

**Dow AgroSciences LLC Study Title:** Field Expression of Cry1F (synpro), Cry1Ac (synpro) and Phosphinothricin Acetyltransferase (PAT) Proteins in Transgenic Cotton Plants, Cottonseed, and Cottonseed Processed Products; and Composition Analysis of Cottonseed and Cottonseed Processed Products, Unpublished Report of Dow AgroSciences LLC (Study ID 010015.03).

## Introduction

The purpose of this study was to determine the levels of Cry1F, Cry1Ac, and phosphinothricin acetyltransferase (PAT) proteins expressed in cotton plants, cottonseed, and the processed products of cottonseed – kernel, hulls, meal and oil. In addition, compositional analysis was performed on terminal leaf, square, cottonseed, and processed products of cottonseed – kernel, hull, meal, and oil to investigate the equivalency between the control and transgenic cotton lines.

**GLP Standards.** This study was conducted in accordance with the US Environmental Protection Agency-FIFRA GLPs Title 40 CFR, Part 160-Federal Insecticide, Fungicide and Rodenticide Act (FIFRA), Good Laboratory Practice Standards (Final Rule) and the Organization for Economic Co-Operation and Development (OECD) OECD Series on Principles of Good Laboratory Practice and Compliance Monitoring, Number 1, OECD Principles on Good Laboratory Practice (as revised in 1977) ENV/MC/CHEM(98)17.

**Quality Assurance.** The study conduct, data, protocol, protocol changes/revisions, and final report were inspected by the Quality Assurance Unit, Dow AgroSciences, Indianapolis, Indiana.

**Archiving.** The final report, field data books, and all raw data are filed in the Dow AgroSciences facility archives, Indianapolis, Indiana.

## Materials and Methods

**Test and Control Substances.** Cotton (*Gossypium hirsutum* L.) was transformed to contain the genes that express full-length synthetic protoxins (synpro) of Cry1F or Cry1Ac from *Bacillus thuringiensis* (B $\theta$ ). Individual Cry1F(synpro) (referred to as Cry1F) and Cry1Ac(synpro) (referred to as Cry1Ac) lines were crossed to produce a stacked product. The Cry1F and Cry1Ac proteins possess insecticidal activity against lepidopteran pests. The transgenic cotton lines also contained an herbicide-resistant selectable marker gene that expressed the protein phosphinothricin acetyltransferase (referred to as PAT). The control substance was seed from the null plants from the F1 segregating generation after stacking Cry1F and Cry1Ac.

**Test System.** Field expression trials of the control, single- and stacked-trait lines were conducted at six test sites located within the major cotton-producing regions of the U.S. These sites represent regions of diverse agronomic practices and a variety of environmental conditions. The trials were located in Arizona, California, Mississippi, North Carolina and two sites in Texas. Samples were collected at various times during the development of the plants.

**Compositional Analysis.** Samples of terminal leaf, squares, delinted cottonseed, and the processed fractions of kernel, toasted meal, refined oil and hulls were analyzed for nutrient content

with a variety of tests. Analyses performed included total protein, moisture, fat, ash, carbohydrate, calories, fiber, minerals, amino acids, fatty acids, tocopherols, gossypol, aflatoxins, cyclopropenoid fatty acids and total polyphenols.

Determination of Cry1F, Cry1Ac and PAT Proteins in Cotton Samples. Samples of cotton were analyzed for the amount of Cry1F (synpro) protein, Cry1Ac (synpro) protein and PAT protein using validated methods. In these methods soluble extractable Cry1F protein, Cry1Ac protein and PAT protein are quantified using enzyme-linked immunosorbent assays (ELISA). Results were reported on a ng protein/mg sample weight basis, with fresh weight used for cottonseed, pollen, nectar and processed products; and dry weight used for all other tissues.

Statistical Treatment of Compositional Analysis. Statistical analysis was performed for cottonseed composition data since these samples were analyzed in replicate for each event. The statistical analysis included calculation of means for Cry1F, Cry1Ac, Cry1F/Cry1Ac stack and control samples, and a comparison of the mean values between the non-transgenic and transgenic lines. Mean values were calculated by averaging data from the six field sites. Analysis of variance was conducted across the six field sites using a mixed model (SAS Institute, 1999). The significance of an overall treatment effect was estimated using a F-test. Transgenic treatments were each compared to the non-transgenic control using a t-test, and again with the P-values adjusted using a Dunnett procedure to maintain the experiment-wide error rate for each analyte at  $\leq 0.05$ . Significant differences were declared at the 95% confidence level.

The results of the nutritional analysis for cottonseed and processed fractions were also compared to values reported in literature (draft OECD, 2002; Berberich et al., 1996; Forster and Calhoun, 1995; Codex 2001; *Cottonseed Oil*, 1990; and Cottonseed Feed Products Guide by NCPA).

## Results and Conclusions

For the Cry1F single and stacked events, all matrices except nectar, meal and oil expressed the Cry1F protein at measurable levels. Average expression levels of Cry1F across matrices ranged from not detected (ND) to 22.8 ng/mg sample weight for the Cry1F event, and ND to 25.3 ng/mg for the stacked event. Expression of Cry1Ac was observed in all matrices from the Cry1Ac and stacked events except for nectar, hulls, meal and oil. Average expression levels of Cry1Ac across matrices ranged from ND to 1.92 ng/mg for the Cry1Ac event, and ND to 1.83 ng/mg for the stacked event samples. Expression results for both Cry1F and Cry1Ac were comparable between the single and stacked event. The average expression levels of PAT across matrices ranged from ND to 0.51 ng/mg for the Cry1F event, and ND to 0.54 ng/mg for the stacked event samples. PAT protein was rarely detected in the Cry1Ac event samples. These data characterize Cry1F, Cry1Ac and PAT expression in cotton tissues grown across environments and demonstrate similarity of expression between the single-trait lines and the stack-trait line.

Statistical analysis of the compositional analysis of cottonseed resulted in a very limited number of instances where the transgenic treatment was statistically different from the control. When comparing results over all sites, the crude fiber content from the stack (Cry1F/Cry1Ac/PAT) and Cry1Ac treatments were found to be slightly lower than the control. The results for manganese and calcium in the Cry1F treatment were also found to be statistically different from the control.

However, for all of these analytes, results for both the control and transgenic plots were within reported literature ranges, so the differences were not considered to be biologically meaningful. A statistical difference between the control and transgenic cottonseed was found for stearic (Cry1F, Cry1Ac and stack) and oleic acid (Cry1F). For both stearic and oleic acid, the transgenic values were closer to the values reported in the literature than the control values, and transgenic values differed from control values by <12%. There also were a limited number of instances when results from compositional analyses were found to be either higher or lower than reported literature ranges. These differences were not considered to be related to the transgenes since results for control and transgenic samples did not differ in these instances. Therefore, the results from the compositional analyses demonstrate substantial equivalence between conventional and transgenic cotton lines.

### Key References

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