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Potential factors impacting season-long expression of Cry1Ac in 13 commercial varieties of Bollgard® cotton

John J. Adamczyk, Jr. and Douglas V. Sumerford

USDA, ARS, Southern Insect Management Research Unit
P.O. Box 346 Stoneville, MS 38776
jadamczyk@msa-stoneville.ars.usda.gov

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Abstract

Thirteen commercial varieties of transgenic Cry1Ac *Bacillus thuringiensis* Berliner (Bt) cotton were examined across two sites in 2000 for potential factors that impact endotoxin expression. In all cases, two varieties (NuCOTN 33B and DP 458B/RR, Delta & Pineland Co., Scott, MS) expressed more Cry1Ac than the other 11 varieties in various plant structures. These two varieties share the same parental background (DP 5415). Furthermore, when the next generation of plants were tested in the greenhouse, the same varietal patterns were exhibited. These data strongly suggest that factors such as parental background had a stronger impact on the expression of Cry1Ac than the environment.

Keywords: transgenic crops, genetically modified organisms (GMO), *Bacillus thuringiensis*, host-plant resistance

Abbreviation:

Bt	*Bacillus thuringiensis*
Cry1Ac	Crystalline ä-endotoxin from Bt, class A, subclass c

Introduction

Transgenic Cry1Ac *Bacillus thuringiensis* (Bt) cotton (Bollgard® in the United States, Ingard® in Australia, Monsanto Co., St. Louis, MO) became commercialized in 1996 as a tool to selectively manage cotton pests. Growers and researchers have noted that many lepidopteran pests are not controlled with this technology alone (Fitt *et al*., 1994, Bacheler and Mott, 1997; Smith, 1997,1998; Fitt, 1998) although it is highly effective against *Heliothis virescens*, and *Pectinophora gossypiella* (Williams, 2000). Supplemental foliar insecticide applications (e.g. pyrethroids, carbamates, and organophosphates) have been used in a number of transgenic Bt cotton fields to control *Spodoptera frugiperda*, *Spodoptera exigua*, *Helicoverpa zea*, *H. armigera* and *H. punctigera* (Bacheler and Mott, 1997; Roof and DuRant, 1997; Fitt, 1998; Smith, 1998; Burd *et al.*, 1999). This technology is highly beneficial to the grower and to the environment by reducing chemical insecticide treatments for target pests, increasing crop yields, and preserving populations of beneficial arthropods (Gianessi and Carpenter, 1999). In addition, the next generation of transgenic Bt cotton will contain multiple or even hybrid *cry* genes to broaden the spectrum of lepidopteran control while reducing the development of transgene resistance (Gould, 1998; Greenplate *et al*., 2000; Sivasupramaniam *et al*., 2001; Stewart *et al*., 2001).

All varieties of transgenic Bt cotton do not provide the same level of lepidopteran control. Cry1Ac expression levels among Bollgard® varieties (all varieties contained the insertion event or construct named '531') have been correlated to survival levels in various Lepidoptera that are intrinsically tolerant to Bt (Adamczyk *et al*., 2001). Differences in larval survival of corn earworms and larval development of fall armyworms were correlated to differential expression of Cry1Ac in various plant parts among commercial varieties of Bt cotton (Adamczyk *et al*., 2001). In addition, profiling season-long expression of Cry1Ac in Bollgard® and Ingard® varieties has shown that the Cry1Ac δ-endotoxin level decreases as the plant ages (Fitt, 1998; Sachs *et al*., 1998; Greenplate *et al*., 2000; Adamczyk *et al*., 2001). Holt, (1998) correlated this decline in Cry1Ac in Ingard® varieties to increased survival of *H. armigera*. Furthermore, season-long expression differences among varieties can vary as much as 2-fold throughout the season (Adamczyk *et al*., 2001) while plant structures, such as terminal leaves, express more Cry1Ac δ-endotoxin compared to certain flower structures (Greenplate, 1999; Greenplate *et al*., 2000; Adamczyk *et al*., 2001; Gore *et al*., 2001). Factors that have been proposed to influence the level of expressed Bt among varieties are still not fully understood, but site-of-gene insertion, cultivar or parental

Table 1. Commercially available transgenic cotton varieties examined in 2000

Cry1Ac [a]	Parental Background	Cry1Ac [a] + Herbicide-Resistance Trait [b]	Parental Background
DP 20B [c]	DP 20 [c]	DP 409B/RR [c]	DP 5409 [c]
DP 50B [c]	DP 50 [c]	DP 422B/RR [c]	DP 20 [c]
NuCOTN 33B [c]	DP 5415 [c]	DP 451B/RR [c]	DP 51 [c]
DP 428B [c]	DP 51 [c]	DP 458B/RR [c]	DP 5415 [c]
ST 4691B [d]	ST 474 [d]	SG 125B/RR [e]	SG 125 [e]
		PM 1218B/RR [f]	PM 1220 [f]
		ST 4892B/RR [d]	ST 474 [d]
		PM 2280B/RR [f]	HS200 [f]

[a] Bollgard®; Monsanto Co., St. Louis, MO).
[b] Roundup Ready®; Monsanto Co., St. Louis, MO).
[c] Delta & Pineland® variety (Delta & Pineland Co., Scott, MS).
[d] Stoneville Pedigree Seed variety (Memphis, TN).
[e] Sure-Grow® variety (Delta & Pineland Co., Scott, MS).
[f] Paymaster® variety (Delta & Pineland Co., Scott, MS).

background, and decreased overall expression of the Cry1Ac δ-endotoxin have been implicated (Sachs *et al*., 1998). The purpose of this research was to profile season-long Cry1Ac expression to determine what potential factors are responsible for differential Bt expression among US commercial varieties.

Materials and Methods

Season-Long Expression Differences

Thirteen transgenic varieties containing Cry1Ac (event 531) were planted in experimental plots on 17 May 2000 near Elizabeth, MS (Table 1). Plots consisted of 4 rows (1.0 m centers) x 30.5 m treatments arranged in a randomized complete block design. Varieties were replicated three times. Only insecticides not active on Lepidoptera were applied to all plots throughout the season as dictated by local management practices. All plots were non-irrigated.

The amount of Cry1Ac present among 13 different transgenic Cry1Ac varieties for 13 sample dates (31 May – 25 August 2000) was determined throughout the season. Because differential expression of Cry1Ac occurs among different plant structures (Greenplate, 1999; Adamczyk *et al*., 2001), a single structure was selected for quantification. For each sample date and for all varieties, a single main-stem terminal leaf (ca. 4.0 cm diameter) was randomly harvested from 10 plants/plot (3 replications/field). Leaves were transported to the laboratory and within 1 h after being harvested, one sample (ca. 5-8 mg) was taken from each leaf using a standard 6.0 mm paper ticket punch. The samples were weighed to accurately determine the initial amount of leaf tissue and combined (i.e. pooled) for each variety/plot into a 2.0 ml microcentrifuge tube containing two 6.4 mm 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 1 min using a mini-beadbeater-8™ and incubated at room temperature for 15 min. The tubes were then centrifuged at 10,000 rpm for 2 min. For each sample, 20µl of supernatant was diluted 1:25 dilution with Cry1Ac extraction buffer. A commercial quantification plate kit then was utilized to quantify the amount of Cry1Ac present for each variety (EnviroLogic, Inc., Portland, ME). This "sandwich" enzyme-linked immunosorbent assay (ELISA) uses a color development step where intensity of color production is proportional to Cry1Ac concentration in the sample extract. For all sample dates, unknowns were plotted against a standard curve with calibrators supplied with the kit. The amount of Cry1Ac was expressed as parts per million after the proper dilution factors were factored into the calculations. Figure 1 shows the typical precision that we obtained in our experiments. Differences in Cry1Ac levels among varieties were analyzed using ANOVA from PROC MIXED, and the means were separated using the LSMEANS option (SAS Institute, 1985). In addition, varietal expression slopes were analyzed using PROC REG (SAS Institute, 1985), and a test for homogeneity of regression coefficients was conducted as described in Steel and Torrie, (1980).

Season-Long Expression Differences Across Sites

The above experiment was repeated in two sites (fields) that differed by soil composition (silt-loam: Site #1; heavy clay: Site #2). Both sites contained 8 transgenic Bt varieties containing Cry1Ac (event 531) (all "DP" or "NuCOTN" varieties; see Table 1) that were planted in experimental plots on 17 May 2000 near Elizabeth, MS (Table 1). Plots consisted of 4 rows (1.0 m centers) x 30.5 m treatments arranged in a randomized complete block design. Varieties were replicated three times. Only insecticides not active on Lepidoptera were applied to all plots throughout the season as dictated by local management practices. All plots were non-irrigated. Quantification of Cry1Ac was conducted exactly as described above. Each experiment was treated as a split-plot. The main unit was 8 varieties, and the subunit was a repeated measure over 7 dates. Differences in Cry1Ac levels among varieties were analyzed using ANOVA from PROC MIXED, and the means were separated using the LSMEANS option (SAS Institute, 1985). Furthermore, variance component analysis was conducted using PROC MIXED (Littell *et al*., 1996).

Correlating Varietal Expression Differences to Different Plant Structures and Generations

G_1 Experiment. Before planting, the amount of Cry1Ac was determined in samples of seed for all 13 varieties (Table 1). Seeds (10) were placed in 10.0 x 15.5 mm zip-lock plastic bags and crushed into a fine powder. Three samples (3 replications) from the bag were then individually weighed to determine the amount of starting material and the amount of Cry1Ac was quantified using

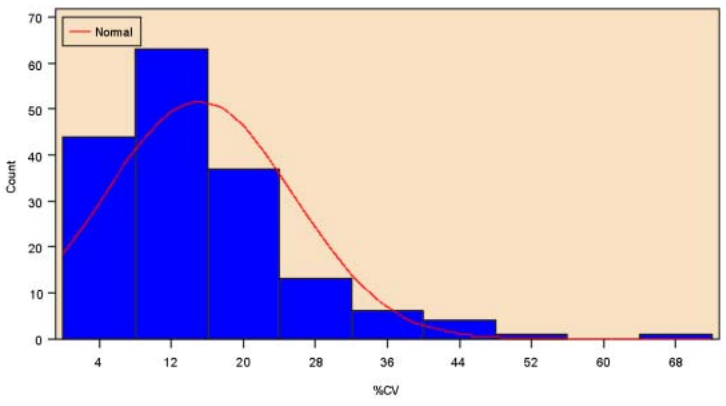

Figure 1. Frequency distribution of %CVs for Cry1A expression for each variety x date combination.

the protocol described above (Envirologix, Inc.) except that the extract was incubated overnight at room temperature to maximize extraction of Cry1Ac.

Seasonal variation of Cry1Ac in terminal leaves among the 13 varieties was correlated to Cry1Ac levels observed in the seed and cotyledon samples. Cry1Ac levels in cotyledons (26 May 2000) were determined for all 13 varieties (Table 1) planted in Site #1 as described above for terminal leaves (PROC CORR, SAS Institute, 1985).

G_2 Experiment. Seeds from all varieties planted in Site #1 were collected from mature bolls at the end of the season for greenhouse plantings and subsequent analysis of the G_2 generation. We collected a random subsample (30-50 seeds/variety) from 30 to 50 lb of seed cotton harvested from each plot. Seeds from all 13 varieties were planted in a strip-plot design in the greenhouse. Seeds (20/variety) or main-stem terminal leaves were analyzed for Cry1Ac levels, and statistical correlations conducted, as described above.

Results and Discussion

Season-Long Expression Differences

Transgenic cotton varieties differed in the amount of Cry1Ac expressed throughout the growing season. Several analyses of the data were compared to model the repeated measure nature of the subunit date, and a model treating date as a striped-split plot was chosen based on –2 log likelihood values. Two varieties (NuCOTN 33B and DP458B/RR) expressed Cry1Ac at significantly higher levels compared to the 11 other Cry1Ac varieties (Figure 2, Table 2). Furthermore, there were no significant differences detected among the 11 other varieties (Table 2). In a previous study, Adamczyk *et al*., (2001) also showed that field plots of the cultivar NuCOTN 33B expressed Cry1Ac at significantly higher levels throughout the season compared to a stacked variety also included in this current study (cv. DP 451B/RR; Delta & Pineland Co., Scott, MS). Both NuCOTN 33B and DP458B/RR are derived from the same parental background (cv. DP 5415). Sachs *et al*., (1998) noted that Cry1Ac concentration was 19% lower in one experimental background (cv. C312/ST213) compared to another (cv. C312/DP61), although the effect on lepidopteran biology was not determined. However, Adamczyk *et al*., (2001) showed that differential expression of Cry1Ac among commercial varieties affected plant resistances to insects.

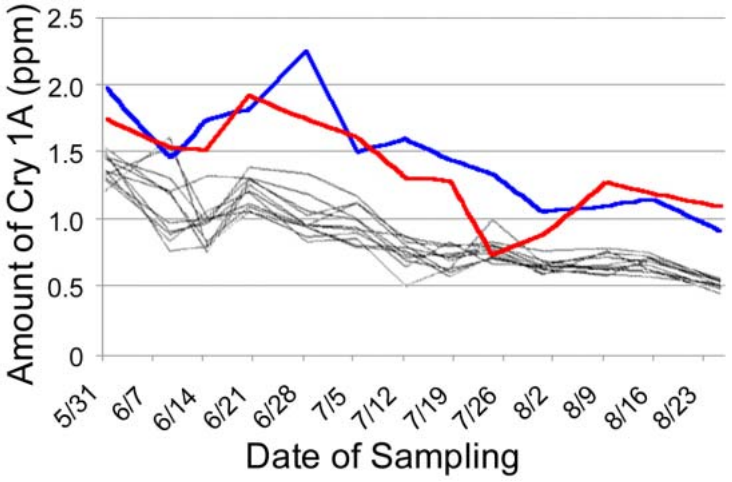

Figure 2. Expression of Cry1A in terminal leaves throughout the growing season for 13 transgenic varieties (see Table 1). Blue line, NuCOTN 33B; red line, DP 458B/RR; black lines, 11 additional Bt varieties.

Table 2. Interaction of variety planted and date of sampling on expression of Cry1A in transgenic varieties for Figure 2. [For full data behind the summary presented in this table, the fully searchable complete table is available for download at http://www.insectscience.org/1.13].

Fixed Effects	Num df	Den df	F-Value	P > F
Variety	12	24	64.50	<.001
Date	12	24	18.39	<.001
Date x Variety	144	288	2.56	<.001
NuCOTN 33B & DP 458B/RR [a] Vs. Other Varieties	1	24	714.07	<.001
Remaining Varietal Effects [a]	11	24	0.004	>.999

Differences in least square means for effects (LSMEANS option of PROC MIXED, SAS Institute, 1985).
Random variables = Rep, Rep x Variety, Rep x Date.
[a] Represents a contrast statement

In the current study, and as described by others (Finnegan *et al*., 1998, Adamczyk *et al*., 2001), Cry1Ac levels decreased consistently throughout the growing season (Figure 2, also see Date Effects, Table 2). Finnegan *et al*., (1998) concluded that part of the decline in Cry1Ac expression was related to reductions in the levels of mRNA production. In a second analysis of our data where date was treated as a linear trend, the slopes among varietal expression lines were similar, which suggests that the decrease of Cry1Ac expression throughout the season was independent of the variety (Table 3).

Season-Long Expression Differences Across Sites

An analysis of variance of the data combined across the two sites was performed. As in the previous experiment, several analyses were compared to model the repeated measure nature of the subunit date and a model treating date as a striped-split plot was chosen based on –2 log likelihood values. Analysis of variance results shown in Table 4 treated site as a fixed effect with different

Table 3. Regression for varietal expression lines in Figure 2.

Variety	Slope	Low CL	High CL	t-value	P > t
DP 20B	-0.0089	-0.0125	-0.0053	-5.396	<0.001
DP 50B	-0.0082	-0.0120	-0.0043	-4.634	<0.001
NuCOTN 33B	-0.0119	-0.0175	-0.0063	-4.687	<0.001
DP 428B	-0.0110	-0.0137	-0.0084	-9.220	<0.001
DP 409B/RR	-0.0065	-0.0093	-0.0038	-5.185	<0.001
DP 422 B/RR	-0.0074	-0.0094	-0.0054	-8.112	0.005
DP 451B/RR	-0.0075	-0.0124	-0.0026	-3.400	0.003
DP 458B/RR	-0.0102	-0.0161	-0.0043	-3.788	<0.001
SG 125B/RR	-0.0100	-0.0133	-0.0067	-6.710	<0.001
PM 1218B/RR	-0.0110	-0.0138	-0.0082	-8.583	<0.001
ST 4691B	-0.0098	-0.0131	-0.0066	-6.721	<0.001
ST 4892B/RR	-0.0084	-0.0118	-0.0051	-5.539	<0.001
PM 2280B/RR	-0.0098	-0.0141	-0.0055	-5.009	<0.001

Test for the homogeneity of regression coefficients (Steel and Torrie, 1980):

F-value	0.8363
P-value	0.6131
Num df	12
Den df	143

Table 4. Interaction of variety planted and date of sampling, while accounting for site, on expression of Cry1A in transgenic varieties for Figure 3. [For full data behind the summary presented in this table, the fully searchable complete table is available for download at http://www.insectscience.org/1.13].

Fixed Effects	Num df	Den df	F-Value	P > F
Variety	7	14	77.19	<0.001
Date	6	24	3.86	0.008
Site	1	23.2	1.69	0.207
Date x Site	6	24	2.23	0.075
Date x Variety	42	168	2.39	<0.001
Variety x Site	7	14	0.79	0.609
Date x Variety x Site	42	168	0.66	0.944

Differences in least square means for various effects (LSMEANS option of PROC MIXED, SAS Institute, 1985).
Random variables: Rep(Site) Rep x Variety, Date x Rep(Site), Variety x Date x Rep, Variety x Site x Rep.

Table 5. Variance component analysis for varieties profiled in Figure 3.

	% of Total [a]	
Source of σ^2	All 13 Varities	Excluding NuCOTN 33B & DP 458B/RR
Variety	51.34	0
Date	3.88	0
Date x Site	6.15	15.69
Date x Variety	5.11	2.09
Rep x Variety	0.53	0
Rep x Date(Site)	13.31	34.74
Rep x Date x Variety	0	0
Rep x Variety x Site	0.17	3.71
Rep(Site)	0	0.19
Site	0	0.33
Variety x Site	0	0
Date x Variety x Site	0	0
Residual	19.51	43.25

[a] Variance component estimates expressed as a percent of total variance.

soil composition. Variety and dates were also treated as fixed effects. Previous researchers have noted that environmental factors, such site, soil moisture, and fertility influence Cry1Ac expression (Sachs *et al*., 1998). However, in our study, site differences did not significantly contribute to variations in Cry1Ac expression, and interactions among variety, date of sampling, and site were not as significant as variety alone (see F-values, Table 4). As in the previous study mentioned above (see Table 2), variety and date effects as well as the date by variety interaction significantly contributed to Cry1Ac expression differences while NuCOTN 33B and DP458B/RR expressed ca. 1.5 to 2.0-fold higher than the other 6 "DP" varieties (Figure 3). In a separate analysis, variety, date, and site were considered random sources of variation in order to measure their relative (percent) importance in the total variability of Bt expression. Transgenic plant variety, especially NuCOTN 33B and DP458B/RR (same parental background, cv. DP 5415), were significant components that contributed to Cry1Ac expression differences (Table 5).

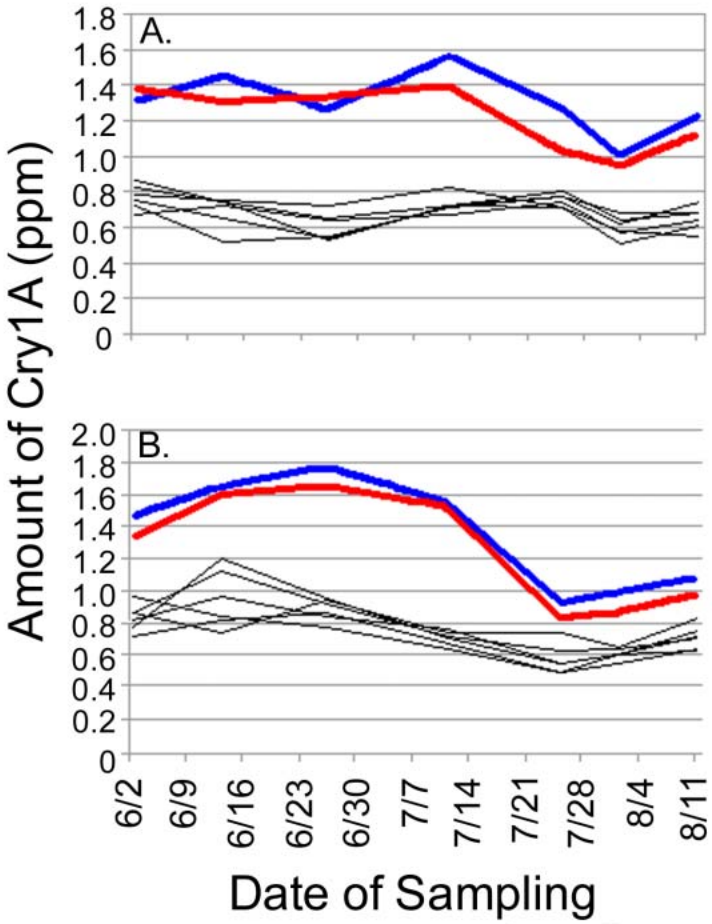

Figure 3. Expression of Cry1A in terminal leaves for 8 transgenic varieties planted at two sites: A) Silt-loam soil, Site#1; B) Clay soil, Site#2. All varieties examined were "DP" or "NuCOTN" (see Table 1). Blue line, NuCOTN 33B; red line, DP 458B/RR; black lines, 6 additional Bt varieties.

Correlating Varietal Expression Differences to Different Plant Structures and Generations

G_1 Experiment. Examining expression levels of Cry1Ac from different plant structures among varieties further supports the conclusion that environmental factors were not as significant as other factors (i.e. parental background). The amounts of Cry1Ac in cotyledon vs terminal leaves were significantly correlated among all 13 varieties for 11 sample dates. In addition, Cry1Ac levels in the cotyledon stage were significantly correlated to mean Cry1Ac levels in terminal leaves for all 13 sample dates. Because, NuCOTN 33B and DP458B/RR accounted for the majority of varietal differences (Table 2), a correlation analysis was conducted in which these two varieties were deleted. Nevertheless, Cry1Ac levels in the cotyledons were significantly correlated to Cry1Ac levels in terminal leaves (Table 6).

G_2 Experiment. As in the G_1 experiment, expression of Cry1Ac was higher in G_2 varieties with the DP5415 background (DP 458B/RR & NuCOTN 33B) compared to the other 11 commercial transgenic varieties (see Table 1). It should be noted that the Cry1Ac extraction protocol is different for seeds than the cotyledon or terminal leaf assay (longer incubation step). Thus the amount of Cry1Ac reported for seeds does not necessarily reflect a greater titer of Bt compared to the other examined plant structures (Figure 4).

Differential expression of Bt among varieties and plant structures has been reported to be the result of the ELISA measuring only soluble protein (Sachs *et al*., 1998, Greenplate *et al*., 2000). It

Table 6. Correlating Cry1A levels in cotyledons to Cry1A levels in terminal leaves among 13 varieties for 13 sample dates.

Julian date	r-coefficient	P-value
152	0.6301	0.0210
161	0.5717	0.0412
166	0.5387	0.0575
172	0.7868	0.0014
180	0.7394	0.0039
187	0.8592	0.0002
194	0.7674	0.0022
200	0.7653	0.0023
206	0.3655	0.2194
213	0.7387	0.0039
222	0.8086	0.0008
228	0.7454	0.0034
238	0.7973	0.0011
Mean among 13 varieties for 13 sample dates	0.7818	0.0016
Mean among 11 varieties for 13 sample dates (w/o NuCotn 33B or DP 458B/RR)	0.6525	0.0295

n = 13 for all dates

was implied that expression differences among varieties are ELISA artifacts rather than quantifiable differences. However, in our study, we have shown that differential expression among these varieties was correlated with different plant structures from the parental generation to the G_2 generation. This correlation also strongly suggested that the reported expression differences among Cry1Ac varieties are indeed quantifiable and are not ELISA artifacts. Although the current study further supports Cry1Ac expression differences among varieties, segregation analyses will determine if these differences are under genetic control. Furthermore, these studies are much needed to determine if transgenic crops can be selected based on their plant-insect resistance traits (i.e. highest expression varieties) in addition to their agronomic traits.

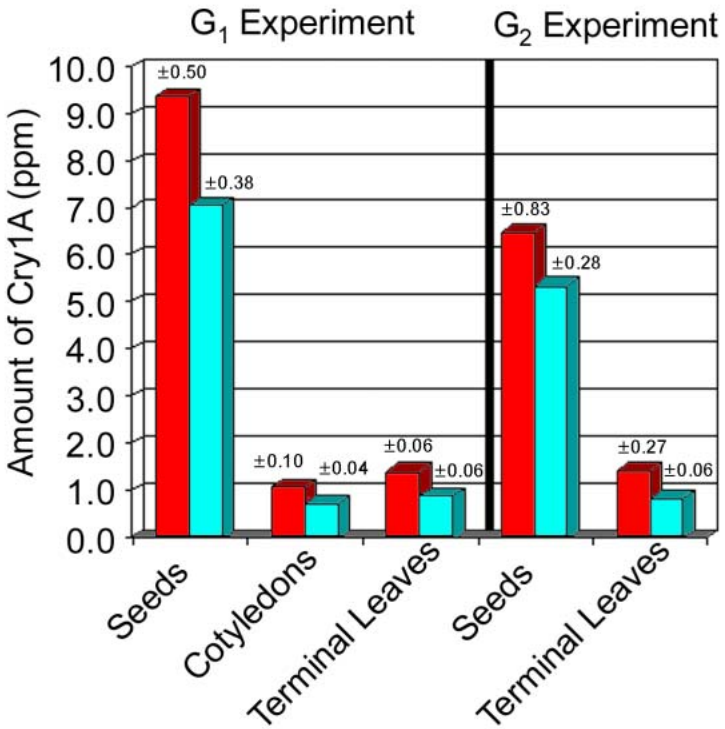

Figure 4. Mean expression (± SE) of Cry1A in various plant structures in the G_1 and G_2 generations of plants. Two varieties (red bars, DP458B/RR & NuCOTN 33B) with the same parental background (DP5415) were compared with the other 11 transgenic varieties (aqua bars) (see Table 1).

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