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Application for an Extension of the Determination of Nonregulated Status for Insect-Resistant, Glufosinate Ammonium-Tolerant Cotton (08-340-01p):

Transformation Event T303-3

OECD Unique Identifier BCS-GHØØ3-6

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January 30, 2012

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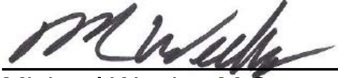
CERTIFICATION

The undersigned certifies, that to the best knowledge and belief of the undersigned, this petition includes all information and views on which to base a determination, and that it includes relevant data and information known to the petitioner which is unfavorable to the petition.



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SUMMARY

Bayer CropScience requests a determination from USDA APHIS that insect-resistant, glufosinate ammonium tolerant cotton event T303-3 and any progeny derived from crosses of this event with traditional cotton varieties, and any progeny derived from crosses of this event with transgenic cotton varieties that have also received a determination of nonregulated status, no longer be considered regulated articles under 7 CFR Part 340, and that APHIS consider this document as an extension to petition 08-340-01p. The subject of petition 08-340-01p, TwinLink™ Cotton, received a determination of non-regulated status on September 23, 2011.

Gossypium hirsutum transformation event T303-3 was produced by *Agrobacterium*-mediated transformation utilizing vector pTDL004 containing a *cry1Ab* gene construct, encoding insect-resistance, and the *bar* gene as a selectable marker conferring tolerance to glufosinate ammonium herbicides. The antecedent organism, cotton event T304-40 as described in petition 08-340-01p, was also generated through *Agrobacterium*-mediated transformation utilizing a slightly different vector (pTDL008). Both events produce the same insecticidal crystal protein (ICP) Cry1Ab (expression product of the *cry1Ab* gene) and PAT protein (expression product of the *bar* gene).

Molecular analysis has demonstrated that event T303-3 contains one stably integrated intact copy of the *cry1Ab* gene and *bar* gene cassettes. Evaluation of the Cry1Ab protein expressed by event T303-3 was found to be comparable to that expressed by event T304-40 as well as that expressed in *E. coli* for the purposes of protein safety assessment in petition 08-340-01p.

Agronomic evaluations demonstrated that no differences in morphology, disease or pest susceptibility were observed when compared to other cultivated cotton varieties. No adverse effects on beneficial organisms were found. Composition analysis of raw agricultural commodities produced by event T303-3 was found to be substantially equivalent to that of its non-transgenic counterpart.

Based on all analyses performed, event T303-3 was found to be comparable to the antecedent organism, event T304-40.

A comparison of characteristics of events T303-3 and T304-40 is summarized in Table 1 and is discussed in the appropriate sections of the petition.

Table 1. Comparison of events T304-40 and T303-3

Characteristic	T303-3	T304-40
Crop	Cotton	Cotton
Recipient Organism	*Gossypium hirsutum*	*Gossypium hirsutum*
Parent Line	Coker 315	Coker 315
Transformation Method	*Agrobacterium*-mediated	*Agrobacterium*-mediated
Trait	Insect-resistant, herbicide-tolerant	Insect-resistant, herbicide-tolerant
Gene Products	Cry1Ab and PAT	Cry1Ab and PAT
Vector	pTDL004	pTDL008
Gene/Donor	*cry1Ab*/*Bacillus thuringiensis berliner* 1715	*cry1Ab*/*Bacillus thuringiensis berliner* 1715
Promoter/Donor	P35S2/Cauliflower Mosaic Virus promoter	Ps7s7/Subterranean Clover Stunt Virus promoter
Terminator/Donor	3' me1/3' untranslated region of NADP-malic enzyme gene of *Flavia bidentis*	3' me1/3' untranslated region of NADP-malic enzyme gene of *Flavia bidentis*
Gene/Donor	Phosphinothricin acetyltransferase (*bar*)/*Streptomyces hygroscopicus*	Phosphinothricin acetyltransferase (*bar*)/*Streptomyces hygroscopicus*
Promoter/Donor	P35S3/Cauliflower Mosaic Virus promoter	P35S3/Cauliflower Mosaic Virus promoter
Terminator/Donor	3' nos/3' untranslated region of nopaline synthase gene from T-DNA of pTiT37	3' nos/3' untranslated region of nopaline synthase gene from T-DNA of pTiT37
Molecular Weight Cry1Ab	~69 kDA	~69 kDA

ACRONYMS AND SCIENTIFIC TERMS

a.i.	Active ingredient
A	Acre
ADF	Acid Detergent Fiber
ANOVA	Analysis of Variance
BC	Backcross
BCS	Bayer CropScience
bp	Base pair
Bt	*Bacillus thuringiensis*
cry1Ab	Insecticidal crystal protein gene from *Bacillus thuringiensis*
Cry1Ab	Insecticidal crystal protein expressed from the *cry1Ab* gene
d.w.	Dry weight
F_1, F_2, etc	Breeding generations denoting product of pollinations and subsequent self-pollinations following the cross of transformation event with conventional germplasm
ELISA	Enzyme-Linked Immunosorbent Assay
FWS	U.S. Fish & Wildlife Service
g	Gram
g	Earth's gravitational acceleration (1 g = 3,530,394 cm / minute^2)
ICP	Insecticidal crystal protein
kDa	Kilodalton
lb	US pound
LB	Left border of T-DNA
µg	Microgram
MQ	Milli-Q water
MW	Molecular Weight
NDF	Neutral Detergent Fiber
ng	Nanogram
ORF	Open Reading Frame
PAT/*bar*	Phosphinothricin Acetyl-Transferase as expressed by the *bar* gene
pg	Picogram
RB	Right border of T-DNA
SD	Standard Deviation
T_1, T_2, etc	Breeding generations denoting self-pollinations of the T_0 plant (transformation event)
T-DNA	Transfer DNA from *Agrobacterium tumefaciens*
TwinLink	Insect-resistant, herbicide-tolerant stacked cotton product (events T304-40 x GHB119)
U.S.	United States

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I. RATIONALE FOR NONREGULATED STATUS

The United States Department of Agriculture (UDSA) Animal and Plant Health Inspection Services (APHIS) is responsible for protection of the US agricultural infrastructure against noxious pests and weeds. Under the Plant Protection Act (7 USC § 7701-7772) APHIS considers plants altered or produced by genetic engineering as restricted articles under 7 CFR 340 which cannot be released into the environment without appropriate approvals. APHIS provides that petitions may be filed under 7 CFR §340.6 to evaluate data to determine that a particular regulated article does not present a plant pest risk. Should APHIS determine that the submitted article does not present a plant pest risk; the article may be deregulated and released without further restrictions.

This petition serves an application for an Extension of the *Petition for Determination of Nonregulated Status for Insect-Resistant, Glufosinate Ammonium-Tolerant cotton: TwinLink™ Cotton (events T304-40 x GHB119)*. The petition for TwinLink Cotton (08-340-01p) received a determination of non-regulated status on September 23, 2011. Event T303-3 expresses the same Cry1Ab and PAT/*bar* proteins as event T304-40 and therefore there are no changes in the rationale from petition 08-340-01p entitled "Petition for Determination of Nonregulated Status for Insect-Resistant and Glufosinate Ammonium-Tolerant Cotton: TwinLink™ Cotton (events T304-40 x GHB119)".

II. THE COTTON FAMILY

The genus *Gossypium* is classified under the tribe Gossypiae, family Malvaceae, subfamily Malvoideae.

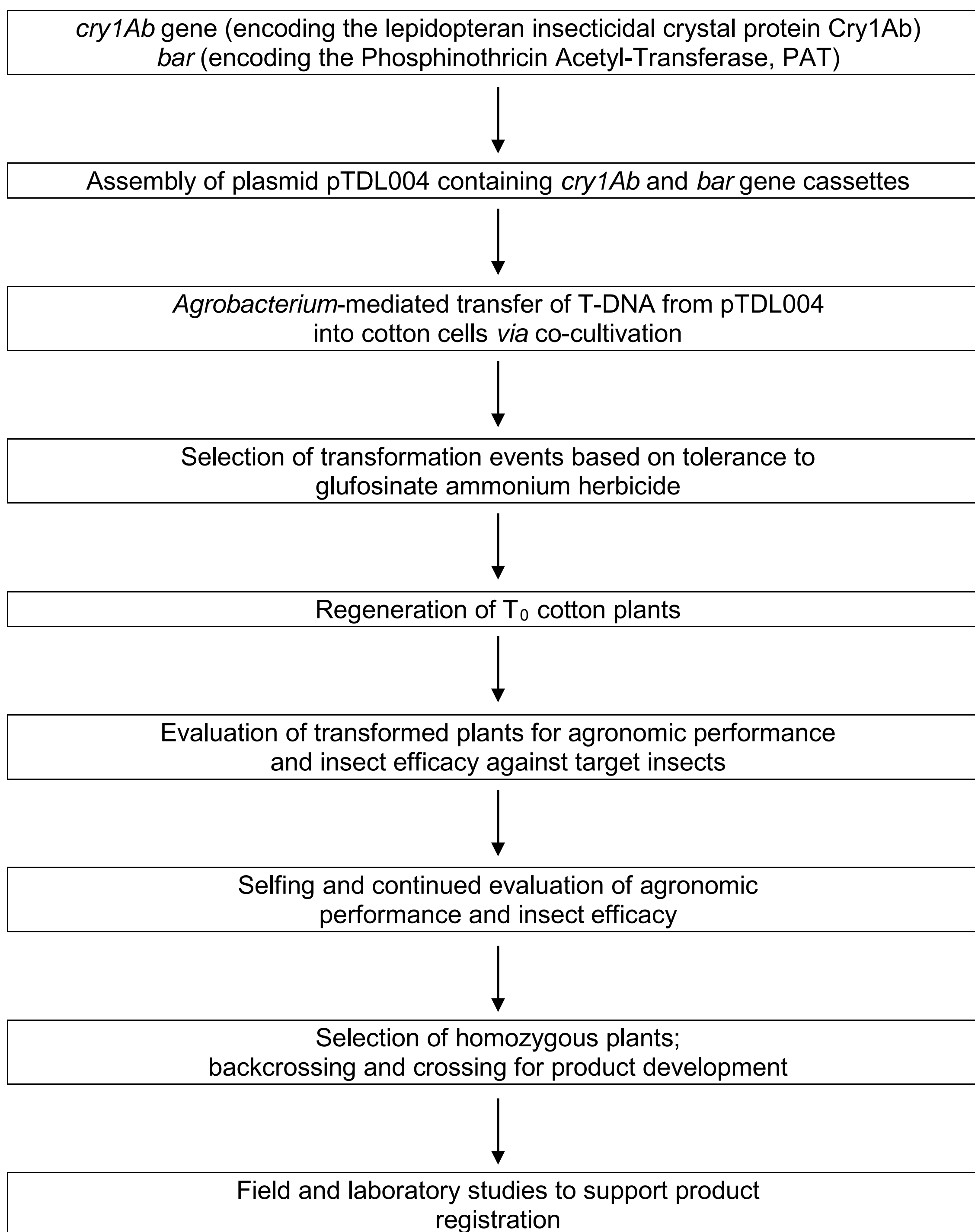
The OECD consensus document on cotton biology (OECD, 2008) provides information pertaining to the following aspects of cotton biology:

- Taxonomy, morphology and uses
- Centers of origin of the species and domestication
- Agronomic practices
- Reproductive biology and dispersal
- Genetics and hybridization
- Interactions with other organisms
- Human health and biosafety

III. DEVELOPMENT OF EVENT T303-3

The T303-3 event was obtained using the same transformation system (*Agrobacterium*-mediated) as was used to obtain the antecedent organism, event T304-40 (USDA, 2008). The parental line used, Coker 315, was the same for event T304-40. A schematic of the development process of event T303-3 is shown in Figure 1.

Figure 1. Schematic of the development process of event T303-3 cotton



III.A. The Transformation System

EventsT304-40 and T303-3 were both generated through *Agrobacterium*-mediated gene transfer of the T-DNAs from pTDL008 and pTDL004, respectively. Each T_0 plant was crossed with conventional cotton in order to obtain homozygous and stable lines.

Agrobacterium-mediated gene transfer of a plasmid results in the transfer of the DNA fragment between the T-DNA border repeats to the plant genome. The left and right border repeats of *A. tumefaciens* are also inserted in the individual events. Even though some of the regulatory elements used in the transformation process were derived from *A. tumefaciens*, a known plant pathogen, the genes that cause crown gall disease were not part of the T-DNA, and therefore were not incorporated into the recipient plant (Deblaere *et al.*, 1985). Section V.C. summarizes the analysis to show the absence of vector backbone in event T303-3.

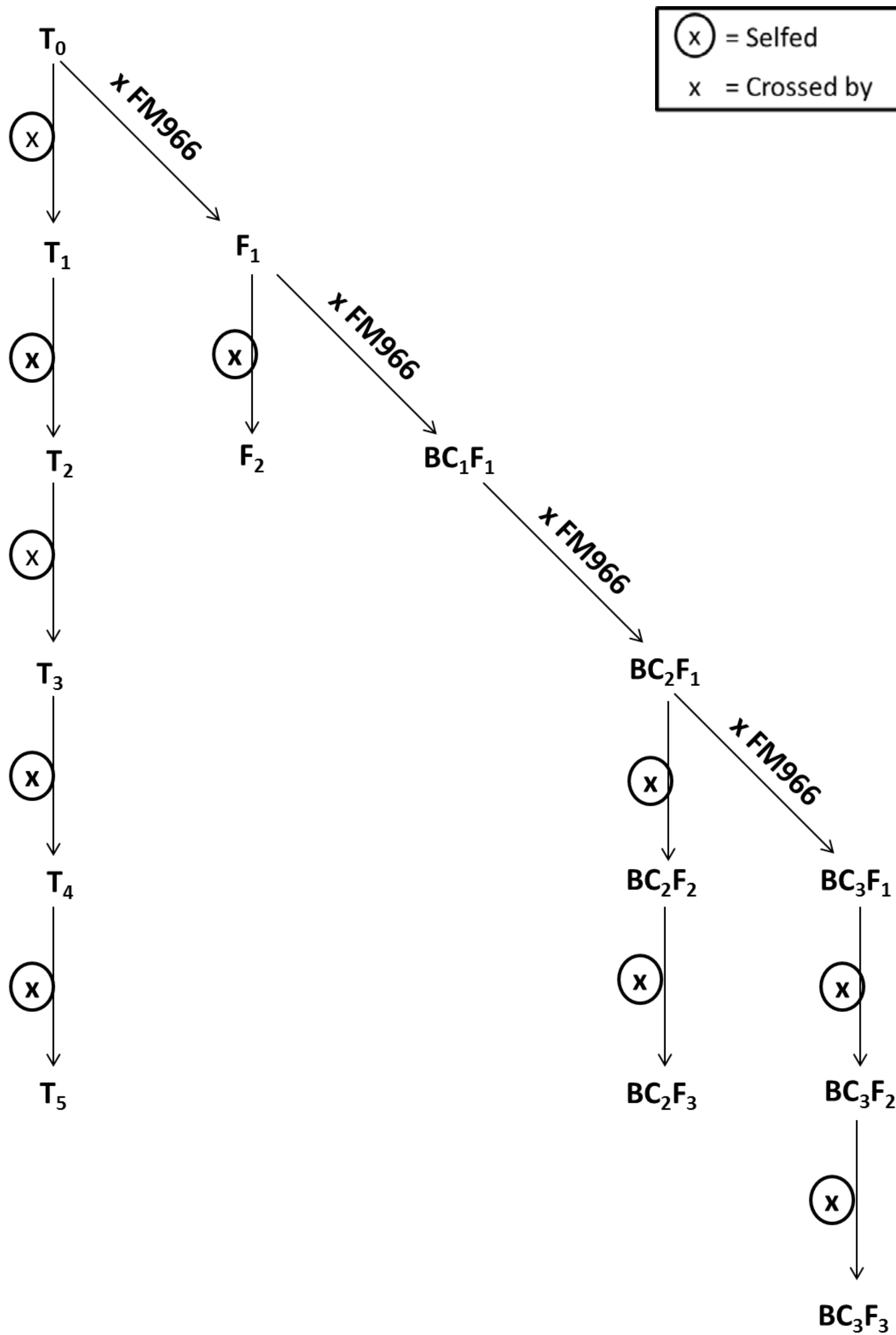
III.B. The Parental Line

The parental line, Coker 315, is an older commercial variety of upland cotton (*Gossypium hirsutum*) which is no longer commercially cultivated. However, Coker 315 is well suited for transformation due to its capacity for regeneration from tissue culture.

III.C. Breeding History

The T_0 transformation event of Coker 315 was both self-pollinated and cross-pollinated with conventional cotton to produce T_1 and F_1 lines, respectively. The T_1 and F_1 lines then underwent a series of self- and cross-pollinations as well as backcrosses to produce stable lines for the various assessments outlined in this petition. See Figure 2 for a schematic of the crossing scheme utilized.

Figure 2. Breeding diagram for event T303-3 cotton



III.D. Generations Used for Analyses

Table 2 provides the generation and genetic background of event T303-3 used for each analyses presented in this petition.

Table 2. Generations and backgrounds used for analysis

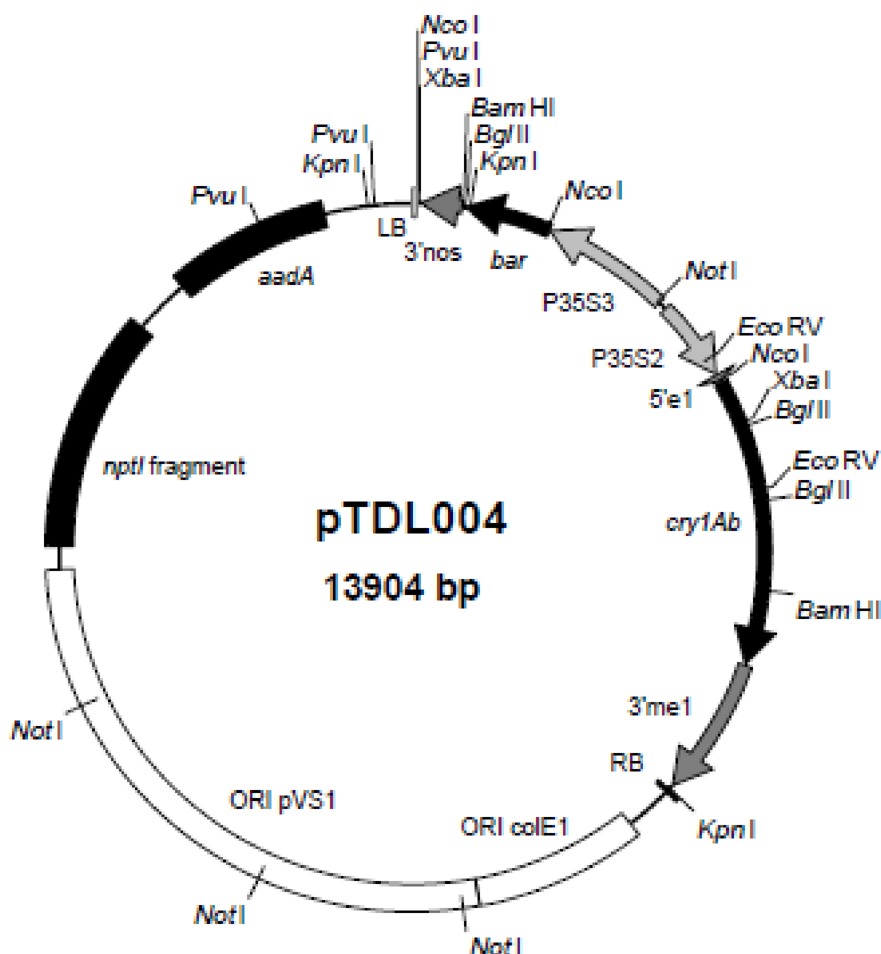
Analysis Conducted	Generation
Insert Characterization by Southern Blot Analysis	BC_2F_2
DNA Sequencing	BC_2F_2
ORF Analysis	BC_2F_2
Absence of Vector Backbone	BC_2F_3
Mendelian Inheritance	T_1, T_2, F_2, BC_3F_3
Protein Expression	T_4
Comparison of Cry1Ab proteins from events T303-3 and T304-40	T_4
2005 Agronomic and Phenotypic Evaluation in Field	T_4
2006 Agronomic and Phenotypic Evaluation in Field	T_5
Composition Analysis	T_4

IV. GENETIC MATERIAL USED FOR TRANSFORMATION

IV.A. Construction of Plasmid pTDL004

The vector pTDL004 is a derivative of the vector pGSV20 in which the *bar* gene cassette coding for the phosphinothricin acetyltransferase protein of *Streptomyces hygroscopicus* (Thompson *et al.*, 1987) was inserted together with the *cry1Ab* gene cassette encoding the Cry1Ab crystal protein of *Bacillus thuringiensis* subsp. *berliner* (Höfte *et al.*, 1986). A map of plasmid pTDL004 is provided in Figure 3.

Figure 3. Vector map of pTDL004



IV.B. Description of Genetic Elements and Regulatory Sequences

Regulatory sequences in the T-DNA plasmid of vector pTDL004 include two copies of the P35s promoter sequence (P35s3 and P35s2), 3'nos terminator sequence, 3'me1 terminator sequence, and the 5'e1 leader sequence. Genetic elements in the T-DNA plasmid of vector pTDL004 are the *bar* and *cry1Ab* (Cry1Ab5PGS3a) gene sequences. Also included in the T-DNA of vector pTDL004 are the left and right border repeats (LB and RB, respectively) from the pTDL004 vector. Other genetic elements of vector pTDL004 occupying nucleotide positions from 5176 bp to 13904 bp (ORI pVS1 and ORI colE1) should not be inserted into the plant genome during the transformation process. For event T303-3 cotton, absence of these genetic elements (i.e., vector backbone) was confirmed (Section V.C.).

IV.C. Identity and Source of Genetic Material

Nucleotide positions within the plasmid pTDL004, orientation, identity and source of genetic elements and regulatory sequences are provided in Table 3.

Table 3. Genetic elements of T-DNA from pTDL004

Nt positions	Orientation	Origin
1-25		**LB**: left border from the TL-DNA from pTiB6S3 (Gielen *et al.*, 1984).
26-335	Counter clockwise	**3'nos**: sequence including the 3'untranslated region of the nopaline synthase gene from the T-DNA of pTiT37 (Depicker *et al.*, 1982).
336-887	Counter clockwise	***bar***: coding sequence of the phosphinothricin acetyltransferase gene of *Streptomyces hygroscopicus* as described by Thompson *et al.* (1987).
888-1771	Counter clockwise	**P35s3**: sequence including the promoter region of the Cauliflower Mosaic Virus 35S transcript (Odell *et al.*,1985).
1772-2304	Clockwise	**P35s2**: sequence including the promoter region from the Cauliflower Mosaic Virus 35S transcript (Odell *et al.*, 1985).
2305-2359	Clockwise	**5'e1**: sequence including the leader sequence of the tapetum specific E1 rice gene (*GE1*) (Michiels *et al.*, 1992).
2360-4213	Clockwise	**Cry1Ab5PGS3a**: sequence coding the Cry1Ab crystal protein gene (MetAlaAsp2...Asp616) from *Bacillus thuringiensis berliner* 1715 (Höfte *et al.*,1986).
4214-5150	Clockwise	**3'me1**: sequence including the 3'untranslated region of the NADP-malic enzyme gene from yellowtop (Flaveria bidentis)(Marshall *et al.*, 1996).
5151-5175		**RB**: right border repeat from the TL-DNA from pTiB6S3 (Gielen *et al.*, 1984).

V. GENETIC CHARACTERIZATION OF EVENT T303-3

Genetic characterization of event T303-3 demonstrated that one copy of the *cry1Ab* gene cassette and one copy of the *bar* gene cassette were inserted as a result of the transformation. The DNA sequence of the pre-insertion locus as well as the insert was determined. The 5' and 3' flanking sequences of the insertion site of event T303-3 and the pre-insertion locus of wild type *G. hirsutum* were determined and no rearrangements of genomic DNA were found. Further investigation confirmed the absence of any elements of the vector backbone.

V.A Insert Characterization

V.A.1. Southern Blot Analyses

Southern blot analyses were used to characterize the insert present in event T303-3 (Habex *et al.*, 2007). To this end, several identically prepared membranes were loaded with samples of event T303-3 genomic DNA digested with nine different restriction enzymes. The appropriate positive control samples (genomic DNA from non-transgenic cotton supplemented with one copy pTDL004 DNA digested with *Not*I) and negative controls (genomic DNA from non-transgenic cotton) were also prepared and included on the membranes. The blots were sequentially hybridized with different probes, either designed to hybridize to a specific component of the *cry1Ab* or *bar* gene cassettes or to the complete T-DNA. A detailed description of the materials and methods employed for the insert characterization of event T303-3 are provided in Appendix 2.A. A summary of the restriction enzymes and T-DNA probes used as well as expected and obtained hybridization fragments is provided in Table 4.

Table 4. Expected and Obtained Hybridization Fragments of Event T303-3

Digest	Probes												Description fragments
	PT020-1: 3'me1		PT021-1: *cry1Ab*		PT022-1: P35S3		PT023-1: *bar*		PT024-1: 3'nos		PT025-1: complete T-DNA		
	Expected hybridization fragments	Obtained hybridization fragments	Expected hybridization fragments	Obtained hybridization fragments	Expected hybridization fragments	Obtained hybridization fragments	Expected hybridization fragments	Obtained hybridization fragments	Expected hybridization fragments	Obtained hybridization fragments	Expected hybridization fragments	Obtained hybridization fragments	
BamHI	> 904 bp	ca. 1500 bp	> 904 bp 2448 bp	ca. 1500 bp 2448 bp	2448 bp > 1307 bp	2448 bp ca. 7000 bp	> 1307 bp	ca. 7000 bp	2448 bp	2448 bp	> 904 bp 2448 bp > 1307 bp	ca. 1500 bp 2448 bp ca. 7000 bp	5' integr. fr. internal fr. 3' integr. fr
BglII	> 1471 bp	ca. 2050 bp	> 1471 bp 474 bp 1429 bp	ca. 2050 bp 474 bp 1429 bp	1429 bp > 1285 bp	1429 bp ca. 7700 bp	> 1285 bp	ca. 7700 bp	1429 bp	1429 bp	> 1471 bp 474 bp 1429 bp > 1285 bp	ca. 2050 bp 474 bp 1430 bp ca. 7700 bp	5' integr. fr. internal fr. internal fr. 3' integr. fr
SacI	> 4659 bp	ca. 14 kb	> 4659 bp	ca. 14 kb	> 4659 bp	ca. 14 kb	> 4659 bp	ca. 14 kb	> 4659 bp	ca. 14 kb	> 4659 bp	ca. 14 kb	Integr. fr
KpnI	> 3410 bp	ca. 14 kb	> 3410 bp	ca. 14 kb	> 3410 bp > 1249 bp	ca. 14 kb ca. 7600 bp	> 1249 bp	ca. 7600 bp	> 3410 bp	ca. 14 kb	> 3410 bp > 1249 bp	ca. 14 kb ca. 7600 bp	5' integr. fr 3' integr. fr.
NcoI	> 2272 bp	ca. 7600 bp	> 2272 bp	ca. 7600 bp	794 bp > 739 bp	794 bp ca. 14 kb	854 bp	854 bp	854 bp	854 bp	> 2272 bp 794 bp 854 bp > 739 bp	ca. 7600 bp 794 bp 854 bp ca. 14 kb	5' integr. fr. internal fr. internal fr. 3' integr. fr
PvuI	> 3075 bp	ca. 4000 bp	> 3075 bp	ca. 4000 bp	> 3075 bp > 1584 bp	ca. 4000 bp > 14 kb	> 1584 bp	> 14 kb	> 1584 bp	> 14 kb	> 3075 bp > 1584 bp	ca. 4000 bp > 14 kb	5' integr. fr 3' integr. fr.
XbaI	> 1976 bp	ca. 2100 bp	> 1976 bp 1103 bp	ca. 2100 bp 1103 bp	1103 bp > 1580 bp	1103 bp ca. 5300 bp	> 1580 bp	ca. 5300 bp	> 1580 bp	ca. 5300 bp	> 1976 bp 1103 bp > 1580 bp	ca. 2100 bp ca. 1100 bp ca. 5300 bp	5' integr. fr. internal fr. 3' integr. fr
EcoRV	> 1546 bp	ca. 5000 bp	> 1546 bp 881 bp	ca. 5000 bp 881 bp	881 bp (1) > 2232 bp	/ ca. 14 kb	> 2232 bp	ca. 14 kb	> 2232 bp	ca. 14 kb	> 1546 bp 881 bp > 2232 bp	ca. 5000 bp 881 bp ca. 14 kb	5' integr. fr. internal fr. 3' integr. fr
NotI	> 4659 bp	> 14 kb	> 4659 bp	> 14 kb	> 4659 bp	> 14 kb	> 4659 bp	> 14 kb	> 4659 bp	> 14 kb	> 4659 bp	> 14 kb	Integr. fr
WT - *NotI*	none	none	none	none	none	none	none	none	none	none	none	none	none
WT + 1 copy pTDL004 - *NotI*	4915 bp	4915 bp	4915 bp	4915 bp	4915 bp 6167 bp	4915 bp 6167 bp	6167 bp	6167 bp	6167 bp	6167 bp	4915 bp 6167 bp	4915 bp 6167 bp	Plasmid fr. Plasmid fr.

(1) This fragments was not visible due to a small overlap with the used probe (overlap of 92 bp)

Based on the obtained hybridization results, the organization of the inserted transgenic sequences of event T303-3 were determined and demonstrated that the insert of event T303-3 has one complete copy of each the *cry1Ab* and *bar* gene cassettes. Figure 4 shows a schematic presentation of the insert organization deduced by hybridization fragment sizes. Restriction enzymes and probes as well as the expected hybridization fragments are indicated.

Figure 4. Event T303-3 insert organization by probe hybridization sizes

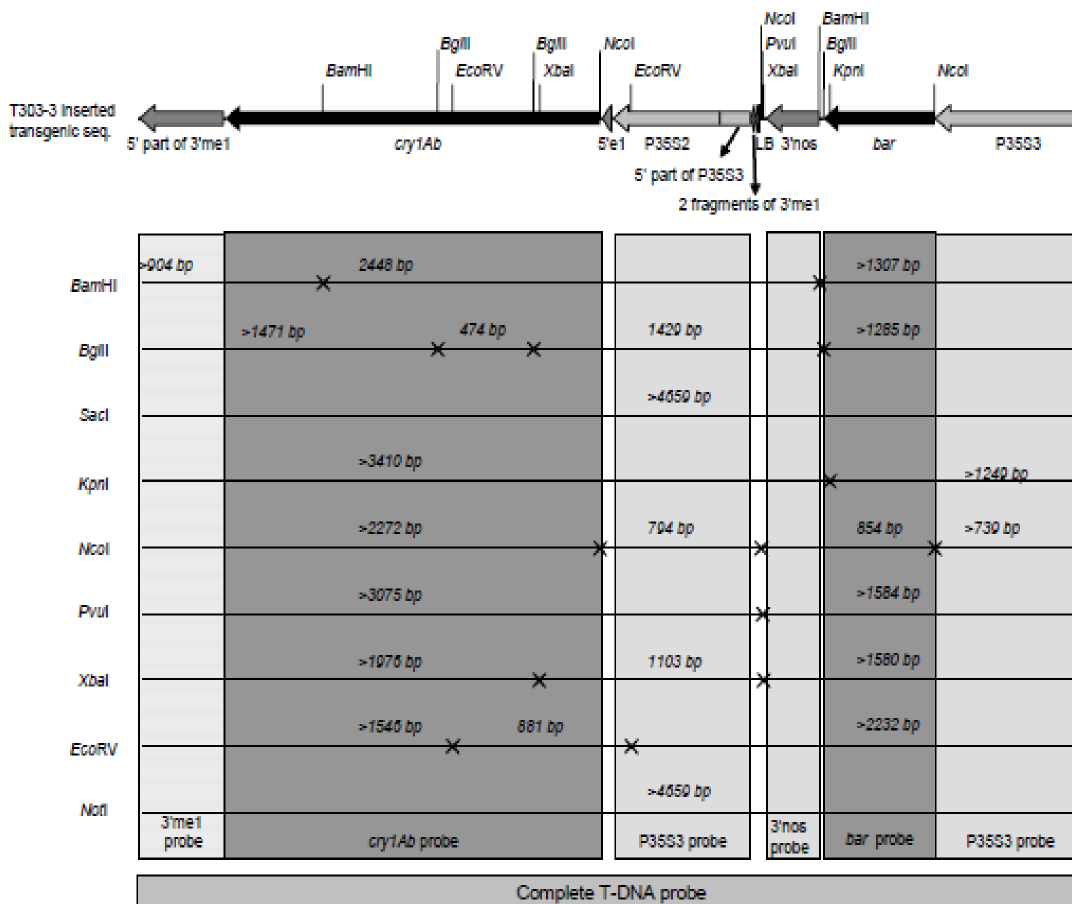
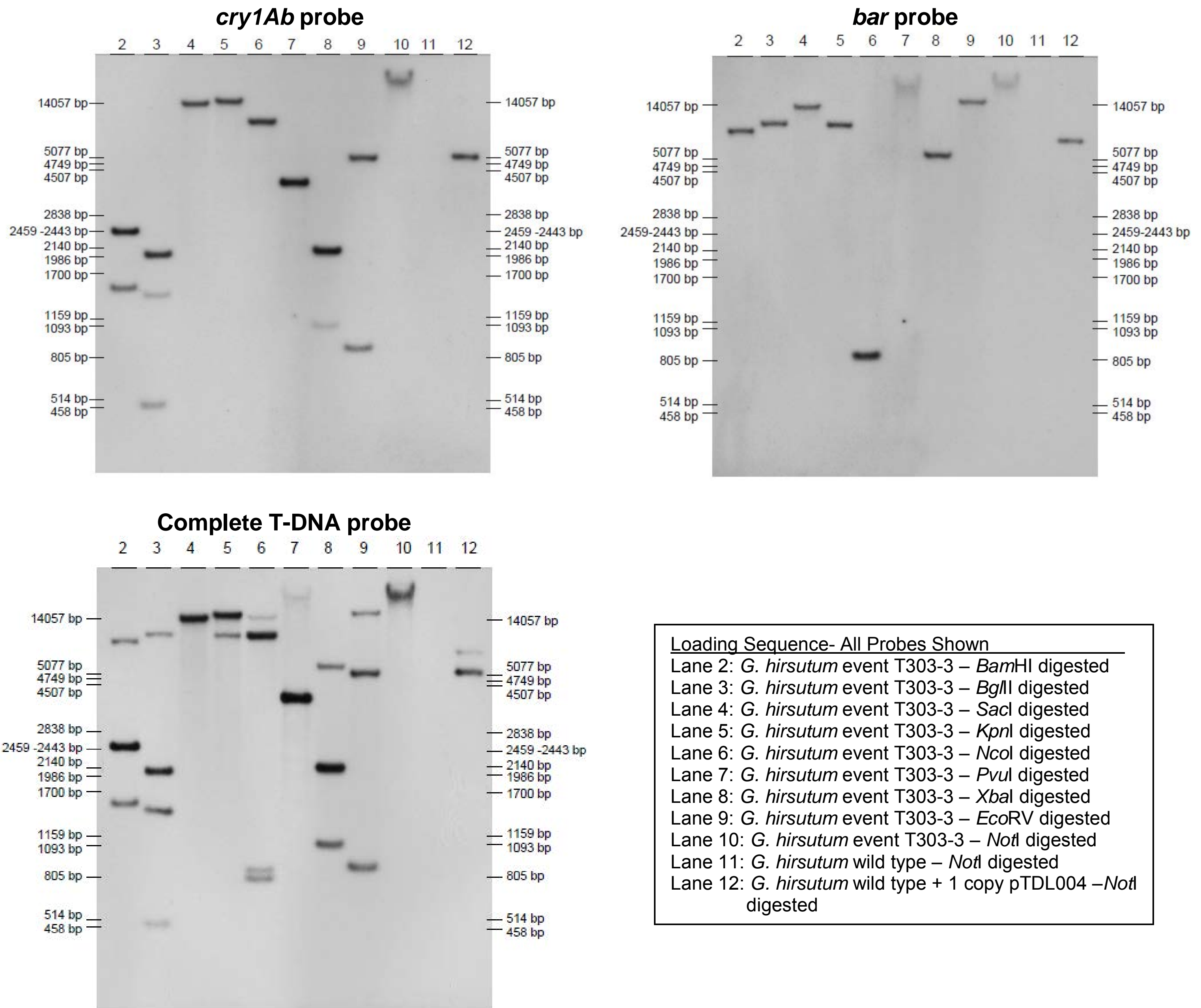


Figure 5 shows the results of the Southern analyses utilizing the *cry1Ab*, *bar*, and complete T-DNA probes. Additional results for all Southern analyses utilized for insert characterization are presented in Appendix 3.A.

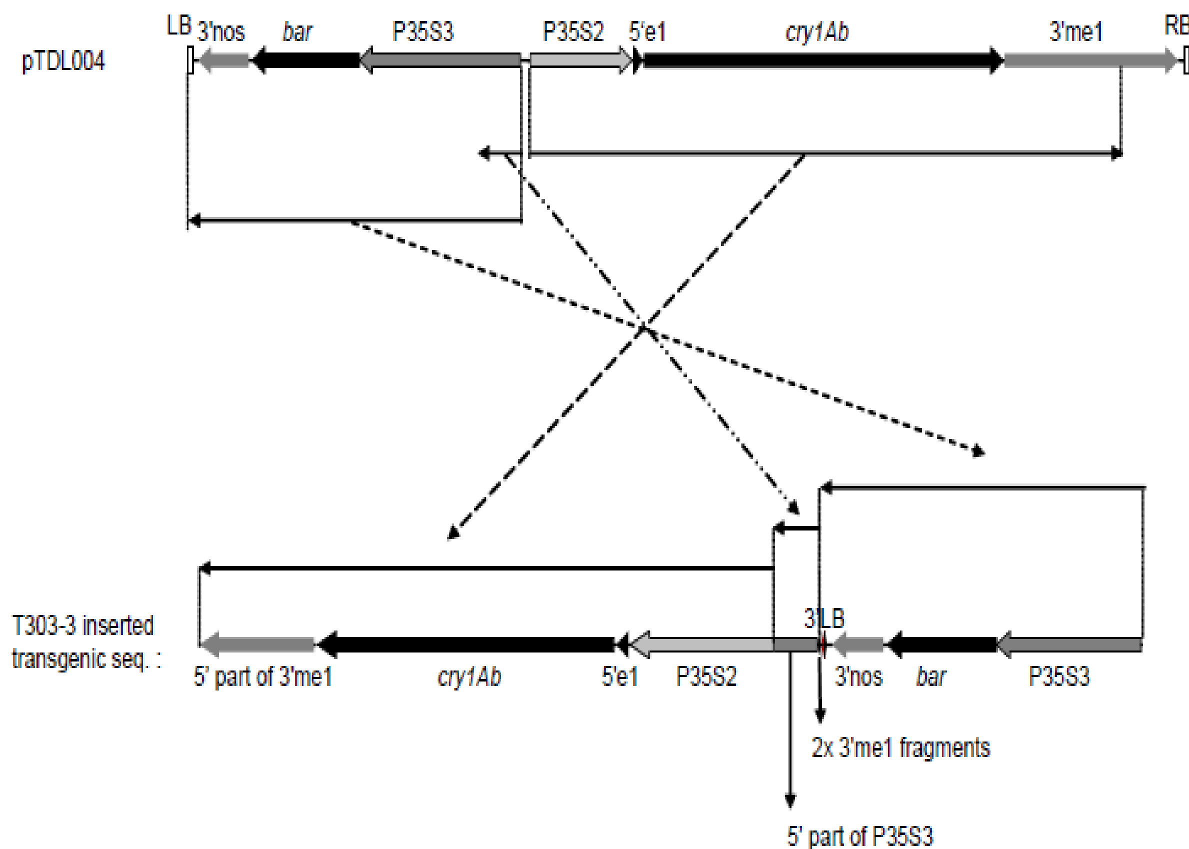
Figure 5. Hybridization results utilizing *cry1Ab*, *bar* and T-DNA probes



V.A.2. DNA Sequencing

DNA sequencing of the transgenic locus confirmed the results of the characterization by Southern analyses which indicated that the inserted sequence of event T303-3 has one intact copy of each the *cry1Ab* and *bar* gene cassettes (Moens et al., 2007). DNA sequencing also confirmed the arrangement of the inserted DNA elements as deduced from Southern analyses (Figure 6). As shown below, the insertion resulted in rearrangements of genetic elements of the T-DNA from pTDL004. However, each of the gene cassettes were inserted as complete and single copies

Figure 6. Insert organization of event T303-3



The pre-insertion locus, the inserted sequences and the flanking sequences were determined by DNA sequence analysis of event T303-3 and non-transgenic *G. hirsutum* (Moens *et al*., 2007). The results showed a target site deletion of 1184 bp. The putative function of the deleted sequence was not explored; however agronomic and composition analyses (Section VII) indicate that event T303-3 is comparable to other *G. hirsutum* varieties.

Event T303-3 flanking sequences of 776 bp at the 5' and 251 bp at the 3' junctions, respectively, were determined and were shown to be identical to sequences at the pre-insertion locus of non-transformed *G. hirsutum*.

V.B. Open Reading Frames and Associated Regulatory Regions

During the transformation process, the T-DNA from plasmid pTDL004 underwent some rearrangements creating several new junctions as shown in Figure 6. For these regions, an investigation was conducted as to whether any novel unexpected proteins could be expressed due to the insertion and rearrangement of the gene cassettes (De Pestel, 2008).

Open reading frame (ORF) analysis and gene search tools were applied to predict the presence of any potential newly created coding sequences in the junction regions. Seven newly created ORFs were identified and characterized. Bioinformatics tools were applied to look for regulatory elements needed for transcription and translation of any potential new ORFs. Four putative promoter regions were identified, however, none of them were found to be in a configuration likely to initiate transcription of the predicted ORFs. Thus, it was concluded that the expression of a new proteins from the seven predicted ORFs was highly unlikely as the regulatory elements necessary to initiate transcription and translation were not present.

In addition, the putative amino acid sequences of the seven ORFs were also compared with sequences of known allergens and toxins using publically available protein databases Uniprot-Swissprot (release 55.6, 2008), Uniprot-TrEMBL (release 37.8, 2008), PDB (release 2007), DAD (release 37.8, 2007), and GenePept (release 165, 2008) (Capt, 2009). Searches included epitope homology search using overlapping eight amino acid sequences and overall homology search utilizing BLASTP algorithm. The matching criteria was 100% identity match over a linear contiguous eight amino acid segment and 35% identity match over the full-length query sequence with a known toxin or allergen for epitope and overall homology searches, respectively. No significant similarities were found between the putative seven ORF sequences and any sequences from the databases.

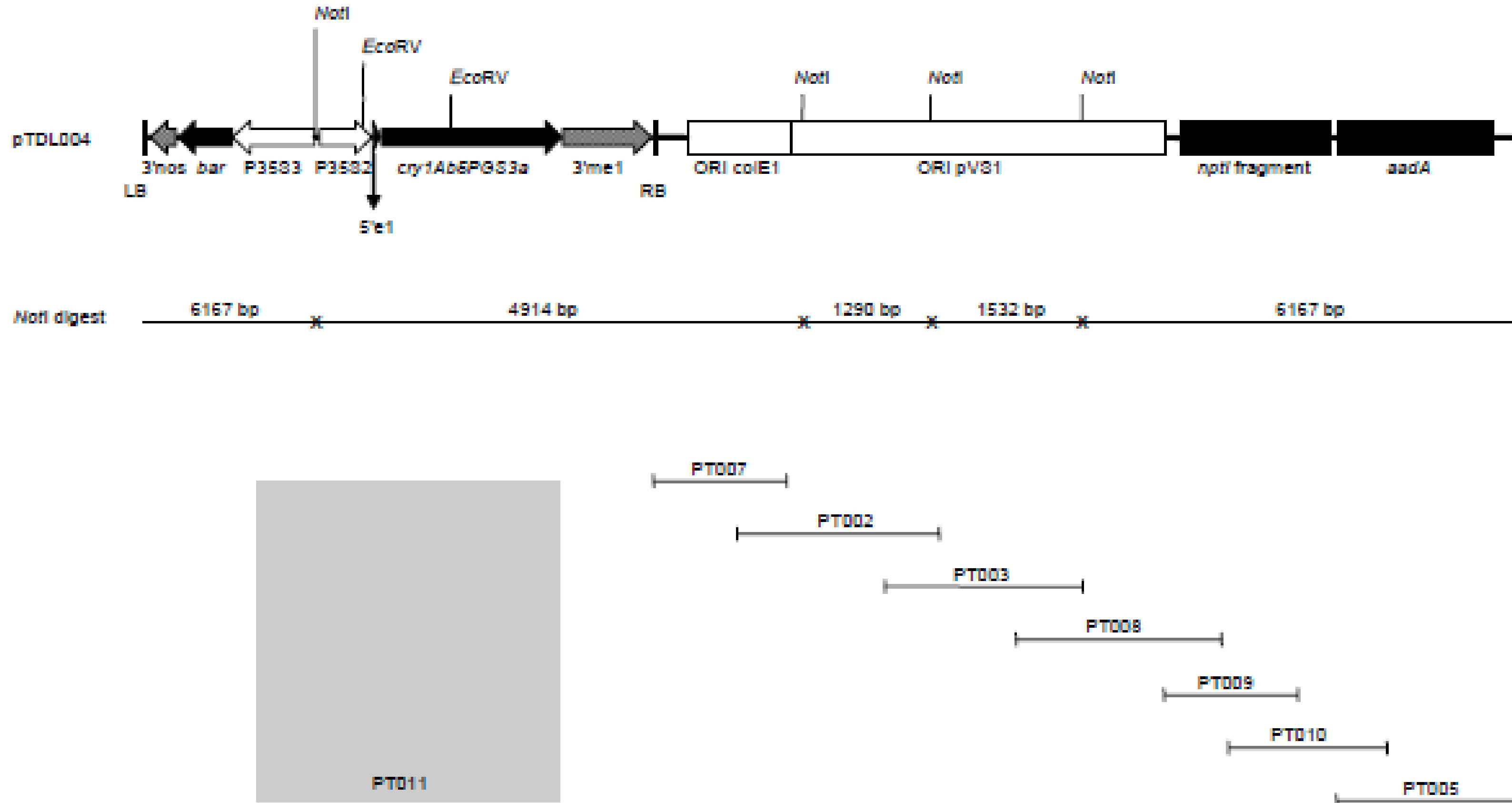
These findings show that the ORF analysis of the insertion present in event T303-3 did not identify any potential for new proteins to be expressed and moreover, putative amino acid sequences did not show homology to any known toxins or allergens.

V.C. Confirmation of the Absence of Vector Backbone

Southern analysis, confirmed the absence of vector backbone in event T303-3 (Habex, 2006). Samples of genomic DNA from event T303-3, non-transgenic control plants (negative control), and DNA from plasmid pTDL004 (positive control) were analyzed using seven overlapping vector backbone probes covering the vector backbone

sequences of plasmid pTDL004. Detailed materials and methods are provided in Appendix 2.B. A schematic diagram illustrating the probe strategy is shown in Figure 7.

Figure 7. Schematic drawing of probe strategy for vector backbone detection

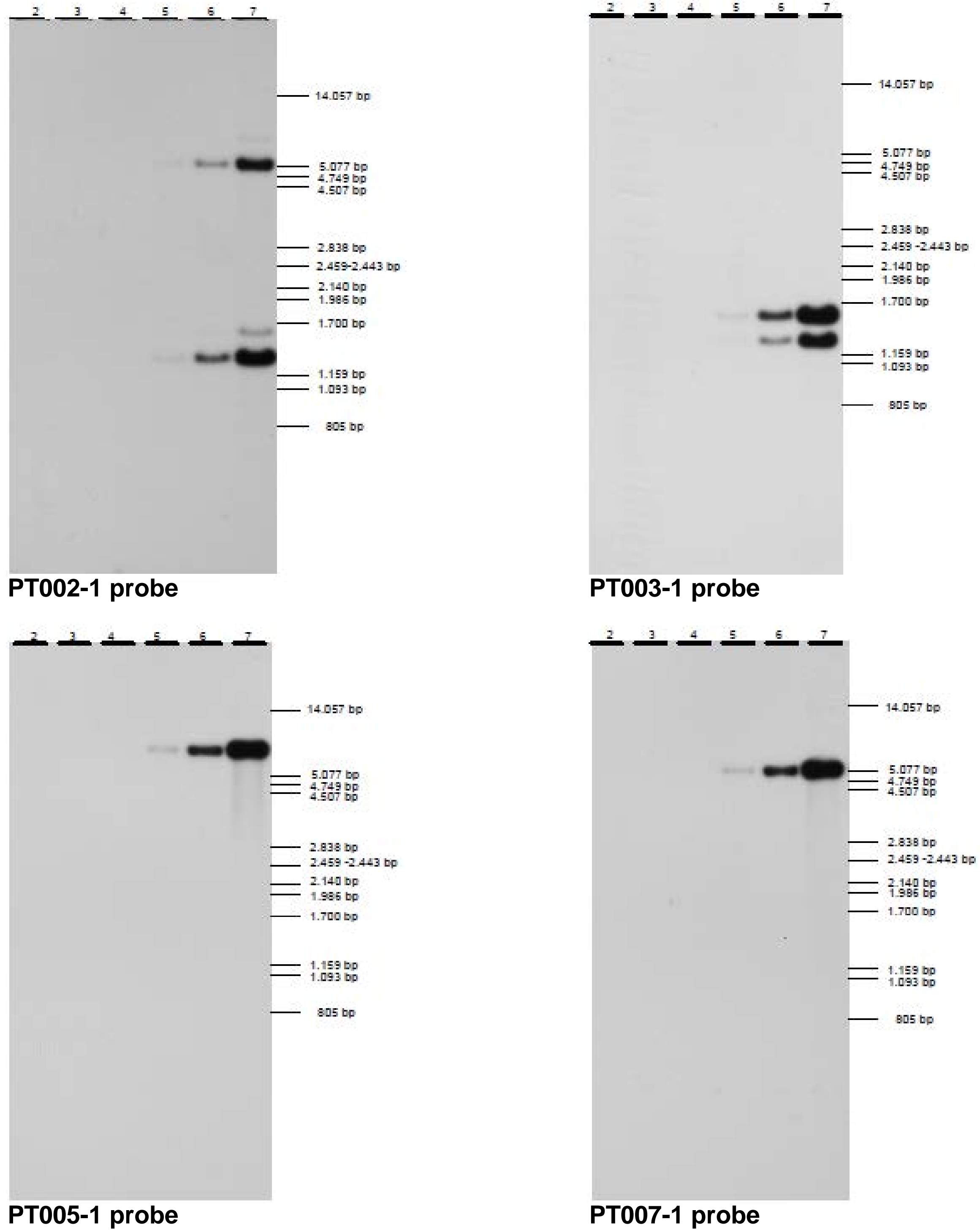


Expected and obtained hybridization fragments by probe, test material, and digest used are provided in Appendix 3 Table 24 (vector backbone probes) and 25 (T-DNA probe).

Genomic DNA from event T303-3 plants and negative control plants showed no hybridization with any of the vector backbone probes. Hybridization occurred with the positive control, demonstrating the conditions were appropriate for hybridization to occur (Figure 8).

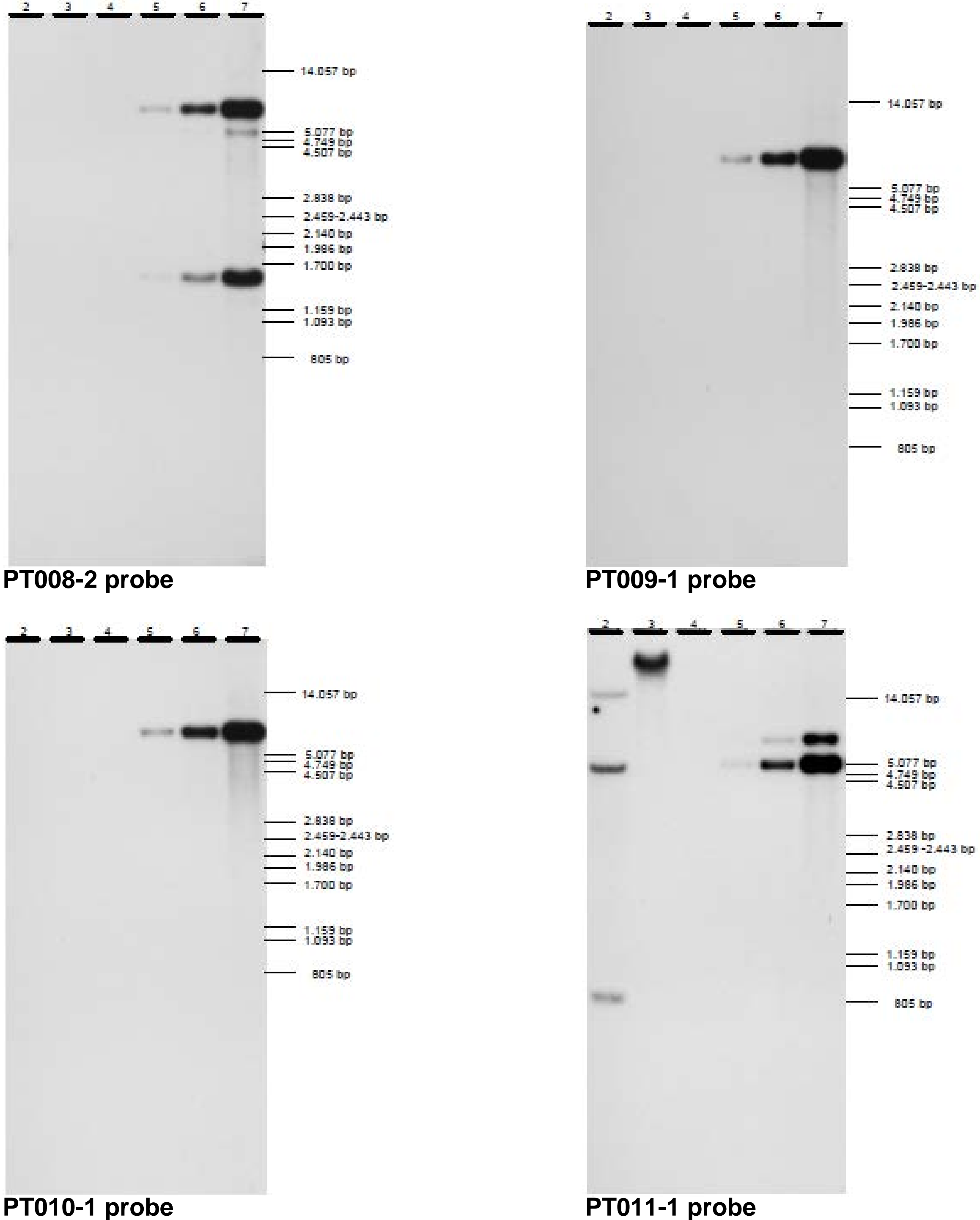
CONTAINS NO CONFIDENTIAL BUSINESS INFORMATION

Figure 8. Event T303-3 verification of absence of vector backbone – Southern blot results



Loading Sequence All Probes
Lane 2: *G. hirsutum* event T303-3 – *Eco*RV digested
Lane 3: *G. hirsutum* event T303-3 – *Not*I digested
Lane 4: *G. hirsutum* wild type – *Not*I digested
Lane 5: *G. hirsutum* wild type – *Not*I digested + 0.1 copy pTDL004 – *Not*I digested
Lane 6: *G. hirsutum* wild type – *Not*I digested + 1 copy pTDL004 – *Not*I digested
Lane 7: *G. hirsutum* wild type – *Not*I digested + 10 copy pTDL004 – *Not*I digested

Figure 8 (continued). Event T303-3 verification of absence of vector backbone – Southern blot results



Loading Sequence All Probes
Lane 2: *G. hirsutum* event T303-3 – *Eco*RV digested
Lane 3: *G. hirsutum* event T303-3 – *Not*I digested
Lane 4: *G. hirsutum* wild type – *Not*I digested
Lane 5: *G. hirsutum* wild type – *Not*I digested + 0.1 copy pTDL004 – *Not*I digested
Lane 6: *G. hirsutum* wild type – *Not*I digested + 1 copy pTDL004 – *Not*I digested
Lane 7: *G. hirsutum* wild type – *Not*I digested + 10 copy pTDL004 – *Not*I digested

V.D. Mendelian Inheritance

The Mendelian segregation of event T303-3 was analyzed, using chi-square analysis, at several stages of introgression of event T303-3 into conventional lines as well as in several inbred lines of the T_0 transformant. To determine whether event T303-3 was segregating as expected each generation was sprayed with glufosinate ammonium herbicide to eliminate herbicide-susceptible plants. The results shown in Table 5 illustrate that event T303-3 behaves as a single dominant allele at one locus, with the expected segregation ratio of 3:1 and is stably integrated within and across generations.

Table 5. Mendelian segregation of cotton event T303-3

Generation	Ratio	Observed		Expected		Chi-Square
		T[c]	S[d]	T[c]	S[d]	
T_1	3:1	40	9	37	12	1.1496[e]
T_2	3:1	23	6	22	7	0.2873[e]
F_2[a]	3:1	16	6	15	5	2.5[f]
BC_3F_3[b]	3:1	18	6	15	9	0.3[f]

[a] Self-pollinated hemizygous plant.
[b] Hemizygous BC_3F_2 plant, self-pollinated.
[c] Tolerant to glufosinate herbicide, positive segregant
[d] Susceptible to glufosinate herbicide, null segregant
[e] The chi-square value at the 95% level is 3.84. To be significantly different, the calculated chi-square value would need to be greater than 3.84.
[f] The chi-square value at the 95% level is 5.99. To be significantly different, the calculated chi-square value would need to be greater than 5.99.

V.E. Conclusions

Results of the molecular characterization of event T303-3 indicate that the event contains a single insert containing the *cry1Ab* and *bar* gene expression cassettes. The results demonstrated that the inserted DNA contains the *cry1Ab* and *bar* genes and their respective regulatory elements in a functional order. The insert has been shown to be stably inherited in several different genetic backgrounds and generations using Mendelian segregation analysis that tracked phenotypic tolerance to glufosinate ammonium herbicide. Those results clearly indicate that the trait is inherited as expected for a single locus. Southern blot analysis also confirmed the absence of the transformation vector backbone. Genomic flanking sequences at the 5' and 3' junctions of the insertion in event T303-3 were sequenced and found to match those of the pre-insertion locus. Bioinformatics analysis of ORFs did not identify any homologies with known allergen and toxin sequences.

With the exception of the promoter for the *cry1Ab* gene, event T303-3 cotton contains the same genetic elements as the antecedent organism. Genetic characterization demonstrates that event T303-3 is no more likely to become a plant pest in the environment than the antecedent organism, event T304-40.

VI. CHARACTERIZATION OF THE INTRODUCED PROTEINS

Event T303-3 and the antecedent organism, event T304-40, were transformed to produce the same insecticidal crystal protein (ICP) Cry1Ab coded for by the *cry1Ab* gene isolated from *Bacillus thuringiensis berliner* and the same PAT protein expressed by the *bar* gene from *Streptomyces hygroscopicus*. Event T304-40 is one of the parental events in TwinLink Cotton (T304-40 x GHB119) and as such has been previously reviewed by USDA APHIS as part of the determination of non-regulated status (USDA-APHIS, 2011).

VI.A Cry1Ab Protein

VI.A.1 History and Background

There are no changes in the history and background of the Cry1Ab protein from the previous petition 08-340-01p entitled "Petition for Determination of Nonregulated Status for Insect-Resistant and Glufosinate-Ammonium-Tolerant Cotton: TwinLink™ Cotton (events T304-40 x GHB119)" (USDA, 2008).

VI.A.2 Characterization of the Cry1Ab Protein

Petition 08-340-01p demonstrated the Cry1Ab protein produced *in planta* by event T304-40 was comparable to the Cry1Ab protein isolated from *E. coli* (USDA, 2008). For the protein safety data provided in the petition to also be applicable to the Cry1Ab protein produced by event T303-3, additional analysis by SDS-PAGE was performed to demonstrate that the Cry1Ab protein produced *in planta* by event T303-3 is comparable to that produced by event T304-40 and the *E. coli*-derived protein.

Because the Cry1Ab protein in T303-3 cotton was shown to be comparable to that of event T304-40 (Section VI.E.), no additional information on the characterization beyond that presented in petition 08-340-01p is provided.

VI.B The PAT/*bar* Protein

PAT protein biochemistry and mode of action are included in the peer-reviewed scientific journal published by Herouet et al. (2005) and in US-FDA BNF 000086 (US-FDA, 2003). Briefly, phosphinothricin (L-PPT) and demethylphosphinothricin (DMPT) are inhibitors of glutamine synthetase. This inhibition results in the accumulation of toxic ammonium ions and a decrease in the amount of glutamine, an essential amino acid used in many anabolic processes. The PAT enzyme is an acetyltransferase that specifically catalyzes the acetylation of both L-PPT and DMPT. Enzymatic properties of the PAT protein are well-characterized, in particular, pH and temperature dependency are well-described and understood. From the perspective of safety, this characterization demonstrates that metabolic effects of the expression of the PAT protein are limited to conferring tolerance to the herbicide glufosinate ammonium.

VI.C Protein Safety

Since the Cry1Ab and PAT proteins expressed in T303-3 cotton are the same as expressed in T304-40 cotton, no additional safety information is available that was not presented in petition 08-340-01p. In summary, in order to assess any potential adverse effects to humans or animals resulting from environmental release of the crops expressing

the Cry1Ab and PAT proteins, Bayer CropScience (BCS) has conducted a detailed safety evaluation based on Codex Alimentarius Commission (Codex Alimentarius, 2003). As a basis, BCS performed a series of safety studies with these proteins, including homology searches of the amino acid sequences with comparison to all known allergens and toxins from large public databases, an *in vitro* digestibility assay of the proteins, and an acute toxicity test in the mouse. Moreover, publicly available review documents issued by regulatory authorities, indicating that similar protein family members are safe, have been used for supporting this safety assessment. The results of studies conducted by BCS are consistent with the published information, confirming that the crops containing these proteins can be safely used as food or feed.

Lack of allergenic potential

- The Cry1Ab and PAT proteins have no amino acid sequence similarity to known allergens, as demonstrated by overall amino acid and epitope homology searches;
- As expected, the Cry1Ab and PAT proteins only have similarities with other Cry or PAT proteins;
- The Cry1Ab and PAT proteins do not share epitopes with known allergens;
- The Cry1Ab and PAT proteins are not glycosylated;
- The Cry1Ab and PAT proteins are not heat stable;
- The Cry1Ab and PAT proteins are degraded by human simulated gastric and intestinal fluids. This minimizes the likelihood that these proteins could survive in the human digestive tract and be absorbed.

Lack of toxic potential

- The Cry1Ab and PAT proteins have no amino acid sequence similarity to known allergens, as demonstrated by overall amino acid homology searches;
- The Cry1Ab and PAT proteins are degraded by human simulated gastric and intestinal fluids. This minimizes the likelihood that these proteins could survive in the human digestive tract and be absorbed;
- There were no mortalities, clinical signs or treatment-related effects in female OF1 mice after an acute oral administration by gavage of the Cry1Ab, Cry2Ae or PAT proteins at 2,000 mg protein/kg body weight.

In conclusion, it is considered that the *cry1Ab* and *bar* genes as well as the Cry1Ab and PAT proteins are not toxic to mammals and do not possess any of the characteristics associated with food allergens. Therefore, no effects on animal and human health are to be expected by consumption of the *cry1Ab* or *bar* genes and the Cry1Ab or PAT proteins.

VI.D. Expression of Introduced Proteins

The content of the Cry1Ab and PAT proteins in ginned cottonseed (also referred to as fuzzy seed) of event T303-3 was determined by enzyme-linked immunosorbent assays (ELISA) specific to each protein (Robinson, 2008). Detailed methods for analysis of expression levels of Cry1Ab in raw agricultural commodity are provided in Appendix 2.C.

Since fuzzy seed is not delinted, it could not be ground into a homogeneous material. A procedure was developed previously to effectively remove the lint coat from the kernel, creating two fractions (kernel and lint coat). These two fractions were analyzed separately for Cry1Ab and PAT content and then added together to re-create the amount in the original fuzzy seed. Seed was collected from event T303-3 plants grown in six field trials in

the U.S. One unsprayed event T303-3 sample and three event T303-3 samples sprayed with glufosinate ammonium from each of the sites were analyzed. Unsprayed, non-transgenic seed (control) from each of the trial sites was also analyzed.

On a dry weight basis, the average Cry1Ab protein content from all test sites of cotton fuzzy seed samples from unsprayed event T303-3 plants ranged from 7.14 µg/g to 17.9 µg/g (overall average value = 11.7 ± 3.74 µg/g) and from 10.9 µg/g to 19.1 µg/g in fuzzy seed samples from sprayed event T303-3 plants (overall average value = 14.3 ± 3.50 µg/g) (Table 6). Cry1Ab protein constitutes 0.005073% of the total crude protein in unsprayed transgenic fuzzy seed and 0.005862% in sprayed transgenic fuzzy seed. No Cry1Ab protein was detected in the non-transgenic control fuzzy seed samples. As a comparison, the Cry1Ab content of event T304-40 in unsprayed fuzzy seed was 1.29 ± 0.52 µg/g d.w. (0.000561% of total crude protein) and 1.54 ± 0.318 µg/g d.w. (0.000561% of total crude protein). These differences in the amount of Cry1Ab protein does not affect the efficacy displayed toward the target pest organisms and the Cry1Ab protein represents a small component of the total protein in events T303-3 and T304-40.

Table 6. Cry1Ab protein levels in event T303-3 fuzzy seed

Sample	Average Cry1Ab Content (µg/g Sample)		Average Cry1Ab Content (as % of total crude protein)	
	No Glufosinate	Glufosinate Sprayed	No Glufosinate	Glufosinate Sprayed
Fuzzy Seed	17.9	17.3	0.007668	0.007442
	11.4	15.4	0.005758	0.006873
	7.14	10.9	0.003004	0.004631
	11.3	11.8	0.004450	0.004436
	9.06	11.3	0.003901	0.004255
	13.7	19.1	0.005659	0.007537
Range in Values	7.14 – 17.9	10.9 – 19.1	0.003004 – 0.007668	0.004255 – 0.007537
Average ± SD	11.7 ± 3.74	14.3 ± 3.50	0.005073 ± 0.001648	0.005862 ± 0.001578

On a dry weight basis, the average PAT protein content from all test sites of transgenic cotton ranged from 56.5 µg/g to 130 µg/g d.w. in unsprayed fuzzy seed (overall average value = 102 ± 26.9 µg/g) and from 89.3 µg/g to 142 µg/g in sprayed fuzzy seed (overall average value = 118 ± 20.7 µg/g) (Table 7). PAT protein constitutes 0.043618% of the total crude protein in unsprayed transgenic fuzzy seed and 0.048027% of the total crude protein in sprayed transgenic fuzzy seed. No PAT/*bar* protein was detected in the non-transgenic unsprayed fuzzy seed. As a comparison, the PAT/*bar* protein of event T304-40 was 163 ± 32 µg/g (0.0728% of total crude protein) in unsprayed transgenic fuzzy seed and 163 ± 27 µg/g (0.0736% of total crude protein) in sprayed transgenic fuzzy seed. These differences in the amount of the PAT/*bar* protein do not affect the level of tolerance to the herbicide between the two events and PAT protein represents a small component of the total protein content of the two events.

Table 7. PAT/*bar* protein levels in event T303-3 fuzzy seed

Sample	Average PAT/*bar* Content (µg/g Sample) ± SD		Average PAT/*bar* Content (as % of crude protein) ± SD	
	No Glufosinate	Glufosinate Sprayed	No Glufosinate	Glufosinate Sprayed
Fuzzy Seed	113	115	0.048430	0.049645
	82.7	101	0.041649	0.045095
	56.5	89.3	0.023766	0.038042
	114	142	0.044902	0.053390
	130	125	0.055968	0.047537
	113	138	0.046993	0.054451
Range in Values	56.5 – 130	89.3 – 142	0.023766 – 0.055968	0.038042 – 0.054451
Average ± SD	102 ± 26.9	118 ± 20.7	0.043618 ±0.010832	0.048027 ±0.006020

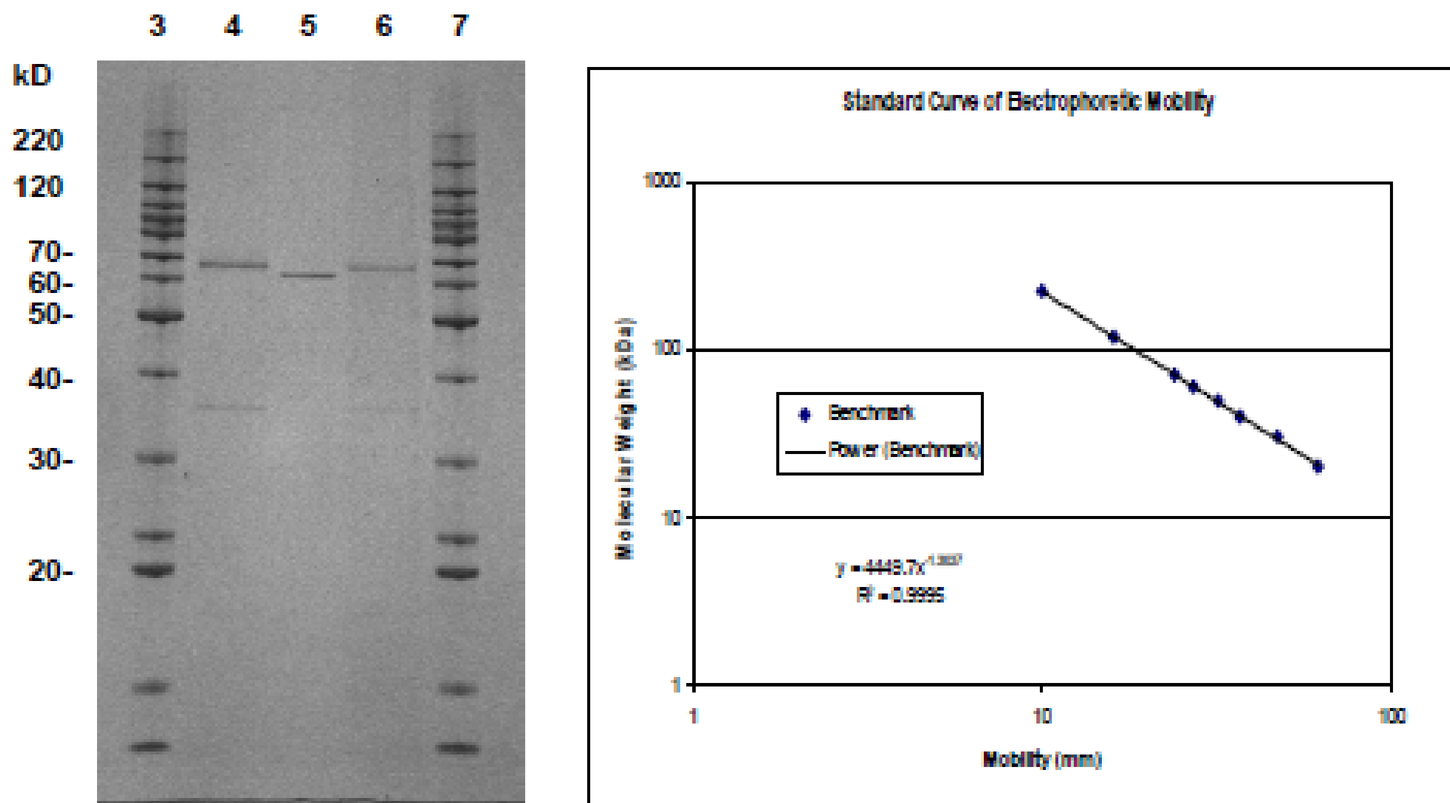
VI.E Comparison of Cry1Ab Proteins from Events T303-3 and T304-40

Safety assessment studies, such as the acute toxicity study, require large amounts of the target protein. The expression of the Cry1Ab protein in event T303-3 cotton tissues is relatively low and thus, purification of sufficient quantities of the protein for safety assessment studies from cotton tissue is not feasible. Thus, Cry1Ab protein was produced using *E. coli*. The protein produced by the bacterium was engineered to match the amino acid sequence of the Cry1Ab protein produced *in planta.* A full protein characterization study (SDS-PAGE, western analysis, glycol-staining, LC/MS, N-terminal sequencing) has been conducted on the Cry1Ab protein, expressed by event T304-40, in the combined event product TwinLink (T304-40 x GHB119) (USDA, 2008). Therefore, a SDS-PAGE analysis was conducted to compare the Cry1Ab protein isolated from event T303-3 cotton plants to the Cry1Ab protein isolated from event T304-40 cotton plants and the microbially-produced Cry1Ab from *E. coli* (Haas, 2009). A summary of the methods is provided in Appendix 2.D.

The Cry1Ab protein isolated from event T303-3, event T304-40 and *E. coli* was analyzed by SDS-PAGE to compare their respective molecular weight. Figure 9 shows the Coomassie stained gel. The Cry1Ab from events T303-3 and T304-40 have equivalent measured mobilities of 25 mm. The mobility of the trypsin digested Cry1Ab from *E. coli* (26mm) is greater than that of the plant-derived Cry1Ab proteins due to the loss of 28 amino acids which were cleaved during the production of the protein.

Bayer CropScience

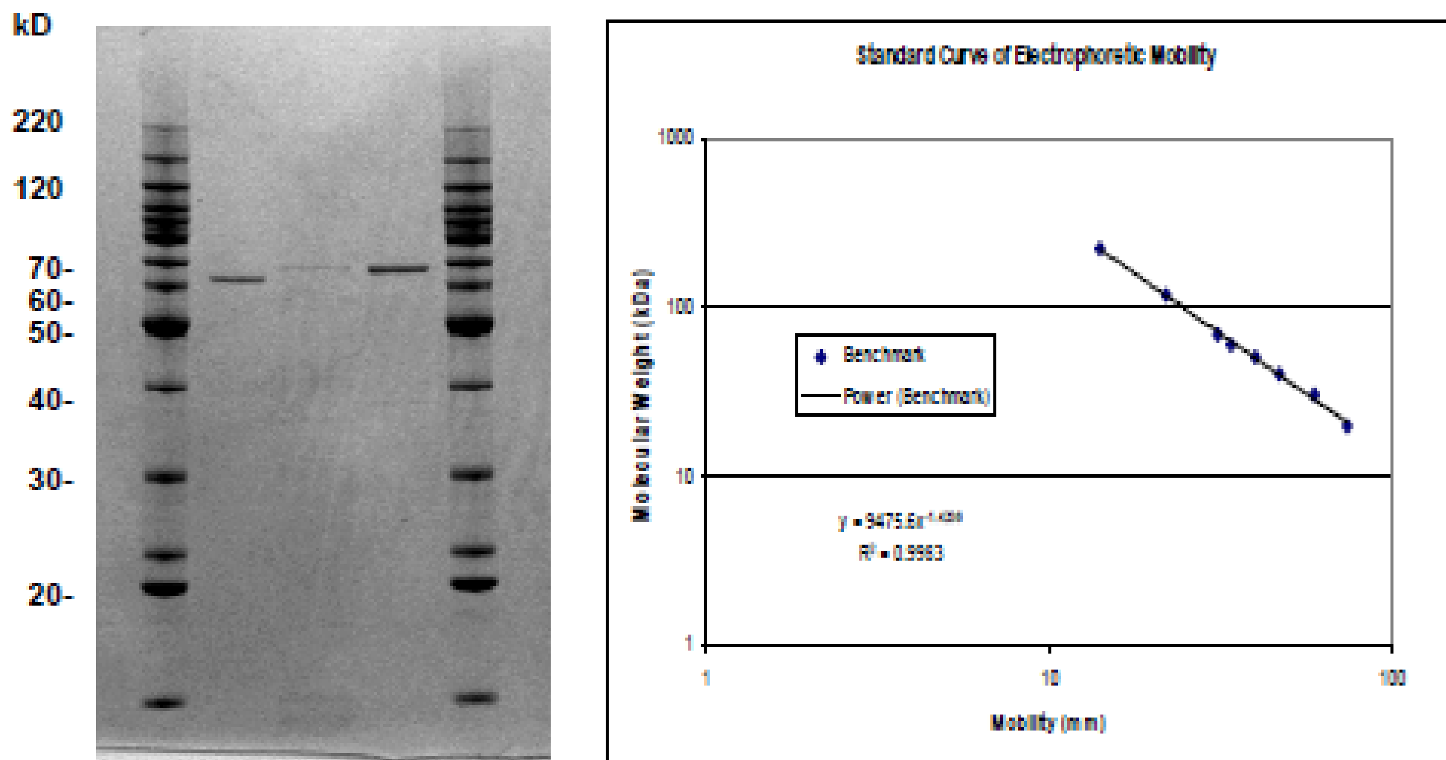
Figure 9. Comparison of the Cry1Ab protein from *E. coli* to the Cry1Ab protein isolated from events T303-3 and T304-40



3: MWM
4: T303-3 / Cry1Ab, est. 206 ng, 10 µl load
5: Trypsin digested Cry1Ab standard from E. coli, 206 ng, Batch 25.05.99.70
6: T304-40 / Cry1Ab. est. 100 ng, 10 µl load
7: MWM

The Coomassie stained gel in Figure 10 shows a comparison of the electrophoretic mobility of trypsin digested Cry1Ab protein from *E. coli* and a thrombin-engineered Cry1Ab protein from *E. coli* to the Cry1Ab isolated from event T304-40 cotton leaves. The thrombin-engineered Cry1Ab protein is representative of the plant form of Cry1Ab expressed in events T303-3 and T304-40 cotton and is converted to the active form when ingested by the target insect. The measured mobility of this protein and the Cry1Ab protein from event T304-40 cotton leaves was 32mm. The measured mobility of the trypsin-digested Cry1Ab was 34mm. Plotting of the electrophoretic mobility *vs*. the respective molecular weight (MW) yielded the approximate molecular weight. For the Cry1Ab protein from the T304-40 cotton leaves and the thrombin protein standard, the approximate MW was 68.2 kDa. The approximate MW for the trypsin-digested protein was 62.6 kDa.

Figure 10. Comparison of trypsin-digested and thrombin-engineered Cry1Ab from *E. coli* to Cry1Ab isolated from event T304-40



3: MWM
4: Trypsin digested Cry1Ab standard from E. coli, 206 ng, Batch 25.05.99.70
5: T304 / Cry1Ab est, 29 ng, 10 µl load
6: Thrombin engineered Cry1Ab standard from E. coli, 206 ng, Batch MIN1418-8
7: MWM

In summary, the SDS-PAGE results indicate that the *E. coli*-produced Cry1Ab proteins are comparable to the Cry1Ab proteins isolated from events T303-3 and T304-40. Therefore, protein characterization and safety data for the Cry1Ab produced by event T304-40 provided in petition 08-340-01p adequately addresses the same criteria for event T303-3.

VII. AGRONOMIC AND PHENOTYPIC EVALUATION

Agronomic and phenotypic characteristics of event T303-3 were evaluated in field trials in multiple locations in typical cotton growing regions of the US in 2005 and 2006. Field trials were conducted according to common agricultural practices for a given growing region.

In addition, all field trials of T303-3 conducted from 2001 to 2006 for increasing research seed as well as agronomic and phenotypic evaluation were observed for unusual or unexpected characteristics in regards to susceptibility to plant disease, weediness potential, effect on non-target organisms, beneficial insects, and the overall environment (Appendix 1).

VII.A History of Field Activities

Field trials for characterization of agronomic and plant product quality (i.e., fiber quality parameters) were conducted in 2005 under acknowledged notification 05-040-06n. In 2006 field trials were conducted under acknowledged notification 06-047-02n to assess agronomics, plant product quality, and phenotypic characteristics (i.e., efficacy). A summary of field trial locations for the assessment of agronomics, plant product quality, and phenotypic characteristics is provided in Table 8. Locations for agronomic and phenotypic evaluation were representative of major portions of the US Cotton growing regions including the Texas High Plains and Gulf Coast, and the Louisiana Delta region.

Table 8. Field trial locations for evaluation of agronomic and phenotypic characteristics

Notification Number	Year	Number of Locations	Locations
05-040-06n	2005	3	Gaines Co., TX Uvalde Co., TX Wharton Co., TX
06-047-02n	2006	5	Bossier Co., LA Gaines Co., TX Lubbock Co., TX Wharton Co., TX (2)[a]

[a] Two sites at this location

Field trials conducted in 2005 for agronomic and plant product quality assessment included homozygous event T303-3 of Coker 315 (T_4 generation) compared with wild type Coker 315.

In 2006, comparisons for agronomics and plant product quality were assessed in four field trials. Phenotype (i.e., efficacy) parameters were assessed in up to five field trials; however fewer field trials were utilized for the assessment of certain parameters. Field trials in 2006 utilized homozygous event T303-3 in the Coker 315 background (T_5 generation) and wild type Coker 315 as the comparator. In each of these trials, entries of homozygous event T303-3 and the comparator were infested or not infested with larva of *Helicoverpa zea* Boddie (cotton bollworm). Plots which were not artificially infested were sprayed with conventional insecticides according to university extension recommendations if natural infestations were observed.

Similar field trials were used for assessment of the antecedent organism, event T304-40, in 2007 and 2008.

VII.B Agronomic and Phenotypic Characteristics Evaluated

A summary and descriptions of agronomic, yield and plant product quality, and phenotypic characteristics evaluated in 2005 and 2006 field trials are provided in Table 9, 10, and 11.

Table 9. Agronomic characteristics evaluated in field trials

Parameter	Description
Emergence	Evaluation of the germination rates and plant population
Height	Average plant height from cotyledonary node to terminal, expressed in inches
Total number of nodes	Number of reproductive nodes present on the main stem of the plant
Height to node ratio (HNR)	Plant height divided by total number of nodes

Table 10. Yield and fiber quality characteristics evaluated in field trials

Parameter	Description
Yield	Productivity expressed as pounds of lint produced per acre
Gin turnout	Lint weight divided by seed cotton weight, expressed as a percentage
Seeds per boll	Average number of seeds per boll
Seed index	Average weight in grams of 100 seed, an indication of seed size and maturity.
Fiber length	Average length of the longer one-half of cotton fibers
Fiber strength	The force in grams required to break a bundle of fibers one tex unit in size (1 tex = weight in grams of 1,000 meters of fiber)
Fiber micronaire	A measure of fiber fineness and maturity as indicated by specific surface area
Fiber uniformity %	Ratio between the mean length and upper half mean length of the fibers expressed as a percentage

Table 11. Phenotypic (efficacy) characteristics evaluated in field trials

Parameter	Description
Flowers- % damaged	A measure of the percent open flowers damaged by insects
Flowers- living larvae	A count of living insect larva present in 25 flowers
Squares- % damaged	A measure of the percent immature flowers (squares) damaged by insects
Squares- living larvae	A count of living insect larva present in 25 squares
Bolls- % damaged	A measure of the percentage of fruit (bolls) damaged by insects
Bolls- living larvae	A count of living insect larva present in 25 bolls

Agronomic, plant product quality, and phenotype parameters were not assessed at all locations each year. Table 12 provides a matrix of which parameters were assessed at each field trial location.

Table 12. Parameters assessed by year and location

Location County/ST	Year	Emergence	Height	Total nodes	HNR	Yield	Gin turnout	Seeds per boll	Seed index	Fiber length	Fiber strength	Fiber micronaire	Fiber uniformity	Flowers- % damaged	Flowers- living larvae	Squares- % damaged	Squares- living larvae	Bolls - % damaged	Bolls - living larvae
Gaines/TX	‘05	■	■	■	■	■	■	■		■	■	■	■						
Uvalde/TX	‘05	■	■	■	■	■	■	■		■	■	■	■						
Wharton/TX	‘05	■	■	■	■	■	■	■		■	■	■	■						
Bossier/LA	‘06													■	■	■		■	
Gaines/TX	‘06	■	■	■	■	■	■	■	■	■	■	■	■			■	■	■	■
Lubbock/TX	‘06	■	■	■	■	■	■	■	■	■	■	■	■			■	■	■	■
Wharton/TX	‘06	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
Wharton/TX	‘06	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■

VII.B.1 Agronomic and Plant Product Characteristics

In 2005, some small differences were observed in agronomic characteristics between homozygous event T303-3 cotton and the comparator. In most cases, event T303-3 cotton was within the ranges observed for TwinLink Cotton (the product of the breeding cross of event GHB119 and event T304-40, the antecedent organism). A summary of agronomic data from 2005 field trials across locations is presented in Table 13 along with range of means for TwinLink Cotton from 2007 and 2008 field trials for the same parameters.

Table 13. Summary of agronomic data from 2005 field trials

Parameter	Coker 315 WT Mean	Coker 315 T303-3 Mean	Standard Deviation	TwinLink Range of Means[a]
Emergence plants / ft (3)[b]	0.44	0.23	0.098	1.36 – 3.57
Height inches (3)	38.81	41.16	2.210	30.33 – 38.17
Total Nodes (3)	22.34	24.67	0.712	16.95 – 23.00
HNR (3)	1.74	1.67	0.040	1.62 – 3.74

[a]Range of means for TwinLink Cotton (GHB119 x T304-40) from petition 08-340-01p
[b]Number of field trials where parameter was measured
WT = Wild type
HNR = Height to node ratio

Generally, event T303-3 Coker 315 and wild type Coker 315 were comparable for most parameters and means were within one standard deviation of each other. Further, agronomic parameters for event T303-3 fell within the range of values observed for TwinLink Cotton, or at most approximately one standard deviation outside that of TwinLink

Cotton. Comparisons of TwinLink Cotton to event T303-3 should be made with the consideration that the data does not come from the same field trials or the same year and therefore variation due to weather and location is expected.

In 2005, emergence was particularly low for both wild type Coker 315 and event T303-3. This was due to poor seed quality as a result of the delinting process for preparing planting seed. The process for preparing planting seed was improved upon for the 2006 field trials as evidenced by markedly improved emergence for both wild type Coker 315 and event T303-3 cotton. In 2006 field trials, all agronomic parameters evaluated were in line with the observed values for TwinLink Cotton from 2007 and 2008 field trials (Table 14).

Table 14. Summary of agronomic data from 2006 field trials

Parameter	Coker 315 WT (I)	Coker 315 WT	Coker 315 T303-3 (I)	Coker 315 T303-3	Standard Deviation	TwinLink Range of Means[a]
Emergence plants / ft (4)[b]	3.53	3.65	3.07	3.49	0.473	1.36 – 3.57
Height inches (4)	38.46	37.43	38.66	38.02	1.196	30.33 – 38.17
Total Nodes (4)	20.67	19.87	21.33	20.88	0.518	16.95 – 23.00
HNR (4)	1.84	1.87	1.80	1.82	0.077	1.62 – 3.74

[a]Range of means for TwinLink Cotton (GHB119 x T304-40) from petition 08-340-01p Appendix 4
[b]Number of field trials where parameter was measured
WT = Wild type
(I) = Infested with *H. zea*
HNR = Height to node ratio

Plant product characteristics were generally comparable among event T303-3 and wild type Coker 315 in 2005 field trials (Table 15). All plant product characteristics of event T303-3, with the exception of micronaire, were within the range of means observed for TwinLink Cotton in 2007 and 2008 field trials. In the case of micronaire, event T303-3 was within one standard deviation of that observed for TwinLink Cotton. Seed per boll was not evaluated in TwinLink Cotton field trials and therefore is not presented in Table 15.

Table 15. Summary of plant product characteristics from 2005 field trials

Parameter	Coker 315 WT Mean	Coker 315 T303-3 Mean	Standard Deviation	TwinLink Range of Means[a]
Yield lb / A (3)[b]	1180.56	749.22	68.657	608.00 – 1434.00
Gin Turnout % Lint (3)	39.74	38.91	0.619	35.97 – 45.40
Seed per Boll (3)	11.06	12.00	0.534	- - -
Fiber Length 1/32nd inch (3)	1.16	1.25	0.001	1.11 – 1.30
Fiber Strength g / TEX (3)	29.20	30.98	0.869	30.05 – 34.77
Micronaire (3)	4.09	3.69	0.249	3.77 – 5.25
Fiber Uniformity % (3)	83.06	85.60	0.403	81.75 – 87.87

[a]Range of means for TwinLink Cotton (GHB119 x T304-40) from petition 08-340-01p
[b]Number of field trials where parameter was measured
WT = Wild type
HNR = Height to node ratio

Again in 2006 field trials, plant product characteristics were generally comparable between wild type Coker 315 and event T303-3 in both the infested and non-infested treatments (Table 16). Means for event T303-3 from either treatment were generally within the ranges of that observed for TwinLink Cotton. Micronaire, fiber strength and yield were within one standard deviation of that observed for TwinLink Cotton. Again seed per boll and seed index were not evaluated in TwinLink Cotton field trials.

Table 16. Summary of plant product characteristics from 2006 field trials

Parameter	Coker 315 WT (I)	Coker 315 WT	Coker 315 T303-3 (I)	Coker 315 T303-3	Standard Deviation	TwinLink Range of Means[a]
Yield lb / A (4)[b]	756.19	942.08	584.36	613.00	73.404	608.00 – 1434.00
Gin Turnout % Lint (4)	38.03	38.29	37.62	37.37	1.092	35.97 – 45.40
Seed per Boll (4)	30.90	32.70	22.42	22.71	1.202	- - -
Seed Index g / 100 seed (4)	7.99	7.69	9.05	8.51	0.749	- - -
Fiber Length 1/32nd inch (4)	1.17	1.16	1.24	1.25	0.020	1.11 – 1.30
Fiber Strength g / TEX (4)	28.65	28.80	29.33	28.87	0.745	30.05 – 34.77
Micronaire (4)	4.27	4.06	3.56	3.46	0.252	3.77 – 5.25
Fiber Uniformity % (4)	82.92	82.37	82.84	83.12	0.405	81.75 – 87.87

[a]Low and high mean for TwinLink Cotton (GHB119 x T304-40) from petition 08-340-01p Appendix 4
[b]Number of field trials where parameter was measured
WT = Wild type
(I) = Infested with *H. zea*

Event T303-3, when compared with wild type Coker 315, consistently had lower yields in both 2005 and 2006 field trials. This in part led to the decision to move forward with the antecedent organism, event T304-40, as the “Cry1Ab component” in the TwinLink Cotton breeding stack. Aside from yield, event T303-3 was comparable to wild type Coker 315 and TwinLink cotton in all other agronomic and plant product characteristics. Though event T303-3 demonstrated lower yield potential, this does not in itself present any greater risk of event T303-3 becoming a plant pest.

VII.B.2 Assessment of Phenotype by Efficacy Against Insect Pests

The efficacy assessments conducted in 2006 field trials showed that the phenotype of event T303-3 was performing as expected and that the Cry1Ab protein expressed in event T303-3 was effective in protecting the plant from cotton bollworm.

A summary of efficacy data is presented in Table 17. As expected, event T303-3 in either the infested or non-infested treatments had lower damage ratings on squares, flowers, or bolls compared to the wild type Coker 315, infested or not infested with cotton bollworm. The same is true in terms of living larvae of cotton bollworm found on squares, flowers, or bolls. For all assessments made, event T303-3 plots that were infested or not infested were similar to each other. These results confirm that the Cry1Ab protein expressed by

event T303-3 effectively controls the target insect pest it was challenged with in these field trials and the phenotype of event T303-3 is comparable to the antecedent organism.

Table 17. Summary of phenotypic characteristics by efficacy against target pests

Parameter	Coker 315 WT (I)	Coker 315 WT	Coker 315 T303-3 (I)	Coker 315 T303-3	Standard Deviation
Flowers % Damaged (3)[a]	8.8	3.8	1.9	1.5	1.984
Flowers Living Larvae (3)	1.73	1.50	0.40	0.20	0.666
Squares % Damaged (5)	19.63	10.86	2.20	1.14	5.716
Squares Living Larvae (4)	3.27	1.77	0.28	0.14	1.708
Bolls % Damaged (5)	5.56	1.97	1.72	0.50	1.477
Bolls Living Larvae (4)	0.34	0.23	0.14	0.06	0.171

[a]Number of field trials where parameter was measured
WT = Wild type
(I) = Infested with *H. zea*

VII.C Disease and Pest Characteristics

Personnel conducting the field trials also visually monitored plant disease and pest resistance characteristics of event T303-3 cotton and non-transgenic controls. There were no differences reported in severity of disease symptoms or insect damage (other than the targeted insects susceptible to the Cry1Ab protein) between the transgenic plants and the non-transgenic cotton plants. For additional information please see the field trial termination reports provided in Appendix 1.

VII.D Composition Analysis

To conduct compositional analysis of event T303-3 cotton, six field trials were conducted in Georgia, Arkansas, Mississippi and Texas, all important cotton-growing regions of the southern U.S. The plants in the trials were grown under conditions typical of cotton production practices. At each site, there were six transgenic plots and three non-transgenic plots. Three of the transgenic plots at each site were sprayed twice with a tank mix of glufosinate ammonium herbicide at 0.52 lb a.i./A and ammonium sulfate at 3.0 lbs/A (target application rate). The other three transgenic plots at each site were left unsprayed.

Nine samples of fuzzy seed (ginned cottonseed) were collected from each of the six field trials (Haas, 2007). There were a total of 18 samples from each of three groups: non-transgenic Coker 315 cotton, T303-3 cotton that was unsprayed and T303-3 cotton that

was sprayed with glufosinate herbicide. Analyses of the seed samples were carried out at Eurofins Scientific Des Moines, IA (Table 18). For list of the analytical methods used refer to Table 21 in Appendix 2.E.

Table 18. Analyses performed on cottonseed of event T303-3 and its non-transgenic counterpart

Matrix	Analyses Performed
Cottonseed (fuzzy seed)	Proximates: moisture, ash, fat, protein, including carbohydrate calculation; acid detergent fiber; neutral detergent fiber, Antinutrients: gossypol (free,"-", "+" and total); phytic acid

VII.D.1 Proximate Analysis

Table 19 shows the results of the proximate analysis of event T303-3 cottonseed. These results are comparable to the proximate analysis of TwinLink (T304-40 x GHB119) as provided in petition 08-340-01p (USDA, 2008).

Table 19. Mean proximate composition in cottonseed of event T303-3

Parameter	Non-Transgenic	Transgenic Not sprayed	Transgenic Sprayed	Reference ranges [a]
	Mean ± SD	Mean ± SD	Mean ± SD	
Moisture %fw	8.64 ± 1.77	9.42 ± 1.34	9.71 ± 1.91	4.0 – 15.9
Protein %dm	23.01 ± 2.06	24.05 ± 1.70	24.63 ± 1.83	11.7 – 34.2
Fat %dm	17.38 ± 2.90	19.05 ± 1.37	19.13 ± 1.46	11.8 – 36.3
Ash %dm	4.26 ± 0.56	4.53 ± 0.45	4.65 ± 0.57	3.2 – 5.0
Total Carbohydrates %dm [b]	55.35 ± 3.29	52.37 ± 2.08	51.60 ± 2.47	36.4 – 74.4
ADF %dm	40.58 ± 4.56	39.48 ± 4.20	36.80 ± 3.46	29.0 – 66.9
NDF %dm	50.02 ± 5.90	48.85 ± 4.03	45.80 ± 4.47	38.1 – 71.4

Data represent an average of three replicate samples at six field test sites.
[a] Reference ranges compiled from OECD (2004), ILSI (2007), USCA (1982), Calhoun et al. (1995), Bertrand et al. (2005), Amann (1999), Lundquist (1995), Berberich et al. (1996) and Nida et al. (1996).
[b] Total carbohydrates calculated as 100% - (protein %dm + fat %dm + ash %dm)

Additional composition data on amino acids, fatty acids, minerals and vitamins is provided in Appendix 3.C. Literature values for the individual analyses of cottonseed are also presented for reference. Values for event T303-3, in either the sprayed or unsprayed treatment, were comparable with those the non-transgenic counterpart and within the reference ranges from the literature.

VII.D.2 Anti-Nutrient Analysis

Fuzzy seed of event T303-3 was also analyzed for the content of anti-nutrients of cotton—gossypol and phytic acid (Haas, 2007). The results are presented in Table 20. The values for gossypol and phytic acid were within the reported literature ranges and were similar for the non-transgenic, the transgenic unsprayed and transgenic sprayed plants and comparable to that of TwinLink Cotton (USDA, 2008).

Table 20. Mean anti-nutrient composition in cottonseed of event T303-3

Parameter	Based on dry matter			Reference ranges[a]
	Non-Transgenic	Transgenic Not sprayed	Transgenic Sprayed	
	Mean ± SD	Mean ± SD	Mean ± SD	
Free gossypol %	0.53 ± 0.12	0.56 ± 0.06	0.53 ± 0.06	0.23 – 1.40
(−)Gossypol %	0.32 ± 0.07	0.32 ± 0.05	0.32 ± 0.04	0.18 – 0.77[b]
(+)Gossypol %	0.41 ± 0.08	0.44 ± 0.04	0.43 ± 0.05	0.28 – 1.22[b]
Total gossypol %	0.73 ± 0.15	0.76 ± 0.08	0.75 ± 0.08	0.46 - 1.99
Phytic acid %	1.63 ± 0.29	1.65 ± 0.25	1.70 ± 0.28	0.85 - 2.57

Data represent an average of three replicate samples at six field test sites.
[a]Reference ranges compiled from OECD (2004), ILSI (2007), Calhoun et al. (1995), Berberich et al. (1996), Nida et al. (1996)., Phelps et al. (1965), and Wozenski and Woodburn (1975)
[b]The proportion of (-)gossypol and (+) gossypol in whole, fuzzy cottonseed was reported as 38.8% and 61.2% of total gossypol (Calhoun et al., 1995); on the basis of the range for total gossypol of 0.46 – 1.99 % dm this equals to 0.18 – 0.77% dm for (-) gossypol and 0.28 – 1.22% dm for (+) gossypol.

VII.E. Conclusion

Overall, these evaluations indicate that event T303-3 does not show any unexpected changes in plant morphology as compared to conventional cotton. Any statistically significant differences observed are unrelated to the introduced trait. The agronomic performance data indicate no biologically meaningful differences between event T303-3 and the non-transgenic counterpart nor the antecedent organism, event T304-40. No differences between the event T303-3 and the non-transgenic control were observed for disease occurrence or severity and response to insect pressure, with the exception of the target pests.

Samples of fuzzy seed of event T303-3 were analyzed for their proximate, amino acids, fatty acids, minerals, vitamins and anti-nutrient composition and compared to a non-transgenic control. Published data ranges were provided as additional points of reference. Plant material was collected from six locations and analyzed using standards methods. The ranges and standard deviations overlapped for event T303-3 and the non-transgenic control and fell within the published data ranges.

VIII. ENVIRONMENTAL SAFETY AND IMPACT ON AGRONOMIC PRACTICES

VIII.A. Potential for Gene Transfer

Only two wild *Gossypium* species are present in the US: *G. thurberi* Todaro found in mountain regions of Arizona at altitudes of 2500 to 5000 feet and *G. tomentosum* which is found in Hawaii. Only *G. tomentosum* is capable of crossing with domesticated cotton that will produce fertile offspring. There is no expected selective advantage conferred by the introduced *cry1Ab* and *bar* genes in event T303-3 if that cross would occur.

Vertical Gene Flow:

Cotton pollination
Gossypium hirsutum is considered to be a self-pollinating crop. Cotton pollen is heavy and sticky thus cross pollination by wind is unlikely. Cotton can, however, be pollinated by insects. Honeybees (*Apis mellifera*) and bumblebees (*Bombus* spp.) are the primary insect pollinators. McGregor (1976) traced the movement of pollen from a cotton field surrounded by a large number of honeybee colonies. Movement of the pollen was traced by means of fluorescent particles. McGregor found that at 150 to 200 feet away from the source plant, only 1.6 percent showed the presence of the fluorescent particles. By comparison, the isolation distances for Foundation, Registered and Certified seeds in 7 CFR Part 201 are 1320, 1320 and 660 feet, respectively. The trend for cross pollination to decrease as the distance from the source increased has been established by several research groups over the years. (Kareiva et al., 1994; Van Deynze et al., 2005).

Outcrossing potential to wild/weedy relatives
The potential for outcrossing can be defined as the ability of gene escape to wild cotton relatives. Previously, the USDA stated in the environmental assessment document of LLCotton25 cotton that "gene flow from genetically engineered cotton into wild cotton relatives are not likely, and if it occurs, would not lead to increased weediness" (USDA, 2003). *G. tomentosum,* found only in Hawaii, is the only species capable of crossing with domesticated cotton that will produce fertile offspring. Outcrossing to *G. tomentosum* is unlikely as there is no cotton production in Hawaii other than winter nursery breeding activities where isolation practices are employed, and therefore the potential for gene flow to these wild relatives is low. There are other wild relatives known to exist in Southern Florida and Puerto Rico that are capable of crossing with cultivated cotton. However, these wild relatives are found hundreds of miles from where cotton production occurs.

Outcrossing potential to feral or cultivated cotton
No feral cotton populations (domesticated plants capable of surviving outside of cultivation) of *G. barbadense* have been found in the US Cotton production area. Seed production fields (production of planting seed) are required to be isolated from other cotton fields to prevent cross pollination. Therefore if any cross pollination were to occur to either *G. barbadense* or *G. hirsutum* it would be from a lint production field where seed is crushed and not propagated.

Potential for Horizontal Gene Flow:
Bayer CropScience is not aware of any reports of incidents of naturally occurring transgene movement from transgenic crops to sexually incompatible species.

VIII.B Weediness Potential

In the United States, cotton (*G. hirsutum*) is not a weed pest and has no sexually compatible weedy relatives except perhaps *G. tomentosum* in Hawaii where there is no commercial cotton production. A number of references confirm the lack of weediness of cotton such as Crockett (1977), Holm et al. (1979), and Muenscher (1980). The USDA has previously determined that "cotton is not considered to be a serious, principal or common weed pest in the US" (USDA, 2003). Previous findings by the USDA of similar herbicide-tolerant cotton during environmental assessment expected no change in weediness potential, and an example is glufosinate ammonium tolerant cotton (LibertyLink), commercially sold today. The largest concern is that of volunteer plants that could become weedy in subsequent years. Volunteers are also limited by the geography in which they may exist as cotton does not survive as a perennial where freezing temperatures are reached during the winter. Volunteers can easily be controlled by crop rotation, tillage and/or pre- or post-emergence herbicides. For example, glufosinate ammonium tolerant cotton volunteers could easily be controlled by using the herbicide glyphosate.

There is limited probability that event T303-3 cotton or any *Gossypium* species containing event T303-3 cotton would become a weed problem. The data presented in this petition did not indicate any significant differences in phenotypic or plant morphological characteristics between event T303-3 cotton and the conventional non-transgenic comparator line that would impact plant pest or noxious weed potential. Based on this data there is no evidence to suggest that T303-3 cotton has a higher likelihood to become a plant pest than conventional cotton or event T304-40 cotton which has been granted non-regulated status as part of the deregulation of TwinLink Cotton on September 23, 2011 (USDA, 2011). There were no instances in which volunteer monitoring after harvest revealed any differences in survival or persistence relative to other cotton varieties.

VIII.C Effects on Non-Target Organisms

An assessment of the risk to non-target species has been performed for the Cry1Ab protein expressed in T304-40 cotton. Since the Cry1Ab protein in T303-3 cotton is the same protein, the assessment for T304-40 informs the assessment of T303-3. The toxicity of the Cry1Ab protein expressed in T303-3 cotton is expected to be similar. Testing with microbially-derived Cry1Ab protein at levels greatly exceeding the expressions levels found in Cry1Ab transgenic plants resulted in no effects on several beneficial species (USDA, 2008). Therefore, the data presented in the previous petition adequately addresses toxicity to humans and non-target organisms for event T303-3 plants.

VIII.D Effects on Endangered Species

The responsibility for endangered species falls to the US Fish & Wildlife Services (FWS) under the Endangered Species Act (ESA) (16 USC §1531). Section 6 of the ESA requires federal agencies who conduct activities which may affect listed species to consult with the FWS to ensure that listed species are protected should there be a potential impact.

It is not anticipated that the use of event T303-3 cotton will impact any currently listed species of concern. Species of concern that may inhabit areas close to commercial cotton operations would not be additionally impacted by the use of T303-3 cotton. Commercial agriculture routinely disturbs the ground in which crops are currently planted. As a result,

perennial vegetative species would not grow in these areas. Additionally, because horizontal gene flow to sexually incompatible species is not an issue, there is negligible potential for exposure to the transgene contained in event T303-3 cotton through sexual reproduction.

Cry1Ab protein is highly specific to lepidoptera, so the only organisms which might be considered to be 'at risk' from T303-3 cotton would be endangered lepidopteran insects. While there are endangered lepidopteran species in cotton growing counties (*e.g.* Kern Primrose Sphinx moth, Saint Francis' Satyr butterfly) the larvae are highly unlikely to be exposed to Cry1Ab protein because their habitats do not overlap with cotton fields and their larvae do not feed on cotton. The amount of pollen that would drift from these cotton plants onto plants fed upon by endangered or threatened species would be very small. It is also therefore highly unlikely that event T303-3 cotton could outcross to any wild or weedy relatives of cotton.

For these reasons, it is not believed that the use of T303-3 cotton in commercial cotton production will adversely impact endangered species of concern.

VIII.E Indirect Effects on Other Agricultural Products

T303-3 cotton is an alternative cotton product that contains insect-resistant and herbicide-tolerant traits. Current agricultural practices already in use for these types of products are not expected to change with the introduction of event T303-3 cotton. Adoption and use of genetically modified cotton can provide positive impacts on agricultural practices. These positive impacts have been detailed in a study by Brookes and Barfoot (2008) and include:

Herbicide-tolerant crops

• Increased management flexibility that comes from a combination of the ease of use associated with broad-spectrum, post-emergent herbicides;
• Compared to conventional crops, where post-emergent herbicide application may result in 'knock-back' (some risk of crop damage from the herbicide); this problem is less likely to occur in herbicide-tolerant crops;
• Facilitation of adoption of no/reduced tillage practices with resultant savings in time and equipment usage;
• Improved weed control has reduced harvesting costs – cleaner crops have resulted in reduced times for harvesting;
• Elimination of potential damage caused by soil-incorporated residual herbicides in follow-on crops.

Insect-resistant crops

• Production risk management/insurance purposes – taking away the worry of significant pest damage occurring;
• A 'convenience' benefit (less time spent on crop walking and/or applying insecticides);
• Savings in energy use – mainly associated with less spraying;
• Savings in machinery use (for spraying and possibly reduced harvesting times);
• Improved health and safety for farmers and farm workers (from reduced handling and use of insecticides);

Genetically modified herbicide-tolerant cotton was first grown commercially in the US in 1996 and by 2010, was planted on 73% of the total cotton plantings (USDA ERS, 2011).

IX. STATEMENT OF GROUNDS UNFAVORABLE

Bayer CropScience knows of no study data and/or observations associated with insect-resistant herbicide-tolerant event T303-3 cotton that will result in adverse environmental consequences for its introduction. TwinLink Cotton (T304-40 x GHB119), which contains the antecedent event T304-40, received nonregulated status in September 2011 (USDA, 2011). Since the Cry1Ab and PAT proteins in event T303-3 cotton are the same as those of the antecedent event, the assessment for T304-40 informs the assessment of T303-3.

The evidence and data provided in this petition supports the conclusion that event T303-3 cotton presents a low risk to human health and the environment and does not pose a plant pest risk.

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Appendix 1. Field Trial Termination Reports 2001-2006

USDA 2001 Termination Report for Bt Cotton

Bayer CropScience LP

Trials Conducted: State (County)

01-075-08n: MS (Washington)
01-075-18n: MS (Washington)
01-292-01n: PR (Juana Diaz)

Trials Not Conducted: State (County)

01-292-01n: PR (Sabana Grande)
01-312-05n: PR (Juana Diaz, Sabana Grande)

Planting Dates

May 25, 2001 (Washington Co., MS) through December 5, 2001 (Juana Diaz, PR)

Harvest and Plot Destruction Dates

November 8, 2001 (Washington Co., MS) through June 28, 2002 (Juana Diaz, PR)

Purpose

Field trials were conducted to test the efficacy as well as breeding of transgenic herbicide-tolerant cotton. Transgenic plants contained the *bar* gene expressing the PAT enzyme, which confers resistance to the broad-spectrum herbicide glufosinate-ammonium, as well as the *Cry1Ab* gene allowing resistance to attack by various lepidopteran pest species.

Observations

Experienced personnel qualified in cotton cultivation performed all plot observations. Recorded observations for transgenic and non-transgenic control plots were provided from first square through open boll growth stages.

Plant emergence patterns were uniform and vigorous within both plot types. Germination rates ranged from 80 to 90% (Washington Co., MS).

No morphological differences were noted between transgenic and non-transgenic plants. The only in-field phenotypic difference observed between the two genotypes was their respective levels of tolerance to lepidopteran feeding pressure.

Insect pest species listed for the Washington Co., MS site included bollworm (*Helicoverpa zea*), plantbugs (*Lygus lineolaris*), and cutoworms (*Agrotis upsilon*). At the Juana Diaz, PR site only bollworm (*Helicoverpa zea*) was reported. There were no differences recorded in either diversity or density of insect pest species found between transgenic and non-transgenic plots.

A single species of beneficial insect, the ladybug (*Hippodamia convergens*), was recorded at the Washington Co., MS site. At Juana Diaz, PR honeybees (*Apis melifera*) and aphid lions (*Chrysopa sp* larvae) were recorded. As with the pest species, no differences were noted between species profiles for the two plot types.

No phtyopathology was recorded at either site.

Post-Trail Monitoring

Volunteer monitoring revealed post-season plants at the Washington Co., MS sites with less than ten plants found on April 17, 2002, and the Juana Diaz, PR site with less than ten plants found on July 24, 2002. These were hand-weeded. Clean inspections were established by May 3, 2002 and August 23, 2002, respectively.

USDA Field Termination Report

Notification No.: 02-058-02n

Applicant No.: BT-2A-Cotton-MR

Permittee: Aventis CropScience (Now Bayer CropScience LP)
Research Triangle Park, NC; 919-549-2655

Regulated Article: Herbicide-tolerant, glufosinate-ammonium-tolerant; Insect-resistant, resistant to Lepidopteran pest species, Cotton (*Gossypium hirsutum*)

Site Release Information: As shown below, one (1) of two (2) sites was planted:

County/Parish/District	State or Territory	Release Status
Washington	MS	Planted
Lubbock	TX	Not Planted

Release information for Washington Co., MS is as follows:

Acreage Planted	Date Planted	Germination Data Transgenic *vs*. Non-transgenic	Date Terminated
0.35	5/20/02	**% Emergence/Seedling Vigor** 70% *vs* 85% on 5/27/02 **Initial Stand Count Percentage** 75% *vs* 90% on 6/4/02 **Final Stand Count** 1.4 plants per foot *vs* 1.6 plants per foot on 6/28/02	11/26/02

Purpose of Release: The purpose of the release was to test the efficacy of the transgenic herbicide-tolerant cotton plants. Transgenic plants contained the *Cry1Ab* gene, allowing resistance to attack by various lepidopteran pest species. The performance of the transgenic cotton with respect to the nontransgenic counterpart plant was also evaluated, as were the overall agronomic characteristics.

Observations: The test site was inspected twelve (12) times during the growing season (5/27/02, 5/31/02, 6/4/02, 6/19/02, 6/20/02, 6/26/02, 6/28/02, 7/8/02, 7/12/02, 8/1/02, 8/9/02, 8/12/02) for agronomic growth characteristics and disease and insect pest infestation.

CONTAINS NO CONFIDENTIAL BUSINESS INFORMATION

Observations were recorded from first square through open boll growth stages on both the transgenic and nontransgenic plants. Transgenic plants did not germinate quite as well as the nontransgenic counterpart, but both grew vigorously.

Two (2) species of insect pests were noted: thrips and plant bugs. Thrips were seen on 5/31/02, and plant bugs were observed on 7/12/02 and 8/1/02. Damage levels ranged from slight to moderate. No differences were recorded in the diversity of insect pest species found between the transgenic and nontransgenic counterpart.

The only beneficial insect observed was the ladybug which was seen on 6/19/02, 7/12/02 and 8/12/02.

No disease susceptibility was noted on the transgenic or non-transgenic plants during any of the visits made on 5/27/02, 6/20/02, 7/8/02 or 11/26/02.

Results: The only in-field phenotypic differences observed between the transgenic and non-transgenic plants were in the level of tolerance to lepidopteran feeding pressure and tolerance to herbicide (glufosinate-ammonium) treatment.

Plant Disposition: The crop was harvested 11/26/02. Following harvest, all remaining plant material was shredded, burned and disked under.

Volunteer Monitoring: The plot area was visually inspected for volunteer cotton plants four (4) times during the following growing season. No volunteer plants were observed during any of the visits.

Weediness Characteristics: No difference in weediness characteristics between the transgenic and non-transgenic cotton lines was observed.

Non-Target Organisms: No adverse effect on non-target organisms from either the transgenic or nontransgenic plants was observed in the trial.

Weather Synopsis: Weather notations indicate the site experienced normal climatic conditions during the growing season, but was usually wet at harvest time.

CONTAINS NO CONFIDENTIAL BUSINESS INFORMATION

Containment Measures:	Transgenic cotton plants were separated from conventional cotton plants by a distance of ~250 feet. A 40-foot wide perimeter of nontransgenic cotton plants bordered the test plot to prevent the flow of pollen from transgenic to nontransgenic plants. The border rows were destroyed at the conclusion of the trial and were monitored as part of the test plot the following growing season.

CONTAINS NO CONFIDENTIAL BUSINESS INFORMATION

USDA Field Termination Report

Notification No.: 02-078-01n

Applicant No.: BT-2B-Cotton-MR

Permittee: Aventis CropScience (Now Bayer CropScience LP)
Research Triangle Park, NC; 919-549-2655

Regulated Article: Herbicide-tolerant, glufosinate-ammonium-tolerant; Insect-resistant, resistant to Lepidopteran pest species, Cotton (*Gossypium hirsutum*)

Site Release Information: Release information for Washington Co., MS is as follows:

Acreage Planted	Date Planted	Germination Data Transgenic *vs*. Non-transgenic	Date Terminated
0.35	5/20/02	**% Emergence/Seedling Vigor** 70% *vs* 85% on 5/27/02 **Initial Stand Count Percentage** 75% *vs* 90% on 6/4/02 **Final Stand Count** 1.4 plants per foot *vs* 1.6 plants per foot on 6/28/02	11/26/02

Purpose of Release: The purpose of the release was to test the efficacy of the transgenic herbicide-tolerant cotton plants. Transgenic plants contained the *Cry1Ab* gene, allowing resistance to attack by various lepidopteran pest species. The performance of the transgenic cotton with respect to the nontransgenic counterpart plant was also evaluated, as were the overall agronomic characteristics.

Observations: The test site was inspected twelve (12) times during the growing season (5/27/02, 5/31/02, 6/4/02, 6/19/02, 6/20/02, 6/26/02, 6/28/02, 7/8/02, 7/12/02, 8/1/02, 8/9/02, 8/12/02) for agronomic growth characteristics and disease and insect pest infestation.

CONTAINS NO CONFIDENTIAL BUSINESS INFORMATION

Observations were recorded from first square through open boll growth stages on both the transgenic and nontransgenic plants. Transgenic plants did not germinate quite as well as the nontransgenic plants, but both grew vigorously.

Two (2) species of insect pests were noted: thrips and plant bugs. Thrips were seen on 5/31/02, and plant bugs were observed on 7/12/02 and 8/1/02. Damage levels ranged from slight to moderate. No differences were recorded in the diversity of insect pest species found between the transgenic and nontransgenic counterpart.

The only beneficial insect observed was the ladybug which was seen on 6/19/02, 7/12/02 and 8/12/02.

No disease susceptibility was noted on the transgenic or non-transgenic plants during any of the visits made on 5/27/02, 6/20/02, 7/8/02 or 11/26/02.

Results: The only in-field phenotypic differences observed between the transgenic and non-transgenic plants were in the level of tolerance to lepidopteran feeding pressure and tolerance to herbicide (glufosinate-ammonium) treatment.

Plant Disposition: The crop was harvested 11/26/02. Following harvest, all remaining plant material was shredded, burned and disked under.

Volunteer Monitoring: The plot area was visually inspected for volunteer cotton plants four (4) times during the following growing season. No volunteer plants were observed during any of the visits.

Weediness Characteristics: No difference in weediness characteristics between the transgenic and non-transgenic cotton lines was observed.

Non-Target Organisms: No adverse effect on non-target organisms from either the transgenic or nontransgenic plants was observed in the trial.

Weather Synopsis: Weather notations indicate the site experienced normal climatic conditions during the growing season, but was usually wet at harvest time.

CONTAINS NO CONFIDENTIAL BUSINESS INFORMATION

Containment Measures:	Transgenic cotton plants were separated from conventional cotton plants by a distance of ~250 feet. A 40-foot wide perimeter of nontransgenic cotton plants bordered the test plot to prevent the flow of pollen from transgenic to nontransgenic plants. The border rows were destroyed at the conclusion of the trial and were monitored as part of the test plot the following growing season.

CONTAINS NO CONFIDENTIAL BUSINESS INFORMATION

USDA Field Termination Report

Notification No.: 02-261-25n

Applicant No.: BT-2D-Cotton-MR

Permittee: Bayer CropScience LP
Research Triangle Park, NC; 919-549-2655

Regulated Article: Herbicide-tolerant, glufosinate-ammonium-tolerant; Insect-resistant, resistant to Lepidopteran pest species, Cotton (*Gossypium hirsutum*)

Site Release Information: Release information for Sabana Grande District, PR is as follows:

Acreage Planted	Date Planted	Germination Data Transgenic *vs*. Non-transgenic	Date Terminated
0.22	11/19/02	**% Emergence/Seedling Vigor** >50% *vs* >50% on 11/30/02 **Initial Stand Count Percentage** >80% *vs* >80% on 12/6/02 **Final Stand Count Percentage** 40-60% *vs* >80% on 12/20/02	4/23/03

Purpose of Release: The purpose of the release was for breeding and seed increase. Transgenic plants contained the *Cry1Ab* gene, allowing resistance to attack by various lepidopteran pest species. The performance of the transgenic cotton with respect to the nontransgenic counterpart plant was also evaluated, as were the overall agronomic characteristics.

Observations: The test site was inspected fifteen (15) times during the growing season (11/26/02, 11/30/02, 12/6/02, 12/8/03, 12/10/02, 12/17/02, 12/20/02, 1/2/03, 2/15/03, 2/23/03, 3/1/03, 3/10/03, 4/5/03, 4/15/03, 4/16/03) for agronomic growth characteristics and disease and insect pest infestation.

CONTAINS NO CONFIDENTIAL BUSINESS INFORMATION

Observations were recorded from first square through open boll growth stages on both the transgenic and nontransgenic plants. Both the transgenic and nontransgenic plants germinated well and grew vigorously.

Four (4) species of insect pests were noted: grasshoppers (12/6/02), leafminers (12/17/02), aphids (12/20/02), and armyworms (1/2/03). Damage levels ranged from slight to moderately severe. No differences were recorded in the diversity of insect pest species found between the transgenic and nontransgenic counterpart.

Two (2) species of beneficial insects were observed: cucumber beetle on 12/20/02 and honeybees on 3/1/03.

No disease susceptibility was noted on the transgenic or non-transgenic plants during any of the visits made on 12/6/02, 2/15/03, 3/10/03 or 4/5/03.

Results: The only in-field phenotypic differences observed between the transgenic and non-transgenic plants were in the level of tolerance to lepidopteran feeding pressure and tolerance to herbicide (glufosinate-ammonium) treatment.

Plant Disposition: The crop was harvested over two (2) days: 4/16/03 and 4/17/03. Following harvest, all remaining plant material was mechanically cultivated 4/23/03.

Volunteer Monitoring: The plot area was visually inspected for volunteer cotton plants five (5) times during the following growing season, until no volunteers had been observed for two (2) consecutive post-season monitoring visits.

Post-Season Volunteer Monitoring

Date	No. Plants Observed/Stage	Method of Destruction
4/30/03	>50 plants/V2	Mechanically Cultivated
5/14/03	>50 plants/V3	Mechanically Cultivated
6/4/03	11 to 50 plants/V2	Mechanically Cultivated
6/24/03	None	Field Mechanically Cultivated
7/18/03	None	Field Mechanically Cultivated

CONTAINS NO CONFIDENTIAL BUSINESS INFORMATION

Weediness Characteristics:	There was no evidence of change in characteristics that would enhance survival of the glufosinate-ammonium-tolerant transgenic cotton plants as compared to the non-transgenic cotton plants. No difference in weediness characteristics between the transgenic and non-transgenic cotton lines was observed.
Non-Target Organisms:	No adverse effect on non-target organisms from either the transgenic or nontransgenic plants was observed in the trial.
Weather Synopsis:	Weather notations indicate the site experienced typical climatic conditions during the growing season.
Containment Measures:	The Sabana Grande test site is a 59-acre farm. The site produces no commercial crops. A 40-foot-wide perimeter of nontransgenic cotton surrounded the test plot to prevent the flow of pollen. At trial conclusion, the border rows were not harvested but were destroyed along with the remnants of the test plot. The border area was monitored (as part of the test plot) for volunteer cotton plants during the period that followed.

CONTAINS NO CONFIDENTIAL BUSINESS INFORMATION

USDA Field Termination Report

Notification No.: 03-070-12n

Applicant No.: BT/HT-3A-Cotton-MR

Permittee: Bayer CropScience LP
Research Triangle Park, NC; 919-549-2655

Regulated Article: Herbicide-tolerant, Glufosinate-ammonium-tolerant; Insect-resistant, resistant to Lepidopteran insect pests, Cotton (*Gossypium hirsutum*)

Site Release Information: All release sites authorized under this notification were planted. Below are acreage amounts, planting dates and termination dates for each site:

County/State	Acreage Planted	Date Planted	Date Terminated
Oktibbeha/MS	2.75	5/27/03	10/24/03
Washington/MS	1.2	5/29/03	12/5/03
Lubbock/TX	0.69	5/26/03 & 6/9/03	1/10/04

Purpose of Release: The trials were established to evaluate the efficacy of the herbicide-tolerant/insect-resistant cotton plants. Transgenic plants contained the *cry1Ab* gene, allowing resistance to attack by various lepidopteran pest species. The performance of the transgenic cotton with respect to the nontransgenic counterpart was also evaluated, as were the overall agronomic characteristics.

Observations: The test sites were visually inspected multiple times during the growing season for agronomic growth characteristics and disease and insect pest infestation. Observations were recorded for the transgenic and nontransgenic plants from emergence through harvest.

County or District/ State or Territory	Germination Data Transgenic *vs.* Non-transgenic
Oktibbeha/MS	Mean germination ranged from 55% to 95.25% between the various cotton lines.
Washington/MS	**% Emergence/Seedling Vigor** 85% *vs.* 85% on 6/5/03 **Initial Stand Count Percentage** 90% *vs.* 90% on 6/13/03 **Final Stand Count Percentage** 90% *vs.* 90% on 9/18/03

CONTAINS NO CONFIDENTIAL BUSINESS INFORMATION

County or District/ State or Territory	Germination Data Transgenic *vs.* Non-transgenic
Lubbock/TX	**% Emergence/Seedling Vigor** 65.3% *vs.* 62% on 7/16/03 **Initial Stand Count Percentage** 65% *vs.* 62% on 7/16/03 **Final Stand Count Percentage** 65% *vs.* 62% on 8/19/03

In Oktibbeha Co., some plots were infested with *H. zea* and some were not. Each week, for a period of six (6) weeks, damage and larvae counts were made.

In general, transgenic cotton plants exhibited normal growth and development at each of the locations. Experimental treatments of glufosinate-ammonium were made. Hail damage necessitated a replant in Lubbock Co.

County/State	Observations/Dates		
	Fungi/Diseases	**Insect Pests**	**Beneficial Insects**
Oktibbeha/MS	None observed 6/11/03, 7/6/03, 8/6/03 and 8/24/03.	Some plots were infested with *H. zea*. No record made of the presence of any other insect species.	No notation was made of the presence of any beneficial insects.
Washington/MS	None observed 6/5/03, 7/8/03, 7/28/03 and 11/24/03.	A light infestation of plant bugs was seen 7/28/03 and 8/2/03.	Lady beetles were observed on 6/5/03, 7/8/03 and 8/2/03.
Lubbock/TX	None observed 7/16/03, 7/28/03 and 8/15/03.	A light infestation of bollworms and leafminers were observed 7/38/03. Aphids were observed 10/20/03.	Ladybugs were observed 7/16/03 and 8/15/03, adult hooded beetles were observed 7/28/03, and lacewings were seen 8/15/03.

Results:

No disease susceptibility or resistance differences were observed between the transgenic cotton plants and its nontransgenic counterpart. Some phenotypic differences were observed between the transgenic and nontransgenic plants in terms of the level of tolerance to herbicide treatment and susceptibility to lepidopteran pests.

Plant Disposition:

The Oktibbeha Co. trial was harvested 10/23/03. Stalks were cut and disked under the following day. In Washington Co., harvest occurred 12/1/03 and 12/2/03. Stalks were mowed, and lint and seed burned on 12/5/03. The Lubbock Co. trial was destroyed 1/10/04 by burning the plot and disking it under.

CONTAINS NO CONFIDENTIAL BUSINESS INFORMATION

Volunteer Monitoring: The plot areas were visually inspected for volunteer cotton plants during the following growing season. The table below summarizes observations made and actions taken to eliminate volunteer plants.

Post-Season Volunteer Monitoring

County/State	Date	No. Plants Observed/Stage	Method of Destruction
Oktibbeha/MS	5/24/04	None	
	6/2/04	None	
	7/14/04	None	
	7/21/04	None	
	7/28/04	None	
Washington/MS	4/5/04	None	
	4/13/04	None	
	4/28/04	None	
	5/6/04	None	
	5/14/04	None	
Lubbock/TX	4/2/04	None	
	4/16/04	1 to 10 plants	Plants were removed by hand and field was mechanically cultivated.
	4/30/04	None	
	5/13/04	1 to 10 plants	Plants were removed by hand and herbicide was applied.
	5/26/04	None	
	6/15/04	None	

Weediness Characteristics: There was no evidence of change in characteristics that would enhance survival of the transgenic cotton plants as compared to the nontransgenic cotton plants. No difference in weediness characteristics was observed between the transgenic and nontransgenic cotton lines.

Non-Target Organisms: No adverse effect on non-target organisms from either the transgenic or nontransgenic plants was observed during any of the trials.

Weather Synopsis: The weather in Oktibbeha Co. was noted to be wet. Conditions for Washington Co. were normal. Lubbock Co. was wet and cold early in the season.

Containment Measures: A 40-foot-wide perimeter of nontransgenic or commercial cotton surrounded the test plots to minimize pollen flow. The border rows were not harvested but destroyed at the conclusion of the trial. The border areas were monitored along with the actual test plot the next growing season for volunteer cotton plants.

CONTAINS NO CONFIDENTIAL BUSINESS INFORMATION

USDA Field Termination Report

USDA Notification Number: 05-040-06n

Applicant Internal Number: BT-5A-Cotton-MR

Applicant: Bayer CropScience LP
Research Triangle Park, NC 27709
(919) 549-2655

Regulated Article: *Gossypium hirsutum;* plant incorporated protectant (PIP); tolerant to glufosinate-ammonium herbicide

Site Release Information: Trials utilizing this trait were conducted at four (4) locations. A fifth site located in Bossier Parish, LA was not planted:

County / State	Acreage Planted	Date Planted	Date Terminated	Isolation Method
Pinal Co., AZ	0.426	5/27/05	11/25/05	660 ft isolation distance
Gaines Co., TX	0.96	5/9/05	11/7/05	40 ft isolation buffer
Uvalde Co., TX	0.2755	4/22/05	9/23/05	660 ft isolation distance
Wharton Co., TX	0.2355	5/5/05	10/6/05	660 ft isolation distance

Purpose of Release: Trials were established to evaluate the performance of Plant Incorporated Protectant traits in various cotton varieties.

Observations:

- Pinal Co., AZ: Observations were made between planting and harvest four times during the season. A non-transgenic check was not present to compare the regulated article to, but cooperator observed no abnormal increases or decreases in insect, disease, or weed pressure given the climate and region the cotton was planted in. Cooperator did indicate that seed germination was lower than normal, but this was most likely due to the variety of plant the transgenic event was tested in.

- Gaines Co., TX: Observations were made between planting and harvest four times during the season. Cooperator observed a difference in the early emergence of the cotton, with the non-transgenic line emerging at 4.88 plants/ft. (81% germination) vs. 2.87 plants/ft. (47% germination) for the transgenic lines at the 1-2 leaf stage of growth. Observations taken one month later showed that this difference was no longer present, with the transgenic lines having caught up to the non-transgenic lines. No other differences in disease, insect pests, or phenotypical differences were seen between the transgenic and non-transgenic lines.

- Uvalde Co., TX: Observations were made between planting and harvest four times during the season. Cooperator observed differences between transgenic and non-transgenic lines with transgenic lines emerging at cotyledon stage at 45% and the non-transgenic counterpart at 7%. This difference was less noticeable within 14 days of the previous observations, but still had a stand which was 5-6% greater than the non-transgenic line. Both lines showed equal infestations of Texas root rot and Boll Dangle, with an equal mortality rate of plants which contracted root rot. Additionally, transgenic lines showed a much lower infestation of cotton bollworms, with infestation rates in the non-transgenic lines ranging from 20-500% greater infestation than the transgenic varieties. No other differences were noted in weediness characteristics, phenotype, or beneficial insect populations.

- Wharton Co., TX: Observations were made between planting and harvest four times during the season. Cooperator noticed little to no differences between transgenic and non-transgenic lines, with a slight decline in one transformation event of this trait (most likely attributable to the plant variety). Cooperator did notice that the transgenic plants appeared to be more of an "open plant type" but this could not be assigned to either the plant variety or the transgenic trait. Bollworm pressure never developed fully enough to evaluate the efficacy of the PIP trait.

Plant Disposition: Plants were harvested between late September and late November of 2005. All field sites were destroyed with no seed retained. Remaining plant material in the field was cultivated into the soil after harvest. All seed sent to cooperators was utilized in the planting of these trials.

Volunteer Monitoring: Volunteer monitoring is currently being conducted on the Pinal Co., Gaines Co., and Uvalde Co. trial sites. Monitoring is scheduled to conclude one year from the dates of harvest. Monitoring was discontinued at the Wharton Co. site on 5/19/06 due to the replant of another regulated article in the same trial area.

Weediness Characteristics: There was no indication of increased weediness characteristics in either the transgenic or non-transgenic varieties.

Non-target Organisms: There was no indication of any adverse effects to non-target insect populations or beneficial insects. Various beneficial insect populations were present with lacewings, assassin bugs, lady beetles, and various coleopteran species observed.

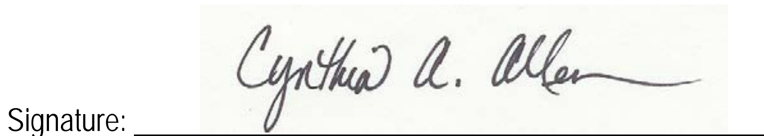
Weather Synopsis:

- Pinal Co., AZ: Reported drier than normal fall season.
- Gaines Co., TX: Reported as normal for this region of Texas.

CONTAINS NO CONFIDENTIAL BUSINESS INFORMATION

- Uvalde Co., TX: Abnormally dry the entire season, with the month of July being abnormally hot.
- Wharton Co., TX: Abnormally hot all season, with dry conditions existing from June to mid-July.

Containment Measures: All plots, with the exception of the site in Gaines Co., TX, were isolated from other sexually compatible species and commercial cotton by a distance of at least 660 feet. The Gaines Co. site was isolated from the surrounding cotton by an isolation border of at least 40 feet. This isolation border was validated by internal audit.



Signature: ______________________ Date: 6/30/06

Cynthia A. Allen
Regulatory Compliance Specialist
Bayer CropScience – Regulatory Affairs BioScience

USDA Field Termination Report

USDA Notification Number: 05-091-08n

Applicant Internal Number: BT-5D-Cotton-MR

Applicant: Bayer CropScience LP
Research Triangle Park, NC 27709
(919) 549-2655

Regulated Article: *Gossypuim hirsutum;* plant incorporated protectant (PIP); tolerant to glufosinate-ammonium herbicide

Site Release Information: Trials utilizing this trait were conducted at six (6) locations:

County / State	Acreage Planted	Date Planted	Date Terminated	Isolation Method
Crittenden Co., AR	0.24	5/24/05	11/1/05	660 ft. spatial isolation
Jackson Co., AR	0.293	5/26/05	11/4/05	660 ft. spatial isolation
Tift Co., GA	0.29	6/21/05	12/14/05	660 ft. spatial isolation
Tate Co., MS	0.17	5/30/05	10/28/05	660 ft. spatial isolation
Hockley Co., TX	0.27	5/26/05	11/21/05	660 ft. spatial isolation
Wharton Co., TX	0.26	6/8/05	11/14/05	660 ft. spatial isolation

Purpose of Release: Trials were established to evaluate the performance of Plant Incorporated Protectant traits in various cotton varieties, and generate samples for residue analysis of cotton varieties.

Observations:

- Crittenden Co., AR: Cooperator noted significant reduction in the germination rate of the transgenic plots throughout the trial period, although non-transgenic checks within the plot were also below average germination indicating possible issues with the evaluated variety. No differences were noted in plant diseases, insect pest populations, beneficial insect populations, or weediness characteristics. Cooperator did note that transgenic variety initially seemed to lag behind the non-transgenic variety in vigor, but later caught up, and was growing normally. There was also a 15-25% difference the boll opening between the transgenic and non-transgenic varieties, which the cooperator attributed to the low plant population of the transgenic variety.

CONTAINS NO CONFIDENTIAL BUSINESS INFORMATION

- Jackson Co., AR: Cooperator noted significant reduction in the germination rate of the transgenic plots throughout the trial period, although non-transgenic checks within the plot were also below average germination indicating possible issues with the evaluated variety. No significant differences were noted in plant diseases, insect pest populations, beneficial insect populations, or weediness characteristics. Cooperator did note that transgenic plants appeared to be shorter and did not appear as healthy as non-transgenic plants, but mentioned this could have been due to the thin plant stand.

- Tift Co., GA: Cooperator noted significant reduction in the germination rate of the transgenic plots throughout the trial period, although non-transgenic checks within the plot were also below average germination indicating possible issues with the evaluated variety. No significant differences were noted in plant diseases or beneficial insect populations. Cooperator did note significant reductions in budworm and bollworm populations in the transgenic variety, and also noted a higher percentage of plants with multiple terminals in the non-transgenic. This trend was noted in the non-transgenic variety as well, but to a lesser degree, indicating this to be a varietial effect, rather than a transgenic one. Cooperator also noted that transgenic variety seemed to outperform non-transgenic variety in growth and fruit set.

- Tate Co., MS: Cooperator noted significant reduction in the germination rate of the transgenic plots throughout the trial period, although non-transgenic checks within the plot were also below average germination indicating possible issues with the evaluated variety. No significant differences were noted in plant diseases, insect pest populations, beneficial insect populations, physical plant characteristics, or weediness characteristics.

- Hockley Co., TX: Cooperator noted significant reduction in the germination rate of the transgenic plots throughout the trial period, although non-transgenic checks within the plot were also below average germination indicating possible issues with the evaluated variety. No significant differences were noted in plant diseases, insect pest populations, beneficial insect populations, physical plant characteristics, or weediness characteristics.

- Wharton Co., TX: Cooperator noted significant reduction in the germination rate of the transgenic plots throughout the trial period, although non-transgenic checks within the plot were also below average germination indicating possible issues with the evaluated variety. No significant differences were noted in plant diseases, insect pest populations, beneficial insect populations. Cooperator did note that transgenic varieties had very low emergence, and did not mature normally throughout the season. This resulted in low retention of fruit, increased vegetative growth, and showed an increased incidence of hard lock.

Plant Disposition: For all six locations, plots were harvested and data taken, with samples sent to Bayer facility for analysis. All remaining harvested material in the field and in the lab was destroyed. Unplanted seed which was not utilized by the cooperator was either returned to Bayer for storage or destroyed.

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Volunteer Monitoring: Volunteer monitoring is ongoing for all trial sites and is scheduled for completion one year from the date of harvest.

Weediness Characteristics: There was no indication of increased weediness characteristics in either the transgenic or non-transgenic varieties for most plots. Multiple terminal growth in both varieties in Tift Co., GA was noted, along with the stunted development of the transgenic variety in Wharton Co., TX. In both instances, this development was attributed to the plant variety.

Non-target Organisms: There was no indication of any adverse effects to non-target insect populations or beneficial insects. Various beneficial insect populations were present with lacewings, assassin bugs, lady beetles, and various coleopteran species observed.

Weather Synopsis:

- Crittenden Co., AR: Below normal rainfall; compensated via irrigation of plots.
- Jackson Co., AR: Normal weather conditions
- Tift Co., GA: Slightly elevated rainfall amounts in late summer
- Tate Co., MS: Below average rainfall
- Hockley Co., TX: Weather was within normal averages for this region; a hail storm was reported early in the season with minimal (<5%) damage to plots.
- Wharton Co., TX: Plots experienced above average temperatures for this region. Minor plot damage resulted from Hurricane Rita on 9/24/05, but an evaluation showed that there was no loss of lint or seed from the plot.

Containment Measures: All plots were isolated from other cotton by a distance of at least 660 ft. from the plot perimeter.

Signature: ______________________ Date: 6/30/06

Cynthia A. Allen
Regulatory Compliance Specialist
Bayer CropScience – Regulatory Affairs BioScience

USDA Field Termination Report

USDA Notification Number: 05-189-07n

Applicant Internal Number: IR-5A-GH-MR

Applicant: Bayer CropScience LP
Research Triangle Park, NC 27709
(919) 549-2655

Regulated Article: *Gossypium hirsutum;* plant incorporated protectant (PIP); tolerant to glufosinate-ammonium herbicide

Site Release Information: Trials utilizing this trait were conducted at one (1) location:

County / State	Acreage Planted	Dates Planted	Date(s) Terminated	Isolation Method(s)
Sabana Grande, PR	1.015	11/15/05	5/4/06	Isolation border & physical barrier

Purpose of Release: This trial was established to evaluate the performance of plant incorporated protectant traits in cotton varieties.

Observations: Cooperator did not note any differences between transgenic and non-transgenic varieties other than effects of the PIP on bollworm populations. No other differences in beneficial insect populations, plant diseases, physical plant characteristics, or weediness characteristics were reported.

Plant Disposition: Cooperator confirmed that all seed sent by Bayer and not used in the creation of the trial was returned. The plot was harvested 5/4/06. All harvested material was sent to the Bayer facility in Lubbock, Texas. Residual plant material was incorporated into the trial site.

Volunteer Monitoring: Volunteer monitoring is currently being conducted and is scheduled for completion one year from the date of harvest.

Weediness Characteristics: There was no indication of increased weediness characteristics in either the transgenic or non-transgenic varieties.

Non-target Organisms: There was no indication of any adverse effects to non-target insect populations or beneficial insects.

Weather Synopsis: Cooperator reported normal growing conditions for this region.

Plot Damage: No damage to plots was reported.

Containment Measures: Plot was contained utilizing a 40 ft isolation border. As a secondary measure to prevent gene flow, a nylon/mesh screen cage was erected which enclosed the transgenic plants. The cage was erected before flowering and remained until harvest.

Signature: ________________________ Date: 6/30/06

Cynthia A. Allen
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CONTAINS NO CONFIDENTIAL BUSINESS INFORMATION

USDA Field Termination Report

USDA Notification Number: 06-047-02n **Applicant**

Reference Number: IR-6A-GH-MR **Applicant**:

Bayer CropScience LP
Research Triangle Park, NC 27709

Regulated Article: *Gossypium hirsutum (cotton)*

Site Release Information: Trials utilizing this trait were conducted in fourteen (14) locations.

County / State	Acreage Planted	Date Planted	Date Terminated	Isolation Method
Bossier/ LA	0.70	5/31/06	11/14/06	Isolation Border Rows
Madison/ LA	1.28	6/05/06	12/05/06	Isolation Border Rows
Coahoma/ MS	1.28	6/06/06	10/06/06	Isolation Border Rows
Oktibbeha/ MS	0.648	5/30/06	10/10/06	Isolation Border Rows
Washington/ MS (2-locations)	2.34	5/30/06 & 6/06/06	11/28/06 & 12/18/06	Isolation Border Rows
Durham & Halifax/ NC	0	-	-	*Trials were not initiated*
Dillon/ SC (2-locations)	3.88	6/02/06 & 5/30/06	12/08/06 & 12/11/06	Isolation Border Rows
Gaines/ TX	0.73	5/26/06	12/05/06	Isolation Border Rows
Lubbock/ TX (1)	0.165	5/24/06	12/14/06	Isolation Border Rows
Lubbock/ TX (2)	0	-	-	*Trial was not initiated*
Uvalde/ TX (2-locations)	1.12	5/25/06	10/30/06 & 10/03/06	Isolation Border Rows
Wharton/ TX (2-locations)	1.2989	6/08/06	11/14/06	Isolation Border Rows

Observations: *listing of characteristics between the transgenic and non-transgenic:*

1. Bossier Pa., LA
   - Cooperator noted no unusual characteristics.
   - Seedling disease was discovered in both transgenic and non-transgenic.
   - No differences in plant disease, insect pest or beneficial insect populations.
   - Non-transgenic slightly taller than transgenic.
   - Transgenic had more "hook-billed" bolls.

2. Madison Pa., LA:
   - No unusual characteristics.
   - No differences in emergence, maturity, flowering, plant disease, insect pest population, weediness or beneficial insect populations.

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3. Coahoma Co., MS:
    - Approximately 120 days after planting the cotton, drift from a nearby field the cooperator was spraying, caused the site to prematurely defoliate into a total loss of yield. Therefore, crop was not harvested and was destroyed with a mechanical shredder.
    - Prior to defoliation there were no significant differences between the transgenic and non-transgenic. Observations were considered normal.

4. Oktibbeha Co., MS:
    - Plots were artificially infested with cotton boll worms.
    - Non-transgenic plots were grown without artificial infestation of the cotton boll worms.
    - No significant differences were noted in plant diseases, insect pest population, beneficial insect populations, weediness or physical plant characteristics.

5. Washington Co., MS:
    - Cooperator noted that there were no significant differences in plant diseases, insect pest and beneficial insect populations.
    - Everything appeared normal between the transgenic and non-transgenic from the first true-leaf stage to maturity.

6. Washington Co., MS:
    - Cooperator noted that during maturity the non-transgenic had a better fruit load than the transgenic.
    - No significant differences were noted in plant diseases, insect pest populations, and beneficial insect populations.
    - No significant differences in emergence, squaring, blooming or maturity.
    - A delay in harvesting due to an unusual cool and wet autumn

7. Durham Co., NC:
    - Study cancelled due to business reasons therefore, planting was never initiated.

8. Halifax Co., NC:
    - Study was cancelled due to receiving seed too late.

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9. Dillon Co., SC (1):
   - Cooperator did note that transgenic varieties had severe delayed maturity and parrot beak non-opened bolls at maturity.
   - Agronomic performance deemed as unacceptable. Efficacy data was biased based on poor crop performance for other physiological aspects of the line tested.
   - No significant differences were noted in plant diseases, insect pest populations or beneficial insect populations.

10. Dillon Co., SC (2):
    - Cooperator noted delayed maturity, parrot beak underdeveloped and non-opening bolls.
    - Agronomic performance deemed as unacceptable.
    - Genetic Effects are unacceptable and many bolls never opened.
    - Efficacy data was biased based on poor crop performance
    - There were no significant differences in plant diseases, insect pest, and beneficial insect populations.

11. Gaines Co., TX:
    - Cooperator noted no significant differences in plant diseases, insect pest, and beneficial insect populations.
    - It was noted that all other visual observations were normal.
    - There was some hail but it did not cause damage or loss to the plot.

12. Lubbock Co., TX (1):
    - Cooperator noted no significant differences in plant diseases, insect pest, and beneficial insect populations.
    - Observations show no significant differences between the transgenic and non-transgenic plots.

13. Lubbock Co., TX (2):
    - Study cancelled due to business reasons therefore, planting was never initiated

14. Uvalde Co., TX (1):
    - No significant differences were noted in plant diseases, insect pest or beneficial populations.
    - Observations show that the transgenic plot had delayed maturity and the cotton bolls were unacceptably small.

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15. Uvalde Co., TX (2):
    - No significant differences were noted in plant diseases, insect pest or beneficial populations
    - It was noted by the cooperator that the boll size for the transgenic was far superior to the non-transgenic

16. Wharton Co., TX (1) & (2):
    - Cooperator noted early boll opening for transgenic and non transgenic.
    - The transgenic plot showed resistance to bollworm, budworm infestations.
    - No significant differences were noted in plant diseases, insect pest or beneficial insect populations.
    - No unusual growth patterns.

Plant Disposition: Plots were harvested and data taken, with samples sent to Bayer facility for analysis. All remaining harvested material in the field was destroyed. Unplanted seed which was not utilized by the cooperator was either returned to Bayer for storage, or destroyed.

Volunteer Monitoring: Volunteer monitoring is currently being conducted on all trial sites, and scheduled to conclude one year from the termination date.

Weediness Characteristics: There was no indication of increased weediness characteristics in either the transgenic or non-transgenic plots.

Non-target Organisms: There was no indication of any adverse effects to non-target insect populations or beneficial insects. Various beneficial insect populations were present with lacewings, assassin bugs, lady bugs, and various coleopteran species observed.

Weather: Weather for all sites was either normal or hot and dry. However, Washington County, Mississippi autumn season was unusually cool and wet causing a delay in harvesting and Wharton County, Texas (2), July and October was extremely wet.

Signature: Date: 9/19/07

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USDA Field Termination Report

USDA Notification Number: 06-068-03n

Applicant Reference Number: IR-6D-GH-MR

Applicant: Bayer CropScience LP
Research Triangle Park, NC 27709

Regulated Article: *Gossypium hirsutum (cotton)*

Site Release Information: Trials utilizing this trait were conducted in ten (10) locations.

County / State	Acreage Planted	Date Planted	Date Terminated	Isolation Method
St. Landry/ LA	1.84	6/12/06	11/16/06	Isolation Border Rows
Tate/ MS (2 locations)	1.46	5/31/06	11/ 10/06	Isolation Border Rows
Washington/ MS	1.49	6/08/06	12/02/06	Isolation Border Rows
Hockley/ TX (2 locations)	1.92	5/30/06 & 6/06/06	12/18/06	Isolation Border Rows
Uvalde/ TX (2 locations)	2.85	5/30/06 & 6/13/06	11/10/06	Isolation Border Rows
Wharton/ TX (2 locations)	4.61	6/13/06 & 6/14/06	11/14/06 & 11/20/06	Isolation Border Rows

Observations: *listing of characteristics between the transgenic and non-transgenic:*

1. St. Landry Co., LA:
   - No unusual characteristics.
   - No difference in emergence, both plots had early flowering, boll set and boll opening with some boll rot.
   - No differences in plant disease, insect pest or beneficial insect populations

2. Tate Co., MS:
   - No significant differences were noted in plant diseases, insect pest and beneficial insect populations.
   - Cooperator did note that maturity of bolls were significantly slower in the transgenic plot as compared to the non-transgenic.
   - Seedling Vigor in the transgenic plot was approximately 40% of the non-transgenic.

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3. Washington Co., MS:
   - Cooperator stated that over-all the plots are similar and there are no significant differences.
   - The plots yield was poor and the transgenic appears to have slightly less fruit set.
   - Misshaped bolls are present in transgenic and non-transgenic plots
   - The hot dry weather seemed to stress the plants and as a result fruit shedding occurred.
   - Regimen A has set more fruit than Regimen B or C.
   - Regimens B and C had poor yields.
   - No significant differences were noted in plant diseases, insect pest, and beneficial insect populations.

4. Hockley Co., TX:
   - Cooperator stated there were no difference in emergence and no significant differences in plant diseases, insect pest populations, beneficial insect populations, emergence, flowering, boll set and boll opening.

5. Uvalde Co., TX:
   - No significant differences were noted in plant diseases, insect pest, beneficial insect and wild life populations.
   - Due to low yield (15%) emergence) a decision was made to re- plant. Replanting took place two (2) weeks later. During the re-planting, previously emerged plants were not removed.
   - It was noted that the transgenic was less tolerant of whiteflies than the non-transgenic. Possible, two-week difference in planting date may have been the cause.
   - It was observed that the transgenic cotton was very light and fluffy, there was no worm presence but the whiteflies made up for the lack of worm presence.

6. Wharton Co., TX:
   - Cooperator noted no unusual characteristics/growth patterns.
   - No significant differences were noted in plant diseases, insect pest or beneficial insect populations.
   - Early bloom for transgenic and non-transgenic.
   - Transgenic emergence was lower than expected and the non-transgenic had good emergence.
   - Bolls in transgenic were smaller than normal

Plant Disposition: Plots were harvested and data taken, with samples sent to Bayer facilities for analyses. All remaining harvested material in the field was destroyed. Unplanted seed which was not utilized by the cooperator was either returned to Bayer for storage, or destroyed.

Volunteer Monitoring: Volunteer monitoring is currently being conducted on all trial sites, and scheduled to conclude one year from the termination date.

Weediness Characteristics: There was no indication of increased weediness characteristics in either the transgenic or non-transgenic plots.

Non-target Organisms: There was no indication of any adverse effects to non-target insect populations or beneficial insects. Various beneficial insect populations were present with lacewings, assassin bugs, lady beetles, and various coleopteran species observed.

Weather: Weather for all sites were either normal or hot and dry. Wharton County, Texas experienced an extremely wet season for the months of July and October.

Signature: Date: 9/19/07

Regina J. Hayes
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Appendix 2. Materials and Methods

2.A. Insert Characterization

Southern analyses were conducted to characterize the DNA insertion in event T303-3 cotton. Genomic DNA was isolated from transgenic and non-transgenic plants and these samples were subjected to Southern analyses using the different components of the transgenic cassette (3'me1, *cry1Ab*, P35S, *bar*, 3'nos, 5'e1) as well as the complete T-DNA fragment as probes.

Test Items, Reference Items, and Standards

Test Item
BC_2F_2 of FM966 event T303-3 was grown in the greenhouse and leaf tissues were harvested from individual plants for genomic DNA extraction.

Control Cotton Material
Non-transgenic cotton variety FM966 seed were planted in the greenhouse and leaf tissues were harvested from individual plants for genomic DNA extraction.

Reference Material
Plasmid DNA from pTDL004 was prepared from *E.coli* strain MC1061.

λ DNA digested with *Pst*I (Fermentas, Burlington, Ontario, Canada) was used as the molecular weight marker on the agarose gels.

Genomic DNA Extraction

Total genomic DNA was prepared from leaf material harvested from event T303-3 cotton and from leaf material of non-transgenic cotton variety FM966. The frozen tissue samples were pulverized and the DNA was extracted using milli-Q (MQ) water and chloroform:isoamylalcohol. To remove all undissolved particles present in the DNA samples, the final DNA samples were centrifuged. All samples had large, viscous pellets. The supernatant was removed to a clean tube and the pellets were dissolved in MQ water overnight. The concentration of the DNA samples was determined.

Restriction Digests of Total Genomic DNA

For each Southern blot, nine aliquots of 10 µg of event T303-3 genomic DNA were digested with the following restriction enzymes: *Bam*HI, *Bgl*II, *Sac*I, *Kpn*I, *Nco*I, *Pvu*I, *Xba*I, *Eco*RV and *Not*I. Two aliquots of 10 µg genomic DNA from the non-transgenic control were digested with the *Not*I restriction enzyme. The total reaction volume was 50 µl and the digests were incubated overnight at 37°C.

Five µg of plasmid pTDL004 DNA were digested with *Not*I. The total reaction volume was 50 µl and the incubation was performed for approximately three hours at 37°C. Plasmid DNA digestion was checked by agarose gel electrophoresis and the four expected fragments (6167bp, 4915bp, 1532bp and 1290bp) were observed. The concentration of the digested plasmid DNA was determined to be 95.33 ng/µl.

Gel Electrophoresis of Digested DNA Samples

Four identical agarose gels were prepared. An appropriate dilution of the *Not*I digested pTDL004 sample was made. Assuming a single copy integration of the transgene into the cotton genome, 10 µg of homozygous genomic DNA corresponds to ~ 30.9 pg of pTDL004 plasmid DNA. The amount representing approximately one plasmid copy per genome was added to 10µg of digested non-transgenic DNA. This served as the positive control and was used to show that the hybridizations were performed under conditions allowing hybridization of the probes with the target sequences.

After addition of the loading dye, each digest was loaded onto the gel. λ DNA digested with *Pst*I was included as the molecular weight standard. Following electrophoresis, the separated DNA fragments were transferred from the agarose gel to a Hybond-N+ membrane.

DNA Probe Hybridization

Probe templates each containing one of the genetic elements of plasmid pTDL004 and the complete T-DNA probe were prepared by PCR. Identical PCR reactions were pooled and following volume reduction by evaporation, the different PCR products were loaded on an agarose gel and purified by gel extraction. To determine the quality of the probe templates, samples of each were loaded on an agarose gel and electrophoresed. The determined molecular weight of each probe template fit with the expected fragment sizes. The concentration of the different probe templates was determined by comparing band intensities of the purified probe templates with band intensities of a low DNA mass ladder (Invitrogen Corp., Carlsbad, CA). The templates were stored in the freezer.

For hybridization, the probe templates were labeled with [α-^{32}P]. Visualization of the hybridizing fragments was achieved via exposure of a BioMax MR film to the hybridized membrane.

Stripping of Probes

Membranes were stripped of the radioactively-labeled probes to prepare the blot for subsequent re-hybridization to additional probes.

Determination of Hybridization Fragment Sizes

Lengths of all hybridization fragments were determined by comparing them with the molecular weight marker. A semi-logarithmic standard curve was constructed using the λ DNA marker with the y-axis being the distance between the gel loading slot and the different fragments of digested marker DNA and the sizes of the fragments of the DNA marker on the x-axis. The length of each hybridization fragment was determined by measuring the migration distance of the fragment and extrapolating it to the standard curve.

2.B. Confirmation of Absence of Vector Backbone

Southern analyses were conducted to confirm the absence of vector backbone from plasmid pTDL004. Genomic DNA was isolated from event T303-3 plant material and

probed with overlapping elements of the vector backbone. Samples were then probed with fragments of the T-DNA (p35S and *cry1Ab*) as a positive control.

Test Items, Reference Items, and Standards

Test Item
BC_2F_3 of FM966 event T303-3 was grown in the greenhouse and leaf tissues were harvested from individual plants for genomic DNA extraction.

Control Cotton Material
Non-transgenic cotton variety FM966 seed were planted in the greenhouse and leaf tissues were harvested from individual plants for genomic DNA extraction.

Reference Material
Plasmid DNA from pTDL004 was prepared from *E.coli* strain MC1061.

λ DNA digested with *Pst*I (Fermentas, Burlington, Ontario, Canada) was used as the molecular weight marker on the agarose gels.

Genomic DNA Extraction

Total genomic DNA was prepared from leaf material harvested from event T303-3 cotton and from leaf material of non-transgenic cotton variety FM966.

Restriction Digests of Total Genomic DNA

Seven samples each of 10 µg of T303-3 genomic DNA were digested with *Not*I or *Eco*RV restriction enzymes and 28 samples each of 10 µg non-transgenic control genomic DNA were digested with the *Not*I restriction enzyme. All digestions were performed in a total reaction volume of 40 µl. The digests were incubated overnight at 37°C.

Five µg of pTDL004 plasmid DNA was digested with *Not*I. The total reaction volume was 50 µl. Incubation was performed overnight at 37°C. To check that the plasmid DNA was totally digested, 200 ng of the digest were loaded on a 1% TAE horizontal submarine agarose gel. All fragments separated as expected (6167 bp, 4915 bp, 1532 bp and 1290 bp). The concentration of the *Not*I –digested plasmid DNA was determined at 98.56 ng/µl by measuring the fluorescence of the Quant-iTTM PicoGreen® dsDNA reagent.

Gel Electrophoresis of Digested DNA Samples

Seven identical agarose gels were prepared. An appropriate dilution of the *Not*I digested plasmid pTDL004 sample was made. Assuming a single copy integration of the transgene into the cotton genome, 10 µg of homozygous genomic DNA corresponds to ~ 30.9 pg of pTDL004 plasmid DNA. The amount representing approximately 0.1, 1 and 10 plasmid copies per genome was added to 10 µg of digested non-transgenic DNA. These preparations served as the positive controls and were used to show that the hybridizations were performed under conditions allowing hybridization of the probes with the target sequences.

Each digest was loaded onto the gel in its appropriate well. λ DNA digested with *Pst*I was included as the molecular weight standard.

Following electrophoresis, the separated DNA fragments were transferred from the agarose gel to a Hybond-N+ membrane.

DNA Probe Hybridization with Vector Backbone Probe Templates

The vector backbone probes were prepared by [α-^{32}P] labeling of probe templates PT002, PT003, PT005, PT007, PT008, PT009 and PT010. Each of the seven membranes was hybridized with a different [α-^{32}P] labeled probe. Visualization of the hybridizing fragments was achieved *via* exposure of a BioMax MR film to the hybridized membrane.

Following the stripping of vector backbone probes from the membranes, each membrane was re-hybridized with the T-DNA probe. The T-DNA probe was prepared by [α-^{32}P] labeling. Visualization of the hybridizing fragments was also achieved *via* exposure of a BioMax MR film to the hybridized membrane.

Determination of Hybridization Fragment Sizes

Lengths of all hybridization fragments were determined by comparing them with the molecular weight marker. A semi-logarithmic standard curve was constructed using the λ DNA marker with the y-axis being the distance between the gel loading slot and the different fragments of digested marker DNA and the sizes of the fragments of the DNA marker on the x-axis. The length of each hybridization fragment is determined by measuring the migration distance of the fragment and extrapolating it to the standard curve.

2.C. Protein (Cry1Ab and PAT) Expression in Raw Agricultural Commodity

Fuzzy seed (ginned seed but not yet de-linted) from event T303-3 cotton were analyzed for the expression of Cry1Ab and PAT proteins using enzyme-linked immunosorbent assays (ELISAs).

Field production of Seed Samples

Generation T_4 event T303-3 cotton seed and non-transgenic Coker 315 cotton seed were grown in six trials in the U.S. All trials were managed according to regional commercial production practices. Samples of fuzzy seed were collected from ginned harvested samples from each plot at each trial site.

Reference Materials

Cry1Ab and PAT proteins, produced in *E.coli*, were used as standards for the ELISA methods.

Processing of Fuzzy Seed Samples

The fuzzy seed was processed by mechanical de-linting. The de-linted seed was mechanically cracked in a roller mill and the hull material was separated from the kernel.

Protein Extraction from Samples

The sample materials were prepared by either grinding in a blender pre-chilled with dry ice or by grinding with a mortar and pestle. The ground sample was mixed with extraction buffer and shaken for 30 min at 4˚C. Insoluble material was removed by centrifugation. The supernatants were then analyzed by the specific ELISAs for the presence of the Cry1Ab and PAT proteins.

Determination of Cry1Ab and PAT Protein Concentrations

The amount of Cry1Ab and PAT protein in the sample extracts were determined by commercial ELISA kits (EnviroLogix, Inc., Portland, ME). All assays resulted in color development in the last step, which was measured by optical density of each microtiterplate well at 450nm. Softmax Pro™ software (Molecular Devices, Version 4.0) was used to derive the concentration of immunoreactive Cry1Ab and PAT proteins. The optical density (OD) values were adjusted for the buffer blank and then any background due to the matrix was subtracted, using the average value from two wells containing non-transgenic extracts, assayed on the same microtiter plate. The OD corrected for the buffer blank and the non-transgenic background was converted to the protein analyte concentration using the standard curve from the same microtiter plate.

2.D. Protein Characterization

Because a full equivalence study (USDA, 2008) was done on the Cry1Ab protein isolated from TwinLink Cotton (where event T304-40, the antecedent organism, is one of the parents in the TwinLink breeding stack), only an SDS-PAGE analysis was done in this study to compare Cry1Ab protein produced in event T303-3 to that produced in *E. coli* and event T304-40.

Test Items, Reference Items, and Standards

<u>Protein expressed *in planta*</u>

Event T303-3 and event T304-40 plants were grown in the greenhouse and leaves were harvested into plastic bags and placed on dry ice. Leaf samples were processed in a Waring blender pre-chilled with dry ice, with small amounts of dry ice added during the blending process to ensure samples remained frozen. Ground samples were stored in a freezer at approximately -20°C overnight to allow for dry ice dissipation prior to extraction of protein.

The Cry1Ab proteins were extracted by mixing ground plant leaves with extraction buffer at a ratio of 25 g of ground leaves to 100 mL of extraction buffer in 5 oz specimen containers. The 850 mL of extraction buffer contained 150 mM NaCl and 100 mM sodium phosphate (pH 7.2), 0.85 g of polyvinylpyrrolidinone (40,000 Mol. Wt), 1 mM PMSF, 1 mM benzamidine/caproic acid and 850 µg of leupeptin/antipain.

After addition of the extraction buffer to the ground leaf material, the 5 oz container with extraction buffer and ground leaf material was continuously inverted on a rotary rocker at ~30 rpm for 20 minutes at room temperature. The extract was filtered through Miracloth® and the filtrate vacuum filtered through a 0.45 µm cellulose acetate membrane.

The Cry1Ab antibody affinity columns used for the purification were prepared using approximately 750 µL of crude rabbit polyclonal antibody serum raised against the Cry1Ab protein. The Cry1Ab antibodies were covalently attached to individual Pierce AminoLink® Plus immobilization columns.

Protein Expressed by *E. coli*

Trypsin-digested Cry1Ab protein (batch no. 25.05.99.70, purity > 85%) expressed by *E. coli* was provided by Bayer BioScience N.V. Molecular and Biochemical Analytical Services- Protein Characterization, Zwijnaarde, Belgium.

Thrombin-engineered Cry1Ab protein from *E.coli* (batch no. Min1418-1, purity > 85%) was provided by Bayer BioScience N.V. Molecular and Biochemical Analytical Services- Protein Characterization, Zwijnaarde, Belgium.

Molecular Weight Markers

Molecular weight markers (Invitrogen BenchMark, Cat# 10747) comprised of a series of recombinant proteins of known molecular weight.

SDS-PAGE

The Cry1Ab protein from *E. coli* and the Cry1Ab protein isolated from events T303-3 and T304-40 cotton leaves were analyzed by SDS-PAGE. The protein from the plants and the corresponding protein from *E. coli* were denatured and analyzed by electrophoresis on a polyacrylamide gel. The gel was stained with Coomassie Brilliant Blue R-250 to visualize the protein bands.

SDS-PAGE analysis was also conducted as described above on trypsin-digested Cry1Ab protein from *E. coli*, the thrombin-engineered Cry1Ab protein from *E. coli*, and Cry1Ab protein isolated from the leaves of event T304-40.

The electrophoretic mobility on a polyacrylamide gel of recombinant proteins of known molecular weight (Molecular Weight Markers) was utilized to generate a standard curve of electrophoretic mobility for each of the SDS-PAGE analyses runs. The standard curves were utilized to determine the molecular weights of the Cry1Ab proteins from *E. coli* (trypsin-digested and thrombin-engineered), event T304-40, and event T303-3.

2.E. Composition Analysis

Samples were analyzed to assess the nutrient and anti-nutrient composition of event T303-3 cotton grain and compare it to that of the non-transgenic control.

Field production of Seed Samples

Generation T_4 event T303-3 and non-transgenic cotton seed (Coker 315) were grown in six trials in primary cotton growing regions of the U.S in 2005. All trials were managed according to regional commercial production practices. Each trial consisted of nine plots.

Six plots were event T303-3 and three were non-transgenic Coker 315. Of the six event T303-3 plots, three were treated twice with a tank mix of glufosinate ammonium herbicide and ammonium sulfate at 0.52 lb ai/A (equivalent to 40 oz Ignite® Herbicide/A) and 3.0 lb/A, respectively. Samples of fuzzy seed were collected from each plot (i.e., three samples T303-3 sprayed, three samples T303-3 nonsprayed, three samples non-transgenic Coker 315) at each trial site and shipped frozen to Bayer CropScience, RTP, NC.

Composition Analysis of Raw Agricultural Commodity

Subsamples of frozen ginned seed were sent to two 3rd party labs for composition analysis, Eurofins Scientific (Des Moines, IA) and Covance Laboratories (Madison, WI). All parameters with the exception of cyclopropenoid fatty acid content were performed by Eurofins Scientific.

Methods utilized by laboratories for each parameter analyzed are shown in Table 21.

Statistical Methods

Analysis was conducted by analyzing for differences between the three treatments (i.e., event T303-3 sprayed, event T303-3 unsprayed, non-transgenic Coker 315) for 55 different components. These components were:

- Proximate and fiber compounds: moisture, protein, crude fat, ash, total carbohydrates, ADF, and NDF
- Minerals: Calcium, Phosphorus, Potassium, Magnesium, Iron, and Zinc
- Tocopherols: alpha-, beta-,gamma-, and delta tocopherol, total tocopherol (Vitamin E)
- Anti-nutrients: free gossypol, "-" and "+" gossypol, total gossypol, phytic acid
- Amino acids: alanine, arginine, aspartic acid, cystine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and valine
- Fatty acids: myristic (C14:0), palmitic (C16:0), stearic C18:0), arachidic (C20:0), behenic (C22:0), lignoceric (C24:0), palmitoleic (C16:1), oleic (C18:1), linoleic (C18:2), linolenic (C18:3)
- Cyclopropenoid fatty acids: malvic acid, sterculic acid and dihydrosterculic acid

Statistical analyses included descriptive statistics (i.e., mean value, standard deviation, min/max values), over all analysis (i.e., ANOVA by treatment, site, and respective interaction terms), and by site analysis (i.e., ANOVA by treatment where significant interactions [$p<0.05$] by site were detected, followed by t-tests). Level of significance was fixed 0.05 (two-sided).

Table 21. Analytical methods used for compositional analysis

Parameter	Laboratory	Method
Moisture	Eurofins Scientific	AOAC 930.15
Crude fat	Eurofins Scientific	AOAC 920.39
Crude protein	Eurofins Scientific	AOCS 990.03
Ash	Eurofins Scientific	AOAC 942.05
Acid Detergent Fiber	Eurofins Scientific	ANKOM ADF 05/03
Neutral Detergent Fiber	Eurofins Scientific	ANKOM NDF 05/03
Carbohydrates	Eurofins Scientific	21CFR - Difference between 100 and the sum of crude protein, fat, moisture and ash
Ca	Eurofins Scientific	AOAC 984.27 and 985.01
P	Eurofins Scientific	AOAC 984.27 and 985.01
K	Eurofins Scientific	AOAC 984.27 and 985.01
Fe	Eurofins Scientific	AOAC 984.27 and 985.01
Mg	Eurofins Scientific	AOAC 984.27 and 985.01
Zn	Eurofins Scientific	AOAC 984.27 and 985.01
Tocopherols	Eurofins Scientific	AOAC 971.30 with HPLC Quantitation
Amino Acids	Eurofins Scientific	AOAC 982.30 Sec. D, F
Fatty Acids	Eurofins Scientific	AOCS Ce 1e-91 (Capillary GC)
Phytic Acid	Eurofins Scientific	Analytical Biochemistry 77, 536-539 (1977)
Gossypol (total)	Eurofins Scientific	AOCS Ba 8-78 (1983, reapproved 1997)
Gossypol (free)	Eurofins Scientific	Method version of AOCS Ba 7-58 (reapproved 1997) and JAOCS vol. 59, no. 12, pp. 546-549 (Dec. 1982) (Modified)
Cyclopropenoid Fatty Acids	Covance Laboratories	Covance method procedure MP-CPFA-MA (HPLC)

Appendix 3. Characterization of Event T303-3

3.A. Insert Characterization

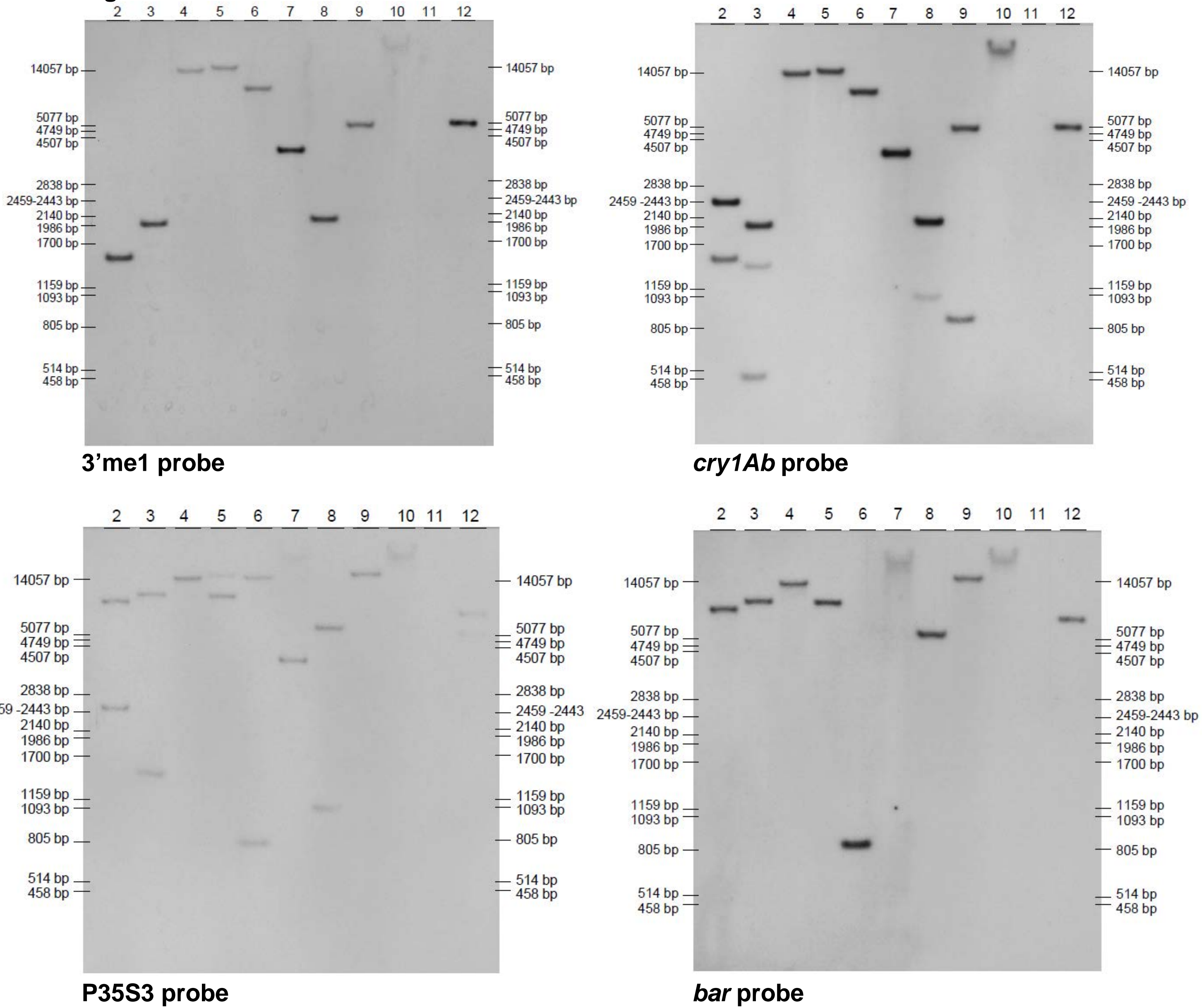
Genomic DNA isolated from event T303-3 cotton was digested with one of the following restriction enzymes: *Bam*HI, *Bgl*II, *Sac*I, *Kpn*I, *Nco*I, *Pvu*I, *Xba*I, *Eco*RV, and *Not*I. Non-transgenic genomic DNA digested with *Not*I was used as a negative control; non-transgenic genomic DNA supplemented with one copy of pTDL004 and digested with *Not*I was used as a positive control. The resulting DNA fragments were analyzed by Southern blot with six different probes, corresponding to the different genetic elements contained in the T-DNA from plasmid pTDL004. Probe information is presented in Table 22. An overview of the obtained Southern blot results is present in Figure 11.

The results of the Southern blot analysis demonstrate that the inserted transgenic sequence in event T303-3 consists of one complete copy of the *cry1Ab* and *bar* genes with the associated regulatory sequences required for expression.

Table 22. Event T303-3 insert verification – probe information

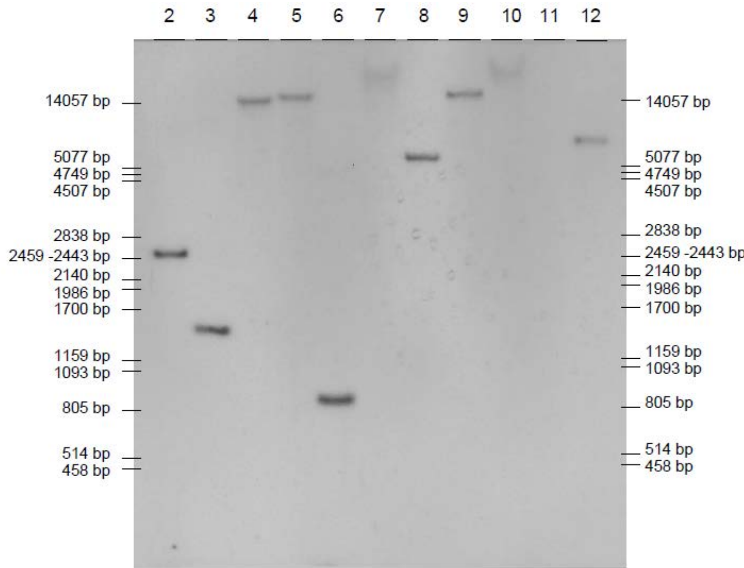
Probe Template ID	Genetic Element	Size Probe Template (bp)
PT020-1	3'me1 probe	859
PT021-1	*cry1Ab* probe	1822
PT022-1	P35S3 probe	801
PT023-1	*bar* probe	425
PT024-1	3'nos probe	214
PT025-1	Complete T-DNA probe	4929

Figure 11. Event T303-3 insert verification – Southern blot results



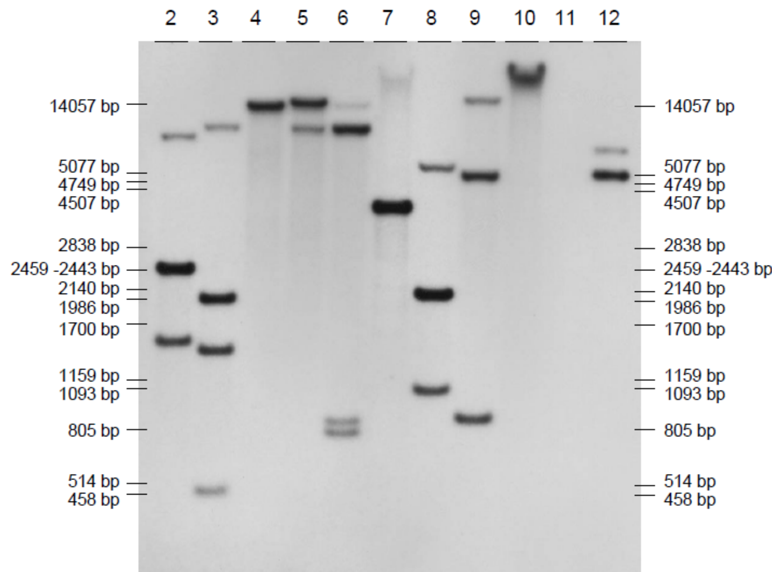
<u>Loading Sequence- All Probes</u>
Lane 2: *G. hirsutum* event T303-3 – *Bam*HI digested
Lane 3: *G. hirsutum* event T303-3 – *Bgl*II digested
Lane 4: *G. hirsutum* event T303-3 – *Sac*I digested
Lane 5: *G. hirsutum* event T303-3 – *Kpn*I digested
Lane 6: *G. hirsutum* event T303-3 – *Nco*I digested
Lane 7: *G. hirsutum* event T303-3 – *Pvu*I digested
Lane 8: *G. hirsutum* event T303-3 – *Xba*I digested
Lane 9: *G. hirsutum* event T303-3 – *Eco*RV digested
Lane 10: *G. hirsutum* event T303-3 – *Not*I digested
Lane 11: *G. hirsutum* wild type – *Not*I digested
Lane 12: *G. hirsutum* wild type + 1 copy pTDL004 –*Not*I digested

Figure 11 (continued). Event T303-3 insert verification – Southern blot results



3'nos probe

Loading Sequence- All Probes
Lane 2: *G. hirsutum* event T303-3 – *Bam*HI digested
Lane 3: *G. hirsutum* event T303-3 – *Bgl*II digested
Lane 4: *G. hirsutum* event T303-3 – *Sac*I digested
Lane 5: *G. hirsutum* event T303-3 – *Kpn*I digested
Lane 6: *G. hirsutum* event T303-3 – *Nco*I digested
Lane 7: *G. hirsutum* event T303-3 – *Pvu*I digested
Lane 8: *G. hirsutum* event T303-3 – *Xba*I digested
Lane 9: *G. hirsutum* event T303-3 – *Eco*RV digested
Lane 10: *G. hirsutum* event T303-3 – *Not*I digested
Lane 11: *G. hirsutum* wild type – *Not*I digested
Lane 12: *G. hirsutum* wild type + 1 copy pTDL004 –*Not*I digested



Complete T-DNA probe

3.B. Confirmation of Absence of Vector Backbone

Genomic DNA isolated from event T303-3 cotton was digested with restriction enzymes *Eco*RV and *Not*I. Non-transgenic genomic DNA digested with *Not*I was used as a negative control; non-transgenic genomic DNA supplemented with 0.1, 1, and 10 copies of plasmid pTDL004 and digested with *Not*I was used as a positive control. The resulting DNA fragments were analyzed by Southern blot with seven overlapping probes, corresponding to the different genetic elements contained in the vector backbone of plasmid pTDL004 as well as the complete T-DNA probe. Probe information is presented in Table 23.

Table 24 and 25 summarizes the expected and obtained hybridization fragments using vector backbone probes and T-DNA probe, respectively.

The results of the Southern blot analysis confirm than event T303-3 contains no elements of the pTDL004 vector backbone.

Table 23. Event T303-3 vector backbone analysis – probe information

Probe Template ID	Genetic Element	Position in pTDL004	Size Probe Template (bp)
PT007-1	Vector Backbone Probe	bp 5135 to 6480	1346
PT002-1	Vector Backbone Probe	bp 5981 to 8019	2039
PT003-1	Vector Backbone Probe	bp 7474 to 9461	1988
PT008-1	Vector Backbone Probe	bp 8794 to 10860	2067
PT009-1	Vector Backbone Probe	bp 10277 to 11629	1353
PT010-1	Vector Backbone Probe	bp 10931 to 12526	1596
PT005-1	Vector Backbone Probe	bp 12019 to 13879	1861
PT011-1	T-DNA probe (P35S + *cry1Ab)*	bp 1619 to 4187	2492

Table 24. Expected and obtained hybridization fragments with overlapping vector backbone probe templates

Probe template ID	T303-3 - *EcoRV*		T303-3 - *NcoI*		WT (FM966) - *NcoI*		WT - *NcoI* + 0.1; 1; or 10 copies pTDL004 - *NcoI*			
	Expected hybridization fragments	Obtained hybridization fragments	Expected hybridization fragments	Obtained hybridization fragments	Expected hybridization fragments	Obtained hybridization fragments	Expected hybridization fragments	Obtained hybridization fragments: 0.1 copy	Obtained hybridization fragments: 1 copy	Obtained hybridization fragments: 10 copies
PT002-1 (H1/05-004/01)	/	/	/	/	/	/	1290 bp 1532 bp* 4915 bp	1280 ± 20 5100 ± 200	1280 ± 20 1550 ± 30 5100 ± 200	1280 ± 20 1550 ± 30 5100 ± 200 6250 ± 100
PT003-1 (H1/05-004/02)	/	/	/	/	/	/	1290 bp 1532 bp	1280 ± 20 1550 ± 30	1280 ± 20 1550 ± 30	1280 ± 20 1550 ± 30
PT005-1 (H1/05-004/03)	/	/	/	/	/	/	6167 bp	6250 ± 100	6250 ± 100	6250 ± 100
PT007-1 (H1/05-004/04)	/	/	/	/	/	/	4915 bp	5100 ± 200	5100 ± 200	5100 ± 200
PT008-1 (H1/05-004/05)	/	/	/	/	/	/	1532 bp 6167 bp	1550 ± 30 6250 ± 100	1550 ± 30 6250 ± 100	1550 ± 30 6250 ± 100 5100 ± 200
PT009-1 (H1/05-004/06)	/	/	/	/	/	/	6167 bp	6250 ± 100	6250 ± 100	6250 ± 100
PT010-1 (H1/05-004/07)	/	/	/	/	/	/	6167 bp	6250 ± 100	6250 ± 100	6250 ± 100

Fragment sizes are provided in bp
*Only 85 bp overlap

Table 25. Expected and obtained hybridization fragments using T-DNA probe

Probe template ID	T303-3 - *EcoRV*		T303-3 - *NotI*		WT (FM966) - *NotI*		WT - *NotI* + 0.1; 1; or 10 copies pTDL004 - *NotI*			
	Expected hybridization fragments	Obtained hybridization fragments	Expected hybridization fragments	Obtained hybridization fragments	Expected hybridization fragments	Obtained hybridization fragments	Expected hybridization fragments	Obtained hybridization fragments		
								0.1 copy	1 copy	10 copies
PT011-1 (H3/05-004/01)	14000 4850 881	ca. 14000 4860 ± 140 880 ± 30	> 4664	>14000	/	/	6167 4915	5100 ± 200	6250 ± 100 5100 ± 200	6250 ± 100 5100 ± 200
PT011-1 (H2/05-004/02)	14000 4850 881	ca. 14000 4860 ± 140 880 ± 30	> 4664	>14000	/	/	6167 4915	5100 ± 200	6250 ± 100 5100 ± 200	6250 ± 100 5100 ± 200
PT011-1 (H2/05-004/03)	14000 4850 881	ca. 14000 4860 ± 140 880 ± 30	> 4664	>14000	/	/	6167 4915	5100 ± 200	6250 ± 100 5100 ± 100	6250 ± 100 5100 ± 100
PT011-1 (H2/05-004/04)	14000 4850 881	ca. 14000 4860 ± 140 880 ± 30	> 4664	>14000	/	/	6167 4915	5100 ± 200	6250 ± 100 5100 ± 200	6250 ± 100 5100 ± 200
PT011-1 (H2/05-004/05)	14000 4850 881	ca. 14000 4860 ± 140 880 ± 30	> 4664	>14000	/	/	6167 4915	5100 ± 200	6250 ± 100 5100 ± 200	6250 ± 100 5100 ± 200
PT011-1 (H2/05-004/06)	14000 4850 881	ca. 14000 4860 ± 140 880 ± 30	> 4664	>14000	/	/	6167 4915	6250 ± 100 5100 ± 200	6250 ± 100 5100 ± 200	6250 ± 100 5100 ± 200
PT011-1 (H2/05-004/07)	14000 4850 881	ca. 14000 4860 ± 140 880 ± 30	> 4664	>14000	/	/	6167 4915	6250 ± 100 5100 ± 200	6250 ± 100 5100 ± 200	6250 ± 100 5100 ± 200

Fragment sizes are provided in bp

3.C. Composition Analysis

Fuzzy seed was harvested from six field trials. Each trial consisted of three replications of event T303-3 not sprayed, event T303-3 sprayed and the non-transgenic Coker 315. Fuzzy seed samples were processed and analyzed for proximate, amino acid, fatty acid, cyclopropenoid fatty acid, mineral, tocopherol, and anti-nutrient composition. Data for composition analysis is presented in Table 26, 27, 28 and 29.

Table 26. Amino acid composition in cottonseed of event T303-3

Total Amino Acids	% Dry matter			Reference ranges [a]
	Non-Transgenic	Transgenic Not sprayed	Transgenic Sprayed	
	Mean ± SD	Mean ± SD	Mean ± SD	
Alanine	0.94 ± 0.08	0.99 ± 0.07	1.00 ± 0.06	0.42 - 1.51
Arginine	2.48 ± 0.33	2.62 ± 0.28	2.65 ± 0.22	1.05 - 4.40
Aspartic acid	2.26 ± 0.21	2.30 ± 0.21	2.33 ± 0.17	1.00 - 3.55
Cystine	0.34 ± 0.04	0.36 ± 0.03	0.37 ± 0.03	0.16 - 0.86
Glutamic acid	4.64 ± 0.55	4.91 ± 0.47	4.97 ± 0.38	1.96 - 8.16
Glycine	0.95 ± 0.09	0.99 ± 0.08	1.00 ± 0.06	0.44 - 1.58
Histidine	0.63 ± 0.07	0.67 ± 0.06	0.67 ± 0.05	0.31 - 1.03
Isoleucine	0.70 ± 0.06	0.73 ± 0.07	0.75 ± 0.05	0.35 - 1.17
Leucine	1.33 ± 0.13	1.40 ± 0.12	1.42 ± 0.10	0.63 - 2.23
Lysine	1.11 ± 0.16	1.23 ± 0.15	1.18 ± 0.10	0.52 - 1.65
Methionine	0.38 ± 0.04	0.41 ± 0.03	0.41 ± 0.03	0.15 - 0.54
Phenylalanine	1.22 ± 0.14	1.28 ± 0.13	1.30 ± 0.10	0.54 - 2.03
Proline	0.91 ± 0.11	0.95 ± 0.09	0.97 ± 0.11	0.41 - 1.39
Serine	1.02 ± 0.10	1.08 ± 0.09	1.09 ± 0.07	0.50 - 1.63
Threonine	0.75 ± 0.06	0.79 ± 0.06	0.80 ± 0.04	0.34 - 1.21
Tryptophan	0.28 ± 0.03	0.29 ± 0.02	0.30 ± 0.02	0.10 - 0.49
Tyrosine	0.56 ± 0.06	0.59 ± 0.06	0.61 ± 0.05	0.32 - 1.17
Valine	0.97 ± 0.10	1.02 ± 0.10	1.03 ± 0.08	0.45 - 1.67

Data represent an average of three replicate samples at six field test sites.
[a]Reference ranges compiled from OECD (2004), ILSI (2007), Lawhon et al. (1976), and Bertrand et al. (2005)

Table 27. Fatty acid composition in cottonseed of event T303-3

Total fatty acids	% Relative			Reference ranges[a]
	Non-Transgeni	Transgenic Not sprayed	Transgenic Sprayed	
	Mean ± SD	Mean ± SD	Mean ± SD	
Saturated				
Myristic C 14:0	0.70 ± 0.12	0.62 ± 0.07	0.61 ± 0.06	0.53 - 1.17
Palmitic C16:0	23.21 ± 1.38	22.43 ± 1.05	22.34 ± 1.07	21.1 - 29.9
StearicC18:0	2.42 ± 0.11	2.52 ± 0.12	2.51 ± 0.13	2.15 - 3.4
Arachidic C20:0	0.30 ± 0.02	0.28 ± 0.03	0.28 ± 0.02	0 - 0.48
Behenic C22:0	0.17 ± 0.05	0.15 ± 0.01	0.15 ± 0.01	0 - 0.27
Lignoceric C24:0	0.12 ± 0.02	0.11 ± 0.01	0.11 ± 0.01	0 - 0.30
Sum saturated	26.92	26.11	26.00	24.1 - 35.5
Mono-unsaturated				
Palmitoleic C16:1	0.51 ± 0.04	0.48 ± 0.04	0.48 ± 0.04	0.46 - 0.86
Oleic C18:1	14.80 ± 0.77	14.61 ± 0.55	14.61 ± 0.50	13.4 - 22.0
Sum mono-unsaturated	15.31	15.09	15.09	13.9 - 22.9
Poly-unsaturated				
Linoleic C18:2	56.20 ± 1.71	57.35 ± 1.62	57.47 ± 1.49	36.3 - 64.0
Linolenic C18:3	0.58 ± 0.14	0.56 ± 0.04	0.55 ± 0.04	< 0.10 - 0.62
Sum poly-unsaturated	56.78	57.91	58.02	36.3 - 64.3
Sum of total fatty acids	99.01	99.11	99.11	-

Data represent an average of three replicate samples at six field test sites
[a]Reference ranges compiled from OECD (2004), ILSI (2007), Bertrand et al. (2005), Berberich et al. (1996), and Nida et al. (1996)

Table 28. Cyclopropenoid fatty acid composition in cottonseed of event T303-3

Parameter	Based on dry			Reference ranges[a]
	Non-Transgeni	Transgenic Not sprayed	Transgenic Sprayed	
	Mean ± SD	Mean ± SD	Mean ± SD	
Malvalic acid %rel.	0.389 ± 0.076	0.361 ± 0.093	0.354 ± 0.088	0.17 - 1.5
Sterculic acid %rel.	0.331 ± 0.045	0.306 ± 0.054	0.324 ± 0.065	0.12 - 0.92
Dihydrosterculic acid %rel.	0.149 ± 0.020	0.150 ± 0.015	0.146 ± 0.016	0.11 - 0.50

Data represent an average of three replicate samples at six field test sites.
[a]Reference ranges compiled from OECD (2004), ILSI (2007), Calhoun et al. (1995), Berberich et al. (1996), Nida et al. (1996), Phelps et al. (1965), and Wozenski and Woodburn (1975)

Table 29. Mineral and tocopherol composition in cottonseed of event T303-3

Parameter	Based on dry matter			Reference ranges[a]
	Non-Transgenic	Transgenic Not sprayed	Transgenic Sprayed	
	Mean ± SD	Mean ± SD	Mean ± SD	
Calcium %	0.15 ± 0.02	0.24 ± 0.05	0.27 ± 0.09	0.09 – 0.33
Phosphorus %	0.62 ± 0.08	0.65 ± 0.07	0.66 ± 0.09	0.31 – 0.86
Potassium %	1.18 ± 0.11	1.20 ± 0.06	1.21 ± 0.07	0.96 – 1.42
Magnesium %	0.38 ± 0.03	0.41 ± 0.03	0.41 ± 0.03	0.27 – 0.49
Iron %	0.0061 ± 0.0015	0.0063 ± 0.0015	0.0072 ± 0.0021	0.0023-0.016
Zinc mg/kg	30.26 ± 3.40	37.04 ± 12.81	35.45 ± 5.01	17.8 – 63.0
αTocopherol mg/kg	128.39 ± 25.47	115.31 ± 17.57	124.56 ± 13.41	16 - 245
β Tocopherol mg/kg	< 6.00	< 6.00	< 6.00	0 – 10
γTocopherol mg/kg	49.00 ± 18.40	49.22 ± 14.50	58.33 ± 13.61	16 – 271
δ Tocopherol mg/kg	< 6.00	< 6.00	< 6.00	0 – 10
Total Tocopherol (vitamin E)[b]	177.53 ± 32.31	164.79 ± 27.02	182.56 ± 20.61	32 – 536

Data represent an average of three replicate samples at six field test sites.
[a]Reference ranges compiled from OECD (2004), ILSI (2007), USCA (1982), Calhoun et al. (1995), Lundquist (1995) and FAO/WHO (2001)
[b]Vitamin E as the sum of all tocopherols in mg/kg dm