two low expression parents (ST 4691B and PM 1218BR) was negligible relative to the high (DP 5415 derived transgenic cultivars; $F = 714.07$, $P < 0.0001$) versus low (remaining 11 cultivars in-

cluding ST 4691B and PM 1218BR; $F = 0.004$, $P > 0.999$) comparisons.

If the selection of cultivars that express the highest amounts of Cry1Ac (i.e. high-dose strategy for all lepidopteran pests) is to be achieved, breeders need to be aware of these problems. Because backcrossing is the primary method used by commercial cotton breeders, the selection and use of donor and/or recurrent parents that will result in a high level of Cry1Ac expression is crucial. Since the expression level in new cultivars is unknown, choosing a high-expressing donor parent and carefully selecting progeny to cross with the recurrent parent(s) is important. Differences in the expression of Cry1Ac among cultivars can be detected in any given plant structure, including seeds (Adamczyk and Sumerford, 2001). Therefore, Cry1Ac expression could be determined and high-expressing progeny selected for crossing at various steps in a backcross transfer program.

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