BOLLGARD II EFFICACY: QUANTIFICATION OF TOTAL LEPIDOPTERAN ACTIVITY IN A 2-GENE PRODUCT

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Abstract

A 4 field site study was performed in which Bollgard II (containing 2 lepidopteran active Bt proteins: Cry1Ac and CryX) and DP50B (original Bollgard® containing only Cry1Ac) cotton tissue samples were collected throughout the growing season and evaluated for total lepidopteran bioactivity using a sensitive *Heliothis virescens* quantitative bioassay which utilized purified Cry1Ac as the quantitative standard. In addition, protein-specific ELISA assays were performed upon the same tissue samples to determine relative levels of both insect control proteins. Total lepidopteran bioactivity, expressed in Cry1Ac equivalents, was greatly increased in Bollgard II tissue samples. Overall means were four times as great for Bollgard II as for DP50B (the "parent" Bollgard® variety); overall means were 3 times as great for terminal foliage and 6 times as great for square tissue. This relative increase in lepidopteran activity was observed at every sampling time (from 4-leaf stage to 6 weeks after first bloom) and at every field site. ELISA evaluations showed that the presence of the second protein (CryX) had no deleterious effect on the levels of the first Bollgard® protein (Cry1Ac) as measured in DP50B. Also, relative levels of the two Bt proteins remained relatively constant over time and across field sites. A main-effect ANOVA determined that, in addition to the Bollgard II-Bollgard® difference, field site, sampling time, and plant tissue type were all significant sources of variability among levels of lepidopteran bioactivity; although the tissue type variability was due solely to differences between terminals and squares within DP50B; when evaluated alone, there was no statistical difference in the lepidopteran activity between Bollgard II squares and terminals. These data strongly suggest that the greatest single effect of the addition of the CryX protein to Bollgard® to produce Bollgard II is likely to be greatly increased lepidopteran activity, especially in reproductive tissues.

Introduction

In the development of the second generation of Bollgard® products, a second insect control gene encoding another Bt protein, qualitatively different from Cry1Ac (called CryX by Monsanto), was used to transform tissue from the current Bollgard® variety DP50B (Delta & Pineland). Cloned plants regenerated from the transformed tissue expressed both the Cry1Ac protein and the CryX protein. These genes also segregated independently. The proposed name for the 2-gene product is Bollgard II; it has not yet received EPA registration. In this study, quantitative evaluations were made to determine levels of lepidopteran activity in specific Bollgard II tissues over time and in comparison with its Bollgard® "parent" variety (DP50B).

Materials and Methods

Cotton tissue samples from 4 field sites were collected and shipped to Monsanto laboratories where they were processed and evaluated in a sensitive quantitative bioassay which utilized purified Cry1Ac as a standard and took advantage of the extreme sensitivity of *Heliothis virescens* to the Cry1Ac protein (Greenplate, 1999). Tissue sample effects on *H. virescens* larval development were compared with effects of known concentrations of Cry1Ac; lepidopteran activity levels in cotton tissues were thereby estimated and expressed as "Cry1Ac equivalents". Within each site, several plants within 4 replicate plots were sampled at 2 week intervals beginning at 4-leaf stage and ending at 6 weeks after first bloom. The specific tissues sampled were main terminal foliage, and pre-candle squares (1st position square 2-3 nodes below main terminal); terminal tissue was sampled from 4-leaf stage to 6 weeks after first bloom; square tissue was sampled from 2 weeks pre-bloom to 3 weeks after first bloom. The JMP® (version 3.1) statistical software (SAS Institute, Cary NC) was used to perform the statistical evaluations on lepidopteran bioactivity values. A main effect ANOVA was used to test the influence of field site, sampling time, tissue type, and replicate plot (within site) on variability among mean Cry1Ac levels. Subsequent mean comparisons were made using Tukey-Kramer HSD (Kramer, 1956). The cotton tissue samples were further evaluated in Cry1Ac- and CryX-specific ELISA tests (Sims *et al* 1996) to determine relative levels of the two proteins over time and across field sites.

Results

A main-effect ANOVA determined that variety (Bollgard® vs Bollgard II), field site, sampling time, and plant tissue type were all significant sources of variability among levels of lepidopteran bioactivity (Table 1); although the tissue type variability was due solely to differences between terminals and squares within DP50B; when evaluated alone, there was no statistical difference in the lepidopteran activity between Bollgard II squares and terminals (Table 3). There was no significant plot effect. Table 2 shows that overall means of lepidopteran activity were 4 times as great in Bollgard II as in Bollgard® (65 and 17 µg of Cry1Ac equivalents per g dry wgt, respectively). There was no significant difference between levels of lepidopteran activity in terminals and

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squares of Bollgard II (67 and 62 µg of Cry1Ac equivalents per g dry wgt, respectively), while Bollgard® terminals contained twice as much lepidopteran activity as corresponding squares (22 and 10 µg of Cry1Ac equivalents per g dry wgt, respectively) (Table 3). Tables 4 and 5 show that overall levels of lepidopteran bioactivity remained significantly higher in Bollgard II tissues at all sampling times and at all field sites.

ELISA evaluations were used to measure relative levels of individual proteins (Figures 1-3). The addition of the second protein (CryX) to Bollgard® to create Bollgard II appeared to have no deleterious effect on levels of the original Bollgard® protein (Cry1Ac) overall (Figure 1), or at various sampling times (Figure 2), or field sites (Figure 3).

Discussion

In this 4 field-site study, total lepidopteran bioactivity, expressed in Cry1Ac equivalents, was greatly increased in Bollgard II tissue samples. Overall means were four times as great for Bollgard II as for DP50B (the "parent" Bollgard® variety); overall means were 3 times as great for terminal foliage and 6 times as great for square tissue (Table 2; Table 3). This relative increase in lepidopteran activity was observed at every sampling time (from 4-leaf stage to 6 weeks after first bloom) and at every field site (Table 4; Table 5). A main-effect ANOVA of total lepidopteran bioactivity determined that, in addition to the Bollgard II-Bollgard® difference, field site, sampling time, and plant tissue type were all significant sources of variability (Table 1); although the tissue type variability was due solely to differences between terminals and squares within DP50B; when evaluated alone, there was no statistical difference in the lepidopteran activity between Bollgard II squares and terminals (Table 3).

Protein-specific ELISA evaluations showed that the presence of the second protein (CryX) had no deleterious effect on the levels of the first Bollgard® protein (Cry1Ac) as measured in DP50B (Figure 1). Also, relative levels of the two Bt proteins remained relatively constant over time and across field sites (Figure 2; Figure 3). It may be observed that the ELISA values for Cry1Ac in DP50B were somewhat lower than Cry1Ac values as estimated in the quantitative bioassay (Table 2; Figure 1). This can be explained to a large degree by the ability of the ELISA to measure only soluble protein. The ability of the ELISA procedure to extract and solubilize Cry1Ac is never complete; some remains insoluble and, therefore, undetected (Sachs *et al* 1998). In addition, combined ELISA values for CryX and Cry1Ac in Bollgard II are considerably greater than values (estimated in Cry1Ac equivalents) for total lepidopteran bioactivity (Table 2; Figure 1); this, although apparently inconsistent, can also be explained. The approximate 10X higher level of CryX over Cry1Ac in Bollgard II (Figure 1) did not result in a 10X (or greater) difference in bioactivity over Bollgard® (Table 2) because the CryX protein is less potent than Cry1Ac against *H. virescens* (Monsanto internal communication). Instead, in Bollgard II the original Cry1Ac with 10X CryX added combined to result in the reported 3-6X increase in the *H. virescens* bioactivity.

As measured in this study, the greatest single effect of the addition of the CryX protein to Bollgard® to produce Bollgard II was greatly increased lepidopteran activity, especially in reproductive tissues.

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Table 1. ANOVA main effects table.

Source	DF	Sum of Squares	F Ratio	Prob>F
Sampling Time	5	23463.43	11.588	<.0001
Line	1	170885.02	421.9796	<.0001
Replicate[Field Site]	12	1563.16	0.3217	0.9851
Tissue Type	1	8863.61	21.8876	<.0001
Field Site	3	11436.97	9.4141	<.0001

Table 2. Mean lepidopteran activity levels (MLA), expressed as µ Cry1Ac equivalents/g dry weight, for Bollgard® and Bollgard II. Means with different letters are statistically different at P = 0.05 as measured by Tukey-Kramer HSD.

Variety	MLA	SEM	
Bollgard II	64.94	2.63	a
Bollgard	16.89	1.04	b

Table 3. Mean lepidopteran activity (MLA), expressed as µ Cry1Ac equivalents/g dry weight, for Bollgard® and Bollgard II terminals and squares. Within columns (tissue type), means with different lower-case letters are statistically different at P = 0.05 as measured by Tukey-Kramer HSD. Within rows (variety), means with different upper-case letters are statistically different at P = 0.05 as measured by Tukey-Kramer HSD.

Variety	Terminal MLA	SEM		Square MLA	SEM	
Bollgard II	66.89	3.91	a A	62.08	3.02	a A
Bollgard	21.55	1.49	b A	10.05	0.7	b B

Table 4. Mean lepidopteran activity (MLA), expressed as µ Cry1Ac equivalents/g dry weight, for Bollgard® and Bollgard II at various sampling times. For every sampling time, Bollgard II and Bollgard® means are statistically different at P = 0.05 as measured by Tukey-Kramer HSD.

Samling Time	Bollgard II MLA	SEM	Bollgard MLA	SEM
4 leaf	57.24	9.76	27.59	4.72
Pre-bloom	78.45	5.30	15.14	10.62
1st bloom	65.67	4.71	18.56	2.07
2 Weeks	65.96	5.52	13.88	1.57
3-4 Weeks	66.11	6.61	16.50	3.08
6 Weeks	29.27	4.54	15.34	3.15

Table 5. Mean lepidopteran activity (MLA), expressed as µ Cry1Ac equivalents/g dry weight, for Bollgard® and Bollgard II at various field sites. For every field site, Bollgard II and Bollgard® means are statistically different at P = 0.05 as measured by Tukey-Kramer HSD.

Field Site	Bollgard II MLA	SEM	Bollgard MLA	SEM
LA	83.34	5.84	17.29	2.07
MS	66.42	3.40	21.87	1.51
SC	35.73	3.67	8.59	0.53
TX	73.35	4.39	19.52	2.68

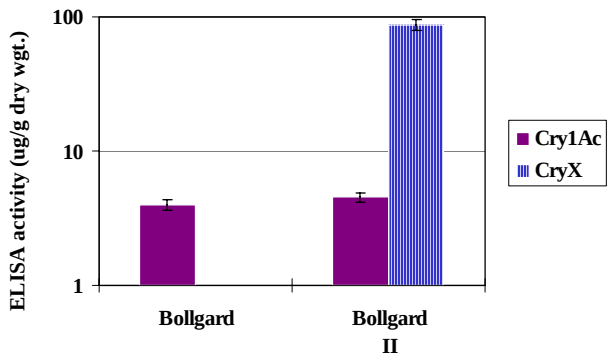

Figure 1. Overall mean concentrations (as measured by ELISA) of Cry1Ac in Bollgard and Bollgard II tissues and CryX in Bollgard II tissues.

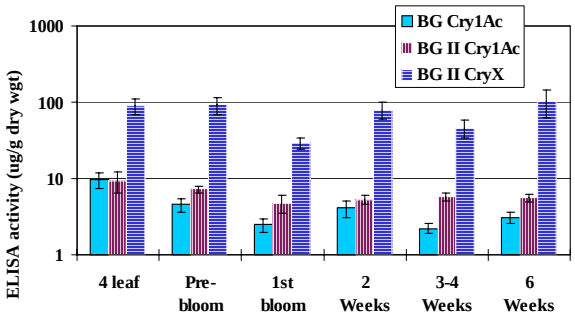

Figure 2. Mean concentrations, from specific sampling times, of Cry1Ac in Bollgard and Bollgard II tissues and CryX in Bollgard II tissues. All means represent ELISA-derived values.

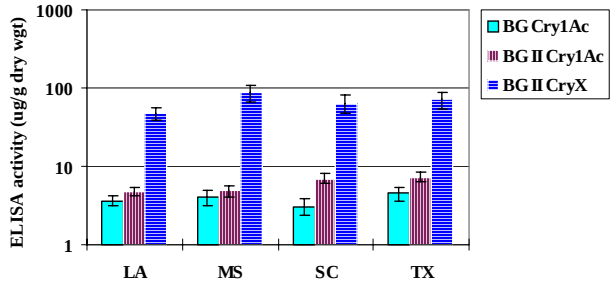

Figure 3. Mean concentrations, from individual field sites, of Cry1Ac in Bollgard and Bollgard II tissues and CryX in Bollgard II tissues. All means represent ELISA-derived values.